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Modular Automated Processing System (MAPS) for higher throughput multistep serum processing

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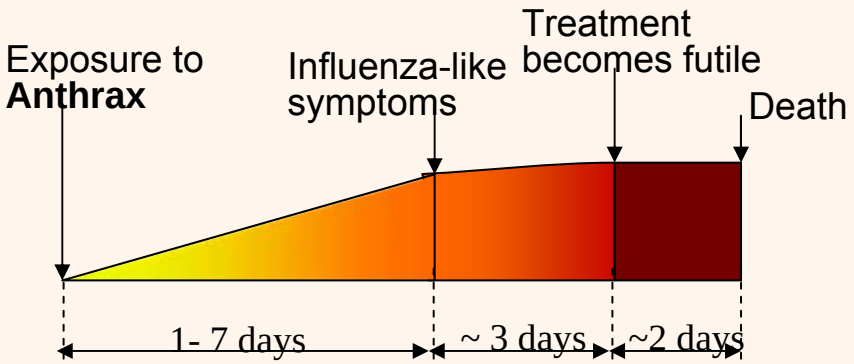
Why biomarker discovery at Sandia ?

Need: Early-stage protein biomarkers of infection to reduce the impact of outbreaks and bioterrorism attacks



- Rapidly evolving
- non-specific symptoms
- 100% mortality if detected late

Discovery of biomarkers of infectious diseases is an extreme bioanalytical challenge

-rapid sequence of biological events

-low concentrations and/or subtle posttranslational modifications

-high background of inflammatory response (specificity problematic)

-human samples scarce, highly pathogenic (small animal studies in BSL2-4 labs)

Challenges of biomarker discovery

technical:

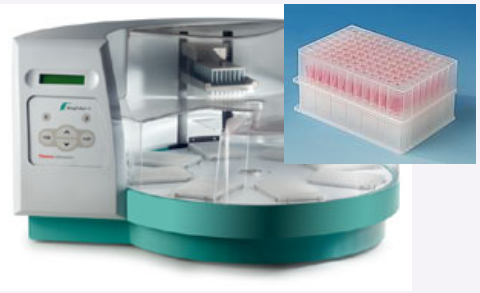
1. Complexity of proteome
2. High background
3. Relevant proteins are low-med abundance
4. Minute post-translational modifications

and biological: 5. Person-to-person variation

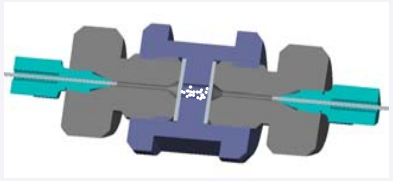
Hundreds of samples need to be processed in extensive clinical trials to identify, verify and validate **robust, specific biomarkers/ biomarker panels**

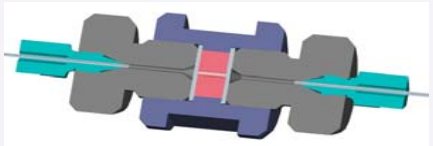
*"**Most biomarkers are not adopted for regulatory use because of absence of needed evidence"** Janet Woodcock, FDA*

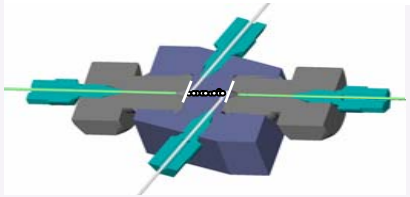
Commercial options for sample processing are benchtop, 1-2 dimesions & off-line

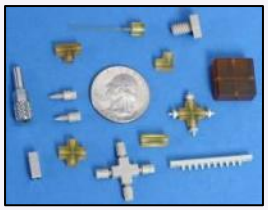


Sandia developed 1µl – 2 ml PEEK cartridges, miniature valves, fittings, micropumps, prototyping board, controllers for on-line low pressure processing in a modular architecture



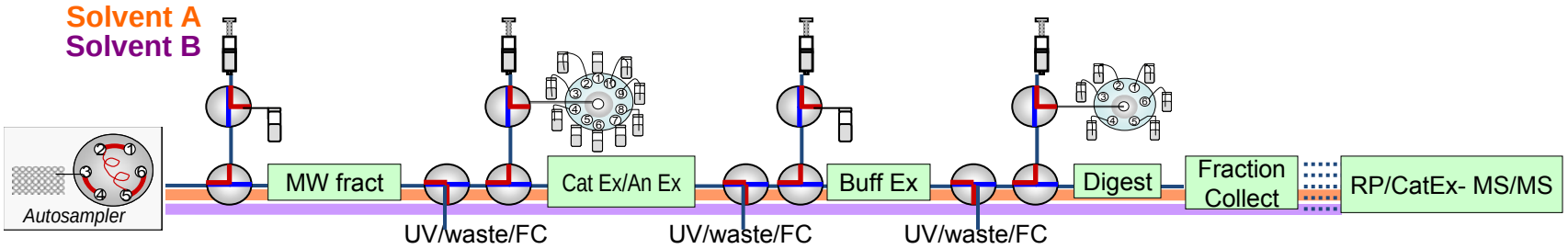


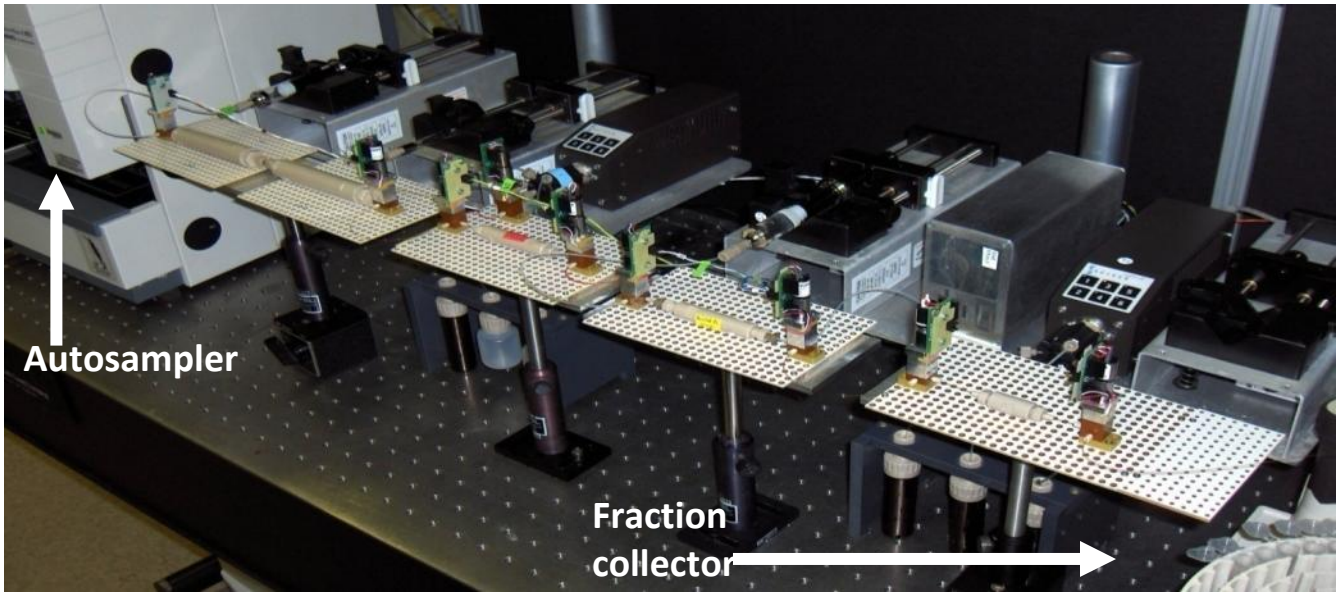




MAPS is a sum of Sandia-developed and commercial hardware for mesofluidic systems (µl- ml)

-**customized workflows** for various sample types (serum, urine, homogenized cells/tissues, etc.)
-**automated** systems = large number of samples, reproducibility, fine tuned optimization
-**multistep** processing

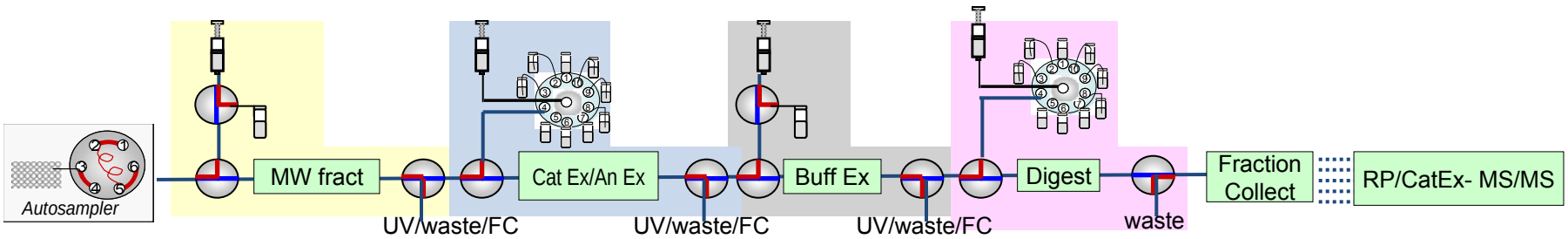




First setup for testing endogenous peptides in mouse serum

MAPS modules adapted and tested for on-line serum analysis

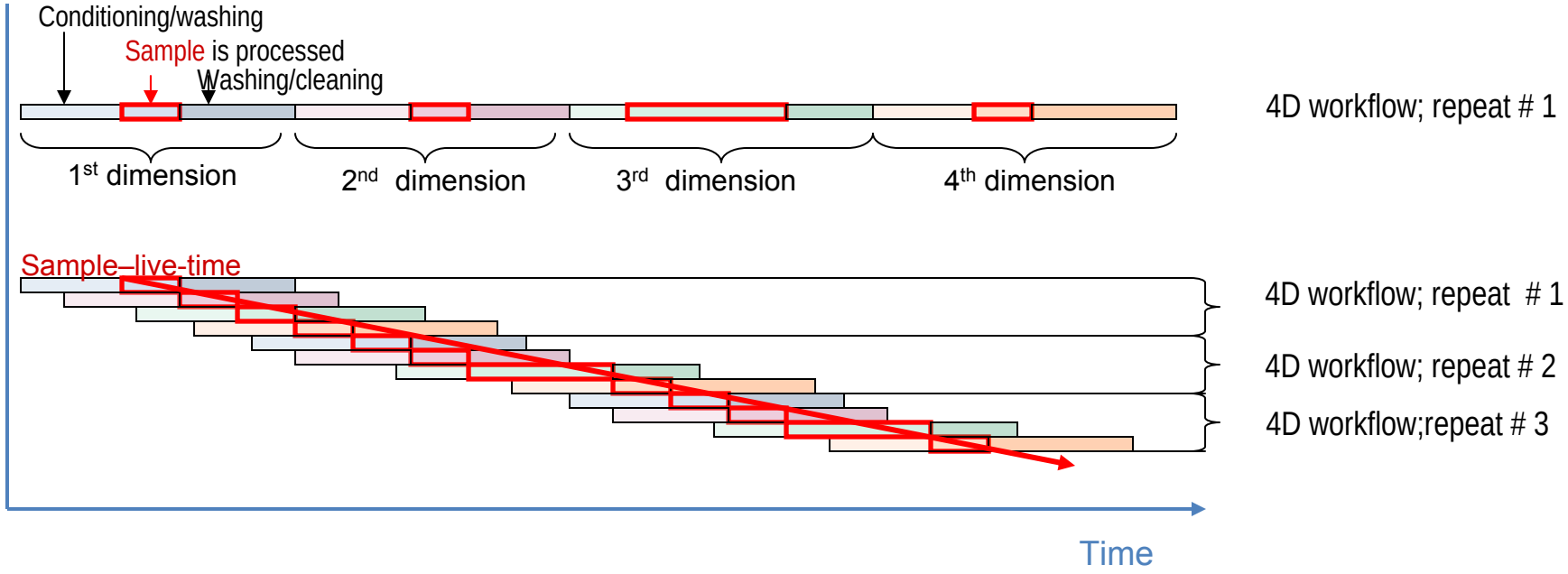
- **Protein concentration**
  - •ion-exchange, reversed-phase/hydrophobic interactions
  - •selective peptide enrichment (RAM CatEx, RAM-RP)
  - •phospho enrichment using IMAC sorbents
  - •glyco enrichment using lectin columns (ConA, WGA)
- **Protein depletion** (HAP removal)
- **Size-based fractionation of HMW/LMW proteins**
- **Desalting, buffer exchange, interferent removal** (size exclusion)
- **On-line enzymatic digestion**
- **Isoelectric fractionation**



Most modules consist of 3 valves +1 column

MAPS enables higher throughput

- Replaces manual labor
- 24/7 unattended operation
- Enables automated method optimization and troubleshooting
- Use of multiple setups (inexpensive, small footprint)
- Stringing “sample-live” processing steps



Multistep processing

WHY multistep?

-complex proteome overwhelms detection

-increasing separation dimensions reveals more proteins, more detail

-specific enrichment of targeted protein groups

Integration is the main challenge with on-line multistep processing

-requires exquisite fluidic control, timing for optimal peak transfer between dimensions

-real time monitoring/troubleshooting of individual/select groups of modules

-minimize peak splitting, carry-over

-**incompatibilities between separation dimensions** arise from

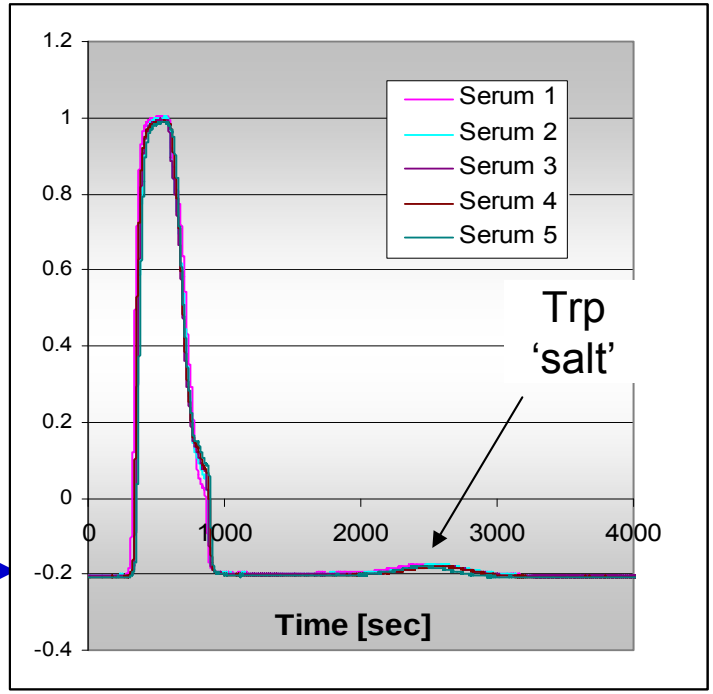
- buffer compositions
- variable capacities, operating parameters
- dispersive vs. concentrating methods

➔Need to make compromises
= thorough method development to check impact on output data

On-line buffer exchange module enables multi-module integration

- Adjustable to 5ul-500ul samples
- High-capacity packing
- Rapid separation
- Minimal sample dilution

Serum's high salt amount (150 mM) incompatible with downstream processing is reproducibly removed



Low pressure size-exclusion cartridge replaces centrifugation-based buffer exchange to amend matrix/buffer incompatibilities between separation methods

Analytical Chemistry 78, 5632

Putting all modules together

MAPS 8.0 software offers:

- **Control/test of 24 microvalves, 6 commercial valves, autosampler, 6 pumps, 2 fraction collectors**
- **Real-time 2 point UV monitoring during processing**
- **Unlimited number of steps (*210 used so far with 24/7 operation*)**

=> multidimensional analysis (6-10 dimensions) in serial and parallel configurations

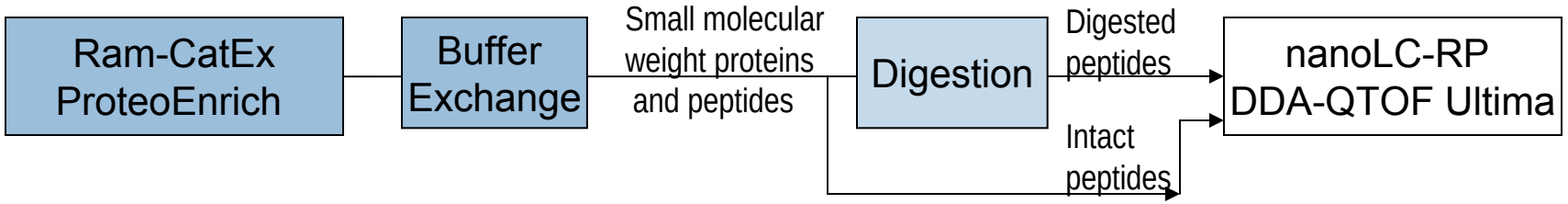
=> rapid prototyping, troubleshooting and method optimization

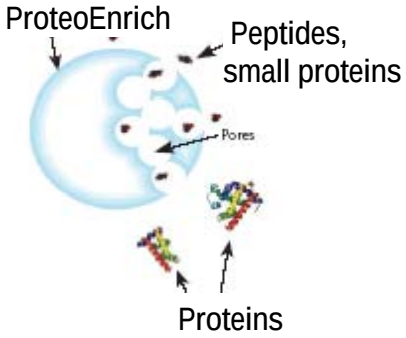
Jim Brennan and Stan Mrowka

VERSION	7.01				
File name					
# of Iterations	2				
comments	sting				
yringe Pump Setup					
Command Dt	10(	ms			
SP ID	(n	Fill Rate	FR units	ReFill Rate	RR units
1	0.	40	uL/min	400	uL/min
2	0.	125	uL/min	500	uL/min
3	4.6	100	uL/min	400	uL/min
4	7.3	125	uL/min	500	uL/min
5	4.6	100	uL/min	400	uL/min
6	4.6	100	uL/min	400	uL/min
Valve Setup (6 or 11 ports)					
Rheodyne #	10				

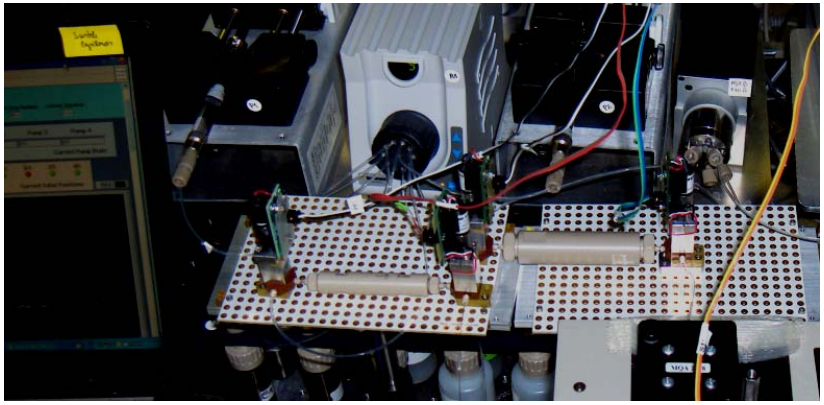
Step	T [s]	RO1	RO2	RO3	RO4	RO5	FC	AS
1	623	g	g	r	g	g	0	0
2	200	g	g	r	g	g	0	0
3	10	g	g	r	g	g	0	0
4	190	g	g	r	g	g	0	1
5	2100	g	g	r	g	g	0	0
6	82	g	g	r	g	g	0	0
7	217	g	g	r	g	g	0	0
8	120	g	r	r	g	g	0	0
9	480	g	g	r	g	g	0	0
10	1320	r	g	g	g	g	0	0
11	870	g	g	r	g	r	0	0
12	85	g	r	r	g	g	0	0
13	260	g	r	r	g	g	0	0
14	190	g	g	r	g	g	0	0
15	180	g	r	r	r	r	0	0
16	120	g	r	r	r	r	1	0
17	320	g	g	r	g	g	0	0
18	230	g	g	r	g	g	0	0
19	640	g	g	r	g	g	0	0
20	85	g	r	r	g	g	0	0
21	310	g	r	r	g	g	0	0
22	190	g	g	r	g	g	0	0
23	180	g	r	r	r	r	0	0
24	460	g	r	r	r	r	1	0
25	320	g	g	r	g	g	0	0
26	90	g	g	r	g	g	0	0
27	980	g	g	r	g	g	0	0
28	85	g	r	r	g	g	0	0
29	310	g	r	r	g	g	0	0
30	190	g	g	r	g	g	0	0
31	180	g	r	r	r	r	0	0
32	650	g	r	r	r	r	1	0
33	220	g	g	r	g	g	0	0
34	110	g	g	r	g	g	0	0
35	1060	g	g	r	g	g	0	0
36	85	g	r	r	g	g	0	0
37	260	g	r	r	g	g	0	0
38	190	g	g	r	g	g	0	0
39	180	g	r	r	r	r	0	0
40	120	g	r	r	r	r	1	0
41	220	g	g	r	g	g	0	0
42	330	g	g	r	g	g	0	0
43	640	g	g	r	g	g	0	0
44	40	g	g	r	g	r	0	0
45	160	g	g	r	g	r	0	0
46	40	g	g	r	g	r	0	0
47	160	g	g	r	g	r	0	0
48	30	g	g	r	g	r		
49	120	g	g	r	g	r		
50	30	g	g	r	g	r		
51	120	g	g	r	g	r	0	0
52	60	g	g	r	g	r	0	0
53	240	g	g	r	g	r	0	0

Workflow 1: Restricted Access Media Cation Exchange, RAM-CatEx

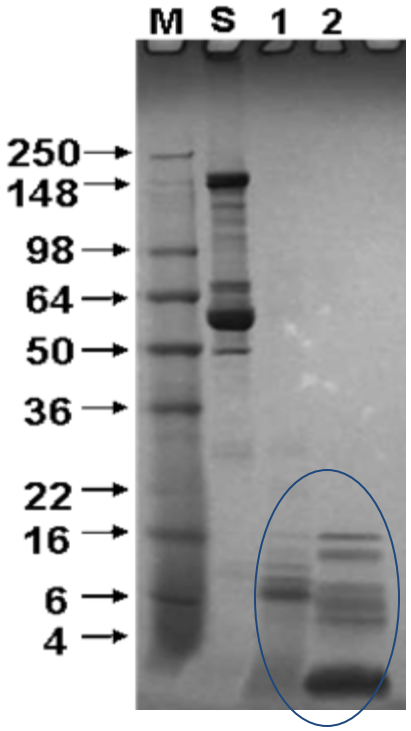




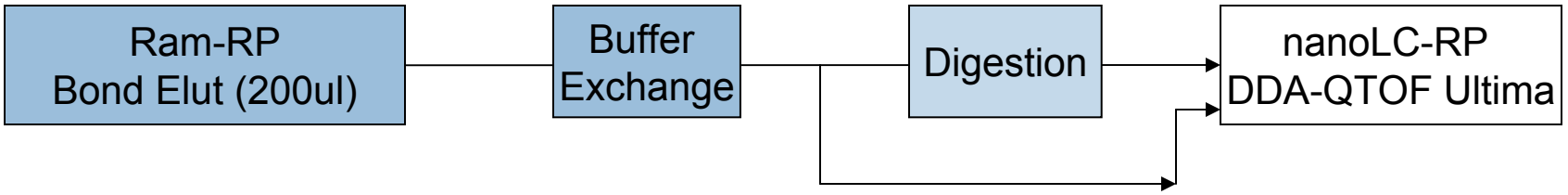
Restricted access media achieves simultaneous separation based on size and adsorption on derivatized inner pores

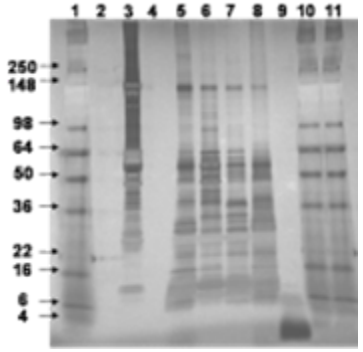


#	ID
M	marker
S	Mouse serum (5 ug)
1	RAM-CatEx-BE fraction 1 (5 ug)
2	RAM-CatEx-BE fraction 2 (5 ug)



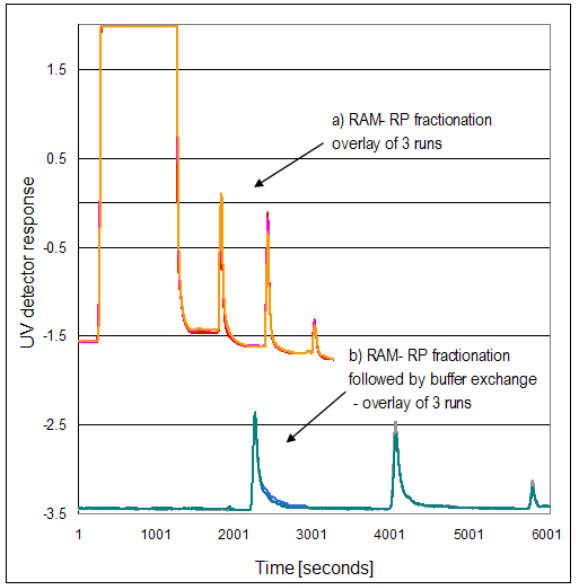
Workflow 2: Restricted Access Media Reversed Phase, RamRP



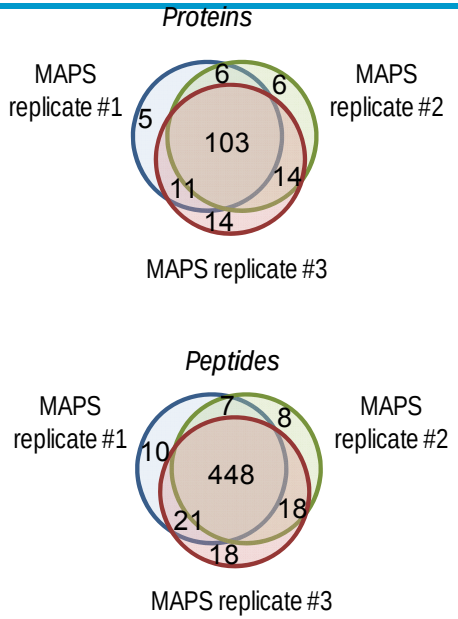


SDS PAGE of RamRP - BE fractions compared to serum.

Lane	
1	marker
2	blank
3	Mouse serum
4	Serum filtrate with 10kDaMWCO
5	RAM-RP 40% Methanol elution peak
6	RAM-RP 60% Methanol elution peak
7	RAM-RP 80% Methanol elution peak
8	RAM-RP 100% methanol elution peak
9	RAM-RP filtrate using 10kD MWCO
10,11	Marker



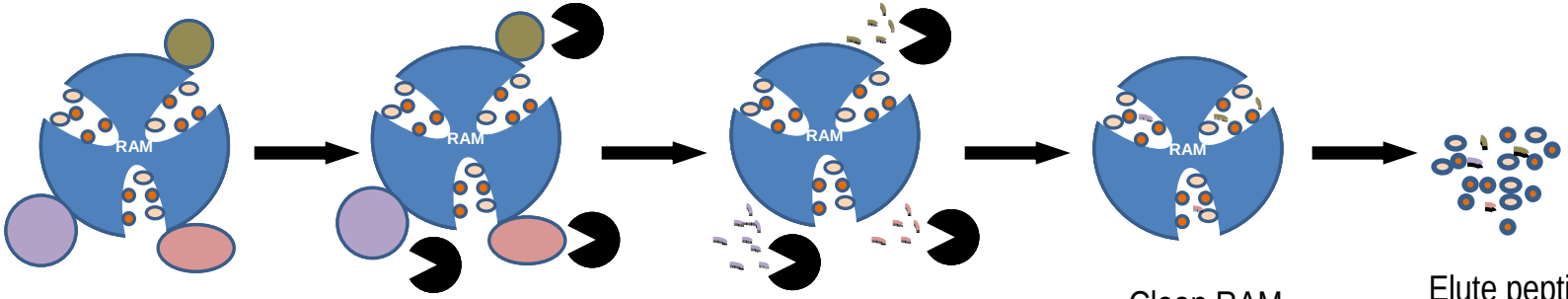
Reproducible processing *(under 6 % relative standard deviation for 19 runs)* requires only 3 replicas maximum protein identification

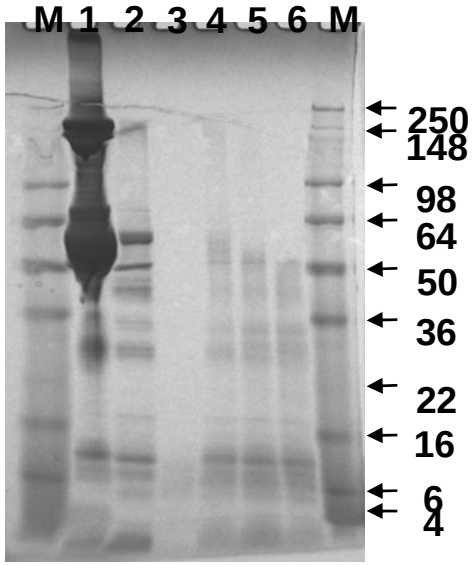


Number of proteins and peptides identified from three technical replicates of the same sample processed on MAPS with Ram-RP-BE.

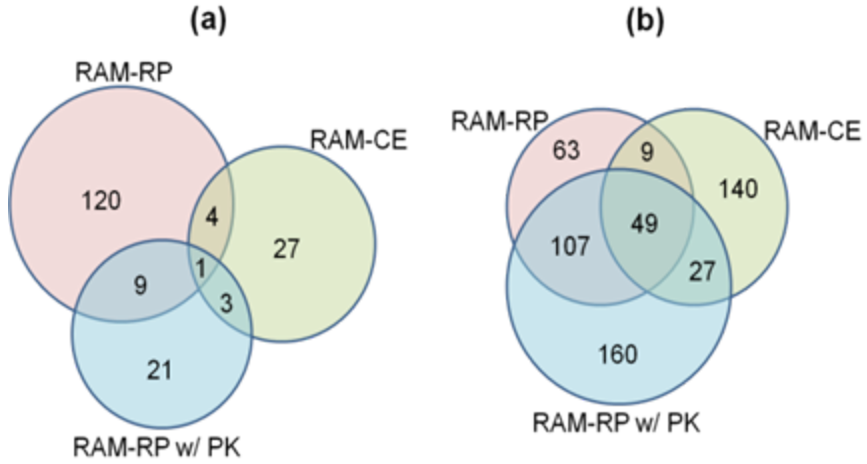
J Chromatography B 879,1112

Workflow 2b: RamRP with Proteinase K treatment (RAM-RP-PK)





M: marker
1: serum
2: RAM-RP
3: Proteinase K
4: PK120s RAM-RP
5: PK160s RAM-RP
6: PK240s RAM-RP



Overlay of identified proteins from
(a) tryptic digested peptides and (b) intact peptides

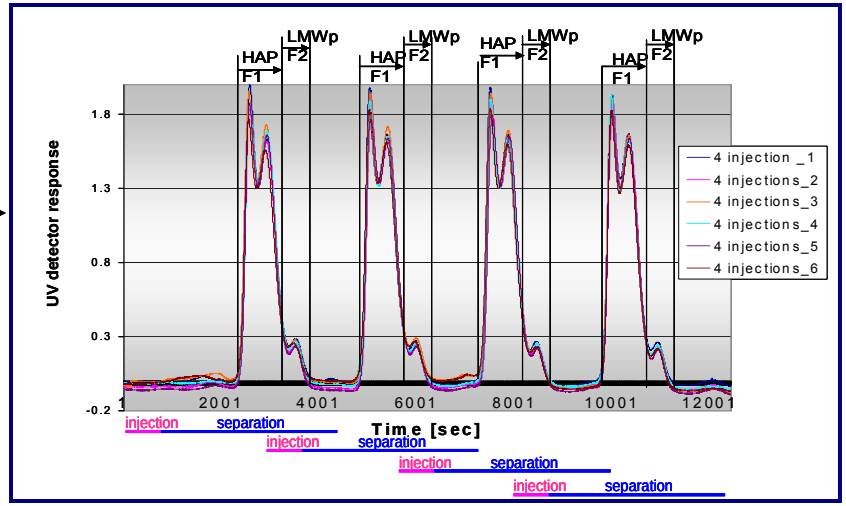
For peptidome analysis size based HAP depletion improves upon the capacity and throughput of immunodepletion

Size-based HAP depletion vs. immunodepletion:

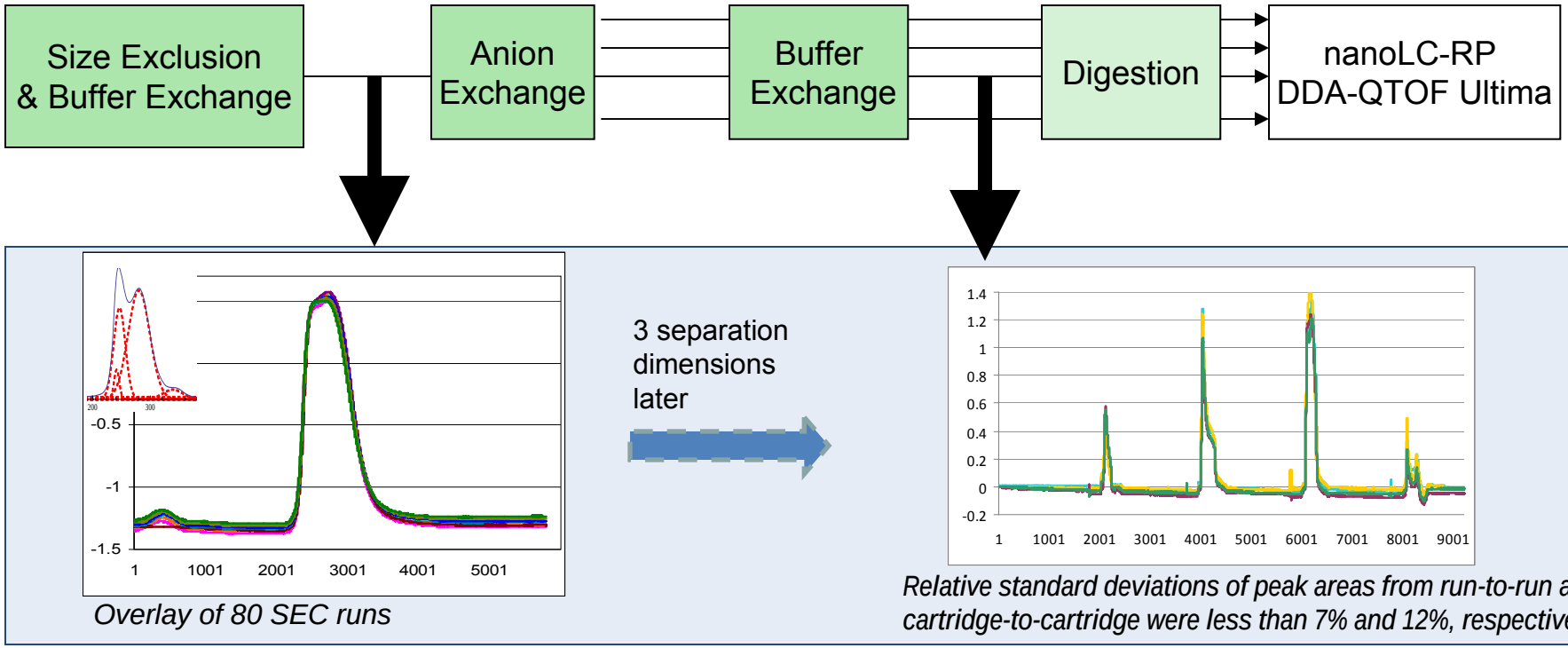
+ similar HAP removal
+ 100x reduction in operating cost
+ higher throughput through 20x higher sample capacity
+ 100x reduction in sample dilution
+ excellent reproducibility for over 100 runs

24 reproducible injections: overlay of 6 runs, each of 4 injections (50 µl serum/injection)

Multithreaded injections reduce the SEC run time with 30%.

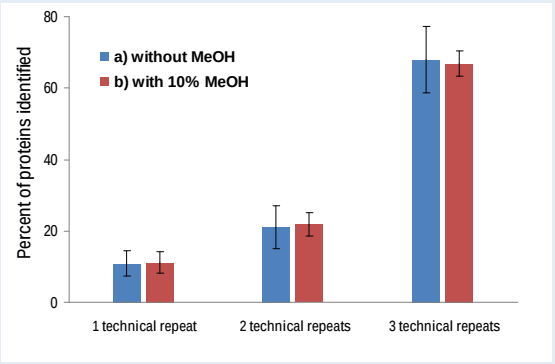


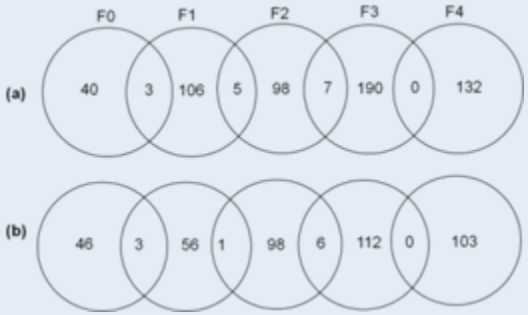
Workflow 3: Size Exclusion – Anion Exchange - Buffer Exchange

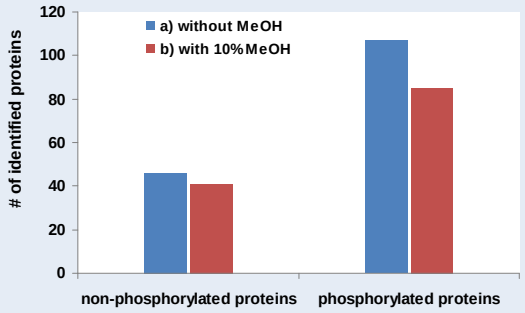


Overlay of 80 SEC runs

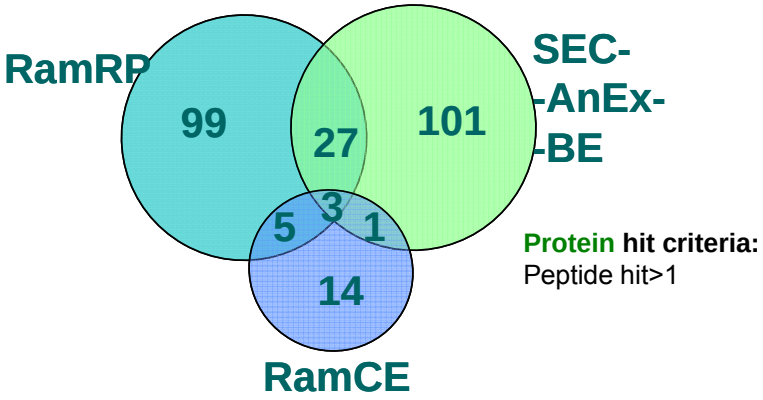
Relative standard deviations of peak areas from run-to-run and cartridge-to-cartridge were less than 7% and 12%, respectively



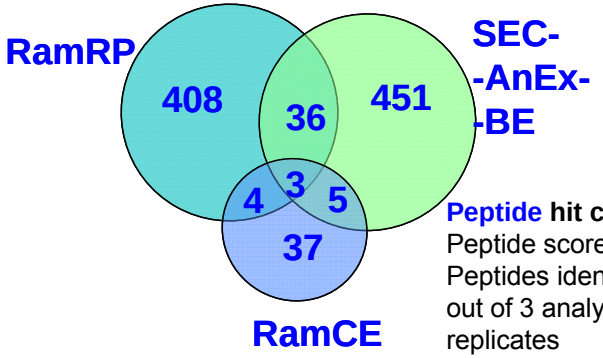




Comparison of protein and peptide overlap between the 3 processing methods



Protein hit criteria:
Peptide hit>1



Peptide hit criteria:
Peptide score>30,
Peptides identified in 2 out of 3 analytical replicates

Only ~10% of the total number of proteins identified were common to all sample prep trains

=> Truly comprehensive proteome studies should consider several processing trains to achieve maximum proteomic coverage

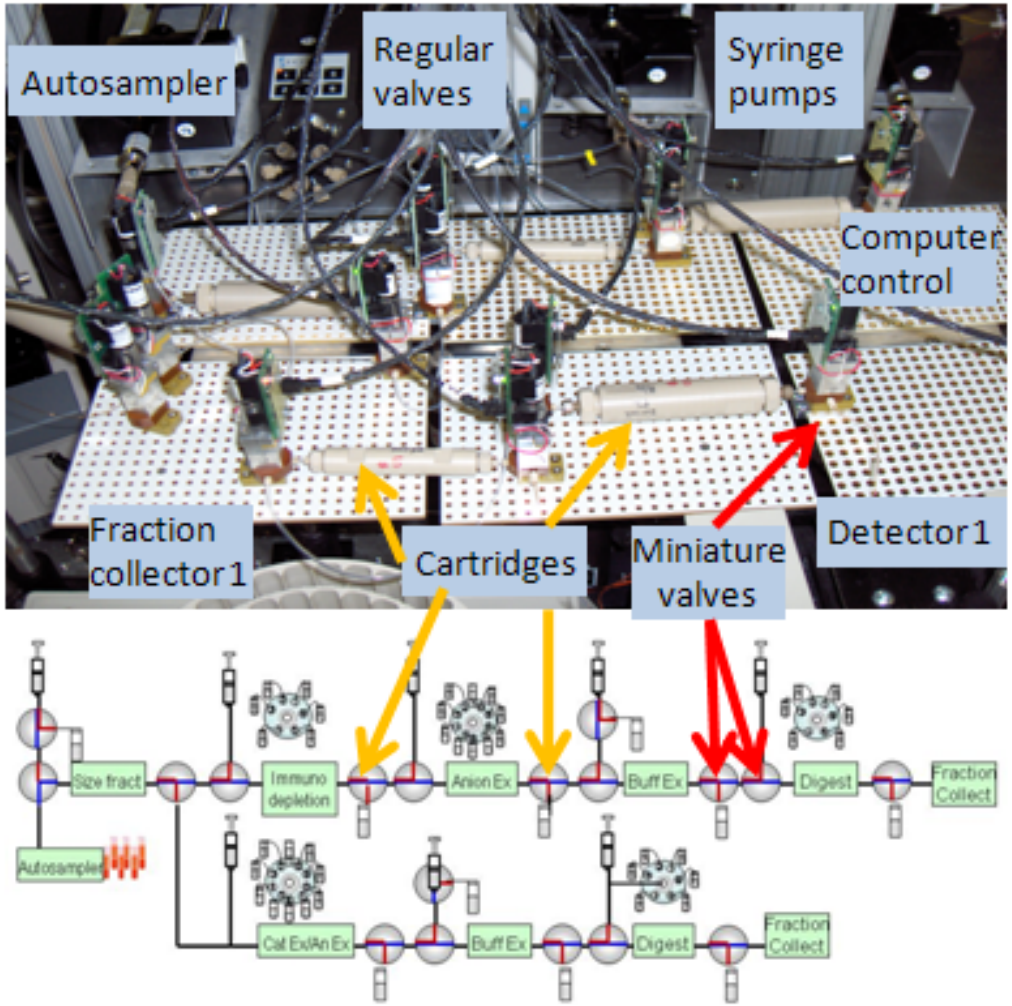
Mice infection with *Bacillus anthracis*

collaboration with Dr Ashok Chopra, Professor of Microbiology and Immunology at UTMB

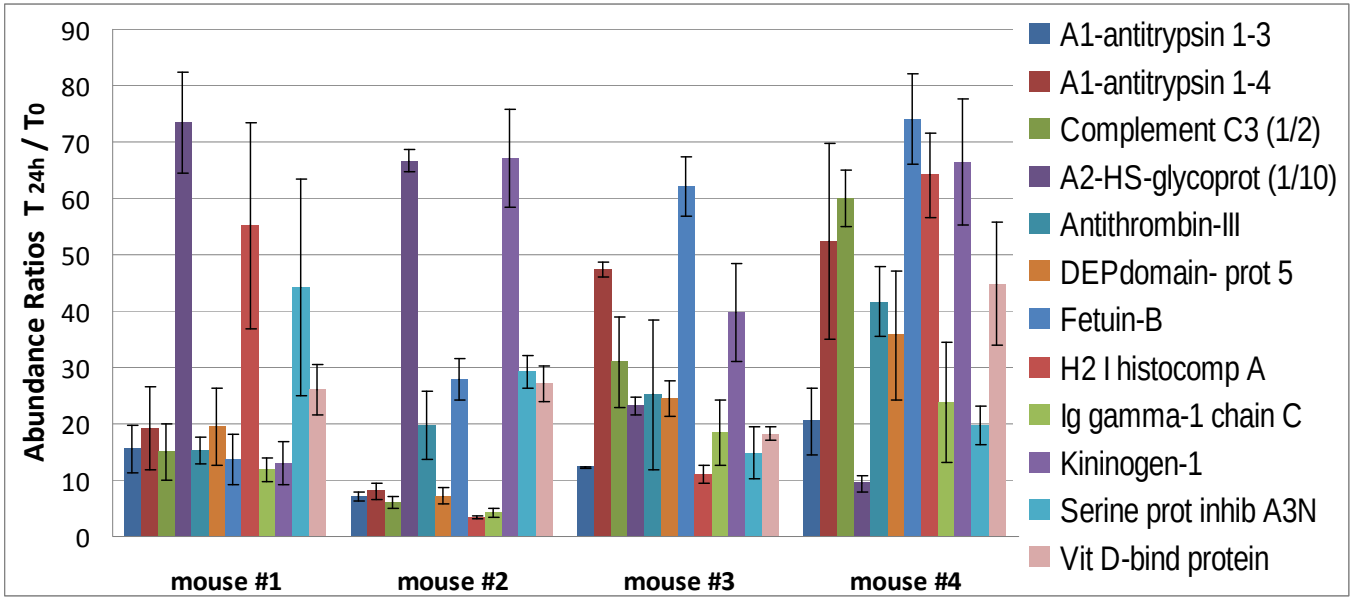
20 mice were inoculated with 5 LD50 (5x10^4 *Bacillus anthracis* Ames spores) in the BSL 3 facility of the Galveston National laboratory

- 24 h after inoculation blood was drawn from 10 mice
- 48 h after inoculation blood was drawn from 4 mice

6 mice died 48 h after inoculation



Mouse proteins showing increased abundance in serum following exposure to *B. anthracis* -*preliminary data*



-serum samples of four individual mice pre- *vs.* post-exposure (T0 *vs.* T24hr) were fractionated in the SEC-AnEx-BE workflow (3 LC-MS/MS technical replicates/sample for identification and semi-quantitative estimate of abundance variance)

-proteins above constitute potential biomarkers (verification/accurate quantitation with targeted proteomics methods typically follows)

- 9 acute inflammatory marker proteins and 6 other proteins showed over 10 fold variation in abundance in 3 out of 4 mice

-Further processing in progress to better asses/constrain mouse-to-mouse variability

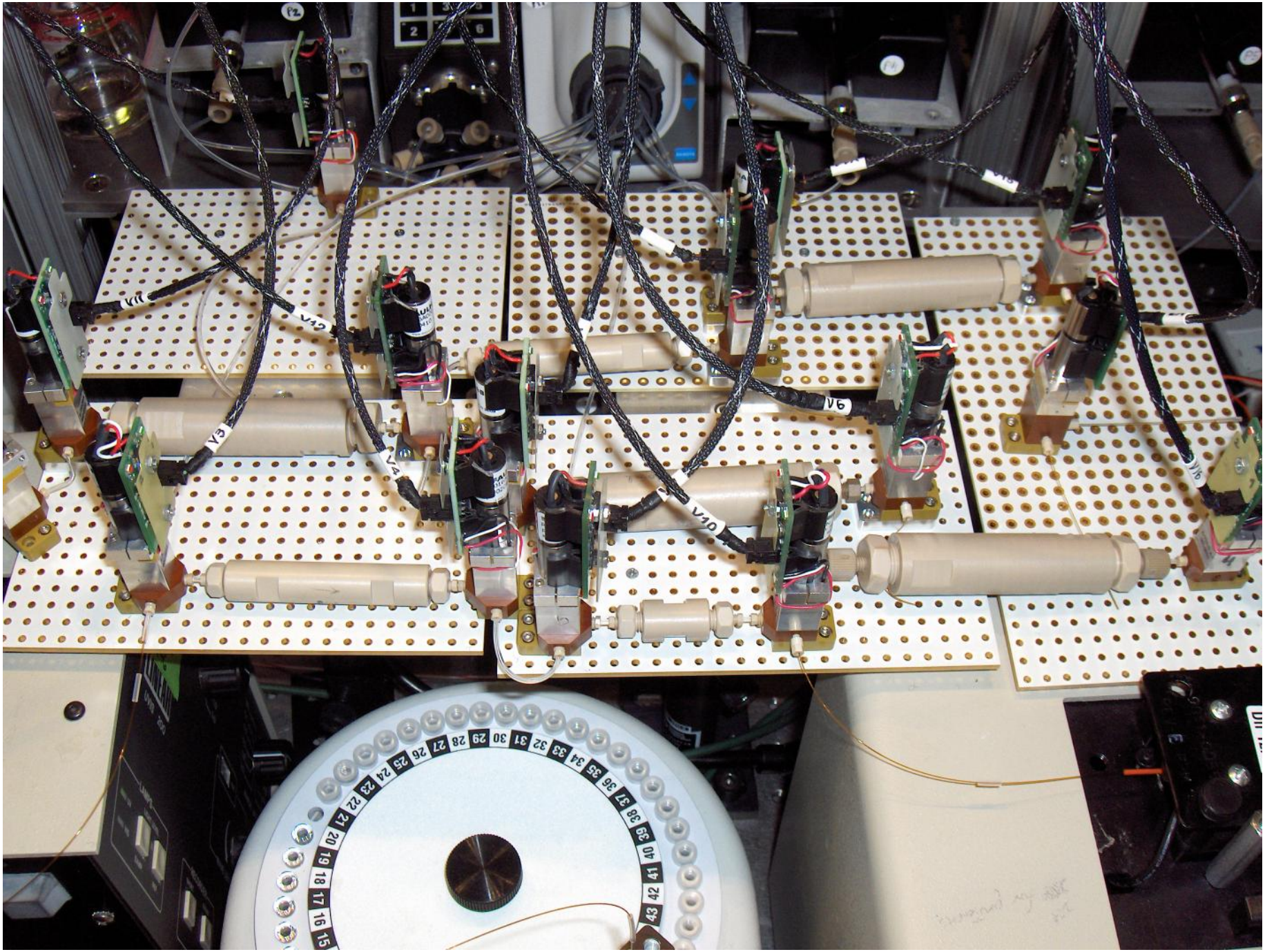
Summary and future directions

MAPS offers

=> high-throughput automated analysis
=> 5-200ul volume sample processing
⇒multidimensional fractionation (size, hydrophobicity, charge, affinity, etc.)
=> universal bioanalysis platform (serum, urine, cell culture, etc.)
=> rapid prototyping and method optimization
=> real-time UV monitoring between processing steps
=> very reproducible → less replicates needed
=>BSL2/BSL3 operation

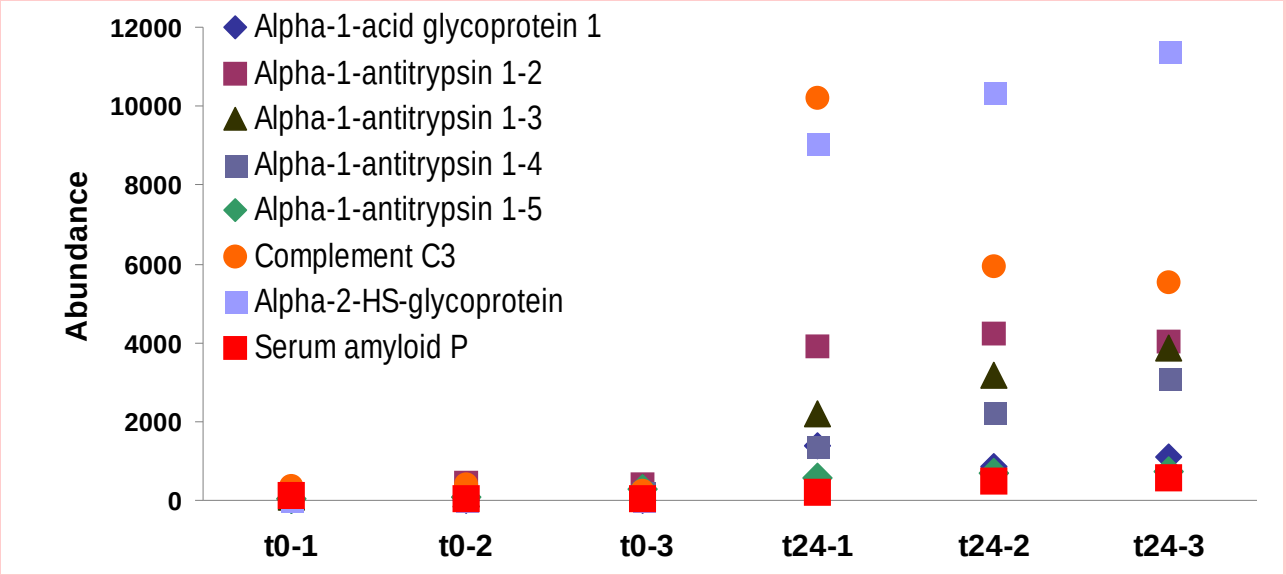
Future directions

⇒Exploit high-throughput capability (clinical proteomics – infectious disease and cancer, time course analysis, testing of new materials/methods for selective enrichment)
⇒Expand processing dimensions (include targeted phospho/glyco selection proteins/digested peptides)
⇒Develop modules to integrate nucleic acids processing
⇒Expand range of samples (tissue, biomass, etc.)

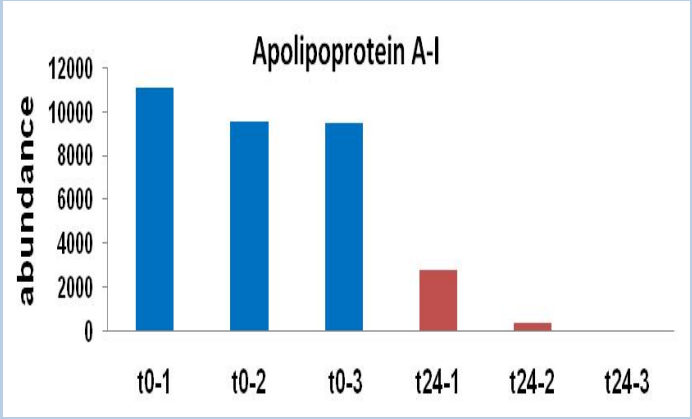

V12
V3
V4
V10
V6
V16
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43

Acute phase inflammation biomarkers
differentially expressed during Anthrax challenge
– preliminary data-

Increased levels



Decreased levels



- Serum amyloid B and Complement C3 bind to bacterial cell walls and mark the target for phagocitosis
- Apolipoprotein A binds to LPS & endotoxins and is a major contributor to HDL anti-endotoxin function
- A1-antitrypsin downregulates inflammation

On-line trypsin digestion using IMER immobilized enzyme reactor (IMER)

F8L variola coat protein

In solution overnight trypsin digestion

MSQQLSPINIETKKAISNARLKPLNIHYNESKPTTI
QNTGKLVRINFKGGYLSGGFLPNEYVLSSLHIYWGK
EDDYGSNHLIDVYKYSGEINLVHWNKKKYSSYEEAK
KHDDGLIIISIFLQVSDHKNVYFQKIVNQLDSIRTA
NTSAPFDSVFYLDNLLPSKLDYFKYLGTTINHSADA
VWIIFPTPINIHSDQLSKFRTLLSLSNHEGKNPHYI
TENYRNPYKLNDDTEVYYSGE

168 identified aa of 236 total

71% coverage

On-line 10min trypsin digestion

20µL cartridge with 7 µM 1000A at 37ºC

MSQQLSPINIETKKAISNARLKPLNIHYNESKPTTI
QNTGKLVRINFKGGYLSGGFLPNEYVLSSLHIYWGK
EDDYGSNHLIDVYKYSGEINLVHWNKKKYSSYEEAK
KHDDGLIIISIFLQVSDHKNVYFQKIVNQLDSIRTA
NTSAPFDSVFYLDNLLPSKLDYFKYLGTTINHSADA
VWIIFPTPINIHSDQLSKFRTLLSLSNHEGKNPHYI
TENYRKNPYKLNDDTEVYYSGE

156 identified aa of 236 total

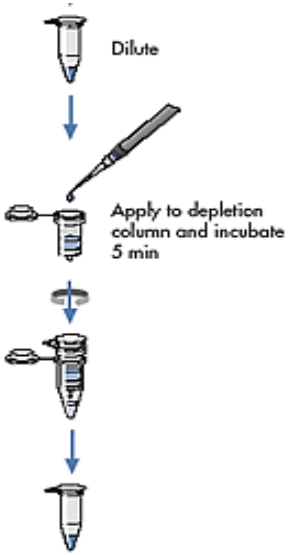
66% coverage

Protein	Digestion method	Coverage
Michrom BSA digest	In solution (alkylated)	36%
BSA	in-house IMER 1000 A, 7 µm (not alkylated)	18%
BSA	in-house IMER 4000 A, 10µm (not alkylated)	25.7%
Beta casein	in-house IMER 1000 A, 7 µm (not alkylated)	52%

IMER works best → smaller proteins
→ larger pores packings

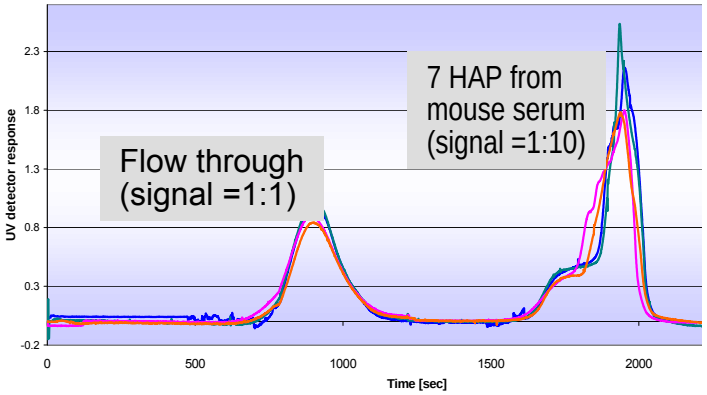
Removal of High Abundance Proteins (HAP) using the Immunodepletion Module

Gold standard : Genway IgYM7 spin column

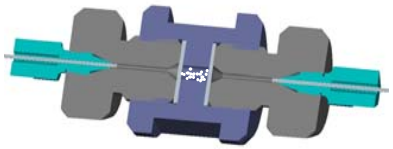


+ removes 85-90% of high abundance proteins
- requires 15-20x serum dilution
- expensive (1,500 $ /1ml cartridge/20µl serum)
- 25% run-to-run variation

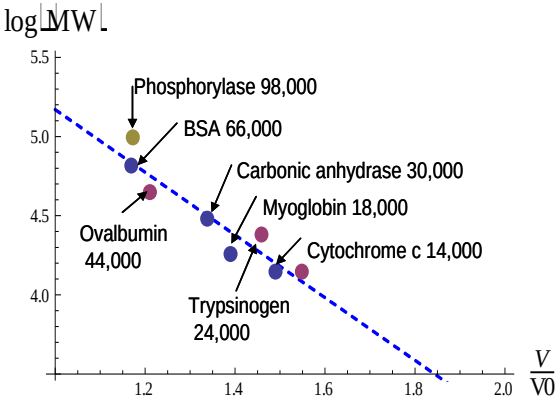
Sandia MAPS module with IgYM7



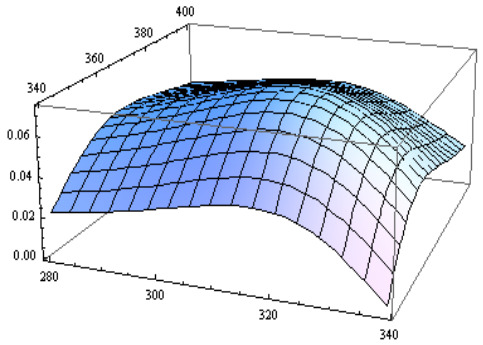
+ removes 85-90% of high abundance proteins
+ extends packing lifetime (use low pressures)
+ improves reproducibility (5%, reused >20x)
+ readily integrated in automatable system



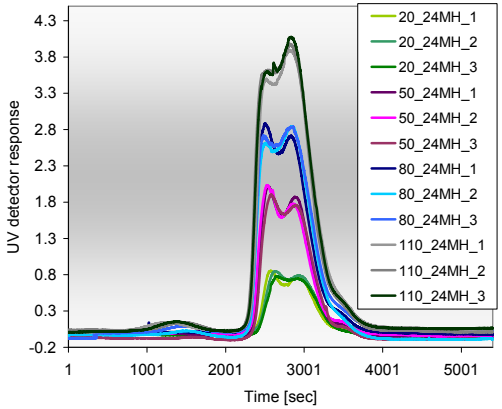
HAP (MW>45kDa) depletion for peptidome analysis using highly reproducible size fractionation module



Predictable module allows fine tuning of fraction collection for specific MW



[Recovery *Conc] of myoglobin as a function of fractionation range

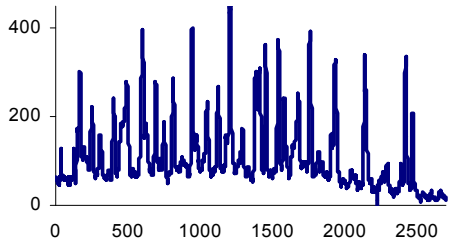


50 µl serum injections at different injection volumes

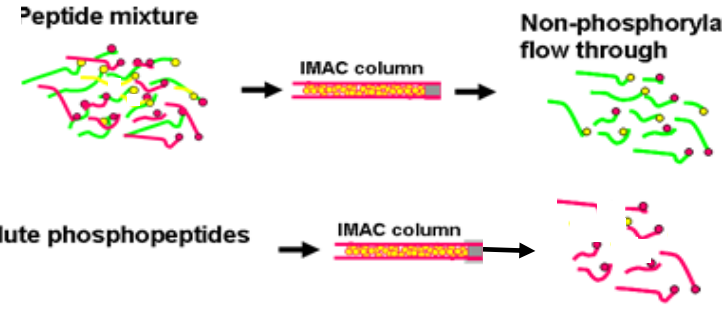
Overall, in our size exclusion module:

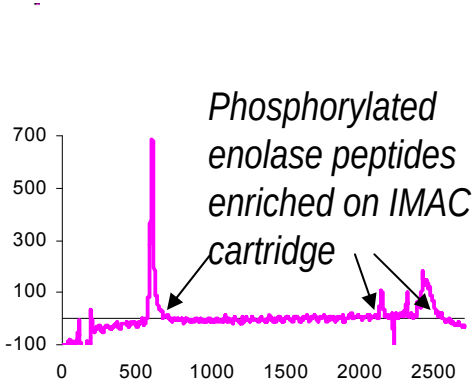
- the **depletion** of albumin and other higher MW proteins is **97%**
- the **recovery** of myoglobin (and other proteins and peptides with MW ~18kDa) is **84%**

Phospho-enrichment module

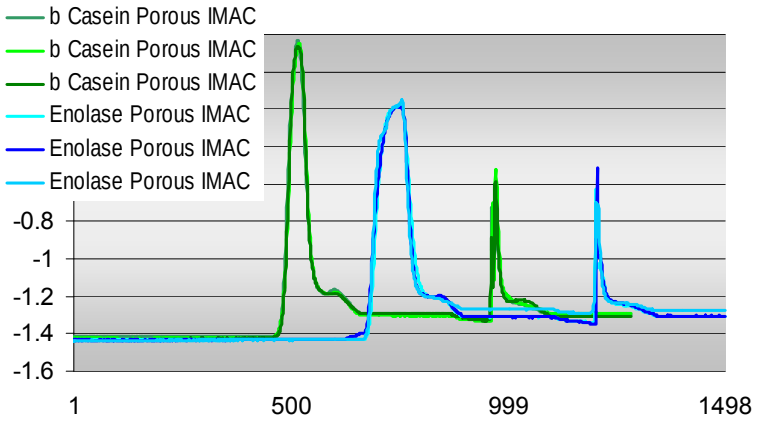


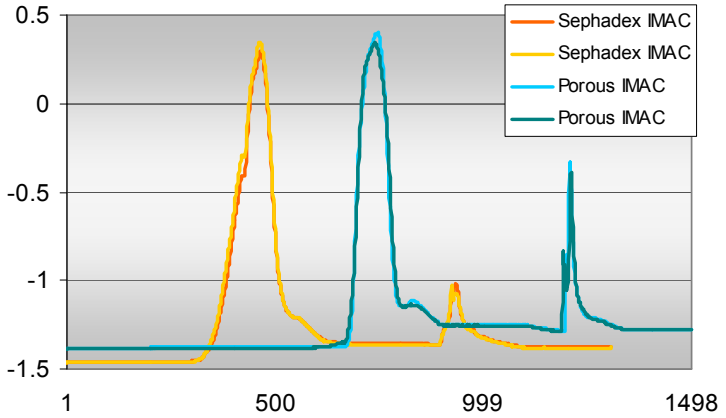
Enolase digest





Phosphorylated enolase peptides enriched on IMAC cartridge

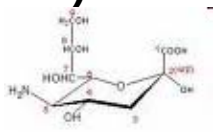


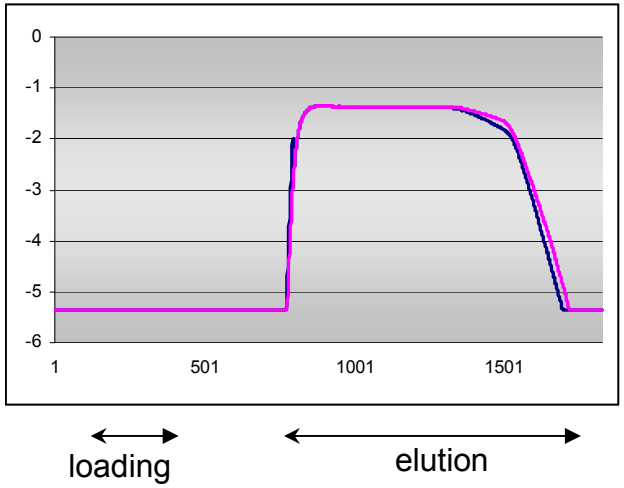


Glyco-enrichment module

Wheat germ agglutinin (WGA)

=lectin which selectively adsorbs sialic acid containing glycosyls

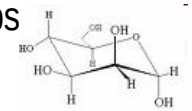


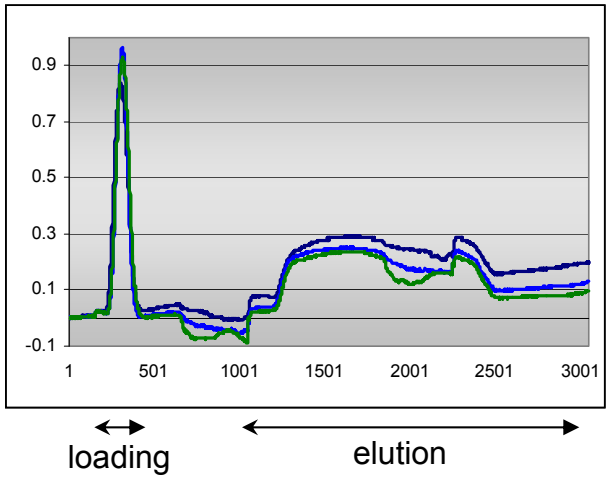


Bovine fetuin loaded on a WGA column was retained and subsequently eluted

Concanavalin A (ConA)

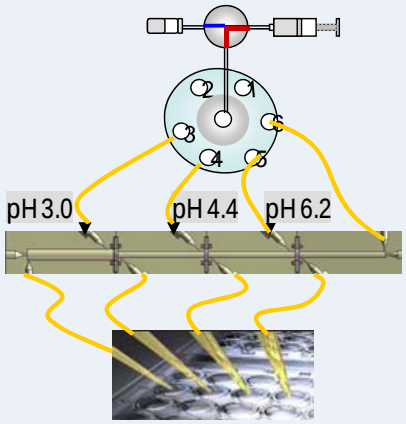
=lectin which selectively adsorbs mannose containing glycosyls

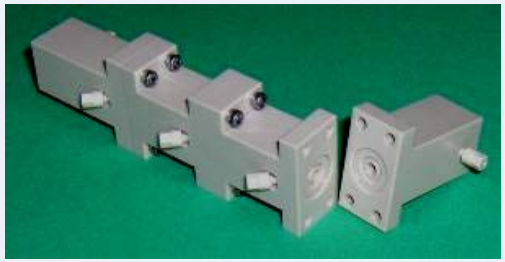




Ovalbumin loaded on a ConA column was partially retained and subsequently eluted

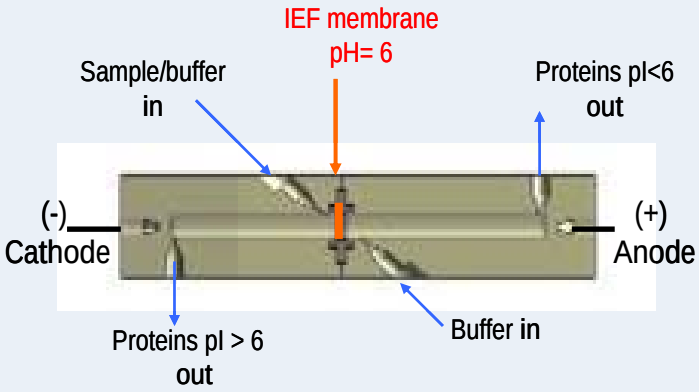
On-line, gel-free isoelectric fractionator





Improves commercial IEF fractionators:

+ on-line, automatable processing and sample handling
+ allows continuous sample loading= process larger sample volumes, higher concentration ratios
+ can adjust the chambers' volume to increase conc ratios
+ select membranes pH/chamber volume as needed for uniform protein mass distribution in chambers



Mixture (yellow) loaded at cathode. Proteins separated according to their pI: at cathode (red) proteins with pI > 6, at anode (blue) proteins with pI < 6