Lipid Profiling Using Sub-2µm Particle CO_2 Based Supercritical Fluid Chromatography Coupled to Mass Spectrometry

Compositional Analysis of Lipids

20-21 June 2013, Het Pand, Ghent, Belgium

Giorgis Isaac, PhD

A Partner Yesterday, Today and Tomorrow

Waters
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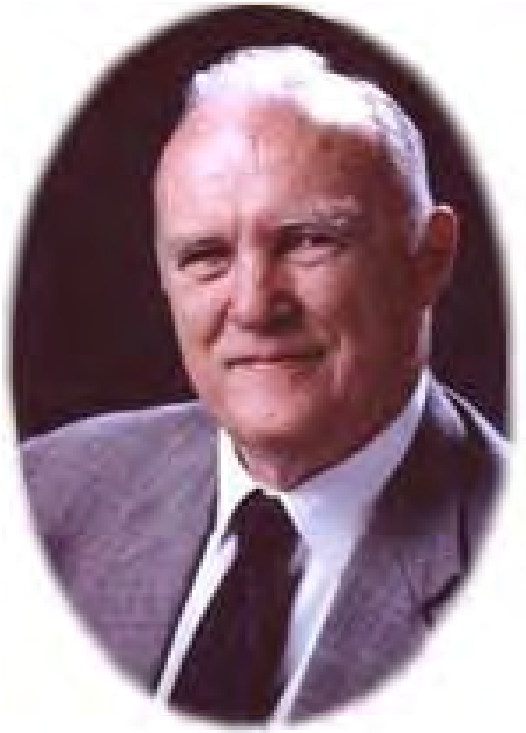
- Global leader focused on Complementary Analytical Technologies
- Year founded: 1958
- Publicly traded (NYSE:WAT)
- Headquartered in Milford, Massachusetts
  - Manufacturing in United States, Ireland, United Kingdom, and Singapore
- Number of Employees Worldwide: 5,700,
  - 2,600 Sales and Service to Maintain Direct Link with Customers



James Waters
Founder

Core Competencies:
A Total Systems Solution Approach

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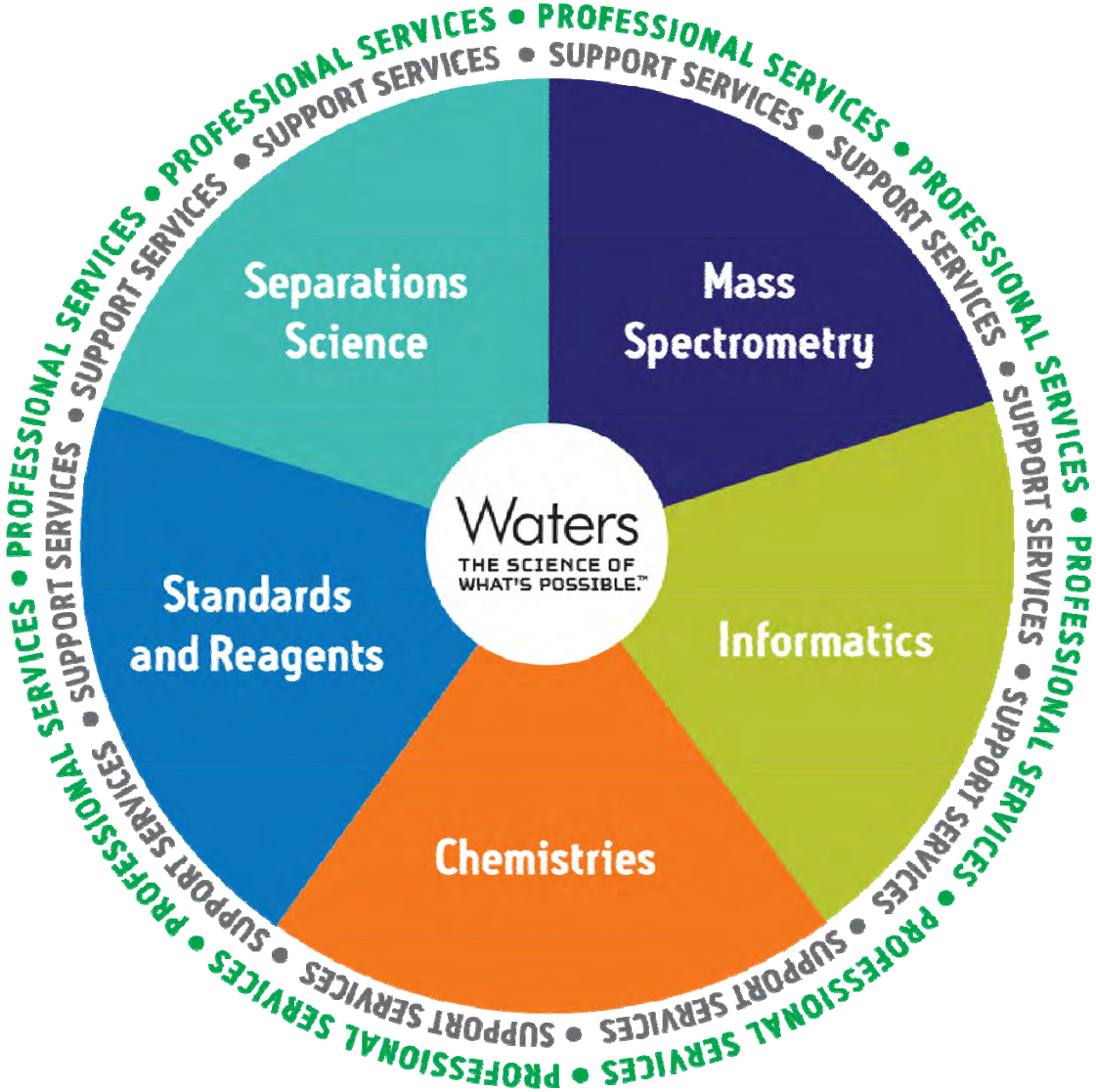

Contents

- Introduction and UPC^2 Overview
  - What is UPC^2?
- UPC^2 Column Screening for Lipid Class Separation
  - Biological Application
- UPC^2 Free Fatty Acid and Neutral Lipid (TG and CE) Method
  - Biological Application
- Conclusions

UltraPerformance Convergence Chromatography

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Convergence Chromatography is a category of separation science that provides orthogonal and increased separation power, compared to liquid or gas chromatography, to solve separation challenges.

UltraPerformance Convergence Chromatography [UPC²] is a holistically designed chromatographic system that utilizes liquid CO_2 as a mobile phase to leverage the chromatographic principles and selectivity of normal phase chromatography while providing the ease-of-use of reversed-phase LC.

The **ACQUITY UPC² System** is built utilizing proven UPLC Technology to enable scientists the ability to address routine and complex separation challenges while delivering **reliability, robustness, sensitivity** and **throughput** never before possible for this analytical technique.

Separation Technology Overview

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Gas Chromatography

GC

Separation achieved by a temperature gradient

- **High efficiency [N]**
  - Virtually no limitation on column length
- **Limited selectivity [α]**
  - Limited stationary phase options

Liquid Chromatography

LC

Separation achieved by a solvent gradient

- **High efficiency [N]**
  - Limited to pressure drop across column
- **Moderate selectivity [α]**
  - Different modes: reversed-phase, normal-phase, SEC, IEX, affinity, ion pair, HILIC, GPC…etc.

Convergence Chromatography

CC

Separation achieved by density/solvent gradient

- **High efficiency [N]**
  - Very low viscosity enables longer columns and smaller particles
- **High selectivity [α]**
  - Wide variety of stationary phase and mobile phase co-solvent and modifier options

Evolution of Separation Technology

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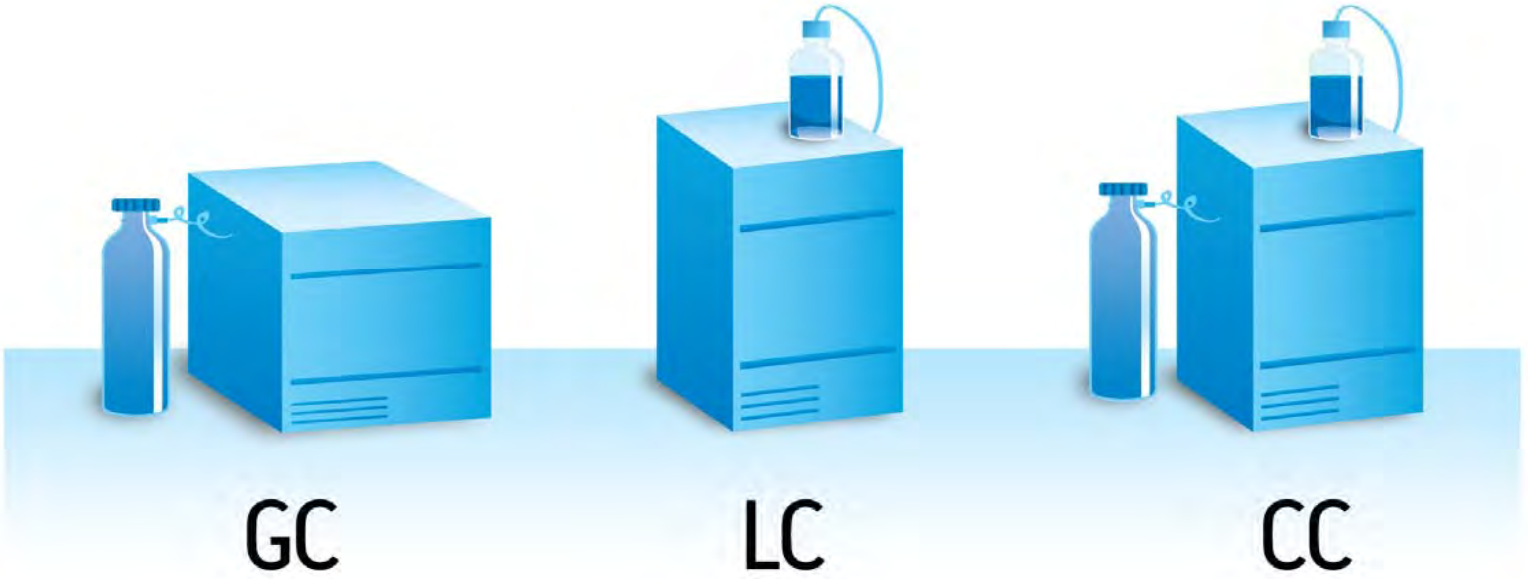

GC
Gas Chromatography

LC
Liquid Chromatography

CC
Convergence Chromatography

GC

↓

Capillary GC

HPLC

↓

UPLC

SFC

↓

UPC²

UPLC and UPC2: Resolution, Sensitivity, Throughput

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Workflow Sample Preparation

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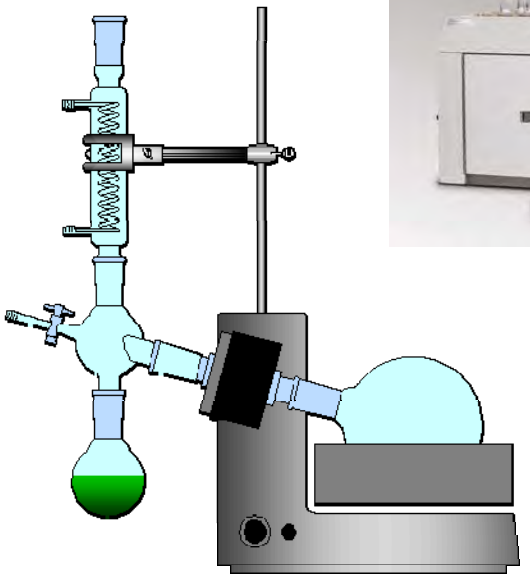
Drying

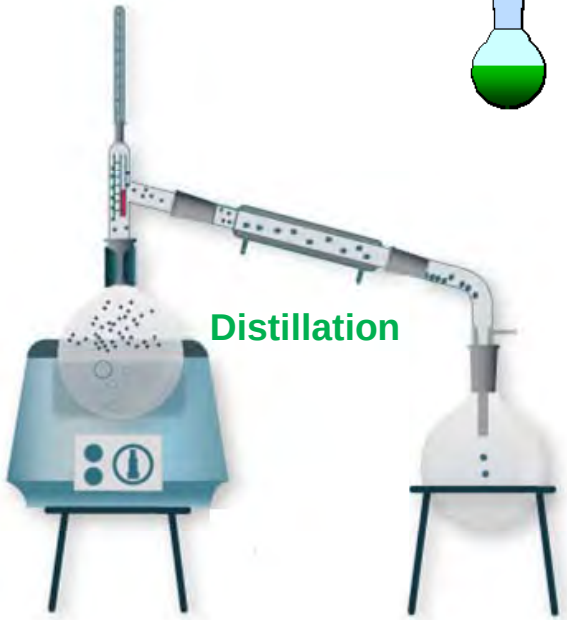
Grinding



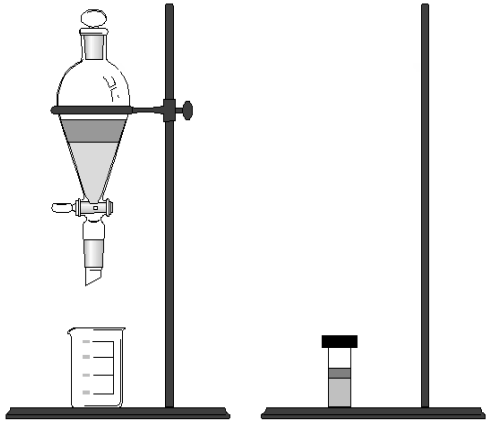
Evaporation

SFE

Soxhlet Extraction



Distillation



Macro Micro

Liquid / Liquid

Sample Preparation

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- Sample preparation is the most often cited area of improvement
- Most sample preparation involves being in an organic phase
  - Liquid/Liquid, PPT, Soxhlet, distillation, evaporation and reconstitution
  - Many matrixes will respond best to organic phases (gels, blisters, ointments, synthesis solvents, etc.)
- Many sample preparation steps then have to go through a phase transfer to put the analytes of interest into a less organic phase to be able to be injected onto reversed phase chromatographic systems
- This phase transfer process can be potentially eliminated by injecting directly onto a UPC^2 system
  - This is where significant cost savings can or have been made by companies
  - The secondary benefits of UPC^2 adding solvent reduction and faster analysis times is a driving factor

Where is UPC² Applicable?

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- Food and environment
  - Vitamins, essential oils, pesticides, lipids, triglycerides, food additives
- Pharmaceutical and Life Sciences
  - Metabolite ID, stability-indicating, impurity profiling
  - Purity assessment, final product analysis
  - Chiral
  - Orthogonal method screening (vs. RPLC)
- Chemical materials
  - Polymers, organometallics, dyes, petroleum, surfactants, petrochemicals, biodiesel
- Clinical research
  - Vitamin D metabolites
  - Steroids

ACQUITY UPC² Columns

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BEH 2-EP (2-Ethylpyridine)

BEH Technology™

- Good retention, peak shape and selectivity

BEH

- Heightened interaction with polar head groups such as phospholipids (use full for lipid class separation)

CSH Fluoro-Phenyl

CSH Technology

- Good retention of weak bases
- Alternate elution orders for acidic and neutral compounds

HSS C_{18} SB

HSS HIGH STRENGTH SILICA

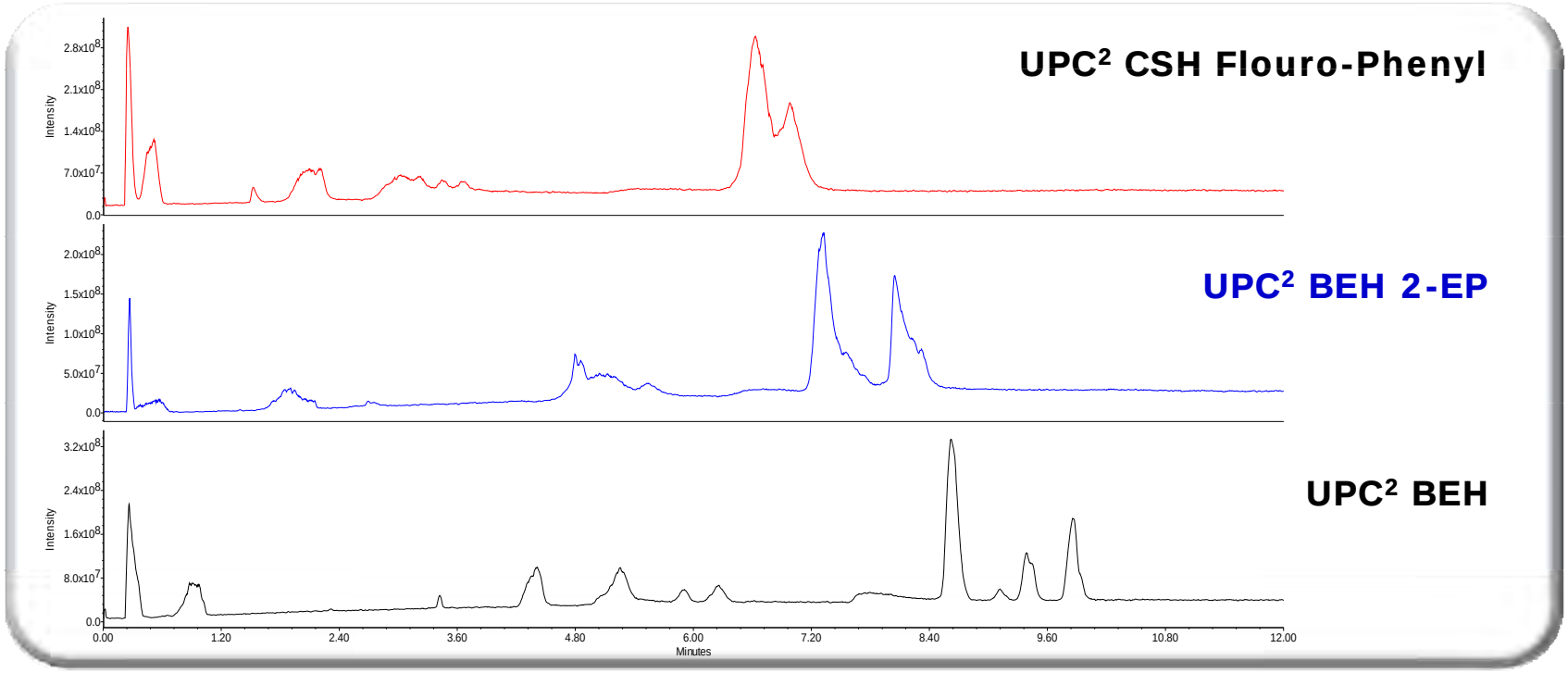
- Analysis across vertical markets (Lipids, Pharmaceutical, Food, Chemical Materials)

UPC² Column Screening for Lipid Class Separation

Sample Information

- Total lipid class extracts were purchased from Avanti Polar Lipids.
- Mix 1 and Mix 2 were Brain (porcine) extracts except for LPC and PG which were Egg (chicken).
- Stocks were prepared in 50:50 chloroform:methanol.
- A working lipid mixtures were prepared as follow:
  - Mix 1: Ceramide, SM, (0.05mg/mL) PG, PE, PC, (0.1mg/mL)
  - Mix 2: LPC, LPE, (0.05mg/mL)
  - Mix 3: 1:1 of [mix 1] and [mix 2]

Column Screening



- *12 minute 10-50%B screening method was used.*
- *Column screen utilizing UPC² available stationary phases performed by injection of the mixture*
- *The 2-EP and PFP gradients were modified 10-30%B to adjust for comparative use of the separation space.*
- *The co-solvent was 2g/L Ammonium Formate in MeOH based on previous reports from Bamba et al [1].*

1. Bamba T, *et al*. High Throughput and Exhaustive Analysis of Diverse Lipids by Using Supercritical Fluid Chromatography-Mass Spectrometry for Metabolomics. J. BioSc BioEng., **105**, 460-469, (2008)

Rapid Screening Separation

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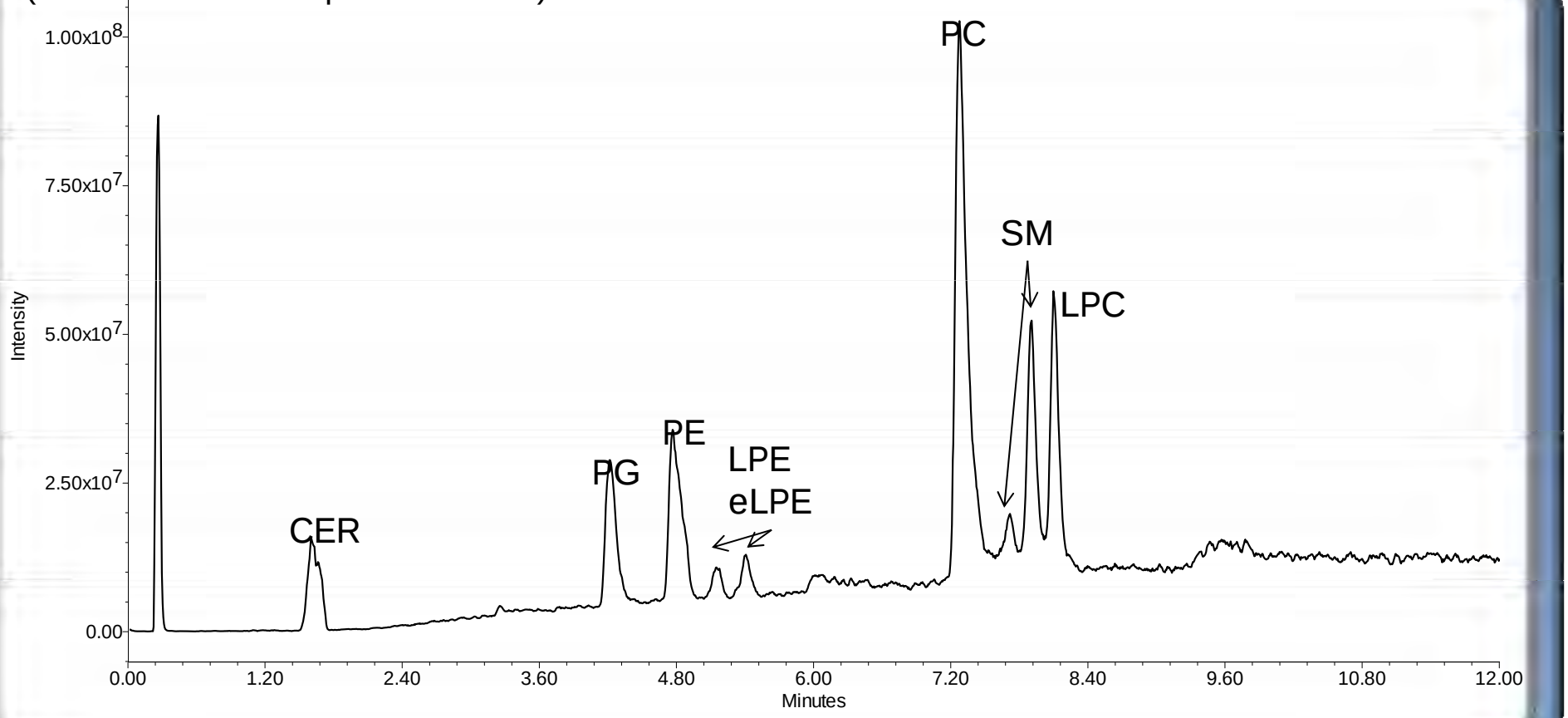
Lipids Mixture 3
(Avanti combined lipid standards)



ACQUITY UPC² BEH

ES+ TIC

Exploring Solvent Mixtures

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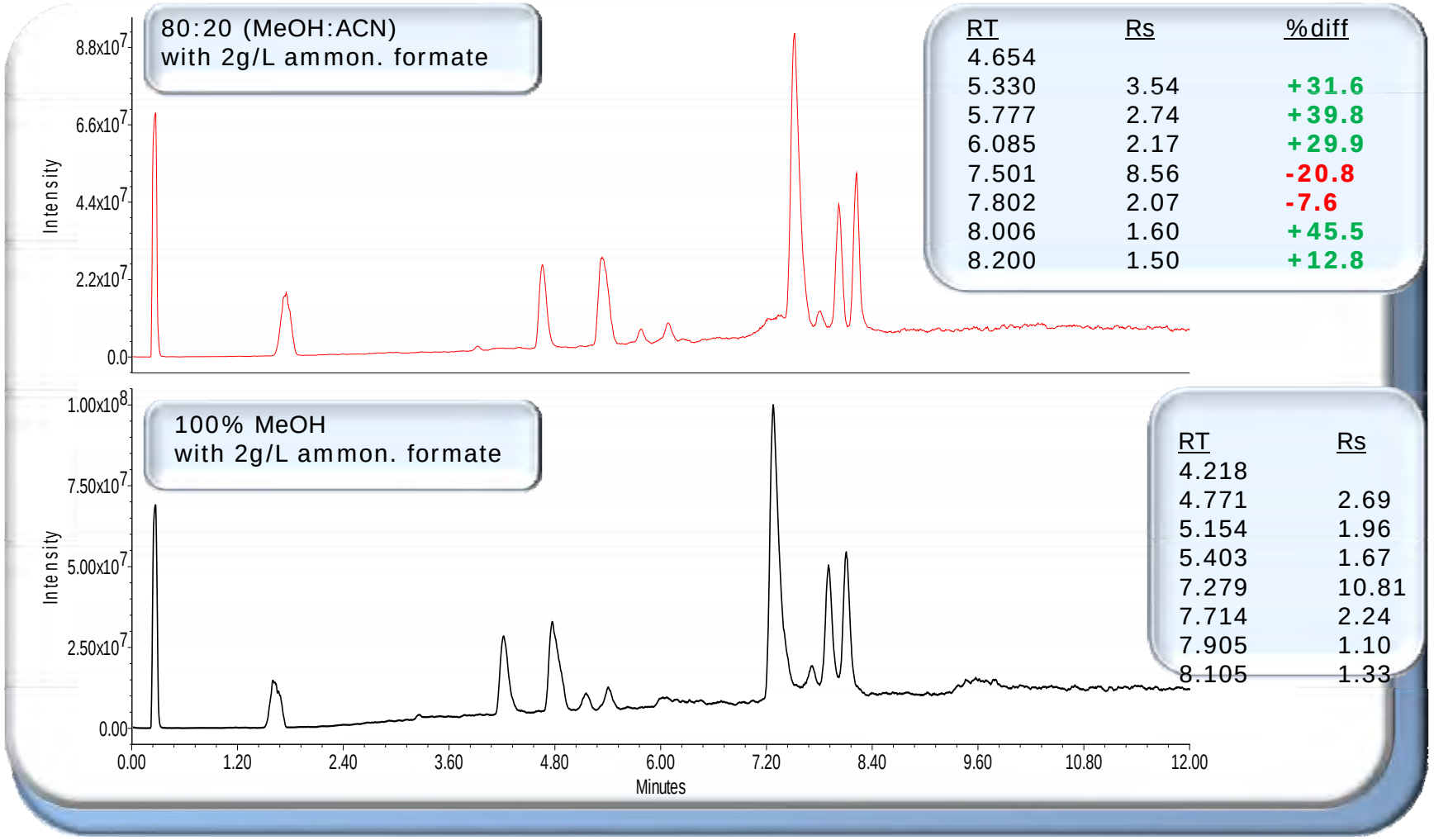
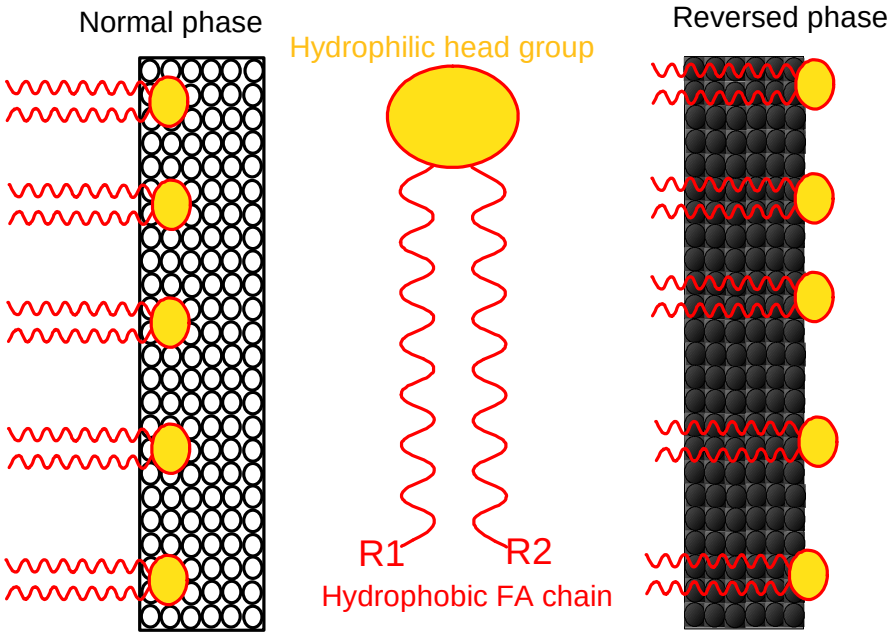


- *Addition of a weaker organic solvent can increase resolution between peaks*
- *No evidence of selectivity changes*

Retention Mechanisms of Lipids for HILIC vs. RP



- Bare silica or silica bonded to polar group such as cyano, amino
- Non polar MP Hex, chloroform
- Separation based on **adsorption of the head group to the NP material for lipid class separation.**

- Silica bonded to nonpolar group such as C18, C8, C4
- Polar MP water, MeOH, CAN
- Separation based on **hydrophobic interaction of the FA chain and RP material for lipid molecular species separation.**

PC Standards: Effects of Double Bond

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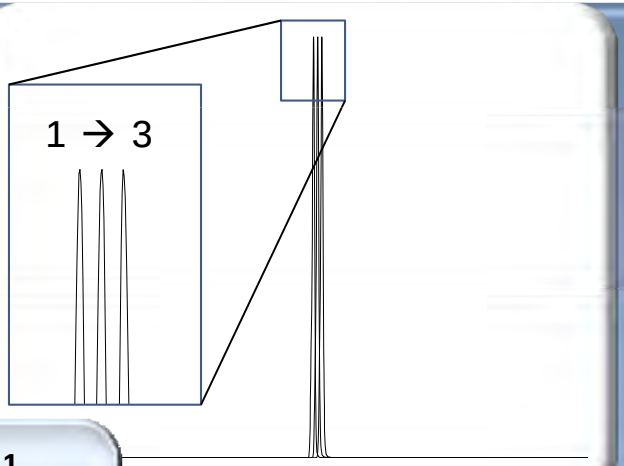
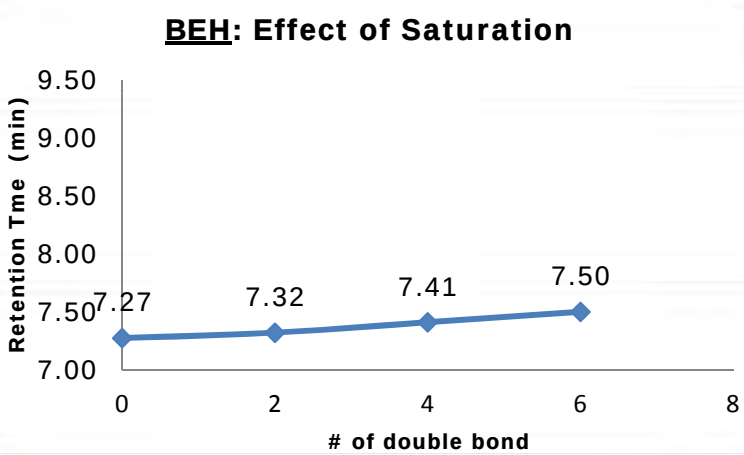
1. PC: 18:1(Δ9 cis) / 18:1 (Δ9 cis)
2. PC: 18:2(Δ9,12 cis) / 18:2 (Δ9,12 cis)
3. PC: 18:3(Δ9,12,15 cis) / 18:3 (Δ9,12,15 cis)





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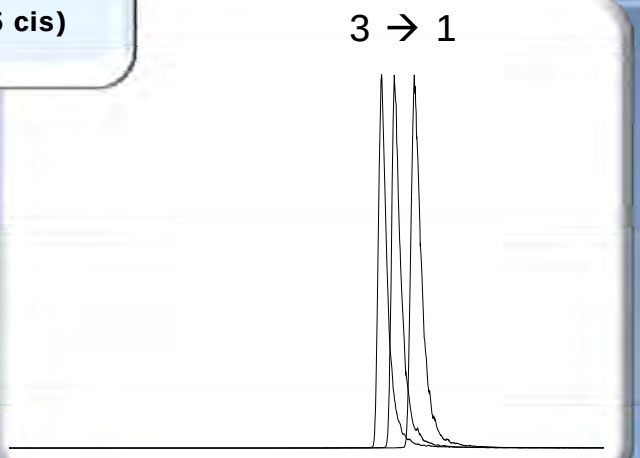

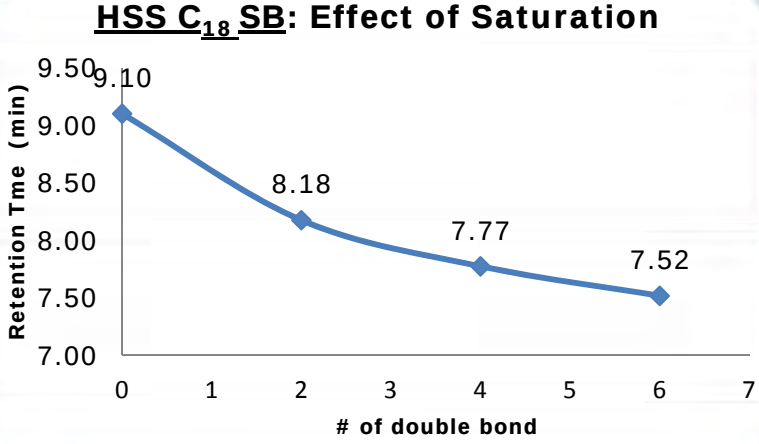

ACQUITY UPC² HSS C_{18} SB

PC Standards: Effects of Acyl Chain

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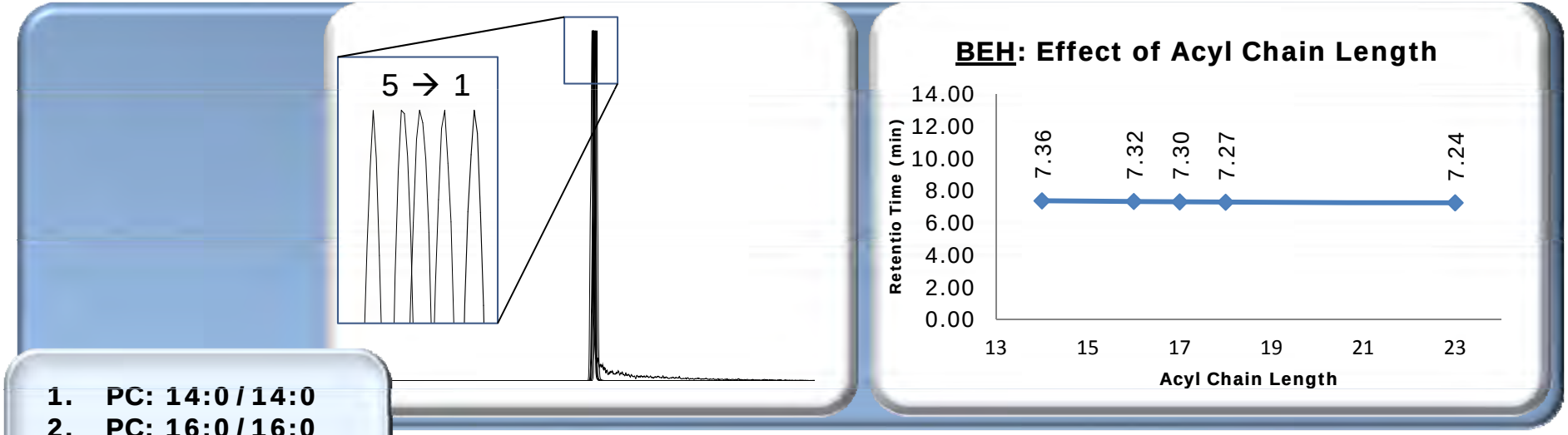
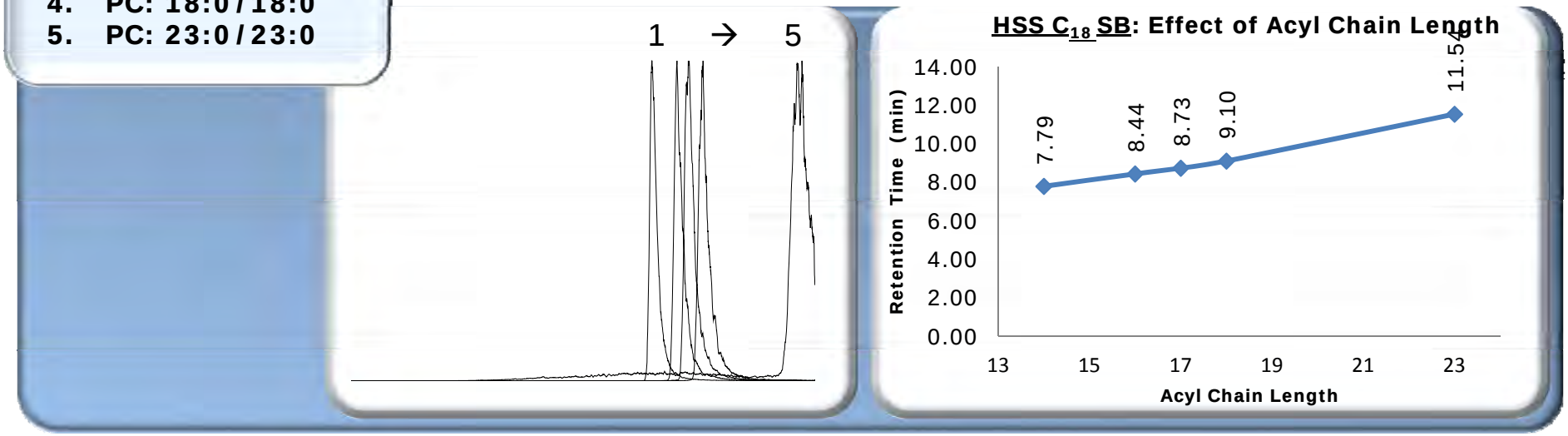


1. PC: 14:0/14:0
2. PC: 16:0/16:0
3. PC: 17:0/17:0
4. PC: 18:0/18:0
5. PC: 23:0/23:0

ACQUITY UPC² BEH



ACQUITY UPC² HSS C_{18} SB

UPC² Analysis of a Mouse Heart Extract

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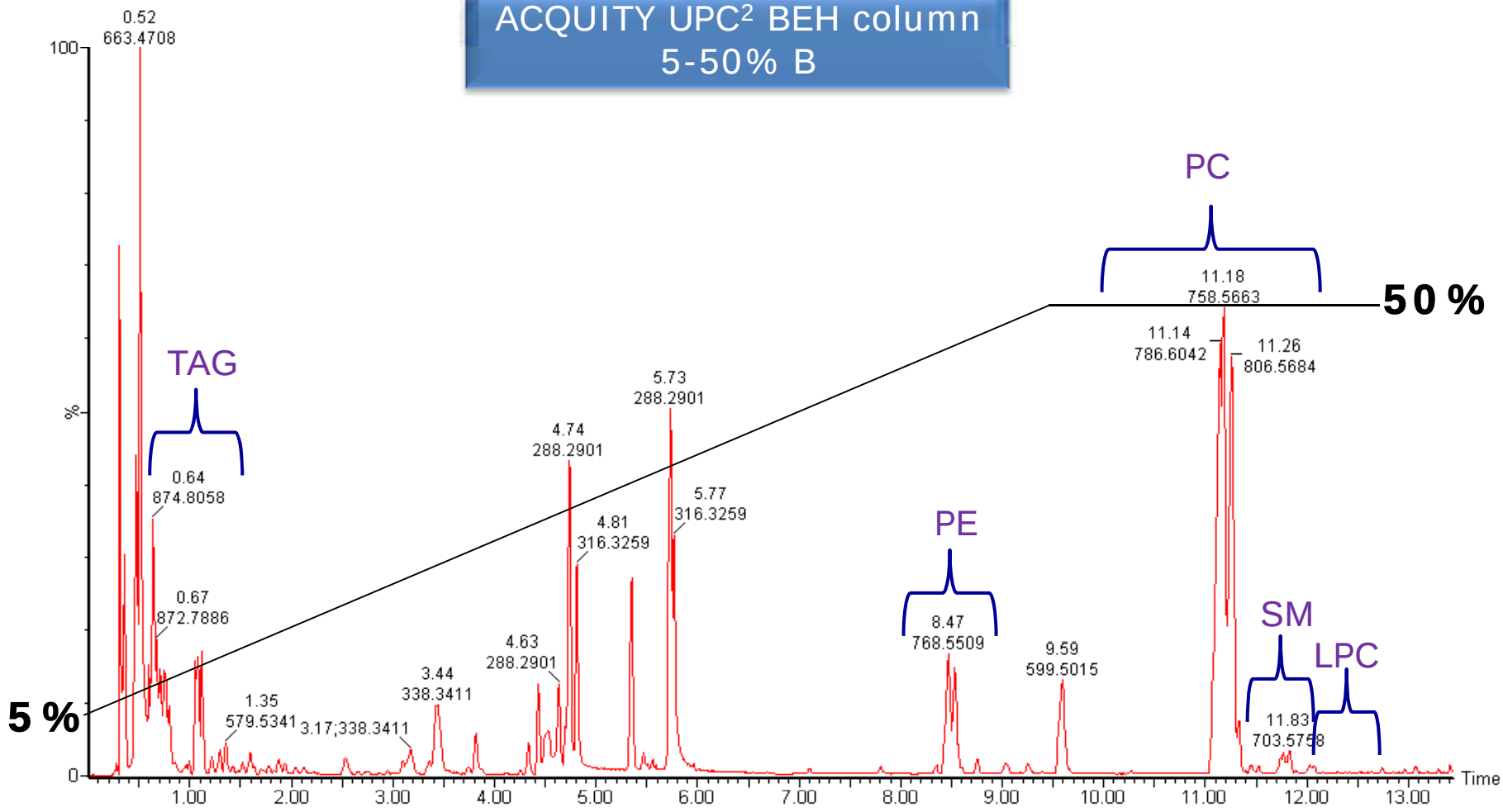
ACQUITY UPC² BEH column
5-50% B



TAG: Triacylglycerides
SM: Sphynogomyelin

PE: Phosphotidylethanolamine
LPC: Lysophosphotidylcholine

PC: Phosphotidylcholine

UPC² Analysis of Cotton Seed Lipid Extract

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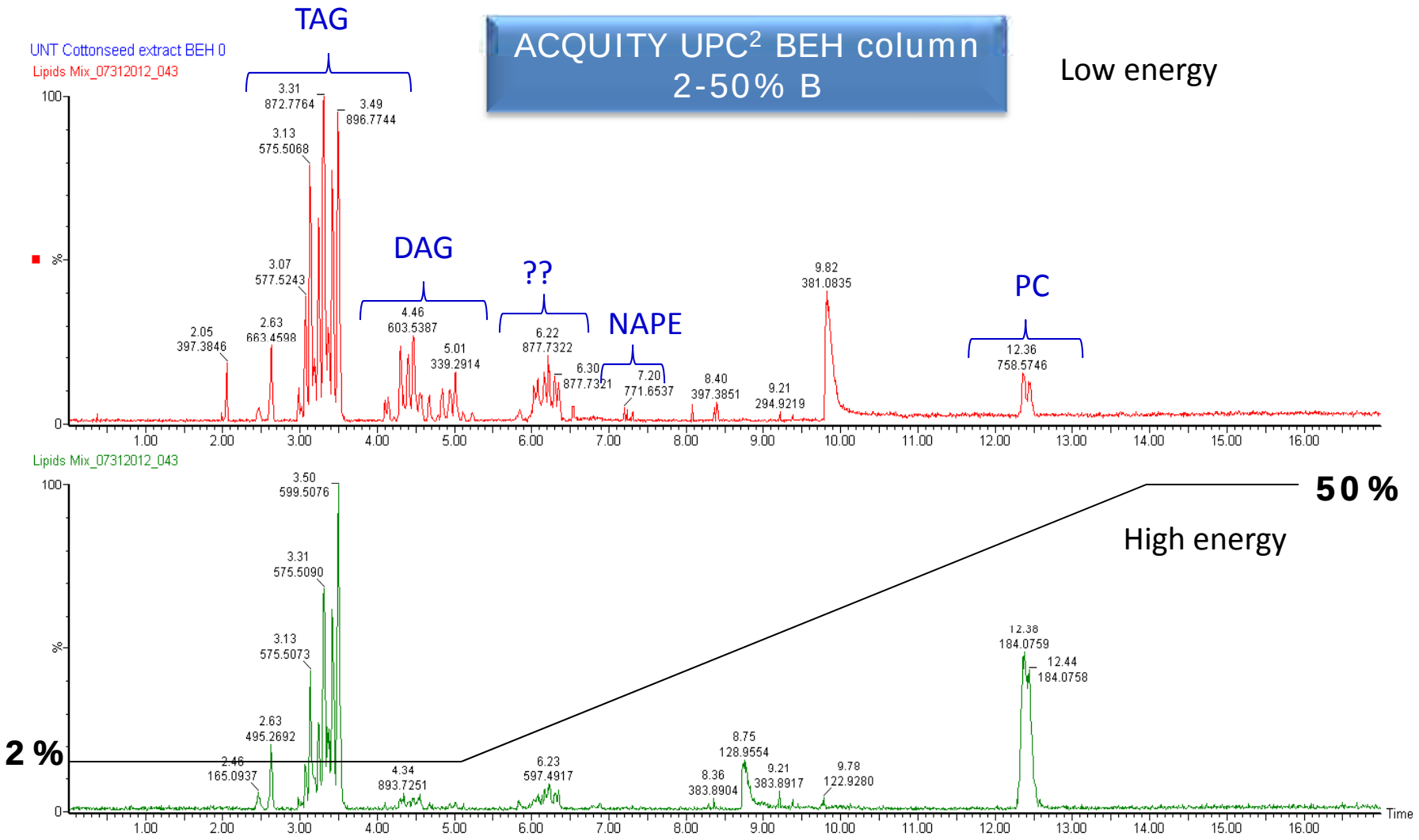
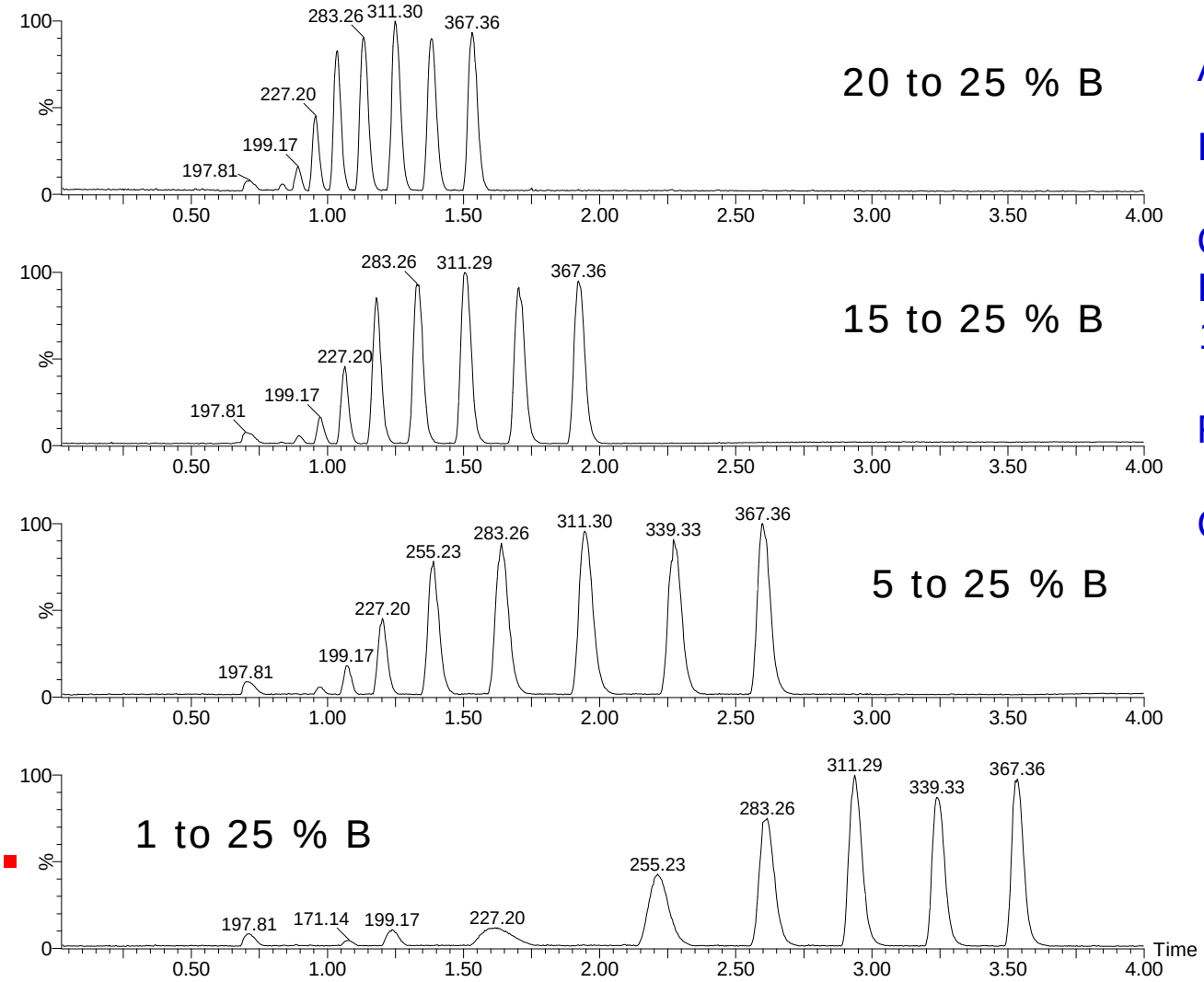


UPC² Free Fatty Acid and Neutral Lipid Method

Even carbon number saturated FFA (C8:0-24:0) mix



A= CO_2

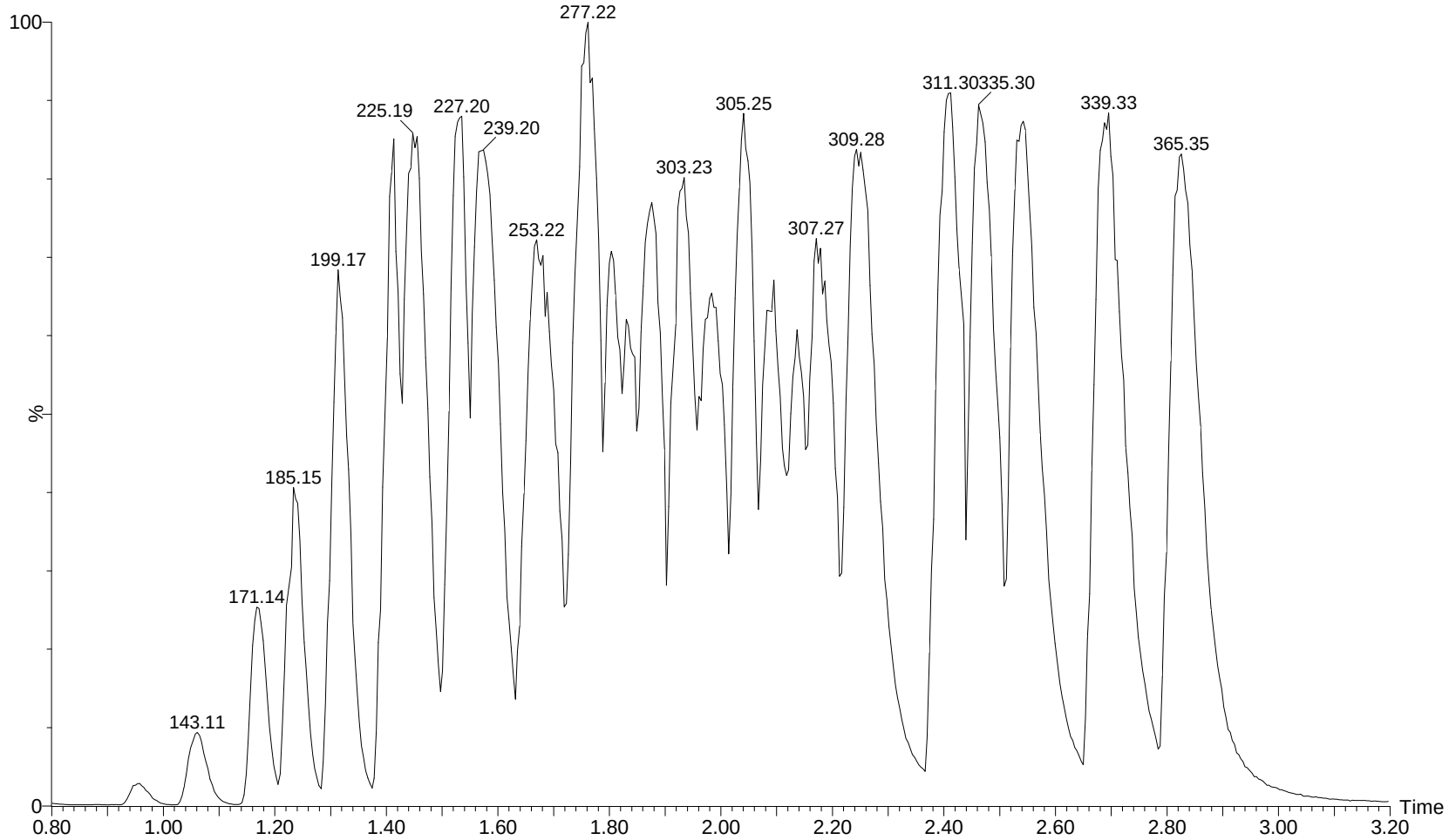
B=MeOH in 0.1% FA

Column= ACQUITY UPC^2 HSS C_{18} SB 1.8µm (2.1 x 150 mm)

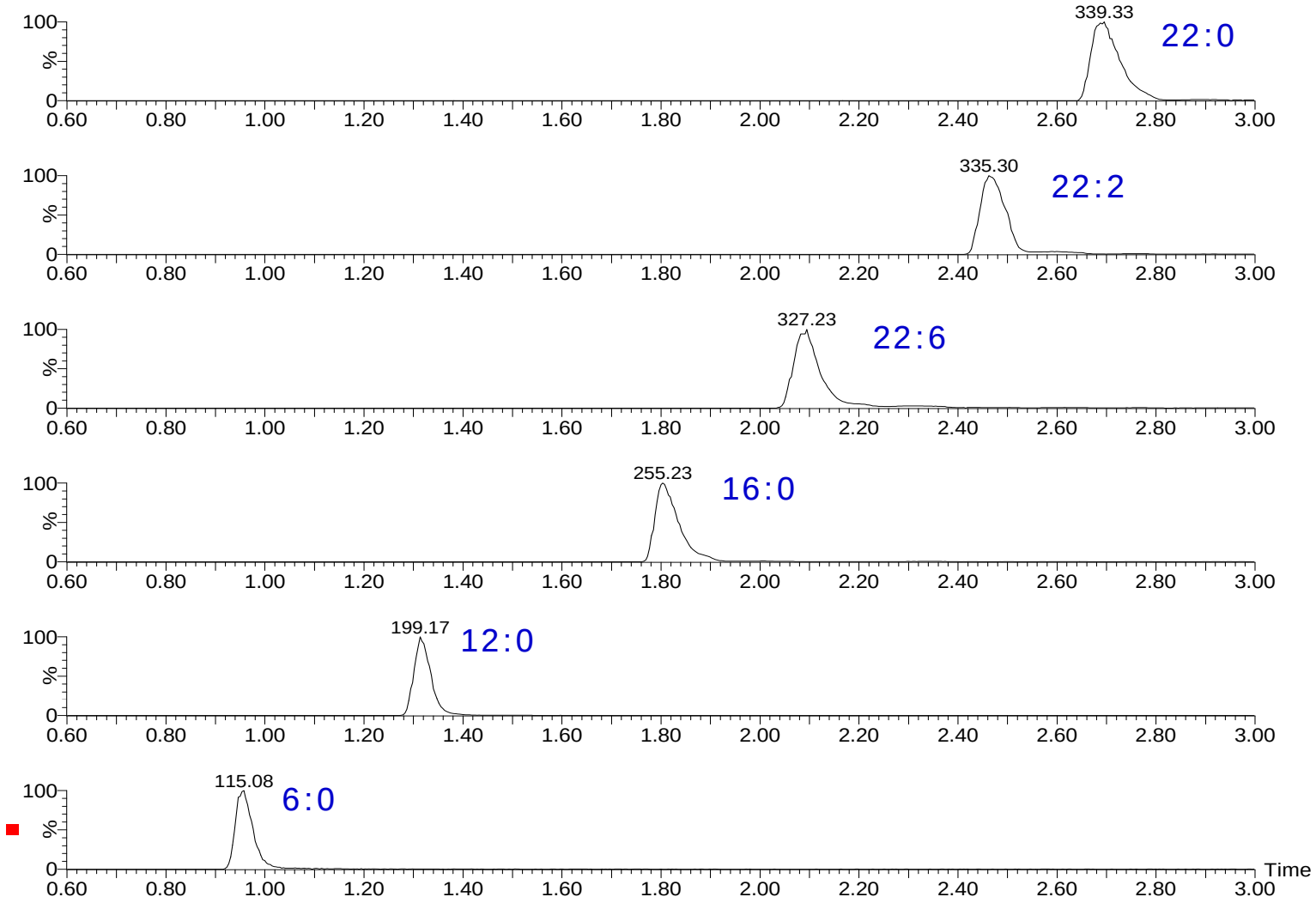
Flow rate= 0.6 mL/min

Column temp= 50 ºC

NU-Check GLC 85 (32 complex std mixture)



Effect of FA chain length and number of double bond on retention time



Extracted ion chromatogram showing the separation of isobaric species based on the position of the double bond

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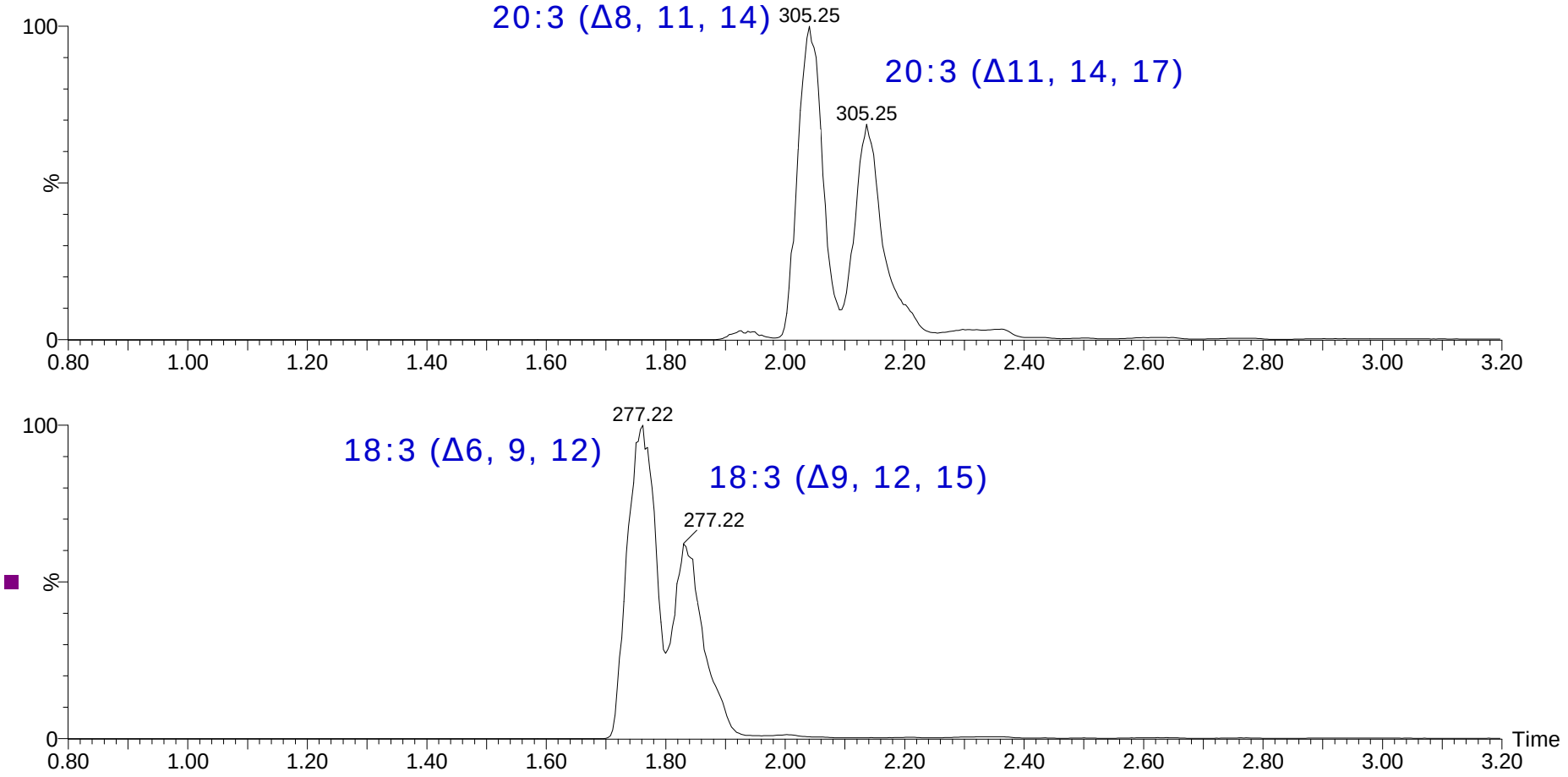

Biological Application

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- Four extracts
  - 2 algae extracts at two pyrolysis temp. (310 and 360 degree)
  - 2 algaenan extracts at two temp. (310 and 360 degree)
- Extracts were dissolved in dichloromethane (1:10 dilution)

- UPC^2 conditions
  - Column: HSS C_{18} SB (2.1 x 100 mm)
  - Mobile phase A: CO_2
  - Mobile phase B: Methanol in 0.2% FA
  - Flow rate: 1.5 mL/min
  - Make up solvent: IPA in 0.1% NH_4OH (flow rate=0.2 mL/min)
  - Gradient: 1 to 10 B in 10 min or 1 to 20 B in 10 min
  - Injection volume: 1 µL

Effect of Modifier Gradient in Retention Time (FFA C8-C36)

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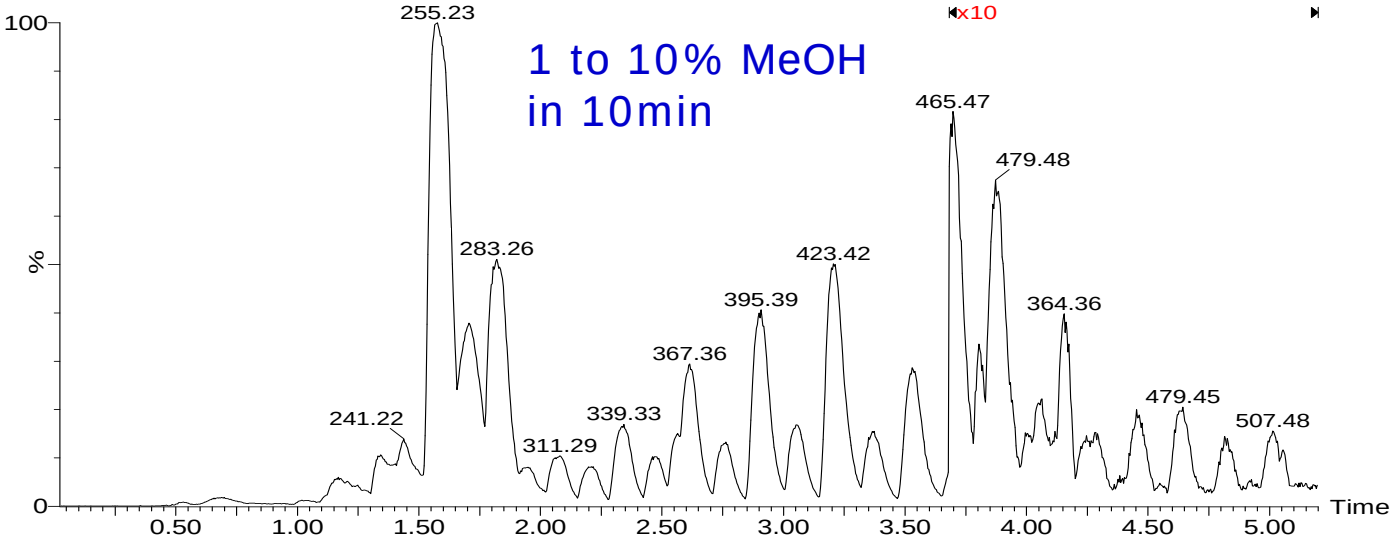

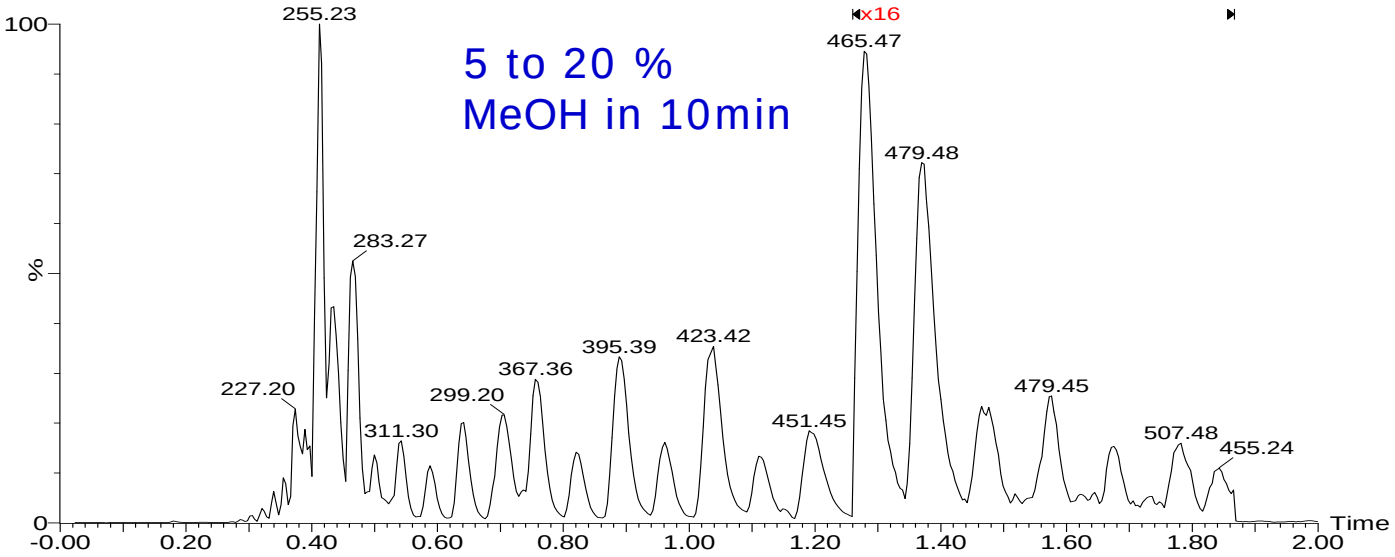

OLD DOMINION UNIVERSITY

TransOmics™ Informatics
...complementary workflows

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PROTEOMICS

HDMS^E ALIGNMENT

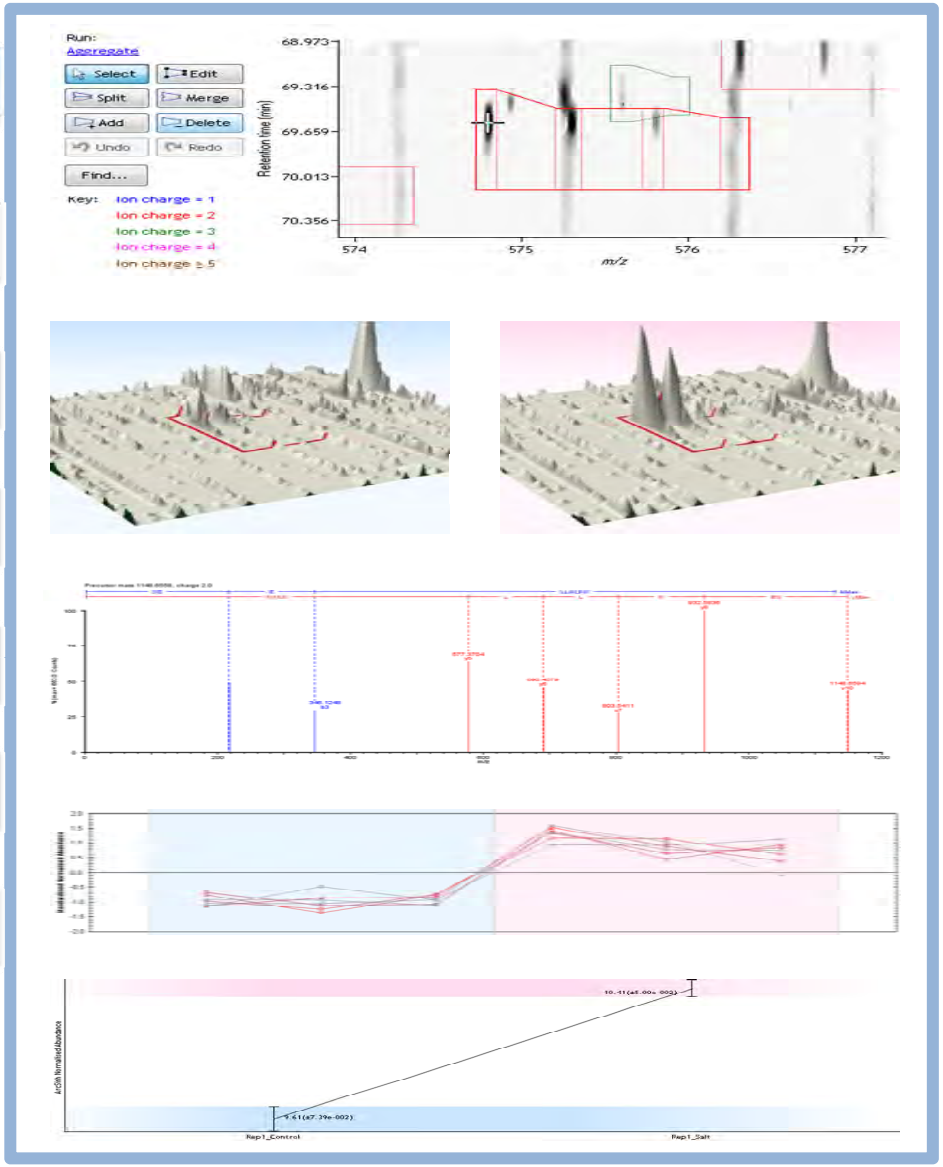
PEAK DETECTION

PEPTIDE QUANT

IDENTIFICATION

PROTEIN QUANT

STATS



METABOLOMICS

HDMS^E ALIGNMENT

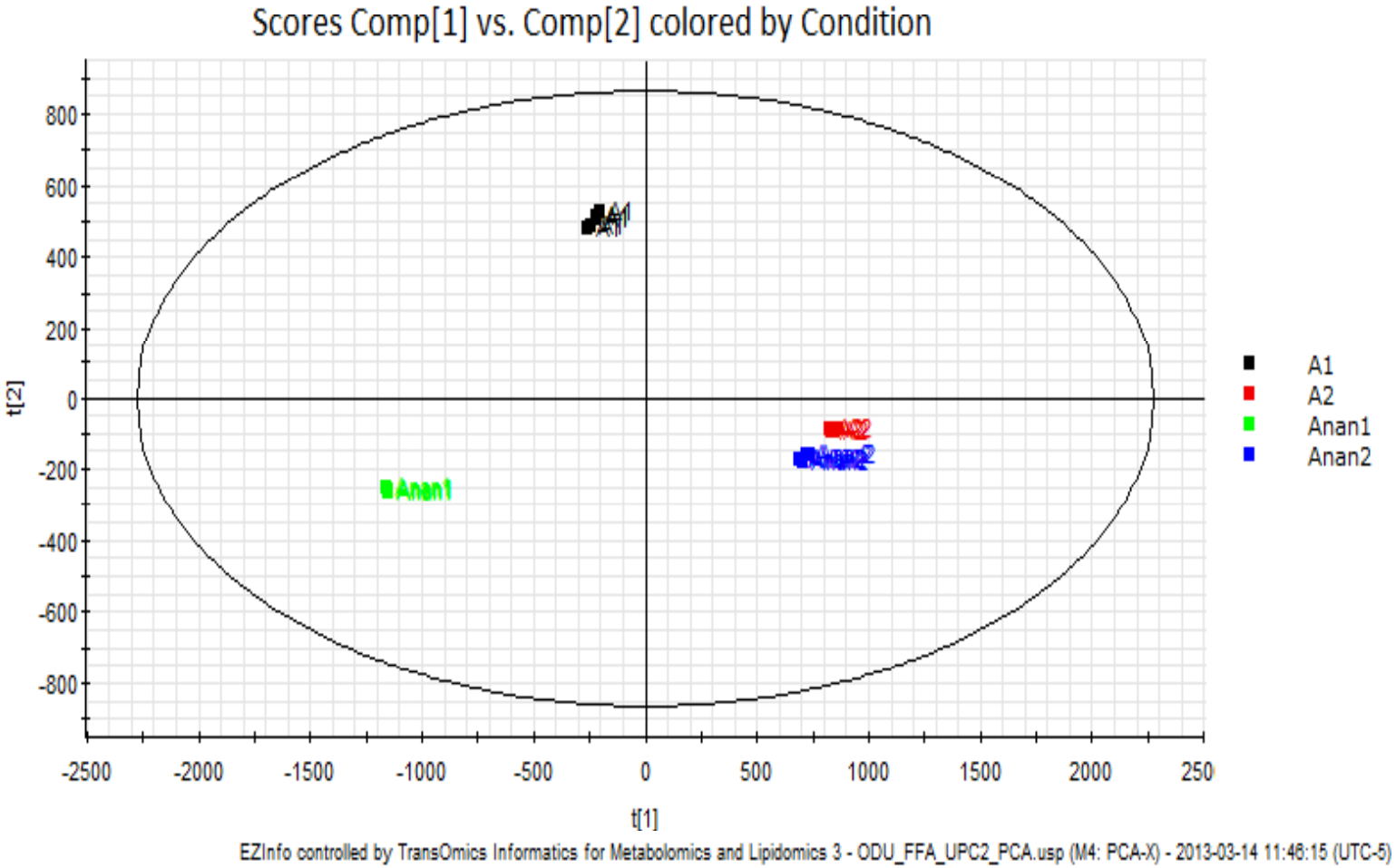
PEAK DETECTION

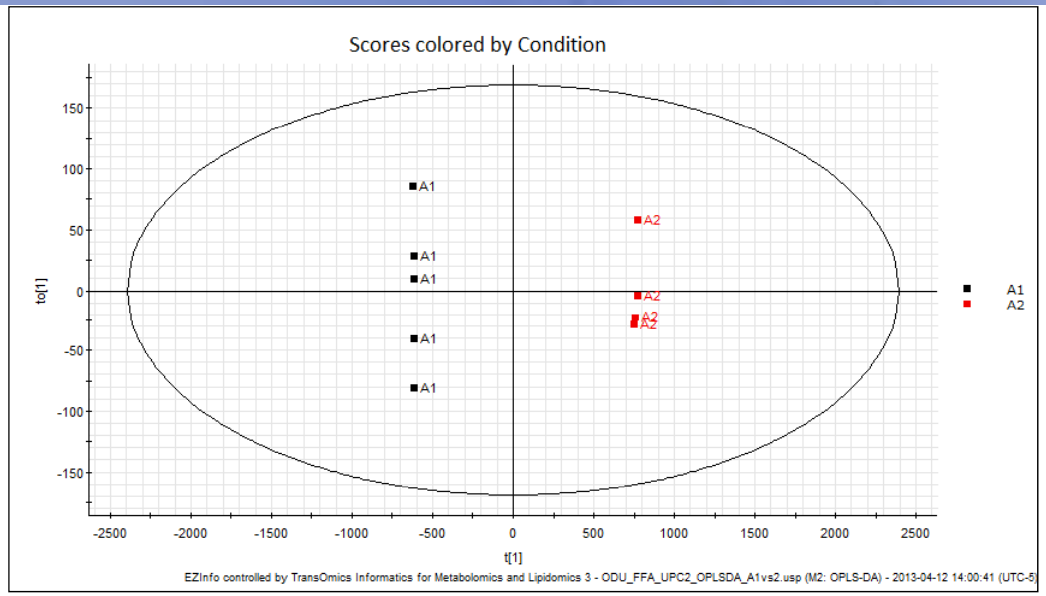
DECONVOLUTION

COMPOUND QUANT

IDENTIFICATION

STATS

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to[1]
t[1]
A1
A2
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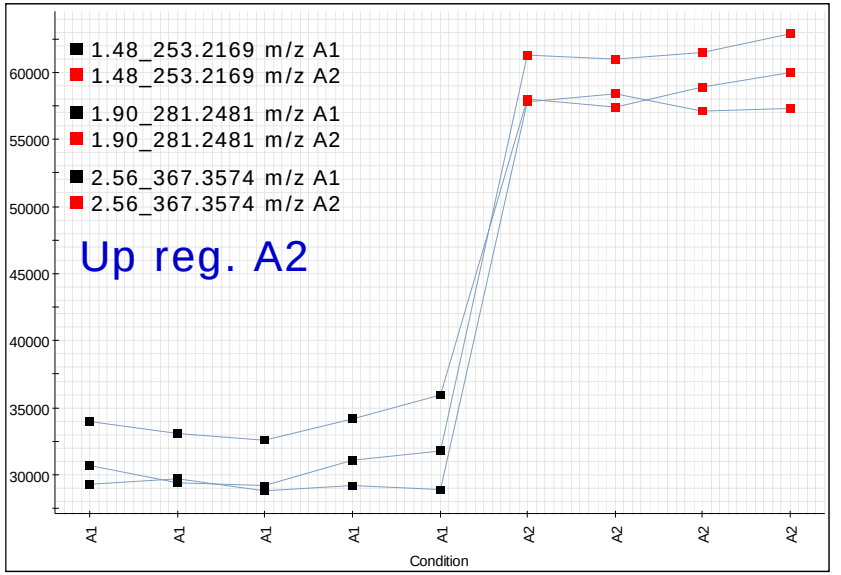
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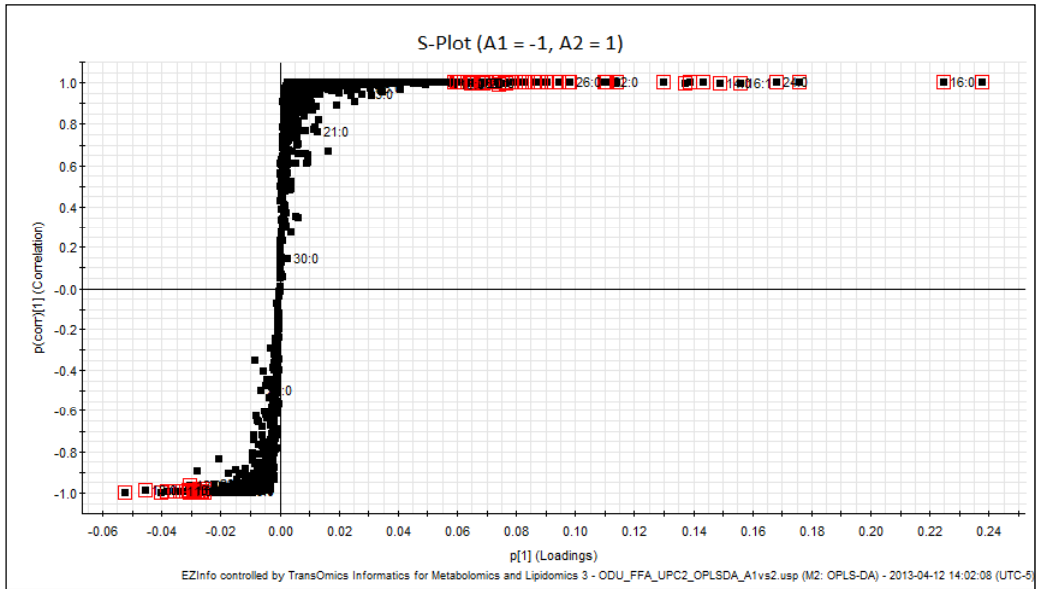
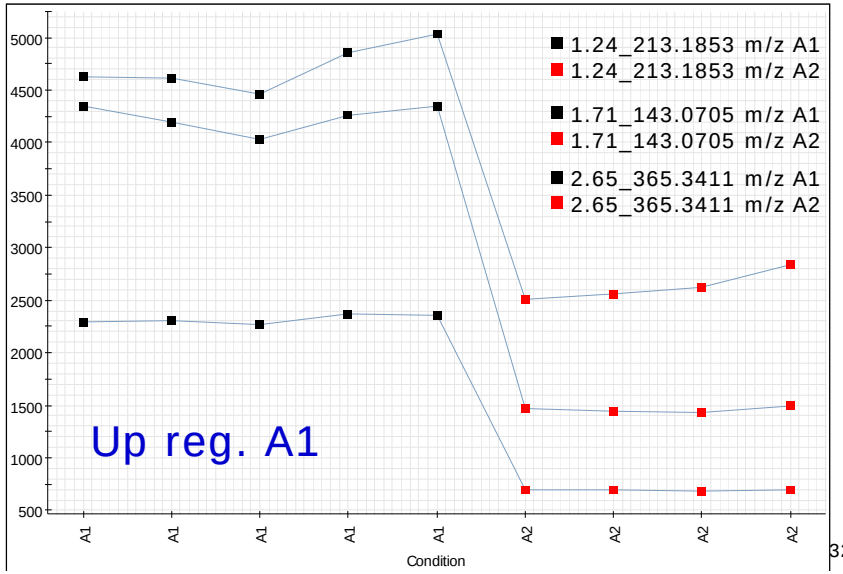
1.90_281.2481 m/z A1
1.90_281.2481 m/z A2
2.56_367.3574 m/z A1
2.56_367.3574 m/z A2
Up reg. A2
Condition


S-Plot (A1 = -1, A2 = 1)
p(corr)[1] (Correlation)
p[1] (Loadings)
21:0
30:0
16:0
EZInfo controlled by TransOmics Informatics for Metabolomics and Lipidomics 3 - ODU_FFA_UPC2_OPLSDA_A1vs2.usp (M2: OPLS-DA) - 2013-04-12 14:02:08 (UTC-5)


1.24_213.1853 m/z A1
1.24_213.1853 m/z A2
1.71_143.0705 m/z A1
1.71_143.0705 m/z A2
2.65_365.3411 m/z A1
2.65_365.3411 m/z A2
Up reg. A1
Condition

3.31_437.4353 (29:0FFA)

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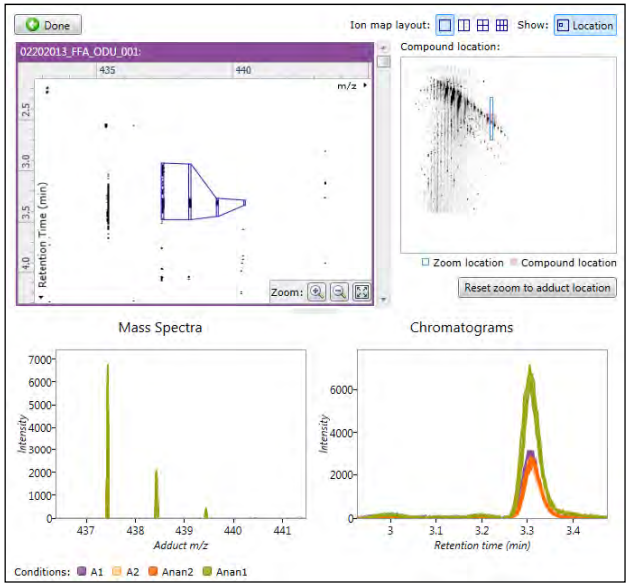
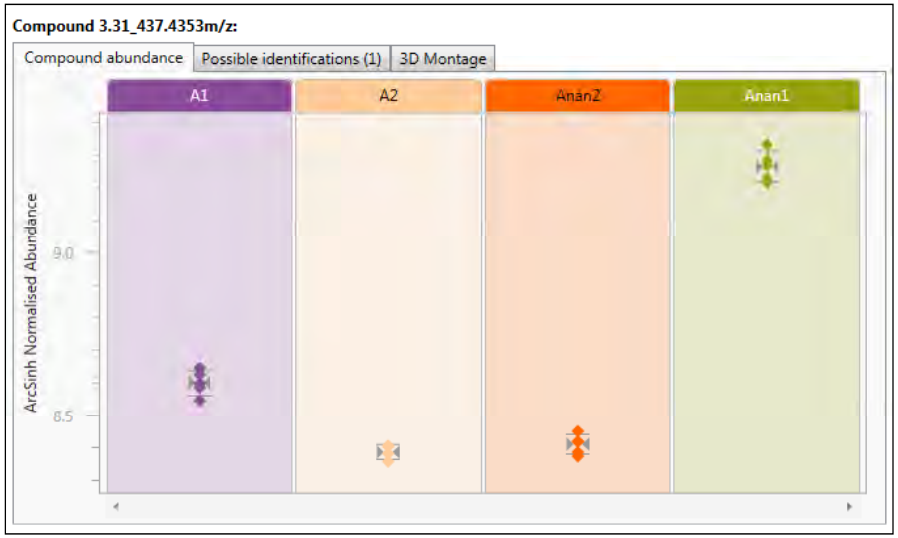


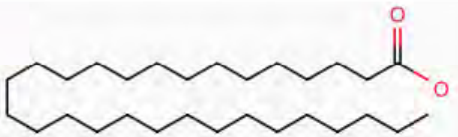


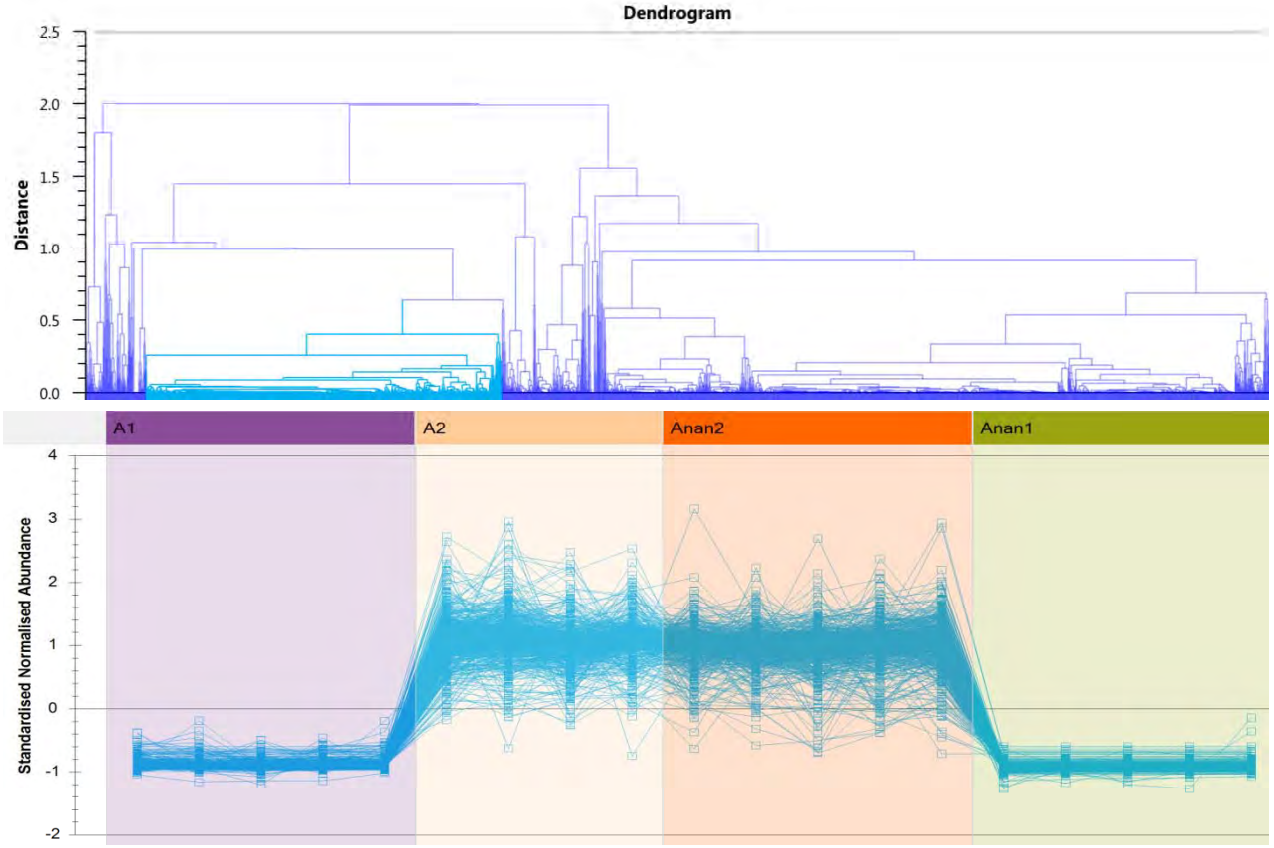
Peak Width	Accepted ID	Identifications	Anova (p)	Max fold change	Highest mean	Lowest mean	Tag	Isotope distribution	Max Abundance	Min CV%	Description
0.22	LMFA01010023	3	2.22E-16	2.47	A2	Anan1			4725.0680	1.95	tricosanoic acid
0.23	LMFA01170029	2	3.33E-16	382	A2	Anan1			3667.2347	0.98	Octadecanedioic acid
0.30	LMFA01010027	3	4.44E-16	2.28	Anan1	Anan2			4214.1367	0.97	Carboceric acid
0.55	LMFA01010029	2	6.66E-16	2.41	Anan1	A2			5290.8416	2.25	nonacosanoic acid
0.05	LMFA01170055	2	2.33E-15	Infinity	A2	Anan1			428.9681	1.98	9Z-Octadecenedioic acid

Compound 3.31_437.4353m/z:

Compound abundance | Possible identifications (2) | 3D Montage

Compound ID	Description	Adducts	Formula	Retentio	Score	Mass error (ppm)	Isotope similarity
LMFA01010029	nonacosanoic acid	M-H	$C_{29}H_{58}O_2$		65.4	-1.24	97.73
LMFA01020313	Mycocerosic acid (C29)	M-H	$C_{29}H_{58}O_2$		65.4	-1.24	97.73





Expression and abundance profile of selected features according to their relative similarity between the different groups.

- FFA containing C8-C36 were analyzed
- Algae 1 (310 ºC) contains elevated level of short (C9:0-C13:0) and long (C31:0-C37:0) chain FFA.
- Algae 2 (360 ºC) contains elevated level of medium (C14:0-C29:0) chain FFA.
- Algaenan 1 (310 ºC) contains elevated level of Long (C28:0-C37:0) chain FFA.
- Algaenan 2 (360 ºC) contains elevated level of short and medium (C9:0-C27:0) chain FFA.

Nu-Check GLC 768 (15 saturated complex TAG mixture)

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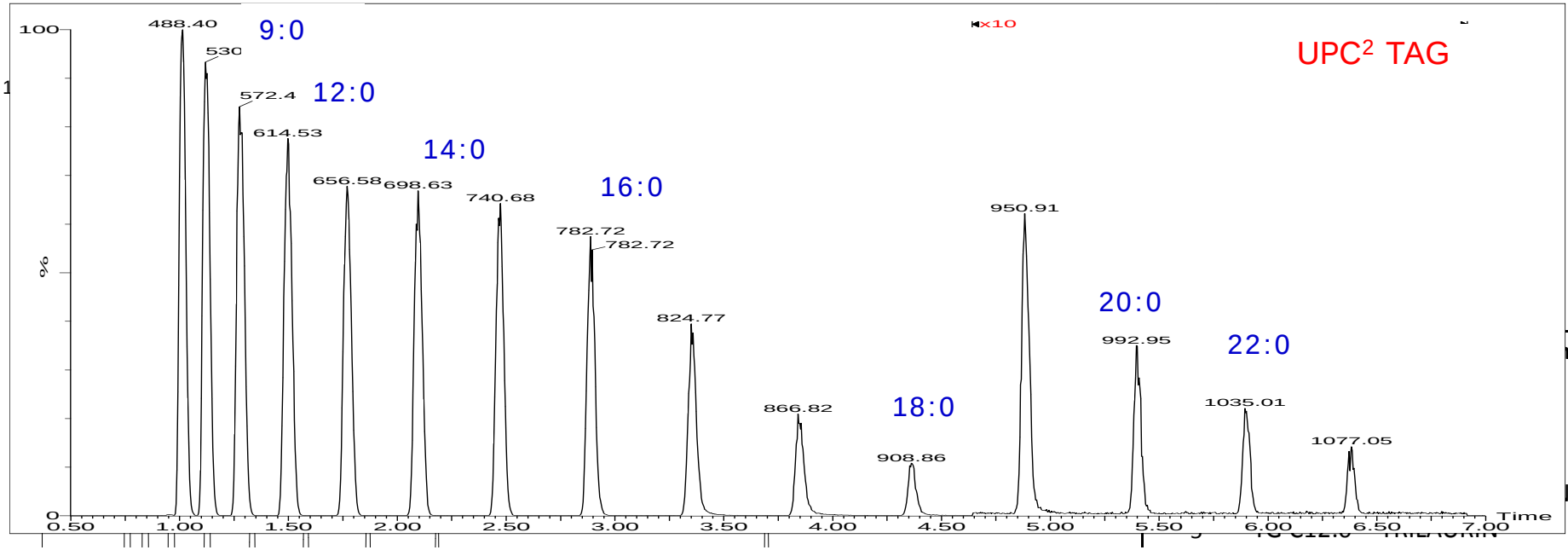
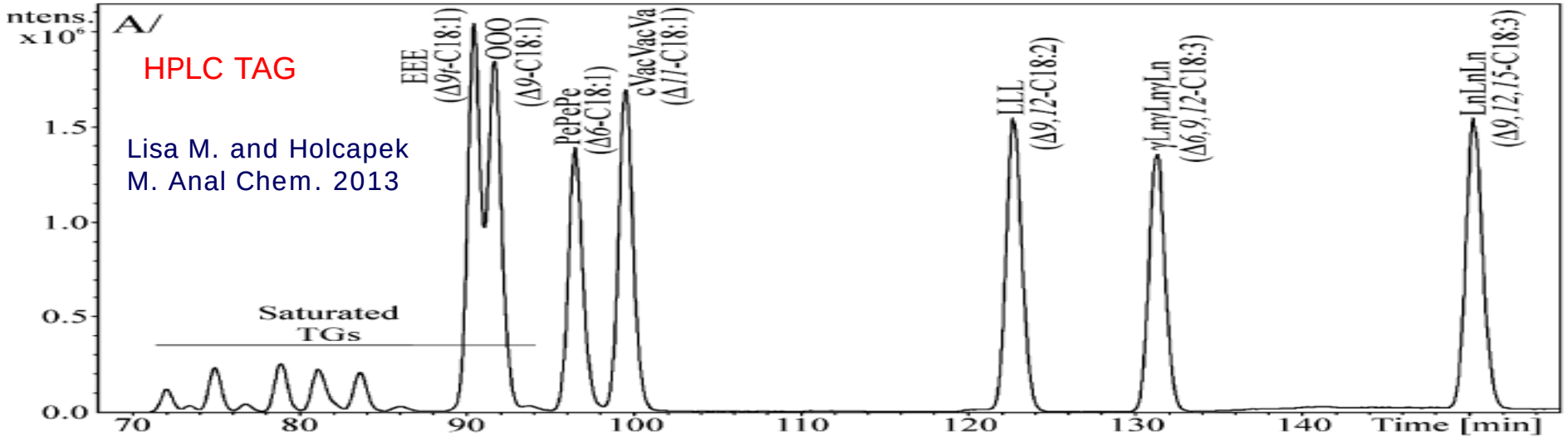
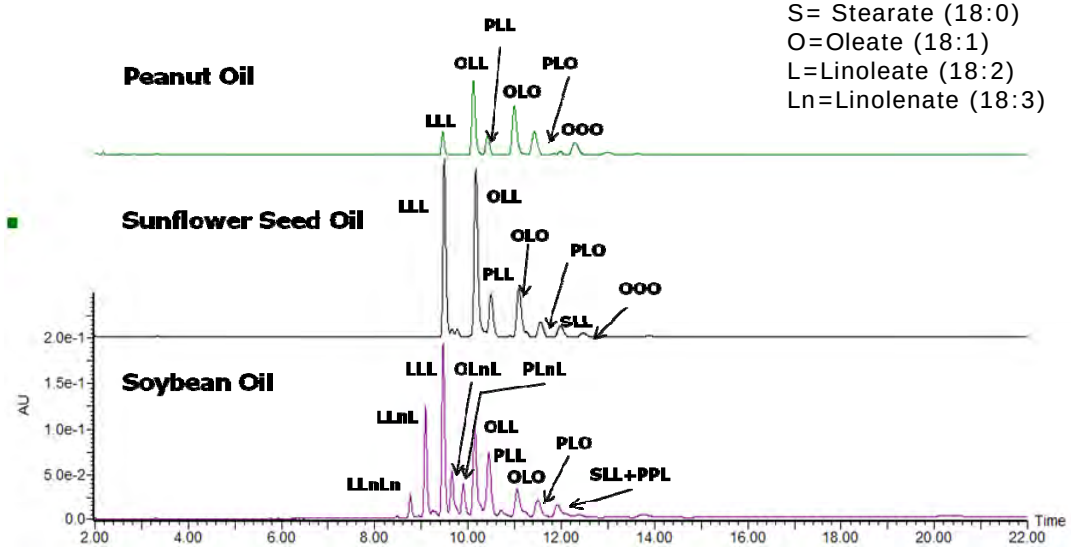




Separation of Edible Oil UPC^2 / PDA



Abbreviation:
P=Palmitate (16:0)
S= Stearate (18:0)
O=Oleate (18:1)
L=Linoleate (18:2)
Ln=Linolenate (18:3)

MP A= CO_2
MP B=ACN
Flow= 1mL/min
Column= ACQUITY UPC^2 HSS C_{18} SB 1.8µm (2.1 x 150 mm)

Gradient

Time	% ACN
0	3
2	3
17	70
22	70
22.5	3

UPC² TG Analysis from Mouse Adipose Tissue Lipid Extract

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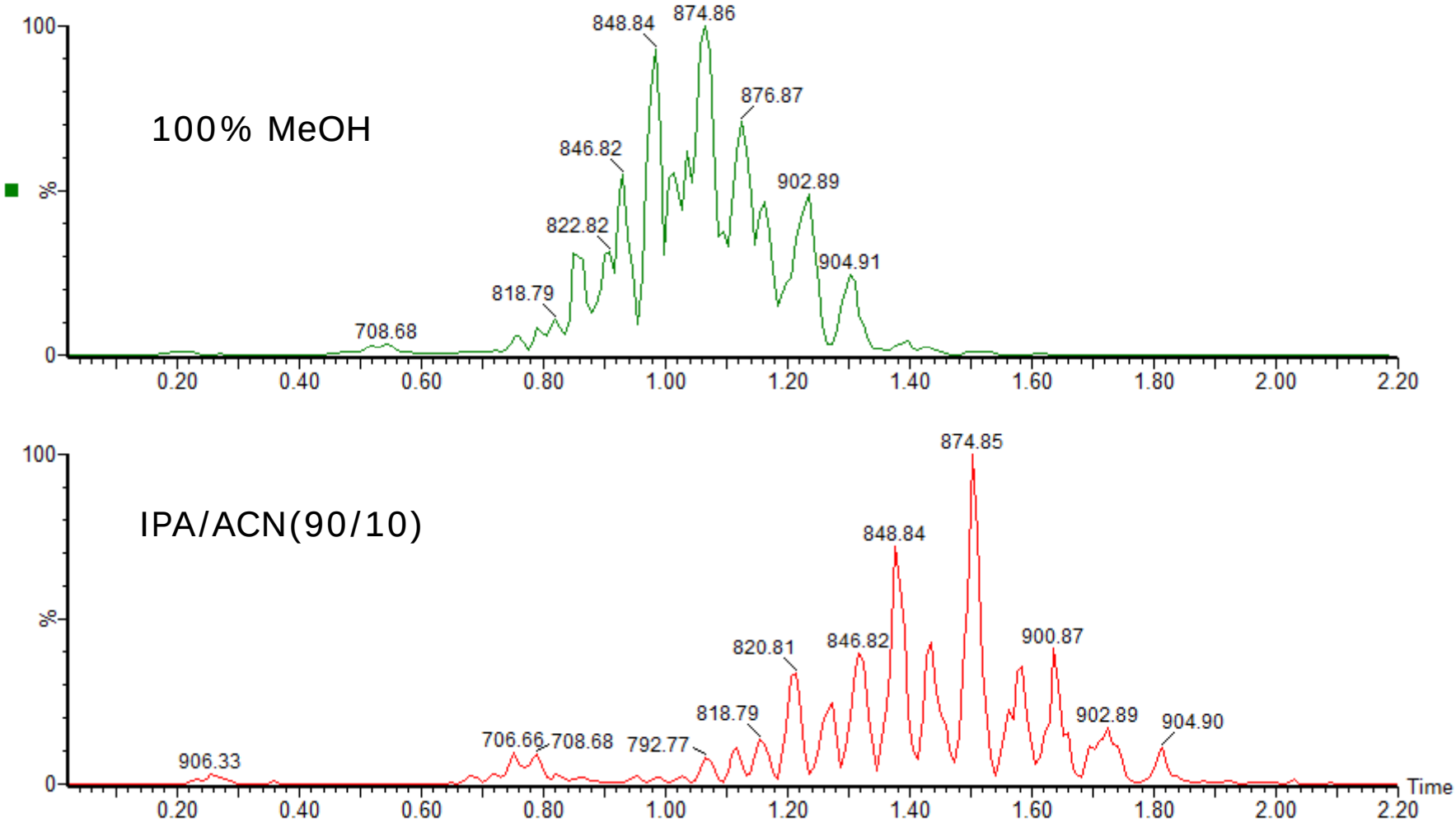
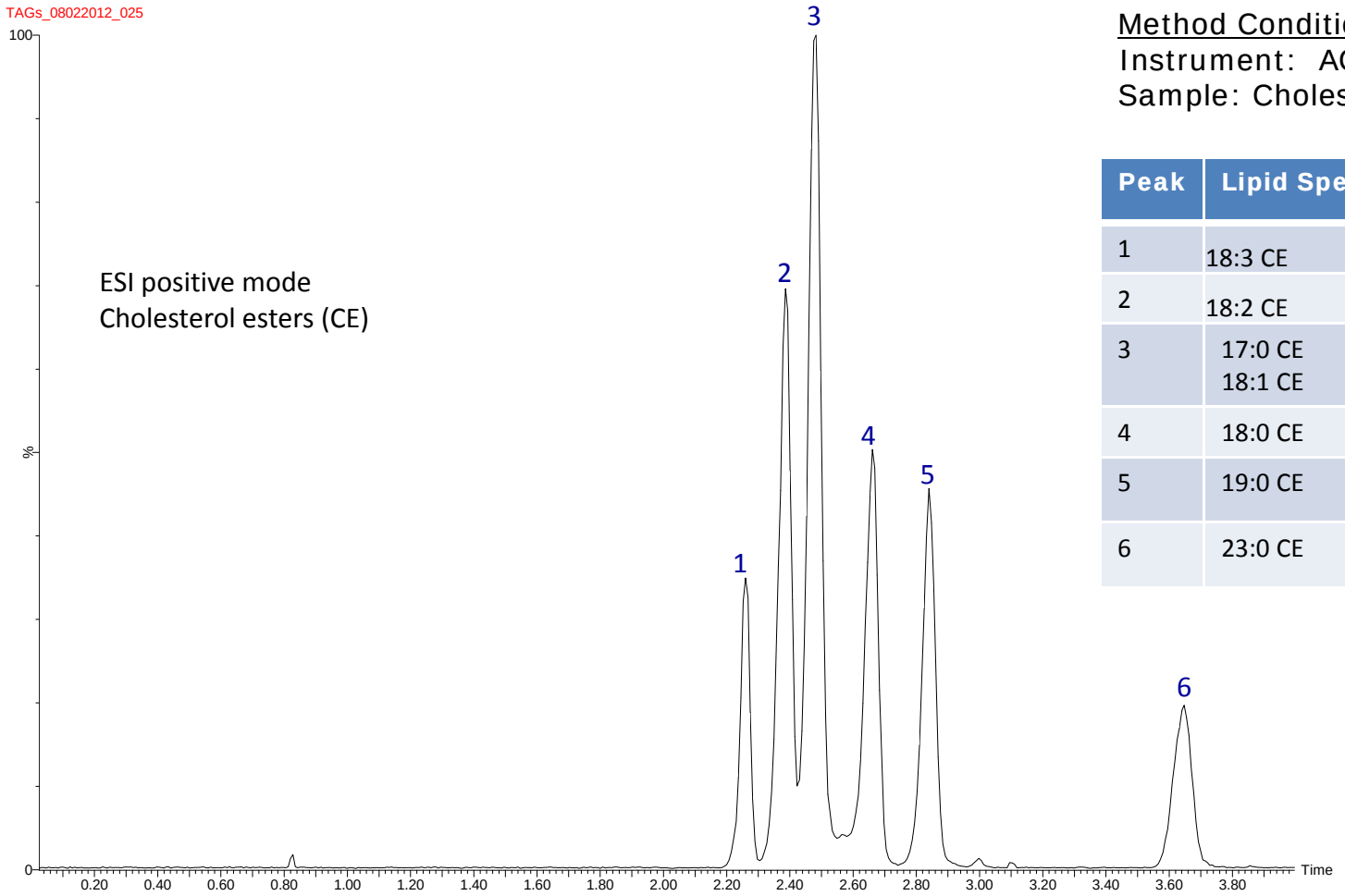


UPC² Cholesterol Esters (CE)



Method Conditions*

Instrument: ACQUITY UPC²

Sample: Cholesterol ester mixture

Peak	Lipid Species
1	18:3 CE
2	18:2 CE
3	17:0 CE 18:1 CE
4	18:0 CE
5	19:0 CE
6	23:0 CE

Separation of Neutral Lipids Based on Chain Length and Double Bond Position

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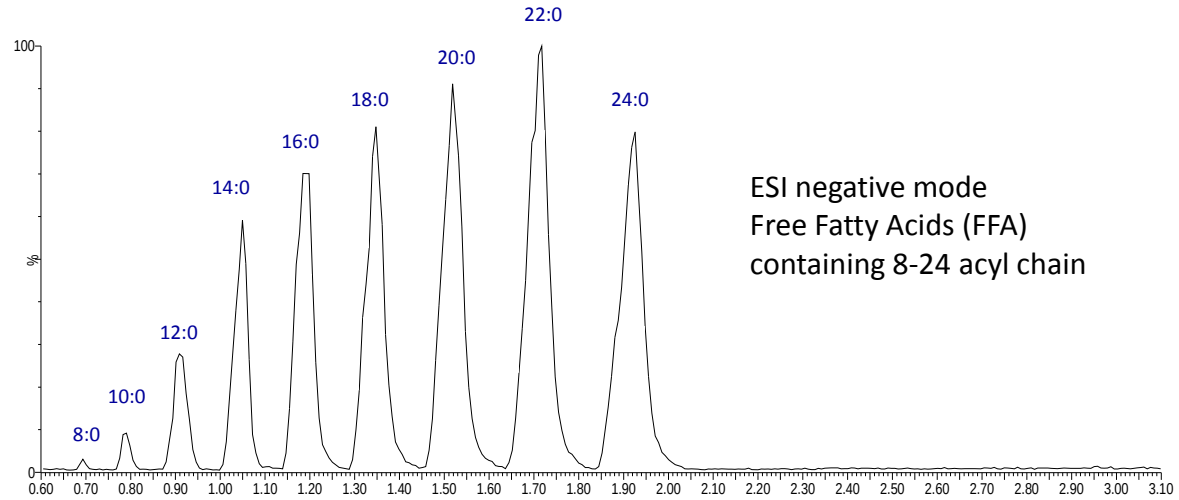
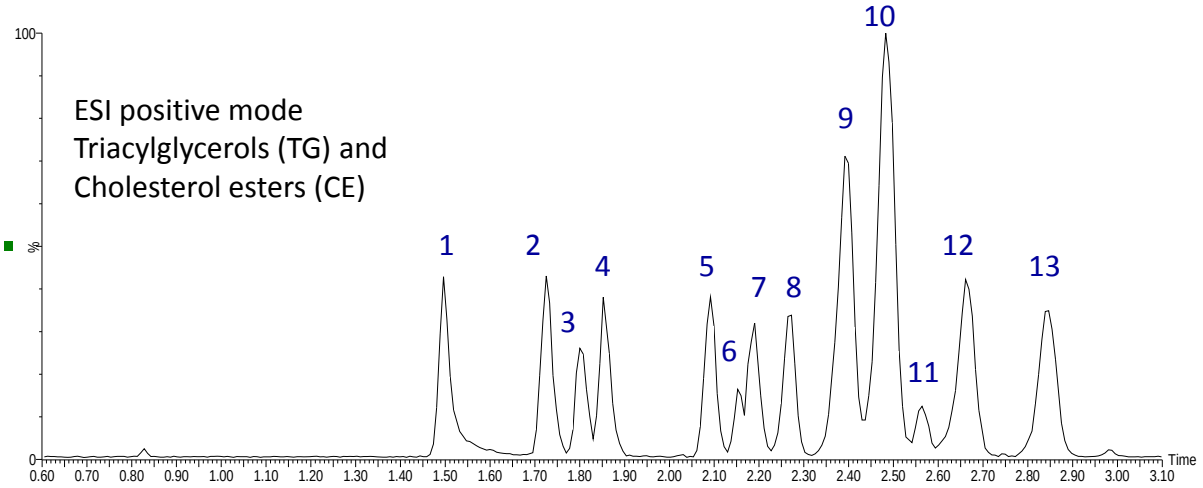
ACQUITY UPC² HSS C18 SB column
1-10% B



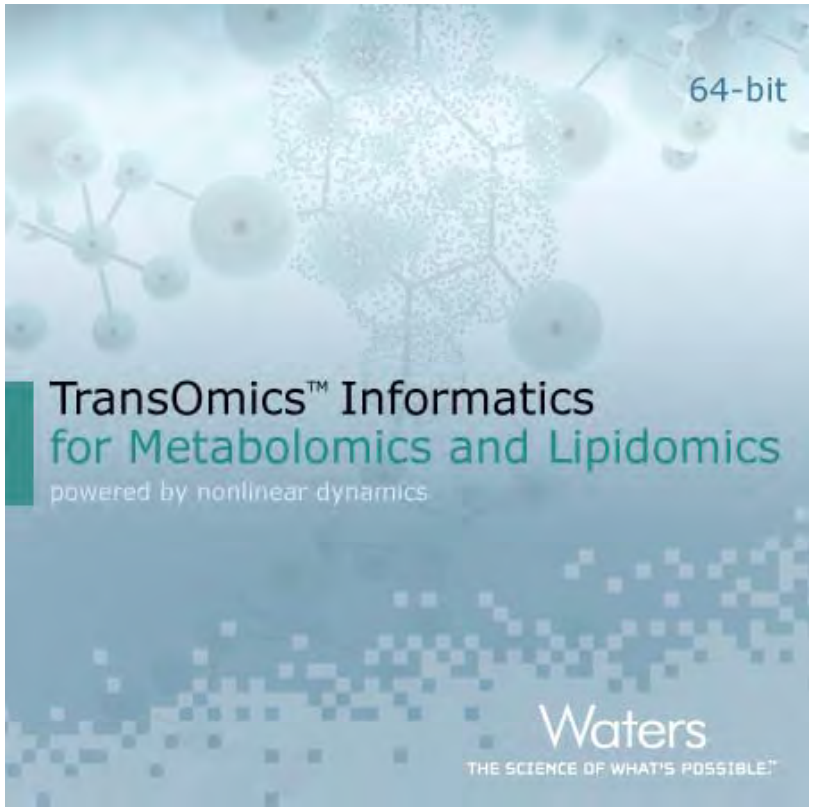


Peak	Lipid Species
1	15:0/15:0/15:0 TG
2	18:3(Δ9,12,15Cis)/18:3(Δ9,12,15Cis)/18:3(Δ9,12,15Cis) TG
3	16:0/16:0/16:0 TG
4	18:2(Δ9,12Cis)/18:2(Δ9,12Cis)/18:2(Δ9,12Cis) TG
5	18:1(Δ9Tr)/18:1(Δ9Tr)/18:1(Δ9Tr) TG
6	17:0/17:0/17:0 TG
7	18:1(Δ9Tr)/18:1(Δ9Tr)/18:1(Δ9Tr) TG
8	18:3 CE
9	18:2 CE
10	17:0 CE 18:1 CE
11	18:0/18:0/18:0 TG
12	18:0 CE
13	19:0 CE

Conclusions

- UPC^2 provides added chromatographic performance such as
  - Speed of separation (10X faster compared to GC/MS)
  - Reduced sample preparation step
  - Reduced solvent use (green chemistry)
  - High cost saving and high throughput
  - Complements to MS due to its low solvent load
- The organic phase lipid extract can be directly injected to the system saving time and reducing cost per analysis
- UPC^2 provides a single technique for separation of polar and non-polar lipids with a simple switch of the column and gradient, thus combining two or three techniques into one.
- No derivitization required for free fatty acid analysis
- Unlike GC/MS, low volatile very long chain fatty acids (>24 carbons) can be easily analyzed with UPC^2.

- Collaboration with biological application
- Test key for TrasnsOmics Informatics for Metabolomics and Lipidomics



Acknowledgments

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