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# GC-MS Analysis of Amino Acid Enantiomers as Their *N(O,S)*-Perfluoroacyl Perfluoroalkyl Esters: Application to Space Analysis

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**ABSTRACT** The target of the in-situ research of optical activity in extraterrestrial samples stimulated an extended investigation of a GC-MS method based on the derivatization of amino acids by using a mixture of perfluorinated alcohols and perfluorinated anhydrides. Amino acids are converted to their *N(O,S)*-perfluoroacyl perfluoroalkyl esters in a single-step procedure, using different combinations of the derivatization reagents trifluoroacetic anhydride (TFAA)-2,2,2-trifluoro-1-ethanol (TFE), TFAA-2,2,3,3,4,4,4-heptafluoro-1-butanol (HFB), and heptafluorobutyric anhydride (HFBA)-HFB. The derivatives obtained are analyzed using two different chiral columns: Chiral-L-Val and  $\gamma$ -cyclodextrin (Rt- $\gamma$ -DEXsa) stationary phases which show different and complementary enantiomeric selectivity. The mass spectra of the derivatives are studied, and mass fragmentation patterns are proposed: significant fragment ions can be identified to detect amino acid derivatives. The obtained results are compared in terms of the enantiomeric separation achieved and mass spectrometric response. Linearity studies and the measurement of the limit of detection (LOD) show that the proposed method is suitable for a quantitative determination of enantiomers of several amino acids. The use of the programmed temperature vaporiser (PTV) technique for the injection of the untreated reaction mixture is a promising method for avoiding manual treatment of the sample and decreasing the LOD. *Chirality* 18:279–295, 2006. © 2006 Wiley-Liss, Inc.

**KEY WORDS:** chirality; amino acids; derivatization; GC-MS; in-situ analysis; planetary exploration

The in-situ search for organic molecules in extraterrestrial environments has been proposed for a better understanding of the solar system origin and the emergence of life on Earth. With this aim, detection of chemical biomarkers, i.e., of organic molecules that play an important role in biochemistry, will be one of the main goals of future space missions. Among the key chemical biomarkers on Earth,<sup>1–3</sup> amino and carboxylic acids which have been synthesized by experimental simulations under plausible prebiotic conditions,<sup>4–6</sup> have been detected in several meteorites<sup>7–13</sup> and in the lunar soil.<sup>14–16</sup> Thus, these chemical species appear to be widespread in the solar system, but they can exist without the presence of life. For this reason, the analysis of their optical activity seems to be a good complementary diagnostic tool to identify the link to a biotic or a prebiotic activity. In fact, almost exclusively L-amino acids are incorporated into proteins during microbial biosynthesis, although various D-amino acids have been found in archaea, yeasts, fungi, plants, insects, mollusks, and other eucaryotic organisms.<sup>17–19</sup> Because there are no evident biochemical reasons why L-amino acids should be

selected over D-amino acids, it is generally assumed that life elsewhere could be based on either L- or D-amino acids, but not both.<sup>20–22</sup> Identifying an excess of D-amino acids in an extraterrestrial sample could provide unambiguous evidence for life that evolves independently from the Earth.<sup>23–24</sup> In addition, a hypothesis on the origin of the source of homo-chiral life on Earth could be made: endogenous if originated on the Earth itself, or exogenous, if the enantiomeric excesses were imported from space. Today, this investigation is encouraged by the extraordinary discoveries made by the recent spatial missions which proved the presence of water ice deposits and methane on Mars, in addition to evidence of past liquid water.<sup>25,26</sup>

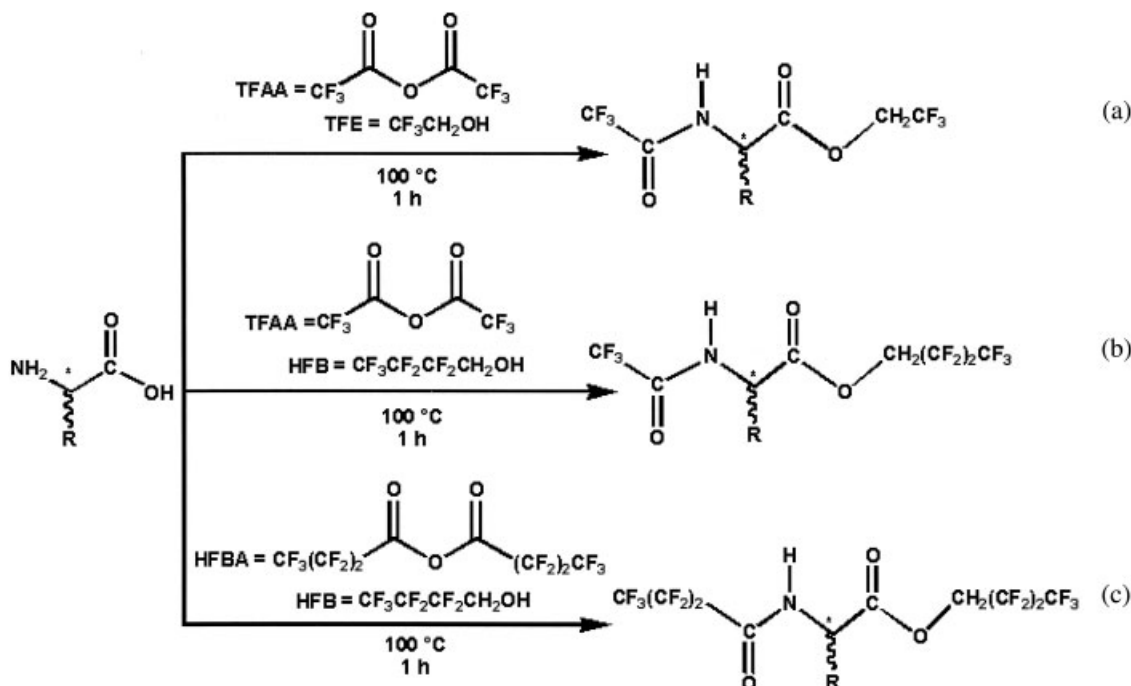
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**Fig. 1.** Simplified diagram of the derivatization reaction for the three combinations of reagents. Three kinds of the obtained perfluoroacyl perfluoroalkyl esters of the amino acids are indicated as (a) trifluoroacetyl trifluoroethyl derivative, (b) trifluoroacetyl heptafluorobutyl derivative, and (c) heptafluorobutyryl heptafluorobutyl derivative.

To date, the GC is the only separative analytical technique which has been used for the in-situ analysis of extraterrestrial environments, such as Venus and Mars. Titan and comet Churyumov Gerasimenko are the present targets of the Cassini-Huygens and Rosetta missions with onboard GC-MS experiments.<sup>27–32</sup> However, amino acids must be chemically transformed to be analyzed by GC because they are not volatile compounds.

The goal of this work is therefore to develop a simple and automated derivatization procedure as a suitable candidate for the GC in-situ analysis of amino acid enantiomers. The main requirements of the method, imposed by space constraints (limitation in volume, weight, power consumption, etc.) are at least as follows:

- (i) Possibility of automation: the process should be a single-step derivatization, simple and fast, achieved under mild reaction conditions. It would also require no mixing devices, no excessive storage area for all the reagents, and no purification procedures.
- (ii) Sensitivity: the amounts of amino acids detected in the extraterrestrial sample found on Earth are in the ppm-ppb range, or sub-ppb level. Moreover, an automated device onboard a space probe must analyze very small samples.
- (iii) Achievement of chemoselective and enantioselective separation: the final goal is the complete separation of many amino acid pairs in one analytical run with good resolution values.

**TABLE 1.** Examples of application of one-pot esterification–acylation using a mixture of perfluoroanhydride–perfluoroalcohol\*

Substrate	Reaction condition
Phe, Tyr, DOPA <sup>40</sup>	PFPA–HFIP, 5:1 v/v, 1 h, 100°C
Glu, GABA <sup>41</sup>	PFPA–HFIP, 2:1 v/v, 1 h, 60°C
GABA <sup>42,43</sup>	TFAA–HFIP, 2:1 v/v, 1 h, rt
Gly <sup>44</sup>	HFBA–HFIP, 2.5:1 v/v, 40 min, 80°C
Gln <sup>45</sup>	PFPA–PFIP, 5:1 v/v, 2 h, 60°C
Tyr and 3-nitrotyrosine <sup>46</sup>	TFAA–TFE, 4:1 v/v, 30 min, 70°C
γ-Glutamyl amino acids <sup>47</sup>	PFPA–PFIP, 5:1 v/v, 15 min, 75°C
Hydroxy acids <sup>48</sup>	PFPA–various perfluoroalcohols, 4:1 v/v, 10 min, 75°C
Opiates, cocaine and its metabolites <sup>49</sup>	HFBA–HFIP, 1.42:1 v/v, 1 h, 80°C
Pesticides containing phosphonic and amino groups <sup>50–51</sup>	TFAA–TFE, 2:1 v/v, 1 h, 100°C

\*Legend: Phe, phenylalanine; Tyr, tyrosine; DOPA, dihydroxyphenylalanine; Glu, glutamic acid; GABA, γ-aminobutyric acid; Gly, glycine; Gln, glutamine; TFAA, trifluoroacetic anhydride; PFPA, pentafluoropropionic anhydride; HFBA, heptafluorobutyric anhydride; TFE, 2,2,2-trifluoro-1-ethanol; PFIP, 2,2,3,3,3-pentafluoropropionic-1-propanol; HFIP, hexafluoroisopropyl alcohol.

**TABLE 2.** Characteristic ion peaks in EI<sup>+</sup> spectra of HFBA–HFB derivatives of amino acids excluding certain *m/z* values (69, 119, 169, 197, and 213)\*

Amino acid	Molecular ion peak ( <i>m/z</i> )	Base peak ( <i>m/z</i> )	M-227	Other important ions ( <i>m/z</i> )
Val	495	55	268	225, 253, 453
Ala	(467)	240	240	220, 270
Leu	(509)	69	282	214, 225, 240, 253, 266, 453
Ile	(509)	69	282	57, 225, 253, 453
Ser (bis-acylated)	679	238	452	210, 254, 266, 268, 282, 466, 480, 482
Pro	493	266	266	296, 474, 493
Thr (mono-acylated)	(497)	54	/	251, 252, 279, 280, 479
Thr (bis-acylated)	(693)	252	466	241, 280, 453, 480, 494
Gly	(453)	226	226	56, 206, 256, 284
Met	527	61	300	75, 253, 266, 315, 453
Asp (bis-esterified)	693	466	466	51, 113, 253, 254, 266, 281, 296, 424, 446, 494
Tyr (bis-acylated)	755	303	528	134, 275, 315, 331, 343, 452, 542, 543
Phe	543	91	316	131, 330
Glu (bis-esterified)	707	252	480	280, 508
Pyr	507	280	280	252, 310, 338
Cys (bis-acylated)	695	285	468	238, 255, 270, 298
Lys (bis-acylated)	720	280	493	67, 84, 226, 239, 253, 268, 295, 520
His	533	81	306	54, 75, 121, 136, 320, 364
Gln (bis-acylated)	(720)	70	(493)	55, 56, 70, 84, 99, 111, 127, 239, 253, 281, 296, 324
Trp (bis-acylated)	778	326	551	129, 565

\*Mass value given in brackets indicates the absence of a molecular ion peak in the spectrum.

At present, the most popular amino acid derivatization procedure, i.e., the silylation with *N*-methyl-*N*-*tert*-butyldimethylsilyl-trifluoroacetamide (MTBSTFA)<sup>33–35</sup> seems unsuitable for the enantioselective separation of amino acids. The amino acid derivitization into *N*(*O,S*)-perfluoroacyl alkyl esters seems the most useful method for their GC enantiomeric analysis on a chiral column.<sup>36–39</sup> The original procedure requires two steps: (1) esterification of the carboxyl group by heating in anhydrous alcoholic acidified medium and subsequent removal of reagents excess; (2) acylation of the  $\alpha$ -amino and other groups by heating with perfluorinated anhydrides. A modified procedure has been proposed which unifies the two processes in one step by simultaneously performing esterification and acylation to obtain the *N*(*O,S*)-perfluoroacyl perfluoroalkyl derivatives by using a mixture of perfluorinated anhydride and perfluoro alcohols.<sup>40–51</sup> This modified procedure, even if already applied to different matrices for determining isolated amino acids or other substrates (see Table 1), was not systematically investigated: in particular, no chromatographic data concerning the determination of the whole series of proteinogenic amino acids are reported.

In this work, the performance of the perfluoroacyl perfluoroalkyl derivatives is investigated for the GC enantioselective separation of amino acids, and suitability of the procedure for automation is checked. In particular, the following topics are considered: (1) the reactivity of 20 proteinogenic amino acids and the MS properties of the derivatives obtained; (2) the retention behavior of the derivatives and their enantiomeric separation on two chiral capillary columns, Chirasil-L-Val and Rt- $\gamma$ -Dexsa;<sup>52,53</sup> (3) the linearity and sensitivity of the analytical method; (4) the possibility of automating the sample treatment

by using a programmed temperature vaporization (PTV) injector.

The interest of the results of this study could be broader than that concerning the specific target here considered: in fact, the enantiomeric separation of amino acids is a very relevant topic in many fields, i.e., pharmacology, medicine, biology, food, archaeological, and soil chemistry.

## EXPERIMENTAL

### Materials

The *D*- and *L*-enantiomers and racemates of 20 proteinogenic tested amino acids, Ala, Asp, Asn, Arg, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val, trifluoroacetic anhydride (TFAA), heptafluorobutyric anhydride (HFBA), 2,2,2-trifluoroethanol (TFE), 2,2,3,3,4,4,4-heptafluoro-1-butanol (HFB), ethyl acetate, acetonitrile, methanol, and methyl dodecanoate (methyl laurate), were purchased from Sigma-Aldrich and Fluka (Strasbourg, France). All of these compounds were analytical-grade reagents. 2 N HCl-methanol solution was obtained by bubbling HCl gas, generated by the slow addition of concentrated aqueous solution of HCl (37%) to a stirred concentrated aqueous solution of H<sub>2</sub>SO<sub>4</sub> (95%), in dry methanol. Anhydrous methanol was prepared by reflux over magnesium turnings before distillation in a glass system.

Amino acids were dissolved either individually or as a mixture up to a concentration of  $1 \times 10^{-2}$  M of each in distilled water or 0.1 N HCl to obtain standard stock solutions.

The internal standard solution was prepared by dissolving methyl dodecanoate in ethyl acetate at the concentration of  $5 \times 10^{-3}$  M.

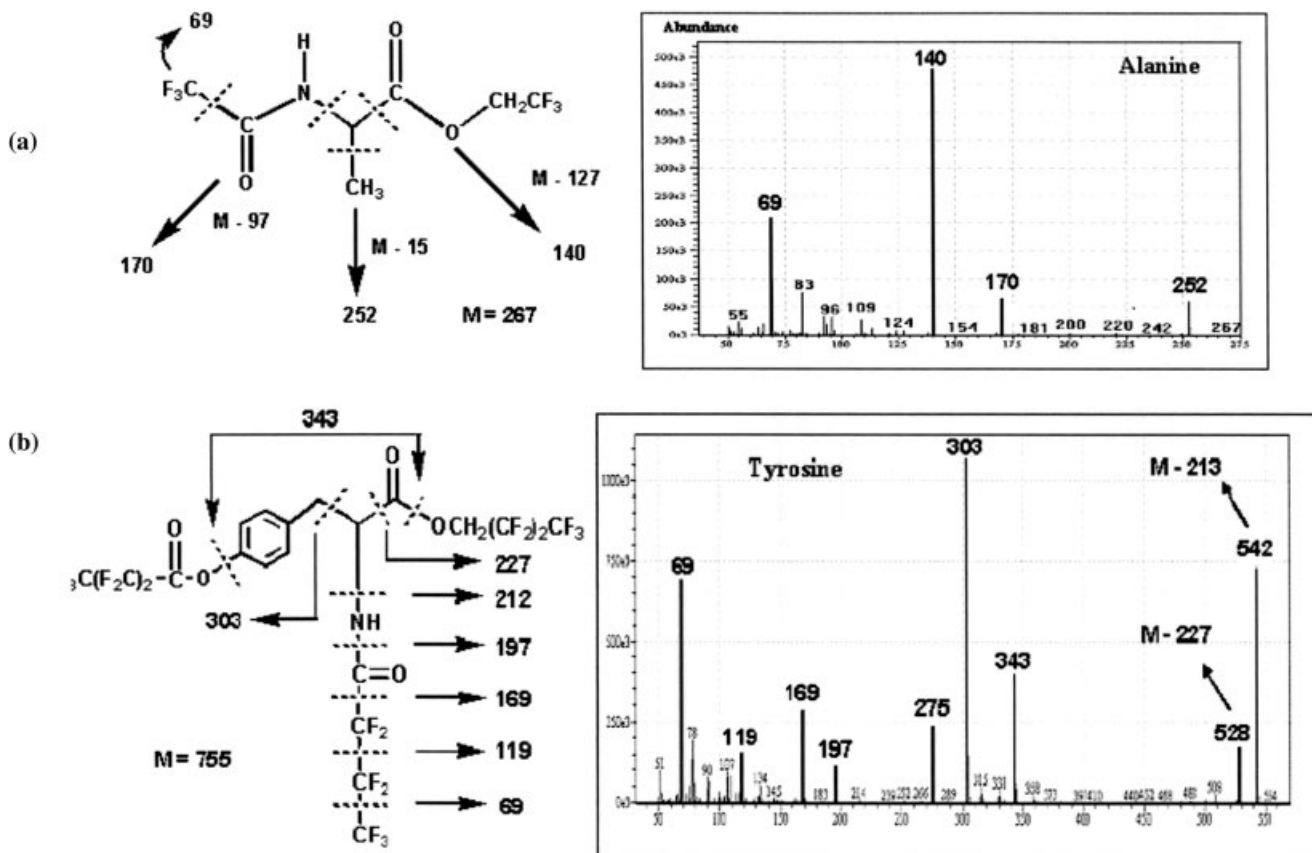


Fig. 2. (a) Mass spectrum of TFAA-TFE Ala, (b) mass spectrum of HFBA-HFB Tyr. Mass spectra were acquired in full scan mode in the range 50–800 amu.

### Derivatization Procedure

The present method used a one-step derivatization. The original two-step procedure was tested for comparison.

Standard amino acid stock solution, usually 48  $\mu\text{l}$  ( $2.4 \times 10^{-7}$  mol), was transferred into a 2-ml prescored ampoule and evaporated to dryness.

**One-step procedure.** The two derivatizing agents (50  $\mu\text{l}$  perfluoroalcohol and 100  $\mu\text{l}$  perfluoroanhydride) were added to the dry residue and the ampoule was sealed and kept at 100°C for 1 h without stirring. After being cooled to room temperature, the reagents were removed using a nitrogen stream.

**Two-step procedure.** For the esterification step, 50  $\mu\text{l}$  of a 2 N HCl-methanol were added to the dry residue, and the ampoule was sealed and kept at 100°C for 30 min without stirring. After being cooled to room temperature, the ampoule was opened, and the reagents were removed using a nitrogen stream. For the perfluoroacylation step, 100  $\mu\text{l}$  of heptafluorobutyric anhydride was added to the dry residue, the ampoule was sealed again and kept at 100°C for 30 min without stirring. After being cooled to room temperature, the ampoule was opened and the reagents were removed using a stream of nitrogen.

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In both procedures, the residues were dissolved in 100  $\mu\text{l}$  of ethyl acetate, and 20  $\mu\text{l}$  of internal standard (IS) solution was added; 1  $\mu\text{l}$  of this solution was injected into the GC-MS.

### Instrumentation

Analysis was performed using a Shimadzu QP5050 GC-MS instrument operating with a quadrupole detection mode with an electron positive ionization ( $\text{EI}^+$ ) source (ionization energy 70 eV). The instrument was autotuned using perfluorobutylamine and operated at 1.2 scans/s in full-scan mode from  $m/z$  50 to 800 amu. It was equipped with a split/splitless injector, a PTV injector and Chemstation software (LabSolutions, GC-MS solutions), all from Shimadzu.

The temperature of the split/splitless injector was 270°C, and the interface temperature was 180°C.

The liner was packed with deactivated glass wool purchased from Restek (France) to prolong column life.

Helium was used as a carrier gas at 1 ml/min and a split injection mode (split ratio 1:20) was used.

Separation was achieved using two different chiral WCOT fused silica capillary columns:

- Chirasil-L-Val (L-valine-*tert*-butylamide modified polydimethylsiloxane, 25 m  $\times$  0.25 mm I.D., Varian, The

**TABLE 3. The chromatographic behavior of the three types of amino acid derivatives on Chirasil-L-Val column: retention time values obtained under the same GC experimental conditions. GC-MS analysis\***

Amino acid	TFAA-TFE <i>t<sub>r</sub></i> (min)	TFAA-HFB <i>t<sub>r</sub></i> (min)	HFBA-HFB <i>t<sub>r</sub></i> (min)
DL-Ala	11.15	<i>13.27</i>	<i>13.99</i>
	11.61	<i>13.75</i>	<i>14.38</i>
DL-Val	11.87	<i>13.47</i>	<i>13.94</i>
	12.23	<i>13.82</i>	<i>14.20</i>
DL-Ile	14.25	15.62	15.97
	14.63	15.98	16.24
<i>DL-Pro</i>	<i>14.54</i>	<i>16.24</i>	<i>17.04</i>
<i>DL-Thr</i> (mono-acylated)	<b>No sep</b>	<i>No sep</i>	<i>No sep</i>
	<i>14.67</i>	<i>16.28</i>	<i>17.02</i>
Gly	<i>No sep</i>	<i>No sep</i>	<i>No sep</i>
DL-Leu	14.88	17.01	18.16
	16.73	18.16	18.38
<b>DL-Asp</b> (bis-esterified)	17.36	18.78	18.93
	<b>21.15</b>	<b>23.28</b>	<b>23.13</b>
DL-Met	<b>No sep</b>	<b>No sep</b>	<b>No sep</b>
	24.50	25.63	25.55
DL-Glu (bis-esterified)	24.91	26.02	25.88
	25.11	<i>27.09</i>	<i>26.86</i>
DL-Phe	25.46	<i>27.42</i>	<i>27.15</i>
	25.73	<i>26.70</i>	<i>26.79</i>
DL-Tyr	26.05	<i>27.00</i>	<i>27.06</i>
	32.17	32.67	33.33
DL-Gln (bis-acylated)	32.41	32.88	33.52
	34.85	34.90	35.20
DL-Lys	35.32	35.38	35.52
	37.12	37.57	36.83
DL-Trp	<b>No sep</b>	<b>No sep</b>	<b>No sep</b>
	39.97	39.91	39.03
	<b>No sep</b>	<b>No sep</b>	<b>No sep</b>

\*Conditions for GC-MS: Column, Chirasil-L-Val; programmed temperature range, 40–200°C, 4°C/min, followed by isothermal conditions; carrier gas, helium; flow rate, 1 ml/min; split ratio, 1:20. Enantiomeric pairs that could not be separated even after optimization of the temperature program are presented in boldface type. Derivatives for which the column showed low selectivity for each combination of reagents are shown in italic type.

Nederlands) with a 0.12- $\mu$ m film coating. The maximum operating temperature was 200°C.

- (ii) RT- $\gamma$ -DEXsa (2,3-di-acetoxy-6-*O*-*tert*-butyl dimethylsilyl  $\gamma$ -cyclodextrin doped into 14% cyanopropylphenyl/86% dimethyl polysiloxane, 30 m  $\times$  0.25 mm I.D., Restek, France) with a 0.25- $\mu$ m film coating with a maximum operating temperature of 230°C.

## RESULTS AND DISCUSSION

The amino acid derivatization reaction based on the one-step perfluoroacylation-perfluoroesterification is investigated (the reaction scheme is reported in Fig. 1). Among the different reaction conditions reported in the literature (Table 1, see Section 1), the procedure employed by MacFarlane et al. to derivatize Phe, Tyr, and DOPA,<sup>40</sup> or by Börjesson et al. for determining glyphosate and (aminomethyl)phosphonic acids in water and soil was chosen<sup>50</sup> (see Section 2.2).

The selection of the most useful anhydrides for the quantitative GC determination of amino acids is based on the published techniques concerning the two-step perfluoroacylation esterification procedure: these results indicate trifluoroacetic (TFAA) and heptafluorobutyric (HFBA) anhydrides.<sup>54,55</sup> The best results were obtained with HFBA, which is a strong acylating agent similar to TFAA, but it is more reactive and forms more stable derivatives than TFAA.<sup>56</sup> TFAA and HFBA were used in combination with the corresponding perfluorinated alcohols TFE (2,2,2-trifluoro-1-ethanol) and HFB (2,2,3,3,4,4,4-heptafluoro-1-butanol) to give three varieties of amino acid derivatives (Fig. 1). The perfluoro alcohols chosen, HFB in particular which has the highest boiling point, were volatile enough to permit their easy removal after the reaction is completed. The derivatives obtained are shown in Figure 1. Note that although the bis-derivatives are schematized in Figure 1, tris-derivatives were also obtained (but not in all case) when the functional groups in the R chain were esterified (in the case of dicarboxylic amino acids) or acylated.

The aim of this study was to find the reagents that yield the best amino acid derivatives on the basis of the following parameters: (1) the best enantiomeric resolution of the derivatives on the two selected chiral stationary phases, displaying different separation mechanisms, Chirasil-L-Val and Rt- $\gamma$ -Dexsa,<sup>52,53</sup> (2) the lowest times of analysis; (3) the highest MS detectability. Therefore, method validation was performed: precision (RSD), detection limits (LOD), and quantification limits (LOQ) were determined. Finally, the possibility of automating the sample treatment using a (PTV) injector was evaluated.

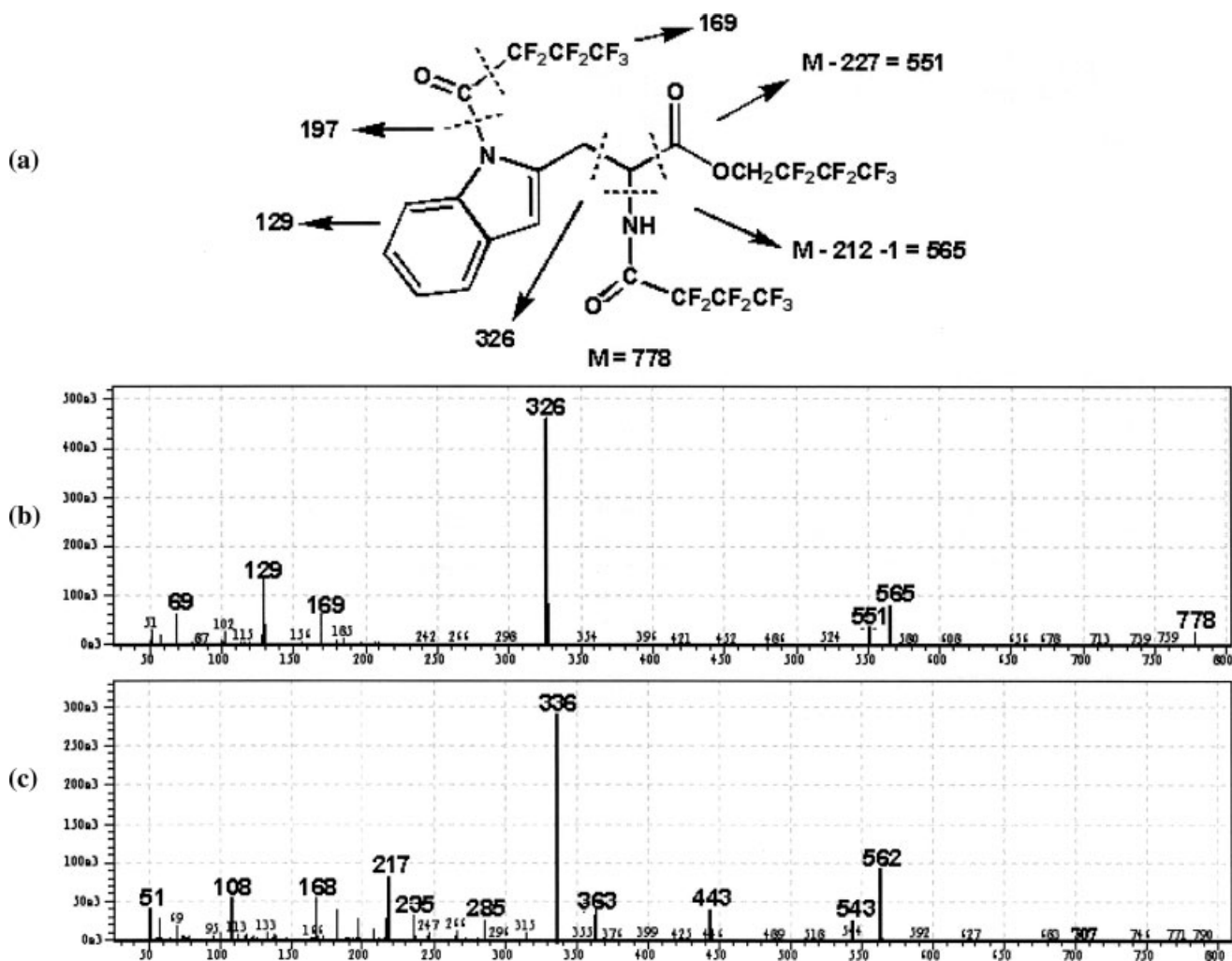
### Structural Information on the Derivatives

Twenty proteinogenic amino acids were derivatized using the reaction conditions indicated above. Mass spectrometric fragmentation for 18 analyzable derivatives are reported in Table 2. The mass spectra were interpreted on the basis of the few examples previously reported (references listed in Table 1). The *N*(*O,S*)-perfluoroacyl perfluoroalkyl derivatives show a characteristic fragmentation pattern: prominent peaks are present at *m/z* = 69, corresponding the ion  $\text{CF}_3^+$ , *m/z* = 119, 169, due to the

**TABLE 4. *R<sub>s</sub>* values for a series of separable amino acid enantiomers derivatives obtained using the three combinations of reagents on Chirasil-L-Val\***

Amino acid	TFAA-TFE <i>R<sub>s</sub></i>	TFAA-HFB <i>R<sub>s</sub></i>	HFBA-HFB <i>R<sub>s</sub></i>
DL-Ala	1.79	1.84	1.71
DL-Val	1.49	2.26	1.20
DL-Ile	1.56	1.93	1.44
DL-Leu	3.22	3.97	4.13
DL-Met	2.25	2.93	2.31
DL-Glu	3.43	2.31	2.07
DL-Phe	2.23	2.27	2.05
DL-Tyr	1.61	1.60	1.46

\*Chromatographic conditions were the same specified in Table 3.



**Fig. 3.** (a) Proposed fragmentation mechanism of bis-acylated HFBA-HFB Trp. (b) Mass spectrum of bis-acylated HFBA-HFB Trp. (c) Mass spectrum of HFBA-HFB Trp by-product.

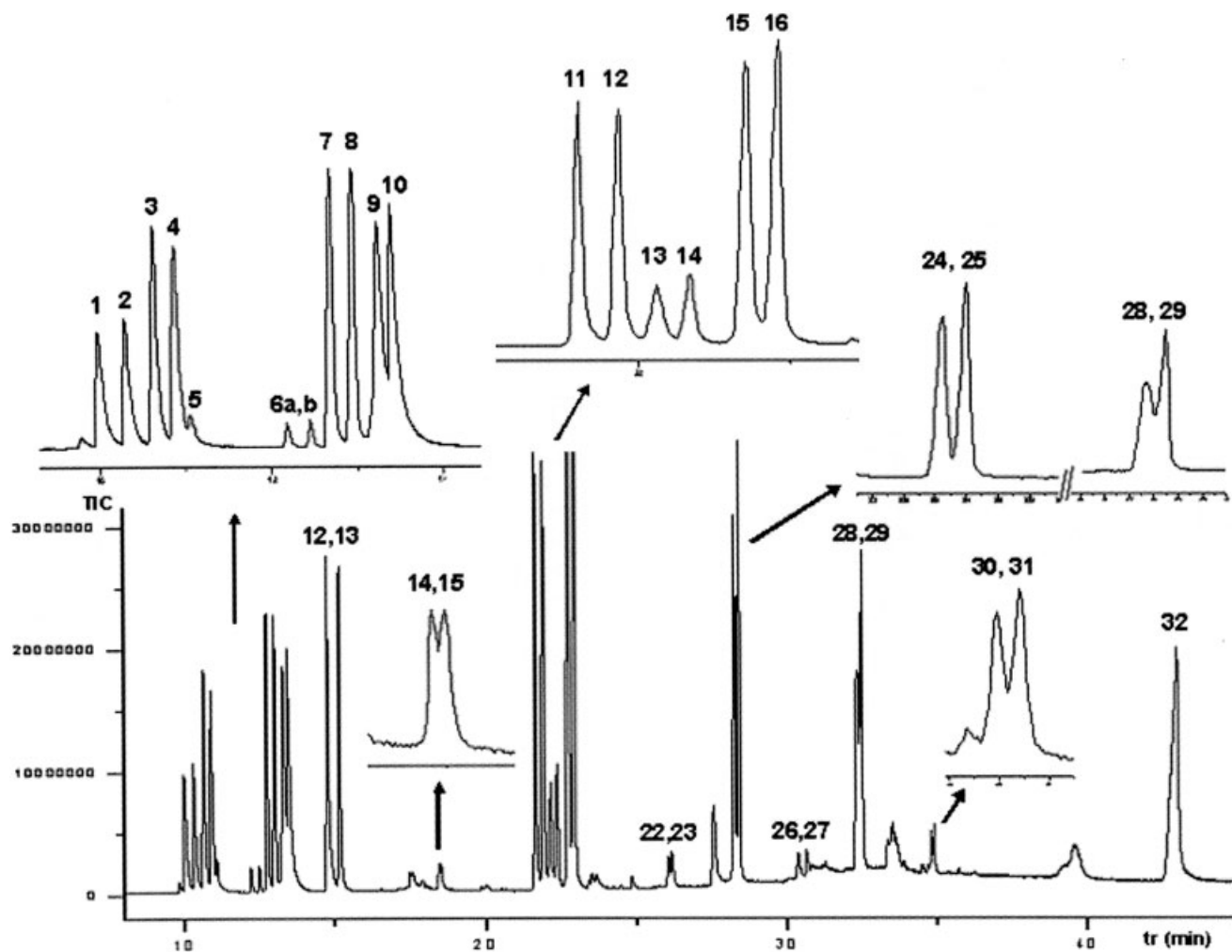
increments of  $\text{CF}_2$ . The other important ions were mainly due to the cleavage of the C—C bond next to C=O with the consequent loss of the ion  $[\text{OCOCH}_2(\text{CF}_2)_n\text{CF}_3]^+$  ( $n = 0$  or 2) and the formation of easily recognizable peaks at  $m/z = M-127$  (for the TFAA-TFE and TFAA-HFB derivatives) and  $m/z = M-227$  for the HFBA-HFB derivatives. Moreover, the mass spectra show the fragments  $[\text{CF}_3(\text{CF}_2)_n\text{CO}]^+$  (97 or 197 amu),  $\text{CF}_3(\text{CF}_2)_n\text{CONH}_2$  (113 or 213), and the corresponding ions resulting from their loss from the molecular ions. The mass spectra of TFAA-TFE derivatives produce identical ion patterns but each fragment shows a 100 amu shift toward lower mass values. The mechanism proposed for the fragmentation of the derivatives is reported in Figure 2.

#### Separation on Chirasil-L-Val

On a Chirasil-L-Val stationary phase, 15 of the 20 proteinogenic amino acids were detected and separated after derivatization using the above specified reaction conditions. The chromatographic behaviour of the three types of Chirality DOI 10.1002/chir

derivatives were compared under the same GC experimental conditions: it was possible to separate 9 enantiomeric pairs giving almost identical retention times (Table 3). In comparison to the TFAA-TFE derivatives, the introduction of two  $\text{CF}_2$  groups (100 amu) using the TFAA-HFB procedure or of four  $\text{CF}_2$  groups (200 amu) in the case of bis-trifluoroacetyl heptafluorobutyl esters, and of four or six  $\text{CF}_2$  groups (300 amu) using the HFBA-HFB method, produced an increment of molecular weight but did not significantly increase the retention time. The analysis of the heaviest amino acid derivatives (Tyr, Gln, Lys, Trp) was possible at a temperature lower than column bleeding limit ( $200^\circ\text{C}$ ). The separation of the TFAA-HFB derivatives shows the greatest problems: coelution of Ala-Val, Pro-Thr, and Glu-Phe enantiomer pairs, while the TFAA-TFE derivatives display the best selectivity on Chirasil-L-Val column.

Comparing the resolution of the three types of derivatives, no substantial differences were observed. In general the HFBA-HFB derivatives displayed the lowest values. The resolution values were equal to or higher than 1.2 when separation occurred (see Table 4). These



**Fig. 4.** Gas chromatogram of TFAA-TFE derivatives of an amino acids mixture prepared from the D and L forms: column, Chirasil-L-Val; temperature program range: 40–200°C, 4.5°C/min, followed by isothermal conditions; carrier gas, He; flow rate, 1 ml/min; split ratio, 20:1. *R<sub>s</sub>* values are reported in parentheses for peak numbers as follows: [1] D-Val (2.211), [2] D-Ala (1.816), [3] L-Val, [4] L-Ala, [5] allo-Thr, [6a,b] allo-Ile, [7] D-Ile (2.032), [8] L-Ile, [9] DL-Pro + DL-Thr, [10] Gly, [11] D-Leu (3.128), [12] L-Leu, [13] D-Asp, [14] L-Asp, [15] D-Met (2.479), [16] L-Met, [18] D-Glu (1.323), [19] L-Glu, [20] D-Phe (1.648), [21] L-Phe, [22] D-Gln (by-pr.), [23] L-Gln (by-pr.), [24] D-Tyr (1.162), [25] L-Tyr, [26] D-Gln (diacylated, 1.230), [27] L-Gln (diacylated), [28] D-Lys (0.696), [29] L-Lys, [30] D-Trp (diacylated, 0798), [31] L-Trp (bis-cylated), [32] Trp (by-product).

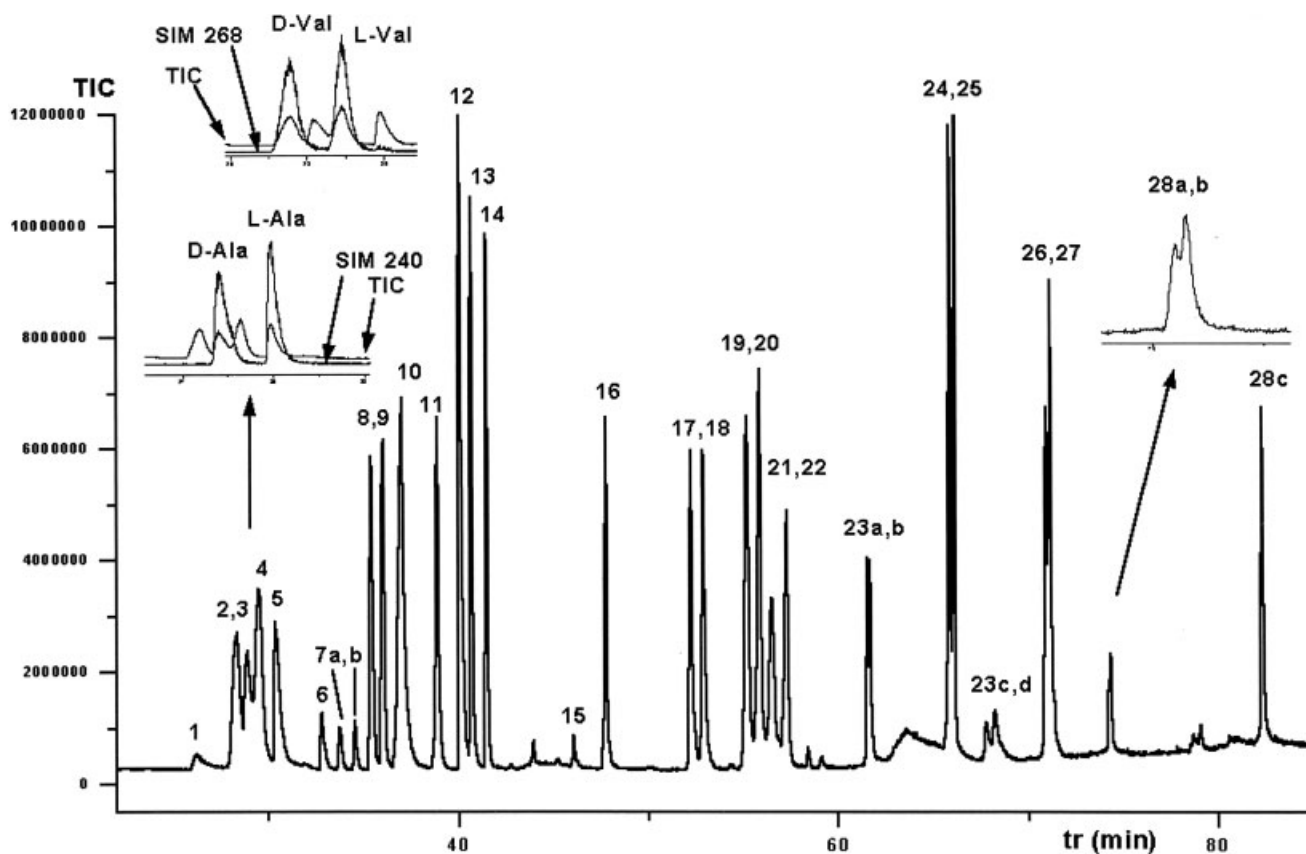
results show that the interaction mechanism with the Chirasil-L-Val stationary phase is almost identical, as it is to be expected for this type of chiral stationary phase. Detection of Gln and Asn was problematic because the amide group is acid-labile and thus was completely or partially destroyed under the acidic conditions of derivatization: in fact, the reaction produces heptafluorobutyric acid that yields glutamic acid and aspartic acid, respectively.<sup>45,56</sup> In the case of Gln also pyroglutamic acid and some by-products were obtained. Asn did not give a detectable signal. Trp yield two peaks corresponding to a mixture of the *N,N*-bis-acylated derivative and a byproduct which seem to be a degradation product.<sup>57,58</sup> The mass spectra of the two derivatives are shown in Figure 3: the by-product mass spectrum is characterized by a base peak at  $m/z = 336$  which shows a 10-amu difference compared to the base peak of the bis-acylated derivative. The by-product was the major product, as it can be seen in the chromatograms reported in Figures 4 and 5: it was

easily identified in TIC or SIM analysis mode, and therefore it can be used to detect and quantitatively determine Trp. The mechanism proposed for the fragmentation of bisacylated HFBA-HFB Trp is presented in Fig. 3a). It was impossible to identify specific peaks for His, Ser, Cys, and Arg.

A mixture of 15 amino acids was prepared, and the corresponding derivatives were analyzed on Chirasil-L-Val. The chromatograms of the TFAA-TFE derivatives resulted more interesting from a qualitative point of view and those of HFBA-HFB derivatives were the most interesting from a quantitative point of view, as it is reported in Section 3.4. After a number of trials, the best separation results were obtained using the chromatographic conditions specified in Figures 4 and 5.

The column showed better selectivity in the case of TFAA-TFE derivatives even if it was not possible to separate DL-Thr from DL-Pro. It was possible to elute all the analytes in about 44 minutes with a linear temperature





**Fig. 5.** Gas chromatogram of HFBA-HFB derivatives of an amino acid mixture prepared from the D and L forms: column, Chirasil-L-Val; temperature program range, 60°C (27 min) to 120°C (10 min) at 3°C/min, to 155°C (5 min) at 10°C/min, to 200°C at 3°C/min, followed isothermal conditions; carrier gas, He; flow rate, 1 ml/min; split ratio, 20:1. *R<sub>s</sub>* values are reported in parentheses as follows: [1] Cys (by-prod.), [2] D-Val (1.730), [3] D-Ala (3.366), [4] L-Val, [5] L-Ala, [6] allo-Thr, [7a,b] allo-Ile, [8] D-Ile (1.990), [9] L-Ile, [10] DL-Pro, [11] DL-Thr, [12] Gly, [13] D-Leu (3.908), [14] L-Leu, [15] Pyr, [16] DL-Asp, [17] D-Met (1.217), [18] L-Met, [19] D-Phe (1.997), [20] L-Phe, [21] D-Glu (1.774), [22] L-Glu, [23a] D-Gln (mono-acylated), [23b] D-Gln (mono-acylated), [24] D-Tyr (1.261), [25] L-Tyr, [23c] D-Gln (bis-acylated, 0.893), [23d] L-Gln (bis-acylated), [28] D-Lys (0.867), [29] L-Lys, [28a] D-Trp, [28] L-Trp, [28c] Trp (by-product).

program. Under these conditions, 11 enantiomeric pairs could be separated with satisfactory *R<sub>s</sub>* values, with the exception of DL-Lys and DL-bis-acylated Trp which were only slightly separated. The *R<sub>s</sub>* values are reported in brackets in Figure 4.

The separation of the HFBA-HFB derivatives, which exhibited the greatest mass spectrometric response, was also studied. Separation, chromatographic conditions, and *R<sub>s</sub>* values are reported in Figure 5.

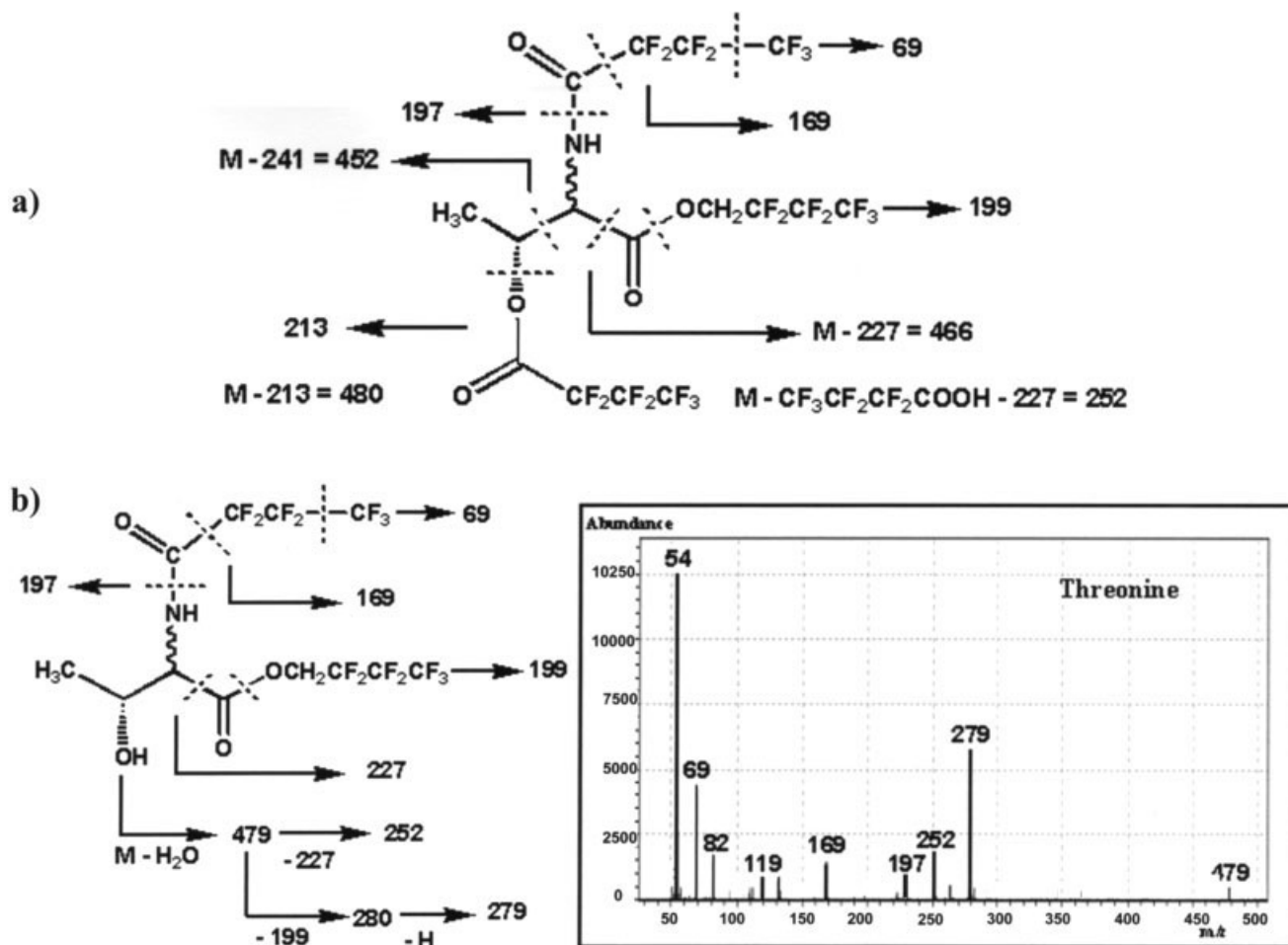
It will be noticed that, under the same flow conditions (1 ml/min), the separation of the same mixture was achieved with more difficulty compared to the TFAA-TFE combination: a long isotherm (27 min) was required to separate Ala enantiomers from Val enantiomers in the TIC detection mode, and a three-stage temperature program was necessary to obtain the other separations. Complete elution was achieved in about 83 min. In this case the problem of the overlap between DL-Ala and DL-Val can be resolved using SIM (selected ion monitoring) analysis, by selecting the *m/z* = 240 and *m/z* = 268 values (see Section 3.1) for Ala and Val, respectively. In conclusion, we were able to elute 15 amino acids derivatives and to separate 10 enantiomeric pairs, 8 of which had *R<sub>s</sub>* values

higher than 1.2. Low resolutions were obtained for DL-Lys, DL-Gln, and DL-diacylated Trp for which the *R<sub>s</sub>* was not measurable.

Using the Chirasil-L-Val column, it was impossible to separate Thr, Pro, and Asp enantiomers for the three kinds of derivatives considered. Thr was detected only in mono-acetylated form (OH-free) as can be seen in Figure 6 showing the proposed fragmentation mechanism.

Figures 4 and 5 show that, for all the studied amino acids, the D-derivatives are eluted faster than the corresponding L-derivatives.

**Separation on a RT- $\gamma$ -DEXsa.** Separation on a RT- $\gamma$ -DEXsa column was studied because it is based on a chiral recognition mechanism different from that of Chirasil-L-Val.<sup>59,60</sup> In the case of modified cyclodextrins, the retention is explained by a host-guest interaction due to the formation of inclusion complexes together with external multiple associations between the substituting groups protecting the hydroxyls of the D-glucose units. The mechanism of chiral recognition of Chirasil-L-Val could be attributed to the hydrogen bonding formed between each enantiomer and the chiral selector: such interactions pre-



**Fig. 6.** (a) Proposed fragmentation mechanism of bis-perfluoroacylated Thr as its HFBA-HFB derivative and its mass spectrum. (b) Proposed fragmentation mechanism of mono-perfluoroacylated Thr as its HFBA-HFB derivative and its mass spectrum.

suppose the presence of a reversible diastereoisomeric association mechanism. It has been demonstrated that the enantiomeric resolution can be affected by the dimensions of the cyclodextrin and of the chiral selectand: the separation factors decrease by reducing the cavity ring of the cyclodextrin or increasing the size of amino acid derivative molecules.<sup>61,62</sup> For these reasons, a modified  $\gamma$ -cyclodextrin was chosen in this study, because it seems to be the most suitable to separate the largest HFBA-HFB derivatives.

RT- $\gamma$ -DEXsa showed a complementary behavior compared to Chirasil-L-Val: on this column 18 of the 20 proteinogenic amino acids tested could be separated, including additional amino acids such as Ser and Cys in bis-acylated form and His in mono-acylated form (see Section 3.1). Moreover, it was possible to detect Thr also in bis-acylated form as a minority product (very low percentage) rather than its mono-acylated form, which could be separated into its enantiomeric pairs. The chromatographic behavior of the three types of derivatives was compared in the same GC experimental conditions, indicated in Table 5. Different to Chirasil-L-Val, on RT- $\gamma$ -DEXsa the HFBA-HFB derivatives exhibited retention times lower than those of the cor-

responding TFAA-TFE and TFAA-HFB derivatives. This effect was more evident for amino acid derivatives eluted in isothermal conditions at the maximum operating temperature of the column (230°C), such as Lys, Trp, His, and Gln.

Table 6 shows that the  $R_s$  values were highly variable and that the best separations were achieved for the smallest TFAA-TFE derivatives. In fact, in fixed GC chromatographic conditions, it was possible to separate 14 TFAA-TFE, 12 TFAA-HFB and 13 HFBA-HFB enantiomeric pairs. In particular, the satisfactory  $R_s$  values obtained for Pro enantiomers must be noted which were not separated on Chirasil-L-Val, but separated as TFAA-TFE and HFBA-HFB derivatives on RT- $\gamma$ -DEXsa. Moreover, Asp enantiomer derivatives, not separated using Chirasil-L-Val, were separated for all the three combinations of reagents. Lys enantiomers showed good  $R_s$  values as TFAA-TFE and TFAA-HFB derivatives, differently from Chirasil-L-Val, which displays only low separation for the three derivatives. Ser and Cys enantiomers, not detected with Chirasil-L-Val, were resolved for all the three derivative forms. From a qualitative point of view, the better results were obtained with the TFAA-TFE derivatives since they show the highest  $R_s$  values between the enantiomers, the

**TABLE 5. Chromatographic behavior of the three types of amino acid derivatives on RT- $\gamma$ -DEXsa column: retention time values obtained under the same chromatographic conditions. GC-MS analysis\***

Amino acid	TFAA-TFE $t_r$ (min)	TFAA-HFB $t_r$ (min)	HFBA-HFB $t_r$ (min)
DL-Val	8.592	8.092	7.125
	8.742	8.267	7.183
DL-Ile	9.542	9.200	8.150
	9.667	9.283	8.233
<b>DL-Thr (OH-free)</b>	<b>10.175</b>	<b>9.842</b>	<b>9.225</b>
	<b>No sep</b>	<b>No sep</b>	<b>No sep</b>
DL-Leu	10.383	<i>10.342</i>	<i>9.242</i>
	10.883	<i>No sep</i>	<i>9.333</i>
DL-Ala	10.700	<i>10.350</i>	8.658
	11.1192	<i>No sep</i>	8.775
DL-Pro	13.333	<b>12.558</b>	11.283
	13.667	<b>No sep</b>	11.417
DL-Thr <sup>a</sup> (bis-acylated)	14.783	13.300	8.943
	15.033	14.142	9.633
Gly	15.442	14.608	13.275
DL-Asp (bis-esterified)	17.033	15.208	14.017
	17.742	16.067	14.208
DL-Ser <sup>a</sup>	17.325	16.025	12.992
	19.300	18.067	13.192
DL-Cys <sup>a</sup>	19.650	18.525	16.200
	20.217	18.658	16.283
DL-Met	<i>20.050</i>	19.383	<i>17.725</i>
	<i>20.475</i>	19.533	<i>17.975</i>
DL-Phe	<i>20.575</i>	19.900	18.825
	<i>20.650</i>	20.183	18.908
DL-Glu (bis-esterified)	20.158	19.117	<i>17.808</i>
	21.133	19.783	<i>17.983</i>
DL-Tyr	27.033	26.342	<b>24.558</b>
	27.183	25.917	<b>No sep</b>
DL-Lys	36.733	35.008	28.850
	36.983	35.417	28.925
DL-Trp (bis-acylated)	36.983	34.642	30.775
	<b>No sep</b>	<b>No sep</b>	<b>No sep</b>
<b>DL-His<sup>a</sup></b>	<b>40.925</b>	<b>38.975</b>	<b>36.192</b>
	<b>No sep</b>	<b>No sep</b>	<b>No sep</b>
DL-Gln (bis-acylated)	52.892	52.583	43.192
	58.217	57.800	44.177

\*GC conditions: Column, RT- $\gamma$ -DEXsa; temperature program range, 120–230°C, 4°C/min, followed by isothermal conditions; carrier gas, helium; flow rate, 1 ml/min; split ratio: 1:20. Enantiomeric pairs that could not be separated even after adjustment of the temperature program are reported in boldface type. Derivatives for which the column showed low selectivity for each combination of reagents are underlined in italic type.

<sup>a</sup>Amino acid derivatives not detected using Chirasil-L-Val.

only coelution pairs are DL-Met and DL-Glu. The Leu, Ala, and Pro enantiomers can be separated using each combination of reagents, with the exception of the TFAA-HFB combination. Also the DL-Leu and DL-Ala pairs coeluted. Using the HFBA-HFB combination, Tyr enantiomers were not separated while Val and Lys enantiomers displayed lower  $R_s$  in comparison with the other derivatization reagents. The DL-Leu and DL-Thr, and DL-Glu and DL-Met pairs show some coelution problems.

A mixture of the 18 amino acids reported above was prepared and the corresponding derivatives were ana-

lyzed on RT- $\gamma$ -DEXsa. The chromatograms of the TFAA-TFE and the HFBA-HFB derivatives are given in Figure 7 and Figures 8 and 9, respectively. In fact, the TFAA-TFE combination is preferable for qualitative analysis, while the HFBA-HFB reagents give the better results for quantitative analysis, also on RT- $\gamma$ -DEXsa, as observed on Chirasil-L-Val (see Section 3.4). As can be seen in Figure 7, detection of some TFAA-TFE amino acid derivatives is difficult because they show a low MS signal: Ser, Cys, His, Gln, and Asp yield a signal comparable to the level of background noise in the range of derivatized quantities studied, between  $2.4 \times 10^{-7}$  and  $9.6 \times 10^{-7}$  mol. Analogous results were obtained for the TFAA-HFB derivatives, while, at the same derivatized concentrations, HFBA-HFB derivatives showed a chromatographic signal significantly higher than the background noise.

After many trials, the best separation results were obtained using the chromatographic conditions specified in Figures 7–9. The column showed better selectivity toward the TFAA-TFE derivatives, even if it was impossible to completely separate L-Ser from D-Glu and L-Met from L-Phe. All the analytes, including the Trp by-product, could be eluted in 67 min using nearly linear temperature programming conditions. In these conditions we were able to separate 14 enantiomeric pairs with satisfactory  $R_s$  values (reported in brackets in Fig. 4), DL-Phe being the exception.

Figures 8 and 9 display the first and second parts of the chromatogram of the HFBA-HFB derivatives.

Under the same flow conditions (1 ml/min), the separation of the same mixture was achieved in about 75 min, including the Trp by-product, employing a three-stage programmed temperature range. Difficulties in separating DL-Thr from L-Leu and L-Ser from Gly in TIC mode can be resolved in SIM mode by selecting the specific detection

**TABLE 6.  $R_s$  values for a series of separable amino acid enantiomers derivatives obtained using the three combinations of reagents on Chirasil-L-Val\***

Amino acid	TFAA-TFE $R_s$	TFAA-HFB $R_s$	HFBA-HFB $R_s$
DL-Ala	4.94	No sep	1.26
DL-Val	1.75	1.82	NM
DL-Leu	5.65	No sep	0.95
DL-Pro	3.68	No sep	2.25
DL-Ile	0.81	0.71	0.92
DL-Met	5.18	1.86	2.93
DL-Glu	11.26	7.51	2.20
DL-Phe	0.84	3.43	0.92
DL-Asp	7.66	9.17	2.80
DL-Tyr	1.86	5.31	No sep
DL-Lys	1.25	2.21	NM
DL-Cys	6.01	2.25	0.98
DL-Ser	21.05	19.63	1.87
DL-Gln	10.31	9.86	2.51

\*Chromatographic conditions were the same as in Table 5. NM, not measurable.

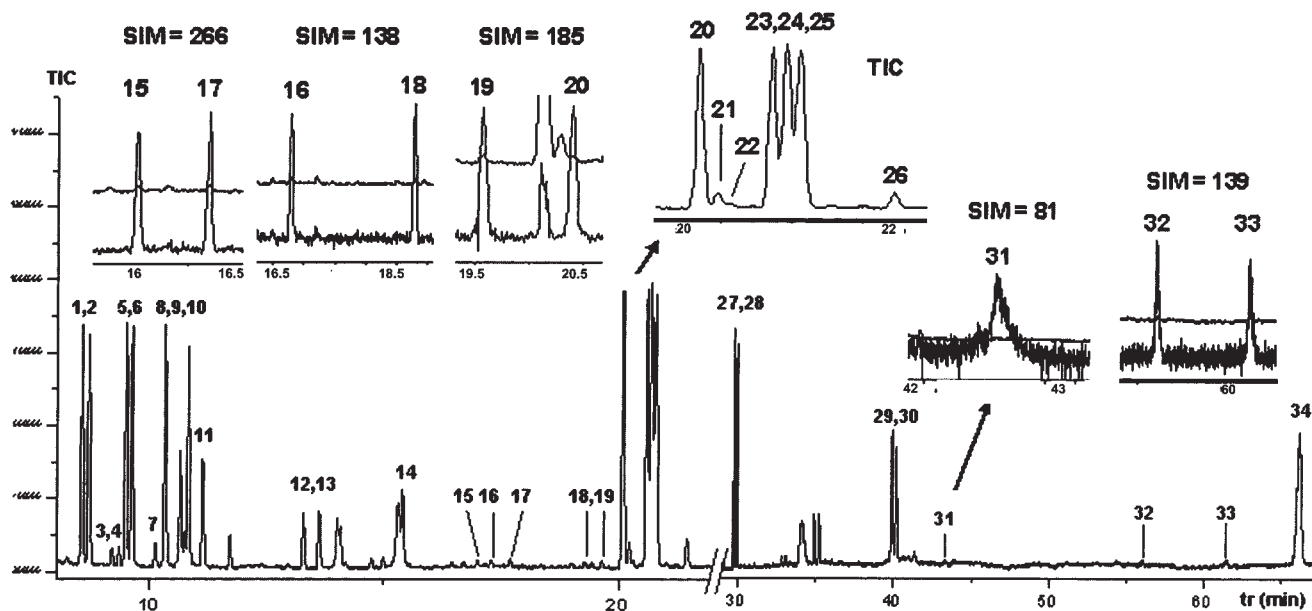


Fig. 7. Gas chromatogram of TFAA-TFE derivatives of an amino acid mixture prepared from the D and L forms: column, Rt- $\gamma$ -DEXsa; temperature program range, 120–195°C (5 min), 4°C/min, to 230°C at 4°C/min, and then isothermal conditions; carrier gas, He; flow rate, 1 ml/min; split ratio, 20:1; *R<sub>s</sub>* values are reported in parentheses for peak numbers as follows: [1] D-Val (1.667), [2] L-Val, [3,4] DL-allo-Ile, [5] D-Ile (1.472), [6] L-Ile, [7] DL-Thr (OH free), [8] D-Leu (5.319), [9] D-Ala (5.281), [10] L-Leu, [11] L-Ala, [12] D-Pro (3.600), [13] L-Pro, [14] Gly, [15] D-Asp (8.364), [16] D-Ser (21.419), [17] L-Asp, [18] L-Ser, [19] D-Cys (7.250), [20] D-Met (5.276), [21] D-Glu (11.189), [22] L-Cys, [23] D-Met, [24] L-Phe (0.781), [25] D-Phe, [26] L-Glu, [27] L-Tyr (2.064), [28] D-Tyr, [29] L-Lys (1.447), [30] D-Lys, [31] DL-His, [32] D-Gln (10.449), [33] L-Gln, [34] Trp (by-product).

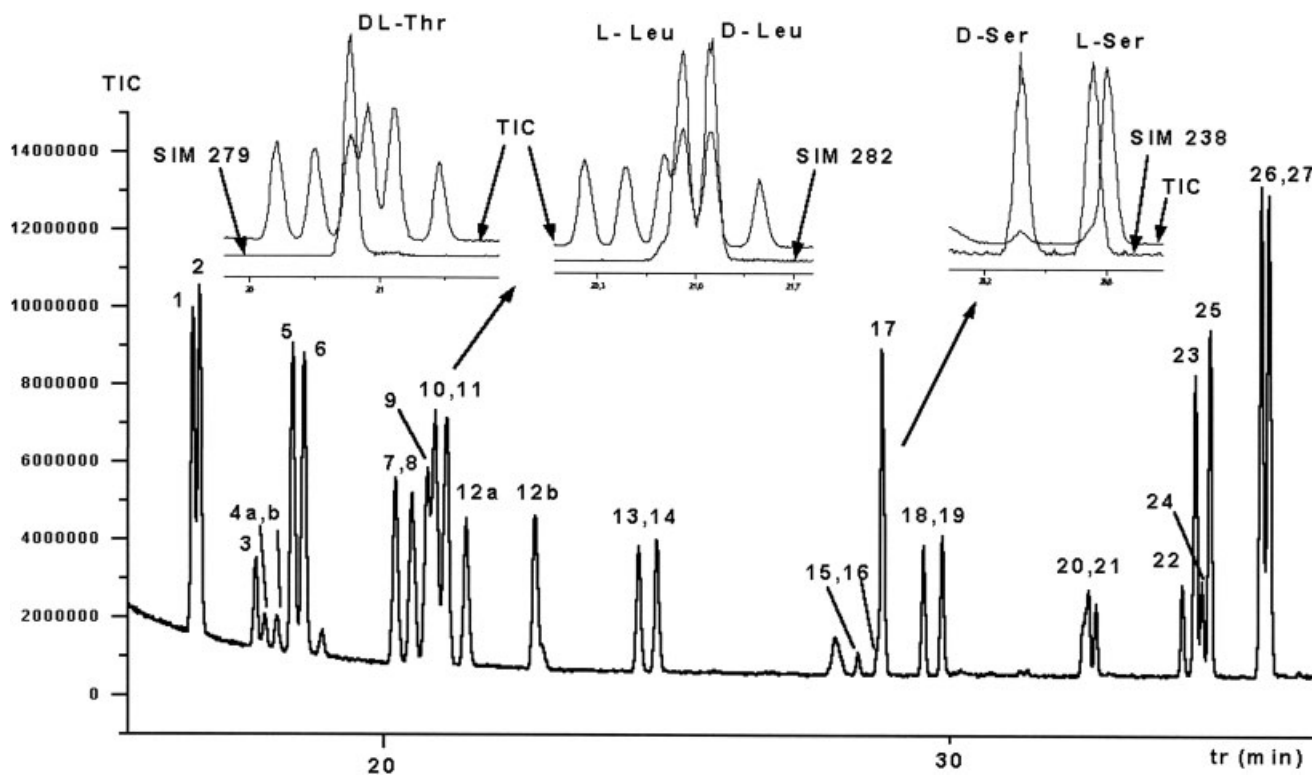
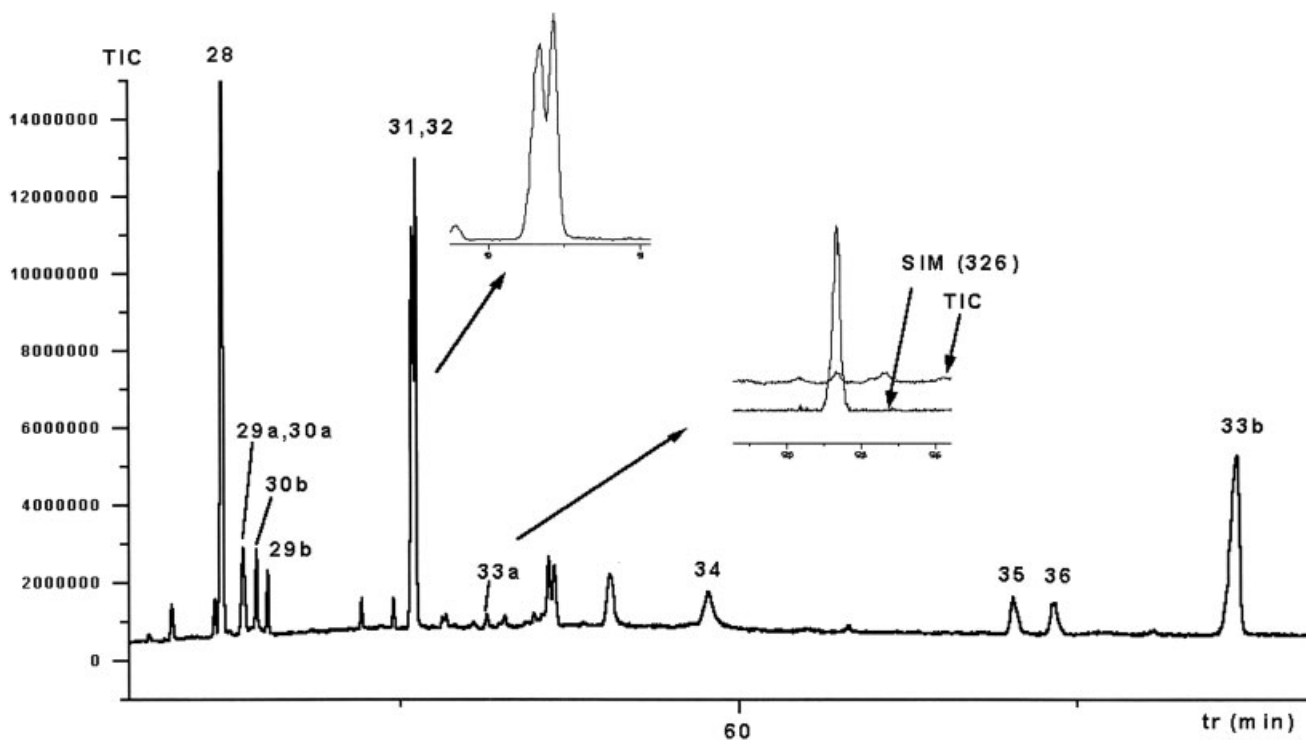


Fig. 8. First part (from 12 to 36 min) of gas chromatogram of HFBA-HFB derivatives of an amino acid mixture prepared from the D and L forms: column, Rt- $\gamma$ -DEXsa; temperature program range, 80–140°C (5 min) at 4°C/min, to 190°C (5 min) at 4°C/min, to 230°C at 4°C/min, and then isothermal conditions; carrier gas, He; flow rate, 1 ml/min; split ratio, 20:1. *R<sub>s</sub>* values for each peak given in parentheses: [1] L-Val (0.805), [2] D-Val, [3] DL-allo-Thr, [4a,b] DL-allo-Ile, [5] L-Ile (1.282), [6] D-Ile, [7] L-Ala (5.280), [8] D-Ala, [9] DL-Thr (OH-free), [10] L-Leu (1.000), [11] D-Leu, [12a,b] DL-Cys (by-product), [13] D-Pro (2.186), [14] L-Pro, [15] D-Ser (2.934), [16] L-Ser, [17] Gly, [18] D-Asp (2.718), [19] L-Asp, [20] D-Cys (1.202), [21] L-Cys (free SH), [22] L-Met (3.153), [23] D-Met, [24] D-Glu (2.177), [25] L-Glu, [26] L-Phe (1.064), [27] D-Phe.



**Fig. 9.** Second part (from 40 to 76 min) of gas chromatogram of HFBA-HFB derivatives of an amino acid mixture prepared from the D and L forms (see Fig. 8).  $R_s$  values are given in parentheses for each peak number: [28] DL-Tyr, [29a,b] Gln (by-prod.), [30a,b] DL-Pyr, [31] L-Lys (0.602), [32] D-Lys, [33a] DL-Trp, [33b] DL-Trp (by-product), [34] DL-His, [35] D-Gln (3.066), [36] L-Gln.

values  $m/z = 279$  for Thr,  $m/z = 282$  for Leu, and  $m/z = 238$  for Ser. In conclusion, it was possible to separate 13 enantiomeric pairs, 9 of which had acceptable  $R_s$  values (higher than 1.2). On this column, DL-Lys showed good  $R_s$  values only as TFAA-TFE and TFAA-HFB derivatives, while DL-Val, DL-Leu, DL-Phe were better separated on Chirasil-L-Val stationary phase.

### Quantitative Analysis

#### Relative response, linearity, and detection limits.

The sensitivity of the method was investigated for the three derivatives, by measuring on the TIC chromatogram the ratio of the peak areas of the considered compound relative to the internal standard, i.e., methyl laurate. The starting amount of amino acid,  $2.4 \times 10^{-7}$  mol, was the same in all the cases, chosen to allow the comparison of the three derivatization methods, from the least to the most quantitative. The results (Table 7) show that the MS response increased with the size of the protecting groups. The best response was obtained with the HFBA-HFB derivatives which also presented the lowest values of relative standard deviation, ranged from 3% to 11%. If attention is focused on the quantitative analysis, the HFBA-HFB combination seems to be the best choice to quantify amino acid enantiomers using both the Chirasil-L-Val and RT- $\gamma$ -DEXsa columns.

Therefore, the HFBA-HFB combination was selected for quantitative studies: the linearity of the method was

investigated, LOD and LOQ were determined on the RT- $\gamma$ -DEXsa column.

The linearity of the method was investigated for a series of amino acids: five different increasing amounts of L-amino acid (the concentration range is reported in Table 8) were submitted to the derivatization procedure and MS analysis. The calibration lines were obtained by plotting the peak height, obtained in TIC mode, as a function of the quantity injected. Each point of the curve represents the average value of three replicates. The equation of the calibration lines were computed by the least-squares method: the regression parameters are reported in Table 8. The good linearity of the calibration lines is proved by the squared correlation coefficient values close to 1 ( $>0.99$ , see Table 8).

The detection limit  $X_{LOD}$  was determined as the analyte concentration yielding a signal value  $Y_{LOD} = \bar{y}_b + 6\sigma_b$ , where  $\bar{y}_b$  is the blank average signal of 10 blank responses and  $\sigma_b$  its standard deviation.<sup>63</sup> This  $X_{LOD}$  value corresponds to a same risk of 0.13% that the blank signal may be misinterpreted as well as the compound may be lost. The quantification limit  $X_{LOQ}$  was determined as the analyte concentration corresponding to a signal value  $Y_{LOQ} = \bar{y}_b + 20\sigma_b$ , to assure a satisfactory measurement precision (RSD = 5%), for the quantitative determination.<sup>63</sup> Consequently, the detection limits and the quantification limits reported in Table 9 were obtained as  $X_{LOD} = 6\sigma_b/S$  and  $X_{LOQ} = 20\sigma_b/S$ , where  $S$  is the sensitivity of the analytical method corresponding to the slope of the calibration line. In order to verify

**TABLE 7. Relative GC-MS responses of the amino acid derivatives relative to an internal standard\***

Amino acid	TFAA-TFE			TFAA-HFB			HFBA-HFB		
	$A/A_{IS}^a$	SD	RSD	$A/A_{IS}^a$	SD	RSD	$A/A_{IS}^a$	SD	RSD
Gly	1.01	0.019	4.4	1.29	0.14	11	1.78	0.21	11
DL-Ala	0.40	0.16	39	1.33	0.13	10	1.36	0.074	5.4
DL-Val	0.91	0.13	14	1.55	0.071	4.6	2.08	0.081	3.9
DL-Ile	1.92	0.25	13	3.80	0.22	5.9	5.06	0.41	8.0
DL-Pro	1.21	0.087	7.2	2.01	0.17	8.6	4.33	0.15	3.4
DL-Leu	1.34	0.35	20	2.43	0.044	1.8	4.37	0.37	8.5
DL-Met	3.09	0.33	10	3.76	0.35	9.2	5.79	0.17	3.0
DL-Glu	1.14	0.16	14	1.00	0.074	7.4	3.53	0.28	8.05
DL-Phe	3.56	0.15	4.2	5.20	0.37	7.1	7.61	0.42	5.6
DL-Tyr	1.67	0.33	20	1.88	0.28	15	4.12	0.29	7.0
DL-Trp (bis-acylated)	0.48	0.045	9.3	0.65	0.021	3.2	1.00	0.17	16
DL-Asp <sup>b</sup>	0.37	0.080	21	0.15	0.043	28	1.92	0.010	5.2

\*Abbreviations:  $A/A_{IS}$ , peak area ratio of a series of amino acids (D+L form) relative to the internal standard; IS, internal standard, methyl dodecanoate (methyl laurate); SD, intra-day standard deviation; RSD, intra-day relative standard deviation.

<sup>a</sup>Mean value of 5 replicated samples analyzed in the same day.

<sup>b</sup>Values determined on RT- $\gamma$ -DEXsa. DL-Asp coeluted with the IS on Chirasil-L-Val. The amount of each enantiomer which reached the detector was about 100 pmol, and that of the IS was about 40 pmol.

the condition of the intercept values statistically equal to zero, the 95% two-sided confidence interval was calculated for each intercept value ( $b_0$ ), and the  $t$ -test was applied at 5% significance. This condition was verified for the most of the cases: the only exceptions were Ala and Pro for which the 95% two-sided confidence interval for the intercept does not include zero, revealing the presence of low systematic errors in the procedure. The values obtained are reported in Table 9: the low detection and quantification limit (at the sub-pmol level) show that the method is compatible with its application to space sample analysis. Moreover, some measurements

were also performed to experimentally determine the real minimum detectable amino acid quantity using the procedure (experimental LOD, 5th column in Table 9). Standard mixtures containing decreasing amounts of amino acid were derivatized and analyzed to obtain a signal-to-noise ratio at least 3 to 1 (values ranging from 3 to 7 were accepted). The obtained results (5th column in Table 9) show experimental LOD at the pmol level, with the exception of L-Ser, L-Cys, L-His, and L-Gln which gave the lowest yields. For these amino acids the experimental LOD were of the order of  $10 \times 10^{-10}$  moles (150, 100, 200, 100 pmol, respectively, corresponding to 360,

**TABLE 8. Calibration lines for a series of HFBA-HFB amino acid derivatives in the L form**

Amino acid	Starting amount <sup>a</sup> (nmol)	Injected amount <sup>b</sup> (pmol)	Regression equation	$R^{2c}$
L-Val	12-288	5-120	$y = 48251x - 104053$	0.9987
L-Ile	12-288	5-120	$y = 91830x + 215692$	0.9990
L-Ala	12-288	5-120	$y = 9886x + 108054$	0.9987
L-Leu	12-288	5-120	$y = 116743x - 70491$	0.9971
L-Thr	24-288	10-120	$y = 26140x - 249153$	0.9956
L-Pro	12-288	5-120	$y = 11693x - 79247$	0.9978
L-Gly	2.4-288	1-120	$y = 148135x + 176365$	0.9976
L-Asp	12-288	5-120	$y = 26006x - 227861$	0.9960
L-Glu	12-288	5-120	$y = 50423x - 123370$	0.9942
L-Met	12-288	5-120	$y = 210855x - 108826$	0.9967
L-Phe	12-288	5-120	$y = 128113x - 873123$	0.9976
L-Tyr	12-288	5-120	$y = 157460x - 136136$	0.9975
L-Lys	12-288	5-120	$y = 36470x - 119308$	0.9979

<sup>a</sup>Original amount of amino acid, given as underivatized nanomoles.

<sup>b</sup>Injected amount of amino acid given as picomoles of underivatized compounds. The injected amount of amino acid was estimated considering the volume of solvent (100  $\mu$ l) added to the derivatization residue, the volume of internal standard solution added (20  $\mu$ l), the injected volume, (1  $\mu$ l), and the split ratio 20:1.

<sup>c</sup> $R^2$  = square correlation coefficient.

**TABLE 9. LOD and LOQ calculated from the calibration curves and experimental LOD of the L-forms of a series of HFBA-HFB amino acid derivatives**

Amino acid	LOD $6\sigma_b/S$ (injected pmol)	LOQ $20\sigma_b/S$ (injected pmol)	Experimental LOD $3 < S/N < 7$ (injected pmol)
L-Val	0.472	1.575	2
L-Ile	0.245	0.817	3
L-Ala <sup>a</sup>	1.961	6.536	4
L-Thr	0.687	2.291	10
L-Leu	0.207	0.691	4
L-Pro <sup>a</sup>	1.126	3.753	3
L-Gly	0.088	0.292	1
L-Asp	0.274	0.914	5
L-Glu	0.356	1.188	3
L-Met	0.182	0.606	5
L-Phe	0.133	0.442	2
L-Tyr	0.147	0.482	5
L-Lys	0.450	1.500	3

<sup>a</sup>In the case of Ala and Pro, the 95% two-sided confidence interval for the intercept does not include zero. The results of the  $t$ -test were  $Ala/t| = 5.968 > 3.182$  and  $Pro/t| = 3.702 > 3.182$ .

**TABLE 10.** Relative GC-MS responses for a series of amino acid derivatives in relation to the internal standard methyl dodecanoate obtained derivatizing the same starting amount,  $2.4 \times 10^{-7}$  mol, of the L form, using the proposed 1-step derivatization method and the common 2-step method\*

Amino acid	HFBA-HFB $A/A_{IS}^a$	HFBA 2N HCl-MeOH $A/A_{IS}$
Ala	0.899	0.140
Val	1.396	0.334
Ile	1.677	0.549
Leu	1.958	1.694
Gly	1.965	0.415
Pro	1.721	1.752
Thr	0.723 <sup>b</sup>	0.603
Ser	—	0.481
Asp	0.606	1.180
Glu	1.537	1.509
Cys	0.021	1.139
Met	2.502	2.134
Phe	3.267	3.652
Tyr	1.944	2.141
Lys	2.604	2.747
Trp	0.057 <sup>c</sup> 0.466 <sup>d</sup>	1.376

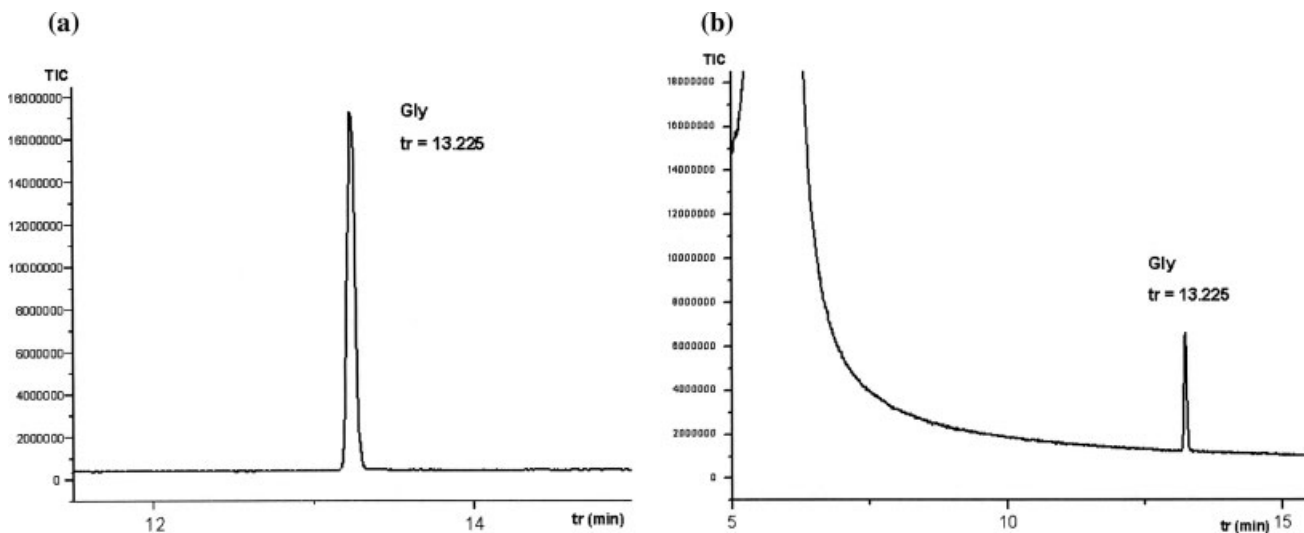
<sup>a</sup>The listed values are the average of at least three replicated samples, determined on an RT- $\gamma$ -DEXsa column.

<sup>b</sup>In the case of the 1-step reaction, the peak ratio relative to the internal standard was calculated considering the sum of the L form and the percentage of the L form derived from racemization when this phenomenon occurred.

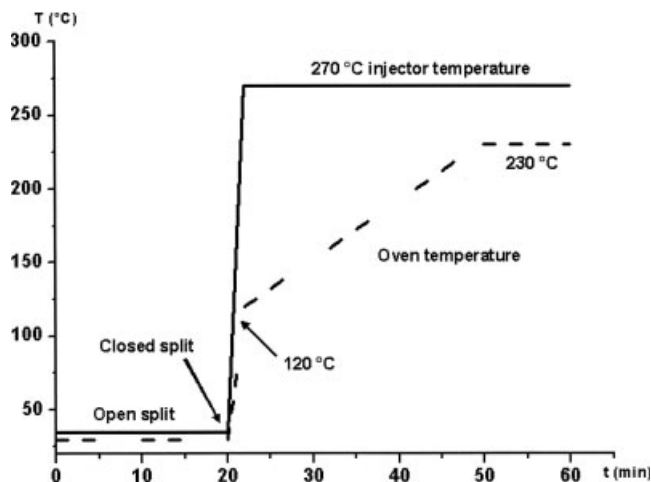
240, 480, and 240 nmoles of initial, not derivatized, amino acid). In meteorites found on Earth, amino acids that are common to biological systems are either present in trace amounts (e.g., Ser, Thr) or absent (e.g., Tyr, Phe, Met, Cys, Lys, His, Arg).<sup>7-16</sup> The principal common amino acids detected were Glu, Asp, Pro, Gly,

$\beta$ -Ala, Ile, Leu, Sar, Ala, Val, and rare or uncommon amino acids:  $\alpha$ -aminoisobutyric acid,  $\alpha$ -amino-*n*-butyric acid, isovaline, other *N*-alkyl amino acids and  $\alpha$ -dialkyl amino acids, more resistant to racemization phenomena than the  $\alpha$ -alkyl amino acids. Therefore most of them were aliphatic amino acids, which are well detected using the proposed 1-step method.

In this study, racemization phenomena were observed: varying from 10% for Ala, Asp, Val, and Leu to 20% for Phe and Lys, to more relevant values for Glu (30%) and Pro (40%). This is a drawback of the procedure because it limits the quantitative determination of the relative abundance of each enantiomer. However, it is a useful tool for detecting the presence of an enantiomeric excess, which is relevant to the search for life by in-situ space analysis. The quantitative aspects of the one-step derivatization procedure was compared to two-step procedure. HFBA-HFB reagents were used for the one-step procedure and HFBA 2N HCl-methanol for two-step procedure. The assays were performed on RT- $\gamma$ -DEXsa column. The same reaction conditions were chosen for both procedures: temperature 100°C, reaction time 1 h. More precisely, in the two-step procedure the reaction time was 30 min for esterification and 30 min for acylation, excluding the time required for all the manual steps, i.e., cooling the esterification reaction mixture, opening the derivatization ampoule, eliminating the reagents by an N<sub>2</sub> stream, adding the acylation reagent, and further closing hermetically the derivatization ampoule. The results obtained using the two different procedures were compared in terms of the peak area ratio relative to the internal standard (Table 10). The data show that the two techniques yield comparable responses, with the exception of Cys and Ser displaying highest responses with the two-steps method. Otherwise, the one-step reaction gives the highest yields for the most volatile amino acids: Ala in particular, Val, Leu, Ile, and Gly. No racemization



**Fig. 10.** (a) Split injection of a treated sample of HFBA-HFB-derivatized Gly,  $2 \times 10^{-3}$  M in ethyl acetate. Volume injected, 1  $\mu$ l; split ratio, 20:1. (b) Split injection of an untreated sample of HFBA-HFB derivatized Gly,  $2 \times 10^{-3}$  M in the reagent mixture. Volume injected, 1  $\mu$ l; split ratio, 20:1.



**Fig. 11.** PTV injection conditions: oven temperature (indicated by the broken line), from 30°C (20 min) to 120°C, 70°C/min, to 230°C (4°C/min); PTV temperature (indicated by the continuous line), from 35°C (20 min) to 150°C, 250°C/min, to 150°C, 150°C/min, to 270°C, 50°C/min, and then isothermal conditions. (The temperature program of the injector was selected with respect to the increase rates accepted by the instrument, indicated by Shimadzu); flow program: split ratio, 245:1; flow rate (45 kPa at 35°C), 1 ml/min for 20 min, then the split vents are closed (splitless) and the head pressure is increased at a rate 35 kPa/min up to 80 kPa corresponding to 1 ml/min at 120°C, and then isobaric conditions.

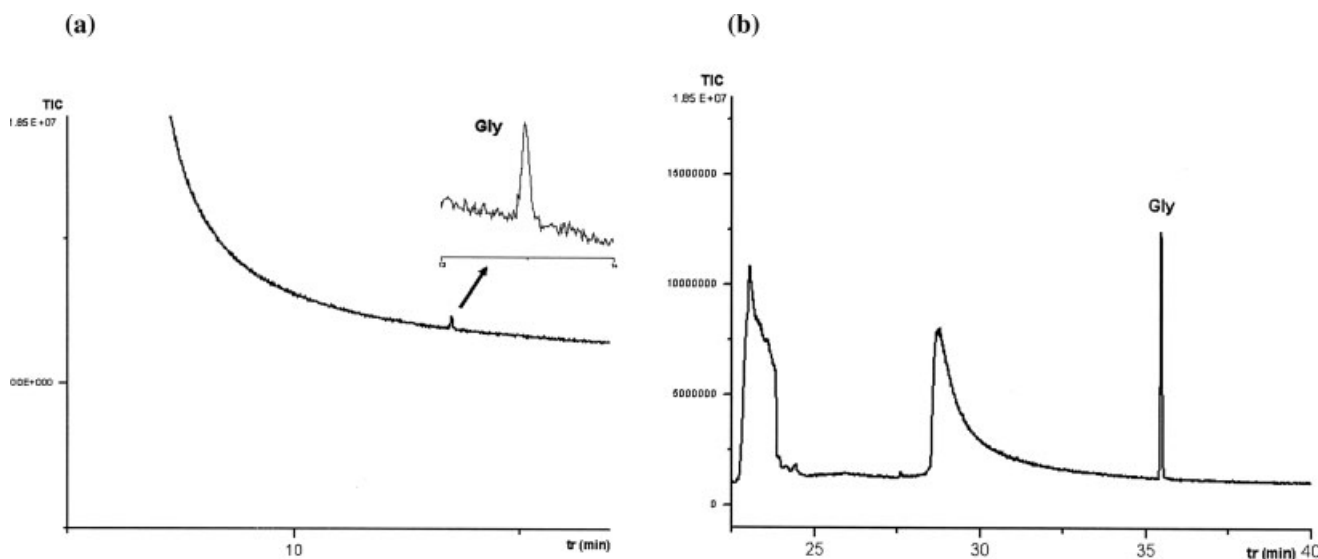
phenomena were observed in the case of the two-step procedure.

**Programmed vaporization temperature (PTV).** The possibility of automating sample treatment was studied. The normal procedure involves the evaporation of the reagent excess after reaction and addition of solvent to dissolve the residue before the GC analysis. This procedure is necessary because the reaction produces a large amount of free perfluoroacid which, in addition to the

excess of reagents, can severely damage the column and produce strong peak tailing. Some preliminary results are reported here: some experiments were performed in order to reduce the manual treatment of the sample by using a programmed temperature vaporization (PTV) injector.

The case studied was Gly derivatized using HFBA-HFB. As first test a normal treated sample and untreated sample were compared in terms of chromatographic signal obtained using the split injection. The starting derivatized amount of amino acid,  $2.4 \times 10^{-7}$  mol, was the same in both the cases. The treated sample was obtained applying the proposed procedure, adding to the dry derivatization residue (see Section 2.2) 120  $\mu$ l of ethyl acetate. The untreated sample was obtained using 80  $\mu$ l of HFBA and 40  $\mu$ l of HFB (120  $\mu$ l of added volume in total) for the derivatization reaction, eliminating the subsequent purification procedure. The molarity of the final solutions, in ethyl acetate or derivatization reagents, was about  $2 \times 10^{-3}$ ; 1  $\mu$ l of the solution was injected in split mode (split ratio 20:1) in both cases. The obtained chromatographic signals, reported in the same TIC scale, are displayed in Figure 10a and b. As can be seen in Figure 10b, the large tailed peak which appears in the first part of the chromatogram of the untreated sample, due to the reagent excess and by-products (i.e., heptafluorobutyric acid or, for example, the products obtained from the competitive reaction between the reagents) caused an increase in background noise and a decrease in intensity of the analyte signal.

The PTV injection seems to be a promising technique for an on-line treatment of the sample for eliminating reagent excess without manual procedures. The untreated sample was injected at low temperature on an adsorbent material (deactivated glass wool) in the liner, and the split vent was opened. The analytes were enriched in the vaporizing chamber, since the reagents, due



**Fig. 12.** (a) Split injection of a non treated sample of HFBA-HFB-derivatized Gly,  $2 \times 10^{-4}$  M, in the reagent mixture, injected volume, 1  $\mu$ l. (b) PTV injection of the same not treated sample indicated in (a). Injection conditions described in Figure 11 were used; injected volume, 1  $\mu$ l.



to their higher volatility, were discharged via the split line using a high flow of carrier gas. The split valve was then closed and the analytes were transferred to the column by rapid heating in splitless mode. Figure 11 shows the programmed injection conditions. In order to avoid the saturation of the capillary column and of the detector, due the splitless conditions of injection, the reaction mixture obtained derivatizing  $2.4 \times 10^{-7}$  mol of Gly, was diluted with the reagents up to the concentration of  $2 \times 10^{-4}$  M, and 1  $\mu$ l injected into the vaporizing chamber. The chromatogram obtained by injecting the same untreated sample in split mode and in PTV mode, recorded in the same TIC scale, are showed in Figure 12a and b respectively. The comparison between the two chromatograms shows a significant decrease in the reagent peak and background noise intensities, with a consequent increase in the Gly signal, in the case of the PTV injection. Using the split injection, the measured signal-to-noise ratio (S/N) was 5 while using the PTV injection, S/N = 120.

### CONCLUSIONS

The one-step derivatization reaction of amino acids to their *N(O,S)*-perfluoroacyl perfluoroalkyl esters results a convenient procedure for the GC-MS analysis of several amino acids. The procedure is simple and fast: the reagents are added at the same time, the reaction occurs without stirring and in absence of particular equipments or precautions. The good linearity of the method and the measure of the LOD show that the procedure is advantageous for amino acid quantification when the combination HFBA-HFB is chosen. The PTV injection technique can eliminate the manual treatment of the reaction mixture, and enhances the sensitivity of the method. The results concerning the enantiomeric analysis show that the *N(O,S)*-perfluoroacyl perfluoroalkyl amino acid derivatives are well separated in their enantiomeric pairs on the two tested GC capillary chiral columns. Since the two columns considered were complementary in their performance, a search for an optimum unique column for the enantiomeric separation is under study and will be the topic of a future work. Some drawbacks of the method represented by the racemization phenomena under selected conditions and for some amino acids are identified and reported. These results show that the employment of this technique for the enantiomeric analysis of amino acids requires careful study of the optimum reaction conditions in order to avoid racemization phenomena or to give precise percents of racemization when an enantiomeric excess is present, especially at low concentration of amino acid. Nevertheless, the presented results can be employed for designing an instrumental method suitable for the in-situ analysis.

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