Journal Homepage: http://ijcpe.uobaghdad.edu.iq
Iraqi Journal of Chemical and Petroleum Engineering
Vol. 26 No. 2 (June 2025) 143 – 151
EISSN: 2618-0707, PISSN: 1997-4884

Studying the chromatographic separation of caffeine, theophylline, and theobromine

Hassanain A. Hassan [a], Miqat Hasan Salih [a, *], Maha H. Alhassani [a]

a Department of Chemical Engineering, College of Engineering, University of Baghdad, Baghdad, Iraq

Abstract

Theobromine, theophylline, and caffeine retention times in a C_{18} HPLC column (stationary phase) were investigated as a function of mobile phase flow rate, mobile phase composition, and column temperature. When the mobile phase flow rate increased from 0.1 to 1 mL/min and the methanol concentration increased from 5 to 30%, the retention time and peak width of these three compounds were found to be reduced. While there was a small influence of increasing the mobile phase flow rate on the resolution, decreasing the methanol concentration in the mobile phase considerably reduced the resolution. In addition, the mobile phase flow rate and composition were determined to have a more significant impact than the column temperature. According to the findings, theobromine, theophylline, and caffeine were most effectively separated by liquid chromatography at a flow rate of 0.5 mL/min with a mobile phase methanol concentration of 15%.

Keywords: HPLC; Mobile phase; Stationary phase; Retention time; Resolution.

Received on 06/04/2025, Received in Revised Form on 10/06/2025, Accepted on 10/06/2025, Published on 30/06/2025

https://doi.org/10.31699/IJCPE.2025.2.15

1- Introduction

Liquid Chromatographic separation is a powerful analytical technique in which different solutes are separated based on their partitioning between the mobile and stationary phase [1-3]. In high performance liquid chromatography (HPLC), the mobile phase (solvent) carrying the compounds to be separated (solutes) is forced by a high-pressure pump to flow through the stationary phase (packed bed) [4-7] as shown in Fig. 1. After the solute elute from the stationary phase, the UV detector detects the solute, and accordingly a chromatogram is generated by the HPLC software [8, 9]. This makes the separation by HPLC to be the most useful method of separating compounds in a mixture for both analytical and industrial purpose [7, 10].

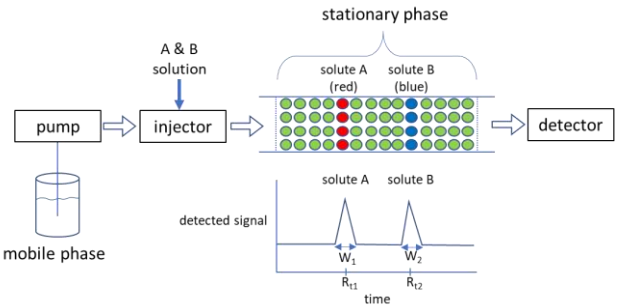

Fig. 1. Schematic diagram for the main elements of high-performance liquid chromatography (HPLC) for the separation of two compounds, A (red) and B (blue)

The separation process by HPLC is controlled by several parameters, such as the column (stationary phase), the mobile phase constituents (solvent) [11], the stationary phase (column), solvent follow rate, and temperature [12, 13]. A good separation method requires choosing the right column (stationary phase) and the corresponding solvent (mobile phase) [14, 15].

Caffeine (CAF) and its naturally occurring derivatives [16], theophylline (TP) and theobromine (TB), which exist in drinks such as coffee and tea [17-22], have been chosen to study their separation by HPLC. The chemical structure of caffeine, theophylline, and theobromine are illustrated [21-23] in Fig. 2.

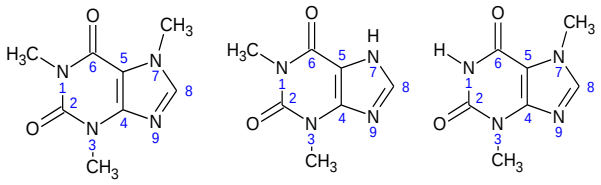

Fig. 2. Chemical structure of caffeine (1,3,7-trimethylxanthine), theophylline (1,3-dimethylxanthine, and theobromine (3,7-dimethylxanthine)

The separated compounds elute from the HPLC column at different retention times (R_t). The separation efficiency

*Corresponding Author: Email: mekat.hassan@coeng.uobaghdad.edu.iq

of two components by HPLC is measured by the resolution, R_s, which is defined as [24]:

$$R_s = \frac{R_{t1} - R_{t2}}{\frac{w_1 + w_2}{2}} \quad (1)$$

Where R_{t1} and R_{t2} are the retention times of component 1 and component 2 respectively, and w_1 and w_2 are the widths of the peaks of component 1 and component 2 respectively as it is shown in Fig. 3. Both retention time and peak width have the units of time as shown in Figure 3. It is obvious from the definition of resolution that the higher the difference between the retention times between two constituents the higher resolution. However, a reasonable width is required for each peak to result in a good separation. Peaks range from very narrow to very wide, but something between is usually wanted for efficient separation. Very wide peaks require longer time to elute from the column due to longer time spent for the solute(s) interacting with the stationary phase. On the other side, very narrow peaks for solute means that these solutes spent short time interacting with the stationary phase. The interacting of solutes with the stationary phase depend on their affinity to type of stationary phase [10, 25].

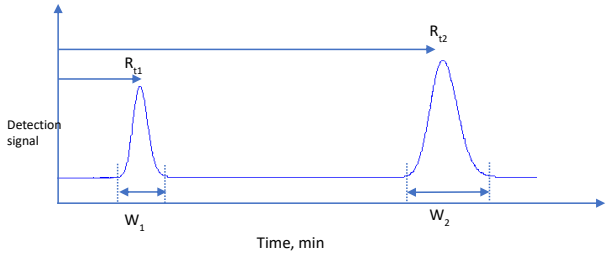

Fig. 3. HPLC chromatogram for two components (two peaks)

A good separation requires enough time to distinct the peak from each other. This means there should be clear peaks separated from each other as shown in Fig. 3. Many researchers studied the analysis ad separation of different compounds using HPLC. Finglas and Faulk described an HPLC method for the analysis of thiamine riboflavin in raw potato and cooked potato using a C_{18} column (stationary phase) and water-methanol solvent (mobile phase) in which they recovered more than 90% of the solutes [26, 27]. Nemutlu et. al., described an HPLC method for the determination of seven quinolones in plasma and amniotic liquid [28]. In their study, they applied an experimental design to optimize the method for the highest resolution and minimum retention time. Sharyn et. al., identified fish species using HPLC protein profiles [29]. Al-Janoobi et. al., developed and validated an HPLC method to quantify theophylline in rabbit plasma using Waters® C_{18} column and (96% water- 4% acetonitrile) mobile phase. They observed a retention time of ~5.2 minute for theophylline [30]. Additionally, Charehsaz et. al., developed another method for the determination of theophylline in urine, saliva, and plasma samples using C18 column and mobile phase consisted of (60% methanol-40% water) [31]. Moreover, Rosario Brunetto et. al., developed another HPLC method for the determination of caffeine, theophylline, and theobromine in cocoa samples [32]. In their study, they used a C_{18} column as the stationary phase and 20% methanol as the mobile phase with a flow rate of 1.4 mL/min.

All previous research focused on analyzing or separating the target compound(s). Therefore, there still need for more understanding of how the retention time changes when the HPLC parameters change. Mobile phase flow rate and its constituents are the most important and economical operational parameters in HPLC analysis. In this study, different mobile phase flow rates and different volume ratios of mobile phase (methanol-water-acetic acid) were used to study how the retention time and resolution change using a Hypersil BDS C_{18} column (4.6 by 50 mm) column. In addition, the effect of the column temperature on the solutes retention times was also examined.

2- Experimental work

2.1. Chemicals and reagents

Caffeine, theophylline, and theobromine were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade-methanol (J.T. Baker, Phillipsburg, NJ, USA) and acetic acid were used in the mobile phase. The chromatographic separation study was performed using Shimadzu Prominence HPLC System that equipped with a Hypersil BDS C_{18} column (4.6 by 50 mm) which served as the stationary phase.

2.2. Preparing the mobile phase

Different mobile phase concentrations were prepared by mixing methanol with distilled water, then adding acetic acid according to the compositions required in the mobile phase. For example, to prepare a bottle of 1000 mL mobile phase (15% methanol, 85% water, and 0.5% acetic acid), 150 mL methanol was added to 850 mL distilled water, then 5 mL acetic acid was added. Finally, the solution was mixed to ensure a homogeneous solution. Then, the mobile phase (in the bottle) was sonicated by Branson 2510 ultrasonic cleaner for one hour to remove any bubbles formed previously during mixing the components. After that, the mobile phase is ready to be used in the HPLC analysis.

2.3. HPLC analysis

The HPLC analysis was performed by injecting 1 µL sample that contained 1 mM caffeine, 1 mM theophylline, and 0.5 mM theobromine. The injection was previously programmed by the HPLC software (EZstart) and performed automatically. The run continued until the separation was completed and a clear chromatogram, showing the peaks for theobromine, theophylline, and caffeine, was produced and recorded.

3- Results and discussion

3.1. Effect of solvent flow rate on the retention time

The retention times for theobromine (TB), theophylline (TP), and caffeine (CAF) were observed at solvent flow rates of 0.1, 0.25, 0.5, 0.75, and 1.0 mL/min. The solvent composition was 15% methanol, 85% distilled water, and 0.5% acetic acid. Table 1 summarized the retention times and peak widths of the HPLC chromatograms for TB, TP, and CAF respectively, at different flow rates. At solvent flow rate of 0.1 mL/min, TB, TP, and CAF eluted at retention times of 15.6, 25.02, and 43.92 minute respectively. When the solvent flow rate was 0.25 mL/min, the retention times were reduced to 6.1, 9.78, and 17.21 minute for TB, TP, and CAF respectively. The retention time for each component clearly decreased after raising the mobile phase flow rate. The reduction in retention time for each component continued to appear when the solvent flow rates were increased to 0.5, 0.75, and 1.0 mL/min as it is shown in Table 1.

Fig. 4 and Fig. 5 show the curves for the reduction in retention time and peak width respectively, for TB, TP, and CAF respectively, as the mobile phase flow rate was increasing. It is obvious that the difference in retention time was higher between any two of the three components, but it became smaller as the flow rate increased. This suggests that separation is more efficient at low flow rates because of the higher difference in retention time. It might be thought that a lower solvent flow rate is economical. However, it takes a quite longer times for the solutes to elute from the column. Therefore, large quantity of solvent might be consumed which is unfavorable to ensure an economical HPLC analysis. Based on these results, a solvent flow rate between 0.5-0.75 mL is economical to achieve an efficient separation of TB, TP, and CAF.

Fig. 5 shows the curves for the peak widths for TB, TP, and CAF respectively, as a function of mobile phase flow rate. Similar behavior was observed (See Table 1), the higher the flow rate the smaller the peak width. However, the peak width has a lower importance than the retention time because if there is a reasonable difference in retention time between any two components, an efficient separation occurs. A smaller peak width means that the solute interaction with the stationary phase takes a short period of time while wider peak width indicates a longer period of time for the solute to interact with the stationary phase. Fig. 5 shows that at all mobile phase flow rates, caffeine peak width was always wider than those of theobromine and theophylline. This means that caffeine had a longer time interacting with the stationary phase.

As it is shown in Table 1, for theobromine, when the solvent flow rate increased from 0.1 to 0.25 mL/min, the percentage reduction in retention time was 61%. This percentage reduction was 50% when the solvent flow rate increased from 0.25 to 0.5 mL/min, 34% was observed when the solvent flow rate increased from 0.5 to 0.75 mL/min, and 23% when the solvent flow rate increased from 0.75 to 1.0 mL/min. Same percentage reductions in retention time were produced for theophylline and caffeine as the mobile phase flow rate increased. By examining Table 1, it is clear that, for all three compounds, as the solvent flow rate increased the retention time became shorter. The reason behind that reduction in retention time is that a higher flow rate caused a lower interaction time between the solute and the stationary phase, which resulted in a shorter retention time. In addition to that, the similar reductions in retention time with the increasing flow rates indicates that the three components have behaved similarly in their interaction with the stationary phase inside the column. This is because that caffeine, theophylline, and theobromine structures are not very different (Fig. 2).

Table 1. Retention time (R_t) and peak width (W) of the chromatograms for theobromine (TB), theophylline (TP), and caffeine (CAF) at different solvent flow rates

Solvent flow rate (mL/min)	TB, min		TP, min		CAF, min	
	R_t	W	R_t	W	R_t	W
0.10	43.92	1.5	25.02	2.5	15.60	4.5
0.25	17.21	1	9.78	1	6.10	2
0.50	8.76	0.5	4.95	0.5	3.06	1
0.75	5.67	0.3	3.22	0.4	2.01	0.7
1.00	4.42	0.2	2.50	0.25	1.54	0.5

3.2. Effect of solvent flow rate on the resolution

From the results shown in Table 1, it is possible to apply the resolution definition, mentioned earlier, at each specific flow rate to find the resolution between any two of the three components by substituting the corresponding retention time and peak width. Fig. 6 shows the change in resolution with the change of the mobile phase flow rate for each couple of the three compounds. It is clear that the highest resolutions resulted were between caffeine and theobromine and the lowest were between theophylline and theobromine. The high resolution (between 7 and 10) for caffeine-theobromine were due to the large difference in retention time between these compounds while the lower resolutions (between 3.5 and 4.5) for theophylline-theobromine were due to the small difference in retention time between them. The large difference in retention times between caffein and theobromine is because caffeine has one more methyl group than theobromine (Fig. 2) which increases the interaction time with the C_{18} stationary phase. The case is opposite with theobromine, has one less methyl group, less interaction time with the column.

Fig. 6 shows that that the resolution decreased when the solvent flow rate increased from 0.1 to 0.25 mL/min, then it increased when the solvent flow rate increased from 0.25 to 0.5 mL/min. Same decrease and increased was repeated when the solvent flow rate was increased from

0.5 to 0.75 mL/min and from 0.75 to 1.0 mL/min respectively. This alternating decrease and increase in the resolution could be explained according the definition of the resolution which depends on the two components' retention time and peak width. According to the resolution definition, the higher the nominator and/or the lower the denominator results in a higher resolution which means a better separation efficiency. For theobromine - caffeine separation, when the solvent flow rate was 0.1 mL/min, the difference in retention time between caffeine and theobromine (R_{t2}-R_{t1}) was 28.32 minute and the average of the width of the two peaks ((w_1+w_2)/2) was 3 minutes. As a result, the resolution was 9.44. When the solvent flow rate was increased to 0.25 mL/min, the retention times for both theobromine and caffeine became shorter (17.21 and 6.1 min), and the width of each peak for both of them became narrower (2 and 1 min). This is due to the reduction in the interaction time for each solute with the stationary phase which is resulted from the increase in the mobile phase flow rate. Therefore, the difference in retention time was 11.11 min and the average of the width of the two peaks was 1.5. Subsequently, the resolution was 7.41. The (7.41) resolution at a solvent flow rate of 0.25 mL/min has a lower value than the (9.44) resolution at a solvent flow rate of 0.1 mL/min because the difference in retention time between caffeine and theobromine was reduced from 28.32 to 11.11 minutes (61% reduction). While the peak widths were 4.5 and 1.5 minutes for caffeine and theobromine respectively at 0.1 mL/min flow rate which were reduced to 2 and 1 minutes when the solvent flow rate was increased to 0.25 mL/min. This means that the average width was reduced from 3 to 1.5 minutes (50% reduction). Since the 61% reduction in the retention time difference was higher than the 50% reduction, then the resolution was decreased. Similarly, the reason behind the increasing resolution when the solvent flow rate was increased from 0.25 to 0.5 mL/min was that the effect of the reduction in the retention time difference was lower than the effect of the reduction in the average width.

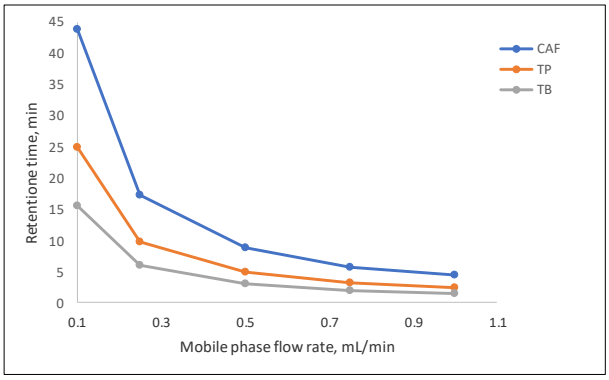

Fig. 4. Retention time for theobromine (TB), theophylline (TP), and caffeine (CAF) as a function to the solvent flow rate

Based on the previous results, a solvent flow rate between 0.25-0.75 mL/min was found to be efficient and economical for good separation. At this range of solvent flow rate, the highest resolutions were recorded at a solvent flow rate of 0.5 mL/min in which the resolutions were 3.8 for TP-TB, 7.6 for CAF-TB, and 5.1 for CAF-TP. Consequently, a solvent flow rate of 0.5 mL/min has been determined to be the optimum solvent flow rate to achieve an efficient economical separation.

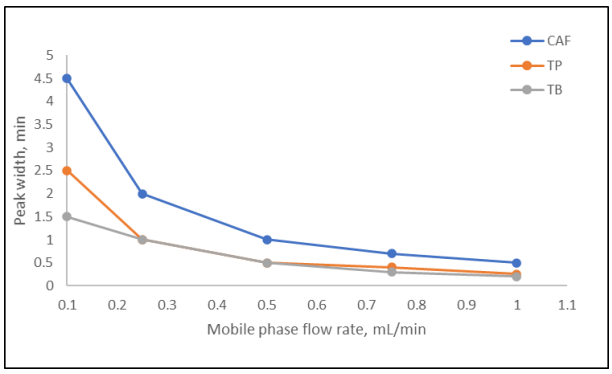

Fig. 5. Peak width for theobromine (TB), theophylline (TP), and caffeine (CAF) as a function to the solvent flow rate

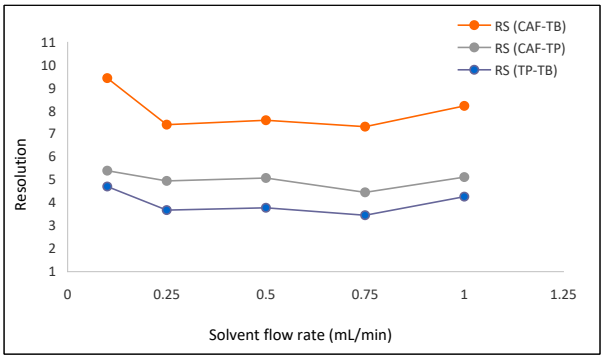

Fig. 6. Resolution as a function of mobile phase flow rate

3.3. Effect of methanol concentration in mobile Phase on the retention time

The mobile phase (solvent) composition has a significant effect on the liquid chromatographic separation. To examine the effect of the solvent composition on the retention time of the three compounds (TB, TP, and CAF), the optimum solvent flow rate (from previous results) of 0.5 mL/min was used. The three compounds entered the HPLC column, eluted at different times, and an HPLC chromatogram was recorded at each run. Different compositions of methanol in the mobile phase were used in each run. These compositions were 5%, 10%, 15%, 20%, and 30% methanol. In each one of those compositions, 0.5% acetic acid was added to control the pH at 3 which was recommended by the HPLC column manufacturer.

Fig. 7 and Fig. 8 show the retention times and peak width for TB, TP, and CAF respectively at different methanol concentrations in the mobile phase, while **Table 2** summarized all results. At a solvent consisted of 5%

methanol and 95% distilled water, the retention times of TB, TP, and CAF where 6.96, 12.38, and 28.88 minute respectively. These retention times dropped to 6.64, 11.90, 27.25 minute for TB, TP, and CAF respectively, when methanol was raised to 10% (90% distilled water) as shown in Fig. 7 and Table 2. This increase in the amount of methanol caused a percentage reduction in retention time of 5, 4, and 6% for TB, TP, and CAF respectively. Although the amount of methanol was doubled (from 5% to 10%), the reduction in retention time for each of the three compounds was quite low. Moreover, raising methanol in the mobile phase to 15% resulted in retention times of 3.06, 4.95, and 8.76 minutes for TB, TP, and CAF respectively (Fig. 7). This means that increasing the percentage of methanol in the solvent from 10 to 15% resulted in a significant reduction in the retention time by the amounts of 54%, 58%, and 68% for TB, TP, and CAF respectively. The reason behind this dramatic decrease in the reduction time for the three solutes is that the polarity of the solvent was reduced significantly when the amount of methanol increased from 10 to 15%. When the polarity of the solvent was reduced, the solvent became more attractive to theobromine, theophylline, and caffeine, and causing them to lower their interaction time with the stationary phase, and eventually eluted from the column at shorter retention times. Furthermore, increasing the composition of methanol from 15% to 20% of the mobile phase resulted in a shorter retention of the solutes molecules inside the column. The retention times for TB, TP, and CAF were 2.12, 3.07, and 4.51 minutes respectively. This is a reduction of 31%, 38%, and 49% in the retention time of TB, TP, and CAF respectively. Similarly, when a methanol composition of 30% in the mobile phase was used, the retention time of the three compounds dropped to become 1.71, 2.18, and 2.77 minute for TB, TP, and CAF respectively. These results were corresponding to a reduction in retention time of 19%, 29%, and 39% for TB, TP, and CAF, which are resulted from increasing methanol composition in the mobile phase from 20% to 30%.

Similarly, peak widths of all three components were decreased when methanol concentration in the mobile phase increased. Fig. 8 shows the effect of methanol composition in the mobile phase on the peak width for the TB, TP, and CAF respectively. When examining Fig. 7 and Fig. 8, we can observe a proportional relationship between the reduction in retention time and the reduction in peak width. This is an indication that both retention time and peak width are dependent on the solute molecule's interactions with the stationary phase, and the shorter they interact the shorter retention times and peak width produced.

Indeed, these results proved that adding more methanol to the mobile phase resulted in shorter retention times for the three compounds to elute from the HPLC column. However, the greatest retention time reduction was observed when methanol concentration was raised to 15% in which the retention times were reduced to 3.06, 4.95, and 8.76 minutes for TB, TP, and CAF respectively. Therefore, 15% methanol composition in the mobile phase was considered to be the best composition for the mobile phase. At that solvent composition, peak widths were 0.5, 0.5, and 1 minute for TB, TP, and CAF respectively (Table 2).

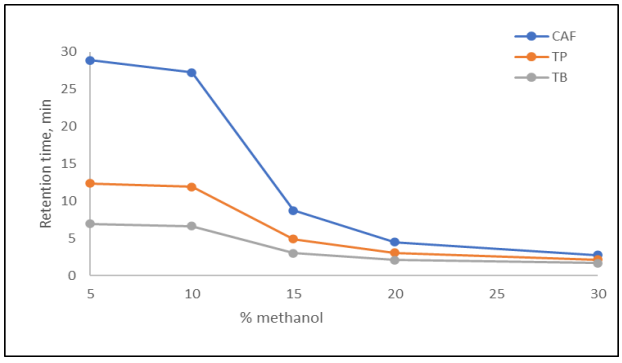

Fig. 7. Retention time of TB, TP, and CAF at different methanol concentration in the mobile phase

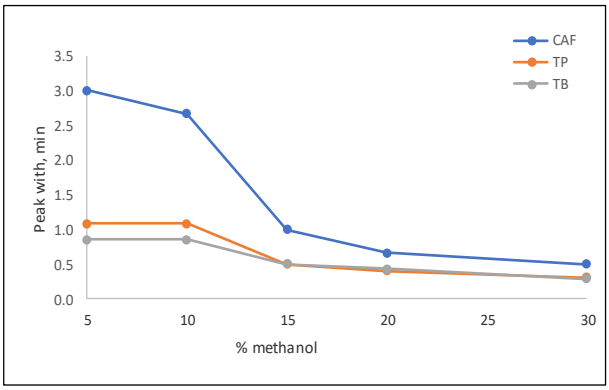

Fig. 8. Peak width of TB, TP, and CAF at different methanol concentration in the mobile phase

Table 2. Retention time (R_t) and peak width (W) of the chromatograms for theobromine (TB), theophylline (TP), and caffeine (CAF) at different methanol composition in the mobile phase

% Methanol	TB, min		TP, min		CAF, min	
	R_t	W	R_t	W	R_t	W
5	6.96	0.86	12.38	1.08	28.88	3.00
10	6.64	0.86	11.90	1.08	27.25	2.67
15	3.06	0.50	4.95	0.50	8.76	1.00
20	2.12	0.43	3.07	0.41	4.51	0.67
30	1.71	0.29	2.18	0.31	2.77	0.50

3.4. Effect of methanol concentration in the mobile phase on the resolution

As it was previously mentioned, for two peaks which represent two solutes, the resolution depends on the retention time difference and the average width of the two peaks. Fig. 9 depicted the resolution of each couple of the three compounds as a function of methanol concentration in the mobile phase. When the concentration of methanol was 5%, the resolution was 5.59 for (TP-TB), 11.36 for (CAF-TB), and 8.09 for (CAF-TP). These values did not strongly change when 10% methanol was used in which the resolutions became 5.43 for (TP-TB), 11.7 for (CAF-TB), and 8.19 for (CAF-TP). There was a significant drop in resolution when a methanol concentration was increased to 15%, in which the resolutions dropped to 3.78 for (TP-TB), 7.6 for (CAF-TB), and 5.08 for (CAF-TP). This reduction in resolutions continued as more methanol was used in the mobile phase. At 20% methanol the resolutions were 2.3, 4.37, 2.68 while they were 1.6, 2.7, and 1.44 for TP-TB, CAF-TB, and (CAF-TP respectively (Fig. 9). Therefore, these results showed that the more methanol added to the mobile phase resulted in reducing the resolution of any couple of the three compounds. This is because the addition of methanol to the mobile phase resulted in reducing the retention time and peak width for the three solutes. Moreover, the gap between each two peaks was also decreased when methanol concentration in the mobile phase was increased. Moreover, it was observed from Figure 9 that the resolutions for (CAF-TP) and (TP-TB) became closed to each other as methanol concentration increased beyond 20%. These results suggest that more methanol concentration is not favorite for the separation, and an efficient separation for all compounds can be achieved by a methanol concentration range between 5% and 20% in the mobile phase. Since 15% methanol concentration within that range, this confirms earlier results of using 15% methanol concentration as an optimum concentration for methanol to achieve an efficient economical HPLC separation.

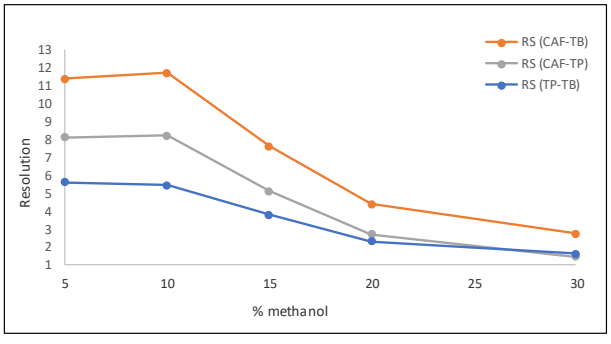

Fig. 9. Resolution (Rs) as a function of % methanol in the mobile phase

3.5. Effect of column temperature on the retention times

Based on previous results, the effect of column temperature on the retention times of TB, TP, and CAF, was examined by a solvent containing 15% methanol at a flow rate of 0.5 mL/min. The column temperature used were 25, 30, 35, and 40 ºC. Fig. 10 and Fig. 11 show the effect of the column temperature on the retention time and peak width of TB, TP, and CAF.

It is clear from Fig. 10 that as the temperature of the column increased, the retention time of each of the three compounds was decreased. For example, the retention time of caffeine was reduced from 3.06 to 2.91, 4.95 to 4.7, and 8.76 to 8.32 minutes for TB, TP, and CAF respectively when the temperature was increased from 25 to 30 ºC. This reduction in retention time continued as the temperature raised to 35 and 40 ºC as it is shown in Fig. 10. A similar behavior was observed for the peak widths for all compounds (Fig. 11).

The reduction in retention time resulted from the temperature increase was not as high as in the case of previous parameters (mobile phase flow rate and composition). Moreover, the reduction in retention time (determined from Fig. 10) for all three compounds was the same which was 5% when the temperature was increased from 25 to 30 ºC, 7.5% when the temperature was increased from 30 to 35 ºC, and 10% when the temperature was increased from 30 to 40 ºC. Therefore, it can be concluded that the effect of temperature is not as significant as the mobile phase flow rate and mobile composition. This confirms that solvent flow rate and composition are the most important parameters in the HPLC separation.

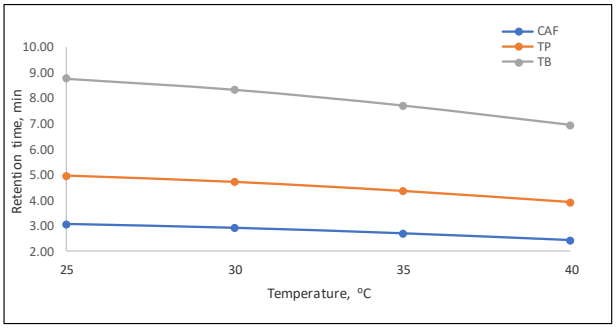

Fig. 10. Retention time of TB, TP, and CAF at different column temperatures

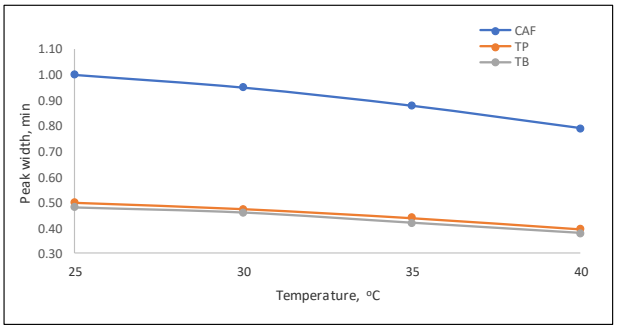

Fig. 11. Peak width of TB, TP, and CAF at different column temperatures

3.6. Effect of acetic acid on the mobile phase pH

It was mentioned earlier that a pH of 3 was recommended by the HPLC column manufacturer. That's

why 0.5% acetic acid was used in the mobile phase in all previous results. However, it is worth looking at how the pH varies if the amount of acetic acid in the mobile phase changes. It is well known that the more acid added produces more hydrogen ions and consequently increasing the acidity of the solution which means lowering the pH. When the mobile phase contained no acetic acid (15% methanol and 85% distilled water), the pH of the solution was 7.76. Adding acetic acid in the amount of 0.5% concentration in the mobile phase reduced the pH to 3.0 as it is shown in Fig. 12. More addition of acetic acid to the mobile phase solution produced a pH lower than 3.0. These results proved how the addition of acetic acid to the mobile phase plays a crucial role in controlling the pH of the solvent. In addition to that, the effect of the concentration of methanol on the pH was also examined as it is shown in Fig. 13. When fixing the concentration of acetic acid at 0.5%, it was found that methanol concentration has no effect on the mobile phase acidity as the pH was around 3.0.

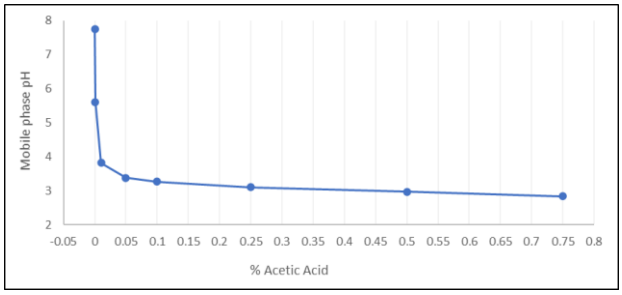

Fig. 12. Effect of the concentration of acetic acid on the mobile phase pH

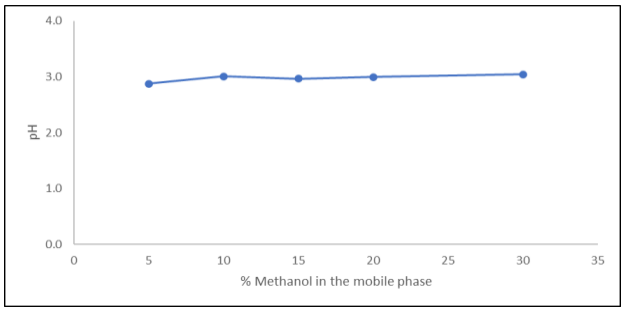

Fig. 13. Effect of the concentration of methanol on the mobile phase pH

4- Conclusion

The liquid chromatographic separation of theobromine (TB), theophylline (TP), and caffeine (CAF) was experimentally studied by a C_{18} HPLC column as the stationary phase and methanol-water system as the mobile phase. The effect of mobile phase flow rate and concentration of methanol in the mobile phase was closely examined. It was found that raising the mobile phase flow rate caused a reduction in the retention time of TB, TP, and CAF. However, increasing the concentration of methanol in the mobile phase caused a reduction in the retention time. It was found that the optimum solvent flow rate and mobile phase composition were 0.5 mL/min and 15% methanol while the column temperature had a lower effect on the retention time.

Conflict of interest

The authors declare that there is no conflict of interest.

References

[1] S. Meirinho, M. Rodrigues, A. Fortuna, A. Falcão, G. Alves, "Liquid chromatographic methods for determination of the new antiepileptic drugs stiripentol, retigabine, rufinamide and perampanel: A comprehensive and critical review", *Journal of Pharmaceutical Analysis*, 11, 2021, 405–421. https://doi.org/10.1016/j.jpha.2020.11.005

[2] A.J.H. Smit, "Chromatography. A Review of Principles and Applications.", *Journal of the American Chemical Society*, 79, 1957, 5586. https://doi.org/https://doi.org/10.1021/ja01577a077

[3] S. Debnath, M. Das, S. Mondal, B.K. Sarkar, G. Babu, "Advances in chromatography: contemporary techniques and applications", *Essential Chem*, 2, 2025 1–27. https://doi.org/https://doi.org/10.1080/28378083.2025.2466624

[4] W.J. Lough, I.W. Wainer, "High Performance Liquid Chromatography: Fundamental Principles and Practice", cRc press, 1995.

[5] B.L. Reuhs, "High-Performance Liquid Chromatography", in: *Food Analysis*, 2017: pp. 213–226. https://doi.org/10.1007/978-3-319-45776-5

[6] K.T. C, "Quality-control analytical methods: High-performance liquid chromatography", *International journal of pharmaceutical compounding*, 8, 2004, 223–227.

[7] B.P. Ismail, S.S. Nielsen, "Basic principles of chromatography", in: *Food Analysis*, 2017. https://doi.org/https://doi.org/10.1007/978-3-319-45776-5_36

[8] S.A.S. Al-azawi, "synthesis and study of some mixed ligand complexes of caffeine", *Baghdad Science Journal*, 3, 2006, 314–320. https://doi.org/10.21123/bsj.2006.705

[9] D. Schieppati, N.A. Patience, S. Campisi, G.S. Patience, "Experimental methods in chemical engineering: High performance liquid chromatography—HPLC", *The Canadian Journal of Chemical Engineering*, 99, 2021, 1663–1682. https://doi.org/10.1002/cjce.24050

[10] T. M, A. S, G. SJ, I. SS, H. A, "Ultra Performance Liquid Chromatography (UPLC) - A Review", *Austin Journal of Analytical and Pharmaceutical Chemistry*, 2, 2015, 1–5.

[11] S.A. Alallaq, I.A. Murtadeh, A.M.S. Hatem, "Determination of Alkaloids Constituents in Some Ferns by Using High Performance Liquid Chromatography Technique – Iraq", *Iraqi Journal of Science*, 64, 2023, 4340–4346. https://doi.org/10.24996/ijs.2023.64.9.3

[12] A.S. Ali, N.A. j. Imran, J.M. Mahmood, S.A. Jassim, "Optimum conditions for ascorbic acid determination in three Iraqi citrus using HPLC technique", *Baghdad Science Journal*, 11, 2014, 1233–1242. https://doi.org/10.21123/bsj.2014.11.3.1233-1242

[13] N.I. Al-Ani, H.N. Borazan, "Interactions of caffeine and theophylline with p-cresol: UV studies", *Journal of Pharmaceutical Sciences*, 67, 1978, 1381–1384. https://doi.org/10.1002/jps.2600671013

[14] K.F. Alsamarrai, S.T. Ameen, "Simultaneous Ratio Derivative Spectrophotometric Determination of Paracetamol, Caffeine and Ibuprofen in Their Ternary Form", *Baghdad Science Journal*, 19, 2022, 1276–1285. https://doi.org/10.21123/bsj.2022.6422

[15] H.M. Jewely, T.Z. Abdul-Jalil, "Extraction, Isolation and Identification of Caffeic Acid and p-Coumaric acid from N-butanol Fraction of Iraqi Osteospermum ecklonis (F. Asteraceae)", *International Journal of Drug Delivery Technology*, 12, 2022, 648–653. https://doi.org/10.25258/ijddt.12.2.31

[16] S.R. Hassan, A.M. Al-Yaqoobi, "Assessment of Ultrasound-Assisted Extraction of Caffeine and its Bioactivity", *Journal of Ecological Engineering*, 24, 2023, 126–133. https://doi.org/10.12911/22998993/157540

[17] D. Wang, S.P. Hong, K.H. Row, "Solid extraction of caffeine and theophylline from green tea by molecular imprinted polymers", *Korean Journal of Chemical Engineering*, 21, 2004, 853–857. https://doi.org/10.1007/BF02705530

[18] G.R. Walle, T. Suzuki, "Biosynthesis and Biodegradation of Caffeine, Theobromine, and tHeophylline in Coffea arabica L. Fruits", *Journal of Agricultural and Food Chemistry*, 32, 1984, 845–848. https://doi.org/10.1021/jf00124a038

[19] H. Ashihara, A. Crozier, "Biosynthesis and Metabolism of Caffeine and Related Purine Alkaloids in Plants", *Advances in Botanical Research*, 1999. https://doi.org/10.1016/S0065-2296(08)60228-1

[20] H. Ashihara, T. Suzuki, "DISTRIBUTION AND BIOSYNTHESIS OF CAFFEINE IN PLANTS", *Frontiers in Bioscience-Landmark*, 9, 2004, 1864–1876. https://doi.org/10.2741/1367

[21] K.H.R. Algharrawi, R.M. Summers, M. Subramanian, "Production of theobromine by N-demethylation of caffeine using metabolically engineered E. coli", *Biocatalysis and Agricultural Biotechnology*, 11, 2017, 153–160. https://doi.org/10.1016/j.bcab.2017.06.014

[22] K.H.R. Algharrawi, M. Subramanian, "Production of 7-methylxanthine from Theobromine by Metabolically Engineered E. coli", *Iraqi Journal of Chemical and Petroleum Engineering*, 21, 2020, 19–27. https://doi.org/10.31699/ijcpe.2020.3.3

[23] K.H.R. Algharrawi, R.M. Summers, S. Gopishetty, M. Subramanian, "Direct conversion of theophylline to 3-methylxanthine by metabolically engineered E. coli", *Microbial Cell Factories*, 14, 2015, 1–12. https://doi.org/10.1186/s12934-015-0395-1

[24] G. Vijayamma, S. Nirmala, "Development of eco-friendly UPLC methods for metformin and empagliflozin by combining green chemistry with analytical quality by design", Acta Chromatographica, 2024, 1–11. https://doi.org/10.1556/1326.2024.01274

[25] S.S. Nielsen, Food Analysis - 4th Ed, 2010. https://doi.org/10.1007/978-3-319-45776-5

[26] P.M. Finglas, R.M. Faulks, "The HPLC analysis of thiamin and riboflavin in potatoes", *Food Chemistry*, 15, 1984, 37–44. https://doi.org/10.1016/0308-8146(84)90037-2

[27] S. Amanipour, H. Rostampour, A. Nezamzadeh Ejhieh, "A Validated Reversed-Phase HPLC Method Toward Quantification of Some Water-Soluble Vitamins and Preservatives in Pharmaceutical Samples", *Iranian Journal of Chemistry and Chemical Engineering*, 42, 2023, 3398–3408. https://doi.org/10.30492/ijcce.2023.561171.5561

[28] E. Nemutlu, S. Kir, Ö. Özyüncü, M.S. Beksaç, "Simultaneous separation and determination of seven quinolones using HPLC: Analysis of levofloxacin and moxifloxacin in plasma and amniotic fluid", *Chromatographia*, 66, 2007, 15–24. https://doi.org/10.1365/s10337-007-0292-9

[29] S.G. Armstrong, D.N. Leach, S.G. Wyllie, "The use of HPLC protein profiles in fish species identification", *Food Chemistry*, 44, 1992, 147–155. https://doi.org/10.1016/0308-8146(92)90328-Y

[30] F.I. Al-Jenoobi, A. Ahad, G.M. Mahrous, M. Raish, M.A. Alam, A.M. Al-Mohizea, "A Simple HPLC-UV Method for the Quantification of Theophylline in Rabbit Plasma and its Pharmacokinetic Application", *Journal of Chromatographic Science*, 53, 2015, 1765–1770. https://doi.org/10.1093/chromsci/bmv094

[31] M. Charehsaz, A. Gürbay, A. Aydin, G. Şahin, "Simple, fast and reliable liquid chromatographic and spectrophotometric methods for the determination of theophylline in urine, saliva and plasma samples", *Iranian journal of pharmaceutical research*, 13, 2014, 431–439.

[32] M. del R. Brunetto, L. Gutiérrez, Y. Delgado, M. Gallignani, A. Zambrano, Á. Gómez, G. Ramos, C. Romero, "Determination of theobromine, theophylline and caffeine in cocoa samples by a high-performance liquid chromatographic method with on-line sample cleanup in a switching-column system", *Food Chemistry*, 100, 2007, 459–467. https://doi.org/10.1016/j.foodchem.2005.10.007

دراسة الفصل الكروماتوغرافي للكافيين والثيوفيلين والثيوبرومين

حسنين عباس حسن [١]، ميقات حسن صالح [١،*]، مها هادي الحسني [١]

١ قسم الهندسة الكيمياوية، كلية الهندسة، جامعة بغداد، بغداد، العراق

الخلاصة

تمت دراسة أزمنة استبقاء الثيوبرومين والثيوفيلين والكافيين في عمود HPLC من نوع C_{18} (الطور الثابت) كدالة لمعدل تدفق الطور المتحرك، وتركيبه، ودرجة حرارة العمود. عند زيادة معدل تدفق الطور المتحرك من ٠,١ إلى ١ مل/دقيقة وزيادة نسبة تركيز الميثانول من ٥% إلى ٣٠٪، لوحظ انخفاض في زمن الاستبقاء وعرض القمة لهذه المركبات الثلاثة. في حين أن زيادة معدل تدفق الطور المتحرك كان لها تأثير طفيف على الدقة، إلا أن تقليل تركيز الميثانول في الطور المتحرك قلل من الدقة بشكل كبير. بالإضافة إلى ذلك، تبين أن معدل تدفق الطور المتحرك وتركيبه لهما تأثير أكثر أهمية من درجة حرارة العمود. وفقًا للنتائج، تم فصل الثيوبرومين والثيوفيلين والكافيين بشكل أكثر فعالية باستخدام الكروماتوغرافيا السائلة عند معدل تدفق ٠.٥ مل/دقيقة وتركيز ميثانول في الطور المتحرك بنسبة ١٥٪.

الكلمات الدالة: HPLC، الطور المتحرك، الطور الثابت، زمن الاستبقاء، الدقة.