FIGURE 1

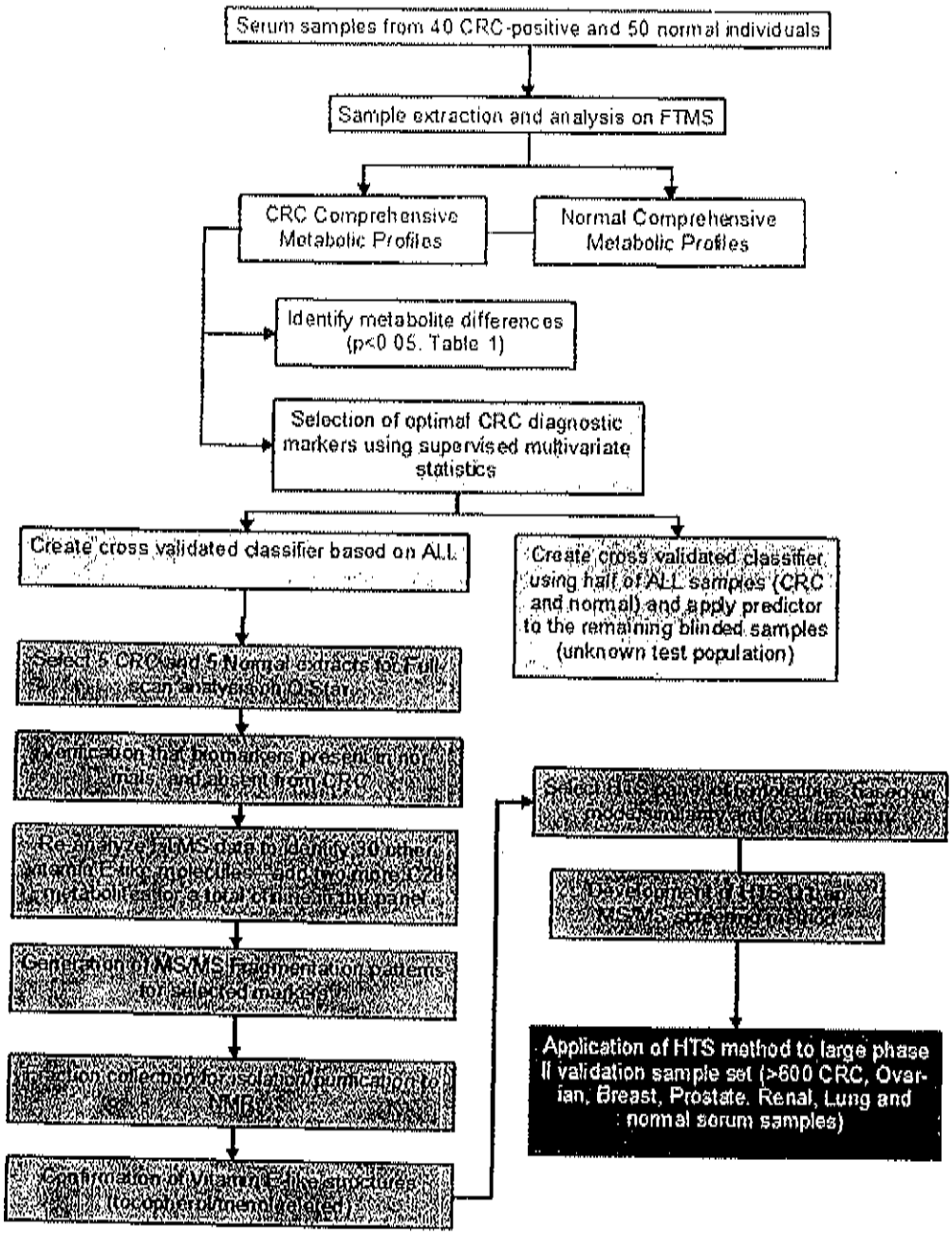
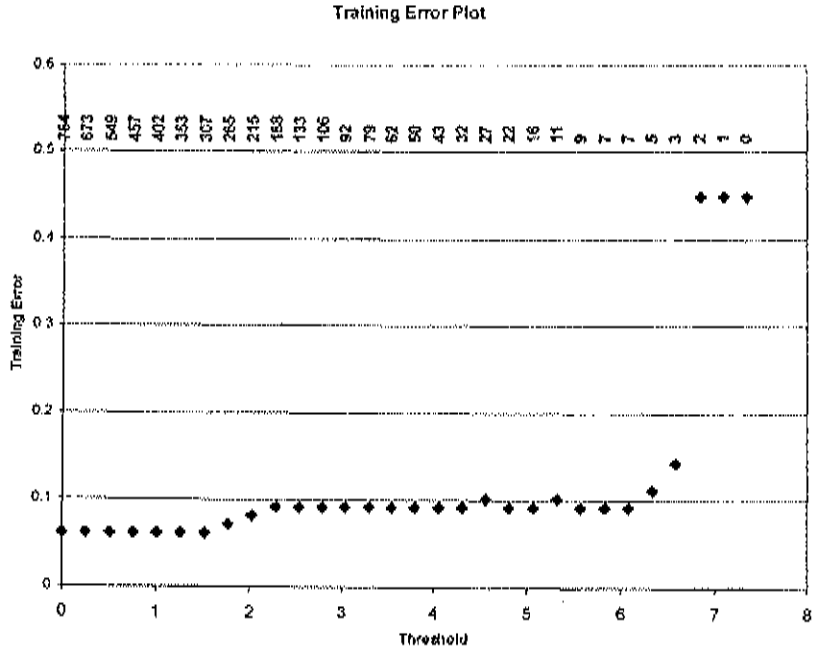

Serum samples from 40 CRC-positive and 50 normal individuals
Sample extraction and analysis on FTMS
CRC Comprehensive Metabolic Profiles
Normal Comprehensive Metabolic Profiles
Identify metabolite differences (p<0.05, Table 1)
Selection of optimal CRC diagnostic markers using supervised multivariate statistics
Create cross validated classifier based on ALL
Create cross validated classifier using half of ALL samples (CRC and normal) and apply predictor to the remaining blinded samples (unknown test population)
Select 5 CRC and 5 Normal extracts for Full Scan analysis on Q-Star
Verification that biomarkers present in normals and absent from CRC
Re-analyze FTMS data to identify 30 other potential biomarkers and two more CRC metabolites for a total of nine in panel
Generation of MS/MS Fragmentation patterns for selected markers
Fraction collection for validation by MALDI
Confirmation of Vitamin E-like structures (tocopherol/tocotrienol related)
Application of HTS method to large phase II validation sample set (>600 CRC, Ovarian, Breast, Prostate, Renal, Lung and normal serum samples)

FIGURE 2

A


Training Error Plot
Training Error
Threshold
764 673 549 457 402 353 307 265 215 168 133 106 92 79 62 50 43 32 27 22 16 11 9 7 7 5 3 2 1 0

B

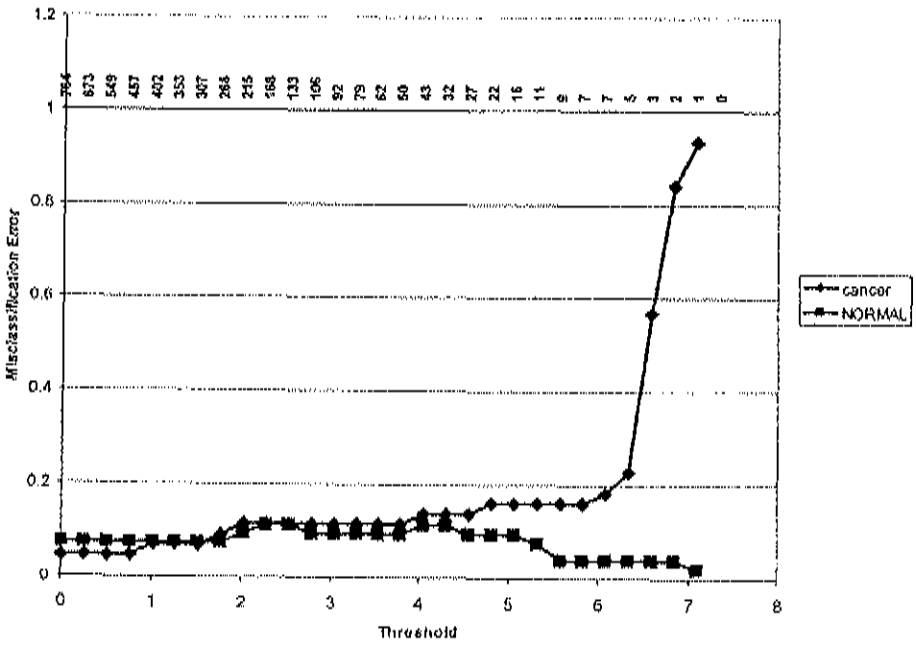

Individual CV Plots
Misclassification Error
Threshold
cancer
NORMAL
764 673 549 457 402 353 307 265 215 168 133 106 92 79 62 50 43 32 27 22 16 11 9 7 7 5 3 2 1 0

FIGURE 3

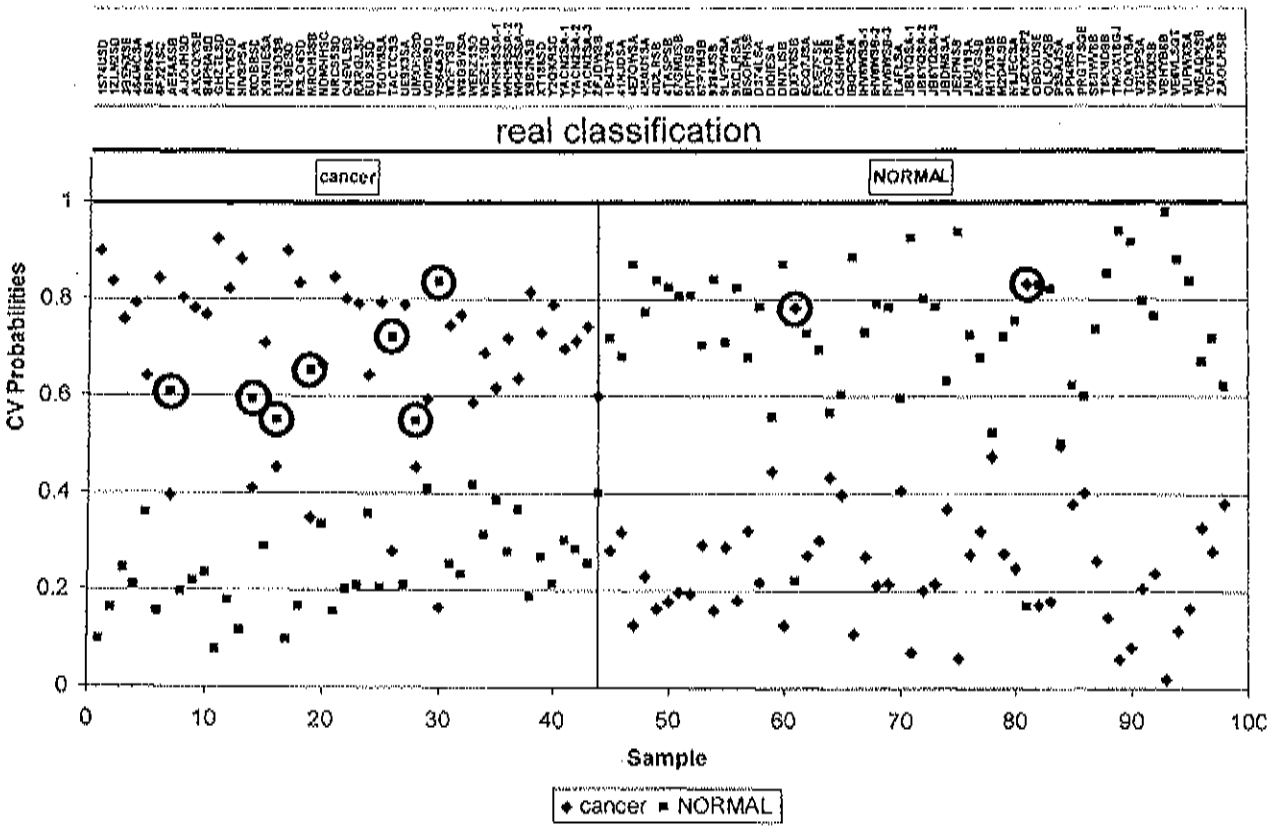

real classification
cancer
NORMAL
CV Probabilities
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
80
90
100
Sample
cancer
NORMAL

FIGURE 4

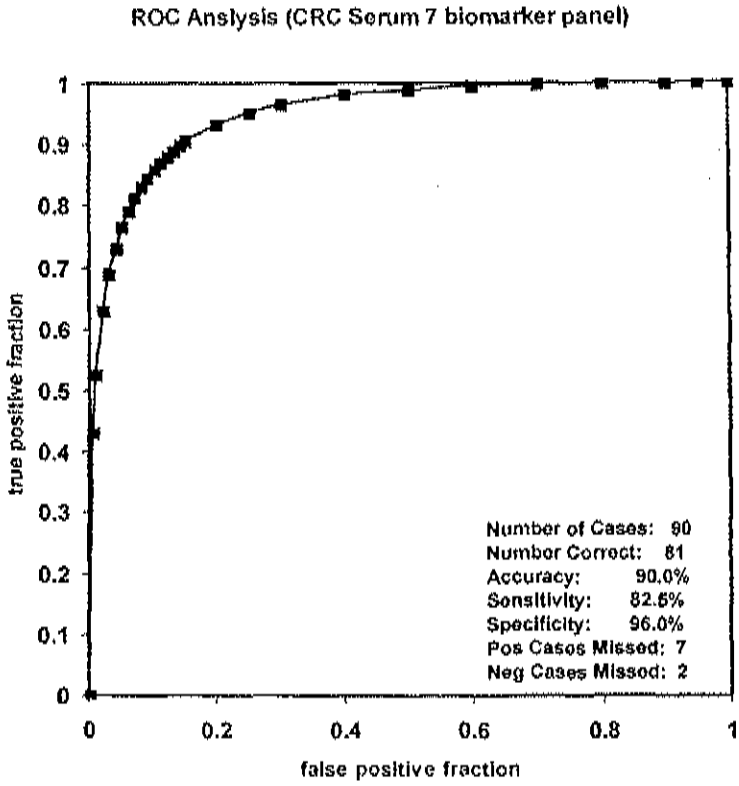

ROC Anslysis (CRC Serum 7 biomarker panel)
true positive fraction
false positive fraction
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
0.2
0.4
0.6
0.8
1
Number of Cases: 90
Number Correct: 81
Accuracy: 90.0%
Sensitivity: 82.5%
Specificity: 96.0%
Pos Cases Missed: 7
Neg Cases Missed: 2

FIGURE 5

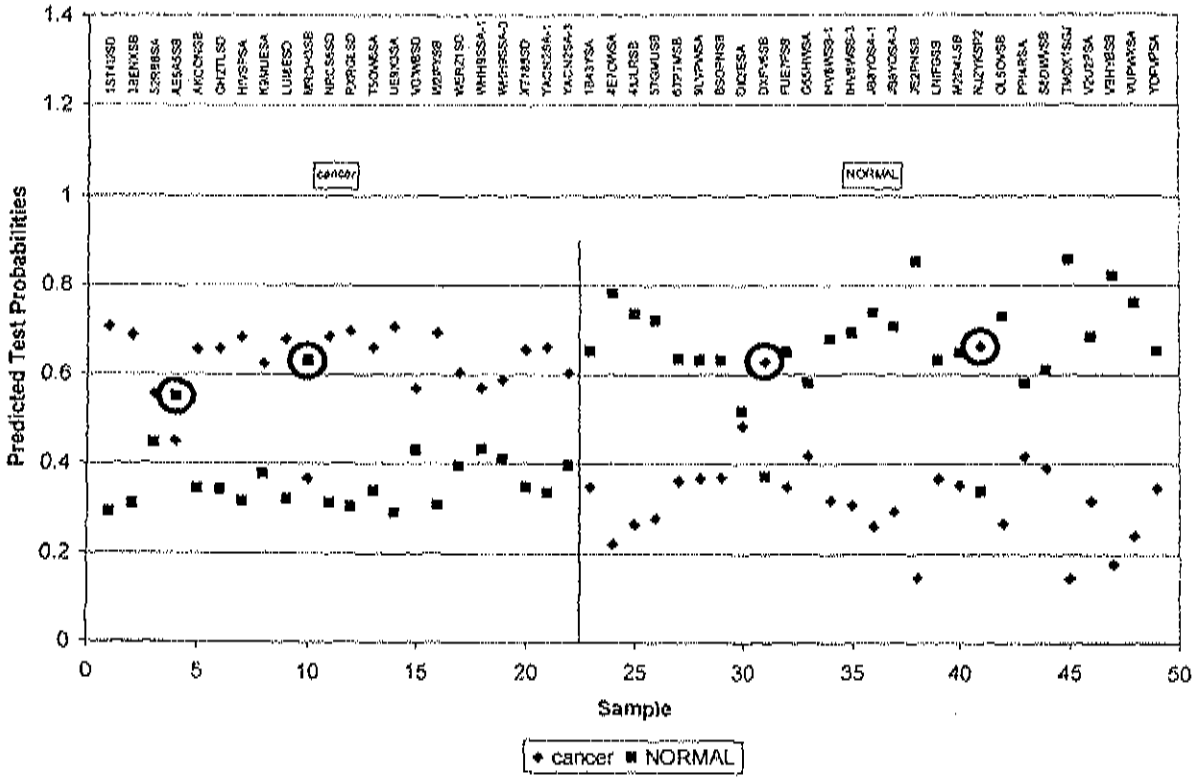

Predicted Test Probabilities
1.4
1.2
1
0.8
0.6
0.4
0.2
0
cancer
NORMAL
0
5
10
15
20
25
30
35
40
45
50
Sample
cancer
NORMAL

FIGURE 6

A

patientID	actual	prediction
1B43YSA	normal	normal
1S74USD		
3BENXSB		
4E7OWSA	normal	normal
4IULRSB	normal	normal
52R86SA		
57GMUSB	normal	normal
67P7MSB	cancer	normal
9LVPWSA	normal	normal
AE5A5SB		normal
AKCQNSB		
BSOPNSB	normal	normal
DIQIESA	normal	normal
DXFV6SB		
FUE7FSB	normal	normal
G55HWSA	normal	normal
GHZTLSD		
HIVSPSA		
IHV6WSB-1	normal	normal
IHV6WSB-3	normal	normal
JB8YQSA-1	normal	normal
JB8YQSA-3	normal	normal
JE2PNSB	normal	normal
K9MUESA		
LNIFGSB	normal	normal

patientID	actual	prediction
LUI8ESD		
M2D4LSB	normal	normal
MRQH3SB		normal
NJZYKSP2	normal	
NRCS5SD		
OL5OVSB	normal	normal
PPI4RSA	normal	normal
RXRGLSD		
S8DWVSB	normal	normal
T5OW8SA		
TMOX1SGJ	normal	normal
UE9X3SA		
V2U2PSA	cancer	normal
VBHYBSB	normal	normal
VDIWBSD		
VUPWXSA	normal	cancer
W2IFYSB		
WERZ1SD		
WHH9SSA-1		
WHH9SSA-3		
XT186SD		
YACN2SA-1		
YACN2SA-3		
YOFVPSA	normal	normal

B

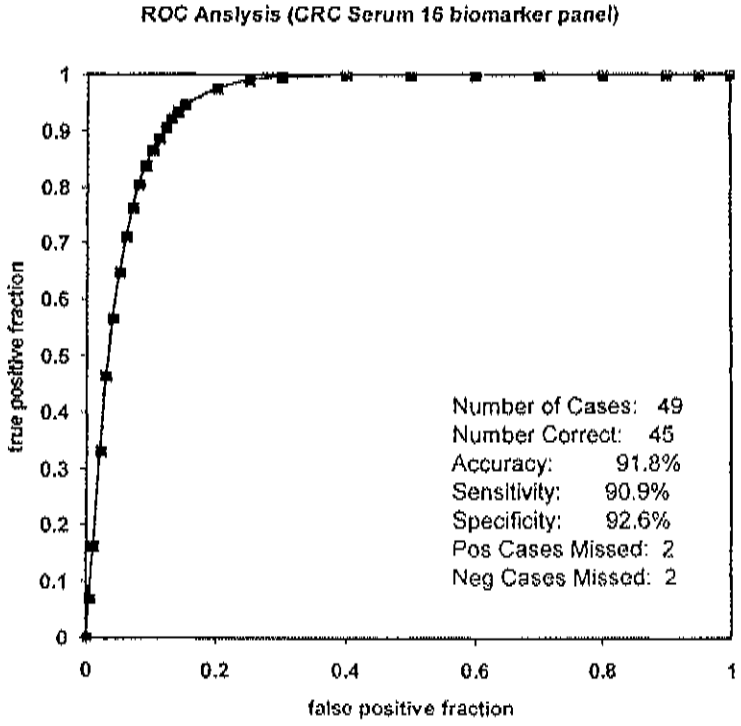


FIGURE 7

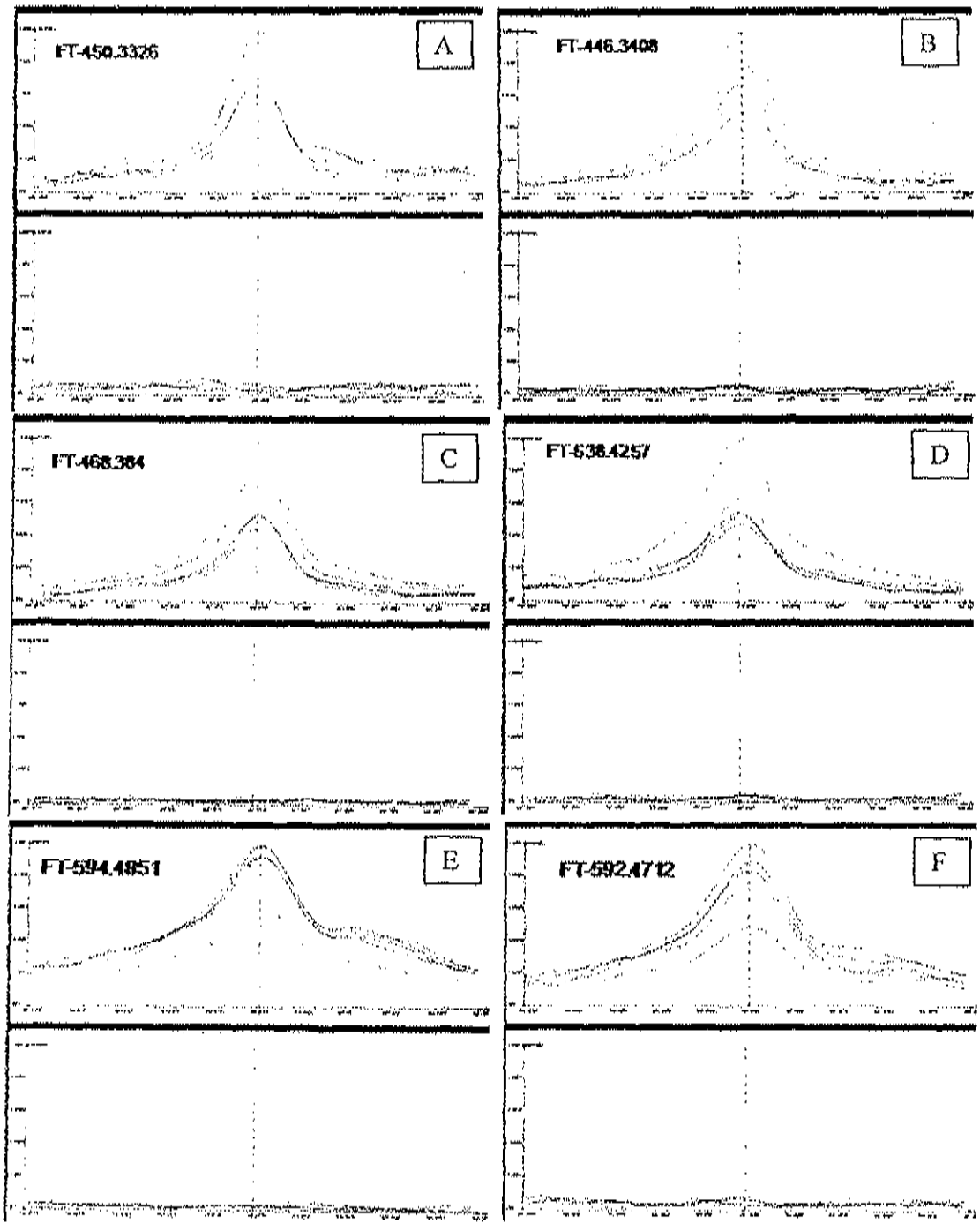

FT-459.3326
A
FT-446.3408
B
FT-468.384
C
FT-638.4257
D
FT-594.4851
E
FT-592.4712
F

FIGURE 8

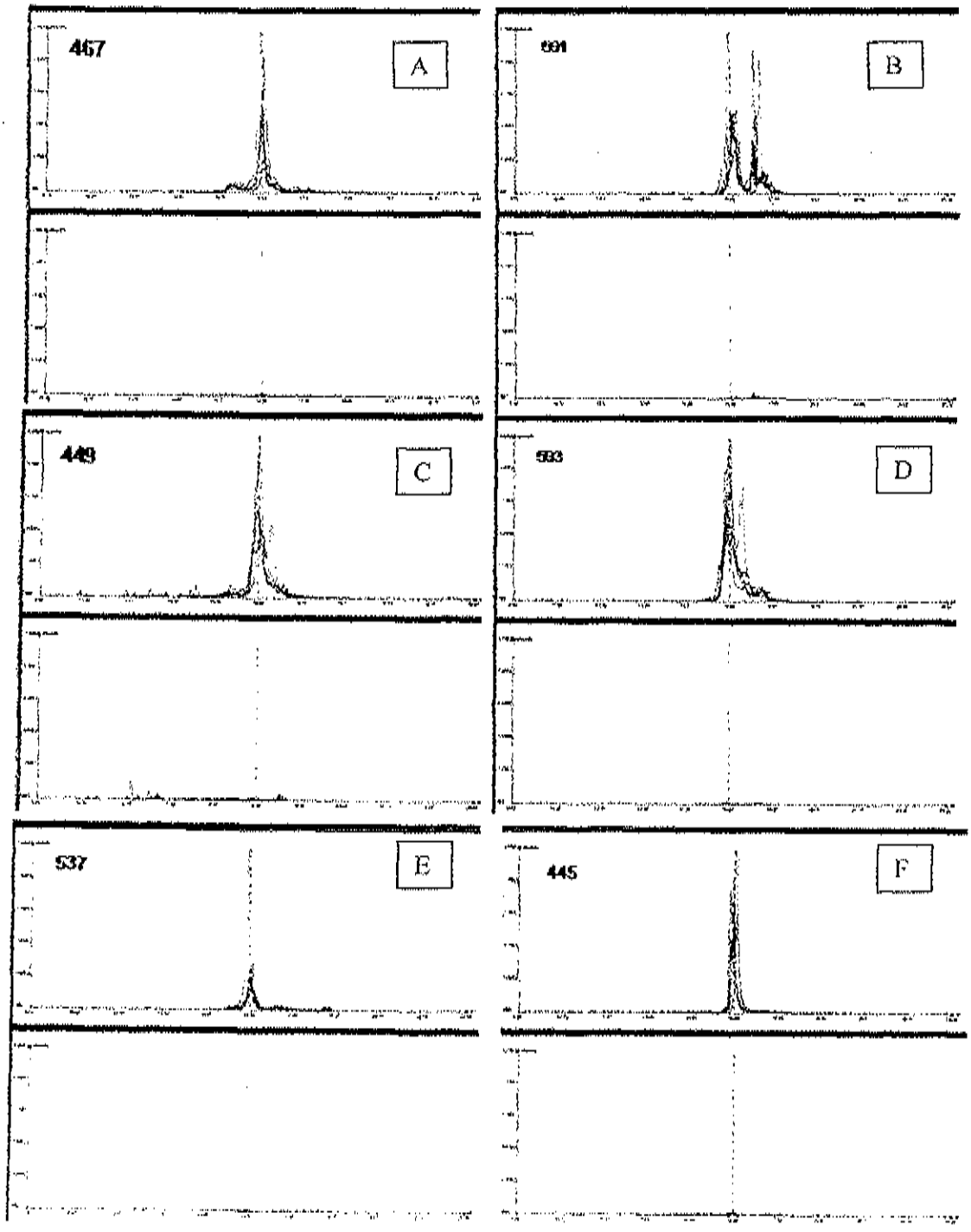


FIGURE 9

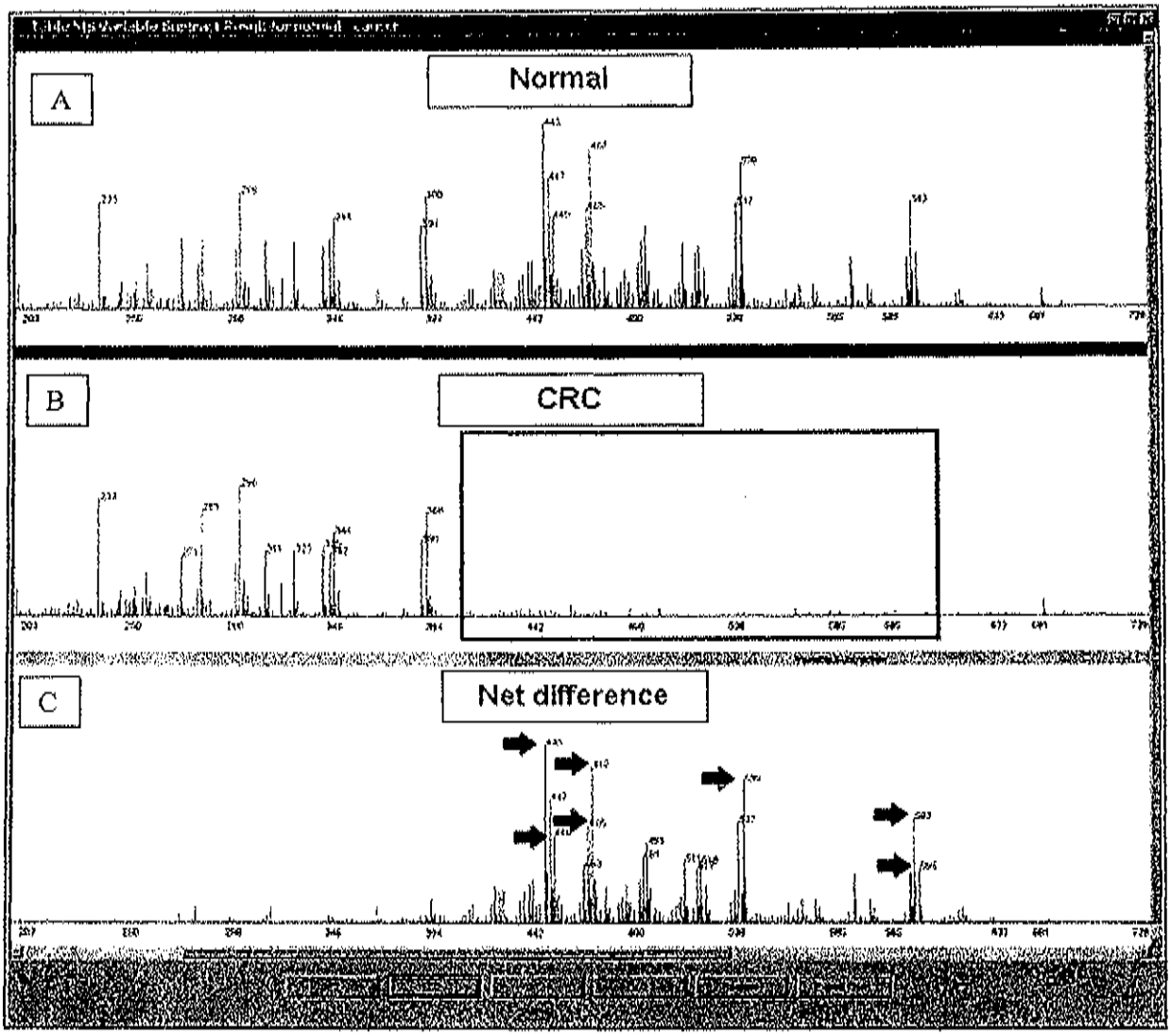
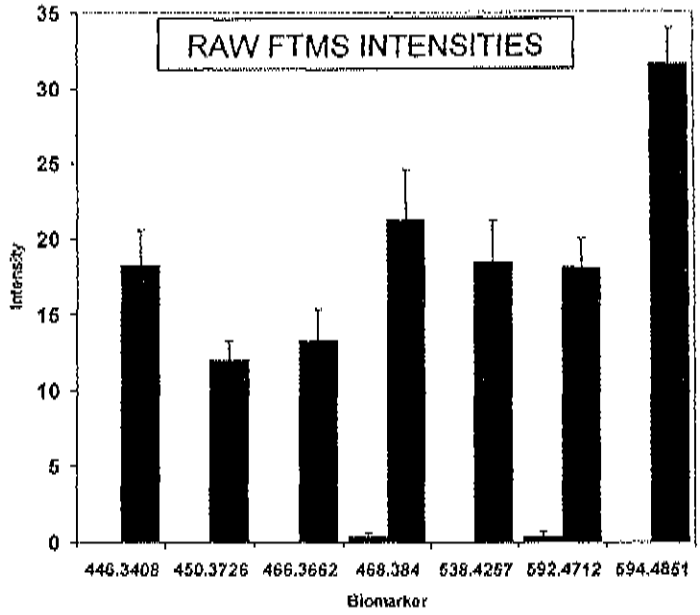


FIGURE 10

A


RAW FTMS INTENSITIES
35
30
25
20
15
10
5
0
Intensity
446.3408
450.3726
466.3662
468.384
538.4257
592.4712
594.4861
Biomarker

B

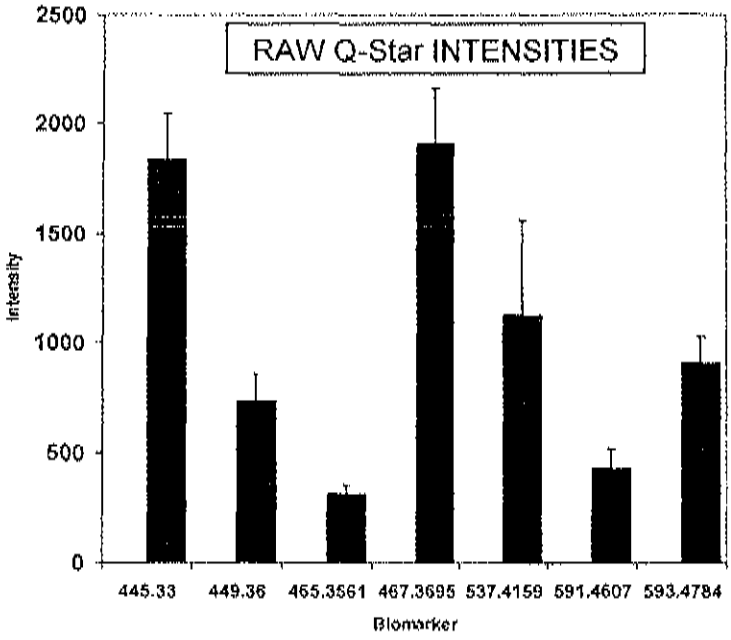

RAW Q-Star INTENSITIES
2500
2000
1500
1000
500
0
Intensity
445.33
449.36
465.3661
467.3695
537.4159
591.4607
593.4784
Biomarker

FIGURE 11

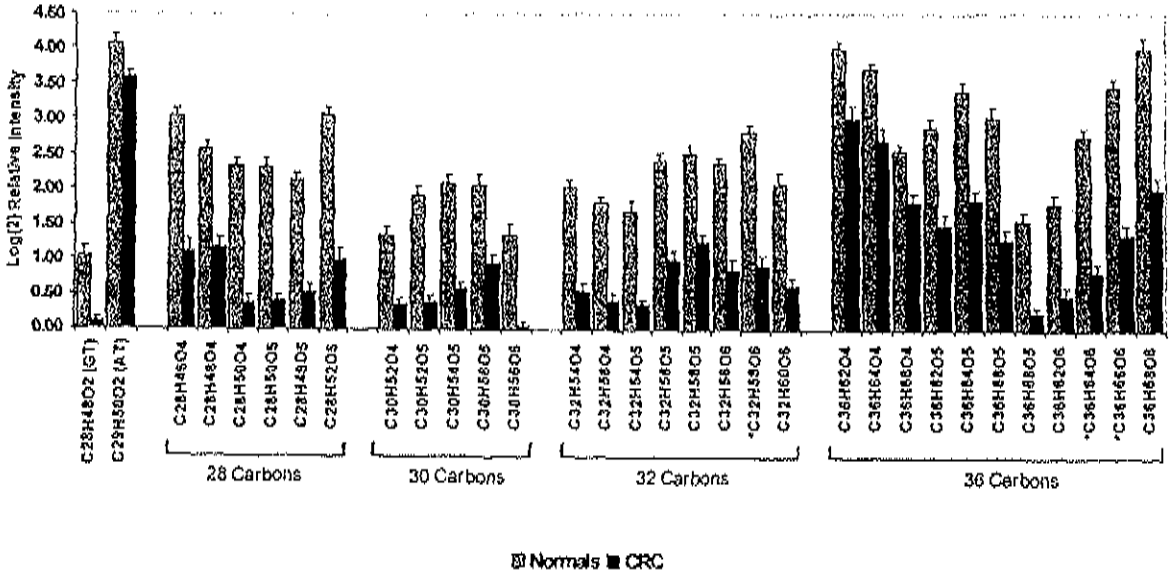
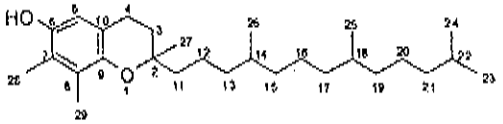
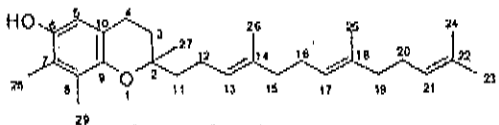

Log(2) Relative Intensity
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
C28H48O2 (GT)
C29H50O2 (AT)
C28H46O4
C28H48O4
C28H50O4
C28H50O5
C28H48O5
C28H52O5
C30H52O4
C30H52O5
C30H54O5
C30H56O5
C30H56O6
C32H54O4
C32H56O4
C32H54O5
C32H58O5
C32H58O5
C32H56O6
*C32H56O6
C32H60O6
C36H62O4
C36H64O4
C36H66O4
C36H62O5
C36H64O5
C36H66O5
C36H68O5
C36H62O6
*C36H64O6
*C36H66O6
C36H68O6
28 Carbons
30 Carbons
32 Carbons
36 Carbons
Normals
CRC

FIGURE 12

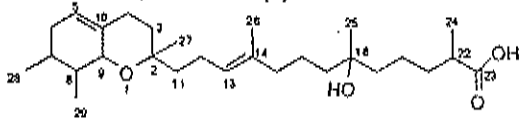
A. 416.3656, C28H48O2, gamma-tocopherol (1)



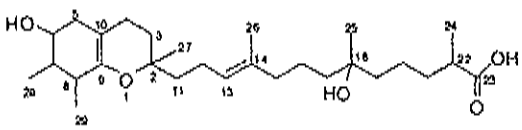
B. 410.3184, C28H42O2, gamma-tocotrienol (2)



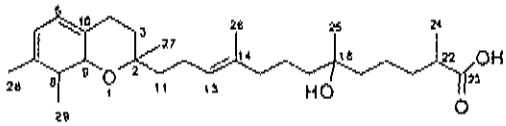
C. 448.3406, C28H48O4 (3)



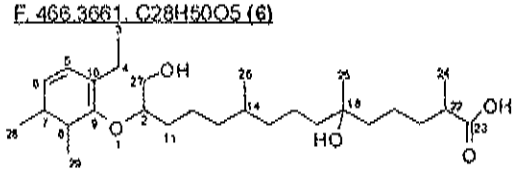
D. 464.3522, C28H48O5 (4)



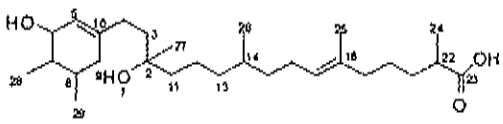
E. 446.3406, C28H46O4 (5)



F. 466.3661, C28H50O5 (6)



G. 450.3726, C28H50O4 (7)



H. 468.3840, C28H52O5 (8)

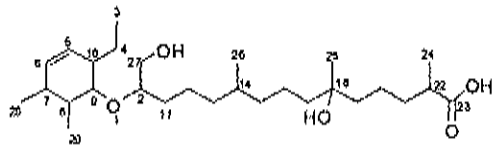


FIGURE 13: Structural assignments for the key MS/MS fragments for m/z 448.3726 (neutral), C28H47O4

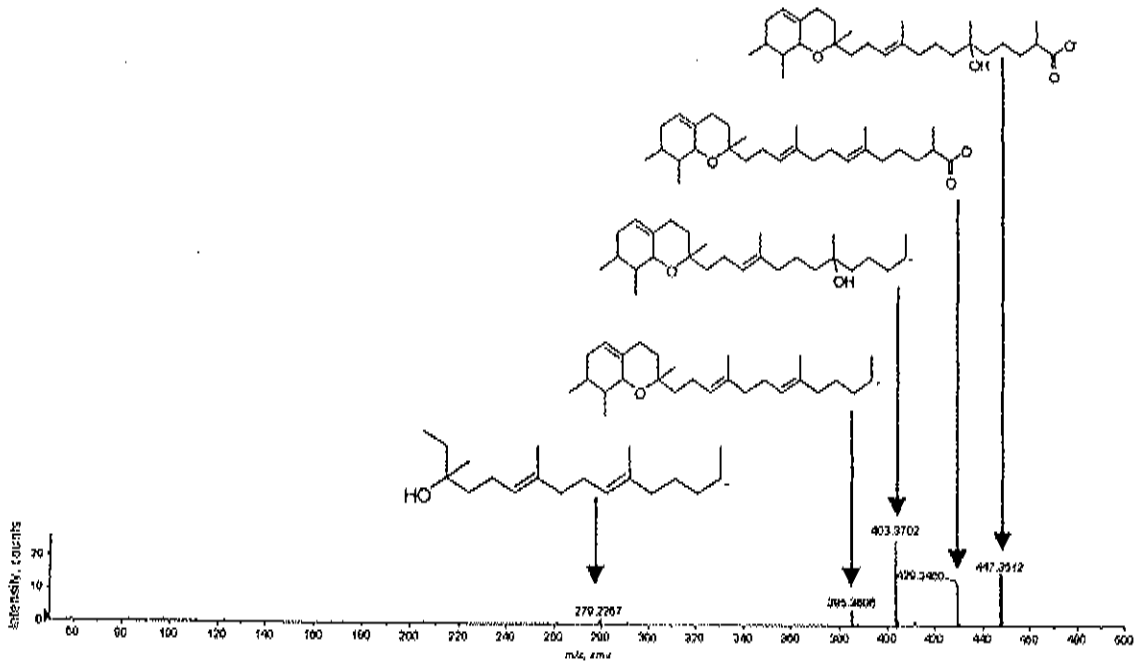


FIGURE 14: Structural assignments for the key MS/MS fragments for 464.3522 (neutral), C28H47O5

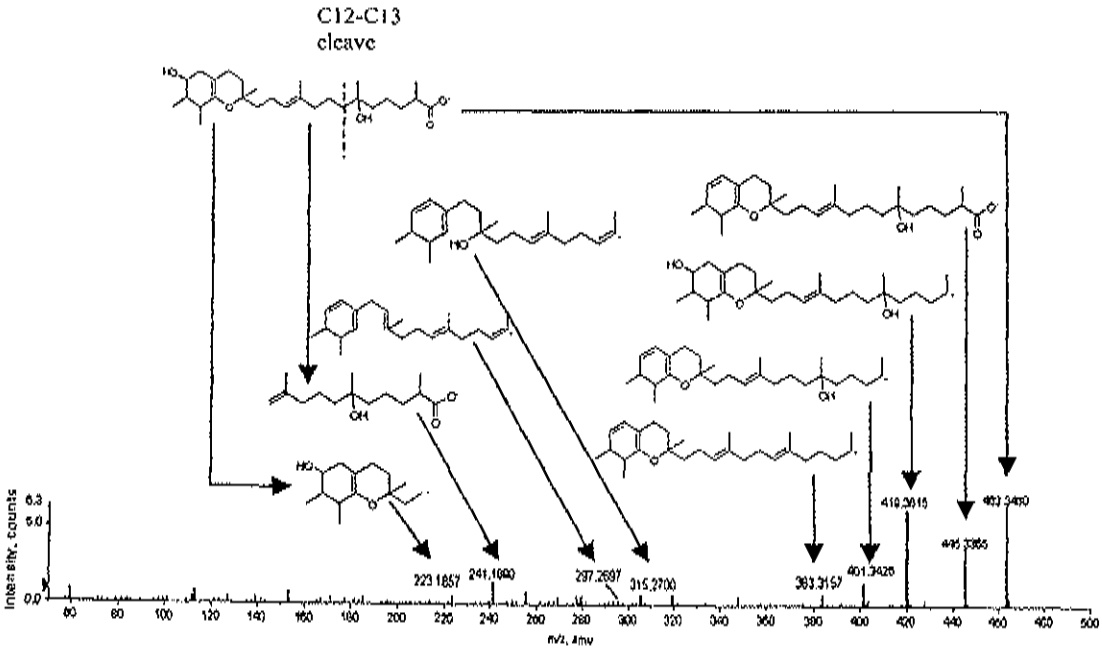


FIGURE 15: Structural assignments for the key MS/MS fragments for 446.3522 (neutral), C28H45O4

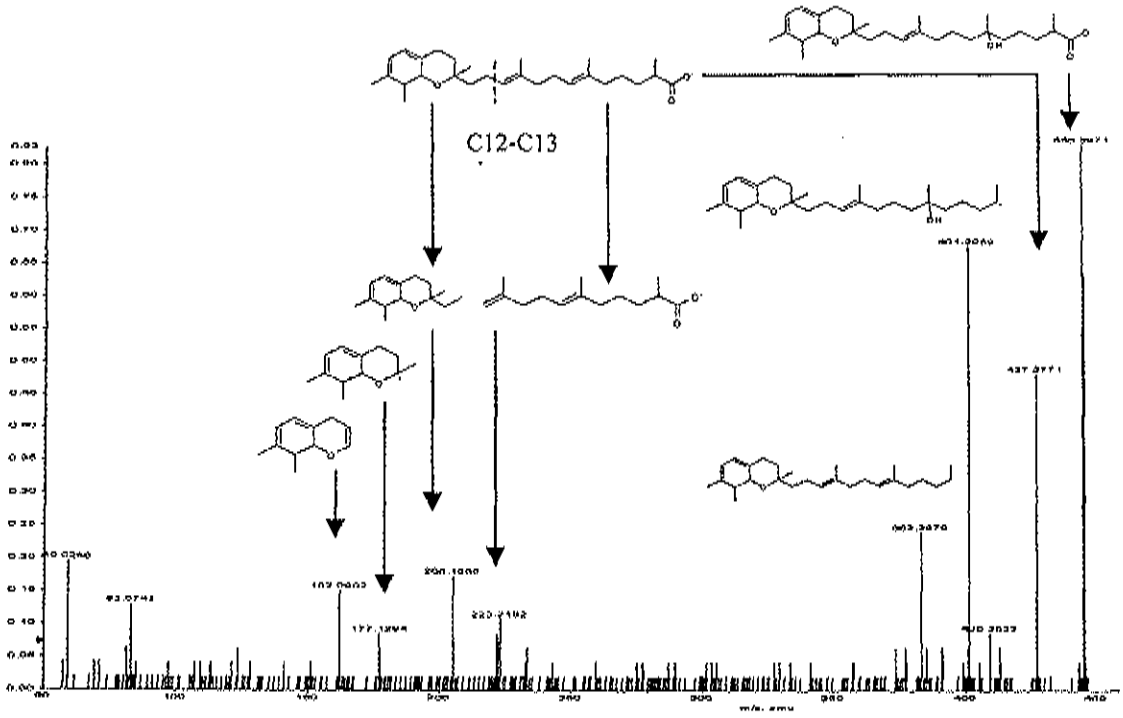


FIGURE 16: Structural assignments for the key MS/MS fragments for 466.3661 (neutral), C28H49O5

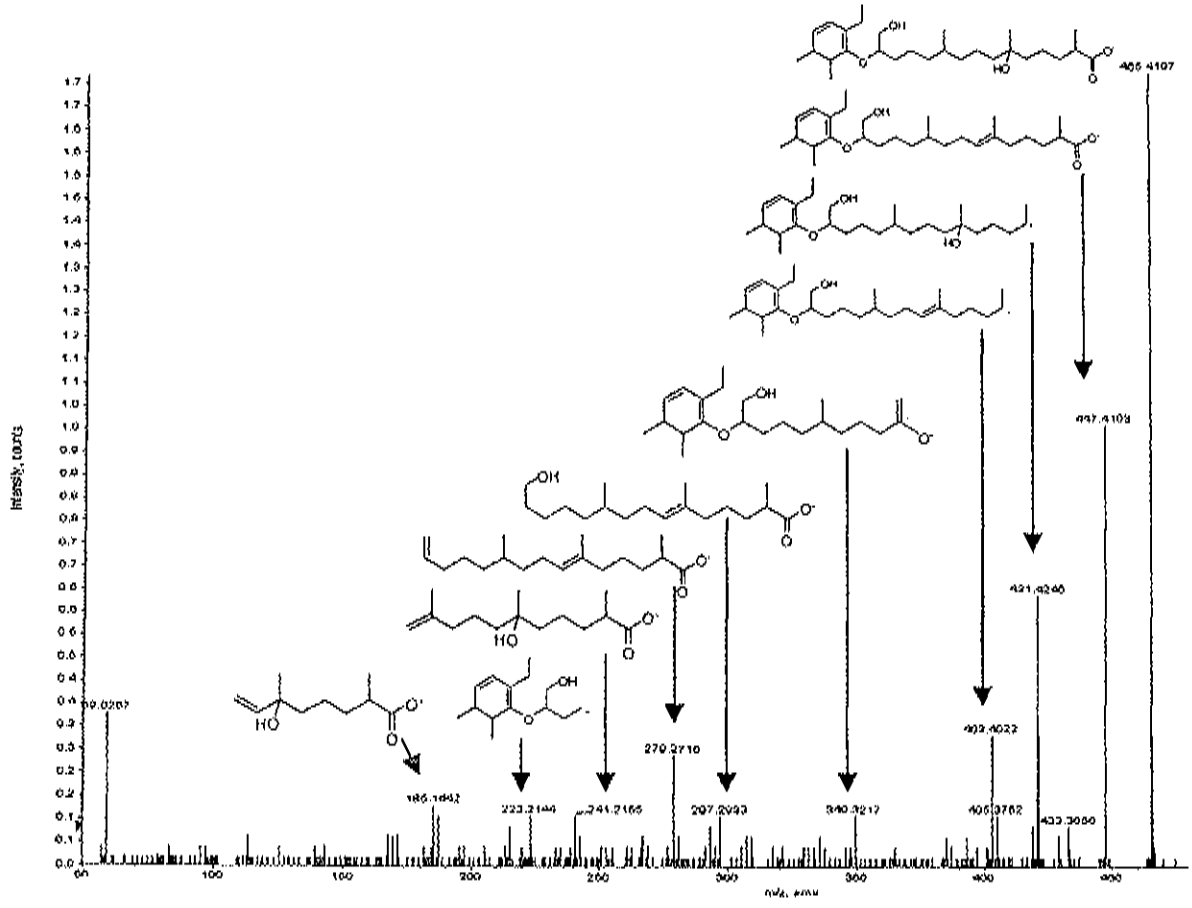


FIGURE 17: Structural assignments for the key MS/MS fragments for 450.3726 (neutral), C28H49O4

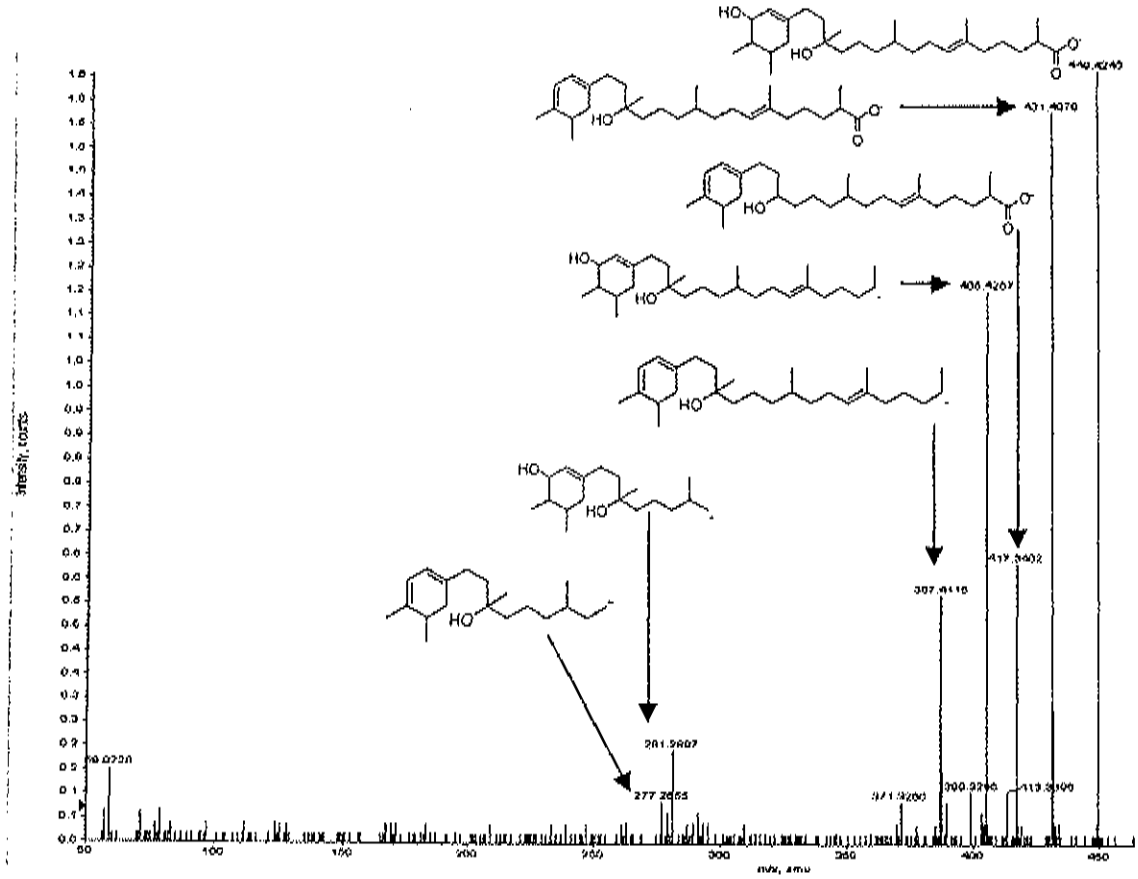


FIGURE 18: Structural assignments for the key MS/MS fragments for 468.3840 (neutral), C28H51O5

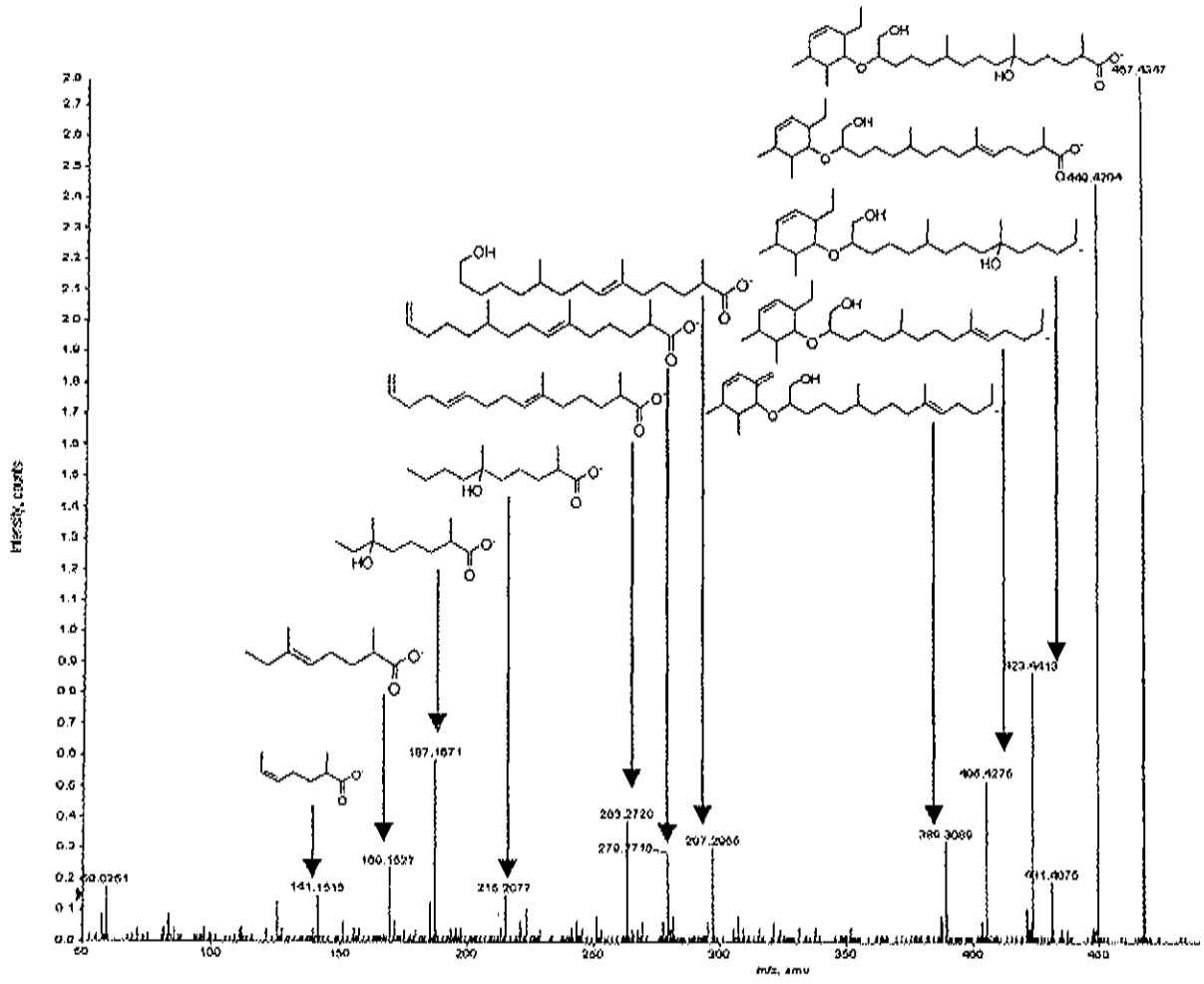
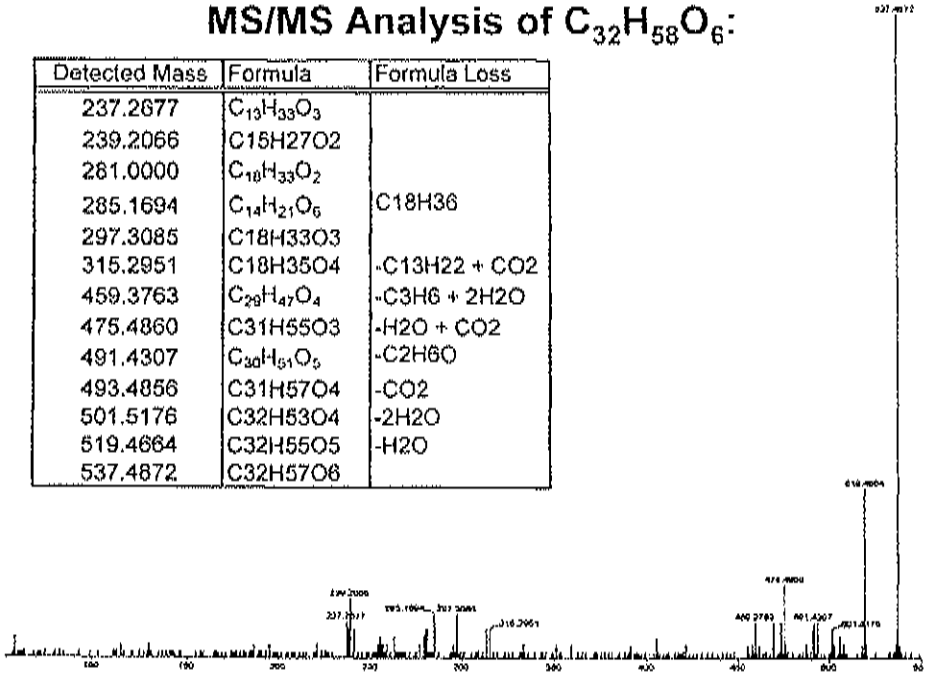


FIGURE 19. Putative structures of key MS/MS fragments for biomarker 538.4259 (neutral) ($C_{32}H_{57}O_6$).

MS/MS Analysis of $C_{32}H_{58}O_6$:

Detected Mass	Formula	Formula Loss
237.2677	$C_{13}H_{33}O_3$	
239.2066	C15H27O2	
261.0000	$C_{18}H_{33}O_2$	
285.1694	$C_{14}H_{21}O_6$	C18H36
297.3085	C18H33O3	
315.2951	C18H35O4	-C13H22 + CO2
459.3763	$C_{28}H_{47}O_4$	-C3H6 + 2H2O
475.4860	C31H55O3	-H2O + CO2
491.4307	$C_{30}H_{51}O_5$	-C2H6O
493.4856	C31H57O4	-CO2
501.5176	C32H53O4	-2H2O
519.4664	C32H55O5	-H2O
537.4872	C32H57O6	



MS/MS Analysis of $C_{32}H_{58}O_6$:

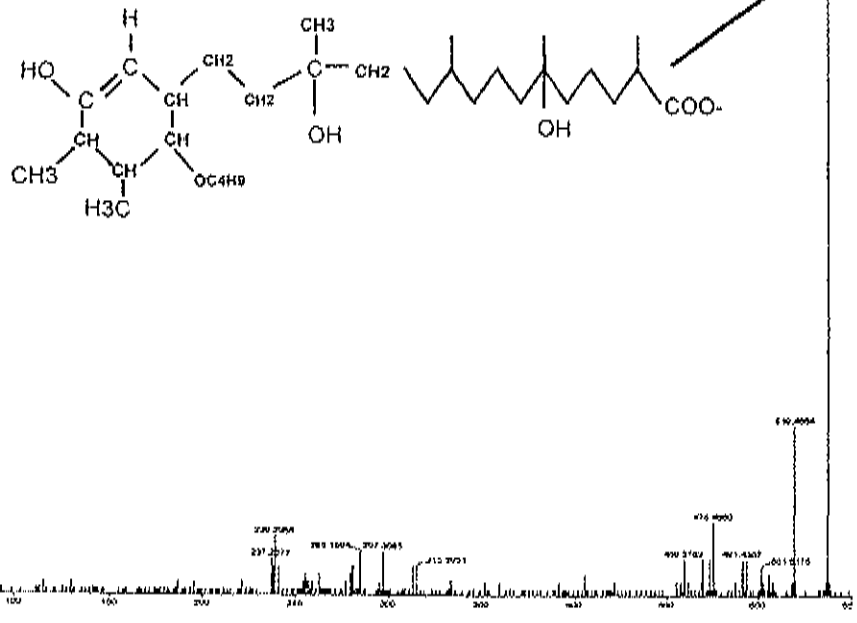
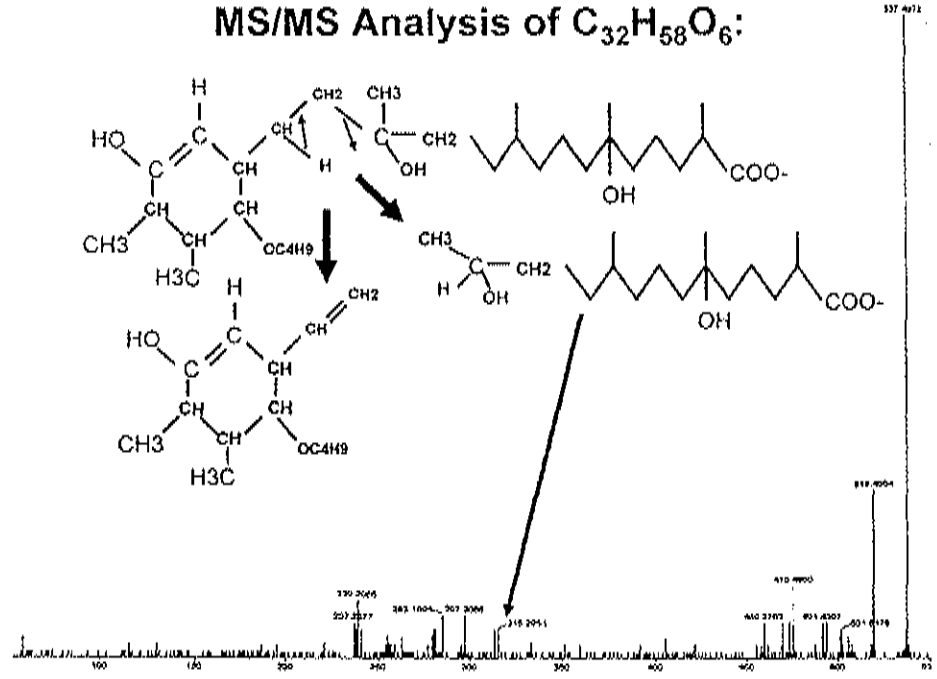


FIGURE 19 CONT'D



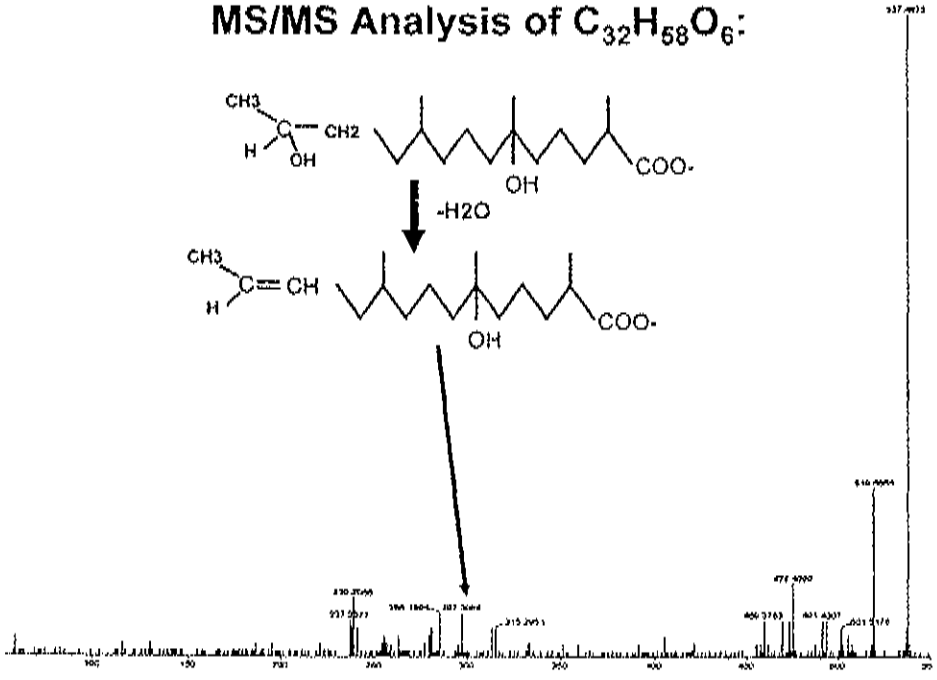
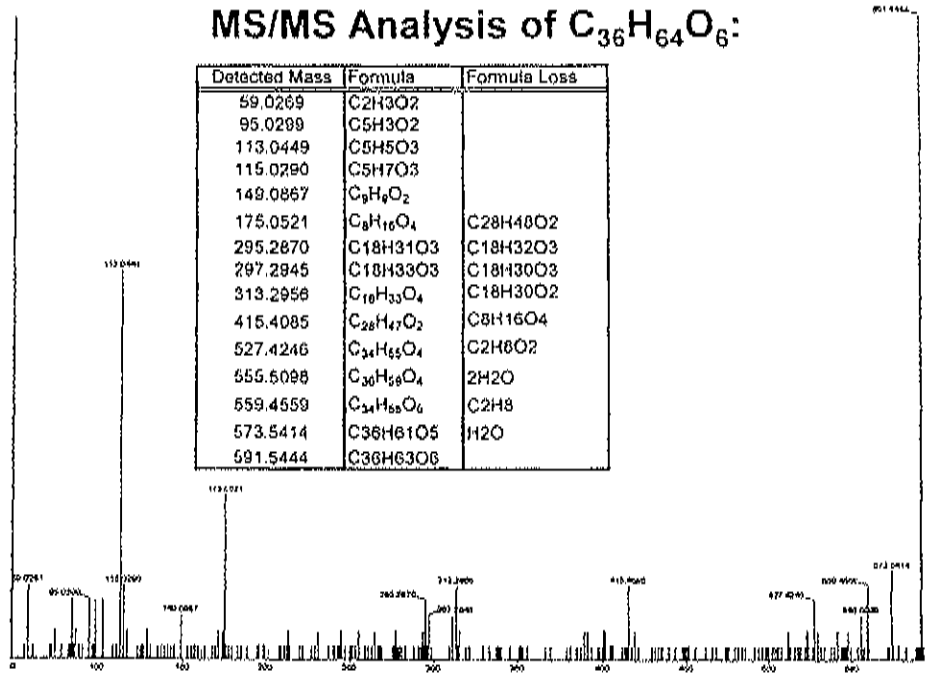


FIGURE 20. Putative structures of key MS/MS fragments for biomarker 592.4711 (neutral) ($C_{36}H_{63}O_6$).



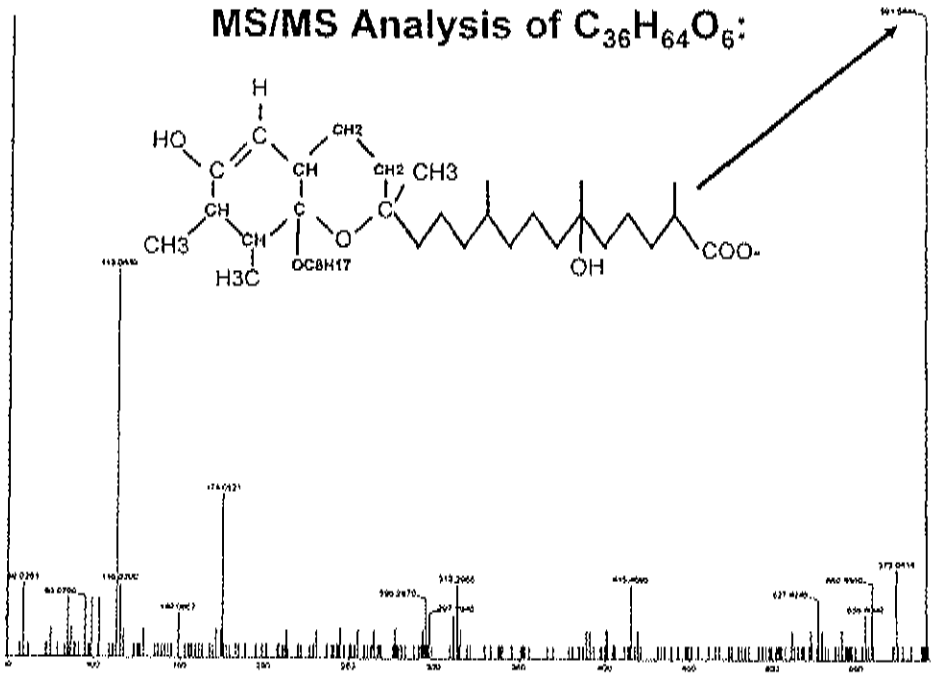


FIGURE 20 CONT'D

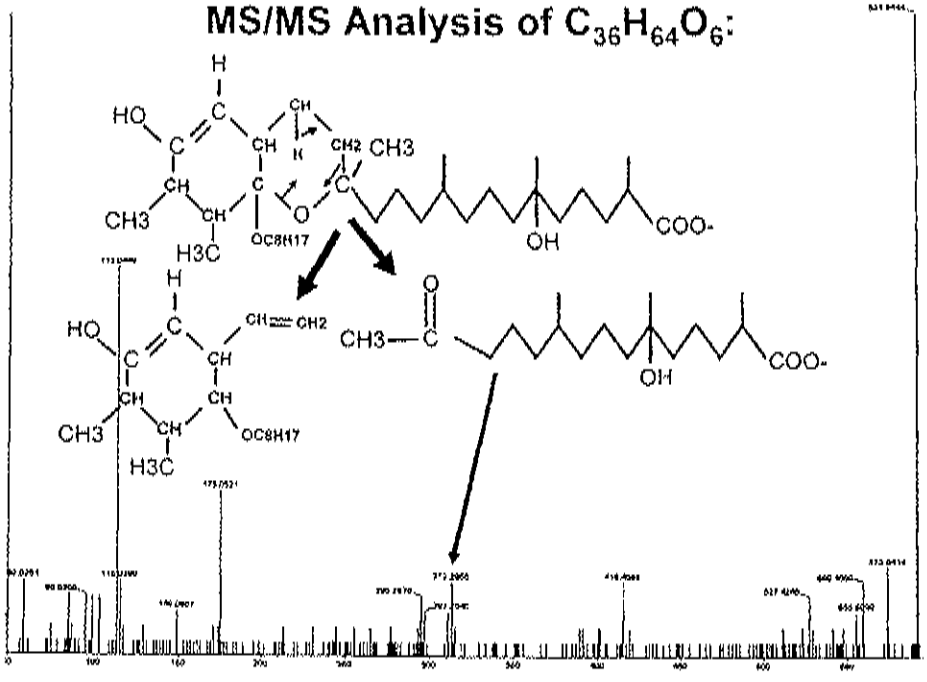
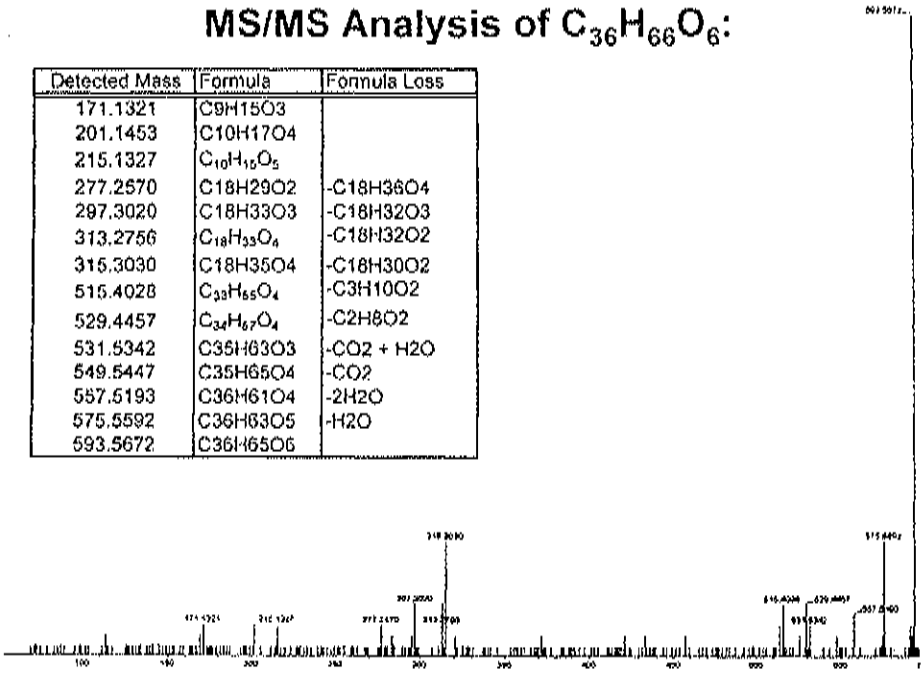


FIGURE 21. Putative structures of key MS/MS fragments for biomarker 594.4851 (neutral) ($C_{36}H_{65}O_6$).

MS/MS Analysis of $C_{36}H_{66}O_6$:

Detected Mass	Formula	Formula Loss
171.1321	C9H15O3	
201.1453	C10H17O4	
215.1327	$C_{10}H_{15}O_5$	
277.2570	C18H29O2	-C18H36O4
297.3020	C18H33O3	-C18H32O3
313.2756	$C_{18}H_{33}O_4$	-C18H32O2
315.3030	C18H35O4	-C18H30O2
515.4028	$C_{33}H_{55}O_4$	-C3H10O2
529.4457	$C_{34}H_{57}O_4$	-C2H8O2
531.5342	C35H63O3	-CO2 + H2O
549.5447	C35H65O4	-CO2
557.5193	C36H61O4	-2H2O
575.5592	C36H63O5	-H2O
593.5672	C36H65O6	



MS/MS Analysis of $C_{36}H_{66}O_6$:

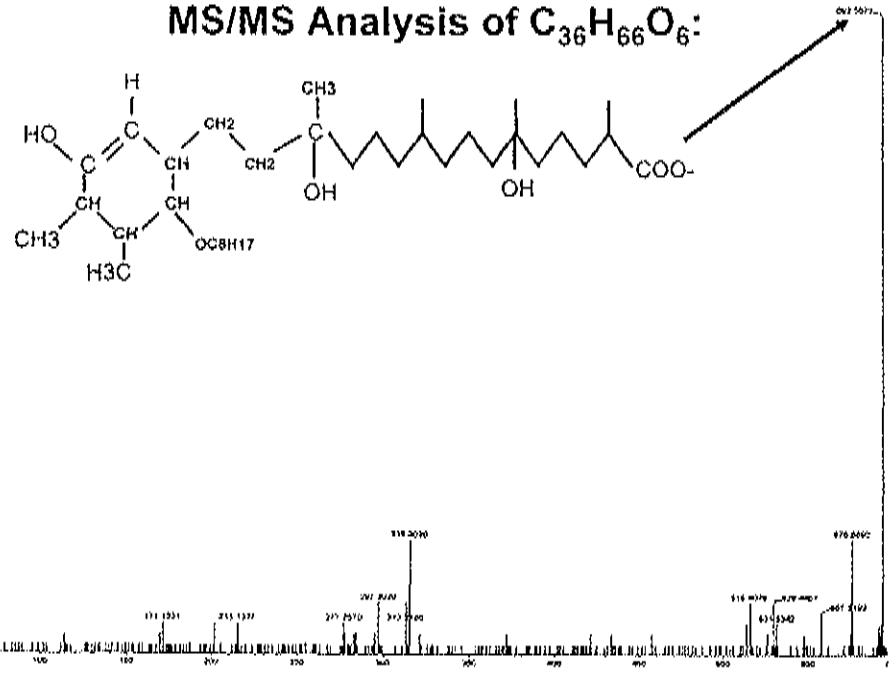
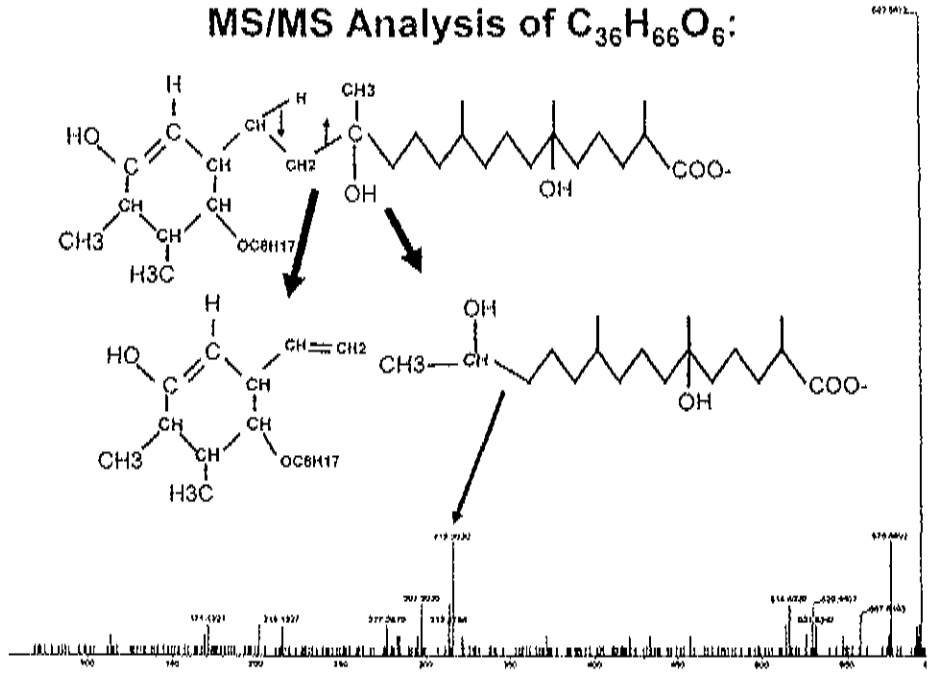


FIGURE 21 CONT'D



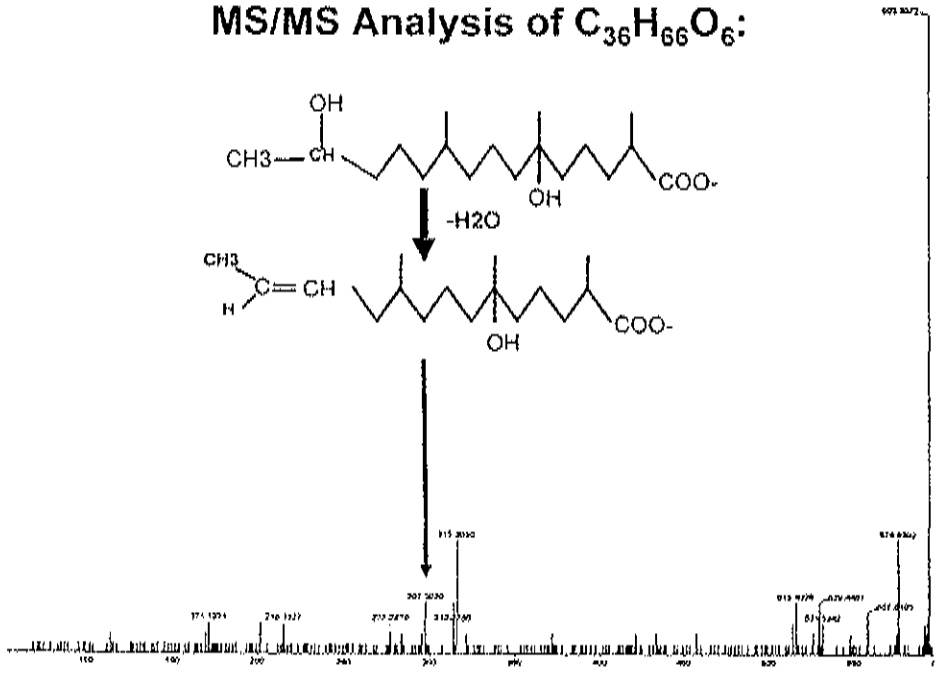


FIGURE 22

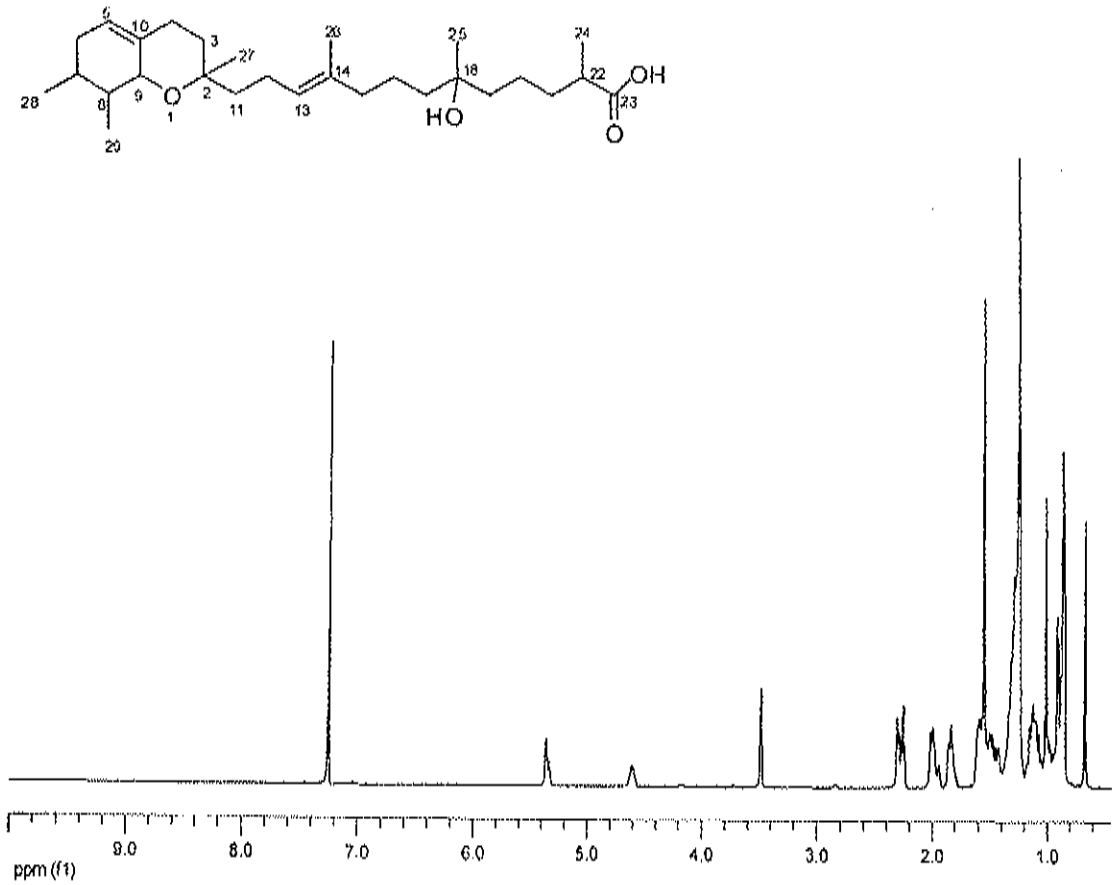
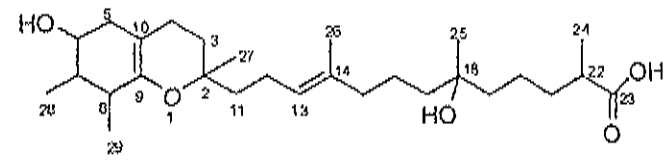


FIGURE 23


HO
5
10
3
27
26
25
24
14
18
22
OH
23
9
O
2
1
11
13
HO
O
28
6
29

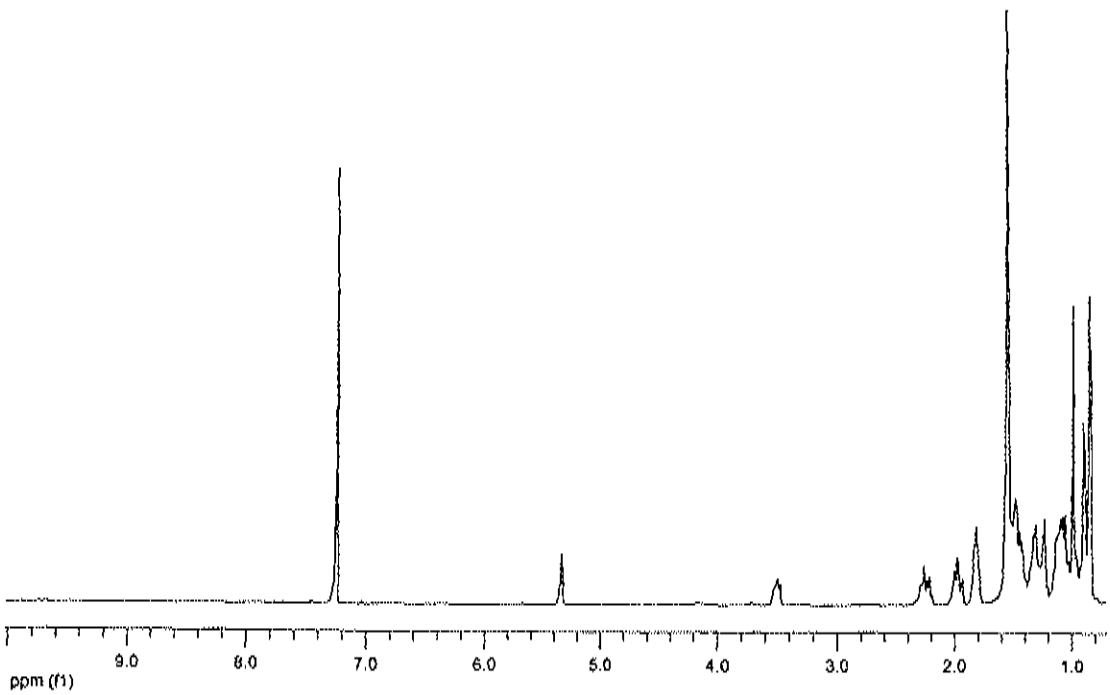
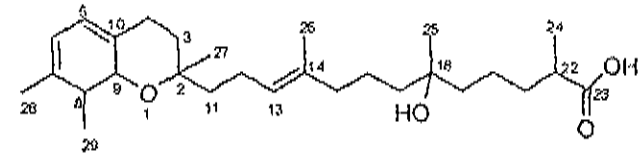

9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ppm (f1)

FIGURE 24


10
3
27
26
25
24
14
18
22
OH
9
O
2
23
28
4
1
11
13
HO
O
29

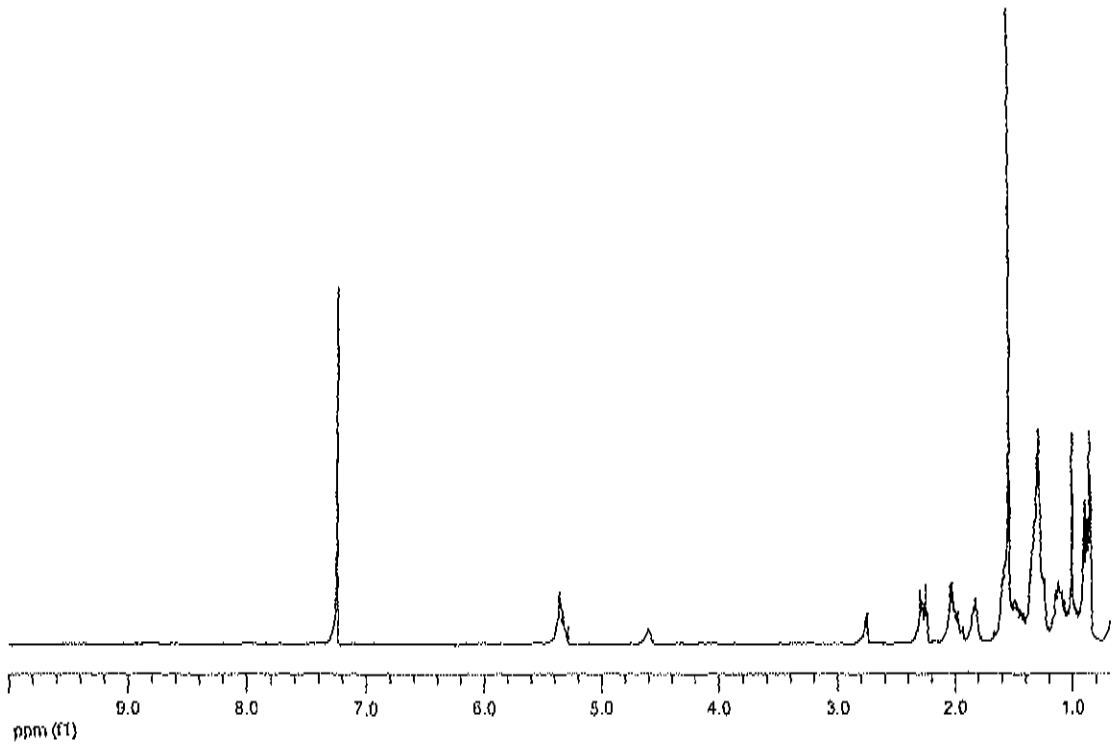

9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ppm (f1)

FIGURE 25

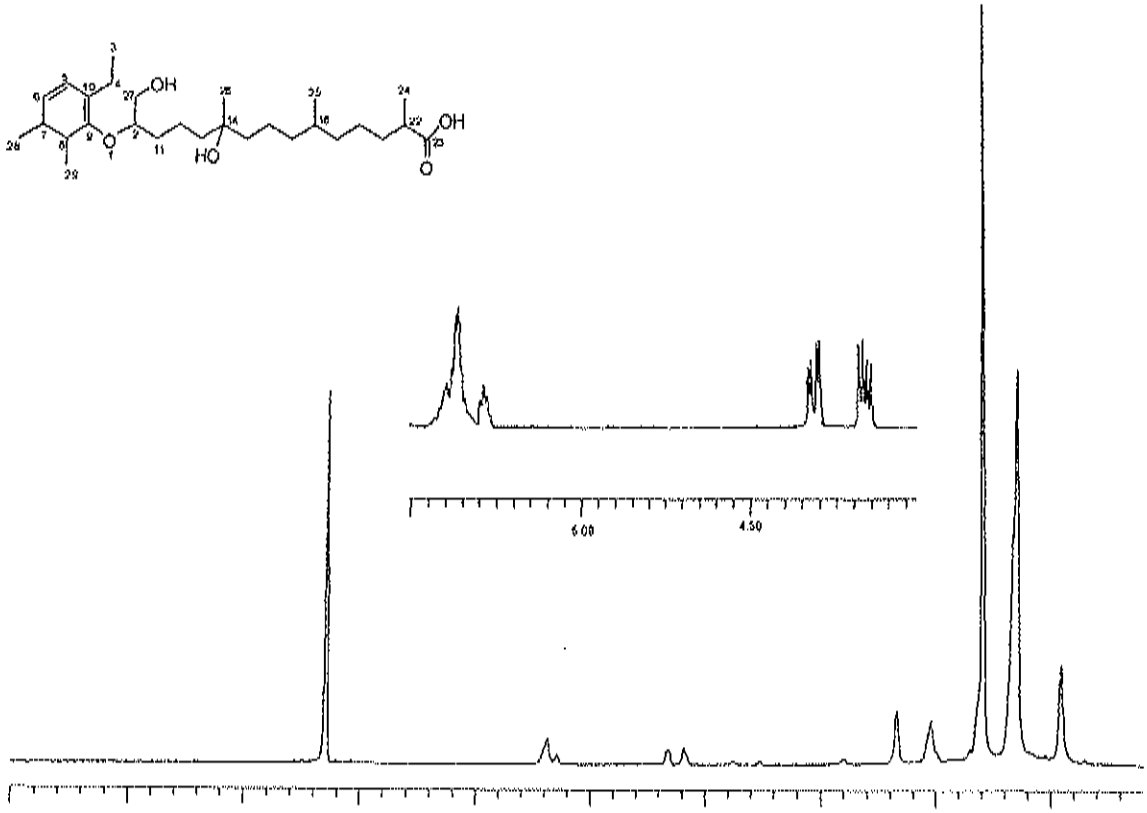


FIGURE 26

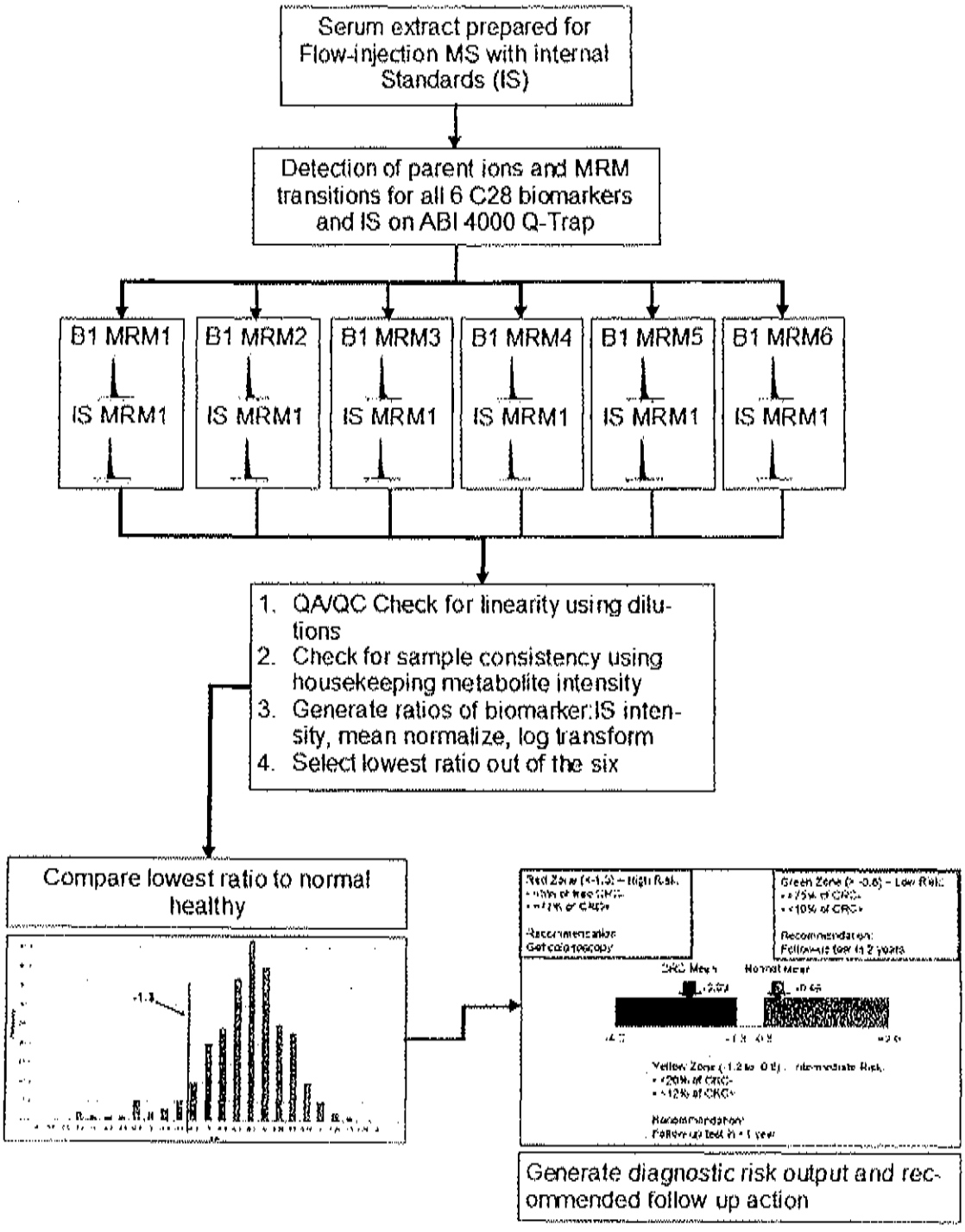

Serum extract prepared for Flow-injection MS with internal Standards (IS)
Detection of parent ions and MRM transitions for all 6 C28 biomarkers and IS on ABI 4000 Q-Trap
B1 MRM1
IS MRM1
B1 MRM2
IS MRM1
B1 MRM3
IS MRM1
B1 MRM4
IS MRM1
B1 MRM5
IS MRM1
B1 MRM6
IS MRM1
1. QA/QC Check for linearity using dilutions
2. Check for sample consistency using housekeeping metabolite intensity
3. Generate ratios of biomarker:IS intensity, mean normalize, log transform
4. Select lowest ratio out of the six
Compare lowest ratio to normal healthy
Generate diagnostic risk output and recommended follow up action

FIGURE 27

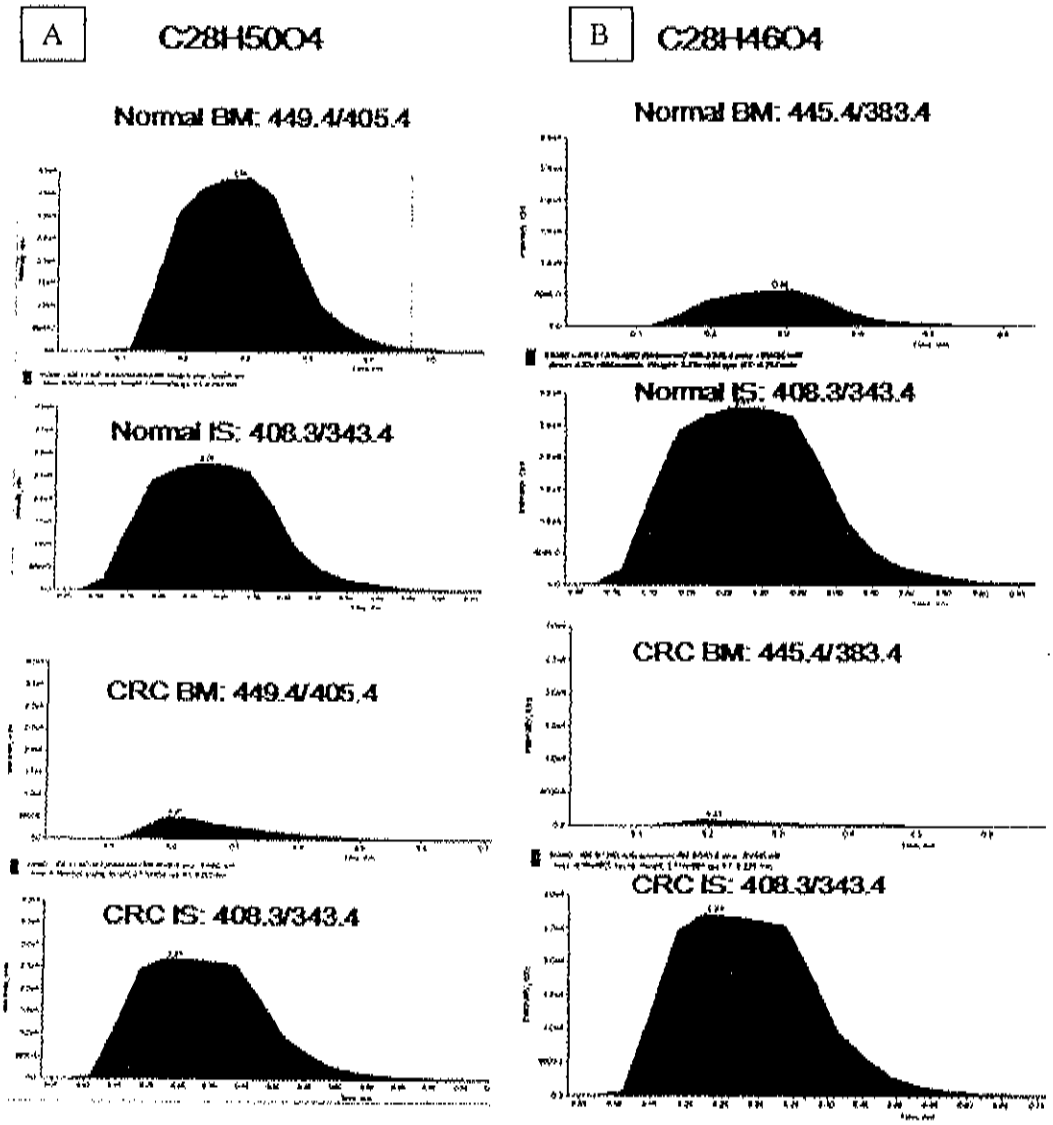
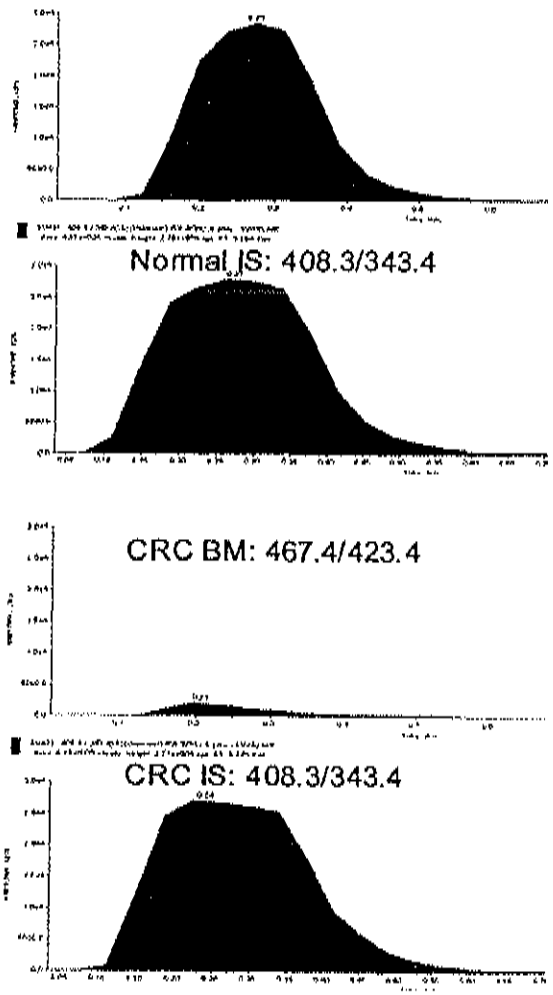

A
C28H50O4
Normal BM: 449.4/405.4
Normal IS: 408.3/343.4
CRC BM: 449.4/405.4
CRC IS: 408.3/343.4
B
C28H46O4
Normal BM: 445.4/383.4
Normal IS: 408.3/343.4
CRC BM: 445.4/383.4
CRC IS: 408.3/343.4

FIGURE 27 CONT'D

C C28H52O5



D C28H48O4

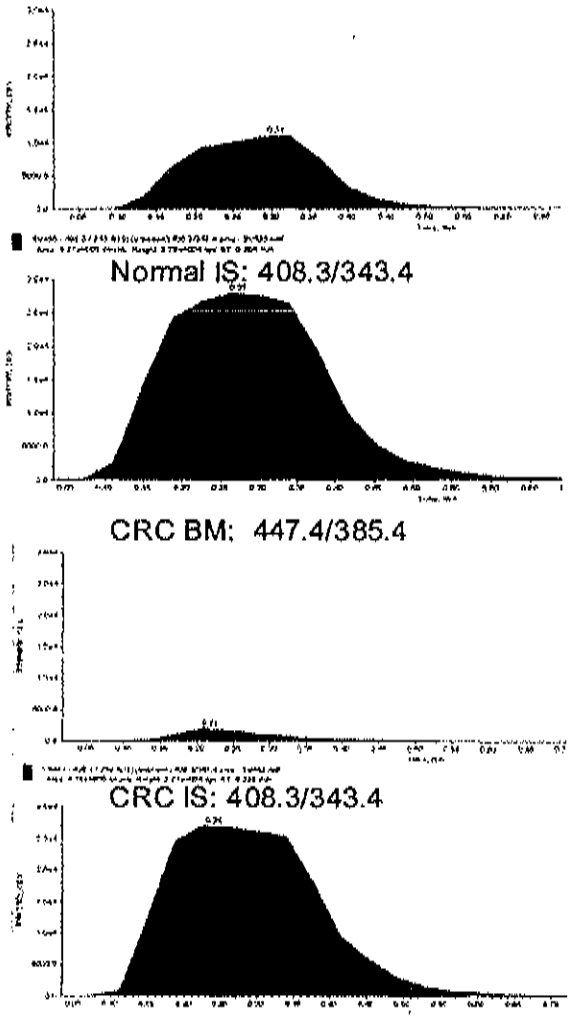
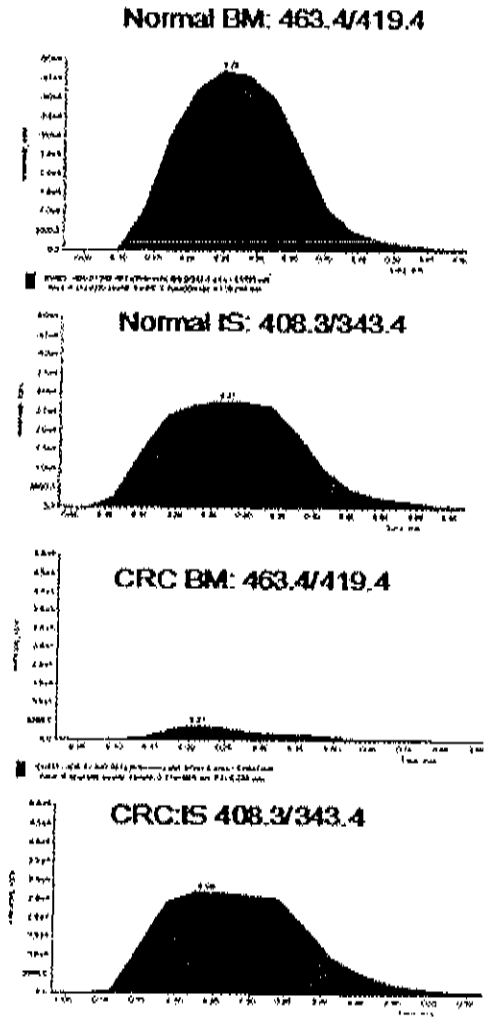


FIGURE 27 CONT'D

E

C28H48O5



F

C28H50O5

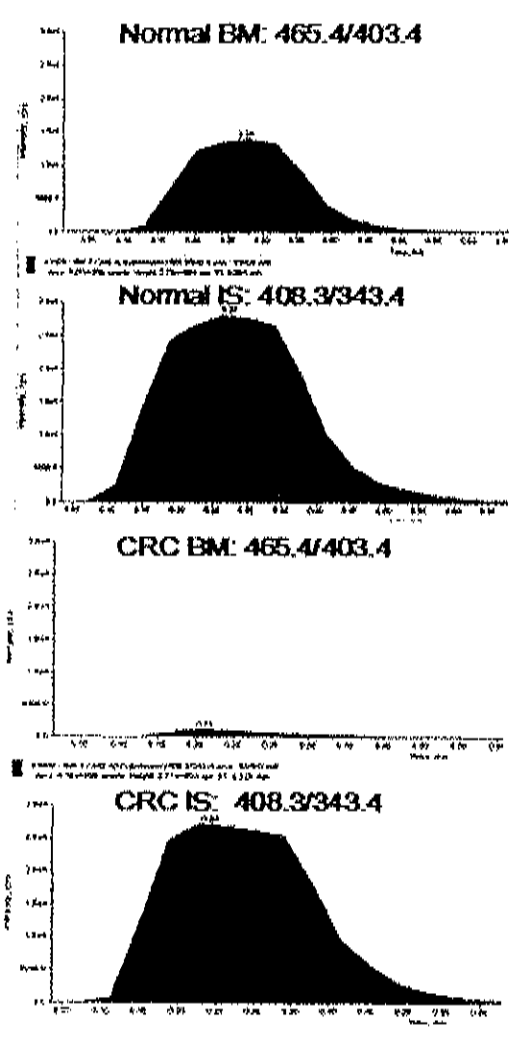
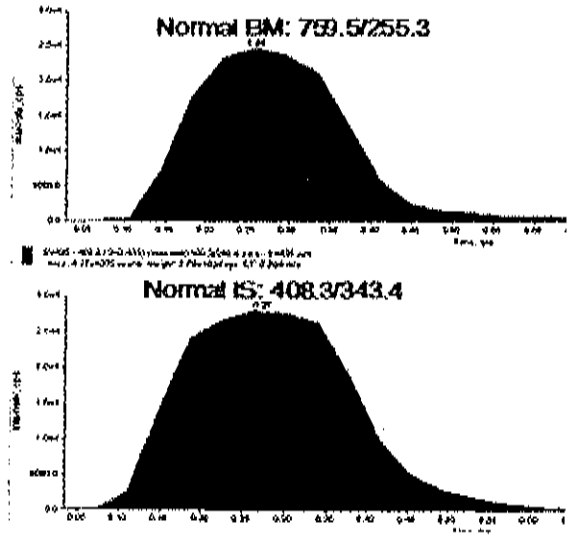


FIGURE 27 CONT'D

G

Housekeeper



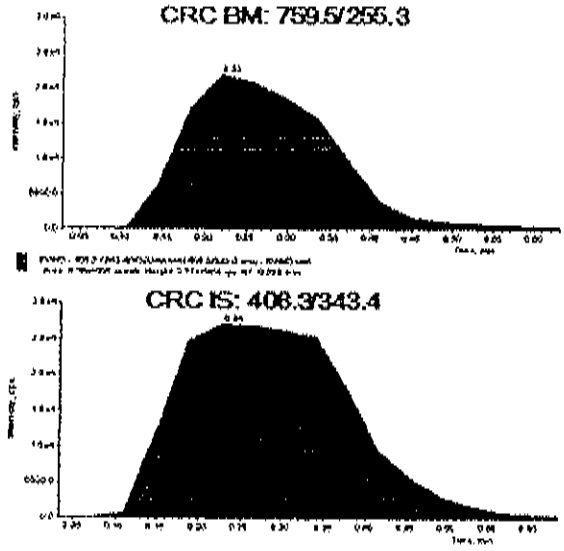


FIGURE 28

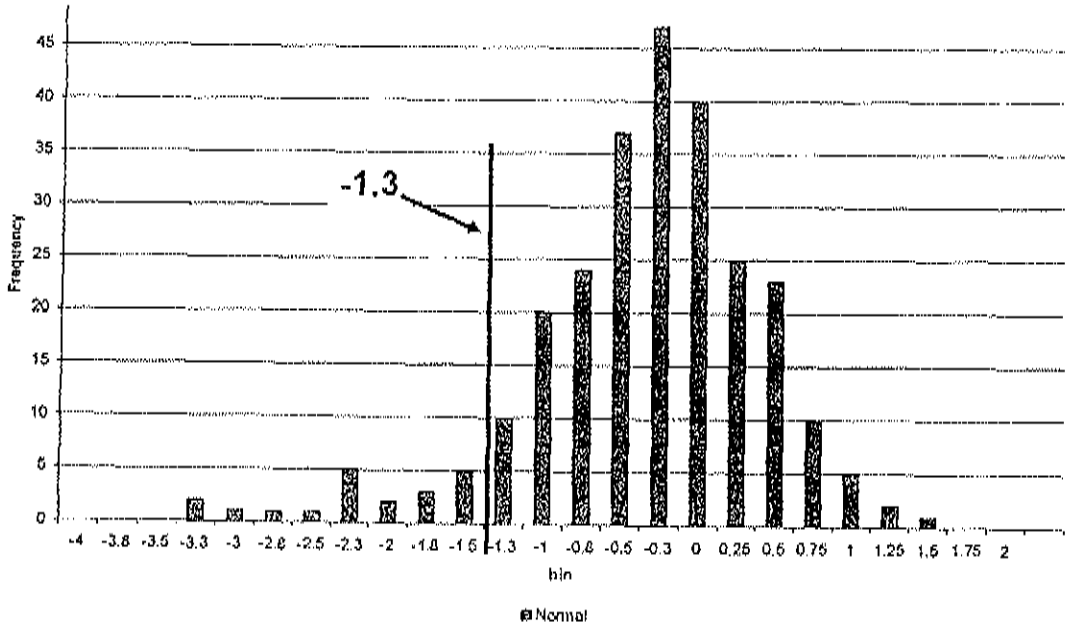

45
40
35
30
25
20
15
10
5
0
Frequency
-1.3
-4 -3.8 -3.5 -3.3 -3 -2.8 -2.5 -2.3 -2 -1.8 -1.5 -1.3 -1 -0.8 -0.5 -0.3 0 0.25 0.5 0.75 1 1.25 1.5 1.75 2
bin
Normal

FIGURE 29

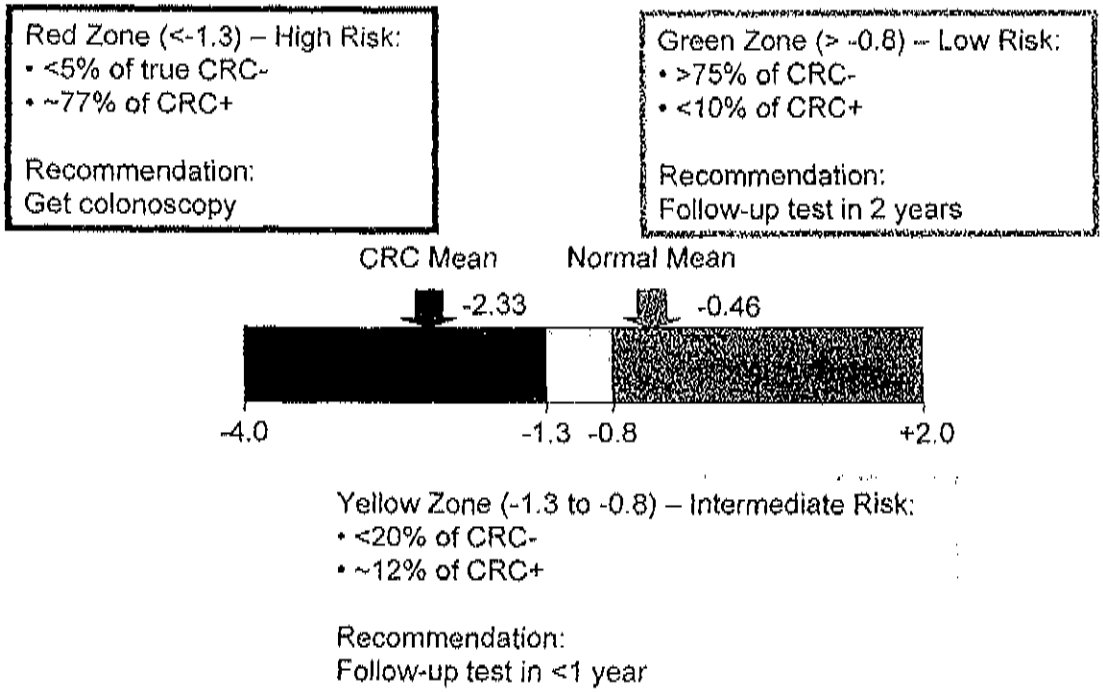

Red Zone (<-1.3) – High Risk:
• <5% of true CRC-
• ~77% of CRC+
Recommendation:
Get colonoscopy
Green Zone (> -0.8) – Low Risk:
• >75% of CRC-
• <10% of CRC+
Recommendation:
Follow-up test in 2 years
CRC Mean
Normal Mean
-2.33
-0.46
-4.0
-1.3
-0.8
+2.0
Yellow Zone (-1.3 to -0.8) – Intermediate Risk:
• <20% of CRC-
• ~12% of CRC+
Recommendation:
Follow-up test in <1 year

FIGURE 30

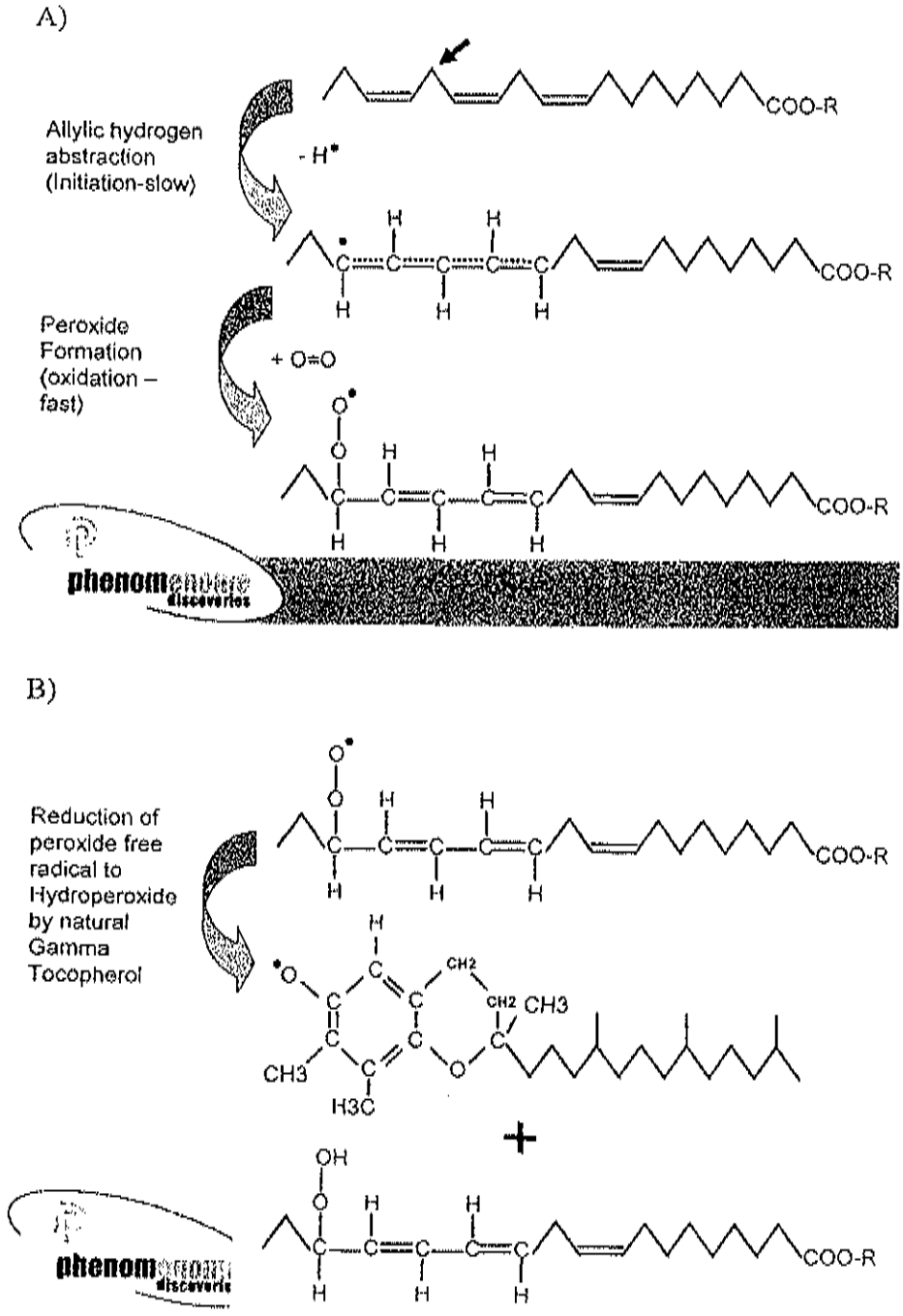
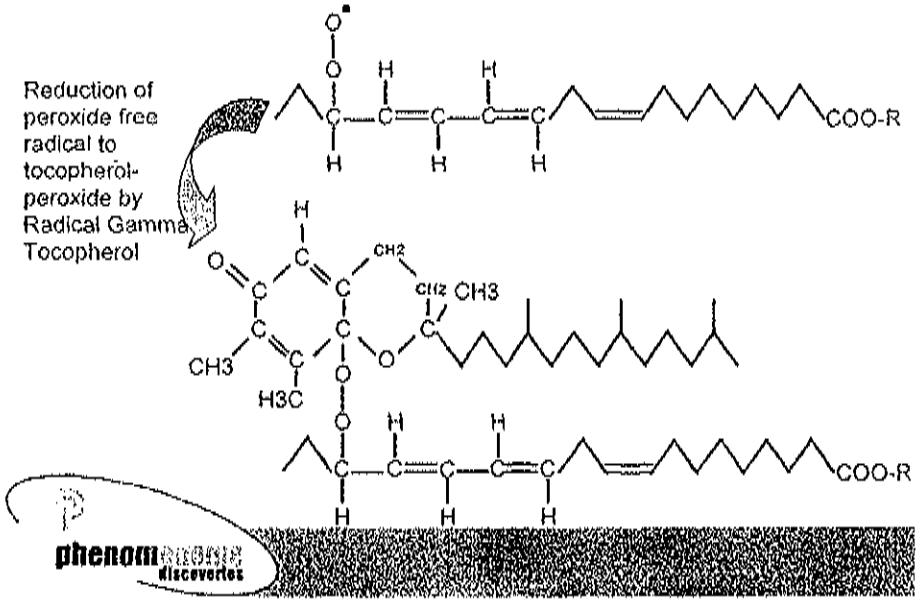

A)
Allylic hydrogen abstraction (Initiation-slow)
- H•
COO-R
Peroxide Formation (oxidation – fast)
+ O=O
B)
Reduction of peroxide free radical to Hydroperoxide by natural Gamma Tocopherol
CH3
H3C
CH2
CH3
OH

FIGURE 30 CONT'D

C)



D)

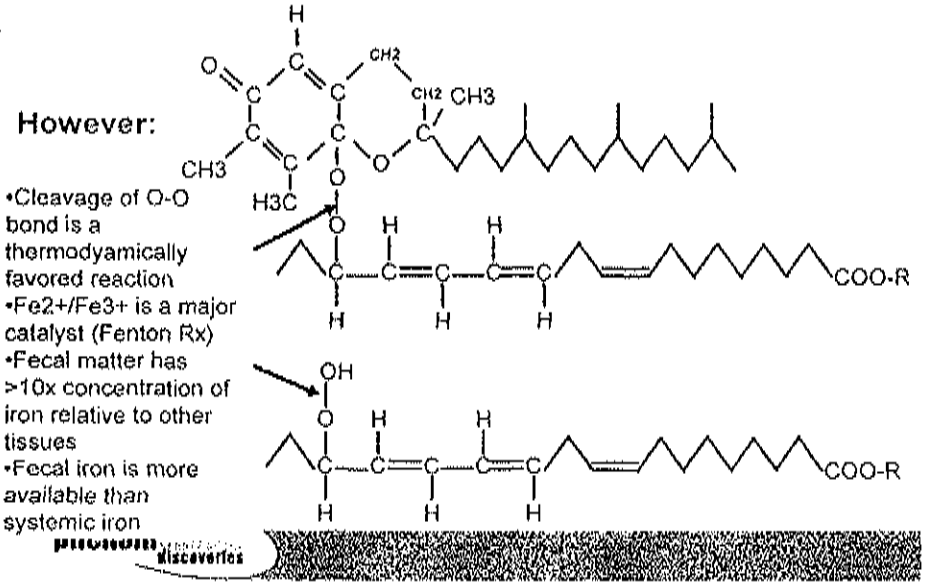


FIGURE 31

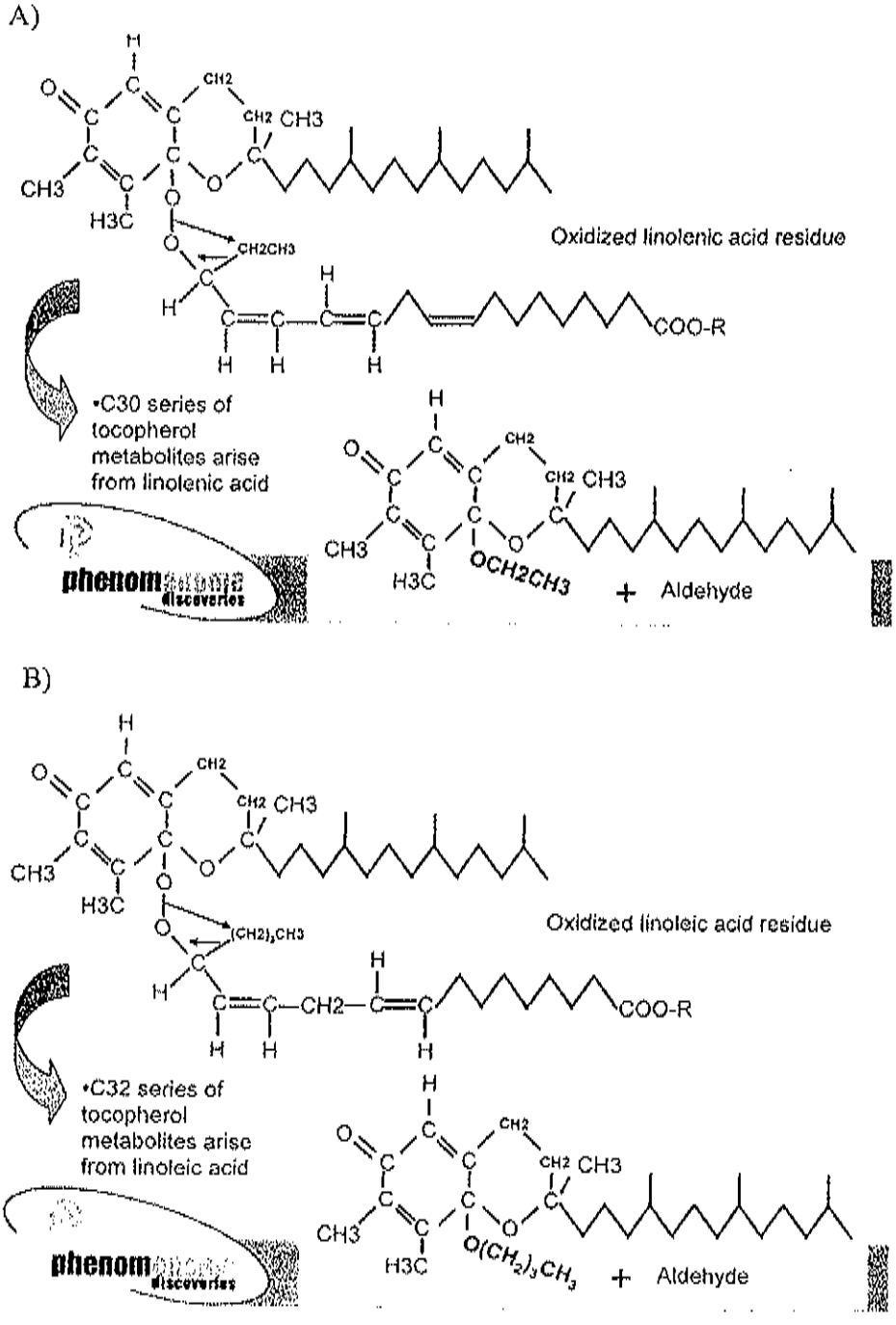

A)
Oxidized linolenic acid residue
COO-R
•C30 series of tocopherol metabolites arise from linolenic acid
+ Aldehyde
B)
Oxidized linoleic acid residue
COO-R
•C32 series of tocopherol metabolites arise from linoleic acid
+ Aldehyde

FIGURE 31 CONT'D

C)

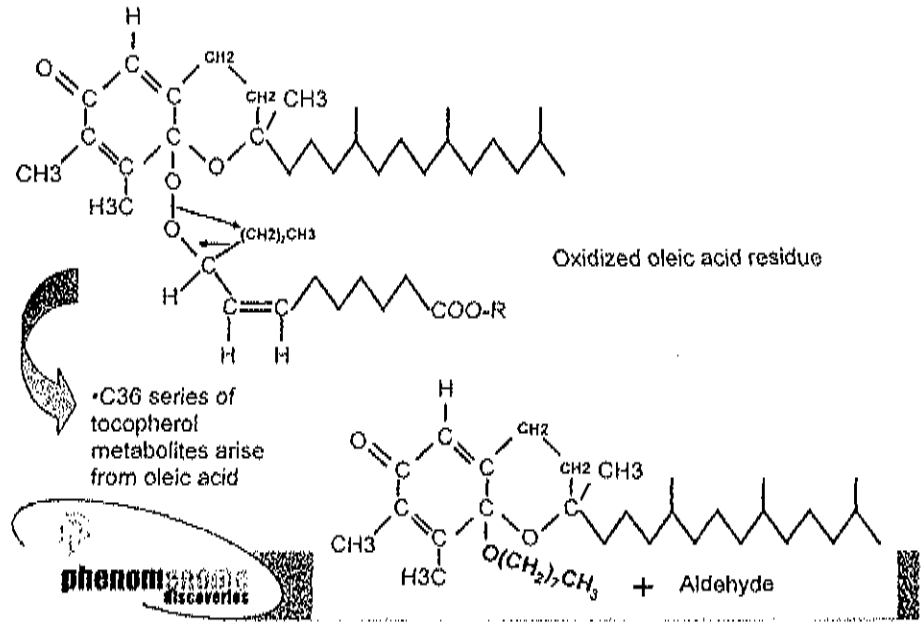


FIGURE 32

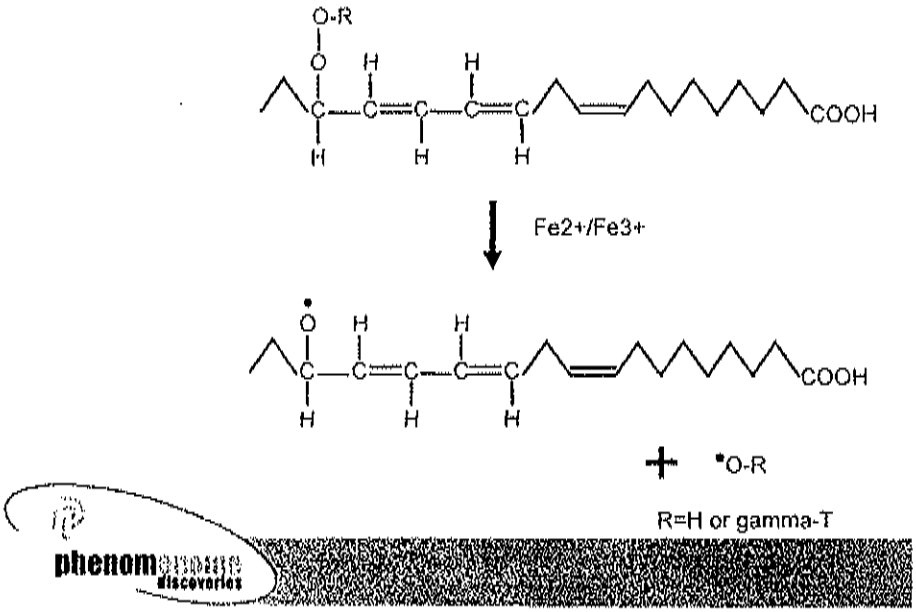


FIGURE 33

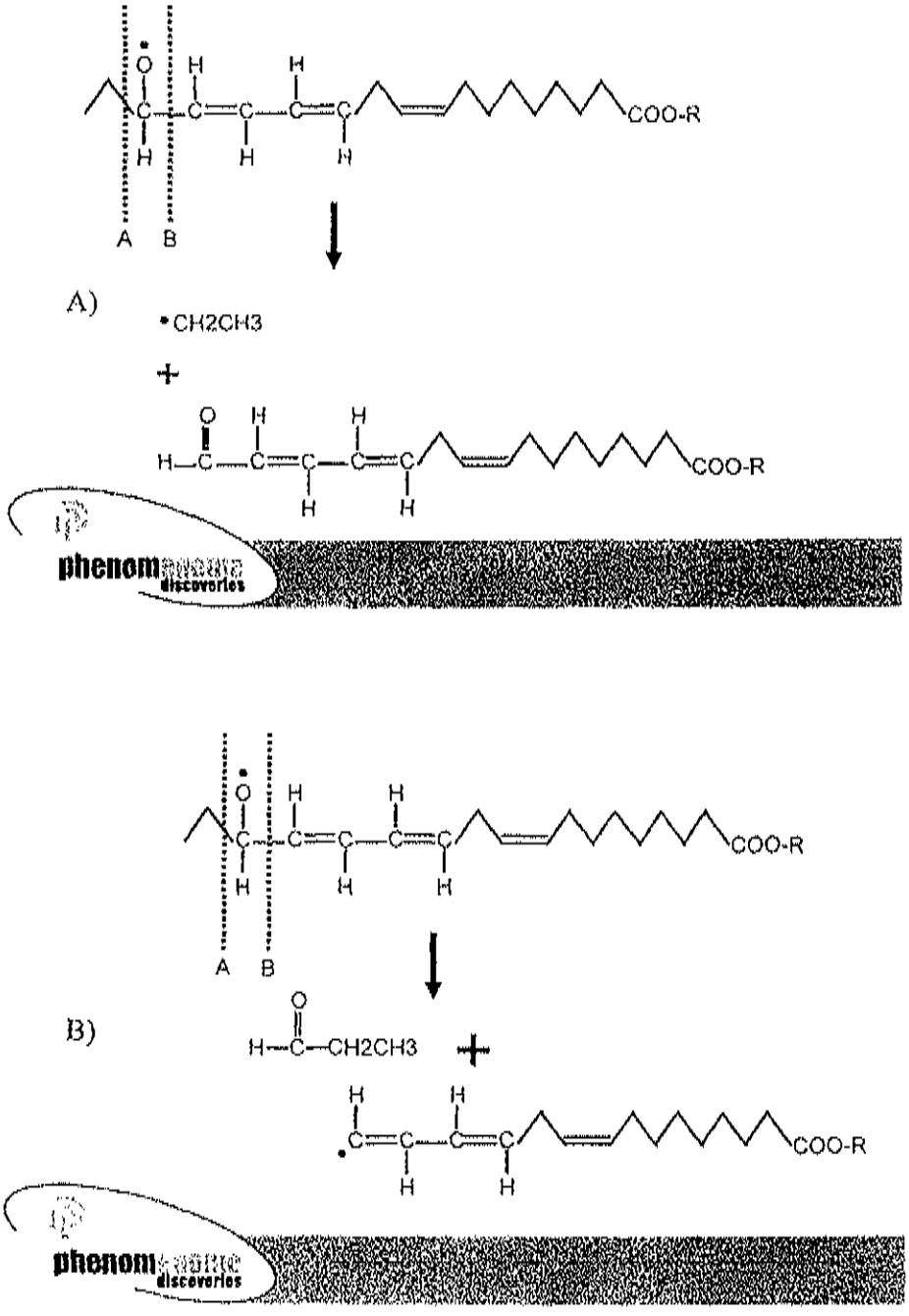
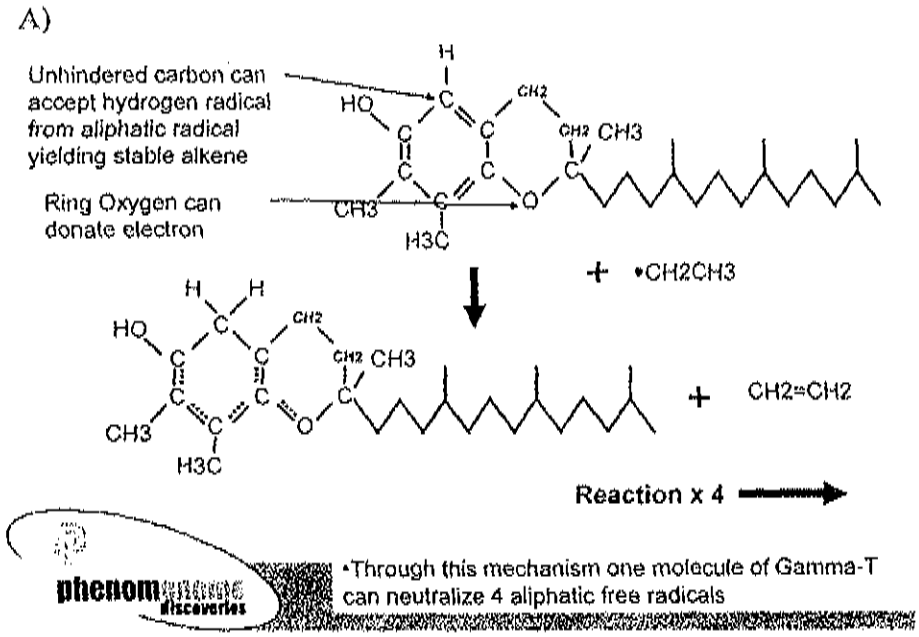


FIGURE 34


A)
Unhindered carbon can accept hydrogen radical from aliphatic radical yielding stable alkene
Ring Oxygen can donate electron
+ •CH2CH3
+ CH2=CH2
Reaction x 4
•Through this mechanism one molecule of Gamma-T can neutralize 4 aliphatic free radicals

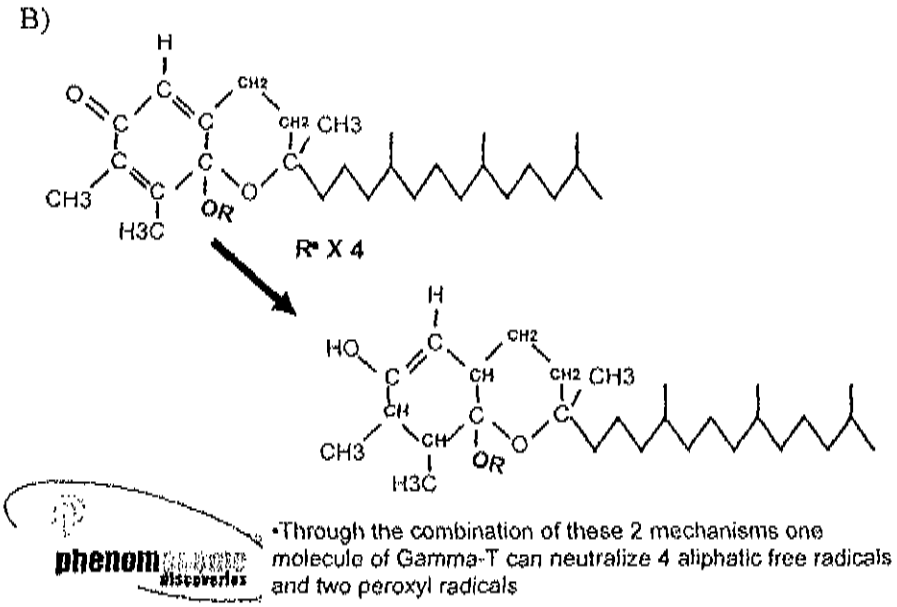

B)
R• X 4
•Through the combination of these 2 mechanisms one molecule of Gamma-T can neutralize 4 aliphatic free radicals and two peroxyl radicals

FIGURE 35

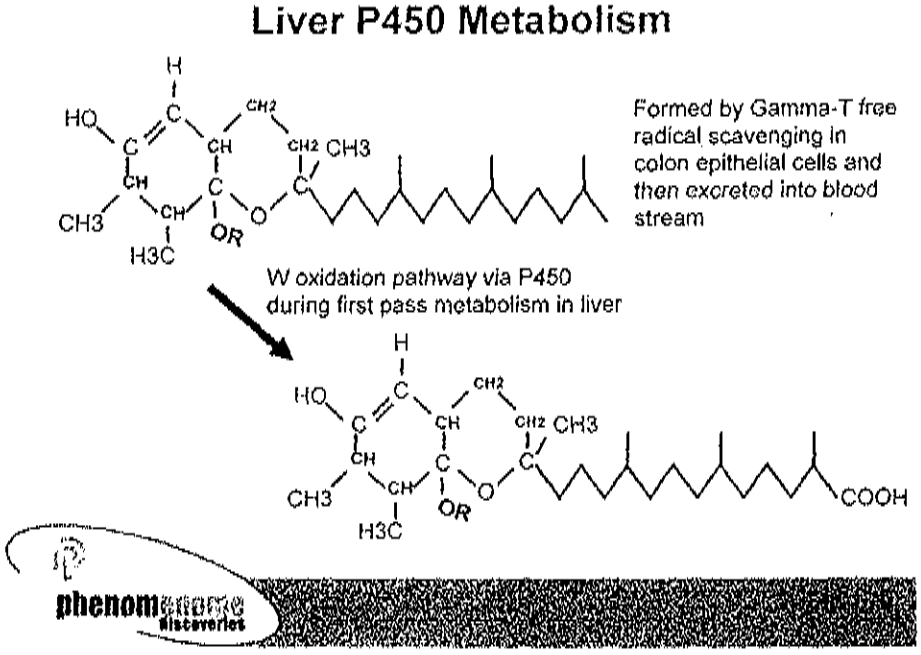
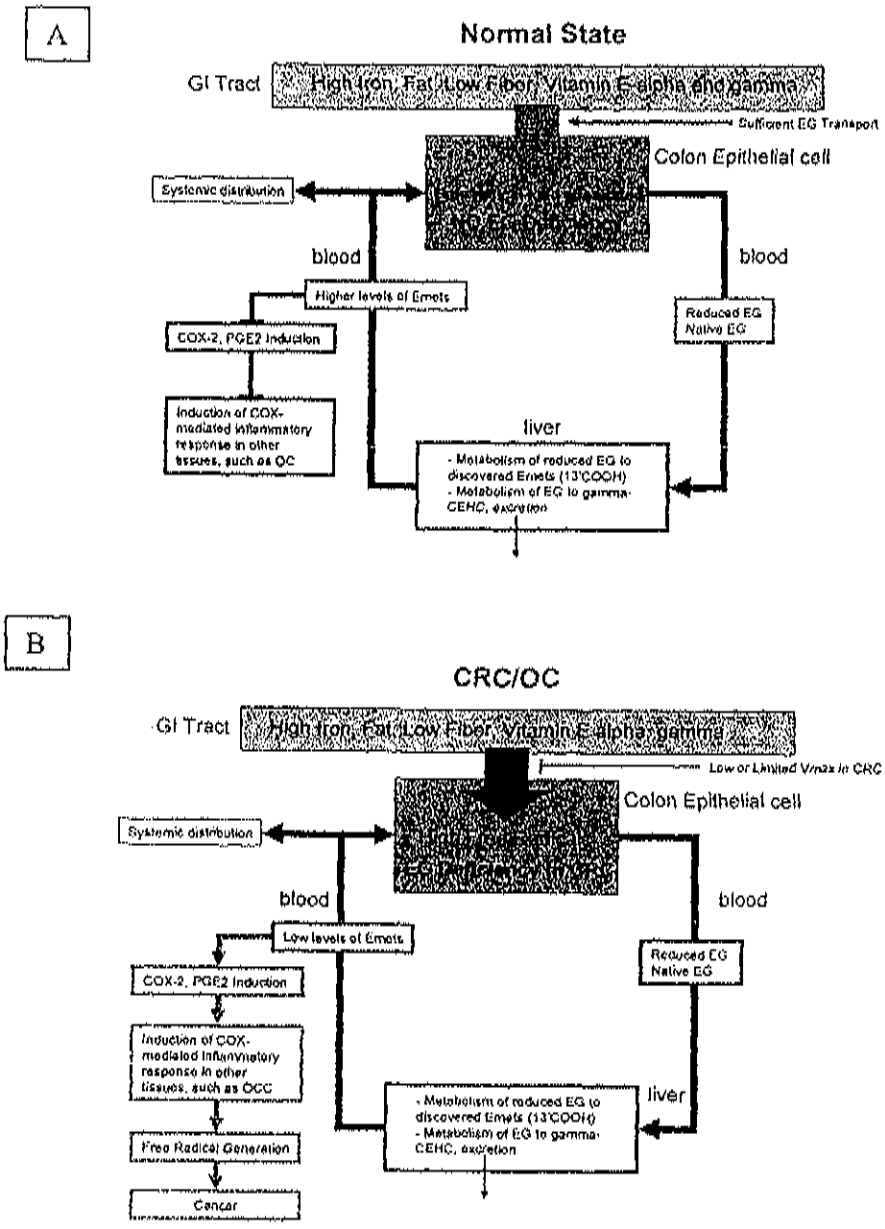

Liver P450 Metabolism
Formed by Gamma-T free radical scavenging in colon epithelial cells and then excreted into blood stream
W oxidation pathway via P450 during first pass metabolism in liver
COOH
phenomenome discoveries

FIGURE 36


A
Normal State
GI Tract
High Iron, Fat, Low Fiber, Vitamin E alpha and gamma
Sufficient EG Transport
Colon Epithelial cell
Systemic distribution
blood
blood
Higher levels of Emts
Reduced EG
Native EG
COX-2, PGE2 Induction
Induction of COX-mediated inflammatory response in other tissues, such as OC
liver
- Metabolism of reduced EG to discovered Emts (13'COOH)
- Metabolism of EG to gamma-CEHC, excretion
B
CRC/OC
GI Tract
High Iron, Fat, Low Fiber, Vitamin E alpha, gamma
Low or Limited Vmax in CRC
Colon Epithelial cell
Systemic distribution
blood
blood
Low levels of Emts
Reduced EG
Native EG
COX-2, PGE2 Induction
Induction of COX-mediated inflammatory response in other tissues, such as OCC
liver
- Metabolism of reduced EG to discovered Emts (13'COOH)
- Metabolism of EG to gamma-CEHC, excretion
Free Radical Generation
Cancer