Method development strategy for Hypercarb®

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Hypercarb is a unique stationary phase for high performance liquid chromatography, with properties diverse from traditional silica gel stationary phases. Having a non-derivatised porous graphitic carbon surface, free from functional groups, means that unique retention and separation of polar compounds as well as geometric isomers can be achieved. We discuss a 'new generic' approach which comprises the starting conditions for obtaining a separation on Hypercarb, as well as discussing new applications.

Porous Graphitic Carbon (Hypercarb) has special characteristics as a stationary phase in HPLC. The physical properties differ significantly from those of traditional silica stationary phases, and thus Hypercarb provides the chromatographer with increased method development options.

Hypercarb particles are spherical and fully porous, showing a particle porosity of around 75%. At the molecular level it is composed of flat sheets of hexagonally arranged carbon atoms. This homogeneous, unmodified surface is highly reproducible and contains no active sites for secondary interactions.

Hypercarb provides unique retention and separation of polar compounds. The surface of Hypercarb is stereo selective with the capability to separate geometric isomers and other closely related compounds. Hypercarb is stable across the entire pH range 0-14, and is not affected by aggressive solvent systems. Its compatibility with most solvent systems enables separation of a wide range of polarities within a single chromatographic run.

The strength of analyte interactions is largely dependent on the size and orientation of the molecular area in contact with the graphite surface, and also on the positioning of the analytes functional groups at the graphite surface. Ross[1] discussed the polarisability of the surface of graphite as the key to understanding the mechanism by which polar compounds are retained (Figure 1).

Ross and Knox[2, 3] calculated values pertaining to the polar retention effect on graphite, by comparing retention behavior of test polar solutes with those of equivalent hydrocarbons. The overall retention on graphite is a combination of two mechanisms: the dispersive interactions between analyte – mobile phase and analyte – graphite surface, and charge induced interactions of a polar analyte with the polarisable surface of graphite, in which the electronic distribution of charge is relevant.

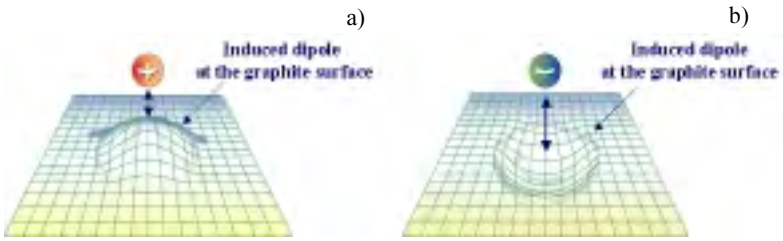


Figure 1. Schematic representation of polar analyte retention in which a) positive and b) negative charges approach the graphite surface, resulting in a charge-induced dipole at the graphite surface.

Generic Method

The unique retention mechanism of Hypercarb requires a different method development strategy. Hypercarb provides increased dispersive interactions relative to other supports, and thus a relatively short 100 x 4.6mm 5µm column is a good starting choice. The elutropic series associated with silica does not always apply to Hypercarb. Methanol and acetonitrile are similar in strength but weaker solvents than 2-propanol (IPA) or dichloromethane and tetrahydrofuran (THF), the latter two being the strongest of the series. The pressure difficulties associated with the use of 2-propanol may be overcome by mixing it with acetonitrile in a proportion of 1:1 or 3:1. This approach increases the relative elution strength of acetonitrile whilst avoiding excessive backpressure.

If compounds with a wide range of polarities or unknown compounds are contained within the sample, then the method described in Figure 2 is proposed as an initial scouting run. In the first 10 min of the run the percentage of the solvent of intermediate strength (acetonitrile/IPA) is increased from 30 to 100% for elution of the ionisable and neutral compounds of high and intermediate polarity. During the following 15 min the gradient of the strongest solvent (dichloromethane) develops from 0 to 75%, with the consequent elution of the less polar and apolar analytes.

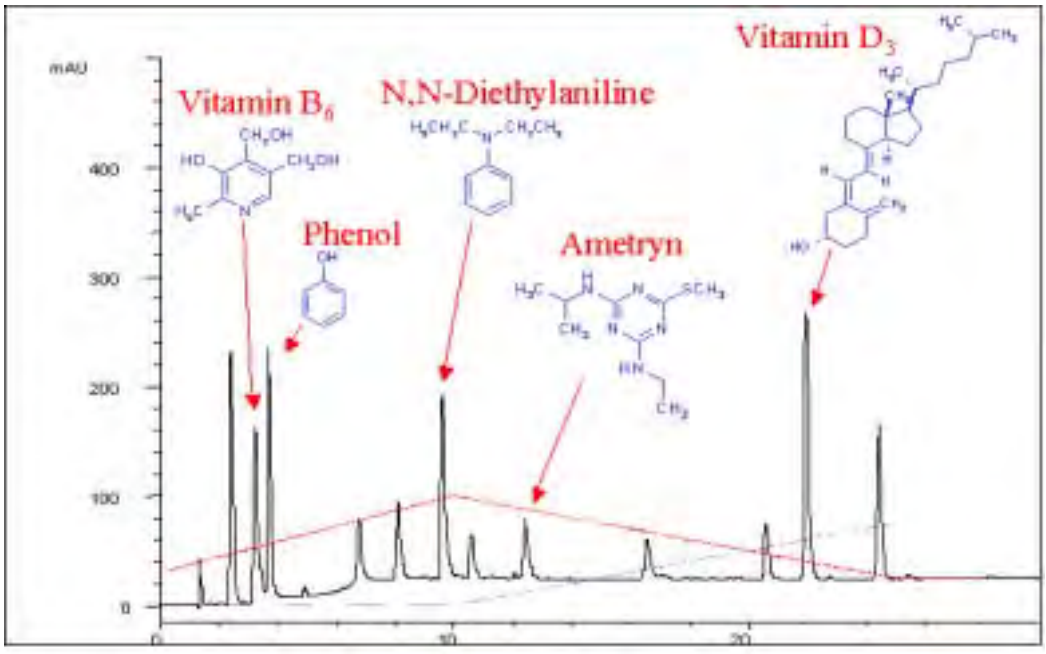


Figure2. 30% to 100% MeCN:IPA(1:1) in 10 minutes, then 0 to 75% DCM in further 15 minutes

Figure 2 shows the successful separation of water soluble vitamins from neutrals, bases, triazines and fat soluble vitamins. This test mix has a wide range of polarities and planarity's. In general, the analysis of a mixture of compound with these characteristics would require two chromatographic systems, one reverse phase one normal phase. The unique properties of the Hypercarb surface allow both solvent systems to be run in tandem, so that the separation can be achieved. Alternatively, if the polarity of the solutes is known, or if in an initial scouting run they all elute from the column in either stage of the gradient, then only one stage of the gradient need be run.

Optimisation of conditions

Example 1. Once the relative retentions of the analytes have been found then the gradient can be optimised for the solutes. Addition of a competitive modifier for the graphite surface, such as TFA, can significantly improve resolution and peak asymmetry for acidic and basic compounds. Figure 3 demonstrates the optimisation of the separation of 4 hippuric acids. In figure 3a a generic gradient of water containing 0.1% TFA to MeCN/IPA (1:3) containing 0.1%TFA, 5% - 100% B in 10 minutes was used. Resolution and run time were improved (figure 3b) by changing to isocratic conditions and by reducing the strength of the organic phase.

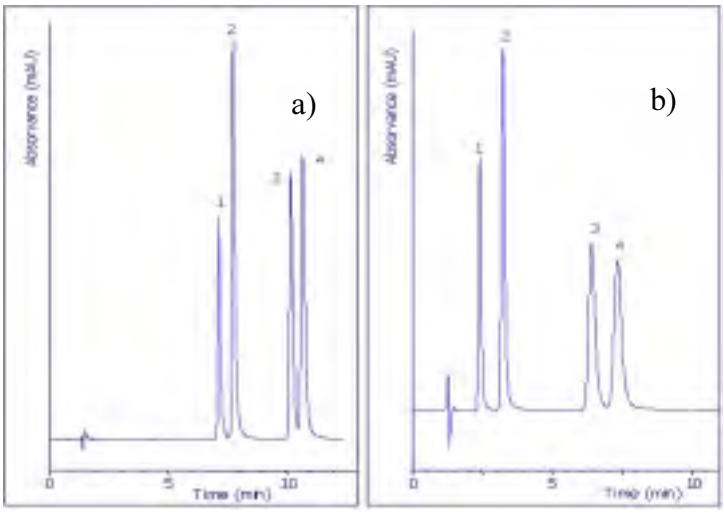


Figure 3. 1) 2-Methylhippuric acid, 2) Hippuric acid, 3) 3-Methylhippuric acid, 4) 4-Methylhippuric acid
a) 0.1% TFA to MeCN:IPA (1:3)+0.1% TFA 5 to 100% B in 10 Minutes
b) 60% MeCN:IPA (1:1) 40% 0.1% TFA

Example 2. The same generic gradient was used for the separation of six ß-Blockers (Figure 4a). Optimisation of the gradient to remove the unnecessary time at the start of the chromatogram and also to baseline resolve the critical pairs of analytes 2,3 and 5,6, means a change in the concentration of TFA to 0.5% and a slight change in the gradient start and finish ratios (Figure 4b). The Hypercarb physical stability allows high concentrations of aggressive buffers such as 0.5% TFA to be safely used with no detrimental effect on the graphite surface.

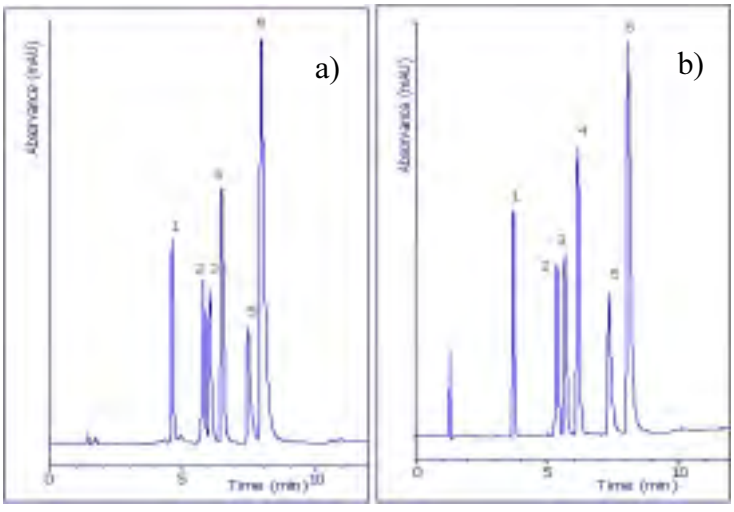


Figure 4. 1) Atenolol, 2) Nadolol, 3) Metoprolol, 4) Timolol, 5) Alprenolol, 6) Pindolol
a) 0.1% TFA to MeCN:IPA (1:3)+0.1% TFA 5 to 100% B in 10 Minutes
b) 0.5% TFA to MeCN:IPA (1:3)+0.5% TFA 15 to 95% B in 10 Minutes

Effect of TFA concentration. Increasing the concentration of TFA in the mobile phase can have significant effects on the peak shape and resolution of analytes as is shown in the separation of procainamide and its metabolites in Figure 5. Concentration was increased from 0.05% to 1.0% TFA, all other conditions remained constant, levels above 1.0% did not show any further improvements for these analytes.

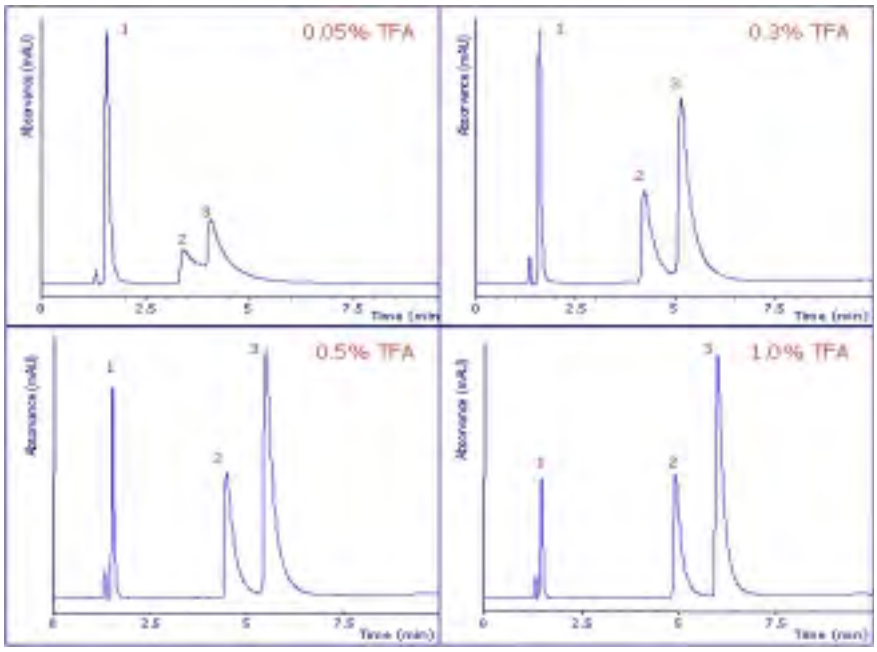


Figure 5. 1)Procainamide, 2) N-Acetyl procainamide 3) N-Propionyl procainamide
x% TFA to MeCN:IPA (1:3) + x% TFA 35 to 95% B in 10 Minutes

Effect of type of modifier. Alternatively for basic compounds switching to 1-methylpiperidine can improve peak asymmetry and resolution if TFA does not give improved results. Figure 6 shows how changing from TFA to 10mM methylpiperidine increased the sensitivity, resolution and peak shape significantly, for six basic anilines. The separation can actually be achieved with no buffer present in the mobile phase, however the methylpiperidine shows an improvement of >20% in peak asymmetry, compared with a non-buffered solution.

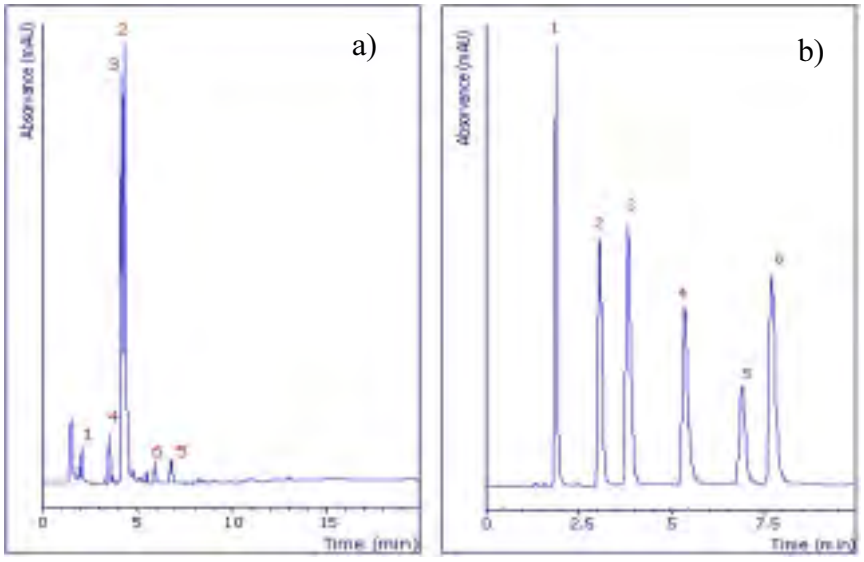


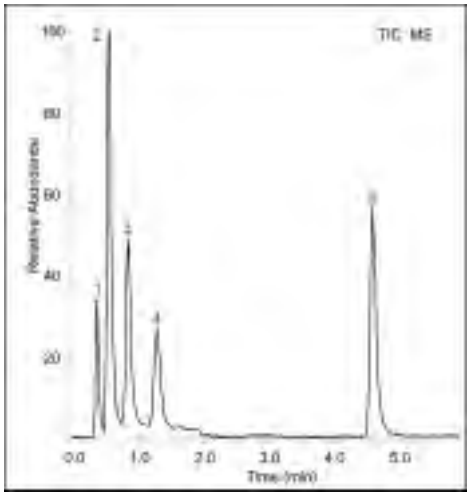
Figure 6. 1) Aniline, 2) 3-Ethylaniline, 3) 2-Ethylaniline, 4) N-Ethylaniline, 5) N,N-Dimethylaniline, 6) N,N-Diethylaniline
a) 0.1% TFA to MeCN:IPA (1:3)+0.1% TFA 5 to 100% B in 10 Minutes
b)10mM 1-Methylpiperidine pH 10.5 to MeCN:IPA (1:1) 50% to 90% B in 10 minutes

[1] P.Ross LC-GC Europe, Vol 13, no.5, May 2000
[2] P.Ross PhD Thesis, Edinburgh University (Edinburgh, Uk, 1999)
[3] J.H.Knox and P.Ross, "Quantification and Correlation of the Energies Associated with Retention of Polar analytes on Porous Graphitic Carbon" poster HPLC 99' Granada, Spain, 30 May- 4 June 1999

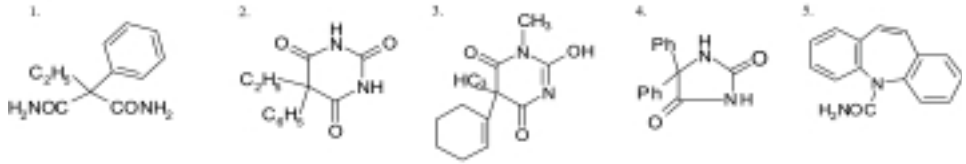
Anticonvulsants

Anticonvulsants are a therapeutic class which includes compounds from several different groups. These include the barbiturates and compounds similar to the tricyclic antidepressants.

Hypercarb has been demonstrated to provide good selectivity of this range of compounds. The strongest retained compounds are predictably the tricyclic aromatics, which exhibit the most planar structure, enabling maximum contact with the porous graphitic surface. Hypercarb is also stable at extreme pHs and does not show dissolution or hydrolysis, as with silica phases outside the normal 2 – 8 pH range.

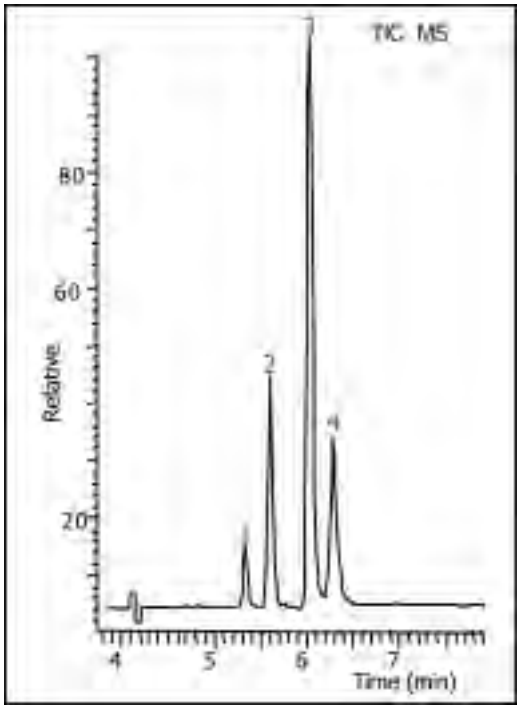


Column:	Hypercarb 30x2.1mm, 5µm
Mobile phase:	A - Ammonium acetate 50mM, pH 9.5
	B - Acetonitrile
Gradient:	27 to 100% B in 5 min
Flow rate:	0.4 ml/min
Temperature:	25°C
Detection:	APCI
	t= 0 to 0.5 min +APCI
	t= 0.5 to 2 min -APCI
	t= 2 to 6 min +APCI
Analytes:	1. 2-Ethyl-2-Phenylmalonamine
	2. Phenobarbital
	3. Hexobarbital
	4. 5,5-Diphenylhydantoin
	5. Carbamazepine



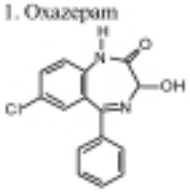
Benzodiazepins

These depress the central nervous system by acting on the GABA (gamma-amino butyric acid) receptor complex which is the main inhibitory neurotransmitter in the CNS. This produces effects such as sedation, hypnosis and the reduction in the severity of convulsions. Benzodiazepins are commonly used in the treatment of psychological disorders including anxiety and insomnia but are also substances of abuse.

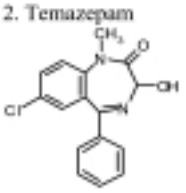


Column:	Hypercarb 50 x 2.1mm 5µm
Mobile phase:	(A) Ammonium acetate 50mM pH9.0
	(B) Acetonitrile/IPA (1:1)
Gradient:	2 to 100% B in 3 min
Flow rate:	0.4 ml/min
Temperature:	25 °C
Detection:	+ESI, Probe Temperature: 500°C, Electrospray voltage: 4.5kV

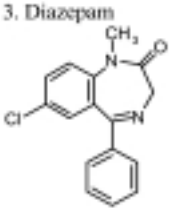
1. Oxazepam



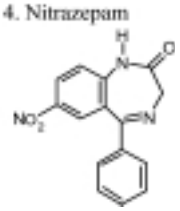
2. Temazepam



3. Diazepam

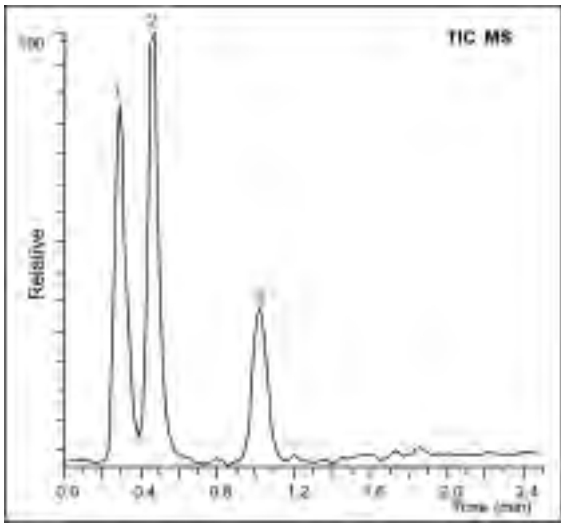


4. Nitrazepam



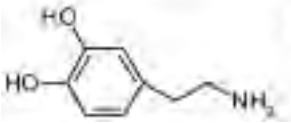
Catecholamines

These biogenic compounds have important functions in the central nervous system, stimulating specific receptors within the CNS, producing a range of physical and biological responses including cardiac stimulation, vasodilation and bronchial relaxation.

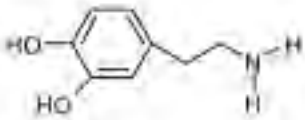


Column:	Hypercarb 50 x 2.1mm 5µm
Mobile phase:	(A) 0.5 % Formic acid + H_2O (B) Acetonitrile/formic acid (99.5:0.5)
Gradient:	13 to 50 % B in 2 min
Flow rate:	0.4 ml/min
Temperature:	25 °C
Detection:	+ESI, Probe temperature: 450°C, Electrospray voltage 4.5 kV

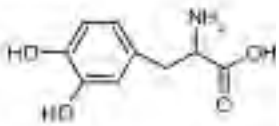
1. Adrenaline (Epinephrine)



2. Dopamine

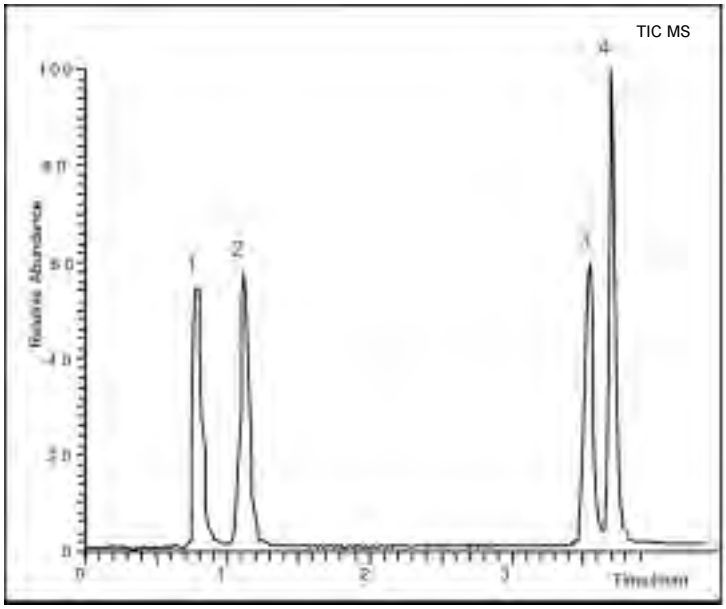


3. L-Dopa



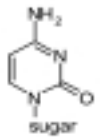
Nucleosides

These are building blocks for nucleic acid chains and consist of a nitrogenous base (purines or pyramidines) bonded to a sugar (ribose or deoxy-ribose). Nucleosides when bound to a phosphate are named nucleotides, which are the 'links' in the nucleic acid chain.

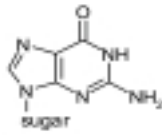


Column:	Hypercarb 50 x 2.1mm 5mm
Mobile phase:	(A) Water (B) Acetonitrile
Gradient:	53 to 100 % B in 2 min
Flow rate:	0.4 ml/min
Temperature:	25 °C
Detection:	-ESI, Probe Temperature 400°C Electrospray voltage 4.0 kV

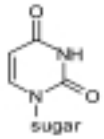
1. Cytidine



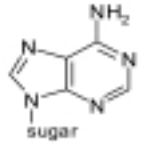
3. Guanosine



2. Uridine



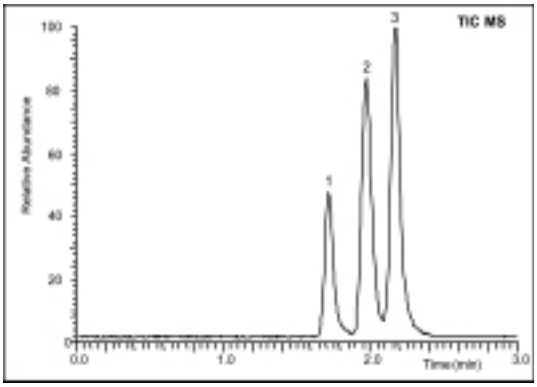
4. Adenosine



Small Polar Peptides

Structural elucidation of proteins is accomplished by a procedure often referred to as peptide mapping. This typically involves the digestion of the protein with a proteolytic enzyme yielding smaller peptides which are then separated using liquid chromatography. However, conventional phases such as C18 are generally too hydrophobic to retain the more polar peptides and these tend to be lost in the solvent front. Polar peptides are generally short and contain polar amino acids (e.g. lysine & arginine).

Hypercarb by virtue of its porous graphitic surface exhibits exceptional retention of short polar peptides. The buffer matrix which is employed during digestion of the parent protein is removed at the solvent front thus enabling further characterisation of the polar peptides (e.g. spectroscopy or Edman degradation).



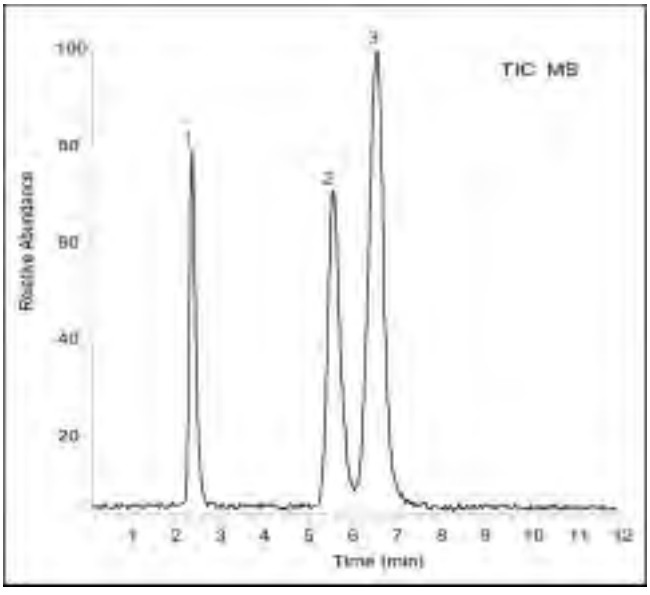
Column:	Hypercarb 100x4.6mm, 5µm
Mobile phase:	A -H_2O + 0.1% methylpiperidine B - Acetonitrile + 0.1% methylpiperidine
Gradient:	20 to 100% B in 10 min
Flow rate:	0.5 ml/min
Temperature:	30°C
Detection:	+ESI, Probe Temperature 400°C Electrospray voltage 4.5 kV

1. Asp-Ser-Asp-Pro-Arg
2. Arg-Gly-Glu-Ser
3. Thr-Ser-Lys

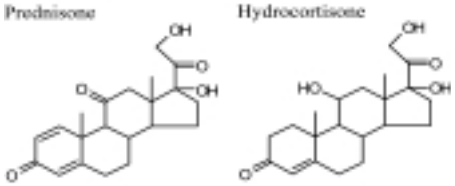
Steroids

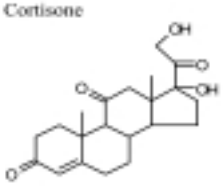
Steroids are a large class of compounds with applications in biology, chemistry and medicine. The analysis of these compounds is commonly carried out using C18 phases with intermediate concentrations of organic modifier (typically 60 %).

Hypercarb is particularly suited to separating large organic molecules such as steroids in mobile phases with high organic contents. Such mobile phases lend themselves particularly well to mass spectroscopic detection.



Column:	Hypercarb 30x3 mm, 5µm
Mobile phase:	A - H_2O + 1% acetic acid B - Methanol + 1% acetic acid Isocratic (5:95) A:B
Flow rate:	0.6 ml/min
Temperature:	40°C
Detection:	+APCI, Probe Temperature 400°C, Corona discharge 4.5kV
Analytes:	1. Prednisone 2. Hydrocortisone 3. Cortisone

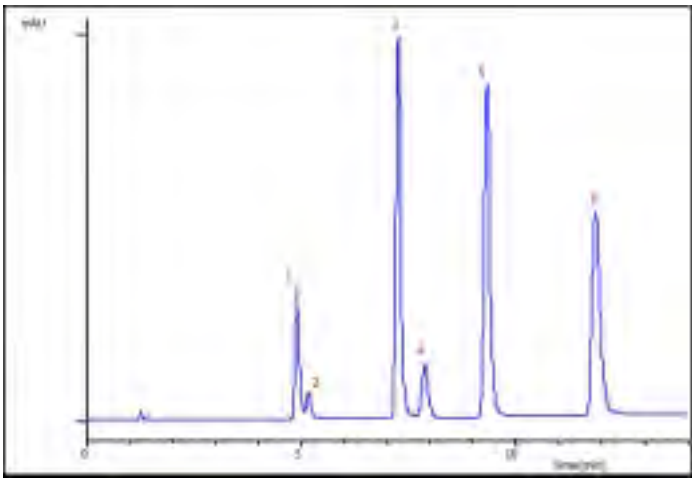




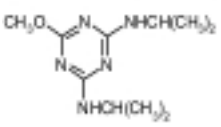
Triazines

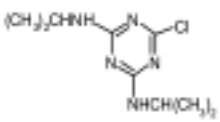
Triazine compounds consist of a six-membered heterocyclic ring containing three nitrogen and three carbon atoms and are predominantly utilised as pesticides in the agricultural sectors.

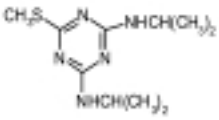
These compounds demonstrate the applicability of Hypercarb to compounds of intermediate polarity and also its high selectivity for similarly substituted structures.

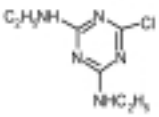


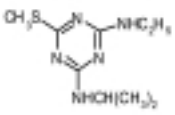
Column:	Hypercarb 100 x 4.6mm, 5µm
Mobile phase:	A - Water B - Acetonitrile / isopropanol (1:3)
Gradient:	T (min) %B 0 35 10 95
Flow rate:	1 ml/min
Temperature:	60°C
Detection (UV):	240 nm

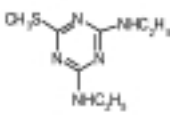
1. Prometon


2. Propazine


3. Prometryn


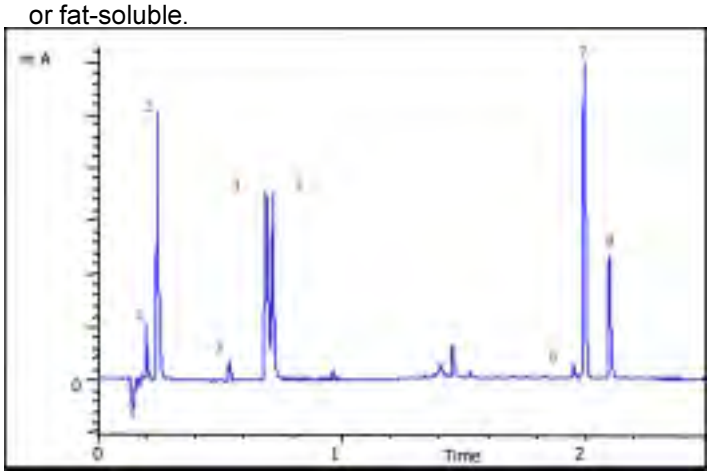
4. Simazine


5. Ametryn


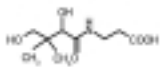
6. Simetryne


Water & Fat Soluble Vitamins

These are small biologically active molecules obtained primarily in the diet in trace quantities. Vitamins are used by the body as coenzymes in biological reactions. They may also be classified as either water or fat-soluble.

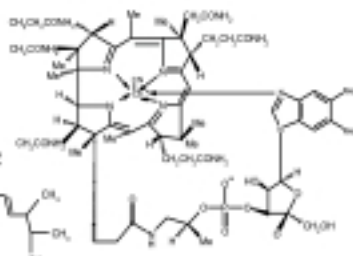


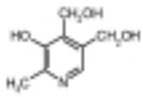
Column:	Hypercarb 100 x 4.6 mm 5mm		
Mobile phase:	(A) Ammonium acetate 50 mM pH 6.0 (B) Acetonitrile/IPA (1:1) (C) Tetrahydrofuran		
Gradient:	Time (min)	%B	%C
	0	7	0
	10	60	0
	12	95	5
	25	0	100
Flow rate:	1.0 ml/min		
Temperature:	25 °C		
Detection:	At 10 min changes from 215 to 275 nm		

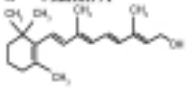
1. Vitamin B5


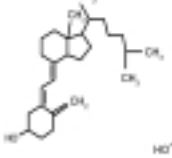
2. Vitamin B3


3. Biotin


4. Vitamin B12


5. Vitamin B6


6. Vitamin A


7. Vitamin D3


8. Vitamin D2