Performance PerSPECtives

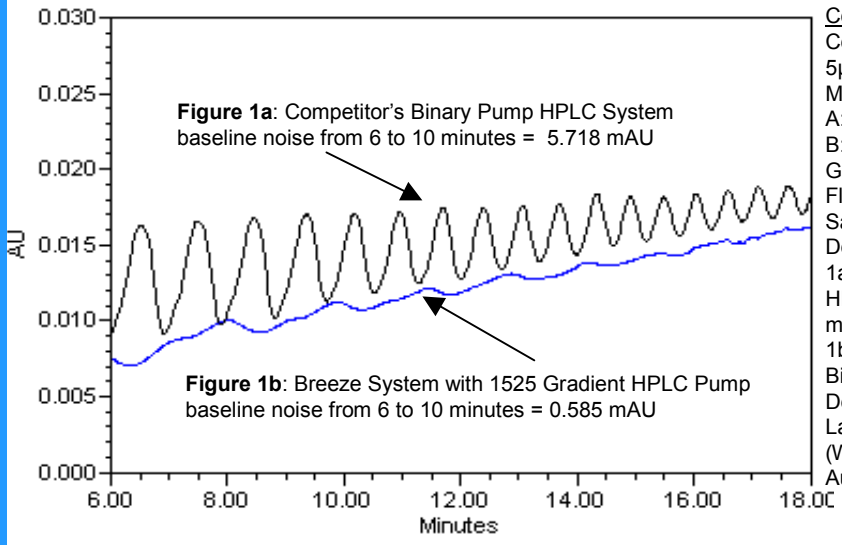
Waters® Breeze™ HPLC System
Peptide Maps Using Acetonitrile/TFA Gradients

Question: I understand how Waters Breeze HPLC System balances simplicity, high performance, reliability and affordability in an easy-to-use HPLC system. Can a Breeze HPLC System also be used to generate peptide maps at analytical flow rates (i.e., 1 mL/min) using acetonitrile/trifluoroacetic acid gradients?

Peptide Maps:
Peptides are a class of biomolecule composed of amino acid residues containing hydrophobic, acidic, basic, and neutral side chains. Reversed-phase HPLC is commonly used to separate and compare complex samples containing peptides of various size and chemical composition. For example, the protein cytochrome *c* can be digested into small peptides by the enzyme trypsin. A "peptide map" can be generated from this protein digest using reversed-phase HPLC on a C8 or C18 column. The separation is performed using a shallow gradient of increasing organic solvent concentration (e.g., 5 to 45% acetonitrile in 40 minutes). Because peptides can contain charged amino acid residues, trifluoroacetic acid (TFA) is added to HPLC eluent A (water) and eluent B (acetonitrile). The TFA serves as a pH modifier by minimizing the presence of undesired negative charges on the reversed-phase column due to presence of free silanols on the base silica material. It also ion-pairs with protonated amino acids residues resulting in increased retention of the peptides on the reversed-phase material.

Challenge Using TFA in HPLC Gradient Chromatography:
HPLC generated peptide maps are monitored at low-UV wavelengths (e.g., 214 nm) due to the high absorptive energy of the peptide bond that connects each pair of amino acids. However, a significant degree of low-UV absorbance is also exhibited by the TFA reagent. Furthermore, the degree of absorbance due to the presence of TFA increases as the concentration of acetonitrile increases during the separation. Consequently unacceptable levels of baseline noise occur if the HPLC solvent delivery system does not adequately mix the eluents during the acetonitrile/TFA gradient generation. Figure 1a shows characteristic baseline-noise ripple on a competitor's binary pump HPLC system due to incomplete mixing of the TFA contained in Eluents A and B. The deleterious effects of this phenomenon on the quality of collected data are detailed in Waters Performance PerSPECtive WPP223. By comparison, Waters Breeze HPLC system is designed to minimize baseline noise by providing adequate TFA mixing (See Figure 1b).

Fig. 1: TFA Gradient on Competitor's Binary System vs. Waters Breeze System with 1525 Binary HPLC Pump.



Conditions:
Column: Symmetry® C18, 5µm, 3.9 x 150 mm at 35° C.
Mobile Phase:
A: 0.1% TFA in Water.
B: 0.1% TFA in Acetonitrile.
Gradient: 5 - 40% B in 35 min.
Flow: 1.0 mL/min.
Sample: Water (10 µL inj. vol.).
Detection: 214 nm at 2 pts/sec
1a: Competitor's Binary Pump HPLC System with gradient mixer and in-line degasser.
1b: Breeze System with 1525 Binary HPLC Pump, In-line Degasser (WAT079700), Large Volume Gradient Mixer (WAT051518), and 717Plus Autosampler.

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How Adequate Eluent Mixing is Accomplished?:
The technology contained in Waters Breeze HPLC Systems make them well suited for gradient chromatography at a variety of operating conditions. (See Waters Performance PerSPECtive WPP 1500 entitled: "Waters Breeze Systems Gradient Chromatography"). In the case of peptide mapping, each solvent required for the gradient elution is delivered to the column via a separate pump. Breeze Software manages solvent flow rate and gradient composition. As indicated in Figure 1b, thorough blending of the TFA occurs after the eluents leave each pump. "High Pressure Mixing" is the term used to describe this technique for non-isocratic solvent mixing and delivery.

Breeze Technology Yields Reproducible Peptide Maps:
The ability of reversed-phase HPLC to separate peptides of very similar chemical composition is well established. However, the capacity of the HPLC system to yield reproducible gradients is critical when this technique is used to compare and contrast peptide samples of different but similar chemically composition. The ability of Waters Breeze technology to generate reproducible peptide maps at analytical flow rates is clearly indicated in Figure 2 and Table 2 making this system well suited for this demanding application.

Fig. 2: Peptide map of a tryptic digest of Cytochrome *c*

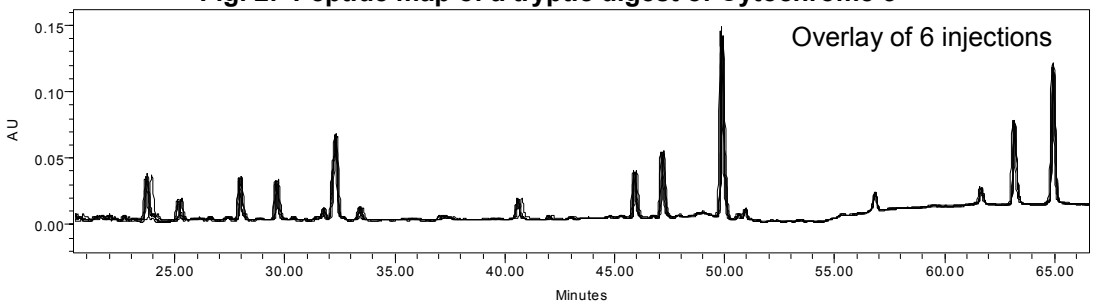

Table 2: Retention time mean (minutes) and standard deviation of 6 injections

Peak #	1	2	3	4	5	6	7	8	9
R.T. Mean	23.74	27.95	29.62	32.30	45.90	47.17	49.87	63.18	64.95
R.T. Std. Dev.	0.101	0.057	0.060	0.037	0.052	0.051	0.046	0.038	0.033

Conditions Used for Figure 2 and Table 2:
Breeze System with 1525 Binary HPLC Pump, In-line Degasser (WAT079700), Large Volume Gradient Mixer (WAT051518), 717Plus Autosampler, and 2487 Dual Wavelength Absorbance Detector.
Column: Symmetry® C18 5 µm, 3.9 x 150 mm at 35º C.
Mobile Phase: A: 0.1% TFA in Water B: 0.085% TFA in Acetonitrile.
Gradient: 0 - 40% B in 80 min.
Flow: 1.0 mL/min.
Column Temperature: 35° C.
Sample: Tryptic digest of bovine cytochrome *c* (20 µL Inj. Vol.).
Detection: 214 nm at 2 pts/sec.

Summary:

• Waters Breeze HPLC System balances simplicity, high performance, reliability, and affordability in an easy-to-use HPLC system.
• The reversed-phase HPLC separation of samples containing compounds of very similar chemical composition challenges the ability of any HPLC system to yield reproducible results.
• Breeze HPLC System provides users with the technology required for demanding applications such as peptide mapping at analytical flow rates.