Subcritical water extraction of amino acids from Mars analog soils

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List of abbreviations:

SCWE – Subcritical Water Extraction

PAH – Polyaromatic hydrocarbon

AAs – Amino Acids

JSC-M1A – JSC Mars-1A

BSA – Bovine Serum Albumin

Nle – Norleucine

Asp – Aspartic acid

Ser –Serine

Glu – Glutamic acid

Gly – Glycine

His – Histidine

Arg – Arginine

Thr – Threonine

Ala – Alanine

Pro – Proline

Cys – Cysteine

Tyr – Tyrosine

Val – Valine

Met – Methionine

Lys – Lysine

Ile – Isoleucine

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Leu – Leucine

Phe – Phenylalanine

AH – Acid hydrolysis

CV – column volumes

DHCl – dilute HCl

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ABSTRACT: For decades, the Martian regolith has stymied robotic mission efforts to catalog the organic molecules present. Perchlorate salts, found widely throughout Mars, are the main culprit as they break-down and react with organics liberated from the regolith during pyrolysis, the primary extraction technique attempted to date on Mars. This work further develops subcritical water extraction (SCWE) as a technique for extraction of amino acids on future missions. The effect of SCWE temperature (185, 200, and 215°C) and duration of extraction (10 – 120 min) on the total amount and distribution of amino acids recovered was explored for three Mars analog soils (JSC Mars-1A simulant, an Atacama desert soil, and an Antarctic Dry Valleys soil) and bovine serum albumin (as a control solution of known amino acid content). Total amounts of amino acids extracted increased with both time and temperature; however, the distribution shifted notably due to the destruction of the amino acids with charged or polar side chains at the higher temperatures. The pure bovine serum albumin solution and JSC Mars 1A also showed lower yields than the Atacama and Antarctic extractions suggesting that SCWE may be less effective at hydrolyzing large or aggregated proteins. Changing solvent from water to a dilute (10 mM) HCl solution allowed total extraction efficiencies comparable to the higher temperature/time combinations while using the lowest temperature/time (185°C/20 min). The dilute HCl extractions also did not lead to the shift in amino acid distribution observed at the higher temperatures. Additionally, adding sodium perchlorate salt to the extraction did not interfere with recoveries. Native magnetite in the JSC Mars-1A may have been responsible for destruction of glycine, as evidenced by its uncharacteristic decrease as the temperature/time of extraction increased. This work shows that SCWE can extract high yields of native amino acids out of Mars analog soils with minimal disruption of the distribution of those amino acids, even in the presence of a perchlorate salt.

INTRODUCTION

Starting with the Viking Landers,[1] and continuing through to the present, the detection of organic molecules on Mars has proven challenging. The discovery of large concentrations of perchlorate on Mars by the Phoenix Lander helped to unravel the mysterious absence of indigenous organics measured by the Viking Landers,[2] as well as the results of the biology experiments.[3] The presence of perchlorate salts during pyrolysis volatilization of samples containing organics was shown to degrade the organics present by reactions with chlorine and oxygen radicals due to the decomposition of perchlorate during heating.[4] The Curiosity mission has also found perchlorate in the samples it has analyzed, which has once again interfered with the detection of organic molecules in the sample.[5,6] The improved capabilities of Curiosity relative to the Viking landers have allowed for identification of indigenous organics, but they were detected in the form of chlorobenzene and several dichloralkanes that had been altered from their original state during pyrolysis heating.[7] In addition, evidence for combustion of reduced organic carbon to CO_2 due to the decomposition

of oxychlorine compounds was also observed.[5] These results, combined with the likelihood of a global perchlorate presence on Mars,[8] make alternative extraction methods desirable for future Mars missions.

One promising technique for extracting a wide variety of organic compound classes is Subcritical Water Extraction (SCWE). SCWE is a subset of the larger field of pressurized liquid extraction.[9] Pressurized liquid extraction aims to take advantage of the faster reaction rates and specific solvent properties while extracting at high temperatures. Pressurized systems are required to keep the solvents in a liquid state while raising the temperature beyond the typical liquid/vapor transition at typical laboratory ambient pressures.

SCWE specifically focuses on water as the extraction solvent. Water has a number of benefits, as a solvent for pressurized liquid extraction. The first is that the dielectric constant of water changes dramatically as temperature rises, so much so that water behaves much more like the less polar solvent acetone at 325°C than like the polar solvent it is at room temperature.[10,11] This changing polarity can then be used to allow compound class specific extraction,[12-14] and for water to extract nonpolar organics such as polyaromatic hydrocarbons (PAHs) that normally would not be soluble. A benefit for spaceflight (and industrial) application is that water is a nontoxic, noncorrosive, easy to purify, store and transport solvent. Finally, it is suitable for promoting hydrolysis reactions such as the breakdown of proteins into their constituent amino acids,[15] or polysaccharides into individual sugars. In fact, SCWE has been widely investigated for the processing of food wastes because of its ability to promote protein hydrolysis.[16] This ability can be very useful for simplifying complex mixtures of biopolymers of a variety of lengths into larger quantities of monomers that can be more easily separated and detected.

SCWE has been used to extract a variety of relevant organic compounds from samples with planetary science applications in mind including amino acids,[15,17] PAHs,[18,19] and aliphatic hydrocarbons.[13] Amino acids (AAs) have long been considered good molecules to search for when trying to look for biochemical signatures of life.[20-23] Creamer et al.[23] recently provided an overview of three different ways that AAs can be used to separate biotic from abiotic processes; by looking at their molecular abundances relative to glycine, by evidence for chiral excess, and by the presence of specific uniquely biotic or abiotically produced (as far as we know) AAs. Even beyond biosignature detection, AAs remain valuable targets as they have been found to be one of the more abundant compound classes present in meteorites,[24] and can help put constraints on our understanding of prebiotic chemical processes.

The gold standard for AA extraction on Earth is acid hydrolysis,[25] and this technique has been used extensively for analysis of extraterrestrial materials (meteorites).[26-28] The problem for spaceflight is the challenge of storing concentrated hydrochloric acid (6N) and then heating it to a minimum of 110°C, in a low mass, low power extraction instrument. Furthermore, additional processing may be required to remove the acid prior to downstream analysis adding complexity. SCWE provides an attractive alternative to acid hydrolysis because of the benefits just described; water is a much easier to store and handle solvent, and the SCWE technique provides the flexibility to extract other classes of compounds with the same extractor by tuning the temperature.

The work presented here focuses on optimizing the extraction of native AAs from Mars analog soils, and is especially concerned with the possible biosignature related to the distribution of AAs relative to glycine in a sample. The high temperatures used in SCWE are capable of degrading AAs and so mitigating any possible disruption of the indigenous AA distribution while still maximizing recovery efficiency is critical. As part of this goal, this work also explores the hydrolysis of a pure protein solution using SCWE to understand

how efficiently polypeptides are broken down and whether the intrinsic AA distribution is disturbed during hydrolysis.

EXPERIMENTAL

Mars Analog Sample Descriptions. JSC Mars-1A (JSC-M1A; Orbitec) was developed as a spectral and physical simulant for testing various instruments and hardware in preparation for Mars missions. Less work has been done characterizing the native organic molecules present to understand its use as a Mars simulant in the search for biosignatures. The closely related JSC Mars 1 simulant (JSC-Mars 1A is a larger collection of material that includes the original JSC-Mars1 site within it) has had measurements of some of the AAs present.[29] JSC-M1A soil simulant has been characterized in detail[30]. Briefly, it is sourced from the Pu'u Nene cinder cone in Hawaii and it is largely made up by amorphous palagonite with different amounts of alteration. X-ray diffraction spectra show a major peak for calcium feldspar and a minor peak for magnetite (The presence of a significant amount of magnetic material is clear from the material that adheres to the magnetic stir bar used in these experiments). No evidence for phyllosilicates is observed.

The Atacama Desert is another widely accepted Mars analog site because of the similarity of soil/regolith formation properties in its hyperarid regions and those on Mars [31-33]. The Atacama soil sample (labeled AT45-A3) is predominantly gypsum, from a large (~30 cm) evaporite mound. The sample was collected a few cm below the ground at site 45 on Yungay 1122 hill in the Chilean Atacama Desert (S 24°03.651'; W 69°52.102') and the site description, sampling procedure, and labeling protocol have been described previously.[34] The same work identified extractable amino acids from another sample a few cm away. The general properties of the soils of the Yungay region have also been described.[32,35]

The cold and dry conditions of the Antarctic Dry Valleys have also long made them one of the best Mars analog environments available on Earth.[36,37] The Antarctic soil was collected from the Wright Valley in the Antarctic Dry Valleys (S 77°31'11.9"; E 161°51'18.1"). Detailed study of the Wright Valley soils has been previously described.[37] Soils were collected in the top 2 cm using sterile techniques and were stored and shipped frozen. These soils are dominated by silicates and salt evaporates. Halite (NaCl) comprises up to 50% of the soil volume at depths of 2 – 4 cm. Plagioclase, quartz and potassium-feldspar dominate the silicate materials. Iron-oxides including magnetite are present but at low levels. Unlike the JSC-M1A, no visible amount of these samples adhered to the magnetic stir bar.

All the samples were sieved and the fraction of <150 μm sized particles were used in the extractions. The primary reason for this was the ability to compare these results with a companion piece of work that developed a fluidic chip based SCWE extractor which could not accept particles of larger sizes.[38] This size fraction is also what the sample handling suite of Curiosity is able to deliver,[39] and hence it is a plausible requirement of a future mission. Finally, smaller particles will have greater overall surface area and overall homogeneity leading to better reproducibility and quantification.

Instrumentation and SCWE Procedure. Sub-critical water extractions and hydrolyses were carried out using an Anton Paar Monowave EDU, a microwave synthesis/hydrolysis instrument. Sample vials were made of borosilicate glass and were sealed with PEEK caps over silicone gaskets. A clean stir bar was added to every run for magnetic stirring during the extractions. Extractions were run in triplicate at 185°C for 20, 40, 60, and 120 minutes, at 200°C for 10, 20, 40, and 60 minutes, and at 215°C for 10, 20, 30, and 60 minutes. After a brief spike in power the average power required to maintain temperatures was ~15 – 30

W, and heating and cooling typically took ~2 minutes (Figure S-1). All glass sample containers were ashed at 500°C for 4 hrs prior to use to remove any residual organics.

Four different samples were extracted/hydrolysed: A pure solution of bovine serum albumin (BSA; Sigma Aldrich lyophilized powder, >98%), and the JSC-M1A, Atacama, and Antarctic samples already described. For the BSA hydrolysis, samples were prepared containing 1.2 nmol of BSA combined with 4 nmol of norleucine (Nle; Sigma…) as an internal standard in a total of 2.2 mL of water. For each of the three soil samples, 0.4g of soil was combined with 0.4 nmol Nle internal standard in 2.2mL water. All samples were sparged with Argon to remove oxygen, a stir bar was added and the tubes were sealed with caps and gaskets. Blank runs consisting only of 0.4 nmol Nle in 2.2 mL water were also run at each temperature/time condition.

Dilute HCl extraction conditions for soils were exactly the same as described except the samples and internal standard were combined in a total volume of 2.2 mL of 10 mM HCl instead of pure water. Similarly the perchlorate extraction runs were also the same except that the samples and internal standard were combined in a total volume of 2.2 mL of 2.5 mM sodium perchlorate (Fisher Scientific) solution. The perchlorate concentration was taken from the results of the Wet Chemistry Lab instrument onboard the Mars Phoenix Lander.[40]

At the completion of each run, the samples were vortexed and the sample tubes were set aside for 10 minutes to allow for particulate settling (For the BSA and blank runs, this step was skipped, and 2 mL of the solution was transferred directly to a microcentrifuge tube). After settling, as much fluid as possible was removed into two microentrifuge tubes (typically 1 mL in each). The tubes were placed in a low speed centrifuge for 2 minutes to remove any residual particulate material, and then 0.9mL of fluid was removed from each of the tubes and combined in a single microcentrifuge tube. All the samples were then frozen at -20°C and lyophilized overnight (Thermo-Fisher Micro Modulyo-115).

The three soil samples were also extracted via room temperature sonication for 1 hr (Bransonic 5510) in either pure water or 10 mM HCL to determine pre-SCWE levels of free amino acids. The water temperature of the bath was monitored and never exceeded 35°C after 1 hr. Samples were prepared and processed in an identical manner to those extracted via SCWE.

Amino Acid Detection. AA separation and detection was performed by reverse phase HPLC (Waters 2695 separation module) and laser induced fluorescence (LIF; Waters 2475 fluorescence detector) using the Waters AccQ-Tag™ method.[41] This method is capable of separating the 17 hydrolysate amino acids (but not their enatiomeric pairs, D/L), Aspartic acid (Asp), Serine (Ser), Glutamic acid (Glu), Glycine (Gly), Histidine (His), Arginine (Arg), Threonine (Thr), Alanine (Ala), Proline (Pro), Cysteine (Cys), Tyrosine (Tyr), Valine (Val), Methionine (Met), Lysine (Lys), Isoleucine (Ile), Leucine (Leu), Phenylalanine (Phe), and our internal standard Norleucine (Nle). Five calibration standards were prepared for each HPLC run (0, 0.2, 0.5, 1.0, 2.0 nmol) using a Pierce amino acid standard mix of the 17 hydrolysate AAs plus Nle.

The samples were prepared for HPLC analysis by redissolving the lyophilized extracts in 160µL of the AccQ-Tag™ borate buffer (The JSC-M1A samples were redissolved in only 80 µL). Next, 80 µL of each were transferred to tubes for labelling, and 20uL of the fluorescent labeling agent were added to each while vortexing. These samples were then transferred to HPLC vials for analysis. For the Atacama samples, after the redissolution step significant insoluble material was present. These samples were then centrifuged at 8000 rpm to pelletize the insoluble material prior to transfer to the labelling tubes.

Peaks were identified and quantified using Empower software (Waters Corp.) by comparisons to the standard curves generated for each analysis run. Values were averaged over the three extractions performed at each condition, and the error bars reported are the one sigma standard deviation from the mean. The blank runs were also averaged and subtracted from the data prior to their normalization to a nanmole per gram (nmol/g) value based on the amount of sample analyzed. Background levels of AAs during SCWE were typically below the limits of detection, but small contributions from Gly, Ala, Pro, and Leu were sometimes measured. Examples of the raw HPLC chromatograms for the 200C/40 min SCWE condition including a blank and standards are shown in Figure S-2.

Acid Hydrolysis Procedure. A liquid acid hydrolysis (AH) protocol was used as the gold standard to determine the total amount of AAs present in each soil sample in order to assess the overall extraction efficiency of the different SCWE conditions. AH was performed at 160°C for 15 min in 2.0 mL of 6N HCl (Fisher Certified), based on the protocol of Stell et al.[42] The protocol was first demonstrated on a 0.04 nmol BSA sample to verify that the modified AH protocol provided sufficient recovery. JSC-M1A, Atacama, and Antarctic soils 0f 0.05, 0.20, and 0.20g respectively were added to 2.2 mL of 6N HCl for AH.

After AH for the BSA sample, it was lyophilized and prepared for analysis in an identical manner to the SCWE samples. However, for the final AH of soil samples it was necessary to desalt them to permit labeling without interference from the leached inorganic species. The desalting procedure followed that prescribed by Amelung and Zhang, 2001.[43] Briefly, 2g of cation exchange resin (Dowex 50W-X8, 100-200 mesh H^+ resin) were prepared in 2 mL Pierce centrifuge columns. The resin was rinsed successively with 2 Column Volumes (CV) of water, 2CV of 2M NaOH, 4CV or water, 2CV of 2M HCl, and then 6CV of water. The AH extracts were then added, and then rinsed with 4CV of water. Finally, 2M NH_4OH was used to elute the AAs under basic conditions, and then samples were lyophilized for analysis.

Control experiments on the desalting procedure using the HPLC AA calibration standard found interference from residual ammonium in the samples with the labeling dye. Redilution of the lyophilized samples in water and an additional lyophilization step improved recovery, for a median recovery of each residue of 0.79. This procedure was then used for the actual soil sample analysis. The absolute AA recoveries were normalized by the individual AA recovery factors determined during the control experiments, and these factors are reported in Table S-1.

RESULTS AND DISCUSSION

Amino Acids detected and their classification. Of the 17 hydrolysate AAs 14 are reported here. His, Cys, and Met were frequently not well separated or below the limits of quantification. Furthermore Cys and Met suffer known degradation during acid hydrolysis,[25] interfering with a reliable efficiency measurement, and was not something that was accounted for in this work.

All of the 14 AAs reported here have had their degradation at elevated temperatures and pressures investigated.[44-51] The results of these investigations show that the AAs can be broadly grouped into three classes: Group 1 are made up of the charged (acidic/basic) AAs, Arg, Lys, Asp, and Glu, as well as the polar side chain AAs Ser and Thr. The AAs in Group 1 are the most susceptible to decomposition at the elevated temperatures of SCWE, and show rapid (< 5 min) decomposition at temperatures ≥ 250°C. Group 2 consists of the AAs with larger hydrophobic side chains including Tyr, Phe, Leu, and Ile. Group 2 is more stable than Group 1 at SCWE temperatures, even at temperatures ≥ 250°C. Group 3 consists of the small aliphatic AAs Gly, Val, Ala, as well as Pro, and are also stable at higher temperatures. Group 3 AAs are

also formed during the decomposition of members of Group 1 and Group 2, and can thus help determine if the extraction conditions are changing the initial AA distribution in the sample.

Acid Hydrolysis of Samples. AH was used to determine the total amount of each AA recoverable. The SCWE extraction results were then compared to the AH results to determine SCWE extraction efficiencies, and any changes to the distribution of AAs. A liquid AH protocol was chosen because it has been shown that vapor methods are sometimes insufficient to fully penetrate soils and sediments.[52] The tradeoff is that liquid AH protocols typically show significantly higher background contamination from AAs in the HCl. This was true for our work as well, the concentrated HCl in the AH extraction had the largest AA background during blank runs (Table 1; Data for each AA is in Table S-2).

Traditional AH has typically been performed at 110°C for 20 – 24 hrs in 6N HCl.[25] However, the ability of microwave systems, such as the Monowave used in the SCWE extractions, to rapidly heat to higher temperatures has allowed the development of more rapid AH protocols such as the 6N HCl at 160°C for 15 minutes protocol used in this work (Initially suggested by the CEM corporation[42]). In order to verify this AH procedure, pure solutions of BSA were hydrolyzed and then compared to their predicted distributions (Figure 1). Overall this method showed >85% recovery of the predicted AAs, no systematic change in the distribution of AAs, and good reproducibility. A longer extraction time of 30 min did not improve the yield. The slightly lower than anticipated total recovery may be related to the tendency of BSA to polymerize at higher temperatures and pressures,[53] making it more robust to hydrolysis. The yield was also comparable to previously reported results that saw less than a quantitative recovery of all AAs.[51] Given the unknown nature of the type and status of proteins or polypeptides in the soil samples, the yield was deemed sufficient for comparing the different soils, and providing a reproducible standard for assessing the SCWE extractions.

The total AA recoveries via AH are presented in Table 1. The Antarctic and Atacama both have low levels of recoverable AAs on the order of tens of nmol/g. The JSC-M1A in contrast is nearly 3 orders of magnitude more rich. The AA recoveries reported here are also 5 – 30 times more abundant (depending on the specific AA) than those reported for AH extraction on the original JSC Mars 1 simulant.[29] That work by Garry et al. demonstrated the importance of size fraction (surface area) for the overall yield of AAs for these simulants. This study uses a smaller size fraction with greater surface area per unit mass which will contribute to the increased amounts of AAs measured. Other factors such as how the samples were collected and processed along with the fact that the JSC-M1A comes from a larger overall area also contribute to the different amounts of AAs measured.JSC-M1A was originally developed as a spectral simulant for Martian soils,[30] and while the mineralogy remains quite relevant (especially the possible role of magnetite as will be discussed), the level of organic content may not make it suitable for the type of low levels of organic content expected to be found on Mars.[7]

Among the AAs analyzed here the most prevalent AAs (the top 5 of Gly, Val, Glu, Ala, and Leu) were the same across all three soils (Figure 2). These AAs are also the most abundant in E. Coli and the Mono lake samples studied by Creamer et al. with the exception of Ser which was found in higher abundance than in this work.[23] This highlights the biological origin of the AAs in these samples, as abiotic distributions of the protein AAs found in the Murchison meteorite show a greater ratio of Gly to thenext most abundant protein AAs..[23,54]

SCWE Extraction Efficiencies and Effect on AA Distributions. Previous work on SCWE of Atacama soils found that 200°C was the optimum temperature for recovery for a limited set of AAs (Gly, Ala, Val,

Ser, Asp, Glu).[15] A major goal of this work was to further explore the effect that SCWE extraction temperature and extraction duration have on the recovery of a broader range of AAs. Using the 200°C as a guide, we tested a range of extraction times at 185°C, 200°C, and 215°C. Pressure was not controlled during the extractions and varied according to the vapor pressure of water at each temperature (11.2, 15.5, and 21.1 Bar). While pressure can affect the extractions, temperature is the dominant variable.[46,51] These temperatures were also guided by the modeled destruction rates of the Group 1, 2, and 3 AAs previously discussed. In addition to optimizing time and temperature, this work also focused on comparing results across different soils and the protein standard, BSA.

Overall the trend for total yield of AAs increased with both increasing temperature and increased extraction time for the BSA, Antarctic and Atacama samples (Figure 3). Peak extraction efficiencies (relative to acid hydrolysis) were in the 50 – 60% range for the Antarctic and Atacama samples, but were lower for the BSA. The extractions were also reproducible, despite the possibility of heterogeneity in the samples, the extractions were performed in triplicate and the 1σ error for almost all conditions was ≤ 20%.

The efficiency of total AA recoveries for the higher organic content JSC-M1A did not follow the same trend, and was typically only on the order of 1% (Figure 4). It is important to note though that a 1% recovery from the JSC-M1A still returned more absolute amounts of AA's than the same extractions on the low biomass samples because of the large amount of extractable AA's to begin with (Table 1).

The lower recovery of BSA relative to the soil samples could be related to two different explanations. The first, as already mentioned in regards to the AH yield, is that the known polymerization of BSA (even in dilute solutions) at high temperature / pressure may shield it from hydrolysis.[53] The second possibility is that the BSA results demonstrate expected recoveries from a protein in this size range, and that the Antarctic and Atacama soils contain a larger abundance of already partially degraded proteins and polypeptides making their hydrolysis into individual AAs more efficient (faster) with respect to the extraction times explored in this work. This second scenario could also help explain the much lower extraction efficiencies of the JSC-M1A, because the clearly biologically rich sample may be made up of predominantly intact large proteins that like the BSA do not break down as fast into their constituent AAs.

As a check on the value of SCWE we also performed extractions via sonication (Table 1). The total AAs recovered via room temperature sonication were at least one order of magnitude lower than the SCWE recoveries.

The total AA recovered does not tell the whole story because of the previously discussed effect of temperature on AA decomposition. It is therefore important to contrast the overall trend of increasing recovery with time and temperature to the observed trends in each of the three groups of AA's that have different resilience to decomposition. A useful way to quickly understand the changes is to compare two time/temperatures at either end of the spectrum with similar total AA extraction recovery. Figure 5 shows the percentage increase or decrease of each AA at the 215°C extraction for 30 min (215C/30m) relative to the extraction at 185°C for 60 min (185C/60min). For the rapidly decomposed AA's in group 1, all of them show a significant decrease during the high temperature extraction because of their susceptibility to decomposition. The more stable group 2 and 3 AA's show the opposite effect and mostly increased at the higher temperature. For group 2, this is likely due to more AA's released during continued protein or polypeptide hydrolysis. The evidence for this is that overall AA recovery increases in the pure BSA solution at higher times and longer temperatures, which must be the result of further protein breakdown. For group 3,

there are two contributions to their increase; the greater extent of hydrolysis similar to group two, but they are also augmented by the decomposition of the AA's in group 1 becoming these simpler AAs.[44]

These trends were not as clear for the higher biomass JSC-M1A where recoveries were mostly flat across changing time and temperature. In fact, one of the few noticeably changing AA's was the decrease in Gly, one of the typically stable and increasing AA moieties (Figure 6). Destruction of Gly in the presence of magnetite under SCWE conditions has been observed previously,[46] and could be occurring here in the JSC-M1A where magnetite makes up a significant fraction of the soil minerals. The significant loss of Gly, and possibly some of the other small group 3 AA's, may explain the lack of the typically observed increasing recovery with time and temperature trend, because their catalytic destruction is offsetting their typical gains via additional hydrolysis and production from other decomposing AA's.

Complete tables of the data for the Antarctic, Atacama, JSC-M1A, and BSA extractions are in tables S-3 – S-6.

The Effect of Dilute HCl and Perchlorate on SCWE. The desire to increase extraction yields, but minimize extraction time and impact on the native distribution of AA's via decomposition, led to the use of a 10 mM "dilute" HCl solution (DHCl) with pH 2 in the SCWE benchtop extractions. The DHCl results show a similar total AA extraction efficiency to the highest temperatures and longest extraction times, and in the case of BSA and JSC-M1A show the best efficiencies by far (Figures 3 – 4). Importantly, the DHCl extractions do not show a systematic enhancement of only one group of AAs, but rather show improved extraction primarily of both groups 1 and 3 relative to the 185C/60m extraction (Figure 7). The fact that the DHCl results were also significantly better for both the JSC-M1A and BSA total AA recoveries indicates that the acidic conditions directly improve hydrolysis, not just liberation of AA's from on or within soil particles. This is also confirmed by the extraction yields for sonication in water compared to DHCl, which are all of the same order of magnitude (Table 1), and both are one order of magnitude lower than their respective SCWE recoveries Overall this makes the DHCl condition very promising as a quick, high efficiency way to perform SCWE without disrupting the native AA distribution.

We additionally looked for any effect of perchlorate on the Antarctic extractions and whether they changed the AA distributions because of the known problems with perchlorate interference during pyrolysis extraction on Mars. No change in AA distribution was observed even at the most extreme extraction conditions used in this study, 215°C for 60 minutes (215C/60m) (Figure 8). For these experiments sodium perchlorate was added, but less thermally stable iron and magnesium perchlorate salts are also expected on Mars.[8] However, the high solubility of perchlorate salts means that during SCWE they will be in solution, not as a solid salt, so the counter-ion should not have the same effect on stability as it does during pyrolysis. Still, future work is warranted to verify that no unexpected behavior occurs with different parent salts.

CONCLUDING REMARKS

This work has demonstrated quantitatively the ability of SCWE to reliably extract AAs from natural samples under a variety of extraction conditions. Changing the solvent to 10 mM HCl improves the overall extraction efficiency and allows shorter extraction time at lower temperature to improve the preservation of the less stabile AAs. These data reinforce the potential utility of SCWE for AA extraction from Martian samples where sample alteration due to a variety of oxidative stresses is likely, but SCWE is not a substitute for standard AH procedures in a laboratory setting that allows for complete hydrolysis of larger intact polypeptides.

While this work did not assess the change in the chiral distributions at different extraction conditions, it seems likely that at conditions where AAs are being destroyed significant racemization is also occurring. It's possible that the use of a dilute acid and optimized extraction time and temperature could also be used to successfully preserve the native chiral distributions as well.

The low recoveries found for the JSC-M1A sample are likely caused in part by the fact that a large amount of the AAs are present in large intact proteins, which the BSA results suggest will result in a lower extraction yield. The presence of magnetite also plays a role because it catalyzes the destruction of glycine and possibly other AAs. Other unidentified properties might also play key roles such as the ionic strength and pH of the extracted solution as well as the presence of other soluble species. Further investigation was deemed outside the scope of this work, primarily because the sample is so rich in organic matter that it is not as useful a Mars analog as the other soils.[7,55,56] From a planetary science perspective, the absolute amount of organic material recovered from the JSC-M1A would provide a treasure trove for analysis from a Martian sample, even if the true efficiency of extraction is low.

However, JSC-M1A extractions were useful in highlighting the potential for interference during extraction by magnetite, a mineral that has been identified in Martian samples.[57,58] This juxtaposition between realistic and unrealistic components of JSC-M1A is representative of the typical types of tradeoffs required when developing planetary analogs.[59] On one side, fully controlled synthetic analogs allow explicit testing of hypotheses, and on the other side uncontrolled but relevant environmental samples allow for proving that techniques can be used on unknown samples that have undergone representative natural processes. Frequently, a middle ground is also used, such as the addition of perchlorate to the extractions performed here of otherwise natural samples. For Mars, where we have more knowledge than other solar system bodies, a high quality suite of synthetic analogs has been developed for testing the survival of microorganisms.[60] The range of different mineral and physiochemical components/conditions of these analogs could provide a good test of possible interference with SCWE extractions as part of future work. Overall, the goal of quantitatively extracting unaltered organic compounds of interest from unknown future planetary samples requires that a variety of different analogs, both more and less controlled are tested. SCWE, and the larger field of pressurized liquid extraction, appear to be promising techniques for pursuing this goal.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest

TABLES

Table 1. Total AAs recovered from extractions (nmol/g)

Extraction type	JSC-M1A	Antarctic	Atacama	Blank*
Acid Hydrolysis	1.91×10^4	8.45×10^1	2.88×10^1	2.00
SCWE (peak)	2.39×10^2	5.45×10^1	1.49×10^1	0.16
Sonication	1.15×10^1	3.80×10^0	1.92×10^0	0.15
SCWE (DHCl)	1.02×10^3	4.83×10^1	7.84×10^0	0.54
Sonication (DHCl)	1.40×10^1	1.21×10^0	Below quan-tification	0.40

*nmol/mL

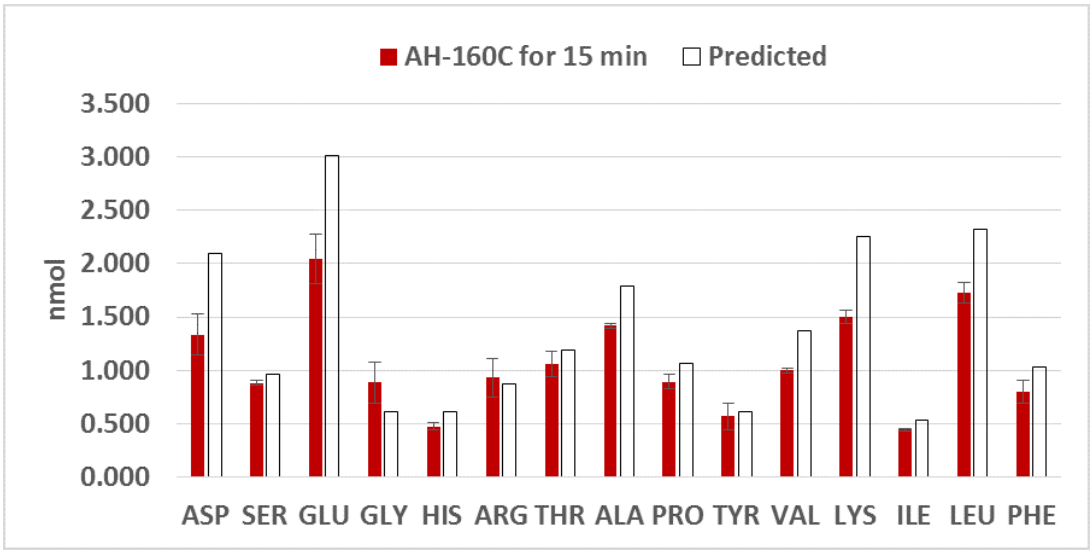

Figure 1. The Waters AH protocol of 160°C for 15 min provided >85% recovery of AAs and the expected distribution.

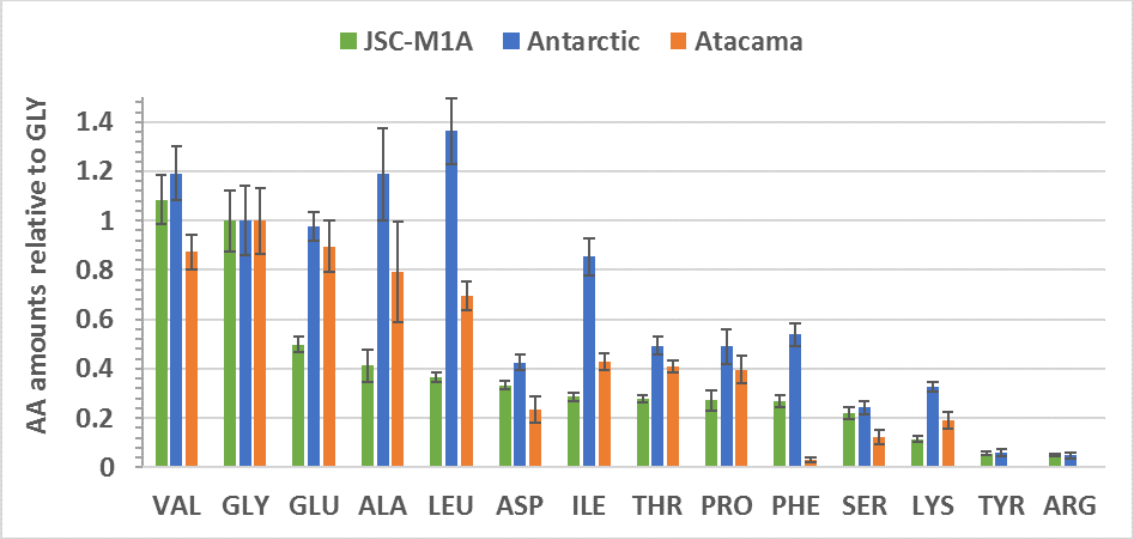

Figure 2. The distribution of AA's relative to Gly in each sample. The top 5 most prevalent AA's are always Val, Gly, Glu, Ala, Leu.

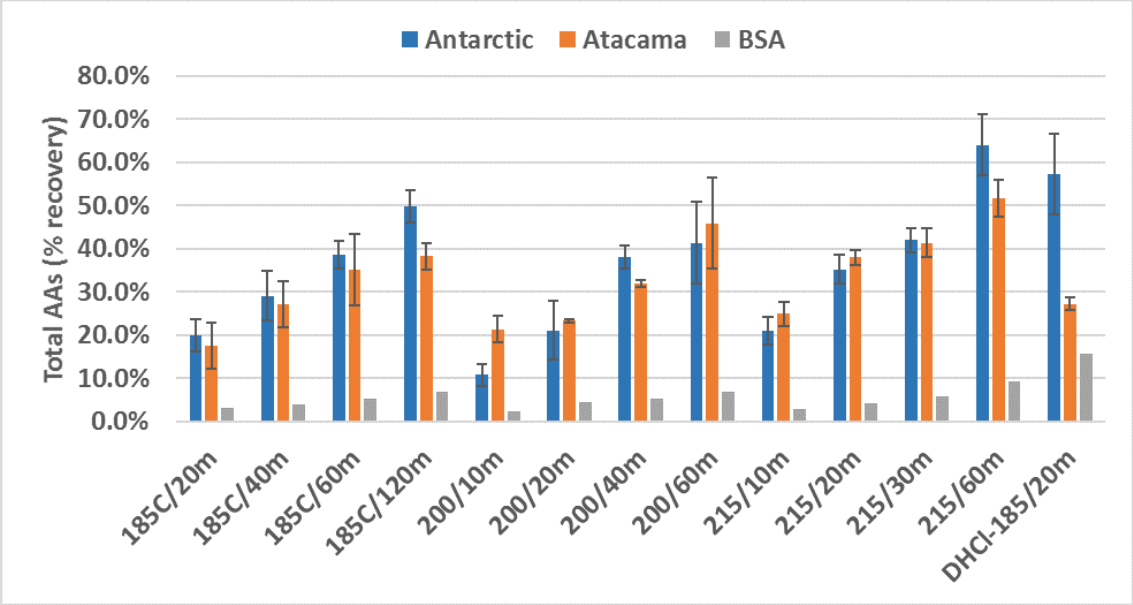

Figure 3. The general trend of increasing recovery with time and temperature is observed for both the low biomass Antarctic and Atacama samples. Adding a small amount of acid (10 mM HCl) leads to much better recovery at lower temperature and time (DHCl-185/20m).

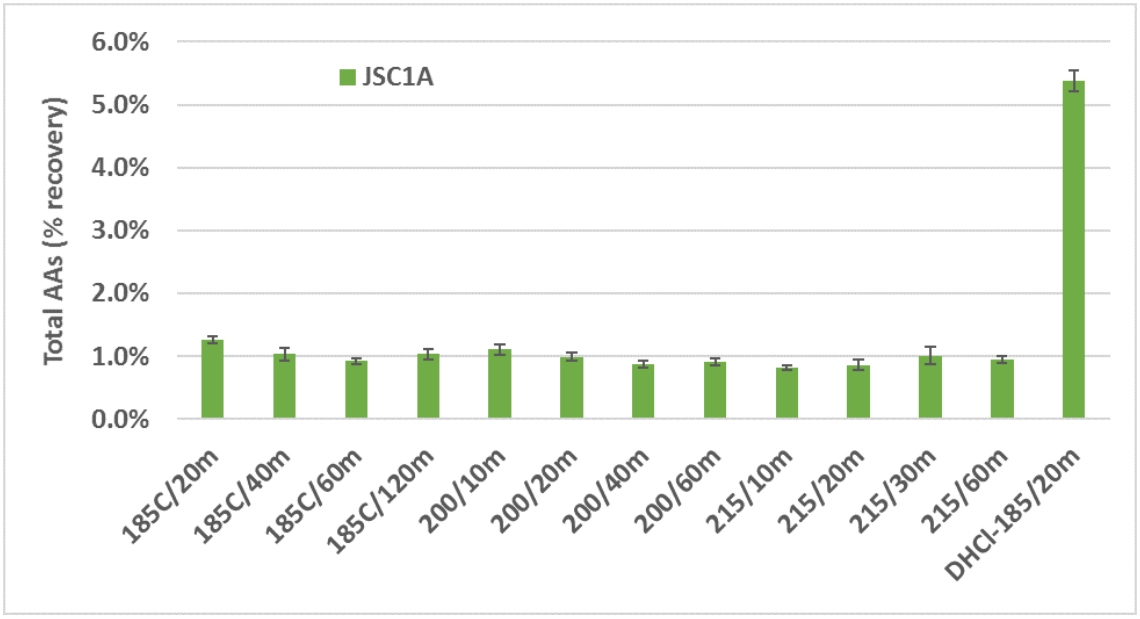

Figure 4. Total amino acid recovery is much lower for the JSC-M1A data. The dilute HCl (DHCl-185/20m) at 185°C for 20 min showed the best recovery, demonstrating its importance to enhancing protein hydrolysis.

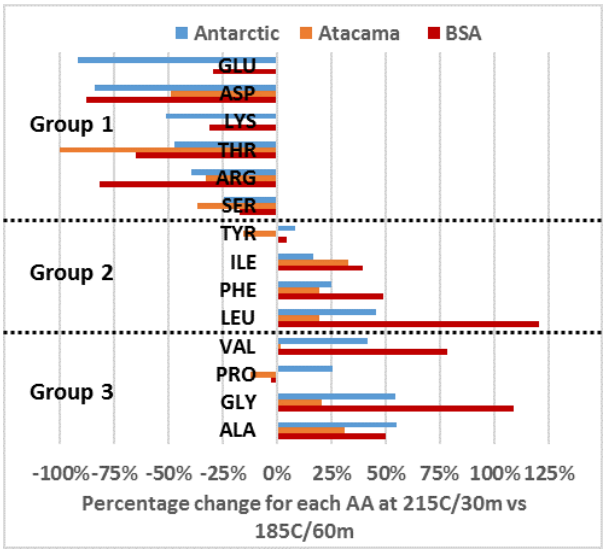

Figure 5. All of the less thermally stable Group 1 AA's were detected at increased levels at 185C compared to 215C (the extraction times picked for comparison have nearly identical total AA recoveries). The more stable Group 2 and 3 AA's show the opposite trend. The simplest AA's in Group 3 tend to show the greatest increase at 215 due to the decomposition of other AA's into the simpler ones.

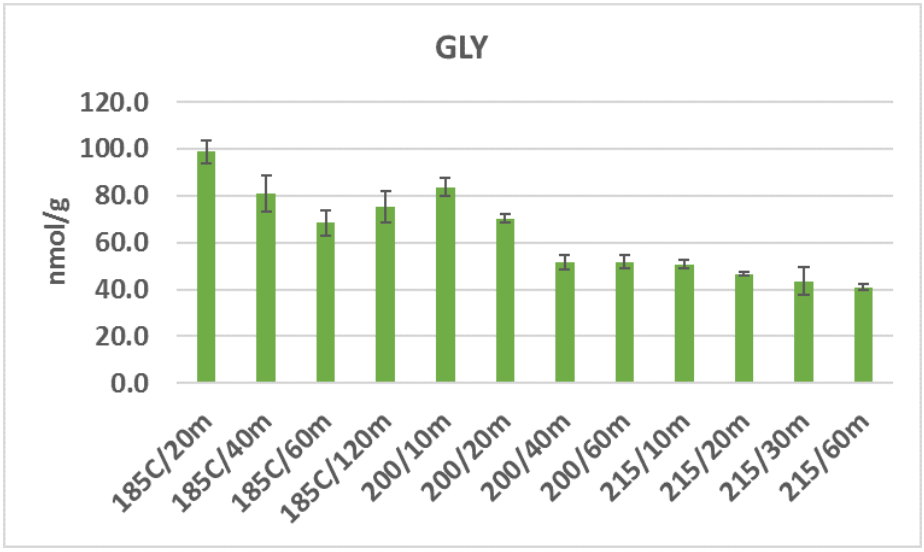

Figure 6. Glycine in JSC-M1A was unexpectedly found to decrease both with increasing extraction time and temperature, counter to its typical pattern in all the other samples. Catalytic mineral surface destruction could be responsible.

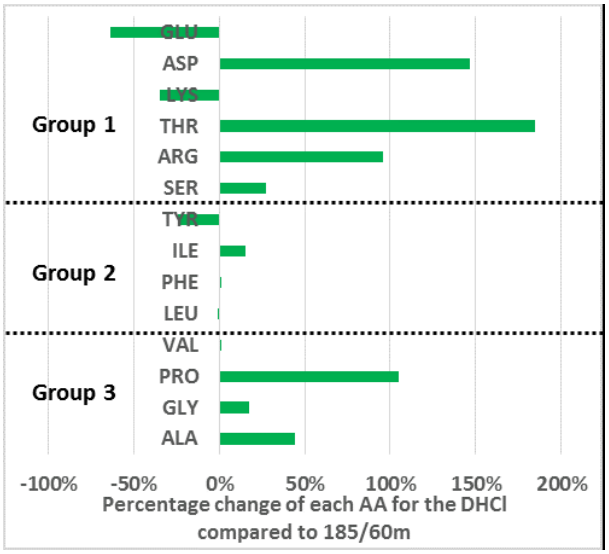

Figure 7. The DHCl extraction conditions improved the total yield of AAs relative to 185C/60m extractions, but unlike the higher temperature extractions does not do so by systematically enhancing only one of the AA groupings.

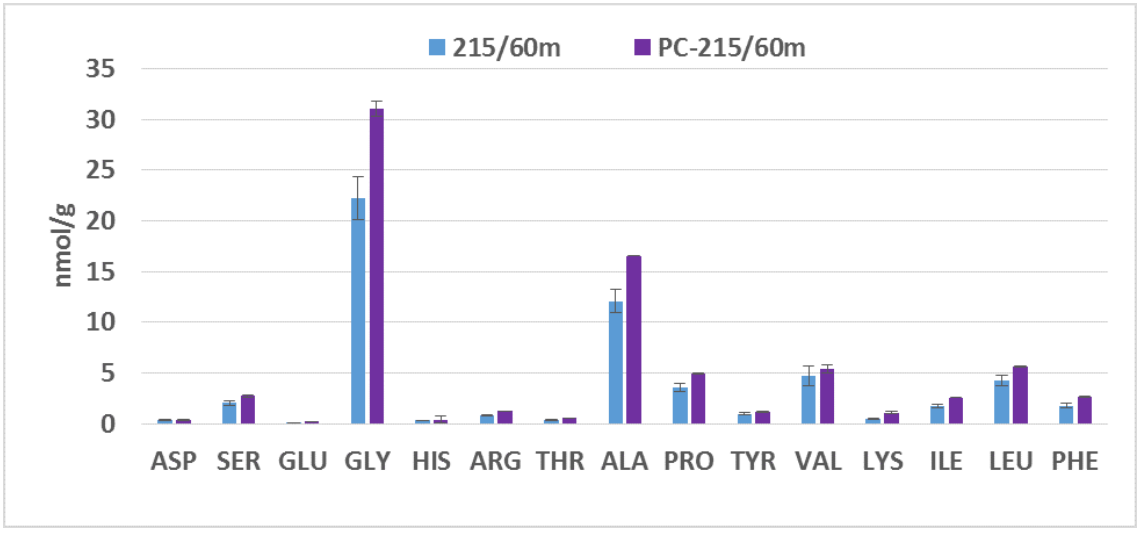

Figure 8. Results from the addition of 2.5 mM sodium perchlorate to the Antarctic sample under the harshest extraction conditions of this study did not change AA recovery of distribution appreciably.