window was used in this method. Although the data demonstrated that over 80% of matrix peptides were removed from the trap column prior to the micro-LC-MS analysis, the assay sensitivity may have been compromised by the significant amount of matrix molecules captured by the trap column that could still co-elute with the target peptide.

[006] Methods and systems described herein utilize 2D-LC with mass spectrometry detection in a manner that, in some aspects, allows for both full automation and an increased sensitivity compared to 1D-LC-MS/MS methods.

SUMMARY

[007] This disclosure relates to methods of detecting an analyte in a sample. In some examples, the methods comprise: (a) performing high pH reversed phase liquid chromatography (RPLC) survey scan with detection by mass spectrometry (MS) on the analyte at previously determined concentration in the presence of an internal standard, to determine the peak apex (T_{apex}) of the analyte; (b) obtaining an experimental sample comprising the analyte; (c) performing high pH RPLC on the sample of (b); (d) collecting at least a portion of the high pH RPLC eluate of (c) corresponding to T_{apex} for the analyte +/- average peak width of the analyte or of the internal standard in the survey scan of (a), wherein the at least a portion is optionally collected in a trap column; (e) performing low pH RPLC on the collected portion of the high pH RPLC eluate of (d); and (f) detecting the analyte by MS. In one aspect, the disclosure provides a method of detecting an analyte in a sample, comprising: (a) performing high pH reversed phase liquid chromatography (RPLC) survey scan with detection by mass spectrometry (MS) on the analyte at previously determined concentration in the presence of an internal standard, to determine the peak apex (T_{apex}) of the analyte; (b) obtaining an experimental sample comprising the analyte; (c) performing high pH RPLC on the sample of (b); (d) collecting at least a portion of the high pH RPLC eluate of (c) corresponding to T_{apex} for the analyte +/- average peak width of the analyte or of the internal standard in the survey scan of (a), wherein the at least a portion is optionally collected in a trap column; (e) performing low pH RPLC on the collected portion of the high pH RPLC eluate of (d); and (f) detecting the analyte by MS. In some examples, the analyte is a peptide.

[008] The disclosure also relates to methods of detecting a peptide analyte in a sample, comprising: (a) obtaining an experimental sample and performing an affinity-based capture to isolate a protein in the sample; (b) digesting the captured protein with a protease to obtain a peptide analyte for detection; (c) performing high pH reversed phase liquid chromatography (RPLC) survey scan with detection by mass spectrometry (MS) on the peptide analyte at previously determined concentration in the presence of an internal standard peptide, to determine the peak apex (T_{apex}) of the analyte; (d) performing high pH RPLC on the digested sample of (b) comprising the peptide analyte; (e) collecting at least a portion of the high pH RPLC eluate of (d) corresponding to T_{apex} for the analyte +/- average peak width of the analyte, the internal standard, or a peptide in the survey scan of (c), wherein the at least a portion

is optionally collected in a trap column; (f) performing low pH RPLC on the collected portion of the high pH RPLC eluate of (e); and (g) detecting the analyte by MS. In one aspect, the disclosure provides a method of detecting a peptide analyte in a sample, comprising: (a) obtaining an experimental sample and performing an affinity-based capture to isolate a protein in the sample; (b) digesting the captured protein with a protease to obtain a peptide analyte for detection; (c) performing high pH reversed phase liquid chromatography (RPLC) survey scan with detection by mass spectrometry (MS) on the peptide analyte at previously determined concentration in the presence of an internal standard peptide, to determine the peak apex (T_{apex}) of the analyte; (d) performing high pH RPLC on the digested sample of (b) comprising the peptide analyte; (e) collecting at least a portion of the high pH RPLC eluate of (d) corresponding to T_{apex} for the analyte +/- average peak width of the analyte, the internal standard, or a peptide in the survey scan of (c), wherein the at least a portion is optionally collected in a trap column; (f) performing low pH RPLC on the collected portion of the high pH RPLC eluate of (e); and (g) detecting the analyte by MS.

[009] In any of the above methods, the high pH RPLC and the low pH RPLC can each be performed at microflow rates. In any of the above methods, the high pH RPLC and/or the low pH RPLC can be performed at a flow rate of 4-10 µL/min, 5-10 µL/min, 6-9 µL/min, 7-10 µL/min, 6-8 µL/min, 5 µL/min, 6 µL/min, 7 µL/min, 8 µL/min, 9 µL/min, or 10 µL/min. In some examples, the high pH RPLC and/or the low pH RPLC are performed on a C18 column. In some examples, both the high pH RPLC and the low pH RPLC are performed on a C18 column. In some examples, the high pH RPLC and survey scan are performed at a pH between 8 and 10, between 8 and 9, between 9 and 10, or at pH 8.0, pH 8.5, pH 9.0, pH, 9.5, or pH 10, and optionally with a mobile phase A comprising ammonium formate and a mobile phase B comprising ammonium formate and acetonitrile (e.g., ammonium formate in 90% acetonitrile). In some examples, the high pH RPLC survey scan and the high pH RPLC performed on the sample or digested sample are each performed at substantially the same flow rate, pH, temperature, pressure, and/or column type (e.g. C8, C18, etc.). In any of the above methods, the low pH RPLC can be performed at a pH between 2 and 4.5, between 2 and 4, between 2 and 3, between 2 and 2.5, between 2.5 and 3, between 3 and 4, or at pH 2.0, pH 2.5, pH 3.0, pH 3.5, pH 4.0, or pH 4.5, and optionally with a mobile phase A comprising formic acid and a mobile phase B comprising formic acid and acetonitrile (e.g., formic acid in acetonitrile).

[0010] In any of the above methods, the sample can be a biological sample, while in some cases, the same is not a biological sample. In some cases, the sample is a biological fluid sample, such as tears, saliva, lymph fluid, urine, serum, cerebral spinal fluid, pleural effusion, ascites, or plasma. In some examples, the sample is a serum sample. In some cases, the sample is a tissue sample. In some cases, the sample is treated to enrich or extract components comprising the analyte prior to use in the method.

[0011] In any of the methods herein, the portion of the high pH RPLC eluate on which the low pH RPLC is performed may correspond to a portion comprising T_{apex} for the analyte +/- 0.25 minutes at a flow rate of 8 µl/min in a C18 column. In some examples, the portion of the high pH RPLC eluate on which the low pH RPLC is performed corresponds to a portion comprising T_{apex} for the analyte +/- 0.3

minutes at a flow rate of 8 µl/min in a C18 column. In some examples, the portion of the high pH RPLC eluate on which the low pH RPLC is performed corresponds to a portion consisting of T_{apex} for the analyte +/- between 0.25 and 0.30 minutes at a flow rate of 8 µl/min on a C18 column.

[0012] In some examples, where the analyte is a peptide, the peptide or a protein comprising the peptide is captured by attachment to particles coated with a binding partner for the protein, such as a ligand or antigen. In some examples, the particles are coated with protein A, protein G, or a protein A and G chimera. In some examples, the protein is an antibody. In some examples, the analyte is a portion of an antibody (e.g., an antigen binding fragment such as a Fab, Fv, scFv, or $F(ab')_2$ fragment). In some cases, the analyte is an antigen or a drug binding target. In some examples, a protein analyte is cleaved into peptides and the peptides are analyzed as analytes in the methods herein. In some such cases, the protease or proteases used for the cleavage comprise one or more of: trypsin, endoproteinase LysC, endoproteinase ArgC, staph aureus V8, endoproteinase GluC, chymotrypsin, or papain.

[0013] In some cases, methods herein detect more than one analyte in the sample. Thus, in some cases, levels of two or more analytes are determined, and in some cases the methods detect a modified and unmodified form of a molecule and/or determine the ratio of a modified and unmodified form of a molecule. In some cases where the methods detect a modified and unmodified form of a molecule, the modified and unmodified forms comprise a modified and unmodified protein, such as an alkylated and nonalkylated protein or a glycosylated and unglycosylated protein, or a molecule and a metabolite of the molecule.

[0014] In some methods herein, the high pH RPLC and low pH RPLC and MS detection are automated. In some cases, detecting the analyte by MS following the low pH RPLC comprises quantitatively determining the amount or concentration of the analyte in the sample. In some examples, the MS is tandem MS ("MS/MS") (such as multiple reaction monitoring (MRM), single ion monitoring (SIM), triple stage quadrupole (TSQ), quadrupole/time of flight (QTOF), quadrupole linear ion trap (QTRAP), hybrid ion trap/FTMS, time of flight/time of flight (TOF/TOF), or tandem in time MS/MS).

[0015] In some examples, the at least a portion of high pH RPLC eluate is diluted in a low pH mobile phase prior to performing low pH RPLC, optionally

further wherein the dilution is automatically controlled. In some examples, the methods herein allow for at least 30-fold or for 30 to 100-fold greater sensitivity in analyte detection than a single dimension HPLC-tandem MS (1D-LC-MS/MS) process at microflow rates, and/or wherein the method allows for at least 100-fold greater sensitivity in analyte detection than a single dimension HPLC-tandem MS (1D-LC-MS/MS) process at analytical flow rates.

[0016] In some examples, the methods allow for detection of an analyte in a tissue sample at a concentration of less than 10 fmol/µg total protein, less than 1 fmol/µg total protein, 1-10 fmol/µg total protein, less than 0.1 fmol/µg total protein, 0.1-1 fmol/µg total protein, or between 0.01 and 0.1 fmol/µg total protein; and/or detection of an analyte in a fluid sample at a concentration of less than 10 ng/mL, less than 1 ng/mL, less than 100 pg/mL, or less than 10 pg/mL, for example, such as 1-10 ng/mL, 100pg/mL to 1 ng/mL, or 10-100 pg/mL.

[0017] This disclosure also encompasses systems for performing the methods herein. In some examples, the system comprises: (a) an injection valve for injecting a sample containing the analyte into the system, wherein the injection valve is connected to a high performance liquid chromatography (HPLC) column and a first dimension pump to control flow of sample and mobile phase through the column; (b) a trap column for collecting at least a pre-determined portion of a first dimension HPLC column eluate for analysis in a second dimension HPLC process; (c) a trap valve for controlling flow path of mobile phases from a first dimension HPLC process to a second dimension HPLC process; (d) a second dimension pump to control flow of mobile phases through a second dimension HPLC column; and (e) a mass spectrometer for analysis of analyte following a second dimension HPLC process. In one aspect, the disclosure provides a system for performing a two-dimensional liquid chromatography method for detecting an analyte in a sample, wherein the system comprises: (a) an injection valve for injecting a sample containing the analyte into the system, wherein the injection valve is connected to a high performance liquid chromatography (HPLC) column and a first dimension pump to control flow of sample and mobile phase through the column; (b) a trap column for collecting at least a pre-determined portion of a first dimension HPLC column eluate for analysis in a second dimension HPLC process; (c) a trap valve for controlling flow path of mobile phases from a first dimension HPLC process to a second dimension HPLC process; (d) a second dimension pump to control flow of mobile phases through a second dimension HPLC column; and (e) a mass spectrometer for analysis of analyte following a second dimension HPLC process, wherein the injection valve and the trap valve positions are automatically controlled during a two-dimensional liquid chromatography process, and wherein the automatic control allows for only a pre-determined portion of eluate from the first dimension to be separated in the second dimension. In some systems, the mass spectrometer is a tandem mass spectrometer. In some cases, the injection valve and the trap valve positions can be automatically controlled during a two-dimensional liquid chromatography process, and wherein the automatic control allows for only a pre-determined portion of eluate from the first dimension to be separated in the second dimension. In some cases, the pre-determined portion of eluate from the first dimension corresponds to a portion comprising T_{apex} for the analyte +/- 0.25 minutes at a flow rate of 8 µl/min in a C18 column. In some cases, the pre-determined portion of eluate from the first dimension corresponds to a portion comprising T_{apex} for the analyte +/- 0.3 minutes at a flow rate of 8 µl/min in a C18 column. In some cases, the pre-determined portion of eluate from the first dimension

survey scan of (c), wherein the at least a portion is optionally collected in a trap column; (f) performing low pH RPLC on the collected portion of the high pH RPLC eluate of (e); and (g) detecting the PD-L1 peptide analyte by MS (e.g., by tandem MS such as multiple reaction monitoring (MRM), single ion monitoring (SIM), triple stage quadrupole (TSQ), quadrupole/time of flight (QTOF), quadrupole linear ion trap (QTRAP), hybrid ion trap/FTMS, time of flight/time of flight (TOF/TOF), or tandem in time MS/MS).

[0022] It is to be understood that both the foregoing description and the following further description are exemplary and explanatory only and are not restrictive of the claims.

[0023] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate one (several) example(s) and together with the description, serve to explain certain principles described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] Figure 1 shows the overall workflow of the reagent-independent, targeted 2D-LC-MS/MS method.

[0025] Figure 2 shows the setup for determining the retention time window for analyte transfer by High-pH RPLC-MS/MD survey scan analysis. The retention time window is defined as T_{apex} +/- 0.3 min, where T_{apex} represents the retention time at the peak apex of the analyte.

[0026] Figure 3 shows the schematic workflow of ultra-targeted 2D-LC-MS/MS with a targeted-2D-with-dilution configuration. In the figure, simple dashed lines show the flow path of the high-pH mobile phase, solid lines show the flow path of low-pH mobile phase, composite dotted and dashed lines show the flow path of a mixture of the high- and low-pH mobile phases, while arrows show the location of the analyte. The method consists of five steps: (Step 1) load sample and equilibrate columns; (Step 2) inject sample and separate the analyte from matrix molecules using a linear high-pH RPLC gradient; (Step 3) transfer analyte fraction/portion within a 0.6-min retention time window (T_{apex} +/- 0.3 min) onto the trap column, and dilute the fraction/portion with the second-dimension low-pH mobile phase A; (Step 4) elute the analyte through the analytical column using a linear low-pH RPLC gradient for MS analysis, and in the meantime elute matrix molecules from the high-pH RPLC column