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Development of amino based columns for affinity chromatography

Geeta Shetty
San Jose State University

DEVELOPMENT OF AMINO BASED COLUMNS FOR AFFINITY CHROMATOGRAPHY

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Geeta Shetty

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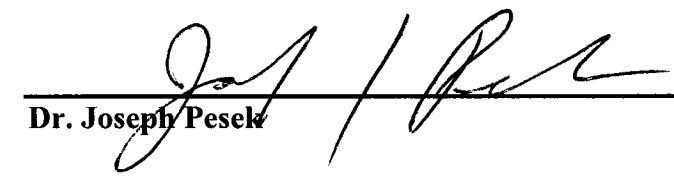
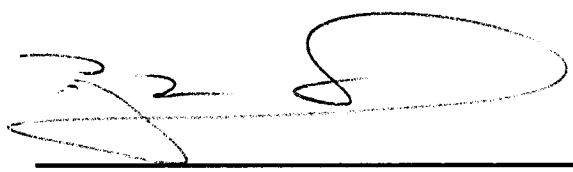
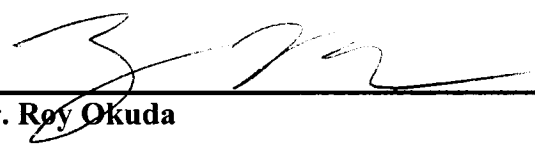
APPROVED FOR THE DEPARTMENT OF CHEMISTRY



Dr. Joseph Pesek



Dr. Roger Terrill



Dr. Roy Okuda

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ABSTRACT

DEVELOPMENT OF AMINO BASED COLUMNS FOR AFFINITY CHROMATOGRAPHY

by Geeta Shetty

Amorphous silica is commonly used as an HPLC support material due to its mechanical strength and high surface area. The chemistry of silica is dictated by the residual silanol groups present on the surface making it very hydrophilic. In this study the silica surface is modified using a silanization/hydrosilation technique. Two organic groups 3-amino-3-methyl-1-butyne and 4-diethylamino-2-butyn-1-ol, were selected as the bonded moiety. In a subsequent step trypsin was bonded to 3-amino-3-methyl-1-butyne for affinity chromatography. The specificity of the trypsin bonded column was tested by retaining trypsin inhibitor. All the bonded phases were characterized using elemental analysis and spectroscopic techniques. The Neue test was done for initial chromatographic evaluation of the bonded phases followed by analysis of sugars and other polar compounds.

ACKNOWLEDGEMENTS

I would like to thank Dr. Joseph Pesek and Dr. Maria Matyska as my research advisors for their direction, assistance and guidance. In particular, Dr. Maria's recommendations and suggestions have been priceless in my research and academic work. My sincere thanks to Dr. Joseph Pesek for keeping me motivated to finish my research work and complete my thesis. I am also grateful to Dr. Roy Okuda and Dr. Roger Terrill for acting as my committee members and for evaluating my research work. I would like to thank Mike Stephens from the Chemistry Department for his technical support.

Special thanks to my student colleagues who helped me in so many different ways. Also, thanks to my parents for loving me and supporting me through this journey. Finally, I would like to thank my beloved husband Girish Shetty who always supported me and encouraged me to go back to school and fulfill my dreams.

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I Introduction

A. Background

Chromatography, which is one of the most popular separation techniques, was first discovered by the Russian scientist Michael Tswett in the year 1906 [1]. Since then there have been some major advances in the field of column chromatography in order to improve the sensitivity and the reproducibility of the technique. The basic principle of chromatography lies in the distribution of the desired components in two different phases, the stationary phase and the mobile phase. Components that have a stronger affinity towards the stationary phase are retained by the column for a longer period of time and the components that have a stronger affinity for the mobile phase are eluted much faster [2].

Depending on the type of the mobile phase, chromatographic methods are further classified as liquid chromatography or gas chromatography. In liquid chromatography the mobile phase is a liquid and in gas chromatography the mobile phase is a gas. The stationary phase in both liquid and gas chromatography can either be a solid or a liquid on a solid adsorbent. One of the shortcomings of gas chromatography is the inability to use non-volatile and ionic samples, which does not pose any problem when these samples are used in liquid chromatography. In the early stages of liquid chromatography the flow of mobile phase was limited, leading to poor efficiency and resolution of the compounds. This problem was resolved by introducing high pressure liquid chromatography (HPLC) [2]. With the feasibility of running samples at pressures of up to 6000 psi, HPLC quickly gained momentum. As the nature of the stationary phases used in chromatography

strongly affected the efficiency of the technique, researchers concentrated on developing new stationary phases coupled with optimizing the mobile phase conditions [3]. The modes of separation in HPLC are broadly classified into normal-phase and reversed-phase depending on the relative polarities of the mobile phase and the stationary phase. In normal-phase, relatively polar materials like amino, cyano and diol bonded silica are used as the stationary phase, hence allowing polar compounds to be retained longer on these columns. In reversed-phase, polar compounds are eluted much faster due to the polar nature of the mobile phase. Alkyl-bonded silica stationary phases are the most popular among the reversed-phase columns [4]. In reversed-phase chromatographic separations the mobile phase conditions can be optimized by addition of organic modifiers in order to achieve good separations of nonpolar compounds. However, these aqueous mobile phases can sometimes damage the alkyl bonded stationary phase [5].

B. Aqueous Normal Phase Chromatography

In normal phase chromatography the mobile phase consists of low to medium polar solvents such as hexane, isopropanol, ethyl acetate, chloroform, heptane or a combination of these solvents. The retention of solutes increases as the amount of non polar solvent increases. In reverse phase chromatography the mobile phase consists of a mixture of water and a polar organic solvent like methanol, acetonitrile or THF. In normal phase an adsorptive mechanism is used to separate polar compounds based on the differences in their relative polarities. The aqueous normal phase (ANP) method uses a silica hydride based stationary phase with an organic group bonded to the silica hydride

surface [6]. The unique feature of ANP is that the mobile phase is both non-polar and aqueous; this is done by combining small amounts of water with an organic solvent [7]. In ANP chromatography polar compounds are retained strongly by increasing the amount of organic solvent in the mobile phase. One can also alter the elution order by modifying the amount of water used in the mobile phase. As the amount of water increases in the mobile phase, the polar compounds are eluted faster [8]. Hence an ANP stationary phase is able to work in both reverse phase and normal phase by varying the amount of water in the mobile phase. The ability of ANP chromatography to retain both polar and non polar compounds over a range of mobile phase compositions distinguishes it from the traditional hydrophilic interaction chromatography (HILIC) technique, which is mainly used to separate polar compounds [9].

C. High Performance Affinity Chromatography

As the name suggests, high performance affinity chromatography (HPAC) relies on the basic principle of HPLC and the interaction of biological molecules to its substrates. In this technique a specific binding agent is used for the separation of the desired target analyte. Due to the highly specific nature of the stationary phase it becomes easier to purify a specific analyte from a complex mixture without interference from other sample components [10]. An immobilized agent, known as the "affinity ligand" is covalently attached to the stationary phase. The affinity ligand can be one of the two interacting pairs and the target molecule is analyzed by injection onto the column using traditional HPLC methods. However as the separation process is highly specific it

becomes important to mimic the natural environment of the affinity ligand and the binding molecule. The general schematic diagram showing the affinity chromatographic process step by step is illustrated in Figure 1 [11]. The mobile phase used for the injection of the target analyte is called the "application buffer". The binding between the target analyte and the affinity ligand is reversible and usually occurs due to electrostatic forces or hydrogen bonding. Once the target analyte is bound to the stationary phase matrix it can then be eluted by using an "elution buffer" [12]. After the target analyte has been eluted, the original buffer can be used to regenerate the column for subsequent analysis.

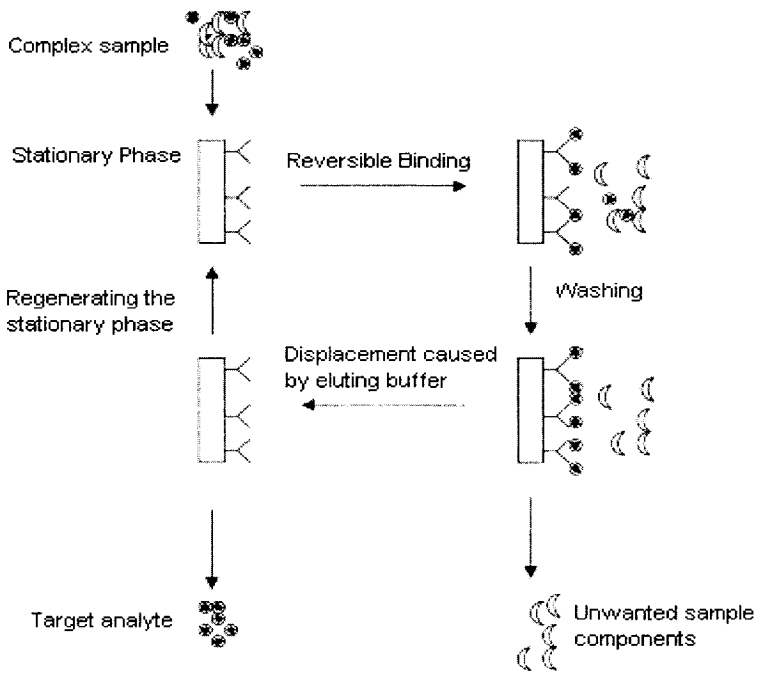


Figure 1: Steps involved in affinity chromatography [reproduced with permission from 11].

In the early stages of development of affinity chromatography, agarose and cellulose were the most commonly used base support material. However agarose is not able to provide good mass transfer properties and cellulose is prone to degradation. Also both agarose and cellulose has poor mechanical stability. Silica as a supporting matrix was able to overcome these problems but could not be used directly due to its strong non specific binding with biological molecules. This problem was resolved by modifying the silica surface with an organic group followed by attaching the desired affinity ligand [13].

D. Common Packing Material

An ideal packing material used for the stationary phase should be inert, mechanically strong and have high surface area. Silica is the most commonly used base support material due to its physical properties and the number of practical applications developed. Other inert materials used are alumina and zirconia. Some of the sought-after properties of silica include good mechanical strength, high efficiency and high internal surface area which favor strong interaction with the analyte molecule. Also, silica is available in a variety of pore sizes and shapes, making them desirable for a multitude of industrial and research applications [14]. The chemical formulation of amorphous silica is expressed as SiO_2 x H_20. It contains a three dimensional framework of silicon atoms joined together with oxygen atoms by siloxane bonds (Si-O-Si) and residual silanol groups (Si-OH). These silanol groups are highly polar and acidic in nature. The silanol groups on the silica surface can either be isolated (free silanols), vicinal or geminal as shown in Figure 2. The isolated silanol has a single bond with the silicon atom and the

silicon atom is connected by three bonds to the bulk matrix. In case of vicinal or bridged silanols two silanols are connected to two different silicon atoms by a hydrogen bond. The geminal silanol has two OH groups bonded to one silicon atom [15].

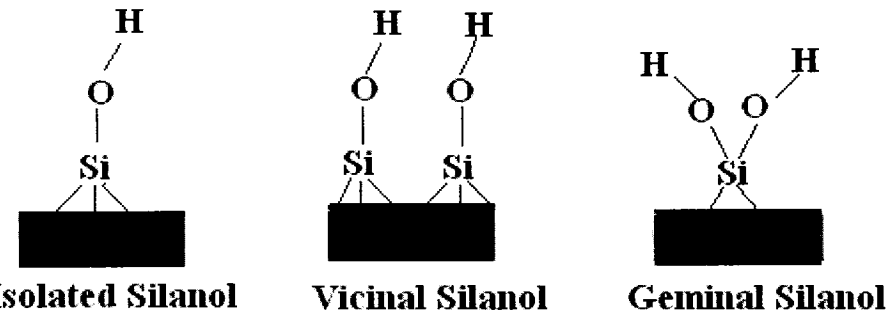


Figure 2: Types of silanol groups on the silica surface [15].

When analyzing polar compounds like proteins these silanol groups present on the silica surface can cause irreversible adsorption of the analyte through hydrogen bonding and dipole-dipole interactions. This causes a lack of precision in the chromatographic technique and peak tailing. Similar problems can also occur with basic compounds, as the silanol groups show an electrostatic interaction with basic solutes leading to poor resolution and poor efficiency. A packing material consisting of silica can be used under both aqueous and non aqueous chromatographic conditions. However due to the hydroscopic nature of the silica support it can strongly retain water. Over time the use of a highly polar solvent like water can decrease the specific surface area of the silica support. Also silica can only be used in the pH range of 2-8, as the pH of the mobile becomes highly acidic (below pH 2) the bonded group can undergo hydrolysis. At a very

basic pH (above pH 9) the silica may dissolve forming silicates. When a binary mobile phase such as water and some organic solvent are used for the analysis, water has the tendency to get adsorbed by organic solvents to a varying degree, depending on the conditions and the solvents used. The stationary phase then absorbs the water from the mobile phase as a result of the silanol groups. This in turn causes the retention time of the analytes to vary, hence making it difficult to precisely determine the sample elution time [16].

E. Modifying the Silica Surface

(i) Common Methods: In order to overcome all the problems associated with surface silanols in amorphous silica researchers have focused into developing techniques to modify the surface by attaching an organic group. These silanol groups serve as a medium to attach the desired organic group [17]. One method used to modify the surface is called an "esterification process". In this procedure an alcohol is reacted with the silanol to produce a silica-oxygen-carbon (Si-O-C) linkage between the silica surface and the organic group as shown in Reaction 1. This technique leads to poor hydrolytic stability and hence cannot be used in many chromatographic applications [18].

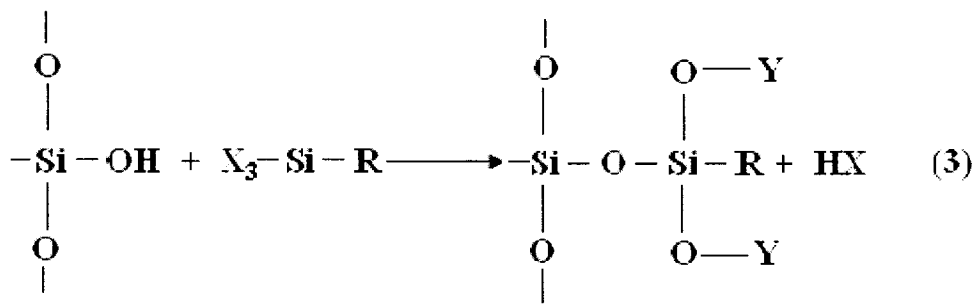
$$\text{Si} - \text{OH} + \text{R} - \text{OH} \rightarrow \text{Si} - \text{OR} + \text{H}_2\text{O} \qquad (1)$$

Another technique used to modify the silica surface is called organosilanization. In this method as shown in Reaction 2, a dimethylalkyl silane (X-SiR'$_2$R) is used as an

organosilanization reagent to form a monomeric bonded phase. The dimethylalkyl silane results in a single point of attachment between the silica surface and the bonded organic group, where X symbolizes a halide group, R' is methyl and R is an organic group that provides the desired property to the bonded phase [19]. This type of reaction creates a silicon-oxygen-silicon-carbon (Si-O-Si-C) linkage between the bonded phase and the silica surface, hence providing both thermodynamic and hydrolytic stability.

$$\text{Si} - \text{OH} + \text{X} - \text{SiR'}_2\text{R} \rightarrow \text{Si} - \text{O} - \text{SiR'}_2\text{R} + \text{HX} \qquad (2)$$

Organosilanization reactions can also be used to form polymeric bonded phases. In polymeric bonded phases trifunctional silanes are used to react with both the surface silanols and the adjacent silane groups as shown in Reaction 3, where Y = Si or H. However if all the adjacent groups do not crosslink as expected, some of the oxygens will end up having hydrogen attached to it instead of silicon atoms. This could pose a bigger problem as basic compounds, including most pharmaceuticals, when analyzed could lead to irreversible adsorption with the residual silanols on the silica surface [19].



(3)

Another approach to forming a monomeric bonded phase is also mentioned in the literature. This method involves a two-step reaction scheme and creates a single point of attachment between silicon and carbon. The first step involves using thionyl chloride in the presence of toluene and produces a silica-halide intermediate as shown in Reaction 4. The second step utilizes either a Grignard reagent or an organolithium reagent to attach the desired organic group as shown in Reactions 4a and 4b. The chances of residual silanol groups are minimal in this type of reaction. However due to the hydrolytic instability of the chlorinated intermediate and the possibility of surface contamination resulting from metal salt by-products, this technique is less favored [19].

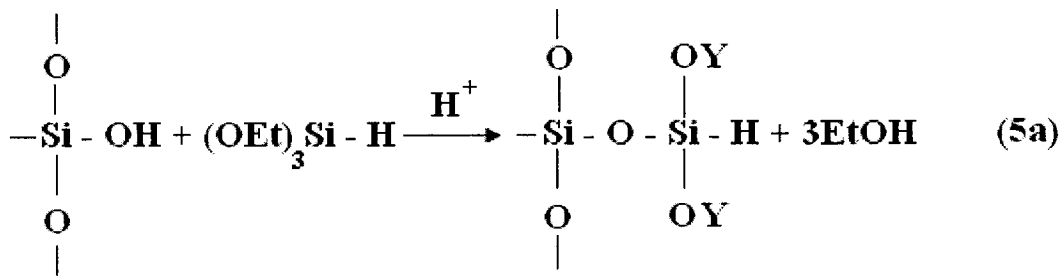
$$\mathbf{Si - OH + SOCl_2 \rightarrow Si - Cl + SO_2 + HCl} \qquad \mathbf{(4)}$$

$$\mathbf{Si - Cl + BrMgR \rightarrow Si - R + MgClBr} \qquad \mathbf{(4a)}$$

$$\mathbf{Si - Cl + Li - R \rightarrow Si - R + LiCl} \qquad \mathbf{(4b)}$$

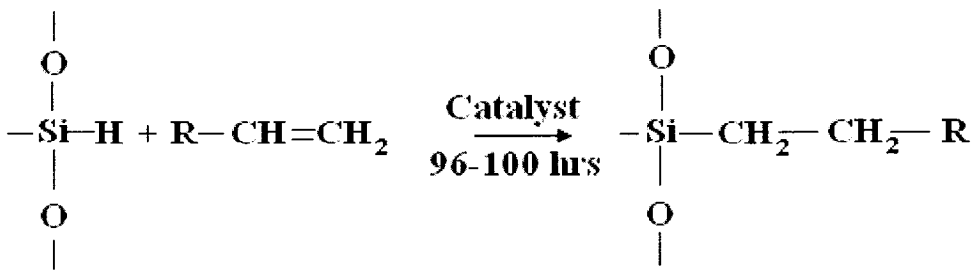
(ii) Silanization/Hydrosilation: A novel technique developed by Pesek, *et al.* is able to resolve the concerns associated with the silica surface modification techniques mentioned above. It is a two step process, where the first step involves the preparation of a silica hydride intermediate and the second step involves the attachment of the desired organic group. In the first step, the silanization reaction, the silanol group (Si-OH) is converted into silica hydride (Si-H) under optimized reaction conditions as shown in Reaction 5a, where Y = Si or H. The goal of this reaction is to replace most of the silanol groups with silica hydride in the presence of triethoxysilane, water, an acid catalyst and a suitable

solvent. Unlike the halide intermediate created in the organometallic reaction, the silica hydride species is hydrolytically stable [20].



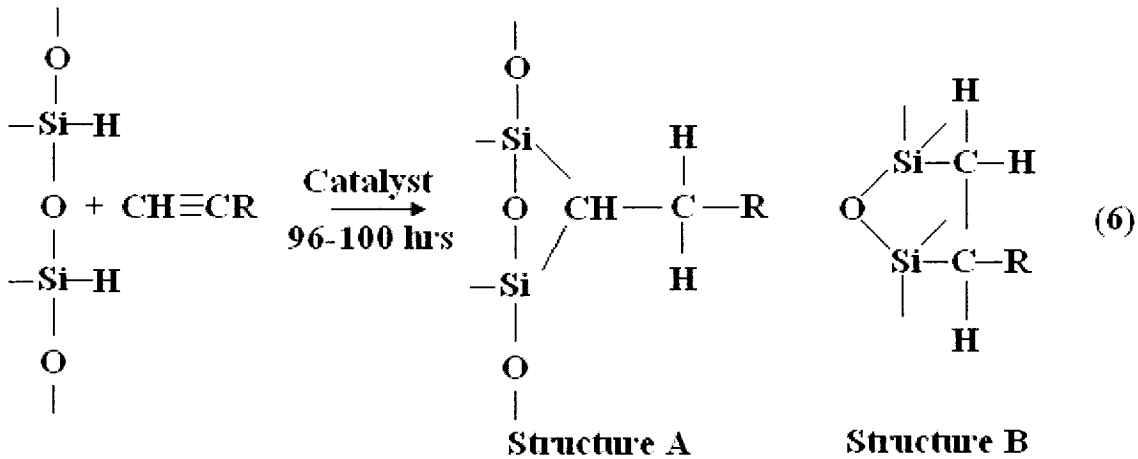
(5a)

In the second step of this reaction a terminal olefin is attached to the silica hydride surface through a hydrosilation reaction. Although terminal olefins are the most commonly used for attachment to the hydride surface, other compounds such as alkynes, nitriles and cyanos can also be bonded in the presence of transition metal complexes. Hexachloroplatinic acid in 2-propanol, also known as Speier's catalyst, is used in this study as the transition metal catalyst as illustrated in Reaction 5b. Other organic and inorganic complexes of rhodium, palladium, ruthenium and nickel can also be used as a catalyst for the hydrosilation reaction [21].



(5b)

During the addition of the organic group in the hydrosilation reaction the Speier's catalyst present in the reaction mixture can form intermediate species with the organic group. This could reduce the product yield, ultimately reducing the effectiveness of the procedure. Also, deposition of platinum metal on the hydride surface could occur during the reaction between Speier's catalyst and silica hydride [22]. Tert-butyl peroxide and Azobisisobutyronitrile (AIBN) are free radical catalysts that can be used as an alternative to Speier's catalyst [23]. In this study two amino compounds, 4-diethylamino-2-butyn-1-ol and 3-amino-3-methyl-1-butyne are bonded to a silica hydride surface during the hydrosilation procedure. The 4-diethylamino-2-butyn-1-ol bonded phase was synthesized using both platinum and free radical catalysts. The platinum catalyst has a tendency to coordinate with the free amino group of 3-amino-3-methyl-1-butyne; hence only a free radical catalyst was used for the bonded phase synthesis of this compound [24]. The NMR studies of this research indicated two possible hydrosilation structures for both the amino compounds as illustrated in Reaction 6.



(6)

F. Research Goals

This research involved a two-part study of chromatographic columns. In the first phase of this study we concentrated on developing HPLC columns and the second phase was wholly devoted to understanding the separation characteristics of the developed columns. Silica was chosen as the base material for the stationary phase of the columns under study. In order to create a stationary phase that caters to wide range of pharmaceutical applications, the silica surface was fabricated by the silanization method as described above. This step involved the conversion of surface silanols into silica hydride. To confirm the presence of the newly synthesized hydride surface, silica hydride was analyzed using FTIR. In the second step, a hydrosilation reaction was carried out to attach an organic compound to the hydride surface. Two amino compounds with alkyne groups were selected as the bonded moieties. In the hydrosilation reaction 4-diethylamino-2-butyn-1-ol and 3-amino-3-methyl-1-butyne were attached to the silica hydride surface, where the alkyne group served as the point of attachment between the hydride surface and the amino group. At this point we were focused into investigating two aspects; whether the organic group was attached to the silica hydride surface and if so, what was the percentage of surface coverage.

To determine the presence of the organic group on the hydride surface, spectroscopic studies such as NMR and FTIR are carried out. The surface coverage is determined by quantitative elemental analysis of carbon. In order to get the maximum surface coverage of the bonded phase, an initial synthesis is carried out in small batches to determine optimized conditions followed by big batch synthesis. Once both the amino

bonded phases were synthesized, a portion of the 3-amino-3-methyl-1-butyne bonded phase was used to create an affinity column. This column was synthesized by first activating the 3-amino-3-methyl-1-butyne with carbonyldiimadazole followed by attachment of trypsin using a suitable buffer. Once the packing material for all the columns was synthesized, they were chromatographically evaluated using different groups of compounds. The final four columns used for chromatography studies were as follows: 4-amino-4-methyl-1-butyne bonded phase using platinum catalyst, 4-amino-4-methyl-1-butyne bonded phase using free radical catalyst, 3-amino-3-methyl-1-butyne bonded phase using free radical catalyst and 3-amino-3-methyl-1-butyne bonded phase + trypsin (affinity column).

For the initial chromatographic characterization method the Neue test was done on all four columns [25]. Next, the amino based columns and the protein column were used to analyze a set of sugar samples. Further evaluation of all four columns was done by analyzing metformin and a few tricyclic antidepressants. All the samples were analyzed under aqueous normal phase conditions. The last part of the research involved determining the affinity aspect of the protein column (3-amino-3-methyl-1-butyne bonded phase + trypsin) using trypsin inhibitor.

II Experimental

A. Materials:

(i) Compounds used for synthesis: Astrosil™ silica, which had a 4.2 μm average particle size, was used in the synthesis of all the stationary phases. Astrosil™ silica with a specific surface area of 340 m^2/g was purchased from Stellar Phases (Langhorne, PA). All the other compounds used in the synthesis of bonded phases are shown in Table 1.

Table 1. Compounds used in the synthesis of stationary phases.

Compound Name	CAS Number	Manufacturing Company
Triethoxysilane	78-07-9	Sigma-Aldrich.
Dioxane	123-91-1	Fisher Chemicals
Toluene	108-88-3	Fisher Chemicals
3-amino-3-methyl-1-butyne	2978-58-7	GFS Chemicals
4-diethylamino-2-butyn-1-ol	10575-25-4	Sigma-Aldrich
Diethyl ether	60-29-7	Sigma-Aldrich
Hexachloro platinic acid	16941-12-1	Sigma-Aldrich
t-butyl peroxide	110-05-04	Sigma-Aldrich
1,1'-Carbonyldiimidazole	530-62-1	Sigma-Aldrich
Trypsin	9002-07-7	Sigma-Aldrich

(ii) Solvents and buffers used as mobile phases: The chromatographic evaluation of stationary phases was done using organic solvents, water and buffers as mobile phases. For the Neue test 35% 20mM K_2HPO_4 / KH_2PO_4 buffer at pH 7 and 65% methanol were used. The sugar analysis was done using different ratios of acetonitrile and water. Metformin and tricyclic compounds were analyzed using different ratios of 20mM K_2HPO_4 / KH_2PO_4 buffer at pH 7 and methanol. Finally, to determine the affinity aspect of the protein column 0.01 M phosphate buffer at pH 7 was used as the application buffer and acetic acid at pH 2.8 was used as the elution buffer. The CAS number and manufacturer of the solvents and buffers used are shown in Table 2.

Table 2: Compounds used in the mobile phases.

Compound Name	CAS Number	Manufacturing Company
K_2HPO_4	7758-11-4	Fisher Chemicals
KH_2PO_4	7778-77-0	J.T. Baker Chemical
Methanol	67-56-1	Fisher Chemicals
Acetonitrile	75-05-8	Fisher Chemicals
NaH_2PO_4	10049-21-5	Fisher Chemicals
Acetic acid	64-19-7	J.T. Baker Chemical

(iii) Samples analyzed in the study: Tables 3, 4, 5 and 6 show a list of all the compounds analyzed in this research.

Table 3: List of samples used for Neue test.

Compound Name	CAS Number	Manufacturing Company
Uracil	66-22-8	Matheson Coleman & Bell
Naphthalene	91-20-3	Sigma-Aldrich
Acenaphthene	83-32-9	Sigma-Aldrich
Amitriptyline	549-18-8	Sigma-Aldrich

Table 4: List of sugars used for chromatographic analysis.

Compound Name	CAS Number	Manufacturing Company
Glucose	50-99-7	Sigma-Aldrich
Sucrose	57-50-01	J.T. Baker Chemical
Fructose	57-48-7	Mallinckrodt
Lactose	63-42-3	Pfanstiehl Laboratories
Xylose	58-86-6	J.T. Baker Chemical Co.
Maltose	69-79-4	Fisher Scientific
Raffinose	512-69-6	Fisher Scientific
Melezitose	597-12-6	Sigma-Aldrich

Table 5: List of pharmaceutical drugs used in this study.

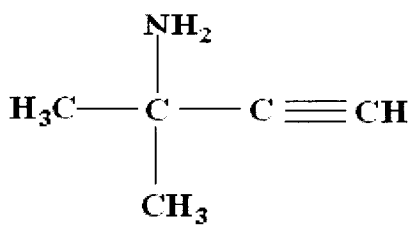
Compound Name	CAS Number	Manufacturer
Metformin	N/A	Donated to SJSU
Desipramine	58-28-6	Sigma-Aldrich
Amitriptyline	549-18-8	Sigma-Aldrich
Doxepin	1229-29-4	Sigma-Aldrich
Clomipramine	17321-77-6	Sigma-Aldrich
Imipramine	113-52-0	Sigma-Aldrich
Nortriptyline	894-71-3	Sigma-Aldrich

Table 6: Protein used in the study of affinity chromatography

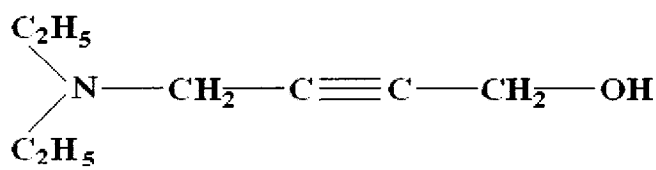
Compound Name	CAS Number	Manufacturer
Trypsin inhibitor	9035-81-8	Sigma-Aldrich

B. Structures:

(i) Amino compounds used in the bonded phase synthesis:

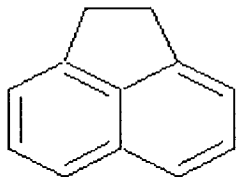


3-amino-3-methyl-1-buytne

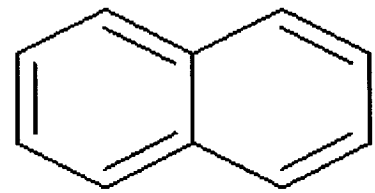


4-diethylamino-2-butyn-1-ol

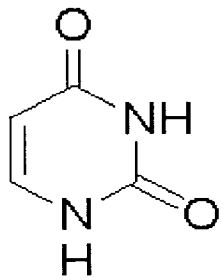
(ii) Structures of the compounds used in Neue test:



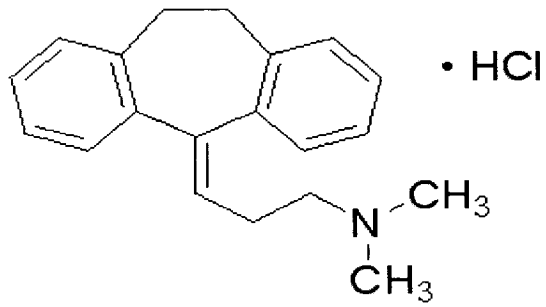
Acenaphthene



Naphthalene

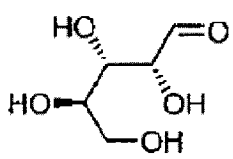


Uracil

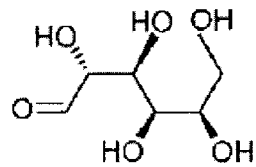


Amitriptyline

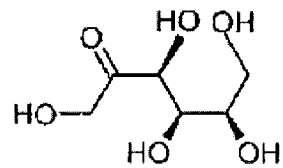
(iii) Structures of sugars used in chromatographic evaluation:



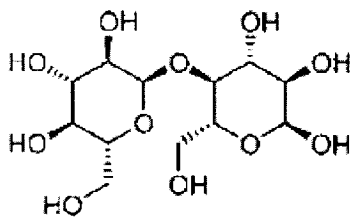
Xylose



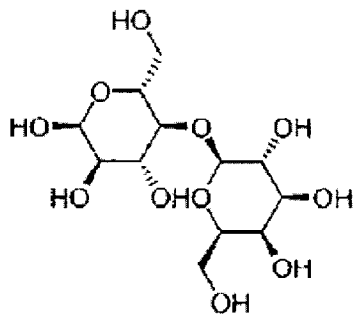
Glucose



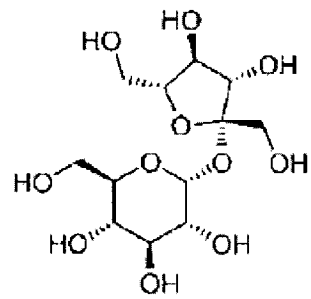
Fructose



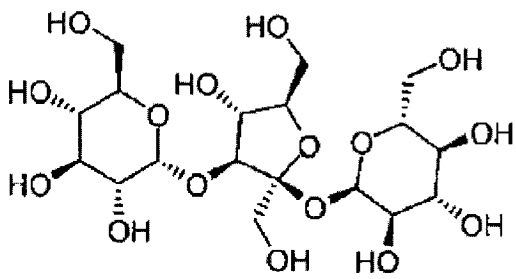
Maltose



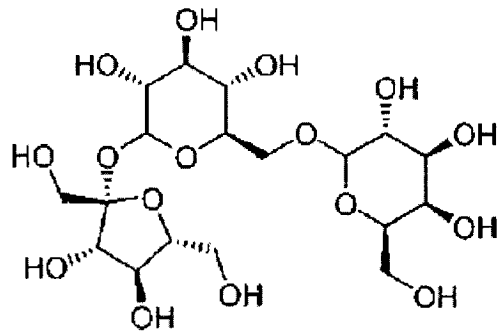
Lactose



Sucrose

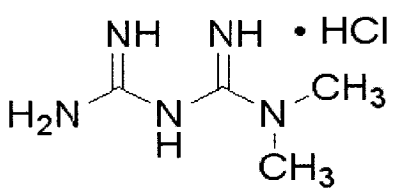


Melezitose



Raffinose

(iv) Structures of pharmaceutical drugs used in chromatographic evaluation:



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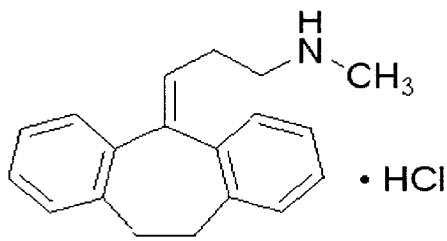

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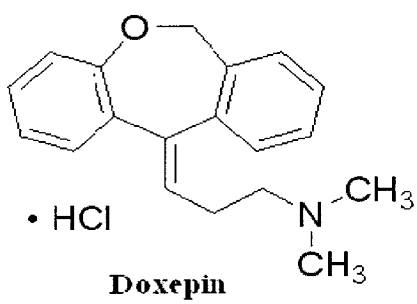
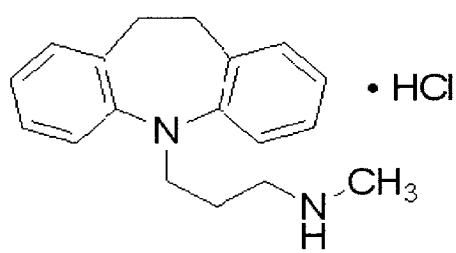
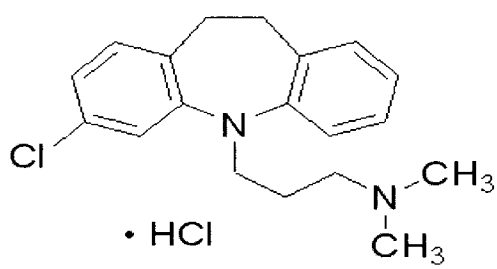


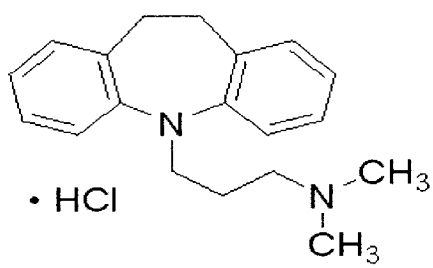
Doxepin



Desipramine



Clomipramine



Imipramine

C. Methods:

(i) Synthesis of silica hydride: In the first step, 15 g of Astrosil silica was weighed out and dried overnight in a vacuum oven at 100 °C. This dried silica was used as the base material for the silanization reaction. Prior to the synthesis, all the necessary glassware was washed and stored in the oven. In the experimental setup, 15 g of dried silica was placed in a 500 ml 3-necked round bottom flask equipped with a condenser and was set on a heating mantle. The heating mantle was connected to a transformer and was placed on a magnetic stirrer. In order to monitor the reaction temperature and stir the reaction mixture a thermometer and a magnetic stirrer are placed in the round bottom flask. After adding the silica, 600 ml of dioxane and 7.29 mL of 2.3 M HCl solution were added to the round bottom flask. This mixture of silica, dioxane and HCl catalyst was heated to 70 °C. Once the reaction temperature was stabilized at 70 °C, 90.15 mL of dioxane and 20.85 mL of triethoxysilane was added to the reaction mixture using an addition funnel. In order to avoid direct addition of TES solution to the reaction mixture, the TES/dioxane mixture was added drop-wise with constant stirring in the presence of argon gas. After the addition of all the TES/dioxane mixture, the temperature was raised to 90 °C. The reaction was complete when the entire reaction mixture was heated at 90 °C for 90 minutes. The silica hydride formed was then cooled and filtered using vacuum suction. The silica hydride was washed three times each with dioxane, toluene and diethyl ether. The final product was kept overnight at room temperature to evaporate the ether, followed by drying the silica hydride at 110 °C in a vacuum oven for 24 hours [20]. The newly synthesized silica hydride surface was characterized using FTIR.

(ii) Synthesis of 4-diethylamino-2-butyn-1-ol bonded phase using free radical catalyst: After the silanization reaction described above, part of the silica hydride was used in the synthesis of bonded phases, via a hydrosilation reaction. The experimental setup for the hydrosilation reaction was similar to the silanization reaction described in the earlier section. It should be noted that the bonded phase was the actual stationary phase packing material and not the silica hydride. Hence it was necessary to optimize the hydrosilation reaction to get the best surface coverage of the bonded phase on the silica hydride surface. In the optimization step only 0.5 g of silica hydride was used to bond 4-diethylamino-2-butyn-1-ol. The characterization of this bonded phase is discussed later and will be henceforth referred as small batch synthesis.

For the final stationary phase (big batch synthesis) 3 g of silica was used as the base material. In this reaction 4.5 ml of 4-diethylamino-2-butyn-1-ol, 60 ml of toluene and 240 μL t-butyl peroxide were placed in a round bottom flask. The reaction mixture was heated at a constant temperature of 70 °C +/- 2 °C for approximately one hour followed by slow addition of 3 g of silica hydride through the third neck of the flask with constant stirring. Then the flask was flushed with nitrogen keeping all the joints properly sealed. After stabilizing the reaction at 70 °C the temperature was raised to 100 °C +/- 2 °C and the mixture was heated for 100 hrs. The bonded phase formed was then cooled and filtered using vacuum suction. The bonded phase was washed two times with toluene, followed by diethyl ether. The final product was kept overnight at room temperature to evaporate the ether and then dried overnight at 110 °C in a vacuum oven [24].

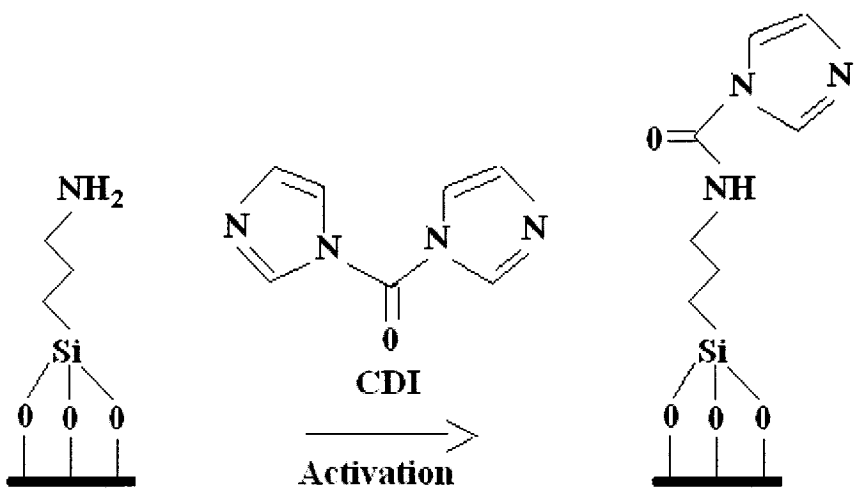
(iii) Synthesis of 4-diethylamino-2-butyn-1-ol bonded phase using Speier's catalyst: The experimental setup for the bonded phase synthesis of 4-diethylamino-2-butyn-1-ol is similar to the silanization reaction. In this hydrosilation reaction, the reaction conditions were optimized by initially creating a small amount of bonded phase followed by big batch synthesis. 0.5 g of silica hydride was used in the small batch synthesis and the characterization results of this bonded phase are discussed later.

For the big batch synthesis, 3 ml of 4-diethylamino-2-butyn-1-ol, 40.02 ml of toluene and 0.78 ml of 10 mM hexachloroplatinic acid in 2-propanol was heated in a 3-necked round bottom flask. The reaction mixture was heated at 70 °C +/- 2 °C for one hour while being stirred. Then the flask was flushed with nitrogen keeping all the joints properly sealed. Once the temperature of the reaction mixture was stabilized and a clear solution obtained, 3 g of silica hydride was added slowly to the third neck of the flask with constant stirring. The reaction temperature was then increased to 100 °C +/- 2 °C and reaction was continued at this higher temperature for 100 hrs keeping all the joints sealed. After completion of the reaction, the flask was cooled to room temperature. The newly formed bonded phase was then vacuum filtered and washed three times with toluene and diethyl ether. The final product was kept overnight at room temperature to evaporate the ether and then dried overnight at 110 °C in a vacuum oven [24]. The 4-diethylamino-2-butyn-1-ol bonded phase was later characterized using elemental analysis, FTIR and NMR. The characterization results of these bonded phases are discussed in later sections.

(iv) Synthesis of 3-amino-3-methyl-1-butyne bonded phase using free radical catalyst: The second amino compound chosen, as the bonding moiety for the silica hydride surface was 3-amino-3-methyl-1-butyne. In the small batch synthesis of the 4-diethylamino-2-butyn-1-ol bonded phase only one reaction condition explained in the previous section was tried out. However for the small batch synthesis of 3-amino-3-methyl-1-butyne bonded phase it was decided to explore three different reaction conditions. The reaction conditions were optimized by using three different amounts of free radical catalyst in three different set-ups. The experimental set-up in all three small batch syntheses was similar to the silanization reaction described earlier. The different amounts of t-butyl peroxide (free radical catalyst) used in the small batch syntheses were 15 μL, 28 μL and 45 μL. The characterization results of all the three small batch bonded phase syntheses are discussed later. After careful review of the characterization data it was decided to use 45 μL of t-butyl peroxide for the big batch synthesis of 3-amino-3-methyl-1-butyne.

The big batch bonded synthesis of 3-amino-3-methyl-1-butyne was carried out according to the standard protocol of the hydrosilation reaction described in the earlier sections. In this experimental setup 2.7 ml of 3-amino-3-methyl-1-butyne, 84.48 ml of toluene and 270 μL of t-butyl peroxide were heated at 70 °C +/- 2 °C for approximately an hour. After the addition of 3 g of silica hydride and flushing with nitrogen the reaction temperature was increased to 100 °C +/- 2 °C and continued for 100 hrs [24]. The final product was filtered, washed and vacuum dried according to the same procedure used in previous synthesis, followed by characterization of the bonded phase.

(v) Activation of 3-amino-3-methyl-1-butyne bonded phase for attachment of protein: After the hydrosilation reaction of 3-amino-3-methyl-1-butyne was completed, part of the bonded phase was used for the synthesis of the trypsin bonded protein column. The covalent immobilization of the protein to the bonded stationary phase is a two-step process. The first step involves the activation of the base material and the second step involves the attachment of the desired biological molecule. In this research the base material used for the activation was the 3-amino-3-methyl-1-butyne bonded phase and the biological molecule attached to the column was trypsin enzyme. The general reaction scheme for this process is shown in Reaction 7.

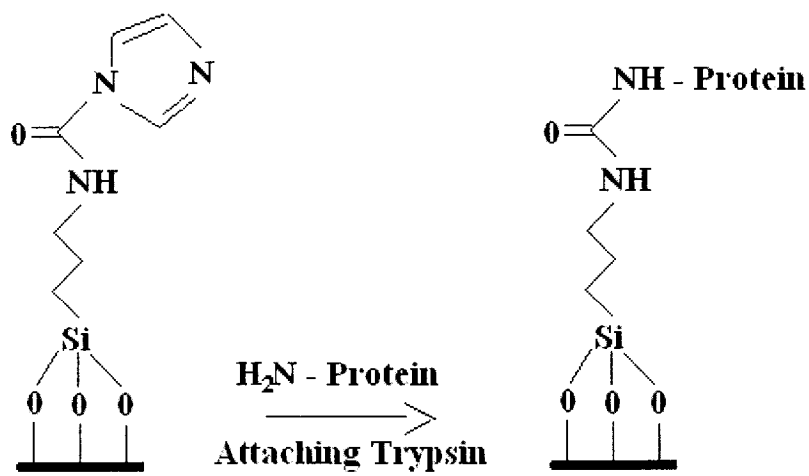


(7)

Prior to the activation process, all the necessary glassware was washed and dried in an oven. The base material, 3-amino-3-methyl-1-butyne bonded silica was dried overnight in the vacuum oven set at 110 °C. Following a written protocol [26], 1.5 g of bonded phase and 2.4 g of 1,1'-carbonyldiimidazole was suspended in 50 ml of dry dioxane. The reaction mixture was stirred for 30 minutes at room temperature. The

activated compound was transferred onto a filter crucible. The final product was washed three times with dioxane, acetone and diethyl ether [26]. The activated bonded phase was dried under reduced pressure in a desiccator. The 3-amino-3-methyl-1-butyne activated bonded material was then used for the synthesis of 3-amino-3-methyl-1-butyne + trypsin bonded phase.

(vi) Coupling of the protein to the activated 3-amino-3-methyl-1-butyne bonded phase: In order to attach the desired protein it is important to mimic the biological environment of the protein. Hence instead of using organic solvents for the coupling of protein to the activated bonded phase, 0.1 M phosphate buffer at pH 7 was selected. In this procedure, a suspension of 1.5 g of bonded silica in 75 ml of 0.1 M phosphate buffer and 0.15g of trypsin was prepared. The reaction mixture was constantly stirred at 4 °C for 48 hrs. The final product was transferred onto a filter crucible and washed with 0.1 M phosphate buffer [26]. A general reaction scheme for the process is shown in Reaction 8.



(8)

Trypsin attached to the 3-amino-3-methyl-1-butyne bonded phase was a single polypeptide chain of 223 amino acid residues. A stable linkage between the protein and the activated bonded phase is formed by a nucleophilic substitution reaction between the activated bonded phase and the primary amines on the protein. In this study, a side chain amino group such as arginine and lysine and/or an N-terminal amino group could be responsible for the covalent immobilization of the protein with the amino bonded stationary phase. [27].

(vii) Column packing: After the syntheses of all the stationary phases were completed, they were characterized spectroscopically and packed into stainless steel columns. The four columns were packed in-house with methanol as the driving solvent, using a Haskel pneumatic pump (Burbank, CA). The 4-diethylamio-2-butyn-1-ol bonded phase with platinum catalyst and the 4-diethylamino-2-butyn-1-ol bonded phase with free radical catalyst were packed into 150 mm x 4.6 mm i.d. stainless steel columns. The 3-amino-3-methyl-1-butyne bonded phase with free radical catalyst and the 3-amino-3-methyl-1-butyne + trypsin bonded phase were packed into 100 mm x 4.6 mm i.d. stainless steel columns. Depending on the length of the column 1.5 g to 2.5 g of bonded phase was suspended into 30 ml of isopropyl alcohol followed by 10 minutes of sonication. This slurry was added to a reservoir tube and the rest of the tube was filled with HPLC grade methanol. The slurry in the reservoir tube was forced into the stainless steel column under high pressure (~ 6000 psi) in the presence of nitrogen gas. All the stainless steel columns were purchased from Alltech (Deerfield, IL).

D. Instrumentation:

(i) Diffuse Reflectance Infrared Fourier Transform (DRIFT) spectroscopy: DRIFT spectroscopy was used to provide qualitative information about the silica hydride surface and the bonded phases. In this technique, the samples were ground finely and irradiated with a radiation ranging in wavelength from 4000 – 400 cm^{-1}. This region provides a unique absorption pattern for organic compounds and hence can be used to study the surface chemistry of the bonded phase. DRIFT spectroscopy provides structural information of a compound or surface and hence can be used to study both the native and the modified form of silica. Infrared absorption occurs when the vibrational modes of the analyte molecule is excited by the radiation [28].

In this study, DRIFT spectra were obtained on an ATI Mattson Infinity Series FTIR^{TM} spectrometer equipped with a deuterated triglycine sulfate (DTGS) detector. The sample was placed in a 3 mm diameter and 2 mm depth diffuse reflectance accessory. The sample for analysis was prepared by mixing 5 mg of KBr with 95 mg of sample and this mixture was dried overnight in a vacuum oven at 110 °C. The dried sample was then finely ground with a mortar pestle and transferred into the diffuse reflectance cup. In order to obtain a smooth surface, the sample was pressed with a slide and the sample scan was referenced to KBr. Before the analysis, the sample compartment was purged with nitrogen gas for 20 minutes to remove any atmospheric CO_2 and water. This CO_2 and water might interfere with the analysis and show up in the spectrum. The spectra were recorded in the infrared region of 4000 cm^{-1} to 400cm^{-1}. The spectra were collected at 4 cm^{-1} resolution and averaged over 150 scans.

(ii) Elemental Analysis: Elemental analysis was done by Desert Analytics (Tucson, AZ). The percentage of carbon of the synthesized bonded phases was determined by a micro-combustion method. The percentage of carbon is used to determine the surface coverage of the bonded phase. The surface coverage of the bonded phase provides quantitative information about the amount of organic group attached to the silica hydride surface. The relationship between the percentage of carbon and the surface coverage of the bonded phase is given by the Berendsen and De Galan equation [29]. Equation 9 shows the parameters used in the calculation of surface coverage.

$$\alpha(\mu mole/m^2) = 10^6 P_c / (10^2 M_c n_c - P_c M_R) S_{BET} \qquad (9)$$

In the above equation, P_C is the carbon percentage difference between the bonded phase and the silica hydride. M_C and M_R represent the atomic weight of the carbon atom and the molecular weight of the bonded organic group. BET is the nitrogen absorption method used to determine the surface area of silica and n_c is the number of carbon atoms present in the bonded phase. S_{BET} is the specific surface area of the silica matrix, which is provided by the manufacturer.

(iii) Nuclear Magnetic Resonance (NMR) spectroscopy: NMR spectroscopy is useful to determine the structure of a chemical species. In this technique the nuclei of the chemical species is placed in a strong magnetic field and irradiated periodically with an intense pulse of radio-frequency radiation. In this study, the pulse width was 6.5 μs and

the pulse interval is approximately 5 sec. As the nuclei begins to relax and return to its equilibrium position a time domain radio frequency called the free induction decay is emitted (FID). The FID is a characteristic of the chemical environment of the sample as it represents all the frequencies emitted by the sample. In solid-state NMR the resolution is very poor. This is a result of faster relaxation of nuclei and due to the anisotropy of the local magnetic field. In the case of ^{13}C nuclei the lines are comparatively much lower in intensity, due to low abundance of the nuclei. To resolve this problem and improve the sensitivity, magic-angle spinning (MAS) with cross polarization (CP) was used [30]. MAS involves rotating the sample rapidly in an air driven rotor at an angle of 60 ° with respect to the applied field. In this study, a Varian NMR Inova 400 spectrometer was used with a spinning rate of 5000 Hz. 150 mg of a solid sample (bonded phase) was packed into a double bearing ZrO_2 rotor and the spectra were obtained with a CP contact time of 5 ms. The spectra were analyzed with VNMR 6.1B software.

(iv) High Performance Liquid Chromatography (HPLC): The HPLC instrument used for the analysis of sugars consisted a SP8800 ternary pump, a chromjet integrator, a laser scattering detector and a Rheodyne manual injector. The laser light scattering drift tube temperature was set at 63 °C. The detection cell temperature was set at 60 °C and the pressure of the driving CO_2 was set at 1.25 kg/cm^2. The flow rate of the mobile phase was set at 0.35 ml/min and the pump pressure was set at a maximum of 2000 psi. The chart speed and the integrator attenuation were set at 0.25 cm/min and 64 respectively. The sugar samples were prepared by dissolving 15 mg of sample in 1 ml of 10:90

methanol/water. The Neue test and the analysis of the pharmaceutical drugs were done using Millennium 2.1 software, a Waters 515 HPLC pump, a Waters 717 plus autosampler and a Waters 991 photodiode array detector. The pharmaceutical drug samples were prepared by dissolving 1 mg of sample in 1 ml of methanol. The mobile phase flow rate was set at 0.5 ml/min. The organic solvents and water used in the mobile phase was degassed using helium gas at a flow rate of 5 ml/min. Deionized water used in the mobile phase was obtained from a Milli-Q^{TM} purification system (Millipore Corp., Bedford, MA). All the samples were filtered with a 20 μm nylon membrane before analysis. All four newly packed columns were flushed with methanol for 10 hours before testing them chromatographically. One of the pharmaceutical drug doxepin, had two peaks instead of a one in the chromatogram. In order to confirm the degradation of the sample, LC/MS analysis was done of this compound on the 3-amino-3-methyl-1-butyne bonded phase column.

The LC/MS analysis of Doxepin was done using a Micromass platform LC mass spectrometer system set consisting of a Micromass LC model M940150DCI APCI probe, an Edwards model E2M30 rotary vacuum pump and a MassLynx 3.4 software. The instrument was purged with liquid nitrogen gas at 100 psi. The parameters used for the analysis were as follows: APCI pin 3.20, cone 30, skimmer 2.0, probe temperature 60 °C, source heater 140 °C, LM and HM resolution 15, gas flow 250 ml/min, ion energy 1.0, multiplier 650 and APCI in the positive mode. 1 ml of 0.5% formic acid was added to 1000 ml of 50:50 water/methanol mobile phase.

III Results and Discussion

A. Elemental Analysis: Elemental analysis was one of the techniques used to confirm the presence and bonding of the amino compounds to the silica hydride surface. The surface coverage of the bonded group α is calculated using Equation 9, described previously. The molecular weight of 3-amino-3-methyl-1-butyne is 83.13 g/mol and the molecular weight of 4-diethylamino-2-butyn-1-ol is 141.21 g/mol. Table 7 shows the results of the small batch syntheses of the bonded phases. For the small batch synthesis of the 3-amino-3-methyl-1-butyne bonded phase, the reaction conditions were optimized by using different amounts of free radical catalyst. For the 4-diethylamino-2-butyn-1-ol bonded phase synthesis both free radical and platinum catalysts were used.

The surface coverage values for the small batch indicated that both the amino compounds were bonded to the silica hydride surface successfully. However the 4-diethylamino-2-butyn-1-ol bonded phase had better surface coverage with both Pt catalyst and free radical catalyst compared to the 3-amino-3-methyl-1-butyne bonded phase with free radical catalyst. For the 3-amino-3-methyl-1-butyne bonded phase synthesis 45 μL of free radical catalyst provided the best surface in comparison to the 15 μL and 28 μL free radical catalyst. Hence, the 45 μL reaction condition was used for the big batch synthesis. The results of the big batch synthesis are shown in Table 8. In the small batch synthesis the 4-diethylamino-2-butyn-1-ol bonded phase with the platinum catalyst showed higher surface coverage. However, in the big batch synthesis 4-diethylamino-2-butyn-1-ol bonded phase with the free radical catalyst showed higher surface coverage.

Table 7: Surface coverage for small batch syntheses

Compound Name	Type of Catalyst	% of Carbon	Surface Coverage $\alpha = \mu mol/m^2$
3-amino-3-methyl-1-butyne	Free radical (15 µL)	1.65	0.83
3-amino-3-methyl-1-butyne	Free radical (28 µL)	1.76	0.88
3-amino-3-methyl-1-butyne	Free radical (45 µL)	2.76	1.41
4-diethylamino-2-butyn-1-ol	Free radical	8.60	3.02
4-diethylamino-2-butyn-1-ol	Platinum catalyst	9.64	3.44

Table 8: Surface coverage for big batch syntheses

Compound Name	Type of Catalyst	% of Carbon	Surface Coverage $\alpha = \mu mol/m^2$
3-amino-3-methyl-1-butyne	Free radical	4.66	2.44
4-diethylamino-2-butyn-1-ol	Free radical	7.71	2.66
4-diethylamino-2-butyn-1-ol	Platinum catalyst	6.23	2.10

B. DRIFT Spectroscopy:

(i) Silica hydride surface characterization: After the silanization reaction the newly synthesized silica hydride surface was characterized using DRIFT spectroscopy. Figure 3 shows the DRIFT spectrum of the silica hydride intermediate. The sharp peak at 2250 cm^{-1} is due to the stretching vibrations of the newly formed Si-H bond, hence confirming the success of the silanization reaction. The broad peak between 3800 cm^{-1} and 3000 cm^{-1} is due to the H-bonded silanol group and adsorbed water. The band near 2890 cm^{-1} is due to the residual ethoxy group from the silanization reaction. The band near 3750 cm^{-1} is due to OH stretching vibrations from the isolated silanol groups. The rest of the peaks in the spectrum can be attributed to various fundamental vibrations of the silica matrix.

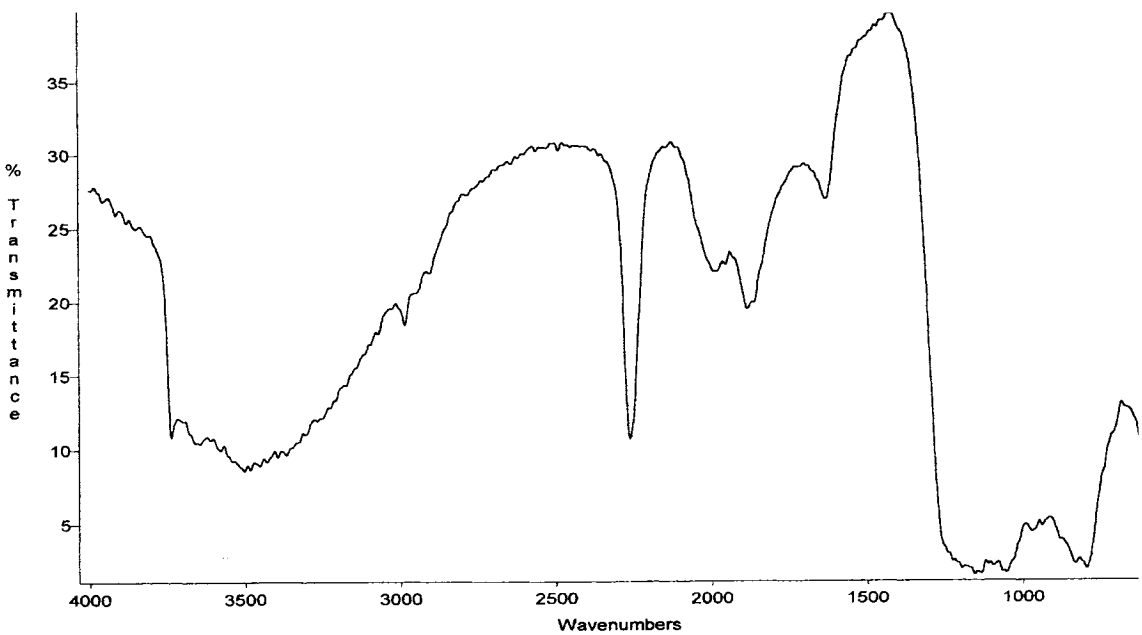


Figure 3: DRIFT spectrum of the silica hydride surface.

(ii) Surface characterization of 3-amino-3-methyl-1-butyne bonded phase: The DRIFT spectrum of the 3-amino-3-methyl-1-butyne bonded phase using a free radical catalyst is shown in Figure 4. After the silanization reaction the DRIFT spectrum shows a strong peak in the 2200 cm^{-1} region. The intensity of this Si-H band is considerably reduced after the silica hydride intermediate, indicating that the organic group has been attached to the surface. The presence of strong C-H stretching bands in the region of 2800 cm^{-1} to 3000 cm^{-1} confirms this assumption. The peak at 3740 cm^{-1} is due to the unreacted silanols present on the silica hydride surface.

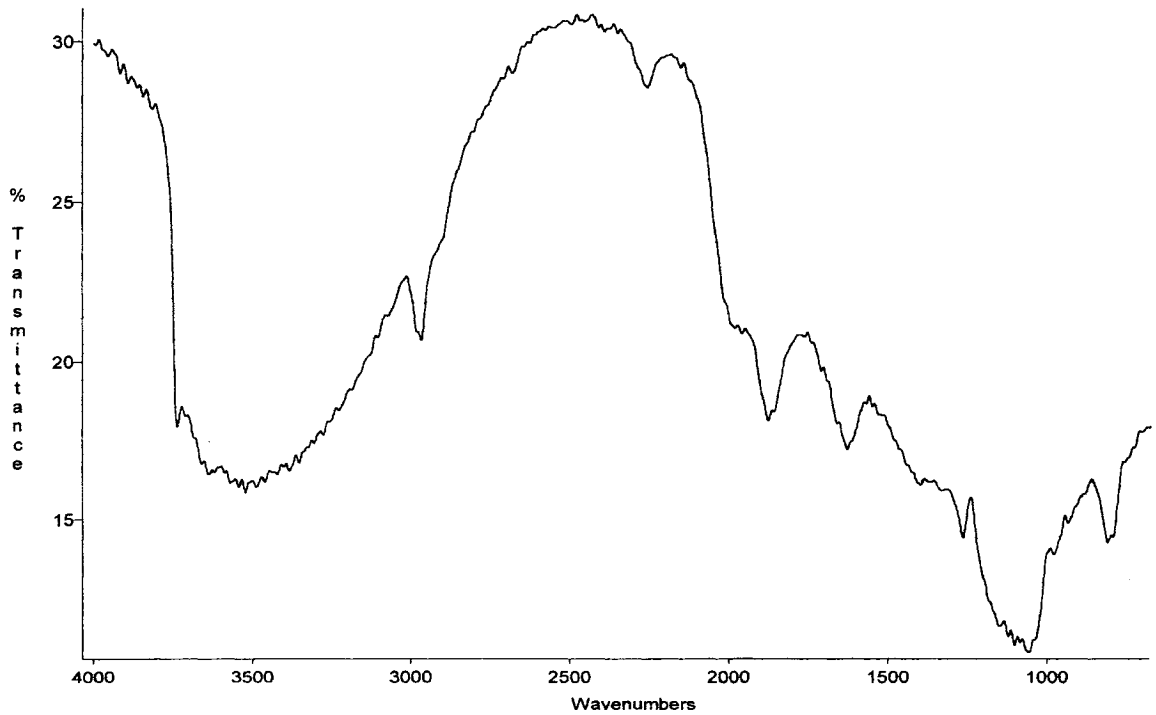


Figure 4: DRIFT spectrum of 3-amino-3-methyl-1-butyne bonded phase.

(iii) Surface characterization of 3-amino-3-methyl-1-butyne + trypsin bonded phase:

The DRIFT spectrum of the 3-amino-3-methyl-1-butyne + trypsin bonded phase is shown in Figure 5. A considerable change can be noticed in the DRIFT spectrum of the trypsin bonded phase compared to the 3-amino-3-methyl-bonded phase. This is a good indication that the surface morphology has changed. Also, the sharp peak at 1700 cm^{-1} represents the C=O frequency of the amide and the groups present on the amino acids of the protein. The other smaller peaks are due to fundamental vibrations of the various organic groups present on the protein bonded surface.

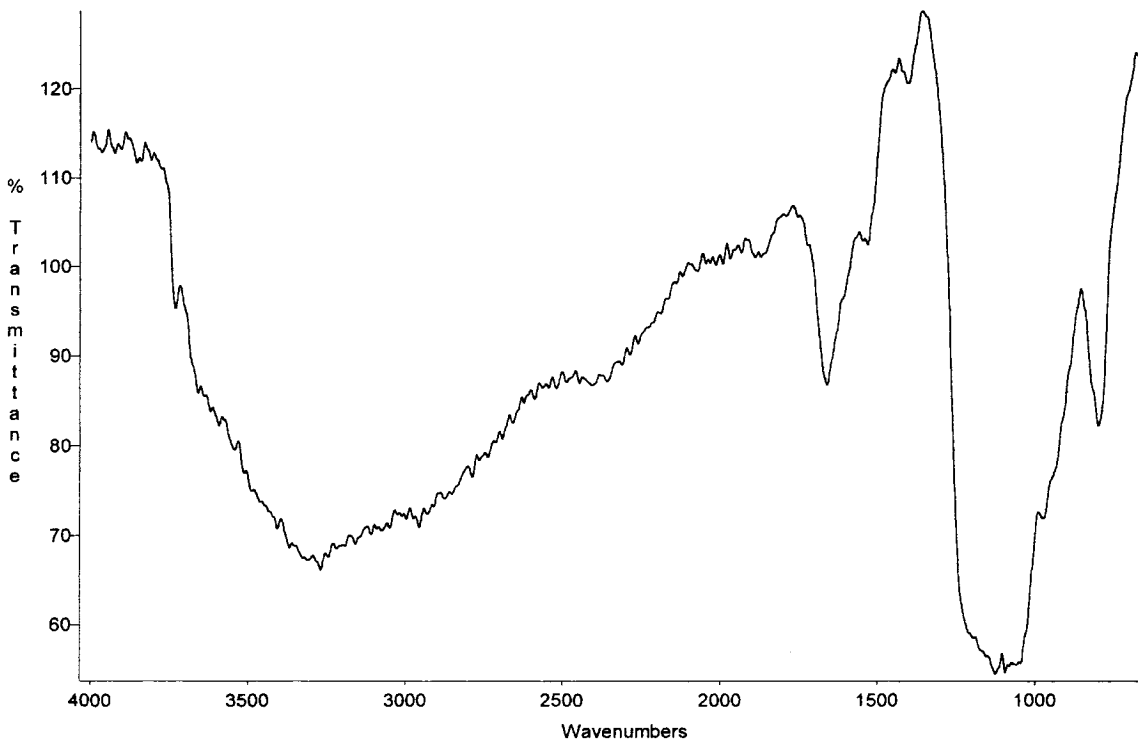


Figure 5: DRIFT spectrum of 3-amino-3-methyl-1-butyne + trypsin bonded phase

(iv) Surface characterization of 4-diethylamino-2-butyn-1-ol bonded phase made with the platinum catalyst: The DRIFT spectrum of the 4-diethylamino-2-butyn-1-ol bonded phase made with the platinum catalyst is shown in Figure 6. The reduction in the Si-H peak at 2250 cm^{-1} is a clear indication of replacement of the hydride intermediate with the bonded group. The peaks at 2980 cm^{-1} and 1450 cm^{-1} correspond to aliphatic C-H stretching and bending vibrations of the bonded group, hence confirming the presence of an organic moiety. The rest of the peaks in the spectrum can be attributed to the vibrations of the silica matrix and adsorbed water.

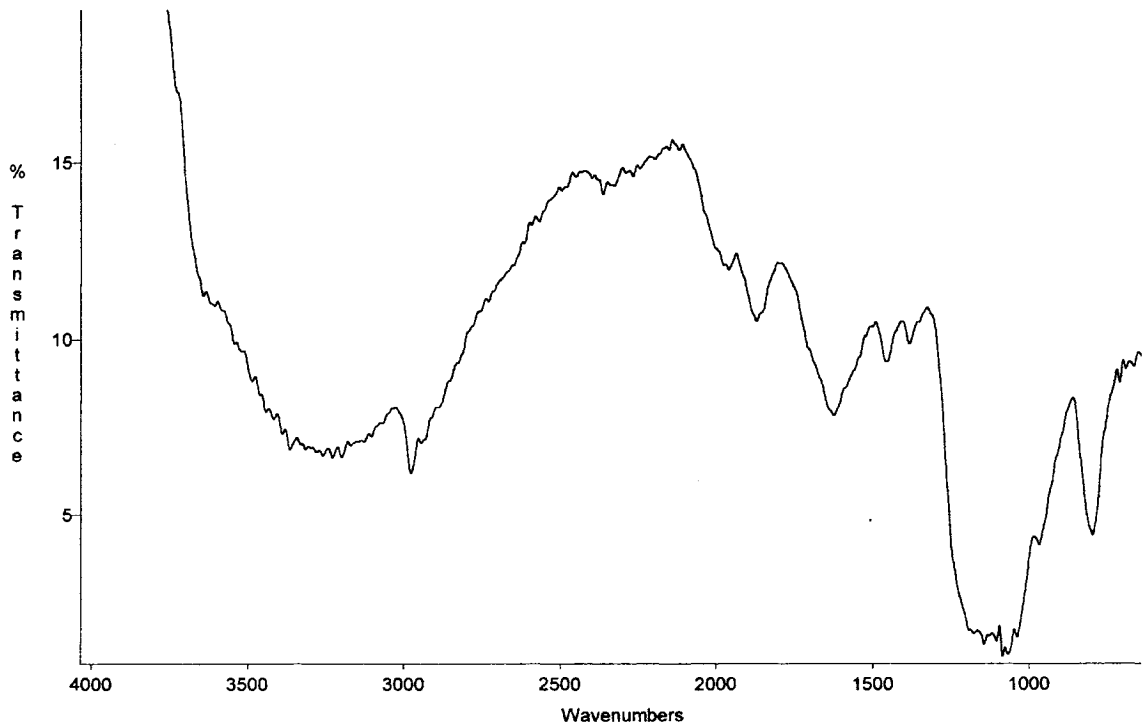


Figure 6: DRIFT spectrum of 4–diethylamino-2-butyn-1-ol bonded phase by platinum catalyst.

(v) Surface characterization of 4-diethylamino-4-butyn-1-ol bonded phase made by the free radical catalyst: The DRIFT spectrum of the 4-diethylamino-2-buty-n-1-ol bonded phase made by the free radical catalyst is shown in Figure 7. The presence of strong C-H stretching bands in the region of 2800 cm^{-1} and 3000 cm^{-1} show the bonding of the organic group to the hydride surface. Also the Si-H peak at 2250 cm^{-1} is reduced in intensity. The C-H peak in Figure 7 is comparatively more intense than Figure 6, indicating more organic group was bonded using free radical catalyst. The elemental analysis data confirms this assumption that the free radical catalyst was able to provide better surface coverage.

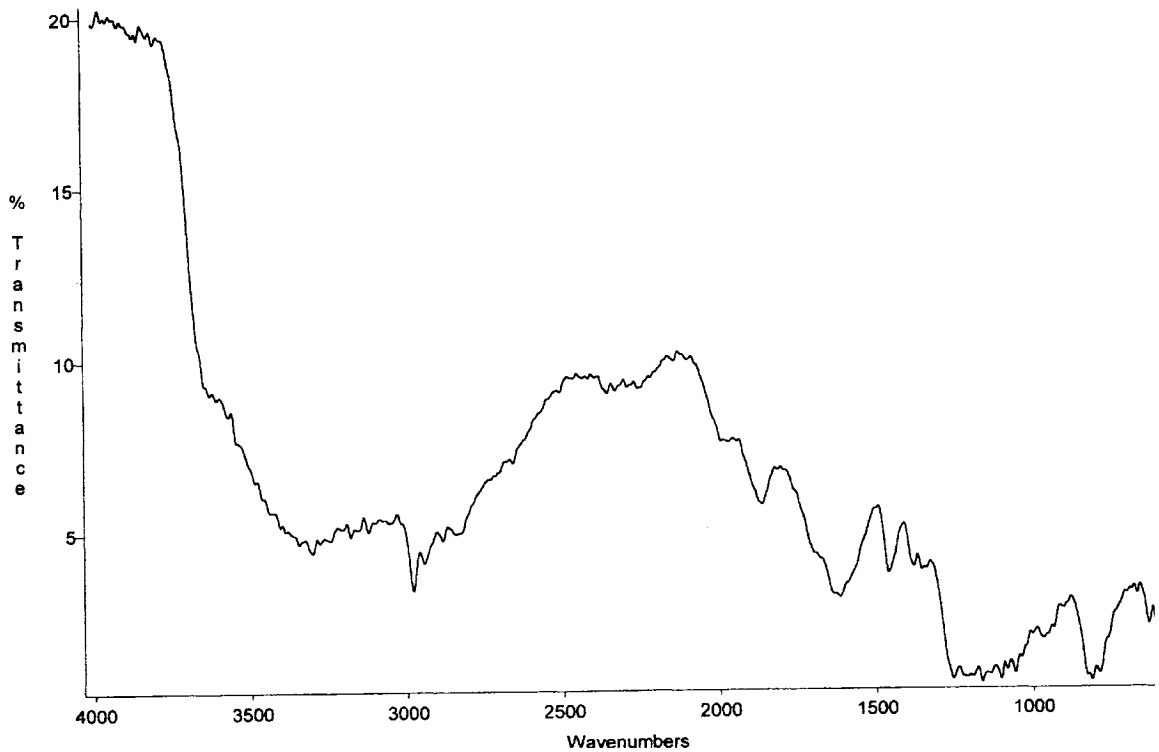


Figure 7: DRIFT spectrum of 4–diethylamino-2-butyn-1-ol bonded phase by free radical catalyst.

C. Solid State ^{13}C CP-MAS NMR Spectroscopic Evaluation:

(i) Characterization of 3-amino-3-methyl-1-butyne bonded phase: Figure 8 shows the ^{13}C CP-MAS NMR spectrum of the 3-amino-3-methyl-1-butyne bonded phase using a free radical catalyst. The sharp peak at 125 ppm indicates that the organic moiety is bonded to the silica hydride surface through an olefin bond. The peaks at 25 ppm and 61 ppm correspond to the methyl and methylene resonances of the residual ethoxy group present on the silica hydride surface. Overall the spectrum indicates bonding of 3-amino-3-methyl-1-butyne to the silica hydride surface.

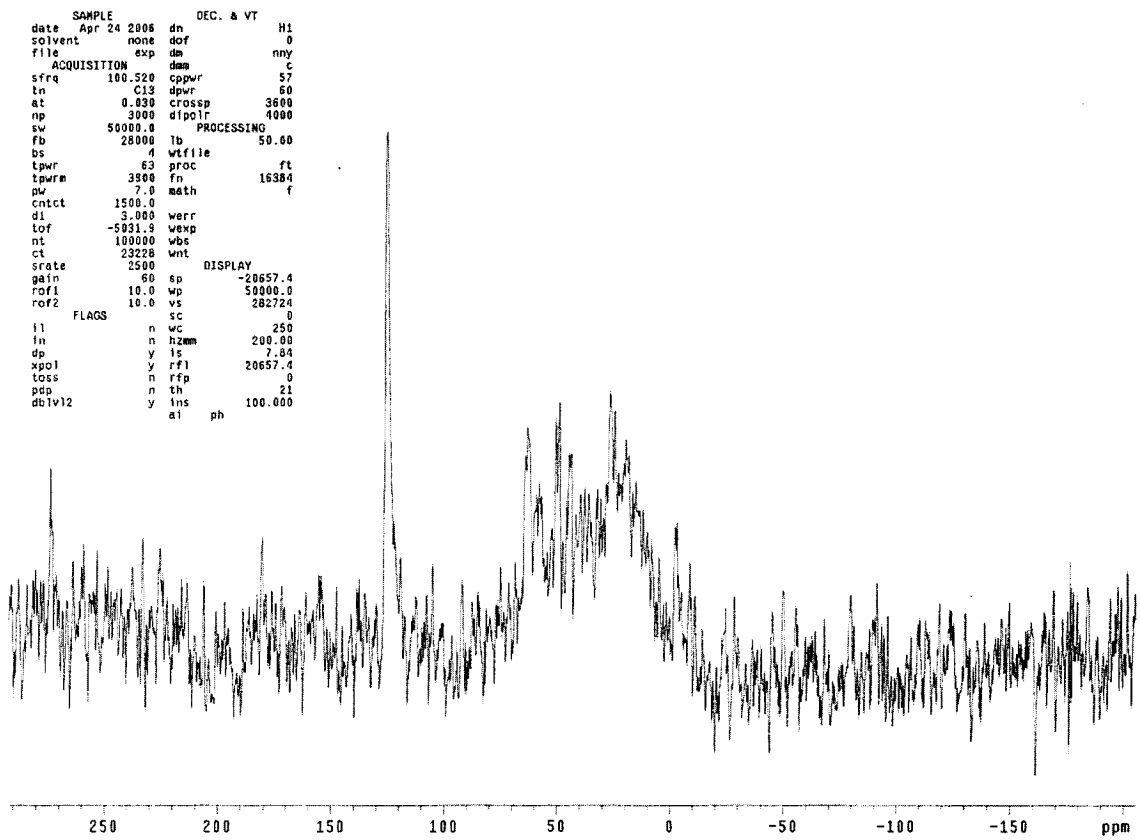


Figure 8: NMR spectrum of 3-amino-3-methyl-1-butyne bonded phase.

(ii) Characterization of 3-amino-3-methyl-1-butyne + trypsin bonded phase: Figure 9 shows the ^{13}C CP-MAS NMR spectrum of the 3-amino-3-methyl-1-butyne + trypsin bonded phase. The peaks between 10 ppm to 42 ppm are due to the various carbons of the protein on the hydride surface. The signal at 173 ppm is due to the C=O from the amide carbonyl groups present in the amino acids, hence confirming the presence of protein on the amino bonded surface. The broad peak between 49 ppm and 59 ppm is due to the remainder of the carbon atoms in the protein bonded amino phase.

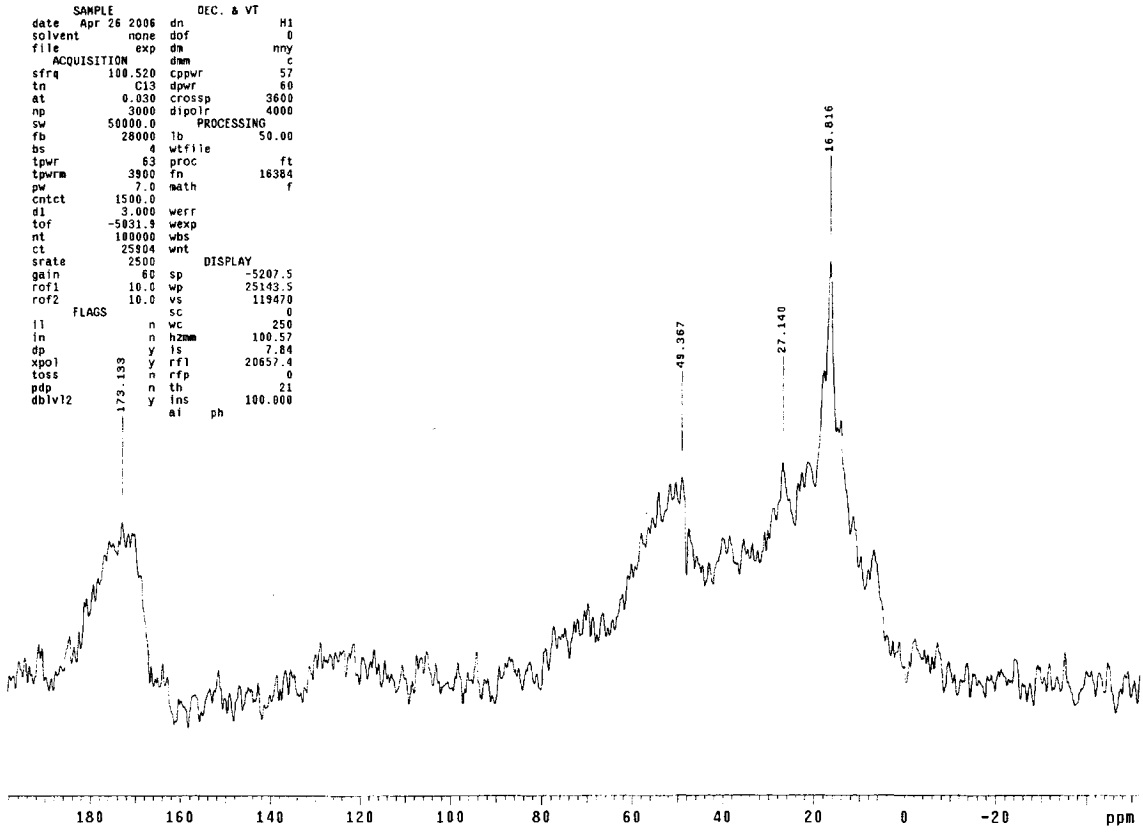


Figure 9: NMR spectrum of 3-amino-3-methyl-1-butyne + trypsin bonded phase.

(iii) Characterization of 4-diethylamino-2-butyn-1-ol bonded phase made by the platinum catalyst: Figure 10 shows the ^{13}C CP-MAS NMR spectrum of the 4-diethylamino-2-butyn-1-ol bonded phase made by the platinum catalyst. The sharp peak at 6.43 ppm is due to the methyl resonance of the bonded moiety, hence confirming the presence of the organic group on the silica hydride surface. The broad peak between +5 ppm and 20 ppm is due to the C-H attached to the surface of silica. The broad peak at 45 ppm is due to the three methylene carbons of the bonded group. Overall the spectrum confirmed the bonding of 4-diethylamino-2-butyn-1-ol to the silica hydride surface.

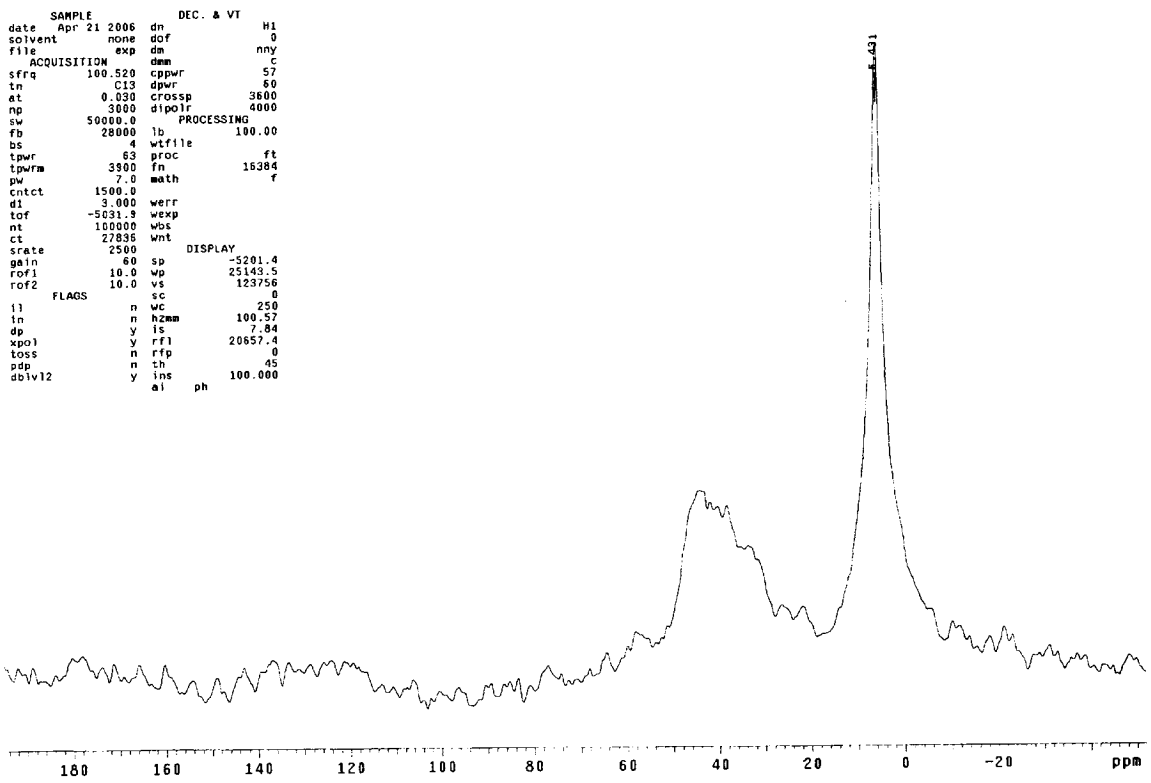


Figure 10: NMR spectrum of 4-diethylamino-2-butyn-1-ol bonded phase with platinum catalyst.

(iv) Characterization of 4-diethylamino-2-butyn-1-ol bonded phase made by the free radical catalyst: Figure 11 shows the ^{13}C CP-MAS NMR spectrum of the 4-diethylamino-2-butyn-1-ol bonded phase made by the free radical catalyst. The spectrum is similar to the one obtained for the synthesis of 4-diethylamino-2-butyn-1-ol bonded phase using the platinum catalyst. The sharp peak at 6.614 ppm is due to the methyl resonance of the bonded moiety and the broad peak underneath it is due to the carbon bonded to the surface. The broad peak at 40 ppm corresponds to the three methylene carbons of the bonded moiety. After looking at the characterization results it was confirmed that 4-diethylamino-2-butyn-1-ol was bonded to the silica hydride surface using both platinum and free radical catalysts.

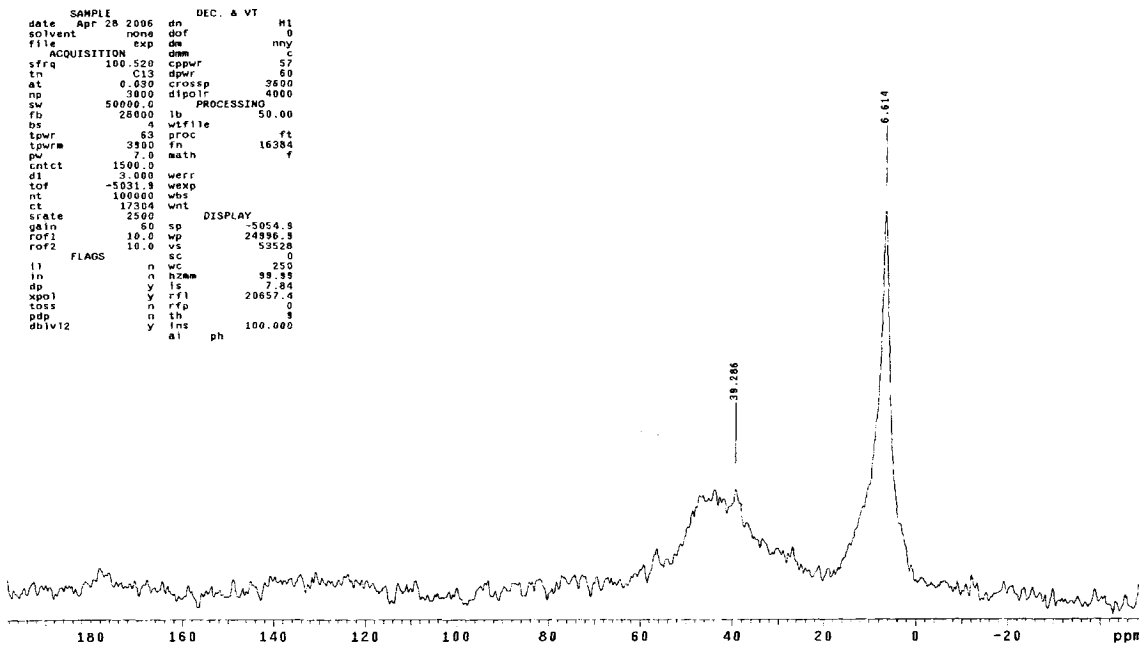


Figure 11: NMR spectrum of 4-diethylamino-2-butyn-1-ol bonded phase with free radical catalyst.

D. Chromatographic Evaluation:

(i) Neue Test: All four stationary phases were initially characterized using the Neue test in order to determine the hydrophobicity and silanophilic activity at pH 7. Two purely hydrophobic analytes, naphthalene and acenaphthene, were used to determine the hydrophobicity of the column. Amitriptyline, a polar basic compound, was used to measure the silanophilic activity of the stationary phases. To determine the dead volume of the column, uracil was used. The compounds were analyzed using 35% 20 mM K_2HPO_4/KH_2PO_4 buffer at pH 7 and 65 % methanol. The flow rate was set to 1 ml/min for both the 4-diethylamino-2-butyn-1-ol columns (column dimensions are 150 mm x 4.5 mm). The flow rate was set at 0.8 ml/min and the injection volume was 1µL for all the columns. The detection wavelength was set at 210 nm for amitriptyline and 254 nm for naphthalene and acenaphthene. Chromatograms on the 3-amino-3-methyl-1-butyne bonded phase are shown in Figures 12 and 13 as an example.

The hydrophobicity can be measured using Equation 10, where V is the molar volume of the compound being analyzed, $^{\vee}$ is the hydrophobicity parameter of the stationary phase and k is the retention factor of the hydrophobic compound.

$$\ln (k) = {}^{\vee} \times V - 3.068 \quad (10)$$

The silanophilic activity at pH 7 can be calculated using Equation 11, where S is the silanophilic activity, $k_{amitriptyline}$ is the retention factor of amitriptyline and $k_{acenaphthene}$ is the retention factor of acenaphthene. The results of the Neue test are shown in Table 9, where t_R is the retention time of the solutes in minutes.

$$S = \ln (k_{amitryptiline}) - 0.7124 \times \ln (k_{acenaphthere}) + 1.9748 \quad (11)$$

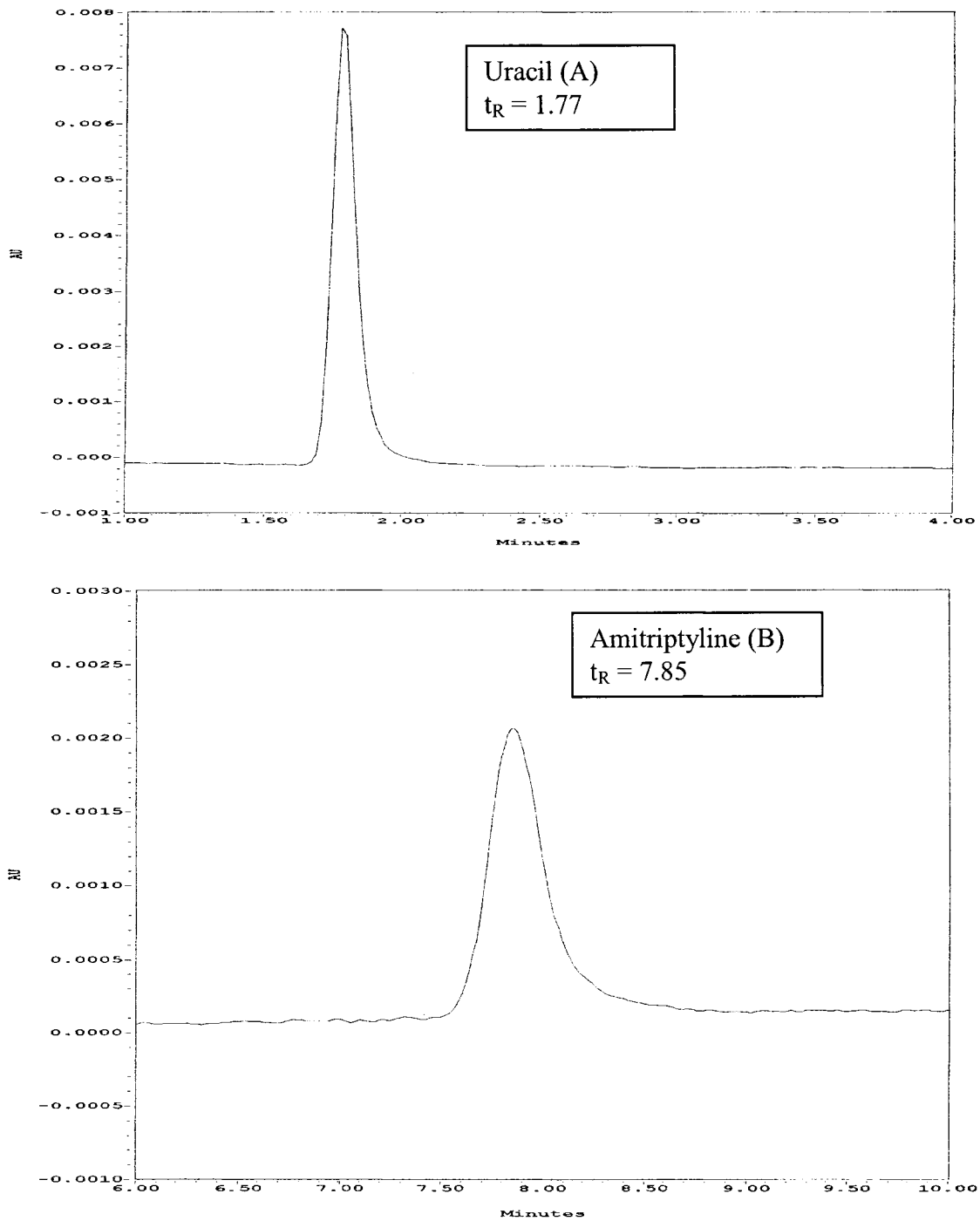


Figure 12: Chromatograms of uracil (A) and amitriptyline (B) on the 3-amino-3-methyl-1-butyne bonded phase.

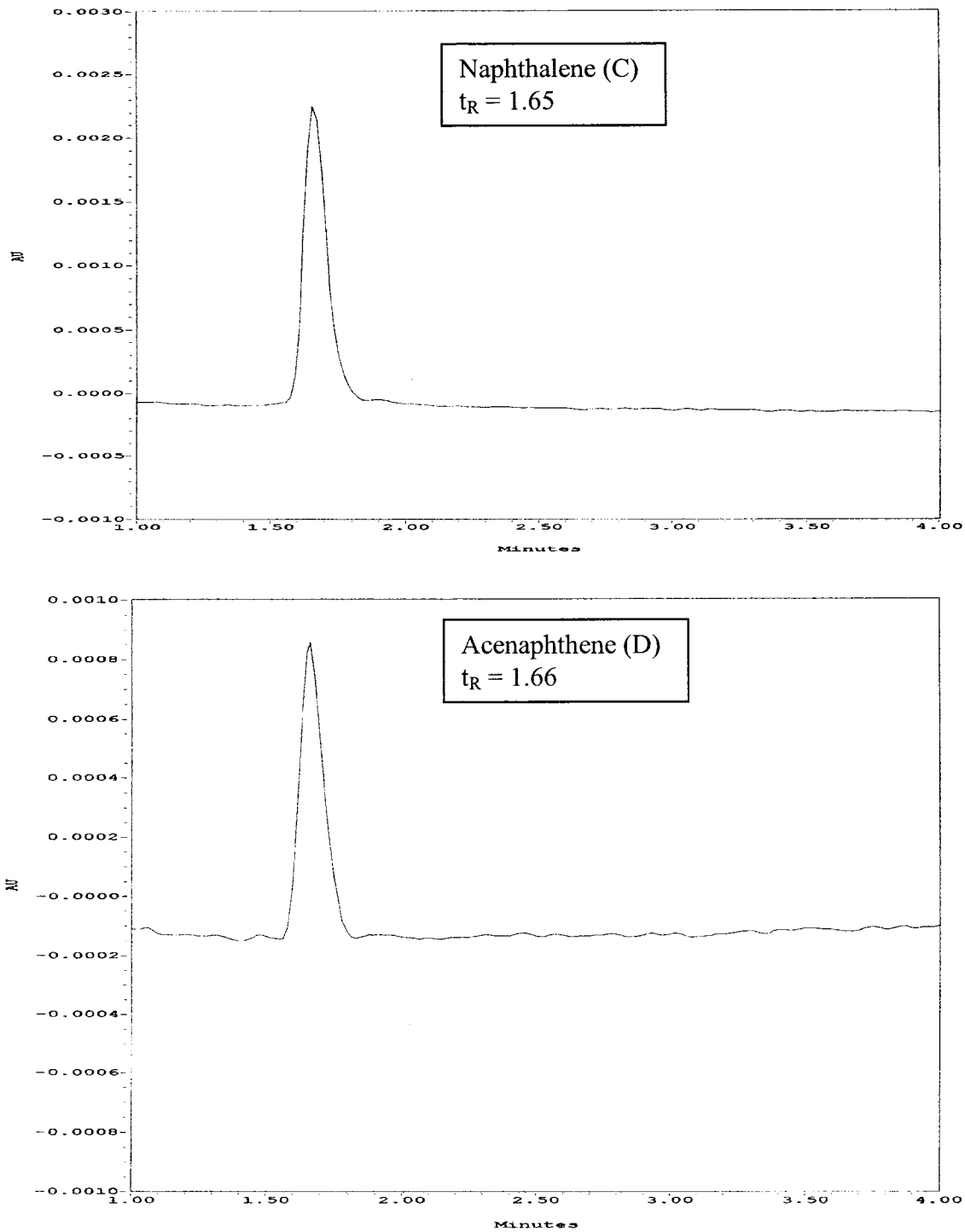


Figure 13: Chromatograms of naphthalene (C) and acanaphthene (D) on the 3-amino-3-methyl-1-butyne bonded phase.

Table 9: Results of the Neue test

Stationary Phase	Uracil (t_R)	Acenaphthene (t_R)	Naphthalene (t_R)	Amitriptyline (t_R)
3-amino-3-methyl-1-butyne bonded phase (FR)	1.77	1.66	1.65	7.85
3-amino-3-methyl-1-butyne + trypsin bonded phase	1.74	1.70	1.72	9.75
4-diethylamino-2-butyn-1-ol bonded phase (Pt)	2.54	2.34	2.36	10.3
4-diethylamino-2-butyn-1-ol bonded phase (FR)	2.46	2.37	2.37	11.2

The retention time of acenaphthene and amitriptyline was less then the void volume on all four columns, hence the retention factor values were negative. The retention of the uracil might be due to the interaction of uracil with the residual Si-H intermediate which was not replaced by the organic group during the hydrosilation step. Also the faster elution of the two hydrophobic compounds confirms the polar nature of the amino bonded phases. The longer retention time of amitriptyline could be attributed to the presence of residual silanol groups that make the surface of the bonded phase silanophilic. However, in this case as all the columns under study are highly polar in nature, the retention of amitriptyline is due to the polar nature of the stationary phase and not necessarily due to the presence of surface silanols. Also the columns under study had different dimensions but the flow rate used for all the analyses was 0.8 ml/min. The 3-amino-3-methyl-1-butyne bonded phase column and the trypsin bonded column were

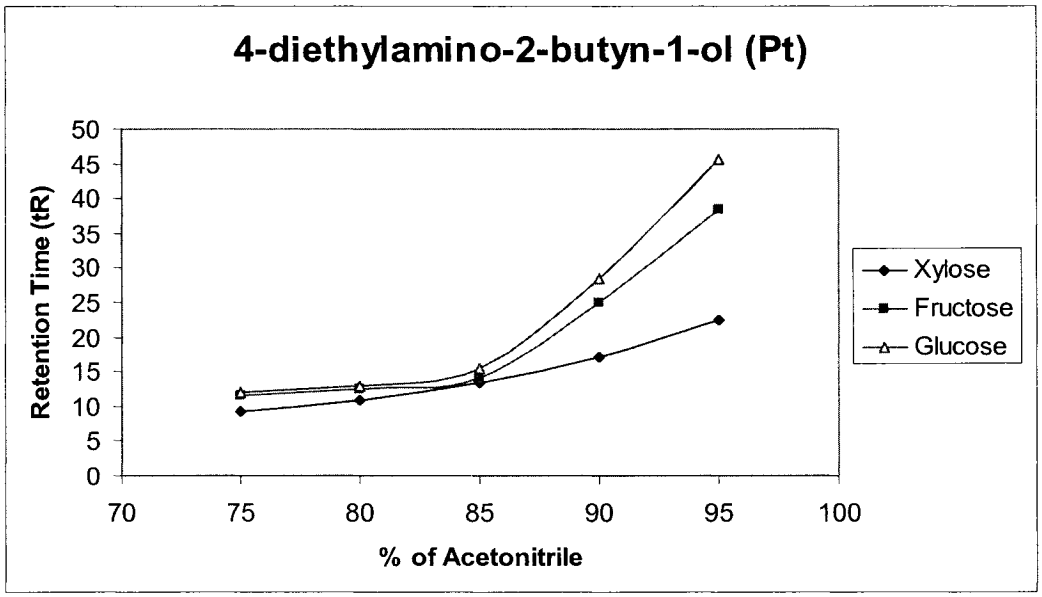
only 100 mm in length. However both the 4-diethylamino-2-butyn-1-ol columns were 150 mm in length. This clearly indicates that the 3-amino-3-methyl-1-butyne bonded phase column and the trypsin bonded column are comparatively more polar in nature due to the presence of free amino group on the bonded phase.

(ii) Sugar Analysis: Chromatographic evaluation of all four columns was done by studying the retention of sugars on these columns. The analysis was carried out in the aqueous normal phase mode using a laser light scattering detector. Five different ratios of acetonitrile and DI water were used, starting from 95 % acetonitrile and 5% water. 15 mg/mL sugar samples in 10:90 methanol/water were injected onto the columns and the flow rate was set to 0.3 ml/min. Initially eight sugars were analyzed on all four amino columns using only 5 % water. The sugars analyzed were maltose, sucrose, glucose, fructose, lactose, xylose, raffinose, and melezitose. As sugars show typical normal phase behavior on these polar columns the retention time of sugars was very high when 95% of acetonitrile was used, making the mobile phase relatively non polar. In fact the retention time of the disaccharides were over 60 minutes and the trisaccharides were over 90 minutes making the analysis time very long even for a single determination. Hence it was decided to analyze just the monosaccharides on all four bonded phases.

For the analysis of monosccharides the concentrations of mobile phase (acetonitrile: water) used were as follows: 95:5, 90:10, 85:15, 80:20 and 75:25. The order of elution for the sugars was based on the number of hydroxyl groups present on each sugar. In normal phase chromatography the least polar compound is eluted first

followed by more polar compounds. On all four columns the least polar sugar xylose was eluted first. Glucose and fructose were eluted very close to each other. The retention of these sugars was due to the polar-polar interaction between the amino groups on the bonded phase and the OH groups of the sugars. The elution order is based on the number of hydroxyl groups on each sugar [31]. Xylose which has only four hydroxyl groups is eluted first, followed by fructose and glucose which have five hydroxyl groups [32]. As both fructose and glucose have five hydroxyl groups sometimes they were eluted as single peak. The best separation was achieved by using a lower amount of water in the mobile phase.

A study of the relationship of the retention time of the sugars with the amount of organic solvent in the mobile phase was done by plotting a retention map on all four bonded phase. As shown in Figure 14 and Figure 15, the retention times of the sugars are directly proportional to the amount of organic solvent. As the amount of organic solvent increases the retention time of the sugars increases. This type of behavior is typical of aqueous normal phase chromatography. This ANP behavior was observed on all four columns. The retention times of sugars on the 4-diethylamino-2-butyn-1-ol column using platinum catalyst and 4-amino-2-butyn-1-ol using free radical catalyst were longer compared to the 3-amino-3-methyl-1-butyne and the protein column. However the column dimensions vary. The 4-diethylamino-2-butyn-1-ol columns are 150 mm long compared to just 100 mm long for the 3-amino-3-methyl-1-butyne and the protein column. The 3-amino-3-methyl-1-butyne and the protein column are more polar in nature due to the presence of the free amino group on the both the stationary phases.



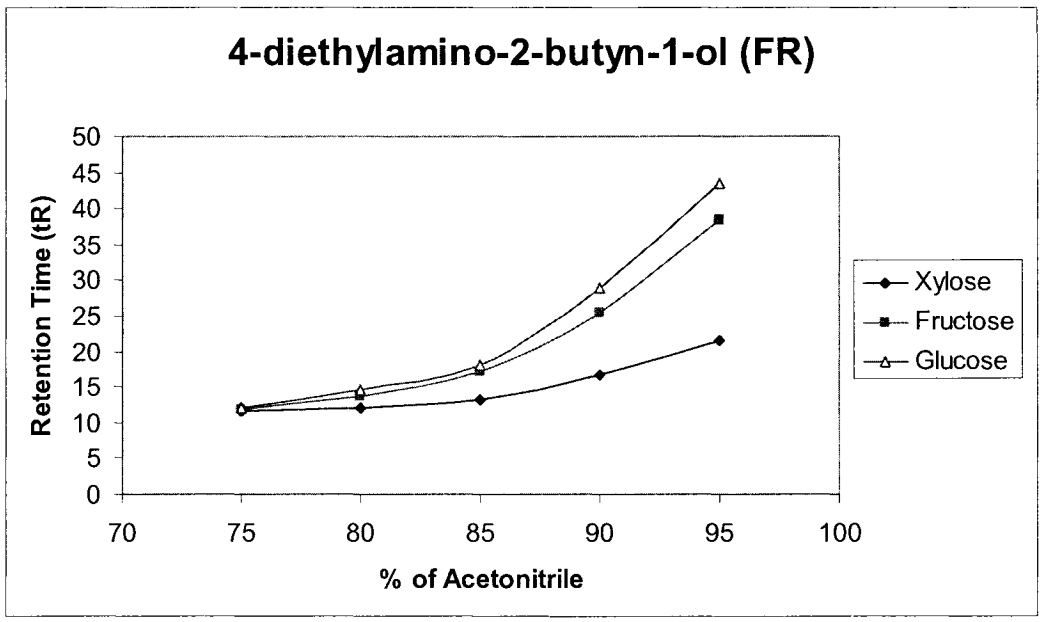


Figure 14: Retention map for sugar analysis on the 4-amino-2-butyn-1-ol bonded phase made with Pt (top) and the 4-amino-2-butyn-1-ol bonded phase made with free radical (bottom) using acetonitrile/water as the mobile phase.

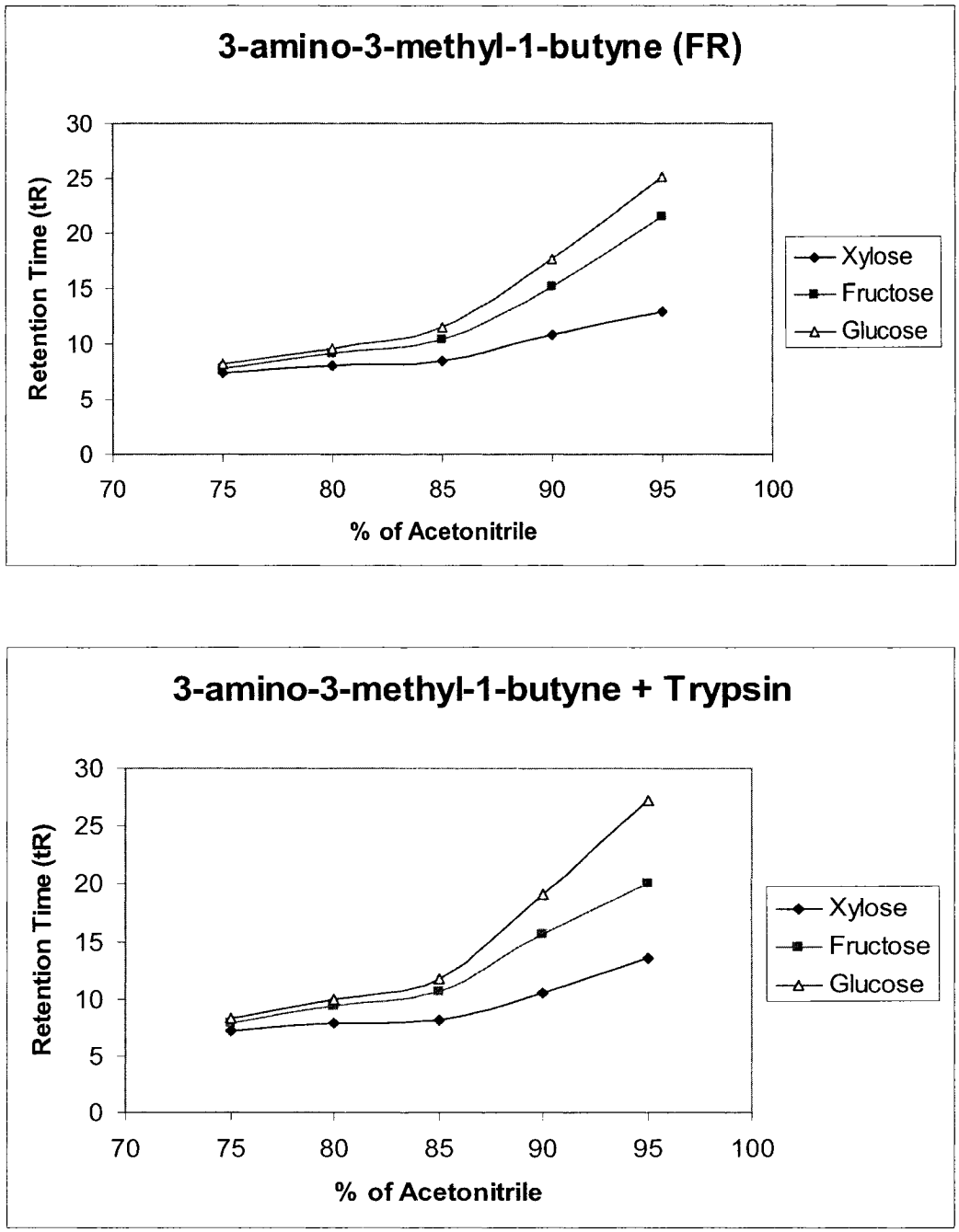


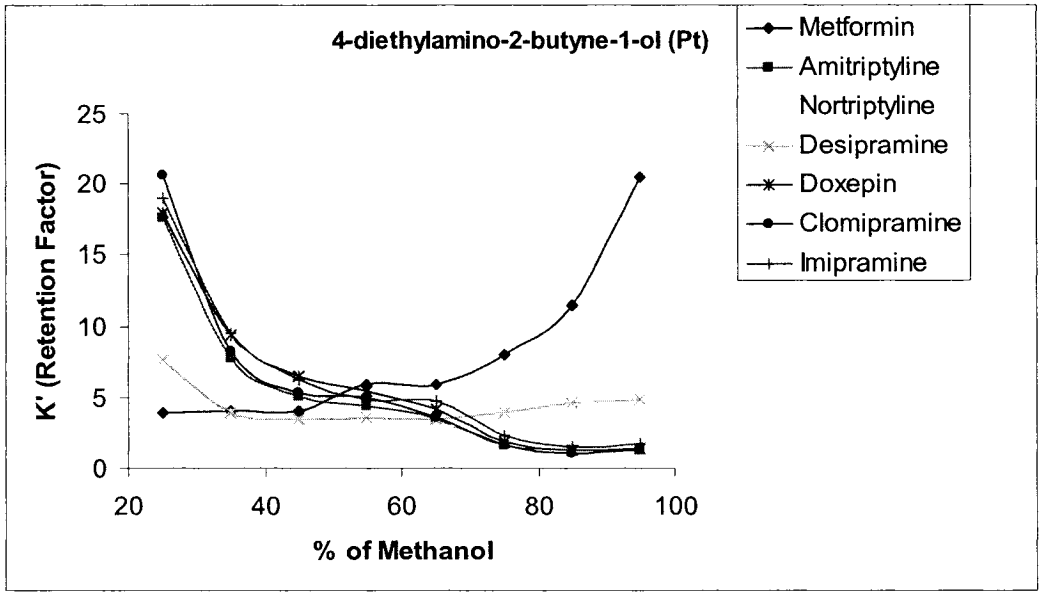
Figure 15: Retention map for sugar analysis on the 3-amino-3-methyl-1-butyne bonded phase made with free radical (top) and the 3-amino-3-methyl-1-butyne + trypsin bonded phase (bottom) using acetonitrile/water as the mobile phase.

(iii) Analysis of Metformin and antidepressants: The four bonded phases used in this part of the study were the 4-diethylamino-2-butyn-1-ol with Pt catalyst, the 4-diethylamino-2-butyn-1-ol with free radical catalyst, the 3-amino-3-methyl-1-butyne bonded phase and the 3-amino-3-methyl-1-butyne + trypsin bonded phase. The seven polar compounds used in the study were as follows: metformin, doxepin, amitriptyline, desipramine, nortriptyline, clomipramine and imipramine. Uracil was used to determine the void volume. Out of the seven compounds metformin is highly polar in nature and while the rest of the compounds are moderately polar. The flow rate was 0.8 ml/min for all four columns and the injection volume was 10 µL. Different ratios of methanol and 20 mM K_2HPO_4/KH_2PO_4 buffer at pH 7 were used as the mobile phase. The concentrations of mobile phase (methanol/buffer) used were as follows: 25:75, 35:65, 45:55, 55:45, 65:35, 75:25, 85:15 and 95:5. The UV detection wavelength was set at 254 nm.

Metformin showed ANP behavior on all four columns. Hence retention increased as the amount of methanol increased as shown in Figures 16 and 17. This might be the due to the interaction of the highly polar amino groups of metformin with the amino bonded phase. Also the degree of ionization depends on the pKa of the drug and the pH of the mobile phase. The pKa of metformin is 12.4, whereas the pKa of all the other six drugs is close is 9.5. In ANP chromatography the retention time for basic solutes increases at lower pH. The analysis was done at pH 7. Hence the highly ionized metformin sample was retained longer on the columns as the amount of organic solvent increased in the mobile phase.

Amitriptyline, imipramine, clomipramine and doxepin showed reverse phase behavior on all four columns. This might be due to the hydrophobic interaction of these compounds with the stationary phase and a comparatively lower pKa value. The pKa of amitriptyline, imipramine, clomipramine is 9.5 and doxepin is 9.0. Nortriptyline and desipramine which have pKa value of 9.7 and 10.5, showed reverse phase behavior with a low amount of methanol and ANP behavior with a higher amount of methanol on all four columns. Although all four columns showed a similar retention mechanism, compounds on the protein column showed a more random pattern. This might be due to complex interactions from the various functional groups present on the amino acid chain.

The retention times of pharmaceuticals on the 4-diethylamino-2-butyn-1-ol column made with the free radical catalyst was longer compared to the 4-diethylamino-2-butyn-1-ol column made with the platinum catalyst. This behavior can be attributed to the fact that 4-diethylamino-2-butyn-1-ol column made with the free radical catalyst had higher surface coverage of the bonded phase as determined by the elemental analysis data. The retention times of pharmaceuticals on the 3-amino-3-methyl-1-butyne and the protein column were shorter compared to both the 4-diethylamino-2-butyn-1-ol columns. This was due to the difference in the column dimensions as explained earlier. All the pharmaceuticals under study showed very good separation and peak resolution on all four columns. The best separation was achieved with equal amounts of organic solvent and buffer. It was difficult to determine the retention time of doxepin, as there were two peaks instead of one in the chromatogram. Hence an LC/MS analysis was done and the result of the analysis is discussed later.



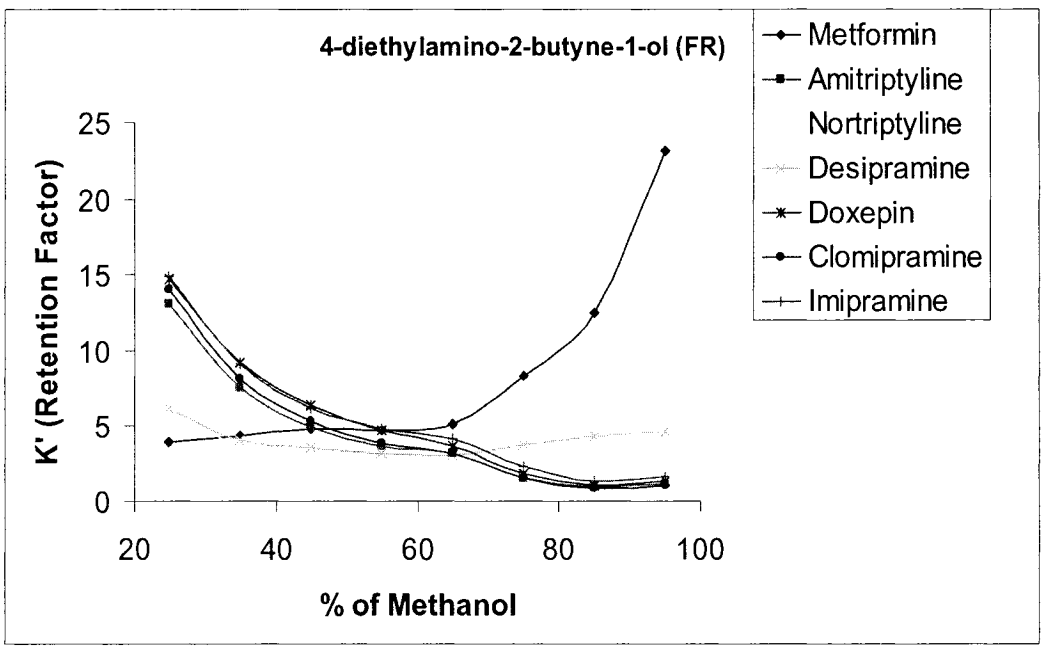
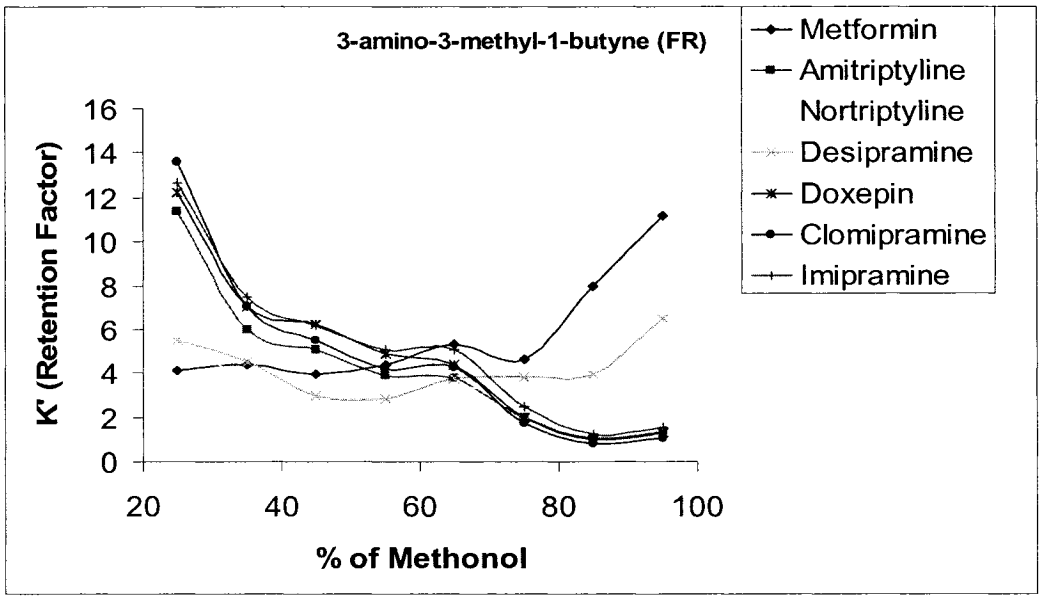


Figure 16: Retention map of metmorfin & antidepressants on the 4-amino-2-butyn-1-ol bonded phase made with Pt (top) and the 4-amino-2-butyn-1-ol bonded phase made with free radical (bottom) using methanol/buffer as the mobile phase.



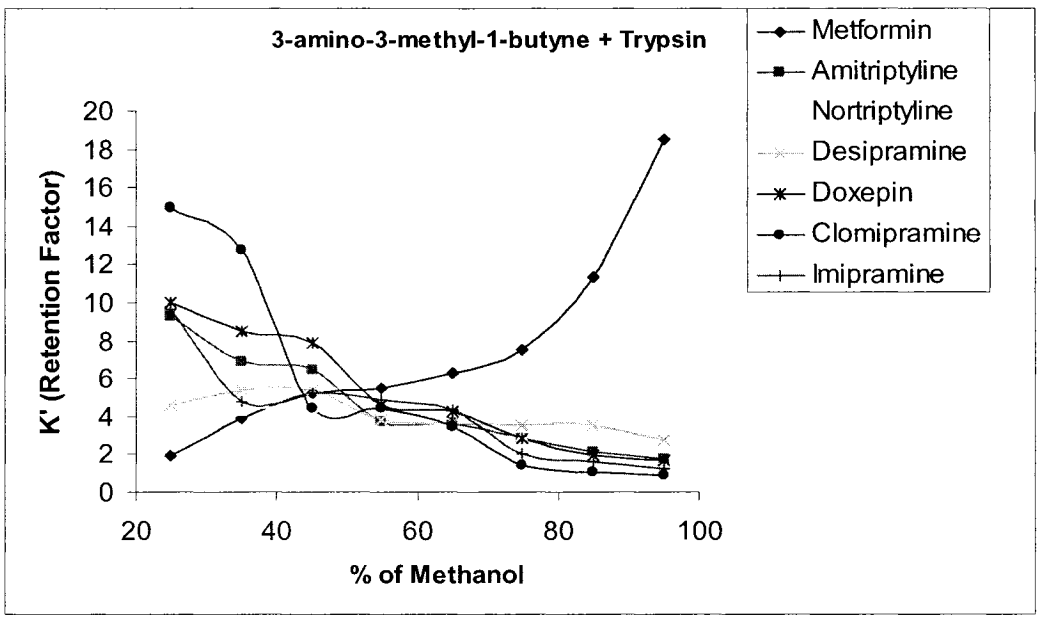


Figure 17: Retention map of metformin & antidepressants analysis on the 3-amino3-methyl-1-butyne bonded phase using free radical (top) and the 3-amino-3-methyl-1-butyne + trypsin bonded phase (bottom) using methanol/buffer as the mobile phase.

(iv) Analysis of Doxepin using LC/MS: During the analysis of the polar antidepressants, the doxepin sample had two peaks in all the chromatograms as shown in Figure 18. It was initially thought a chiral separation of the two doxepin isomers was being observed. Hence it was important to do an LC/MS analysis in order to get more insight into the structural details of the compounds being eluted. Also it was difficult to confirm the retention time of doxepin due to the presence of two peaks in the chromatogram. The mobile phase contained 50:50 methanol/water with 0.5% formic acid. The flow rate was set to 0.5 ml/min. The ion source used in the study was APCI which produces a singly charged protonated ion via a proton transfer mechanism. The 3-amino-3-methyl-1-butyne bonded phase column was used as the stationary phase.

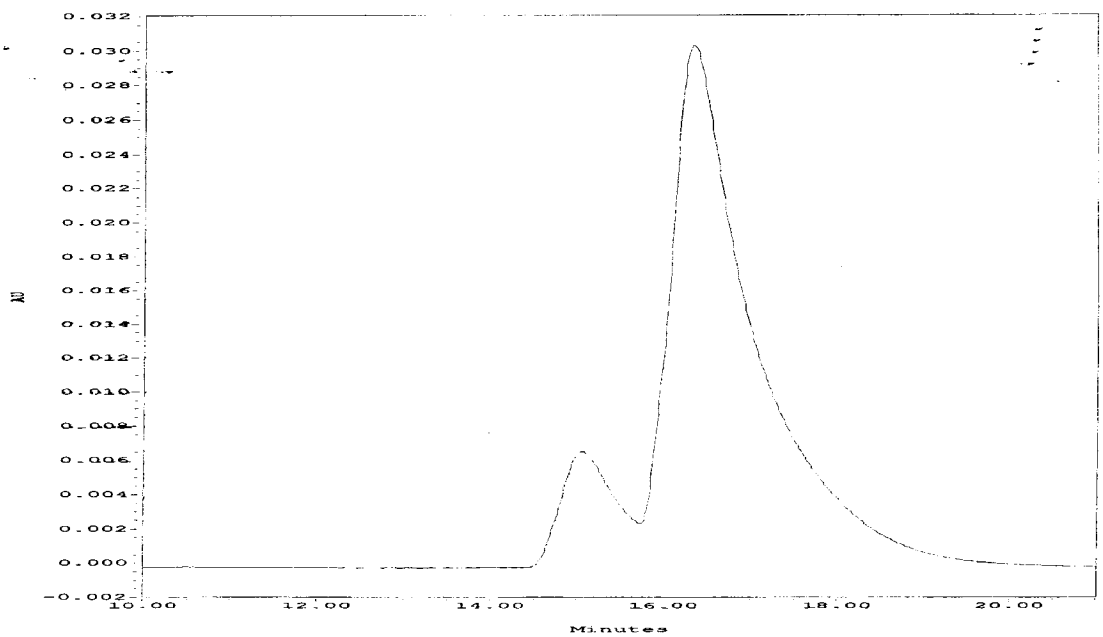


Figure 18: Analysis of doxepin on the 3-amino-3-methyl-1-butyne column using 45% methanol and 55% phosphate buffer (UV @ 254 nm).

The doxepin sample formed ions in the methanol and formic acid. The most intense peak is exhibited as the $[M+H]^+$ ion. The molecular weight of doxepin is 279.37 g/mol. The mass spectrum of the larger peak from Figure 18 is shown in Figure 19 and the mass spectrum of smaller peak is shown in Figure 20. The most intense peak observed in Figure 18 was for ion 280.37 m/z with 100 % relative intensity confirming the presence of protonated doxepin ion. Figure 19 had peaks for ions at 123.03 m/z, 122.43 m/z and other peaks with small intensities. This might be due to the degradation of the sample over time. Hence, the larger peak from Figure 18 was noted as the retention time was doxepin sample. The mass spectrum revealed that the two peaks observed in the chromatogram are due to the partial degradation of the sample and not due to chiral separation as originally suspected.

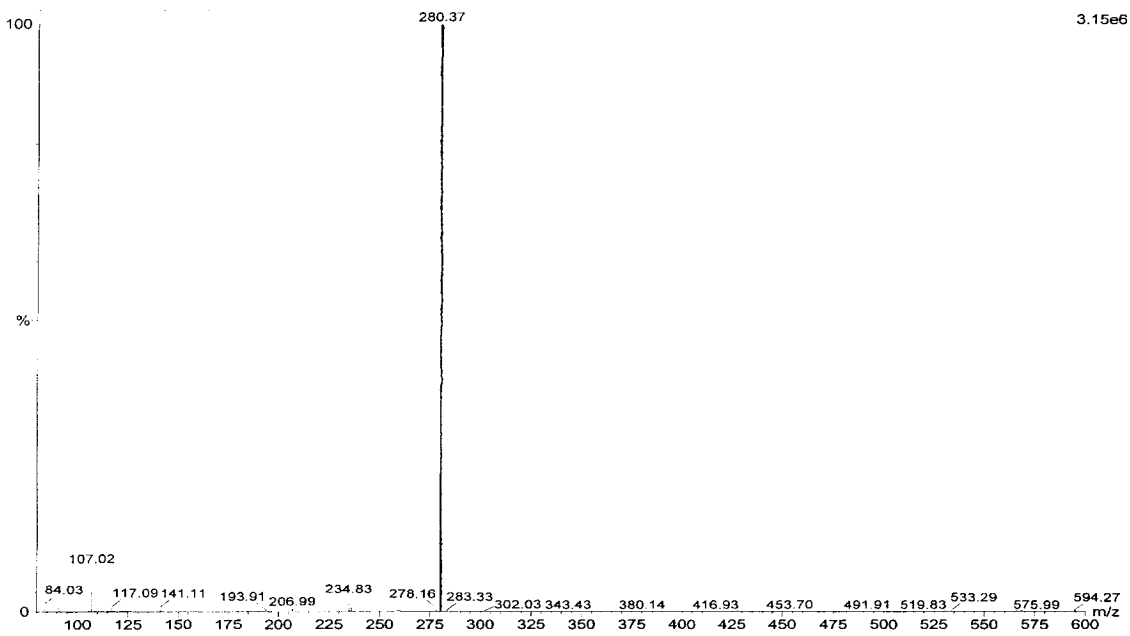


Figure 19: Mass spectrum of the larger peak from the doxepin chromatogram.

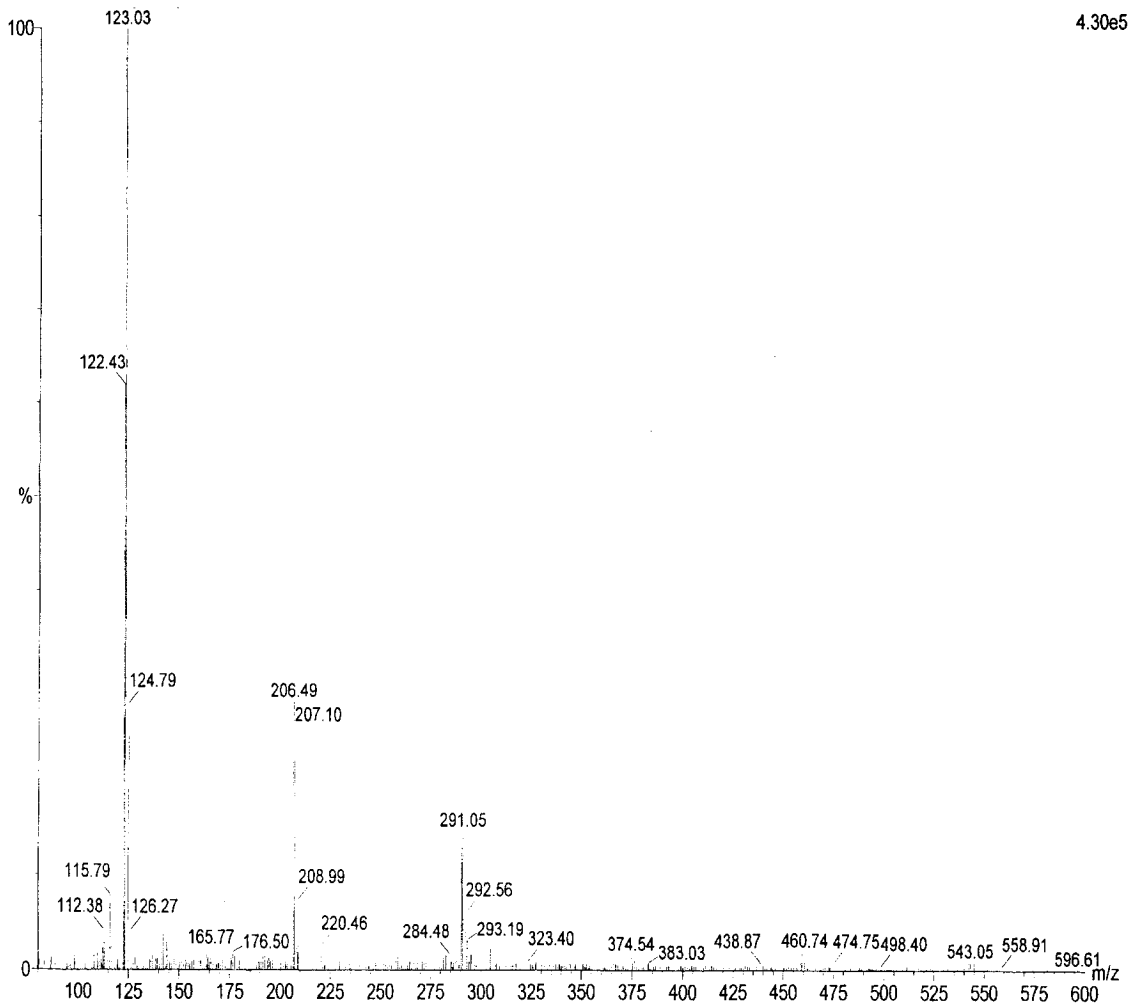


Figure 20: Mass spectrum of the smaller peak from the doxepin chromatogram.

(v) Affinity Chromatography: One of the goals of this research was to study the affinity aspect of the protein column. The column evaluated was the 3-amino-3-methyl-1-butyne bonded phase + trypsin. Type III-O trypsin inhibitor from chicken egg white was used to test the affinity aspect of the amino bonded trypsin column. The trypsin inhibitor contains 129 amino acids and was eluted through the column using two different mobile phases. A 0.01M phosphate buffer at pH 7 was used as the attaching buffer and acetic acid at pH 2.8 was used as the eluting buffer. The phosphate buffer helps to form the affinity complex between the trypsin and the trypsin inhibitor as shown in Figure 21 (A). A 20 μL sample of trypsin inhibitor was injected into the amino bonded trypsin column and run for 35 minutes using 0.01 M phosphate buffer as the mobile phase in order to form the affinity complex. The flow rate was set to 0.8 ml/min and the detection wavelength was set at 280 nm. The lack a of chromatographic peak under the phosphate buffer conditions is a good indication of the attachment of the inhibitor to the enzyme.

The acetic acid buffer helps to release the retained trypsin inhibitor as shown in Figure 21 (B). Once the inhibitor was attached to the protein column using the phosphate buffer the mobile phase was changed to the acetic acid buffer. The flow rate was 0.8 ml/min and the analysis was run for 10 minutes. A sharp peak for the trypsin inhibitor was observed after 5.64 minutes indicating the release of the inhibitor from the coupled trypsin enzyme. Due to the highly specific nature of affinity chromatography, it can be confirmed that the trypsin enzyme was attached to the 3-amino-3-methyl-1-butyne bonded phase.

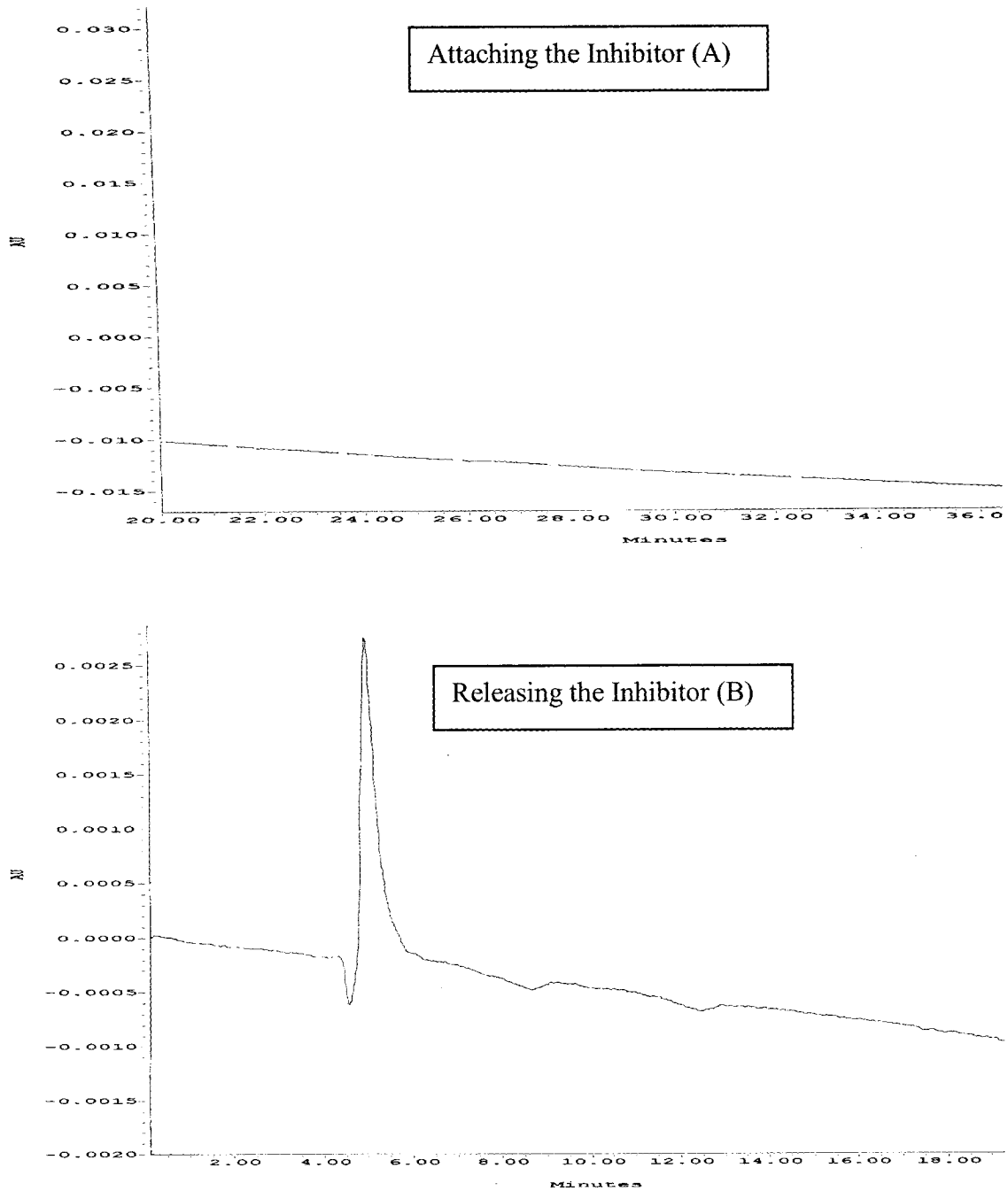


Figure 21: Affinity chromatography of trypsin inhibitor on trypsin column. Mobile phase for attaching the inhibitor is phosphate buffer at pH 7 (A) and acetic acid buffer at pH 2.8 is used for releasing the inhibitor (B).

(vi) Overall column performance: Figure 22 shows a typical separation of mixture of polar compounds on the 4-diethylamino-2-butyn-1-ol bonded phase made with the platinum catalyst. Each sample was injected three times before running the mixture and the difference in the retention time was +/- 0.01 min. Hence the results indicated good reproducibility. As shown in Figures 22, 23, 24 and 25 all four columns showed good separation and peak resolution. On all four stationary phases the best separation of the polar compounds was obtained when the mobile phase had almost equal amounts of buffer and organic solvent.

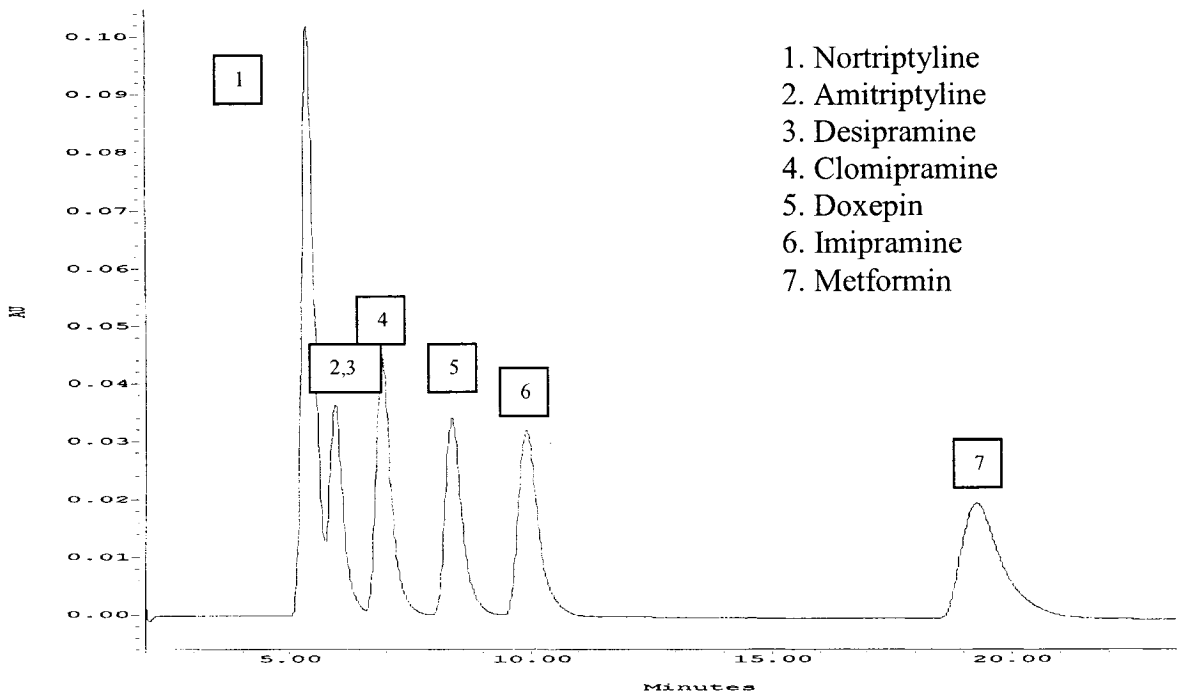


Figure 22: Separation of polar compounds on the 4-diethylamino-2-butyn-1-ol bonded phase (Pt). Mobile phase: 45% (20 mM K_2HPO_4/KH_2PO_4 buffer pH 7): 55% (Methanol), UV: @ 254nm.

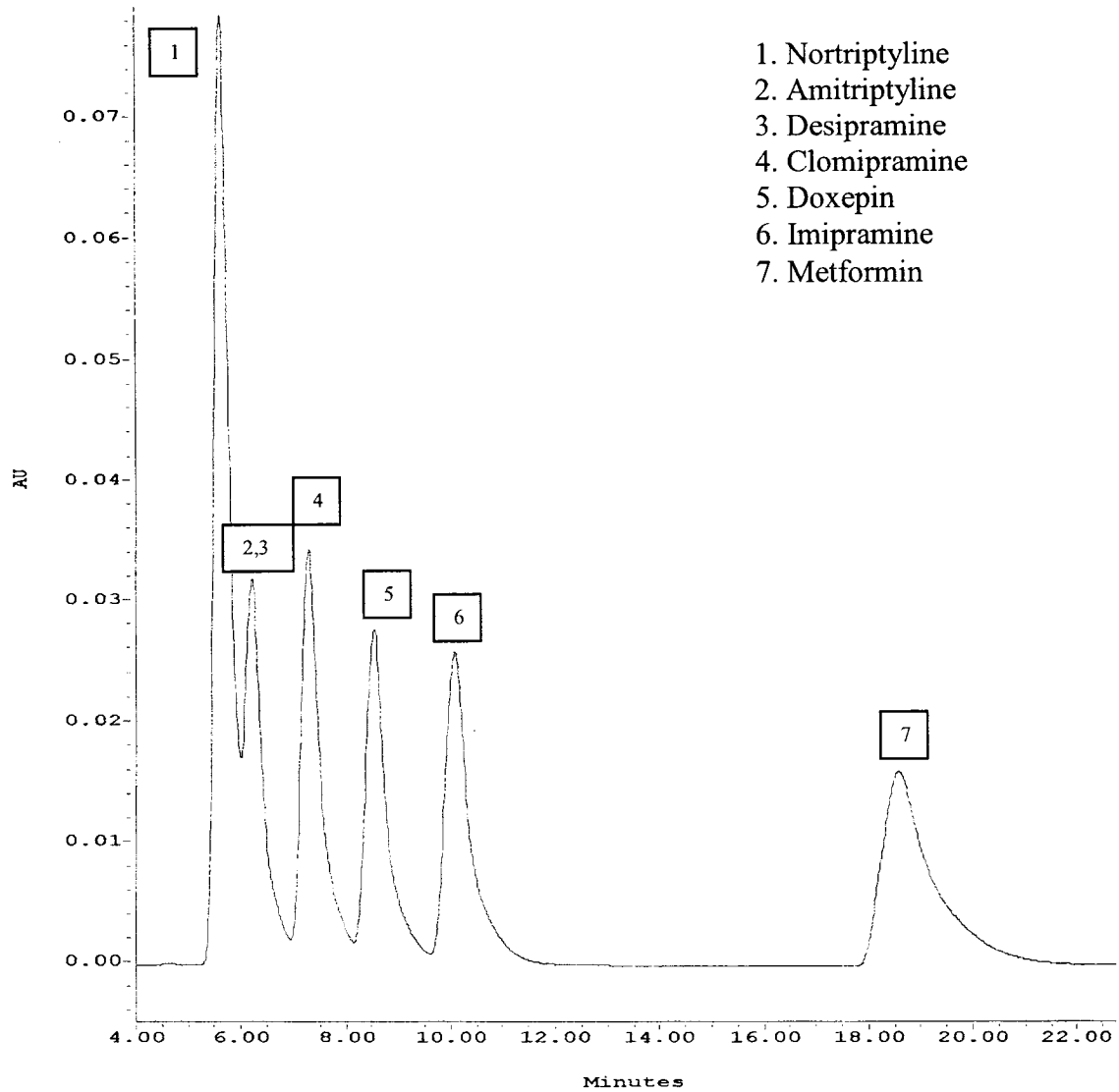


Figure 23: Separation of polar compounds on the 4-diethylamino-2-butyn-1-ol bonded phase (FR). Mobile phase: 45% (20 mM K_2HPO_4/KH_2PO_4 buffer pH 7): 55% (Methanol), UV: @ 254nm.

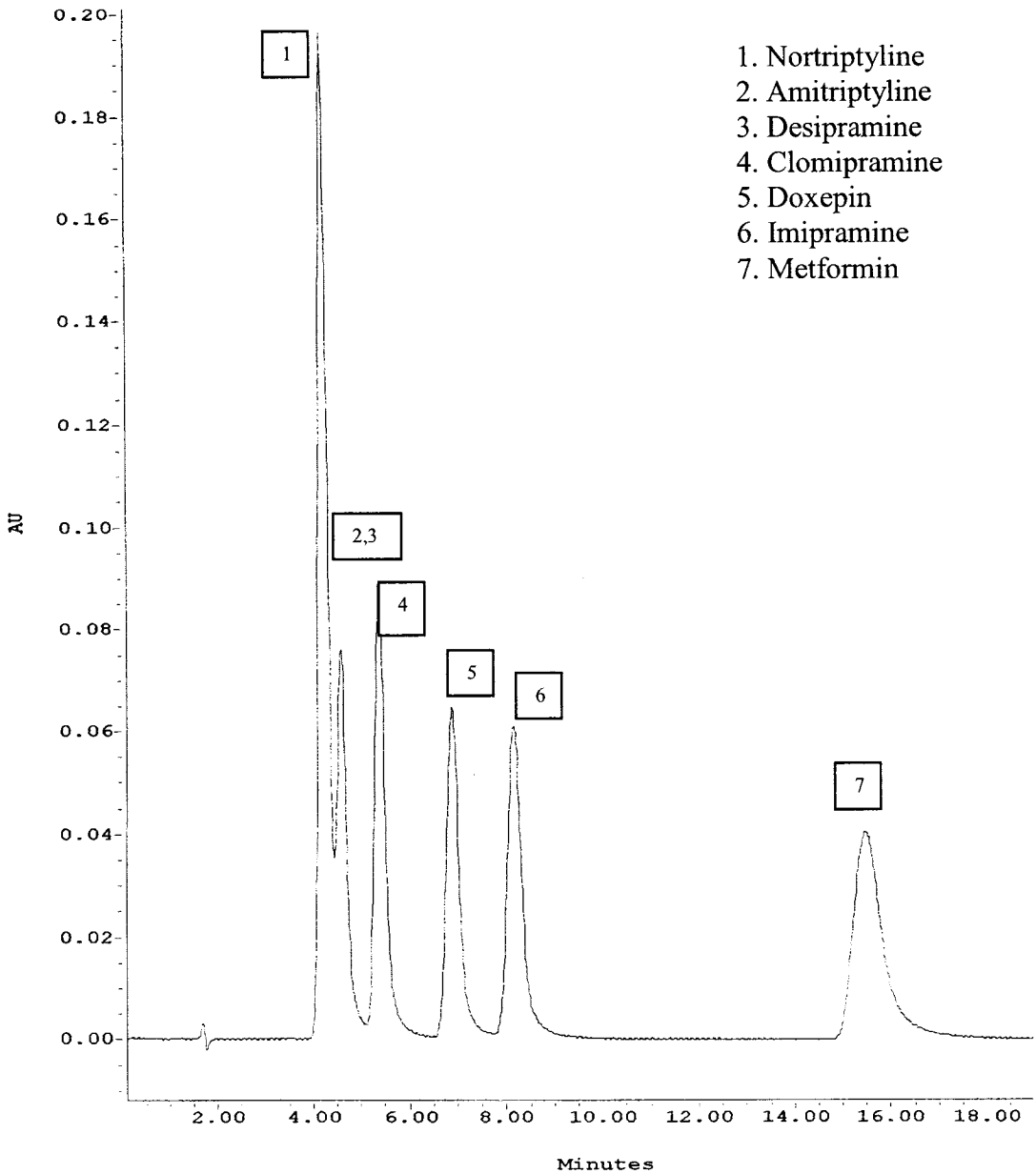


Figure 24: Separation of polar compounds on the 3-amino-3-methyl-1-butyne (FR). Mobile phase: 45% (20 mM K_2HPO_4/KH_2PO_4 buffer pH 7): 55% (Methanol), UV: @ 254nm.

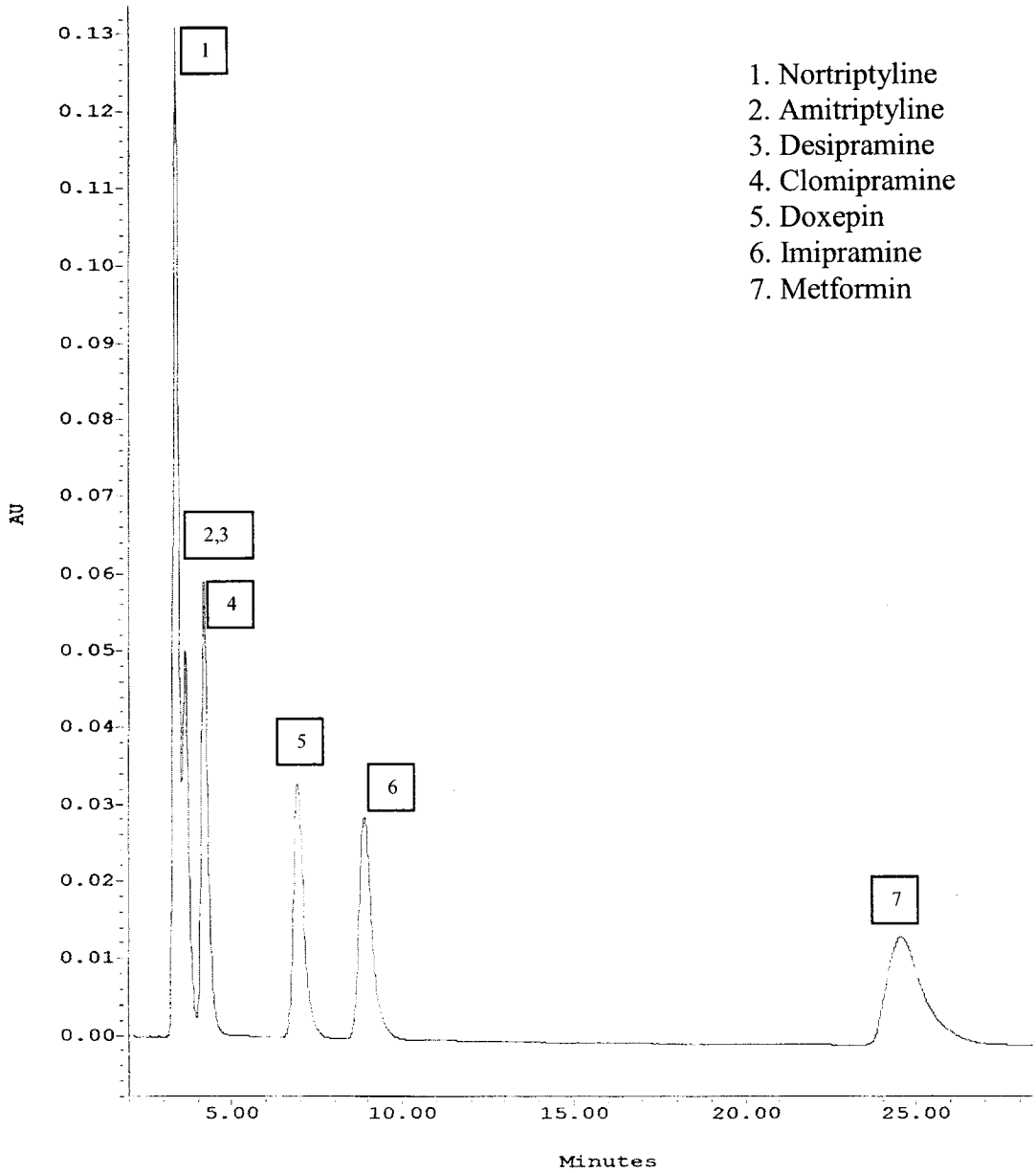


Figure 25: Separation of polar compounds on the 3-amino-3-methyl-1-butyne + trypsin bonded phase. Mobile phase: 45% (20 mM K_2HPO_4/KH_2PO_4 buffer pH 7): 55% (Methanol), UV: @ 254nm.

IV Conclusion

The two main goals of this research were the synthesis of the stationary phases and the characterization of the stationary phases. The spectroscopic characterization of the amino bonded phases and the protein column using DRIFT and solid state NMR confirmed the attachment of the desired organic group. The elemental analysis indicated that the hydrosilation reaction using the free radical catalyst provided better surface coverage of the bonded phase. The sugars showed ANP behavior on all four columns and hence confirmed the polar nature of the bonded phase as determined by the Neue test. The sugars were retained much longer on the 3-amino-3-methyl-1-butyne bonded phase and the protein bonded phase. This behavior was attributed to the presence of the free amino groups in both the bonded phases. Also of the two 4-diethlamino-2-butyn-1-ol columns the one synthesized using a free radical catalyst was able to retain sugars longer. This is due to the better surface coverage of the bonded phase as evidenced by the elemental analysis results.

When metformin and tricylic drugs were analyzed, metformin showed ANP behavior on all four columns. Amitriptyline, doxepin, imipramine and clomipramine showed classic reverse behavior where the retention of the solute decreases as the amount of organic solvent increases. Nortriptyline and desipramine showed both reverse phase and ANP behavior under different mobile phase conditions. The affinity aspect of the trypsin column was confirmed by attaching and eluting trypsin inhibitor under different mobile conditions. Future work can focus on analyzing melamine, a basic compound and hence these columns can find application in the quality control studies of food products.

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