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Chemical derivatization of amino acids for in situ analysis of Martian samples by gas chromatography

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Abstract

Three different methods of derivatization are tested in order to select and optimize one for the in situ analysis of amino acids in Martian samples. The silylation procedure can easily be automated with a high yield and a linear response in a large range of concentrations. The alkylation method is simple and easily automated, but irreproducible data are obtained for the reaction in the GC liner at quite a high temperature (300°C). Moreover by-products of the reaction interfere in the GC chromatograms and mass spectrometry detection is needed for product identification. The chloroformate derivatization has several advantages such as one-step reaction and short time analysis. The main problem of this procedure is the shaking step which difficult to develop in space application.

Keywords: Derivatization, GC; Martian samples; Amino acids

1. Introduction

Among the planets and satellites of the solar system, Mars, the red planet, is currently one of the best candidates for space exploration to evaluate the possibility of extinct or evoluted forms of extraterrestrial life. One of the main objectives of the Viking mission to Mars was to search for traces of life in the Martian soil. The U.S. National Aeronautics Space Administration (NASA) sent two identical spacecraft and successfully placed two Viking landers on the surface of Mars in 1976. Among the scientific instruments aboard each lander was a gas chromatograph–mass spectrometer (GC–MS). Biemann [1] was responsible for the MS instrument mainly designed for the detection of organic compounds in the GC–MS mode. This instrument was also used to determine the composition of the minor constituents of the lower atmosphere. The MS detector was connected to a sub-system GC, in order to analyze the organic compounds in the Martian surface material. In addition, biological investigations were carried out on board the landers. Oyama and Berdhal [2] used a GC in the gas exchange experiment (GEX) to determine the gas composition changes above a soil sample which was incubated in the presence of a moistened or aqueous nutrient. No organic compound was detected above the part per billion (ppb) level in the upper few centimetres of the Martian surface. The results of other experiments on board the landers, however, led to the conclusion

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that the Martian surface is saturated with an oxidant of unknown type, and thus any organics deposited in the Martian surface layer would be destroyed in short timescales. However, the oxidizing layer may only extend a few meters below the surface, so the preservation of Martian organics in the subsurface is possible.

Gas chromatography (GC) is still the best available space instrumentation for in situ analysis of organic molecules. The method is well suited for the analysis of a wide spectrum of prebiotic molecules [3–6]. New technologies and methods directly focused on exobiology science goals are currently being developed and work is continuously undertaken in order to satisfy space research requirements [7–9].

Any exobiological strategy for investigating whether organic molecules are present in extraterrestrial environments should focus on compounds that (i) are readily synthesised under plausible prebiotic conditions, (ii) are abundant in carbonaceous meteorites, and (iii) play a major role in biochemistry. Among the few types of organic substances that fulfil these requirements are the amino acid compounds [10].

The analysis of amino acids by GC requires chemical derivatization procedures. Polar organic compounds that contain labile hydrogen atoms (e.g. hydroxyl, amino and thiol groups) are typically of low volatility due to their tendency to self-associate or to associate with polar liquid or solid media through hydrogen bond formation [11]. The derivatization of the polar compound yields a more volatile analyte and increases its solubility in non-polar fluids (e.g. organic solvents, supercritical fluids).

Derivatization processes need handling of liquids which is difficult in space environments but was however successfully performed on Viking missions [2]. Although laboratory-based derivatization techniques are routinely performed, the procedure was never used in space missions and an automatic derivatization system compatible with flight instrumentation constraints has yet to be developed. The aim of this work is to test the various derivatization procedures and to select one that will best fit the scientific needs for spacecraft operation: automation, short analysis time and low energy consumption. The study was conducted on capillary columns that fulfil the requirements of spacecraft instrumentation: resistance to vibrations, radiation and thermal cycles under vacuum, from −50 to 200°C, close to the conditions encountered during transfer flights [3,9].

2. Experimental

2.1. Reagents

Equimolar solutions of 17 protein amino acids in 0.1 *M* hydrochloric acid and tryptophan were purchased from Fluka (Buchs, Switzerland). A standard solution containing 10^{-3} *M* of the 17 amino acids and tryptophan was prepared. Methyl chloroformate, tetramethylammonium hydroxide, dimethylformamide, pyridine, chloroform, methanol, and pyrene were from Fluka and *N*-methyl-*N*-(*tert*.-butyldimethylsilyl)-trifluoroacetamide was from Pierce (Rockford, IL, USA).

2.2. Preparation and analysis of derivatives

2.2.1. Silylation

A sample of 10 μl of the standard amino acid solution was dispensed into a 1-ml vial and excess solvent was evaporated at 35°C using a stream of dry nitrogen. Then 30 μl *N,N-tert*.-butyl(dimethylsilyl)trifluoroacetamide (MTBSTFA), and 10 μl dimethylformamide were added and the vial was heated at 75°C for 30 min. After cooling, 0.1 μl of the reaction mixture was injected directly into the gas chromatograph on a CPSIL 5 CB (100% dimethylpolysiloxane, 0.25-μm film thickness) capillary column (15 m×0.25 mm) from Chrompack (Middelburg, The Netherlands).

For the response linearity study, aliquots of the standard solution containing 10^{-3} *M* of amino acid were diluted with pure water so that 40, 25, 10, 4, 2.5, 1, and 0.4 nmol of each amino acids were injected in four replicates. The response ratio of amino acid to pyrene plotted against the mass of amino acids injected should result in a straight line if no loss of the amino acid derivatives occurs due to a derivative-packing interaction during chromatographic analysis.

2.2.2. Alkylation

A sample of 0.1 μl of the standard amino acid solution was injected simultaneously with 0.2 μl of tetramethylammonium hydroxide (25% w/v in methanol) at 300°C on a BPX5 (5% diphenyl–95% dimethylpolysiloxane, 0.25-μm film thickness) capillary column (25 m×0.22 mm) from SGE (Ringwood Victoria, Australia).

2.2.3. Acylation

A sample of 15 μl of the standard amino acid solution was diluted to 60 μl with water, 40 μl of methanol–pyridine (4:1) and 5 μl of reagent were added and mixed briefly shaking the tube (3–5 s). Gas liberation (carbon dioxide) usually occurs. Then, 100 μl of chloroform (containing 1% MCF) were added and the derivatives were extracted into the organic phase. Next 0.1 μl of the reaction mixture was injected directly in the gas chromatograph on a CPSIL 19 CB (14% cyanopropyl–phenyl, 86% dimethylpolysiloxane, 1-μm film thickness) capillary column (15 m×0.25 mm) from Chrompack (Middelburg, The Netherlands).

2.3. Instruments

The analyses were performed with a Shimadzu QP5050 GC–MS instrument operating with a quadrupole detection mode. The temperature of the split/splitless injector was 300°C and the temperature of the detector was 270°C.

The analyses were performed on three different capillary columns with increasing stationary phase polarity, i.e. from methyl- through phenyl- to cyanopropylsilicones. Helium was used as carrier gas.

3. Results and discussion

Three main types of derivatization reactions have been tested: alkylation, alkylation–acylation and silylation reactions. The analysis were focused on the amino acids found in Martian meteorites: glycine, alanine, valine, serine, proline, aspartic acid, and glutamic acid [12–14]. The quadrupole MS detection mode, available in spacecraft instrumentation [15], was used in this study.

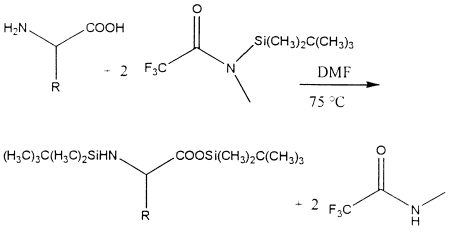
3.1. Silylation

In general, the advantage of the silylation reactions over some other derivatization methods is that it is a single-step procedure which does not require separation of the derivatives prior to GC analysis [11].

The reactivity of silylation reagents towards active hydrogen atoms decreases in the order of hydroxyl (aliphatic>phenolic>carboxyl), amine, to amide. Silylation reactions involve the nucleophilic attack by the analyte heteroatom (O, N or S) on the Si atom of the silylation reagent. Silylation reagents must have a good leaving group in order to stabilize the loss of a silyl group and to avoid the competition with derivatized group of the analyte in a back reaction.

Although a large range of silylation reagents is available, e.g. hexamethylsilazane (HMDS), trimethylchlorosilane (TMCS) and *N*,*O*-bis-(dimethylsilyl)trifluoroacetamide (BSTFA), previous work indicates that TMS derivatives suffer from instability and require strong anhydrous conditions. Soils with moisture content greater than 0.4% could be found in some extraterrestrial environments [1]. This residual humidity could reduce the reaction yields and the analyte recoveries [14]. To avoid an additional drying step, the *N*,*N*-*tert*.-butyl(dimethylsilyl)trifluoroacetamide (MTBSTFA) derivative agent was selected as it is less sensitive to hydrolysis than the other reagents. Typical yields obtained with this reagent are >96% [16].

The reaction and the gas chromatographic conditions of this silylation procedure on amino acids have been optimised by MacKenzie et al. [17]:



The reaction time is 30 min at 75°C. This time cannot be decreased by increasing temperature as the molar responses of some amino acids (lysine, histidine, glycine, proline, methionine and cystine) are decreased [17].

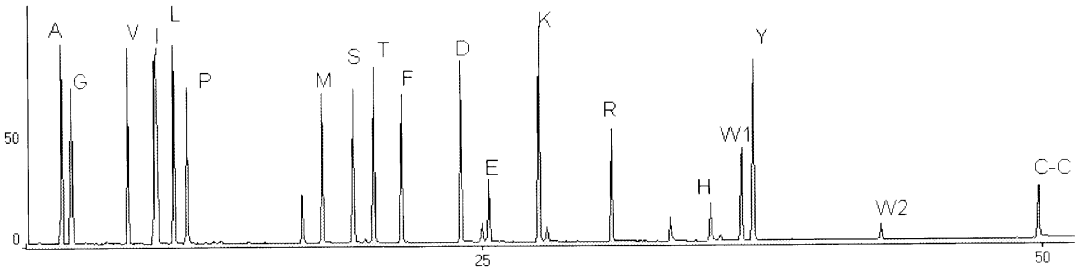


Fig. 1. GC–MS analysis of the amino acids standard mixture (100 μ*M* each). A 15-m×0.25-mm CPSIL 5 CB fused-silica WCOT column, operated in the split mode (1:100) was programmed at 3°C/min from 120 to 270°C with an inlet internal helium pressure of 19.5 kPa. The letter represent the standard single-letter convention for the amino acids. W1, W2 are two *tert*.-butyldimethylsilyl derivatives of tryptophan.

Fig. 1 shows the separation of the mixture of the derivatized amino acids we obtained on a CPSIL 5 CB capillary column. The separation of the silylated derivatives of the low molecular weight amino acids detected in Martian meteorites (from glycine to glutamic acid) is achieved on a capillary column in less than 30 min.

Calibration graphs for all of the amino acid derivatives, obtained by plotting the ratios of their peak areas to that of internal standard, showed good linearity in the range 40–0.4 nmol of amino acids injected. The precision and accuracy of this method as a means of quantitating free amino acids is shown in Table 1. Excellent linear fits of the data were obtained for each amino acid. The molar responses relative to pyrene are given in Table 1. Four replicate samples were analysed. The amount of each amino acid injected was 10 nmol and that of pyrene (I.S.) was 5 nmol. The relative standard deviations (RSD) were less than 5% ($n=4$) for all of the amino acid derivatives, except for lysine, histidine, tryptophan and cystine derivatives, which showed higher values but <10%. Detection limits are given in Table 1. The arginine and histidine are not detected below 5 pmol. Detection of 0.5 pmol of other amino acids was achieved, except for alanine and leucine, and isoleucine detected at 50 fmol ($4<S/N<8$). The within-day and between-day variability were deter-

Table 1
Relative molar responses (RMRs) of amino acids as their silylated derivatives with corresponding standard deviation, linearity of the detector response (coefficient of regression: r^2) and detection limits

Amino acid	RMR	SD	RSD (%)	r^2	Detection limit (pmol)
Alanine (ala-a)	1.971	0.059	2.99	0.992	0.05
Glycine (gly-g)	1.508	0.039	2.58	0.992	0.5
Valine (val-v)	2.109	0.044	2.06	0.999	0.5
Leucine (leu-l)	2.093	0.043	2.04	0.999	0.03
Isoloeucine (ile-i)	2.111	0.042	2.01	0.992	0.05
Proline (pro-p)	1.562	0.021	1.33	0.992	0.5
Methionine (met-m)	0.722	0.019	2.64	0.996	0.5
Serine (ser-s)	1.588	0.029	1.82	0.983	0.5
Threonine (thr-t)	1.629	0.026	1.57	0.995	0.5
Phenylalanine (phe-f)	1.654	0.025	1.52	0.995	0.5
Aspartic acid (asp-d)	1.461	0.046	3.14	0.996	0.5
Glutamic acid (glu-e)	1.627	0.078	4.77	0.995	0.5
Lysine (lys-k)	1.871	0.127	6.78	0.995	1
Arginine (arg-r)	0.815	0.037	4.53	0.994	5
Histidine (his-h)	0.670	0.054	8.06	0.999	5
Tyrosine (tyr-y)	1.372	0.048	3.47	0.993	0.5
Tryptophan (Tryp-w)	1.462	0.095	6.50	0.979	0.5
Cystine (cys-cys-c-c)	0.698	0.067	9.58	0.999	5

Table 2
Within day and between days precision of the silylation method

Amino acid	Concentration (nmol)	Within-day precision ($n=4$)		Between-day precision ($n=20$)	
		SD	RSD (%)	SD	RSD (%)
Alanine (ala-a)	10.25	0.41	4.04	0.56	2.80
Glycine (gly-g)	12.32	0.59	4.79	0.64	2.64
Valine (val-v)	9.30	0.22	2.36	1.01	5.41
Leucine (leu-l)	9.26	0.78	8.37	0.92	4.76
Isoloeucine (ile-i)	10.37	0.50	4.84	0.46	2.27
Proline (pro-p)	11.93	0.48	4.02	0.68	2.78
Methionine (met-m)	10.06	0.73	7.28	0.61	3.15
Serine (ser-s)	10.56	0.39	3.65	0.90	4.28
Threonine (thr-t)	9.79	0.21	2.15	0.63	3.19
Phenylalanine (phe-f)	9.94	0.04	0.37	0.45	2.28
Aspartic acid (asp-d)	9.27	0.33	3.55	0.31	1.72
Glutamic acid (glu-e)	10.71	0.03	0.30	0.17	0.80
Lysine (lys-k)	11.22	0.25	2.22	0.89	3.91
Arginine (arg-r)	10.10	0.58	5.74	0.47	4.66
Histidine (his-h)	9.78	0.09	0.95	0.38	1.96
Tyrosine (tyr-y)	11.37	0.65	5.74	0.54	2.46
Tryptophan (trp-w)	9.52	0.98	10.29	0.84	8.8
Cystine (cys-cys-c-c)	9.89	0.21	2.07	0.61	3.11

mined by repeated analysis of four quality control samples (10 nmol injected) on the same day and on 5 different days. The data given in Table 2 indicate that the method is precise within and between days.

The maximum column temperature used for the elution of glutamic acid is 203°C. This quite high temperature may be difficult in space GC instrumentation because of energy consumption. In addition, the low stability of the reagent implies keeping the reagent at <4°C during the whole time of the mission (flying to the planet and landing). This means additional costs and use of special equipment for the derivatization package. The main concern of this method is that the derivatives produced have a large molecular weight that may be difficult to detect with a space mass spectrometer instrument. Assuming an MS of the type used for Cassini-Huygen (2–146 amu) or Viking (10–220 amu) in the mission [18], the structural identification is limited to the derivatives of alanine, glycine, valine and proline. However as the derivatization procedure did not induced interfering peaks, identification on the basis of retention time analysis could be proposed with a universal detection system.

Otherwise, this derivatization procedure can be automated as it just requires solubilization of the amino acids in MTBSTFA/DMF and heating at a moderate temperature.

3.2. Alkylation

The alkylation reagents and their corresponding catalysts are interesting for amino acid derivatization in space instrumentation. This procedure is not commonly used in GC for the analysis of amino acids but has been developed for analysis in thermochemolysis [19] and in supercritical fluid extraction [20], and has been proposed for derivatisation of carboxylic acids in space research [21].

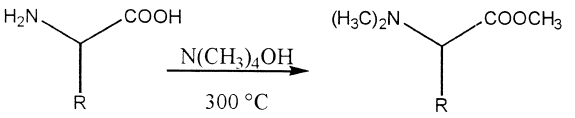
The alkylation reagents displace the active hydrogen in hydroxyl (–OH), carboxyl (–COOH), amine (NH), amide (COHN) and thiol (–SH) groups by nucleophilic displacement to form esters, ethers, thioethers, *N*-alkyl amines and *N*-alkyl amides, respectively. Alkylation reagents include acidic methanol, alkyl halides such as methyl iodide and pentafluorobenzyl bromide (PFBBr), dimethylformamide dialkylacetals and tetraalkylammonium salts. On-column alkylation using tetraalkylammonium salts is a commonly used method for the derivatization of acidic and phenolic substances for GC analysis [22]. Dimethylformamide dialkylacetals react with amino

acids in a complete reaction at 100°C [23] but give low yield (25%) with some *N*-alkyl amino acids [24].

In view of the short time needed for the derivatization procedure and the minimum energy consumption required, tetraalkylammonium salts were the reagents selected for this study. Tetraalkylammonium ion-pairs react under high temperature conditions to form the alkylated derivative and a volatile trialkylamine reaction by-product. Tetraalkylammonium ion-pair reactions occur in thermochemolysis extraction or in the injection port of the gas chromatograph. In addition the reagent is also compatible with the presence of water that could arise from extraction of moistened samples [25]. Typical yields of on-column methylation range from 70 to 80% [26].

Fig. 2 shows the separation of the methyl derivatives of the amino acids studied (100 pmol injected). The elution of amino acids up to glutamic acid is achieved in less than 10 min on a BPX5 capillary column. The column temperature required to elute glutamic acid is quite high (200°C) and this may be, as we already said, a difficulty for applications in space instrumentation.

On the basis of the resulting MS spectra of the derivatives (Table 3), the most probable scheme is:



One can notice that a different mass fragmentation of amino acids derivatives is obtained with an ion trap MS detection: protonated molecular ions [M + 1] are observed as a result of self-chemical ionization. This phenomenon was already described for fatty acid methyl esters [27].

Many by-products of reaction interfere in the total ion current chromatogram of the methylated derivatives. This clearly demonstrates that identification on the basis of retention time analysis is not reliable. MS detection is required for the in situ analysis and identification of the products resulting from simple derivatization reactions. However reproducible val-

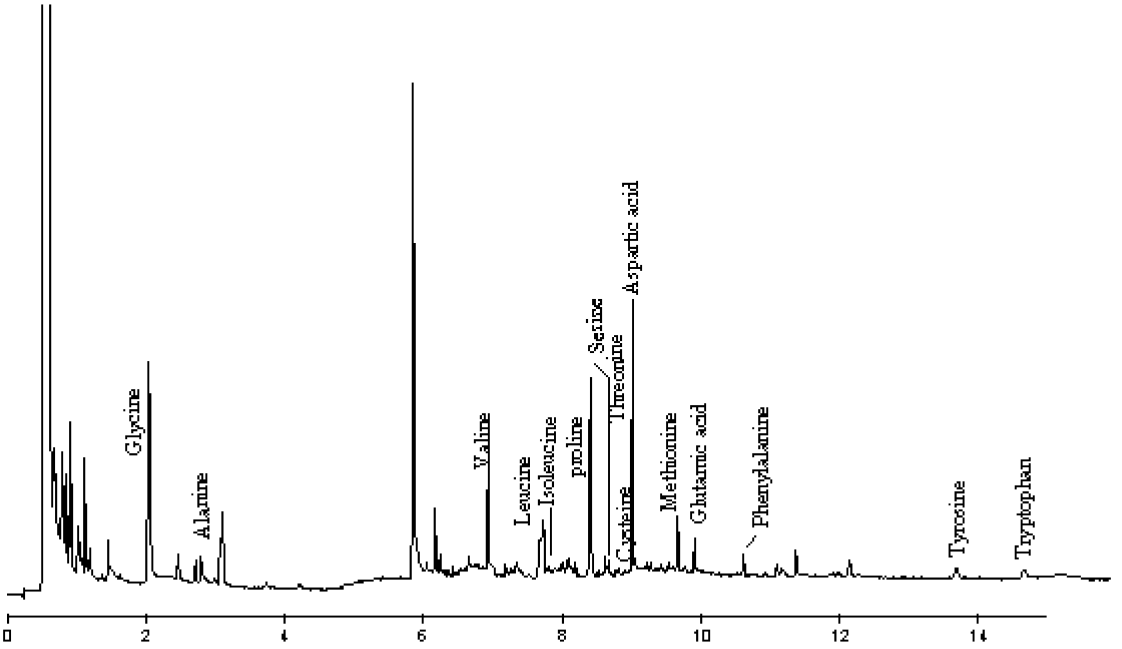


Fig. 2. A chromatogram of methylated amino acids. A 25-m×0.22-mm BPX5 fused-silica WCOT column was used, operated in the split mode (1:20) at 25°C/min from 40 to 200°C with an inlet internal helium pressure of 38.5 kPa.

Table 3
Mass spectrometric fragmentation of the *N,N*-dimetylated amino acids obtained on a quadrupole GC–MS instrument

N,N-Dimethyl amino acid derivatives	MW[a]	Fragments (intensities)
Glycine	117	117(2) 58(100) 42(38)
Alanine	131	131(16) 116(5) 73(25) 72(100) 70(22) 56(31) 42(45)
Valine	159	159(1) 116(50) 100(100) 85(18) 84(15) 70(10) 56(19) 42(38)
Leucine	173	173(1) 116(16) 115(13) 114(100) 98(2) 58(10) 42(7)
Isoleucine	173	173(1) 116(16) 115(13) 114(100) 72(12) 85(15)
Proline	143	143(1) 84(100) 42(25)
Serine	161	161(2) 116(50) 103(5) 102(100)
Threonine	175	175(1) 116(100) 84(15) 58(17)
Cysteine	177	177(2) 118(35) 116(100) 103(3) 98(1) 84(10) 71(57) 70(19) 56(49)
Aspartic acid	189	189(2) 130(100) 116(28) 98(30) 56(12)
Methionine	191	191(9) 133(15) 132(100) 116(22) 84(90) 70(21) 61(81)
Glutamic acid	203	203(2) 172(8) 145(10) 144(100) 116(8) 85(9) 84(69) 70(8) 56(6)
Phenylalanine	207	148(32) 116(100) 91(11) 84(4) 77(9) 65(4) 63(2) 56(11)
Tyrosine	237	178(15) 163(4) 121(9) 117(10) 116(100) 102(16) 77(9) 63(1)
Tryptophan	260	260(1) 144(30) 116(100) 101(1) 77(5) 63(1)

[a] MW, molecular weight.

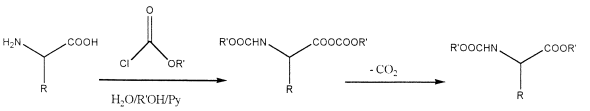
ues for methylation were not obtained: chemical reactions occurred in the injector liner requiring its replacement every 20 injections. Unreproducible results are thus obtained preventing a quantitative analysis of the data and limit of detection measurements.

This derivatization method has several advantages for space instrumentation. It is simple, easily automated and requires only the solubilization in the reagent of the organic compounds to be studied. The temperature of the reaction is quite high (300°C) and further studies are needed to test the performance of the system. In view of space considerations, the reagent does not need special storage and the low molecular weight derivatives obtained are compatible with the range of MS detectors for space instrumentation. If the problem of analysis non-reproducibility could be solved this simple on-column derivatization procedure would be attractive for GC space applications.

3.3. Acylation–alkylation

The derivatization reaction of acids, hydroxy acids [28] and amino acids [29–32] at room temperature with chloroformates has proved to be simple and fast. The method is well suited for GC analysis and gives a high yield in the preparation of derivatives (>96%) [33]. A judicious choice of the alkyl group of the chloroformate and the alcohol solvent allows the formation of a single derivative in high yield [30]. Rapid analysis of amino acid enantiomers by chiral-phase capillary gas chromatography has also been achieved using ethyl chloroformates [34] which permit in situ chiral separation.

The acidic group of amino acids reacts with chloroformate to give, via decarboxylation, an ester derivative and the amino group is derivatized into the carbamate form in the presence of the alcohol via an acetylation procedure.



A quantitative and reproducible derivatization of proteins amino acids was developed by Husek using methyl chloroformate and ethyl chloroformate. The yield of the chloroformate reaction is high and quantitative analysis was demonstrated by the analysis of protein amino acids derivatives [30]. This derivatization method was used in this study, selecting methyl chloroformate as the reagent in the presence of methanol. The molecular weight of the resulting derivatives is lower than that obtained with ethyl chloroformate. Moreover, low temperature storage is not required with methyl chloroformate.

The above procedure was simplified as the extraction of the derivatives with chloroform was not performed. This extraction step is not required as MS detection permits an unambiguous identification of the derivatives. Fig. 3 illustrates the fragmentogram of the analysis performed in less than 7 min on the CPSIL 19 CB capillary column. A poor detection limit is achieved as the extraction step is not performed: a large background noise is then observed. Low molecular weight amino acid derivatives (alanine, glycine, valine, proline) are detected at 2 pmol injected whereas others are detected when 20 pmol were injected.

A vigorous shaking of chloroformate is needed for quantitative analysis. If this step is omitted the yield of reaction decreased (<37%). At present, the chloroformate derivatisation method can not be proposed for space GC application, as the technology for mixing and homogenisation is not yet available.

One can notice that the detection limit is decreased to the femtomole range for the pentafluorobenzyl amino acid derivatives with the best MS detection conditions (electron capture negative ionization (ECNI) mass spectrometry and SIM mode) and an extraction procedure decreasing the background level [35]. It would be possible to obtain a better detection limit by using this detection mode and a fluorinated reagent. At present this approach is not possible because (i) the ECNI/MS detector has not yet been developed for space instrumentation, (ii) the reagent is unstable at greater than −20°C and (iii) an extraction procedure is needed. Moreover, a shaking step is required for a high reaction yield.

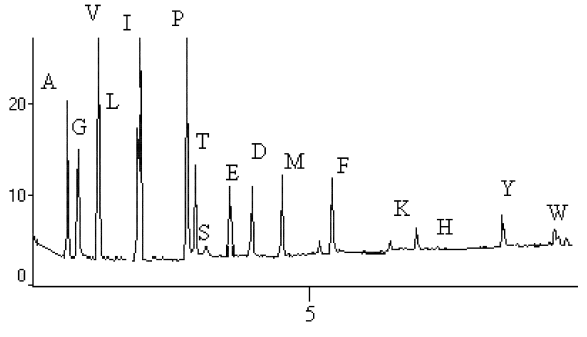


Fig. 3. GC–MS fragmentogram of the amino acids standard mixture (100 μ*M* each). A 15-m×0.25-mm CPSIL 19 CB fused-silica WCOT column, operated in the split mode (1:20) was programmed at 25°C/min from 120 to 280°C with an inlet internal helium pressure of 49.5 kPa. The letter represent the standard.

4. Conclusion

Gas chromatography is a powerful analytical instrument for exobiology science and extraterrestrial environment exploration. The technology on board spacecraft is far from that existing in current Earth experiments but remains in continuous development. Future space missions will have to take into account amino acids analysis. The technique to be preferred is silylation as quantitative analysis in a large range of concentrations can be achieved. This procedure can lead to automation using the available space instrumentation. Complementary studies are however needed concerning the extraction procedure of the amino acids from inorganic matrices.

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