# Stationary Phase Selection for Nucleotides and Nucleosides Using High Performance Liquid Chromatography

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# Abstract

Mixtures of nucleotides, nucleosides and their respective purine or pyrimidine bases are difficult to analyze by reversed phase high performance liquid chromatography (RP HPLC). These compounds cover a wide range of polarities and functionalities, from the acidic nucleotides to the basic purines and pyrimidines, making it very difficult to retain and resolve all of them with conventional alkyl stationary phases. Traditional HPLC analyses of these compounds often use a combination of reversed phase- ion pairing (RP-IP) and/or ion exchange (IEX) mode.

# Abstract (cont.)

Nucleotides are often analyzed by anion exchange while nucleosides are sometimes analyzed by cation exchange modes. These methods are not compatible with all the solutes in these mixtures and lack ruggedness.

This study is important in demonstrating that all three classes of compounds (nucleotides, nucleosides, bases) can be analyzed by RP-HPLC using one column and the same simple isocratic mobile phase. This provides greater convenience, reproducibilty and ruggedness in developing methods for these mixtures.

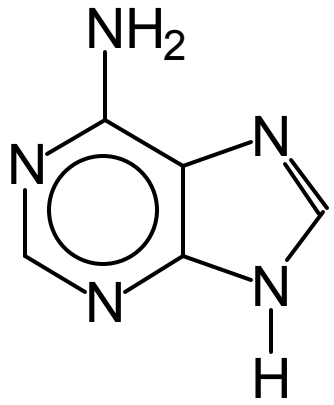
# Abstract (cont.)

By using a unique, intrinsically base deactivated stationary phase (Ultra IBD), simple RP-HPLC conditions were identified which resolve any common purine or pyrimidine base from its related ribonucleoside and mono-, di- and triphosphate nucleotides.

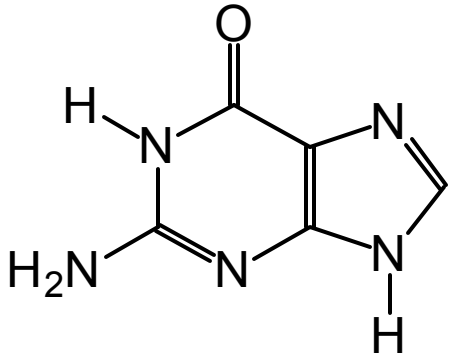
# Introduction

A nucleoside consists of a nitrogenous base linked to a pentose (sugar). A nucleotide is composed of a nucleoside plus one or more phosphate groups. The nitrogenous bases of nucleosides and nucleotides are either purines or pyrimidines. Figure 1 shows the structures of the major purines (adenine, guanine) and pyrimidines (cytosine, uracil, thymine). Figure 2 shows the structures of four common ribonucleosides in which a base is linked to ribose. Figure 3 shows the structures of three important nucleotides, ATP, ADP, and AMP, which are composed of the nucleoside adenosine plus three, two and one phosphate group(s), respectively.

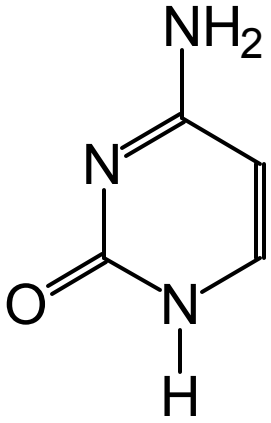
Figure 1. Purine and Pyrimidine Bases



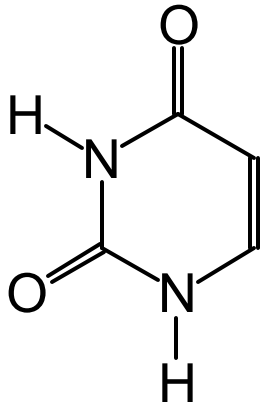

Adenine



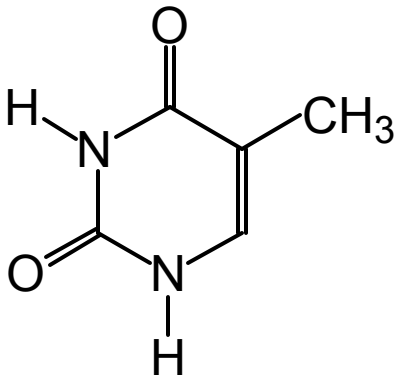

Guanine




Cytosine

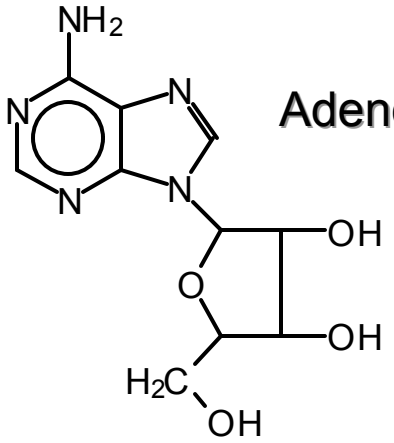



Uracil

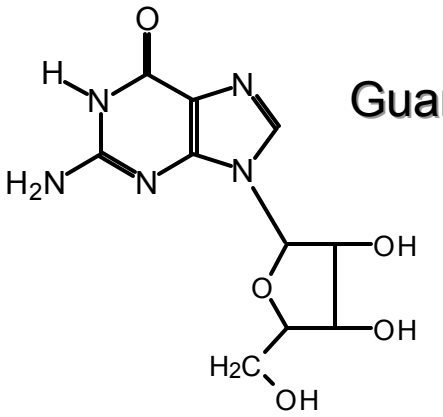



Thymine

Figure 2. Nucleosides

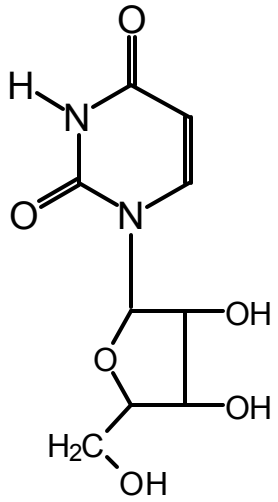



Adenosine




Guanosine

Uridine




Cytidine

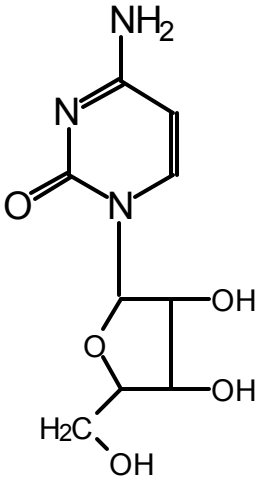
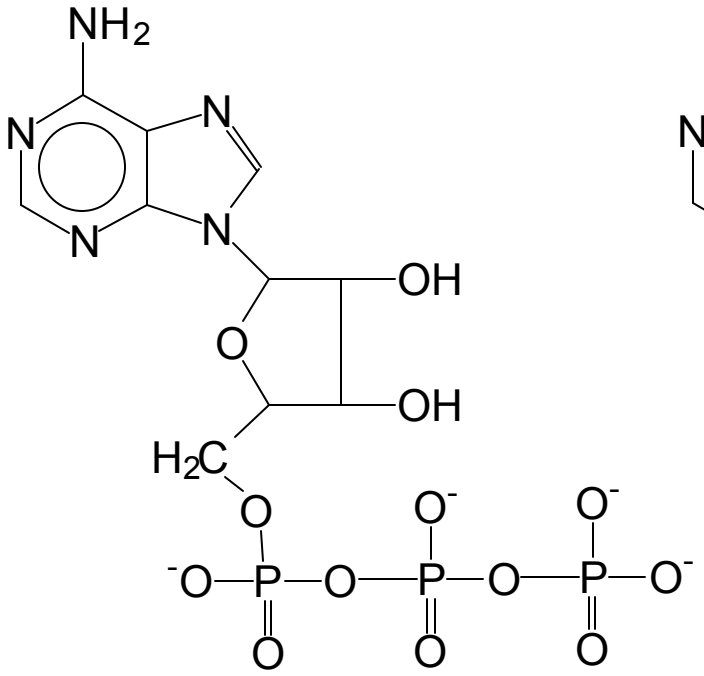
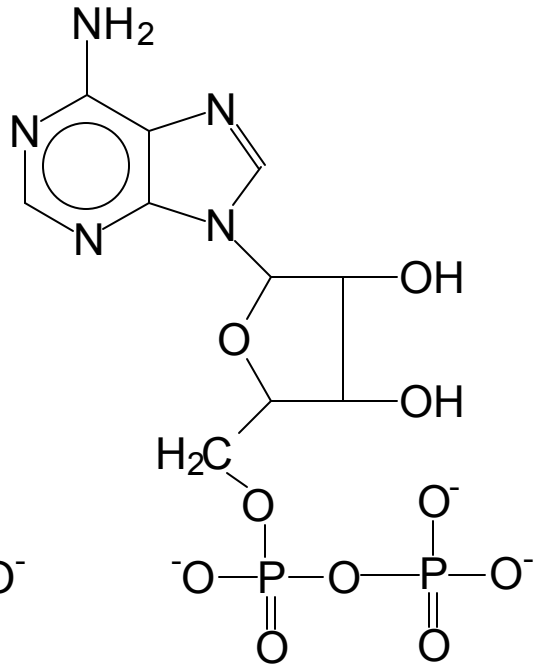


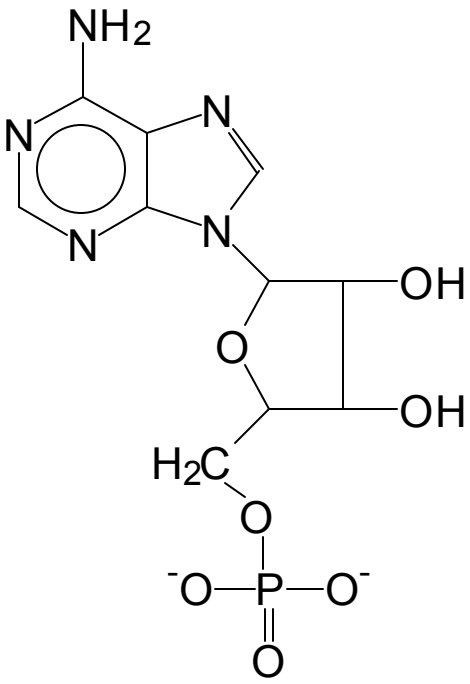
Figure 3. Nucleotides




5'-ATP




5'-ADP




5'-AMP

# Introduction (cont.)

The Ultra IBD (intrinsically base-deactivated) column is particularly effective for retaining and resolving complex mixtures of nucleotides, nucleosides, and purine and pyrimidine bases. The unique stationary phase of the Ultra IBD is composed of a polar group within, or intrinsic to, an alkyl chain. The polar group gives extra retention for many polar analytes as well as unique selectivity, a very high level of base deactivation, and compatibility with highly aqueous mobile phases. The Ultra IBD is ideal for LC/MS because it can often resolve acidic, basic, zwitterionic and/or neutral compounds in a single analysis using simple mobile phases.

# HPLC Conditions

- Column: Ultra IBD, 150x4.6mm, 5um (Restek Corporation)
- Instrument: HP 1100
- Mobile Phase: 97.5:2.5 (v/v) 20mM Ammonium acetate, pH 5.8: MeOH
- Flow Rate: 1.0 mL/min
- Detection: UV 260nm
- Temperature: 35°C
- Injection Volume: 20µl

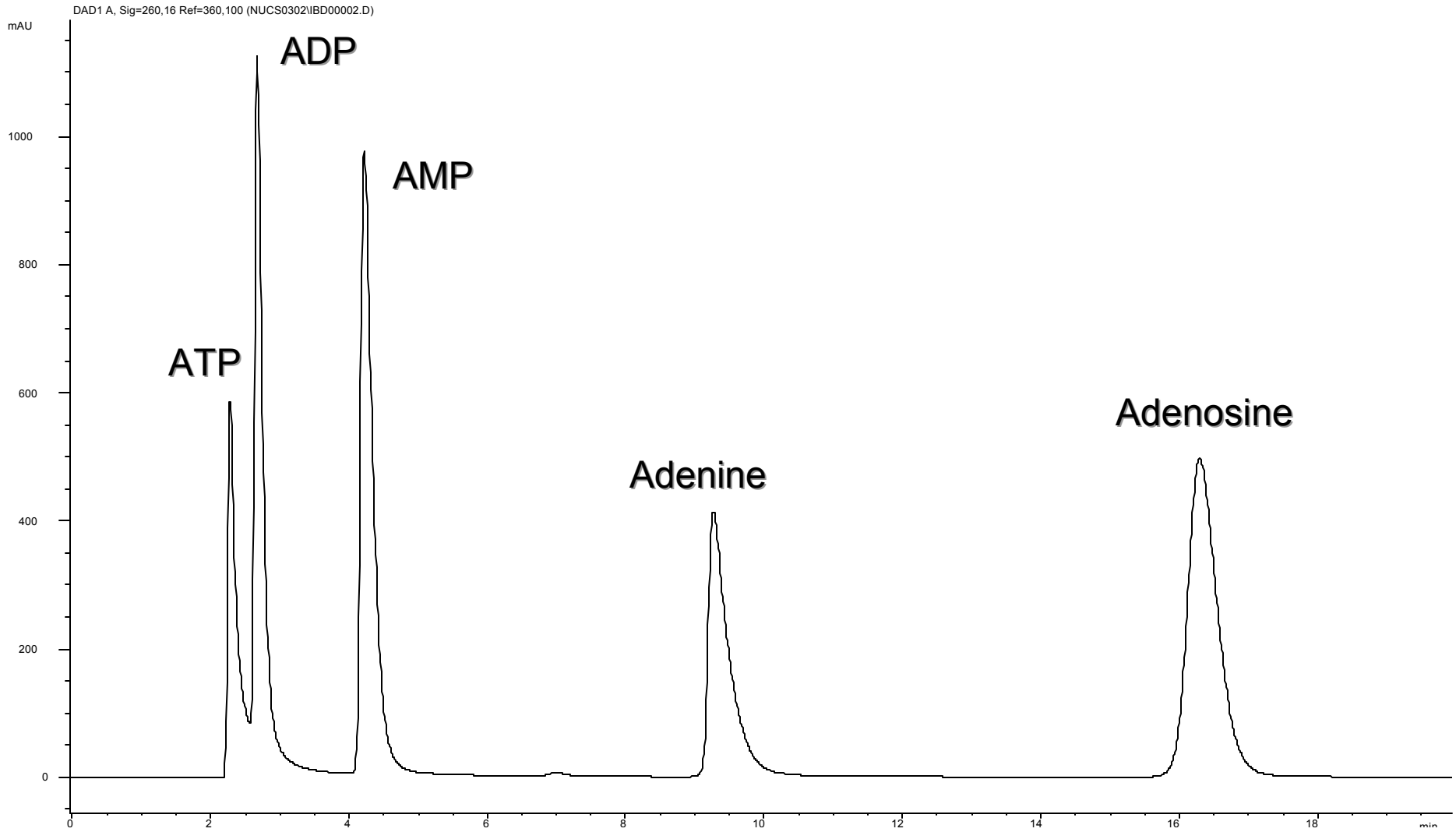
# HPLC Conditions (cont.)

Samples: Mixtures of standards dissolved in 20mM Ammonium acetate, pH 5.8. Concentrations of individual components were approximately 80-360 µg/mL for the five component mixtures and approximately 40-160 µg/mL for the 11 component mixture.
All nucleotides had the phosphates linked to the C5 hydroxyl of ribose (ie. 5'-ATP).

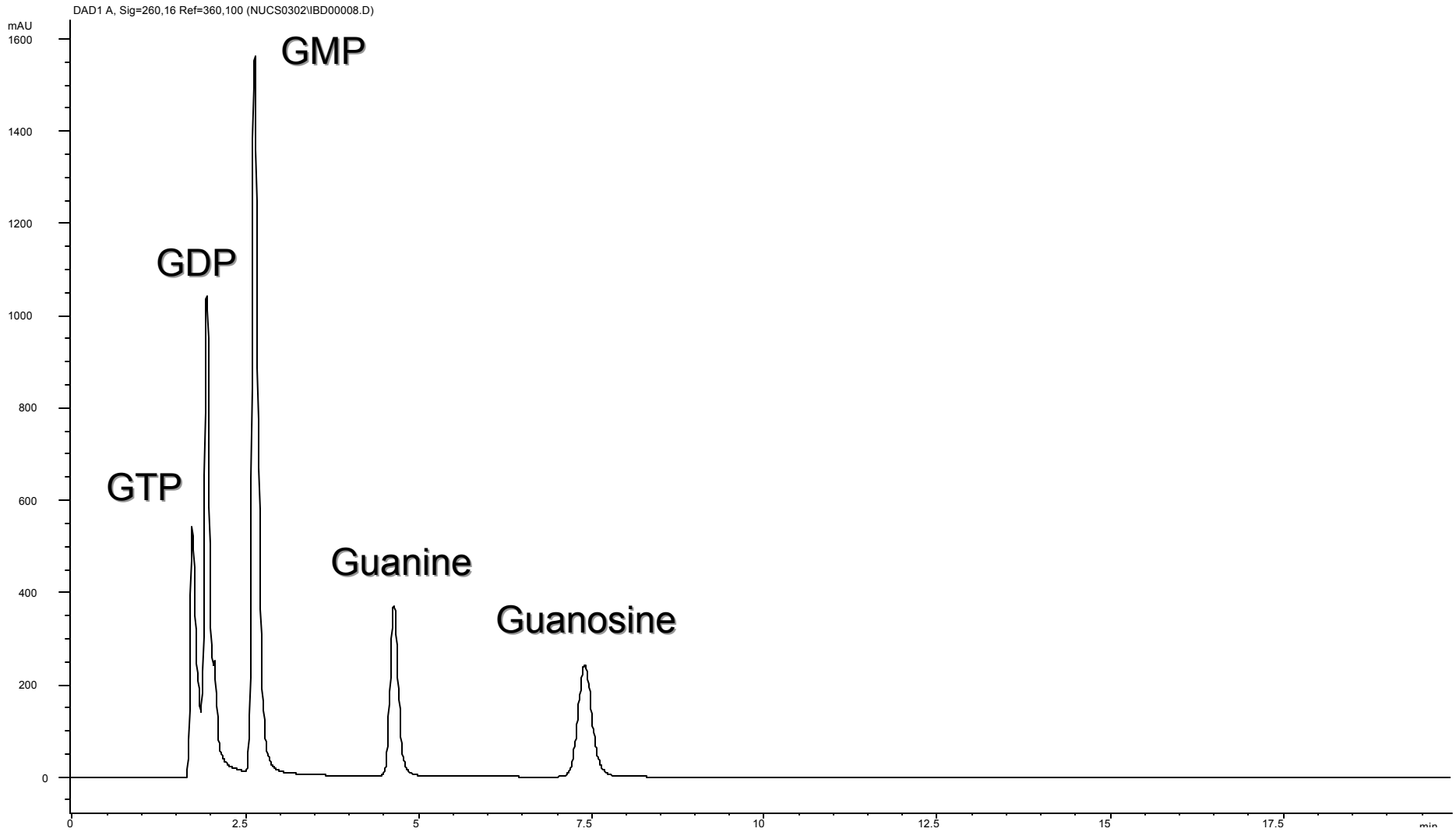
# Results

Figures 4 through 8 each show a separation of one of the major purines or pyrimidines from its respective ribonucleoside and mono-, di-, and triphosphate nucleotide. Note that each of these separations was achieved using the same conditions and that in each case the order of elution is triphosphate, diphosphate, then monophosphate nucleotide, followed by the base, and lastly the nucleoside. There are slight "shoulders" on the peaks for GDP (Figure 5), TTP (Figure 7), and UMP (Figure 8). These were present in the GDP, TTP, and UMP standards, respectively and were presumed to be impurities or degradation products.

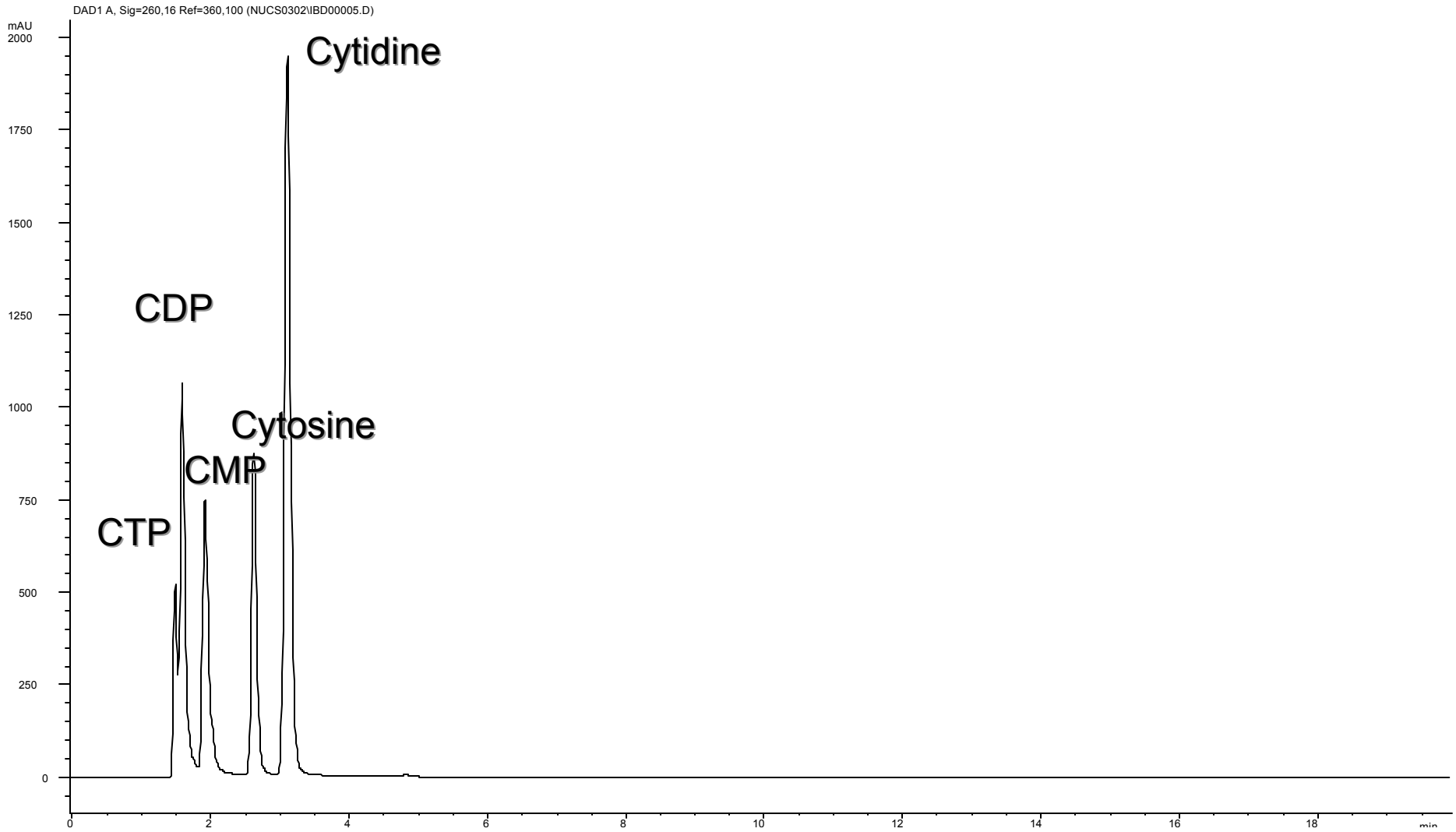
# Figure 4. Adenine Family



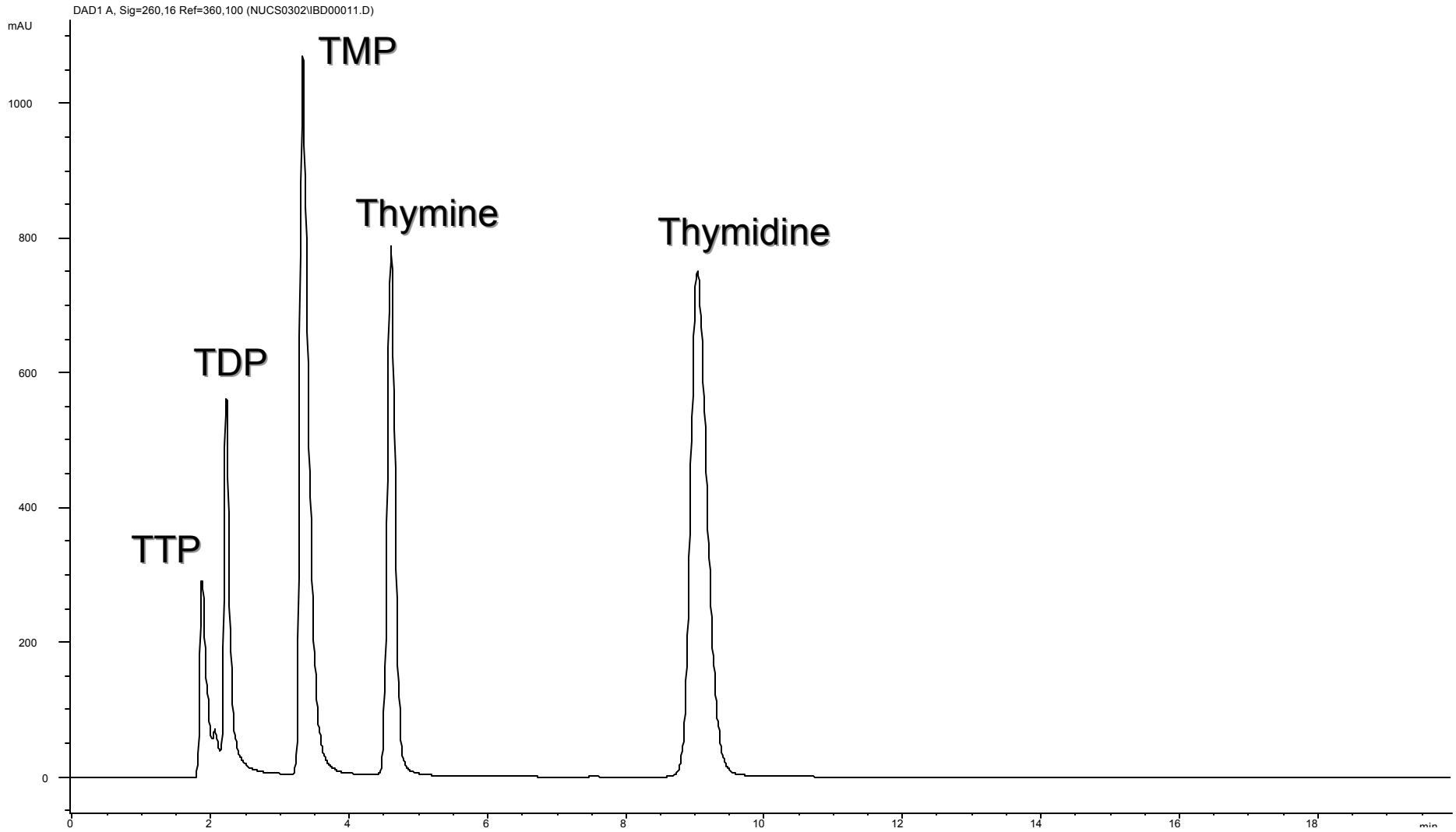
# Figure 5. Guanine Family



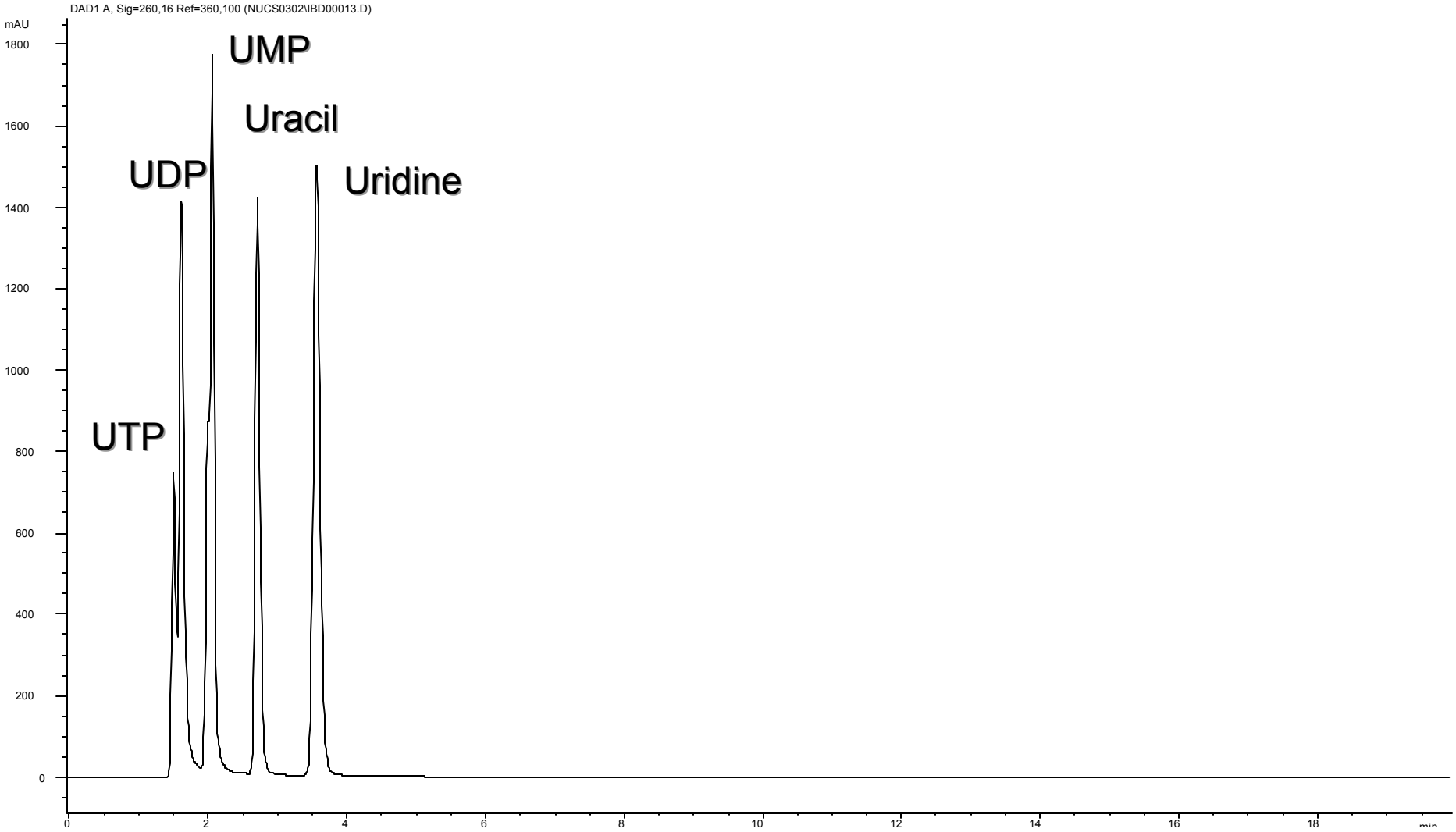
# Figure 6. Cytosine Family



# Figure 7. Thymine Family



# Figure 8. Uracil Family



# Results (cont.)

Table 1 lists the typical retention times obtained for all 25 of the compounds separated in Figures 4 – 8. While not all 25 compounds can be resolved in a single run, it would be possible to analyze all of these compounds using these chromatographic conditions with MS or MS/MS detection. Note that the mobile phase is compatible with MS detection, as all of its components are volatile. Figure 9 shows the resolution of a mixture of 11 various nucleotides, nucleosides, and bases.

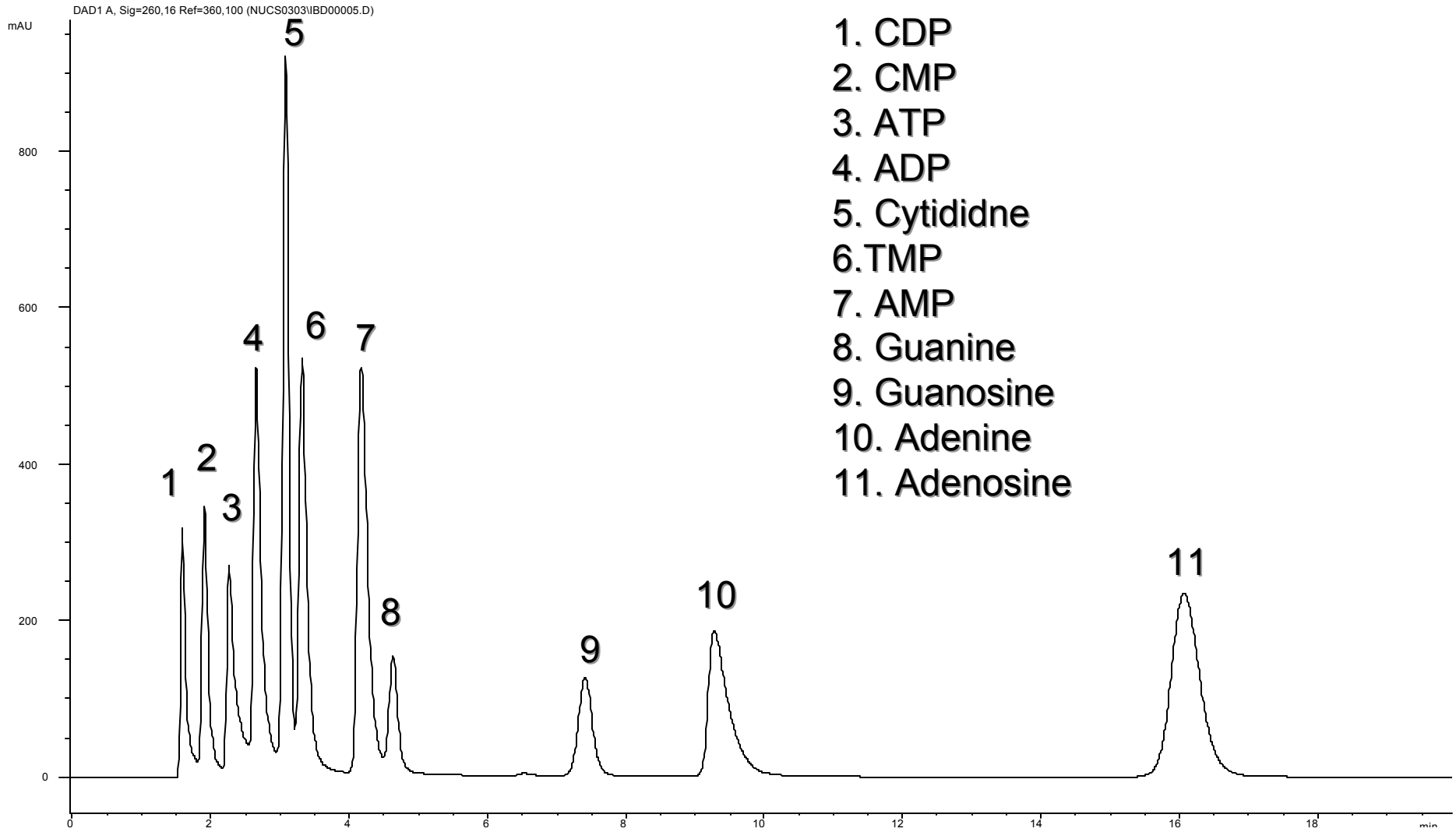
Table 1. Typical Retention Times For Common Nucleotides, Nucleosides, and Purine and Pyrimidine Bases.

| Compound | Ret.(min) | Compound | Ret.(min) |
|---|---|---|---|
| CTP | 1.5 | TDP | 2.2 |
| UTP | 1.5 | ATP | 2.3 |
| CDP | 1.6 | GMP | 2.6 |
| UDP | 1.6 | Cytosine | 2.6 |
| GTP | 1.7 | ADP | 2.7 |
| TTP | 1.8 | Uracil | 2.7 |
| CMP | 1.9 | Cytidine | 3.1 |
| GDP | 1.9 | TMP | 3.3 |
| UMP | 2.0 | Uridine | 3.6 |

Table 1. Typical Retention Times For Common Nucleotides, Nucleosides, and Purine and Pyrimidine Bases (Cont.).

| Compound | Ret.(min) |
|---|---|
| AMP | 4.2 |
| Guanine | 4.6 |
| Thymine | 4.6 |
| Guanosine | 7.4 |
| Thymidine | 9.0 |
| Adenine | 9.3 |
| Adenosine | 16.2 |

# Figure 9. Eleven Component Mixture of Nucleotides, Nucleosides, and Related Bases



# Conclusions

The unique stationary phase of the Ultra IBD column can retain and resolve mixtures of nucleotides, nucleosides, and purine and pyrimidine bases by RP HPLC, using isocratic elution with a simple, volatile mobile phase. A single set of chromatographic conditions was identified which can resolve any of the common purine or pyrimidine bases from its respective ribonucleoside and mono-, di-, and triphosphate nucleotides. Future work will focus on MS detection.