CHEMICAL VOLATILIZATION AS A TECHNIQUE FOR THE DETECTION OF EXTRATERRESTRIAL BIOPOLYMERS AND POSSIBLE METABOLIC PRODUCTS*

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Abstract. The utility of wet chemical reaction sequences, suitable for automation and telemetered control, as a means of detecting biopolymers is explored and its potential assessed with respect to extraterrestrial life detection. A variety of depolymerisation reagents and derivatisation are examined. Additionally, the importance of detecting carbon – either inorganic or organic – is discussed and the relevance of this approach to the detection and indentificaton or inorganic carbon via reactor head space analysis is discussed.

Since 1969 the search for extraterrestrial life has been intensified. This has resulted from three important areas of research: (1) the organic analyses of the returned lunar surface materials from Apollo 11, 12 and 14; (2) organic analyses of meteorites and particularly the Murchison meteorite; and (3) the radio-telescopic search for organic molecules, ions and radicals in interstellar space. The results from all three areas have, in general, heightened scientific interest in the NASA Viking program to place a soft landing automated scientific package on the Martian surface in 1976. The key objective of the Viking project is to determine whether there is, or has been, or could be life on Mars (NASA Report, 1969). Techniques and instrumentation based on pyrolysis Gas Chromatograph-Mass Spectrometry (GC-MS) have been developed for a preliminary analysis of the Mars surface. However, the adequency of this technique for such a complex problem as life detection is the subject of some debate, especially in view of the results already obtained from lunar, meteoritic and Precambrian organic analyses.

In recent years much use has been made of *n*-alkane and fatty acid distribution patterns, presence of 'biological markers' such as pristane, phytane, etc., in Precambrian (Eglinton *et al.*, 1964, 1966; Johns *et al.*, 1966; Van Hoeven *et al.*, 1963; Oró and Nooner, 1967) and meteoritic analysis (Han *et al.*, 1969; Nooner and Oró, 1967). But much has also been made of abiotic syntheses such as the Fischer-Tropsch reaction which has been shown to produce many compounds and distributions of compounds similar to naturally occurring mixtures (Gelpi *et al.*, 1970; Studier *et al.*, 1968). The situation at present appears to be that the presence of particular distributional char-

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acteristics and individual biologically important molecules *indicate*, but do not prove a biogenic origin. The characteristics of ancient life forms and their residues are not well defined on Earth as evidenced by Precambrian analyses which have somewhat tentatively shown the existence of life 3.7×10^9 yr ago (Eglinton *et al.*, 1964, 1966; Johns *et al.*, 1966; Van Hoeven *et al.*, 1969; Oró and Nooner, 1967), but the origin of life is still unknown and the processes of chemical and biochemical evolution are still unsubstantiated by experimental findings. Therefore, the detection, recognition and quantitation of extraterrestrial life, especially by telemetered automatic instrumentation are not simple objectives which are readily realized, even if life did or does exist at appropriate location under investigation.

The preliminary analysis of the Martian surface during the proposed NASA Viking mission for 1975–6 will consist of pyrolysis as a technique for volatilizing organic molecules or polymers and detection of the products by GC-MS.

Thermal analyses as a general rule are destructive processes which alter the chemical architecture of molecules whose very specificity is the key to biogenicity and which randomize biological distribution patterns which are again another indicator of biological activity. These processes of alteration, randomization and destruction increase in efficiency with increasing applied energy. Temperatures of 300–600 °C are sufficient to degrade proteins, amino acids (Hare, 1969), steroids and terpenoids (Steel *et al.*, 1971), fatty acids (Eisma and Jurg, 1969), sugars (Swain, 1969) and even alkanes (Holman *et al.*, 1966; Henderson *et al.*, 1968) to products which are irresolvable from the products of abiogenic synthesis such as the Fischer-Tropsch reaction. The importance of this effect is probably inversely proportional to the concentrations at which the molecules occur. Nevertheless, if there are indeed organic molecules present in the Martian soil, either volatile or non-volatile high molecular weight polymers, then pyrolysis-GC-MS should yield useful data and act as the basis for follow-up scientific mission design. However, it should also be stated that in the absence of detectable organic molecules pyrolysis-GC-MS (at the temperatures proposed, $\leqslant 500\,^{\circ}C$) will not yield any information regarding the nature of that 'non-organic' carbon indigenous to Mars. The latter situation would be very similar to the organic analyses of returned lunar samples presently being conducted in several laboratories throughout the world. A short summary of the results of extraterrestrial organic analysis of lunar materials, meteorites and the radio-telescopic search for interstellar organic molecules will serve to emphasize the difficulties involved in designing a telemetered life detection package for surface analysis of extraterrestrial bodies.

1. Analysis of Returned Lunar Surface Materials (Apollo 11 and 12)

Organic analysis investigators are now in general agreement that there is <1 ppm indigenous organic molecules in the lunar surface (*Proceedings of the Apollo 11 Lunar Science Conference, 1970; Proceedings of the 2nd Lunar Science Conference, 1971*). There is still doubt as to the absolute value and the composition of this small quantity of lunar carbon. The main research activity now centers on the definition and identifica-

tion of the nature of the lunar carbon, and despite the efforts of approximately 10 research groups throughout the world, this is still an unsolved problem. Certainly it has been shown that carbides (Chang *et al.*, 1971; Henderson *et al.*, 1971), graphite and perhaps some carbonate (Henderson *et al.*, 1971), are present and a small amount of indigenous methane (Chang *et al.*, 1971; Cadogan *et al.*, 1971) has been shown to exist, but nevertheless no carbon mass balance has been achieved.

2. Organic Analyses of Meteorites

In recent years many carbonaceous chondrites have been subjected to organic analyses (Gelpi *et al.*, 1970; Studier *et al.*, 1968). The results have often been equivocal with respect to biogenicity and at least two distinct schools of thought exist: (1), that any organic molecule thus far isolated and identified from a meteorite is either contamination or the product of abiotic syntheses during the thermal history of the meteorite (Anders, 1962; Hayes, 1967); (2), that at least some portion of the proven organic content is indigenous and indicative of a biogenic origin (Anders, 1962; Hayes, 1967; Nooner and Oró, 1967). Only the latest research results on the Murchison meteorite where both D and L amino acids have been isolated and identified, apparently have any real credibility (Kvenvolden *et al.*, 1970). However, even these results are not unequivocal and the problems of terrestrial contamination by bacteria, abiotic synthesis and racemization of enantiomers have not been completely excluded. Nevertheless, this work represents possibly the most definitive study of extraterrestrial organic chemistry in terms of biogenicity up to the present time.

3. Radio-telescopic Identification of Organic Molecules in Space

The last year or so has seen several very interesting organic molecules and fragments identified in interstellar space, always associated with prestellar dust clouds. Such molecules as formaldehyde (HCHO), cyanoacetylene ($HC{\equiv}C{-}C{\equiv}N$), hydrogen cyanide (HCN), water, hydrogen, CH^+, NH_3, CH_3OH, CO, and HCO_2H have been identified (Snyder and Buhl, 1970). Many of these have been investigated as the probable constituents of primordial atmospheres and have been shown to produce, under suitable excitation conditions, many important biochemicals such as purines, pyrimidines, amino acids, sugars and polypeptides (Lemmon, 1970).

Furthermore, these identifications in interstellar space suggest that molecules such as HCN, HCHO, NH_3 and $HC{\equiv}C{-}C{\equiv}N$ or their polymers could be present on every cosmic body. One reservation which should be introduced is that since these molecules have only been identified in association with dust clouds it is possible that they would not have survived the highly energetic processes of the collapse of such a proto-star (Conference on the Relationchip of Interstellar Molecules to the Origin of Life, 1971). Furthermore, statistical analyses reveal that, even if survival was possible, the rates of destruction and the frequency of intermolecular collisions are such that the synthesis of high molecular weight organic molecules in interstellar space is at best

unlikely (Conference on the Relationship of Interstellar Molecules to the Origin of Life, 1971).

The main conclusions from these results appear to be that:

(1) The organic analysis of sub-ppm concentrations of organic compounds in a silicate/mineral matrix, even in modern laboratories is difficult. The problems of contamination are extremely significant and the sensitivity limits of the techniques used are at their maxima.

(2) In the absence of significant quantities of organic molecules, biogenic or abiogenic, identification and quantitation of the nature of the carbon in a mineral/silicate matrix is a difficult problem. Carbon chemistry, as opposed to organic chemistry, is one of the less well-developed areas of elemental analysis, thus necessitating much method and instrument development. The nature of the carbon is a significant and important question which is a key point in understanding chemical and biochemical evolution, and therefore, attempts should be made to design an automated package which under these circumstances could yield useful data which would impact upon chemical evolution (i.e., define the abundance and nature of the indigenous Martian carbon), but which would also be capable of sophisticated analyses of biopolymers if they are present.

(3) The presence of significant quantities of organic molecules in any extraterrestrial sample is important, but that in itself will not unequivocally indicate the existence of life forms. After identification has been made the question of biogenicity vs. abiogenicity has to be resolved. Therefore, an immediate requirement for any organic analysis, whether in a laboratory or by an automated instrument package, is for a specific and unambiguous analytical method for determining the biogenicity of any multi-carbon containing molecules.

(4) The radio-telescopic identifications of low molecular weight organic molecules and radicals in interstellar space, together with the identification of enantiomeric amino acids in the Murchison meteorite support to some extent the theories that life must exist elsewhere in the Universe and that the processes of chemical and biochemical evolution cannot be unique to the Earth.

This report presents preliminary results from experiments whose objectives were to provide chemical techniques which would tackle some of the problems outlined above. In particular, a major objective was to design a chemical system which would yield data on the nature of Martian carbon, whether it is organic or 'inorganic' in character and which would be non-destructive with respect to biological specificity in the biosynthesis of organic molecules.

4. Chemical Volatilization-Objectives, Constraints, and Methods

The technical objective of this investigation was to develop a chemical system which will specifically derivatize and volatilize non-volatile organic molecules and polymers in suitable forms for GC-MS analysis. The method should be generally applicable to proteins, polysaccharides, polyesters (e.g. 'kerogen'-like polymers), free amino acids

and organic molecules with functionalities with labile hydrogen atoms. A second objective was to dissolve any silicate matrix such that trapped gases or reactive minerals, e.g., carbides would be detected by reactor head-space analysis.

Since the system must comply with the engineering requirements of a remote extra-terrestrial experiment, constraints were placed on the system. Thus a single reactor must be used for all operations to eliminate transfers and the reaction mixture and products should be analyzed directly. The preferred system would be a single reagent which would simultaneously depolymerize polymers and convert resultant and indigenous monomeric units to volatile derivatives. A multi-step procedure must be restricted to simple, successive addition of reagents by telemetered control.

Amino acids, dipeptides and polypeptides were chosen as the model compounds for evaluation of potential chemical reactions. Silylation reactions were selected for the derivatization of the monomers (Pierce, 1968; Gehrke and Leimer, 1970).

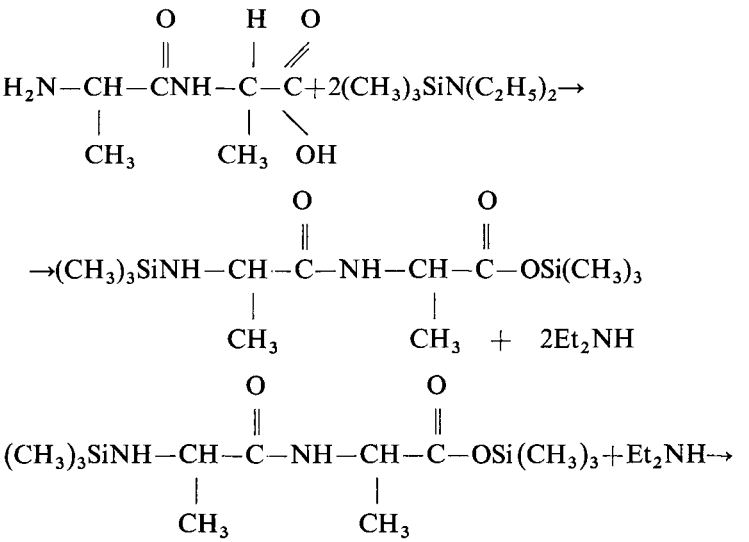
Two general routes were selected for combined depolymerization and monomer derivatization:

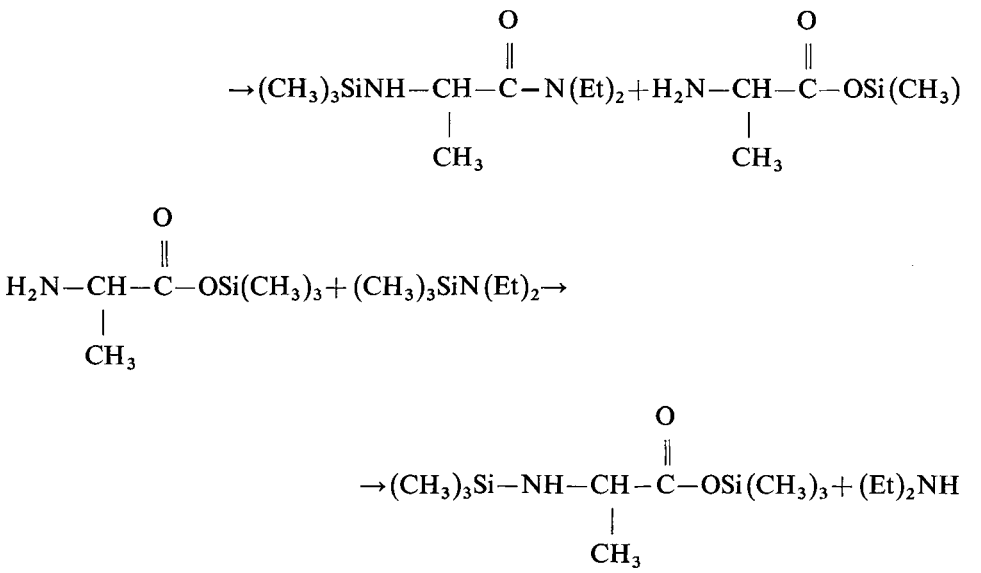
(1) *Single-step route* – Use of a silylation reagent which liberates a nucleophile (or is compatible with an added one) which cleaves the peptide bond with or without a catalyst.

(2) *Multi-step route* – Use of modified conventional methods of peptide cleavage (i.e. acid and base hydrolysis) followed by direct derivatization of the reaction mixture.

A. SINGLE-STEP ROUTE

Alanylalanine was used as a model polypeptide to evaluate the silylation of the cleaved polypeptide. Trimethylsilyldiethyl amine (TMSDEA) was used as the silylation reagent (Hils *et al.*, 1966; Mason and Smith, 1966; Ruhlmann, 1959, 1961), since it liberates diethyl amine, a nucleophile capable of cleaving the peptide bond by amide interchange (transamidation). The reaction scheme is as follows:

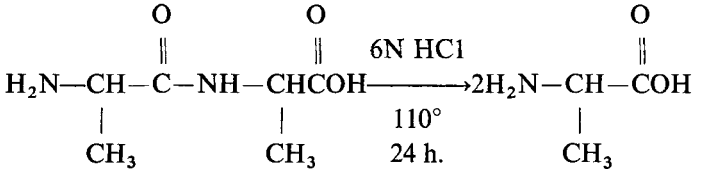


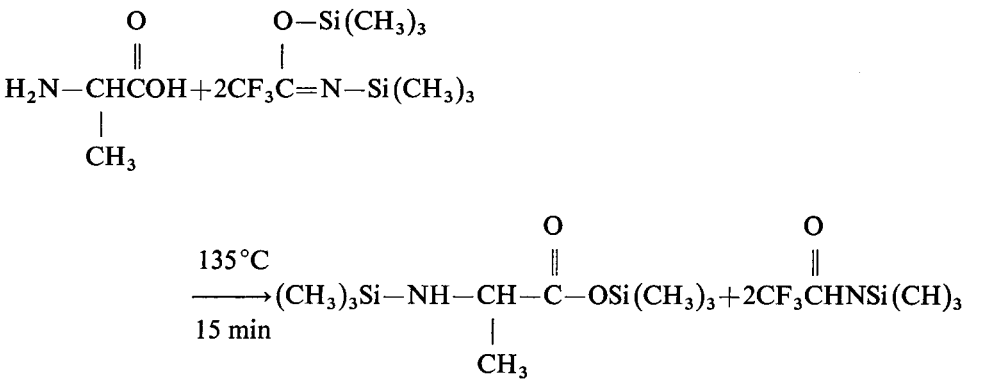


A series of screening reactions were carried out with and without added acid and diethyl amine (DEA). All reactions were run in a teflon lined screw-cap vial heated in a sand bath at 135 °C for 45 min. Alanylalanine (1.0 mg) was treated with trimethylsilyldiethylamine (TMSDEA) (0.10 ml), with and without additional reagents as described in Table I. For each set of reaction media three runs were made – one with alanylalanine to evaluate peptide cleavage, one with alanine to evaluate derivatization, and one with no sample for background. At the end of each run, the solution was analyzed directly for alanine-$(TMS)_2$ by GC using a standard prepared by a known route with N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) (Gehrke and Leimer, 1970) (GC conditions: $6' \times 1/16''$ column packed with 3% JXR on Gas Chrom Q 60/80 mesh; temperature, 90 °C; helium flow rate, 10 ml min^{-1}, flame ionization detector).

B. MULTI-STEP ROUTE

This route was based upon aqueous acidic (or basic hydrolysis) of polypeptides by known routes with minimal amounts of water followed by direct derivatization of the hydrolysis mixture using the extremely reactive reagent, N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) (Gehrke and Leimer, 1970). The reaction is illustrated using the model polypeptide, alanylalanine:





Since the hydrolysis reaction is a well-known procedure for amino acid analysis preliminary evaluation was performed by determining the feasibility of the crucial second step. A series of screening reactions were carried out with alanine in acidic, basic, and neutral aqueous solutions and with aqueous acid (6N HCl) neutralized by added pyridine. Reaction vessels and analyses were similar to those described for the single-step route. The composition of the reaction media and the results are presented in Table II.

The following conclusions may be drawn from the results from the single-step experiments shown in Table I:

(a) TMSDEA alone neither cleaves the peptide bond nor derivatizes the free amino acid.

(b) Addition of small amounts (approx. 1% by volume of TMSDEA) of acids such as trifluoroacetic acid, trichloroacetic acid, and trimethylchlorosilane do not cause peptide bond cleavage but do promote alanine-$(TMS)_2$ formation.

(c) Addition of small amounts of DEA (approx. 1–2% by volume of TMSDEA) and trimethylchlorosilane ($\sim$1% by volume of TMSDEA) causes a small amount (approx. 2%) of peptide bond cleavage along with alanine-$(TMS)_2$ formation, however an excess of the amine inhibits both reactions.

The following conclusions may be drawn from the results from the multi-step feasibility experiments shown in Table II:

(a) No alanine-$(TMS)_2$ is formed in 6N HCl.

(b) Quantitative conversion to alanine-$(TMS)_2$ is achieved in pure water and in 6N HCl neutralized by stoichiometric amounts or small excess (1 mole) of pyridine. A large excess of pyridine inhibits the reaction.

(c) Approximately a 50% conversion to alanine-$(TMS)_2$ was achieved in a saturated barium hydroxide solution used for basic hydrolysis of polypeptides.

(d) In all cases where alanine-$(TMS)_2$ was formed sufficient BSTFA had to be added to consume all of the water. The hexamethyldisiloxane formed in this reaction is not an active silylating reagent in this medium.

The success in the multi-step feasibility experiments allowed the application of the entire reaction procedure to several model biopolymers, i.e., alanylalanine, a decapeptide and a collagen (bovine achilles tendon).

TABLE I

Data for screening reactions-single-step route

Model compounds (mg.-compound)	TMSDEA[a] (ml)	BSTFA[b] (ml)	Diethyl amine (ml)	H_2O (ml)	Trimethyl-chlorosilane (ml)	Trifluoro acetic acid (ml)	Trichloro acetic acid (ml)	Aceto-nitrile (ml)	Detection of Alanin $(TMS)_2$
1.0-alanine		0.10							+
1.0-alanylalanine		0.10							−
1.0-alanine	0.10								−
1.0-alanylalanine	0.10								−
1.0-alanine	0.10				0.01				+
1.0-alanylalanine	0.10				0.01				−
1.0-alanine	0.10					0.01			+
1.0-alanylalanine	0.10					0.01			−
1.0-alanine	0.10						0.01		+
1.0-alanylalanine	0.10						0.01		−
1.0-alanine	0.10		0.01		0.01				+
1.0-alanylalanine	0.10		0.01		0.01				+
1.0-alanine	0.10		0.10		0.01				−
1.0-alanylalanine	0.10		0.10		0.01				−
1.0-alanine	0.10			0.01	0.01				−
1.0-alanylalanine	0.10			0.01	0.01				−
1.0-alanine	0.10		0.01		0.01				+
1.0-alanylalanine	0.10		0.01		0.01				+
1.0-alanine	0.10			0.01	0.01			0.10	−
1.0-alanylalanine	0.10			0.01	0.01			0.10	−
1.0-alanine	0.10		0.015		0.01				+
1.0-alanylalanine	0.10		0.015		0.01				+ (2.3 % conv.[c])
1.0-alanine	0.10		0.10[d]		0.01[d]				+
1.0-alanylalanine	0.10		0.10[d]		0.01[d]				+

[a] Trimethylsilyldiethylamine

[b] N, O-bis-trimethylsilyltrifluoroacetamide

[c] Conversion of alanylalanine to alanine-$(TMS)_2$

[d] Heated at 135 °C for 0.5 h with simultaneous evolution of reagents. Then 0.10 ml TMSDEA was added to the dry residue and heated at 135 °C for 45 min in a sealed vial.

TABLE II

Data for screening reactions-multi-step route

Model Compounds (mg.-compound)	BSTFA[a] (ml)	6N HCl (ml)	H_2O (ml)	Sat aq. $Ba(OH)_2$ (ml)	Pyridine (ml)	Acetonitrile (ml)	Dimethylformamide (ml)	Yield of Monomer – TMS (%)
1.0-alanine	0.10	0.01						0
1.0-alanine	0.10	0.01				0.10		0
1.0-alanine	0.10	0.01			0.10			0
1.0-alanine	0.10	0.01			0.20			0
1.0-alanine	0.30	0.01			0.20			0
1.0-alanine	0.50		0.01					present[b]
1.0-alanine	0.50	0.01						0
1.0-alanine	0.50		0.01		0.50			$\leqslant 1$
1.0-alanine	0.50		0.01				0.50	$\leqslant 1$
0.20-alanine	0.10	0.0020			0.0010			96
0.20-alanine	0.10	0.0020			0.0015			109
0.20-alanine	0.10	0.0020			0.0020			100
0.20-alanine	0.10		0.0020					110
0.20-alanine	0.10			0.0020				53

[a] N,O-bis-trimethylsilyltrifluoroacetamide

[b] Qualitative analysis only.

5. Multi-Step Chemical Volatilization of Peptides

Alanylalanine (0.0899 g) in 6N HCl (1.0 ml) was placed in a sealed tube and heated for 24 h at 110 °C. A 0.002 ml aliquot was neutralized with pyridine (0.002 ml) followed by addition of BSTFA (0.10 ml) and heating at 135 °C for 15 min in a sealed tube. Analysis for alanine-$(TMS)_2$ was conducted as before. The results are presented in Table III and Figure 1.

TABLE III

Quantitative data for alanylalanine hydrolysis and derivatization

Sample	% Conversion[a]
Alanine TMS in hydrolysis medium	97.0
Alanylalanine hydrolysate TMS	99.2

[a] To alanine TMS relative to the alanine TMS standard prepared in pure BSTFA.

In a similar manner, the decapeptide cyclo-L-valyl-L(N-tosyl)-lysyl-L-leucyl-D-phenylalanyl-L-prolyl-L-valyl-L(N-tosyl)lysyl-L-leucyl-D-phenylalanyl-L-prolyl (0.1039 g) in 6N HCl (1.0 ml) was hydrolyzed and derivatized. The analysis for amino acid $(TMS)_2$ derivatives was similar to the other analyses except for the GC column temperature conditions which were 80 °C for 5 min followed by temperature programming to 250 °C at 6° min^{-1}. The results are presented in Table IV and Figure 2.

Collagen (0.10 g) was hydrolyzed in 6N HCl (1.0 ml), derivatized and analyzed as

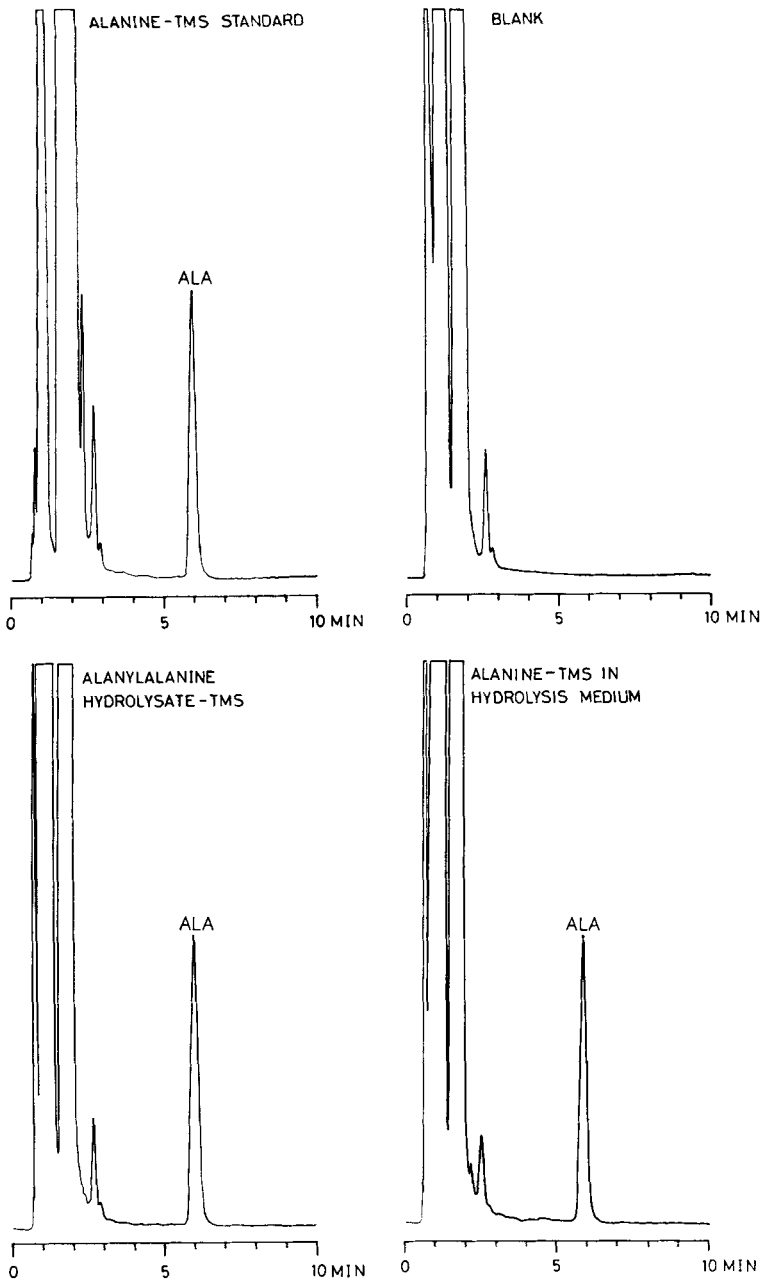


Fig. 1. Gas chromatographic records of the alanylalanine hydrolysis products and control experiments. Conditions: 3% JXR column (6 ft × 1/16 in.; 60/80 mesh Gas Chrom Q); column temperature 90°C; helium flow rate 10 ml min^{-1}; flame ionization detector.

TABLE IV
Quantitative data for the decapeptide hydrolysis and derivatization

Sample	Percent Conversion[a]				
	Valine	Leucine	Proline	Phenyl-alanine	Lysine
Amino Acid mixture TMS in hydrolysis medium	109	107	109	96.2	37.1
Decapeptide hydrolysate TMS	106	96.7	90.8	38.7	0

[a] To the amino acid TMS derivatives relative to the amino acid mixture TMS standard prepared in pure BSTFA.

previously described. A gas chromatographic trace of the reaction products as TMS derivatives is shown in Figure 3.

The quantitative results shown in Tables III and IV for the depolymerization and derivatization of peptides in a single reactor indicate the feasibility of such a system for life detection by telemetry on a planetary surface has a high potential value. With the exception of lysine, good conversion of the constituent amino acids to their TMS derivatives was achieved. Qualitatively, the products of collagen hydrolysis emphasize the potential of this approach. Therefore, the multi-step route using sequential reactions in a single closed reactor can be developed into a suitable system for the analysis of polypeptides and amino acids in Martian soil. The feasibility of the route using acid hydrolysis followed by neutralization and derivatization has been demonstrated. It is possible that this route could be expanded to include other biopolymers such as polysaccharides and polyester linkages (Pierce, 1968) as well as the specific derivatization of functionalities in organic molecules of lower molecular weight which are typical metabolic products of living systems. Furthermore, this method might be compatible with demineralization of silicate matrices in soil (using aqueous HF) which would release any small molecules or polymers bound or trapped in the inorganic matrix. Thus, by reactor head space analysis prior to derivatization, the presence or absence of such molecules as CO, CO_2, CH_4, $HC{\equiv}CH$, $H_2C{=}CH_2$, NH_3, HCN, etc., could be established. The stereoisomerism of amino acids is preserved during acid hydrolysis and therefore gas chromatographic resolution of stereoisomers would be possible (Kvenvolden *et al.*, 1971; Oró *et al.*, 1971; Parr *et al.*, 1971) with its attendant advantages with regard to identification of biological characteristics.

The presence of polypeptides in Martian soil could not be determined unequivocally by the acid hydrolysis derivatization route since complete depolymerization occurs and the resulting amino acid derivatives would be indistinguishable from free amino acids originally present. Since polypeptide formation is a prerequisite for the evolution of life as we know it, their presence is an important question. Currently, we are evaluating a system analogous to the acid hydrolysis route based upon hydrazinolysis of polypeptides, a well-known procedure in protein analysis. In this procedure all amino acids in the polypeptide are converted to their respective hydrazides except the terminal

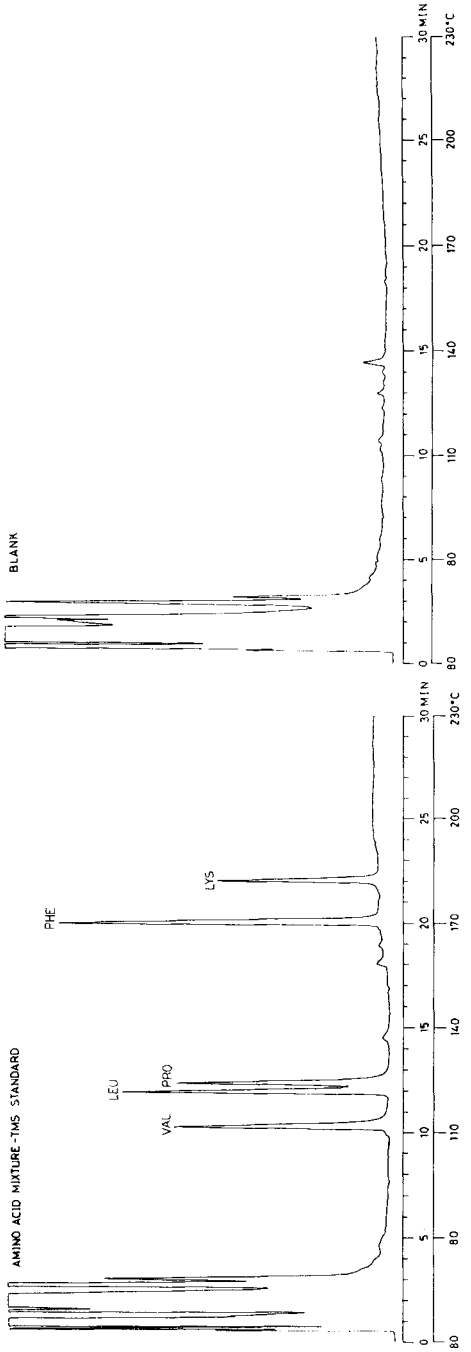


Fig. 2. Gas chromatographic records of the decapeptide hydrolysis products and control experiments. Conditions: 3% JXR column (6 ft×1/16 in.; 60/80 mesh Gas Chrom Q; column temperature 80 °C isothermal for 5 min, followed by temperature programming to 250 °C at 6° min^{-1}; helium flow rate 10 ml min^{-1}; flame ionization detector.

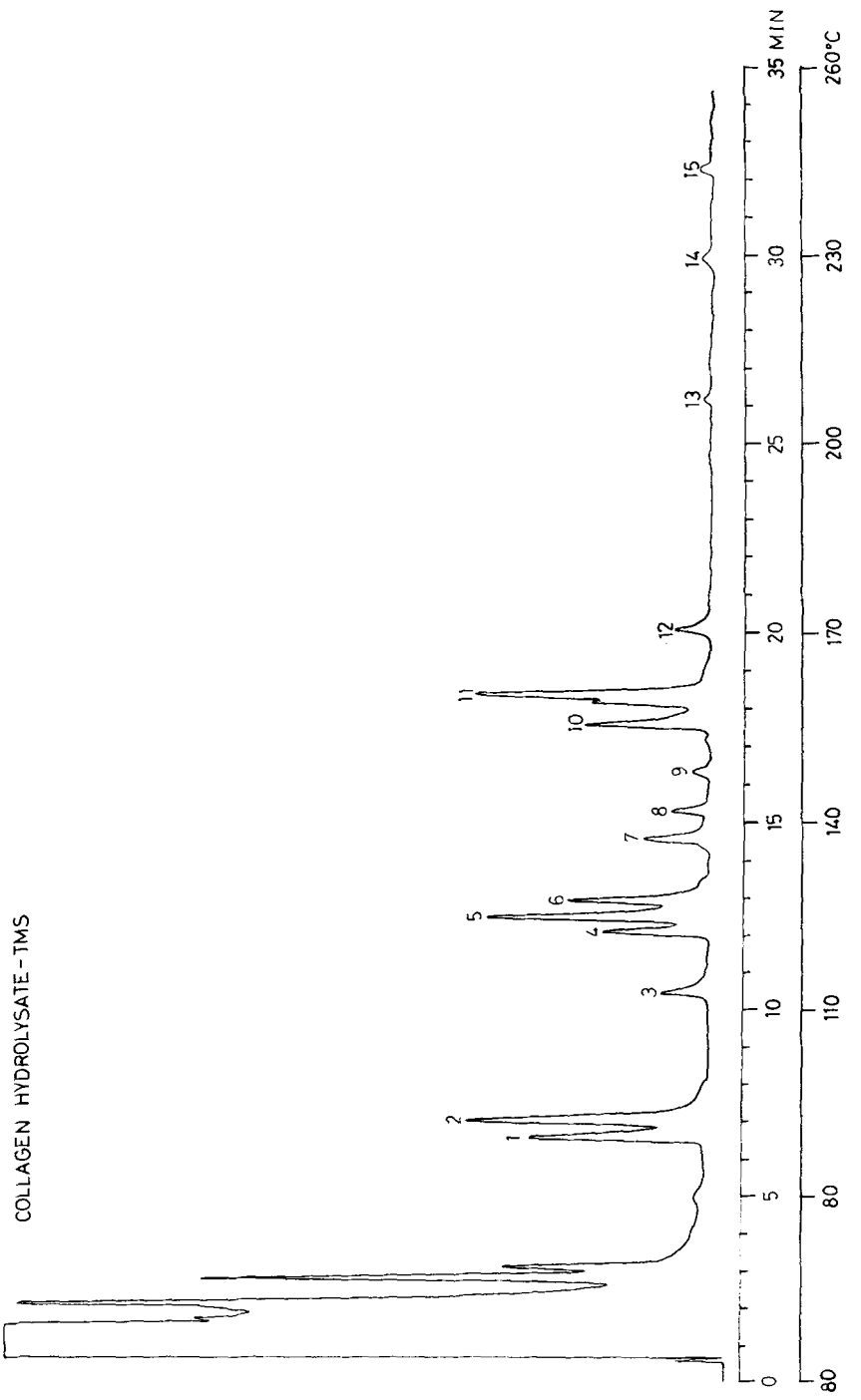


Fig. 3. Gas Chromatographic record of the collagen hydrolysis products – conditions as described in Figure 2.

amino acid with a free carboxyl group. These hydrazides could be volatilized by derivatization with BSTFA and analyzed by GC-MS. Their detection would constitute compelling evidence for the presence of polypeptides and their abundance relative to amino acid derivatives would provide structural information, i.e., minimum molecular weight, gross chain composition, end group structure.

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