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Development of a semi-preparative C_{30} HPLC column for carotenoids separation

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DEVELOPMENT OF A SEMI-PREPARATIVE C_{30} HPLC COLUMN FOR CAROTENOIDS SEPARATION

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ABSTRACT

DEVEOPMENT OF A SEMI-PREPARATIVE C_{30} HPLC COLUMN FOR CAROTENOIDS SEPARATION

by Paul Lee

Carotenoids are a group of antioxidant compounds that are naturally found in many plants and microorganisms. Due to their antioxidant properties, carotenoids have many pharmaceutical applications. Carotenoids also have industrial and commercial production value as they are often used as nutrient supplements and food colorants.

In this study a semi-preparative C_{30} HPLC column was developed to separate the carotenoids β-carotene, lycopene, and lutein. The development process consisted of synthesizing the HPLC column's stationary phase, characterization of the stationary phase, packing the stationary phase, and evaluating the efficiency of the column.

Characterization of the stationary phase with nuclear magnetic resonance spectroscopy (NMR) and FTIR indicated successful synthesis of the packing material. The semi-preparative column was tested with reverse phase and normal phase runs. Separation of the three compounds was achieved in the normal phase.

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CHAPTER 1
INTRODUCTION

1.1 Carotenoids

Carotenoids are typically red, yellow, or orange color pigments that are widely distributed in nature. Most carotenoids are isomers of 40 carbon chain atoms as shown in Figure 1. It is estimated 100 million tons of carotenoids are naturally produced each year [1]. In many fruits and vegetables such as tomatoes, mangos, and carrots have carotenoids in them. Carotenoids are responsible for bright distinctive color in each plant. The pigments are also present in animals. The reddish-orange color spots on salmon, pink feathers on flamingoes, and yellow yolk in eggs are due to the presence of carotenoids. Currently there have been more than 600 types of carotenoids discovered [2]. Certain plants and photosynthetic microorganisms naturally produce these color pigments. Plants and microorganisms use carotenoids to protect against photooxidative damage by quenching photosensitizers, reacting with singlet oxygen, and neutralizing peroxy radicals [3].

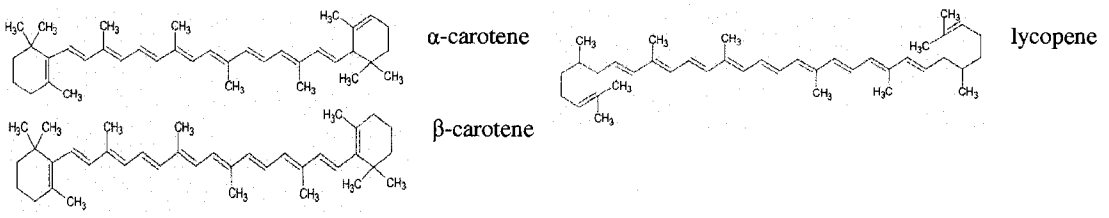


Figure 1. Chemical structures of α-carotene, β-carotene, and lycopene. Adapted from Packer *et al* [4].

From a health benefit perspective carotenoids serve as anti-oxidants, are shown to reduce the risk of cancer, enhance the immune system, and have numerous pharmaceutical applications [5,6]. In many mammals, including humans, 38 different

types of carotenoids have been identified to be precursors to vitamin A, a compound required for good health and vision [7]. Some of the pharmaceutical uses include making supplement pills with carotenoids, treatment of erythropoietic protoporphyria (photosensitivity disorder), and application in clinical studies of cancer and immune response [5].

Carotenoids also have industrial and commercial interest. The carotenoid pigment, β-carotene, is used as a nutrient supplement and food colorant. Astaxanthin, another form of a carotenoid, is added to fish feed for many farmed fish and shellfish [8].

There is a major global market for carotenoids. In 1999 the global market for carotenoids was $786 million with a 2.9% annual average growth rate (AAGR) [9]. With these figures the numbers reached $935 million in 2005 [9]. Animal feed constitutes the majority of the market with an increase of $462 to $527 million from 1999-2005 with a 2.2% AAGR. The pharmaceutical market is 15% of the total sector, and jumped from $115-$173 million within the same six year span with a 7.0% AAR as shown in Table 1. β-carotene and astaxanthin together account for about 56% of all carotenoids sold while canthaxantin is about 19%. All the other types of carotenoids account for the remaining 25% [9].

There have been numerous chemical methods developed to synthesize carotenoids. There are also various separation methods to obtain carotenoids from natural sources, such as plants and microorganisms. Some microorganisms, like certain algae and bacteria, have been genetically modified to produce carotenoids for human use and pharmaceutical studies [6].

Table 1. Global market for carotenoids from 1999-2005 [9].

	1999	2005	AAGR %
Food	209	235	2.0
Animal Feed	462	527	2.2
Pharmaceutical	115	173	7.0
Total	786	935	2.9

The analytical separation and detection of carotenoids, a process called high performance liquid chromatography (HPLC), will be discussed later on. Next, the basics of HPLC will be discussed.

1.2 HPLC

High performance liquid chromatography is an important tool for compound separation and detection. It is often employed in chemical, molecular biology, veterinary, agricultural & food, environmental analysis, and other similar fields. A typical HPLC instrument typically consist of a solvent delivery system containing a pump, a detector unit, injection port (manual or automated), data analysis unit, a column, and a reservoir for solution and waste deposit container as shown in Figure 2.

HPLC evolved from liquid chromatography (LC). Like liquid chromatography, the two main components of HPLC are the stationary phase and the mobile phase. The stationary phase is the solid packing material inside the HPLC column. The mobile phase is a solvent or solvent mixture and passes through the stationary phase. When injected, the compounds that are to be separated move to the stationary phase via the

mobile phase. Once reaching the stationary phase there are two mechanisms for separation of components. The mechanisms are adsorption and partition [10]. As a mixture of components flows through the stationary phase certain components of the mixture will adsorb more strongly to the stationary phase than others due to their different chemical properties. The stronger the adsorption the longer it takes for that particular component to elute from the column and vice versa for weakly adsorbed components as seen in Figure 3 [10]. Thus, absorption strength is the basis for separation. In partition, separation is achieved by distribution of the component mixture between the stationary phase and the mobile phase [10].

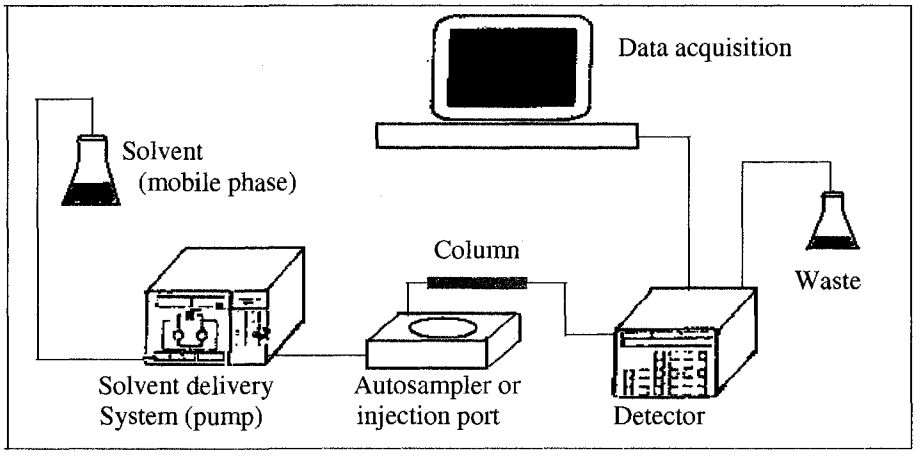


Figure 2. Schematic of a HPLC system. Adapted from Kitipat [11].

HPLC gradually came about in the late 1960s with the discovery of new packing materials that were considerably smaller in particles size compared to LC. It was discovered that reducing the particle size greatly enhanced column separation and efficiency. The diameters of these new packing materials ranged from 3-10 micrometers [12]. The reduction in diameter resulted in a greater resistance to flow requiring the

solvents to be pumped through the column. HPLC is unlike LC in that the mobile phase flows through the LC column due to low pressure by gravity feeding or pumping.

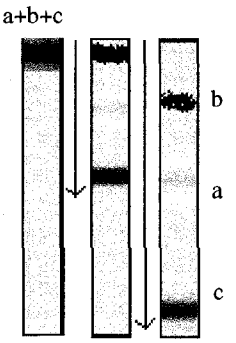


Figure 3. Elution of components A and B through a HPLC column. Adapted from Medtechnica [13].

There are various HPLC column sizes. Microbore HPLC columns are about 2 mm in diameter. The typical dimensions for an analytical HPLC column are 4.6 mm diameter and 10 to 25 cm in length. Larger columns used for semi-preparative HPLC and preparative HPLC are 10 mm and 20 mm in diameter [14]. Column sizes are chosen depending on the purpose of the separation. Analytical columns are used in small scale separations to determine qualitative or quantitative information about a sample. For example, analytical columns can be use to determine the elution profile of a sample or determined the number of components present in a sample. Usually, once the components are analyzed with an analytical column it flows directly to a waste container and no further test or analysis is done with the sample. Larger columns such as semi-preparative and preparative are used for large scale separation to isolate and collect, enrich, or further purify components of interest. Fractions eluting from the semi-preparative or preparative can be collected and further purified with the same column or other similar columns [15].

There are typically two types of solvents used in HPLC. One is reverse phase and the other is normal phase. In reverse phase the mobile phase is polar (such as methanol or water) and the stationary phase is nonpolar (usually an alkyl groups). In normal phase the stationary phase is polar (such as silica or silica modified with a polar organic group) and the mobile phase is nonpolar (such as hexane or benzene) [13].

1.2.1 Scaling Up

Scaling up of a HPLC run is often done to purify compounds of interest in larger quantities. Usually, the scaled-up process is optimized from analytical scale. HPLC analytical runs are usually performed first to establish separation efficiency due to cost effectiveness of operating with smaller columns. This includes determining the best stationary phase, mobile phase, flow rate, temperature for separation, and HPLC parameters with the smaller column. Once these conditions and parameters are established, a larger semi-preparative or preparative column can be used.

The amount of packing material, flow rate, and load sample required for the larger column is determined based on the ratio of the diameters and lengths of the two columns. For example, if the amount of packing material needed for an analytical column is known, the amount required for the larger column can be determined with Equation 1.

$$M_p = M_a (V_p/V_a) \qquad \text{Equation 1}$$

where M_p is the amount of a particular material required to synthesize the stationary phase for the semi-preparative column. M_a is the known amount used when synthesizing the packing material for the analytical column. V_p is the volume of the semi-preparative column. V_a is the volume of the analytical column.

Likewise, the flow rate can be scaled up according to Equation 2 [15].

$$F_p = F_a (r^2_p/r^2_a * L_p/L_a) \qquad \text{Equation 2}$$

where F_p is the flow rate of the semi-preparative column, F_a is the flow rate of the analytical column, r_p is the radius of the semi-preparative column, and r_a is the radius of the analytical column, L_p is the length of the semi-preparative column, and L_a is the length of the analytical column.

Similar to the previous two equations, the load sample can also be scaled according to Equation 3 [15].

$$X_p = X_a (r^2_p/r^2_a * L_p/L_a) \qquad \text{Equation 3}$$

where X_p is the load amount for the semi-preparative column, and X_a is the known load amount for the analytical column.

Analytical columns usually have particle diameters of about 3-5 microns. Semi-preparative columns typically have particle diameters of 7 microns or greater [13].

1.2.2 HPLC Parameters

There are several parameters that quantitatively measure how well a HPLC column separates the components of interest. These parameters will vary based on the dimension of the column, type of column, type of mobile phase or stationary phase used, and HPLC instrument.

The retention time (t_A) is the time it takes for component A to reach the detector once it has been injected into the system. Adjusted retention time (t_A') is a more accurate measurement of retention time, and is found by subtracting the dead volume, t_0, which is

the time it takes an unretained compound to reach the detector. The adjusted retention time is given in Equation 4 [16].

$$t_A' = t_A - t_0 \qquad \text{Equation 4}$$

The capacity factor (k) measures the retention volume of a component specific to a particular stationary and mobile phase. It is an indication of how long it takes component A to elute out of the column relative to an unretained component. The capacity factor is defined according to Equation 5 [17].

$$k = (t_A - t_0)/t_0 \qquad \text{Equation 5}$$

Selectivity or separation factor (α) measures the relative separation of any two components when component A elutes first followed by component B. Separation factor is the ratio of capacity factors for components A and B as defined in Equation 6 [17].

$$\alpha = (t_B - t_0)/(t_A - t_0) \qquad \text{Equation 6}$$

The selectivity factor is always 1 or greater.

Resolution (R) measures the degree of separation between two consecutive eluting components from the column. Resolution is given by Equation 7 [17].

$$R = (t_B - t_A) / (0.5 (W_A + W_B)) \qquad \text{Equation 7}$$

Where t is the retention time and W is the width of the peak at baseline. A resolution value of 1.5 indicates complete separation of two components [17].

When molecules of a particular component travel through the column they disperse and the band broadens during the flow process. Band broadening increases with more time the molecules remain in the system [17]. It is preferable to minimize the broadening process. Less broadening reduces likelihood of peak overlap, thus, the more

efficient the column. Column efficiency is measured in terms of numbers of theoretical plates (N) or as height equivalent to theoretical plate (H). Numbers of theoretical plates and height equivalent to theoretical plate are defined in Equations 8 and 9 [17].

$$H = L/N \qquad \text{Equation 8}$$

$$N = 16\,(t/W)^2 \qquad \text{Equation 9}$$

where L is length of column, t is the retention time, and W is peak width at baseline.

1.3 Silica

The most commonly used packing material for a HPLC column is silica gel. Silica gel is often used because it has a high surface area, good mechanical strength, and is cost effective. Another important property of silica is that it is available in different particle sizes and shapes [14]. The matrix of a silica particle consists of silicon atoms bonded together through oxygen linkages. On the surface of a silica particle there are several active sites that can potentially be used to modify the surface of silica for better separation properties. Silica gel surfaces can have free silanol groups on which OH groups are attached to Si atoms on the surface. Geminol is the term used when there are two OH groups attached to one Si atom on the surface. Another active group is called a siloxane linkage. This is when an oxygen atom is bonded to two Si atoms on the surface. Bound water is the term used to describe the attachment of a water molecule in which hydrogens on adjacent free silanol forms hydrogen bonds with a water molecule [18].

1.3.1 Modification of Silica

Silica in its native form is typically not used as a stationary phase because it does not separate analytes well. To achieve better separation, efficiency, and reduce the

polarity of the surface, there have been attempts to chemically attach different organic moieties to the silica surface. This is generally done with attachment to the free silanols. The surface can be modified depending on the components to be separated. The first and most basic method is called esterification. The reaction involves a silanol group reacting with an alcohol to form a Si-O-C bond [19]. This primitive method is not used often since the linkage is unstable in aqueous conditions. Another method of attachment is an organosilanization reaction. This reaction occurs between an organosilane reagent and the silanols forming Si-O-Si-C linkages [18]. Another method involves chlorination of the silanols followed by a Grignard reagent (BrMgR) or organolithium (LiR) reaction to form Si-C linkages. This method is not preferred since it requires extremely dry conditions.

A more recent and favorable method was developed for modifying the silica surface. The process is a two-step method termed silanization followed by hydrosilation. In silanization the reaction involves silanols with a reagent like triethoxysilane (TES) to form a Si-H monolayer (silica hydride). Silanization, the second reaction, involves the reaction of silica hydride with Speier's catalyst, hexachloroplatinic acid, and the organic moiety of interest to form a monolayer of Si-C [19]. This method has many advantages over the others. The linkage formed is more stable since acid is not formed during the reaction. In the first step the silica hydride monolayer is not acidic unlike the organosilanization method. This method also removes more of the silanols group than organosilanization making the bonded phase less polar which is advantageous when separating certain molecules. A summary of these reactions is shown in Figure 4.

Type of Reaction	Chemical Reaction	Surface Linkage (Bonded Phase)
Esterification	Si-OH + R-OH → Si-OR + H_2O	Si-O-C
Organosilanization	A) Si-OH + X-SiR'$_2$R → Si-O-SiR'$_2$R +HX B) Si-OH + X_3-Si-R → Si-O-Si(OY)-R + 3 HX Y=Si or H	Si-O-Si-C
Chlorination followed by reaction of Grinard reagents and organolithium compounds	Si-OH+$SOCl_2$ $\xrightarrow[\text{reflux}]{\text{Toluene}}$ Si-Cl + SO_2+HCl A) Si-Cl + BrMg-R → Si-R + MgClBr or B) Si-Cl + Li-R → Si-R + LiCl	Si-C
A) TES Silanization B) Hydrosilation	A) Si-OH + TES(-O-Si-H(-O)-O) $\xrightarrow[\text{catalyst}]{}$ Si-O-Si-H B) Si-H + CH_2=CH-R → Si-CH_2-CH_2-R	A) Si-H (monolayer) B) Si-C

Figure 4. Various reaction types. Adapted from Nguyen [19].

1.4 Surface Characterization Technique

Various characterization techniques are used to evaluate native silica, silica hydride, and the bonded phase. These techniques determine whether or not the organic moiety of interest was successfully bonded onto the silica hydride surface and to what degree the bonding occurred. Four types of characterization methods will be discussed next.

1.4.1 Diffuse Reflectance Fourier Transform Infared Spectroscopy (DRIFT)

Diffuse Reflectance Fourier Transform Infrared Spectroscopy (DRIFT) is a widely used spectroscopic method. DRIFT detects stretching and bending vibrations of

functional groups present in a compound when an IR beam is applied [11]. Functional groups such as C-C,C=C, O-H, N-H, C-O,C=O,C-N, and C-H will absorb a distinctive frequency of IR radiation [20]. For example, free silanols on the silica surface absorb at 3700 cm^{-1}, Si-H at 2260 cm^{-1}, C-H at 3000-2800 cm^{-1}, and water at 4000-3000 cm^{-1}[20]. By comparing FTIR spectra of native silica, silica hydride, and the bonded phase compound the success of bonding can be qualitatively determined.

1.4.2 Cross-Polarization, Magic Angle Spinning NMR (CP-MAS NMR)

Solid-state NMR, also known as cross-polarization magic angle spinning NMR (CP-MAS NMR), is another common technique for characterization. When conventional NMR is used on solid compounds the problem of broad spectra lines and low signal intensity is common. Broad spectra lines are due to chemical shift anisotropy (CSA) and low signal intensity is due to dipolar coupling [11].

CSA is orientation-dependent and occurs when the motions of atoms in solids are restricted. The lack of motion results in an electronic shielding effect within a solid molecule. Magic angle spinning (MAS) overcomes the CSA problem by spinning the sample rapidly (2 kHz or greater) at 57.4 degrees with respect to the applied magnetic field [11, 19].

Dipolar coupling between protons and carbons in the solid sample contribute to low signal intensity. The cross-polarization (CP) method overcomes the problem of low signal intensity by decoupling of protons. Cross-polarization enhances the ^{13}C signal by excitation of the ^{1}H spins and then transferring the signal to the ^{13}C spin system [11]. As a result, CP-MAS^{1}H NMR or CP-MAS^{13}C NMR is able to achieve high-resolution for

solid compounds. This is an advantage CP-MAS ^{13}C NMR has over solution NMR for solid compound analysis.

1.4.3 Elemental Analysis

Elemental analysis is often employed to determine the carbon content of the bonded phase. The results obtained from elemental analysis can be used to determine the surface coverage (α_R) of the bonded organic moiety using the equation proposed by Berendsen and DeGalan as show in Equation 10 [21]:

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

In Equation 10 α_R is the surface coverage of bonded organic moiety, P_c is the percentage of carbon by weight of bonded phase, M_c is the atomic weight of carbon, n_c is the number of carbons in the bonded group, M_R is the molecular weight of the bonded organic moiety weight, and S_{BET} is the specific area (m^2/g). There are roughly about 8 $\mu mol/m^2$ of silanol groups available for bonding on the silica surface [22]. Therefore, the surface coverage can not exceed this amount.

1.5 Environmental and Safety Concerns

When working with HPLC or semi-preparative HPLC environmental and safety concerns need to be taken into consideration. Environmental concerns include the possibility of ozone destruction and solvents deposited onto landfills. There are also laboratory safety concerns such as dealing with solvent vapor and proper handling of solvents. Safety and environmental issues related to HPLC will be briefly discussed next.

Running a HPLC instrument is relatively safe. However, safety protocols should still be followed in order to reduce the chance of injuries to the experimenter or

bystanders. Safety items and equipment including eyes wash sink, safety showers, first aid kit, fire blanket, fire extinguishers, and emergency flashlights should be in place prior to any lab work. All the items should be stocked and the equipment should be in proper working order. Safety goggles and latex gloves must be worn at all times when handling chemicals. This is important since HPLC runs and stationary phase synthesis involves the use of many types of solvents and chemicals.

Acetonitrile, a common solvent, is often used as a mobile phase during HPLC runs. This solvent can be absorbed through oral, dermal, and vapor inhalation. Once inside the body acetonitrile can metabolized into hydrogen cyanide and thiocyanate both of which are highly toxic [23]. Another chemical that poses a serious health risk is triethoxysilane (TES). TES can irritate the skin, eyes, nose, throat, and can even cause blindness with long term exporsure. Aside from the health concerns TES is highly flammable and should only be handled inside a well ventilated hood [24].

Solvents that are used as mobile phases are often degassed with a stream of helium. The degassing process accelerates the vaporization of solvents, especially the ones used in normal phase. Besides being harmful to the human body the vapors are flammable. Normal phase solvents that are used during HPLC runs should be placed under a ventilated hood for vapor removal.

In addition to the adverse health risks due to exposure and laboratory safety concerns, such as explosions, there are also harmful environmental impacts from improper solvent disposal. When released as a vapor into the atmosphere some HPLC solvents are relatively safe. The vapors are diluted with air. However, most halogenated

solvents such as methylene chloride are hazardous since they are known to cause ozone depletion. As the vapor of chlorinated solvents are dispersed into the stratosphere where the compounds are broken down and chlorine radicals are formed. The radicals destroy the ozone layer by reacting with ozone (O_3) forming ClO and O_2 [25].

The depleting of stratospheric ozone also has a secondary effect. As the amount of ozone becomes less there is an increase in UV radiation. At ground level the radiation increases the photo reaction that generates smog [25]. When volatile organic solvents (VOCs), commonly defined as solvents containing more than one carbon atom, are emitted the solvents reacts with nitrogen oxide. In the presence of sunlight the reaction produces smog [25].

Solvents can also have detrimental effects if deposited on landfills. The solvents can leach into ground water. In addition, storm water discharge can carry the solvents into rivers or oceans harming land and aquatic life. Studies have shown that exposure to methanol can kill birds or fish and reduce the growth rate in plants [26].

Due to the potential impact on the environment chemicals and solvents should be disposed of in designated waste bottles. The type of discarded solvents or chemicals should be labeled on the bottle. Chemicals or chemicals that are highly toxic should be discarded in its own separate bottle. The waste bottles should be properly disposed or treated at RCRA approved site.

CHAPTER 2
LITERATURE REVIEW

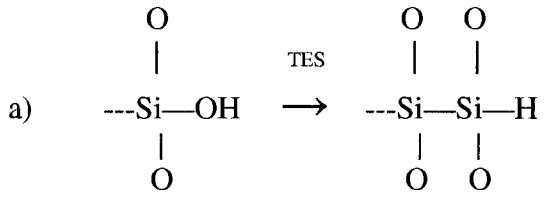
As mentioned previously, traditionally, a method of silica modification was chlorination of the silanols followed by a Grignard reagent (BrMgR) or organolithium (LiR) reaction to form a Si-C linkage as discussed by Sandoval *et al* [22]. However, there are drawbacks to this type of reaction. The procedure is usually time and labor-intensive. Furthermore, the reaction requires an extremely dry condition [22]. The presence of moisture can cause the Si-Cl bonds to hydrolyze back to S-OH [27].

2.1 Silanization/Hydrosilation Method of Modification

Chu *et al* studied a more efficient method for attaching an organic group to a silica surface [27]. The process is a two-step procedure termed silanization followed by hydrosilation as shown in Figure 5. In silanization the reaction involves surface silanols with a reagent like triethoxysilane (TES) to form a Si-H monolayer (silica hydride) as explained by Chu *et al.* Hydrosilation is the term used to describe the second reaction involving silica hydride for attaching the organic group of interest to form a monolayer of Si-C, the bonded phase [18]. The second step can be initiated with a free radical reaction or hexachloroplatinic acid which serves as a catalyst [27].

Chu *et al* discovered advantages to this method. The process is simpler, more efficient, and more economical compared to the traditional chlorination/reduction method. The time it takes to synthesize silica hydride is significantly reduced from 3-4 days to about 4 hours [27]. The need for an extremely dry condition to produce silica hydride is eliminated. More importantly, Chu *et al* noted that the silica hydride coverage is 4 times greater with silanization/hydrosilation than the traditional

chlorination/reduction procedure [27]. The greater silica hydride coverage allows for more organic moiety attachment.



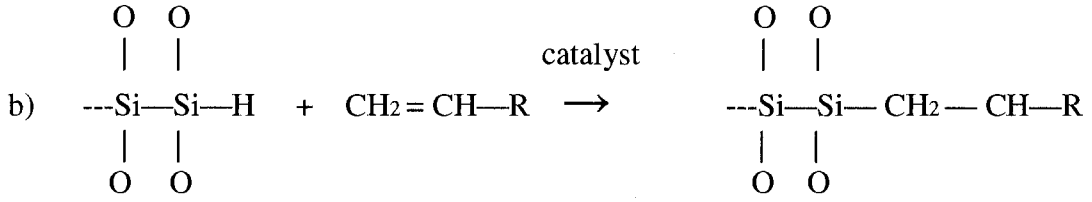


Figure 5. Silanization follow by hydrosilation [18].

Diffuse infrared Fourier transform (DRIFT) and solid state NMR (CP-MAS^{13}C NMR) were used to prove that a greater amount of Si-H bonds was produced with the silanization/hydrosilation method of modification compared to the chlorination/reduction method. Partisil-40 and Vydac TP were the two types of silicas were used in the DRIFT analysis as shown in Figure 6. Chu et al noted in the TES silanization reaction (graph C) Si-H peaks were also observed with less percent transmittance as compared with the chlorination/reduction (graph B) procedure which indicates more silica hydride bond formation with the silanization/hydrosilation method. For comparison, in Figure 6 spectrum A is unmodified silica which is expected to have no Si-H bonds.

CP-MAS^{13}C NMR was also used to verify an increase in Si-H coverage as shown in Figure 7. Native silica samples, samples obtained with the chlorination/reduction

procedure, and samples from the silanization method were all analyzed with CP-MAS^{13}C NMR. The study showed that the peaks at -84 ppm corresponding to Si-H was greatest in the silanization method.

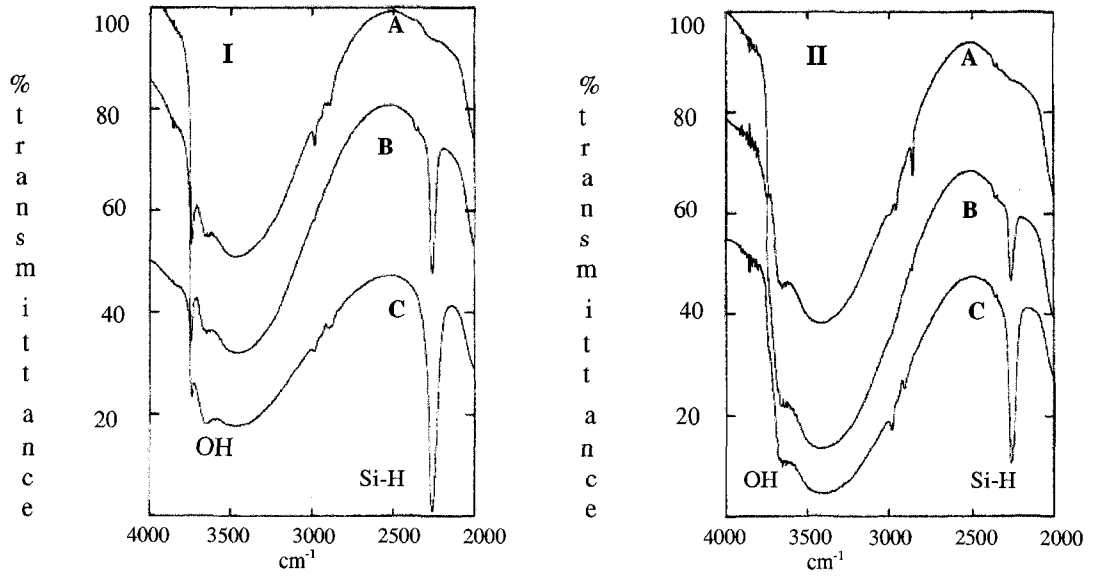


Figure 6. DRIFT analysis for modification of silica into silica-hydride: I) Partisil-40; II) Vydac TP. A) silica (unmodified); B) chlorination/reduction; C) TES silanization [27].

The results of FTIR and CP-MAS^{13}C NMR illustrated that the silanization/hydrosilation method is superior to the chlorination/reduction since there are more Si-H bonds produced with the new method.

2.2 Silanization/Hydrosilation Method for Attaching C_{22} and C_{30} Alkyl Groups

An experiment was conducted by Pesek *et al* in which the silanization and hydrosilation procedures were used to attach the organic moieties (C_{22}) and triacontyl (C_{30}) to Vydac TP 106 silica for various separations including alpha and β-carotene [28]. Similarly, DRIFT and CP-MAS^{13}C NMR were also used for characterization analysis. The DRIFT spectrum in Figure 8 has several peaks within the 2750-3000 cm^{-1} range

corresponding to C-H stretching. This confirms bonding of the alky groups to silica hydride. The peak at 2250 cm^{-1} is due to stretching of Si-H bonds, and is present since not all alkyl groups will react with silica hydride. In a similar experiment Olivia noted only about 20 % of all the Si-H bonds react with alkyl groups [29].

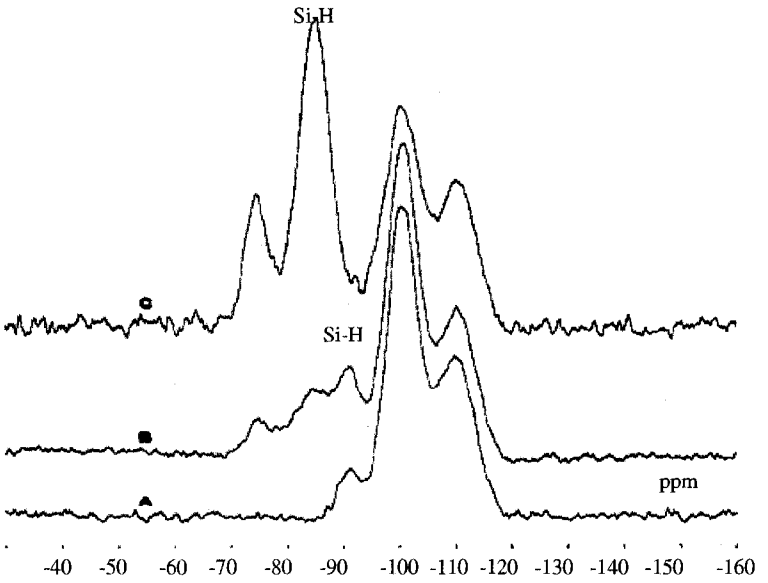


Figure 7. CP-MAS^{13}C NMR graph of mofified Partisil-40: A) Native silica; B) chlorination; C) TES silanization [27].

In addition, CP-MAS^{13}C NMR was used to confirm the bonding of the alky group to silica hydride. The highly visible peak at 30 ppm is due to the methylene groups with trans/gauch configuration [28]. The smaller peak at 32 ppm corresponds to methylene groups with all trans configuration as show in Figure 9.

Both columns (C_{22} and C_{30}) were able to resolve aromatic hydrocarbons including a mixture of benzene, toluene, ethylbenzene, isopropylbenzene, t-butylbenzene, and anthracene. The C_{30} column had a slightly longer retention time [28]. Vitamin mixtures including delta-tocopherol, gamma-tocopherol, and alpha-tocopherol were also tested.

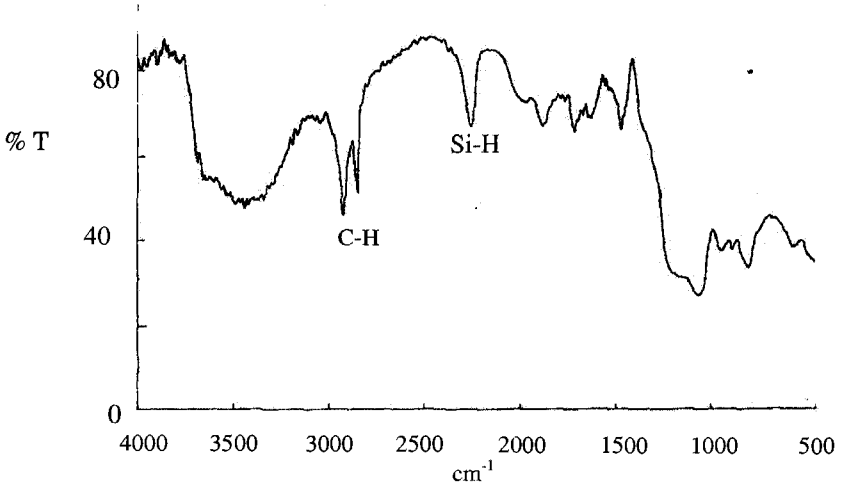


Figure 8. DRIFT graph for C_{22} bonded to silica by hydrosilation using hexachloroplatinic acid [28].

Both columns successfully resolved the three compounds with the C_{30} column having slightly better separation [28]. However, there were compounds in which there were measurable differences in the separation between the two columns, such as alpha and β-carotene. It was noted that the separation was satisfactory with the C_{30} column but the C_{22} column separation ability was not as good. The difference in performance may be attributed to the lower surface coverage on the C_{22} column [28]. Figure 10 shows a chromatogram with the C_{30} column for a mixture of alpha and β-carotene.

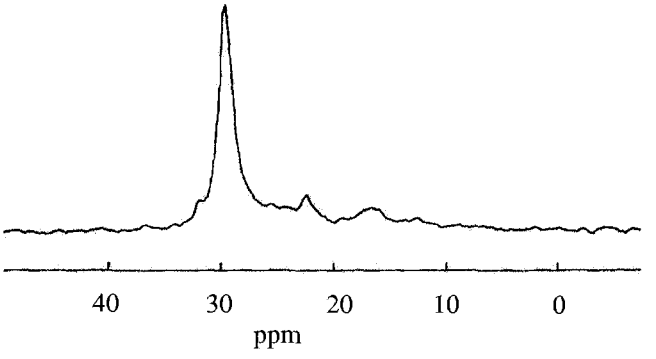


Figure 9. CP-MAS^{13}C NMR spectrum of C_{22} bonded to silica hydride [28].

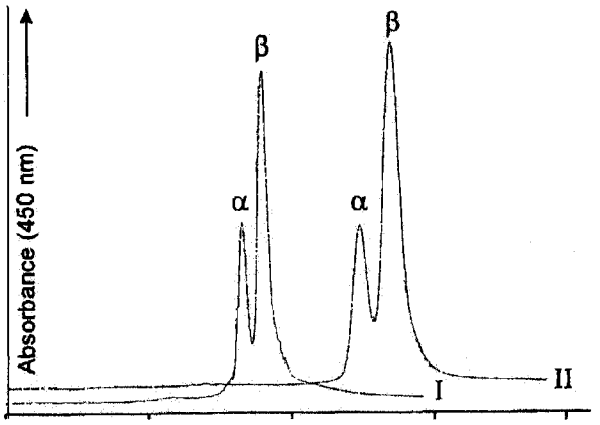


Figure 10. Separation of alpha and β-carotene with C_{30} column: I) Mobile phase = 90:10 acetonitrile/water; II) Mobile phase = 93:07 methanol/water [28].

2.3 Semi-Preparative Separation of Lycopene

Hakala and Heinonen conducted a study of the separation of lycopene from tomato puree using a three-step separation process [3]. In the first step they extracted carotenoids with petroleum ether (PE). Next, solid phase extraction with a silica cartridge packing was used to purify the extracted portion from the previous step. A distinctive red band corresponding to lycopene was collected and subsequently used for the next step. Next, the lycopene was further purified using semi-peparative HPLC with a Bondapak C_{18} column. Analytical HPLC was used to check the purity and recovery during every step.

After the extraction with PE, but prior to the silica cartridge step, the chromatogram displayed peaks for xanthophylls, lycopene, and β-carotene as shown in Figure 11a. This is expected since no purification steps were taken. After the third fractionation with the preparative HPLC column the xanthophylls and β-carotene were no longer present. Thus, the semi-peparative column was successful in separating the three

compounds. However, it was noted that there was an unknown peak eluting just before the lycopene peak and a cis isomer of lycopene peak eluting as a shoulder of the lycopene peak as shown in Figure 11b. It was suggested that a study should be done with normal phase [$Ca(OH)_2$-stationary phase] semi-preparative HPLC to separate the cis-lycopene and the unknown.

2.4 Effect of Endcapping and Bonded Group Length on Separation Efficiency

A study was done by Sander and associates into the development of a highly selective HPLC column [30]. Their aim was to engineer a column with high selectivity for polar and nonpolar carotenoids compared to what was commercially available at the time. In addition, endcapping and length of bonded groups were tested for enhancing selectivity of columns.

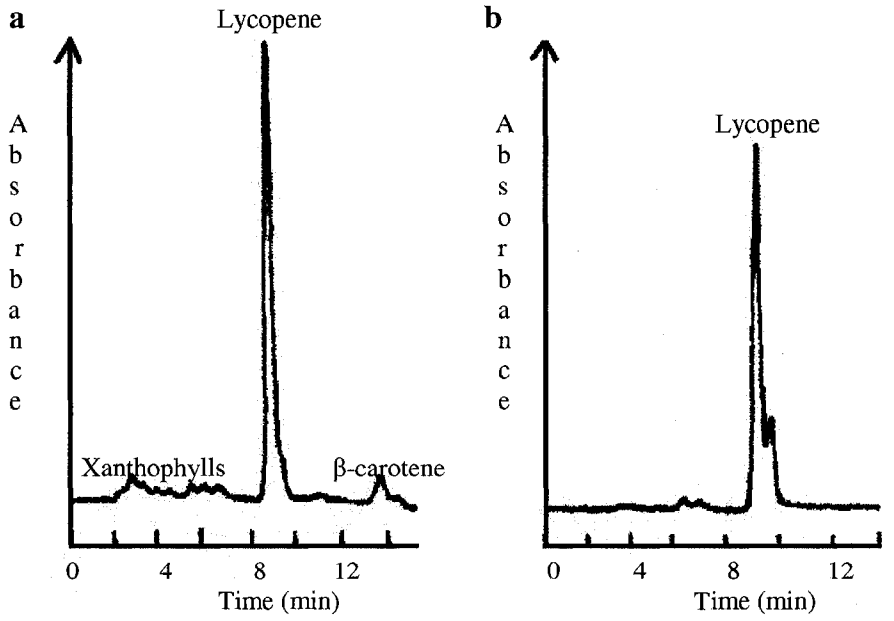


Figure 11. Chromatograms of lycopene after: a) Extraction with PE; b) Third fractionation with using a C_{18} semi-preparative HPLC. Adapted from Heinonen *et al* [3].

In the encapping study one polymeric C_{18} column was endcapped with trimethylchlorosilane (TMS) and another polymeric C_{18} column was endcapped with hexamethyldisilazane (HMDS). For comparison, a third polymeric C_{18} column was not endcapped. Comparisons of the three column types are shown in Figure 12a, b, and C. Figure 12a shows three chromatograms of the non-endcapped C_{18} column. Figure 12b shows three chromatograms of the TMS endcapped C_{18} column and Figure 12c shows three chromatograms of the HMDS endcapped C_{18} column. It was discovered that endcapping had no influence on nonpolar carotenoids, as shown in column 3 of Figures 12a, b and c as where there are three distinct peaks for α-carotene, β-carotene, and lycopene in each of the three chromatograms. However, for polar carotenoids, endcapping did not increase separation efficiency as seen in Figure 12b and c column 2. There are two distinct peaks in column 2 of Figure 12a corresponding to lutein and zeaxanthin for the non-endcapped C_{18} column indicating complete separation. However, for the encapped C_{18} columns shown in column 2 of Figure 12b and c the two peaks are closer together, an indication that separation was not as good.

It was concluded that silanols on the particle surface had minimal interaction with nonpolar carotenoids. As a result, regardless of whether or not there is encapping, the silanol groups would not have much influence on the separation of nonpolar carotenoids. In contrast, polar carotenoids such as lutein contain an OH group. Since the free silanols on the particle surface are also polar there will be some attractive forces. Therefore, endcapping would reduce these forces and greatly decrease separation for polar carotenoids [30].

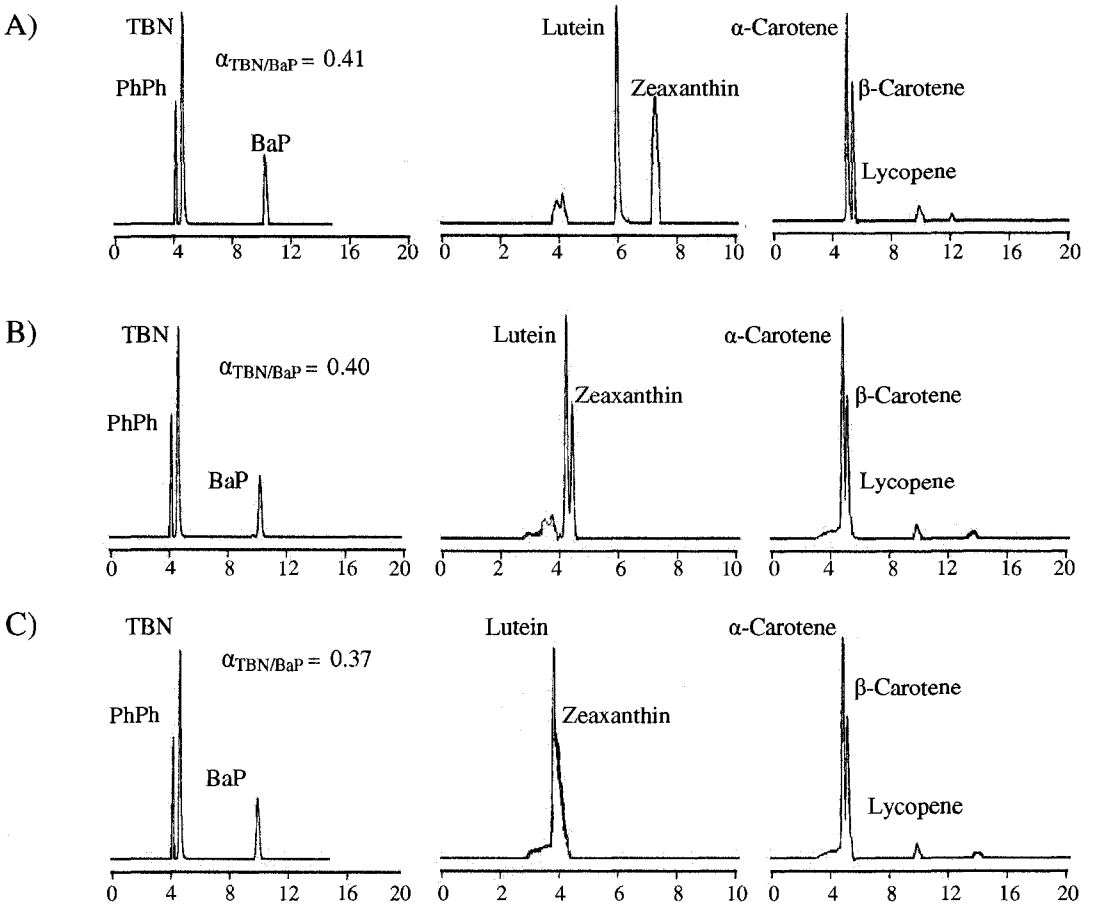


Figure 12. Comparison of three column types: A) polymeric C_{18} column, non-endcapped; B) polymeric C_{18} column, endcapped with TMDS; C) polymeric C_{18} column, endcapped with HMDS. Adapted from Sander et al [30].

In addition to endcapping Sander *et al* studied how separation was influenced by the length of the substrate attached to the silica particle [30]. The types of columns used were a monomeric C_{18}, a polymeric C_{18}, and an engineered polymeric C_{30}. It was observed that most polar carotenoids were separated in both monomeric and polymeric C_{18} columns with the exception of lutein and zeaxanthin. The C_{30} column was more efficient in separating all the polar carotenoids. Differences in performance were more apparent with nonpolar carotenoids. Many nonpolar carotenoids had overlapping peaks

or peaks very close together as show in Figure 13 with the C_{18} monomeric column [30]. In contrast, the polymeric C_{18} column was only able to partially separate the nonpolar carotenoids. The C_{30} column had well separated peaks for both polar and nonpolar carotenoids. From these observations it appears that longer bonded alkyl chains produce better separation for carotenoids.

Separation of carotenoids with analytical HPLC can be achieved as demonstrated in these studies. The general findings from these two investigations are that longer bonded alky groups on silica, and greater surface coverage of the alkyl groups result in better separation of carotenoids.

Most of the previous reviews focused on analytical HPLC development. Equally as important is the development of a scaled-up HPLC process from analytical to semi-preparative or preparative. The next two reviews focus on the scaled-up process of HPLC from analytical to semi-preparative.

2.5 Scale-up Separation of Caffeine, Theophylline, Dicloxacillan, Cloxacillan and Oxacillan

Majors conducted a study into the development of scaling up from analytical to preparative HPLC for separating caffeine and theophylline using reverse phase HPLC with a C_{18} stationary phase [31]. The solvent used was water:acetonitrile (90:10). The same type of packing material was used for both columns each having 5 micron diameter particles. The method development process started at the analytical scale. Once the best conditions for separation were determined on the analytical column it was scaled to preparative size. For the larger column load volume and flow rate were increased with

formulas equivalent to Equations 2 and 3. The two components were separated with both columns as demonstrated in Figure 14. First the column was overloaded to determine the effect on the retention time. It was noted that as the mass of injection increased the detection band broadens and there was a slight decrease in retention time with the analytical column [31]. In the analytical column there are two peaks corresponding to the overloading of caffeine and theophylline as shown in Figure 14A.

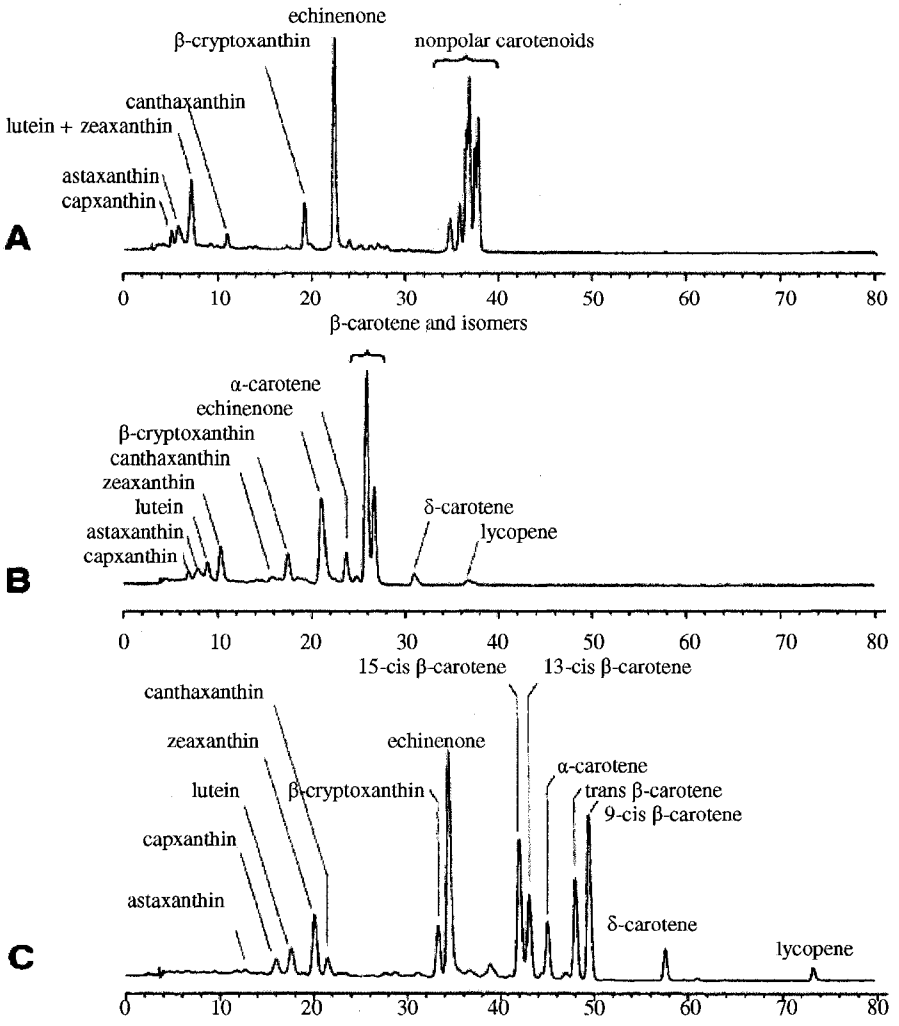


Figure 13. Three chromatograms displaying the effects of organic moiety length on separation: A) Commercial C_{18} monomeric column; B) commercial polymeric column; C) engineered polymeric C_{30} column. Adapted from Sander *et al* [30].

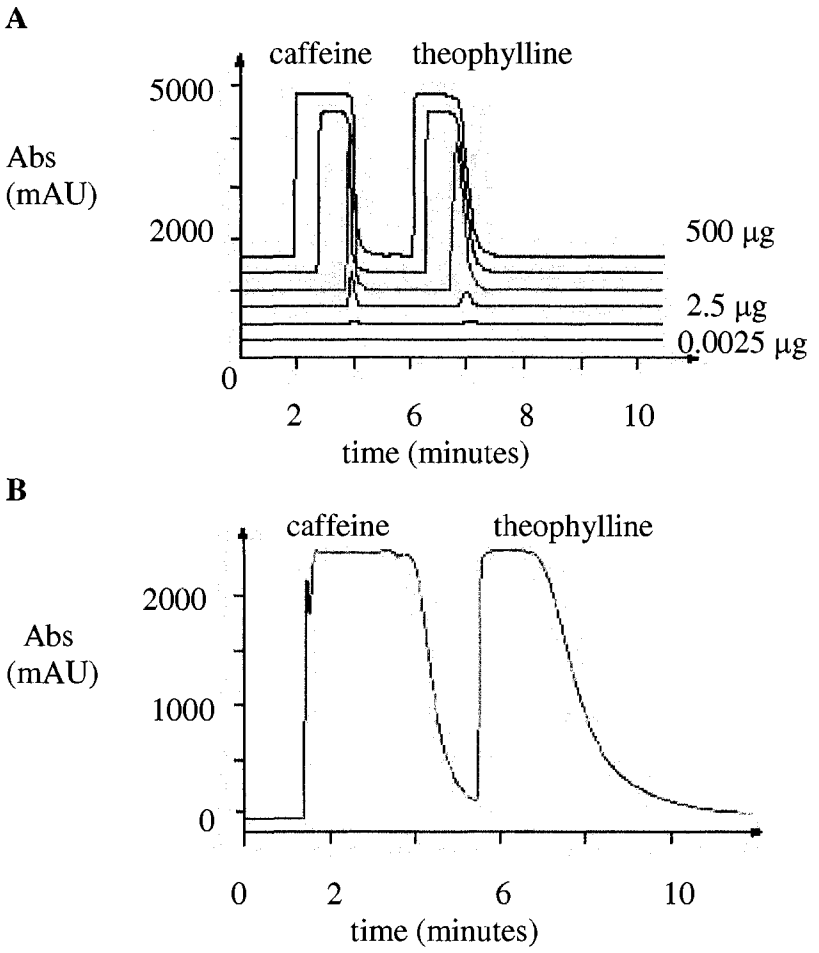


Figure 14. Scaled-up of reverse phase separation of caffeine and theophylline: A) Analytical separation; B) Preparative separation resulting from scale-up calculation. Adapted from Majors *et al* [31].

In a similar study Majors and Long also conducted an experiment into the scale-up development of three antibiotics [32]. The antibiotics are dicloxacillan, cloxacillan, and oxacillan. The process was also done in reverse phase with a C_{18} column. A mobile phase consisting of water:acetonitrile (65:35) was used. Similarly, the preparative method was determined first on the analytical scale. Both the analytical and the preparative columns had the same packing material. It was noted that separation was

achieved for both the analytical and preparative column. The chemical structures of the three antibiotics and chromatograms from the analytical columns are show in Figure 15.

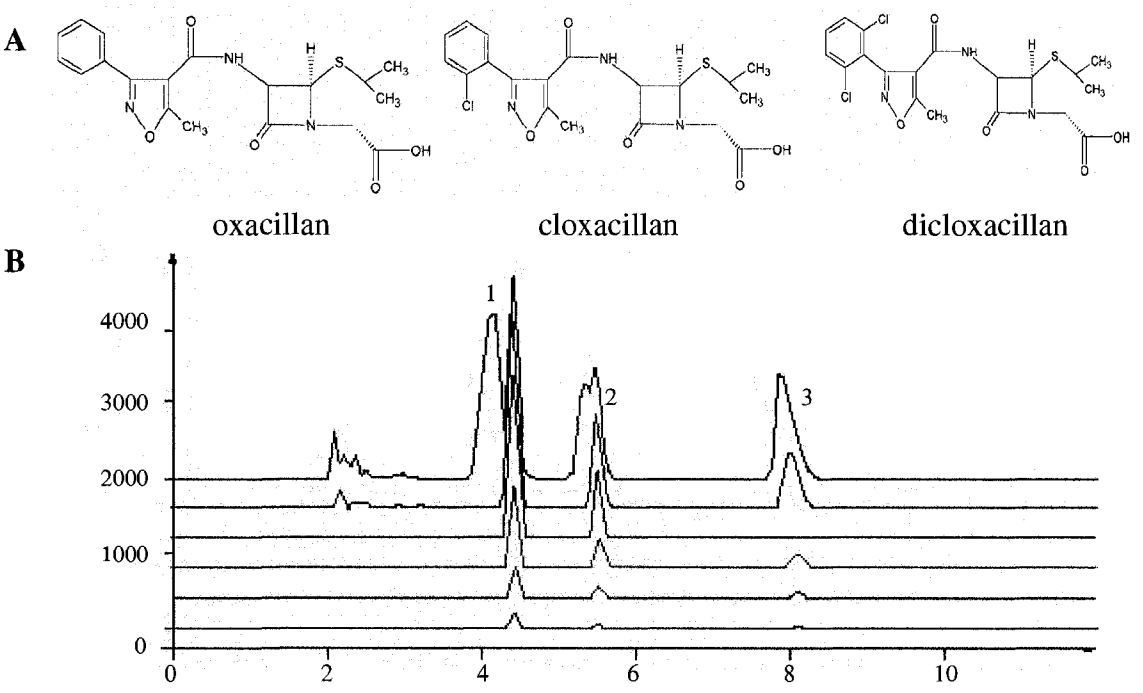


Figure 15. Scaled-up separation of three types of antibiotics: A) Structure of antibiotics; B) Separation on the analytical column. Adapted from Majors *et al* [32].

There has been considerable progresses over the years in the area of carotenoids separation with HPLC. Chu *et al* utilized the silanization/hydrosilation method of silica modification [27]. The technique improved the efficiency of HPLC columns by the presence of silica hydride bonds, increasing the surface coverage, decreasing silanols on the particle surface, and reducing the overall synthesis time.

Pesek conducted a study in which 1-dococene (C_{22}) and triacontyl (C_{30}) were attached to Vydac TP 106 silica using the silanization/hydrosilation procedure [28]. Various compounds were tested with each column. Both columns separated various compounds with the C_{30} performing only slightly better. However, the discrepancy

between the two was evident in the separation of alpha and β-carotene. There was better separation with the C_{30} column.

An experiment by Hakala and Heinonen was conducted into the development of a process to separate lycopene from tomato puree [3]. LC and a Bondapak C_{18} semi-peparative HPLC column were used to separate lycopene. It was discovered that the C_{18} column was not able to separate the cis-lycopene and an unknown carotenoid. The authors concluded by suggesting a normal phase [$Ca(OH)_2$-stationary phase] semi-preparative HPLC column should be tested to check for the separation of the cis-lycopene and the unknown compound.

Sander *et al* studied the effect of endcapping and substrate length on column efficiency [30]. It was determined endcapping did not influence the separation of nonpolar compounds. For polar carotenoids, endcapping reduce separation efficiency. Sander concluded the increase in substrate length from C_{18} to C_{30} gave better separation for certain compounds such as carotenoids.

Scale-up development from analytical to preparative HPLC was studied by Majors and Long *et al* [31,32]. Separation of caffeine, theophylline, and three antibiotics were successfully separated on preparative C_{18} columns using an analytical HPLC to first determine the best conditions for separation.

CHAPTER 3
EXPERIMENTAL

3.1 Research Objectives

The objective of this study was to develop a scaled-up separation process for a triacontyl (C_{30}) semi-preparative HPLC column for the separation of the carotenoid compounds lycopene, β-carotene, and lutein. The development process included synthesizing the packing material of the semi-preparative column, characterization of the packing material, and evaluating the semi-preparative column for separation efficiency. The best conditions for separation were first to be determined with a smaller analytical column. The same conditions would then be use in scaling to the semi-preparative column.

3.2 Analytical HPLC Runs

As stated earlier, the first part of the experiment was to establish the best conditions for carotenoid separation using a smaller analytical C_{30} column before scaling up to the semi-preparative column. The carotenoids to be separated were β-carotene, lycopene, and lutein. The structures of the three compounds along with α-carotene and astaxanthin are shown in Table 2. The pure carotenoid standards of β-carotene and lycopene were purchased from Sigma Aldrich (St. Louis, MO). Lutein, in the form of dietary pills, was purchased from a local nutritional supplement retailer. Several HPLC runs were initially conducted using the analytical C_{30} column. The column was tested for separation efficiency in both the revere phase and normal phase. The reverse phase instrument consisted of a Waters 515 HPLC pump, a Waters 991 photodiode array detector, and a Waters 717 plus autosampler. The normal phase instrument consisted of a

Hitachi L-6200 intelligent pump, a Hitachi L-400 UV detector, a Hewlett Packard 35900 interface, and manual injection port. In reverse phase, isocratic mobile phases were tested. Solvents that are polar were used in reverse phase. For normal phase, both isocratic and gradient mobile phases were tested for separation efficiency. Nonpolar solvents were used in normal phase. The choices of available solvents are shown in Table 3 listed in increasing polarity order.

Table 2. Chemical structures of α-carotene, β-carotene, lycopene, astaxanthin, and lutein.

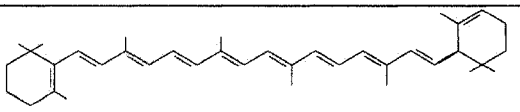
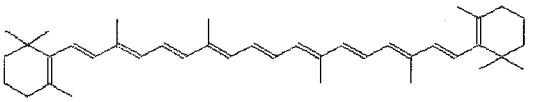
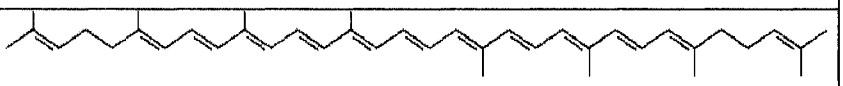
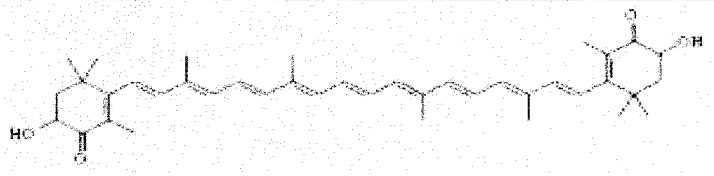
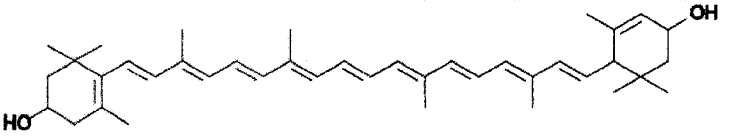
Name	Chemical Structure
α-carotene	
β-carotene	
Lycopene	
Astaxanthin	
Lutein	

Table 3. Polarity index of various solvents.

Chemical name	Polarity index	Chemical name	Polarity Index
n-pentane	0.0	tetrahydrofuran	4.2
n-hexane	0.0	ethyl acetate	4.3
pentane	0.23	methyl isobutyl ketone	4.5
octane	0.4	dioxane	4.8
carbon tetrachloride	1.6	ethanol	5.2
xylene	2.5	pyridine	5.3
toluene	2.3	acetone	5.4
di-ethyl ether	2.8	acetic acid	6.2
benzene	3.0	acentonitrile	6.2
methyl chloride	3.4	dimethyl sulfoxide	6.5
chloroform	3.4-4.4	methanol	6.6
ethylene dichloride	3.7	water	9.0
n-propanol	4.0		

In reverse phase, solvents with high polarity were used. Several solvents including acetonitrile and methanol were mixed with highly purified water for isocratic runs. A Milli-Q apparatus (Millipore, Bedford, MA, USA) was used for all water purification. The ratio of solvent to water was initially kept high and then decreased in an attempt to obtain the best separation and retention time of the compounds. For normal phase, including both isocratic and gradients runs, solvents that are less polar relative to reverse phase were evaluated. These solvents included hexane, methylene chloride, 1-butanol, acetonitrile, and methanol.

3.3 Synthesis of Silica Hydride Via Silanization

Prior to synthesis, 9.65 grams of Vydac TP silca (7.6 μm diameter, 280.4 m^2/g surface area, and 0.54 cc/g pore volume) was dried in a vacuum oven overnight. All glassware used was likewise thoroughly washed and dried overnight in an oven. The

9.65 grams of silica and a magnetic stirrer were placed in a 3-necked round bottom (RB) flask. The RB is equipped with a condenser, a drying tube, a thermometer, and an additional funnel. The entire apparatus was placed on top of a heating mantle and magnetic stirrer plate. Next, 343 mL of dioxane was added to the RB followed by 4.2 mL of 2.3 M HCl. The mixture was heated to approximately 70°C. After the temperature became stable at 70°C, TES solution (10 mL of TES and 51.5 mL of dioxane) was added drop wise using the additional funnel. After all the TES solution was added the mixture was refluxed for 90 minutes at 90°C. After the cooling period, the product was transferred to a filter crucible attached to a vacuum. The silica hydride was washed two times with dioxane, three times with toluene, and three times with diethyl ether. Then, the silica hydride was dried overnight at room temperature to remove the ether. The next day the product was placed overnight in a vacuum oven at 120°C.

3.4 Synthesis of Bonded Phase Via Hydrosilation

Prior to hydrosilation, 9.65 grams of the silica hydride was stored in the vacuum oven overnight at 120°C. All glassware was washed and dried in the oven overnight. A 250 mL 3-necked round bottom flask (RB) was fitted with a thermometer in one neck, a condenser equipped with a drying tube in the second neck, and a glass stopper in the third neck. A magnetic stirrer was placed inside the RB. The entire apparatus was put on a heating mantle and stirrer plate. Next, 126 mL of toluene was added to the RB flask followed by 31.7 grams of the triacontyl compound and 1 mL of 10 mM hexachloroplatinic acid. The mixture was heated to 60-70°C and stirred for 1 hour. Then, silica hydride was slowly added to the RB flask through the open flask after

removing the stopper. The temperature was adjusted to 105°C for 110 hours. After the cooling period the bonded phase was washed 4 times with toluene, 2 times with methylene chloride, and three times with diethyl ether. The product was dried in the hood at room temperature overnight. The next day the product was transferred to a vacuum oven and kept at 120°C for two days.

3.5 Characterization Analysis

3.5.1 Diffuse Reflectance Infared Transform Spectroscopy (DRIFT)

Surface DRIFT analysis prior to, and after silanization/hydrosilation were done using an ATI Mattson Infinity Series FT-IR spectrophotometer (Madison, WI) equipped with a TGS detector. Approximately 0.005 gram of KBr was added to 0.095 gram of the sample. The mixture was placed in a 2 mm sample cup and the surface was smoothed with a glass plate. Pure KBr was first scanned as a reference prior to analysis of the sample. Each sample was scanned 112 times at a resolution range of 4-8 cm^{-1} over a spectral range of 4000-450 cm^{-1}. The spectra were normalized to 100% transmittance. The data was analyzed with a Perkin-Elmer Series 7500 computer.

3.5.2 Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR spectra were measured on a Varian Inova NMR 400 MHz spectrometer. The organic moiety (15 mg) was loaded into a thin glass walled NMR tube. Deuterated benzene was added to the sample prior to NMR analysis.

3.6 Column Packing

The triacontyl bonded silicas were packed into an empty semi-preparative stainless steel column (Alltech, Deerfiled, IL). The dimensions of the column are 7.5 mm

ID x 250 mm length. During packing the column was attached to a Haskell Pump (Burbank, CA) column packer. About 9.56 grams of the bonded phase was added to a solution of carbon tetrachloride:methanol (9:1) forming a homogenous suspension. The mixture was poured into the reservoir of the column packer. The packing process was done using nitrogen under a pressure of about 6000 psi with methanol as the driving solvent.

3.7 Scaling Up to Semi-Preparative HPLC

The amount of starting material (silica) required for synthesizing the bonded phase for the semi-preparative column was scaled-up using previously established procedures from analytical column synthesis. Scaling up of the required starting material was done according to Equation 1.

$$M_p = M_a (V_p/V_a) \qquad \text{Equation 1}$$

where M_p is the amount of a starting material required to synthesize the bonded phase for the semi-preparative column. M_a is the known amount used during synthesis of the starting material for the analytical column. V_p is the volume of the preparative column. V_a is the volume of the analytical column. Basically, the amount of required material for the larger column is increased by the ratio of the volume of the two columns.

The flow rate was also scaled-up. This was done according to Equation 2 [15].

$$F_p = F_a (r^2_p/r^2_a * L_p/L_a) \qquad \text{Equation 2}$$

where F_p is the flow rate of the semi-preparative column, F_a is the flow rate of the analytical column, r_p is the radius of the semi-preparative column, and r_a is the radius of the analytical column.

The load sample was also be scaled according to Equation 3 [15].

$$X_p = X_a (r^2_p/r^2_a * L_p/L_a) \qquad \text{Equation 3}$$

where X_p is the load amount for the semi-preparative column, X_a is the load amount for the analytical column, r_p is the radius of the semi-preparative column, r_a is the radius of the analytical column, L_p is the length of the semi-preparative column, and L_a is the length of the analytical column.

3.8 Semi-Preparative HPLC Runs

Semi-preparative runs were initially evaluated using scaled-up flow rates and load amounts according to equations 2 and 3. However, higher pressure was generated at the scaled-up flow rate with the larger column. Consequently, a lower flow rate had to be used. The will be explained in further detail in the Results and Discussion section. It was originally thought that the mobile phase which resulted in the best separation with the analytical HPLC would also be tested on the semi-preparative column. However, limited runs were conducted with the smaller column due to lack of component retention. This will also be discussed in the Results and Discussion chapter.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 DRIFT Analysis for TES Silanization

DRIFT analyses were taken before and after silanization reaction. Figure 16 is a FTIR spectrum of native Vydac silica prior to silanization. Most native silica have a profile similar to Figure 16. The peak at 3400 cm^{-1} is due to stretching of O-H bonds of silanol groups on the silica surface and adsorbent water.

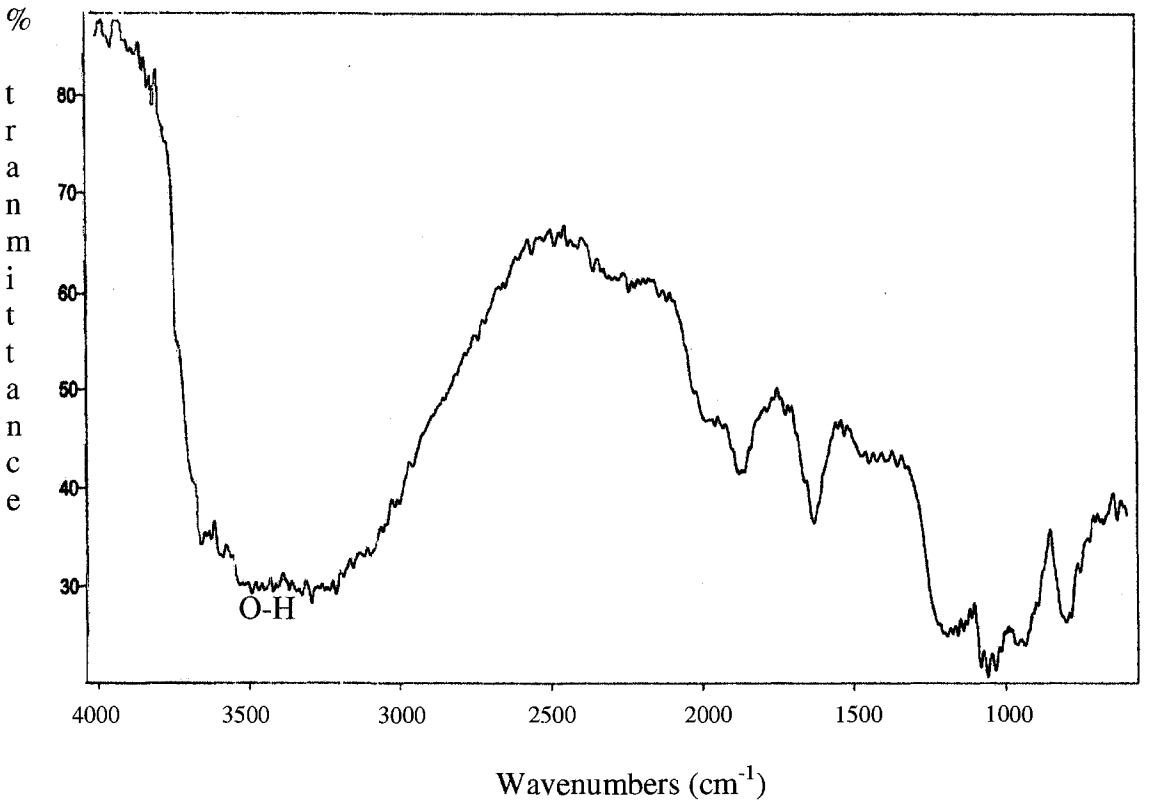


Figure 16. FTIR spectrum of native silica.

After the silanization reaction silica hydride is formed. The FTIR spectrum of silica hydride is show in Figure 17. There is sharp peak at 2250 cm^{-1} corresponding to the silica hydride bond (Si-H).

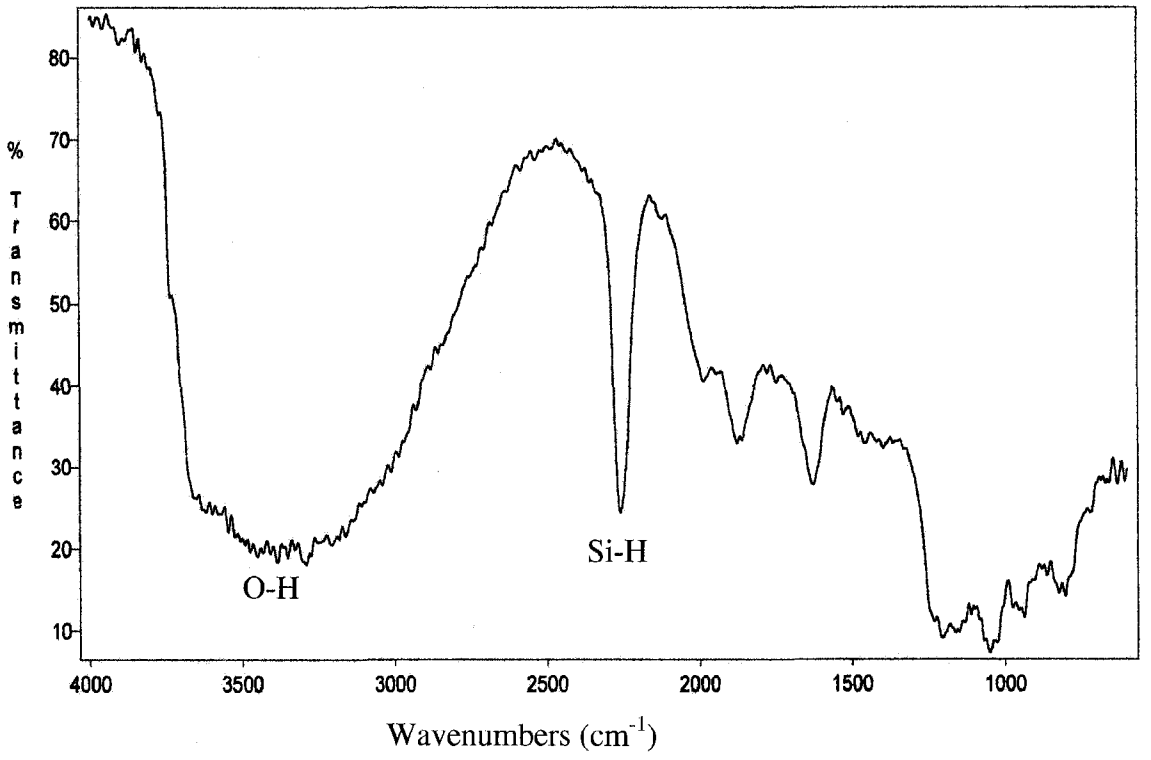


Figure 17. FTIR spectrum of silica hydride.

For comparison both spectra (Figure 16 & 17) are shown in Figure 18. Native silica is displayed in the top spectrum and the bottom spectrum is silica hydride. The stretching peaks due to O-H bonds are still present in the silica hydride spectrum since not all silanol groups will react to form Si-H bonds.

4.2 FTIR Spectrum for Hydrosilation

Bonded phase synthesis via hydrosilation reaction was also analyzed with FTIR. Figure 19 is the FTIR spectrum of the C_{30} moiety bonded to silica hydride. The peaks from 2750 cm^{-1} to 2850 cm^{-1} are signals generated due to stretching of C-H bonds. The Si-H bonds are still present at 2250 cm^{-1} due to some unreacted hydride groups on the surface.

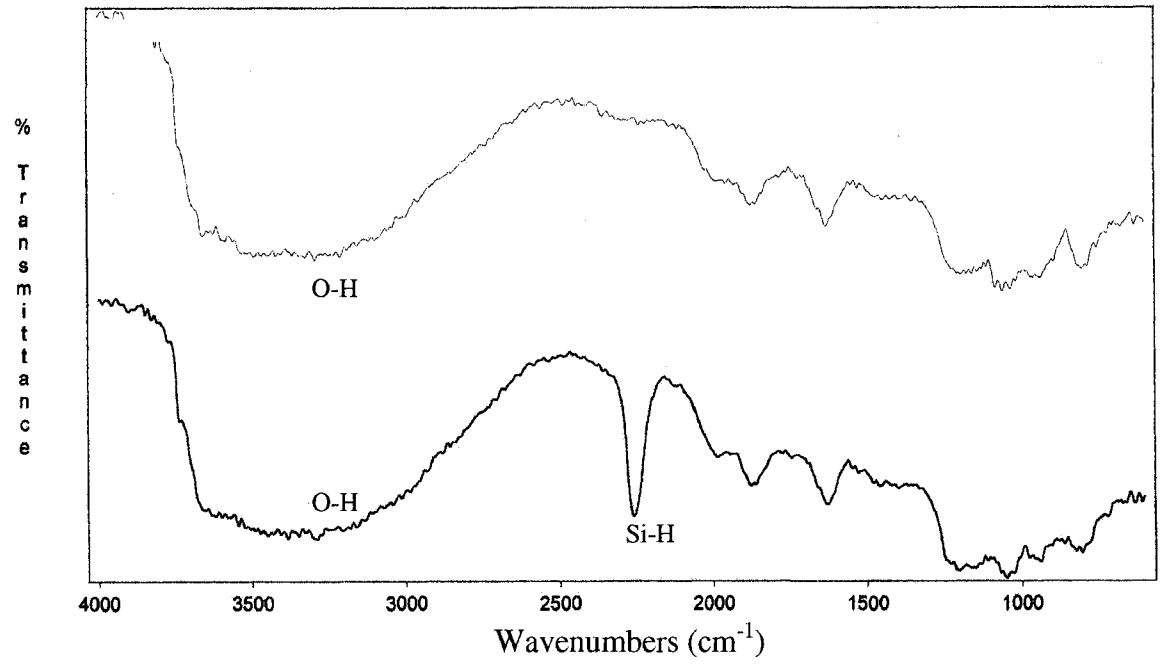


Figure 18. FITR spectra of native silica and silica hydride.

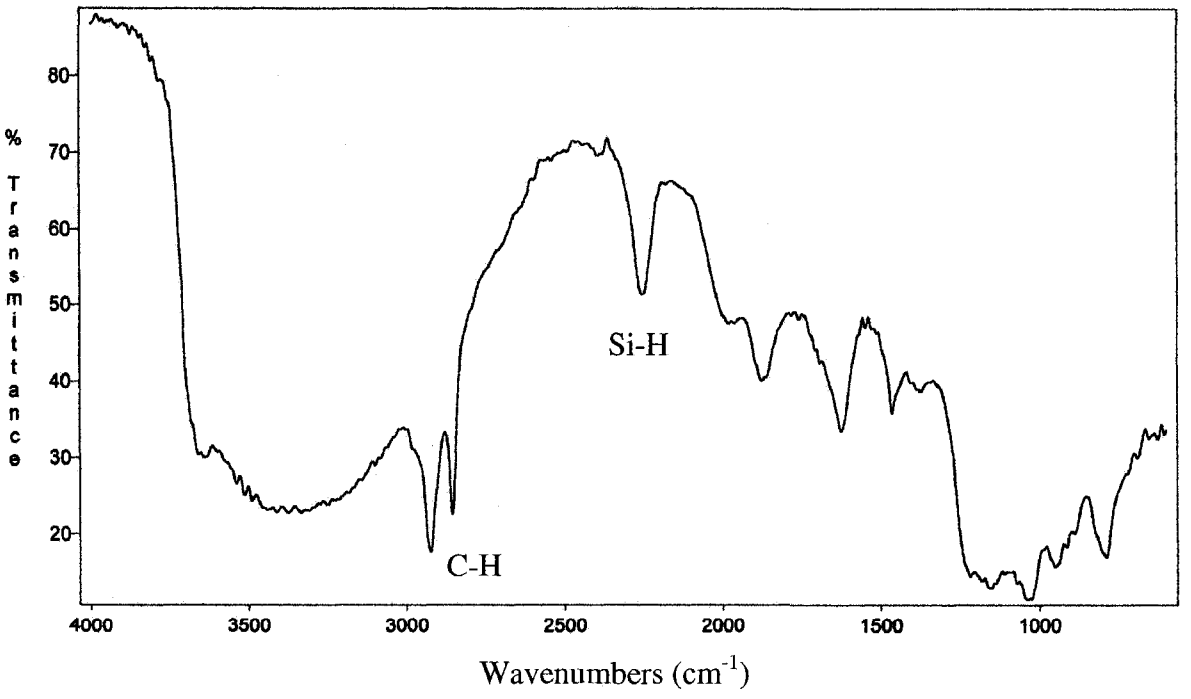


Figure 19. FTIR Spectrum C_{30} of bonded phase.

Two FTIR spectra illustrating the modification of the bonded phase before and after hydrosilation are shown in Figure 20 by combining Figures 17 and 19. The top spectrum is silica hydride prior to hydrosilation reaction. The bottom spectrum is the bonded phase that was formed after hydrosilation. In the top spectrum there are no peaks corresponding to C-H bonds since no C_{30} groups have bonded to silica hydride. In the bottom spectrum the peaks corresponding to stretching of C_{30} groups are apparent. This is an indication that some of the C_{30} groups had attached to silica hydride during the hydrosilation reaction. The spectrum has a noticeable peak at 2250 cm^{-1} due to Si-H bonds. This peak is also present in the bonded phase spectrum but the intensity of the peak is reduced. The reduction in Si-H bonds is expected since the alkyl group attaches directly to the silica hydride bonds. As a result, hydrosilation reaction with Si-H groups on the surface is converted to Si-C_{30} linkages.

4.3 Nuclear Magnetic Resonance Spectrum for Hydrosilation

Nuclear magnetic resonance spectroscopy was employed for characterization of the organic moiety group. The NMR spectrum is shown in Figure 21. The noticeable peaks clustered around the 30 ppm point are due to the main carbon backbone chain consisting of methylene groups $(CH_2)_n$ in the gauche configuration [33, 34]. The peak at 32.2 ppm represents the methylene groups in trans configuration. In previous literature cited, at temperatures above 30°C the gauche configuration dominates the spectrum. At temperatures below 20°C the trans configuration is dominant [33, 34]. The peak at 23 ppm represents signal splitting of the carbon atoms in the 2^{nd} and 29^{th} position on the main chain [33]. Another peak of interest is at 14.2 ppm, which represents the carbon

atoms at the 1st and 30th position [30]. The lower intensity at these two peaks is probably due to the restriction in motion of the methylene groups.

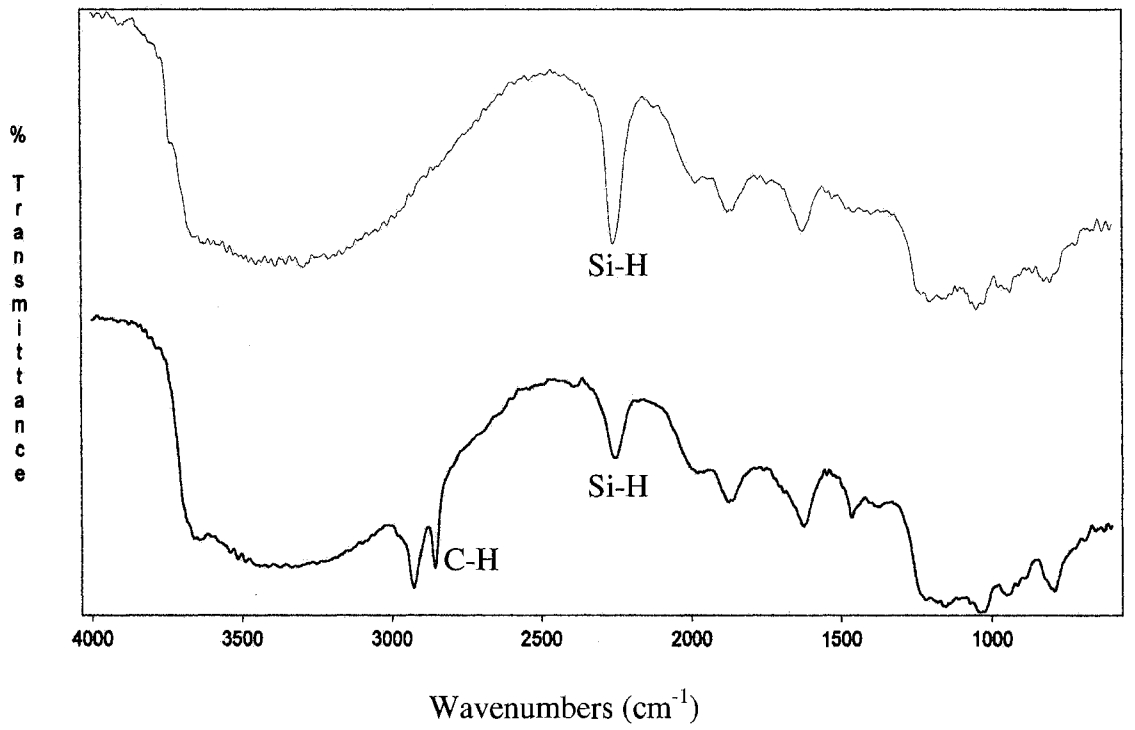


Figure 20. FTIR spectra of silica hydride and bonded phase.

4.4 Analytical Chromatographic Evaluation

HPLC runs using an analytical C_{30} column were evaluated in both reverse and normal phases. The standard detection wavelength was 450 nm. Pure standards of β-carotene, lycopene, and lutein were evaluated. In both phases, uracil was used to determine the dead volume.

4.4.1 Reverse Phase Analytical Evaluation

Analytical HPLC runs were first conducted in reverse phase. Various solvents were tested including methanol, acetonitrile, and ethanol. In addition, various ratios of

solvent to water were also evaluated. Regardless of the type of solvents tested, virtually no separation was achieved for the three carotenoids. The retention time for all three carotenoids did not vary significantly. The chromatogram for β -carotene and uracil is shown in Figure 22. A mobile phase consisting of acetonitrile and water (90:10 ratio) was used for both β-carotene and uracil with a flow rate of 1.0 mL/min.

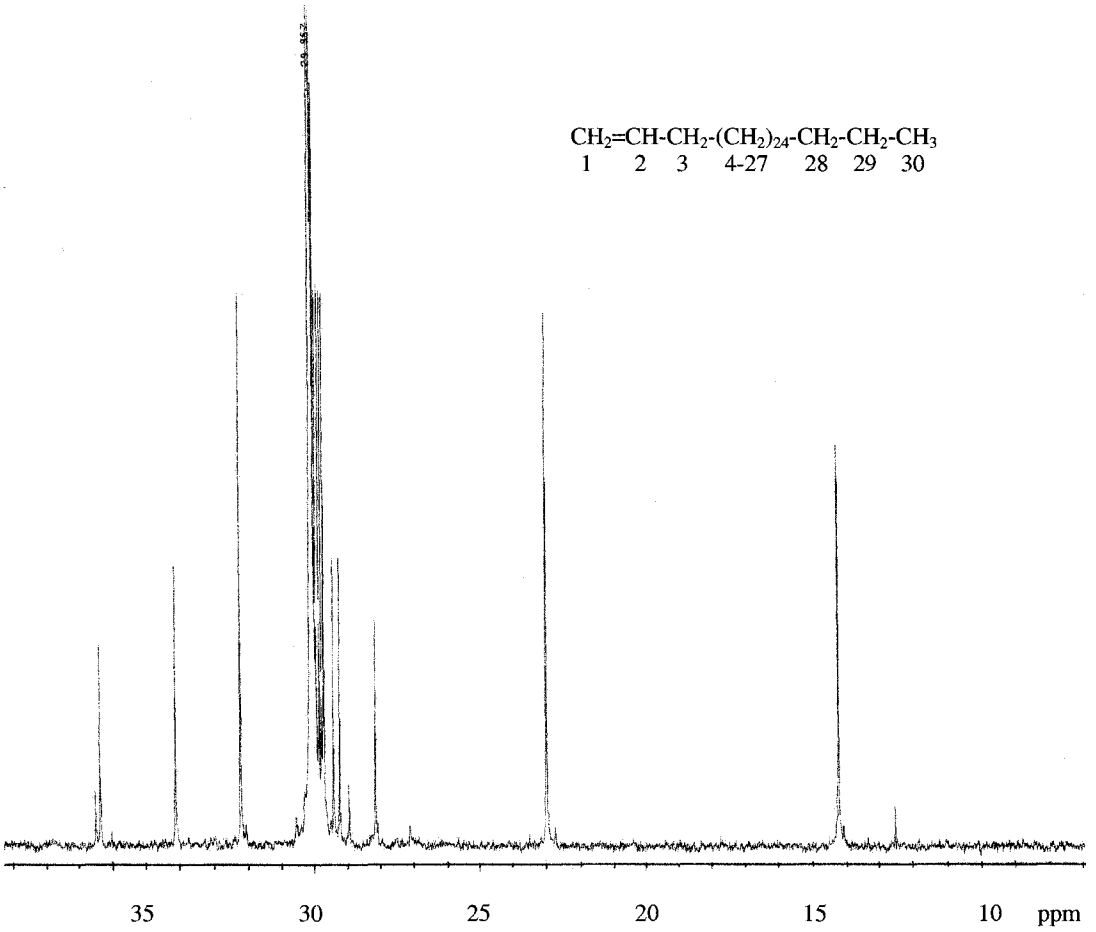


Figure 21. Nuclear magnetic spectroscopy spectrum of triacontyl (C_{30}).

Both β-carotene and uracil had a retention time of 3.5 minutes. Lycopene and lutein also had a retention times very close to 3.5 minutes. Using Equation 5, since the

retention time is known, the capacity factor (k) is calculated to be 0 for β-carotene. Column efficiency, in term of number of theoretical plates (N), was also determined using Equation 9. Theoretical plates are calculated using retention times and W, the width of the peak at baseline. The width was determined by extending a tangent line from the peak to the baseline. Both capacity factor (k) and N for β-carotene are shown in Table 4.

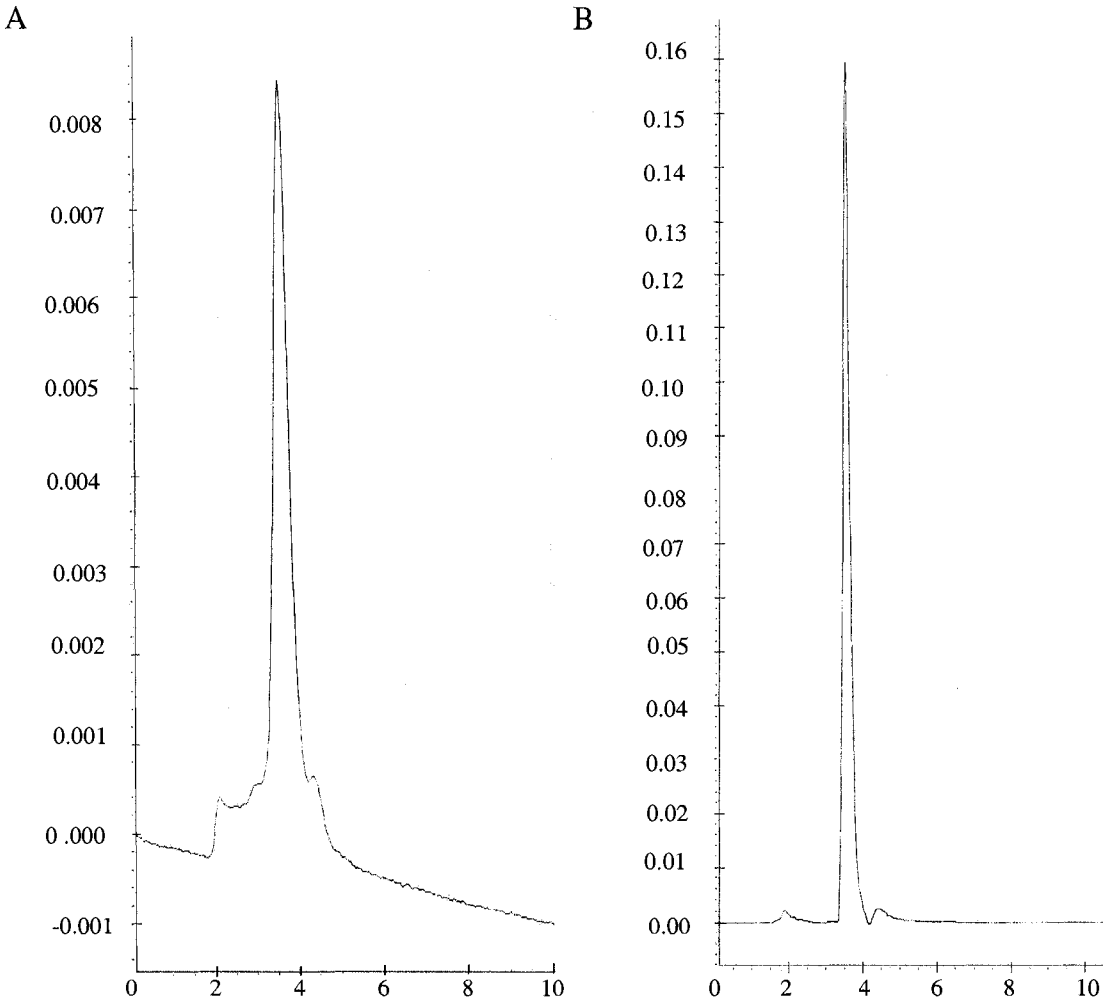


Figure 22. Reverse phase analysis using solvents acetonitrile/water (90:10): A) Chromatogram of β-carotene; B) Chromatogram of uracil. Flow rate = 1.0 mL/min and detection wavelength at 450 nm.

Table 4. Capacity factor (k) and theoretical plates (N) values for β-carotene. Mobile phase acetonitrile/water (90:10). Flow rate = 1.0 mL/min.

t_{bet} (retention time of β-carotene)	t_0 (retention of uracil)	$k=(t_A-t_0)/t_0$	W (width of peak at Baseline)	$N=16(t_A / W)^2$
3.5 minutes	3.5 minutes	k= 0	0.41 minutes	N= 410

The capacity factor is an indication of how well a component is retained in the column relative to uracil, a component that is known to not have any significant retention within the column [17]. With a capacity factor of zero, the retention time of β-carotene is the same as uracil. This is an indication that β-carotene is not retained within the column. Lycopene and Lutein are also not retained since the retention times are very similar to β-carotene. Although not shown here, the resolution or degree of separation between the peaks is zero. The resolution formula in Equation 7 has a minimum value of zero. Zero is an indication of complete peak overlap and a value of 1.5 or greater is an indication of complete peak separation. In this situation, a zero value not only indicates peak overlap but also poor retention of the components. Varying the mobile phase composition of acetonitrile to water from 90:10 to 80:20, 70:30, and 60:40 did not significantly increase the retention times of the three carotenoids and uracil. When determining the capacity factor and resolution values for acetonitrile and water ratio of 80:20, 70:30, and 60:40, the values were still essentially zero.

4.4.2 Reverse Phase Analytical Analysis of Tomato Extract

Tomatoes were thinly sliced and extracted with acetonitrile. The extracts were analyzed with the analytical C_{30} column. The mobile phase was acetonitrile/water (90:10) with a flow rate of 1 mL/mn and a detection wavelength of 450 nm. The chromatogram for tomato extract is shown in Figure 23. Three main peaks can be

observed on the chromatogram. The tallest peak has a retention time of 3.5 minutes. This is the same retention time of the pure β-carotene standards in Figure 22. However, it is not conclusive that this peak is β-carotene due to the poor retention properties of the column. Poor retention is also evident as it is observed that three peaks elute prior to 3.5 minutes, the retention time of uracil.

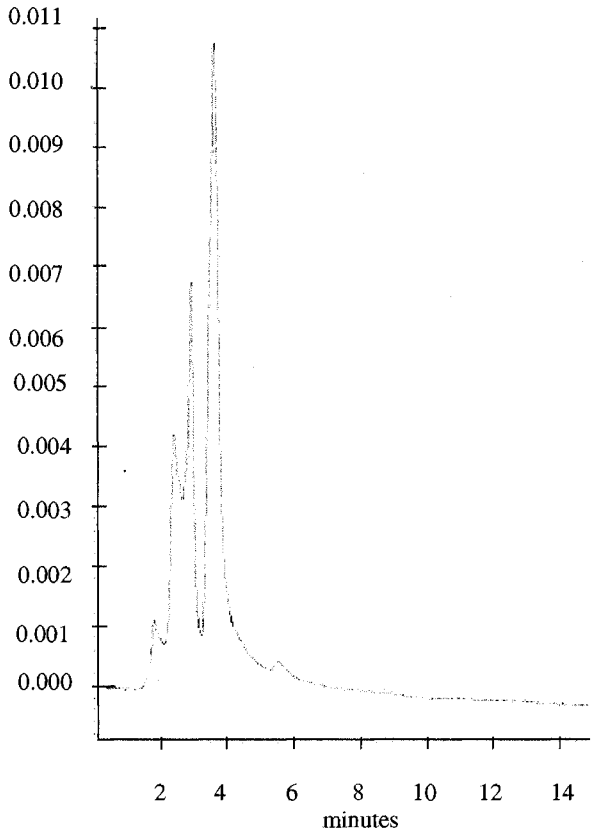


Figure 23. Analysis of tomato extract using solvents actetonitrile/water (90:10), flow rate = 1mL/mn, and detection wavelength of 450 nm.

4.4.3 Normal Phase Analytical Evaluation

Analytical HPLC runs were also conducted under normal phase. Different solvents were tested in trying to achieve the best separation. Despite testing various solvents the same issue was apparent as in reverse phase, specifically, the poor retention

of the compounds. β-carotene, lycopene, and lutein had very similar retention time to that of uracil. In some cases these compounds even eluted from the column slightly before uracil. Figure 24 is a chromatogram of β-carotene and uracil. The mobile phase consisted of acetonitrile:1-butanol (70:30), flow rate is 1 mL/mn, and detection wavelength is 450 nm.

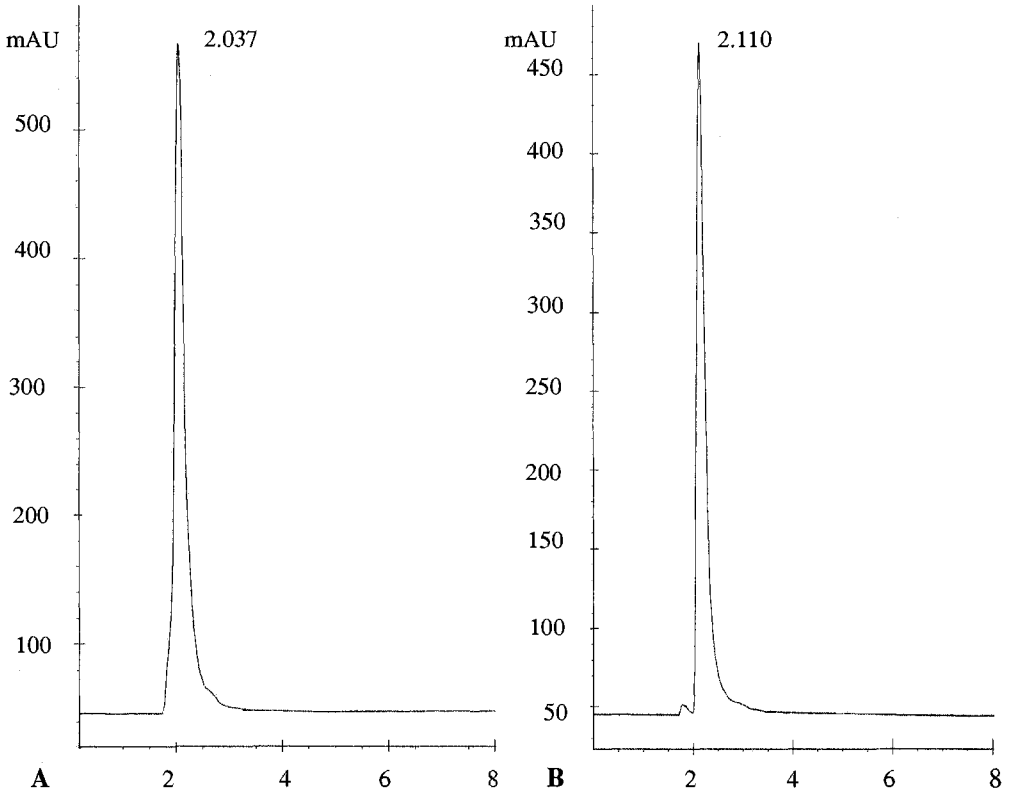


Figure 24. Normal phase analysis with solvents acetonitrile:1-butanol (70:30): A) Chromatogram of β-carotene; B) Chromatogram of uracil. Flow rate = 1 mL/mn and detection wavelength is 450 nm.

The retention time for β-carotene is 2.037 minutes and uracil had a retention time 2.11 minutes. Although the chromatograms for lycopene and lutein are not show here the retention times for these two compounds were very similar eluting at 1.97 and 2.0 minutes. With these retention times the capacity factor (k) for all compounds are

negative values. However, for practical purposes the capacity factor will be considered zero which again indicates the compounds are not being retained in the column.

Theoretical plates were also calculated. With a retention time of 2.037 minutes and a baseline peak width of 0.552 minutes the number of theoretical plates (N) was calculated to be 218.

Both reverse and normal phase indicated poor retention times with all three compounds with the analytical C_{30} column. The compounds essentially had the same retention time as uracil. It was initially suspected that the loss of retention was due to column contamination. Consequently, the column was flushed overnight with different solvents including methanol and acetonitrile with no improvement in retention. Poor retention may also be due to loss or deterioration of the bonded phase. This particular analytical column was developed by a former student more than thirteen years ago. Repetitive use over the years, using the column under harsh solvent conditions, and the age of the column can result in loss of bonded phase. Loss of retention can also occur if there is contaminant build-up in the column, or if the column is used with aqueous or high polar solvents [35, 36]. In the latter case, the alkyl chains can fold upon itself or the neighboring alkyl chain in a phenomenal called phase collapse [36]. During phase collapse the separating components will not be able to adsorb to the stationary phase resulting in retention loss, increased tailing, and retention irreproducibility [36]. Poor retention may also be evident when evaluating the number of theoretical plates (N). Column efficiency is measured in terms of plate numbers. Efficiency increases with the number of plates. Most analytical columns on average have between 2000-6000

theoretical plates [37]. This analytical C_{30} column has 410 and 218 theoretical plates when evaluated in reverse and normal phase which is indicative of the low efficiency of the column.

4.5 Semi-Preparative Chromatographic Evaluation

Analytical runs were first conducted in reverse phase with the intent of scaling-up to semi-preparative runs using parameters established with the analytical column. However, with the incapability of the analytical column to retain the carotenoids, a true scale-up process could not be established since some relevant data could not be determined. Another reason scaling-up could not be done was due to the large pressure drop in the instrument. With the increased size of the column and increased flow rate the pressure drop exceeded the limit of the pump. Initially, to scale-up the flow rate Equation 2 was used. The flow rate for the analytical column was 1 mL/min. The equivalent flow rate for semi-preparative column is 3.86 mL/min which generated a high pressure drop within the system. Even after reducing the flow rate high pressure was still being produced. Due to the high pressure the semi-preparative column was not evaluated in reverse phase due to concerns of possible damage to the system's pump.

Without being able to develop a scale-up procedure the performance of the smaller analytical column could not be evaluated in greater detail and compared to the larger column. In a scale-up process the objective is to replicate performance of the larger column starting with the smaller column. Performance is measured in terms of the HPLC parameters such as resolution (R), selectivity (α), retention time, and theoretical

plates (N). Since a scale-up process could not be established it is unknown as to whether or not the two columns perform similarly.

4.5.1 Normal Phase Semi-Preparative Analysis

Various solvents were evaluated under normal phase conditions as shown in Table 5. The solvent combination that achieved the best separation was acetonitrile and 1-butanol which was also evaluated with the analytical column. Although complete separation among all the peaks was not achieved, overall resolution using this solvent combination was better compared to other solvents tested.

Table 5. Retention time (t_R) for, lutein, lycopene, and β-carotene with various solvents.

Solvent: Flow rate = 2mL/min	Retention time (min) t_{lu}	Retention time (min) t_{ly}	Retention time (min) t_{bet}
Methanol: Acetonitrile (70:30)	6.96	7.02	7.01
Methanol: Acetonitrile (50:50)	6.57	6.75	6.70
Methanol: Acetonitrile (30:70)	5.93	6.23	6.46
Acetonitrile: 1-butanol (70:30)	4.80	5.40	6.57
Methyl Chloride: Hexane (70:30)	3.45	3.77	3.89
Chloroform: Benzene (70:30)	4.21	4.40	Peak undetected

The flow rate first tested was 3.86 mL/min with the semi-preparative column. This flow rate is equivalent to 1 mL/min with the analytical column. However, high pressure-drop in the system and poor separation were observed. Decreasing the flow rate not only lowered the pressure drop as expected, but also gave better separation of the three compounds with the acetonitrile and 1-butanol solvent mixture. Figure 25 displays three chromatograms showing how separation is improved as the flow rate is decreased

from 1.5 mL/min to 0.5 mL/min using the solvent ratio of acetonitrile and 1-butanol (70:30) and a detection wavelength of 450 nm. The first peak in all three chromatograms corresponds to lutein followed by lycopene and β-carotene.

Resolution (R), shown in Equation 7, measures the degree of separation between two consecutive peaks on a chromatogram. Resolution was first calculated for the adjacent peaks of lutein and lycopene and then for lycopene and β-carotene. Resolution values and retention times for lutein, lycopene, β-carotene and uracil are shown Table 6. Table 6 shows quantitatively that there is better separation between the first (lutein) and second peak (lycopene) as flow rate decreases. With a flow rate of 1.5 mL/min the resolution was 0.24. Resolution increased to 0.51 and 0.80 with as flow rate is decreased to1.0 mL/min and 0.5 mL/min. However, separation was not completely achieved with lutein and lycopene. A resolution of 1.5 indicates complete separation [17]. The highest resolution with lutein and lycopene was only 0.80 as flow rate is varied. Incomplete separation is also apparent from Figure 25 as there is significant overlap between the two peaks observed in all three chromatograms. Resolution also improved for lycopene and β-carotene as the mobile phase is decreased from 1.5 mL/min to 0.5 mL/min. With flow rates 1.0 mL/min and 0.5 mL/min there is complete separation between the two peaks as resolution for both is greater than 1.5. For flow rate 1.5 mL/min the resolution value of 1.24 indicates incomplete separation. However, chromatogram A in Figure 25 shows complete separation. The discrepancy may be due to errors from how the resolution value is obtained. The time period at baseline between the peaks two (lycopene) and three (β-carotene) were obtained by extending a tangent line from the side of each peak to

the baseline. The time period obtained from this extension method may not represent the true value leading to inaccuracies in calculating the resolution.

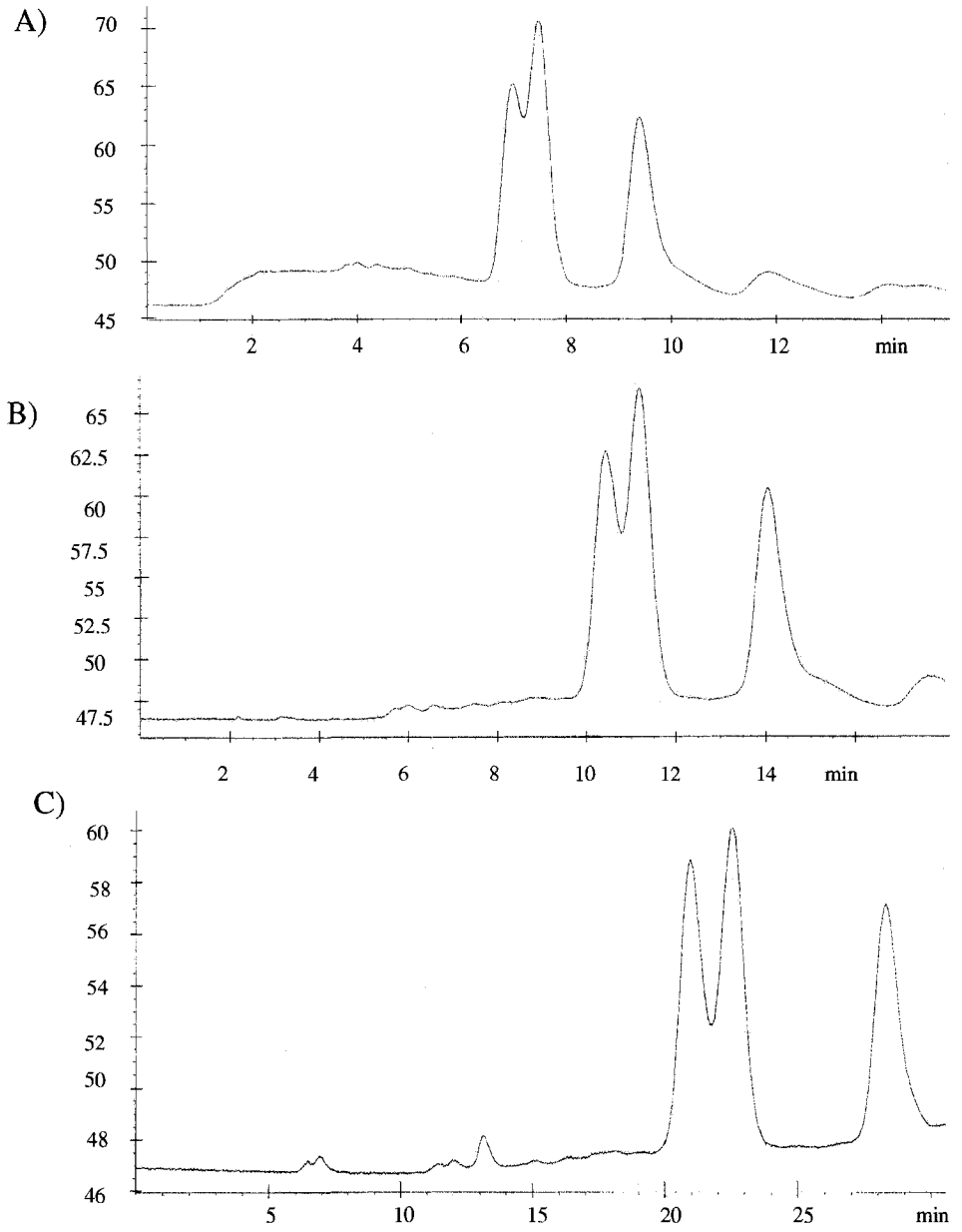


Figure 25. Three chromatograms displaying the improvement of separation as flow rate is decreased: A) Chromatogram with a flow rate of 1.5 mL/min; B) Chromatogram with a flow rate of 1.0 mL/min; C) Chromatogram with a flow rate of 0.5 mL/min. Mobile phase consisted of acetonitrile:1-butanol (70:30).

As shown in Table 6 the retention time for uracil was less than the retention times of the three carotenoids with each of the three different flow rates. For example, for a flow rate of 1.5 mL/min the retention time for uracil is 4.27 minutes and 6.97 minutes for the earliest eluting carotenoid, lutein. This is an indication that the compounds are better retained in the semi-preparative column in comparison to the analytical column for which uracil and each of the carotenoids have essentially the same retention times.

Table 6. Retention time (t_R) and resolution (R) values for lutein-lycopene and lycopene-β-carotene at flow rates of 1.5 mL/min, 1.0 mL/min, and 0.5 mL/min with mobile phase acetonitrile:1-butanol (70:30).

Acetonitrile:1-butanol (70:30); Flow rate = 1.5 mL/min	Acetonitrile:1-butanol (70:30); Flow rate = 1.0 mL/min	Acetonitrile:1-butanol (70:30); Flow rate = 0.5 mL/min
Retention time $t_{lu} = 6.97$ min $t_{ly} = 7.47$ min $t_{bet} = 9.41$ min $t_{ura} = 4.27$	Retention time $t_{lu} = 10.43$ min $t_{ly} = 11.82$ min $t_{bet} = 14.05$ min $t_{ura} = 6.41$	Retention time $t_{lu} = 20.94$ min $t_{ly} = 22.53$ min $t_{bet} = 28.27$ min $t_{ura} = 12.66$ min
Resolution $R_{lu\text{-}ly} = 0.24$ $R_{ly\text{-}bet} = 1.24$	Resolution $R_{lu\text{-}ly} = 0.51$ $R_{ly\text{-}bet} = 2.38$	Resolution $R_{lu\text{-}ly} = 0.80$ $R_{ly\text{-}bet} = 2.96$

The reason for better separation with a lower flow rate may be due to the increased amount of time the solutes interact with the stationary phase. This interaction is the basis for separation. With a slower flow rate there is more time for interaction which results in better separation. Based on the observation that a slower flow rate achieves better separation the decision was made to conduct runs with flow rates of 0.5 mL/min despite the fact that band broadening will occur at such a flow rate.

After determining that acetonitrile and 1-butanol gave the best resolution, the process was refined further by varying the ratios of acetonitrile to 1-butanol. With an initial ratio of 70:30 of acetonitrile to 1-butanol, the ratio was changed by decreasing

acetonitrile by increments of 10 and increasing 1-butanol by increments of 10. Consequently, changing the solvent ratio makes the mobile phase less polar. The final ratio of acetonitrile to 1-butanol tested was 40:60. Figure 26 displays four chromatograms with different ratios of the mobile phase. From the chromatograms it is noticeable that retention times are reduced going from a solvent ratio of 70:30 to 40:60. Similar to before, in all the chromatograms, the first detected peak is lutein followed by lycopene and β-carotene.

Table 7 shows the retention time, resolution, and theoretical plates for the components. Retention times are reduced as the mobile phase becomes less polar due to the change in solvent composition. From the chromatograms in Figure 26 it is observed that the visual separation between lutein and lycopene is improved going from a solvent ratio of 70:30 to 40:60. This is also evident as the resolution is improved from 0.86 to 1.33 as shown in Table 7. Despite the improvement of separation going from a solvent ratio of 70:30 to 40:60, full separation between lutein and lycopene could not be achieved. The highest resolution was 1.33. The opposite effect is observed with lycopene and β-carotene, as the two peaks are moving closer to one another as the solvent composition changed from 70:30 to 40:60. Despite the fact that the lycopene and β-carotene peaks are getting closer to each other there still seems to be full separation with the exception of the 40:60 ratio which had a resolution of 1.32. A decision was made not to test the solvent ratio of 30:70 acetonitrile to 1-butanol since separation between lycopene and β-carotene would likely not be improved, and also the retention time for lutein would approach that of uracil as the mobile phase decreases in polarity.

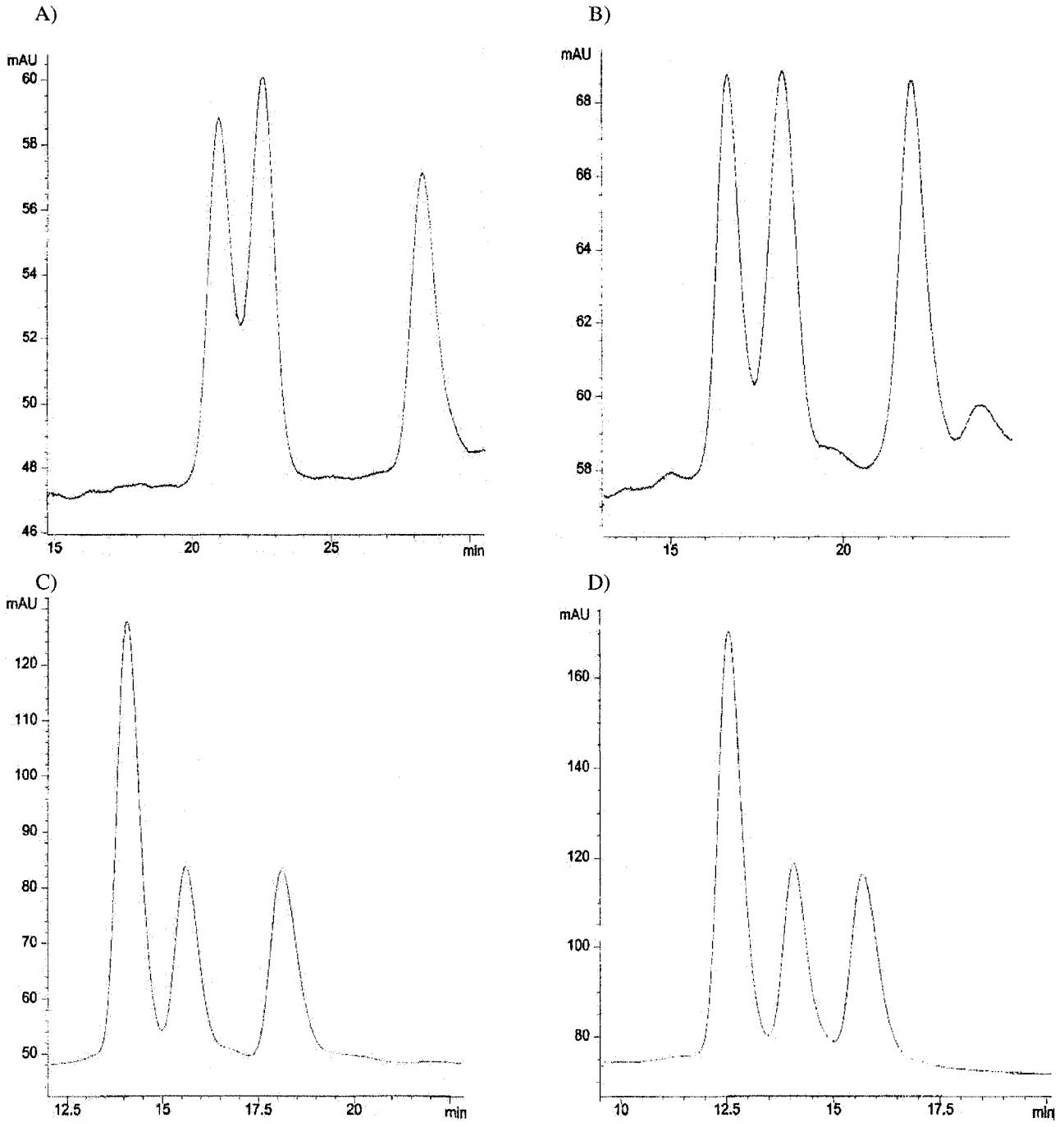


Figure 26. Chromatograms displaying the improvement of separation with various ratios of acetonitrile to 1-butanol: A) Chromatogram with a mobile phase of 70:30 acetonitrile to 1-butanol; B) Chromatogram with 60:40 acetonitrile to 1-butanol; C) Chromatogram with 50:50 acetonitrile to 1-butanol; D) Chromatogram with 40:60 acetonitrile to 1-butanol. Flow rate = 0.5 mL/min.

Table 7. Retention times (t_R) and resolution (R) values for lutein-lycopene and lycopene-β-carotene with different ratios of the mobile phase. Flow rate = 0.5 mL/min.

Acetonitrile:1-butanol (70:30); Flow rate = 0.5 mL/min;	Acetonitrile:1-butanol (60:40); Flow rate = 0.5 mL/min;	Acetonitrile:1-butanol (50:50); Flow rate = 0.5 mL/min;	Acetonitrile:1-butanol (40:60); Flow rate = 0.5 mL/min;
Retention time t_{lu} = 20.94 min t_{ly} = 22.53 min t_{bet} = 28.27 min t_{ura} = 11.79 min	Retention time t_{lu} =16.65 min t_{ly} = 18.24 min t_{bet} = 21.97 min t_{ura} = 11.79 min	Retention time t_{lu} = 14.07 min t_{ly} = 15.61 min t_{bet} = 18.12 min t_{ura} = 11.79 min	Retention time t_{lu} = 12.53 min t_{ly} = 14.07 min t_{bet} = 15.69 min t_{ura} = 11.79 min
Resolution $R_{lu\text{-}ly}$ = 0.86 $R_{ly\text{-}bet}$ = 1.24	Resolution $R_{lu\text{-}ly}$ = 1.0 $R_{ly\text{-}bet}$ = 2.21	Resolution $R_{lu\text{-}ly}$ = 1.17 $R_{ly\text{-}bet}$ = 1.81	Resolution $R_{lu\text{-}ly}$ = 1.33 $R_{ly\text{-}bet}$ = 1.32
Theoretical Plates N_{lu} = 2256 N_{ly} = 2611 N_{bet} = 4111	Theoretical Plates N_{lu} = 2543 N_{ly} = 2186 N_{bet} = 3718	Theoretical Plates N_{lu} = 2500 N_{ly} = 3077 N_{bet} = 2442	Theoretical Plates N_{lu} = 2214 N_{ly} = 2787 N_{bet} = 2473

Despite not being able to fully separate lutein and lycopene, the column does separate the components well enough to indicate which carotenoids are present in a mixture of the three compounds. However, with larger columns, such as semi-preparative and preparative, it is sometimes necessary to not only separate but also collect a component within a mixture. In this situation it is favorable to have all the components completely separated. Even though lutein and lycopene were not completely separated it should still be possible to collect each of the two components. This can be done by collecting fractions of the components prior to and after the overlap of the two peaks. The only drawback is that some amount of each component would be lost since fractions of the peaks corresponding to overlapping are not collected.

To determine the efficiency of the column theoretical plates (N) were also calculated. Theoretical plate values ranged from 2186 to 4111 as shown in Table 6. Theoretical plates determine efficiency by measuring how narrow the chromatographic

peaks are [17]. Since a low flow rate of 0.5 mL/min was used, the peaks are somewhat broader as compared with a higher flow rate. However, increasing the flow rate does not always increase theoretical plate values. The theoretical plate equation in Equation 9 is proportional to retention time. Increasing the flow rate reduces the retention time therefore, the number of theoretical plates are also reduced. Due to the decrease in retention time a theoretical plate value of 2500 was calculated for a flow rate of 1.5 mL/min with the 70:30 solvent composition. Although the peaks were narrower, the theoretical plate values are less compared to the slower flow rate of 0.5 mL/min for the same 70:30 solvent ratio (2500 vs 4111).

HPLC columns, on average, have about two to six thousand theoretical plates [37]. However, more importantly, when trying to evaluate the overall performance of a column, other HPLC parameters such as resolution (R) and selectivity factor (α) should also be considered. In this particular experiment, being able to separate the components was one of the objectives. Therefore, having an acceptable value for resolution takes priority over achieving high theoretical plate values.

It should be noted that uracil was used with the four different ratios of the mobile phase in Table 6. The retention time varied for each run. For the ratio of 70:30 the retention time was 12.66 minutes and decreased to 11.79 minutes with the 40:60 ratio although flow rate was the same for all four mobile phases. The difference is almost one full minute. This is an indication that there is some degree of retention with uracil. However, since uracil was used to determine the void time, the shortest retention time among the different solvent ratios will be used as the universal void time.

The difference in retention time was almost one minute going from a 70:30 solvent composition to 40:60 which indicates uracil is retained as stated earlier. Deuterium oxide (DO_2) was also tested as a dead volume marker to see if it was less retained than uracil. However, neither a peak nor deflection was detected. As a result, the retention time for deuterium oxide could not be evaluated as the dead volume.

CHAPTER 5
CONCLUSION

The first part of this experiment consisted of synthesizing and characterizating the stationary phase. The two-step synthesis procedure included silanization followed by hydrosilation. The starting material for synthesis was silica. During silanization, silica reacted with the reagent triethoxysilane to form a silica hydride monolayer. The monolayer was confirmed with FTIR analysis by the presence of a sharp peak at 2250 cm^{-1}. The second step of the synthesis process involved hydrosilation. During hydrosilation the organic moiety groups were attached to the silica hydride surface forming the stationary phase of the column. FTIR and NMR also confirmed the bonding of the C_{30} group.

In the second part of the experiment, the original intent was to first establish the best conditions for carotenoid separation with the smaller analytical column, and then scale-up to the larger semi-preparative column. Conditions such as flow rate and load volume would have been scaled-up with Equations 2 and 3. However, due to poor retention time in the analytical column possibly due to the loss of bonded phase or phase collapse, scaling from the analytical column to the semi-preparative column could not be established. Therefore, it is uncertain whether or not the performance of the two columns, in terms of HPLC parameters, is similar when scaling-up. In an ideal case when scaling up or down, HPLC parameters such as resolution (R), selectivity (α), retention time, and theoretical plates (N) should be similar for both columns. In this

experiment it is unknown whether or not the parameters would have been similar since limited runs were conducted with the analytical column.

The semi-preparative column was tested for carotenoid separation with little guidance or starting points from the analytical column in regards to which flow rates or mobile phase would achieve the best separation. Various flow rates were evaluated and the low flow rate of 0.5 mL/min resulted in the best separation. Similarly, of all the mobile phase systems tested it was determined that a combination of acetonitrile and 1-butanol achieved the best separation. However, there was not a complete separation between lutein and lycopene. The resolution (R) for lutein and lycopene was 1.33. There was complete separation between lycopene and β-carotene and lutein and β-carotene.

CHAPTER 6
FUTURE EXPERIMENTS

Characterization methods such as FTIR and NMR were used to analyze the intermediate and final bonded phase product during the synthesis process. While these two techniques are useful they do not give a quantitative analysis of surface coverage of the bonded C_{30} group. In the future elemental analysis using a micro-combustion technique would give a quantitative analysis of the percentage of C_{30} group on the surface of the silica particle. This is important since greater surface coverage should result in better separation characteristics in the column.

Due to the high pressure-drop in the instrument the column was not tested in reverse phase. In the future, the column should be tested in reverse phase to determine the separation efficiency of the components with polar solvents. The column should also be evaluated on other carotenoids. In this experiment only three carotenoids were evaluated.

The possibility of using experimental design should be explored in future work involving stationary phase synthesis. Variables that can be vary include time, temperature, and duration of reaction. Although it will be labor intensive, experimental design will give a statistical analysis of which combination of variables will achieve the best result for surface coverage of the bonded group.

In this experiment the semi-preparative column was developed to be analyzed for carotenoid separation. The smaller analytical column was developed 13 years earlier and over time the column lost its ability to retain the analytes. In any future work involving scaling, the packing material for the analytical column should be synthesized,

characterized, and packed simultaneously with the packing material for the larger column. This will ensure that similar steps were taken during the development process. This will allow a more in depth study into whether the two columns are scalable.

Since some degree of separation was achieved with the three carotenoids using the semi-preparative column it is worthwhile to pursue a scale-down procedure. This would involve the development of a smaller analytical column. The stationary phase could be synthesized, characterized, and packed into the column. For scaling down the same mobile phase as the semi-preparative column should be used. The only difference is that the load volume and flow rate will be reduced according to the smaller column volume. The semi-preparative column was tested again after several months and chromatograms similar to Figure 26C were obtained. This is an indication that the semi-preparative has not lost any retention properties and can be evaluated in scaling studies.

In this experiment uracil was used as a dead volume indicator. There are indications that uracil is retained in the column. A less retained dead volume indicator should be evaluated with this semi-preparative column.

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