Controlling On-Chip Gradient Elution Microchannel Electrochromatography Using the LabSmith HVS448

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Separations of complex peptide mixtures can take too long to elute without the ability to apply a solvent gradient.

One solution for applying a gradient is called Microchannel Electrochromatography, or MEC. In reversed phase MEC buffers with aqueous and organic components comprise the mobile phase of a separation through a solid and porous stationary phase localized in a microfluidic device. MEC requires precise and automated control of three important experimental parameters:

1. **At least two applied electric field gradients**
2. **Timing and control of the sample injection/switch to sample elution**
3. **The electroosmotic flow (EOF) velocity during buffer gradient formation.**

The LabSmith HVS448 High Voltage Sequencer is the only solution on the market capable of the precise, accurate voltage control and timing required for on-chip MEC. With the HVS448 in their toolkit, Professor Aaron Wheeler and Ph.D. students Michael Watson and Jared Mudrick of The University of Toronto could focus on a solution to the third critical problem of implementing MEC: control of the EOF velocity.

Described below is a guide to implementing their velocity-matched buffer approach to the on-chip gradient MEC elution of a protein digest using the HVS448 (Figure 1d), first published in *Analytical Chemistry* (*Anal.Chem.*, 2009, 81, 3851-3857).

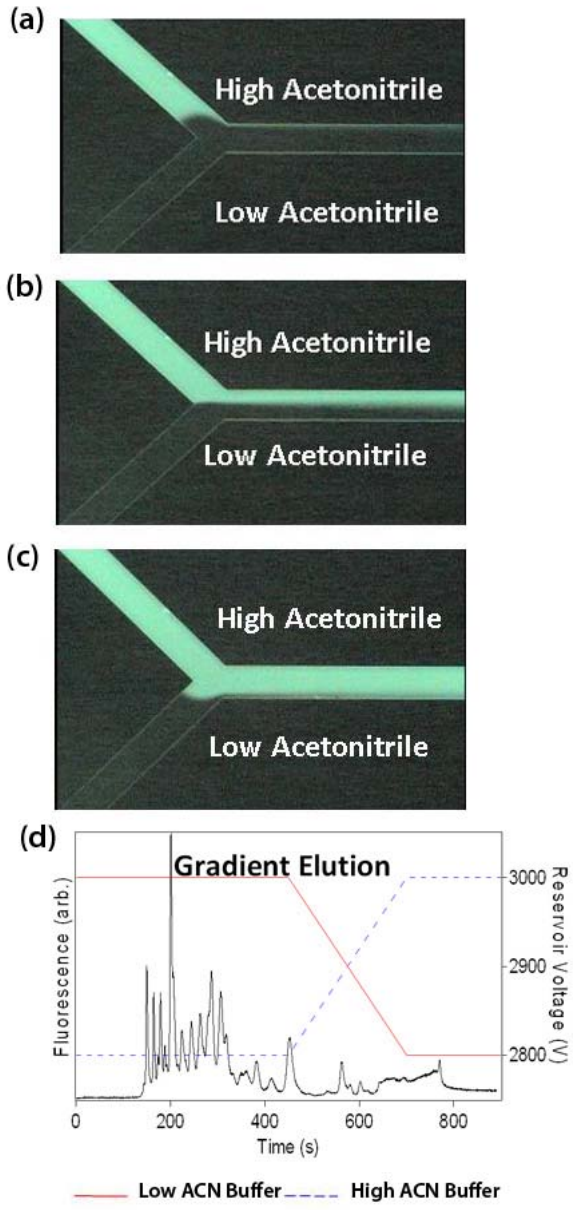

Figure 1. On-chip Gradient MEC. (a-c) Images of the solvent gradient generated on chip with fluorescein dye incorporated in the high acetonitrile buffer. (d) Gradient MEC elution of trypsin digest of casein.

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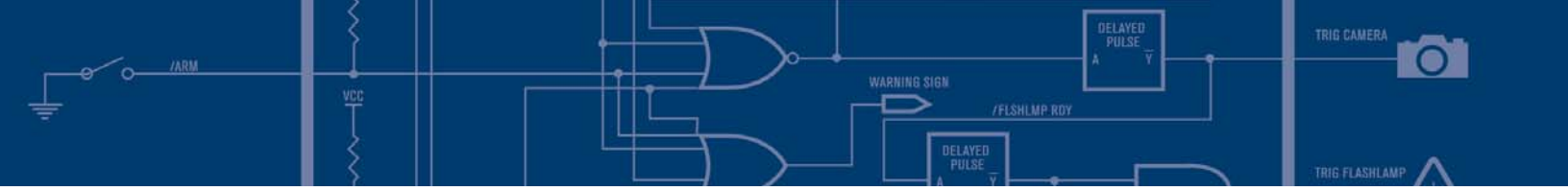


Introduction

From biomarker discovery to benchmarking, monitoring and diagnostics, chromatography remains a powerful tool for peptide and protein analysis *(1)*. Microchannel Electrochromatography, or MEC, makes it possible to minimize dispersion due to pressure-driven flow while keeping the plate number advantage given by chromatography, resulting in increased resolution at higher speeds *(2)*. In addition to a performance advantage, MEC simplifies instrumentation by using only electric fields to generate flow, which eliminates the need for high pressure mechanical pumps, fittings, and valves. With the advent of lab-on-a-chip platforms for separations, MEC has moved from promise to reality *(3)*. However, for complex samples such as peptides and proteins, it becomes necessary to use gradient elution, with the solvent composition changing in time, for efficient separations (Figure 1).

On-Chip Gradient Electrochromatography Method

The Wheeler group's technique required the titration of the buffer salt concentration in a pair of elution buffers, one containing high acetonitrile (60% vol) and the other low acetonitrile (2% vol). Buffers with differing organic modifier compositions migrate at different linear EOF velocities, prohibiting solvent gradient creation *(4)*. To create a velocity-matched buffer pair, buffer salt was titrated into the faster low acetonitrile buffer. For a stationary phase the Wheeler group utilized porous polymer monoliths (PPMs), but this approach applies to any stationary phase including open tubular capillary electrochromatography methods.

Microfluidic Chips

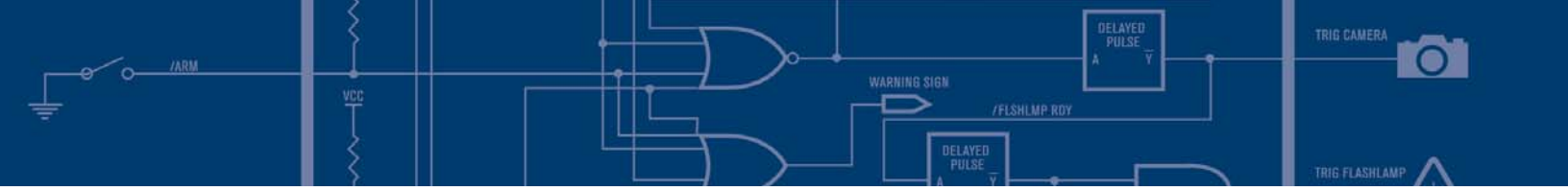
The glass chips used in the procedure were wet-etched with channels 20 μm deep and 100 μm wide. The chips used a custom, 5-reservoir design with two buffer inlets, one sample inlet, and two outlets (Sample Waste and Buffer Waste). Figure 2 shows an example of the custom chip design (Micralyne, Edmonton, AB) as well as the channels from the LabSmith HVS448 that were used to control each reservoir. A serpentine channel of 4.75 cm separates the buffer inlets from the injector and separation column. The length allows for complete mixing of the aqueous and organic elution solvents. The separation channel length was 7.5 cm long, with the length to detector of 7.0 cm.

Figure 2. Gradient MEC Chip. Channels A and C contain the two elution buffers. Channel B is the Sample, Channel D is Sample Waste, Channel E is Buffer Waste.

Stationary Phase Preparation

Porous polymer monoliths were prepared by *in situ* photopolymerization of a casting solution in dry channels, pretreated (silanized for crosslinking to monolith) with trimethylsilylpropionic acid (TMSPA). The pretreatment procedure requires activation of the channel walls by pumping through 200 mM NaOH at 2μL/min for 1.5 h, followed by a deionized water rinse, followed by a methanol rinse. TMSPA (20% vol in methanol) was applied at the same rate and time, followed by a methanol rinse. The channels were then dried at room temperature under nitrogen. A monomer/photoinitiator mixture was formed by measuring the solid reagents, 2,2-dimethoxy-2-phenlacetophenone (DMPA, 2.5 mg) and 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS, 1.2 mg) into an amber glass vial, followed by liquid reagents, butyl acrylate (BA, 278.5 μL), 1,3-butanediodiacrylate (BDDA,150 μL), lauryl acrylate (LA, 69 μL), and trimethylsilylpropionic acid (TMSPA, 2.5 μL).

Monolith formation was initiated by pipetting the casting solution into the buffer outlet reservoir such that it filled the channel network by capillary action. The flow was balanced after filling by dispensing equivalent volumes from the casting solution into the remaining reservoir and covering them to prevent evaporation. Electrical tape at the bottom surface formed a crude photomask beginning 50 μm downstream of the injectors. Devices were illuminated by UV radiation (100 W, 365 nm, 5 min) from below using a UV lamp (UVP, Upland, CA). During polymerization, a small fan was used to cool the lamp and device. After polymerization the reservoir solutions were replaced with 94 mM phosphate buffer (pH 6.8, 30% acetonitrile), and the unreacted casting solution was driven from the channels by EOF (150 V/cm, 120 min) *(4)*.

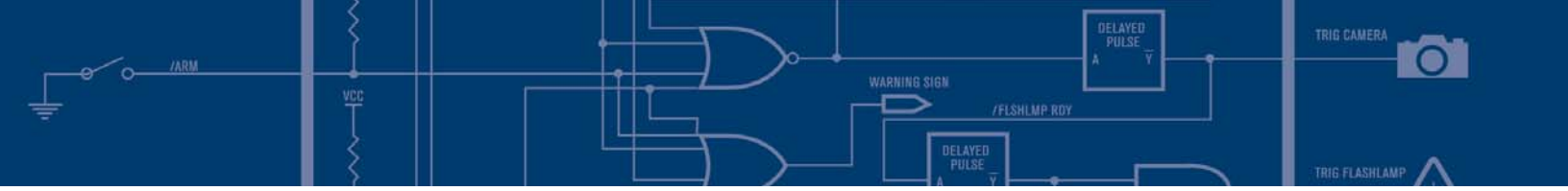


Buffer Velocity Matching

A velocity-matched buffer pair was determined experimentally by the current monitoring method. Briefly, a voltage divider circuit setup was created by placing a resistor (1 MΩ) in series with the microchannel device and ground of the HVS448. This setup allowed for the voltage drop across the fixed resistor to be measured as a higher condictivity buffer (eg. 20 mM Borate) was replaced with a lower condictivty buffer (eg. 18 mM Borate). By this method, two optimum buffer compositions were determined for generating the buffer gradient. A low organic buffer (180 mM borate, pH9, 2% acetonitrile) and a high organic buffer (20 mM borate, pH 9, and 60% acetonitrile) both had an EOF velocity of ~0.8 mm/s when a 245V/cm electric field was applied *(4).* Programmable acetonitrile gradient (see below) were then created using the velocity-matched buffer pair. As proof of principle work Watson *et al.* successfully demonstrated the separtions of peptide standards under differing gradient slopes (not shown) and a gradient

Table 1. HVS448 Sequence Software Wizard Settings for Gradient CEC
Sequence Software Gradient Program Download **(Michael Watson)**

Step Name	Step A "PreLoad"	Step B "DoStepB"	Step C "DoStepC"	Step D "Load"	Step E "DoStepE"
Switch Step					
After Delay	1.0 ms	250000.0 ms	Only Manually	60000.0 ms	450000.0 ms
Next Step	Step D	Step C		Step E	Step B
Channel A					
Input DC (V)	3000.000	-	3000.000	1180.000	2725.000
Waveform	-	Sawtooth (RIGHT)	-	-	-
Offset (V)		2880			
Amplitude (V)		120			
Period (ms)		250000			
Phase Delay		0			
# V Settings/Cycle		500			
Channel B					
Input DC (V)	1500.000	1500.000	1500.000	0.000	1500.000
Channel C					
Input DC (V)	2825.000	-	2825.000	1250.000	3000.000
Waveform	-	Sawtooth (LEFT)	-	-	-
Offset (V)		2900			
Amplitude (V)		100			
Period (ms)		250000			
Phase Delay		0			
# V Settings/Cycle		500			
Channel D					
Input DC (V)	1500.000	Unchanged	1500.000	1500.000	1500.000
Chanel E					
Input DC (V)	0.000	0.000	0.000	1500.000	0.000



separation of a tryptic digest of casein (Figure 1) in less than five minutes *(4).*

Using the HVS448 High Voltage Sequencer for Electrochromatography

The LabSmith HVS448 High Voltage Sequencer is a multi-channel device for precisely timing, sourcing and sensing current and/or high voltage. It is used in conjunction with LabSmith's Sequence™ software, which provides a simple, step-wise environment for creating complex experimental control programs.

The HVS448 was used to apply and control electric fields and monitor currents. In this work LabSmith's Sequence software was used to program and control the buffer gradients. Analytes were loaded using a "pinched" injection mode over the double tee channel element and then injected onto the column using the voltages listed in Table 1. Gradient separations were programmed using matched increasing/decreasing voltage programs ranging between 3.0 and 2.8 KV for the low acetonitrile and high acetonitrile buffers.

The Sequence software was programmed to linearly ramp the low-organic phase buffer from 3.0 to 2.8kV while the high-organic phase buffer was ramped from 2.8 to 3.0 kV. The program settings in the Sequence Wizard are listed in Table 1. A link to the downloadable program is also embedded.

Detection

Analytes were detected by laser-induced fluorescence using an inverted microscope (Olympus IX-71). Excitation was accomplished using an argon ion laser (Melles Griot, Carlsbad, CA) at 488 nm (20 mW) as the excitation wavelength and a 60x objective. The fluorescent emission was collected by the same lens and filtered optically with a 536/ 40 nm band-pass and 488 nm notch filter and filtered spatially using a 500 µm pinhole then imaged onto a photomultiplier tube (PMT, Hamamatsu, Bridgewater, NY). The PMT current was converted to a voltage using a picoammeter (Keithley Instruments, Cleveland, OH) and collected using an A-to-D converter and a PC running a custom LabVIEW (National Instruments, Austin, TX) program.

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