OraChrom, Inc.
The Vanguard of Liquid Chromatography.

10-B Henshaw Street Woburn, MA 01801 USA
Phone (781) 932 0151 Fax (781) 932 0787
E-mail: *info@orachrom.com* *www.orachrom.com*

APPLICATION NOTE

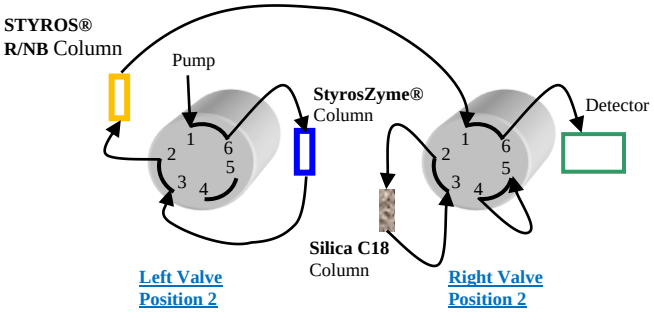
Casein Digestion.
TPCK Derivatized Compared with Non-Derivatized Trypsin in Digesting Fat Free Casein from bovine milk.

OraChrom has now a well-established automated digestion setup of proteins or proteinaceous species to use in Application Notes for its various enzymes.

Fat free Casein from bovine milk is considered here to find out additional factors involved in the online digestion and the capability to control them.

We have shown so far, in Application Notes 142 and 153, that the enzyme column remains stable, and it is not affected during the digestion of the Trypsin itself in solution.
That is now a controlled digestion of the enzyme in solution and not the immobilized enzyme of the enzyme reactor.

The following shows the starting position of the valves used:



Setup 1

In this first position, the StyrosZyme® enzyme column and the polymeric STYROS® R/NB column 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.
Both polymeric columns can tolerate high pH.

The buffers used are as follow:

Buffer A: 0.8 % TFA in H2O: ACN 98:2 (for mapping)
Buffer B: 0.8 % TFA in H2O: ACN 30:70 (for mapping)
Buffer C: 0.1 M Tris, pH = 8.8 (for digestion)

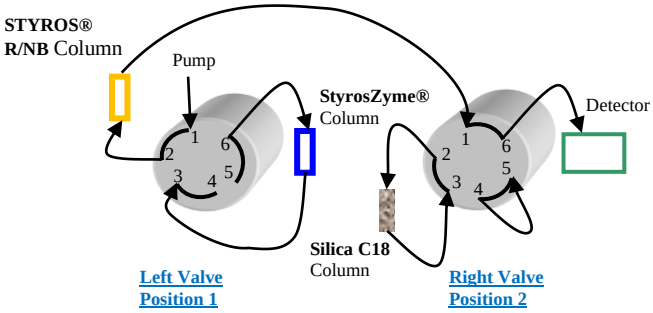
1-Equilibrate the enzyme column with all columns except the silica column, in line, as shown in Setup 1.

Time	% Of buffer C	Flow rate (ml/min)
0	100	0.2
0.01	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 Casein in digestion buffer C 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	100	0.2
10	100	0.2

In the second setup, the left valve is switched to position 1 with only the STYROS® R reversed phase column online to equilibrate it with the starting 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
1	5	95	0.2
10	5	95	0.2

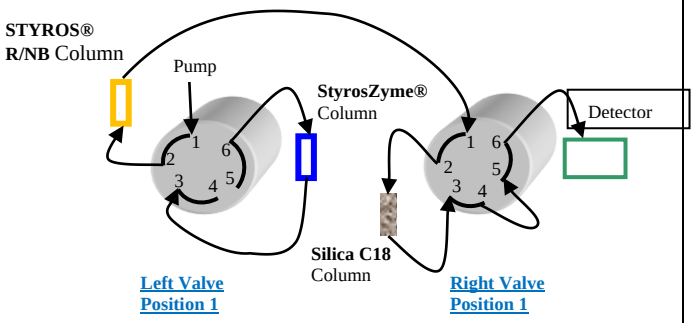
4-The digested peptides are now trapped on the polymeric reversed phase column and can be mapped following a gradient.
The setup is now as Setup 2.

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0	5	95	0.2
0.1	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
0.1	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.

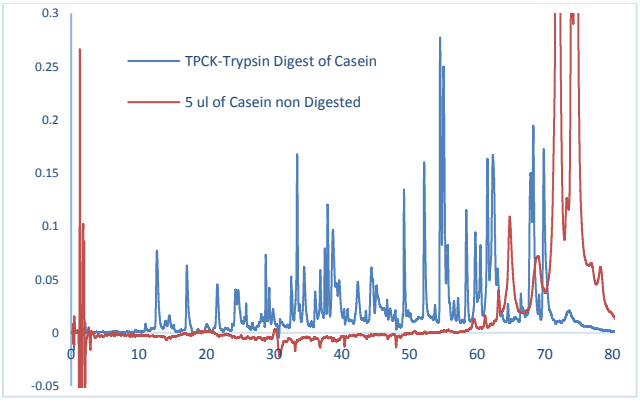
The setup remains Setup 3.

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0	5	95	0.02
0.01	60	40	0.2
1	100	0	0.2
2	100	0	0.2
4	5	95	0.2
8	5	95	0.2

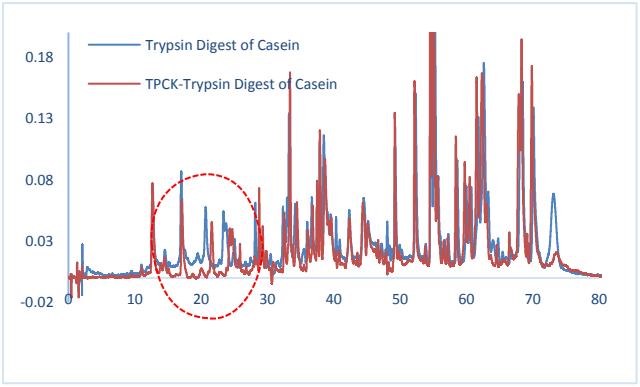
The system is now ready for the next cycle to check the reproducibility of the digestion.
The temperature is set at 37°C for all sequences. The absorbance is monitored by the PDA at 214 nm.

During this application the primary focus has been the difference between the digestion with Trypsin compared with TPCK-Trypsin.
The conclusion at a liquid chromatography level shows that the difference is in the less hydrophobic peptides that result from the digestion with the 2 types of Trypsin used. The hyphenation with a mass spectrometer can provide additional information as to the peptides generated specifically in the digestion of Lysozyme.

The first chromatogram shows the complete digestion of Casein compared with the non-digested casein in the same amount.



In the second chromatogram we have compared the digestion with Trypsin to its TPCK-derivatized species



As this setup lends itself to the proper hyphenation with a mass spectrometer it provides a good example of study of protein digestion without the shortcomings resulting from the instability of the matrix, its leaching and therefore depleting of the enzyme or even batch digestion froth with autolysis of the digesting enzyme itself.