1. **Questions**

1.1. Define resolution for MS and for chromatography. Resolving power or resolution may be defined for one peak relative to another or simply based on a single peak.

<table>
<thead>
<tr>
<th>Resolution</th>
<th>MS</th>
<th>CHROMATOGRAPHY</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Δm$ defined arbitrarily by distinguishability of 2 peaks</td>
<td>$α = t_{R2} - t_{R1}$</td>
<td></td>
</tr>
</tbody>
</table>

1.2. What is an internal standard and what does it accomplish analytically.

Internal standards are standards added intentionally to all samples, standards and blanks. The ideal internal standard is completely resolved from the unknown analyte by the measuring instrument but is chemically ‘identical’ to the analyte. Identical means that it experiences the same losses during sample preparation, the same matrix effects and responds to changes in instrument sensitivity in the same way as the analyte.

1.3. Does an internal standard account for losses during sample preparation? If so, how?

The internal standard ideally is lost at the same rate during sample preparation by accidental dilutions, chemical degradation, evaporation, pipeting losses, adsorption to surface etc. This is not so hard to imagine as the IS is mixed with the sample before the sample preparation is begun.

1.4. Discuss isotope dilution from the perspective of a ‘perfect internal standard’.

Mass spec is unique in its ability to distinguish identical chemicals of different mass (isotopomers). Most isotopomers have chemical and physical properties that are indistinguishable. For example, $\text{CH}_3^{37}\text{Cl}$ and $\text{CH}_3^{35}\text{Cl}$ have indistinguishable boiling points, electronic spectra, dielectric constants, reactivities, solubilities etc., but are completely resolved in even inexpensive mass spectrometers.
1.5. **Distinguish between a stable isotopic label and a radioisotopic label. Why are stable isotopic labels used in MS?**

Radioisotopes are those that decay by alpha, beta, gamma, electron capture, positron emission etc. All but pure gamma emitters change chemical identities and masses during this transition. The transition can make the radioisotope detectable by a variety of means (scintillation counting, x-ray film etc.) But these mass changes defeat the resolution desired in MS isotope dilution. Radioisotopes are dangerous, expensive and require special handling.

1.6. **Define proteomics.**

Proteomics refers to the measurement of the broad inventory of proteins expressed in a given organism or cell line. For example, proteomics may refer to the identification, detection and quantitation of all of proteins and post-translational modifications observed in a given cell line at a given time.

1.7. **Define polynucleotide, polypeptide and protein.**

DNA nucleotides comprise adenine (A), guanine (G), thymine (T) and cytosine (C). Polynucleotides comprise polymers with variable A,G,T and C subunits linked by phosphate esters of deoxyribose (RNA uses uracil (U) and ribose). Oligonucleotides are short (less than about 20 or so units) of AGTC. Proteins are formed from amino acids linked by peptide (amide) bonds. Proteins are made by living cells – polypeptides are short (< about 20?) chains of amino acids that may or may not be biogenic.

1.8. **Would you describe the contents of a cell as:**

1.8.1. A solution of one or two different proteins.
1.8.2. A solution of a few polynucleotides and polypeptides.
1.8.3. A solution with a few mono and polysaccharides, lipids, glycolipids, proteins, glycoproteins, peptides, RNA, DNA, small molecule electrolytes and metabolites and basically a very complex thing.

1.9. **How does chromatography help to simplify the analysis of a cell’s contents?**

Analysis of a cellular contents is a very challenging problem – usually requiring a variety of fractionations such as centrifugation to separate organelles, salting out of proteins, dialysis to remove salts and small molecules etc. This may reduce the dimension of the problem to just 10’s to hundreds of abundant chemical species. Chromatography (HPLC in one of its many incarnations) can then reduce this by a factor of ten or better.

1.10. **How does mass spectrometry help?**

MS gives sensitively and possibly quantitatively reports the masses of the biomolecule constituents of the mixture. If two biomolecules have different masses then they are resolved even if they co-elute. Mass may not seem like the most amply descriptive property of a biomolecule, but it’s not bad! Fragmentation patterns from protein databases can be identified by computer analysis rapidly and faithfully.
Questions that are more specific to MS:

1.11. How is **product ion scanning** superior to single quadrupole analysis of a protein mixture for the purpose of identifying or quantifying a given component of a complex biological sample?

In the product ion scan, one can screen the mixture for the mass of the component of interest in Q1, fragment it in Q2 and then scan for the fingerprint of the fragmentation products in Q3. This is powerful if you know what you are looking for! Even if Q1 admits some interfering species, the fingerprint in Q3 is likely to be interpretable.

1.12. Given the exact structure of a small molecule, say, CO, and the mass spectrum comprising just the monocation, CO+, how many peaks do you expect to find in the MS of this molecule? Assume you can measure within about 100:1 signal to noise ratio. If more than one, then with what relative intensities?

It’s kind of a weak question, I admit – but the idea is that CO⁺ should show up in four places corresponding to $^{12}\text{C}^{16}\text{O}^+$, $^{13}\text{C}^{16}\text{O}^+$, $^{12}\text{C}^{18}\text{O}^+$ and $^{13}\text{C}^{18}\text{O}^+$. The abundance of $^{13}\text{C}$ is about 1%, so this one should be seen at SNR=100. But $^{18}\text{O}$ is rarer and the isotopomers with $^{18}\text{O}$ would not normally be observed unless the sample were enriched in $^{18}\text{O}$. One to two peaks should be observed for CO⁺.

1.13. Apply the same reasoning to the analysis of a 5 kDa protein (not in detail, just discuss the general idea).

The basic idea then is this – the 5 kDa peak will have many sidebands comprising small and variable amounts of $^{13}\text{C}$ and $^2\text{H}$. So, for example, if the resolving power of an instrument is 10,000 at 5kDa, then while it is conceivable that one might resolve the centers of the distributions formed by two different proteins of nominal mass 5000 Da and a 4,998 Da proteins, their individual isotoper masses will not be resolved. Take home message: Proteins will produce isotopic distributions around their nominal masses that are normally unresolved. Even if an instrument has the resolving power to distinguish two proteins, their actual resolution may be difficult to these distribution functions.
1.14. Contrast quadrupole MS detection with fluorescence and absorbance spectroscopy in terms of:

<table>
<thead>
<tr>
<th>Figure of Merit</th>
<th>q-Mass Spec</th>
<th>Fluorescence</th>
<th>Absorbance</th>
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</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>+++</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>ng (10^{-9} g)</td>
<td>yg (10^{-21} g)</td>
<td>nM conc. DL</td>
</tr>
<tr>
<td>Selectivity</td>
<td>++++++++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Especially with fragmentation pattern recognition.</td>
<td>Fluorescence is not that common.</td>
<td>Many interferants normally possible.</td>
</tr>
<tr>
<td></td>
<td>Pretty much anything can give a ion fragment!</td>
<td>Only selective if a fluorophore can be attached selectively to your target.</td>
<td>Lots of molecules exhibit a UV abs., all have IR abs.</td>
</tr>
<tr>
<td>Destructive or non?</td>
<td>destructive</td>
<td>semi-destructive through label</td>
<td>non-destructive</td>
</tr>
<tr>
<td>Sample Size Required</td>
<td>nL (MALDI) to 100 uL (ESI)</td>
<td>pL + microscope</td>
<td>0.1 mL – 1.0 mL</td>
</tr>
<tr>
<td>Speed</td>
<td>Medium to fast (instrument dependent, sample prep dependent)</td>
<td>Slow (assume biolabeling to make selectivity)</td>
<td>Fast (simple measurement)</td>
</tr>
<tr>
<td>Cost</td>
<td>High (instrument)</td>
<td>Medium (instrument + enzymes + labor)</td>
<td>low</td>
</tr>
<tr>
<td>Instrument size</td>
<td>big</td>
<td>med</td>
<td>Small</td>
</tr>
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</table>
Questions we have answered:

Chem 250 Fall 2008
Answers to questions given in class 8.27.08

1.15. A strong cation exchange resin contains sulfonic acid (a strong acid) residues. Assume the solution pH is 7.5.

1.15.1. Will all proteins bind to this resin?

No, a cation exchange resin has anionic (strong acid) groups on the surface such as R-SO3-. Therefore it will bind proteins that are either net cationic or that have at least some patches of positive charge on the surface. It will not bind purely anionic proteins. It will not bind proteins with a pI above the buffer pH = 7.5.

1.15.2. How will proteins bind to this resin?

Electrostatic attraction between cationic (lysine and arginine) groups on the protein’s periphery and sulfonates on the resin.

1.15.3. Will this be a mono- or multi-valent binding interaction? (Note that multivalent interactions such as DNA duplexation have characteristically sharp association isotherms.)

The likelihood is that this will be multivalent. The more lysine groups on the protein surface, the more points of attraction and the stronger the binding.

1.15.4. Why would increasing ionic strength of, e.g. NaCl decrease the strength of this binding interaction?

Increasing ionic strength will tend to elute the protein. One can think of this as a competitive binding effect (like the Na⁺ in the solution competes with lysine⁺ for the sulfonate) or as a charge screening effect wherein at high ionic strength ions in solution simply tend to cancel electric fields and prevent the normal electrostatic interactions from extending beyond a few angstroms.

1.15.5. On what basis will this separation work?

The separation will work on the basis of the strength of the cation-anion attraction balanced against the solvating (enthalpic) and liberating (entropic) energies of releasing the protein.

1.16. On what basis does reverse-phase chromatography separate analytes?

Hydrophobicity – or, the tendency of non-polar components of the solution to stick to or partition into the oily coating on the chromatographic stationary phase.
1.17. Will ionic strength have a dominant influence on reverse-phase chromatographic separation carried out in 100% aqueous buffer?

Ionic strength will only slightly modify the retention in the reverse phase system compared to the hydrophobic/hydrophilic quality of the protein (solute / analyte). Even if it does change the retention properties, the idea is that the basis of the separation on the reverse phase column will be different to that of the ion exchange column.

1.18. How does the combination of strong cation exchange followed by reverse-phase LC equate to a 2-D separation? Just sketch the outcome.