ADDITIONAL CHARACTERIZATION OF ADENOSINE NUCLEOSIDASE FROM ALASKA PEA SEEDS

by

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I dedicate this research to my husband, my dear parents and my family. I love you all.

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First thanks to God for allowing me to complete this project. I would like to express my deep sense of gratitude to my advisor, Dr. Paul C. Kline, for his guidance, support, and ongoing encouragement throughout this project. I also wish to thank my committee members, Dr. Donald A. Burden and Dr. Justin M. Miller, for their advice and insightful comments.

Also, I would like to thank my husband, and I want to say to him "I could not have reached my goals without your help and support at all times." Finally, I would like to express my thanks to my parents for their encouragement and love.

ABSTRACT

Adenosine nucleosidase was purified from Alaska pea seeds six days after germination. A 3-fold purification has been reached with a 0.56 % recovery. The purification scheme involved ammonium sulfate precipitation between 30% and 60% saturation, followed by ion exchange chromatography on a DEAE column. A final chromatography step used a hydroxyapatite column. The subunit molecular weight of adenosine nucleosidase was determined by SDS-PAGE to be 26.7 kD. The Michaelis constant, K_m, and the maximum velocity, V_{max}, for inosine were determined to be 377.7 ±141.7 µM and 0.00078 ± 0.000106 µM/min respectively.

The substrate specificity of the enzyme was investigated using 2'-deoxyinosine, allopurinol riboside, 3-deazauridine, and adenine- β-arabinofuranoside. Based on the observed substrate specificity, adenosine nucleosidase from Alaska pea seeds hydrolyzes both purines and pyrimidines. While these results place adenosine nucleosidase in the non-specific inosine-uridine nucleoside hydrolases (IU-NH), significant differences in its substrate specificity compared to the lead enzyme of the class, (IU-NH) from *Crithidia fasciculata* were found.

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CHAPTER I

INTRODUCTION

Enzymes are biological molecules that act as catalysts for biochemical reactions (*1*). Enzymes achieve this function by providing a lower activation energy pathway for a reaction, and therefore, the reaction occurs at a faster rate *(1).* Another characteristic is enzymes do not alter the position of the equilibrium of the reaction, but instead speed up the rate of the reaction *(2).* Enzymes are proteins which are building blocks for all living organisms. Cells contain numerous proteins, and their functions determine the types of reactions or activities that are carried out within the cell. The binding of a substrate or starting material to an enzyme is very specific. The sequence in which the amino acids in a protein are arranged in the polypeptide chain is primarily responsible for the specificity of the enzymes (*1*). The substrate binds to the active site, which is often grooves or channels found on the surface of protein. The enzyme's sequential arrangement of amino acids is responsible for the three-dimensional structure typical of all proteins *(2).*

Most enzymes are multi-subunit complexes consisting of individual chains of amino acids. Some proteins require a non-protein component known as a co-factor to facilitate their functions. These cofactors may be inorganic ions such as Zn^{2+}, Mg^{2+}, Mn^{2+}, or organic or metallorganic molecules commonly referred to as coenzymes *(1).* Coenzymes combine with the enzyme-substrate complex during reactions, albeit temporarily, and help speed up the reaction. There are six classes of enzymes, namely: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases *(2).*

Nucleotides are molecules that carry out a variety of functions, one of their principal functions being to give the main thermodynamic thrust for various chemical reactions. This is mainly done by adenosine triphosphate (ATP), but guanosine triphosphate (GTP) is used for some reactions, UTP is used in complex carbohydrate biosynthesis, and CTP is used in complex lipid synthesis (*3*). The terms ATP, GTP, CTP, and UTP refer to nucleoside triphosphates that contain ribose. Nucleoside triphosphates (NTP) are necessary for life, as they provide the building blocks of nucleic acids (DNA and RNA) and have many other roles in cell metabolism and regulation. NTPs provide energy and phosphate groups for phosphorylation (*3*). Nucleotides are composed of three major parts, namely a sugar containing five carbons, a nitrogenous base, and one or more phosphate groups (Figure 1). The sugar can either be ribose or deoxyribose. Nucleotides derive their names from the nitrogenous base they contain and the sugar attached to the base *(4*). A nucleoside is the part of the nucleotide that consists of the sugar and the base (*4*).

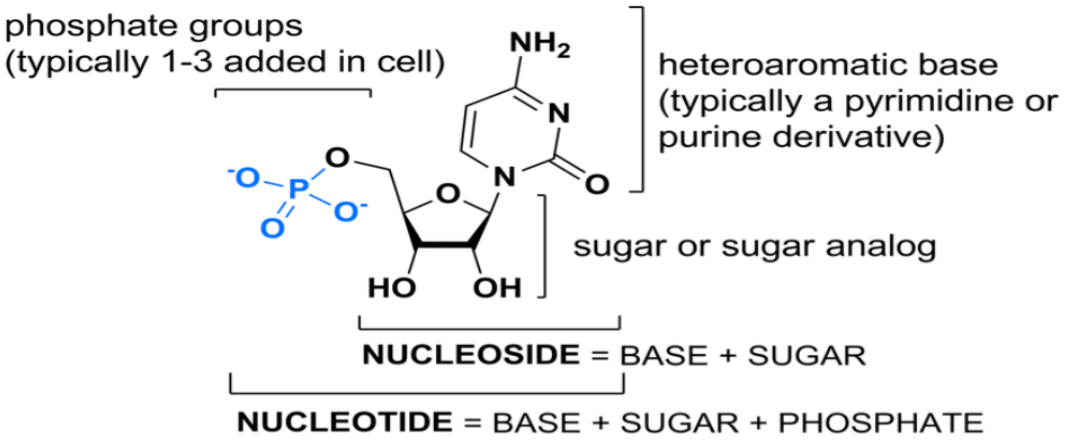


Figure 1. Structure of a nucleotide and a nucleoside (4)

Nucleotide metabolism is a feature in the cells of all living organisms. In plants, nucleotides can be synthesized from 5-phosphoribosyl-1-pyrophosphate (PRPP) and simple molecules such as carbon dioxide, amino acids, and tetrahydrofolate *(5)*. Nucleotides can also be synthesized from preformed nucleosides and nucleobases through salvage reactions. Nucleotides can be metabolized into simpler compounds. This process allows the recycling of phosphates, carbon, and nitrogen into key metabolic pools *(5)*.

Free nucleotides play an integral part in metabolism and cell signaling. These structures carry metabolic energy and other crucial high-energy electrons. As shall be discussed later, there are five major nitrogenous bases. Examples of nucleotides include adenosine triphosphate (ATP), flavin adenine dinucleotide (FAD), and nicotinamide adenine dinucleotide (NAD), which perform vital functions in a cell. Nucleotides are also metabolic regulators. For example, cyclic-adenosine monophosphate (cAMP) controls hormonal actions (*6*).

Purines and Pyrimidines

Purines and pyrimidines are two kinds of nitrogen-containing bases found in living organisms. Purines are aromatic compounds composed of a six-membered nitrogen-containing ring fused to a five-membered nitrogen-containing ring *(7)*. Pyrimidines consist of a single six-membered nitrogen-containing ring. The major nitrogenous bases are adenine, thymine, cytosine, guanine, hypoxanthine, or uracil. Purines and pyrimidines participate in many biochemical processes in plants. These two groups of aromatic compounds are precursors for the synthesis of primary products, such

as sucrose, polysaccharides, phospholipids, as well as some secondary products such as antibiotics and pigments (8). Nucleotides are building blocks for nucleic acids, and act as energy sources *(8).*

These functions demonstrate the integral role purines and pyrimidines play in the growth and development of plants. The common pyrimidine bases include cytosine, thymine, and uracil that correspond to the nucleosides cytidine, thymidine, and uridine *(9).* Cytosine has an exocyclic amino group at the C4 position, uracil has exocyclic carbonyl groups at the C4 and C2 positions, and thymine has an exocyclic methyl group at the C5 position and exocyclic carbonyl groups at its C4 and C2 positions (Figure 2). Purines include adenine, guanine, and hypoxanthine which are the nitrogenous bases for adenosine, guanosine, and inosine respectively. Guanine has an exocyclic amino group at the C2 position, while at the C6 position there is a carbonyl group. Adenine, on the other hand, has an exocyclic amino group at the C6 position of the purine ring. Hypoxanthine has an exocyclic carbonyl group at the C6 position of the purine ring. (Figure 2). DNA has rarely been found to contain hypoxanthine, while hypoxanthine/ inosine is an essential component of some RNA (*10*). During the synthesis of purines, inosine monophosphate (IMP) is a common intermediate in the synthesis of the other purines, AMP and GMP *(8).* Adenosine is a purine nucleoside in which adenine is attached to a ribose moiety by a β-N_9-glycosidic bond (Figure 3). Inosine is a purine nucleoside in which hypoxanthine is attached to a ribose ring by a β-N_9-glycosidic bond. Guanosine is a nucleoside in which guanine is attached to a ribose moiety by a β-N_9-glycosidic bond (Figure 3). Cytidine is a pyrimidine nucleoside in which cytosine is attached to a ribose

ring by a β-N_1-glycosidic bond. Uridine is a nucleoside in which uracil is attached to a ribose ring by a β-N_1-glycosidic bond. Thymidine is a nucleoside in which thymine is attached to 2$^{'}$-deoxyribose by a β-N_1-glycosidic bond (Figure 3).

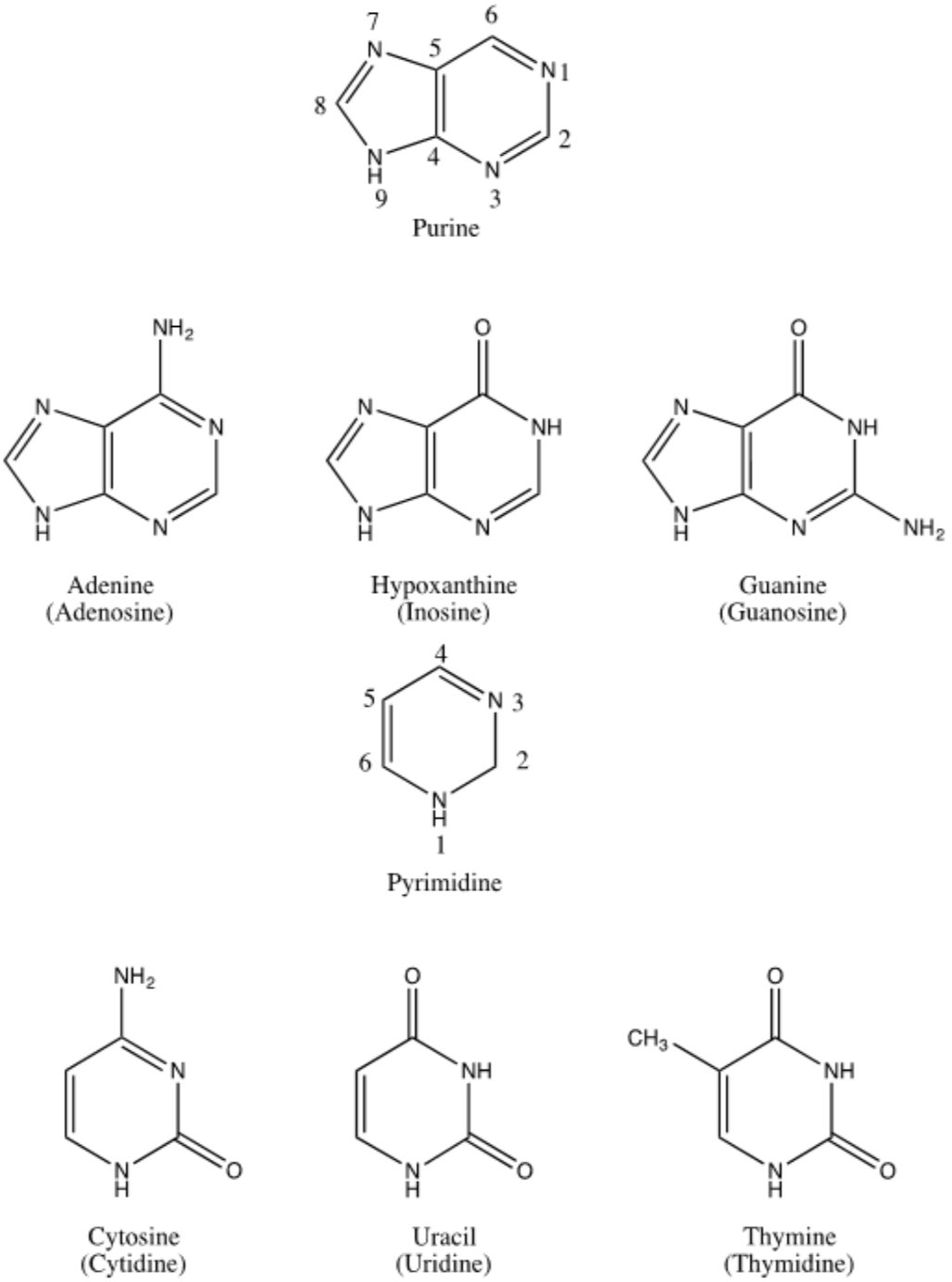


Figure 2. Purine and pyrimidine bases. The corresponding nucleoside name is in parentheses. The common numbering systems of purines and pyrimidine are also shown.

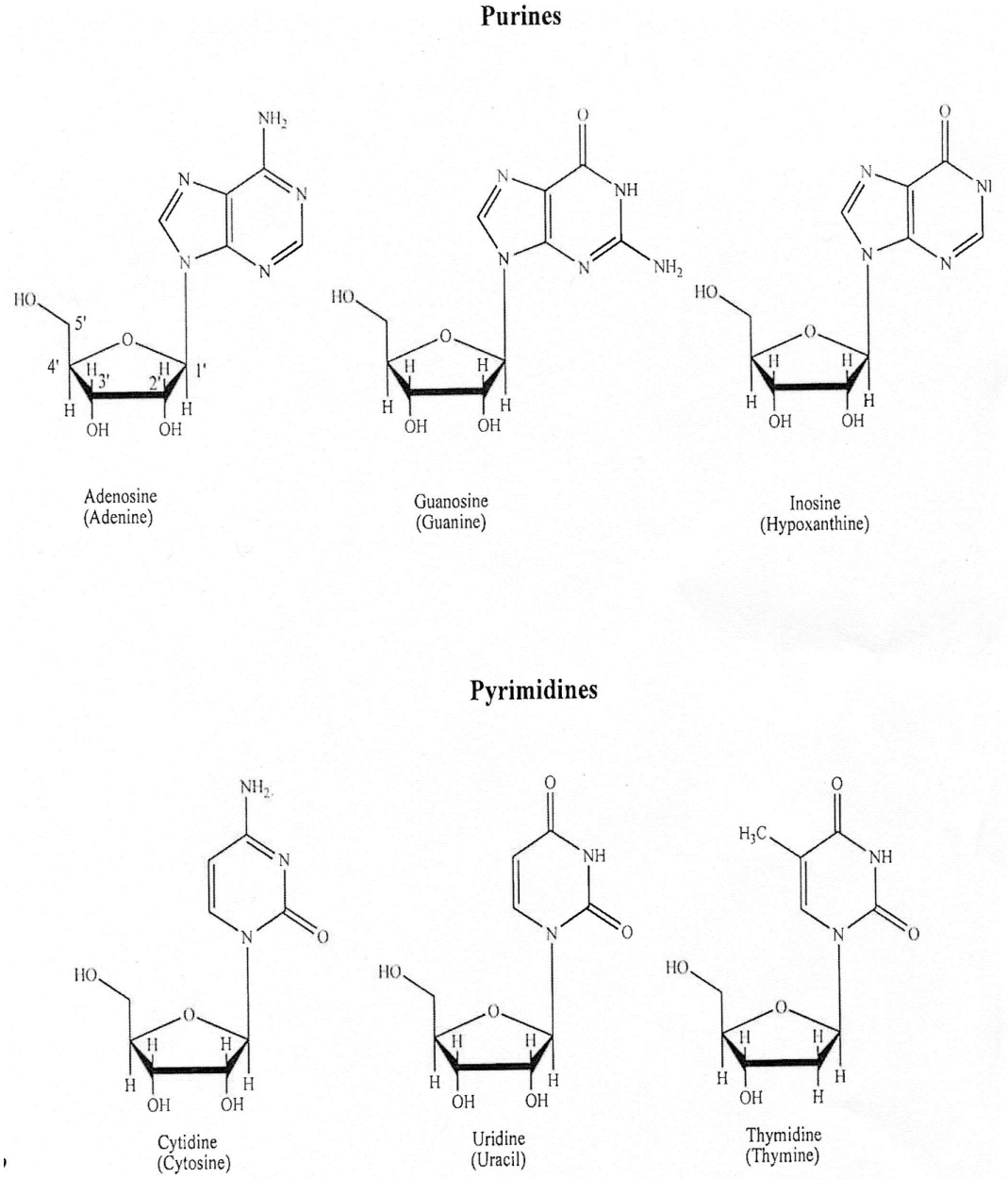


Figure 3. Common nucleosides.The corresponding base name is in parentheses. The numbering system for the sugar moiety is shown. Note the sugar of thymidine is 2'-deoxyribose, rather than ribose as seen in the other nucleosides.

Purine and Pyrimidine Metabolism

Nucleotides are crucial cellular components for plant growth, development, and metabolism. In a plant cell, the pathway for the synthesis of nucleotides is the same as those found in animals and microorganisms *(2).* Nucleotides are synthesized from amino acids and other small molecules via the *de novo* pathways or recovered by salvage pathways where free nucleobases and nucleosides are recovered and remade. Salvage pathways recycle nucleosides and free bases (Figure 4) *(9).*

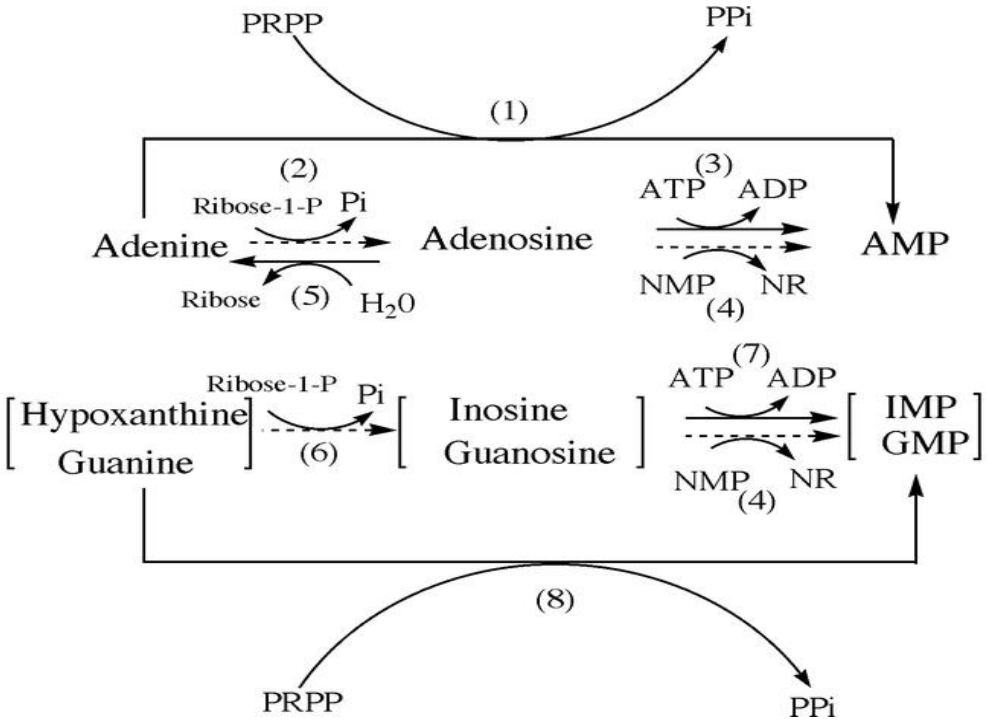


Figure 4. Salvage pathway for purine nucleotide (*9*).

The *de novo* pathway enzymes build purine nucleotides using small molecules including the amino acids glutamine, glycine, and aspartate, the activated ribose precursor 5-phosphoribosyl-1-pyrophosphate (PRPP) and bicarbonate. This is an energy intensive pathway in which 6 ATPs are required in the purine biosynthetic pathway from ribose-5-phosphate to the branch point involving IMP. However, seven high-energy phosphate bonds are consumed because α-PRPP formation in the first reaction followed by PPi release in the next reaction represents the loss of 2 ATP equivalents. The *de novo* purine nucleotide synthesis pathway has a higher requirement for energy than the salvage pathway (*5).*

The pyrimidine nucleotide *de novo* biosynthetic pathway, also referred to as the "orotate pathway," synthesizes UMP from carbamoyl phosphate (CP), aspartate, and PRPP (*5*). The orotate pathway consists of six enzymatic reactions not shown in (Figure 5). The initial reaction catalyzed by CP synthetase (CPSase) produces CP by a combination of carbonate, ATP, and an amino group from glutamine. To form the pyrimidine ring from CP, three additional reactions are necessary. The phosphoribosyl group of PRPP is added to the pyrimidine base orotate, creating orotidine 5'-monophosphate (OMP) which is decarboxylated to yield UMP, the first pyrimidine nucleotide. UMP is phosphorylated to UDP and UTP. In the synthesis of CTP, an amino group is transferred from glutamine to UTP by CTP synthetase *(5).* To produce dTMP, dUTP (formed from UMP) is first hydrolyzed to dUMP by deoxyuridine phosphatase. Finally, dTDP is converted to dTTP by a nucleoside diphosphate kinase (NDK) (*11*).

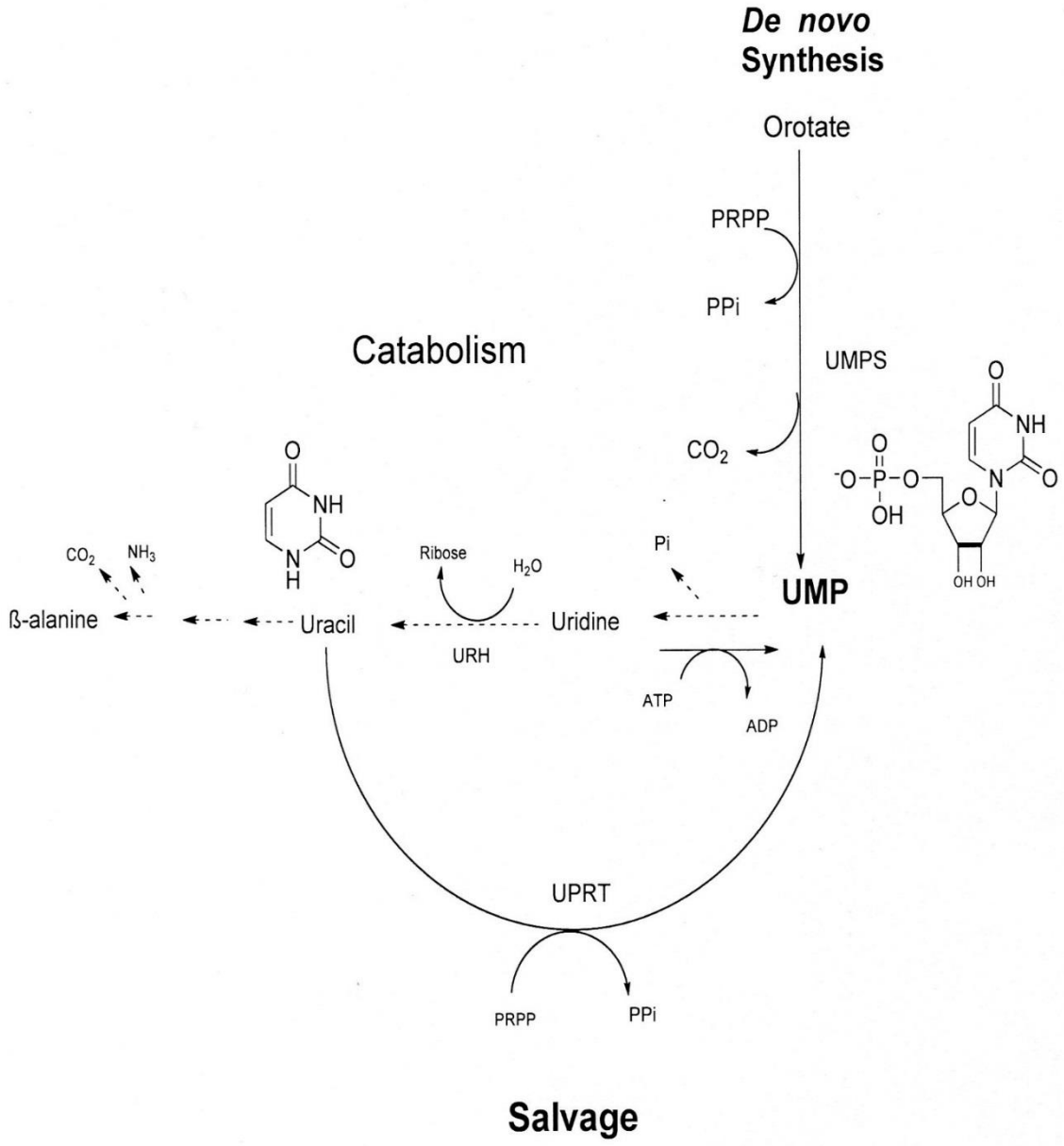


Figure 5. *De novo* UMP synthesis and catabolism of uridine. Orotate is converted to UMP by uridine monophosphate synthetase (UMPS), uridine is converted to uracil by uridine nucleosidase (URH). UMP and uracil are interconverted by uracil phosphoribosyltransferase (UPRT) (*12*).

UMP synthesis leads to a decreased *de novo* pyrimidine synthesis accompanied by a stimulation of the less energy consuming salvage pathway (Figure 5). The formation of carbamoyl phosphate is the rate controlling step in the pyrimidine synthesis pathway *(9*). Conversion of carbamoyl phosphate, catalyzed by aspartate transcarbamoylase, forms carbamoyl aspartate by reaction with aspartate. Formation of the pyrimidine ring is catalyzed by dihydroorotase with an addition of a hydrogen ion forming dihydroorotate *(5).*

An intermediate step in nucleoside and nucleobase salvage pathways is the hydrolysis of nucleosides to their nucleobases. This is solely accomplished by nucleosidases, also known as nucleoside hydrolases in a plant (*13*). As already discussed, nucleotides can be synthesized *de novo* from amino acids and small molecules. Salvage pathways can also recover and recycle nucleosides and free bases (Figures 4 and 5). The nucleotide *de novo* synthesis pathway consumes more energy. Thus, some cells employ a strategy to reuse preformed nucleosides and nucleobases through the pyrimidine salvage pathway. Uridine kinase, cytidine kinase, deoxycytidine kinase, and thymidine kinase catalyze the conversion of uridine, cytidine, deoxycytidine, and thymidine, into UMP, CMP, dCMP, and dTMP respectively *(14).* This method conserves energy, unlike the *de novo* process. The bases and nucleosides are transformed into nucleoside monophosphates with assistance from phosphoribosyltransferases and nucleoside kinases. These processes allow the cleaving of the nucleobases from the sugar, and recycling of the base as a nucleotide monophosphate *(14).*

Plants with reduced or enhanced nucleoside hydrolase expression exhibit slower germination or growth as compared to other plants, thereby indicating an interference

with normal nucleotide metabolism, which is usually required in large amounts during this period of growth (*15*). This enzyme is found in the cytoplasm of root vasculature, root meristems, mature pollen cells, and guard cells *(15).*

Cytokinins are chemical messengers specifically found in plants that participate in the regulation of cell cycles among other developmental processes (*16*). Cytokinin hormones were discovered in the 1950s with the first to be identified being kinetin (6-furfurylaminopurine), an adenine derivative (Figure 6) (*16*). Kinetin was initially misidentified as a product of the degradation of DNA. Due to their functions, cytokinins are present in all plant tissues although they are primarily concentrated in the root tips, the apex of the shoots, and seeds that are yet to mature (*17*). The hormones can act over long distances or near the cells that produced them in a process known as paracrine signaling (*16*). The action of these hormones can also be directed towards the cells that produced them, a process known as autocrine signaling. Cytokinins also regulate germination of seeds, expansion of cotyledons, chlorophyll differentiation, leaf senescence, and plant-pathogen interactions among other processes in the life cycle of the plant (*16*).

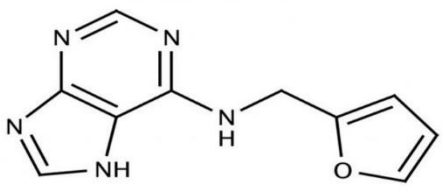


Figure 6. Structure of Kinetin (6-furfurylaminopurine) (*16*).

Cytokinins are primarily adenine derivatives having a side chain at the N6-position. The biological activity of the cytokinin can be affected by the structure and conformation of the attached N6 side chain (*18*). Structure and formation of the chain have led to classifications of cytokinins into aromatic and isoprenoid cytokinins (*18*). The latter are the most abundant type and are classified further as either zeatin-type cytokinins with a hydroxylated isopentenyl N6 side chain or isopentenyl (IP)-type containing an isopentenyl N6 side chain (*16*). The aromatic class of cytokinins has an aromatic benzyl group at N6. Besides these two groups, there are also synthetic cytokinins, which do not occur naturally but are quite active (*17*). Examples of synthetic cytokinins include diphenylurea and thidiazimin (Figure 7) (*19*).

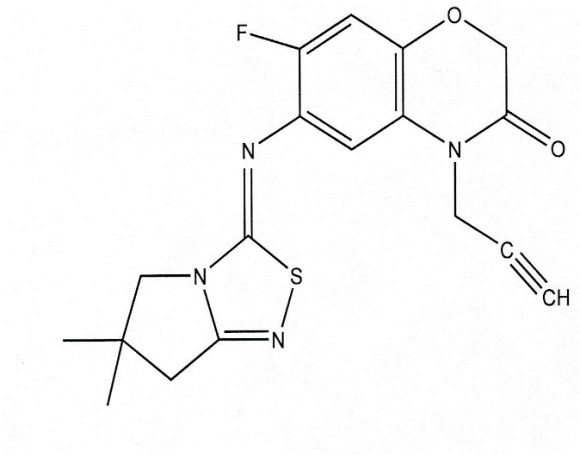


Figure 7. Structure of the synthetic cytokinin, thidiazimin (19).

In the biosynthesis and metabolism of cytokinins, transport processes, the rate of *de novo* synthesis, metabolic breakdown, and interconversion all affect the homeostasis of cytokinins (*17*). The metabolism of cytokinins primarily involves various conversions

among its bases, ribotides, ribosides, and modification of the side chain, cytokinin degradation, conjugation, and hydrolysis reactions of the conjugates (*16*).

Cytokinins have two types of biosynthetic pathways. The first one is the direct pathway that transfers the isopentenyl moiety from dimethylallyl pyrophosphate (DMAPP) to adenosine monophosphate (AMP), adenosine diphosphate (ADP) or adenosine triphosphate (ATP) (*17*). This reaction is facilitated by IPT (isopentenyl transferase). AMP, ADP, and ATP serve as substrates in the reaction and result in the formation of isopentenyl derivatives of AMP, ADP, and ATP,which are the initial active molecules of cytokinins (*17*). To form zeatin-type cytokinins, the isopentenyl side chain undergoes hydroxylation. The second pathway is the indirect pathway, in which cytokinins are released by turnover of tRNA containing cis-zeatin. tRNAs have been identified as a source of cytokinins because the tRNAs in most organisms have isopentenylated adenine among other structural derivatives (*17*). Aromatic cytokinins have an aromatic benzyl or hydroxybenzyl group at N^6 for example, N^6-benzyladenine (BA), *meta*-topolin (mT). Aromatic cytokinins exist as free bases and the corresponding ribosides and ribotides (*20*).

The interconversion of cytokinin bases, nucleosides and nucleotides is a major feature of cytokinin metabolism, and the enzymes that regulate the interconversion have been identified (*17).* The base in the cytokinin is the most active part of the hormone. Ribosides and ribotides are formed by respective attachment of ribose or ribose-5'-phosphate to the N9 atom found in the adenine ring (*17*). Interconversion is instrumental in the regulation of the concentration of active cytokinins in the cells (*18*). Enzymes

responsible for the metabolism of adenine, AMP, and adenosine may also influence the process.

Inactivation of cytokinins is also possible either permanently or transiently by glycosylating the side chain or purine ring (*16*). The N3, N7, and N9 positions of the purine ring can be glycosylated (*17*). The most commonly conjugated sugar molecule is glucose. Some transient forms of cytokinins include the N3 and O- conjugates that can be hydrolyzed despite being inactive biologically. That means N3 and O-conjugates become active again after hydrolysis. Conjugation by glycosylation helps to regulate activity levels of cytokinins in various tissues and species of plants (*18*).

An enzymatic oxidative cleavage of the N6-side chain has been determined to be a method of irreversibly degrading the cytokinins (*16*). This reaction is mediated by the oxidases or dehydrogenases of cytokinins that contain an FAD cofactor. The products of the reaction are an aldehyde and adenine, while the preferred substrates are isopentenyl adenine, zeatin, and their respective ribosides (*16*).

Cytokinins are transported to the shoots in the xylem from the roots and the back to the roots in the phloem (*16*). Such transported hormones play a significant role in coordinating development in the roots and shoots especially concerning the availability of nutrients. Studies conducted to investigate this transport have indicated that H+-coupled transport systems of high-purine affinity are responsible for transporting cytokinins (*16*). Signaling by cytokinins is perceived and transduced in a several step phosphorelay system through an elaborate two-component system (*16*). Two-component systems are a common method to transmit a signal. *Arabidopsis* uses a two-component signaling system to mediate the response to cytokinins (*16*).

Nucleoside Hydrolase from Plants

Nucleoside hydrolases are a group of enzymes that catalyze the hydrolysis of the N-glycosidic bond between the nitrogenous base in a nucleoside and the pentose sugar (*21*). They exist in living organisms such as bacteria *(22),* yeast *(23),* protozoa *(24),* insects *(25),* mesozoa *(26)* and plants *(13).* Genes that contain the specific NH fingerprint sequence DXDXXXDD are also present in plants, amphibians and fish *(21).* Mammals do not contain any nucleoside hydrolases *(21).* An example of a nucleoside hydrolase is adenosine nucleosidase, which catalyzes the hydrolysis of adenosine to adenine and ribose. These enzymes are characterized based on their substrate specificity *(27).* There are nonspecific hydrolases that catalyze the hydrolysis of both purines and pyrimidines. There are also enzymes specific for purine nucleosides. Another group of nucleoside hydrolases consists of six-oxo purine specific inosine-guanosine preferring hydrolases *(28).* Nucleoside hydrolases have previously been isolated and purified from plants such as coffee leaves, barley leaves, wheat germ, spinach beet, tea leaves, tomato roots and leaves (*29*). The nucleoside hydrolases obtained from previously have been shown to have a variety of molecular weights, pH optima, and subunit structures.

The structure of Inosine-Uridine nucleoside hydrolase (IU-NH) from *Crithidia fasciculata* (Cf NH) and the crystal structure of IU-NH from *Leishmania major* (LmNH) were solved by Schramm *et al*. using X-ray crystallography (*30, 31*). The enzyme was in complex with the inhibitor *para*-aminophenyliminoribitol. A crystal structure of Inosine-Adenosine-Guanosine nucleoside hydrolase (IAG-NH) from *Trypanosoma vivax* has also been solved. The enzyme was in complex with the inhibitor 3-deaza-adenosine (*32*).

IAG-NH from *Trypanosoma vivax* is a homodimer (Figure 8a) while IU-NH from both *Crithidia fasciculata* and *Leishmania major* are homotetramers (Figure 8b) (*21*). The monomeric subunits of both groups exhibit the same architecture, and have a single globular domain (Figure 8c, d). The IU-NH tetramers and the IAG-NH dimers are organized in various quaternary structures, which have different subunit-subunit interfaces. The α/β core of the NH monomer is made of eight-stranded mixed β sheets, where seven of the strands are parallel and one is anti-parallel (Figure 8e, f) (*21*). The first six β sheet stands resemble a dinucleotide-binding or Rossmann fold (*21*). At the C-terminal end of the inner sheet is located an active site. In Figure 8c, d loop I and loop II are centered on either side of the active site (*21*). When para-aminophenyliminoribitol (pAPIR) binds to the *Crithidia fasciculata* NH enzyme both loops change their conformation to move certain side-chains into the active site to restrict access of the solvent (*21*).

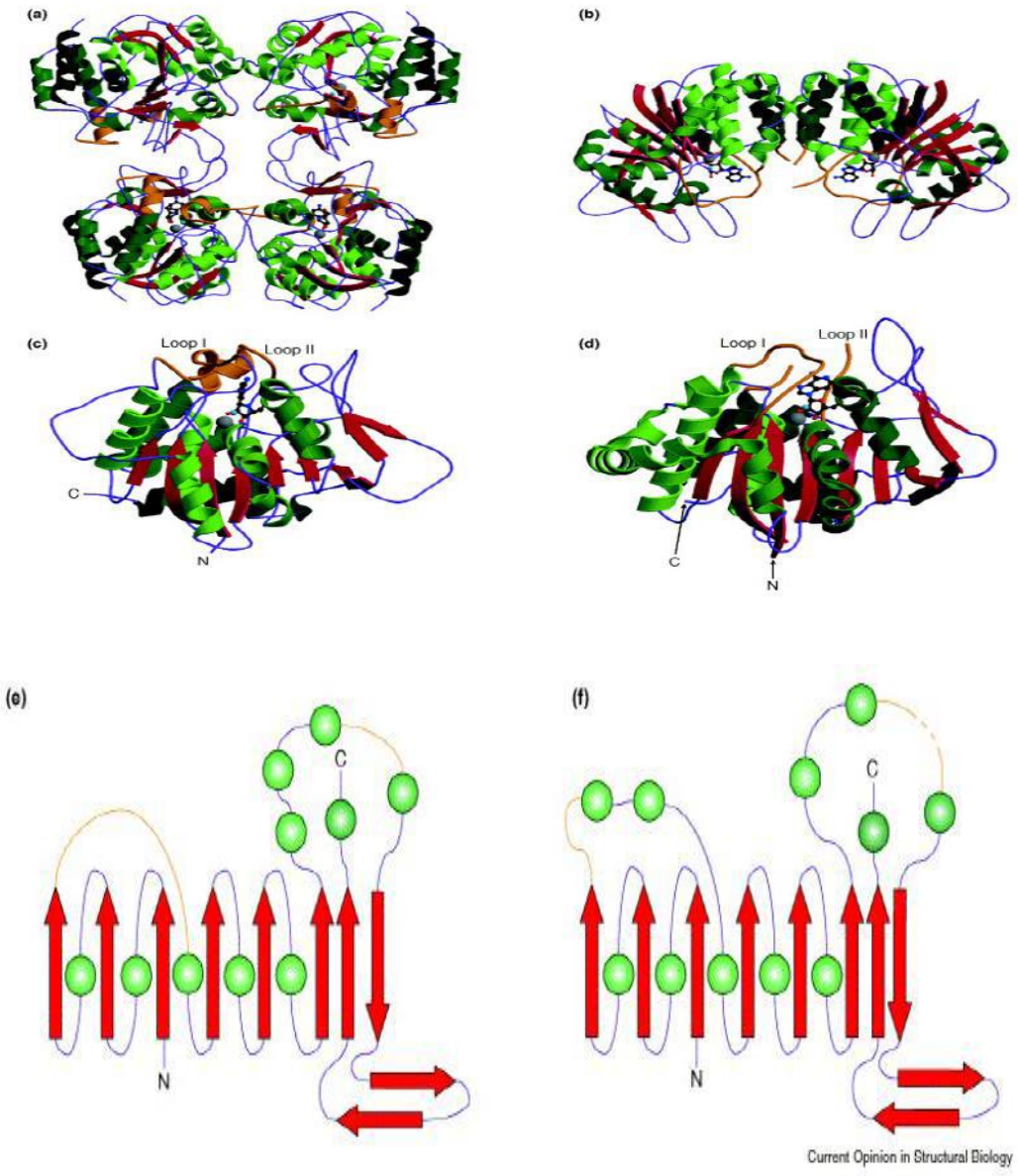


Figure 8. Nucleoside hydrolase from different structures containing a common fold. Quaternary nucleoside hydrolase structures, tertiary structures and topologies of IU-NH of *C .fasciculata* (a, c, e) IU-NH (Cf NH in complex with pAPIR; PDB code 2MAS) and (b, d, f) of IAG-NH of *T. vivax*. β PDB code 1HP0 strands are shown in red while α helices are in green. A gradient from light to dark green when going from the N towards C-terminus represent the sequence of different helices. Two recurring loops next to the active site are presented in yellow. The active sites of the enzymes are occupied by Ca^{+2} ion (gray sphere) and a nucleophilic water molecular (blue sphere). The ball and stick models represent the ligands. Reprinted from reference 21 with permission of Elsevier.

Adenosine Nucleosidase

Adenosine nucleosidase (EC 3.2.2.7) catalyzes the reaction between adenosine and water to yield ribose and adenine (Figure 9). This is an irreversible hydrolysis reaction (*27*).

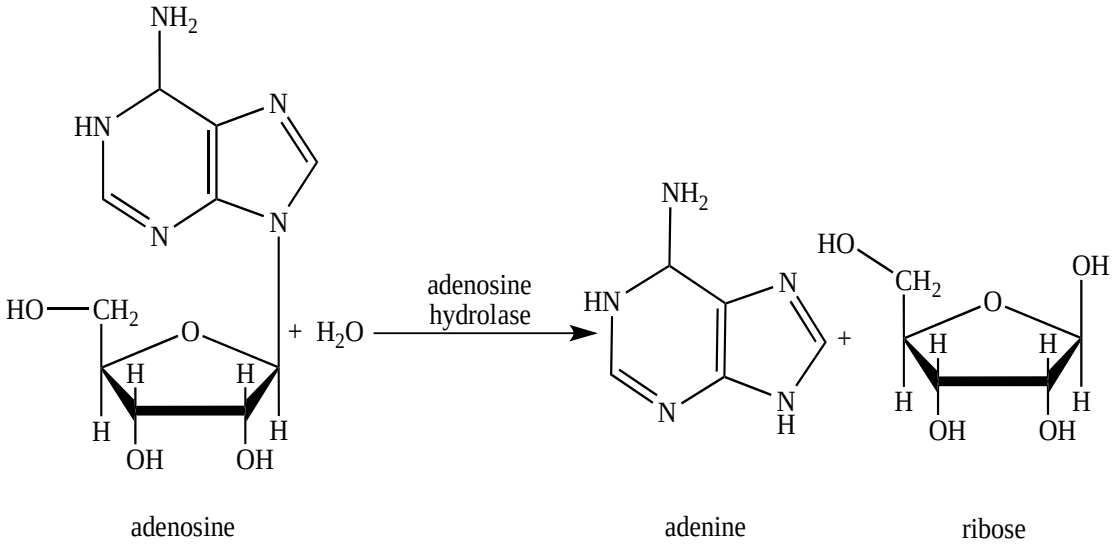


Figure 9. Hydrolysis of adenosine to ribose and adenine by adenosine nucleosidase (*27*).

Adenosine belongs to a subclass of nucleosidase hydrolases, the glycosylases, which hydrolyze N-glycosyl compounds. The enzyme isolated from tea leaves and spinach beet leaves has an optimum pH for hydrolysis of 4.5, and it is not inhibited or activated by the presence of phosphate ions as is the case with inosine nucleoside *(33).* It is an enzyme in the purine salvage pathway in plants. Research has suggested that adenosine nucleosidase controls the levels of cytokinins, which play a significant role in the growth of plants (*27*). However, cytokinins have not been shown to be substrates for adenosine nucleosidase, and thus, the enzyme indirectly influences the levels of

cytokinins by regulating the levels of adenine, which can be used to synthesize cytokinins *(28).* The protein was first purified from soybean leaves by Miller and Evans *(34)* and has been purified from other plant sources. These plant sources include barley leaves *(27),* wheat germ *(28),* yellow lupin seeds *(33),* spinach beet leaves *(35),* tea leaves *(36),* Jerusalem artichoke *(37),* and Alaska pea seeds (38).

Adenosine nucleosidase was purified from barley leaves and determined to have a molecular weight of 66,000 daltons by gel filtration chromatography *(27).* Further analysis by SDS-PAGE showed that the enzyme was a dimer with a subunit molecular weight of 33,000 daltons. The pH optima were 4.7 and 5.4 for citrate and (N-morpholino) ethane sulphonic acid buffers, respectively. The K_m for adenosine was found to range between 0.8 to 2.3 μM with the value dependent on temperature and buffer system. Substrates for the enzyme were determined, in addition to adenosine, to be adenosine N-oxide, 2'- deoxyadenosine and purine riboside. Adenine and cytokinins were found to be inhibitors of the enzyme *(27).*

Abusamhadneh *et al.* purified adenosine nucleosidase from yellow lupin seeds (*33*). The purification achieved a percent yield of 2.3%, and the enzyme's molecular weight was found to be 72,000 daltons by size exclusion chromatography *(33).* The enzyme was determined to have an optimum pH of 7.5 and the K_m value was 4.7 μM for adenosine. Significant activity was observed for adenosine, 2'-deoxyadenosine, guanosine, cytidine, and inosine, *(33).*

Adenosine nucleosidase partially purified from spinach beet leaves was found to have an optimum pH of 4.5, and the K_m value for adenosine was found to be 11 μM *(35).*

In the analysis, the enzyme demonstrated significant specificity for adenosine and did not use other nucleosides as substrates. Some activity was recorded with adenosine N-oxide, which is produced by oxidizing adenosine to form a nitrogen monoxide bond *(35).*

Purification of adenosine nucleosidase from tea leaves resulted in three types of the protein (I, II, and III) (*36*). Each protein was determined to have a molecular weight of approximately 68,000 daltons by gel filtration using Sephadex G-100. Enzymes I and II had a pH optimum of 4, while adenosine nucleosidase III had a pH optimum of 4.5. All three proteins exhibited similar substrate specificity. Naturally occurring nucleosides hydrolyzed by the three adenosine nucleosidases included adenosine and 2'-deoxyadenosine with a higher rate of hydrolysis being shown by 2'-deoxyadenosine *(36).*

Adenosine nucleosidase has also been purified from Jerusalem artichoke shoots *(37).* Besides adenosine, inosine and guanosine nucleosidase activities were also observed. The properties of the enzyme include high stability, optimal pH from 5 to 7 and high affinity for substrates. The substrate specificity differentiates the two enzymes (adenosine and inosine-guanosine nucleosidase). The addition of adenosine had no effect on the rate when either inosine or guanosine acted as a substrate. The hydrolysis rates of inosine and guanosine were inhibited by adding 6-mercaptopurine riboside. Adenosine had a K_m value of 17 μM, while guanosine and inosine had values of 8.5 μM and 2.5 μM respectively *(37).*

Shamsuddin isolated another form of adenosine nucleosidase from Alaska pea seeds *(38).* The enzyme was determined to belong to the non-specific inosine-uridine nucleoside hydrolases (IU-NH). The molecular weight was 26,000 daltons by SDS-

PAGE and 26,103 daltons by mass spectrometry. A yield of 1.3 % with 4-fold purification was accomplished. The K_m value using adenosine as the substrate was 137 ± 48 μM, and V_{max} was 0.34 ± 0.02 μM/min. Table 1 are the nucleosides tested for activity *(38).*

Table 1. Substrate specificity of adenosine nucleosidase from Alaska pea seeds. "Yes" represents were hydrolyzed as determined by HPLC and were classified as a substrate *(38).*

Nucleosides	Substrate	Nucleosides	Substrate
Adenosine	Yes	2'-deoxyadenosine	Yes
Guanosine	No	3'-deoxyadenosine	Yes
Inosine	Yes	5'-deoxyadenosine	Yes
Uridine	Yes	7'-Deazaadenosine (Tubercidin)	No
Cytidine	Yes	5-Methyluridine	Yes
Purine riboside	No		
Thymidine	Yes		

Adenosine nucleosidase has been purified from tomato leaves and roots (*39*). In tomato roots, R1 and R2 adenosine nucleosidases were found. Lf, another form of adenosine nucleosidase was found in plant leaves. The optimum pHs were found to be

5.0, 6.0, and 6.0, for R1, R2 and Lf respectively. Using adenosine as the substrate, the K_m values were determined to be 25 μM, 9 μM, and 6 μM for R1, R2, and Lf. R2 was determined to be the primary component in the root, and its molecular weight was determined to be 68,000 daltons. Significant similarities were observed between R2 and Lf. Cytokinin ribosides were found to be competitive inhibitors of all the three enzymes *(39).*

Calcium-stimulated guanosine-inosine nucleosidase from yellow lupin (*Lupinus luteus*) has been isolated *(40).* The enzyme functions as a monomeric 80 kDa polypeptide most effectively between pH 4.7 and 5.5. Of various mono- and divalent cations tested, Ca^{2+} appeared to stimulate enzyme activity (*40*). The nucleosidase was activated 6-fold by 2 mM exogenous $CaCl_2$ or Ca $(NO_3)_2$ with K_a = 0.5 mM (estimated for $CaCl_2$). The K_m values were 8.5 μM for guanosine and 2.5 μM for inosine. Guanosine was hydrolyzed 12% faster than inosine while adenosine and xanthosine were poor substrates. 2′-Deoxyguanosine, 2′-deoxyinosine, 2′-methylguanosine, pyrimidine nucleosides and 5′-GMP were not hydrolyzed *(40).*

Inosine nucleosidase has been purified from yellow lupin (*Lupinus Luteus* L.) seeds in which the enzyme hydrolyzed inosine to hypoxanthine and ribose *(41).* The extraction was carried out using ammonium sulfate fractionation, and chromatography on aminohexyl-Sepharose, Sephadex G-100, and hydroxyapatite. By gel filtration, the molecular weight of the enzyme was 62,000 dalton *(41).* The enzyme was determined to have an optimum activity around pH 8, and the K_m value for inosine is 65 μM.

Nucleosides that were substrates for the enzyme are xanthosine, purine riboside (nebularine), 6-mercaptopurine riboside, 8-azainosine, adenosine, and guanosine *(41).*

CHAPTER II

MATERIALS AND METHODS

Equipment and Instrumentation

High-performance liquid chromatography (HPLC) was performed on a Dionex UltiMate 3000 Standard LC System and the modules that make up the system include, LPG-3400SD quaternary analytical pump, WPS-3000TSL analytical auto sampler, TCC-3000SD column oven and VWD-3100 variable wavelength UV detector. UV-Vis spectroscopy was carried out on a Hitachi U-2900 spectrophotometer. Protein purification was carried out on an AKTA Fast Protein Liquid chromatography (FPLC) system from GE Healthcare.

Materials and Reagents

Alaska (Wilt Resistant) pea seeds were obtained from Ferry-Morse Garden Seed Company. Protamine sulfate salt, DL-dithiothreitol (DTT), protease inhibitor cocktail for plant cells, DEAE Sepharose™ Fast Flow column, and various nucleosides and bases were purchased from Sigma-Aldrich. Bio-Red protein assay standard II, Bio-Rad protein assay dye reagent. Bio-Scale™ Mini CHT™ 40 µm hydroxyapatite, cartridge Precision Plus Protein™ Unstained Standards, 10x Tris/Glycine/SDS buffer, and 2x Laemmli sample buffer were obtained from Bio-Rad Inc. Ammonium sulfate, Tris base for molecular biology and Lonza 15 % Gold Precast gels, GelCode ™ Blue Safe Protein Stain, and methanol (HPLC Grade) were obtained from Fisher Scientific. Kinetex EVO

C18 (150 x 4.6 mm) column was purchased from Phenomenex, all other chemicals were reagent grade.

Measurement of Enzyme Activity

Reducing Sugar Assay

Enzyme activity of column fractions was determined by reducing sugar assay (*42*). Adenosine (1mM) in 10 mM Tris pH 7.2 (1 mL) and 100 µL of solution from each column fraction was added to each tube. In the control, 100 µL of DI water was added instead of enzyme. Fractions were incubated four hours at room temperature. Copper reagent (300µL) (4% Na_2CO_3 1.6% glycine, and 0.045% $CuSO_4.5H_2O$) and neocuproine reagent (300 µL) (0.12% neocuproine, pH 3.0) were added to each tube. The tubes were placed in a boiling water bath for 7 minutes. After allowing the tubes to cool, the absorbance of each fraction was measured at 450 nm. The control was used to set the absorbance at zero. The amount of ribose was determined by comparison to a standard calibration curve (Figure 10).

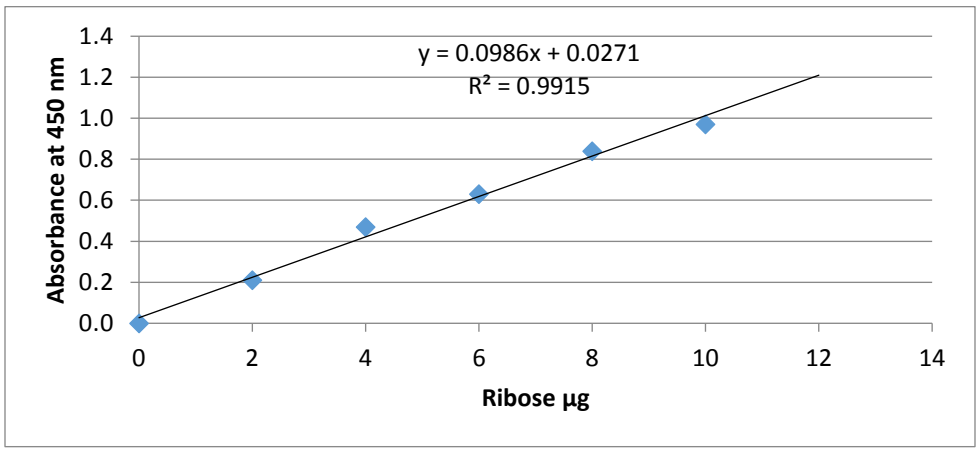


Figure 10**.** Standard reducing sugar assay calibration curve. The activity of the enzyme was measured by the appearance of ribose as the nucleoside was hydrolyzed.

HPLC Analysis to Determine Enzyme Activity

HPLC analysis was also used to determine enzyme activity by measuring the relative amounts of nucleoside and base as a function of time. To measure activity, 1 mM adenosine in 10 mM Tris buffer pH 7.2 (1000 µL) was placed in a HPLC vial. To initiate the reaction 50 µL of the enzyme was added to the vial. The reaction mixture was incubated at room temperature. To determine the activity 10 µL was injected onto the Kinetex EVO C18 (150 x 4.6 mm). The mobile phase was 98% 10 mM ammonium phosphate pH 5.4 and 2% methanol at a flow rate of 0.6 mL/min. The temperature of the column oven was 30.0 °C. The eluent was monitored at 254 nm. The retention time of adenosine was 13.1 minutes, adenine was 4.9 minutes, inosine was 4.8 min and hypoxanthine was 3.1 min (Figures 11, 12, 13, 14 respectively). The amount of adenosine and adenine present were calculated based on their respective peak areas. Activity was calculated by the disappearance of adenosine as a function of time.

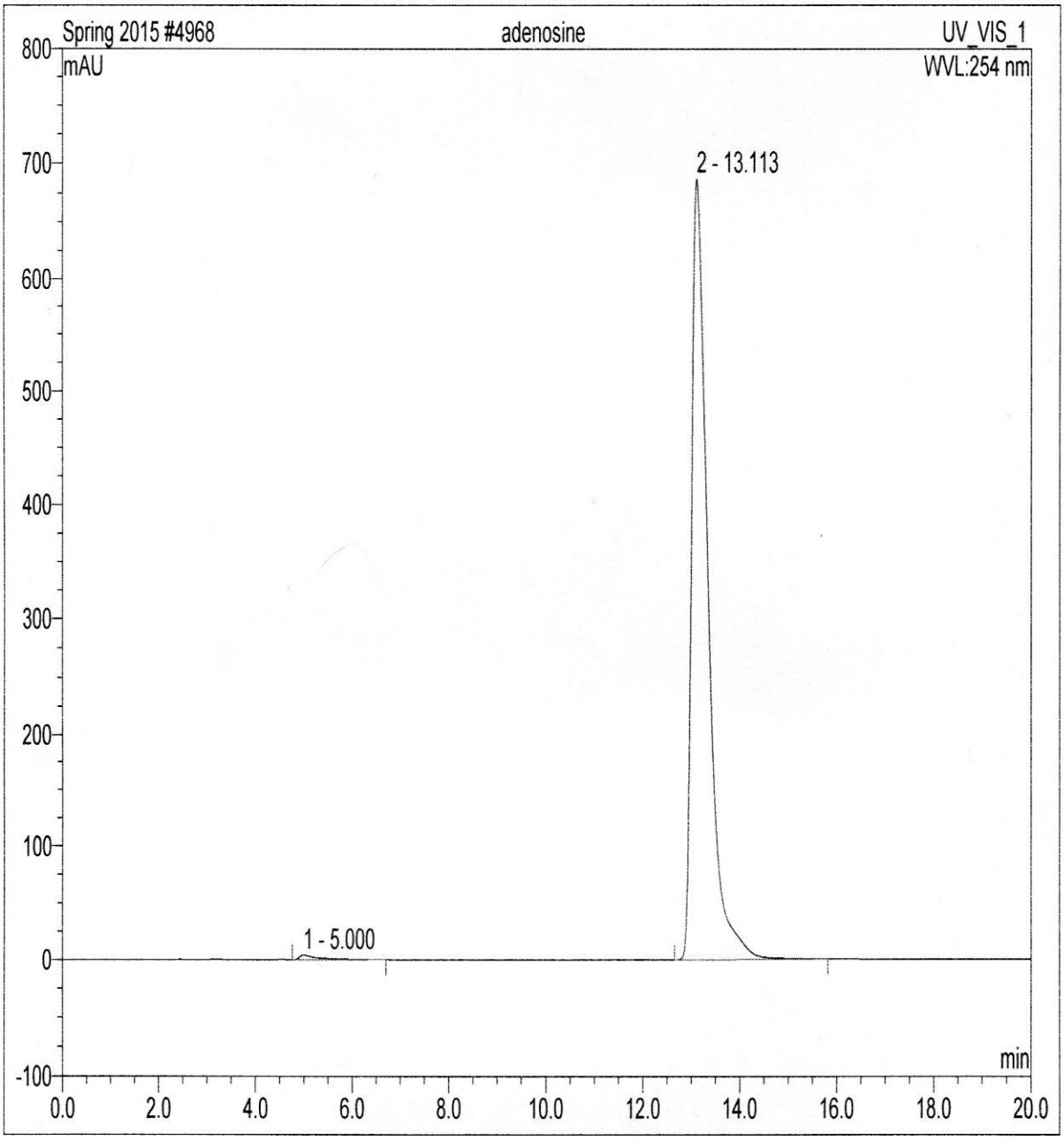


Figure 11. HPLC analysis of adenosine. The standard consisted of 1 mL 1 mM adenosine in 10 mM Tris pH 7.2.

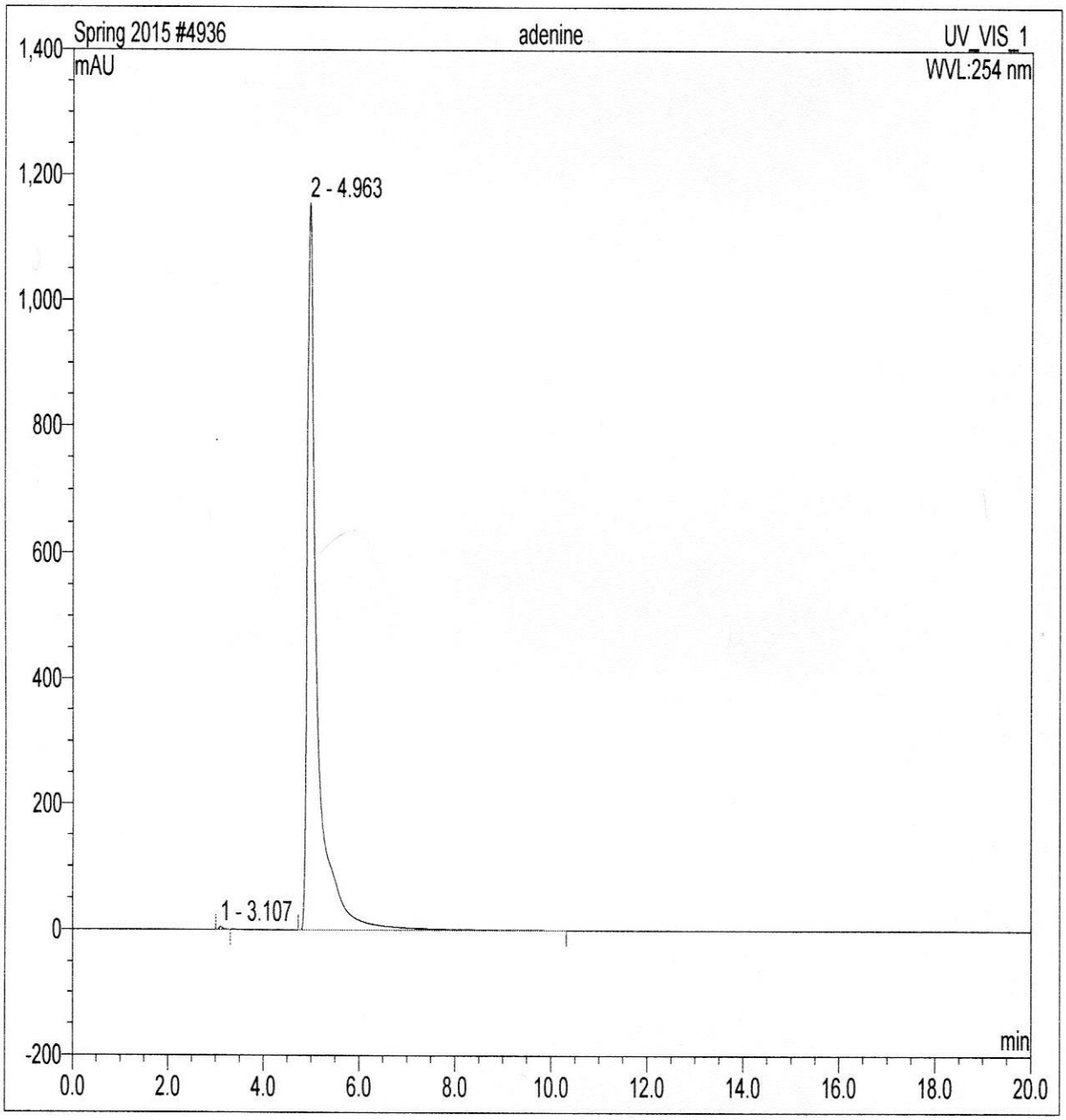


Figure 12. HPLC analysis of adenine. The standard consisted of 1 mL 1 mM adenine in 10 mM Tris pH 7.2.

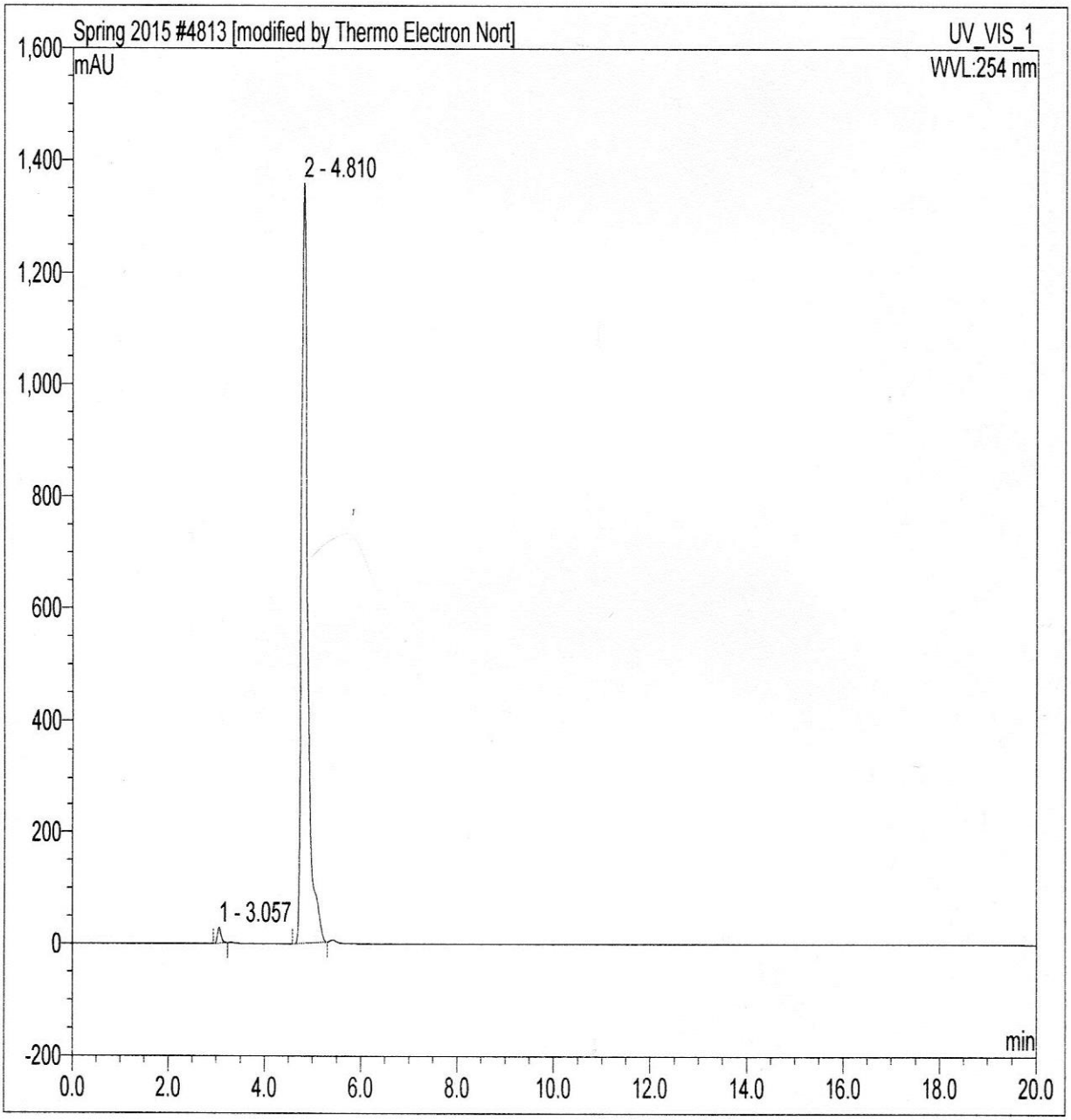


Figure 13. HPLC analysis of inosine. The standard consisted of 1 mL 1 mM inosine in 10 mM Tris pH 7.2.

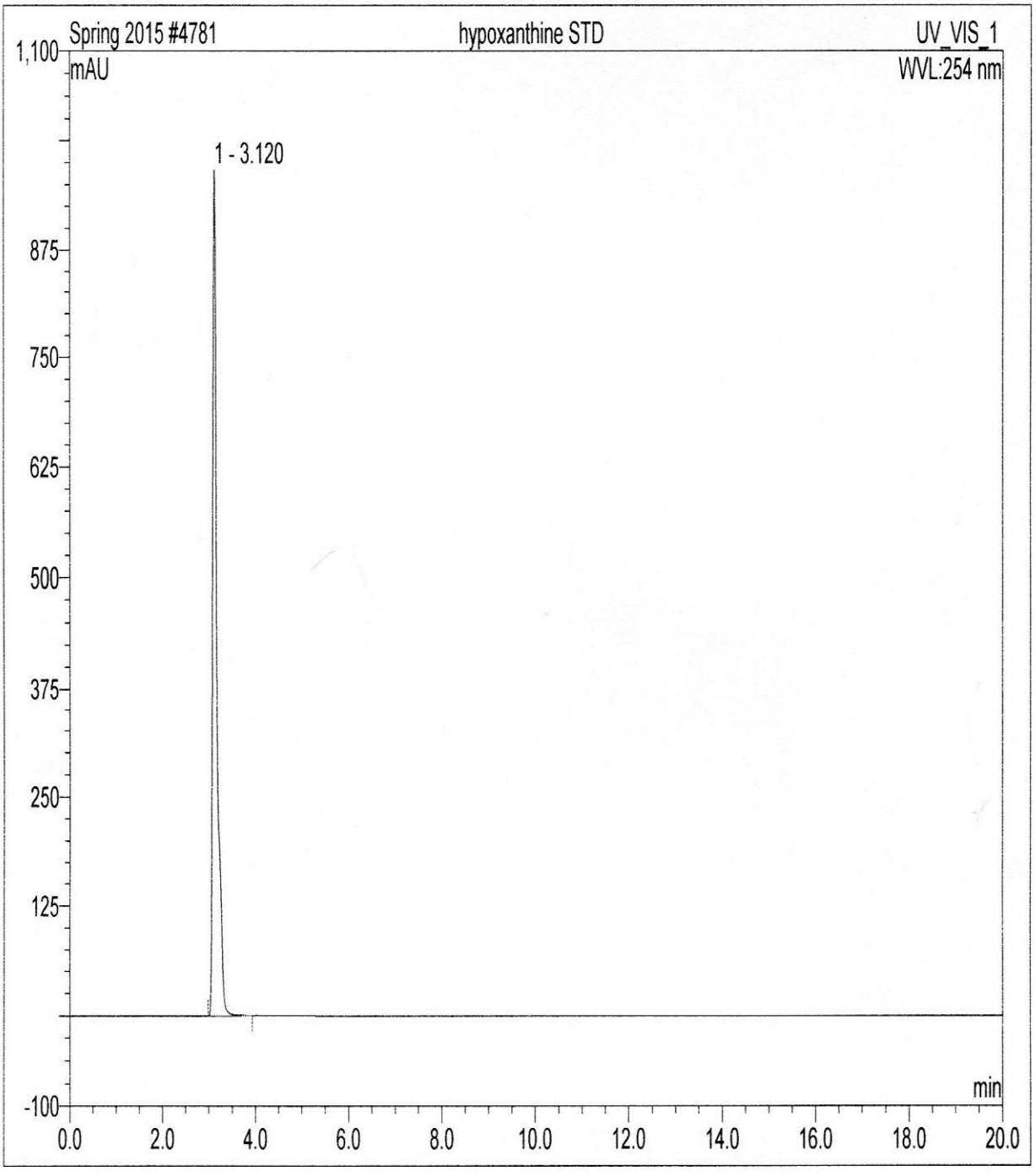


Figure 14. HPLC analysis of hypoxanthine. The standard consisted of 1 mL1 mM hypoxanthine in 10 mM Tris pH 7.2.

Determination of Protein Concentration

To determine the concentration of protein, 5 µL of sample and 795 µL of DI water was mixed with 200 µL of dye reagent. The absorbance of the sample was measured at 595 nm. Protein concentration was determined using a calibration curve prepared with Bio-Rad protein assay standard II bovine serum albumin (Figure 15). Bio-Rad assay was carried out after each ammonium sulfate precipitation step and pool #2 and pool #1, from both DEAE and hydroxyapatite columns respectively. The protein concentration of fraction from DEAE (pool #2) and hydroxyapatite (pool #1) columns was determined at 280 nm.

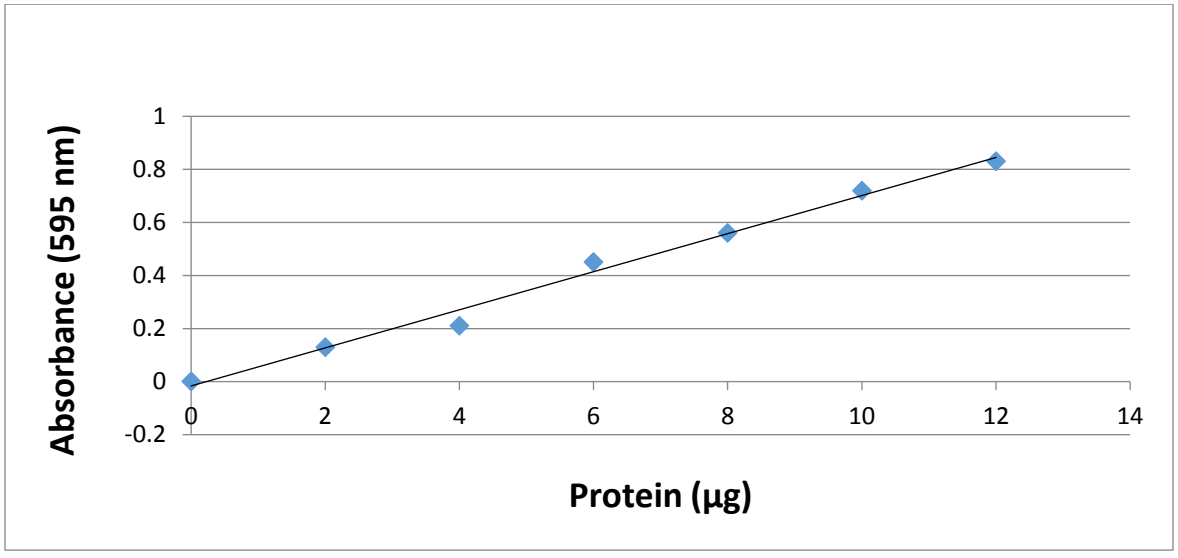


Figure 15. Calibration curve of Bio-Rad protein assay standard II bovine serum albumin.

Enzyme Purity and Molecular Weight Determination Using Gel Electrophoresis

To determine the molecular weight and purity of the protein (pool#1 and pool #2), the pools were assayed by SDS-PAGE using 15% Lonza Precast gel. To prepare for electrophoresis, the samples (10 µL) were added to 10 µL of 2X Laemmli sample buffer containing β-mereaptoethanol. The sample was centrifuged for 40 seconds and incubated at 95 °C for 5 minutes. The sample was centrifuged again. The sample was loaded onto the gel along with 10 µL Precision Plus Protein™ Unstained Standards. The gel ran for 60 minutes at 118 volts. The gel was placed in a container and washed with DI water 3 times for five minutes each. Enough GelCode™ Blue Safe Protein Stain was added to cover the gel and the gel stained overnight. The gel was washed with DI water. A calibration curve to relate log MW to distance traveled was constructed using the Precision Plus Protein™ Unstained molecular weight marker. The subunit molecular weight was determined by comparing the distance traveled to the calibration curve.

Enzyme Extract from Alaska Pea Seeds

Alaska pea seeds (108.91 g) were soaked in bleach (100 mL) for 5 minutes and thoroughly washed with tap water three times. The seeds were placed on moistened paper towels to allow the seeds to germinate for six days at room temperature. The paper towels were kept moist with tap water. The seeds were placed in a blender and 600 mL of 10 mM Tris buffer, pH 7.2 and 50 µL of plant protease inhibitor, 100 mg of dithiothreitol and 2 g of protamine sulfate added. The seeds were homogenized for one minute with a two-minute rest. The homogenation was repeated 5 times. The homogenation was carried

out in cold room at 4 ºC. The initial extract was centrifuged for 30 minutes at 10,000 xg at 4 ºC.

Purification of Adenosine Nucleosidase

After centrifugation, the supernatant was saved and transferred to a graduated cylinder to measure the volume (610 mL). Ammonium sulfate (107.4 g) was slowly added to the supernatant to bring the solution to 30% saturation. After dissolving the ammonium sulfate, the solution was incubated overnight at 4 ºC.

The next day, the solution was centrifuged in an Allegra 64R centrifuge for 30 minutes at 10,000*xg* at 4 ºC. The supernatant (660 mL) was saved and the pellet discarded. Ammonium sulfate (128.7 g) was slowly added to the solution to bring the solution to 60% saturation. The solution was left overnight in the cold room at 4 ºC. After this step, the sample was centrifuged again at 10,000*xg* at 4 ºC. The pellet was saved and dissolved in 15 mL of 10 mM of Tris buffer, pH 7.2. The solution was dialyzed against 1 L of 10 mM Tris buffer pH 7.2 two times.

Diethylaminoethyl (DEAE) Ion Exchange Chromatography

The DEAE Sepharose^{TM} Fast Flow column was prepared by washing with high salt (1 L of 1M NaCl) followed by washing a second time with 1 L of DI water. The column was washed a final time with low salt (1 L of 10 mM Tris buffer pH 7.2). The sample (18 mL) was loaded onto the gravity column. The column was washed with 250 mL of 10 mM Tris pH 7.2. Bound protein was eluted with a sodium chloride (NaCl)

linear gradient from zero to 1 M NaCl in 10 mM Tris buffer pH 7.2 (250 mL). The fraction size was 10 mL. Fractions were assayed for activity by reducing sugar assay. Fractions 16-21 and 51-64 were labeled pool #1 and pool #2 respectively. All pools were assayed for activity by HPLC.

Hydroxyapatite Chromatography

After DEAE column Pool # 2 was dialyzed with 1 L 10 mM phosphate buffer pH 7.2 overnight. Dialysis was repeated twice, replacing the phosphate buffer two times. After dialysis, the sample was concentrated to 5 mL using an Amicon centrifugal concentrator on an Eppendorf 5810R centrifuge to load onto the hydroxyapatite column.

The column used was a Bio-Scale™ Mini CHT™ 40 µm hydroxyapatite cartridge. The pressure was 0.35 MPa, and the flow rate was 2 mL/min. The column was washed with 5 mL of DI water. Buffer A (low salt buffer) was 10 mM potassium phosphate buffer pH 6.8. Buffer B (high salt buffer) was 400 mM potassium phosphate buffer pH 6.8. The fraction size was 5 mL. After loading the sample the column was washed with 500 mL (5 column volumes) of Buffer A. A linear gradient was run from zero percent Buffer B to one hundred percent Buffer B over 100 mL (20 column volumes). The effluent from the column was monitored at 280 nm. After the fractions, had been collected (30 fractions) from the hydroxyapatite column, the activity of these fractions was determined using the reducing sugar assay, and the fractions were incubated three hours. The fractions that had a high activity were pooled. Fractions 3 and 4 were pooled (Pool #1) and Fractions 16, 17 and 18 were pooled (Pool#2).

Steady State Kinetic Analysis

The kinetic constants, K_m and V_{max} of the enzyme were measured using inosine as the substrate. The progress of the reaction was monitored by HPLC as described above. An aliquot of the enzyme solution (25 µL) was added to reaction mixtures containing variable concentrations of inosine in 50 mM Tris pH 7.2. For the control, 25 µL of water was added instead of the enzyme solution. Ten (10) µL aliquots were injected at intervals and the relative amounts of base and nucleoside determined until approximately 25 % of inosine converted to hypoxanthine. Concentration of hypoxanthine was determined using a calibration curve, (Figure 16). The velocity was calculated by taking the slope of a plot of hypoxanthine produced vs reaction time. The concentrations of inosine used were approximately 50, 100, 250, 500, 750, 1000, and 1250 µM. All activities were run in duplicate.

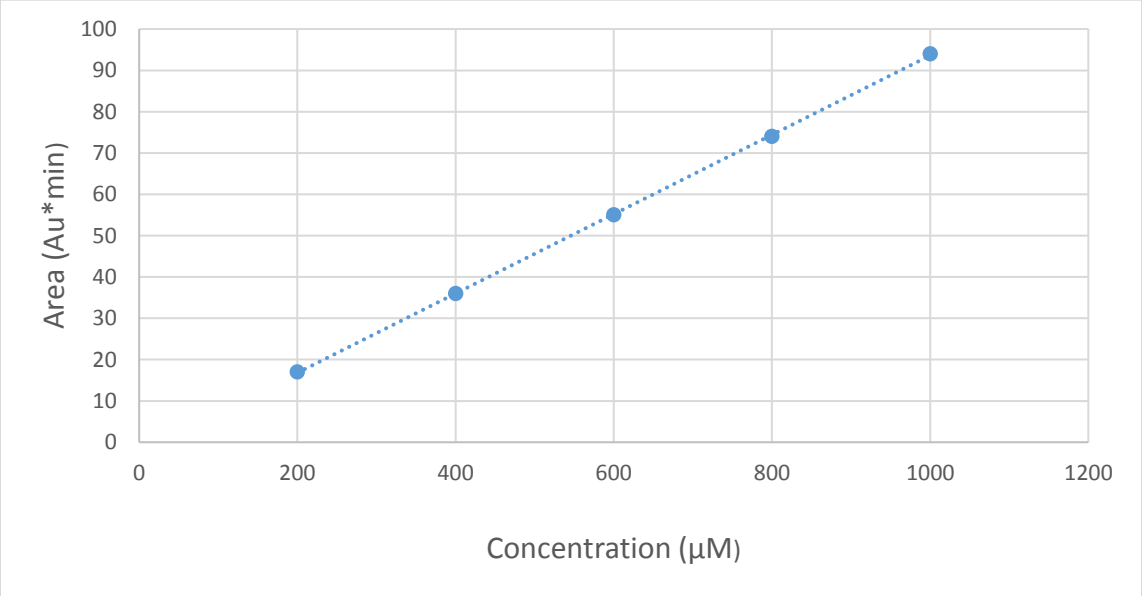


Figure 16. Calibration curve of hypoxanthine.

Determination of Substrate Specificity

Several substrates were tested for activity against the enzyme. Nucleoside (1mM) in 10 mM Tris pH 7.2 (1000 µL) was added to an HPLC vial along with 25 µL of the enzyme. The control consisted of 25 µL of DI water instead of enzyme. The same chromatography conditions were used as was used for the HPLC activity assay for adenosine. The appearance of the base and/or disappearance of the nucleoside as a function of time were used to determine activity. The analysis was carried out from two days to four days to depending on the sample. The following nucleosides as substrates were tested with adenosine nucleosidase: 2'-deoxyinosine, allopurinol riboside, 3'-deazauridine and adenine- β-arabinofuranoside.

CHAPTER III

RESULTS AND DISCUSSION

The hydrolysis of adenosine to ribose and adenine is catalyzed by adenosine nucleosidase, an enzyme in the purine metabolism in plants. Adenosine nucleosidase is hypothesized to affect the levels of cytokinins (*9*). Cytokinins are hormones in the plant that are derivatives of adenosine. Adenosine nucleosidase can help in controlling cytokinins indirectly by altering the amount of adenosine available to produce cytokinins. This would affect the plant life cycle, since cytokinins are involved in many plant processes. Cytokinins are mainly involved in cell growth and cell differentiation. They also help in apical dominance, the formation of leaves, development of buds, and aid in providing a medium of cell division by mitosis. In the absence of cytokinins, cell division through mitosis would be impossible.

Adenosine Nucleosidase Purification

The goal of this project is to characterize adenosine nucleosidase from germinated Alaska pea seeds. After germination, Alaska pea seeds (108.91g) were homogenized and centrifuged to remove insoluble material. The activity of initial extract was determined by HPLC using 1 mM adenosine in 10 mM Tris buffer pH 7.2 and 50 µL of initial extract. There are multiple peaks in the chromatogram that correspond to adenosine, inosine, adenine, and hypoxanthine (Figure 17). Based on the appearance of

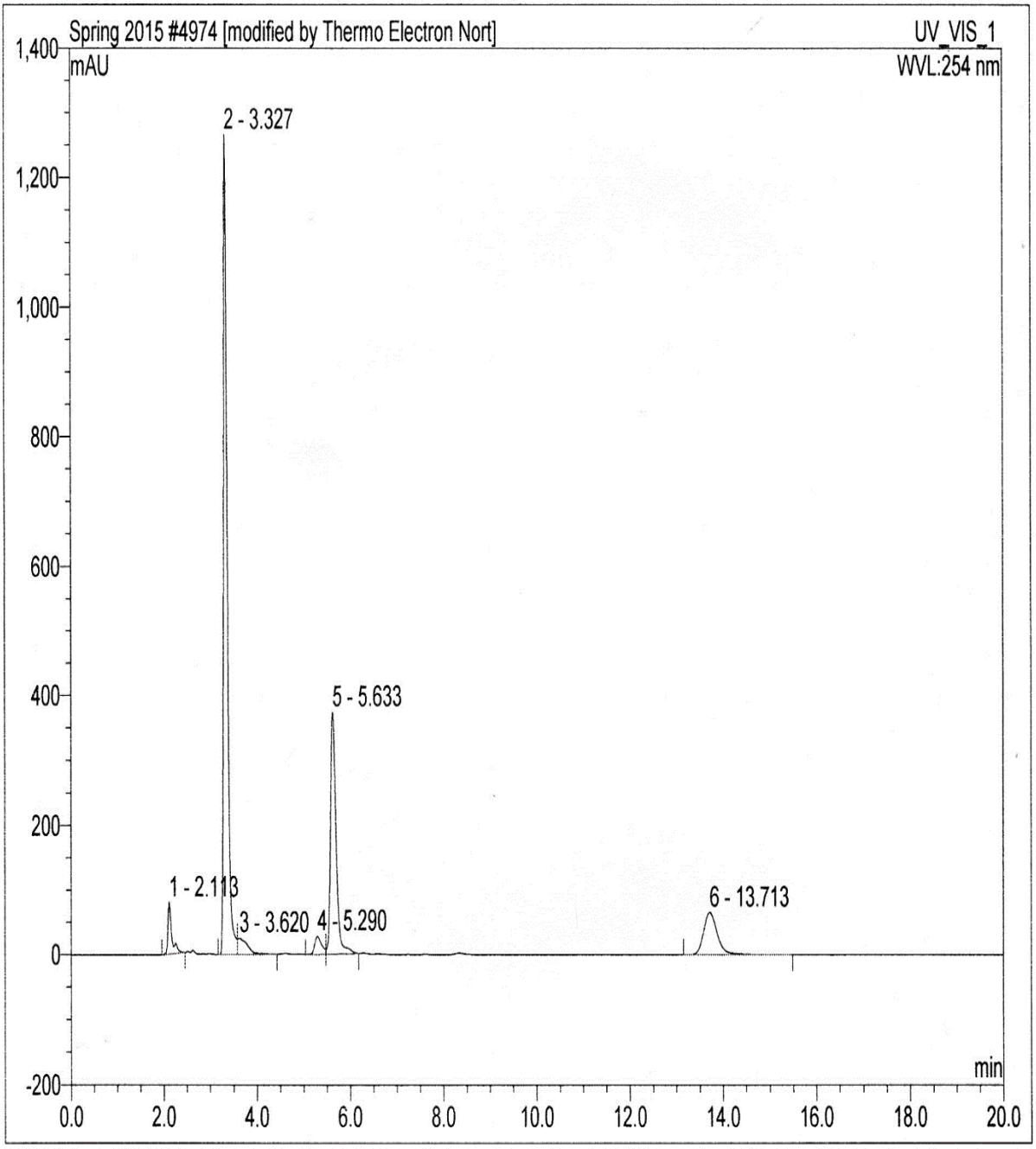


Figure 17. HPLC analysis of the activity of the initial extract. The reaction mixture consisted of 1 mL 1 mM adenosine in 10 mM Tris pH 7.2 and 50 μL of initial extract. After 5 hours, the chromatogram shows adenosine (13.71 min), adenine (5.63 min), inosine (5.29) and hypoxanthine (3.32 min).

nucleosidase. The specific activity of the initial extract was 5.66 x 10^{-3} μmol/min/mg based on the disappearance of adenosine.

Ammonium sulfate was added to bring its concentration to 30% saturation. The addition of the ammonium sulfate required 15 minutes to avoid local high concentrations. The solution was incubated in the cold room at 4 ºC overnight. The solution was centrifuged to remove precipitated proteins, and the 30% supernatant and the pellet were assayed for activity by HPLC (Figures 18-19 respectively). Ammonium sulfate was added to increase the concentration to 60% saturation. The solution stored in the cold room at 4 ºC overnight. The sample was centrifuged to separate the pellet and the supernatant. The pellet was dissolved in 15 mL of 10 mM Tris buffer pH 7.2.

The 60% supernatant and the 60% pellet were assayed for activity by HPLC. The chromatogram of both showed multiple peaks (Figures 20-21). The HPLC of the 60% saturation shows almost no adenosine nucleosidase activity in the supernatant. The results of the reaction for the 60% pellet show two nucleoside metabolizing enzymes. The first one, adenosine deaminase, converts adenosine to inosine, and the second, adenosine nucleosidase, and converts adenosine to adenine and inosine to hypoxanthine. The specific activity of 30% supernatant was 6.01 x 10^{-3} μmol/min/mg, and the specific activity of 60% saturation pellet was 5.26 x 10^{-3} μmol/min/mg.

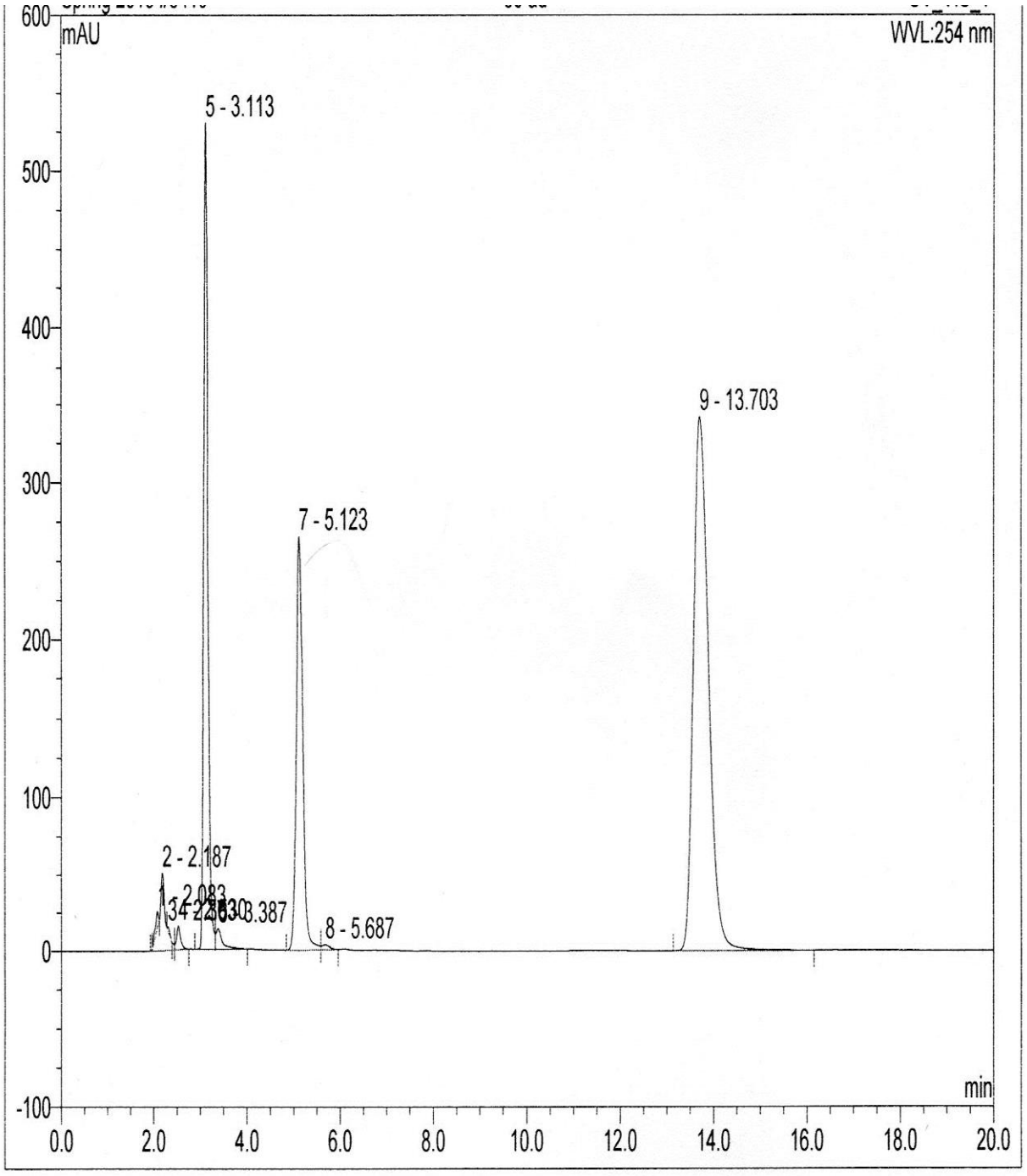


Figure 18. HPLC analysis of the activity of 30% saturation supernatant. The reaction mixture consisted of 1 mL 1 mM adenosine in 10 mM Tris pH 7.2 and 50 µL of 30% saturation sample. After 7 hours, the chromatogram shows adenosine (13.70 min), adenine/inosine (5.12 min), and hypoxanthine (3.11 min).

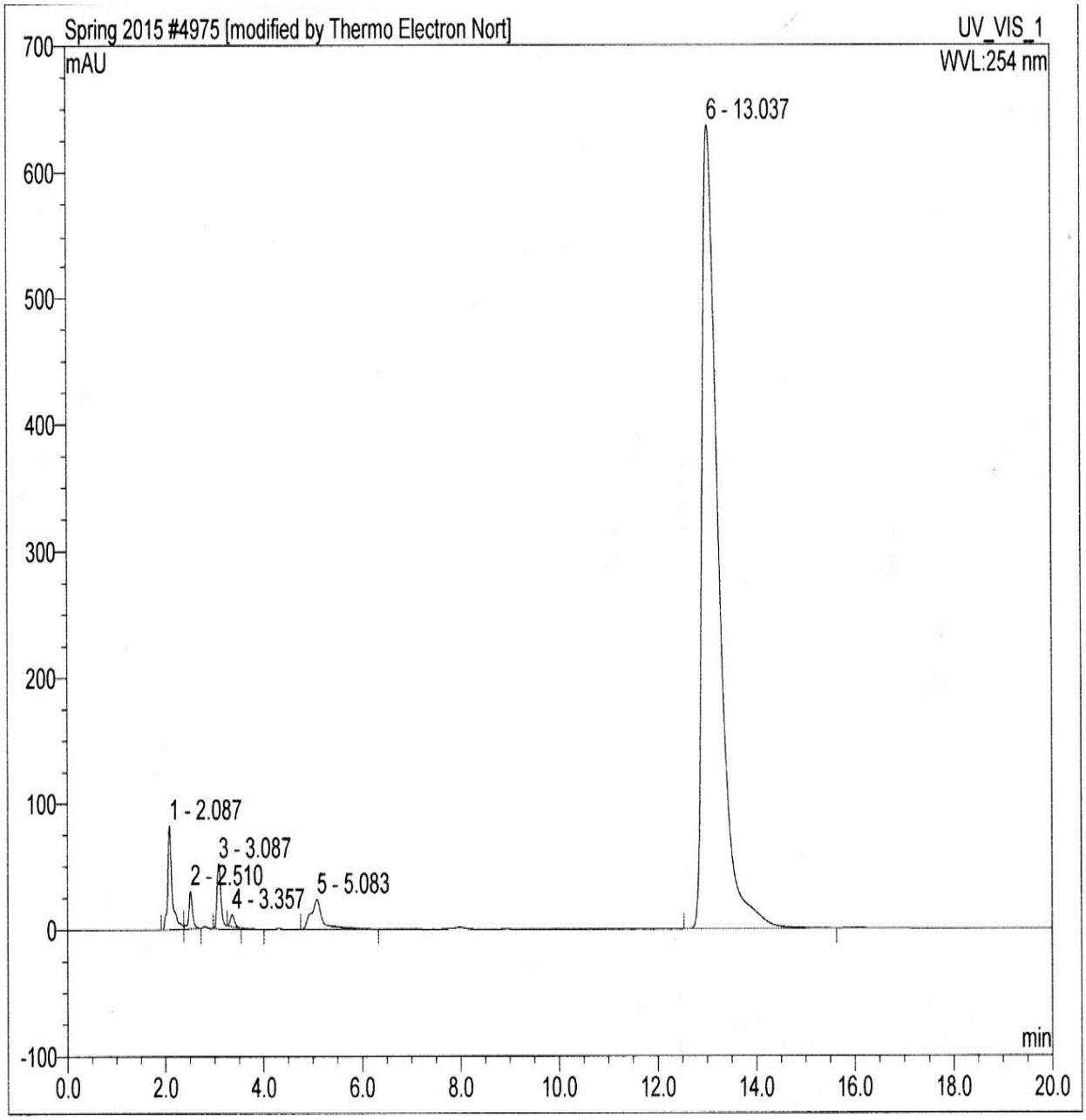


Figure 19. HPLC analysis of the activity of 30% saturation pellet. The reaction mixture consisted of 1 mL 1 mM adenosine in 10 mM Tris pH 7.2 and 50 µL of 30% saturation sample. After 6 hours, the chromatogram shows adenosine (13.03 min), adenine/inosine (5.08 min), and hypoxanthine (3.08 min).

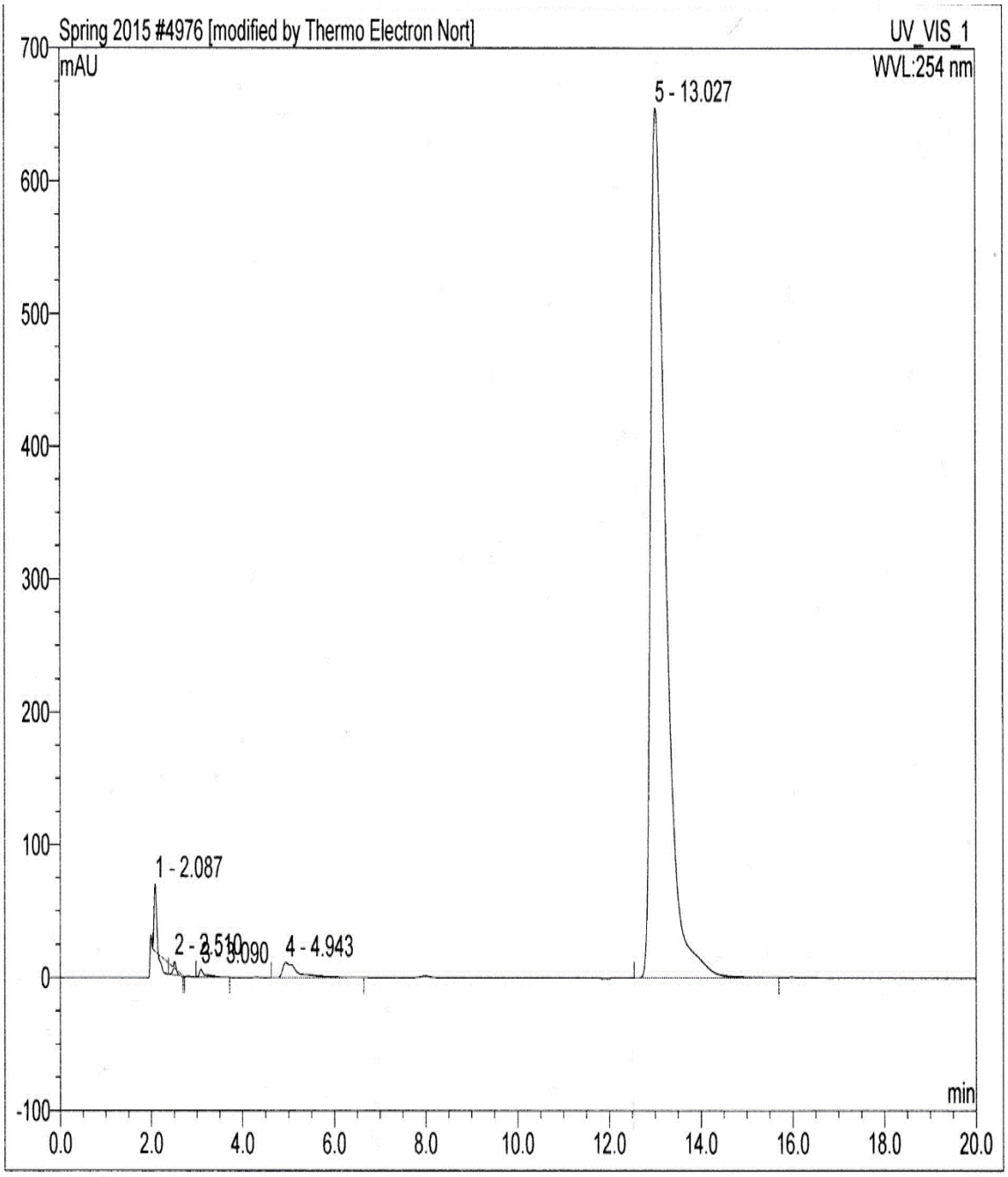


Figure 20. HPLC analysis of the activity of 60% supernatant sample. The reaction mixture consisted of 1 mL of 1 mM adenosine in 10 mM Tris pH 7.2 and 50 μL of 60% saturation sample. After 6 hours, the chromatogram shows adenosine (13.02 min), adenine/inosine (4.94 min).

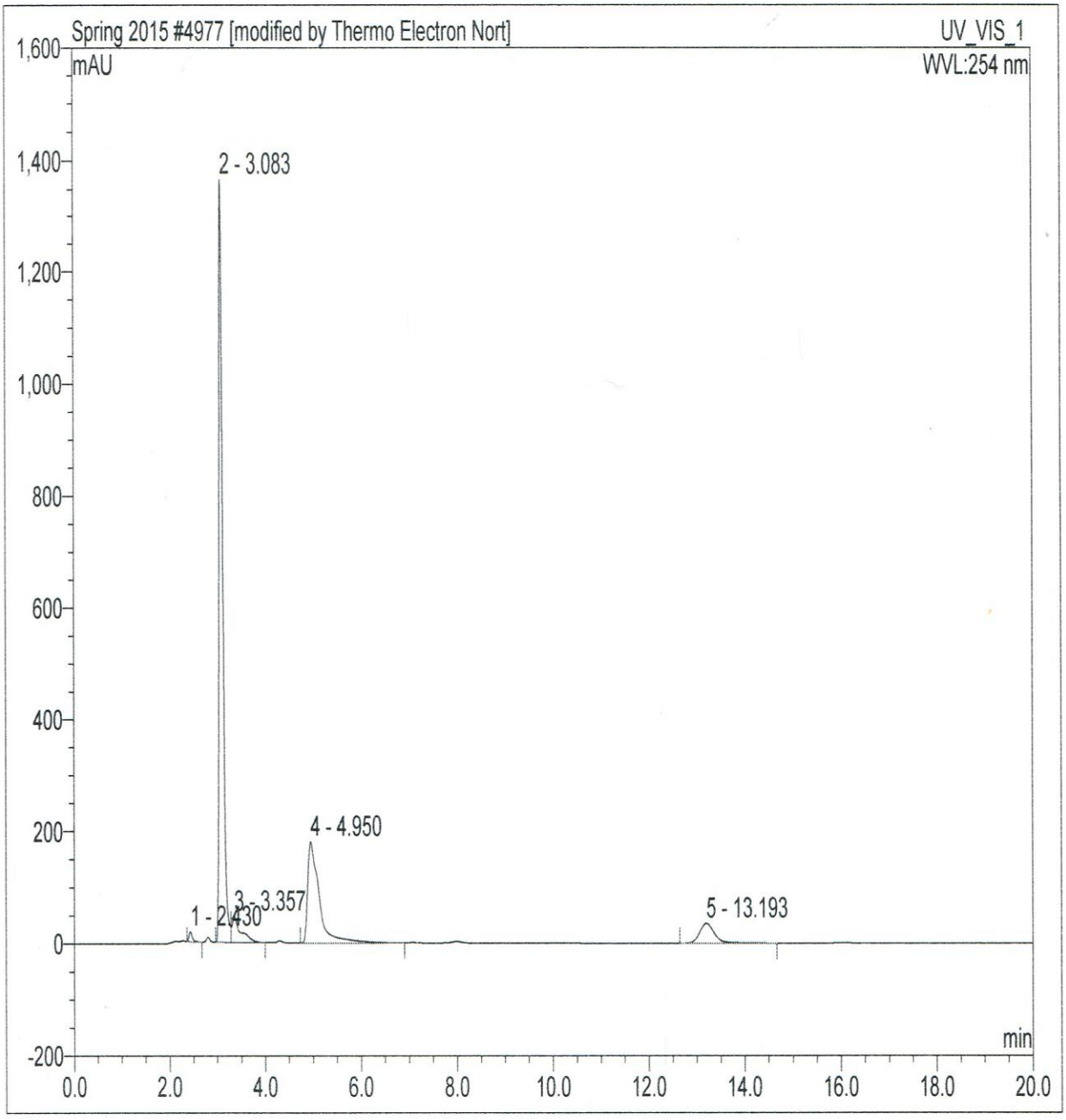


Figure 21. HPLC analysis of the activity of 60% saturation pellet. The reaction mixture consisted of 1 mL of 1 mM adenosine in 10 mM Tris pH 7.2 and 50 µL of 60% saturation pellet. After 6 hours, the chromatogram shows adenosine (13.19 min), adenine/ inosine (4.95 min), and hypoxanthine (3.08 min).

After dialyzing the 60% pellet solution, the sample was loaded onto a DEAE column. Ion-exchange chromatography (IEC) is one of the most popular methods to separate charged biological molecules such as proteins, peptides, amino acids, or nucleotides (*43*). The amino acids that make up proteins have positively and negatively charged side chains. Based on the pH of their environment, proteins may carry a net positive charge, a net negative charge, or no charge. The pH at which a molecule has no net charge is its isoelectric point, or pI (*43*). The sample (18 mL) was loaded onto the DEAE column. The column was washed with 10 mM Tris pH 7.2 to elute proteins that did not bind to the column. Bound protein was eluted with a linear gradient from zero to 1 M NaCl in 10 mM Tris pH 7.2 after Fraction 25. The Fractions were measured for activity by reducing sugar assay.

The fractions that produced the most ribose were pooled and concentrated (Figure 22). Fractions 16-21 and 51-64, labeled as pool #1 and pool #2 respectively, were assayed for activity by HPLC using adenosine as the substrate (Figures 23 -24). This step gave a 3 fold-purification. The overall purification after this step was 2.72- fold with 13.6 % percent recovery. The specific activity increased to 1.54×10^{-2} μmol/min/mg.

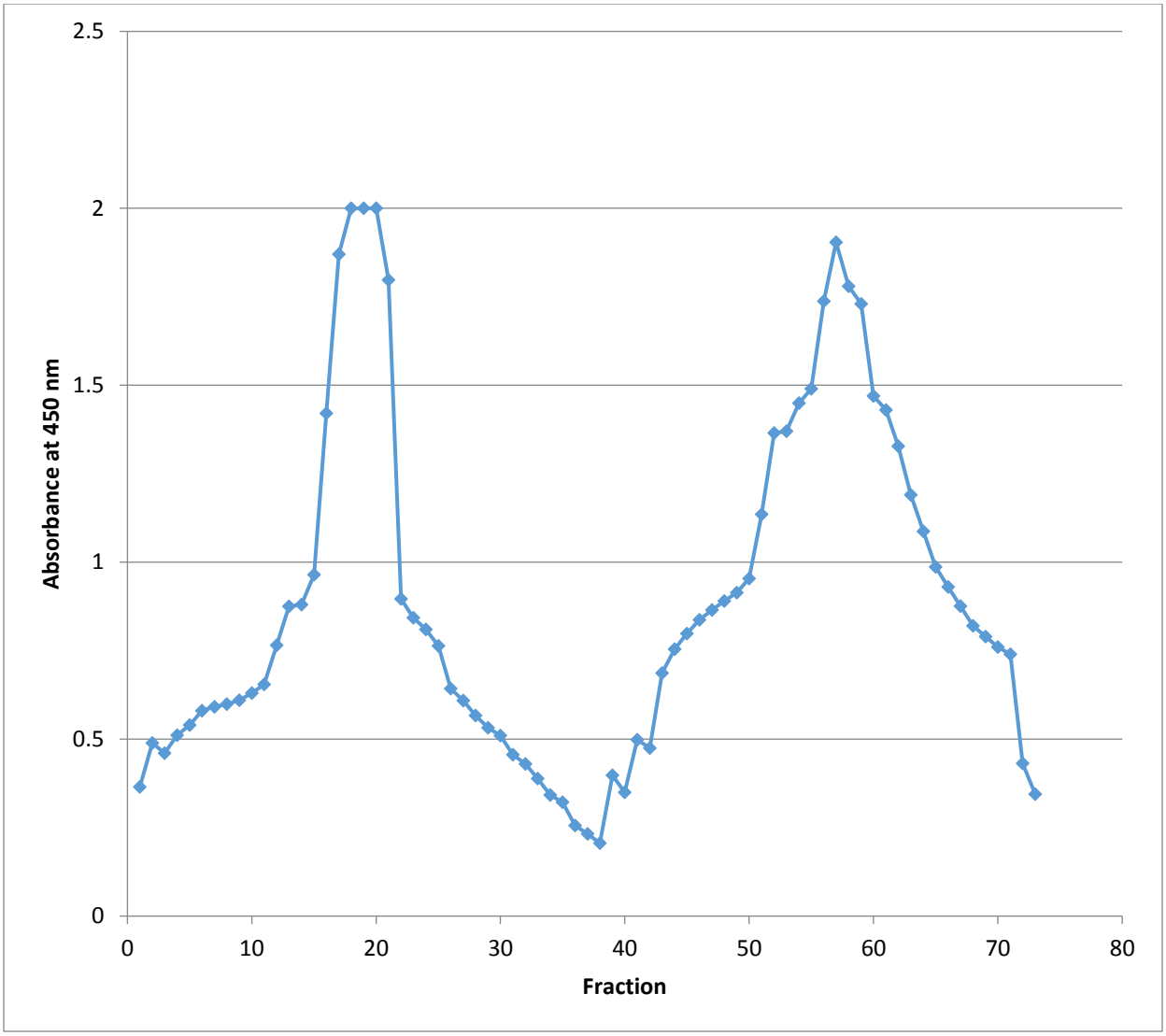


Figure 22. Activity tested by reducing sugar assay for fractions from DEAE column. Adenosine (1 mM) reaction mixture and 100 µL of enzyme from DEAE column. Fraction 16-21 and 51-64 were labeled as pool #1 and pool #2 respectively. The sodium chloride gradient started at Fraction 25.

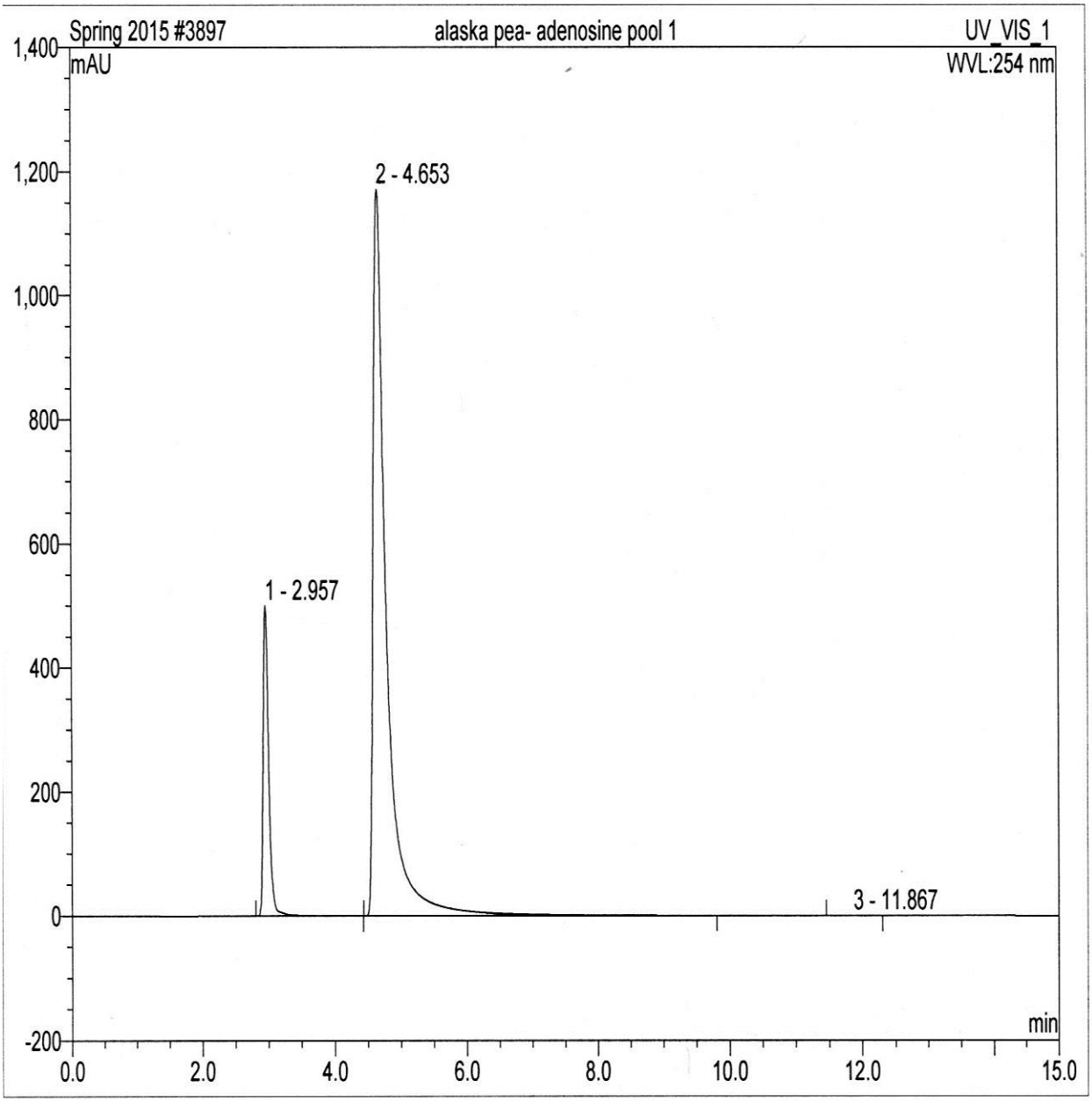


Figure 23. HPLC analysis of the activity of pool #1 from DEAE column. The reaction mixture consisted of 1 mL 1 mM adenosine in 10 mM Tris pH 7.2 and 50 μL of pool #1 from DEAE column. After 5 hours, the chromatogram shows no peak for adenosine retention time.the peaks represent the products adenine/inosine (4.65 min), and hypoxanthine (2.95 min).

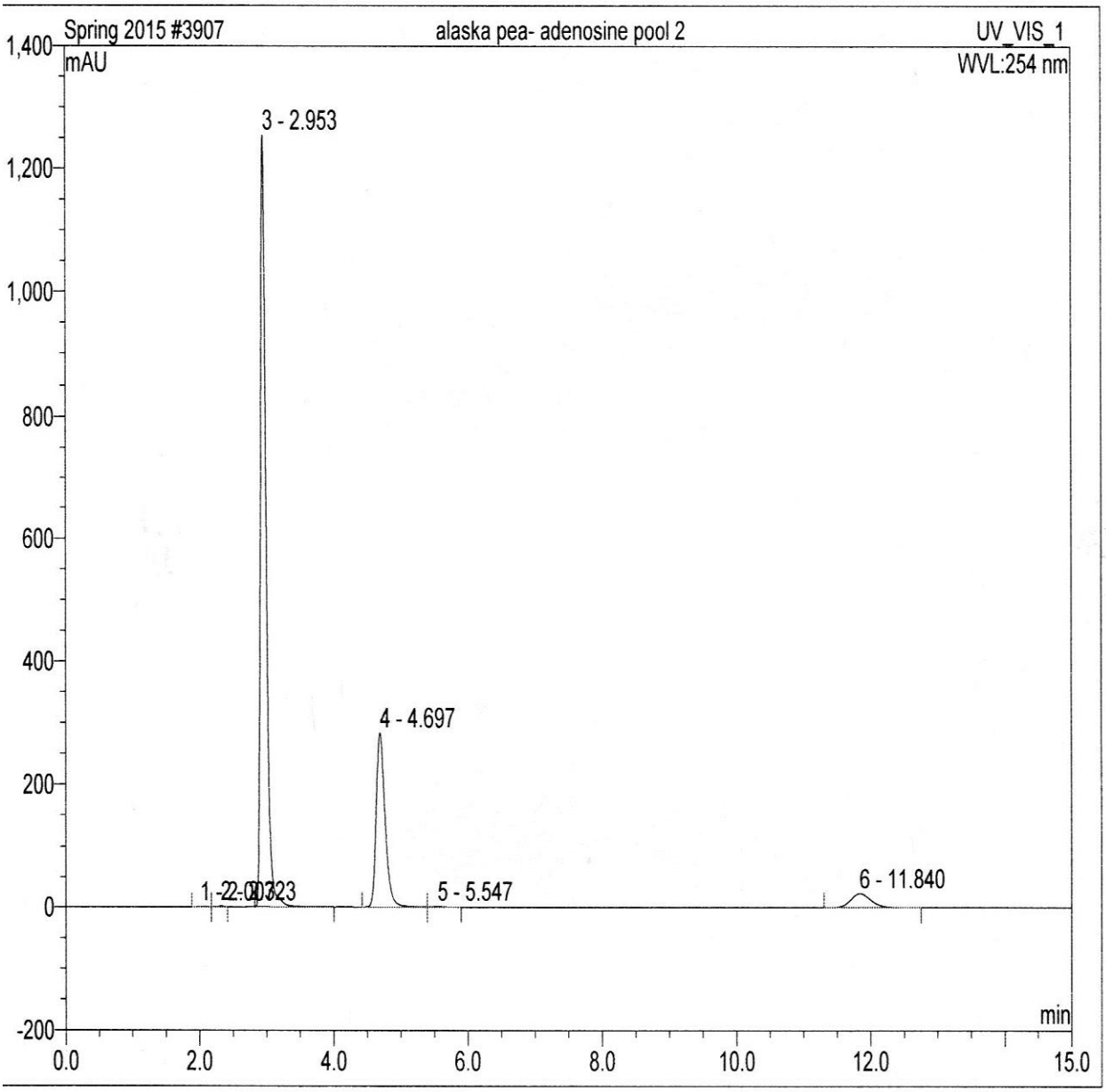


Figure 24. HPLC analysis of the activity of pool #2 from DEAE column. The reaction mixture consisted of 1 mL 1 mM adenosine in 10 mM Tris pH 7.2 and 50 µL of pool #2 from DEAE column. After 8 hours, the chromatogram shows a small peak for adenosine (11.84 min), and major peaks for the products adenine/inosine (4.69 min), and hypoxanthine (2.95 min).

The last step in purifying the enzyme was to use a hydroxyapatite column. Hydroxyapatite ($Ca_5(PO_4)_3OH)_2$, is a form of calcium phosphate used in the chromatographic separation of biomolecules (*44*). It is used to separate proteins that are homogeneous by electrophoretic and other chromatographic techniques. Hydroxyapatite chromatography has been used in the purification of subclasses of monoclonal and polyclonal antibodies, antibodies that differ in light chain composition, antibody fragments, isozymes, and single-stranded DNA from double-stranded DNA (*44*).

Pool# 2 from DEAE column was concentrated to 5 mL to load it onto the hydroxyapatite column. The column was washed with five column volumes of low salt buffer A (10 mM potassium phosphate buffer pH 6.8). A linear gradient was run from 0 % to 100 % 400 mM potassium phosphate buffer pH 6.8. Two peaks were observed in the elution profile of the sample based on UV absorbance at 280 nm (Figure 25). A reducing sugar assay of the fractions collected was carried out (Figure 26). The elution profile shows two peaks of activity that coincide with the UV absorbing peaks. The fractions that produced the most ribose were pooled and concentrated (Figure 26). Fractions 3 and 4 were pooled and named pool#1, and Fractions 16-18 were pooled and named pool# 2. The activities of Fractions 3-4 (Figures 27-28) and Fractions 16-17-18 (Figures 29, 30 and 31 respectively) were tested by HPLC using adenosine as a substrate. The overall purification after this step for pool#1 was 3-fold with 0.56 % recovery. The specific activity increased to 1.71×10^{-2} μmol/min/mg.

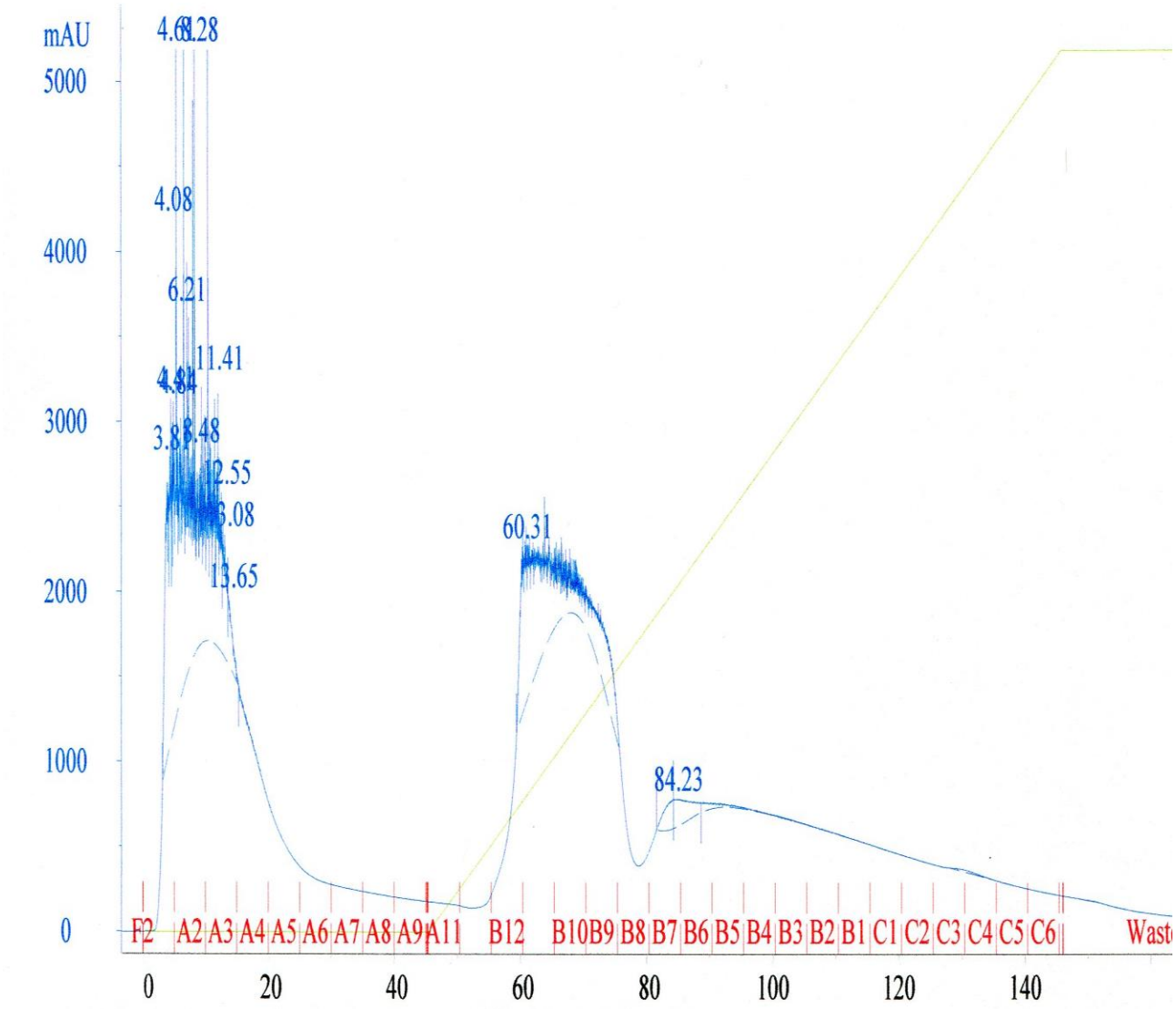


Figure 25. Elution profile from hydroxyapatite chromatography. The column used was Bio-Scale™ Mini CHT™ 40 µm hydroxyapatite cartridge. The flow rate was 2 mL/min. After the sample was loaded, the column was washed with five column volumes of low salt buffer. The low salt buffer, buffer A, was 10 mM potassium phosphate buffer pH 6.8, and the high salt buffer, buffer B, was 400 mM potassium phosphate buffer pH 6.8. The fraction size was 5 mL. A linear gradient was run from 0% buffer B to 100% buffer B over 20-column volumes

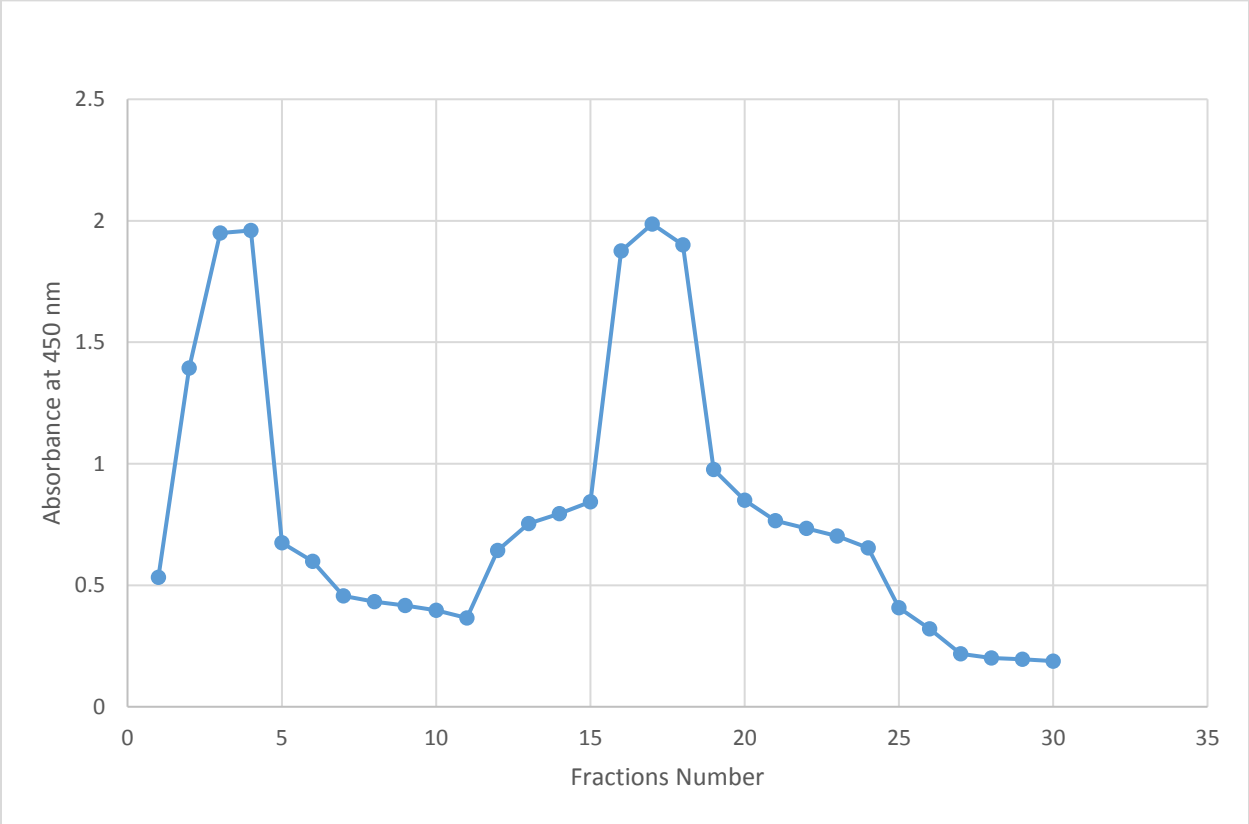


Figure 26. Activity tested by reducing sugar assay for Fractions from hydroxyapatite column. Adenosine (1mM) in 10 mM Tris pH 7.2 reaction mixture and 100 µL of the enzyme from Fractions of hydroxyapatite column.

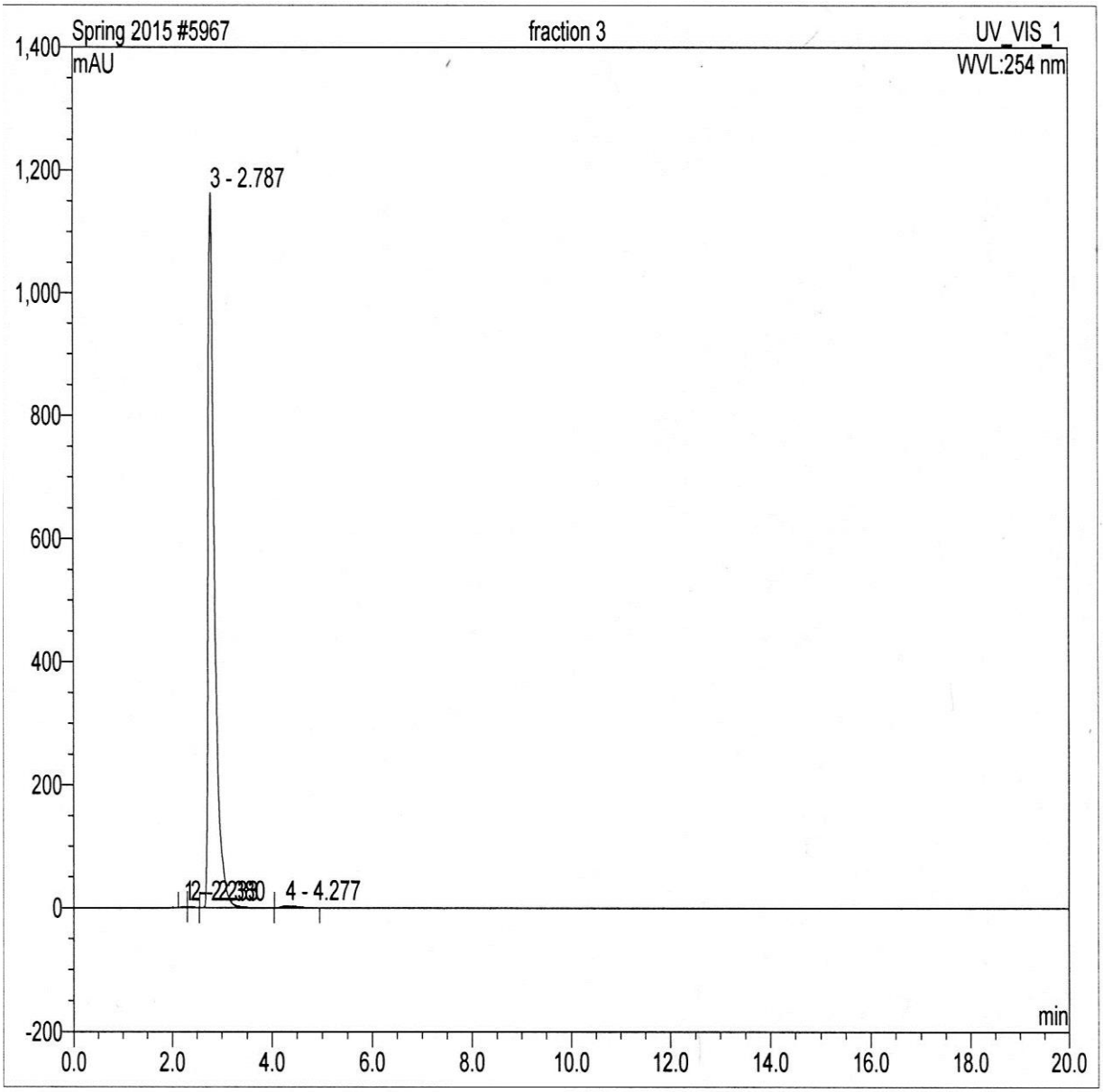


Figure 27. HPLC of analysis of the activity of Fraction 3 from hydroxyapatite column. The reaction mixture consisted 1 mL 1 mM adenosine in 10 mM Tris pH 7.2 and 100 μL of Fraction 3 from hydroxyapatite column. After 18 hours, the chromatogram shows adenosine has disappeared. Small amount of adenine was present (4.27 min), while the majority was hypoxanthine (2.78 min).

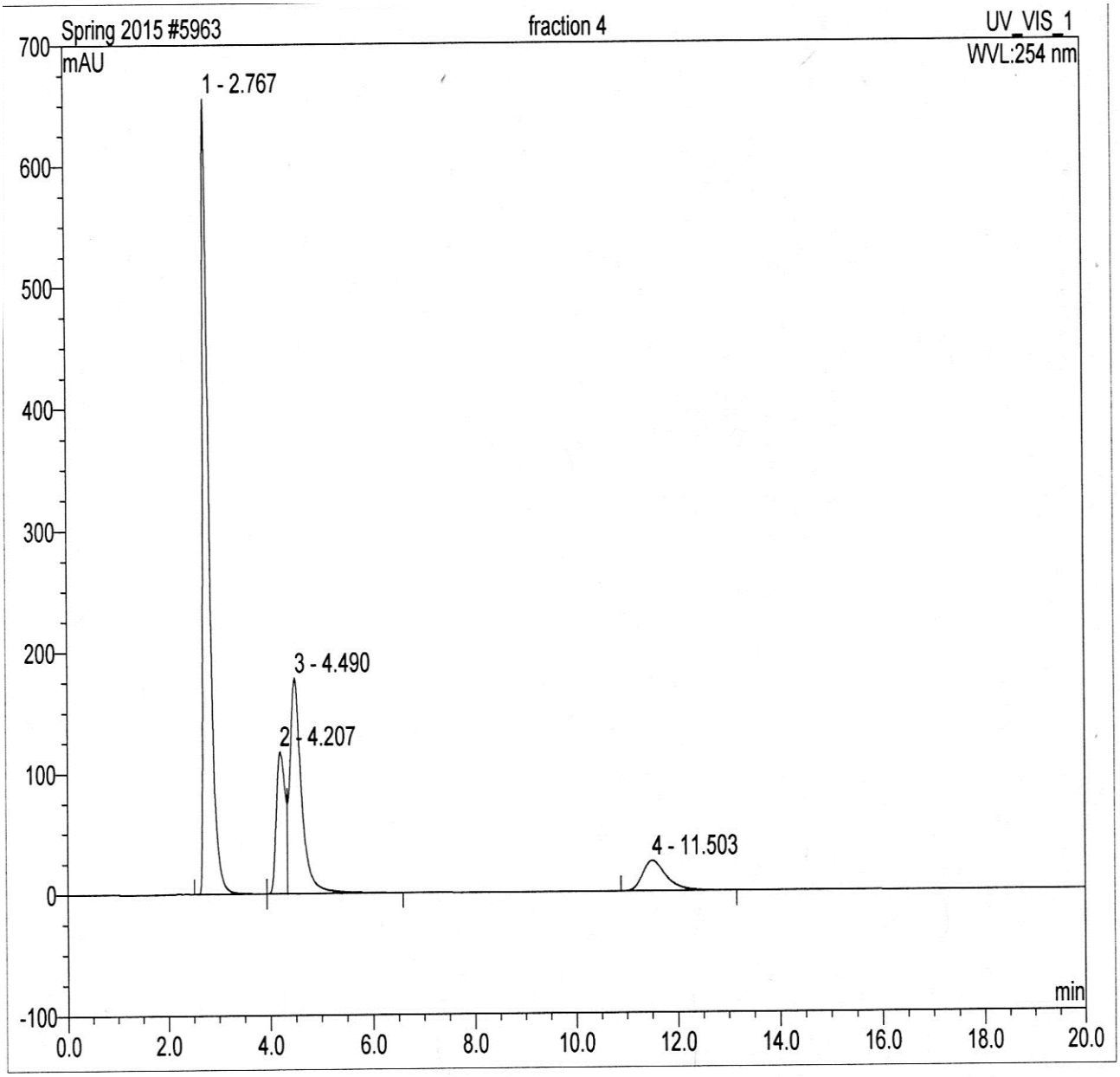


Figure 28. HPLC of analysis of the activity of Fraction 4 from hydroxyapatite column. The reaction mixture consisted 1 mL 1 mM adenosine in 10 mM Tris pH 7.2 and 100 μL of Fraction 4 from hydroxyapatite column. After 18 hours, the chromatogram shows a small amount of adenosine (11.50 min), adenine (4.49 min), inosine (4.20 min), and hypoxanthine (2.76 min).

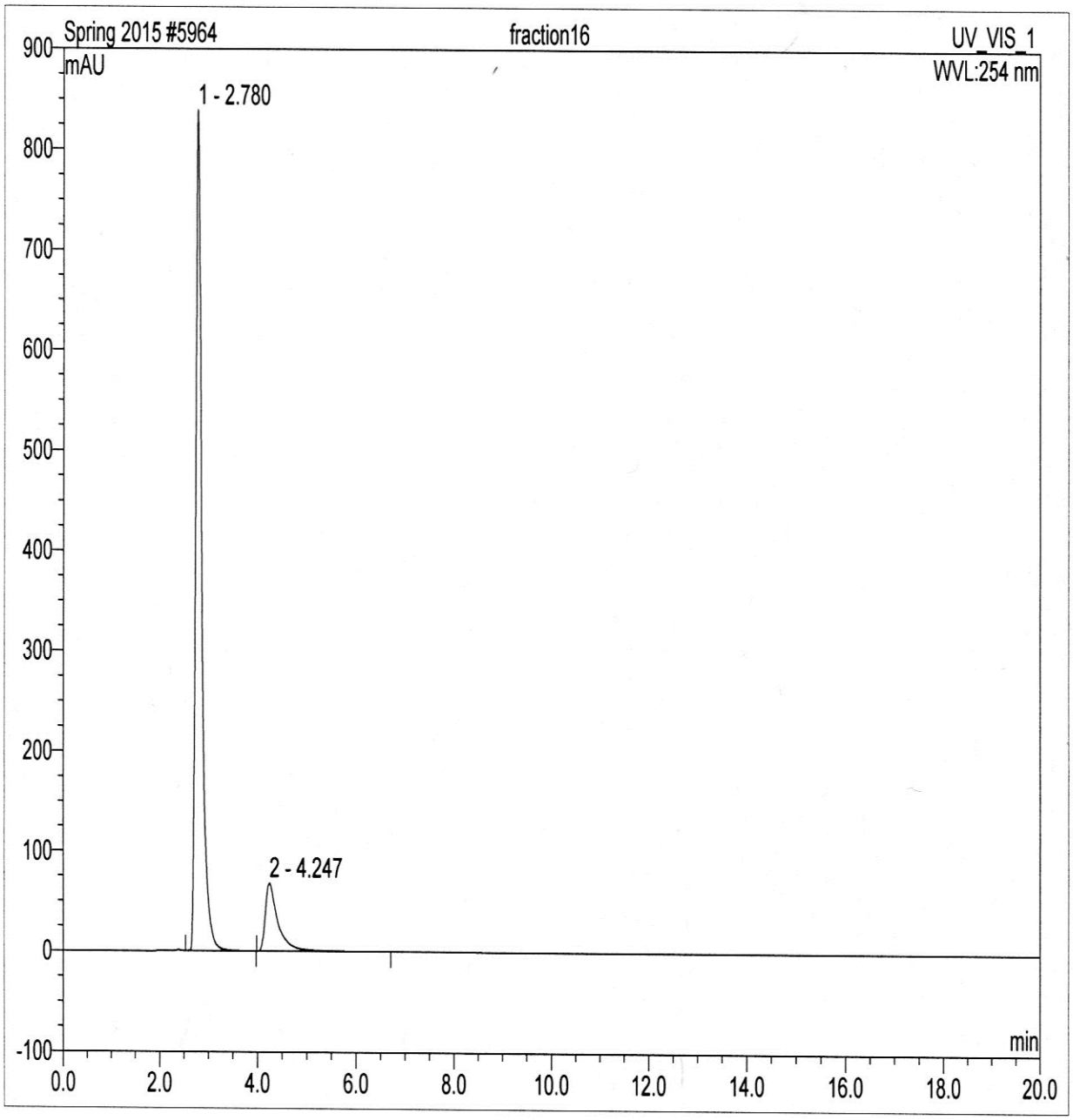


Figure 29. HPLC of analysis of the activity of Fraction 16 from hydroxyapatite column. The reaction mixture consisted 1 mL 1 mM adenosine in 10 mM Tris pH 7.2 and 100 μL of Fraction 16 from hydroxyapatite column. After 19 hours, the chromatogram shows adenosine has disappeared. The products show a small amount of adenine was present (4.24 min), while hypoxanthine (2.78min) was the major product present.

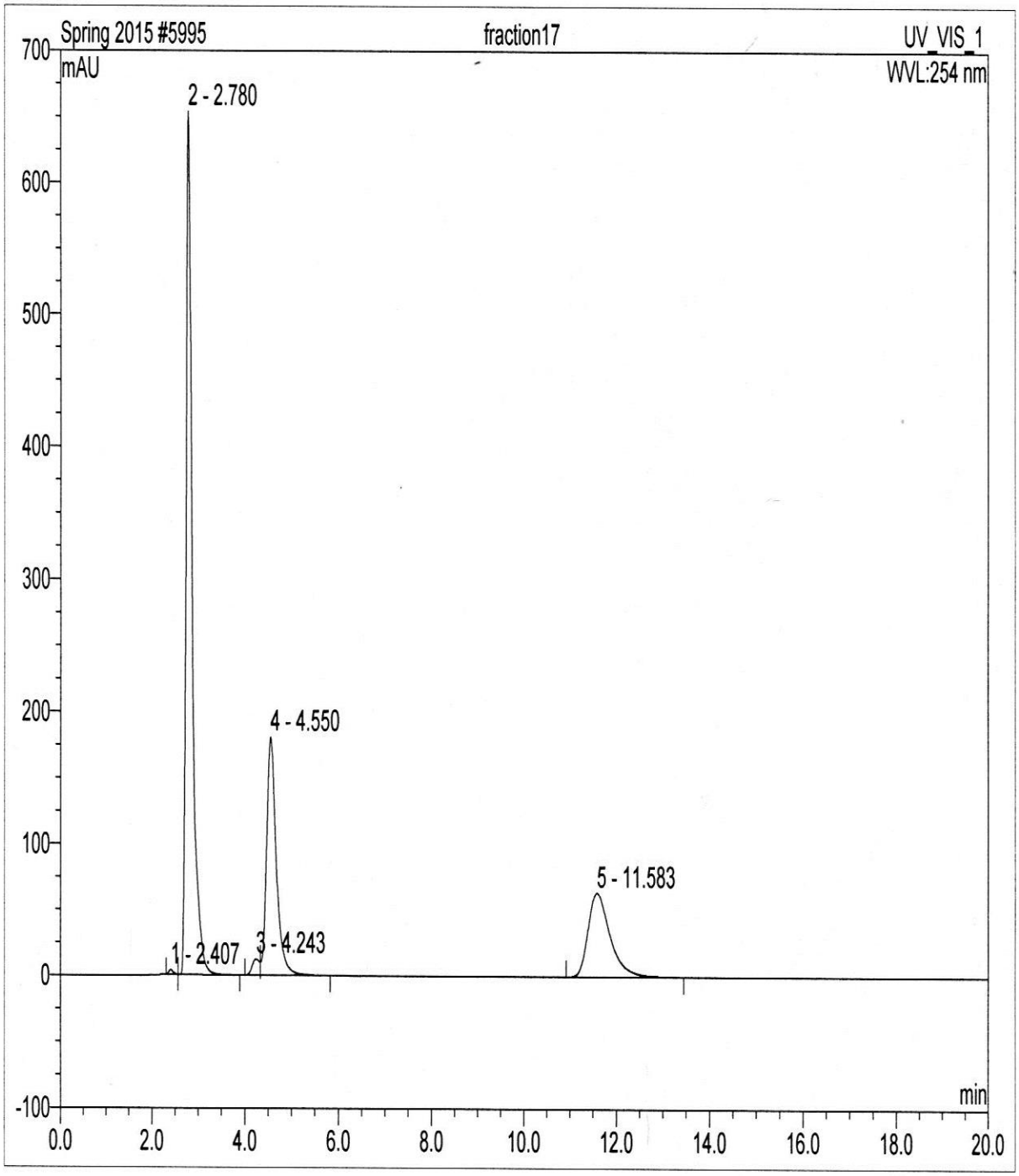


Figure 30. HPLC of analysis the activity of Fraction 17 from hydroxyapatite column. The reaction mixture consisted 1 mL 1 mM adenosine in 10 mM Tris pH 7.2 and 100 μL of Fraction 17 from hydroxyapatite column. After 20 hours, the chromatogram shows adenosine (11.58 min), adenine/ inosine (4.55 min), and hypoxanthine (2.78 min).

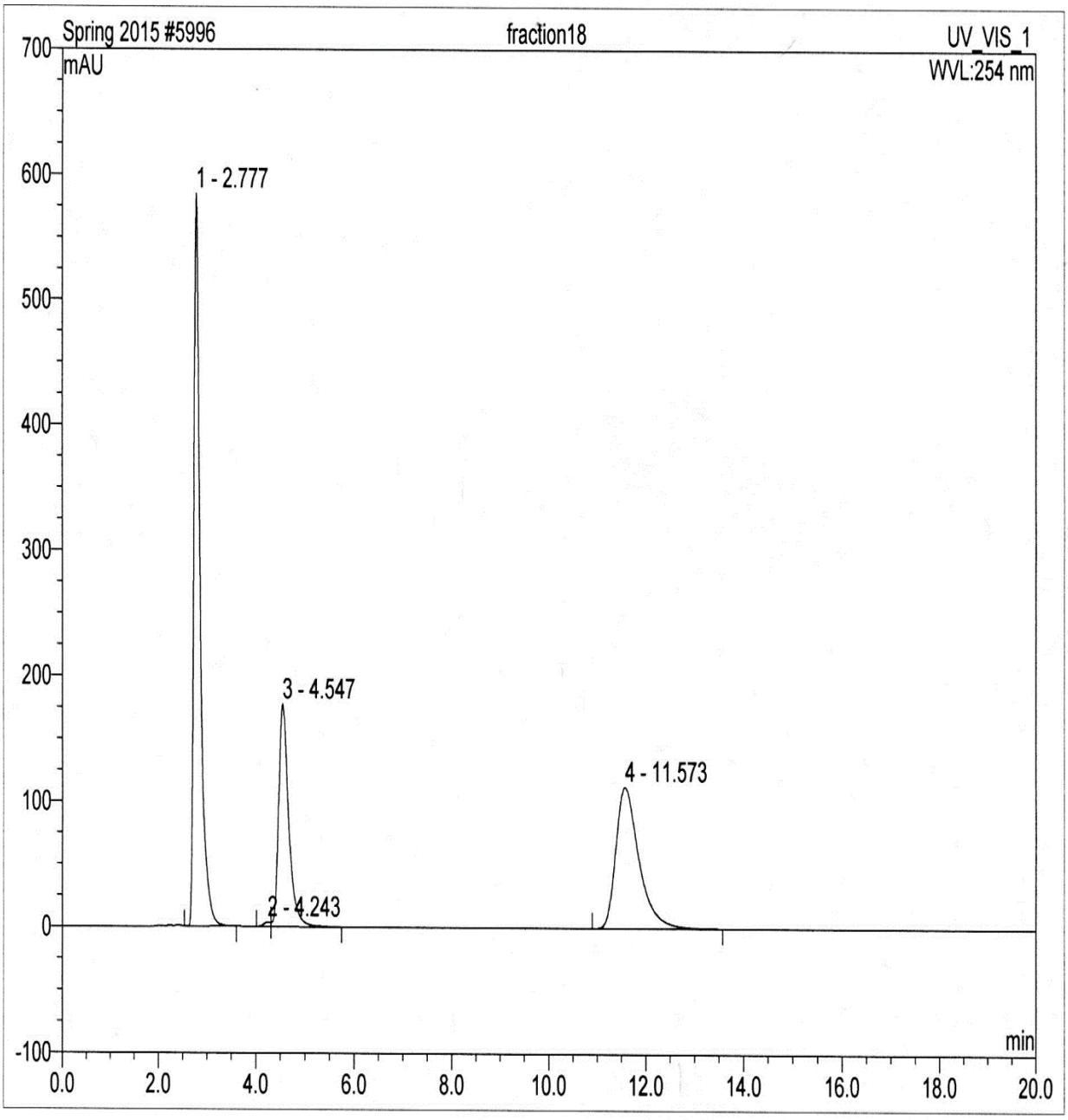


Figure 31. HPLC of analysis the activity of Fraction 18 from hydroxyapatite column. The reaction mixture consisted 1 mL of 1 mM adenosine in 10 mM Tris pH 7.2 and 100 µL of Fraction 18 from hydroxyapatite column. After 20 hours, the chromatogram shows adenosine (11.57 min), adenine/ inosine (4.54 min), and hypoxanthine (2.77 min).

Table 2. Summary of adenosine nucleosidase purification from Alaska pea seeds.

Step	Total volume (mL)	Total protein (mg)	Activity (μmol/min)	Total Activity (μmol/min)	Specific Activity (μmol/min*mg)	Purification Fold	% Recovery
Initial Extract	610	5813.3	$2.7x10^{-3}$	32.94	$5.66x10^{-3}$	1	100
Ammonium sulfate 30% Supernatant	660	5484.6	$2.5x10^{-3}$	33	$6.01x10^{-3}$	1.06	100.1
Ammonium sulfate 60% Pellet	95	8.73	$2.3x10^{-3}$	4.37	$5.26x10^{-3}$	0.92	13.26
DEAE (Ion exchange) (Pool #2)	70	4.15	$3.2x10^{-3}$	3.56	$1.54x10^{-2}$	2.72	10.80
Hydroxyapatite (Pool #1)	8.5	1.53	$5.6x10^{-4}$	0.23	$1.71x10^{-2}$	3.02	0.56

Molecular Weight of Adenosine Nucleosidase

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was run on Fractions 3-4 and Fractions 16-18 from the hydroxyapatite column to determine the molecular weight and to determine if the enzyme was pure or not. To calculate the molecular weight Precision Plus Protein™ Unstained Standards were used to construct a calibration curve (Figure 32). The molecular weight for both fractions was approximately 26,000 daltons.

In the gel, the protein in Fractions 3-4 appears to be~95% pure while Fractions 16-18 appear to be 90% pure (Figure 33). The molecular weight subunit of the purified enzyme is like adenosine nucleosidase molecular weight (26,103 daltons) that was purified by Shamsuddin from Alaska pea seeds (*38*).

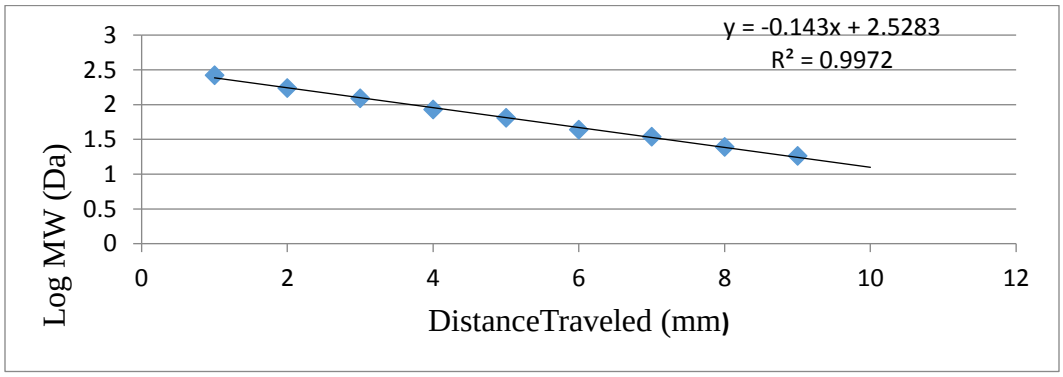


Figure 32. SDS-PAGE calibration curve. To determine the molecular weight based on Precision Plus Protein™ unstained standards. The band from Fractions 3 and 4 traveled 7.70 mm and the band from Fractions 16, 17 and 18 traveled 7.80 mm. The subunit molecular weight for adenosine nucleosidase from Fractions 3 and 4 and Fractions 16, 17 and 18 were~26.7 kD and~25,.8 kD respectively.

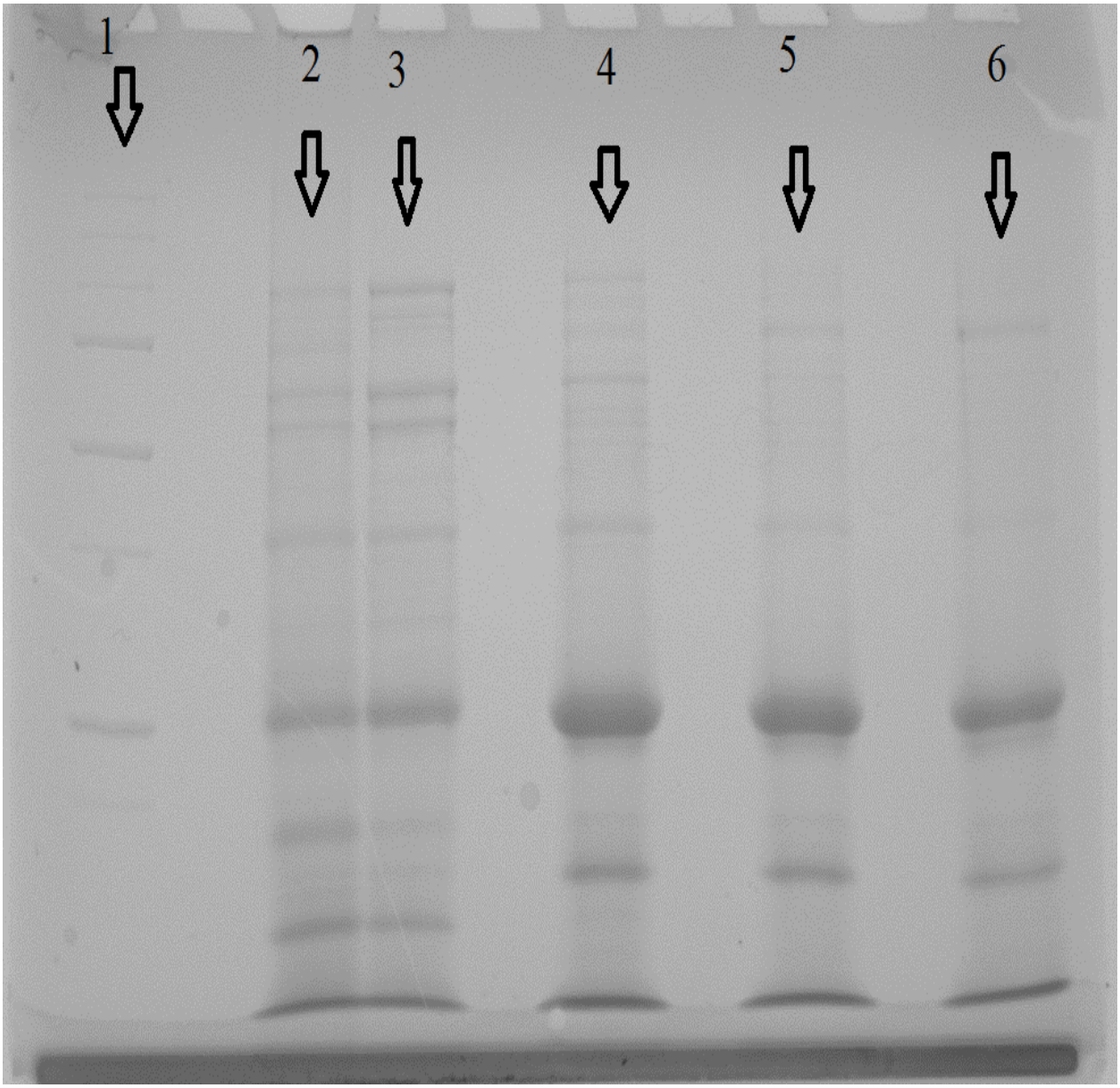


Figure 33. Determination of the subunit molecular weight of adenosine nucleosidase. From Fractions 3-4 and Fractions 16-18 from hydroxyapatite column using SDS-PAGE. Lane 1 is Precision Plus Protein™ Unstained Markers. Lanes 2 and 3 are Fractions 3 and 4 respectively from hydroxyapatite column. Lanes 4, 5 and 6 Fraction 16, 17 and 18 respectively from hydroxyapatite column. Based on observation of the intensity of bands Fractions 3-4 appear to be~95% pure while Fractions 16-18 are~90% pure.

Kinetic Analysis

Some enzymes follow Michaelis-Menten kinetics, which is described by the Michaelis-Menten equation.

$$V_{\max} = k_{cat}\left[E_T\right]; K_M = \frac{k_{off} + k_{cat}}{k_{on}}$$

$$v = \frac{V_{\max}\left[S\right]}{K_M + \left[S\right]}$$

Here, V_{max} represents the maximum rate achieved by the system at saturating substrate concentration. The Michaelis constant, $K_{M,}$ is the substrate concentration at which the reaction rate is half of V_{max} (*45*). The plot of the Michaelis-Menten equation predicts the reaction velocity as a function of substrate concentration (*45*). The velocity-substrate concentration profile was determined by measuring the velocity of the reaction at different substrate concentrations and plotting the data according to the Michaelis-Menten equation. A nonlinear regression of the Michaelis-Menten plot found the K_M for inosine to be 377.78±141.77 μM, with a V_{max} of 0.00078±0.000106 μM/min (Figure 34). A comparison of this value to the K_M of inosine nucleosidase from other sources is shown in Table 3.

y = m1*x/(m2+x)		
	Value	Error
m1	0.00078025	0.00010625
m2	377.78	141.77
Chisq	1.5248e-8	NA
R	0.96165	NA

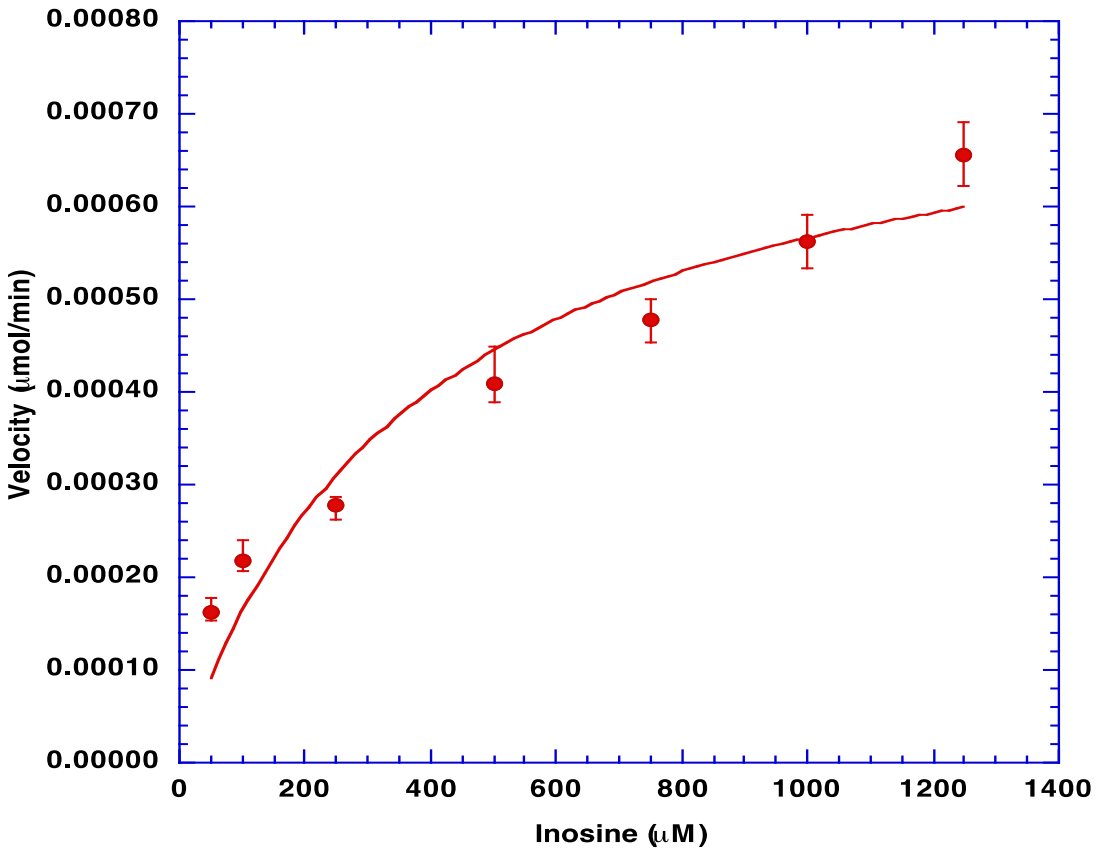


Figure 34. Kinetic analysis of adenosine nucleosidase from Alaska pea seeds. The substrate, inosine, concentrations used were approximately 50, 100, 250, 500, 750, 1000, and 1250 µM. The Michaelis-Menten equation yields a K_M of 377.78 ±141.77µM. The assay was run in duplicate.

Table 3. Characterization of adenosine nucleosidase from various plant sources.

Tissue	K_m (μM)	Molecular Weight (Da)	Reference
Wheat germ cells	1.43	59,000	33
Barley leaves	0.8-2.3	66,000	29
Yellow lupin seeds	4.7	177,000	34
Jerusalem artichoke shoots	17	----	32
Tea leaves	----	68,000	31
Alaska pea seeds	137	26,000	27

Substrate Specificity of Adenosine Nucleosidase

Substrate specificity is the ability of an enzyme to bind to compounds that have similar molecular shapes. The three-dimensional shapes of the substrate complement the three-dimensional shape of the active site (*46*). Several substrates were tested for activity with adenosine nucleosidase. The nucleosides tested were 2'-deoxyinosine, allopurinol riboside, 3'-deazauridine and adenine- β-arabinofuranoside. The reaction mixture consisted of 1 mM nucleoside in 10 mM Tris pH 7.2. Addition of 25 µL of the enzyme started the reaction. The control consisted of 25 µL of DI water instead of the enzyme solution. The reaction was incubated for 13 hours at 32 ºC. To determine if the nucleosides were a substrate or not, the appearance of the base and nucleoside were monitored by HPLC. The retention time of the nucleosides and bases were determined using standard samples (Table 4). All base/ nucleoside pairs were well resolved under the conditions used.

The adenosine nucleosidase from Alaska pea seeds hydrolyzed both purine and pyrimidines. Based on substrate specificity it belongs to the non-specific inosine-uridine nucleoside hydrolases (IU-NHs). Substrate specificity can be used to determine the importance of the number and the position of hydroxyl groups on ribose in nucleosides along with the importance of other groups on the nucleoside. Adenosine nucleoside from Alaska pea seeds did not depend on the number and position of hydroxyl groups on the ribose in nucleoside. For example, 2'-deoxyinosine is a substrate with adenosine nucleosidase from Alaska pea seeds. When comparing 2'-deoxyinosine and inosine structures, the hydroxyl group on carbon 2'-deoxyinosine is absent (Figure 35).

Therefore, the 2'-hydroxyl group is not critical for the reaction. Adenine-β-arabinofuranoside (Figure 36) is also a substrate for adenosine nucleosidase from Alaska pea seeds. Adenosine has the opposite stereochemistry from that of adenine-β-arabinofuranoside on the 2'-hydroxy group. Both are substrates.

Table 4. Retention time of nucleosides and bases. They were determined using standard samples.

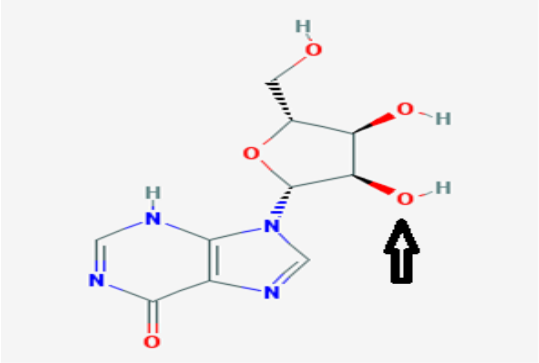
Nucleoside	Retention Time (min)	Base	Retention Time (min)
2'-Deoxyinosine	6.25	Hypoxanthine	3.11
Allopurinol Riboside	5.39	Allopurinol	4.06
3'-Deazauridine	4.46	----------	----------
Adenine-β-arabinofuranoside	10.49	Adenine	4.91

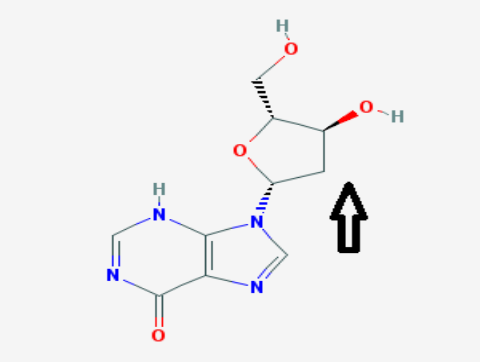
The structure of the base was critical for the reaction between the enzyme and the substrate. Purine riboside is not a substrate with adenosine nucleosidase from Alaska pea seeds and allopurinol riboside is not a substrate. In the base of allopurinol riboside, the N7 nitrogen is absent. In the proposed mechanism N7 is protonated promoting the destabilization of the N-glycosidic bond. Because allopurinol is not a substrate this is further evidence for this mechanism for plant nucleoside hydrolases. On the other hand, the difference between uridine and 3'-deazuridine is the absence of a nitrogen in position

3 and carbonyl group in carbon number 4. Uridine is a substrate with adenosine nucleosidase while 3'-deazuridine is not a substrate.

Table 5. Adenosine nucleosidase substrate specificity.

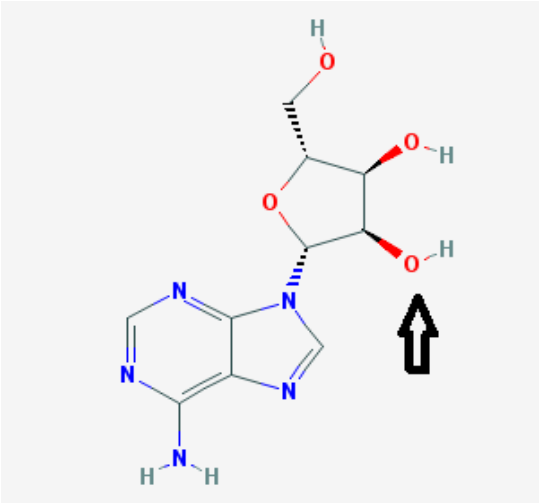
Nucleosides	Substrates
2'-Deoxyinosine	Yes
Allopurinol Riboside	No
3'-Deazauridine	No
Adenine-β-arabinofuranoside	Yes

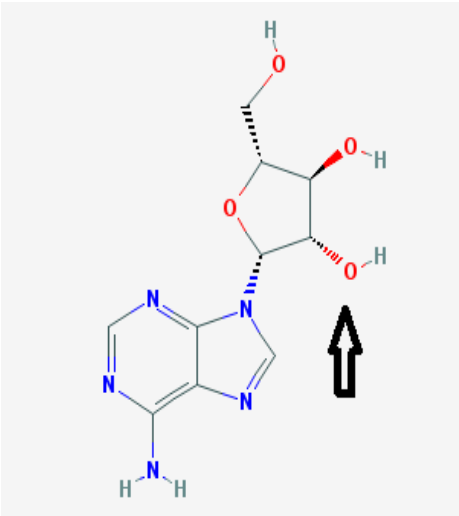




Inosine	2'-deoxyinosine
Substrate	Substrate

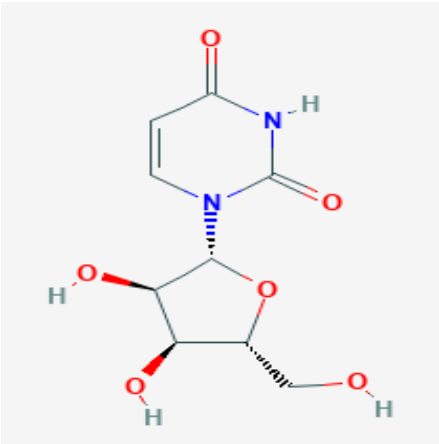
Figure 35. Structure of inosine and 2'-deoxyinosine (*19*).

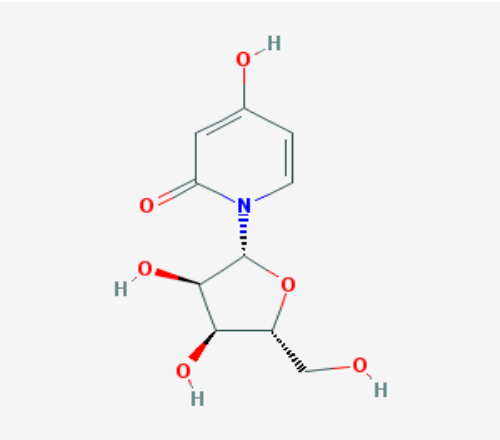




Adenosine	Adenine-β-arabinofuranoside
Substrate	Substrate

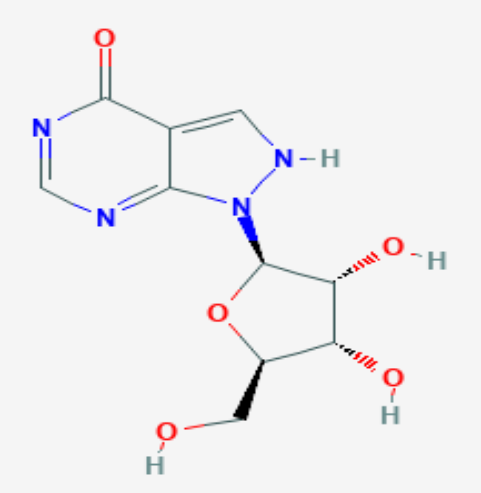
Figure 36. Structure of adenosine and adenine-β-arabinofuranoside (*19*).

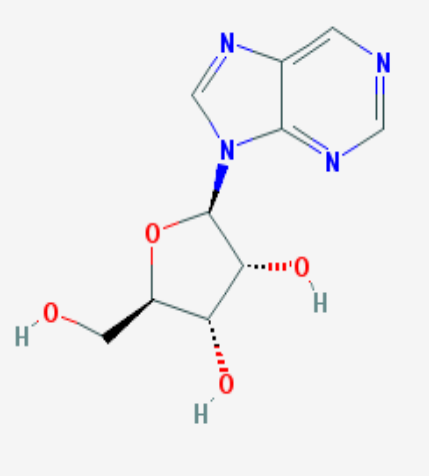




Uridine	3-deazauridine
Substrate	Not a substrate

Figure 37. Structure of uridine and 3-deazauridine (*19*).





Allopurinol riboside	Purine riboside
Not a substrate	Not a substrate

Figure 38. Structure of allopurinol riboside and purine riboside (*19*).

In the chromatogram below, the retention time of the control 2'-deoxyinosine was (6.25 min) while adding the 25 µL of adenosine nucleoside from Alaska pea seeds, the peak of hypoxanthine (3.1 min) was appeared after 25 hours. 2'-Deoxyinosine was a substrate with adenosine nucleosidase (Figures 39-40). On the other hand, the retention time of the control allopurinol riboside was (5.3 min) while adding the same amount of adenosine nucleoside from Alaska pea seed, the peak of allopurinol did not appear after 45 hours (Figures 41-42. So, allopurinol riboside was not a substrate with adenosine nucleoside. In the chromatogram, the 20% of adenine-β-arabinofuranoside converted to adenine (Figures 43-44). So, adenine-β-arabinofuranoside was a substrate with adenosine nucleoside from Alaska pea seeds. The retention time of 3- deazauridine was (4.4 min)

after adding the adenosine nucleoside, the chromatogram did not show any peak except the peak of 3-deazauridine (Figures 45-46).

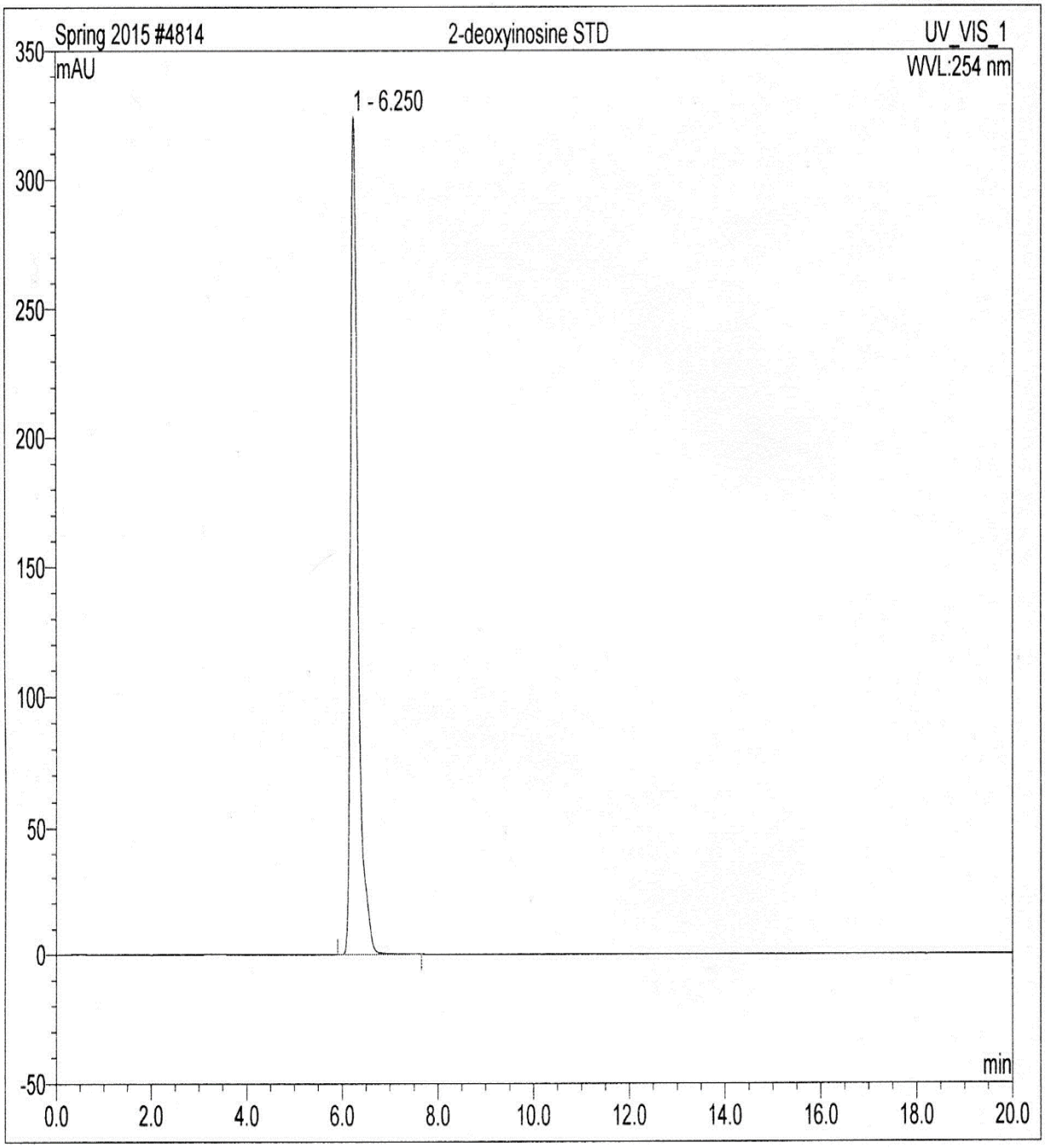


Figure 39. HPLC of analysis 2'-deoxyinosine. The standard consisted of 1 mL 1 mM 2'-deoxyinosine in 1 M Tris pH 7.2. The chromatogram shows 2'-deoxyinosine (6.25 min).

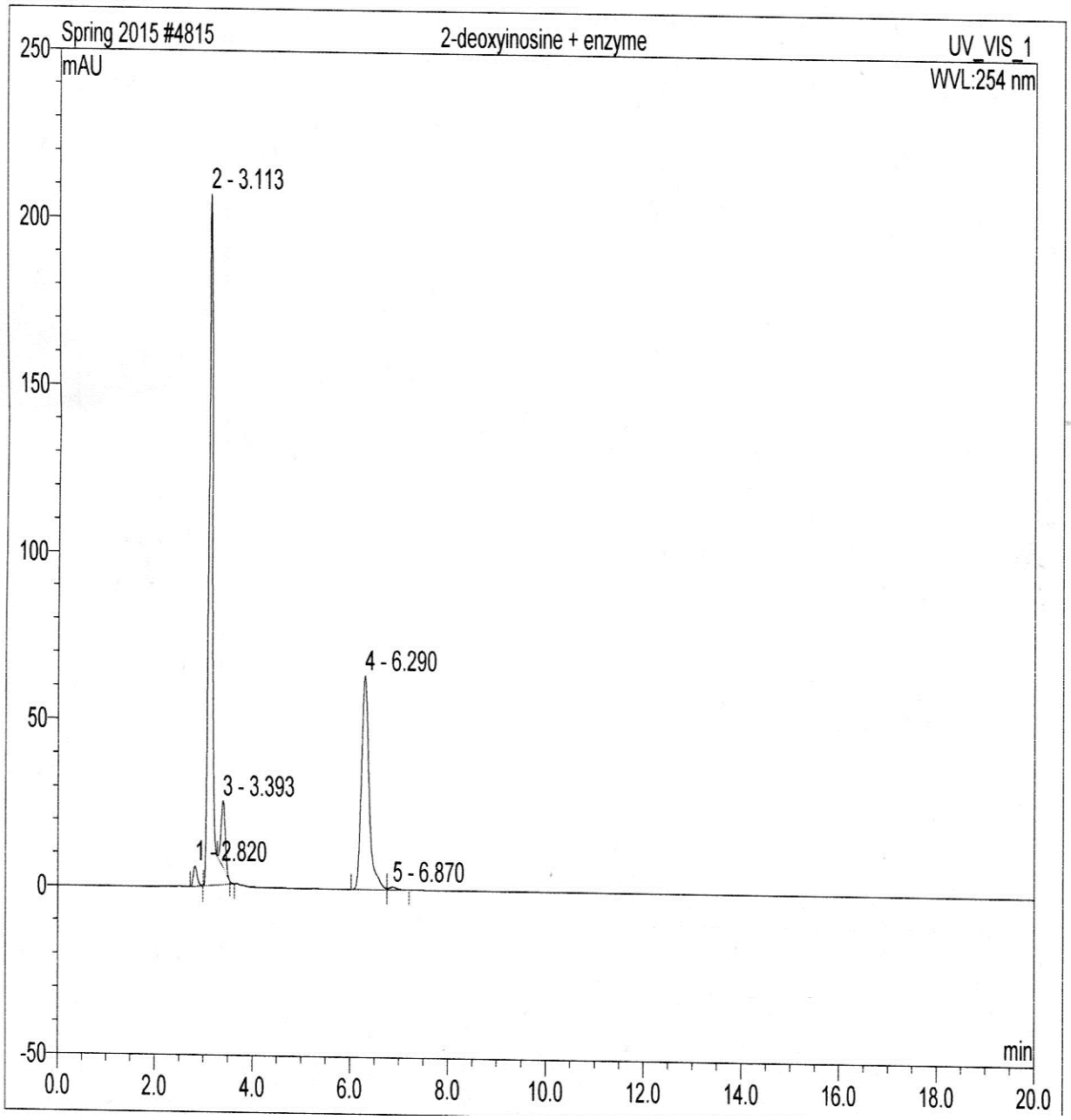


Figure 40. HPLC analysis of the activity of 2'-deoxyinosine. The reaction mixture consisted of 1 mL 1 mM 2'-deoxyinosine in 1 M Tris pH 7.2 and 25 µL of adenosine nucleosidase from Alaska pea seeds. After 25 hours, the chromatogram shows 2'-deoxyinosine (6.29 min) and hypoxanthine (3.11 min).

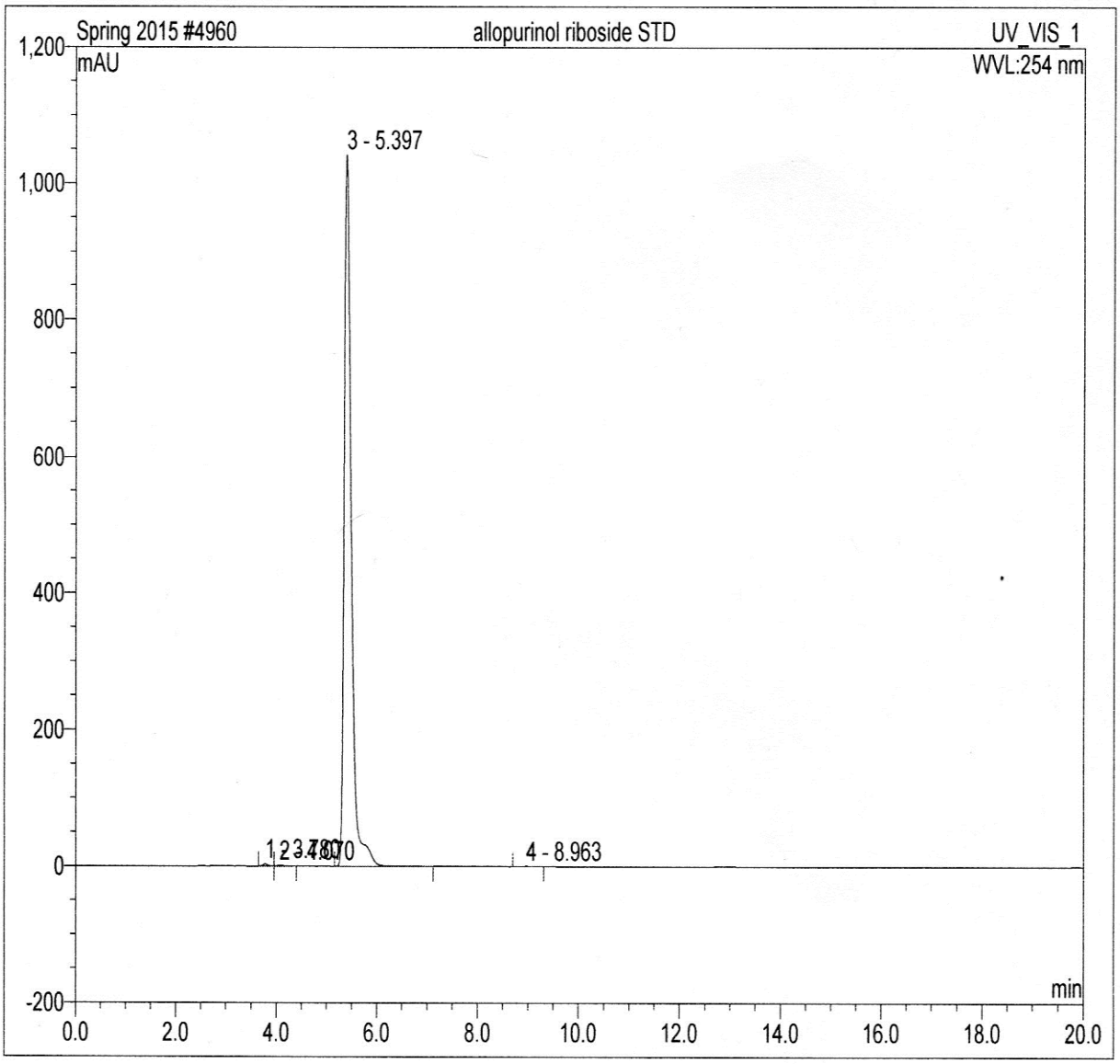


Figure 41. HPLC of analysis allopurinol riboside. The standard consisted of 1 mL 1 mM allopurinol riboside in 1 M Tris pH 7.2. The chromatogram shows allopurinol riboside (5.39 min).

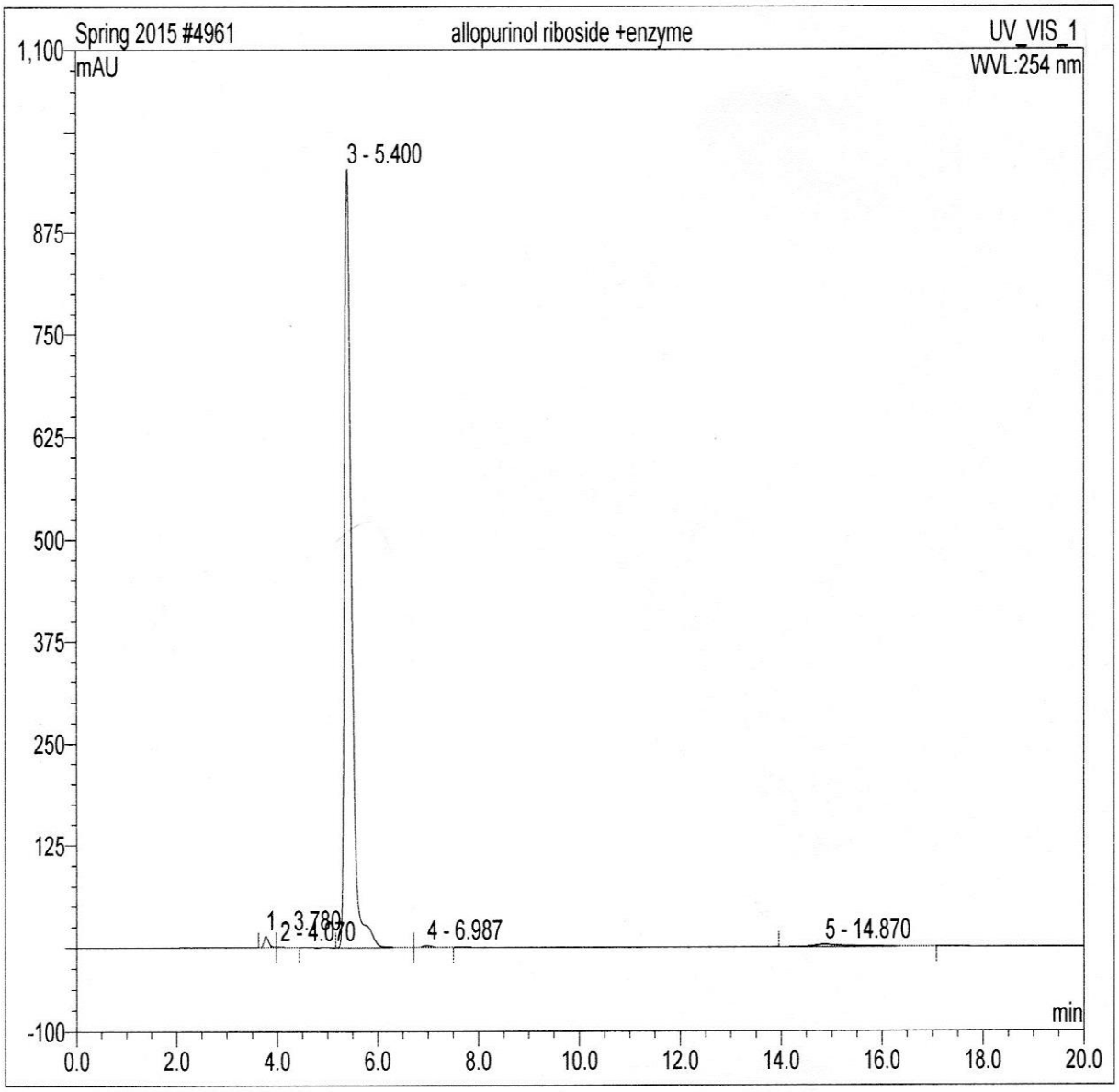


Figure 42. HPLC analysis of the activity of allopurinol riboside. The reaction mixture consisted of 1 mL 1 mM allopurinol riboside in 1 M Tris pH 7.2 and 25 μL of adenosine nucleosidase from Alaska pea seeds. After 45 hours, the chromatogram shows allopurinol riboside (5.40 min).

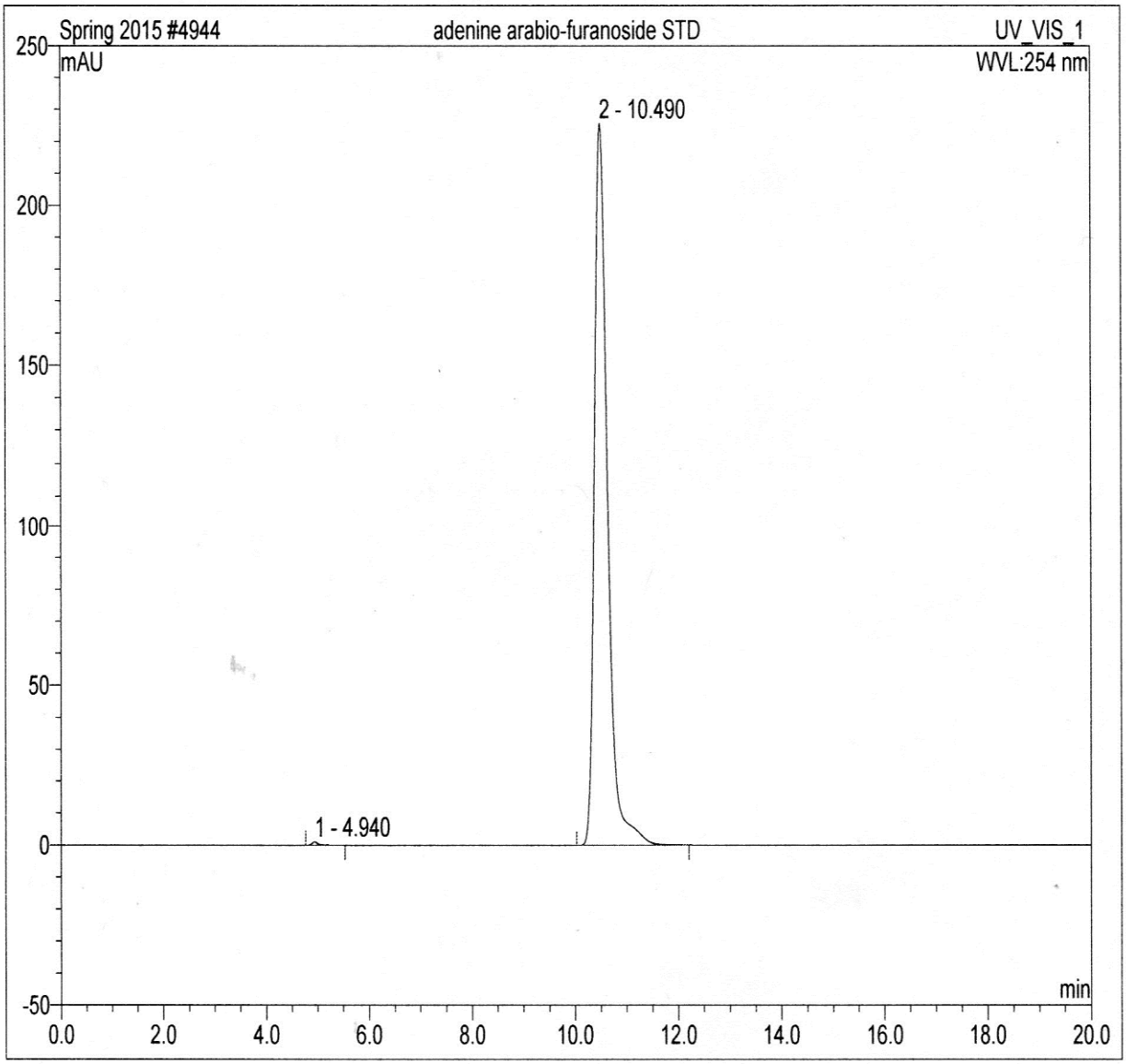


Figure 43. HPLC analysis of adenine-β-arabinofuranoside. The standard consisted of 1 mL 1 mM adenine-β-arabinofuranoside in 1 M Tris pH 7.2. The chromatogram shows adenine-β-arabinofuranoside (10.50min).

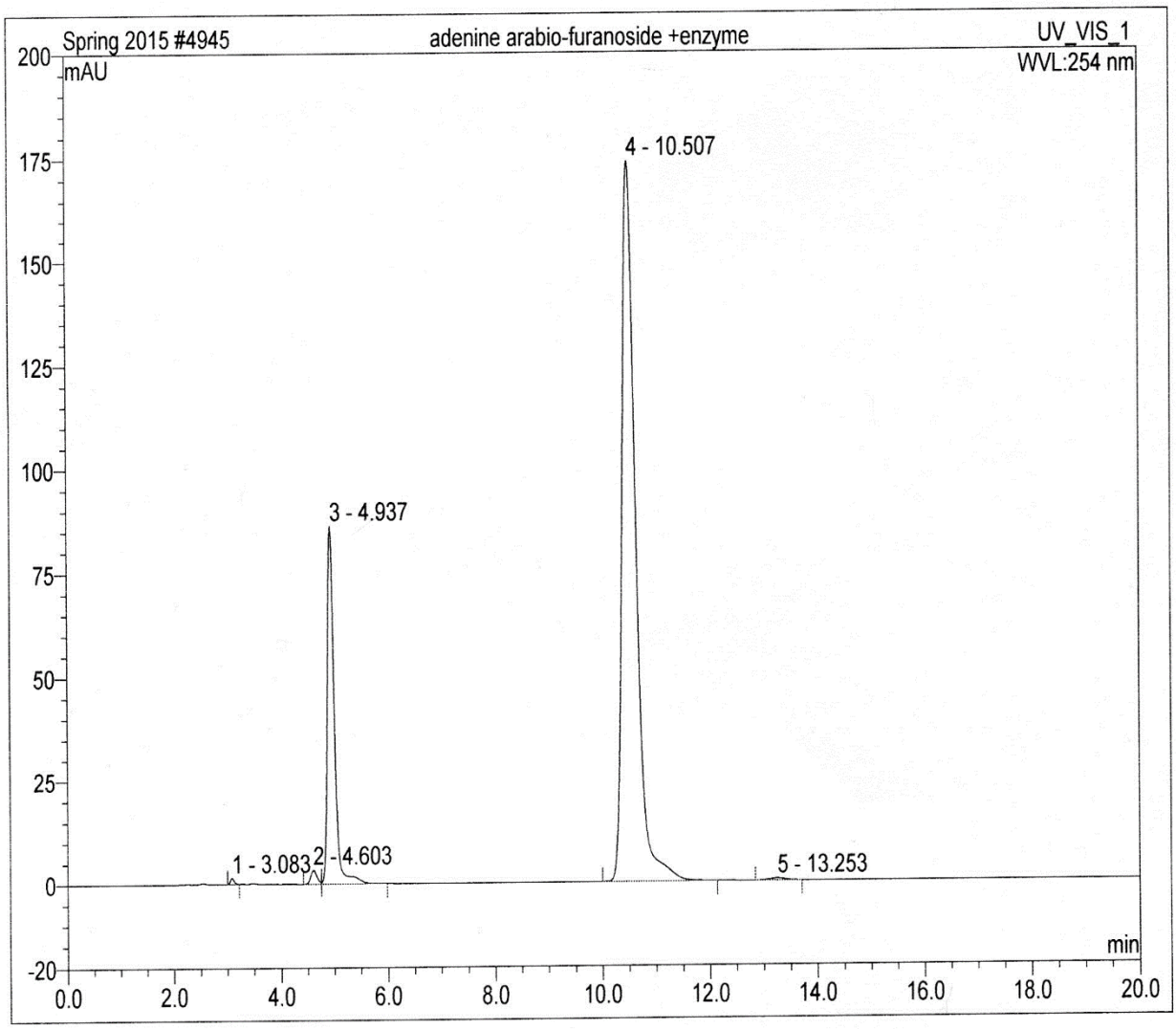


Figure 44. HPLC analysis of the activity of adenine-β-arabinofuranoside. The reaction mixture consisted of 1 mL 1 mM adenine-β-arabinofuranoside in 1 M Tris pH 7.2 and 25 μL of adenosine nucleosidase from Alaska pea seeds. After 58 hours, the chromatogram shows adenine-β-arabinofuranoside (10.50 min) and 20% of adenine-β-arabinofuranoside converted to adenine (4.93 min).

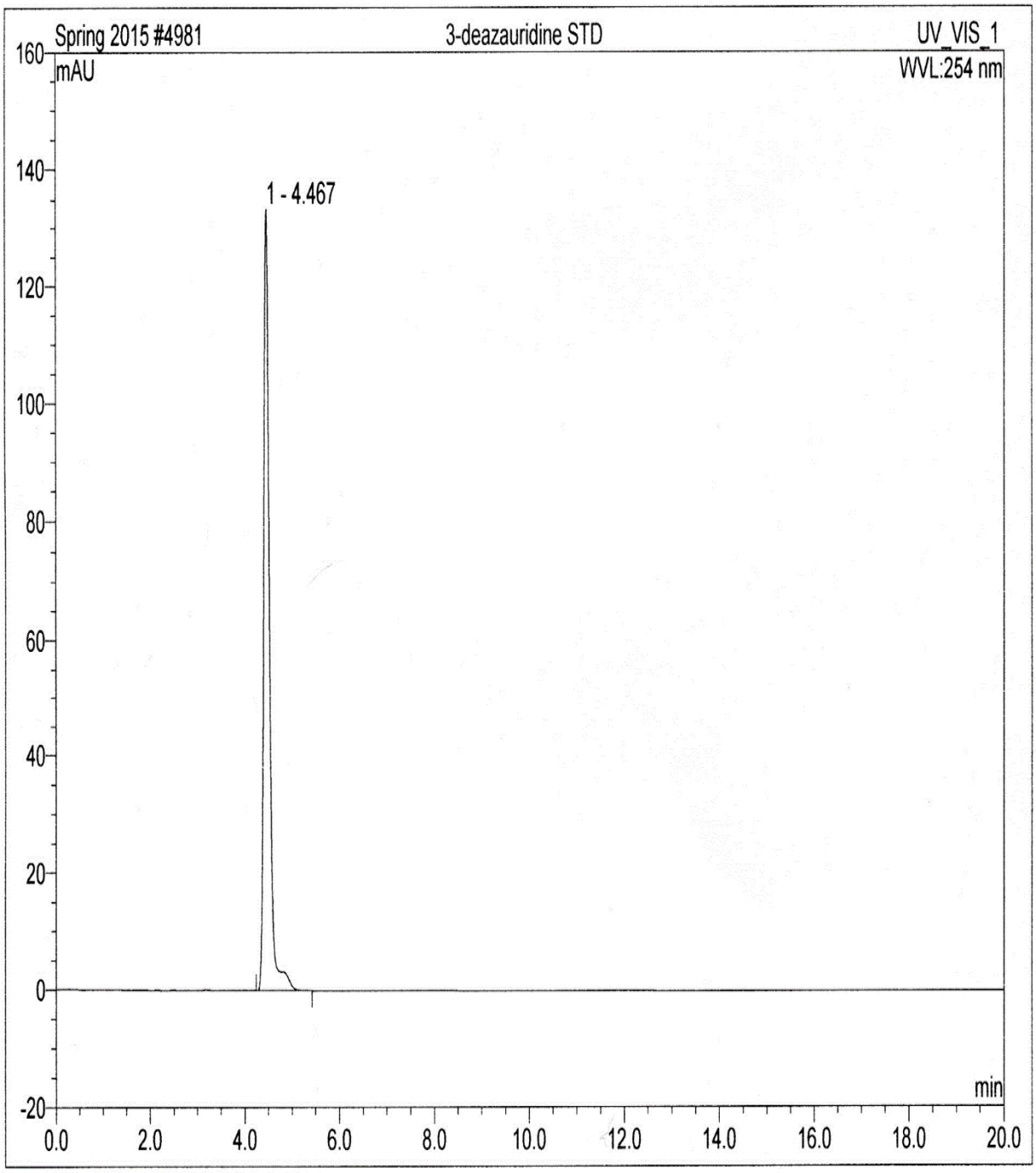


Figure 45. HPLC analysis of 3-deazauridine. The standard consisted of 1 mL 1 mM 3'-deazauridine in 1 M Tris pH 7.2. The chromatogram shows 3'-deazauridine (4.46 min).

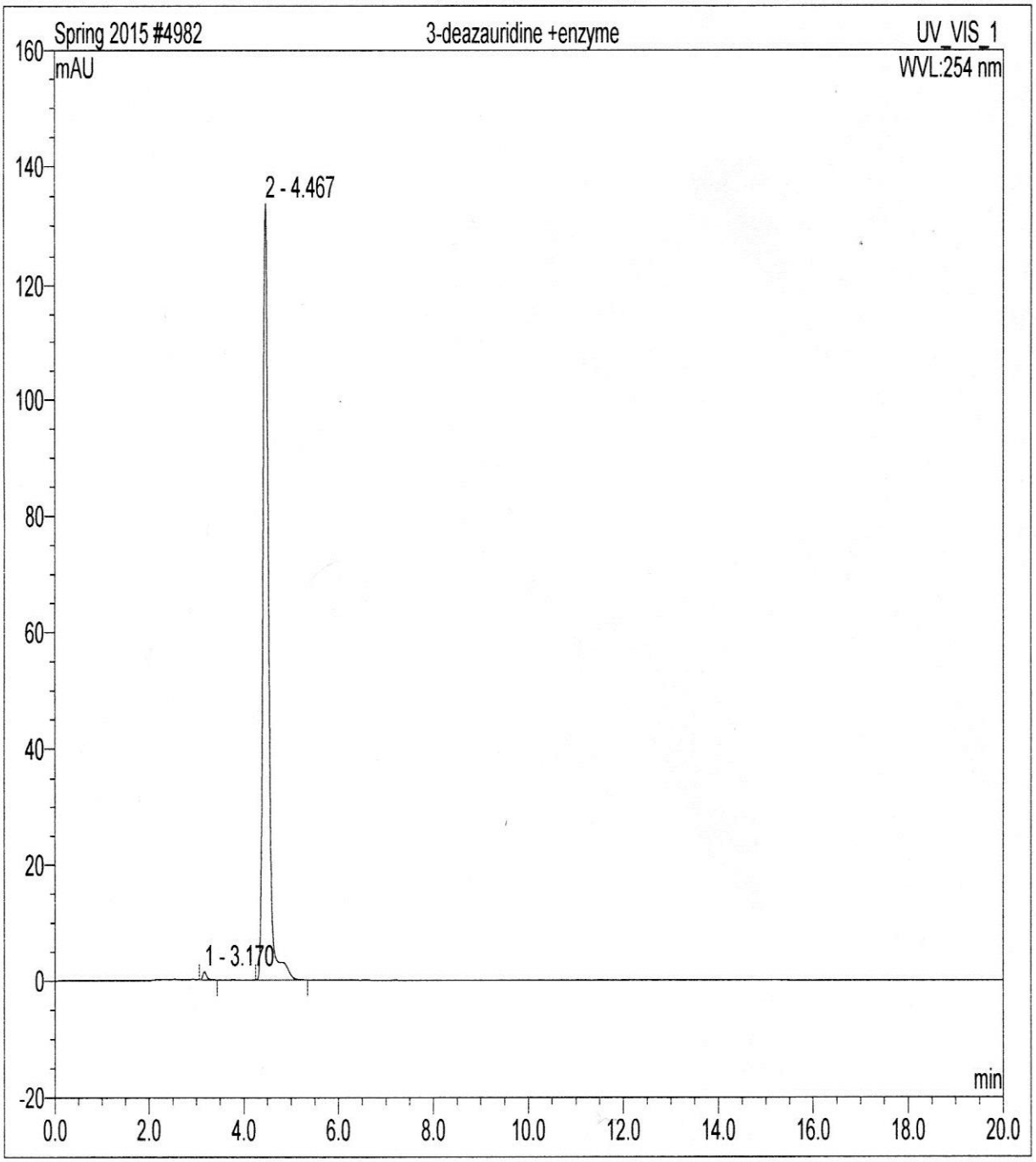


Figure 46. HPLC analysis of the activity of 3-deazuridine. The reaction mixture consisted of 1 mL 1 mM 3-deazuridine in 1 M Tris pH 7.2 and 25 μL of adenosine nucleosidase from Alaska pea seeds. After 19 hours, the chromatogram shows 3-deazuridine (4.46 min).

CHAPTER IV

CONCLUSION

A family of enzymes referred to as nucleoside hydrolases catalyze the hydrolysis of the N-glycosidic bond in pyrimidine and purine to yield the corresponding base and ribose. For instance, adenosine nucleosidase catalyses the hydrolysis of adenosine's glycosidic bond resulting in ribose and adenine. The enzyme, which is part of pyrimidine and purine recovery, enables the base to be reused without the need for extra energy expenditure required to synthesize it. Nucleoside hydrolases have been widely studies in parasitic protozoans.

Adenosine nucleosidase was purified from Alaska pea seeds. It is most active after six days from germination of the seeds, with a 3-fold purification and 0.56 % recovery. The subunit molecular weight was calculated by SDS-PAGE to be~26 kD that is a similar with the result of Shamsuddin (38). The K_M, or Michaelis constant, and V_{max}, or maximum velocity, using inosine as a substrate were calculated to be 377.78 ±141.7 μM, and 0.00078 ±0.000106 μM/min respectively. The substrate specificity show that the purified enzyme, belongs to the non-specific inosine-uridine nucleoside hydrolases (IU-NHs). Adenosine nucleosidase from Alaska pea seeds did not require a 2'-deoxy group on the ribose moiety to catalyze the reaction. 2'-Deoxyinosine was a substrate with adenosine nucleosidase from Alaska pea seeds while allopurinol riboside was not a substrate. Adenine-β-arabinofuranoside was a substrate, because 20% of adenine-β-

arabinofuranoside was converted to adenine. 3-Deazauridine was not a substrate with adenosine nucleosidase from Alaska pea seeds.

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