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Enantiomeric resolution of biomarkers in space analysis: Chemical derivatization and signal processing for gas chromatography–mass spectrometry analysis of chiral amino acids

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ABSTRACT

The work compares two GC–MS methods for enantioselective separation of amino acids as suitable candidate for stereochemical analysis of chiral amino acids on board spacecrafts in space exploration missions of solar system body environments. Different derivatization reagents are used: a mixture of alkyl chloroformate–alcohol–pyridine to obtain the alkyl alkoxy carbonyl esters and a mixture of perfluorinated alcohols and anhydrides to form perfluoroacyl perfluoroalkyl esters. 20 proteinogenic amino acids were derivatized with the two procedures and submitted to GC–MS analysis on a Chirasil-L-Val stationary phase. The results were then compared in terms of the enantiomeric separation achieved and intensity of MS response. The combination of methyl chloroformate (MCF) and heptafluoro-1-butanol (HFB) allows separation of 14 enantiomeric pairs, five of which display a resolution ($R_s \geq 1.2$) supposed to be sufficient to quantify the enantiomeric excess. Three mixtures of trifluoroacetic (TFAA) and heptafluorobutyric (HFBA) anhydrides were combined 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 different reagents (TFAA–TFE, TFAA–HFB, HFBA–HFB): the derivatives obtained show separation of the same number of proteinogenic amino acids (14 of 20) at a temperature lower than column bleeding limit (200 °C) and 8 of them give a separation with $R_s \geq 1.2$. Linearity study and limit of detection (X_{LOD}) computation show that both methods are suitable for quantitative determination of several amino acid diastereomers at trace level ($X_{LOD} \approx 0.5$ nmol as derivatized quantity). Both the procedures were coupled with automatic data handling to increase their suitability for space analysis: the simplified data treatment is especially helpful to handle the low quality data recovered from space experiments and labor and time are saved, as imposed by the space experiments requiring a rapid delivery of the results. To achieve this aim, a chemometric approach based on the computation of the Autocovariance Function (ACVF) was applied to extract information on the enantiomeric pairs present in the sample and the enantioseparation achieved on the chiral column.

1. Introduction

The detection of organic molecules, such as amino acids and sugars, in extraterrestrial environments is one of the most challenging goals for future space missions, since they can be biomarkers indicating life, both extant and extinct [1,2]. Furthermore, it is known that only one on the two enantiomeric structures of these molecules are used to build the biotic macromolecules (L for amino acids and D for sugars) whereas racemic mixtures (L and D in equal parts) are present in abiotic systems. Therefore, the search for homochirality and characterization of the enantiomeric excess in amino acids are of primary relevance as organic signatures of present or extinct life in space [2–5].

Particular efforts are being devoted to Mars because intense exploration, started in the 1990s, revealed that all the ingredients required for life to emerge (liquid water, organic molecules, and energy) should have gathered early in Martian history [6–11].

The analytical instruments suitable for space missions must meet the severe requirements imposed by flight conditions: automation, remote control operations, short analysis times and low energy consumption. Moreover, they must also provide the lowest possible detection limit since biomarker concentrations in extraterrestrial environments are not well known. Gas chromatography (GC) has proved to be the best analytical technique for in situ search for organic molecules in extraterrestrial environments [7–14], among the many conventional bench-top scale instrumentations available for chiral separation of amino acids—including

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HPLC, GC and CE, also applicable to lab-on-a-chip system [15]. Recently, a chiral stationary phase formed by a mixed binary chiral selector has been developed for the simultaneous GC enantioseparation of racemic compounds of exobiologic interest to be used in future space experiments [16].

The GC analysis of low-volatile compounds, such as amino acids, requires a preliminary derivatization step to convert them into more stable, volatile compounds suitable for GC separation [16–18]. This is the basis for space instrument sub-systems developed for the in situ analysis of extraterrestrial atmosphere and soil: i.e., COSAC (COmetary Sampling And Composition experiment) of the Rosetta space mission [4,19] and SAM (Sample Analyses at Mars) on the 2009 Mars Science Laboratory (MSL) rover [8,10,20]. However, further search for suitable procedure for space application has to be developed, since the procedures applied do not allow enantiomer separation (MTBSTFA, used for the COSAC experiment) or make it possible with poor detection sensitivity (DMF-DMA used the SAM experiment). For this reason, other derivatization techniques were investigated as possible candidates for future space experiments to yield derivatives preserving the enantiomeric configuration of amino acid pairs and avoiding racemization phenomena in order to achieve identification and quantitation of an enantiomeric excess [16–18].

To meet these requirements for analyzing amino acids enantiomers in space, two derivatization procedures for enantiomeric separation of amino acids have been recently developed as simple, automatic GC methods that may be suitable candidates for in situ space analysis. One method is based on a derivatization reaction that employs an alkyl chloroformate–alcohol–pyridine mixture to obtain the N(O,S)-alkyl alkoxy carbonyl esters of amino acids [21]. The other is a one-step procedure that obtains the N(O,S)-perfluoroacyl perfluoroalkyl derivatives by using a mixture of perfluorinated anhydride and perfluoro alcohols to simultaneously perform esterification and acylation [22]. The separation was performed on a commercially available GC column coated with Chirasil-Val: its advantage is the availability of the stationary phase in the D- and L-forms, thereby making it possible to reverse the elution order of the enantiomers [23]. Moreover, this type of column is used in the COSAC experiment which currently flies to a comet because it was demonstrated to resist to the space constraints [4,19].

In this paper the derivatization reactions are investigated and compared on the basis of the following properties:

(1) enantiomeric resolution of the derivatives on the Chirasil-L-Val chiral stationary phase under energy saving conditions (short analysis time, low analysis temperature);
(2) analytical performance in terms of MS detectability, i.e., detection (X_{LOD}) and quantification limits (X_{LOQ}).

The possibility of automating data handling is also investigated as an helpful tool to increase the method suitability for high-throughput analysis of the data from space mission experiments: it facilitates the treatment of the low quality data recovered from in situ space analysis and saves labor and time in the data treatment, as imposed by the space experiments requiring a rapid delivery of the results. In addition to the conventional GC–MS data analysis, a chemometric approach was applied to handle complex signals and extract all the analytical information hidden therein, in particular those concerning the enantiomeric pair composition of the sample and the enantioseparation on the chiral column.

2. Signal processing procedure based on Autocovariance Function

In space research, the interpretation of complex chromatographic signals and the extensive amounts of data generated by hyphenated techniques is particularly helpful in decoding chromatograms recovered from space missions, as well as in designing analytical equipment for future space missions [24–32]. In particular, the chromatograms resulting from analytical procedures involving derivatization steps may be crowded with peaks since, besides the intrinsic complexity of the sample containing other interfering organics, artifacts can even result from sample chemical derivatization [31].

Among the many signal processing procedures developed for this problem, a chemometric approach based on the AutoCovariance Function (ACVF) of the chromatographic signal has been developed by the Authors and widely applied to experimental chromatograms [24–32]. The method has proved to be a powerful tool for interpreting chromatograms of complex mixtures, extracting accurate information on the mixture composition and the presence of classes of compounds with correlated structures.

The chemometric approach studies the Experimental Autocovariance Function (EACVF) that can be directly computed from the experimental chromatogram acquired, in digitized form, using the following expression [27]:

$$\mathrm{EACVF}(\Delta t) = \frac{1}{M}\sum_{j=1}^{N-k}(Y_j - \hat{Y})(Y_{j+k} - \hat{Y}) \quad k = 0, 1, 2, \ldots \mathrm{M} - 1 \qquad (1)$$

where Y_j is the digitized chromatogram signal, $\hat{Y}$ its mean value, M the truncation point in the EACVF computation. The correlation time Δt is the interdistance between the subsequent digitized positions, and assumes discrete values with k ranging from 0 to $(M-1)$:

$$\Delta t = k\tau \qquad (2)$$

where τ is the time interval between the subsequent digitized positions.

EACVF values can be plotted as a function of the time interdistance Δt to obtain the EACVF plot: as an example, the EACVF plot computed on the GC–MS signal of Fig. 1a is reported in Fig. 1b. The EACVF study makes it possible to characterize chromatographic signal complexity in terms of a set of statistical parameters describing both the sample complexity and the chromatographic separation. In particular, the following information can be obtained [27–32]:

1. *The mean peak standard deviation*, σ: The first region in the EACVF plot ($\Delta t \leq 4\sigma$) resembles half of a Gaussian peak showing a shape averaged over the shape of all the peaks present in the chromatogram: in the simplified approach, a constant width was assumed as this can be experimentally obtained under optimized programmed temperature conditions [27]. The mean peak standard deviation can be estimated from the width of the EACVF peak close the origin $\Delta t = 0$ using the simple equation (see Fig. 1b):

$$\sigma = d_{h/2}/1.665 \qquad (3)$$

where $d_{h/2}$ is the half-height width of the EACVF peak.

2. *The number of single components* (SC) present in the mixture, m_{tot}, can be estimated from the value of EACVF at the origin ($\Delta t = 0$) using the following equation (Fig. 1b):

$$m_{tot} = \frac{A_T^2(\sigma_h^2/a_h^2 + 1)}{\mathrm{EACVF}(0)d_{h/2}2.129\ X} \qquad (4)$$

where A_T^2 and X are the total area and the total time range of the chromatogram, respectively. The value σ_h^2/a_h^2 is the peak maximum dispersion ratio derived from the mean, a_h, and the

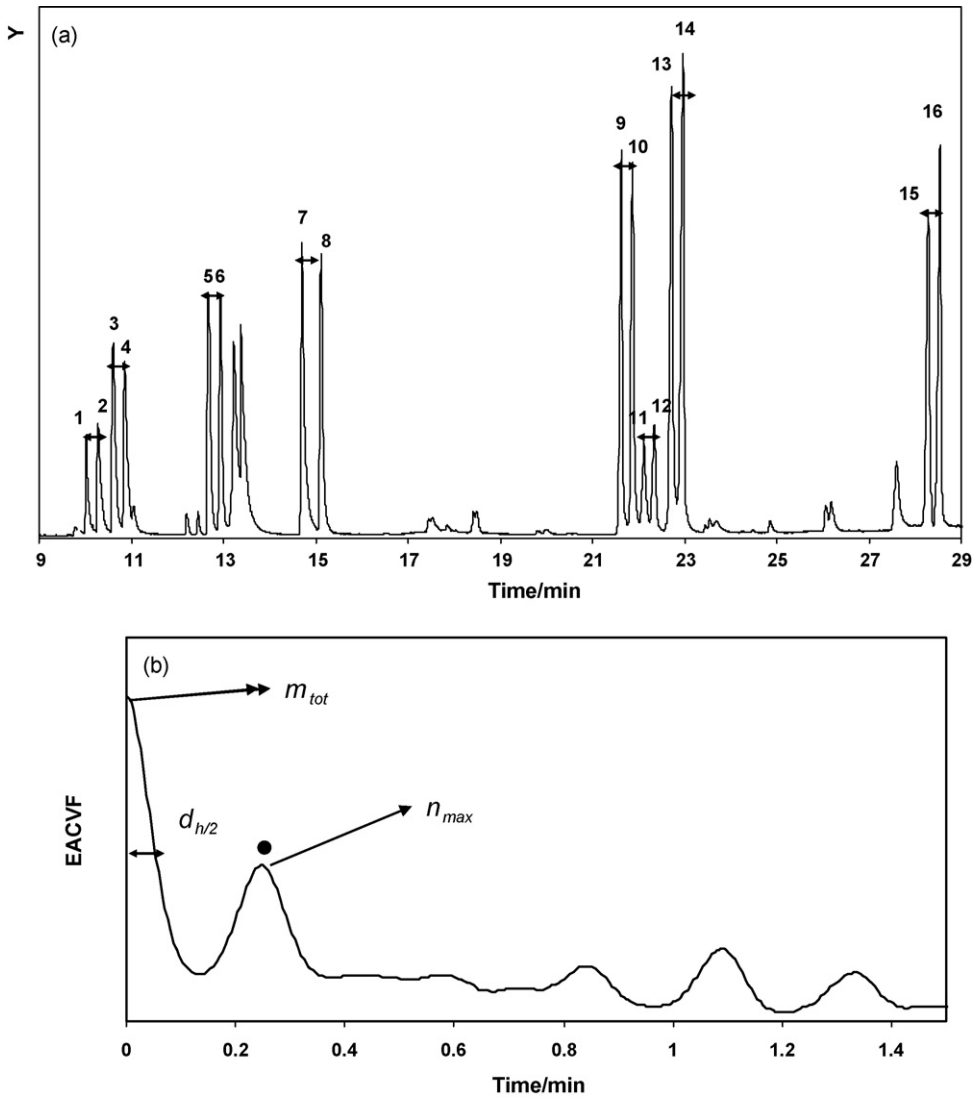

Fig. 1. Separation of TFAA–TFE derivatives. (a) GC–MS signal obtained under the optimized conditions: linear temperature increasing from 40 to 200 °C at 4.4 °C/min rate, followed by isothermal conditions. Arrows: constant interdistance $\Delta t = 0.25$ min between the enantiomeric pairs. D,L-Val [1,2], D,L-Ala [3,4], D,L-Ile [5,6], D,L-Leu [7,8], D,L-Met [9,10], D,L-Glu [11,12], D,L-Phe [13,14], D,L-Tyr [15,16]. (b) EACVF plot computed on the GC–MS signal.

variance, σ_h^2, of peak height computed from the separated peaks observed in the chromatogram [27].

3. *Information on the separation pattern*: The second part of the EACVF plot, for $\Delta t \geq 4\sigma$, shows a specific pattern dependent on the distribution of SC peak positions over the separation axis [28]. In particular, the EACVF method has proved efficient in identifying the presence of retention repetitivities inside the chromatogram, i.e., peaks located at constant interdistance values b repeated in the chromatogram (arrows in the chromatogram of Fig. 1a). Such order can be related to structural regularity in the molecular properties of the mixture components—i.e., a common molecular scaffold or constant structural modifications to yield constant interdistances $\Delta t = b$ in different regions of the chromatogram. In this case, the EACVF plot displays well-defined deterministic peaks located at interdistance b (first deterministic peak, signed by the point in Fig. 1b) and multiple values $\Delta t = bk$, if the interdistance is repeated k times in the chromatogram, as in the case of homologous series. The appearance of these peaks is diagnostic to identify the presence of ordered structures in the chromatogram, and their height, i.e., EACVF(bk) value computed at $\Delta t = bk$, is related to the abundance of the repetitiveness in the chromatogram, i.e., the combination of the number of repeated peaks and their heights. From the height of the first peak, the EACVF(b) value, the number of compounds n_{max} located at constant interdistance $\Delta t = b$ can be estimated according to the equation (see Fig. 1b) [28–30]:

$$\mathrm{EACVF}(b) = \frac{\sqrt{\pi}\sigma a_h^2 n_{\max}}{X}\left[\frac{\sigma_h^2}{a_h^2} + 1\right] \quad (5)$$

3. Experimental

3.1. Amino acids and reagents

Twenty proteinogenic amino acids were studied: Ala, Val, Pro, Ile, Leu, Asp, Thr, Asn, Met, Cys, Glu, Gln, Phe, His, Lys, Tyr, Ser, Arg, and Trp in their D- and L-enantiomeric forms plus glycine. They were purchased from Aldrich (Milan, Italy) and from Fluka (Milan, Italy). Stock standard solutions of the individual pairs of D- and L-amino acids were prepared in a concentration range from 1×10^{-2}

to 4×10^{-2} M using deionized water or 0.1 M HCl in some cases (Asp, Glu, Tyr, and Trp).

A standard solution of methyl laurate 5×10^{-3} M in acetonitrile was prepared as internal standard (IS). Methyl laurate (methyl dodecanoate, 97%) was purchased from Fluka (Milan, Italy).

The derivatization reagents were: 2,2,3,3,4,4,4-heptafluoro-1-butanol (HFB, 95%), methyl chloroformate (MCF, 97%), ethyl chloroformate (ECF), pyridine (Py, 99%), trifluoroacetic anhydride (TFAA), heptafluorobutyric anhydride (HFBA), 2,2,2-trifluoroethanol (TFE). They were purchased from Sigma–Aldrich (Milan, Italy) and Fluka (Milan, Italy). Also the solvents, chloroform (99.8%), methanol, ethanol, acetonitrile, ethyl acetate and acetonitrile, were purchased from Fluka (Milan, Italy). All these compounds were analytical grade reagents.

3.2. GC analysis

GC–MS analysis was performed on a QMD 1000 GC–MS system (Fisons, Milan, Italy). The Electron Impact ionization mode operated at 70 eV. The MS system operated in scan mode (mass range 38–550 u at 1 scan/s, solvent delay: 5 min). The detector and injector temperatures were 250 °C, the carrier gas Helium at a flow rate of 1 ml min^{-1}. Split injection was used with a split ratio of 1:20.

The chromatographic column was a Chirasil-L-Val fused-silica (L-valine-tert-butylamide modified polydimethylsiloxane) 25 m × 0.25 mm I.D. capillary column (Varian, The Netherlands) with a 0.12 μm film coating. Its maximum operating temperature was 200 °C. The enantiomeric resolution of the chloroformate and anhydride derivatives was investigated and compared under the same column temperature program: temperature was linearly increased from 60 to 180 °C at a rate of 4 °C/min and held at 180 °C for 15 min. These conditions present a compromise between short retention times, low column temperature (imposed by energy saving constraint) and good resolution for almost all amino acids [21,22]. To obtain reliable and reproducible quantitative data, the internal standard procedure was used, by selecting methyl laurate as IS: detector response was expressed as peak area value (A_{aa}) relative to IS peak area (A_{IS}), i.e., A_{aa}/A_{IS}.

3.3. Derivatization procedure

3.3.1. Chloroformate derivatization procedure

Standard amino acid stock solutions (25 μl plus 25 μl of IS) were transferred into a silanized screw capped 2 ml vial and the reagents were added in the following order: 60 μl of heptafluoro-1-butanol (HFB), 15 μl of pyridine and 15μl of methyl chloroformate (MCF). For each enantiomeric pair the analyzed quantity ranged from 1×10^{-6} to 5×10^{-7} moles. The previously described procedure was followed [21]. The mixture was immediately shaken for 1 min in an ultrasound bath kept at a constant temperature of 25 °C. The MCF derivatives were extracted from the reactive mixture by adding 200 μl of chloroform and a small volume (20 μl) of saturated NaCl solution. The solution was then shaken for 10 s and, after waiting 2 min to reach phase separation, 1 μl of the bottom chloroform phase was injected into the GC system.

3.3.2. Anhydride derivatization procedure

Derivatization was performed according to the procedure previously described in Ref [22]. Standard amino acid stock solution was transferred into a 2-ml ampoule and evaporated to dryness. For each enantiomeric pair the analyzed quantity was 2.4×10^{-7} moles. The two derivatizing agents (50 μl of perfluoroalcohol and 100 μl of 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. Then, the residues were dissolved in 100 μl of ethyl acetate, and 20 μl of IS solution was added; 1 μl of this solution was injected into the GC–MS.

4. Results and discussion

The two derivatization procedures were applied to twenty proteinogenic amino acids and their performance was investigated and compared by GC–MS analysis of the obtained derivatives in terms of enantiomeric resolution and quantitative sensitivity for the target amino acid pairs.

For both the procedures the chromatographic separation was optimized by selecting proper temperature program conditions to obtain the best enantiomeric resolution for most of the enantiomeric pairs.

4.1. Enantiomeric resolution

20 proteinogenic amino acids were derivatized with chloroformate and perfluoroacylated anhydrides and submitted to GC–MS analysis on a Chirasil-L-Val stationary phase. The same column temperature program (linear increase from 60 to 180 °C at 4 °C/min) was used to investigate and compare the enantiomeric resolution obtained for both the derivative classes. Among different combinations of chloroformates (methyl chloroformate, ethyl chloroformate and isobutyl chloroformate) and alcohols having an identical or different alkyl chains, the combination methyl chloroformate (MCF) and heptafluoro-1-butanol (HFB) was chosen since it allows operation at a lower column temperature and this ensures better chiral separation and reduced energy consumption [21,33].

Among the 20 proteinogenic amino acids analyzed, 14 enantiomeric pairs could be separated in these operating conditions with a constant elution order since the D form always eluted first. Most of these derivatives (6 out of 14) were strongly retained – with retention time longer than 20 min – displaying, in general, lowest resolution for the most retained compounds. Six enantiomeric pairs displayed good resolution values R_s higher than 1.4: Ala, Val, Ile, Leu, Met, and Glu (1st column in Table 1). Eight pairs showed lower resolution ($0.5 \geq R_s \geq 1.5$): they were the heaviest amino acids (Thr, Phe, Lys, Tyr, and Trp) or compounds yielding the bi-esterified (Asp) and bis-acylated (Gln, Ser) derivative as a more stable product. No enantiomeric separation was obtained for Pro, mono-acylated Ser, His and Asn. Arginine was not detected at the studied concentration level because its derivatization yield was very low, given the low reactivity of the guanidine group in the molecule under these derivatization conditions. Glutamine is not reported since it is converted into glutamine acid during the derivatization reaction [34].

Table 1
Enantiomeric resolution of amino acid pairs after chloroformate (1st column) and perfluoro anhydride derivatization (2nd–4th columns).

Amino acids	HFB/MCF	TFAA-TFE	TFAA-HFB	HFBA-HFB
	R_s			
DL-Ala	2.11	1.79	1.84	1.71
DL-Val	2.39	1.49	2.26	1.20
DL-Ile	6.24	1.56	1.93	1.44
DL-Leu	3.88	3.22	3.97	4.13
DL-Met	2.43	2.25	2.93	2.31
DL-Glu	1.38[a]	3.43	2.31	2.07
DL-Phe	0.93	2.23	2.27	2.05
DL-Tyr	0.85[a]	1.61	1.60	1.46

Comparison between R_s values on Chirasil-L-Val under the same program temperature conditions: linear increase from 60 to 180 °C at a rate of 4 °C/min. Absolute quantity submitted to derivatization: 5×10^{-7} moles for chloroformate; 2.4×10^{-7} moles for perfluoroanhydride reaction.

[a] bis-Esterified derivative.

Table 2
X_{LOD} and X_{LOQ} (derivatized nmol) values calculated from the calibration curves of the L-forms of a series of HFB/MCF and HFBA–HFB derivatives of 9 amino acids.

Amino Acids	X_{LOD} (derivatized nmol) HFB/MCF	X_{LOQ} (derivatized nmol)	X_{LOD} (derivatized nmol) HFBA–HFB	X_{LOQ} (derivatized nmol)
L-Ala	1.64	5.47	4.70	15.6
L-Val	0.72	2.4	1.13	3.77
L-Ile	1.28	4.27	0.58	1.93
L-Pro	6.68	22.3	2.71	9.03
L-Leu	6.08	20.3	0.50	1.66
L-Met	3.92	13.0	0.43	1.43
L-Glu	6.28	20.9	0.86	2.86
L-Phe	2.56	8.53	0.31	1.03
L-Tyr	5.8	19.3	0.36	1.20

Trifluoroacetic (TFAA) and heptafluorobutyric (HFBA) anhydrides have been found the most useful reagents for esterification–acylation reaction for quantitative GC determination of amino acids: they are both strong, highly reactive acylating agents that form stable derivatives [35,36]. The procedure was applied using three different combinations of TFAA and HFBA anhydrides 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 derivative combinations (TFAA–TFE, TFAA–HFB, HFBA–HFB, Table 1) [22]. In addition to mono-derivatives, the bis- and tris-derivatives were also obtained when the functional groups were esterified (Asp, Glu) or acylated (Gln, Trp).

The retention behavior of the derivatives and their enantiomeric separation was investigated on chiral Chirasil-L-Val capillary column (linear temperature program from 60 to 180 °C at 4 °C/min). 14 of the 20 proteinogenic amino acids could be separated at a temperature lower than the column bleeding limit (200 °C), even the heaviest amino acid derivatives (Tyr, Gln, Lys, and Trp). Comparison among the obtained results shows that three reagent combinations display similar retention time patterns (11–40 min range) and chiral separations (Table 1, 2nd–4th columns). For all three combinations, 8 enantiomeric pairs could be well separated yielding a good resolution ($R_s \geq 1.4$): Ala, Val, Ile, Leu, Met, Glu, Phe, and Tyr (Table 1, 2nd–4th columns). Other amino acids gave poorly separated peaks (Pro, Thr, Asp, and Lys) or low signals due to the formation of by-products or degradation products (Gln, Trp). In general, the TFAA–TFE derivatives displayed the best selectivity on the Chirasil-L-Val column, while the TFAA–HFB compounds presented the greatest separation problems, in particular coelution of Ala–Val, Pro–Thr, and Glu–Phe enantiomer pairs. This motivates the selection of the TFAA–TFE derivatization for qualitative analysis [22]. However, it must be underlined that the separation of all the studied compounds is far to be achieved under these conditions: a promising alternative may be the use of GC columns coated with different selectors such as one cyclodextrin (CD) or their binary mixture in combination of chiral selectors with complementary enantioselectivity [16,22].

4.2. Quantitative analysis

The performance of the two methods for quantitative analysis was investigated and compared in terms of sensitivity and linearity.

The relative sensitivity was investigated by comparing the response factor measured as the relative peak area A_{aa}/A_{IS}. For the chloroformate derivatives using the HFB/MCF combination, a comparable sensitivity has been found for most of the studied amino acids: the exceptions were lysine and histidine (containing an additional aminic group), serine and glutamic acid (containing the additional hydroxyl group) which gave the lowest reaction yields [21].

Since MS response increases with the size of the protecting groups, when the esterification–acylation procedure was used, the HFBA–HFB derivatives yielded the highest response, making this combination the reagent of choice for quantifying amino acid enantiomers. Comparable sensitivity was found for most of the studied amino acids: L-Met and L-Phe displayed the highest sensitivity which was nearly triple that of L-Pro and L-Trp (bis-acylated) which was the least sensitive [22].

For quantitative analysis, linearity and sensitivity were evaluated by computing the calibration lines: the amino acids studied were L-Ala, L-Val, L-Ile, L-Pro, L-Leu, L-Met, L-Glu, L-Phe and L-Tyr. Different quantities of the L-form (30–500 nmol for HFB/MCF and 3–300 nmol for HFBA–HFB reagents) were submitted to derivatization and MS analysis. From the calibration lines, displaying good linearity over the wide concentration range exploited, the detection limit X_{LOD} and quantification limit X_{LOQ} were determined: X_{LOD} was computed as the analyte concentration yielding a signal value of $X_{LOD} = \bar{y}_b + 6\sigma_b$, where $\bar{y}_b$ is the blank average signal of 10 blank responses and σ_b its standard deviation. The quantification limit X_{LOQ} was determined as the analyte concentration corresponding to a signal value $X_{LOQ} = \bar{y}_b + 20\sigma_b$ to ensure that the quantitative determination gave satisfactory measurement precision (RSD% ≤5%) [37].

The obtained results show the comparable sensitivity of the methods, yielding low detection and quantification limits, X_{LOD} ≤6 nmol and X_{LOD} ≤20 nmol (Table 2). In particular, the chlorofor-

Table 3
Enantiomeric resolution R_s values for amino acid pairs under GC optimized temperature program. Absolute quantity submitted to chloroformate and perfluoroanhydride derivatization: 5×10^{-7} moles. Operating conditions for chloroformate derivatives (1st column): isotherm at 90 °C for 10 min, linear increase to 160 °C at 4 °C/min, isotherm at 160 °C for 10 min, linear increase to 180 °C at 4 °C/min, isotherm at 180 °C for 15 min. Operating conditions for TFAA–TFE perfluoro anhydride derivatives (2nd column): linear increase from 40 to 200 °C at 4.4 °C/min rate, followed by isothermal conditions; operating conditions for HFBA–HFB perfluoro anhydride derivatives (3rd column): isotherm at 60 °C for 27 min, linear increase to 120 °C at 3 °C/min, isotherm at 120 °C for 10 min, increase to 155 °C at 10 °C/min, isotherm at 155 °C for 5 min, increase to 200 °C at 3 °C/min, followed isothermal conditions.

Amino acids	HFB/MCF	TFAA–TFE	HFBA–HFB
	R_s		
DL-Ala	2.11	2.19	2.42[a]
DL-Val	2.39	2.21	2.52[a]
DL-Ile	6.24	2.03	2.30
DL-Pro	NR	LR	LR
DL-Thr	LR	LR	LR
DL-Leu	3.88	2.11	2.91
DL-Asp	0.81	LR	LR
DL-Met	2.43	2.08	2.28
DL-Glu	1.38[a]	1.88	2.14
DL-Phe	0.93[a]	1.95	2.24
DL-Tyr	0.85	2.06	1.96
DL-Gln (bis-acylated)	LR	1.23	0.89
DL-Lys	0.73	0.69	0.87
DL-Trp (bis-acylated)	LR	0.80	0.65

LR: resolution value $R_s \leq 0.6$.
[a] Enantiomeric resolution is possible only under SIM detection.

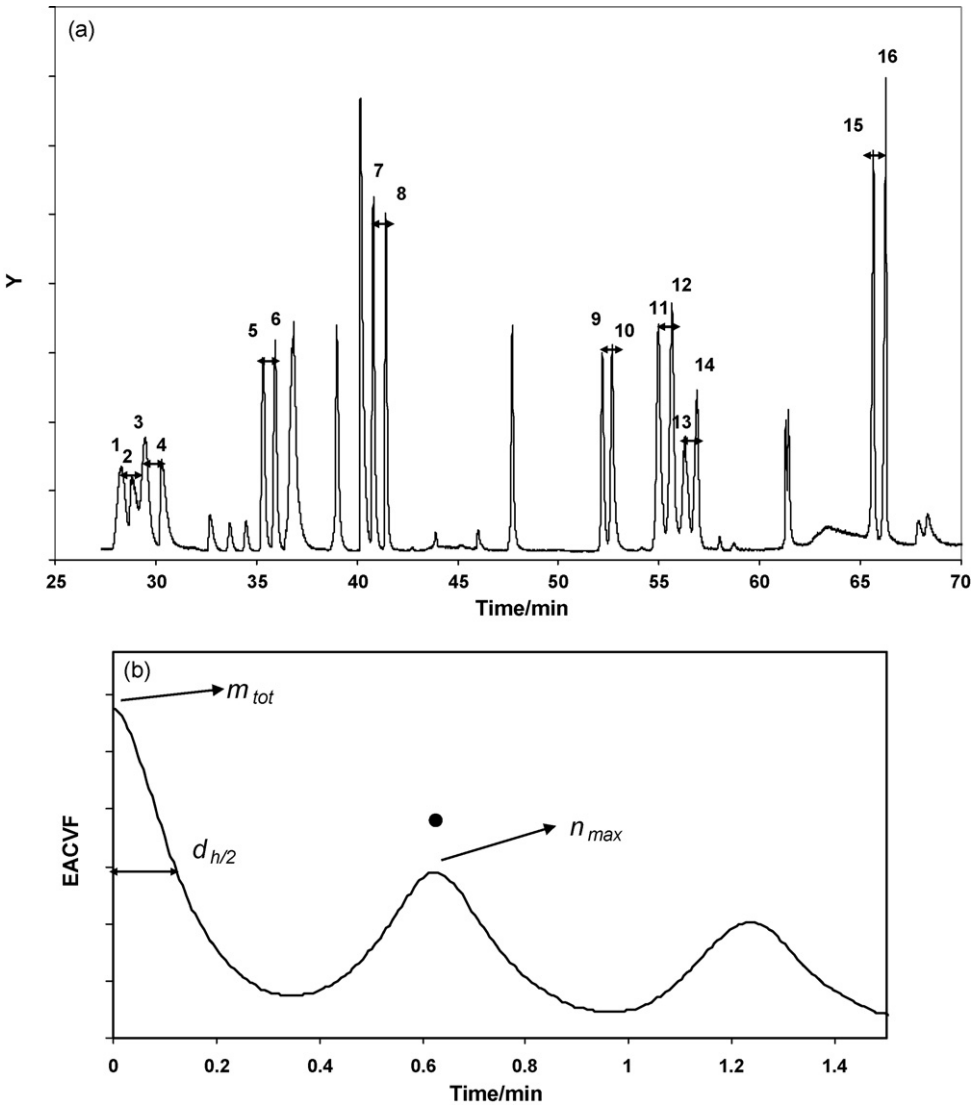

Fig. 2. Separation of HFBA–HFB derivatives. (a) 25–70 min region of the GC–MS signal obtained under the optimized conditions: an isotherm at 60 °C for 27 min followed by a three-stage temperature program: from 60 to 120 °C at 3 °C/min, 10 min at 120 °C, from 120 to 155 °C at 10 °C/min, 5 min at 155 °C, from 155 to 200 °C at 3 °C/min, followed isothermal conditions. Arrows: constant interdistance $\Delta t = 0.63$ min between the enantiomeric pairs. D,L-Val [1,2], D,L-Ala [3,4], D,L-Ile [5,6], D,L-Leu [7,8], D,L-Met [9,10], D,L-Phe [11,12], D,L-Glu [13,14], D,L-Tyr [15,16]. (b) EACVF plot computed on the GC–MS signal.

mate derivatives showed higher sensitivity for the lighter amino acids, L-Ala and L-Val, achieving $X_{LOD} \approx 1$ nmol, while the HFBA–HFB combination showed higher sensitivity for the least volatile amino acids, L-Met, L-Phe and L-Tyr, giving $X_{LOD} \leq 0.4$ nmol derivatized quantity. The obtained X_{LOD} and X_{LOQ} values are compatible to in situ analysis of extraterrestrial environments, where amino acids are expected to be present at the sub-nmol trace level, as suggested by the concentration level found in meteorites on Hearth [1–3].

4.3. Enantiomeric separation of amino acid mixtures

The performance of the described methods in separating amino acid enantiomers was checked on a mixture containing 15 amino acids: Gly and enantiomeric pairs Ala, Val, Pro, Ile, Leu, Asp, Thr, Met, Phe, Gln, Glu, Lys, Tyr and Trp (the absolute quantity submitted to derivatization was 5×10^{-7} moles for each amino acid enantiomer). After chloroformate and anhydride derivatization, the derivatives were submitted to GC–MS analysis on Chirasil-L-Val column: a number of trials were performed to select the best temperature program conditions yielding the best separation of the highest number of enantiomeric pairs.

For the chloroformate derivatives, the best separation was achieved in nearly 1 h by adding an initial isotherm at 90 °C for 10 min and inserting an isotherm step (160 °C for 10 min) in the linear temperature program (from 90 to 180 °C at a rate of 4 °C/min). Under these conditions, 13 of the 14 enantiomeric pairs were separated, in addition to Gly (R_s values reported in Table 3, 1st column), by combining the resolution power of the Chirasil-L-Val column and the high selectivity of the SIM (selected ion monitoring) detection mode. The overlapped peak formed by co-eluting derivatives of Phe and Glu can be solved by operating in SIM detection at $m/z = 91$ for Phe and $m/z = 84$ for Glu. Two esterification–acylation procedures were investigated: the TFAA–TFE derivatives yielding the best separation and the HFBA–HFB compounds giving the highest signal response for quantitative determination. After a number of trials, the proper chromatographic conditions were identified to yield acceptable enantiomeric separations for 14 of amino acid pairs (R_s values reported in Table 3, 2nd and 3rd columns).

The best separation of the TFAA–TFE derivatives was achieved under a linear temperature increasing from 40 to 200 °C at a rate of 4.4 °C/min, followed by isothermal conditions. The GC–MS signal obtained under these conditions (chromatogram in Fig. 1a) shows

that 8 enantiomeric pairs can be properly separated with $R_s \approx 2$ and 3 amino acids, DL-Gln, DL-Lys, and DL-Trp, satisfactory separated with $R_s \approx 0.8$ (R_s data in Table 3, 2nd column).

Separation of the HFBA–HFB derivatives of the same mixture is more difficult: a long analytical run of 85 min is required for the complete elution of all compounds. An isotherm at 60 °C for 27 min is introduced to separate DL-Ala from DL-Val enantiomers followed by a three-stage temperature program: from 60 to 120 °C at 3 °C/min, 10 min at 120 °C, from 120 to 155 °C at 10 °C/min, 5 min at 155 °C, from 155 to 200 °C at 3 °C/min, followed by isothermal conditions. The GC–MS chromatogram of the region 25–70 min containing most of the separated amino acid pairs is reported in Fig. 2a. Also, for these derivatives, 8 enantiomeric pairs can be satisfactory separated with $R_s \geq 2$, while low resolution ($R_s \approx 0.9$) was obtained for DL-Lys, DL-Gln and DL-Trp di-acylated derivatives (R_s data in Table 3, 3rd column). To solve the peak overlapping between DL-Ala and DL-Val pairs, the SIM detection mode was applied by selecting specific $m/z = 240$ value for Ala, and $m/z = 268$ for Val.

4.4. GC–MS signal processing using EACVF

The EACVF approach was applied to handle the complex GC–MS signals of the optimized separation of the derivatized 29-amino acid mixture (Gly and 14 enantiomeric pairs). Under these conditions a constant interdistance $\Delta t = b$ between the separated enantiomeric pairs can be experimentally achieved. The EACVF peak at $\Delta t \leq 4\sigma$ contains information on the separation parameters—the standard deviation σ and the number of components m_{tot} (Eqs. (3) and (4))—while the EACVF peak at $\Delta t = b$ gives information to characterize the enantioseparation achieved—number of separated chiral compounds (Eq. (5)) and mean R_s values.

Unlike the chloroformate derivatives, the perfluoroalkyl esters display a common retention behavior for the different amino acids in the test mixture, hence, under optimized conditions, most of the separated enantiomers exhibited the same chiral separation. This may be an experimental evidence that the chiral interactions between L-Val selector and chiral analyte moiety involved in the recognition process are similar for the studied amino acids.

Among the TFAA–TFE derivatives, 8 amino acids show a nearly constant interdistance $\Delta t = 0.25$ min between the separated enantiomeric pairs: DL-Val, DL-Ala, DL-Ile, DL-Leu, DL-Met, DL-Glu, DL-Phe and DL-Tyr (signed by arrows in Fig. 1a).

The $EACVF_{tot}$ was computed on the chromatographic signal in the 9–29 min region containing 25 amino acids, including the 8 enantiomeric pairs resolved ($EACVF_{tot}$ plot Fig. 1b). From the half-height width of the first EACVF peak, the mean peak standard deviation is estimated as $\sigma = 0.03$ min. From the EACVF (0) value the number of components present in the sample, m_{tot}, can be estimated as $m_{tot} = 28 \pm 5$, according to Eq. (3). The presence of a well-defined peak at $\Delta t = 0.25$ min can be used to identify the presence of enantiomeric pairs and, from its value, the number of the separated enantiomers $n_{max} = 15$ can be correctly estimated.

The HFBA–HFB derivatives of the same enantiomeric pairs (DL-Val, DL-Ala, DL-Ile, DL-Leu, DL-Met, DL-Phe, DL-Glu, and DL-Tyr) are separated by a constant interdistance $\Delta t = 0.63$ min (signed by arrows in the GC–MS signal reported in Fig. 2a). As a consequence, the EACVF plot computed on the signal (reported in Fig. 2b) clearly shows a well-defined peak at $\Delta t = 0.63$ min that is diagnostic for the presence of separated enantiomeric pairs. From the half-height width of the first EACVF peak, the mean peak standard deviation is estimated as $\sigma = 0.07$ min; such a high σ value, describing low separation efficiency, may be expected under these conditions of slow separation (all the retention times are higher than 28 min). From the EACVF(0) value the number of components present in the sample, m_{tot}, can be estimated as $m_{tot} = 29 \pm 5$, according to Eq. (3). From the height of the EACVF peak at $\Delta t = 0.63$ min, the number of the separated stereoisomers can be correctly estimated as $n_{max} = 15$.

Since the computed Δt and σ parameters measure the mean properties of the chromatogram, the ratio $\Delta t/4\sigma$ represents the mean resolution R_s for enantiomeric pairs: Δt is the mean interdistance between the separated enantiomeric pairs and 4σ the mean peak width. For the chromatograms of the TFAA–TFE and HFBA–HFB derivatives (Figs. 1a and 2a) R_s values, respectively, of $R_s = 2.08$ and $R_s = 2.38$ are obtained. These results perfectly agree with the values computed as average on R_s for each separated enantiomeric pair (data in Table 3, 2nd–3rd columns): $R_s = 2.06$ and $R_s = 2.35$ for the TFAA–TFE and HFBA–HFB derivatives, respectively. These results are proof of the reliability and robustness of the EACVF method in evaluating complex GC separations and its applicability to identify and characterize specific retention patterns such as the enantiomeric separation.

5. Conclusions

The results show that the described procedures enable the enantiomeric separation and quantification of 14 enantiomeric pairs of amino acids by combining the chiral selectivity of the commercially available Chirasil-L-Val capillary column and the high selectivity of the SIM detection mode.

Both the methods are simple and fast procedures, based on one-step derivatization reactions, and both display a wide linearity range at trace level (nmol detection limits) for quantitative determinations: these properties make the methods suitable candidates for designing instrumental devices for the in situ analysis of chiral organic compounds of exobiological interest onboard space exploration probes. The space suitability can be enhanced by applying a signal processing method to interpret the data recovered from space GC–MS experiments. Information on the chemical composition of samples collected in space missions, in particular characterization of the enantiomeric excess in amino acids, can be extracted with a simple and automatic procedure reducing the labor and time required as well as the subjectivity introduced by human intervention.

A subsequent challenge is full automation of the entire procedure, making it compatible with remote control conditions. Then, it could be integrated into space instrument sub-systems used to perform extraction, evaporation and derivatization in a single reactor coupled to GC–MS for in situ analysis in extraterrestrial environments [7–9]. Further developments of the enantioseparation system will also focus on instrument miniaturization or implementation into two-dimensional GC × GC apparatus for the pre-separation of achiral components prior to enantiomer resolution [38].

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