1998

Synthesis and characterization of C-10 bonded silica surfaces for HPLC

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SYNTHESIS AND CHARACTERIZATION OF C-10 BONDED SILICA SURFACES FOR HPLC

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

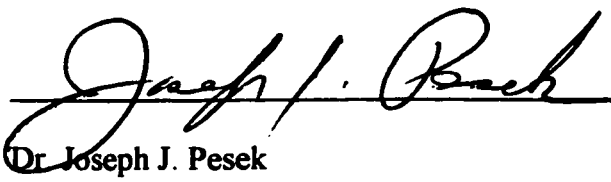
Master of Science

by

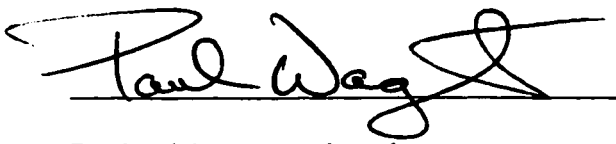
Seema J. Prabhakaran

August 1998

APPROVED FOR THE DEPARTMENT OF CHEMISTRY



Dr. Joseph J. Pesek



Dr. Paul S. Wagenknecht

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ABSTRACT

SYNTHESIS AND CHARACTERIZATION OF C-10 BONDED SILICA SURFACES FOR HPLC

by Seema J. Prabhakaran

The goal of this research was to provide practical experience in the use of acetylene and olefin compounds in the modification of silica surfaces to form very hydrophobic reverse phase materials for High Performance Liquid Chromatography (HPLC). In this study 1,9-decadiyne and 1,9-decadiene reverse phase materials which consist of C10 alkyl groups attached to the surface of 300 angstrom pore diameter silica were packed into HPLC columns. A comparative study was devised because these compounds have very similar structures.

A hydrosilation reaction was used to prepare the bonded phases. Infrared Fourier Transform in the Diffuse Reflectance Mode (DRIFT) and Cross Polarization Magic Angle Spinning Nuclear Magnetic Resonance (CP-MAS-NMR) were used for characterization. Most interestingly, this study illustrates the effects of mobile phase and pH on the retention and selectivity of drugs, Polycyclic Aromatic Hydrocarbons (PAH's) and basic compounds. Both isocratic and gradient elution were used to evaluate column efficiency.

ACKNOWLEDGEMENTS

I would like to show my greatest appreciation to my advisor, Dr. Joseph Pesek and his project scientist Dr. Maria Matyska. With their tremendous help, guidance and support, I have been able to fulfill my goals in this thesis work. I have learned a lot under their supervision.

I would also like to thank my committee members Dr. Pamela Stacks and Dr. Paul Wagenknecht for their valuable time in reviewing my manuscript. Their suggestions and advice have been a great help.

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CHAPTER I

INTRODUCTION

A. Silica Gel

Silica-based materials are extensively used as solid supports for HPLC systems. Silica supports are superior to other oxides (ex., titania, thoria and zirconia) in terms of efficiency, rigidity and performance. Unlike polymer supports, they do not shrink or swell in different solvents. Silica has a very porous nature and its high internal surface area enhances the analyte-stationary phase interactions. A number of reactions can and have been used for the attachment of organic ligands to silica, which are responsible for a range of surface properties varying from hydrophobic to hydrophilic and ionic in nature. The reactions that are used to modify silica give rise to supports of fairly high mechanical strength and reproducibility (i.e. the column selectivity and sample resolution remains the same over a long period) [1]. Silica has an extremely porous nature and bears silanols (Si-OH) as well as siloxane linkages (Si-O-Si). The silanols are considered very strong adsorption sites and siloxanes are usually hydrophobic. The hydrophobic interactions between the solute and siloxane are responsible for the retention of non-polar molecules on its site. Also, bonded silica equilibrates rapidly to new solvent conditions. However, the main drawback of silica supports is that it causes severe peak tailing in the separation of basic compounds. It has very limited pH stability as it is highly unstable below pH 2 and above pH 9. Above pH 9 the silica supports are susceptible to dissolution in aqueous solutions. Below pH 2.0 the siloxane linkage is labile, and the functional groups on the surface will begin to cleave, changing the sorptive properties in a non-reproducible

fashion [2]. However, bonded silica is chemically stable to virtually all organic solvents. Silica has a wide range of physical properties. The one used in this study is the Vydac-TP 106. This silica has a particle size of 6.5 microns, pore diameter of 300 angstroms and surface area of 106 m^2/g.

Vydac's 300 angstrom pore diameter TP silica has been the standard for separation for more than a decade. A diameter of 300 angstroms has repeatedly been proven superior to smaller pore silica for most applications. Not only do these large pores allow large molecules to enter the support interior but also the surface of the large pore silica seems to be better suited to the separation of even small molecules.

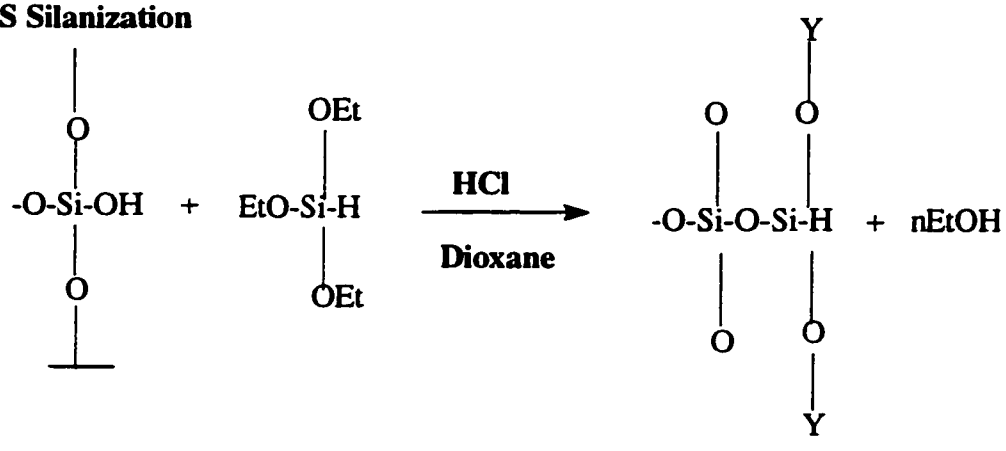
1. Modification Procedures of Silica

Different modification procedures can and have been used for the past 25 years and many types of such bonded phases have been studied using HPLC. Since most of the bonded phases utilize silica as the support material, the bonding of the organic moiety depends on the chemistry of silanols. One type of reaction, is an esterification reaction that involves a reaction between an alcohol and the silanols on the surface to form an Si-O-C linkage. This reaction is not used frequently because the Si-O-C linkages are hydrolytically unstable [3]. Another type of reaction is an organosilanization reaction, between an organosilane reagent and the silanols. The method gives rise to Si-O-Si-C linked surfaces [3]. Another method involves chlorination followed by reaction of a Grignard reagent (BrMgR) or organolithium (LiR) compounds. The reaction requires extremely dry conditions and hence is less preferred. More recently, a silanization reaction followed by hydrosilation reaction has been used. This reaction pathway which

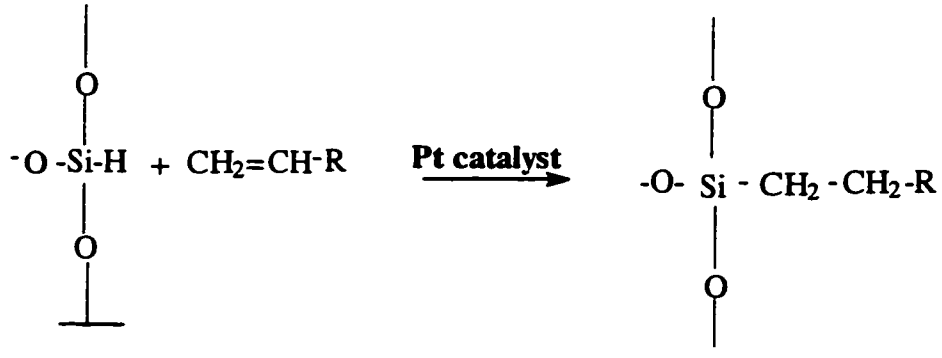
was first introduced by Chu et al., consists of two steps [4]. The first reaction is silanization where the surface is treated with an appropriate reagent, like triethoxysilane (TES) to give an Si-H monolayer as is shown below [5]. The second reaction, hydrosilation, involves the reaction of an inorganic silica hydride with the multiple bonds of an organic compound forming a stable product. An anti-Markownikov addition takes place across the double bond forming an Si-C linked bonded surface [5]. Hexachloroplatinic acid, commonly known as Speier's catalyst, introduced by Speier and coworkers are used to facilitate the reaction [6].

The reaction sequence for attachment of an olefin is as follows:

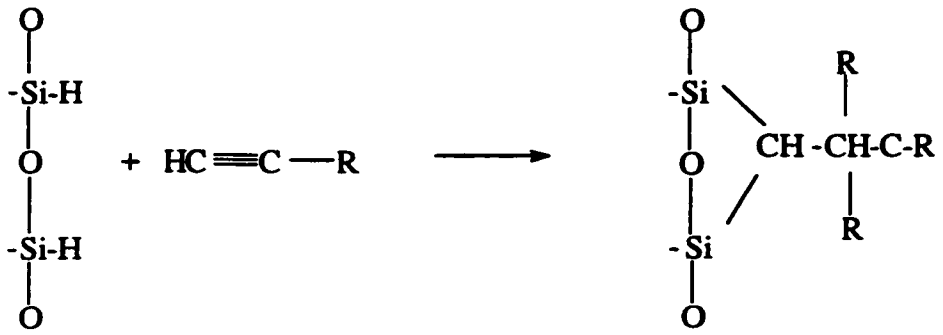
(1) **TES Silanization**



(2) **Hydrosilation**

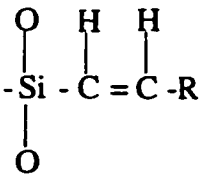


An interesting possibility for the hydrosilation of acetylene compounds is the formation of double Si-C linkage between the surface and the bonded organic moiety. One such bonded material that would result in a high degree of stability is shown below.

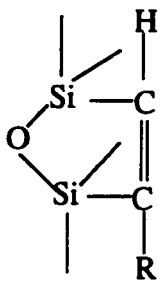


Structure I

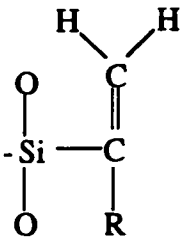
The structure of three other possible bonded species containing an olefin group are as shown below.



Structure II



Structure III



Structure IV

2. Free Radical Initiation Reaction

As an alternative method of catalysis, free radical initiators can be used in place of the transition metal catalyst (Speier's catalyst). Even though many different compounds could be used, this study mainly uses azoisobutyronitrile, AIBN. This experiment was done to determine if free radical initiators are viable for hydrosilation reactions in heterogeneous reactions [5,7].

The main advantages of hydrosilation are that it is a synthetically versatile technique and a large number of functional groups can be bonded. Moreover, more than one type of catalyst can be used [5]. Also, the TES used in the silanization reaction considerably reduces the number of silanols on the surface which would otherwise interfere with column efficiency.

B. High Performance Liquid Chromatography

1. Theory of Operation

a. Stationary Phase: refers to the solid support contained within the column, through which the mobile phase continuously flows. While flowing it also carries the sample of interest and the components in that sample will migrate according to the non-covalent interactions with the stationary phase. The chemical interactions between the solute and the stationary phase determine the degree of separation of the different components in the sample [8].

b. Mobile Phase: refers to the solvent being continuously applied to the stationary phase. The sample that is injected through the injector port is carried by the mobile phase through the column. The most commonly used mobile phase types include isocratic

and gradient. In isocratic elutions, compounds are eluted using a constant mobile phase composition. Whereas, in gradient elutions the compounds are eluted by increasing the strength of the organic solvent in a stepwise or linear fashion.

c. Injector: samples are usually introduced into the column through the injection port that consists of an injection valve and a sample loop [8].

d. Pump: is responsible for delivering the mobile phase into the column from the solvent reservoir [8].

e. Detector: this is the unit that generates a response due to the eluting compound and subsequently produces a peak on the chromatogram. These devices are directly interfaced with the column to detect the compounds that elute from the column [8].

C. Non-polar Interactions in Reverse Phase HPLC

Non-polar interactions usually occur between the carbon-hydrogen bonds of the organic moiety present on the stationary phase and the carbon-hydrogen bonds of the analyte. These forces are also known as "van der Waals" or "dispersion" forces. Since most organic molecules have some non-polar structure, non-polar interactions or hydrophobic interactions are often used to retain analytes on the surface of the stationary phases. Non-polar interactions are very effective for isolating groups of compounds that are dissimilar in structure. Also, these interactions are often the mechanism of choice for applications where the maximum possible number of analytes with differing chemical properties are to be resolved, as in many environmental applications.

Non-polar interactions between non-polar stationary phases and the non-polar analytes are facilitated by mobile phase environments that are very polar in nature. The

reason being that the polar mobile phase-support environment is generally not capable of disrupting the dispersion forces of the non-polar interaction. Even where the analyte has polar groups, any significantly non-polar part of the compound will interact with non-polar functional groups of the support in a polar environment. The best example of a solvent that facilitates retention due to non-polar interactions is water [8].

D. Acetylene Bonded Silica

In the beginning of this study, five different compounds were selected and their bonding patterns on the silica surface were studied. They are listed in Table 1.

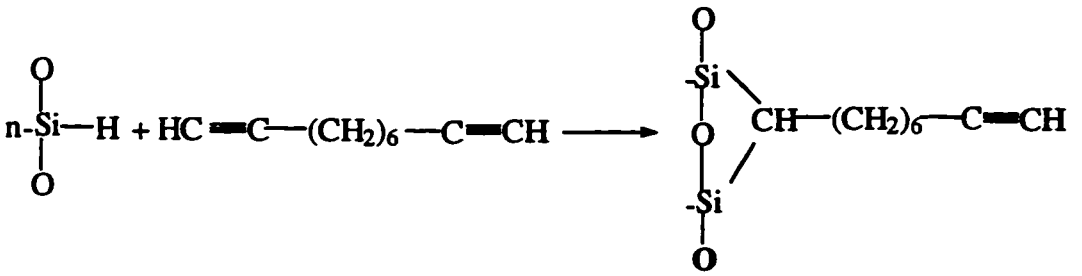
Table 1: Organic Compounds Data Table

Compound	Molecular Formulae	Molecular Weight
1,9-decadiyne	$HC{\equiv}C\text{-}(CH_2)_6\text{-}C{\equiv}CH$	134.22
1,9-decadiene	$H_2C{=}CH\text{-}(CH_2)_6\text{-}CH{=}CH_2$	138.25
3,9-dodecadiyne	$CH_3\text{-}CH_2\text{-}C{\equiv}C\text{-}(CH_2)_4\text{-}C{\equiv}C\text{-}CH_2\text{-}CH_3$	162.27
Propiolic acid	$HC{\equiv}C\text{-}COOH$	70.05
1-dimethylamino-2-propyne	$(CH_3)_2N\text{-}CH_2\text{-}C{\equiv}CH$	83.13

From the percentage of surface coverage results it was proven that 1,9-decadiyne and 1,9-decadiene had good bonding to the silica surface. The C10 alkyl chain in the bonded group was expected to exhibit greater selectivity that would facilitate separation. Selectivity is the primary measure of column performance. A column must separate key components to be useful for an assay or purification. It was thought that this project could prove useful for investigating the potential advantages of using acetylene compounds over olefin compounds.

Hydrosilation reactions of all the compounds listed in Table 1 and their possible products are shown below:

1. 1,9-decadiyne



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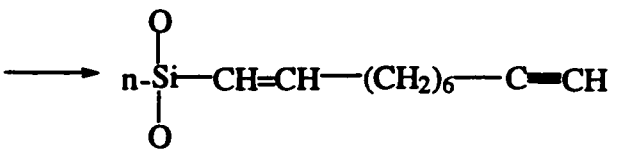
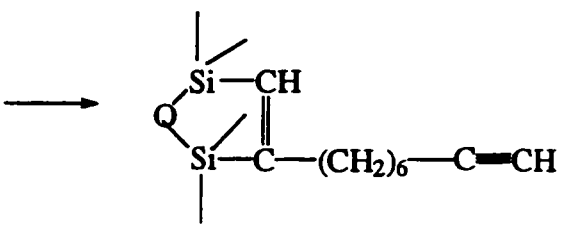
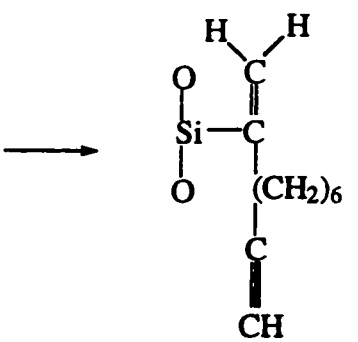


Structure II

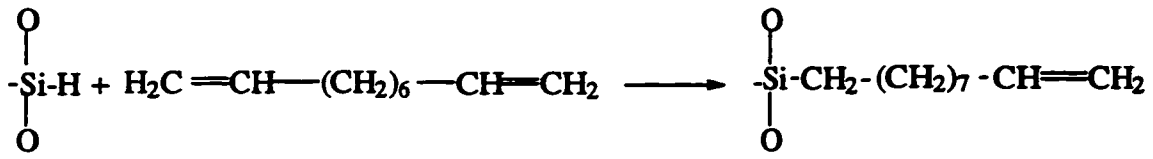


Structure III



Structure IV

2. 1,9-decadiene



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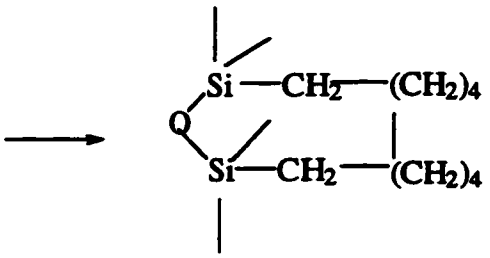

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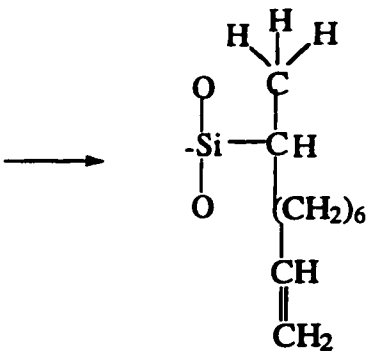
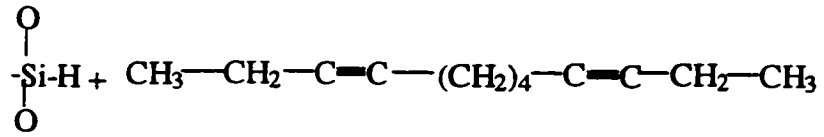
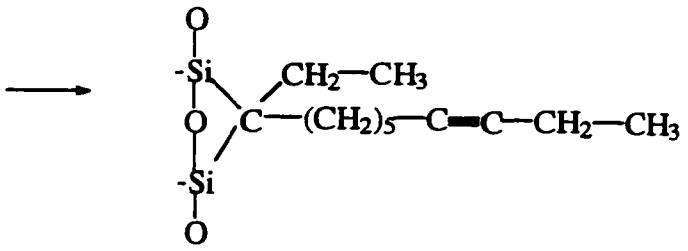


Structure III

3. 3,9-dodecadiyne





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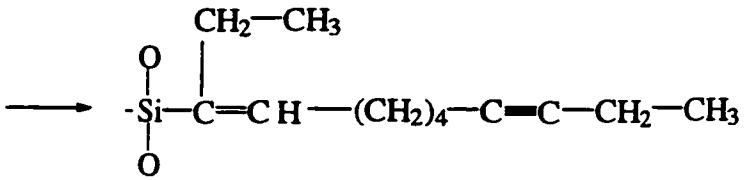

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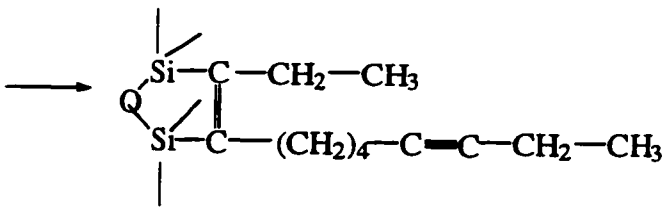
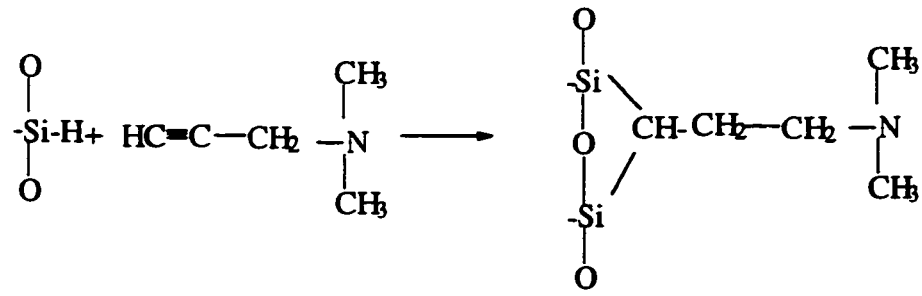


Structure III

4. 1-dimethylamino-2-propyne



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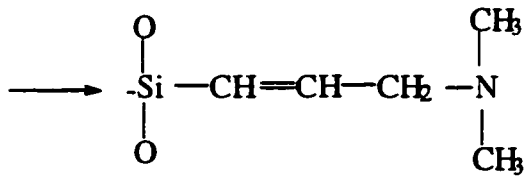
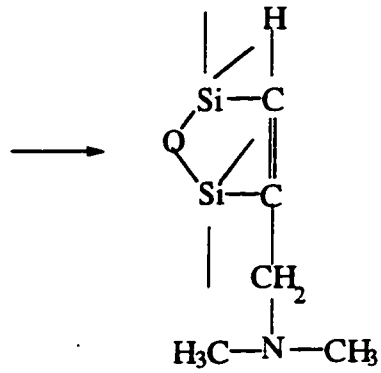


Structure II



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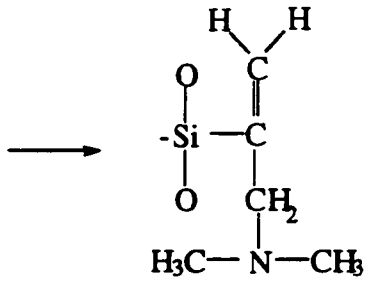
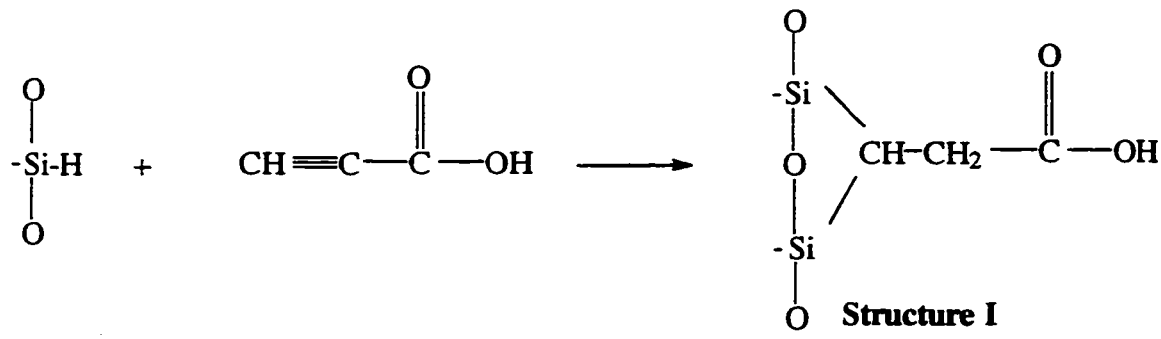


Structure IV

5. Propiolic acid



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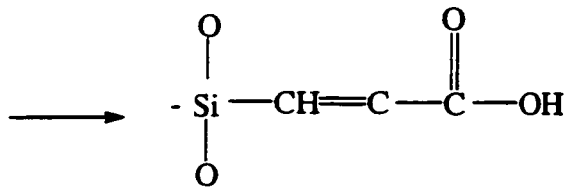

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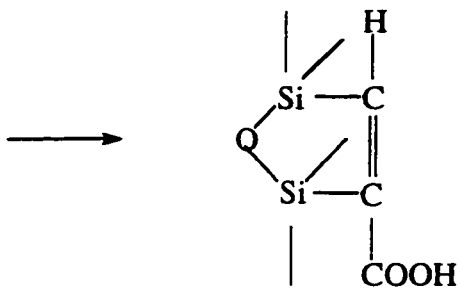

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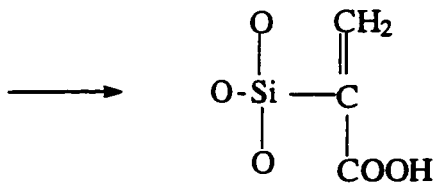


Structure IV

E. CHARACTERIZATION METHODS

Two main characterization methods have been used to determine the success of bonding of organic compounds to the silica surface.

1. Fourier Transform Infrared Spectroscopy (FT-IR)

The most important feature of IR spectroscopy is that it gives structural information about a molecule. The absorption of each type of bond, for example N-H, C-H, O-H, C=O, C-O, C-C, C=C, C≡C, C≡N, are found in certain small portions of the vibrational infrared region. Hence it is very easy to relate the absorption frequencies that are characteristic of certain functional groups in a compound. Since chemical reactions can only take place at the silanols (Si-OH), and they are mainly distributed at the surface, there would be distinct changes in the IR spectrum. Therefore, IR is an excellent tool for studying the surface chemistry of porous oxide materials. For high surface area materials an ordinary transmission spectra can be obtained by mixing KBr to improve light throughput. When working with lower surface area materials it is necessary to signal average, which is possible by acquiring the spectrum in the Fourier Transform mode. Also, an increase in the sensitivity is achieved via diffuse reflectance. A small range of absorption can be defined for each type of bond. Thus, an IR spectrum provides a unique signature for a particular chemical entity [9].

2. Cross Polarization Magic Angle Spinning Nuclear Magnetic Resonance Spectroscopy (CP-MAS NMR)

Besides IR spectroscopy, NMR is another method for determining the structure of an unknown molecule. While IR reveals information about the type of functional groups

present in the molecule, NMR gives information about the number of each type of atom. It also gives information regarding the nature of the immediate environment of the atom of interest. Hence, the combination of IR and NMR spectroscopic methods can provide a very efficient tool for determining the chemical and physical nature of an oxide and modified surfaces.

2a. CP-MAS Carbon-13 NMR

For chemically modified surfaces CP-MAS ^{13}C NMR is often used for characterization. It is usually possible to identify the success of bonding the organic moiety to the silica surface. This is possible by analyzing the resonance of the carbons on the surface. By comparing it with the shifts of the non-bonded species, it is possible to assign general ranges for all the carbons and to specifically identify one or more individual carbons. Hence the verification of the structure using CP-MAS ^{13}C NMR is quite certain [10].

2b. CP-MAS Silicon-29 NMR

CP-MAS ^{29}Si NMR is the most viable technique to study the oxide surfaces that are used in chemically modified stationary phases for HPLC. It has been successful for indicating all the different types of groups on the surface of the silica. It can also tell whether the species has undergone any chemical modification [10].

3. Elemental Analysis

Elemental analysis was done by the conventional method known as micro-combustion. This method is a very sensitive test to monitor the carbon loading on the modified silica. All the bonded silica were analyzed to determine the carbon percentage.

The carbon percentage value is useful in determining the alkyl density on the silica surfaces.

Goals

The first step in this study was to synthesize the silica hydride monolayer and identify the Si-H bonds that were present. To confirm that the organic compounds underwent hydrosilation, different characterization methods could be used; namely Diffuse Reflectance Infrared Fourier transform spectroscopy (DRIFT) and Cross Polarization Magic angle Spinning Nuclear Magnetic Resonance Spectroscopy (CP-MAS NMR). The final goal of this project was to demonstrate the potential of these reverse phase materials in the separation of drugs and other compounds. These columns were expected to improve column stability and allow separations that have not been done before.

CHAPTER II

EXPERIMENTAL

A. Materials

1. Chemicals

Most of the samples for analyses were purchased from Sigma-Aldrich. Their Chemical Abstract Service (CAS) registry numbers are listed in Table 2.

Table 2. The CAS Registry Numbers of Chemicals Used.

Chemical Name	CAS Registry Numbers
2,2-azobisisobutyronitrile	[4187-87-5]
1-dimethyl-2-aminopropyne	[7223-38-3]
1,9-decadiyne	[1720-38-3]
3,9-dodecadiyne	[612827-38-3]
1,9-decadiene	[647-16-1]
Acetonitrile	[75-05-8]
Anthracene	[120-12-7]
Benzene	[71-43-2]
Chlorobenzene	[108-90-7]
Chlorodiazepoxide	[58-25-3]
Clonazepam	[1622-61-3]
Diazepam	[439-14-5]
Diethyl ether	[60-29-7]
Diethanolamine	[111-42-2]
Hexachloroplatinic acid (38-40% Pt)	[16941-12-1]
Hydrochloric acid	[7647-01-0]
Methanol	[67-56-1]

Monoethanolamine	[141-43-5]
Nitrazepam	[146-22-5]
N-nitrosodimethylamine	[62-75-9]
N-nitrosodiethylamine	[55-18-5]
N-nitosodithanolamine	[1116-54-7]
Oxazepam	[604-75-1]
Potassium Bromide	[7758-02-3]
Propiolic Acid	[471-25-0]
Sodium hydroxide	[1310-73-2]
Temazepam	[846-50-4]
Toluene	[108-88-3]
Triethoxysilane (TES)	[998-30-1]
Triethanolamine	[102-71-6]
Tetrahydrofuran	[109-99-8]

2. Chemicals for Preparing Stationary Phases

Propiolic acid, 1,dimethylamino-2-propyne, 1,9-decadiyne, and 3,9-dodecadiyne were purchased from Farchan Laboratories (Gainesville, FL) while 1,9-decadiene was purchased from Aldrich (Milwaukee, WI). All were used as received. Hexachloroplatinic acid hydrate was purchased from Strem Chemicals (Newbury Port, MS). Triethoxysilane (TES) was obtained from Huls Petrarch Systems (Huls, Bristol, PA). Most of the solvents, toluene (EM Science, Gibstown, NJ), diethyl ether (Fisher Scientific, Fair Lawn, NJ), dioxane (Fisher Scientific, Fair Lawn, NJ) and tetrahydrofuran, THF (J.T. Baker Chemicals Co. Phillipsburg, NJ) were obtained in reagent grade. A millipore apparatus was used for the filtration of some of the solvents. The silica used was Vydac TP 106 (The Separations Group, Hesperia, CA) with a particle

diameter of 6.5 micron, a pore diameter of 300 angstroms and a surface area of 106.5 m^2/g.

B. Experimental Methods

1. Preparation of Speier's catalyst

In a nitrogen filled glove box, 0.4 g of chloroplatinic acid hexahydrate (Strem Chemicals, Newbury Port, MS) was added to 100 mL of isopropanol at 93° C [4]. The catalyst, 10 mM Speier's reagent was stored in the refrigerator.

2. Preparation of Silica Hydride via Silanization

The following reactions were done according to Chu et al., [4]. All the glassware was dried before use at 110° C overnight. Typically 10 g of silica was dried under vacuum at 110° C for 24 h.

In a glove box that was thoroughly flushed with nitrogen, 8.45 mL of 1 mM triethoxysilane (TES) was measured into a 50 mL volumetric flask and distilled 1,4-dioxane was added up to the mark. A 500 mL, 3 necked round bottom flask was equipped with a West condenser, addition funnel, a stopper and a magnetic stirring bar. To this 10 g of silica was transferred, followed by 245 mL of dioxane and 10 mL of 2.3 M HCl solution. The joints were sealed with parafilm. The reaction mixture was heated to reflux at 93° C for almost an hour. TES was added dropwise to the flask from the addition funnel over a period of 25 to 30 minutes. After complete addition of TES the reaction mixture was heated at reflux for an hour.

After the apparatus was cooled to room temperature, p-dioxane was poured off and replaced with toluene. The mixture was equally distributed into four centrifuge tubes

(IEC-HNS) that were equipped with stirrer bars. The mixture was washed by stirring for 10 minutes and centrifuged (Damon, IEC div. Model. HNS, 116 Volts). The washing procedure was repeated three more times with fresh 1:1 (v/v) tetrahydrofuran, twice with tetrahydrofuran-water mixture and lastly with ether. Finally the product, silica hydride was transferred to a beaker and covered with a watch glass and placed overnight in the hood to evaporate the solvents. The next day the silica hydride was placed in the drying oven (VWR Scientific) at 110° C for at least one day and was stored in airtight vials.

3. Preparation of Bonded Phases via Hydrosilation

The following reactions were done according to Chu et al., [4]. A 250 mL, 3 necked round bottom flask was equipped with a thermometer, a West condenser and a nitrogen venting system. One hundred mL of toluene, 5 mL of acetylene compound and 100 μL of 10 mM Speier's catalyst were added to the 250 mL flask and refluxed for 1 hour at 100° C. Through the open neck of the flask that was sitting on a heating mantle and a magnetic stirrer, 5 g of silica hydride was added. The flask was then fitted with a glass stopper and sealed with parafilm. The apparatus was refluxed for 100 h at 100° C (close to the boiling point of toluene, 105° C). The contents of the flask were then centrifuged and the solid was collected. The solid was washed three times each with 15-20 mL portions of THF, THF-water mixture and diethylether. The product, derivatized silica, was left to dry overnight in the hood. The next day it was placed in a vacuum oven and dried at 110° C for at least two days. The success of bonding was determined using DRIFT and NMR spectroscopy.

4. Free Radical Initiation Reaction Scheme

A 50 mL round bottom flask was equipped with a thermometer, West condenser and a stirrer. To the flask, 0.5 mL of 1-dimethylamino-2-propyne was carefully added to the flask followed by 10 mL of toluene and 40 μL of AIBN. AIBN was the required catalyst for the reaction. The apparatus was refluxed at 90° C for an hour and 0.5 g of silica was added. The reaction mixture was refluxed at 100° C for 100 hours. Toluene and diethyl ether were used to wash the product. The solid was dried under vacuum at 100° C for 24 hours and was characterized using DRIFT and NMR spectroscopy.

5. Column Packing

1,9-decadiyne, and 1,9-decadiene bonded silica were chosen to be packed into individual stainless steel columns (15 cm x 4.6 mm internal diameter, Alltech Associate Inc., Deerfield, IL). A Haskell Pump (Haskell, Burbank, CA) column packer was used. HPLC-grade methanol (Fisher Scientific, Fair Lawn, NJ) was used as the solvent to pack the modified silica. About 1.9 g of the modified silica was mixed with carbon tetrachloride-methanol (90:10) and the mixture was poured into the pump reservoir. The material was packed under high pressure using nitrogen. After packing, there was a relaxation period before the column was removed from the apparatus. Columns were capped with plastic fittings after removal from the column packer.

C. Surface Characterization Instrumentation

1. Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFT)

The infrared spectra of the various silica samples were obtained using a Perkin Elmer Model 1800 FT-IR spectrophotometer (Perkin Elmer, Norwalk, CT), with a diffuse

reflectance accessory (Spectra Tech., Stamford, CT). The sample was diluted 50% with IR-grade KBr and was placed in a 2 mm sample cup. Spectra consisting of 200 scans referenced against KBr over a range of 4000-450 cm^{-1} were obtained at a resolution of 2 cm^{-1}. All the spectra were normalized to 100% transmittance. A Perkin Elmer Series 7500 technical computer was interfaced with the unit along with a plotter (Hewlett Packard 7475 A, Palo Alto, CA). Some samples were run on the ATI Mattson Infrared Series FT-IR spectrometer. The computer (Hewlett Packard Venturis FX-2) provided with Winfirst software was used to process the signals. The plotter model was the Desk Jet 722C by Hewlett Packard. The procedure was the same except that the amount of the sample was increased to 95%.

2. Cross-Polarization Magic Angle Spinning Nuclear Magnetic Resonance Spectroscopy (CP-MAS NMR)

The CP MAS ^{13}C and ^{29}Si NMR spectra were scanned on a Bruker (Billerica, MA) MSL 300 MHz spectrometer. Solid samples (200-300 mg) were loaded into a rotor and spun at 4700-5200 Hz at room temperature. The ^{13}C and ^{29}Si CP-MAS spectra were recorded with a 3 ms and 5 ms contact time respectively. A repetition rate of 5 s was used for both nuclei. Glycine was used as the external reference for ^{13}C and poly(hydrido)siloxane for ^{29}Si.

3. High Performance Liquid Chromatography Instrumentation

The HPLC pump was a 200 LC series pump from Perkin Elmer (Perkin Elmer, Norwalk, CT). The stationary phase was packed into a stainless-steel column (15 cm L x 4.6 mm i.d., Alltech Associate Inc., Deerfield, IL). A Perkin Elmer LC 295, a

multiwavelength uv absorbance detector was used to detect the components eluting from column. The signals from the detector were processed by an integrator (Hewlett Packard, Model 3395). Helium gas, flowing at 50 mL/min was used to degas the system in order to prevent air bubbles from entering the column. For gradient elution of compounds, the system consisted of pump, HP Series 1050, an auto sampler, HP Series 1050 and a multi-wavelength detector, HP Series 1050 (Hewlett Packard, Palo Alto, CA) HP Chem Station Software (Hewlett Packard, Palo Alto, CA) was used for data analysis.

The Standard Reference Mixture, SRM 869, was donated by the National Institute of Standards and Technology (NIST, Gaithersburg, MD). The Perkin Elmer universal test mix was purchased from Perkin Elmer (Perkin Elmer, Norwalk, CT). Methanol and tetrahydrofuran were purchased from Fisher Scientific. Acetonitrile was obtained from J.T. Baker Chemicals (Phillipsburg, NJ). Water was purified by a millipore apparatus. All reagents used were HPLC grade. One set of samples, benzodiazepines were donated by Hoffman-La Roche, Switzerland. All the nitrosamine samples were purchased from Sigma-Aldrich (Aldrich Chemical Co. Milwaukee, WI)

4. Elemental Analysis

A combustion analysis was done to determine the carbon content. Samples were analyzed by Desert Analytics (Tuscon, AZ) and the percentage for each sample was recorded. These results were useful in calculating the surface coverage of the bonded silica.

D. Surface Coverage

The surface coverage, α_R was calculated using equation (1)

$$\alpha_R(\mu mol/m^2) = \frac{10^6 P_c}{(100 M_c n_c - P_c M_R) S_{BET}} \qquad (1)$$

P_c is the carbon percentage by weight of the modified silica. M_c is the atomic weight of carbon. n_c is the number of carbon atoms in the bonded group. M_R is the molecular weight of the organic compound, and S_{BET} (Brunauer, Emmet and Teller) is the specific surface area (m^2/g) of the silica substrate [11]. The maximum surface coverage value that can be calculated is 8 $\mu mol/m^2$. This value is the average amount of silanols at the surface [12].

E. Stability of Modified Silica

Approximately, 100 mg of the derivatized silica was place in a centrifuge tube (IEC-HNS) fitted with a stirrer and a magnetic bar. To this 5 mL of 10 mM hydrochloric acid was added and stirred continuously for 24 hours at room temperature. The resulting mixture was centrifuged and the supernatant was discarded. The solid was washed with 5 mL portions of diethylether and the washing process was repeated three times. The product was dried under the hood for overnight and then dried in the oven for at least one day.

CHAPTER III

RESULTS AND DISCUSSION

A. Elemental Analysis Results

From the percentage carbon values the surface coverage of the bonded phases have been calculated. Table 3 denotes the surface coverage and the percent carbon for the different bonded phases that were produced by either Platinum or AIBN catalyst.

Table 3. Surface Coverage and Carbon Percentage Data Table

Compound	Surface Coverage, α_R ($\mu mol/m^2$)	% of Surface Covered	Percent Carbon %C
1,9-decadiyne	4.31	53.88	5.17
1,9-decadiene	3.28	41.00	3.98
3,9-dodecadiyne	2.99	37.38	3.48
1-dimethylamino-2-propyne	2.67	33.38	1.66
Propiolic acid (AIBN)	4.22	52.75	1.56

B. Characterization of Silica Hydride

1. DRIFT Spectra

Figure 1 shows the DRIFT spectrum of silica after it has been treated with TES. The main feature of this spectrum is the presence of an intense band at 2250 cm^{-1}. This band is representative of the Si-H bond stretching [13].

2. CP-MAS ^{29}Si NMR Spectra

Figure 2 shows the ^{29}Si NMR spectra for the silanized product. Peak A at –110 ppm is due to silicon atoms which have four siloxane linkages. Peak B near –100 ppm is assigned to silicon atoms having three siloxane linkages and a hydroxyl group [3]. There are more backbone silicon atoms than silanols. However, the intensity of

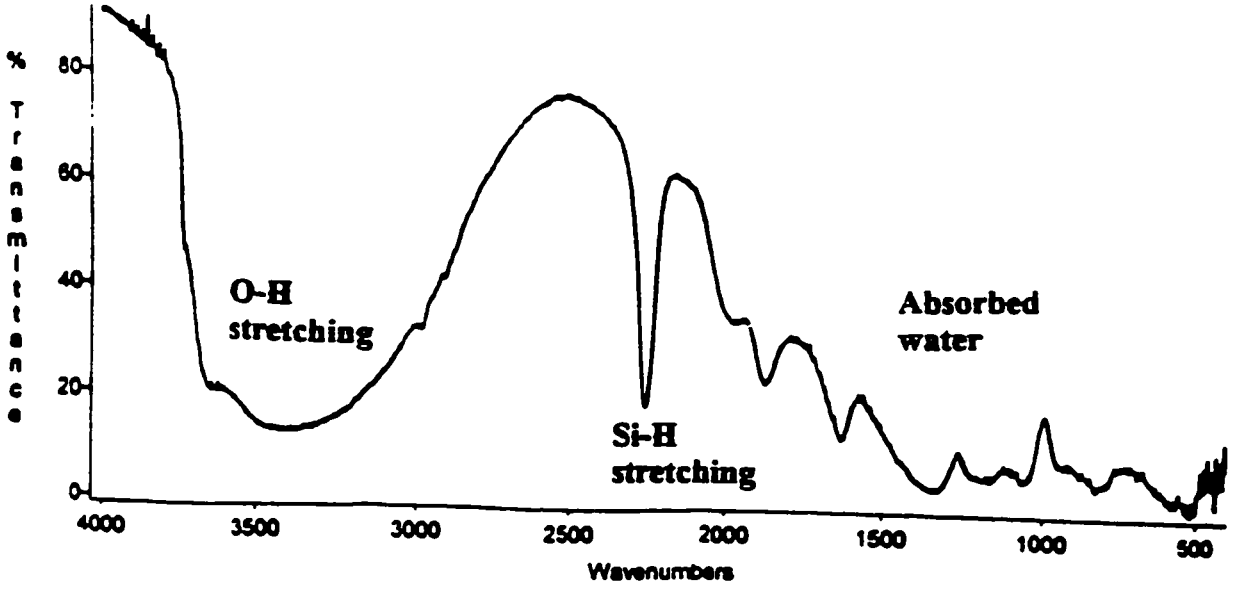


Figure 1. The DRIFT Spectrum of Vydac Silica Hydride.

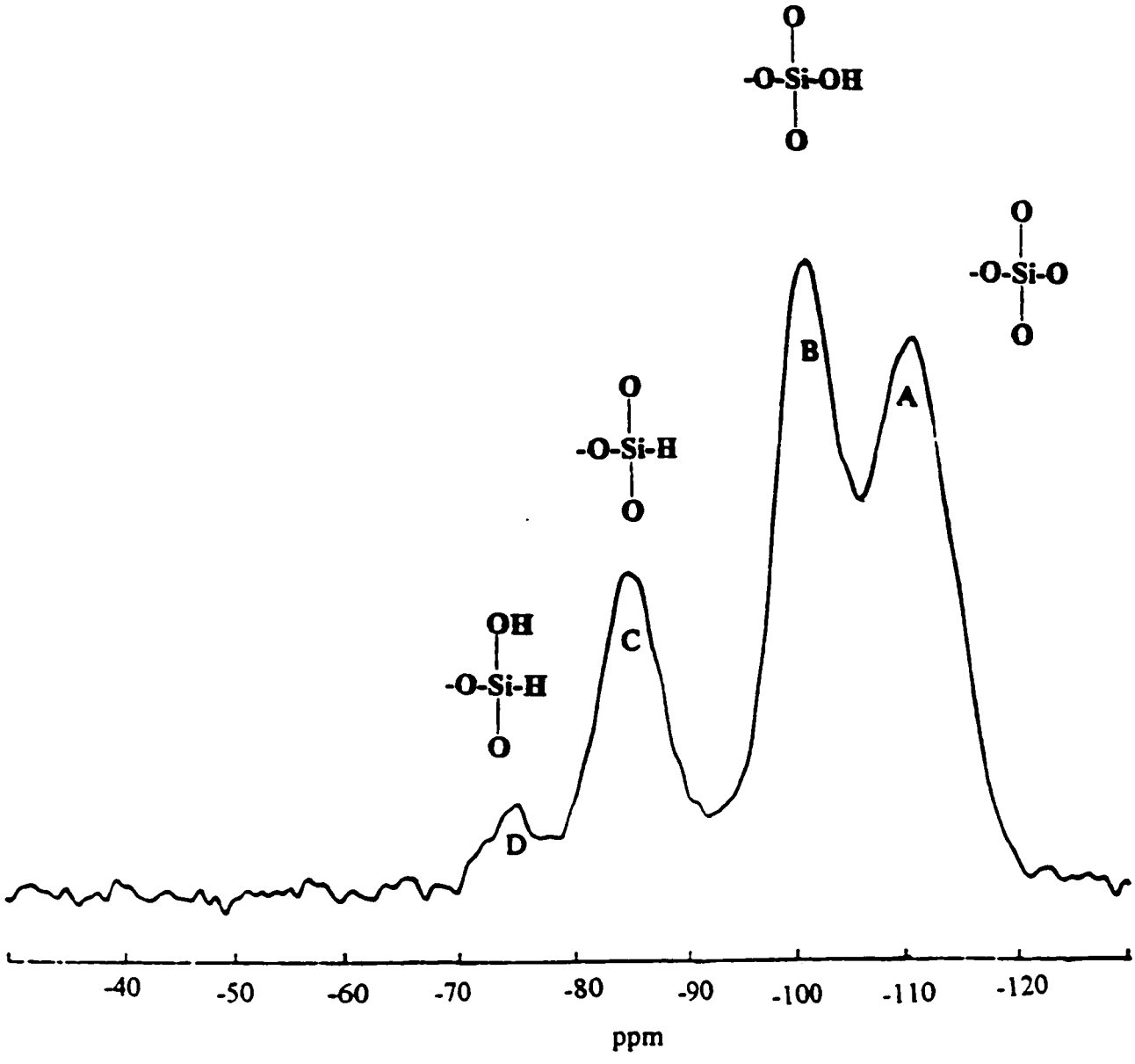


Figure 2. The CP-MAS ^{29}Si NMR Spectrum of Vydac Silica Hydride.

peak A is lower than peak B because these silicon atoms have no hydrogens nearby which would facilitate cross-polarization [3]. The peak at –75 ppm represents the silica hydride (Si-H) peak with two siloxane linkages and a hydroxyl group, while the peak at –85 ppm is due to the silica hydride and the three siloxane linkages [13].

3. CP-MAS ^{13}C NMR Spectra

Figure 3 shows the ^{13}C NMR spectra of Vydac hydride. The main features are the two peaks at 16 and 60 ppm. These two peaks are due to the methyl and methylene moieties of the ethoxy groups respectively. They are very persistent for most bonded phases synthesized from Vydac hydride [13].

C. Confirmation of 1,9-decadiyne

1. DRIFT Spectra

Figures 4a and 4b are DRIFT spectra of 1,9-decadiyne bonded silica and the silica hydride intermediate. The stretching band at 2250 cm^{-1} is due to the Si-H bond stretching. The main difference of this spectra as compared to that of silica hydride is the decrease in the intensity of the Si-H bond stretching at 2250 cm^{-1} and the appearance of a stretch at 3000 cm^{-1}. This stretching frequency at 3000 cm^{-1} is due to the C-H bond stretching frequencies of the attached organic moiety. The weak stretch at 1600 cm^{-1} is due to the C=C bond frequencies. This spectrum is a proof that the Si-H group has undergone a chemical reaction. Figure 4c and 4d are the DRIFT spectra of the acid and the base hydrolyzed 1,9-decadiyne bonded silica respectively. The spectra contains the same features as that of the original 1,9-decadiyne bonded silica, as the C-H and Si-H

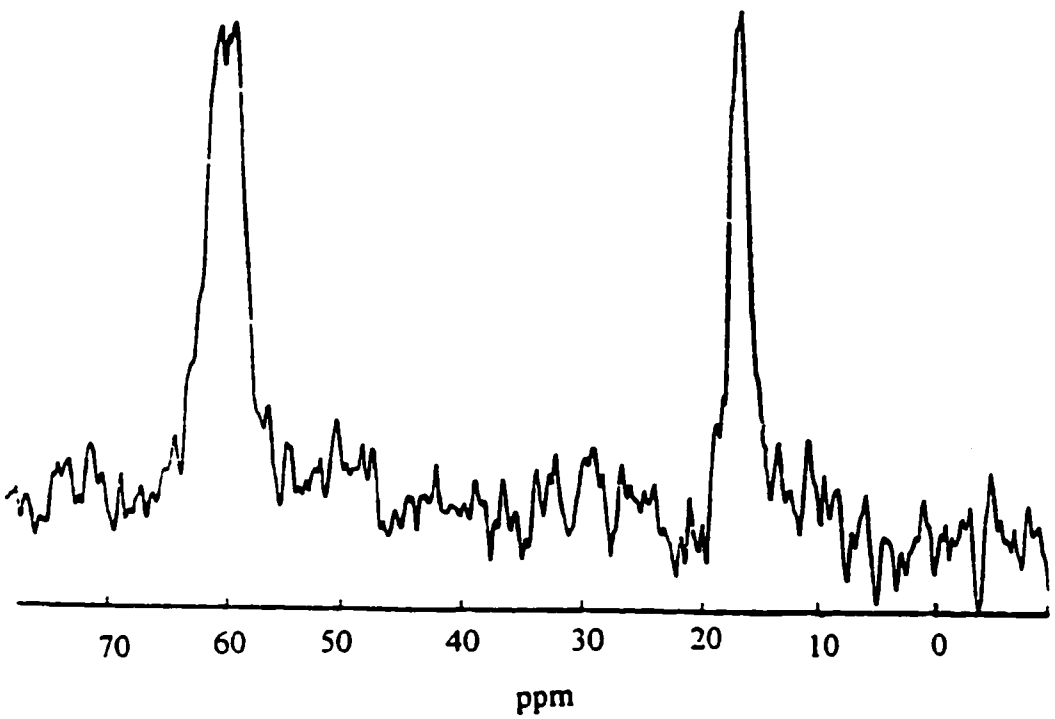


Figure 3. The CP-MAS ^{13}C NMR Spectrum of Vydac Silica Hydride.

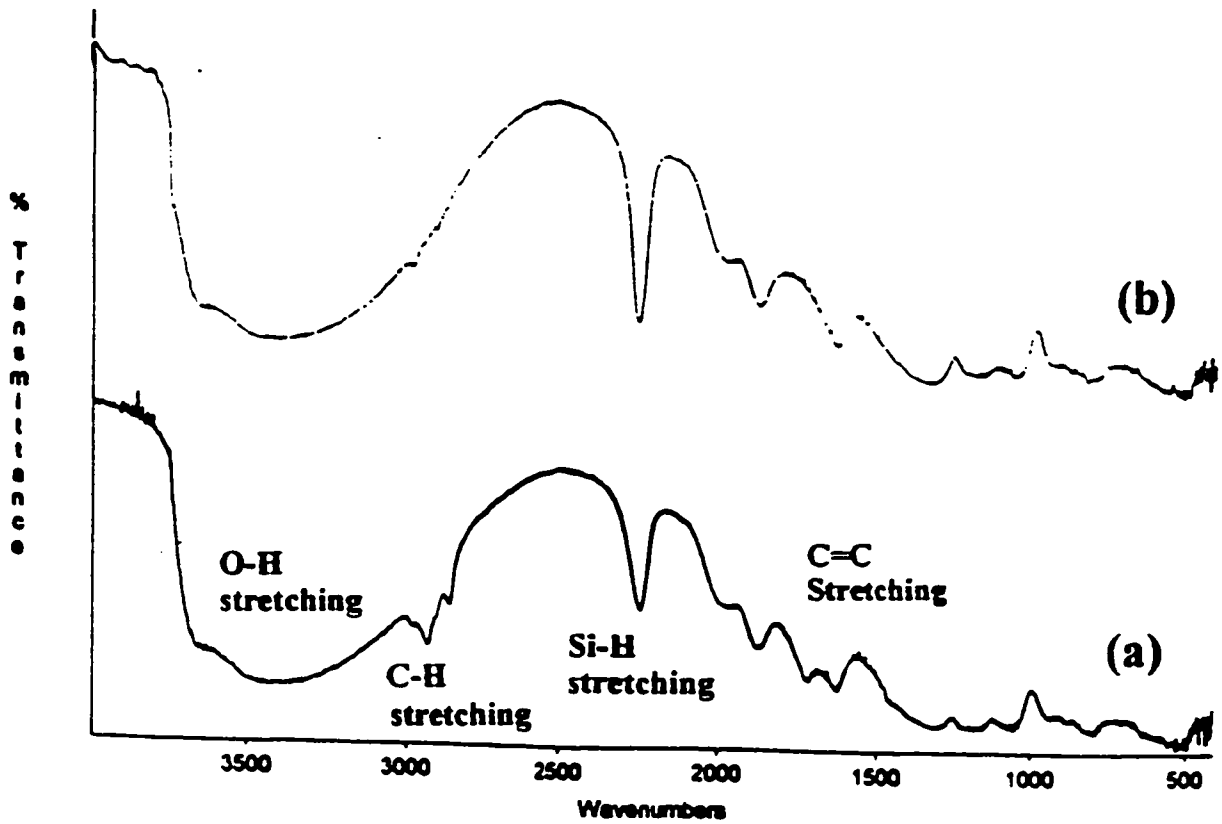


Figure 4a. The DRIFT Spectrum of 1,9-decadiyne Bonded Silica.

b. The DRIFT Spectrum of Vydac Silica Hydride.

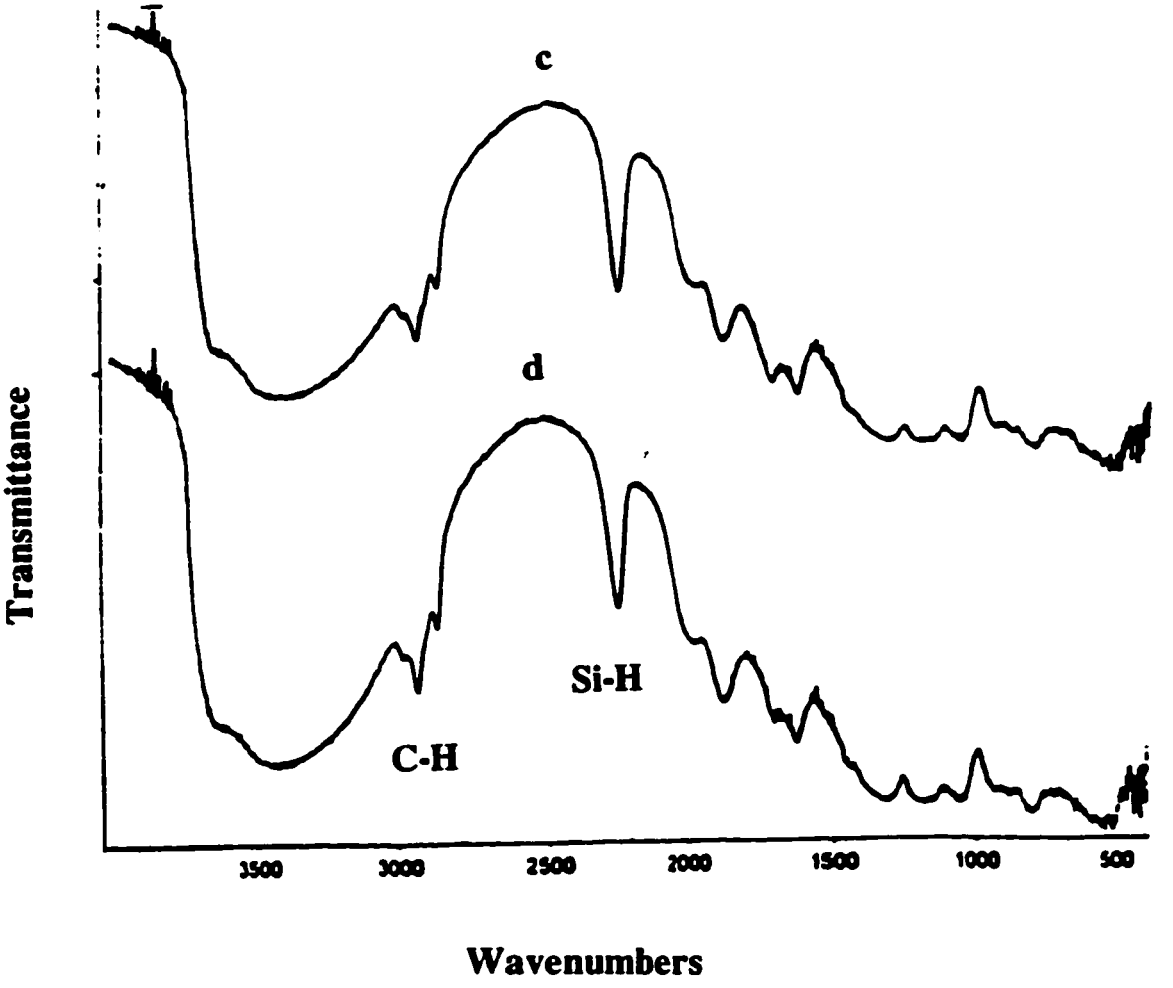


Figure 4c. The DRIFT spectrum of 1,9-decadiyne Bonded Silica after Acid Hydrolysis.

Figur 4d. The DRIFT spectrum of 1,9-decadiyne Bonded Silica after Base Hydrolysis.

stretches are still very prominent. Based on the IR spectra, it can be established that 1,9-decadiyne bonded silica is resistant to hydrolysis.

2. CP-MAS ^{29}Si-NMR Spectra

The ^{29}Si NMR spectrum of 1,9-decadiyne bonded silica is also used to indicate the success of bonding the organic moiety to silica surface. Figure 5 shows the ^{29}Si NMR of 1,9-decadiyne bonded silica. When this is compared to Figure 2, a decrease in the Si-H peak is observed. This is a good indication for a chemical reaction to have occurred. Peak B is due to the silicon atoms with a residual hydroxide group and peak C, due to the silicon atoms in the siloxane backbone. Typically, a new peak representing the Si-C linkage should have appeared at –65 ppm [14]. It could be possible that the Si-C peak is shifted further upfield and is partially hidden under the Si-H peak or is just very low in intensity because of poor cross-polarization [15].

3. CP-MAS ^{13}C NMR Spectra

In order to determine which of the possible alternative structures are present in the 1,9-decadiyne bonded silica, further spectroscopic analysis was conducted. The CP-MAS ^{13}C NMR was used to elucidate the exact structures. The ^{13}C spectrum of 1,9-decadiyne bonded silica is shown in Figure 6. The chemical shifts at 119, 125 and 128 ppm can be assigned to olefin carbons (C=C) [10]. Hence it is not possible for us to eliminate any of the olefin containing structures of II, III and IV. But it is very unlikely that IV is present mainly because it requires the bonding to the carbon at position 2, when it has been known that terminal carbons have been almost exclusively found at the attachment of organic moieties to hydride-base supports. So it is best to consider that the

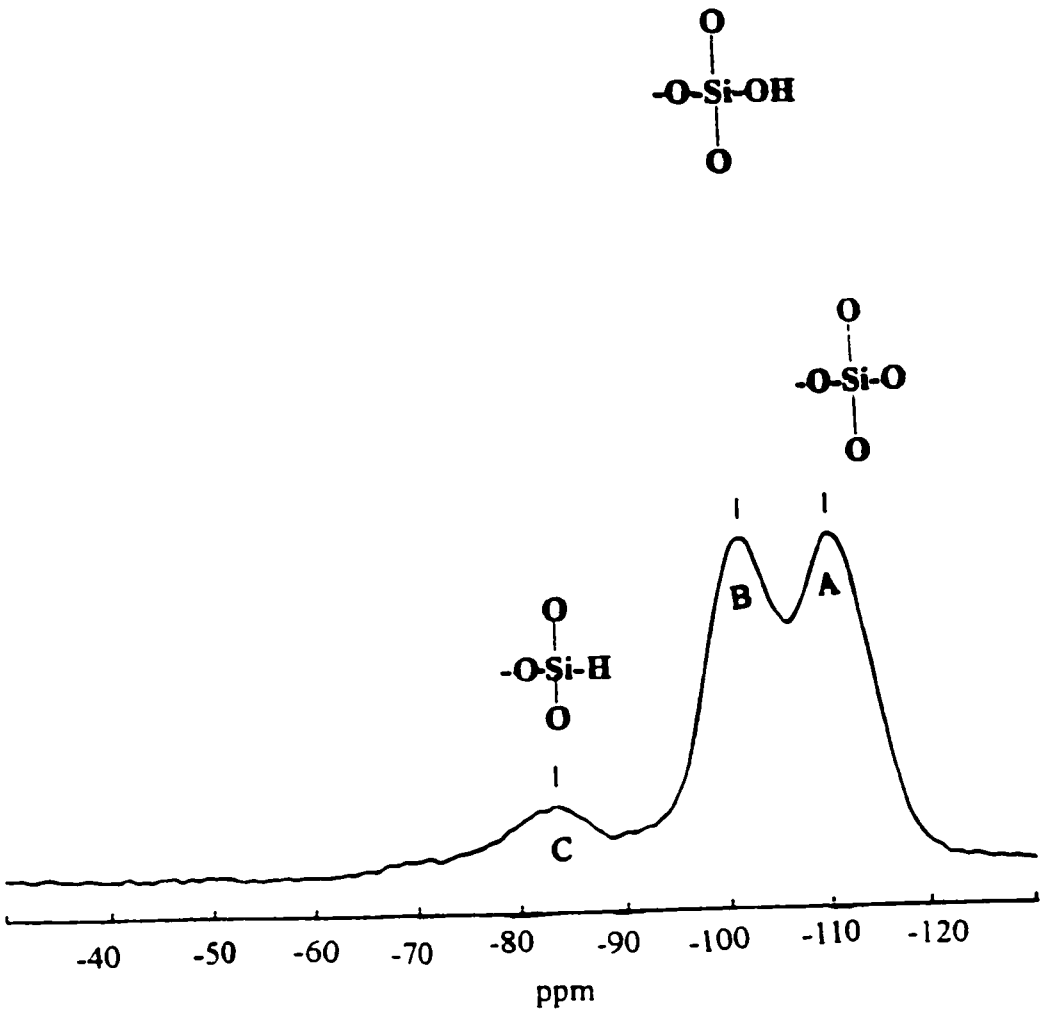


Figure 5. The CP-MAS ^{29}Si NMR Spectrum of 1,9-decadiyne Bonded Silica.

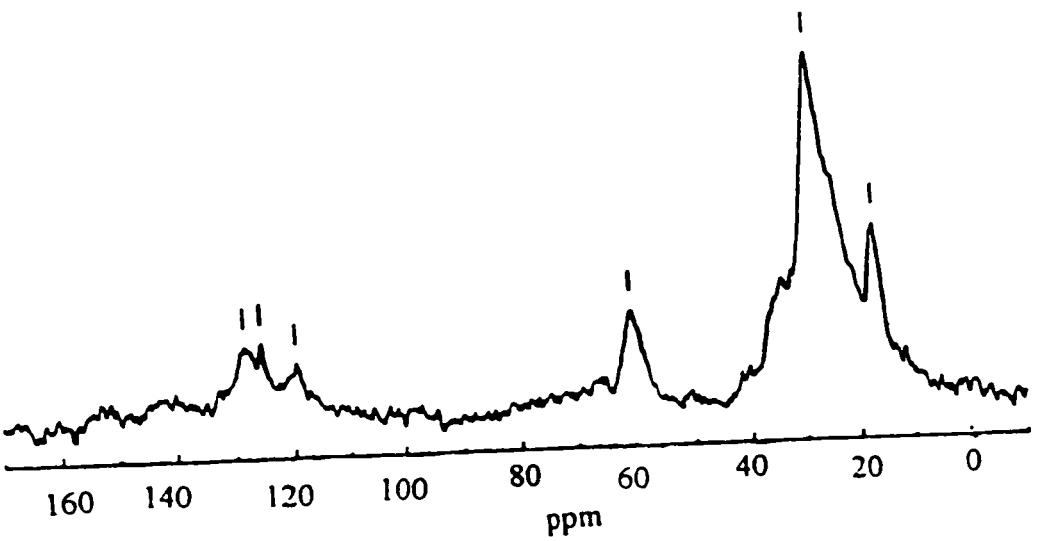


Figure 6. The CP-MAS ^{13}C NMR Spectrum of 1,9-decadiyne Bonded Silica.

chemical shifts in this region are due to II and III or most of the bonded phases could be II. The presence of three peaks could be accounted for by the combined existence of its *cis* and *trans* isomers at the double bonds. The peak at 27 ppm is due to the methylene carbons and hence could be assigned to structure I, II and III. The peak at 60 ppm is due to the methylene moiety of the ethoxy groups. Also, the peak at 18 ppm is due to the methyl moiety of the ethoxy group. These peaks are very characteristic of Vydac silica hydride where some ethoxy group remain after TES silanization process [16]. The only missing peaks are due to the carbons that are directly attached to the silica and also that are attached to two silicon atoms instead of one. However, due to steric constraints Si-C resonances are too low to detect. These studies suggest that structures I, II and III can be present. The power of NMR as a tool to study the chemically modified surfaces is evident from the spectra obtained after modification of silica oxide surfaces.

D. Confirmation of 1,9-decadiene

1. DRIFT Spectra

Figure 7a shows the DRIFT spectra of the product obtained from the reaction of 1,9-decadiene with silica hydride. This material also displays a decrease in the Si-H stretching band at 2250 $cm^{-1}$and a concomitant appearance of the C-H stretching band at 3000 cm^{-1}. This band at 3000 cm^{-1} suggests the success of the hydrosilation reaction.

2. CP-MAS ^{29}Si NMR Spectra

Figure 8 is illustrates the ^{29}Si NMR spectrum of 1,9-decadiene. This is very similar to Figure 5. The success of bonding can only be confirmed by the disappearance of the Si-H peaks at –75 and –85 ppm. All the other peaks are as marked on the spectrum [10].

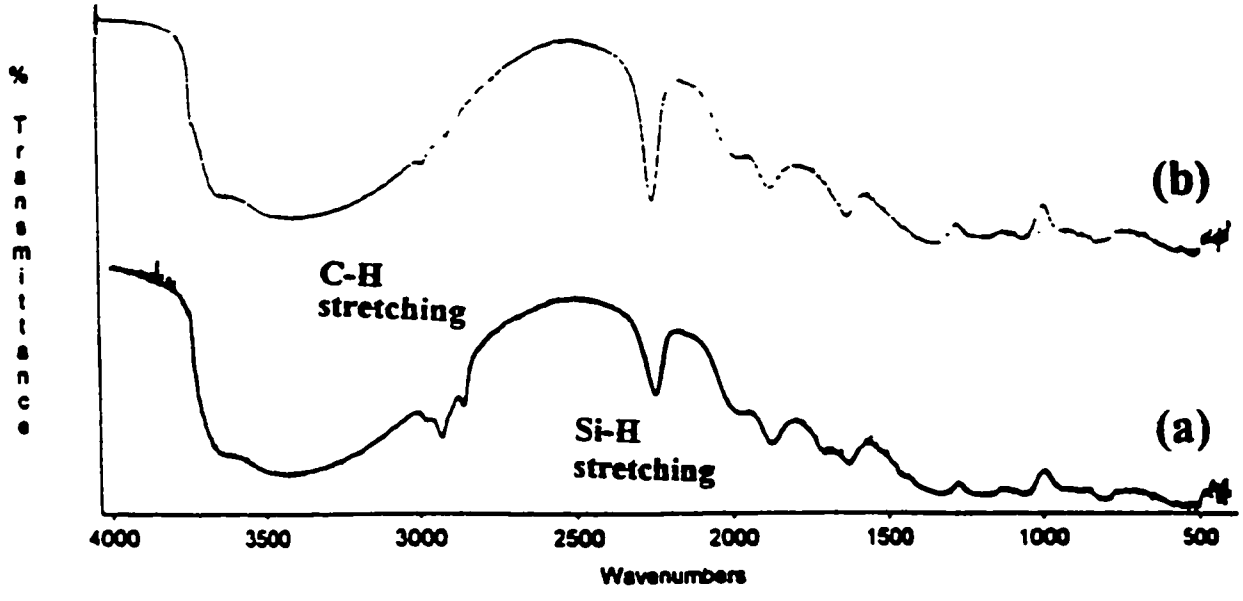


Figure 7a. The DRIFT Spectrum of 1,9-decadiene Bonded Silica.

b. The DRIFT Spectrum of Vydac Silica Hydride.

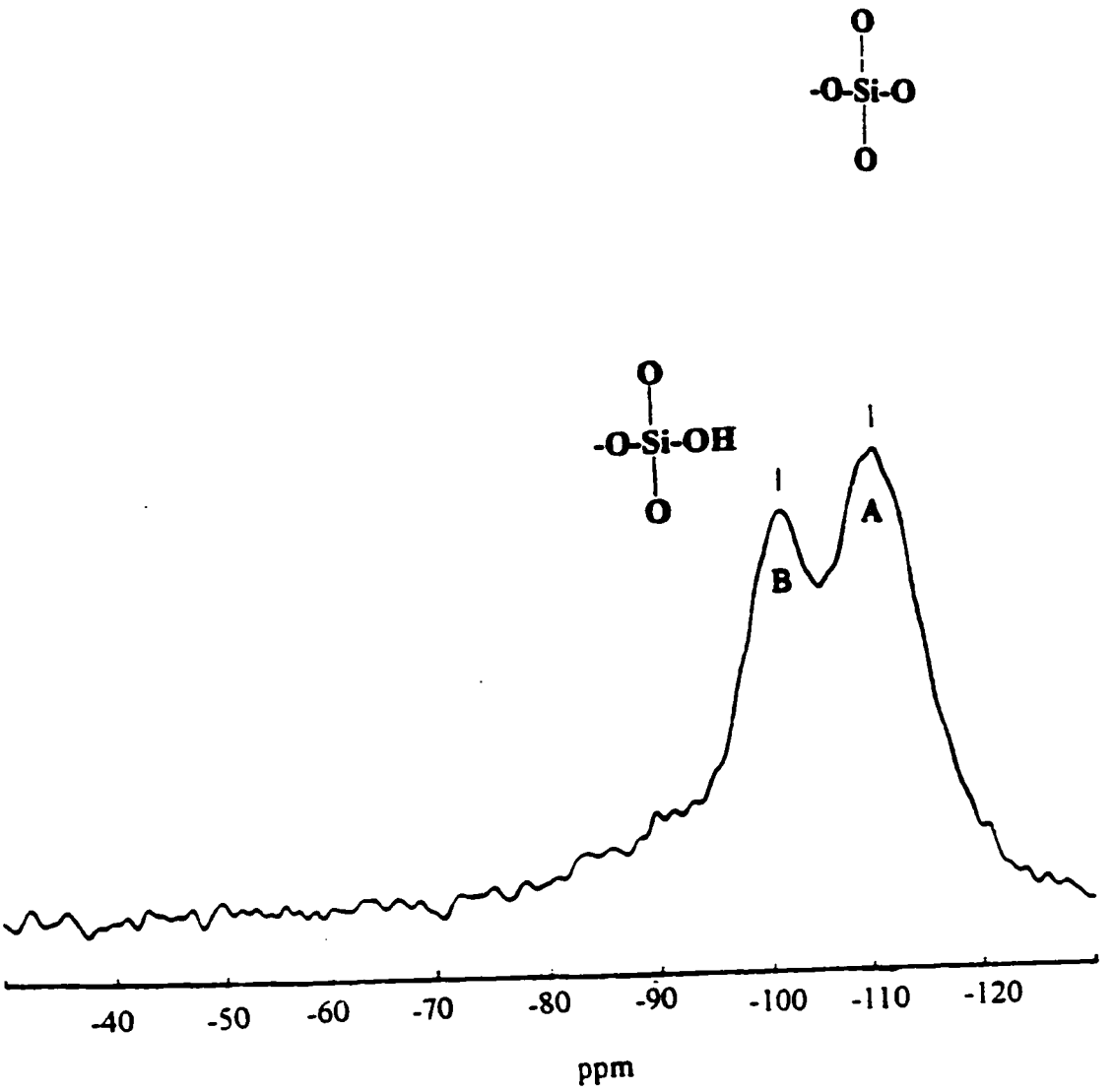


Figure 8. The CP-MAS ^{29}Si NMR Spectrum of 1,9-decadiene Bonded Silica.

3. CP-MAS ^{13}C NMR Spectra

Figure 9 is the ^{13}C NMR spectrum of 1,9-decadiene bonded silica. The ^{13}C CP-MAS NMR spectrum of the bonded materials contains olefinic peaks at 129 ppm indicating that not every double bond has reacted. This suggests that the ring structure (II) is not the only product. The peaks at 29 and 22 ppm are from the methylene carbons (I). Whereas, the small peak at 12 ppm is due to the methylene carbon resonance that is directly attached to silicon. The peaks at 16 and 60 are due to the ethoxy groups from the TES [16]. The ^{13}C CP-MAS NMR of the product of 1,9-decadiene bonded silica suggests that only one type of bonding is possible with olefinic carbons [5].

E. Analysis of Propiolic Acid

1. DRIFT Spectra

For propiolic acid prepared by an AIBN initiation reaction, the DRIFT spectrum is shown in Figure 10. Besides the usual Si-H bond stretching at 2250 cm^{-1}, a new band at 2000 cm^{-1} appears. This could be assigned to the carbonyl stretch from the carboxylic acid moiety [7]. However, the C-H bond stretching frequencies are not as intense as in the previous spectra. This suggests that propiolic acid appears to have limited bonding to silica hydride.

2. CP-MAS ^{13}C NMR Spectra

Figure 11 is the ^{13}C NMR spectrum of propiolic acid bonded silica via AIBN. The chemical shifts in this spectrum can be compared to that of the silica hydride intermediate (Figure 3). The peaks at 17 and 60 ppm are the only prominent chemical shifts in this spectrum. They can be assigned to the ethoxy moiety, which are persistent even after the

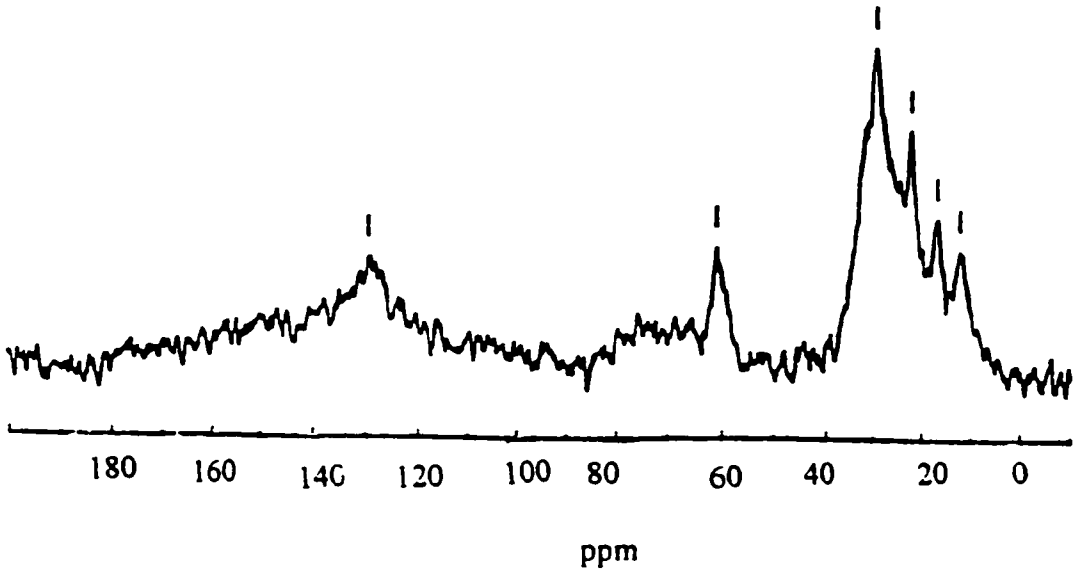


Figure 9. The CP-MAS ^{13}C NMR Spectrum of 1,9-decadiene Bonded Silica.

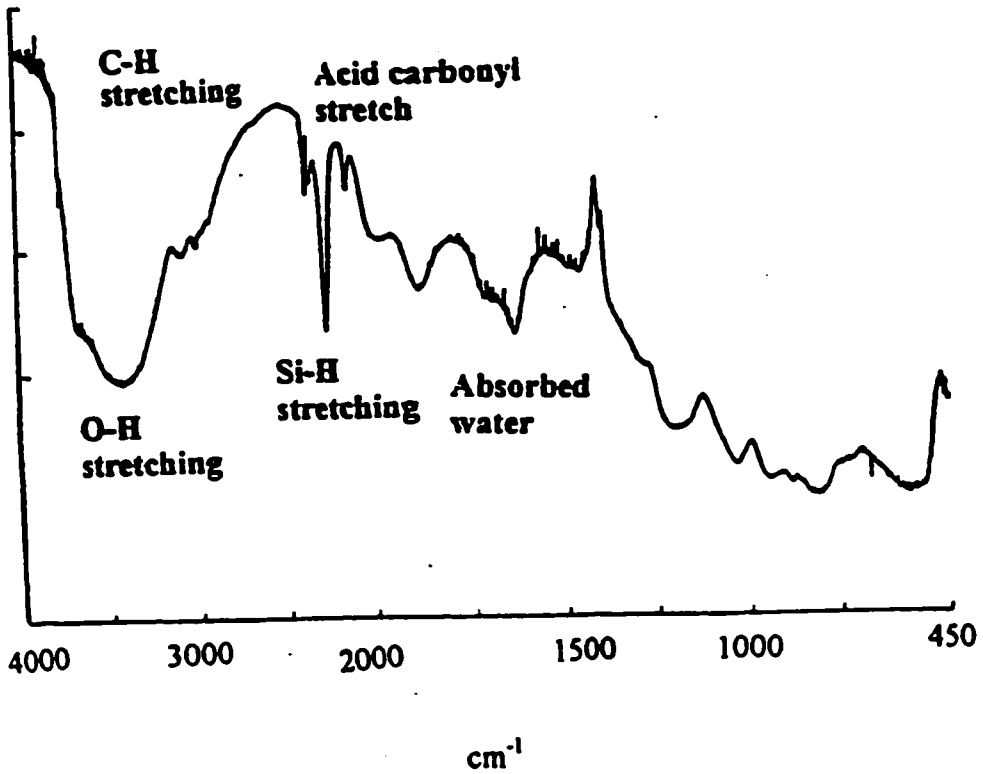


Figure 10. The DRIFT Spectrum of Propiolic Acid Bonded Silica.

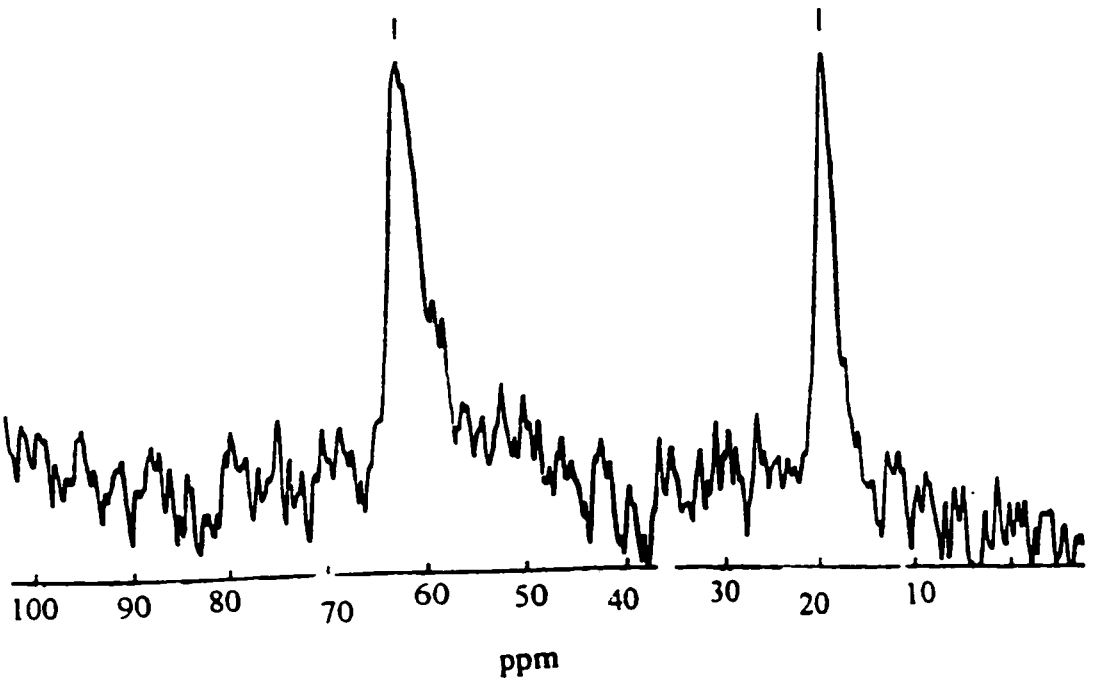


Figure 11. The CP-MAS ^{13}C NMR Spectrum of Propiolic Acid Bonded Silica.

hydrosilation reaction. As no fragment owing to a carbonyl moiety or any other carbons are seen. This observation suggests that no bonding has occurred. This bonding process has been proven to not be successful. Therefore, propiolic acid was not used for further testing by HPLC.

F. Evaluation of 1-dimethylamino-2-propyne bonded silica

1. DRIFT Spectra

The DRIFT spectrum of 1-dimethylamino-2-propyne is shown in Figure 12. There are no corresponding frequencies for Si-H or C-H or C-N bond stretching as is seen in other DRIFT spectra. This indicates the failure of hydrosilation reaction. Moreover, the stretching bands at 3000 cm^{-1} are also not very intense.

2. CP-MAS ^{13}C NMR Spectra

Figure 13 shows the ^{13}C NMR spectrum of 1-dimethylamino-2-propyne bonded to silica. The peak at 127 ppm implies that there are olefinic carbons. This chemical shift could be assigned to II and III. Structure IV is not viable because it again requires the attachment to carbon in the second position. The resonance at 163 ppm could be from the C-N bonds. The peaks at 35 ppm and 44 ppm can be assigned to methyl and methylene groups respectively. The peaks at 16 and 60 ppm are due to the ethoxy groups which are always present in Vydac silica.

G. Evaluation of 3,9-dodecadiyne bonded silica

1. DRIFT Spectra

The spectrum of 3,9-dodecadiyne bonded silica is shown in Figure 14. The bands are very similar to that of 1,9-decadiyne.

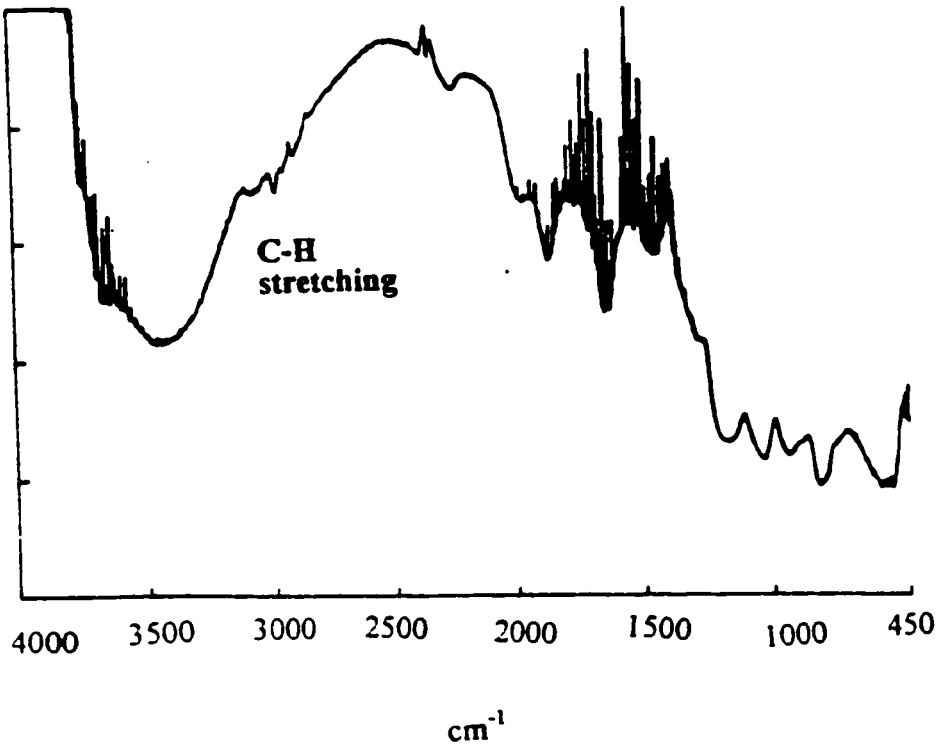


Figure 12. The DRIFT Spectrum of 1-dimethylamino-2-propyne Bonded Silica.

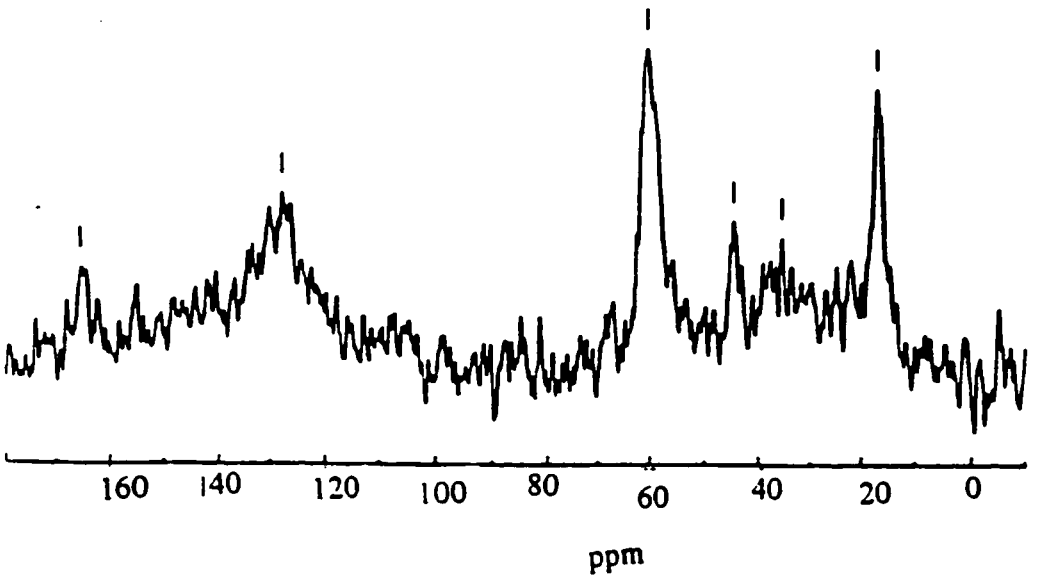


Figure 13. The CP-MAS ^{13}C NMR Spectrum of 1-dimethylamino-2-propyne Bonded Silica.

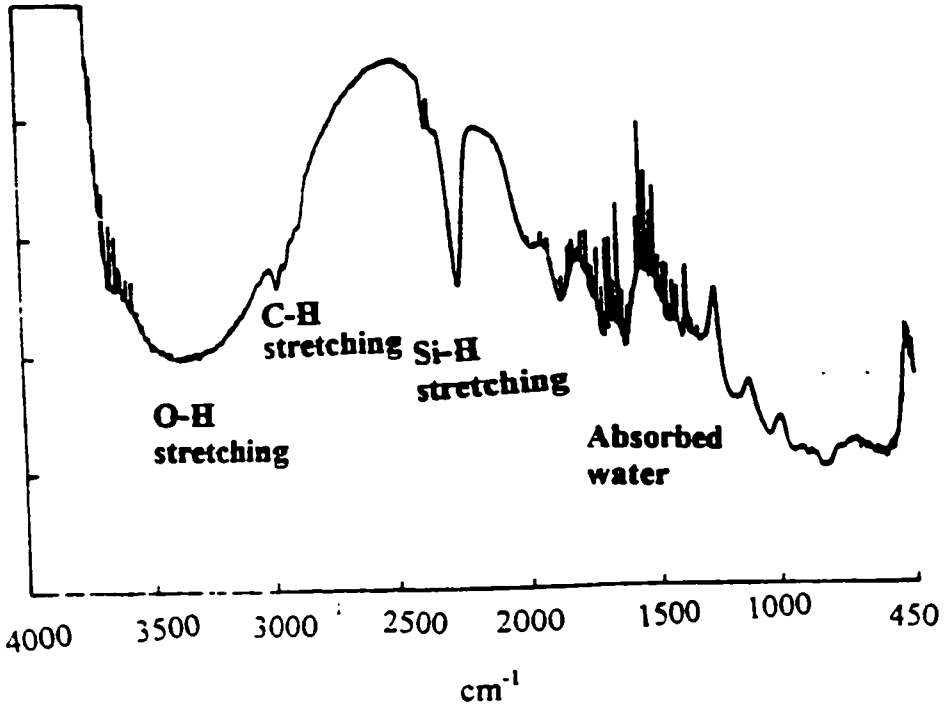


Figure 14. The DRIFT Spectrum of 3,9-dodecadiyne Bonded Silica.

A decrease in the Si-H bond stretching and the appearance of a new stretch at 3000 cm^{-1} corresponds to the C-H bond stretching frequencies. However, the C-H bands are not as intense as in Figure 5. This indicates that bonding is not as extensive as 1,9-decadiyne. The sharp spikes are due to the water molecules absorbed on the surface of silica.

2. CP-MAS ^{29}Si NMR Spectra

The CP-MAS ^{29}Si NMR spectrum of 3,9-dodecadiyne bonded to silica is shown in Figure 15. The Si-H peak A is at –85 ppm. The other two peaks at –101 ppm and –111 ppm are due to silanols and siloxanes present on the surface.

3. CP-MAS ^{13}C NMR Spectra

The ^{13}C NMR spectrum of 3,9-dodecadiyne bonded silica is shown in Figure 16. The lowest field peak at 11 ppm is due to the methyl groups (I, II, III). The chemical shifts at 20 ppm and 27 ppm are from the methylene groups and hence could be assigned to I, II and III. The main shortcoming of this spectrum is the two huge peaks at 16 and 60 ppm which are from the ethoxy groups of the TES intermediate. The chemical shifts at 127 and 134 can be assigned to olefinic carbons (II, III). This study suggests that hydrosilation reaction is possible for alkynes, with triple bonds at other than the terminal position in the alkyl chain [13]. The bonding was considered less extensive as there were two huge peaks from the TES silanzed intermediates. In order to elucidate the structure and extent of bonding of the organic compound, further analyses of the bonded phases were necessary.

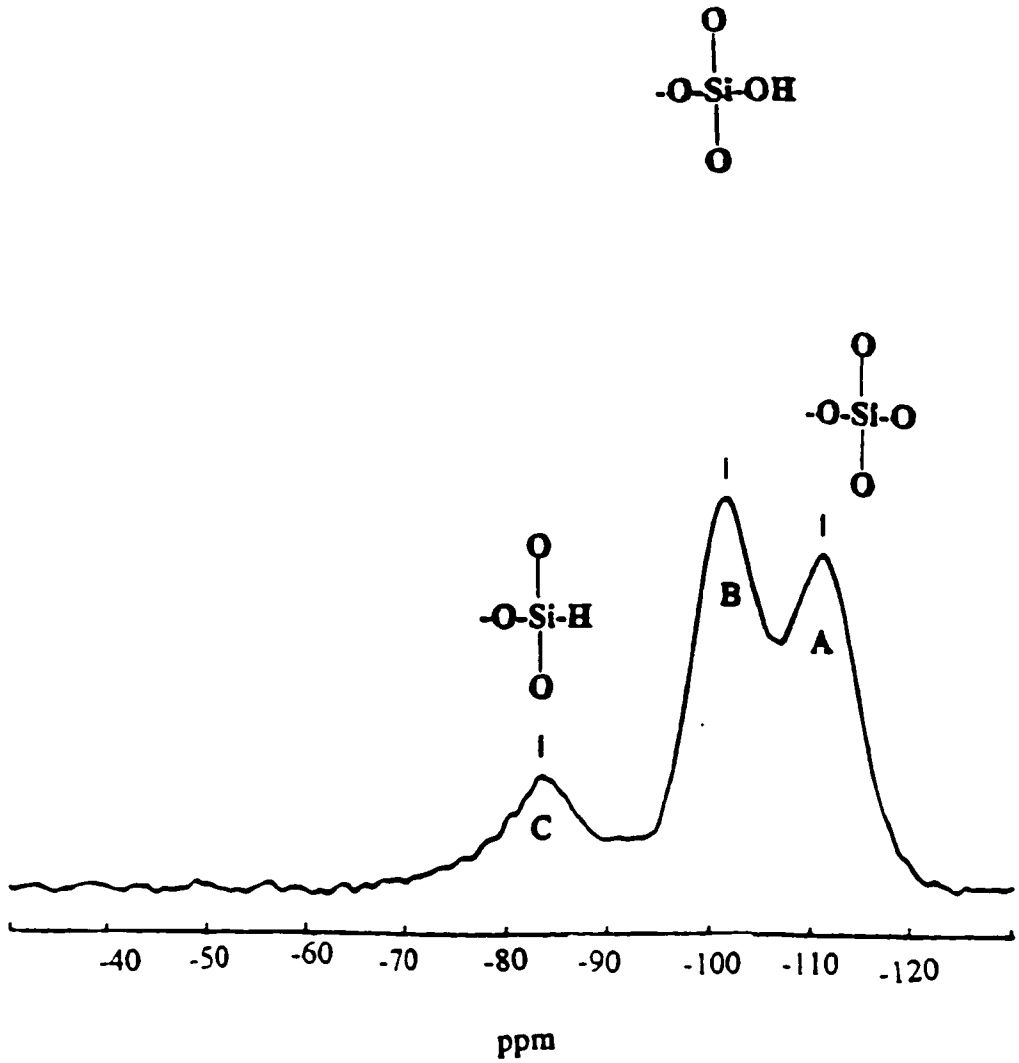


Figure 15. The CP-MAS ^{29}Si NMR Spectrum of 3,9-dodecadiyne Bonded Silica.

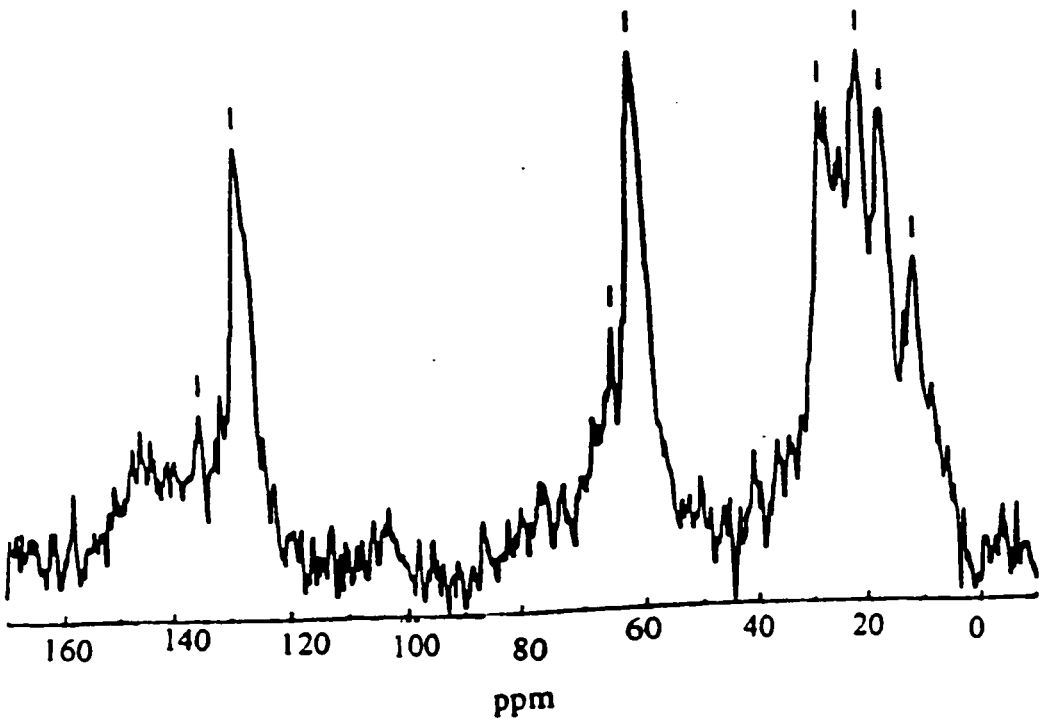


Figure 16. The CP-MAS ^{13}C NMR Spectrum of 3,9-dodecadiyne Bonded Silica.

H. Chromatographic Studies

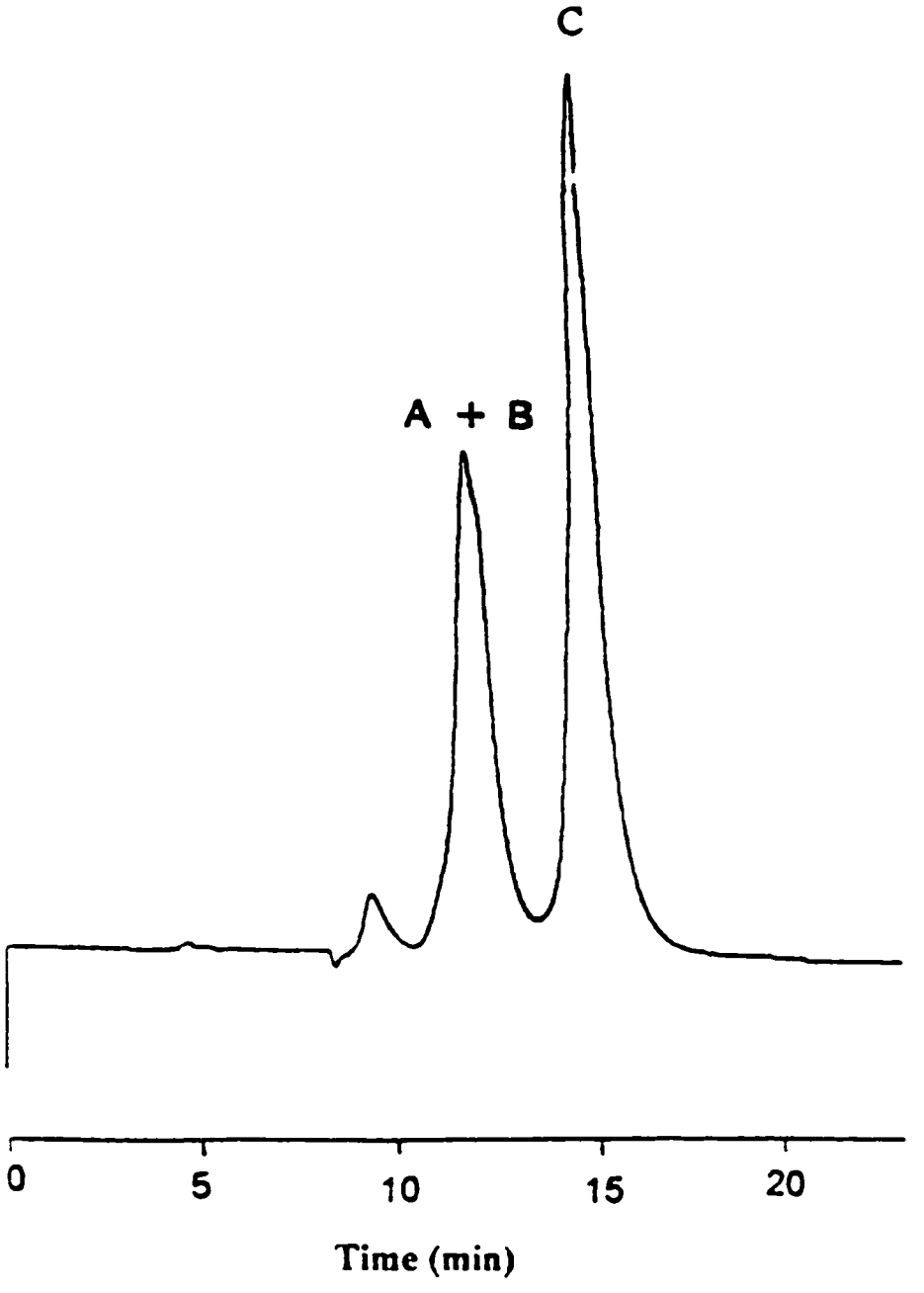
1. 1,9-decadiyne column

a. Isocratic Separation of Three Polycyclic Aromatic Hydrocarbons

Standard reference mixture, SRM 869, is a three component test compound consisting of polycyclic aromatic compounds, benzo[a]pyrene (BaP), phenanthro[3,4-c]-phenanthrene (PhPh) and tetrabenzonaphthalene (TBN). This test mixture has been developed by NIST (National Institute of Standard and Technology) for certifying the performance characteristics of reverse phase HPLC columns (Figure 17). SRM 869 mixture was injected into a 1,9-decadiyne column with methanol-water mixture (90:10, v/v) at a 0.2 mL/min flow rate. The elution order of this mixture is shown in the chromatogram in Figure 17. Further, the separation factor of TBN and BAP, was calculated to be 1.93 which is typical of a highly loaded monomeric phase. The material, 1,9-decadiyne bonded silica, are classified as monomeric bonded phases because there is only a single point of attachment to the surface. An α value greater than 1.7 is considered to be of a monomeric phase [17]. The separation factor αTBN/BaP, is calculated from the retention factor of the two compounds TBN (k_1') and BaP (k_2') [18].

The retention factor, k_1' and k_2' is a measure of the relative retention of each compound on the column, $k' = (t_r - t_o)/t_o$, where t_r are the retention of the solute and t_o is the elution time of the unretained compound (ex., solvent). The elution order is also typical of a highly loaded monomeric bonded phase [19]. Monomeric bonded phases only have a single point of attachment to the silica surface. It is also observed that for

Figure 17. Reversed phase separation of NIST mix (SRM 869) on a 1,9-decadiyne column. Mobile phase: methanol-water, v/v (90:10), at 0.2 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A =Benzo[a]pyrene; B = Phenanthro[3,4-c] phenanthrene; C = Tetrabenzonaphthalene.



aromatic compounds the retention increases with the number of aromatic rings and the linearity of the rings, so that k′ of TBN > PhPh > BaP [20]. Separations were also possible using acetonitrile-water as the mobile phase (Figure 18). Most interestingly, BaP is one of the designated primary pollutants found in the environment. So this technique can be used for monitoring and controlling the presence of such polycyclic aromatic hydrocarbons (PAH's) in air, water and food.

b. Isocratic Separation of Perkin Elmer Universal Test Mix

In order to test the reverse phase mode of operation, another test mixture was used. The Perkin Elmer test mix consists of seven compounds that are aromatic, polar as well as non-aromatic in nature. As the degree of interaction was high, the seven components could be separated. The separation (baseline) obtained with a methanol-water mobile phase (50:50, v/v) is shown in Figure 19. The elution order is typical of a reverse phase mode [21]. Hydrophobic interaction allows the non-polar components to be retained more than the polar components. Figure 20 shows that the selectivity of the column was high even with acetonitrile-water (40:60, v/v), but was found to decrease when tetrahydrofuran was used. It is evident that the 1,9-decadiyne bonded stationary phase is highly selective for compounds with differences in polarity. The peak shape is good indicating minimal residual accessible silanols on the surface.

c. Isocratic Separation of Benzodiazepines

Benzodiazepines are commonly prescribed drugs and are known for sedative, hypnotic and anticonvulsant effects. As their potential for abuse or overdose is high, their analysis is useful in forensic laboratories. Previously, various studies had been done

Figure 18. Reversed Phase separation of NIST mix (SRM 869) on a 1,9-decadiyne column. Mobile phase: acetonitrile-water, v/v (70:30), at 0.2 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A =Benzo[a]pyrene; B = Phenanthro[3,4-c] phenanthrene; C = Tetrabenzonaphthalene.

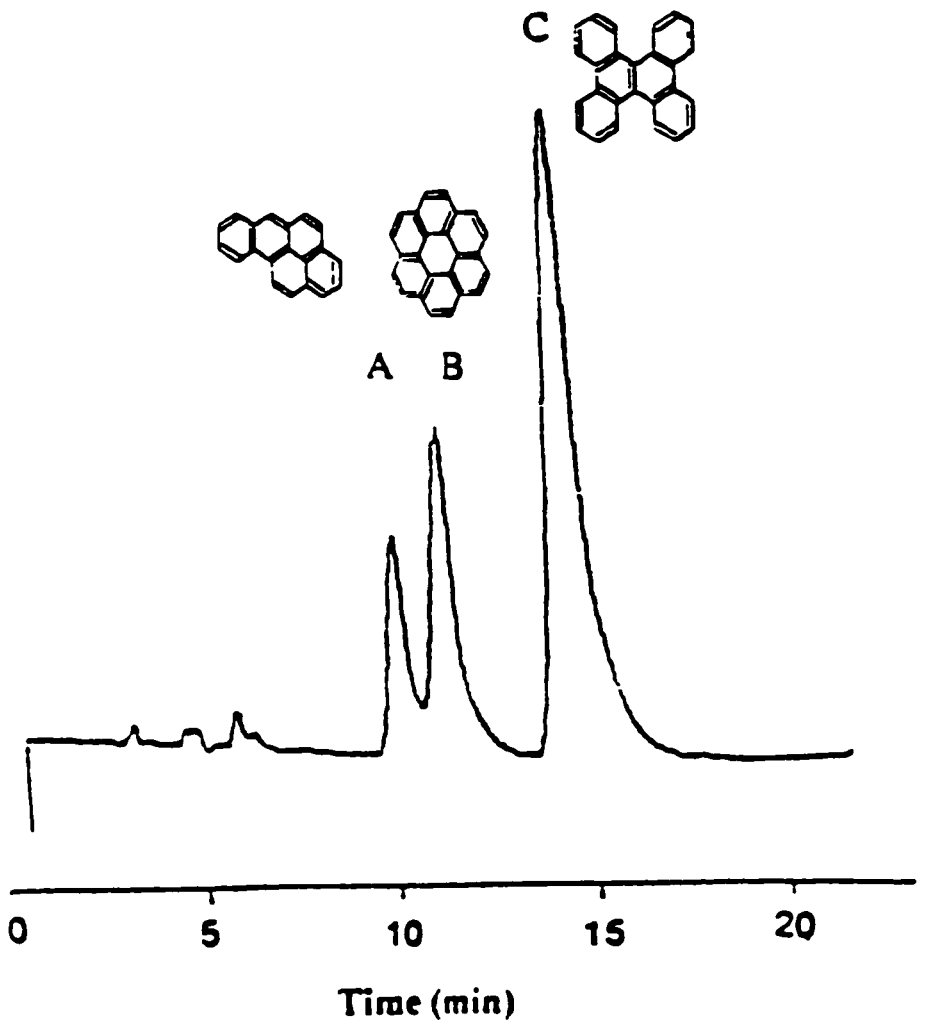


Figure 19. Reversed phase separation of PE universal test mix on a 1,9-decadiyne column. Mobile phase: methanol-water, v/v (50:50), at 0.3 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A = sodium chloride; B = benzene; C = toluene; D = ethylbenzene; E = isopropylbenzene; F = t-butylbenzene; G = anthracene.

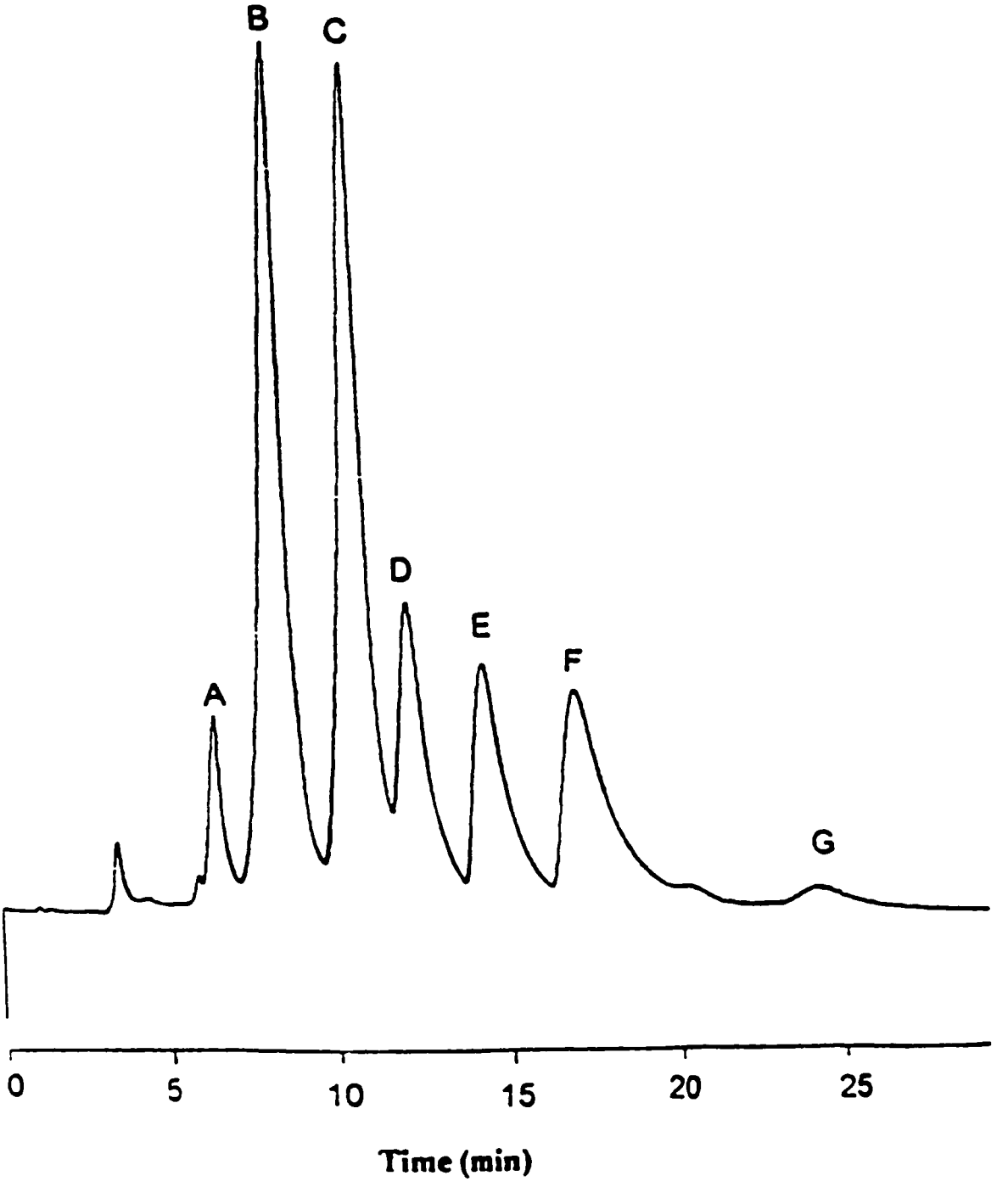
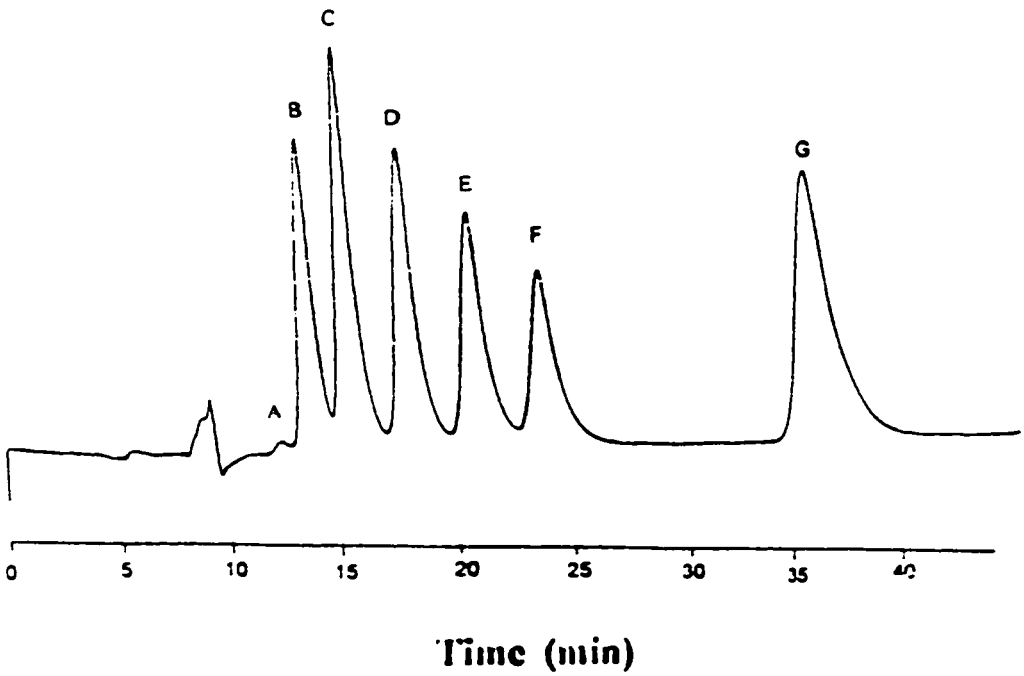


Figure 20. Reversed phase separation of PE universal test mix on a 1,9-decadiyne column. Mobile phase: acetonitrile-water, v/v (40:60), at 0.2 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A = sodium chloride; B = benzene; C = toluene; D = ethylbenzene; E = isopropylbenzene; F = t-butylbenzene; G = anthracene.



where a series of such drugs were analysed in forensic samples and were separated using isocratic reverse phase liquid chromatography [22].

Six of the most commonly used benzodiazepine drugs were chosen to be analyzed. Their names and structures are shown in Table 4. Samples were prepared by dissolving the powdered tablets in pure tetrahydrofuran. This mixture was then centrifuged and the supernatant was stored in the refrigerator. For HPLC analysis the detector was set to 254 nm, because most of these drugs have their uv maxima in that range [22]. They were individually injected into the column with methanol-water at a composition of 90:10 (v/v). Since methanol is a polar solvent, it exhibits specific selectivity towards the polar components in this mixture [23]. Table 5a shows the retention factor for the individual drugs. As there is extensive overlap in the retention times between various benzodiazepines, this particular composition of the mobile phase was considered to be less selective. The goal here was to optimize the conditions for the separation of these drugs. So various compositions of the mobile phase were tried and it was found that by decreasing the organic modifier in the eluent gave unique retention times for each compound. A methanol-water mixture (50:50, v/v) was highly selective for these drugs. The retention times of each of these drugs and their retention factors are shown in Table 5b. Similarly the effect that different solvents have on the retention of these drugs was studied. It was observed that acetonitrile-water at 30:70 showed good resolution power as compared to acetonitrile-water 80:20, v/v (Table 6a and 6b). Tetrahydrofuran-water was highly selective at 40:60,v/v (Table 7a and 7b). A mixture of six benzodiazepines was separated successfully by this column in the isocratic elution

TABLE 4.

Molecular Structure of the Studied Benzodiazepines:

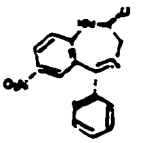
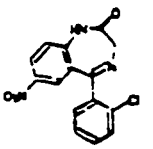
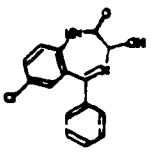
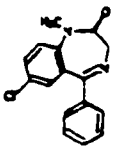
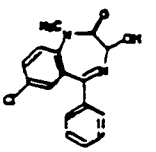
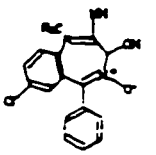
Compound	Structure
Nitrazepam	
Clonazepam	
Oxazepam	
Diazepam	
Temazepam	
Chlorodiazepoxide	

TABLE 5a

Column: 1,9-decadiyne
Mobile Phase: Methanol-Water (90:10, v/v)
Flow Rate: 0.4 mL/min
Pressure: 50 Bar

Analyte	Tablet Concentration(mg)	Sample Dilution (x times)	Retention Factor, k'
Nitrazepam	5	25	0.71
Oxazepam	10	50	0.71
Clonazepam	2	10	0.71
Temazepam	10	50	0.86
Chlordiazepoxide	5	25	0.83
Diazepam	2	10	0.89

TABLE 5b

Column: 1,9-decadiyne
Mobile Phase: Methanol-Water (50:50, v/v)
Flow Rate: 0.4 mL/min
Pressure: 90 Bar

Analyte	Tablet Concentration (mg)	Sample Dilution (x times)	Retention Factor, k'
Nitrazepam	5	25	0.76
Oxazepam	10	50	1.65
Clonazepam	2	10	1.73
Temazepam	10	50	0.26
Chlordiazepoxide	5	25	0.47
Diazepam	2	10	1.60

TABLE 6a

Column: 1,9-decadiyne
Mobile Phase: Acetonitrile-Water (80:20, v/v)
Flow Rate: 0.3 mL/min
Pressure: 43 Bar

Analyte	Tablet Concentration(mg)	Sample Dilution (x times)	Retention Factor, k′
Nitrazepam	5	25	0.46
Oxazepam	10	50	0.46
Clonazepam	2	10	0.42
Temazepam	10	50	0.50
Chlordiazepoxide	5	25	0.48
Diazepam	2	10	0.52

TABLE 6b

Column: 1,9-decadiyne
Mobile Phase: Acetonitrile-Water (30:70, v/v)
Flow Rate: 0.3 mL/min
Pressure: 66 Bar

Analyte	Tablet Concentration(mg)	Sample Dilution (x times)	Retention Factor, k′
Nitrazepam	5	25	2.01
Oxazepam	10	50	1.43
Clonazepam	2	10	2.81
Temazepam	10	50	1.94
Chlordiazepoxide	5	25	1.33
Diazepam	2	10	4.20

TABLE 7a

Column: 1,9-decadiyne
Mobile Phase: Tetrahydrofuran-Water (90:10, v/v)
Flow Rate: 0.5 mL/min
Pressure: 39 Bar

Analyte	Tablet Concentration(mg)	Sample Dilution (x times)	Retention Factor, k'
Nitrazepam	5	25	0.11
Oxazepam	10	50	0.08
Clonazepam	2	10	0.15
Temazepam	10	50	0.07
Chlordiazepoxide	5	25	0.29
Diazepam	2	10	0.17

TABLE 7b

Column: 1,9-decadiyne
Mobile Phase: Tetrahydrofuran-Water (40:60, v/v)
Flow Rate: 0.5 mL/min
Pressure: 95 Bar

Analyte	Tablet Concentration(mg)	Sample Dilution (x times)	Retention Factor, k'
Nitrazepam	5	25	2.14
Oxazepam	10	50	2.32
Clonazepam	2	10	2.57
Temazepam	10	50	2.43
Chlordiazepoxide	5	25	3.03
Diazepam	2	10	6.21

mode with methanol-water (50:50, v/v). Figure 21 shows that nitrazepam and clonazepam could barely be separated from each other even at high percentages of water. A chromatogram of the same mixture using tetrahydrofuran-water (40:60) is shown in Figure 22. The methanol-water solvent was more practical because it could separate all the six components in less than 25 min. The reason being that the OH and NO_2 substituents (nitrazepam and oxazepam) mainly affect solute polarity and are best separated by methanol, whereas the effects of acetonitrile and tetrahydrofuran are low. On the other hand the selectivity of tetrahydrofuran for CH_3 and Cl substituents (diazepam, temazepam and chlorodiazepoxide) is high. These groups give rise to variations in the molecular volume [23].

d. Separation of Diazepam and its Decomposition Products

The isolation of diazepam from its hydrolysis products was investigated. The hydrolysis products, 2-amino-5-chlorobenzophenone (ACB) and 2-methylamino-5-chlorobenzophenone (MACB), are usually detected in the urine samples of patients who have taken benzodiazpine in overdose [24]. In an acidic environment, benzodiazepines easily hydrolyze to its corresponding benzophenones. The diagram in Figure 23 shows the decomposition of diazepam to its metabolite temazepam and oxazepam and eventually to MACB and ACB [24].

For chromatographic analysis of these products, MACB and ACB (Hoffmann-La Roche) samples, diluted in tetrahydrofuran, were injected into the column individually and their retention behavior was investigated. The mobile phase that had good selectivity was tetrahydrofuran-water (40:60) at a flowrate of 0.4 ml/min. Separation of diazepam

Figure 21. Reversed phase separation of benzodiazepines on a 1,9-decadiyne column. Mobile phase: methanol-water, v/v (50:50), at 0.4 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A = nitrazepam; B = oxazepam; C = clonazepam; D = temazepam; E = chlorodiazepoxide; F = diazepam.

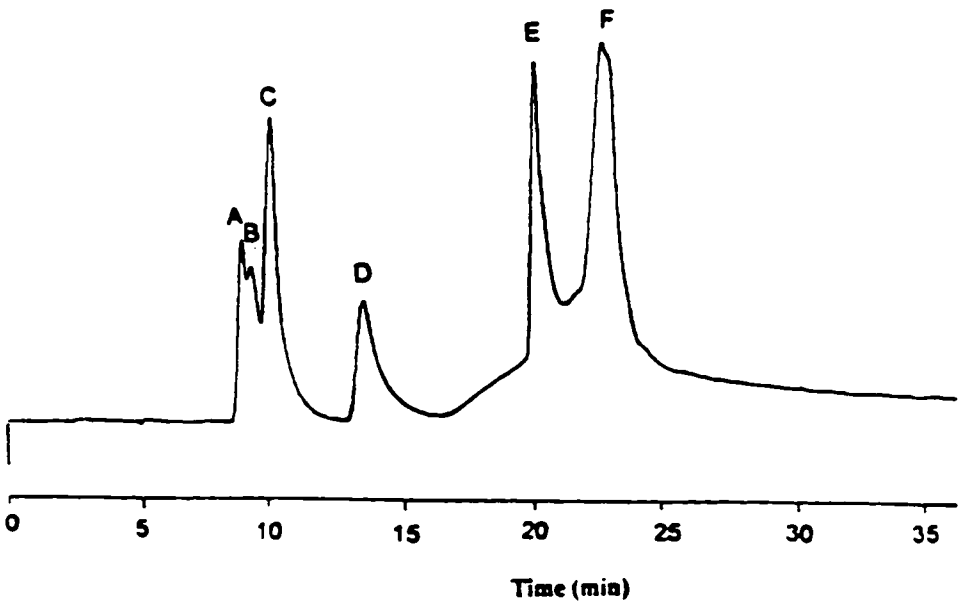
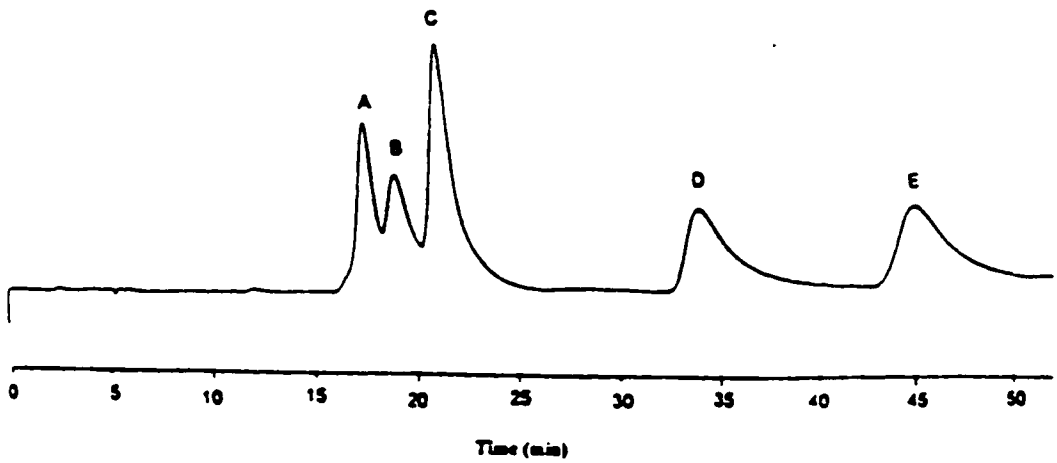


Figure 22. Reversed phase separation of benzodiazepines on a 1,9-decadiyne column. Mobile phase: tetrahydrofuran-water, v/v (40:60), at 0.4 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A = nitrazepam; B = oxazepam; C = temazepam; D = chlorodiazepoxide; E = diazepam.



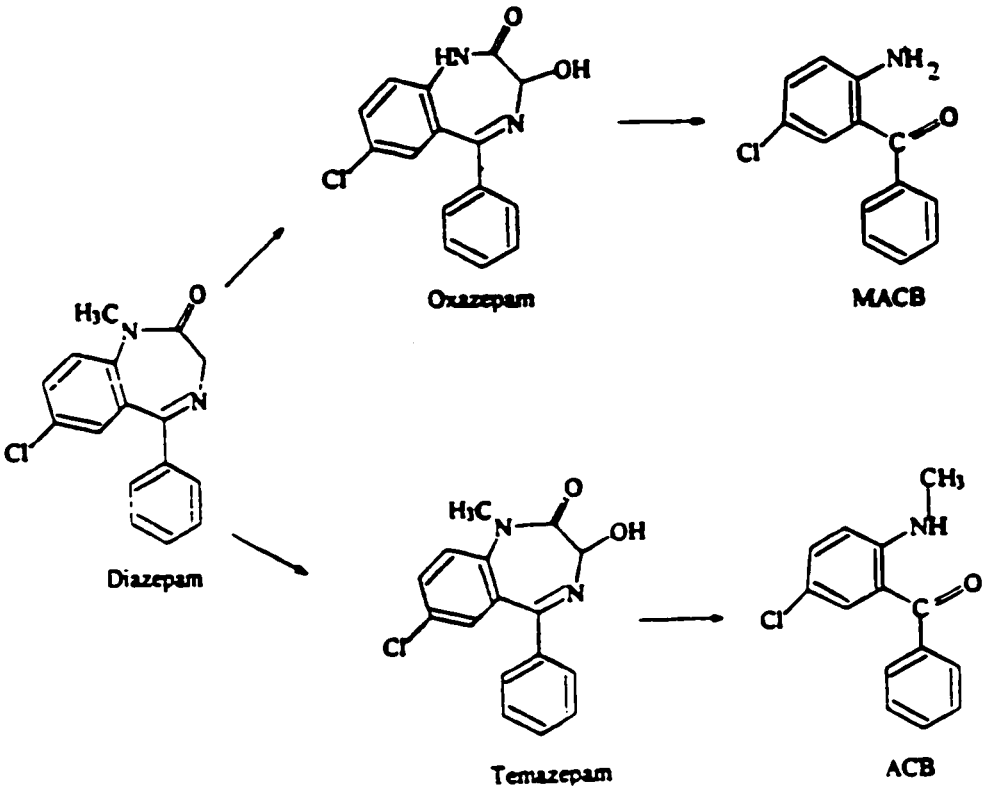


Figure 23. Metabolism of Diazepam and its Hydrolysis Product

from its metabolites temazepam and MACB is shown in Figure 24. Figure 25 shows the separation of diazepam from oxazepam and ACB.

e. Separation of Nitrazepam and Decomposition Products

Similarly nitrazepam breaks down to clonazepam and ANB as shown in Figure 26 [24]. ANB was also diluted in tetrahydrofuran and injected into the column. A mixture consisting of nitrazepam, clonazepam and ANB was prepared and analyzed. Tetrahydrofuran-water (40:60) provided excellent selectivity at 0.4 ml/min (Figure 27). The resolution between the peaks is not as good as in diazepam, because clonazepam and nitrazepam are very similar in retention characteristics.

f. Gradient Elution of Benzodiazepines

Gradient elution was performed on a mixture of four of the benzodiazepines. The gradient was from 40 to 100% methanol in 15 minutes with water at 0.5 mL/min. The gradient did not seem to work very well for this mixture as the base line was too unsteady due to very high pressure with this column. A wavy or undulating baseline could be due to the temperature changes in the room. One way of eliminating this problem is by insulating the column or by using a column oven to cover the detector to keep out air currents. The chromatogram in Figure 28 shows that the trailing peaks are only a shoulder and are unresolvable. This is because of the coelution of two or more components. The peaks at 13.46 and 18.76 minutes are from the impurities present in the mixture.

Figure 24. Reversed phase separation of metabolites and benzodiazepines on a 1,9-decadiyne column. Mobile phase: tetrahydrofuran-water, v/v (40:60), at 0.5 mL/min; sample injection 20 μL; detection: UV at 254 nm: Peaks: A = temazepam; B = diazepam; C = 2-methylamino-5-chlorobenzophenone.

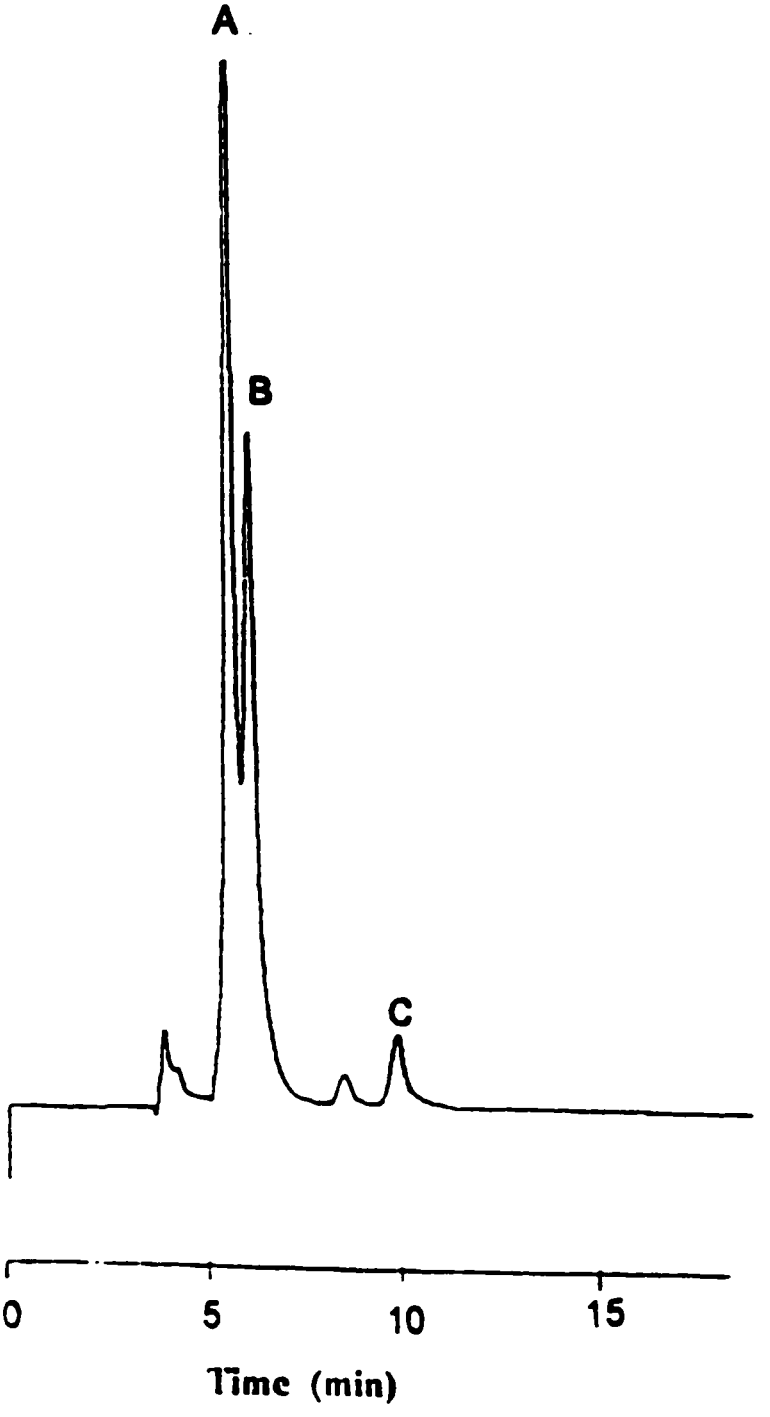
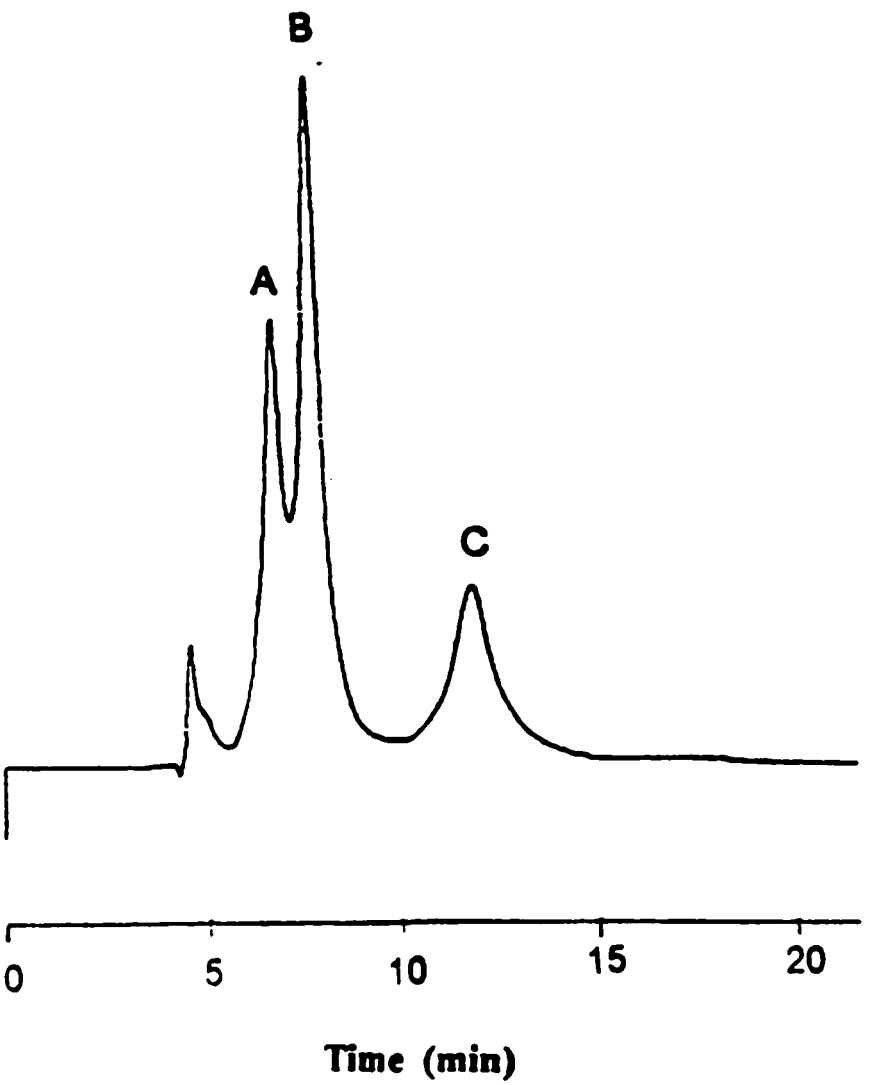


Figure 25. Reversed phase separation of metabolites and benzodiazepines on a 1,9-decadiyne column. Mobile phase: tetrahydrofuran-water, v/v (40:60), at 0.4 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A = oxazepam; B = diazepam; C = 2-amino-5-chlorobenzophenone.



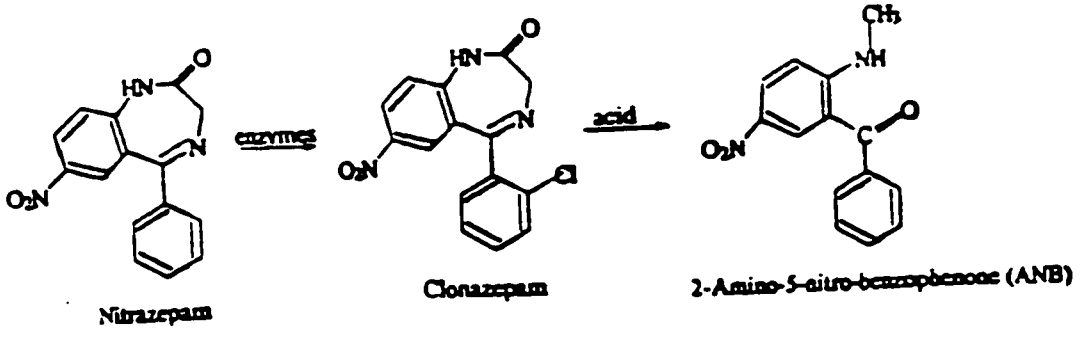


Figure 26. Metabolism of Nitrazepam and its Hydrolysis Product

Figure 27. Reversed phase separation of metabolites and benzodiazepines on a 1,9-decadiyne column. Mobile phase: tetrahydrofuran-water, v/v (40:60), at 0.4 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A = nitrazepam; B = clonazepam; C = 2-amino-5-nitrobenzophenone.

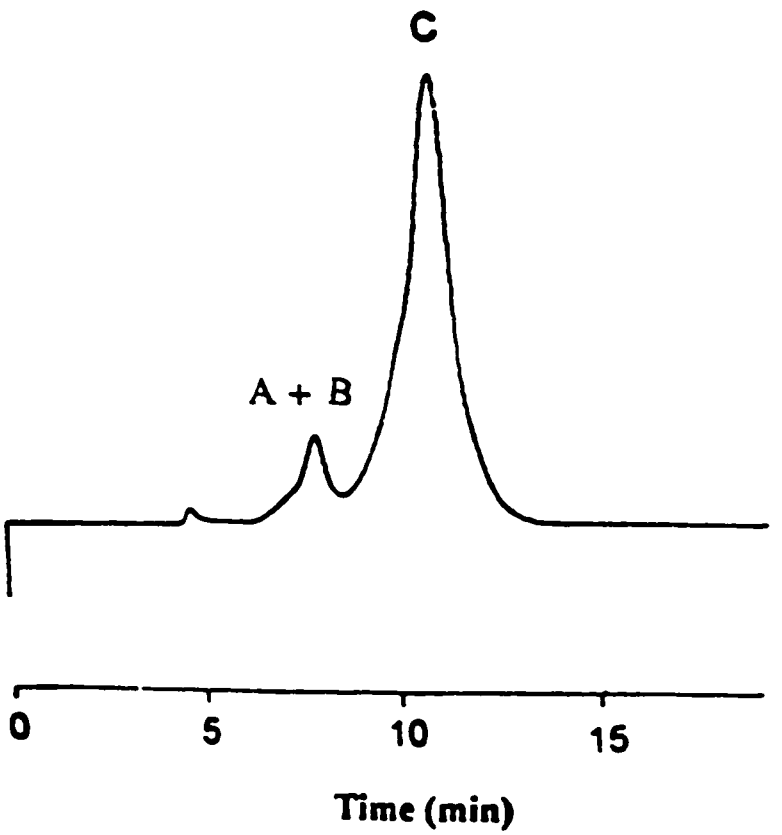
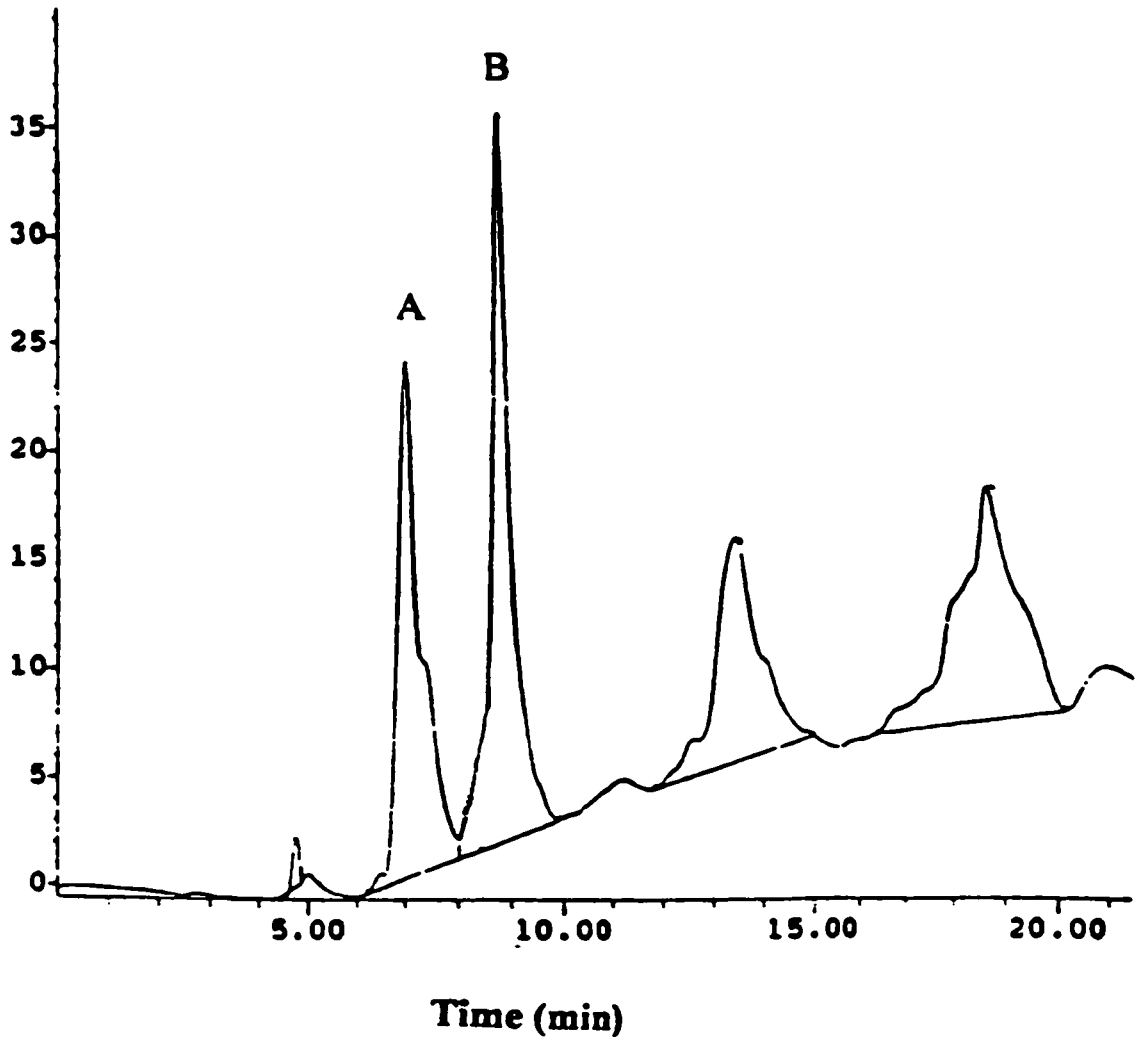


Figure 28. Gradient elution of benzodiazepines on a 1,9-decadiyne column. Mobile phase: gradient from 40 to 100% methanol for 15 min with water at 0.5 mL/min; sample injection 5 µL; detection: UV at 254 nm: Peaks: A = oxazepam; B = temazepam.



2. 1,9-decadiene Column

a. Isocratic Separation of Three Polycyclic Aromatic Hydrocarbons

The three compound test compound, SRM 869, was injected into the 1,9-decadiene column using a methanol-water mixture (80:20) as the mobile phase at a flow rate of 0.3 ml/min to determine the reverse phase characteristics (Figure 29). Acetonitrile-water (70:30) at 0.3 ml/min also had selectivity towards these hydrocarbons (Figure 30). Elution of the three components resulted in a chromatogram that is characteristic of a highly loaded monomeric phase. The separation factor, α for TBN and BaP with methanol-water is around 1.76 and is smaller than the α value of the 1,9-decadiyne column [17, 18].

b. Perkin Elmer Universal Test mix

A standard mixture of aromatic hydrocarbons (benzene, toluene, ethylbenzene, isopropylbenzene and anthracene) was separated in 28 min with methanol-water (50:50) at 0.3 mL/min (Figure 31) and in 20 min for an acetonitrile-water (40:60) mobile phase (Figure 32). The most interesting feature of this chromatogram is the separation between the last two solutes, t-butylbenzene and anthracene. The separation factor, α, for methanol-water and acetonitrile-water is around 2.3 and 1.56 respectively. This high α value can be explained by the increased solute-stationary phase interaction, which increases the retention for planar compounds and solutes with large length to breadth ratios. This result is consistent with good reverse phase properties.

Figure 29. Reversed phase separation of NIST mix (SRM 869) on a 1,9-decadiene column. Mobile phase: methanol-water, v/v (80:20), at 0.3 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A =Benzo[a]pyrene; B = Phenanthro[3,4-c] phenanthrene; C = Tetrabenzonaphthalene.

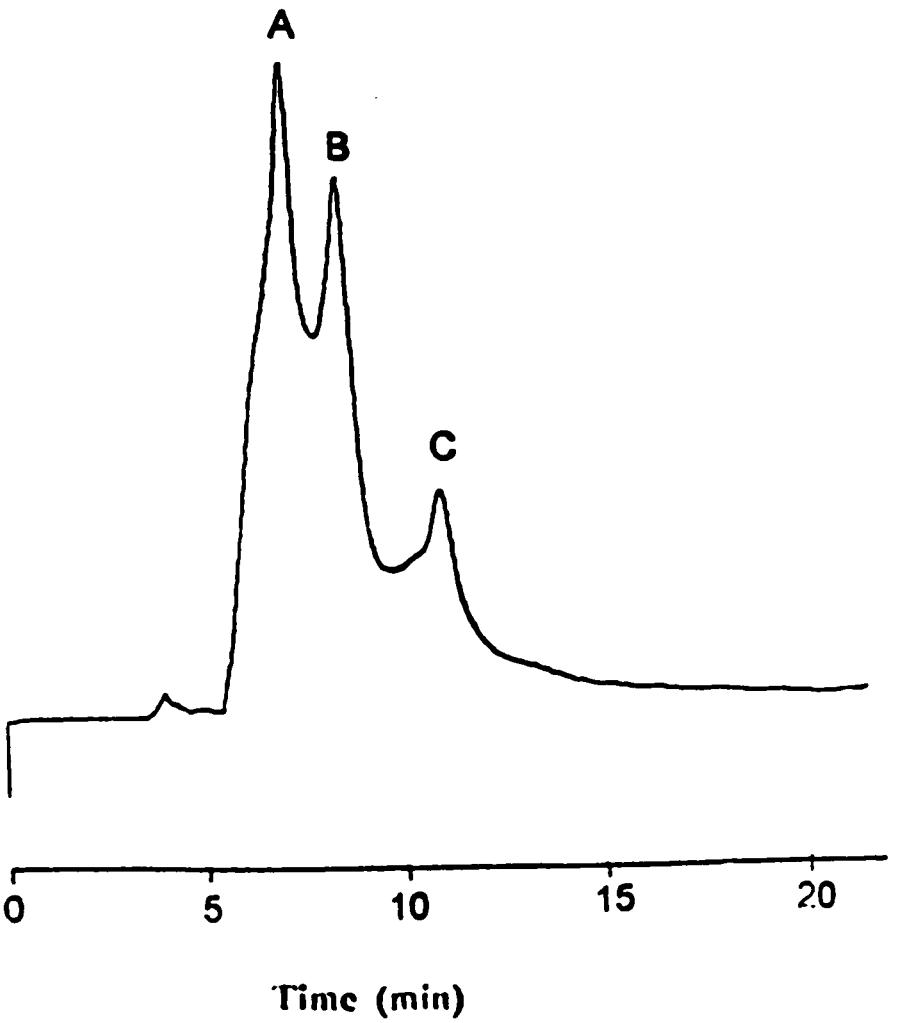


Figure 30. Reversed phase separation of NIST mix (SRM 869) on a 1,9-decadiene column. Mobile phase: acetonitrile-water, v/v (70:30), at 0.3 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A =Benzo[a]pyrene; B = Phenanthro[3,4-c] phenanthrene; C = Tetrabenzonaphthalene.

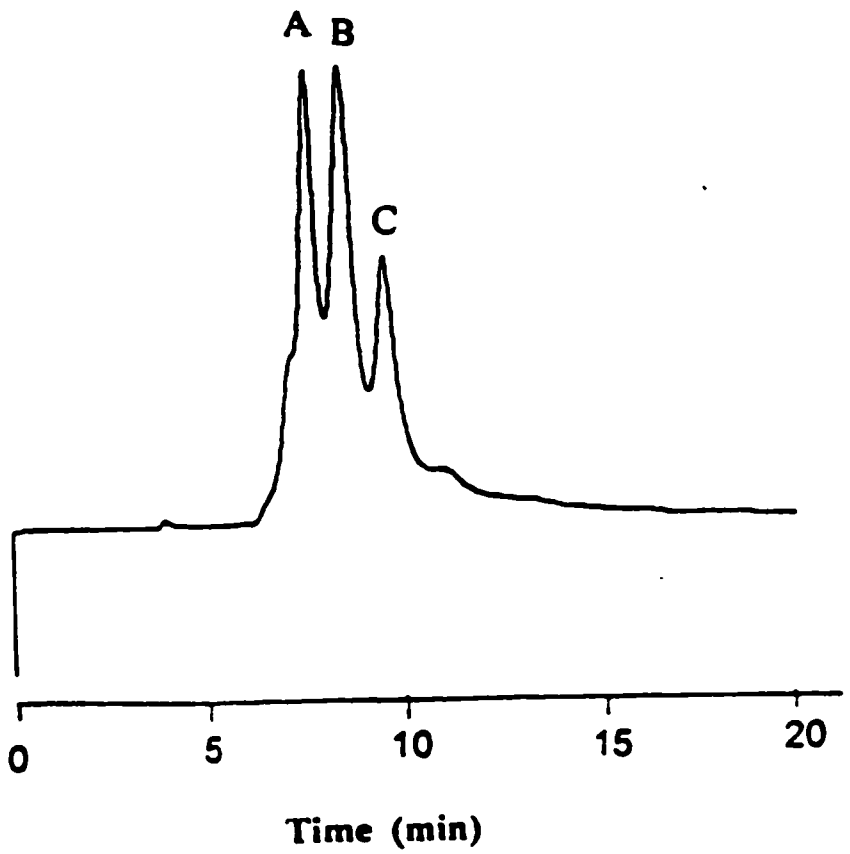


Figure 31. Reversed phase separation of PE universal test mix on a 1,9-decadiene column. Mobile phase: methanol-water, v/v (50:50), at 0.3 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A = sodium chloride; B = benzene; C = toluene; D = ethylbenzene; E = isopropylbenzene; F = t-butylbenzene; G = anthracene.

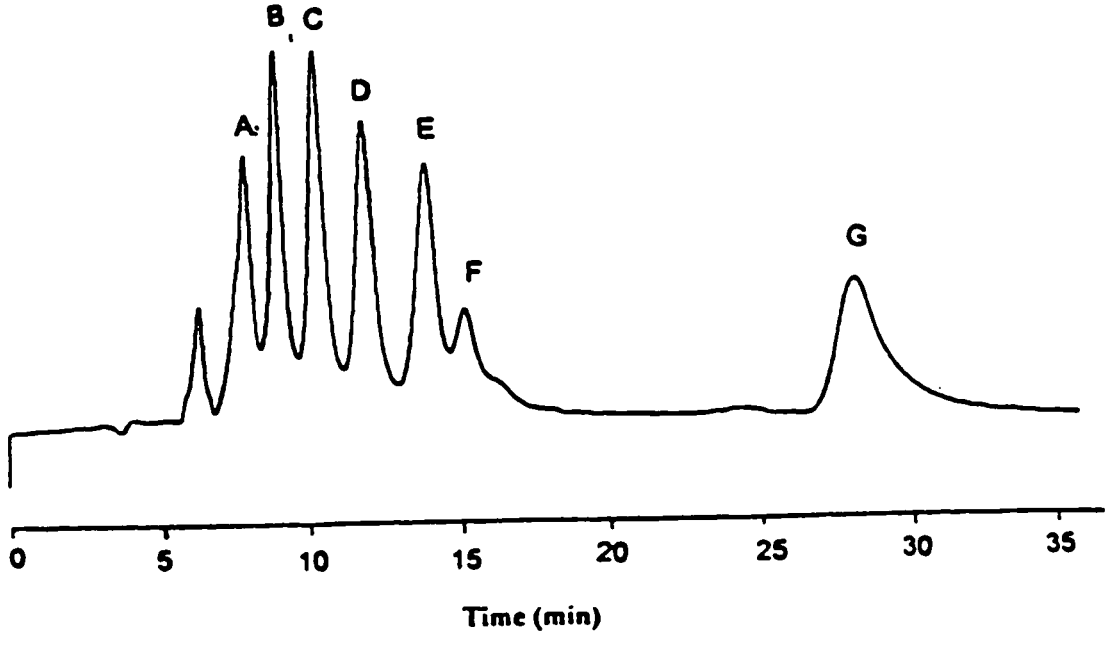
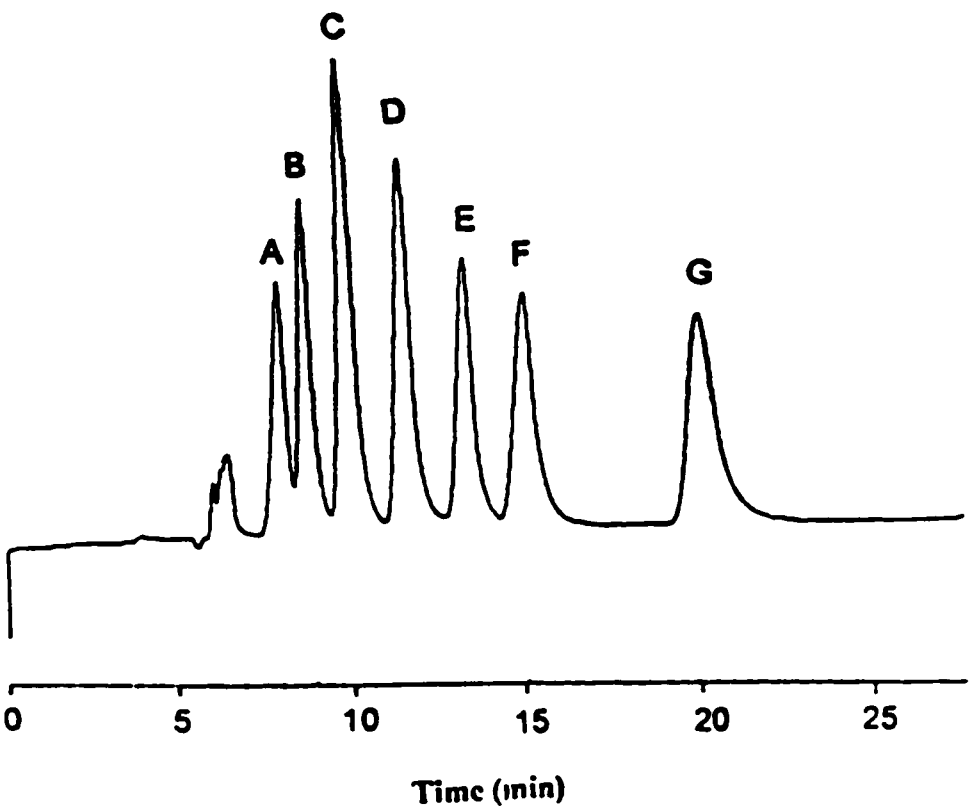


Figure 32. Reversed phase separation of PE universal test mix on a 1,9-decadiene column. Mobile phase: acetonitrile-water, v/v (40:60), at 0.3 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A = sodium chloride; B = benzene; C = toluene; D = ethylbenzene; E = isopropylbenzene; F = t-butylbenzene; G = anthracene.



c. Benzodiazepine Analysis

The separation in Figure 33 a, is the mixture of five benzodiazepines (oxazepam, nitrazepam, temazepam, clonazepam and chlorodiazepoxide) with methanol-water (90:10) at 0.3 ml/min. Baseline separation is obtained for the above compounds by increasing the water percentage in the mobile phase to 60%. Some examples of benzodiazepine separation are shown in Figures 33 b, c, d, e and f. Optimum conditions for these mixtures were found to be methanol-water (40:60) and acetonitrile-water (30:70) at 0.3 ml/min. Baseline or better separation is achieved for all the components. Tables 8a and b shows the increase in retention factor of the individual benzodiazepines with methanol-water (40:60). An increase in the retention factor for these compounds in presence of acetonitrile-water at 30:70 are shown in Tables 9a and b. The retention factors for benzodiazepines with tetrahydrofuran-water are shown in Table 10a and b.

d. Separation of Diazepam and its Decomposition Products

It has already been demonstrated that methanol-water (40:60), acetonitrile-water (30:70) are appropriate for use in benzodiazepine separation. Under similar conditions, diazepam and its metabolites such as temazepam and oxazepam as well as its hydrolysis products (Figure 23) showed excellent retention on 1,9-decadiene columns. Figures 34 a, b and c are the chromatograms showing the baseline separation of temazepam, diazepam and MACB in the presence of different solvents. Chromatograms of oxazepam, diazepam and ACB under different solvents are shown in Figures 35 a, b, and c. In addition, tetrahydrofuran-water (40:60) was also highly selective for these compounds.

Figure 33 a. Reversed phase separation of benzodiazepines on a 1,9-decadiene column. Mobile phase: methanol-water, v/v (90:10), at 0.3 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A = oxazepam + nitrazepam; B = temazepam; C = chlorodiazepoxide; D = diazepam.

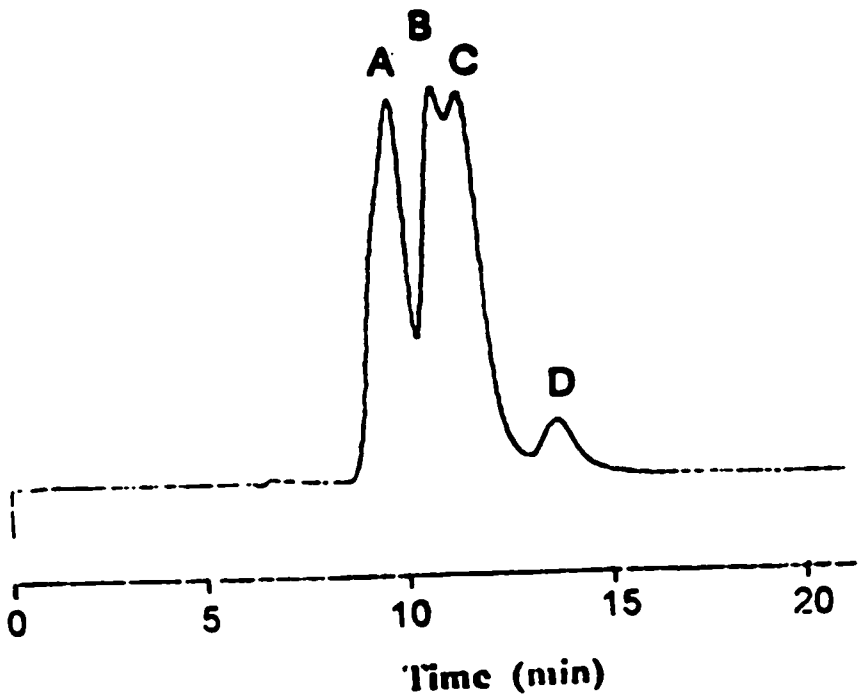


Figure 33 b. Reversed phase separation of benzodiazepines on a 1,9-decadiene column. Mobile phase: methanol-water, v/v (40:60), at 0.3 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A = oxazepam; B = temazepam; C = chlorodiazepoxide; D = diazepam.

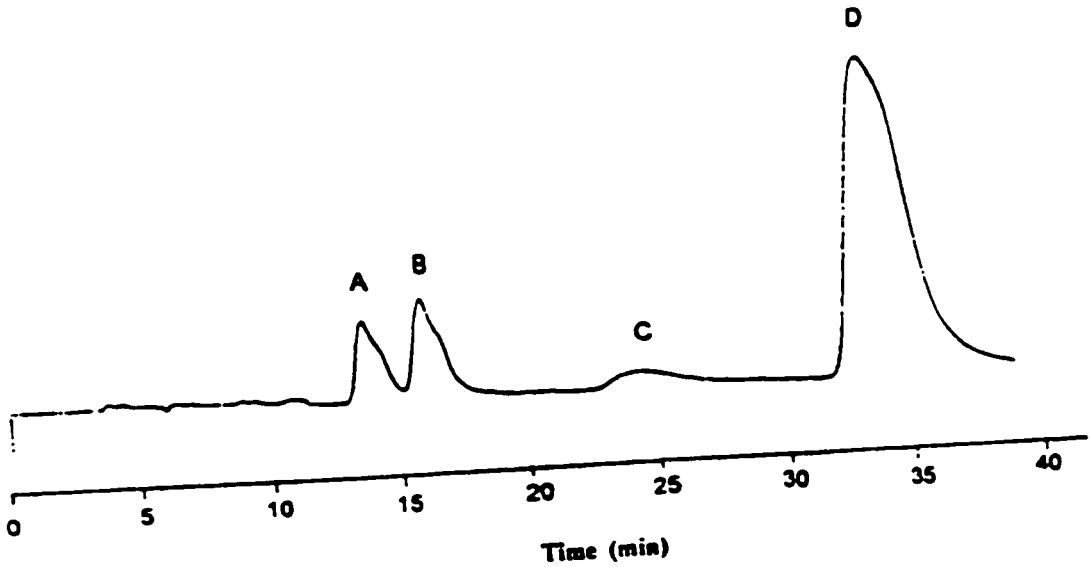


Figure 33 c. Reversed phase separation of benzodiazepines on a 1,9-decadiene column. Mobile phase: methanol-water, v/v (30:70), at 0.3 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks A = oxazepam; B = temazepam; C = chlorodiazepoxide; D = diazepam.

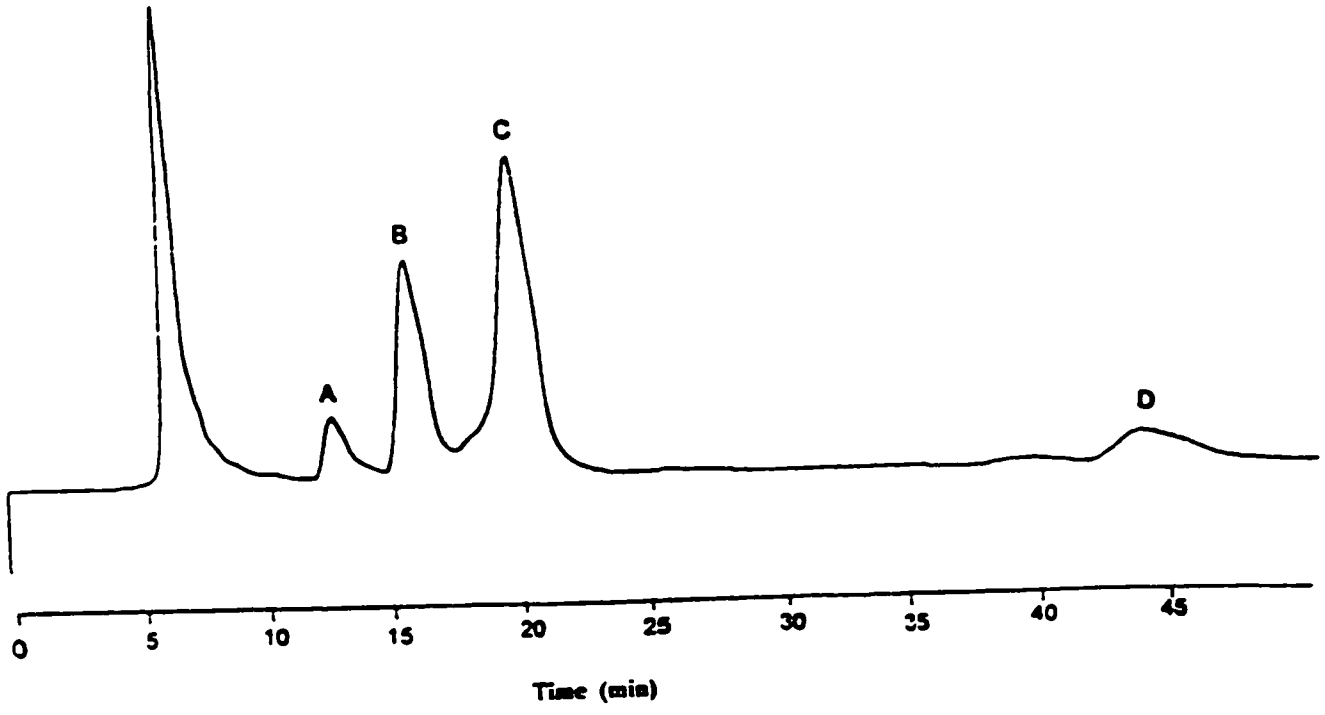


Figure 33 d. Reversed phase separation of benzodiazepines on a 1,9-decadiene column. Mobile phase: methanol-water, v/v (40:60), at 0.3 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks A = oxazepam; B = temazepam; C = clonazepam; D = chlorodiazepoxide.

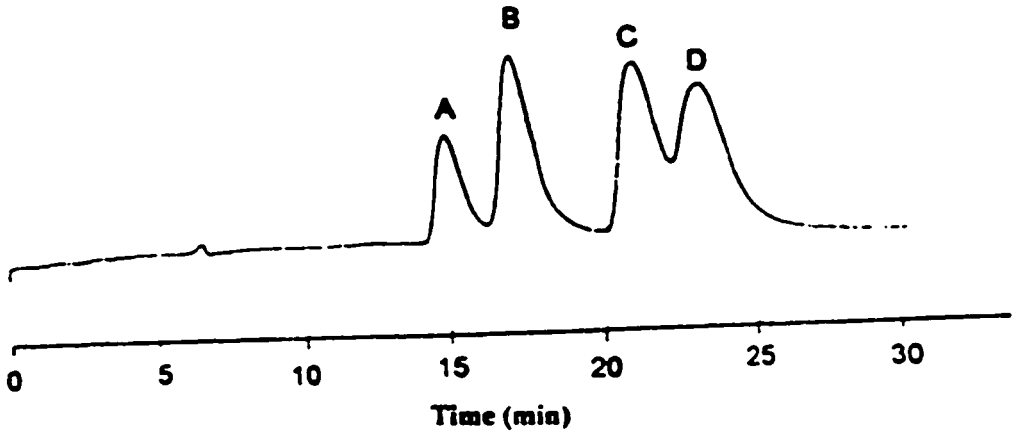


Figure 33 e. Reversed phase separation of benzodiazepines on a 1,9-decadiene column. Mobile phase: methanol-water, v/v (40:60), at 0.3 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks A = nitrazepam; B = oxazepam; C = temazepam; D = chlorodiazepoxide.

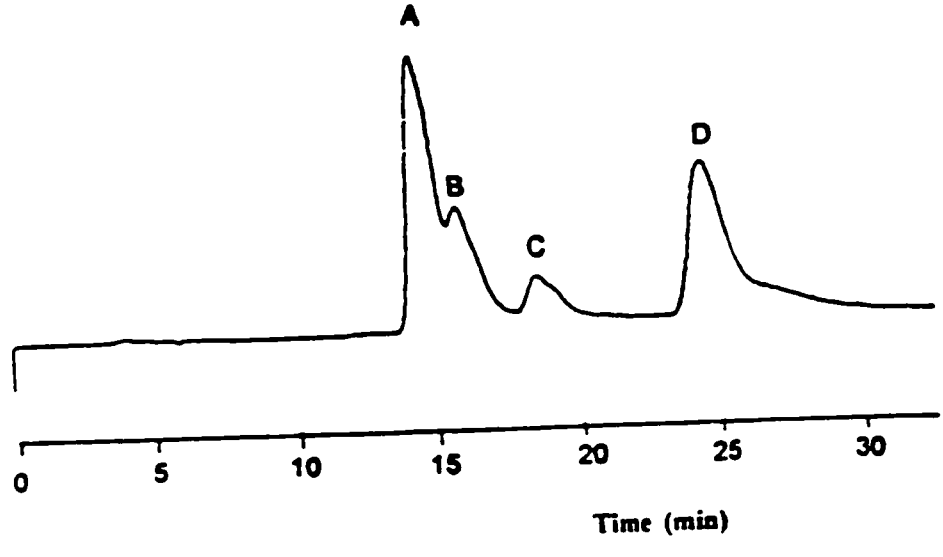


Figure 33 f. Reversed phase separation of benzodiazepines on a 1,9-decadiene column. Mobile phase: methanol-water, v/v (40:60), at 0.3 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks A = nitrazepam; B = temazepam; C = chlorodiazepoxide; D = diazepam.

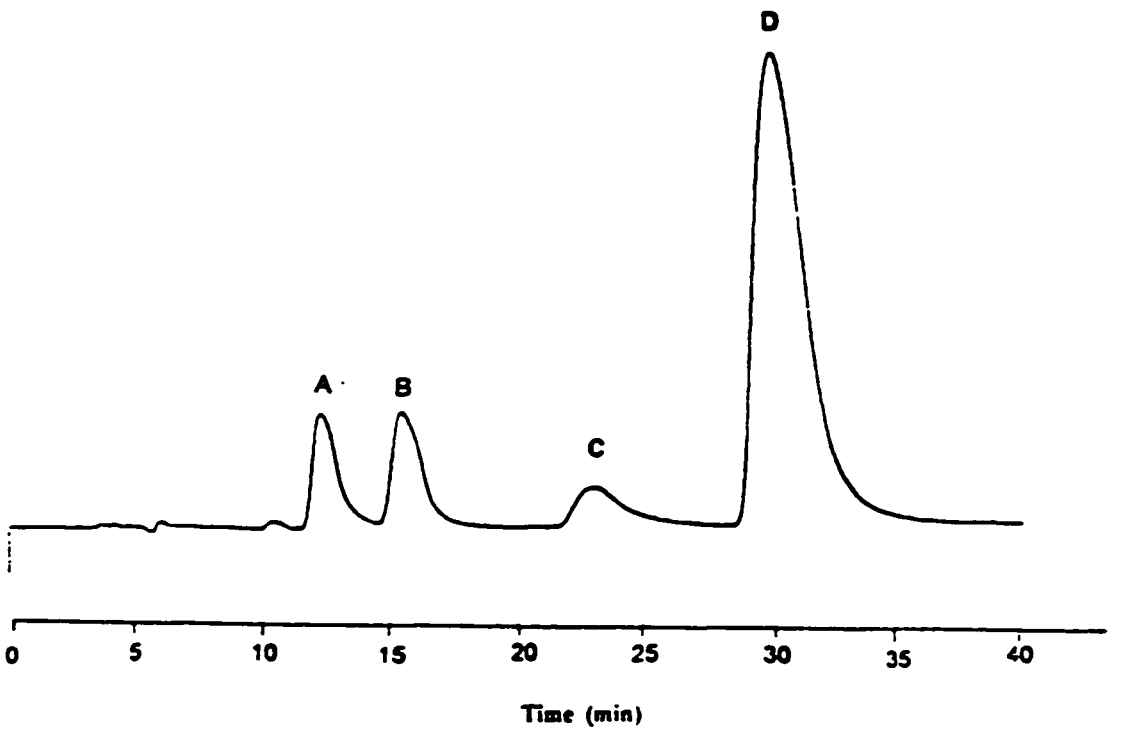


TABLE 8a

Column: 1,9-decadiene
Mobile Phase: Methanol-Water (70:30, v/v)
Flow Rate: 0.3 mL/min
Pressure: 19 Bar

Analyte	Tablet Concentration(mg)	Sample Dilution (x times)	Retention Factor, k'
Nitrazepam	5	25	0.84
Oxazepam	10	50	0.78
Clonazepam	2	10	0.68
Temazepam	10	50	0.73
Chlordiazepoxide	5	25	1.08
Diazepam	2	10	0.91

TABLE 8b

Column: 1,9-decadiene
Mobile Phase: Methanol-Water (40:60, v/v)
Flow Rate: 0.3 mL/min
Pressure: 20 Bar

Analyte	Tablet Concentration (mg)	Sample Dilution (x times)	Retention Factor, k'
Nitrazepam	5	25	1.28
Oxazepam	10	50	1.80
Clonazepam	2	10	1.71
Temazepam	10	50	2.20
Chlordiazepoxide	5	25	2.69
Diazepam	2	10	4.31

TABLE 9a

Column: 1,9-decadiene
Mobile Phase: Acetonitrile-Water (80:20, v/v)
Flow Rate: 0.3 mL/min
Pressure: 7 Bar

Analyte	Tablet Concentration(mg)	Sample Dilution (x times)	Retention Factor, k'
Nitrazepam	5	25	2.41
Oxazepam	10	50	2.48
Clonazepam	2	10	2.51
Temazepam	10	50	2.45
Chlordiazepoxide	5	25	2.43
Diazepam	2	10	2.12

TABLE 9b

Column: 1,9-decadiene
Mobile Phase: Acetonitrile-Water (30:70, v/v)
Flow Rate: 0.3 mL/min
Pressure: 12 Bar

Analyte	Tablet Concentration(mg)	Sample Dilution (x times)	Retention Factor, k'
Nitrazepam	5	25	1.33
Oxazepam	10	50	1.04
Clonazepam	2	10	2.19
Temazepam	10	50	1.53
Chlordiazepoxide	5	25	5.38
Diazepam	2	10	6.15

TABLE 10a

Column: 1,9-decadiene
Mobile Phase: Tetrahydrofuran-Water (80:20, v/v)
Flow Rate: 0.2 mL/min
Pressure: 12 Bar

Analyte	Tablet Concentration(mg)	Sample Dilution (x times)	Retention Factor, k'
Nitrazepam	5	25	0.08
Oxazepam	10	50	0.09
Clonazepam	2	10	0.07
Temazepam	10	50	0.11
Chlordiazepoxide	5	25	0.14
Diazepam	2	10	0.13

TABLE 10b

Column: 1,9-decadiyne
Mobile Phase: Tetrahydrofuran-Water (40:60, v/v)
Flow Rate: 0.3 mL/min
Pressure: 21 Bar

Analyte	Tablet Concentration(mg)	Sample Dilution (x times)	Retention Factor, k'
Nitrazepam	5	25	0.55
Oxazepam	10	50	0.42
Clonazepam	2	10	0.40
Temazepam	10	50	0.48
Chlordiazepoxide	5	25	0.63
Diazepam	2	10	0.61

Figure 34 a. Reversed phase separation of metabolites and benzodiazepines on a 1,9-decadiene column. Mobile phase: methanol-water, v/v (40:60), at 0.5 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A = temazepam; B = diazepam; C = 2-methylamino-5-chlorobenzophenone.

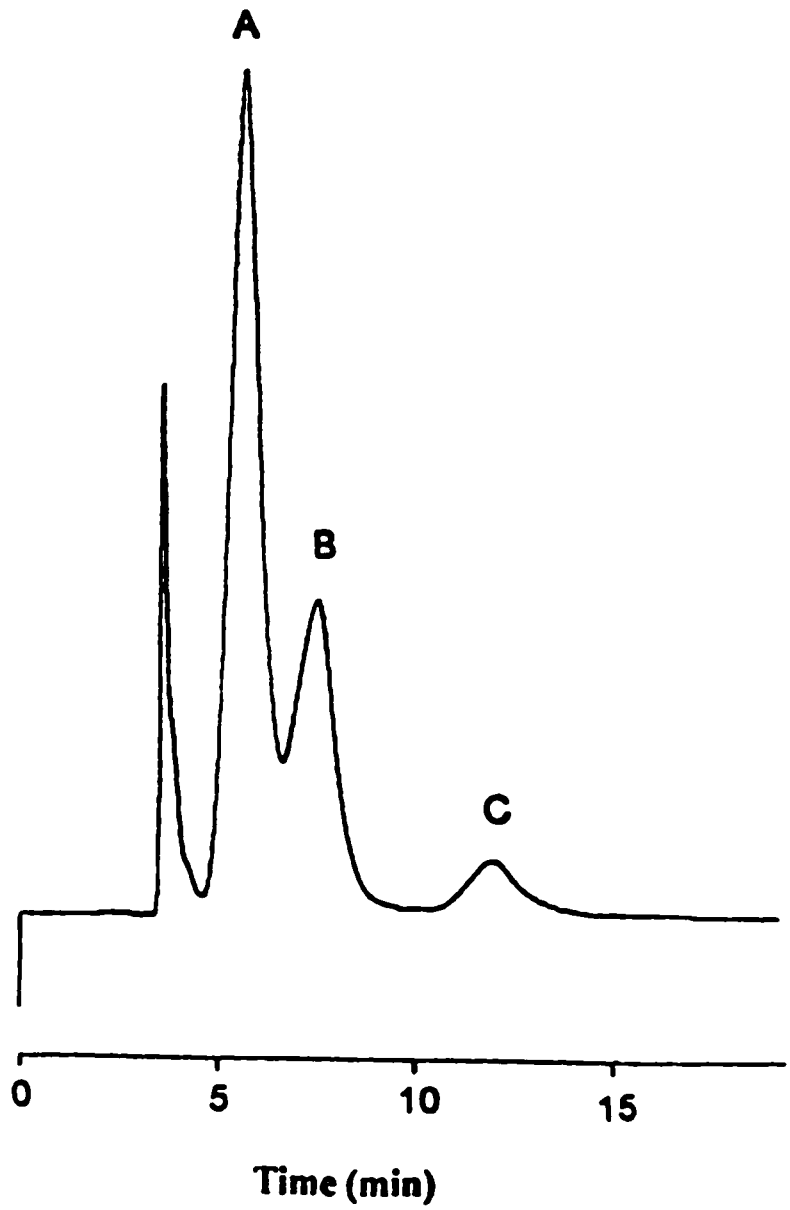


Figure 34 b. Reversed phase separation of metabolites and benzodiazepines on a 1,9-decadiene column. Mobile phase: acetonitrile-water, v/v (30:70), at 0.5 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A = temazepam; B = diazepam; C = 2-methylamino-5-chlorobenzophenone.

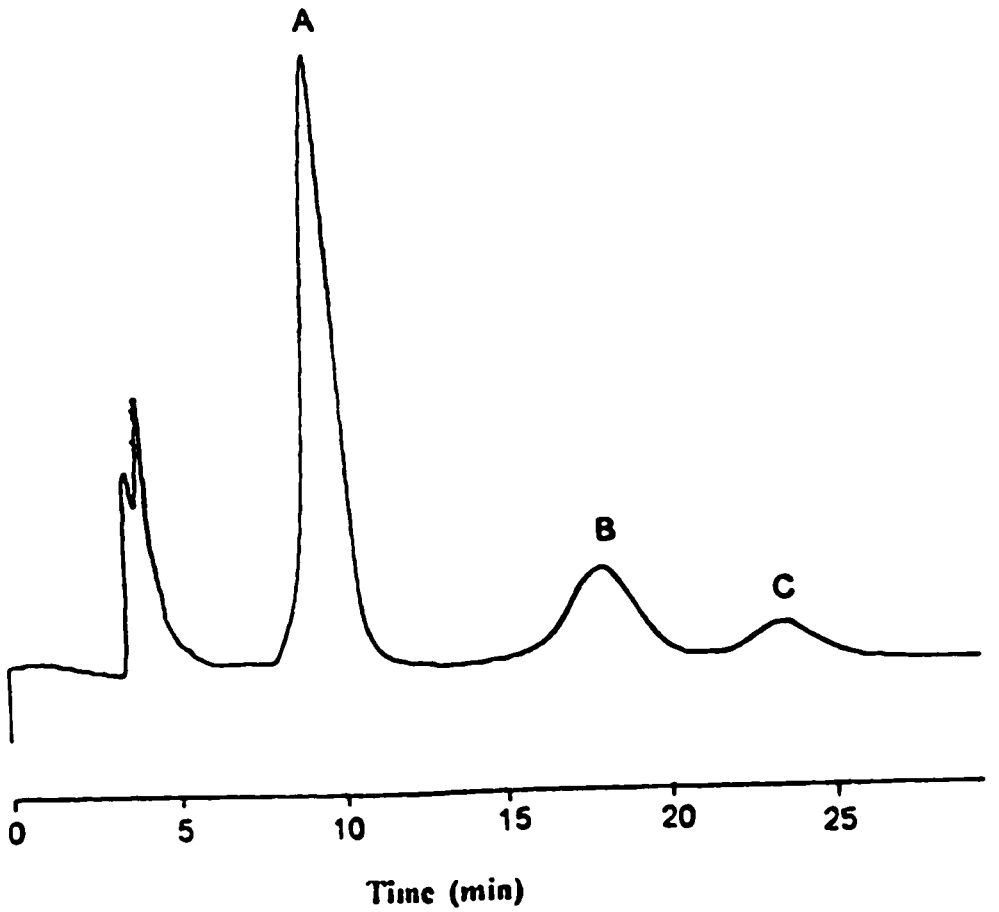


Figure 34 c. Reversed phase separation of metabolites and benzodiazepines on a 1,9-decadiene column. Mobile phase: tetrahydrofuran-water, v/v (40:60), at 0.3 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A = temazepam; B = diazepam; C = 2-methylamino-5-chlorobenzophenone.

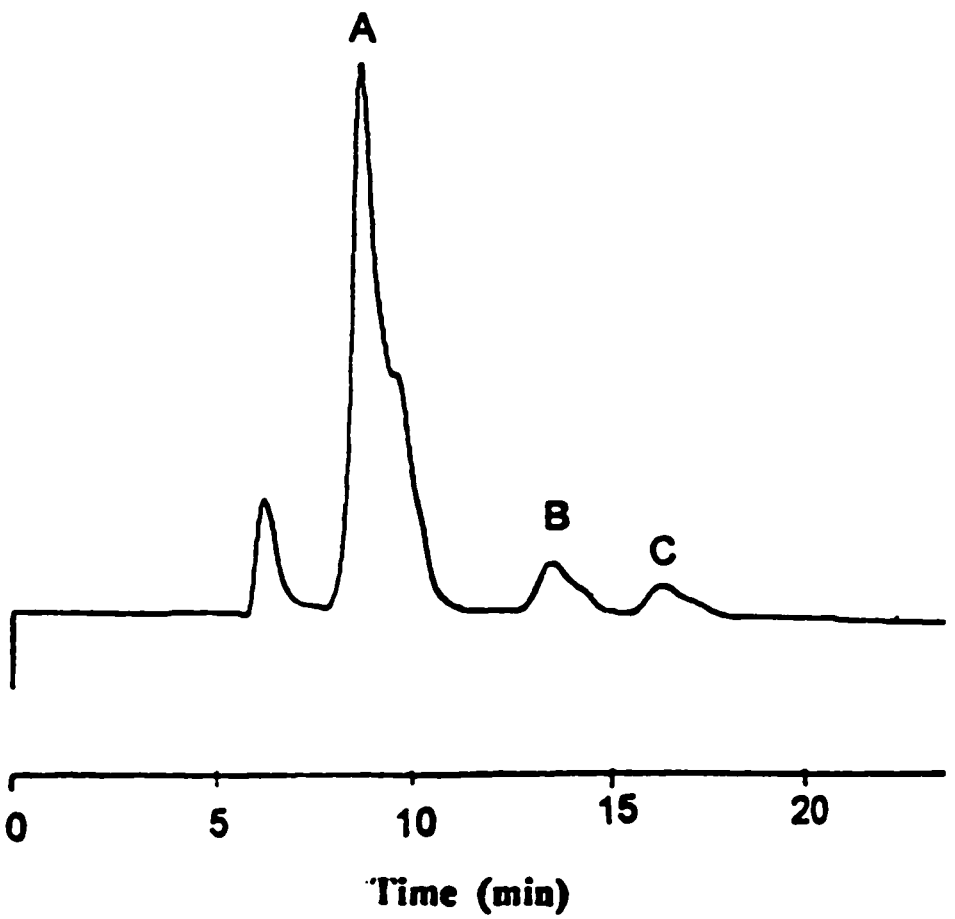


Figure 35 a. Reversed phase separation of metabolites and benzodiazepines on a 1,9-decadiene column. Mobile phase: methanol-water, v/v (40:60), at 0.5 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A = oxazepam; B = diazepam; C = 2-amino-5-chlorobenzophenone.

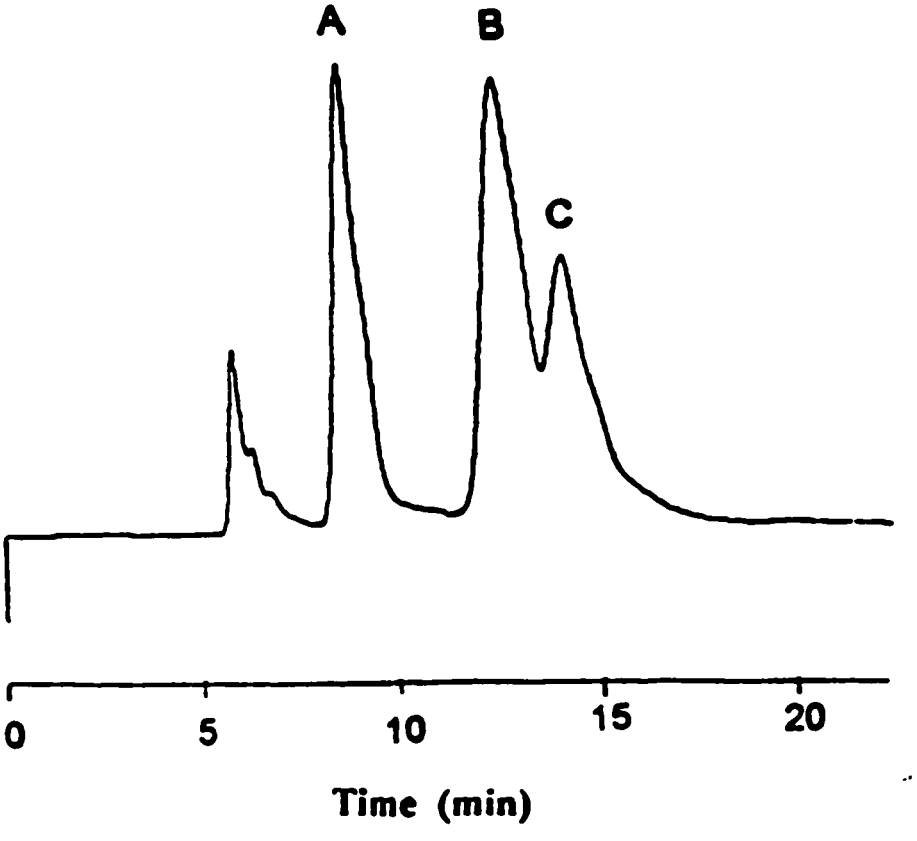


Figure 35 b. Reversed phase separation of metabolites and benzodiazepines on a 1,9-decadiene column. Mobile phase: acetonitrile-water, v/v (30:70), at 0.5 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A = oxazepam; B = diazepam; C = 2-amino-5-chlorobenzophenone.

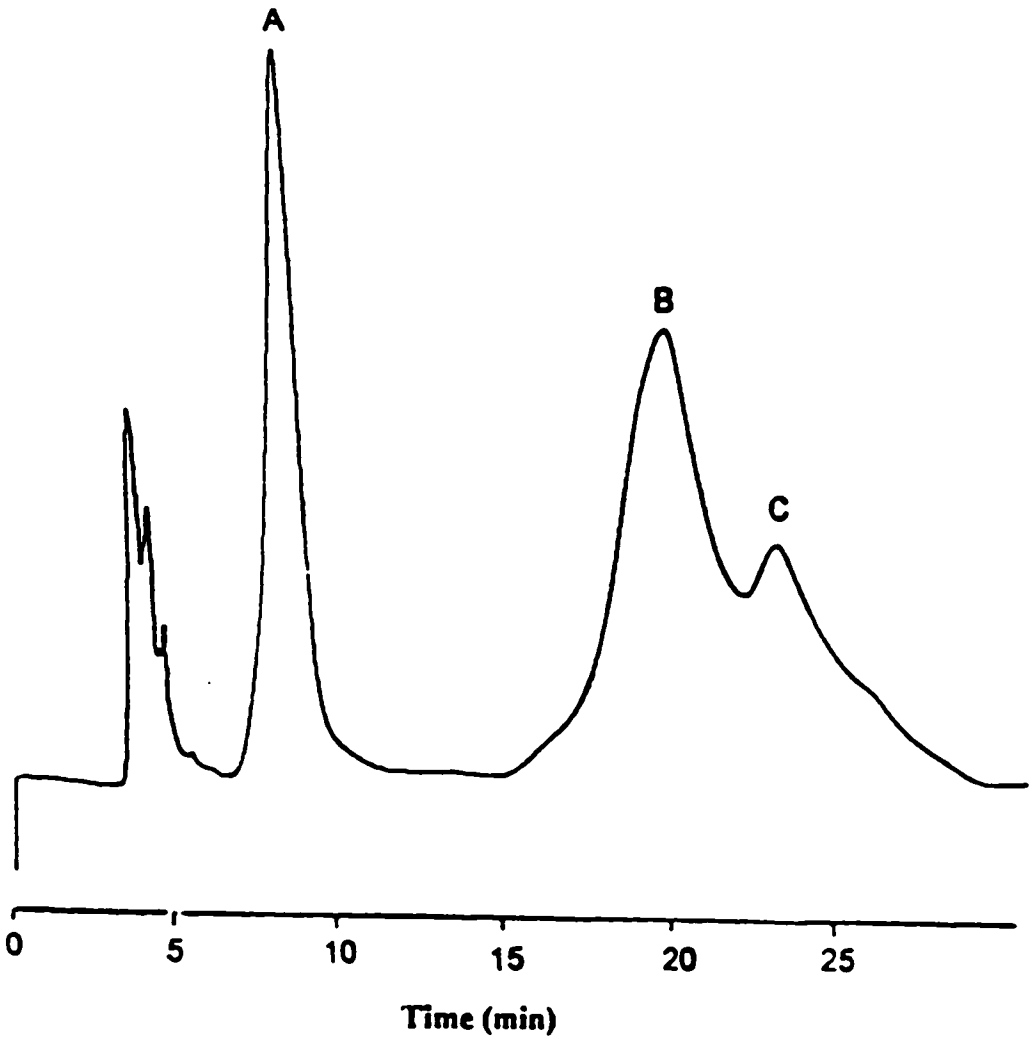
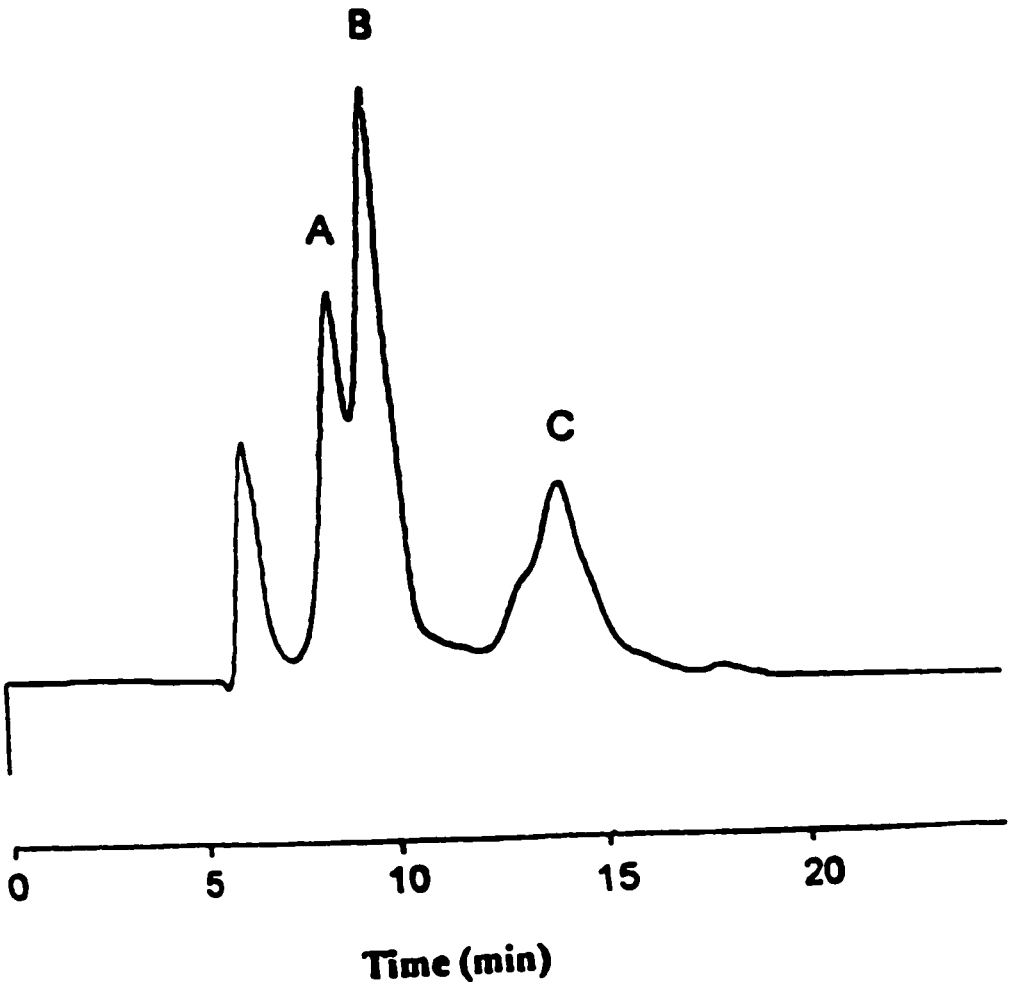


Figure 35 c. Reversed phase separation of metabolites and benzodiazepines on a 1,9-decadiene column. Mobile phase: tetrahydrofuran-water, v/v (40:60), at 0.3 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A = oxazepam; B = diazepam; C = 2-amino-5-chlorobenzophenone.



The increased retention of the hydrolysis products was consistent with the results obtained on the 1,9-decadiyne column.

e. Separation of Nitrazepam and its Decomposition Products

Figures 36 a, b and c show the separation of the three component mixture containing the compound nitrazepam, its metabolite clonazepam and its hydrolysis product, 2-amino-5-nitrobenzophenone (ANB). ANB was completely separated using methanol-water (40:60, v/v) at 0.5 mL/min. The clonazepam peak appears only as a shoulder and does not show any significant separation from nitrazepam.

Tetrahydrofuran-water (40:60, v/v) at 0.3 mL/min and acetonitrile-water (30:70, v/v) also showed high selectivity and good peak shapes. The order of elution is opposite to that obtained in the normal phase mode.

f. Gradient Elution of Benzodazepines

Equal amounts of four of the benzodiazepines were pooled and the sample was injected into the 1,9-decadiene column with methanol and water as the mobile phase. The gradient was from 40 to 100% methanol with water for 15 minutes at 0.5 mL/min. The chromatogram (Figure 37) showed reasonable separation of the four peaks in less than nine minutes. The excellent peak shape of these drugs allows the detection of small impurities which elute just after diazepam at 12.27 minutes. Normally, the impurity would be masked by the preceding diazepam peak. The use of a gradient resulted in sharper, more symmetrical peaks, better selectivity and faster analysis as the time was reduced from thirty-three minutes to less than nine minutes.

Figure 36 a. Reversed phase separation of metabolites and benzodiazepines on a 1,9-decadiene column. Mobile phase: methanol-water, v/v (40:60), at 0.5 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A = nitrazepam; B = clonazepam; C = 2-amino-5-nitrobenzophenone.

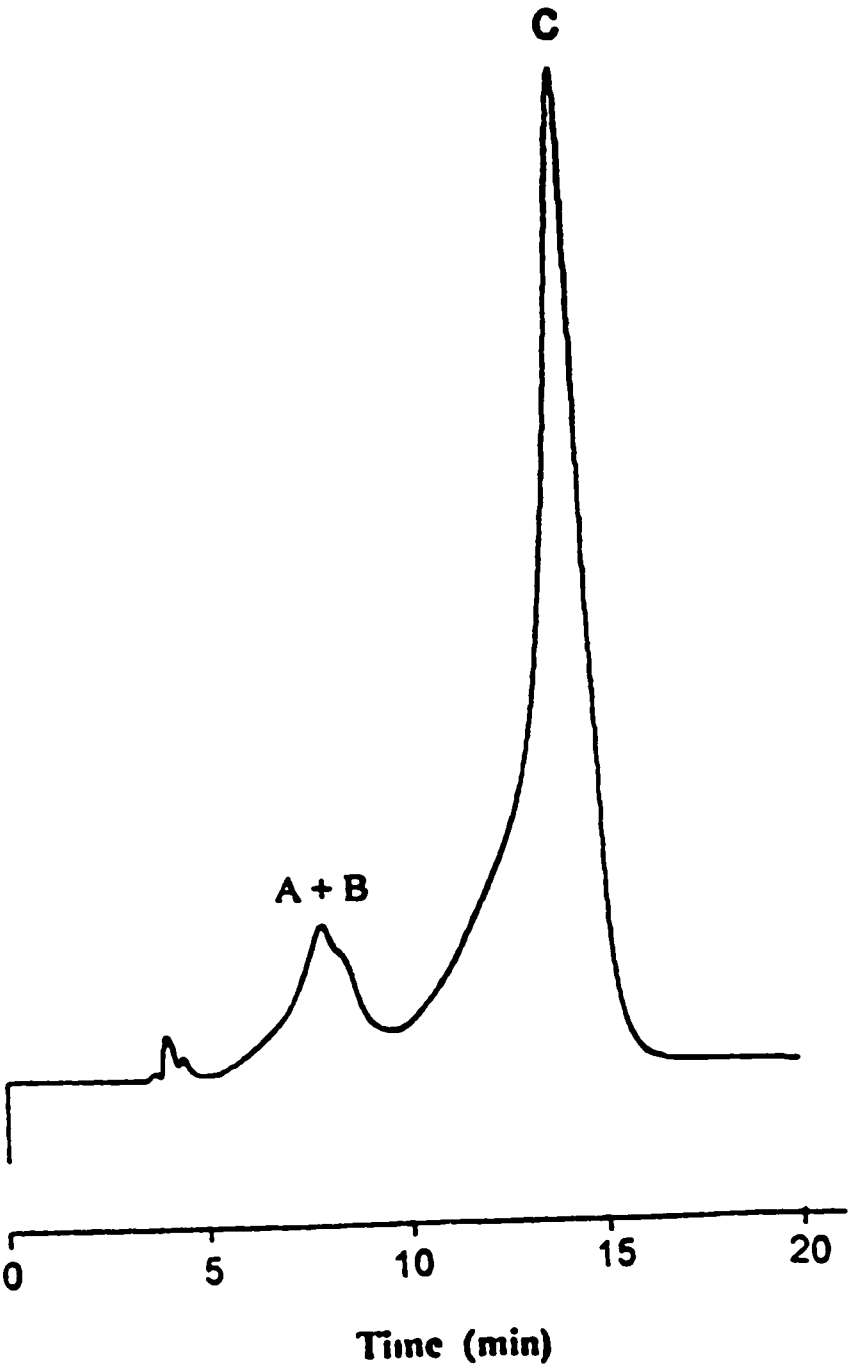


Figure 36 b. Reversed phase separation of metabolites and benzodiazepines on a 1,9-decadiene column. Mobile phase: acetonitrile-water, v/v (30:70), at 0.5 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A = nitrazepam; B = clonazepam; C = 2-amino-5-nitrobenzophenone.

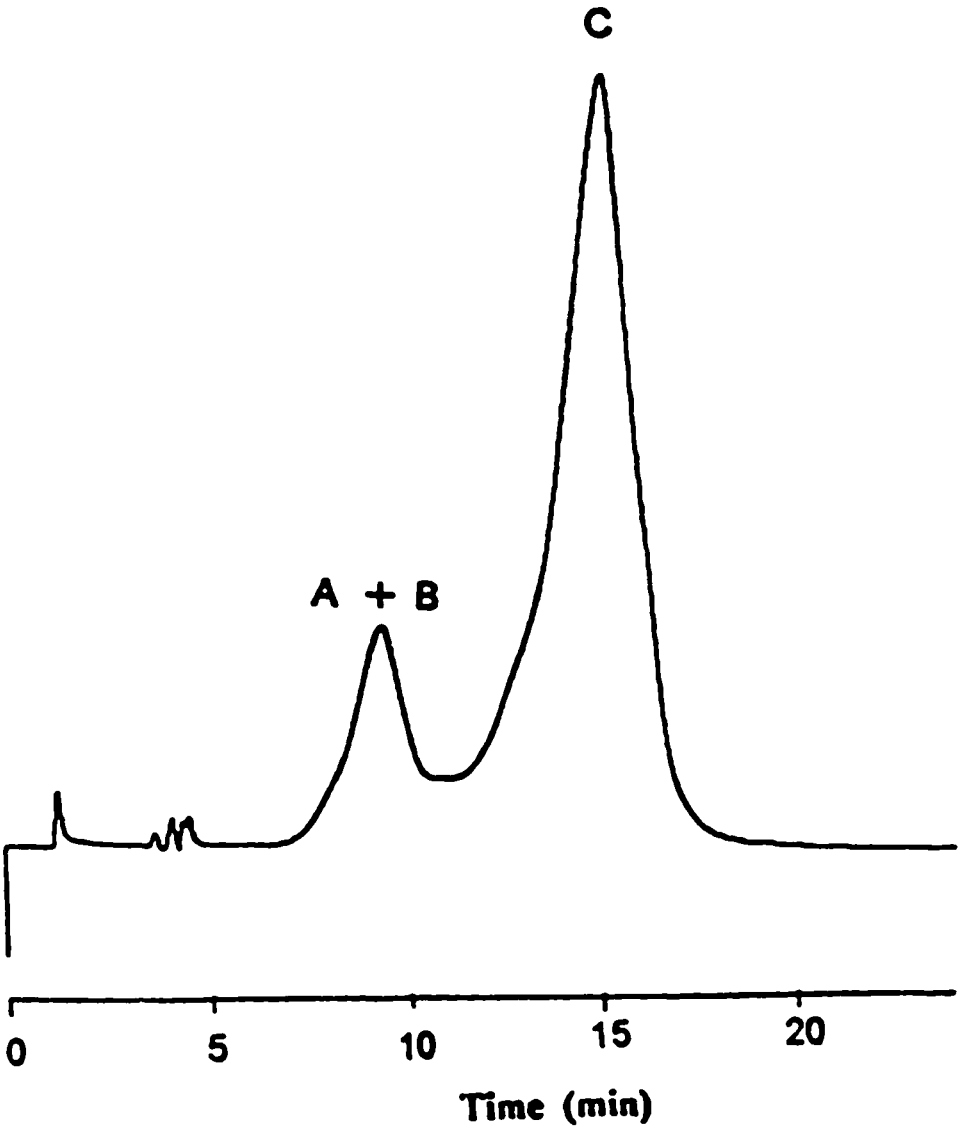


Figure 36 c. Reversed phase separation of metabolites and benzodiazepines on a 1,9-decadiene column. Mobile phase: tetrahydrofuran-water, v/v (40:60), at 0.3 mL/min; sample injection 20 µL; detection: UV at 254 nm: Peaks: A = nitrazepam; B = clonazepam; C = 2-amino-5-nitrobenzophenone.

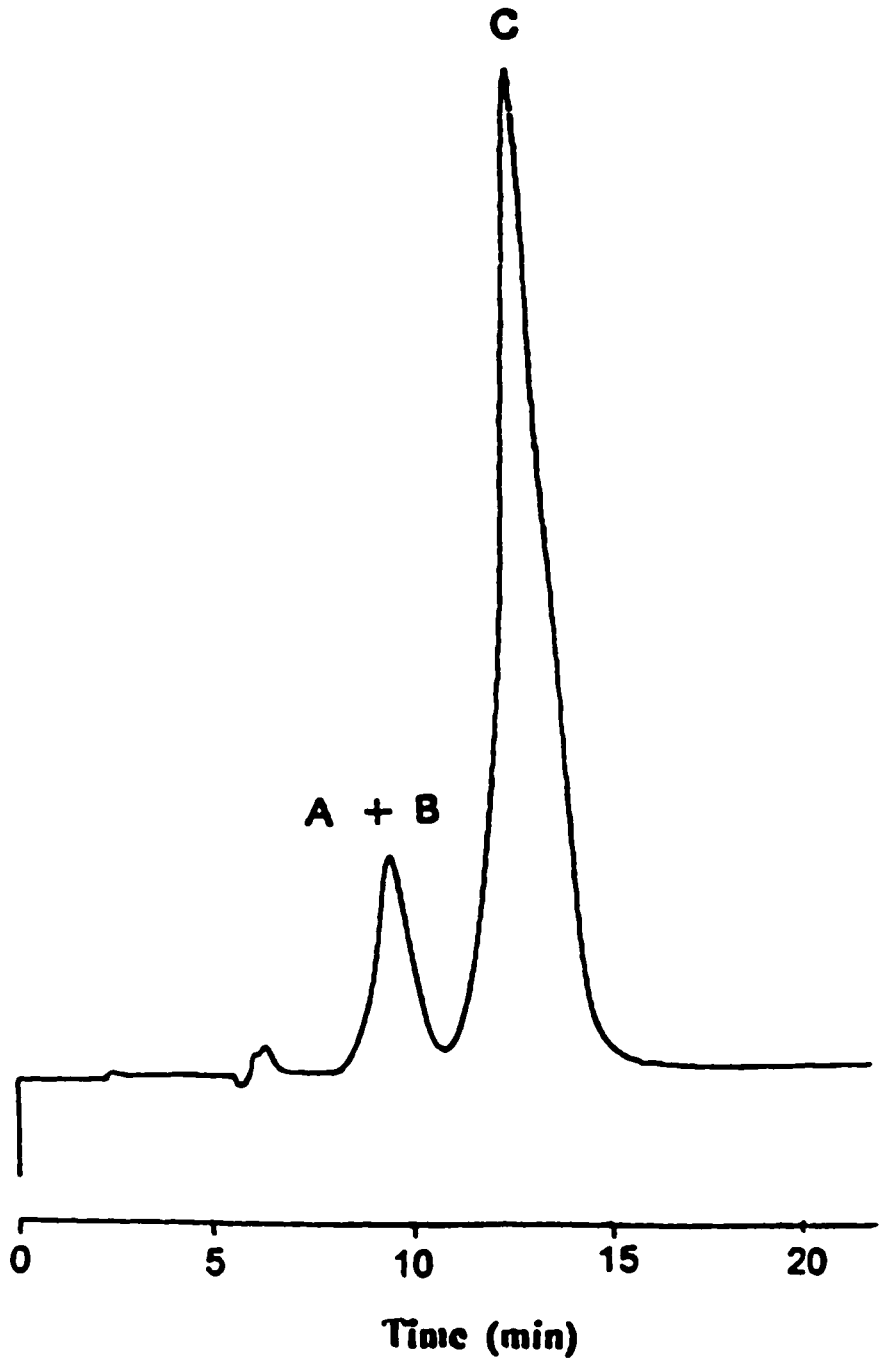
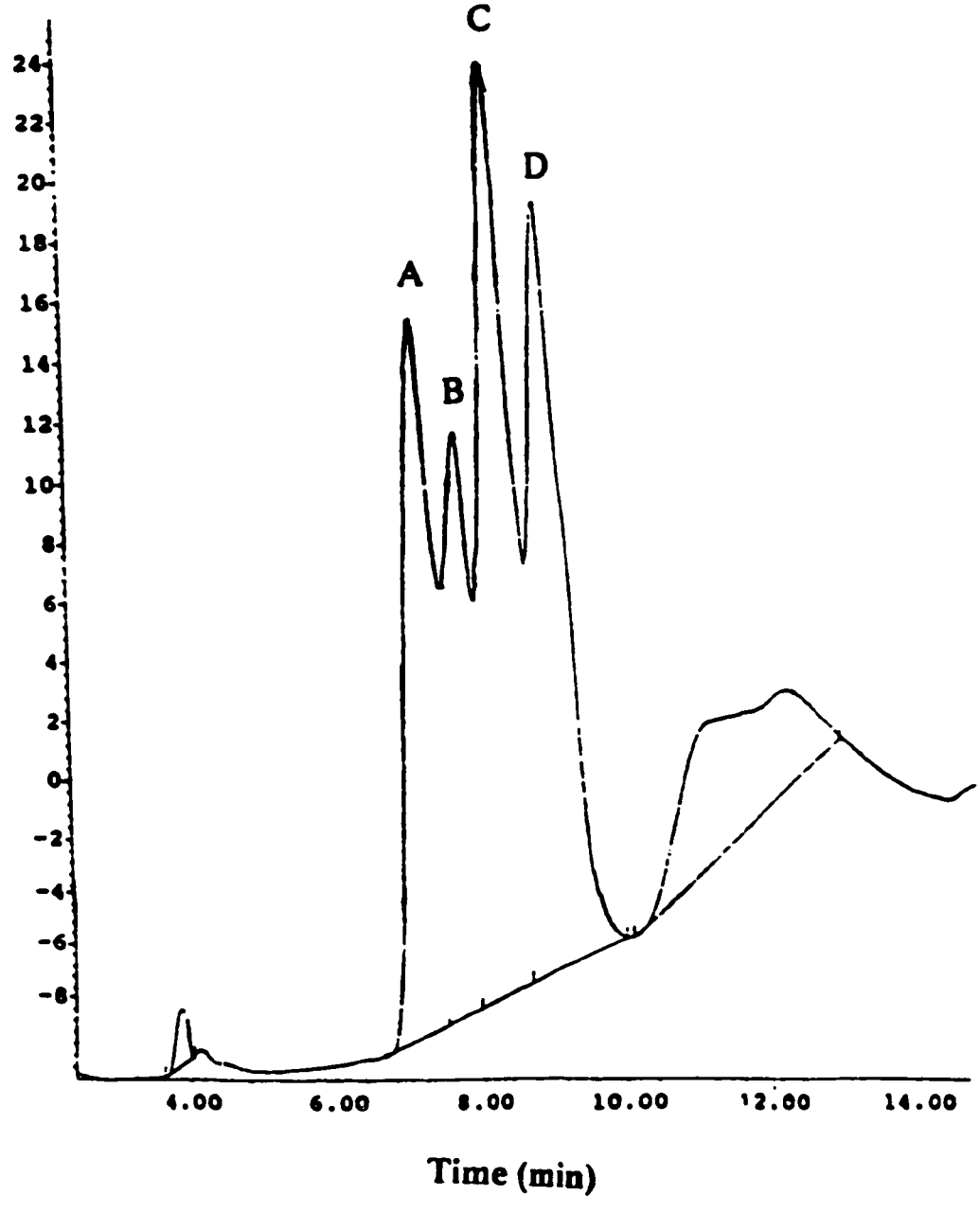


Figure 37. Gradient elution of benzodiazepines on a 1,9-decadiene column. Mobile phase: gradient from 40 to 100% methanol for 15 min with water at 0.5 mL/min; sample injection 5 µL; detection: UV at 254 nm: Peaks: A = oxazepam; B = temazepam; C = chlorodiazepoxide; D = diazepam.



g. Effect of pH on Nitrosamine Separation

The goal of this experiment was to investigate the viability of 1,9-decadiene columns in the separation of nitrosamines. Nitrosamines are the primary contaminants in cosmetics as well as its raw materials. It is known that N-nitroso compounds are mutagenic as well as carcinogenic [25]. The structures of the nitrosamines that were tested in this study are shown in Table 11.

Table 11 :Nitrosamines and their Structure

Nitrosamines (Group A)	**Structure (Group A)**
Monoethanolamine (MeA)	$HOCH_2CH_3NH_2$
Diethanolamine (DeA)	$(HOCH_2CH_3)_2NH$
Triethanolamine (TeA)	$(HOCH_2CH_3)_3N$
Nitrosamines (Group B)	**Structure (Group B)**
N-nitrosodimethylamine (NDMA)	$(CH_3)_2NNO$
N-nitrosodiethylamine (NDEA)	$(CH_3CH_2)_2NNO$
N-nitrosodiethanolamine (NDElA)	$(HOCH_2CH_3)NNO$

The samples were diluted 100 to 5000 times using methanol and considerable care was taken to avoid any kind of contact with these compounds. Samples were injected into the column with methanol-water (70:30, v/v) as the mobile phase and it was found that presence of water in the solvent resulted in peak tailing of most of the group A compounds. Poor efficiency due to peak tailing is due to the presence of free silanols that attract the positively charged amino moiety on nitrosamines [26].

A high resolution reverse phase separation could be optimized by careful adjustment of solvent pH. For instance, by using a buffer (0.02 M, KH_2PO_4) at pH 7.0, the nitrosamines converts from a plus one charge to uncharged, affecting the resolution

between similar amines [27]. The affect of pH on the resolution of a three component mixture of group B nitrosamines (N-nitrosodimethylamine, N-nitrosodiethanolamine, N-nitrosodiethylamine) is illustrated by the separation of a small peak trailing in Figure 38. The trailing peak is only a shoulder for unbuffered-gradient separation but is separated much better at pH 7.0 (Figure 39).

Figures 40 a and b are the isocratic and gradient elution of the three component mixture of Group A nitrosamines. Peak A is completely separated from peak B but peak C only appears as a shoulder for both the isocratic as well as gradient modes. This result shows that a buffered medium did not improve the separation of diethanol from triethanolamine. The method of separation of nitrosamines can be useful in the quantitation of nitrosamine contaminants present in certain cosmetics products as well as raw materials.

Figure 38. Gradient elution of nitrosamines on a 1,9-decadiene column . Mobile phase: gradient from 40 to 100% methanol for 20 min with water at 0.5 mL/min; sample injection 5 µL; detection: UV at 254 nm: Peaks: A = NDEA; B = NDMA; C = NDEIA.

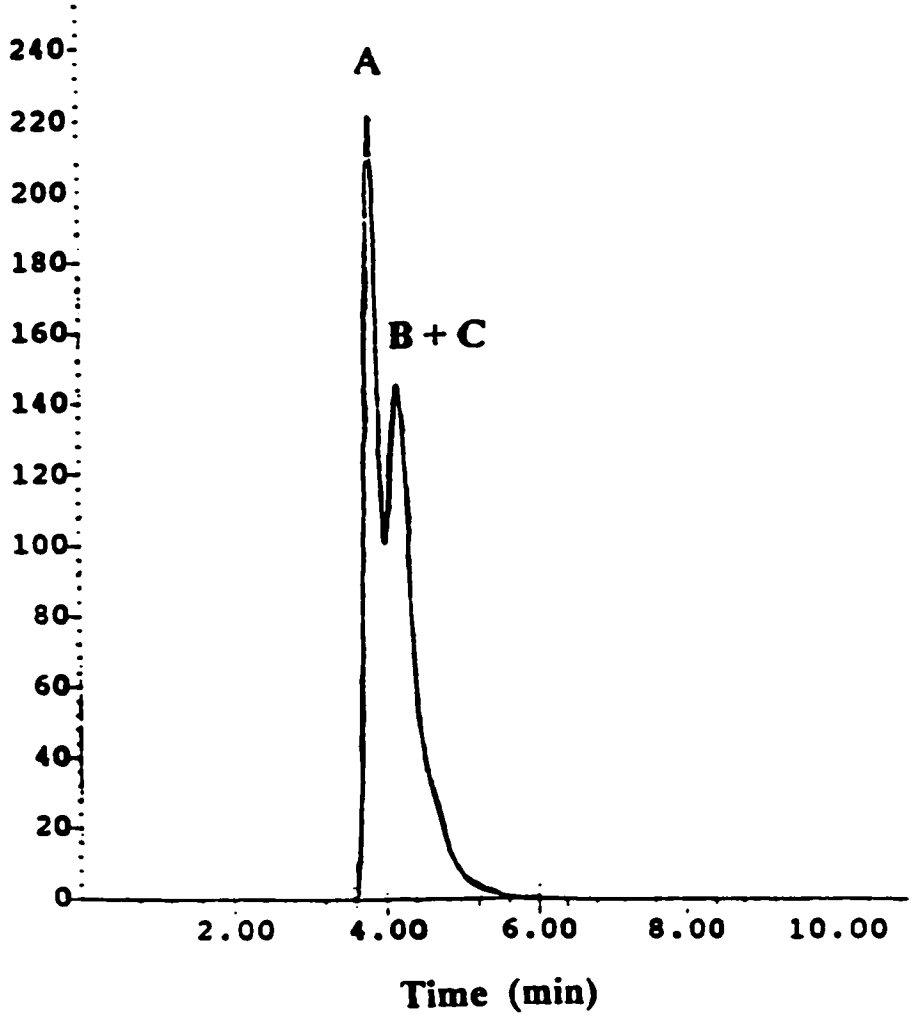


Figure 39. Gradient elution of nitrosamines on a 1,9-decadiene column using buffer. Mobile phase: gradient from 5 to 50% methanol for 15 min with KH_2PO_4 (pH 7.0) at 0.5 mL/min; sample injection 5 µL; detection: UV at 254 nm: Peaks: A = NDEA; B = NDMA; C = NDEIA.

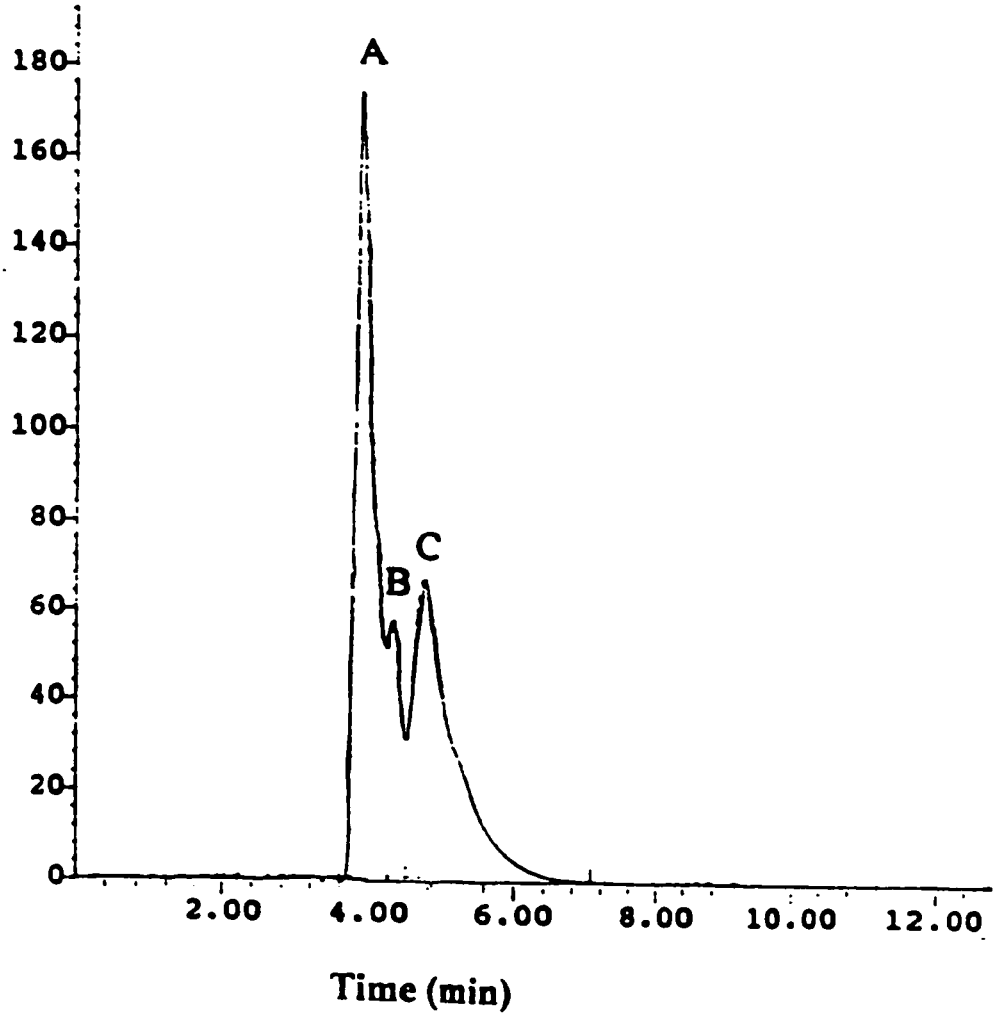


Figure 40 a. Reversed phase separation of nitrosamines on a 1,9-decadiene column using buffer. Mobile phase: methanol-KH_2PO_4 at pH 7.0 (20:80) for 15 min with at 0.5 mL/min; sample injection 5 µL; detection: UV at 254 nm: Peaks: A = MeA; B = DeA; C = TeA.

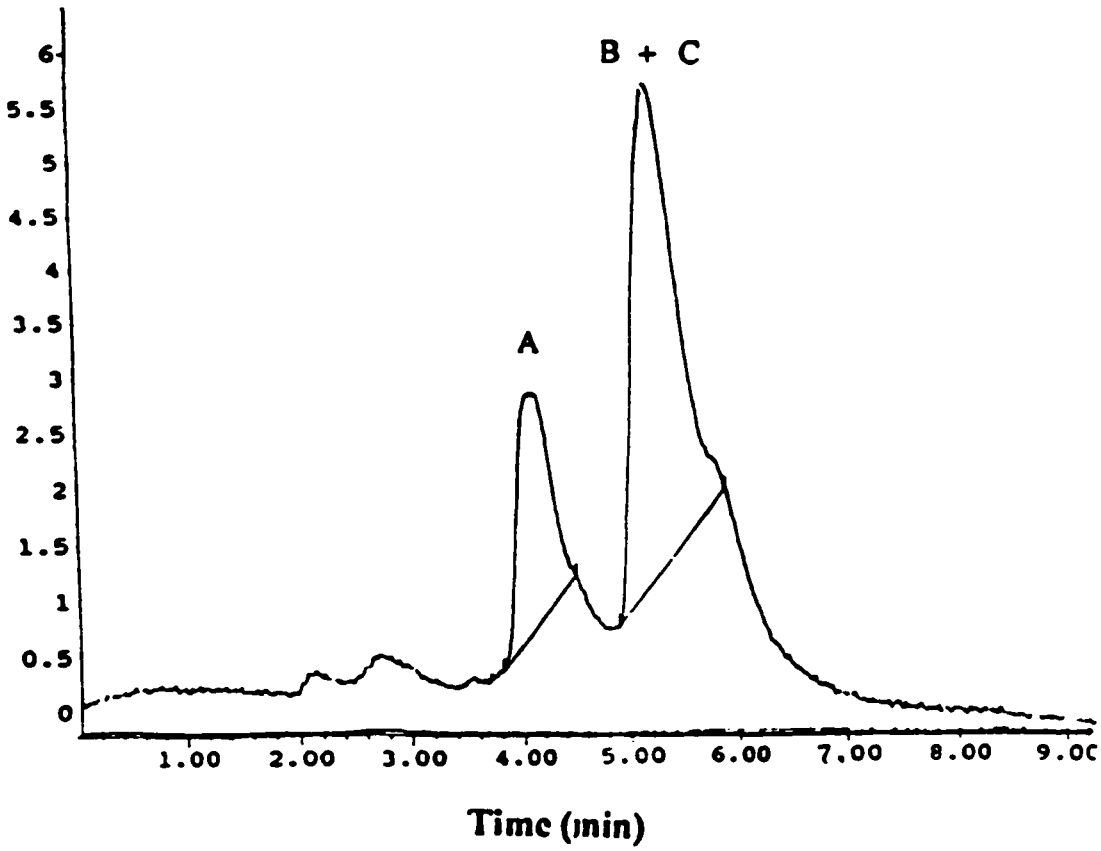
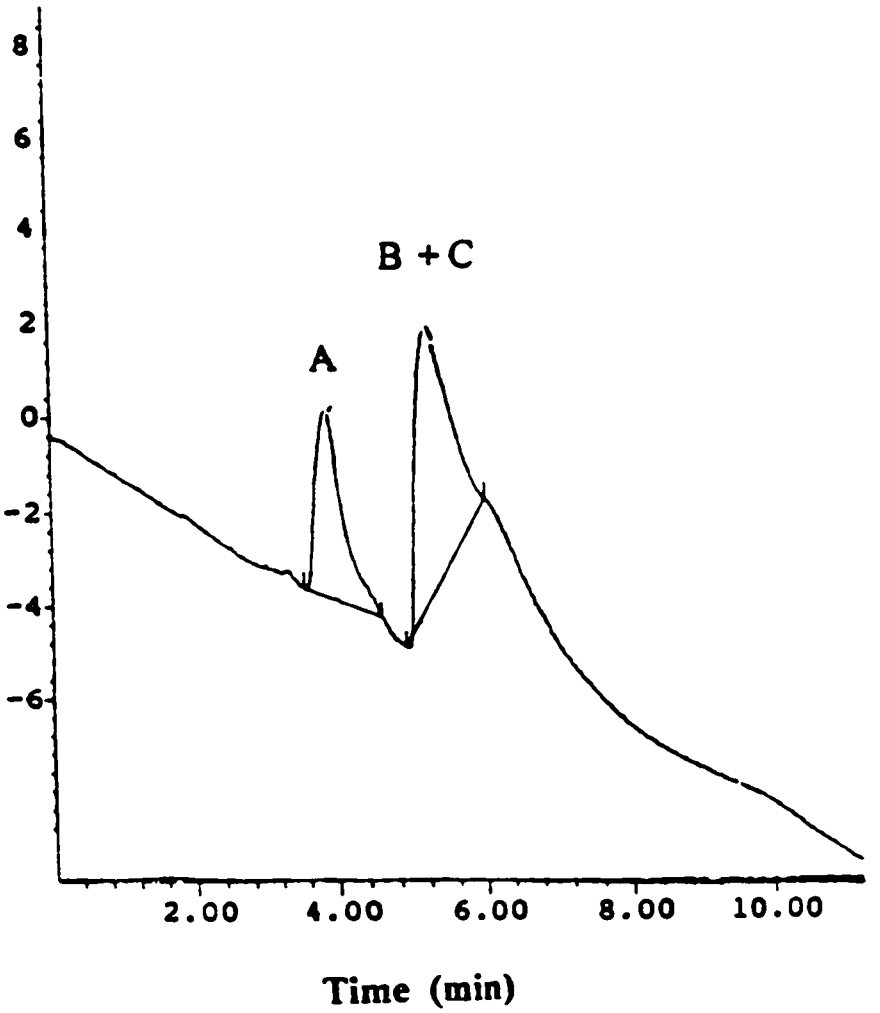


Figure 40 b. Gradient elution of nitrosamines on a 1,9-decadiene column using buffer. Mobile phase: gradient from 5 to 50 % methanol for 15 min with KH_2PO_4 (pH 7.0) at 0.5 mL/min; sample injection 5 µL; detection: UV at 254 nm: Peaks: A = MeA; B = DeA; C = TeA.



CHAPTER IV

CONCLUSION

State-of-the-art reverse phase materials have been synthesized by the TES silanization followed by the hydrosilation reaction method. The efficiencies of two C-10 compounds were studied. Diffuse Reflectance Infrared Fourier Transform spectroscopy, Cross-Polarization Magic Angle Spinning Nuclear Magnetic Resonance spectroscopy and HPLC were used to characterize the bonded phases synthesized in this study. The results showed that it is possible to bond acetylene and olefin compounds to silica hydride surfaces via hydrosilation. Surface coverage is quite high for both 1,9-decadiyne and 1,9-decadiene bonded silica surfaces. Each lot of the bonded material was tested for selectivity with compounds typical of the intended applications. Increased pH stability may be the result of a double Si-C coupling at the surface. The use of solvents at neutral or slightly basic pH added another dimension to the separation of nitrosamines. Amines that exhibit peak tailing or co-elute at one pH, will often be separated at a different pH, a powerful tool in the purification of basic compounds. Vydac 106 TP-C10 reverse phase materials display good peak shape and selectivity for the separation of hydrophobic compounds and benzodiazepine drugs for both isocratic and gradient elution. Reproducibility of the column was determined using benzodiazepines, for which the order of elution remained unchanged from run to run. Hence 1,9-decadiyne and 1,9-decadiene reverse phase materials are ideal for developing high-resolution procedures for pharmaceuticals. Their selectivity for pollutants like PAH's and nitrosamines can not

only be applied to regulation presently in force, but also to new regulations on the horizon.

Future work is focused on extending the applications of these materials to proteins and peptides and their tryptic digests. Also conditions need to be optimized for certain cosmetics that have been spiked with nitrosamines. This would also include sample clean up procedures using Solid Phase Extraction (SPE), identification of the impurities and quantitation using calibration analysis. Eventually, batch-to-batch reproducibility of the reverse phase columns needs to be investigated by replacing the old column with a new column.

CHAPTER V

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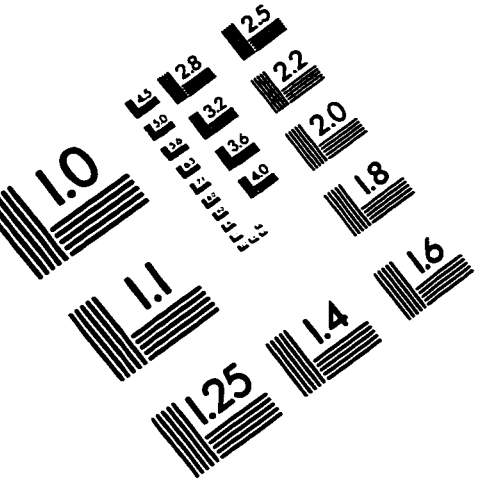
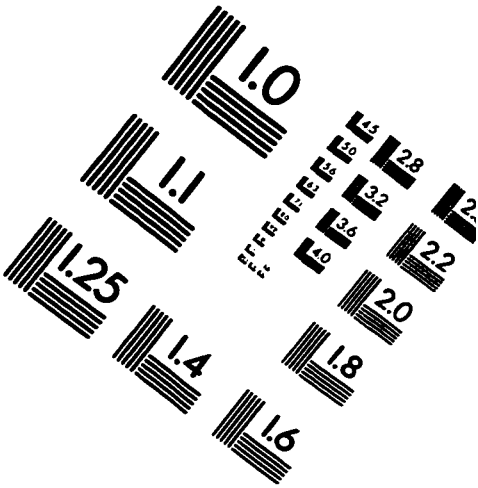
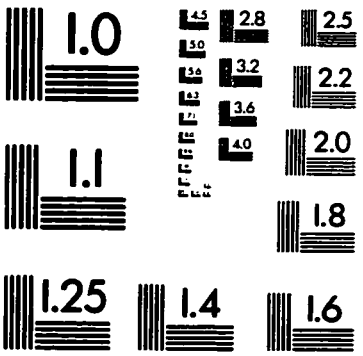
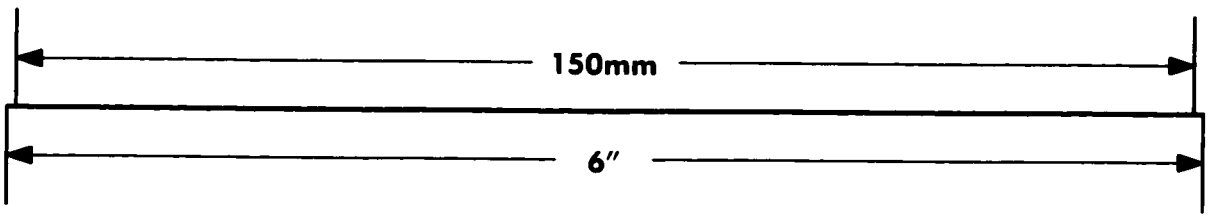
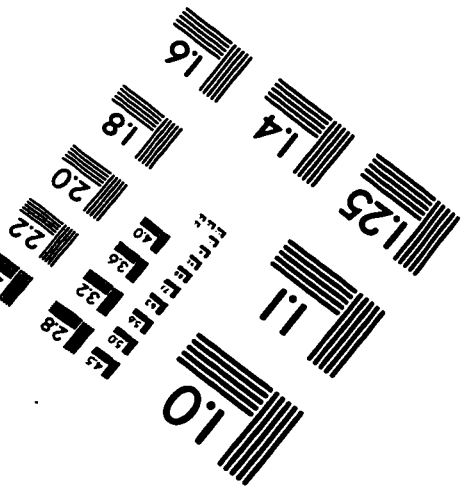
IMAGE EVALUATION TEST TARGET (QA-3)

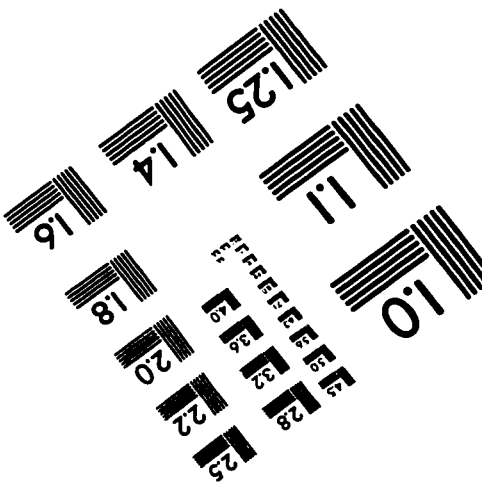












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