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APPLICATION NOTE

Scale Up of the Enzymatic Process Using Stable Immobilized Enzymes. 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.

The use of enzyme is common in biomanufacturing.
The production of mRNA vaccines process is composed of a 2-step enzymatic reaction in continuous form, followed by two multimodal chromatography steps, one in bind-elute mode for the intermediate purification, and a second in flowthrough mode for **polishing**.
This term is synonymous as further attempt to pursue the non-completed purification as the previous step is admittedly termed Intermediate.
We have shown in the previous Application Notes the new polymeric media on which the enzyme is tethered, to be stable in high pHs as well.
The present Application Note underlines the protein load for the digestion using samples from a solution of (10 mg/ml) Cytochrome C from bovine heart.

Keep in mind that the complete, intact mRNA molecule is essential for its potency as a vaccine.
Even minor degradation would have no impact or negative impact on patients.

In their Special Topic Commentary in 2020 Daan J.A. Crommelin et all have clarified the issues that need to be addressed in this area (Journal of Pharmaceutical Sciences 110 (2021) 997-1001.

"…….as mRNA vaccines take a prominent place in global strategies to successfully fight the COVID-19 pandemic, we believe it is short-sighted and unwise to wait for another pandemic before solving the storage stability issues of this versatile, rapidly deployable vaccine platform technology. Instead, a better understanding of the causes and mechanisms of the instability of mRNA vaccine formulations, combined with rational selection of appropriate stabilization technologies, will undoubtedly lead to improvements in mRNA vaccine stability. Such second-generation mRNA vaccine formulations with optimized stability, i.e., enabling shipping and storage at refrigerated or ambient temperatures across the entire vaccine supply chain, should be developed now to facilitate more rapid worldwide distribution of mRNA vaccines in the future."

The term "POLISHING" has become entrenched and ingrained in mindsets during more than a century using unstable media in this field.
No manufacturer can claim to possess a stable media.

Chromatography includes multitudes of phases that are used during the downstream processing of biopharmaceuticals.
It makes close to 80 % of the cost in the downstream.
Rather than resorting to distractions, the manufacturers need to keep the focus on it.
And so have we, in successfully using stable polymerics and tethering enzymes on them and testing them for stability as well as endurance.
They can be made in full variations including affinities, ion exchangers, hydrophobic and hydrophilic.
They are stable. Can be used during many runs and are reproducible as Styros® and StyrosZyme® Simulated-Monolith™.
We will then reach the stage where SMB can be used to automate the full process while making it economical and reproducible and most importantly avoid leaching which is the source of contamination.

Every process in which enzyme are used can become a single stage where an immobilized enzyme gets involved no longer requiring costly recombinant enzymes to satisfy the non-animal origin of it and becoming the only reproducible and non-contaminating step of the manufacturing process.
That is the future of the industry:
Stable polymeric media, not only to have stable immobilized enzymes but the entire range and variations of chromatographic media.

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.

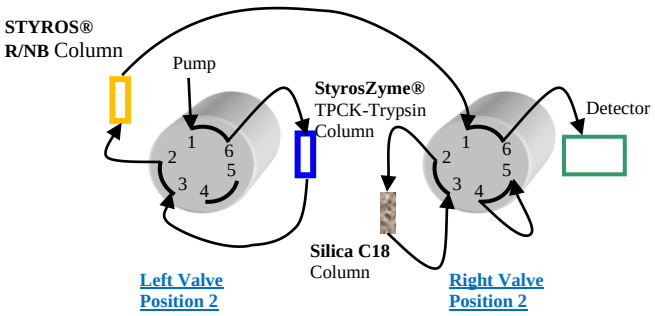
So far, the drug manufactures were not able to address it.

Unless and until we can remove the enzyme or avoid including it uncontrollably in the mixture in the first place, we will remain challenged in the proper manufacture of the clean and pure vaccine.

The enzyme column used here 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 (2.1x50mm) column as well as a Silica C18 column (Acquity UPLC® BEH C18 1.7 µm 2.1x50mm 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= 9.5 (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.

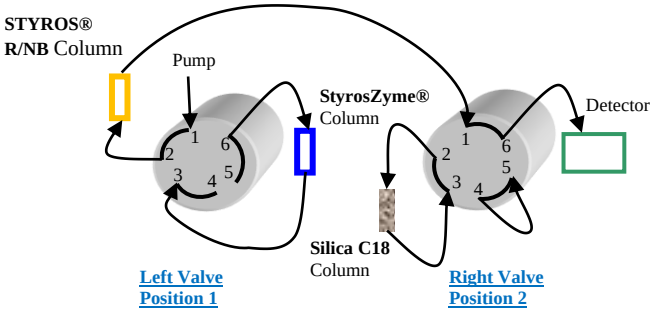
1-Equilibrate the enzyme column with all columns except the silica column, in line, as shown in Setup 1.

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 and 8 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

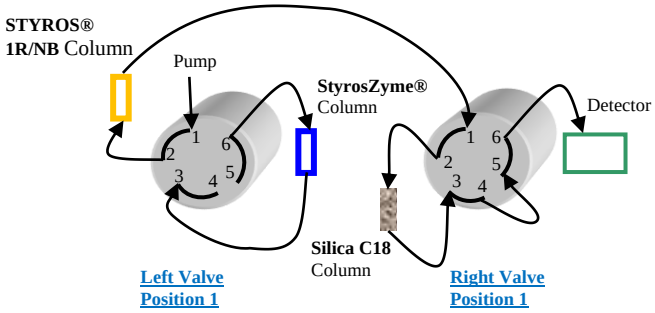
Once the conditions of the digestion are satisfactory, one can use the second switching valve to bring the Silica C18 column online as shown in Setup 3.

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.

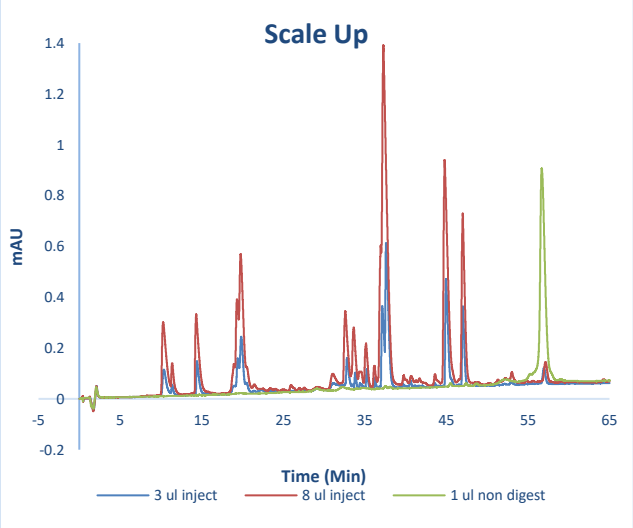
5- Both reversed phase columns are preequilibrated to the initial low organic prior to getting in contact with the digestion buffer.
The setup is now Setup 3.

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 and 8 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 showed the digestion can be carried at different pH's.

The present Application Note clearly shows the reproducibility of the digestion using different loads.
This is a clear assessment of the stability of the enzyme as well as the polymeric Simulated-Monolith™ support on which it is covalently tethered to.
Not only can it be used as a simple way to assess the stability of a candidate media, but it would also be a way to make sure it would not be a source of leaching and contamination avoiding costly additional steps of purifications.



These superimposed chromatograms are a clear testament of the reproducibility of the process of digestion using Cytochrome c.
Regardless of the injected amounts, same ratios of non-digested protein remain, and similar ratios of peptides are generated.

As mentioned before 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, that are Leaching and Contaminating.

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 modifies 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 domestic groups 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.

That is 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 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 while the specter of lingering pandemic is moving past its lull to rear its head back and again.