a journey
Two-dimensional (2D) UPLC

Enhancing the power and practicality of UPLC-MS/MS bioanalysis

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Fig. 2.  
2D TLC analysis of $^{32}$P phospholipids. Extracts from C41–pet (A), C41–MprF2 (B), and C41–MprF1 (C) are shown. $^{32}$P Phospholipids were visualized by phosphorimaging. o, origin; x, unidentified spot specific to the strain C41–MprF1.

“Why bother with 2D LC?”
Column switching is complicated!
our target
reasons
reasons

1. eliminate matrix effects
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2. improve ruggedness
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2. improve ruggedness
3. gain selectivity
1. eliminate matrix effects
2. improve ruggedness
3. gain selectivity
4. allow MP flexibility
UPLC® benefits

✓ speed
✓ separation power
✓ sensitivity
Waters Ethylene-Bridged Hybrid (BEH) Columns

“Bioanalysis is stressful!”
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Blood, plasma, urine, tissue extracts contain:

- Cells
- Proteins
- Salts
- Vitamins
- Nutrients
- Hormones
- Lipids
- Etc...
- Matrix effects
- Ion suppression, sensitivity drift
- Back-pressure buildup
- Reduced column life, retention shift
- Limited assay ruggedness
- Restricted to MS-friendly mobile phases
Phospholipids

Natural Phosphatidylcholine
95% Lecithin (Egg)
Avanti Number 131601
CAS Number 97281-44-2
Egg PC 95%

Representative structure
Zwitterion form -
Chemical Formula: C42H82NO8P
Exact Mass: 759.578
Molecular Weight: 760.076

Representative structure
Protonated form -
Chemical Formula: C42H83NO8P+
Exact Mass: 760.585
Molecular Weight: 761.084

An extensive selection of Natural Lipid Extracts is available.
Visit pages 371-376 for details

Note. Due to the high transition temperatures of the saturated products, it may be necessary to heat the solvent to solubilize the compound. Also, a small amount of methanol (0.1%) and water (0.05%) may be needed to hydrate the compound.

start
Simple
“You can always recognize truth by its beauty and simplicity.”
— Richard Feynman
“Things should be made as simple as possible, but no simpler.”

— Albert Einstein
Simple Configurations
Simple Configurations

- Elute-Filter
- Trap & elute
- “Peak heart-cut”
To order from Avanti refer to page 4. Utilize the links for the latest prices.

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**Phospholipids**

**Natural Phosphatidylcholine**

- **95% Lecithin (Egg)**
  - Avanti Number 131601
  - CAS Number 97281-44-2
  - Egg PC 95%

[Link to current prices and additional information about this product](#)

**Representative structure**

- **Zwitterion form**
  - Chemical Formula: C₄₂H₈₂NO₈P
  - Exact Mass: 759.578
  - Molecular Weight: 760.076

- **Protonated form**
  - Chemical Formula: C₄₂H₈₃NO₈P⁺
  - Exact Mass: 760.585
  - Molecular Weight: 761.084

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**Configuration 1**

1. Elute through pre-column (filter) to analytical column

2. **Back-flush** to clean pre-column
Peptide A in human plasma

WCX μElution SPE and back-flushed guard

Double blank

LLOQ (5 pg/mL)
Peptide A in human plasma
WCX μElution SPE and back-flushed guard

Double blank

LLOQ (5 pg/mL)

Columns:
Waters VanGuard BEH C18, 2.1 x 5 mm, 1.7 µm
Waters AQUITY BEH C18, 2.1 x 100 mm, 1.7 µm

Flow Rates:
0.2 mL/min for analysis
0.4 mL/min for column wash
1.2 mL/min for guard back-flush
Octreotide in human plasma

WCX μElution SPE and back-flushed guard

No valve switch

Valve switch at 2.0 min

Precolumn: Waters VanGuard BEH C18, 2.1 x 5 mm, 1.7 μm
Analytical: Waters ACQUITY UPLC BEH C18, 2.1 x 100 mm, 1.7 μm
Configuration 1

Back-flushed precolumn

Pros
✓ forward flow transfer more selective, may gain useful precolumn separation
✓ fast and efficient back-flush of precolumn, may remove particles from inlet frit
✓ a favorite

Cons
- no solvent front divert to waste
Configuration 2

1. Load precolumn to waste

3. Fore-flush to clean precolumn

2. Fore-flow to transfer
Configuration 2

Fore-flushed precolumn

Pros
✓ divert to waste during loading, good for desalting
✓ forward flow transfer more selective, may gain useful precolumn separation
✓ often preferred

Cons
- no cleaning of precolumn inlet frit
- longer to fore-flush precolumn
"We’re getting closer..."
**Targeted peak heart-cut**

1. Load column 1 to waste

3. Fore-flush to clean column 1

2. **Heart-cut** transfer
C₈ column 1 separation profile (1D)

- Octreotide
- Desmopressin
- Vasopressin
- Phospholipids
- Lyso-GPChos
- GPChos

MRM of 8 Channels ES+
TIC
5.37e6

PPD Proprietary & Confidential
2D separation and online clean-up concept

Load and separate peptides on C$_8$ column 1

(0.25 min)
Transfer 2 peptides to phenyl column 2

Octreotide

Desmopressin
2D RP-RP separation using C$_8$ and Phenyl columns

Desmopressin

Octreotide

No Phospholipids
Targeted peak

Heart-cut transfer

Considerations
- transfer “single peak” or narrow band
- column 2 more retentive than column 1
- low dead volume/minimum tubing critical

Issues
- peak dispersion (band-broadening) during transfer
- high back-pressure when both columns in-series
- difficult to clean column 1
more Complex setups
more

Complex setups

Peak heart-cut with intermediate dilution-trapping to refocus
...it’s not this complicated.
Position A
Load sample on Column 1 and separate analyte band from salts, lipids, and other interferences. Flush Column 2 to clean and re-equilibrate.
Intermediate dilution-trapping

**Position B**
Transfer analyte band from Column 1, dilute and refocus on Trap Column. Little to no flow on Column 2.
Position A
Transfer analyte peak from Trap Column, separate on Column 2 and elute to MS.
Fore-flush Column 1 to clean and re-equilibrate.
2D RP-RP using C₈ and C₁₈ columns with refocus

C₈ column (1st D)

Vasopressin

Desmopressin

Octreotide

C₁₈ column (2nd D)

Desmopressin

Octreotide

PPD Proprietary & Confidential
2D HILIC-RP Concept

- Early eluting hydrophobic diverted to waste
- Analyte band to “heart-cut”
- Phospholipids
Peptide B in human plasma
HILIC-low pH RP

Plasma Blank with I.S.

LLOQ (10 pg/mL)
Peptide B in human plasma

HILIC-high pH RP

Plasma Blank with I.S.

LLOQ (10 pg/mL)
Highlights
- hydrophobic interferences (e.g. lipids) flushed to waste before analyte transfer to RP
- high organic extract (e.g. PPT, SPE) can be directly injected. *Good for adsorptive analytes.*

Issues
- tubing lengths and volumes can affect peak dispersion/dilution (depending on setup)
- carryover may increase due to high aqueous dilution to refocus band before RP
Another interesting approach...
In previously reported two-dimensional methods, an additional 30–50% of runtime is added for completion of regeneration and equilibration steps. In our application, the second column, the second column has already completed its regeneration and equilibration before the peak heart-cut of the compound of interest from the first column is transferred to the second column. However, at the end of the switching window, a mandatory anti-carryover flush was performed at the end of the method. The valve was switched again to the 2-column position (SW2, 16.2 min) and the contents of the second column were directed to waste. Since an additional valve cycle to perform anti-carryover was not necessary, only a single flush/rinse cycle was used.

Figure 1. 2-D RP–RP instrumental set-up. C-peptide peak heart-cut is transferred from the first to the second column (valve position 2). While the analyte on the second column undergoes gradient elution to the mass spectrometer, the first column is regenerated (valve position 1).


...and several related papers by Rogatsky, et al.
The composition of human plasma is stabilized (salt) and was postspiked into 200 μL of plasma. In our experiments, 100 ng of a pure synthetic glucagon (4 + charge state ion at m/z 871.4) was postspiked into SPE-purified plasma demonstrated to be a more efficient approach. 2-D LC/SIM analysis of matrix samples represents 150 pg of IS on column.

The purpose of our research was to evaluate the separate contributions of optimized 1-D and 2-D chromatography/SIM. The efficiency of the 2-D chromatography in reducing matrix effects was compared to MS/MS mode and implemented 2-D chromatography in reducing matrix effects, in the present study we focused on the following questions: (i) Is MS/MS (MRM) always preferential toward reduction of matrix effects during peptide determination from complex biological samples? (ii) What are the contributions of chromatography in reducing matrix effects and signal suppression from biological matrices?

Authors reported an LOD of 1 ng/mL achieved by their LC/MS method and concluded that 1-D (single column) chromatography/SIM is a more efficient strategy to increase assay sensitivity. Under these conditions, a potentially more sensitive approach is to omit MS/MS mode and implement 2-D chromatography/SIM.

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Figure 4. Typical C-peptide matrix sample analyzed by 2-D chromatography/SIM.

Figure 5. Typical C-peptide matrix sample analyzed by 2-D chromatography/MRM.
2D UPLC benefits

✓ Eliminates matrix effects
✓ Orthogonal separations
✓ Enhances sensitivity
✓ Allows MS-unfriendly mobile phases
✓ Extends column life
✓ Keeps MS ion source clean
2D opportunities
2D opportunities

RP-RP
HILIC-RP
NP-RP
IEX-RP
SEC-RP
etc...
2D greatly benefits MS-based bioanalysis, whether using HPLC$^2$, HPLC-UPLC or UPLC$^2$
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“Yes, there’s a learning curve, but it’s well worth it.”
There are the rushing waves... mountains of molecules, each stupidly minding its own business... trillions apart...yet forming white surf in unison.

– Richard Feynman
a few photo credits...

Waters
THE SCIENCE OF WHAT'S POSSIBLE
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