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General sample preparation procedure for LDTD-MS/MS analysis

Serge Auger, Alex Birsan
Phytronix Technologies, Québec, QC

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- **Sample preparation**
- **High-throughput**
- **DBS Extraction**
- **LDTD**

Overview

This document summarizes commonly applied sample preparation techniques and can be used as a reference guide to help improve your extraction method. All of the following sample preparation procedures have demonstrated excellent results and reproducibility in LDTD-MS/MS sample analysis.

Four sample preparation methods will be described:

- Protein precipitation
- Solid phase extraction
- Liquid-Liquid extraction
- Dry blood spot extraction.

Additional procedures will be described for molecules containing a carboxylic acid group and for volatile compounds containing amines.

Finally, we propose different ways to obtain more specific mass spectra analysis and some useful tools for extraction method development.

1) Instrumentation

Phytronix Technologies LDTD ion source :

- Model T-960 : Thermo system
- Model S-960 : Sciex system
- Model WX-960 : Waters Xevo system

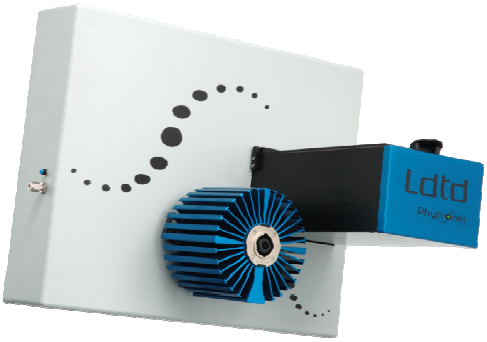

Fig.1 LDTD system

2) LDTD ionization process

The LDTD ion source uses an infrared laser diode to desorb samples that have been dried onto a 96-well LazWell™ plate. The rapid desorption produces neutral species which are carried into a corona discharge region to undergo ionization. The formed ions are subsequently transferred directly into the mass spectrometer for detection.

3) Protein precipitation procedure

Organic Protein Precipitation

This is the most basic type of extraction. Organic solvent (e.g.: acetonitrile, methanol, etc.) is added to an aliquot of matrix (e.g. Plasma, Buffer+BSA, etc.) to induce sample precipitation. The solution is then mixed and the solid particles (non-soluble protein) are removed by centrifugation or filtration.

For LDTD analysis, optimal results are obtained using the following procedure:

- 1 part of sample
- 3 parts of precipitation agent (example: Acetonitrile containing Internal Standard).
- Vortex the sample and remove the solid part by centrifugation or filtration.
- Spot 2 to 5 µl of solution directly into LazWell.

It is important to remove all particles (efficient centrifugation or good filtration) to avoid interference during LDTD analysis.

A larger ratio of precipitation agent to sample will give good results but the samples will be more dilute (useful for highly concentrated molecules).

Improved protein precipitation for LDTD

This improved procedure is similar to the organic protein precipitation but will remove hydrophilic compounds from the solution, thus producing a cleaner and more concentrated extract.

To separate the target molecule from hydrophilic impurities, a salting out technique is used. The improved protein precipitation technique is as follows:

- 1 part of sample
- 3 parts of Acetonitrile (containing Internal Standard).
  - Mix the sample
- Add 1 part of NaCl (saturated solution in water)
- Mix the sample and centrifuge.
- Two phases are obtained:
  - Upper phase: Acetonitrile phase containing the analyte
  - Lower phase: Aqueous phase containing solid particles and hydrophilic impurities.
- Spot upper organic phase directly in LazWell.

This approach resembles a very polar liquid-liquid extraction (LLE) which will yield a cleaner protein precipitation sample as well as a more concentrated sample (since it is not diluted with the aqueous part).

What about Sodium Adducts?

Ionization within the LDTD ion source occurs in a dry environment and therefore avoids the formation of sodium adducts. No formation of sodium adducts was ever observed using this sample preparation on LDTD analysis; contrary to traditional LC-MS/MS analysis which has a high chance of sodium adduct formation.

4) Solid Phase Extraction (SPE) procedure

All solid phase extraction procedures can be performed for LDTD analysis:

- Reverse phase
- Normal phase
- Cation exchange
- Anion exchange
- Mix mode system

A very useful characteristic of the solid phase extraction for LDTD analysis is the ability to spot the elution part directly in the LazWell (evaporation and reconstitution are not needed).

However, if more concentrated samples are needed, the elution part may be evaporated and reconstituted. The best reconstitution solutions for the LDTD-MS/MS analysis is a mixture of **Methanol:Water (75:25) or Acetonitrile:Water (75:25).** (Except for Volatile Amines, see note below)

5) Liquid-Liquid Extraction (LLE) procedure

A liquid-liquid extraction is a useful technique to obtain a clean sample. For the LDTD sample preparation, there is no manipulation difference between organic protein precipitation and the liquid-liquid procedure. The upper layer is spotted directly onto the plate - no evaporation step is required. This approach is less expensive compared to the solid phase approach. The most popular solvents used for liquid-liquid are:

- Ethyl acetate
- Chlorobutane (density lower than water)
- Methyl-ter-butyl ether (MTBE)
- Hexane
- Mixture of Hexane:Ethyl acetate

Typical liquid-liquid extractions are:

- 1 part of sample
- 1 part of buffer with internal standard
  - Mix the sample
- 3 parts of Organic solvent
  - Mix the sample and centrifuge
- Two phases are obtained:
  - Upper phase: Organic phase containing the analyte
  - Lower phase: Aqueous phase containing hydrophilic impurities.
- Spot upper phase directly in LazWell.

If you need more concentrated samples, evaporate a volume of the organic part and reconstitute. The best reconstitution solutions for LDTD-MS/MS analysis are **Methanol:Water (75:25) or Acetonitrile:Water (75:25).**
(Except for Volatile Amines, see note below)

In a liquid-liquid extraction method, the pH of the aqueous phase is very important to obtain good recovery. You may need to adjust the pH of the aqueous phase so that the molecule has the best hydrophobic propriety. To determine the polarity of the molecule, *in silico* estimation of logD value at different pHs can be performed.

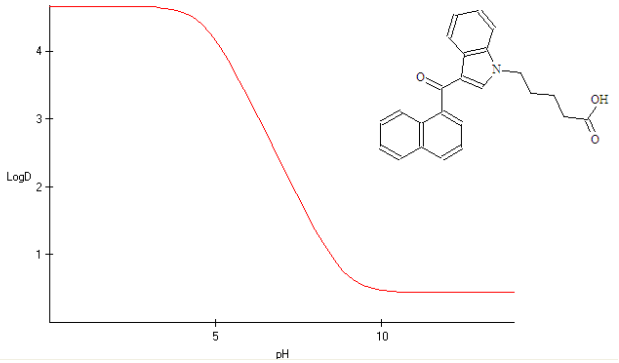

Fig.2 LogD estimation of acidic compound using Pallas Software™

For example, the molecule (Figure 2) has a high log D value at low pH and is therefore less polar at acidic pH (0 to 4) as opposed to a higher pH (>10). The extraction method for this molecule, in order to obtain a good recovery, is: Perform a liquid-liquid extraction (LLE) at acidic pH (2-4).

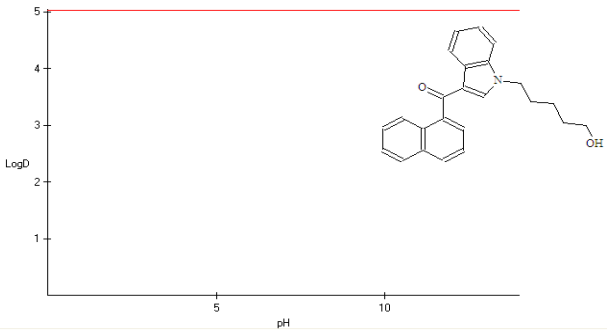

Fig.3 LogD estimation of neutral compound using Pallas Software

Second example, the molecule (Figure 3) is a neutral molecule. This molecule has a high log D value along all the pH range; hence we expect to have a good recovery at any pH. The strategy for this molecule is: Test liquid-liquid extraction (LLE) at acidic, neutral and basic pH with different organic solvents. Select the extraction condition where you obtain the best blank sample.

The important points for LogD estimation are:

- High log D value = More hydrophobic molecule
- Log D value < 0. Molecule is too polar for liquid-liquid extraction. Use another sample preparation strategy.
- Verify if the molecule is stable at the extraction pH suggested. Sometimes a pH can be good for extraction but bad for stability.

6) Dry blood spot extraction procedure

Two different type of cards work well with the LDTD-MS/MS system. Example of these cards is: FTA-DMPK-A (containing denaturizing agent) and FTA-DMPK-C (no denaturizing agent).

Best performance is achieved with the FTA-DMPK-C (without denaturing agent). A direct dissolution using Water / Methanol or Water/Acetonitrile mixture can be used. In the case of a direct dissolution, it is recommended to add the water first followed by Methanol or Acetonitrile (mix and centrifuge). By adding the water first, you minimize the hematocrit effect. The Salting Out procedure described in section 3 may be applied for a better clean-up.

With the FTA-DMPK-A, the use of a liquid-liquid extraction or salting out approach is necessary. The two phases (organic phase and aqueous) keep the denaturing agent into the aqueous phase in order to avoid a suppression effect during LDTD-MS/MS analysis. Two typical FTA-DMPK-A extraction procedures are:

First, you have to spot the sample on the card. Dry blood spot samples consist of a drop of blood (between 15 to 25 µL of blood) spotted on DBS paper and dried. A punch (3 to 6 mm) of DBS paper is extracted to quantify the targeted compound.

DBS extraction 1 (LLE)

- Add 3mm punch in Eppendorf tube (0.5ml)
- 25 µL of Internal standard (water or buffer at a specific pH)
  - Vortex 0.5 min.
- 75 µL of organic solvent
  - Vortex 0.5 min. and centrifuge 2 min. at 14000g.
- Transfer 5 µL of upper phase onto LazWell™
- Perform LDTD-MS/MS analysis

DBS extraction 2 (Salting out)

- Add 3mm punch in Eppendorf tube (0.5ml)
- 25 µL of NaCl (sat) solution in water
  - Vortex 0.5 min.
  - 50 µL of Internal Standard (in Acetonitrile).
  - Vortex 0.5 min. and centrifuge 2 min. at 14000g.
- Transfer 5 µL of upper phase onto LazWell™
- Perform LDTD-MS/MS analysis

The second extraction procedure is a more generic procedure. This procedure can be used for polar and non-polar molecules.

7) Carboxylic Acid Group Procedure

Different molecules contain one or many carboxylic acid groups (COOH) in their structure. With these molecules we noticed a special desorption pattern. At high drug concentration a good linearity was observed, but this was not the case at low concentration. This acidic group seems to interact with the surrounding material. To minimize the molecule`s interaction, EDTA can be added to the final solution or used to pre-coat the Lazwell™ plate before depositing the analyte. Final pH of the solution should be around 9.

You can use the EDTA in two ways:

I. EDTA in extraction solution: A mixture of MeOH/Water/NH_4OH (75/20/5) with different spiked concentrations of EDTA (200, 20 or 2 µg/ml) can be used as reconstituant solution. This mixture can be used to solubilise dry sample extracts or can be added directly to the extraction solution. 20 µg/ml is the generic value.

II. Coating solution: A mixture of MeOH/Water/NH_4OH (75/20/5) with different spiked concentrations of EDTA (200, 20 or 2 µg/ml) are added in LazWell (8 µL) and dried down. The extracted sample (molecule with a carboxylic acid group) is added to the coated plate. It may be necessary to try each EDTA concentration with the target molecule to determine the optimal concentration to obtain acceptable linearity and low background. NH_4OH is added to this mix to keep EDTA in solution and to ensure a uniform coating.

8) Volatile Amines Procedure

Given the low boiling point of these compounds, they tend to vaporize in the LDTD source prior to the completion of a laser power cycle.

In order to stabilize these molecules, perform any of the Solid Phase (SPE) or Liquid-Liquid Extractions (LLE) mentioned in this application note followed by a sample evaporation and reconstitution with: **Methanol:Water (75:25) + 0.01 to 0.1 N HCl.** Be careful, the signal may decrease as the concentration of the acid rises.

9) Mass Spectrometer Optimization

During your extraction method optimization, it is recommended to verify the ion and product ion transitions in positive and negative mode systematically and select the one that gives you the best signal-to-noise response.

The LDTD source charges molecules using a corona needle in a dry environment (no mobile phase and only low quantity of water from carrier gas). Given these conditions, the ionization processes in LDTD differs from the traditional APCI ionization using LC-MS/MS therefore the user should always verify both polarities to be certain of choosing the right one for the molecule.

10) Extraction Method: Development Tip

We recommend the use of a *blank react* (i.e. replace the matrix by a neat solution such as water) when you observe a peak in the blank matrix sample. With the *blank react*, you may be able to determine if the unknown peak in your blank is caused by tube contaminants or the extraction solution.

Conclusion

This document summarizes the observations of many method development and extraction experiments. We hope that these observations provide an added reference to help improve your experience using the LDTD-MS/MS as a high-throughput analysis solution.

