

HyPURITY® Columns

TG01-11



A specialized packing which has
polar embedded character

Analyze • Detect • Measure • Control™

Thermo
ELECTRON CORPORATION

HyPURITY® Columns

HyPURITY Columns

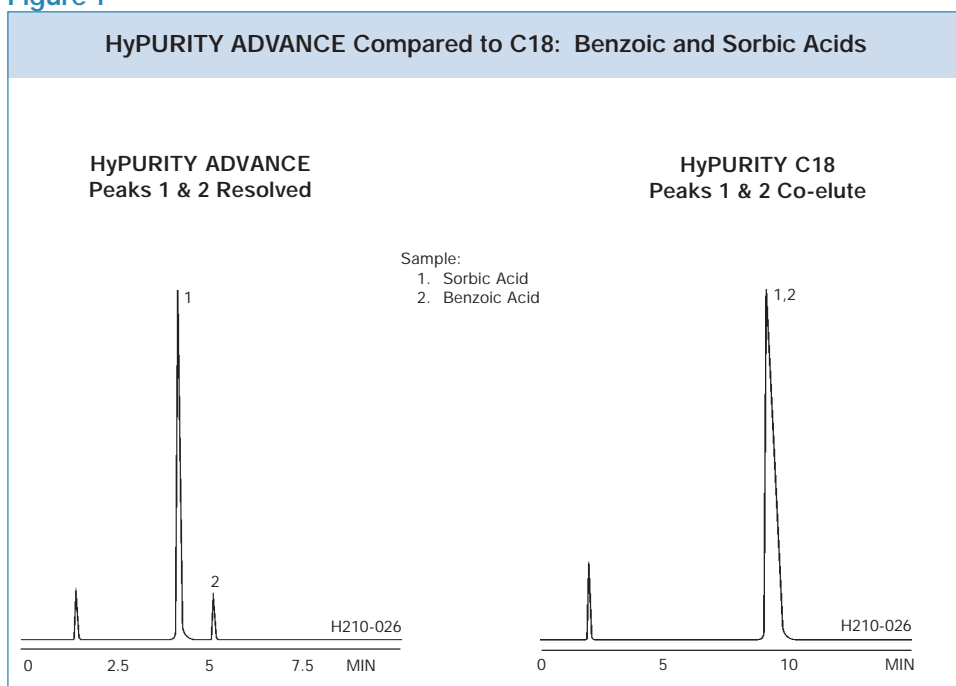
Thermo Hypersil-Keystone is dedicated to the design and manufacture of high quality HPLC media and columns. Over 20 years of experience has resulted in the development of an excellent quality program and accreditation with ISO9001. The HyPURITY family, launched in 1995, has established new standards in column characterization and validation. The quality assurance program has a variety of highly diagnostic chromatographic tests for the investigation of both primary and secondary interactions, ensuring exceptional column-to-column and batch-to-batch reproducibility. The range of stationary phases bonded to the HyPURITY silica includes C18, C8, C4, Cyano and the unique ADVANCE phase. Physical properties are outlined in Table 1.

The HyPURITY ADVANCE phase is a specialized packing which has polar embedded character. It provides excellent performance for compounds showing poor peak shape with traditional alkyl bonded phases such as C18. The HyPURITY ADVANCE phase also provides unique selectivity and is a very useful phase where alternative selectivity is required (Figure 1). The full details concerning characteristics and applications of the HyPURITY ADVANCE column can be found in a separate Technical Guide (TG 01-09).

Table 1

Specifications:				
Packing	Particle Size	Surface Area (m ² /g)	Pore Size (Å)	%Carbon
C18	3 and 5µm	200	180	13%
C8	5µm	200	180	8%
C4	5µm	200	180	4.5%
Cyano	5µm	200	180	4%
ADVANCE	3 and 5µm	200	180	Proprietary

Figure 1



Columns: 5µm, 150x4.6mm,
 Eluent: 80% 25mM KH₂PO₄, pH 2.3 / 20% ACN
 Flow: ADVANCE: 1.5 mL/min
 C18: 1.0 mL/min
 Detector: UV @ 254
 Temp.: 25°C

The Ultimate in Chromatographic Validation

A series of physical and chromatographic studies have been developed to fully characterize the chromatographic surface of the HyPURITY® C18 packing material. The chromatographic probes employed are designed to cover the broad range of possible analyte/stationary phase surface interactions, and have been chosen only after an in-depth literature survey and extensive consultation.

Most current HPLC column manufacturers offer some evidence of stationary phase characterization in terms of physical properties and/or information on column performance, such as efficiency and peak shape parameters. There is little information offered with respect to quality control procedures for batch-to-batch or column-to-column reproducibility.

The following chromatographic tests are used to fully characterize the chromatographic properties of every batch of HyPURITY C18 packing material:

- Steric selectivity
- Hydrophobicity
- Hydrogen bonding
- Ion exchange capacity
- Analysis of bases at pH 7
- Analysis of acids, alcohols and chelators

Every column is tested for efficiency, peak shape and capacity factor. A brief introduction to each of the tests is presented in Table 2. More detailed information is provided in the discussion that follows.

Table 2

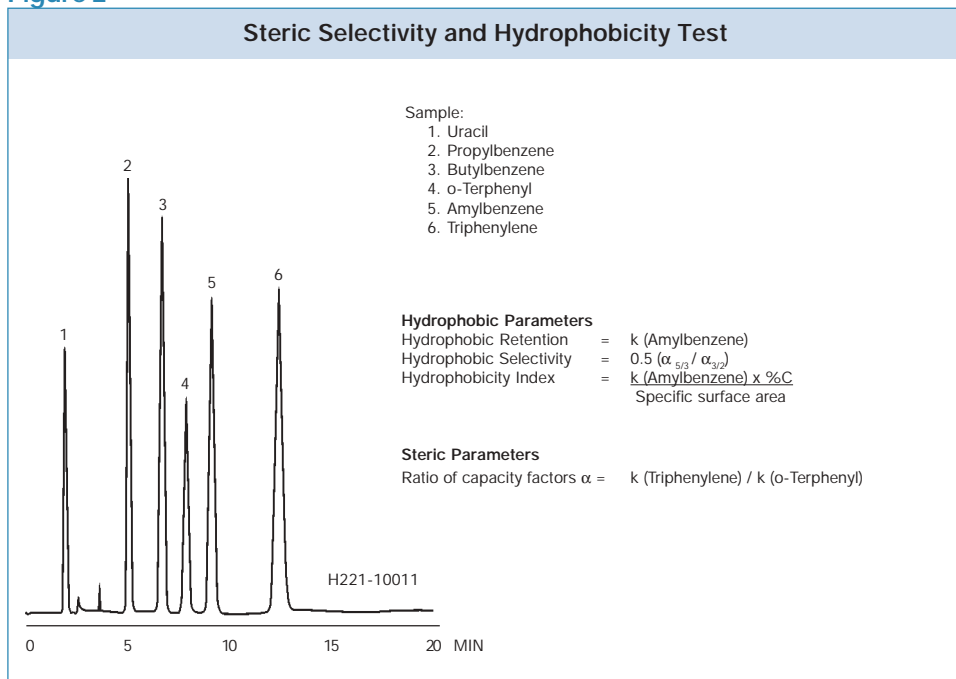
Test	Characteristic Tested	Method
Steric selectivity	Phase selectivity	Relative retention of polyaromatic hydrocarbons
Hydrophobicity	Hydrophobic character and surface coverage of bonded phase	Retention and selectivity of non-polar hydrocarbons
Hydrogen bonding capacity	Measure of residual silanol groups at silica surface	Selectivity factor of caffeine relative to phenol
Ion exchange capacity at pH 2.7	Peak tailing caused by ion exchange sites	Retention of benzylamine
Ion exchange capacity at pH 7.6	Peak tailing caused by ion exchange and dissociated silanol sites	Retention of benzylamine
Analysis of bases at pH 7	Effect of dissociated silanols on basic analytes	Analysis of tricyclic antidepressants, alpha selectivity parameters
Analysis of chelators	Peak tailing caused by active metal/silanol sites and analyte selectivity sensitive to changes in surface silanol content	Chromatography of acids and chelators

Steric Selectivity

Steric selectivity refers to the ability of the stationary phase to recognize and differentiate between molecules with similar structures but different shapes. The nature and orientation of the alkyl ligand (e.g. C18) can effect the extent to which steric selectivity plays a part in a separation. Steric selectivity is often indicative of the surface coverage of the bonding chemistry, and also provides a characteristic by which different HPLC packings can be compared.

Sander and Wise¹ have shown that compounds of similar size and functionality, such as polyaromatic hydrocarbons, may differ in their retention characteristics due to their relative ability to bend and twist out of shape. o-Terphenyl and triphenylene have been selected as probes because the former has the ability to twist and bend while the latter has a fairly rigid structure and will be retained quite differently. A measure of the relative retention of these compounds is indicative of the steric selectivity of the HyPURITY[®] C18 phase (Figure 2).

Figure 2



HyPURITY C18, 5 μ m, 150x4.6mm
Eluent: 80% MeOH / 20% H₂O
Flow: 1.0 mL/min
Detector: UV @ 254

Hydrophobicity

The test probes used to determine the hydrophobicity characteristics of HyPURITY packings have been combined with those of the steric selectivity test. Various hydrophobic parameters are measured by comparative retention of a series non-polar hydrocarbons (alkylbenzenes). In a simple binary eluent of methanol and water, the capacity factors of amylbenzene, 1-butylbenzene and n-propylbenzene give a broad measure of hydrophobic retention and selectivity.

Individual packing materials have different levels of bonded phase coverage and specific surface area. The hydrophobicity index gives a measure of hydrophobic coverage that relates all the factors that can contribute to the overall hydrophobic retention in a column, including surface area, %carbon and chromatographic retention of amylbenzene. The hydrophobicity index provides an indication of the hydrophobic character of the column per unit area.

To quantify these measures of hydrophobicity in the HyPURITY C18 phase the following calculations are made:

- (a) **Hydrophobic Retention** = k (amylbenzene)
- (b) **Hydrophobic Selectivity** = $.05 \alpha$ (amylbenzene, 1-butylbenzene) / α (1-butylbenzene, n-propylbenzene)
- (c) **Hydrophobicity Index** = $\frac{k \text{ (amylbenzene)} \times \%C}{\text{specific surface area}}$

Where %C is the percentage carbon loading as measured by the LECO EC-12 Carbon Analyzer. A typical %carbon value for the HyPURITY C18 packing material is 13%.

Hydrogen Bonding Capacity

The retention of caffeine is normalized against the retention of phenol to provide an indication of residual silanol groups and hydrogen bonding interactions that can occur at the silica surface².

The separation factor k (caffeine) / k (phenol) = α (caffeine / phenol) will vary depending on the hydrogen bonding capacity of the phase.

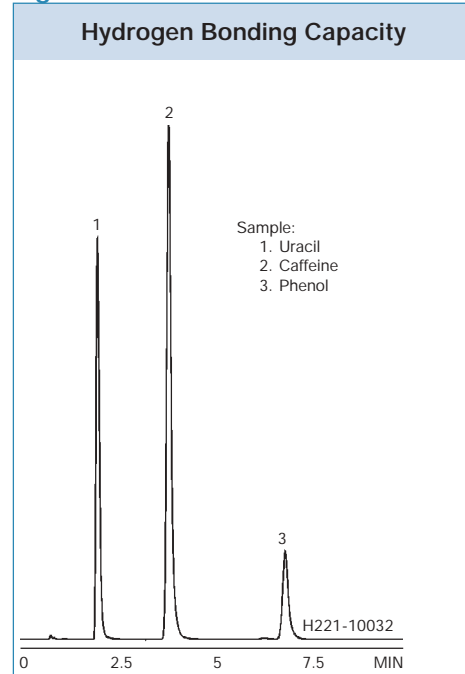
An α (caffeine / phenol) > 0.6 is said to represent a high capacity for hydrogen bonding, and an α (caffeine / phenol) < 0.6 is said to represent low hydrogen bonding capacity. Phenol is used only as a marker in this test and eliminates other column effects when the caffeine capacity factor is measured relative to it.

The test is sensitive for the reproducibility of the C18 bonding process, and provides a useful measure of the hydrogen bonding capacity available for analyte interactions. Figure 3 shows a typical chromatogram of the hydrogen bonding capacity test.

Specification for HyPURITY C18

α (caffeine / phenol)	$0.34 < \alpha < 0.38$
------------------------------	------------------------

Figure 3



HyPURITY C18, 5 μ m, 150x4.6mm
 Eluent: 70% H₂O / 30% MeOH
 Flow: 1.0 mL/min
 Detector: UV @ 254

Ion Exchange Capacity

Tanaka et al² showed that the retention of protonated amines at pH < 3 could be used to determine a measure of the ion exchange sites on the silica surface. The majority of silanol groups (Si-OH) are undissociated at pH < 3, and therefore do not contribute to the retention of protonated amines. Acidic silanols still remaining on the silica surface will be in the dissociated form (SiO⁻). These acidic silanols contribute to the retention of protonated amines by ion exchange.

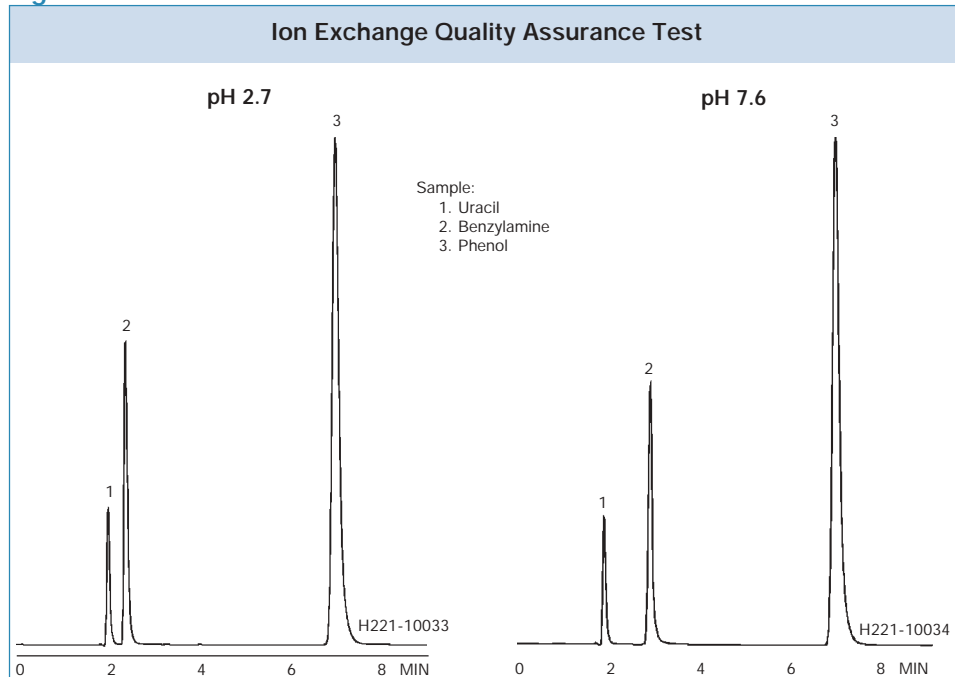
At pH > 7, all of the surface silanol groups are dissociated to form ion exchange sites that increase the retention of protonated amines. To accurately determine the ion exchange character of a bonded phase, retention of amines should be measured at both high and low pH.

The retention of benzylamine is measured and normalized with respect to phenol at pH values of 2.7 and 7.6 to investigate the ion exchange behavior of the HyPURITY[®] C18 phase. Measuring the relative retention of benzylamine in this way shows important ion exchange characteristics of the packing that can be measured on a batch-to-batch basis in order to ensure reproducibility.

Specifications for HyPURITY C18

Surface Parameter Measured	pH	α (benzylamine / phenol)
Ion Exchange Sites and Silanol Effects	7.6	$0.17 < \alpha < 0.19$
Ion Exchange Sites	2.7	$0.07 < \alpha < 0.09$

Figure 4



HyPURITY C18, 5 μ m, 150x4.6mm
 Eluent: 70% 20mM KH₂PO₄ / 30% MeOH
 Flow: 1.0 mL/min
 Detector: UV @ 254

Basic Analytes

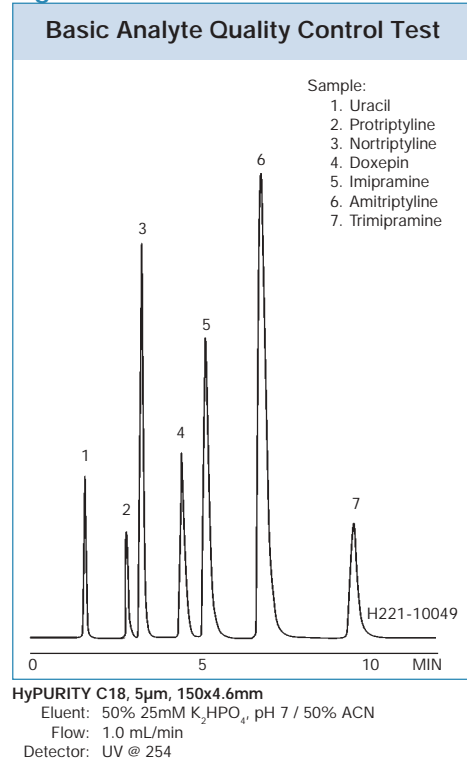
The analysis of basic compounds such as tricyclic antidepressants (TCAs) that are notorious for poor peak shapes and irreversible adsorption provides a further test to ensure lot-to-lot reproducibility. To further challenge the performance of the HyPURITY® C18 phase, the analytes are run at pH 7 where traditional C18 packings typically show poor performance for these analytes.

The HyPURITY C18 column provides good peak shape and resolution of all the analytes. The high surface coverage and minimal ion exchange interactions of the phase contribute to the excellent peak shape and chromatographic performance illustrated in Figure 5.

The capacity factor of these solutes is indicative of the overall performance of the column. A shift in peak retention can easily result from any of the secondary interactions already highlighted in the previous tests, and consequently the TCA test is a very sensitive one.

Test	Specifications
α (Trimipramine/Amitriptyline)	$1.51 < \alpha < 1.71$
α (Amitriptyline/Imipramine)	$1.38 < \alpha < 1.56$

Figure 5



Analysis of acids, alcohols and chelators

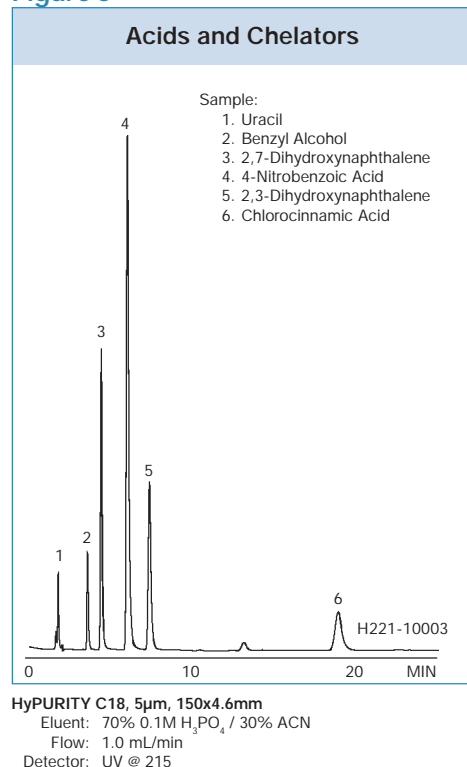
Surface metal interaction can cause changes in selectivity or peak shape for solutes which are able to chelate. Euerby et al³ have shown that the presence of metals arise not only from the base silica itself but also from the column hardware. Contributions from the latter depend greatly on the conditions under which the column is stored. A useful chromatographic test used to identify the presence of metal ions in the column is to compare the peak symmetry of two regioisomers, 2,3- and 2,7-dihydroxynaphthalene (DHN). The former possesses the ability to chelate while the latter does not. The closer the ratio of the peak symmetries of the 2,7 / 2,3-dihydroxynaphthalene is to 1.0, the lower the metal content.

A mixture of acids, alcohols and chelators is employed to illustrate the applicability of the HyPURITY C18 packing for a wide range of acidic and chelating analytes, where both chelating interactions and hydrogen bonding secondary interactions are possible. If not carefully controlled, both interactions will cause changes to the overall selectivity of the column or performance in terms of peak shape.

The ratio of asymmetry values for 2,7-DHN/2,3-DHN on the HyPURITY C18 phase must be >0.9 to pass specification.

Figure 6 shows the separation of acids, alcohols and chelators on a HyPURITY C18 column.

Figure 6



Reproducibility

Each lot of HyPURITY® C18 packing must conform to a range of specifications for quality control. The reproducibility of each lot test is then monitored on an ongoing basis.

Figure 7 shows the reproducibility of %carbon observed for 27 batches of HyPURITY C18 packing manufactured over the last six years. The %carbon is measured by Leco Carbon analyzer and is accurate to within plus or minus 0.1%. Note the continuous tightening of the results over the last year, indicating Thermo Hypersil-Keystone's strong commitment to continuous improvement of product quality.

Figure 8 illustrates batch-to-batch reproducibility for two of the more sensitive chromatographic selectivity parameters. Only once a batch has passed all the Quality Assurance specifications can it be used for column packing. In this example capacity factors for four of the test analytes (from the acid and chelator test) are used to calculate alpha values. These are then recorded and must conform to a narrow window of specification.

Alpha values represent a ratio of capacity factors (k values measured for two different analytes, k_3/k_2 within a given test mixture). In Figure 7 the alpha values measured are α (4/3) and α (6/5).

Where

$$\alpha (4/3) = \frac{k (4\text{-Nitrobenzoic acid})}{k (2,7\text{-Dihydroxynaphthalene})}$$

$$\alpha (6/5) = \frac{k (\text{Chlorocinnamic acid})}{k (2,3\text{-Dihydroxynaphthalene})}$$

Figure 7

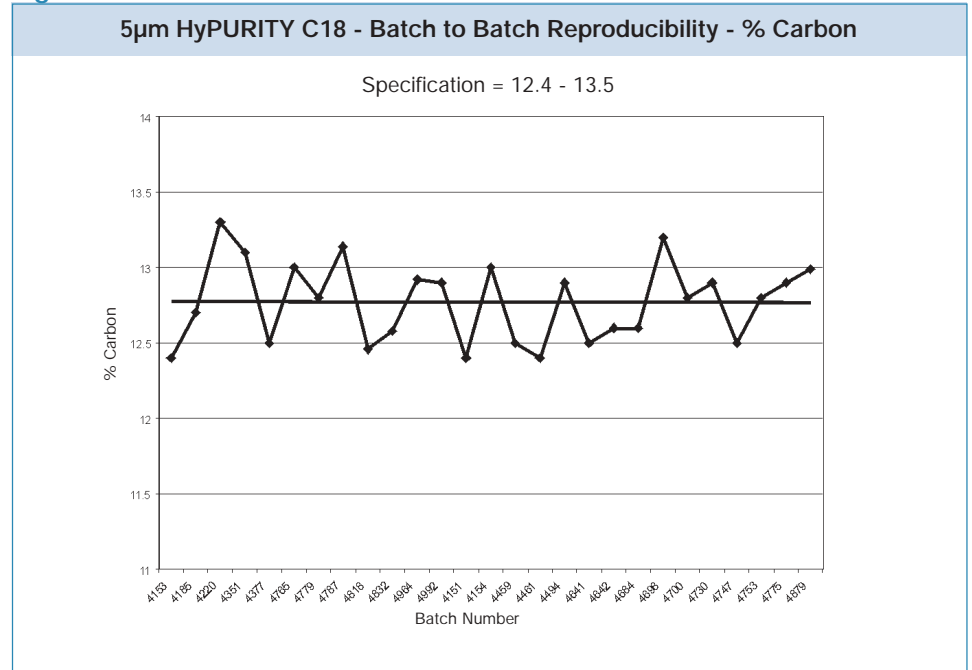
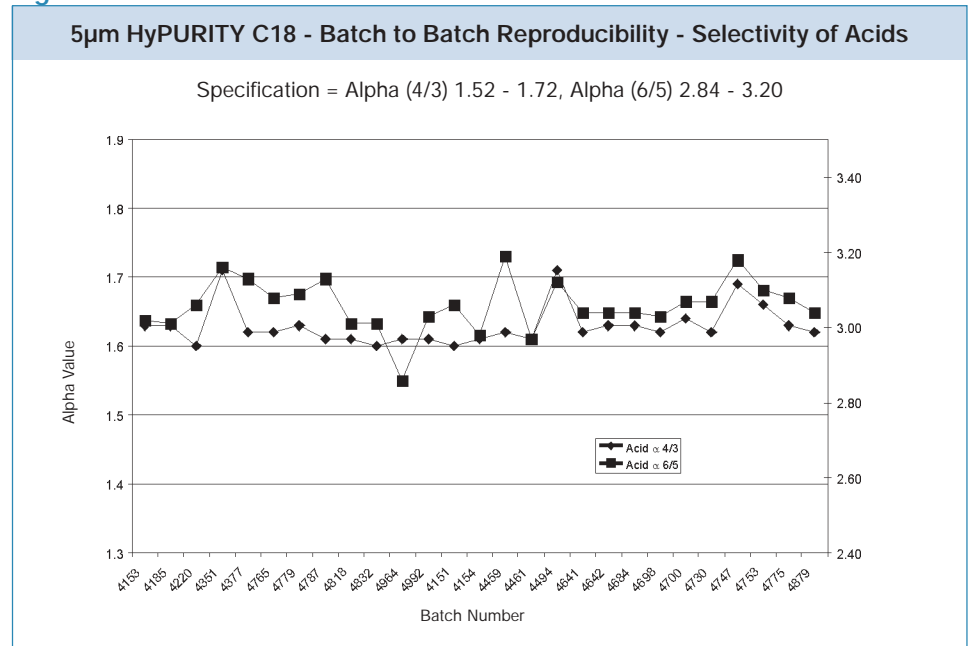


Figure 8



After each lot of HyPURITY® C18 packing material has passed all specifications, it is suitable to be packed into columns. Packed columns undergo additional chromatographic testing to ensure the column has been packed efficiently and that it meets only the highest standards. A typical certificate of analysis for this testing is given in Figure 9. Packed column reproducibility is monitored in terms of peak tailing (asymmetry) and column efficiency. Figure 10 demonstrates how both of these parameters are used to monitor column performance.

Peak Asymmetry

Peak asymmetry (peak tailing) gives a useful measure of the quality of a packed column. Peak tailing is usually observed when a column deteriorates but may also be observed if the column is poorly packed. The asymmetry ratio for a given peak is calculated at 10% of the peak height. To pass specification, asymmetry must fall within 1.2 >0.9. Note how the trend line demonstrates continual improvement in quality as the average asymmetry value now approaches 1.0, as shown in Figure 10.

Column Efficiency

Poorly packed columns will often result in chromatograms that show broad peaks. The column efficiency parameter measures the number of theoretical plates in a column (N). It provides a measure of the degree to which band spreading occurs as an analyte travels along the column length. Poorly packed columns give rise to increased band spreading and low efficiency values. Each 5µm column must have a column efficiency of greater than 75,000 plates per meter. The trend line demonstrates a continual attention to improving quality, as average HyPURITY C18 column efficiency approaches 90,000 plates per meter (Figure 10).

References:

1. Sander and Wise, Synthesis and Characterization of Polymeric C18 Stationary Phases for Liquid Chromatography, Anal. Chem, 56 (1984) 504-510
2. K. Kimata, K. Iwaguchi, S. Onishi, K. Jinno, R. Eksteen, K. Hosoya, M. Araki and N. Tanaka, Journal of Chromatographic Science, 27, 721-728(1989)
3. M.R. Euerby, C.M. Johnson, I.D. Rushin, D.A.S.S. Tennekoon, Journal of Chromatography A, 705 (2), 229-245 (17) -- (1995)

Figure 9

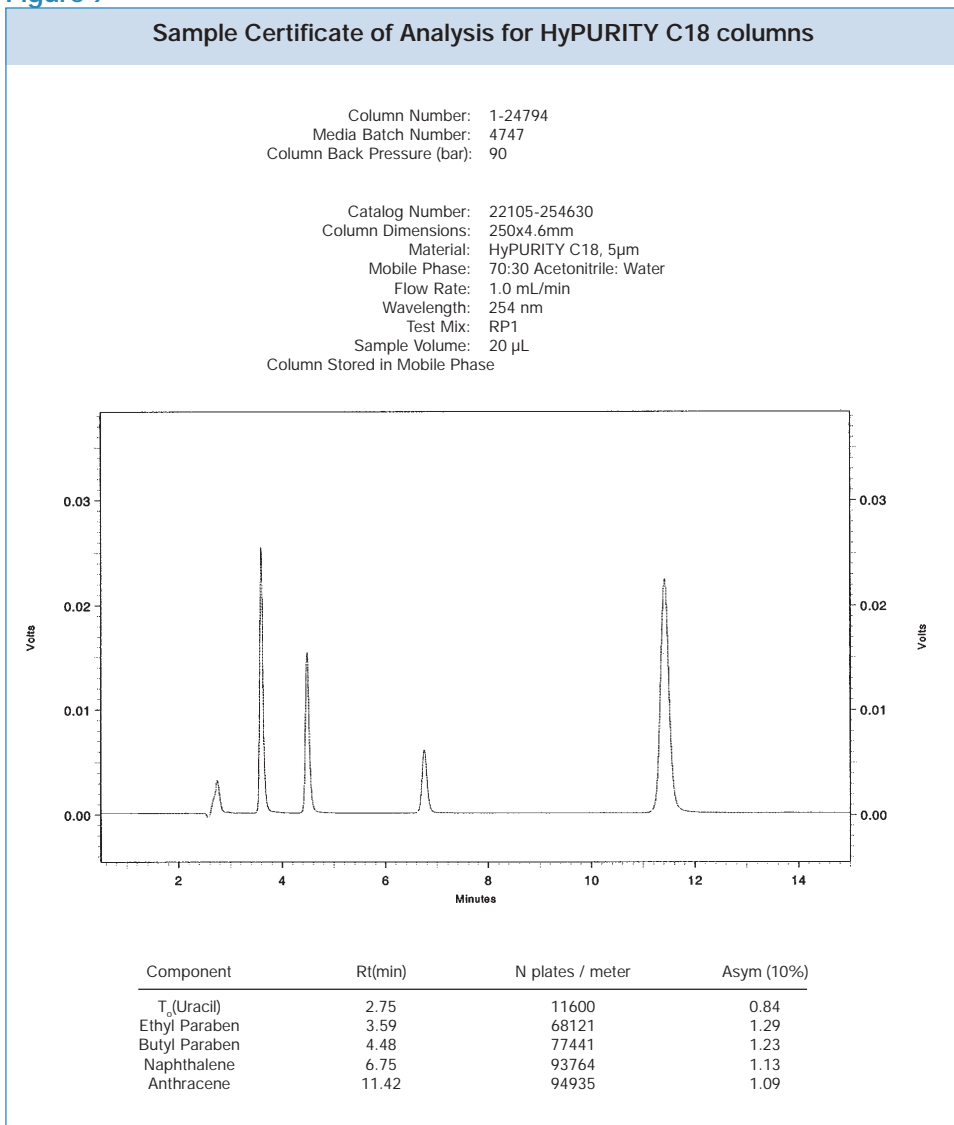
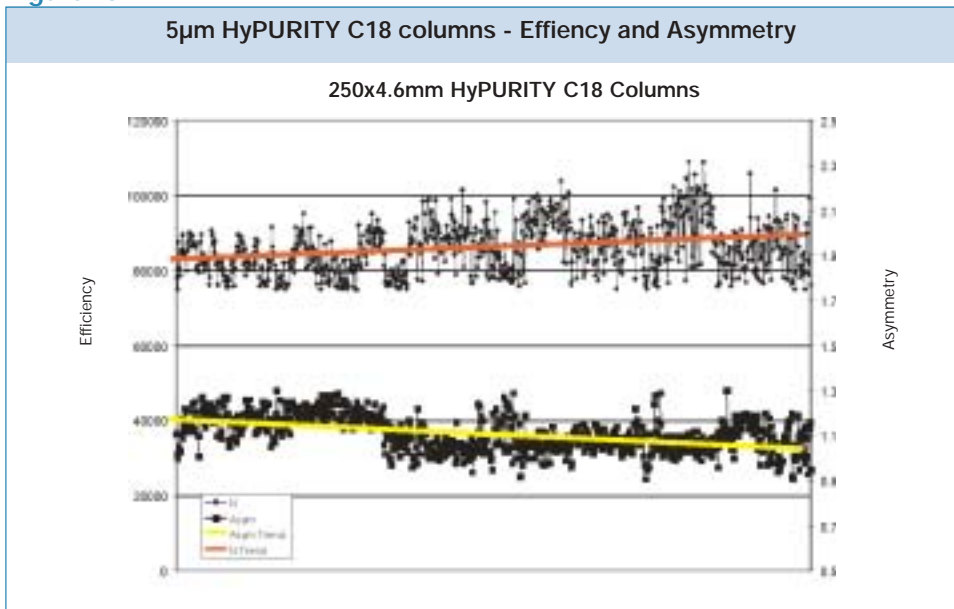
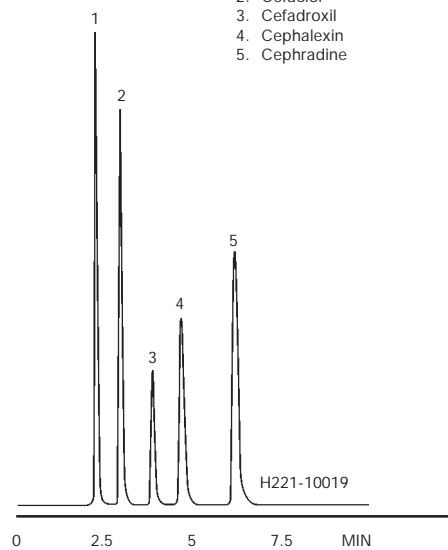


Figure 10



Cephalosporin Antibiotics

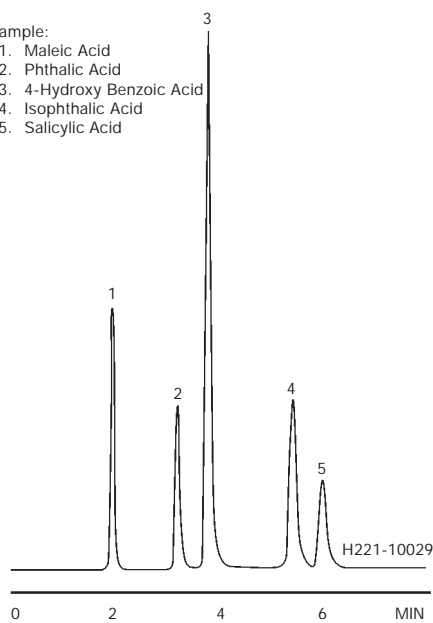
- Sample:
1. Cefazolin
 2. Cefaclor
 3. Cefadroxil
 4. Cephalixin
 5. Cephradine



HyPURITY® C18, 5µm, 150x4.6mm
 Eluent: 85% 0.5% CH₃COOH / 15% ACN
 Flow: 1.0 mL/min
 Detector: UV @ 254

Fungicides / Preservatives

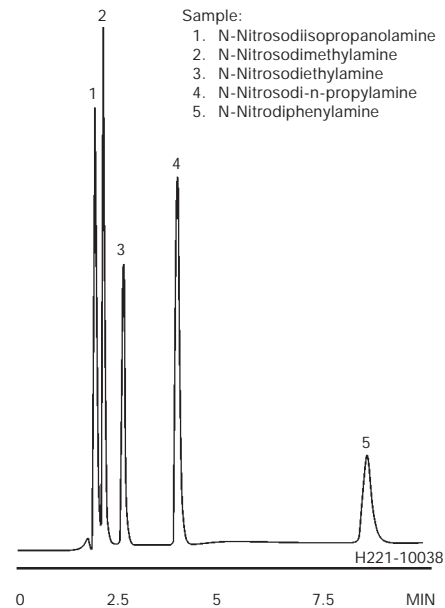
- Sample:
1. Maleic Acid
 2. Phthalic Acid
 3. 4-Hydroxy Benzoic Acid
 4. Isophthalic Acid
 5. Salicylic Acid



HyPURITY C18, 5µm, 150x4.6mm
 Eluent: 67% 25mM KH₂PO₄ / pH 3.35 / 33% MeOH
 Flow: 1.0 mL/min
 Detector: UV @ 210

Nitrosamines

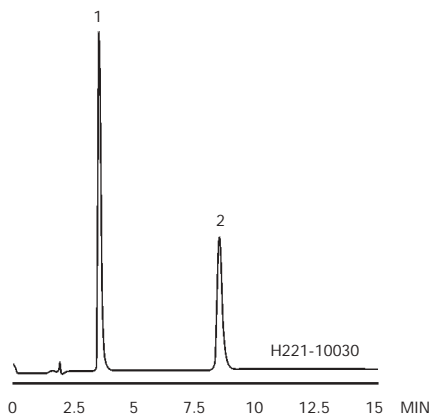
- Sample:
1. N-Nitrosodisopropanolamine
 2. N-Nitrosodimethylamine
 3. N-Nitrosodiethylamine
 4. N-Nitrosodi-n-propylamine
 5. N-Nitrodiphenylamine



HyPURITY C18, 5µm, 150x4.6mm
 Eluent: 50% 10mM K₂HPO₄ / pH 7 / 50% ACN
 Flow: 1.0 mL/min
 Detector: UV @ 230

Glucocorticoids

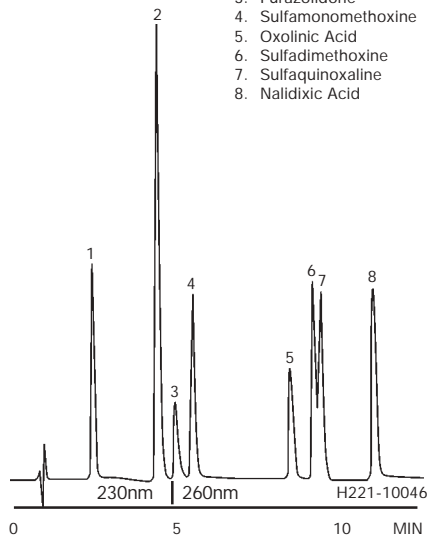
- Sample:
1. Hydrocortisone
 2. Hydrocortisone-21-acetate



HyPURITY C18, 5µm, 150x4.6mm
 Eluent: 35% ACN / 65% H₂O
 Flow: 1.0 mL/min
 Detector: UV @ 254

Synthetic Antibacterials

- Sample:
1. Sulfamerazine
 2. Thiamphenicol
 3. Furazolidone
 4. Sulfamonomethoxine
 5. Oxolinic Acid
 6. Sulfadimethoxine
 7. Sulfaquinoxaline
 8. Nalidixic Acid



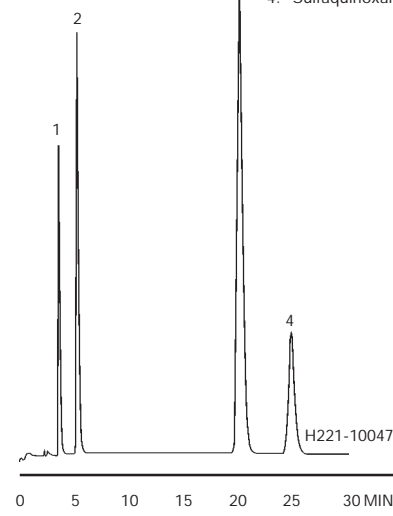
HyPURITY C18, 5µm, 150x4.6mm
 Gradient: A: ACN
 B: 20mM H₃PO₄

Time (mins)	%B
0	90
4	90
20	70

 Flow: 1.0 mL/min
 Detector: 0 - 9.55 mins UV @ 230
 9.55 - 25 mins UV @ 260

Sulfur Drugs

- Sample:
1. Sulfathiazole
 2. Sulfamethiazole
 3. Sulfadimethoxine
 4. Sulfaquinoxaline



HyPURITY C18, 5µm, 150x4.6mm
 Eluent: 72% 5mM Sodium-1-heptanesulfonate / 28% MeOH
 Flow: 1.0 mL/min
 Detector: UV @ 254

HyPURITY is a registered Trademark of Thermo Hypersil-Keystone.
 ©2002 Thermo Hypersil-Keystone.
 All Rights Reserved.

Innovators in life and laboratory sciences, Thermo Electron Corporation provides advanced analytical technologies, scientific instrumentation, laboratory informatics solutions, and laboratory consumables to help scientists and clinicians to discover new drugs, improve manufacturing processes, and diagnose illness and disease. Unparalleled in our capabilities, we can help you every step of the way – from sample preparation and sample analysis through interpretation of results.

Thermo Electron Corporation has direct subsidiary offices in North America, Europe and Japan. To complement these direct subsidiaries, we maintain a network of representative organizations throughout the world. Use this reference list or visit our web site to locate the representative nearest you.

France
Tel. (33) 1 60 92 48 00

Germany
Tel. (49) 6103 4080

United Kingdom
Tel. (44) 1928 581000

United States
Tel. (01) 800 437 2999

For more information on our products and services, please visit our website at: www.thermo.com/hypersil-keystone

Technical information contained in this publication is for reference purposes only and is subject to change without notice. Every effort has been made to supply complete and accurate information; however, Thermo Electron assumes no responsibility and will not be liable for any errors, omissions, damage, or loss that might result from any use of the information contained therein (even if this information is properly followed and problems still arise).

Reference to specifications supersedes all previous information and are subject to change without notice.

ADVANCE, BetaBasic, BetaMax, BETASIL, BioBasic, DASH, DELTABOND, Duet, Fluophase, Hyperbond, Hypercarb, KAPPA, HOT POCKET, HyperGEL, HyperREZ, HyperSEP, Hypersil, HyPURITY, HyPURITY AQUASTAR, Javelin, KEYSTONE, MultiSEP PIONEER, PRISM, Retain, SLIPFREE, UNIGUARD, UNIPHASE, and Verify are trademarks of Thermo Electron Corporation.

AquaSil™ Silicizing Fluid for treating glass surfaces is sold by Pierce Chemical Co., Rockford, IL.

All other trademarks are the property of their respective owners.