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# Hydrophilic interaction chromatography of nucleoside triphosphates with temperature as a separation parameter

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ABSTRACT

Eight deoxynucleoside triphosphates (dNTPs) and nucleoside triphosphates (NTPs): ATP, CTP, GTP, UTP, dATP, dCTP, dGTP and dTTP, were separated with two 15 cm ZIC-*p*HILIC columns coupled in series, using LC–UV instrumentation. The polymer-based ZIC-*p*HILIC column gave significantly better separations and peak shape than a silica-based ZIC-HILIC column. Better separations were obtained with isocratic elution as compared to gradient elution. The temperature markedly affected the selectivity and could be used to fine tune separation. The analysis time was also affected by temperature, as lower temperatures surprisingly reduced the retention of the nucleotides. dNTP/NTP standards could be separated in 35 min with a flow rate of 200 μL/min. In *Escherichia coli* cell culture samples dNTP/NTPs could be selectively separated in 70 min using a flow rate of 100 μL/min.



## 1. Introduction

A significant challenge in the analysis of nucleoside triphosphates (NTPs) and deoxynucleoside triphosphates (dNTPs) is their extreme hydrophilicity, which makes them difficult to chromatograph using well-established reversed phase (RP) chromatography. Instead, methods have been developed for separating dNTP/NTPs using a strong anion exchange column (SAX) [1–6]. However, these methods will not satisfactorily separate all 8 dNTP/NTPs, or have very long run times/cycles. Validated LC–MS methods have been presented, e.g. using ion pair interactions [7] or alternative stationary phases such as porous graphite [8]. However, these methods cannot separate all dNTP/NTPs with chromatography alone. Our goal was to develop a method that could separate all 8 dNTP/NTPs using simple instrumentation. A logical choice was to use hydrophilic interaction chromatography (HILIC) [9,10], as it has in recent years been used extensively for separating hydrophilic compounds [10]. Several mechanisms have been suggested for the HILIC separation mechanism: partitioning between an immobilized water layer in the stationary phase and the mobile phase [9,10], normal phase/adsorption interactions [10,11], electrostatic interactions [12,13], hydrogen bonding [11] and even reversed-phase interactions [14]. However, studies attempting to determine the relative importance of one interaction over the other are inconclusive [14]. It is likely that several/all of these mechanisms are more or less involved, depending on the conditions (type and amount of buffer, amount of water in mobile phase etc.) and column used. This makes HILIC highly versatile, but not straightforward to optimize, and many may find HILIC phases "hard to tune" for their particular application. In this paper we present a separation method for dNTP/NTPs ATP, CTP, GTP, UTP, dATP, dCTP, dGTP and dTTP using commercial polymer-based "ZIC-*p*HILIC" columns with simple LC–UV instrumentation. ZIC-*p*HILIC columns were used instead of silica-based ZIC-HILIC columns since they can be operated with a wider pH range and were consequently easier to "tune". The effect of organic modifier, ionic strength and temperature on the chromatography has been investigated. We found that NTPs and dNTPs behaved anomalously regarding temperature and solvent gradients, and we will discuss these findings. The use of ZIC-*p*HILIC columns for separating dNTP/NTPs in *Escherichia coli* cell culture samples is demonstrated.

## 2. Experimental

### 2.1. Chromatography

The LC–UV system consisted of a Hitachi L-7110 isocratic pump (Merck, Darmstadt, Germany), a Waters 486 tunable absorbance detector (Waters, Milford, MA, USA), ZIC-HILIC columns or ZIC-*p*HILIC columns (2.1 × 150 mm, 5 μm) (Merck Sequant, Umeå,

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Sweden). A Vici 6-port injector (Valco, Schenkon, Switzerland) with a 5 μL loop was used for manual injections. Data was acquired using a computer with TotalChrom Workstation 6.2.1 software from Perkin Elmer (Waltham, MA, USA). ZIC-HILIC columns have a zwitterionic 3-sulfopropyldimethylalkylammonio (also call a sulfobetaine group) inner salt as a functional group [10]. For column heating a Mistral column oven (Spark Holland, (Groningen, Netherlands) was employed. For ZIC-HILIC columns, the mobile phase components were 100 mM ammonium acetate (pH 5.3), and ACN (Rathburn, Walkerburn, Scotland). The ACN percentages examined were between 50% and 85%. Mobile phase solvent components for ZIC-*p*HILIC columns were 100 mM ammonium carbonate (pH 8.9), and ACN. With this column the mobile phase consisted of 70% ACN and 30% of the ammonium carbonate solution unless specified otherwise. In effect, the overall salt concentration in the mobile phase was 30 mM. Alternatively, ethanol and isopropanol (Rathburn) were used instead of ACN.

Stock solutions of the dNTP/NTPs, AMP and ADP (Sigma, St. Louis, MO, USA) were prepared by dissolving 10 μL of a 100 mM standard solution in 5 mL of the appropriate mobile phase, consisting of 70% ACN (alternatively EtOH or isopropanol) and 30% 100 mM buffer, to a final concentration of 100 μg/mL. Working solutions were made by appropriate dilution of the stock solutions with the mobile phase.

### 2.2. Cell culture sample preparation

*E. coli* cells (wild type strain MG1655, provided by Jon Beckwith, Harvard Medical School, Boston, USA) were grown in AB minimal medium [15] supplemented with 1 μg/mL thiamine, 0.2% glucose and 0.5% casamino acids. Mass growth was monitored by measuring the absorbance at 450 nm using a spectrophotometer. The cells were grown exponentially at 37 °C to $A_{450}$ = 0.15 followed by filtration of 300 mL cell culture through a 47 mm, 0.45 μm HAWP04700 nitrocellulose filter (Millipore, Billerica, MA, USA). After filtration the filter was placed cell-side down in a beaker containing 4 mL ice-cold 1 M formic acid and kept for 30 min on ice. Then the filter and extract were transferred to a 50 mL tube and vortexed to remove all cells from the filter. The extract was divided among 10 tubes, centrifuged and the supernatant was completely dried in a Savant AES1010 SpeedVac (GMI, Minneapolis, MN, USA) to remove the formic acid. Pellets from 150 mL cell culture (5 tubes) were dissolved in 200 μL methanol (MeOH)/$H_2O$ (60/40, *v/v*) and vortexed for 2 min. To ensure a selective determination of the NTPs using a (non-specific) UV detector, a somewhat comprehensive 2-step extraction procedure was employed; the first SPE step was performed with 100 mg "SAMPLIQ" $C_{18}$-endcapped cartridge (Agilent Technologies, Santa Clara, CA, USA). The SPE cartridge was conditioned with 1 mL of ACN and 1 mL of $H_2O$/ACN (99/1, *v/v*). A 200 μL sample was loaded onto the SPE cartridge, and the dNTP/NTPs were washed off the SPE cartridge with 1 mL $H_2O$. The eluate was collected and evaporated to dryness under nitrogen. The residue was reconstituted in 200 μL MeOH (60%), vortexed for 2 min and centrifuged for 10 min at 13,000 rpm. The second SPE was performed with a 60 mg "SAMPLIQ" WAX cartridge (Agilent Technologies). The SPE was conditioned with 2 mL MeOH, 2 mL of 50 mM ammonium acetate ($NH_4OAc$) (pH adjusted to 4.5 with acetic acid (AcOH)). 200 μL sample solution was loaded onto the SPE cartridge. The SPE cartridge was washed with 2 mL of 50 mM $NH_4OAc$, pH 4.5. The analytes were eluted from the SPE cartridge with 2 mL of a solution containing MeOH/$H_2O$/$NH_4OH$ (80/15/5, *v/v/v*) The eluate was collected and evaporated to dryness with nitrogen gas. The residue was reconstituted in 50 μL of mobile phase (ACN/100 mM ammonium carbonate, pH 8.9, 70/30, *v/v*), vortexed for 2 min and centrifuged for 10 min at 13,000 rpm, and 15 μL of the supernatant was loaded onto the injector, overfilling the 5 μL loop 3 times prior to injection.

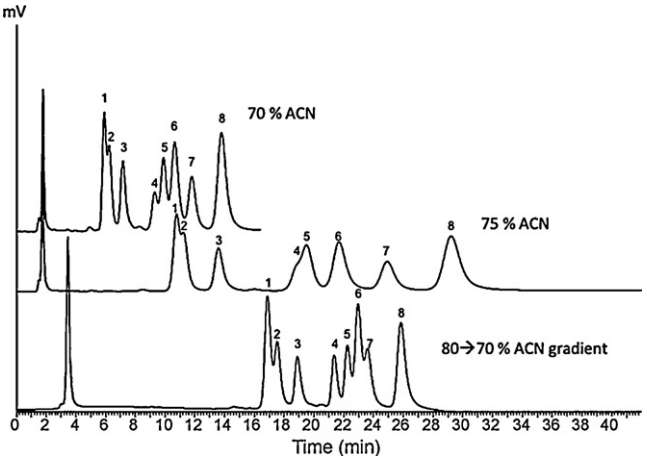


**Fig. 1.** Separation of eight nucleotide standards (12 μg/mL) on a ZIC-*p*HILIC column (2.1 × 150 mm, 5 μm). The mobile phase consisted of 100 mM $(NH_4)_2CO_3$ (*aq*), pH 8.9 (A) and ACN (B). The two chromatograms on the top show isocratic elution with 70% B (total ionic strength 30 mM) and 75% B (total ionic strength 25 mM) at 200 μL/min. The third shows a gradient from 80% to 70% B in 10 min at 200 μL/min. The injection volume was 5 μL and the UV absorbance was set to 254 nm. The elution order is (1) dATP, (2) dTTP, (3) ATP, (4) dCTP, (5) UTP, (6) dGTP, (7) CTP and (8) GTP.

## 3. Results and discussion

### 3.1. Choice of HILIC stationary phase

With a silica ZIC-HILIC stationary phase, the analytes could not be separated, even with weak mobile phases, or by varying the buffer composition (results not shown). The chromatographic peaks were extremely broad and asymmetrical, as commented by Marrubini et al. in their paper describing a successful ZIC-HILIC separation of purine, pyridine and nucleosides [16]. An organic polymer ZIC-HILIC column (ZIC-*p*HILIC) gave markedly improved chromatographic performance, as the column could be used at higher pHs, resulting in symmetrical peaks, increased retention factors and improved separation of the nucleotides. Other reasons for improved chromatography may be that there are no residual silanol groups on the organic polymers, which can be a source of secondary interactions/tailing (although it has been reported that this should not be an issue with silica-based ZIC-HILIC either [10]). The vast difference in performance found between a silica-based and an organic based stationary phase serves as yet another example of how crucial the choice of HILIC column can be for a particular set of compounds.

### 3.2. Solvent composition adjustment for improving separation

Although the ZIC-*p*HILIC column gave improved peak shape and partial separation with a mobile phase containing 70% ACN, the 8 analytes were still not satisfactorily separated (Fig. 1, top). Weaker mobile phases were examined (Fig. 1, middle), but their separation was still not satisfactory, and at the expense of significantly longer run times. Solvent gradients were also explored, in hope of obtaining the moderately improved separations of weaker solvents but at reduced run times. However, the separation actually worsened compared to isocratic conditions (Fig. 1, bottom). As the organic solvent content decreases, the prominence of the various interactions involved in HILIC may vary. These interactions during a gradient may counteract each other, resulting in poorer separation of the nucleotides. In other words, it seems that the ZIC-*p*HILIC

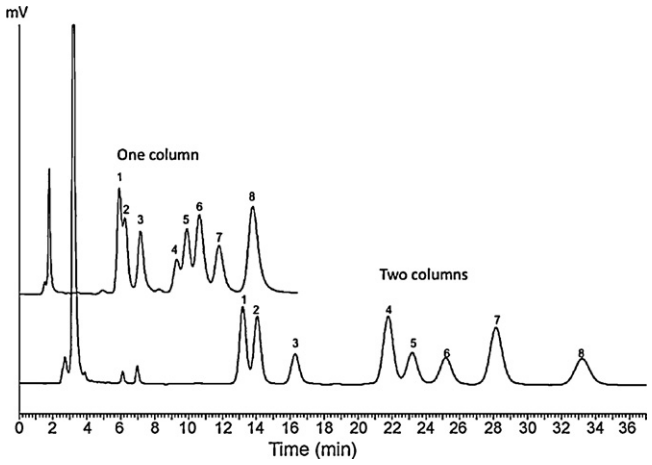


**Fig. 2.** Separation of eight nucleotide standards (12 μg/mL) on two ZIC-pHILIC columns compared to one column. Isocratic elution was performed with 70/30 (*v*/*v*) ACN/$(NH_4)_2CO_3$ (pH 8.9, 100 mM) at 200 μL/min. The injection volume was 5 μL and the UV absorbance was set to 254 nm.

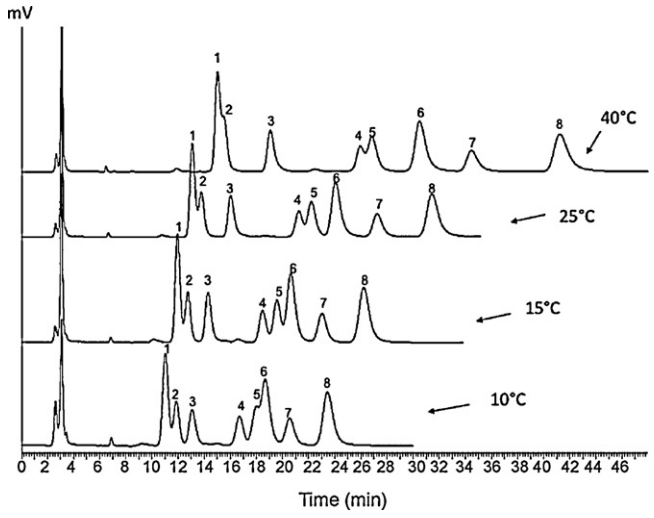


**Fig. 3.** Separation of the eight nucleotides in an *E. coli* cell sample (wild type strain SMG3) on two ZIC-*p*HILIC columns at 15 °C at 100 μL/min. Chromatogram recording began 15.0 min after injection. For other conditions see Fig. 2.

column's selectivity for nucleotides changed during the gradient. Others have also reported effects on HILIC selectivity as function of organic solvent content, e.g. Jandera and coworkers who showed that the elution order under isocratic conditions may change as a function of the ACN mobile phase content [17], and Sandra and coworkers who observed that a gradient could increase the resolution of some compounds but decrease the resolution of others [18].

Salt concentration and buffer type are believed to affect the separation due to e.g. ion pairing with the solutes, thickening of the adsorbed water layer, shielding of ion exchange sites and dipole–dipole interactions [16,19]. The pH 8.9 ammonium carbonate buffer concentration (effectively 22.5–30–37.5 mM) was thus investigated as a parameter to optimize separations. 30 mM ammonium carbonate produced the best efficiency (*N*), and higher ammonium carbonate concentrations gave longer retention times. The increase in retention time with salt concentration is likely due to a shielding of negatively charged sites, lessening electrostatic repulsion, as observed previously [12]. Separation did not improve significantly with increased salt content, and at the cost of longer retention times.

### 3.3. Columns in series for improving separation

Since solvent optimization did not result in complete separations of the 8 dNTP/NTPs, two identical ZIC-*p*HILIC columns were coupled in series (Fig. 2) to increase the number of plates from 1941 to 4252 (*N*/*m* = 12,927 and 14,159, respectively). The flow rate could be doubled from the recommended 100 μL/min to 200 μL/min with the pressure staying well below 200 bar. At this elevated flow rate the efficiency (*N*) was reduced by one third, but overall the separation performance improved compared to using one column at optimal flow rate. Under these conditions, the dNTP/NTP standards (with the possible exception of dATP and dTTP, peak 1 and 2, respectively) were satisfactorily separated in less than 35 min.

### 3.4. Temperature as a separation parameter

Initially, the column temperature was 25 °C, but was raised to 40 °C in an attempt to further improve the separation of dATP and dTTP. The efficiency did increase with temperature at the flow rate used (Fig. 3), but the separation of dATP and dTTP worsened. By reducing the temperature to 15 °C, the separation of dATP and dTTP improved, but instead UTP and dGTP (peaks 5 and 6) began to co-elute. Hence, temperature affects the HILIC selectivity for dNTPs/NTPs.

Interestingly, the retention times increased with temperature (Fig. 3 and van't Hoff plot Fig. 4). An increase in temperature usually leads to a shift in the equilibrium towards the mobile phase, shortening the retention time [18,20]. Inverse relationships between temperature and retention has been found and studied mostly regarding RP phases, and has been related to e.g. varying structural conformities [21], type of stationary phase, solvent and buffer [22,23], and if mobile phase pH is close to the p*K*a of the buffer and solute [24]. However, few studies have discussed this phenomenon in HILIC.

A review by Hao et al. [22] discussed several examples of inverse van't Hoff slopes with various HILIC stationary phases and proposed explanations related to e.g. interactions with residual silanol groups when using silica-based stationary phases. However, the ZIC-HILIC variant has as mentioned been described as being largely free from silanophilic retention properties [10]. Temperature can affect ion exchange effects on retention [25]. It could be speculated that the temperature affects the hydration of the analytes, affect-

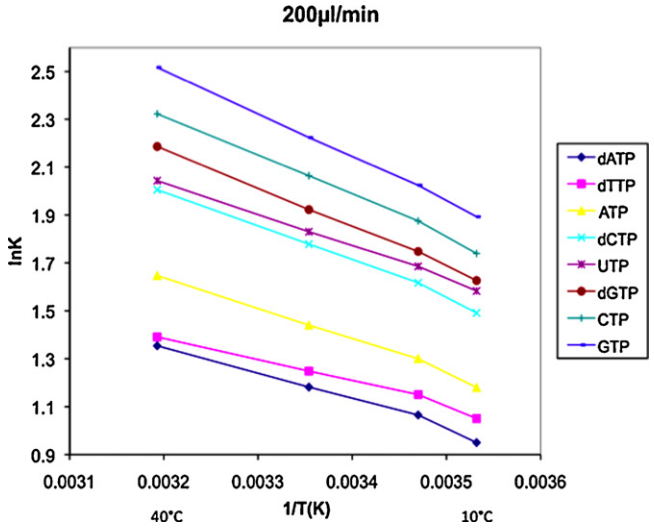


**Fig. 4.** Van't Hoff plot of the eight nucleotides standards chromatographed on two ZIC-pHILIC columns at 40, 25, 15 and 10 °C (*n* = 1 per compound per plot).

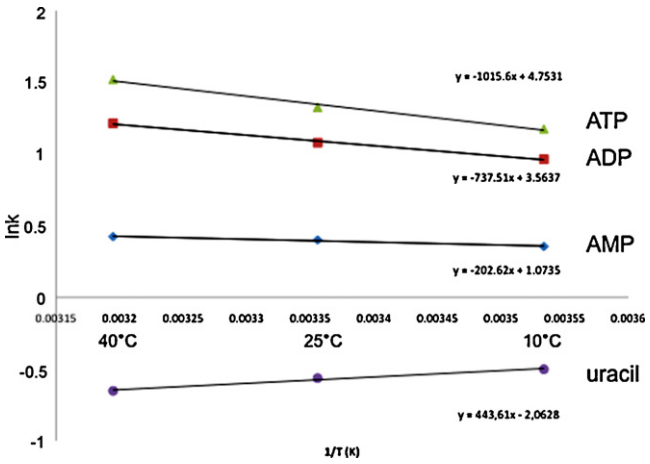


**Fig. 5.** Van't Hoff plot of AMP, ADP, ATP and uracil chromatographed on two ZIC-pHILIC columns at 10, 25 and 40 °C. For other conditions see Fig. 2.

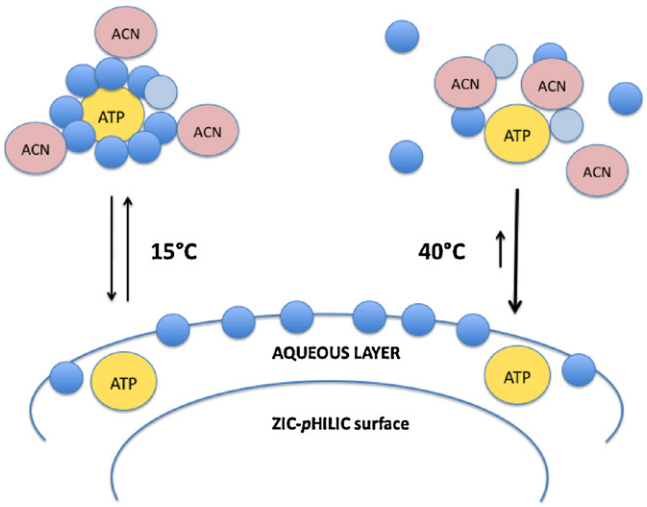


**Fig. 6.** Proposed mechanism of disassociation of NTP/water cluster, leading to increased retention times at higher temperature.

ing the retention [25,26]. It is unlikely that a temperature increase affects the mobile phase pH to the degree that the analytes become protonated/deprotonated, since the mobile phase pH of 8.9 is not near the p*K*a of the phosphate groups or the nucleoside hydroxyl groups [27].

Since hydrophilic interactions seem to be significantly stronger than ionic interactions when the mobile phase contains predominantly the organic component with high amounts of buffer [12], we believe that the retention mechanism for dNTPs/NTPs on the ZIC-pHILIC column is largely a hydrophilic partitioning process. This is in accordance with the retention order triphosphate (most hydrophilic) > diphosphate > monophosphate (least hydrophilic), and that a deoxytriphosphate has a shorter retention time than a more hydrophilic triphosphate. We speculate that increased temperature promotes the hydrophilic interaction with the aqueous layer surrounding the stationary phase zwitterionic groups, as the van't Hoff curve steepness were also of the order triphosphate > diphosphate > monophosphate (Fig. 5). One explanation may be that the very hydrophilic nucleotides cluster with salt and/or water molecules, partially shielding the nucleotides from the predominantly organic mobile phase. This would be analogous to the formation of the hydrophilic layer around a HILIC stationary phase, and the formation of solvent clusters in ACN and water mixtures [28–30]. The significance of solute distribution in mobile phase clusters regarding retention has previously been described [28]. At elevated temperatures, we speculate it becomes increasingly favorable for the NTP/water cluster to dissociate, as is the case for pure water clusters in ACN [29], and the nucleotides become more exposed to the more weakly solvating ACN. Consequently, the equilibrium shifts towards the more hydrophilic layer surrounding the stationary phase particles (Fig. 6). Since the degree of hydration (water clustering) of dNTP/NTPs has been shown to depend upon the base (A, G, C, T) [31], the cluster size may also contribute regarding dNTP/NTP separation selectivity.

According to this model, cluster effects on retention should be less pronounced regarding polar compounds that form water clusters less readily, or form smaller water clusters. This should also be the case for compounds that dissolve well in ACN. Such compounds should have flatter or positive Van't Hoff curves compared to the strongly water clustering NTPs. This was shown to be the case in Fig. 5 with a diphosphate and a monophosphate; model calculations showed that NTPs would form a larger water cluster (results not shown). Positive curvatures in the van't Hoff curves were indeed observed with nucleobase uracil (U) (Fig. 5), for which model calculations showed that it attracts fewer water molecules than nucleotides. The results above show that retention, under the same conditions, can both increase and decrease with temperature, depending on the solute. We therefore hypothesize that with our very polar solutes, the temperature effect on retention is primarily related to level of solute hydration (water clustering) rather than the level of hydration of the *p*HIILIC stationary phase. If a solute/water cluster mechanism is indeed occurring, this should be perturbed in the presence of a hydrogen-bonding organic mobile phase component, as this will demote the clustering of solvent components and solutes. This was found to be the case, as the van't Hoff curve steepness of ATP was reduced by 50% when using isopropanol instead of ACN, and the curve was actually weakly positive when using ethanol (Fig. 7). No correlation was found between the steepness of ATP van't Hoff plots when varying the amount of ACN and ion strength simultaneously (see Fig. 8). Although the data shown here seem to support the cluster/temperature hypothesis, firm conclusions cannot yet be made. Additional experiments can be performed to further try the hypothesis, i.e. using ultra-high pressure instrumentation (not available in this study); Fallas et al. observed that with HILIC, retention factor *k* decreases with pressure at constant temperature [32]. They hypothesize that solutes are

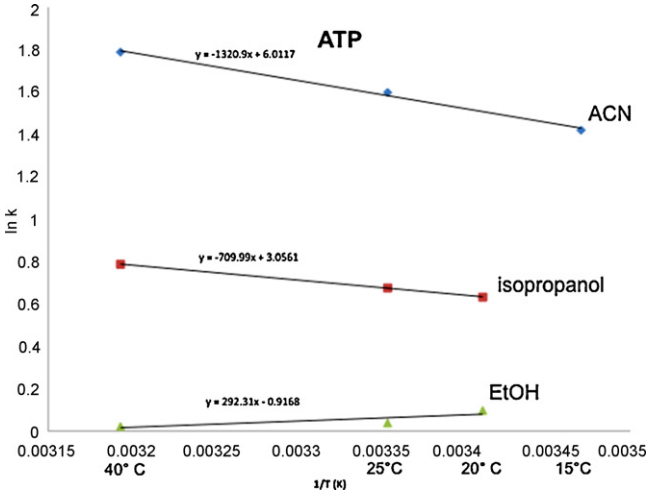


**Fig. 7.** Van't Hoff plot of ATP chromatographed on two ZIC-*p*HILIC columns with ACN, isopropanol or ethanol (EtOH) as the organic solvent. With ACN the temperature was set to 15, 25 and 40 °C. (With isopropanol and EtOH it was not possible to do the analysis at 15 °C due to high backpressure, hence 20 °C was used instead). For other conditions see Fig. 2.

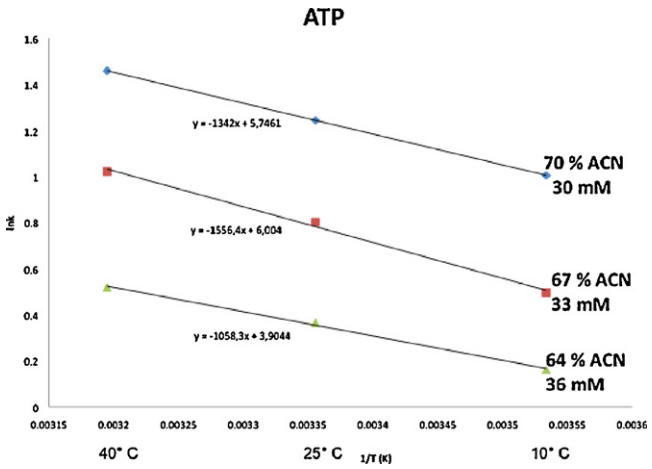


**Fig. 8.** Van't Hoff plot of ATP chromatographed on two ZIC-pHILIC columns at 10, 25 and 40 °C. The ACN content was 64%, 67% or 70%, and the total salt concentration was 36, 33 and 30 mM, respectively. For other conditions, see Fig. 2.

more hydrated (i.e. increased molecular volume) in the stationary phase water layer than in the mobile phase, shifting the equilibrium towards the mobile phase when operating at ultra-high pressures. The increase in a nucleotide/water cluster retention factor with pressure should be more prominent at elevated temperatures, as the molecular volume of the cluster will be smaller.

### 3.5. NTPs and dNTPs in cell culture

The flow rate was set to 100 μL/min when separating dNTP/NTPs in cell culture samples, in order to separate the analytes from other closely eluting compounds (Fig. 9), and to ensure complete separation of dTTP from ATP, which was significantly higher in concentration. The temperature was set to 15 °C, as this temperature arguably gives the best overall separation (Fig. 3).

### 3.6. Comparison with other methods

Using ZIC-*p*HILIC columns, the 8 dNTP/NTP standards could be separated in 35 min. In complex cell culture samples the dNTP/NTPs could be selectively separated (also from other matrix compounds) in 70 min.

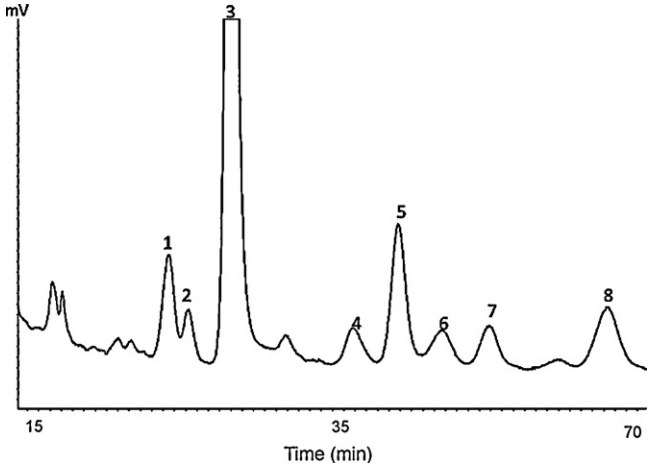


**Fig. 9.** Separation of the eight nucleotides in an *Escherichia coli* cell sample (wild type strain SMG3) on two ZIC-pHILIC columns at 15 °C at 100 μL/min. Chromatogram recording began 15.0 min after injection. For other conditions see Fig. 2.

Decosterd et al. gradient separated all 8 nucleotides in 95 min, with a total analysis cycle of 160 min using an ion-pairing method [6]. Huang et al. separated all 8 nucleotides in less than 55 min, but with what appears to be a large variation in retention time [33].

A highly accurate gradient LC–MS method developed for dNTP/NTPs takes 50 min using a PGC column (total cycle time 68 min), but required a mass spectrometer (MS) to distinguish all the compounds [8]. Several published methods are unable to separate ATP and dGTP [7,34,35], and in a previous paper, the very large ATP co-eluted with dTTP in cell samples [36]. Using ZIC-*p*HILIC columns, ATP is well resolved from neighboring dNTP/NTPs, facilitating arguably easier determination.

## 4. Conclusions

Two 15 cm ZIC-*p*HILIC columns coupled in series isocratically separated all 8 dNTP/NTPs in 35 min (standards), while an analysis time of 70 min was needed for their determination in a cell extract. ZIC-*p*HILIC columns provided better separations than silica-based ZIC-HILIC columns, confirming the differences in performance and selectivity of various HILIC columns. Best separation was achieved in isocratic mode, coupling two identical ZIC-*p*HILIC columns in series. Nucleotide separation can be fine-tuned using temperature as a variable, and interestingly, the retention times of the dNTP/NTPs increased with temperature. We have proposed an explanation based on dissociation of analyte/water clusters with elevated temperature.

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