Analysis of Twelve Amino Acids in Biological Fluids by Mass Fragmentography

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A computerized method has been developed for the simultaneous quantitation of 12 amino acids in biological fluids using the technique of quadrupole mass fragmentography. The amino acids were determined as their *n*-butyl ester *N*-trifluoroacetyl derivatives and a commercially available mixture of deuterated amino acids was used as an internal standard. The combination of individual internal standards with the utilization of the mass spectrometer as a specific ion detector enabled several sources of error inherent in currently used chromatographic procedures to be eliminated. Furthermore, the use of a quadrupole mass spectrometer coupled with an on-line data system allowed the continuous and accurate monitoring of many ions (up to 25 in the present case) over the complete mass range (0 to 750) of the mass spectrometer. Reduction of stored data (establishment of background levels and peak location and calculation of results) was achieved by a totally operator-independent computer analysis program. Analysis on 50-μl samples of plasma and urine were reproducible with a standard deviation of less than 10% over 5 determinations and this precision was obtained at a lower limit of quantitation of approximately 1 nanogram of an amino acid.

Mass fragmentography is rapidly gaining wide acceptance as an accurate and extremely sensitive technique for the simultaneous identification and quantitation of picogram levels of biologically important compounds. Most approaches (*1-4*) have involved the use of sector mass spectrometers as detectors and consequently are severely restricted in the range of the m/e continuum and in the number of different ions that can be successively monitored. Recent publications have reported the use of a quadrupole mass spectrometer as an analog signal detector (*5, 6*) with the significant advantage of a greatly increased range of mass values that can be repeatedly scanned.

The ease of application of on-line data systems to a quadrupole mass spectrometer (*7*) points to an obvious and important extension of this work. Initially, we reported (*8*) the computer controlled operation of the quadrupole mass filter for mass fragmentography and its application to the determination of phenylalanine in serum, and subsequently (*9*) to the simultaneous quantitation of 10 amino acids in soil extracts. We have now made substantial improvements to the method whereby a computer program completes the entire analysis of the data collected. As a demonstration of the utility of the method, we wish to report its application to the simultaneous determination of 12 amino acids in biological fluids.

EXPERIMENTAL

Reagents. A deuterated amino acid mixture was supplied by Merck Laboratory Chemicals, Rahway, N.J. The 1.25*N* HCl in *n*-butanol, 25% (v/v) trifluoroacetic anhydride in methylene chloride, and Tabsorb column packing (EGA on chrom W) were obtained from Regis Chemical Co., Morton Grove, Ill. A standard amino acid solution was purchased from Pierce Chemical Co., Rockford, Ill.

Equipment. GLC separations were carried out using a 6-foot by 4-mm (i.d.) coiled glass column packed with Tabsorb, and using helium as carrier gas (60 ml/min). The gas chromatograph, a Varian Model 1200, was coupled *via* an all-glass membrane separator to a Finnigan 1015 Quadrupole mass spectrometer which in turn was interfaced to the ACME computer system of the Stanford University Medical Center.

Interface hardware is that previously described (*7*). The data acquisition software assumes an operating cycle of: (a) Transmission of a control number, N, from the computer to the interface controller which sets the quadrupole mass analyzer to the specified point in the m/e continuum. (b) Integration of the ion signal for a pre-set period, T, (8 milliseconds in our work), and (c) Computer reading of the integrated ion signal with a 12 bit A → D conversion. Characteristics of the IBM 360/50 to IBM 1800 data path of the ACME computer system dictate that data points be buffered into groups of 250 and, therefore, in our operation, ions are monitored in multiples of 25 with 10 data points or cycles per mass value. The first of the 10 cycles serves only to direct the quadrupole electronics to the approximate mass region of interest. The remaining nine cycles collect a series of ion current integrations 0.5 amu about the m/e being monitored. The nine points are then smoothed with a five-point quadratic function (*10*), and the highest is then selected as the intensity of the particular ion. This procedure allows for small drifts in instrument calibration to be corrected at each mass on every scan. A "spectrum" of precision intensities is collected and filed on disk at 2-second intervals and typically 750 such passes are made in the direction of each sample run. A sum of the precision intensities in each "spectrum" can be used to construct the total ion monitor shown in Figure 1.

Data Analysis Program. The mass fragmentogram data are reduced by a computer program requiring no operator intervention. The program has as its input the series of mass fragmentogram pairs illustrated in Figure 2. Each fragmentogram is represented as a series of 12-bit digital samples measuring the ion current at that mass as a function of time. The program locates candidate peaks in the various mass fragmentograms, selects those peak pairs corresponding to the deuterated and undeuterated analogs of each derivatized amino acid, and measures the area ratio for each pair to quantitate the test mixture for the various amino acids. A sample print-out of the results of this process is shown in Figure 3.

Background Removal. As illustrated in Figure 1, the gas chromatograph effluent has two components: 1) a gently rising background arising from column bleed and other continuously present materials and 2) the test sample and internal standard component peaks. One of the first steps in the data processing is to construct an approximation to this background component so that it can be removed. This is important both to facilitate the detection

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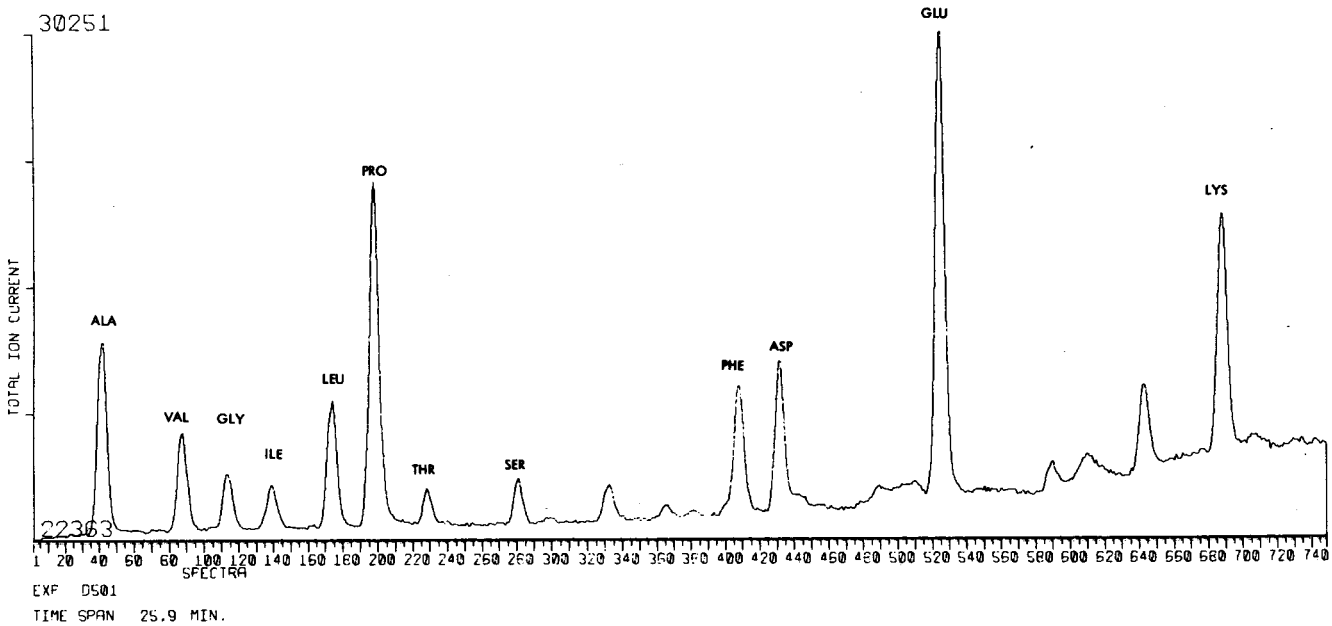

Figure 1. Total ion current from a normal plasma

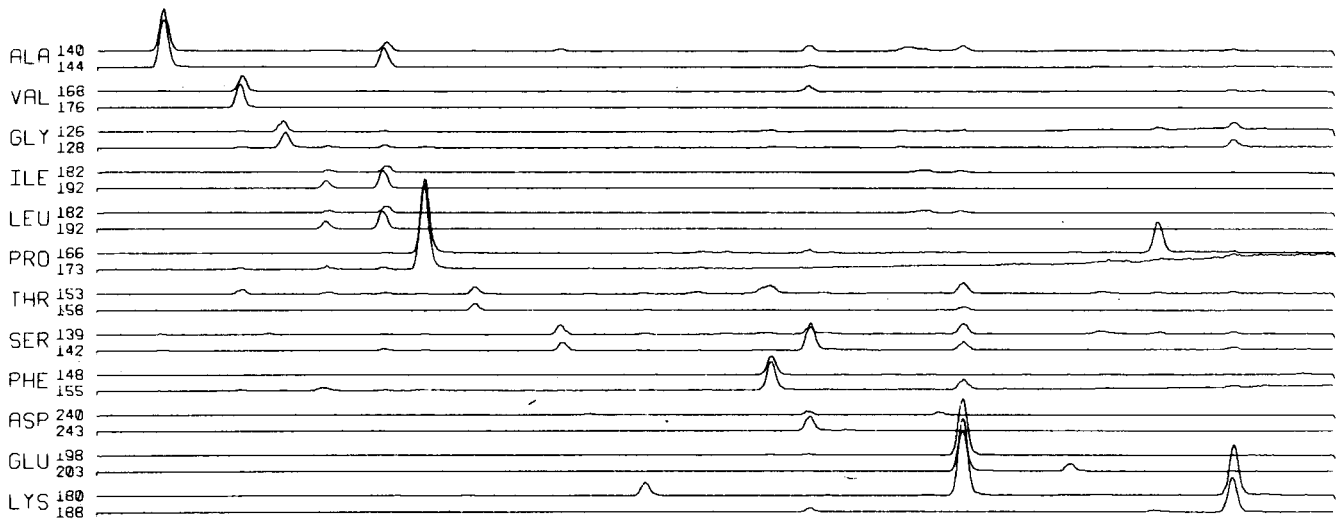

Figure 2. Individual ion chromatograms of monitored fragments

of sample peaks (this is easier in the presence of a flat background rather than a time-varying background) and to make the peak area measurements more accurate (areas of small peaks would be significantly influenced by a background component of comparable size). The plots in Figure 2 (particularly the fragmentograms for masses 166 and 173 used to measure proline and proline-d_7) illustrate the fact that this background varies with elution time. Thus the program approximates the background of each fragmentogram independently.

The local minima of each trace provide a basis for the approximation. For the most part, the sequence of local minima is representative of the background although points between unresolved peaks, for example, may give rise to local minima deviating significantly from the actual background curve. To eliminate the effects of such points, we construct the background approximation as a two-step process. First a "piece-wise least squares fit" through all of the relative minima is constructed [Figure 4(*a*)]. The minima whose signal amplitudes fall above this first approximation (such as between unresolved peaks) are then eliminated prior to a second pass. The piece-wise LS fit resulting from this second analysis is taken to represent the background portion of the signal [Figure 4(*b*)].

The "piece-wise LS fit" is a procedure used to develop a smooth approximation to the irregularly positioned signal minima and to provide an interpolation for points between successive minima. Rather than attempt to fit the entire background by a single polynominal or other function, we construct local least squares fit polynomial approximations to successive small groups of points. These local solutions are then joined together in a way which guarantees continuity of the function and its first derivative. We consider sequences of N minima,

$$S_i = [(y_i,t_i),(y_{i+1},t_{i+1}),\cdots,(y_{i+N-1},t_{i+(N-1)})]$$

where y_i is the signal amplitude at the local minimum at time t_i. Through the points in the sequence S_i, we least squares fit a polynomial, $P_i(t)$ (in our case we use a straight line approximation through 9 points). Successive polynomials, *e.g.*, through S_i and S_{i+1}, are joined together so that at times between $t_{i+(N-1)/2}$ and $t_{i+(N+1)/2}$ (the midpoints of S_i and S_{i+1} respectively, assuming N is odd), the background, $B(t)$, is approximated by

$$B(t) = f(\epsilon)P_i(t) + [1 - f(\epsilon)]P_{i+1}(t)$$

where

$$f(\epsilon) = 1 - 3\epsilon^2 + 2\epsilon^3$$

and

$$\epsilon = \frac{t - t_{i+(N-1)/2}}{t_{i+(N+1)/2} - t_{i+(N-1)/2}}$$

The joining function, $f(\epsilon)$, ensures continuity through the first derivative. This procedure has produced reasonable background approximations, even to quite complex chromatograms.

Having constructed a background function, this component is removed so that the fragmentogram peaks ride on a "flat" back-

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D501
730426

CRT display (Y or N) =?n
mass 140, amp  31.24
mass 144, amp  36.92
Amino Acid ALA   Masses 140/144   Areas   8390.1/ 10036.3     RATIO =   0.8360  Location Error =  0
mass 168, amp  11.98
mass 176, amp  18.48
Amino Acid VAL   Masses 168/176   Areas   3115.1/  4672.0     RATIO =   0.6668  Location Error =  6
mass 126, amp   8.26
mass 128, amp  12.00
Amino Acid GLY   Masses 126/128   Areas   2168.5/  3357.7     RATIO =   0.6458  Location Error =  2
mass 182, amp   1.79
mass 192, amp   5.90
Amino Acid ILE   Masses 182/192   Areas    456.2/  1476.4     RATIO =   0.3090  Location Error =  1
mass 182, amp   5.47
mass 192, amp  14.02
Amino Acid LEU   Masses 182/192   Areas   1360.7/  3552.5     RATIO =   0.3830  Location Error =  5
mass 166, amp  56.99
mass 173, amp  65.63
Amino Acid PRO   Masses 166/173   Areas  16074.4/ 18638.5     RATIO =   0.8624  Location Error =  1
mass 153, amp   5.33
mass 158, amp   5.19
Amino Acid THR   Masses 153/158   Areas   1307.9/  1204.6     RATIO =   1.0857  Location Error =  2
mass 139, amp   7.53
mass 142, amp   6.48
Amino Acid SER   Masses 139/142   Areas   1879.4/  1558.1     RATIO =   1.2062  Location Error =  1
mass 148, amp  14.68
mass 155, amp  22.44
Amino Acid PHE   Masses 148/155   Areas   4221.4/  6180.2     RATIO =   0.6830  Location Error =  4
mass 240, amp   2.86
mass 243, amp  11.00
Amino Acid ASP   Masses 240/243   Areas    705.1/  2936.8     RATIO =   0.2401  Location Error =  0
mass 198, amp  43.69
mass 203, amp  31.23
Amino Acid GLU   Masses 198/203   Areas  12399.6/  8524.1     RATIO =   1.4547  Location Error =  4
mass 180, amp  38.79
mass 188, amp  26.54
Amino Acid LYS   Masses 180/188   Areas  11357.7/  7587.3     RATIO =   1.4969  Location Error =  5
 FINAL ANALYSIS ( mg/100ml)
   AMINO ACID       FOUND
   ALA                2.96
   VAL                1.97
   GLY                2.20
   ILE                0.66
   LEU                1.48
   PRO                2.20
   THR                1.94
   SER                3.17
   PHE                1.55
   ASP                1.09
   GLU                4.73
   LYS                2.06
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Figure 3. Terminal output of calculations and results

ground which has a mean value slightly above zero [Figure 5(*a*)]. The new background is not precisely zero because the approximation to the original data background was fitted through the data MINIMA. Thus, since the background itself contains small fluctuations (caused by noise or very small effluent peaks), these will, in general, fall slightly above the new signal zero. This fact can be used to advantage to assist in setting a more adaptive threshold for peak detection which is the next step in the process.

Peak Detection. The signal peaks are separated from the flattened background by means of a threshold, above which a signal is considered to belong to a component peak and below which to the background. This threshold is set so as to exclude the majority of the background fluctuations while minimizing the truncation of the true peaks. Since background fluctuations will vary in amplitude from run to run (*e.g.*, because of different instrument set-up gain), we have found it desirable to set this threshold based on actual data statistics. If one plots a histogram of the frequency of occurrence of each amplitude level in the flattened fragmentogram, one sees a peak just above zero where most of the new background values lie. Since the sample peaks are relatively few in number and are spread over higher amplitude values, they appear as a "tail" on the high side of the background histogram peak [see Figure 5(*b*)]. Thus, by detecting the mode of the background histogram peak and measuring its width (standard deviation), a data-adaptive threshold value can be set (threshold = mode + 2.5 × standard deviation). The multiplier value of 2.5 is chosen based on an assumption of Gaussian background statistics and a 1% probability that a point above the threshold belongs to the background. This then serves to isolate candidate effluent peaks [Figure 5(*c*)].

These isolated peaks are further screened by a minimum width criterion to eliminate any wideband electronic noise which may be present (actual peaks tend to be at least 10 samples wide because of the way we have set up the data system sampling rate). The remaining peak candidates are then tested to make sure they contain only one maximum—*i.e.*, that they are fully resolved peaks. If not, they are subdivided at successive minima into singlet peaks. This latter problem is rare in the present application. In more complex situations, more sophisticated peak-resolving algorithms can be utilized to get better area and location approximations.

After sorting out the various component peaks in each of the fragmentograms, the members of each fragmentogram pair are compared to each other and only peaks with an analog at the same time position are considered further. The locations of peaks are determined by fitting a parabola about the peak maximum and using the parabola maximum as the peak position. Analogous peaks must coincide in position to within 10 time units.

Peak Identification and Quantitation. Finally, given the set of coincident peaks between the two mass fragmentograms for an

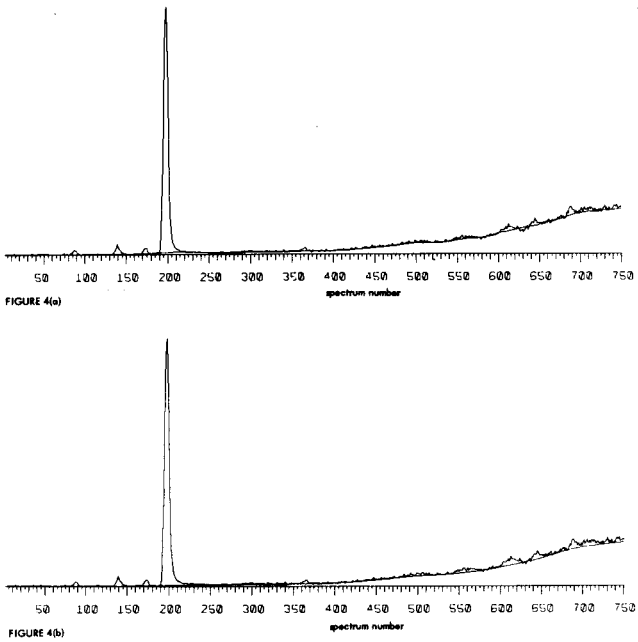


Figure 4. (*a*) First approximation to the fragmentogram background for *m/e* 173. (*b*) Second approximation to the fragmentogram background for *m/e* 173

amino acid, the appropriate peak pair is selected on the basis of position relative to those found previously. We know for the GC column and temperature profile approximately where each amino acid should be eluted in time. The time differences between successive amino acids were measured and stored as a table in the program as the result of a system calibration. For actual data runs, this table is consulted to determine where to search for each particular amino acid, having determined those preceding it in time. About each such projected location, a search is made (within 30 time units) for the largest candidate peak pair. This is assumed to represent the appropriate amino acid. If no peak pair is found within this search window, that amino acid is assumed to be undetectable in the test sample. If a particular amino acid is missing (or undetectable) in a run, its estimated location is used in projecting for the locations of the remaining acids.

Each of these displacements is relative to a previous peak position and thus, a start-up procedure is necessary. We use the fact that alanine comes off our column first and for at least the deuterated analog standard (mass 144), will be the largest peak within the first 100 time units. This algorithm has proved reliable in automatically starting the analysis procedure and accommodates a certain amount of fluctuation between when spectrum recording begins relative to column injection. Using the previous peak screening procedure, this algorithm would not work if alanine were undetectable in the test sample—in this case, the deuterated analog peak would be cast out for lack of a co-located sample peak. Thus, for this special case, we search for the location of the deuterated analog peak in the fragmentogram for mass 144 to locate the starting position and apply the previous screening procedure subsequently for quantitation.

This program is coded to run on the ACME 360/50 time-shared computer facility in the Stanford Medical Center using a PL/1-like programming language. The time to analyze a run varies depending on the loading of the computer facility and the 360/50 clock does not allow us to measure CPU time for this program alone. Using estimates of running times on a relatively unloaded system, several minutes (wall clock time) are required to analyze a run for the 12 amino acids currently being quantitated.

Procedure. Fifty microliters of body fluid (plasma, urine), 25 μl of the deuterated standard amino acid solution (1 mg/ml) and 25 μl of 0.1*N* HCl were mixed (plasma samples were treated with 1 ml of absolute ethanol, the precipitated protein removed by centrifugation and the supernatant liquid evaporated to dryness *in vacuo*) and made up to approximately 2 ml with water. This solution was passed through a 5-cm^3 bed volume column of Bio-Rad AG-50W-X12 (50–100) cation exchange resin. After washing with 20 ml of water to remove neutral and acidic components, the amino acids were eluted with 20 ml of 3*N* ammonia solution. The eluate was evaporated to dryness *in vacuo* and the residue refluxed with 1.25*N* HCl in *n*-butanol for 15 min and evaporated to dryness. Trifluoroacetic anhydride in methylene chloride (1:1, 0.5 ml) was added to the residue and the solution heated in a sealed tube at 60 °C for 10 min. The solvent was removed under a stream of dry nitrogen and the residue taken up in 50 μl of ethyl acetate. An aliquot (1–2 μl) was injected into the injection port of the gas chromatograph (oven temperature 100 °C) and, after a 2-minute delay, the oven was programmed at 4 °C/min (to 220°C). Data acquisition was commenced when the oven temperature reached 115 °C. The mass spectrometer was operated using a ionizing voltage of 70 eV and an ionizing current of 250 μA.

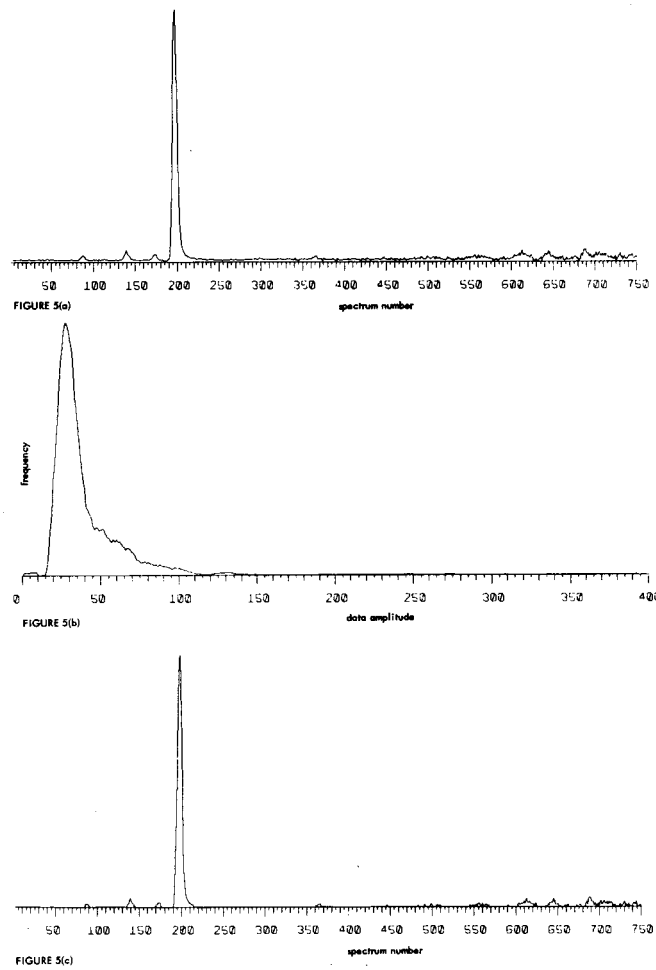


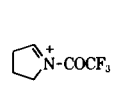
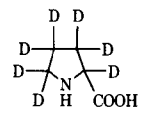
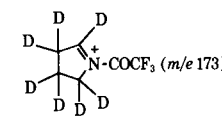
Figure 5. (*a*) Flattened fragmentogram for *m/e* 173, obtained by subtracting the background from 4b. (*b*) Histogram of flattened fragmentogram amplitudes. (*c*) Result of thresholding flattened fragmentogram with background found by histogram 5(*b*)

Calibration of Internal Standards. Six aliquots (500 μl) of normal plasma were each mixed with 250 μl of the deuterated amino acid solution (1 mg/ml) and to 5 of these in turn were added aliquots (10, 20, 30, 40, and 50 μl) of the Pierce standard amino acid solution (1.25 μmole of each amino acid per ml of solution). The six solutions were deproteinized with absolute ethanol and the free amino acids recovered and derivatized as above. Each solution was processed in the GC–MS–computer system and the ratio of the areas of the selected fragment ion and its deuterated analog for each amino acid, plotted *vs.* the amount of that amino acid added. The slopes of each of the straight line graphs obtained were used to calculate a calibration factor for each of the amino acids in the deuterated mixture.

RESULTS AND DISCUSSION

Table I summarizes the structures and *m/e* values of the selected characteristic fragment ions of the normal and deuterated amino acid TAB derivatives. The combination of *O,N*-trifluoroacetyl,*n*-butyl ester derivatization with gas chromatography on EGA on Chrom W (Tabsorb) was chosen because of the excellent separation and peak

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)
THR	$CH_3CH{=}CH\overset{+\cdot}{N}HCOCF_3$ (m/e 153)	$CD_3{-}CDOH{-}CD(NH_2)COOH$	$CD_3CD{=}CD{-}\overset{+\cdot}{N}HCOCF_3$ (m/e 158)
SER	$CH_2{=}CH{-}\overset{+\cdot}{N}HCOCF_3$ (m/e 139)	$CD_2OH{-}CD(NH_2)COOH$	$CD_2{=}CD{-}\overset{+\cdot}{N}HCOCF_3$ (m/e 142)
PHE	$C_6H_5CH{=}CHCOOH]^+$ (m/e 148)	$C_6D_5CD_2CD(NH_2)COOH$	$C_6D_5CD{=}CDCOOH]^+$ (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)

shape characteristics obtained with this system (*11*). One serious disadvantage, however, is that the derivatives of arginine, cysteine, and histidine decompose under these chromatographic conditions. Although the problem has been under study by several groups (*12, 13*), there is at present no combination of derivatization procedure and conventional packed GC column conditions which can give rise to a complete peak separation of all protein amino acids on a single column. A complete separation of all the amino acids would be an ideal but not a necessary prerequisite for a quantitative amino acid analysis by mass fragmentography. Complete separations are only necessary for those amino acids for which it is difficult or impossible to select fragments with non-interfering m/e values. For example the TAB derivatives of alanine, serine, threonine, and valine all have the ion at m/e 126 in common and therefore must all be completely separated from TAB glycine where this is the only ion suitable for monitoring. Leucine and isoleucine, because of their isomerism, also have to be completely separated. With larger amino acids there is usually little problem in finding non-interfering ions since these show several prominent unambiguous fragments as compared with the one or two shown by the smaller compounds.

Table II shows the amino acid determinations for a normal plasma, plasma of a patient with Maple Syrup Urine Disease (*14*), and a normal urine. The results shown are the means and standard deviations obtained from five separate analyses and are expressed in mg/100 ml. Some amino acids analyzed consistently better than others (*e.g.*, phenylalanine, alanine, valine, and lysine) but, in all cases, the standard deviations were less than 10% of the mean. This compares favorably with other methods of amino acid analysis currently in general use (*15*). Replicate injections of the same solution gave results with a standard deviation of less than 4% of the mean over five determinations for each amino acid. Under routine operating conditions, 1 nanogram of an amino acid can be quantitated with this precision. Extension of the method to cover more than 12 amino acids is primarily dependent on obtaining appropriate deuterated standards. Sufficient amounts of arginine and histidine are present in the commercially available mixture used for the present work. Tyrosine-d_7 is also commercially available while other labeled amino acids would have to be chemically synthesized. As the internal standard is itself calibrated *vs.* a

Table II. Amino Acid Determinations, mg/100 ml

	Normal plasma	MSUD plasma	Normal urine
Alanine	2.56 ± 0.12	8.58 ± 0.24	3.47 ± 0.04
Valine	1.82 ± 0.12	5.62 ± 0.23	0.56 ± 0.03
Glycine	1.82 ± 0.06	6.06 ± 0.04	13.89 ± 0.38
Isoleucine	0.68 ± 0.05	5.00 ± 0.45	0.42 ± 0.04
Leucine	1.40 ± 0.08	21.29 ± 1.09	1.02 ± 0.05
Proline	2.15 ± 0.09	8.49 ± 0.24	5.59 ± 0.26
Threonine	1.75 ± 0.16	8.13 ± 0.24	4.29 ± 0.17
Serine	2.05 ± 0.06	9.23 ± 0.30	5.37 ± 0.53
Phenylalanine	1.50 ± 0.06	5.74 ± 0.11	0.94 ± 0.03
Aspartic acid	1.10 ± 0.04	2.31 ± 0.14	3.28 ± 0.16
Glutamic acid + glutamine	4.32 ± 0.33	12.62 ± 0.27	3.72 ± 0.11
Lysine	1.98 ± 0.08	9.65 ± 0.14	10.08 ± 0.54

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standard amino acid solution, it is not necessary to know either the degree of isotope incorporation or the amount of each deuterated compound in the standard solution.

The use of a separate deuterated internal standard for each amino acid being analyzed allows several errors inherent in commonly used methods of amino acid analysis to be eliminated. These include loss of material from nonquantitative transfer, derivatization and column (ion exchange and GLC) recovery; loss of the very volatile derivatives of alanine, valine, glycine, etc. during concentration of the derivatized sample prior to injection on the gas chromatograph (*16*); and loss of basic amino acids which are co-precipitated with protein during plasma work-up (*17*). Furthermore the chances of errors arising through co-elution of interfering compounds in the conventional GC or ion exchange methods of amino acid analysis are significantly reduced since the mass spectrometer detects only those ions known to be specific to the mass spectrum of the amino acid being analyzed.

The fact that the quantitative result obtained for the amino acid composition or soil samples compared very favorably with results obtained from an amino acid analyzer (*9*) suggests quadrupole mass fragmentography will find wide application for the analysis of amino acids in the future. It will be particularly useful for determinations on neonatal plasma and amniotic fluid samples where low sample size or low amino acid content dictates that optimum sensitivity is an important consideration.

The time taken for one complete analysis using this computer directed mass fragmentography system, exclusive of derivatization, is 40 minutes for data collection with an additional 10 minutes before the computer presents the final analytical result.

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