THE SIMULTANEOUS QUANTITATION OF TEN AMINO ACIDS IN SOIL EXTRACTS

BY MASS FRAGMENTOGRAPHY

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The analysis of amino acids from terrestrial and extraterrestrial sources is becoming increasingly important (1-5). The need for a specific, sensitive and rapid method of quantitation is desirable. The methods currently employed for amino acid analysis involve ion exchange procedures (6,7) or gas chromatography (8-10). These techniques, although of immense value, are limited by their non-specificity for the absolute identification of any substance responsible for a gas chromatographic peak.

In the present communication we report an absolute, unambiguous method for the positive identification and quantitation of ten amino acids present in soil extracts using GLC-mass fragmentography. In mass fragmentography the mass spectrometer is used only to detect certain preselected ions known to be characteristic for each compound being quantitated, and the internal standard. The technique of mass fragmentography using sector mass spectrometers is usually restricted to the simultaneous monitoring of up to three integer mass values (11, 12), although with one instrument five ions were used (13). Using a quadrupole mass spectrometer up to eight ions have been selected and their respective analog signals monitored (14). We now wish to report the modification of the gas chromatography-quadrupole mass spectrometer-computer system previously described (15) for the simultaneous monitoring under computer control of the ion currents from 25 pre-selected integer mass values. If required this number could be increased by suitable alteration of the computer control programs. Specifically we wish to report the application of this system to the quantitation of ten of the amino acids present in soil extracts.
METHODS

Reagents: A deuterated amino acid mixture was supplied by Merck Laboratory Chemicals (New Jersey). 1.25N HCl in n-butanol, 25% (v/v) trifluoroacetic anhydride in methylene chloride and Tabsorb column packing were obtained from Regis Chemical Co., Illinois. A standard amino acid solution was purchased from Pierce Chemical Co., Illinois.

Equipment: A Varian model 1200 gas chromatograph was coupled by an all glass membrane separator (16) to a Finnigan 1015 Quadrupole mass spectrometer which was interfaced to the ACME computer system of the Stanford University Medical School (15). GLC separations were conducted using a 6 foot by 4 mm. (I.D.) coiled glass column packed with Tabsorb (Regis Chemical Co.). The flow rate of the carrier gas (helium) was 60 ml/minute.

Procedure

1 g of sieved, air-dried soil (Stanford University garden soil) was refluxed with 6N HCl (10 ml) for 20 hrs. The mixture was filtered and the residue washed with 1N HCl (5 ml). The combined filtrate and washings were extracted with chloroform (4 x 10 ml) and the aqueous phase evaporated to dryness. The residue is dissolved in water (5 ml) and passed through a column of "Ion Retardation Resin" AG 11-A8 (50-100 mesh, 1 x 21 cm). The amino acids were eluted with water (50 ml) and the eluate evaporated in vacuo to dryness. The residue is dissolved in water (5 ml) and placed on a column of
cation exchange resin (AG 50W-X12, 50-100 mesh, 1 x 21 cm) and washed with water (50 ml) to remove neutral and anion contaminants. The amino acids were eluted with 4N NH₄OH (80 ml) and the eluate evaporated to dryness. The residue was dissolved in water and made up to a volume of 4 ml. A portion of this solution (1 ml) was used for the amino acid analysis using an amino acid analyser. To another 2 ml of the processed solution was added 2 ml of the deuterated amino acid standard solution (100 mg in 100 ml of 0.1N HCl) and the mixture evaporated to dryness. The residue was refluxed with 1.2 N HCl in n-butanol (1 ml) for 30 min. and evaporated to dryness in vacuo. To the residue trifluoroacetic anhydride in methylene chloride (0.75 ml) was added and refluxed for 10 min. The solution was evaporated to dryness at room temperature and the residue dissolved in ethyl acetate (100 µl). An aliquot (1 µl) was injected into the injector port of the gas chromatograph and the oven kept at 100° for 1 min. when it was programmed at 4°/min. to 220°.

To each of 4 tubes containing 2 ml of the deuterated amino acid standard solution (100 mg in 100 ml of 0.1N HCl) was added 150, 200, 250 and 300 µl respectively of a standard amino acid solution (2.5 µmoles of each amino acid per ml). The solutions were mixed and evaporated to dryness. Each residue was derivatized by the above method and an aliquot of each (1 µl) injected into the gas chromatograph which was operated under the conditions described above. This procedure was used to construct a standard curve for the quantitation of each amino acid. A typical standard curve is shown (Figure 1) for glutamic acid.
RESULTS

The N-TFA, O-n-butyl derivative was chosen for the derivatization of amino acids for two reasons. Firstly, these derivatives have excellent glc separation characteristics (17) and secondly the selected characteristic fragment ions of the deuterated and non-deuterated derivatives do not interfere with each other, nor with other α-amino acids. Table I records the individual ions monitored for quantitation in the mass spectra of each of the deuterated and non-deuterated amino acids. The computer integrates the intensity of the deuterated and non-deuterated ion currents with time and quantitation is achieved by calculation of the ratio of their respective peak areas.

Our results of a typical soil analysis are compared with those from an amino acid analyser in Table II. The higher value obtained with lysine by the amino acid analyser is due to a ninhydrin positive substance in soil interfering with the quantitation of lysine. In this respect mass fragmentography is superior to the amino acid analyser in that using a mass spectrometer as detector only characteristic pre-selected ions are detected and quantitated and any impurity present under the same gas chromatographic peak is not measured. A summation of 20 such characteristic ions was plotted as an ion chromatogram of a derivatized soil sample and is shown in Fig. 2.

Preliminary experiments showed that when the deuterated amino acid mixture was added directly to the soil sample extensive hydrogen-deuterium exchange occurred during acid hydrolysis of the soil extract. The removal of the isotopic label was catalysed by the hot mineral acid in presence of excess mineral used in the soil hydrolysis step. Fox
and collaborators have reported (4) a similar finding concerning the
decomposition of amino acids in soil upon direct acid hydrolysis. In
the present work the deuterated amino acid mixture was added just before
derivatization (i.e. after hydrolytic extraction of the soil) in order
to avoid this problem. However, in cases where it is necessary to
quantitate the free amino acid content of complex mixtures, such as
in serum or urine samples, the deuterated amino acid mixture may be added
directly to the sample before processing without any deleterious
effects (18).

Although only ten amino acids present in soil were quantitated
the method can be extended to all the normal amino acids found in
protein. The deuterated analogs of arginine, histidine, serine,
threonine and tyrosine are commercially available. Appropriate
deuterated analogs of methionine, tryptophane, cysteine and cystine
would have to be chemically synthesized from the appropriate precursors.
In these instances at least two deuterium atoms should be incorporated
in non-exchangeable positions so that for the characteristic ion
chosen the P + 2 peak is separate from the $^{13}$C isotope contribution
of the unlabeled amino acid. Furthermore, the deuterium substitution
need not be quantitative (>90%) provided the same characteristic ion
of that deuterated analog is used for the construction of a standard
curve such as Figure 1.
Instrument analysis time is approximately one hour and with our system we have been able to achieve accurate quantitation with samples containing as little as 10 nanograms of an amino acid.

SUMMARY

A specific and sensitive method for the identification and simultaneous quantitation by mass fragmentography of ten of the amino acids present in soil has been developed. The technique uses a computer driven quadrupole mass spectrometer and a commercial preparation of deuterated amino acids is used as internal standards for purposes of quantitation. The results obtained are comparable with those from an amino acid analyser. In the quadrupole mass spectrometer-computer system used up to 25 pre-selected ions may be monitored sequentially. This allows a maximum of 12 different amino acids (one specific ion in each of the undeuterated and deuterated amino acid spectra) to be quantitated. The method is relatively rapid (analysis time of approximately one hour) and is capable of the quantitation of nanogram quantities of amino acids.

ACKNOWLEDGMENTS

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REFERENCES


Table I. CHARACTERISTIC FRAGMENT IONS SELECTED FOR MASS FRAGMENTOGRAPHY OF UNDEUTERATED AND DEUTERATED N-TFA-0-n-BUTYL-AMINO ACIDS.

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>Fragment Ion</th>
<th>Deuterated Amino Acids</th>
<th>Fragment Ion</th>
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<tbody>
<tr>
<td>ALA</td>
<td>CH₃=NHCO CF₃ (m/e 140)</td>
<td>CD₃CD(NH₂)COOH</td>
<td>CD₃CD=NHCO CF₃ (m/e 144)</td>
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<tr>
<td>VAL</td>
<td>i-C₃H₇CH=NHCO CF₃ (m/e 168)</td>
<td>1-C₃D₇CD(NH₂)COOH</td>
<td>1-C₃D₇CD=NHCO CF₃ (m/e 176)</td>
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<tr>
<td>GLY</td>
<td>CH₂=NHCO CF₃ (m/e 126)</td>
<td>NH₂CD₂COOH</td>
<td>CD₂=NHCO CF₃ (m/e 128)</td>
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<tr>
<td>ILEU</td>
<td>C₂H₅CH(CH₃)CH=NHCO CF₃ (m/e 182)</td>
<td>C₂D₅CD(CD₂)CD(NH₂)COOH</td>
<td>C₂D₅CD(CD₂)CD=NHCO CF₃ (m/e 192)</td>
</tr>
<tr>
<td>LEU</td>
<td>i-C₃H₇CH₂CH=NHCO CF₃ (m/e 182)</td>
<td>1-C₃D₇CD₂CD(NH₂)COOH</td>
<td>1-C₃D₇CD₂CD=NHCO CF₃ (m/e 192)</td>
</tr>
</tbody>
</table>
| PRO         | \[
\begin{array}{c}
\text{N-COCF₃} \\
\text{(m/e 166)}
\end{array}
\] | | \[
\begin{array}{c}
\text{N-COCF₃} \\
\text{(m/e 173)}
\end{array}
\] |
<p>| PHE         | C₆H₅CH=CHCOOH⁺ (m/e 148) | C₆D₅CD₂CD(NH₂)COOH | C₆D₅CD=CDCOOH⁺ (m/e 155) |
| ASP         | BuOOCCH₂CH=NHCO CF₃ (m/e 240) | HOOCCD₂CD(NH₂)COOH | BuOOCCD₂CD=NHCO CF₃ (m/e 243) |
| GLU         | HOOCCH₂CH₂CH=NHCO CF₃ (m/e 198) | HOOCCD₂CD₂CD(NH₂)COOH | HOOCCD₂CD₂CD=NHCO CF₃ (m/e 203) |
| LYS         | CH₂=CHCH₂CH₂CH=NHCO CF₃ (m/e 180) | NH₂(CD₂)₄CD(NH₂)COOH | CD₂=CDCD₂CD₂CD=NHCO CF₃ (m/e 188) |</p>
<table>
<thead>
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<tr>
<td></td>
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<tr>
<td>Ala</td>
<td>206.5</td>
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<tr>
<td>Lys</td>
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<td>115.3</td>
</tr>
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</table>
Fig. 1.  \( \frac{\text{GLU}}{D_5-\text{GLU}} \) Added
LEGENDS TO FIGURES

Fig. 1. Standard curve for the quantitation of Glutamic acid.

Fig. 2. Typical ion chromatogram of soil amino acids.