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APPLICATION NOTE

Enzyme auto-digestion during batch mode processes. Assessment of the level of contamination comparing it to the digests of Cytochrome c from equine as an example.

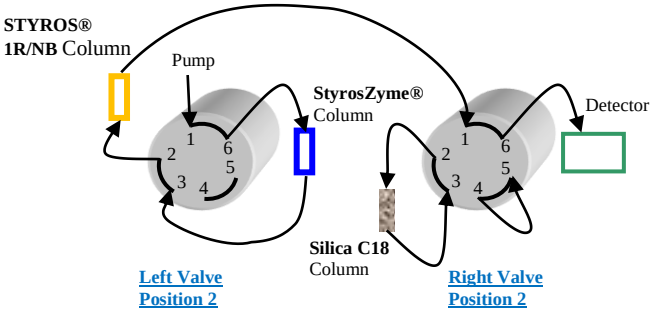
In addition of being tedious and lengthy, the process of batch digestion of proteinaceous species is also froth with the contamination of enzymes auto digests.

It is well understood that as the digestion proceeds in batch, the auto-digested enzymes no longer have a similar behavior as the intact enzymes.

Moreover, batch digestion is an anachronistic process that should no longer remain in the lexicon of today's concept of real-time modus operandi.

To highlight the point, we have plotted the previously digested Trypsin on the immobilized Trypsin reactor, along with the digests of Cytochrome c from equine using similar conditions in both cases.

Cytochrome c and Trypsin samples are made in buffer A with a concentration of 10 mg/ml.
Buffer A: 0.1 % DFA in DI H2O: ACN 98: 2 (for peptide mapping)
Buffer B: 0.1 % DFA in ACN: H2O, 70: 30 (for peptide mapping)
Buffer C: 0.1 M TRIS, pH= 8.5 (for digestion).
The columns are:
A Narrow Bore enzyme column of 2.1x50 mm stainless Steel (StyrosZyme® TPCK-Trypsin), with the same size reversed phase Narrow Bore column (STYROS® 1R) ending with the Silica C18 column (Acquity UPLC® BEH C18 1.7 µm 2.1x50 cm column).
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 columns only, are online and the Silica C18 column is not exposed to any high pH aqueous buffer that is used for the digestion.
It is therefore possible to safely scout the right digestion buffer as well as the optimum conditions for the full digestion prior to the use of the silica column.

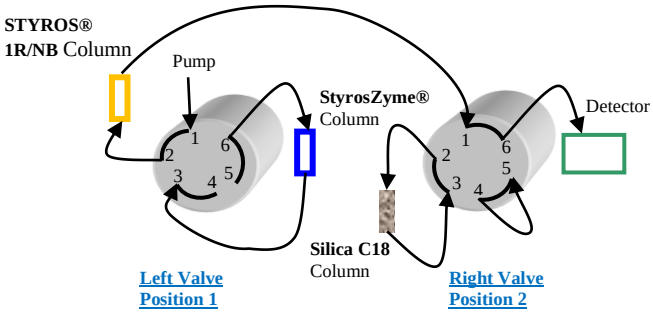
1-Equilibrate the enzyme column with both columns in line as shown in Setup 1.

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, the Cytochrome c sample (3 µl) is injected, and the resulting digests are dumped on the polymeric reversed phase column using the following method:

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

In the second set up, the left valve is switched to position 1 still with only the STYROS® 1R polymeric reversed phase column online in order to equilibrate it with the starting solvent gradient.



Setup 2

3-The reversed phase column only is now online 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	0.2
15	0	100	0.2

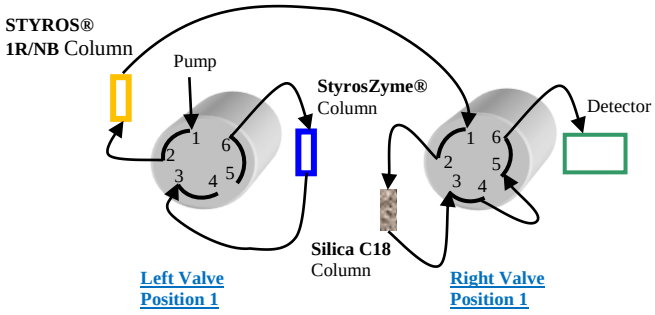
4-The digested peptides are now trapped on the polymeric reversed phase column and can be mapped following a gradient.
The setup is now as Setup 2.

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

When 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:

4'-The digested peptides trapped on the polymeric reversed phase column can now be mapped on the C18 Silica column.
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	60	40	0.2



Setup 3

To re-equilibrate both reversed phase columns that are now online, a similar step than step 5 is used.

5- Both reversed phase columns are pre-equilibrated 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	0.2
15	0	100	0.2

6-In the final step the line and the reversed phase column are flushed clean from any leftover organic going to the enzyme column, still avoiding the Silica column. The setup therefore remains as setup 2.

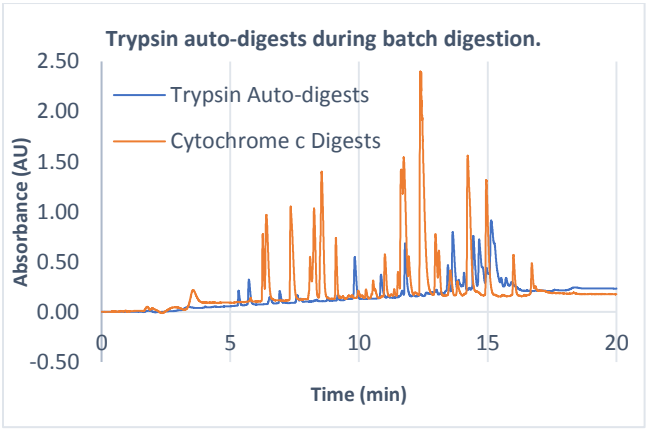
Time	% of digestion buffer C	Flow rate (ml/min)
0		0
0.01	100	0.2
6	100	0.2

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

The use of Narrow Bore columns requires minimal use of solvents therefore the automated digestion and mapping can be run around the clock without any concern of running out of buffers or overfilling the waste.

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

The following shows the superimposed chromatograms of Cytochrome c from equine along with the digests of Trypsin on the same column run separately.
Both digestions are complete.



This process is now run in real time, in a reproducible mode and without contamination of the digests of the target protein.
Fully automated using a non-leaching matrix stable at all pH's.

Same as before the method was run on the Acquity UPLC *I* class Plus and the Acquity UPLC® BEH C18 1.7 µm 2.1x50 cm column.