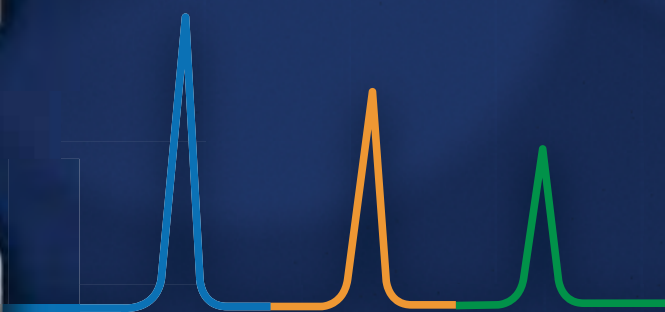
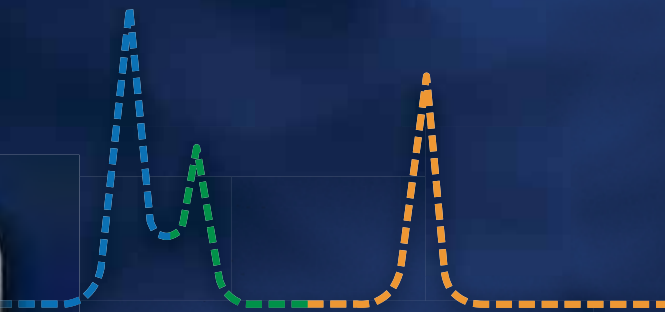


HALO®

GUIDEBOOK ON REVERSED PHASE CHEMISTRIES
& UTILIZING SELECTIVITY FOR HPLC SEPARATIONS



HALO[®]

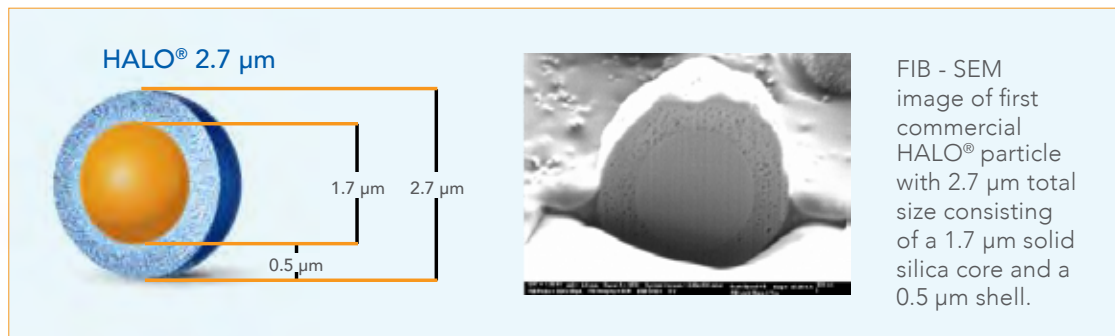
GUIDEBOOK ON REVERSED PHASE CHEMISTRIES
& UTILIZING SELECTIVITY FOR HPLC SEPARATIONS

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Introduction To HALO® Fused-Core® Technology

Since 2006 when Advanced Materials Technology was the first to commercialize HALO® superficially porous particle (Fused-Core®) technology, separation scientists have been benefiting from the advantages of faster chromatography that maintains high resolution without the back pressure consequences of sub-2 μm fully porous particle column technology.

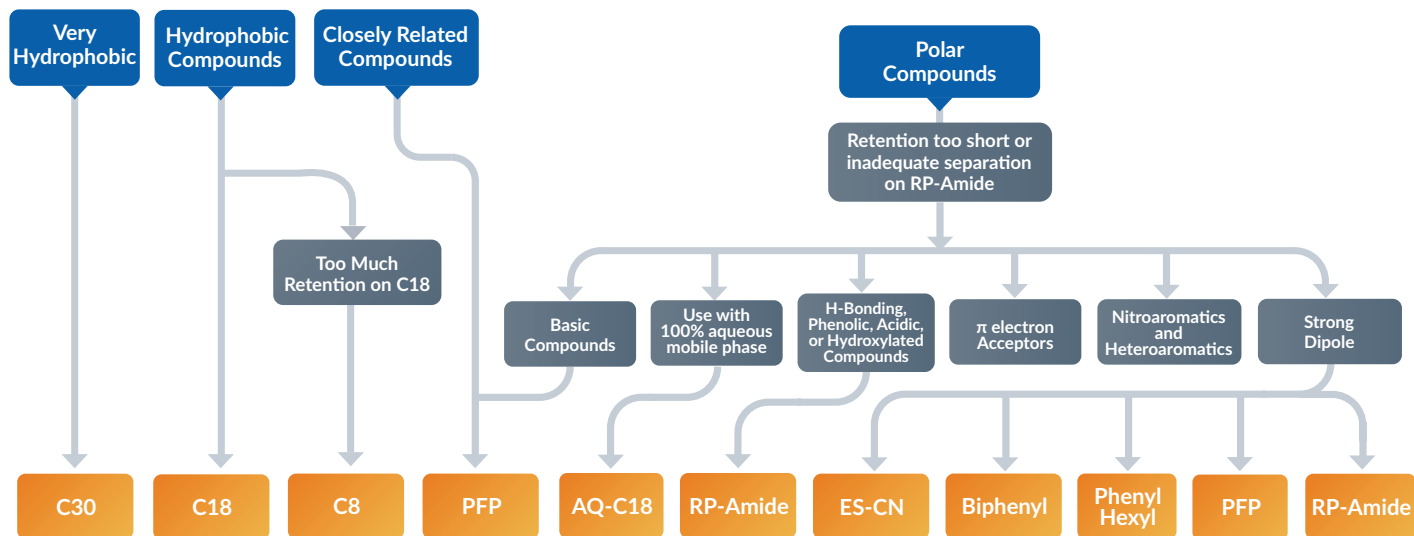


AMT's founding principal scientists, notably Dr. Jack Kirkland, Dr. Joseph DeStefano, and Tim Langlois are regarded as innovators in silica technology with a deep understanding of chromatographic separations. The combination of these talents has enabled the HALO® product line to evolve with multiple options for particle size, particle morphology, and stationary phase.

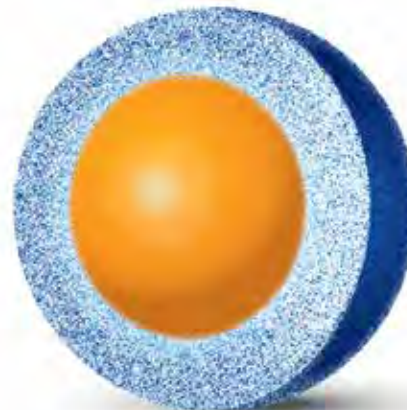
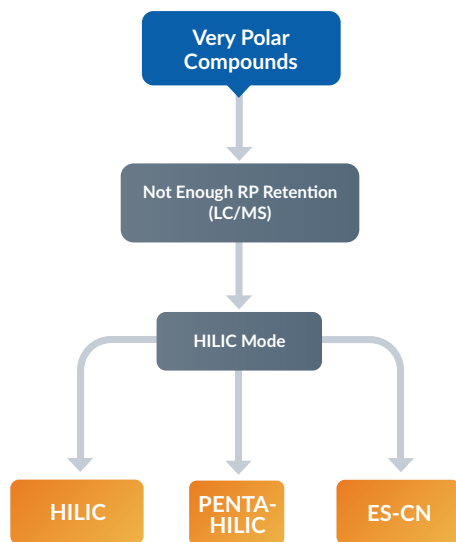


THE PURPOSE OF THIS GUIDE

The purpose of this guide is to introduce you to the HALO® reversed phases for small molecules and how to utilize these various stationary phases to optimize selectivity and ultimately improve your reversed phase chromatographic separations!



Depending on the nature of the analytes that need to be separated, one can use the flow chart as a guide for choosing the most appropriate HALO® stationary phase.



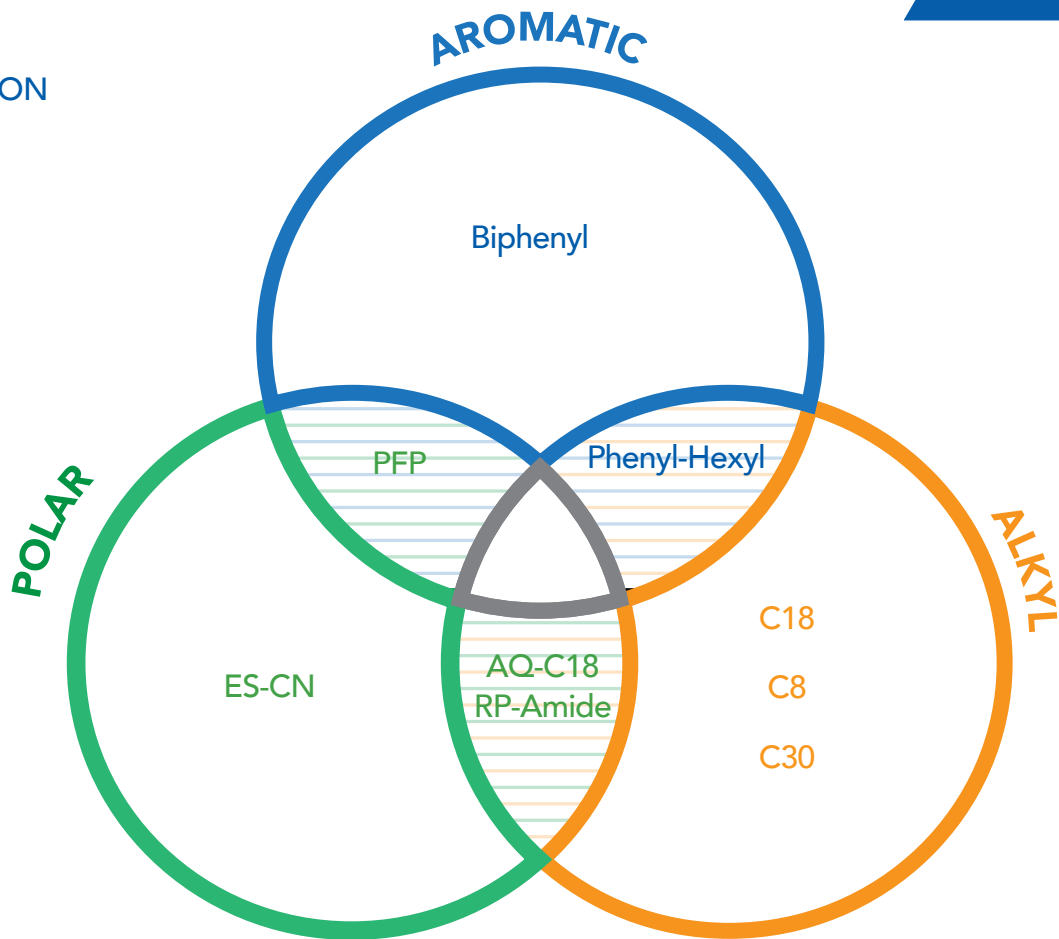
Classification and Characterization of HALO® Reversed Phase Options

What HALO® phases are available for reversed phase analysis of small molecules and when should I use them? These questions are frequently asked by HALO® customers. At the time of this publication, nine different HALO® reversed phase options are available to meet the most challenging separations. For very polar compounds, the reversed phase separation mode may not be the best choice and a change to using Hydrophilic Interaction Liquid Chromatography (HILIC) phases may be preferred. In those cases, HALO® HILIC, HALO® Penta-HILIC, and HALO® ES-CN would be used. These HALO® phases for HILIC mode of separation are available but are beyond the scope of this guide.

The HALO® phases may be classified by three main chemistry designations: alkyl, aromatic, and polar. Non-aromatic alkyl chemistry has the general formula of C_nH_{2n+1} . Aromatic chemistry contains one or more 6-carbon rings with the general formula C_6H_6 . Polar chemistry contains any of the following functional groups or element: amide, cyano, or fluorine. C18, C8, and C30 are all in the alkyl designation. Phenyl-Hexyl, while aromatic, demonstrates alkyl characteristics. Solely aromatic is Biphenyl. PFP shares characteristics of both aromatic and polar chemistries. In the polar section is ES-CN.

Finally, in the overlap section between polar and alkyl are AQ-C18 and RP-Amide. They are mostly polar, but often exhibit some hydrophobic selectivity. When selecting screening phases for method development, it is good practice to choose phases from each main chemistry designation or from overlap sections if necessary to achieve your separation.

CHEMICAL
CLASSIFICATION



HYDROPHOBIC - SUBTRACTION MODEL

The hydrophobic-subtraction model (HSM) is a means by which the selectivity of reversed phase HPLC columns is characterized. Five different parameters (**H**, **S***, **A**, **B**, and **C**) are used to describe the physico-chemical properties of a chromatographic bonded phase.

H measures the hydrophobicity of the phase.

S* measures the resistance of the stationary phase to penetration by a solute molecule.

A measures the hydrogen-bond acidity of the phase.

B measures the hydrogen-bond basicity of the phase.

C measures the interaction of the phase with ionized solute molecules and is measured at pH 2.8 and 7.0.

The combination of these parameters along with the parameters that characterize a solute (η , σ , β , α , κ) is then related to the solute's retention (k_x) relative to the retention of ethylbenzene (k_{EB}) by the equation below:

$$\log \left(\frac{k_x}{k_{EB}} \right) = \eta H - \sigma S^* + \beta A + \alpha B + \kappa C$$

where:

η = solute hydrophobicity

σ = bulkiness of the solute molecule

β = hydrogen-bond basicity of the solute

α = hydrogen-bond acidity of the solute

κ = ionization state of the solute molecule

The HALO® phases have been evaluated using the HSM and the data is available via hplccolumns.org and via the USP website apps.usp.org/app/USPNF/columnsDB.html. In Table 1 are listed the HALO® bonded phases, the USP designations, and the HSM coefficients. HSM parameters are used to evaluate how similar or different (orthogonal), HPLC phases are. The phase parameters may be compared using an F_s value (See next page for equation). Two phases are considered orthogonal when their F_s value is > 12 with the larger the F_s value, the more orthogonal the phase. The F_s values relative to HALO® C18 are listed in Table 1. For more information about the HSM, see references 1 and 2.

Table 1. HSM values

F_s	Phase	USP type	H	S^*	A	B	C (pH 2.8)	C (pH 7.0)
0	HALO C18	L1	1.100	0.040	0.000	-0.050	0.050	0.040
10.04	HALO C8	L7	0.910	0.020	-0.130	0.000	-0.010	0.180
12.07	HALO AQ-C18	L1	1.000	-0.036	0.099	-0.048	0.156	0.864
17.35	HALO Phenyl-Hexyl	L11	0.780	-0.090	-0.230	0.000	0.100	0.450
17.43	HALO C30	L62	0.938	-0.046	-0.140	0.023	0.170	0.350
22.78	HALO ES-CN	L10	0.566	-0.110	-0.344	0.021	0.126	1.150
26.76	HALO Biphenyl	L11	0.708	-0.183	-0.279	0.028	0.047	0.990
52.83	HALO RP-Amide	L60	0.850	0.080	-0.380	0.190	-0.410	0.310
94.45	HALO PFP	L43	0.702	-0.117	-0.073	-0.062	1.170	0.972

*Note HSM values listed are for 2.7 μ m particle size.

REFERENCES

1. *The Hydrophobic-Subtraction Model for Reversed-Phase Liquid Chromatography: A Reprise*, Sept. 01, 2016, John W. Dolan, Lloyd R. Snyder, LCGC North America, Volume 34, Issue 9, 730–741.
2. *The hydrophobic-subtraction model of reversed-phase column selectivity*, L.R. Snyder, J.W. Dolan, P.W. Carr, *Journal of Chromatography A*, 1060 (2004) 77–116.

F_s

$$= \sqrt{(w_H(H_1 - H_2))^2 + (w_S(S^*_1 - S^*_2))^2 + (w_A(A_1 - A_2))^2 + (w_B(B_1 - B_2))^2 + (w_{C_{2.8}}(C_{2.81} - C_{2.82}))^2}$$

Where:

$H_1 - H_2$ is the difference between the hydrophobicity parameters of columns 1 and 2

$S^*_1 - S^*_2$ is the difference between the steric parameters of columns 1 and 2

$A_1 - A_2$ is the difference between the hydrogen bond acidity parameters of columns 1 and 2

$B_1 - B_2$ is the difference between the hydrogen bond basicity parameters of columns 1 and 2

$C_{2.81} - C_{2.82}$ is the difference between the charge interaction parameters (at pH 2.8) of columns 1 and 2

w_H is the weighting factor for the difference in hydrophobicity

w_S is the weighting factor for the difference in steric interactions

w_A is the weighting factor for the difference in hydrogen bond acidity

w_B is the weighting factor for the difference in hydrogen bond basicity

$w_{C_{2.8}}$ is the weighting factor for the difference in charge interactions at pH 2.8

Overview of HALO® Family of Reversed Phases

The HALO® small molecule product line consists of various bonded phases available in three particle sizes (2 μm , 2.7 μm , 5 μm) with a 90 \AA pore size.

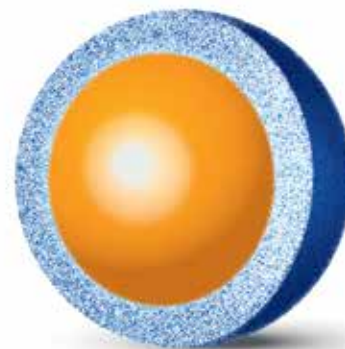
SMALL MOLECULE



90 \AA 2 micron particle



90 \AA 2.7 micron particle

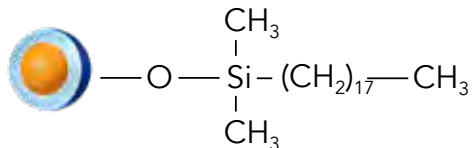


90 \AA 5 micron particle

*C30 is available on 160 \AA , 2.7 μm particles.



HALO[®] C18



Ligand: dimethyloctadecylsilane

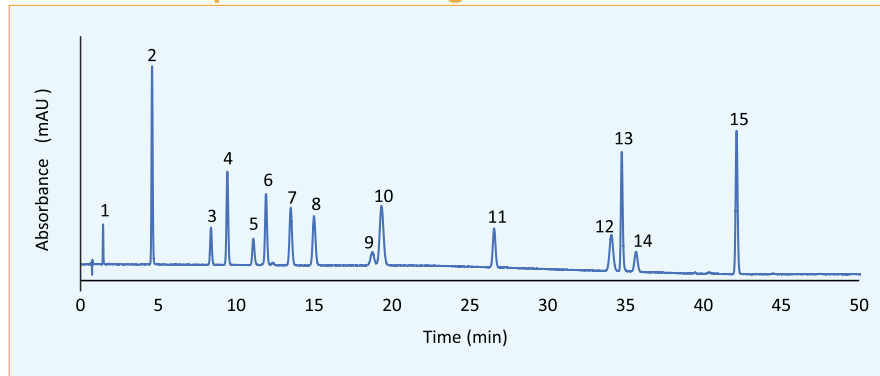
USP Designation: L1

Chemistry Classification: Alkyl

BENEFITS AND BEST APPLICATIONS:

- Universal phase for most applications
- High performance for broad range of polarities
- Excellent peak shape for acids, bases and neutrals

Paracetamol Separation According To EP 9.4



PEAK IDENTITIES (IN ORDER): Impurity K, Paracetamol, Impurity A, Impurity B, Impurity F, Impurity C, Impurity D, Impurity E, Impurity M, Impurity G, Impurity H, Impurity I, Impurity L, Impurity J, Impurity N

TEST CONDITIONS:

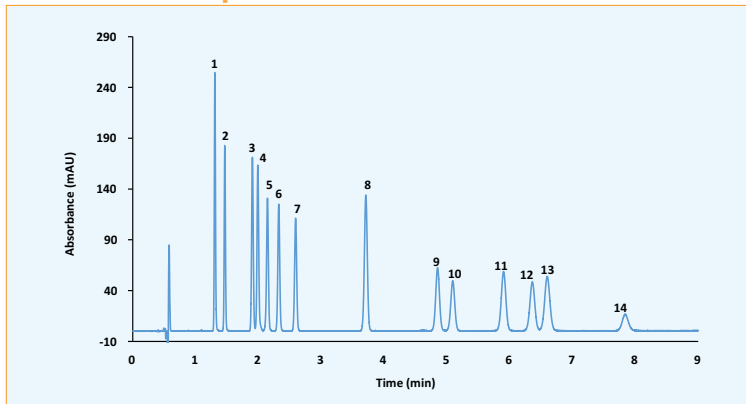
Column: HALO 90 Å C18, 2.7 μ m, 2.1 x 100 mm
Guard Column: HALO 90 Å C18, 2.7 μ m, 2.1 x 5 mm
Mobile Phase A: pH 7 Phosphate Buffer
Mobile Phase B: Methanol
Gradient: 5-34% B in 50 min
Flow Rate: 0.3 mL/min
Initial Pressure: 246 bar
Temperature: 30 °C
Detection: 254 nm, PDA
Injection Volume: 1 μ L

PEAK IDENTITIES (IN ORDER): Cannabidiol (CBD), Cannabidiol acid (CBDA), Cannabigerol (CBG), Cannabigerol acid (CBGA), Cannabidiolol (CBDL), Tetrahydrocannabinol (THC), Cannabinol (CBN), delta-9-Tetrahydrocannabinol (Δ 9-THC), delta-8-Tetrahydrocannabinol (Δ 8-THC), Cannabicyclol (CBL), Cannabichromene (CBC), delta-9-Tetrahydrocannabinolic acid A (THCA), Cannabichromenic acid (CBCA)

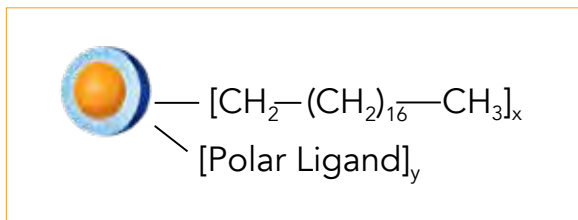
TEST CONDITIONS:

Column: HALO 90 Å C18, 2.7 μ m, 3.0 x 150 mm
Mobile Phase A: Water/0.1% Formic acid
Mobile Phase B: Acetonitrile/0.085% Formic acid
Isocratic: 25/75 A/B
Flow rate: 1.0 mL/min
Pressure: 350 bar
Temperature: 30 °C
Detector: UV 220 nm
Injection Volume: 0.6 μ L

Fast Isocratic Separation Of 14 Cannabinoids



HALO® AQ-C18



Ligand: Polar modified dimethyloctadecylsilane

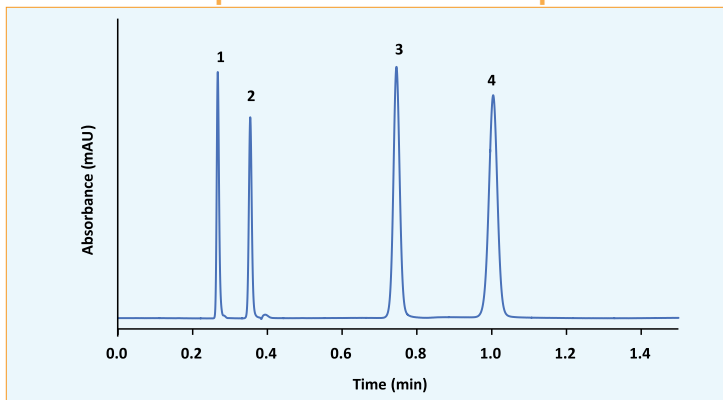
USP Designation: L1

Chemistry Classification: Alkyl / Polar

BENEFITS AND BEST APPLICATIONS:

- Polar analytes
- Alternate selectivity
- 100% aqueous compatible

Nucleobases Separation Under 100% Aqueous Conditions



PEAK IDENTITIES (IN ORDER): Thiourea, 5-Fluorocytosine, Adenine, Thymine

TEST CONDITIONS:

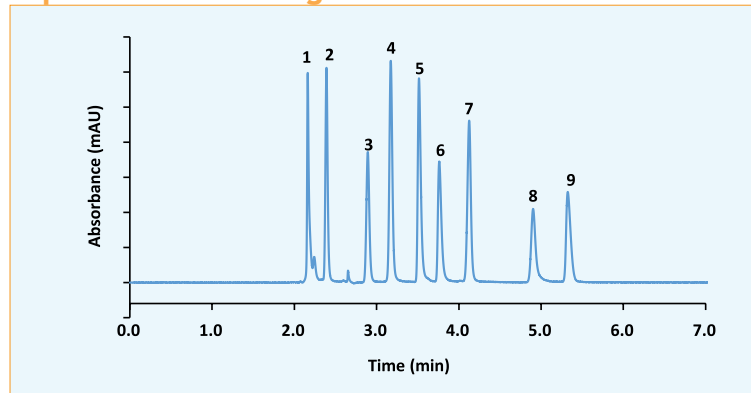
Column: HALO 90 Å AQ-C18, 2.7 μm , 4.6 x 50 mm
 Isocratic: Water, 0.1% TFA
 Flow Rate: 2.0 mL/min
 Pressure: 290 bar
 Temperature: 30 °C
 Detection: UV 254 nm, PDA
 Injection Volume: 0.5 μL
 Sample Solvent: Water, 0.1% TFA

PEAK IDENTITIES (IN ORDER): Oxalic acid, Tartaric acid, Malic acid, Ascorbic acid, L-Lactic acid, Acetic acid, Citric acid, Succinic acid, Fumaric acid

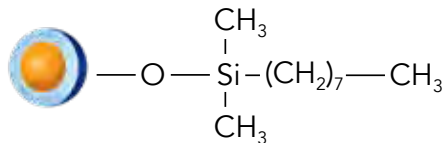
TEST CONDITIONS:

Column: HALO 90 Å AQ-C18, 2.7 μm , 4.6 x 250 mm
 Isocratic: 20 mM Potassium Phosphate Buffer, pH 2.7
 Flow Rate: 1.0 mL/min
 Pressure: 307 bar
 Temperature: 40 °C
 Detection: UV 214 nm, PDA
 Injection Volume: 20 μL
 Sample Solvent: Mobile phase

Separation of Polar Organic Acids



HALO[®] C8



Ligand: Dimethyloctylsilane

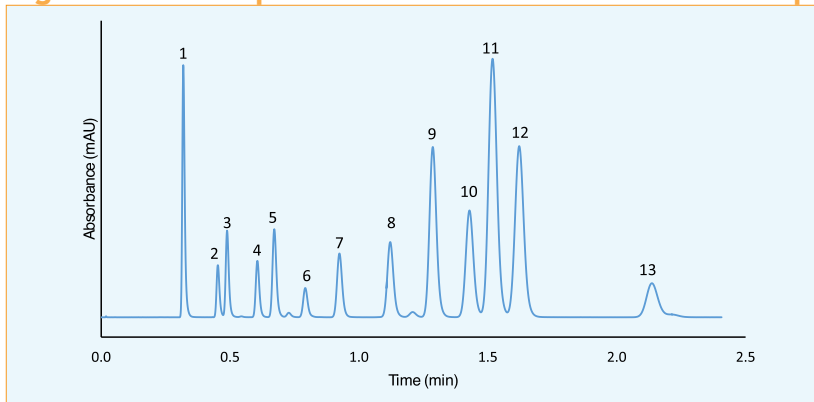
USP Designation: L7

Chemistry Classification: Alkyl

BENEFITS AND BEST APPLICATIONS:

- Ideal for broad range of analytes
- Less hydrophobic than C18
- Recommended for moderately hydrophobic compounds

High Resolution Separation Of Phthalates And Neutral Compounds



PEAK IDENTITIES (IN ORDER): Uracil, 1-indanol, dimethylphthalate, anisole, diethylphthalate, benzophenone, naphthalene, dipropyl phthalate, hexano phenone, phenanthrene, anthracene, 3-phenyltoluene, dibutylphthalate

TEST CONDITIONS:

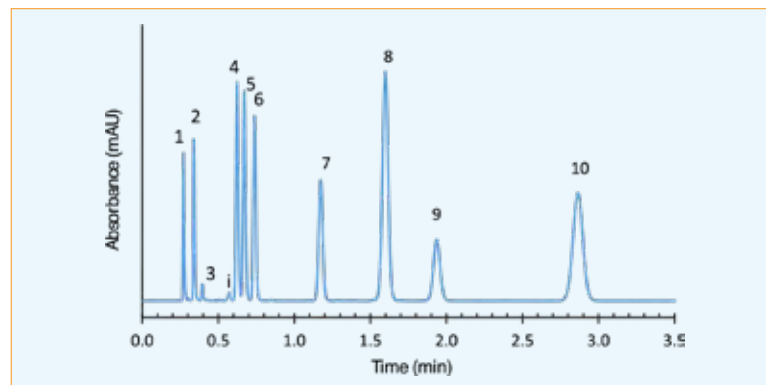
Column: HALO 90 Å C8, 2.7 μ m, 4.6 x 50 mm
 Mobile Phase: 32/68- A/B
 A= Water
 B= 70/30 Acetonitrile/Methanol
 Flow Rate: 2.0 mL/min
 Pressure: 136 Bar
 Temperature: 27 °C
 Detection: UV 254 nm, VWD
 Injection Volume: 1.0 μ L

PEAK IDENTITIES (IN ORDER): Acetaminophen, Aspirin, Salicylic acid, Tolmetin, Ketoprofen, Naproxen, Fenoprofen, Diclofenac, Ibuprofen, Mefenamic acid, i=impurity

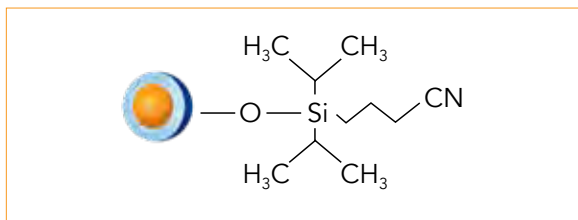
TEST CONDITIONS:

Column: HALO 90 Å C8 2.7 μ m, 4.6 x 50 mm
 Mobile Phase: 35/65- A/B
 A= 0.02 M Sodium Phosphate Buffer, pH 2.5
 B= Methanol
 Flow Rate: 2.0 mL/min
 Pressure: 277 Bar
 Temperature: 35 °C
 Detection: UV 254 nm, VWD
 Injection Volume: 1.0 μ L

Separation Of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)



HALO® ES-CN



Ligand: diisopropylcyanopropylsilane

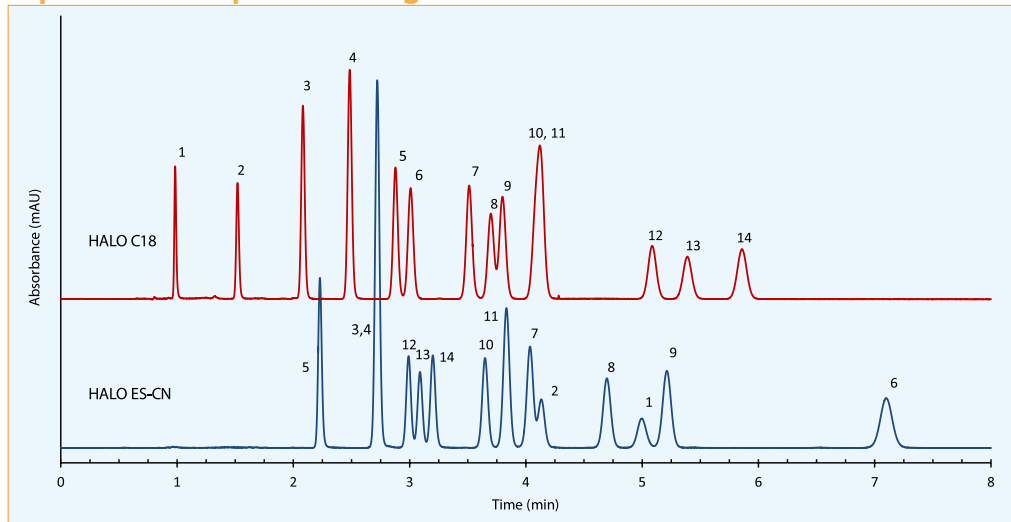
USP Designation: L10

Chemistry Classification: Polar

BENEFITS AND BEST APPLICATIONS:

- Ideal for polar analytes
- Alternate selectivity to alkyl phases
- 100% aqueous compatible

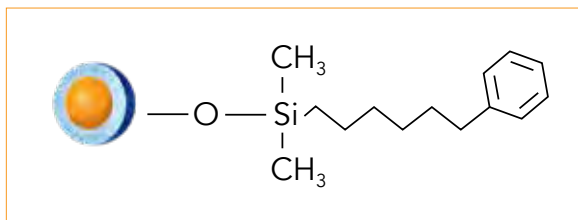
Separation Of Explosives Using Two Phase Chemistries



PEAK IDENTITIES (IN ORDER):
 HMX, RDX, 1,3,5-TNB, 1,3-DNT,
 nitrobenzene, Tetryl, 2,4,6-TNT,
 4-amino-2,6,-DNT, 2-amino-
 4,6-DNT, 2,6-DNT, 2,4-DNT,
 2-nitrotoluene, 4-nitrotoluene,
 3-nitrotoluene, TNB=trinitrobenzene,
 DNT=dinitrotoluene,
 TNT=trinitrotoluene

TEST CONDITIONS:
 Columns: HALO 90 Å C18, 2.7 µm
 HALO 90 Å ES-CN, 2.7 µm
 both 4.6 x 100 mm
 Mobile phase: 50/50:Methanol/Water
 Flow Rate: 1.25 mL/min
 Pressure: about 300 bar
 Temperature: 30 °C

HALO[®] PHENYL-HEXYL



Ligand: dimethylphenyl-hexylsilane

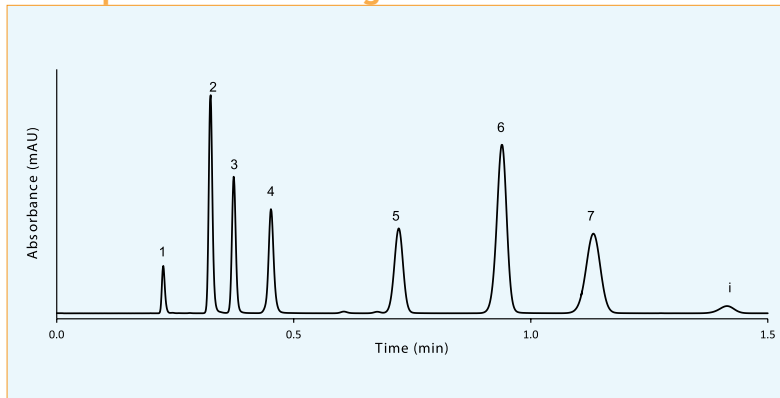
USP Designation: L11

Chemistry Classification: Aromatic / Alkyl

BENEFITS AND BEST APPLICATIONS:

- Ideal for aromatic (pi-pi) compounds (ketones, nitriles, alkenes)
- Alternate selectivity to alkyl phases
- 100% aqueous compatible

Fast Separation Of Anticoagulants



PEAK IDENTITIES (IN ORDER): Uracil, 4-Hydroxycoumarin, Coumarin, 6-Chloro-4-hydroxycoumarin, Warfarin, Coumatetralyl, Coumachlor i=impurity

TEST CONDITIONS:

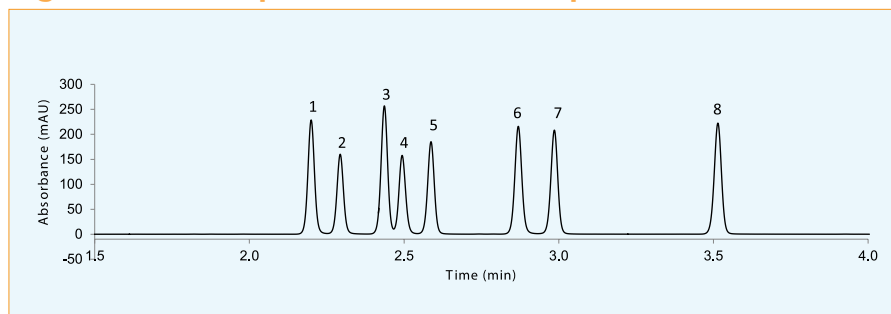
Column: HALO 90 Å Phenyl-Hexyl, 2.7 μ m, 4.6 x 50 mm
 Mobile phase: A/B--40/60
 A=0.1% Formic acid, pH 2.6
 B=Methanol/Acetonitrile-50/50
 Flow Rate: 2.0 mL/min
 Pressure: 210 Bar
 Temperature: 45 °C
 Detection: UV 254 nm
 Injection Volume: 1 μ L

PEAK IDENTITIES (IN ORDER): Oxazepam, Lorazepam, Nitrazepam, Alprazolam, Clonazepam, Temazepam, Flunitrazepam, Diazepam

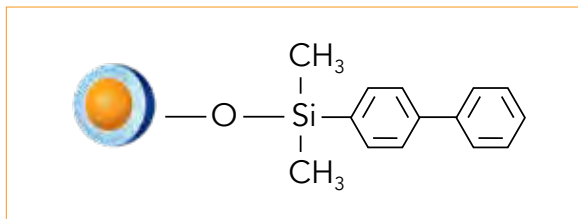
TEST CONDITIONS:

Column: HALO 90 Å Phenyl-Hexyl, 2.7 μ m, 4.6 x 50 mm
 A = 25 mM Ammonium Acetate
 B = Acetonitrile
 Flow rate: 1.5 mL/min
 Gradient: 34–63% B in 3.5 min
 Pressure: 200 bar
 Temperature: 35 °C
 Detection: 254 nm
 Injection Volume: 1 μ L

High Resolution Separation Of Benzodiazepines



HALO® BIPHENYL



Ligand: dimethylbiphenyl

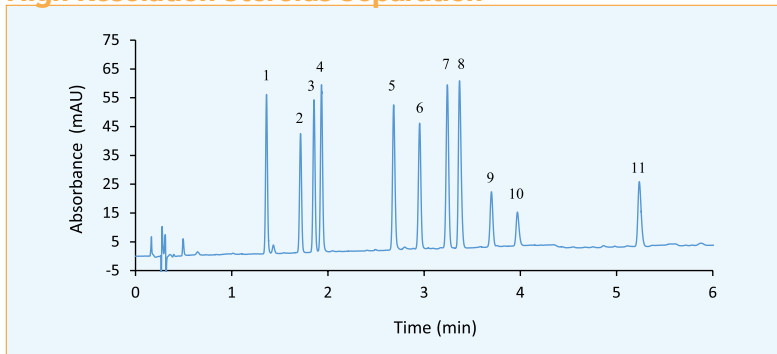
USP Designation: L11

Chemistry Classification: Aromatic

BENEFITS AND BEST APPLICATIONS:

- Ideal for aromatic (pi-pi) compounds (ketones, nitriles, alkenes)
- Alternate selectivity to alkyl phases
- 100% aqueous compatible

High Resolution Steroids Separation



PEAK IDENTITIES (IN ORDER): Estriol, Hydrocortisone, Prednisone, Cortisone, Corticosterone, β -Estradiol, Cortisone Acetate, Testosterone, 17 β -Hydroxyprogesterone, 11-Deoxycorticosterone, Progesterone

TEST CONDITIONS:

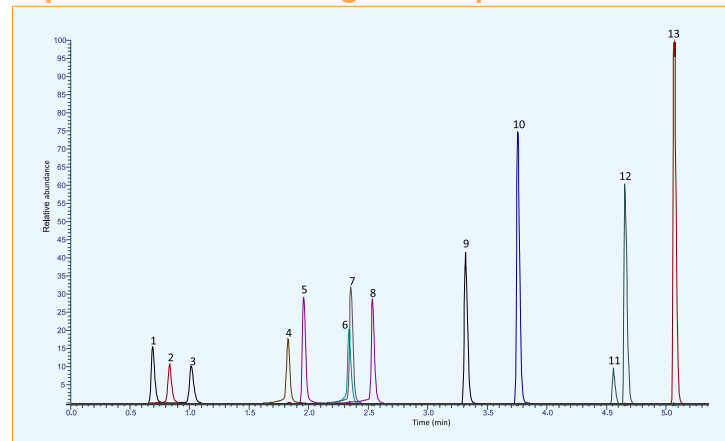
Column: HALO 90 Å Biphenyl, 2.7 μ m, 4.6 x 50 mm
 Mobile Phase A: Water
 Mobile Phase B: Acetonitrile
 Gradient: 20-60% B in 6 min
 Flow Rate: 1.85 mL/min
 Initial Pressure: 344 bar
 Temperature: 30 °C
 Detection: UV 215 nm, PDA
 Injection Volume: 4 μ L

PEAK IDENTITIES (IN ORDER): Morphine (m/z 286), Oxymorphone (m/z 302), Hydromorphone (m/z 286), Naloxone (m/z 328), Codeine (m/z 300), Naltrexone (m/z 342), Oxycodone (m/z 316), Hydrocodone (m/z 300), cis-Tramadol (m/z 264), Meperidine (m/z 248), Fentanyl (m/z 337), Buprenorphine (m/z 468), (+/-) - Methadone (m/z 310)

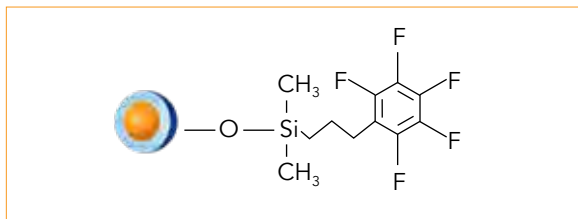
TEST CONDITIONS:

Column: HALO 90 Å Biphenyl, 2 μ m, 2.1 x 100mm
 Mobile Phase A: Water/0.1% Formic acid
 Mobile Phase B: Acetonitrile/0.1% Formic acid
 Gradient: 10-60% B in 5.5 min
 Flow Rate: 0.4 mL/min
 Initial Pressure: 325 bar
 Temperature: 40 °C
 Injection Volume: 1 μ L
 Sample Solvent: 95/5 Water/Acetonitrile
 Detection: +ESI MS

Separation Of Pain Management Opiates



HALO® PFP



Ligand: pentafluorophenylpropylsilane

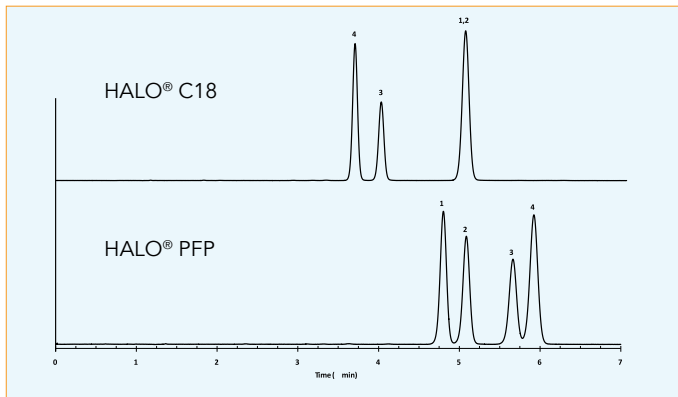
USP Designation: L43

Chemistry Classification: Aromatic / Polar

BENEFITS AND BEST APPLICATIONS:

- Ideal for aromatics and electron-rich compounds
- Alternate selectivity to alkyl phases
- Useful in reversed phase and HILIC modes

Structurally Similar Steroid Separation



PEAK IDENTITIES (IN ORDER): Hydrocortisone, Prednisolone, Cortisone, Prednisone

TEST CONDITIONS:

Columns: HALO 90 Å C18, 2.7 μ m, 4.6 x 100 mm

HALO 90 Å PFP, 2.7 μ m, 4.6 x 100 mm

Mobile Phase: A/B–50/50

A = Water

B = Methanol

Flow Rate: 1.0 mL/min

Pressure: 230 Bar

Temperature: 35 °C

Detection: UV 240 nm

Injection Volume: 0.5 μ L

PEAK IDENTITIES (IN ORDER): δ -Tocotrienol, β -Tocotrienol, γ -Tocotrienol, α -Tocotrienol, δ -Tocopherol, β -Tocopherol, γ -Tocopherol, α -Tocopherol, α -Tocopherol acetate, α -Tocopherol nicotinate, i = impurity

TEST CONDITIONS:

Column: HALO 90 Å PFP, 2.7 μ m, 4.6 x 150 mm

A= Water

B= Methanol

Gradient: 0-95% B in 5.0 min

Flow Rate: 1.5 mL/min

Pressure: 380 bar

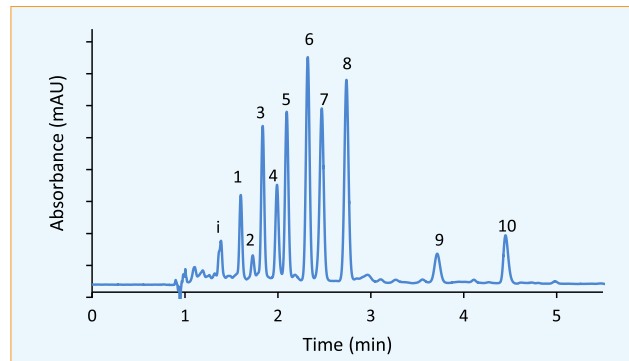
Temperature: 25 °C

Injection Volume: 5 μ L

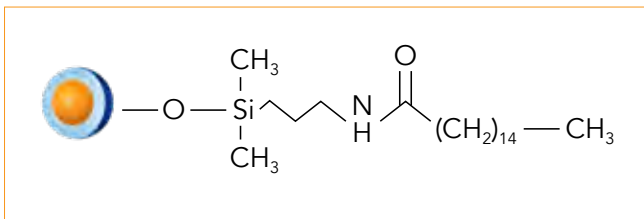
Sample Solvent: Ethanol

Detection: UV 290 nm, PDA

High Resolution Separation Of Vitamin E Congeners



HALO® RP-AMIDE



Ligand: C16 amide

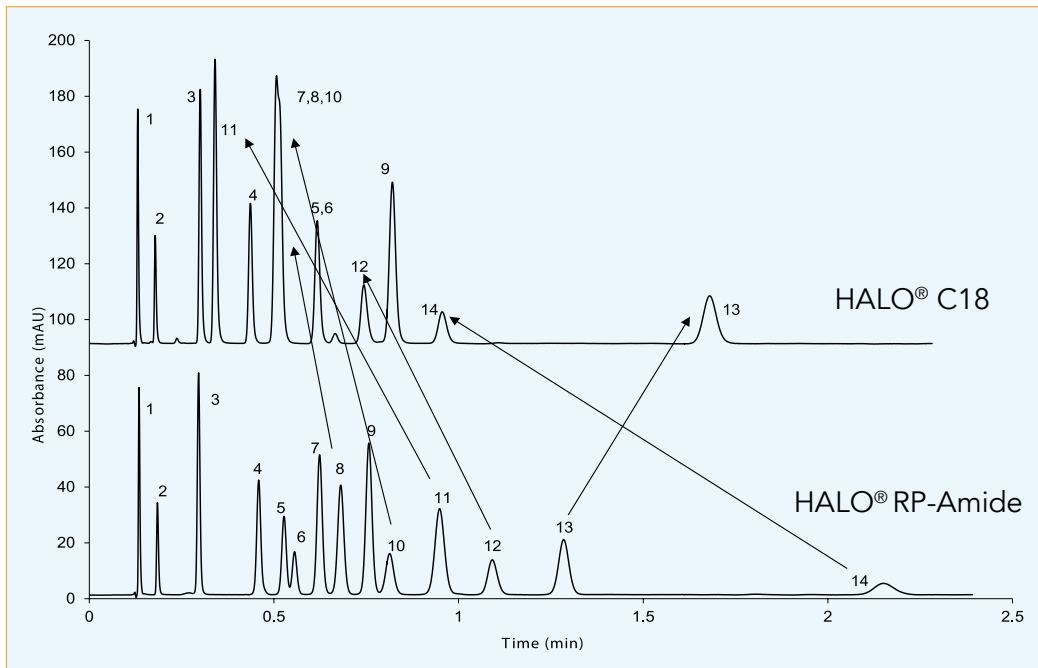
USP Designation: L60

Chemistry Classification: Alkyl / Polar

BENEFITS AND BEST APPLICATIONS:

- Ideal for basic compounds (alcohols, acids, phenols, catechins)
- Alternate selectivity to alkyl phases
- 100% aqueous compatible

Improved Resolution Of Polar Compounds



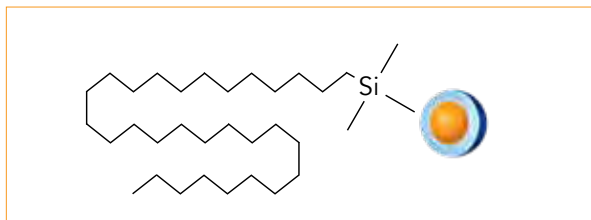
PEAK IDENTITIES (IN ORDER): Uracil, Benzamide, Aniline, Cinnamyl Alcohol, Dimethylphthalate, Phenylacetonitrile, 2-Nitroaniline, 4-Bromoacetanilide, Benzylbenzoate, 2,2'-Biphenol, 4,4'-Biphenol, 3-Ethylphenol, N,N-dimethylaniline, Bisphenol A

TEST CONDITIONS:

Columns: HALO 90 Å C18, 2.7 μ m, 4.6 x 50 mm
 HALO 90 Å RP-Amide, 2.7 μ m, 4.6 x 50 mm
 Mobile Phase: 35/65 ACN/20 mM Phosphate Buffer, pH 7.0
 Flow Rate: 3.0 mL/min
 Pressure: 310 bar
 Temperature: 26 °C



HALO® C30



Ligand: triacontyldimethylsilane

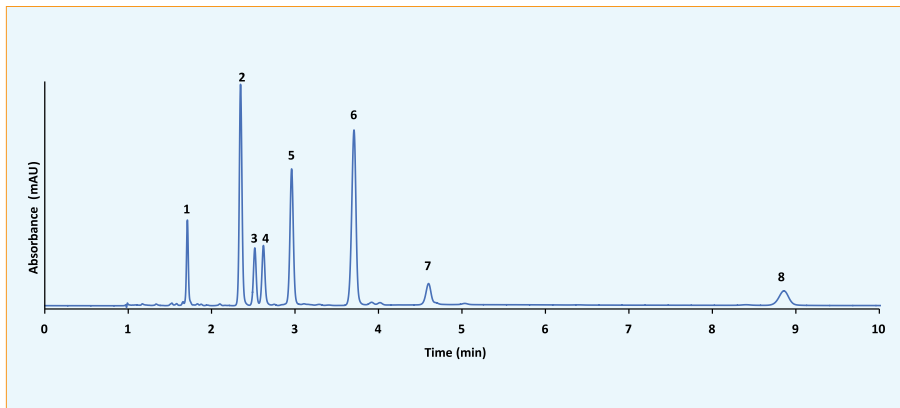
USP Designation: L62

Chemistry Classification: Alkyl

BENEFITS AND BEST APPLICATIONS:

- Ideal for hydrophobic, long chain, structurally related isomers
- Alternate alkyl phase with high shape selectivity
- 100% aqueous compatible

High Resolution Of Fat Soluble Vitamins



PEAK IDENTITIES (IN ORDER): Retinyl acetate (A), Delta tocopherol (E), Ergocalciferol (D2), Cholecalciferol (D3), Alpha tocopherol (E), DL-alpha-tocopherol acetate (E), 2,3-trans-phyllloquinone (K), Retinyl palmitate (A)

TEST CONDITIONS:

Column: HALO 160 Å C30, 2.7 µm, 4.6 x 150 mm
 Isocratic: 100% Methanol
 Flow Rate: 1.5 mL/min
 Pressure: 262 bar
 Temperature: 30 °C
 Detection: UV 280 nm, PDA
 Injection Volume: 2.0 µL
 Sample Solvent: Methanol

TEST CONDITIONS:

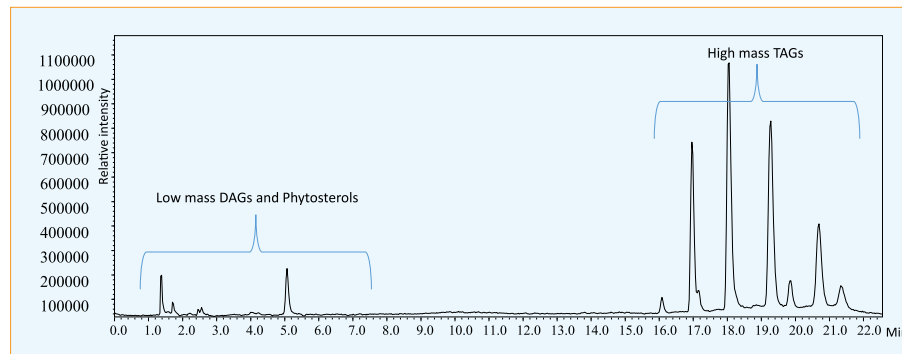
MS TEST CONDITIONS:

MS system: Shimadzu LCMS-2020
 Ionization: +ESI
 Spray voltage: 4.50 kV
 Drying line temp: 300 °C
 Heat Block: 450 °C

LC TEST CONDITIONS:

Column: HALO 160 Å C30, 2.7 µm, 2.1 x 150 mm
 Mobile Phase A: Methanol
 Mobile Phase B: IPA/0.1% Formic acid
 Gradient: 0-40% B in 22 min
 Flow Rate: 0.3 mL/min
 Initial Pressure: 325 bar
 Temperature: Ambient
 Injection Volume: 2 µL
 Sample Solvent: MeOH
 LC System: Shimadzu Nexera X2

Separation Of Triacylglycerides (TAGs) In Corn Oil

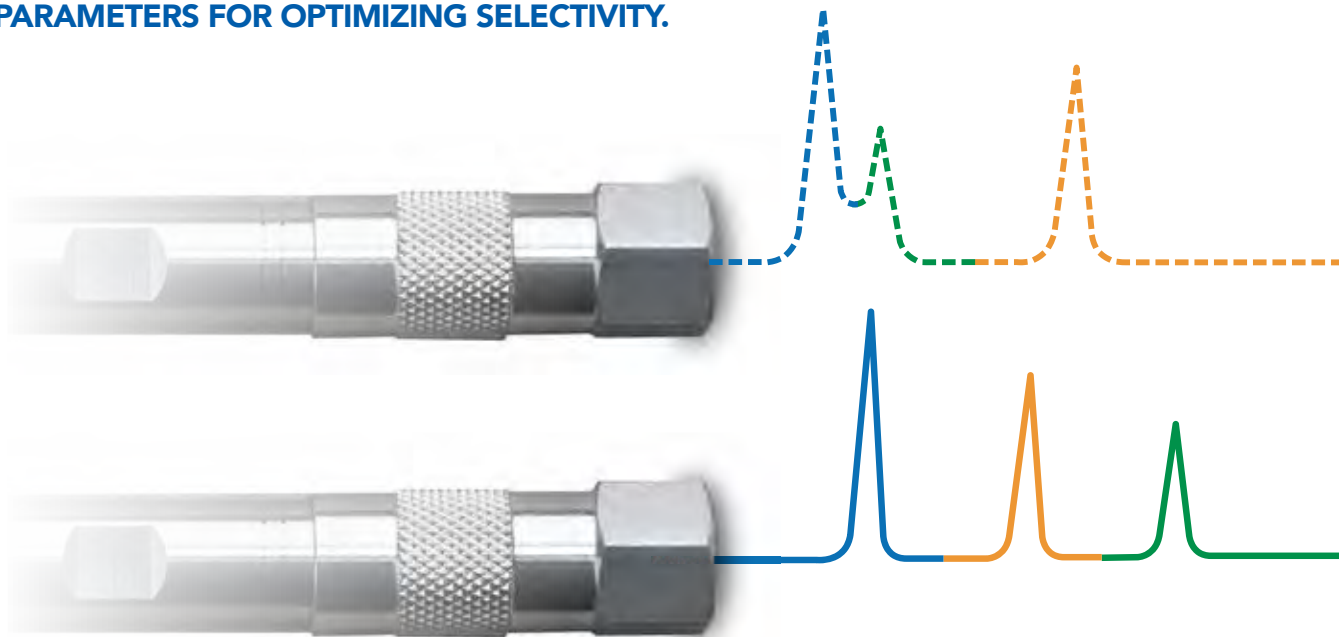


Selectivity

WHAT IS SELECTIVITY?

HOW DOES SELECTIVITY INFLUENCE RESOLUTION?

PARAMETERS FOR OPTIMIZING SELECTIVITY.



WHAT IS SELECTIVITY?

Selectivity (α) (also known as peak spacing or separation factor) is the space between the peaks in an HPLC separation. Mathematically, it is defined as the ratio of two retention factors:

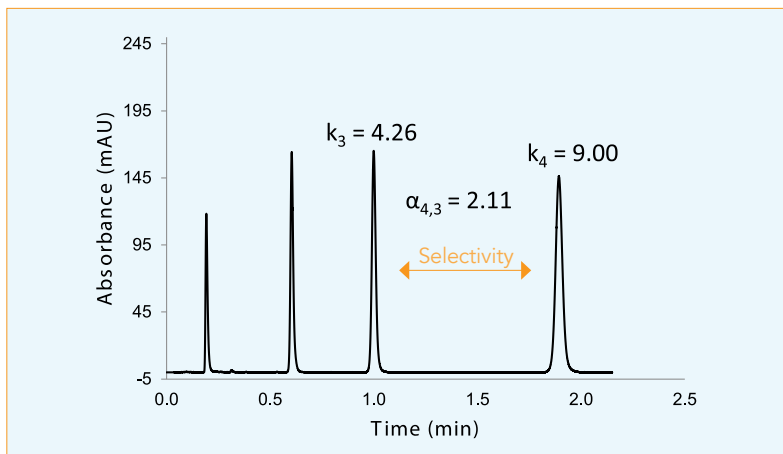
$$\alpha = k_2 / k_1$$

An example chromatogram indicating the retention factors of two adjacent peaks and the selectivity between the two peaks is shown below.

PEAK IDENTITIES (IN ORDER): uracil, pyrene, decanophenone, dodecanophenone

TEST CONDITIONS:

Column: HALO 90 Å C18, 2 μ m 2.1 x 50 mm
Mobile Phase: 85/15 ACN/Water
Flow rate: 0.5 mL/min
Temperature: Ambient (~25 °C)
Detector: UV 254 nm
Sample Volume: 0.2 μ L



HOW DOES SELECTIVITY INFLUENCE RESOLUTION?

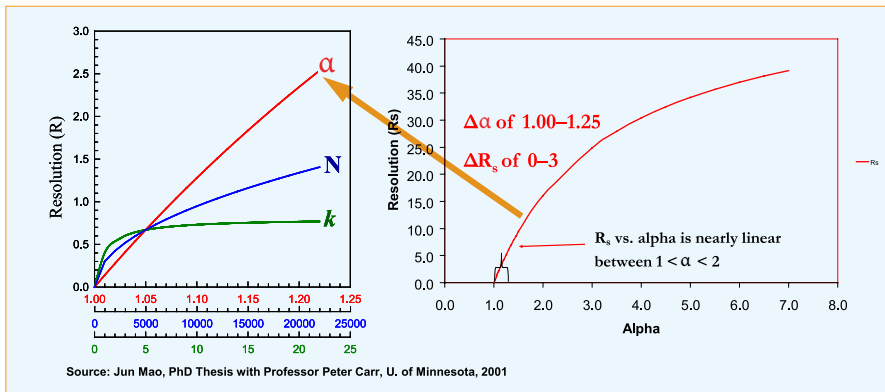
The master resolution equation is comprised of an efficiency term, a selectivity term, and a retention term.

$$R_s = \left(\frac{\sqrt{N}}{4} \right) \times \left[\frac{(\alpha - 1)}{\alpha} \right] \times \left[\frac{k_2}{(1 + k_2)} \right]$$

Efficiency
Selectivity
Retention

N = plates
 α = selectivity
 k = retention factor

Of these three, selectivity is the most effective parameter to change to increase resolution.

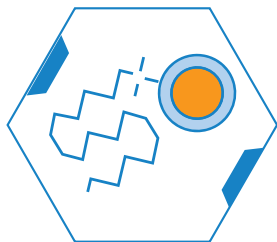


PARAMETERS FOR OPTIMIZING SELECTIVITY

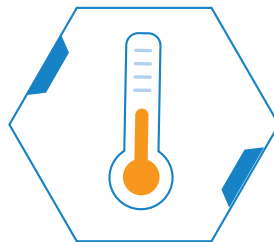
There are several parameters that can be modified in order to optimize selectivity; this guide focuses on mobile phase, bonded phase, temperature and pH. Other parameters, which will not be discussed, include ion pair concentration, % B (organic) solvent/gradient and buffer concentration.



MOBILE PHASE



BONDED PHASE



TEMPERATURE



pH

The list above was adapted from "Introduction to Modern Liquid Chromatography", 3rd Edition, L. R. Snyder, J. J. Kirkland, J. W. Dolan; p. 29, 2010, John Wiley & Sons, Inc.

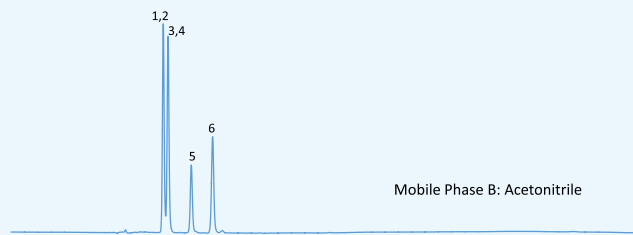




1. MOBILE PHASE

The organic component of the mobile phase has a significant impact on the selectivity of an HPLC separation. A separation that shows coelutions with 100% acetonitrile may benefit from a switch to methanol or a combination of acetonitrile and methanol as shown below:

ACETONITRILE



PEAK IDENTITIES (IN ORDER):

Prednisone, Cortisone, Prednisolone, Hydrocortisone, Dexamethasone, Corticosterone

TEST CONDITIONS:

Column: HALO 160 Å C30 2.7 μ m, 4.6 x 150 mm

Mobile Phase A: Water

B: see chromatogram

Isocratic: 50% B

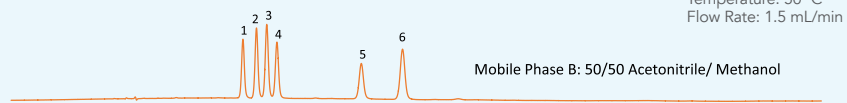
Detection: 220 nm

Injection: 0.5 μ l

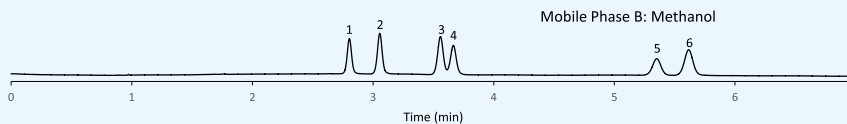
Temperature: 50 °C

Flow Rate: 1.5 mL/min

**ACETONITRILE/
METHANOL**



METHANOL



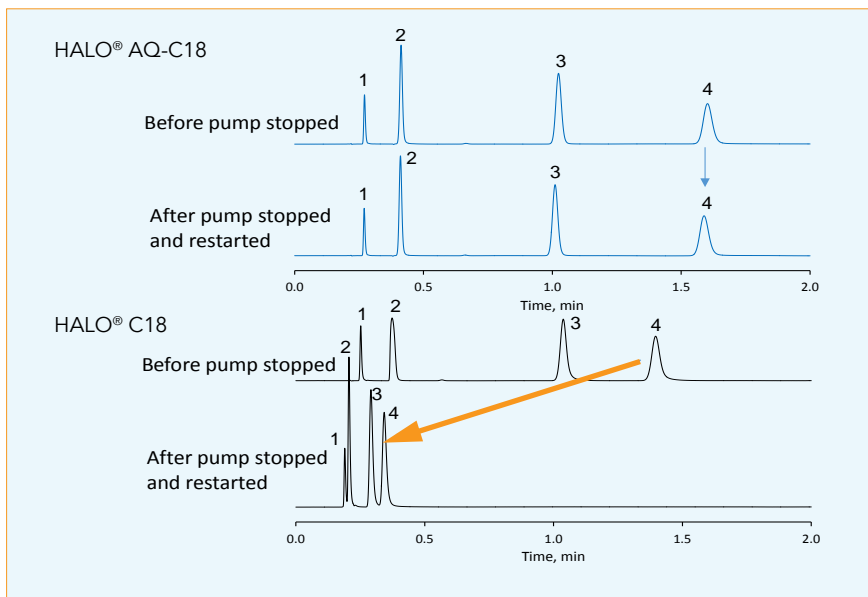
The top chromatogram (1) uses 100% acetonitrile while the bottom chromatogram (3) uses 100% methanol. Neither of these separations provides adequate resolution for all six components. However, a 50/50 mixture of acetonitrile and methanol (2) enables baseline resolution for all of the analytes in the sample along with a compromise in terms of the total time of the separation.

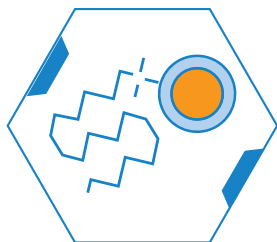
PHASE DEWETTING

Another item that must be considered when using mobile phase to optimize selectivity is phase dewetting. Dewetting occurs when the stationary phase is highly hydrophobic and the mobile phase is changed from one with a high amount of organic solvent component (> 40% ACN or MeOH) to one that is entirely aqueous or mostly aqueous. When the column is under pressure, the aqueous mobile phase is forced into the porous structure where most of the retention occurs. When the solvent delivery pump is stopped, the aqueous mobile phase is no longer forced into the stationary phase pores and is expelled from the interior of the particles. Restarting the solvent delivery pump will not force the aqueous mobile phase back into the pores since the phase is hydrophobic. The retention of the sample components drastically decreases and resolution is lost.



The figure below is a demonstration of what happens to a separation when dewetting occurs with the highly hydrophobic C18. In contrast, the HALO® AQ-C18 phase has an added amount of polar characteristic that prevents it from dewetting. Even when the pump is stopped and restarted, the retention and resolution are both maintained with the HALO® AQ-C18 column. All of the HALO® phases except HALO® C18 and HALO® C8 may be used under 100% aqueous conditions without dewetting.





2. BONDED PHASE

Screening different bonded phases while keeping the mobile phase constant is another way to optimize selectivity. A column switching valve can be added to most HPLC instrumentation for automated screening.

ORTHOGONALITY

A separation of paracetamol (acetaminophen) and its 14 impurities, following EP 9.4, was compared using three orthogonal phases (HALO® C18, HALO® RP-Amide, and HALO® Phenyl-Hexyl) to demonstrate selectivity differences of bonding phases. Interesting elution order changes were observed between HALO® C18 and HALO® RP-Amide. Several impurity components (in particular F and M) were retained longer on the HALO® RP-Amide column compared to HALO® C18 since RP-Amide has more retention for phenol-containing compounds.

MOST SIMILAR (TO C18)

C18

C8

AQ-C18

C30

PFP

Phenyl-Hexyl

ES-CN

Biphenyl

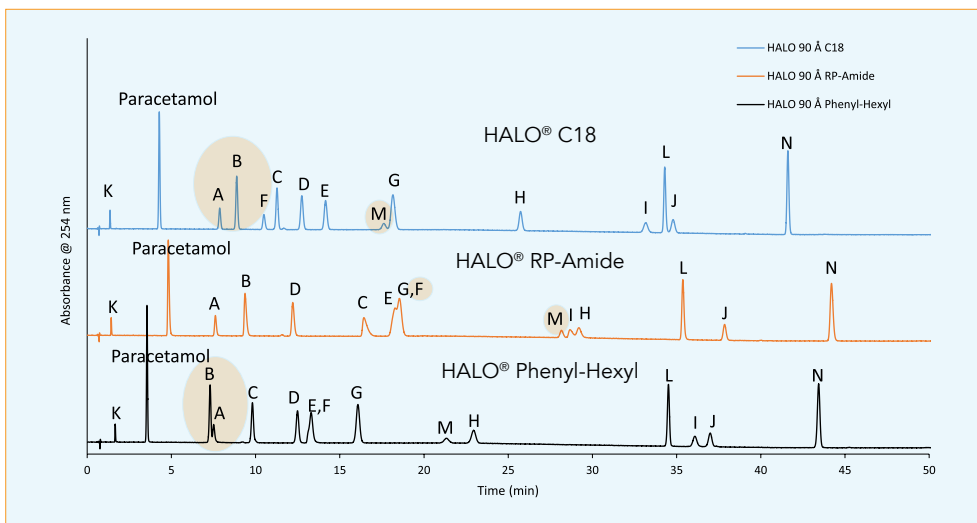
RP-Amide

@ pH 7

MOST ORTHOGONAL (TO C18)



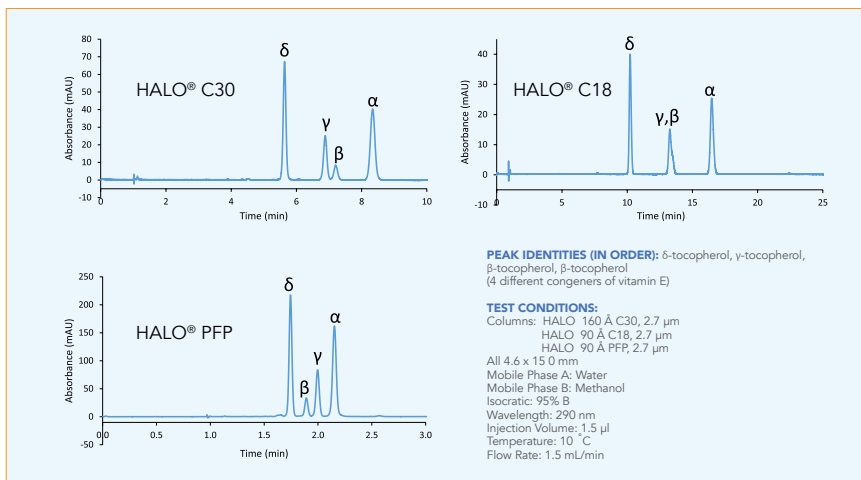
Additionally, there was an elution order switch between impurity A and B on HALO® C18 compared to HALO® Phenyl-Hexyl. These compounds differ by only a methyl group and the position of the hydroxyl group on the phenyl ring so it is reasonable that C18 would retain impurity B more than Phenyl-Hexyl. There were two complete coelutions on the HALO® RP-Amide and HALO® Phenyl-Hexyl phases, which seem to have occurred due to these compounds being retained longer on these two phases. However, this should not be considered a discouraging result since other mixes may benefit from the selectivity of these phases. This is why having multiple bonded phases available for screening is highly recommended.



*Test Conditions are the same as on P. 11

SHAPE SELECTIVITY

Another example of the benefit in screening different bonded phases is shown below, where HALO[®] C18, HALO[®] C30, and HALO[®] PFP were all used to screen the same sample of four tocopherols. While the retention was the highest with HALO[®] C18, there was no resolution between the beta and gamma-tocopherols. For this particular sample, shape selectivity had more impact than hydrophobicity. The highest resolution and least retention was observed with the HALO[®] PFP phase followed by the HALO[®] C30 phase. An elution order reversal was also observed between the HALO[®] PFP and the HALO[®] C30 phases.



These examples highlight the importance of understanding your sample components and properties and the necessity for several column phases when undertaking method development.

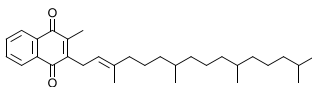




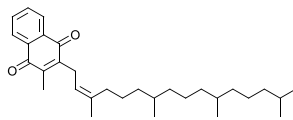
3. TEMPERATURE

As sample matrix and component properties are impacted with temperature, it is another way to optimize selectivity. In the case of macromolecular separations (both biological and industrial polymers), often more desirable results are achieved at higher temperatures (60 °C and higher). This is due to the fact that zone broadening is reduced with higher temperature. In contrast, with isomer separations of vitamins, lower temperatures are of benefit.

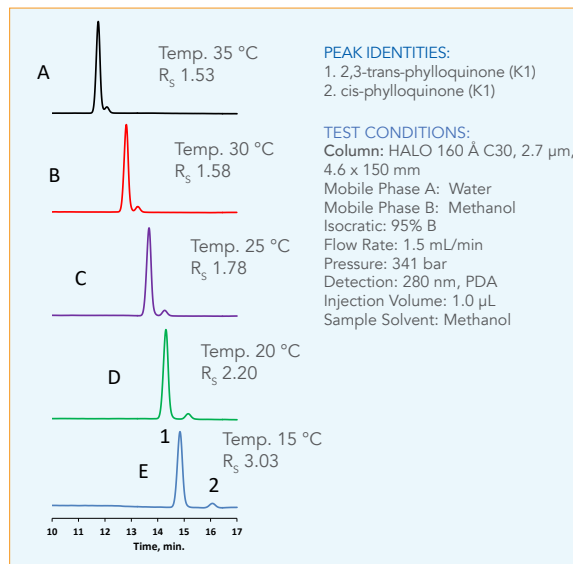
In particular with HALO® C30, running sub-ambient conditions has been shown to increase the resolution by increasing retention. The lower the temperature, the better the resolution as is shown in this vitamin separation. The trans and cis isomers of vitamin K1 are resolved the best at 15 °C on the HALO® C30 column compared to the resolution of the separation at 35 °C.



Vitamin K1: 2,3-trans-phyloquinone



Vitamin K1: cis-phyloquinone





4. pH

Weak acids (**HA**) and bases (**B**) are partially dissociated in water. They exist in an equilibrium between their neutral and charged/ionized states as shown in the following equations:



More retained

Less retained

Acid Ionized
 $pH > pK_a$

Acid Neutral
 $pH < pK_a$

Base Ionized
 $pH < pK_b$

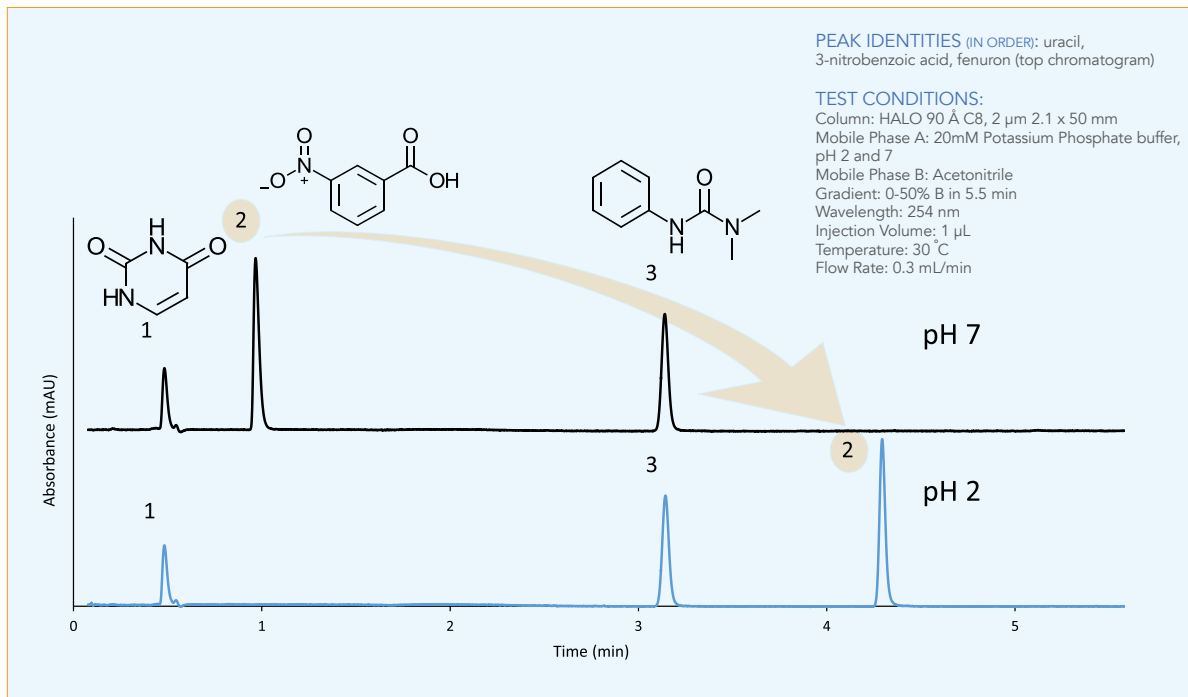
Base Neutral
 $pH > pK_b$

With an increase in pH, retention for an acid decreases while retention for a base increases.

Depending upon the nature of the analytes, changing the pH of the mobile phase can have a large impact on the selectivity of the separation. The pK_a of 3-nitrobenzoic acid is 3.46. At pH 7 (top chromatogram), 3-nitrobenzoic acid is in its anionic/charged form which explains its lower retention compared to when the compound is neutral at pH 2 (bottom chromatogram). The retention of fenuron is unaffected whether the pH is 2 or 7. One must be careful to use a pH that is 2 units away from the pK_a of the analytes in the separation. Or else, the compound will exist in two different forms which could show itself as split peaks.



pH vs Resolution



Conclusion

As has been described, there are several approaches that can be used to optimize selectivity. These approaches can be applied individually or in combination. Screening multiple HALO® phases, followed by organic mobile phase optimization is a good option for method development. Another approach is to hold the bonded phase constant and vary gradient time and temperature followed by use of a commercially available method optimization software package.

While HALO® C18 is a good all-purpose phase for beginning method development, the other HALO® phases offer alternate selectivities. It is good practice to screen multiple phases to ensure the best possible separations.

Additional applications and technical information can be found at [fused-core.com](https://www.fused-core.com)

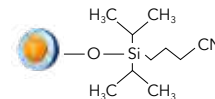
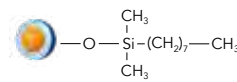
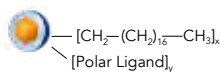
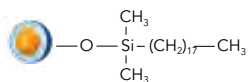
For conversion tips to superficially porous particles from fully porous particles methods, please consult The Method Conversion Guidebook located on the website or by contacting your local HALO® distributor.



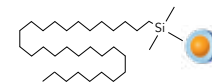
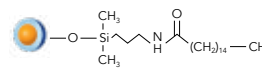
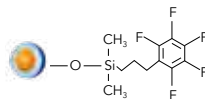
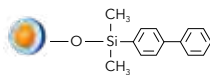
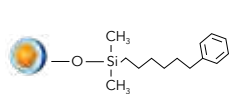
HALO®

PRODUCT SPECIFICATIONS SECTION

Product Specifications



Bonded Phase	C18	AQ-C18	C8	ES-CN
USP Designation	L1	L1	L7	L10
Particle Size(s) (μm)	2 2.7 5	2 2.7 5	2 2.7 5	2 2.7 5
Pore Size (\AA)	90	90	90	90
Carbon Load (%)	7.2 7.7 5.4	6.5 6.7 5.6	4.8 5.4 3.7	3.4 3.5 2.5
Surface Area (m^2/g)	120 135 90	120 135 90	120 135 90	120 135 90
Low pH/T Limit	2/60 $^{\circ}\text{C}$	2/60 $^{\circ}\text{C}$	2/60 $^{\circ}\text{C}$	1/80 $^{\circ}\text{C}$
High pH/T Limit	9/40 $^{\circ}\text{C}$	9/40 $^{\circ}\text{C}$	9/40 $^{\circ}\text{C}$	8/40 $^{\circ}\text{C}$
Endcapped	Yes	Yes	Yes	Yes



Phenyl-Hexyl	Biphenyl	PFP	RP-Amide	C30
L11	L11	L43	L60	L62
2 2.7 5	2 2.7 5	2 2.7 5	2 2.7 5	2.7
90	90	90	90	160
6.3 7.1 5.2	6.7 7.0 5.5	5.3 5.5 3.9	7.3 8.2 5.1	4.5
120 135 90	120 135 90	120 135 90	120 135 90	90
2/60 °C	2/60 °C	2/60 °C	2/60 °C	2/60 °C
9/40 °C	9/40 °C	8/40 °C	9/40 °C	9/40 °C
Yes	Yes	Yes	Yes	Yes

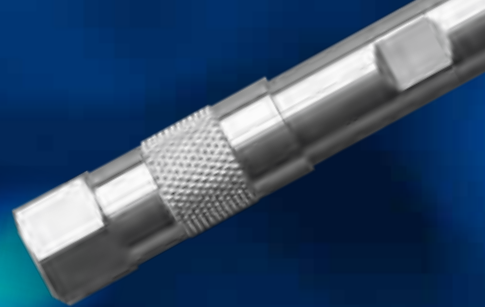




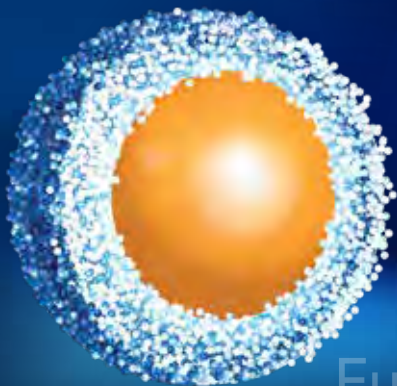
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Highest efficiency RP-Amide ES-CN
C8 C18 Biphenyl Penta-HILIC PFP
2 μ m Phenyl-Hexyl HILIC 5 μ m AQ-C18
Fused-Core[®] 2.7 μ m Small molecule

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