Investigation of Low Molecular Weight Serum Components by an Automated Two-Stage NanoLC-MS Method

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Overview

A unique trap and column arrangement enables the automated analysis of complex mixtures of diverse compound types by nanoLC-MS. This method has been applied to the analysis of non-protein serum components, demonstrating the identification of both peptides and small molecules.

Introduction

Much emphasis has been placed on studying the serum proteome. However, the serum "peptidome" may not be addressed in standard proteomics experiments because it yields few meaningful tryptic peptides; non-polypeptide molecules are overlooked altogether.

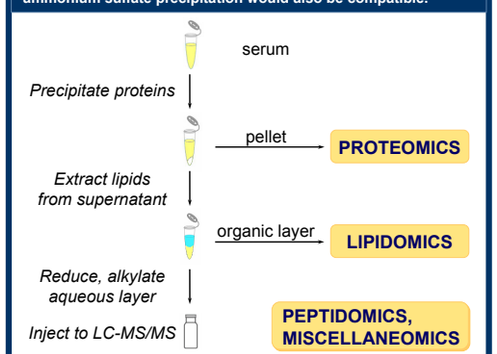
This smaller world is investigated by nano-LC-ESI-MS. Because of the diversity of compounds, no single HPLC stationary phase suffices. The Hypercarb stationary phase is able to retain hydrophilic and highly charged molecules which pass through the initial C18 phase.

Retention time reproducibility, accurate mass, and MS/MS are key elements of identification, particularly for the non-peptide compounds.

Methods

Sample Prep: Human serum was deproteinized and delipidated prior to use in this study. Serum was adjusted to 0.1% formic acid, then 75% acetonitrile, to precipitate proteins. After centrifugation, the supernatant was dried and resuspended in water. Lipids were removed by chloroform/methanol extraction[1]. The resulting aqueous phase was dried and resuspended in 10 mM TRIS pH 7.8, then reduced with DTT and carboxymethylated.

SCHEME 1. Sample preparation workflow allows for study of serum proteins and lipids, as well as the soluble serum fraction. Alternate methods for protein removal such as ammonium sulfate precipitation would also be compatible.



Methods *(continued)*

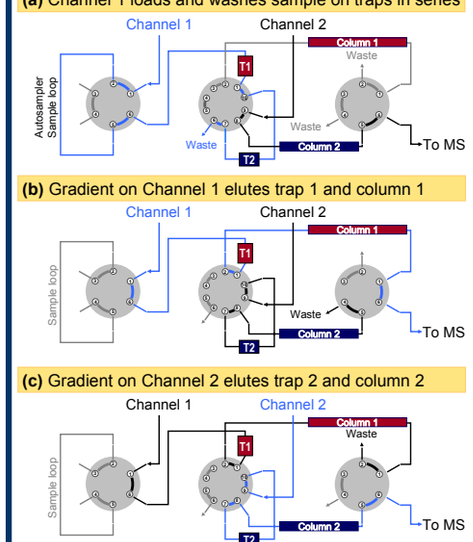
After appropriate dilution, samples were subjected to nanoLC-MS/MS analysis.

HPLC: The automated 2-phase HPLC method was set up using an Eksigent NanoLC-2D, as described in Scheme 2. Traps and columns were self-packed:
- Trap 1: 10 μm Poros (ABI), 0.150 x 15 mm
- Column 1: 5 μm Vydac C18 (Vydac), 0.075 x 250 mm
- Trap 2: 5 μm Hypercarb (Thermo), 0.150 x 15 mm
- Column 2: 5 μm Hypercarb, 0.075 x 100 mm

MS: An Advion NanoMate HD operating in LC mode was interfaced to a Micromass Q-Tof API US mass spectrometer. MS only and MS/MS survey data sets were acquired by means of duplicate injections. Mascot (www.matrixscience.com) and ProteinLynx were used to investigate the peptide data.

SCHEME 2. HPLC setup: Eksigent NanoLC-2D configured with two sets of low-flow gradient pumps, channel 1 and channel 2. The 10-port valve switches between loading traps and eluting columns; an additional 6-port valve directs the appropriate column effluent to the mass spectrometer.

(a) Channel 1 loads and washes sample on traps in series: 0.1% formic acid (FA), approx. 5 μL/min. (b) Channel 1 gradient elutes trap 1 and column 1: 350 nL/min, 0-80% 1B; 1A is 0.1% FA in water, 1B is 0.1% FA in acetonitrile. (c) Channel 2 gradient elutes trap 2 and column 2: 400 nL/min, 0-100% 2B; 2A is 20 mM aqueous ammonium acetate pH 6, 2B is acetonitrile.



Results

FIGURE 1. Low MW serum fraction. Elution from C18 is recorded from 0-60 minutes, elution from Hypercarb is recorded from 60-120 minutes. Compounds were affected differently by protein precipitations at neutral vs. acidic pH: some were more abundant in the acidic precipitation, and vice versa.

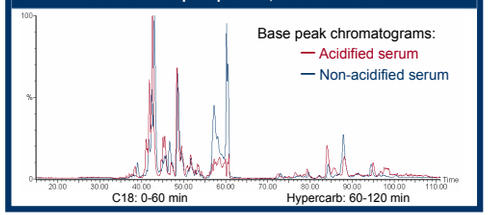


FIGURE 2. Fibrinogen peptides are readily identified. (a) EICs of Fibrinopeptide A and several variants. Modifications: phospho-serine, pyro-glutamic acid. MS/MS confirms that the phosphopeptides are observed on both stationary phases. (b) MS/MS spectra of corresponding modified peptides.

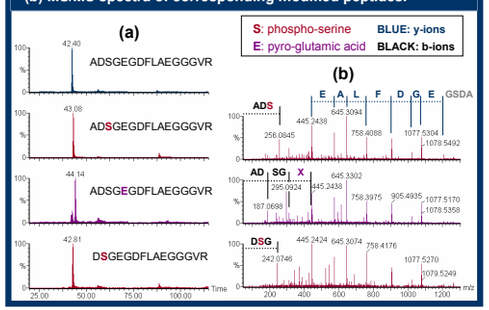


FIGURE 3. "Ragged" peptides. Coverage of fibrinogen is confined to two distinct sections of the fibrinogen sequence[2]. The families of peptides covering the two sections are listed. A similar phenomenon is observed with peptides matching one region of the Complement C3 sequence (see red box).

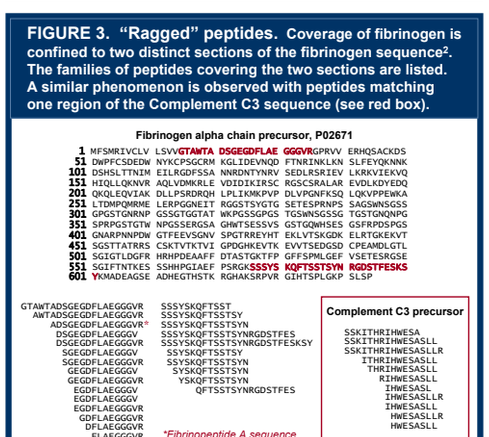
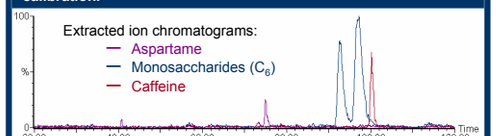
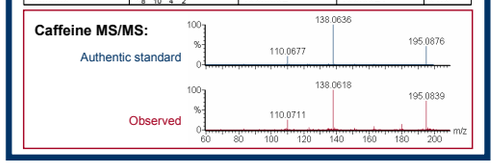


FIGURE 4. Small molecules on Hypercarb. A number of small molecules flow through the reverse phase and retain exclusively on the Hypercarb. Data were acquired with external calibration.



Compound	Molecular Formula	Theoretical (H+)	Observed (H+)	ppm
Aspartame	$C_{14}H_{18}N_2O_5$	295.1294	295.1247	15.9
Monosaccharides	$C_6H_{12}O_6$	181.0712	181.0684	15.5
Caffeine	$C_8H_{10}N_4O_2$	195.0882	195.0821	31.3



Conclusions

Methods are needed to investigate the smaller, but complex world of the low molecular weight serum fraction. The use of complementary stationary phases enables one to survey a broader range of compounds than either phase alone. Searchable libraries of small molecule electrospray MS/MS data would be extremely valuable in this application.

Further studies:

- Sample prep is key to meaningful results – compare sample prep procedures: use protease inhibitors; try alternate deproteinization methods and treatments to dissociate protein-bound components prior to protein removal; omit reduction and alkylation.
- Extend complementary phase concept to different stationary phases and solvent systems.
- Optimize method to focus on compounds of interest: parent ion discovery experiments coupled with peak parking can be a powerful combination for studying specific classes of compounds.

References

[1] Christie, W.W. *et al. Lipid Technology*, **1993**, *5*, 18-19.

[2] Similar peptide variants covering amino acids 19-35 of fibrinogen have been observed in human cerebrospinal fluid: Yuan, X., *et. al. J. Mass Spectrometry*, **2005**, *40*, 176-181.

Acknowledgements

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