Label free high throughput screening of amino acid based assays: old tricks, new speed using LDTD-MS/MS

Pierre Picard, Sylvain Letarte, Jean Lacoursière and Serge Auger
Phytronix Technologies, Québec, CANADA

OVERVIEW

Purpose
- Evaluation of derivatization to analyze amino acids in LDTD-MS/MS

Method
- Testing of different reagents
- Optimization of reaction conditions
- Quantification in cell buffer matrix

Conclusion
- FMOC derivatization is the most efficient
- Minimal sample preparation step
- Analysis at 6 seconds sample to sample compatible with High Throughput Screening speed

INTRODUCTION

Mass spectrometry is considered as the best analytical technique for sensitive and specific measurements. Nevertheless, in screening assays, the throughput must be sufficiently high to keep the cost and time in a reasonable range for the pharmaceutical laboratories. An analyzing speed of 0.9 second per sample has been demonstrated with LDTD-MS/MS for CYP inhibition. Assays with amino acid markers pose a difficulty for a thermal desorption technique as their volatility is limited. The enhancement of vaporization by derivatization was employed for the analysis of amino acids with a GC-MS system in the early 70s. Several of those reagents were tested and optimized to achieve a LDTD-MS/MS analysis of amino acids in accordance with the complete workflow throughput required.

LUXON Ionization Source:

The LUXON Ion Source (**Figure 1**) is the second generation sample introduction and ionization source based on the LDTD® technology for mass spectrometry. LUXON ion source uses Fiber-Coupled Laser Diode (**Figure 2**) to obtain unmatchable thermal uniformity giving more precision, accuracy and speed. The process begins with dry samples which are rapidly evaporated using indirect heat. The thermally desorbed neutral molecules are carried into a corona discharge region. High efficiency protonation and strong resistance to ionic suppression characterize this type of ionization, and is the result of the absence of solvent and mobile phase. This thermal desorption process yields high intensity molecular ion signal in less than 1 second sample to sample and allows working with very small volumes.

Figure 1 LUXON Ion Source

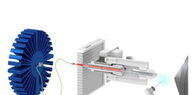


Figure 2 Schematic of the LUXON ionization source

METHOD

Reagent Test

- Dansyl Chloride:
  - 100 µL sample
  - 100 µL carbonate buffer 0.1 M
  - 200 µL Dansyl chloride at 1 mg/mL
  - React 40 minutes at 40 °Celsius
  - LLE using Hexane Ethyl acetate
  - 2 µL deposit into Lazwell plate
- TMPAH
  - 2 µL sample in Lazwell plate
  - 2 µL of 0.005 M TMPAH in MeOH
  - Dry
- O-phthalaldehydes (OPA)
  - Reagent: 5mg of OPA + 100µL of pure Ethanol + 5µL of β-2-mercaptoethanol + 10mL of 50mM carbonate buffer pH10.5
  - 2 µL sample in Lazwell plate
  - 2 µL of reagent
  - Dry in dark
- FMOC
  - Reagent: Borate buffer pH8 at 10 mM mix 1:1 with FMOC at 1 mg/mL in MeCN
  - 2 µL of Sample into Lazwell
  - 2 µL of reagent
  - Dry at room temperature
  - Add 1 µL of EDTA-BSA solution at 100 µg/mL in MeOH-H2O

pH optimization
- Used for FMOC determined as best reagent
  - Borate buffer at 10 mM is adjusted with boric acid or NaOH to cover pH from 6 to 10.5

MS method

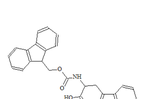
Instrumentation
- LDTD model: LUXON
- MS: Sciex 5500 QTrap®

LDTD Parameters
- Laser power pattern :
  - Increase laser power to 45 % in 3 sec
  - Decrease laser power to 0 %
- Carrier gas flow : 3 L/min (Air)

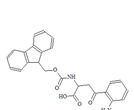
MS Parameters
- APCI (+/-)
- DP: 100 V
- Dwell: 50 msec
- MRM mode **(Table 1)**

Table 1 MRM transitions

Compound	Q1	Q3	CE
Tryptophan	427	159	23
Tryptophan D5	432	193	23
Kynurenine	431	146	23
N-Formyl-Kynurenine	459	174	23
3-Hydroxy-Kynyrenine	485	246	-33
3-Hydroxy-Kynyrenine $N^{15}2C^{13}$	488	248	-33

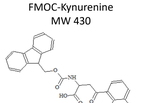


FMOC-Tryptophan
MW 426



FMOC-Kynurenine
MW 430

FMOC-N-Formyl-Kynurenine
MW 458



FMOC-3-Hydroxy-Kynurenine
MW 486

RESULT

Reagent Evaluation
- Dansyl Chloride requires a long reaction time and extra sample treatment to eliminate the reagent from the analysis
- O-phthalaldehydes (OPA) produces thermally fragile derivatized molecules. Quantification is difficult
- TMPAH derivatized molecules desorb quantitatively but use of this reagent increases the background noise
- FMOC produces the most stable and sensitive derivatized product with simple sample preparation

FMOC pH optimization
- Optimal observed pH value of 8 is presented in figure 3. This corresponds to literature review average.

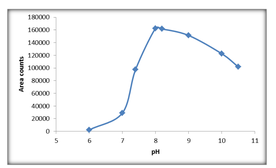


Figure 3 Area count as function of pH

Quantification
- Calibration curve of 10 to 10000 ng/mL was prepared in cell culture buffered solution
- Good accuracy (88.7 to 103.5 %) and precision (3.7 to 9.7 %) were obtained and excellent linearity (R^2= 0.99721 to 0.99910) was achieved for Tryptophan, Kynurenine and N-Formyl Kynurenine
- 3-OH-Kynurenine measurement was obtained using IS ratio from 20 to 10000 ng/mL as shown in Figure 4
- 3-OH-K accuracy (85.6 to 114.3 %) and precision (2.7 to 13.7 %) were obtained with linearity of R^2= 0.9981

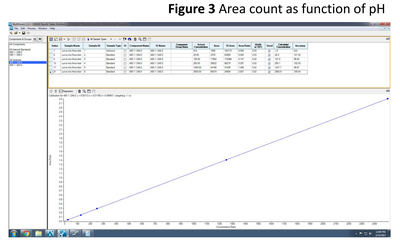


Figure 4 3-Hydroxy-Kynurenine calibration curve

Discussion

- Method conditions were optimized to get the fastest and simplest sample treatment. Minimal concentration of FMOC was found to be 250 µg/mL when mixed 1:1 with the sample in aqueous matrix
- Derivatization occurs during the drying time of 3 minutes. Increasing duration does not improve reaction efficiency
- Derivatized amino acid molecules still present a carboxylic acid function that is known to require EDTA for good thermal desorption
- 1 µL of EDTA solution at 100 µg/mL is added to allow better vaporization. Pre-coating of the plate is not possible because it reduces the derivatization efficiency
- 3-OH-Kynurenine derivatization includes molecular rearrangement with the addition of FMOC at the site of the second amine group
- Use of marked compound with $2C^{13}$, N^{15} and high resolution was necessary to identify the final structure
- Desorption of 3-OH-Kynurenine shows more variability in area count compared to the other compounds but is well corrected by internal standard
- **FMOC** derivatization is accomplished during the drying step of sample preparation. This is a minimal modification of the regular LDTD sample preparation

CONCLUSION

- **First efficient quantification of amino acid in Laser Diode Thermal desorption**
- **Good linearity and reproducibility in all experiments**
- **FMOC derivatization shows the best results with minimal changes in sample preparation**
- **Workflow allows the use of this method in High-Throughput screening**
- **Sample to sample analysis of 6 seconds**