Specialist course on Lipids in Ruminants:

Methods for the fatty acid analysis and their confirmation

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Research Group

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Quality and Safety of Foods from Animal Origin

Vitoria-Gasteiz

European Green Capital 2012

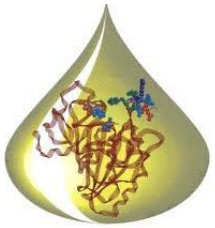
Lipid research - remember

- Analytical methods are **tools** we use to obtain results.

- The results are only **as good as the total sum of all our mistakes**.

- Only accurate analytical results provide the basis for **meaningful interpretations.**

Outline



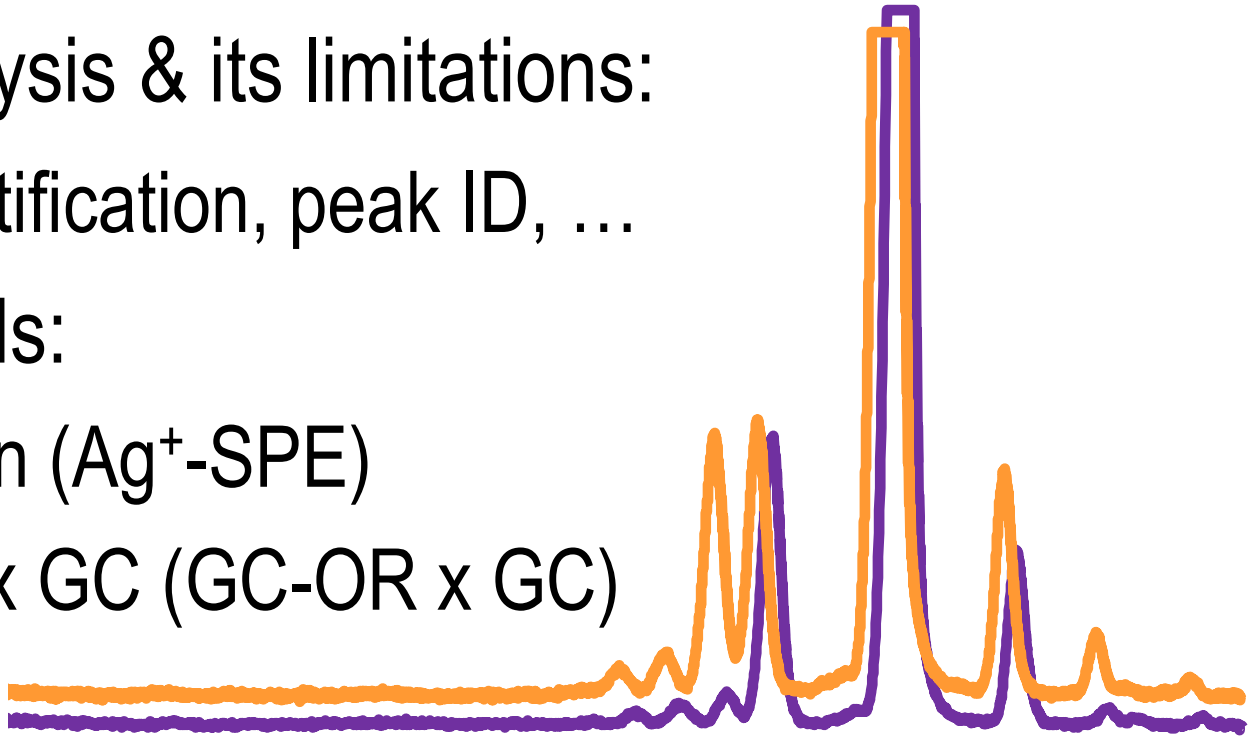
- Lipid extraction
- Derivatization (methylation)
- Fatty acid methyl ester (FAME) purification
- Normal GC/FID analysis & its limitations:

  separations, quantification, peak ID, …
- Confirmatory methods:

  Silver ion fractionation (Ag^+-SPE)

  GC-online reduction x GC (GC-OR x GC)

  GC/MS



Several methods:

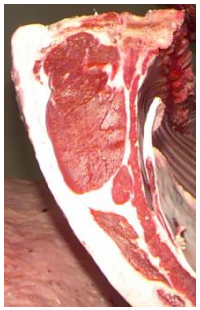
Liquid

- solvent extraction → methylation
- direct methylation

Freeze-dry & pulverize

- solvent extraction → methylation
- direct methylation

Tissue



Homogenize

- solvent extraction → methylation
- direct methylation

Freeze-dry & pulverize

- solvent extraction → methylation
- direct methylation

FAME

Nature of sample

Type of lipid structures common in ruminant samples and their relative amounts (% of total lipids)

Samples	TG	CLA	CE	*O*-acyl PL	*O*-alkenyl PL*	*N*-acyl	FFA
Milk	98	0.7	0.2	0.5	-	0.2	<0.1
Meat	70	0.7	0.5	20	4	4	<0.1
Blood	35	0.7	5	50	3	3	1
Rumen fluids/feces	10-20	0.2	-	1	-	<0.1	80**

* Glycerol-O-CH=CH-R

** Salts and complex glycolipids

Extraction – nature of sample

Solvent systems that include chloroform:

Folch *et al*. (1957)

(JBC 226: 497)

A SIMPLE METHOD FOR THE ISOLATION AND PURIFICATION OF TOTAL LIPIDES FROM ANIMAL TISSUES*

By JORDI FOLCH, M. LEES,† and G. H. SLOANE STANLEY‡

Bligh & Dyer (1959)

(Can.J.Biochem.Physiol. 37: 911)

A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION[1]

E. G. Bligh and W. J. Dyer

Extraction– other solvent mixtures

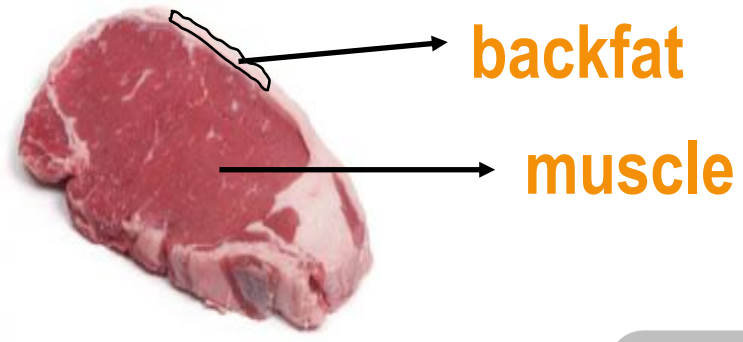
- Hexane or heptane/isopropanol (Hara & Radin, 1976)
- Dichloromethane/methanol (Marmer & Maxwell, 1981)

Note:

- Hexane will only extract neutral lipids, **not phospholipids (PL)**.

- Suitable for sample types that consist mainly of TG (vegetable oils, adipose tissue).

- **Animal tissues that contain PL require polar solvent extraction**.



Derivatization

1) Acid-catalyzed methylation
2) Base-catalyzed methylation
3) Combining acid- & base-methylations
4) Direct methylation

Derivatization

Suitability of catalysts for the methylation of specific lipid classes

(Cruz-Hernandez *et al.*, 2006)

Type of Lipids	*Structures*	*Catalysts*[a]			
		HCl	*BF_3*	*$NaOCH_3$*	*DAM*
Esters	RCH_2-CO-OR´	Y	Y	Y	No
Free Fatty Acids	RCH_2-COOH	Y	Y	No	Y
Amides	RCH_2-CO-NHR´	Y,L	Y,L	No	No
Alk-1-enyl ethers	RCH_2-O-CH=CHR´	Y	Y	No	No
Phospholipid esters	$R(PO_4X)(OOCR´)$	Y	Y	Y	No
Cholesterol esters	RCOO-cholesteryl	Y,L	Y,L	No	No
Conjugated bonds[b]	RCH=CH-CH=CHR´	Isom	Isom	Stable	Stable

DAM: diazomethane. **Y**: catalyst suitable; **No**: catalyst not suitable; **L**, longer reaction times & generally higher temperatures are required.

Derivatization

1) Acid-catalyzed methylation:

- **Quantitative**: methylation of all common lipid classes including:

  FFA, *O*-acyl (esters, glycosides), *N*-acyl (sphingomyelin), alk-1-enyl ethers (plasmalogenic lipids).

Example:

- 5% (w/v) solution of anhydrous methanolic HCl:

  Methylation of all lipids is complete in 1h at 80ºC.

Bubbling dry HCl gas into anhydrous methanol (Stoffel et al., 1959).

Adding acetyl chloride into dry methanol (Lepage & Roy, 1986).

Commercial 0.5N or 3N methanolic HCl preparations can be purchased (Sigma-Aldrich).

Derivatization

1) Acid-catalyzed methylation (cont.):

Major disadvantages:

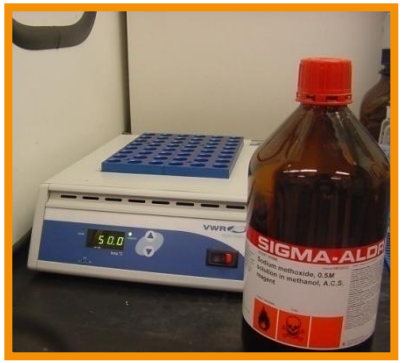
- Isomerization of *c,t*-, *t,c*- and *c,c*- to *t,t*-CLA isomers (Kramer *et al*., 1997).
- Formation of methoxy artifacts depending on sample type (Yurawecz *et al*., 1994; Kramer *et al*., 1997).

- Lowering the methylation temperature to 60ºC (Park *et al*., 2001) or room temp (Werner *et al*., 1992), decreases CLA isomerization somewhat (Park *et al*., 2001), but under these milder conditions methylation may not be complete (Kramer *et al*., 1997; Cruz-Hernandez *et al*., 2006).

Derivatization

2) Base-catalyzed methylation:

- **Not-quantitative**: converts only acyl moieties to FAME, but not FFA, *N*-acyl lipids and alk-1-enyl ethers (Kramer *et al*., 1997; Cruz-Hernandez *et al*., 2004, 2006).



Example:

- $NaOCH_3$/methanol (0.5N methanolic base #33080, Sigma-Aldrich)

  Rapid methylation (15min at 50ºC) without CLA isomerization (Kramer *et al*., 1997).

Derivatization

3) Combining acid- & base-methylations:

Option A:

- Perform the **two methylations** in sequence.

Option B:

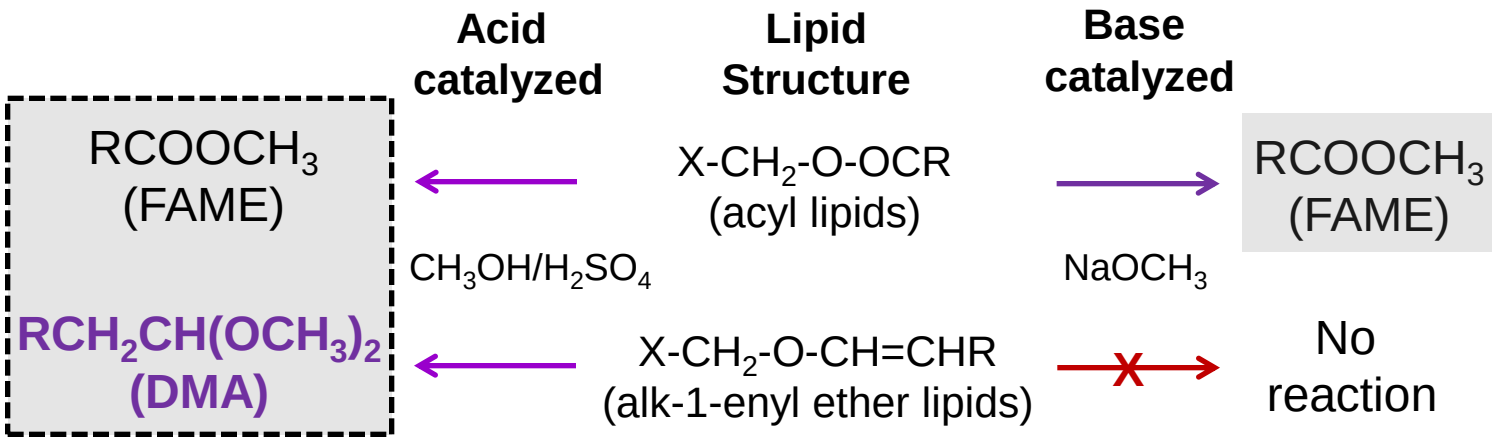
- Perform the **2 methylations** separately and merge the results.

Derivatization

3) Combining acid- & base-methylations (cont.):

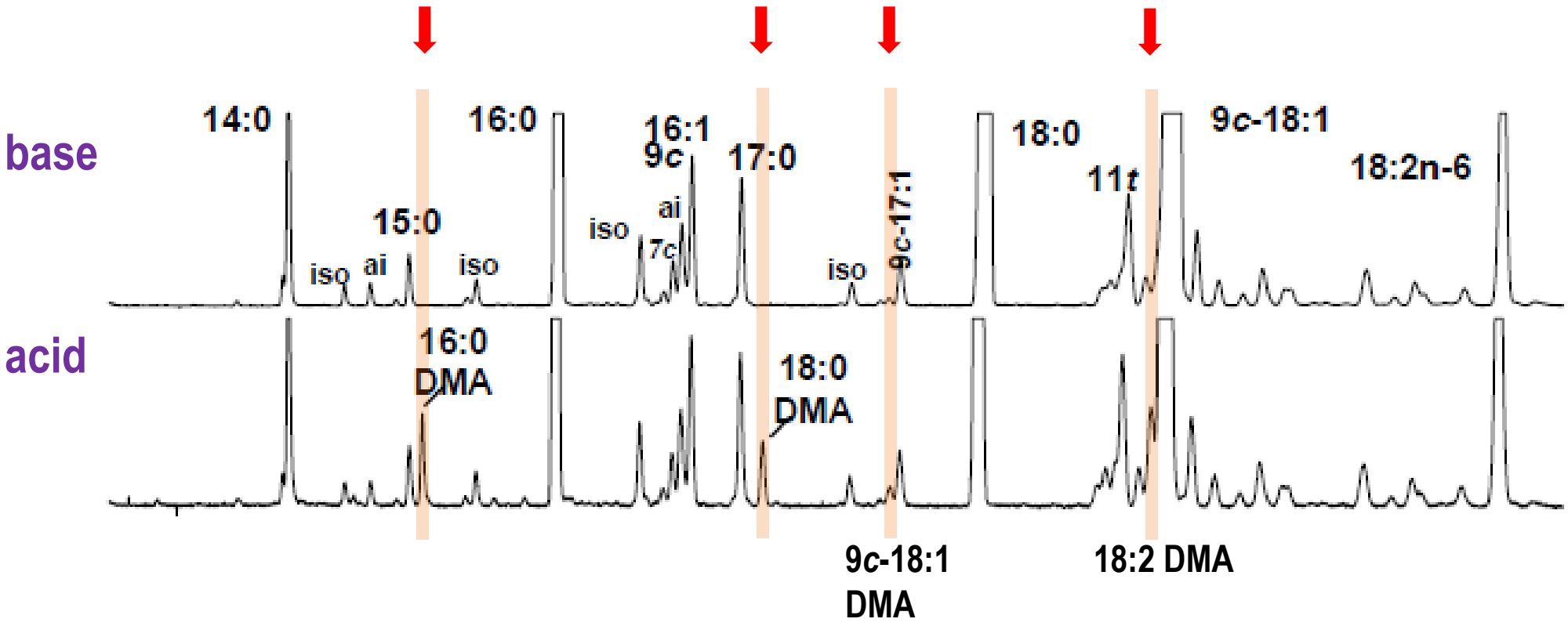
Option B: Use acid-catalyzed results for quantitative purposes and correct specific regions, such as CLA region, from the base-catalyzed results (Cruz-Hernandez *et al*., 2006; Kramer *et al*., 2008).

Products of acid- and base-catalysts permits the identification of acyl and plasmalogenic lipids.



Derivatization

Partial GC chromatogram of total sheep lipids methylated using a base- or an acid-catalyzed procedure



Derivatization

4) Direct methylation: quick

- Direct transesterification of the lipids, **without prior extraction,** with acid or base catalysts.

Note of caution:

- Analysis of different lipid classes is not possible.

- Information is limited since it provides only the total FAME composition.

- A base catalyst does not methylate a number of lipid classes.

Derivatization– rumen samples

- **Rumen digesta** samples are unique in that they contain high levels of **FFAs**.

Options:

Direct saponification (5% NaOH or 0.5M $NaOCH_3$ in ethanol)

and :

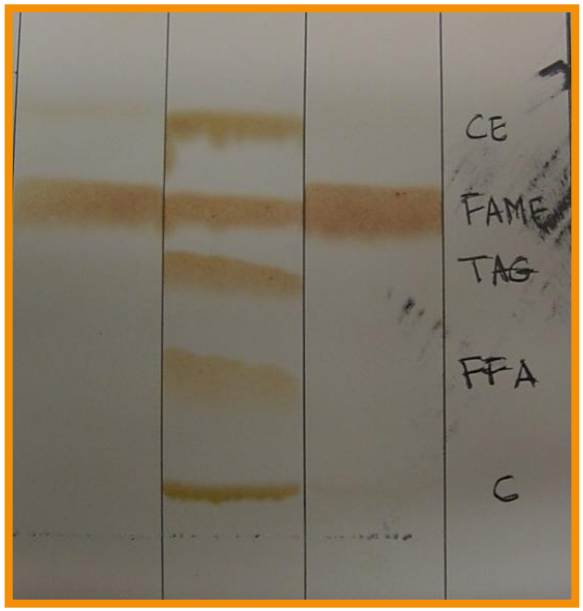
1) acid-catalyzed **methylatio**n (HCl in methanol) or

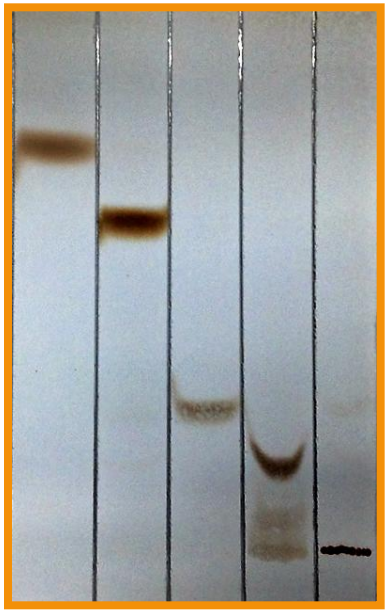
2) **methylation** with trimethylsilyl-diazomethane (TMS-DAM), if the sample contains acid labile components.

FAME purification / clean up

… will depend on the sample type

- Examine the FAMEs for purity & completeness of methylation (i.e., digesta & grass samples)
- By **TLC** (**T**hin **L**ayer **C**hromatography) silica plates:





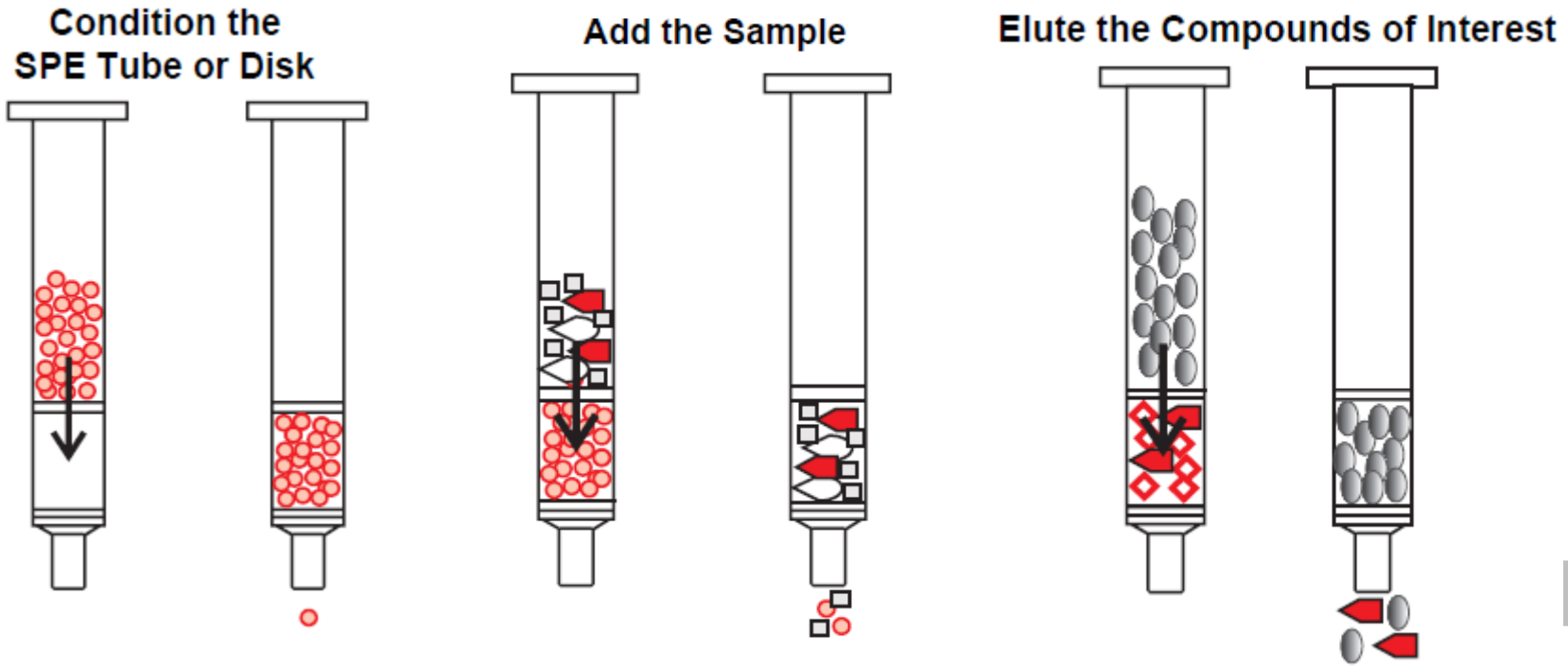
CE TG FFA C PL

FAME purification / clean up

… will depend on the sample type

- Examine the FAMEs for purity & completeness of methylation

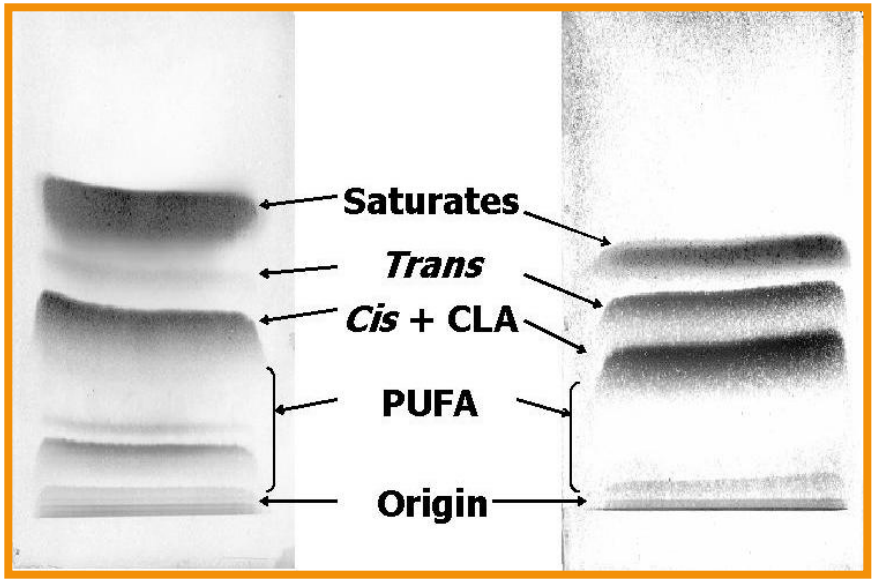
- By **SPE** (**S**olid **P**hase **E**xtraction) silica cartridges :



TLC or SPE - other applications

- **Separate specific lipids:** polar & neutral lipids, polar lipid classes, 3D separations of all lipid classes (Kramer *et al*., 1983), …

- With **silver ion (Ag^+-)**: fractionation of FAMEs



GC/FID – improved separations

Selection of a GC column:

- The choice of GC column **depends on the sample type** but there is general agreement among researchers to use:

100m highly polar (100% cyanopropyl polysiloxane) **capillary columns: SP-2560** (Supelco) **or CP-Sil88** (Varian)

New 100m ionic liquid column (SLB-IL111, Supelco**)**

(significantly more polar)

GC/FID – improved separations

SP-2560 (Supelco) or CP-Sil88 (Varian):

- **100m highly polar** column provides improved separation of:

18:1 region (Molekentin & Precht, 1995; Precht & Molkentin, 1996, 1997, 1999, 2000b, 2003; Wolff *et al*., 1995; Ratnayake, 2001, 2004; Ratnayake *et al*., 2006, 2009; Kramer *et al*., 2001, 2002, 2008; Wolff & Precht, 2002; Cruz-Hernandez *et al*., 2004, 2006; Shingfield *et al*., 2006; Destaillats *et al*. 2007)

16:1 region (Precht & Molkentin, 2000a; Kramer *et al*., 2008)

18:2 region (Precht & Molkentin, 1997, 2003; Kramer *et al*., 2008)

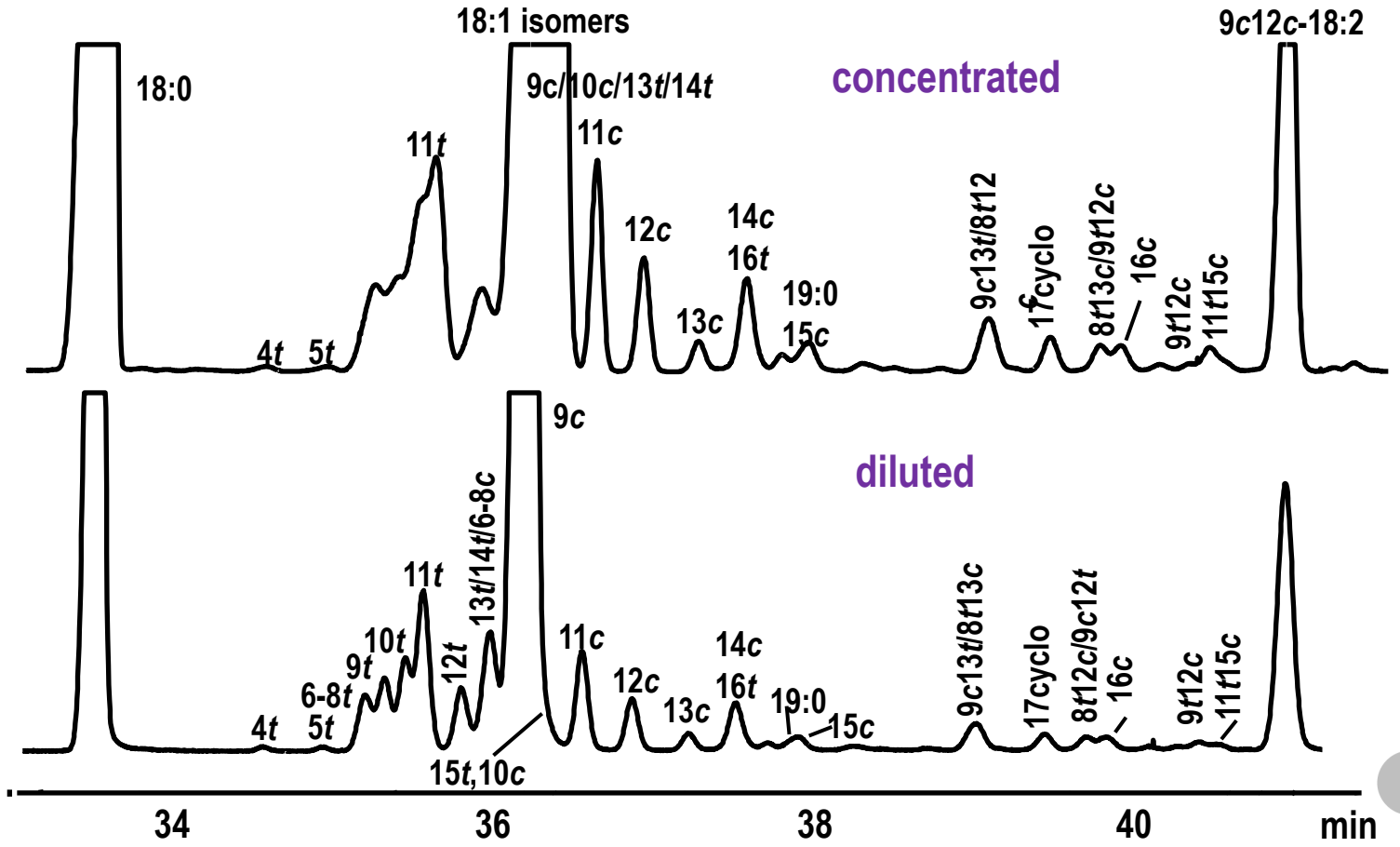
20:1-18:3 region (Wolf 1994; Precht & Molkentin, 1999; Kramer *et al*., 2008)

CLA region (Roach *et al*., 2000, 2002; Cruz-Hernandez *et al*., 2004, 2006; Shingfield *et al*., 2006)

GC/FID – improved separations

Adjustment of sample load:

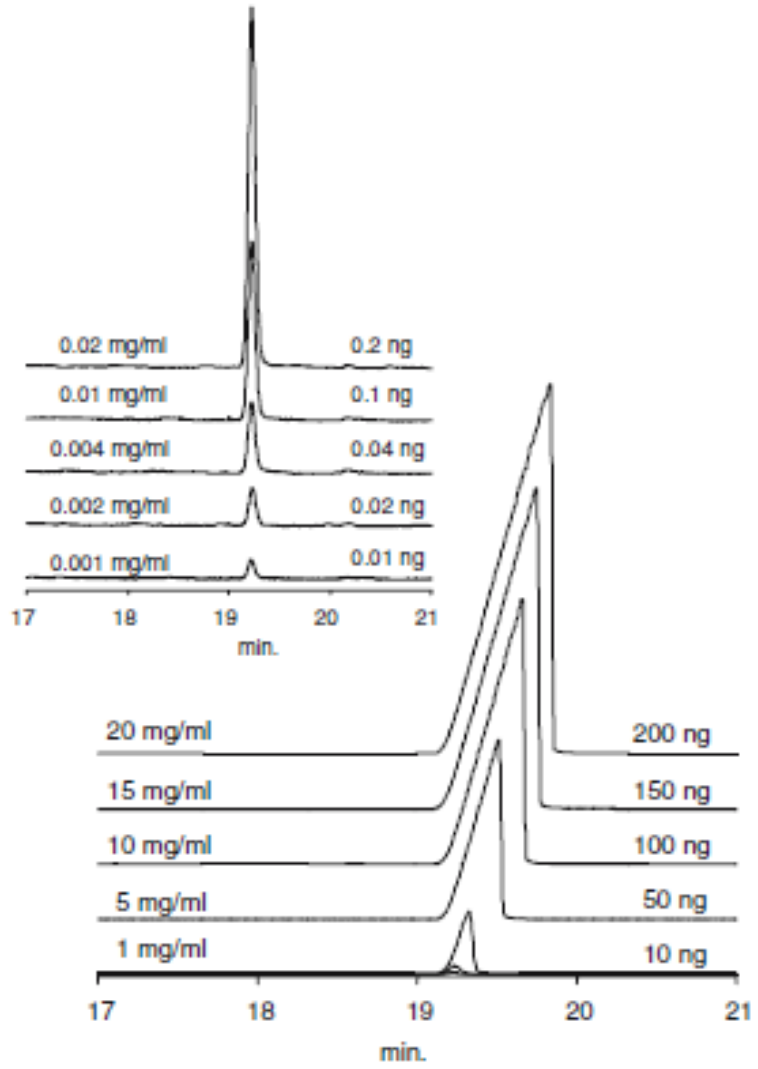
- Reduce sample load onto GC column if sample is too concentrated:



GC/FID – improved separations

Adjustment of sample load:

Partial GC chromatogram of 9*c*-18:1 injected at different concentrations



The **relative retention time shifts (peak maximum)** with increased sample load. However, the **start of the peak remains the same**.

(Delmonte & Rader, 2007)

GC/FID – improved separations

GC conditions: temperature program *vs* isothermal?

- **Depends largely on the nature of the samples**. The analysis of wide range of FAMEs is impossible under isothermal conditions.
- **Temperature programs:** incorporates specific needs to resolve either short- or long-chain FAMEs, and at the same time include a **temperature plateau** that provides maximum resolution of specific FAME regions.

- **Ruminant fats:** at least 2 complementary GC temperature programs are proposed **with plateaus at 175ºC** & **150ºC** to improve the resolution of the 18:1 & other FAME regions (Kramer *et al*., 2008).

GC/FID – improved separations

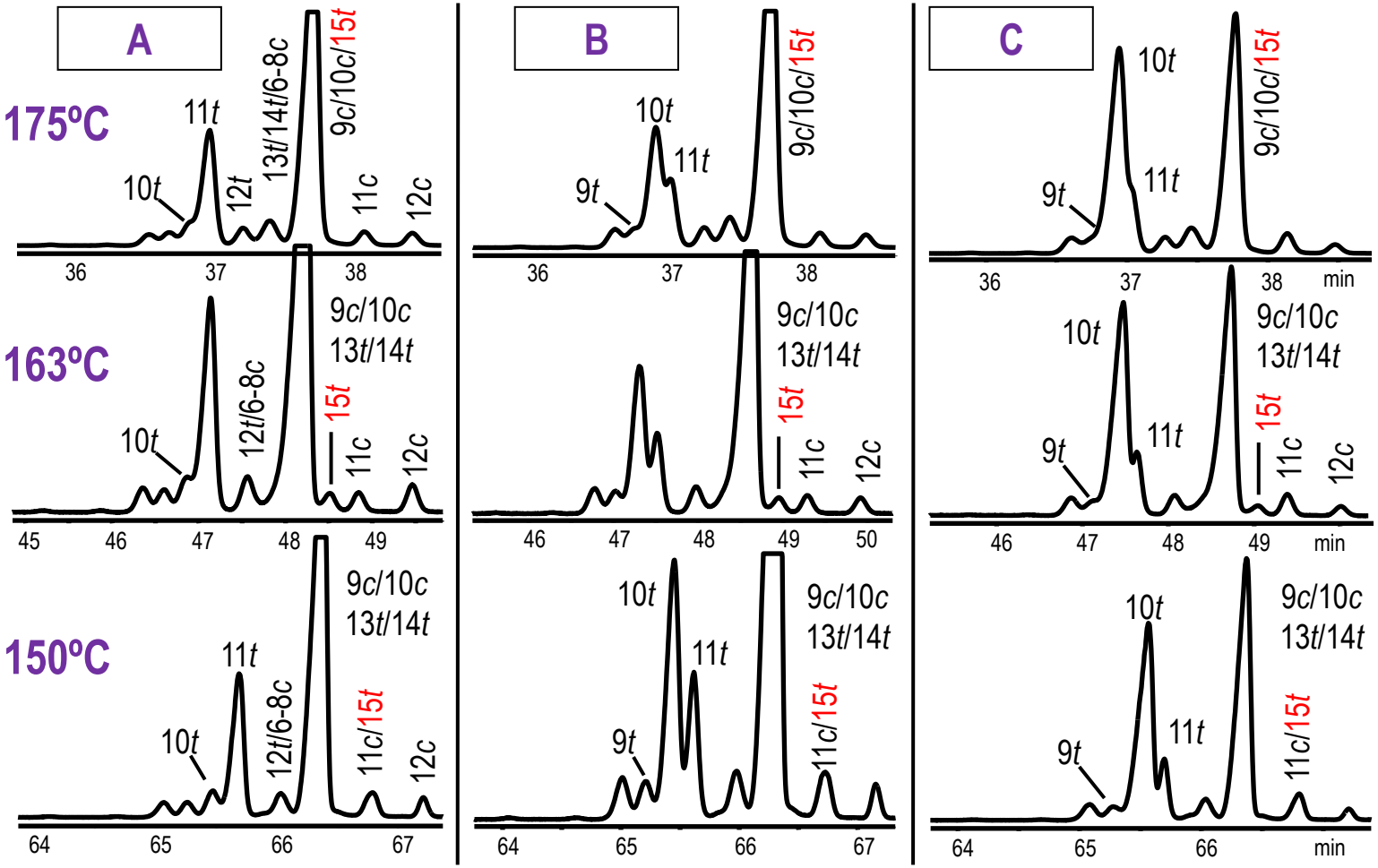
Details of the 3 temperature programs evaluated:

Descriptions	175 °C	163 °C	150 °C
Initial temperature (°C)	45	45	45
Time (min)	4	4	4
First rate of increase (°C/min)	13	13	13
Plateau temperature (°C)	175	163	150
Time (min)	27	37	47
Second rate of increase (°C/min)	4	4	4
Final temperature (°C)	215	215	215
Time (min)	35	40	35
Total time (min)	86	103.8	110.33

(Kramer *et al.*, 2008; Lipids 43:259-273)

GC/FID – improved separations

Separation of 3 types of fat samples with different proportions of *cis*- and *trans*-18:1 isomers affected by temperature



(Kramer *et al*., 2008)

GC/FID – improved separations

(Delmonte *et al.*, 2011)

Ionic liquid column (SLB-IL111):

- Resulted in **enhanced separation** of:

  - *cis*- & *trans*-FA isomers 14:1 to 20:1.

  - 20:1 & 18:3 isomers.

  - CLA isomers: the first GC column able to resolve the isomeric CLA pair **9*c*,11*t*-** and **7*t*,9*c*-CLA** (previously achieved only by Ag^+-HPLC).

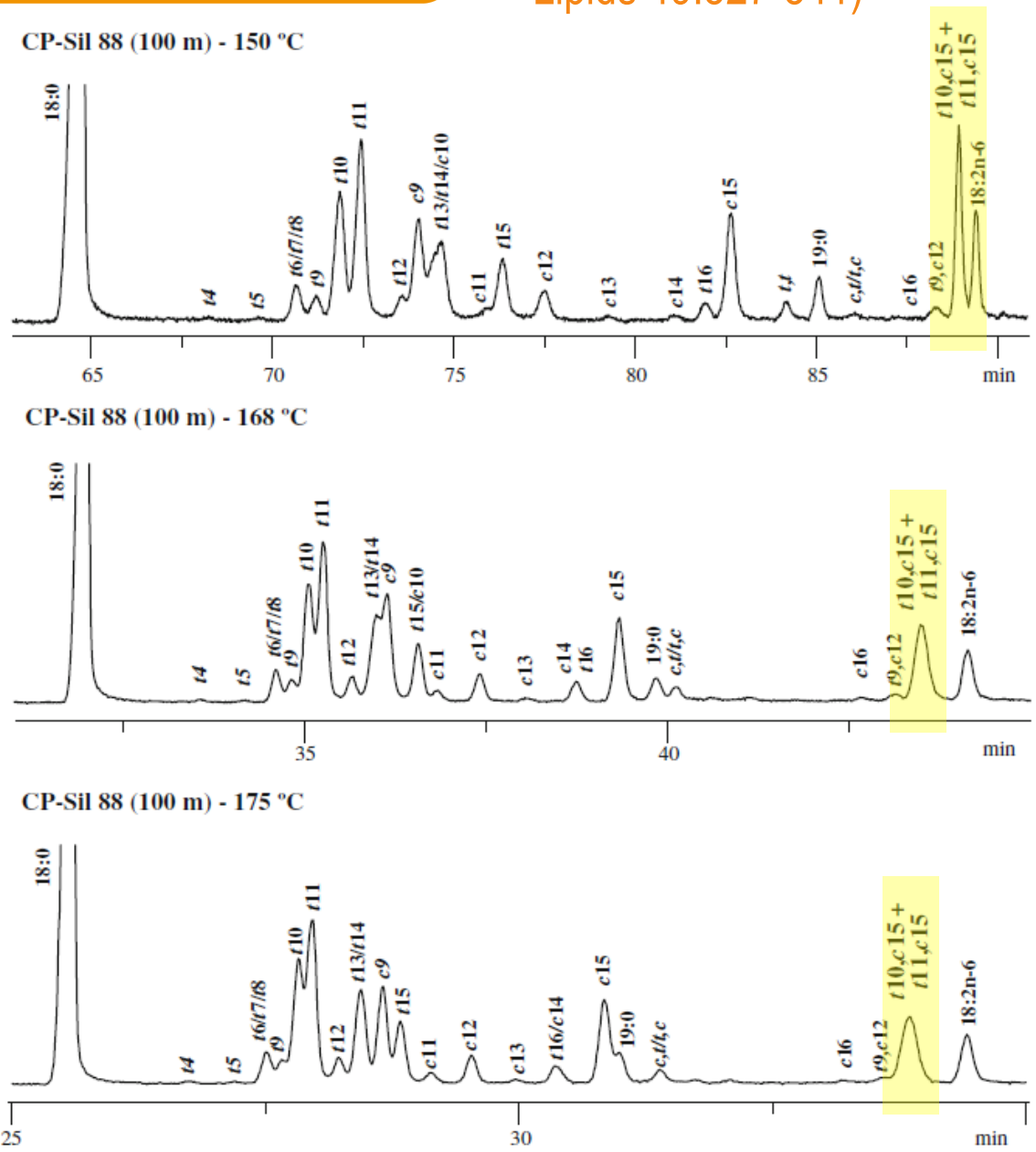
Disadvantage:

- Elution of the SFAs in the region between the *trans*- & *cis*- FA isomers.

GC/FID – improved separations

(Alves & Bessa, 2014; Lipids 49:527-541)

Differentiation of missing intermediate (10*t*,15*c*-18:2) of 10*t*-shifted rumen bio-hydrogenation pathway from 11*t*,15*c*-18:2.



GC/FID – improved separations

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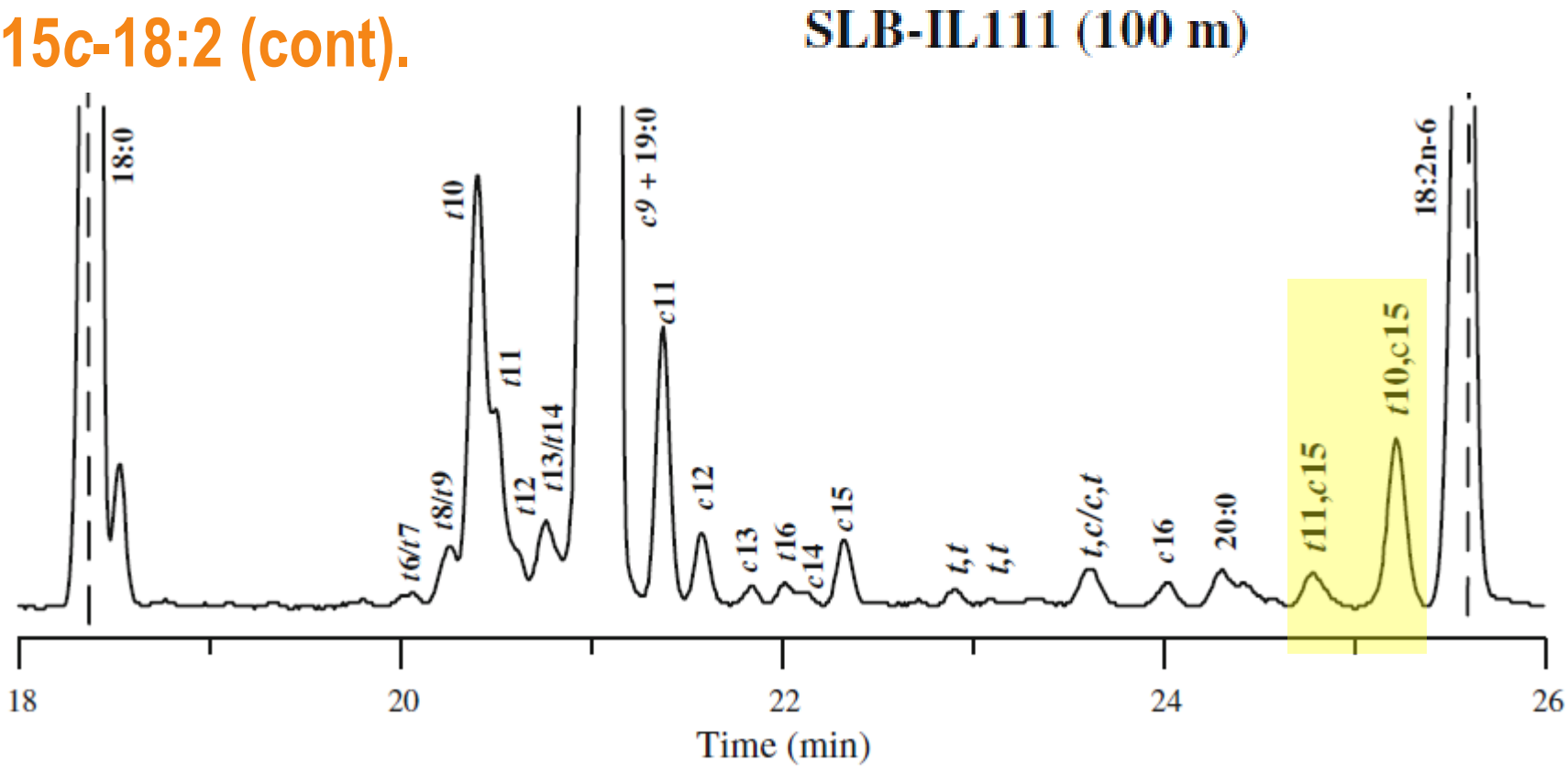
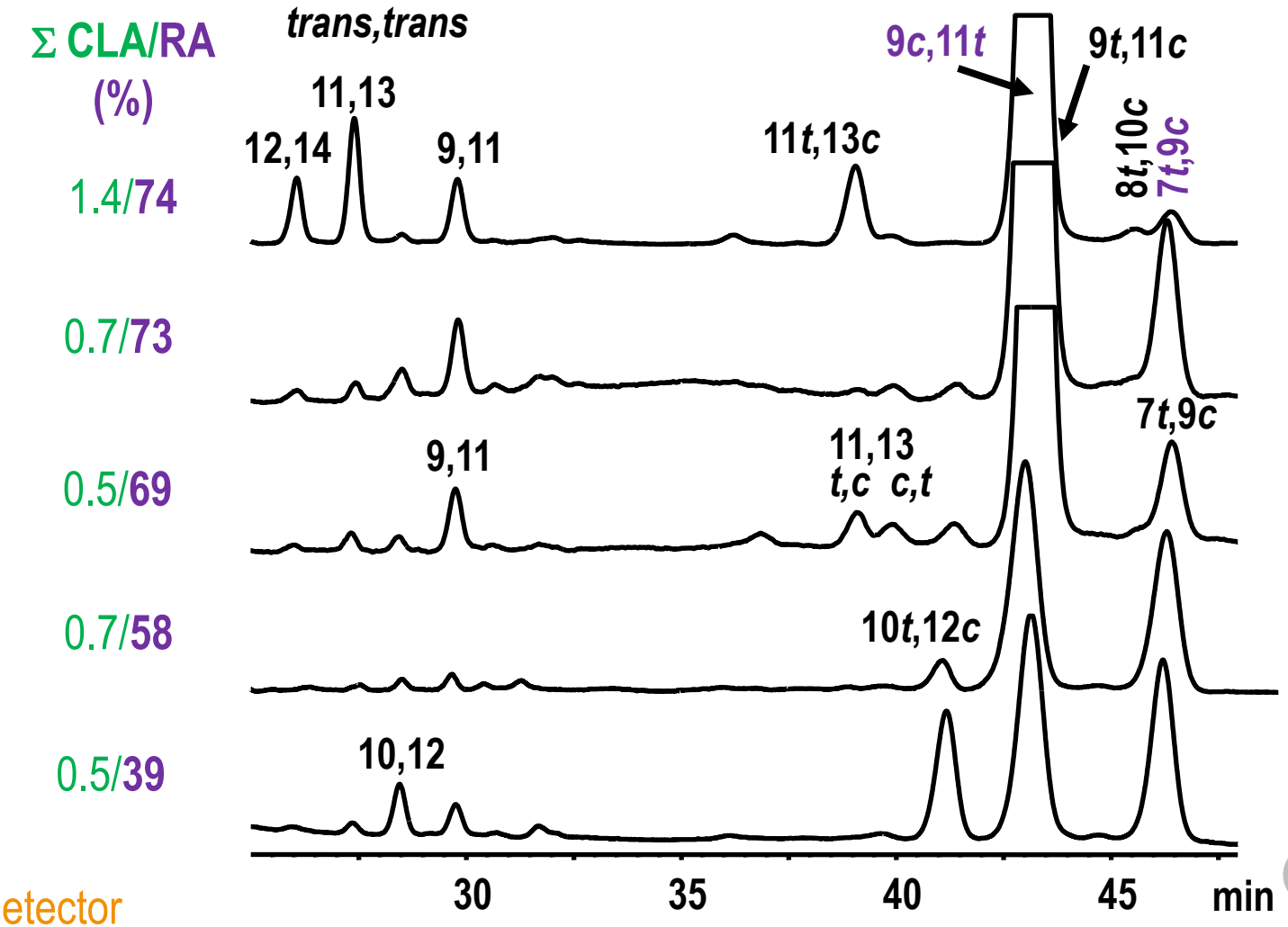


GC/FID – improved separations

Ag^+-HPLC (DAD; 233nm): CLA isomer separation

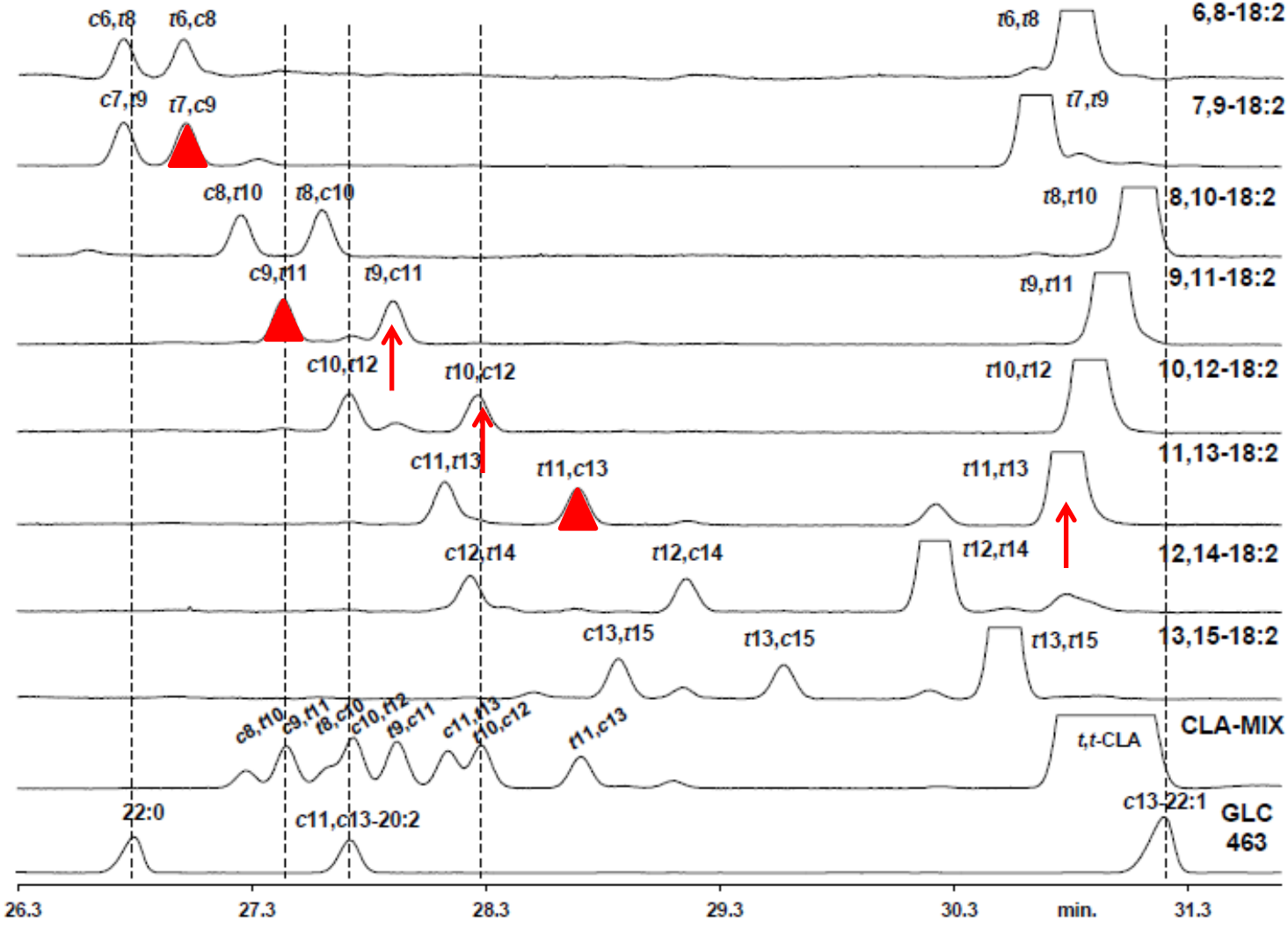


RA: rumenic acid

DAD: diode array detector

GC/FID – improved separations

Partial GC chromatogram of CLA isomers with double bond positions from 6,8- to 13,15-18:2 using the ionic column.



(Delmonte *et al.*, 2011)

GC/FID – peak ID

Standards (STD):

- Lack of authentic standards for some (or many) FAMEs.

  Commercial suppliers:

  - Nu-Chek Prep, Inc., MN, USA.
  - Matreya, PA, USA.
  - Larodan Fine Chemicals, Sweden.
  - Sigma-Aldrich Co., USA.

- Need to use methods/references where ID has been verified → Use retention times & elution orders from literature.

GC/FID – peak ID

Make your own standards:

- *Trans* FAME from a partially hydrogenated oil
- Vegetable & fish oils
- Simple synthesis: isomerization with *P*-toluene sulfinic acid or with I_2, reduction with hydrazene, conjugation with alkali, … (Delmonte *et al*., 2009).

- In-house mixtures:

Good STD:

#463 (Nu-Chek) + 21:0 + 23:0 + CLA mixture #UC-59M (Nu-Chek) + other LC-SFAs

GC/FID – quantification (IS)

When / which to add?

- For large tissue portions gravimetric determination of the total lipids extracted is just as accurate as adding large amounts of IS to the whole sample, and is more economical.
- Ideally, IS should **not be present** in the sample and should be **within the range** of FA chain length found in the sample.

  1) Examine the total lipid profile without addition of an IS.

  2) Finding a FA not present in ruminant fat is virtually impossible, therefore a compromise is necessary.

  Chose one with less interference.

GC/FID – quantification (IS)

Most commonly used IS:

TAG, FAME or FFA of 13:0, 15:0, 17:0, 19:0; 21:0, & 23:0.

- 13:0 – reasonable. Present in only minor amounts.
- 15:0, 17:0 – not recommended. Present in animal fats and elute in a region that is already crowded with many other FAME isomers.
- 19:0 – not recommended. Overlaps with 15*c*-18:1 and some *t,t*-18:2.
- 21:0 – not recommended. Elutes among the CLA isomers.
- 23:0 – reasonable. Present only in trace amounts and well resolved on SP-2560 & ionic columns, but there may be solubility problems.

Confirmatory methods

Silver ion fractionation (Ag^+-SPE)

- Rapid method to confirm structure based on **number of double bonds** & **geometric configuration** of FAME fractions.

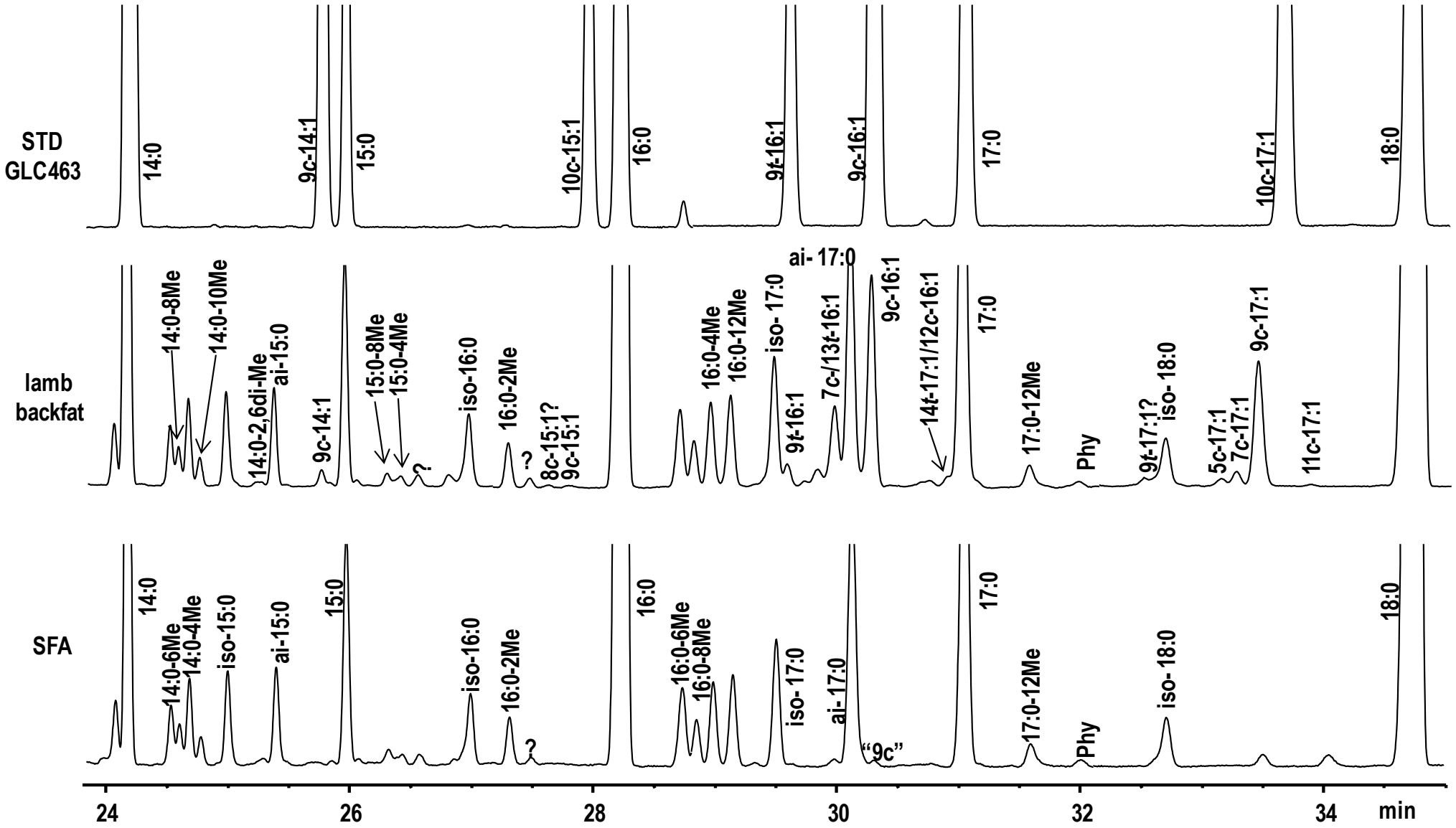
  Kramer *et al*. (2008)

- Use Ag^+-SPE cartridges with **glass** casings.

  Belaunzaran *et al*. (2014, 2016)

Confirmatory methods

GC program:
45-**175**-215ºC
(Bravo-Lamas *et al.*, 2016)



Confirmatory methods

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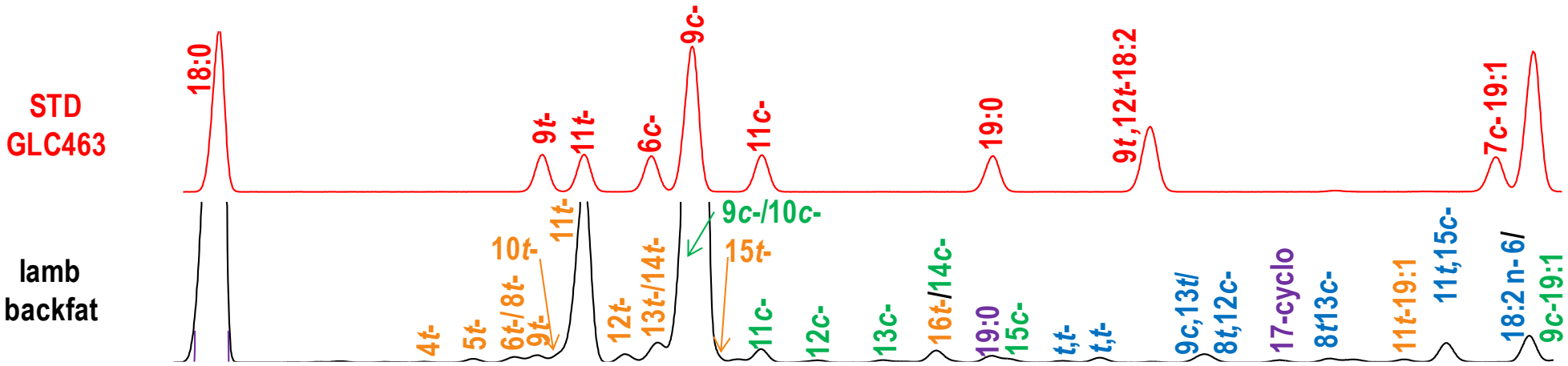
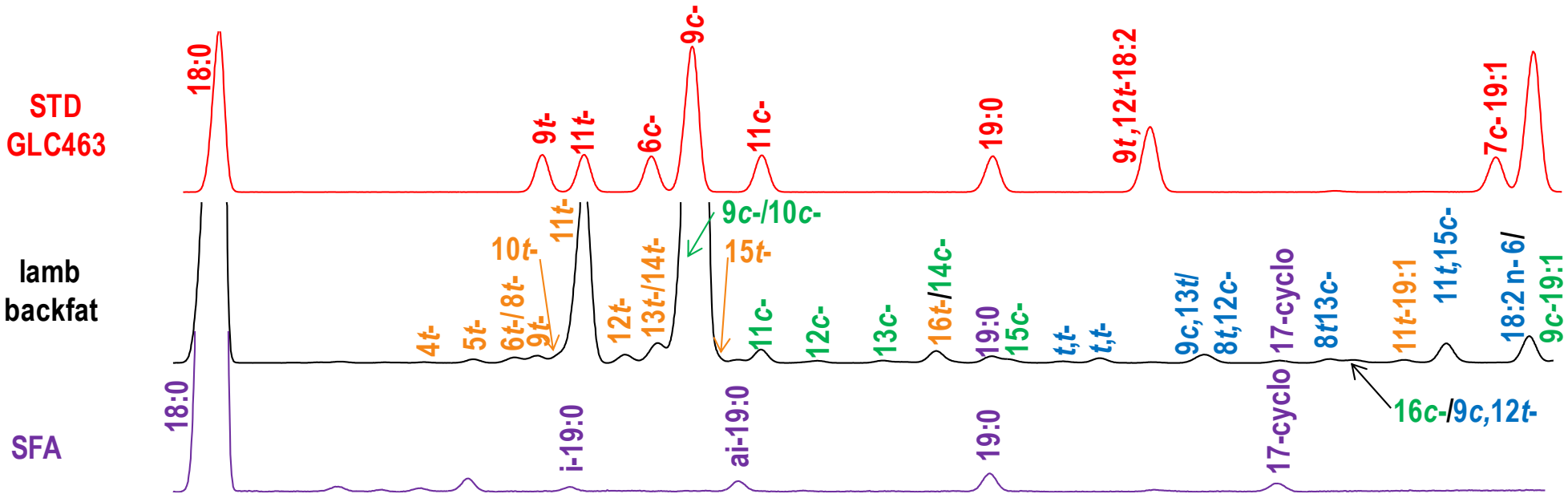


Confirmatory methods

GC program:
45-**175**-215ºC



Confirmatory methods

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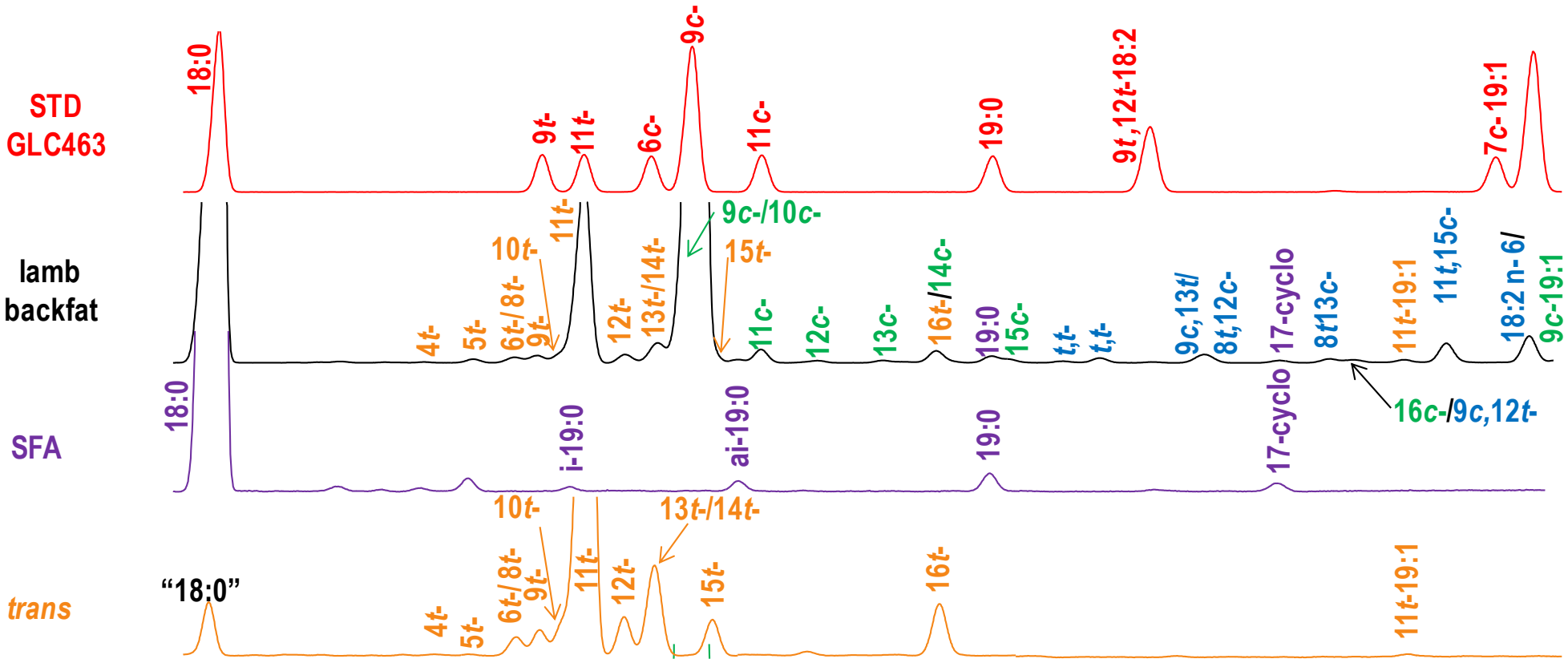

Confirmatory methods

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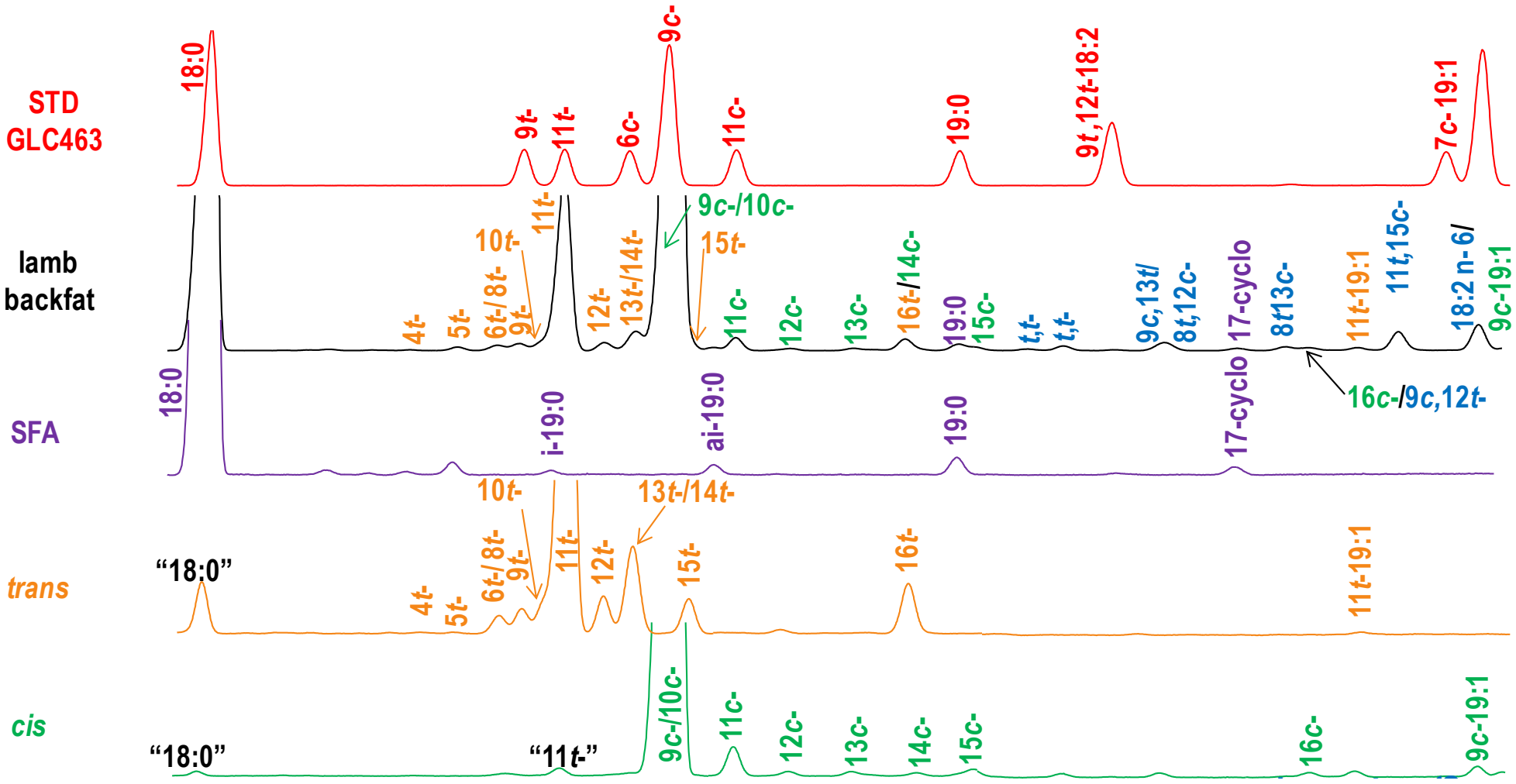
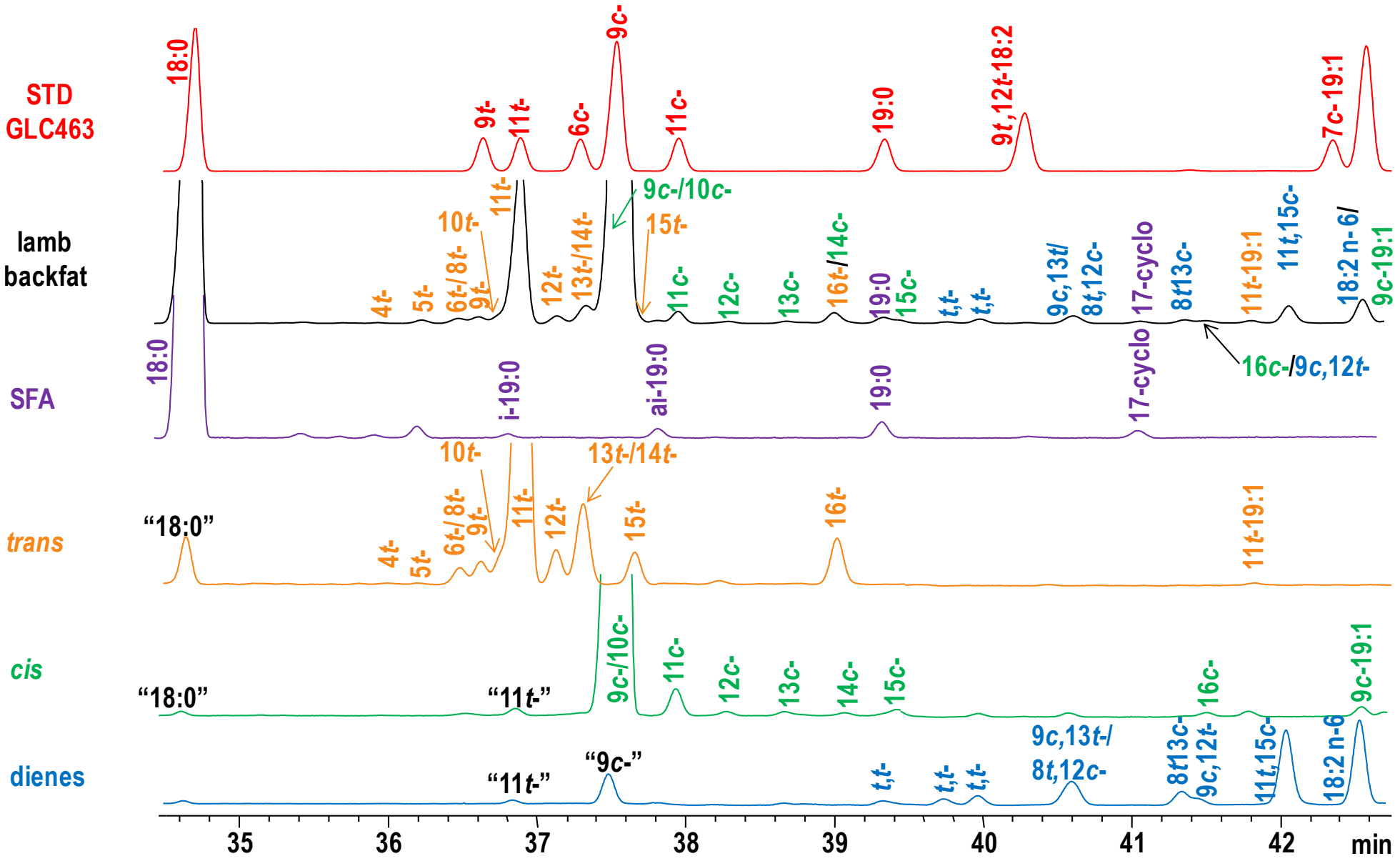


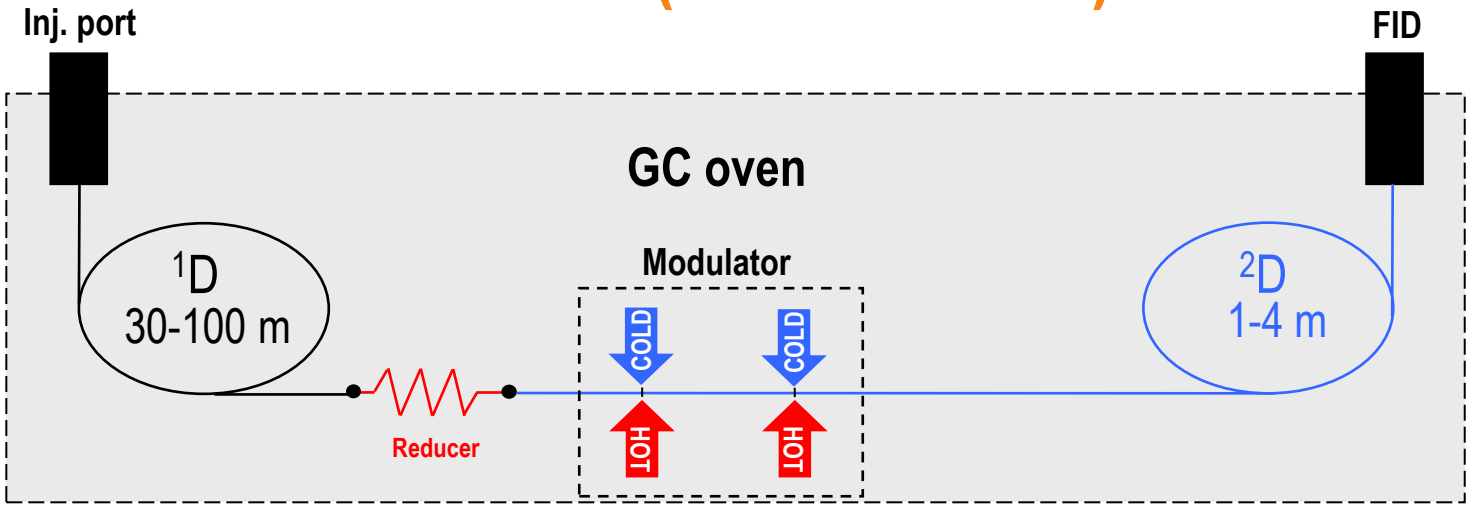
Confirmatory methods

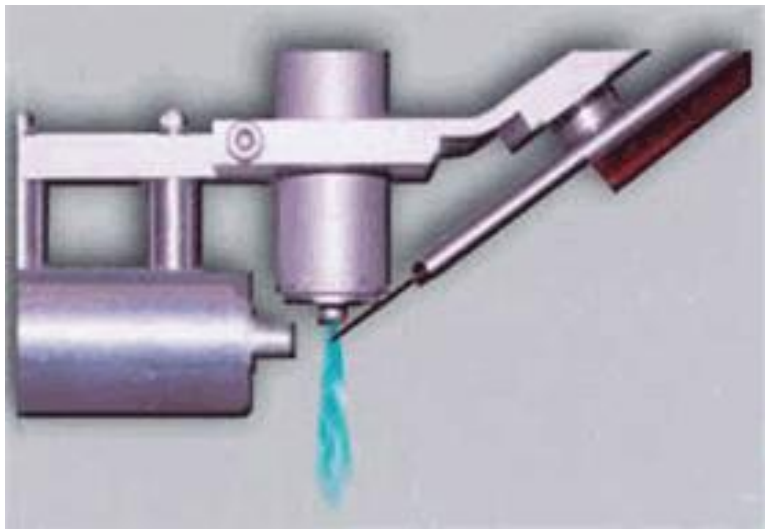
GC program:
45-**175**-215ºC



Confirmatory methods

GC - online reduction x GC (GC-OR x GC)

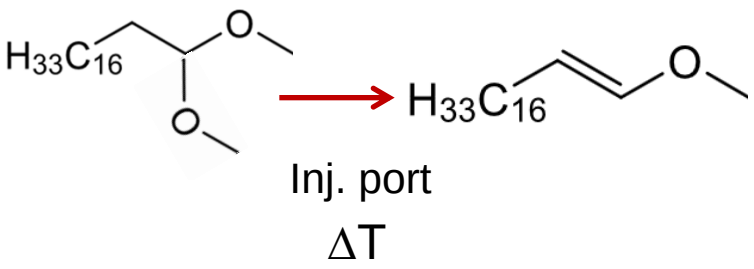


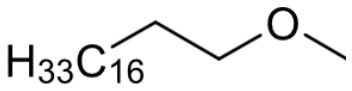


GC–OR x GC

Principle:

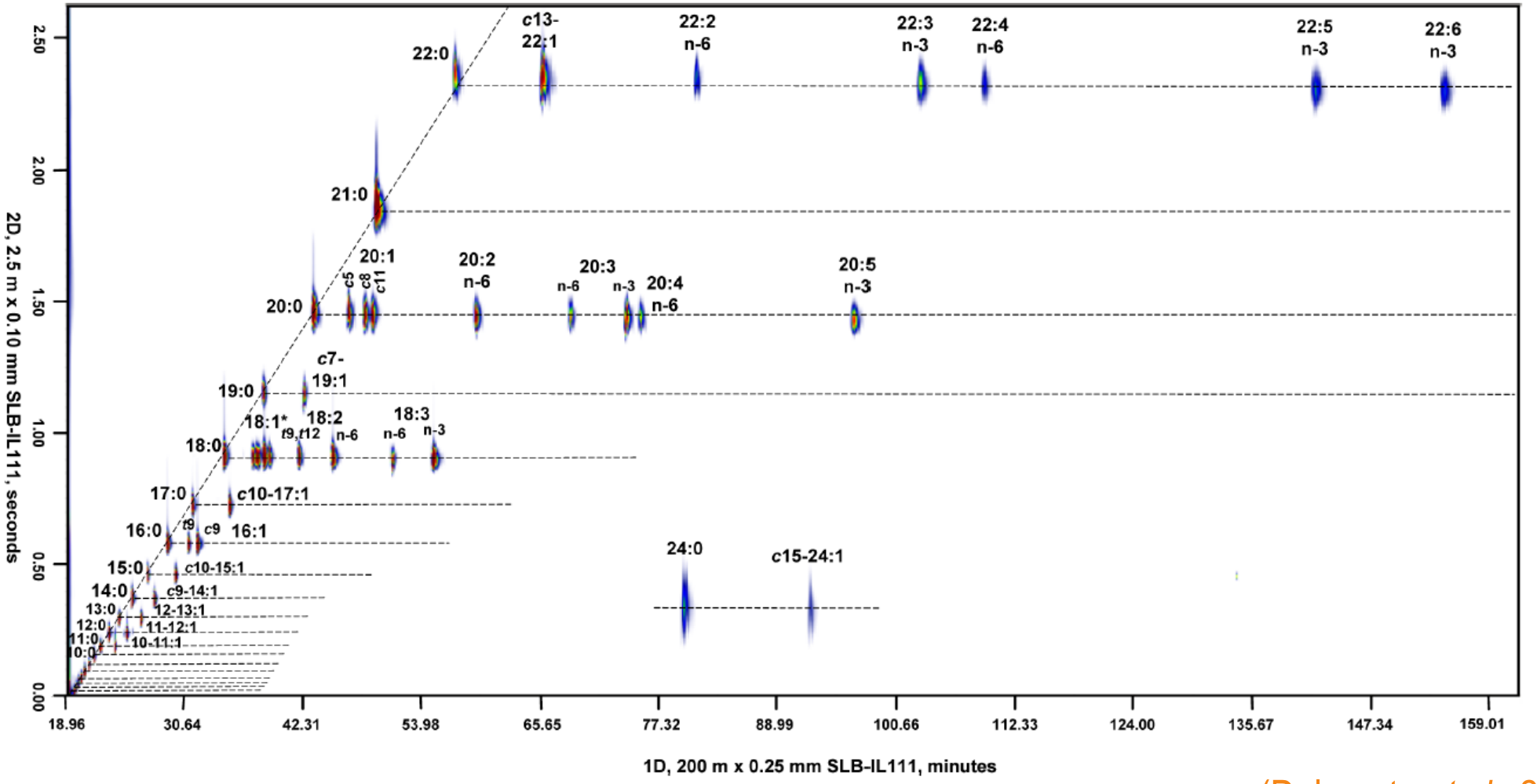
^{1}D separation	Reduction	^{2}D separation
……		……
16:0, 16:1, 16:3, 16:4		16:0
18:0, 18:1, 18:3, 18:4	Pd capillary reducer	18:0
20:0, 20:1, 20:3, 20:4, 20:5, …	(H_2, carrier gas)	20:0
22:0, 22:1, 22:2, 22:3, 22:4, …		22:0
24:0, 24:1, …		24:0
……		……





GC–OR x GC

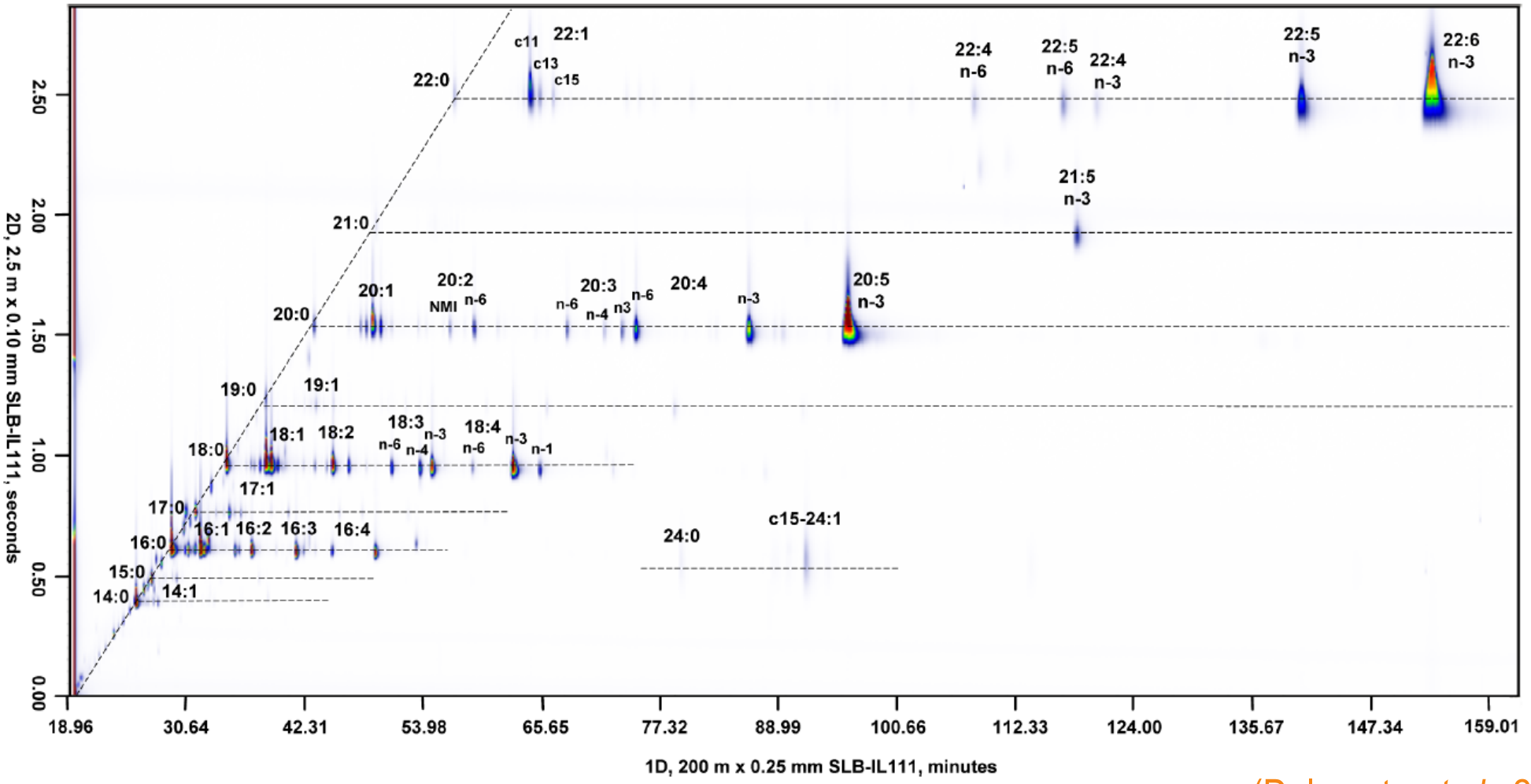
Reference mixture GLC463 (Nu-chek Prep., Inc.)



(Delmonte *et al.*, 2013)

GC–OR x GC

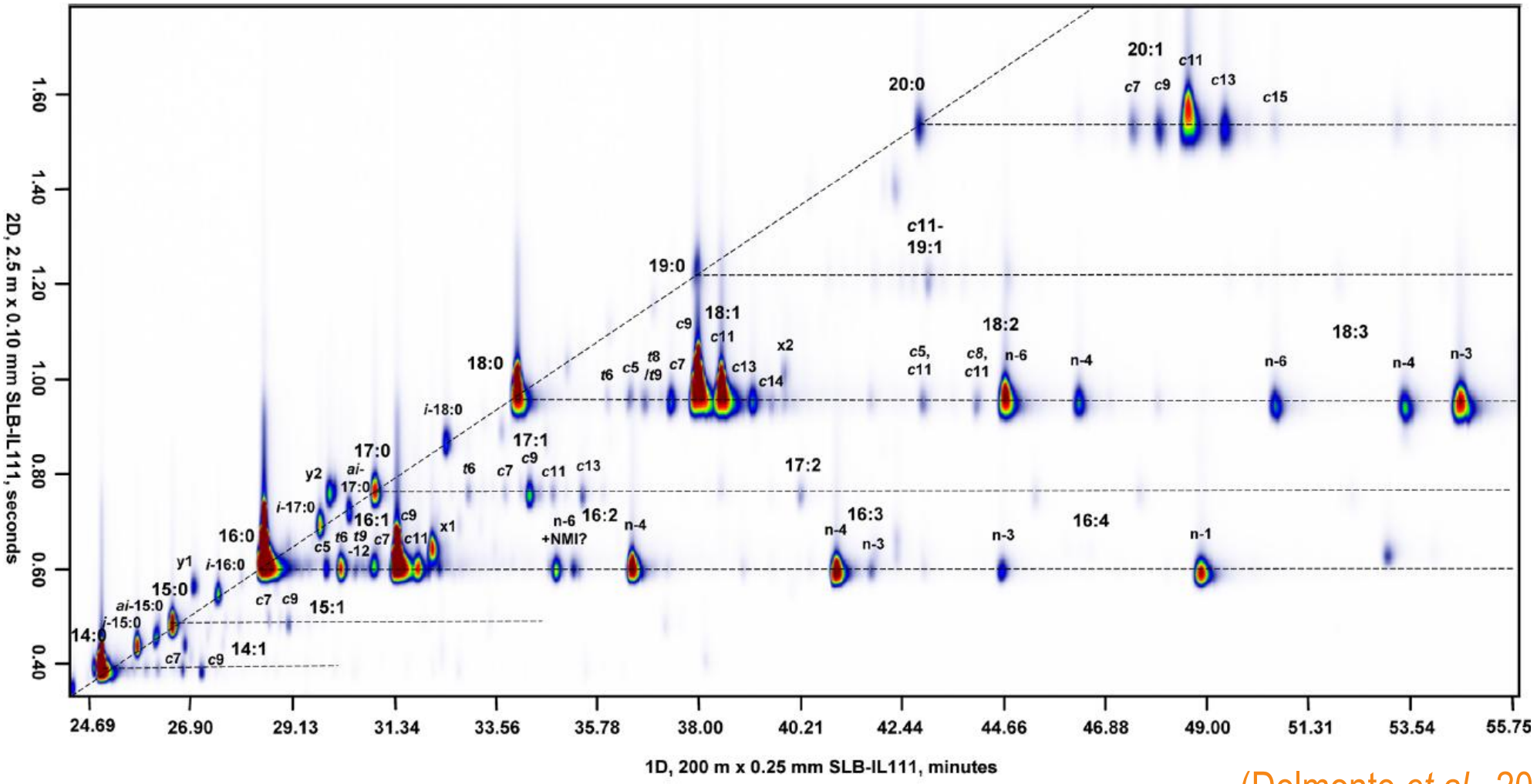
Menhaden fish oil sample



(Delmonte *et al.*, 2013)

GC–OR x GC

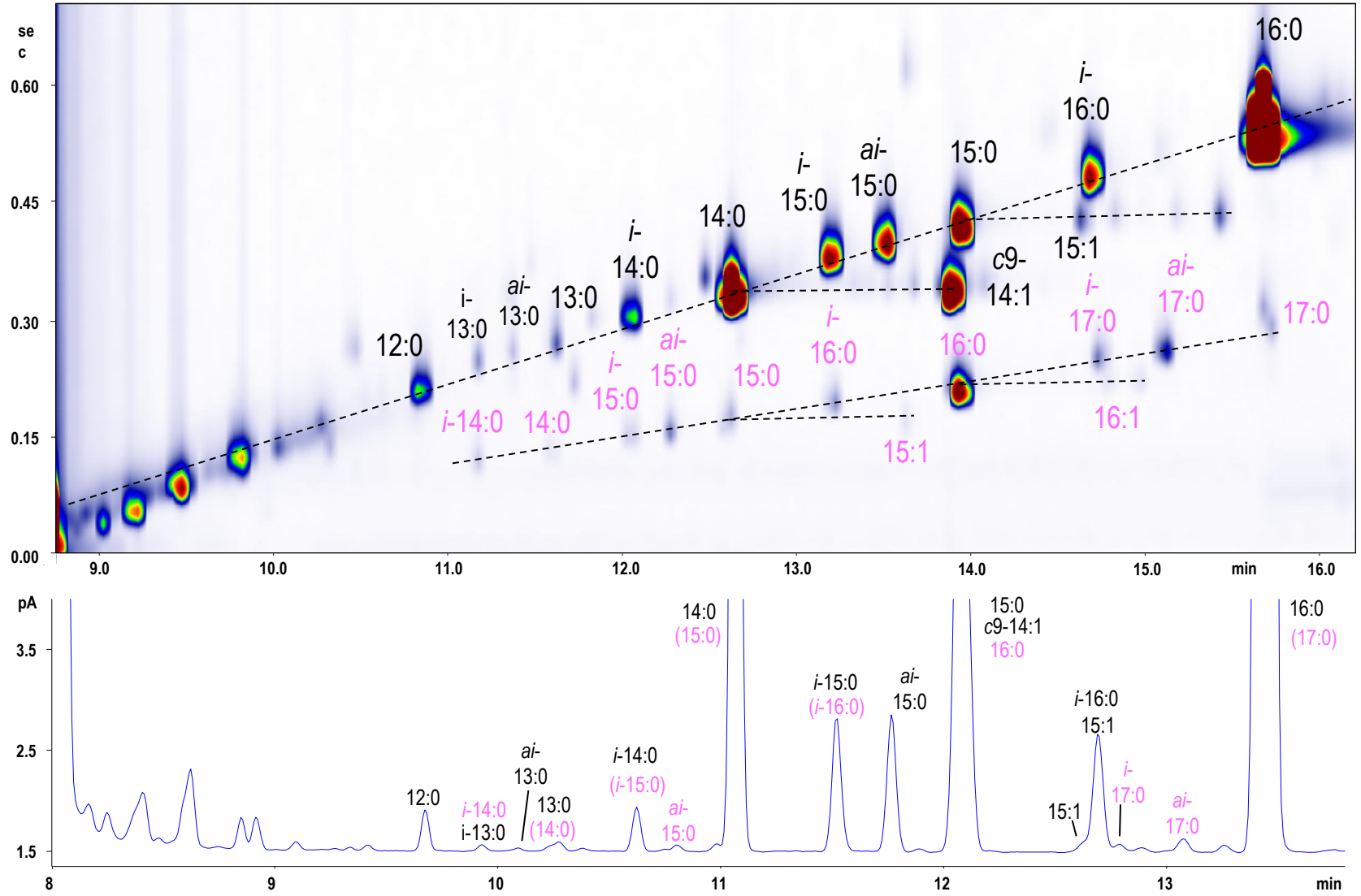
Menhaden fish oil sample (14:0 to 18:3n-3 region)



(Delmonte *et al.*, 2013)

GC–OR x GC

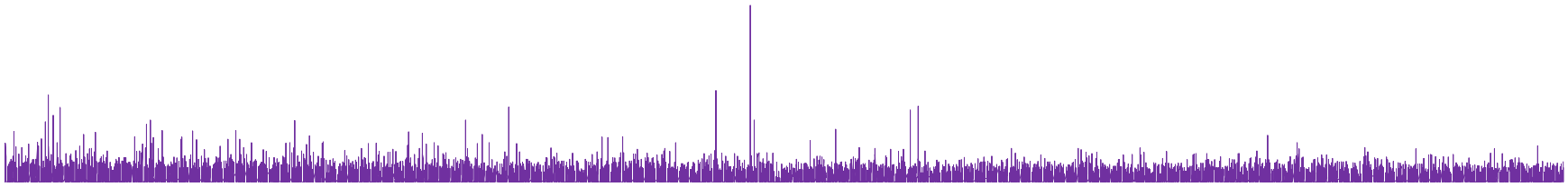
Bovine muscle (solvent front-16:0 region)



Confirmatory methods

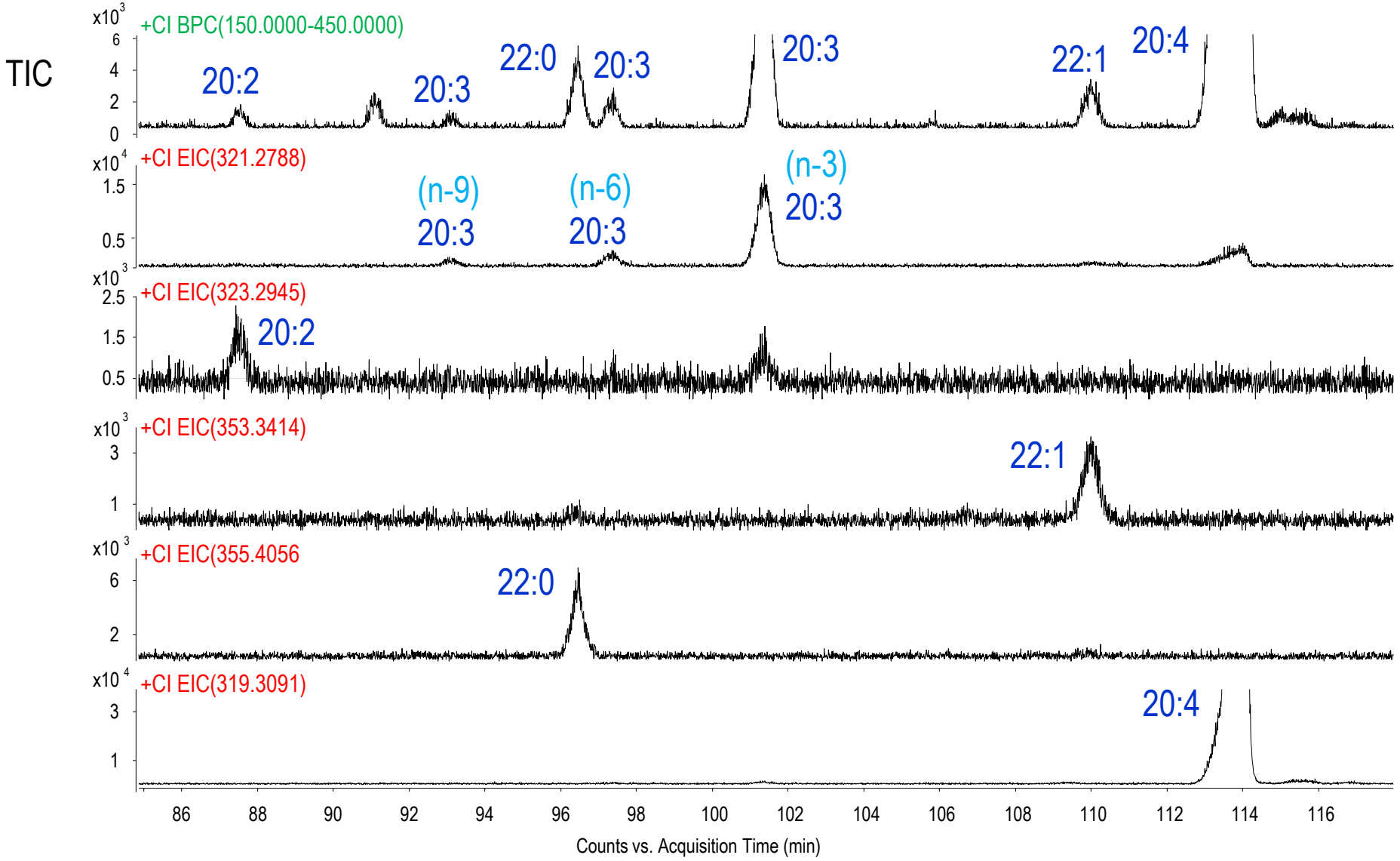
GC – QTOF/MS

- Column: SP2560, 100m x 0.25mm
- Carrier: Helium at 1.1mL/min constant flow
- Elution temperature: 180ºC isothermal
- Ionization: **chemical ionization** with **isobutane (Cl^+)**



GC–QTOF/MS

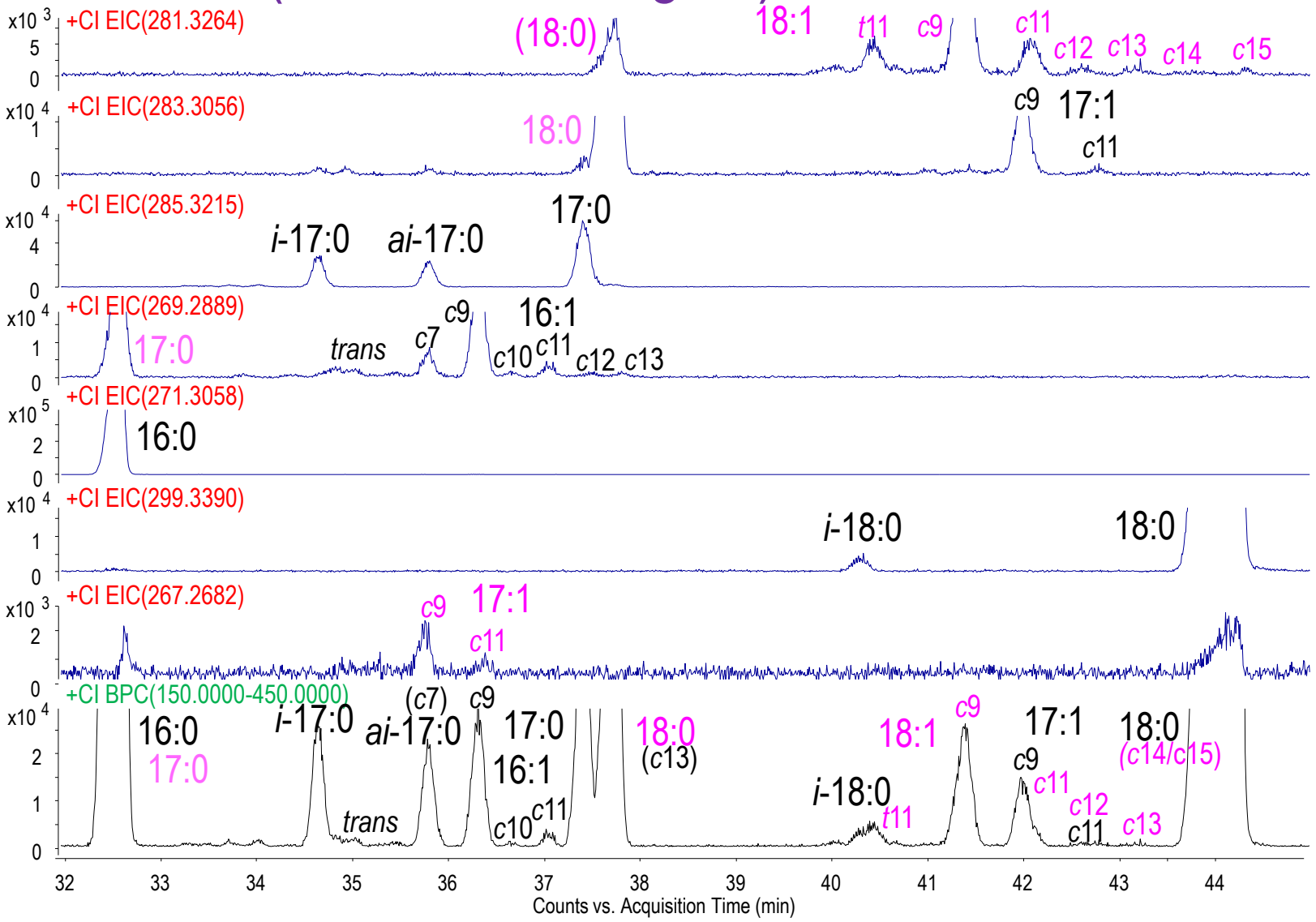
Menhaden fish oil



GC–QTOF/MS

DMA FAME

Bovine muscle (16:0 to 18:0 region)



Final remarks

1) Perform a complete lipid extraction (requires chloroform).
2) Methylate using both acid & base catalysts.
3) Purify FAMEs when necessary.
4) Apply proper sample load (concentration) onto GC column.
5) Use 100m columns (SP2560, ionic).

Final remarks (cont.)

6) Ensure good separations of 16:1, *trans*-18:1, 18:3 and CLA regions, and specific critical pairs:

*iso*17:0 // 8*t*- & 9*t*-16:1

*anteiso*17:0 // 7*c*- & 9*c*-16:1

10*t*-18:1 // 11*t*-18:1

10*t*,15*c*-18:2 // 11*t*,15*c*-18:2

21:0 // CLA

EPA // 24:0, …

Final remarks (cont.)

7) Remember 9*c*,11*t*- is not resolved from 7*t*,9*c*-CLA using SP-2560 or CP Sil88 columns. However, these and other CLA isomers can be resolved using ionic GC column or Ag^+ -HPLC.

8) Deal with plasmalogens and sphingomyelin in meat and blood products.

9) Use confirmatory methods to establish peak ID.

Bibliography

-Sebedio & Christie, W.W. (1998) Trans Fatty Acids in Human Nutrition. The Oily Press, Dundee, Scotland.

-Christie, W.W. (2003) Lipid Analysis. Isolation, Separation, Identification and Structural Analysis of Lipids. 3rd Ed. The Oily Press, Bridgwater, England.

-Cruz-Hernandez, C., J.K.G. Kramer, J. Kraft, V. Santercole, M. Or-Rashid, Z. Deng, M.E.R. Dugan, P. Delmonte, and M.P. Yurawecz. (2006) Systematic analysis of *trans* and conjugated linoleic acids in the milk and meat of ruminants. Pages 45-93 in Advances in Conjugated Linoleic Acid Research, Volume 3. M.P. Yurawecz, J.K.G. Kramer, O. Gudmundsen, M.W., Pariza, and S. Banni, eds. AOCS Press, Champaign, IL.

-Mossoba, M.M. & Kramer, J.K.G. (2009) Official Methods for the Determination of Trans fat. 2nd Ed. AOCS Press, Urbana, Illinois.

Bibliography (cont.)

-Ratnayake, W.M.N. & Cruz-Hernandez, C. (2009) Analysis of trans fatty acids of partially hydrogenated vegetable oils and dairy products. Pp. 105-146 in Trans Fatty Acids in Human Nutrition, 2nd Ed. F. Destaillats, J.L. Sebedio, F. Dionisi & J.M. Chardigny, eds. The Oily Press, Bridgwater, UK.

-Aldai N; Kramer JKG; Cruz-Hernández C; Santercole V; Delmonte P; Mossoba MM; Dugan MER. (2012) Appropriate Extraction and Methylation Techniques for Lipid Analysis. Pp. 249-290 in *Fats and Fatty Acids in Poultry Nutrition and Health* (Eds., G. Cherian & R. Poureslami), Nottingham Press, UK.

http://www.cyberlipid.org
http://lipidlibrary.aocs.org
http://library.med.utah.edu/NetBiochem

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Research group
www.ehu.es/lactiker

Thank you for the attention

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