CYCLOBOND Series HPLC Column Operating Instructions

Important: CYCLOBOND columns should never be left idle in aqueous buffer systems even for short periods. Wash the buffer out of the column with 50/50:HPLC Grade Water/Acetonitrile (20-25 mL for 25 cm columns) and then either anhydrous isopropanol or acetonitrile (20-25 mL). Isopropanol or acetonitrile are the only recommended solvents for storage. Storage in methanol is not recommended even for short periods.

Introduction

CYCLOBOND I 2000 columns are packed with unique chromatographic stationary phases produced by chemically bonding cyclodextrins to a high purity 5 µm silica gel. The cyclodextrins are linked to the silica through a spacer arm of optimum chain length by a patented process which yields a stable, non-hydrolytic, non-nitrogen containing ether bond. At this time, there are two native cyclodextrin bonded phases available in the series, CYCLOBOND I 2000 (β-cyclodextrin) and seven derivatives and CYCLOBOND II (γ-cyclodextrin) and one derivative.

CYCLOBOND I 2000 (β-cyclodextrin) is the most versatile of the native products. The macrocyclic molecule contains 7 glucopyranose units arranged in the shape of a hollow truncated cone in which the interior cavity, 7.5 Å in diameter, is relatively hydrophobic being comprised of essentially the methylene and glucoside linkages producing a high electron density. The exterior faces are hydrophilic. The larger opening is surrounded by the secondary hydroxyls to form a right handed screw. The primary hydroxyls constitute the smaller end of the cone.

From the CYCLOBOND I 2000, seven derivatives have been prepared by reacting the hydroxyls at the mouth of the cavity selectively with various reagents. These new derivatives are designed to optimize interactions with the center of chirality of various enantiomers as well as accommodate various steric interactions for a variety of analytes. For a comprehensive review of these interactions see the **CYCLOBOND Handbook**, 6th Edition.

CYCLOBOND II is bonded with the gamma form of cyclodextrin consisting of 8 glucopyranose units arranged in the same truncated cone shape as the beta form producing an internal diameter of 9.5 Å.

Derivatized Cyclodextrins

CYCLOBOND I 2000 DNP is the first π acidic dinitrophenyl group ever to be successfully anchored to a bonded cyclodextrin demonstrating a high degree of stability. In the majority of cases, this new derivative has enhanced selectivity for a wide variety of analytes over the conventional CYCLOBOND I 2000. The dominant selectivity has been in the reversed phase mode but a variety of separations have been successfully run in both normal phase and the typical polar organic mode originally designed for cyclodextrin phases. For a successful application in normal and polar organic phases, the analyte must have a π basic structure.

CYCLOBOND I 2000 DM is prepared by the methylation of the 2 and 3 position hydroxyl groups of the bonded β-cyclodextrin. This chemistry effectively eliminates the chiral hydrogen bonding normally associated with the bonded native β-cyclodextrin and instead, establishes a weak dipole effect in normal phase systems. This phase can now more effectively separate structural and positional isomers in a reversed phase system and, for bulky molecules, create steric effects. In the normal phase mode, weak dipoles can be effected that result in chiral recognition.

CYCLOBOND I 2000 SP, RSP, and HP-RSP results from a reaction with either pure 'S' or racemic propylene oxide. The result is additional flexible hydrogen bonding groups extending the interactive potential for sterically hindered or extended chiral centers from the aromatic or included portion on the analyte.

The RSP has demonstrated unique selectivity for certain chiral amines and neutral molecules unattainable currently on any other chiral stationary phase. The 'S' form has increased stereospecific hydrogen bonding capabilities over the racemic.

CYCLOBOND I 2000 AC is the peracetylated form of the bonded beta. This reaction has the effect of enlarging the cavity dimensions as well as providing specific interactions with the included analyte. The most common selective effect has been seen with cis/trans structures and with amine and hydroxyl functions on an alpha or beta carbon. In the normal phase mode this stationary phase might be considered analogous to acetylated cellulose.

CYCLOBOND I 2000 DMP is prepared by the reaction with 3,5-dimethylphenylisocyanate. In addition to π-π interactions, there are also present the hydrogen donor and acceptor sites yielding results very similar to typical cellulose derivatives only in this case the groups are permanently bonded to the stable CYCLOBOND I 2000 structure.

CYCLOBOND II AC is the peracetylated form of the bonded gamma. Specific chiral interactions are produced with the hydroxyl groups and amines extending out of the included portions of the analyte. Naphthylamines and sterols are common applications.

Column Installation

Astec columns are packed at very high pressures leading to excellent bed stability. Flow directions can be reversed, therefore, to increase column use. However, it is important to note that when a column is reversed, contaminants on the frit and the head of the column exists. It is important that before washing of the column it should be disconnected from the detector. The direction of flow on the column is presented as a reference point only.

Column Conditioning

CYCLOBOND columns are shipped in IPA for long term stability. Before use the column should be washed with 20 mL of HPLC grade water at 0.8 mL/min. IPA and water have a high viscosity so it is important to lower the flow rate. If the column is to be used in a normal or polar/organic phase solvent system then the column is next washed with 20 mL of ethanol. Ethanol is compatible with all common HPLC solvents. If the column is to be used in the reversed phase mode then subsequent to water washing, condition the column with organic/buffer composition that is appropriate. For long life it is important to never leave a column without flow in buffer. The column should be washed with 50:50 water:acetonitrile for several column volumes to remove salts and returned to IPA for storage. Acetonitrile can be used for short term (overnight) storage.

Mobile Phase Design
There are three distinct ways to develop separations on cyclodextrin based phases. They are

1. Polar Organic Phase Mode
   Acetonitrile/Methanol/Triethylamine/Acetic Acid
   Typical composition: 95/5/0.1/0.1
2. Normal Phase Mode
   Hexane/IPA
   Typical composition: 90/10
3. Reversed Phase Mode
   Acetonitrile/Buffer
   Typical composition: 20/80 (pH 3.0-7.5)

Choice of conditions vary with solubilty and analyte structure. It is necessary to read the CYCLOBOND HANDBOOK to develop expertise in these areas. To obtain a copy of this handbook, complete and return the business reply card included with your column.

Cyclodextrin phases are stable in all known solvents, however, halogenated solvents form strong inclusion complexes and should be avoided both as a mobile phase solvent and solubilizing solvent for analytes.

Buffers
The efficiency of CYCLOBOND separations has been substantially increased in certain instances with the use of buffers. To obtain reproducible separations in the reversed phase mode, some buffer should always be present even with neutral compounds.

Triethylamine acetate (TEAA) buffer has been used successfully and can be prepared by neutralizing a 0.1% solution of triethylamine with glacial acetic acid. For the derivatives of amino acids and peptides pH 4.0 TEAA substituted for water in 60/40:MeOH/H_2O system has produced a four-fold increase in efficiency.

Other buffers can and have been used such as TFA, ammonium acetate and ammonium nitrate. It has been demonstrated that the type and concentration of buffers used can affect selectivity. Phosphate buffers are not recommended. If they must be used, pass through reversed phase columns, as certain impurities have been found to strongly include in the CYCLOBOND cavity. Ammonium phosphate is less aggressive than either the K or Na salt on the siloxane bond chemistry and is recommended.

Never Store Columns Even For Short Periods Of Time In Any Buffer. Wash The Column With Water, Then With Either Methanol Or Acetonitrile.

pH
This is the most important parameter in chiral separations in the reversed phase mode. All CYCLOBOND derivatives have been run in the reversed and the normal phase mode confining the pH of the buffers between 4.0 and 6.5 for the CYCLOBOND I 2000 Acetylate and 3.0-7.5 for all others. The stability of cyclodextrin inclusion complexes is dependent on the charge of the guest molecule. In general, the binding strength of the formally charged species is usually smaller than that of the corresponding neutral species (presumably due to diminished hydrophobic interactions between the charged guest molecule and the nonpolar cyclodextrin cavity). Therefore, the retention as well as the selectivity of the separations of molecules that possess ionizable acidic or basic functional groups can be affected by altering the pH. A strategy may be to take advantage of a difference in pKa, that is, causing one compound to be neutral and, therefore, strongly included while keeping other components ionized and obviously poorly retained. Substituted phenols are an exception to this condition. Phenolate anions bind better than the corresponding neutral phenols. Because of the complexities of these interactions, it is necessary to observe the retention and resolution as a function of pH, usually testing at pH 4 and pH 7 or 0.5 pH units above and below the pK value.

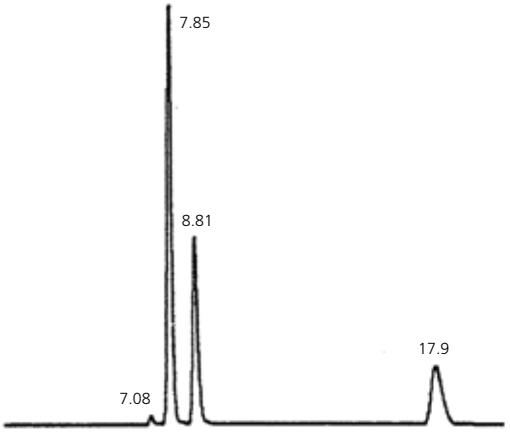
Temperature Effects
Changes in temperature have a greater affect on the retention of solutes on CYCLOBOND I 2000 columns than on comparable reversed phase columns. This is because the binding constant of a solute to the cyclodextrin involves several interactive mechanisms that dramatically change with temperature. Lower temperatures enhance the weaker bonding forces. The net result is that the chromatographer has an additional powerful means to control selectivity and retention. Column reproducibility can be established by maintaining a constant temperature condition within 1 °C. Maximum temperature is 50 °C.

Sample Solvents
Expect best results when mobile phase is used as the sample solvent. When the sample solvent has stronger eluotropic properties than the mobile phase, components of the sample may deposit on the head of the column causing poor peak shape and reduced column lifetime. Even if sample adsorption does not occur, incompatibilities between the sample solvent and mobile phase may cause peak distortion, especially in early-eluting compounds.

Injection Volumes And Concentrations
Two distinct examples of column capacity have been observed for CYCLOBOND I 2000 phases whether run in reversed phase, normal phase or polar organic phase mode. The first example relates to a predominant inclusion effect. In this case, the separation is dependent upon sample volume and concentration. Typical examples of 1-5 µL of 1 mg/mL concentration are required for good resolution. In other cases, the load volume and concentration may be 5 to 10 times higher without affecting resolution. Therefore, begin the separation study at the lowest volumes and concentrations until a proper determination can be made of its effect.

Stability
The stability of CYCLOBOND to pH is in the range of 3.0 to 7.5. Strong acids attack the cyclodextrin structure while strong alkaline conditions attack the silica base. Pure water can cause column deterioration over long periods of time. Use of 90% aqueous mobile phases showed no change over many injections. A precolumn (before injector) of silica should be used when operating with buffers greater than pH 6.5. Successful runs of pH 3.0 buffer have been used for short periods. Immediately wash the column with water followed by acetonitrile after the pH 3.0 buffer run. To establish column reproducibility it is recommended that the separation of ortho, meta, para nitroaniline be run in 40/60: MeOH/H_2O. The selectivity between the ortho and para establishes the integrity of the bonded cyclodextrin. See following figure.

CYCLOBOND I 2000
Selectivity for m-, o-, p-Nitroaniline
40/60:MeOH/H_2O; 1.0 mL/min.



Flow Rate
Initial flow rates may be set at 1.0 mL/min. unless the effects of mass transfer are clearly visible. Generally, lower flow rates (0.25 to 1.0 mL) give higher efficiency and greater resolution for enantiomers. Non-chiral separations based on inclusion complexing behave similarly to typical reversed phase columns. See **CYCLOBOND HANDBOOK** on flow rate effects.

Astec Columns May Be Operated From Either Direction Without Loss Of Performance Due To The Uniform Packing System That Produces Uniform Packing Density. This Is True For All Astec Columns.

Pressure
Operating pressure for CYCLOBOND columns is generally in the range of 800 to 1000 psi (100 mm) and 2000 to 2500 psi (250 mm) at 1.0 mL/minute for 40/60:MeOH/H_2O. As with standard reversed phase columns the higher the water content, the higher the back pressure with a maximum at 50/50. Care should always be exercised in prefiltering and degassing the water and solvent used with these columns.

Regeneration
Columns showing decreased resolution can sometimes be regenerated by passing several column volumes of 50:50 acetonitrile:water. Acetonitrile may be used for final displacement and storage. Long term storage (> 24 hours) is best done in isopropanol.

Storage
Subsequent to the quality control test the column is conditioned with isopropanol for storage and shipment. When analysis is complete the column should be returned to this solvent to ensure long life.

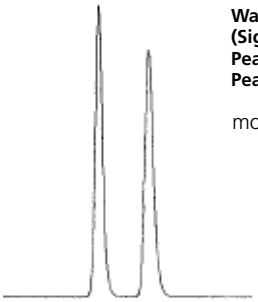
Column Assessment Parameters
Column Assessment Parameters provide useful data as to the repeatability of column performance and can be used to evaluate your column for signs of deterioration. Each column is individually tested and assigned a serial number for traceability of all column components.

Since virtually every molecule, organic or inorganic, can be "included" no useful marker has been found to measure void volume. The retention volume of the solutes is used as a relative measure of consistent packing.

Note: CYCLOBOND columns should be tested before use. The analyte, its source and the operating conditions can be found on the Certificate of Analysis that is included with each column. This will ensure repeatable performance and no false negative results.

Performance Testing of CYCLOBOND Columns

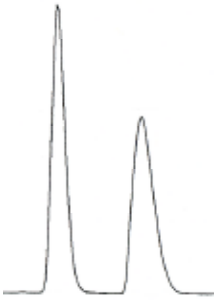
CYCLOBOND I 2000



Warfarin
(Sigma #A2250)
Peak 1 – 6.89 min.
Peak 2 – 7.90 min.

mobile phase: 100/0.3/0.2 ACN/ HOAc/TEA
flow rate: 1 mL/min.
det.: 254 nm
injection: 2 µL
sample: 5 mg/mL

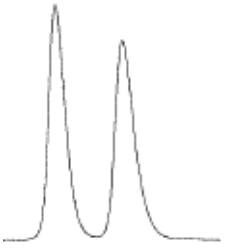
CYCLOBOND I 2000 DNP



Hydrobenzoin
(Aldrich #30142-6)
Peak 1 – 7.06 min.
Peak 2 – 8.29 min.

mobile phase: 20/80:ACN/0.1% TEAA, pH 4.1
flow rate: 1 mL/min.
det.: 254 nm
injection: 1 µL
sample: 5 mg/mL

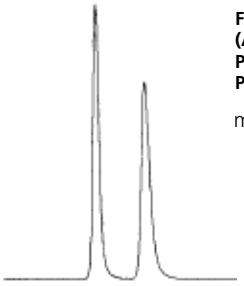
CYCLOBOND I 2000 SP



Chlorthalidone
(Sigma#C2775)
Peak 1 – 11.16 min.
Peak 2 – 12.65 min.

mobile phase: 10/90:ACN/0.1% TEAA, pH 4.1
flow rate: 1.5 mL/min.
det.: 230 nm
injection: 2 µL
sample: 5 mg/mL

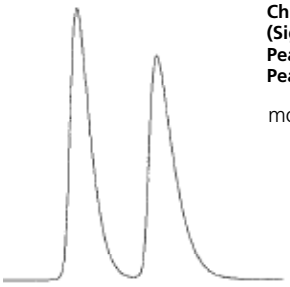
CYCLOBOND I 2000 DMP



Flavanone
(Aldrich #10,203-1)
Peak 1 – 5.41 min.
Peak 2 – 6.51 min.

mobile phase: 20/80:EtOH (denatured)/ hexane
flow rate: 1 mL/min.
det.: 254 nm
injection: 2 µL
sample: 5 mg/mL

CYCLOBOND I 2000 RSP



Chlorthalidone
(Sigma #C2775)
Peak 1 – 12.04 min.
Peak 2 – 13.73 min.

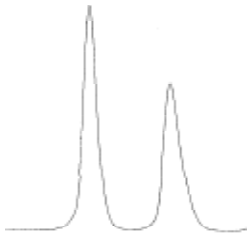
mobile phase: 10/90:ACN/ 0.1% TEAA, pH 4.1
flow rate: 1.5 mL/min.
det.: 230 nm
injection: 2 µL
sample: 5 mg/mL

CYCLOBOND I 2000 AC

Norphenylephrine
(Aldrich #11,372-7)
Peak 1 – 4.18 min.
Peak 2 – 4.74 min.

mobile phase: 10/90:MeOH/ 0.5% NaAc, pH 5.5
flow rate: 1 mL/min.
det.: 254 nm
injection: 2 µL
sample: 5 mg/mL

CYCLOBOND I 2000 DM



Warfarin

Peak 1 – 8.20 min.
Peak 2 – 10.20 min.

mobile phase: 20/80:ACN/ 0.1% TEAA, pH 4.1
flow rate: 1.5 mL/min.
det.: 254 nm
injection: 2 µL
sample: 5 mg/mL