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Search for evidence of life in space: Analysis of enantiomeric organic molecules by N,N-dimethylformamide dimethylacetal derivative dependant Gas Chromatography–Mass Spectrometry

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ABSTRACT

Within the context of the future space missions to Mars (MSL 2011 and Exomars 2016), which aim at searching for traces of life at the surface, the detection and quantitation of enantiomeric organic molecules is of major importance. In this work, we have developed and optimized a method to derivatize and analyze chiral organic molecules suitable for space experiments, using N,N-dimethylformamide dimethylacetal (DMF-DMA) as the derivatization agent. The temperature, duration of the derivatization reaction, and chromatographic separation parameters have been optimized to meet instrument design constraints imposed upon space experiment devices. This work demonstrates that, in addition to its intrinsic qualities, such as production of light-weight derivatives and a great resistance to drastic operating conditions, DMF-DMA facilitates simple and fast derivatization of organic compounds (three minutes at 140 °C in a single-step) that is suitable for an *in situ* analysis in space. By using DMF-DMA as the derivatization agent, we have successfully identified 19 of the 20 proteinic amino acids and been able to enantiomerically separate ten of the potential 19 (glycine being non-chiral). Additionally, we have minimized the percentage of racemized amino acid compounds produced by optimizing the conditions of the derivatization reaction itself. Quantitative linearity studies and the determination of the limit of detection show that the proposed method is also suitable for the quantitative determination of both enantiomeric forms of most of the tested amino acids, as limits of detection obtained are lower than the ppb level of organic molecules already detected in Martian meteorites.

1. Introduction

To search for traces of life in extraterrestrial settings, it is mandatory to analyze obtained extraterrestrial samples for known terrestrial organic biomarkers. Ideally, presence of these biomarkers will provide non-disputable evidence that the sample's planetary source has supported some form of life. Thus, terrestrial biomarkers must be either a remnant, or a characteristic of life, and must indicate life only. There are several known classes of organic compounds that could be used as possible life indicators: amino acids, the monomer building blocks of proteins, biologically active nucleic acids and their associated sugars such as the monomers of DNA and RNA, and lipids or their carboxylic acids degradation products. While these compounds are not functional biomarkers in and of themselves, the analysis of their optical activity/chirality should be a good complementary diagnostic tool from which to derive evidence of biotic or prebiotic activity. It is important to note that unlike the amino or nucleic acid classes, carboxylic acid compounds are highly resistant to decomposition in oxidizing soils like those predicted on Mars [1,2]. As such, detecting lipid-derived carboxylic degradation products in extraterrestrial samples is the most likely scenario.

Because of the conditions under which they are operated, space instruments for *in situ* analysis must meet several stringent design requirements. Miniaturization (small size and mass), a low energy consumption/power intake, high mechanical and shock strength, resistance to vibration, and simplicity of construction [3,4] are all necessary if an instrument is to be included in a space mission. Gas Chromatography (GC) coupled with mass spectrometry [5] instruments fulfils these requirements. Given the compatibility of GC instrumentation with space experimentation demands it is unsur-

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prising that GC has already been selected for inclusion into the next generation of Mars surface exploration rovers, i.e. the NASA/MSL with SAM experiment and the ESA/Exomars with MOMA experiment. Unfortunately, GC instruments can only be used to analyze molecules which are volatile and thermally stable. As such, in their unadulterated state, few or none of the proposed terrestrial organic biomarkers would be compatible with a GC–MS experiment. However, it has been demonstrated that refractory molecules can be derivatized prior to GC analysis to facilitate their detection by GC–MS [8]. Within the context of the proposed NASA/MSL and the ESA/Exomars missions, all GC experimentation designed for terrestrial biomarker detection will thus include a pre-experiment chemical derivatization procedure [6,7].

To date, the most popular derivatizing agent used in conjunction with GC–MS experimentation is (N-(t-butyldimethylsilyl)-N-methyltrifluoroacetamide, MTBSTFA) [2,6,9–13]. However, MTBSTFA is unsuitable for enantiomeric separation of amino acids. To separate amino acid enantiomeric pairs, other alternate derivatizating procedures have been developed. Alkylalkoxycarbonylation-esterification (alkyl chloroformate) [14–16] is one such example. A one-step reaction mechanism, alkylalkoxycarbonylation-esterification effectively esterifies the carboxylic group of the amino acid residue but protects the amino terminus (or other groups susceptible of nucleophilic substitutions) from derivatization. However, while the alkylalkoxycarbonylation-esterification reaction is effective in derivatizing amino acids so as to facilitate their enantiomeric separation, unfortunately, the alkylalkoxycarbonylation-esterification reaction is not compatible with the experimental restrictions imposed upon space experiments. First, the rapidity of the reaction time of the alkylalkoxycarbonylation-esterification reaction assumes a *sine qua non* step of vigorous stirring. As the yield of recovery decreases with an increase in the reaction time, without perpetual shaking to ensure homogenization, recovery rates from the reaction decrease dramatically. The type of perpetual reaction agitation needed to maximize product recovery rates cannot be achieved for *in situ* analyses in conjunction with space missions. Moreover, the alkylalkoxycarbonylation-esterification reaction produces heavy molecular weight derivatives and by-products, the detection of which can only be achieved in a relatively high mass range of the mass spectrometer. Space mass spectrometers are often mass (m/z) limited, and thus, can detect only low mass derivatives. The respective weights of the derivatives and the by-products produced from the alkylalkoxycarbonylation-esterification reaction extend beyond the range of detection for this class of mass spectrometric instruments. Additionally, the reaction products generated using the alkylalkoxycarbonylation-esterification reaction can be separated by Chirasil-val stationary phases only. In fact, the reaction mixture physically deteriorates the commonly used cyclodextrin-based columns. Finally, the reagents used in the Alkylokoxycarbonylation-esterification reaction need to be stored at low temperatures, making the reaction especially incompatible with space missions [17].

An alternate derivatization technique used to facilitate enantiomeric separation of amino acids is perfluoroacylation/perfluoroesterification. This reaction can be performed automatically, in a single step, and requires no mixing device. Additionally, the technique displays a detection sensitivity that is compatible with space sample analysis [18,19]. Despite its advantages over the alkylalkoxycarbonylation-esterification method, however, there are several major features of the perfluoroacylation/perfluoroesterification method that make it ill-suited for space experimentation. First, the derivatization generates a high rate of racemized amino acid products, ranging from 10 percent to 40 percent of the parent product. Like the alkylalkoxycarbonylation-esterification reaction, perfluoroacylation/perfluoresterification generates derivatives of higher molecular weight than would be optimal for detection by space mass spectrometers. Moreover, the perfluoro-agent is highly corrosive and potentially damaging to both the GC-column and instrument. Lastly, reproducibility of the perfluoroacylation/perfluoroesterification reaction ranges from 3 percent to 11 percent, far lower than would be ideal for a space mission [18–22].

DMF-DMA dependant derivatization has been proposed as alternate chiral-based separation technique for amino acid compounds [23–25]. In fact, in March 2004, when the ESA/Rosetta mission was launched, the mission incorporated a novel, single step DMF-DMA-based strategy for amino acid derivatization developed by Meierhenrich et al. [27]. This technique represents the first derivatization technique to be incorporated into a space mission. Currently, this workflow has been incorporated in the COSAC experiment [28] based on a multi-capillary GC coupled with mass spectrometric instrumentation [29,30], and it should allow for the derivatization and subsequent chiral separation of any amino acids present at the surface of comet 67P/Churyumov-Gerasimenko. However, information regarding the applicability of using this technique for space applications is largely missing from the field [10,26]. While Meierhenrich co-workers did report the specific type of chiral columns used during chemical separation [28] and determined whether the selected columns could resist space constraints [31], no mention was given to the nature of their exact procedure or the choice of their specific experimental parameters. It has been well demonstrated that the DMF-DMA reaction is largely unaffected by high temperature (DMF-DMA derivatization still occurs at temperatures >190 °C when heat is maintained for more than one hour), leads to the production of low molecular weight products required for experimentation compatible with space mission analyses (limiting the MS instrument mass range required for detection), and the reaction can be readily automated. Thus, even despite the dirth of direct research on the topic, one can conclude that the DMF-DMA reaction is well-suited for space applications. DMF-DMA as a derivatization agent is therefore of great interest for use in potential analytical experiments proposed for future Mars exploration missions and it is only a lack of data that has prevented evaluating the potential of incorporating the technique into future Martian probes.

The aim of this paper is to study the mechanism of DMF-DMA-based derivatization and to use said analysis to develop a simple, competitive, and automated chiral derivatization procedure to be incorporated into the SAM and MOMA Mars experiments. The designed derivatization procedure must enable the *in situ* analysis of molecules of exobiological interest, such as amino acids, within the constraints of a space experiment. As our work shows, using DMF-DMA derivatization, we were able to detect and characterize all 20 proteinic amino acids by GC using the chiral capillary column (Chirasil-Dex) that will be used in the SAM and MOMA experiments. In fact, the Chirasil-Dex column proved to be the most effective capillary column for separation of the derivatives we generated with the DMF-DMA reaction [26].

2. Experimental

2.1. Chemicals and materials

For all experiments within this study, all 20 proteinic amino acids, in both L and D forms, purchased from Sigma–Aldrich and Fluka (Strasbourg, France). Sigma–Aldrich and Fluka also supplied the derivatization grade N,N-dimethylformamide dimethylacetal (DMF-DMA) used. Methyl Laurate (Fluka), diluted to 10^{-2} mol L^{-1} in ethyl acetate was used as the internal standard solution. One μL of the diluted methyl laurate solution was added to each derivatized

sample with a Research Eppendorf P10 pipette, precision of ±2.5 percent.

2.2. Derivatization process

Prior to derivatization, all amino acid samples were dissolved in distilled water to obtain standard stock solutions with concentrations ranging between 10^{-2} and 10^{-1} mol L^{-1}. The labile hydrogen of water molecules reacts with DMF-DMA, leading to an overreaction of the derivatization mechanism. To prevent amino acid degradation as a result of this overreaction, before addition of the derivatization solvents, water within the amino acid solutions was completely evaporated under constant dry nitrogen flow. Placing the reaction under constant nitrogen flow served to increase the rate of the evaporation process. The temperature of the evaporation process was kept sufficiently low so as to not induce degradation of any amino acid compounds. For technical facilities, the temperature of evaporation was fixed at 40 °C.

Our proposed DMF-DMA derivatization reaction is a simple one-step procedure. For each reaction performed, 20 μL of the derivatizing agent, DMF-DMA, was added directly to a small, one mL glass jar containing the dried amino acid sample. The reaction vial was then immediately placed in an oil-bath for incubation. Temperature of the oil-bath varied from 80° to 160°, depending on the specific parameters of the experiment. The derivatization reaction was also time-dependant; experimental samples were placed in the heated oil-bath for times ranging from 30 s to 60 min. Immediately after the reaction vial was removed from the oil-bath, one μL of the standard solution (methyl laurate 10^{-2} mol L^{-1} in ethyl acetate) was added to the reaction mixture. The resultant sample was injected into the GC–MS for analysis. Because of the derivatization reaction, the injected molecules were volatile, and therefore vaporizable, thus enabling GC–MS analysis. The derivatization reaction scheme for amino acids is illustrated in Fig. 1. For amino acid samples, the derivatization reaction leads to formation of an amidine ester.

2.3. Gas chromatography mass spectrometric analysis

All experiments were performed using a Shimadzu Gas Chromatography/Mass Spectrometer QP 5050A. For each GC–MS analysis, a one μL syringe from Hamilton was used to inject one μL of solution into the GC–MS. Before each injection, the syringe was carefully washed ten times with acetone to avoid chemical contamination, and then again with ethanol to prevent biological contamination. The QP 5050A GC/MS instrument was operated in quadrupole detection mode with the absolute detector voltage set at one kV. The GC–MS was equipped with a split/splitless injector, the temperature of which was set to 200 °C. For all experiments, the QP 5050A GC–MS was outfitted with Varian's CP-Chirasil DEX CB capillary column (Chirasil-β-dex (eptakis-(2,3,6-tri-O-methyl)-β-cyclodextrine), WCOT Ultimetal type, 30 m length, 0.25 mm internal diameter, and 0.25 μm film thickness. Maximum operating temperature of the column never exceeded 200 °C. To enhance overall lifetime of the Chirasil-Dex column, the liner was coated with DMCS-treated glass wool purchased from Varian (France). Pure helium (99.999 percent) was used as the carrier gas for the GC–MS. Shimadzu LabSolutions GC–MS solution Version 1.01. Real Time Analysis software controlled data acquisition and Shimadzu GC–MS Post run Analysis software was used to facilitate data analysis.

To determine each of the amino acids' unique spectral characteristics, each amino acid was initially characterized separately on the Chirasil-Dex column. Results generated were used to expand the existing NIST mass spectral library of DMF-DMA amino acids derivatives.

3. Results and discussion

As previously explained, DMF-DMA has already been used to derivatize amino acids, but no systematic study of the reaction mechanism for *in situ* analyses of chiral organics has been published [10]. Given the potential applicability of the derivatization reaction for space experimentation, DMF-DMA derivatization seems very promising for future *in situ* analyses, like the planned Exomars Martian mission. Indeed, the derivatization reaction is simple, and easily automated. DMF-DMA remains active for over an hour at temperatures as high as 190 °C, and, contrary to all other derivatizating agents, has a low mass, leading to the formation of low mass derivative products. Given that space mass spectrometers are often mass (m/z) limited and can thus only detect low mass derivatives, this last feature makes DMF-DMA especially attractive as a space derivatization agent. The proposed aim of this study was to determine the best analytical conditions for a derivatization reaction for space applications. To this end, we studied the following parameters: reaction temperature and duration, derivative efficiency enantiomeric resolution, analysis time, and MS detectability. Method validation was completed by investigating the precision, detection (LOD), and quantification limits (LOQ) of our technique. By studying the relative effect of different reaction parameters, such as the time and temperature of derivatization, the chromatographic separation, the quantitative aspects of the method, the structural information of the derivatives, and the mass spectra generated, we have been able to ensure that the parameters of the proposed reaction produce optimal results.

3.1. GC optimization

To ensure all experiments were completed in the shortest time possible without compromising chromatographic resolution, MS detection sensitivity, or the MS detection range, operating temperature parameters and instrument settings were determined prior to experimental data collection. The main factors tested to determine experimental settings were those thought to be most critical for analyte separation. Following experimentation to determine optimal settings for the instrument's helium carrier gas flow rate, split ratio, initial column temperature, and subsequent temperature profile, the following parameters were adopted: the split ratio was set to 80, and the temperature profile consisted of a starting temperature of 70 °C held for duration of five minutes, a linear gradient increasing 3 °C/min, and a ten minute temperature plateau held at the maximum temperature of 190 °C. Once temperature

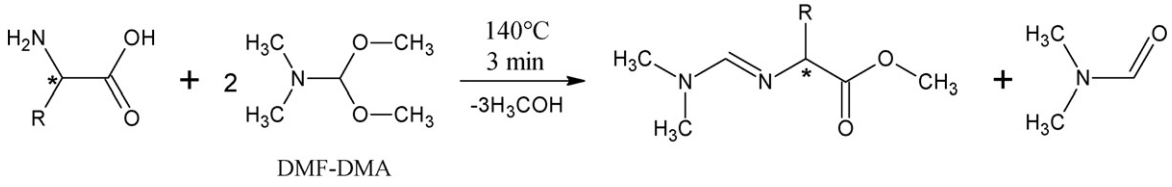

Fig. 1. Scheme of the derivatization reaction between amino acid and DMF-DMA. If a chiral center (*) exists, then it is conserved.

parameters were selected, optimum mobile phase velocity was determined by searching for the minimum height equivalent to a theoretical plate (HETP). Optimizing mobile phase velocity can dramatically decrease the time needed to complete an analysis, a key requirement of space experimentation. Typically, the HETP calculation depends upon the nature of the solute, the stationary phase used, the column geometry imposed, and the temperature during operation. For our study, the HETP was determined by calculating the ratio HETP = L/N with L, the column length and N the number of theoretical plates, N being determined through this equation: $N = 5.54(t_R/w_h)^2$, with the half-height of the chromatographic peak, and t_R the retention time. The HETP was then plotted as a function of the helium carrier gas velocity to provide Golay curves for three separate amino acids: Alanine, Leucine and Phenylalanine. Each measurement was taken three times to ensure satisfactory precision. As Golay curves are plotted for isothermal measurements, reference temperatures were selected for each amino acid. From our previously determined temperature programming, Alanine, Isoleucine, and Phenylalanine elute at 20.2, 28.2 and 44.6 min, respectively. Elution temperatures for these three amino acids were thus selected at 118, 146 and 190 °C, respectively. The Golay curves obtained are plotted in Fig. 2. The maximum efficiency obtained for all three tested amino acids was with a linear velocity ranging from about 30 to 42 cm s^{-1}. As the aim of our optimization study was to limit the time of analysis, we selected the highest velocity, i.e. 42 cm s^{-1}, as the carrier gas velocity for all subsequent studies. As the graph and chromatograms indicate, a carrier gas velocity of 42 cm s^{-1} is equivalent to a GC flow rate of 1.2 mL min^{-1}. Using this flow rate for all experimentation ensured efficient separation of all amino acid species without compromising rapid instrument analysis and runtime.

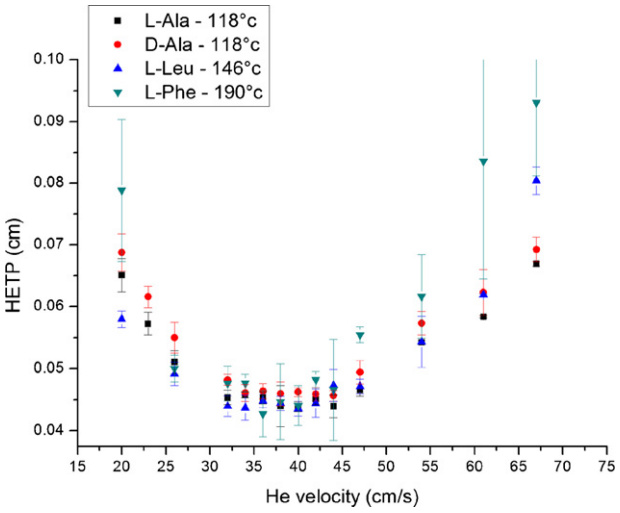

Fig. 2. Evolution of HETP versus U_{mean} different amino acids derivatized with DMF-DMA. The points represent the mean of the experimental data and the lines represent the error bars statistically estimated.
Derivatization with 20 μL DMF-DMA, 3 min at 140 °C. Injection of 1 μL of the mixture. GC settings: injector 250 °C, split mode 1/80, detector 200 °C. Isothermal temperature is depending of the amino acid studied: 118, 146 and 190 °C respectively for alanine, leucine and phenylalanine. Mass detector range, m/z from 40 to 350 u.

3.2. Mass spectra library and structural information on the derivatives

Our experimental results have been added to the pre-existing NIST mass spectral library for all 20 amino acids derivatives. Because the DMF-DMA derivatization reaction generates spurious by-products in addition to the primary amino acid product, many parent amino acid compounds were represented on the GC chromatogram as multiple derivatized products. Thus, to confidently identify and distinguish each derivatized amino acid, it was necessary to analyze each DMF-DMA derivative reaction one at a time. The mass spectra generated for all derivatives are listed in Table 1.

All DMF-DMA derivatives show a similar, characteristic fragmentation pattern. Prominent peaks are present in all MS/MS spectra at $m/z = 44$ amu and $m/z = 42$ amu, corresponding to the ion $(CH_3)_2N^+$ generated from the amine part of the molecule and the iminium ion $(CH_2)N^+(CH_2)$ respectively. Other ions present in

Table 1
Retention time for the L and D enantiomers, and characteristic peaks of mass spectra of the amino acid derivatives.

Amino acid	Retention time (min)	Mass fragments (m/z) in u and their relative intensity
Threonine	18.32–19.01, 22.3–23.2	84(100) 57(71) 42(20) 44(16) 56(14) 40(12) 71(12)
Serine	18.42–19.57	70(100) 42(85) 43(30) 44(25) 40(20) 69(18) 41(16) 101(13). 129(4)
Alanine	20.14–20.94	44(100) 99(62) 42(25) 56(13) 158(6)
Glycine	22.44	44(100) 42(39) 85(36) 112(8) 144(7)
Valine	24.42–24.55	44(100) 143(45) 127(34) 42(25) 46(14) 57(11) 41(10)
Leucine	28.19	44(100) 141(53) 156(32) 42(30) 85(24) 40(15) 73(14) 99(13)
Isoleucine	28.13	44(100) 143(55) 42(28) 141(27) 156(24) 73(19) 41(16)
Proline	35.40–35.77	98(100) 70(94) 43(44) 41(43) 68(19) 42(17) 44(13) 129(10) 157(7)
Aspartic acid	35.92–36.08	44(100) 157(41) 42(30) 99(18) 115(14) 156(11) 57(11) 216(9)
Asparagine	35.92–36.08	44(100) 157(40) 40(30) 42(29) 143(18) 99(15) 115(14) 156(10) 57(10) 45(9)
Cysteine	36.47–36.59	44(100) 143(50) 42(20) 46(11) 40(9) 57(5) 159(5) 204(2)
Methionine	40.01	44(100) 157(45) 42(35) 61(33) 111(28) 112(28) 144(23) 40(19) 143(18) 84(17) 57(16) 45(13) 97(12) 159(11) 218(6)
Glutamic acid	40.32	44(100) 40(44) 42(40) 111(38) 171(26) 57(17) 157(16) 46(16) 143(15) 73(14) 230(9) 199(8)
Glutamine	40.32	44(100) 111(69) 42(49) 171(36) 157(26) 41(23) 46(23) 57(23) 84(19) 170(19) 230(14) 199(13)
Phenylalanine	44.73–44.88	44(100) 143(63) 40(30 42(21) 45(11) 175(9) 73(6) 57(5)
Lysine	46.89	44(100) 70(42) 46(41) 42(33) 85(28) 99(23) 40(23) 185(17) 199(11) 142(10) 257(7) 242(7) ET 44(100) 115(62) 42(45) 46(41) 40(25) 143(18) 242(18) 157(17) 257(7)
Tyrosine	47.68–47.77	121(100) 44(57) 40(38) 192(19) 42(16) 143(16) 130(12) 45(10) 122(10) 73(8) 251(4)
Histidine	49.42	44(100) 42(93) 57(52) 192(52) 40(52) 112(39) 96(21) 137(15) 84(14) 164(8)
Arginine	51.38	44(100) 42(40) 98(34) 184(27) 99(26) 100(22) 43(21) 72(21) 46(21) 57(15) 125(14) 256(13)

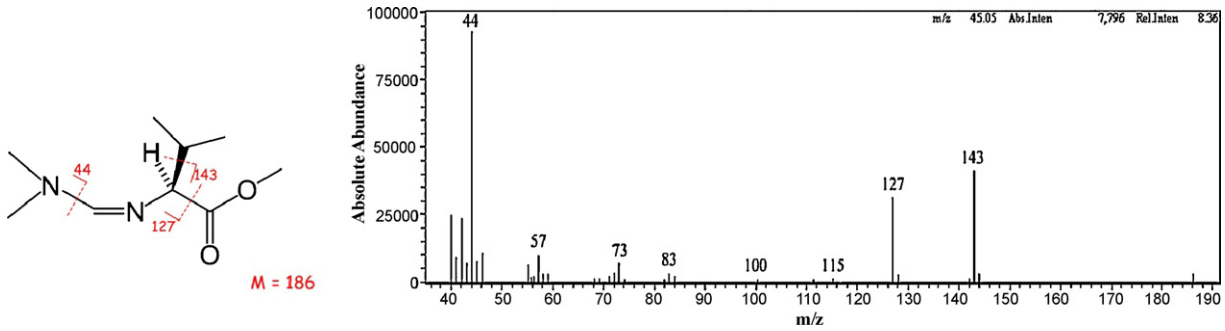

Fig. 3. Mass spectrum of valine amidine ester, and its proposed fragmentation pattern (the collision energy is 70 eV).

MS/MS spectra are detected at $m/z = 59$ amu, or can be attributed to an α-C-C cleavage of the carbonyl group with consecutive loss of the ion C^+OOCH_3, or from internal cleavage at various points within the molecular ion itself. An example of the fragmentation mechanism proposed for the amidine ester of Valine is reported in Fig. 3. Fragmentation of the amidine ester of Valine represents the simplest model observed. For a few of the derivatized amino acid products, derivatization and subsequent GC–MS/MS analysis generated multiple chemically separate species. These species can be attributed to either an incomplete derivatization of the parent amino acid or a molecular rearrangement of the molecule in the gas phase. Lysine is one such example. As the Lysine residue contains two amine groups (NH_2) and one carboxylic group, Lysine derivatives can be either mono or bi-amidinated. These two possible Lysine derivative species elute at different chromatographic retention times and produce different mass spectra. Similarly, Arginine is represented on the chromatogram by several different chromatographic peaks over a wide elution period. In cases where several peaks were observed from a single amino acid, the most prominent peak (largest peak area) within the reference spectra was chosen as the signature reference peak to be used in the rest of the study to identify the amino acid from the amino acid mixture.

3.3. Optimization of the derivatization procedure

To determine the optimal reaction conditions needed to produce maximum product yield with the lowest yield of racemization, the influence of temperature and reaction length on the efficiency of the derivatization reaction was studied. To ascertain a reaction's response sensitivity, the peak areas of the considered compound and the internal methyl laurate standard were determined on the Total Ion Chromatogram (TIC) (obtained using the instrument's full-scan mode) and subsequently compared. For each amino acid, the relative efficiency of the derivatization reaction has been estimated for reactions occurring at temperatures ranging from 80 to 160 °C for 30 s to 60 min. The obtained results show that similar trends exist as a function of varied temperature or reaction time for most of the amino acids studied. Here, we present results for three of the studied 20 amino acids (Fig. 4). As demonstrated in Fig. 4, DMF-DMA is able to derivatize all of the amino acids at temperatures as high as 160 °C. However, it must be mentioned that heating the DMF-DMA reaction to 160 °C generates more spurious by-product species than when the reaction is heated at lower temperatures. At 120 °C, the relative rate of derivatization increases for the first six seconds of the reaction (Fig. 4a), and then slowly decreases as the reaction is prolonged. Optimal reaction time can thus be determined; for our experiments, this reaction time was reached in about nine minutes. For higher reaction temperatures (140 and 160 °C), a similar pattern is observed (Fig. 4a and b), but an exponential-shaped increase in derivatization production occurs, followed by a drastic drop in the concentration of derivatized prod-

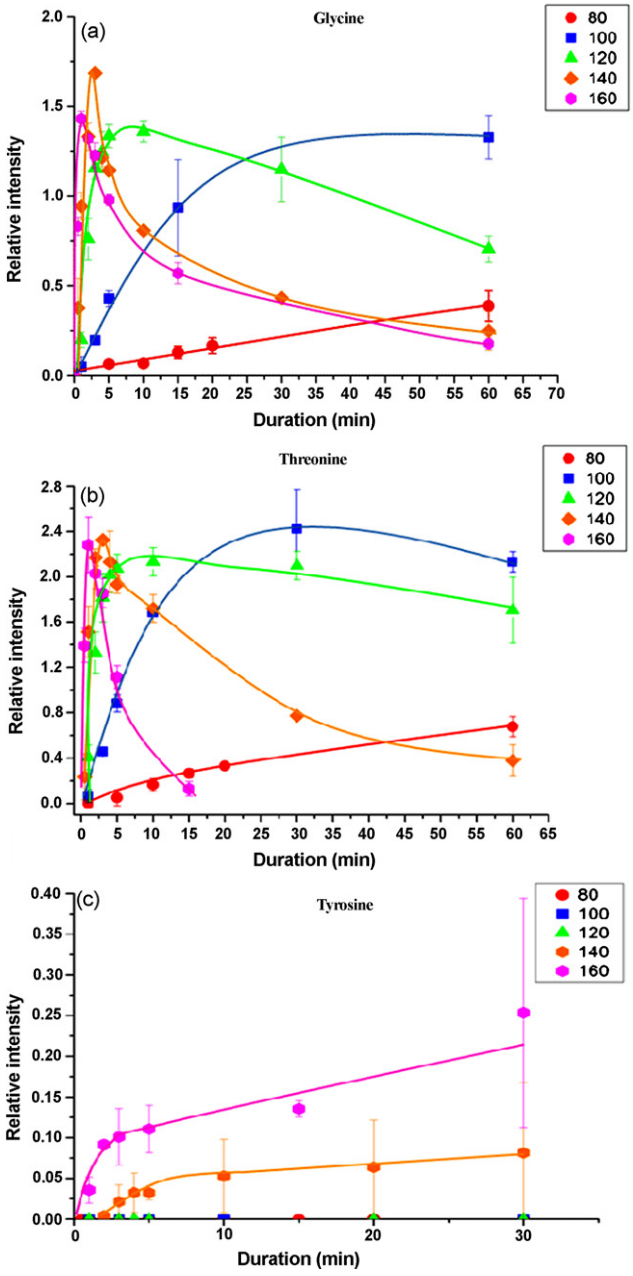

Fig. 4. (a, b and c) Plots of relative efficiency of derivatization (A/AIS) depending on the duration of derivatization (in min) for glycine, threonine and tyrosine. 0.5 μL of Gly 10^{-1} mol L^{-1} has been derivatized (20 μL of DMF-DMA) for each point. Each set of points represents a temperature of derivatization. There are 3 repetitions for each point and the mean is represented on the plot.

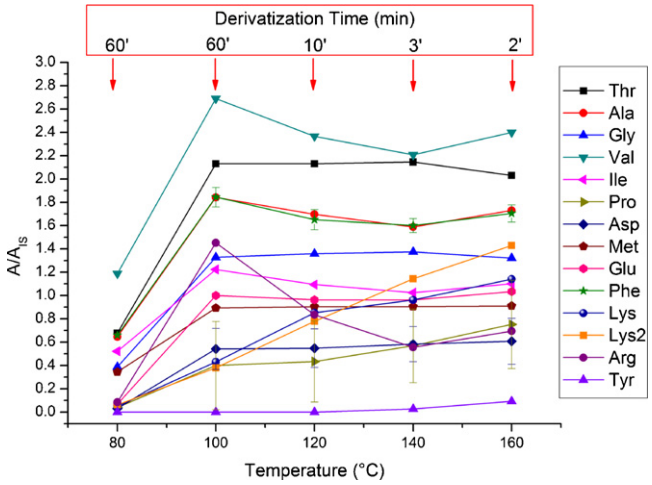

Fig. 5. Relative efficiency of derivatization depending on the temperature of derivatization, from 80 to 160 °C. For each temperature, the optimal duration of derivatization has been determined. 80 °C-60 min, 100 °C-60 min, 120 °C-10 min, 140 °C-3 min and 160 °C-2 min.
Error bar represents the racemization percentage of amino acids at the studied temperatures.
From the mother solution at 10^{-2} mol L^{-1}, amino acid solution are made as following: 5 + 5 μL L,D-Thr, 10 + 10 μL L,D-Ser, 0.5 μL Gly, 2 + 2 μL L,D-Ala, 0.5 + 0.5 μL L,D-Val, 0.5 + 0.5 μL L,D-Ile, 5 + 5 μL L,D-Pro, 2 + 2 μL L,D-Asp, 30 + 30 μL L,D-Cys, 0.2 + 0.2 μL L,D-Met, 1 + 1 μL L,D-Glu, 25 + 25 μL L,D-Tyr, 0.1 + 0.1 μL L,D-Phe, 8 + 8 μL L,D-Lys, 50 + 50 μL L,D-His, 10 + 10 μL Arg. After water evaporation, derivatization has been done with 20 μL DMF-DMA. Each point is the mean of at least 3 repetitions.

uct caused by amino acid degradation. At higher temperatures, degradation of the parent amino acid compound can be observed for even the shortest derivatization durations. With the exception of reactions incubated at 80 °C, for all reactions studied, the maximum derivatization rate was obtained for reactions at every temperature point. Thus, we can conclude that an optimal derivatization duration does exists for most of the temperatures tested. If the derivatization reaction is allowed to extend beyond this time, parent compound degradation occurs, leading to an overall decrease in the relative derivatization yield. Our determined optimum is reported in Fig. 5 as a function of reaction time and temperature. In the 100–160 °C temperature range, the maximum rate of derivatization is relatively constant regardless of the reaction temperature. For ExoMARS experiments, then, the temperature used will be dictated by the time allocated for the derivatization step. For example, if shorter times for the derivatization reaction are required, it will be necessary to use higher temperatures. For temperatures lower than 100 °C, we observe that the derivatization yield increases continuously regardless of reaction time. For example, as shown in Fig. 4a, for derivatization of Glycine at 80 °C, the percent product yield never reaches a maximum within experimental conditions (60 min). Thus, we can conclude that for the time parameters used in our study, the maximum derivatization yield cannot be reached for temperatures lower than 100 °C.

However, even if this is the general trend, not all amino acids demonstrate this characteristic behaviour. Some amino acids are more resistant to derivatization than others. Indeed, Tyrosine (Fig. 4c) is the extreme case. At lower temperatures (80 or 100 °C), regardless of reaction time, no derivatization of the Tyrosine parent species has been observed. Even at the highest temperature and longest reaction time tested, the rate of formation of the Tyrosine derivative continued to increase, never reaching a maximum point. The highest reaction yield we were able to generate was obtained using the upper limits of our experiment; reaction conditions consisting of 160 °C heat applied for 60 min. If Tyrosine is present in a sample collected during space experimentation, derivatization with DMF-DMA will be incomplete, and detection of the residue will be hindered.

In order to determine the best temperature and reaction duration settings, we have also studied the evolution of the derivatization rate as a function of the temperature (Fig. 5). To simplify results, we have only reported how derivatization of each amino acid varies as a function of the previously determined optimal temperature and reaction time (Fig. 4). As our data demonstrates, the maximum derivatization yield is achieved when the derivatization reaction proceeds at a temperature of 100 °C. With the exception of Tyrosine and Lysine derivatives, at a temperature of 100 °C, the rate of formation of derivatized products plateaus midway through the reaction. From our analysis, we can deduce that our derivatization technique is well-suited for reaction temperatures kept higher than 100 °C, and that an increase in the temperature of the reaction can limit the overall time required for the derivatization process. Before making a final decision regarding the temperature and duration of the derivatization step, however, several important points needed to be considered. First, for use as a space application, *in situ* derivatization needs to be both minimally energy and time consuming. Second, the derivatization reaction must be applicable for detection of enantiomeric excess. Lastly, reaction conditions must minimize the possibility of generating racemized amino acid products. As reaction temperatures increase, so to does the risk of generating racemized products. The same phenomenon is observed with derivatization duration. Thus, before determining temperature parameters and reaction duration, it became necessary to study the influence of DMF-DMA derivatization upon racemized amino acid formation.

3.4. Racemization

For all chiral amino acids, any asymmetric carbon bound to hydrogen and adjacent to an electron-withdrawing group, such as the oxygen-rich carboxyl of organic acids, will easily exchange that hydrogen when in the presence of an H_2O or OH^- molecule. Additionally, this alpha-hydrogen atom may also be attacked by another base. During hydrogen exchange or base attack, the three remaining functional groups of the amino acid molecule will rearrange, producing a planar molecular ion. A hydrogen ion can then bond on either side of the plane with equal likelihood, thus racemizing the compound, i.e. converting the molecule from one configuration to the other [32]. This racemization process will only be enhanced during reactions with increasing temperature and heating duration.

To investigate the probability of generating racemized amino acids during the DMF-DMA derivatization process, each amino acid compound has been injected onto the GC-column in its L-form only. Error bars present on Fig. 5 represent the rate of racemization reported for each value. Excepted for Proline and Aspartate, the two amino acids that display significant racemization, no racemization has been observed for derivatization reactions heated to 80 °C, regardless of the duration with which the reaction was allowed to proceed (Fig. 5). As shown in Fig. 5, racemization is a kinetically controlled reaction. Indeed, at higher reaction temperatures, an increase in the duration of derivatization is directly linked to an increase in the rate of racemization. The higher the reaction temperature, the faster the rate of racemization increases as a function of the reaction's duration. For most of the amino acid compounds tested, when the DMF-DMA derivatization reaction proceeds at 140 °C, less time is needed to fully derivatize the parent amino acid, highest yield for most amino acids is detected, and products from the derivatization reaction separate better under the chromatographic conditions of the experiment than when the derivatization reaction is allowed to proceed at different temperatures. However, under these conditions, some hardly derivatized amino acids can still be observed. When the reaction proceeds at

lower temperatures, however, derivatized Tyrosine groups cannot be detected at all, and Lysine and Proline residues are only partially derivatized, thus making their detection difficult. At higher reaction temperatures (160 °C), a high percent yield of racemized product occurs, making these higher temperature reactions incompatible with experimental objectives (Fig. 5).

To facilitate the most advantageous compromise between racemization and derivatization yield, reactions were allowed to proceed for three minutes at a temperature of 140 °C. In fact, for all 20 amino acids, these reaction settings produced the most optimal products in regards to product yield, number of amino acids derivatized, and percent racemization. Indeed, using a higher reaction temperature ensured that the derivatization reaction occurred when the energetic barrier was intense; the short reaction duration helped prevent both degradation of the thermally fragile components and racemization.

An extensive study was undertaken to determine how to minimize the occurrence of racemization for the three minute, 140 °C reaction. In Table 2, we have reported the L/D ratio and the enantiomeric excess, calculated as followed by Eq. (1).

Enantiomeric excess:

$$E_e\,(\%) = 100\frac{L - D}{L + D} \quad (1)$$

As demonstrated in Table 2, racemized products were not observed for any amino acid except for Asn, Asp, Pro, Ser and Tyr. Four of those amino acids (Asn, Asp, Pro and Ser) displayed partial racemization; only Tyrosine displayed complete racemization. For Asn, Asp, Pro and Ser, racemization generated by derivatization will not pose a problem for space applications if the racemization rate is well determined. Indeed, identifying the exact rate of racemization allows the initial value of enantiomeric excess to be elucidated from a simple back-calculation.

It has been suggested that oxygen pressure could affect racemization rate [29]. We thus derivatized our amino acid compounds in a nitrogen-enriched environment. Like Borjesson et al. [33], we also detected no significant difference in percent racemization between derivatization reactions within the presence or absence of oxygen. As such, we decided not to continue in oxygen-free conditions.

Table 2
Enantiomeric excess and D/L ratio of all the separated amino acids. Standard deviation (SD) has been calculated on 3–7 repetitions depending on the AA. Derivatization conditions are 140 °C during 3 min.

Amino Acid	E_e (percent)	L/D ratio	SD
L-Threonine	100	∞	ND
D-Threonine	100	0	ND
L-Serine	40.7	2.43	0.51
D-Serine	38.6	0.41	0.09
D-Alanine	100	0	ND
L-Alanine	100	∞	ND
D-Valine	100	0	ND
L-Valine	100	∞	ND
L-Proline	43.9	3.22	1.31
D-Proline	45.1	0.38	0.10
D-Aspartic acid	77.2	0.13	0.01
L-Aspartic acid	85.3	12.7	1.16
D-Asparagine	55.2	0.29	0.03
L-Asparagine	61.9	4.34	0.91
D-Cysteine	100	0	ND
L-Cysteine	100	∞	ND
D-Phenylalanine	100	0	ND
L-Phenylalanine	100	∞	ND
D-Tyrosine	0	1	ND
L-Tyrosine	0	1	ND

We are using an L/D ratio of 0.94 to 1.06 as the ratio equivalent for racemic mixture, theoretically determined at 1.00. Indeed, the mean of the typical uncertainty for the L/D ratio measurement on four to six repetitions of randomly picked amino acids is about 0.06 and thus a L/D ratio of 0.94 to 1.06 would be statistically indistinguishable from a racemic mixture. This value is a mean, it can vary for each amino acid studied from two to nine percent, and a detailed analysis of each compound should be completed to determine the enantiomeric excess analysis of spectra.

3.5. Chromatographic separation of the amino acids enantiomeric pairs

All the proteinic amino acids have been separated using the chromatographic conditions previously selected (Fig. 6). However under these conditions, some of derivatized amino acids co-elute:

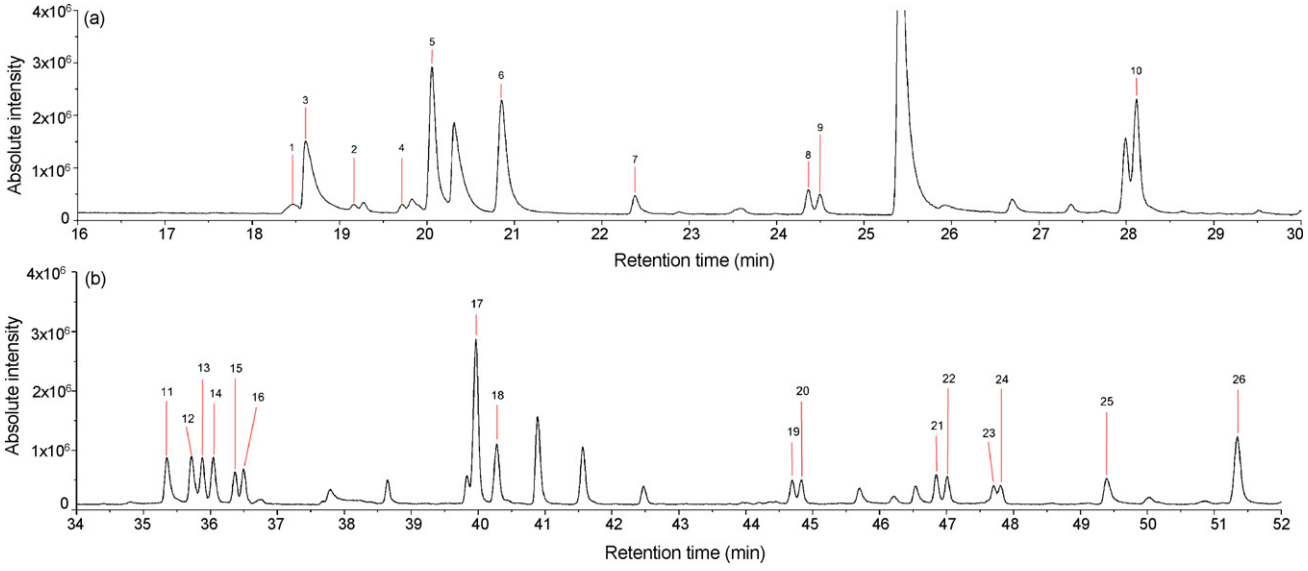

Fig. 6. (a, b) Chromatograms with all the separated amino acids. Derivatization with 20 μL DMF-DMA, 3 min at 140 °C. Injection of 1 μL of the mixture. GC settings: injector 250 °C, split mode 1/80, detector 200 °C, column 70 °C 5 min, increasing temperature rate 3 °C/min up to 190 °C, isotherm 10 min at 190 °C. Mass detector range, *m/z* from 40 to 350 uma. Amino acids have been run, in the concentrations described in Fig. 5.
1: L-Thr, 2: D-Thr, 3: L-Ser, 4: D-Ser, 5: D-Ala, 6: L-Ala, 7: Gly, 8: D-Val, 9: L-Val, 10: D + L Ile, 11: L-Pro, 12: D-Pro, 13: D-Asp, 14: L-Asp, 15: D-Cys, 16: L-Cys, 17: D + L Met, 18: D + L Glu, 19: D-Phe, 20: L-Phe, 21: D + L-Lys, 22: D + L Lys, 23: D or L Tyr, 24: L or D Tyr, 25: D + L His, 26: D + L Arg.

Table 3
Order of enantiomeric separation for the 10 observed amino acids. Retention time with the defined parameters of the GC run, D/L order and resolution.

Amino acid	Order L/D	R_s
Threonine	L D	4.73
Serine	L D	6.36
Alanine	D L	6.59
Valine	D L	0.83
Proline	L D	1.67
Aspartic acid	D L	0.97
Asparagine	D L	0.85
Cysteine	D L	0.60
Phenylalanine	D L	0.84
Tyrosine	ND*	0.70

* Complete racemization.

Glutamine co-elutes with Glutamate, Asparagine with Aspartic Acid, and Lysine co-elutes with Isoleucine. All but one proteinic amino acid is detected (Fig. 6). As Tryptophan is the heaviest of the 20 amino acids, and contains no derivatizable side chain groups, Tryptophan does not elute of the Chiral-Dex column at even the upper temperature limit (200 °C) of the column. To determine the chromatographic separation between the L and D forms of each amino acid, mixtures consisting of both members of the enantiomeric pair were injected onto the GC–MS and analyzed. Ten of the tested amino acids display chiral separation on the chromatogram (Fig. 6): Thr, Ser, Ala, Val, Pro, Cys, Asn, Asp, Phe and Tyr (Glycine is not a chiral amino acid). Threonine and Isoleucine have two asymmetric carbons. As such, Threonine is detected as two pairs of eluted compounds. The diastereoisomers of Isoleucine (R,R or S,S) and allo-Isoleucine (R,S or S,R) can be distinguished chromatographically, but do not separate enantiomerically. Due to low reactivity of its Guanidine group, Arginine is hardly detected. Glutamine and Glutamic Acid display identical elution profiles, as do Glutamine and Glutamic Acid, but each amino acid produces slightly different mass spectra. However, thanks to SIM chromatographic analysis, it is possible to identify even co-eluted amino acids.

Among all amino acids detected, our proposed method enables us to clearly separate the enantiomers of Threonine, Serine, Alanine, Valine, Proline, Aspartic Acid, Asparagine, Cysteine, Phenylalanine and Tyrosine. This chiral separation is relevant to space applications as Aspartic Acid, Serine, Glutamic Acid, Alanine, Valine, and Glycine have already been detected in micrometeorites [34].

For each amino acid, the resolution $R_S = 2(t_{r_2} - t_{r_1}/\omega_1 + \omega_2)$, which measures the quality of separation with ω the width of the peak at its base, has been evaluated for each (Table 3). The elution order for each enantiomeric pair was determined by injecting first the racemic mixture and then, the isolated L or D form (Table 3). For Threonine, Serine and Proline, the L form elutes before the D form. The opposite is true and experimentally observed for Alanine, Valine, Aspartic Acid, Asparagine, Cysteine, and Phenylalanine whereby the D-form elutes prior to the L-form. For Isoleucine, Leucine, Methionine, Glutamine, Glutamic Acid, Histidine and Arginine, only one peak is detected, the two forms are superimposed on the chromatogram and co-elute. No clear relation has been found between the physicochemical properties of an amino acid (acidity, size of the side-chain, etc.) and its chromatographical characteristics (order of elution, peak area, etc.). Some heavy compounds present in the reaction process produced several detectable by-products. Fortunately, for our experimental aims, these by-products did not interfere with analysis. However, if these compounds are the product of thermal degradation, during space experimentation, signal from the native molecule may become suppressed, and the detection limit of the experiment will be decreased. In conclusion, ten enantiomeric pairs have been separated with satisfactory R_S values (Table 3), we were able to elute 19 of a potential 20 amino acid compounds, and of those 19, 10 compounds could be quantitatively separated into distinguishable enantiomeric pairs.

3.6. Quantitative analysis: relative response, linearity and detection and quantification limits

For quantitative analysis, a calibration curve has been plotted with standard solutions for each of the derivatized amino acids, Using the previously determined chromatographic and derivatization conditions, 0.3 pmol to 2.3 nmol of each amino acid have been analyzed. Here we have investigated the linearity of the response, and also calculated the limits of detection and quantification from our experimental measurements.

The mother solution of each amino acid consisted of a concentration equal to 10^{-1} mol L^{-1}. Once obtained, one μL (equalling 10^{-7} mol) of the mother solution was evaporated under constant nitrogen flow prior to the introduction of the derivatization solvents. The derivatization reaction thus consisted of dried amino acid residue, 20 μL of DMF-DMA, plus a volume of one μL of standard solution. The final solution injected into the GC–MS instrument has a concentration of $4.8.10^{-3}$ mol L^{-1}. To elucidate the limit of detection for the GC–MS instrument, this initial solution was then serially diluted until detection of the derivatized product was no longer possible.

Table 4
Linear regression and standard deviation for each amino acid.

AA	Initial amount (nmol)	Injected amount (pmol)	Regression equation	R^2
D-Ala	2–800	1.1–460	$Y = 0.51893x - 0.06266$	0.998
L-Ala	2–800	1.1–460	$Y = 0.27684x - 0.02633$	0.999
Gly	2–800	1.1–460	$Y = 0.29966x + 0.00569$	0.999
D-Val	1–400	0.6–220	$Y = 0.26205x - 0.06192$	0.997
L-Val	1–400	0.6–220	$Y = 0.25717x - 0.06424$	0.997
Ile	1–400	0.6–220	$Y = 0.30053x - 0.08292$	0.996
L-Pro	10–4000	5.7–2280	$Y = 0.3175x - 0.09295$	0.988
D-Pro	10–4000	5.7–2280	$Y = 0.28725x - 0.05132$	0.991
D-Asp	1–400	0.6–220	$Y = 0.17784x + 0.01751$	0.995
L-Asp	1–400	0.6–220	$Y = 0.1926x - 0.0228$	0.999
D-Phe	0.5–200	0.3–100	$Y = 0.21815x - 0.07822$	0.997
L-Phe	0.5–200	0.3–100	$Y = 0.19868x - 0.06207$	0.998
L-Thr	3–1200	1.7–680	$Y = 0.04324x + 0.04853$	0.951
D-Thr	3–1200	1.7–680	$Y = 0.04544x + 0.05349$	0.955
L-Ser	5–2000	2.8–1140	$Y = 0.11878x - 0.00322$	0.999
D-Ser	5–2000	2.8–1140	$Y = 0.01648x + 0.02357$	0.926

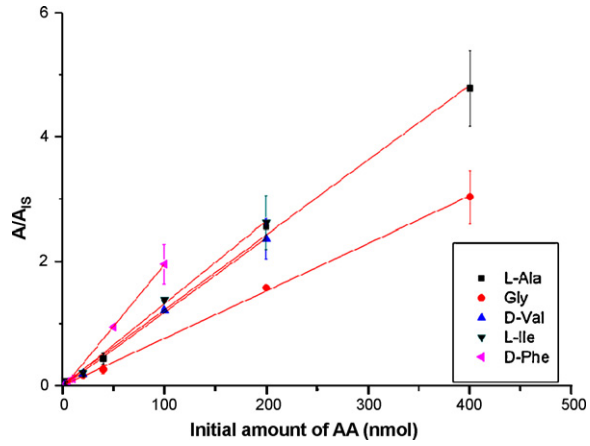

Fig. 7. Calibration curves of response of some amino acids (Gly, L-Ala, D-Val, L-Ile, D-Phe) at different concentrations.

Table 5
Experimental and theoretical limit of detection and limit of quantification of the separated Amino Acids derivatized with DMF-DMA.

Amino acid	LOD*	LOQ*	Experimental LOD 3 < S/N < 10	
			pmol	ppm
D-Ala	0.6	2.0	2.3	0.18
L-Ala	1.2	3.8	2.3	0.18
Gly	1.1	3.6	2.3	0.15
D-Val	0.6	2.0	1.1	0.12
L-Val	0.6	2.1	1.1	0.12
L-Ile	0.5	1.8	1.1	0.13
D-Ile	0.5	1.8	1.1	0.13
L-Pro	4.8	16.2	11.4	1.3
D-Pro	5.4	17.9	11.4	1.3
D-Asp	0.9	3.0	1.1	130
L-Asp	0.8	2.8	1.1	130
D-Phe	0.4	1.2	0.5	0.08
L-Phe	0.4	1.3	0.5	0.08
L-Thr	11.6	38.6	3.4	0.36
D-Thr	10.8	36.0	3.4	0.36
L-Ser	6.7	22.4	5.7	0.6
D-Ser	49.8	166.1	5.7	0.6

* Injected pmol.

3.7. Linearity/calibration curve

As a series of five sequential experiments, increasing amounts (starting amounts equal to 12–288 nmol, injected amounts equal to 5–120 pmol) of derivatized amino acid product were injected into the GC–MS for analysis. To obtain calibration curves for each experiment, the peak area of each eluted compound, obtained in the MS TIC mode, was plotted as a function of the amount of amino acid product injected. Each point in the resultant calibration curves represents the average value of three replicate measurements. To determine peak area, computational analysis was completed using the least-squares method, the regression parameters of which are reported in Table 4. The strong linearity of the calibration curves is demonstrated by the determined squared correlation coefficient values, all of which are close to 1. Calculated calibration curves are shown in Fig. 7.

3.8. Detection limit and quantification limit

The linearity of the applied method allows us to infer the resolution and quantification limits of the experiment. Once inferred, the resolution limit of the experiment can be used to predict the minimal quantity of amino acid needed for detection and analysis. The detection limit X_{LOD} was determined as a function of the analyte concentration, yielding a signal value $Y_{LOD} = y_b + 6\sigma_b$, whereby y_b is the blank average signal generated from ten blank responses. Following determination of the $X_{LOD,}$ the LOD could then be experimentally derived (Table 5).

For each amino acid tested, we determined the limit of detection for our technique to be between 80 ppb to 1.3 ppm. This LOD range is consistent with the concentration found in micrometeorites (from 100 ppb to 100 ppm each amino acid [30,35] or meteorites (from 4 to 60 ppm all together) [36–40]. From the meteoritic flux that we suppose on Mars, we can infer that 1 ppb to 250 ppm of each amino acid will be present on the planet. If this calculation is correct, using our DMF-DMA derivatization reaction, our GC–MS instrument will be sufficiently sensitive to detect the Martian amino acid compounds.

4. Conclusions

When compared to other potential derivatizating agents such as methylchloroformate derivatives or perfluoroacyl/perfluoroalkyl esters, DMF-DMA provides significant advantages for space-related applications. Table 6 highlights the advantages of the DMF-DMA

Table 6
Advantages and drawbacks of 3 derivatizating agents, with regards to spatial application.

	Advantages	Drawbacks
Methyl chloroformate [24]	• One-step, fast reaction • Non-selective reagent for a wide range of analyte • Reaction in aqueous medium • Within mild conditions • Reproducible	• Requires vigorous stirring. • High molecular weight derivatives • Many reagents required • Reagent storage at low temperature • Deteriorates cyclodextrin based-columns • By-products
Perfluoroacylation/perfluoroesterification Alkyl anhydre [14]	• One-step procedure • Low detection and quantification limit	• Racemization • High molecular weight derivatives • Poor reproducibility (3–11 percent) • Very corrosive
DMF-DMA	• Simple and fast one-step reaction • Low detection and quantification limit • Non-selective reagent for a wide range of analyte • Stability (>160 °C) • No corrosive • Low mass derivatives • Short analysis time • Low racemization	• Between-run reproducibility • By-products

technique for space use when compared to the two other methods optimized and adapted in our laboratory for the same objectives. As detailed in Table 6, reliance upon DMF-DMA derivatization gives rise to lower weight derivatized molecules most compatible with space mass spectrometry performance. Moreover, the derivatization reaction facilitates the enantiomeric-based separation of ten of the 19 potential amino acids when all 19 are eluted during a single column run. DMF-DMA is stable and still active at high temperatures (up to 190 °C) essential for space analysis. Similarly, the low time and energy consumption of the DMF-DMA technique facilitates its implementation in the MOMA experiment. Additionally, DMF-DMA derivatization is a rapid one-step reaction (without any cofactor) that can occur under relatively low temperatures (140 °C), allowing for injection of the derivatives obtained directly onto the GC–MS system. Because the reaction occurs in one step, the entire process can easily be automated. Reagents needed for the reaction will be stored at the bottom of the reactor (oven) and isolated from the rest of the oven through a smelted foil. DMF-DMA will then be released at the chosen temperature (140 °C) and contact the Martian soil samples in order to extract and derivatize all targeted organic compounds. Analysis of the target organic compounds will occur rapidly and reproducibly. The three-minute incubation at 140 °C adopted for the procedure represents a compromise between the efficiency of derivatization, the number of derivatives generated, and the destruction rate and/or racemization of these derivatives. The universality of DMF-DMA derivatization and its overall utility and efficiency in chiral-based separation has been demonstrated using the widest possible range of amino acid compounds. In conclusion, it is the culmination of all of these features that makes DMF-DMA the best chemical to use in the *in situ* search for life.

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