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APPLICATION NOTE

Controlling the Automated Digestion of Cytochrome c by Controlling the Linear Velocity of the Flow Rates During the Online Digestion.
StyrosZyme® TPCK-Trypsin, Immobilized Enzyme on Polymeric Hard Gel Simulated-Monolith™ Enzyme Reactor was used with the Acquity UPLC *I* class Plus and Final Silica C18 mapping.

The present mRNA vaccines are the first to have ever been authorized for emergency use by the Food and Drug Administration.
To date all attempts in using mRNA pharmaceuticals have failed and abandoned.
The mere fact that sub-zero temperatures are crucial to preserve them is a clear indication that leftover enzymes need to be kept frozen to prevent spoiling the vaccine.

Indeed, to Dr Margaret Liu, the former chair of the International Society for Vaccines explains the issue as being the ease with which mRNA is destroyed and broken apart by leftover enzyme that purification and POLISHING with the present media cannot remove. Thus, the use of sub-zero temperatures.

Despite being fully aware of it, the drug manufactures were not able to address it.
Unless and until we can remove the enzyme, Trypsin likely, or avoid including it in the mixture in the first place, we will remain challenged in the proper manufacture of the present mRNA, and any future ones required any time and on short notice.

In the present Application Note we show how effective the immobilized Trypsin is in digesting Cytochrome c even at high linear flow rates.

The optimum pH for Trypsin is 9 and the optimum temperature is 65°C. However, these numbers hold true when the digestion proceeds in batch.

In the case of online digestion, we were able to achieve full digestion of the proteins at 37°C and pH of 9.85 using 0.1 M Tris buffer (see also application notes 153 and 170).

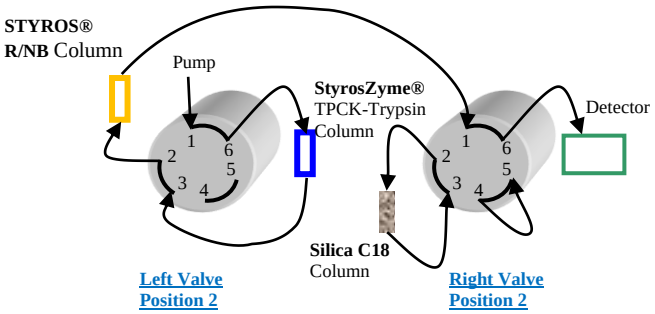
The tandem reversed phase column in such set up needs to tolerate high pH's.
Polymeric reversed phase columns such as STYROS® R are highly recommended, especially when the effluents are used to feed a mass spectrometer.

The proteins that are difficult to digest in solution will be difficult to digest online. It is necessary to assist the unfolding of the protein by denaturing agents and prevent the refolding by using reducing or alkylating agents.

Cytochrome c from Equine heart was used to assess its digestion at different linear flow rates and find out how fast the online digestion can be run and the extent of control one has over the process.
Small columns were used to minimize high solvent consumption as well as the generation of waste.

Two enzyme columns: a 2.1x50 mm and a 4.6x50 stainless Steel (StyrosZyme® TPCK-Trypsin) were used separately to run the digestion at different linear flow rates.
Two narrow bore reversed phase columns, one polymeric (STYROS® R) with high capacity, high pH tolerance and high performance (2.1x50 cm) column as well as a Silica C18 column (Acquity UPLC® BEH C18 1.7 µm 2.1x50 cm column) with high performance.

The buffers used are:
Buffer A: 0.1 % TFA in DI H2O: ACN 98:2 (for peptide mapping)
Buffer B: 0.1 % TFA in ACN: H2O, 70:30 (for peptide mapping)
Buffer C: 0.1 M Tris pH= 9.85 (for digestion).
The UPLC *I* class Plus is fitted with 2 six port valves.
The following shows the starting position of the valves used:



Setup 1

In this first position, the StyrosZyme® TPCK-Trypsin column and the polymeric STYROS® R/NB column only, are online and the Silica C18 column is not exposed to any excessive pH used for the digestion. It is therefore possible to safely scout the right digestion buffer as well as the optimum conditions for the full digestion prior to the use of the silica column. Both polymeric columns, StyrosZyme® TPCK-Trypsin and STYROS® R reversed phase polymeric can tolerate high pH.

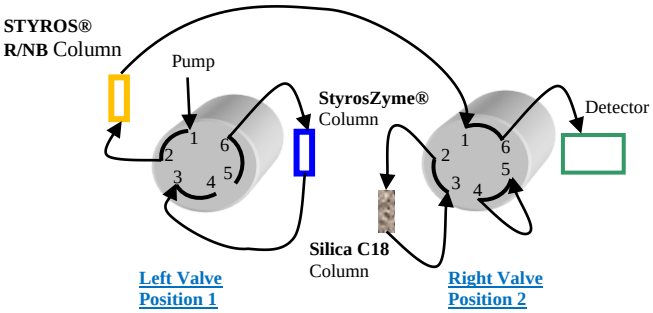
1-Equilibrate the enzyme column with all columns except the silica column, in line, as shown in Setup 1.

Time	% Of buffer C	Flow rate (ml/min)
0	100	0.2
5	100	0.2

2-With both columns in line as in Setup 1, 3 µl of a solution of 10 mg/ml protein in buffer A is injected, and the resulting digests are dumped on the reversed phase polymeric column using the following method:

Time	% Of buffer C	Flow rate (ml/min)
0	100	0.2
5	100	0.2

In the second set up, the left valve is switched to position 1 with only the STYROS® 1R reversed phase column online to equilibrate it with the starting solvent gradient.



Setup 2

3-The reversed phase Polymeric column only is now in line as in Setup 2 to wash the leftover salt and prepare it for the reversed phase mapping.

It is also ready for hyphenation with a mass spectrometer.

Time	% Of buffer B	% of buffer A	Flow rate (ml/min)
0	5	95	0.2
0.01	5	95	0.2
10	5	95	0.2

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

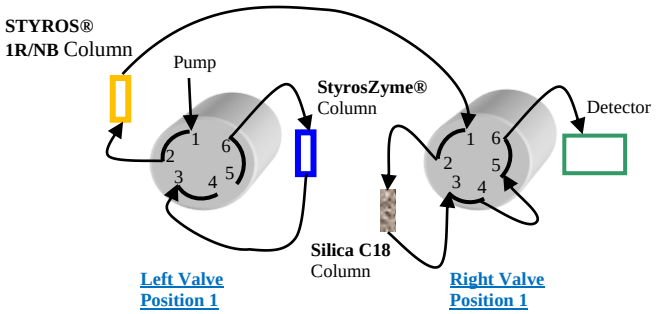
Time	% Of buffer B	% of buffer A	Flow rate (ml/min)
0	5	95	0.2
0.01	5	95	0.2
90	70	30	0.2

Once the conditions of the digestion are satisfactory, one can use the second switching valve to bring the Silica C18 column online as shown in Setup 3.
The same gradient as in step 4 can be used for the mapping to run the separation on the C18 Silica column in addition:

4'-The digested peptides trapped on the polymeric reversed phase column can now be mapped on the C18 Silica column in addition.

The setup is now as Setup 3.

Time	% Of buffer B	% Of buffer A	Flow rate (ml/min)
0	5	95	0.2
0.01	5	95	0.2
90	70	30	0.2



Setup 3

4''-In this step, using the same setup 3, the columns are washed with high organic to make sure they are devoid of any leftover proteinaceous species.
The setup remains Setup 3.

Time	% Of buffer B	% Of buffer A	Flow rate (ml/min)
0	5	95	0.2
0.01	100	0	0.2
4	100	0	0.2
4.1	5	95	0.2
8	5	95	0.2

To re-equilibrate both reversed phase columns that are now in line, the following step 5 is used.

5- Both reversed phase columns are preequilibrated to the initial low organic prior to getting in contact with the digestion buffer.
The setup is now Setup 3.

Time	% Of buffer B	% Of buffer A	Flow rate ml/min
0	5	95	0.2
0.01	5	95	0.2
5	5	95	0.2

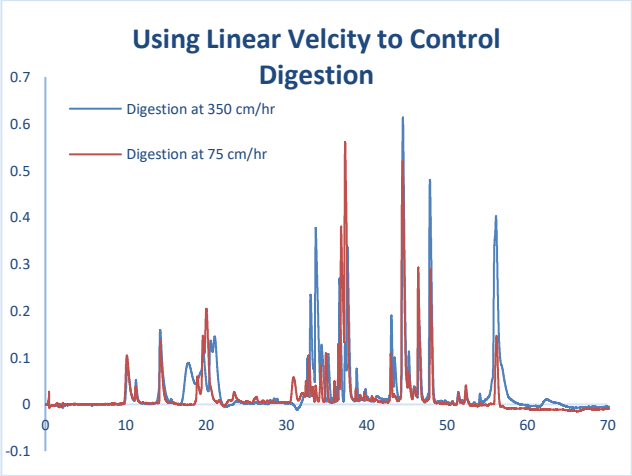
The system is now ready for the next cycle to check the reproducibility of the digestion under the present conditions.

We have used 3 µl of a solution of 10 mg/ml of protein in buffer A as sample to digest.
The temperature is set at 37°C for all sequences and the absorbance is monitored by the PDA at 214 nm.

The digestions are done under similar conditions
The enzyme columns of 2.1 x 50 mm and 4.6 x 50 mm have volumes to 0.173 ml and 0.831 ml respectively as empty columns. During the digestion, with the same volumetric flow rate of 0.2 ml/min the linear flow rate would be 75 cm/hr. for the larger column of 4.6. mm ID and 350 cm/hr. for the smaller column of 2.1 mm ID calculated at the empty stage.
These numbers are higher with the fully packed columns although as Simulated-Monoliths™ the pressure drops are extremely low matching the low pressures of soft gel media.

A total of 1 ml is run through them during the digestion with the same volumetric flow rate of 0.2 ml/min.

The performance of the STYROS® 1R polymeric is crucial in the final separation of the digested peptides,



These are the missing parts in the arsenal of the manufacturers to use unequivocally rather than their slow, leaching and contaminating soft gel.

These polymerics are unique, and so far, have been elusive in research, in academia as well as the entire manufacturing community.

It is understood that the present pandemic is only a warning of what is to follow.

Ignoring the concerns of countries that are resisting the use of the presently tainted vaccines, the manufacturers have effectively created a universal viral supply to keep replenishing the pool and giving the opportunistic virus to be morphed into its most virulent variant.

The view of an expert in the field cannot be ignored especially now that the tools are within reach:

The problem, says Liu, is that mRNA is "really easily destroyed, and that's because there are many, many enzymes that will just break it apart."

Inc.