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APPLICATION NOTE

Controlling the Automated Digestion of Cytochrome c by Controlling the pH 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.

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 or change it altogether to a new entity with different outcome.

Indeed, 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.

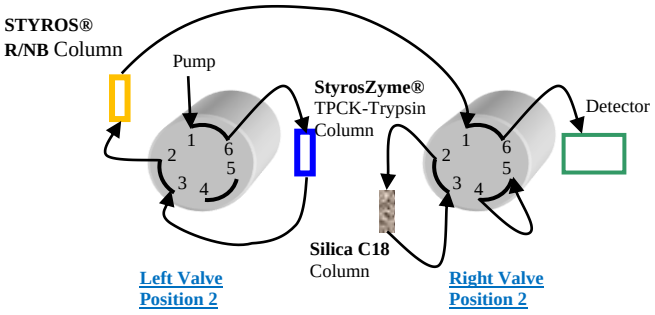
So far, the drug manufactures were not able to address it.
Unless and until we can remove the enzyme, Trypsin likely, or avoid including it uncontrollably in the mixture in the first place, we will remain challenged in the proper manufacture of the present mRNA, and any future ones required any time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

In this additional Application Note we show how effective the immobilized Trypsin is in digesting Cytochrome c at different pH, in a dynamic setting, without even mixing it with the vaccine, and therefore having to remove it afterward during a number of additional steps and without any success.

The enzyme column used is a 4.6x50 stainless Steel (StyrosZyme® TPCK-Trypsin) to run the digestion at different pH.
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 and similar size.

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= 7, 9.5 and 11 (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 high pH used for the digestion.
It is therefore possible to safely scout the right digestion buffer as well as the optimum conditions for the controlled 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.

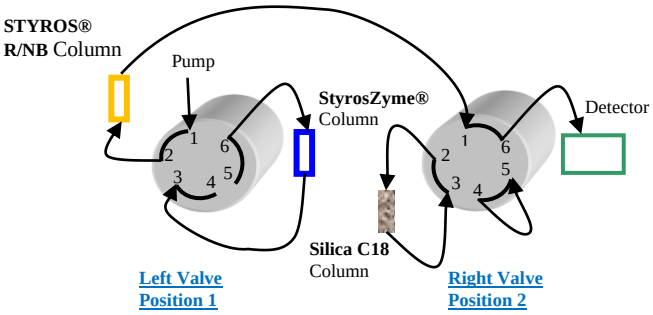
1-Equilibrate the enzyme column with all columns except the silica column, in line, as shown in Setup 1.

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.

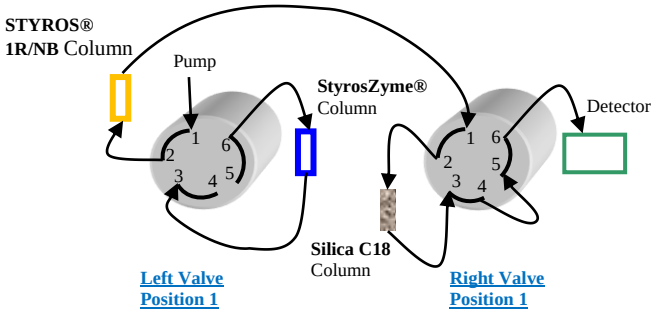
Time	% Of buffer B	% of buffer A	Flow rate (ml/min)
0	5	95	0.2
0.01	5	95	0.2
90	70	30	0.2

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.

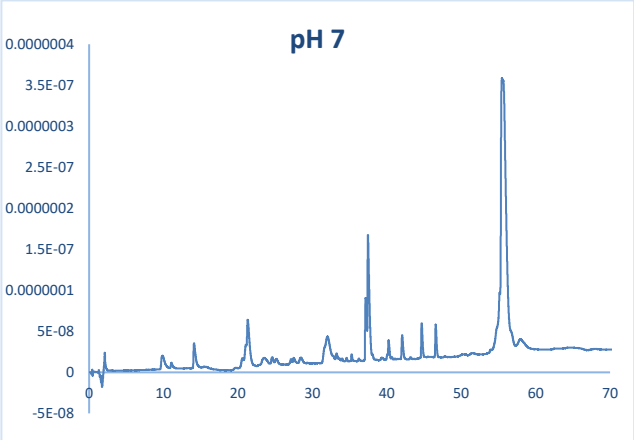
Time	% Of buffer B	% Of buffer A	Flow rate (ml/min)
0	5	95	0.2
0.01	100	0	0.2
4	100	0	0.2
4.1	5	95	0.2
8	5	95	0.2

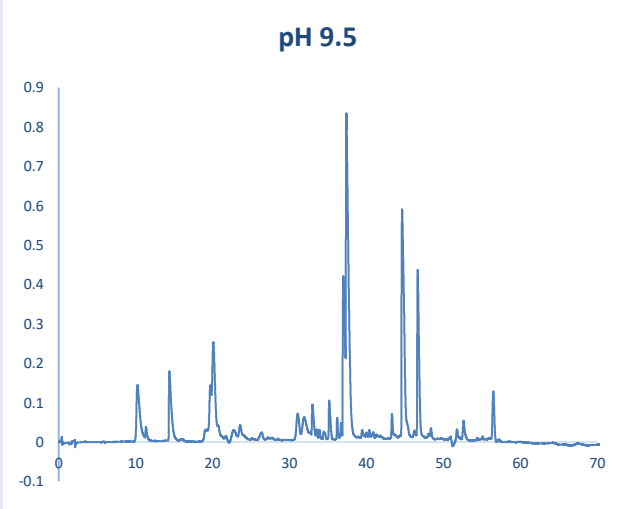
To re-equilibrate both reversed phase columns that are now in line, the following step 5 is used.

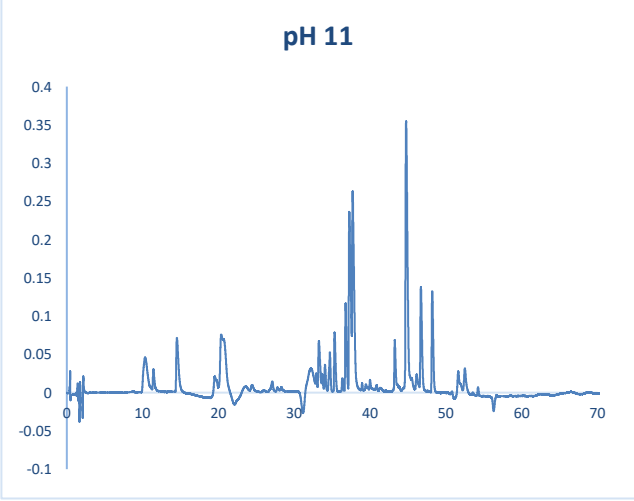
5- Both reversed phase columns are preequilibrated to the initial low organic prior to getting in contact with the digestion buffer.
The setup is now Setup 3.

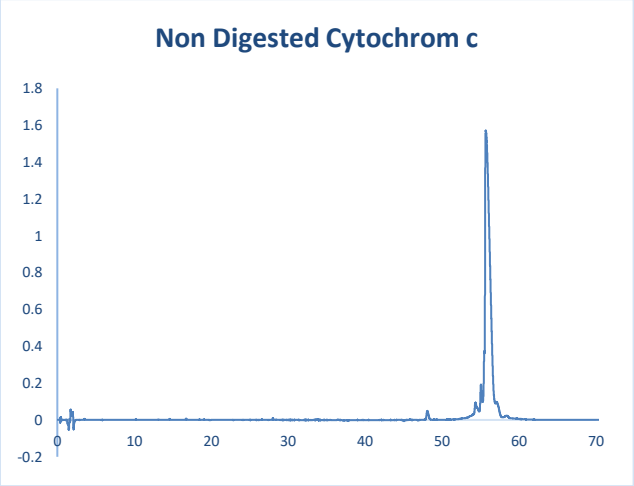
Time	% Of buffer B	% Of buffer A	Flow rate ml/min
0	5	95	0.2
0.01	5	95	0.2
5	5	95	0.2

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
Except for the pH, other conditions remain the same.
A total of 1 ml is run through the enzyme reactor during the digestion with the same volumetric flow rate of 0.2 ml/min.
The following chromatograms depict the peptides resulting from the digestions at different pH's.









Comparing the chromatograms with one another and the last one, we notice the digestion to be complete only at pH 11

It is clear now that the soft gel media manufacturer whose goal was to make it exclusive for the entire biopharmaceutical industry is now facing the unsurmountable challenge to access a different chromatographic media that could solve the problem that it is now facing:
leaching and contamination and even the unintended outcome of a completely different vaccine.
Indeed, the leached enzyme during purification makes the vaccine unstable and easily destroys or modify its mRNA content.
Dr. Margaret Liu, the former chair of the International Society for Vaccines believes the vaccine is easily destroyed and broken apart by enzymes.

Ignoring the concerns of countries or even small domestic group that are resisting the use of the presently tainted vaccines, with irrefutable evidence, 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 more than 100 Application Notes OraChrom has offered here are to convince BARDA (Biomedical Advance Research and Development Authority) in charge of the development of new vaccines for the Project Bioshield.
These are effectively the solution to the challenge we are now facing.

Inc.