HPLC Method Development Guidelines

REVERSED PHASE HPLC METHOD DEVELOPMENT PARAMETERS

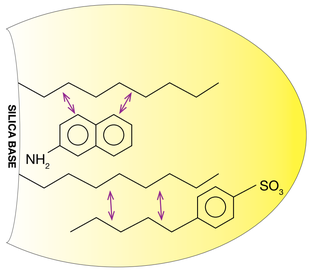
- **Analyte structure and pK_a**
- **Mobile phase composition**
- **HPLC column choice (optimal stationary phase)**

RETENTION MECHANISMS

Hydrophobic / Non-polar / Reversed Phase

Typical Phases: C18, C8, Phenyl, Polymer (i.e. SDVB)

Interactions with alkyl chains

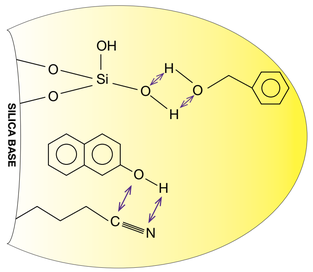


↔ Van der Waals Forces

Hydrophilic / Polar / Normal Phase

Typical Phases: Silica, NH_2, CN

Interactions with silanols and polar functional groups

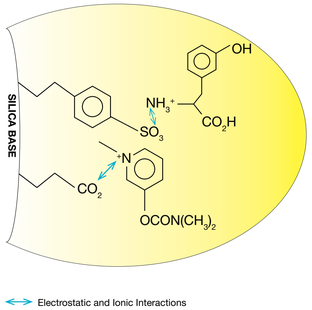


↔ Hydrogen bonding and dipole-dipole interactions

Ion-Exchange

Typical Phases: SCX, WCX, SAX, NH_2

Interactions with ionic functional groups and strong/weak ion-exchangers



↔ Electrostatic and Ionic Interactions

Bond Energies Involved with Retention Mechanisms

Physical Interaction	Types of Compounds	Energy (kJ/mol)
Van der Waals Forces	Alkyl chains, aromatic rings	2 – 10
Dipole-Dipole Interactions	Carbonyls, heterocyclic	8 – 15
Hydrogen Bond	Alcohol, amine, acid	20 – 50
Coulomb Forces	Ionic compounds	100 – 400
Covalent Bond	Nonmetals	> 300

TIP:

For aromatic compounds, phenyl phases offer a different selectivity then alkyl phases (C18, C8). Based on the interactions between the pi-electrons of the stationary phase's phenyl ring and the double bonds in the aromatic ring(s) of your analyte, retention time and elution order can change significantly and thus have a very positive effect on your method.

You can further adjust the selectivity of a phenyl stationary phase by using methanol instead of acetonitrile within the mobile phase. The pi-electrons of acetonitrile can interact with both the phenyl phase and aromatic functional groups in a competing way, thus leading to a reduced analyte retention time.

MOBILE PHASE COMPOSITION: BUFFER AND ORGANIC SOLVENTS

Henderson-Hasselbalch

By using a pH value which is at least two units above or below the pK_a value of analyte you ensure that your compound is either fully charged or uncharged.

Reminder: A buffer always consists of an acid or base and the corresponding salt. Typical concentrations are usually in the range of 10-50 mM depending on the needed ionic strength and solubility of the buffer system. See below for more buffer details.

Buffer, pK_S and pH

Calculated according to the Henderson-Hasselbalch dissociation constant (K_a)

$$K_a = \frac{[H^+][A^-]}{[HA]}$$

$$pH = pK_a - \log \frac{[HA]}{[A^-]}$$

$[A^-]/[HA]$	pH
100:1	pK_a + 2
10:1	pK_a + 1
1:1	pK_a
1:10	pK_a - 1
1:100	pK_a - 2

Buffer Selection

Buffer*	pK_a	Buffer Range (pH)	MS Compatibility
Trifluoroacetic Acid	**< 2**	**< 2.5**	•**
Phosphoric Acid (pK_1)	2.1	1.5 - 2.7	
Citric Acid (pK_1)	3.1	2.5 - 3.7	
Formic Acid	**3.8**	**2.8 - 4.8**	•
Citrate (pK_2)	4.7	4.1 - 5.3	
Acetic Acid	**4.8**	**3.8 - 5.8**	•
Citrate (pK_3)	5.4	4.8 - 6.0	
Carbonate (pK_1)	**6.4**	**5.4 - 7.4**	•
Phosphate (pK_2)	**7.2**	**6.6 - 7.8**	
Triethanolamine	7.8	6.8 - 8.8	•
TRIS	8.3	7.3 - 9.3	
Diethanolamine	8.9	7.9 - 9.9	•
Ammonia	**9.2**	**8.2 - 10.2**	•
Ethanolamine	9.5	8.5 - 10.5	•
Carbonate (pK_2)	**10.3**	**9.3 - 11.3**	•
Diethylamine	10.5	9.5 - 11.5	•
Triethylamine	**11.0**	**10.0 - 12.0**	•
Piperidin	11.1	10.1 - 12.1	

Sources:
Practical HPLC Method Development; L.R. Snyder, JJ Kirkland, and JL Glajch. Wiley Interscience, 1997.
Introduction to Protein and Peptide HPLC; TP Bradshaw, Phenomenex, 1998.

* Common buffers are in bold
** Low concentrations of TFA may be used in LC/MS applications, however it can affect sensitivity.

Solvent Strength

WEAK

Water
Methanol
Iso-propyl Alcohol
Acetonitrile
Acetone
Ethyl Acetate
Ethyl Ether
Tetrahydrofuran
Methylene Chloride
Chloroform
Toluene
Iso-octane
Hexane

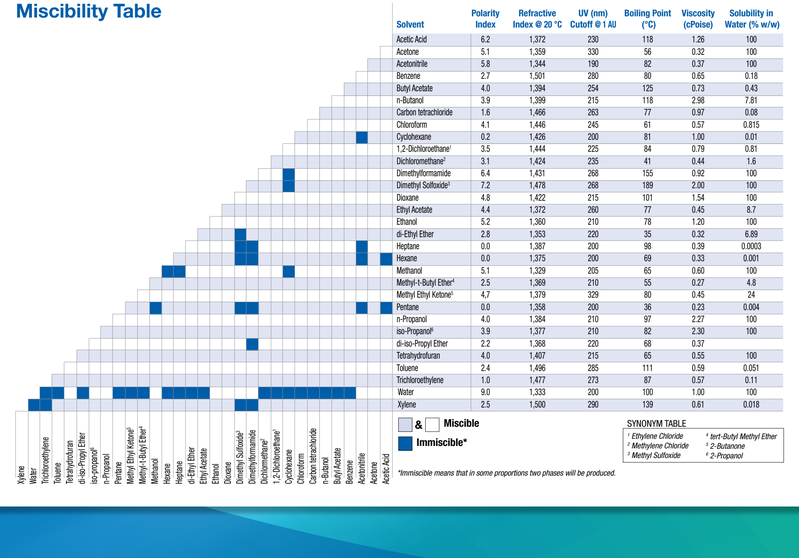
STRONG

TIP:

Use both methanol and acetonitrile to vary the selectivity.

Miscibility Table

Solvent	Polarity Index	Refractive Index @ 20 °C	UV (nm) Cutoff @ 1 AU	Boiling Point (°C)	Viscosity (cPoise)	Solubility in Water (% w/w)
Acetic Acid	6.2	1,372	230	118	1.26	100
Acetone	5.1	1,359	330	56	0.32	100
Acetonitrile	5.8	1,344	190	82	0.37	100
Benzene	2.7	1,501	280	80	0.65	0.18
Butyl Acetate	4.0	1,394	254	125	0.73	0.43
n-Butanol	3.9	1,399	215	118	2.98	7.81
Carbon tetrachloride	1.6	1,466	263	77	0.97	0.08
Chloroform	4.1	1,446	245	61	0.57	0.815
Cyclohexane	0.2	1,426	200	81	1.00	0.01
1,2-Dichloroethane[1]	3.5	1,444	225	84	0.79	0.81
Dichloromethane[2]	3.1	1,424	235	41	0.44	1.6
Dimethylformamide	6.4	1,431	268	155	0.92	100
Dimethyl Solfoxide[3]	7.2	1,478	268	189	2.00	100
Dioxane	4.8	1,422	215	101	1.54	100
Ethyl Acetate	4.4	1,372	260	77	0.45	8.7
Ethanol	5.2	1,360	210	78	1.20	100
di-Ethyl Ether	2.8	1,353	220	35	0.32	6.89
Heptane	0.0	1,387	200	98	0.39	0.0003
Hexane	0.0	1,375	200	69	0.33	0.001
Methanol	5.1	1,329	205	65	0.60	100
Methyl-t-Butyl Ether[4]	2.5	1,369	210	55	0.27	4.8
Methyl Ethyl Ketone[5]	4.7	1,379	329	80	0.45	24
Pentane	0.0	1,358	200	36	0.22	0.004
n-Propanol	4.0	1,384	210	97	2.27	100
iso-Propanol[6]	3.9	1,377	210	82	2.30	100
di-iso-Propyl Ether	2.2	1,368	220	68	0.37	
Tetrahydrofuran	4.0	1,407	215	65	0.55	100
Toluene	2.4	1,496	285	111	0.59	0.051
Trichloroethylene	1.0	1,477	273	87	0.57	0.11
Water	9.0	1,333	200	100	1.00	100
Xylene	2.5	1,500	290	139	0.61	0.018



& Miscible
Immiscible*

*Immiscible means that in some proportions two phases will be produced.

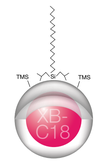
SYNONYM TABLE

[1] Ethylene Chloride
[2] Methylene Chloride
[3] Methyl Sulfoxide
[4] tert-Butyl Methyl Ether
[5] 2-Butanone
[6] 2-Propanol

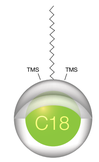
ACHIEVE THE BEST RESOLUTION with proper selectivity

Kinetex® Core-Shell Phases

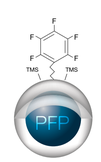
Use these different selectivities for the analysis of small compounds up to 10 kDa



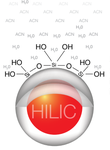
This unique C18 phase yields increased hydrogen bonding with hydrophobic selectivity, resulting in improved peak shape for basic compounds and increased retention of acidic compounds



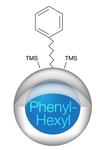
Balanced C18 phase that provides the highest degree of hydrophobic selectivity relative to the other Kinetex phases



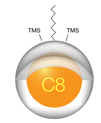
Pentafluorophenyl phase offers a high degree of steric interactions for improved separation of structural isomers, and the electronegative fluorine groups can offer increased retention of polar basic compounds



Used under HILIC running conditions, this phase provides the highest polar selectivity for retention and separation of hydrophilic compounds



Aromatic and moderate hydrophobic selectivity result in the great retention and separation of aromatic hydrocarbons



Moderate hydrophobic and steric selectivity is offered, bringing ultrahigh performance to USP L7 and other octyl silane methods

Aeris™ Core-Shell Phases

Use the Aeris PEPTIDE for the peptides and proteins up to 10 kDa and use the Aeris WIDEPORE for proteins larger than 10 kDa

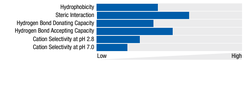
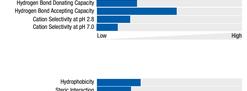
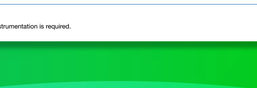
- Aeris PEPTIDE and WIDEPORE
- Hydrophilic peptides and proteins
- PEGylated proteins
- High temperature separations
- Alternate selectivity for peptide mapping

- Aeris WIDEPORE
- Large proteins
- Moderately hydrophobic proteins
- Monoclonal antibodies
- High temperature separations

- Aeris WIDEPORE
- Very large proteins
- Very hydrophobic proteins
- Membrane proteins
- Least retentive

PERFORMANCE GAINS ON ANY LC SYSTEM

Phases

Ligand	Description	Selectivity Profile
	**Kinetex XB-C18** Di-isobutyl side chains differentiate this C18 column. Low ligand density and an inactive surface make this column a great hydrogen acceptor. This phase will demonstrate improved peak shape for basic compounds and increased retention of acids.	
	**Kinetex C18** Very well balanced column providing some selectivity through steric, hydrogen, and cationic pathways. This is a great starting point for ultra-high efficiency separations.	
	**Kinetex C8** Brings the benefits of core-shell technology to USP L7 methods. The phase will provide moderate hydrophobicity and good steric and hydrogen donating selectivity.	
	**Kinetex Phenyl-Hexyl** Aromatic and moderate hydrophobic selectivity result in the great retention and separation of aromatic hydrocarbons.	
	**Kinetex PFP** This pentafluorophenyl column provides a very high degree of steric selectivity to separate structural isomers. The electronegative fluorine groups offer high selectivity for cationic compounds.	

Columns are pH stable from 1.5-10 under isocratic conditions. Columns are pH stable 1.5-8.5 under gradient conditions.
2.1 mm ID Kinetex columns are pressure stable up to 1000 bar. When using Kinetex 1.3 µm or 1.7 µm, increased performance can be achieved, however high pressure-capable instrumentation is required.

phenomenex®
...breaking with tradition℠

Don't hesitate to contact us with any questions!
Web: www.phenomenex.com / Email: info@phenomenex.com

TR0706613_W