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 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, wherein the protein is captured by attachment to particles coated with a binding partner for the protein, wherein the particles are coated with protein A, protein G, or a protein A and protein G chimera, wherein the digesting is carried out on the particles, and wherein the protein is an antibody or an antibody-antigen complex.

[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

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 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

What is Claimed is:

1. 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,

wherein the protein is captured by attachment to particles coated with a binding partner for the protein, wherein the particles are coated with protein A, protein G, or a protein A and protein G chimera,

wherein the digesting is carried out on the particles, and

wherein the protein is an antibody or an antibody-antigen complex.

2. The method of claim 1, wherein the high pH RPLC and the low pH RPLC are each performed at microflow rates.

3. The method of claim 1 or 2, wherein the high pH RPLC is performed on a C18 column, the low pH RPLC is performed on a C18 column, or both the high pH RPLC and the low pH RPLC are performed on a C18 column.

4. The method of any one of claims 1-3, wherein 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.

5. The method of any one of claims 1-4, wherein 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.

6. The method of any one of claims 1-5, wherein the low pH RPLC is 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.

7. The method of any one of claims 1-6, wherein the sample is a biological sample.

8. The method of claim 7, wherein the sample is a biological fluid sample, optionally comprising tears, saliva, lymph fluid, urine, serum, cerebral spinal fluid, pleural effusion, ascites, or plasma, or wherein the sample is a tissue sample; optionally wherein the sample is treated to enrich or extract components comprising the analyte prior to use in the method.

9. The method of any one of claims 1-8, wherein 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 +/- 0.25 minutes at a flow rate of 8 µl/min in a C18 column, T_{apex} for the analyte +/- 0.3 minutes at a flow rate of 8 µl/min in a C18 column, or T_{apex} for the analyte +/- between 0.25 and 0.30 minutes at a flow rate of 8 µl/min on a C18 column.

10. The method of any one of claims 1-9, wherein the protease comprises one or more of: trypsin, endoproteinase LysC, endoproteinase ArgC, staph aureus V8, endoproteinase GluC, chymotrypsin, or papain.

11. The method of any one of claims 1-10, wherein the method detects more than one analyte in the sample, optionally wherein levels of two or more analytes are determined, and optionally wherein the more than one analyte comprises a modified and unmodified form of a molecule.

12. The method of claim 11, wherein the method detects a modified and unmodified form of a molecule, wherein the modified and unmodified forms comprise a modified and unmodified protein.

13. The method of any one of claims 1-12, wherein detecting the analyte by MS following the low pH RPLC comprises quantitatively determining the amount or concentration of the analyte in the sample.

14. The method of any one of claims 1-13, wherein the MS is tandem MS (MS/MS), 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.

15. The method of any one of claims 1-14, wherein the method allows for at least 30-fold or for 30 to 100-fold greater sensitivity in analyte detection than a single dimension high performance liquid chromatography-tandem (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.

16. The method of any one of claims 1-15, wherein the method allows 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 wherein the method allows for 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, less than 10 pg/mL, or at a concentration of 1-10 ng/mL, 100pg/mL to 1 ng/mL, or 10-100 pg/mL.