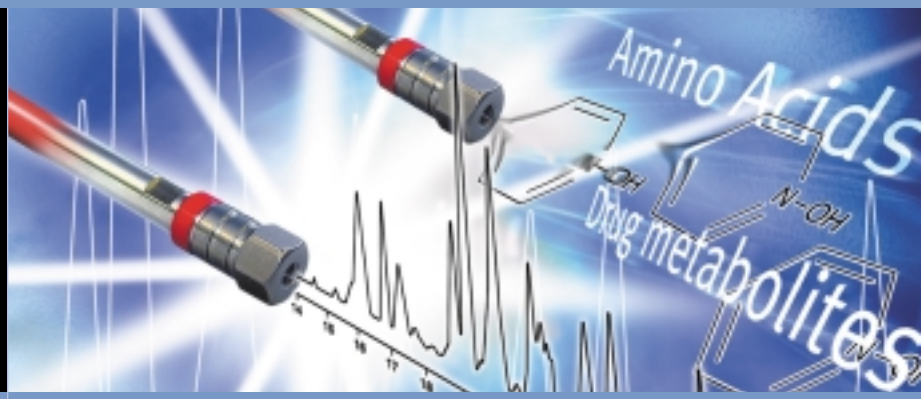
Hypercarb™ HPLC Columns Technical Guide



The Solution for Problem Separations

Analyze • Detect • Measure • Control™

Thermo
ELECTRON CORPORATION

Hypercarb HPLC Columns

The Unique Phase in Liquid Chromatography

Hypercarb porous graphitic carbon (PGC) is a unique material for HPLC manufactured by Thermo Electron Corporation. The Hypercarb material is composed of fully porous individual spherical particles. At the molecular level, it is composed of flat sheets of hexagonally arranged carbon atoms. These carbon atoms have a fully satisfied valence.

The selectivity of the Hypercarb packing is different than the selectivity of silica-based and polymeric phases. The Hypercarb material excels at the separation of highly polar compounds with closely related structures.

The chemical characteristics of the material are also different from conventional HPLC stationary phase materials. The Hypercarb packing is totally stable across the entire pH range of 0-14. It can be used for both normal and reversed phase separations, and the robust nature of the material ensures exceptional column lifetimes.

- **Unique 100% porous graphitic carbon (PGC) material**
- **Excellent for the separation of highly polar compounds**
- **Stable across the entire pH range 0-14**
- **Excellent batch-to-batch reproducibility**
- **Homogeneous crystalline surface**
- **Enhanced selectivity for closely related compounds, including geometric isomers and diastereoisomers**

Phase	Particle Size	% Carbon	Pore Size
Hypercarb	3, 5 and 7µm	100%	250Å

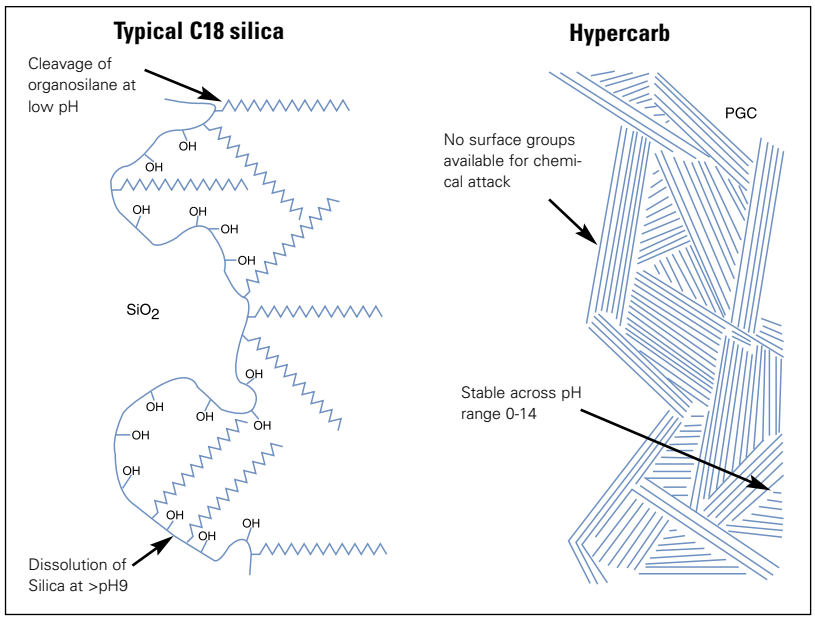


Figure 1: Surface Comparison Between C18 Bonded Silica and Hypercarb Porous Graphitic Carbon

Use Hypercarb Columns For:

- Polar solutes
- Geometric isomers
- Diastereoisomers
- Oligosaccharides
- PCBs

The Hypercarb packing has unique properties as a stationary phase that have been used to provide solutions to a wide range of problematic HPLC separations. On the following pages we consider:

- the retention of very polar analytes not normally retained on C18 silica and,
- the separation of structurally similar compounds such as diastereoisomers that are not separated on C18 silica.

We first briefly review the history associated with the use of PGC in HPLC and the special surface characteristics which give rise to its unique separating properties.

Relative to Other Supports, The Hypercarb Packing Shows:

- Physical properties similar to a wide pore spherical silica.
- Unique mechanism of retention.
- Increased retention of non-polar compounds.
- Increase selectivity towards structurally-related compounds.
- A polar retention effect, whereby analytes of increasing polar functionality show an unexpectedly high affinity toward the carbon surface.
- A lack of any eluotropic series. The eluotropic series for oxide supports, such as silica and alumina, is based on the ability of the solvent to hydrogen bond with the surface. No such interactions can take place at the surface of graphite.
- Stability at extreme conditions of pH (10M acid to 10M alkali), salt concentration and temperature.

We will review several of these observations later in this section.

Physical Properties Related to Retention

The internal surface of the Hypercarb material, with a specific surface area of 120m²/g, is composed of flat sheets of hexagonally-arranged carbon atoms (as in a very large polynuclear aromatic molecule). The surface is crystalline and highly reproducible with a complete absence of micropores. The flat and highly adsorptive surface shows unusual discrimination of closely related compounds and diastereoisomers. If an optically active additive is present, chiral separations are readily achieved. The strength of interaction for individual analytes is largely determined by the molecular area of the analyte in contact with the surface, and also on the nature of the functional groups present at the point of interaction with the flat surface.

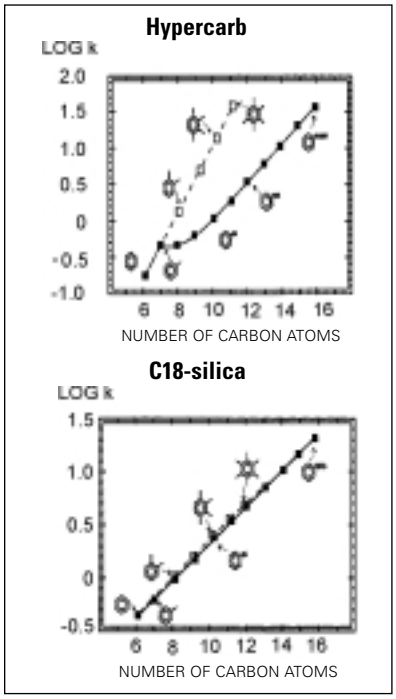


Figure 2: Selectivity: Hydrophobic Solutes

Retention Behavior for Non-Polar Compounds Compared to Silica Based or Polymeric Reversed Phase Media

Kriz, *et al*[1] compared plots of different homologous series, $CH_3(CH_2)_{(n-1)}Ar$ with a series of ortho-substituted xylenes, $Ar(CH_3)_{(n+1)}$. The ortho substituted xylenes gave an alpha value of 0.17 on the Hypercarb column, compared to 0.22 for a Hypersil™ ODS column. This illustrates that the Hypercarb column shows significantly increased discrimination based on the number of methyl substituents present compared to C18 silica. A tabulated summary of Kriz's results is given in Table 1.

Table 1 and Figure 2 show the increased resolving power of the Hypercarb packing. Where no discrimination between methylene and methyl groups are observed for C18 and very little for silica and alumina, considerable resolving power is observed for the Hypercarb column.

The improved discrimination for both methylene and methyl substitution over C18 silica allows improved resolution and selectivity for more complex analytes that may be difficult to separate on C18 silica due to their similar hydrophobicity. When comparing analytes of the same molecular mass with different spatial configuration, the flatter molecule that can more closely align with the flat Hypercarb surface will be retained longer. For example, n-propylbenzene is retained more strongly than iso-propylbenzene.

Using Analyte Functionality to Control Retention

Controlling the pH of the mobile phase, and hence the degree of ionization of the analyte, can be a strong tool in the development of a chromatographic method on a Hypercarb column. The effect on the retention and resolution of isomeric compounds with various substituents in the benzene ring was investigated by Wan *et al*[2]. They show that where the substituents of benzene isomers are either acidic or basic, the position and degree of ionization of such functional groups affects the order of elution in a consistent manner.

The experimental data shown in Figure 3 illustrates the effect of pH on the retention of simple ionizable compounds. The retention of the analyte follows the curve expected for a change in ionic state, where the ionized form is significantly less retained than the non-ionized form. At an appropriate pH (<3) the isomers are easily separated.

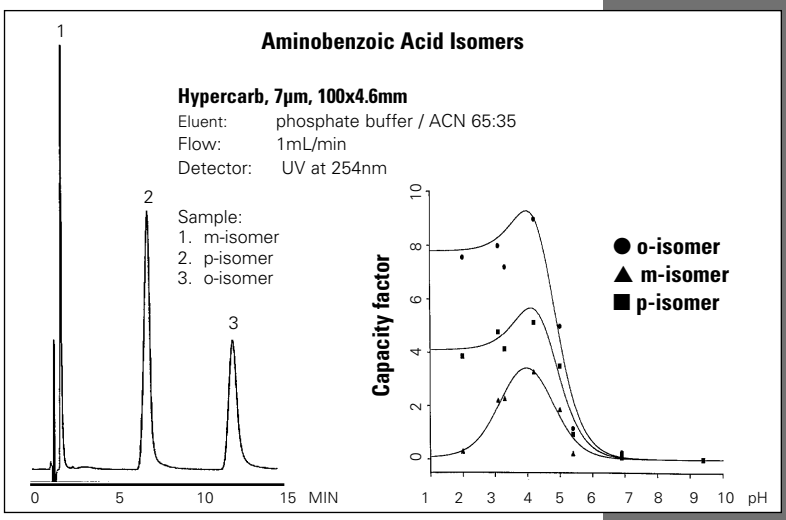


Figure 3: Separation of Geometric Isomers

Support	α (CH_3)	α (CH_2)	Eluent
Hypercarb PGC	0.46	0.22	Methanol
C18 Silica	0.17	0.17	Methanol: Water (80:20w/w)
Phenyl	0.10	0.1	Methanol: Water (70:30w/w)
Silica	0.046	0.1	Pentane
Alumina	0.195	0.00	Pentane

Table 1: Comparison of (CH_3) and (CH_2) selectivity on different stationary phases

The Column of Choice for Problem HPLC Separations

Problem 1: Retention of Polar Analytes in Reversed Phase Systems

In any reversed phase system, analyte retention increases as its hydrophobic properties increase. This is due to the increased dispersive interactions that take place between the stationary phase and the analyte. Conversely, as the polarity of the analyte increases, analyte-solvent interactions begin to dominate and retention is reduced. This simple observation holds true for all reversed phase systems with the exception of the Hypercarb phase. For Hypercarb columns, it has been observed that retention often increases as the polarity of the analyte increases. We have called this effect "the polar retention effect on graphite" or PREG. The PREG makes Hypercarb columns particularly useful for the separation of highly polar compounds which are difficult to retain on C18 phases.

For ionizable compounds, ionized solutes, carbohydrates and compounds containing numerous OH, COOH, and NH groups, the Hypercarb packing should be the stationary phase of first choice.

Coquart and Hennion [3, 4, 5] have carried out in-depth studies on the retention of polar environmental compounds. Their work demonstrates the difference in retention for polar compounds on Hypercarb columns compared to C18-silica and Hamilton® PRP-1, a polymer-based reversed phase material (see Table 2). By measuring log k values for a range of compounds in their mono-, di- and tri-substituted forms at different methanol:water compositions, it is possible to extrapolate these values to give log k data in pure water, i.e. log kw.

The results show that the retention of monosubstituted benzenes may be similar for C18-silica, PRP-1 and Hypercarb columns. However, increasing the polarity by a di- or tri-hydroxyl substitution to the benzene ring significantly increases retention on the Hypercarb column, but decreases retention on C18-silica and PRP-1 (Figure 4). In the case of C18-silica, the di-and tri-hydroxyphenols are not retained at all.

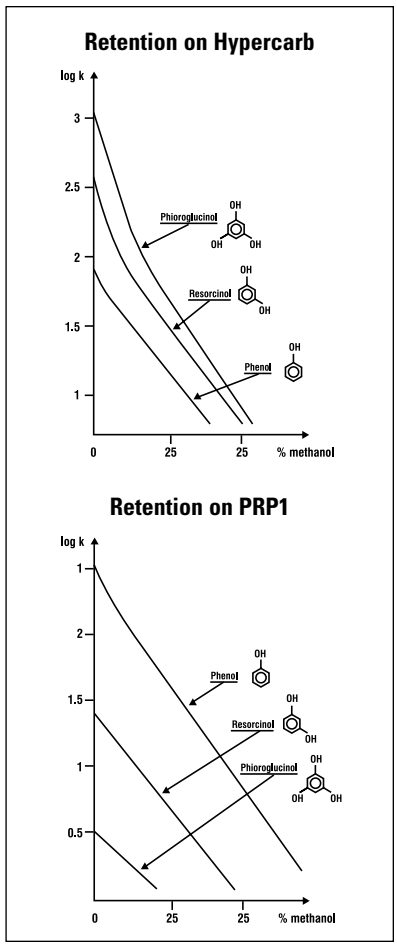


Figure 4: Polar Retention Effect
(Courtesy V. Coquart and J. Henion, J.Chrom., 1992)

	C18 Silica	PRP-1	Hypercarb
Phenol	1.55	2.40	1.80
1,3-dihydroxybenzene	-	1.35	2.35
1,3,5-trihydroxybenzene	-	0.5	2.70

Table 2: Log k in pure water for polyhydroxy phenols

Now Available in 3µm

Thermo Electron has added a 3µm particle size to popular 5 and 7µm columns. This smaller particle size offers the ability to accurately detect and quantify much lower concentrations of analytes. Peak height according to Snyder *et al* should increase by 30% on the substitution of a 5µm for a 3µm particle as illustrated, where an increase >30% is observed in peak height for some neurotransmitters.

The smaller 3µm particle also allows greater resolution to be obtained between critical pairs, as seen in Figure 5 by the extra impurity (peak 7) resolved successfully from the mixture using the 3µm Hypercarb column.

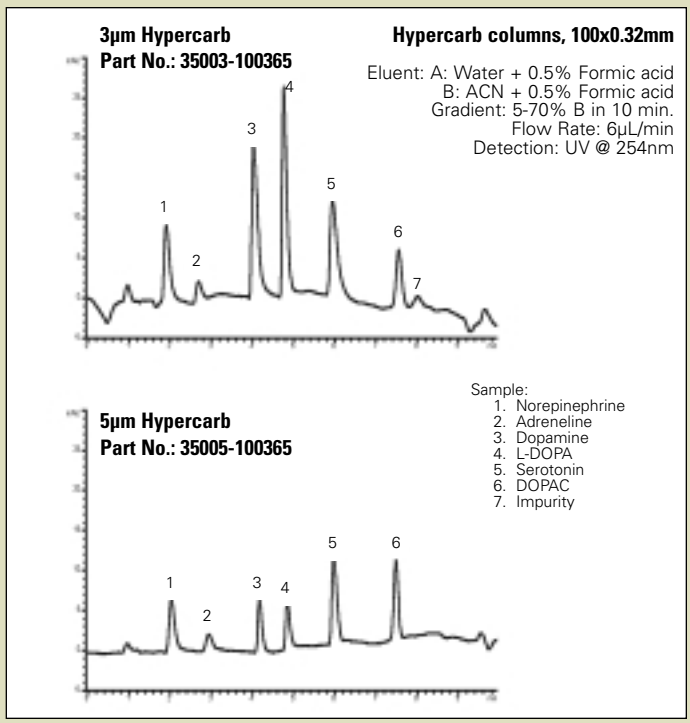


Figure 5: Increase in Sensitivity and Resolution with 3µm Hypercarb Columns

Ref.: Practical HPLC Method Development, Snyder, Kirkland and Glajch

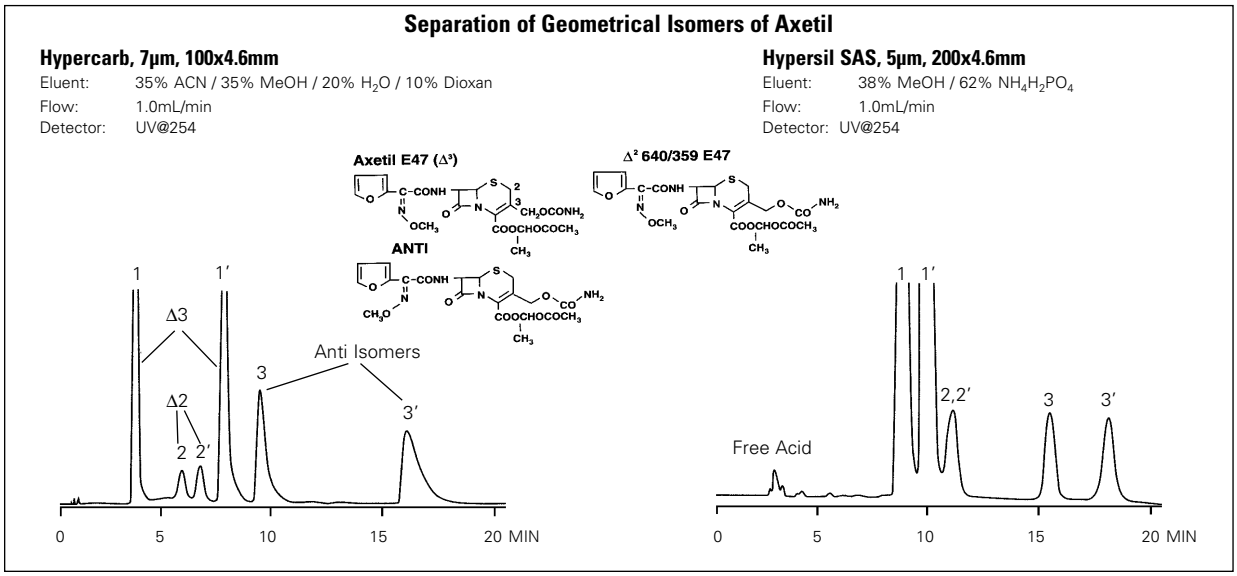


Figure 6: Separation of Geometrical Isomers of Axetil (Author: Norman Smith, Glaxo Group Research, Greenford)

Problem 2: Selectivity for Structurally Related Compounds

The combination of PREG and the differentiation of analytes based on their fit to the graphite surface means that Hypercarb columns are ideally suited to the separation of compounds that are very similar in structure. One of the first to demonstrate this was Norman Smith (Glaxo Group Research) with the complete separation of the diastereoisomers of the antibiotic Axetil. The antibiotic has a chiral center and exists as a pair of diastereoisomers, the ratio of which must be monitored. Figure 6 demonstrates the improved resolution on the Hypercarb column against the column previously found to give the best results.

Problem 3: pH Stability

Figure 7 shows the stability of the Hypercarb phase at pH12. In this study the column has been left to stand for a total of 93 days in 0.1M sodium hydroxide/methanol. At intermittent periods the column has been flushed with the methanol/water and it's selectivity and retention measured. Both retention and selectivity remained consistent over the duration of the study, as shown in Figure 8.

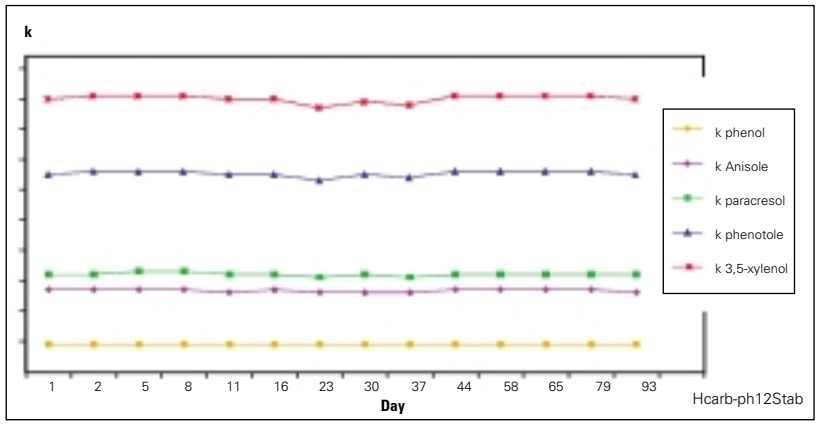


Figure 7: pH 12 Stability Study

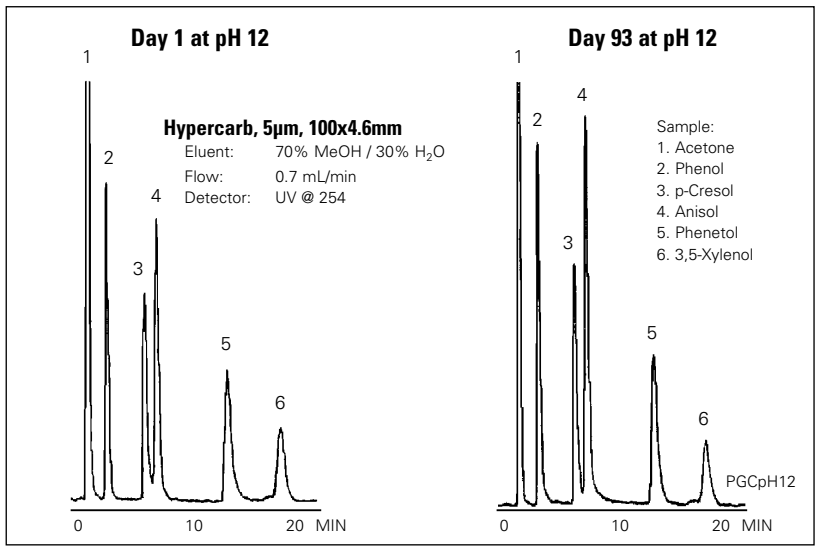


Figure 8: Hypercarb pH 12 Stability Trial

Applications on Hypercarb

Three reviews [6,7,8] covering applications using Hypercarb columns have been published since 1996. Both stress application areas where Hypercarb provides an improvement over existing technology, or where the analysis requirements fall outside those normally associated with silica-based stationary phases. These include pH restrictions, polar retention or improved selectivity for closely related compounds. These applications generally fall into one of the following categories:

- Retention of highly polar and ionized solutes
- Separations of carbohydrates and glucuronides
- Separations of geometric isomers and closely related compounds
- Enantiomeric separations
- Residue analysis
- Solid-phase extraction using Hypercarb packing

Retention of Morphine and its Metabolites

Barrett *et al* [10] report a method for the analysis of morphine-based opiates. The Hypercarb column is shown to separate both the parent drug and its conjugated metabolites in the same chromatographic run. This usually requires separate isocratic methods when using C18-silica, since some of the metabolites such as the glucoronide or sulphate are completely unretained under conditions which allow adequate retention of the parent drug. In this separation the author makes use of the polar retention effect and the ability of the Hypercarb column to separate compounds closely related in structure. Optimum separation was achieved by close control over pH (Figure 9).

Analysis of Hydrophobic and Polar Compounds in a Single Run[9]

Polyethelene glycols (PEGs) and polyethoxylated alkylphenols (PEAPs) are widely used as non-ionic surfactants. The characterization of these surfactants is difficult due to the complexity of the mixture and the lack of chromophores. The time-consuming derivatization procedure required for UV detection is avoided by using evaporative light scattering detection.

PEGs can be present in a surfactant mixture as by-products having no surfactant properties. In RPLC these by-products are eluted close to the void volume, while in NPLC they are strongly retained on the polar support. PEGs not retained with organic mobile phase on silica are easily separated on a Hypercarb column by using an aqueous-organic eluent. In order to analyze the mixture of PEG and PEAP, a gradient elution in two parts (water-acetonitrile then acetonitrile-dichloromethane) enables two distinct fingerprint profiles for this mixture to be obtained, as shown in Figure 10.

Hypercarb, 5µm, 100x4.6mm
Eluent: 40% Ammonium Acetate/ 60% MeOH, pH 9
Flow: 1.0 mL/min
Detector: UV @ 220
Ref.: Barrett D. & Taylor S., University of Nottingham, UK

Sample:
1. Normorphine
2. Morphine-3-glucuronide
3. Morphine-6-glucuronide
4. Morphine
5. Codeine

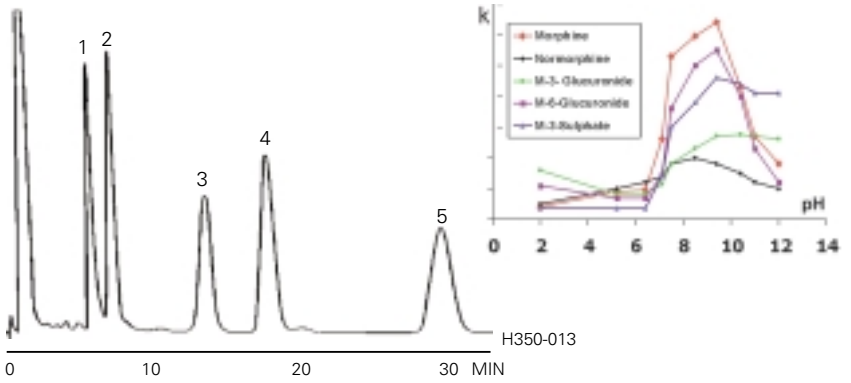


Figure 9: Morphine and Metabolites

Hypercarb, 7µm, 100x4.6mm
Gradient: A: H_2O
B: ACN
C: CH_2CL_2
from 20% B to 100% B in 15 min then 0% C to 80% C in 25 min
Flow: 1.0 mL/min
Detector: ELSD

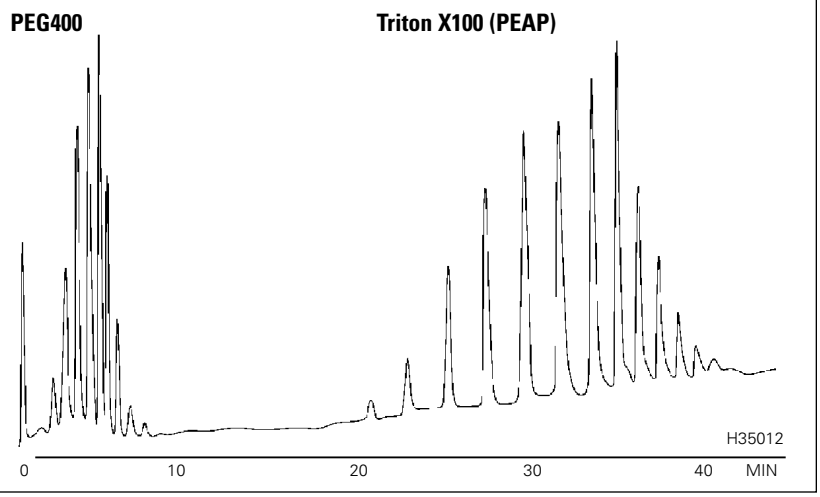
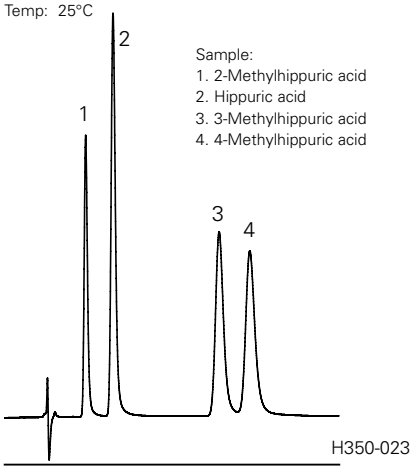


Figure 10: PEGs and PEAPs

Hippuric Acid and Isomers

Hypercarb, 5µm, 100x4.6mm

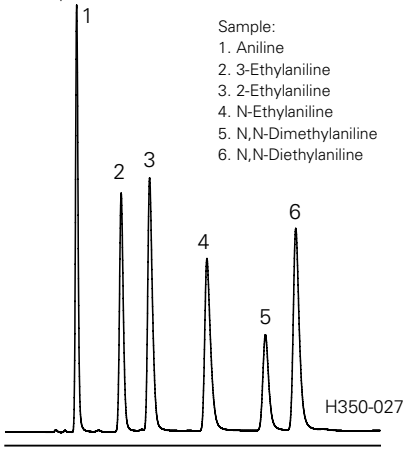
Eluent: A: H_2O + 0.1% TFA
B: ACN/IPA (1:1) + 0.1% TFA
Isocratic: A:B (40:60)
Flow: 1 mL/min
Detection: UV at 225nm
Temp: 25°C



Anilines (Organic Bases)

Hypercarb, 5µm, 100x4.6mm

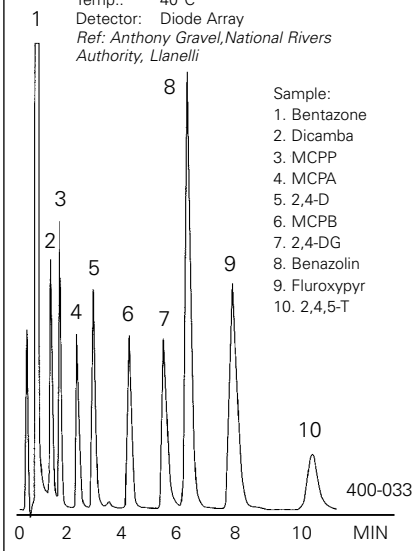
Eluent: A: 10 mM 1-methylpiperidine (pH 10.5)
B: ACN/IPA (1:1)
Gradient: 50-90%B in 10min.
Flow: 1 mL/min
Detection: UV at 270nm
Temp: 25°C



Phenoxy Acids

Hypercarb, 7µm, 100x4.6mm

Eluent: 85% ACN / 15% 1%TFA in H_2O
Flow : 1.0 mL/min
Temp.: 40°C
Detector: Diode Array
Ref: Anthony Gravel,National Rivers Authority, Llanelli

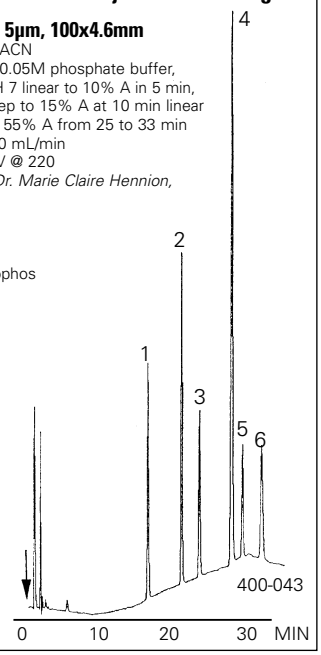


Pesticides-Direct Injection of 150 ng

Hypercarb, 5µm, 100x4.6mm

Gradient: A:ACN
B:0.05M phosphate buffer, pH 7 linear to 10% A in 5 min, step to 15% A at 10 min linear to 55% A from 25 to 33 min
Flow : 1.0 mL/min
Detector: UV @ 220
Courtesy of Dr. Marie Claire Hennion, ESPCI, Paris

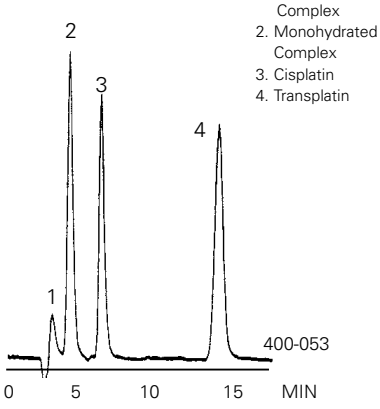
Sample:
1. Oxamyl
2. Monocrotophos
3. Methomyl
4. DEA
5. Aminocarb
6. Fenuron



Cisplatin and its Mono- and Dihydrated Complexes

Hypercarb, 7µm, 100x4.6mm

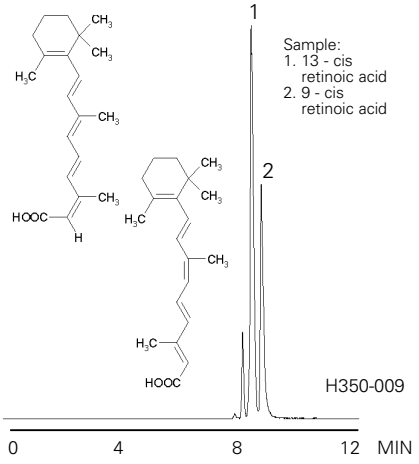
Eluent: 0.001M NaOH
Flow : 0.5 mL/min
Detector: Mass Spec
Temp.: 20°C
Ref.: Anal. Chem. 1995, 67, 3608-3611



Retinoic Acid Isomers

Hypercarb, 5µm, 50x2.1mm

Gradient: A: H_2O + 0.1% DEA
B: ACN / IPA (1:1) +0.1% DEA
3 to 100% B in 10 min
Flow : 0.4 mL/min
Detector: - ESI, 500°C,
4.0 kV, 20, Scan 200 - 400



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10. Barrett D.A. *et al* Chromatographia Vol. 47, No. 11/12, June 1998

Hypercarb Columns

DESCRIPTION	LENGTH (mm)	4.6 mm ID	3.0 mm ID	2.1 mm ID
3 µm Hypercarb	30	35003-034630	35003-033030	35003-032130
	50	35003-054630	35003-053030	35003-052130
	100	35003-104630	35003-103030	35003-102130
5 µm Hypercarb	30	35005-034630	35005-033030	35005-032130
	50	35005-054630	35005-053030	35005-052130
	100	35005-104630	35005-103030	35005-102130
7 µm Hypercarb	50	35007-054630	35007-053030	35007-052130
	100	35007-104630	35007-103030	35007-102130

Other column dimensions are also available. Please call Customer Service for more information. To order standard columns with integral guard (COLUMNPLUS Guard or CPG), please change the last 2 digits of the part number above to 31.

Hypercarb Drop-In Guard Cartridges (pk/2)

DESCRIPTION	LENGTH (mm)	4.6 mm ID	3.0 mm ID	2.1 mm ID
3 µm Hypercarb	10	35003-014001	35003-013001	35003-012101
5 µm Hypercarb	10	35005-014001	35005-013001	35005-012101
7 µm Hypercarb	10	35007-014001	35007-013001	35007-012101
UNIGUARD Direct-Connect Drop-in Guard Cartridge Holder		850-00	852-00	852-00

Note: 4.6 mm Drop-Ins are used for both 4.0 and 4.6 mm analytical columns.

Hypercarb KAPPA Capillary Columns

DESCRIPTION	LENGTH (mm)	500 µm ID	320 µm ID	180 µm ID
3 µm Hypercarb	50	35003-050565	35003-050365	35003-050265
	100	35003-100565	35003-100365	35003-100265
5 µm Hypercarb	50	35005-050565	35005-050365	35005-050265
	100	35005-100565	35005-100365	35005-100265

Other column dimensions are also available. Please call Customer Service for more information.

Hypercarb KAPPA Capillary Guard Columns

DESCRIPTION	LENGTH (mm)	500 µm ID	320 µm ID	180 µm ID
3 µm Hypercarb	30	35003-030515	35003-030315	35003-030215
5 µm Hypercarb	30	35005-030515	35005-030315	35005-030215

Other Hardware Designs for Hypercarb Columns

PIONEER™ Columns		Direct connection columns
Javelin™ Guard Columns		Direct connection column protection
DASH™ Columns		Short, fast columns for high throughput analyses
SLIPFREE™ Column Connectors		Easy to use, void and leak free connectors
Preparative Columns		Available in 10, 21.2, 30, 40, 50 and 100 mm ID

ISO 9001:2000
FM 76231

Thermo Electron Corporation, Bellefonte, PA is ISO Certified.

Thermo Hypersil Ltd., Runcorn, UK is ISO Certified.

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