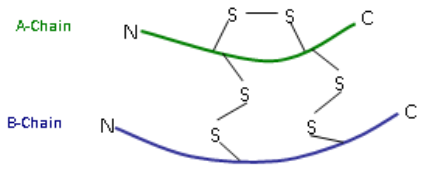
OraChrom, Inc.
The Vanguard of Liquid Chromatography.

10-B Henshaw Street Woburn, MA 01801 USA Phone (781) 932 0151 Fax (781) 932 0787
E-mail: info@orachrom.com www.orachrom.com

APPLICATION NOTE

Automated Digestion with StyrosZyme® TPCK-Trypsin, Immobilized Enzyme on Polymeric Hard Gel Simulated-Monolith™.
Effect of Linear Velocity and Column Length on the Digestion of Oxidized Insulin B Chain.

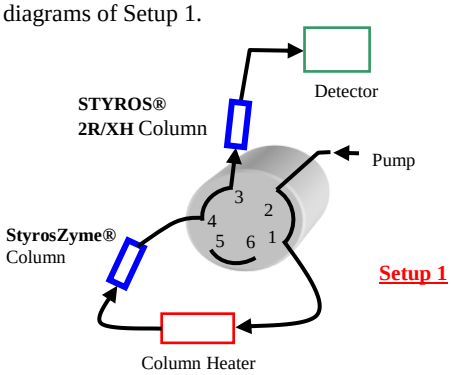
In Application Note 132, the automated digestion of Cytochrome c from equine heart was described using a basic HPLC instrument and the OpenLab Software from Agilent Technologies. The digestion and mapping proceeds in under an hour.
Although the speed of digestion is high considering the linear velocities in a narrow bore column of 2.1 mm ID, the question is how fast, within practical flow rates, is the enzyme able to digest the substrate?
This is the subject of the present Application Note.
A review of the substrate shows the following:
The active form of insulin consists of 2 chains (A-Chain and B-Chain) connected by disulfide bonds between cysteine side chains:



Oxidized B-Chain consists of the following amino-acid sequence:
Phe-Val-Asn-Gln-His-Leu-Cys-(SO3H)-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-(SO3H)-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala

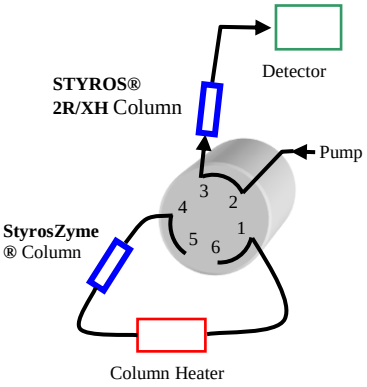
A typical digestion of insulin in solution with high grade Trypsin requires at least 18 hours at 37 °C.
StyrosZyme® TPCK-Trypsin can achieve the digestion on line at 37 °C, in 2 minutes at 0.5 ml/min or 10 minutes at 0.2 ml/min.
The resulting peptides can be mapped directly after digestion by using a STYROS® 2R reversed phase column connected in series to the immobilized enzyme column in 6 minutes.
The setups for the automated digestion are described below.

A 6-port valve connects the enzyme column to the polymeric reversed phase STYROS® 2R column in series as shown in the schematic diagrams of Setup 1.



Setup 1

In the second position, the enzyme column is removed from the line and only the reversed phase STYROS® 2R column remains.



Setup 2

The 3 buffers needed are as follows:
Buffer A: 0.1 % Formic acid in DI H2O (for peptide mapping)
Buffer B: 0.1 % Formic acid in Acetonitrile: H2O, 95:5(for peptide mapping)
Buffer C: 0.1 M TRIS, pH= 8.5 (for digestion)

The temperature is set at 37°C for all sequences.
Narrow bore enzyme columns of two different lengths are used: a 2.1x50 and a 2.1x100 mm stainless Steel column (StyrosZyme® TPCK-Trypsin).
The reversed phase column is a 4.6x50 mm STYROS® 2R.
As a polymeric reversed phase Simulated-Monolith™ column, it can withstand extremes in pHs and has low pressure drops.
A sequence consisting of 6 steps is used in the following order:

1-Equilibrate the enzyme column with both columns in line as shown in Setup 1.

Time	% of buffer C	Flow rate (ml/min)
0		0
0.01	100	1
2	100	1
2.1		0

2-With both columns in line as in Setup 1, 5 µl of a solution of 3 mg/ml of Insulin chain B in buffer A is injected and the resulting digests are dumped on the reversed phase column using the following sequence:

Time	% of buffer C	Flow rate (ml/min)
0		0
0.01	100	0.5
2	100	0.5

3- The enzyme column is removed as shown in Setup 2 to wash off the salt and prepare the reversed phase column for mapping. It is also ready for hyphenation with a mass spectrometer.

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0			0
0.01	10	90	1
3	10	90	1
6	10	90	1

4-The digested peptides are now trapped on the reversed phase column and can be mapped following a gradient. The setup remains as Setup 2.

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0			0
0.01	10	90	1
6	50	50	1

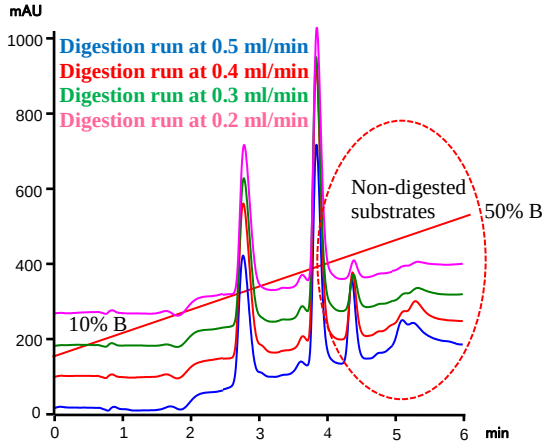
5- The reversed phase column is washed in this step to remove any leftover peptides from the previous digestion. The setup remains as Setup 2.

Time	% of buffer B	% of buffer A	Flow rate ml/min
0			0
0.01	45	55	1.5
1	60	40	1.5
2	60	40	1.5
3	45	55	1.5
3.1	10	90	1.5
5	10	90	1.5

6-In the final step the reversed phase column is pre-equilibrated with the digestion buffer to wash off all organics from the line.
The setup remains as Setup 2.

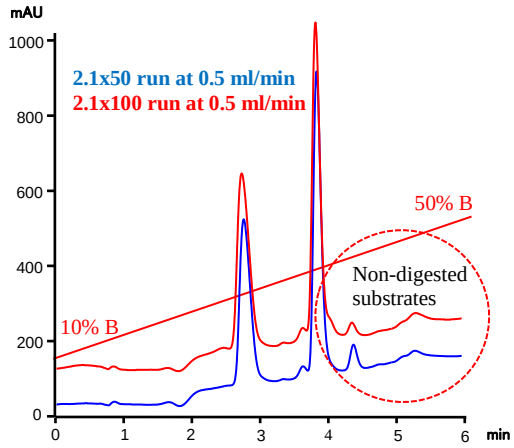
Time	% of buffer C	Flow rate (ml/min)
0		0
0.01	100	1
4	100	1
4.1	100	0

The result of the automated digestion and mapping of the immobilized enzyme (TPCK-Trypsin) in a 2.1x50 mm column at different linear velocities is shown and compared to one another here.

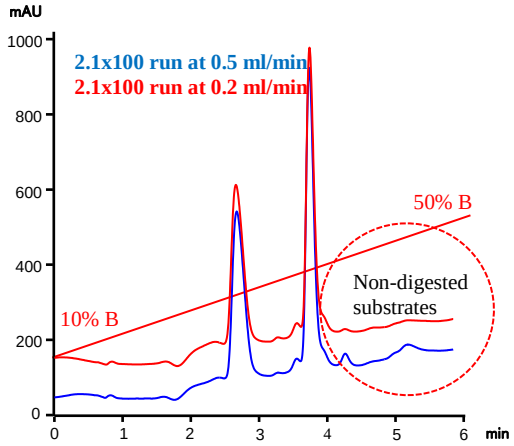


The digestion increases as the linear velocity decreases from 900 cm/hr. to 400 cm/hr. based on an empty column of 2.1mm ID.

One can see a similar phenomenon by increasing the column length from 50 to 100 mm at the same flow rate of 0.5 ml/min during digestion.



If the longer column (100 mm) is run at 0.2 ml/min, the digestion goes to completion:



It is reasonable to conclude that the full digestion of Oxidized B-Chain at a set temperature of 37°C and at a pH 8.5 using 0.1 M Tris buffer depends on both the length of the column used and the linear velocity of the digestion.

Inc.