Comparison of Phenyl- and C18 Bonded Phases with Peptide Mixtures

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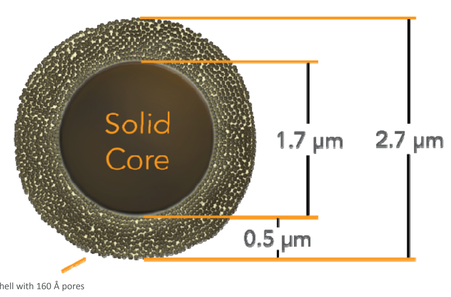
Overview

Octadecyl-silane (ODS,C18) columns are one of the most common bonded-phases for reversed-phase separations of protein and peptide mixtures. In many cases, resolution between two peaks are critical and need to be distinguished from each other. Mobile phases, organic modifiers, and temperature can help with this separation however, different bonded phases may be needed to get the desired selectivity and resolution. Three Phenyl- silane bonded phases were compared to the HALO ES-C18 phase to see if there are any advantages between them. These superficially porous silica particles have a 160Å pore size, ideal for peptides and polypeptides up to 20 kDa. Each phase has a unique silane bonding that could cause different interactions on the stationary phases. This could show differences in selectivity and resolution and potentially give an advantage in the separation.

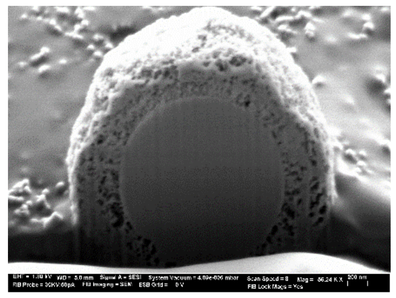
Materials and Methods

Columns of HALO 160Å ES-C18 and Phenyl phases were produced at Advanced Materials Technology, Inc. (Wilmington, DE). SEM images were obtained using a Zeiss (Jena, Germany) Auriga 60 High Resolution Focused Ion Beam & Scanning Electron Microscope at the University of Delaware (Newark, DE).
Mobile phase modifiers were obtained from Pierce and Synquest Laboratories. (TFA/DFA). Acetonitrile was MS grade (JT Baker) and HPLC grade (EMD Millipore). Peptides were from Sigma-Aldrich and AnaSpec. Analytical peptide separations used the Shimadzu Nexera LC-30 components (100 µL mixer), with the SPD M30A UV detector. An Orbitrap Velos Pro ETD (ThermoScientific, Inc.) was used for MS detection. MS was scanned from 300-2000 m/z; the ESI source was operated at 3.5 kV.

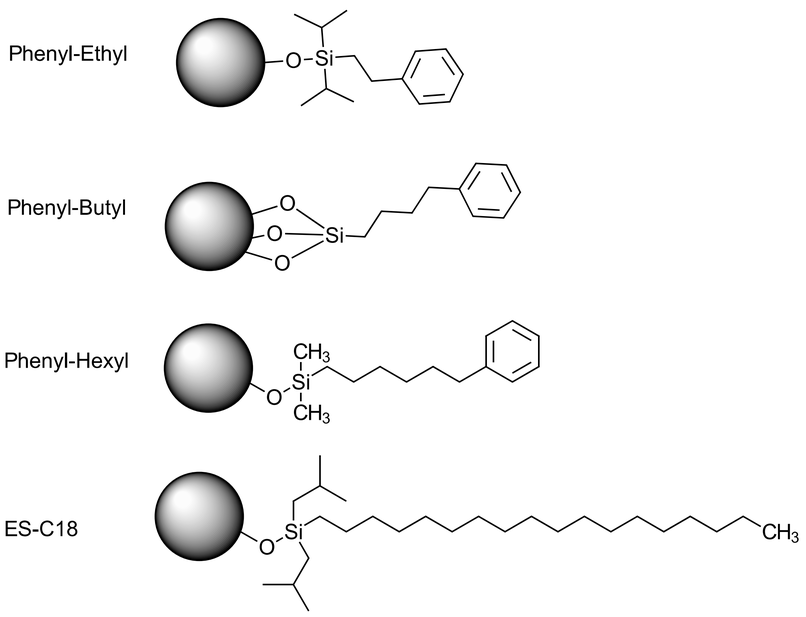
HALO Superficially Porous Particles



Shell with 160 Å pores

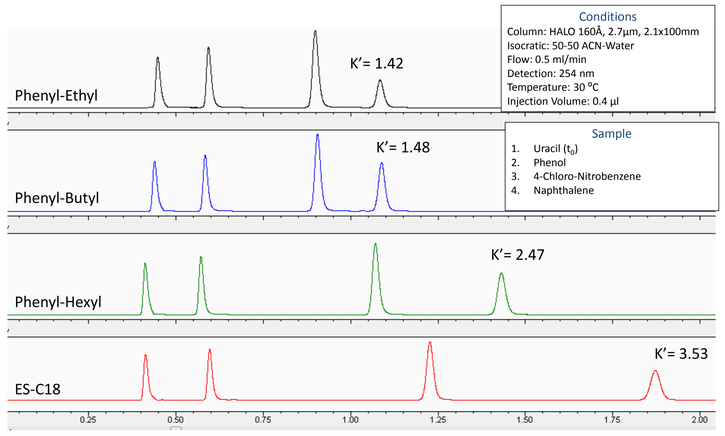


The HALO 160Å particle is a superficially porous particle made with a 1.7µm solid core and a 0.5µm porous shell. The shell consists of 160Å pores, ideal for peptides and polypeptides up to 20 kDa. These particles show excellent stability and provide lower overall back pressure when compared to totally porous particles.



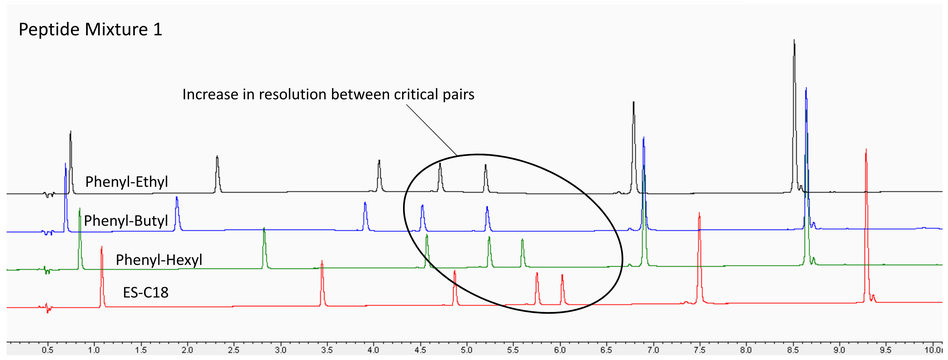
Three different Phenyl phases were compared to the commercialized HALO ES-C18 phase. Both Phenyl-Ethyl and ES-C18 have sterically protecting groups while the Phenyl-Butyl and Phenyl-Hexyl phases are tri-functional and mono-functional, respectively. These unique bondings interact with peptides differently and could give an advantage in the separation. All three Phenyl phases have been end-capped, while the ES-C18 phase is not.

Comparison with Peptides and Small Molecules



Peptide	Sequence	Molecular weight (g/mol)	pI
Gly-Tyr	GY	238.24	5.98
Val-Tyr-Val	VYV	379.45	5.98
Tyr–Tyr–Tyr	YYY	507.54	5.97
Methionine Enkephalin	YGGFM	573.67	5.98
Leucine enkephalin	YGGFL	555.63	5.98
Angiotensin (1–7) Amide	DRVYIHP-NH_2	898.02	7.37
Angiotensin II	DRVYIHPF	1046.19	7.37
Angiotensin 1–12 (human)	DRVYIHPFHLVI	1508.79	7.52
Neurotensin	QLYENKPRRPYIL	1672.92	9.94
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	2846.50	11.85
β-Endorphin	YGGFMTSEKSQTPLVTLFKNAIIKNAYKKGE	3465.03	9.05
Sauvagine	XGPPISIDLSLELLRKMIEIEKQEKEKQQAANNRLLLDTI-NH_2	4599.31	5.08
Bovine Insulin	FVNQHLCGSHLVEALYLVCGERGFFYTPKA	5733.49	6.6

Small molecule testing including neutral compounds shows an increase in retention as the alkyl chain gets longer, as expected.



Phenyl-Ethyl

Peak#	Ret. Time	Width(50%)	Tailing F.	Resolution(JP)
1	0.745	0.02218	1.3	--
2	2.318	0.03185	1.335	34.364
3	4.056	0.02987	1.235	33.227
4	4.711	0.03193	1.23	12.5
5	5.199	0.02932	1.271	9.411
6	6.789	0.03082	1.119	31.195
7	8.514	0.02838	1.261	34.375

Phenyl-Butyl

Peak#	Ret. Time	Width(50%)	Tailing F.	Resolution(JP)
1	0.69	0.02028	1.386	--
2	1.885	0.03425	1.455	25.866
3	3.907	0.0324	1.296	35.789
4	4.521	0.03387	1.289	10.935
5	5.216	0.03177	1.363	12.5
6	6.897	0.02997	1.171	32.13
7	8.641	0.03153	1.422	33.471

Phenyl-Hexyl

Peak#	Ret. Time	Width(50%)	Tailing F.	Resolution(JP)
1	0.843	0.0235	1.399	--
2	2.823	0.02868	1.466	44.768
3	4.569	0.02872	1.332	35.897
4	5.238	0.02938	1.349	13.598
5	5.595	0.02727	1.359	7.433
6	6.901	0.02923	1.185	27.271
7	8.642	0.02963	1.438	34.892

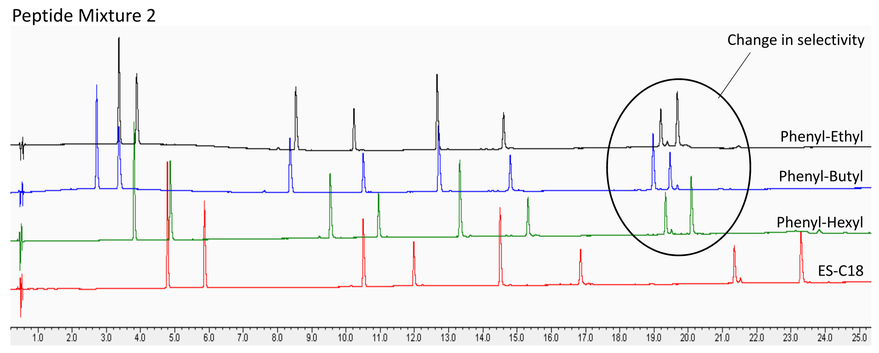
ES-C18

Peak#	Ret. Time	Width(50%)	Tailing F.	Resolution(JP)
1	1.08	0.02293	1.358	--
2	3.444	0.0265	1.375	56.437
3	4.868	0.02745	1.281	31.155
4	5.751	0.02905	1.323	18.429
5	6.023	0.02637	1.509	5.797
6	7.494	0.03132	1.158	30.092
7	9.286	0.02892	1.394	35.103

Resolution between critical pairs also improves with the phenyl-phases. Certain peptides show different behaviors on the bonded phases. Especially the Phenyl-Butyl compared to the Phenyl-Ethyl. Some peptides are less retained while others are more retained. This could have to do with the different silane bondings. (sterically protected vs. tri-functional)

Conditions
Column: HALO 160Å, 2.7µm, 2.1x100mm
Mobile Phase:
A: Water/ 0.1% TFA
B: 70-30 ACN/ Water/ 0.1% TFA
Gradient: 5-55% B in 10 min.
Flow: 0.5 ml/min.
Detection: 220 nm
Temperature: 60°C
Injection Volume: 1.7 µl

Peptide Mixture 1
1. Gly-Tyr (238.24 g/mol)
2. Val-Tyr-Val (379.46 g/mol)
3. Methionine Enkephalin (573.67 g/mol)
4. Angiotensin II (1046.20 g/mol)
5. Leucine Enkephalin (555.62 g/mol)
6. Bovine Ribonuclease A (13.7 kDa)
7. Bovine Insulin (5733.49 g/mol)



Phenyl-Ethyl

Peak#	Ret. Time	Width(50%)	Tailing F.	Resolution(USP)	Area
1	3.365	0.0442	1.255	--	416857
2	3.884	0.05403	1.493	6.257	332155
3	8.533	0.05385	1.6	50.883	293148
4	10.231	0.04725	1.282	19.836	177293
5	12.661	0.05247	1.5	28.769	346896
6	14.603	0.052	1.233	21.894	191883
7	19.194	0.05033	1.311	52.825	188326
8	19.678	0.0565	1.281	5.34	267025

Phenyl-Butyl

Peak#	Ret. Time	Width(50%)	Tailing F.	Resolution(USP)	Area
1	2.715	0.04488	1.295	--	415609
2	3.362	0.06102	1.644	7.08	330595
3	8.358	0.06278	1.866	46.365	294705
4	10.503	0.05035	1.374	21.975	176731
5	12.706	0.05883	1.649	23.331	353714
6	14.8	0.05265	1.169	21.882	174198
7	18.97	0.05493	1.401	45.545	274204
8	19.463	0.0498	1.363	5.499	199799

Phenyl-Hexyl

Peak#	Ret. Time	Width(50%)	Tailing F.	Resolution(USP)	Area
1	3.807	0.04032	1.413	--	416255
2	4.865	0.04815	1.65	14.032	329522
3	9.537	0.05288	1.813	53.785	293167
4	10.949	0.046	1.415	16.811	172364
5	13.324	0.05157	1.686	28.689	347167
6	15.316	0.04855	1.391	23.316	194021
7	19.333	0.04583	1.512	50.291	195403
8	20.087	0.05363	1.454	8.93	271712

ES-C18

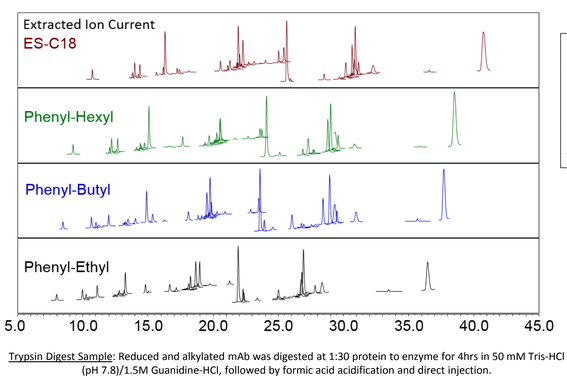
Peak#	Ret. Time	Width(50%)	Tailing F.	Resolution(USP)	Area
1	4.782	0.03792	1.312	--	416091
2	5.864	0.0441	1.652	15.506	332341
3	10.502	0.05062	1.817	57.207	302774
4	11.981	0.04527	1.395	18.153	178743
5	14.499	0.05087	1.67	30.838	345223
6	16.852	0.05178	1.261	26.901	168743
7	21.346	0.05012	1.583	51.762	197522
8	23.292	0.0604	1.481	20.62	281186

Another mix of peptides are examined with the different bonded phases. In this case, resolution between critical pairs decreases. However, the Phenyl-Butyl phase shows a change in selectivity between the last two peptides of the mix. (sauvagine and melittin) This could be very useful in some cases where peak coelution could be an issue.

Conditions
Column: HALO 160Å, 2.7µm, 2.1x100mm
Mobile Phase:
A: Water/ 0.1% TFA
B: 80-20 ACN/ Water/ 0.085% TFA
Gradient: 5-55% B in 25 min.
Flow: 0.5 ml/min.
Detection: 220 nm
Temperature: 60°C
Injection Volume: 2 µl

Peptide Mixture 2
1. Tyr-Tyr-Tyr (507.54 g/mol)
2. Angiotensin (1-7) Amide (898.02 g/mol)
3. Angiotensin II (1046.20 g/mol)
4. Angiotensin (1-12) human (1508.79 g/mol)
5. Neurotensin (1672.92 g/mol)
6. β-Endorphin (3465.03 g/mol)
7. Sauvagine (4599.31 g/mol)
8. Melittin (2846.46 g/mol)

Analysis of a trastuzumab (IgG1) tryptic digest using Mass Spectrometry



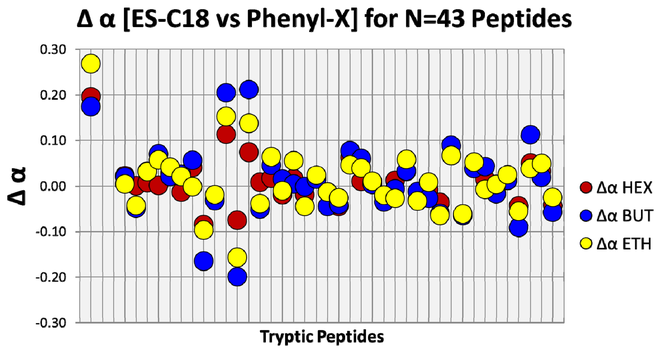
Trypsin Digest Sample: Reduced and alkylated mAb was digested at 1:30 protein to enzyme for 4hrs in 50 mM Tris-HCl (pH 7.8)/1.5M Guanidine-HCl, followed by formic acid acidification and direct injection.

Conditions
Column: HALO 160Å, 2.7µm, 2.1x100mm
Mobile Phase:
A: Water/ 10 mM DFA
B: Acetonitrile/ 10 mM DFA
Gradient: 2-50% B in 60 min.
Flow: 0.5 ml/min.
Detection: 220 nm
Temperature: 60°C

$k = (t_1 - t_0)/t_0$

$\alpha = k_2/k_1$

$\Delta\alpha = |\alpha_{phenyl} - \alpha_{c18}|$



Selectivity: @ Constant Mobile Phase	Hexyl:C18	Butyl:C18	Ethyl:C18
I Σ Δα I	0.044	0.072	0.064
SD	0.062	0.100	0.103
Δα min	0.001	0.002	0.001
Δα max	0.361	0.592	0.623

Conclusions

- Phenyl bonded phases show subtle, but useful selectivity differences compared to ES-C18
- Resolution of critical pairs has been improved by newer application-directed phenyl phases
- A wider range of useful operating conditions could take advantage of improvements in bonded phases for LC and LC-MS applications.
- The range of HALO Fused-Core materials continues to expand, with newer SPP material developments and an expanding range of surface chemistries