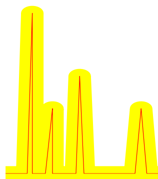

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APPLICATION NOTE

Gamma Globulin from bovine blood, Gamma Globulins from human blood digested with immobilize StyrosZyme® Pepsin, Immobilized Enzyme on Polymeric Hard Gel Simulated-Monolith™ Enzyme reactor with the Acquity UPLC *I* class Plus and Final Silica mapping.

Using Pepsin, an acidic protein with a pI of 1.0, we have digested online and in a fully automated setting, 2 separate Gamma globulins of different origin.

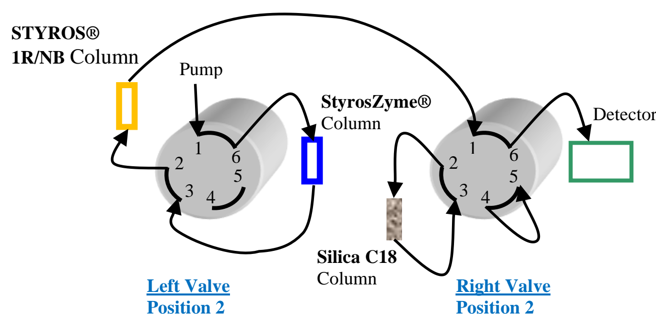
Gamma-globulins are the most heterogeneous globulins. Although most have a molecular weight of approximately 150,000 kDa, that of some, called macroglobulin, is as high as 800,000 kDa. Because typical antibodies are of the same size and exhibit the same electrophoretic behaviors as γ-globulins, they are called immunoglobulins. The designation IgM or gamma M (γM) is used for the macroglobulin; the designation IgG or gamma G (γG) is used for γ−globulins of molecular weight 150,000 kDa.
In the present experiment Gamma globulin from bovine blood were compared with Gamma globulin from Human serum (Cohn Fraction II, III) after online digestion on a StyrosZyme® Pepsin enzyme reactor.
Clear differences of the outcome can be noticed.
To be noted that the results of the digestions are not contaminated with fragments of enzyme auto-digests.
Below pH 6, pepsin preferentially cleaves on the carboxyl side of L-Phenylalanine, L-Leucine, or L-Tyrosine, where the amino side residue is preferably, but not limited to, an amino acid containing a hydrophobic side chain.

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 Phosphate, pH= 2.5 (for digestion).

The 3 columns used are all narrow bore, therefore minimizing the use of high volumes of solvent as well as avoiding the generation of large volume of waste
An Enzyme column of 2.1x100 mm stainless Steel (StyrosZyme® Pepsin).

Two narrow bore reversed phase columns. One polymeric (STYROS® 1R) with high capacity and high pH tolerance and one Silica C18 column (Acquity UPLC® BEH C18 1.7 µm 2.1x50 cm column) with high performance.

The following shows the starting position of the valves used:



Setup 1

In this first position, the StyrosZyme® enzyme column and the polymeric STYROS® 1R/NB column only, are online and the Silica C18 column is not exposed to any extreme pH aqueous buffer that is used for the digestion. It is therefore possible to safely scout the right digestion buffers as well as the optimum conditions for the full digestion prior to the use of the silica column.
Both polymeric columns, StyrosZyme® Pepsin and STYROS® R reversed phase polymeric can tolerate extreme pH's.

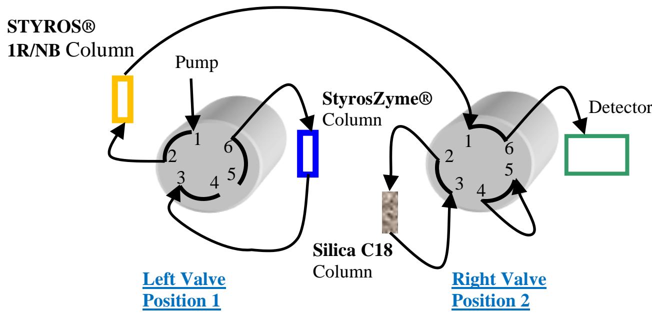
1-Equilibrate the enzyme column with all columns except the silica column, in line, as shown in Setup 1.

Time	% of buffer C	Flow rate (ml/min)
0		0
0.01	100	0.2
6	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		0
0.01	100	0.1
10	100	0.1

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			0
0.01	0	100	0.3
5	0	100	0.3

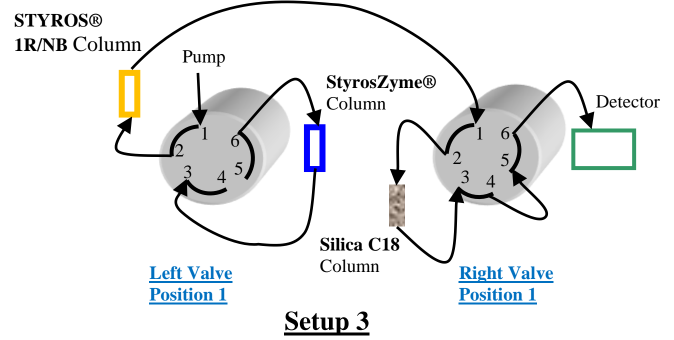
4-The digested peptides are now trapped on the polymeric reversed phase column and can be mapped following a gradient.
The setup is now as Setup 2.

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0			0
0.01	0	100	0.2
20	100	0	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			0
0.01	0	100	0.2
20	100	0	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			0
0.01	60	40	0.3
2	100	0	0.3
4	0	100	0.3
8	0	100	0.3

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			0
0.01	0	100	0.3
10	0	100	0.3

The system is now ready for the next cycle to check the reproducibility of the digestion.

The temperature is set at 37°C for all sequences.

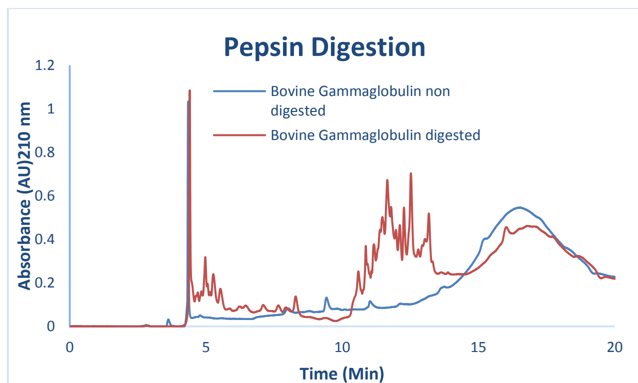
We have compared 2 γ−globulins: human and bovine.
The digestions are done under similar conditions
The enzyme column of 2.1 x 100 mm has a volume to 0.346 ml. A total of 1 ml is run through it during the digestion at a volumetric flow of 0.1 ml/min.

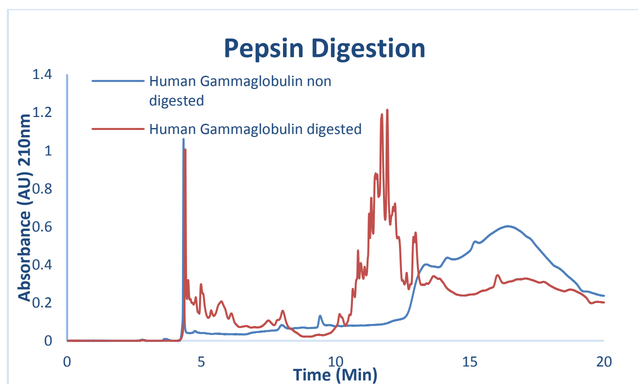
The digests with Pepsin reveal both their differences as well as their similarities.

The performance of the STYROS® 1R polymeric is crucial in the final separation of the digested peptides,
Indeed, a less than optimal performance of it can negatively affect the results of the separation.

To properly assess the trace of the chromatograms we have chosen the 210 nm wavelength.

It is important to note that each gamma globulin is identified as ≥ 95 % pure by Agarose gel electrophoresis.





Compared with one another the digestion of the two globulins shows similarities and differences.