ADVANCES IN FOOD ANALYSIS

SELECTED LC × LC APPLICATIONS: FOOD & NATURAL PRODUCTS

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OUTLINE

1. NP-LC×RP-LC analysis of lipidic matrices

2. HILIC×RP-LC analysis of antioxidants and lipids

3. RP-LC×RP-LC analysis of antioxidants and peptides

4. Conclusions

Impact of LC × LC on food and natural product analysis

- Food and natural products are very complex mixtures containing many nutrients of organic and inorganic nature.

- Natural organic bio-actives represent secondary plant metabolites fundamental for the sensory and nutritional quality of fruits, vegetables and other plants.

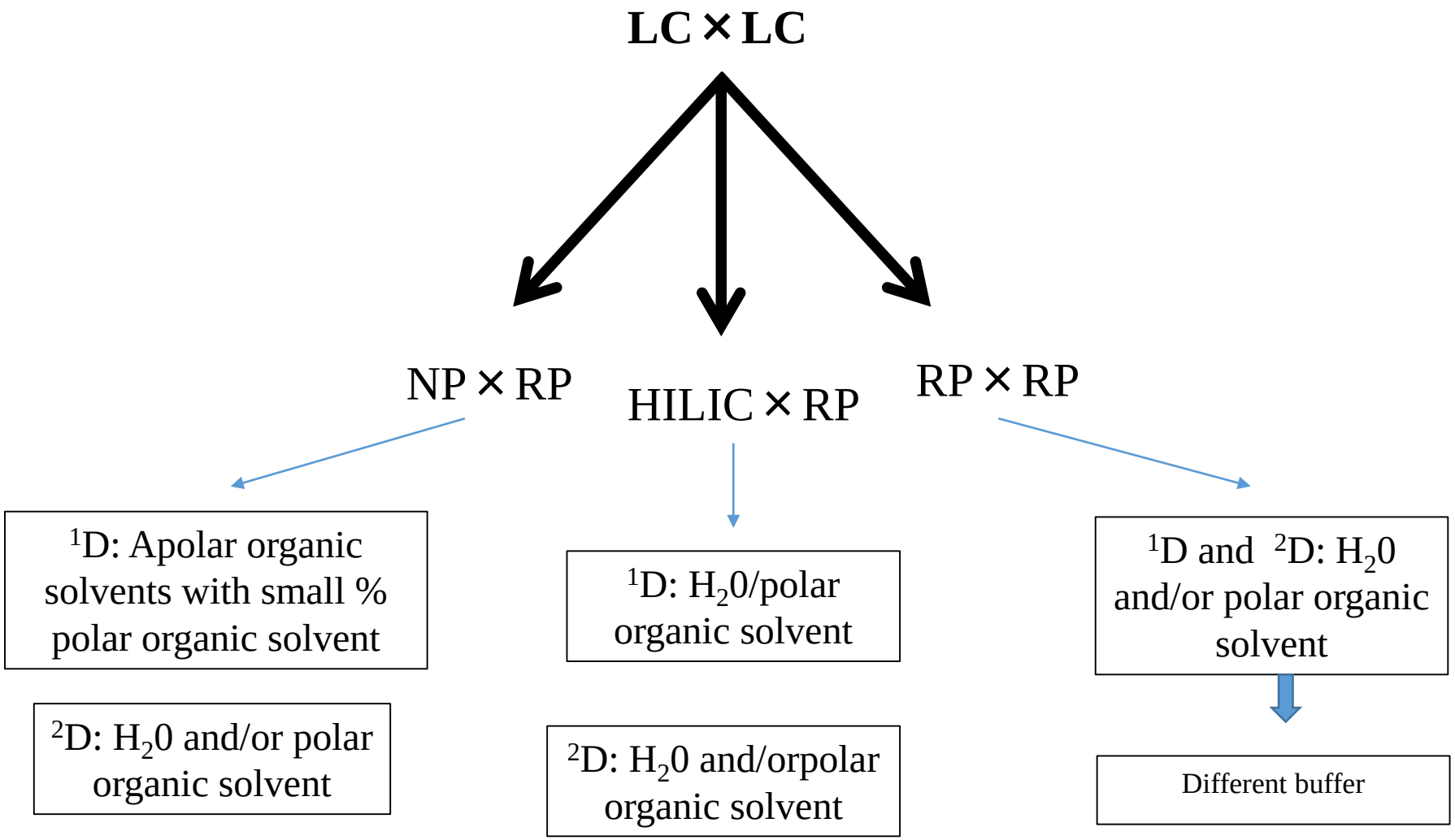
- In many cases the LC-MS, and especially the LC × LC-MS hyphenation, generates valuable tools capable of providing a profound view on the overall composition of food products.

- The main LC × LC-MS applications to food molecules can be considered essentially as "untargeted" ones and have been applied to:

- triacylglycerols (TAGs)
- phospholipids (PLs)
- carotenoids
- polyphenols
- peptides.

F. Cacciola, P. Donato, D. Sciarrone, P. Dugo, L. Mondello, Anal. Chem. 2017, 89, 414-429

Need of LC × LC for food and natural product analysis



Method Development in LC × LC

Selected combinations can be chosen on the basis of analytes properties:
NP × RP; RP × RP; IEX × RP; HILIC × RP; SEC × RP; SEC × NP

NP × RP: a difficult combination (mobile phase immiscibility and peak focusing" issues)
Remedy: need for "mobile phase adjstements"

HILIC × RP: Compatible BUT high solvent mismatch
Remedy: need for "peak focusing" adjustment

RP × RP: Compatible BUT high correlation
Remedy: need for "orthogonality" tuning

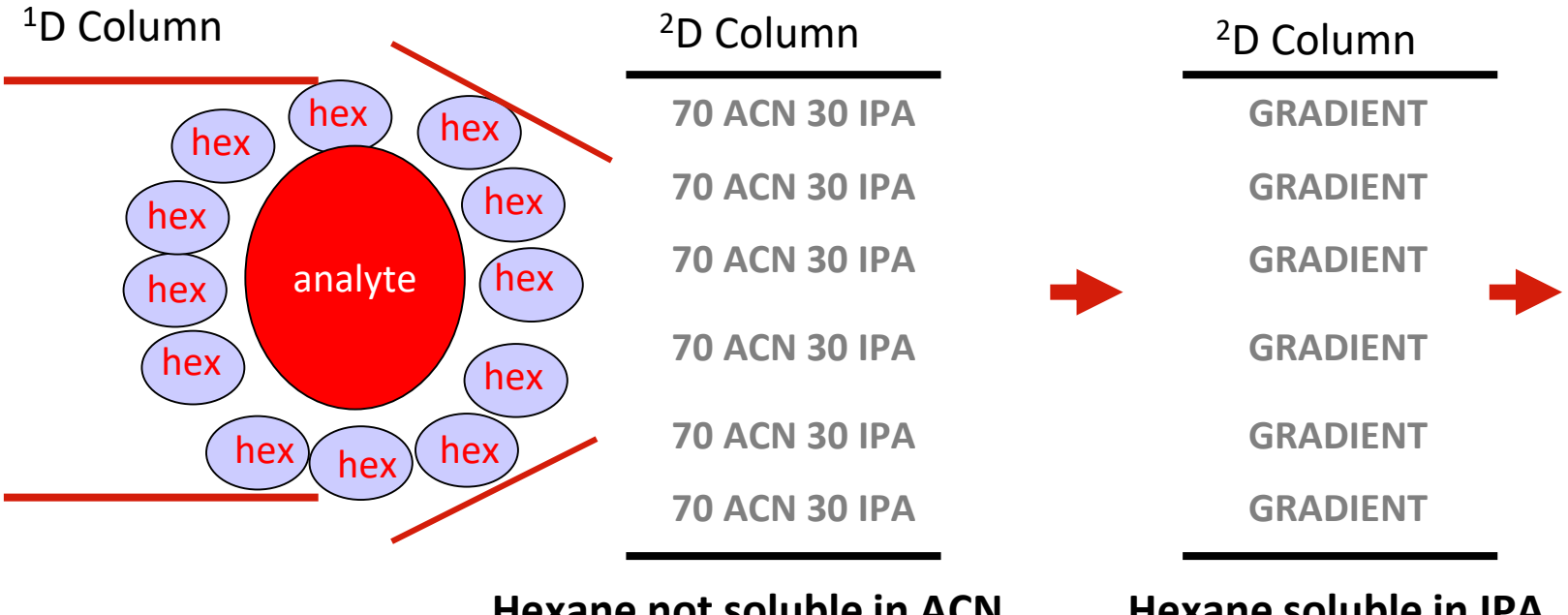
NP-LC × RP-LC applications

Optimization of a NP-LCxRP-LC method

PEAK FOCUSING AT THE HEAD OF THE SECONDARY COLUMN

- Mobile phase used in the NP-LC separation is always stronger than the mobile phase at the head of the secondary column (RP-LC).
- To obtain an effective focusing of the sample in the secondary column, the initial eluent strength has to be maintained low.

FOCUSING EFFECT AT THE ENTRANCE OF THE ^{2}D COLUMN

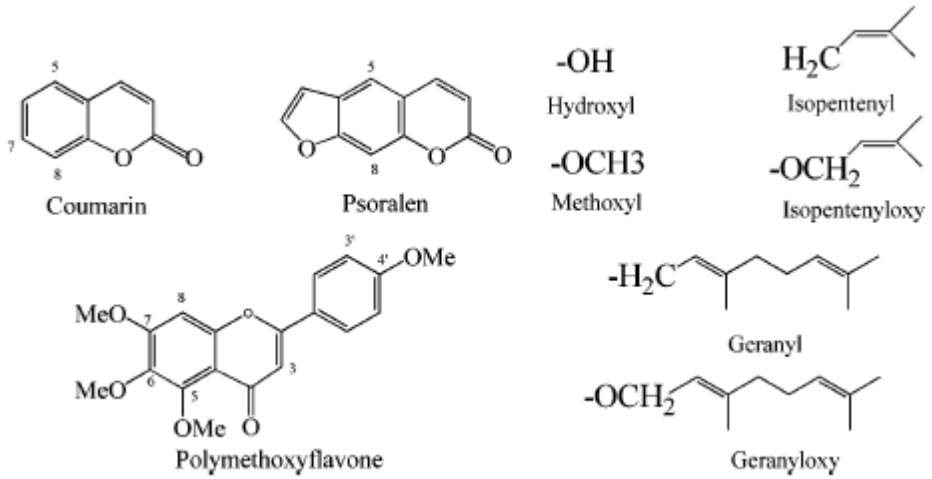


Determination of the oxygen heterocyclic components in the non volatile residue of cold-pressed *Citrus* oils

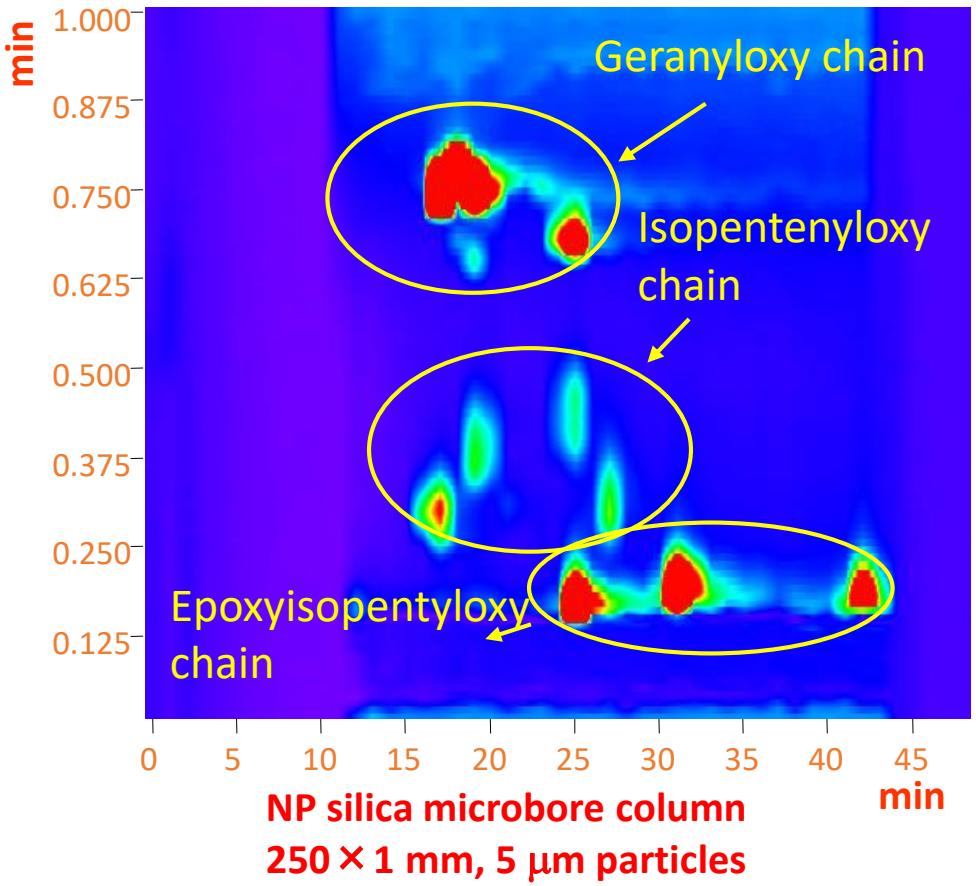
❖ *Citrus* oils contain several oxygen heterocyclic components of similar structures.

❖ These components have an important role in the characterization of cold-pressed *Citrus* oils, since their qualitative and quantitative composition of the fraction is characteristic of each oil.

❖ The analysis of these components is usually carried out by HPLC, using both normal- and reversed-phase modes. However, with both methods, some coelutions may occur.



First application: Comprehensive NP-LC (Adsorption) × RP-LC analysis of the oxygen heterocyclic fraction of a cold-pressed lemon oil



RP monolithic column 3x4.6 mm

Components are separated on the basis of side chain

Isomers are well separated;
homologs are not well separated
Components of different polarity are well separated

Paola Dugo et al., Anal. Chem. 76 (2004) 2525-2530

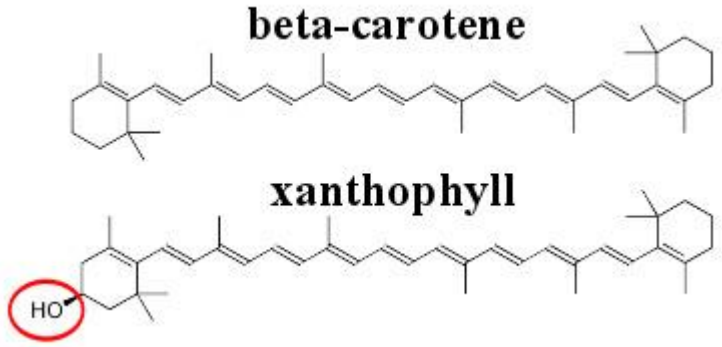
Analysis of carotenoids by LC × LC

- Carotenoids, the most diffuse pigments in nature, are made up of 8 isoprenoid units, with various end groups at the C40 skeleton.

Classified into:

Carotenes (hydrocarbons)

Xantophylls (oxygenated carotenoids)



- They are found in plants in free form or as fatty acid esters. Mono- or dihydroxylated carotenoids often occur in the more stable esterified form.

- Determination of the complete carotenoid composition is complicated, costly and time-consuming, mainly due to their extreme unstability, their complex composition and great structural diversity.

- Commonly used method for carotenoid profile analysis is HPLC in RP (C18 and C30) and NP mode.

- In order to obtain the complete determination of carotenoids in complex samples, multiple separation mechanisms or systems can be considered the most effective.

Separation of carotenoid esters by LC × LC
Method: NP-LC × RP-LC-APCI-MS

First dimension

NP-HPLC (cyano column):

Carotenoids are separated into groups of different polarity (hydrocarbons, esters of mono-ols, monoesters of diols, diesters of diol monoepoxides; diesters of diol diepoxides; monoesters of di-ol diepoxides, …free xantophylls).

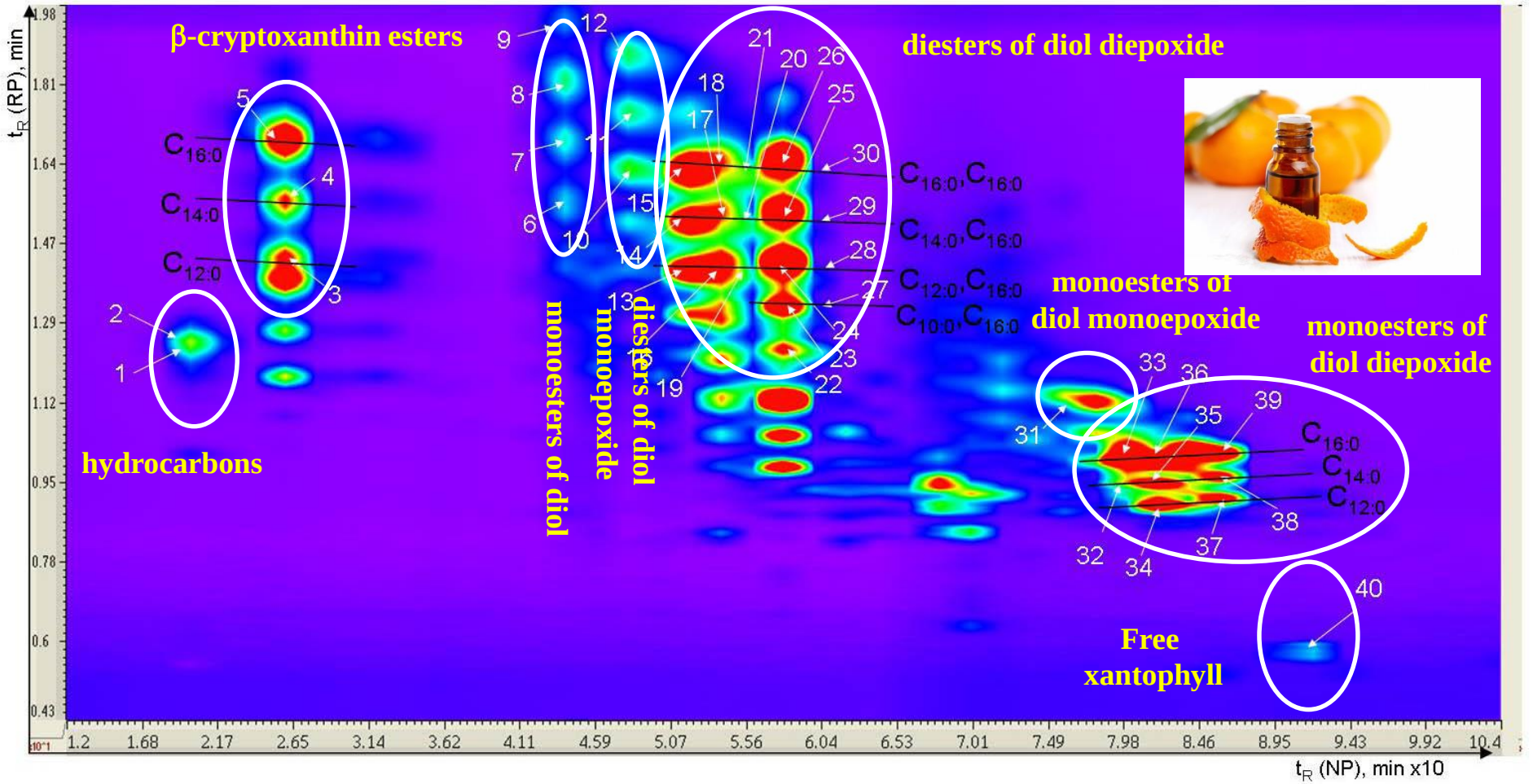
Second dimension

RP-HPLC (C18 column):

Carotenoid esters are eluted according to their increasing hydrophobicity and decreasing polarity (for components of the same class, elution order increases with the number of carbon atoms of the FA chain).

LC × LC analyses of carotenoid esters in orange essential oil

Column set-up:
Cyano 300x1 mm, 5um × Chromolith Performance RP-18 100x4,6mm



Mono-ols: β-cryptoxanthin Diols: lutein; Diol monoepoxides: antheraxanthin

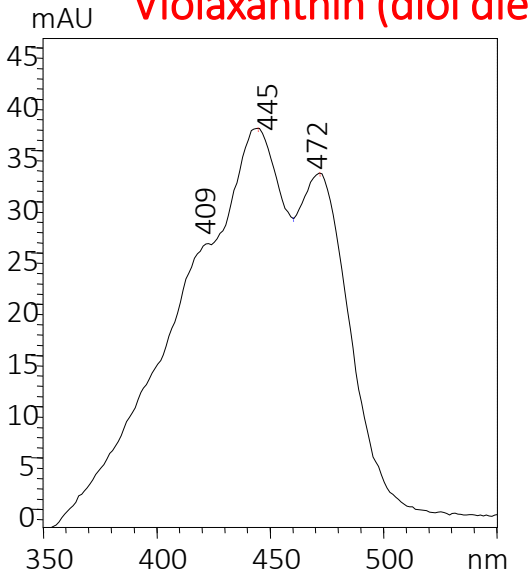
Diol diepoxides: violaxanthin; luteoxanthin; auroxanthin

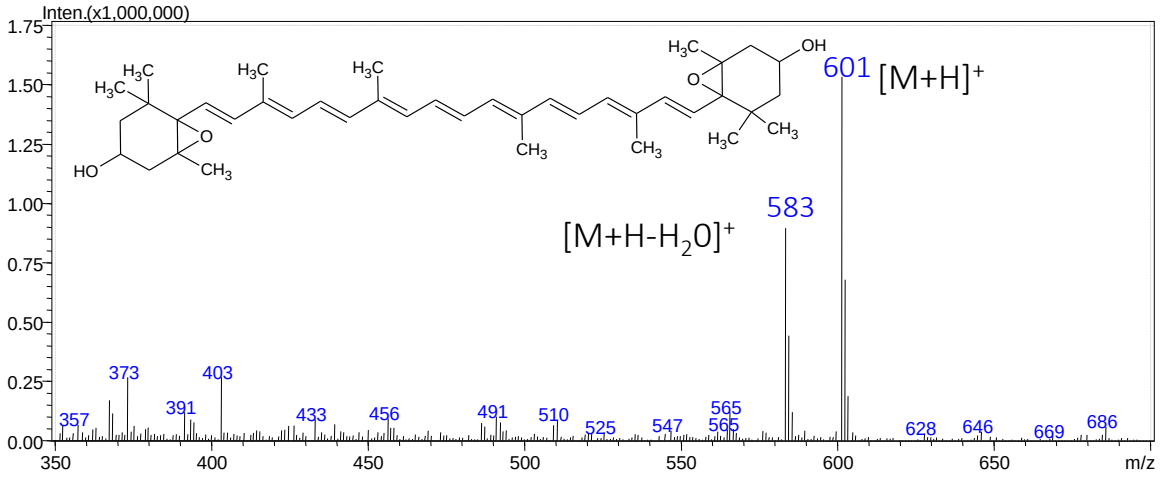
Paola Dugo et al., J. Agric. Food Chem. 56 (2008) 3478-3485

Combination of multiple detection in LC × LC analyses of carotenoids

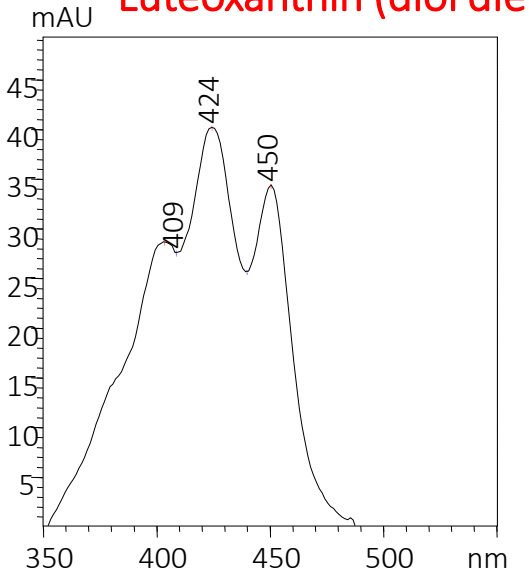
SAME MS SPECTRA BUT DIFFERENT UV SPECTRA

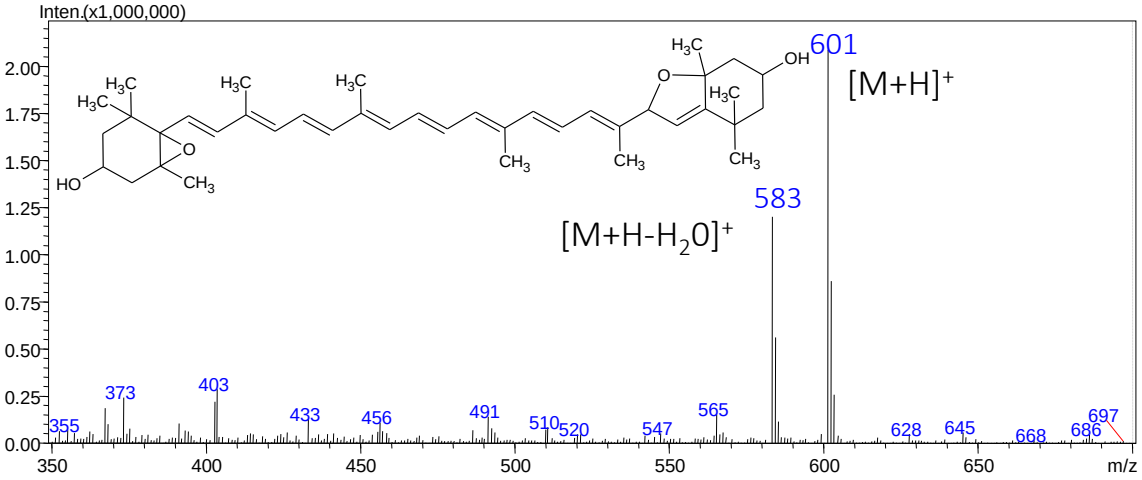
Violaxanthin (diol diepoxide)





Luteoxanthin (diol diepoxide)





Combination of multiple detection in LC × LC analyses of carotenoids

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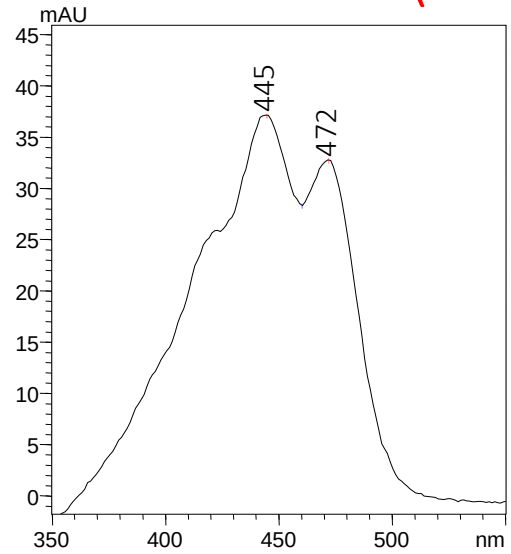
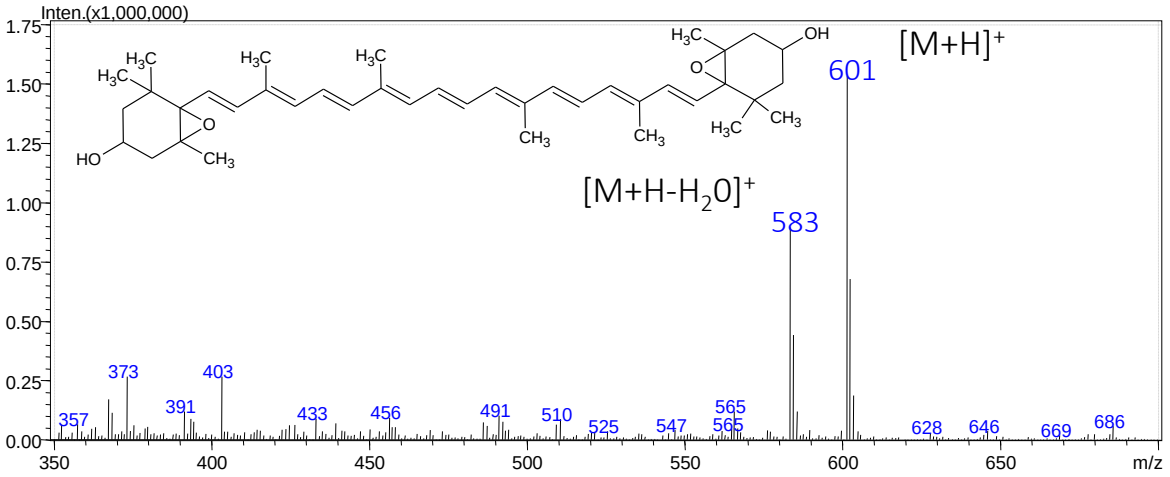
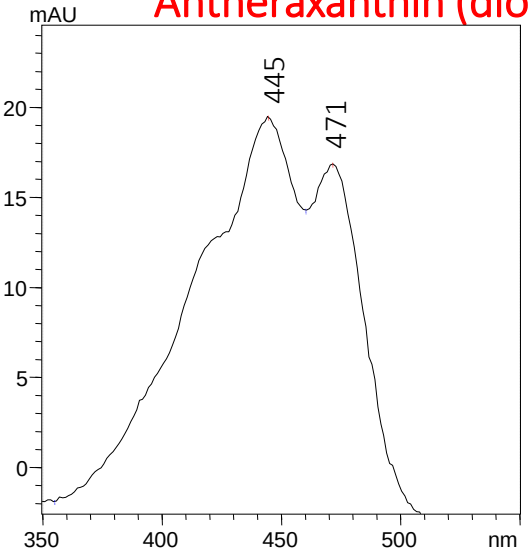
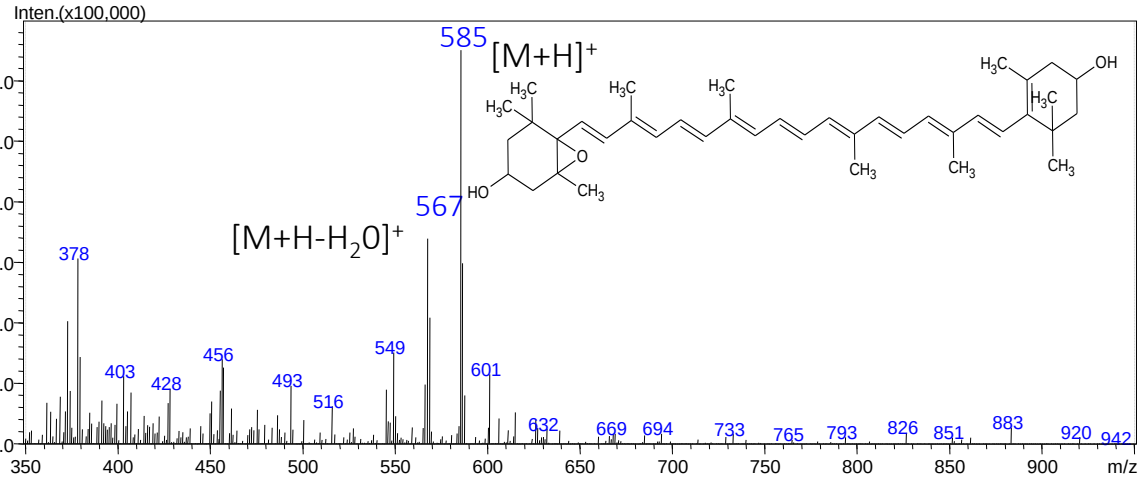




Antheraxanthin (diol monoepoxide)





Analysis of lipids by LC × LC

- Major food constituents: triacylglycerols (TAGs), phospholipids (PLs), fat-soluble vitamins, monoglycerols, diglycerols, waxes, sterols, ...

- Biological functions: energy storage, structural capability (cell membranes), and signaling.

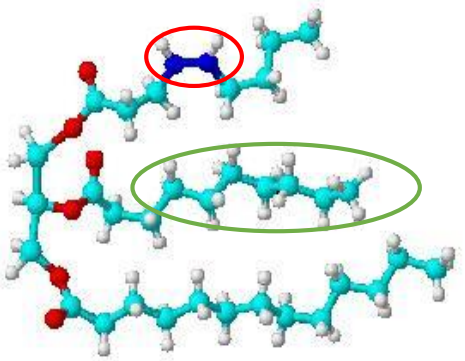
- PLs are mainly involved in many CNS disorders (Alzheimer's, Parkinson's, Multiple sclerosis, and schizophrenia) and deregulated metabolism injuries.

- Total cholesterol, HDL-cholesterol, LDL-cholesterol, and TAGs are involved in cardiovascular diseases (coronary heart disease by blockage of blood vessels).

- TAGs are the dominant lipids in the diet (90-95% of the total dietary fat energy).

Analysis of triacylglycerols by NP-LC × RP-LC

✓ the possible FA combination on the glycerol backbone can lead to an enormous TAG number.



Theoretical TAGs number

$$Z = n^3 + n^2/2$$

Z : number of possible TAGs

n : number of different FAs

e.g. if n = 10

$Z = 10^3 + 10^2/2 = 1050$

Such a number is reduced by biosynthetic preference

Analysis of triacylglycerols by LC × LC

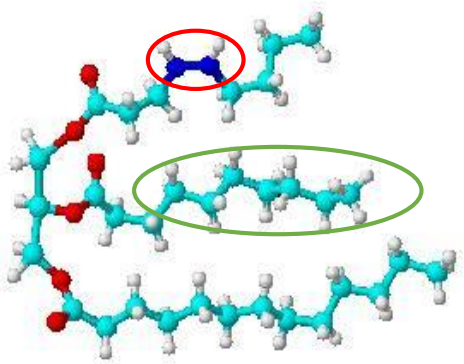
Classical methods for TAG analysis are:

Ag^+-LC: separation occurs mainly on the basis of unsaturation degree. Usually applied as pre-separation, not as stand alone analytical approach

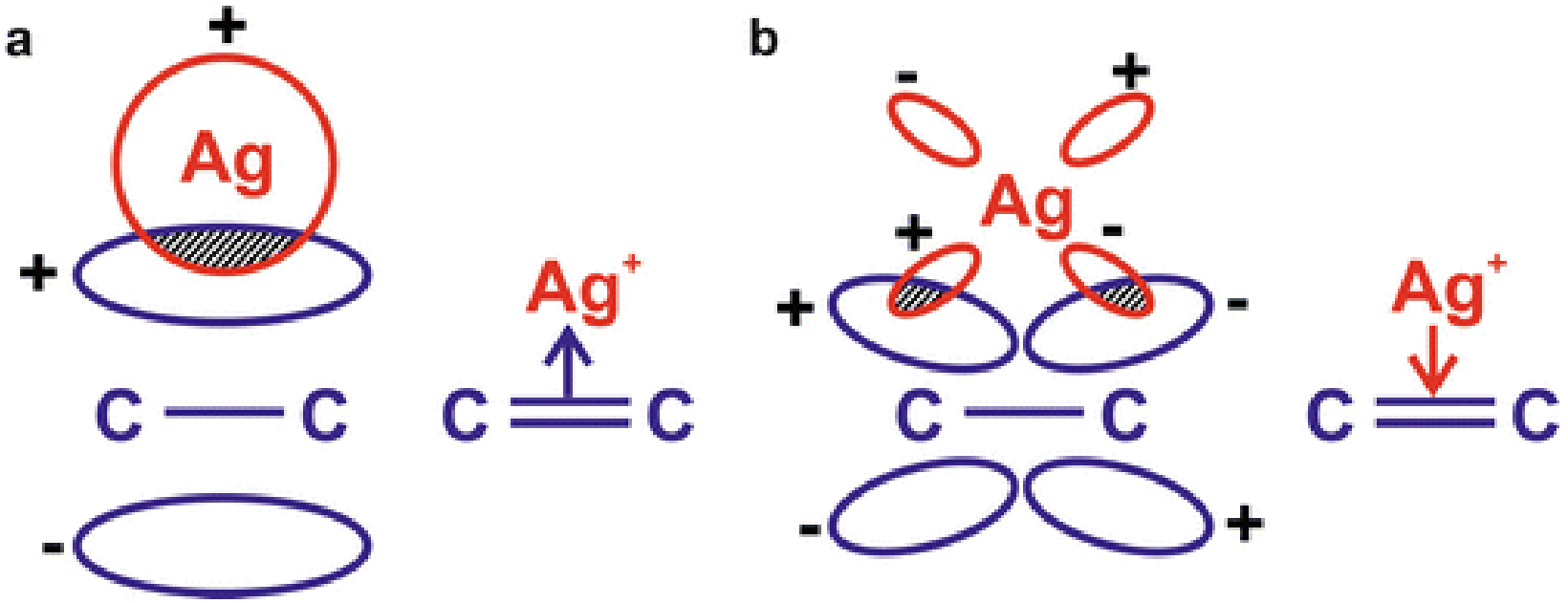
RP-LC: separation occurs on the basis of hydrophobicity, thus TAGs are eluted according to increasing PN number (PN=CN-2DB)*.

Mass spectrometry (MS) with APCI interface, coupled to LC represents by far the most common tool for TAG determination.

*PN: partition number
CN: carbon number
DB: double bonds



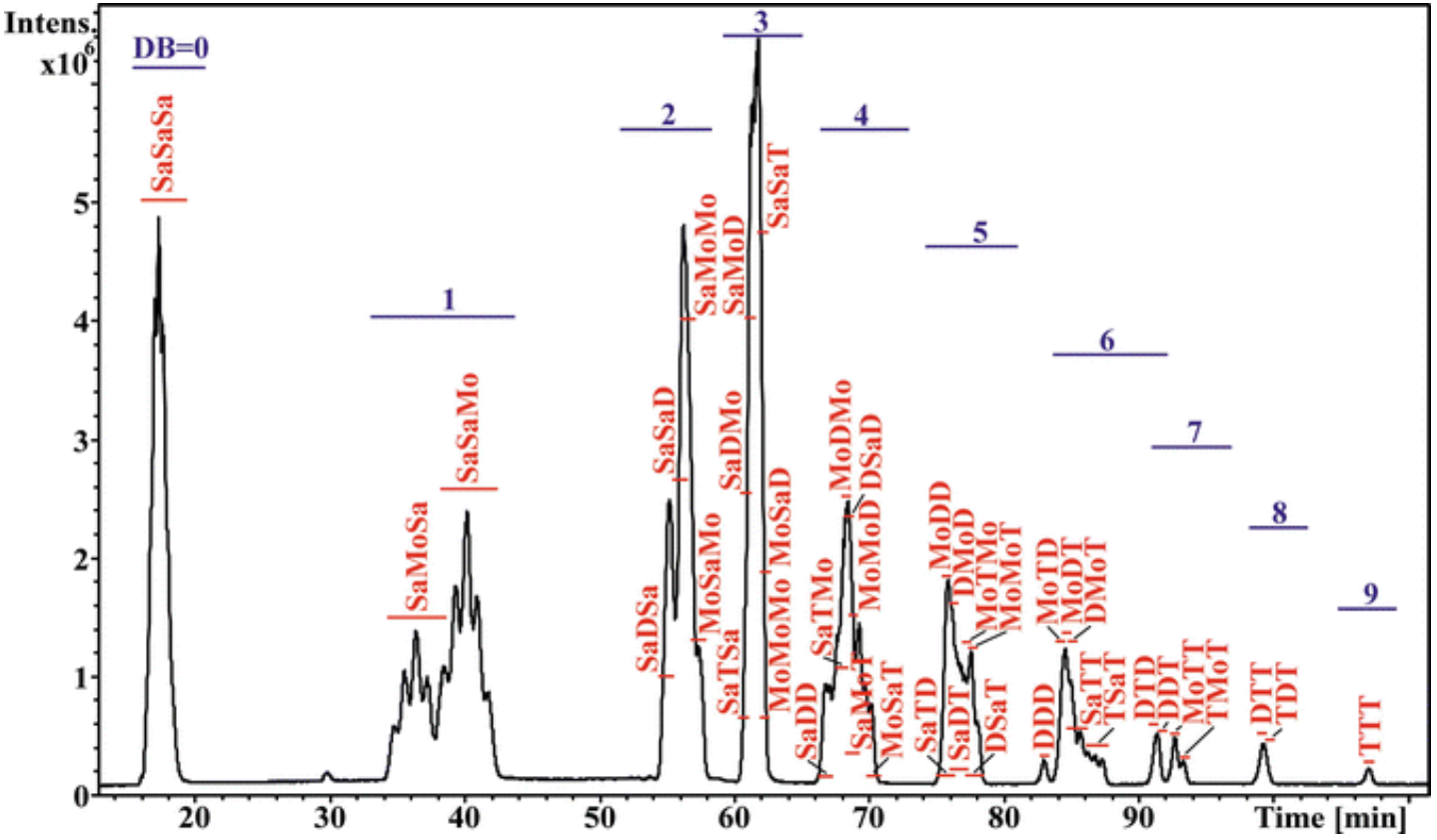
Ag-LC column: analyte interaction mechanism



Description of complex bonding between silver ions and DB by the Dewar-Chatt-Duncanson model: (**a**) σ-donation and (**b**) π-back-bonding interactions between the metal and DB

Dewar JS. A review of the pi-complex theory. Bull Soc Chim Fr. 1951;18:C71–9.

Ag-LC column: TAG elution order



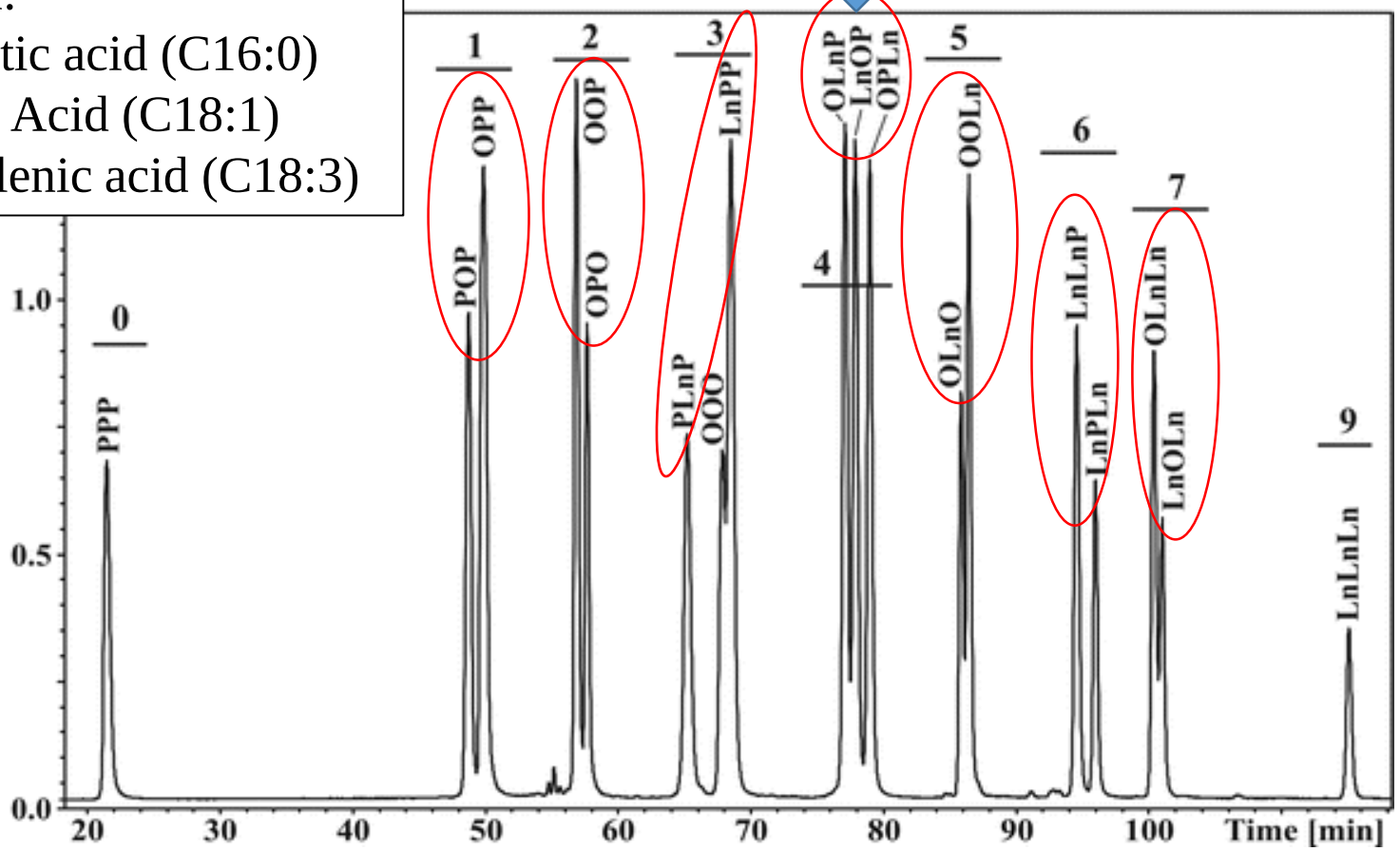
- Ag-HPLC/APCI-MS chromatogram of the randomized mixture of PPP (C48:0), SSS (C54:0), OOO (C54:3), LLL (C54:6), LnLnLn (C54:9), and AAA (C20:0). Peak annotation: Sa saturated, Mo monounsaturated, D diunsaturated, T triunsaturated, DB double bond number

Holčapek M et al, 2009 JSS

Ag-LC column: regioisomeric TAG separation

3 different C52:4 regioisomeric TAGs separated

Legenda:
P: Palmitic acid (C16:0)
O: Oleic Acid (C18:1)
Ln:Linolenic acid (C18:3)



- Ag-HPLC/APCI-MS analysis of randomized mixture of PPP(C48:0)/OOO(C54:3)/LnLnLn(C54:9)

Lísa M et al, Anal. Chem. 2009

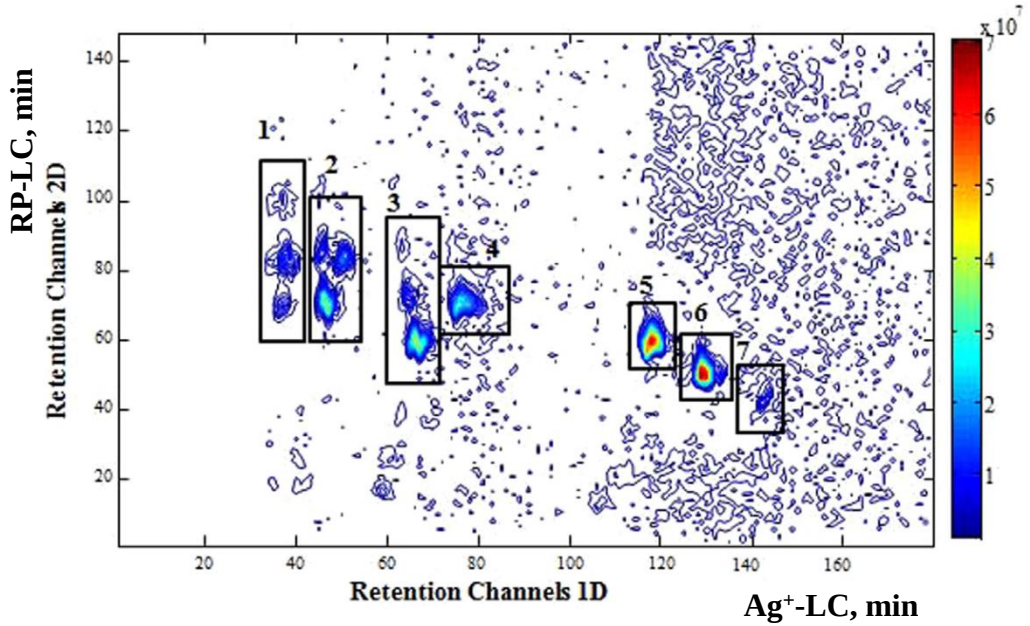
Ag-LC column: regioisomeric TAG separation

Silver-Ion Chromatography of Glycerolipids, Table 1 Regioisomeric occupation of *sn*-2 position for saturated (palmitic), monounsaturated (oleic), and diunsaturated (linoleic) FA in TG of plant (sunflower, olive, and palm) oils and animal fat (lard)

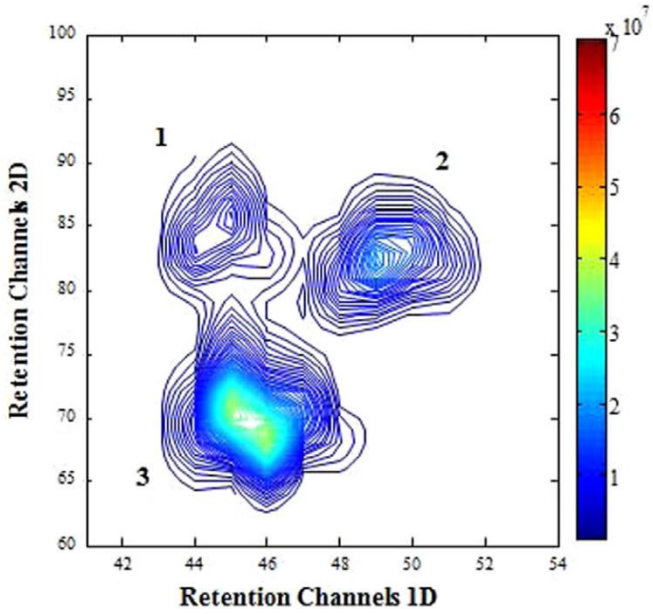
	Sunflower oil			Olive oil	Palm oil	Lard	
Regioisomers	(Lisa et al. 2009)	(Leskinen 2010)	(Herrera et al. 2010)	(Herrera et al. 2010)	(Leskinen 2010)	(Lisa et al. 2009)	(Leskinen et al. 2007)
POP/OPP	100/0	–	99/1	97/3	86/14	8/92	0/100
OOP/OPO	98/2	91/9	100/0	98/2	95/5	12/88	4/96
PLP/LPP	100/0	–	–	–	–	1/99	–
LLP/LPL	97/3	–	–	–	–	9/91	–
OLP/LOP/OPL	63/36/1	–	–	–	–	3/12/85	–

Ag^+-LC × RP-LC-APCI-MS of corn oil TAGs

RESOLUTION OF TRIACYLGLYCEROL STRUCTURAL ISOMERS



Bidimensional chromatogram of the corn oil sample. Seven important chromatographic regions are marked and numbered.



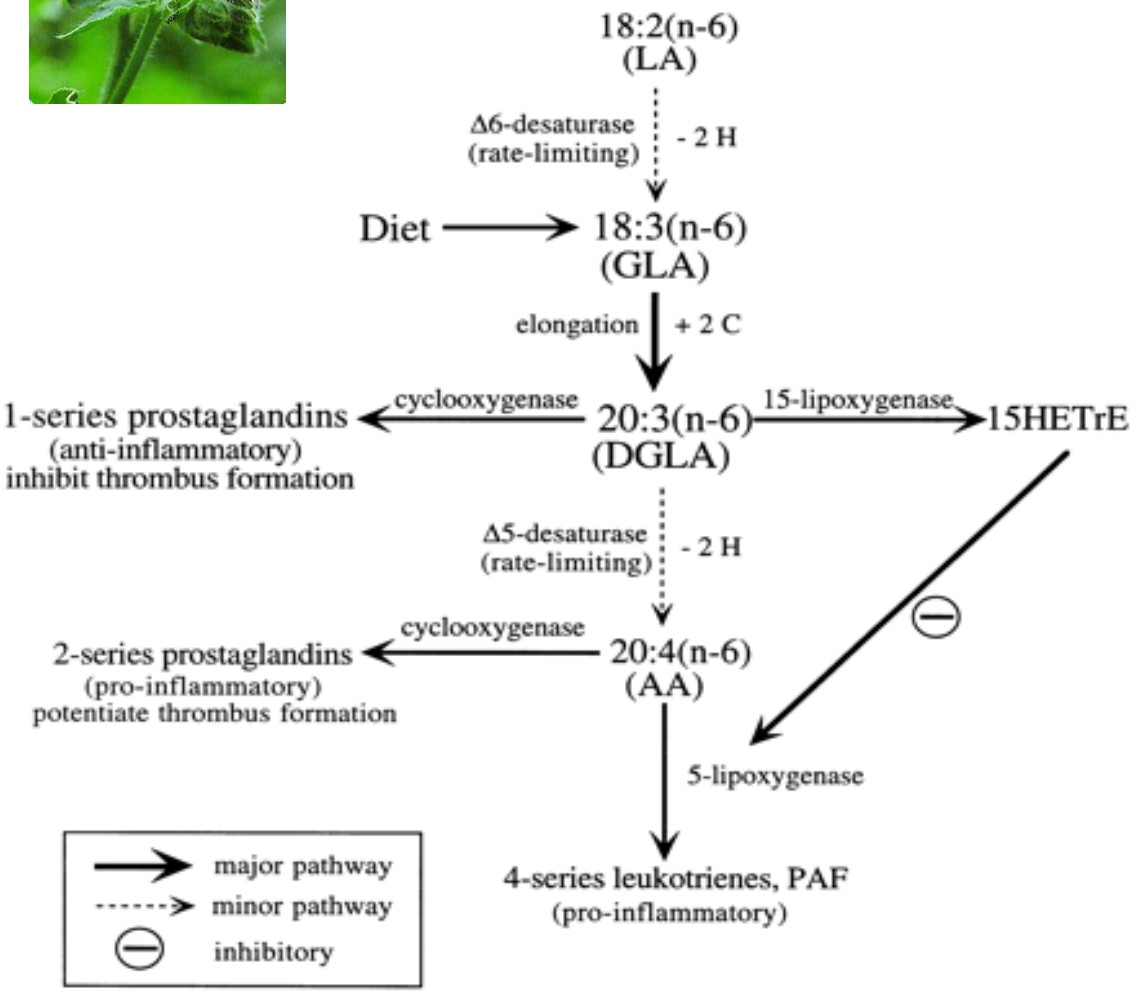
Zoomed view of region of interest number 2 with the three important peaks numbered (peak 1 is SOL, peak 2 is SLO and peak 3 corresponds to POL and PLO).

Meritxell Navarro-Reig et al., Talanta 160 (2016) 624-635

TAGs analysis by Ag^{+}-LC × RP-LC

in vegetable and fish oils

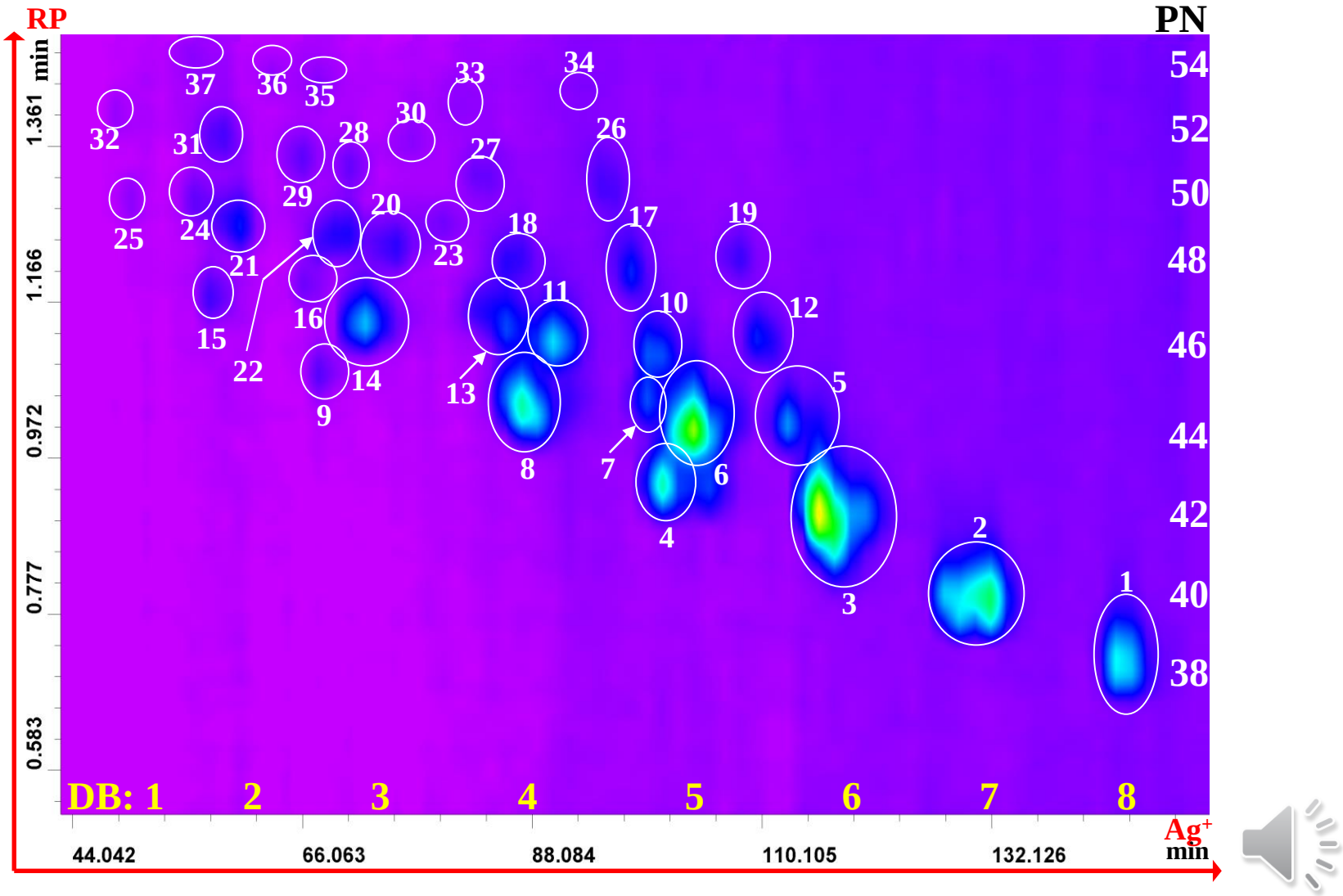
Ag-LC × RP-LC/APCI-IT-TOF-MS of triacylglycerols
Borage oil



γ-linolenic acid ***Metabolism***

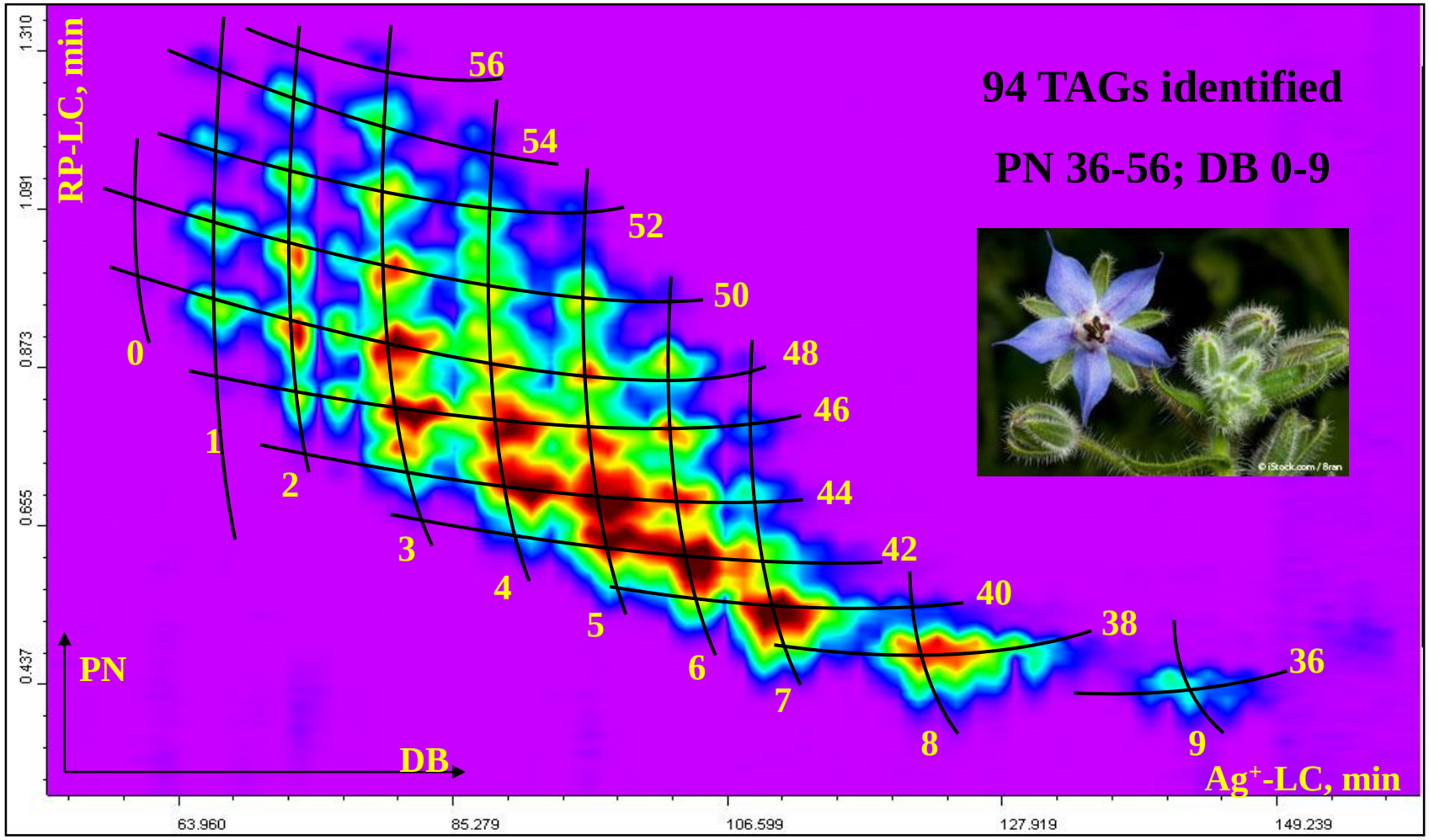
FAMEs	A %	Symbol
C16:0	10.67	P
C16:1	0.14	Po
C17:0	0.05	E
C18:0	3.55	S
C18:1n-9c	16.27	O
C18:1n-7	0.60	V
C18:2n-6	38.24	L
C18:3n-6	***21.67***	***γLn***
C18:3n-3	0.29	Ln
C20:0	0.21	A
C20:1n-9	3.68	G
C20:2	0.18	Es
C22:0	0.12	B
C22:1n-9c	2.27	C22:1
C24:0	0.05	Lg
C24:1n-9	1.36	C24:1

First application: Ag^+-LC × RP-LC-ELSD of triacylglycerols in Borage oil

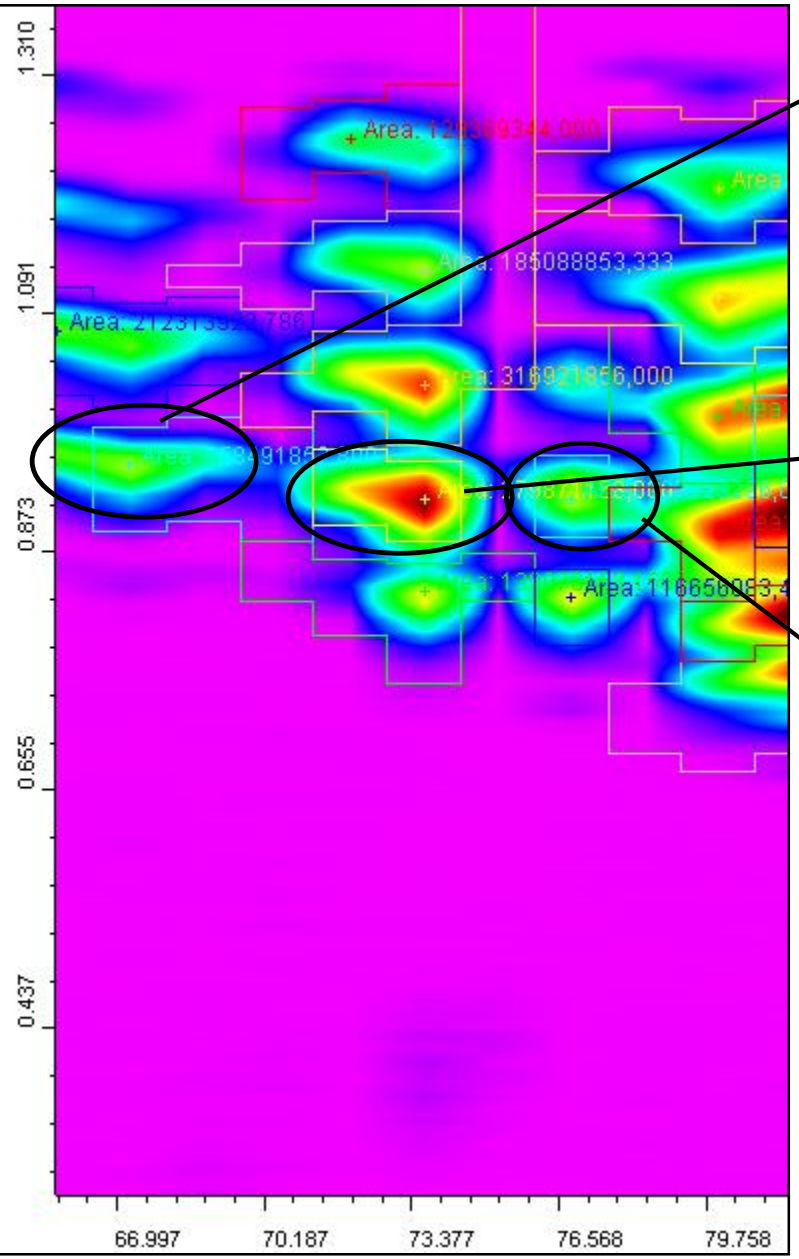


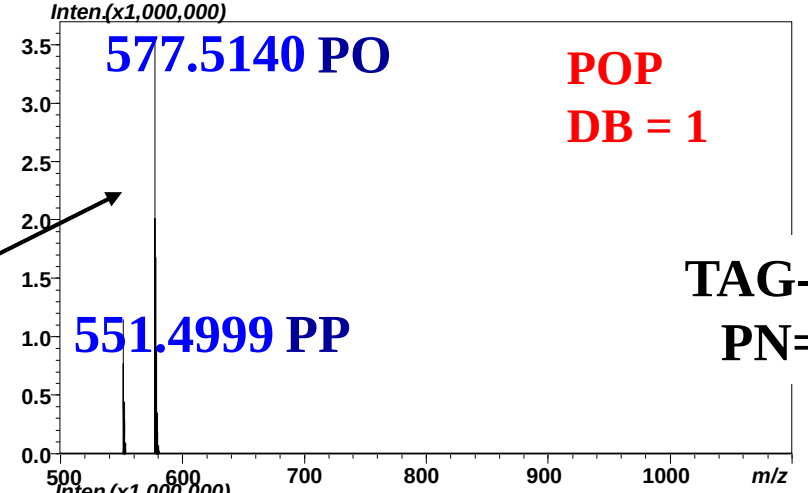
L. Modello & M. Beccaria et al, JSS, 2011

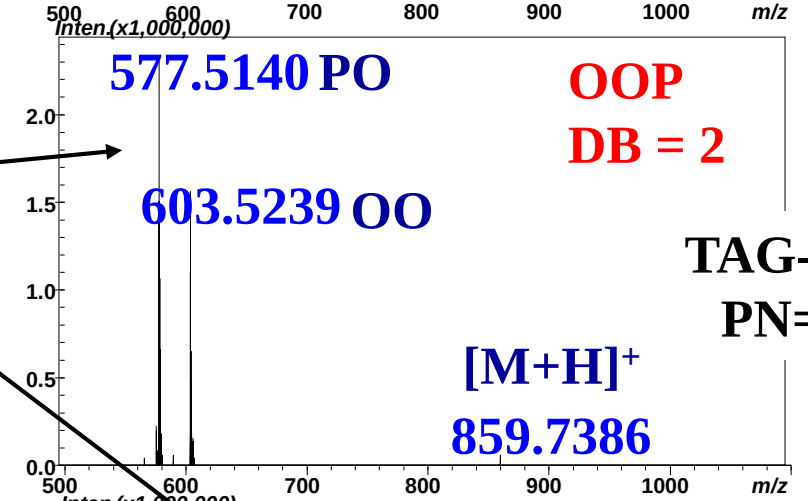
Ag^+-LC × RP-LC/APCI-IT-TOF-MS of triacylglycerols
Borage oil

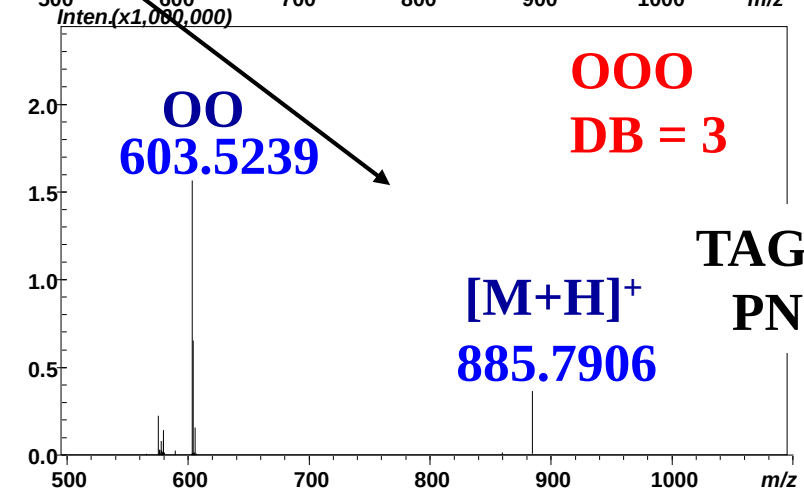


Increasing DB
TAG Separation (same PN)









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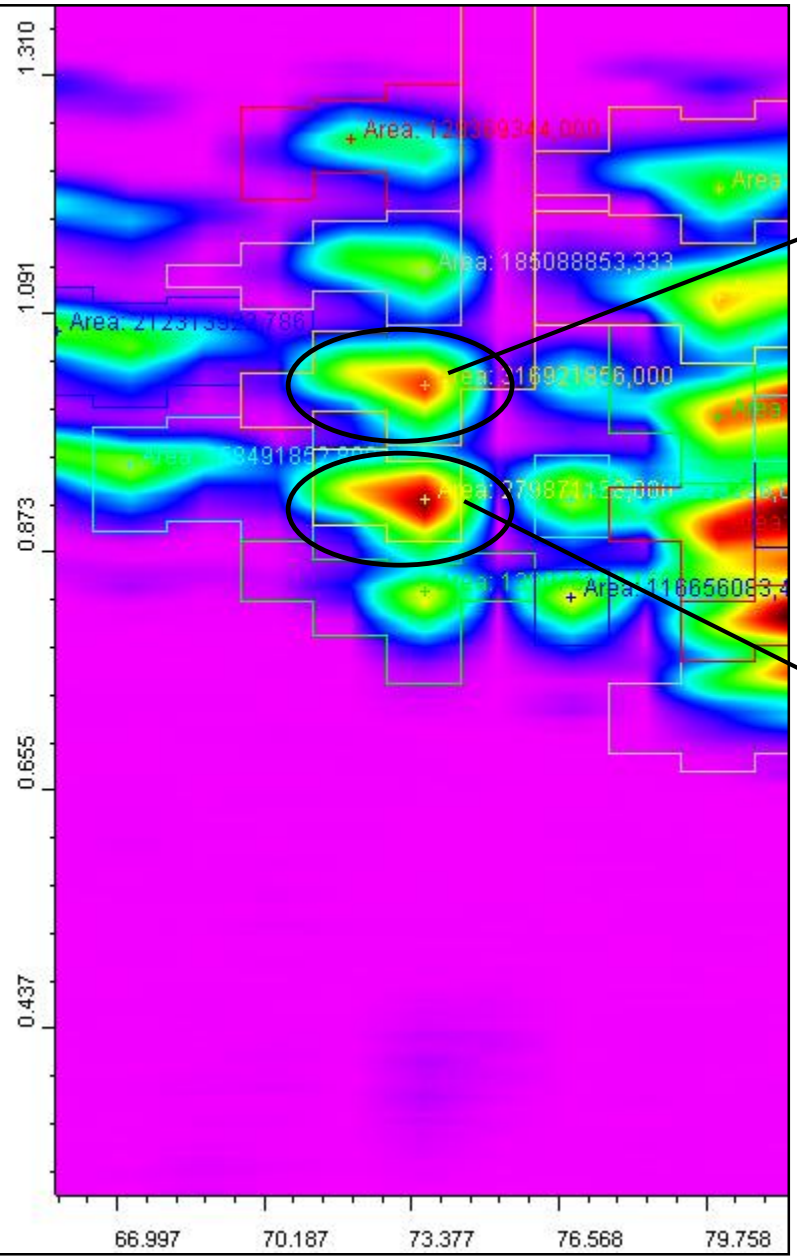
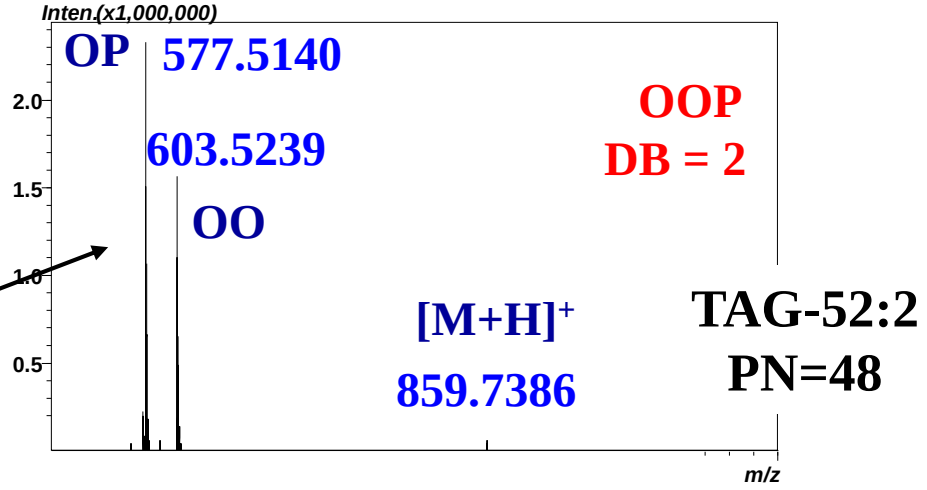
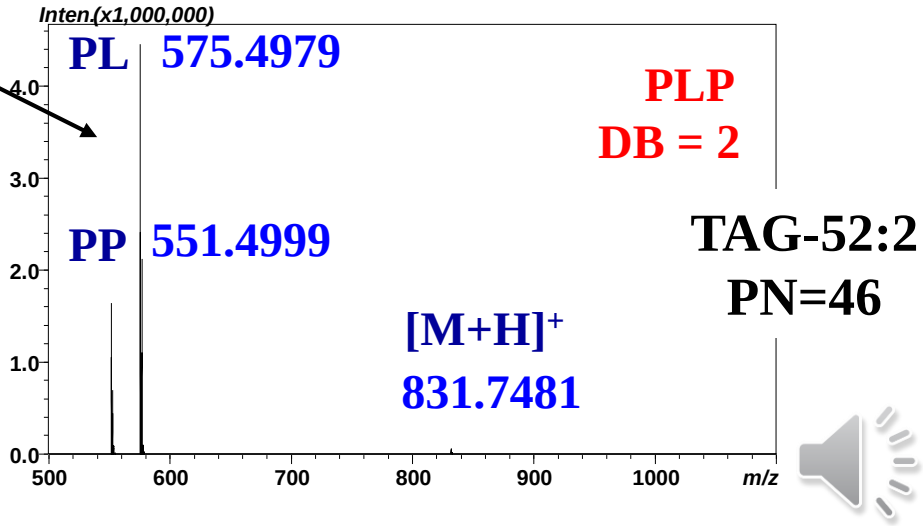






“Continuous” on-line LC × LC

may fail for very complex samples as fish oil

↓

on-line “Stop-flow” and/or off-line LC × LC

^{2}D separation cannot keep up with ^{1}D sampling frequency**

↓

A longer ^{2}D column can be employed

** Tran, B. Q., Lundanes, E., Greibrokk, T., *Chromatographia* 2006, *64*, 1-5.

Case odf Study: Characterization of Fish Oils

❖ Fish and fish oils are gaining increasing importance in the diet due to their health benefits. Such properties are mainly related to their fatty acid composition and in particular for their high amount in PUFA.

❖The characterization of their lipidic fraction represents a very challenging task. This is due to the great number of FA and possible FA combination on the glycerol backbone, leading to an enormous TAG number.

AIM OF THE WORK

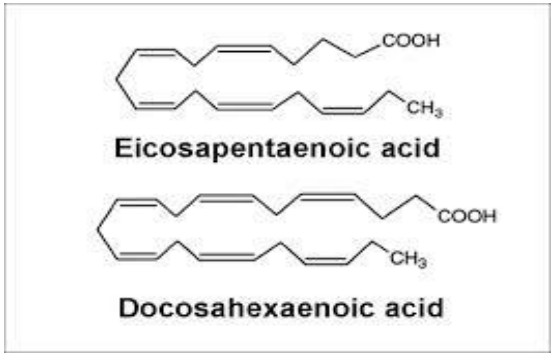
Development of highly efficient 2D-LC methods for TAGs charcaterization of complex samples

Case study:
Fish oil

- *Brevoortia tyrannus* oil, better known as **menhaden oil**, represents one of the most complex ones. Due the high content of omega-3 PUFAs, it may represent a viable source of dietary lipids.

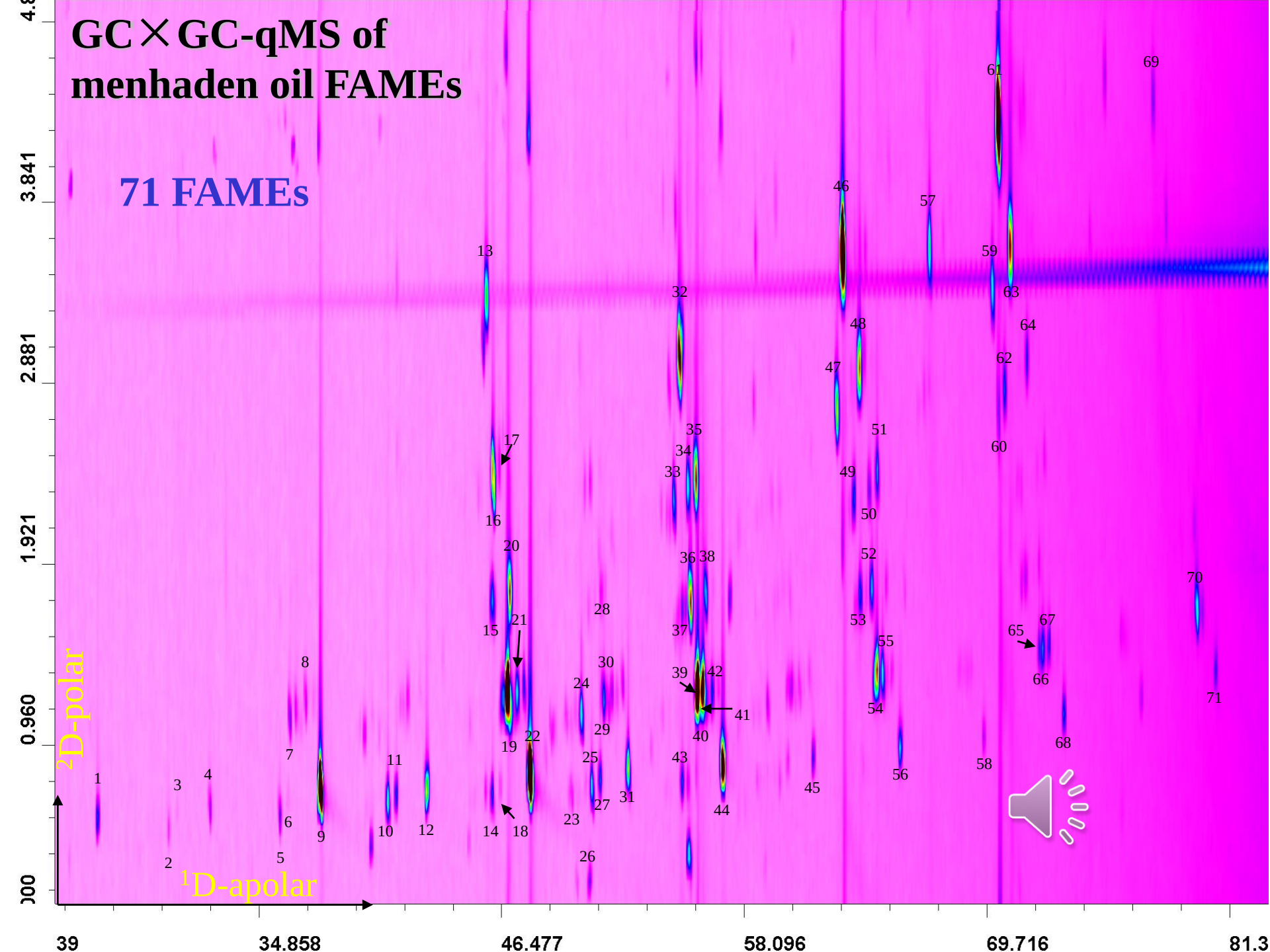
20:5(ϖ3)

22:6(ϖ3)



- Until 2015, only a few selected fractions were analyzed by supercritical fluid chromatography (SFC) and RP-LC, in off-line mode *.

*I. Francois, A. dos Santos Pereira, P. Sandra, J. Sep. Sci., 33 (2010) 1504.


GC×GC-qMS of menhaden oil FAMEs
71 FAMEs
2D-polar
1D-apolar
4.8
3.841
2.881
1.921
0.960
000
39
34.858
46.477
58.096
69.716
81.3

Theoretical TAGs number

$$Z = n^3 + n^2/2$$

Z : number of possible TAGs

n : number of different FAs

With n = 71

$Z = 71^3 + 71^2/2 =$ **>360,000 different TAGs**

Such a number is reduced by biosynthetic preference (different according to the fish species*), however it remains very high

* Nwosu CV, Boyd LC (1997) J Food Lipids 4:65–74

Ag^+-LC × NARP-LC-APCI-qMS stop-flow of a menhaden oil

First dimension

Column: Nucleosil ion-exchange loaded with silver ions* (150 mm × 1.0 mm, 5 um)

Mobile phase: (A) 1.0 % BCN in Hex; (B) 10.0 % BCN in Hex

Linear gradient: 0 min 0% B; 100 min 100% B

Flow-rate: 30.0 mL/min

Vol. Inj. : 2 uL

Sample: menhaden oil (from Supelco) 40 mg in 10 mL Hex

Second dimension

Column: Ascentis Express C18 Fused-core (150 x 2.1 mm, 2.7 um)

Mobile phase: (A) ACN; (B) IPA

Gradient: 0 min 0% B; 3.0 min 35% B; 4.5 min 35% B; 9.0 min 50% B; 10 min 70% B; 10.51 min 0% B

Flow-rate: 0.5 mL

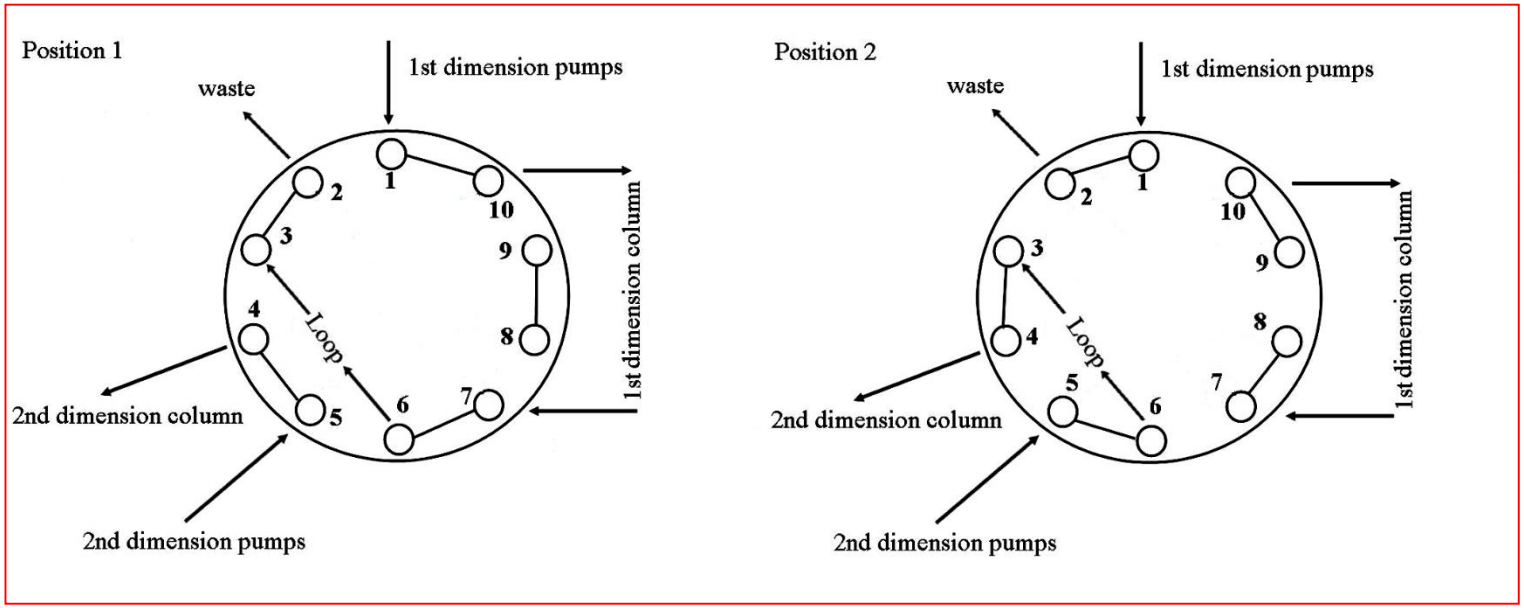
Modulation time: 12 min

Detector: MS ($APCI^+$)

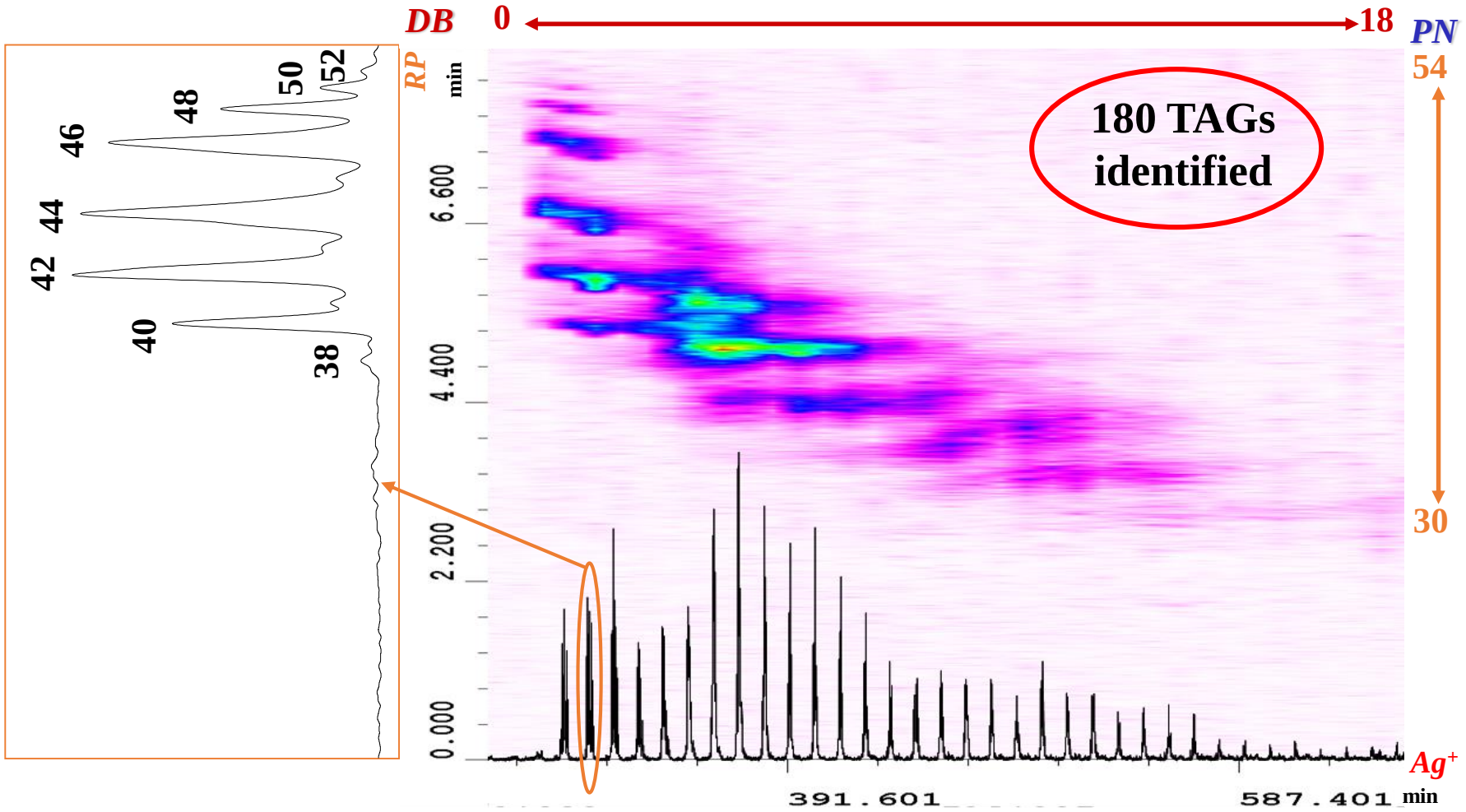
* ChristieWW (1987) J High Resolu tChromatogr Commun 10:148–150

Scheme of the ten-port valve

(position one: flow; position two: stop)



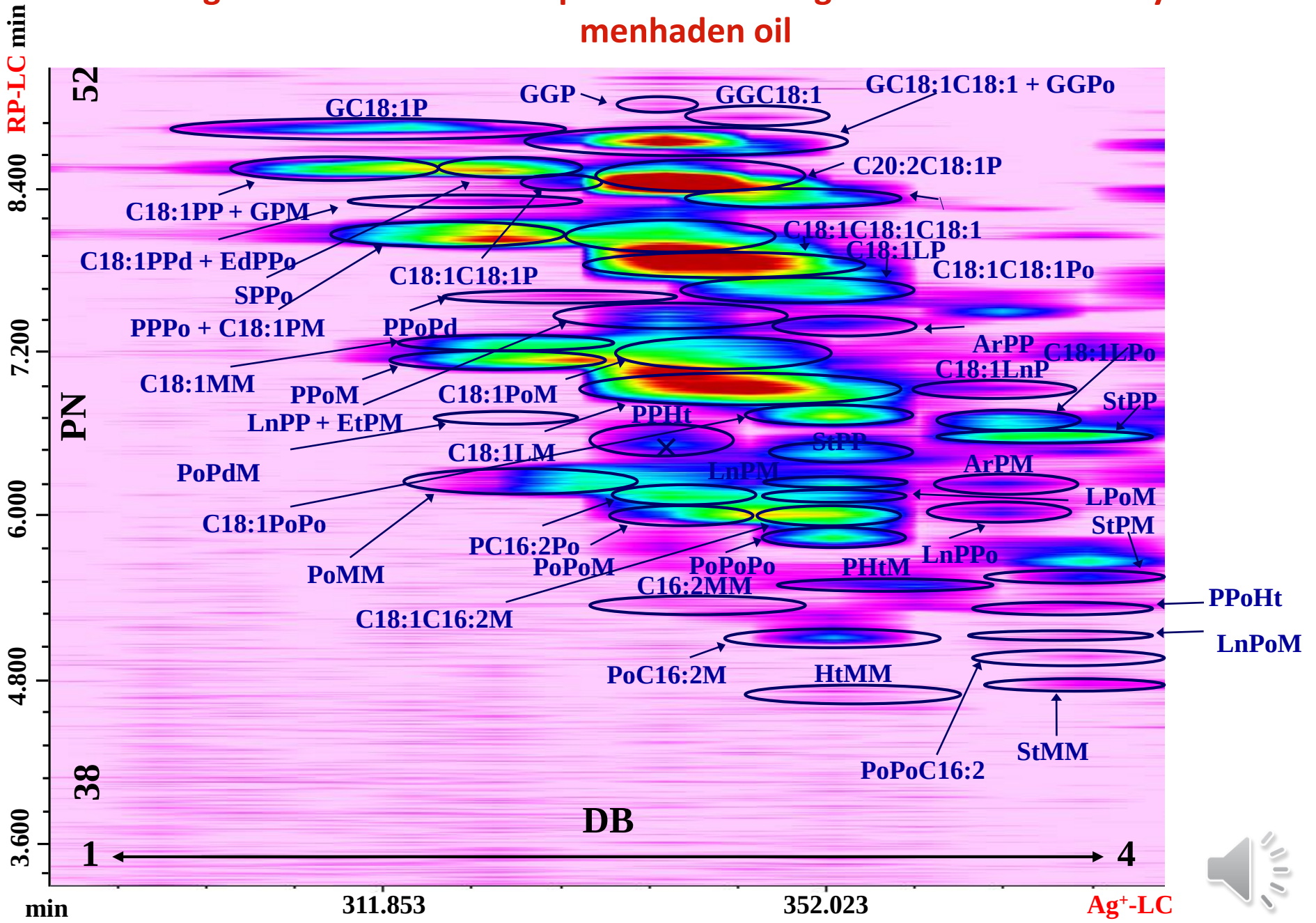
“Stop-flow” Ag^+-LC × RP-LC-APCI-MS of menhaden oil



Marco Beccaria et al., Anal. Bioanal. Chem. 407 (2015) 5211-5225

First comprehensive LC × LC *on-line* separation reported of the TAG fraction in fish (menhaden) oil.

Enlargement of the contour plot for the 2D Ag^+-LC × RP-LC-MS analysis of menhaden oil



Off-line comprehensive LC (Ag^+-LC × NARP-LC-APCI-qMS)

First dimension

Column: Nucleosil ion-exchange loaded with silver ions* (250 mm × 4.0 mm, 5 um)

Mobile phase: (A) 1.0 % BCN in Hex; **(B)** 5.0 % BCN in Hex

Linear gradient: 0 min 0% B; 100 min 100% B

Flow-rate: 0.8 mL/min

Vol. Inj. : 20 uL

Sample: menhaden oil (from Supelco) 40 mg in 10 mL Hex

Second dimension

Column: Ascentis Express C18 Fused-core (150 x 4.6 mm, 2.7 um)

Mobile phase: (A) ACN; **(B)** IPA

Linear gradient: 0 min 0% B; 50 min 70% B

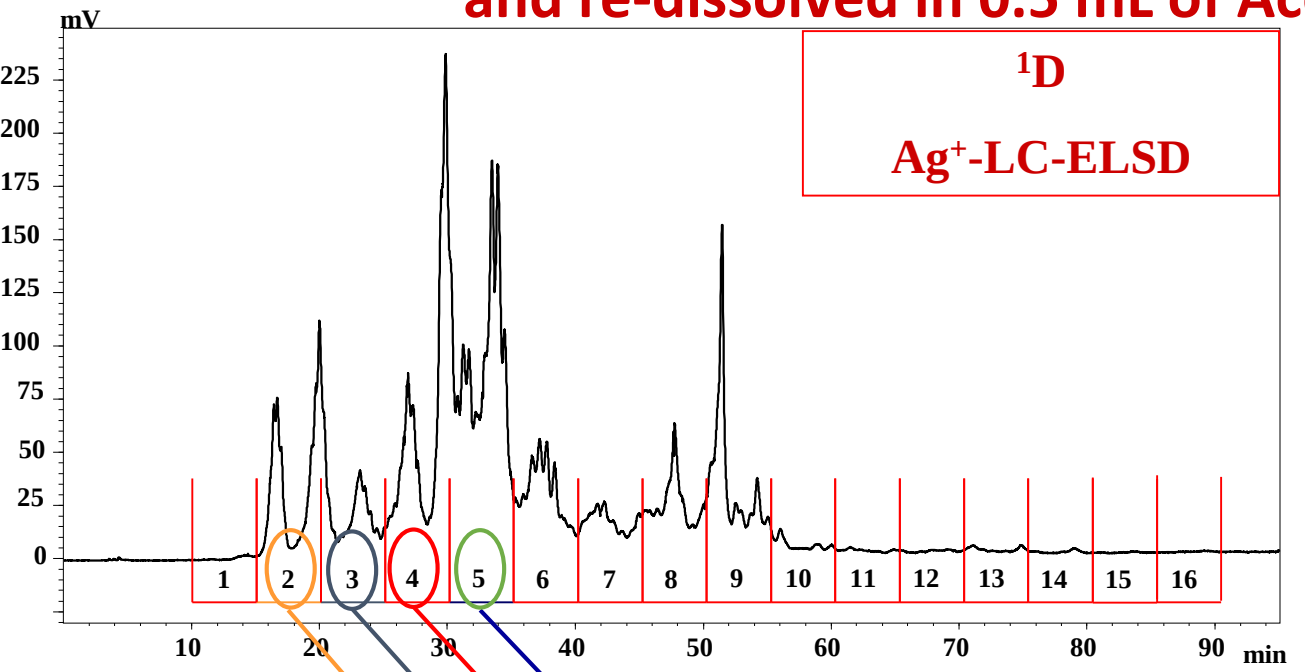
Flow-rate: 1.0 mL

Vol. Inj.: 10 uL

Detector: qMS ($APCI^+$)

* ChristieWW(1987)JHighResolutChromatogrCommun10:148–150

The 16 fractions were evaporated to dryness under a nitrogen stream and re-dissolved in 0.5 mL of Acetone

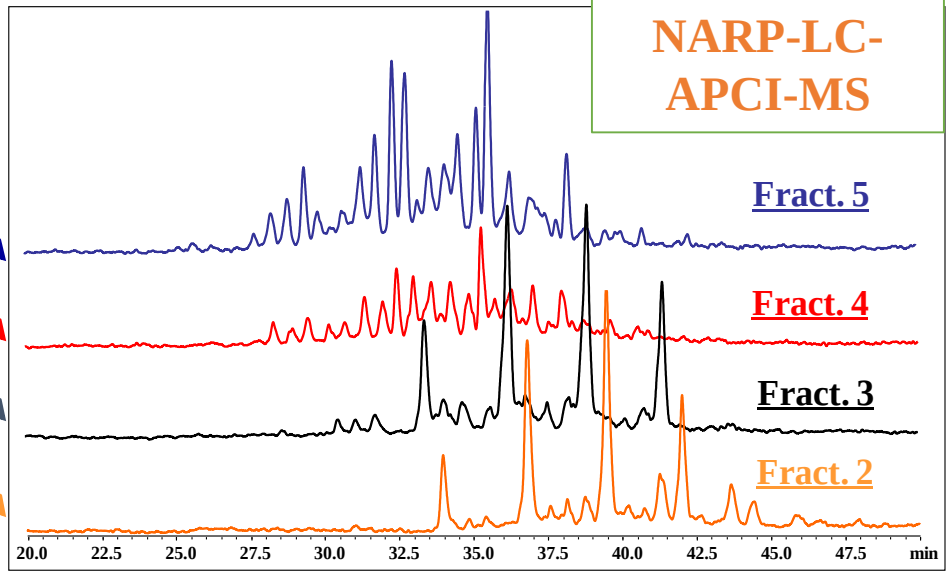


^{1}D

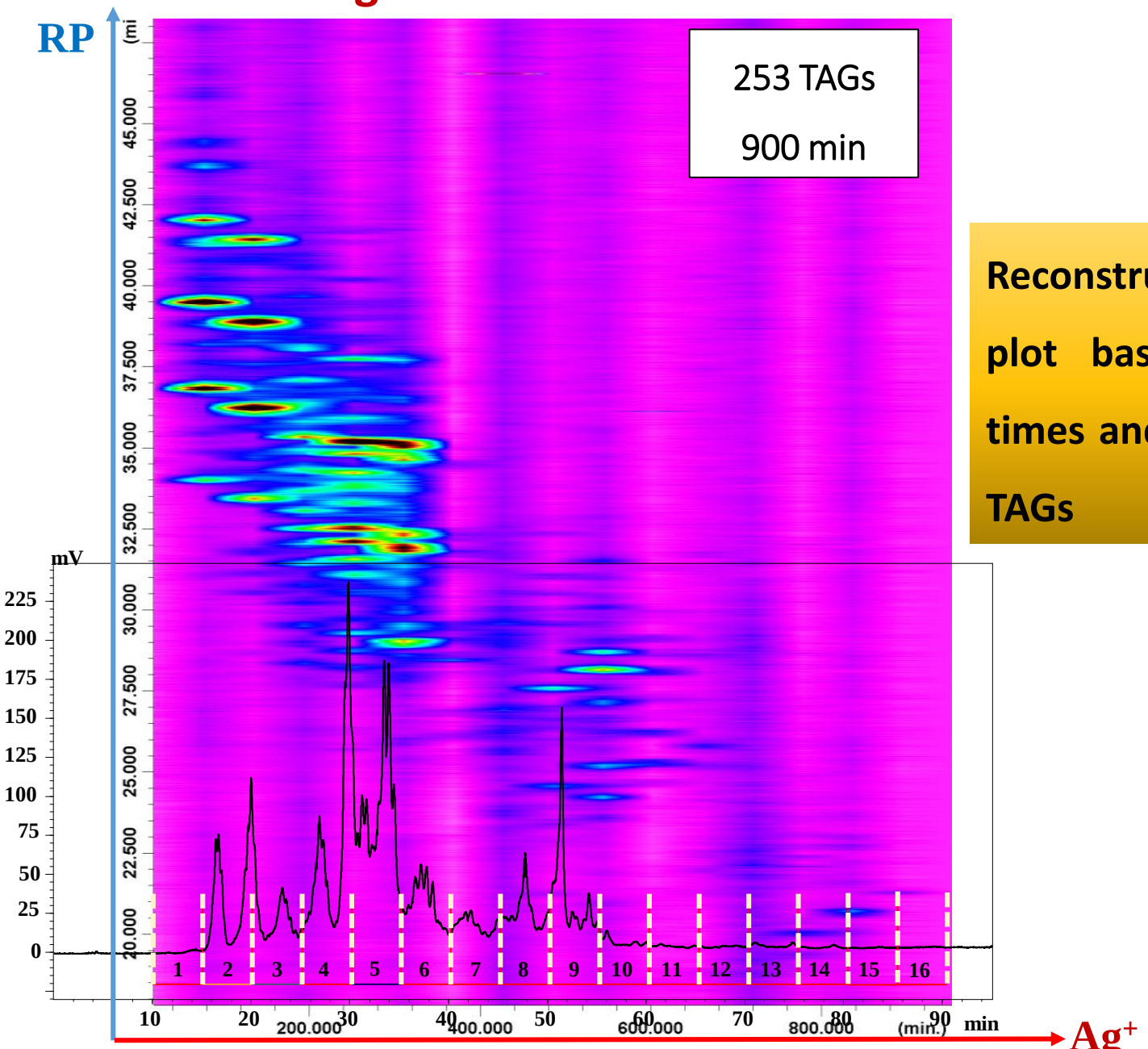
Ag^{+}-LC-ELSD

^{2}D

NARP-LC-APCI-MS



Off-line Ag^+-LC × RP-LC-APCI-MS of menhaden oil TAGs



Reconstruction of the 2D-LC plot basing on retention times and MS intensities of TAGs

Marco Beccaria et al., Anal. Bioanal. Chem. 407 (2015) 5211-5225

HILIC × RP-LC applications

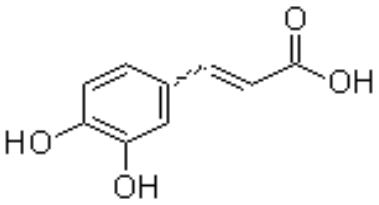
Analysis of polyphenols by HILC-LC × RP-LC

❖ Polyphenols are a structural class of mainly natural, but also synthetic or semisynthetic, organic chemicals characterized by the presence of large multiples of phenol structural units.

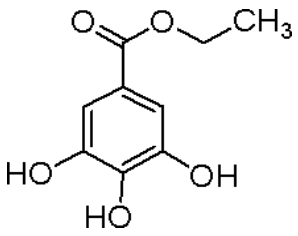
❖ The phenolic content in natural matrices can largely vary with the variety, location, environment, degree of ripeness, and type of extraction.

❖ Phenolic compounds are essential to the quality and nutritional properties, affecting shelf life and sensorial properties: colour, astringency, bitterness, and flavour.

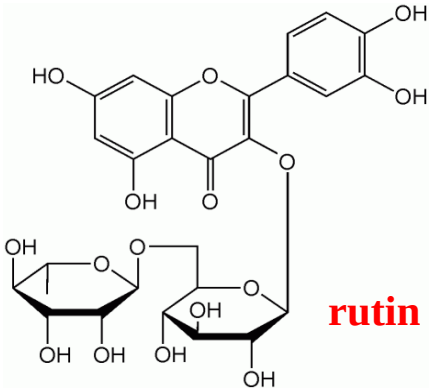
❖ Beneficial effects of these natural molecules on human health include antioxidants, anti-inflammatory as well as the protection on risk factors for cardiovascular diseases.



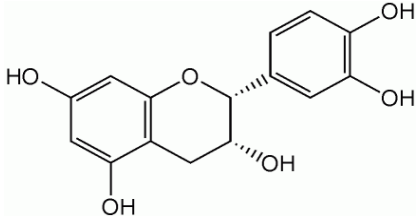
Caffeic acid



Ethyl gallate



rutin



epicatechin

Separation of polyphenols by HILIC × LC

Method: HILIC × RP-LC-ESI-MS

First dimension

HILIC (Amide column):

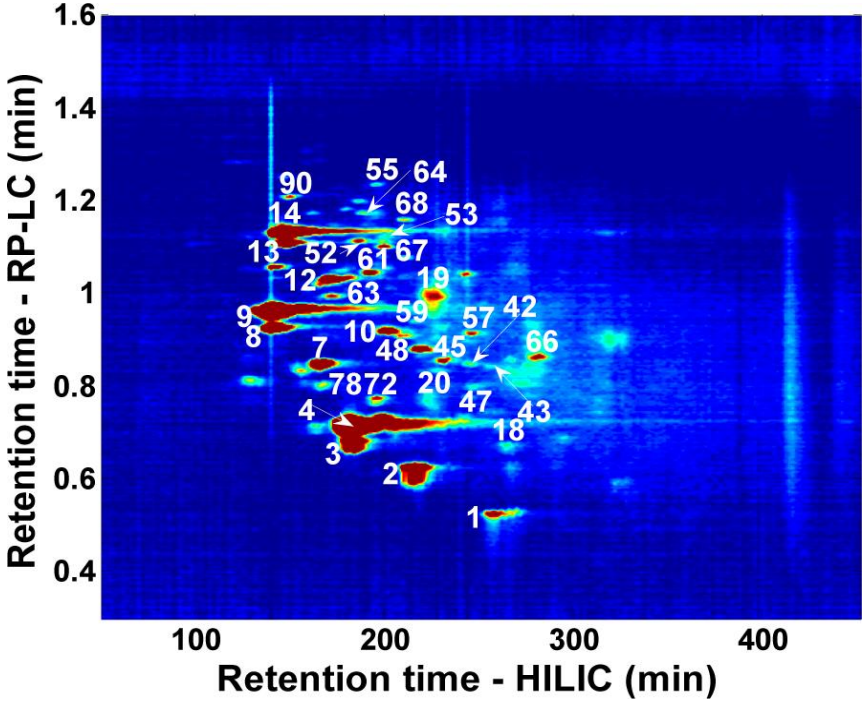
Polyphenols are separated according to different polarity (in case of anthocyanins separation separation occurs on the basis of glycosylation and acylation degree, although isomeric compounds were not resolved).

Second dimension

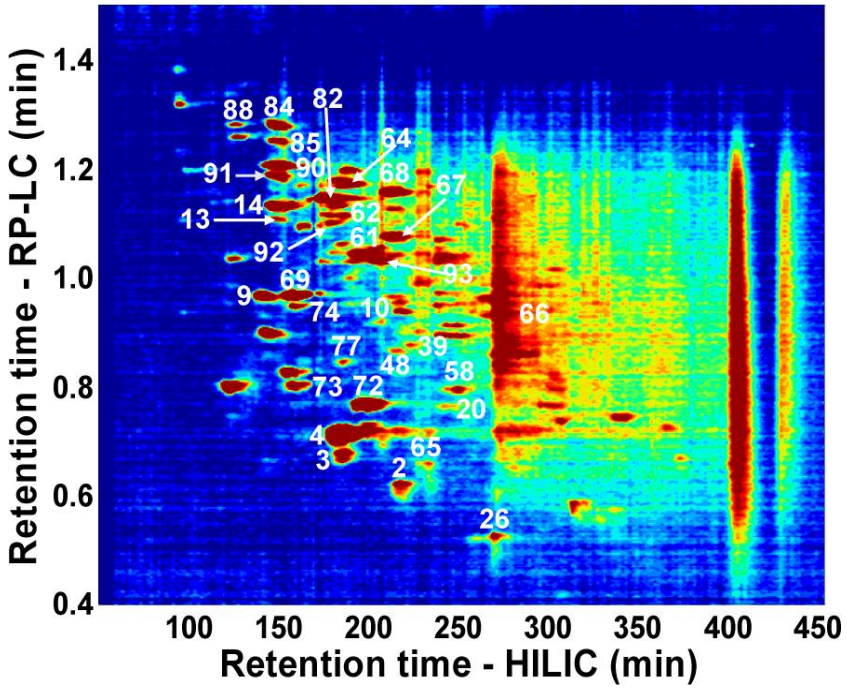
RP-HPLC (C18 column):

Polyphenols are eluted according to their increasing hydrophobicity and decreasing polarity

First application: HILIC × RP-LC-UV-MS Analysis of Anthocyanins and Derived Pigments in Red Wine



2013 Pinotage wine

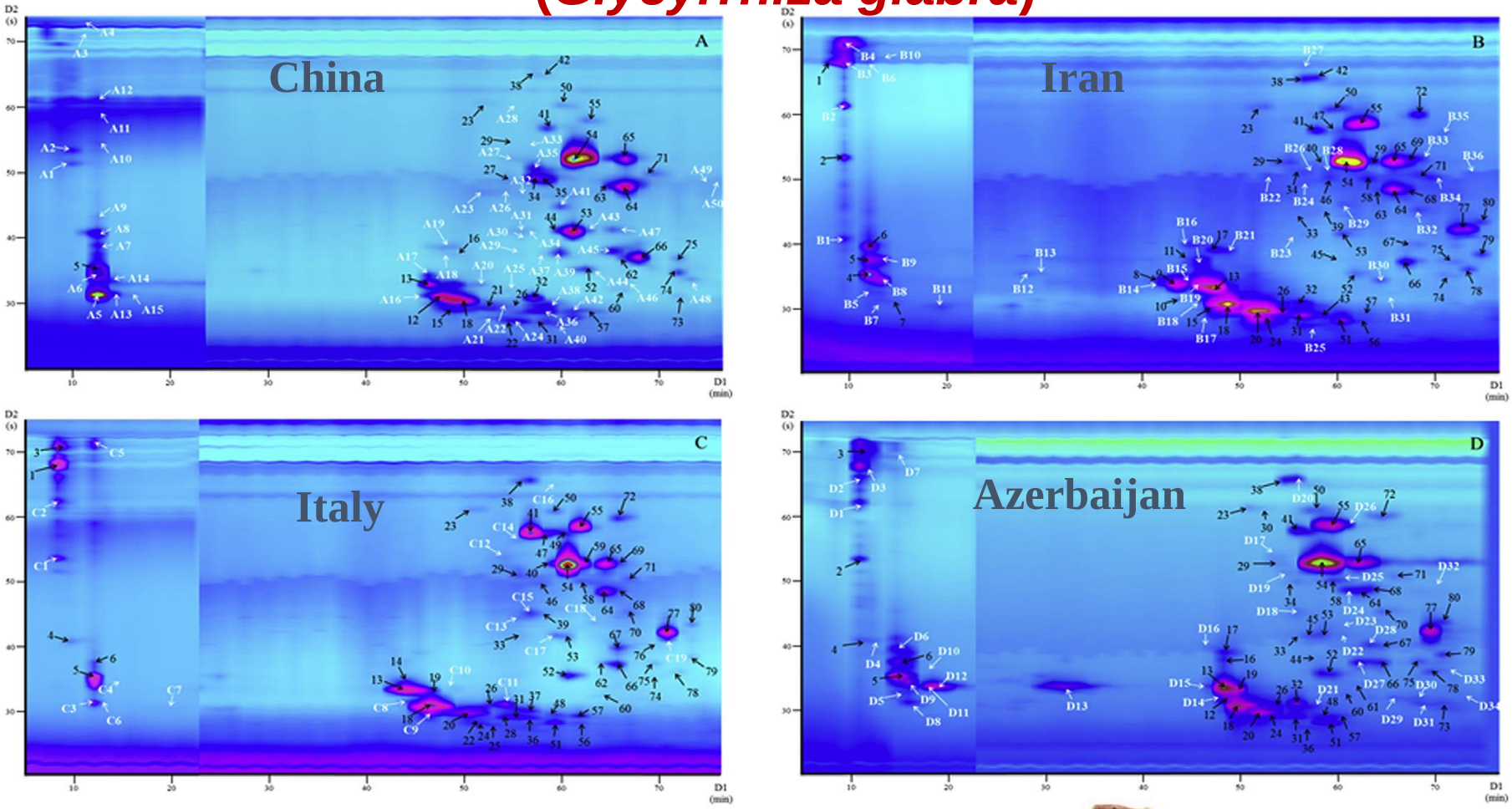


2008 Pinotage wine

Free anthocyanins, diglucosides and oligomeric anthocyanins decrease rapidly with age. In both wines a total of 94 compounds were tentatively identified.

André de Villiers and co-workers, Anal. Chem. 87 (2015) 12006-12015.

HILIC × RP-LC-UV-MS Analysis of licorice samples (*Glycyrrhiza glabra*)

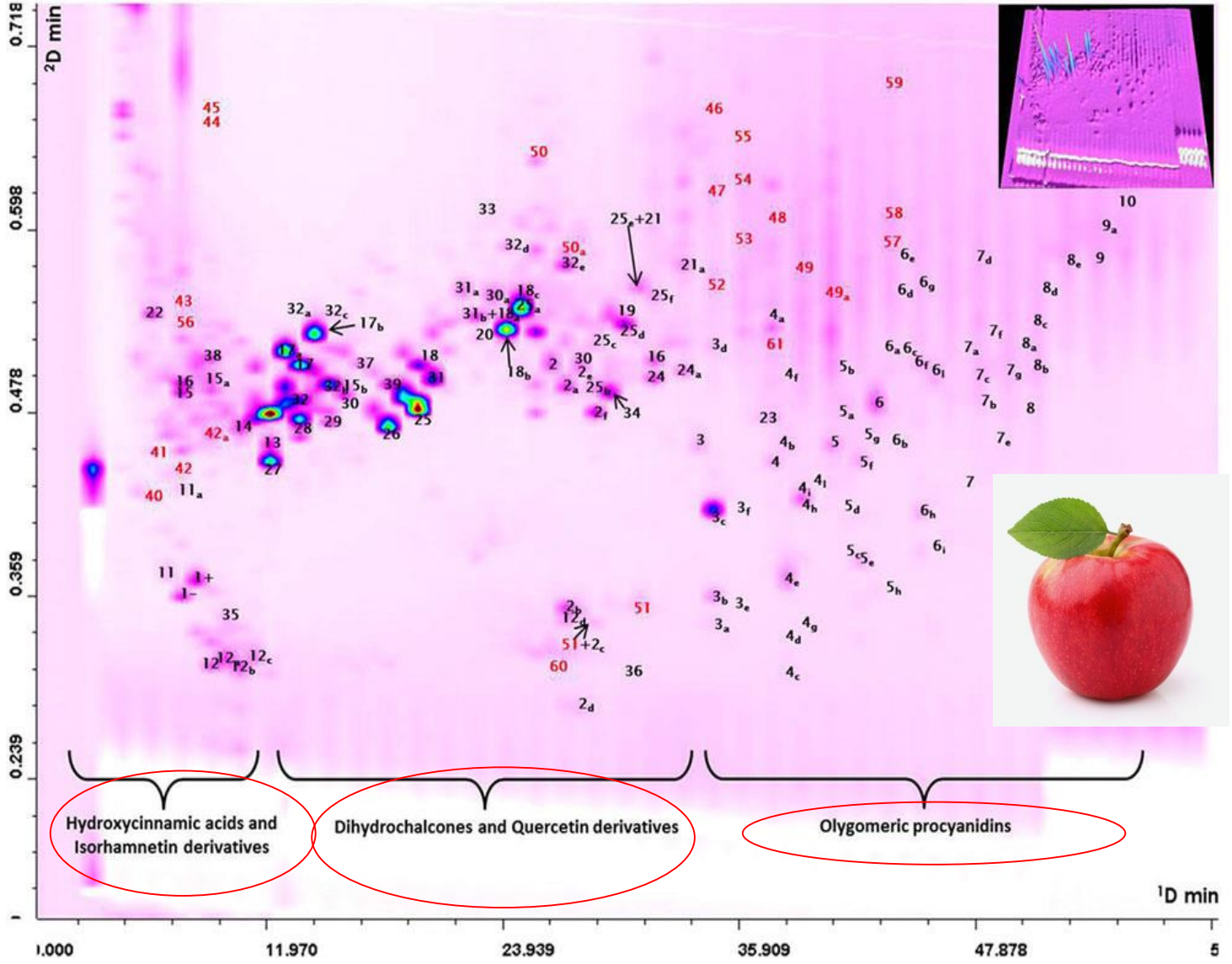


The developed method was capable to separate a large number of compounds (up to 89 in the Iranian sample)

The HILIC × RP-LC-UV-MS generated patterns could be potentially used to assess their geographical location and authentication

Miguel Herrero and co-workers, Anal. Chim. Acta 913 (2016) 145-159.

HILIC × UHP(RP)-LC-UV-MS multiclass polyphenolic analysis in apple

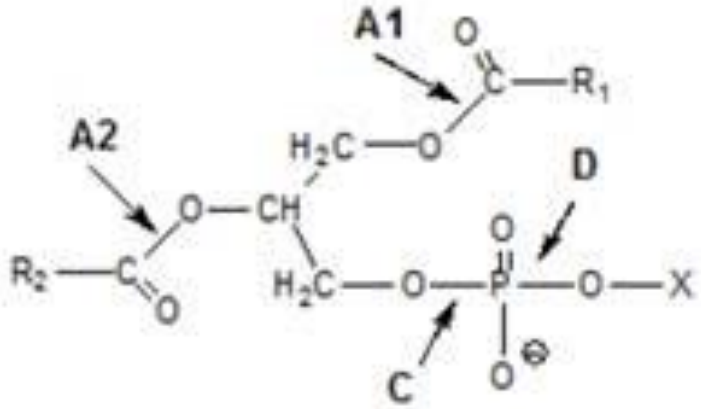


Simultaneous separation of multiple polyphenolic classes, including oligomeric procyanidins, up to degree of polymerization of 10 was accomplished. Tentative identification of 121 analytes in total was achieved.

Eduardo Sommella et al., J. Sep. Sci. 40 (2017) 2188-2197.

Separation of phospholipids by HILIC × LC

Method: HILIC × RP-LC-ESI-MS

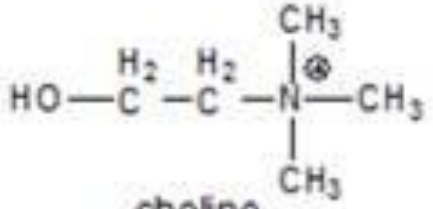


X = choline (phosphatidylcholine or PC)

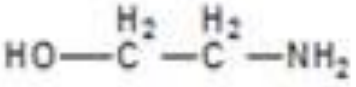
X = ethanolamine (phosphatidylethanolamine, PE)

X = inositol (phosphatidylinositol or PI)

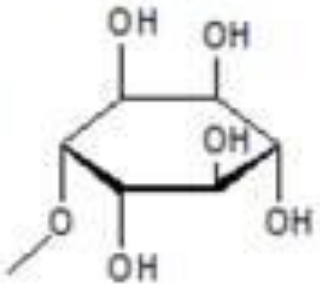
X = hydrogen (phosphatidic acid or PA)



choline



ethanolamine



inositol, link in 1-position

- **Phospholipids** (PL) are a class of lipids having a hydrophilic "head" containing a phosphate group, and two hydrophobic "tails" derived from fatty acids, joined by an alcohol residue. The phosphate group can be modified with simple organic molecules such as choline, ethanolamine or serine

Separation of phospholipids by HILIC × LC

Method: HILIC × RP-LC-ESI-MS

^{1}D-HILIC

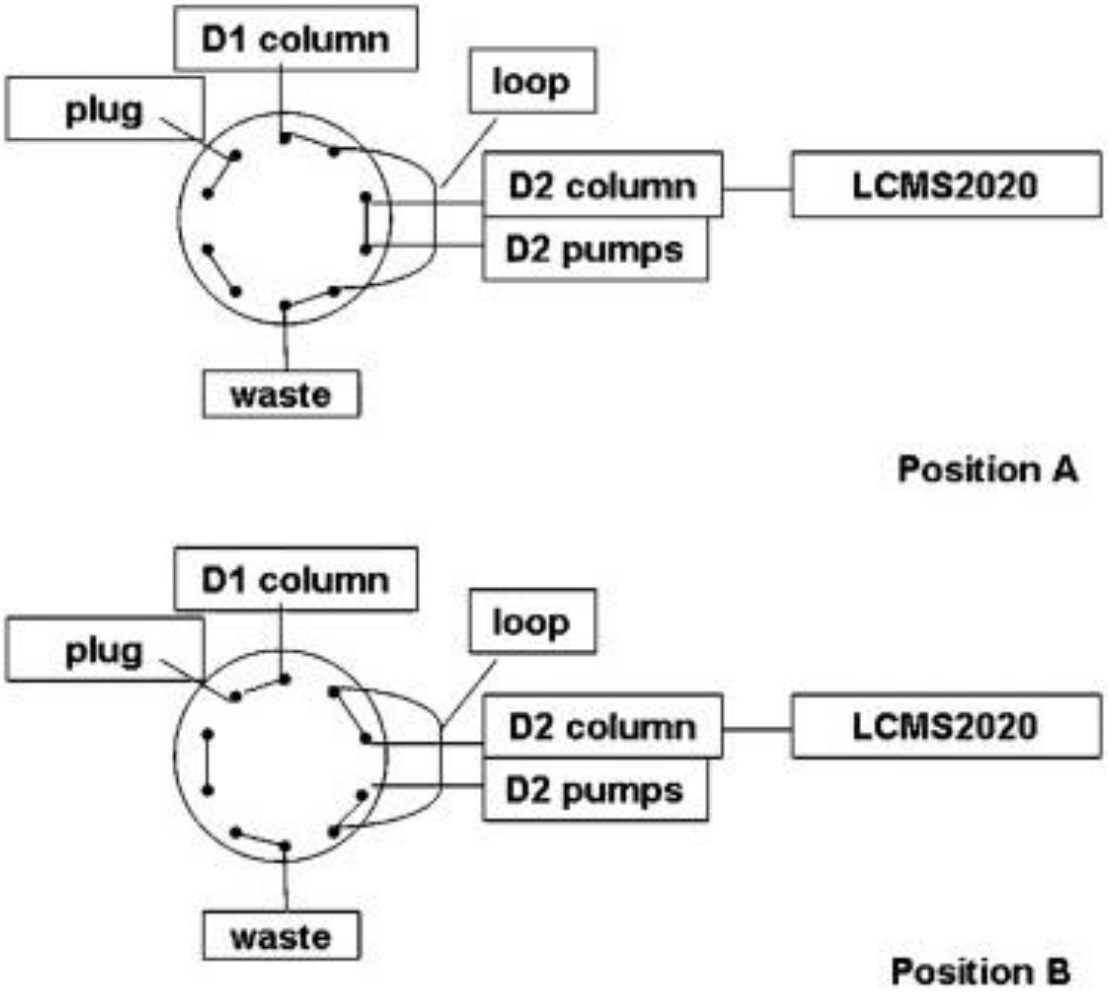
- Phospholipids are separated according to their polar head group e.g.
- Phosphatidylinositol (PI)
- Phosphatidylethanolamine (PE)
- Phosphatidylserine (PS), etc.

^{2}D-RP-LC

- Phospholipids molecular species are separated according to their FA chain lengths and level of unsaturation.

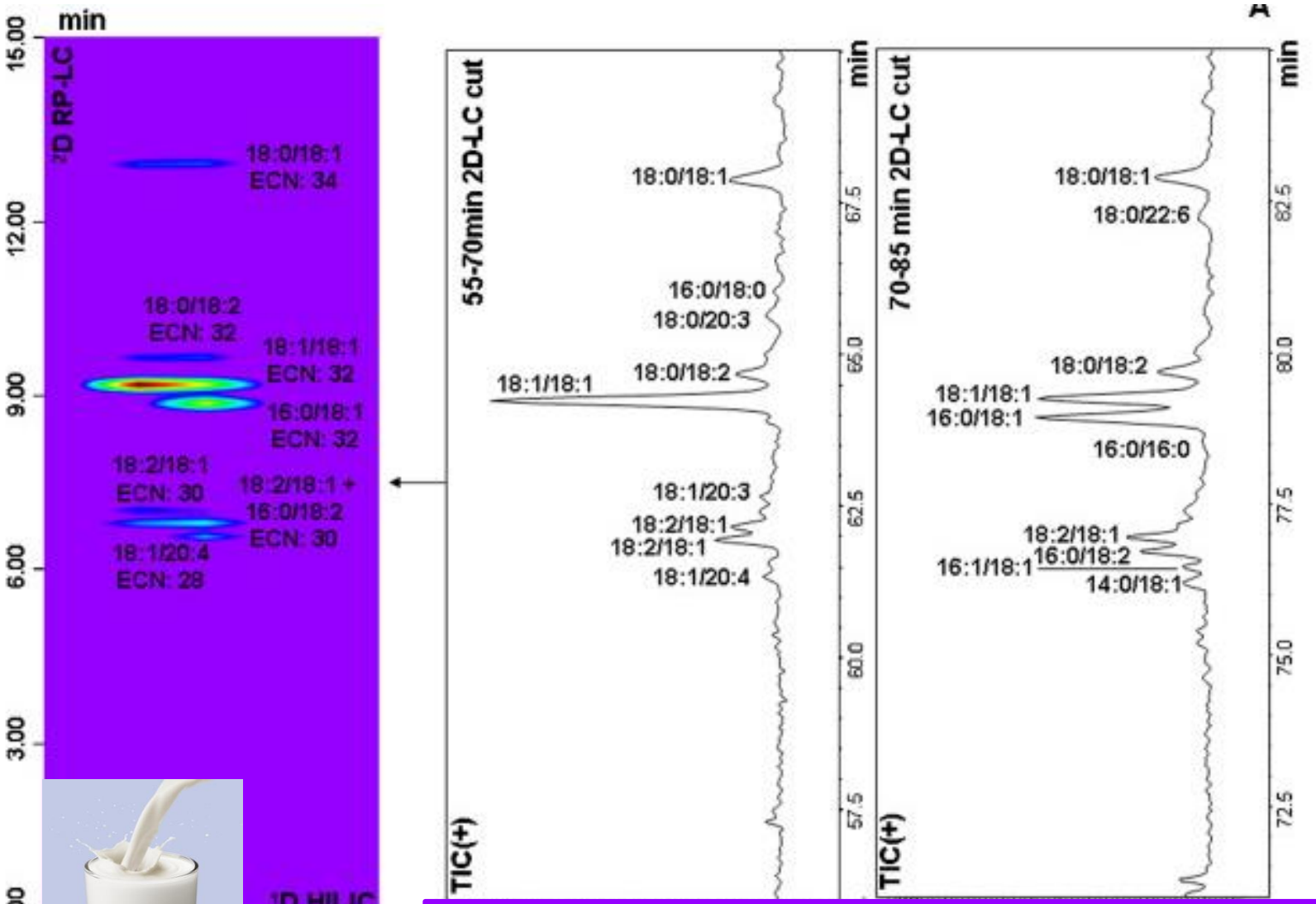
Stop-flow HILIC × RP-LC for PC molecular species in cow's milk

Valve configuration



Paola Dugo et al., J. Chromatogr. A 1278 (2013) 46-53

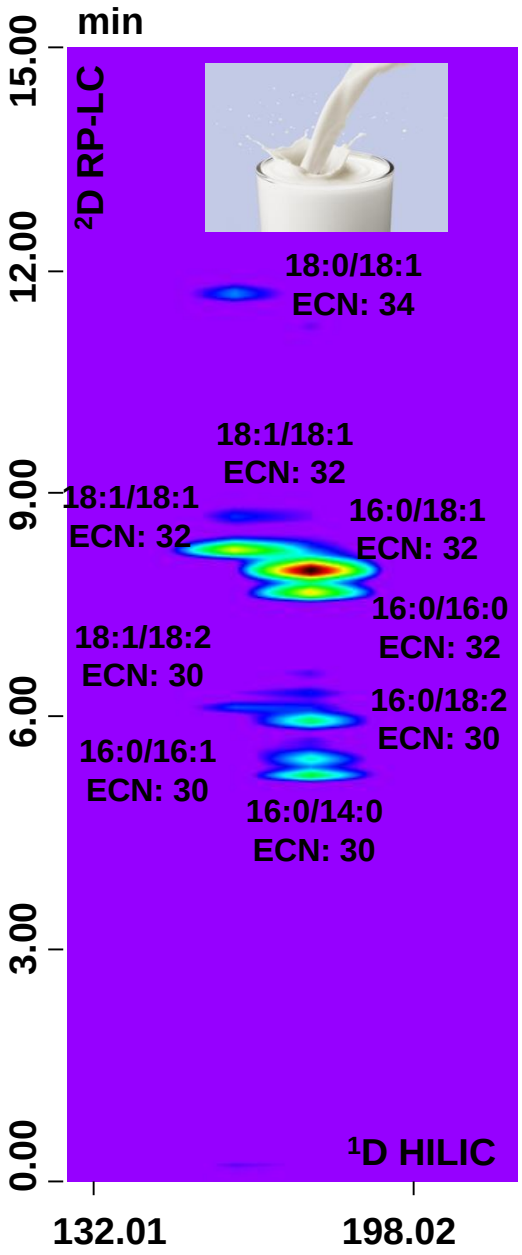
Stop-flow HILIC × RP-LC for PE molecular species in cow's milk

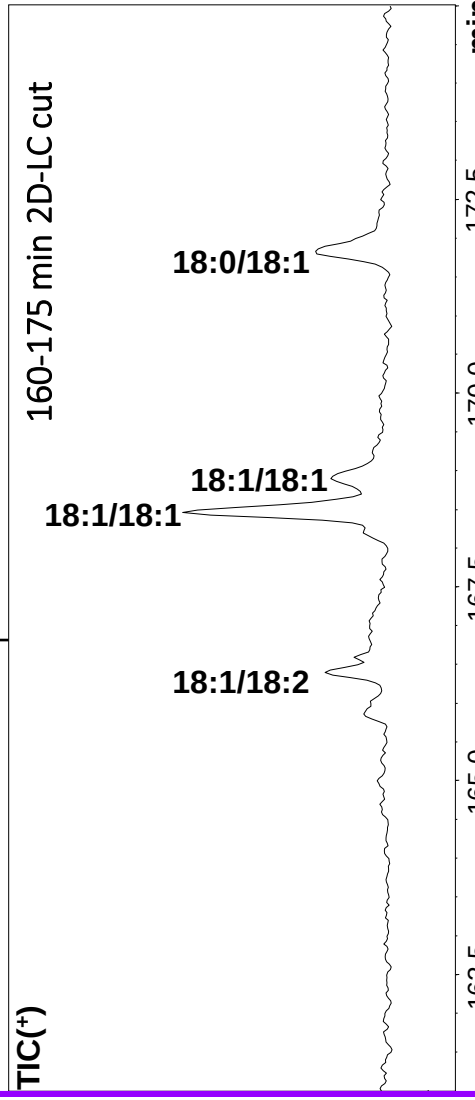


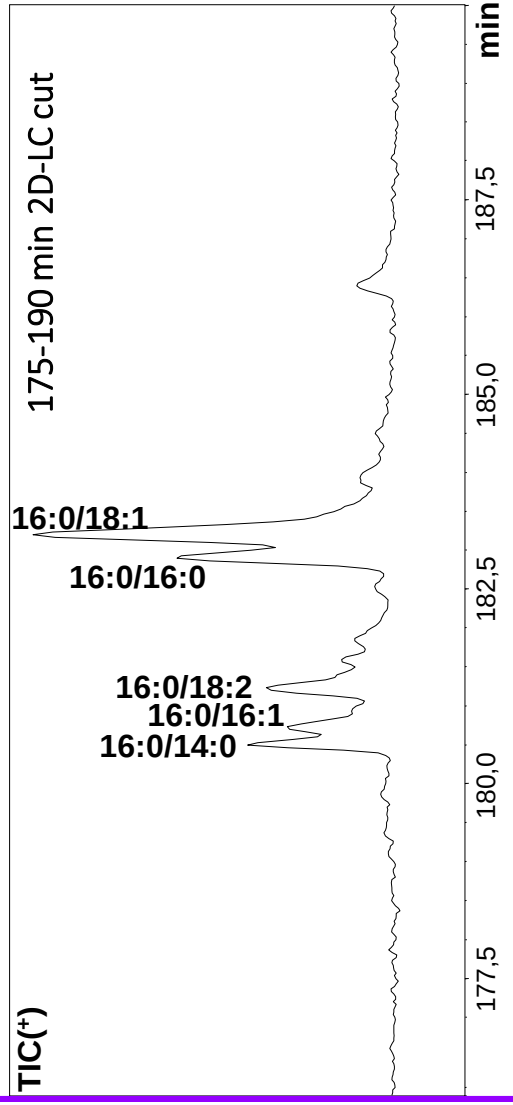
Enlargement of the HILIC × RPLC–ESI-MS contour plot for separation of the PEs molecular species, along with the corresponding ^{2}D raw data of contained in the cow's milk sample.

Paola Dugo et al., J. Chromatogr. A 1278 (2013)

Stop-flow HILIC × RP-LC for PC molecular species in cow's milk



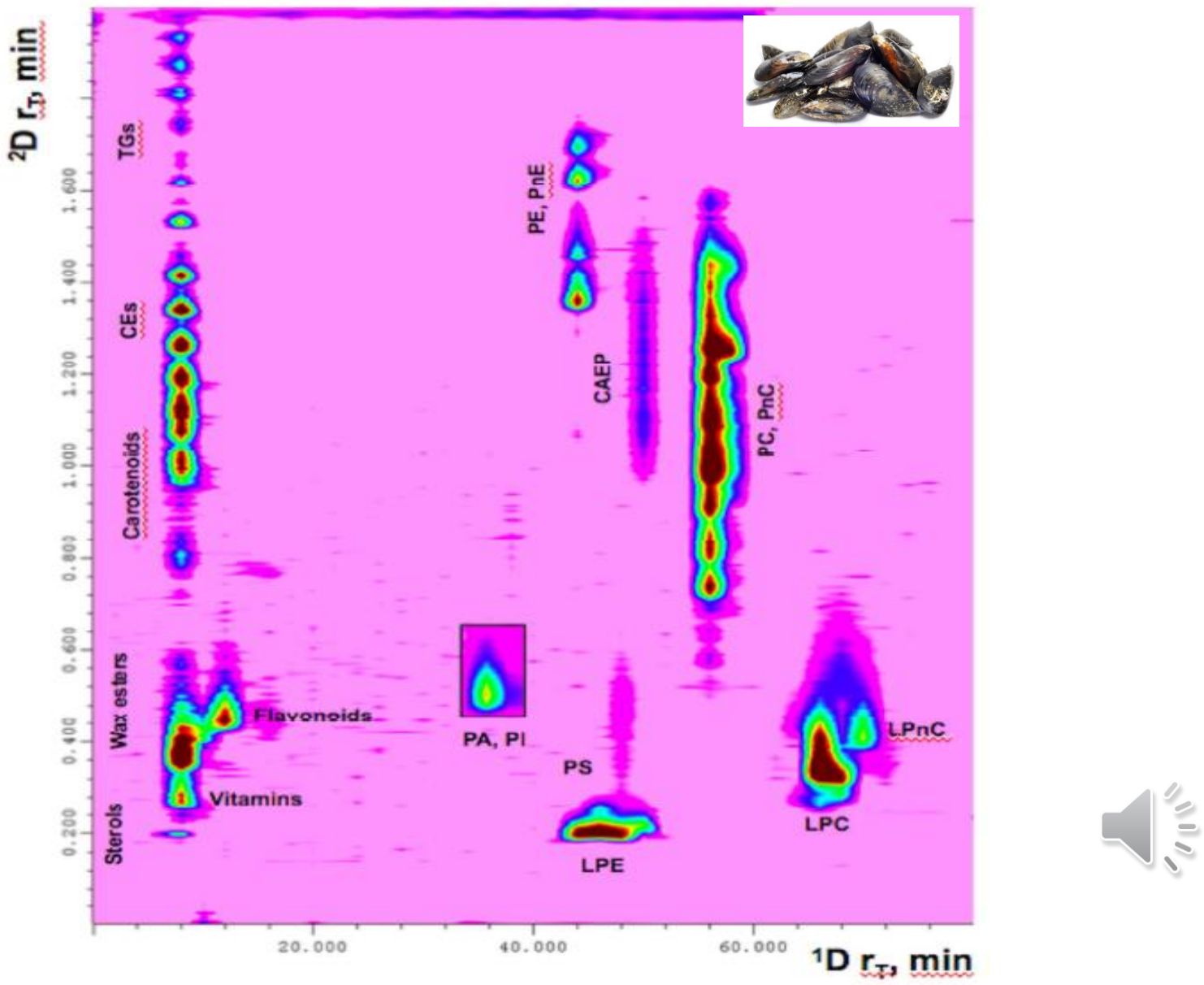




Enlargement of the HILIC × RPLC–ESI-MS contour plot for separation of the PCs molecular species, along with the corresponding ^{2}D raw data of contained in the cow's milk sample.

Paola Dugo et al., J. Chromatogr. A 1278 (2013)

Lipidomics analylis by HILIC × RP-LC-MS of Mussel Lipids



P. Donato et al., Anal. Bioanal. Chem., in press 2018 DOI 10.1007/s00216-018-1045-3

RP-LC × RP-LC applications

Analysis of peptides by LC × LC

- Different strategies have been used by researchers in designing LC × LC systems for proteomic studies.

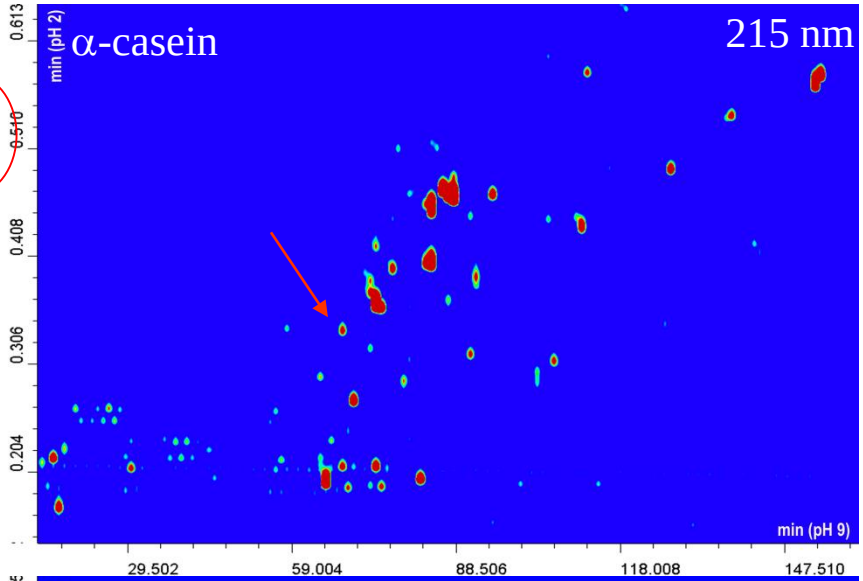
- A first distinction can be made between the directly coupled-column methods and column switching methods.

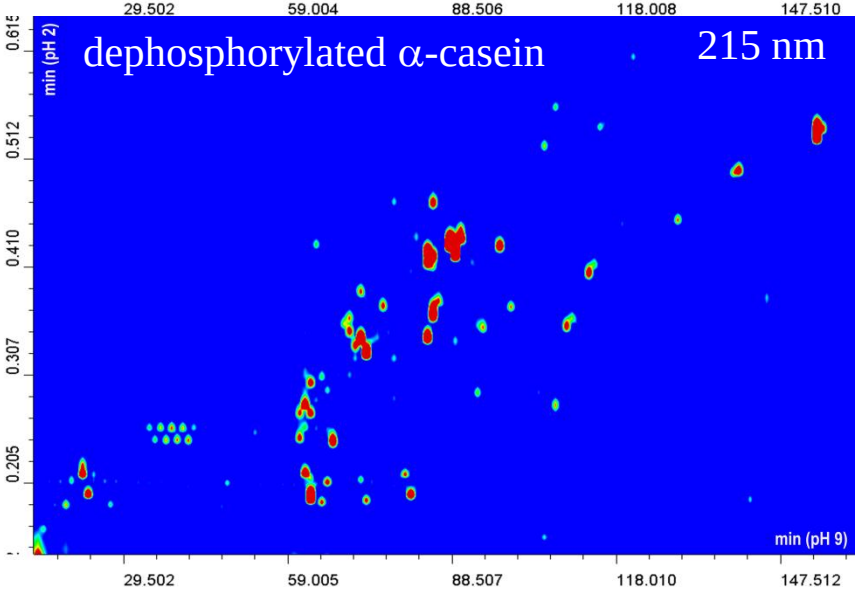
I. First approach: two columns with orthogonal separation selectivities, typically based on strong cation-exchange (SCX) and reversed-phase (RP) materials, are packed in tandem into a single capillary (MudPIT)

II. Second approach: cation-exchange column in the first dimension and C18 column in the second dimension. Pitfall: Desalting step prior to MS detection is needed.

III. Third approach: two C18 columns in both dimensions. Orthogonality is ensured by the use of different pH values in the first and second dimension.

RP-LC × RP-LC of tryptic digested proteins





pH 2

Four serially coupled C18 partially porous

pH 9

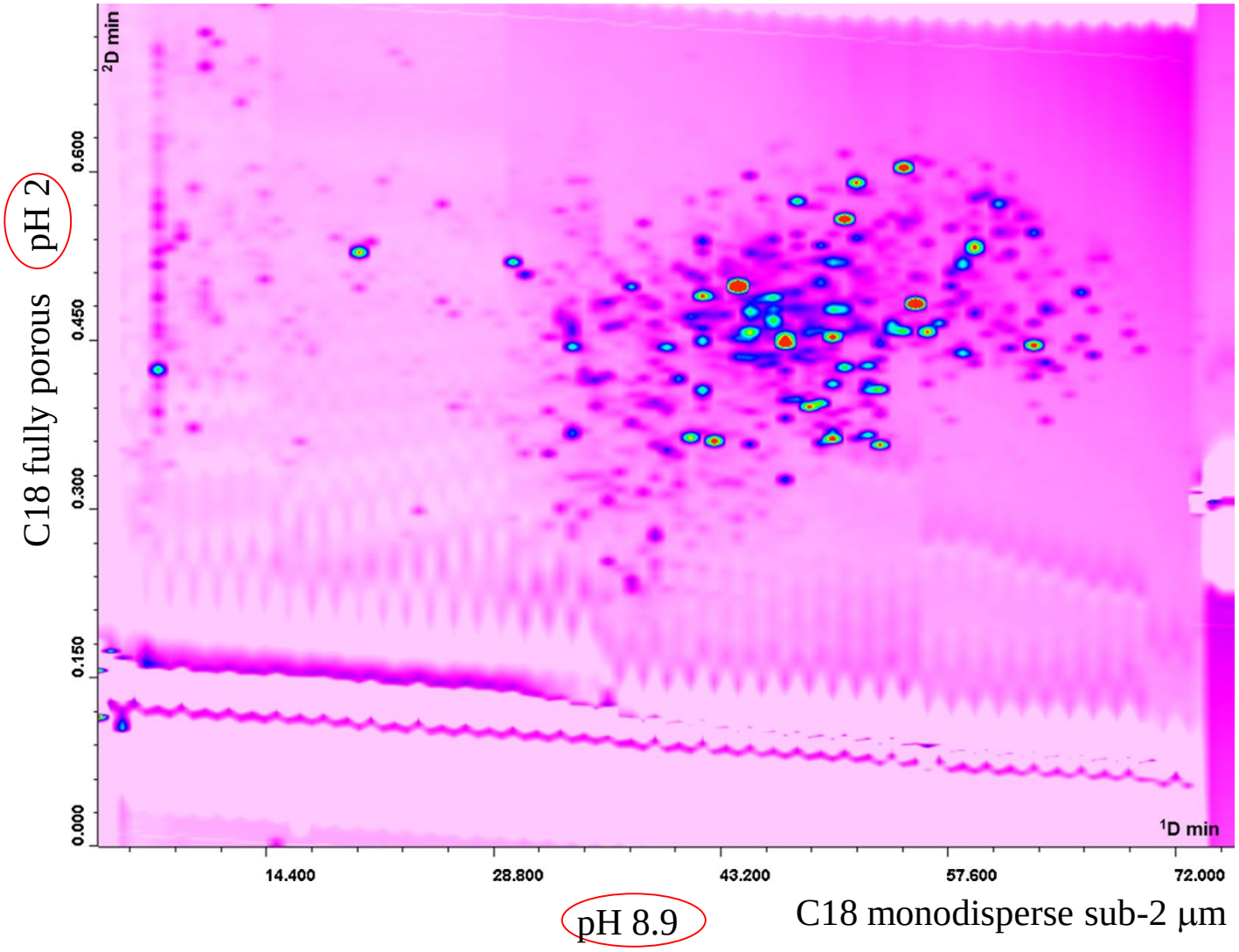
C18 partially porous

A phosphor peptide is indicated by the arrow in the upper plot

A theoretical peak capacity of ca. 8500 was calculated for the setup!

Paola Donato et al., Anal. Chem. 83 (2011) 2485-2491

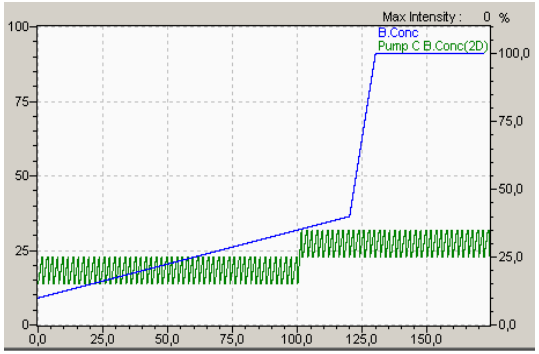
RP-LC × RP-LC of milk peptides after expiration date



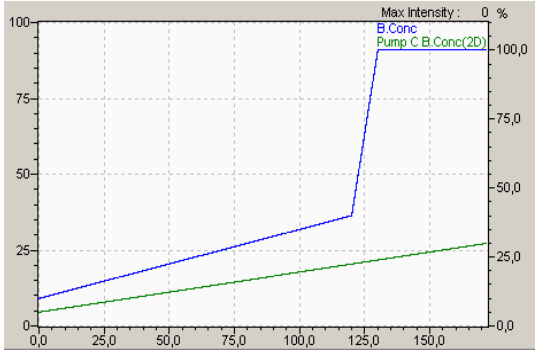
Eduardo Sommella et al., J. Chromatogr. A 1375 (2015) 54-61

Strategies for improving RP × RP separations in ^{2}D

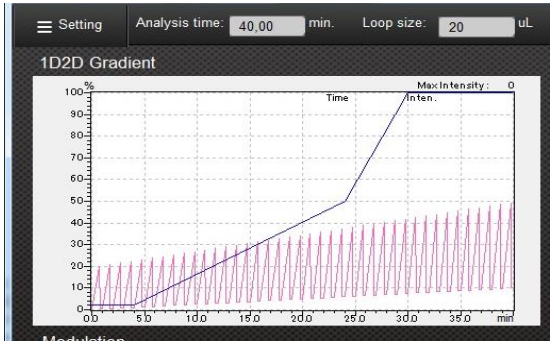
"**Segmented gradient**" (at least two generic and steep mobile phase range in each repeated ^{2}D run)
Advantages:
• Provides significant bandwidth suppression effects.
•The probability of "wrap-around" effects is reduced.



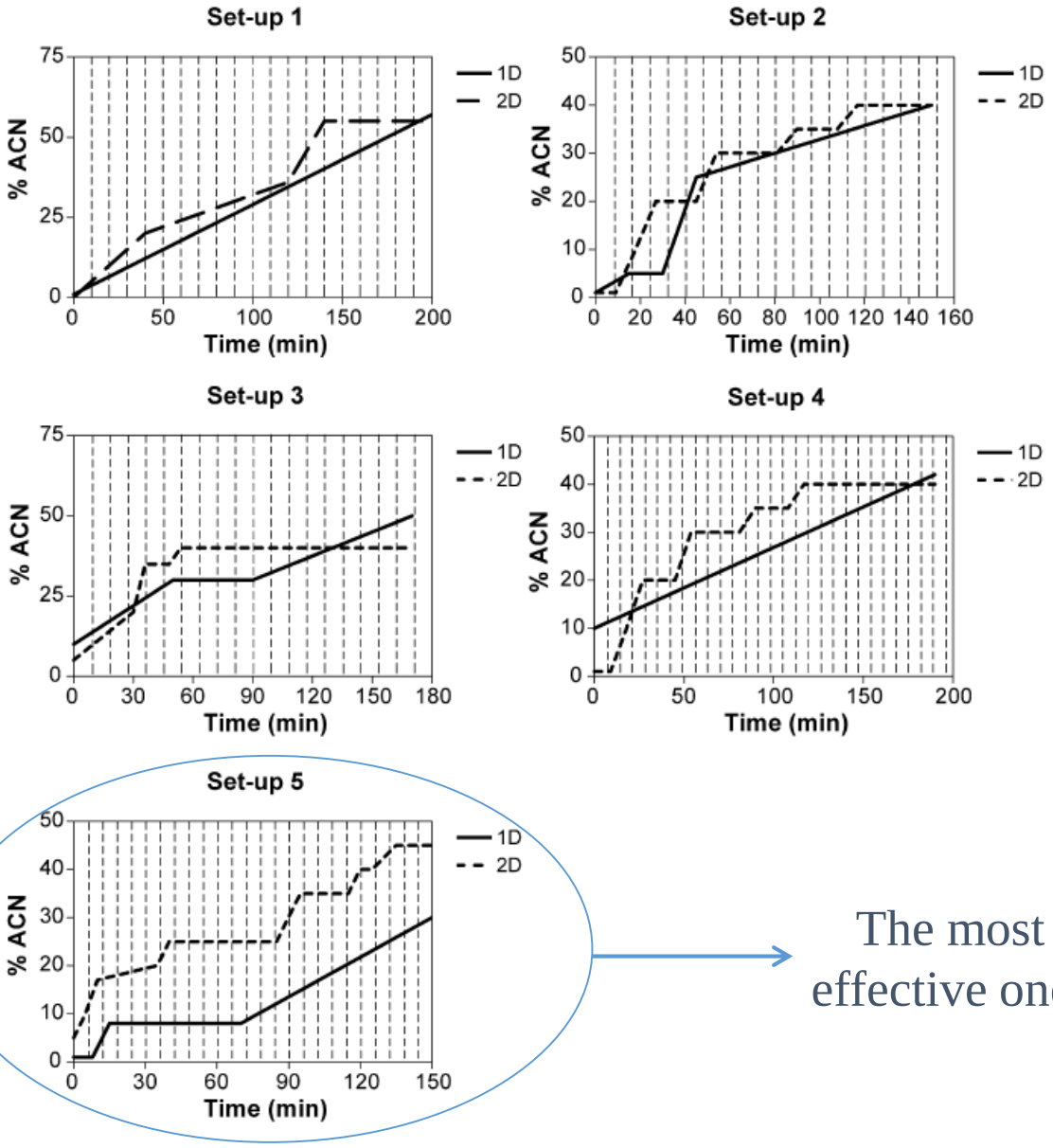
"**Parallel gradient**" (only one single ^{2}D gradient)
Advantages:
•Longer ^{2}D elution time (post-gradient equilibration is not necessary)
• It can be employed in highly correlated RP-LC × RP-LC systems.



"Shift gradient" (continuous change of the gradient)
Advantages:
•Reduces the likelihood of "wrap-around" phenomena.
•As for the "segmented gradient" the concentration of the organic solvent can be adjusted to suit the sample retention.



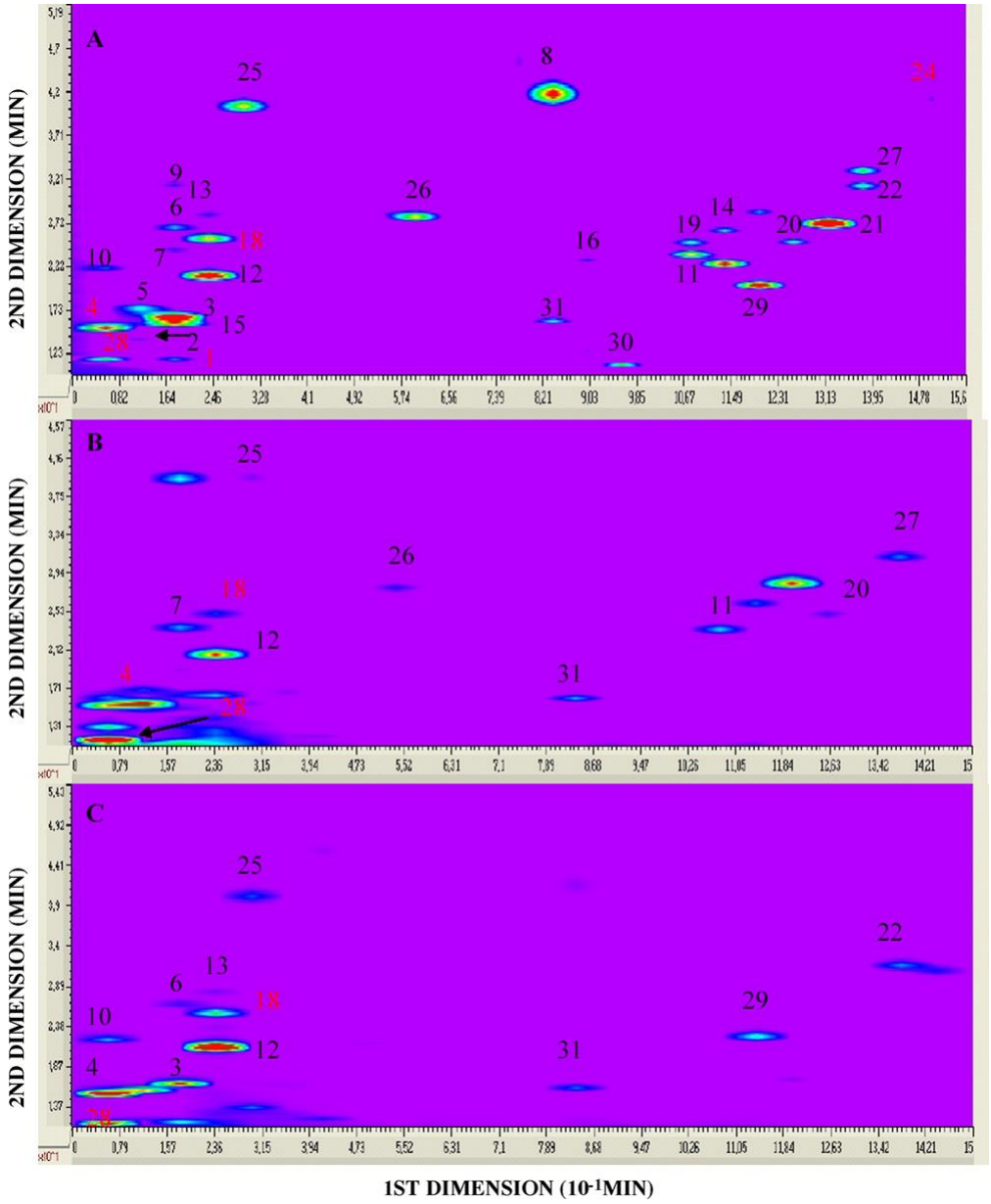
Comparison of different “Parallel gradient” in LC × LC



The most effective one!

Pavel Jandera and co-workers, J. Chromatogr. A 1149 (2007) 73-87

First application: RP-LC × RP-LC separation of polyphenolic antioxidants



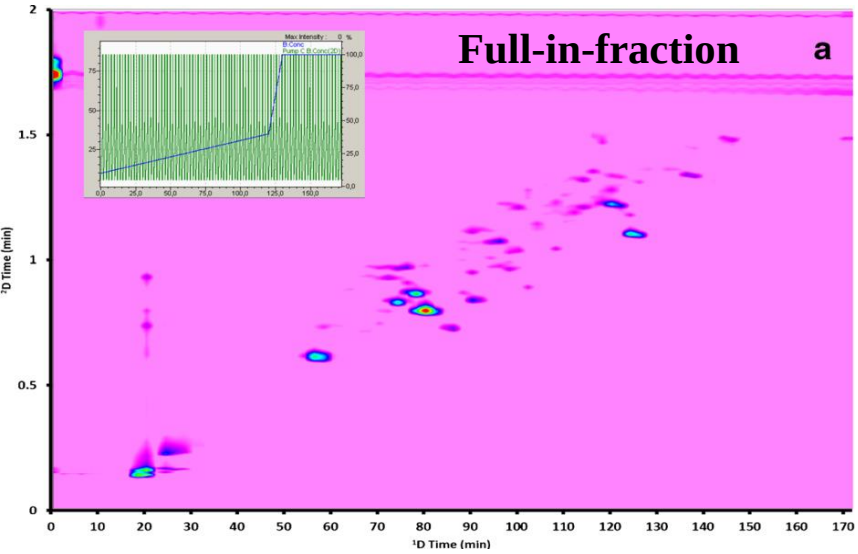
Phenolic and flavone standards

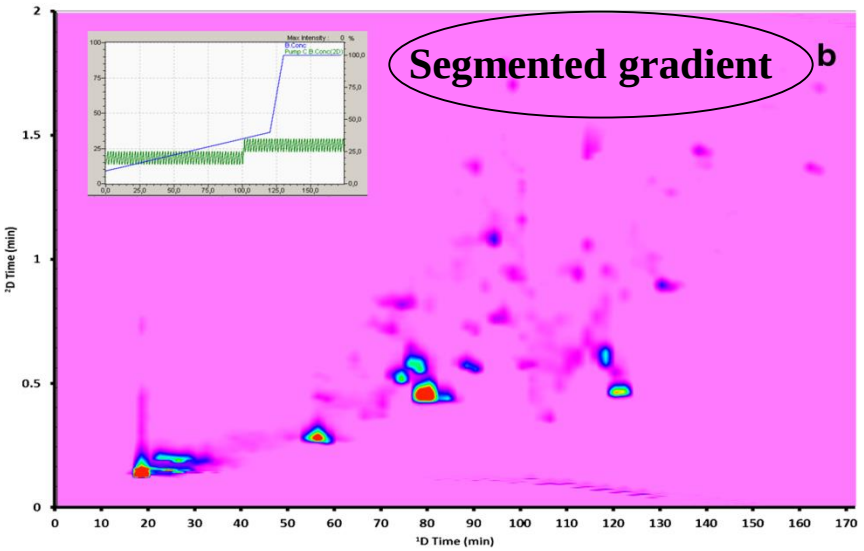
Beer sample

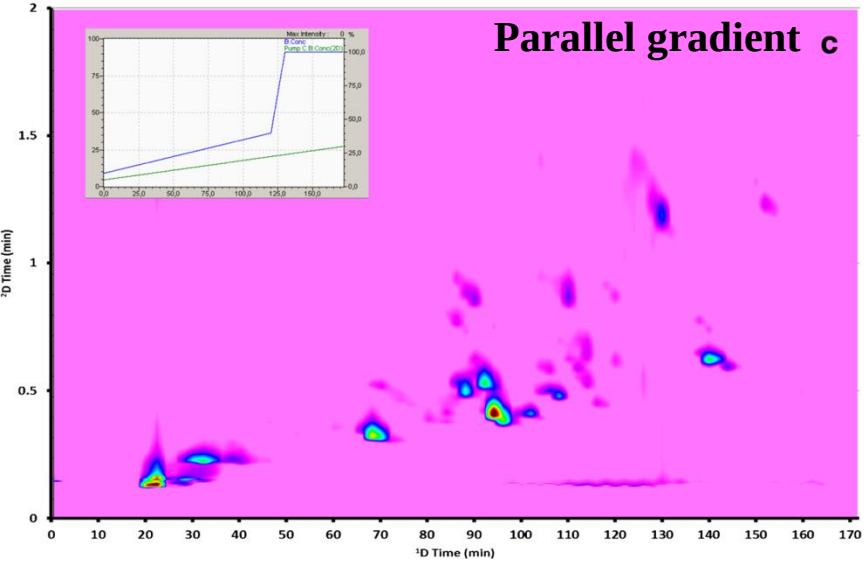
Merlot red wine

Pavel Jandera and co-workers, J. Chromatogr. A 1149 (2007) 73-87

Comparison of RP-LC × RP-LC set-ups for a sugarcane leaf extract





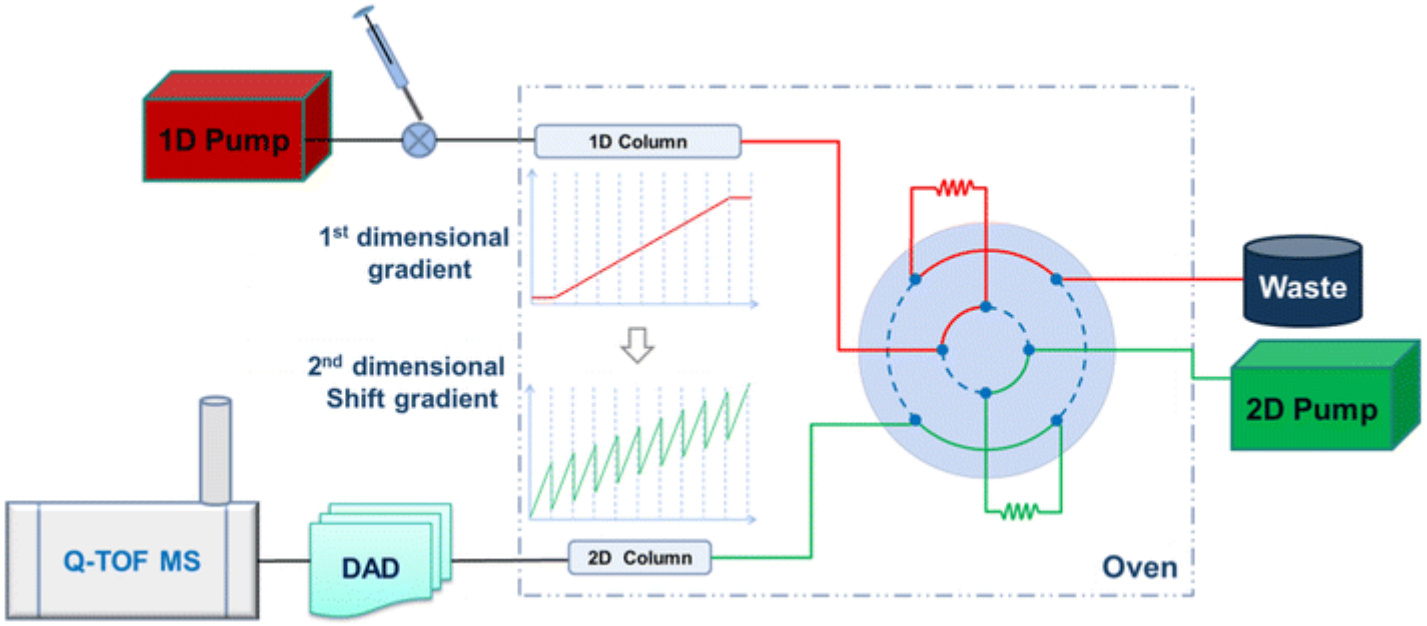


The most effective one, yielding the highest peak capacity, allowing to detect 38 polyphenolic compounds as flavonoid O-glycosides and C-glycosides

Gabriel Mazzi Leme et al., Anal. Bional. Chem 406 (2014) 4315-4324

First application: RP-LC × RP-LC with a "shifted gradient" for analysis of *Hedyotis diffusa*

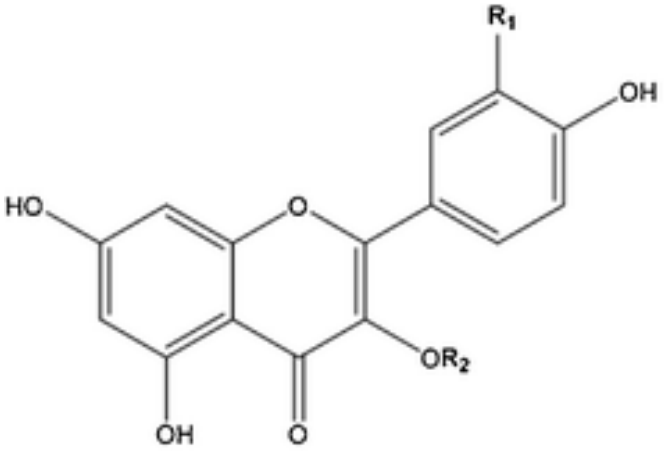
SCHEME OF RP × RP SYSTEM COUPLED WITH DAD AND QTOF-MS



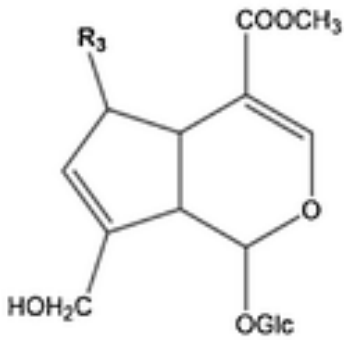
Duxin Li & Oliver J. Schmitz, Anal. Bioanal. Chem. 407 (2015) 231-240

First application: RP-LC × RP-LC with a "shifted gradient" for analysis of *Hedyotis diffusa*

TARGET COMPOUND CLASSES ANALYZED BY RP × RP-DAD/MS



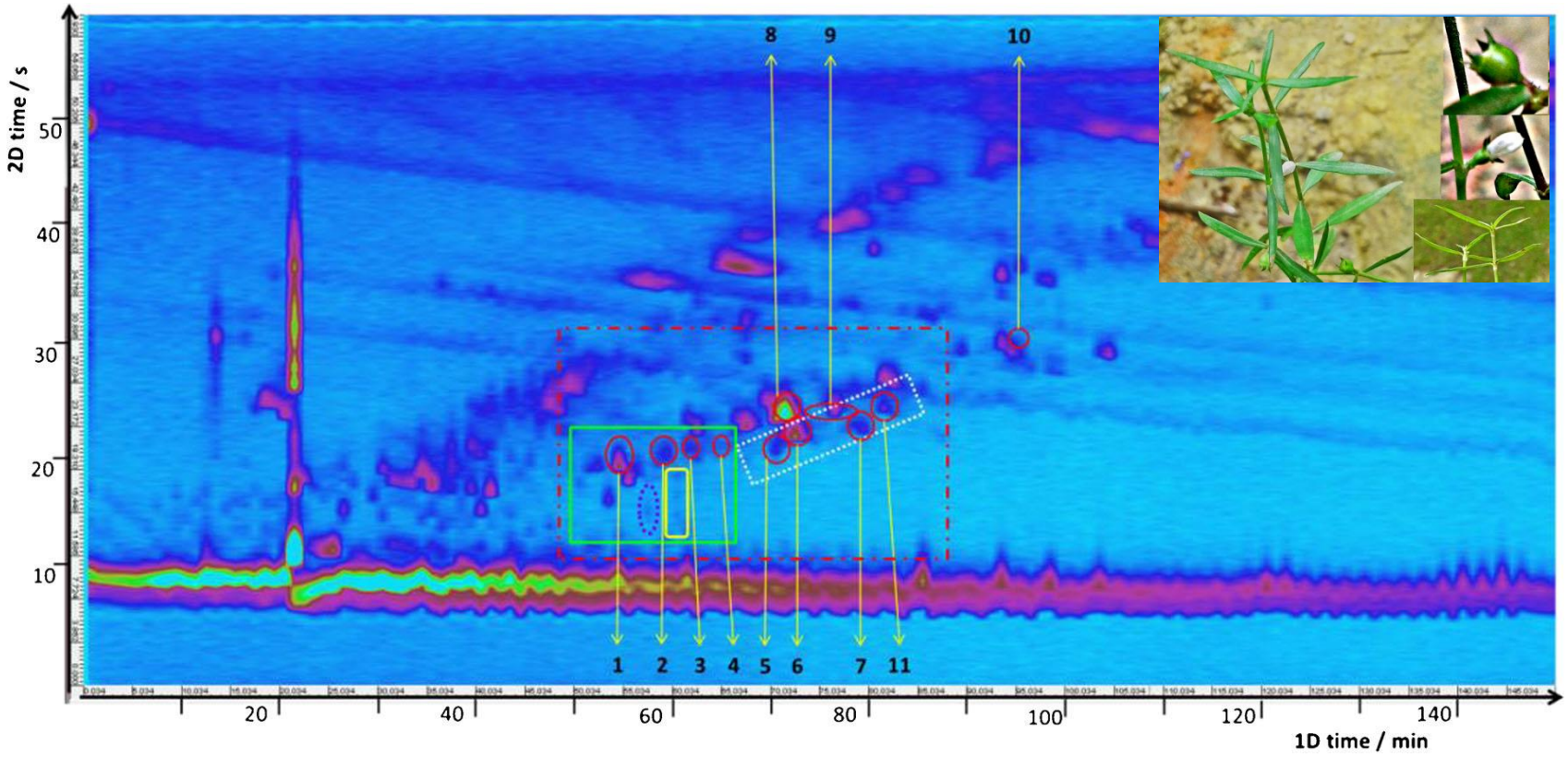
Flavonol glycosides



Iridoid glycosides

Molecular structure of flavonoids (**1**–**7**, **20**) and iridoid glycosides (**8**–**10**); compounds: R_1 = OH, R_2 = Glc - Glc (**1**); R_1 = OH, R_2 = Sambubioside (**2**); R_1 = OH, R_2 = Rutinose (**3**); R_1 = OH, R_2 = Glc (**4**); R_1 = OH, R2 = Glc - Gal-*E*-sinapoyl (**5**); R1 = OH, R2 = Glc - Glc-*E*-feruloyl (**6**); R1 = H, R2 = Glc – Gal - *E*-feruloyl (**7**); R3 = 6-*O*-p-coumaroyl (**8**); R3 = 6-*O*-feruloyl (**9**); R3 = 6-*O*-*p*-methoxycinnamoyl (**10**); R_1 = H, R_2 = Glc - Gal (**20**)

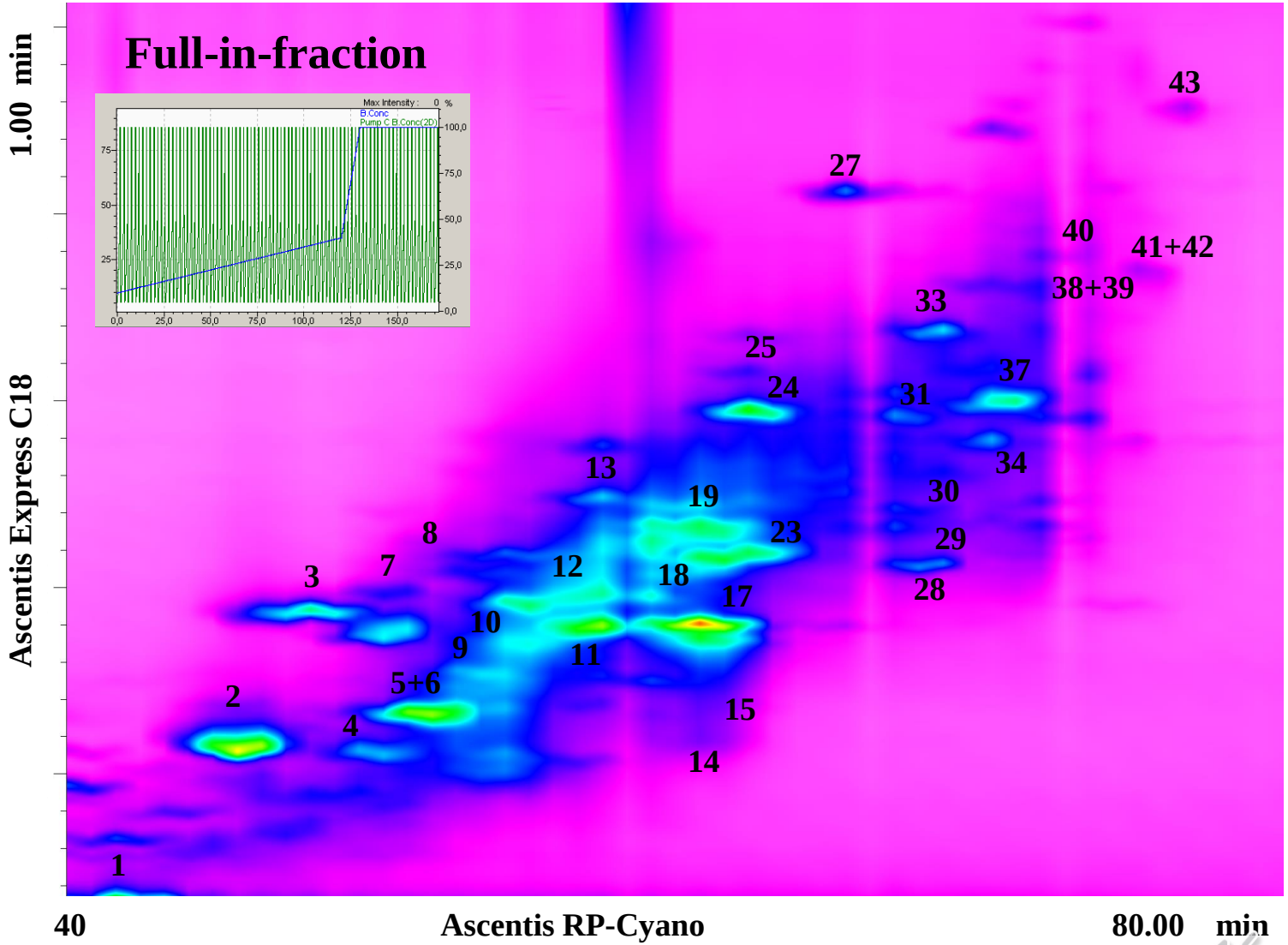
First application: RP-LC × RP-LC with a "shifted gradient" for analysis of *Hedyotis diffusa*



A clear classification of flavonol glycosides (FGs), acylated FGs, and iridoid glycosides (IGs) was achieved

Duxin Li & Oliver J. Schmitz, Anal. Bioanal. Chem. 407 (2015) 231-240

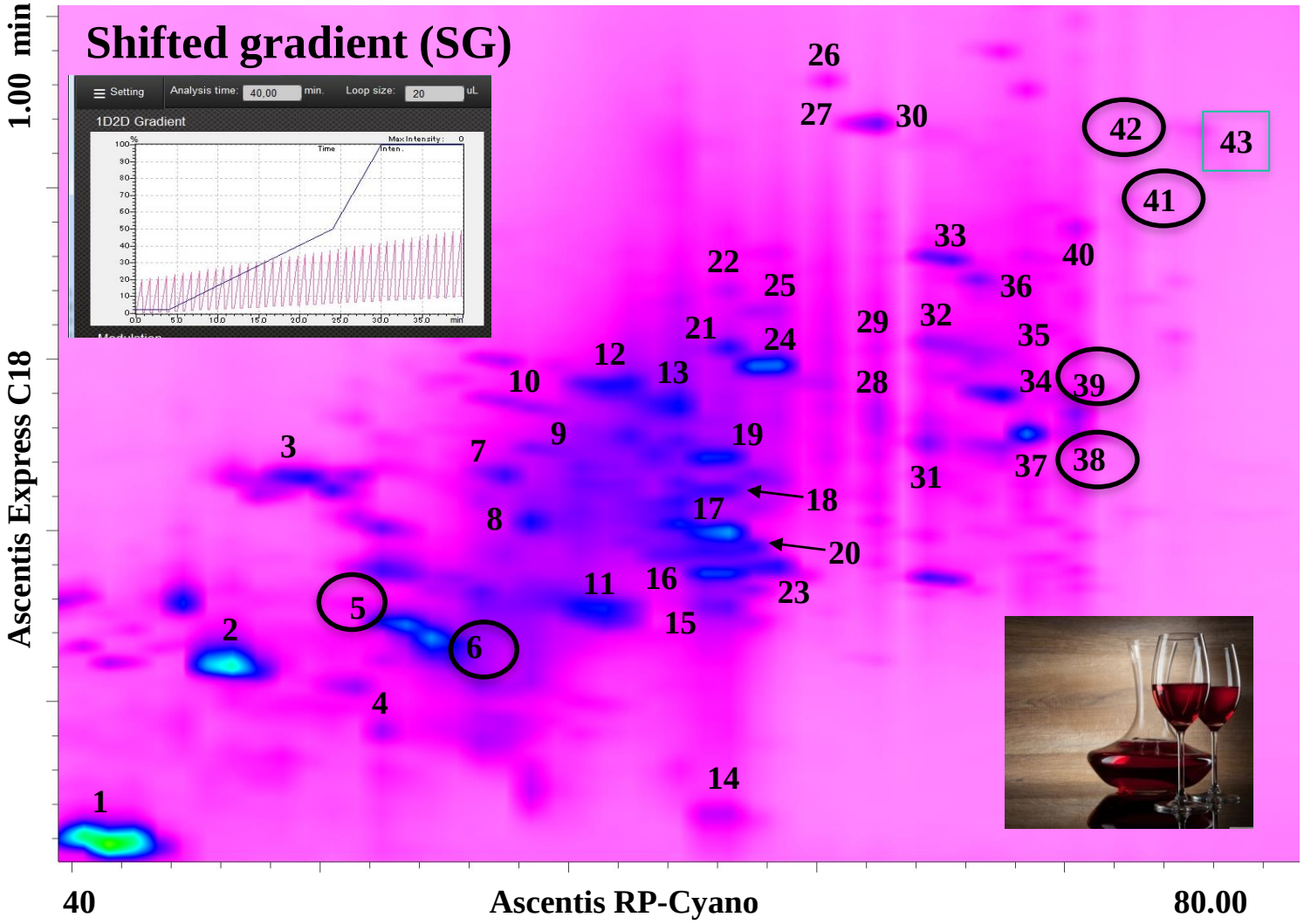
RP-LC × RP-LC Plot of a red wine: Full-in-fraction *vs* Shifted gradient



A total of 35 different polyphenolic compounds were positively identified

Paola Donato et al., J. Chromatogr. A 1458 (2016) 54-62.

RP-LC × RP-LC Plot of a red wine: Full-in-fraction *vs* Shifted gradient



A total of **43** compounds were identified!!!

Paola Donato et al., J. Chromatogr. A 1458 (2016) 54-62.

Evaluation of the system performance

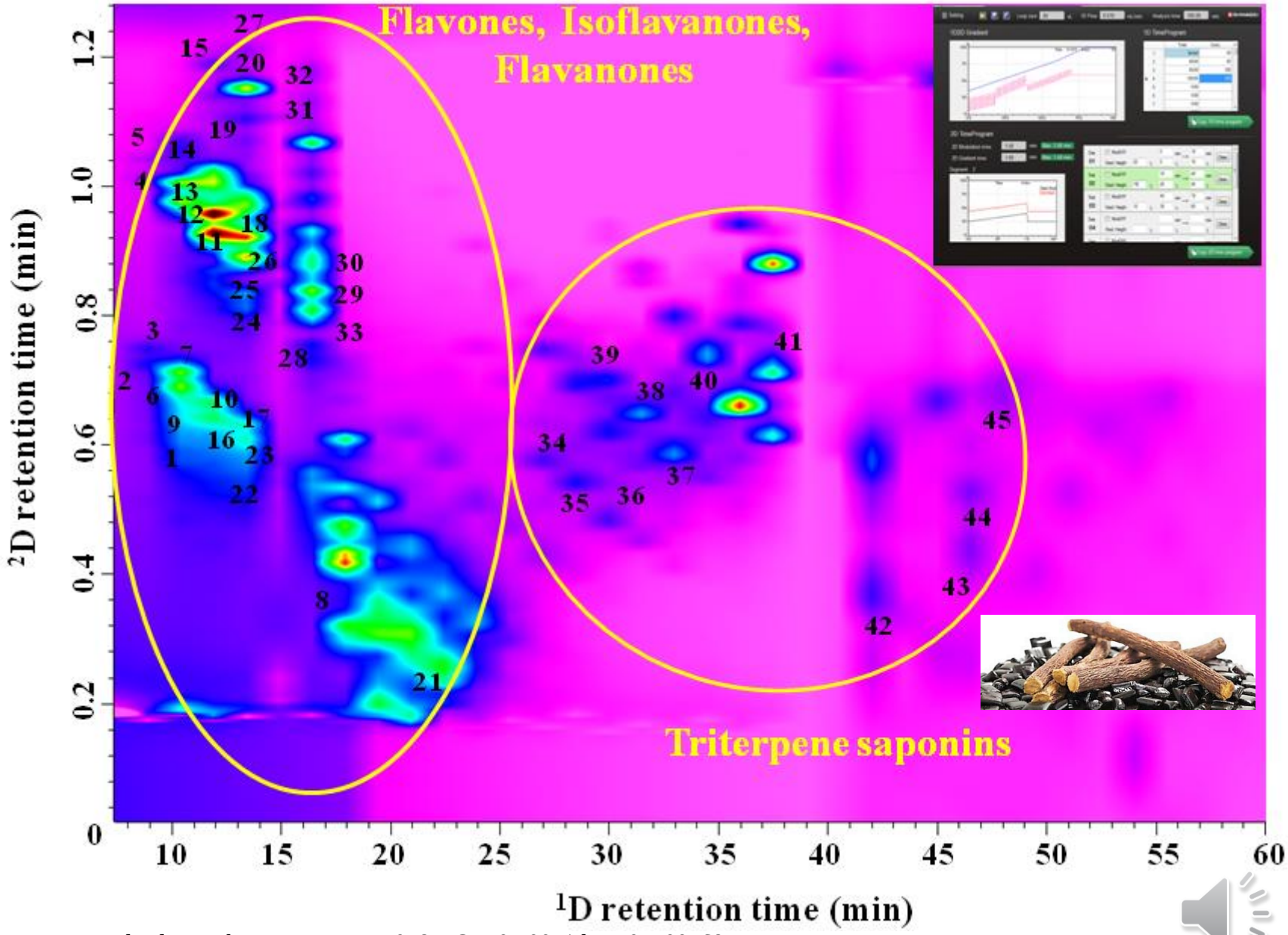
		FULL in FRACTION	SHIFTED GRADIENT
^{1}D	Column	250x1.0 mm, 5.0 μm	250x1.0 mm, 5.0 μm
	Gradient	100 min	100 min
	n_C	15	15
^{2}D	Column	30x4.6 mm, 2.7 μm	30x4.6 mm, 2.7 μm
	Gradient	60 s	60 s
	n_C	46	38
2D	Mod. time	1 min	1 min
No. of ^{1}D transferred fractions		100	100
Theoretical[a] 2Dn_C		690	570
Practical[b] 2Dn_C		75	216

[a] $^1n_C \times {}^2n_C$

[b] *corrected for under-sampling and orthogonality*

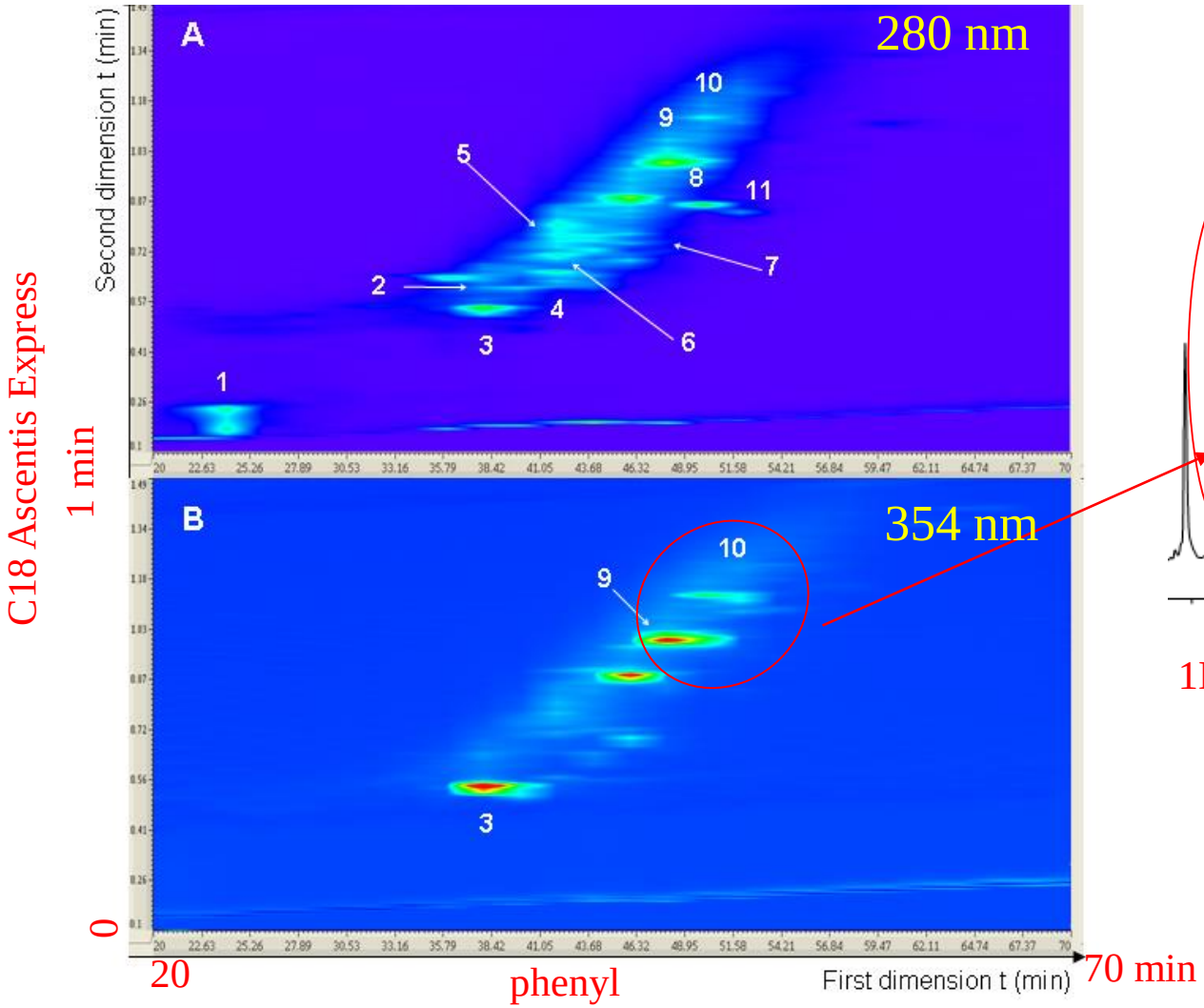
Paola Donato et al., J. Chromatogr. A 1458 (2016) 54-62.

^{2}D-Multi-segmented Shifted Gradient (MSG) RP-LC × RP-LC Plot of licorice



Yong Foo Wong et al. Electrophoresis in press 2018 DOI 10.1002/elps.201700469

Case of study: Quantification by RP-LC × RP-LC of polyphenols in Nero d'Avola red wine



1: gallic acid; 2; procyanidin; 3: caftaric acid; 5: epicatechin; 6: catechin; 7: caffeic acid; 8: ethylgallate; 9: rutin; 10: Isoquercitrin; 11; p-coumaric acid.

Paola Dugo et al. J. Chromatogr. A 1216 (2009) 7483-7487

Parameters of linear regression and retention times: (t_R)*, LOD, LOQ for the studied compounds by RP-LC-PDA…

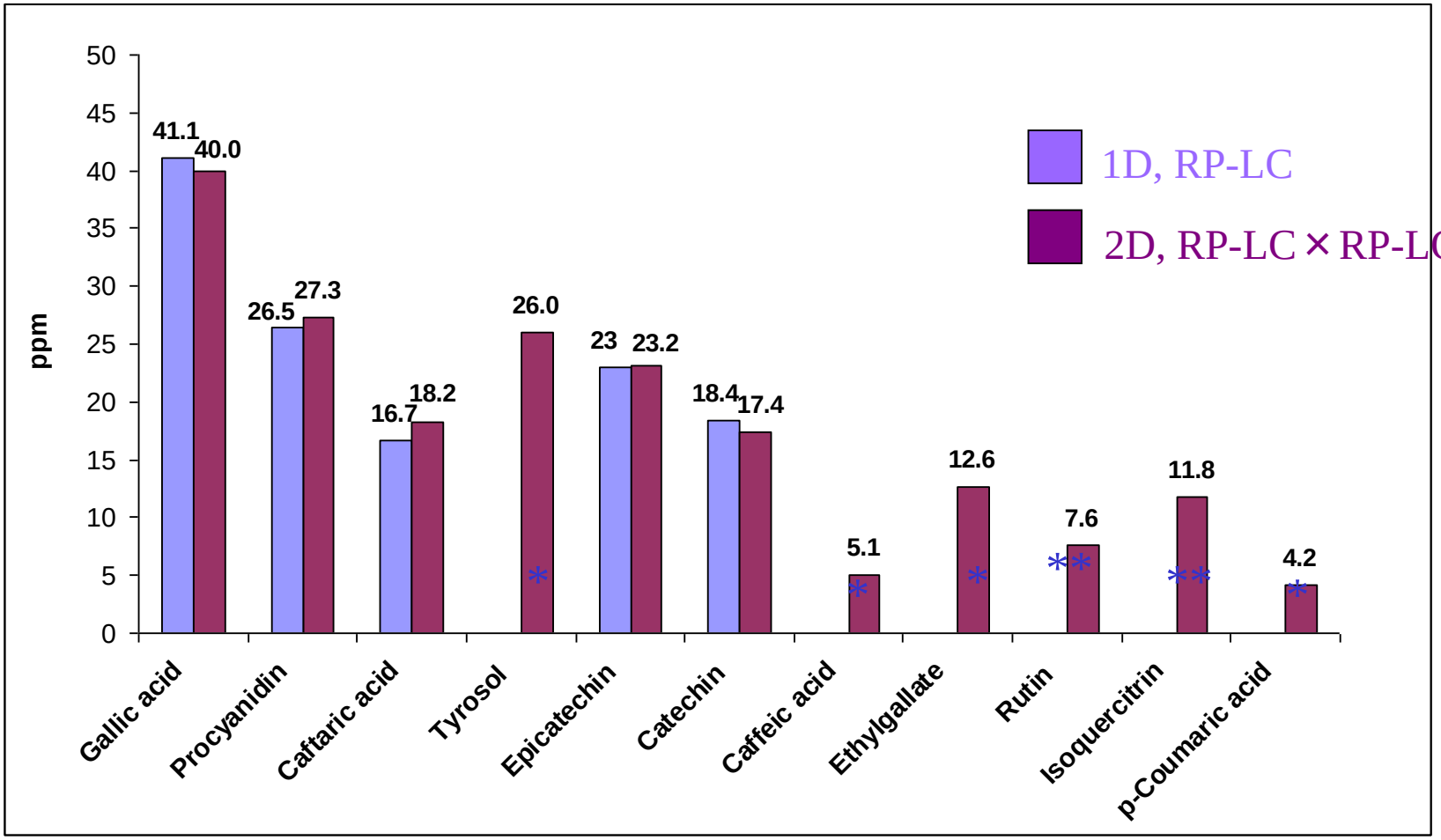
Trivial name	UV nm	t_R (min) ± RSD (%)	Regression equation	LOD (μg/mL)	LOQ (μg/mL)
Gallic acid Benzoic acid - like	270	9.36 ± 1.21	y =77099x-10301 R^2 = 0.9993	0.03	0.11
Ethylgallate Benzoic acid ethyl ester – like	270	36.71 ± 0.26	y = 149614x + 101394 R^2 = 0.9994	0.02	0.05
Tyrosol Phenyl-ethyl alcohol – like	278	28.60 ± 0.29	y =16150x+15453 R^2 = 0.9991	0.13	0.45
Epicatechin Flavan-3-ol - like	278	34.72 ± 0.18	y = 20025x + 6789 R^2 = 0.9991	0.16	0.52
Caffeic acid Cinnamic acid - like	323	32.66 ± 0.24	y =135327x+72215 R^2 = 0.9995	0.02	0.05
Rutin Flavonol-glycoside - like	354	38.58 ± 0.14	y = 44317x + 36978 R^2 = 0.9996	0.03	0.10

…and by RP-LC × RP-LC-PDA

Trivial name	t_R (min)* ± RSD (%)	Regression Equation	R^2	LOD (μg/mL)	LOQ (μg/mL)
Gallic acid	0.25 ± 4.51	y = 203438x – 669334	0.9988	0.09	0.30
Ethylgallate	0.89 ± 2.47	y = 294735x – 337701	0.9998	0.25	0.85
Tyrosol	0.64 ± 1.12	y = 50848x – 35086	0.9999	0.95	3.17
Epicatechin	0.67 ± 0.96	y = 60965x – 259742	0.9895	0.79	2.63
Caffeic acid	0.72 ± 1.27	y = 594002x – 1350198	0.9900	0.10	0.33
Rutin	0.99 ± 1.80	y = 77958x – 172259	0.9930	0.30	1.01

* the retention times (t_R) are the mean of twenty-four replicates corresponding to the second dimension.

Comparative quantitative results (mg/L) for polyphenolic compounds by RP-LC and RP × RP-LC-PDA in Nero d'Avola red wine



* Not separated from the rest of the matrix

**Coeluted in mono-dimensional analysis

Paola Dugo et al. J. Chromatogr. A 1216 (2009) 7483-7487

Concluding Remarks

- ✓ Comprehensive chromatography is a powerful separation tool in the field of food product analysis, allowing to separate, identify and quantify compounds with very similar molecular structures.
- ✓ Commercial instrumentations and softwares make the technique easy to use.
- ✓ Use of different ^{2}D elution strategies in LC × LC for plant derived-extracts is beneficial for elucidating different phytochemical classes.
- ✓ Employment of multi-segmented shifted gradients in ^{2}D represents a powerful strategy for improving the metabolite compound coverage, maximizing separation power.
- ✓ The use of combined analytical tools can help in the characterization of complex food samples, giving information on the presence of components with possible biological activity, target components used to assess origin, authenticity and quality.