OraChrom, Inc.
The Vanguard of Liquid Chromatography.

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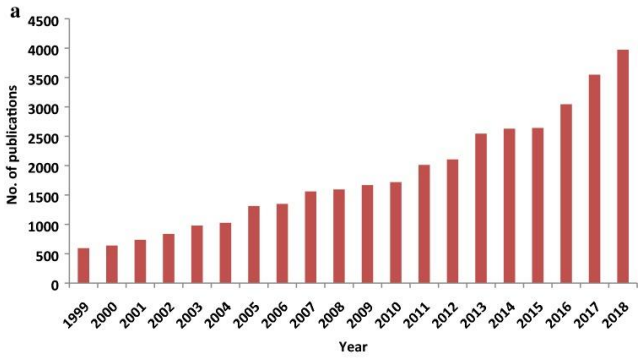
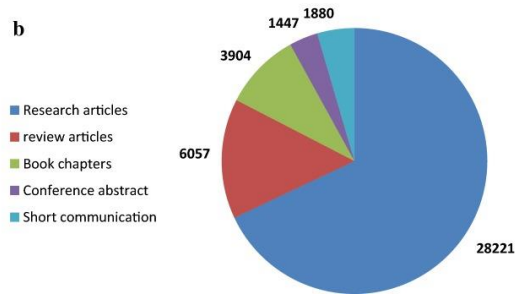
APPLICATION NOTE

Controlling the Online Digestion of Cytochrome c, by Controlling the Linear Velocity.

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 interest in immobilized enzyme has been growing through the years.

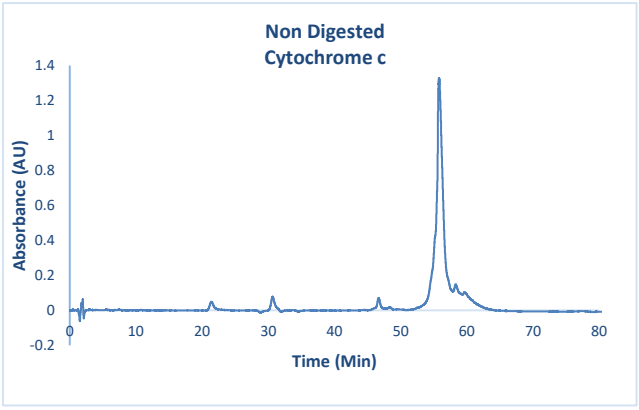




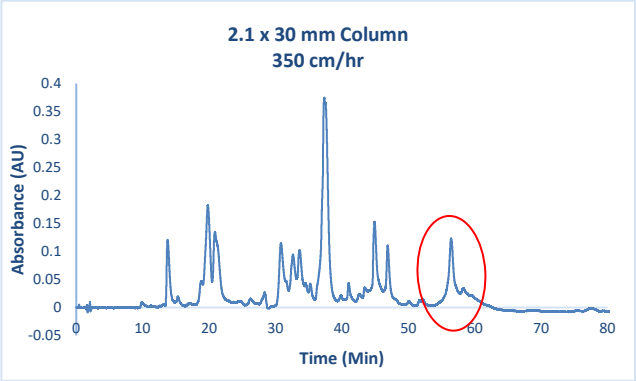
Enzyme immobilization is a technology widely used in various fields and industries such as bioremediation, environmental monitoring, biotransformation, food industry, textile industry, detergent industry, pharmaceutical industry, diagnostics etc. Not only can it lower the cost of the process, but it has also economic and technical advantages and controls. Immobilization provides operational stability. However, there is no universal material that can be used as support. They are all unstable and leach and no support has had a consistent or repeated following.

The standard we have adopted is to immobilize an enzyme on our special polymeric support and test its endurance as well as its function during a long period in order to evaluate it.
To move beyond the present day applications and move in areas where purity becomes essential, one needs to consider a stable polymeric that can withstand all pH's, have favorable flow characteristics and can be manufactured readily and in large quantities to satisfy ALL sectors of the industry, that includes pharmaceuticals, vaccines in particular, that continue to be a challenge to purify as witnessed by their subzero requirements.

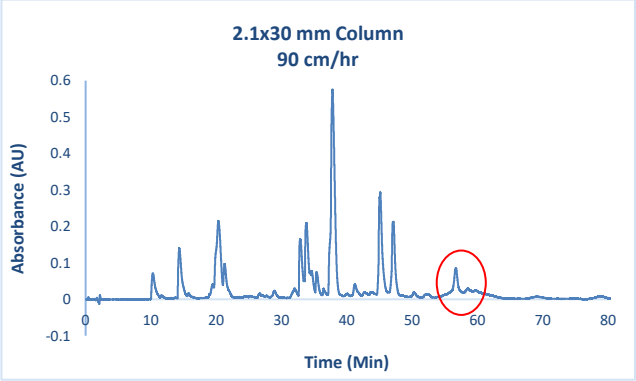
A non-digested sample of Cytochrome c was run to compare with a digested one.



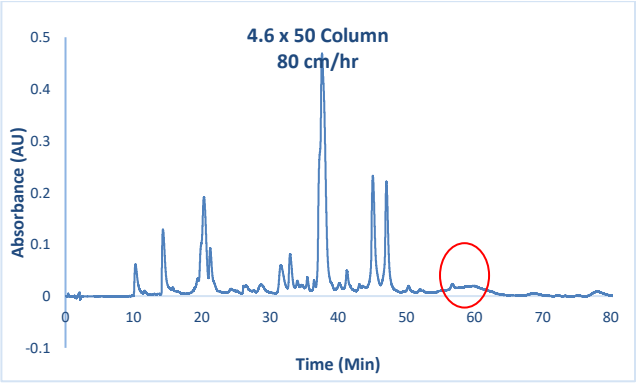
Using a narrow bore column of 2.1 mm ID and volumetric flow rates of 0.2 ml/min (that is a linear flow rate of over 350 cm/hr) the column was able to digest the bulk of the sample without the need for any denaturation or additional pretreatment.



Reducing the flow rate to 0.05 ml/min (that is a linear flow rate of over 90 cm/hr)



The digestion was more advanced with some clear variation of the resulting peptide digests.
Even a modest decrease of the speed during the digestion yields a major difference as noticed in the following chromatogram during the use of a normal bore column of 4.6 mm ID.



The digestion is now complete at this dynamic flow rate.
The vaccine manufacturers have stayed away from using immobilized enzyme as they don't have access to stable media, as they are non-existent.
Having a clear understanding that the benefits of using immobilized enzyme during the manufacture would save them a considerable amount in the downstream operation and they would finally succeed in providing the pure vaccine that would no longer require any subzero temperatures.
The polymeric media specified here as Simulated-Monolith™ is like monolith where the process has changed from slow diffusion to fast convection avoiding their shortcomings as a major impediment that only Simulated-Monoliths™ can circumvent.

The only logical answer to such scientific and technical challenge is therefore StyrosZyme® series offered by OraChrom Inc.
indeed, this is the starting solution.

In the present application note two enzyme reactors were used, one a short narrow bore column of 3 cm length and 2.1 mm of diameter and the other a normal bore column of 4.6 mm diameter and 50 mm length.
They are run at different volumetric flow rate of 0.2 and 0.05 ml/min that corresponds to 80 to 350 cm/hr of linear flow rate calculated for an empty column of 2.1 mm of diameter. The actual linear flow rate of the column is far higher considering the void volume of the column. The normal bore column allows to have linear flow rates of 80 cm/hr at 0.2 ml/min of volumetric flow.
Suffice it to say that such a short contact between the immobilized enzyme and the substrate is sufficient to digest the full amount injected
It is not realistic to consider the digestion to resort to any catalytic triad to proceed.
There is, however, room to consider a shear factor to be involved in assisting the breaking of bonds at specific lysing sites that are clearly detected and are reproducible regardless of the amounts of samples injected as substrates. The superimposed chromatograms in previous application notes provides clear demonstration of this point.

The purification of vaccines continues to be an unsuccessful operation as witnessed by the continued use of subzero temperatures; the increase in contaminated individuals of all ages and despite the loss of more than 1 million death so far in this country alone is indicative of such problem.

The stability and non-leaching of the stationary phase to be used as a support for enzyme immobilization remains the only viable alternative.

The bigger challenges are no longer scientific and technical, 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.

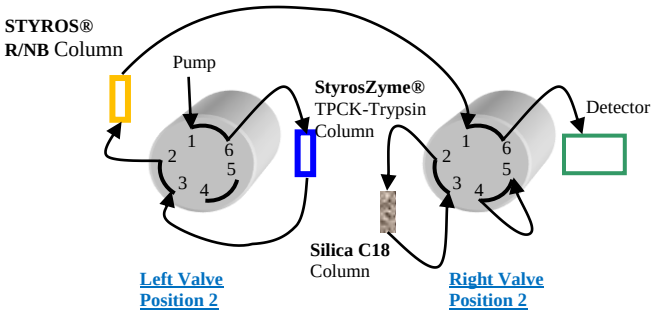
An issue BARDA is presently tasked with in its Project Bioshield.
The multitudes of application notes provided by OraChrom is to convince BARDA that the answer that the entire world has been hoping for is within our grasp here in the USA.
By successfully using stable polymerics and immobilizing enzymes 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.

Two enzyme columns are used here. A narrow bore column of 2.1x30 mm stainless steel (StyrosZyme® TPCK-Trypsin) and a normal bore column of 4.6x50 mm stainless steel to alternatively 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.

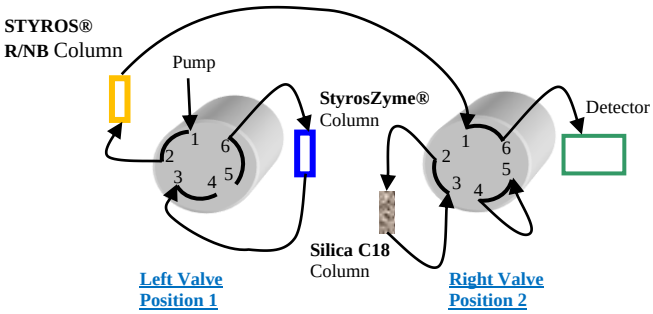
1-Equilibrate the enzyme column with all columns except the silica column, in line, as shown in Setup 1.

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, 3 µl of a solution of 10 mg/ml protein in buffer C 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 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.

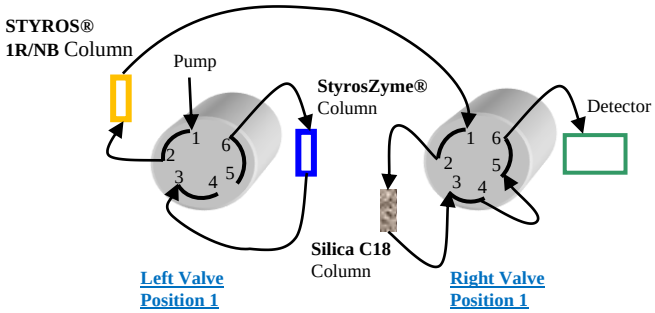
Time	% Of buffer B	% of buffer A	Flow rate (ml/min)
0	5	95	0.2
0.01	5	95	0.2
90	70	30	0.2

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 C 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.

It is important to keep in mind that mRNA technology is unique and has taken decades to mature. It needs all the attention needed to yield the results it deserves and so far, we have been far short of providing it to.

Simulated-Monolith™ support on which any enzyme can be covalently tethered to is the only way available at the present time to address the issue of purity of vaccines.
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.

It is comforting to know that he "Lincoln Law" remains in force to oversee the industry.