THE SIMULTANEOUS QUANTITATION OF TEN AMINO ACIDS IN SOIL EXTRACTS BY MASS FRAGMENTOGRAPHY

W. E. Pereira, Y. Hoyano, W. E. Reynolds, R. E. Summons and A. M. Duffield

Department of Genetics, Stanford University Medical Center

Stanford, California

Running Title: Mass Fragmentography of Amino Acids

Address for Proofs: Dr. A. M. Duffield
Department of Genetics
Stanford University School of Medicine
Stanford, California 94305

Received

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 NH_4OH (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~~ was 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

This research was funded by the Planetology Program Office, Office of Space Science, NASA Headquarters under grant NGR-05-020-004.

REFERENCES

1. POCKLINGTON, R., Anal. Biochem. 45, 403 (1972).
2. TAYLOR, R. and DAVIES, M. G., Anal. Biochem. 51, 180 (1973).
3. HALPERN, B., WESTLEY, J. W., LEVINTHAL, E. C. and LEDERBERG, J., Life Sciences and Space Research - North Holland, Amsterdam, 239 (1967).
4. HARE, P. E., HARADA, K. and FOX, S. W., Proc. Apollo 11 Lunar Science Conf. 2, 1799 (1970).
5. KVENVOLDEN, K., LAWLESS, J. G., PERING, K., PETERSON, E., FLORES, J., PONNAMPERUMA, C., KAPLAN, I. R. and MOORE, C., Nature, 228, 923 (1970).
6. SPACKMAN, D. H., STEIN, W. H. and MOORE, S., Anal. Chem. 30, 1190 (1958).
7. HAMILTON, P. B., Anal. Chem. 35, 2055 (1963).
8. GEHRKE, C. W., KUO, K. and ZUMWALT, R. W., J. Chromatog. 57, 193 (1971).
9. GEHRKE, C. W., ZUMWALT, R. W. and WALL, L. L., J. Chromatog. 37, 398 (1968).
10. JÖNSSON, J., EYEM, J. and SJÖQUIST, J., Anal. Biochem. 51, 204 (1973).
11. HAMMAR, C. G., HOLMSTEDT, B. and RYHAGE, R., Anal. Biochem. 25, 532 (1968).
12. HAMMAR, C. G. and HESSLING, R., Anal. Chem. 43, 298 (1971).
13. SNEDDEN, W., PARKER, R. B. and WATTS, R. E., Int. Conf. on Mass Spectrometry Brussels, 31 Aug. - 4 Sept. 1970, Vol. 5, Institute of Petroleum, London, and Elsevier, Amsterdam, 1971. p. 742.
14. KNIGHT, J. B., Finnigan Spectra, 1, No. 1 (1971).
15. REYNOLDS, W. E., BACON, V. A., BRIDGES, J. C., COBURN, T. C., HALPERN, B., LEDERBERG, J., LEVINTHAL, E. C., STEED, E. and TUCKER, R. B., Anal. Chem. 42, 1122 (1970).

16. HAWES, J. E., MALLABY, R. and WILLIAMS, V. P., J. Chromatog. Sci. 7, 690 (1969).

17. GEHRKE, C. W. and STALLING, D. L., Separation Sci. 2, 101 (1967).

18. PEREIRA, W. E., BACON, V. A., HOYANO, Y., SUMMONS, R. and DUFFIELD, A. M., Clin. Biochem. (In Press).

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

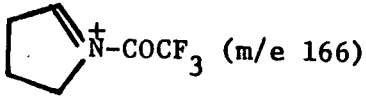
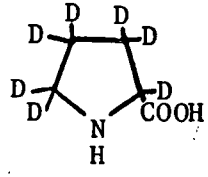
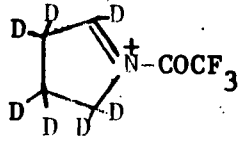
Amino Acids	Fragment Ion	Deuterated Amino Acids	Fragment Ion
ALA	$CH_3CH=\overset{+}{N}HCOCF_3$ (m/e 140)	$CD_3CD(NH_2)COOH$	$CD_3CD=\overset{+}{N}HCOCF_3$ (m/e 144)
VAL	$i\text{-}C_3H_7CH=\overset{+}{N}HCOCF_3$ (m/e 168)	$i\text{-}C_3D_7CD(NH_2)COOH$	$i\text{-}C_3D_7CD=\overset{+}{N}HCOCF_3$ (m/e 176)
GLY	$CH_2=\overset{+}{N}HCOCF_3$ (m/e 126)	NH_2CD_2COOH	$CD_2=\overset{+}{N}HCOCF_3$ (m/e 128)
ILEU	$C_2H_5CH(CH_3)CH=\overset{+}{N}HCOCF_3$ (m/e 182)	$C_2D_5CD(CD_3)CD(NH_2)COOH$	$C_2D_5CD(CD_3)CD=\overset{+}{N}HCOCF_3$ (m/e 192)
LEU	$i\text{-}C_3H_7CH_2CH=\overset{+}{N}HCOCF_3$ (m/e 182)	$i\text{-}C_3D_7CD_2CD(NH_2)COOH$	$i\text{-}C_3D_7CD_2CD=\overset{+}{N}HCOCF_3$ (m/e 192)
PRO	 (m/e 166)		 (m/e 173)
PHE	$C_6H_5CH=CHCOOH\rceil^+$ (m/e 148)	$C_6D_5CD_2CD(NH_2)COOH$	$C_6D_5CD=CDCOOH\rceil^+$ (m/e 155)
ASP	$BuOOCCH_2CH=\overset{+}{N}HCOCF_3$ (m/e 240)	$HOOCCD_2CD(NH_2)COOH$	$BuOOCCD_2CD=\overset{+}{N}HCOCF_3$ (m/e 243)
GLU	$HOOCCH_2CH_2CH=\overset{+}{N}HCOCF_3$ (m/e 198)	$HOOCCD_2CD_2CD(NH_2)COOH$	$HOOCCD_2CD_2CD=\overset{+}{N}HCOCF_3$ (m/e 203)
LYS	$CH_2=CHCH_2CH_2CH=\overset{+}{N}HCOCF_3$ (m/e 180)	$NH_2(CD_2)_4CD(NH_2)COOH$	$CD_2=CDCD_2CD_2CD=\overset{+}{N}HCOCF_3$ (m/e 188)

Table II. ANALYSIS OF AMINO ACIDS IN SOIL (μg/g SOIL)

Amino Acid	Amino Acid Analysis	Mass Fragmentography #1	#2	#3
Ala	206.5	198.7	202.7	198.3
Val	148.3	151.0	150.5	149.9
Gly	215.4	196.8	201.6	201.3
Ileu	95.4	100.4	100.2	92.3
Leu	154.2	152.1	149.7	154.2
Pro	143.4	141.4	142.8	141.2
Phe	80.3	80.5	80.7	80.0
Asp	218.3	217.1	219.8	219.9
Glu	227.0	217.2	215.6	214.1
Lys	129.7	115.3	113.5	114.9

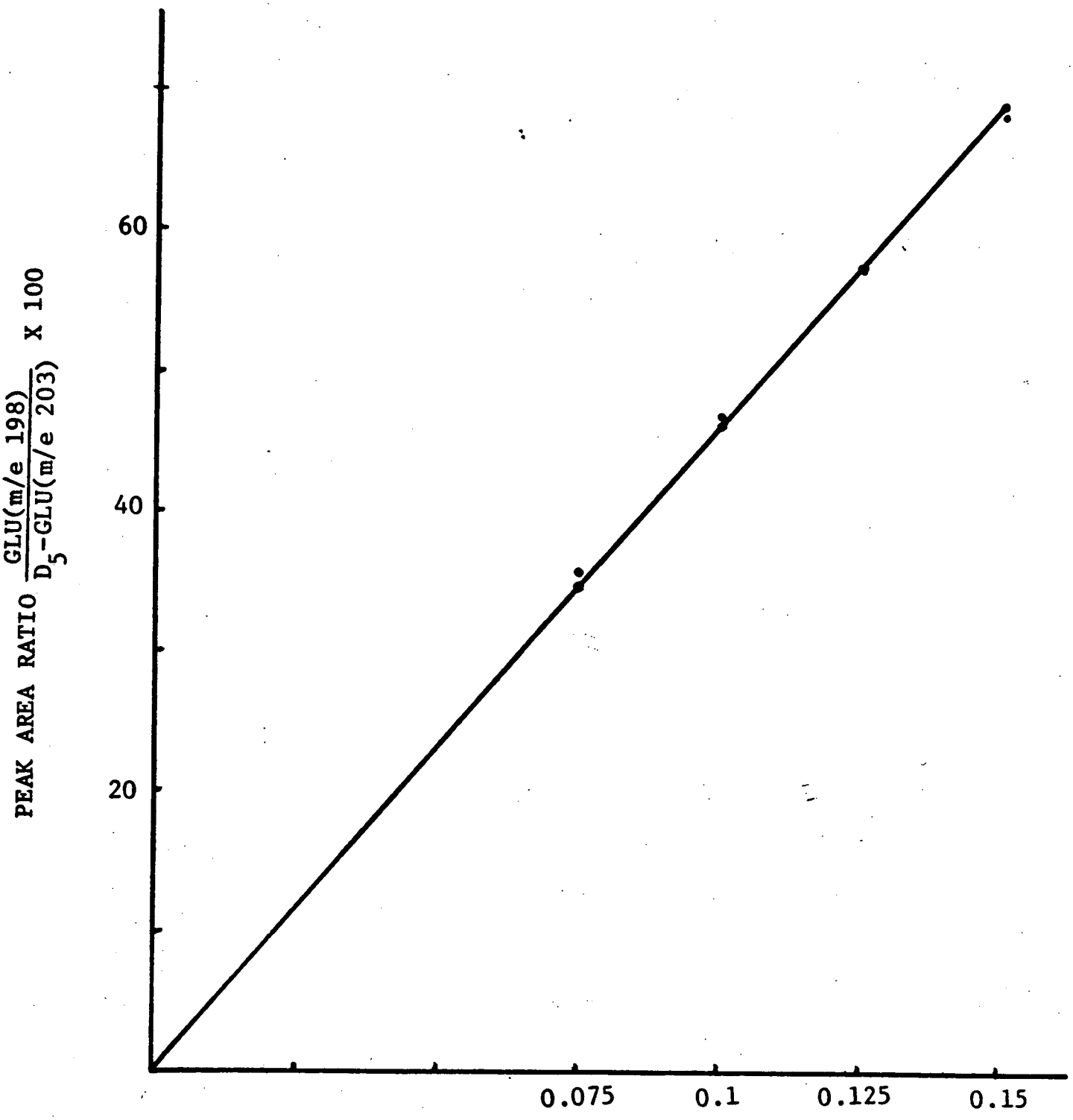

PEAK AREA RATIO $\frac{GLU(m/e\ 198)}{D_5\text{-}GLU(m/e\ 203)}$ X 100
60
40
20
0.075
0.1
0.125
0.15
$\frac{GLU}{D_5\text{-}GLU}$ Added

Fig. 1.

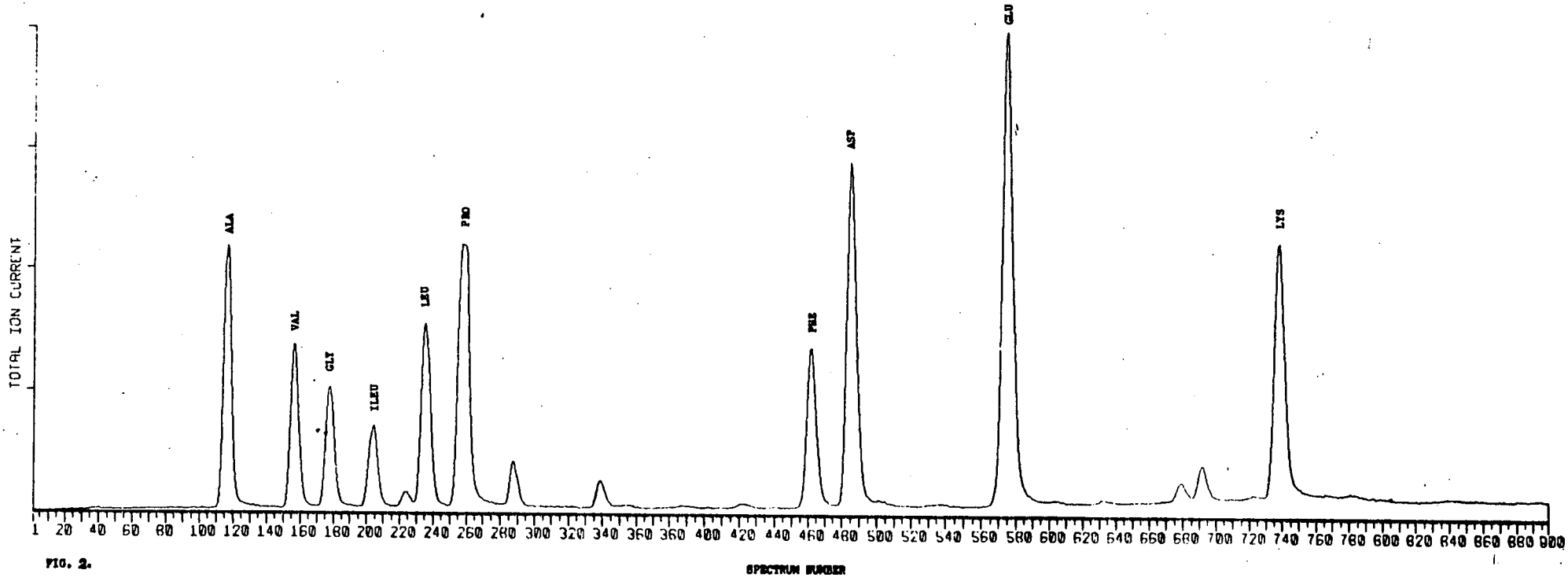

TOTAL ION CURRENT
ALA
VAL
GLY
ILEU
LEU
PRO
PHE
AST
GLU
LYS
1 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 460 480 500 520 540 560 580 600 620 640 660 680 700 720 740 760 780 800 820 840 860 880 900
SPECTRUM NUMBER

FIG. 2.

LEGENDS TO FIGURES

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

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