

Development of a Method for Separation of Adenine Nucleotides

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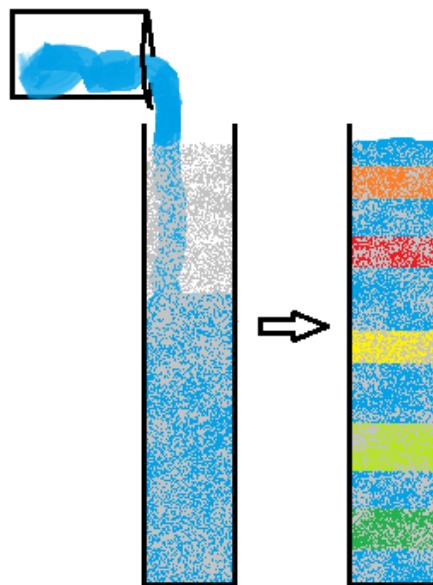
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Kromatografisk magi kan separera snarlika molekyler

På den ryske botanikern Mikhail Semyonovich Tsvets gravsten står det ”Han uppfann kromatografin, separerade molekyler men förenade folk”. Hans metod för att separera molekyler används än idag - till exempel i detta arbete.

Tsvets försökuppställning bestod av en glascylinder fylld med kolsyrad kalk, samt en lösning som innehöll olika färgpigment från växter. En stund efter att han hållt pigmentblandningen i den kalkfyllda cylindern visade det sig att de olika färgerna hade delat upp sig som på bilden. Pigmenten hade nämligen olika kemiska egenskaper och hann därför rinna olika långt genom cylindern innan de fastnade i kalken. Tsvet kallade sin teknik för *kromatografi* efter grekiskans *färgskrift*. Idag utförs kromatografi med avancerade apparater under högt tryck, och kalk används inte längre som packningsmaterial, men principen är fortfarande densamma.



Ibland kan ämnen med väldigt olika biologiska funktioner ha liknande kemiska egenskaper. Olika nukleotider, som till exempel adenosintrifosfat (ATP), är sådana molekyler. ATP minns du säkert som kroppens energivaluta, men det finns även mindre välkända nukleotider, exempelvis diadenosintetrafosfat (Ap4A). Ap4A liknar ATP i sin kemiska struktur, men tros till skillnad från ATP påverka utvecklingen av diabetes. För att undersöka denna molekyl måste man först separera den från andra nukleotider med hjälp av kromatografi, men eftersom kromatografi bygger på molekylers *olika* egenskaper är detta en utmaning. I mitt arbete har jag använt en speciell kromatografisk metod som kallas för *HILIC* - ungefär *hydrofil kromatografi* - för att utnyttja små skillnader mellan olika nukleotider för att separera dem. Efter mycket arbete och en gnutta kromatografisk magi i labbet lyckades jag - nästan.

Abstract

Adenosine tetraphosphate, or Ap4A, is a nucleotide with very interesting proposed biological effects. Previous studies have indicated that blood glucose levels in mammals might increase in the presence of Ap4A, and that Ap4A might antagonize pancreatic ATP channels. In order to study these effects further, a reliable method of separating Ap4A from other nucleotides in biological samples is required. This is made difficult by the high polarity and the high structural resemblance of different nucleotides. The aim of this study was to develop a method for separation of Ap4A from ATP and AMP on the Agilent Poroshell 120 HILIC-Z column, using conditions compatible with mass spectrometry.

A method previously employed to separate simple nucleotides, using 10 mM ammonium acetate buffer at pH 9, a column temperature of 25°C, a flow rate of 300 $\mu\text{L min}^{-1}$, and a re-equilibration time of 33.3 minutes was used as a starting point. [1] In order to investigate the retention mechanism of the separation, an array of parameters (column temperature, mobile phase flow rate, re-equilibration time, buffer concentration, and mobile phase pH) were varied one by one while all the other parameters were held constant.

Out of all evaluated parameters, increasing the buffer concentration to 20 mM was the only adjustment that had a positive effect on the separation. In this method two peaks were observed; one corresponding to ATP + AMP and the other corresponding to Ap4A, with a resolution of about 1.3. Furthermore, it was found that reproducible results could be achieved with a re-equilibration time as short as 8.3 minutes.

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1 Background

Nucleosides are highly important compounds within the human cell. They consist of a nitrogenous base and a sugar, and when they are coupled with one to three phosphate groups, they are referred to as *nucleotides*.

The most well known nucleotide to the non-biochemist is perhaps adenosine triphosphate - or ATP - with its celebrity status as *the* energy molecule of the cell. Other nucleoside triphosphates, such as uridine triphosphate (UTP), cytidine triphosphate (CTP), and guanosine triphosphate (GTP) are also well known for their involvement in DNA biosynthesis and metabolism. Nucleoside mono- and diphosphates are naturally also important in metabolic processes since they form through dephosphorylation of their respective triphosphates.

As it turns out, the biological functions of nucleotides are well investigated, but there is still much to learn about dinucleosides such as diadenosine tetraphosphate (Ap4A). In mammals, it has been shown that it has an effect on cardiovascular function, neuronal signalling, and fertility. [2] It has also been proposed that Ap4A might act as an antagonist on pancreatic ATP channels, affecting insulin secretion, however the mechanism of this requires further study. [2] It has also been found that the concentration of glucose in the blood increases in the presence of Ap4A. [3] Therefore, it is relevant to study the function of Ap4A in relation to metabolic disorders such as diabetes, and a method of separating it from other nucleoside phosphates is required. Because of the high polarity of nucleoside phosphates as well as their high structural resemblance, however, this has proved a challenging task.

Several methods for separating nucleotides are to be found in literature, but none for isolating Ap4A. Moreover, many of the previously developed methods come with significant drawbacks. In a study from 2010, nucleotides were separated using reversed phase liquid chromatography on a C18 column, using a mobile phase of 20 mM sodium dihydrogen phosphate buffer with an ion pairing reagent. [4] The use of phosphate buffer makes this method unsuitable for mass spectrometry detection. In another study from 2018, adenine nucleotides from cultured cells were successfully separated

on a SUPELCOSIL™ LC-18-DB column using a 25 mM pyrophosphate buffer as mobile phase, however this buffer is also incompatible with mass spectrometry. [5]

The aim of this study is to develop a method of separating Ap4A from two of the most common nucleotides in the human cell - AMP and ATP - using a zwitterionic HILIC column. It can then be assumed that adenosine diphosphate (ADP) would elute in between AMP and ATP. UV detection was used in the method development in this study, however the goal was to eventually make the method compatible with mass spectrometry.

1.1 Research questions

The main research question of this work is whether Ap4A can be separated from other adenine nucleotides in a robust, reproducible, and efficient manner, while using buffers compatible with mass spectrometry. In order to answer this question, several more specific questions were considered; how do chromatographic parameters such as column temperature, flow rate, re-equilibration time, buffer concentration, and pH influence the resolution of such a separation?

2 Theory

2.1 Hydrophilic interaction liquid chromatography (HILIC)

Nucleosides are too polar to be retained by an apolar column and can therefore not be separated by reversed phase liquid chromatography (RPLC). An alternative that has been previously used in order to separate these polar metabolites is hydrophilic interaction liquid chromatography (HILIC) [6]. In this technique, the stationary phase is polar; for instance bare silica columns, amide columns, or zwitterionic columns can be used. The strong solvent is always water, and the weak solvent is a polar organic solvent. [7]

Although the HILIC technique was discovered in the early 1990s, its retention mechanism has yet to be fully determined. In the currently accepted retention model it is suggested that the water content of the mobile phase causes an aqueous layer to be formed over the polar surface of the packing material of the column. Polar analytes will then partition between this very polar water-rich layer and the less polar mobile phase. However, partitioning is likely not the only retention mechanism present in HILIC; electrostatic interactions and hydrogen bonds are also believed to contribute to adsorption of the solutes to the stationary phase. [7] Especially when separating charged analytes on a zwitterionic column - as in this study - the contribution of ionic interactions to the retention is of interest.

In order for an aqueous layer to be formed over the stationary phase, the mobile phase needs to contain at least 5% water at all times, and thus the weak solvent needs to be miscible with water. Moreover, in order for the mobile phase not to interfere with the hydrogen bonding and other interactions between the analytes and the stationary phase, use of an aprotic solvent is recommended. For this reason, and because of its relatively low toxicity, acetonitrile is the most common choice for a weak solvent in HILIC separations. [8]

Although they might seem similar, HILIC is not to be confused with normal phase liquid chromatography. These two techniques are differentiated by the mobile phase used as well as the retention mechanism. In normal phase mode, the mobile phase

usually consists of an apolar solvent mixture only containing a small amount of polar modifier, whereas in HILIC the weak solvent naturally needs to be miscible with water. The retention mechanism is also different, being mainly adsorption in normal phase mode, and a mix of partitioning and adsorption in HILIC, as described above. [7]

2.1.1 Re-equilibration time

When running gradient separations in HPLC, one cannot simply start the gradient again immediately after a previous run has ended. In order to obtain robust and repeatable results it is important to flush out the column after returning the mobile phase to the starting composition of the gradient. The time it takes for the column to reach full equilibrium - meaning that the aqueous layer is completely in place, and that further flushing would not change the conditions at all - is known as the *equilibration time*. In reversed phase liquid chromatography, this is mainly done in order to flush out the system and to make sure that the mobile phase that enters the column actually has the desired starting composition of the gradient. Compensation also has to be made for the time it takes for the mobile phase to travel from the pump to the column inlet. [9] In HILIC separations, however, the situation is a little more complex. As recalled from section 2.1, HILIC retention is believed to be based on partitioning between a water layer adsorbed to the stationary phase. This means that this water layer somehow needs to stabilize in between runs in order to ensure identical starting conditions for each injection. [10] It has generally been believed that HILIC columns require extraordinarily long equilibration times between runs in order for the water layer to fully stabilize. How long time is needed depends on the stationary phase chemistry and the mobile phase, but values as high as 40 column volumes have been suggested for complete re-equilibration. [11] Given the column used in this study and a flow rate of $300\text{ }\mu\text{L min}^{-1}$, 40 column volumes corresponds to a re-equilibration time of approximately 46 minutes, which makes the chromatography very time consuming. In recent studies, however, it has been shown that complete re-equilibration in HILIC might not be necessary. For instance, McCalley showed in 2018 that partial re-equilibrations as short as 5 minutes might be enough in order to achieve reproducible results on a zwitterionic HILIC column. [12]

2.2 Retention of nucleotides

Nucleotides are negatively charged at physiological pH, as can be seen in figure 1. The pK_a of ATP, for instance, is 6.5 [13], and it is given by equation 5 that it is fully protonated at pH = pK_a + 2 = 8.5. The pH of the mobile phase then needs to be selected so that the desired degree of protonation is achieved. Of course, it must also be accounted for that the pH of the mobile phase might affect the charge of the stationary phase. The ionic strength of the mobile phase also needs to be monitored, as a high concentration of charged species might shield the electrostatic interactions between the analytes and the stationary phase.

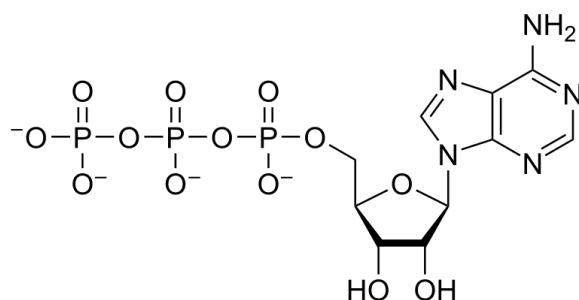


Figure 1: The structure of adenosine triphosphate at physiological pH. (Image licensed under Creative Commons CC0 1.0.)

As the pH reaches below 4.3, a nitrogen in the adenosine component of ATP starts getting protonated as illustrated by figure 3. Since Ap4A has two adenosine components (figure 2), this can possibly be used to affect the separation of these nucleotides.

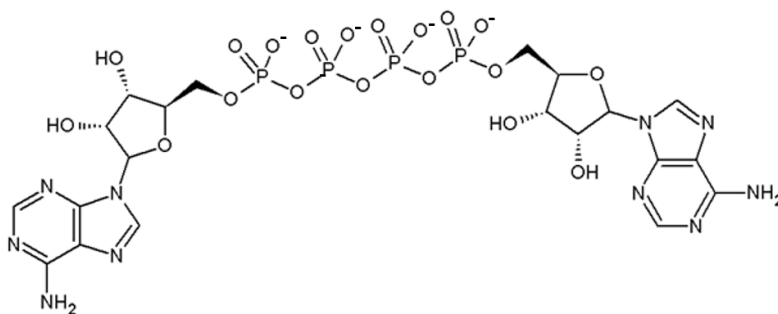


Figure 2: The structure of diadenosine tetraphosphate (Ap4A) at physiological pH. (Image licensed under Creative Commons CC0 1.0.)

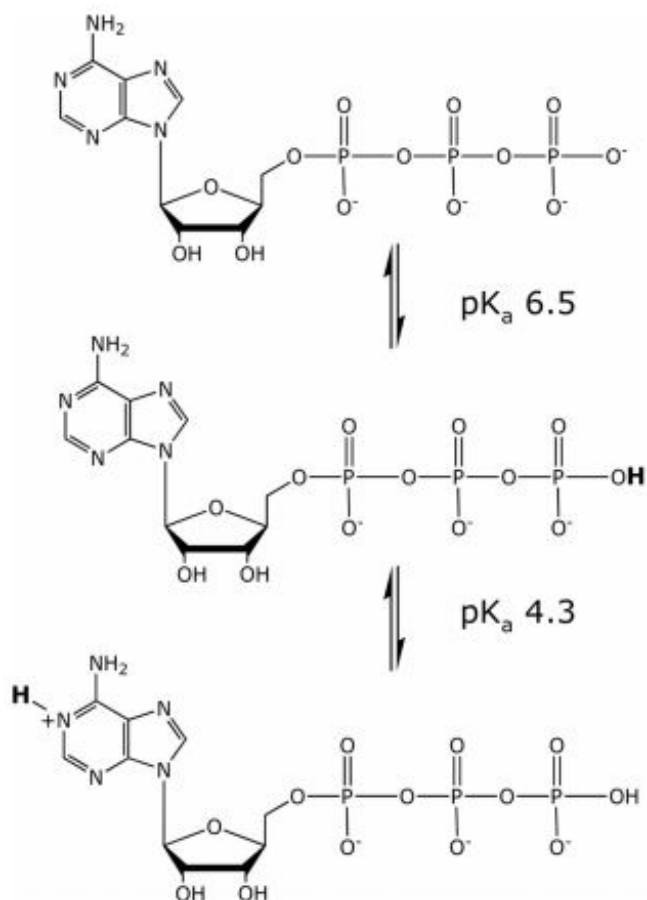


Figure 3: Protonation of ATP. Image borrowed from *The Intrinsic Reactivity of ATP and the Catalytic Proficiencies of Kinases Acting on Glucose, N-Acetylgalactosamine, and Homoserine* by Stockbridge and Wolfenden. [13]

Temperature is an interesting parameter in any HPLC separation. When the temperature of the column oven is increased, the viscosity of the mobile phase travelling through the column decreases, which in turn decreases the back pressure. This way the flow rate of the mobile phase can be increased without exceeding pressure limits. In HILIC, the temperature also has an effect on the adsorbed aqueous layer on the stationary phase. At higher temperatures, the hydrogen bonds between the water molecules in the aqueous layer are weaker, which might reduce the thickness of the layer. [14]

The column investigated here is the Agilent Poroshell 120 HILIC-Z column. The composition of the stationary phase of this column is kept a secret by the manufacturer, providing only the information that it consists of a "zwitterionic phase bonded onto superficially porous silica particles." [15] Therefore, the effect of different parameters on the separation had to be studied in detail.

2.3 Summary of equations

The resolution of the chromatographic peaks was calculated using equation 1,

$$R_S = \frac{0.589\Delta t_r}{w_{1/2av}} \quad (1)$$

where Δt_r is the difference in retention time between the two peaks, and $w_{0.5av}$ is the average half height width of the peaks.

The resolution is given by

$$R_S = \frac{1}{4}\sqrt{N}\frac{k}{1+k}\frac{\alpha-1}{\alpha} \quad (2)$$

where N is the plate number, k is the retention factor, and α is the separation factor. k in equation 2 is given by

$$k = \frac{t_R - t_0}{t_0} \quad (3)$$

where t_R is the retention time of the peak, and t_0 is the dead time.

α in equation 2 is defined as the ratio of the retention factors of two peaks according to equation 4.

$$\alpha = \frac{k_2}{k_1} \quad (4)$$

The ratio of the protonated form of a species in solution to its deprotonated form is related to the pH of the solution by the Henderson-Hasselbalch equation,

$$\text{pH} = \text{p}K_{\text{a}} + \log \frac{[\text{A}^{-}]}{[\text{HA}]} \quad (5)$$

where A^{-} is the deprotonated form of species A, and HA is the protonated form of species A.

3 Experimental

3.1 Chemicals

- Acetonitrile (for LC-MS, HiPerSolv CHROMANORM®[®], $\geq 99.9\%$)
- Ammonium acetate (for LC-MS, LiChropur[™])
- Ammonium formate (for LC-MS, LiChropur[™], $\geq 99.0\%$)
- Ammonia solution 25% (For LC-MS, LiChropur[™])
- Formic acid (LC-MS grade, Fisher Chemical, $\geq 99\%$)
- 1 mM standard solutions of ATP, AMP, and Ap4A

3.2 Instrumentation and chromatographic conditions

Separation was carried out on a Dionex Ultimate 3000 HPLC system with a DAD detector and Chromeleon v.8 software. The column used was an Agilent Poroshell 120 HILIC-Z, 2.1x100 mm, particle size 2.7 μm . Analytes were detected at 267 nm.

3.3 Preparation of mobile phases

For the mobile phases at pH 9.0, a 100 mM ammonium acetate solution was prepared. 1.927 g of ammonium acetate was dissolved in 250 ml of Milli-Q water. The pH was set to 9.0 by dropwise addition of 25% ammonia solution. Mobile phases of concentrations 5, 10, and 20 mM were then prepared by diluting the stock solution 20, 10, and 5 times with acetonitrile (A) and Milli-Q water (B). Mobile phases at pH 9.3 and 9.6 with a buffer concentration of 10 mM were prepared analogously.

For the mobile phases at pH 3.75, a 100 mM ammonium formate stock solution was prepared. Two solutions of formic acid and ammonium formate respectively were initially prepared: 0.472 ml of formic acid was diluted to 125 ml, and 0.788 g ammonium formate was dissolved in 125 ml Milli-Q water. These two solutions were

mixed together, forming a 100 mM stock solution of ammonium formate at a pH of 3.75. 10 mM mobile phases were then prepared by diluting stock solution 10 times with acetonitrile (A) and Milli-Q water (B).

3.4 Preparation of standard solutions

10 ppm AMP solution was prepared by diluting 0.040 ml 0.1 mM AMP aqueous stock solution to 1.5 mL using a mixture of acetonitrile:H₂O (85:15).

10 ppm ATP solution was prepared by diluting 0.030 ml 0.1 mM ATP aqueous stock solution to 1.5 mL using a mixture of acetonitrile:H₂O (85:15).

10 ppm Ap4A solution was prepared by diluting 0.018 ml 0.1 mM Ap4A aqueous stock solution to 1.5 mL using a mixture of acetonitrile:H₂O (85:15).

A mixture with 10 ppm *each* of AMP, ATP, and Ap4A was prepared by pipetting the above mentioned quantities of each nucleotide stock solution into a vial and then diluting the mixture to 1.5 mL.

3.5 Method development

As a starting point in the method development (Method 1 in Table 1), a method from the application note *Monitoring of Mammalian Cell Culture Media with HILIC LC/MS* [1] from Agilent Technologies, Inc. was used. In this Agilent method, an InfinityLab Poroshell 120 HILIC-Z column with dimensions 2.1 mm × 50 mm and a particle size of 2.7 µm was used. The column used for the method development in the present project was identical apart from its length of 100 mm, therefore the gradient was linearly scaled up accordingly.

In order to develop an optimal method for separation of Ap4A from AMP and ATP from there, an array of chromatographic parameters as given by Table 1 were evaluated one by one. A standard solution mix consisting of 10 ppm of each of AMP, ATP, and Ap4A was injected 3-4 times for each of methods 1-14 (Table 1). The gradient was linearly scaled to keep the linear flow rate constant as given by Table 2.

Whenever the conditions were changed, two blank injections were performed prior to analysis in order to re-condition the column.

Table 1: Chromatographic methods that were evaluated. *For method 9, formic acid and ammonium formate (10 mM) were used in place of ammonium acetate buffer.

Method	V_{inj} (μL)	T_{col} ($^{\circ}\text{C}$)	v_{flow} ($\mu\text{L min}^{-1}$)	Buffer	pH	I (mM)	t_{eq} (min)	Grad.
1	1	25	300	Am. ac.	9.0	10	33.3	1
2	1	70	300	Am. ac.	9.0	10	33.3	1
3	1	50	300	Am. ac.	9.0	10	33.3	1
4	1	35	300	Am. ac.	9.0	10	33.3	1
5	1	50	500	Am. ac.	9.0	10	24.0	2
6	1	50	500	Am. ac.	9.0	10	32.0	3
7	1	25	600	Am. ac.	9.0	10	16.7	4
8	1	25	300	Am. ac.	9.0	10	8.33	5
9	1	25	300	Am. for.	3.75	10	8.33	5*
10	1	25	300	Am. ac.	9.0	20	8.33	5
11	1	25	300	Am. ac.	9.0	5.0	8.33	5
12	0.5	25	300	Am. ac.	9.0	20	8.33	5
13	1	25	300	Am. ac.	9.3	10	8.33	5
14	1	25	300	Am. ac.	9.6	10	8.33	5

Table 2: Evaluated gradients. Mobile phase A: Acetonitrile/buffer (90:10) in water ($\text{pH}_{\text{aq}} = 9$). Mobile phase B: buffer in water, pH 9.

Gradient 1	0 min: 5%B, 3.33 min: 5%B, 20 min: 50%B, 21.67 min: 5%B, 51.67 min: 5%B.
Gradient 2	0 min: 5%B, 2.0 min: 5%B, 12 min: 50%B, 13 min: 5%B, 35 min: 5%B.
Gradient 3	0 min: 5%B, 2.0 min: 5%B, 12 min: 50%B, 13 min: 5%B, 43 min: 5%B.
Gradient 4	0 min: 5%B, 1.67 min: 5%B, 10 min: 50%B, 10.84 min: 5%B, 25.84 min: 5%B.
Gradient 5	0 min: 5%B, 3.33 min: 5%B, 20 min: 50%B, 21.67 min: 5%B, 26.67 min: 5%B.

3.6 Statistical analysis

In order to determine whether the investigated parameters had a statistically significant effect on the separation, one way ANOVA followed by Tukey's multiple comparisons tests were performed using GraphPad Prism version 8.3.0 for Windows, GraphPad Software, La Jolla California USA.

4 Results

The results that were obtained from methods 1-14 (as given by Table 1) follow in Table 3. The corresponding chromatograms are presented in appendix A.

Table 3: Data as obtained from methods 1-14: Retention time for the AMP + ATP peak. Retention time, height, area, and width at half height for the Ap4A peak (n = 3-4). Average over the replicates $\pm$ standard deviation.

Method	$t_{\text{R, AMP+ATP}}$ (min)	$t_{\text{R, Ap4A}}$ (min)	h_{Ap4A}	A_{Ap4A}	$w_{1/2, \text{Ap4A}}$ (min)
1	13.2 $\pm$ 0.2	14.3 $\pm$ 0.3	65.8 $\pm$ 23.0	11.1 $\pm$ 2.8	0.217 $\pm$ 0.021
2	13.9 $\pm$ 0.0	15.5 $\pm$ 0.3	0.96 $\pm$ 0.04	1.40 $\pm$ 0.43	3.36 $\pm$ 1.46
3	13.4 $\pm$ 0.7	14.6 $\pm$ 0.3	7.90 $\pm$ 9.34	3.28 $\pm$ 2.10	1.85 $\pm$ 1.17
4	13.1 $\pm$ 0.1	14.1 $\pm$ 0.1	6.55 $\pm$ 1.52	3.27 $\pm$ 0.56	0.583 $\pm$ 0.045
5	9.58 $\pm$ 2.09	10.2 $\pm$ 2.2	10.3 $\pm$ 7.4	3.26 $\pm$ 2.02	0.448 $\pm$ 0.116
6	8.23 $\pm$ 0.25	8.82 $\pm$ 0.06	4.31 $\pm$ 1.41	1.27 $\pm$ 0.27	0.425 $\pm$ 0.095
7	-	-	-	-	-
8	13.4 $\pm$ 0.2	14.1 $\pm$ 0.2	1.99 $\pm$ 0.45	0.903 $\pm$ 0.098	0.673 $\pm$ 0.093
9	-	-	-	-	-
10	7.66 $\pm$ 0.03	9.86 $\pm$ 0.04	40.7 $\pm$ 6.2	9.46 $\pm$ 0.14	0.290 $\pm$ 0.030
11	14.8 $\pm$ 0.2	15.4 $\pm$ 0.2	8.04 $\pm$ 0.79	3.99 $\pm$ 0.17	0.557 $\pm$ 0.033
12	8.27 $\pm$ 0.37	10.1 $\pm$ 0.2	35.6 $\pm$ 0.6	7.58 $\pm$ 0.03	0.260 $\pm$ 0.010
13	13.4 $\pm$ 0.0	13.9 $\pm$ 0.0	11.1 $\pm$ 0.6	3.29 $\pm$ 0.14	0.393 $\pm$ 0.029
14	13.9 $\pm$ 0.2	14.4 $\pm$ 0.2	12.3 $\pm$ 1.9	3.82 $\pm$ 0.17	0.393 $\pm$ 0.058

Table 4: Data as obtained from methods 1-14: Asymmetry factor (A_s as calculated by Chromeleon 6.8), and resolution (R_s , equation 1) for the Ap4A peak (n = 3-4). Average over the replicates $\pm$ standard deviation.

Method	A_s	R_s
1	3.67 $\pm$ 0.63	1.23 $\pm$ 0.14
2	2.51 $\pm$ 0.57	0.311 $\pm$ 0.023
3	3.72 $\pm$ 0.46	0.524 $\pm$ 0.365
4	5.09 $\pm$ 0.20	0.606 $\pm$ 0.068
5	4.16 $\pm$ 0.91	0.569 $\pm$ 0.255
6	5.06 $\pm$ 0.38	0.493 $\pm$ 0.136
7	-	-
8	4.43 $\pm$ 0.65	0.451 $\pm$ 0.100
9	-	-
10	3.05 $\pm$ 0.19	1.27 $\pm$ 0.15
11	6.31 $\pm$ 1.28	1.26 $\pm$ 0.12
12	3.58 $\pm$ 0.12	1.33 $\pm$ 0.04
13	3.78 $\pm$ 0.34	0.524 $\pm$ 0.028
14	4.11 $\pm$ 0.63	0.421 $\pm$ 0.094

5 Discussion

Two peaks were observed in the chromatograms throughout the method development; one confirmed to correspond to AMP + ATP, the other confirmed to correspond to Ap4A. The results from all the runs can be viewed in table 3 in 4.

5.1 Column temperature and flow rate

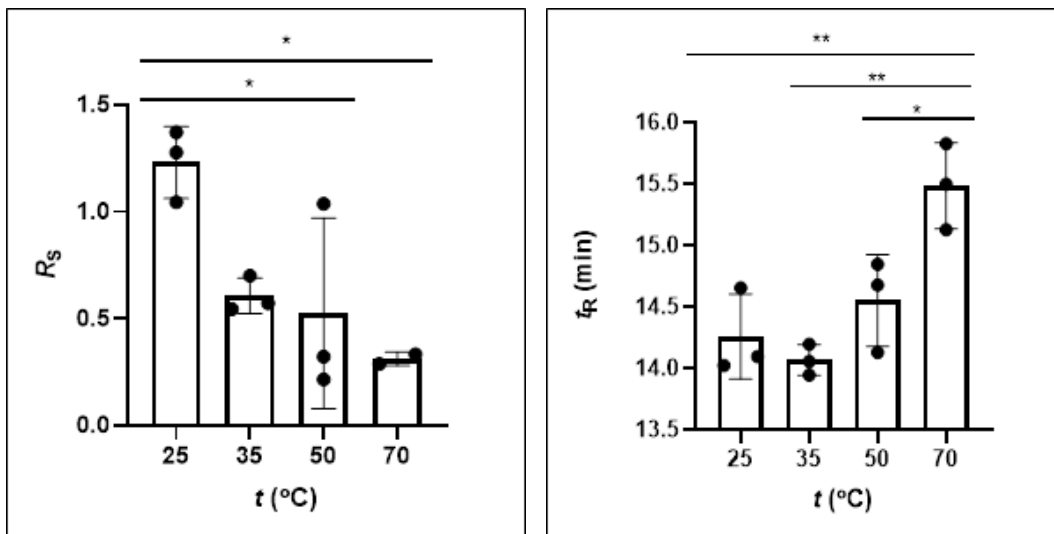
As a first step, the effect of temperature on the separation was studied. Runs were performed with the column oven set to 25, 35, 50, and 70 °C while holding all other parameters constant, according to methods 1 to 4 in table 1. The results are presented in tables 3 and 4 in section 4, as well as in figure 4.

The influence of temperature on resolution, retention time of the Ap4A peak, and the asymmetry factor for the Ap4A peak are presented in figure 4 below. Baseline separation ($R_s > 1.5$) was not achieved in any of these methods. In terms of resolution, a statistically significant difference was observed between the 25 and 50°C runs, and between the 25 and 70°C runs (figure 4a). This clearly indicates that the resolution decreased with increased temperature. Zone spreading due to longitudinal diffusion as well as degradation of Ap4A might have caused this decrease.

A significant increase in retention time with increased temperature was observed over almost all of the runs, as given by figure 4b. This seems counter-intuitive since the retention time is expected to decrease with increased temperature, especially in HILIC, where the adsorbed water layer is believed to decrease with increased temperature. The increase in retention time might be due to insufficient re-equilibration or an unstable adsorbed water layer at the higher temperatures.

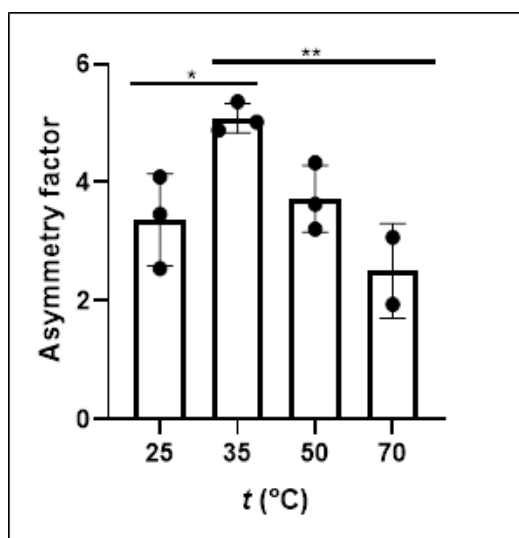
The asymmetry factor of the Ap4A peak (figure 4c) worsened significantly between the 25 and 35°C runs, and improved significantly between the 35 and 70°C runs. In other words, secondary interactions between Ap4A and the stationary phase seem to increase with a 10 degree increase in temperature, and decrease again when the temperature is raised an additional 35 degrees. Peak tailing in HPLC is commonly caused by unwanted secondary interactions between the analyte and the stationary

phase. Since there is a maximum in asymmetry factor at 35 °C, the retention mechanism was probably in its "most mixed" state at this temperature, consisting of both partitioning and adsorption. As discussed in section 2.2, the adsorbed water layer in HILIC is thinner at higher temperatures. Therefore, the dominating retention mechanism at higher temperatures is likely adsorption, and at lower temperatures (where the water layer is thicker) it is likely partitioning.



(a) Influence of temperature on the resolution of the two peaks.

(b) Influence of temperature on the Ap4A retention time.



(c) Influence of temperature on the Ap4A asymmetry factor.

Figure 4: Influence of temperature on the separation. * $p < 0.05$, ** $p < 0.01$

Above it has been made clear that an increase in temperature decreased the resolution of the chromatography. In order to decrease the longitudinal diffusion of the analytes, a natural next step was to at the same time increase the flow rate. Since the peak

tailing seemed to be at its highest at 35°C, and to be able to increase the flow rate without a drastic increase in back pressure, a temperature of 50°C was chosen, and the flow rate was increased to 500 $\mu\text{L min}^{-1}$. The only statistically significant difference that was observed in this experiment was a decreased retention time for the Ap4A peak (figure 5), which is expected for a higher flow rate.

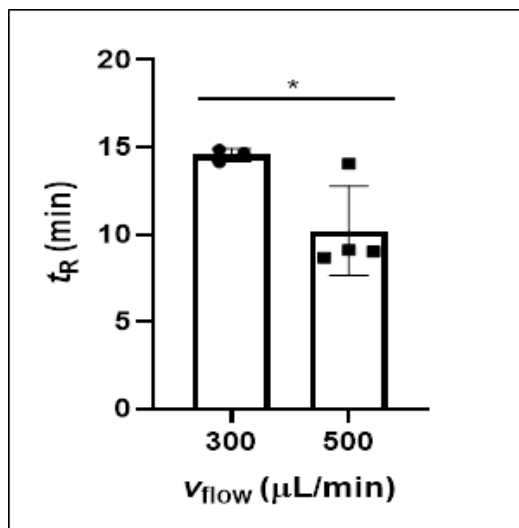


Figure 5: Influence of flow rate on the Ap4A retention time. * $p < 0.05$

A 50°C run with a flow rate of 500 $\mu\text{L min}^{-1}$ and a re-equilibration time increased by 8 minutes (method 6) was then performed, however no significant difference was observed as compared to method 3. A run with a temperature of 25°C and a flow rate of 600 $\mu\text{L min}^{-1}$ (method 7) was also performed, yielding an uninterpretable chromatogram (appendix A) due to unstable back pressure.

5.2 Re-equilibration time

Since saving time by increasing the flow rate and temperature did not seem to be possible, the influence of the re-equilibration time was investigated. As discussed in section 2.1.1, highly reproducible data has previously been shown to be achievable on a column in partial equilibrium. When comparing method 1 ($t_{\text{eq}} = 33.3$ min) and method 8 ($t_{\text{eq}} = 8.33$ min), a significant decrease in resolution was observed when decreasing the re-equilibration time by 25 minutes (figure 6), however no difference in neither asymmetry factor nor retention time was observed. When comparing the standard deviations of the retention times of the replicate runs between these two methods with an F test, no significant difference on a 95% confidence level was observed. Therefore method 8 was developed further.

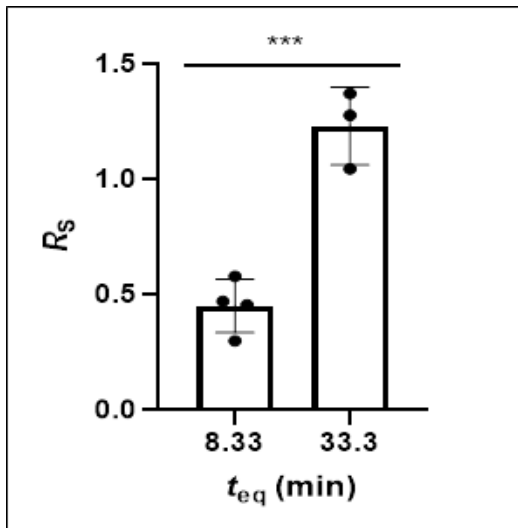
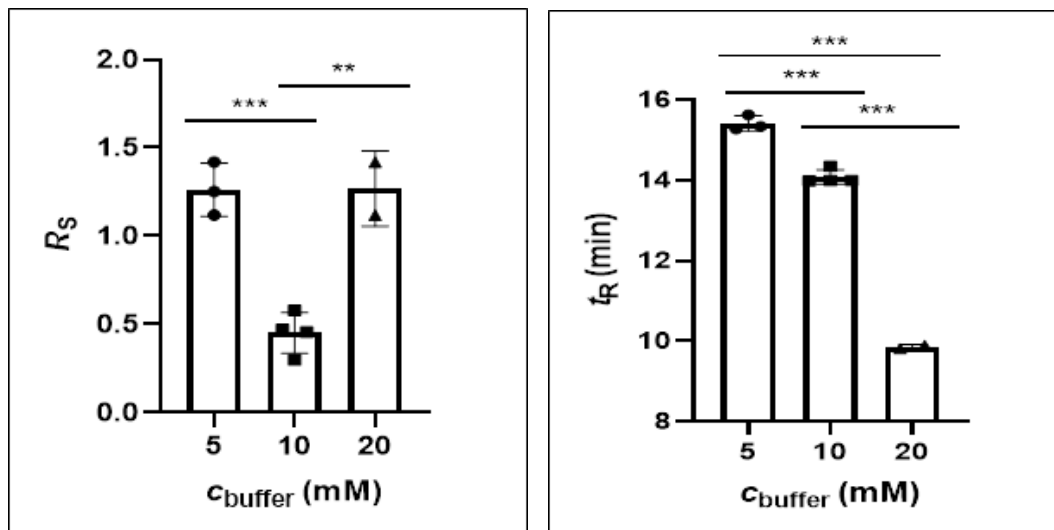


Figure 6: The influence of re-equilibration time on resolution. *** $p < 0.001$

5.3 Buffer concentration

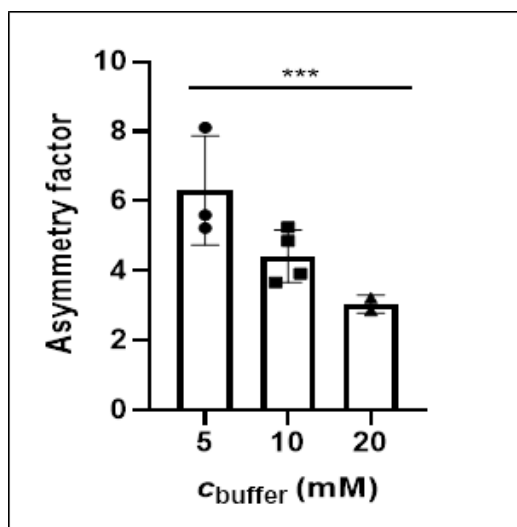
The next parameter to be investigated was the concentration of ammonium acetate in the mobile phase. As discussed in section 2.2, a higher concentration of ions in the mobile phase is expected to shield the interactions between the zwitterionic stationary phase and the ionic analytes. An increased buffer concentration also means better buffer capacity, making the mobile phase more robust to pH fluctuations. Therefore,

three different buffer concentrations were tested; 5, 10, and 20 mM, methods 8, 10, and 11, respectively (figure 7).



(a) Influence of buffer concentration on the resolution of the two peaks.

(b) Influence of buffer concentration on the Ap4A retention time.



(c) Influence of buffer concentration on the Ap4A asymmetry factor.

Figure 7: Influence of buffer concentration on the separation. ** $p < 0.01$, *** $p < 0.001$

The resolution is significantly higher in both the 5 mM method and the 20 mM method than in the 10 mM method (figure 7a). Resolution depends on retention as well as plate number (equations 2 and 3), which in turn depends on the base peak width, which is affected by peak tailing. A lower buffer concentration gives more retention (figure 7b) which then increases the resolution, explaining the increased resolution at 5 mM. On the other hand, a higher buffer concentration gives rise to a better asymmetry factor, which explains the increased resolution at 20 mM.

The retention time decreased significantly with increased buffer concentration (figure 7b), which is expected due to ionic shielding. Moreover, increased buffer concentration decreased the asymmetry factor of the Ap4A peak (figure 7c). Since adsorption of analyte to the stationary phase might make peaks less symmetrical, the decrease is likely caused by ionic shielding. Another contributing factor to the decreased asymmetry factor might be the higher buffer capacity in the 20 mM method.

5.4 pH

The pK_a value of ammonium is about 9.25. [16] In order to investigate the robustness of the method in regard to pH fluctuations, the separation was run with 3 different pH values around this pK_a value; pH = 9.0, 9.3, 9.6 (methods 8, 13, and 14).

The only significant difference between these three methods was a difference in Ap4A retention time between the pH 9 run and the pH 9.6 run. Figure 8 shows that the Ap4A retention time in method 14 (pH = 9.6) was significantly higher than the Ap4A retention time in method 8 (pH = 9.0). Since the strongest basic pK_a of Ap4A as predicted by ChemAxon is 5.29, this increase in pH is not expected to change the characteristics of the analyte. It can however be speculated that some deprotonation might occur in the unknown stationary phase.

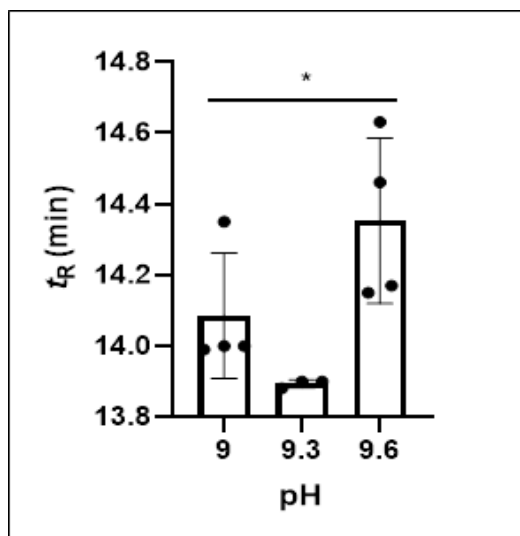


Figure 8: Influence of mobile phase pH on the Ap4A retention time. * $p < 0.05$, *** $p < 0.001$

As discussed in section 2.2, protonation of the adenine part of ATP starts occurring at pH 4.3, leaving it with a positive charge. Since this could possibly be used as a tool for separating ATP and AMP from Ap4A, the gradient was run at pH = 3.75 with ammonium formate buffer (method 9). However, no peaks were obtained under these conditions (see chromatogram 7 in appendix A). In the chromatogram it looks like the peaks might have coeluted with the void, but the lack of peaks could also be caused by the Ap4A "zwitterion" being irreversibly adsorbed to the zwitterionic stationary phase.

6 Conclusions

Separating Ap4A from ATP and AMP on the Agilent Poroshell 120 HILIC-Z column proved more challenging than expected, especially since the composition of the stationary phase remains a mystery.

Given all of the parameters discussed in the previous section, altering the buffer concentration alone seemed to have a positive effect on the separation. Increasing the ammonium acetate concentration to 20 mM or decreasing it to 5 mM significantly increased the resolution, however 20 mM seems preferable due to the low asymmetry factor of the Ap4A peak for this method. A higher concentration of buffer also makes the method more robust in case of pH fluctuations. The highest resolution was obtained in method 12 with $R_s = 1.33 \pm 0.04$.

The resolution initially decreased in response to a decreased re-equilibration time, although the value obtained when the buffer concentration was increased in method 12 ($R_s = 1.33 \pm 0.04$) is actually very close to the value obtained in method 1 ($R_s = 1.23 \pm 0.14$). As a next step, in order to obtain optimal resolution, the re-equilibration time in method 12 should therefore be increased.

The close elution of the nucleotides makes investigation using a diode array detector challenging. Therefore, the next step is to use mass spectrometry detection, which is facilitated by the fact that the developed method uses mobile phases compatible with mass spectrometry. With more specific and selective detection it would also be interesting to introduce more nucleotides containing several different nitrogenous bases. This would bring the project closer to the long term goal of isolating Ap4A from biological samples with a complex matrix.

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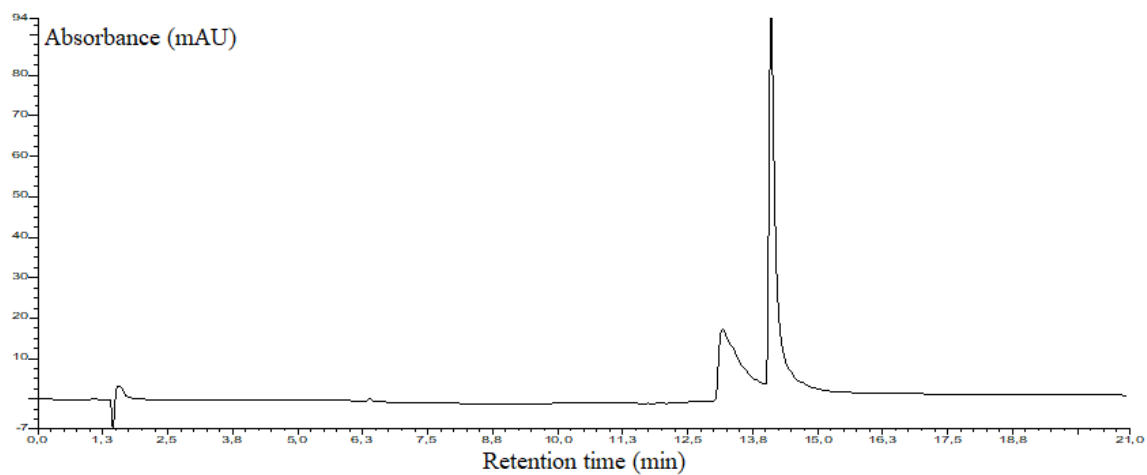
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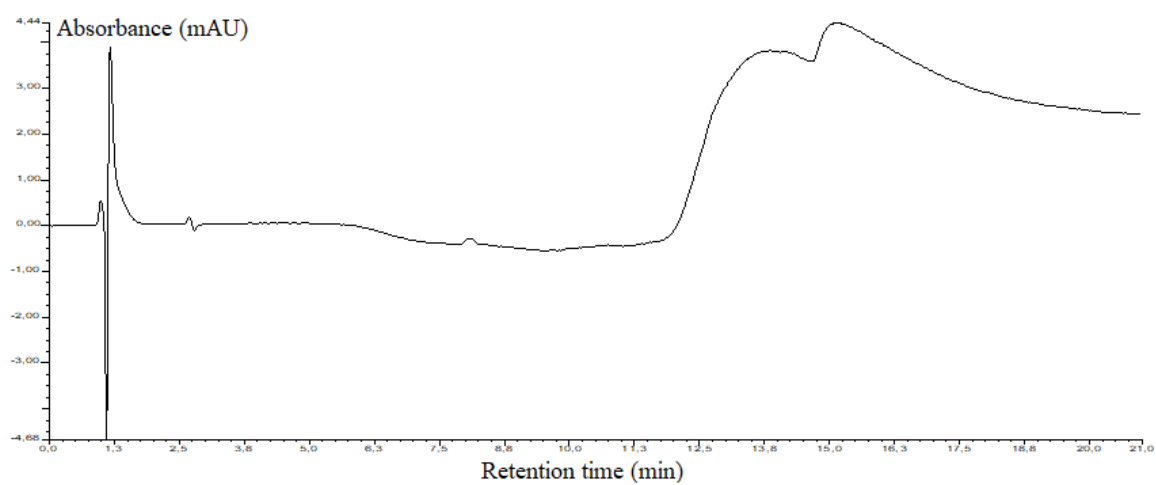
Appendix A Chromatograms for methods 1-14

The chromatograms resulting from methods 1 to 14 follow in Table 5.

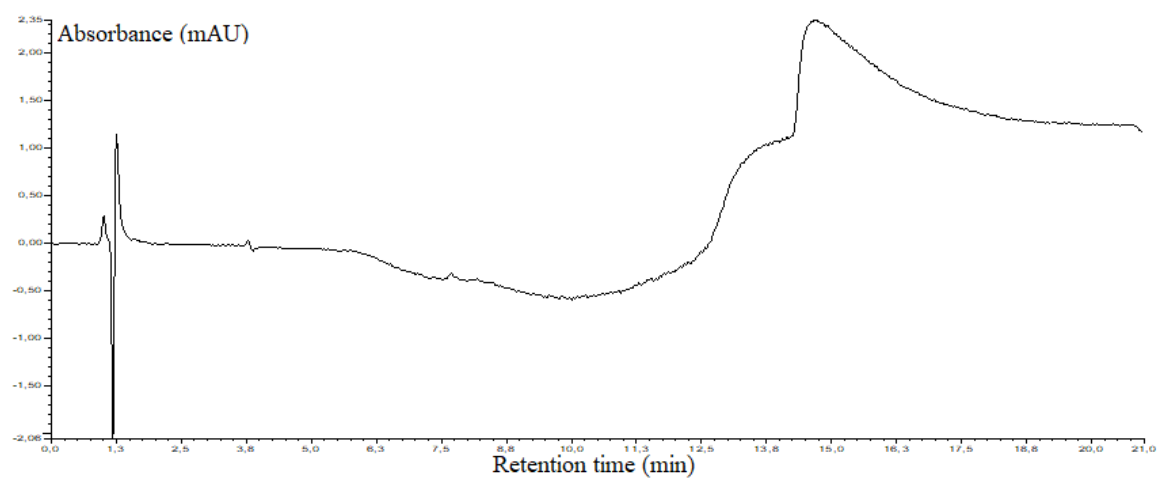
Table 5: The best chromatogram from each method is presented here.



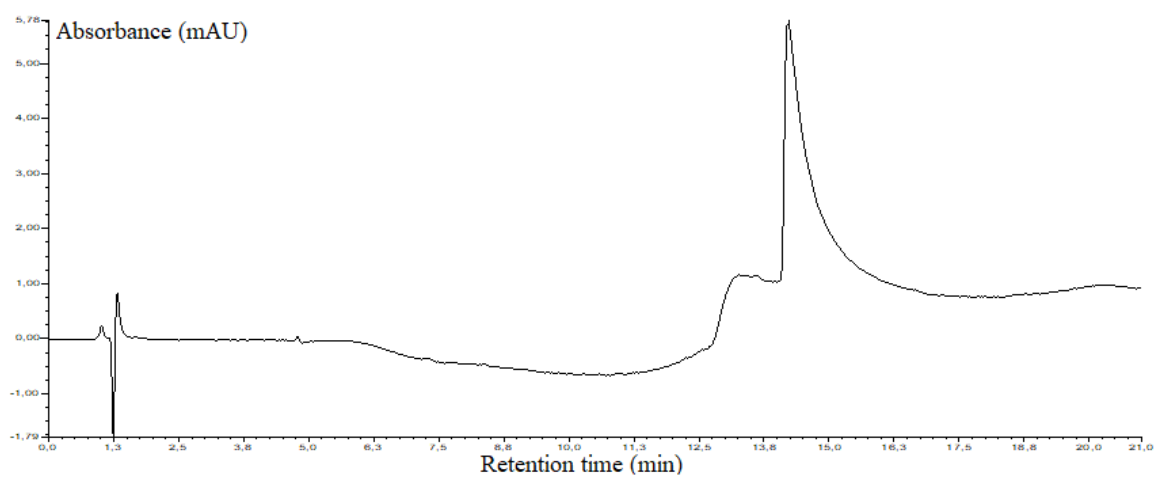
Method 1.



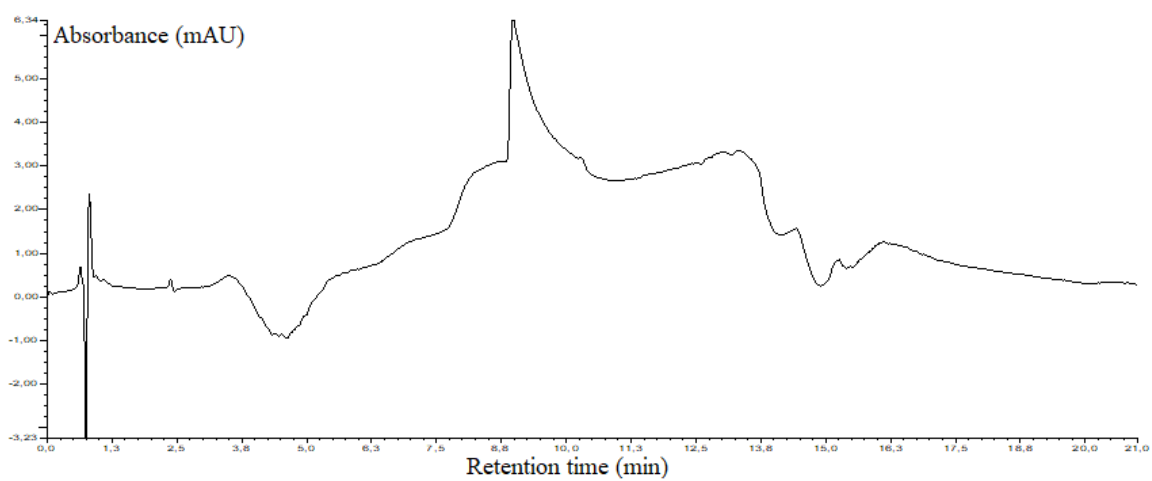
Method 2.



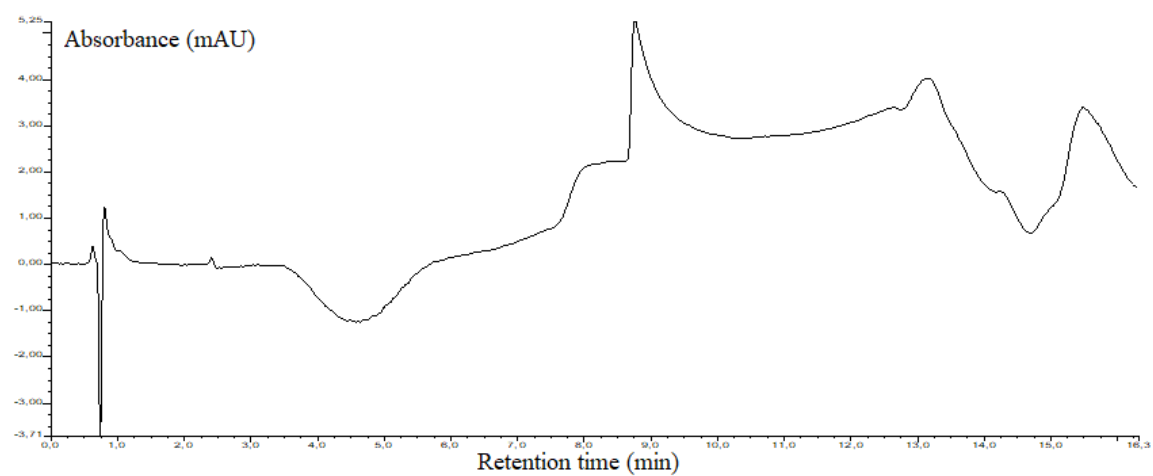
Method 3.



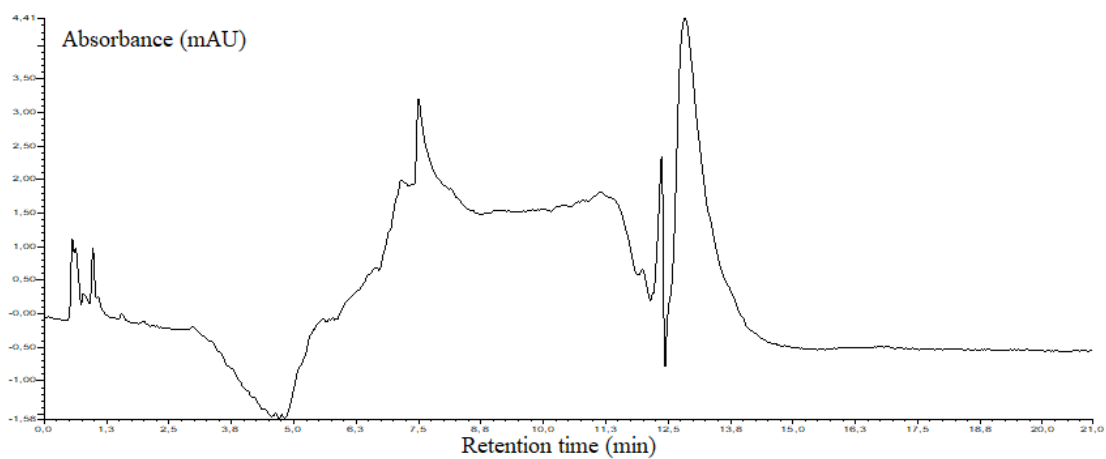
Method 4.



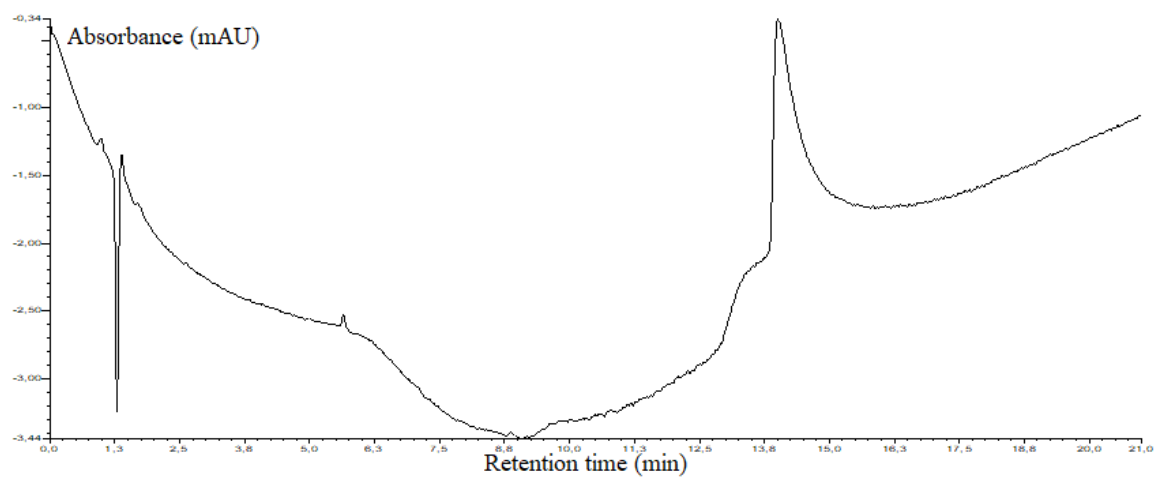
Method 5.



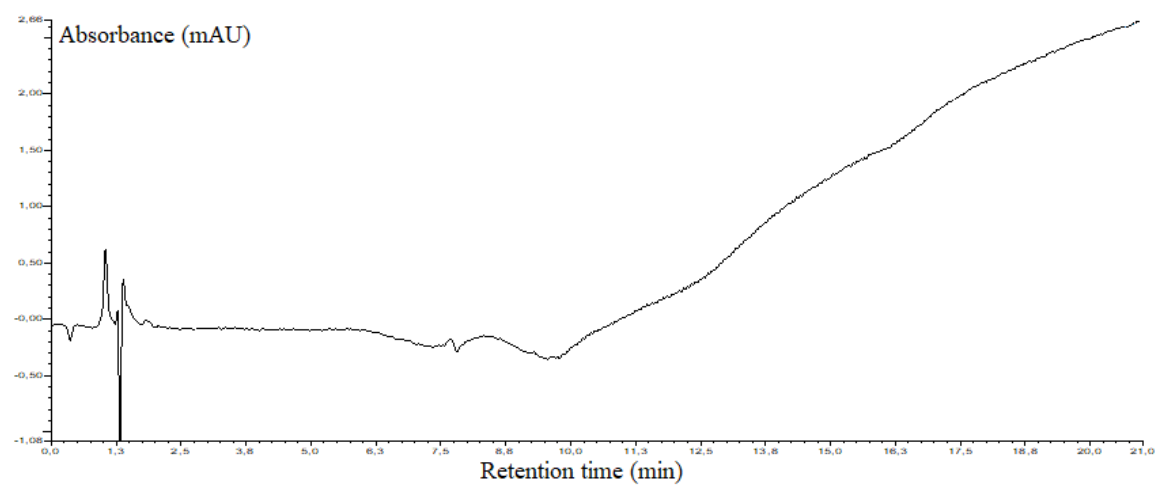
Method 6.



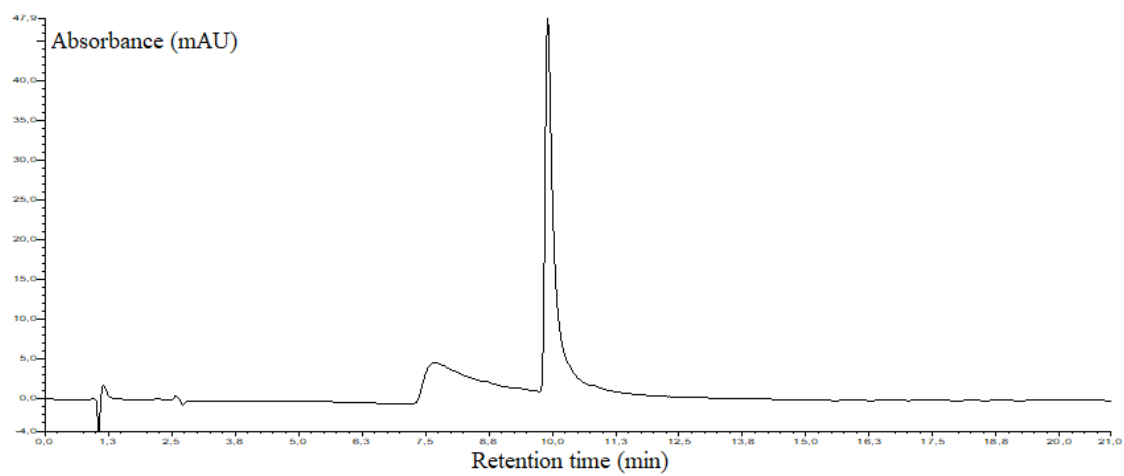
Method 7.



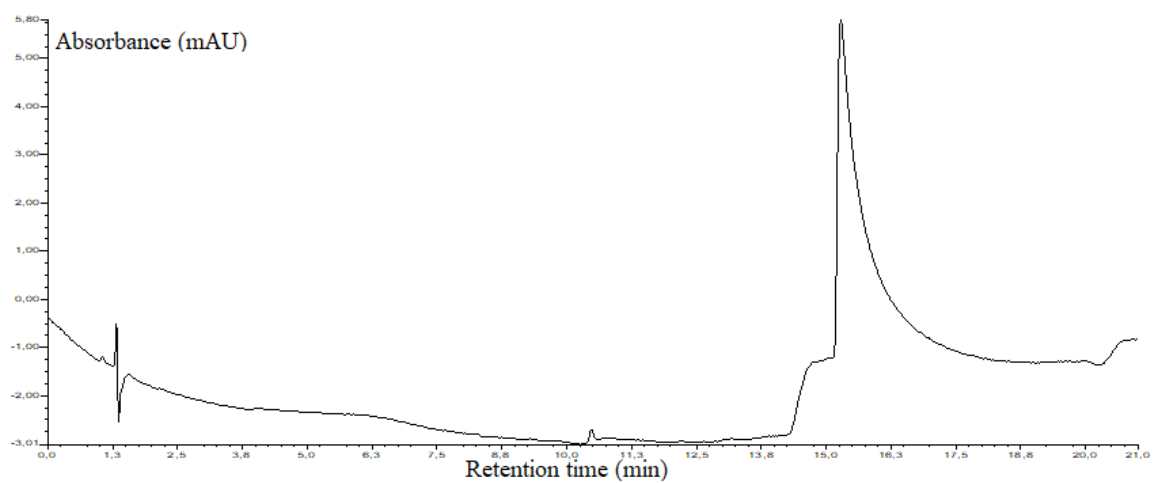
Method 8.



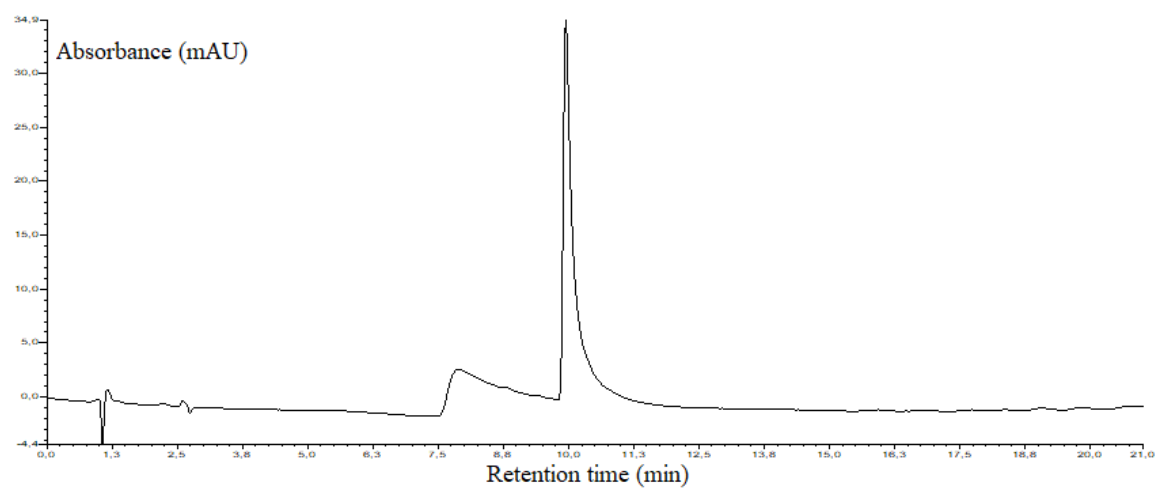
Method 9.



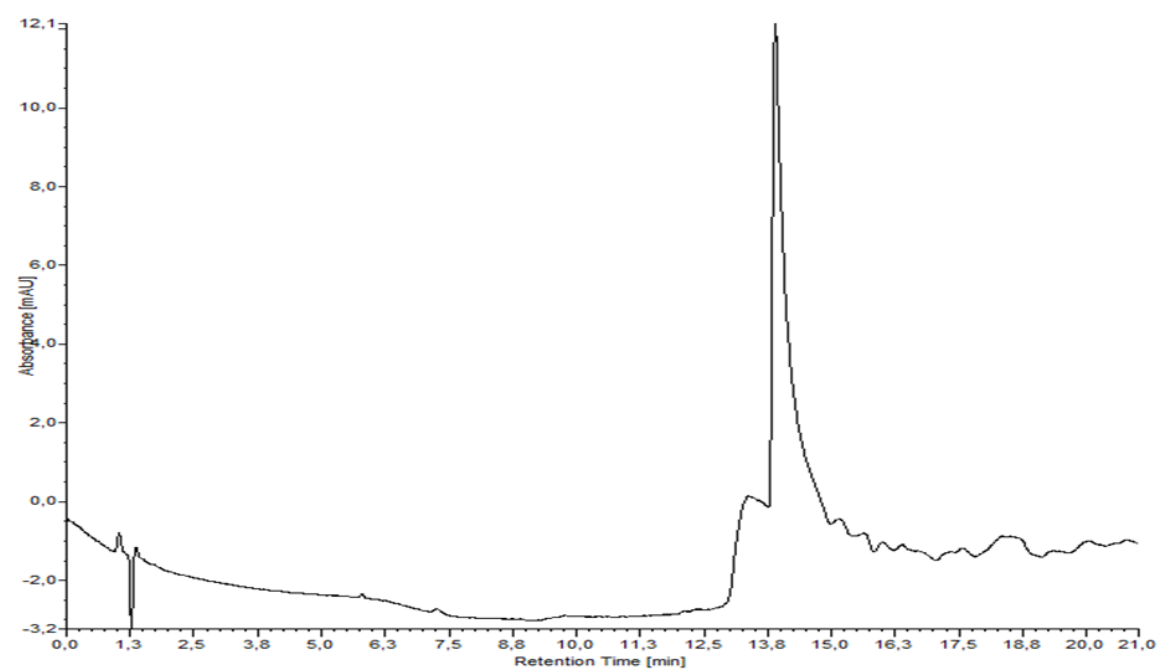
Method 10.



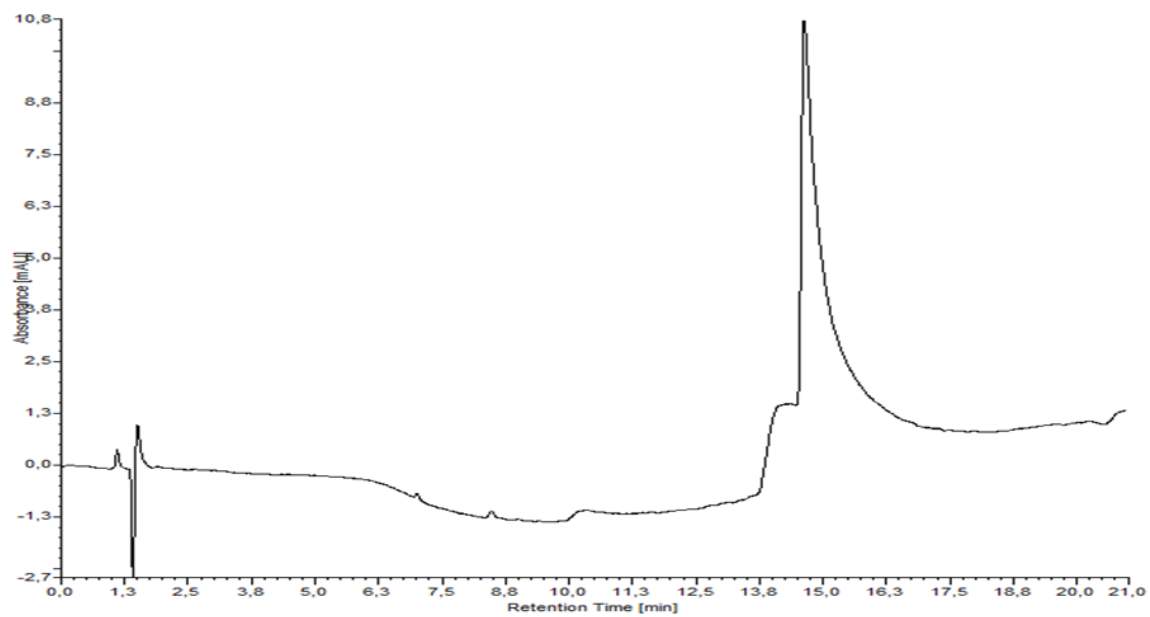
Method 11.



Method 12.



Method 13.



Method 14.