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Evaluation of a Silica Hydride-Based Undecynoic Acid Stationary Phase For High Performance Liquid Chromatography

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EVALUATION OF A SILICA HYDRIDE-BASED UNDECYNOIC ACID
STATIONARY PHASE FOR HIGH PERFORMANCE LIQUID
CHROMATOGRAPHY

A Thesis

Presented to

The Faculty of the Department of Chemistry
San José State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Veena Menon Kozhikote

December 2016

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The Designated Thesis Committee Approves the Thesis Titled

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CHROMATOGRAPHY

by

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ABSTRACT

EVALUATION OF A SILICA HYDRIDE-BASED UNDECYNOIC ACID STATIONARY PHASE FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

by Veena Menon Kozhikote

Undecynoic acid (UDA) attached to a silica hydride surface has been found to exhibit weak cationic exchange properties and an aqueous normal phase (ANP) type of retention. ANP is a mechanism similar to normal phase chromatographic retention, except for the use of a polar solvent such as water in the mobile phase. In ANP, the retention of a polar molecule is increased with a higher concentration of the non-polar mobile phase solvent (usually acetonitrile). Non-polar molecules exhibit retention behavior as in reverse phase (RP) chromatography. The goal of this research was to characterize the chromatographic retention pattern of an undecynoic based silica hydride column by studying various polar and nonpolar analytes. Also investigated were the effects of varying the buffer concentrations -formic acid and ammonium acetate and the effect of temperature on the retention of selected compounds. It has been established from the current work that a silica hydride based UDA column can be used for separation and analysis of nucleotides and nucleosides as well as phenolic acid components in pomegranate peel samples. A phenyl hydride column was additionally used to complete the study on the peel samples since the UDA column was not effective in separating isobaric compounds found in the peels. In conclusion, silica hydride based UDA column has been found to exhibit dual retention capabilities for polar and non-polar molecules.

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LIST OF ABBREVIATIONS

ACN - acetonitrile
AMP- adenosine mono phosphate
ATP - adenosine triphosphate
A ° - Angstrom
ANP - aqueous normal-phase
CI - chemical ionization
DI - deionized
DNA - deoxyribonucleic acid
DEDTP - diethyl dithiophosphate
DEP - diethyl phosphate
DETP - diethyl thiophosphate
DMDTP - dimethyl dithiophosphate
DMP - dimethyl phosphate
DMTP - dimethyl thiophosphate
DAD - diode array detectors
ECD - electrochemical detectors
EI - electron impact
ESI - electron spray ionization
ELSD - evaporative light scattering detectors
FAB - fast atom bombardment
9- fluorenyl methoxy carbonyl chloridefluorophenylmethoxy chloroformate - Fmoc
GC - gas chromatography
HETP - height equivalent to theoretical plate
HPLC - high performance liquid chromatography
HPTLC - high performance thin layer chromatography
HILIC- hydrophilic interaction liquid chromatography

id - internal diameter
IEX - Ion exchange chromatography
MS - mass spectrometer
MALDI - matrix assisted laser desorption
MeOH - Methanol
 μm - micrometers
MN - Molla Nepes
NAD - nicotinamide dinucleotide
NADP - nicotinamide dinucleotide phosphate
NP - normal phase
ONP - organic normal-phase
OPA - ortho pthalaldehyde
PA - peak area
PTH - phenyl thiohydantoin
PAH - polycyclic aromatic hydrocarbons
PH - Purple heart
Q-TOF - Quadrupole-time of flight
RI - refractive index
RT - retention time
RP - reverse phase
RNA - ribonucleic acid
TCC - thermostatted column compartment
TLC - Thin layer chromatography
TOF - time-of-flight
UV - Ultraviolet
UDA - undecynoic acid column
USDA - United States Department of Agriculture
VWD - Variable wavelength detectors

v/v - volume/volume

I. INTRODUCTION

A. Background of chromatography

This method of separation was first observed by Mikhail S Tswett while trying to separate the different components present in chlorophyll.¹ It was initially believed that the green plant pigment chlorophyll was comprised of two pigments. However, the experiments conducted by Tswett that consisted of preparing homogenized plant extracts, passing the mixture into a column of calcium carbonate/chalk, and further addition of a mobile phase such as petroleum ether demonstrated that chlorophyll actually consisted of eight pigments. With the passing of the ether through the column, many colored bands were obtained with intermittent regions free of any color.

A book was published in 1910 describing the above technique of “chromatography,” as coined by Tswett (“chroma” meaning color and “graphy” meaning writing in Greek). Later in 1931, the German chemist Richard Kuhn and his student, Edgar Lederer, reported the use of this technique to separate many biologically active molecules.

There exist many types of chromatography and the classification depends on many factors such as the physical state of the stationary and mobile phase, principle of separation, chemical nature of the mobile and stationary phase, and format of the stationary phase. Based on the physical state of the stationary phase and mobile phase, chromatography is classified as homogenous and heterogeneous chromatography. In the former type the physical states of the stationary and mobile phase are the same while in the latter the physical states are different. An example of homogenous chromatography is liquid-liquid chromatography while liquid-solid, gas-liquid, and gas-solid

chromatography belong to the class of heterogeneous chromatography.² According to the principle of separation there exists adsorption and partition chromatography. The underlying principle for adsorption chromatography is the adsorption of the analyte molecule on the stationary phase. Compounds that are strongly adsorbed on the stationary phase elute slowly while those weakly adsorbed elute faster. Partition chromatography is based on the difference in the dissolution of the analytes in the stationary/mobile phase. For example in the case of liquid-solid chromatography, the partition arises between the liquid mobile phase and the quasi liquid layer on the stationary phase.²

Normal phase (NP) and reverse phase (RP) chromatography are other types of chromatography and this classification is based on the chemical nature of the mobile and stationary phase. While NP employs a polar stationary phase and a non-polar mobile phase, in RP a non-polar stationary phase and a polar mobile phase are used. Polar analyte/hydrophilic compounds are retained longer on the column, while non-polar/hydrophobic compounds elute at or near the void/dead volume in NP as opposed to RP chromatography wherein hydrophilic/polar compounds elute near the dead volume. Some examples of polar stationary phases used in NP are bare silica, or compounds bonded to the surface containing either cyano or amino groups. The non-polar mobile phase usually comprises solvents such as hexane, heptane, or ethyl acetate. Some examples of non-polar stationary phases and polar mobile phases used in RP are columns containing a C8/C18 group and water, respectively. Yet another classification of chromatography based on the format of the stationary phases are column and planar

chromatography. While in column chromatography the stationary phase is columnar shaped, it is planar or flat in planar chromatography. High performance liquid chromatography (HPLC) and gas chromatography (GC) are examples of the former type while thin layer chromatography (TLC), paper chromatography, and high performance thin layer chromatography (HPTLC) belong to the latter type of chromatography.²

Some additional types of chromatography include size exclusion and ion-exchange chromatography (IEX). In size exclusion chromatography, the stationary phase has pores and the analytes are separated based on their molecular size. While larger molecules are unable to pass through, the small or medium sized molecules pass through the pores and thus takes a longer time to elute. In IEX, the stationary phase is charged and this enables the stationary phase to have an electrostatic attraction with oppositely charged species present in the sample. IEX is classified into cation exchange and anion exchange based on the charge of the stationary phase. Thus in cation exchange chromatography, the stationary phase is negatively charged and in anion exchange chromatography the stationary phase is positively charged.

B. High performance liquid chromatography (HPLC)

Chromatography paved the way for better separation of compounds with a reduction in the analysis time.³ The technique of HPLC was initially termed “high pressure liquid chromatography” but eventually it was replaced by the term “high performance liquid chromatography.” In HPLC, the eluent is delivered by a pump that can withstand high pressures on the order of 6000 psi. The particle size used in HPLC

(around 3-10 μm) is far smaller than what was used for earlier chromatographic techniques.

Some of the advantages of this technique are a) higher separation capacity with batch analysis of multiple components, b) superior quantitative capability, c) reproducibility, d) easy preparative separation, e) higher sensitivity, and f) lower sample consumption. Figures 1 and 2 below depict a flow diagram of an HPLC and a typical HPLC instrument, respectively.

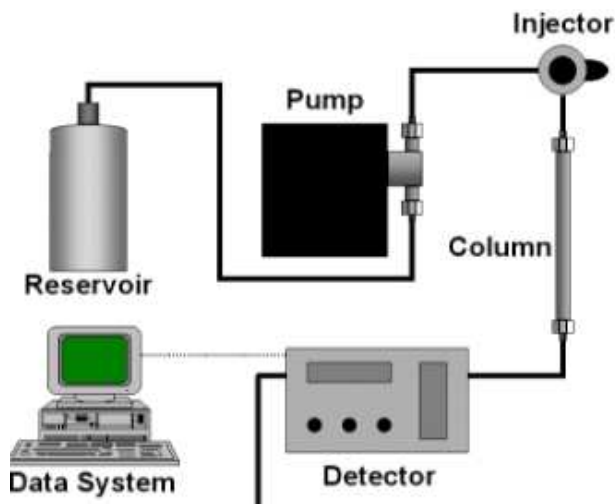


Figure 1. Flow diagram of HPLC⁷ (Reprinted with permission from LCResources.com)



Figure 2. Representative photograph of HPLC instrument (© Agilent Technologies, Inc. 2014. Reproduced with permission, Courtesy of Agilent Technologies, Inc.)

Some of the applications of HPLC include analysis of medical products (drugs and antibiotics), quality control studies in food products (vitamins, food additives, sugars, and organic acids), environmental sample analysis, and other organic industrial products.³

1. A typical HPLC instrument consists of the following elements:

1.1. An ideal solvent delivery system should possess qualities like withstanding high pressure, have small pulsations accompanying pressure fluctuation, and an ability to accommodate a wide flow rate setting with accuracy in flow rate.³ Typical solvents used are deionized (DI) water and HPLC/special grade organic solvents such as acetonitrile, methanol, acetone, and isopropanol.

The desirable properties of an HPLC grade solvent are inertness, low viscosity, high purity, and low toxicity.⁴ A highly viscous solvent tends to increase the back pressure in the column and capillaries, leading to heat generation and subsequent peak broadening.⁴ Additionally, an ideal solvent should also be transparent/invisible in the detector. For UV detectors, this means that the solvent should not absorb light at the wavelength selected. Further, an ideal solvent should be miscible with the other solvents, as immiscible solvents lead to unstable baselines and fluctuating/high pressures.⁴ Solvents are also to be filtered prior to their use in the HPLC instrument to prevent valve and column blocks.⁴ Acetonitrile (ACN) is generally the preferred solvent over methanol (MeOH) or isopropanol.

1.2. Degassing unit: Dissolved air and bubbles in the solvent lead to large baseline shifts in the detector cell and the retention times and peak areas may not be stable.⁴ It is thus required to get rid of air bubbles and other dissolved gases before starting a chromatographic run.³ Some of the techniques used to degas are freeze thaw cycles under vacuum, ultra-sonication, or helium sparging.⁴ Some HPLC instruments also have an inbuilt degassing unit in the flow path between the solvent reservoir and pump. The basic principle of degassing includes applying vacuum to the solvent that is separated from an empty chamber by a semi permeable membrane for removing gas bubbles and other dissolved gases.⁴ The membrane permits the passage of small air molecules, while the larger solvent molecules remain in the liquid phase itself.

1.3. Sample injector unit: This component introduces the sample into the flow path.⁴ Some desirable properties of an injector are to permit free adjustment of injection

volume with minimal sample loss, minimal sample band broadening, superior durability, and pressure resistance.^{3,4} A minimum delay volume high accuracy/precision of the injected sample, and fast injection cycle are some other useful features.⁴ Manual injectors and auto samplers are two types of commonly used injectors. While the manual injector enables a basic and affordable solution to introduce the desired volume of the sample to the flow path, standard auto samplers automatically inject the samples stored in vials.⁴

1.4. Column oven /thermostatted column compartment (TCC): An ideal column oven /TCC should maintain a constant temperature for reproducible operation of the instrument.

1.5. Pumps: Pumps are an essential component of the HPLC instrument as a stable flow of the solvent and pressure are essential for reproducible chromatography. Pumps should also be able to withstand a range of pressures and flow rates, and various compositions of the mobile phases.⁴

1.6. Detectors: An ideal detector is required to be extremely sensitive to detect even small concentrations of the sample, be extremely selective, and have a low baseline noise.⁴ Detectors are broadly categorized into a) those that measure bulk property and b) those that detect specific properties. Some examples of the former class are refractive index detectors (RI), evaporative light scattering detectors (ELSD), and conductivity detectors. While these detectors are almost universal and measure bulk property, they have low sensitivity. The second class of detectors measure specific properties of the

analytes and some examples are ultraviolet absorption (UV) detector, mass spectrometer (MS) detector, and fluorescence and electrochemical detector (ECD).

The most commonly used detectors are UV. Though UV detectors are simple and robust they require analyte molecules to bear a chromophore group in order to be detected.^{4,5} Hence compounds that lack such a chromophore group either go undetected or need to be chemically converted to a chromophore bearing form before they can be detected. MS detectors on the other hand are highly specific and sensitive. However these detectors are very expensive and also require the sample to be ionizable in order to be detected. The two kinds of detectors that have been used in this project are UV and MS and a detailed explanation of their principle of operation is given in the following section.

C. Background on detectors

1.1. The UV detector works on the principle of the Beer-Lambert law that states the absorbance of electromagnetic radiation is proportional to the concentration of a compound in the cell and the cell length.⁶ Electromagnetic radiation either excites electrons from a lower energy level (ground) to a higher energy level (excited) or results in an increased rotation/vibration of the molecule. Either of these alterations results in a decrease in the intensity of the transmitted light and thus a measure of the absorbance helps estimate the concentration of the sample.

UV and visible radiation results in excitation of pi/non-paired electrons of some functional groups. Some types of UV detectors are fixed wavelength detectors, variable wavelength detectors (VWD), and diode array detectors (DAD).⁶ VWD's use a

monochromatic slit and grating to select one wavelength to pass through a sample cell.⁷ DAD's enable a sample to be subjected to light of all wavelengths generated by a lamp and are the most versatile of all the UV detectors and hence most frequently used in method development.^{6,7} A typical UV detector permits a user to use wavelengths between 190 and 380 nanometers (nm) with 254 nm being most commonly used.⁸

In DAD, light from a broad emission source is collimated by an achromatic lens to concentrate the light exiting through a detector cell, onto a holographic grating. The light spread from the grating falls onto a diode array that contains hundreds of diodes. The output from any of the diode can be selected and a chromatogram be developed using the UV wavelength that falls on that particular diode, thus producing a real time chromatogram.⁶ In spite of the ease and low cost of UV detectors, these detectors require the molecule to be tested to have a chromophore (color bearing) group in order to be detected. Figures 3 and 4 below show a schematic diagram of a VWD and DAD respectively.

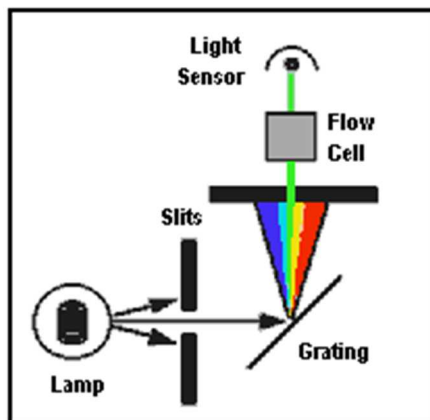


Figure 3. Arrangement of a variable wavelength detector⁷ (Reprinted with permission from LCResources.com)

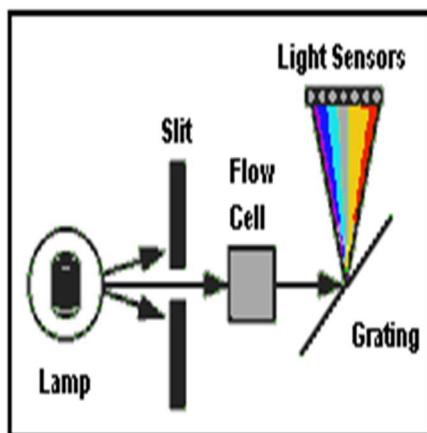


Figure 4. Instrumental setup of a diode array detector⁷ (Reprinted with permission from LCResources.com)

1.2. Mass spectrometers are the ultimate choice for any detector as they are highly selective and sensitive. The only downside of these detectors are their cost and need for a sample to be ionizable for detection.⁹ The three essential components of any mass spectrometer are an ion source, a mass analyzer, and a detector. An ion source, usually a

high energy beam of electrons ionizes the sample to produce ions.⁹ Some of the diverse methods employed to achieve ionization include electron impact (EI), chemical ionization (CI), electron spray ionization (ESI), fast atom bombardment (FAB), and matrix assisted laser desorption (MALDI).¹¹ The mass analyzer separates the ions according to the mass /charge ratio, by focusing the accelerated ions in a beam that is bent by an external magnetic field.^{9,11} Some of the different kinds of mass analyzers are quadruple mass filters, quadruple ion traps and time-of-flight (TOF). Lastly, a detector records the charge-induced current that is created when an ion hits the surface.¹¹ Figure 5 shows the layout of a mass spectrometer.¹⁰

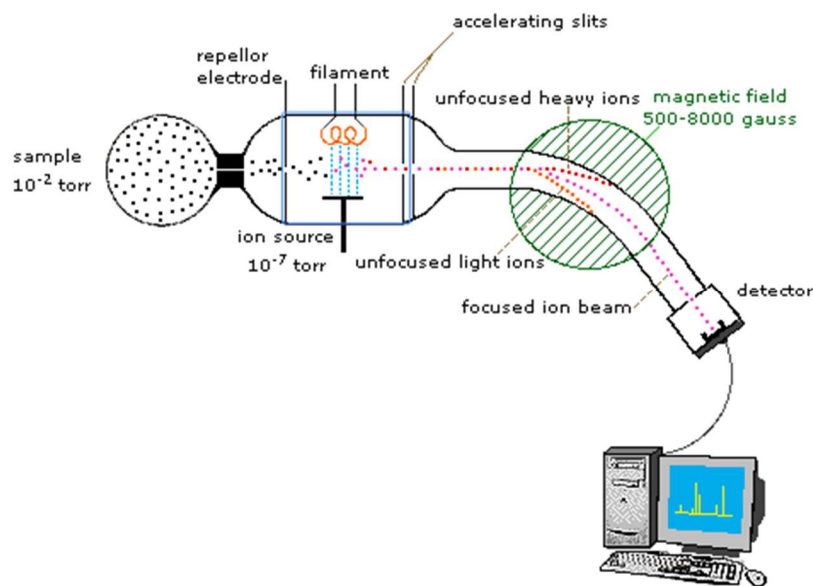


Figure 5. Layout of mass spectrometer¹⁰ (Reprinted with permission from William Reusch)

Quadrupole mass filters are utilized for a large number of studies in organic chemistry due to their high reproducibility, ease to operate, and robustness. The quadrupole mass filters can also be interfaced with a variety of inlet systems. However, they lack high resolution. The instrumentation consists of four parallel rods of hyperbolic cross section set up symmetrically. By applying an appropriate ratio of radio frequency and DC voltage to the rods, desired ions are focused to pass through the vacuumed quadrupoles towards an electron multiplier detector and a plot of ion counts vs ion mass is recorded.¹²

D. Chromatographic parameters

Some of the parameters needed to be considered during chromatographic studies are capacity factor (“k”), selectivity (“ α ”), resolution (“Rs”), and plate height (“H”).

1.1.Capacity factor /retention factor (“k”) measures the retention time of compounds relative to an unretained compound. “k” is calculated using Equation (1) below:

$$k = (t_R - t_0) / t_0 \quad \text{Equation 1}$$

While t_R denotes the retention time for the sample compound, t_0 represents the elution time of an unretained compound. For an unretained compound that elutes with the solvent front, k equals 0.

A higher retention factor indicates a strong retention of the compound on to the column. The capacity factor (“k”) is a dimensionless quantity and is also independent of column dimensions. The most common way to alter retention factor is to adjust the solvent strength of the mobile phase. A “k” value less than 1 is unreliable as the analytes

may be co eluting with other sample components, while a higher “k” may also be undesirable as it may require a long time for the sample to elute, thus involving more time and solvent expenditure. It is also observed that as the retention factor increases, the peak height decreases and peak width increases. An ideal value for “k” is between 2 and 10.

1.2. Selectivity (“ α ”) is a measure of the system to chemically distinguish between sample components and is given by Equation (2). It is measured as a ratio of the retention factors of two peaks and is visualized as the distance between apices of two peaks as is illustrated in Figure 6.¹³

$$\alpha = (k_2/k_1) = (t_{R2}-t_0)/(t_{R1}-t_0) \quad \text{Equation 2}$$

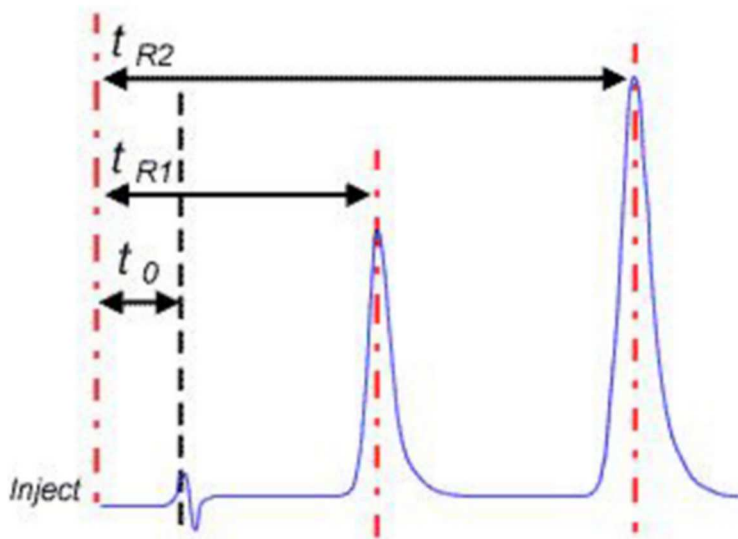


Figure 6. Illustration of resolution [Reprinted with permission from Chromacademy.com]

As can be clearly seen from Figure 6, the second compound has a longer retention time (“ t_{R2} ”) and thus “ k_2 ” is larger than “ k_1 ”. A good separating power (high “ α ”) is indicated by an adequate separation between the apices of adjacent peaks. However, this

is not directly indicative of good resolution and “ α ” also depends on the properties of the analyte, stationary phase, and the mobile phase. Any of these three factors may be altered to optimize the selectivity of an HPLC separation. Additionally the solvent strength, additives, and temperature also affect the selectivity factor. The pH of the mobile phase is another key factor especially when analyzing molecules having an ionizable group.

1.3. Efficiency (“N”) /plate height (“H”) measures the dispersion of the analyte band as it travels through the HPLC system/column and is mathematically given in Equations (3) and (4). Column performance is reflected by the value of “N.” A large “N” indicates a highly efficient column.¹³ The column is divided into theoretical plates and the plate height denotes the distance over which the sample components achieve an equilibration between stationary and mobile phases in the column. The plate height is also referred to as “height equivalent to theoretical plate/HETP” and is given by Equation (5) below. For a highly efficient column, the distance between two successive plates (H) is small and the number of theoretical plate (N) is large.

$$N = 16 (t_R/t_w)^2 \quad \text{Equation 3}$$

$$N = 5.54 (t_R/t_{w0.5})^2 \quad \text{Equation 4}$$

$$H = L/N \quad \text{Equation 5}$$

1.4. Resolution (“Rs”) is a quantitative measure of how well two peaks can be differentiated in a chromatographic separation and is defined as the difference in retention times between two peaks divided by the combined width of the elution peaks and is given in Equation (5). In Equation (6), B has a longer retention time than A and

“ w_A ” and “ w_B ” denote the peak widths of peaks A and B respectively. The fundamental formula for resolution involving all the parameters is given in Equation (7).

$$R_S = 2 [(t_R)_B - (t_R)_A] / W_B + W_A \quad \text{Equation 6}$$

$$R_S = \frac{1}{4} \sqrt{N} \times [(\alpha - 1)/\alpha] \times [k/(k + 1)] \quad \text{Equation 7}$$

E. Silica surface-surface modification

Silica has been traditionally used as a solid support for chromatography due to its high mechanical strength and the ability to accurately fabricate particles of desirable pore size or diameter.¹⁴ However, irregularly shaped silica used initially restricted the flexibility of chromatographers to the usage of organic solvents for organic normal phase chromatography leading to poor efficiency and irreproducible results. Reverse-phase chromatography employed silica bonded to low polarity organosilanes using siloxane bonding technology (Si-O-Si-C).¹⁴ The hydrolytic susceptibility of this bond also became problematic while working at high or low pH, strong buffers, and ion pairing reagents. Subsequently, spherical shaped silica particles that were more stable, uniformly shaped, sized, and those that gave better precision began to be used. However even with these type of silica frequent tailing problems were observed while separating basic compounds at an acceptable pH and this led to the development of Type B silica materials.¹⁴

Type B silica of high purity demonstrated increased pH stability, improved packed-bed stability, and improved peak shape for chromatographic separations.¹⁴ The minimal trace metal content in the lattice also aided in reducing the effect of free silanol interactions when the solute was ionized.

End-capping the silica surface by reacting the silica surface with a small reagent such as trimethylchlorosilane deactivates the silanol groups on the surface and also helps improve peak shapes for some compounds. It also serves the purpose of preventing the rapid dissolution of the silica at a higher pH.¹⁴ However, even after end-capping many undesirable effects were observed during chromatographic separations like the loss of retention at a higher pH from the disintegration of the underlying silica bed and resulted in the loss of bonded material and instability of the packed columns. The hygroscopic nature of type A and B silica also led to retention time variability in normal phase separations and necessitated the careful control of the water content in the mobile phase while using these types of silica-based stationary phases.¹⁴ To overcome the disadvantages that result from using Type A and B silica, a novel grade of materials Type C silica was synthesized and characterized by Dr. Joseph Pesek of San Jose State University.

F. Type C silica-silica hydride

Silica hydride opened newer avenues for chromatographic /electro chromatographic analysis and separation by overcoming many challenges that were previously observed while using Type A and Type B silica.¹⁵ The novelty of Type C silica resulted from the replacement of polar silanol (-OH) groups by a non-polar hydride species (-H) that imparted many useful chromatographic properties.^{14,16,17} The silica hydride surface possess nearly 10 times less silanol activity as compared to an end-capped Type B silica.^{14,15,17,18} Figures 7a and 7b below depict the structural differences between Type B and C silica.¹⁵

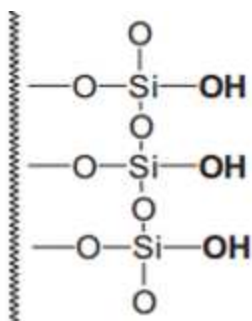


Figure 7a. Type B silica with silanol group on Si surface ¹⁵ (Reprinted from from *TrAC Trends in Analytical Chemistry*,42, Pesek, J. J., Matyska, M. T.,Boysen, R. I., Yang, Y., & Hearn, M. T,Aqueous normal-phase chromatography using silica-hydride-based stationary phases, 64-73,2012, with permission from Elsevier)

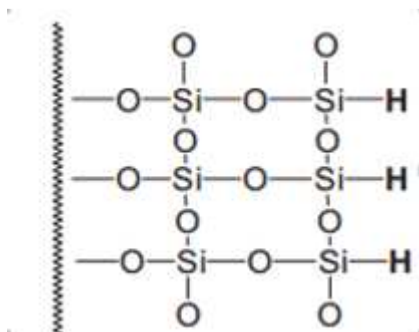


Figure 7b. Type C silica with silica hydride on Si surface¹⁵ (Reprinted from from *TrAC Trends in Analytical Chemistry*,42, Pesek, J. J., Matyska, M. T.,Boysen, R. I., Yang, Y., & Hearn, M. T,Aqueous normal-phase chromatography using silica-hydride-based stationary phases, 64-73,2012, with permission from Elsevier)

The lack of a polar group on the silica surface in Type C silica also resulted in little or no water adsorption, thus making it a good choice for organic normal phase chromatography with a variety of solvents and improved speed.^{14,19} The Type C silica surface also provided stable chromatographic results when used between pH of 2 to 8.¹⁴ It was also possible to conduct chromatography over a wide range of mobile phase compositions ranging from 100% aqueous to pure organic solvents when utilizing a silica

hydride-based column.^{14,19,20} Further, no phase dewetting/phase collapse was observed while using a pure aqueous mobile phase on a silica hydride based column as opposed to what is observed on an RP column.^{15,19} This new class of stationary phase could also be used in any of the three modes of chromatography, organic normal-phase (ONP), aqueous normal-phase (ANP), and reverse-phase chromatography (RP) and it exhibited minimum hysteresis while changing from one mode to another.^{14,17,19,20,21} This unique property also helped silica hydride stationary phases to retain both polar and nonpolar compounds, thus providing an experimental flexibility not available with other stationary phases based on organosilane chemistry.^{15,17,19} The equilibration time when changing from RP to NP or vice versa was also minimal and in some cases, both the NP and RP mechanisms operated simultaneously in a single isocratic run.¹⁷ The reproducibility between successive chromatographic runs and runs on different batches of the same Type C based stationary phase was also found to be excellent.^{17,19}

G. Fabrication of silica hydride

There are two synthetic approaches to the fabrication of silica hydride materials:

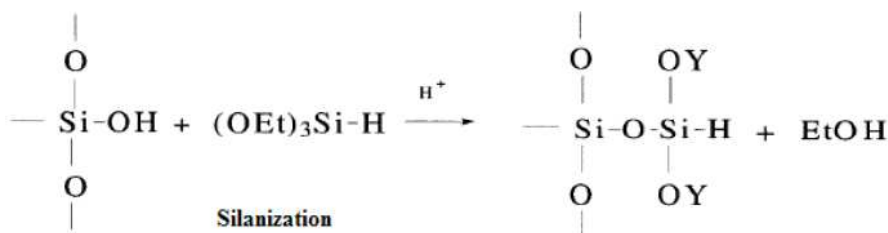
1) Conversion of silicon hydroxide to a silicon chloride intermediate using thionyl chloride (SOCl_2) and subsequent reduction with lithium aluminum hydride (LiAlH_4).

The reaction is conducted in a moisture free environment as the silicon-chlorine bond is hydrolytically unstable and reverts to silicon hydroxide in the presence of water.^{17,21}

2) The second approach is a single step process involving a condensation reaction between silanols and triethoxysilane in the presence of an aqueous acid catalyst. The relative number of silica hydride groups to residual silanols depends on a number of

reaction parameters. However, ^{29}Si -CP-MAS-NMR studies reveal hydride coverage greater than 95%.²¹

The second approach has been used to synthesize the column used in the current research. A silanization reaction converts the silanol group on the silica surface to a hydride group and a subsequent hydrosilation reaction between the silica hydride intermediate and an unsaturated group, such as olefin in the presence of a catalyst (hexachloroplatinic acid /Spiers catalyst) or a free radical initiator (tertiary-butyl peroxide), gives a surface with the desired organic moiety attached.^{15,20} Figures 8 and 9 presents the scheme of silanization and hydrosilation reactions respectively.



Y = Si or H depending
on extent of crosslinking

Figure 8. Silanization reaction (Reprinted from *Journal of Chromatography A*, Pesek, J. J., Matyska, M. T., Oliva ,Michelle., & Evanchic, Marc., Synthesis and characterization of bonded phases made via hydrosilation of alkynes on silica hydride surfaces,145-154,1998,with permission from Elsevier)

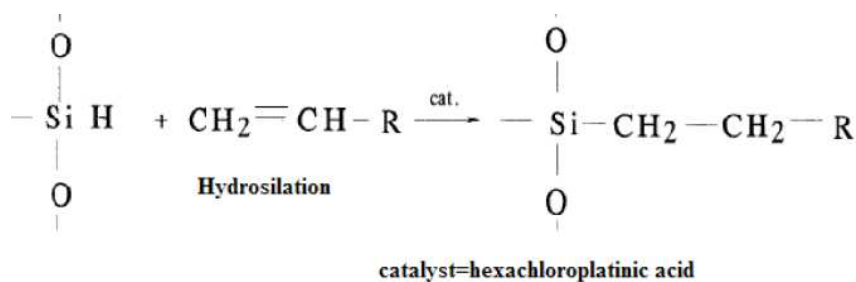


Figure 9. Hydrosilation reaction¹⁵ (Reprinted from *TrAC Trends in Analytical Chemistry*, 42, Pesek, J. J., Matyska, M. T., Boysen, R. I., Yang, Y., & Hearn, M. T., Aqueous normal-phase chromatography using silica-hydride-based stationary phases, 64-73, 2012, with permission from Elsevier)

The specific property of the separation medium that has a stable Si-C bond (hydrophobic, hydrophilic, or ion exchange property) is reflected by the nature of the organic moiety attached to silica hydride surface in the hydrosilation reaction.^{15,19,20,21} Though terminal olefins are usually employed in the hydrosilation step, it is also possible to bond olefins with a non-terminal double bond, alkynes or cyano groups to the silica hydride surface and in the case of alkynes, a bidentate attachment to the surface is observed.²¹ Table 1 below provides examples of silica hydride stationary phases and their applications to analysis of various compounds.¹⁵

Table 1. Examples of silica hydride stationary phases and their application in analyzing various compounds¹⁵ (Reprinted from from *TrAC Trends in Analytical Chemistry*, 42, Pesek, J. J., Matyska, M. T., Boysen, R. I., Yang, Y., & Hearn, M. T., Aqueous normal-phase chromatography using silica-hydride-based stationary phases, 64-73, 2012, with permission from Elsevier)

Functional group at the surface	Compounds
Small alkyl (Diamond Hydride)	Amino acids, carbohydrates, small organic acids and other metabolites, peptides, propantheline bromide, fluoxetine, nucleotides
Phenyl	Propantheline bromide, fluoxetine
Cholesterol	Nucleotides, quinolinedione, tetramic acid, benzopyran, choline, tobramycin, peptides, ribose nucleosides, cytidine derivatives
Octadecyl	Metformin, glyburide, amphotericin, peptides, glucosamine, tobramycin, triacetyl derivatives, carvone, loratadine
Alkyl carboxylic acid	Amino acid, nucleic acids, theophylline-related compounds, anilines, benzoic acids, choline and tobramycin

H. Aqueous normal phase chromatography

ANP chromatography is a novel separation mechanism displayed by silica hydride based stationary phases using an acidic / neutral pH of mobile phase showing a retention pattern similar to that of normal phase chromatography.^{16,18} However, in ANP the mobile phase also contains a small amount of polar solvent such as water in addition to the organic solvent.^{17,18} This type of chromatography exhibits “dual retention” behavior by retaining polar and non-polar compounds on the same column. The retention of a polar compound increases with an increase in the concentration (60% or more) of the organic solvent, usually ACN and the retention of the hydrophobic/non-polar compound increases with an increase in the concentration of the polar component in the mobile phase.^{17,18} In ANP chromatography, a mobile phase with a high pH is not necessary in order to separate polar and non-polar compounds as opposed to RP. Additives such as formic acid, acetic acid, ammonium acetate, ammonium formate are also present in the mobile phase to serve the purpose of a buffering reagent and to improve peak shapes.¹⁸

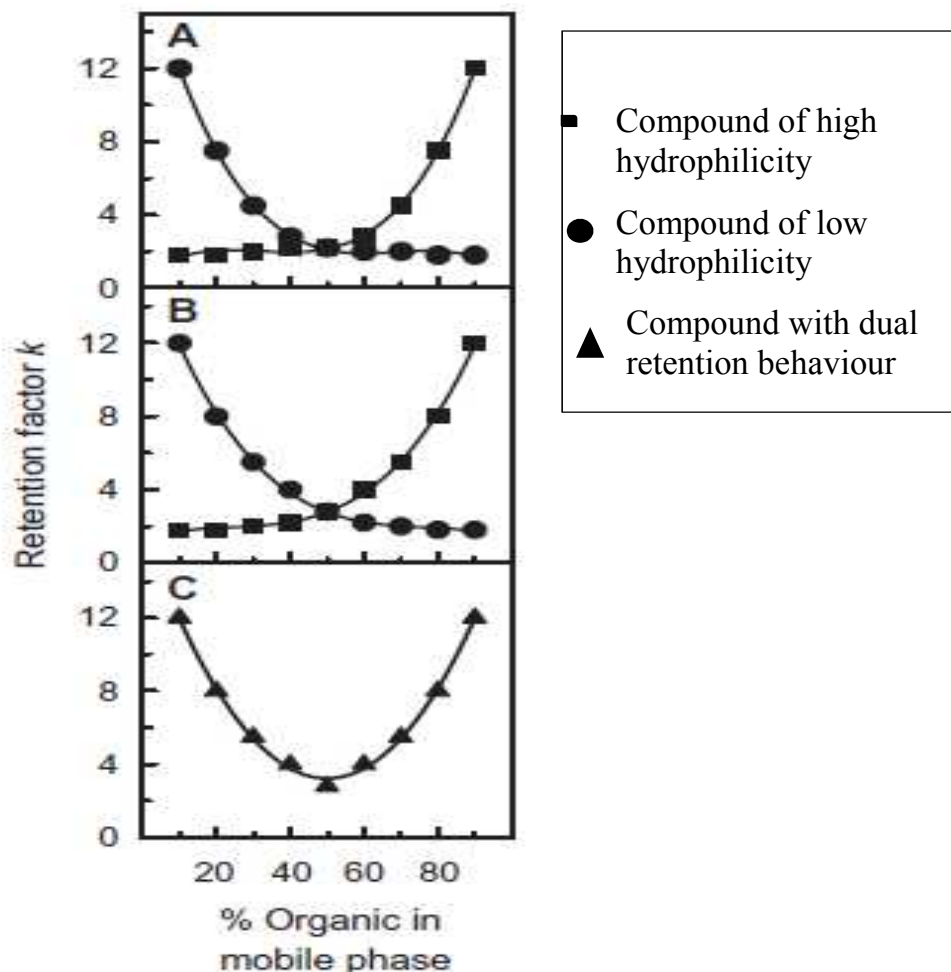
In the RP retention mechanism, the hydrophobic interaction between the organic moiety on the stationary phase and the analyte tends to dominate the mechanism with silica hydride materials.¹⁷

ANP can be coupled with mass spectrometer detectors for analysis of metabolites (amino acids, small organic acids, and carbohydrates) in physiological samples.¹⁶ Some of the other advantages observed in the ANP retention mechanism are excellent reproducibility and minimum column equilibration time.¹⁶

The partition mechanism between the mobile phase and a hydration shell on the silica surface is observed for typical hydrophilic interaction liquid chromatography (HILIC) chromatography. Partitioning occurs between the mobile phase and a thin layer of water (hydration shell) on the silica surface. The hydration shell formed as a result of the interaction between the polar silanol groups and the water molecules in the eluent has a greater affinity for polar analyte molecules than the non polar analytes and is thus responsible for the NP retention displayed by HILIC materials.¹⁷ Type C silica that has half of a monolayer of water cannot function by such a partition mechanism and an adsorption mechanism, wherein an adsorbed water molecule or a component derived from the mobile phase is displaced by solute molecules is more likely.¹⁷ However, the exact mechanism of ANP retention has not been fully established.

HILIC materials separate only polar compounds and no separation can be achieved while using mixtures containing polar and non-polar compounds on such a material, while a type C based stationary phase material exhibits dual retention or retention of both polar and non-polar compounds.¹⁵ Figures 10a,10b, and 10c shown

below illustrate the three kinds of retention observed in the ANP chromatography separation mechanism.¹⁵



Figures 10a, 10b, and 10c. The three kinds of retention mechanism observed in ANP¹⁵(Reprinted from *TrAC Trends in Analytical Chemistry*,42, Pesek, J. J., Matyska, M. T.,Boysen, R. I., Yang, Y., & Hearn, M. T,Aqueous normal-phase chromatography using silica-hydrate-based stationary phases, 64-73,2012, with permission from Elsevier)

In Figure 10a, clear NP and RP mechanisms with no overlap over any mobile phase composition range is observed. At a higher water content, the non-polar /hydrophobic molecules are retained while the polar molecules/hydrophilic species elute at void volume. A reverse trend is observed at higher concentration of the nonpolar

component. Figure 10b shows both RP and NP operating with a small range of mobile phase concentrations (around 45-65% organic content) thus exhibiting an overlap of the two retention mechanisms and enabling the separation of mixtures of polar and non-polar compounds under isocratic conditions.¹⁵ Gradient elution can also be used to improve the efficiency and decrease retention times.¹⁵ The retention as illustrated in Figure 10c is observed for solute molecules having polar and non-polar groups hence permitting either the RP or ANP mode to operate by varying the mobile phase composition.^{15,17,19} The “U” shaped retention profile is typical of ANP retention and can be explained as a switch in the retention mechanism from RP to NP.¹⁹

I. Types of samples used in the evaluation of HPLC column

In this research, small molecules such as amino acids, nucleotides, nitrogenous bases (purine and pyrimidine), organic acids, ergogenic acids, organic amines, and polycyclic aromatic hydrocarbons (PAH) have been used. The first class consists of amino acid molecules such as L- phenylalanine, L- tryptophan and phenyl thiohydantoin (PTH) derived amino acids such as PTH asparagine, PTH aspartic acid, and PTH Isoleucine. Amino acids are important biological molecules that exist as zwitterions at normal physiological pH (pH between 6 and 8) and they constitute the monomeric units of peptides.²¹ Amino acids are composed of an amino group (-NH₂) at one end and a carboxylic acid group (-COOH) at the other end and this multiple polarity makes it difficult to separate them on RP based columns.²² The dual retention capacity observed in ANP makes these small molecules a suitable candidate for study.

Of the 22 amino acids only tyrosine, tryptophan and phenylalanine can be detected by a UV detector. The remaining amino acids need to be derivatized before they can be detected in the UV-visible range. Some of the compounds used for derivatizing amino acids are dansyl chloride, ortho-phthalaldehyde (OPA), 9- fluorenylmethoxy carbonyl (FMOC), and phenyl thiohydantoin (PTH). In this project, three PTH derived amino acids have been used besides L-phenylalanine and L-tryptophan.

The second class of compounds tested includes nitrogenous bases and nucleosides that make up the backbone of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). The retention of nucleotides such as adenosine triphosphate (ATP), adenosine mono phosphate (AMP), nicotinamide dinucleotide phosphate (NADP), and nicotinamide dinucleotide (NAD) that are found in cell extracts and used as biomarkers for various diseases have also been studied in this project.²³ The determination of nucleotide concentration is significant in order to realize the degree of cellular metabolic stress and inherited diseases of purine and pyrimidine nucleotide metabolism²³. The NAD/NADP ratio is thus an indication of the metabolic activity and health of the cells.²³ The highly charged and polar nature of these molecules makes retention difficult on RP columns. Further, the incompatibility of other chromatographic techniques with MS detectors also makes it challenging to analyze these molecules. Thus, they become suitable solutes for ANP retention study.

PAH's such as naphthalene, fluorene, and acenaphthene are expected to exhibit reverse phase retention. An ergogenic acid such as creatinine that is obtained as a breakdown product of creatine and vitamin B12 (thiamine hydrochloride) have also been

found to be relevant candidates for this study. Lastly, the retention behavior of theophylline, caffeine, 7-(2,3-dihydroxypropyl) theophylline and other some small organic compounds such as benzoic acid, p-amino benzoic acid, p-nitro aniline, and p-amino ethyl benzoate have also been investigated in this project.

J. Silica hydride based undecynoic acid (UDA) column

The silica hydride based UDA had been previously synthesized in our lab and packed into columns of the following dimensions: 75cm x 4.6 mm id and 50 cm x 2.1 mm id. The former column was used while conducting studies on a UV detector while the latter was used for MS studies.

UDA is an 11 carbon chain molecule terminating in a carboxylic acid (-COOH) group. It has a bidentate attachment to the silica hydride surface and the column displays weak cation exchange properties in addition to the ANP behavior. The weak cationic exchange property of the silica hydride based UDA column is attributed to the presence of a carboxylic acid group on the organic moiety. The (-COOH) group is either neutral or negatively charged based on the pH of the mobile phase. While under acidic conditions the carboxylic acid group is fully protonated and neutral, at a pH above the pKa the acidic group is deprotonated and capable of exhibiting the ion exchange property. The ion exchange property of the UDA provides additional selectivity in cases where ANP retention fails to provide full resolution.²⁴

K. Research goals

The primary goal of this research was to study the normal and reverse phase retention ability of the silica hydride based UDA column using HPLC. In order to

establish the dual retention property, a number of small molecules like amino acids, nucleosides, nucleotides, ergogenic acids, nitrogenous bases, and other small organic molecules were tested and their retention times documented. UV detectors were used for the isocratic runs of these molecules and the ANP retention behavior of the UDA column was established. The solvents used for the retention study were DI water and ACN both containing formic acid at a 0.1% volume/volume (v/v) concentration.

The second goal was to evaluate the change in retention behavior of some of the polar and non-polar compounds with a change in the buffer concentration, using 0.05 %, 0.075%, 0.2%, and 0.3% (all concentrations expressed as v/v) formic acid. Yet another goal of the current research was aimed at testing the compatibility of a new buffer such as ammonium acetate and to study the retention time of few polar compounds with a change in the concentration of ammonium acetate.

A further objective was to examine the retention behavior of some selected nucleotides on the UDA column. The biological significance of studying nucleotide concentration makes this an important application of using a silica hydride based undecynoic acid column. Another objective of this project was to analyze the effect of a change in column temperature on the retention time of some polar compounds.

Additional objectives were directed at analyzing urine samples from farmers to check them for the presence of certain pesticides as well as their quantitative determination and examining pomegranate peel samples for the presence of certain phenolic and other compounds. Pomegranate peels contain a large variety of compounds such as phenols, flavonoids, tannins and besides their nutritive values have also been

found to have many features such as antibacterial, antiamoebic, analgesic, and anthelmintic properties.²⁵ The UDA column and an MS detector have been used to estimate the various compounds present in the pomegranate peel samples.

II. EXPERIMENTAL

A. Column

The analytical columns used in this investigation were synthesized and characterized previously by another research student. The dimensions of the columns used for HPLC- UV detection and LC-MS detection are 7.5 cm x 4.6 mm id and 50 x 2.1 mm id column, respectively. The particle size of both columns is 4 μm (micrometers) with a pore size of 100 A° (Angstrom).

The silica hydride based UDA column exhibits weak cationic exchange properties in addition to the RP/ANP retention mechanism. The terminal carboxylic acid group contributes to the additional selectivity of the column. The (-COOH) group is between 90% and 99% negatively charged when using a mobile phase at pHs of 5.9 and 6.9, respectively. The cationic nature of the column is exhibited only at a pH range of 6 or higher and is completely absent below pH 4.9. Table 2 gives details on some of the column specifications of the Cogent UDA columns manufactured by MicroSolv technologies (Eatontown, NJ). The maximum pressure that the columns can bear is 4500 psi and 8000 psi for the 4 μm and 2 μm particle sizes, respectively.²⁶

Table 2. Column specifications of Cogent UDA column²⁶ (Reprinted with permission from MicroSolv Technology)

Bonded Phase	Pore Size	Surface Area	Pore Volume	Particle Size	End-Capping	Carbon Load	pH Range	Max Temp
UDA™	100Å	390 +/- 30m ² /gm	1.00 +/- 0.10 mL/g	4µm	N/A	14-15%	2.0-8.0	80°C
UDA 2.0™	120Å	340 +/- 20m ² /gm	1.00 +/- 0.10 mL/g	2.2µm	N/A	14-15%	2.0-8.0	80°C

B. Compounds and mobile phase used for characterization studies

All the compounds used for this research were stored in the refrigerator and sample stock solutions with a 1 mg/mL concentration were prepared for the HPLC-UV and HPLC-MS studies. The mobile phases and the chemical compounds used for column characterization are shown in Tables 3, 4.1, 4.2, 4.3, 4.4, and 4.5 below. The chemical structures of all the compounds are shown in Figure 13.

Table 3. Chemicals used as mobile phase

Chemical name	Manufacturing company
Acetonitrile (Lot no: 981414, CAS-75—05-8)	Fisher Chemicals Fair Lawn, NJ
Formic acid (Lot # 127 F-3436)	Sigma Chemical Company, P.O Box 14508, St. Louis, MO – 63178
Ammonium acetate (GFS lot no: P997392)	Matheson Coleman & Bell, Rutherford, NJ

Table 4.1. Nitrogenous bases, nucleosides, nucleotides and ergogenic acids

Chemical name	Manufacturing Company
NADP	Sigma Chemical Company, P.O Box 14508, St. Louis, MO – 63178
NAD	Sigma Chemical Company, P.O Box 14508, St. Louis, MO – 63178
Creatinine	Sigma Chemical Company, P.O Box 14508, St. Louis, MO – 63178
Cytosine	Nutritional Biochemical Corp. Cleveland, OH
Uracil	Sigma Chemical Company, P.O Box 14508, St. Louis, MO – 63178
Adenine	Sigma Chemical Company, P.O Box 14508, St. Louis, MO – 63178
Thymine	Sigma Chemical Company, P.O Box 14508, St. Louis, MO - 63178
Adenosine	CALBIOCHEM Co. LA
Thymidine	Nutritional Biochemical Corp. Cleveland, OH
ATP	Sigma Chemical Company, P.O Box 14508, St. Louis, MO - 63178
AMP	Sigma Chemical Company, P.O Box 14508, St. Louis, MO - 63178

Table 4.2. Amino acids

Chemical name	Manufacturing Company
L-tryptophan	Sigma Chemical Company, P.O Box 14508, St. Louis, MO – 63178
L-phenylalanine	J.T. Baker Chemical Co. Phillipsburg, NJ- 08865
PTH Asparagine [Lot no: 71F-7714]	Sigma Chemical Company, P.O Box 14508, St. Louis, MO – 63178
PTH Isoleucine [Lot no: 90F-9018]	Sigma Chemical Company, P.O Box 14508, St. Louis, MO – 63178
PTH Aspartic acid [EEC no : 227-055-5]	Sigma Chemical Company, P.O Box 14508, St. Louis, MO – 63178

Table 4.3. Organic acids and amines

Chemical name	Manufacturing Company
Benzoic acid	Sigma Chemical Company, P.O Box 14508, St. Louis, MO - 63178
p-amino benzoic acid	Sigma Chemical Company, P.O Box 14508, St. Louis, MO - 63178
p-nitro aniline	Sigma Chemical Company, P.O Box 14508, St. Louis, MO - 63178
Ethyl p-amino benzoate	Sigma Chemical Company, P.O Box 14508, St. Louis, MO - 63178

Table 4.4 Polycyclic aromatic hydrocarbons

Chemical name	Manufacturing Company
Naphthalene	Sigma Chemical Company, P.O Box 14508, St. Louis, MO – 63178
Fluorene	Sigma Chemical Company, P.O Box 14508, St. Louis, MO – 63178
Acenaphthene	Sigma Chemical Company, P.O Box 14508, St. Louis, MO – 63178

Table 4.5. Xanthine-related and other miscellaneous compounds

Chemical name	Manufacturing Company
Caffeine	Sigma Chemical Company, P.O Box 14508, St. Louis, MO – 63178
Theophylline	Sigma Chemical Company, P.O Box 14508, St. Louis, MO – 63178
7-(2,3-dihydroxypropyl) theophylline	Sigma Chemical Company, P.O Box 14508, St. Louis, MO – 63178
Thiamine HCl	Matheson Coleman & Bell, Cincinnati, Ohio

C. Chemical structures of the chemical compounds analyzed

Figure 11.1-11.8 shows the chemical structures of all different classes of compounds tested on the column.

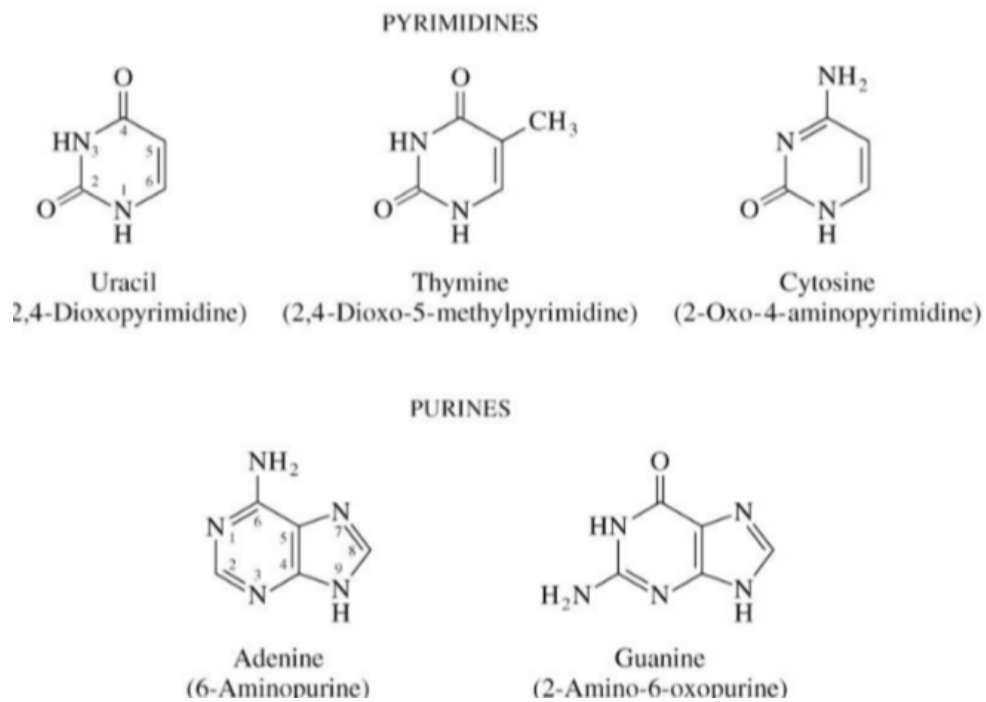


Figure 11.1. Chemical structures of nitrogenous bases

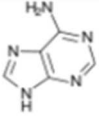
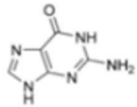
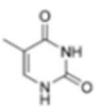
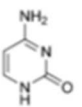
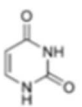
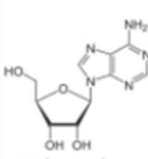
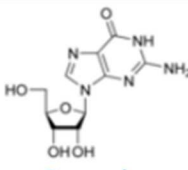
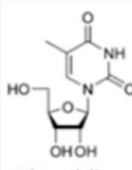
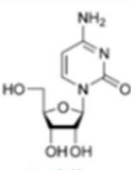
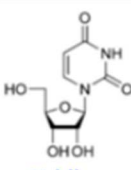
Nucleobase	 Adenine	 Guanine	 Thymine	 Cytosine	 Uracil
Nucleoside	 Adenosine A	 Guanosine G	 Thymidine T	 Cytidine C	 Uridine U

Figure 11.2. Chemical structures of nucleosides

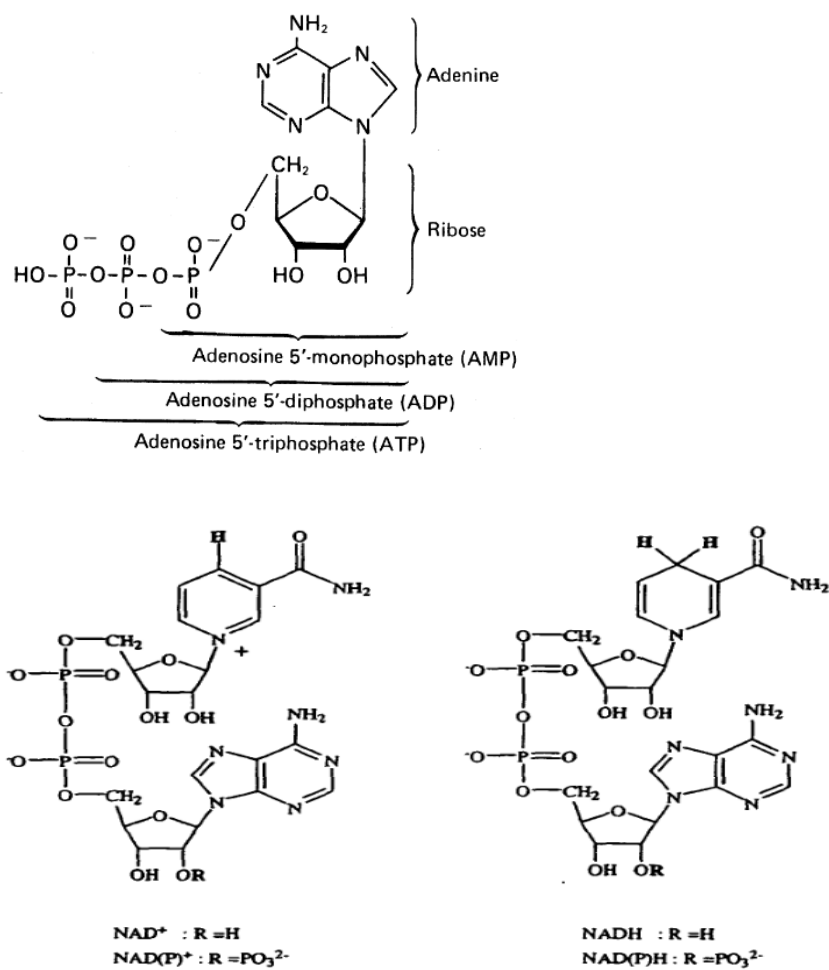
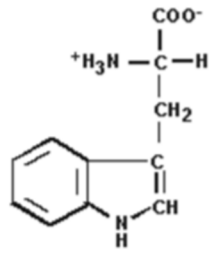
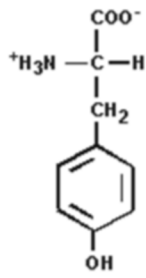


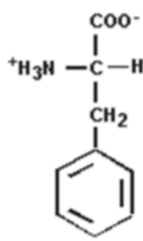
Figure 11.3. Chemical structures of nucleotides and coenzyme



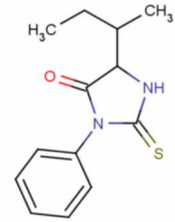
Tryptophan



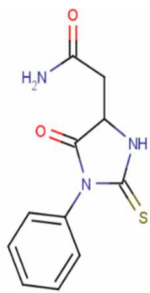
Tyrosine



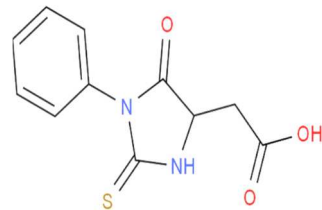
Phenylalanine



PTH Isoleucine



PTH Asparagine



PTH Aspartic acid

Figure 11.4. Chemical structures of amino acids

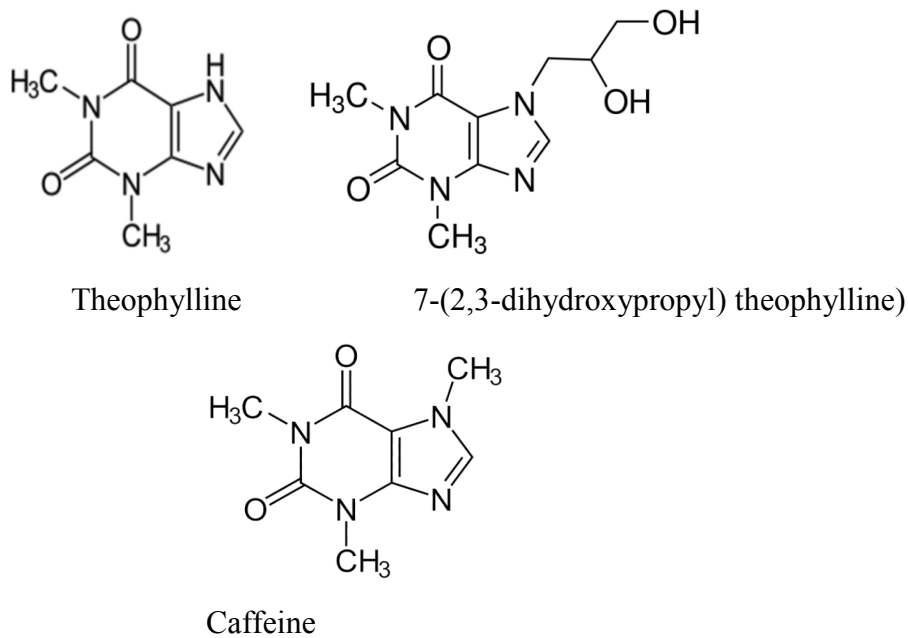


Figure 11.5. Chemical structures of xanthine-related compounds

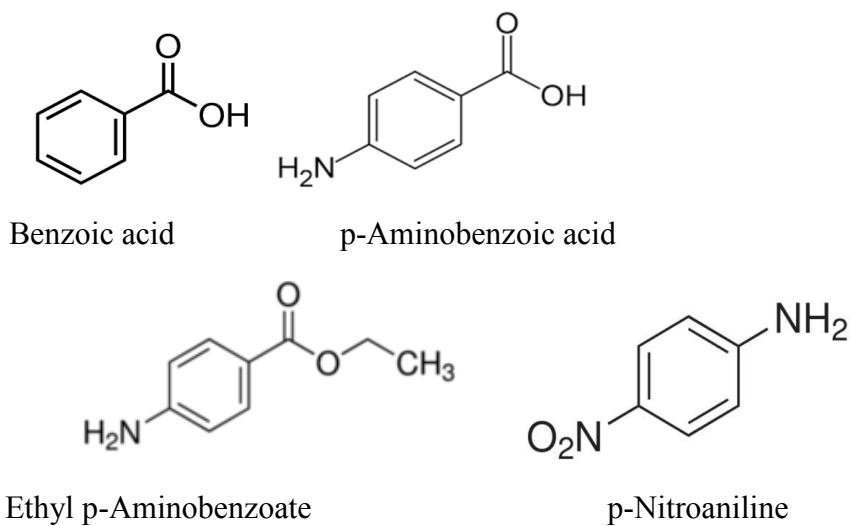


Figure 11.6. Chemical structures of small organic molecules

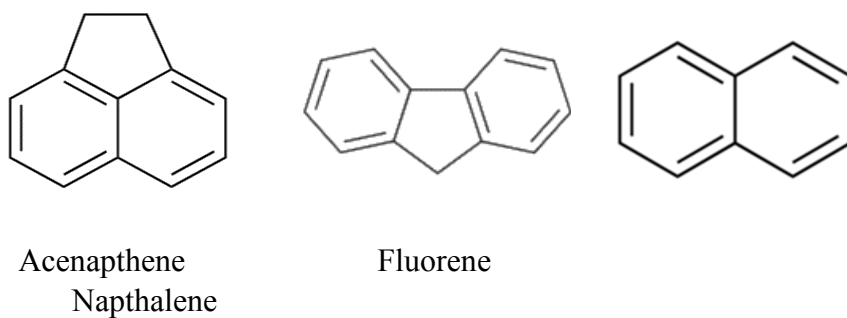
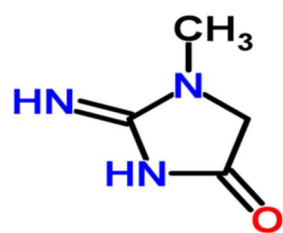
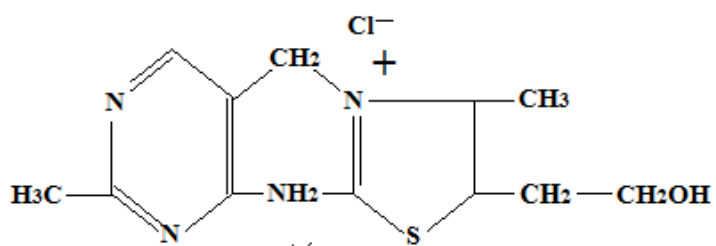


Figure 11.7. Chemical structures of Polycyclic aromatic hydrocarbons



Creatinine



Thiamine hydrochloride (Vitamin B1)

Figure 11.8. Chemical structures of miscellaneous compounds

D. HPLC and MS Instrumentation

Three types of instruments attached to three different kinds of detectors were employed to complete the current research. The first kind of HPLC instrument used for UV detection was a Hewlett Packard 1090 series which was used for column characterization and analyte retention studies. This instrument was introduced in the early 1980s. The 1090 series has a high performance solvent delivery system (DR5) with 1, 2 or 3 active solvent channels.²⁷ Each solvent channel contains a reservoir and a dual syringe metering pump with a rotary valve. Helium (He) degassing is essential and there are provisions for either manual injection or auto sampler injection. While it is only possible to do one injection at a time for the former, the latter permits up to 99 injections in a sequence. The oven temperature can be set to any range from 0-100 °C. Nitrogen gas connected to the instrument provided the pressure for the injector needle to move up and down and it was set at a pressure of 70 psi. For the purpose of instrument control and data collection, Chem Station software was used. For all UV detection studies, the wavelength of the detector was set to 214 nm and 254 nm, the injection volume to 2 µL and flow rate at 0.5 mL/min. The oven temperature for all retention studies was 40 °C. However, for the retention study with varying column temperature, the temperature was increased from 30-55 °C with a 5 °C increment at each step. The DI water used as a solvent was prepared on a Milli-QTM purification system (Millipore Corp., Bedford, MA). Figure 12 below illustrates a typical HP 1090 series instrument.



Figure 12. A view of the HP 1090 series HPLC-UV Instrument (Reprinted with permission from Labequip.com)

The second type of instrument used for pesticide analysis was an Agilent 1100 series LC system (Santa Clara, CA, USA), including a degasser, binary pump, and temperature controlled column compartment. The mass spectrometer was an Agilent 6220 MSD (time-of-flight) with a dual sprayer electrospray ionization source (ESI). The final mobile phase obtained after method development used a gradient. Solvent A was 10 mM ammonium acetate in DI water and solvent B was acetonitrile with 0.1% formic acid (v/v). The gradient program started with 100% B and was gradually decreased to 95% B over the next two minutes and held at 95% B for another six minutes, and then linearly increased to 100% B over the next one minute. The flow rate was maintained at 0.4 mL/min and the injection volume at 1 μ L. The analysis was done using m/z for $[M+H]^+$ in the positive ion mode. Data acquisition and instrument control was done

using Mass Hunter software. Figure 13 below illustrates the arrangement of a 1100 series LC system coupled with a 6220 series TOF detector.



Figure 13. 1100 series LC system-6220 series TOF detector (Reprinted with permission from University of Florida)

The third instrument employed for the study on the pomegranate peel samples was an Agilent 1200 SL series LC system (Santa Clara, CA, USA), including a degasser, binary pump, temperature controlled, and temperature controlled column compartment . The mass spectrometer was an Agilent 6520 MSD TOF with a dual sprayer electrospray ionization source (ESI). The drying gas temperature was set at 250 °C, flow rate set at 0.4 mL/min and the injection volume at 1 μ L. Solvent A was DI water with 0.1% formic acid (v/v) and solvent B was acetonitrile with 0.1% formic acid (v/v). Two different columns were employed for this analysis. The UDA column used had the same dimensions as stated earlier. The gradient program started at 15% B and was gradually

increased to 80% B over the next four minutes and held at 80% B for another two determination was done using m/z for $[M+H]^+$ in the positive ion mode. Data acquisition and instrument control was done using Mass Hunter software. Figure 14 shows the layout of a 1200 SL series LC system coupled with a 6520 Q-TOF detector (Quadrupole- time of flight).



Figure 14. 1200 series LC system-6520 series Q-TOF detector (Reprinted with permission from University of Washington)

III. EXPERIMENTS AND DISCUSSION

Column characterization studies

The main purpose of the research was to characterize the silica hydride-based undecynoic acid column. The dimension of the column used for this purpose was 7.5cm x 4.6mm id. Two different types of detectors were used for this purpose, an HPLC-UV detector (HP 1090 series) and an MS detector. Each HPLC run was repeated three times for the purpose of reproducibility and recorded. Graphs were plotted with retention time in minutes along the y-axis and % composition of organic component in the mobile phase on the x-axis. In studies where retention pattern was determined using a different concentration of the buffer/additive, the concentration was plotted on the x-axis and retention time in minutes along the y-axis. Further, in the characterization studies done at different oven temperatures, the column temperature was plotted on the x-axis and the retention time in minutes along the y-axis.

A. Chromatographic evaluation of HPLC column using UV detection

Amino acids, nitrogenous bases, nucleosides, nucleotides, ergogenic acids, xanthine-related compounds, and other small organic molecules were used to characterize the column. A series of runs were conducted by varying the mobile phase composition of the organic solvent from 30-90%, and each run was repeated three times. The injection volume for each sample was 2 μ L and the instrument flow rate and temperature were set at 0.5 mL/min and 40 $^{\circ}$ C, respectively. Stock solutions of the samples were prepared by dissolving 1 mg in 1 mL of the solvent. Further, the solutions were sonicated to dissolve the solute completely and obtain a clear solution. A fixed amount (100 μ L) of the stock

solution was micro-pipetted into a vial and diluted with 900 μL of the solvent to be injected into the HPLC instrument. In all of the characterization studies, the polar solvent (A) was DI water and the organic solvent (B) was ACN. The buffer used for all the characterization studies was formic acid (0.1% v/v) premixed with solvents A and B.

1.1. Evaluation of nitrogenous bases

All three pyrimidine bases (uracil, thymine, and cytosine) and a purine base (adenine) were run on the column with ACN compositions ranging from 30-90%. A mixture of 50:50 DI water:ACN premixed with 0.1% v/v of formic acid was used to dissolve all samples and prepare the stock solution. The retention data and retention plots for these compounds are provided in Table 5 and Figure 15. The order of retention time observed for these nitrogenous compound is as expected on a cationic exchange column with maximum retention observed for adenine and minimum for uracil. The retention times of thymine and uracil were found to be very close to each other. The presence of the amino group on adenine could probably account for the strong interaction and hence longer retention on the column. The retention time is relatively long for cytosine also; however, it is also slightly lower than adenine and this could be due to the presence of an electron withdrawing moiety (keto group). Thymine and uracil have similar retention times, which can be explained by the similarity in their chemical structures. Thymine with an additional methyl group in the ring structure, is slightly less polar and thus displays a lower retention time than uracil.

Table 5. Retention time data for nitrogenous bases

Concentration (%)		Retention time (Minutes)			
DI Water + 0.1% Formic acid	ACN + 0.1% Formic acid	Thymine	Cytosine	Uracil	Adenine
70	30	1.888	1.852	1.769	2.049
60	40	1.834	1.877	1.744	2.076
50	50	1.800	1.951	1.729	2.207
40	60	1.811	2.143	1.742	2.506
30	70	1.844	2.582	1.790	3.086
20	80	1.933	3.710	1.879	4.804
10	90	2.150	4.971	2.865	11.823

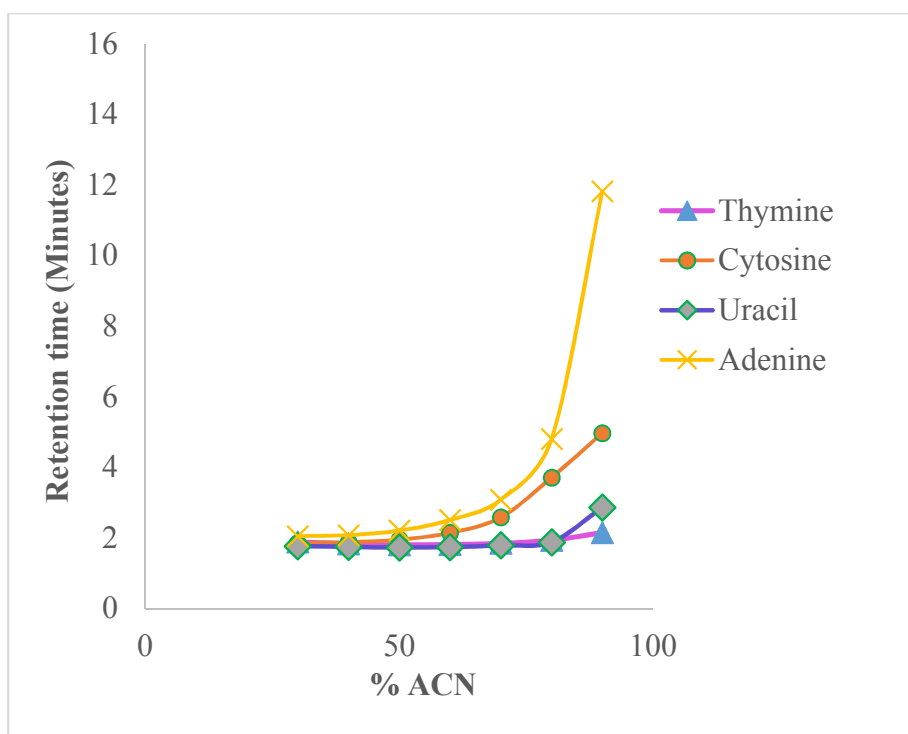


Figure 15. Retention time plot for nitrogenous bases

1.2. Nucleosides and nucleotides

Nucleosides consist of a nitrogenous base such as adenine, cytosine, guanine, and thymine along with a deoxyribose sugar, in the case of DNA. In RNA, uracil is found instead of thymine and each of the nitrogenous bases is attached to a ribose sugar for RNA.²⁸ Nucleotides are monophosphate, diphosphate, or triphosphate nucleosides wherein either one, two, or three sugars are phosphorylated, respectively. The atoms and groups on the nucleobases are responsible for the interaction. The nucleosides used for this project were adenosine and thymidine. Adenosine and thymidine were dissolved in 50:50 DI water/ACN premixed with 0.1% v/v formic acid and this was used to dissolve the samples and prepare the stock solution. The retention time data and plots for nucleosides are given in Table 6 and Figure 16. The higher polarity of adenosine as

compared to the thymidine results in the stronger retention on the UDA column and thus the retention time of adenosine is larger than thymidine.

Table 6. Retention time data for nucleosides

Concentration (%)		Retention time (Minutes)	
DI Water + 0.1% Formic acid	ACN + 0.1% Formic acid	Adenosine	Thymidine
70	30	2.078	1.852
60	40	1.847	1.753
50	50	1.835	1.703
40	60	1.871	1.676
30	70	1.952	1.686
20	80	2.166	1.720
10	90	2.670	1.781

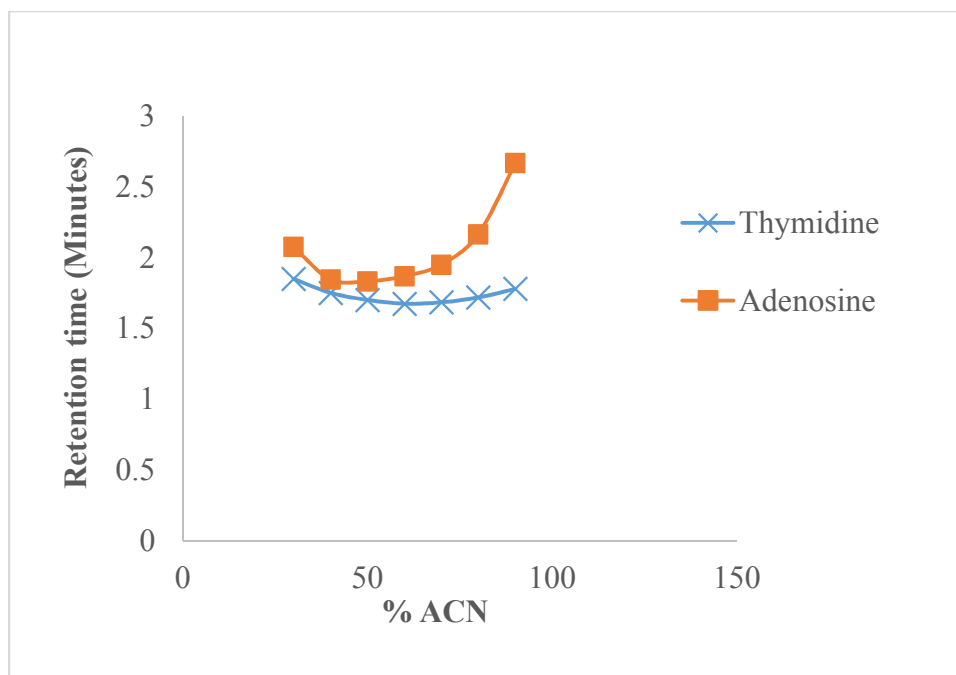


Figure 16. Retention time plot of nucleosides

1.3. Underivatized and PTH derivatized amino acids

L-phenylalanine, L-tryptophan, PTH asparagine, PTH aspartic acid, and PTH isoleucine were dissolved in 50:50 DI water/ACN premixed with 0.1% formic acid (v/v). Isocratic runs were conducted between 30% and 80% concentrations of ACN. The general order of elution of PTH derived amino acids was found to be PTH isoleucine < PTH asparagine < PTH aspartic acid and this follows the general pattern: acidic < neutral < basic.

For underivatized amino acids such as L-phenylalanine and L-tryptophan, L-tryptophan was retained slightly longer than phenylalanine at each of the mobile phase compositions. Among the 22 amino acids, only the 3 aromatic amino acids, tyrosine, tryptophan and phenylalanine, absorb light in the UV range and the maximum absorbance is observed for tryptophan.²⁹ Phenylalanine is the most hydrophobic while

the phenolic and indole rings of tyrosine and tryptophan make them somewhat polar.²⁹ The retention time data is provided in Table 7 and the retention plot is shown in Figure 17. The hydrophobicity of L-phenylalanine is explained by the possibility of ring stacking (lack of substituents on the ring and equal sharing of electrons between the carbon atoms in the ring). In tyrosine, a hydroxyl group (-OH) on the phenolic ring permits hydrogen bond thus making it more polar and hydrophilic. L-tryptophan has a more complex structure with a “nitrogen” containing indole ring that can additionally engage in hydrogen bonds accounting for its maximum polarity.³⁰

Table 7. Retention time data for amino acids

Concentration (%)		Retention time (Minutes)				
DI Water + 0.1% Formic acid	ACN + 0.1% Formic acid	L- phenylala nine	L- tryptophan	PTH Isoleucine	PTH Aspartic acid	PTH Asparagine
70	30	1.954	2.079	6.949	2.492	2.442
60	40	1.897	1.977	3.747	2.082	2.100
50	50	1.845	1.889	2.610	1.869	1.914
40	60	1.919	1.981	2.113	1.754	1.815
30	70	2.129	2.203	1.879	1.694	1.770
20	80	2.238	2.796	1.775	1.644	1.762
10	90	2.764	5.147	1.704	1.652	1.750

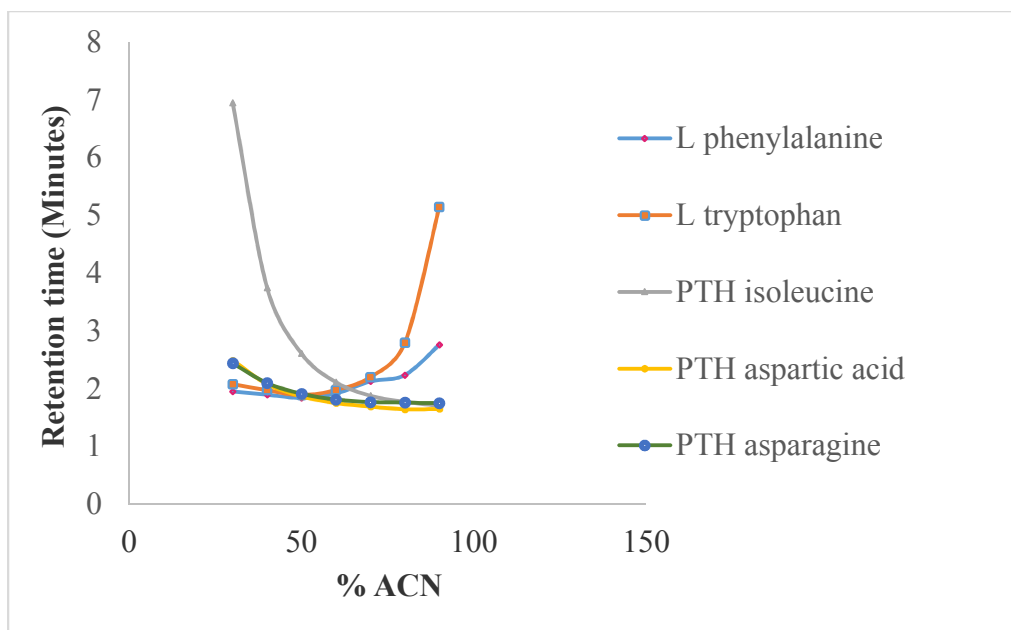


Figure 17. Retention time plots for amino acids

1.4. Xanthine-related compounds

All the analytes were dissolved in DI water to make the stock solution. The stock and sample concentrations were the same as used for the other studies. Theophylline has bronchodilation properties and is used to manage obstructive asthma while caffeine is the most active xanthine in stimulating the central nervous system. Lufyllin /diprophylline /7-(2,3-dihydroxypropyl) Theophylline is used to treat asthma, cardiac dyspnea, and bronchitis. It also serves as an adenosine receptor antagonist and phosphodiesterase inhibitor.³¹ The retention time data for these compounds is shown in Table 8. The retention data has also been plotted in Figure 18. Isocratic runs between 20 and 90% ACN were conducted and in each of the runs 7-(2,3-dihydroxypropyl) Theophylline eluted first followed by theophylline and caffeine. The longer retention of caffeine can be substantiated due to the hydrophobic interaction of the molecule with the large non

polar region of the stationary phase. Theophylline is slightly acidic and thus elutes faster from the UDA column.

Table 8. Retention time data for xanthine-related compounds

Concentration (%)		Retention time (Minutes)		
DI Water + 0.1% Formic acid	ACN + 0.1% Formic acid	Theophylline	Caffeine	7-(2,3-dihydroxyprop- yl)Theophylline
80	20	2.538	3.468	1.926
70	30	2.168	2.635	1.844
60	40	2.014	2.315	1.785
50	50	1.935	2.168	1.747
40	60	1.919	2.104	1.757
30	70	1.950	2.096	1.804
20	80	2.043	2.154	1.900
10	90	2.309	2.348	2.128

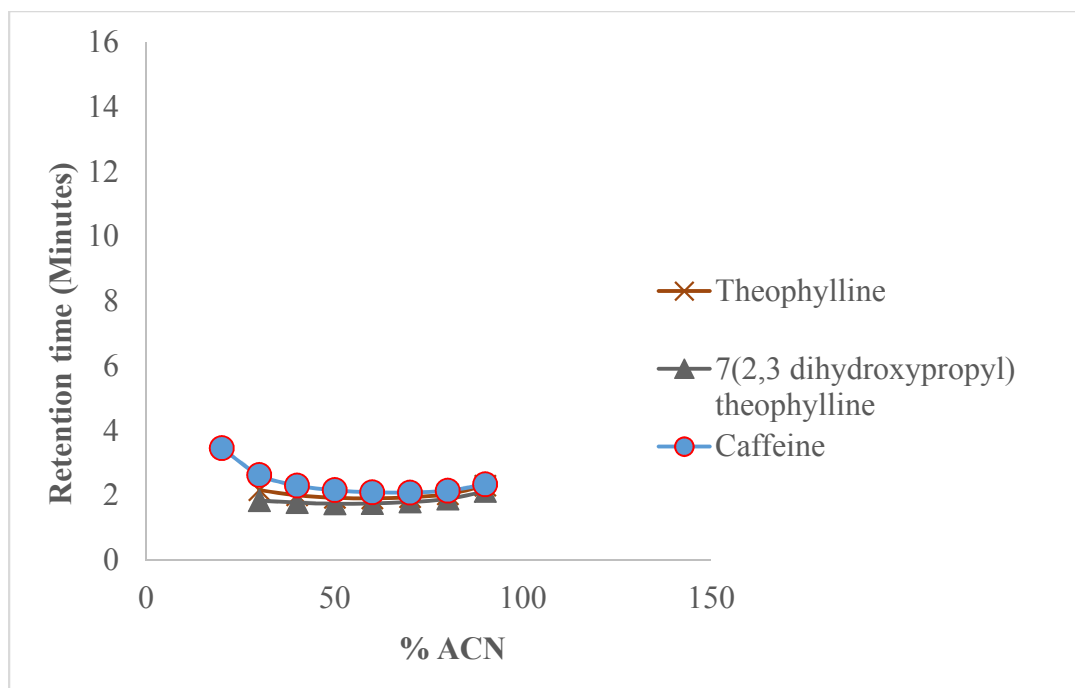


Figure 18. Retention time plots of xanthine-related compounds

1.5. Small organic molecules

Small organic molecules such as benzoic acid, p-amino benzoic acid, p-nitro aniline, and ethyl p-amino benzoate were also used for column characterization. All stock and sample solutions were prepared in 50:50 DI water/ ACN premixed with 0.1% v/v formic acid. For each of the four molecules the retention time was found to decrease with an increase in non-polar component of the mobile phase. The retention table and the corresponding plots are given in Table 9 and Figure 19 respectively.

Table 9. Retention time data for small organic molecules

Concentration (%)		Retention time (Minutes)			
DI Water + 0.1% Formic acid	ACN + 0.1% Formic acid	Benzoic acid	p-amino Benzoic acid	p- nitroaniline	Ethyl p-amino benzoate
70	30	3.582	2.489	4.256	5.073
60	40	3.100	2.250	3.560	3.790
50	50	2.393	1.982	2.392	2.565
40	60	2.008	1.868	2.080	2.181
30	70	1.887	1.818	1.911	1.977
20	80	1.841	1.815	1.837	1.881
10	90	1.728	1.815	1.715	1.730

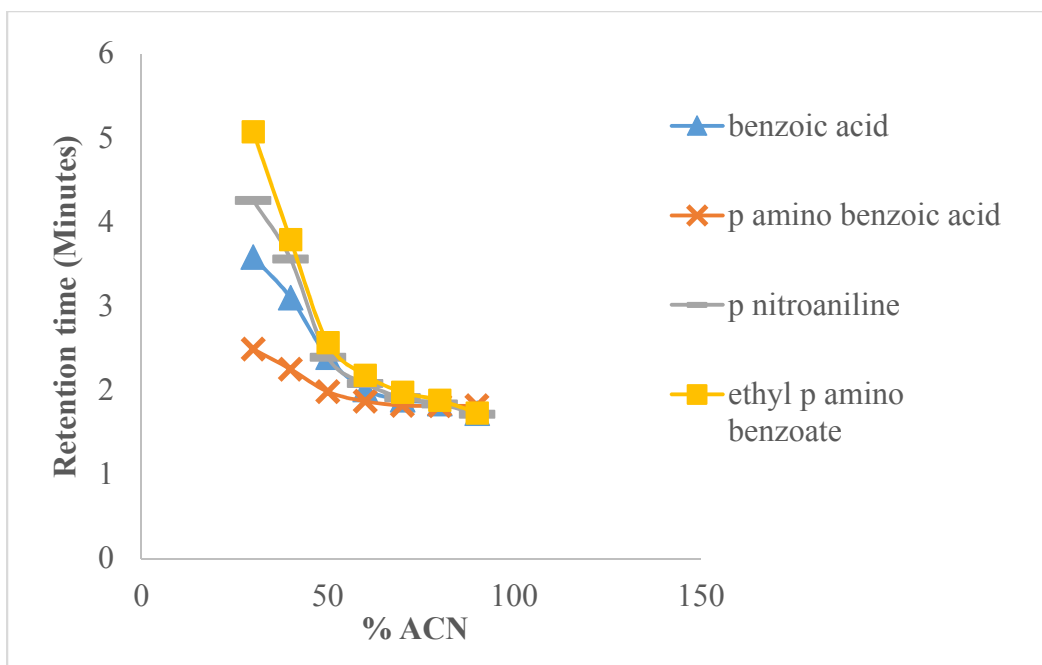


Figure 19. Retention time plot of small organic molecules

1.6. Polycyclic aromatic hydrocarbons (PAHs)

Stock solutions of PAH's such as naphthalene, fluorene, and acenaphthene were prepared by dissolving 1mg/mL of the samples in solvent. Sample solutions to be used on the HPLC were made from this stock solution. The solvent used for dissolving acenaphthene and fluorene was 100% ACN while naphthalene was dissolved in 50:50 (DI water/ACN premixed with 0.1% formic acid. Isocratic runs were conducted on all three molecules between 40% and 90% concentration of ACN. The least retention time was observed for naphthalene followed by fluorene and acenaphthene. The retention time data and plots for the PAH's are shown in Table 10 and Figure 20. As can be seen from the plots, the retention times of all three molecules decreases with an increase in concentration of ACN in the mobile phase, thus confirming the reverse-phase retention mechanism for these non-polar compounds.

Table 10. Retention time data for polycyclic aromatic hydrocarbons

Concentration (%)		Retention time (Minutes)		
DI Water + 0.1% Formic acid	ACN + 0.1% Formic acid	Naphthalene	Fluorene	Acenaphthene
60	40	8.747	13.746	*
50	50	4.772	6.334	13.457
40	60	3.220	3.751	6.381
30	70	2.507	2.703	3.833
20	80	2.146	2.156	2.770
10	90	1.972	1.864	2.256

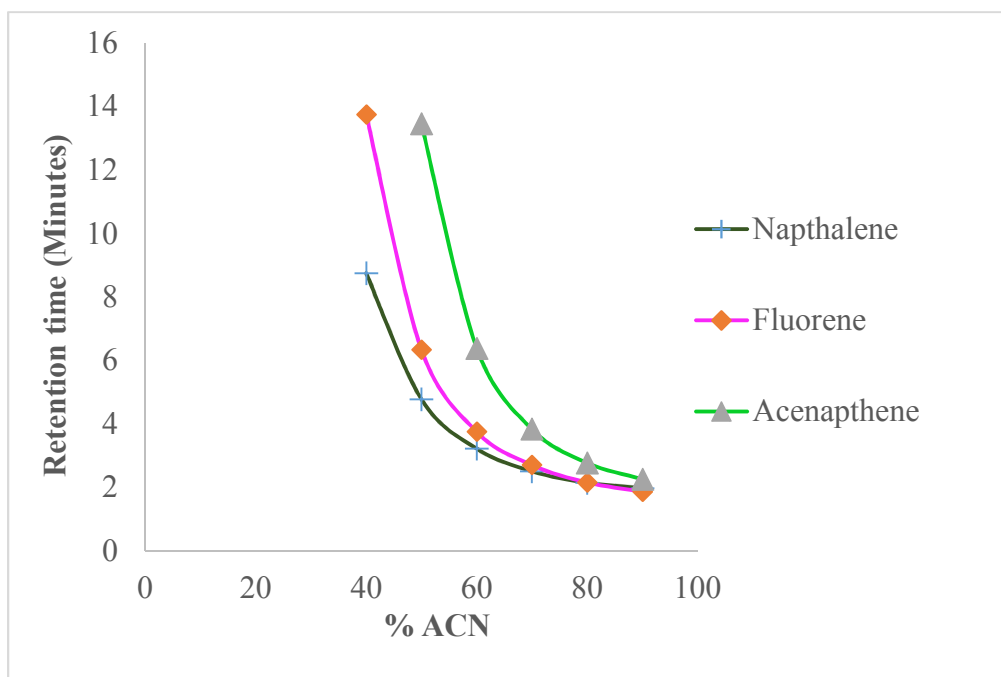


Figure 20. Retention time plot for polycyclic aromatic hydrocarbons

1.7. Miscellaneous

The last set of compounds tested on the columns were thiamine hydrochloride and creatinine. Creatinine is the breakdown product of creatine and belongs to the class of ergogenic acids. Creatinine is chiefly filtered out of the blood by the kidneys and their levels in physiological samples such as urine and blood is indicative of the kidney health of an individual.

Thiamine (vitamin B1) is an essential vitamin for aerobic metabolism, cell growth, transmission of nerve impulses, and acetylcholine synthesis. Vitamin B1 is also essential to maintain a healthy heart and nerves and lack of it may lead to heart failure/nervous problems. Both the samples were dissolved in 50:50 DI water/ACN premixed with 0.1% formic acid (v/v) and isocratic runs were conducted between 30-80 %

compositions of ACN. Both the samples clearly showed a normal phase retention mechanism, with an increase in retention observed as the concentration of ACN was increased in the mobile phase as shown in Figure 21. The retention time data is given in Table 11.

Table 11. Retention time data for miscellaneous compounds

Concentration (%)		Retention time (Minutes)	
DI Water + 0.1% Formic acid	ACN + 0.1% Formic acid	Creatinine	Thiamine HCl
70	30	1.754	1.821
60	40	1.915	1.856
50	50	1.925	1.972
40	60	1.884	2.438
30	70	2.245	3.753
20	80	2.742	8.494
10	90	4.031	9.876

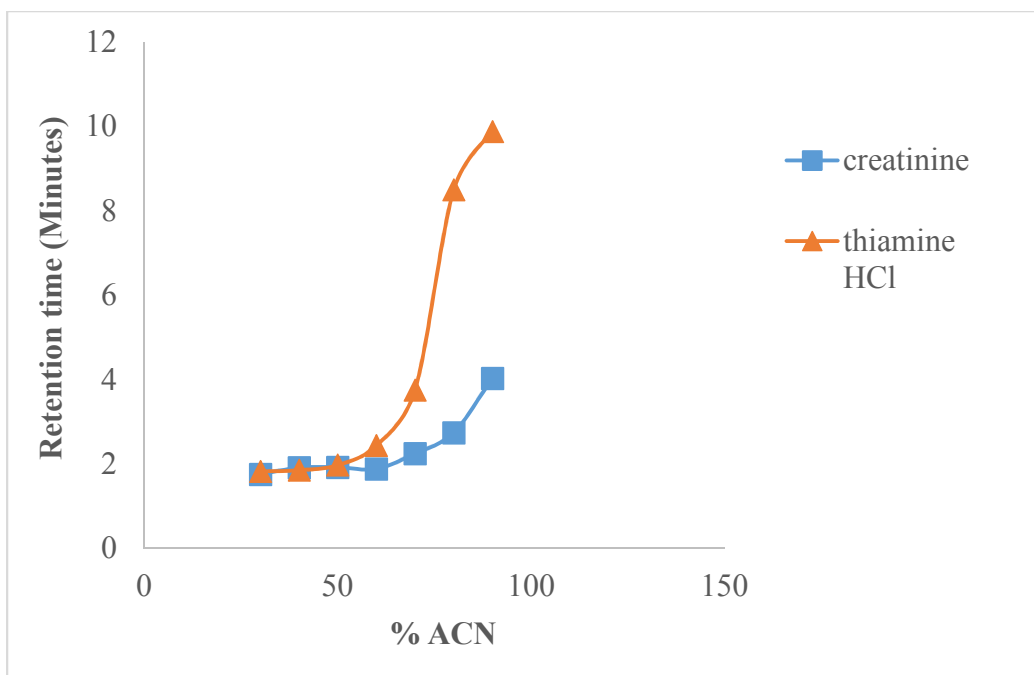


Figure 21. Retention time plots for miscellaneous compounds

B. Retention study with a change in the additive concentration

Polar compounds such as L-tryptophan, adenine, cytosine, and thiamine and non-polar compounds such as naphthalene and fluorene were used to study the effect of varying buffer/additive concentrations on the retention time. Stock solutions of all solutes were prepared by dissolving 1 mg of the compound in 1 mL of the solvent. The solvents used were the same as for the characterization studies. Sample solutions used for the retention time determinations were prepared by pipetting 100 μ L of the stock and mixing with 900 μ L of the solvent. The instrument parameters used were the same as earlier.

DI water (solvent A) and ACN (solvent B) were prepared with different concentrations of formic acid. The amounts of the formic acid used were 0.05%,

0.075%, 0.1%, 0.2%, and 0.3% v/v. L-tryptophan, adenine, and cytosine were studied at a 20:80 (A: B) composition while a 30:70 (A: B) was used for thiamine for each new additive concentration. The non-polar compounds were tested at a 60:40 (A: B). The retention time data and plots for the polar compounds are given in Table 12 and Figure 22. Table 13 provides the retention time data and Figure 23 shows the retention map for the non-polar compounds. While no significant trend can be inferred for the non-polar compounds, there was a slight decrease in retention time observed with an increase in the additive concentration for the tested polar compounds. This trend observed was opposite to that of hydrophilic interaction liquid chromatography (HILIC), that is based on type B silica based columns and separates only polar compounds.³² In the case of HILIC, when the formic acid concentration in the mobile phase increases, it readily partitions into the water layer on the polar silanol surface thereby causing an increase in the polarity of the surface and resulting in an increase in retention time for the polar compounds. For non-polar compounds, the interaction happens with the silica hydride surface that has very low or negligible polar silanol group, and thus the retention is unaffected by the change in concentration of the formic acid. This experiment thus proves that the ANP retention is based on an adsorption mechanism as opposed to a partition mechanism suggested for HILIC.

Table 12. Retention time data for polar compounds at varying formic acid concentrations

% Formic acid	Retention time (Minutes)			
	L-tryptophan 80B	Adenine 80B	Cytosine 80B	Thiamine HCl 70B
0.050	2.936	4.983	3.435	4.481
0.075	2.700	4.381	2.862	3.277
0.100	2.796	4.804	3.710	3.753
0.200	2.481	3.893	2.529	2.568
0.300	2.293	3.361	1.966	1.918

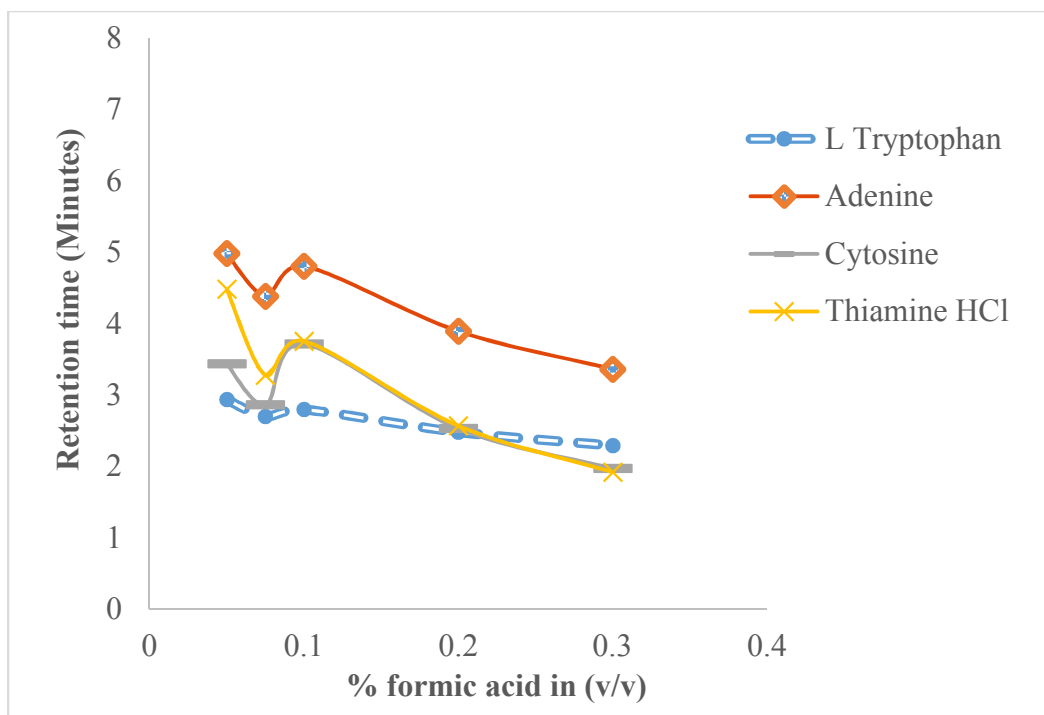


Figure 22. Retention time plots for polar compounds at various concentrations of formic acid

Table 13. Retention time data for non-polar compounds at varying formic acid concentrations

% Formic acid	Retention time (Minutes)	
	Naphthalene 40B	Fluorene 40B
0.050	8.760	13.753
0.075	8.811	13.868
0.100	8.747	13.746
0.200	8.833	13.887
0.300	8.780	13.740

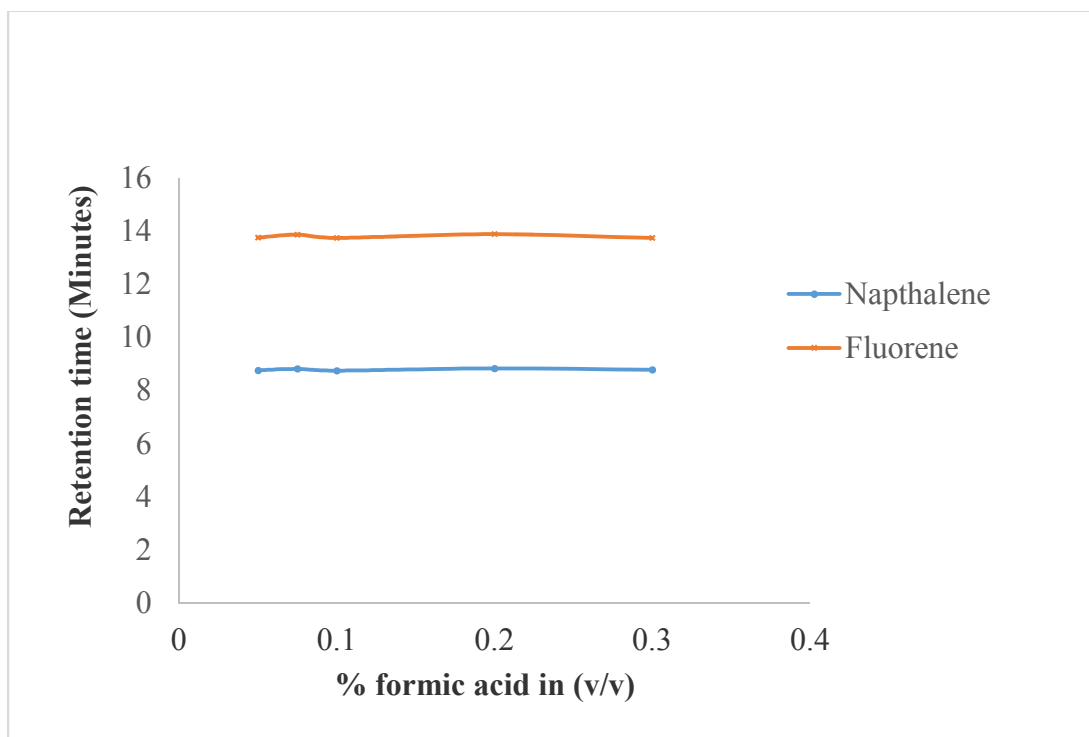


Figure 23. Retention time plots for non-polar compounds at various concentrations of formic acid

C. Repeat study with various formic acid concentrations

In order to reconfirm the observed decrease in retention time with an increase in concentration of the formic acid, the runs were repeated for some selected polar compounds such as L-tryptophan, adenine, and cytosine. The results obtained were found to be similar to those obtained earlier, thus demonstrating the reproducibility of the silica hydride based UDA column and the previously observed trend. The retention data and plots for the repeat study are shown in Table 14 and Figure 24.

Table 14. Retention time data for selected polar compounds at varying formic acid concentrations-repeat study

% Formic acid	Retention time (Minutes)		
	L-Tryptophan 80B	Adenine 80B	Cytosine 80B
0.050	2.733	5.206	3.305
0.075	2.534	4.710	2.842
0.100	2.806	5.372	3.380
0.200	2.279	4.137	2.491
0.300	2.071	3.640	2.207

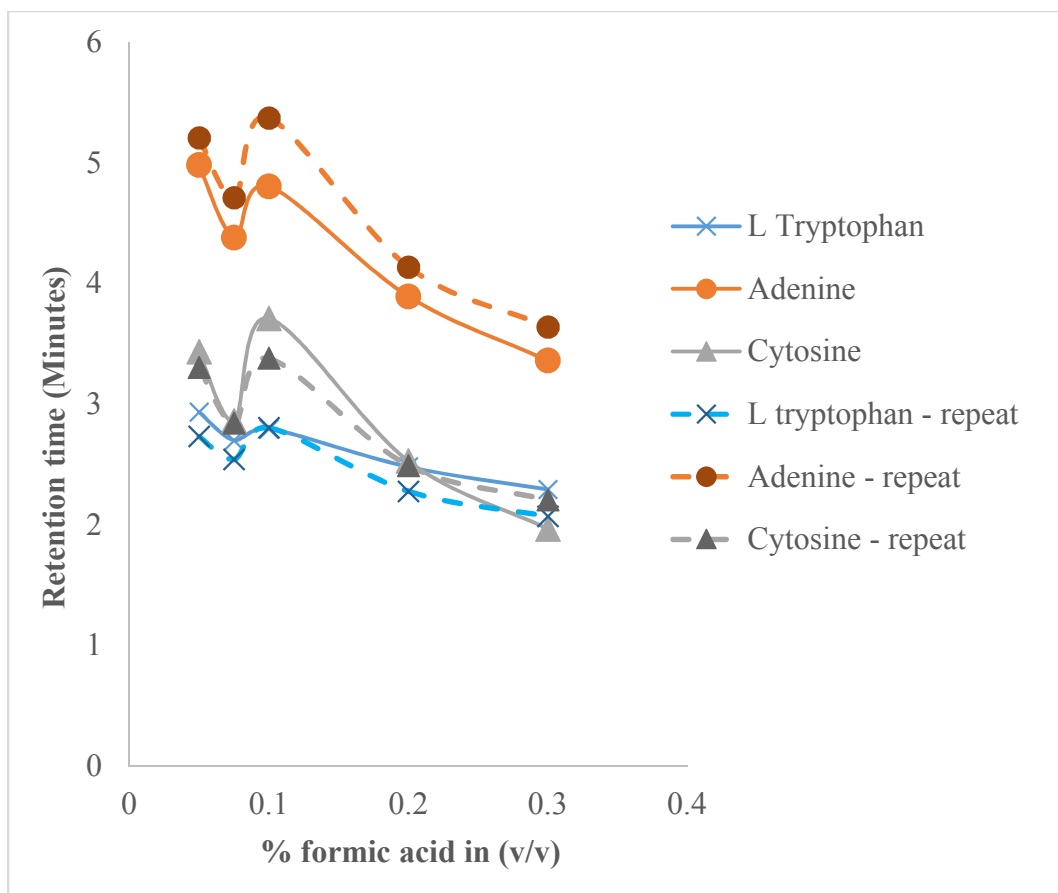


Figure 24. Retention time plots for selected polar compounds at various concentrations of formic acid – repeat study

D. Compatibility of ammonium acetate as an additive

A stock solution of ammonium acetate with a concentration of 128.72 mM was made by diluting ammonium acetate in water and stored in the refrigerator. Sample solutions of polar compounds such as L-tryptophan, adenine, cytosine and uracil were prepared from a stock solution having a concentration of 1 mg/mL. A non-polar compound, acenaphthene was also tested. Solvent A was DI water and solvent B acetonitrile and each of the solvents was diluted with the required volume of stock ammonium acetate solution to get the desired concentration of 10 mM ammonium

acetate. Isocratic runs were conducted at mobile phase compositions ranging from 50% and 85% acetonitrile and each run was repeated three times and the retention time measured. The data is tabulated in Table 15 and the retention time plots are shown in Figure 25.

For each of the polar compounds, an increase in retention time was observed with an increase in concentration of acetonitrile displaying a typical normal phase retention mechanism, while for acenaphthene there was a decrease in retention time with an increase in the ACN concentration in mobile phase and this was typical of reverse-phase retention. For both polar and non-polar compounds the trend observed with ammonium acetate additive was similar as seen in the case of formic acid additive. These studies indicate the compatibility of ammonium acetate as an additive with the silica hydride-based UDA column.

Table 15. Retention time data for compounds at 10 mM ammonium acetate concentration

ACN (%)	Retention time (Minutes)				
	L-tryptophan	Adenine	Cytosine	Uracil	Acenaphthene
50	2.237	2.867	2.258	1.756	6.906
60	2.339	2.955	2.402	1.798	3.985
65	2.313	3.320	2.605	1.817	3.353
70	2.806	3.219	2.733	1.868	2.837
75	3.040	3.251	2.952	1.871	2.554
80	3.634	3.693	3.304	2.022	2.096
85	5.350	4.095	3.897	2.131	1.924

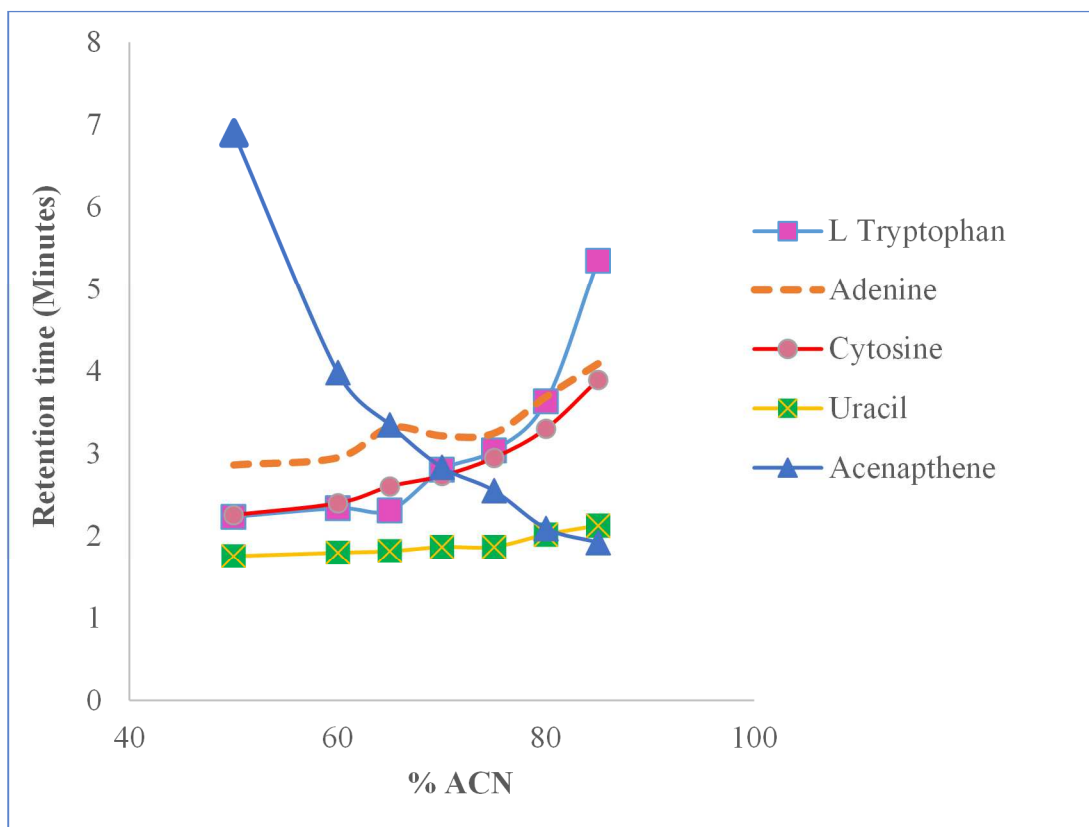


Figure 25. Retention time plots for compounds at 10 mM ammonium acetate concentration

E. Retention study with varying concentrations of ammonium acetate additive

Various concentrations of the buffer were pipetted from the stock solution and added to each of the solvents (DI water and ACN) to get strengths of 1 mM, 2 mM, 5 mM, and 15mM. Four different polar compounds, adenine, cytosine, uracil, and L-tryptophan were studied at a 30:70 (A: B) solvent ratio for each different concentration of ammonium acetate. No pronounced trend could be inferred from such a retention study as can be seen from Table 16 and Figure 26 shows the retention time and plot for such data respectively. All the compounds showed a reduction in retention time for 2 mM concentration and this increased at 5 mM and thereafter not much of a significant

change could be seen. A possible explanation for the above trend could be the buffer overpowering the analyte for adsorption on the stationary phase surface with an increase in pH as the strength of the buffer is increased.

At each of the above concentrations, the pH of the solvent was found to be in the 6.5-7 range. Above a pH of 6, the ion-exchange property of the column is also expected to play a major role in the retention. With a change in pH, the surface properties of the stationary phase is thus expected to change depending on the extent of ionization of the carboxylic acid group of the undecynoic acid and this could also be another factor for the observed trend for polar and non-polar compounds. At an acidic pH, the carboxylic acid group on the undecynoic acid is protonated (unionized) and polar compounds are retained by interacting with the lone pair electrons on the (-COOH) group of the undecynoic acid. At a basic pH, the carboxylic acid group on the UDA column is ionized and in such a case, retention of polar compounds takes place by the interaction of the opposite charges on the stationary phase and the analyte molecules. In each of the above case, retention of non-polar compounds takes place by interacting with the alkyl group on the stationary phase.

A large retention is observed for adenine and the smallest for uracil. The order of retention time in decreasing order is adenine > cytosine > uracil > L-tryptophan. For non-polar compounds such as acenaphthene the retention time decreases initially and then increases at about 15 mM.

Table 16. Retention time data for compounds at varying ammonium acetate concentration

Concn of ammonium acetate (mM)	Retention time (Minutes)			
	L-tryptophan	Adenine	Cytosine	Uracil
1	2.487	4.555	3.956	1.853
2	2.091	3.611	2.786	1.785
5	3.351	3.269	2.930	1.956
10	2.806	3.219	2.733	1.868
15	2.422	3.063	2.610	1.894

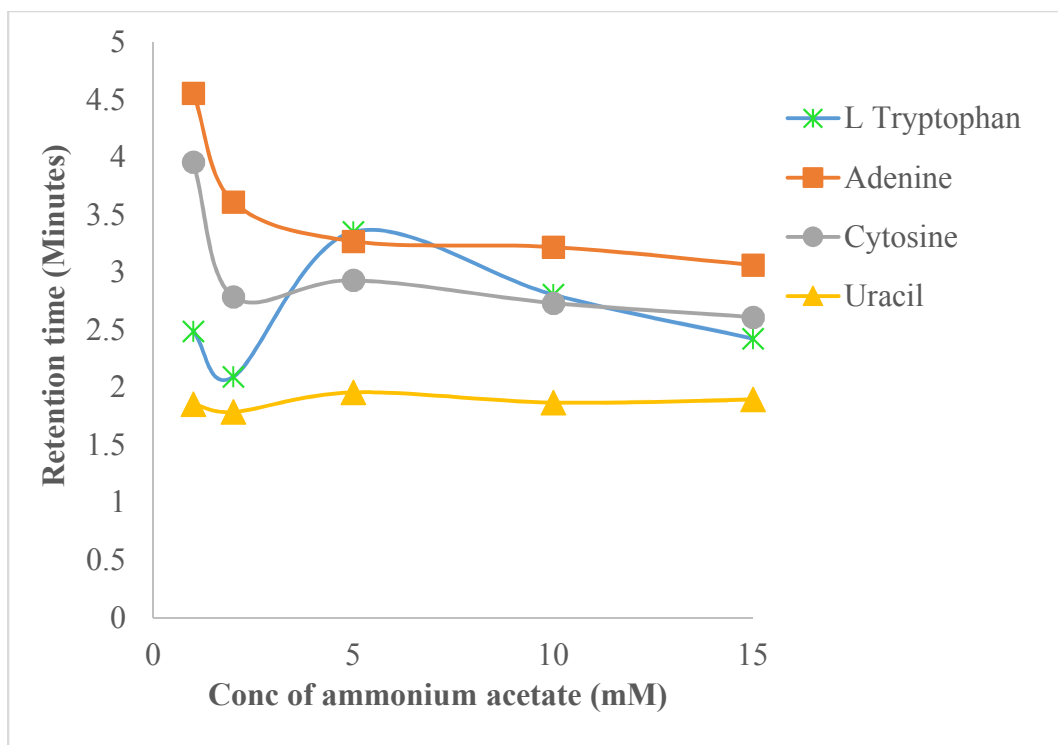


Figure 26. Retention time plots for compounds at varying concentrations of ammonium acetate

F. Effect of temperature on retention

In order to study the effect of retention with varying temperature, the column oven was varied from 30-55°C and the variation in retention time was studied. The retention data is tabulated in Table 17 and the retention plots are shown in Figure 27. The column temperature is plotted on the x-axis and the retention time along the y-axis in each case. It can be inferred from the table and the graph that as the temperature is increased, there is a slight decrease in retention time for one compound. This can be explained as a result of a decrease in viscosity or retention mechanism with an increase in temperature.

Table 17. Retention time data for compounds at different oven temperature

Oven temperature (°C)	Retention time (Minutes)		
	L-tryptophan	Adenine	Cytosine
35	2.350	4.068	2.562
40	2.306	3.892	2.515
45	2.278	3.704	2.506
50	2.278	3.586	2.497
55	2.257	3.515	2.510

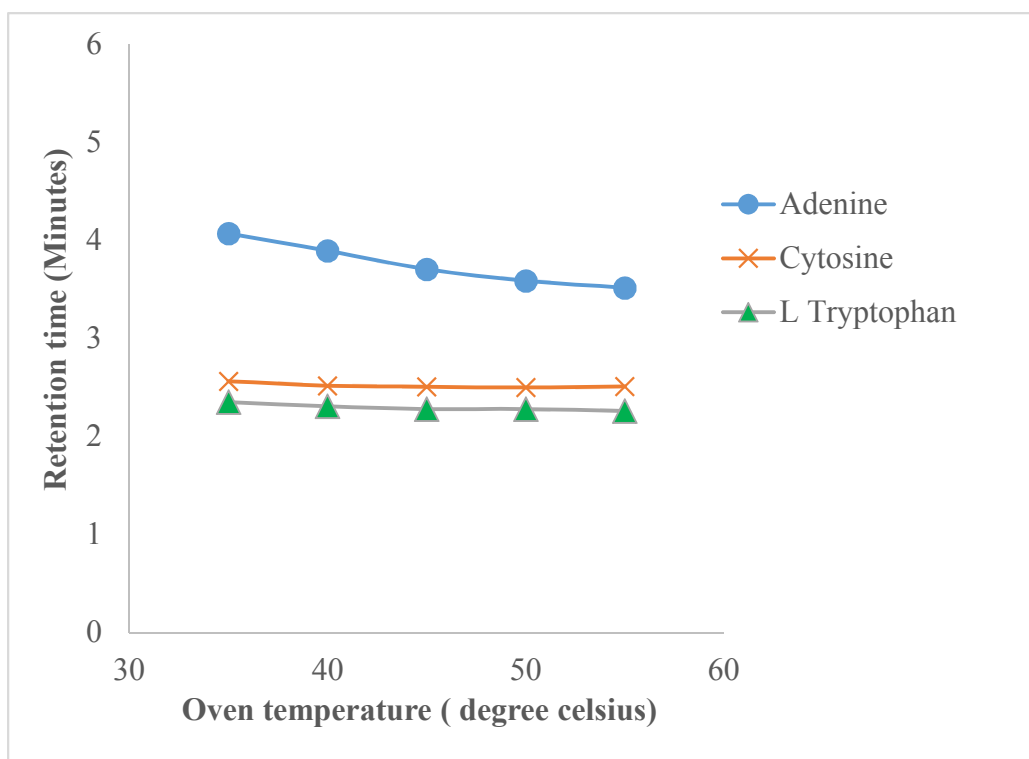


Figure 27. Retention time plots for varying temperature

IV. APPLICATIONS

1.1. Nucleotide characterization

The nucleotides used for characterization studies of the current column were adenosine monophosphate (AMP) and adenosine triphosphate (ATP) that are the mono and tri phosphorylated forms of adenine, respectively. The retention mechanisms of NAD and NADP that are important coenzymes found in cells were also studied. NAD participates in many cellular redox reactions such as glycolysis and the citric acid cycle of cellular respiration, while NADP is NAD with a third phosphate group is a reducing agent produced in the light reaction of photosynthesis and plays a role in many anabolic reactions of plants and animals. The retention time data and plots for the nucleotides and coenzymes are given in Table 18 and Figure 28.

The stock and sample solutions of all the nucleotides were prepared by their dissolution in DI water. Isocratic runs were conducted at ACN concentrations of 30-85%. For each of the nucleotides, the retention time was found to be higher at the beginning (30% ACN) and gradually decreased at 50% ACN for ATP and 60% ACN for NAD and NADP, respectively and again increased at 80% ACN. From the above set of runs, it is clear that these class of compounds are well retained on the UDA column and it is also suitable even to separate their complex mixtures.

Table 18. Retention time data for nucleotides and coenzymes

Concentration (%)		Retention time (Minutes)			
DI Water + 0.1% Formic acid	ACN + 0.1% Formic acid	AMP	ATP	NAD	NADP
70	30	1.900	1.471	1.513	1.521
60	40	1.813	1.461	1.510	1.518
50	50	1.549	1.455	1.487	1.502
40	60	1.567	1.484	1.478	1.478
30	70	1.554	1.524	1.487	1.487
20	80	1.538	1.579	1.533	1.547
10	90	1.582	1.598	1.601	1.605

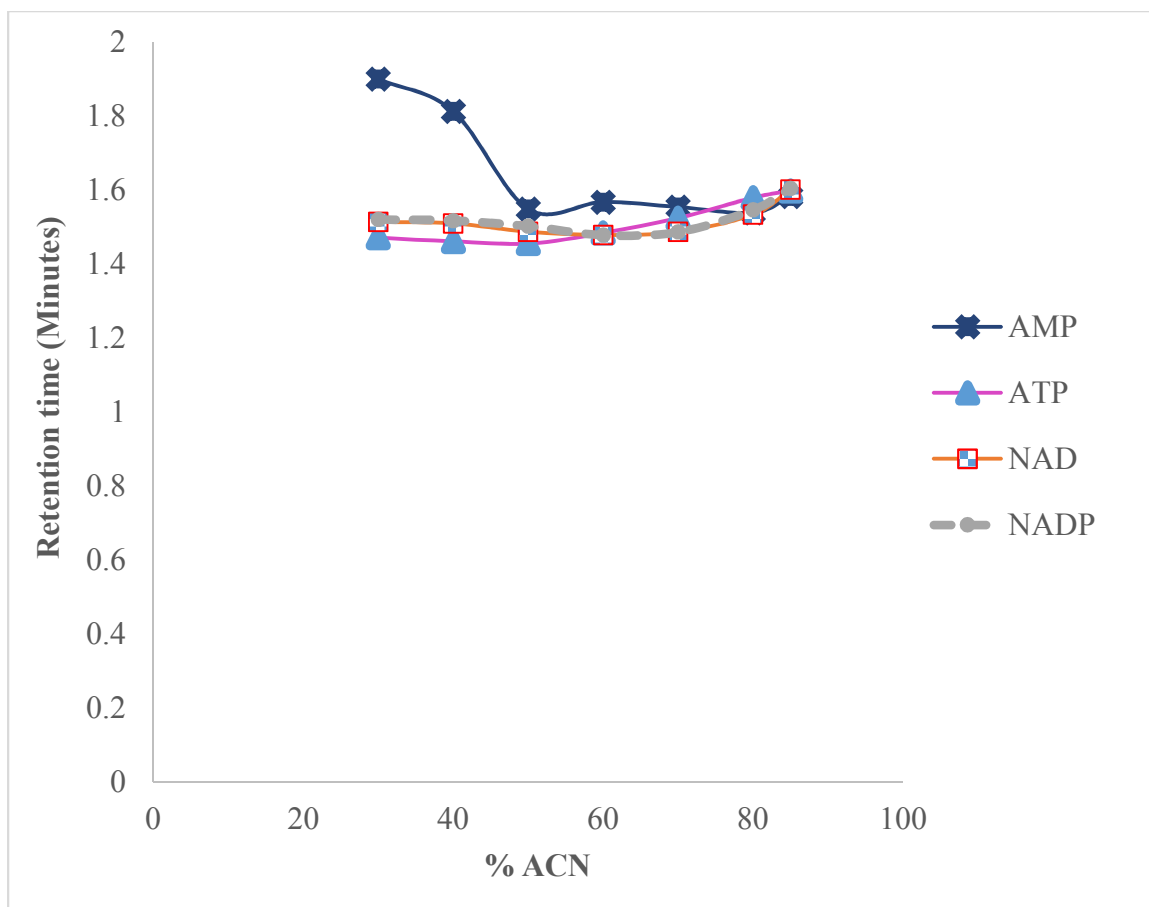


Figure 28. Retention time plot of nucleotides and coenzymes

1.2. Retention of ATP and NADP at varying formic acid concentrations

ATP and NADP were used to study the effects of varying additive concentrations on the retention time. Stock solutions of all solutes were prepared by dissolving 1 mg of the compound in 1 mL of the solvent. The solvents used were the same as for the characterization studies. Sample solutions used for the run were prepared by pipetting 100 μ L of the stock in 900 μ L of the same solvent. The instrument parameters used were the same as earlier.

DI water (solvent A) and ACN (solvent B) were prepared with different concentrations of formic acid. The amounts of the formic acid used were 0.05%, 0.075%, 0.1%, 0.2%, and 0.3% v/v. ATP and NADP were studied at a 20:80 (A:B) composition. The retention time data and plots for the selected compounds are given in Table 19 and Figure 29. While no significant trend can be inferred for ATP, there was a slight increase in retention time observed with an increase in the additive concentration for NADP. Based on the previous set of experiments conducted for the characterization studies, the retention time was found to decrease with an increase in concentration of formic acid, thus clearly distinguishing the ANP and HILIC retention mechanisms. The difference between these results and those obtained earlier is most likely the effect of the carboxylic acid group on the bonded moiety. Thus, ion-exchange effects may also contribute to the retention mechanism.

Table 19: Retention time data for ATP and NADP

% Formic acid	Retention time (Minutes)	
	ATP	NADP
0.050	1.509	1.481
0.075	1.510	1.484
0.100	1.504	1.482
0.200	1.517	1.482
0.300	1.492	1.677

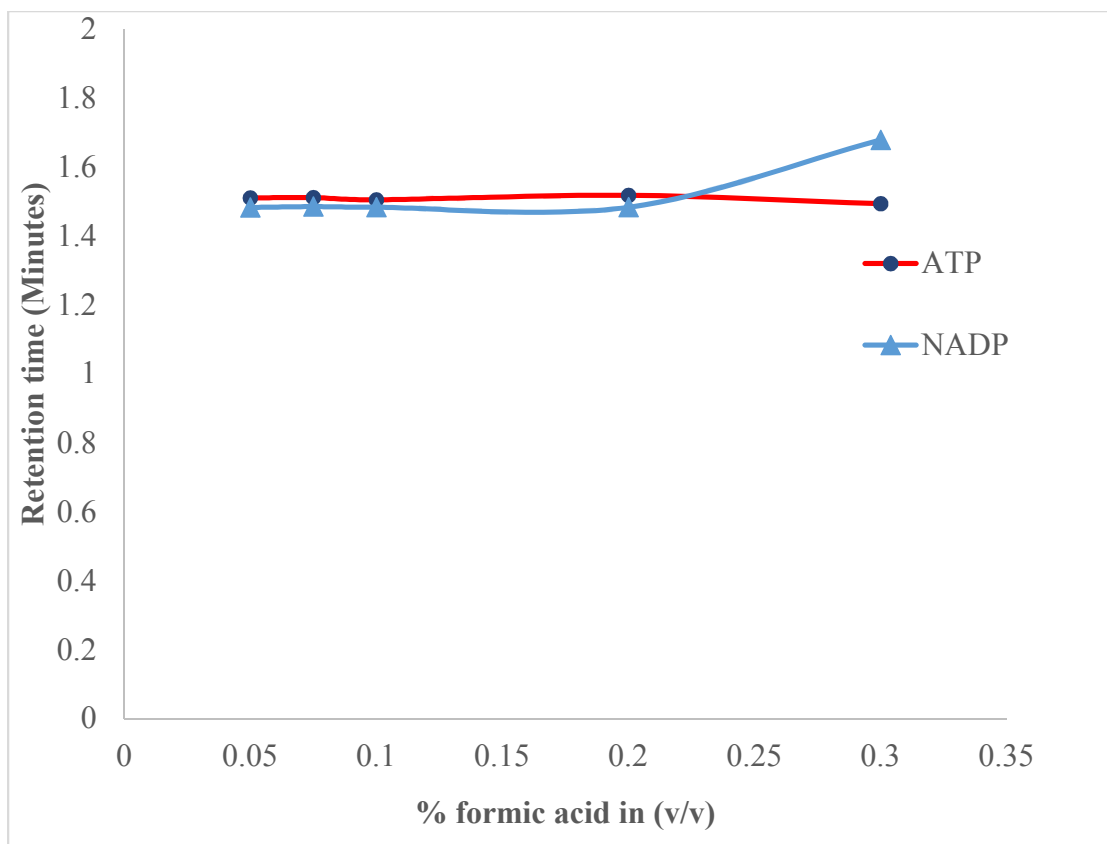


Figure 29. Retention time plot of ATP and NADP

B. Pesticide analysis in urine sample

Urine samples of farmers were collected for testing the presence of various types of chemicals found in pesticides, such as dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), dimethyldithiophosphate (DMDTP), diethyl phosphate (DEP), diethyl thiophosphate (DETP), diethyldithiophosphate (DEDTP), and the deuterated derivatives of all these compounds. The chemical structures of DMP, DMTP, DMDTP, DEP, DEDP, and DEDTP are shown in Figure 30.

The (m/z) and [M-H]⁻ of all the compounds and their deuterated derivatives are given in Table 20. A fixed volume (1 μL) of each of the compound was injected into an

Agilent 1100 series LC coupled with a Model 6220 TOF detector. The extracted ion chromatogram obtained is shown in Figure 31. As is clearly evident from the extracted ion chromatogram, only DMP-d6 and DEDTP-d10 was detected. These compounds are standards spiked in the samples and are not found in the real samples. This could either indicate the complete absence of the compounds in the urine sample or a lack of sensitivity of the TOF detector. A follow-up study on the MS-MS instrument is suggested to further confirm the results.

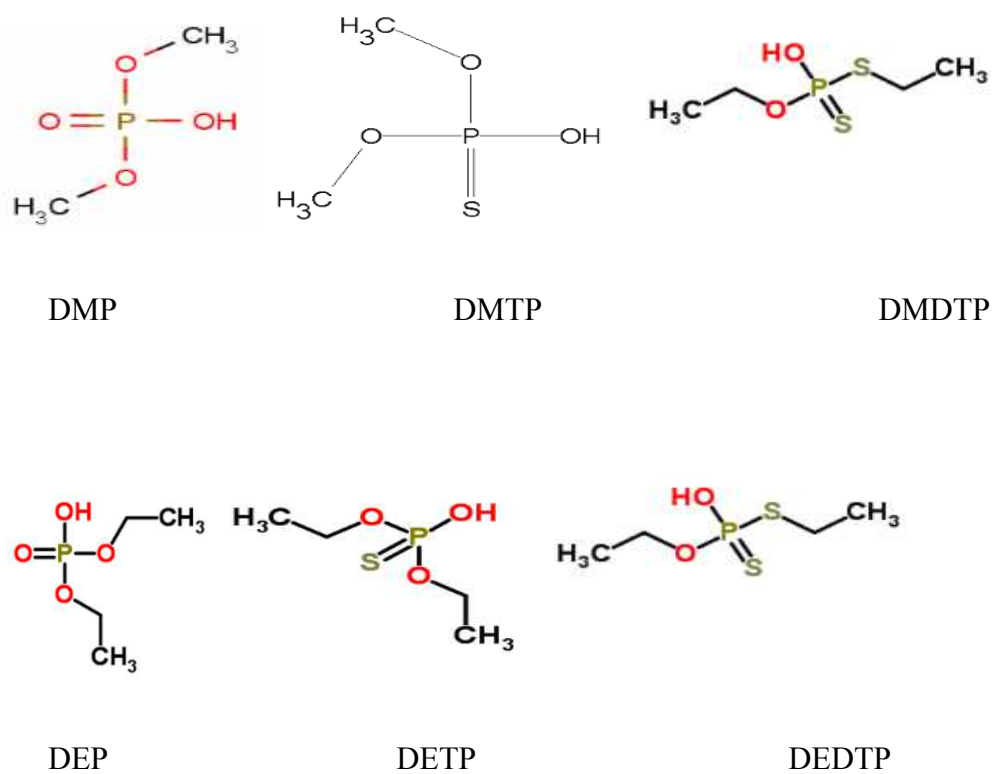
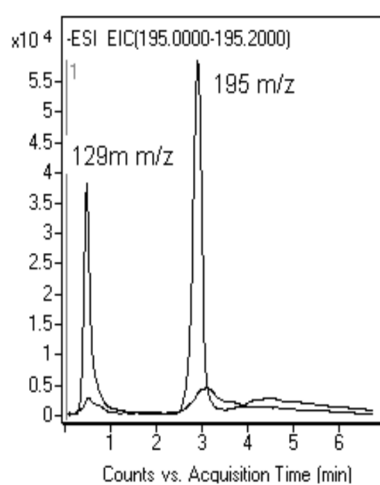


Figure 30. Chemical structures of non-deuterated forms of the compounds analyzed in the pesticide sample

Table 20. (m/z) and [M-H]⁻ of deuterated and non-deuterated components analyzed

Name of compound	Formula of anion	Monoisotopic mass (m/z)	[M-H]⁻
DMP	C ₂ H ₆ O ₄ P ₁	125.0003	123.9931
DMTP	C ₂ H ₆ O ₃ S ₁ P ₁	140.9775	139.9702
DMDTP	C ₂ H ₆ O ₂ S ₂ P ₁	156.9546	155.9474
DEP	C ₄ H ₁₀ O ₄ P ₁	153.0316	152.0244
DETP	C ₄ H ₁₀ O ₃ S ₁ P ₁	169.0088	168.0015
DEDTP	C ₄ H ₁₀ O ₂ S ₂ P ₁	184.9859	183.9794
DMP-d6	C ₂ D ₆ O ₄ P ₁	131.0380	130.04
DMTP-d6	C ₂ D ₆ O ₃ S ₁ P ₁	147.0151	146.0172
DMDTP-d6	C ₂ D ₆ O ₂ S ₂ P ₁	162.9923	161.9944
DEP-d10	C ₄ D ₁₀ O ₄ P ₁	163.0944	162.1026
DETP-d10	C ₄ D ₁₀ O ₃ S ₁ P ₁	179.0715	178.0798
DEDTP-d10	C ₄ D ₁₀ O ₂ S ₂ P ₁	195.0487	194.057

Scan Frag=140.0V UDA-2x50-Pesticides_A-W-10mMaac_BACNFA_Gr3_neg_284.d



UDA column 2.1x50 mm
A: DI water\10 mM ammonium acetate
B: acetonitrile\0.1% formic acid
Flow rate: 0.4 mL/min

Gr 3

Time (min)	%B	Flow rate mL/min
0.00	100.0	0.400
2.00	95.0	0.400
8.00	95.0	0.400
9.00	100.0	0.400

129 m/z [M-H]⁻ = DMP-d6
195 m/z [M] = DEDTPd10

Figure 31. Extracted ion chromatograms of standards spiked in urine sample

C. Quantification of phenolic acids in pomegranate peel samples on UDA column

Five different varieties of lyophilized pomegranate peel samples that were grown around Davis, California (CA) were obtained from the UC Davis pomegranate germplasm and forwarded by the United States Department of Agriculture (USDA). The details (names, accession identification numbers (ID's), and place of origin) of these five samples are provided in Table 21. The samples were used to confirm the presence of eight phenolic acid compounds; gallic acid, caffeic acid, catechin, epicatechin, chlorogenic acid, ellagic acid, punicalagin, and rutin. Additionally the phenolic acids were also quantified in the peel samples where they were detected. All the standards were provided by Chromadex Inc, 10005 Muriands Blvd, Ste G, Irvine, California-92618, U.S.A.

Table 21. Accession ID's, names, and place of origin of the various pomegranate peel samples

Accession ID	Pomegranate Cultivar	Country of Origin
DPUN0014	Molla Nepes	Turkmenistan
DPUN0015	Parfianka	Turkmenistan
DPUN0117	Vkusnyi	Turkmenistan
DPUN0056	Purple Heart	United States
DPUN0081	Wonderful	United States

Each of the lyophilized samples (1.0565 g of Molla Nepes, 1.0229 g of Wonderful one, 0.9881 g of Purple heart, 1.0959 g of Parfionka, and 1.0129 g of Vkunysi) were milled using a coffee grinder and sieved through a 100 mesh/standard

sieve. The powdered samples were extracted with 15 mL of 80% (v/v) aqueous methanol at room temperature and sonicated for 30 minutes. The solutions were then centrifuged at 2000 g (gravity) for 10 minutes and the supernatant was collected and extracted three times with 80% aqueous methanol. The combined supernatant was rotavapored to remove the methanol, and it was further reconstituted to 50 mL with 80% aqueous methanol, filtered, and transferred into vials.³³

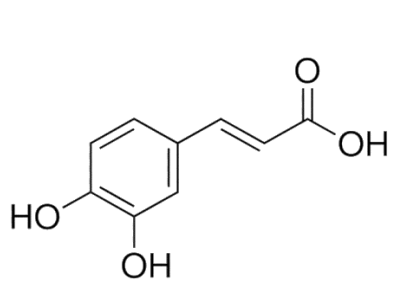
Stock solutions of all the standards (except ellagic acid) were prepared by dissolving 1 mg of the solute in 1 mL of the solvent. The weights of the standards and the solvents used to dissolve each of the 7 standards are provided in Table 22. Table 23 lists the chemical formulae, (m/z)'s in the positive, and negative ionization modes, and the UV absorbance of each of the 7 phenolic acid standards. The chemical structures of these eight compounds are shown in Figure 32 below.

Table 22. Weights and solvents used for the phenolic acid standards

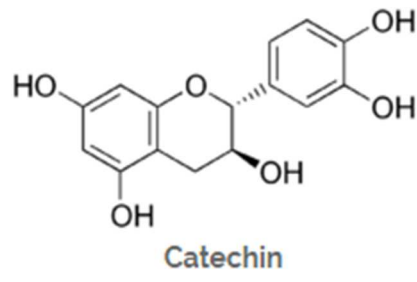
Solute	Weight	Solvent used
Gallic acid	10 g in 10 mL	50 ACN+ 0.% formic acid : 50 DI water + 0.1% formic acid
Caffeic acid	10.3 g in 10 mL	50 ACN+ 0.% formic acid : 50 DI water + 0.1% formic acid
Epicatechin	13. 1g in 10 mL	50 ACN+ 0.% formic acid : 50 DI water + 0.1% formic acid
Rutin	1.4 mg in 1.4 mL	100 % methanol
Catechin	1.3 mg in 1.3 mL	100 % ethanol
Punicalagin	1.3 mg in 1.3 mL	100 % methanol
Chlorogenic acid	1.7 mg in 1.7 mL	100 % ethanol

Table 23. Chemical formulae, (m/z)'s in positive, and negative ionization modes, and UV absorbance of the phenolic acid standards

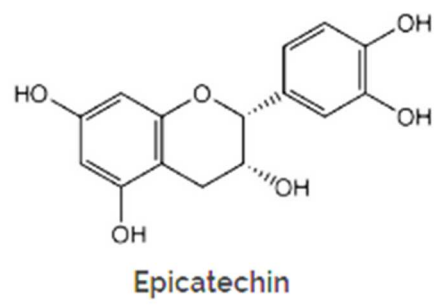
Phenolic compound	Formula	[M+H]⁺	[M+H]⁻	UV(nm)
Gallic acid	C ₇ H ₆ O ₅	171.0288	169.0142	270
Punicalagin	C ₄₈ H ₂₈ O ₃	1085.0738	1083.0593	270 (255)
Catechin	C ₁₅ H ₁₄ O ₆	291.0863	289.0718	270
Chlorogenic acid	C ₁₆ H ₁₈ O ₉	355.1024	353.0878	320
Caffeic acid	C ₉ H ₈ O ₄	181.0495	179.0350	320
Epicatechin	C ₁₅ H ₁₄ O ₆	291.0863	289.0718	270
Rutin	C ₂₇ H ₃₀ O ₁₆	611.1607	609.1461	270
Ellagic acid	C ₁₄ H ₆ O ₈	303.0135	300.9990	255



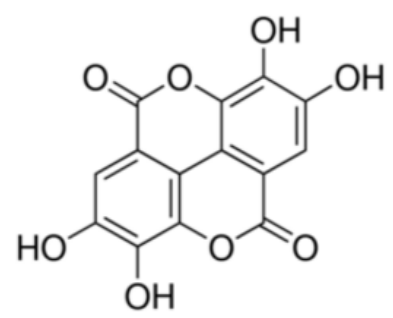
Caffeic acid



Catechin



Epicatechin



Ellagic acid

Figure 32. Chemical structures of the phenolic acids being identified and quantified

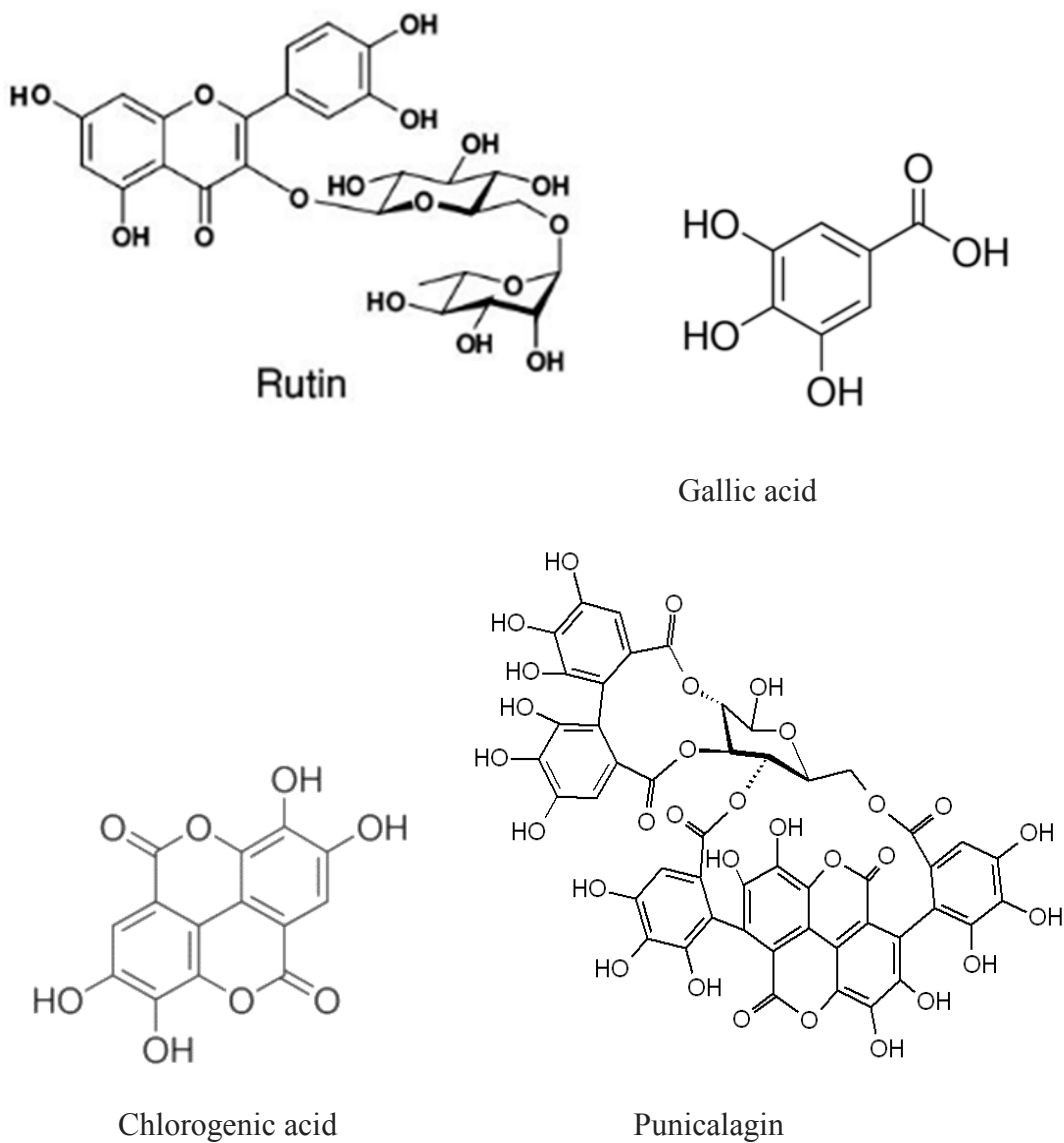


Figure 32. Chemical structures of the phenolic acids being identified and quantified

A definite amount (100 μL) of each of the 7 standards were dissolved in 300 μL of DI water and this standard mix was used to spike each of the pomegranate peel samples. The five pomegranate samples were transferred to vials and run on the 6520 series Q TOF. The retention time (RT) and peak area (PA) were noted for the phenolic acids in each analysis. Tables 24 (a-e) show the retention times and peak areas of the standards, unspiked, and spiked versions of Molla Nepes (MN), Wonderful, Purple heart (PH), Parfionka, and Vkunysi samples respectively. The injection volume in each case was 5 μL and the solvents used were DI water + 0.1% formic acid (solvent A) and ACN + 0.1% formic acid (solvent B). The flow rate was kept at 0.4 mL/min. The method used for all solutions was a gradient that started at 15% B that was increased to 80% B over a period of 4 minutes and then held at 80 % B for another two minutes, and later decreased to 15% B over the next three minutes. A fixed amount (100 μL) of the standard stock solution was micro pipetted and diluted with 900 μL of the respective solvent and the RT's and PA's were noted in this case too. The presence of a phenolic acid compound was confirmed by comparing the (m/z) of that particular phenolic acid compound in the pomegranate peel sample with the (m/z) of that phenolic acid standard. An illustrative example is shown in Figure 33, where the (m/z) observed for the caffeic acid standard is also seen in the Vkunysi sample, clearly confirming the presence of this compound in the peel sample.

In order to further confirm that the observed peak for the peel samples was due to the phenolic acid compound, 450 μL of each pomegranate peel sample was spiked with 50 μL of the standard mix and recording the RT and PA. An increase in the peak area of

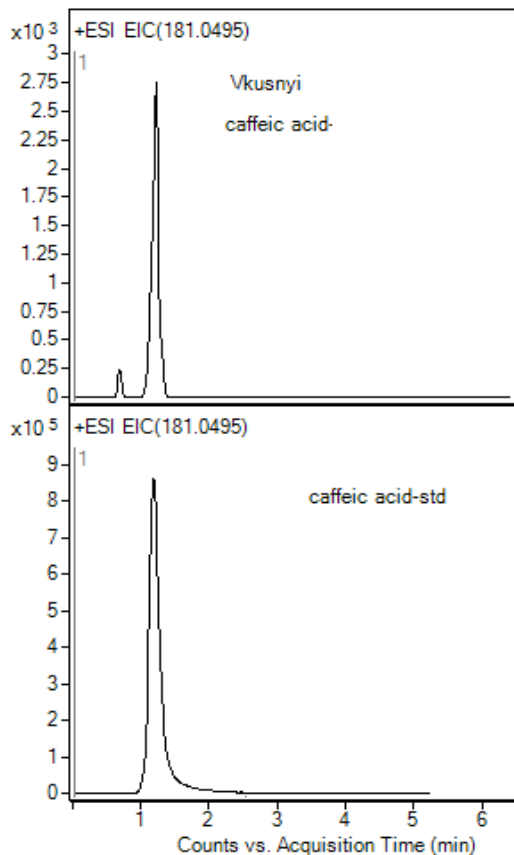
the detected compound confirmed that the observed (m/z) in the peel sample was due to the presence of the compound being investigated. In some cases the phenolic acid compounds were not detected in the peel samples, but were observed after spiking the samples with the standards. Some surprising results were also seen in samples for certain phenolic acid compounds where there was either a decrease in peak areas or total disappearance of the previously observed peaks after spiking. There has been no reasonable explanation for this observation. Figures 34 and 35 are representative graphs illustrating the comparison of the observed (m/z)'s in two of the pomegranate peel samples (Parfionka and Vkunysi) with the standards (rutin and chlorogenic acid). It is clearly evident from these figures that though rutin is detected in both the peel samples, chlorogenic acid is absent in the Parfionka sample as the characteristic peak of this compound (seen in the standard) is absent in this sample.

For the Molla Nepes sample, while there was an increase in the peak areas of gallic acid, rutin, and ellagic acid there was a decrease in the peak areas observed for catechin, epicatechin, punicalagin, and chlorogenic acid. The peak that was observed for caffeic acid was absent after spiking. For the Wonderful sample, while there was an increase in the peak areas of punicalagin, chlorogenic acid, and rutin, a decrease in the peak areas was observed for catechin, epicatechin, and ellagic acid. No chlorogenic acid was present in the peel, while the peak for gallic acid was seen after spiking. For the PH sample too, no gallic acid was present in the peel, while the peak for chlorogenic acid was seen after spiking and there was a decrease in the peak areas observed for all the other phenolic acid compounds. It was interesting to note that there was a drastic

reduction in the peak areas of catechin and epicatechin after spiking the sample with the standards.

For the Parfianka sample, while there was an increase in the peak areas of rutin, catechin, and epicatechin; there was a decrease in the peak areas observed for gallic acid and punicalagin. The peak for ellagic was visible after spiking while gallic acid and punicalagin was completely absent. For the Vkunysi sample, there was an increase in the peak areas of gallic acid, and there was a decrease in peak areas for all other compounds except caffeic acid. Two surprising observations for this sample was the disappearance of the caffeic acid after spiking and a great decrease in peak areas of chlorogenic acid after spiking.

UDA_2x50_Vkusnyi_AFA_BFA_VeenaRPGGr1_5uL_04.d



UDA 2.1 x 50 mm

Gr 1

A: DI water/0.1%formic acid

B: acetonitrile/0.1% formic acid

0.4 mL/min

0 min 15%B

4 min 80%B

6 min 80%B

9 min 15%B

Figure 33. Comparison of caffeic acid standard with Vkusnyi sample

UDA_2x50_Vkusnyi_AFA_BFA_VeenaRPG1_5uL_04.d

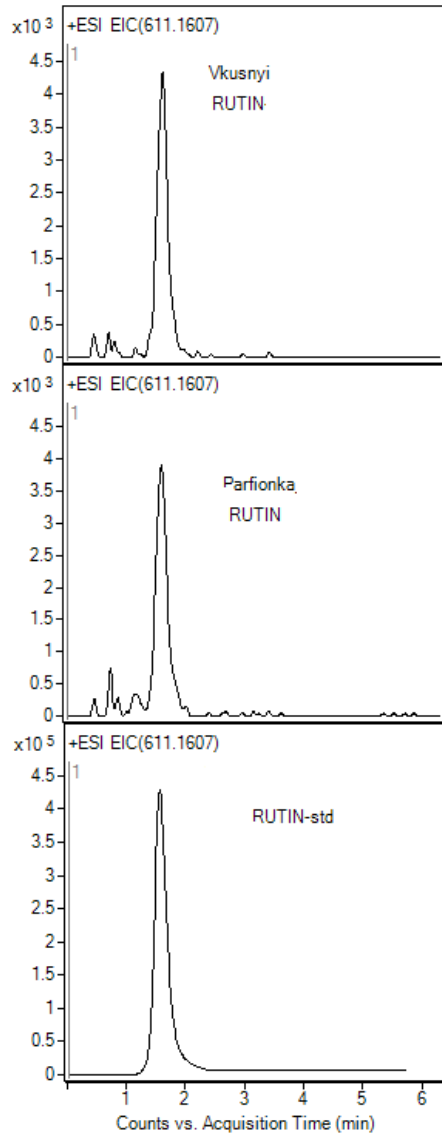


Figure 34. Comparison of Vkusnyi and Parfionka sample with rutin standard

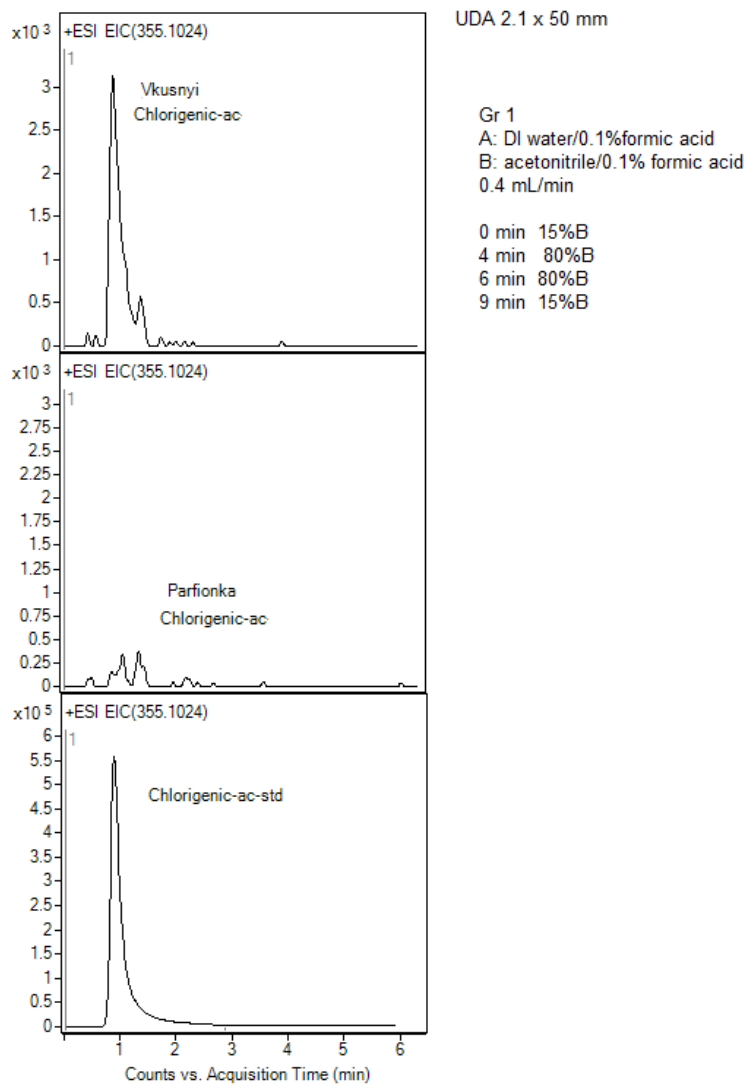


Figure 35. Comparison of Vkusnyi and Parfionka sample with chlorogenic acid standard

Another interesting observation from the UDA column was that no separation of the isomers, catechin and epicatechin was achieved and this is shown in Figure 36. It is very clear from Figure 36 that the retention time of catechin and epicatechin are very close to each other. A correct quantification of all the eight phenolic acid compounds was thus not possible on the UDA column leading to the use of a phenyl hydride column for investigating if these isomers could be well resolved.

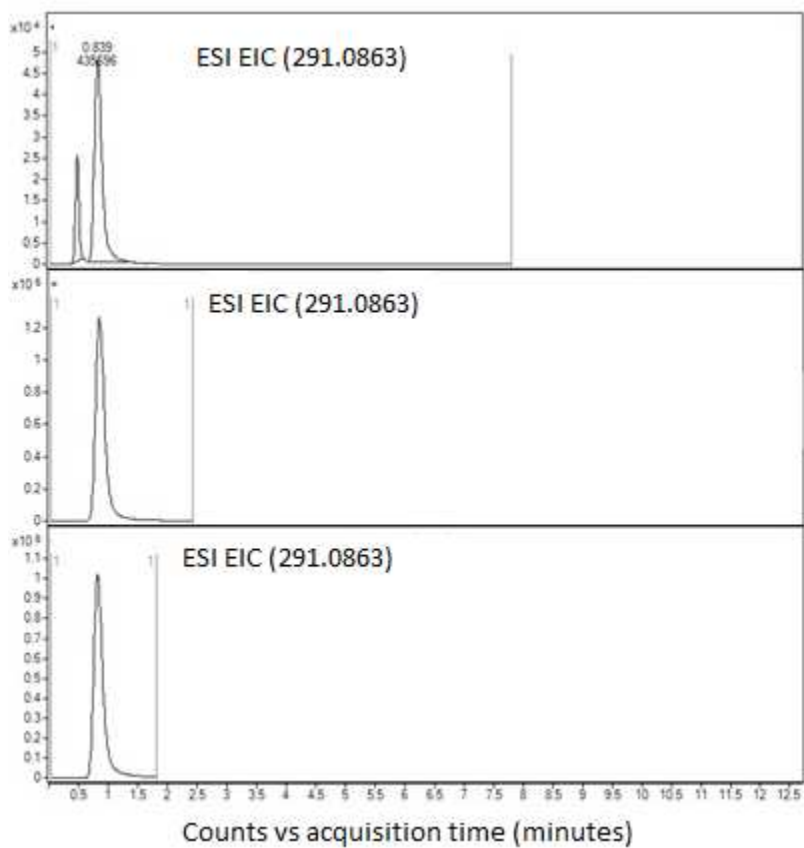


Figure 36. Comparison of retention times of catechin, epicatechin, and spiked Molla Nepes sample

Table 24a. Retention time and peak areas of standards, unspiked, and spiked Molla Nepes (MN) sample on the UDA column

Phenolic acids (RT,PA)	Standards	Unspiked MN (5 μ L)	Spiked MN (5 μ L)
Gallic (RT,PA)	0.655,5819408	0.648, 8491	0.651,8961
Punicalagin (RT,PA)	0.688,126608	0.730, 90319	0.721,69518
Catechin (RT,PA)	0.811,10693244	0.848, 461640	0.839,435696
Chlorogenic Acid (RT,PA)	0.895,6549993	0.977, 4567	0.851,1924
Caffeic Acid (RT,PA)	1.191,8893976	1.259, 320	no peak
Epicatechin (RT,PA)	0.837,13309604	0.848, 461640	0.839,435696
Rutin (RT,PA)	1.568,6309965	1.635, 3022	1.638,4966
Ellagic Acid (RT,PA)	*	1.165, 119549	1.144,138560

Table 24b. Retention time and peak areas of standards, unspiked, and spiked Wonderful sample on the UDA column

Phenolic acids (RT,PA)	Standards	Unspiked Wonderful (5 μ L)	Spiked Wonderful (5 μ L)
Gallic (RT,PA)	0.655,5819408	no peak	0.78113486
Punicalagin (RT,PA)	0.688,126608	0.718, 91259	0.722,93271
Catechin (RT,PA)	0.811,10693244	0.848, 773038	0.849,706388
Chlorogenic Acid (RT,PA)	0.895,6549993	no peak	no peak
Caffeic Acid (RT,PA)	1.191,8893976	1.177, 591	1.250,835
Epicatechin (RT,PA)	0.837,13309604	0.848, 773038	0.849,706388
Rutin (RT,PA)	1.568,6309965	1.635, 72890	1.638,73305
Ellagic Acid (RT,PA)	*	1.130, 177628	1.133,171244

Table 24c. Retention time and peak areas of standards, unspiked, and spiked Purple heart (PH) sample on the UDA column

Phenolic acids (RT,PA)	Standards	Unspiked PH(5 μ L)	Spiked PH(5 μ L)
Gallic (RT,PA)	0.655,5819408	no peak	no peak
Punicalagin (RT,PA)	0.688,126608	0.736,98424	0.736,89184
Catechin (RT,PA)	0.811,10693244	0.853,504190	0.866,431504
Chlorogenic Acid (RT,PA)	0.895,6549993	no peak	0.948,80044
Caffeic Acid (RT,PA)	1.191,8893976	1.255,956	1.253,235
Epicatechin (RT,PA)	0.837,13309604	0.853,504190	1.253,235
Rutin (RT,PA)	1.568,6309965	1.664,20350	1.653,17077
Ellagic Acid (RT,PA)	*	1.147,196183	1.148,183239

Table 24d. Retention time and peak areas of standards, unspiked, and spiked Parfionka sample on the UDA column

Phenolic acids (RT,PA)	Standards	Unspiked Parfionka(5 μ L)	Spiked Parfionka(5 μ L)
Gallic (RT,PA)	0.655,5819408	0.70,46628	0.700,46208
Punicalagin (RT,PA)	0.688,126608	0.724,101351	0.723,72768
Catechin (RT,PA)	0.811,10693244	0.841,482477	0.841,484938
Chlorogenic Acid (RT,PA)	0.895,6549993	no peak	no peak
Caffeic Acid (RT,PA)	1.191,8893976	no peak	no peak
Epicatechin (RT,PA)	0.837,13309604	0.841,482477	0.841,484938
Rutin (RT,PA)	1.568,6309965	1.629,51360	1.628,58388
Ellagic Acid (RT,PA)	*	no peak	1.135,113979

Table 24e. Retention time and peak areas of standards, unspiked, and spiked Vkunysi sample on the UDA column

Phenolic acids (RT,PA)	Standards	Unspiked Vkunysi(5 μ L)	Spiked Vkunysi(5 μ L)
Gallic (RT,PA)	0.655,5819408	0.736, 15188	0.727,23222
Punicalagin (RT,PA)	0.688,126608	0.724, 64471	0.715,54791
Catechin (RT,PA)	0.811,10693244	0.854, 751274	0.821,612191
Chlorogenic Acid (RT,PA)	0.895,6549993	0.901, 38984	0.891,7572
Caffeic Acid (RT,PA)	1.191,8893976	1.229, 18570	no peak
Epicatechin (RT,PA)	0.837,13309604	0.854, 751274	0.821,612191
Rutin (RT,PA)	1.568,6309965	1.655, 54829	1.573,36328
Ellagic Acid (RT,PA)	*	1.147. 133440	1.491,93343

D. Quantification of phenolic acids in pomegranate peel samples on phenyl hydride column

The pomegranate peel samples prepared earlier were run on the phenyl hydride column. The instrumental parameters and the solvents were the same as for the UDA column. The gradient method that was used for the UDA column was not effective for this column, hence a different gradient (gradient 4) was used where the concentration of the organic solvent was kept at 5% for ten minutes and gradually changed to 80% over the next three minutes and finally decreased to the initial concentration over the course of the next one minute. The spiking in this case was carried out by mixing 825 μ L of each sample with 25 μ L of each of the seven standards (except ellagic acid) and noting the RT and PA.

The RT and PA were tabulated for the unspiked and spiked samples and are shown in Tables 25 (a-e). It is very clear from Tables 25(a-e) that in each of the samples, catechin and epicatechin had different retention times thus proving that the phenyl hydride column was effective in carrying out the separation. Figure 37 shows the separation of catechin and epicatechin in the same standard mix as was used for the UDA column. Figure 38 shows the separation of catechin and epicatechin in the Molla Nepes and Parfianka sample.

Table 25a. Retention time and peak areas of unspiked and spiked Molla Nepes (MN) sample on the phenyl hydride column

Phenolic acids (RT,PA)	Unspiked MN	Spiked MN
Gallic(RT,PA)	1.535,81611	1.611,1065050
Punicalagin(RT,PA)	(7.280,13.519), (415712,268964)	(7.097,13.488), (507100,21159)
Catechin(RT,PA)	6.235,575582	6.204,3000083
Chlorogenic Acid(RT,PA)	8.749,465	8.237,1947904
Caffeic Acid(RT,PA)	no peak	6.568,2135079
Epicatechin(RT,PA)	8.15,91694	8.013,3909163
Rutin(RT,PA)	13.953,43897	13.982,183613
Ellagic Acid(RT,PA)	no peak	no peak

Table 25b. Retention time and peak areas of unspiked and spiked Wonderful sample on the phenyl hydride column

Phenolic acids (RT,PA)	Unspiked Wonderful	Spiked Wonderful
Gallic(RT,PA)	1.448,219262	1.624,1077140
Punicalagin(RT,PA)	(7.134,13.443), (1505561,907779)	(7.028,13.454), (981385,637275)
Catechin(RT,PA)	6.123,20330	6.17,3603784
Chlorogenic Acid(RT,PA)	no peak	6.262,1838922
Caffeic Acid(RT,PA)	no peak	6.593,16074077
Epicatechin(RT,PA)	8.003,76780	7.98,3759658
Rutin(RT,PA)	13.971,44519	3.983,1098566
Ellagic Acid(RT,PA)	no peak	no peak

Table 25c. Retention time and peak areas of unspiked and spiked Purple heart sample on the phenyl hydride column

Phenolic acids (RT,PA)	Unspiked PH	Spiked PH
Gallic(RT,PA)	1.46,150852	1.596,975720
Punicalagin(RT,PA)	(7.04,13.455), (33337,845942)	(7.073,13.476), (945722,567538)
Catechin(RT,PA)	6.23,721939	6.216,3102462
Chlorogenic Acid(RT,PA)	no peak	8.283,1949716
Caffeic Acid(RT,PA)	no peak	6.592,2622042
Epicatechin(RT,PA)	no peak	8.025,3709687
Rutin(RT,PA)	15.64,50096	13.981,1125633
Ellagic Acid(RT,PA)	no peak	no peak

Table 25d. Retention time and peak areas of unspiked and spiked Parfionka sample on the phenyl hydride column

Phenolic acids (RT,PA)	Unspiked Parfionka	Spiked Parfionka
Gallic(RT,PA)	1.615,149771	1.603,956752
Punicalagin(RT,PA)	(7.067,13.446), (842323,777695)	(7.242,13.481), (650150,433272)
Catechin(RT,PA)	6.162,854715	6.22,3183409
Chlorogenic Acid(RT,PA)	no peak	83,232,048,343
Caffeic Acid(RT,PA)	no peak	6.596,2293162
Epicatechin(RT,PA)	7.912,207004	8.018,3940811
Rutin(RT,PA)	13.975,45778	13.986,1262438
Ellagic Acid(RT,PA)	no peak	no peak

Table 25e. Retention time and peak areas of unspiked and spiked Vkunysi sample on the phenyl hydride column

Phenolic acids (RT,PA)	Unspiked Vkunysi	Spiked Vkunysi
Gallic(RT,PA)	1.626,1044853	1.6,1070255
Punicalagin(RT,PA)	(6.959,13.456), (2712,37950)	(7.228,13.478), (338302,249900)
Catechin(RT,PA)	6.149,908005	6.194,3197456
Chlorogenic Acid(RT,PA)	no peak	8.262,2059708
Caffeic Acid(RT,PA)	no peak	6.582,2245427
Epicatechin(RT,PA)	7.958,70129	8.015,3987484
Rutin(RT,PA)	13.973,52292	13.983,1210974
Ellagic Acid(RT,PA)	no peak	no peak

From Tables 25 (a-e) it can be inferred that for the Molla Nepes sample, there was an increase in the peak areas of all the seven phenolic compounds while caffeic acid and chlorogenic acid was absent initially, but appeared after spiking. For the Wonderful sample, while there was an increase in the peak areas of all compounds except punicalagin. Chlorogenic acid and caffeic acid were not present in the original sample, however they were seen after spiking. For the PH sample too, all the compounds except chlorogenic acid, caffeic acid, and epicatechin were present initially. After spiking, the initially absent compounds showed up and there was a substantial increase in the peak areas of the other compounds as well. In the Parfianka and Vkunysi samples chlorogenic acid and caffeic acid were absent and the other compounds tested showed an increase in peak areas after spiking. Figures 39 and 40 illustrates the absence or presence of chlorogenic acid and rutin respectively in the unspiked and spiked Molla Nepes samples. Figure 42 shows the presence of the characteristic peaks for punicalagin in each of the five unspiked pomegranate peel samples.

The amount of each of the phenolic acid compound detected in the peel samples were calculated using Equation 7 given below.

$$C_b = \frac{A_b \times C_s \times \Delta V}{[(A_c \times V_{tot}) - (A_b \times V_b)]} \quad \text{Equation 7}$$

In the above equation the terms denote: C_b - concentration of the analyte in the sample in parts per million (ppm), A_b – peak area of the unspiked sample, A_c - peak area of the spiked sample, C_s – concentration of the standard used for spiking the sample in ppm, ΔV – volume of the standard used for spiking the sample, V_{tot} - volume of sample+ volume of standard used for spiking the sample, and V_b - volume of sample. From the

values of Cb obtained for each of the component, their amounts in 1 gram of each of the pomegranate peel sample was determined and this is tabulated in Table 26. Figure 42 shows the bar graph showing the amount of each of the phenolic acid components in the five peel samples. From the bar graph we can infer that Vkunysi sample is one variety that has most of the phenolic acid components being analyzed. Caffeic acid and chlorogenic acid were not detected in any of the five samples. Thus it can be concluded from this study that Vkunysi is an ideal sample, although Wonderful is commercially available in the market.

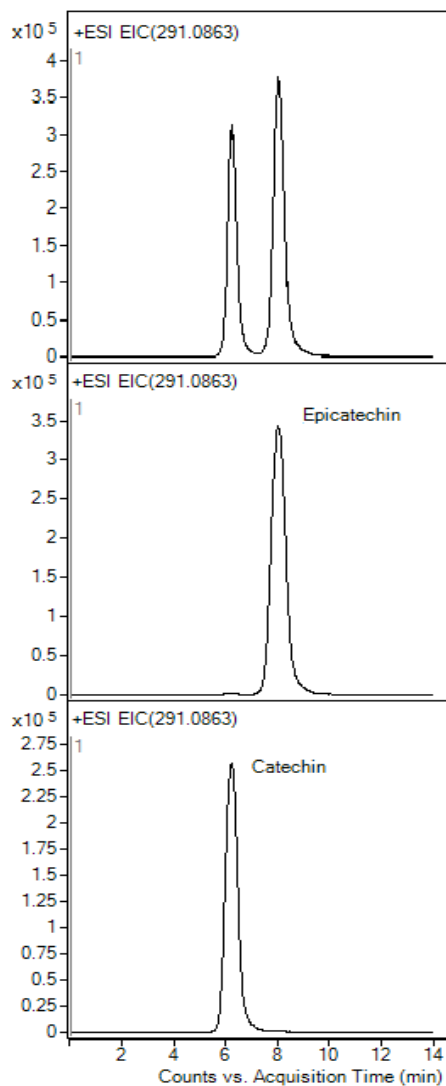
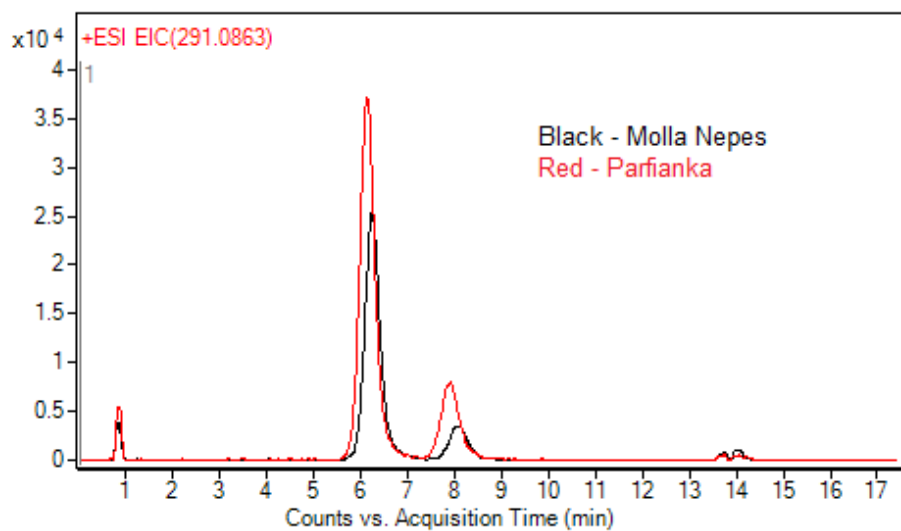


Figure 37. Separation of catechin and epicatechin in the standard mix on phenyl hydride column

Phenyl-2x100_PARFIANKA_5 uL_Veena-RP-Gr4-5uL_01.d



Black - Molla Nepes

Figure 38. Separation of catechin and epicatechin on phenyl hydride column in Molla Nepes and Parfianka sample

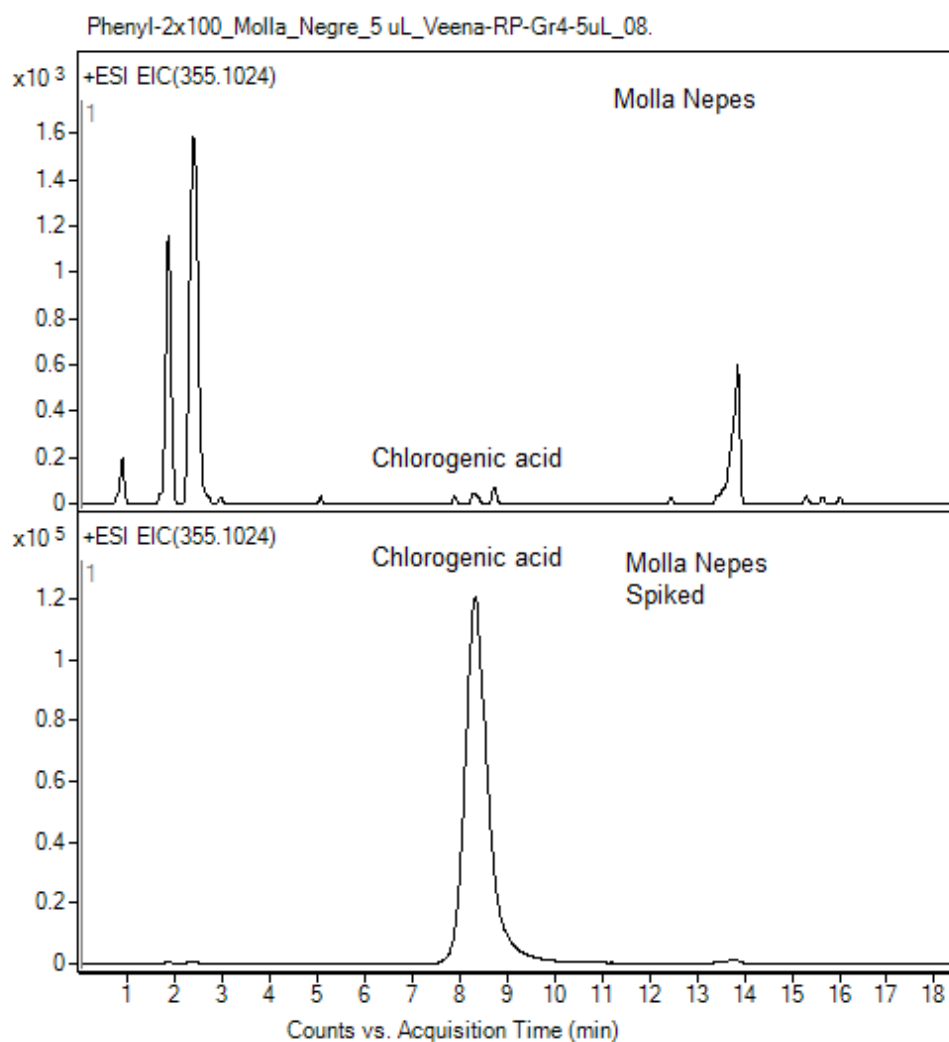


Figure 39. (m/z) of chlorogenic acid absent in the original sample and clearly seen in the spiked Molla Nepes sample

Scan Frag=175.0V Phenyl-2x100_Molla_Negre_5 uL_Veena-RP-Gr4-5uL_08.d

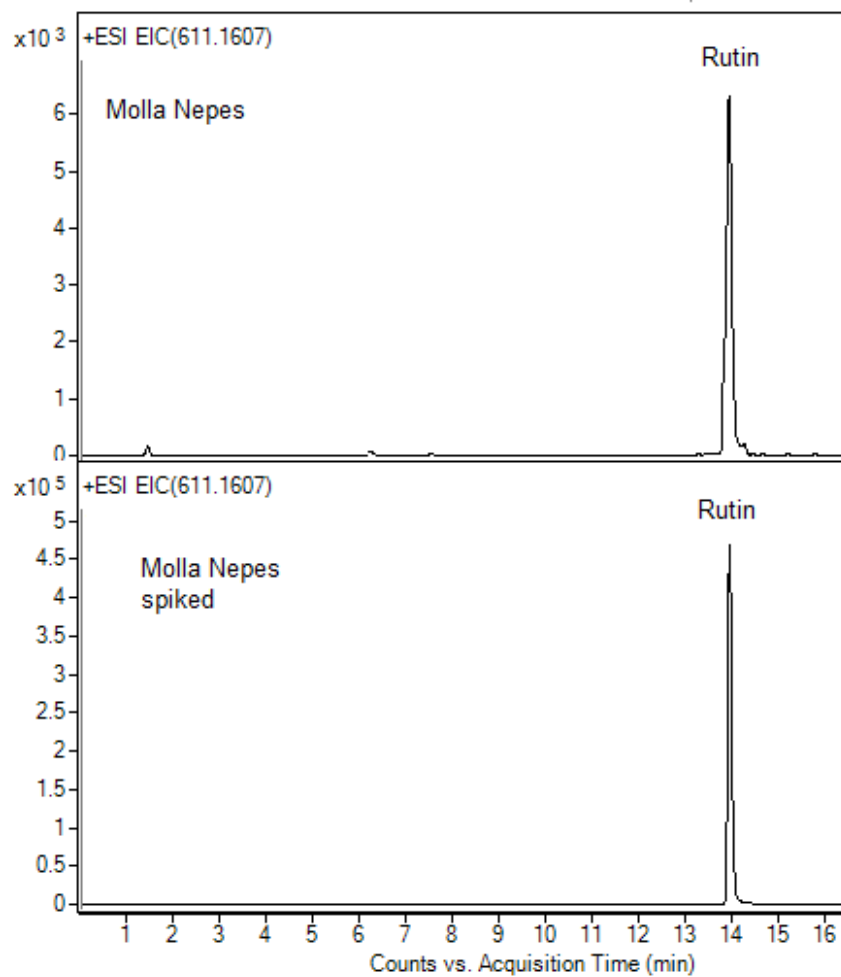


Figure 40. (m/z) of rutin in the unspiked and spiked Molla Nepes sample

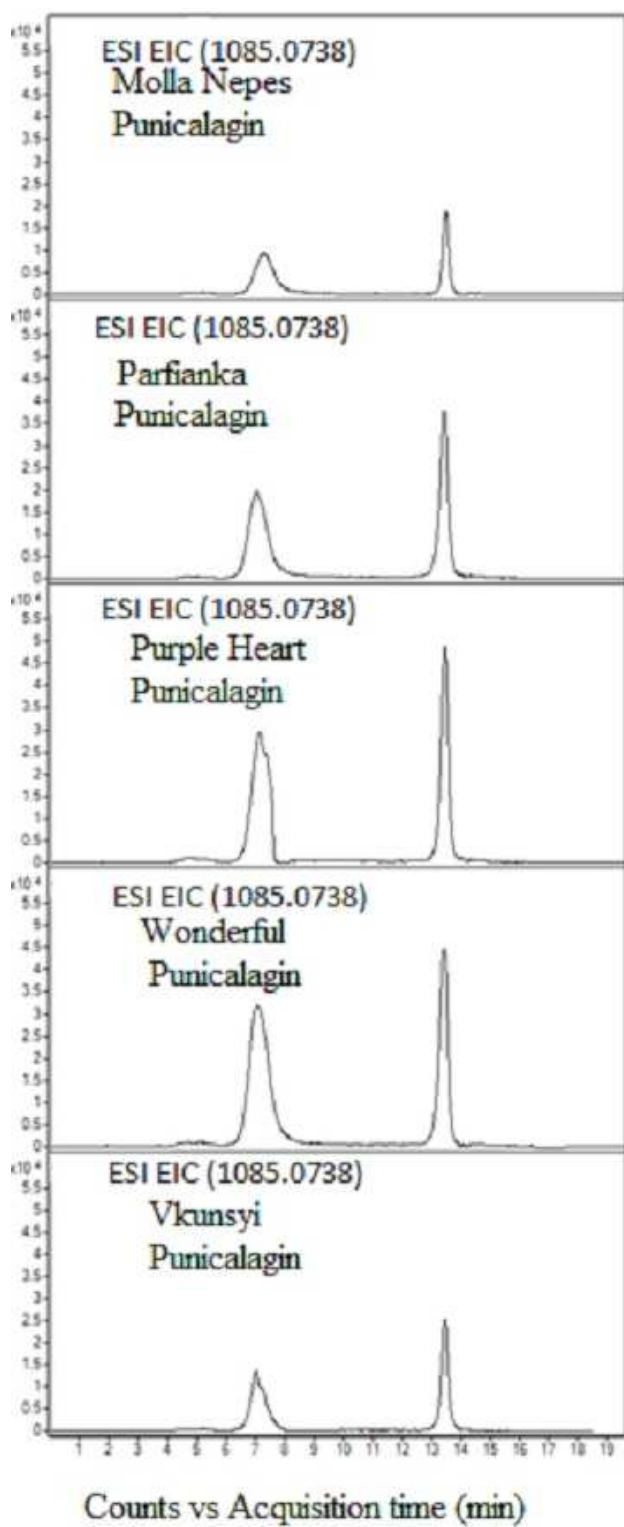


Figure 41. (m/z)'s for punicalagin in each of the five unspiked pomegranate peel samples

Table 26. Amount of each of the phenolic acid component in the pomegranate peel samples in $\mu\text{g/g}$

Amount of phenolic acid compounds ($\mu\text{g/g}$)	Names of the pomegranate samples					
	MN	Parfianka	PH	Wonderful	Vkunsyi	
Gallic acid	97.809	210.714	230.300	310.348	25026.19	
Punicalagin	*	*	*	*	11438.93	
Catechin	362.993	539.293	494.994	9.011	630.027	
Chlorogenic acid	0.480	0	0	0	0	
Caffeic acid	0	0	0	0	0	
Epicatechin	479.484	82.093	0	33.102	28.707	
Rutin	516.368	60.027	82.397	72.185	77.884	
Ellagic acid	*	*	*	*	*	

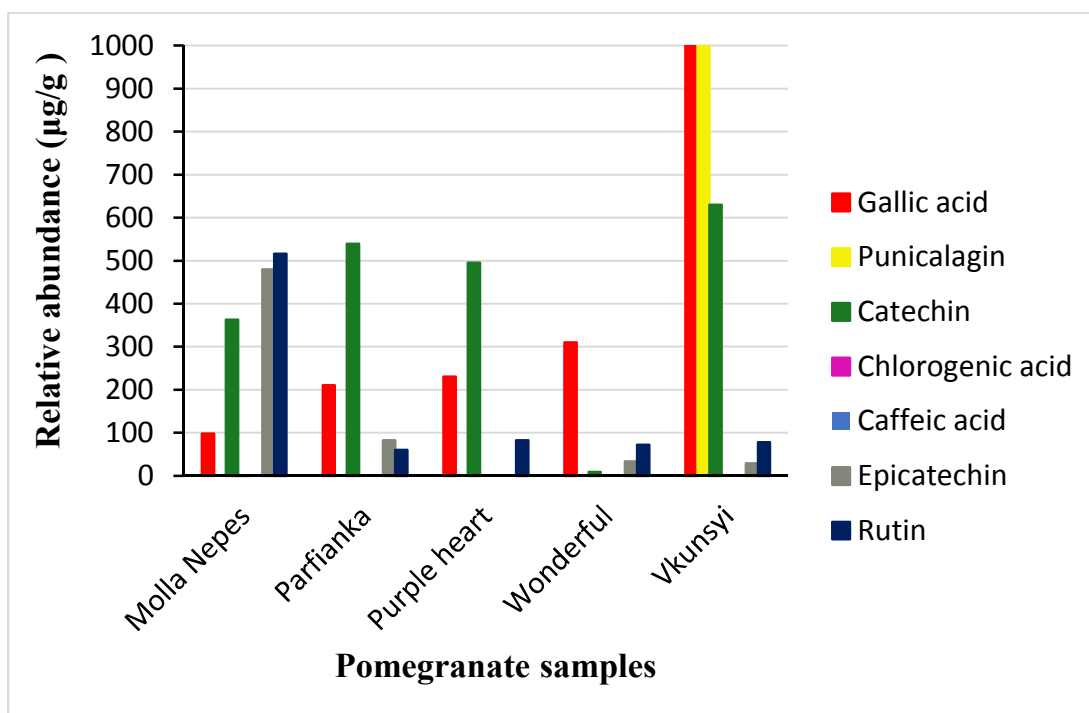


Figure 42. Bar graph showing the amount of each of the phenolic acid component in the pomegranate peel samples

V. CONCLUSION

One of the primary goals of this project was to characterize the silica hydride UDA column and establish the dual retention profile. Polar compounds were retained by the ANP mechanism at higher concentrations of the organic component in the mobile phase, while non-polar compounds displayed the usual RP mechanism. The ANP and RP data for the polar and non-polar compounds displayed a typical U shaped retention map when overlaid that is similar to other silica hydride based stationary phase.

Another aspect of the current project was to investigate the effect of varying concentrations of formic acid buffer. While polar compounds showed a decrease in retention as the concentration of the buffer was increased, no such trend was observed for non-polar compounds. The observed trend for polar compounds was clearly different from the HILIC retention mechanism [32], thus clearly distinguishing the ANP and HILIC retention mechanisms.

The compatibility of another buffer, ammonium acetate was also tested and established. The effect of varying the ammonium acetate buffer was also studied. No significant conclusion was made from the varying buffer study and this could be possibly due to the effect of ionization of the carboxylic acid group on the UDA above a pH of 6.

Lastly, characterization of the silica hydride based UDA column was done by varying the column oven temperature to study its effect on the retention of some compounds. The retention time of all compounds tested was found to vary slightly with varying temperature, thus proving the temperature dependence on retention time.

In addition to characterization studies, applications of the UDA column were also investigated. Several nucleotides and coenzymes, urine samples to check for the presence of pesticides, and the separation and quantification of the phenolic acid components in pomegranate peel samples were studied. The UDA column was found to be a suitable candidate to separate nucleotides and the pesticide samples. Complete separation of isomeric compounds in the pomegranate peel samples could not be achieved, and this led to the use of a phenyl hydride column that gave more promising results.

The data obtained from the current studies could be useful for future investigations of the silica hydride-based UDA column. However, at this point, a detailed account of the separation mechanism and weak cationic nature of the UDA column has not been established. Further goals include studying the retention of more non-polar compounds especially at a concentration greater than 50%, organic solvent in the mobile phase, and checking for any change in retention time beyond this concentration. It would also be interesting to look into the retention of amino acids and how the presence of acidic/basic groups and hydrophobic portions influence retention by studying these molecules under a range of concentrations of the mobile phase, in both RP and ANP mechanisms. Yet another future goal would be to analyze the separation of the nucleotides in a more complex physiological matrix. The urine sample analysis could also be done using an MS-MS detector to check for the presence of additional compounds. Lastly, the use of different gradients to separate the isomeric compounds, catechin, and epicatechin could also be studied.

REFERENCES

1. Skoog, A. D.; Holler, F. J.; Nieman, T. A. Principles of Instrumental Analysis, 5th ed.; Saunders College Publishing: Orlando, Florida, 1998, Chapters 26, 28.
2. Ranganr.Types of Chromatography| Based on Different Techniques & Methods <http://www.rajaha.com/types-chromatography-experiments/> (accessed Dec 10, 2015).
3. Kupiec, T. Quality-control analytical methods: High-performance liquid chromatography. *Int.J.Pharm.Compnd.* **2004**, *8*, 223-227.
4. Agilent Technologies. Overview on a chromatographical system. http://www.team-cag.com/support/theory/chroma/hplc_bas_at/chromatography/chromatographicalSystem.html (accessed 10 Dec , 2015).
5. Swartz, M. Seeing is Believing: Detectors for HPLC. *LC GC North America.* **2010**, *28*, 880-889.
6. Taylor, Tony. Important aspects of UV detection for HPLC. *LC GC North America.* **2015**, *33* ,870.
7. Resources. Getting Started in HPLC Section 2E. HPLC Detector <http://www.lcresources.com/resources/getstart/2e01.htm> (accessed Dec 10, 2015).
8. Lendia, B.E.; Meyer, V.R. The UV Detector for HPLC- An ongoing success story. *LC GC Europe.* **2005**, *18*, 156-163.
9. El-Aneed, A.; Cohen, A.; Banoub, J. Mass spectrometry, review of the basics: electrospray, MALDI, and commonly used mass analyzers. *Appl. Spectrosc.Rev.* **2009**, *44*, 210-230.
10. William Reusch . Mass spectrometry. <http://www2.chemistry.msu.edu/faculty/reusch/virttxtjml/spectrpy/massspec/masspec1.htm> (accessed 24 February 2016).
11. Finehout, E. J.; Lee, K. H. An introduction to mass spectrometry applications in biological research. *Biochem. Mol. Biol. Educ.* **2004**, *32*, 93-100.

12. Miller, P. E.; Denton, M. B. The quadrupole mass filter: basic operating concepts. *J. Chem. Educ.* **1986**, *63*(7), 617-622.
13. Bill Champion. Tips and tricks of HPLC separations.
[http://www.agilent.com/cs/library/eseminars/public/Tips%20and%20Tricks%20of%20HPLC%20Separation%20\(4-12-2011\).pdf](http://www.agilent.com/cs/library/eseminars/public/Tips%20and%20Tricks%20of%20HPLC%20Separation%20(4-12-2011).pdf) (accessed 29 Decemebr 2015).
14. Brown, L.; Ciccone, B.; Pesek, J. J.; Matyska, M. T. An evolution in separation media for HPLC. *Am Lab.* **2003**, *35*, 23-29.
15. Pesek, J. J.; Matyska, M. T.; Boysen, R. I.; Yang, Y.; Hearn, M. T. Aqueous normal-phase chromatography using silica-hydrate-based stationary phases. *TrAC, Trends Anal. Chem.* **2013**, *42*, 64-73.
16. Pesek, J. J.; Matyska, M. T.; Loo, J. A.; Fischer, S. M.; Sana, T. R. Analysis of hydrophilic metabolites in physiological fluids by HPLC-MS using a silica hydrate based stationary phase. *J. Sep. Sci.* **2009**, *32*, 2200-2208.
17. Pesek, J. J.; Boysen, R. I.; Hearn, M. T.; Matyska, M. T. Hydrate-based HPLC stationary phases: a rapidly evolving technology for the development of new bio-analytical methods. *Anal. Methods.* **2014**, *6*, 4496-4503.
18. Boysen, R. I.; Yang, Y.; Chowdhury, J.; Matyska, M. T.; Pesek, J. J.; Hearn, M. T.; Simultaneous separation of hydrophobic and hydrophilic peptides with a silica hydrate stationary phase using aqueous normal phase conditions. *J. Chromatogr. A.* **2011**, *1218*, 8021-8026.
19. Pesek, J. J., Matyska, M. T. *Hydrophilic interaction liquid chromatography (HILIC) and advanced applications*; Wang J. Perry; He Weixuan, Taylor and Francis group: Florida, U.S.A, **2011**, Chapter 1, pp 1-26.
20. Yang, Y.; Boysen, R. I.; Kulsing, C.; Matyska, M. T.; Pesek, J. J.; Hearn, M. T. Analysis of polar peptides using a silica hydrate column and high aqueous content mobile phases. *J. Sep. Sci.* **2013**, *36*, 3019-3025.
21. Pesek, J. J.; Matyska, M. T. Our favorite materials: Silica hydrate stationary phases. *J. Sep. Sci.* **2009**, *32*, 3999-4011.
22. Bayer, E.; Grom, E.; Kaltenecker, B.; Uhlmann, R. Separation of amino acids by high performance liquid chromatography. *Anal. Chem.* **1976**, *48*, 1106-1109.
23. Matyska, M. T.; Pesek, J. J.; Duley, J.; Zamzami, M.; Fischer, S. M. Aqueous normal phase retention of nucleotides on silica hydrate based

columns: Method development strategies for analytes relevant in clinical analysis. *J. Sep. Sci.* **2010**, *33*, 930-938.

24. Microsolv technologies. Cogent UDA™ HPLC column use and application. <http://kb.mtc-usa.com/article/AA-02490/99/>(accessed on 10 December 2015).

25. Al-Rawahi, A. S.; Edwards, G.; Al-Sibani, M.; Al-Thani, G.; Al-Harrasi, A. S.; Rahman, M. S. *Eur. J. Med.Plants.* **2014**, *4*, 315-331.

26. Microsolv technologies. Cogent UDA™ HPLC columns: weak cationic exchange on silica. <http://mtc-usa.com/cogent-uda.aspx>(accessed 24 February, 2016).

27. GMI.HPLC systems. HP 1090 series HPLC systems. <http://www.gmi-inc.com/hp-1090-series-hplc-system.html>(accessed on 10 December 2015).

28. Dewey, C. N., & Pachter, L. Evolution at the nucleotide level: the problem of multiple whole-genome alignment. *Hu. Mol. Gen.* **2006**, *15*, 51-56.

29. Dougherty, D. A. Cation- π interactions involving aromatic amino acids. *J. Nutr.* **2007**, *137*, 1504-1508.

30. Lieberman, M., Marks, A. D., Smith, C. M., & Marks, D. B. *Marks' essential medical biochemistry*; Lippincott Williams & Wilkins: Baltimore, Maryland USA, 2007; Chapter 4, pp 53-62.

31. Ribeiro, J. A.; Sebastiao, A. M. Caffeine and adenosine. *J. Alzheimers. Dis.* **2010**, *20*, 3-15.

32. Pesek, J. J.; Matyska, M. T.; Natekar, H. Evaluation of the dual retention properties of stationary phases based on silica hydride: Perfluorinated bonded material *J. Sep. Sci.* **2016**, *39*, 1050-1055

33. Wang, B. N.; Liu, H. F.; Zheng, J. B.; Fan, M. T.; Cao, W. *J. Agric. Food.Chem.* **2011**, *59*, 1288-1292.