SYSTEMATIC OPTIMIZATION OF UPLC PEPTIDE MAPS WITH THE ACQUITY UPLC H-CLASS SYSTEM

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INTRODUCTION

The analysis of protein structure is routinely based on enzymatic digestion to more manageable fragments. The separation of these fragments, peptide mapping, has long been a fundamental tool for structural analysis. Reversed-phase HPLC has become the preferred tool for peptide mapping. The chromatographic separation is coupled to mass spectral detection for identification of the chromatographic peaks. The analysis of authentic protein samples is, however, more complicated because all samples are mixtures of native and modified or damaged proteins. Low abundance peptides, representing these trace modifications, may co-elute with major components. The analysis benefits therefore from the use of the most highly resolving chromatographic techniques.

UPLC® peptide mapping routinely gives higher resolution than is possible with HPLC. UPLC is used in different ways to meet the requirements of specific analyses. First, the inherently high resolution can be used to ensure that all components in a sample are detected. Second, the high resolution can be used to reduce run time while preserving the resolution of a well established map. Third, a very fast assay for particular diagnostic peptides can be developed. All three strategies will be illustrated with protein digests. The various options for improving selectivity, including gradient slope, temperature, and mobile phase modifier will be tested. Several new UPLC packing materials will also be compared in terms of their effects on selectivity in peptide maps. The systematically optimized chromatography improves the overall sensitivity and dynamic range of MS characterization, thus allowing accurate quantitation of modified proteins.

The use of UPLC for peptide mapping optimizes resolution by reducing band broadening, that is by increasing efficiency. While this approach leads to better resolution, sensitivity, and speed, it is still necessary to exploit the chemical and chromatographic operational variables that can be used to optimize retention and selectivity. The variables include nature of the organic solvent, pH, modifier, and concentration. Exploring this separation space requires preparation of many solvent mixtures. We have now developed a new UPLC instrument that makes it easier to manipulate the parameters that can further enhance resolution. The ACQUITY UPLC® H-Class System provides quaternary solvent blending that can be used with the Auto•Blend™ technique to continuously vary to mobile phase composition used.

METHODS

Materials

ACQUITY UPLC BEH 130 C18 1.7 µm 2.1 x 100 mm Column
ACQUITY UPLC BEH 300 C18 1.7 µm 2.1 x 100 mm Column

Trifluoroacetic Acid; Pierce
Water; MilliQ
Acetonitrile; Fisher Optima
Isopropanol; Fisher HPLC Grade

Samples

Native digest:
MassPREP™ Phosphorylase b Digestion Standard
MassPREP Enolase Digestion Standard
MassPREP Hemoglobin Digestion Standard
MassPREP Peptide Standards

Instruments

Waters ACQUITY UPLC H-Class System including:
ACQUITY UPLC Quaternary Solvent Manager
ACQUITY UPLC Sample Manager (FTN-SM)
ACQUITY UPLC Column Heater (CH-A)
ACQUITY UPLC TUV Detector at 214 nm

Chromatography

Mobile phase A: Water
Mobile phase B: Acetonitrile
Mobile phase C: Isopropanol
Mobile phase D: 1% TFA in Water
Needle wash: 50:50 Acetonitrile/Water
Flow rate 0.2 mL/min
Injection: 10 µL

Waters ACQUITY UPLC H-Class System.

AUTO•BLEND

Controlling Chromatographic Selectivity

Mobile phase modifiers in peptide mapping:

- Reduce charge on peptides
- Neutralize acidic side chains by lowering pH
- Neutralize basic side chains by ion pairing

Organic Solvents in Peptide Mapping:

- Acetontrile preferred
  - Lowest viscosity
    - Best peak shape
    - Lowest backpressure
  - Best UV transparency
  - Easiest to evaporate
- Alcohols for special cases
  - Ethanol for biocompatibility
  - Propanol for hydrophobic
- Protic vs. non-protic for selectivity

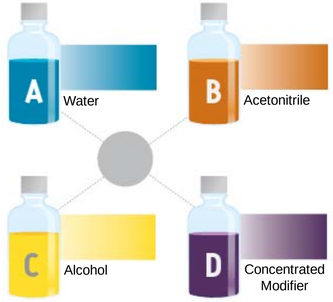

Figure 1. Most chromatographic separations are done with a strong and weak solvent that are pre-mixed. Preparing the array of solvents used to optimize a peptide map can be a time-consuming and cumbersome process. With 4 solvent bottles, we can use each of those 4 solvent bottles as a pure solvent and use one of them as concentrated modifier. We can then draw any percentage we want from any of those bottles for adjusting the separation or actually creating on demand the mobile phase that we require for the separation.

RESULTS

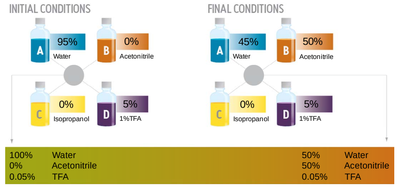

Figure 2. Many reverse phase separations are dependent on the concentration of organic solvent, on the identity of the organic solvent, the pH, and the ionic strength of the buffer. As shown here, we configure our Auto•Blend system with a bottle of pure water, pure acetonitrile, pure isopropanol, and a concentrated solution of trifluorocetic acid.

Our initial concentration for making the injection is a largely water solution that contains a variable amount of TFA. We can change the TFA from 2 to 5 to 10% on the D line to get different concentrations. The final conditions are generated in the same way. Running a gradient of increasing acetonitrile by drawing from the water and the acetonitrile bottle while holding the TFA concentration independent, we can observe the effect on the separation if we draw different percentages from that D line.

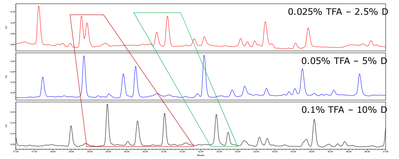

Figure 3. The enolase digestion standard was analyzed with three different concentrations of TFA programmed as different percentages from the D line; the peptides rearrange elution position. The first two peaks that are circled are partially resolved at the low concentration of TFA and are much better resolved as the TFA is increased. The second group of peaks outlined in green actually merge together as TFA increases and then reverse their elution order with continued to increases in the TFA concentration.

To do this experiment with a binary liquid chromatograph, 6 different of mobile phases, a bottle of water, and a bottle of acetonitrile in each of the three concentrations of TFA are required. With Auto•Blend, only one concentrated bottle of TFA and three pure solvent stocks are required. This technique is very convenient and makes it very easy to explore the separation space.

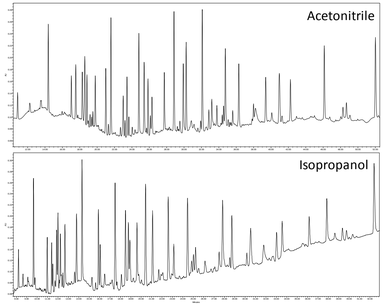

Figure 4. In a similar experiment, the TFA was held constant at 0.05%, that is 5%D with a gradient of increasing isopropanol delivered as a gradient from Line A to Line C instead of the usual acetonitrile gradient from line A to Line B. The change in selectivity is obvious.

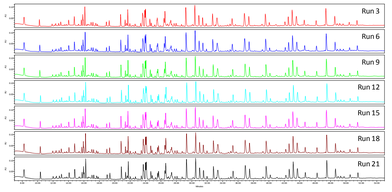

Figure 5. In this 60-minute gradient running at just 2 tenths of a mL per minute, we can see the large number of peaks. We've chosen samples at regular intervals throughout the weekend. And you can see we have preserved the resolution and retention time of that long shallow gradient over the relatively long series of runs that carried us through the weekend.

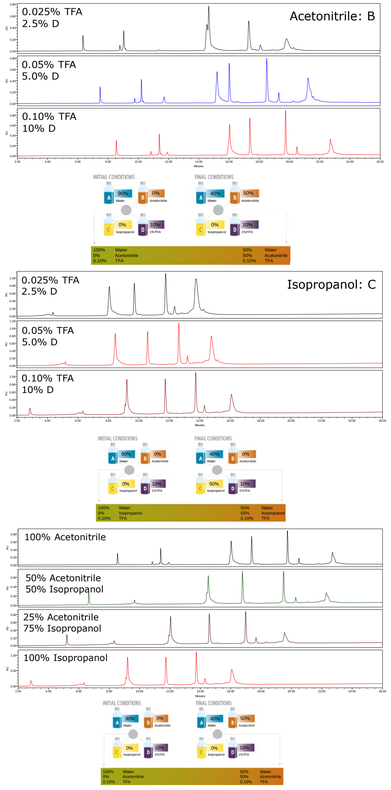

Figure 6. AutoBlend for Protein Separations. Concentration of acid, nature of organic solvent, and proportions of organic solvent were varied in an automated protocol with bottles of pure solvent.

CONCLUSIONS

- **The new ACQUITY UPLC H-Class System is suitable for the reversed-phase separation of biomolecules**
- **Auto•Blend provides an efficient way to adjust selectivity in peptide mapping**
- **The same solvent blending principles can be applied to protein separations**
- **Auto•Blend methods have the same reproducibility and consistency as binary methods**

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