2023

Investigation of factors affecting reverse-phase high performance liquid chromatography

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Faculty Sponsor - Dr. Michael Hunnicutt

Abstract

This study focused on the application of reverse-phase high-performance liquid chromatography (RP-HPLC). RP-HPLC is used for the purification of organic molecules, including pharmaceuticals, natural products, and proteins. We developed a modified version of a United States Pharmacopeia (USP) procedure to separate and purify the chemical components found in Excedrin tablets. The goal was to investigate the relationship between retention time (Tr) and the capacity factor (k') of solutes. By varying the composition of the mobile phase and stationary phase, we experimentally determined the capacity factor of acetaminophen (APAP), caffeine, and aspirin. Our results show that the capacity factor (k') of all solutes increased as the percentage of water in the mobile phase increased. This trend was consistent across all three columns tested. We also observed co-elution of solutes in the C8 and phenyl columns at lower water compositions. For superior separation and baseline resolution of all peaks, we found that the C18 column, as specified in the USP monograph, provided excellent results at all mobile phase compositions tested. Additionally, we investigated the solute retention as a function of mobile phase acidity. When using an acidified mobile phase with the C18 column (3% acetic acid), all solutes exhibited excellent peak shape and symmetry. However, the removal of acid from the mobile phase resulted in poor peak symmetry (non-Gaussian) for solutes containing a carboxylic acid moiety, such as aspirin and benzoic acid. Conversely, more polar solutes like APAP and caffeine maintained their Gaussian peak shape.In conclusion, our study highlights the successful implementation of RP-HPLC for the purification of organic molecules and reveals the relationship between retention time and the capacity factor of solutes. The choice of column and mobile phase composition played a crucial role in achieving optimal separation and peak symmetry.

Introduction

High-performance liquid chromatography (HPLC) is a widely used analytical technique for the separation and quantification of complex mixtures. In HPLC, a mobile phase is passed through a stationary phase that separates the components of a sample based on their chemical and physical properties [1]. HPLC has gained popularity over other separation techniques due to its high sensitivity, reproducibility, and accuracy, making it ideal for qualitative and quantitative analyses. Reverse-phase high-performance liquid chromatography (RP-HPLC) is a type of high-performance liquid chromatography (HPLC) that uses a stationary phase with a non-polar surface and a mobile phase with a polar solvent. This reversed-phase environment allows for the separation of analytes based on their hydrophobicity [3]. Reverse-phase high-performance liquid chromatography is actively used in various fields, including pharmaceuticals, biotechnology, food and beverage, and environmental sciences. In the pharmaceutical industry, RP-HPLC is utilized for drug discovery and development, including the analysis of drug impurities, degradation products, and drug metabolites. RP-HPLC is also used for quality control and assurance in drug manufacturing. However, achieving optimal separation and resolution in RP-HPLC is not always straightforward and can be influenced by several factors, including the composition of the mobile phase, the properties of the stationary phase, and the operating conditions of the instrument. Understanding and controlling these factors is critical for achieving reliable and reproducible results in RP-HPLC. This paper aims to investigate the factors that affect RP-HPLC and their impact on the separation and resolution of complex mixtures. By exploring these factors, we can gain a better understanding of RP-HPLC and develop strategies to improve the performance and reliability of this powerful analytical technique.

Experimental Method

A modified version of the United States Pharmacopeia (USP) method was used for the introduction of a high-performance liquid chromatography (HPLC) in CHEZ 309 [1]. The % label claim of APAP, caffeine and aspirin for an over-the-counter analgesic product was determined (Excedrin Extra Strength). As specified by the USP monograph, the C18 column served as the stationary phase. Isocratic elution was used to separate the solutes with a mobile phase composition of 69% water, 28% methanol and 3% glacial acetic acid. A single point calibration standard which included an internal standard, benzoic acid, was used for quantitation.

To investigate the relationship between k' of the active ingredients (i.e., solutes) and % water in the mobile phase, the mobile phase composition of methanol and water was varied between 25-75% respectively. The mobile phase was acidified with 3% glacial acetic acid in all cases. Each of these mobile phase compositions were used to determine the k' of the solutes for the C18, C8 and phenyl columns. Moreover, to examine the effect of acidifying the mobile phase, a binary system consisting of water and methanol was used.

Results and Discussion

Introduction of HPLC in CHEZ 309

To verify the success of introducing an HPLC experiment in CHEZ 309, a table containing students' data (n=50) was compiled. The average % label claim of the three active ingredients ranged between 97-98%. The average standard deviation of the % label claim for all three active ingredients was between 9-11%. (See Table 1). Generation of high % label claims and low standard deviation values indicated that the students understood the modified USP procedure and were able to perform the experiment successfully. Students became well

acquainted with an HPLC instrument. They were able to select and load different analysis methods, set up sequence files to analyze samples and correctly make injections. They also learned how to quantitate the label claim of each active ingredient using equations provided by the USP monograph.

Table 1. CHEZ 309 student data.

Students (N=50)	**Acetaminophen**	**Caffeine**	**Aspirin**
Product Label Claim	250 mg/tablet	65 mg/tablet	250 mg/tablet
Assay Result (Avg)	244	63	244
% Label Claim (Avg)	97	97	98
%Rel. Std. Dev. (Avg)	9	11	10

Additionally, students were able to demonstrate their knowledge regarding solute retention with respect to a varying mobile phase composition. They investigated this relationship by generating a k' plot for APAP and aspirin against % water in the mobile phase. The k' plot formed by students depicted that an increase in % water in the mobile phase had a greater effect on the less polar molecule aspirin than the more polar molecule APAP (Figure 1).

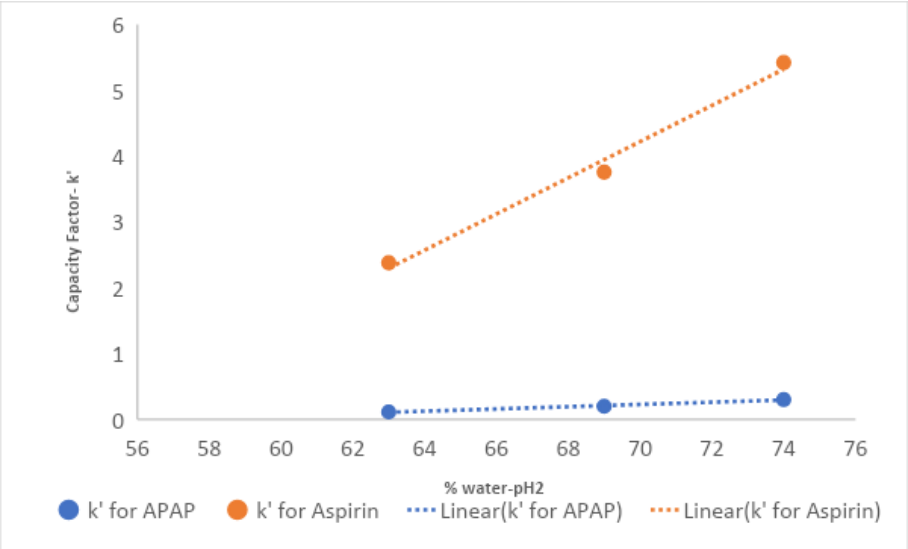

Figure 1. *k' plot for APAP and aspirin vs. % water*

APAP and aspirin's interaction with the stationary phase, at varying mobile phase compositions, is displayed through their respective slopes in Figure 1. APAP's flat slope indicates decreased retention on the hydrophobic stationary phase, whereas aspirin's steep slope is indicative of greater retention on the hydrophobic column. The difference in the k' trendlines for aspirin and APAP is due to the principle of 'like dissolves like.' In partition chromatography, solutes partition between the stationary phase and mobile phase. Increasing the % water in the mobile phase increases its polarity. The more non-polar aspirin is less soluble in this polar medium. As a result, increasing the % water in the mobile phase causes aspirin to partition into the hydrophobic stationary phase to a greater extent. This allows for stronger retention on the column and a greater k' value. APAP is a more polar molecule and as % water in the mobile phase increases, it favors the polar medium much more than aspirin. Therefore, increasing the % water in the mobile phase does not greatly affect the k' value of APAP.

Investigation of factors influencing retention and selectivity in reverse phase-HPLC

In HPLC, partition of solutes occurs between the mobile phase and stationary phase. The partition coefficient (K_{eq}) of this process is defined as the ratio of the concentration of solutes in the stationary phase to the concentration of the solutes in the mobile phase (equation 1). The octanol-water coefficient (K_{ow}) determines the ratio of concentration of solutes in a non-polar, hydrophobic octanol environment to the concentration of solutes in a polar, hydrophilic water environment (equation 2).

$$K_{eq} = \frac{[C]_{Stationary\ phase}}{[C]_{Mobile\ phase}} \qquad (2) K_{ow} = \frac{[C]_{octanol}}{[C]_{Water}}$$

*Where [C] is the concentration of solutes.

This is analogous to the K_{eq} of a reverse-phase HPLC system, where the C18 column serves as a non-polar medium and the mobile phase serves as a polar medium. By establishing a relationship between K_{eq} and $K_{ow,}$ the K_{ow} values can be used to predict the order of elution of solutes [2].

Polar nitrogen containing compounds (APAP, caffeine) and less polar benzoic acid containing compounds (aspirin, benzoic acid) were used in all studies. Figure 2 contains the structures of all the solutes with their associated log K_{ow} values.

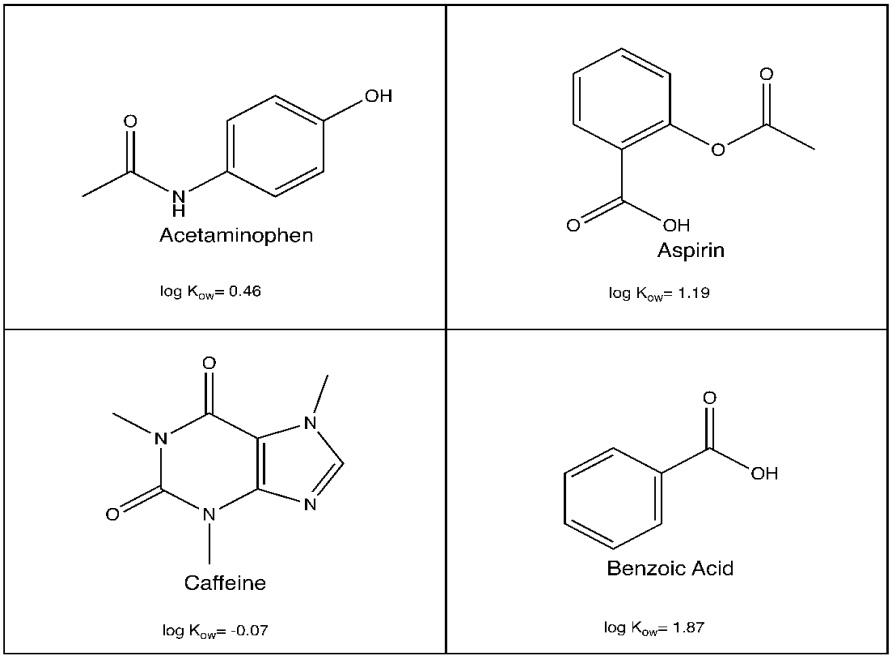

Figure 2. *Structures of solutes and internal standard with their log K_{ow}values*

Based on the log K_{ow} values mentioned in Figure 2, the order of elution predicted from first to last is as follows: caffeine, APAP, aspirin, and benzoic acid. Caffeine with the lowest octanol-water coefficient indicates that it favors a polar environment and is, therefore, predicted to elute first. However, experimentally caffeine was retained longer on the column and APAP eluted first (Figure 3). This is because log K_{ow} is consistent with the order of elution of solutes in a classic binary system comprised of methanol and water. The inclusion of acid in the mobile phase disrupted the formation of a classic hydrophobic-hydrophilic system.

The difference in order of elution and log K_{ow} is also due to the solute structure and their hydrogen-bond acidity. Large variations in the solute polarizability can affect the linear relationship between K_{eq} and log K_{ow} which leads to an incorrect prediction of solute elution [3].

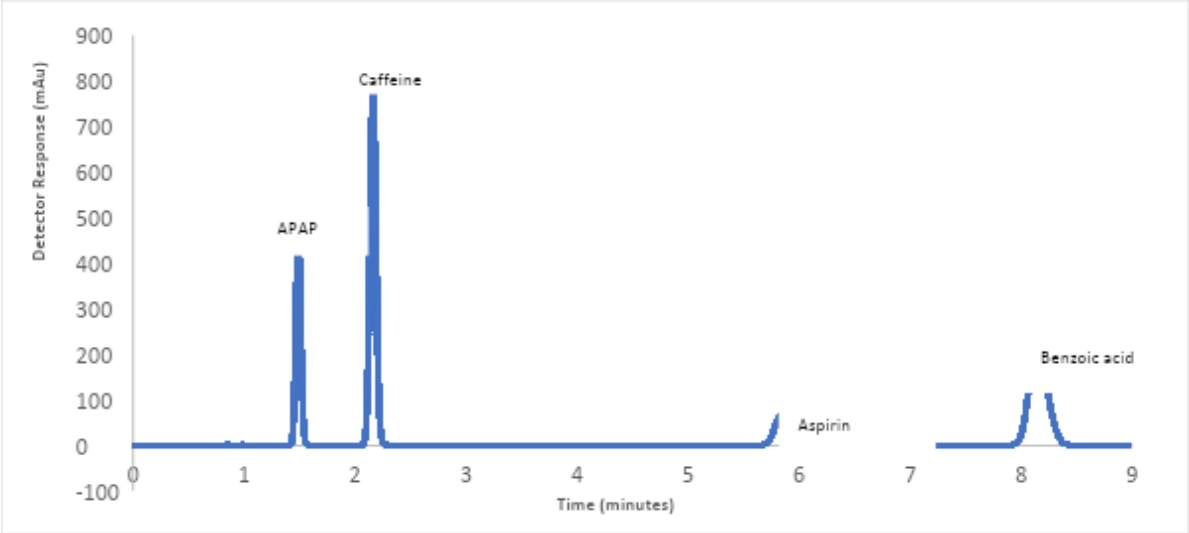

Figure 3. *Chromatogram of solutes on C18 column @69%H20,28%MeOH, 3%GAA*

A capacity factor (k') study was conducted to observe the behavior of each solute at varying mobile phase and stationary phase compositions. The capacity factor determines how long a solute is retained on any column. Specifically, it is a ratio of the retention time of the solute in the stationary phase to the retention time of the solute in the mobile phase (equation 3). The time for an unretained compound to pass through the column and enter the detector indicates the retention time of a solute in the mobile phase (t_m). Acetone was used to generate values for t_m as this compound has very little affinity for the stationary phases used.

$$(3) k' = \frac{t_r - t_m}{t_m} = \frac{t_s}{t_m}$$

*Where t_s is the time a solute spends in the stationary phase, t_m is the time a solute spends in the mobile phase.

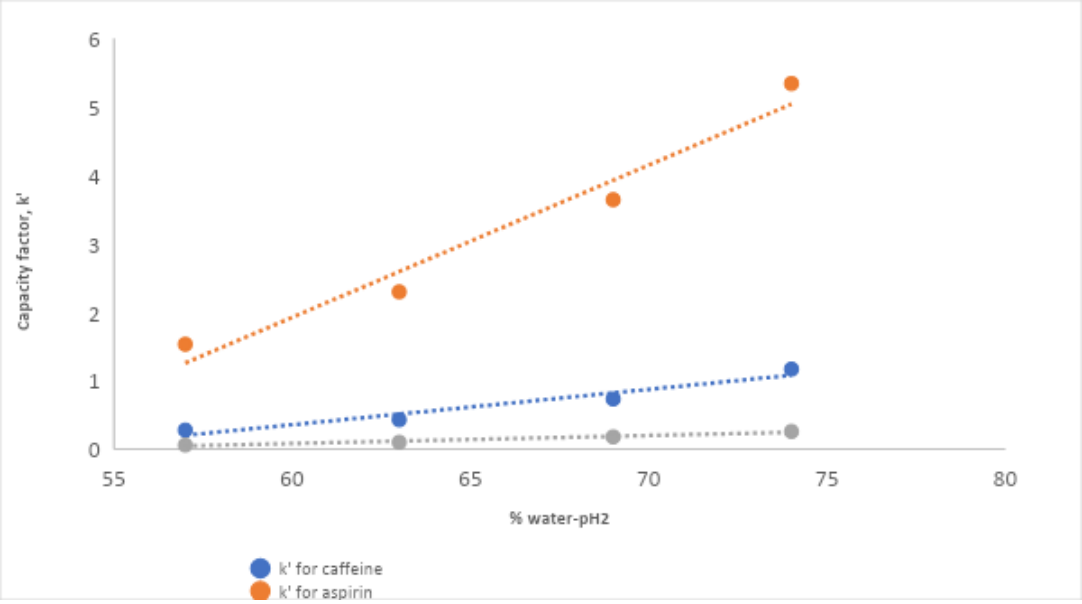

Figure 4. *k' of solutes on C18 column*

The k' of solutes were measured at a range of mobile phase compositions using a C18 column. Increasing the % water in the mobile phase from 57-74% caused the k' of the more non-polar solute (i.e., aspirin) to increase significantly as shown in Figure 4. The retention of aspirin was previously described on page 5. APAP and caffeine were less affected than aspirin by changes in the mobile phase composition. Increasing the % water in the mobile phase caused a steady increase in APAP and caffeine's k' values. This is because the more polar solutes (i.e., APAP, caffeine) were more soluble in the polar mobile phase than aspirin. Increasing the % water did not induce the more polar solutes to partition out of the mobile phase to the same extent as the more non-polar aspirin. This generated small k' values for APAP and caffeine. Caffeine's k' values were also seen to be slightly greater than APAP's. The difference in APAP and caffeine's k' values is due to their difference in structure and polarity as seen in Figure 5.

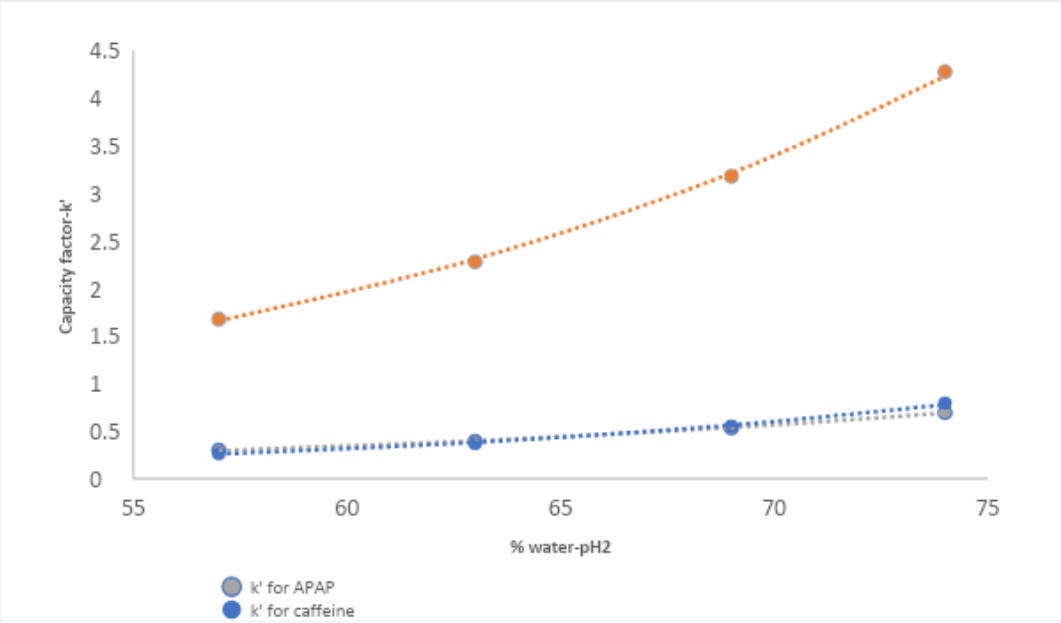

Figure 5. *k' of solutes on C8 column*

The k' of solutes were measured using a C8 column as shown in Figure 5. Aspirin's k' increased with increasing % water in the mobile phase. However, the increase observed in aspirin's k' values were not as great as with the C18 column. The more polar solutes, APAP and caffeine, co-eluted at all mobile phase compositions except at the composition with the highest % water (i.e., 74%). The more polar solutes, possibly, had similar interactions with the C8 stationary phase. Co-elution was a result of the C8 stationary phase not being sufficiently hydrophobic to separate these two solutes.

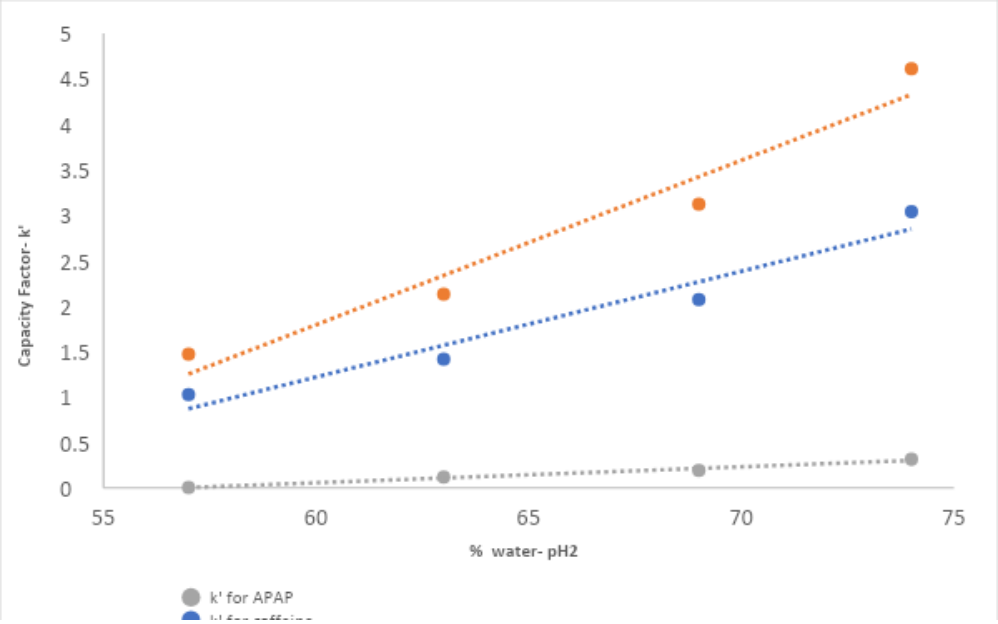

Figure 6. *k' of solutes on phenyl column*

The k' of solutes were measured using a phenyl column as shown in Figure 6. Increasing the % water in the mobile phase showed the same effect on aspirin as with the C18 and C8 column. The phenyl column was able to separate all solutes being analyzed at all mobile phase conditions, unlike the results generated using the C8 column. An increase in caffeine's k' values indicates that it interacts more strongly with the phenyl stationary phase than APAP. This could possibly be due to differences in polarizability between the two polar molecules. Another possible reason is due to the number of rings associated with the two polar solutes and their interaction with the phenyl stationary phase. The structures of caffeine and APAP can be seen in Figure 2.

Table 2. Capacity factor of solutes at highest % water.

Solute	C18 column	Phenyl Column	C8 column
APAP	0.26	0.31	0.70
Caffeine	1.17	3.04	0.79
Aspirin	5.34	4.61	4.28

Mobile phase: 74% H20, 23%MeOH,3% GAA

The k' values for each column, at the highest % water, are summarized in Table 2. At the highest % water, all solutes were separated on each column and followed the same order of elution. The k' of aspirin, for each column, increased with increasing % water in the mobile phase. The C18 stationary phase had the highest k' value of aspirin, followed by phenyl and C8 stationary phase. The C18 column had the highest k' value of aspirin due to the column's increased hydrophobicity. C18 chains bonded to silanol groups attributed to the creation of a very non-polar stationary phase, promoting strong retention of the non-polar solute aspirin. The C8 column comprised of C8 chains bonded to silanol groups decreased the hydrophobicity of the stationary phase. The hydrophobicity further decreased with the phenyl column, where silanol groups were attached to propyl chains linked to polarizable benzene rings.

Caffeine's k' value on the phenyl column was greater than its k' value on the C18 column. This is because caffeine had more favorable interactions with the polar phenyl column than the non-polar C18 column. A possible explanation for this occurrence is that caffeine and the phenyl column's polarity are very similar.

APAP's k' values for all three columns were the lowest, indicating that APAP was the least retained solute. Out of the three stationary phases analyzed, the C8 column had the highest

k' value of aspirin. It can be hypothesized that the C8 column's polarity was very similar to APAP's polarity, hence why APAP was retained longest on the C8 column.

The selectivity factor (α) measures the ability of a column to separate a pair of solutes (A and B) under certain mobile phase conditions. The selectivity factor measures the degree of separation between two solutes based on the ratio of their capacity factors, where B elutes after A (equation 4).

$$(4) \quad \alpha = \frac{k'_B}{k'_A}$$

Table 3. Selectivity factor of solutes at highest % water.

Column	**α Caffeine/APAP**	**α Aspirin/Caffeine**	**Total Run time (min)**
C18	4.54	4.57	10.50
Phenyl	9.67	1.52	4.50

Mobile phase: 74% H_20, 23%MeOH,3% GAA

The selectivity factor of solutes on the C18 and phenyl column are shown in Table 3. In comparison to the C18 column, the phenyl column showed excellent separation and selectivity not only between caffeine and APAP, but also between aspirin and caffeine.

The phenyl column was able to separate all solutes at a shorter run time, whereas the C18 column took twice the amount of time than the phenyl column. However, in the presence of benzoic acid in the sample, using a phenyl column, aspirin and benzoic acid were seen to co-elute at 69% water. The presence of carboxyl group moieties in aspirin and benzoic acid, in combination with the less hydrophobic phenyl column, resulted in inadequate separation of the two compounds. The selectivity factor for caffeine-APAP and aspirin-caffeine, for the C18

column, were seen to be almost identical. Evaluation of the three columns indicated that the C18 column served as the superior column as it provided sufficient and consistent separation of all active ingredients and benzoic acid while generating sharp, gaussian peaks.

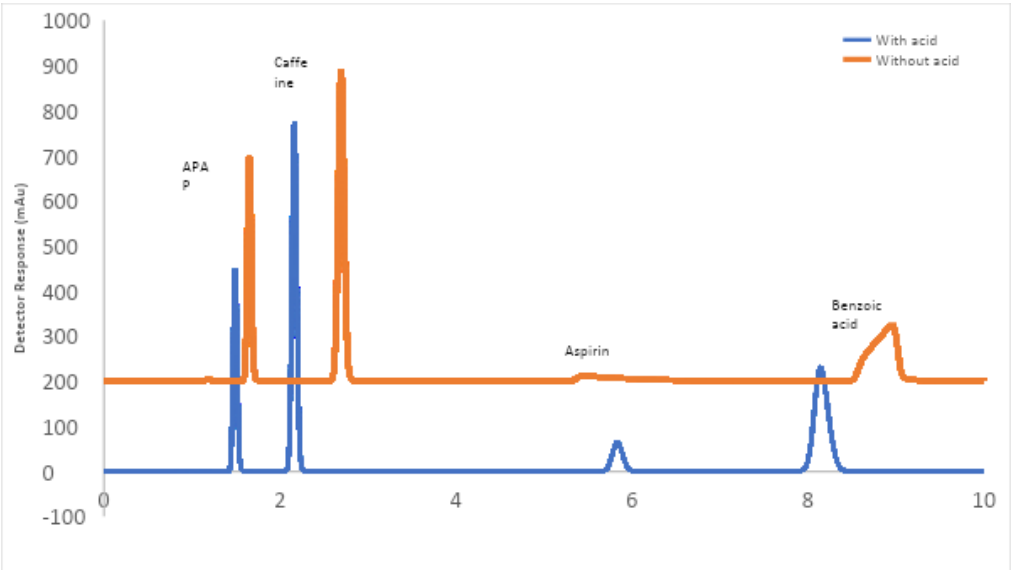

Figure 7. *Chromatogram of solutes and internal standard with and without acid*

Results from investigating the effect of acidifying the mobile phase can be seen in Figure 7. In the absence of acid, the resolution and peak shape of the less polar solutes (i.e., aspirin, benzoic acid) was lost. The more polar solutes (i.e., APAP, caffeine) were seen to preserve their peak symmetry. APAP, caffeine, and benzoic acid were retained slightly longer on the C18 column, whereas aspirin eluted slightly earlier. Acidification of the system caused residual unmodified silica particles on the stationary phase to be chemically modified with acetic acid, creating a more classic reverse phase-HPLC system. This allowed the stationary phase to be uniformly non-polar with C18 and C3 chains interacting with the solutes. Acidification of the mobile phase also suppressed the ionization of the solutes which allowed for solely hydrophobic interactions between the solutes and the stationary phase.

In the absence of acid, solutes containing a benzoic acid moiety (i.e., aspirin, benzoic acid) were able to undergo ionization. Acetic acid was also no longer present to bind with the silanol group. This possibly resulted in unmodified silanol groups interacting with the ionized solutes. The unmodified silanol groups increased the polarity of the stationary phase and allowed for polar interactions with the ionized solutes. This polar interaction is believed to have affected the rate of mass transfer of solutes between the stationary phase and mobile phase, resulting in poor peak symmetry seen in the form of peak tailing and fronting.

When acid was included in the mobile phase, a classic reverse phase system was formed which possibly increased mass transfer of solutes between the two phases. These results indicate that acidification of the mobile phase is essential to generate sharp, gaussian-shaped peaks. Gaussian-shaped peaks are important as these peaks are reproducible and can be easily integrated. Sharp gaussian peaks are required if the concentration of the solutes is to be measured quantitatively. Broad asymmetric peaks are difficult to accurately integrate and reproduce, and thereby cannot be used for quantification.

Future Work

Results from this study show the phenyl stationary phase to serve as a superior column than the C18 stationary phase. This is because the phenyl column would be able to separate all solutes with good selectivity and, with a shorter run time. A shorter run time can reduce the amount of chemical waste produced and allow more samples to be analyzed at a given time. The reduction in the generation of chemical waste and faster results can make phenyl a more cost-efficient option.

Further work can also be conducted to see if there is a relationship between the co-elution of the more polar solutes (i.e., APAP, caffeine) and the C8 column. Similarly, research can be done to see if there is a relationship between the co-elution of the more non-polar solutes (i.e., aspirin, benzoic acid) and the phenyl column.

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