

# Factors Affecting Signal Levels in HPTLC/DESI-MS of Dipeptides and Tryptic Peptides

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## OVERVIEW

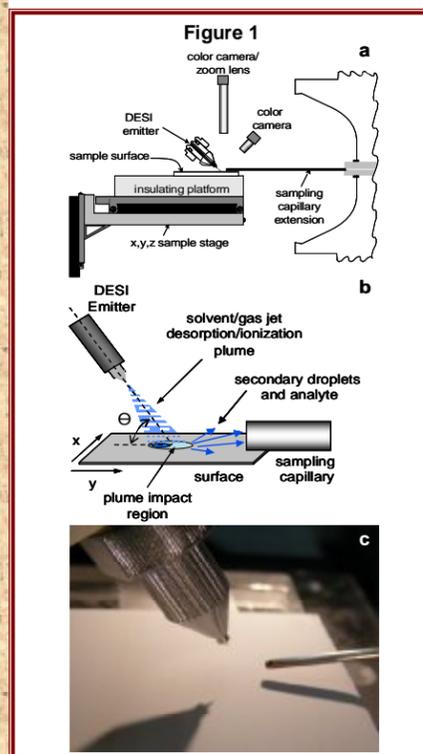
- Thin layer chromatography (TLC) is a quick and simple technique for separating mixtures, and as a planar technique, lends itself well to desorption electrospray ionization (DESI) analysis.
- Here we investigate the utility of high performance (HP)TLC/DESI-MS for characterization of dipeptides and mixtures of tryptic peptides.
- Goals:
  - Evaluate factors that influence signal levels of small biological molecules in DESI-MS
  - Explore effects of spray solvent composition, TLC solid phase, and hydrophilicity of analyte on ionization efficiency
  - Examine the effect of amino acid hydrophilicity on desorption ionization of dipeptides and tryptic peptides
  - Compare DESI and electrospray ionization (ESI)
  - Characterize the utility of HPTLC/DESI-MS for characterization of a complex peptide mixture

## INTRODUCTION

- DESI-MS is a developing atmospheric pressure surface sampling/ionization technique that combines easy sample preparation and high throughput analysis with the high sensitivity and selectivity of mass spectrometry-based detection [1].
- Our group has been examining the use of DESI as a means to couple TLC and MS [2,3]. One focus of DESI-MS research has been the separation and subsequent characterization of mixtures of peptides from a protein tryptic digestion [4,5]. Recent work in our group showed that peptide ionization efficiencies in HPTLC/DESI-MS varied dramatically for a single protein digest, and that the nature of the separation phase and DESI spray solvent were important variables [5].
- To systematically examine the reasons for these observations, a series of leucine-containing dipeptides as well as digests of five common proteins were spotted or separated on different separation phases and analyzed using different DESI spray solvents.

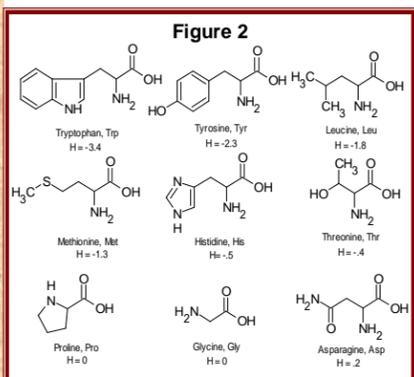
## EXPERIMENTAL

- Materials. HPLC grade methanol and acetonitrile were obtained from Burdick and Jackson (Muskegon, MI). HPLC grade water, glacial acetic acid, and ammonium hydroxide (28-30% by mass) were obtained from Mallinckrodt Baker (Phillipsburg, NJ). n-Butyl alcohol was purchased from EM Science (Savannah, Georgia). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and used as received. ProteoChrom<sup>®</sup> HPTLC plates were obtained from EMD Chemicals (Gibbstown, NJ).
- Thin Layer Chromatography. For dipeptide analysis, samples were applied to TLC plates with a 2.5  $\mu$ L Biohit autopipet. Samples were applied in 1  $\mu$ L volumes. The plates were developed with Merck's recommended solvent compositions of butyl alcohol/pyridine/NH<sub>4</sub>OH/ water (14/17/5/13, v/v/v/v) for the silica plates, butyl alcohol/pyridine/acetic acid/water (15/10/3/12, v/v/v/v) for the cellulose plates, and 70/30 methanol/0.1 M ammonium acetate for the reversed phase plates. All plates were run in triplicate unless otherwise noted, and subsequently cut into appropriate sizes for DESI-MS analysis. Protein tryptic digests separated on ProteoChrom<sup>®</sup> HPTLC Silica gel 60 F<sub>254s</sub> and ProteoChrom<sup>®</sup> HPTLC Cellulose plates were received as a gift from Merck KGaA. Migration distance on all plates was 5 cm, and development times ranged from 45 to 80 minutes.
- DESI-MS. The DESI-MS setup used for these experiments has been described in detail elsewhere [3]. The mass spectrometer used was a ThermoFinnigan LCQ Deca ion trap (Thermo-Finnigan, San Jose, CA, USA). The manual- and computer-controlled x, y, z sample stage is shown in Figure 1a. The DESI plume region is shown in Figure 1b. A photo of the DESI emitter and extension capillary is shown in Figure 1c.



## RESULTS AND DISCUSSION

- Dipeptide Series. The series of dipeptides investigated in this study was chosen to evaluate a range of hydrophilicities and differ by only one amino acid. Hydrophobic peptides have been shown to have stronger signals in ESI [6]. Leucine, with its large non-polar surface area, was chosen as the common amino acid in the series. Figure 2 shows the individual amino acids in the series with their hydrophilicity constants (H). H is defined as the free energy transfer from water to methanol in kcal/mol [7].



- Thin Layer Chromatography. The dipeptides were developed on three different HPTLC plates.

- ProteoChrom<sup>®</sup> HPTLC Silica Gel 60 F<sub>254s</sub>
- ProteoChrom<sup>®</sup> HPTLC Cellulose
- ProteoChrom<sup>®</sup> HPTLC RP-18 F<sub>254s</sub>

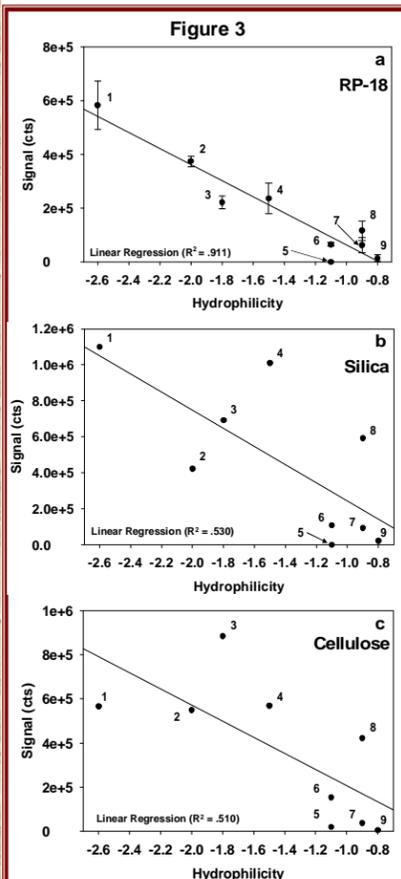
| Dipeptide | ID | H <sup>a</sup> | R <sub>f</sub> Values |        |           |
|-----------|----|----------------|-----------------------|--------|-----------|
|           |    |                | C-18                  | Silica | Cellulose |
| Leu-Trp   | 1  | -2.6           | 0.58                  | 0.98   | 0.91      |
| Tyr-Leu   | 2  | -2             | 0.71                  | 0.99   | 0.89      |
| Leu-Leu   | 3  | -1.8           | 0.62                  | 0.99   | 0.94      |
| Met-Leu   | 4  | -1.5           | 0.62                  | 0.99   | 0.91      |
| His-Leu   | 5  | -1.1           | 0.32                  | 0.93   | 0.66      |
| Thr-Leu   | 6  | -1.1           | 0.71                  | 0.98   | 0.78      |
| Gly-Leu   | 7  | -0.9           | 0.67                  | 0.92   | 0.70      |
| Pro-Leu   | 8  | -0.9           | 0.61                  | 0.92   | 0.79      |
| Leu-Asn   | 9  | -0.8           | 0.76                  | 0.93   | 0.61      |

<sup>a</sup>Expressed as the average free energy transfer from water to methanol (in kcal/mole) [7] for the two given amino acids.

- Table 1 lists the nine dipeptides used in the study paired with assigned average hydrophilicity constants and measured R<sub>f</sub> values on all three plates.

- The least hydrophilic dipeptides have lower R<sub>f</sub> values on the non-polar C-18 plates and higher R<sub>f</sub> values on the two normal-phase plates.
- The C-18 plate gave the best separation, with peptides ranging over nearly half of the development lanes.
- Separations on the silica plate were least effective.

- HPTLC/DESI-MS of Dipeptides. Figure 3 shows results of DESI-MS analysis of the three developed TLC plates.



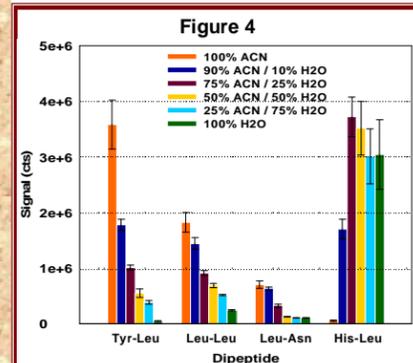
The effect of hydrophilicity on signal intensity for nine dipeptides on (a) ProteoChrom<sup>®</sup> HPTLC RP-18, (b) ProteoChrom<sup>®</sup> HPTLC Silica gel 60 F<sub>254s</sub>, and (c) ProteoChrom<sup>®</sup> HPTLC Cellulose plates. The data in (a) represents the average of three TLC developments, and the data in (b) and (c) represent data from single developments. Acetonitrile was used as a spray solvent.

- A clear trend between hydrophilicity and signal response is evident for C-18 plates when acetonitrile is used as a spray solvent (Figure 3a). However, the trend is less pronounced on silica and cellulose plates (Figures 3b, c).

- Silica plates have polar silanol groups and cellulose plates have free carboxyl groups exposed on their surfaces, both of which can participate in additional hydrogen bonding with amino acid side-chains. These additional chemical groups can alter the dipeptide's affinity for the solid phase.

- Water was not used as a spray solvent because it destroyed the C-18 stationary phase.

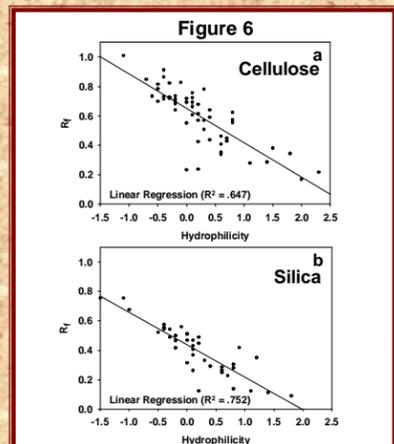
- Spray Solvent Composition. Figure 4 shows signal levels for four dipeptides obtained using various spray solvent compositions. Tyr-Leu, Leu-Leu, and Leu-Asn are listed in order of increasing hydrophilicity. His-Leu is the only dipeptide that showed higher signal levels in water than in acetonitrile.



Effect of spray solvent composition on HPTLC/DESI-MS signal levels for four dipeptides. Samples were deposited on a normal-phase ProteoChrom<sup>®</sup> HPTLC Silica gel 60 F<sub>254s</sub> plate, and sprayed with the indicated solvent mixture. Five sample spots were averaged for each data point over the range when the analyte signal was > 10% of the maximum signal observed for the [M+H]<sup>+</sup> ion.

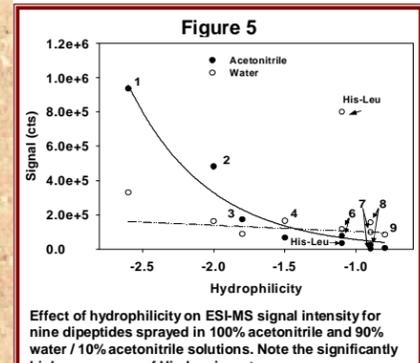
- A decrease in signal intensity with an increase in spray solvent % H<sub>2</sub>O composition was observed for the first three peptides, while the opposite was true for His-Leu. In general, as dipeptide hydrophilicity increased, the signal in 100% acetonitrile spray solvent decreased and the signal in 100% H<sub>2</sub>O spray solvent increased.

## Tryptic Digests



Correlation between R<sub>f</sub> and hydrophilicity for tryptic peptides identified by MS/MS spectra developed on (a) HPTLC ProteoChrom<sup>®</sup> Cellulose and (b) ProteoChrom<sup>®</sup> Silica gel 60 F<sub>254s</sub> plates. Peptides were identified using DBDigger [8] and the MASPIC scoring system [9].

- ESI-MS of Dipeptides. Electrospray ionization involves the production of charged droplets from an analyte-containing solvent spray. These droplets are aimed into a heated sampling capillary, where desolvation occurs. Figure 5 shows the effect of hydrophilicity on signal levels of the dipeptides in two solvents.

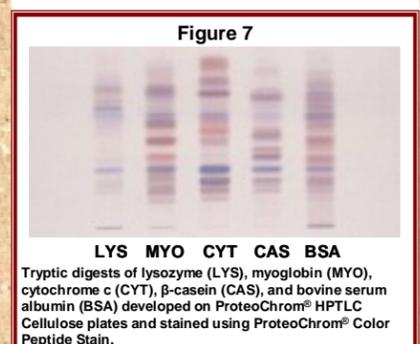


Effect of hydrophilicity on ESI-MS signal intensity for nine dipeptides sprayed in 100% acetonitrile and 90% water / 10% acetonitrile solutions. Note the significantly higher response of His-Leu in water.

- The same general correlation between hydrophilicity and signal response is seen for ESI and for DESI on C-18 plates, indicating a similarity in ionization mechanisms between the two techniques.
- The signal in ESI showed a more dramatic response to hydrophilicity when acetonitrile was used than when water was used as a solvent.
- His-Leu shows the same preference for water in both techniques. The dipeptide's ability to gain or lose a proton on its His side-chain may increase its ability to ionize.
- Signal levels when acetonitrile was used as a spray solvent were not dramatically higher than when water was used, contrasting with DESI results (Figure 4).

- Figure 6 shows that increased peptide hydrophilicity correlates with increased retention (lower R<sub>f</sub> values) on both (a) cellulose and (b) silica plates.

- No correlation between hydrophilicity or R<sub>f</sub> values and peptide signal response was found in HPTLC/DESI-MS of peptide mixtures.
- Numerous peptides had similar R<sub>f</sub> values, evident in the closely spaced bands visible in Figure 7.



## CONCLUSIONS

- Solvent composition and TLC solid phase are important parameters in HPTLC/DESI-MS of dipeptides.
- Hydrophobic dipeptides showed higher signal response than hydrophilic peptides, and acetonitrile as a spray solvent gave a higher signal than water in general.
- Hydrophilicity effects on signal were suppressed on normal phase plates, but a clear correlation between decreasing hydrophilicity and increasing signal was observed on reversed-phase plates.
- The same trend between hydrophilicity and signal for a set of dipeptides was observed in both DESI and ESI, indicating a similarity in ionization mechanisms between the two methods.
- Hydrophilicity values were useful in predicting retention behavior on TLC for protein tryptic digests, but did not correlate with signal intensity.

## FUTURE WORK

- Altering the pH of the DESI spray solvent and TLC development solvent could affect the charge of the peptides, improving ionization of basic and acidic peptides.
- Solvent evaporation plays a role in transporting analyte ions from surfaces to the gas phase – perhaps increasing surface and sampling temperature can improve peptide ionization efficiency.
- In both ESI and DESI, a method is needed that limits the amount of peptide-peptide signal suppression in mixture characterization.

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