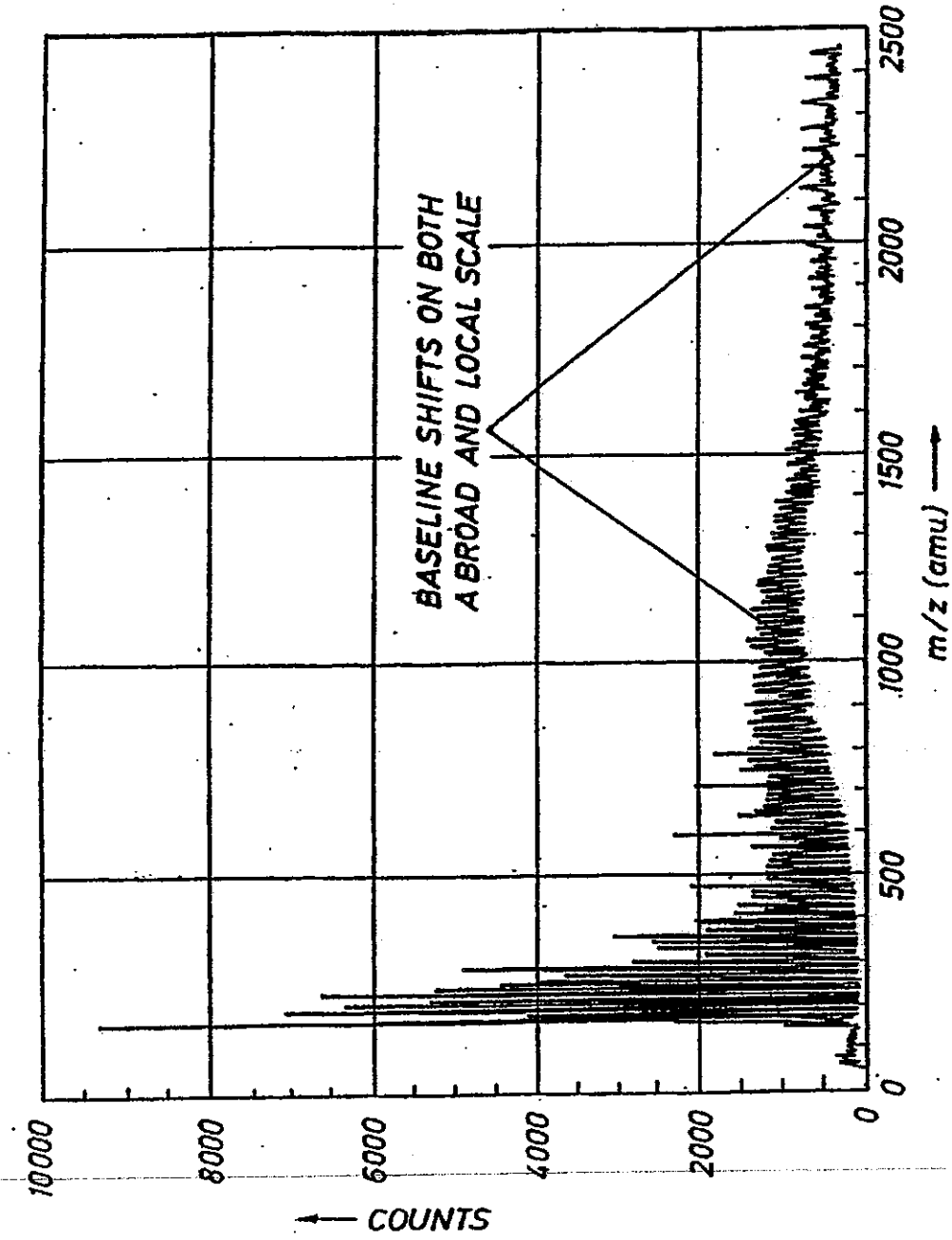

BASELINE SHIFTS ON BOTH
A BROAD AND LOCAL SCALE
10000
8000
6000
4000
2000
0
COUNTS
0
500
1000
1500
2000
2500
m/z (amu)

FIG. 1

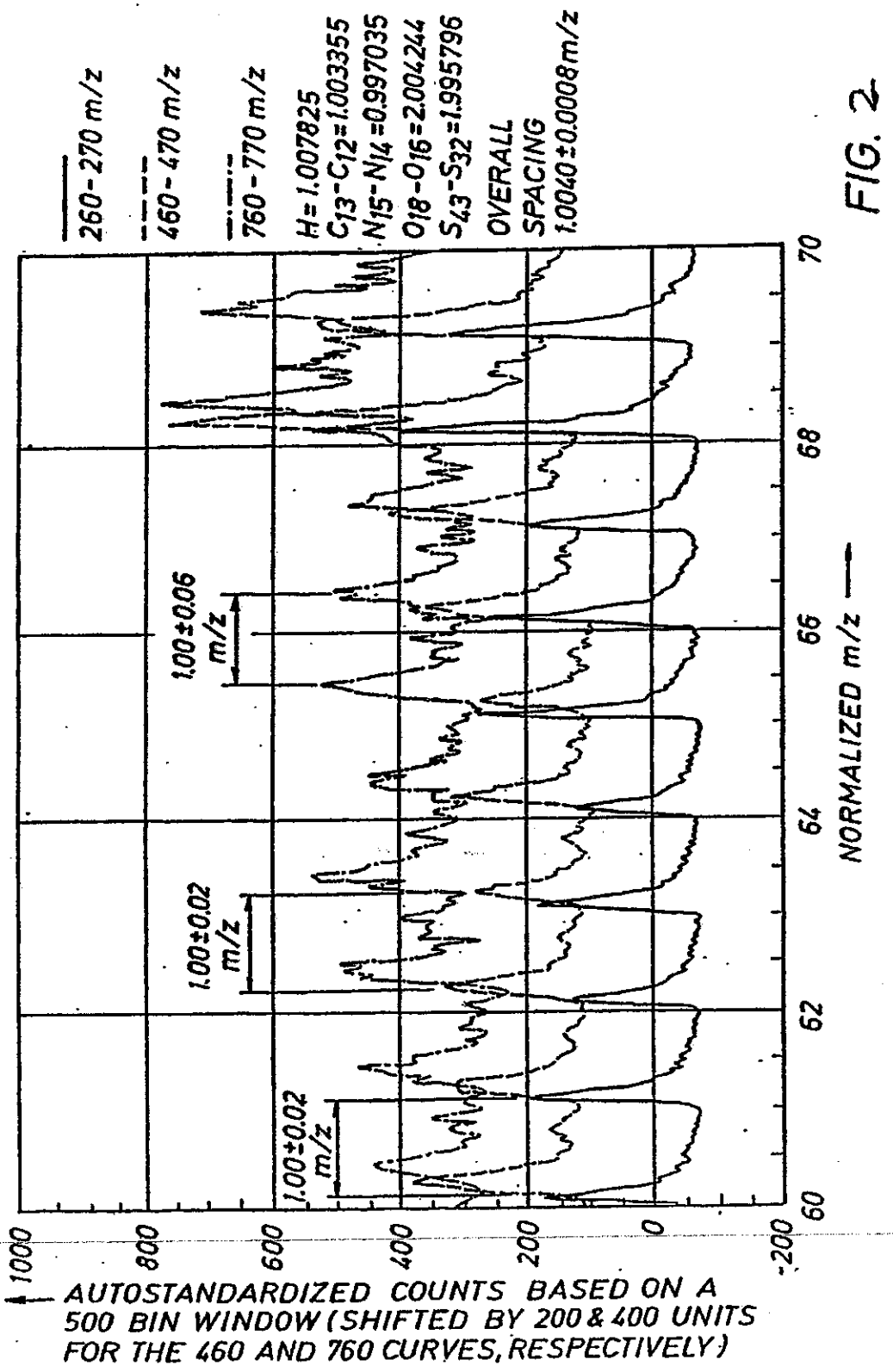
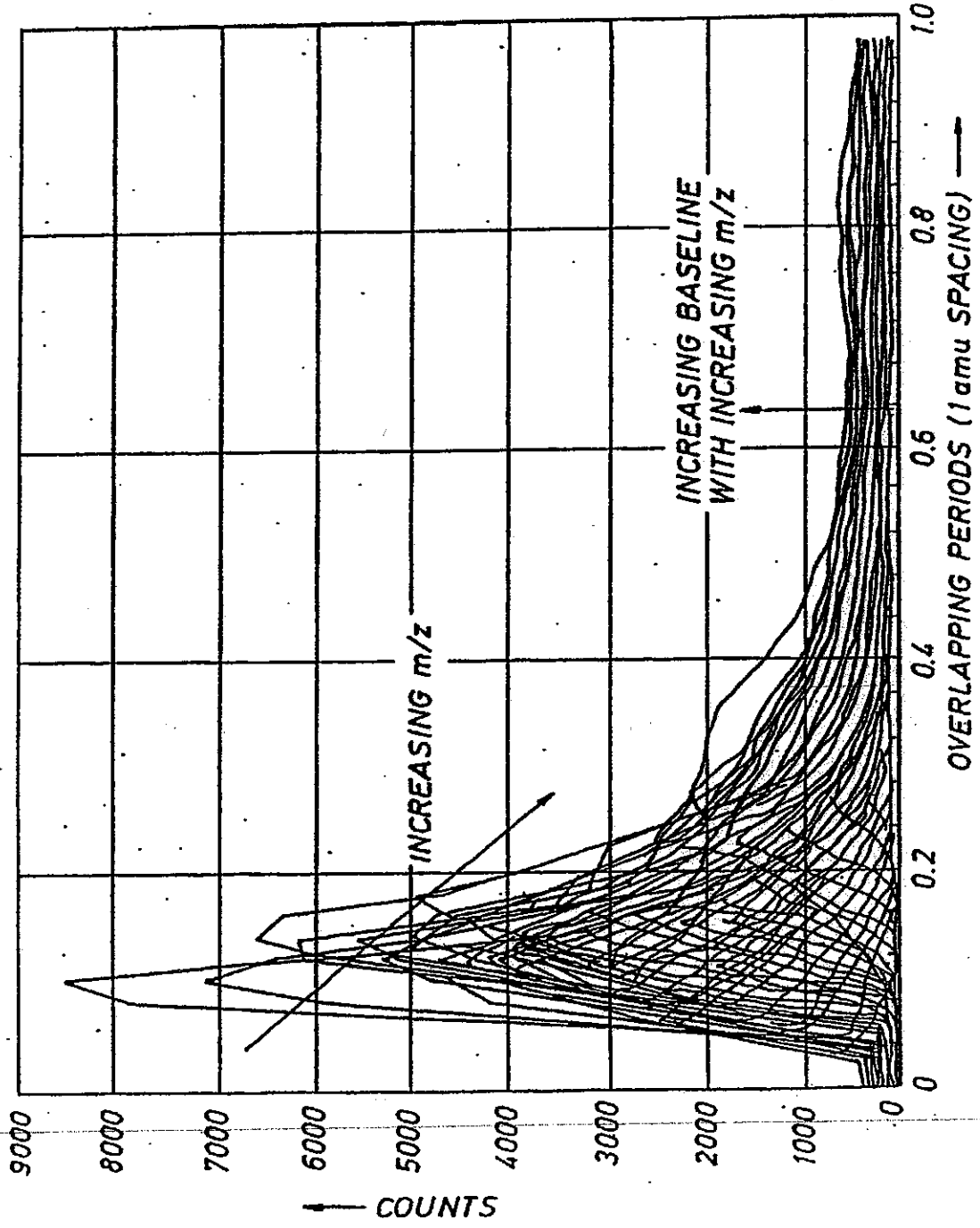

260-270 m/z
460-470 m/z
760-770 m/z
H=1.007825
C13-C12=1.003355
N15-N14=0.997035
O18-O16=2.004244
S43-S32=1.995796
OVERALL SPACING
1.0040±0.0008m/z
1.00±0.06 m/z
1.00±0.02 m/z
1.00±0.02 m/z
NORMALIZED m/z
60
62
64
66
68
70
1000
800
600
400
200
0
-200
AUTOSTANDARDIZED COUNTS BASED ON A 500 BIN WINDOW (SHIFTED BY 200 & 400 UNITS FOR THE 460 AND 760 CURVES, RESPECTIVELY)

FIG. 2

FIG. 3


INCREASING m/z
INCREASING BASELINE WITH INCREASING m/z
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
COUNTS
0
0.2
0.4
0.6
0.8
1.0
OVERLAPPING PERIODS (1 amu SPACING)

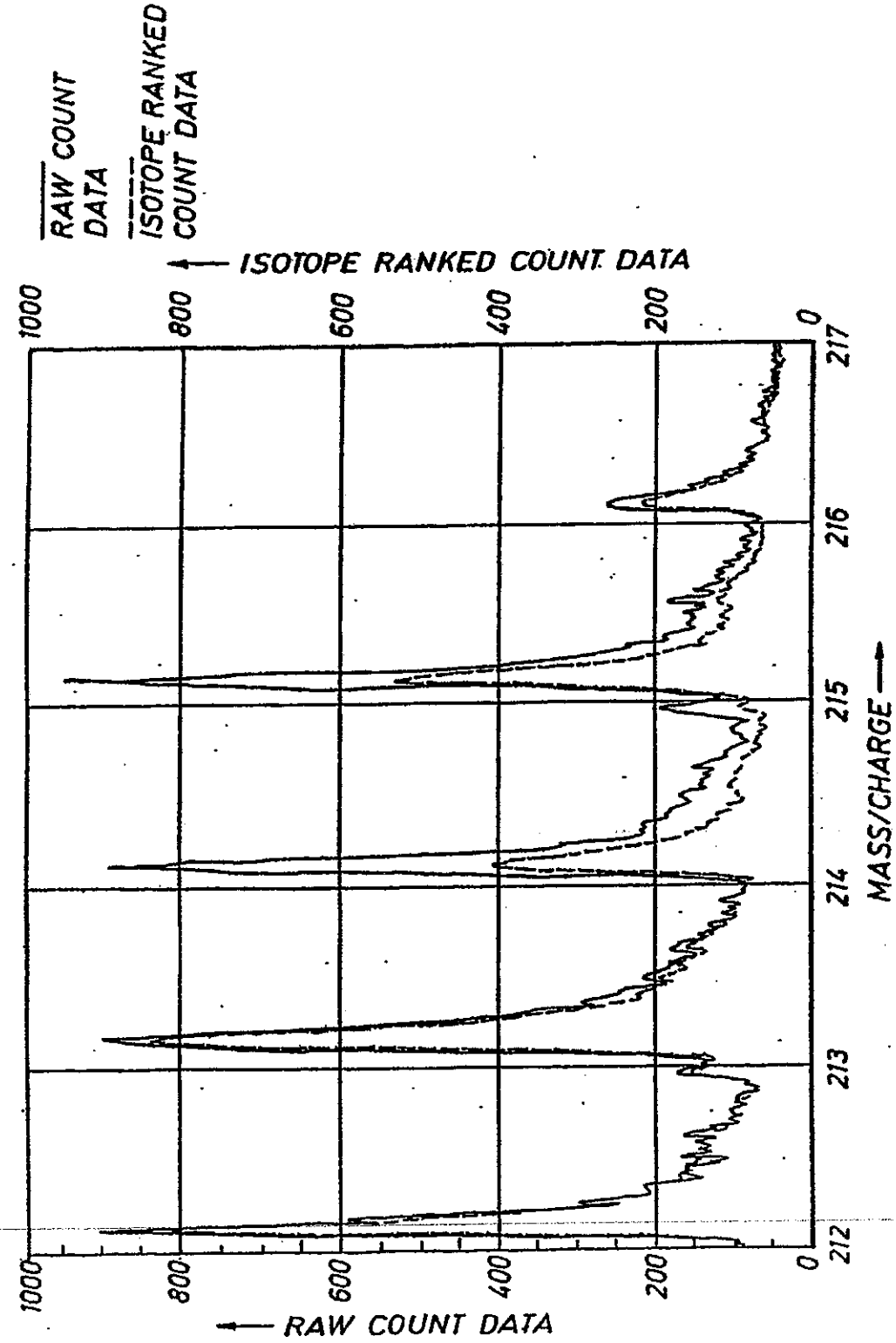

RAW COUNT DATA
ISOTOPE RANKED COUNT DATA
ISOTOPE RANKED COUNT DATA
1000
800
600
400
200
0
217
216
215
214
213
212
MASS/CHARGE
RAW COUNT DATA

Fig. 4

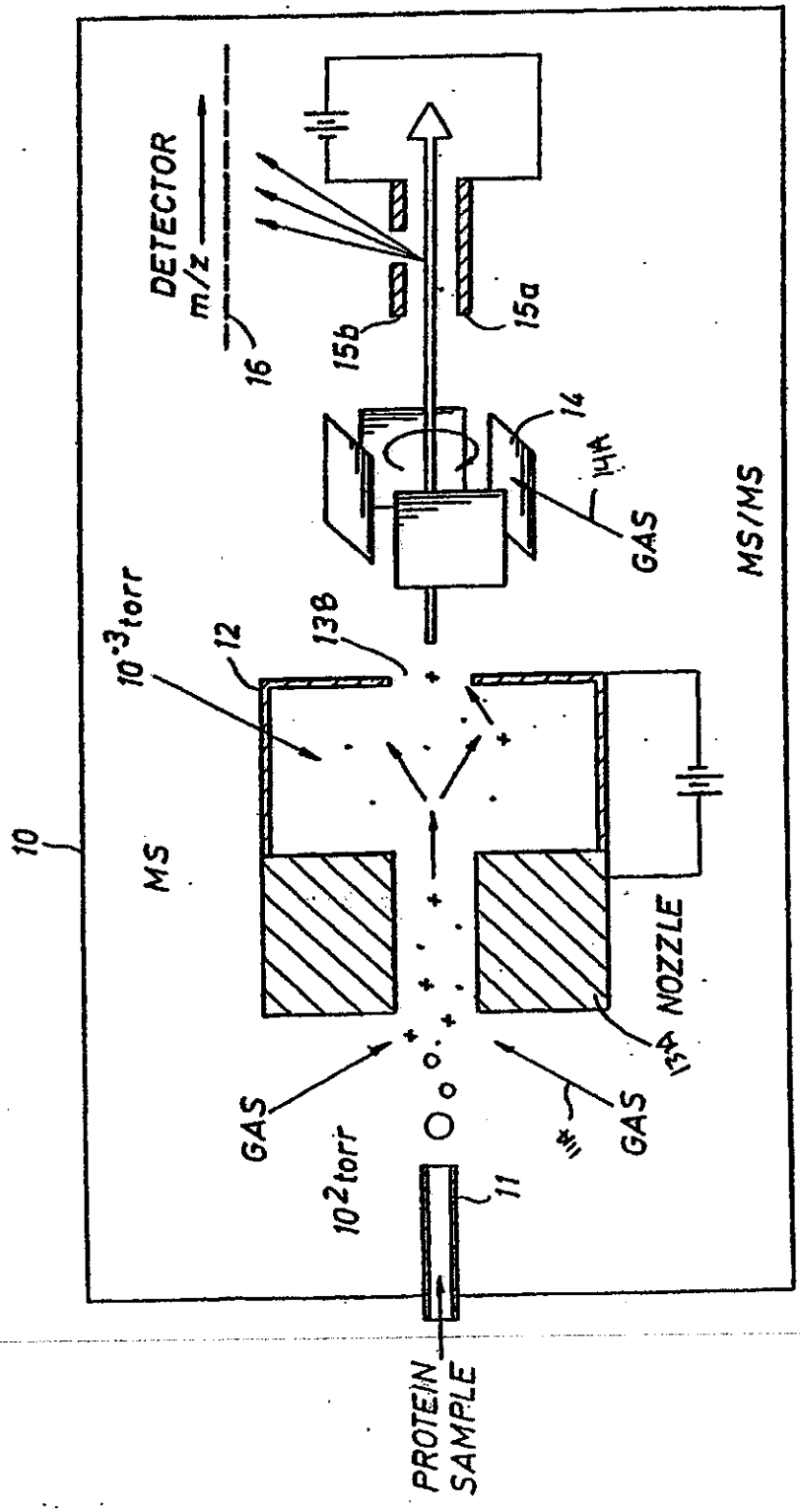

DETECTOR
m/z
16
15b
15a
14
14A
GAS
MS/MS
10^{-3}torr
12
13B
10
MS
NOZZLE
13A
GAS
GAS
10^{2}torr
11
PROTEIN
SAMPLE

FIG. 5

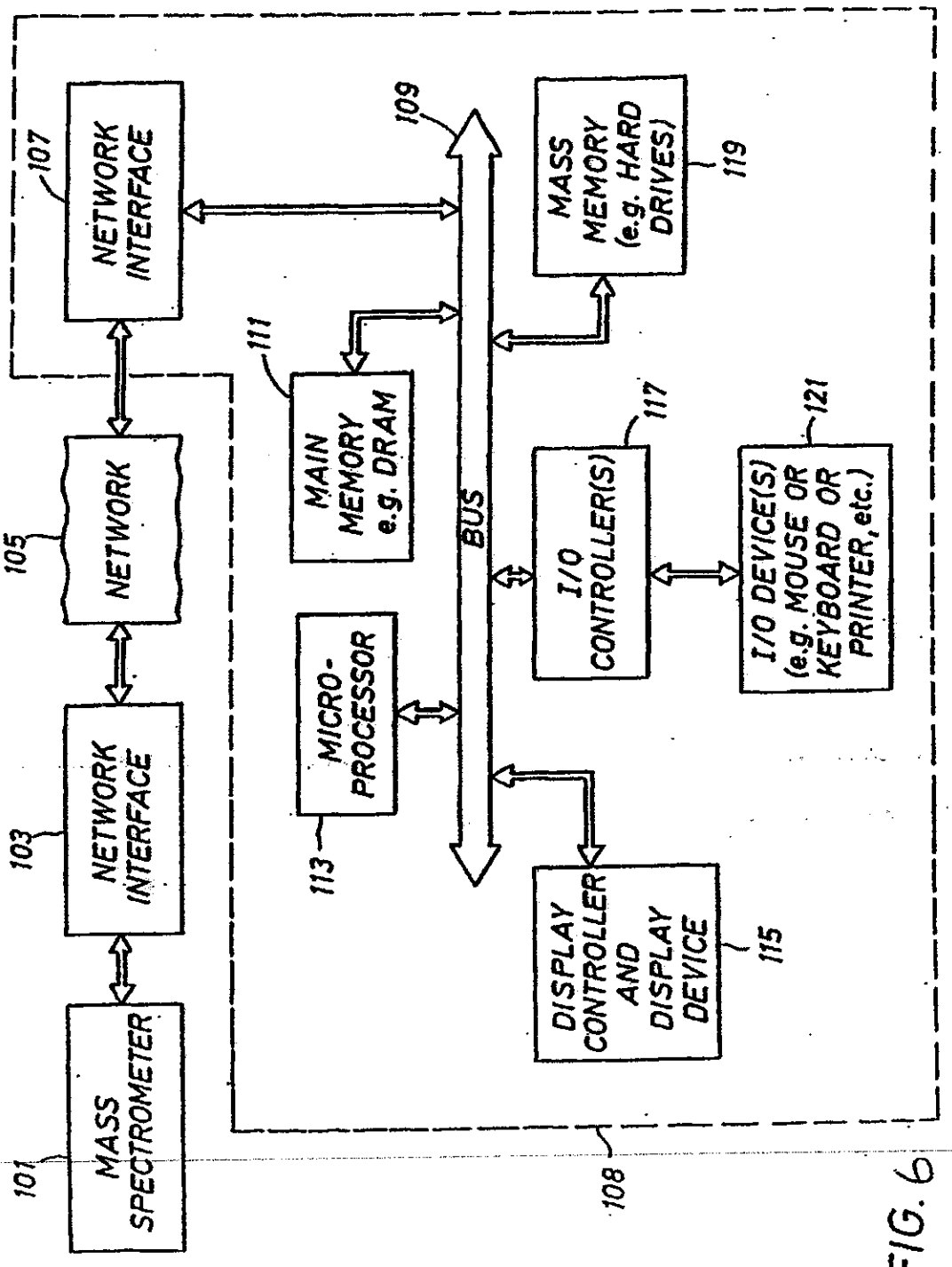

MASS SPECTROMETER
101
NETWORK INTERFACE
103
NETWORK
105
NETWORK INTERFACE
107
108
BUS
109
MAIN MEMORY e.g. DRAM
111
MICRO-PROCESSOR
113
DISPLAY CONTROLLER AND DISPLAY DEVICE
115
I/O CONTROLLER(S)
117
MASS MEMORY (e.g. HARD DRIVES)
119
I/O DEVICE(S) (e.g. MOUSE OR KEYBOARD OR PRINTER, etc.)
121

FIG. 6

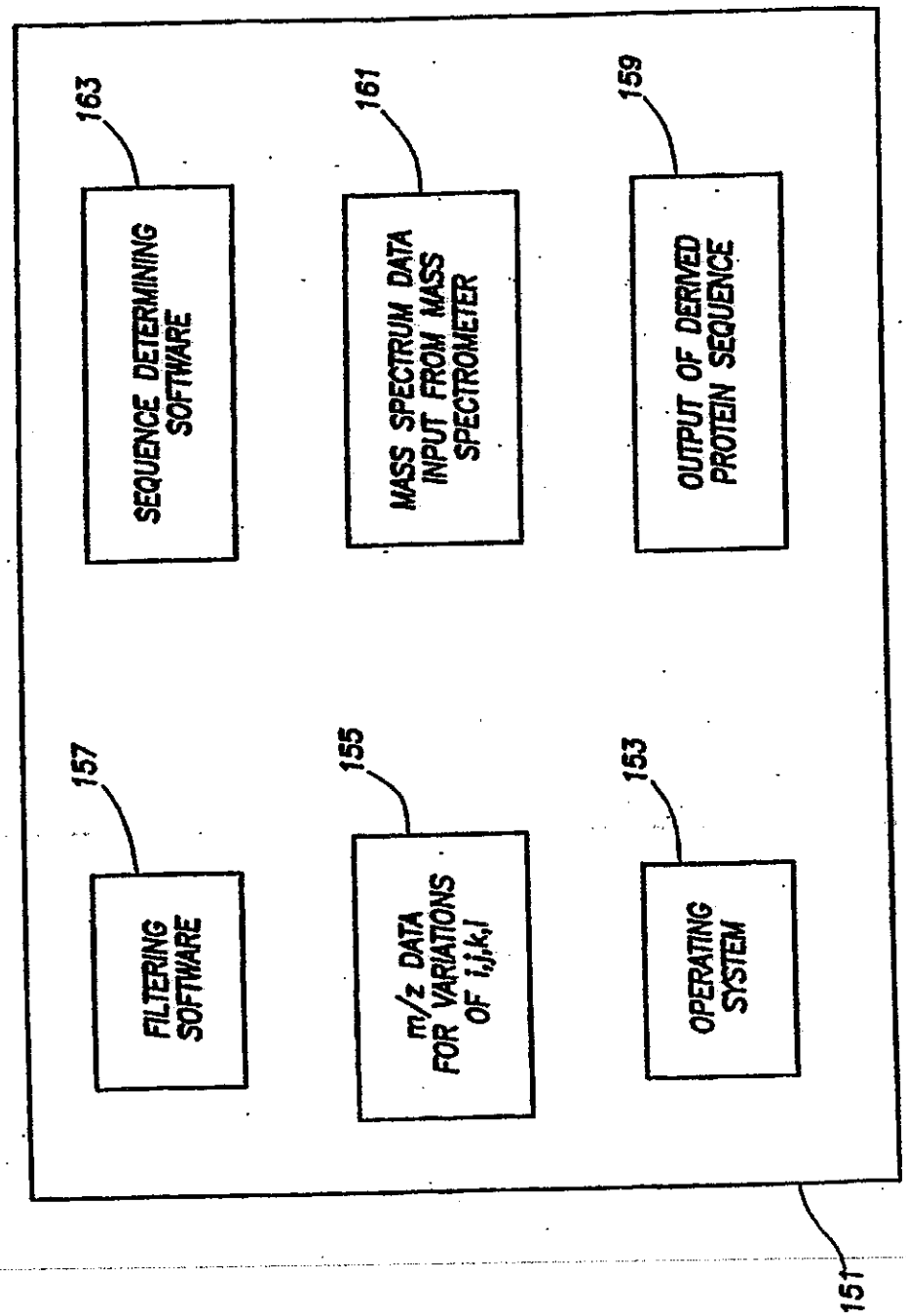
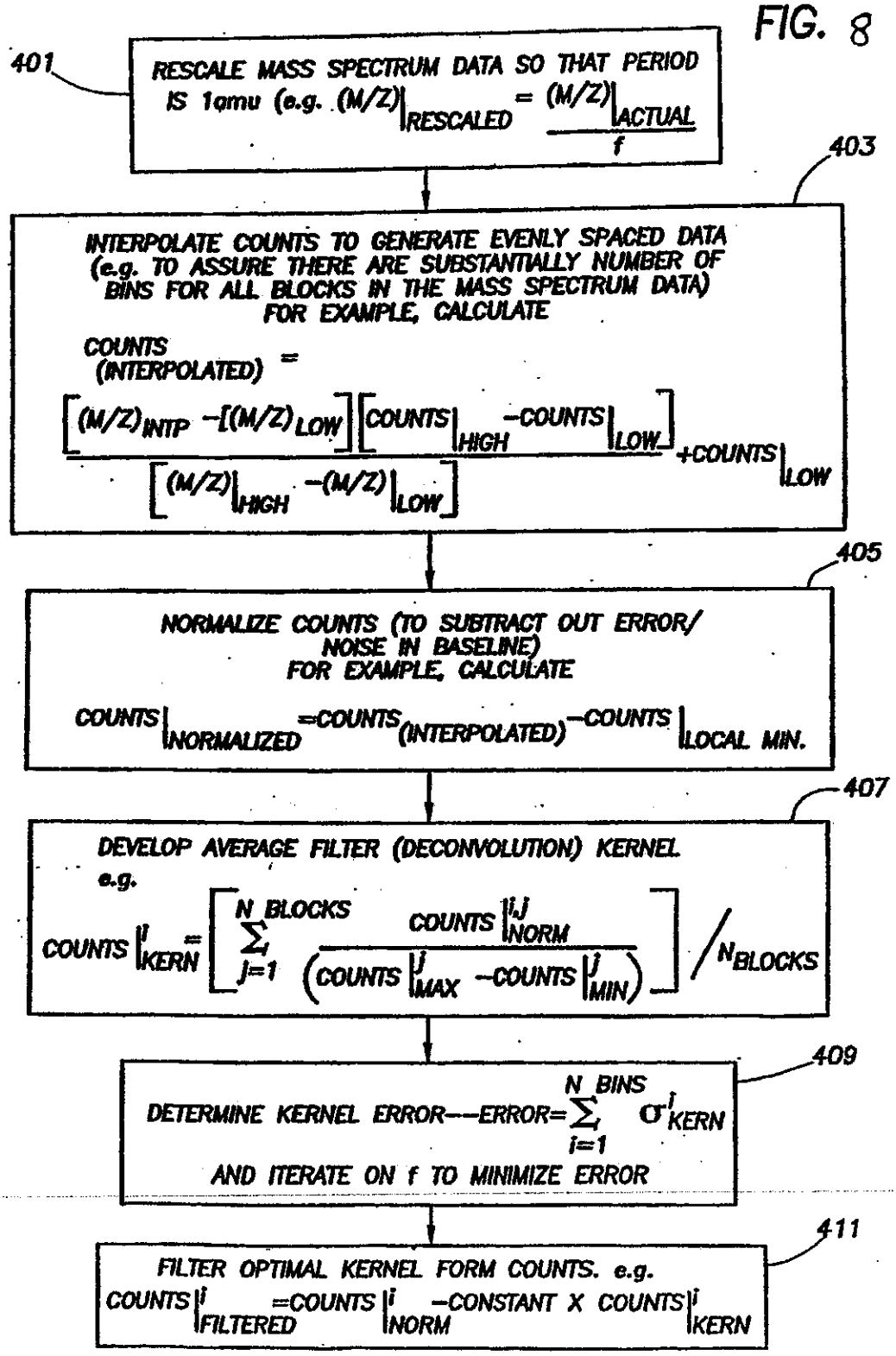

163
SEQUENCE DETERMINING SOFTWARE
161
MASS SPECTRUM DATA INPUT FROM MASS SPECTROMETER
159
OUTPUT OF DERIVED PROTEIN SEQUENCE
157
FILTERING SOFTWARE
155
m/z DATA FOR VARIATIONS OF i,j,k,l
153
OPERATING SYSTEM
151

FIG. 7

FIG. 8


401
RESCALE MASS SPECTRUM DATA SO THAT PERIOD IS 1amu (e.g. $(M/Z)|_{RESCALED} = \frac{(M/Z)|_{ACTUAL}}{f}$)
403
INTERPOLATE COUNTS TO GENERATE EVENLY SPACED DATA (e.g. TO ASSURE THERE ARE SUBSTANTIALLY NUMBER OF BINS FOR ALL BLOCKS IN THE MASS SPECTRUM DATA) FOR EXAMPLE, CALCULATE
$COUNTS_{(INTERPOLATED)} = \frac{[(M/Z)_{INTP} - (M/Z)_{LOW}][COUNTS|_{HIGH} - COUNTS|_{LOW}]}{[(M/Z)|_{HIGH} - (M/Z)|_{LOW}]} + COUNTS|_{LOW}$
405
NORMALIZE COUNTS (TO SUBTRACT OUT ERROR/ NOISE IN BASELINE) FOR EXAMPLE, CALCULATE
$COUNTS|_{NORMALIZED} = COUNTS_{(INTERPOLATED)} - COUNTS|_{LOCAL\ MIN.}$
407
DEVELOP AVERAGE FILTER (DECONVOLUTION) KERNEL e.g.
$COUNTS|^{j}_{KERN} = \left[\sum_{j=1}^{N\ BLOCKS} \frac{COUNTS|^{i,j}_{NORM}}{(COUNTS|^{j}_{MAX} - COUNTS|^{j}_{MIN})}\right] / N_{BLOCKS}$
409
DETERMINE KERNEL ERROR---$ERROR = \sum_{i=1}^{N\ BINS} \sigma^{i}_{KERN}$
AND ITERATE ON f TO MINIMIZE ERROR
411
FILTER OPTIMAL KERNEL FORM COUNTS. e.g.
$COUNTS|^{i}_{FILTERED} = COUNTS|^{i}_{NORM} - CONSTANT\ X\ COUNTS|^{i}_{KERN}$

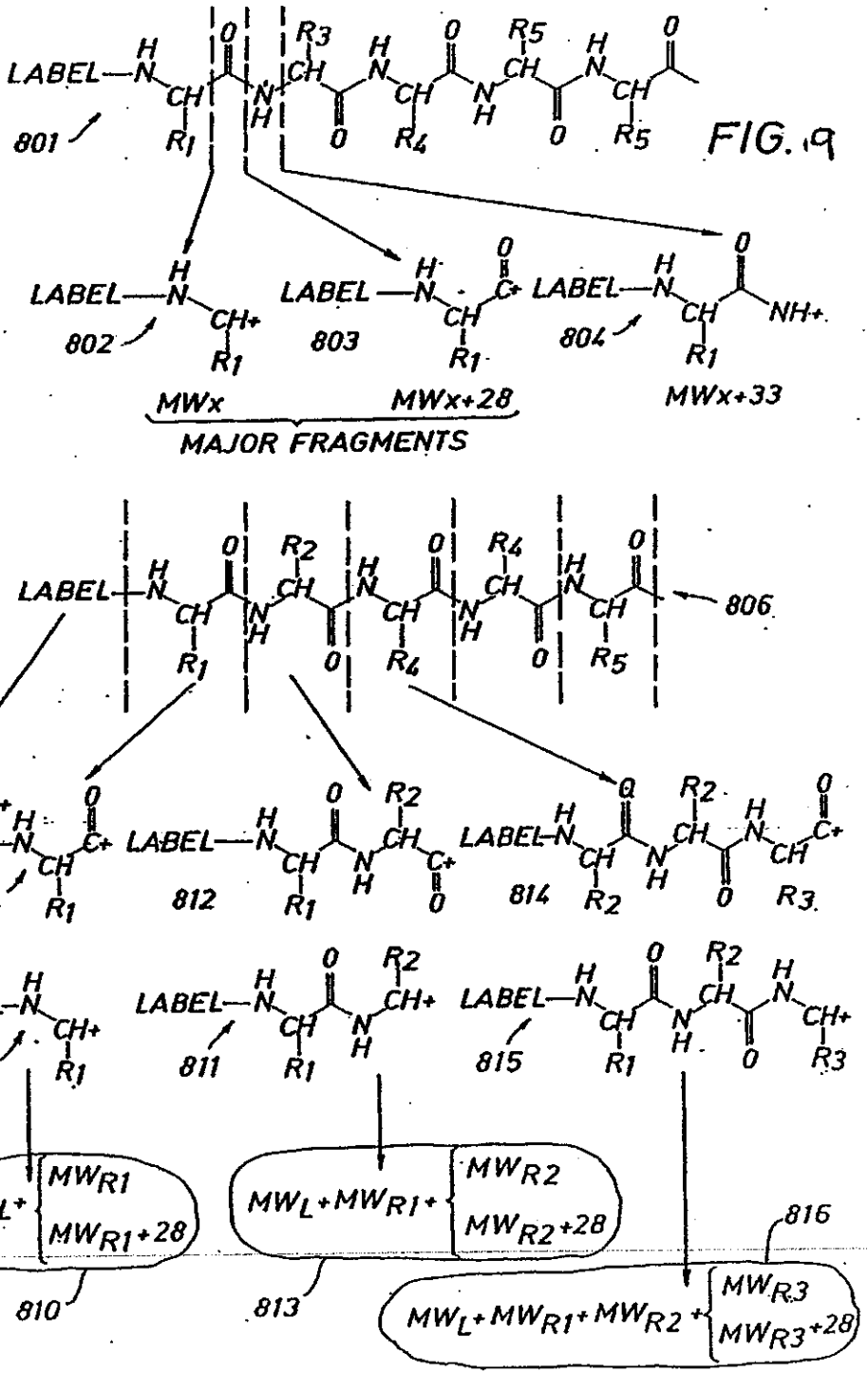

LABEL
801
802
803
804
MWx
MWx+28
MWx+33
MAJOR FRAGMENTS
806
807
808
812
814
809
811
815
816
810
813

FIG. 9

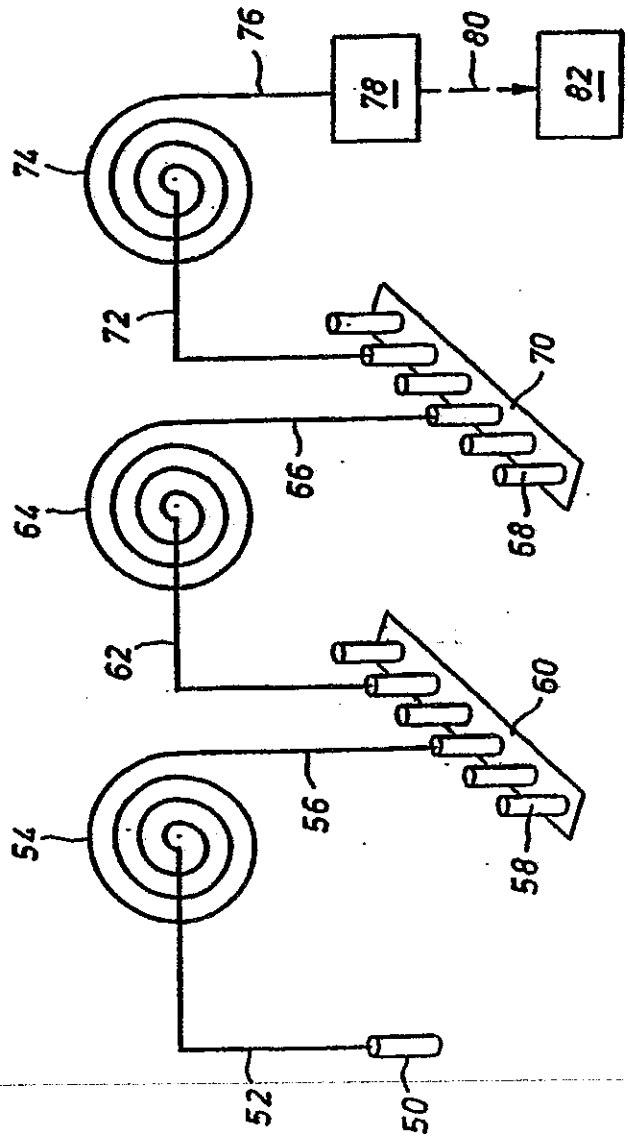


FIG. 10

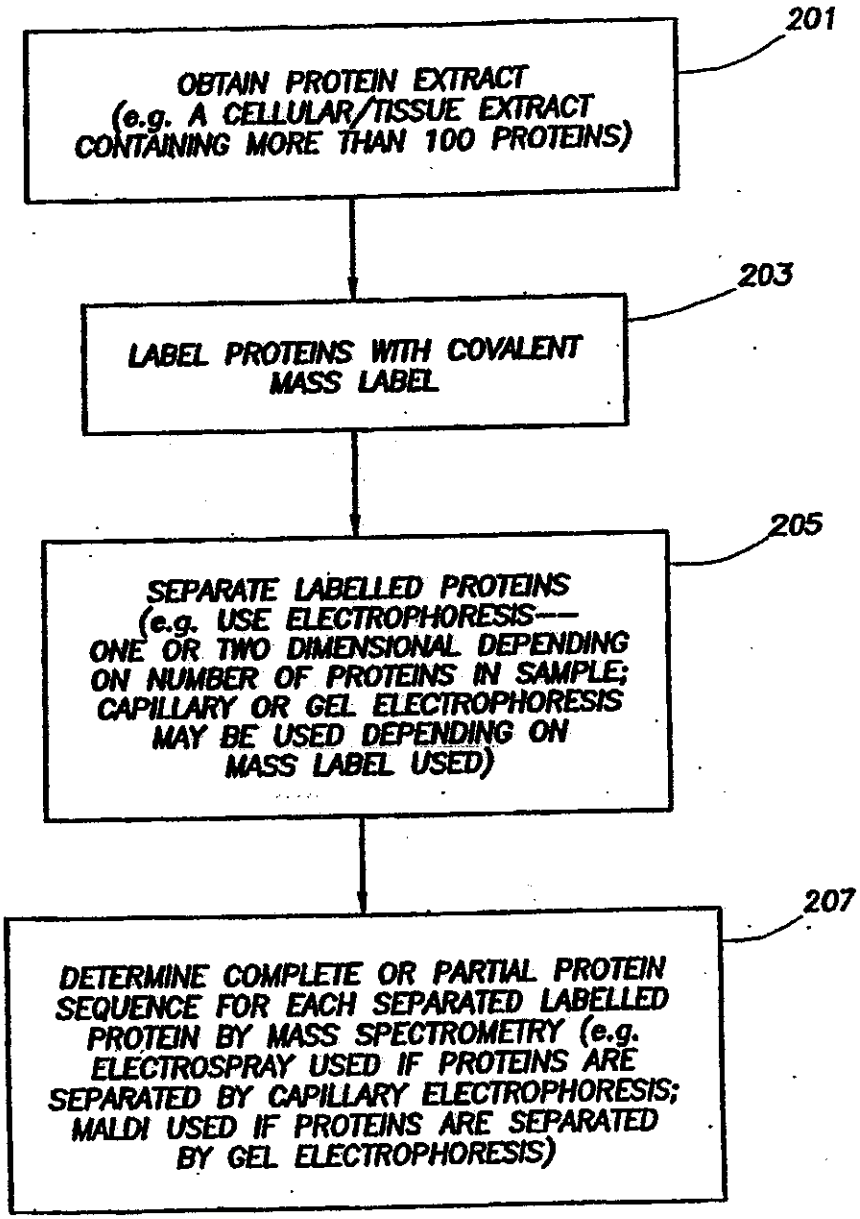

201
OBTAIN PROTEIN EXTRACT (e.g. A CELLULAR/TISSUE EXTRACT CONTAINING MORE THAN 100 PROTEINS)
203
LABEL PROTEINS WITH COVALENT MASS LABEL
205
SEPARATE LABELLED PROTEINS (e.g. USE ELECTROPHORESIS— ONE OR TWO DIMENSIONAL DEPENDING ON NUMBER OF PROTEINS IN SAMPLE; CAPILLARY OR GEL ELECTROPHORESIS MAY BE USED DEPENDING ON MASS LABEL USED)
207
DETERMINE COMPLETE OR PARTIAL PROTEIN SEQUENCE FOR EACH SEPARATED LABELLED PROTEIN BY MASS SPECTROMETRY (e.g. ELECTROSPRAY USED IF PROTEINS ARE SEPARATED BY CAPILLARY ELECTROPHORESIS; MALDI USED IF PROTEINS ARE SEPARATED BY GEL ELECTROPHORESIS)

FIG. 11

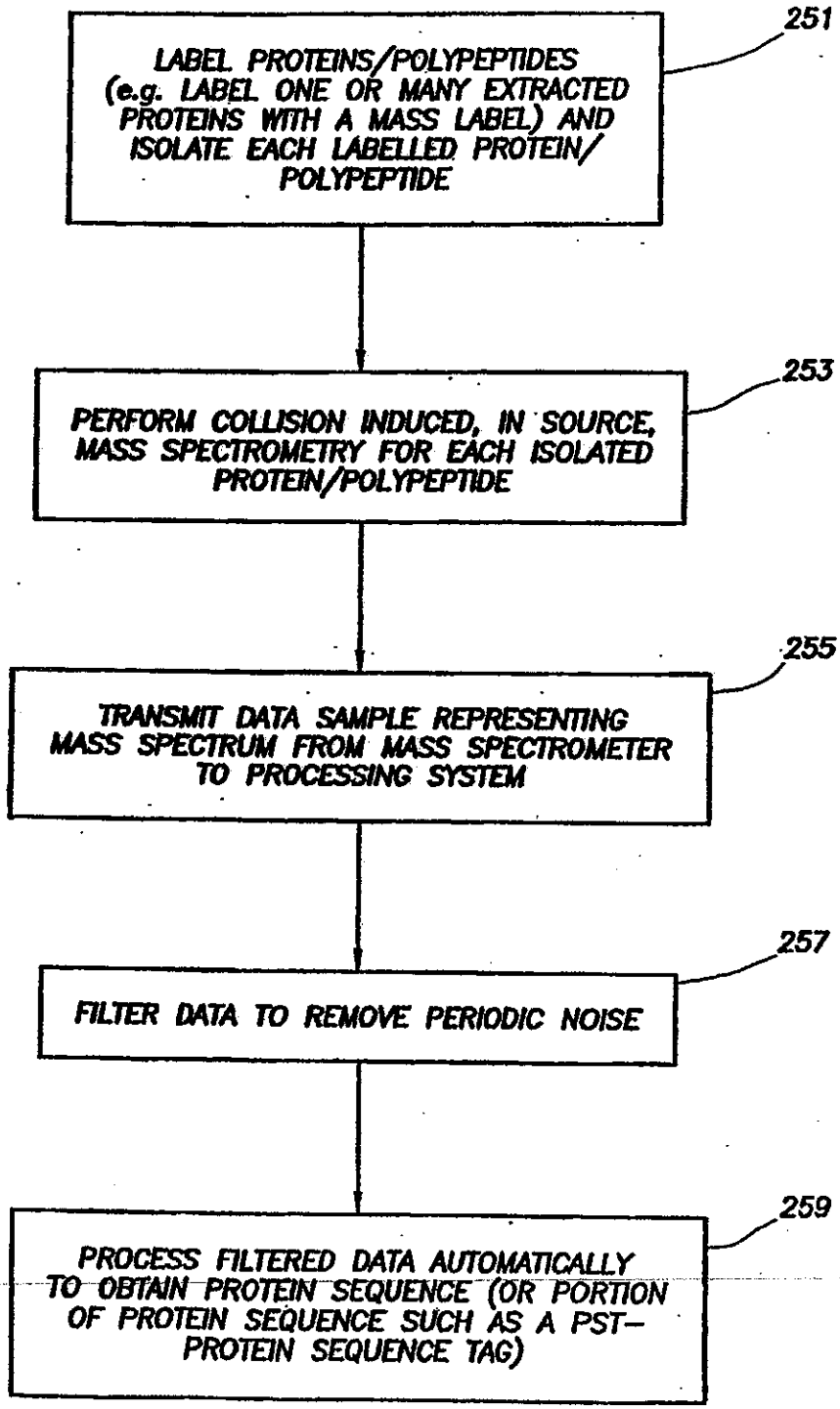

LABEL PROTEINS/POLYPEPTIDES (e.g. LABEL ONE OR MANY EXTRACTED PROTEINS WITH A MASS LABEL) AND ISOLATE EACH LABELLED PROTEIN/ POLYPEPTIDE
251
PERFORM COLLISION INDUCED, IN SOURCE, MASS SPECTROMETRY FOR EACH ISOLATED PROTEIN/POLYPEPTIDE
253
TRANSMIT DATA SAMPLE REPRESENTING MASS SPECTRUM FROM MASS SPECTROMETER TO PROCESSING SYSTEM
255
FILTER DATA TO REMOVE PERIODIC NOISE
257
PROCESS FILTERED DATA AUTOMATICALLY TO OBTAIN PROTEIN SEQUENCE (OR PORTION OF PROTEIN SEQUENCE SUCH AS A PST-PROTEIN SEQUENCE TAG)
259

FIG. 12

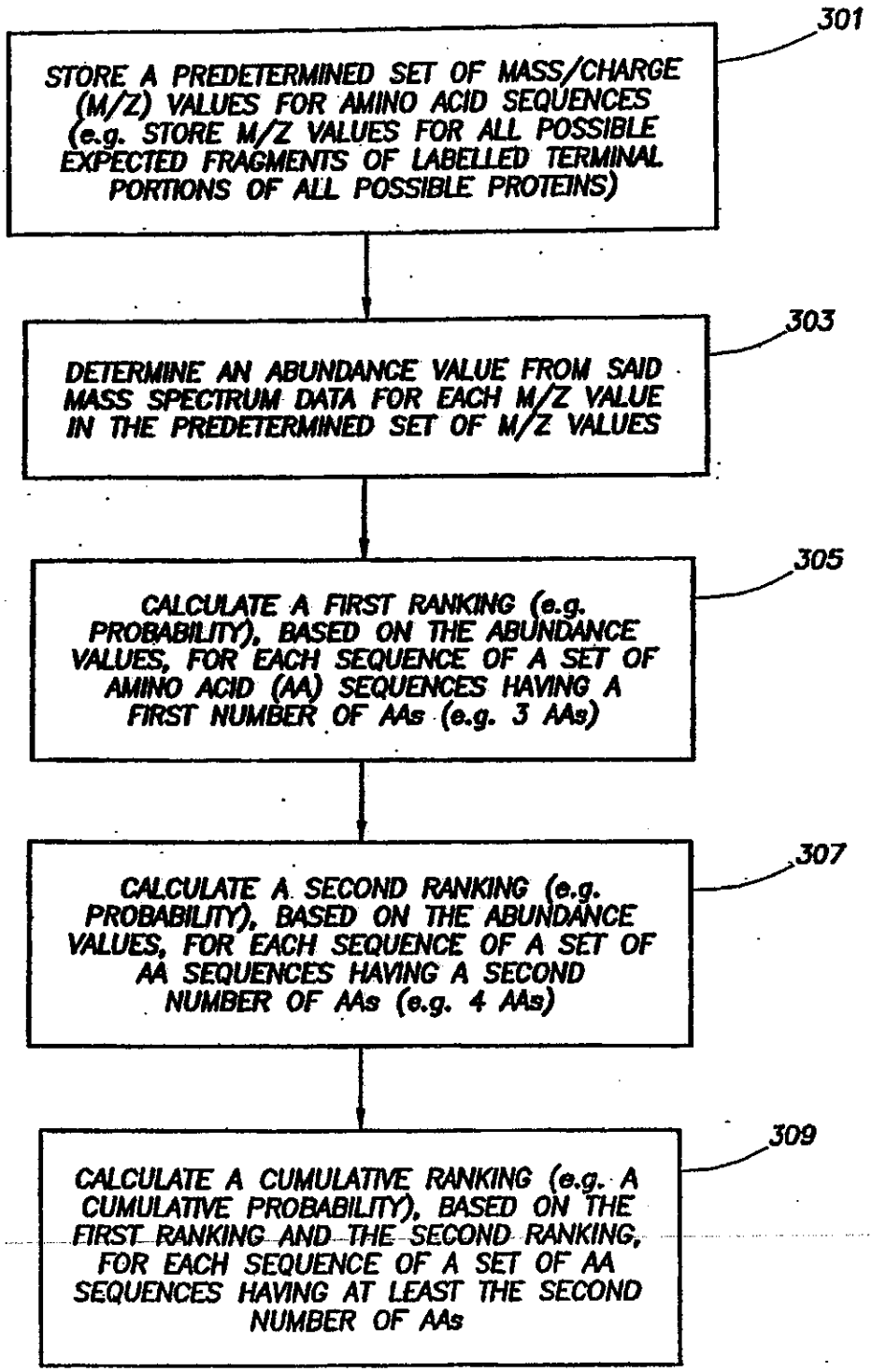

301
STORE A PREDETERMINED SET OF MASS/CHARGE (M/Z) VALUES FOR AMINO ACID SEQUENCES (e.g. STORE M/Z VALUES FOR ALL POSSIBLE EXPECTED FRAGMENTS OF LABELLED TERMINAL PORTIONS OF ALL POSSIBLE PROTEINS)
303
DETERMINE AN ABUNDANCE VALUE FROM SAID MASS SPECTRUM DATA FOR EACH M/Z VALUE IN THE PREDETERMINED SET OF M/Z VALUES
305
CALCULATE A FIRST RANKING (e.g. PROBABILITY), BASED ON THE ABUNDANCE VALUES, FOR EACH SEQUENCE OF A SET OF AMINO ACID (AA) SEQUENCES HAVING A FIRST NUMBER OF AAs (e.g. 3 AAs)
307
CALCULATE A SECOND RANKING (e.g. PROBABILITY), BASED ON THE ABUNDANCE VALUES, FOR EACH SEQUENCE OF A SET OF AA SEQUENCES HAVING A SECOND NUMBER OF AAs (e.g. 4 AAs)
309
CALCULATE A CUMULATIVE RANKING (e.g. A CUMULATIVE PROBABILITY), BASED ON THE FIRST RANKING AND THE SECOND RANKING, FOR EACH SEQUENCE OF A SET OF AA SEQUENCES HAVING AT LEAST THE SECOND NUMBER OF AAs

FIG. 13

FIG. 14A

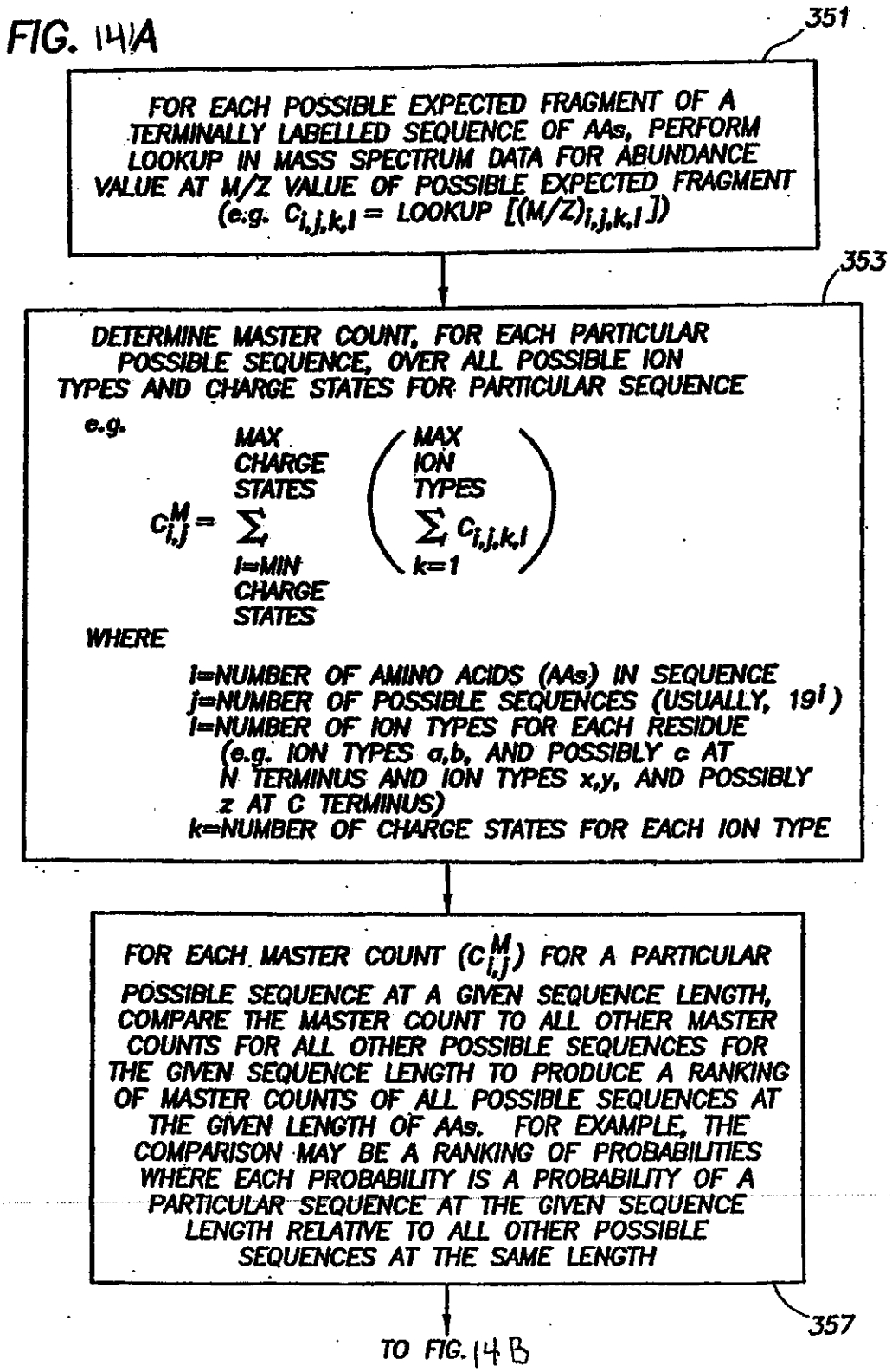

351
FOR EACH POSSIBLE EXPECTED FRAGMENT OF A TERMINALLY LABELLED SEQUENCE OF AAs, PERFORM LOOKUP IN MASS SPECTRUM DATA FOR ABUNDANCE VALUE AT M/Z VALUE OF POSSIBLE EXPECTED FRAGMENT (e.g. $C_{i,j,k,l}$ = LOOKUP $[(M/Z)_{i,j,k,l}]$)
353
DETERMINE MASTER COUNT, FOR EACH PARTICULAR POSSIBLE SEQUENCE, OVER ALL POSSIBLE ION TYPES AND CHARGE STATES FOR PARTICULAR SEQUENCE
e.g.
$C_{i,j}^{M} = \sum_{l=\text{MIN CHARGE STATES}}^{\text{MAX CHARGE STATES}} \left(\sum_{k=1}^{\text{MAX ION TYPES}} C_{i,j,k,l} \right)$
WHERE
i=NUMBER OF AMINO ACIDS (AAs) IN SEQUENCE
j=NUMBER OF POSSIBLE SEQUENCES (USUALLY, 19^i)
l=NUMBER OF ION TYPES FOR EACH RESIDUE (e.g. ION TYPES a,b, AND POSSIBLY c AT N TERMINUS AND ION TYPES x,y, AND POSSIBLY z AT C TERMINUS)
k=NUMBER OF CHARGE STATES FOR EACH ION TYPE
FOR EACH MASTER COUNT ($C_{i,j}^{M}$) FOR A PARTICULAR POSSIBLE SEQUENCE AT A GIVEN SEQUENCE LENGTH, COMPARE THE MASTER COUNT TO ALL OTHER MASTER COUNTS FOR ALL OTHER POSSIBLE SEQUENCES FOR THE GIVEN SEQUENCE LENGTH TO PRODUCE A RANKING OF MASTER COUNTS OF ALL POSSIBLE SEQUENCES AT THE GIVEN LENGTH OF AAs. FOR EXAMPLE, THE COMPARISON MAY BE A RANKING OF PROBABILITIES WHERE EACH PROBABILITY IS A PROBABILITY OF A PARTICULAR SEQUENCE AT THE GIVEN SEQUENCE LENGTH RELATIVE TO ALL OTHER POSSIBLE SEQUENCES AT THE SAME LENGTH
357
TO FIG. 14B

FIG. 14 B

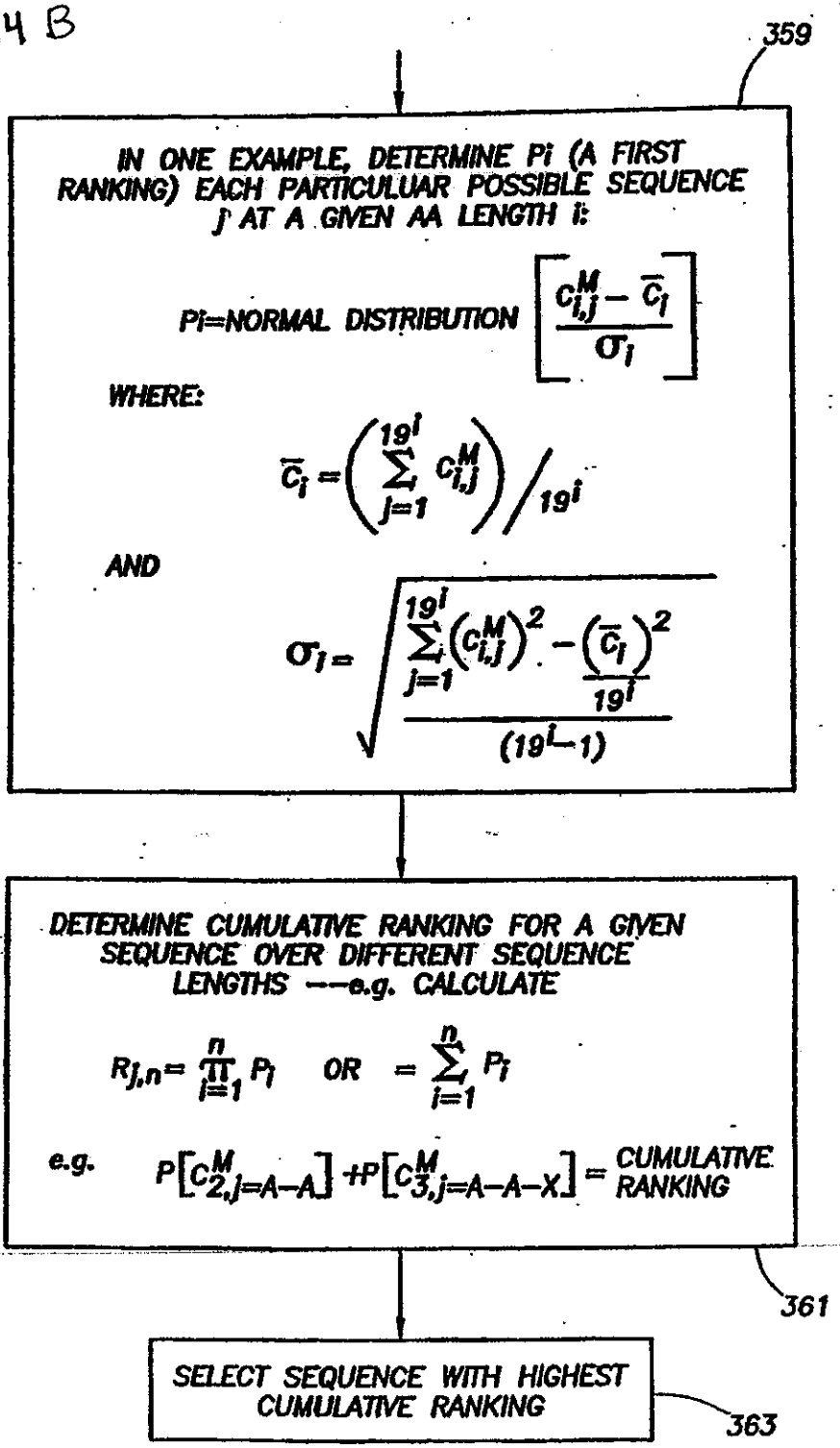
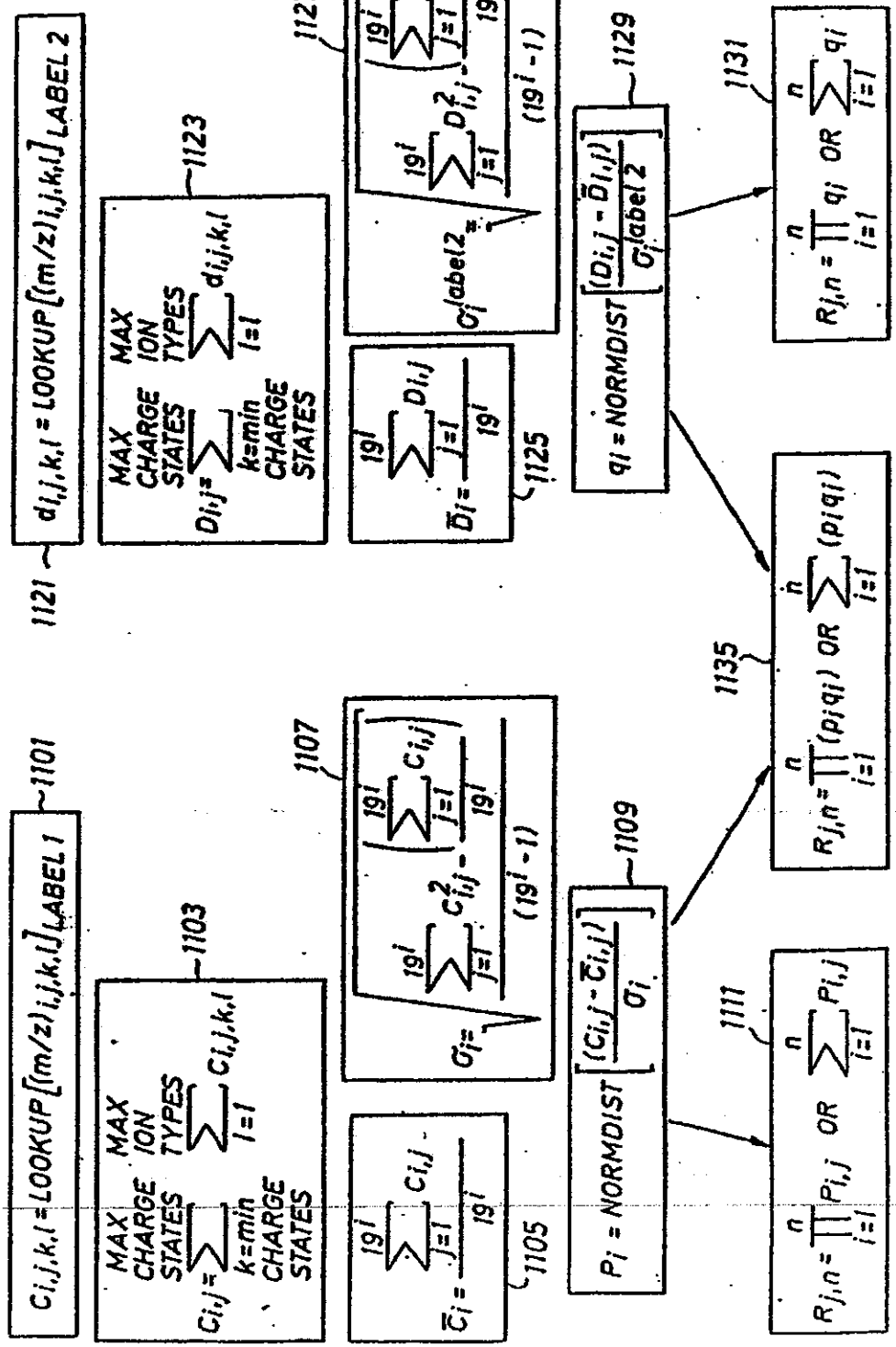

359
IN ONE EXAMPLE, DETERMINE Pi (A FIRST RANKING) EACH PARTICULUAR POSSIBLE SEQUENCE j AT A GIVEN AA LENGTH i:
Pi=NORMAL DISTRIBUTION $\left[\frac{c^M_{i,j} - \bar{c}_i}{\sigma_i}\right]$
WHERE:
$\bar{c}_i = \left(\sum_{j=1}^{19^i} c^M_{i,j}\right) / 19^i$
AND
$\sigma_i = \sqrt{\frac{\sum_{j=1}^{19^i}\left(c^M_{i,j}\right)^2 - \frac{\left(\bar{c}_i\right)^2}{19^i}}{(19^i - 1)}}$
DETERMINE CUMULATIVE RANKING FOR A GIVEN SEQUENCE OVER DIFFERENT SEQUENCE LENGTHS ---e.g. CALCULATE
$R_{j,n} = \prod_{i=1}^{n} P_i$ OR $= \sum_{i=1}^{n} P_i$
e.g. $P\left[C^M_{2,j=A-A}\right] + P\left[C^M_{3,j=A-A-X}\right]$ = CUMULATIVE RANKING
361
SELECT SEQUENCE WITH HIGHEST CUMULATIVE RANKING
363

FIG. 15


LABEL 1
LABEL 2
$C_{i,j,k,l}$ = LOOKUP $[(m/z)_{i,j,k,l}]_{LABEL\ 1}$
1101
$d_{i,j,k,l}$ = LOOKUP $[(m/z)_{i,j,k,l}]_{LABEL\ 2}$
1121
$C_{i,j} = \sum_{k=min\ CHARGE\ STATES}^{MAX\ CHARGE\ STATES} \sum_{l=1}^{MAX\ ION\ TYPES} C_{i,j,k,l}$
1103
$D_{i,j} = \sum_{k=min\ CHARGE\ STATES}^{MAX\ CHARGE\ STATES} \sum_{l=1}^{MAX\ ION\ TYPES} d_{i,j,k,l}$
1123
$\bar{C}_i = \frac{\sum_{j=1}^{19^i} C_{i,j}}{19^i}$
1105
$\sigma_i = \sqrt{\frac{\sum_{j=1}^{19^i} C_{i,j}^2 - \frac{\left(\sum_{j=1}^{19^i} C_{i,j}\right)}{19^i}}{(19^i - 1)}}$
1107
$\bar{D}_i = \frac{\sum_{j=1}^{19^i} D_{i,j}}{19^i}$
1125
$\sigma_i^{label2} = \sqrt{\frac{\sum_{j=1}^{19^i} D_{i,j}^2 - \frac{\left(\sum_{j=1}^{19^i} D_{i,j}\right)}{19^i}}{(19^i - 1)}}$
1127
P_i = NORMDIST $\left[\frac{(C_{i,j} - \bar{C}_{i,j})}{\sigma_i}\right]$
1109
q_i = NORMDIST $\left[\frac{(D_{i,j} - \bar{D}_{i,j})}{\sigma_i^{label2}}\right]$
1129
$R_{j,n} = \prod_{i=1}^{n} P_{i,j}$ OR $\sum_{i=1}^{n} P_{i,j}$
1111
$R_{j,n} = \prod_{i=1}^{n} (p_i q_i)$ OR $\sum_{i=1}^{n} (p_i q_i)$
1135
$R_{j,n} = \prod_{i=1}^{n} q_i$ OR $\sum_{i=1}^{n} q_i$
1131

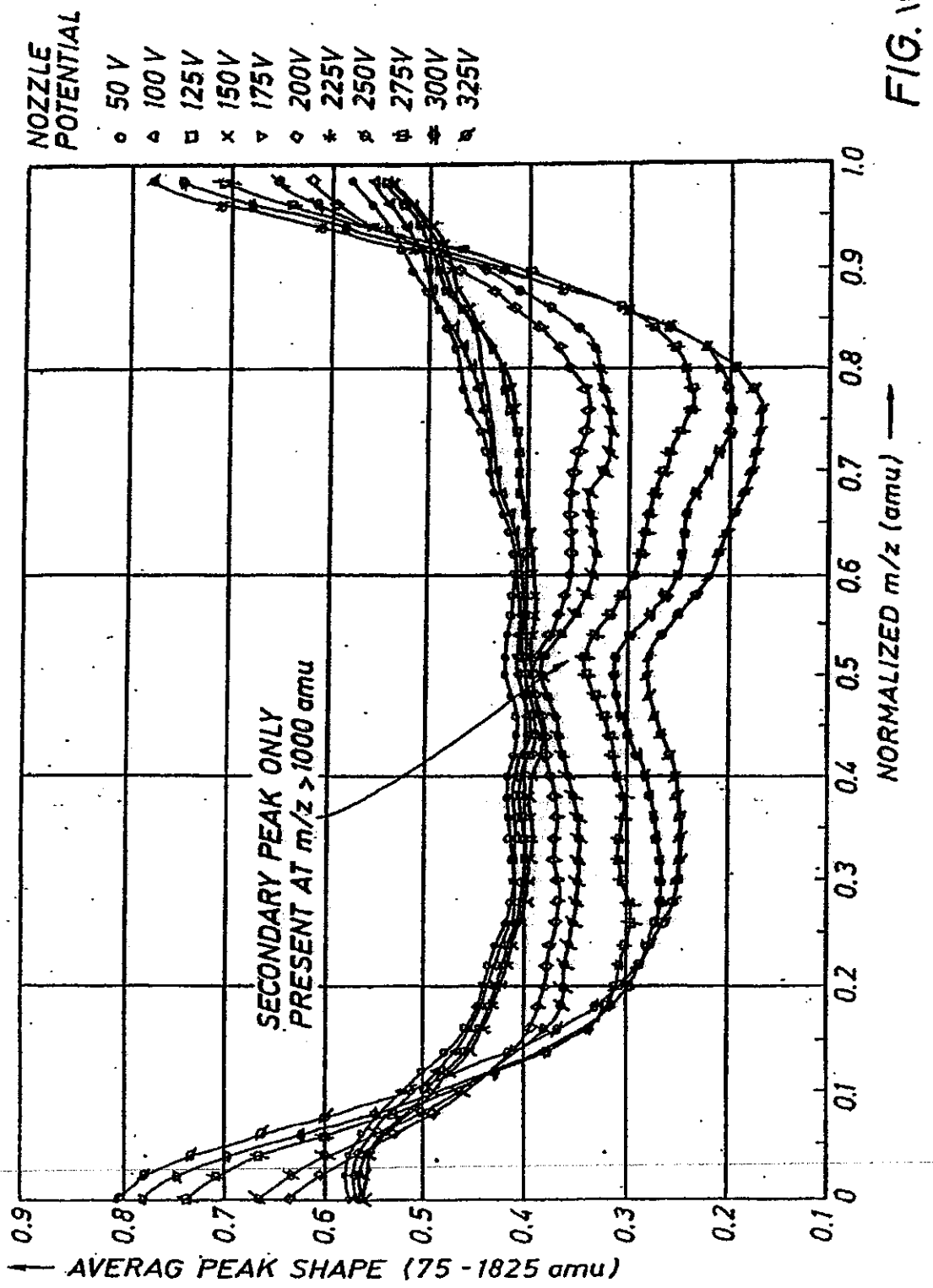

NOZZLE POTENTIAL
50 V
100 V
125V
150V
175V
200V
225V
250V
275V
300V
325V
SECONDARY PEAK ONLY PRESENT AT m/z >1000 amu
NORMALIZED m/z (amu) →
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1
← AVERAG PEAK SHAPE (75 - 1825 amu)

FIG. 16

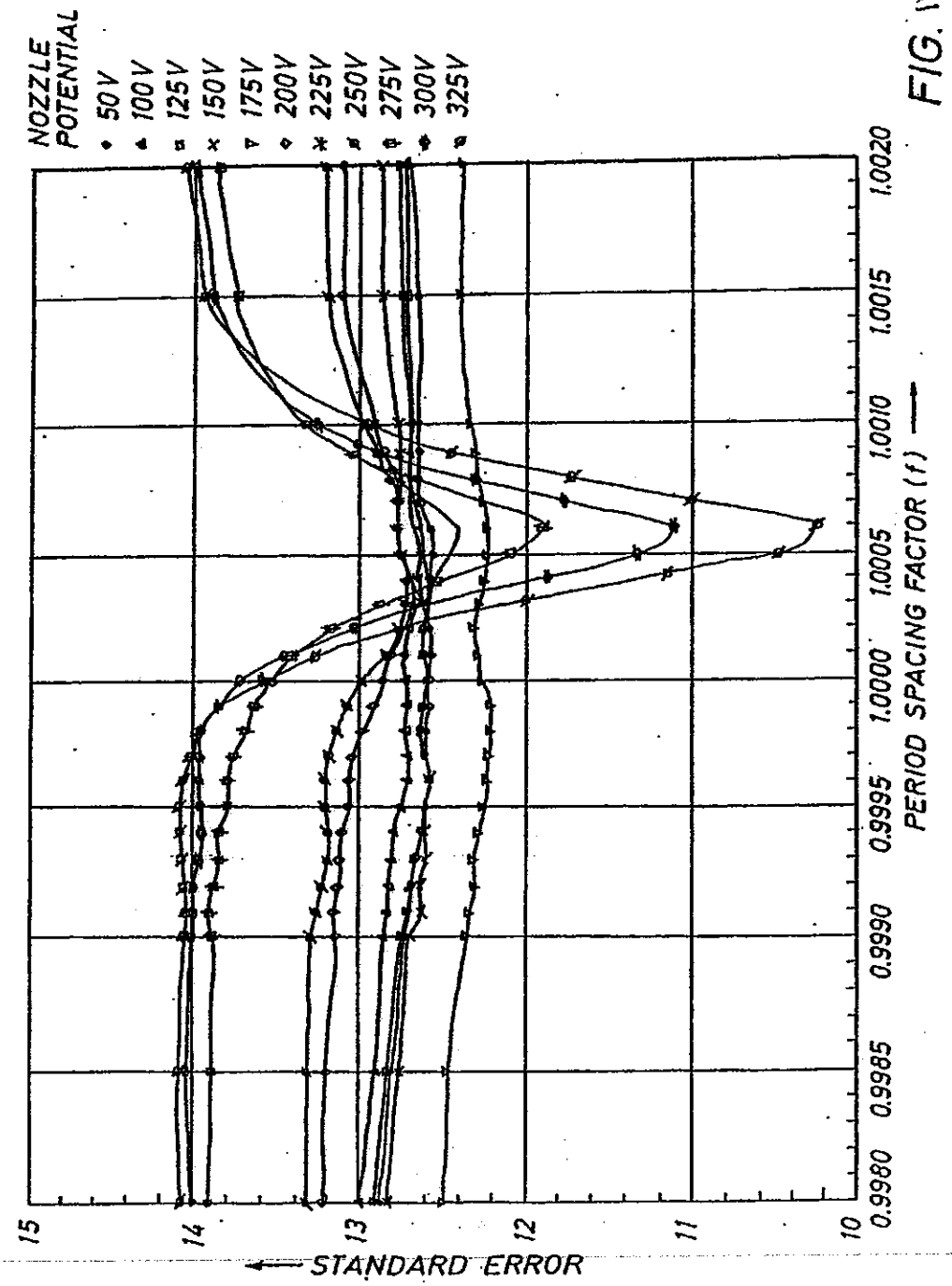
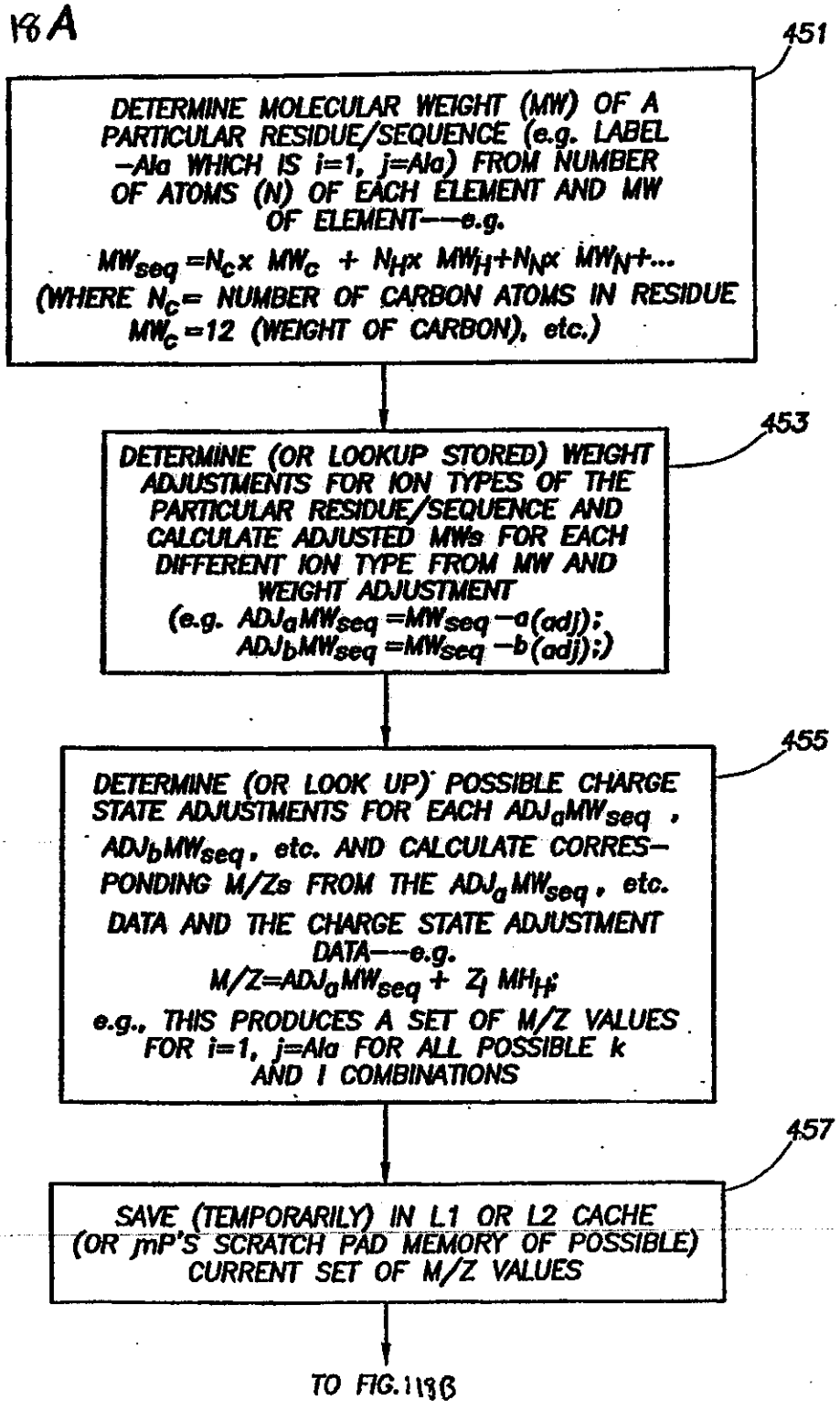

NOZZLE POTENTIAL
50 V
100 V
125 V
150 V
175 V
200 V
225 V
250 V
275 V
300 V
325 V
PERIOD SPACING FACTOR (f) —
0.9980
0.9985
0.9990
0.9995
1.0000
1.0005
1.0010
1.0015
1.0020
15
14
13
12
11
10
STANDARD ERROR

FIG. 17

FIG. 18A


451
DETERMINE MOLECULAR WEIGHT (MW) OF A PARTICULAR RESIDUE/SEQUENCE (e.g. LABEL -Ala WHICH IS i=1, j=Ala) FROM NUMBER OF ATOMS (N) OF EACH ELEMENT AND MW OF ELEMENT--e.g.
$MW_{seq} = N_C x\ MW_C + N_H x\ MW_H + N_N x\ MW_N + \ldots$
(WHERE N_C= NUMBER OF CARBON ATOMS IN RESIDUE
MW_C=12 (WEIGHT OF CARBON), etc.)
453
DETERMINE (OR LOOKUP STORED) WEIGHT ADJUSTMENTS FOR ION TYPES OF THE PARTICULAR RESIDUE/SEQUENCE AND CALCULATE ADJUSTED MWs FOR EACH DIFFERENT ION TYPE FROM MW AND WEIGHT ADJUSTMENT
(e.g. $ADJ_a MW_{seq} = MW_{seq} - a(adj)$;
$ADJ_b MW_{seq} = MW_{seq} - b(adj)$;)
455
DETERMINE (OR LOOK UP) POSSIBLE CHARGE STATE ADJUSTMENTS FOR EACH $ADJ_a MW_{seq}$, $ADJ_b MW_{seq}$, etc. AND CALCULATE CORRESPONDING M/Zs FROM THE $ADJ_a MW_{seq}$, etc. DATA AND THE CHARGE STATE ADJUSTMENT DATA--e.g.
$M/Z = ADJ_a MW_{seq} + Z_l MH_H$;
e.g., THIS PRODUCES A SET OF M/Z VALUES FOR i=1, j=Ala FOR ALL POSSIBLE k AND l COMBINATIONS
457
SAVE (TEMPORARILY) IN L1 OR L2 CACHE (OR mP'S SCRATCH PAD MEMORY OF POSSIBLE) CURRENT SET OF M/Z VALUES
TO FIG. 18B

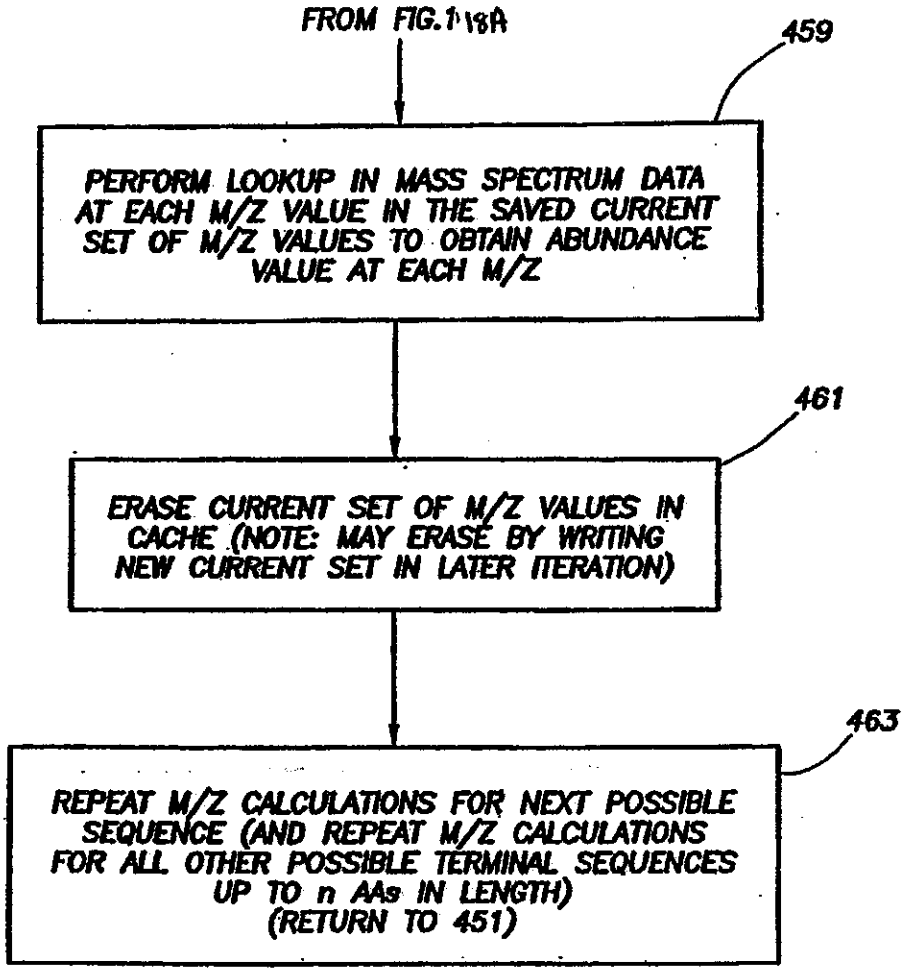

FROM FIG. 18A
459
PERFORM LOOKUP IN MASS SPECTRUM DATA AT EACH M/Z VALUE IN THE SAVED CURRENT SET OF M/Z VALUES TO OBTAIN ABUNDANCE VALUE AT EACH M/Z
461
ERASE CURRENT SET OF M/Z VALUES IN CACHE (NOTE: MAY ERASE BY WRITING NEW CURRENT SET IN LATER ITERATION)
463
REPEAT M/Z CALCULATIONS FOR NEXT POSSIBLE SEQUENCE (AND REPEAT M/Z CALCULATIONS FOR ALL OTHER POSSIBLE TERMINAL SEQUENCES UP TO n AAs IN LENGTH)
(RETURN TO 451)

FIG. 18B

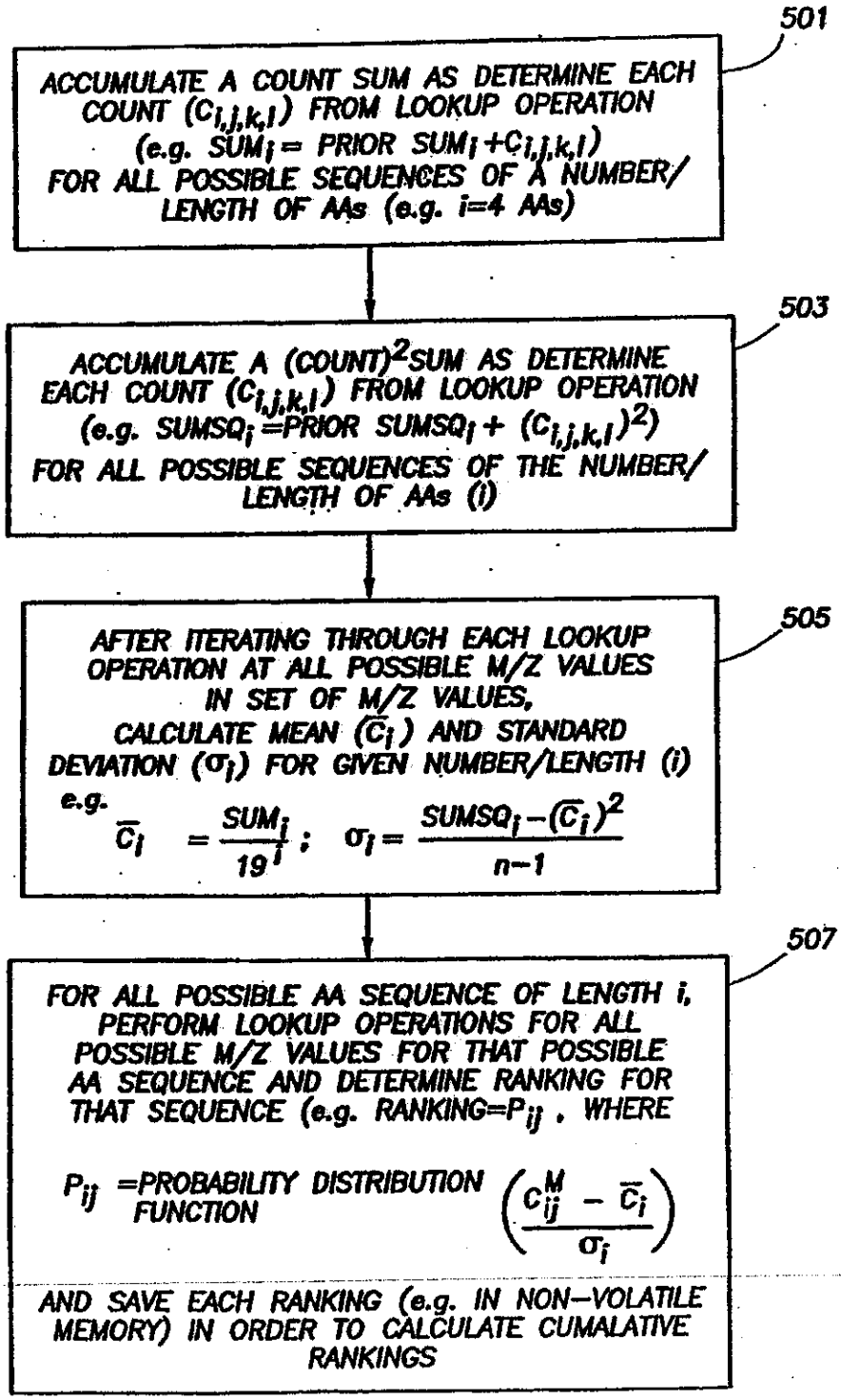

501
ACCUMULATE A COUNT SUM AS DETERMINE EACH COUNT ($C_{i,j,k,l}$) FROM LOOKUP OPERATION (e.g. SUM_i = PRIOR $SUM_i + C_{i,j,k,l}$) FOR ALL POSSIBLE SEQUENCES OF A NUMBER/ LENGTH OF AAs (e.g. i=4 AAs)
503
ACCUMULATE A $(COUNT)^2$SUM AS DETERMINE EACH COUNT ($C_{i,j,k,l}$) FROM LOOKUP OPERATION (e.g. $SUMSQ_i$ =PRIOR $SUMSQ_i + (C_{i,j,k,l})^2$) FOR ALL POSSIBLE SEQUENCES OF THE NUMBER/ LENGTH OF AAs (i)
505
AFTER ITERATING THROUGH EACH LOOKUP OPERATION AT ALL POSSIBLE M/Z VALUES IN SET OF M/Z VALUES, CALCULATE MEAN ($\overline{C_i}$) AND STANDARD DEVIATION (σ_i) FOR GIVEN NUMBER/LENGTH (i)
e.g. $\overline{C_i} = \frac{SUM_i}{19^i}$; $\sigma_i = \frac{SUMSQ_i - (\overline{C_i})^2}{n-1}$
507
FOR ALL POSSIBLE AA SEQUENCE OF LENGTH i, PERFORM LOOKUP OPERATIONS FOR ALL POSSIBLE M/Z VALUES FOR THAT POSSIBLE AA SEQUENCE AND DETERMINE RANKING FOR THAT SEQUENCE (e.g. RANKING=P_{ij} , WHERE
P_{ij} =PROBABILITY DISTRIBUTION FUNCTION $\left(\frac{C_{ij}^M - \overline{C_i}}{\sigma_i}\right)$
AND SAVE EACH RANKING (e.g. IN NON-VOLATILE MEMORY) IN ORDER TO CALCULATE CUMALATIVE RANKINGS

FIG. 19

FIG. 20A

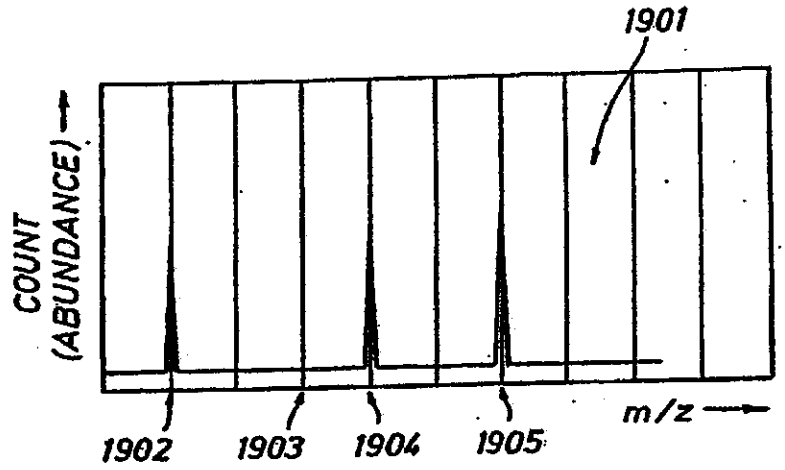
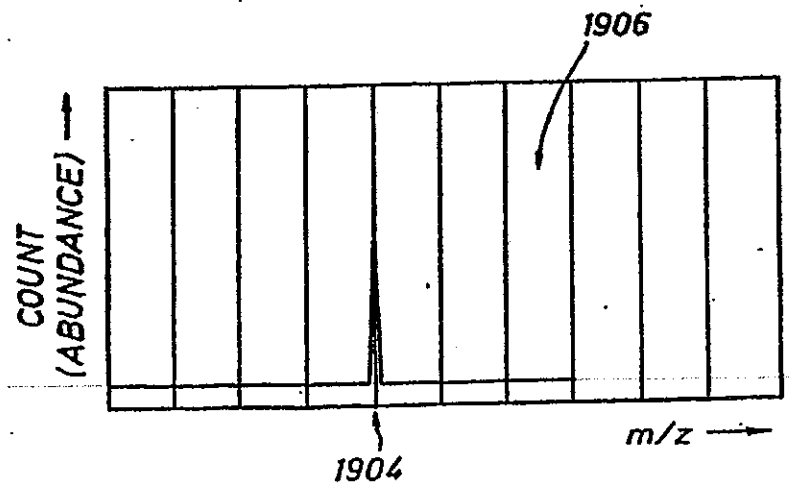


FIG. 20B



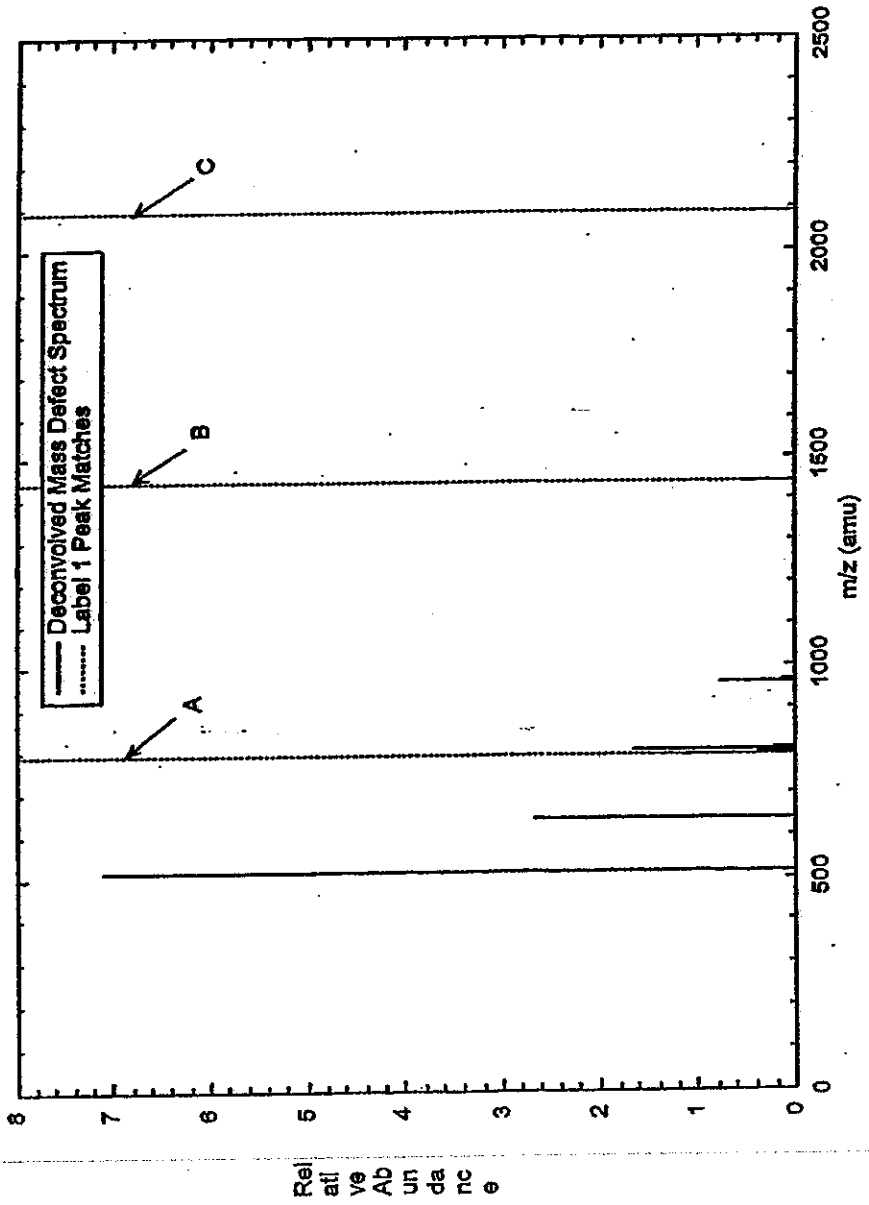

Deconvolved Mass Defect Spectrum
Label 1 Peak Matches
A
B
C
0
500
1000
1500
2000
2500
m/z (amu)
0
1
2
3
4
5
6
7
8
Relative Abundance

FIG. 21

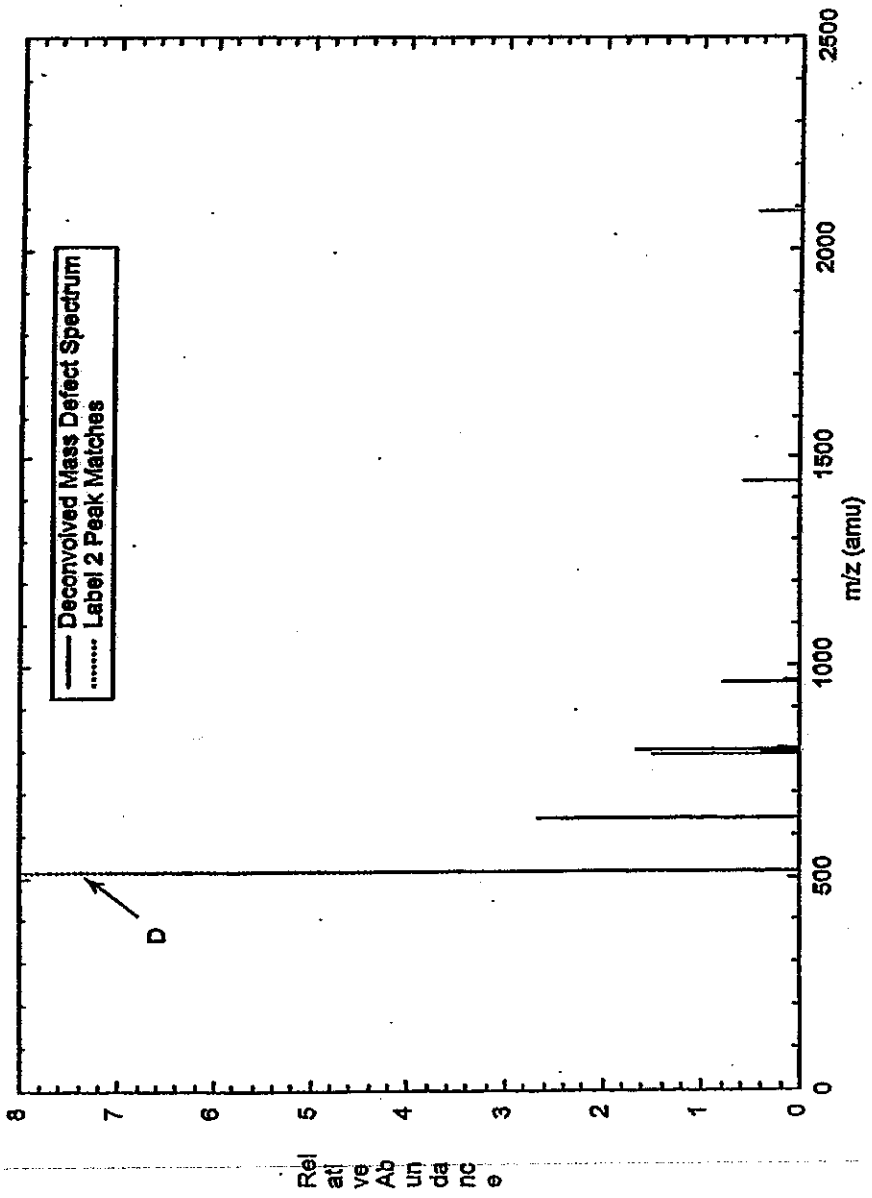

Deconvolved Mass Defect Spectrum
Label 2 Peak Matches
D
Relative Abundance
m/z (amu)
0
1
2
3
4
5
6
7
8
500
1000
1500
2000
2500

FIG. 22

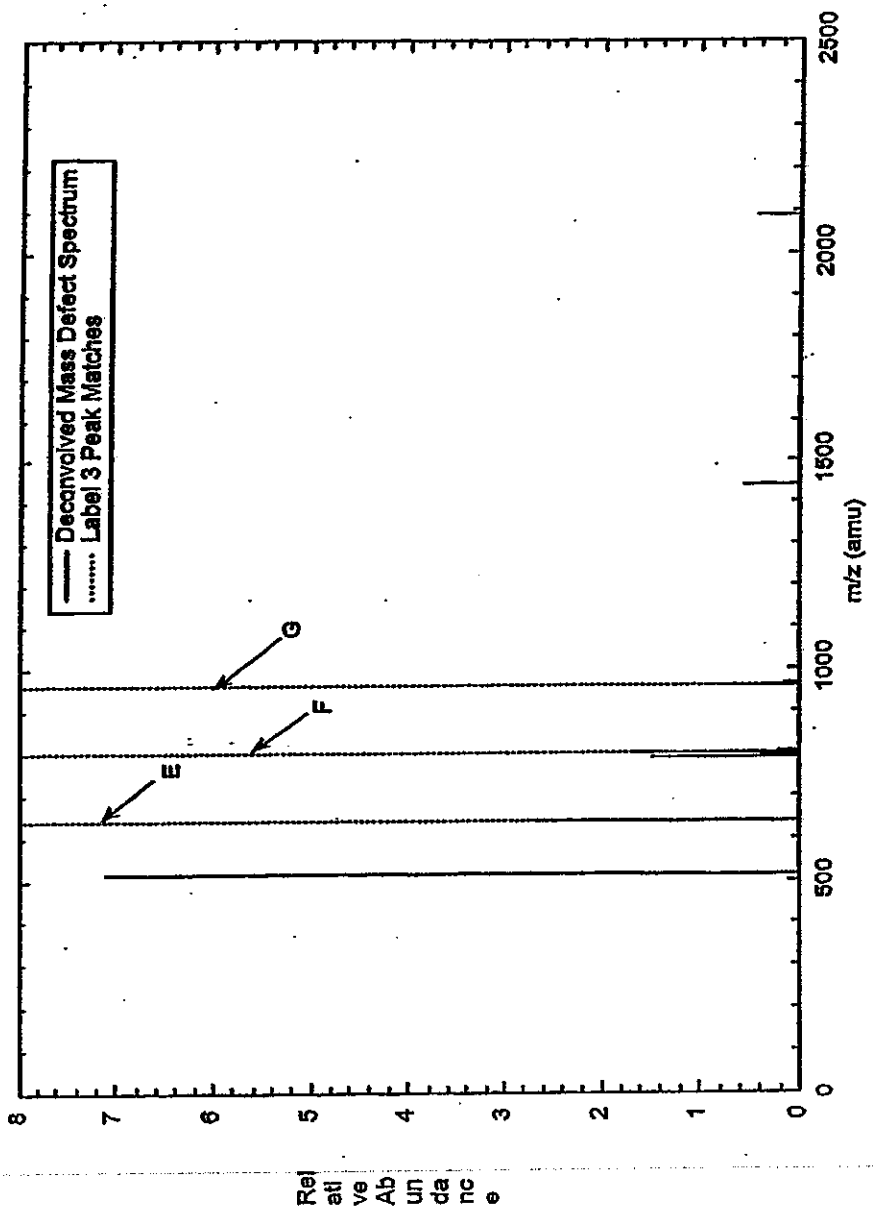

Deconvolved Mass Defect Spectrum
Label 3 Peak Matches
G
F
E
0
500
1000
1500
2000
2500
m/z (amu)
0
1
2
3
4
5
6
7
8
Relative Abundance

FIG. 23

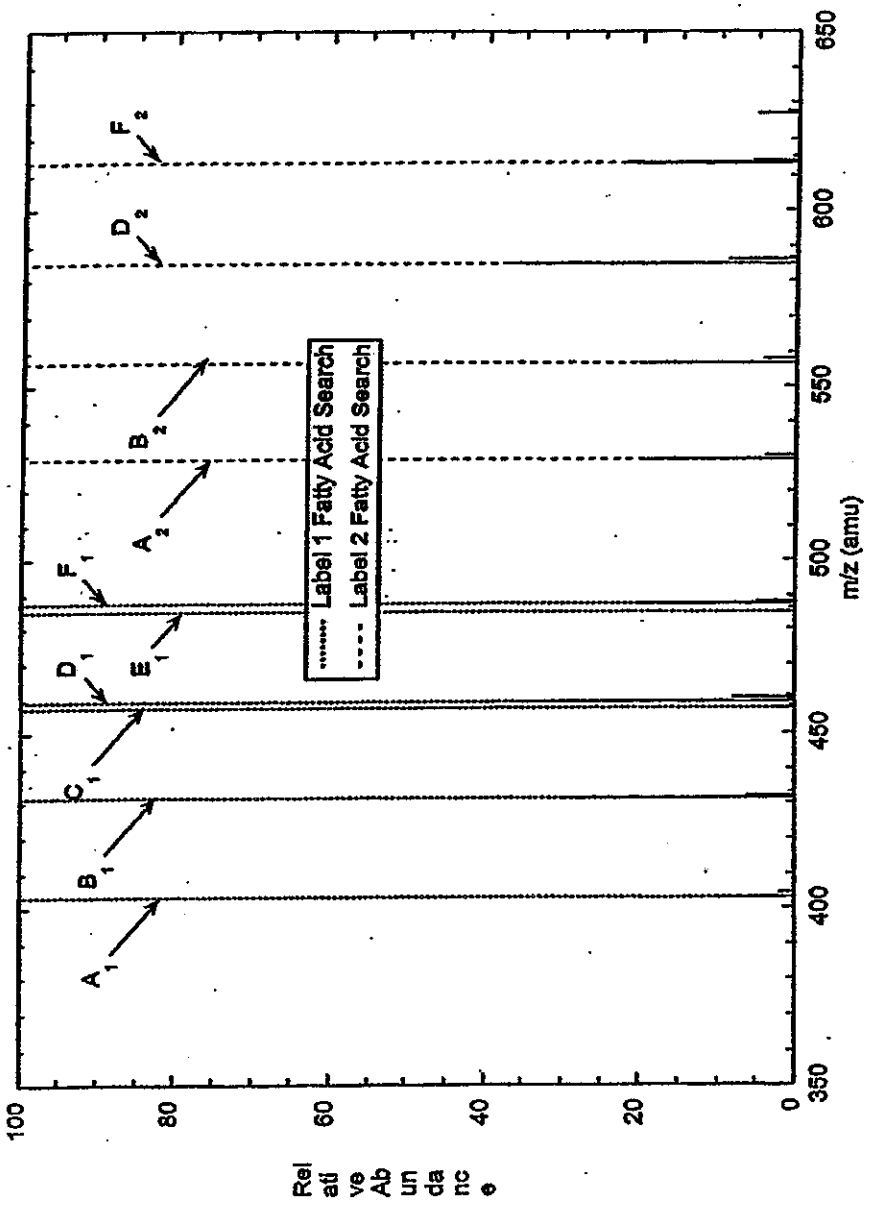
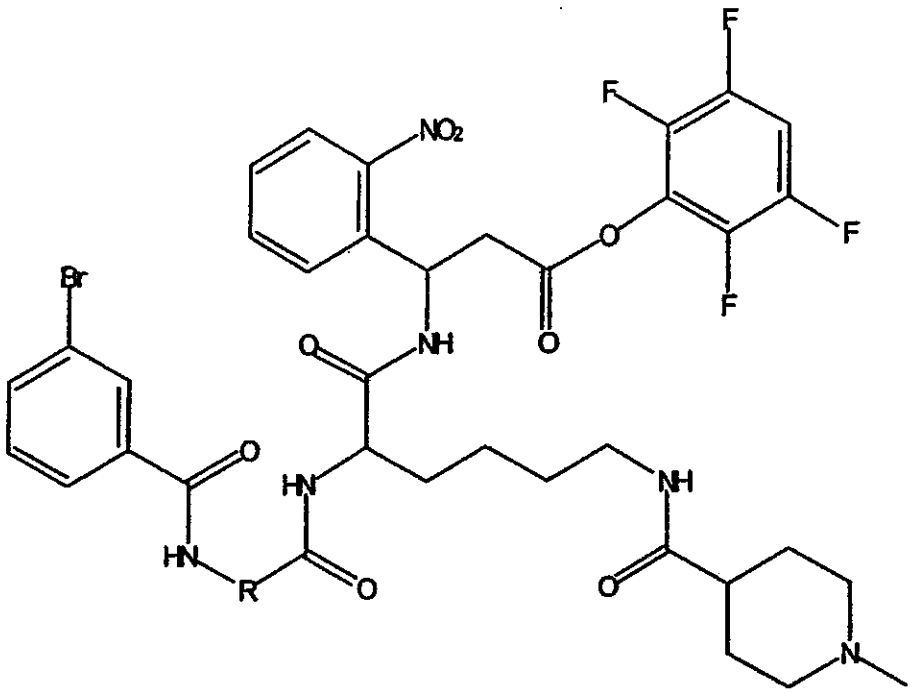

Label 1 Fatty Acid Search
Label 2 Fatty Acid Search
A1
B1
C1
D1
E1
F1
A2
B2
D2
F2
m/z (amu)
Relative Abundance

FIG. 2.4

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The general structure of the photocleavable mass defect tag where Br is the mass defect element that is linked through the amino acid (R) to the remainder of the tag.

FIG.25

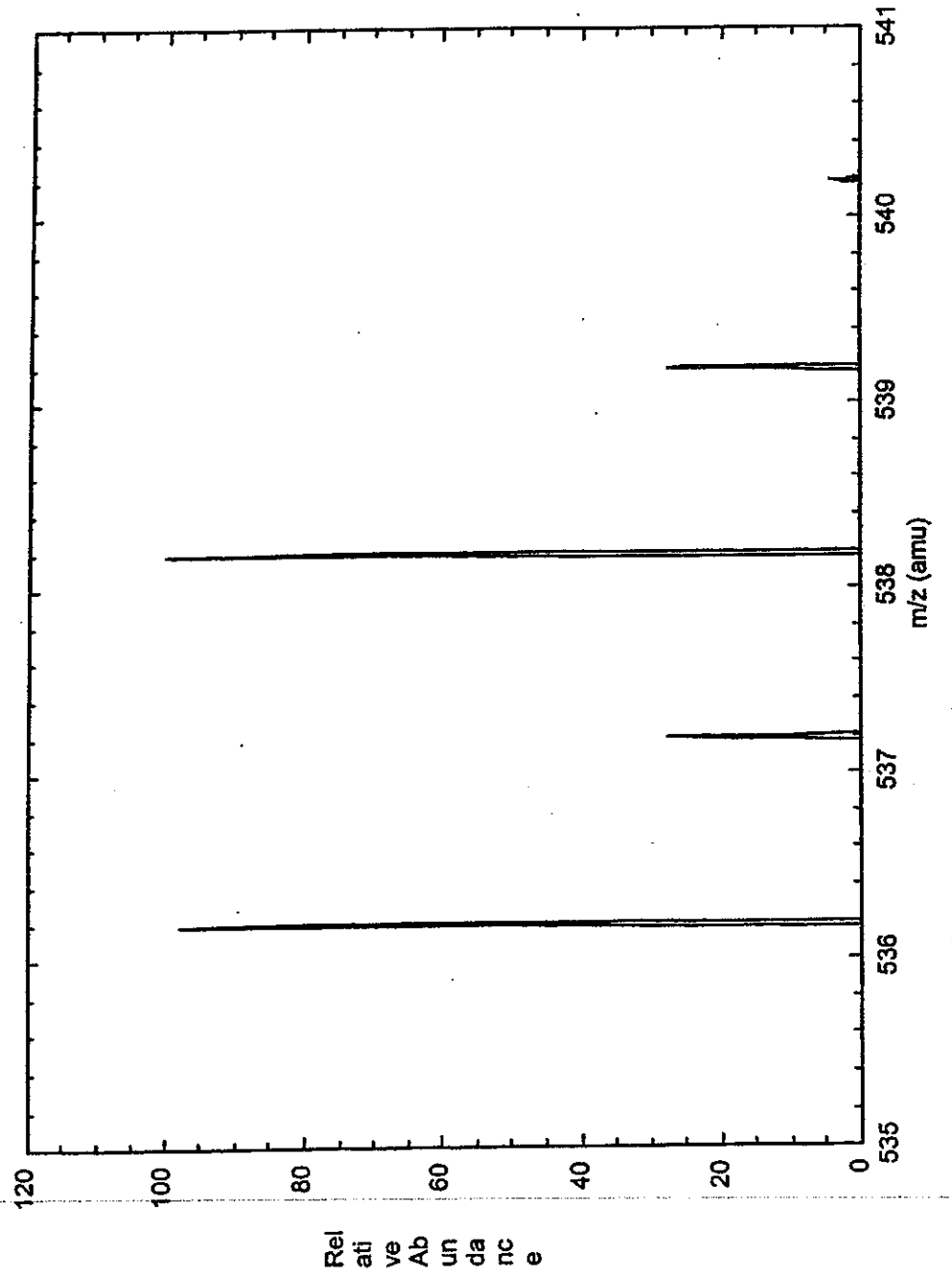

Relative Abundance
120
100
80
60
40
20
0
535
536
537
538
539
540
541
m/z (amu)

FIG 26

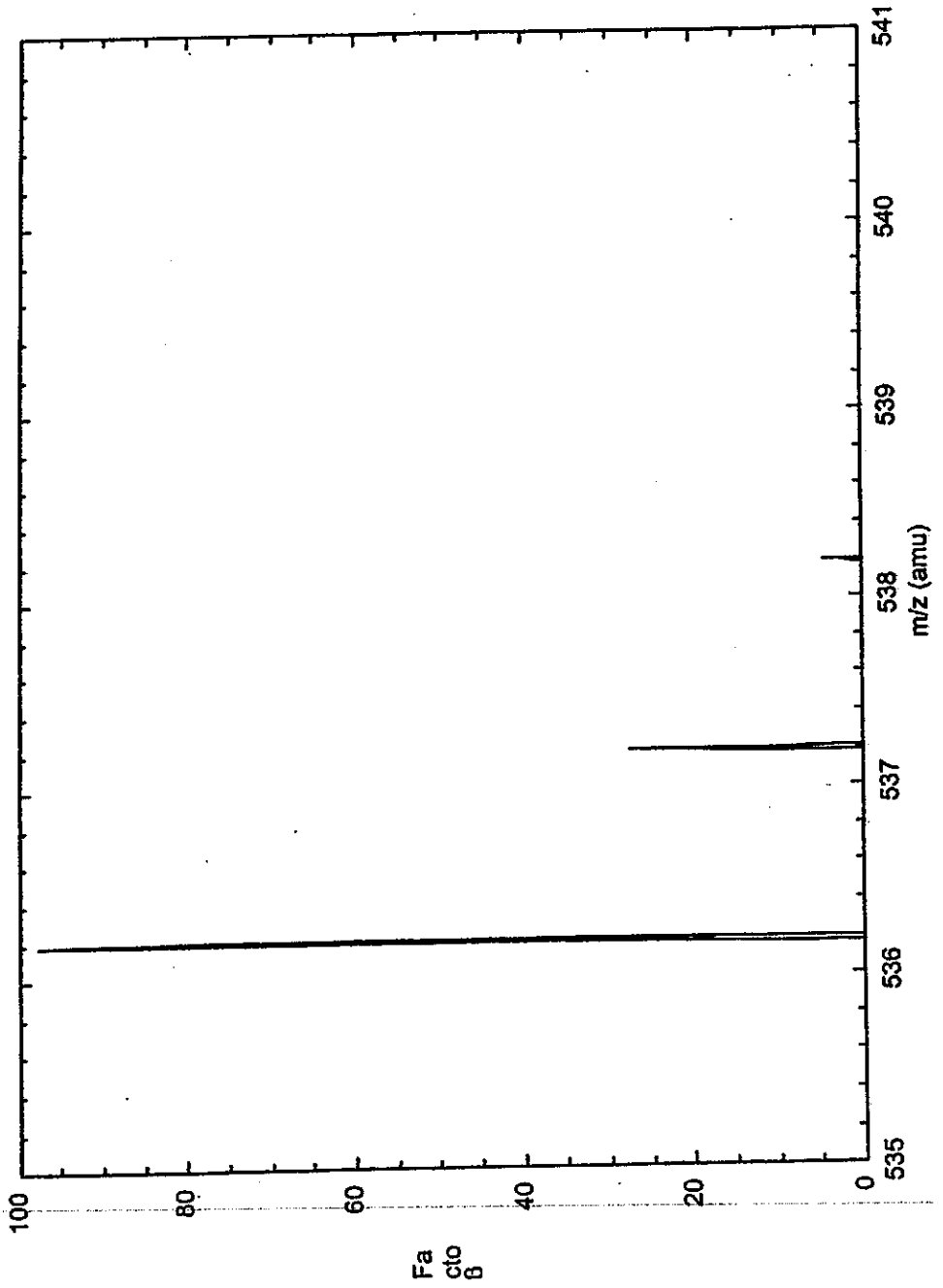


FIG 27

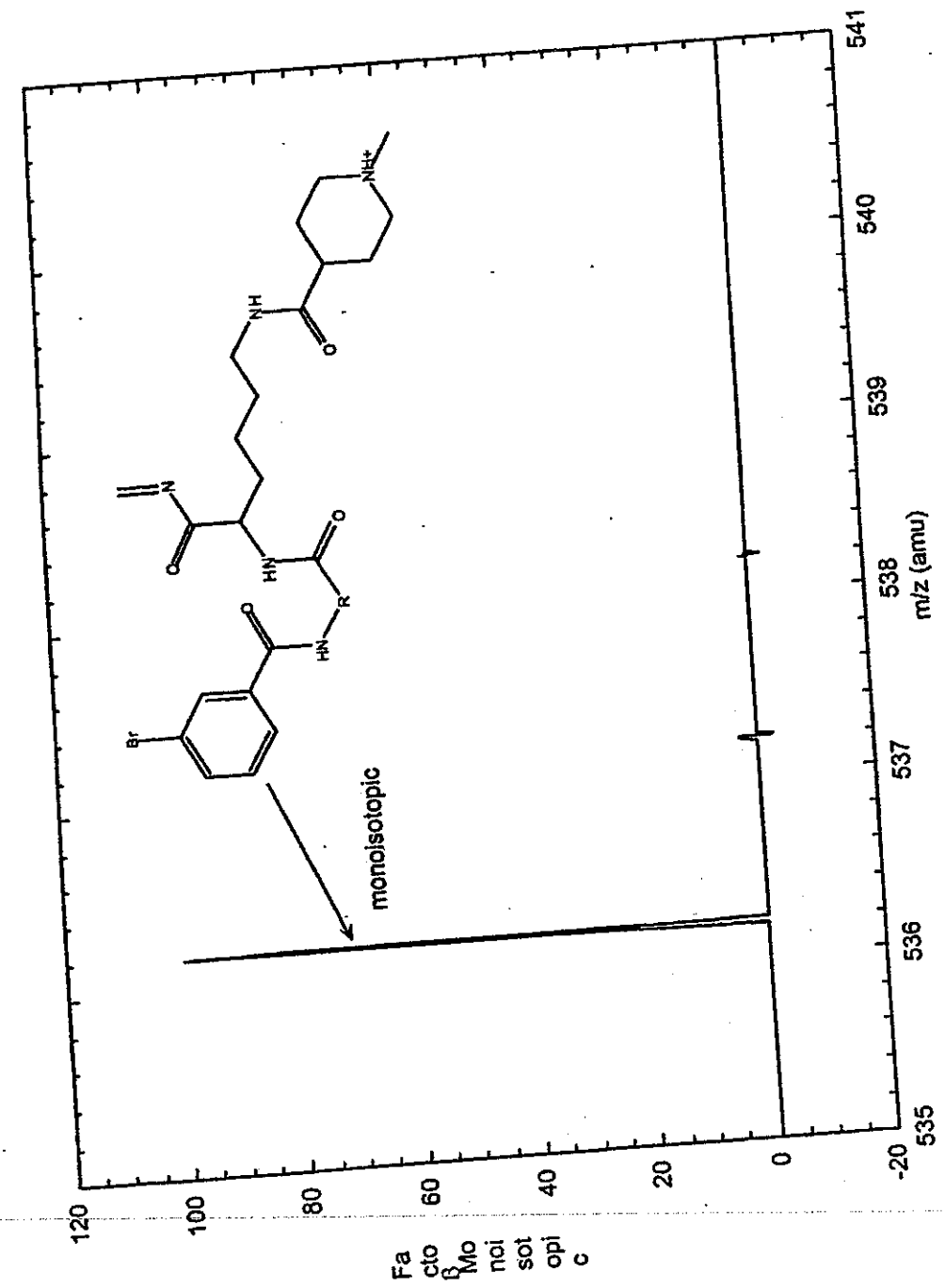

monoisotopic
Br
NH+
NH
HN
R
120
100
80
60
40
20
0
-20
535
536
537
538
539
540
541
m/z (amu)
Fa
cto
β
Mo
noi
sot
opi
c

FIG 28

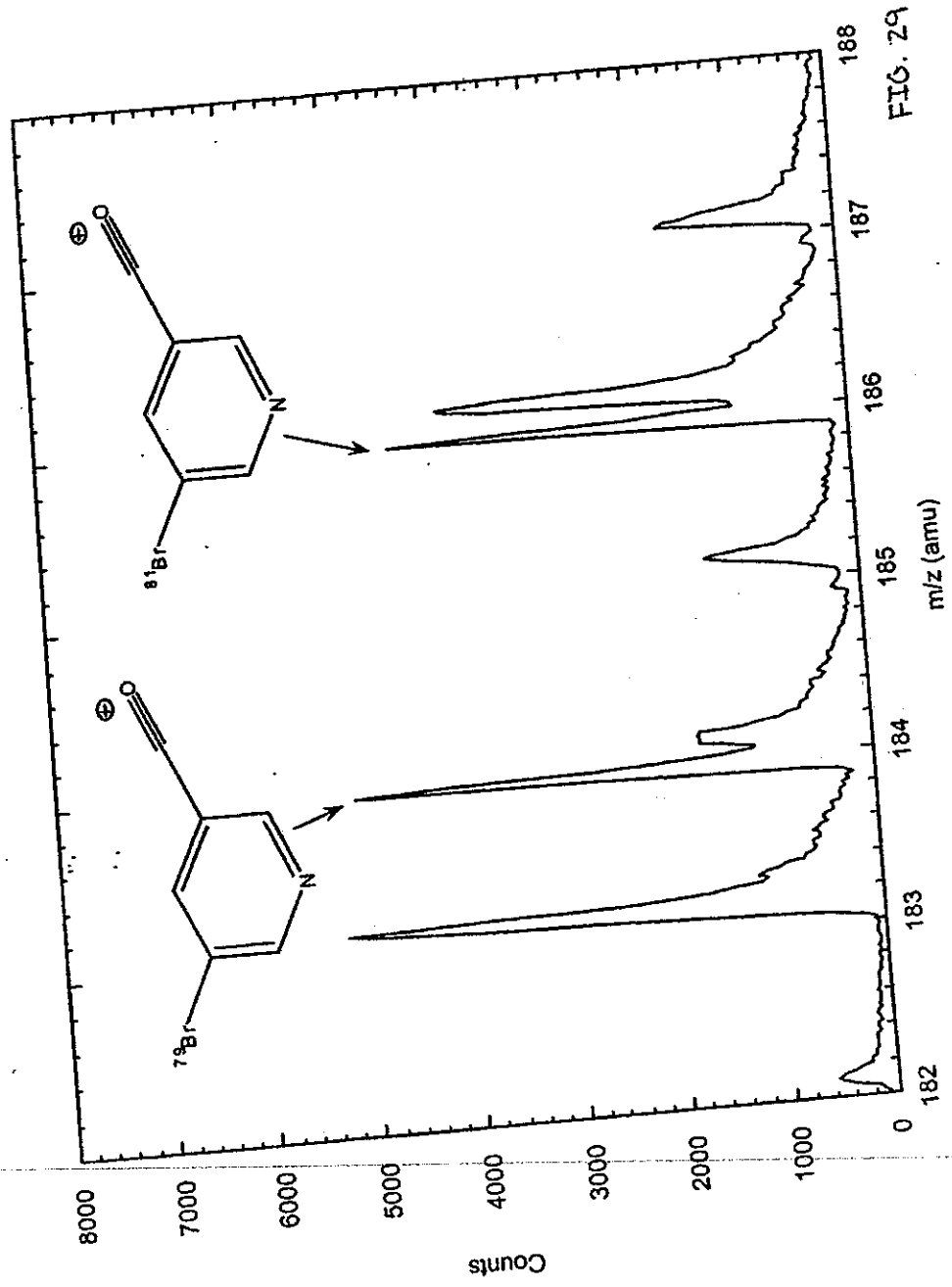

Counts
8000
7000
6000
5000
4000
3000
2000
1000
0
182
183
184
185
186
187
188
m/z (amu)
^{79}Br
^{81}Br

FIG. 29

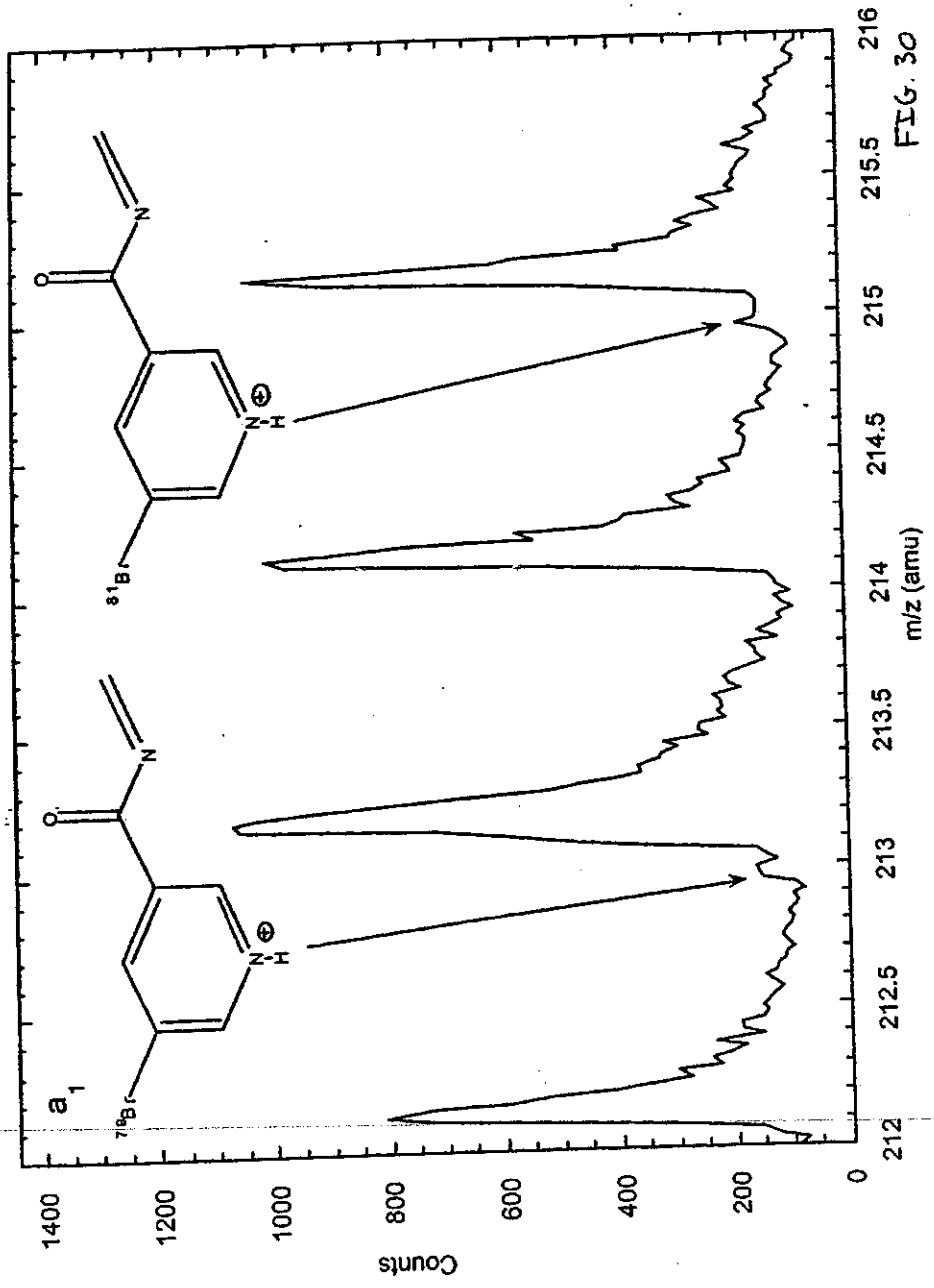

a1
79Br
81Br
Z-H
Counts
m/z (amu)
0
200
400
600
800
1000
1200
1400
212
212.5
213
213.5
214
214.5
215
215.5
216

FIG. 30

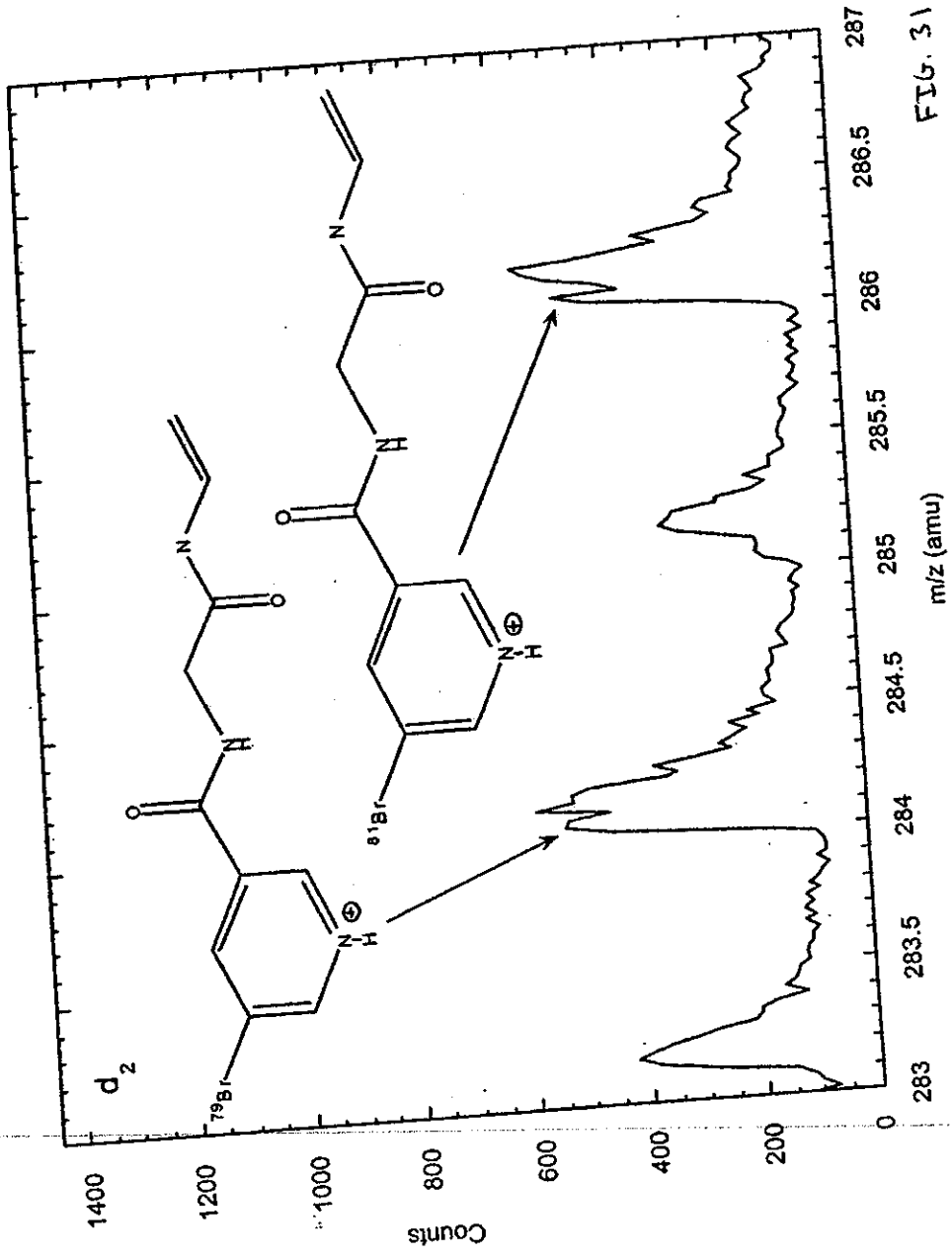
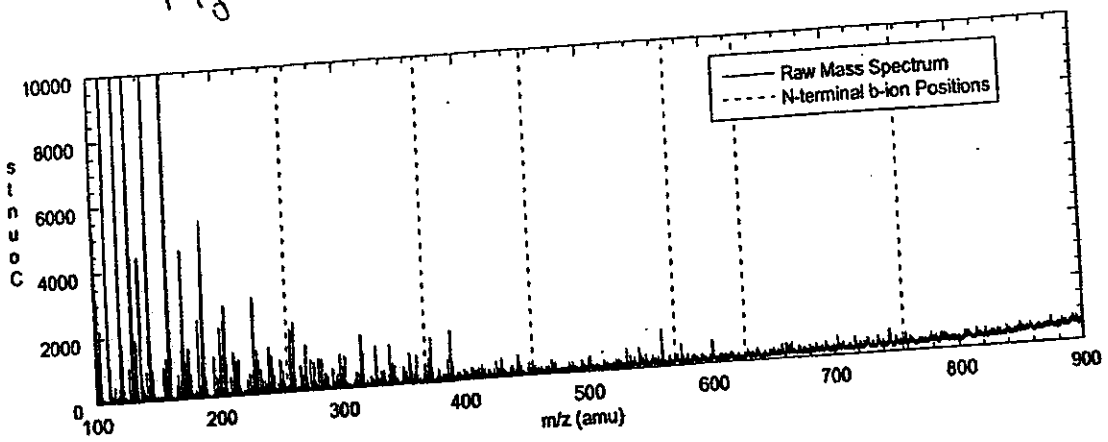
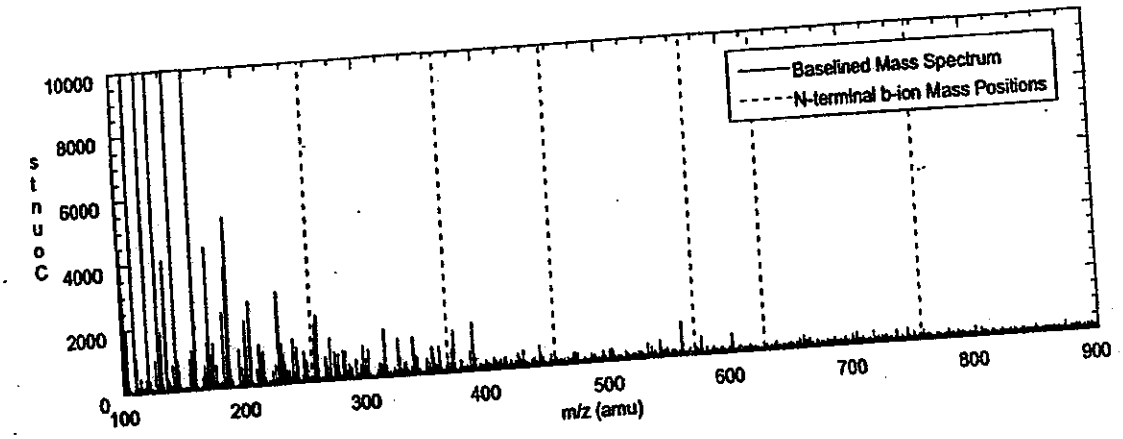
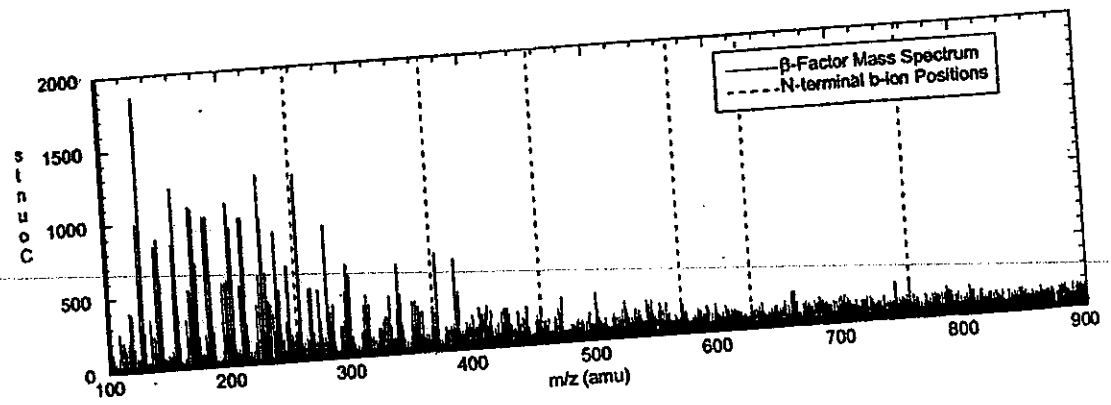

d2
79Br
81Br
N-H
N-H
Counts
0
200
400
600
800
1000
1200
1400
283
283.5
284
284.5
285
285.5
286
286.5
287
m/z (amu)

FIG. 31

Fig. 32


Raw Mass Spectrum
N-terminal b-ion Positions
Count
10000
8000
6000
4000
2000
0
100
200
300
400
500
600
700
800
900
m/z (amu)


Baselined Mass Spectrum
N-terminal b-ion Mass Positions
Count
10000
8000
6000
4000
2000
0
100
200
300
400
500
600
700
800
900
m/z (amu)


β-Factor Mass Spectrum
N-terminal b-ion Positions
Count
2000
1500
1000
500
0
100
200
300
400
500
600
700
800
900
m/z (amu)

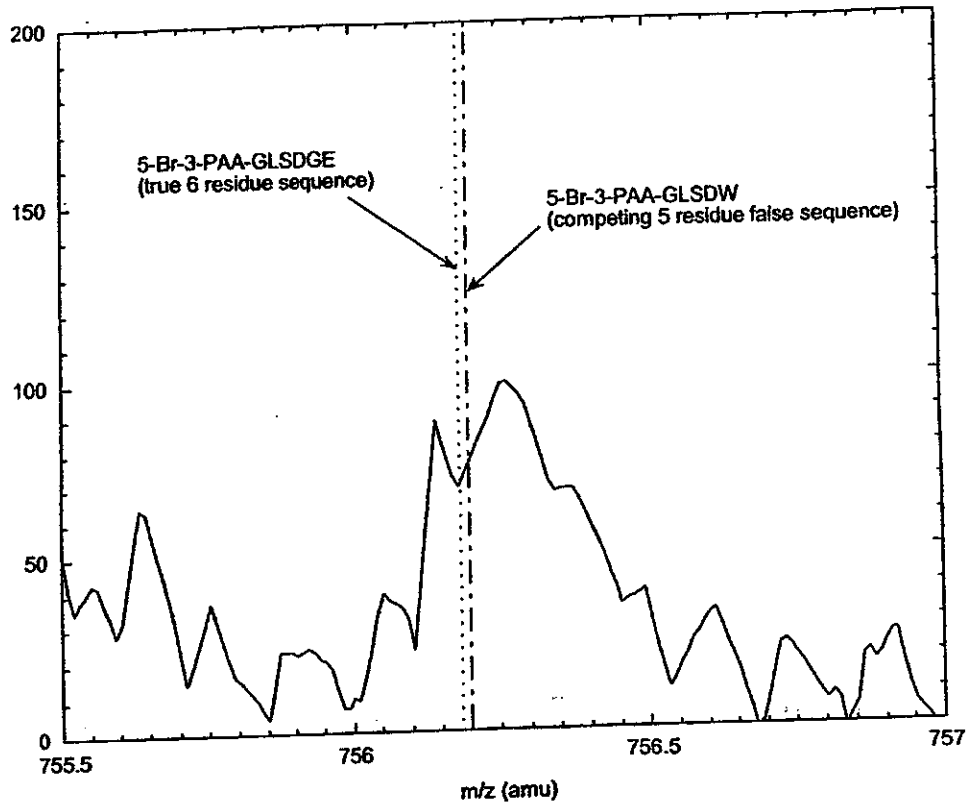

200
150
100
50
0
5-Br-3-PAA-GLSDGE
(true 6 residue sequence)
5-Br-3-PAA-GLSDW
(competing 5 residue false sequence)
755.5
756
756.5
757
m/z (amu)

FIG. 33

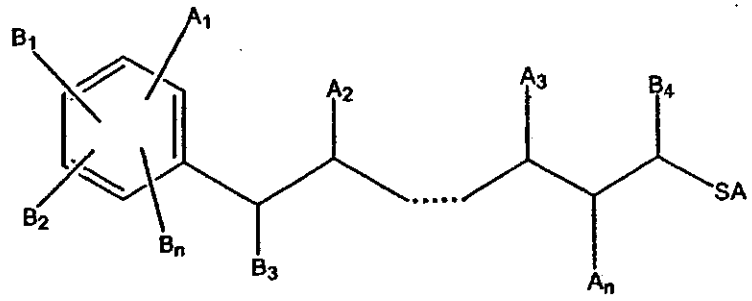


FIG. 34

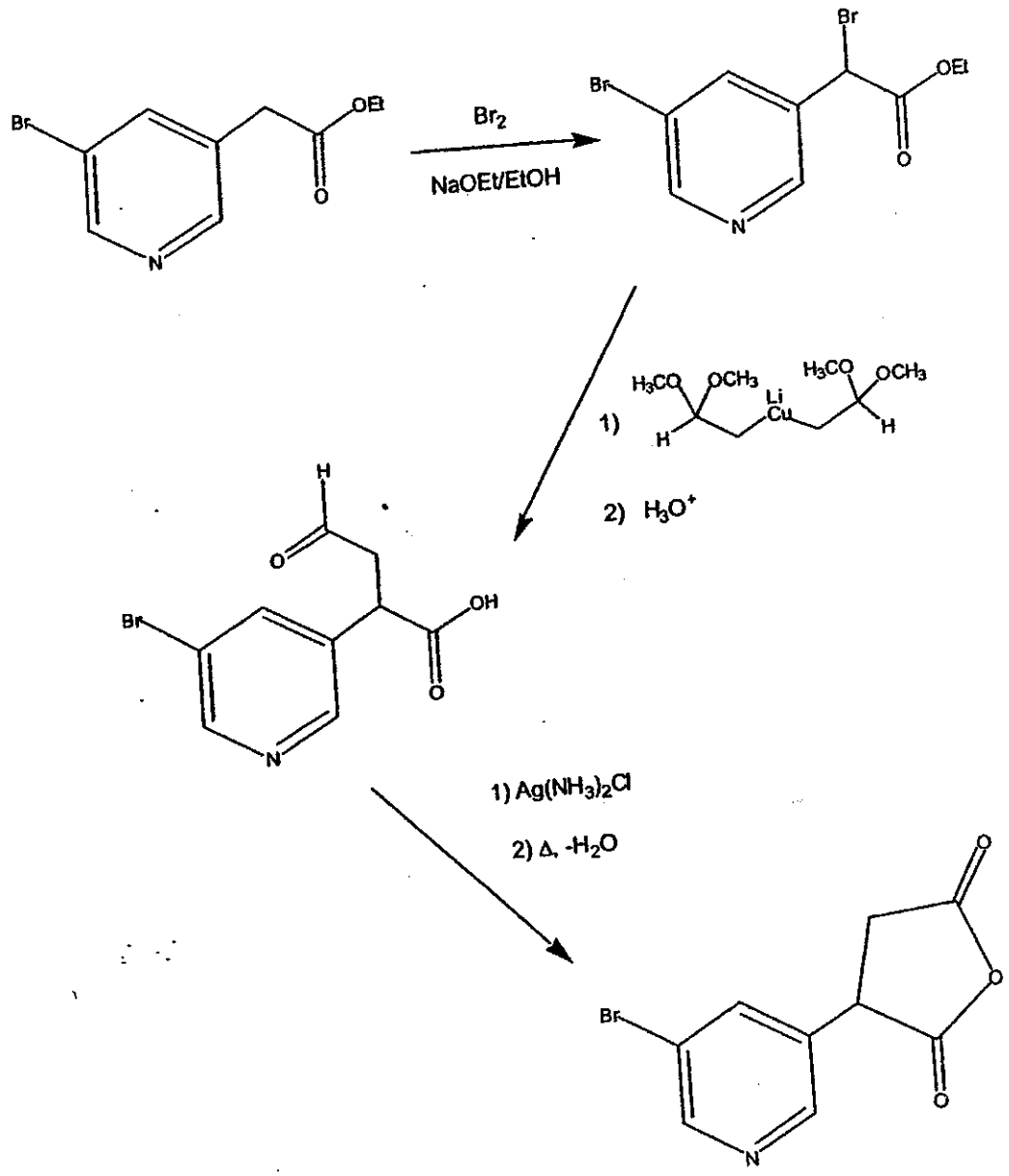
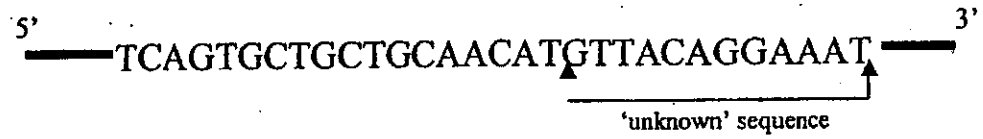

Br
OEt
N
O
Br_2
NaOEt/EtOH
Br
OEt
O
N
1)
H_3CO
OCH_3
Li
Cu
H
2) H_3O^+
H
O
OH
Br
N
1) $Ag(NH_3)_2Cl$
2) Δ, $-H_2O$
O
O
O
Br
N

FIG. 35



M13 primer



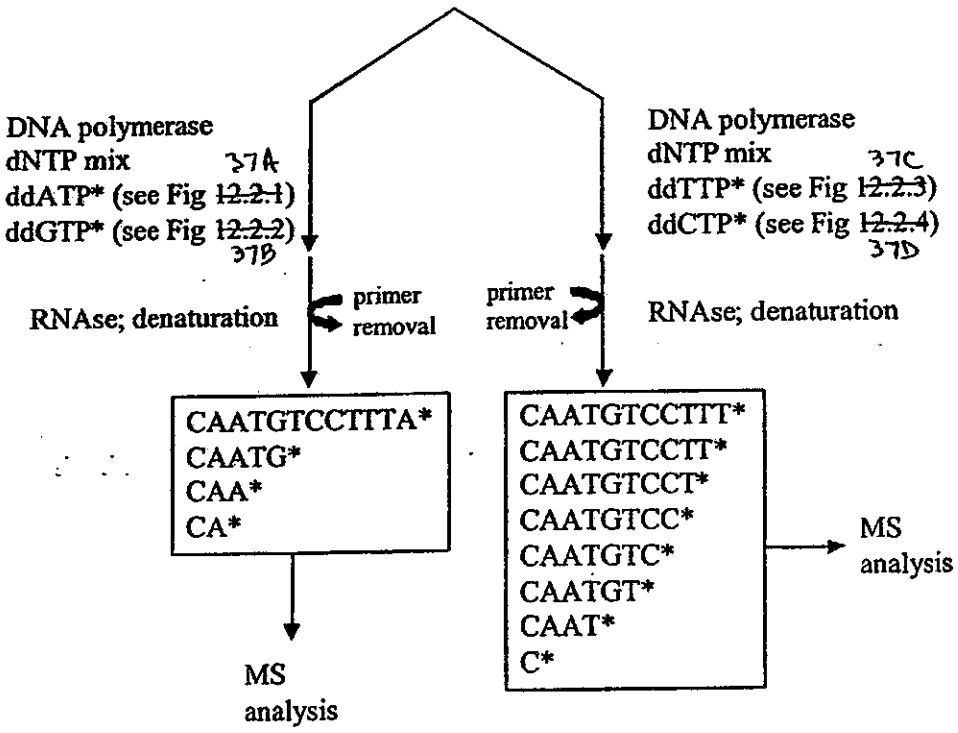
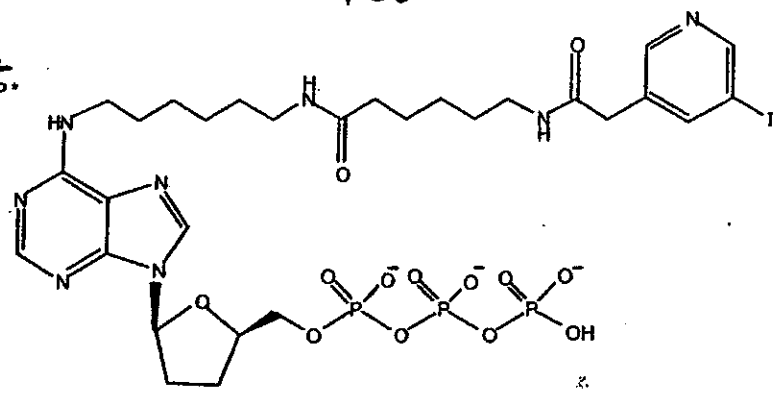


FIG. 36

FIG. 37

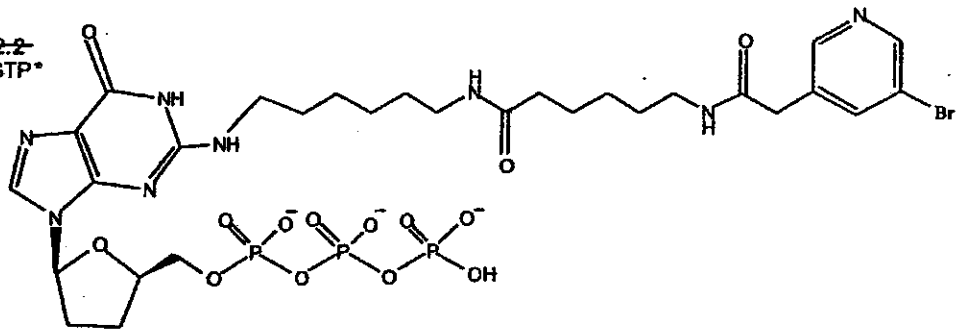
~~12.3.1~~
ddATP*

A



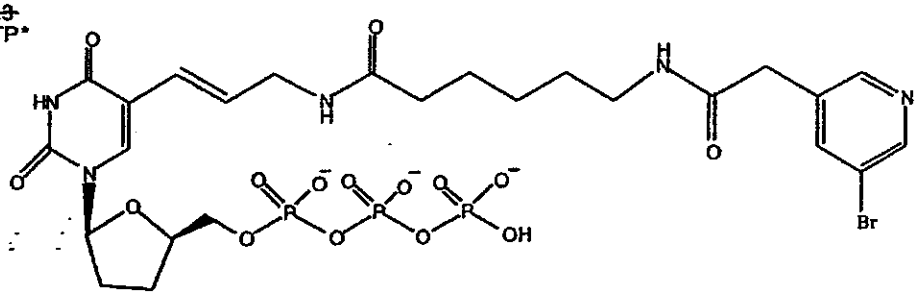
~~12.2.2~~
ddGTP*

B



~~12.2.3~~
ddTTP*

C



~~12.2.4~~
ddCTP*

D

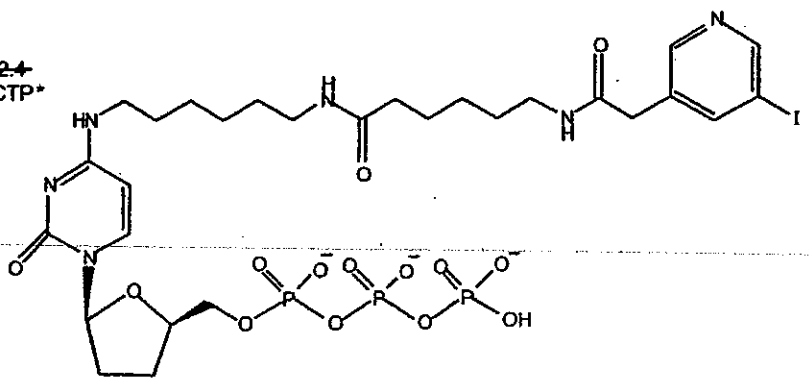


FIG. 38

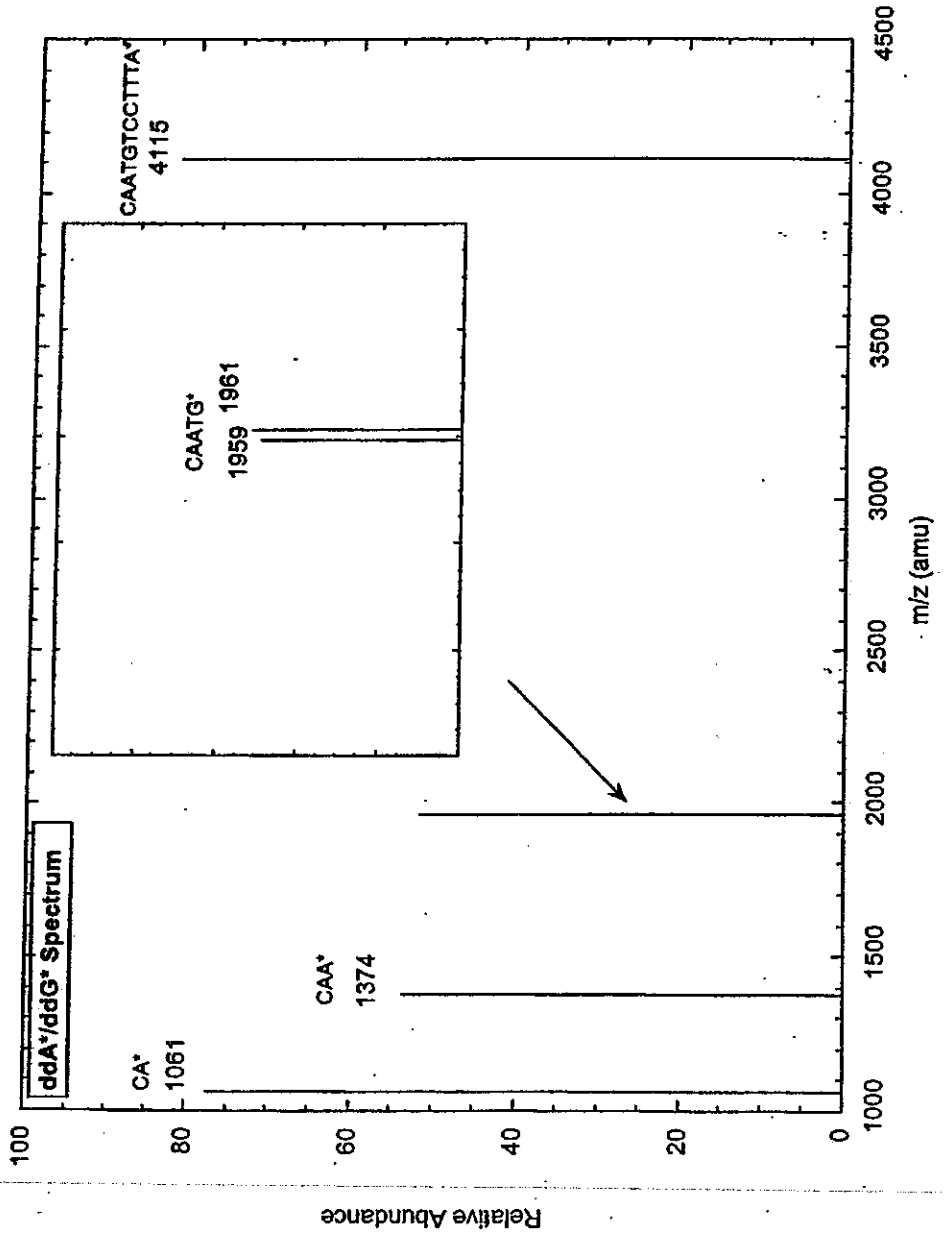
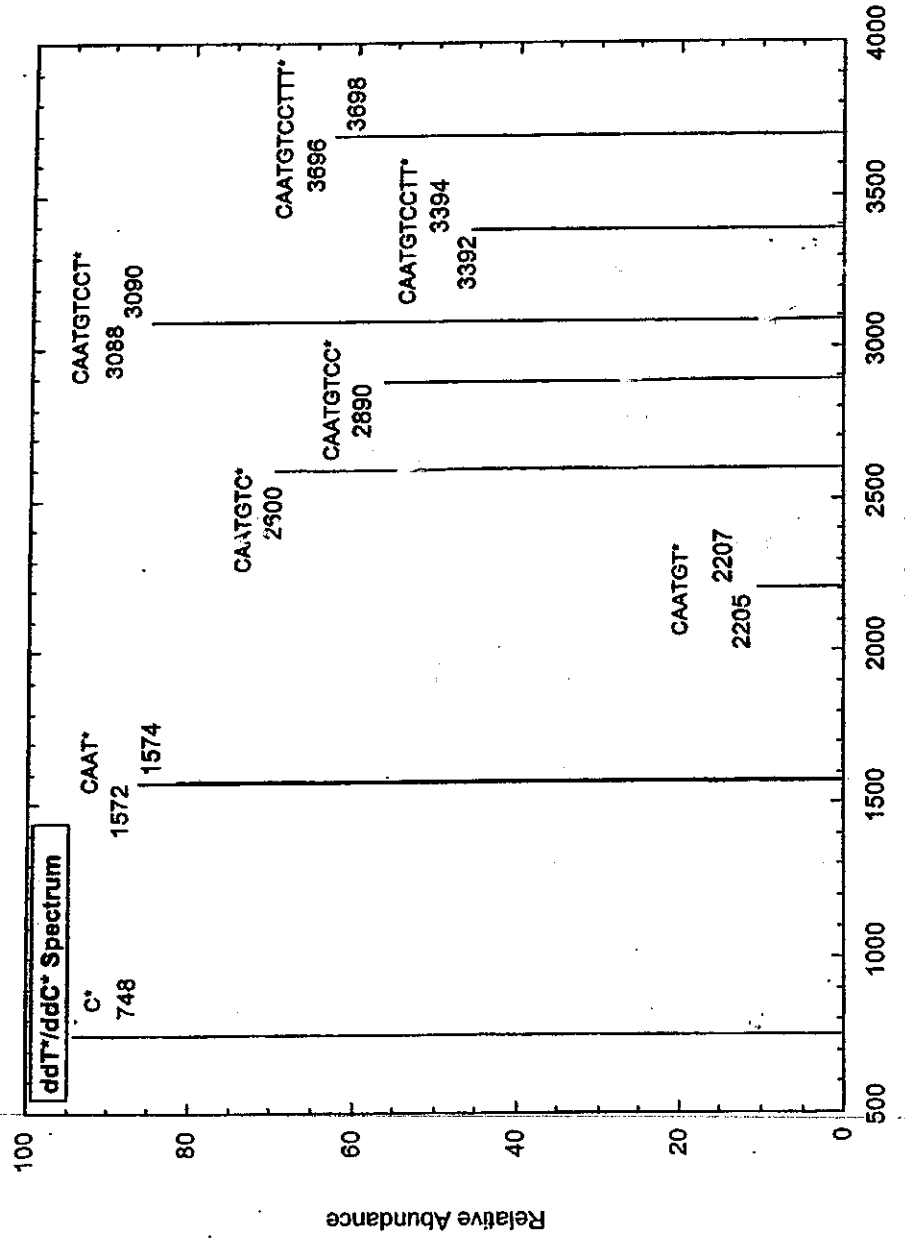

ddA*/ddG* Spectrum
CA*
1061
CAA*
1374
CAATG*
1959
1961
CAATGTCCTTTA*
4115
Relative Abundance
m/z (amu)
0
20
40
60
80
100
1000
1500
2000
2500
3000
3500
4000
4500

FIG. 39


ddT*/ddC* Spectrum
C*
748
CAT*
1572
1574
CAATGTCCT*
3088
3090
CAATGTC*
2600
CAATGTCC*
2890
CAATGTCCTT*
3394
3392
CAATGTCCTTT*
3696
3698
CAATGT*
2205
2207
Relative Abundance
m/z (amu)
0
20
40
60
80
100
500
1000
1500
2000
2500
3000
3500
4000