Integration of O-18 labeling and solution isoelectric focusing in a shotgun analysis of mitochondrial proteins

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Nov. 27, 2007

Introduction

- Proteome: the time- and cell-specific protein complement of the genome
- Proteomics: large-scale study of proteins concurrently and rapidly
  - Bridge the gap between the genomic information and biological function
  - Search for disease biomarker, develop better diagnostic tests and identify therapeutic targets

Challenges

Sample preparation

- The number of proteins in a human cancer cell: >100,000
- Dynamic range: as high as 10^{12}
- Specific classes of proteins, e.g. high pI, MW or hydrophobicity

Quantitation

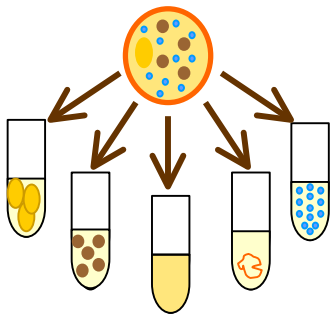
- Facile methods for comparative proteomics

Proteomic Analysis

Protein preparation → **Protein separation and quantitation** → **Protein analysis**

Fractionation:

e.g. subcellular fractions



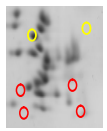
1. Separation:

--- Gel electrophoresis

--- In solution separations:

~ liquid chromatography
~ electrophoresis
~ multidimensional separation

2. Quantitation

Gel-base densitometry 

Chemical labeling 

Enzyme-catalyzed isotope labeling (O-18)

Mass spectrometry
&
Bioinformatics

The Shotgun Approach

- The protein mixture is digested into peptides with proteases
- The peptides are separated and resolved

- Easier to be handled and separated
- Easier to fragment in the MS
- Challenging: Assemble peptides into proteins : robust separation, computational analysis and interpretation of the data

Objective

To perform a comparison study of the mitochondrial proteins between MCF-7 drug susceptible and drug resistant cell lines

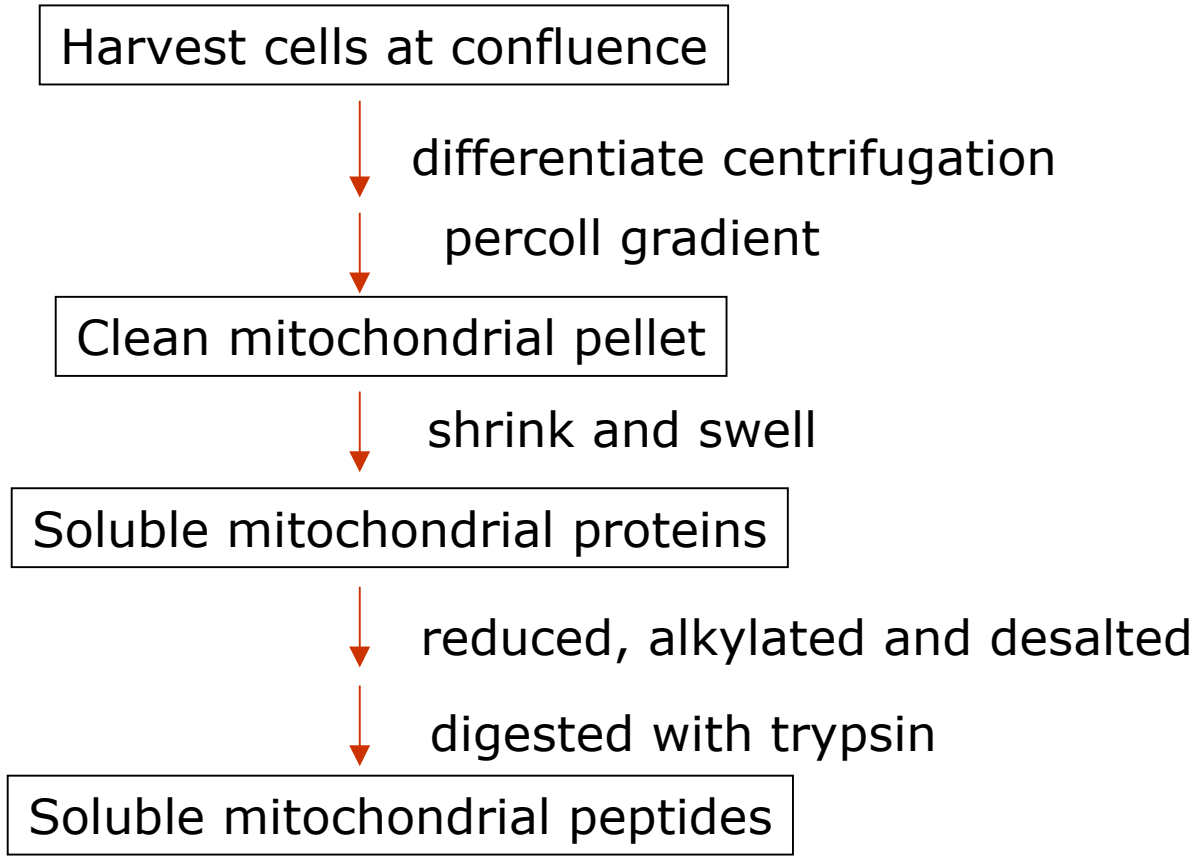
- Drug resistance is the most challenging problem in chemotherapy treatment of cancer cells
- Proteomics approach for study of mechanism of drug resistance
  - increase in drug efflux (P-gp)
  - block apoptosis
- Why mitochondria?
  - central to apoptosis
  - energy factory

Outline

- Develop a reproducible method to extract soluble mitochondrial proteins from MCF-7 cells
- Combine solution isoelectric focusing (solution IEF) and reversed-phase HPLC-MS for shotgun analysis of mitochondrial peptides
- Integration of O-18 labeling with this shotgun strategy to detect changes in protein abundances between drug susceptible and mitoxantrone resistant MCF-7 cancer cells
- Evaluate this result with reverse O-18 labeling
- Consider functions of those altered proteins in drug resistance

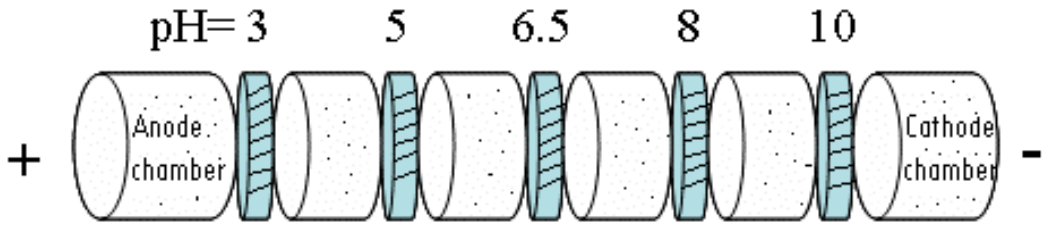
Methods

Preparation of mitochondrial peptides



Two-dimensional separation strategy

1st dimension separation: solution IEF



The IEF device is made of multiple chambers separated by a series of microporous membrane with defined pH values.

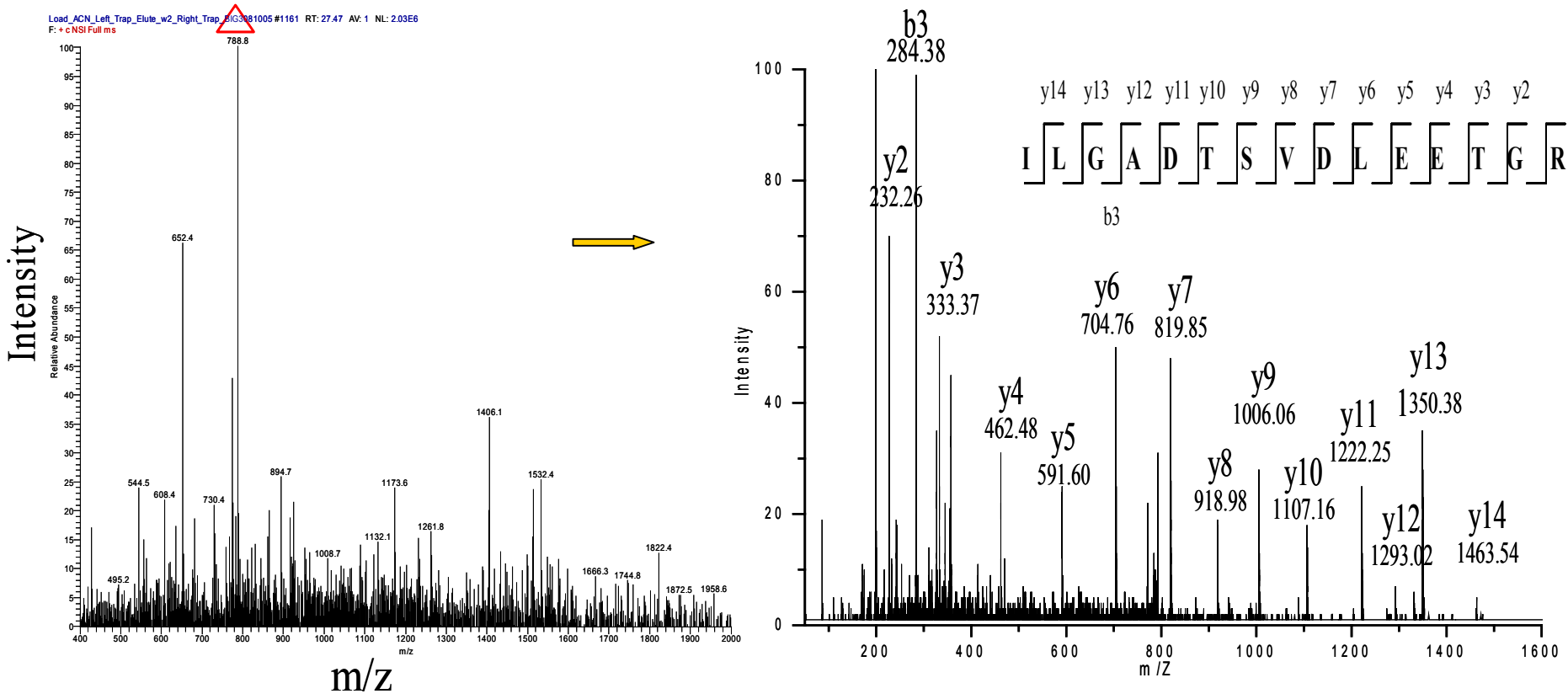
2nd dimension separation: reversed-phase µLC-MS/MS

NanoLC was performed using C-18 column in a liner gradient mode

Mass Spectrometry and Protein Identification

Tandem Mass Spectrometry (MS/MS)

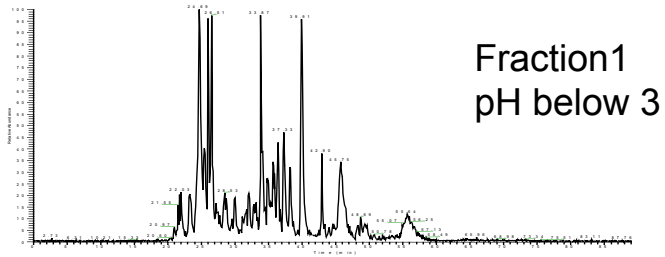
- Mass spectrometry of fragment ions of peptides
- Very confident protein identifications



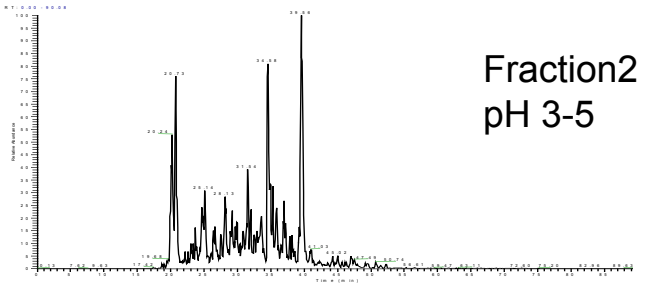
Results

Evaluation of solution IEF

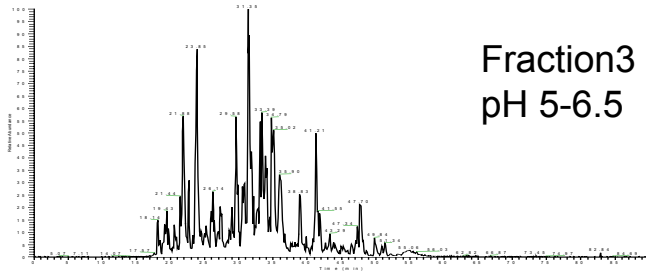
? Each chamber contains different peptides



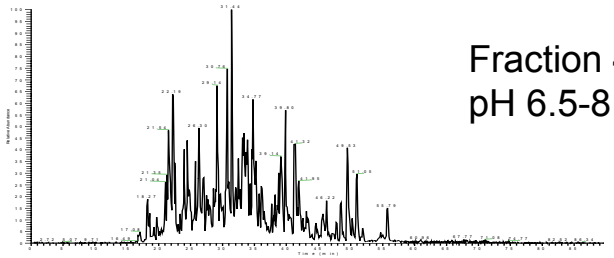
Fraction1
pH below 3



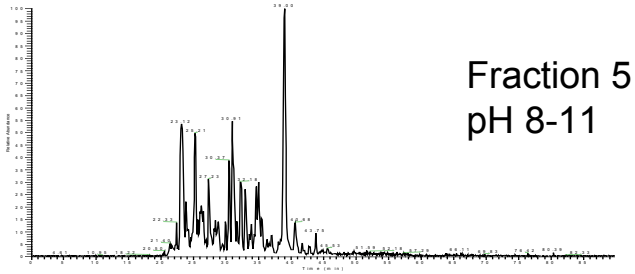
Fraction2
pH 3-5



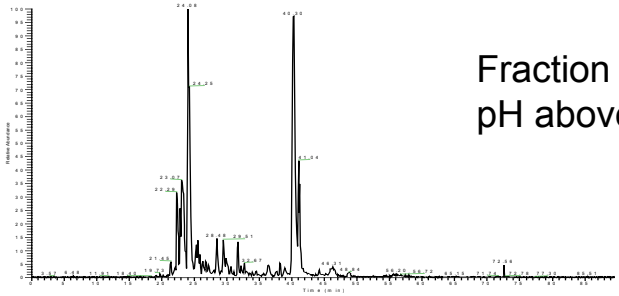
Fraction3
pH 5-6.5



Fraction 4
pH 6.5-8

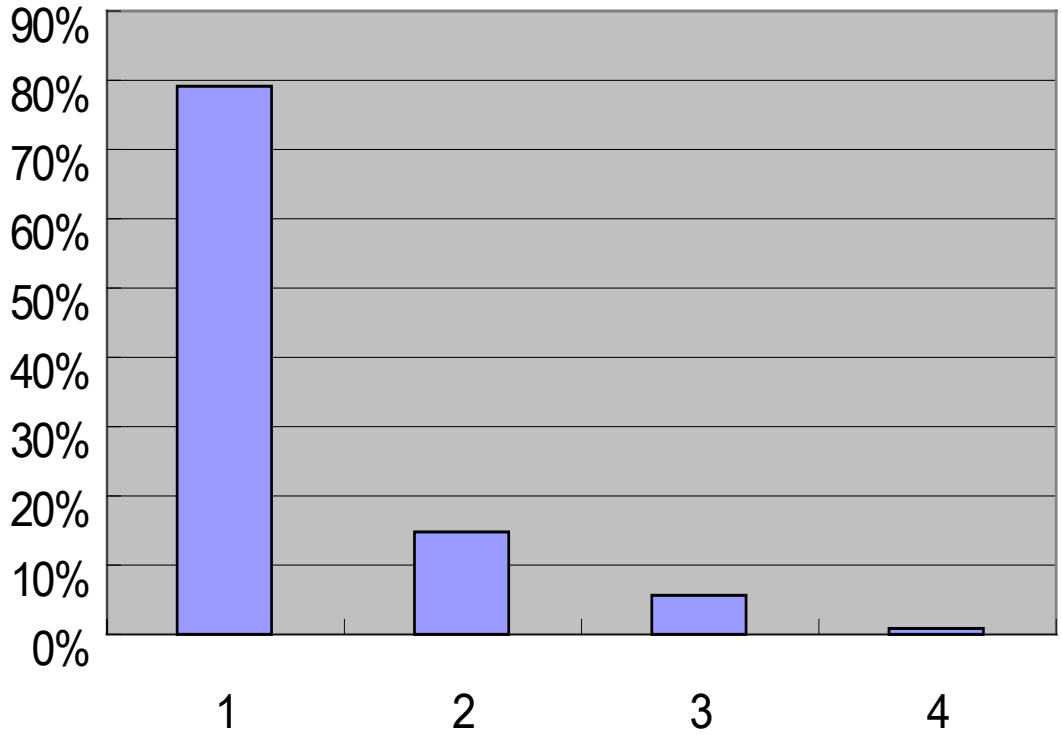


Fraction 5
pH 8-11

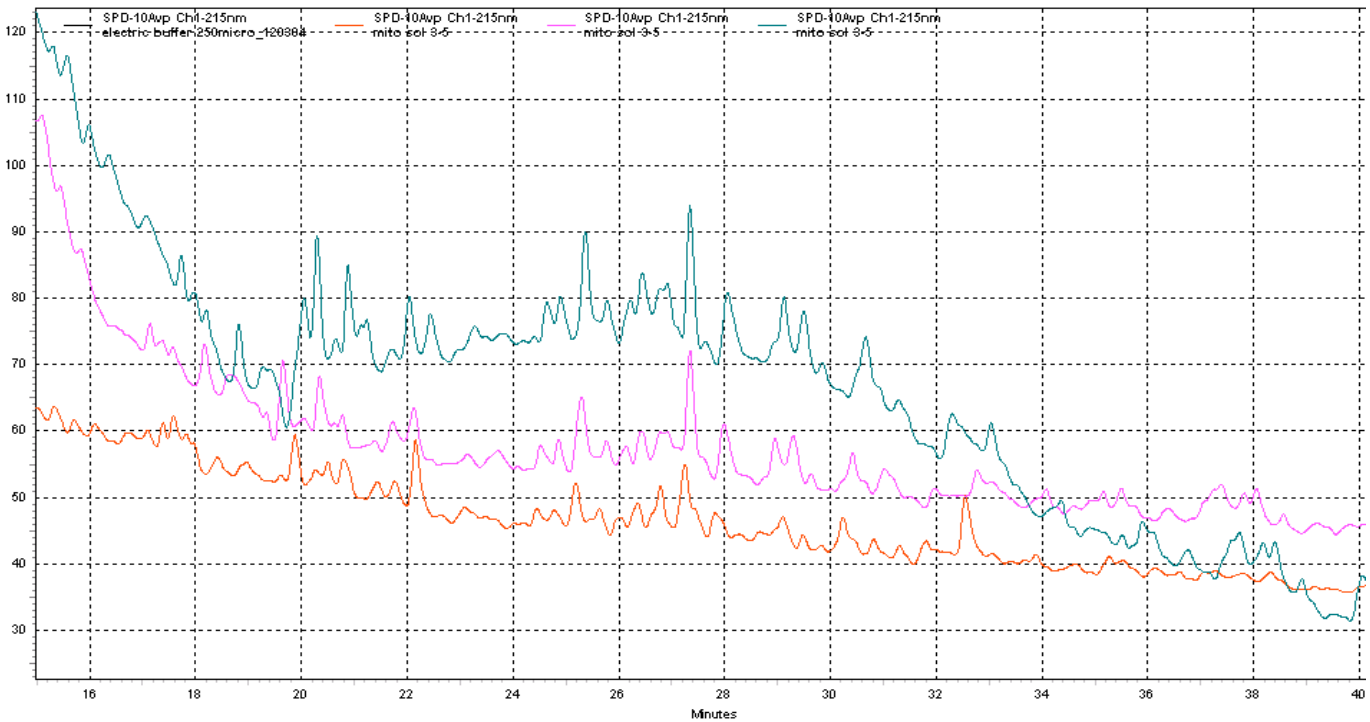


Fraction 6
pH above 11

? Redundant peptide distribution

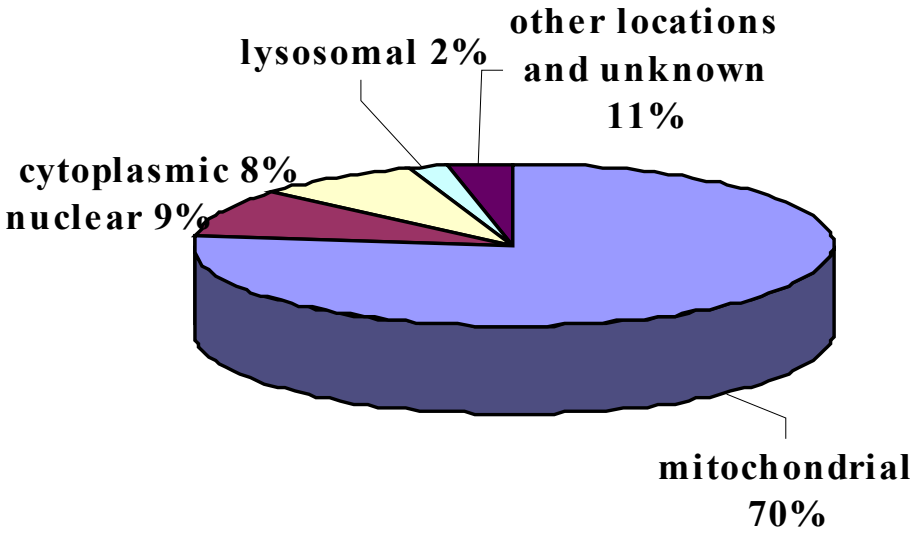


? Reproducibility

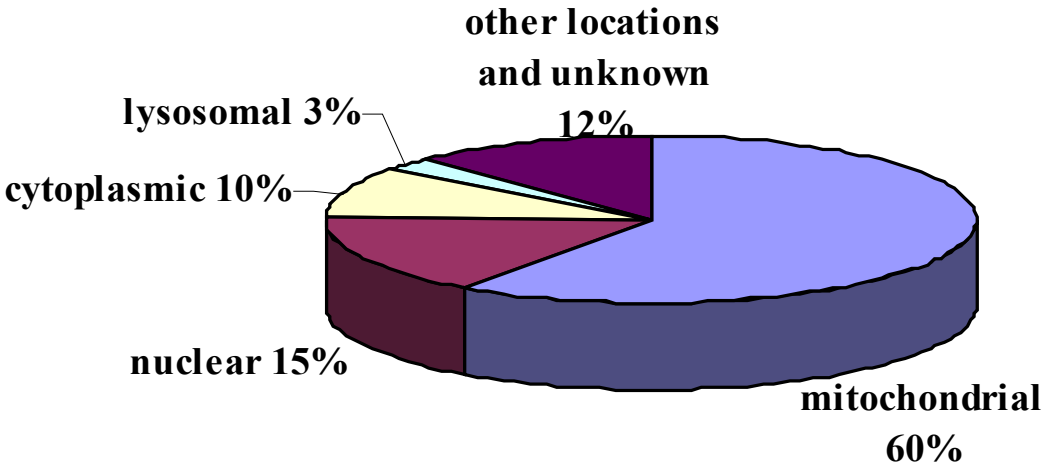


UV (214nm) HPLC chromatogram of a single fraction (pH3-5) from three solution IEF separations

? Distribution of identified peptides and proteins by organelles

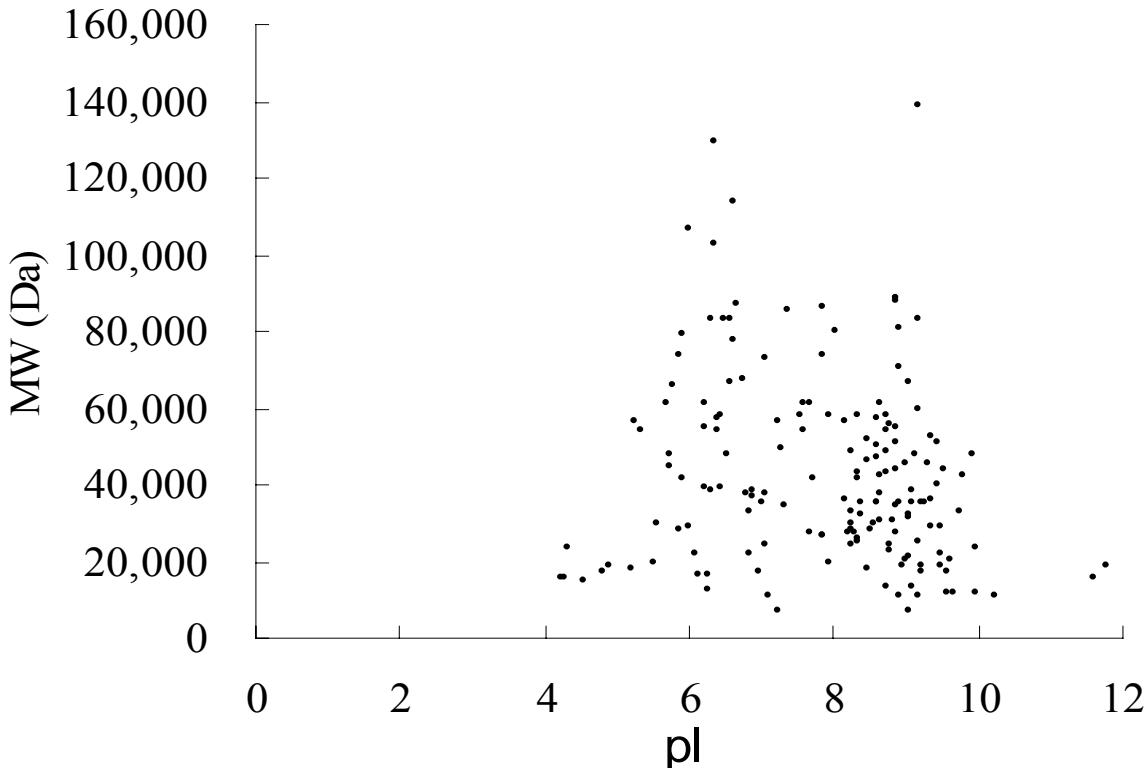


637 peptides were identified, of which 446 peptides originated from mitochondria

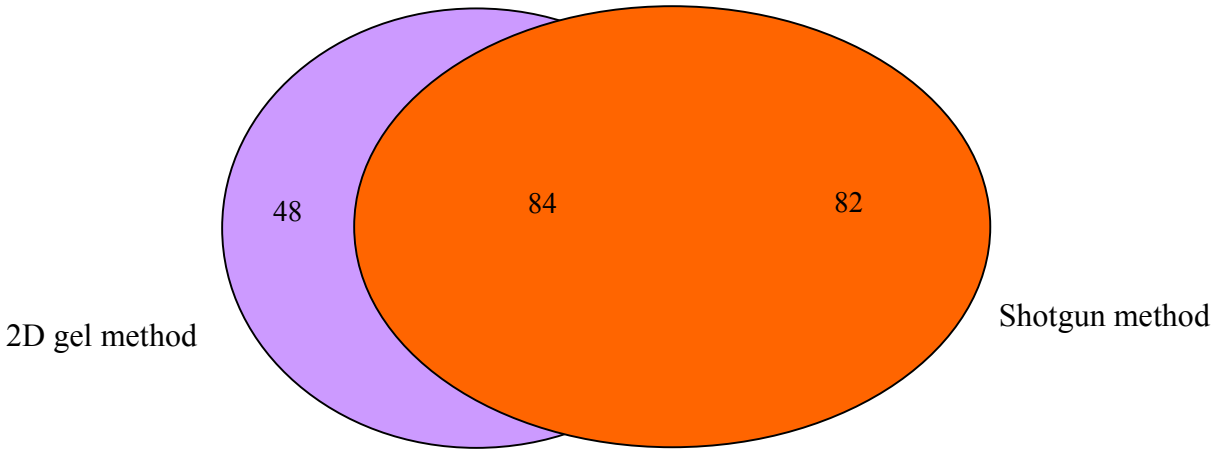


Subcellular distribution of the 278 proteins

? Distribution of pI and MW of identified mitochondrial proteins

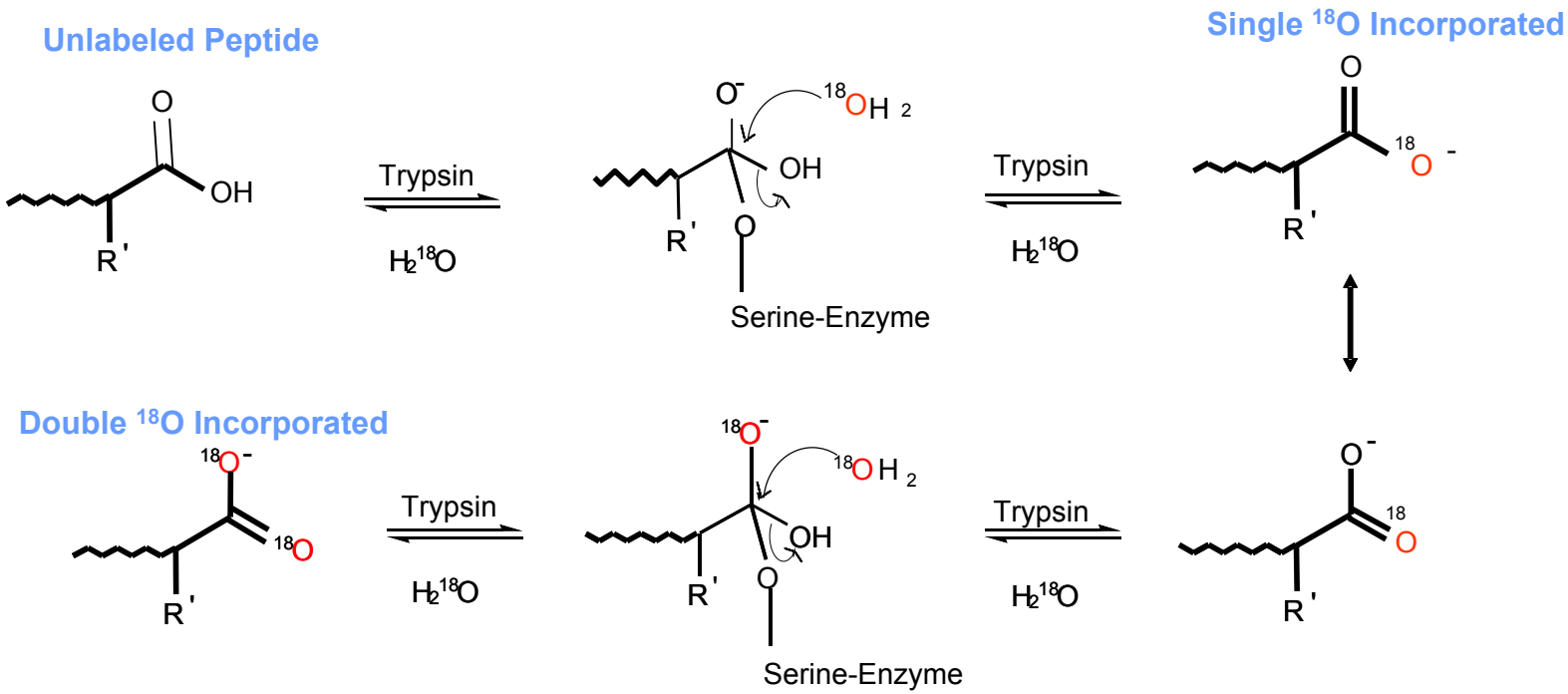


?Comparision of identified proteins in 2D gel and this study



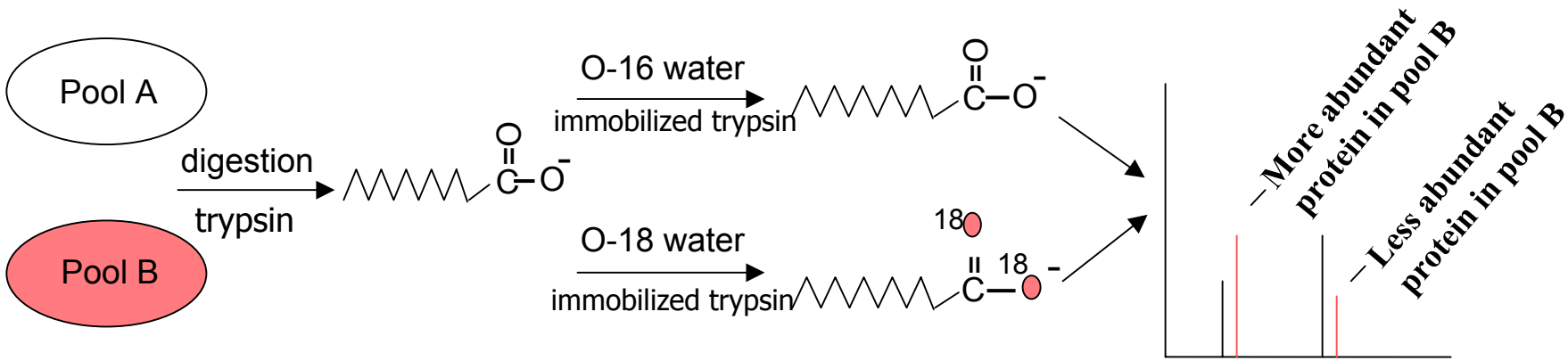
Quantitative Comparison

Enzymatic O-18 incorporation



Modified from Yao & Fenselau Analytical Chemistry 2001, 73, 2836-42

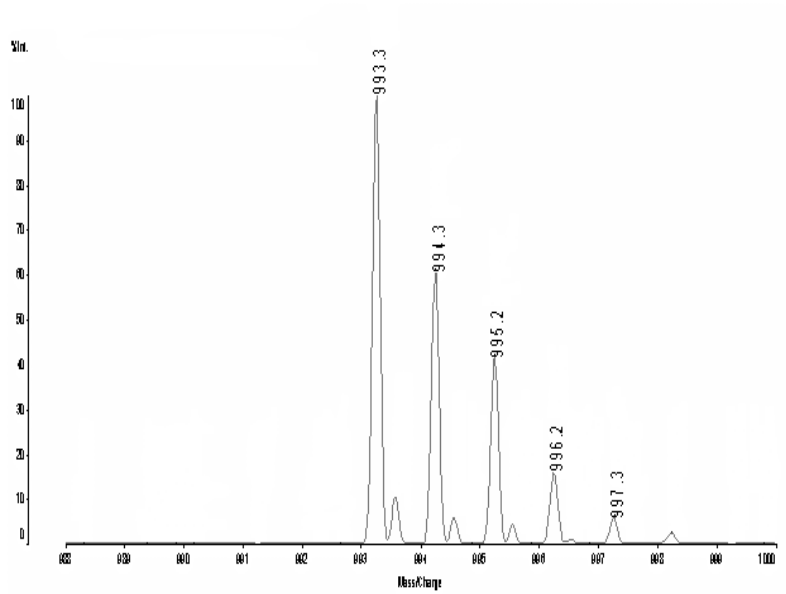
Relative quantitation with O-18 labeling



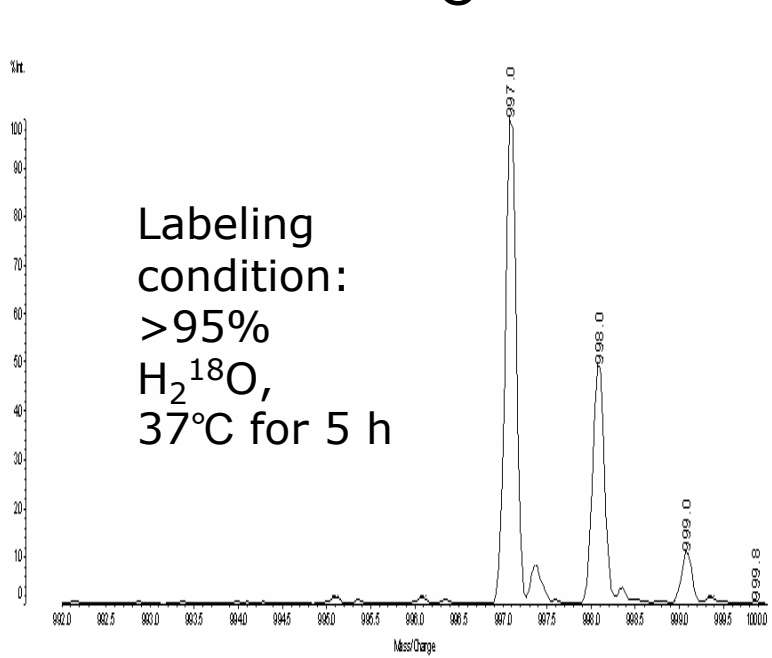
- Global labeling strategy (exception: peptide from C-terminus of the protein)
- Compatible with analysis of proteins from tissue and other limited samples
- The by-product is water and the immobilized catalyst can be readily removed.
- Compatible with peptide separation strategies

O-18 Labeling Efficiency

^{16}O



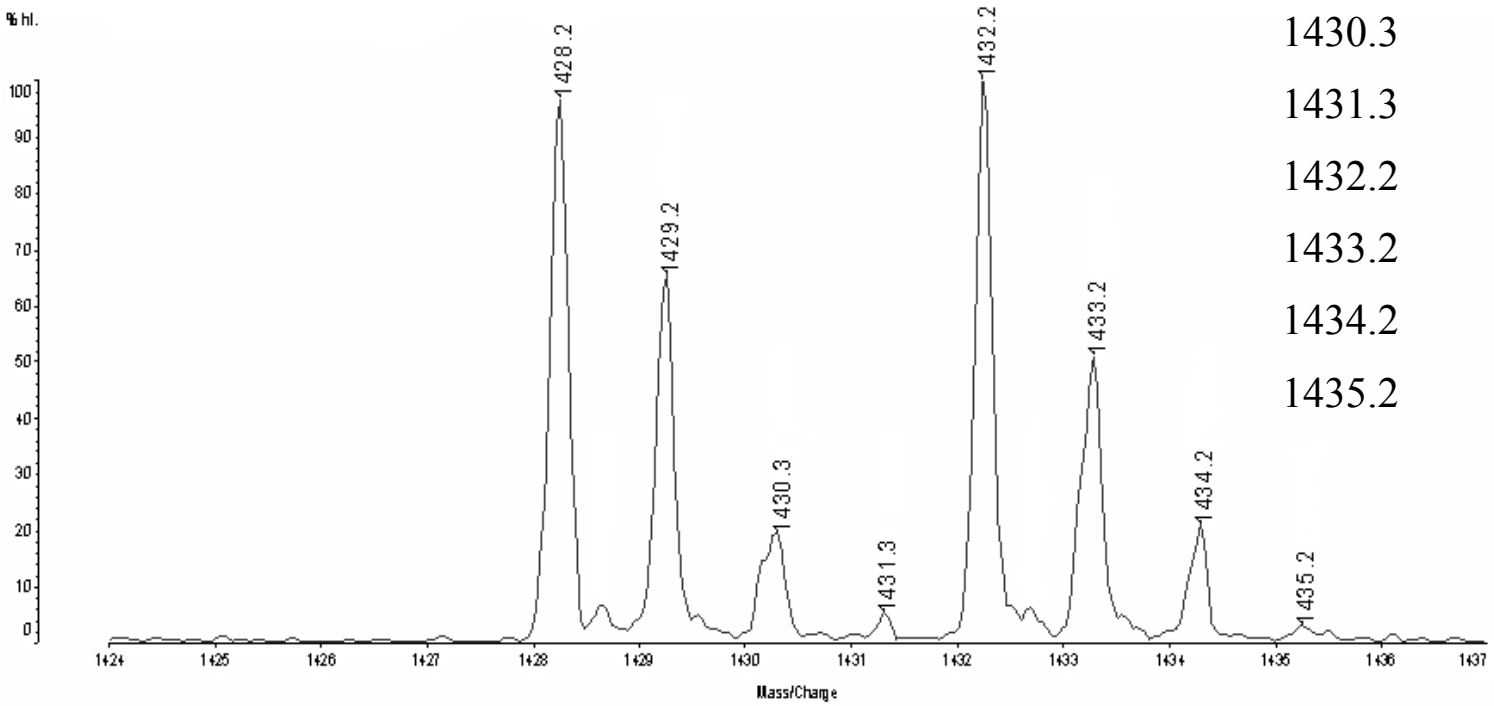
^{18}O



Labeling condition: >95% $H_2{}^{18}O$, 37℃ for 5 h

Lysozyme peptide: WWCNDGR

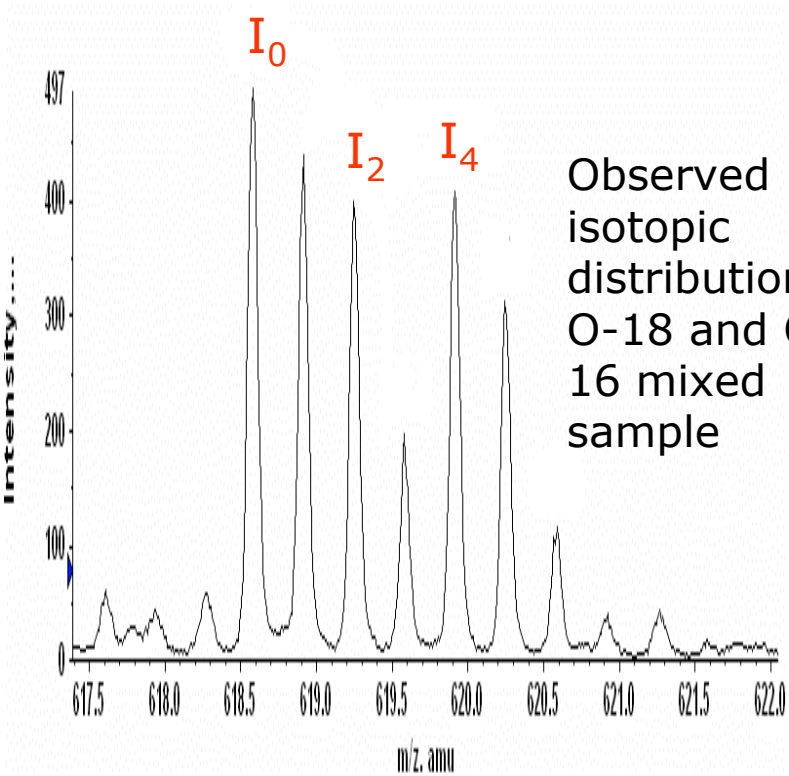
Spectrum of $^{16}O/^{18}O$ mixture (1:1)



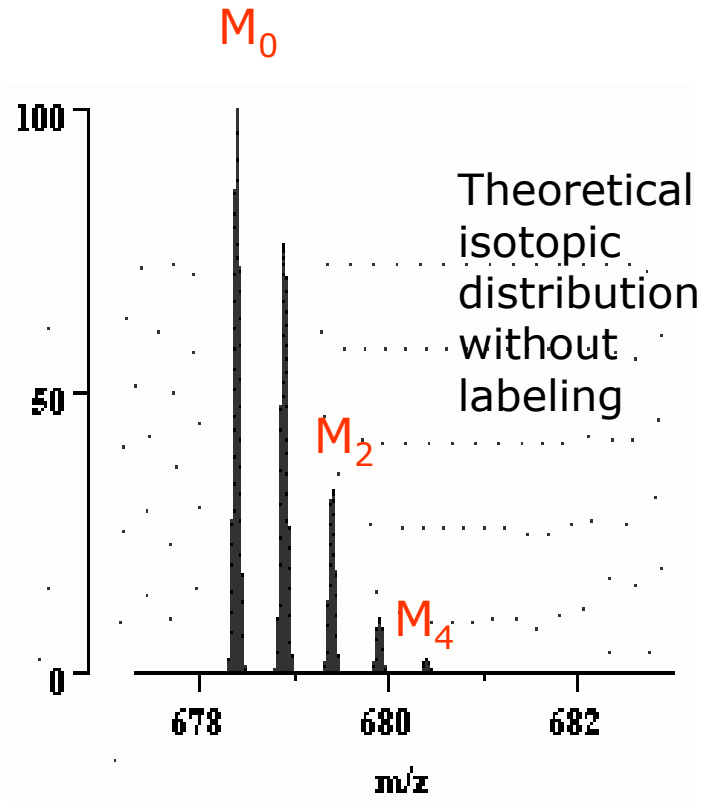
Mass	% Area
1428.2	97.6
1429.2	70.1
1430.3	26.9
1431.3	4.9
1432.2	100.0
1433.2	61.3
1434.2	23.0
1435.2	4.2

Quantitation of $^{18}O/^{16}O$ ratios

Ratios are calculated from mass spectral peak area



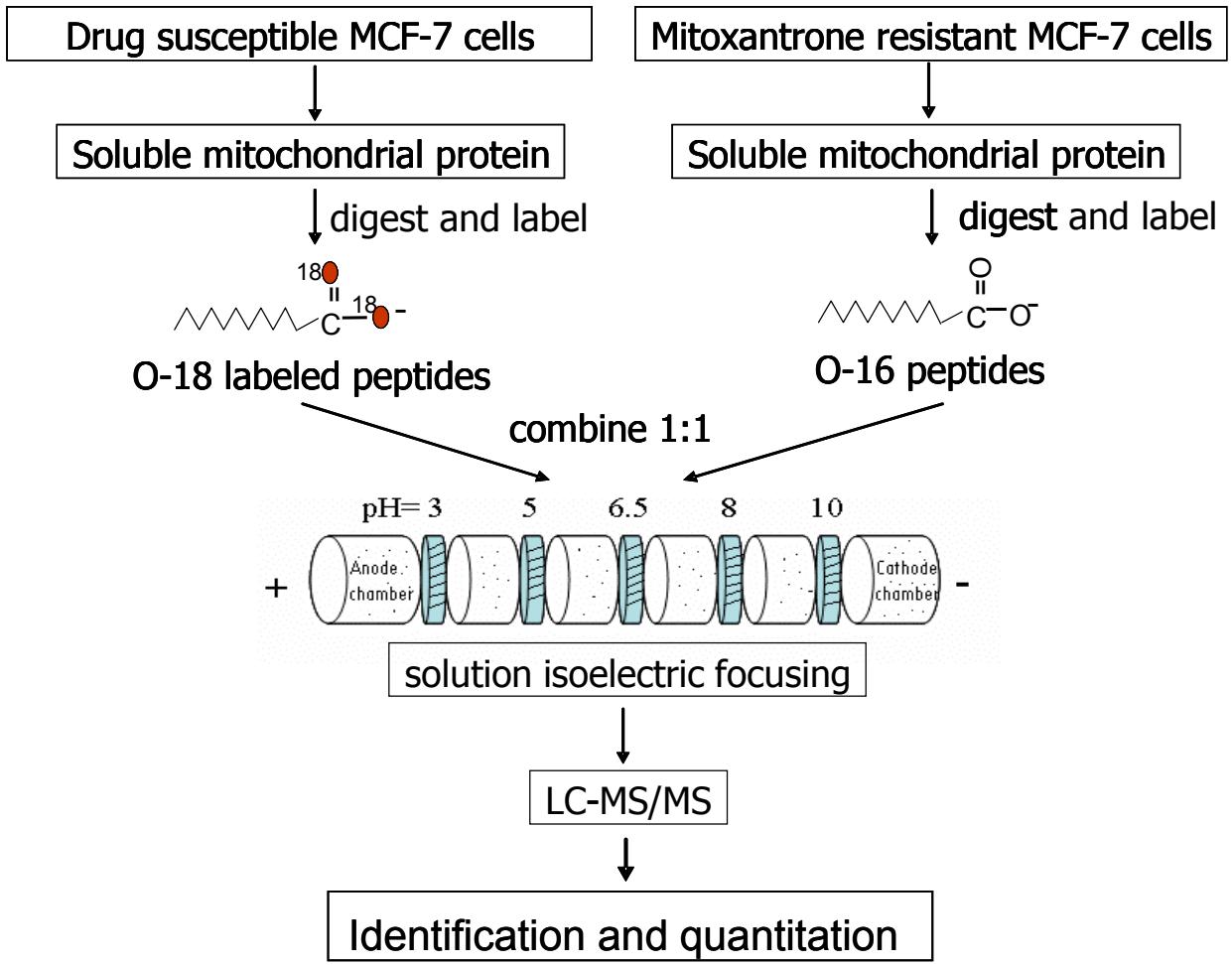
Observed isotopic distribution of O-18 and O-16 mixed sample



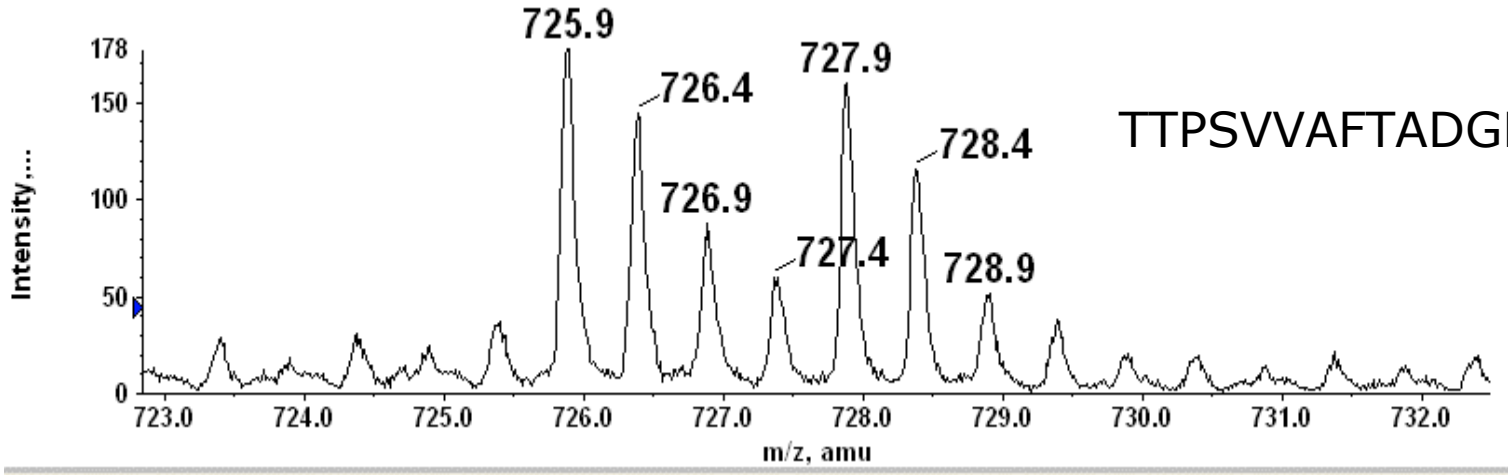
Theoretical isotopic distribution without labeling

$$^{18}O/^{16}O=\{I_4-(M_4/M_0)I_0-M_2/M_0[I_2-(M_2/M_0)I_0]+[I_2-(M_2/M_0)I_0]\}/I_0$$

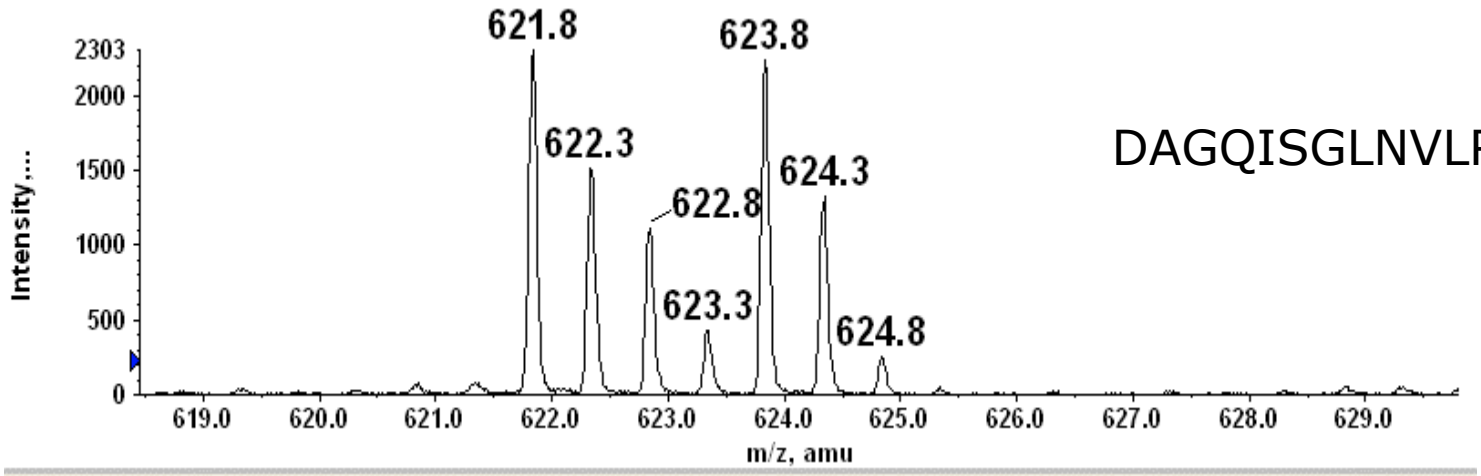
Integration of O-18 labeling with two-dimensional separation strategy



Unchanged abundance in drug resistant cells



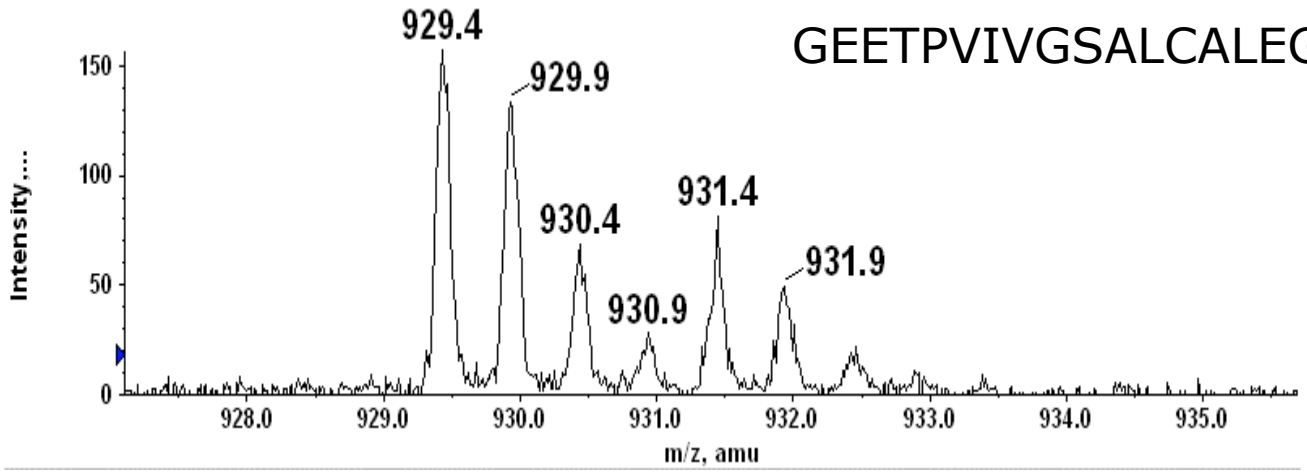
TTPSVVAFTADGER (+2)

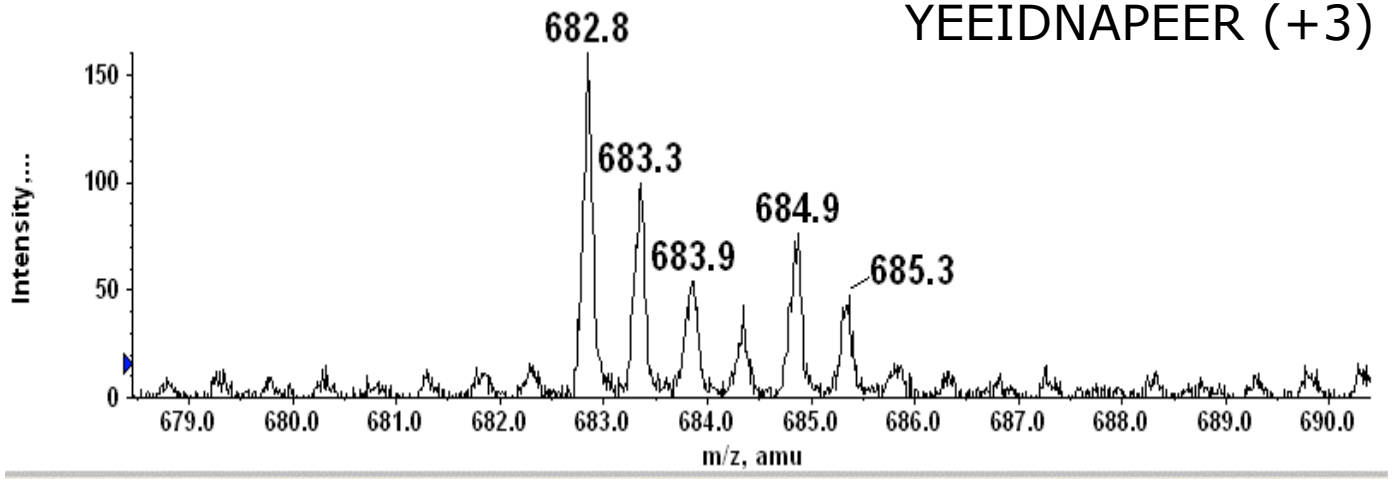


DAGQISGLNVLR (+2)

Two pairs of peptides from heat shock protein 70

Altered abundance in drug resistant cells



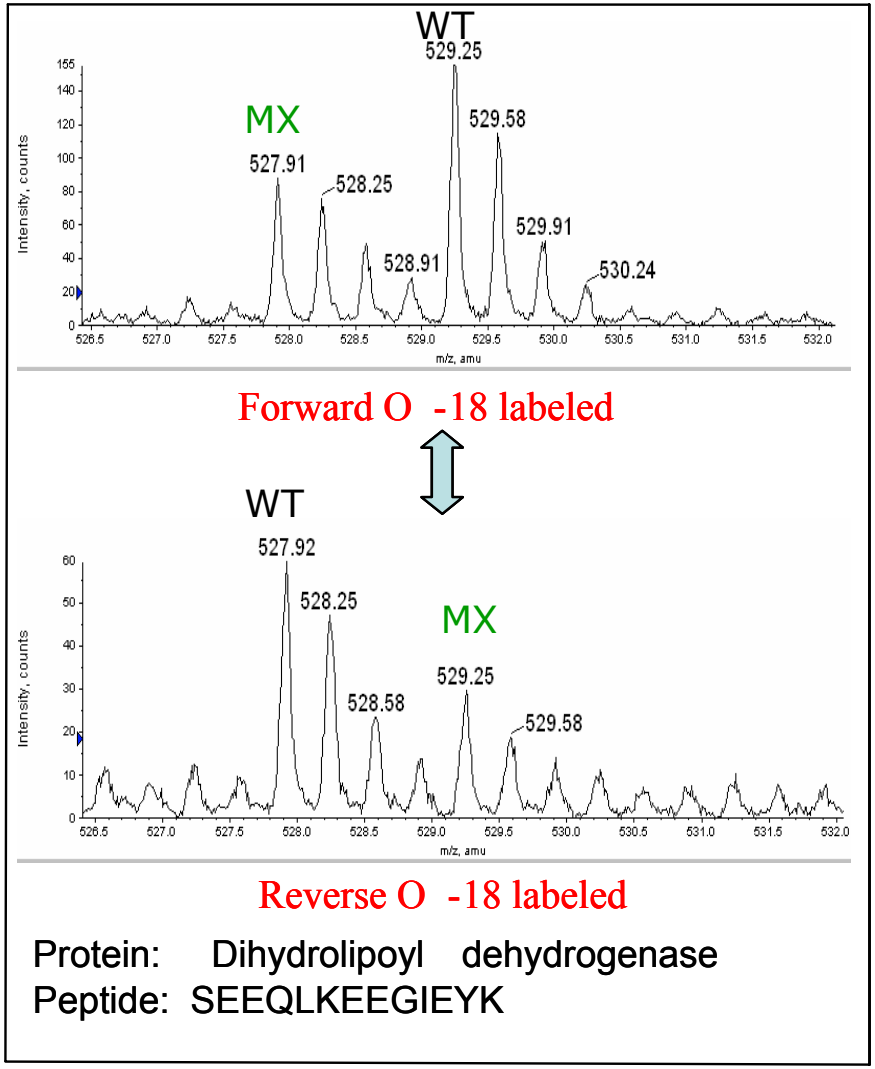


Two pairs of peptides from elongation factor Tu

Reverse O-18 labeling

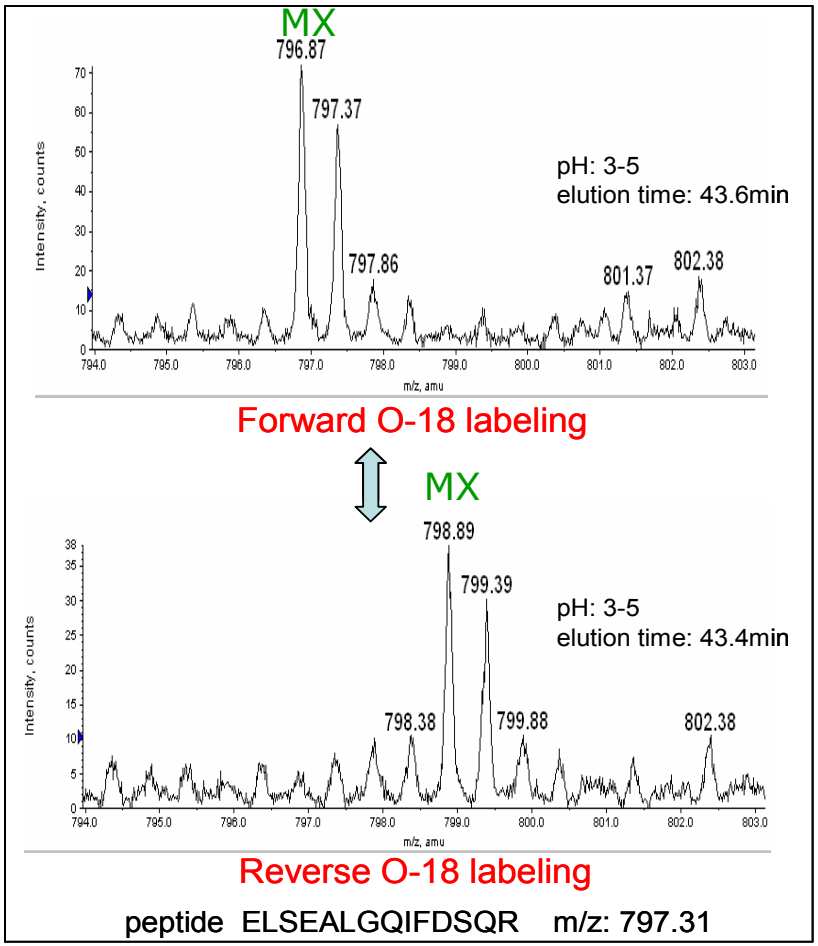
- **Forward labeling**: Peptides from drug susceptible cell line were labeled with O-18 water.
- **Reverse labeling**: Peptides from drug resistant cell line were labeled with O-18 water

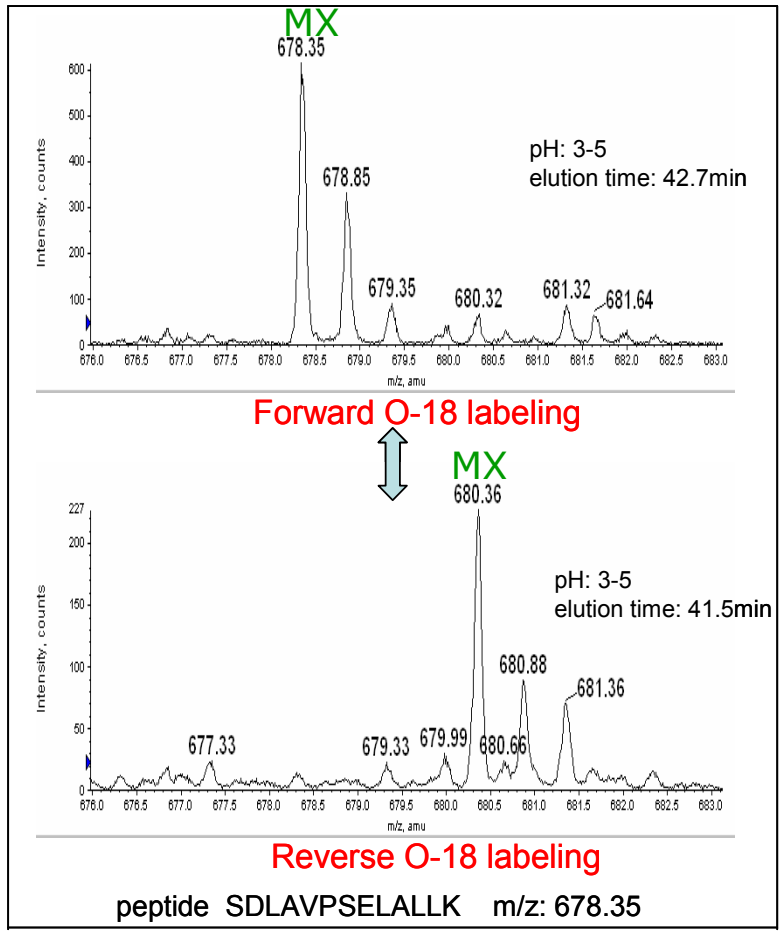
- Estimate the precision and confirm the behavior of “on/off” proteins
- Help interpretation of tandem mass spectra and thus provide peptide identification



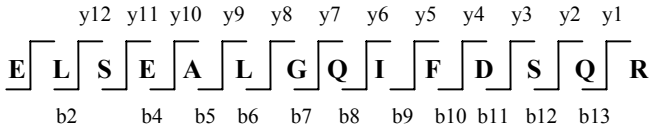
Example of quantitative comparisons based on peptide

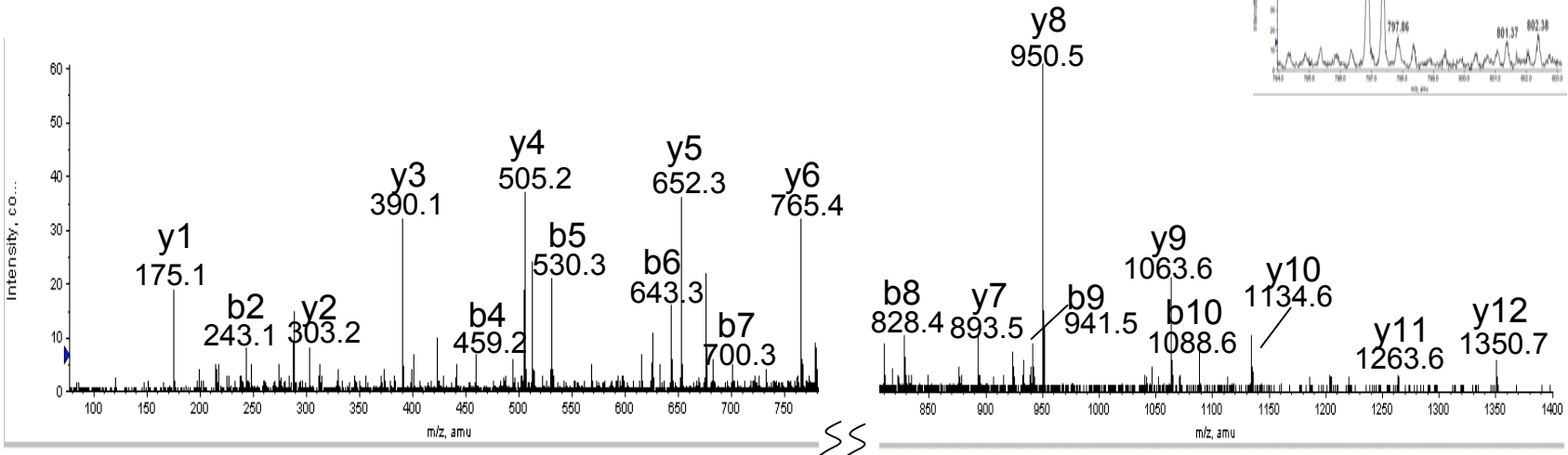
MX: mitoxantrone-resistant cells, WT: drug-susceptible cells



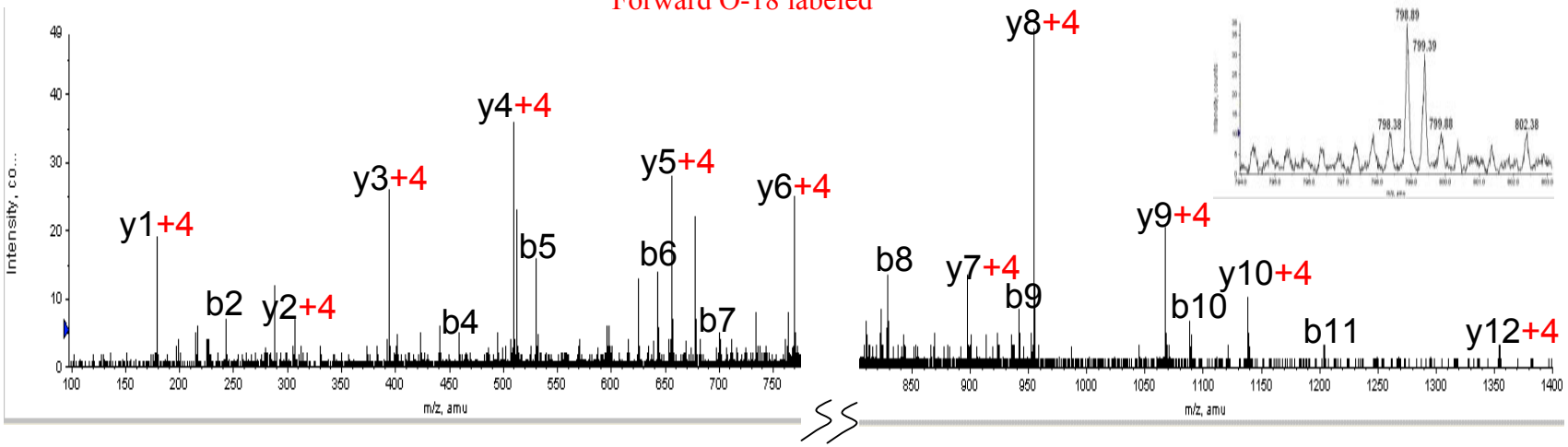


Both peptides come from protein: Galectin-3 binding protein – “on/off”





Forward O-18 labeled



Reverse O-18 labeled

Galectin-3 binding protein

Proteins with altered abundances in drug resistance

Accession number	Protein name	Abundance ratio (MX/WT) in forward labeling	Abundance ratio (MX/WT) in reverse labeling
Q9HCCO	Methylcrotonoyl-CoA carboxylase beta chain	2.0±0.6	1.9±0.1
P49411	Elongation factor Tu	2.4±0.4	2.4±0.5
P35232	Prohibitin	2.9±0.3	2.4±0.1
Q92665	Mitochondrial 28S ribosomal protein S31	3.3±0.5	2.5±0.5
Q9BZZ5	Apoptosis inhibitor 5	3.3±0.1	2.4±0.2
P20674	Cytochrome C oxidase polypeptide Va	3.5±0.6	2.0±0.4
Q16698	2, 4-dienoyl-CoA reductase	3.6±0.3	2.1±0.0
Q08380	Galectin-3 binding protein precursor	Only present in MX	Only present in MX
P09622	Dihydrolipoyl dehydrogenase	0.46±0.12	0.42±0.11
Q9HAV7	GrpE protein homolog 1	0.48±0.12	0.42±0.07

Biological implications of abundance changes

Apoptosis

- **Galectin-3 binding protein (“on/off” protein, only in MX)**

  -- tumor-secreted antigen in human breast cancer cells

  -- galectin-3 may confer chemotherapy and apoptosis resistance

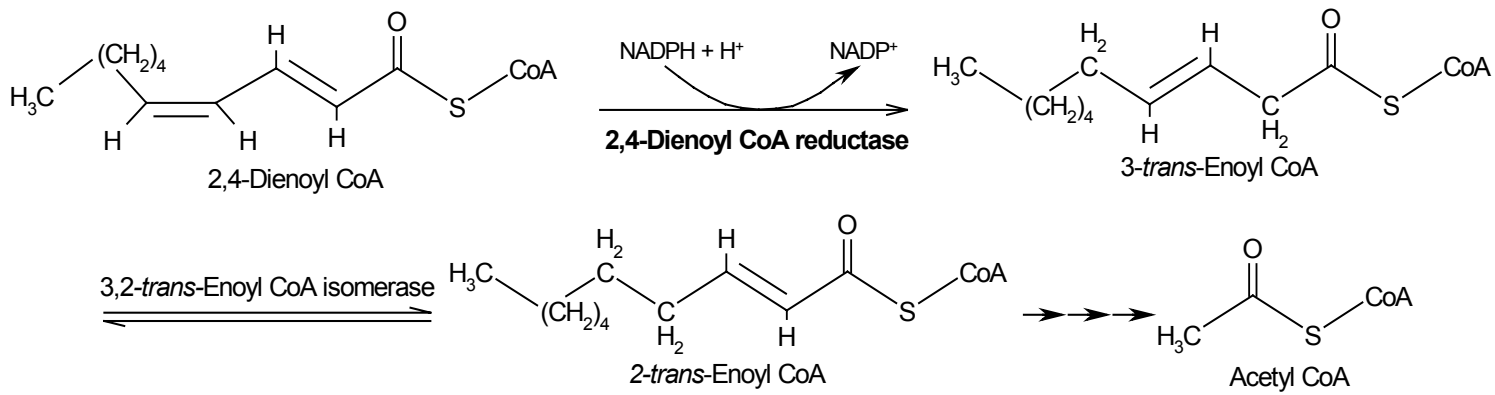
  -- galectin-3 translocated to the mitochondrial membranes and inhibited cytochrome c release following a variety of apoptotic stimuli

- **Apoptosis inhibitor 5 (↑)**

  -- a potent suppressor of E2F-dependent apoptosis

Fatty acid metabolism

- **2, 4-dienoyl-CoA reductase (↑)**



-- Warburg hypothesis: cancer cells consistently rely on glycolytic pathway to convert glucose to ATP (reducing ROS ?)

-- fatty acid oxidation was shown to be reduced in cancer cells

-- higher use of fatty acid for fuel in mitochondria in drug-resistant cells

Conclusion

- A two-dimensional solution phase separation method combined with forward and reverse O-18 labeling strategy has been established and successfully applied for protein qualitative and quantitative analysis

- The overall strategy identified ten proteins with abundances altered significantly in drug resistant cells. They are active involved in apoptosis, fatty acid metabolism, TCA cycle, etc. , and may be associated with drug resistance

Acknowledgments

- Dr. Catherine Fenselau
- Dr. Peter Gutierrez
- Dr. Nathan Edwards
- The group members
- NIH