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MASS-DIRECTED PURIFICATION OF PROTEINS AND PEPTIDES*

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OVERVIEW

Experiments were carried out to evaluate the fractionation of proteins and peptides according to mass. Model mixtures were separated by either reversed-phase or ion-exchange chromatography with mass spectrometry-compatible (MS-compatible) mobile-phase additives. Fraction collection was triggered by the mass-to-charge ratio (m/z) of each one of the components of the mixture. Chromatography was additionally monitored with a UV/Vis detector in order to compare the new technique with one generally accepted in separations. The results indicated that superior purification is achieved by this new technique. Fraction collection triggered by changes in the m/z ratio reduces sample handling and analysis time. This study demonstrates the utility of mass-directed fractionation of peptides and proteins when MS-compatible mobile-phase additives are used.

METHODS

Equipment:
FractionLynx™ AutoPurification System:
Waters® 2767 Sample Manager,
Waters® 2525 Binary Gradient Module,
Waters® 2996 PDA Detector and a
Micromass® ZQ™ Mass Spectrometer

MATERIALS

Bovine pancreas insulin chain B (oxidized form), oxytocin, vasopressin, bovine pancreas ribonuclease A, horse cytochrome C, bovine heart cytochrome C, chicken egg lysozyme, ammonium formate and formic acid were purchased from Sigma (St. Louis, MO, USA).

INTRODUCTION

The purification of peptides and proteins usually requires the separation of the compounds of interest from impurities that are very similar in chemical characteristics. Chromatography is the predominant mode of isolation and purification of proteins and peptides. The most commonly used modes of chromatography for peptides and proteins are reversed-phase (RP) chromatography (for high resolution) and ion-exchange chromatography (exposes the compounds to relatively mild conditions). Traditional analyses consist of UV-Vis monitoring, collection of a rather large number of fractions and then analysis of those fractions in order to identify and verify the presence of the targeted compounds. Mass spectrometry (MS) has been extensively used for the analysis of peptides, proteins and their digests, as well as for the purification of combinatorial libraries. The benefit of MS directed purification does not only rest in the fact that compounds are identified on-line by their mass but also that the number of fractions is significantly reduced to the relevant fractions containing the compounds needed.

Reversed-Phase Chromatography

Model Mixture 1   (bovine pancreas insulin chain B (oxidized form), oxytocin and vasopressin)
Columns: Symmetry® C_{18} 4.6 x 50 mm and 7.8 x 50 mm
Mobile Phase A: 90:10 Water:1% Formic Acid
Mobile Phase B: 90:10 ACN: 1% Formic Acid
Gradient: 90% A to 60 % A over 10 min
Sample Concentration: 1 mg/mL (0.3 mg/mL of each compound)
Analytical Column: 1.8 mL/min and 1000 µL injection
Prep Column: 5.3 mL/min and 2875 µL injection
Detection/Collection: UV at 215 nm and fraction collection was carried out at a m/z ratio of 543, 1007.5 and 1148 for vasopressin, oxytocin and insulin bovine chain B, respectively.

Model Mixture 2   (horse cytochrome C and ribonuclease A)
Columns: Symmetry300™ C_{18}, 4.6 x 50 mm and 7.8 x 50 mm
Mobile Phases: as for Model Mixture 1
Gradient: 80% A to 60% A over 10 minutes
Sample Concentration: 1 mg/mL (0.5 mg/mL of each protein)
Analytical Column: 1.8 mL/min and 30 µL
Prep Column: 5.3 mL/min and 90 µL
Detection/Collection: UV @ 215 nm and fraction collection was carried out at a m/z ratio of 687.8 and 1053.8 for horse cytochrome C and ribonuclease A, respectively.

Ion-Exchange Chromatography:

Model Mixture 3   (bovine cytochrome C and lysozyme)
Column: Protein-Pak™ SCX 5 x 50 mm, 8 µm
Initial conditions: 525 mM ammonium formate, pH 4.5, fixed linear gradient of 4 mL/min
Sample Concentration: 2.5 mg/mL and 5 mg/mL of bovine cytochrome C and lysozyme, the injection volume was varied from 10 to 200 µL
Flow Rate: 1 mL/min
Detection/Collection: UV @ 280 nm and fraction collection was carried out at m/z ratio of 825.2 and 1101.9 for cytochrome C and lysozyme, respectively.

Isocratic Ion-Exchange Chromatography:

Model Mixture 3   (bovine cytochrome C and lysozyme)
Mobile Phase: Ammonium formate buffer, pH 4.5, various salt concentrations
Flow rate: 0.5 mL/min and 10 µL injections.
Detection: UV @ 280 nm.

**Mass Spectrometer Conditions (ES+):**

Capillary: 3.5 kV	Source: 150 ℃
Cone: 25 V	Desolvation: 350 ℃
Extractor: 3V	Desolvation Gas Flow: 500 L/hr
Rf lens: 0.3 V	Cone Gas Flow: 50 L/hr

RESULTS

— Model Mixture 1

Analytical results of Model Mixture 1. The compounds are 1) vasopressin, m/z 543; 2) oxytocin, m/z 1007.5; and 3) insulin chain B (oxidized form), m/z 1148. A) UV-Vis trace at 215 nm and B) Total ion chromatogram (ES+).
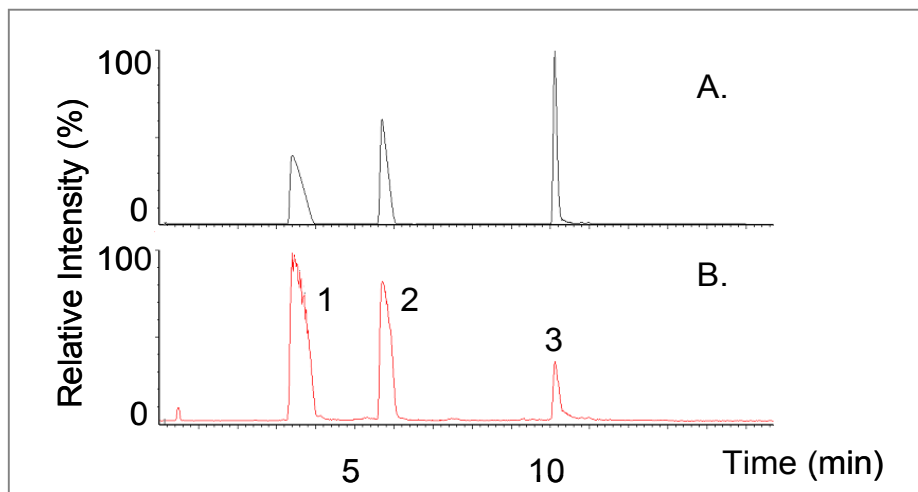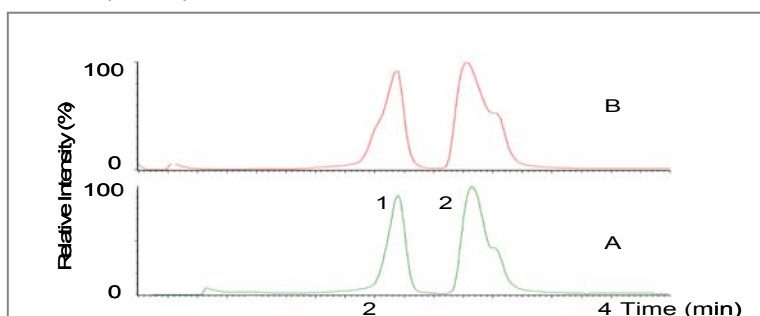

Mass-directed preparative results of Model Mixture 1 under ES+ ionization mode. A) Extracted ion chromatogram of vasopressin at m/z 543 (2 charge state). B) Extracted ion chromatogram of oxytocin at m/z 1007.5 (1 charge state). C) Extracted ion chromatogram for insulin bovine chain B at m/z 1148 (3 charge state). Results illustrate the ease of collection as well as the on-line identification of each one of the components.
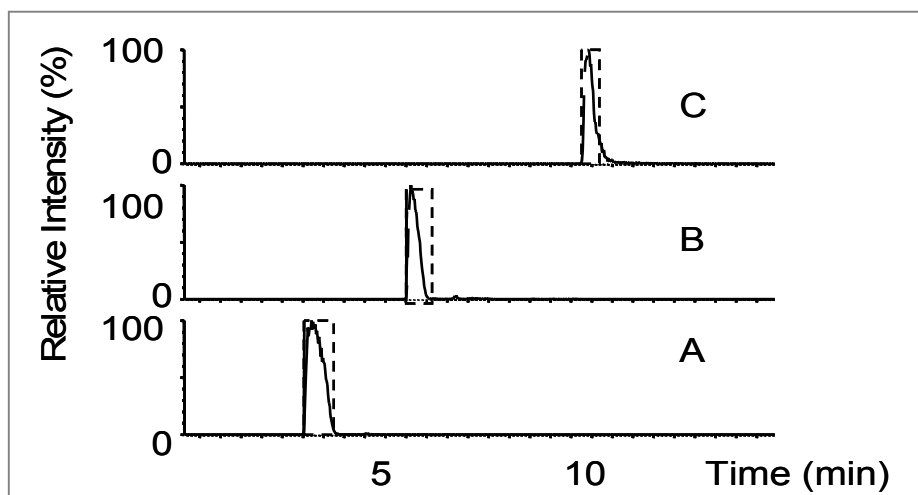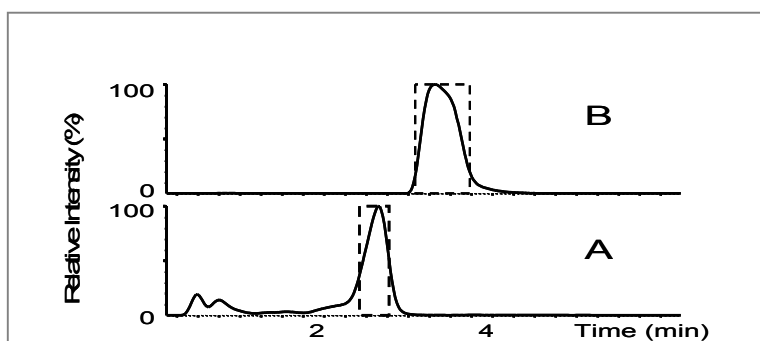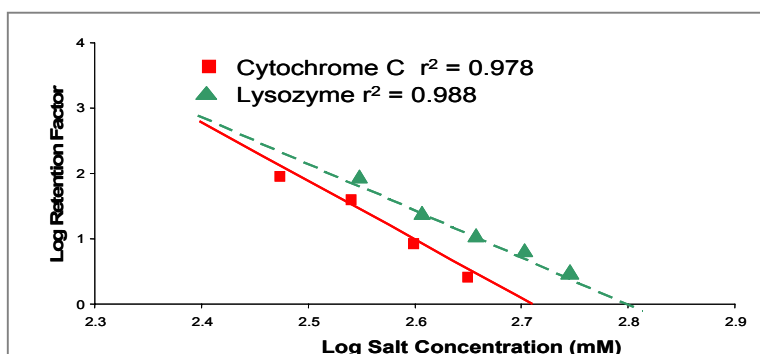
RESULTS -Model Mixture 2

UV/Vis traces of both the analytical and preparative scale chromatograms corresponding to Model Mixture 2 monitored at 215 nm. The compounds are: 1) ribonuclease A and 2) horse cytochrome C. A) Symmetry® 300 C18 4.6 x 50 mm and B) Symmetry 300™ C18 7.8 x 50 mm.


Mass-directed preparative results of Model Mixture 2 employing Symmetry® 300 C_{18} 7.8 x 50 mm column at the m/z ratio corresponding to each one of the compounds of the mixture. A) ribonuclease A, m/z 1053.3 and B) horse cytochrome C, m/z 687.8. Baseline resolution is observed and results in pure collected fractions.


RESULTS—Model Mixture 3

Individual isocratic runs of horse cytochrome C and chicken egg lysozyme were made, and the resulting log retention-salt concentration plot is shown below. The results indicate that the proteins have a diverging log(k)-salt plot. These types of problem mixtures separate successfully under linear gradient chromatography.

Electrospray spectra of the proteins in Model Mixture 3 indicating the maximums that were then selected for the mass-directed purification of the compounds. A) horse cytochrome C; m/z 825.3 and B) chicken egg lysozyme, m/z 1101.9.
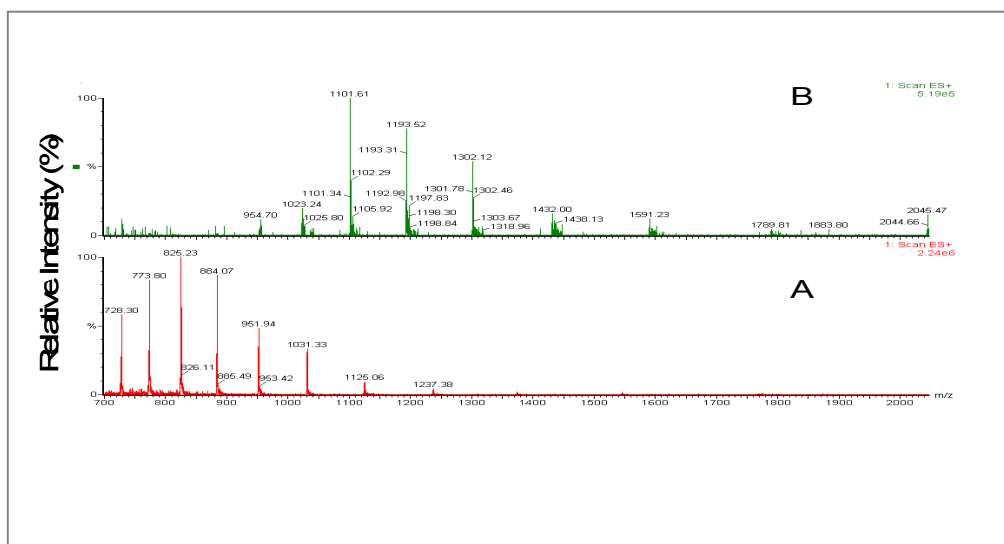
Results depicting the resolution between the proteins in Model Mixture 3 employing Protein-Pak™ SCX 5 x 50 mm column and a linear gradient. A) Extracted chromatogram of chicken egg lysozyme, m/z 1101.9. B) Extracted chromatogram of horse cytochrome C, m/z 825.3 and C) UV-Vis trace at 280 nm. Amount of material recovered is 95.7%. Dashed lines indicate fraction collected.

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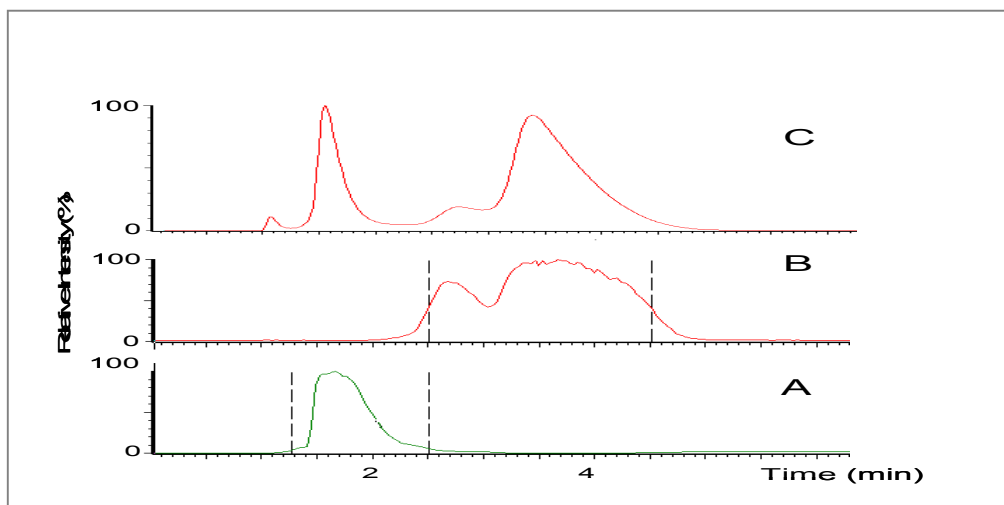
CONCLUSIONS

This work demonstrates the utility of purifying proteins and peptides by using reversed-phase or ion-exchange chromatography with MS-compatible mobile phases. Fraction collection is triggered by a mass spectrometer. Using this technique, a decrease in the number of fractions collected is possible compared to classical fraction collection techniques. In addition, *in-situ* verification of the fractions as well as automation is easily achieved. Further, by employing on-line identification of the eluting peaks, it is indeed possible to eliminate the requirement of analysis of the collected fractions.