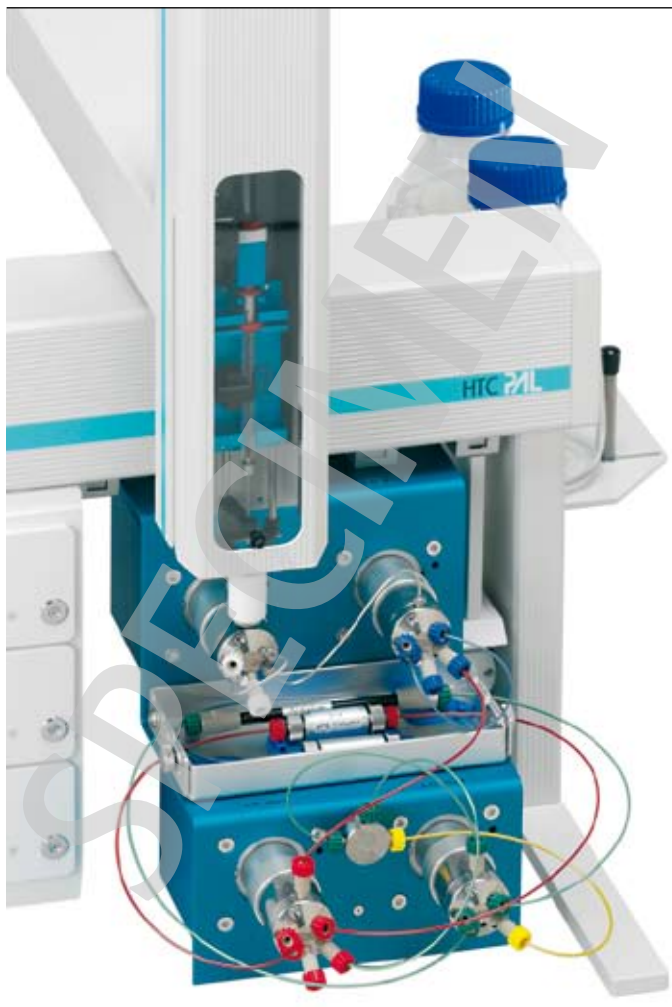


High SPEed™ On-line Multidimensional SPE-SPE-LC



Platform for Sample Preparation
and undisturbed LC-MS-MS Analysis
for Basic Drugs in Biofluids.

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Abstract

High SPEed™ On-line Multidimensional SPE-SPE-LC

High SPEed™ is a dedicated platform for fully automated on-line SPE-SPE-LC sample preparation and analytical separation technique.

The generic approach for basic drugs (e.g. anti depressants and metabolites) is well proven in clinical routine analysis. The multidimensional platform using a column switching concept allows a fully automated clean-up of raw biofluids such as plasma or urine. The first step consists of a size selective fractionation on restricted access material (RAM) combined with reversed phase chromatography. Second step represents a chemo-selective fractionation relying on cation-ion exchange and hydrophobic interaction chromatography.

The fourth valve in the system is intended for various special tasks, e.g. in the method development phase for the so-called "Post Column Infusion" to monitor the influence of the matrix in respect of the ion suppression of the MS signal. Furthermore does the fourth valve enhance the flexibility for the routine analysis with possibilities like "In-Line Dilution" or "early Back-flush of the Analytical Column".

The "On-Line SPE-SPE" clean-up procedure for basic target analytes present biofluids such urine and plasma (human or animal) eliminates all matrix effects influencing the ionization process which is a major problem in bioanalytical LC-MS analysis. Eliminating matrix effects ensures a high quality identification and quantification of the various target analytes and allows to speed up the chromatographic process. Typical cycle times are 7 to 12 minutes.

The concept of High SPEed™ is a unique and flexible platform for routine analysis in clinical-chemical and other bioanalytical labs. The concept is based on the work of Prof. Boos and his co-workers*.

A step-by-step method development guide is provided to establish the valve switching times and recoveries of the various classes of basic drugs.

* In cooperation with Prof. Dr. Dr. K.S. Boos, Dr. Rosa Morello and co-workers.
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Overview of complete system
High SPEed™ On-Line Multidimensional SPE-SPE-LC Technique.
Target Analytes Basic Drugs in Biofluids

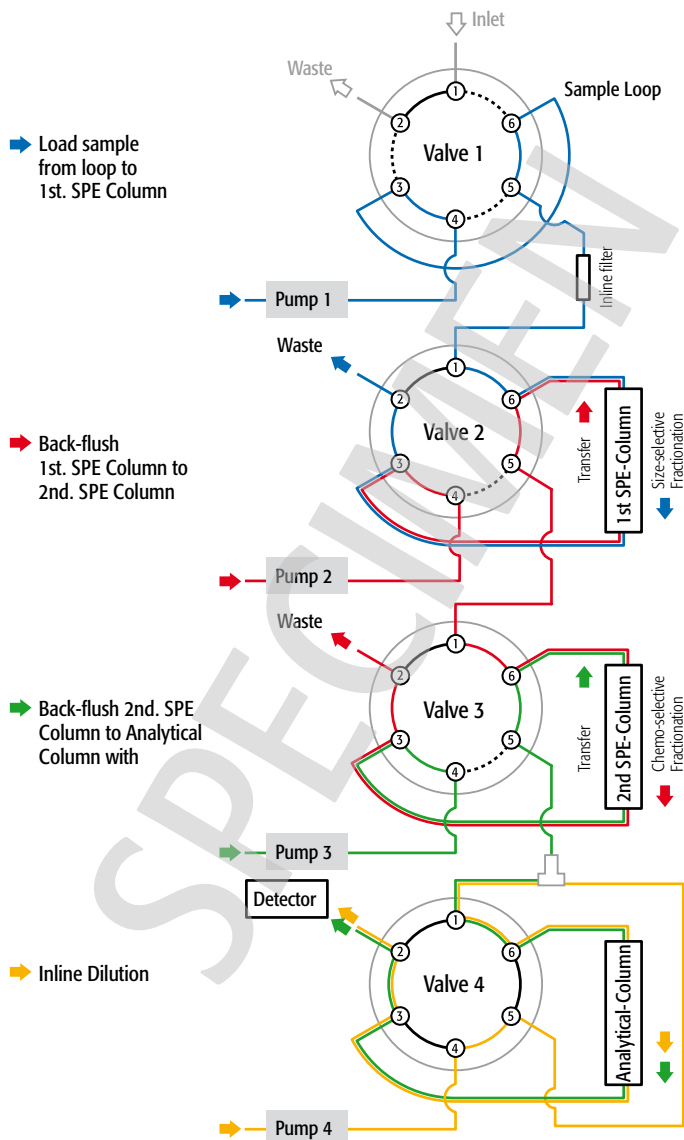


Figure 1: High SPEed™ Flow Schematic for full System Overview (including “In-Line Dilution” option).

Basic Steps Overview

1. Method Development

Step 1 Analyte Separation

Despite of being the last step in an on-line procedure this step should be evaluated at first.

Step 2-5 Determination of Switching Time of Valve 2

Elution Profile of Matrix and Analytes

Step 6-9 Determination of Switching Time of Valve 3

Depletion of low and high molecular weight components which otherwise would disturb the electrospray ionization process in LC-MS/MS analysis.

Step 10-13 Evaluation of Analyte(s) Recovery

The recovery of the analyte(s) gives a good indication of the efficiency of the 2 clean-up steps.

2. Advanced Method

In-Line Dilution

If the amount of organic modifier used to back-flush the second SPE column is too high the analyte(s) can not be focused at the top of the analytical column. An enrichment is possible by adding Water from Pump 4 via the T-piece at the entrance of Valve 4.

Post Column Infusion

To monitor any suppression or enhancement of the ionization of the analyte by the matrix constituents is a constant flow of a diluted analyte added to the sample stream.

Early Back-flush of Analytical Column

An early back-flush would allow increasing the throughput if the later eluting part is of no interest.

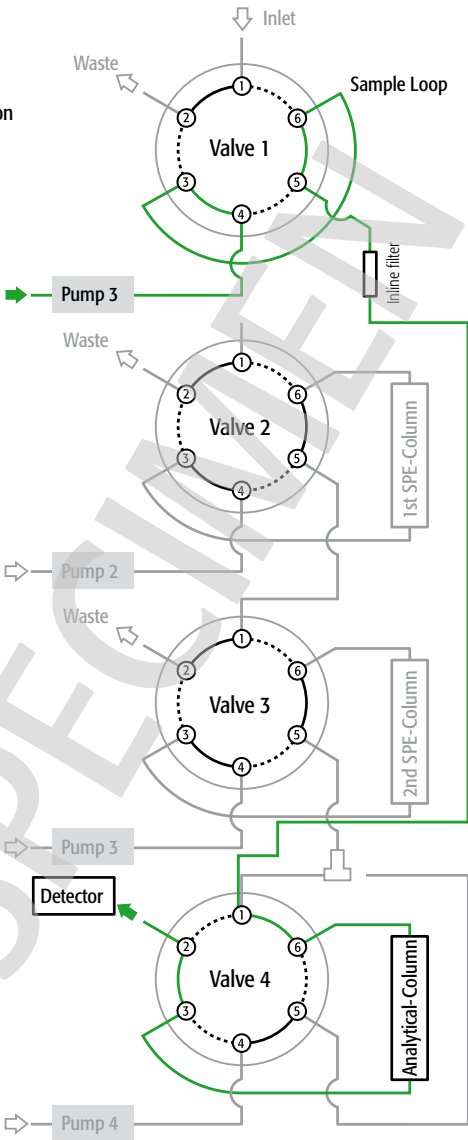
3. On-line SPE-LC

In the literature, several applications are described where only one SPE column is used. For more details see "On-line SPE-LC".

Method Development - Step 1

Separation of Analyte(s) on Analytical Column

➔ Inject Standard Solution directly on Analytical Column



Step 1: Analytes Separation
Figure 2

Method Development - Step 1

Separation of Analyte(s) on Analytical Column

The separation of the analyte(s) on the analytical LC-column should be evaluated at first.

In case of a reversed phase LC-column the separation should be optimized in such a way that the analytes are not eluted too early. This enables the use of a higher amount of organic modifier for the transfer step.

The described SPE columns can be operated to a flow rate of up to 3 ml/min. The flow rate in the system depends on:

- the amount of injected bio fluidic sample
- the parameter values like t_{M1} and t_{M2} (see below)
- enrichment (recovery) of analytes respectively value t_A

If a splitting after the column is needed for the MS Detector depends on the type of the LC-MS detector and the mode operated.

At this stage of method development it is not necessary to inject a spiked sample (including the full matrix). A standard mix of the analytes is sufficient to establish the separation on the analytical column. In contrary, matrix loaded samples would block the inlet frit of the column.

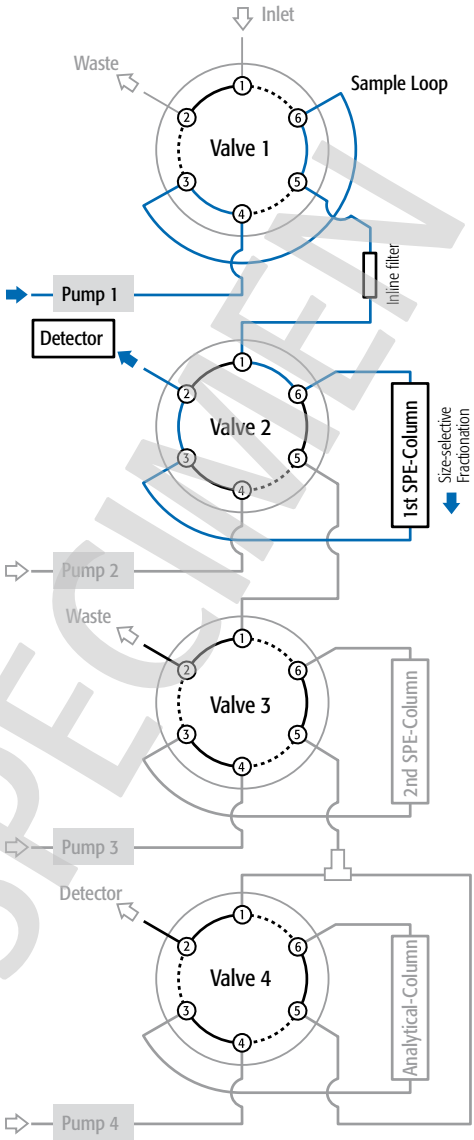
Connect the Pump 3 to Valve 1, a gradient pump with the corresponding mobile phase.

The detector can be a UV detector, select a wavelength in the range of 240 to 280 nm.

In the situation where the amount of organic modifier needed for the transfer from the second SPE column (Valve 3) to the analytical column is too high so no analytical separation is achieved, then a SPE-column with weaker retentive power (RP-4) should be used or an in-line dilution (see advanced steps In-line Dilution) should be performed.

Method Development - Step 2
Determination of t_{M1}
Elution Profile of Matrix on 1st SPE Column

➔ Elution Profile or breakthrough of Matrix on 1st. SPE Column



Step 2: Determination of t_{M1}
Figure 3

Method Development - Step 2

Determination of t_{M1}

Elution Profile of Matrix on 1st SPE Column

In order to determine parameter value t_{M1} the SPE-column is directly coupled to an appropriate detector. For that purpose port no. 2 of valve no. 2 (Fig. 3) is connected with the detector. The sample is injected onto the SPE-column and the elution profile of the sample matrix is recorded (Fig. 4). For monitoring a protein matrix a UV detector set at 280 nm is recommended.

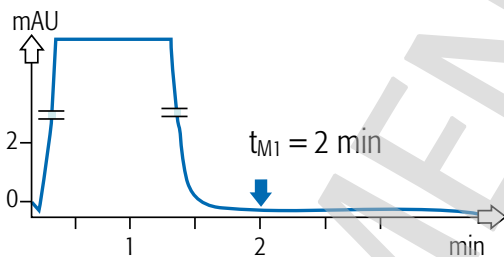


Figure 4: Determination of t_{M1}

Example:	
1 st SPE-column :	RAM- RP-18 (25 x 4 mm)
Mobile phase :	water / acetonitrile (98/2, v/v)
Flow-rate :	1 ml/min
Sample :	100 μ l human plasma
Detection :	UV 280 nm

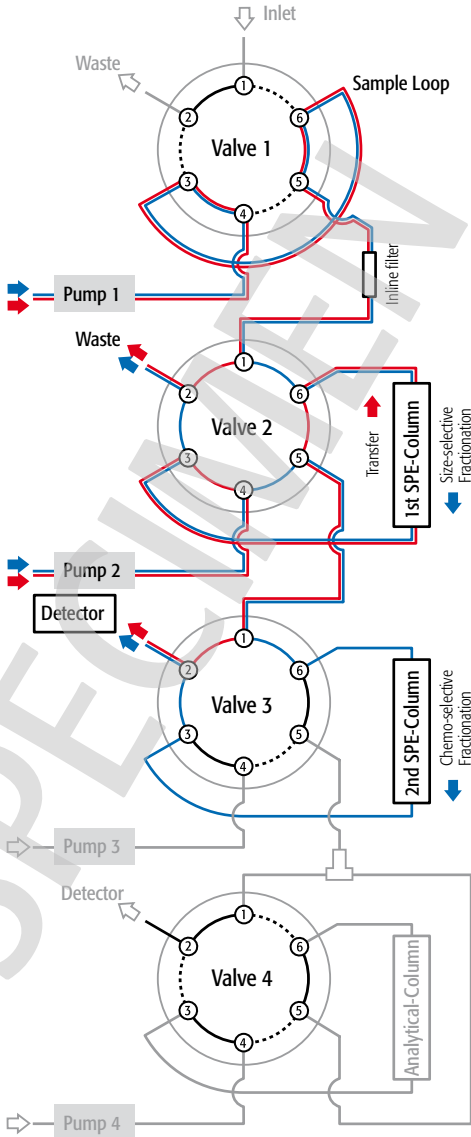
When eluting a protein matrix, care should be taken to ensure that the mobile phase does not possess denaturing properties. With regard to the amount of organic modifier it is recommended to use less than 15 vol% methanol, less than 10 vol% acetonitrile and less than 5 vol% 2-propanol. When processing samples containing lipids, e.g. blood plasma one should always add an organic solvent which solubilises the lipids and prolongs the lifetime of the SPE-column. For the displacement of protein bound analytes the addition of 2 to 5 vol% of acetonitrile or methanol is recommended.

To protect the SPE-column from being clogged by particulate matter present in a sample a dedicated In-line filter (stainless steel sieve) has to be installed between port no. 6 of valve no. 1 and port no. 1 of valve no. 2.

Method Development - Step 3
Determination of t_{A1}
Elution Profile of Analytes on 1st SPE Column

➔ Elution Profile with back-flush of Analyte on 1st SPE Column

➔ Back-flush of Analyte from 1st SPE Column to Detector



Step 3: Determination of t_{A1}
 Fig 5

Method Development - Step 3

Determination of t_{A1}

Elution Profile of Analytes on 1st SPE Column

Determination of t_{A1} uses the same instrumental set-up as for t_{M1} . A standard solution of the analyte(s) having approx. the same concentration as expected in the actual sample is injected onto the SPE-column. Then the elution profile is recorded using the same flow-rate and composition of the mobile phase as for t_{M1} .

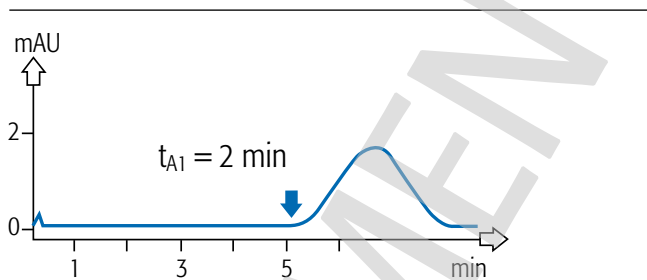


Figure 6: Determination of t_{A1}

Example:

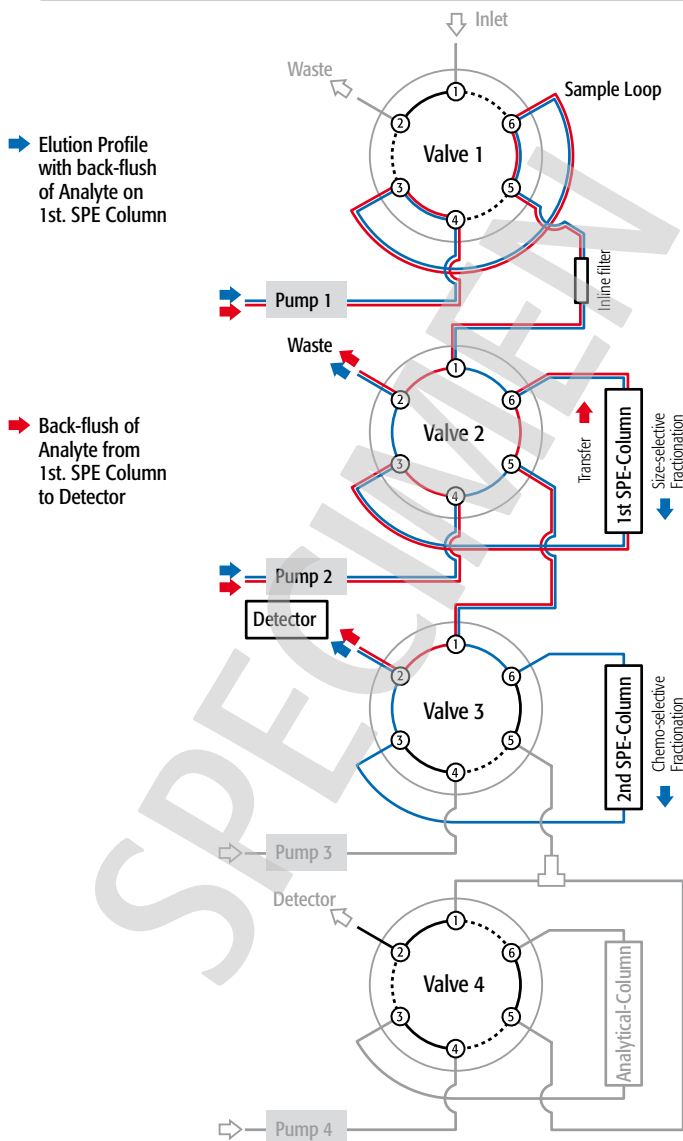
1 st SPE-column :	RAM-RP-18 (25 x 4 mm)
Mobile phase :	water / acetonitrile (98/2, v/v)
Flow-rate :	1 ml/min
Sample :	50 μ l standard solution of drug XY
Detection :	UV 240 nm

One goal with regard to the optimization of the fractionation step is to achieve a time interval between t_{M1} and t_{A1} as large as possible. This allows extending the time for the elution of the matrix and ensures a quantitative extraction of the analyte(s) as well as an effective depletion of the sample matrix

Method Development - Step 4

Determination of t_{T1}

Transfer of Extracted Analytes on 1st SPE Column (Back-flush)



Method Development -Step 4

Determination of t_{T1}

Transfer of Extracted Analytes on 1st SPE Column (Back-flush)

The instrumental set-up for the determination of t_{T1} is as depicted in Fig.7.

First, a standard solution of the analyte(s) is injected onto the 1st SPE-column. The SPE-column, then is flushed with the mobile phase delivered by pump 1 for a time (volume) smaller than t_{A1} . At this point of time valve 2 is rotated. The resulting valve configuration allows the following: (1) elution of the analyte(s) from the SPE-column with an appropriate mobile phase under reversal of the flow-direction (back-flush) (2) monitoring of the elution profile by an appropriate detector.

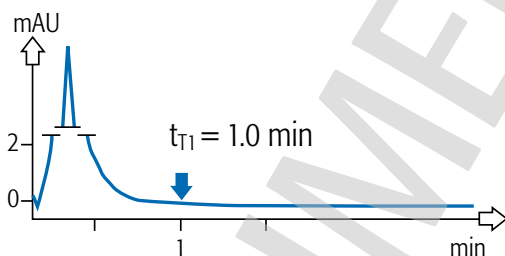


Figure 8: Determination of t_{T1}

Example:	
1 st SPE-column :	RAM-RP-18 (25 x 4 mm)
Mobile phase :	acetonitrile/0,01 M ammonium acetate (60/40, v/v)
Flow-rate :	1 ml/min
Sample :	50 μ l standard solution of drug XY
Detection :	UV 240 nm

The transfer step should always be optimized by adjusting the composition of the mobile phase in such a way that the desorption of the analyte(s) takes places in a volume as small as possible. This measure diminishes peak broadening on the 2nd SPE column.

The transfer of the analyte(s) should only be initiated after complete elution of the sample matrix. Otherwise, the 2nd SPE and analytical column may become contaminated with matrix components, resulting in an irreversible increase in back-pressure and in a significant decrease of capacity and selectivity.

For safety reasons, a "guard" column should always be placed in front of the analytical LC-column.

Method Development

How to read and interpret the Valve schematics?

For all valve schematic is basically the same skeletal structure shown, representing the plumbing for the routine analysis as laid out in Figure 1.

For the method development steps is in certain situations only part of the full system used. All other parts are greyed out. Note that in some diagrams is the detector or a pump moved to another position. In the basic structure is the original position shown but greyed out. Example Figure 7.

The line from Pump 4 to the T-piece is in certain situation disconnected. This shall make the user aware that the Pump 4 is not used for this experiment. The T-piece can be removed or at least plugged at the entry. Example Figure 14.

The Valve 1 is in all diagrams in the "Inject-Position". The position "Load" to fill the loop is a logical and self-explaining step.

The colours in the diagram actually represent two functions:

- Flow of the Mobile Phase from the individual pumps
- The timed steps are differentiated as well by the colour.

Example Figure 7:

- ➡ Pump 1 delivers the Mobile Phase through the loop to Valve 2 in the forward flow direction to the 1st SPE column. In this mode delivers the Pump 2 a mobile phase to support the 2nd SPE column. This line is shown to make the user aware that the 2nd SPE column has to have a flow at this particular time.
- ➡ At the moment the Valve 2 is switched is the flow from Pump 1 directed to "Waste" at Valve 2. The mobile phase from Pump 2 does back-flush the 1st SPE column and flows to Valve 3 where the Detector is connected to port 2.

Example Figure 1:

- ➡ The loop content is in the first step washed onto the 1st SPE column to eliminate macromoleculare matrix.
- ➡ In the second step transfers the mobile phase form Pump 2 the analyte(-s) in the back-flush mode onto the 2nd SPE column.
- ➡ The third timed step is the back-flushing of the extracted analyte(-s) onto the analytical column.
- ➡ If the elution power of the mobile phase from Pump 3 is too strong can the "In-line Dilution" with Water form Pump 4 weaken the strength to focus the analyte(-s) at the analytical column head. This step would be simultaneously timed with step 3.