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The Vanguard of Liquid Chromatography.

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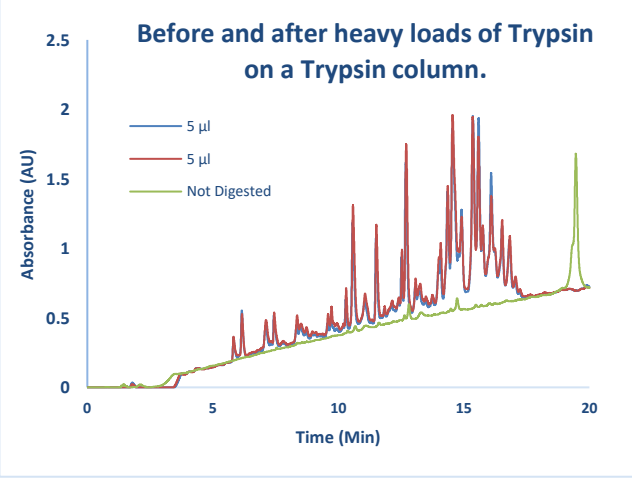
APPLICATION NOTE

Digesting Free Enzymes with Immobilized Enzymes.

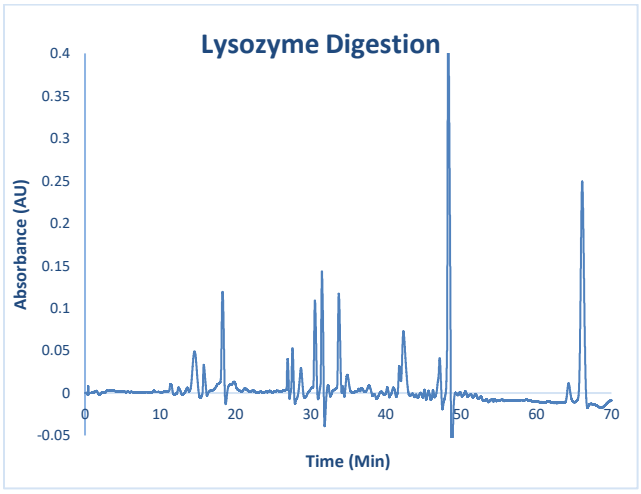
Case Study: StyrosZyme® TPCK-Trypsin, Immobilized Enzyme on Polymeric Hard Gel Simulated-Monolith™ Enzyme Reactor used with the Acquity UPLC *I* class Plus and Final Silica C18 mapping.

We have shown in Application Note 153 that the injection of Trypsin in solution results in its full digestion by running it through a column packed with immobilized Trypsin during a 10-minute run at a volumetric flow rate of 0.1 ml/min. in a 4.6mm ID column of 50 cm length.
Regardless of the injected amounts and the frequency of injections, the enzyme reactor will remain stable and active, and is not negatively affected.
The peptides generated can therefore be considered the autolysis of Trypsin in a dynamic setting.
To note that no denaturation is needed prior to the digestion and the process is simple, reproducible, and fast. Minutes rather than hours or overnight.

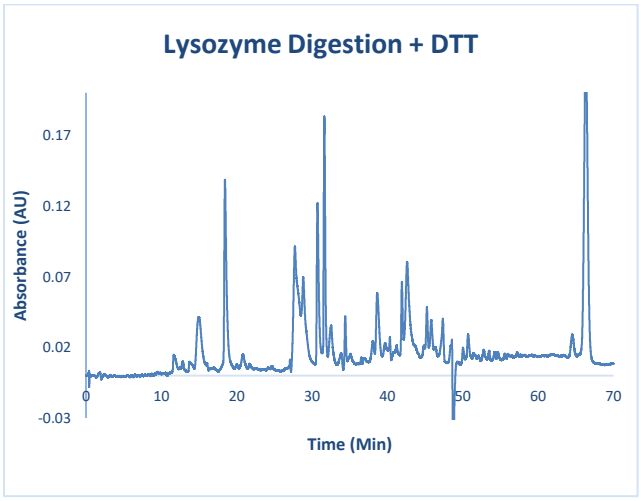


In the present Application Note, we have chosen Lysozyme, a protein with enzymatic activity using the same Immobilized Trypsin column with the same settings.

Although the digestion remains incomplete, and some non-digested entities can be detected, it is similarly done without denaturing Lysozyme that is from chicken egg.



Using small amounts of DTT to break the disulfide linkages of the cysteine residues of the enzyme, it is possible to further push the process of digestion further as seen in the following chromatogram.



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

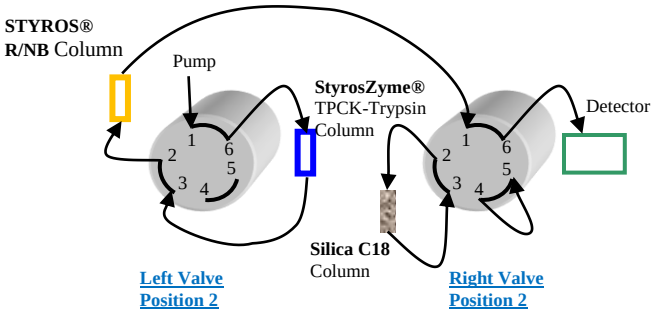
Two narrow bore reversed phase columns, one polymeric (STYROS® R) with high capacity, high pH tolerance and high performance (2.1x50mm) column as well as a Silica C18 column (Acquity UPLC® BEH C18 1.7 µm 2.1x50mm column) with high performance and similar size.

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= 8.8 (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.

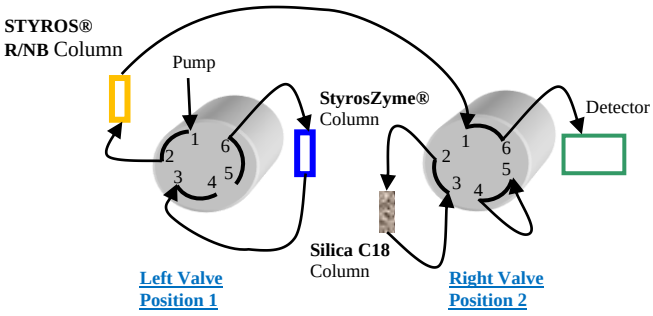
1-Equilibrate the enzyme column with all columns except the silica column, in line, as shown in Setup 1.

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, 5 µl of a solution of 5 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
10	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 mapping gradient solvent.



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.

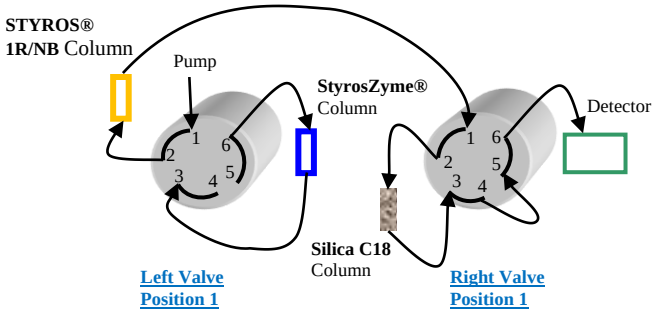
Time	% Of buffer B	% of buffer A	Flow rate (ml/min)
0	5	95	0.2
0.01	5	95	0.2
90	70	30	0.2

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.
5µl of a solution of 5 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

StyroZyme® TPCK-Trypsin column is a Simulated-Monolith™ polymeric support on which the enzyme is covalently tethered to.
Compared to conventional supports with superficial pores that operate by slow diffusion, monolithic supports, and Simulated-Monoliths™ supports operate by fast convection.
The back pressures are considerably less, and it is therefore possible to have several of them in series and online without exceeding the pressure limits of the instruments.
It is also helpful to know that any candidate media to be used as a support to immobilize enzyme needs to be tested in such a process and checked for its stability and endurance.
Indeed, the purpose of these Application Notes remains to make this very point as any unstable media is prone to leach and contaminate the final product.
The very low temperatures (-70°C) in the present vaccines is a clear indication and proof of their impurities.
As the level of leftover enzyme impurity increases the degrees of temperature should decrease.
In the expert opinion of Dr Margaret Liu, the former chair of the International Society for Vaccines, mRNA is easily destroyed and broken apart by leftover enzyme that purification and "POLISHING" with the present media cannot remove.
mRNA vaccines have been intensely tested for HIV, rabies, zika and influenza among others and failed to yield any breakthrough despite the low threshold of 50 % requirement by FDA.
Although the science backing mRNA is well founded, its purity remains questionable and only the virus itself can convince the players to adjust their course before time runs out.

Our primary focus has been on the enzyme Trypsin as it is considered a universal enzyme.
It is present both in prokaryotes as well as eukaryotes.

To circumvent the subsequent cleaning and removal of the leftover, specifically for biopharmaceuticals, one requires to resort to immobilized enzyme to prevent their inclusion at any stage of the process.
This would avoid all ensuing operations in attempting to purifying the resulting digests.
Additionally, the use of any free enzyme would be avoided, so would enzymes with non-animal origin.
The stability and non-leaching of the stationary phase to be used as a support for enzyme immobilization is the initial criteria to be considered.
At present, no stationary phase on the market can satisfy such basic criteria.
The fact that no immobilized enzyme is being used by biomanufacturers to make purity sensitive vaccines is the obvious proof of such assertion.
Trypsin, as a universal enzyme requires high pHs that polymerics can tolerate.

Moving further, we notice that the slow pace of diffusion needs a dramatic update to the fast rhythm of convection offered by monoliths.
However, the shortcomings of monoliths are a major impediment that only Simulated-Monoliths™ can overcome.
The only logical answer to such scientific and technical challenges is therefore StyrosZyme® series offered by OraChrom Inc. with no sales pitch detected anywhere in the literature.

The use of enzyme is common in biomanufacturing where purity is not the focus., trypsin, and elastase is required for the enzyme digestion to takes place.
It lowers the bonding energy of the lysing sites to be ruptured and for the digestion process to occur.

The bigger challenges are no longer scientific, rather purely financial as well as administrative:
How to convince BARDA to approach this simple but decisive issue in getting the manufacturers to rethink the over 80 % profit earned by using leaching products that cannot be used to immobilize enzyme, or provide purity, the key to circumvent all issues related to contamination.
An issue BARDA is presently tasked with in its Project Bioshield.
The multitudes of application notes provided by OraChrom is to convince BARDA that the answer that the entire world has been hoping for is within our grasp here in the USA.
By successfully using stable polymerics and immobilizing enzymes on them we have demonstrated that indeed it is possible to make every process in which enzymes are used to become a single stage where immobilized enzyme are replacing the batch digestion, no longer requiring costly recombinant enzyme to satisfy the non-animal origin nor require any after and additional purification and re-purification euphemistically called “polishing” to run the purification.

Stable polymeric media is the future of the industry specifically for biopharmaceuticals, where purity is essential.