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APPLICATION NOTE

Assessing Chromatography Media's Stability by Tethering Enzymes on them. Case Study: StyrosZyme® TPCK-Trypsin, Immobilized Enzyme on Polymeric Hard Gel Simulated-Monolith™ Enzyme Reactor used with the Acquity UPLC *I* class Plus and Final Silica C18 mapping.

To assess the stability of a chromatography media an enzyme can be tethered on its surface and then used as an enzyme reactor to check its performance as well as its endurance under harsh conditions.

The present application note is indeed the distillate of a large number of runs during which a multitude of proteins, including the immobilized enzyme itself, have been digested showing the reproducibility of the digestion as well as its endurance during runs.
Noteworthy is the fact that the only Immobilized enzyme on polymeric (PorosZyme TPCK-Trypsin) was discontinued due to leaching and instability.
The one left on the market is now StyrosZyme® with several versions of it: TPCK-Trypsin, Pepsin, and Papain.

The soft gel industry (derivatives of Agarose) has tried to disseminate the wrong impression that immobilized enzymes are unstable by "nature".

StyrosZyme® immobilized enzyme is the uncontested proof that it is the base on which the enzyme is being immobilized that is unstable.

The data shows the immobilization in fact increases the stability of the enzyme, brings the process of digestion under control, faster and addresses the contamination of final products by deleterious enzyme contamination which continues to be a detriment to all biopharmaceuticals including and specifically vaccines.

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 specimen with unknown 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, very likely Trypsin, 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.

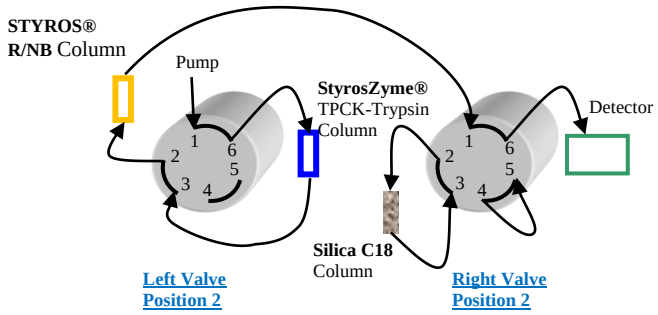
In the previous Application Note we showed how effective the immobilized Trypsin is in digesting Cytochrome c at different pH, in a dynamic setting.

Same process can be done with vaccines. Without the need to mix it and having to quench and purify it afterward.

The enzyme column used is a 4.6x50 stainless Steel (StyrosZyme® TPCK-Trypsin) to run the digestion.
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.

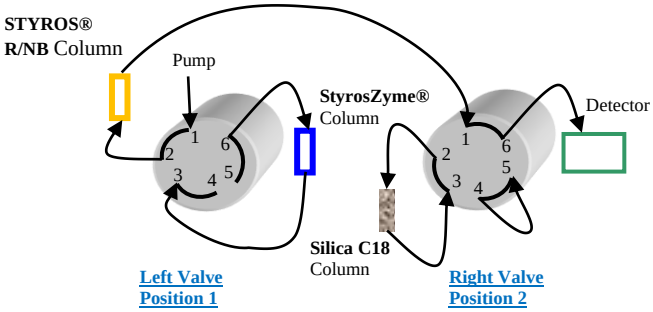
1-Equilibrate the enzyme column with all columns except the silica column, in line, as shown in Setup 1.

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.

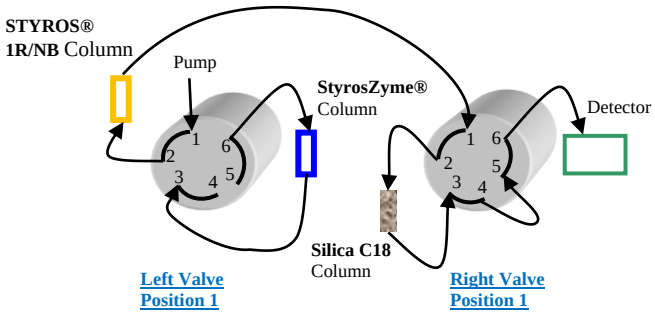
Time	% Of buffer B	% of buffer A	Flow rate (ml/min)
0	5	95	0.2
0.01	5	95	0.2
90	70	30	0.2

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.

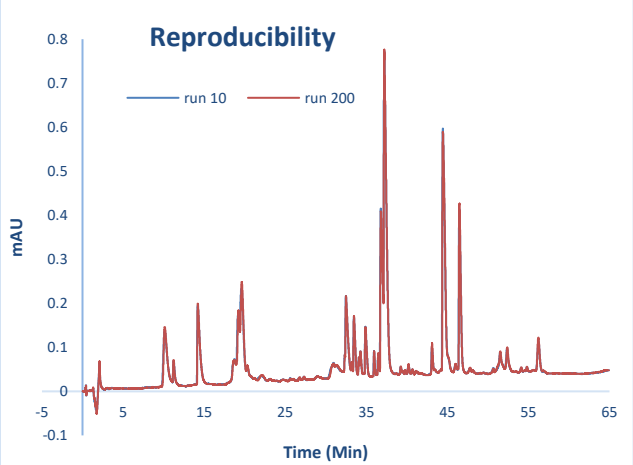
Time	% Of buffer B	% Of buffer A	Flow rate ml/min
0	5	95	0.2
0.01	5	95	0.2
5	5	95	0.2

The system is now ready for the next cycle to check the reproducibility of the digestion under the present conditions.
3 µl of a solution of 10 mg/ml of protein in buffer A is injected 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

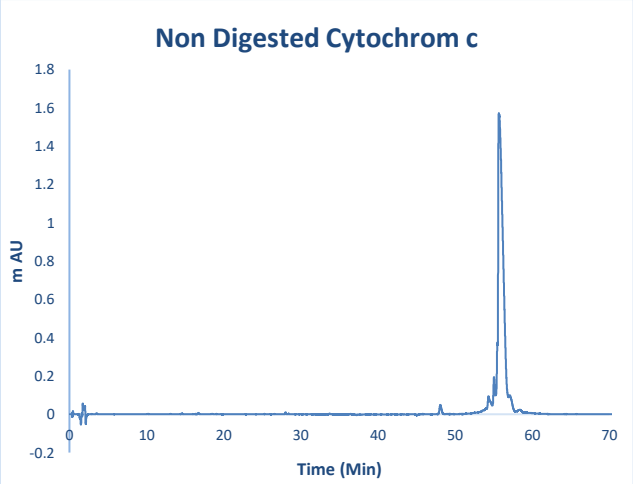
The previous Application Note (192) showed the digestion can be carried at different pH's.
The present Application Note clearly shows the reproducibility of the digestion over multiple runs.
This is a clear assessment of the stability of the enzyme as well as the polymeric Simulated-Monolith™ support on which it is covalently thethered.

This can therefore be used by manufacturers to evaluate any chromatography media as a candidate for their applications to either consider it or move to the next and make sure there is no leaching as" POLISHING" is not an option to fully purify.

Note the reproducibility of runs depicted on the following superimposed chromatograms.



And compare it with the non-digested protein under the same conditions.



The notes from the previous Application Note apply here as well to get the attention of the vaccines manufactures.

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 chromatography media that could solve the problem associated with Agarose derivatives:

Leaching and contamination.

Added to it is now the unintended outcome of a completely different vaccine that results from the presence of contaminated enzyme.

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 the ability to morph 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.

An inexcusable shortcoming of the Authority.

These are effectively the solution to the challenge we are now facing.

Affinity chromatography such as Protein A, in addition to its exorbitant cost and leaching problems resulting in contamination, is being replaced by other media using different ligands.
The problem of choosing a stable chromatography media still persists.

Until and unless FDA increases its requirement of only 50 percent efficiency to win approval no manufacturer will invest in stable media and no entity has the technology to get funding by BARDA to mass produce a stable media to move past the persistent issues of vaccine and drug contamination.