# Concepts for Development of an Analytical Method to Determine CLA Composition in Foods, Dietary Supplements and Reference Materials


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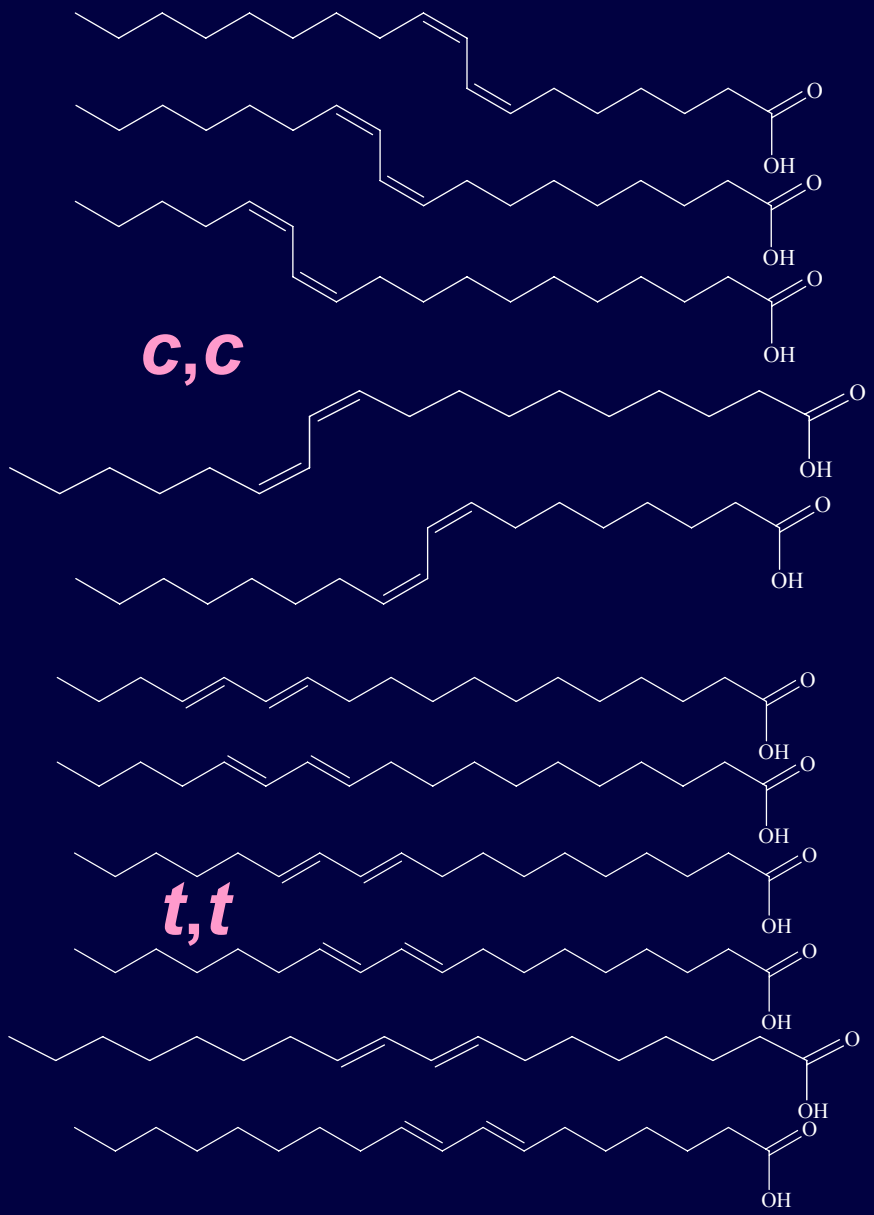
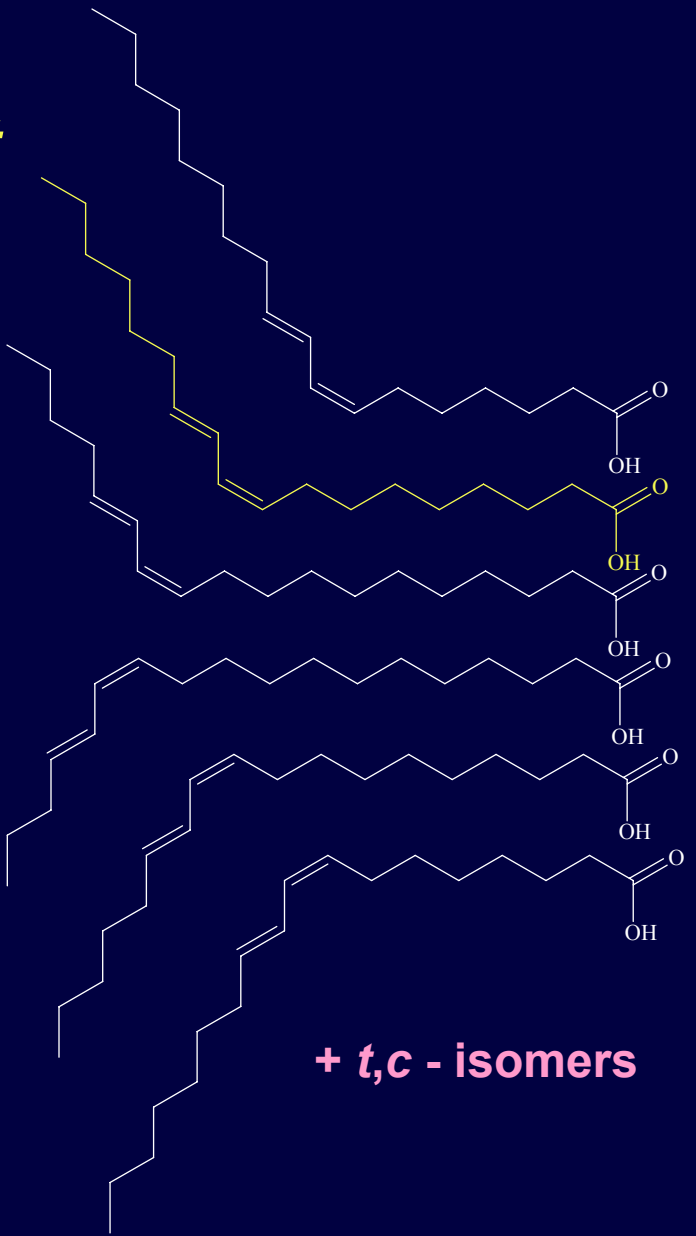
# Geometrical and positional CLA Isomers



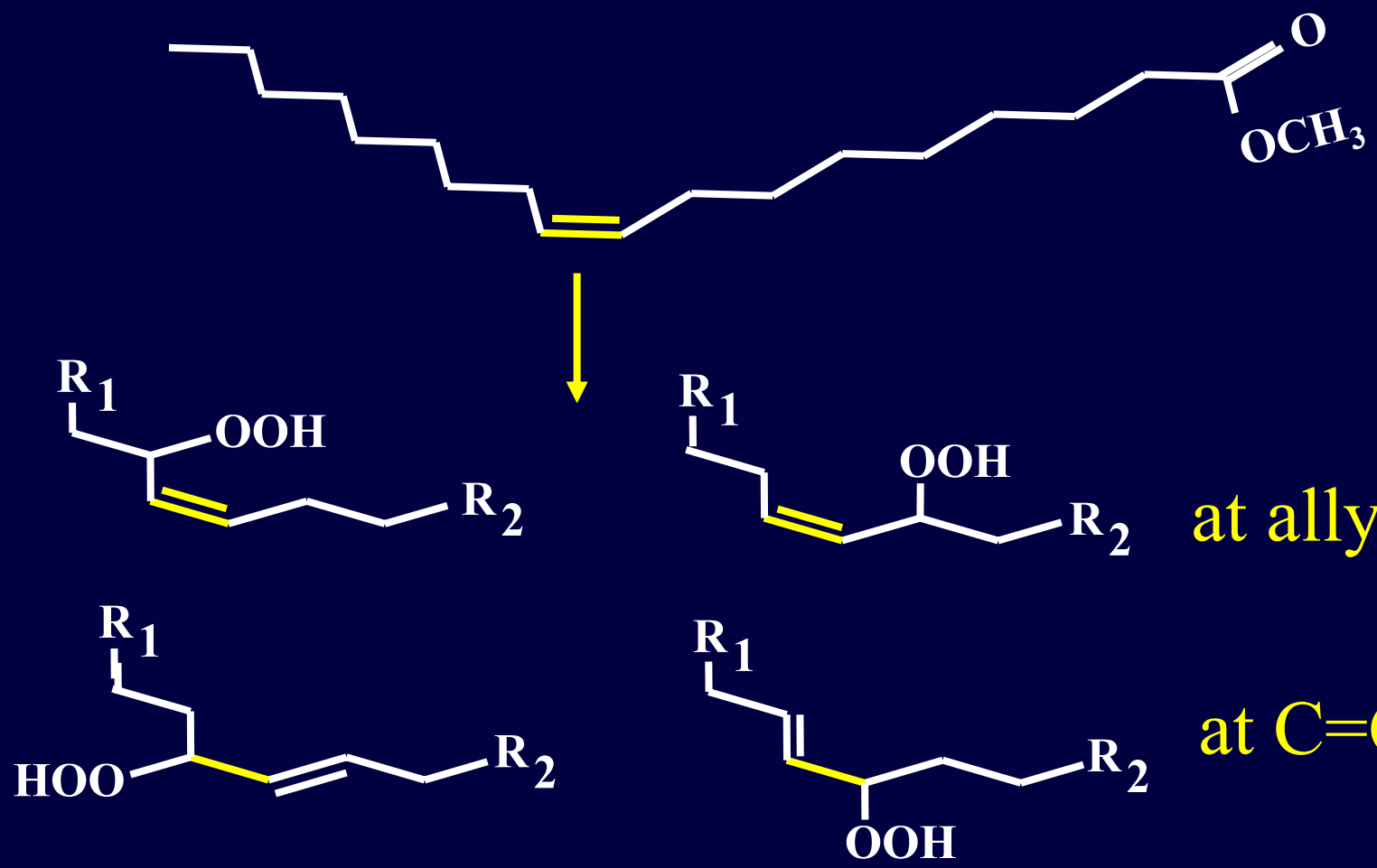



# Detection of CLA isomers

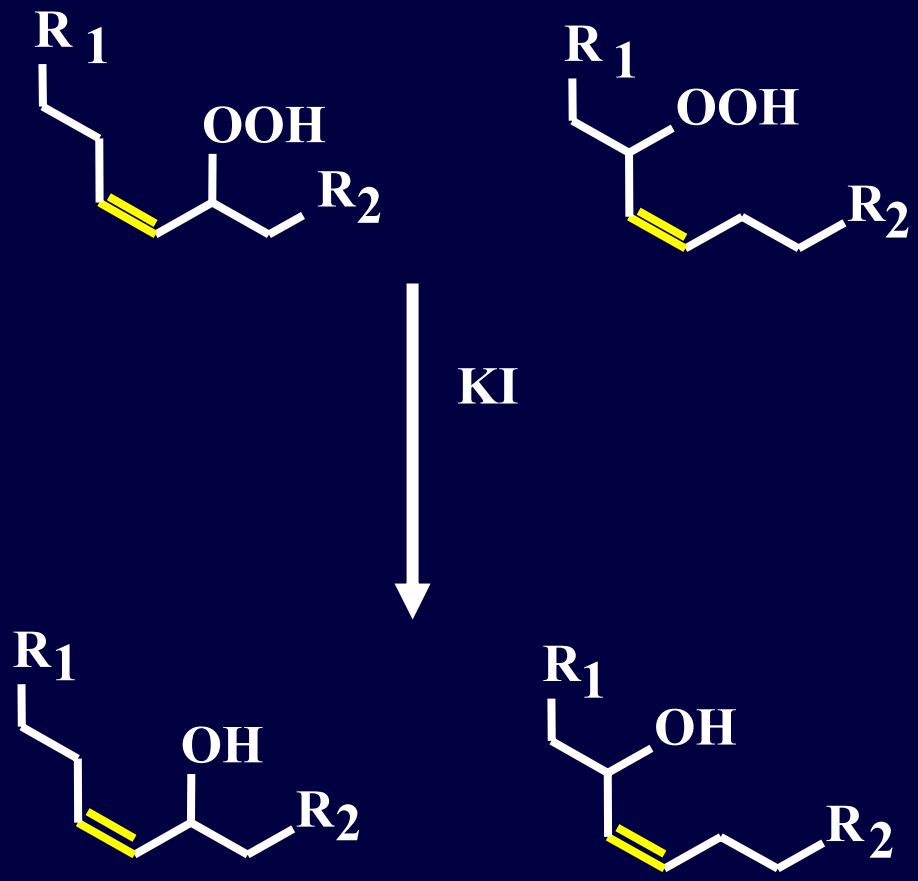
- **GC/FID - non-selective detector**
- **GC/DD/FTIR - semi-selective detector. *c/t* at 985 and 947 (+/- 4 $cm^{-1}$). The wave number of the carbonyl band was used as reference.**
- **$Ag^{+}$-HPLC - selective detector for conjugated double bonds at 233 nm.**
- **High resolution GC/MS - selective detector for 18:2 FAME at 294.2559 *m/z*.**

# Methylation procedures

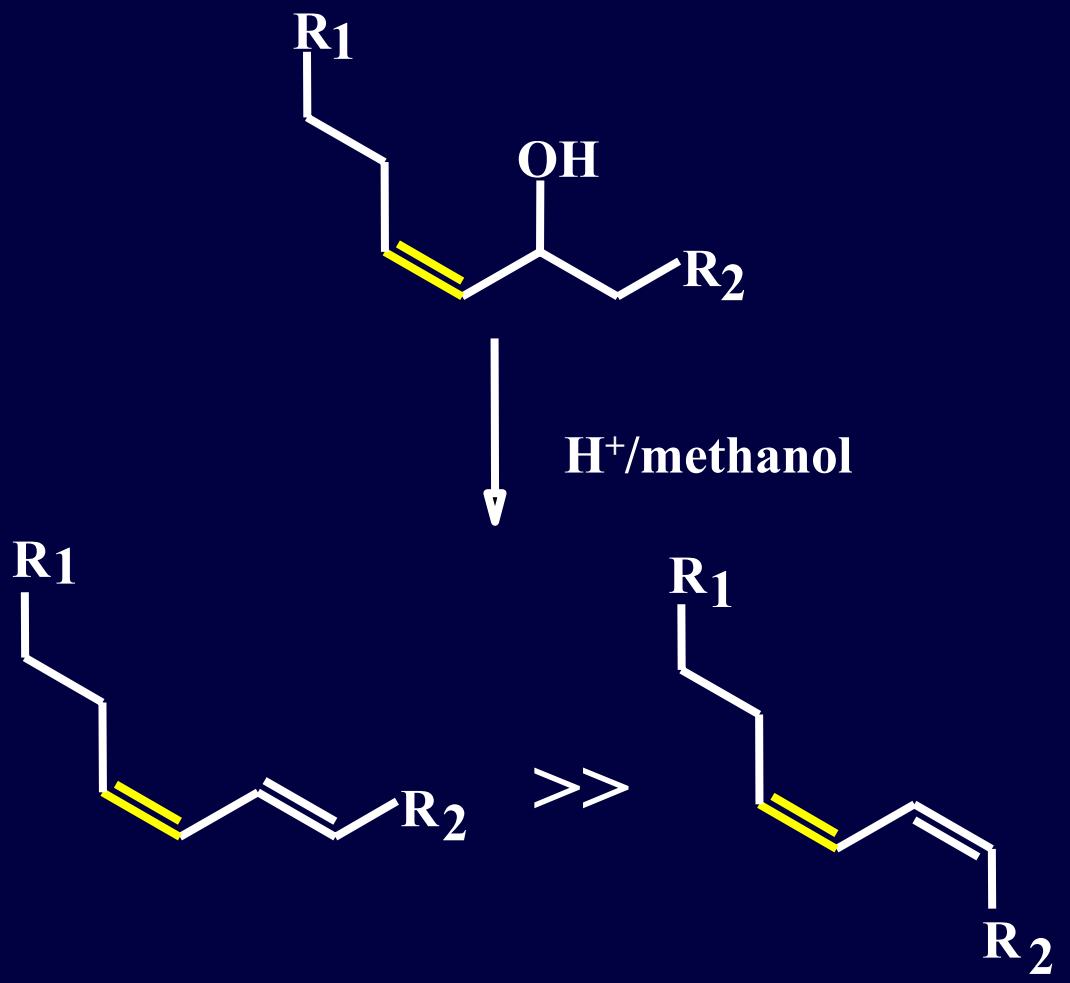
# Oxidation of Monoenes



# Reduction of oxidized monoenes

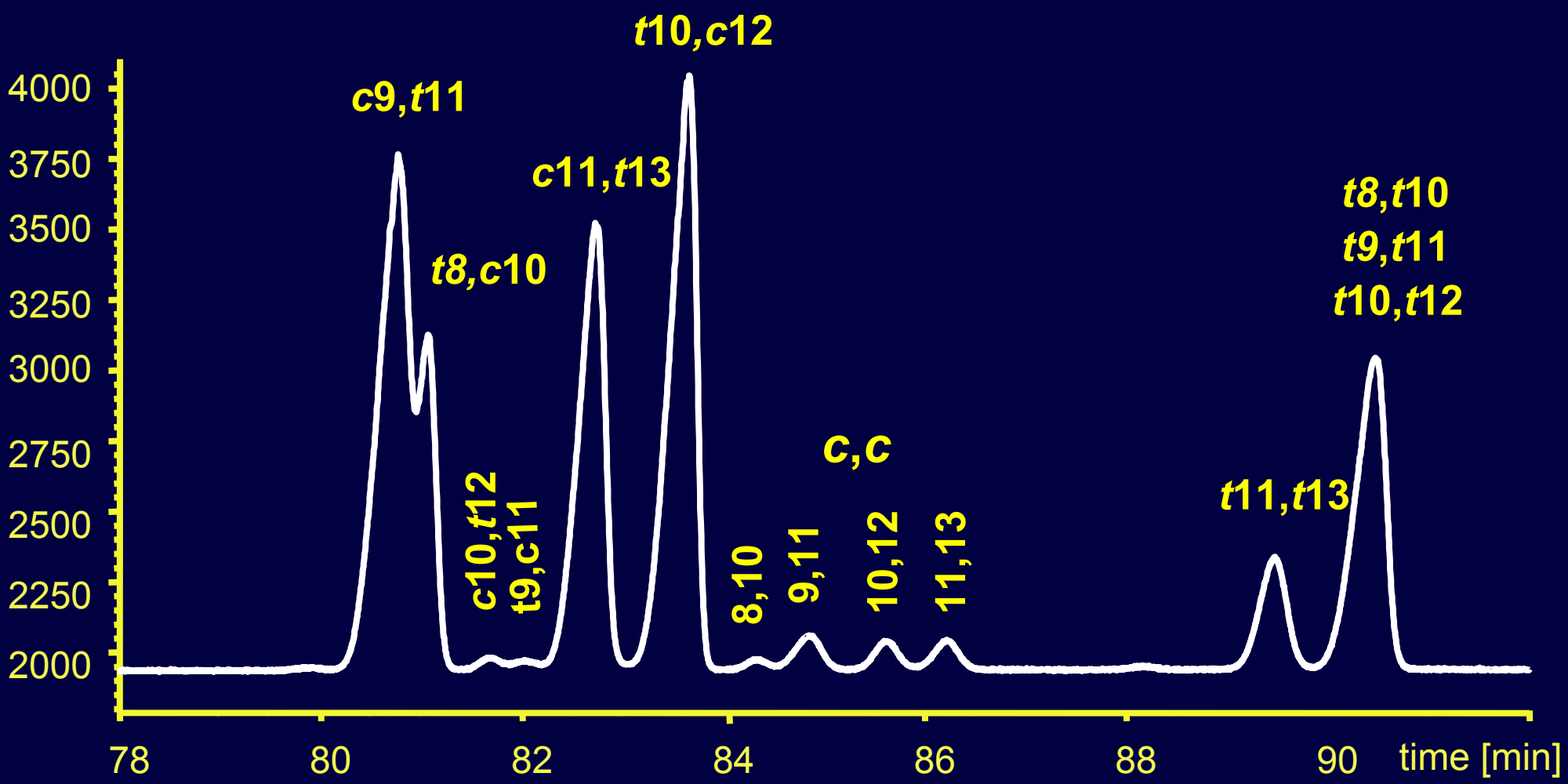


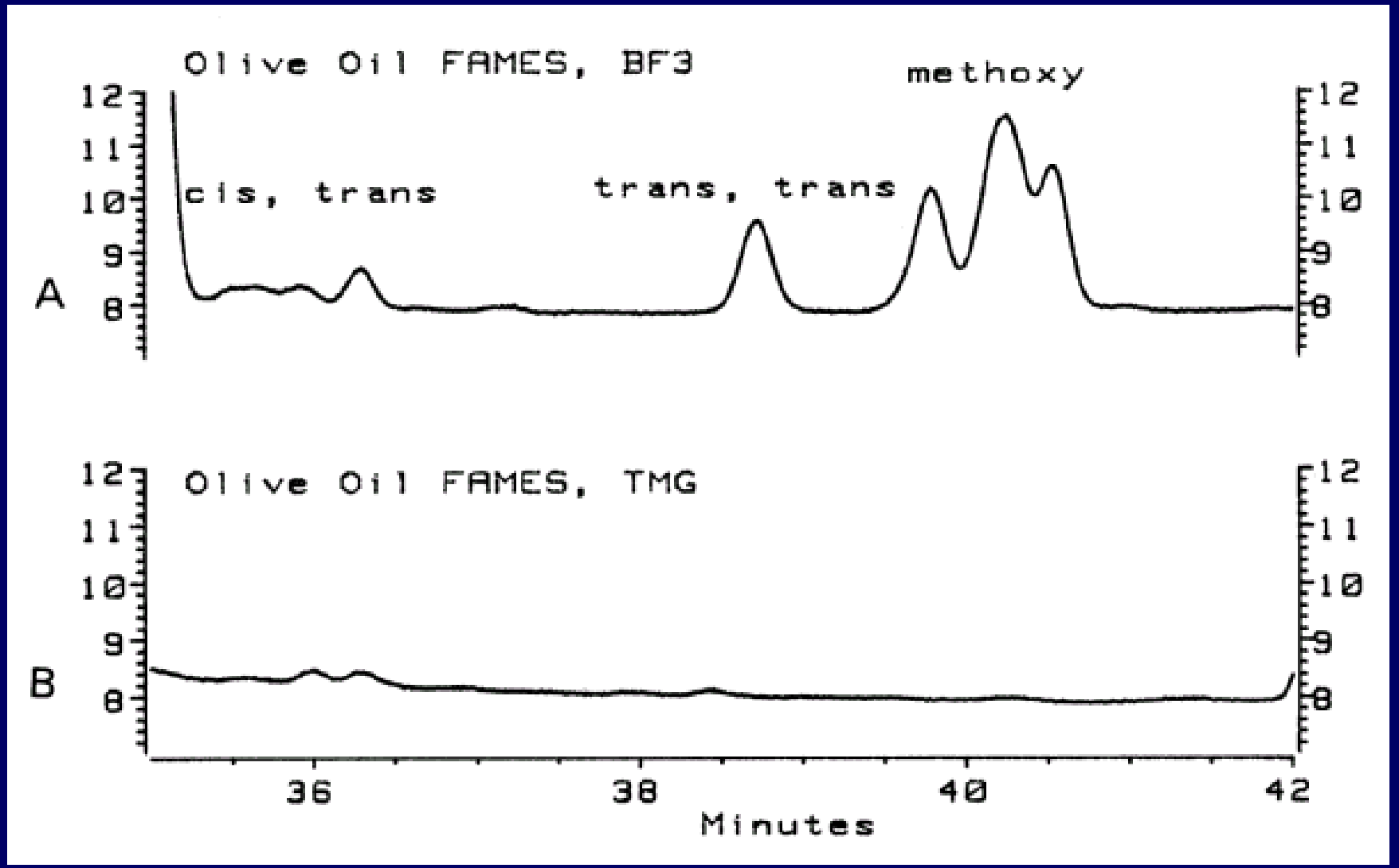
# Dehydration of LOH




**Plus other products**

# GC chromatogram of a CLA reference




Olive Oil FAMES, BF3
methoxy
cis, trans
trans, trans
A
12
11
10
9
8
Olive Oil FAMES, TMG
B
36
38
40
42
Minutes

# Methylation Procedure

- Weigh 5-30 mg of test sample into a screw capped test tube
- Dissolve test sample in benzene or toluene (<1 mL)
- Add 2 mL of 0.5 N NaOMe/methanol (Supelco Inc.)
- Store overnight in the dark or heat for 15 minutes at 50°C
- Add $H_2O$ to make a methanol:$H_2O$ mixture (95:5) which expels hexane
- Add 2-3 mL hexane, mix and remove hexane layer containing the FAME
- Dry hexane layer over $Na_2SO_4$ and analyze directly by GC or HPLC

# TrimethylsilylDiazomethane Methylation

- Weigh 5-25 mg FFA into a screw capped test tube.

- Add 1 mL 20% methanol/benzene and 0.5 mL of 10% TMS- diazomethane in hexane solution.

- Let stand for 30 min with occasional gentle shaking or constant stirring.

- Add glacial acetic acid dropwise with gentle swirling to remove excess TMS-diazomethane. (If color does not disappear after 10 drops, proceed to the next step.)

- Add 5 mL $H_2O$ and 5 mL petroleum ether; shake for 30 s, dry petroleum ether over $Na_2SO_4$. Use petroleum ether solution directly for GC or HPLC analysis.

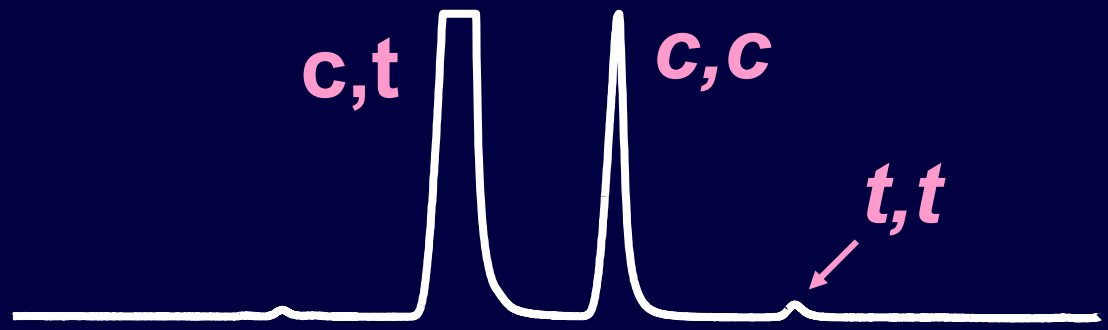
# Synthesis

# Isomerization with iodine

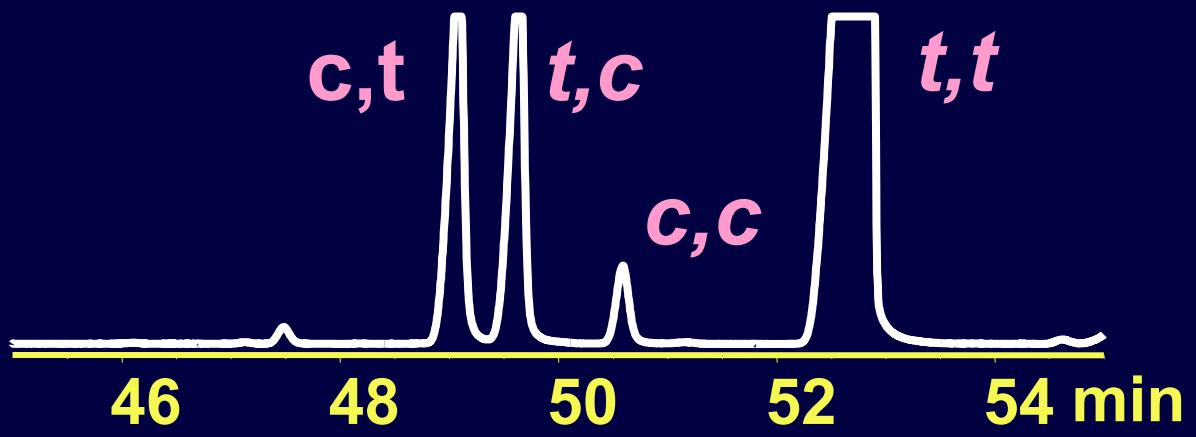
- **Dissolve CLA FAME in hexane**
- **Add a few crystals of iodine**
- **Expose to daylight in closed flask**
  **(flush with $N_2$ to prevent oxidation)**
- **Remove iodine with $Na_2S_2O_3$**
  **(wash thoroughly)**
- **Dry hexane solution with $Na_2SO_4$**

# Isomerization of 9,11-CLA [GC]

Before Isomerization



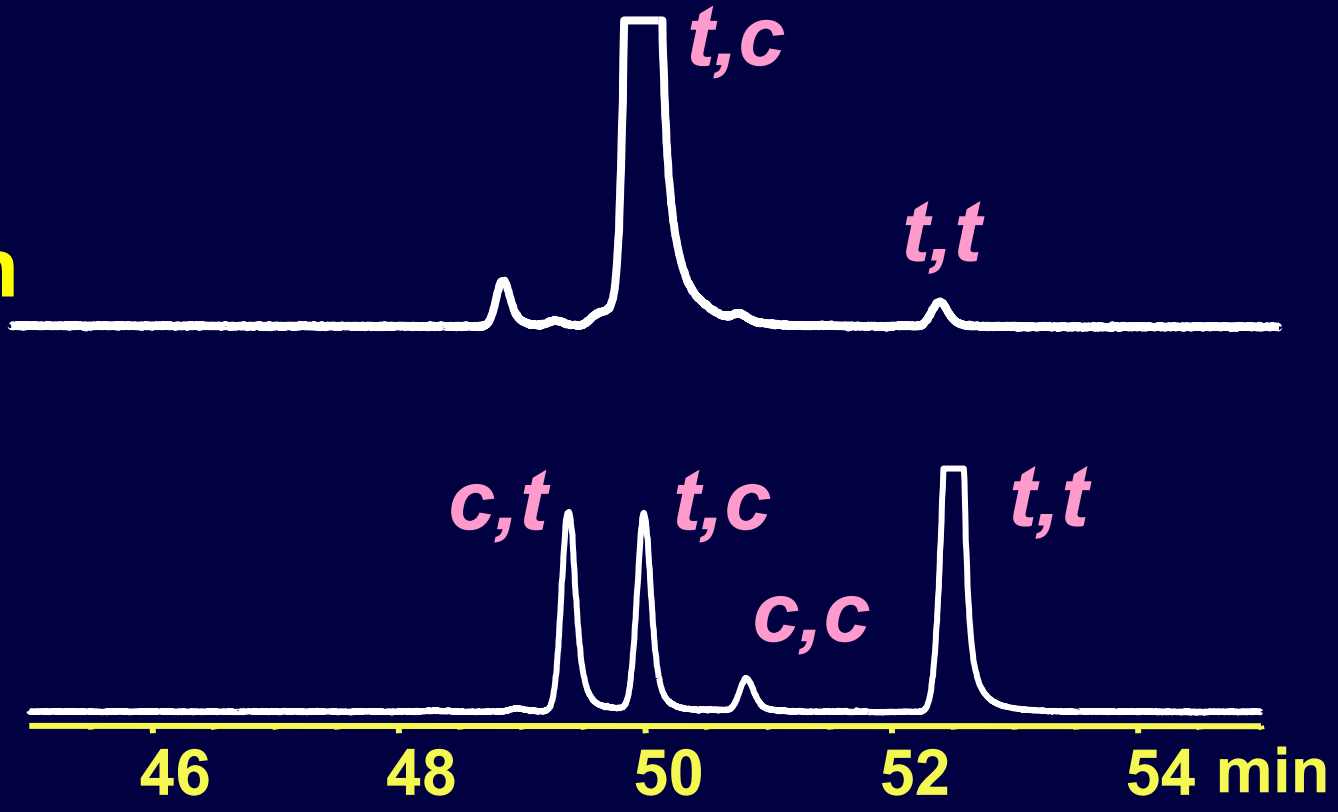

After Isomerization



# Isomerization of 10,12-CLA [GC]

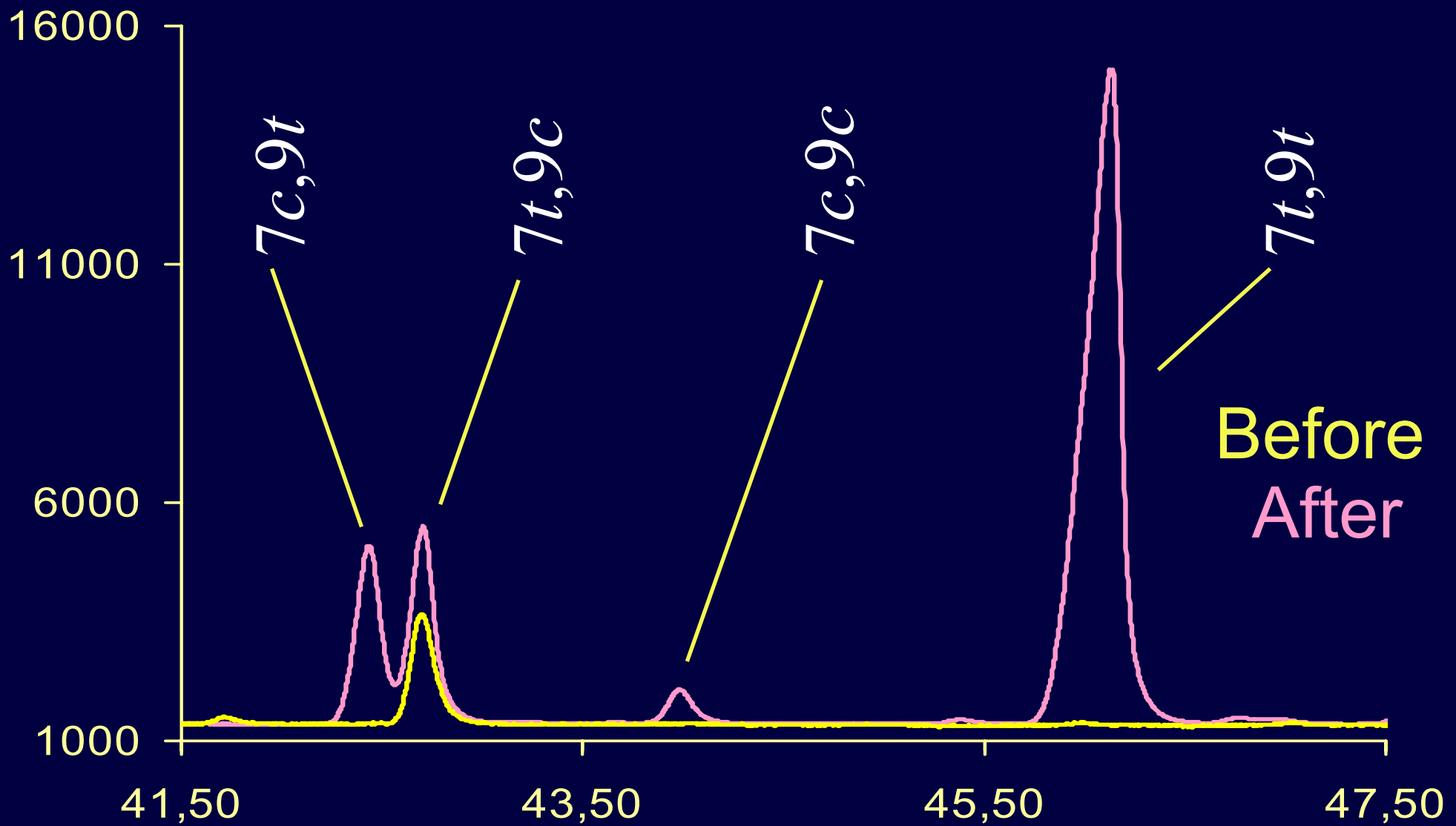
Before Isomerization

After Isomerization



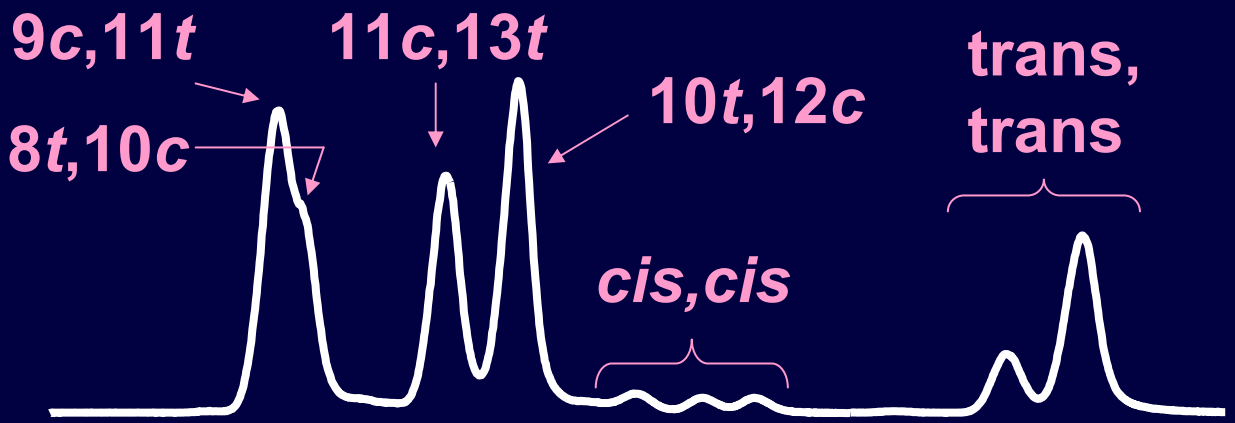
# 7*t*,9*c* $I_2$ isomerized.

GC

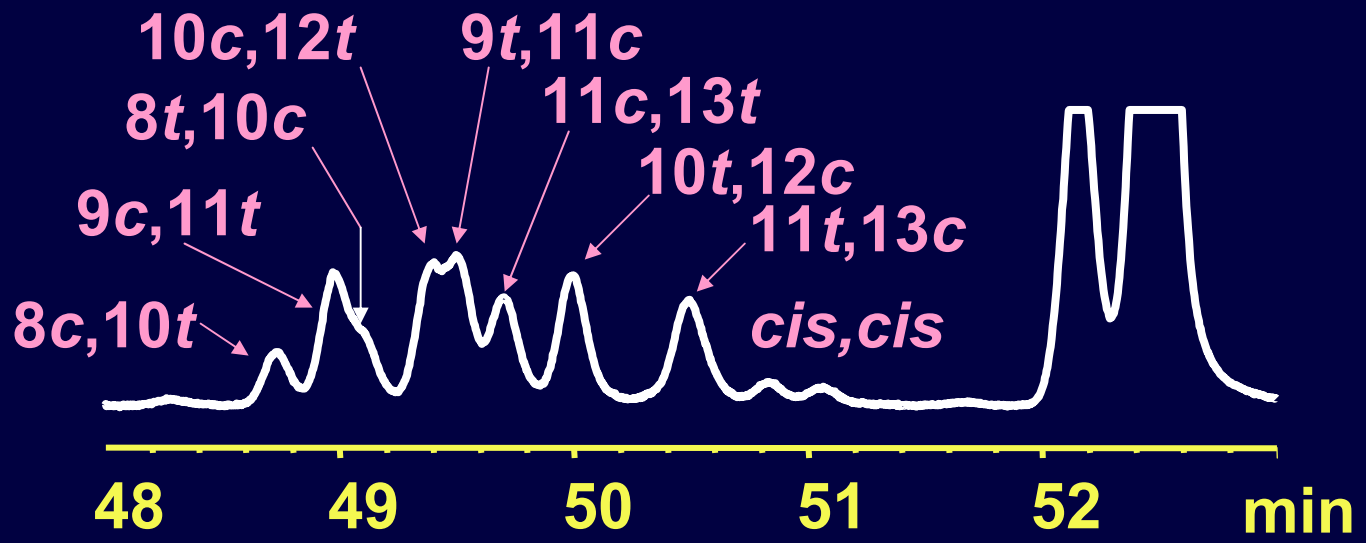


# Isomerization of Nu-Chek FAME CLA (GC)

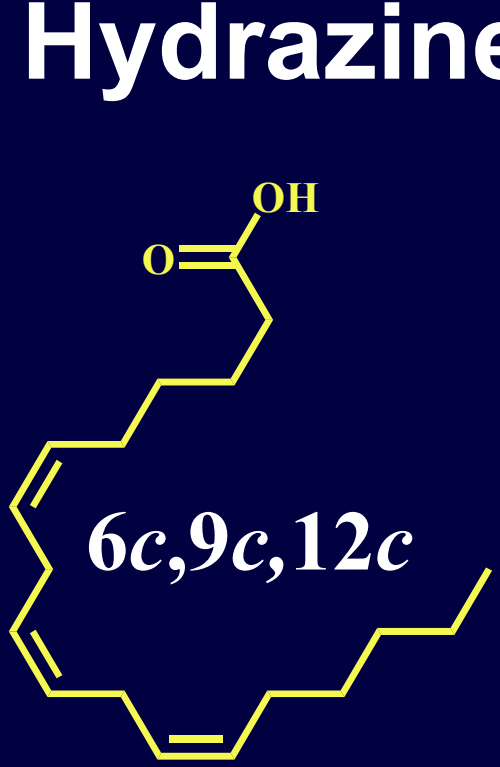
**Before Isomerization**




**After Isomerization**

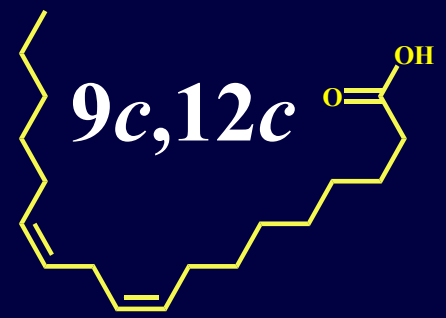


# Reduction of γ-Linolenic Acid with Hydrazine.




**10% Hydrazine in Methanol**

**45°C**, 120 min.



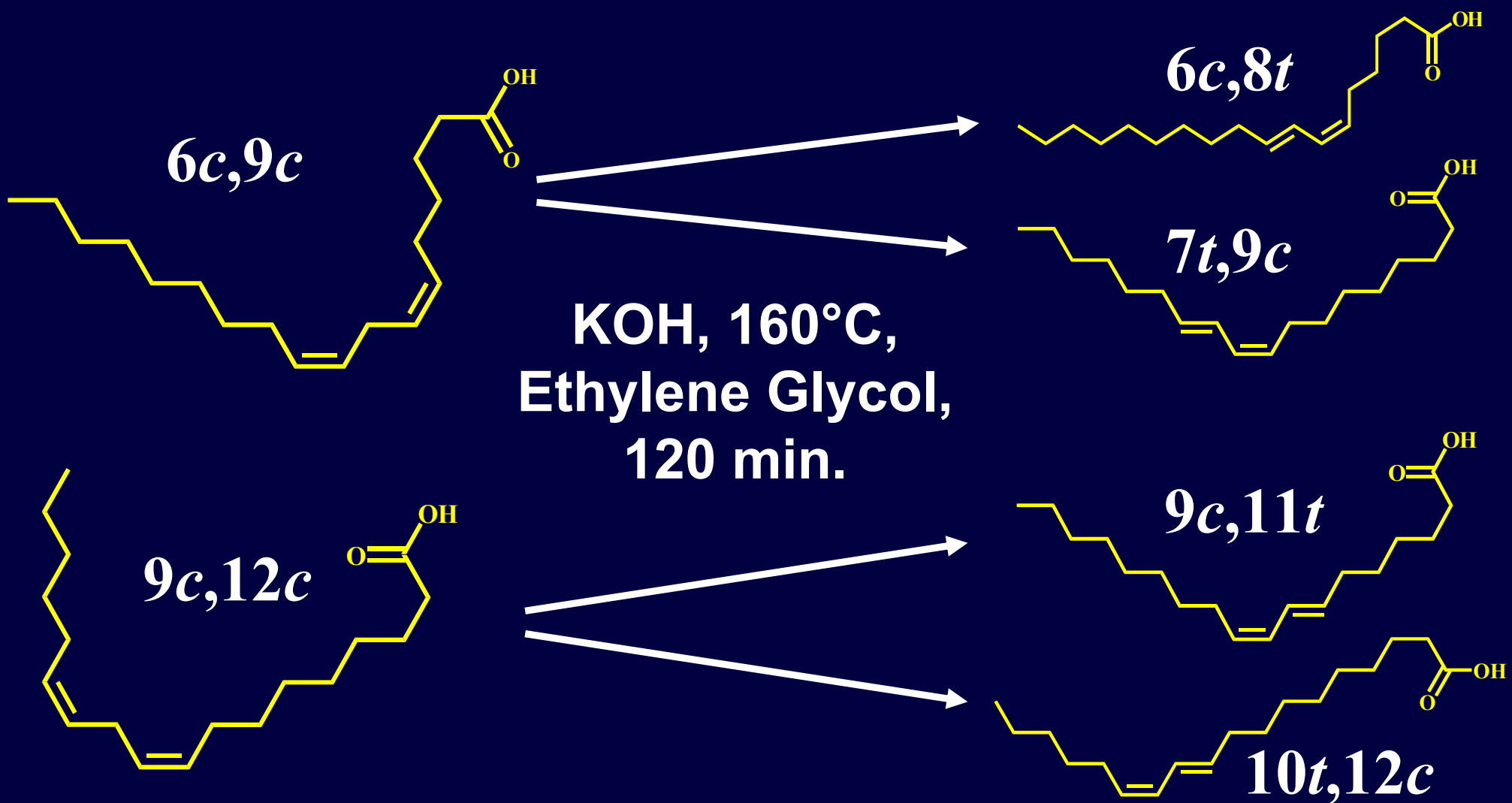
**γ-Linolenic Acid**

**(6c,9c,12c-Octadecatrienoic acid)**

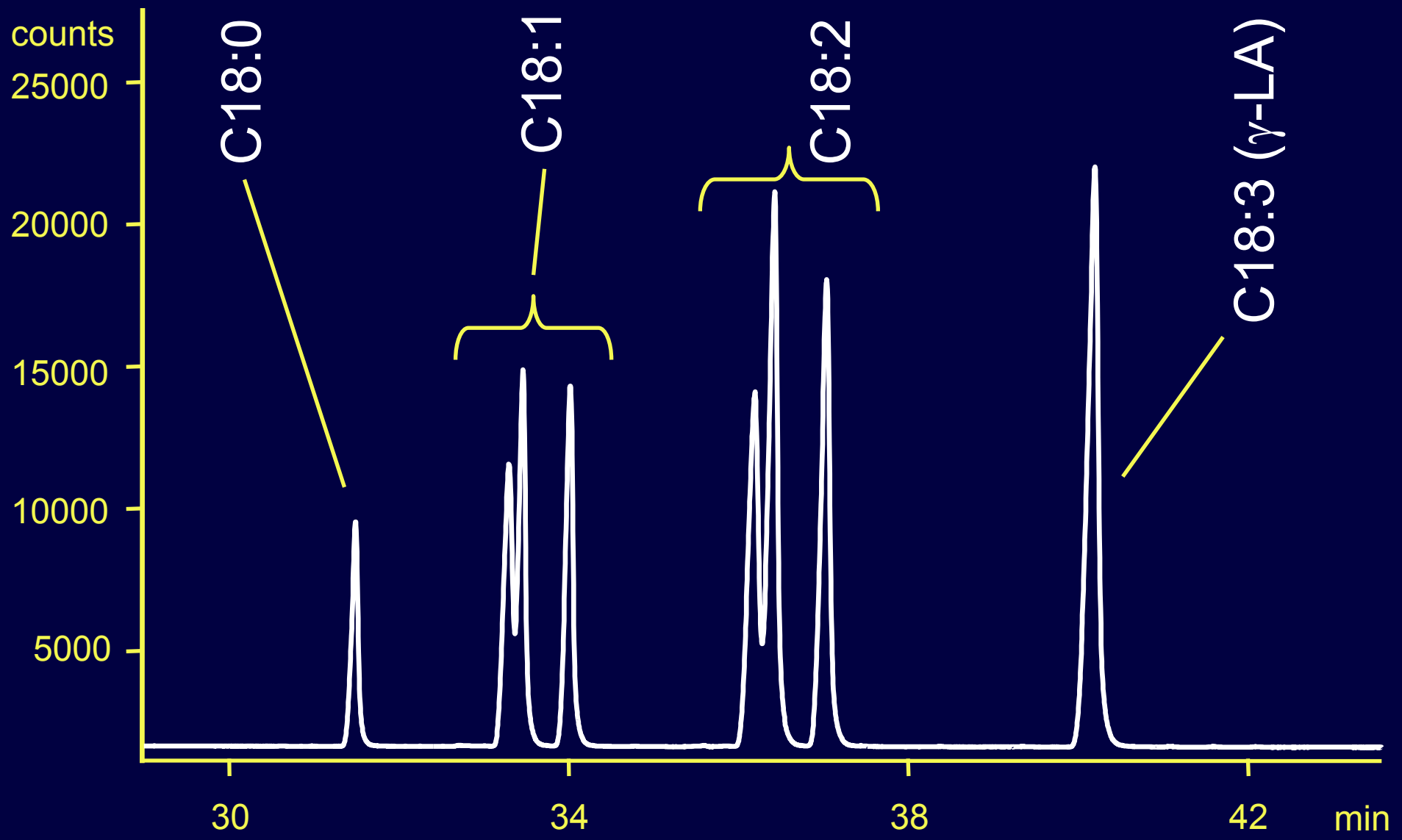
\+

**6*c*-**C18:1, **9*c*-**C18:1
**12*c*-**C18:1, C18:0

# Alkali Isomerization of dienes.

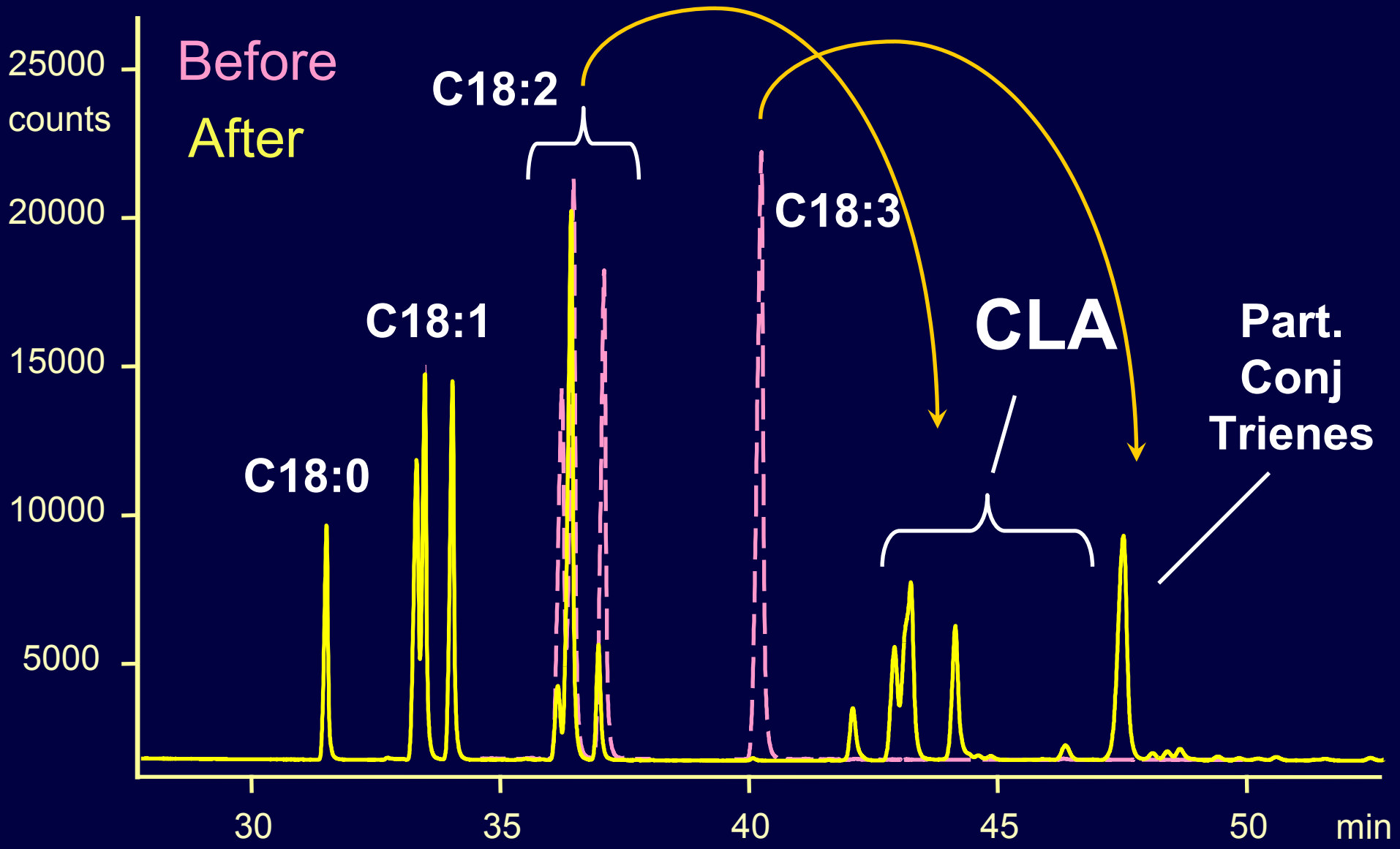


# Reduction with hydrazine.

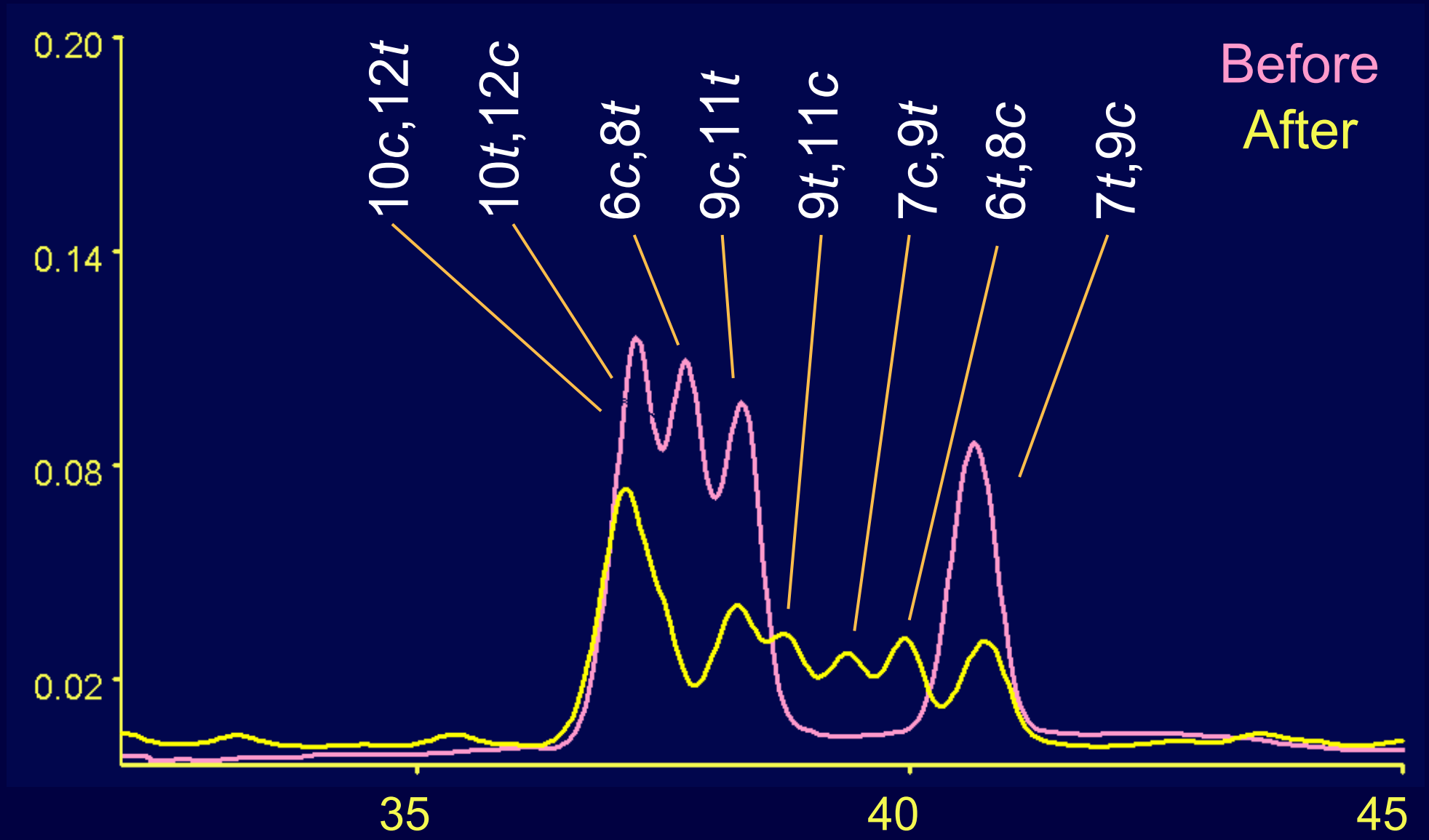


# Isomerization with alkali.

GC



# Reaction products $I_2$ isomerized (*c*,*t*). HPLC



# Ag+ HPLC

# 3X Ag+ HPLC

- **3 ChromSpher 5 lipid columns used in series.**
- **Mobile phase, (0.1 % MeCN, 0.5 % ethyl ether)/hexane, isocratic, 1.0 mL/min.**
- **Column was pretreated daily by eluting with 1% acetonitrile/hexane for 30 minutes prior to analysis.**
- **Column was equilibrated with the elution solvent for at least 30 minutes prior to test portion injection.**
- **Typical injections of test portion volumes were 5-40 μL, representing < 250 μg lipid.**
- **PDA-detection 200-300 nm, chromatogram extracted at 205, 229-234 nm.**

# Some Factors Effecting Ag+ HPLC

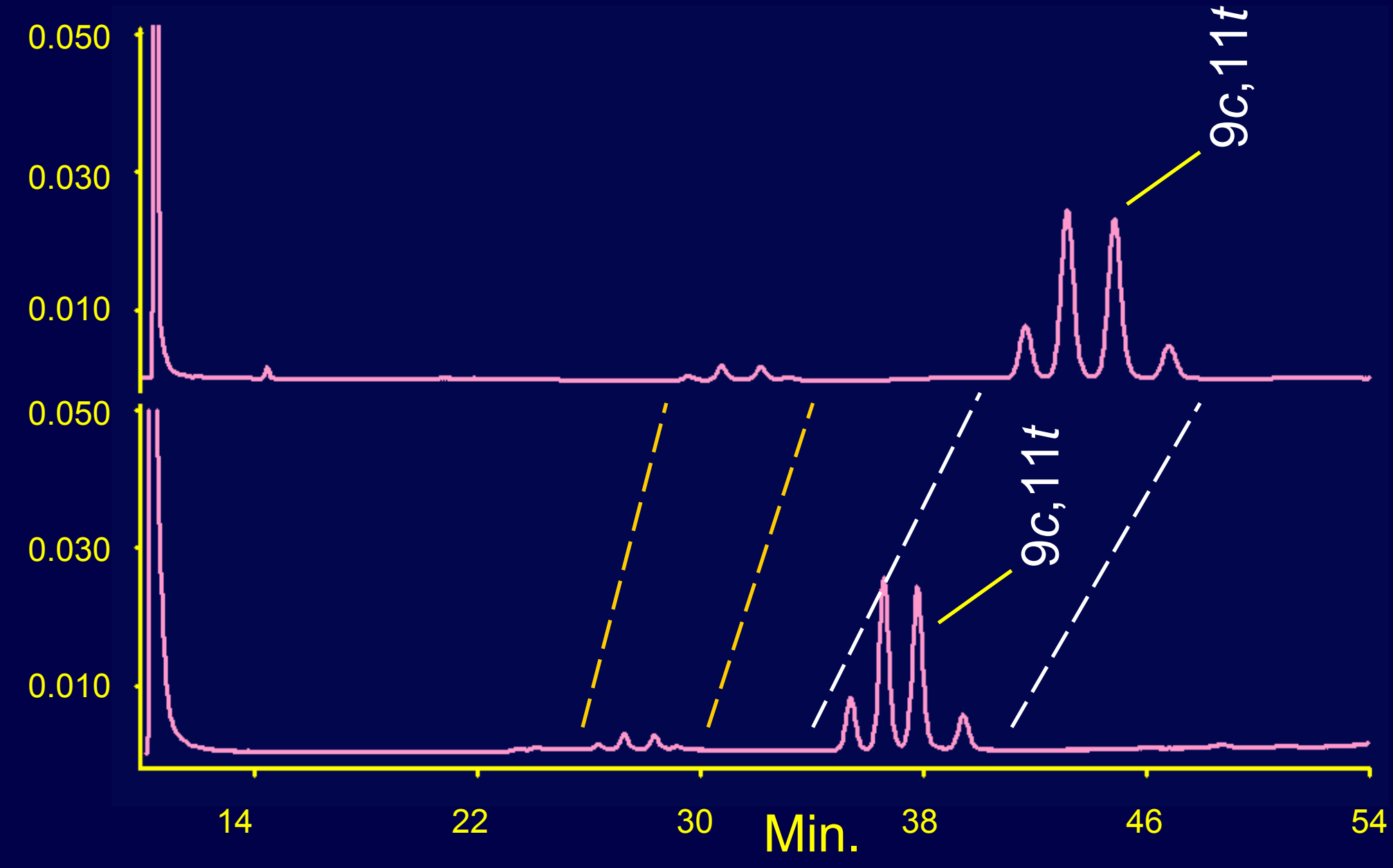
- Column temperature: Hold constant.
- Conditioning: Needed for reproducible RVs.
- Sample load: Overload causes loss of resolution. The overload on the Ag+ may be from components that do not have a chromophore.

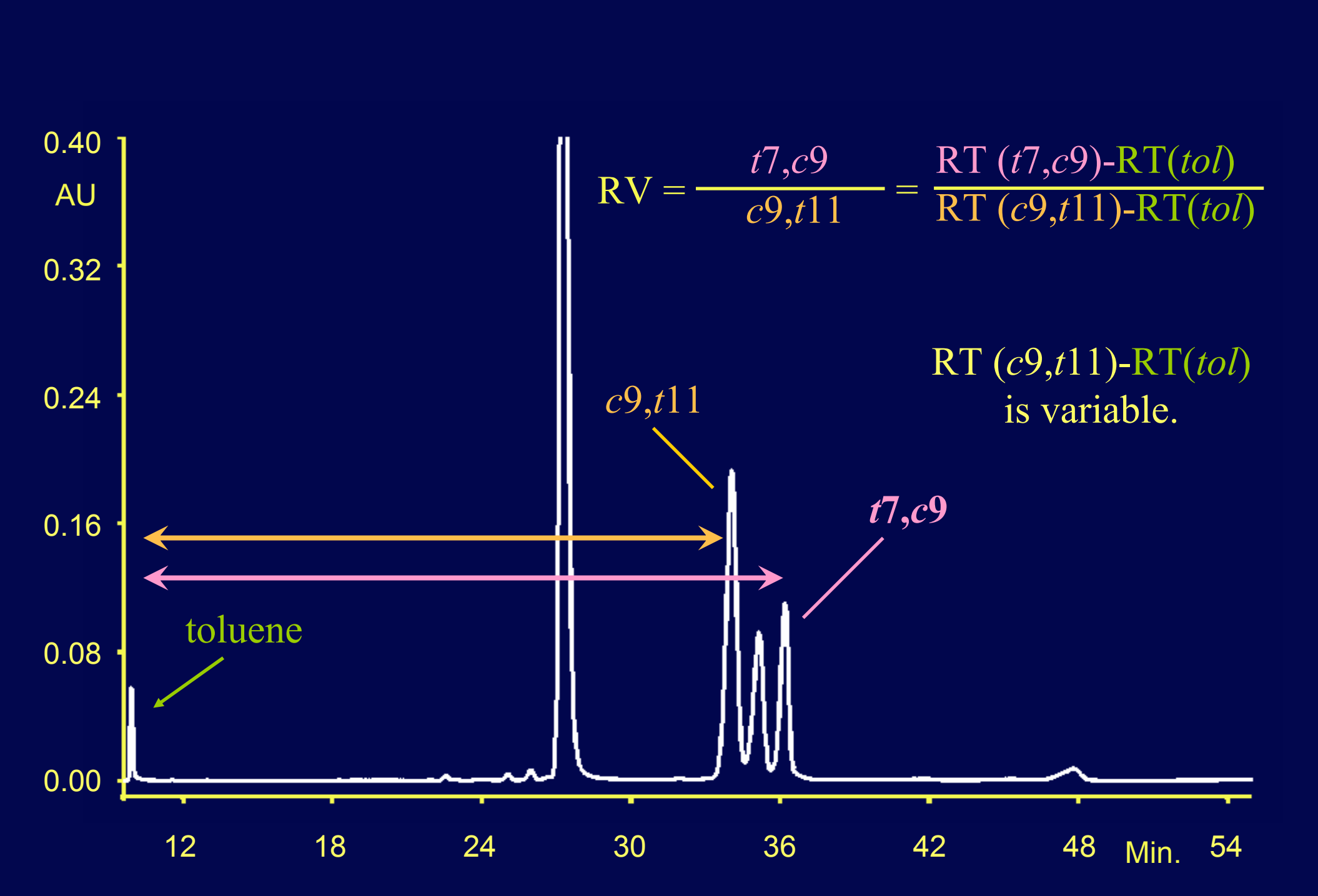

Reference:

(1) R. O. Adlof. (1997) In: New Techniques and Applications in Lipid Analysis. Eds. R. E. McDonald, M. M. Mossoba, AOCS Press, Champaign IL (USA) pp 256-265.

(2) B. Nikolova-Damyanova, S. Momchilova, W. W. Christie (2000) *J. High Resol. Chromatogr.* **23** 348-352.

# Retention volume drift

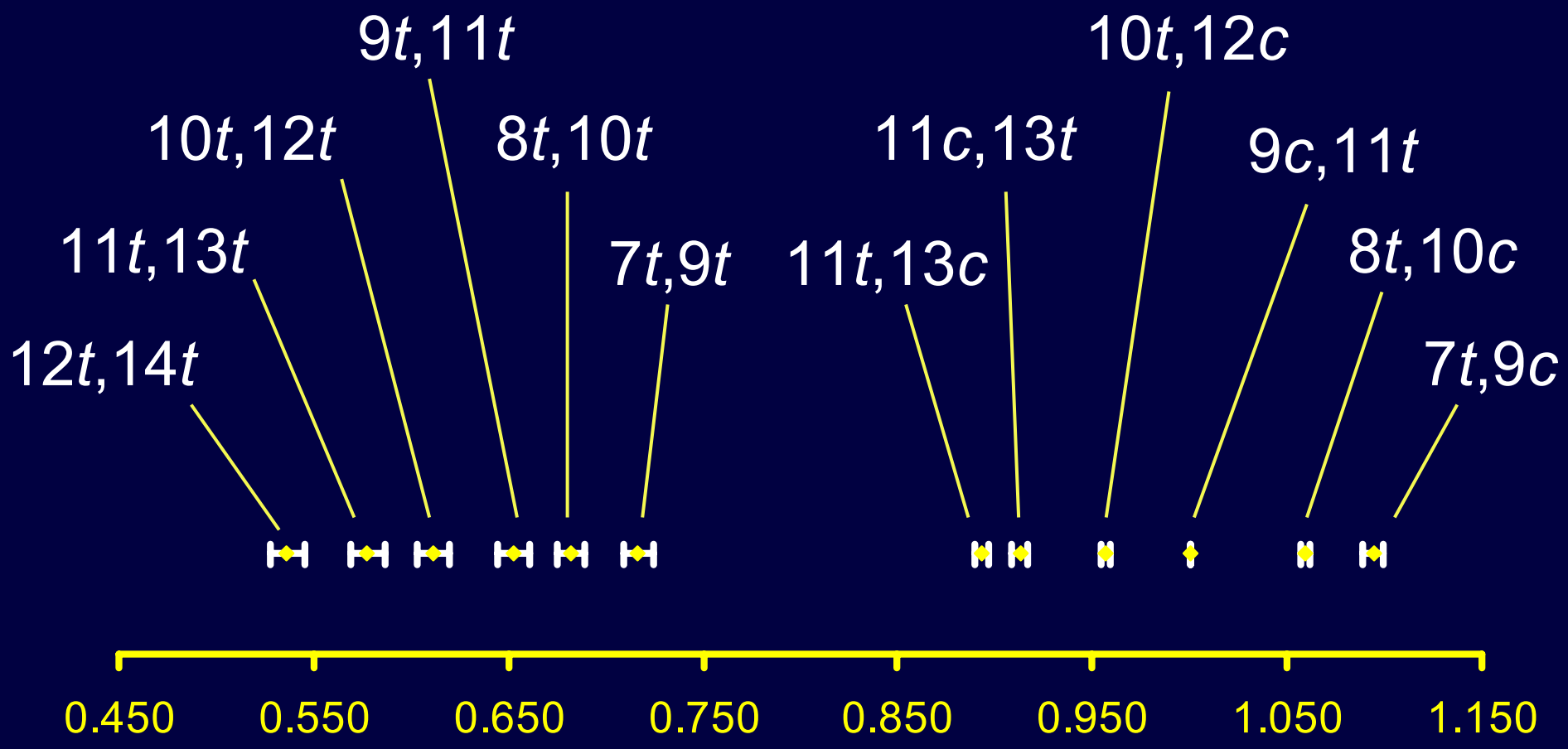



0.40
AU
0.32
0.24
0.16
0.08
0.00
12
18
24
30
36
42
48
Min.
54
RV = t7,c9 / c9,t11 = RT (t7,c9)-RT(tol) / RT (c9,t11)-RT(tol)
RT (c9,t11)-RT(tol) is variable.
c9,t11
t7,c9
toluene

# Rel. Ret. Vol., 88 samples

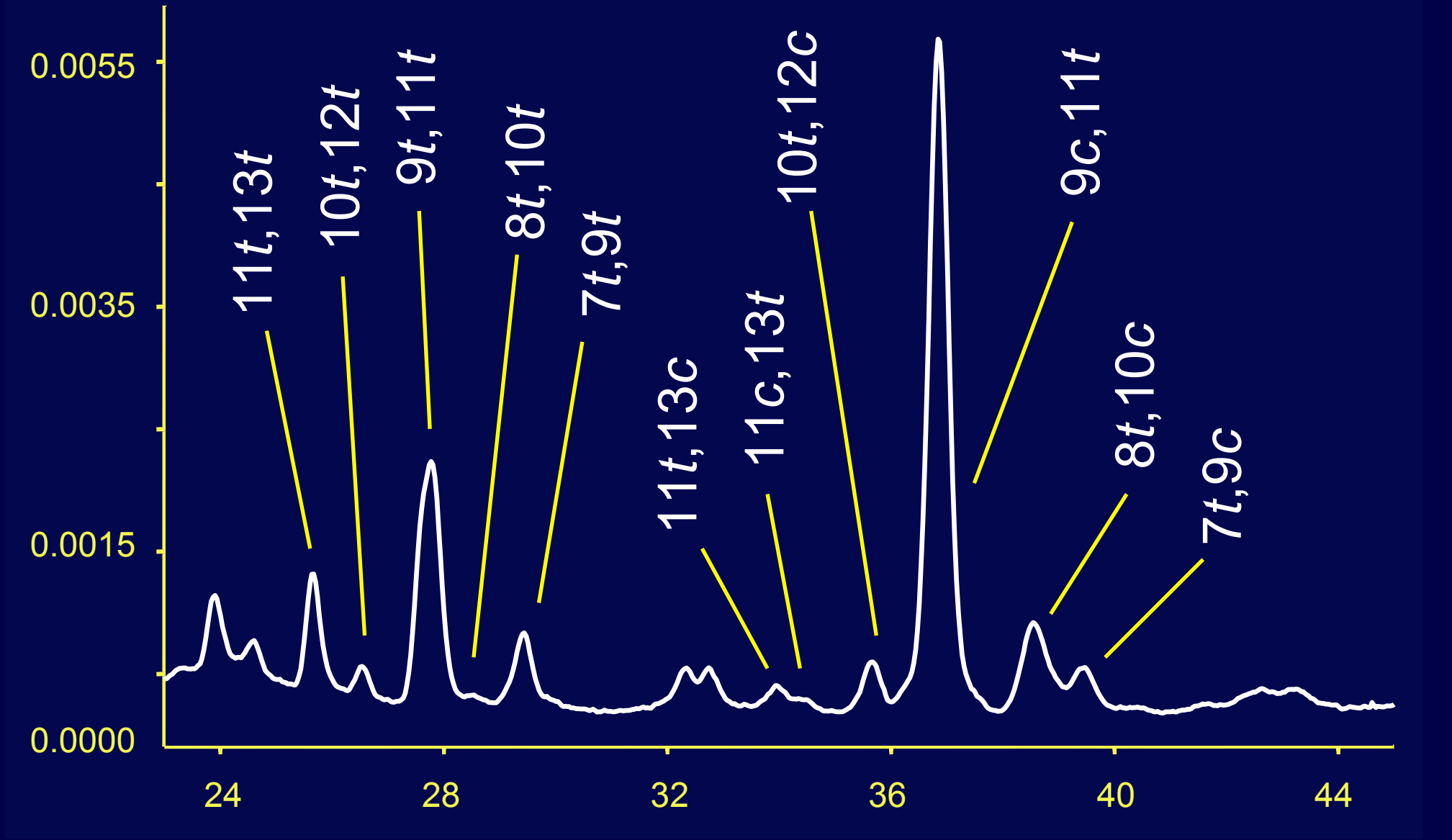
| | *Average Rel. Ret. Vol.* | *Std. Dev.* | *C.V. %* |
|---|---|---|---|
| *12t,14t* | 0.536 | 0.009 | 1.625 |
| *11t,13t* | 0.578 | 0.008 | 1.459 |
| *10t,12t* | 0.611 | 0.008 | 1.337 |
| *9t,11t* | 0.653 | 0.008 | 1.265 |
| *8t,10t* | 0.682 | 0.007 | 1.041 |
| *7t,9t* | 0.717 | 0.008 | 1.091 |
| *11t,13c* | 0.893 | 0.004 | 0.444 |
| *11c,13t* | 0.913 | 0.004 | 0.483 |
| *10t,12c* | 0.957 | 0.003 | 0.268 |
| *9c,11t* | 1.000 | 0.000 | 0.000 |
| *8t,10c* | 1.059 | 0.002 | 0.221 |
| *7t,9c* | 1.094 | 0.005 | 0.471 |

# Rel. Ret. Vol., 88 samples

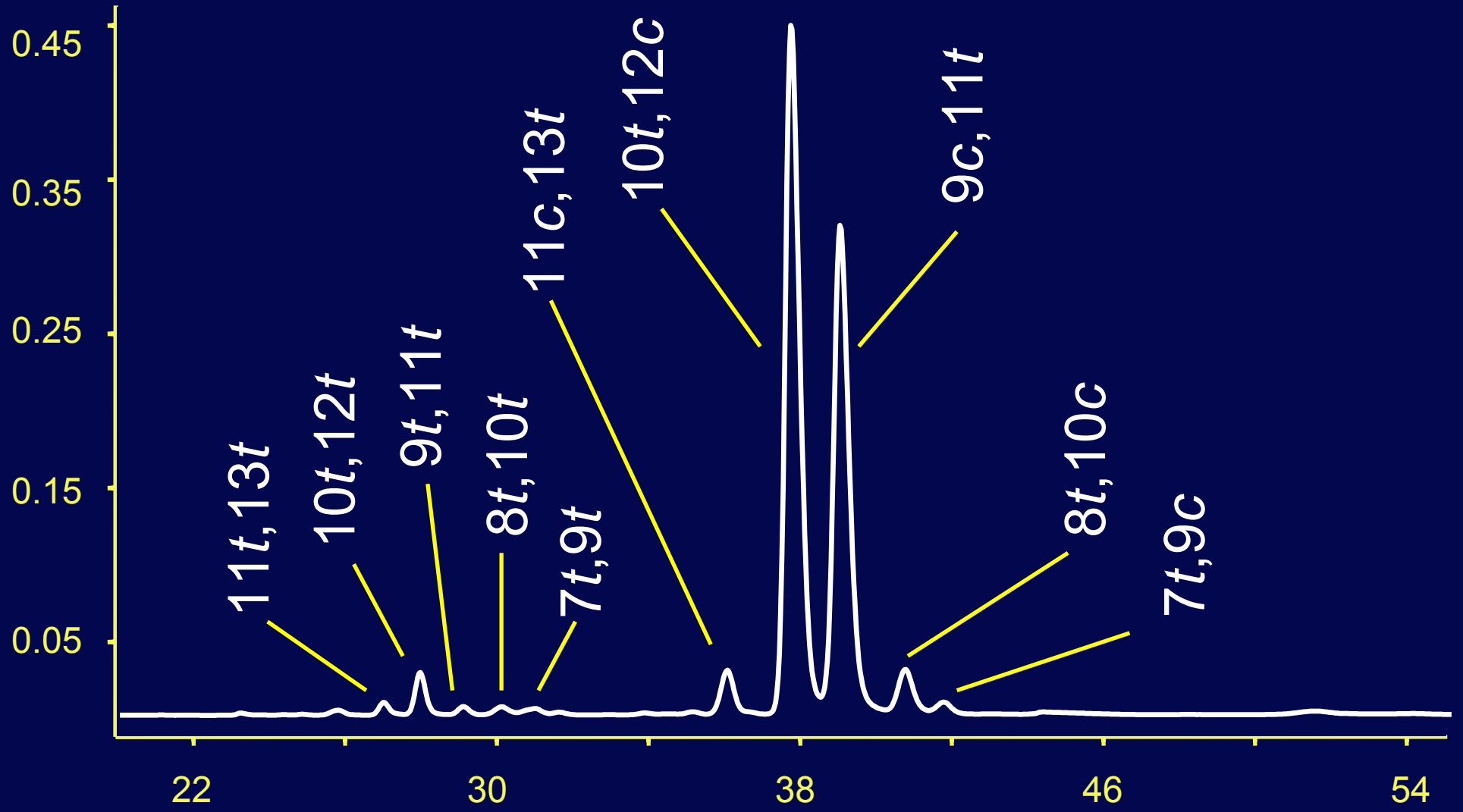


# Cow's Blood sample.

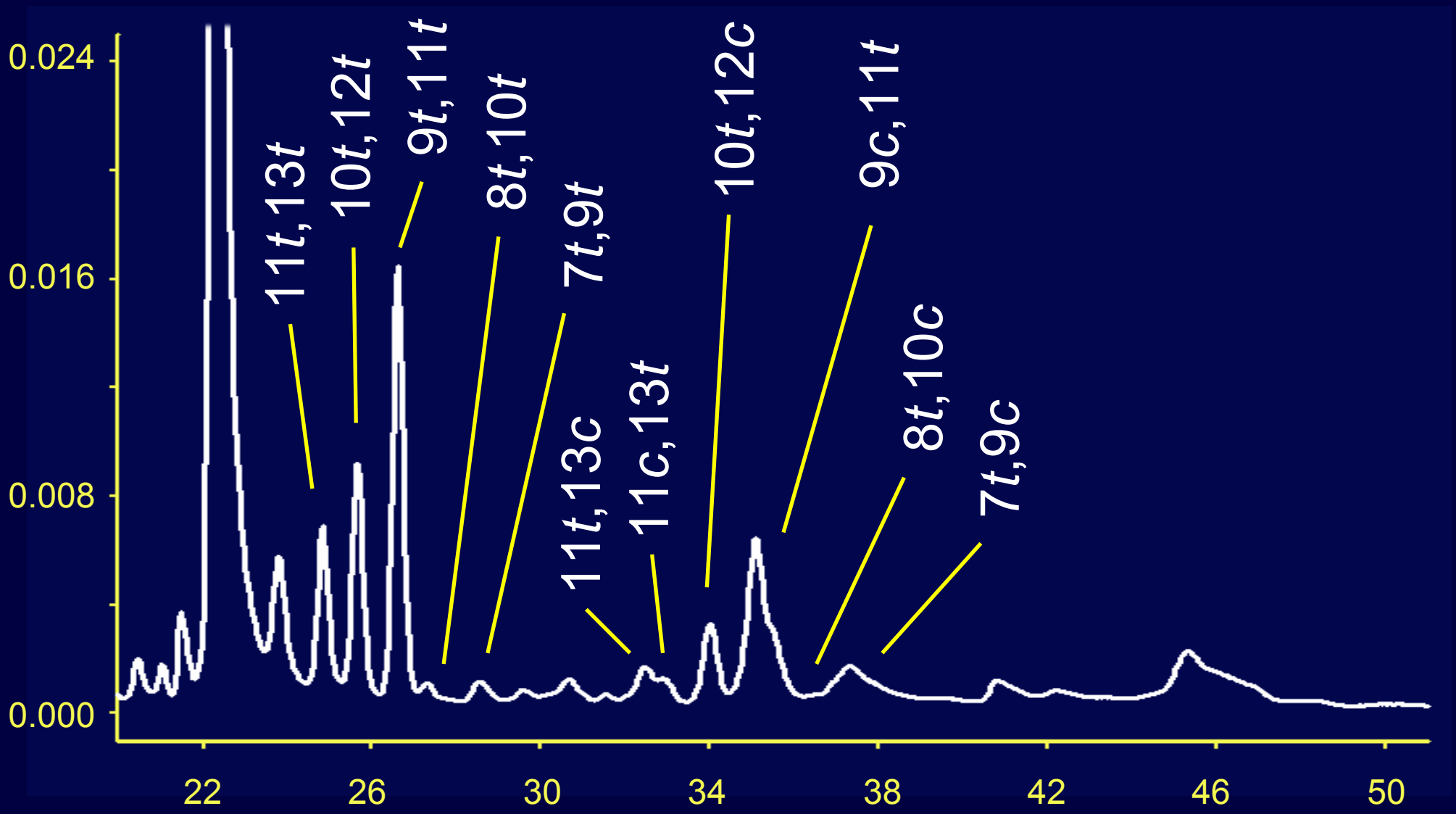
HPLC



# Milk sample with high 10*t*,12*c*



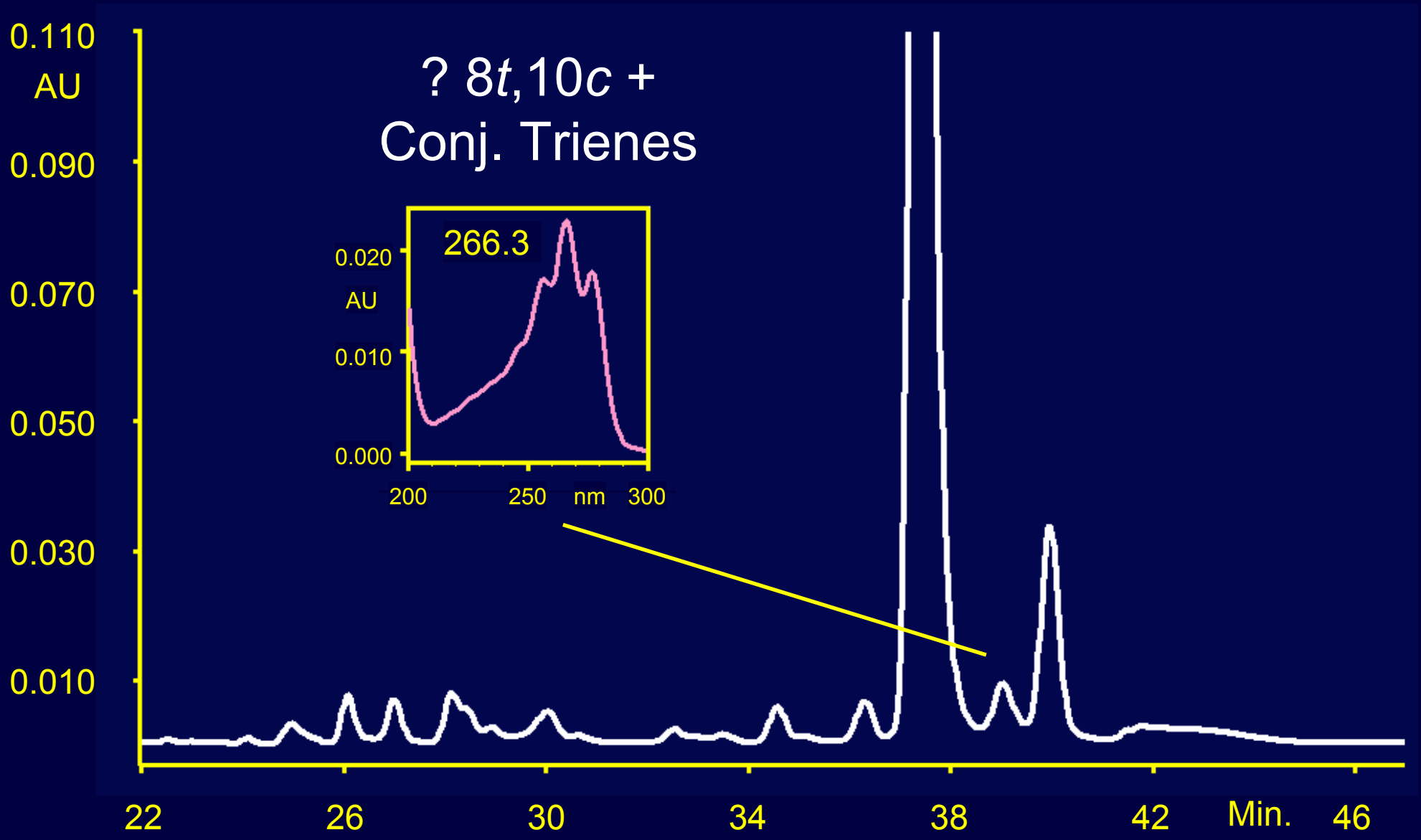
# Cow's Rumen Fluid sample.



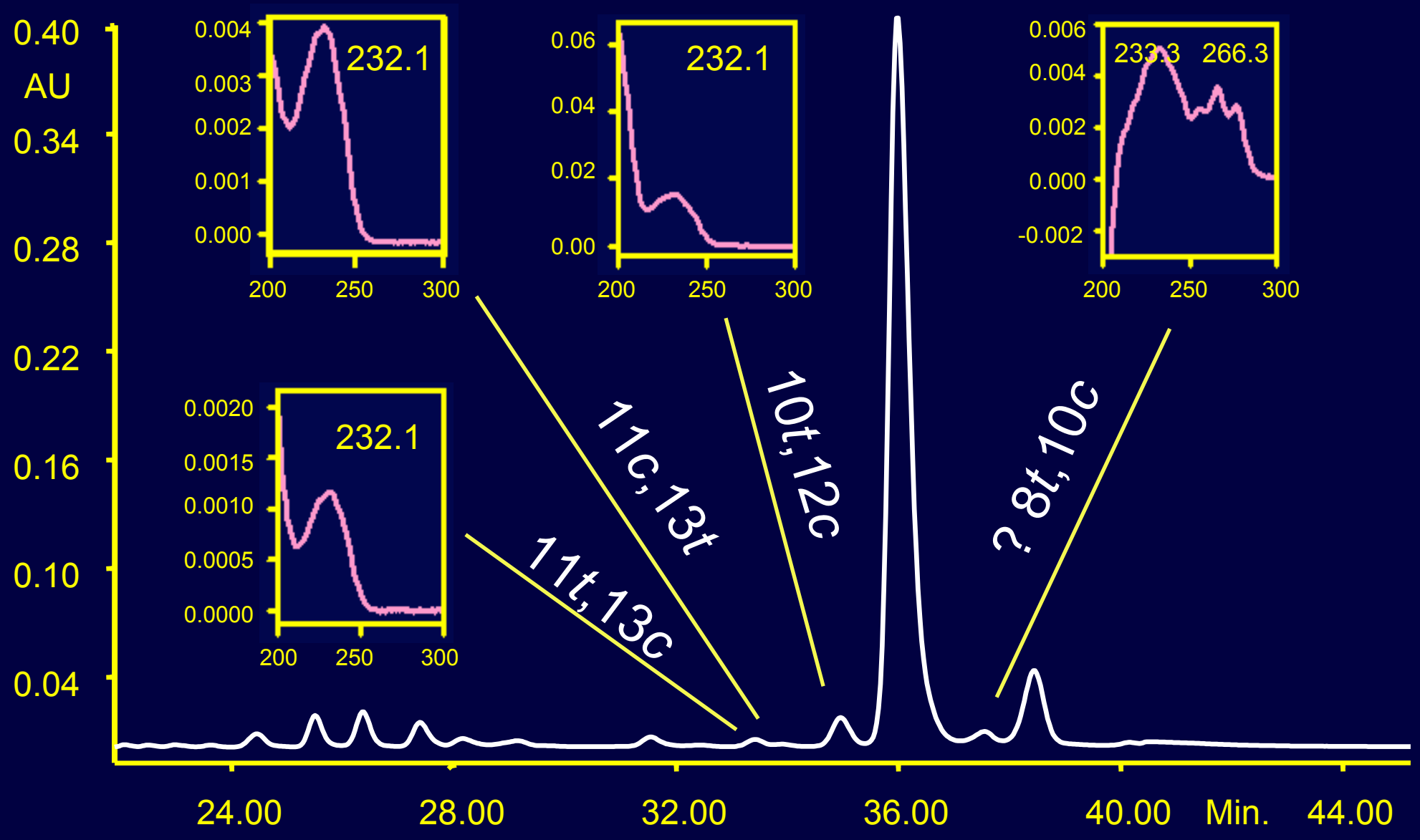
# Relative retention volumes ($RV_{tol.}$)

| Isomer | RV | Isomer | RV |
|---|---|---|---|
| *Toluene* | *0.000* | *6c,8t* | *0.975* |
| *13t,15t* | *0.543* | *9c,11t* | *1.000* |
| *12t,14t* | *0.543* | *9t,11c* | *1.015* |
| *11t,13t* | *0.587* | *8t,10c* | *1.058* |
| *10t,12t* | *0.621* | *7c,9t* | *1.046* |
| *9t,11t* | *0.659* | *6t,8c* | *1.065* |
| *8t,10t* | *0.687* | *7t,9c* | *1.095* |
| *7t,9t* | *0.714* | *13c,15c* | *1.180* |
| *6t,8t* | *0.712* | *12c,14c* | *1.180* |
| *15c,12t* | *0.818* | *11c,13c* | *1.302* |
| *12c,14t* | *0.854* | *10c,12c* | *1.394* |
| *11t,13c* | *0.895* | *9c,11c* | *1.478* |
| *11c,13t* | *0.913* | *6c,8c* | *1.505* |
| *10t,12c* | *0.956* | *8c,10c* | *1.539* |
| *10c,12t* | *0.956* | *7c,9c* | *1.576* |

# $Ag^{+}$HPLC of milk FAME

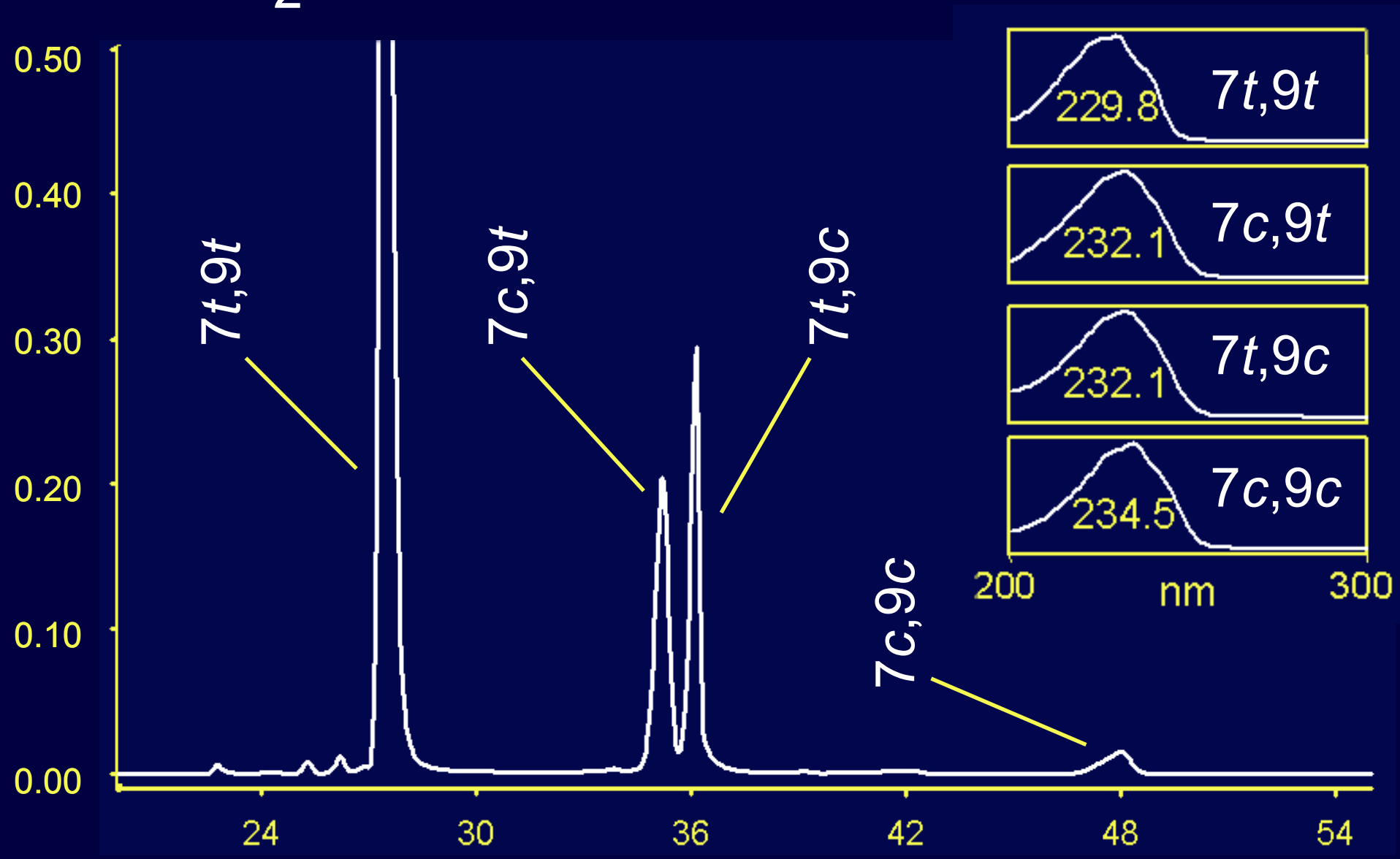


# $Ag^{+}$HPLC of milk FAME

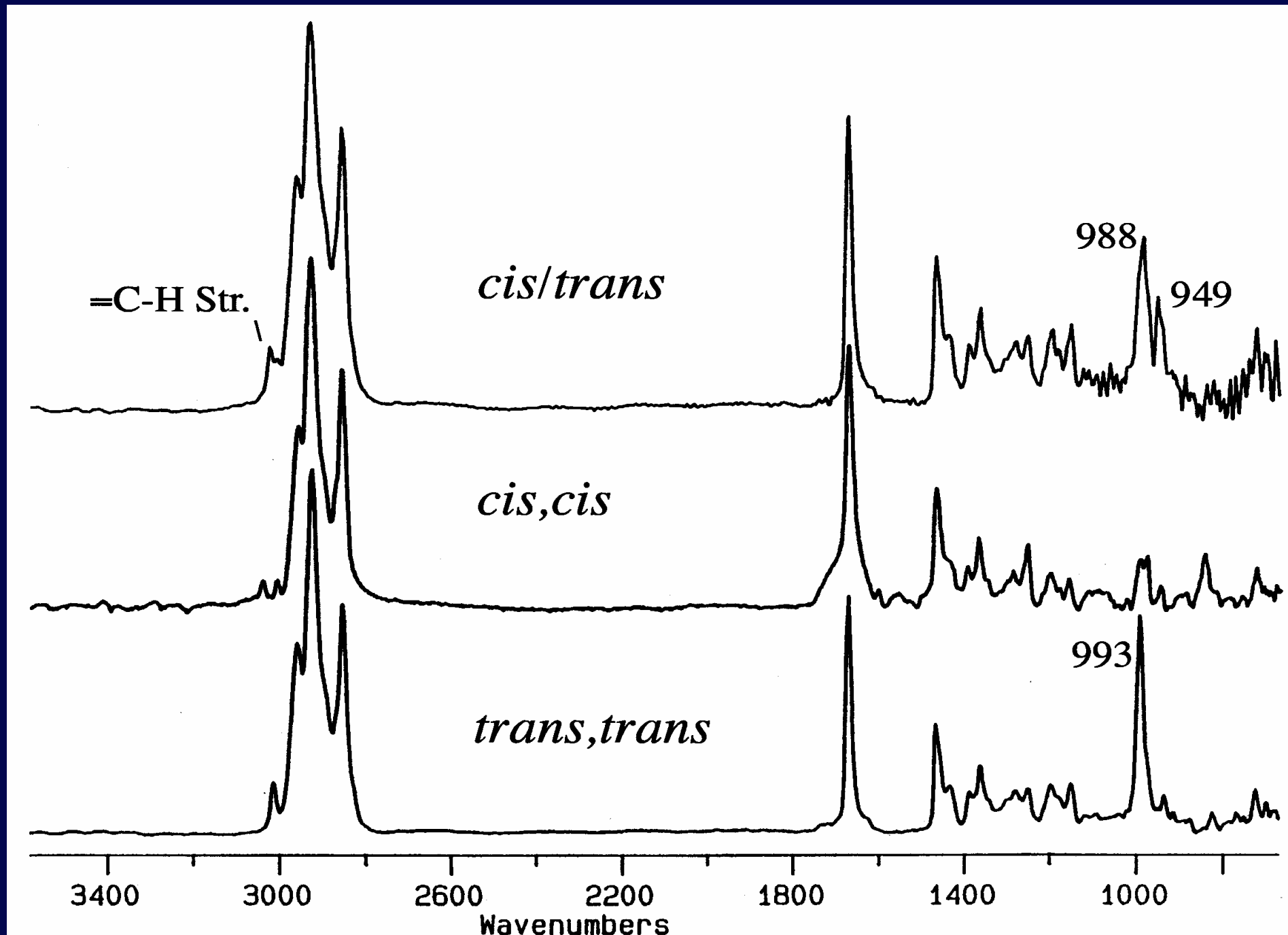


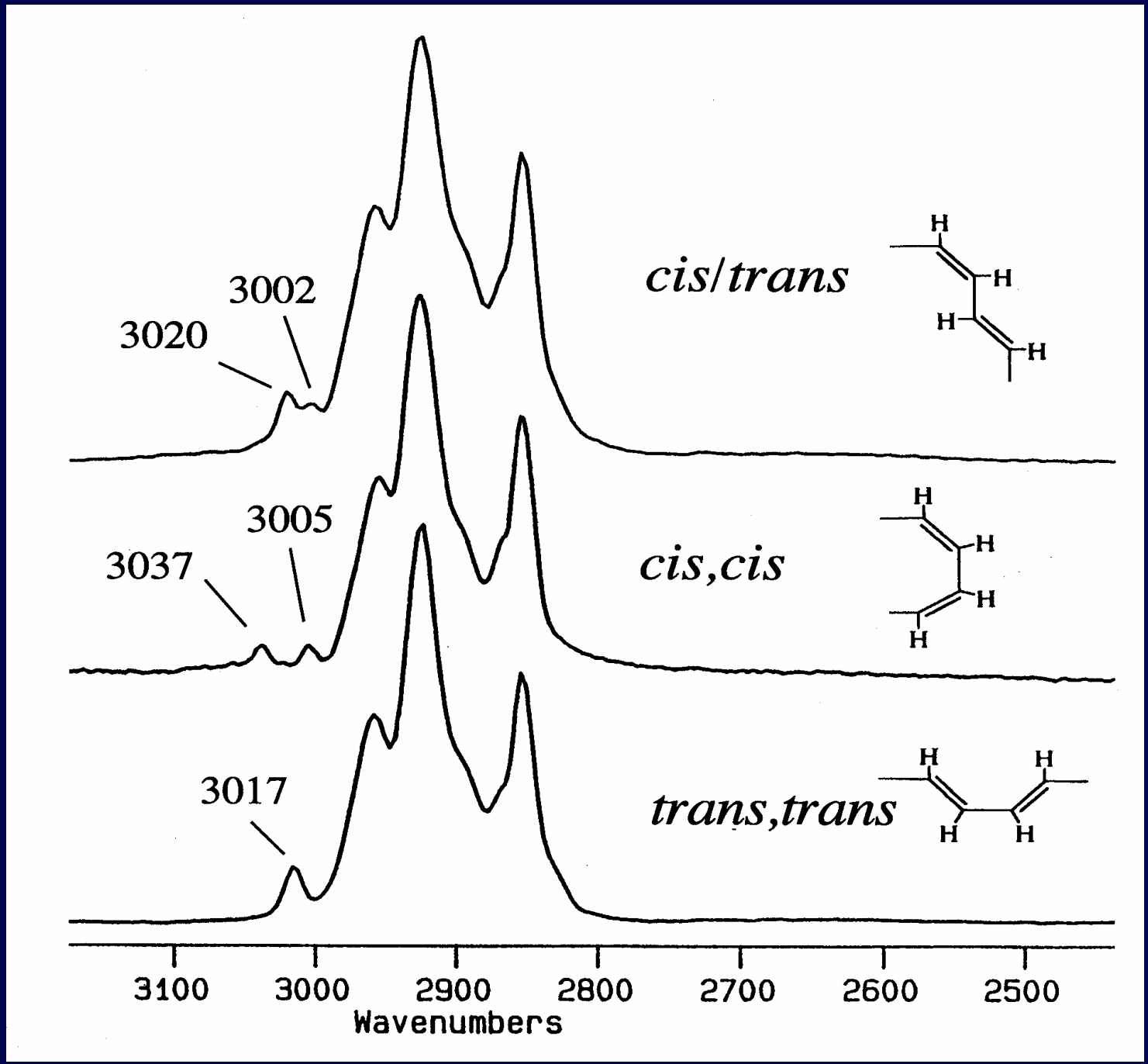
# 7*t*,9*c* $I_2$ isomerized.

HPLC

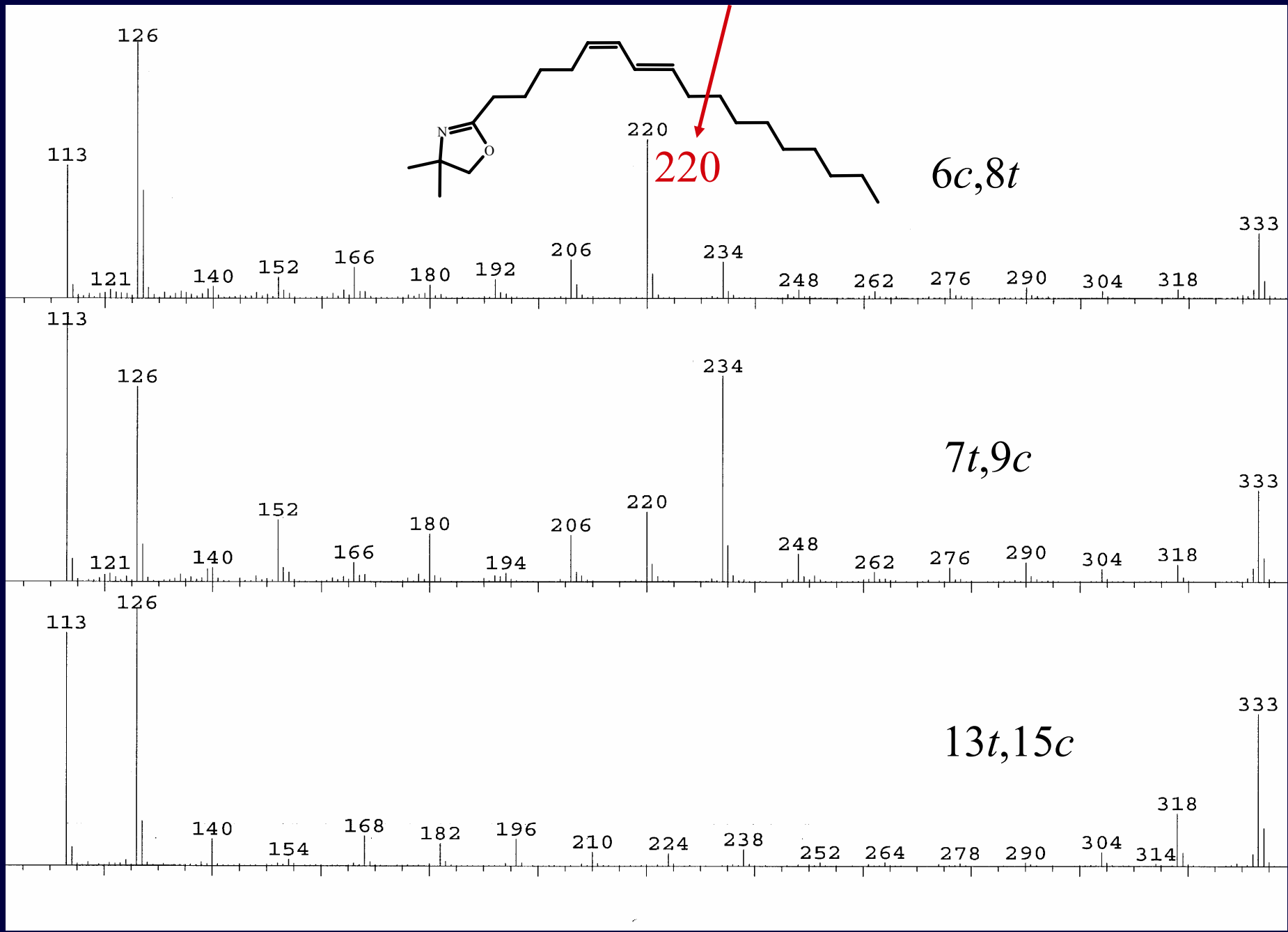


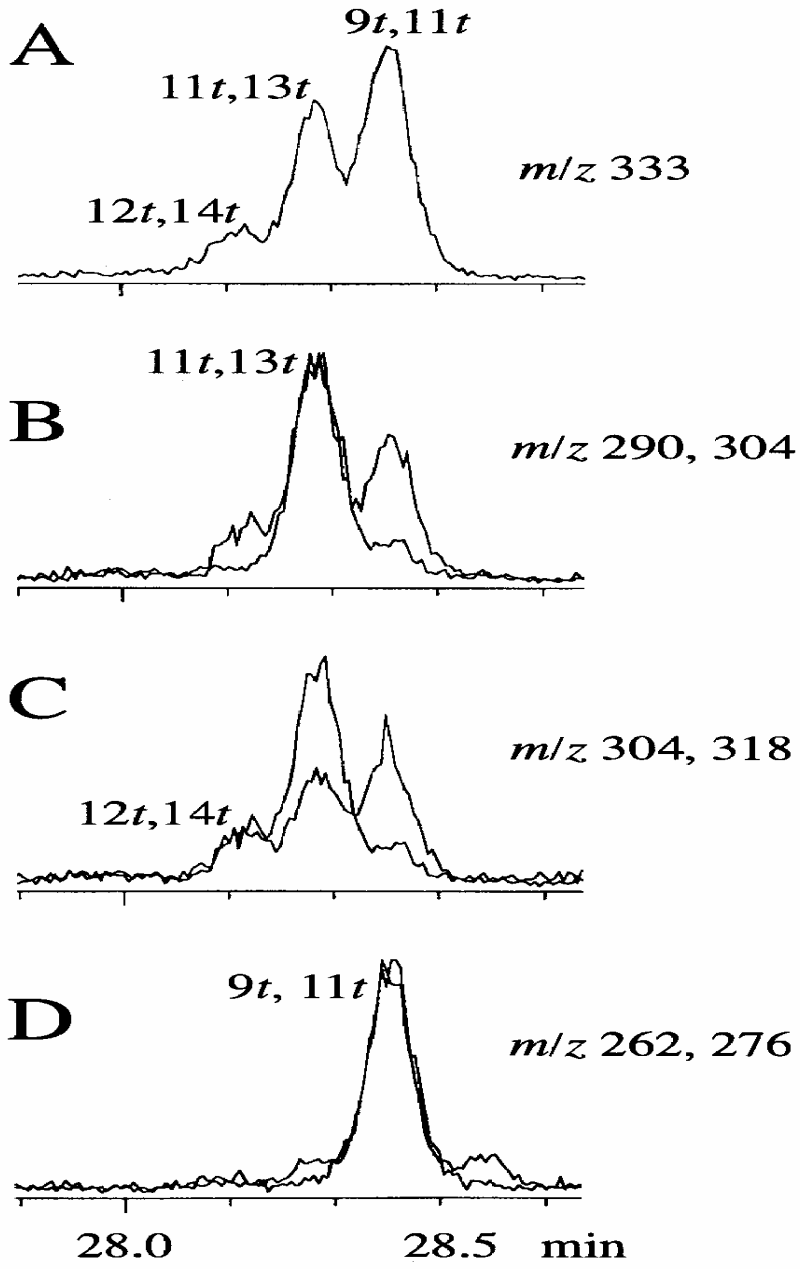
# GC/FTIR

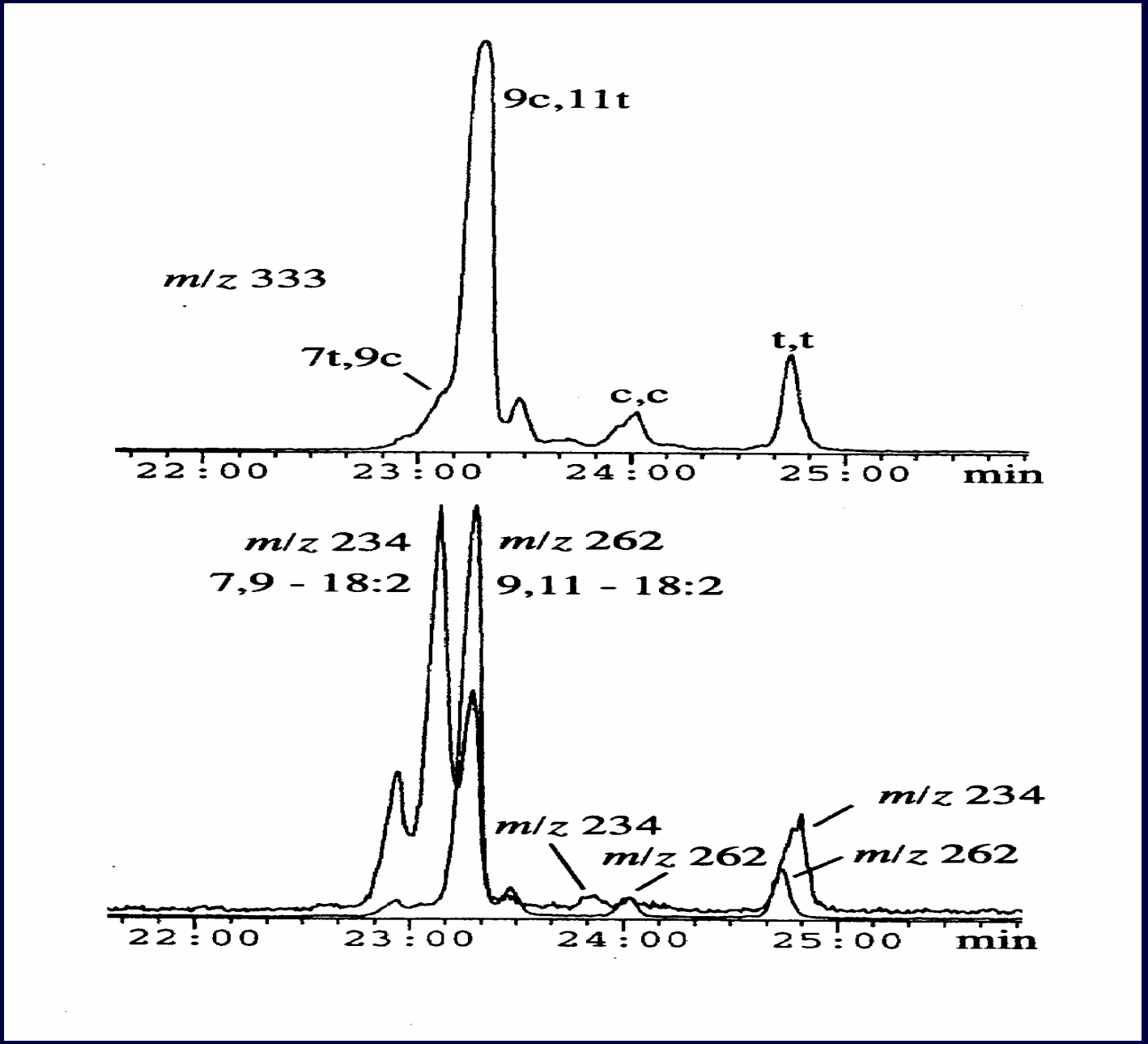

=C-H Str.
cis/trans
988
949
cis,cis
993
trans,trans
3400
3000
2600
2200
1800
1400
1000
Wavenumbers

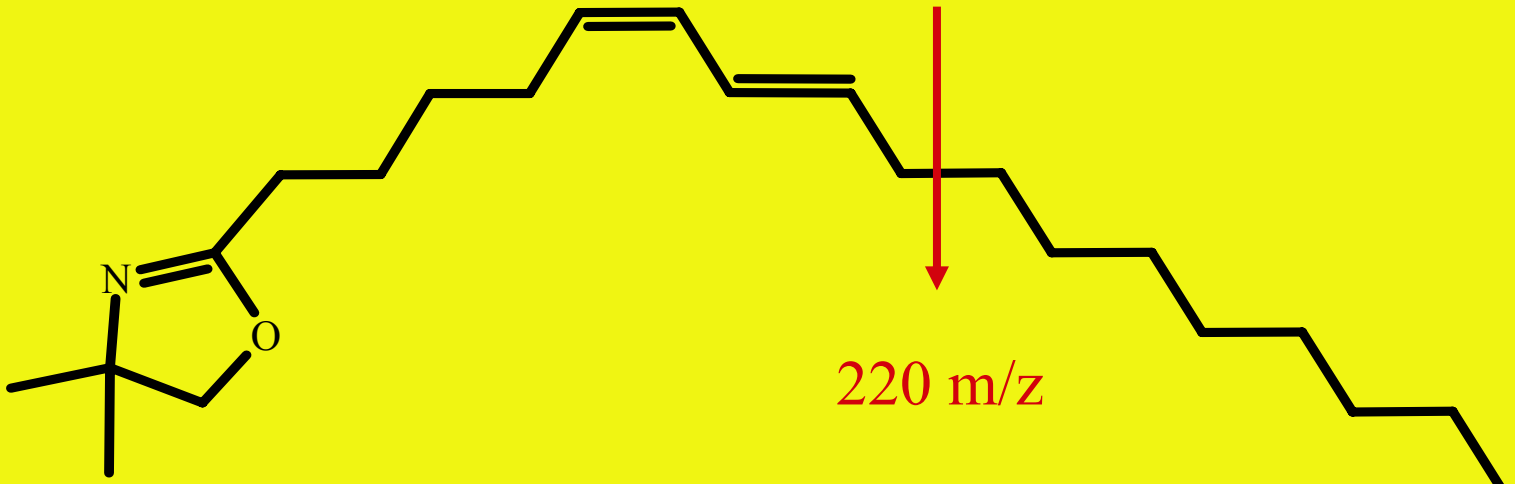

3020
3002
cis/trans
3037
3005
cis,cis
3017
trans,trans
3100
3000
2900
2800
2700
2600
2500
Wavenumbers

# GC/MS


126
113
121
140
152
166
180
192
206
220
234
248
262
276
290
304
318
333
220
6c,8t
113
126
234
7t,9c
152
180
206
220
121
140
166
194
248
262
276
290
304
318
333
113
126
13t,15c
140
154
168
182
196
210
224
238
252
264
278
290
304
314
318
333

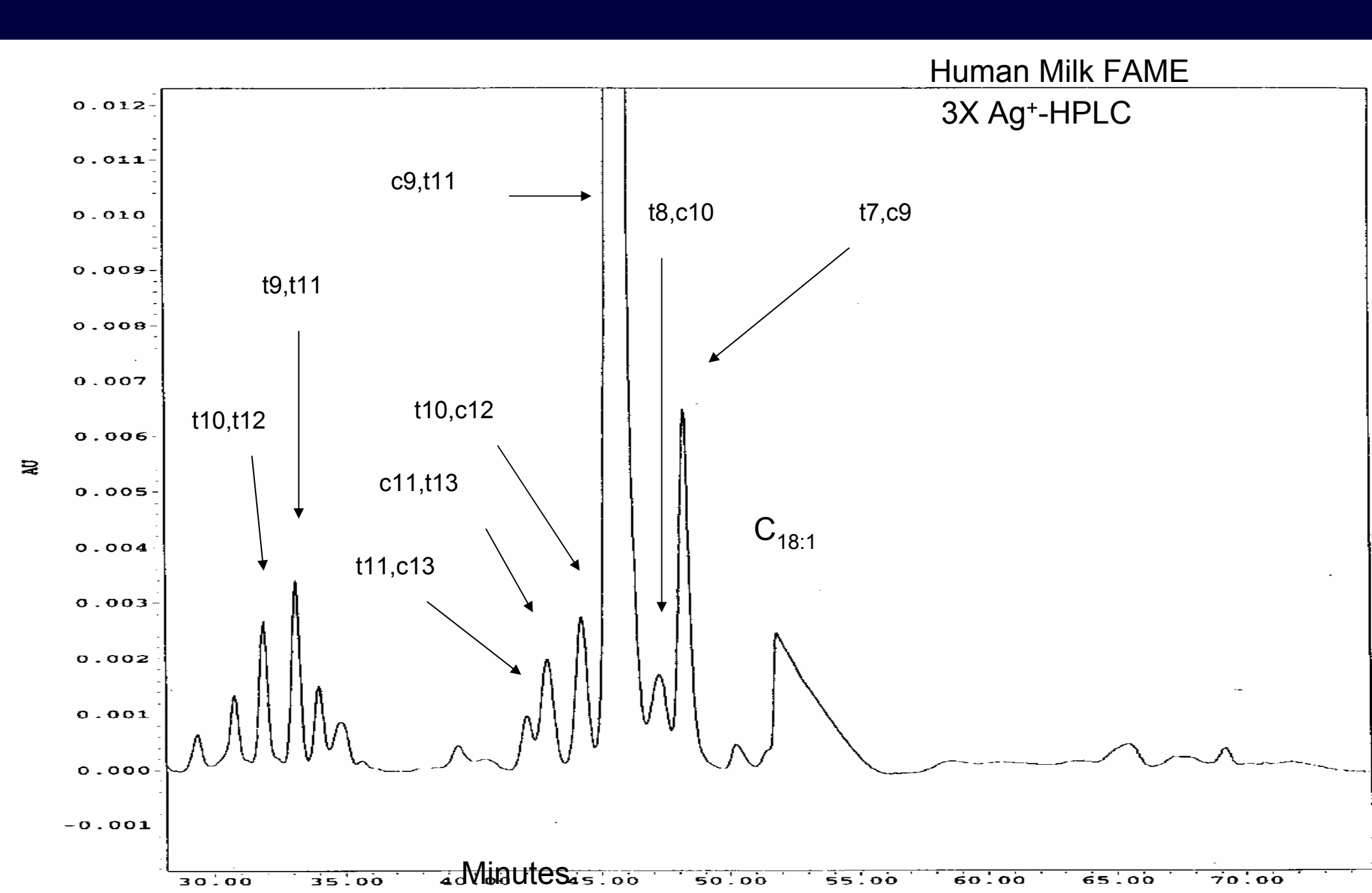

A
9t,11t
11t,13t
12t,14t
m/z 333
B
11t,13t
m/z 290, 304
C
12t,14t
m/z 304, 318
D
9t, 11t
m/z 262, 276
28.0
28.5
min

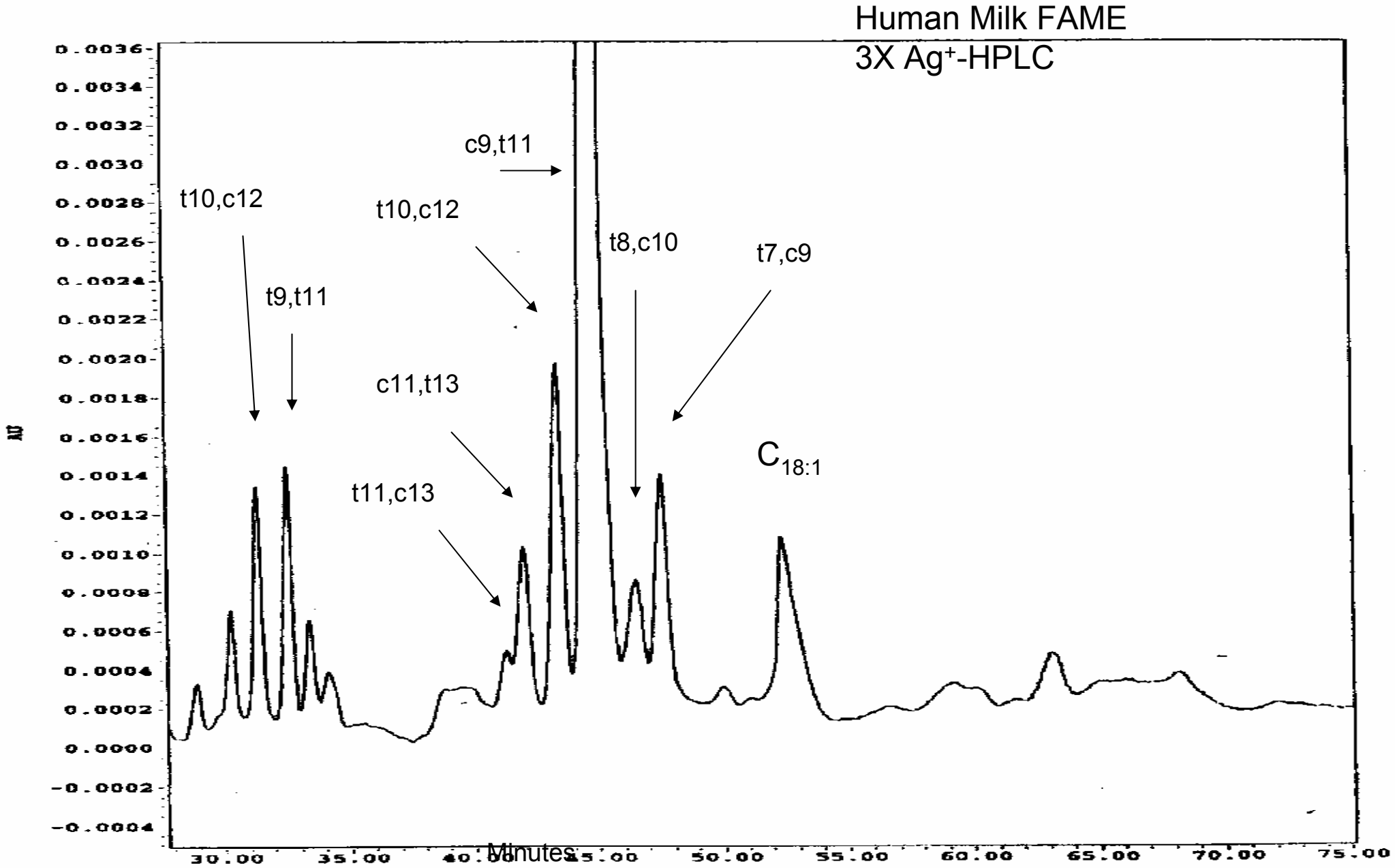

9c,11t
m/z 333
7t,9c
c,c
t,t
22:00
23:00
24:00
25:00
min
m/z 234
7,9 - 18:2
m/z 262
9,11 - 18:2
m/z 234
m/z 262
m/z 234
m/z 262
22:00
23:00
24:00
25:00
min

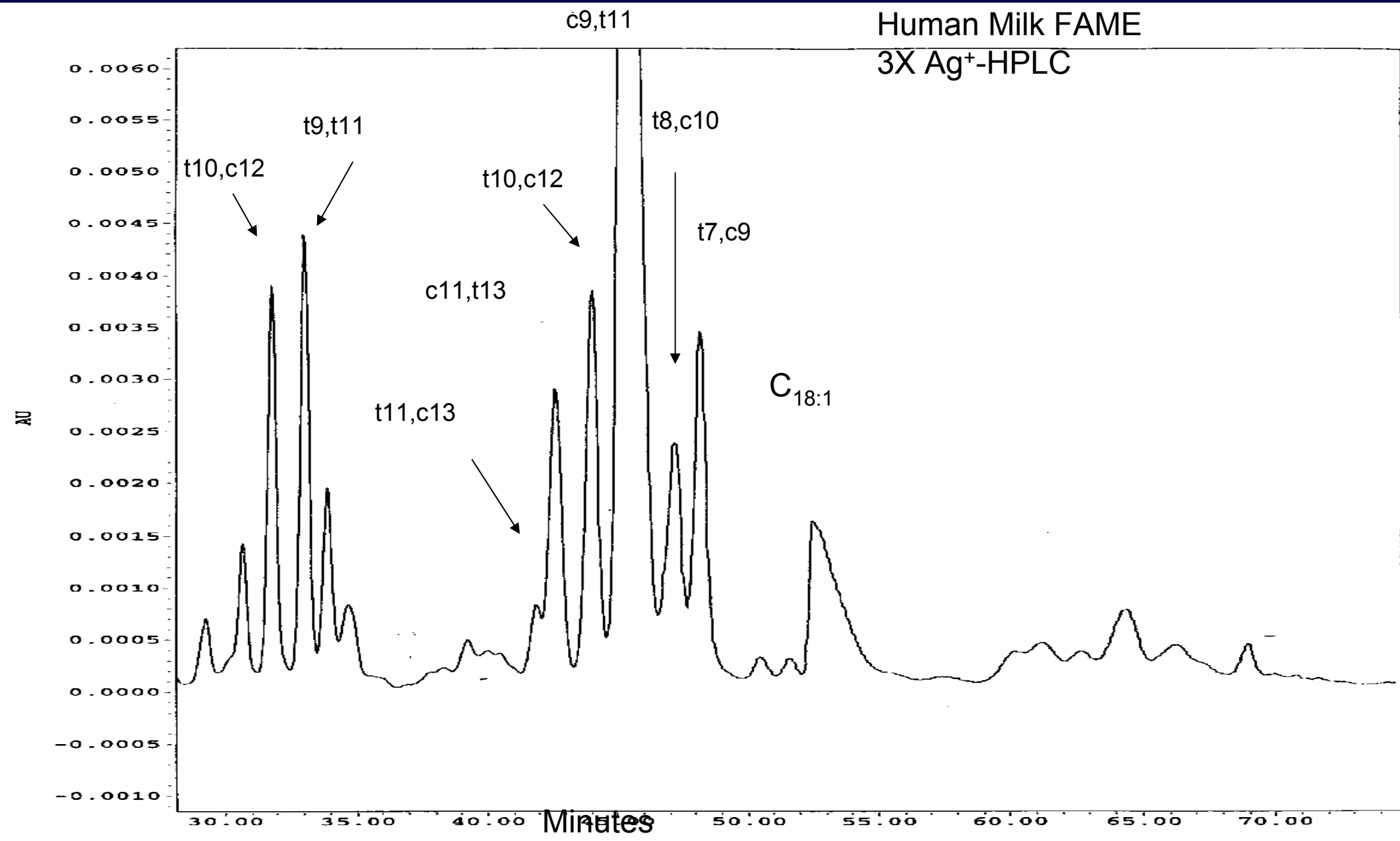



| *Isomer* | *n-2* | *Loss of 12 Da* | *m+2* | *m+3* |
|---|---|---|---|---|
| ***6,8*** | 140 | 154, 166, 180, 192 | **220** | 234 |
| ***7,9*** | 154 | 168, 180, 194, 206 | **234** | 248 |
| ***8,10*** | 168 | 182, 194, 208, 220 | **248** | 262 |
| ***9,11*** | 182 | 196, 208, 222, 234 | **262** | 276 |
| ***10,12*** | 196 | 210, 222, 236, 248 | **276** | 290 |
| ***11,13*** | 210 | 224, 236, 250, 262 | **290** | 304 |
| ***12,14*** | 224 | 238, 250, 264, 276 | **304** | 318 |
| ***13,15*** | 238 | 252, 264, 278, 290 | **318** | 332 |

# CLA in Human Milk


Human Milk FAME
3X Ag+-HPLC
c9,t11
t8,c10
t7,c9
t9,t11
t10,t12
t10,c12
c11,t13
t11,c13
C18:1
AU
0.012
0.011
0.010
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
-0.001
30.00
35.00
40.00
45.00
50.00
55.00
60.00
65.00
70.00
Minutes


Human Milk FAME
3X Ag+-HPLC
t10,c12
t9,t11
t10,c12
c9,t11
t8,c10
t7,c9
c11,t13
t11,c13
C18:1
AU
Minutes
0.0036
0.0034
0.0032
0.0030
0.0028
0.0026
0.0024
0.0022
0.0020
0.0018
0.0016
0.0014
0.0012
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
-0.0002
-0.0004
30.00
35.00
40.00
45.00
50.00
55.00
60.00
65.00
70.00
75.00


Human Milk FAME
3X Ag+-HPLC
c9,t11
t9,t11
t10,c12
t10,c12
t8,c10
t7,c9
c11,t13
t11,c13
C18:1
AU
0.0060
0.0055
0.0050
0.0045
0.0040
0.0035
0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
-0.0005
-0.0010
30.00
35.00
40.00
45.00
50.00
55.00
60.00
65.00
70.00
Minutes

# CLA Isomer in Human Milk

**Average total CLA by GC is 0.54 %**

**3X $Ag^+$-HPLC (% CLA)**

| Isomer | mean |
|---|---|
| t12t14 | 0.61 |
| t11t13 | 1.70 |
| t10t12 | 2.72 |
| t9t11 | 3.30 |
| t8t10 | 1.56 |
| t7t9 | 1.30 |
| 11,13 c/t1.12 | |
| 11,13 c/t2.95 | |
| 10,12 c/t4.20 | |
| 9,11 c/t | 71.15 |
| 8,10 c/t | 1.92 |
| 7,9 c/t | 7.44 |

**GC (% CLA)**

| Isomer | mean |
|---|---|
| 7,9 c/t (?) | 3.6 |
| c9t11 (7,9 c/t:8,10 c/t) | 62.4 |
| t9c11(c10t12) | 5.0 |
| c11t13 (x) | 3.5 |
| t10c12 | 3.8 |
| t11c13/c9c11 | 3.0 |
| c11c13 | 3.9 |
| t12t14 (?) | 0.3 |
| t11t13 | 5.6 |
| t10t12/t9t11/t8t10 | 7.4 |
| t7t9 | 1.6 |