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APPLICATION NOTE

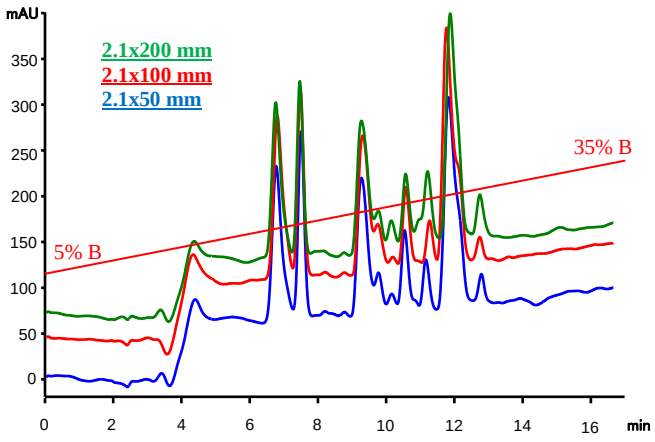
StyrosZyme® TPCK-Trypsin, Immobilized Enzyme on Polymeric Hard Gel Simulated-Monolith™. Effect of Different Variables on Automated Digestion.

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 proceeds in under an hour.

Note that the protein was not denatured and used in its native from.
In this Application Note additional variables are considered.
Among other:

- The length of the enzyme column and its effect on the digestion,
- The amount of substrate and its effect on the digestion,
- Increasing the interaction time between the substrate and the enzyme by reducing the flow rate during digestion.
- Increasing the ID of the enzyme column and therefore modifying the linear velocity during digestion.
- Decreasing the temperature and running the digestion at room temperature of 20°C.
- Reproducibility of the automated digestion.
- The optimum linear velocity for the digestion in a given setting?

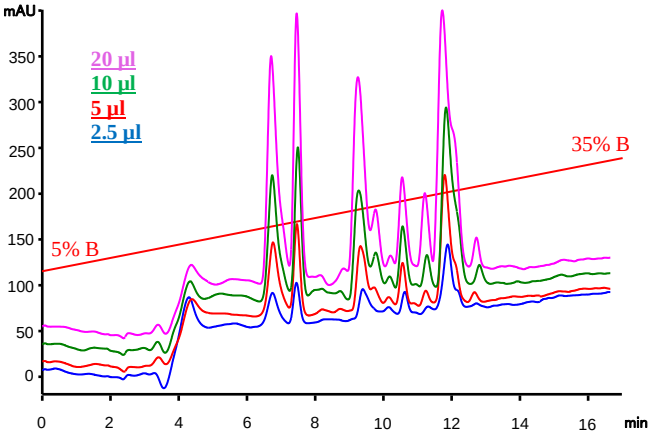
-To assess the **variability in enzyme column lengths**, we retained the narrow bore format of 2.1 mm ID and compared 3 columns of 50, 100 and 200 mm long.
The amount of substrate is the same for all columns (10 µl)
The results are shown in the following chromatogram.



The digestions are similar. This suggests that a 50 mm column digests the protein to its equilibrium and no change occurs should the end user use a longer column.

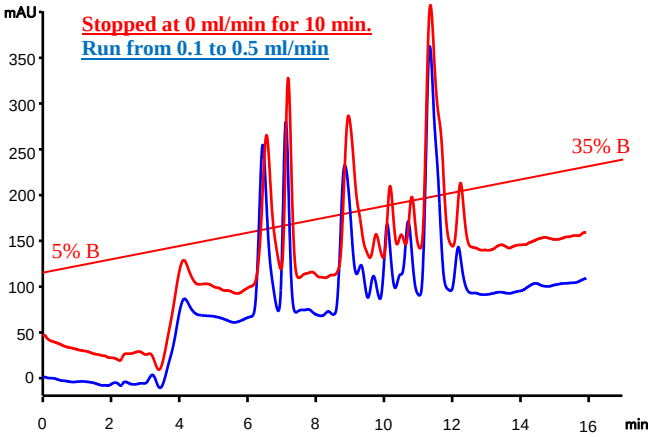
-**The amount of substrate** and its effect on the digestion.

Using increasing amounts of sample protein and monitoring the effect on the digestion:



The digestions are similar. This suggests that the load of the enzyme column can accommodate substrates from 2.5 µl to 20 µl without any sign of saturation.

-Increasing the interaction time between the substrate and the enzyme by reducing the flow rate during digestion.
The digestion was run previously from 0.1 to 0.5 ml/min for 2 minutes. The flow was stopped for 10 minutes instead:

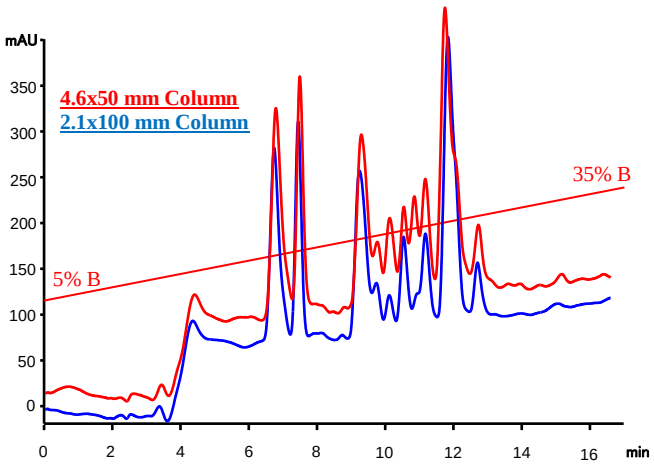


The digestions remain similar. The presence or absence of flow does not affect the digestion.

-**Increasing the ID of the enzyme column** and therefore modifying the linear velocity during digestion.

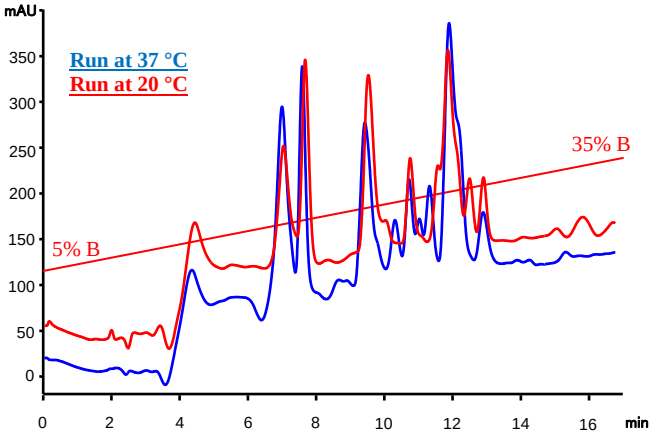
A 2.1x 100 mm column of 0.35 ml volume was compared to a column of 4.6 x 50 mm with a volume of 0.83 ml.

The narrow bore column of 2.1 mm ID and a cross section of 0.035 cm^2 has a linear velocity that decreases to 43 cm/hr at the volumetric flow of 0.025 ml/min compared with the initial flow of 0.1 ml/min.
At the same flow rate of 0.025 ml/min, the normal bore column of 4.6 mm ID and a cross section of 0.17 cm^2 has a linear velocity of 9 cm/hr.
This is almost 5 times slower than the narrow bore and therefore more time for the substrate to interact with the enzyme.



Yet the digestions remain similar within the range explored here.

-**Decreasing the temperature** and running the digestion at room temperature of 20°C.
The previous digestions were run at 37 °C. It would be appropriate to know the importance of maintaining such temperature for the digestion to reach its equilibrium when it is run at a lower temperature such as room temperature.

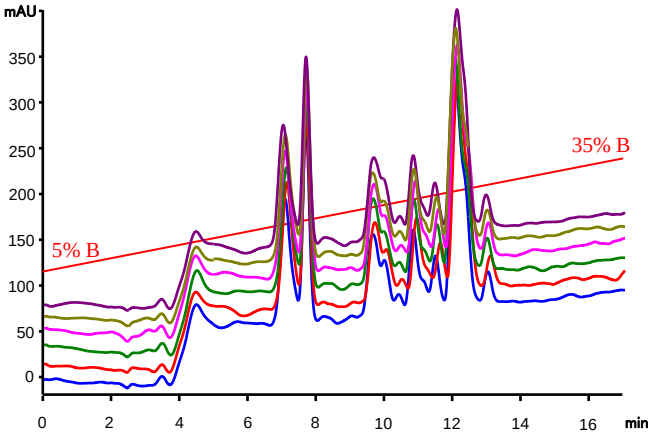


Lowering the temperature does affect the digestion.

-Reproducibility of the automated digestion is also important as it is one of the goals of the exercise.

A 4.6x50 mm StyrosZyme® TPCK-Trypsin column is used at a volumetric flow rate of 0.5 ml/min during the digestion. This is equivalent to 180 cm/hr of linear velocity for an empty column.

Several consecutive injections were run during an automated sequence and the results were compared:



The automation yields reproducible results.

The operating parameters remain the same as in Application Note 132:

HPLC System.	Agilent 1100 with thermostatted column compartment and a 6-port valve.
Columns	**StyrosZyme® TPCK-Trypsin** 2.1 X 100 mm/ **StyrosZyme® TPCK-Trypsin** 4.6 X 50 mm **STYROS® 2R/XH** 4.6 X 150 mm
Mobile phase.	A: 0.1 % Formic Acid B: 0.1 % Formic acid in Acetonitrile: H2O 95:5 C: 0.1 M Tris, pH=8.5
Flow rates	As indicated in various steps
Gradient	As indicated in various steps
Temperature	37°C
Detection	214 nm
Injection volumes	10 µl
Sample:	3 mg/ml of Cytochrome c in buffer A.

It is also important to look further into the process of digestion in the present set up of automation and explore additional variables: How fast can the digestion reach its equilibrium by using the linear flow rates for columns of different diameters.
This is the subject of Application Note 134.

Inc.