



**Evaluation of phospholipids effect on ionization in  
LDTD-MS/MS analysis of human plasma extracts  
from protein precipitation, SPE and LLE**

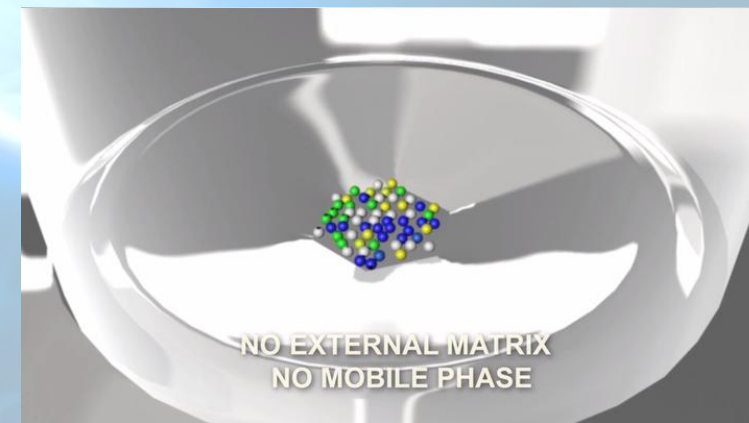
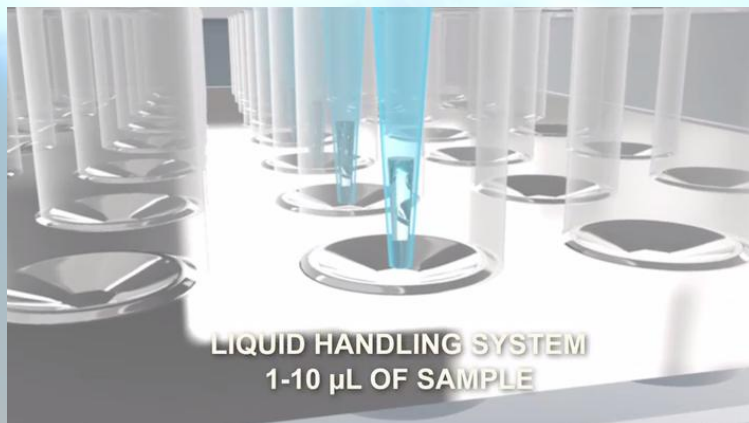
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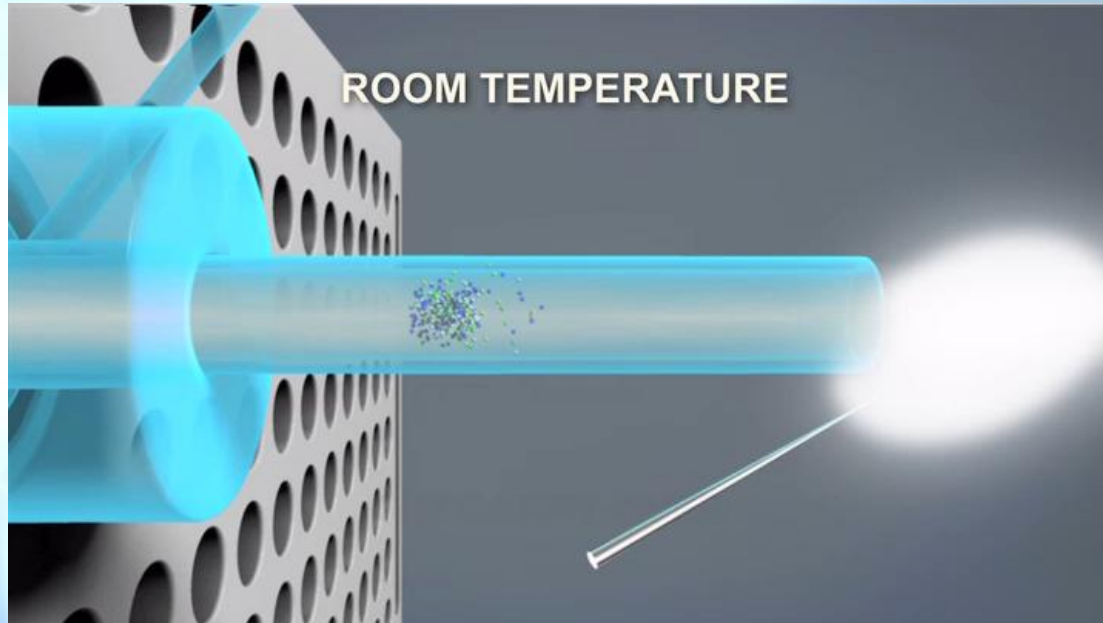
# LDTD: Laser Diode Thermal Desorption

- Thermal Probe are used for several years in mass spectrometry
- LDTD is an improvement of this technique:
  - Use of Laser Diode to control precisely the thermal energy transferred to the sample
  - Sample holder allow nanoscale deposit format\*
  - Gas dynamics optimized to transfer the vaporized sample to the ionization region
  - APCI ionization parameterization
- The results is an ion source witch have:
  - Good reproducibility and precision
  - Sensitivity equivalent to LC-MS
  - Linearity up to  $10^6$
  - Speed : typically 10 seconds sample to sample

# LDTD: Sample deposition



# LDTD: vaporization and transport



# LDTD: APCI ionization



- APCI gas composition: Air with ppm level of water
- No solvent, no mobile phase
- $\text{H}_3\text{O}^+$  ( $\text{H}_2\text{O}$ )<sub>n</sub> cluster distribution\* center at n=3
- Gas temperature 30 Celsius

\* 57<sup>th</sup> ASMS Mechanisms involved in positive atmospheric pressure chemical ionization (APCI) of LDTD source.

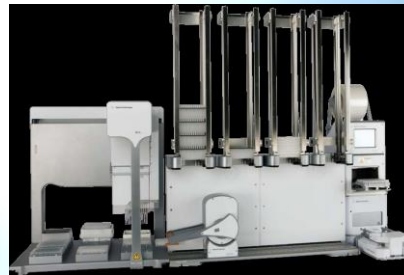
# Phospholipids Effects: Description of experimentation

- Phospholipids used to evaluate their influence on LDTD analysis:
  - **18:0-18:2 PC** 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine
  - **16:1 PE** 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphoethanolamine
  - **18:1 PS** 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine
  - **18:1 PA** 1,2-dioleoyl-*sn*-glycero-3-phosphate
- Qualitative analysis of the Phospholipids in LDTD source
- Application on different extraction methods with human plasma:
  - Protein Precipitation
  - Liquid-Liquid Extraction
  - Solid Phase Extraction
- Phospholipids effects on multiple compounds spiked in protein precipitation extract

# Instrumentation and functional conditions

## Instrumentation

- Agilent extraction system, Bravo
- Phytronix Technologies LDTD ion source A-3840
- Agilent 6410 mass spectrometer system



## MS conditions

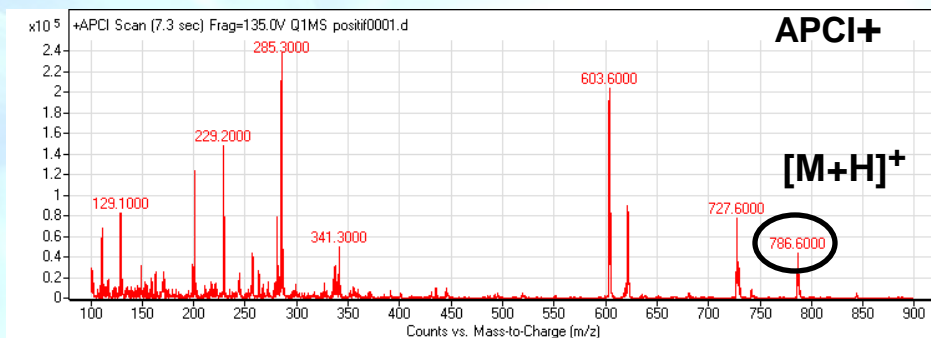
- Fragmentor = 135
- MS Gas flow = 5.0 l/min at 325 Celsius
- Corona current = 3  $\mu$ A

## LDTD conditions (generic)

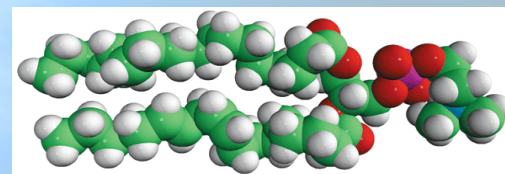
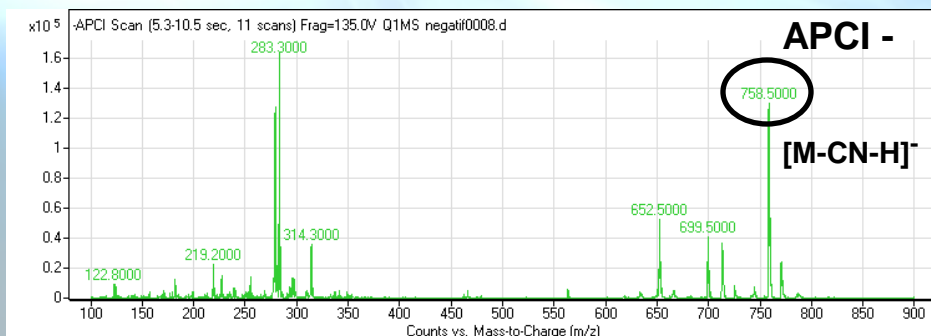
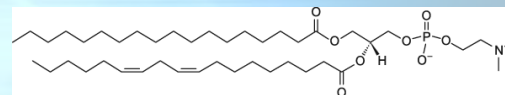
- Carrier gas flow = 3.0 l/min
- Laser Pattern = 2 second ramp from 0 to 35 % + 2 sec plateau

# Phospholipids: Ionization in LDTD source

- Full Scan spectra of PC 18:0, 18:2

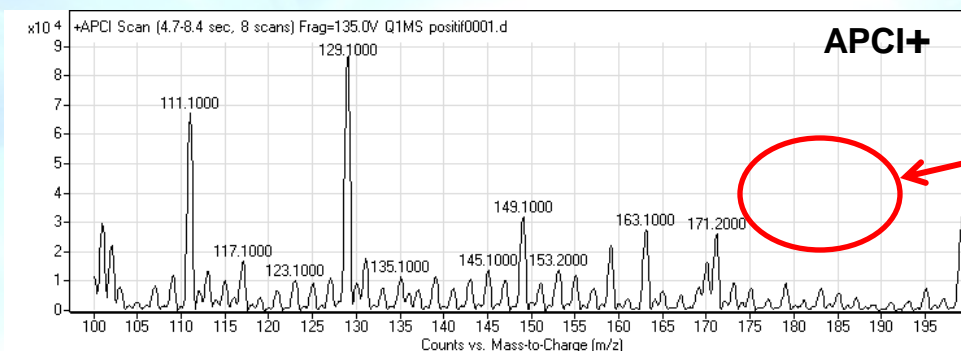


**MW: 785.593**



# Phospholipids: Ionization in LDTD source

- Zoom of the Full Scan spectra of PC 18:0, 18:2



Absence of the mass 184

MRM	Monitoring transitions		
Compound Name	Precursor Ion	Product Ion	Collision Energy
PE	547.5	237.0	25
PA	621.6	339.3	30
PS	638.6	339.1	30
PC	786.6	603.3	25

# Phospholipids in human plasma: Assay composition

- Dextrorphan and Dextrorphan-D3 (ISTD) into all plasma
- Standard curve from 1 to 800 ng/ml
- 6 different plasma matrix spiked at medium QC level
- The amount of phospholipids added is the expected concentration in plasma (~1 mg/ml) adjusted for the dilution ratio: 160 µg/ml

## Liquid-liquid and protein precipitation

Mid QC extract preparation spiked with:

- PC
- PA
- PE
- PS
- MeOH as reference

## Solid phase extraction

Mid QC extract preparation spiked with:

- Mix of phospholipids (70%PC, 15% PS, 10%PE, 5%PA)
- MeOH as reference

<b>MRM</b>	<b>Monitored transitions</b>		
<b>Compound Name</b>	<b>Precursor Ion</b>	<b>Product Ion</b>	<b>Collision Energy</b>
Dextrorphan	258.2	157.2	38
Dextrorphan_D3	261.3	157.2	38

# Phospholipids in human plasma: Sample preparation

## Protein Precipitation

20 µl of spiked plasma  
+  
100 µl Acetonitrile

Vortex 10 seconds

Centrifuge 12 minutes

Deposit of 2µl supernatant  
onto Lazwell plate

Dry completely

## Liquid-liquid Extraction

20 µl of spiked plasma  
+  
100 µl Ethyl Acetate

Vortex 10 seconds

Centrifuge 12 minutes

Deposit of 2µl supernatant  
onto Lazwell plate

Dry completely

## Solid Phase Extraction

50µL sample + 50µL IS+ 200 µl  
water

Waters MCX, µElution plate

Activation 200µL MeOH  
200µL FA (0.1% in water)

Load :300µL

Wash 1: 200µL FA (0.1% in  
water)

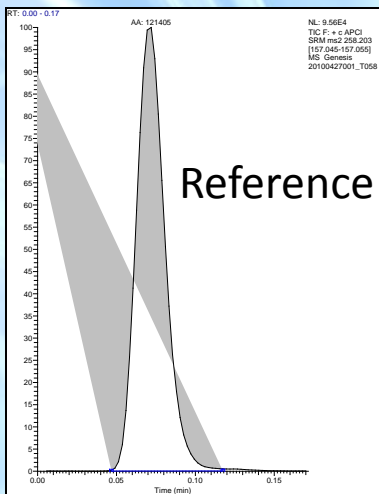
Wash 2 : 200µL MeOH

Elution : 75µL MeOH :NH<sub>4</sub>OH  
(98 :2) + dilution with 250µL  
FA(0.1% in water)

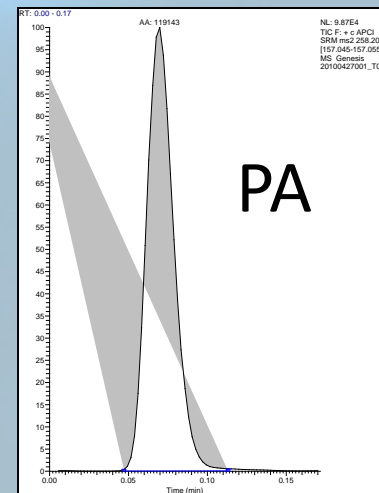
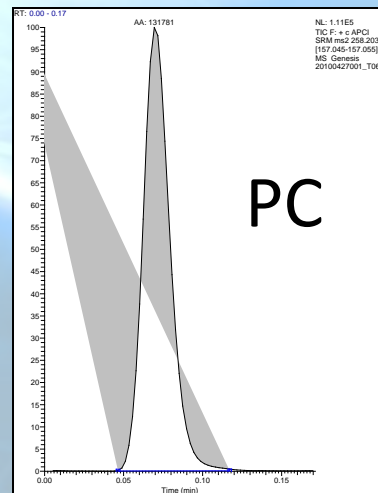
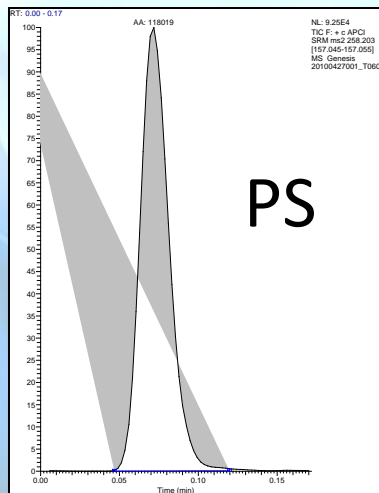
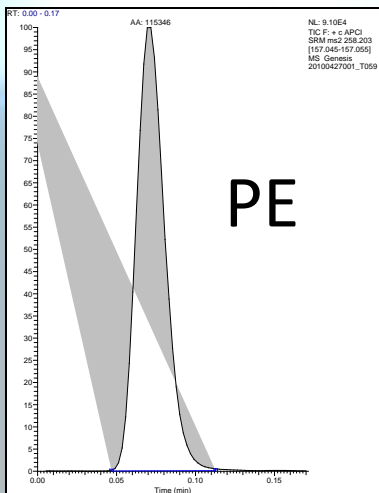
Deposit of 2µl onto Lazwell plate

Dry completely

# Phospholipids in human plasma: results



	Area Protein Precipitation Matrix #1				
	MeOH reference	PE	PS	PC	PA
Dextrorphan	121405	115346	118019	131781	119443
difference		5.0%	2.8%	-8.5%	1.6%



# Protein precipitation results

$$\text{Area difference} = \frac{100\% \times (\text{reference} - \text{fortified})}{\text{reference}}$$

- Bilateral unpaired T-Test show no statistical difference in a confidence interval of 95%
- Tested phospholipids does not interfere in the protein precipitation methods
- Use of internal standard correct for the pipetting volume error and instrumental variation

Area difference in % (n=3)

Matrix	PE	PS	PC	PA
M1	7.3	-9.2	3.2	5.8
M2	-5.2	-1.5	-5.8	-6.2
M3	-7.6	0.3	-6.3	1.2
M4	8.0	1.4	4.1	-2.3
M5	-10.3	-5.3	-3.2	7.2
M6	-5.5	7.7	7.2	3.9

Ratio difference in % (n=3)

Matrix	PE	PS	PC	PA
M1	0.2	2.9	3.4	3.7
M2	-0.6	-5.4	0.4	-4.1
M3	0.3	1.2	-0.8	1.1
M4	0.9	0.4	3.6	0.4
M5	-0.4	-1.5	-3.7	-0.5
M6	-1.1	1.6	6.0	0.7

# Liquid-liquid extraction results

- Bilateral unpaired T-Test show no statistical difference in a confidence interval of 95% except for one analysis
- Tested phospholipids does not interfere in the protein precipitation methods
- Use of internal standard correct for the pipetting volume error and instrumental variation

Area difference in % (n=3)				
Matrix	PE	PS	PC	PA
M1	-9.3	-17.7	7.2	-6.3
M2	8.3	-15.4	-5.9	-15.2
M3	-9.5	0.9	-8.5	5.1
M4	-7.6%	14.6	12.2	4.6
M5	-11.9	10.8	-7.2	13.9
M6	-6.6	7.9	-19.3	3.8

Ratio difference in % (n=3)				
Matrix	PE	PS	PC	PA
M1	-0.6	3.2	4.0	3.1
M2	-0.6	-4.3	-0.8	-0.3
M3	0.4	-6.5	0.3	-4.2
M4	0.9	-0.2	3.3	-0.8
M5	0.0	-1.5	2.7	-0.8
M6	-1.8	-2.8	-3.5	-3.0

# Solid phase extraction results

- Mix of phospholipids representing the average proportion of concentration in human plasma
- Tested phospholipids does not interfere in the SPE methods
- Use of internal standard correct for the pipetting volume error and instrumental variation

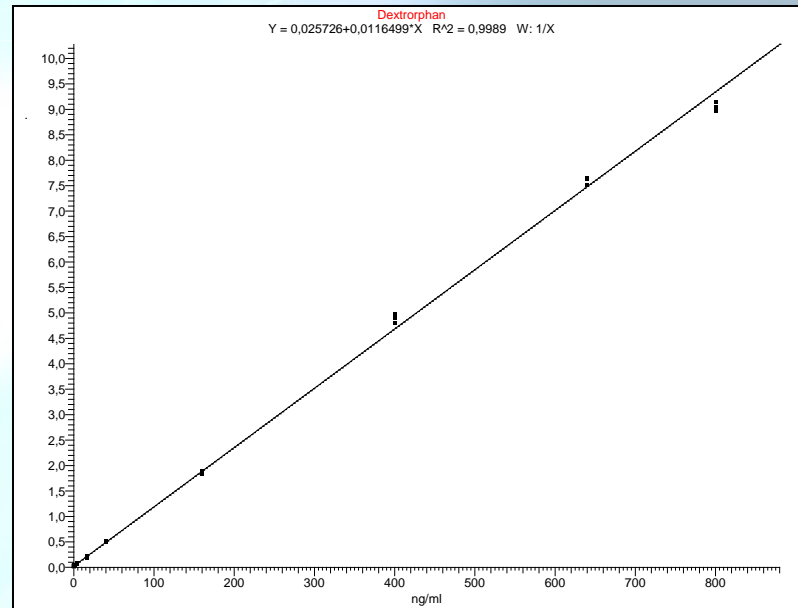
Matrix	Area difference in % (n=4)	Ratio difference in % (n=4)
M1	15.8	1.6
M2	6.9	-0.2
M3	-4.1	-1.3
M4	6.1	-1.7
M5	0.6	-0.6
M6	-6.0	1.2

Matrix M6 replicate	Liquid handling reproducibility	
	Area difference in % (n=4)	Ratio difference in % (n=4)
1	1.1	-0.8
2	18.4	-3.8
3	10.4	-0.1
4	-6.0	1.2
5	0.6	-0.8
6	7.3	0.7

# Phospholipids in human plasma: results

Linearity	
Extraction Method	R <sup>2</sup>
Protein Precipitation	0.9989
Liquid-liquid extraction	0.9954
Solid phase extraction	0.9994

Protein Precipitation results		
Nominal concentration (ng/ml)	Accuracy	%CV n=4
1.6	102.3	13.1%
4.0	98.6	11.1%
16.0	97.3	9.6%
40.0	103.1	3.0%
160.0	98.5	1.2%
400.0	103.9	1.7%
640.0	101.7	0.8%
800.0	96.9	0.8%


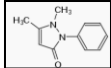
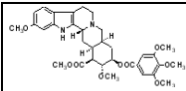
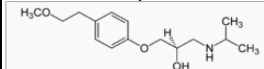
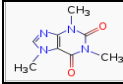
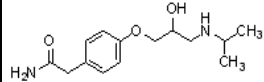
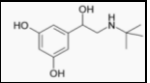
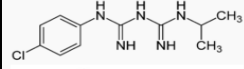


Protein precipitation mid QC 200 ng/ml nominal (n=3)											
Matrix	Reference		PE		PS		PC		PA		
	Measured (ng/ml)	CV (%)	Measured (ng/ml)	CV (%)	Measured (ng/ml)	CV (%)	Measured (ng/ml)	CV (%)	Measured (ng/ml)	CV (%)	
<b>M1</b>	188	0.9	183	1.0	205	0.5	189	4.2	202	1.1	
<b>M2</b>	211	3.8	202	1.9	185	1.1	210	3.1	197	0.8	
<b>M3</b>	205	1.6	217	1.9	203	3.4	185	1.7	212	0.7	
<b>M4</b>	197	1.4	201	0.1	188	0.8	210	2.0	190	1.4	
<b>M5</b>	195	2.8	187	4.0	200	1.2	201	1.9	205	3.5	
<b>M6</b>	203	5.7	193	2.4	195	2.0	184	1.4	193	0.2	

# Phospholipids: Effect on multiples compounds

Protein precipitation (1:5 v/v ACN) supernatant spiked with drugs at 200 nM split in two parts:

- Fortified with phospholipids mix to get a concentration of 200 µg/ml
- Add equivalent volume of MeOH to the second part

Name	Structure	Log D (pH 7)	Log P	MW	Area Variation
Carbamazepine		3.16	3.16	236.3	2.4%
Antipyrine		1.43	1.43	188.2	3.3%
Reserpine		0.98	4.42	608.7	-1.2%
Metoprolol		-0.97	1.85	267.4	-0.5%
Caffeine		-1.76	-0.46	194.2	-4.4%
Atenolol		-1.93	0.76	266.3	8.8%
Terbutaline		-2.42	0.43	225.3	33.0%
Proguanil		-3.92	1.58	253.7	7.1%

# Discussion and conclusion

- Low influence of phospholipids in LDTD ion source even if there is no separation
- The robustness of LDTD to phospholipids is compare in equivalent conditions with ESI and APCI by a loop injection of Dextrorphan with the same level of phospholipids. ESI show 92% of suppression and APCI 79%
- No statistical difference between the three sample extraction procedures
- Six different sources of plasma give the same results fortified or not by phospholipids
- Application to 8 different compounds with various Log D, Log P and molecular weight show same behavior as Dextrorphan
- Multivariable analysis should identify trends in problematic compounds