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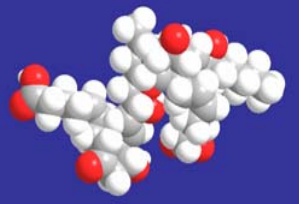
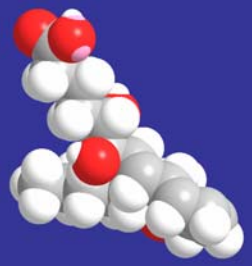
LIPID MAPS Lipidomics Workshop

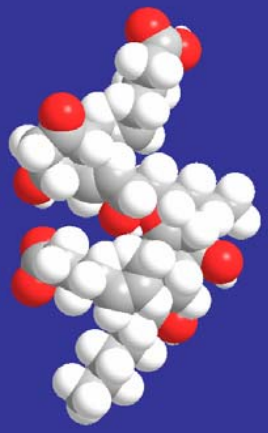
April 28, 2007





Eicosanoids

Richard Harkewicz



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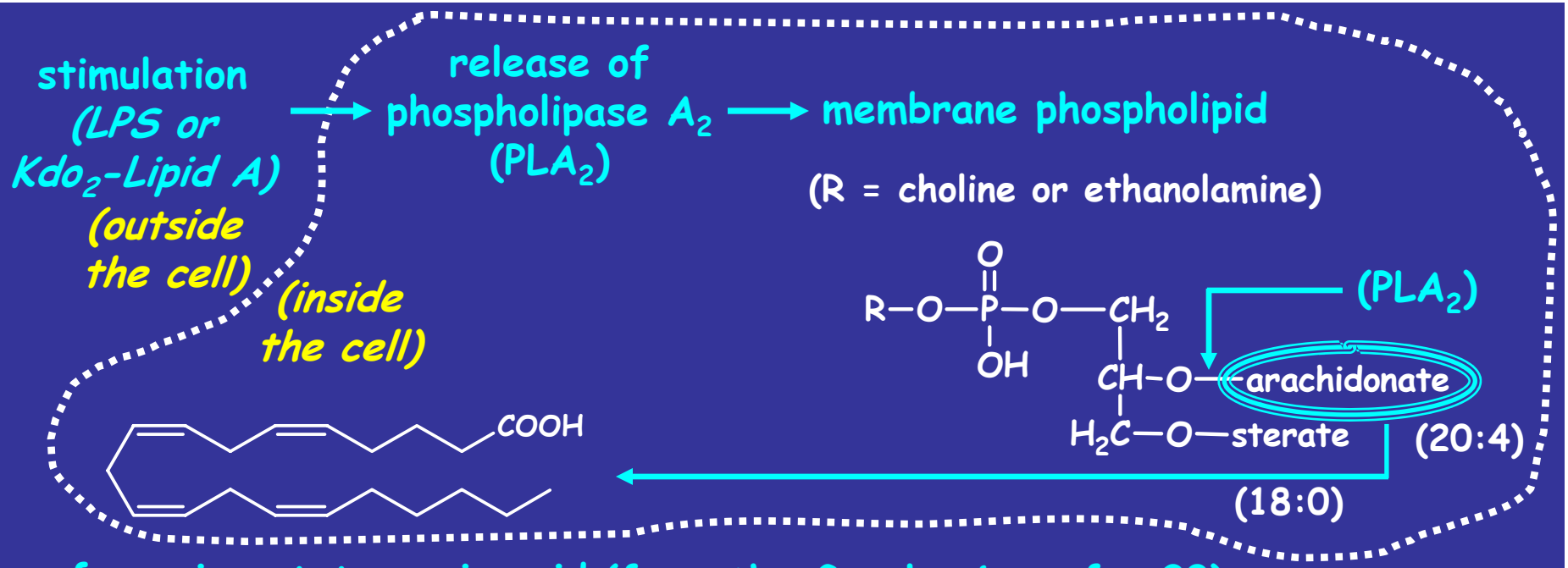
Outline

- Brief description of eicosanoids
- Sample preparation/extraction
- Analytical methodology: LC-MS
- Library of eicosanoid standards
- Chiral chromatography – *enzymatic or nonenzymatic?*
- DIMPLES/MS: A stable isotope substrate labeling strategy enabling the search for novel eicosanoids
- Comparison of LIPID MAPS eicosanoid approach with others in literature
- Future plans

brief description of eicosanoids (depicted on next slide)

When cells are stimulated, for example by the endotoxin LPS or Kdo_2-Lipid A, this initiates a cascade of events which involves the release *within the cell* of the enzyme phospholipase A_2 (PLA_2). PLA_2 then acts on membrane phospholipids which contain a polar phosphoryl head group and 2 nonpolar fatty acid side chains. Most commonly, the sn1 position contains a saturated fatty acid such as steric acid and the sn2 position an unsaturated fatty acid such as arachidonic acid.

The PLA_2 hydrolytically excises the sn2 position fatty acid making it freely available within the cell. NOTE that *free AA* is not normally available within the cell – it is esterified to the phospholipid and only made available in free-form through such actions as endotoxin stimulation.



free eicosatetraenoic acid (from the Greek *eicosa* for 20)

AKA arachidonic acid (20:4, n=6)

Eicosanoids *- powerful inflammatory mediators that are derived from arachidonic acid and act in autocrine and paracrine fashion (signal at or immediately adjacent to their site of synthesis)*

- Once they are made, they are quickly secreted from the cell
- Transcription factor: Can also enter cell's nucleus, binding and activating nuclear receptors

Sometimes referred to as *local hormones*

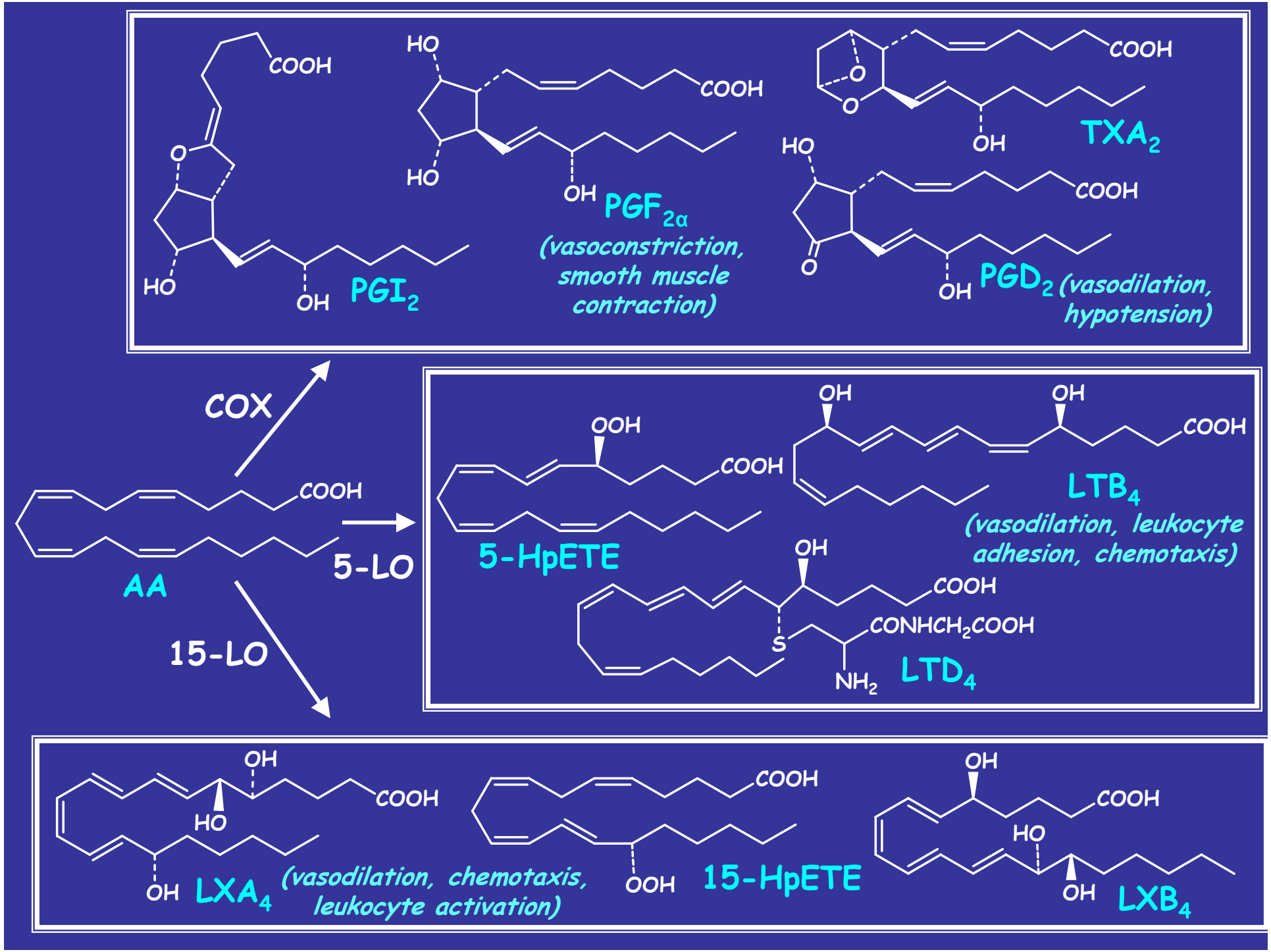
brief description of eicosanoids (cont.)
(depicted on next slide)

Within the cell, the free AA can serve as a substrate for the cyclooxygenase (COX) enzymes – and *with additional downstream enzymes* can lead to various prostaglandins – noted by their 5 carbon membered-ring.

The free AA can also serve as a substrate for the 5-lipoxygenase (5-LO) enzyme leading to, for example, the leukotrienes.

Additionally, the free AA can serve as a substrate for the 15-lipoxygenase (15-LO) enzyme, leading to the lipoxins.

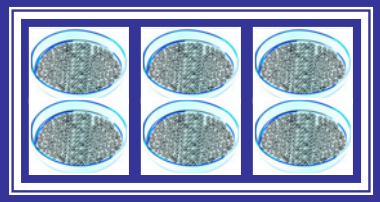
Please NOTE only a few examples of the *many* possible eicosanoid products are shown, along with a few of their physiological roles.


COOH
O
HO
OH
PGI2
HO
HO
COOH
OH
PGF2α
(vasoconstriction, smooth muscle contraction)
O
O
COOH
OH
TXA2
HO
COOH
O
OH
PGD2 (vasodilation, hypotension)
COX
COOH
AA
5-LO
15-LO
OOH
COOH
5-HpETE
OH
OH
COOH
LTB4
(vasodilation, leukocyte adhesion, chemotaxis)
OH
COOH
S
CONHCH2COOH
NH2
LTD4
OH
HO
COOH
OH
LXA4
(vasodilation, chemotaxis, leukocyte activation)
COOH
OOH
15-HpETE
OH
COOH
HO
OH
LXB4

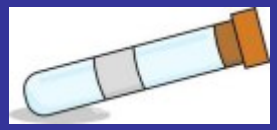
sample preparation and extraction

0 h → 24 h → 48 h → LC-MS analysis

- 0 h: Plate Cells
- 24 h: LPS *or* Kdo_2-Lipid A [100 ng/ml]
- 48 h: Collect Medium
- Solid phase extraction



1. Plate cells
   ↓ 24 h
2. Stimulate (LPS or Kdo)
   ↓ 24 h
3. Remove medium (≈2 ml per well) (≈2e6 cells per well)



1. Spike medium with deuterated internal standards (10 ng/100 µl)
2. Add EtOH to obtain 10% EtOH medium solution
3. Centrifuge 5 min @ 3000 rpm

1. Solid phase extraction (Phenomenex® Strata-X 8B-S100-UBJ)
2. Prewash SPE column with 2 ml MeOH then 2 ml H_2O
3. Apply sample to column then wash with 2 ml 10% MeOH
4. Elute eicosanoids with 1 ml MeOH
5. Dry under vacuum and resuspend in HPLC solvent A

analytical methodology: LC-MS (depicted on next slide)

The first structural characterization of eicosanoids occurred in the early 1960's and these studies depended heavily upon gas chromatography-mass spectrometry (GC-MS). GC-MS continued to play a very central role in eicosanoid analyses many years after.

More recent advances in electrospray ionization (ESI) mass spectrometry coupled to high performance liquid chromatography have offered extremely sensitive and quantitative assays for most of the eicosanoids *without the need for chemical derivatization* prior to analysis *as is required by GC-MS techniques*.

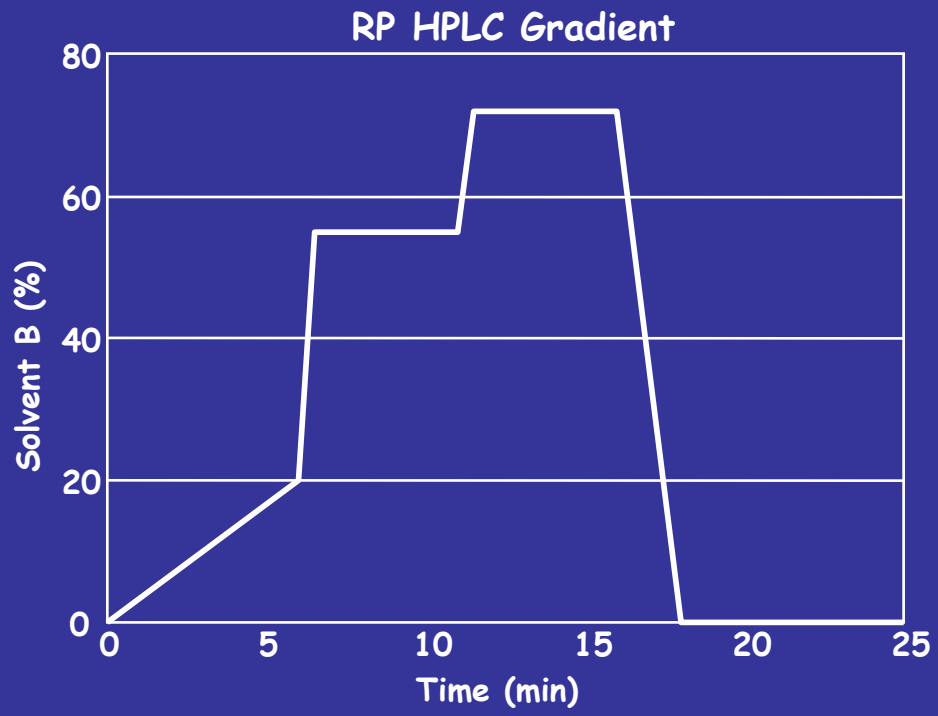
Shown here is the platform we are using for our eicosanoid surveys, employing reversed-phase liquid chromatography (RPLC) and negative electrospray ionization. With negative electrospray ionization, a proton is removed from the eicosanoid's carboxyl group, making a negative ion. The mass spectrometer we use is the Applied Biosystems 4000 QTrap.

column
Vydac® 201TP52
2.1 mm X 250 mm

Solvent A
H_2O/ACN/formic acid:
63/37/0.02

Flow rate
300 µl/min

Solvent B
ACN/IPA:
50/50



solvent A

solvent B

LC pump

auto sampler

RPLC C_{18} column

(-)ESI

collision gas (N_2)

ABI Analyst software

EM detector

4000 QTRAP™
LC/MS/MS System

Q1 mass analyzer

q2 collision cell

Q3 *linear trap* mass analyzer

Applied Biosystems® 4000 QTrap
Quadrupole mass spectrometer

analytical methodology: LC-MS (cont.) (depicted on next slide)

A specialize form of tandem mass spectrometry is known as *Multiple Reaction Monitoring or MRM*.

Here ions are introduced into Q1. Using Q1 we isolate one specific mass to-charge ratio or *m/z*. Ion's having this specific *m/z* are then fragmented in Q2. Q3 is set to pass only one specific *m/z* fragment – a fragment having an *m/z* that is correlated to the *m/z* of the precursor that it was produced from. Therefore for a specific molecule, we have a specific MRM pair associated with it.

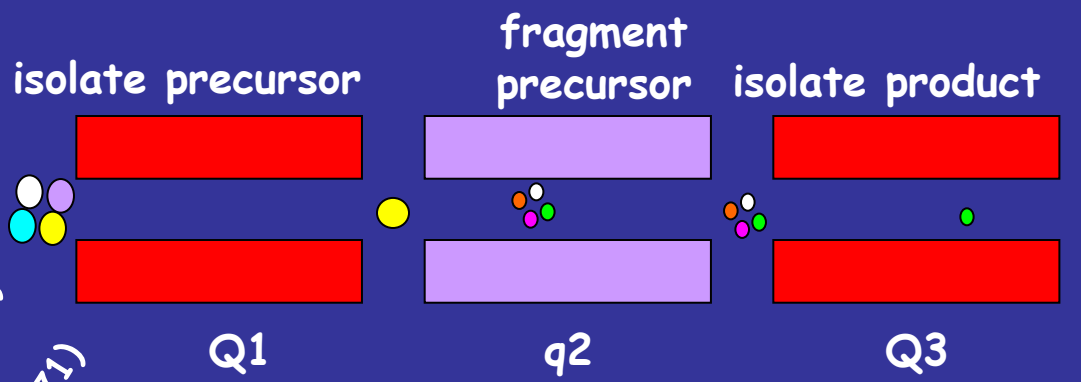
Coupling a liquid chromatography separation to the mass spectrometer and running the mass spec in MRM mode, the mass spectrometer is used as *a highly selective and highly sensitive HPLC detector*.

Shown here is the chromatogram obtained using a mixture of 11 eicosanoid standards along with their specific MRM pairs.

Eicosanoid Standard Mixture LC/MS - MRM

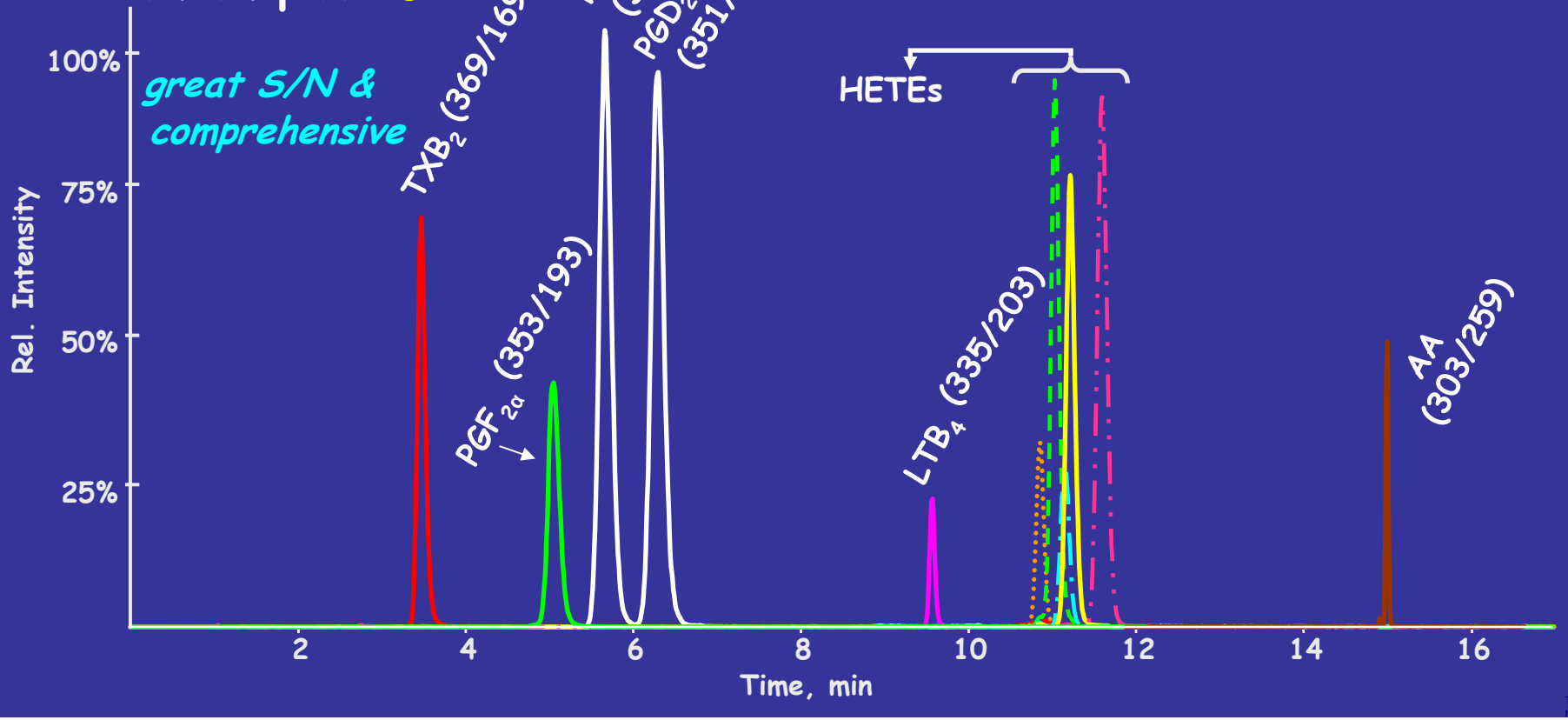
***M**ultiple **R**eaction **M**onitoring*

"specialized" form of MSMS



MRM pair: ● + ●

great S/N & comprehensive

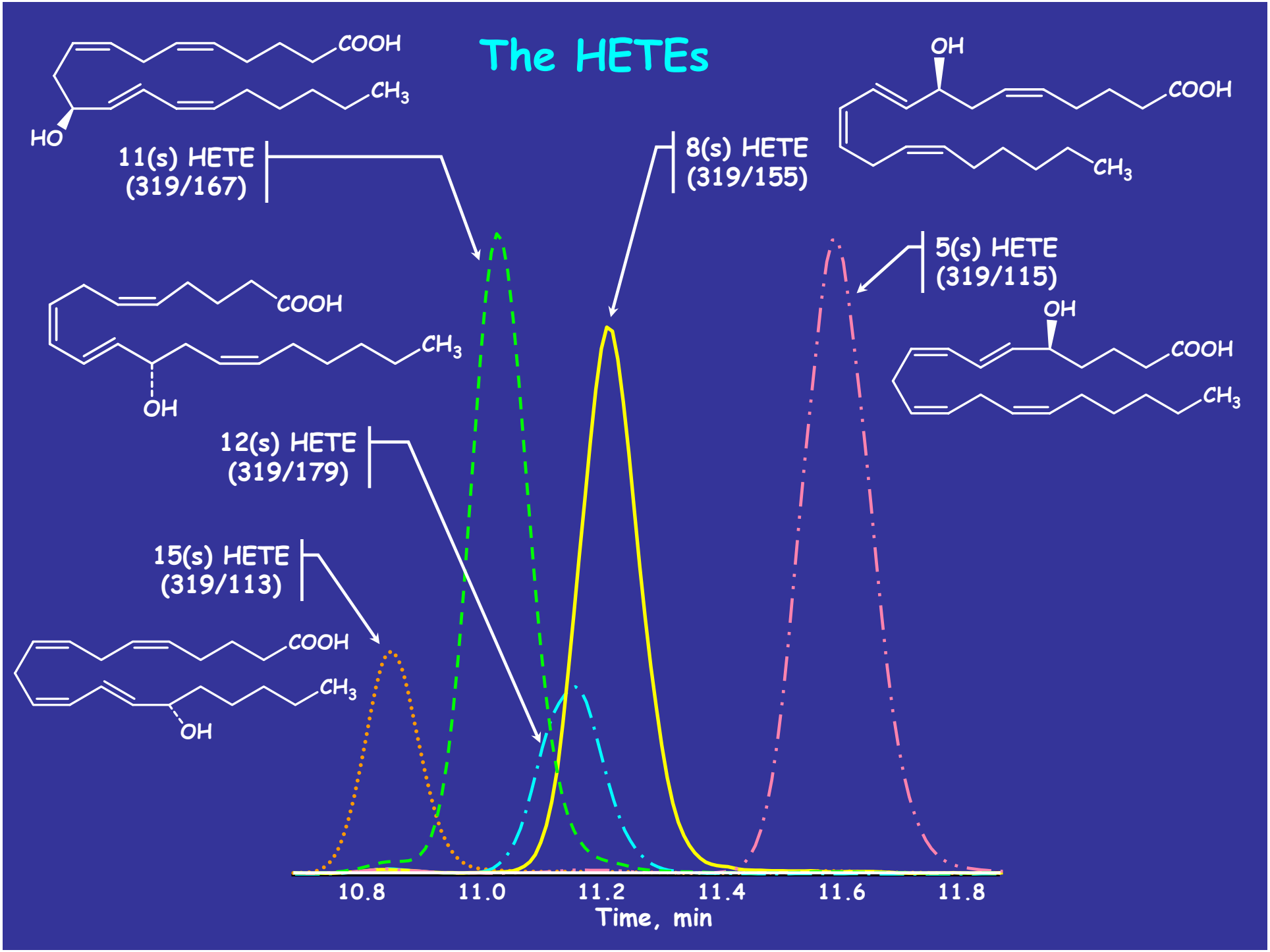


analytical methodology: LC-MS (cont.)
(depicted on next slide)

The great thing about using the mass spec's MRM mode is we can create a method that can monitor over 100 species, according to their specific MRM pair, in a single analysis (16 min). The mass spectrometer cycles through all the MRM pairs repeatedly, from the beginning of the analysis to the end. If a specific molecule is eluting from the chromatography column at any time during the analysis, the MRM pair will detect it.

This provides a great signal-to-noise ratio and the method is extremely comprehensive.

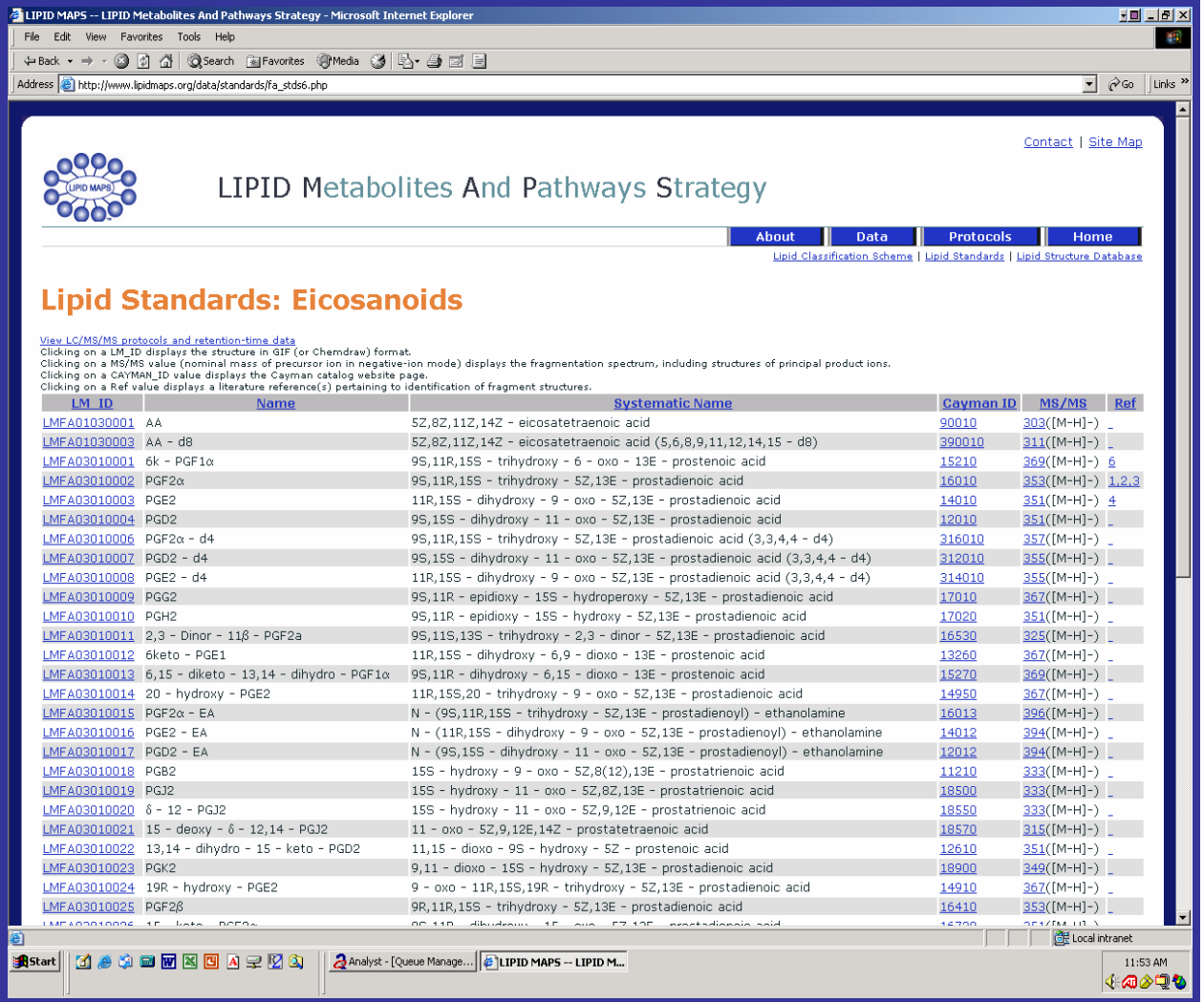
Looking at the area of the hydroxyeicosatertaenoic acid or HETE compounds in more detail we see that *combining LC retention time and MRM specificity,* we can clearly resolve the very similar members of this family of positional isomers.


The HETEs
COOH
CH3
HO
11(s) HETE
(319/167)
OH
COOH
CH3
8(s) HETE
(319/155)
OH
COOH
CH3
5(s) HETE
(319/115)
OH
COOH
CH3
12(s) HETE
(319/179)
OH
COOH
CH3
15(s) HETE
(319/113)
OH
10.8
11.0
11.2
11.4
11.6
11.8
Time, min

Library of Eicosanoid Standards
(http://www.lipidmaps.org)

Provides:

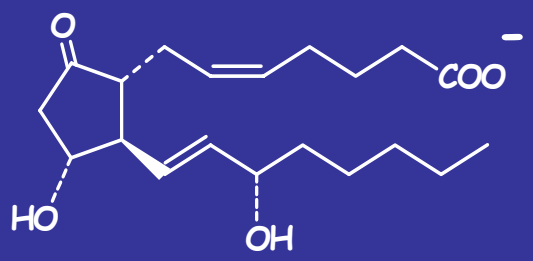
- Chemical structure in ChemDraw® format
- LC and MS protocols
- MSMS fragmentation spectra
- LC retention times for given set of conditions
- Web-link to Cayman Chemical for each eicosanoid



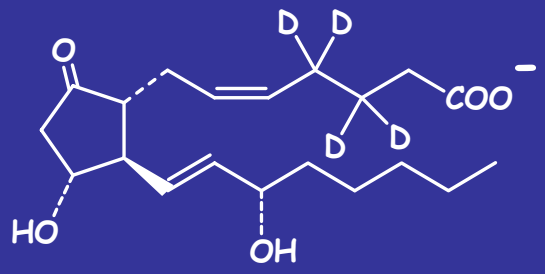
LM_ID	Name	Systematic Name	Cayman ID	MS/MS	Ref
LMFA01030001	AA	5Z,8Z,11Z,14Z - eicosatetraenoic acid	90010	303([M-H]-)	
LMFA01030003	AA - d8	5Z,8Z,11Z,14Z - eicosatetraenoic acid (5,6,8,9,11,12,14,15 - d8)	390010	311([M-H]-)	
LMFA03010001	6k - PGF1α	9S,11R,15S - trihydroxy - 6 - oxo - 13E - prostenoic acid	15210	369([M-H]-)	6
LMFA03010002	PGF2α	9S,11R,15S - trihydroxy - 5Z,13E - prostadienoic acid	16010	353([M-H]-)	1,2,3
LMFA03010003	PGE2	11R,15S - dihydroxy - 9 - oxo - 5Z,13E - prostadienoic acid	14010	351([M-H]-)	4
LMFA03010004	PGD2	9S,15S - dihydroxy - 11 - oxo - 5Z,13E - prostadienoic acid	12010	351([M-H]-)	
LMFA03010006	PGF2α - d4	9S,11R,15S - trihydroxy - 5Z,13E - prostadienoic acid (3,3,4,4 - d4)	316010	357([M-H]-)	
LMFA03010007	PGD2 - d4	9S,15S - dihydroxy - 11 - oxo - 5Z,13E - prostadienoic acid (3,3,4,4 - d4)	312010	355([M-H]-)	
LMFA03010008	PGE2 - d4	11R,15S - dihydroxy - 9 - oxo - 5Z,13E - prostadienoic acid (3,3,4,4 - d4)	314010	355([M-H]-)	
LMFA03010009	PGG2	9S,11R - epidioxy - 15S - hydroperoxy - 5Z,13E - prostadienoic acid	17010	367([M-H]-)	
LMFA03010010	PGH2	9S,11R - epidioxy - 15S - hydroxy - 5Z,13E - prostadienoic acid	17020	351([M-H]-)	
LMFA03010011	2,3 - Dinor - 11β - PGF2a	9S,11S,13S - trihydroxy - 2,3 - dinor - 5Z,13E - prostadienoic acid	16530	325([M-H]-)	
LMFA03010012	6keto - PGE1	11R,15S - dihydroxy - 6,9 - dioxo - 13E - prostenoic acid	13260	367([M-H]-)	
LMFA03010013	6,15 - diketo - 13,14 - dihydro - PGF1α	9S,11R - dihydroxy - 6,15 - dioxo - 13E - prostenoic acid	15270	369([M-H]-)	
LMFA03010014	20 - hydroxy - PGE2	11R,15S,20 - trihydroxy - 9 - oxo - 5Z,13E - prostadienoic acid	14950	367([M-H]-)	
LMFA03010015	PGF2α - EA	N - (9S,11R,15S - trihydroxy - 5Z,13E - prostadienoyl) - ethanolamine	16013	396([M-H]-)	
LMFA03010016	PGE2 - EA	N - (11R,15S - dihydroxy - 9 - oxo - 5Z,13E - prostadienoyl) - ethanolamine	14012	394([M-H]-)	
LMFA03010017	PGD2 - EA	N - (9S,15S - dihydroxy - 11 - oxo - 5Z,13E - prostadienoyl) - ethanolamine	12012	394([M-H]-)	
LMFA03010018	PGB2	15S - hydroxy - 9 - oxo - 5Z,8(12),13E - prostatrienoic acid	11210	333([M-H]-)	
LMFA03010019	PGJ2	15S - hydroxy - 11 - oxo - 5Z,8Z,13E - prostatrienoic acid	18500	333([M-H]-)	
LMFA03010020	δ - 12 - PGJ2	15S - hydroxy - 11 - oxo - 5Z,9,12E - prostatrienoic acid	18550	333([M-H]-)	
LMFA03010021	15 - deoxy - δ - 12,14 - PGJ2	11 - oxo - 5Z,9,12E,14Z - prostatetraenoic acid	18570	315([M-H]-)	
LMFA03010022	13,14 - dihydro - 15 - keto - PGD2	11,15 - dioxo - 9S - hydroxy - 5Z - prostenoic acid	12610	351([M-H]-)	
LMFA03010023	PGK2	9,11 - dioxo - 15S - hydroxy - 5Z,13E - prostadienoic acid	18900	349([M-H]-)	
LMFA03010024	19R - hydroxy - PGE2	9 - oxo - 11R,15S,19R - trihydroxy - 5Z,13E - prostadienoic acid	14910	367([M-H]-)	
LMFA03010025	PGF2β	9R,11R,15S - trihydroxy - 5Z,13E - prostadienoic acid	16410	353([M-H]-)	

The eicosanoid library provides information from which a comprehensive method (LC-MRM-MSMS) is created and used to survey eicosanoid release from stimulated cells

use of deuterium labeled internal standards allows absolute quantitation for a number of eicosanoids

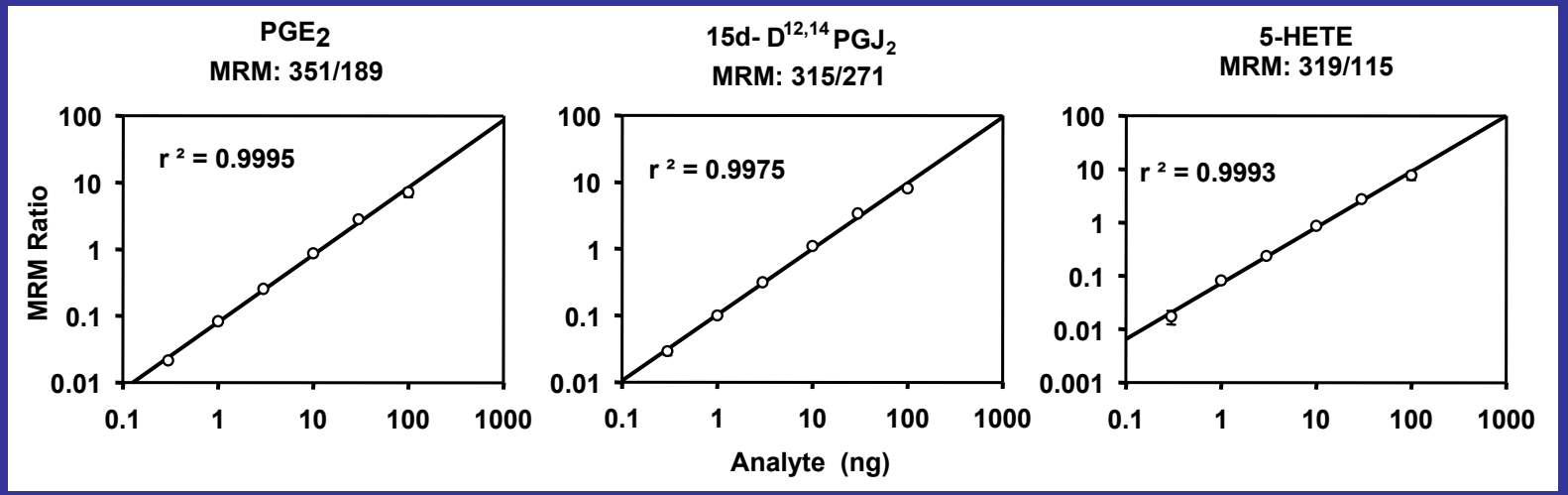


PGE_2 $[M-H]^-$ = 351



PGE_2 - d4 $[M-H]^-$ = 355

example of internal standard calibration curves used for quantitation



chiral chromatography aids in determining enzymatic vs. nonenzymatic origin

atmospheric pressure chemical ionization (APCI) used

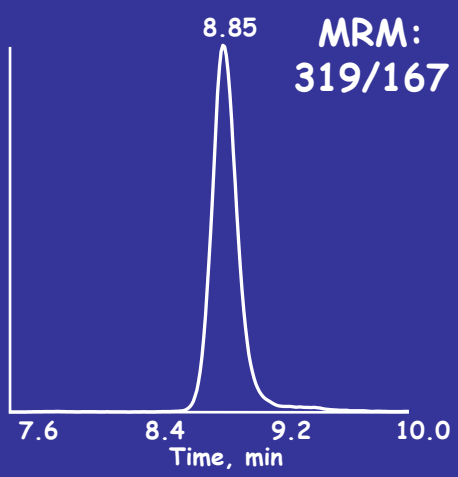
column
Chiralpak® AD-H
4.6 mm X 250 mm

Flow rate
500 µl/min

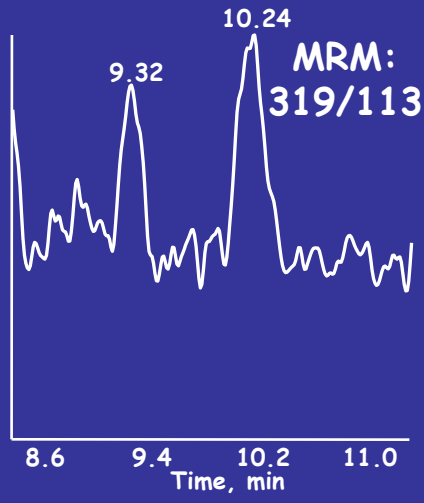
Solvent A
hexane/EtOH/H_2O/formic acid:
96/4/0.08/0.02

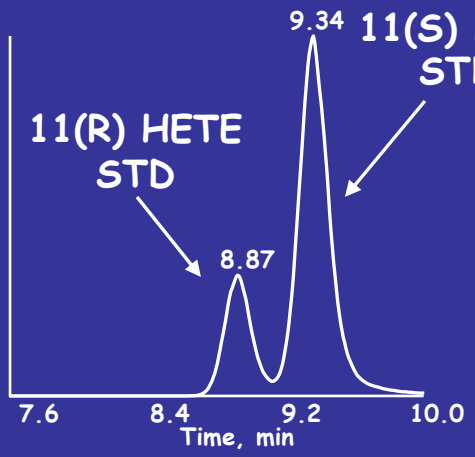
Solvent B
EtOH : 100

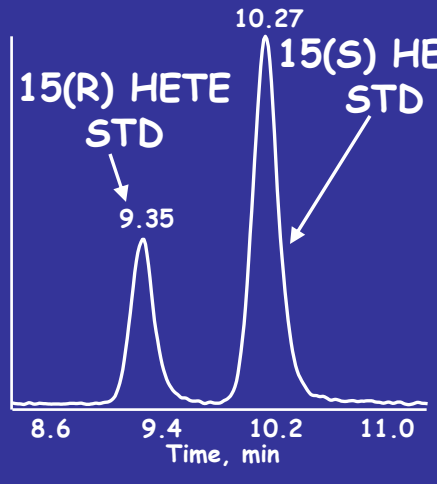
Kdo_2-Lipid A stimulated RAW cells



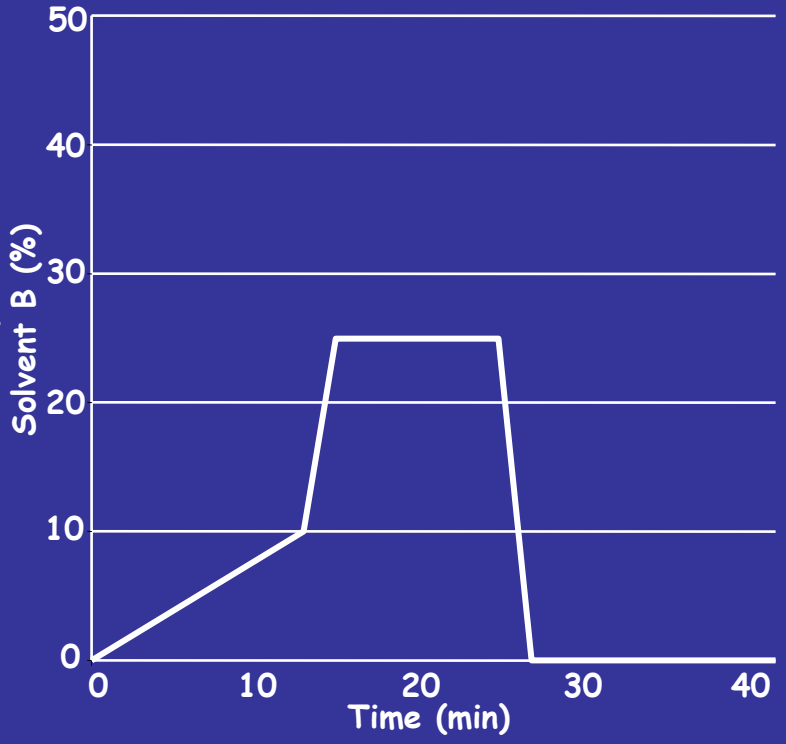
Kdo_2-Lipid A stimulated RAW cells







NP HPLC Gradient



Are biologically significant eicosanoids being overlooked?

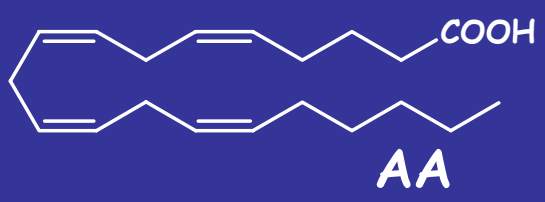
Are there species for which we have no prior knowledge of or expectation of their presence and, hence, no available MRM pairs or chromatography retention times that would be required for their detection?

To address these concerns a mass spectral based stable isotope labeling strategy has been developed

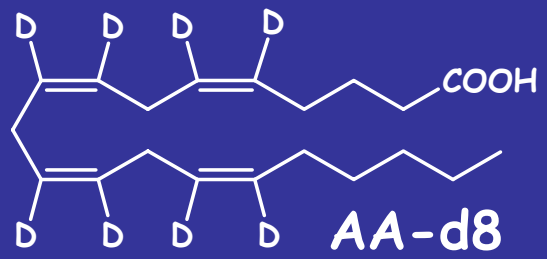
DIMPLES/MS: Diverse Isotope Metabolic Profiling of Labeled Exogenous Substrates using Mass Spectrometry

Harkewicz et al. (2007) J. Biol. Chem. 282 pp. 2899-2910

Incubation of cells in medium supplemented with deuterium-labeled arachidonic acid (AA-d8)



AA

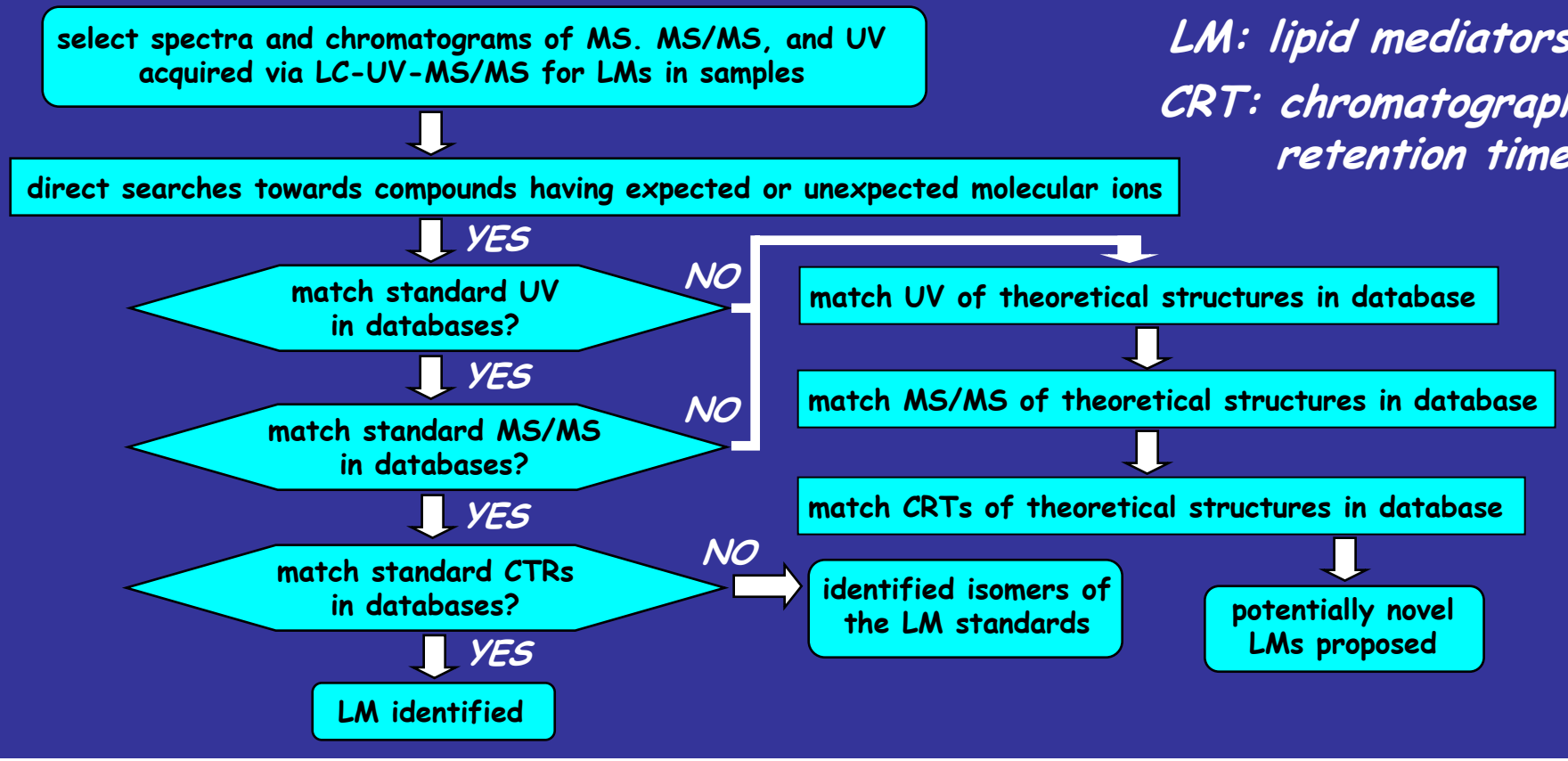


AA-d8

another approach to eicosanoid lipidomics and the search for novel eicosanoids

Serhan Lab developing theoretical databases and algorithms based on virtual LC-UV spectroscopy-tandem mass spectrometry and chromatograms for identifying potential eicosanoids without synthetic or authentic products as standards

Y. Lu et al. (2005) J. Lipid Res. 46 pp. 790-802

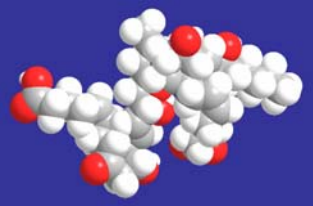


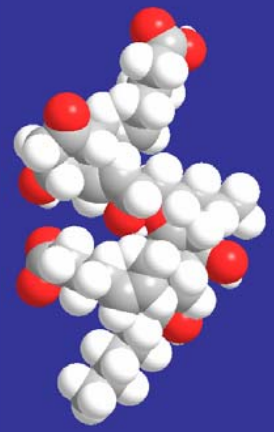
LM: lipid mediators

CRT: chromatographic retention time

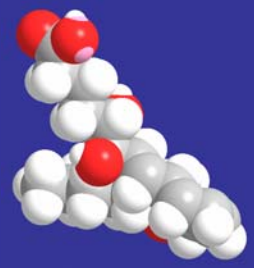
www.lipidmaps.org

future plans





- Continue to expand eicosanoid library
- Incorporate UV detection and analyses into eicosanoid surveys



- Expand search for novel eicosanoids
- Similar studies with Ω-3 fatty acid supplementation
  EPA (20:5 n-3) and DHA (22:6 n-3)