

HPTLC/DESI-MS Imaging of Tryptic Protein Digests Separated in Two Dimensions

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OVERVIEW

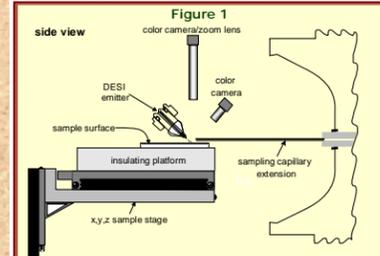
- Desorption electrospray ionization mass spectrometry (DESI-MS) is rapidly developing as a surface sampling/ionization source for the interrogation of a wide variety of analytes on a broad range of surfaces under ambient conditions.
- Here we investigate HPTLC/DESI-MS in an imaging mode for detection and identification of tryptic peptides from two-dimensional separations of cytochrome c and myoglobin digests.
- Data-dependent tandem mass spectra and protein database searches were used to identify peptide ions and peptide distributions were mapped for each separated protein digest.
- Sequence coverages determined from the HPTLC/DESI-MS/MS data were 81% for cytochrome c and 74% for myoglobin, comparing well to those determined using the standard HPLC/ESI-MS/MS approach (92% and 84%, respectively).
- Use of more sensitive mass spectrometry instrumentation leads to improved detection of low R_f peptides, and should allow decreased plate loadings and improved sequence coverages.

INTRODUCTION

- DESI has been shown to have great potential as a means to couple thin-layer chromatography (TLC) and mass spectrometry (MS) for the analysis of analytes separated on TLC plates.¹⁻⁴
- In a previous report we demonstrated the potential of high performance (HPTLC/DESI-MS/MS) for the analysis and identification of protein tryptic digests separated in one dimension (1D) on HPTLC plates.⁵
- Results suggested that two-dimensional (2D) HPTLC separations would provide less peak overlap and less potential for signal suppression, thus improving the number and quality of the peptide identifications by DESI-MS/MS.
- Imaging via multiple equally-spaced line scans is an all-encompassing discovery tool, and can be used even when the spatial locations of analyte spots on a TLC plate cannot be accurately determined.
- Manual or automated spot sampling can only be used if the band is visible or the spatial location of the band on the plate is otherwise known.
- The research presented here is focused on the further development of DESI-MS as a tool for readout of 2D separations on TLC plates.
- In this work we
 - Describe the use of DESI-MS/MS in an imaging mode for detection and identification of tryptic peptides from two 2D TLC separations of cytochrome c and myoglobin digests on ProteoChrom[®] HPTLC Cellulose layers.
 - Determine protein sequence coverages and map the two-dimensional distributions of identified peptides for each separated protein digest.

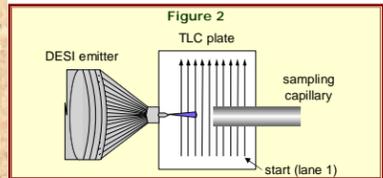
EXPERIMENTAL

- Materials and Reagents.** 2-butanol, pyridine, acetic acid, ammonia, ninhydrin, ammonium bicarbonate, and trypsin were obtained from Merck KGaA. HPLC grade water was purchased from J. T. Baker. Bovine heart cytochrome c (CAS 9007-43-6) was obtained from Sigma-Aldrich. Horse skeletal muscle myoglobin (CAS 100684-32-0) was obtained from Calbiochem. The ProteoChrom[®] HPTLC Cellulose sheets used for the separations and the ProteoChrom[®] Color Peptide Staining Kit used for staining the developed plates were obtained from Merck KGaA.
- Tryptic Digest.** Each model protein was dissolved in 25 mM ammonium bicarbonate buffer. The final protein concentration was ~2 µg/mL. Trypsin was added to the protein buffer mixtures such that the trypsin:protein ratio was 1:100. The mixtures were incubated for 15 h at 37 °C.
- Thin-Layer Chromatography.** Separations were performed on ProteoChrom[®] HPTLC Cellulose sheets. Samples were applied as 1 mm bands using an ATS 4 fully automated sample applicator. The total sample volume was 10 µL, giving a total of ~20 µg protein per band. Development was carried out in a normal flat bottom chamber using 2-butanol/pyridine/acetic acid/water (45/30/9/36, v/v/v/v) for the first dimension, and butanol/pyridine/ammonia/water (39/34/10/26, v/v/v/v) for the second dimension. The migration distance, achieved in ~45 min, was 50 mm in both dimensions. The ProteoChrom[®] Color Peptide Staining Kit was used to stain the cellulose sheets, following the manufacturer's guidelines.
- HPLC-MS.** HPLC separations were conducted on an Agilent 1100 Series Capillary LC System. Separation was achieved on an Acclaim PepMap 100 C18 column (1 mm x 150 mm; 3 µm particle size; Applied Biosystems) at ambient temperature. The column was equipped with a ColumnSaver[™] precolumn filter (MacMod Analytical Inc.). The mobile phase consisted of water (0.1% formic acid) (A) and 80% acetonitrile/20% water (0.1% formic acid) (B). The mobile phase was applied in a gradient elution starting at 0 min with 96% A : 4% B and changed over the next 30 min to 80% A : 20% B, and then held at 80% A : 20% B for 20 min. Each run was followed by a 20 min equilibration period. The flow rate was 30 µL/min and the injection volume was 1 µL. The LC system was coupled to a LCQ Deca ion trap (Thermo Scientific, San Jose, CA) operated using Xcalibur version 1.3 software.
- DESI-MS.** The manual- and computer-controlled x, y, z sample stage is shown in Figure 1. The mass spectrometer used was either a ThermoFinnigan LCQ Deca ion trap or the LTQ portion of a LTQ FT Ultra Hybrid Mass Spectrometer (Thermo Scientific, San Jose, CA, USA). The extended atmospheric sampling heated capillary allowed the instruments to be interfaced with automated MS2000 x, y, z robotic platforms (Applied Scientific Instrumentation Inc., Eugene, OR) for scanning purposes. For more details on instrumental setup see ref. 2 and 4. Water was used as a spray solvent at a flow rate of 5 µL/min.



DATA ACQUISITION

- Multiple Lane Scans and Data Acquisition.** A spatially-resolved chemical image of the peptides separated onto the surface of the TLC plate was accomplished by conducting a series of computer-controlled unidirectional lane scans through the area of interest on the plates (Figure 2).



Mass spectrometric data acquisition was started at the beginning of every lane scan by a trigger signal from the software controlling the stage.⁶ Acquisition time was set according to the time necessary to scan a lane. Automatic gain control was used for all measurements. The full scan mass spectra (m/z 200–2000) and data dependent tandem mass spectra (MS/MS product ion spectra, dynamic exclusion disabled) for each lane scan were stored as separate data files. The two most abundant peaks in every full scan mass spectra were subjected to MS/MS analysis at a normalized collision energy of 35%. Three microscans were acquired per spectrum. The lane spacing was 0.7 mm for cytochrome c and 0.75 mm for myoglobin.

- Peptide Identification and Spatial Location Assignment for Image Generation.** After all lanes were scanned, MS/MS spectra were extracted from the collected RAW files, converted to MS2 format,⁷ searched using the proteomics database search program DSDigger⁸ including the MASPIC⁹ scoring scheme, and filtered using the DTASelect algorithm¹⁰ with ACN of at least 0.08, and cross-correlation (X_{corr}) scores of at least 20 (+1), 25 (+2) and 40 (+3). For identified peptides, a surface position was calculated corresponding to the location at which the MS/MS spectrum was acquired. The x coordinate was calculated by multiplying the surface scan rate and the time at which the specific MS/MS spectrum was acquired during a particular lane scan. The y coordinate was calculated using the lane spacing and the lane number corresponding to the MS2 file containing the specific MS/MS spectrum.

RESULTS AND DISCUSSION

- 1D separations**
 - Faster and easier to scan using DESI-MS.
 - Overlapping peptide bands may lead to signal suppression and lower sequence coverages.
- 2D separations**
 - Most effective 2D separations will have peptides distributed over the entire area of the plate.
 - In the 2D separations shown in Figures 3 a and b, separation selectivity was similar for the two solvent systems used in the separations.
- HPTLC/DESI-MS of 2D separations**
 - Only unstained separations were imaged.
 - Imaging was used instead of spot sampling because the precise location of each peptide spot on an unstained plate could not be located.
 - A benefit of imaging is that any peptide spot can be scanned multiple times for a "peak parking" effect.

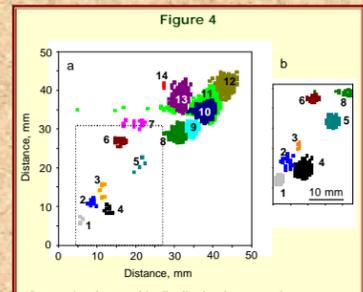


Figure 4. 2D map showing peptide distribution for a cytochrome c tryptic digest separated on a ProteoChrom[®] HPTLC Cellulose sheet acquired using an LCQ Deca Ion Trap mass spectrometer. Inset: peptide distribution acquired using an LTQ FT Ultra Hybrid.

Table 1. Peptides detected, spectral counts, charge states and sequence coverages for cytochrome c tryptic digests separated using 2D HPTLC and analyzed using DESI-MS/MS and separated using HPLC and analyzed using ESI-MS/MS. Identifying numbers in column 2 refer to Figure 4.

Peptide	ID	Charge State	Spectral Counts		
			HPTLC/DESI-MS/MS (LCQ)	HPTLC/DESI-MS/MS (FT)	HPLC/ESI-MS/MS
KRGER	1	+1	1	20	---
KRER	2	+2	11	20	---
KRNE	3	+1	7	7	2
KK	4	+1	8	32	---
KTQAGPSYDANK	5	+2	4	44	4
KRGRDLAYLK	6	+2	15	25	4
KRRRDLAYLK	7	+2	12	---	---
KVPIPK	8	+1	41	---	---
KIPVK	9	+2	38	---	14
KIPVK	9	+2	34	---	4
TEPNIHLGK	10	+2	100	---	6
IPVVK	11	+1	122	---	4
MRGAK	12	+2	75	---	3
EDLAIYK	13	+1	90	---	8
GTYWELTMEYLNK	14	+2	3	---	10
IPVQAGSYDANK	---	+2	---	---	1
ITQAGPSYDANK	---	+2	---	---	2
YTDANKNGITWGRLEMEYLNK	---	+1	---	---	2
KETWGRLEMEYLNK	---	+2	---	---	1
GTYWELTMEYLNK	---	+2	---	---	2
GTYWELTMEYLNK	---	+2	---	---	1
YKPIK	---	+1	---	---	1
YKPIK	---	+1	---	---	1
KRGRDLAYLK	---	+2	---	---	1
KRGRDLAYLK	---	+2	---	---	1
Sequence coverages, from MS/MS data	---	---	81%	---	89%

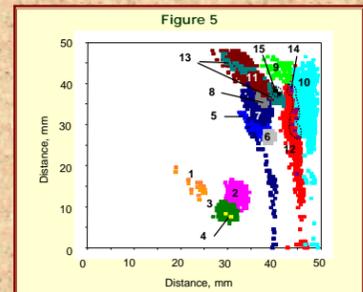


Figure 5. 2D map showing peptide distribution for a myoglobin tryptic digest separated on a ProteoChrom[®] HPTLC Cellulose sheet acquired using an LCQ Deca Ion Trap mass spectrometer.

Table 2. Peptides detected, spectral counts, charge states and sequence coverages for myoglobin tryptic digests separated using 2D HPTLC and analyzed using DESI-MS/MS and separated using HPLC and analyzed using ESI-MS/MS. Identifying numbers in column 2 refer to Figure 5.

Peptide	ID	Charge State	Spectral Counts		
			HPTLC/DESI-MS/MS	HPTLC/DESI-MS/MS	HPLC/ESI-MS/MS
ASLELAK	1	+1	9	---	---
KRRRDLAYLK	2	+2	81	---	---
KRRRDLAYLK	2	+2	28	---	---
KRRRDLAYLK	2	+2	24	---	---
KRRRDLAYLK	2	+2	36	---	---
KRRRDLAYLK	2	+2	75	---	---
KRRRDLAYLK	2	+2	---	---	4
KRRRDLAYLK	2	+2	16	---	2
KRRRDLAYLK	2	+2	30	---	2
KRRRDLAYLK	2	+2	---	---	2
KRRRDLAYLK	2	+2	77	---	---
KRRRDLAYLK	2	+2	98	---	4
KRRRDLAYLK	2	+2	---	---	5
KRRRDLAYLK	2	+2	181	---	---
KRRRDLAYLK	2	+2	57	---	2
KRRRDLAYLK	2	+2	97	---	2
KRRRDLAYLK	2	+2	---	---	2
KRRRDLAYLK	2	+2	204	---	---
KRRRDLAYLK	2	+2	48	---	8
KRRRDLAYLK	2	+2	---	---	4
KRRRDLAYLK	2	+2	15	---	2
KRRRDLAYLK	2	+2	11	---	---
KRRRDLAYLK	2	+2	243	---	4
KRRRDLAYLK	2	+2	55	---	2
KRRRDLAYLK	2	+2	9	---	---
KRRRDLAYLK	2	+2	---	---	7
KRRRDLAYLK	2	+2	---	---	2
KRRRDLAYLK	2	+2	---	---	3
KRRRDLAYLK	2	+2	---	---	2
KRRRDLAYLK	2	+2	---	---	2
KRRRDLAYLK	2	+2	---	---	2
KRRRDLAYLK	2	+2	---	---	2
KRRRDLAYLK	2	+2	---	---	4
KRRRDLAYLK	2	+2	---	---	4
Sequence coverages, from MS/MS data	---	---	74%	---	84%

- Characteristics of DESI-MS spectra affecting peptide identifications**
 - Lower incidence of multiple charging seen in DESI-MS spectra of proteins and peptides relative to spectra acquired using ESI-MS
 - Large peptides (>2000 Da) may fall outside the mass range of the mass spectrometer
 - Doubly-charged ions more readily identified from sequencing algorithms than singly-charged ions
 - Greater abundance of sodiated ions seen in DESI-MS spectra relative to ESI-MS spectra
 - Calcination reduces signal from desired protonated ions
 - Fragmentation patterns differ from protonated analogues

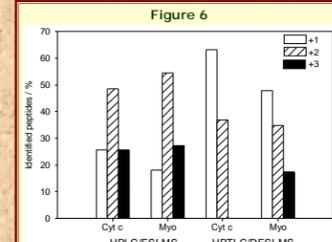


Figure 6. Percent identified peptides in the +1, +2, and +3 charge states for cytochrome c (Cyt C) and Myoglobin (Myo) tryptic digests separated using HPTLC and detected using DESI-MS and separated using HPLC and detected using ESI-MS.

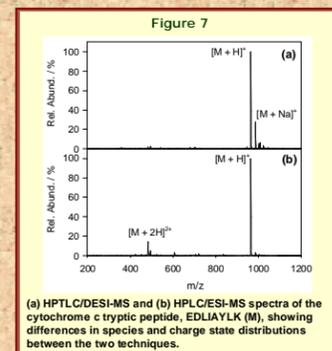


Figure 7. (a) HPTLC/DESI-MS and (b) HPLC/ESI-MS spectra of the cytochrome c tryptic peptide, EDLAIYK (M), showing differences in species and charge state distributions between the two techniques.

CONCLUSIONS

- We have demonstrated the potential of HPTLC/DESI-MS/MS imaging for determining the spatial distribution of peptides resulting from 2D separations of protein tryptic digests on HPTLC plates.
- Data-dependent tandem mass spectra and protein database searches were used to identify peptide ions, and peptide distributions were mapped for each separated protein digest.
- Sequence coverages for cytochrome c and myoglobin were 81% and 74%, respectively, and compare well to those from HPLC/ESI-MS/MS.

FUTURE WORK

- Use more sensitive mass spectrometry instrumentation to improve detection levels.
- Understand and improve desorption/ionization of low R_f peptides.
- Develop methods to improve abundances of multiply-charged ions.
- Investigate HPTLC/DESI-MS for analysis of proteins separated on HPTLC plates.

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