STUDY OF NUCLEOSIDE DEGRADING ENZYME ACTIVITIES IN BEAN, ORGANIC BEAN, OKRA, ORGANIC OKRA, SQUASH AND ORGANIC SQUASH

by

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I dedicate this research to my parents, my sisters, and my brothers. I love you all.

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Finally, I would like to express my thanks to my family and my friends for their support and love. Special thanks to my loving parents, my brothers and my sisters for their prayer, concern and kind words over the years.

ABSTRACT

Pyrimidine and purine nucleotide metabolism are essential for development and growth of all organisms. Nucleoside degradation reactions have been found in virtually all organisms. Many enzymes are involved in the degradation and salvage of nucleotides, nucleobases and nucleosides. Deaminases contribute in interconversion of one nucleoside into another by removing amino groups from the base. Nucleoside hydrolase is a glycosidase that catalyzes the cleavage of the N-glycosidic bond in nucleosides to facilitate recycling of nucleobases.

The nucleoside degrading enzyme activity was tested by adding a crude protein extract from 5-9 day old bean, organic bean, okra, organic okra, squash and organic squash seedlings to reaction mixtures containing either inosine, uridine, cytidine, adenosine, or 2'-deoxyadenosine. The activities of the enzyme were determined on a Dionex Ultimate 3000 High Performance Liquid chromatography system (HPLC). Nucleosidases were found to be active in all six plants and specific activities are reported.

There are variations in the level of activities observed between bean, organic bean, okra, organic okra, squash and organic squash. Based on the specific activities the highest activity was recorded for uridine, cytidine and adenosine in different types of seeds while inosine and 2'deoxyadenosine had the lowest activity. Bean was the lowest in activity with all five nucleosidases compared to nucleosidase metabolizing activities in organic bean, okra, organic okra, squash and organic squash.

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CHAPTER I

INTRODUCTION

Nucleotides are important molecules since they are major sources of energy and subunits of nucleic acids. They are also the precursors for the synthesis of cofactors such as nicotinamide adenine dinucleotide (NAD) and *S*-adenosyl methionine (SAM).[1] Nucleotides participate in cell metabolism, development and growth. Nucleotides are composed of three parts: a five-carbon sugar (ribose or 2-deoxyribose), nitrogenous base (purine and pyrimidine), and at least one phosphate group. Nucleotides without phosphate groups are nucleosides. The nitrogenous bases, purines or pyrimidines, in nucleosides are linked to the pentose sugar through a ß-N-glycosidic bond that is formed between carbon 1 of the sugar and N9 of a purine and or N1 of a pyrimidine (Figure 1). The sugar in DNA is 2-deoxyribose. It differs from ribose in RNA since the second carbon lacks a hydroxyl group (Figure 2).[1]

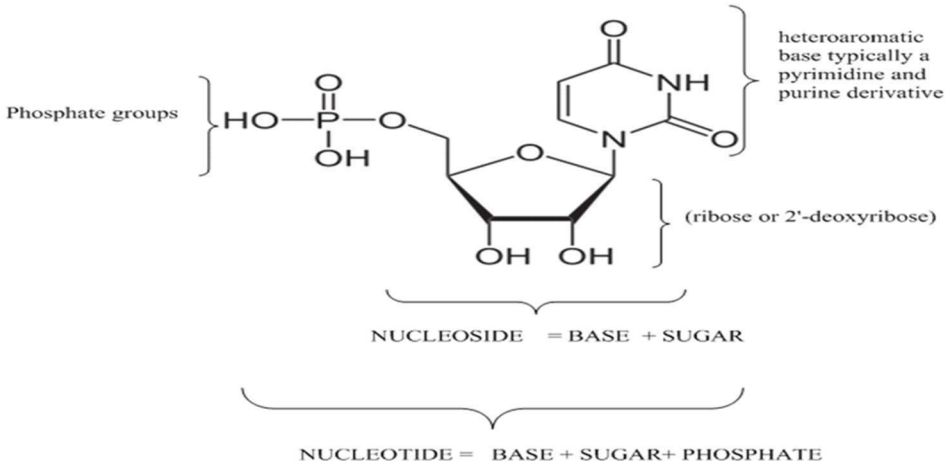


Figure 1: Structural elements of nucleosides and nucleotides.

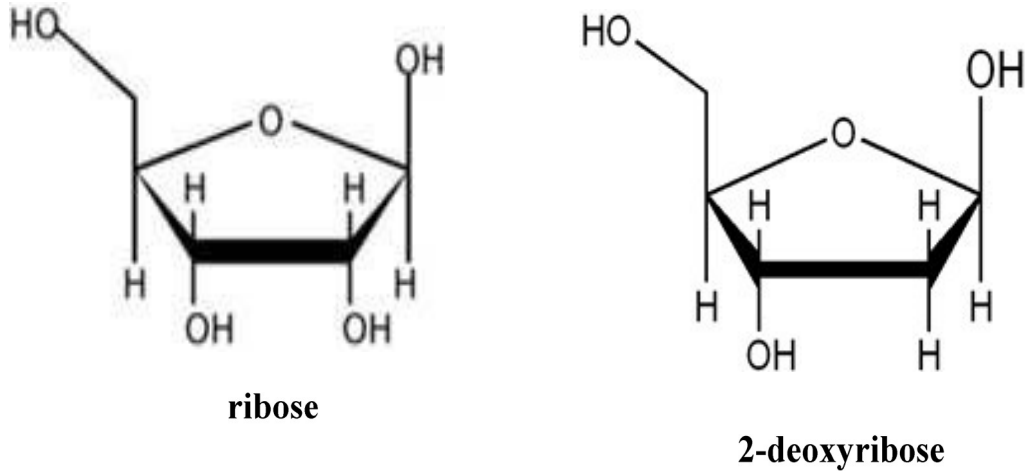


Figure 2: The structures of ribose in RNA and 2-deoxyribose in DNA.

Purines and Pyrimidines

Purines and pyrimidines are the two major families of nitrogenous bases, which are required for the construction of genetic materials in all organisms. Common purine bases include adenine, guanine and hypoxanthine corresponding to the nucleosides adenosine, guanosine, and inosine, respectively. Each purine consists of a double ring with a heterocyclic five-atom ring, fused on one side to a heterocyclic six-atom ring. Pyrimidine bases consist of uracil, thymine and cytosine corresponding to the nucleosides uridine, thymidine and cytidine, respectively, each having a single 6-member ring (Figure 3).

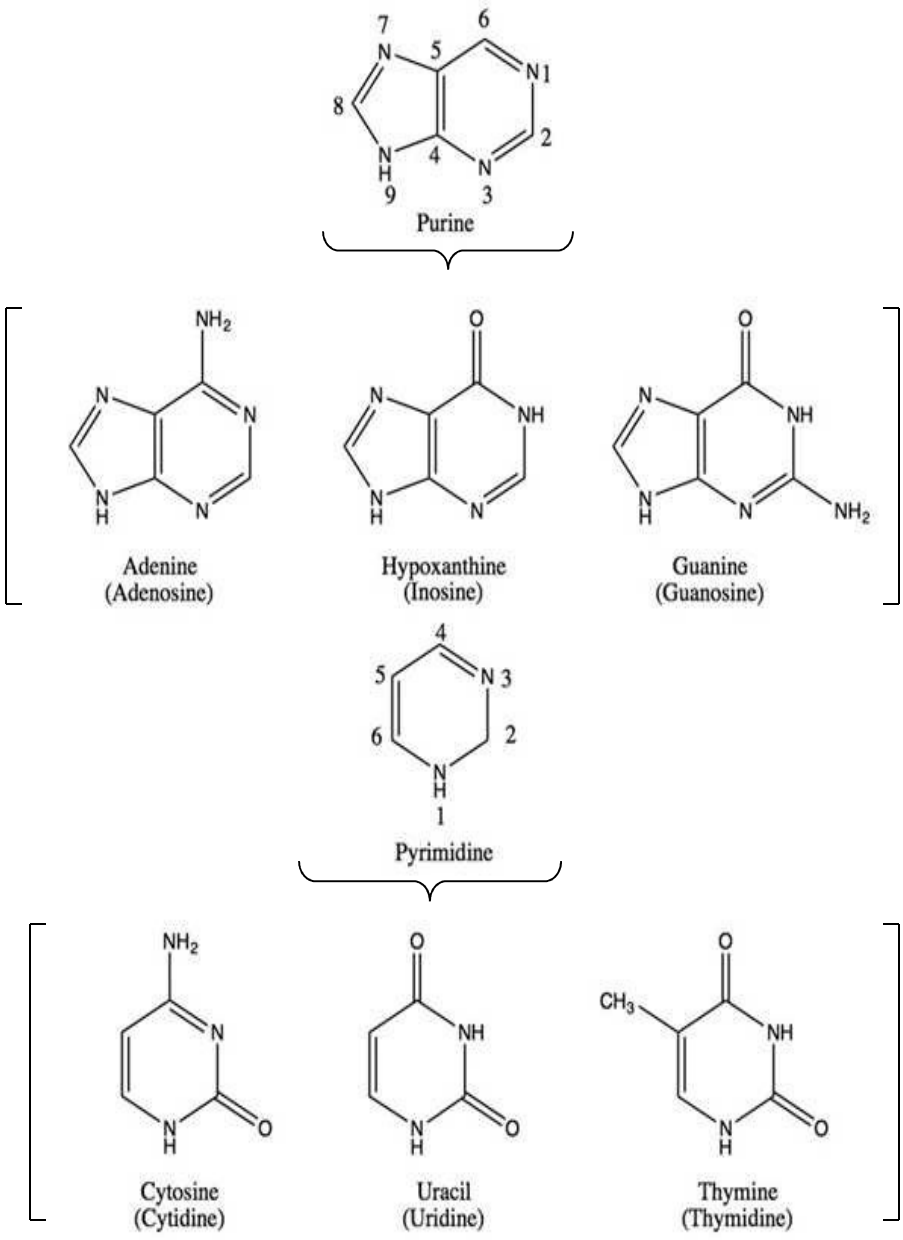


Figure 3: Common purine and pyrimidine bases with their numbering schemes.

Purine and Pyrimidine Metabolism

Metabolism of nucleotides can be categorized into (a) *de novo* synthesis, (b) nucleotide degradation, (c) salvage pathways, which recycle nucleotides as well as free bases, and (d) phosphotransfer reactions.[1] It has been shown that the pathways for synthesizing purine and pyrimidine nucleotides in plants are similar to those found in animals.[2] Additionally, specific nucleotides are transformed, for example to deoxynucleotides in the case of DNA synthesis or by adding side chains to form unusual nucleotides, such as constituents of tRNA. Each nucleotide and its intermediates are also precursors in the synthesis of hormones and secondary metabolites. For instance, UTP and UDP involved in the degradation and synthesis of sucrose and UDP-glucose as co-substrates.[1]

De novo biosynthesis of pyrimidines involves uridine monophosphate (UMP) formation from carbamoyl phosphate (CP), 5-phosphoribosyl-1-pyrophosphate (PRPP), and aspartate. *De novo* biosynthesis of pyrimidine nucleotide is also called the orotate pathway.[1] Pyrimidine metabolism involves six enzymatic steps (Figure 4 steps 1– 6).[1] Carbamoylphosphate synthase (CPSase) forms carbamoyl phosphate (CP) from adenosine triphosphate (ATP), carbonate, and an amino group from glutamine. Carbamoyl phosphate (CP) is utilized in the synthesis of both pyrimidines and as an arginine biosynthesis precursor. In the second step, aspartate transcarbamoylase catalyzes CP condensation with aspartate to produce carbamoyl aspartate (CA), which is unique to the biosynthesis of pyrimidines.[1]

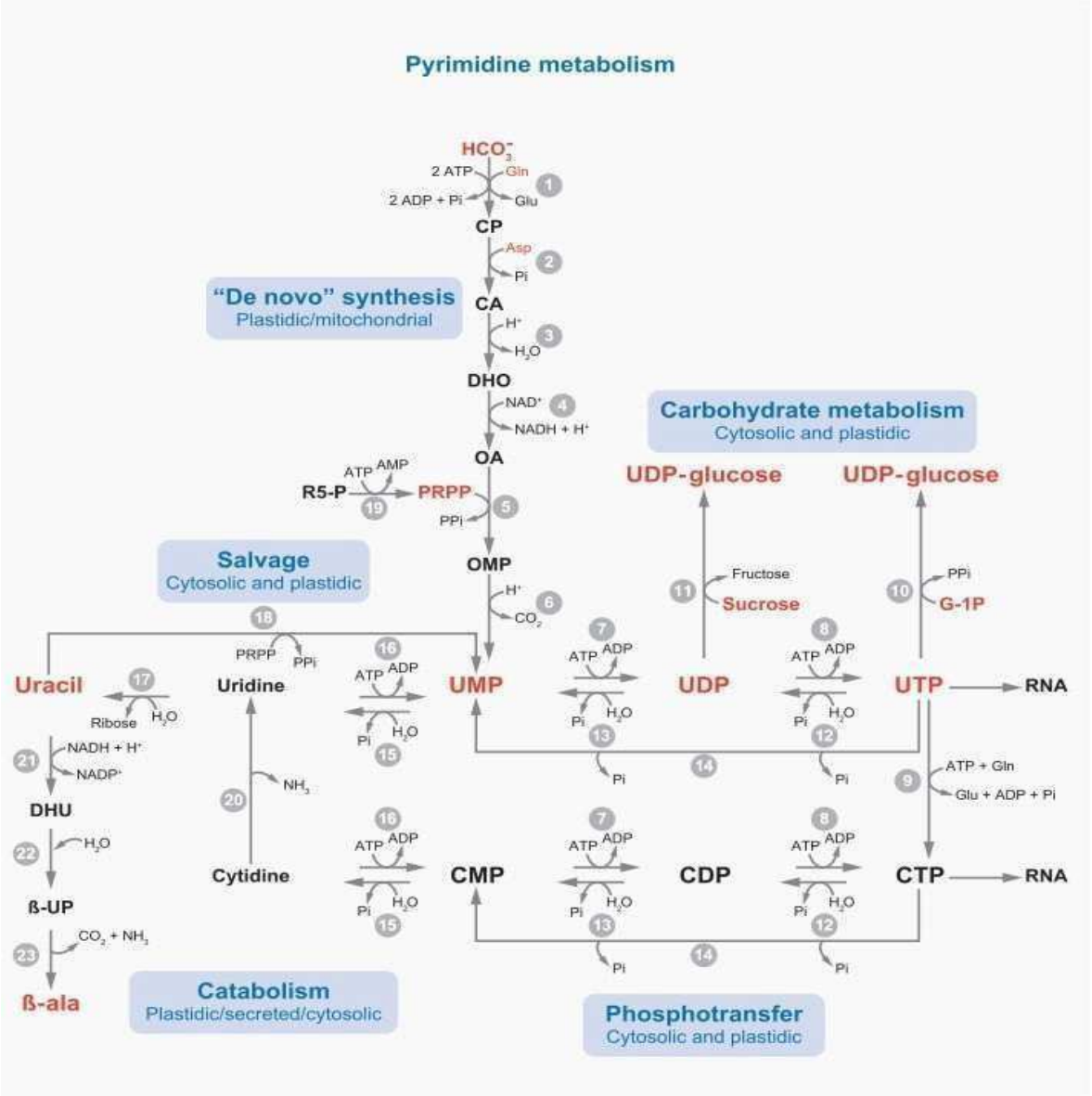


Figure 4: A general diagram of plant pyrimidine metabolism. The metabolic components shown are: 5-phosphoribosyl-1-pyrophosphate (PRPP), glutamate (Glu), glutamine (Gln), adenosine triphosphate (ATP), adenosine diphosphate (ADP), ribose-5-phosphate (R5-P), carbamoyl phosphate (CP), carbamoyl aspartate (CA), inorganic phosphate (Pi), dihydroorotate (DHO), orotic acid (OA), orotidine 5' –monophosphate (OMP), uridine monophosphate (UMP), (CDP) cytosine monophosphate (CMP), cytosine triphosphate uridine diphosphate (UDP), uridine triphosphate (UTP), uridine diphosphoglucose (UDPglucose), cytosine diphosphate (CTP), dihydrouracil (DHU), ß-ureidopropionate (ß –UP), ß -alanine (ß -ala), pyrophosphate (PPi), glucose-1-phosphate (G-1P), aspartate (Asp), and adenosine monophosphate (AMP).Taken from reference 1.

The next step is carbamoyl aspartate (CA) cyclization to form the pyrimidine ring yielding dihydroorotate is normally catalyzed by dihydroorotase (DHOase). Afterward,

dihydroorotate, oxidized by dihydroorotate dehydrogenase (DHODH) to produce orotate.[1] 5-Phosphoribosyl-1-pyrophosphate condenses with orotate to yield orotidine 5monophosphate (OMP) and inorganic pyrophosphate by the enzyme OPRTase (orotate phosphoribosyltransferase) which is then decarboxylated by orotidylate decarboxylase to yield uridine-5'- monophosphate (UMP). Uridine monophosphate (UMP) is phosphorylated to uridine diphosphate (UDP) by UMP kinase, which is converted by uridine diphosphate kinase to form uridine triphosphate (UTP). Then UTP is converted to cytidine triphosphate (CTP) by (CTP) synthase.[1]

Pyrimidine nucleotides are normally catabolized through subtraction of phosphate groups to yield a nucleoside, in a reaction catalyzed by 5'-nucleotidases. Afterward, the nucleosides are changed to free bases through removal of the ribose group in a reaction catalyzed by nucleosidases. Because animals and plants lack cytosine deaminase, the deamination involves the removal of the amino group from the nucleoside cytidine to yield uridine catalyzed by CDA (cytidine deaminase). Uridine is then metabolized to uracil. The bases thymine and uracil are degraded through a reductive pathway by three consecutive reactions, which are catalyzed by dihydrouracil dehydrogenase (PYD1), dihydropyrimidinase (PYD2), and ß-ureidopropionase (PYD3) (steps 21-23 of Figure 4).[1]

Alternatively, uracil can be transformed to nucleotides through the salvage pathways. Chemically, this degradative pathway can be described as a reversion or degeneration synthesis of the *de novo* pathway, which results in β-aminobutyrate or βalanine formation via the release of CO_2 and NH_3.[1] (Figure 4).

Purine metabolism is necessary for living cells. Purine *de novo* synthesis occurs from small molecules, including the amino acids glutamine, glycine, and aspartate, along

with 10-formyl tetrahydrofolate, the activated ribose precursor 5-phosphoribosyl1pyrophosphate, and carbon dioxide (Figure 5).[1] Biosynthesis of purine begins with phosphoribosyl amine (PRA) formation from 5-phosphoribosyl-1-pyrophosphate (PRPP) as well as glutamine (Figure 5). This first reaction is catalyzed by 5-phosphoribosyl1pyrophosphate ATase. Glycine amide ribonucleotide (GAR) synthetase hydrolyzed an ATP and GAR formation is achieved by attaching glycine through an amide bond to phosphorybosyl amine.[1] Glycine amide ribonucleotide is then transformylated using 10THF to yield FGAR (formylglycinamide ribonucleotide). The subsequent step is catalyzed by the formylglycinamidine ribonucleotide synthetase (FGAMS) which consumes glutamine and ATP, and results in the production of, formylglycinamidine ribonucleotide (FGAM), which then goes through ring closure to yield AIR (5aminoimidazoleribonucleotide) using another ATP.[1] The reaction is catalyzed by AIRS (5-aminoimidazole ribonucleotide synthetase).

In building the second ring of the purine skeleton, carbon dioxide, aspartate, and another molecule of 10-formyltetrahydrofolate are inserted to form the imidazole ring. 5aminoimidazole ribonucleotide (AIR) is carboxylated by aminoimidazole ribonucleotide carboxylase to 4-carboxy aminoimidazole ribonucleotide (CAIR). The addition of aspartate forms N-succinyl-5-aminoimidazole-4carboxamide ribonucleotide (SAICAR).

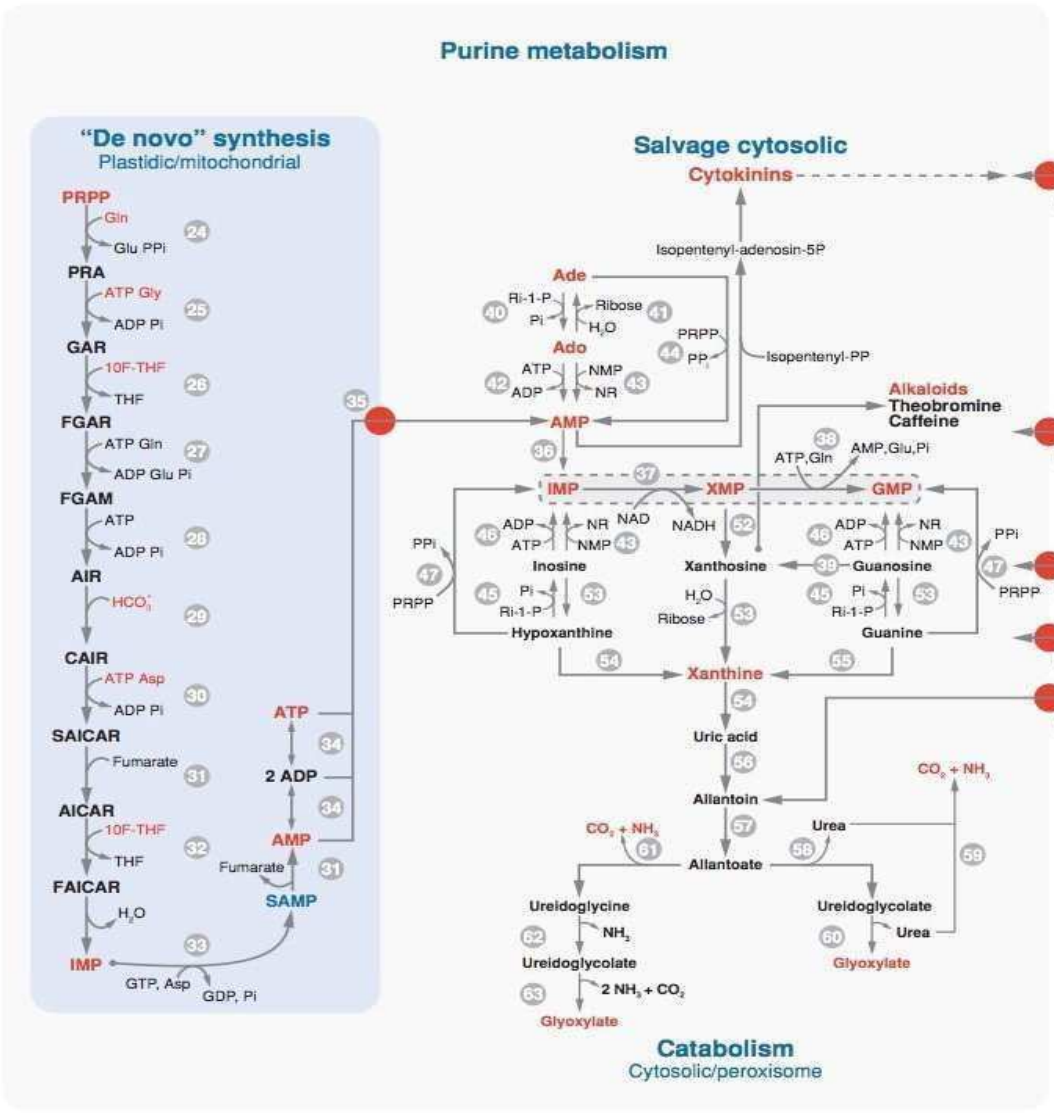


Figure 5: A general diagram of plant purine metabolism. The metabolic components shown are: 5-phosphoribosyl-1-pyrophosphate (PRPP), glutamine (Gln), glutamate (Glu), 5-phosphoribosylamine (PRA), pyrophosphate (PPi), glycine (Gly),glycinamide ribonucleotide (GAR), 10-formyl tetrahydrofolate (10F-THF),formylglycinamide ribonucleotide (FGAR), formylglycinamidine ribonucleotide (FGAM), 5-aminoimidazole ribonucleotide (AIR), 4-carboxy aminoimidazole ribonucleotide (CAIR), aspartate (Asp), N-succinyl-5-aminoimidazole-4-carboxamide ribonucleotide (SAICAR), 5aminoimidazole-4- carboxamide ribonucleotide (AICAR), 5-formaminoimidazole4carboxamide ribonucleotide (FAICAR), inosine monophosphate (IMP), adenylosuccinate (SAMP), adenosine monophosphate (AMP), xanthosine monophosphate (XMP), guanosine monophosphate (GMP), adenine (Ade), and adenosine (Ado).Taken from reference 1.

The next step is catalyzed by adenylosuccinate lyase (ASL) and produces fumarate to form 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR).

The last carbon of the purine ring, is provided by 10-formyltetrahydrofolate to form 5-formaminoimidazole-4-carboxamide ribonucleotide (FAICAR), which goes through dehydration as well as ring closure to form the first complete purine molecule inosine monophosphate (IMP).[1] Biosynthesis of purines after IMP divides into two pathways. One pathway forms adenosine monophosphate (AMP), and the other forms guanosine monophosphate (GMP). Adenosine monophosphate (AMP) is synthesized by removing the carbonyl group at the sixth carbon and replacing it with an amino group. Aspartate provides the amino group and GTP (guanosine triphosphate) donates energy through the hydrolysis of a phosphate bond, to form adenylosuccinate (SAMP). Adenylosuccinate synthase catalyzes the reaction. Adenylosuccinate lyase (ASL) catalyzes the removal of fumarate to create AMP. The second pathway leading to guanosine monophosphate (GMP) is triggered by IMP oxidation followed by an amino group insertion, which is provided by the amide nitrogen of glutamine. Xanthosine monophosphate (XMP) is created by inosine-5′-monophosphate dehydrogenase (IMPDH) using NAD^+ as the hydrogen acceptor. GMPS (GMP synthase) catalyzes the last step to form GMP.[1]

There are two possible paths for the catabolism of adenine nucleotides, after IMP synthesis from AMP by AMP deaminase.[1] In the first pathway, IMP can be dephosphorylated to inosine by phosphatases or 5'-nucleotidases, and then hydrolyzed to hypoxanthine through guanine/inosine nucleosidase and later transformed to xanthine through XDH (xanthine dehydrogenase). In the second pathway IMP is changed to XMP

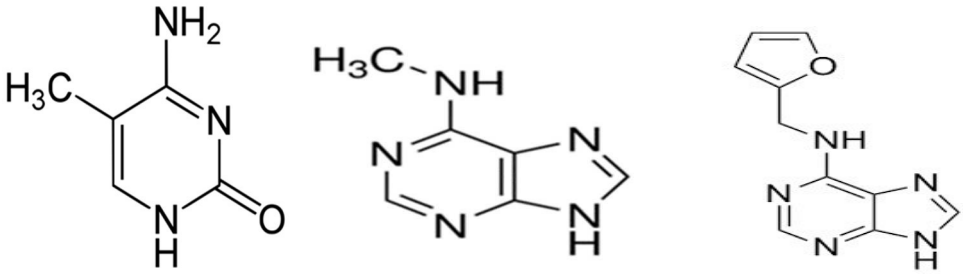
by use of IMPDH and then metabolized to xanthosine by 5'-nucleotidases that is then converted by guanine/inosine nucleosidase to xanthine (Figure 5).[1]

Guanosine deaminase has been found in plants. This indicates that guanosine is produced by guanine nucleotide degradation through dephosphorylation. Guanosine is either transformed to guanine or deaminated to xanthosine through inosine/guanine nucleosidase, which then gives xanthine

Xanthine degradation begins with xanthine dehydrogenase, which results in uric acid formation. Allantoin is formed from uric acid by uricase wich is then transformed to allantoate catalyzed by allantoinase.[1] Allantoic acid is converted to ureidoglycine or ureidoglycolate that are further metabolized to the end products of purine catabolism, CO_2, NH_3, and glyoxylate. These final products can be reused within photosynthesis (CO_2) or can be reassimilated through the photorespiratory glycolate plus glutamine oxoglutarate aminotransferase (GOGAT) cycle.[1]

Unusual bases are modified forms of the normal DNA or RNA bases.[3] The normal bases include adenine, cytosine, guanine, and uracil. Examples of unusual bases include 5-methyl cytosine and N^6-methyladenine (Figure 6).[3] They are found in tRNA and are formed after the post transcription modification of a normal base.[3] A particular important nucleoside derivative in plants are the cytokinins. The first cytokinin was isolated as a DNA degradation product, an adenine (aminopurine) derivative named kinetin (6 furfurylaminopurine) (Figure 6).[4] Cytokinins are found in all plant tissues. Adenine or adenosine derivatives are the building blocks of cytokinins. Cytokinins affect the physiology of plants in several ways. Cytokinins are plant specific chemical regulators

that regulate the plant cell cycle, among other developmental features. The structure and conformation of the side chain plays a huge role in the biological activity of the cytokinin.[4]



5-methylcytosine N6-methyladenine kinetin

Figure 6: Chemical structures of the mentioned unusual bases.

Nucleoside Hydrolases

Nucleoside hydrolases (NHs) are enzymes that cleave the N-glycosidic bond of ribonucleosides to produce a free base, either a pyrimidine (uracil, thymine and cytosine) or a purine (adenine, guanine and hypoxanthine) and ribose.[5] Nucleoside hydrolases have been found in different organisms such as bacteria,[6,7] protozoa,[8,9] mesozoa,[10] insects,[11] and yeast.[12] They have also been found in plants such as *Arabidopsis thaliana*,[13] Alaska pea seeds,[14] and soybean leaves.[15] However, NHs have not been found in mammals.[5]

There exist major types of parasitic protozoan nucleoside hydrolases from organisms such as *Leishmania donovani, Crithidia fasciculata* and *Trypanosoma vivax*. Due to evolution, these enzymes have been further classified into three sub-groups based on their substrate specificity.[5] The subgroups include the non-specific inosine-uridine preferring nucleoside hydrolases (IU-NHs),[16,17] the purine specific inosineadenosineguanosine preferring nucleoside hydrolases (IAG-NHs)[18,19] and the 6-

oxo purine-specific inosine-guanosine preferring nucleoside hydrolases (IG-NHs).[5] However, current studies have indicated that the classification is artificial and inadequate since there are NHs that do not fit into the subgroups.[6,10]

There are also nucleosidases that have been found in *Leishmania donovani*.[21] The first enzyme found in *Leishmania donovani* was purine 2'-deoxyribnucleosidase with a molecular weight of 33,000 Da as determined by gel filtration using Sephadex G100 resin and a pI value of 4.3.[26] The second hydrolase was purine ribonucleosidase (EC 3.2.2.1) with a molecular weight of 205,000 Da as determined by gel filtration on Sephadex G200 and a 4.4 pI value. The third hydrolase was pyrimidine ribonucleosidase (EC. 3.2.2.8) with a molecular weight of 180,000 Da from gel filtration on Sephadex G200 and a 6.3 pI value.[20]

The most widely studied inosine/uridine nucleoside hydrolase (IU-NH) is found in *Crithidia fasciculata.*[21] This enzyme catalyzes the hydrolysis of inosine or uridine to produce hypoxanthine or uracil and ribose. Inosine/uridine nucleoside hydrolase from *C. fasciculata* was cloned by polymerase chain reaction techniques. This enzyme has k_{cat}/K_m ranging between

10^3 -10^5 M^{-1} s^{-1} and gave a major mass peak of 34 194 amu by mass spectrometry.[22]

Guanosine-inosine nucleoside hydrolase has also been purified from extracts of *C. fasciculata*.[23] The molecular weight of GI-nucleoside hydrolase was 40,800 Da, determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The V_{max} for

guanosine and inosine as substrates is 365 mol/min/mg and 148 mol/min/mg respectively and the K_m values are 77 m and 16 M, respectively.[23]

Several crystal structures of nucleoside hydrolases have been determined. The structure of IU-NH from *C. fasciculata* is a homotetramer (Figure 7a). However, the IAG-NH from *Trypanosoma vivax* crystallized as a homodimer (Figure 7b). The core of the NH monomer is formed as illustrated in the diagram (Figure 7a, 7b). They are composed of a characteristic eight-stranded mixed sheet, which has seven parallel and one anti-parallel strand. There are several other strands surrounding helices. The monomeric subunits of both subgroups are similar in architecture and topology (Figure 7c, 7d). They consist of a single globular domain. However, the subunits of IU-NH tetramers and IAG-NH dimer have different quaternary architectural arrangements, which involves different subunits and subunit interfaces.

The first six strands of the sheet are arranged in a way that resembles a dinucleotide-binding (Figures 7e, 7f). The active site of the enzyme is located towards the end of the central sheet. There are two flexible loops positioned on each side of the active site of the enzyme. They change conformation to position chains in the active site when binding the inhibitor para-aminophenyliminoribitol (pAPIR) to the *C. fasciculata* NH enzyme.[5]

The NHs have a deep narrow active site in every subunit. A Ca^{2+} ion is firmly bound or attached at the active site (bottom) (Figure 8).5 This metal (octacoordinated) is chelated via a conserved system of interaction involving the oxygen sidechains: Asp 10, Asp 15, as well as Asp 261, the carbonyl oxygen main chain of Thr 137 as well as three molecules of water. After substrate binding, the moiety of ribose is fixed firmly within active site cleft. Two Ca^{2+} which are bound to molecules of water are substituted with the 2'-and 3'-hydroxyl groups of the sugar. The only remaining Ca^{2+} chelated H_2O molecule interacts or combines with aspartate (conserved Asp10).[5]

The of inflexible specificity nucleoside hydrolases for ribose is created via an elaborate system of conservative interactions that involves the 2'-, 3'- and 5' hydroxyls of the sugar and Asp 14, Asn 173, Asn 186, Glu 184, and Asp 261 conserved residues, and Ca^{2+}ion (Figure 8).

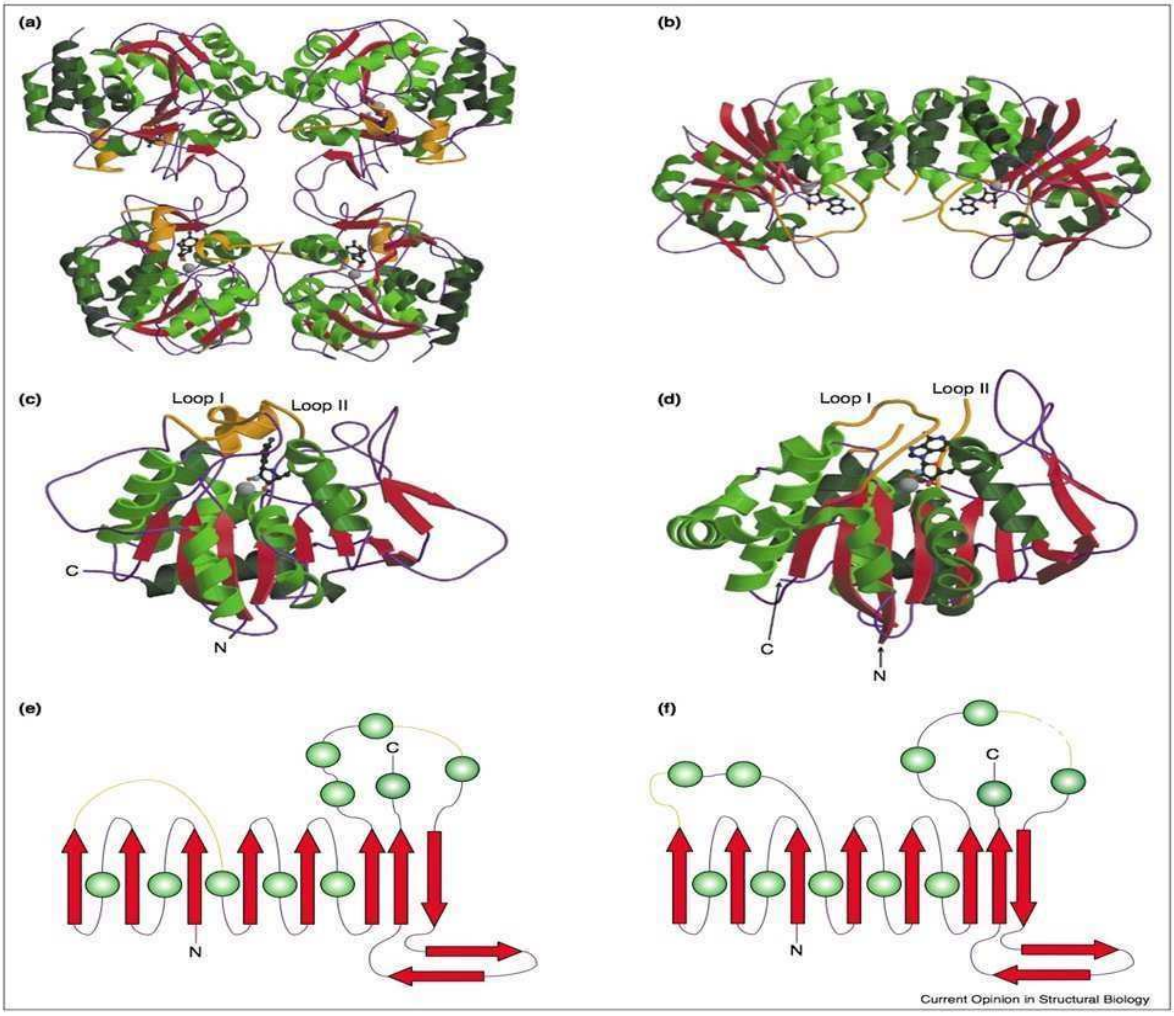


Figure 7: Different quaternary nucleoside hydrolase structures are built from a common fold. The topologies, tertiary structures, and quaternary structures of (b,d,f) an IAG-NH (TvNH in complex with 3-deaza-adenosine; PDB code 1HP0) and (a,c,e) an IU-NH (Cf NH in complex with pAPIR; PDB code 2MAS) are compared. The order of the helices is indicated by going from light to dark green when going from the N towards the C terminus. α helices are shown in green and β strands in red. Two flexible loops in the area of the active site are shown in yellow. The nucleophilic water molecule and Ca^{2+} ion are located in the active site pockets of the enzymes. The nucleophilic water molecule is shown as a blue sphere and the Ca^{2+} ion is shown as a grey sphere. The ligands are represented as stick and models. Taken from reference 5.

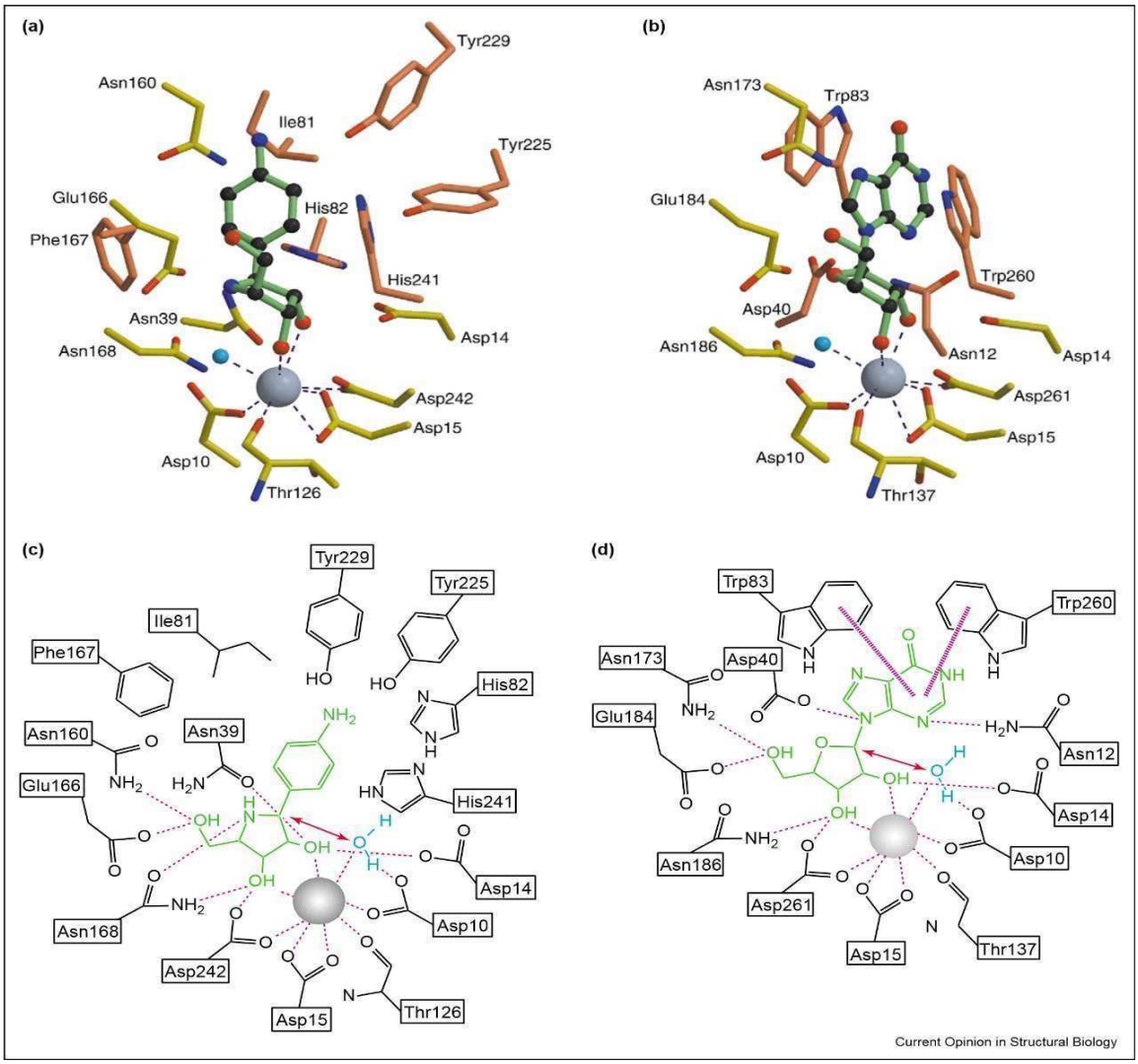


Figure 8: Comparison between the active sites. (a,c), an IU-NH (*Cf NH* in complex with pAPIR) and (b,d) an IAG-NH (*TvNH* in complex with inosine). The Ca^{2+} ion is shown as a grey sphere, the enzyme bound ligands are indicated in green and the nucleophilic water molecule is shown as a bluesphere. In (a,b), The ribose are shown in yellow. The active site residues that have strong interactions with the Ca^{2+} ion, while the residues of the nucleobase are shown in light brown. The red arrows indicate the nucleophile and the electrophilic center of the substitution reaction. The intermolecular interactions are represented schematically in (c,d). Other possible interactions are indicated by the dotted lines. The poor analogy of the p-aminophenyl group with real nucleobases does not allow a direct and quick analysis of catalytic interactions in the nucleobase-binding pocket of Cf NH (c).The nucleobase in the TvNH–inosine complex is arranged between the side chains of Trp83 and Trp260 (d). Taken from reference 5.

Uridine Nucleosidase

Uridine nucleosidase (EC 3.2.2.3) has been purified from commercial bakers' yeast.[24] Uridine nucleosidase is an enzyme that catalyze the hydrolysis of uridine to produce uracil and ribose (Figure 9).[7] It is located in the cytoplasm. The molecular weight of uridine nucleoside was approximately 32,500 Da, determined by gel filtration. Uridine nucleosidase is active for both uridine and 5-methyluridine with an optimal pH around 7.0 and K_m of 0.86×10^{-3} M and 1.66×10^{-3} M, respectively.[24]

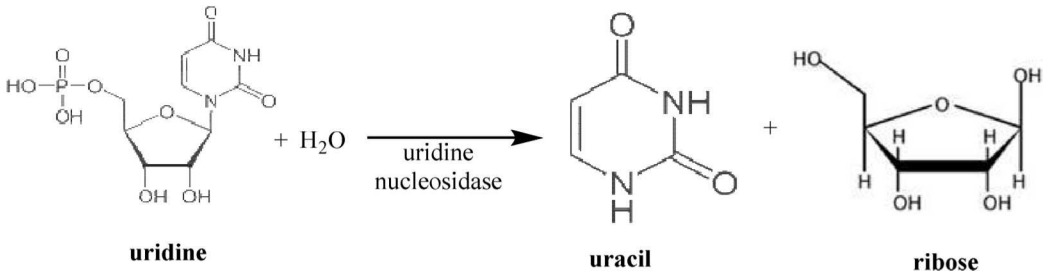


Figure 9: Hydrolysis of uridine to uracil and ribose by uridine nucleosidase.

Inosine Nucleosidase

Inosine nucleosidase (EC3.2.2.2) has been purified from a number of sources including *yellow lupin (Lupinus luteus).*[25] Inosine nucleosidase is an enzyme that catalyzes the hydrolysis of inosine to produce hypoxanthine and ribose *(*Figure 8).[25] The inosine nucleosidase is found in both lupin seeds and seedlings. The molecular weight of the native inosine nucleosidase is 62,000 Da as determined by Sephadex G- 100 gel filtration. Its K_m value has been computed to be about 65 μM. The enzyme achieves optimum activity at pH 8.[25]

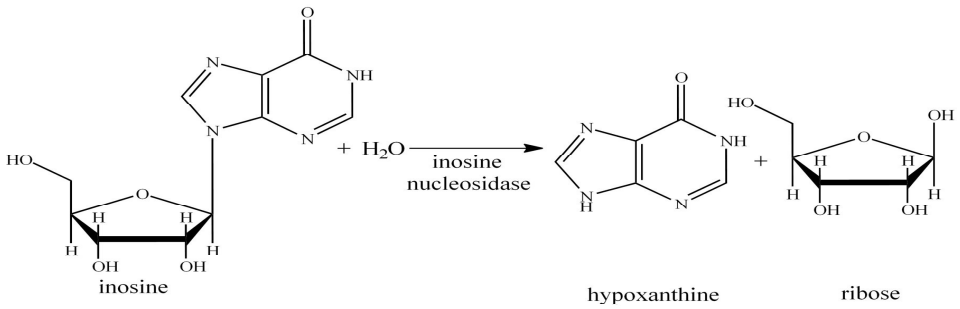


Figure 10: Hydrolysis of inosine to hypoxanthine and ribose by inosine nucleosidase.

Adenosine Nucleosidase

Adenosine nucleosidase (EC 3.2.2.7) is an enzyme that catalyzes the hydrolysis of adenosine to produce adenine and ribose. It has been purified from many plants such as soybean leaves,[15] spinach beet leaves,[26] wheat germ,[27] tea leaves,[28] barley leaves,[29] *yellow lupin,*[30] Jerusalem artichoke shoots,[31] and tomato roots and leaves.[32]

Adenosine nucleosidase belongs to the glycosylase enzymatic group, known for the hydrolysis of N-glycosyl compounds.[30] The enzyme has an optimum pH for hydrolysis at 4.5 and phosphate ions do not inhibit or activate its functions as compared to inosine nucleoside.[33]

Adenosine nucleosidase has been purified from *yellow lupin* with molecular weight 72 kDa, determined by gel-filtration on a Sephacryl S200 16/60 column. It has two subunits with a molecular weight of 33 kDa per subunit as determined by SDS-PAGE. The K_m for adenosine was 4.8 μM and the pH optimum was 7.5.[30]

Adenosine nucleosidase has been purified from leaves of *Coffea arabica.* The specific activity of adenosine nucleosidase was 8333 nkat/mg for adenosine. The

molecular weight of the adenosine nucleosidase was 72 kDa estimated by gel filtration, and subunit molecular weight was determined by SDS-PAGE to be 34.6 kDa. This enzyme has optimum activity at pH 6.0, with the K_m at this pH of 6.3 μM, and V_{max} of 9.8 nkat.[34]

Adenosine nucleosidase was purified from barley leaves. The K_m for adenosine from barley leave depending on buffer system and temperature was 0.8 to 2.3 μM. The molecular weight was determined by native gel filtration to be 66.0 ± 3.0 kDa and subunit molecular weight was 33.0 kDa estimated by SDS-PAGE. The pH optima are 5.4 in an ethanesulphonic acid buffer and 4.7 in a citric acid buffer.[29]

Purification of the enzyme from Alaska pea seeds resulted in a molecular weight of 26,103 Daltons as estimated through mass spectrometry and its K_m value was determined to be 13 μM.[14]

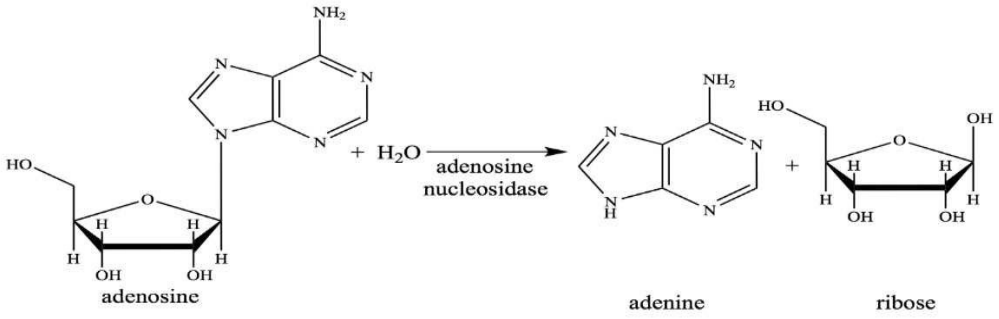


Figure 11: Hydrolysis of adenosine to adenine and ribose by adenosine nucleosidase.

Cytidine Nucleosidase

Cytidine nucleosidase facilitates the conversion of cytidine to cytosine (Figure 12). Ribose is also formed during the reaction. It has been purified from *Arabidopsis thaliana* and has a molecular weight of 63,000 Da. Its K_m value was found to be 150 μM for cytidine.[35]

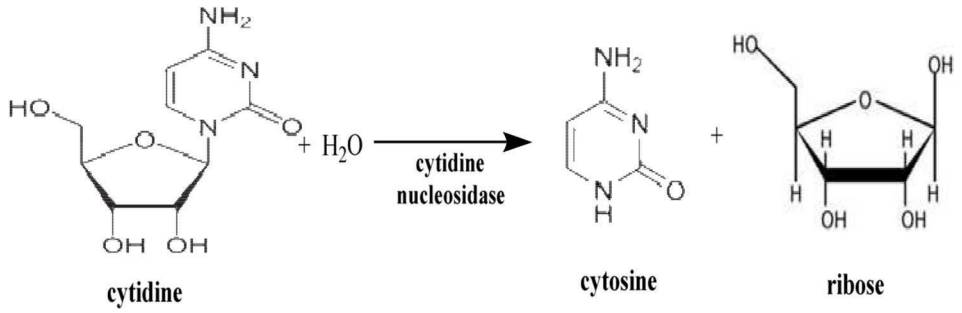


Figure 12: Hydrolysis of cytidine to cytosine and ribose by cytidine nucleosidase.

Nucleoside Deaminases

Adenosine Deaminase

Adenosine deaminase (EC 3.5.4.4) is an enzyme from the purine metabolism pathway. Through this enzyme, inosine or deoxyinosine can be synthesized from adenosine or deoxyadenosine (Figure 13).[35] This enzyme is instrumental in the formation of nucleotides in mammals either by salvage or *de novo* pathways.[36] Nucleic acids and signaling molecules can also be formed with the help of this enzyme. The enzyme has been purified from animals such as cattle, pigs, camels, and even humans.[37] Adenosine deaminase purified from humans has an approximate molecular weight of 38,200 Da by Stokes radius and a pH optimum ranging between 7.0 and 8.0. The enzyme was found to have a K_m value of 52 μM. In potato tubers no adenosine deaminase activity was found.[39]

Twenty different seeds, such as beans, onions, buck wheat, oats, beet, mallow and celery, were tested for adenosine deaminase activity. No adenosine deaminase activity was found in these seeds.[38] Adenosine deaminase in barley seeds had activity in both germinated and fresh seeds after fifteen hours of incubation. However no activity was found after short incubation times.[38]

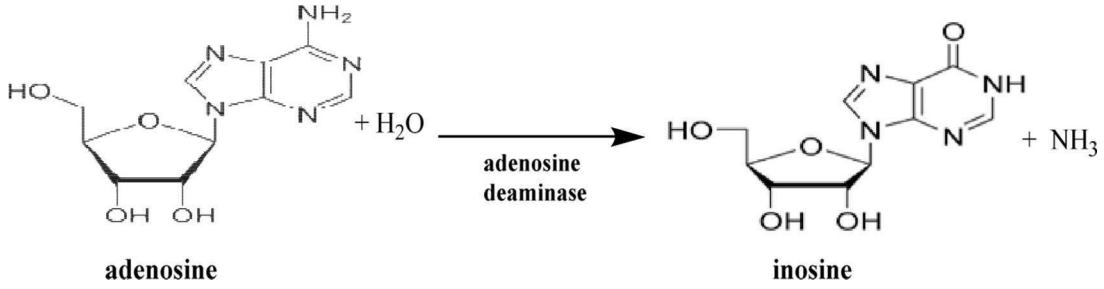


Figure 13: Adenosine deaminase converts adenosine to inosine and releases NH_3.

Cytidine Deaminase

Cytidine deaminase (EC 3.5.4.1) is an enzyme from the pyrimidine salvage pathway. This pathway is involved in the synthesis of uracil and ammonia from cytosine.[3] However, this enzyme is yet to be isolated from higher organisms, but has been found in plants, fungi, and bacteria. There are significant differences between the bacterial (*Escherichia coli*) and fungal (*Saccharomyces cerevisiae*) enzymes in their molecular mass, amino acid sequences and structures.[40] The molecular mass of *Saccharomyces cerevisiae* enzyme was approximately 35 kDa and has a homodimer structure with 158 residues.[40] Cytidine deaminase has been cloned, expressed and purified from *Arabidopsis thaliana*.[35] The enzyme was >98% pure using ion-exchange chromatography.[35] This enzyme was found to be a dimer composed of two identical subunits with molecular mass was 63 kDa.[40]

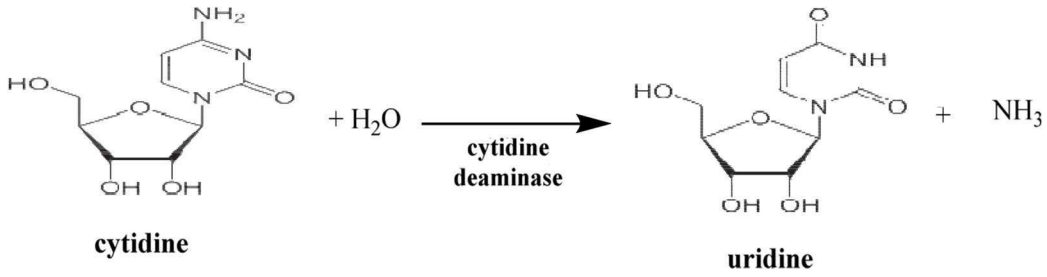


Figure 14: Cytidine deaminase converts cytidine to uridine and releases NH_3.

Current Research

The goal of this thesis work is to demonstrate the presence or absence of hydrolyzing and deaminase enzymes involved in nucleoside catabolism in germinated seedlings of bean, organic bean, okra, organic okra, squash and organic squash. The purine nucleosides adenosine, 2'-deoxyadenosine, and inosine were used to determine whether purine nucleosidases were present. The pyrimidines uridine and cytidine were used to determine whether pyrimidine nucleosidases were present.

CHAPTER II

MATERIALS AND METHODS

Equipment and Instrumentation

The activities of the enzyme were determined on a Dionex Ultimate 3000 High Performance Liquid chromatography system (HPLC). The system consisted of a thermostatted column holder, autosampler, quaternary analytical pump and a variable wavelength UV detector.

Materials and Reagents

Okra (Clemson Spineless #80), bean (Blue Lake 274 Bush) and squash (Early Summer Crookneck) were purchased from Ferry-Morse seed company. Inosine, uridine, cytidine, adenosine, 2'deoxyadenosine, and the corresponding nucleobases were purchased from Sigma Aldrich. Dithiothreitol (DTT), protease inhibitor cocktail for plant, poly(vinylpolypyrrolidone) (PVPP) and protamine sulfate salt from salmon (Grade X) were also obtained from Sigma Aldrich. Tris Base salt for molecular biology was purchased from Fisher BioReagents and was used to make all stock solutions. Ammonium phosphate was obtained from J.T. Baker Chemical Co. Protein assay dye reagent concentrate and protein standard II were obtained from Bio-Rad. All other reagents were reagent grade.

Methods

Seed Germination

All seeds were soaked in bleach for 5 minutes and then extensively washed with tap water. The seeds were then placed in a tupperware container on a moist paper towel

and covered with a second moist paper towel. The top was placed on the container and then left to germinate at room temperature. The seedlings were harvested on the 7th day after germination. The seedling material was homogenized in 10 mM Tris buffer pH 7.2 (1 mL for every 1 g of biomass) using a Waring commercial blender at 4°C. Dithiothreitol (DTT) (15 mg), protease inhibitor cocktail (10 uL), protamine sulfate (2 g) and, polyvinylpolypyrrolidone (2 g) were added to the Tris buffer. The homogenate was centrifuged at 13,000*x*g for 30 minutes at 4°C. The supernatant was centrifuged again at 8,500*x*g for 15 minutes at 4°C. The resulting supernatant was filtered through a double layer of cheesecloth to remove the remaining particulates, and the crude extract was stored at 4°C.

Measurement of Enzyme Activity

A reaction mixture consisting of 1 mM nucleoside in 10 mM Tris pH7.2 was prepared and 100 µL of crude extract was added. The solution was incubated at room temperature and aliquots withdrawn at timed intervals.

The nucleoside and base present were separated by HPLC on a Kinetex 5µ, EVO C18 reverse phase (150 x 4.6 mm) column eluted with 98% 10 mM ammonium phosphate pH 5.4 and 2% methanol. The temperature of the column oven was 30.0 °C. The amount of nucleoside and base present were determined from the area of each peak measured at 254 nm. The flow rate was 0.6 mL/min. Every injection was set at 10 µL. Samples were incubated on the autosampler at 32 °C. The disappearance of parent nucleoside was used to calculate enzyme activity. The identity of the base and nucleoside were based upon their retention times and the activity was calculated by dividing the

amount of base produced or loss of starting nucleoside by the reaction time. Specific activity was determined by dividing reaction velocity by protein concentration.

Determination of Protein Concentration

The protein concentration was determined using a Bio-Rad protein Assay. The assay mixture consisted of enzyme solution and water equal to a total volume 800 µL, to which 200 µL of Bio Rad dye was added. The protein concentrations were estimated by comparing the results to a standard curve that was constructed using the Bio-Rad protein assay standard bovine serum albumin (1.45 mg/mL). The absorbance were measured at 595 nm (Figure 15).

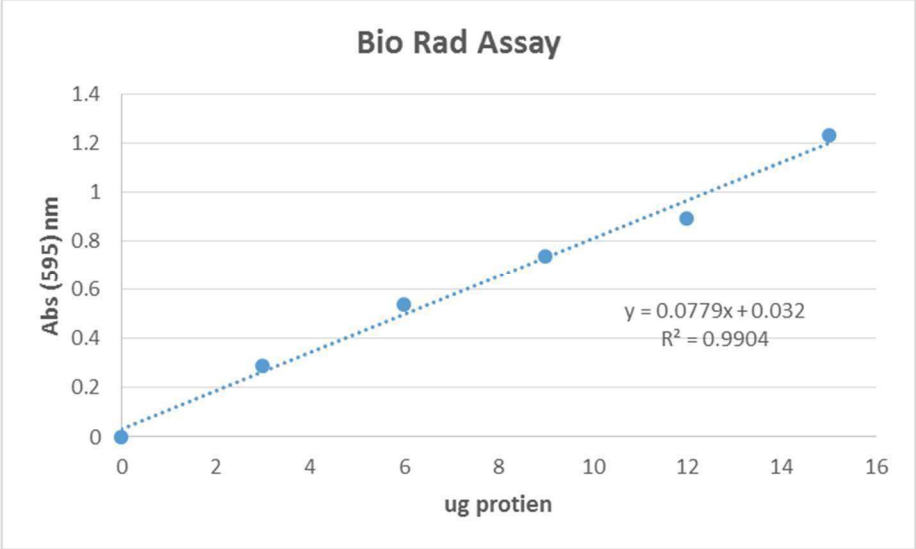


Figure 15: Protein concentrations calibration curve. Determined at 595 nm using BioRad protein assay standard bovine serum album.

CHAPTER III

RESULTS AND DISCUSSION

Purines and pyrimidines are integral components of the nucleic acids. Besides comperising RNA and DNA, they are involved in various biological functions, including signal transduction pathways, immunological responses and as key components for regulation of enzymatic pathways.[41] The maintenance of the right balance of nucleotides is essential for the survival of the cell. If some compounds are left to accumulate in the cell, they may turn out to be toxic. The balance between nucleotide and nucleoside pools is achieved through the breakdown of nucleotides to their corresponding nucleosides and nucleobases.[36]

An intermediate step in nucleoside salvage pathways is the hydrolysis of nucleosides to their nucleobases.[36] Nucleoside hydrolase is a glycosidase that catalyzes the cleavage of the N-glycosidic bond in nucleosides to facilitate recycling of nucleobases. Nucleotide metabolism is facilitated through metabolic pathways such as the *de novo* synthesis, nucleotide degradation, and the salvage pathways. These pathways are facilitated by enzymes and are specific to either purine or pyrimidine metabolism.[33]

This paper discusses some of the nucleoside enzymes that are involved in the degradation of nucleosides and their activities. Cytidine deaminase is an interconversion enzyme that is responsible for the hydrolytic deamination of cytidine to uridine. Adenosine nucleosidase is an important enzyme in the purine metabolism of plants. The enzyme has been purified from a number of plants and has shown different characteristics, substrate specificity, optimum pH, and K_m values. Inosine nucleosidase is

a purine catabolism pathway enzyme and has been purified from both plants and animals.[31] In plants, it has been purified from *yellow lupin* seeds. Adenosine deaminase is a purine metabolism pathway enzyme that catalyzes the conversion of adenosine or deoxyadenosine to adenine. In the mammalian tissue, the enzyme is useful in the *de novo* or salvage pathway synthesis of nucleotides.[42]

Other enzymes discussed in this paper include uridine nucleosidase and deoxyadenosine nucleosidase. Uridine nucleosidase takes part in pyrimidine metabolism. It is located in the cytoplasm. Uridine nucleosidase has been purified from commercial bakers' yeast.[36] Deoxyadenosine nucleosidase is a purine metabolism enzyme that participates in the degradation process and removes ribose from the nucleoside (Figure 16). There was also a less common purine base xanthine, corresponding to the nucleoside xanthosine, evaluated in this study and it could be one of the products of enzymes in the in plant extract (Figure 17).

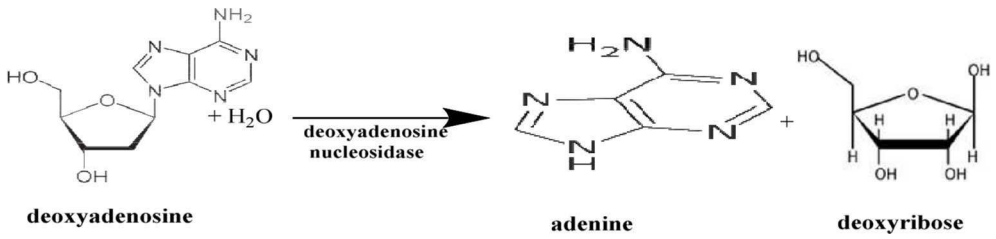
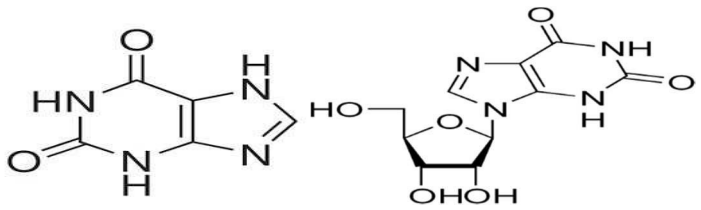


Figure 16: Hydrolysis of deoxyadenosine to adenine and deoxyribose by deoxyadenosine nucleosidase.



xanthine xanthosine

Figure 17: Structures of additional nucleosides and bases.

Crude protein extracts prepared 5-9 days after germination of okra, organic okra, bean, organic bean, squash and organic squash seeds, were evaluated for presence of catabolic enzymes that participate in the salvage of nucleosides. The seedling material was homogenized in 10 mM Tris buffer pH 7.2 (1 mL for every 1 g of biomass). The extract was tested for the presence of two groups of enzymes; nucleosidases (hydrolases), and deaminases. Nucleosidases remove the ribose hydrolytically. Deaminases contribute in interconversion of one nucleoside into another by removing amino group from the base. The activities of all nucleosidases were measured by observing the disappearance of the parent nucleosides and appearance of the nucleobases.

All reaction samples were monitored using the same HPLC method. The reaction mixture consisted of 1 mM nucleoside in 10 mM Tris pH 7.2 and 100 μL of initial extract. The solution was incubated at room temperature. The amounts of nucleoside and base present were determined on HPLC by measuring the absorbance at 254 nm of peaks eluted from a Kinetex 5μ EVO C18 column using 98% 10 mM ammonium phosphate pH 5.4 and 2% methanol. The nature of enzymatic activity was assessed quantitatively and based on the peak area. The identity of the starting product nucleoside and nucleobases was based on their retention time.

The retention times for both parent nucleoside and base were obtained and are listed in Table 1.The disappearance of parent nucleoside, measured by the decrease in peak area, was used to calculate enzyme activity. The activity was calculated by dividing the amount of nucleoside reduction by the reaction time. Specific activity was determined by dividing reaction velocity by protein concentration. The specific activity may contain multiple activities including hydrolases and deaminases activities. At this point the multiple activities cannot be separated Therefore, the activity calculation was based on the decrease of starting materials.

Table 1. Summary of retention times of nucleosides and nucleobase. Retention times of nucleosides and bases were determined using a 98 % 10 mM ammonium phosphate pH 5.4, 2 % methanol mobile phase on a Kinetex C18 (150 x 4.6 mm) HPLC column at 254 nm.

Nucleosides	Retention Time (min)	Nucleobase	Retention Time (min)
Inosine	6.4	Hypoxanthine	3.4
Uridine	3.6	Uracil	2.6
Cytidine	3.0	Cytosine	2.4
Adenosine	14.8	Adenine	2.7
2'-Deoxyadenosine	21.0	Adenine	2.7
Xanthosine	6.6	Xanthine	3.8
2'-Deoxyadenosine	21.0	2'-Deoxyinosine	7.7

This study was to determine if there was a difference in the nucleoside metabolizing activities of regular and organic seeds. Organic seeds are produced from plants that have not been exposed to any chemicals during growth in the field. Regular seeds are often exposed to variety of chemicals during growth. These chemicals are used to increase growth, ward off bacteria or insects, and protect against fungi. A third type of seed, not included in this study are genetically modified seeds. These seeds have foreign genetic material artificially inserted into their genomes.

Enzyme Activities in Okra (Clemson Spineless #80) and Organic Okra (Clemson Spineless #80).

Okra (Clemson Spineless #80) and organic okra (Clemson Spineless #80) is particularly popular in Africa, India, Brazil, Turkey, Spain and the southern USA. The okra plant has nutritional value. Its seeds can be used as a healthy substitute for coffee because they do not contain caffeine.[43] The activities of a number of metabolizing enzymes were determined from okra and organic okra. Using uridine as the substrate, a robust nucleosidase activity in both okra and organic okra was reveled (Figures 18-19). Significant disappearance of uridine commenced within the second hour after reaction initiation for okra and after five hours for organic okra.

No additional products were observed during the reaction. Activity levels were similar in both okra and organic okra. The calculated specific activities for okra and organic okra reactions containing 1 mM uridine were 0.017 μmol/(min mg) and 0.025 μmol/(min mg) respectively. The specific activity was similar with the results of uridine nucleosidase from corn which is 0.031 μmol/(min mg) reported by Ustynov.[42]

Inosine was degraded to hypoxanthine within three hours of the reaction initiation for both okra and organic okra with specific activity at a similar level (Figures 20-21). The specific activity for inosine hydrolyzing enzymes in okra and organic okra were 0.0096 μmol/(min mg) and 0.0039 μmol/(min mg), respectively, lower than that for uridine. The specific activity for inosine in okra and organic okra were similar to the results of inosine nucleosidase from yellow lupin and corn which are 0.0077 μmol/(min mg) and 0.0044 μmol/(min mg), respectively.[42]

Cytidine nucleosidase and deaminase have not been found in plants.[41] Cytidine deaminase was active in both okra and organic okra (Figures 22-23). Cytidine degradation reaction in okra and organic okra revealed multiple activities: cytidine nucleosidase, cytidine deaminase and uridine nucleosidase. Resolution between cytidine, uridine, uracil and cytosine was baseline, and allowed for clear identification of peaks based on the retention times of the controls.

Uridine was formed within the first two hours of the reaction by cytidine deaminase and was converted to uracil by the action of uridine nucleosidase. Cytidine was also degraded quickly to cytosine by the action of cytidine nucleosidase. The reactions in organic okra took a substantially longer time to initiate than okra. The specific activity for enzymes catalyzing degradation of cytidine were 0.014 μmol/(min mg) in okra and 0.0052 μmol/(min mg) in organic okra.

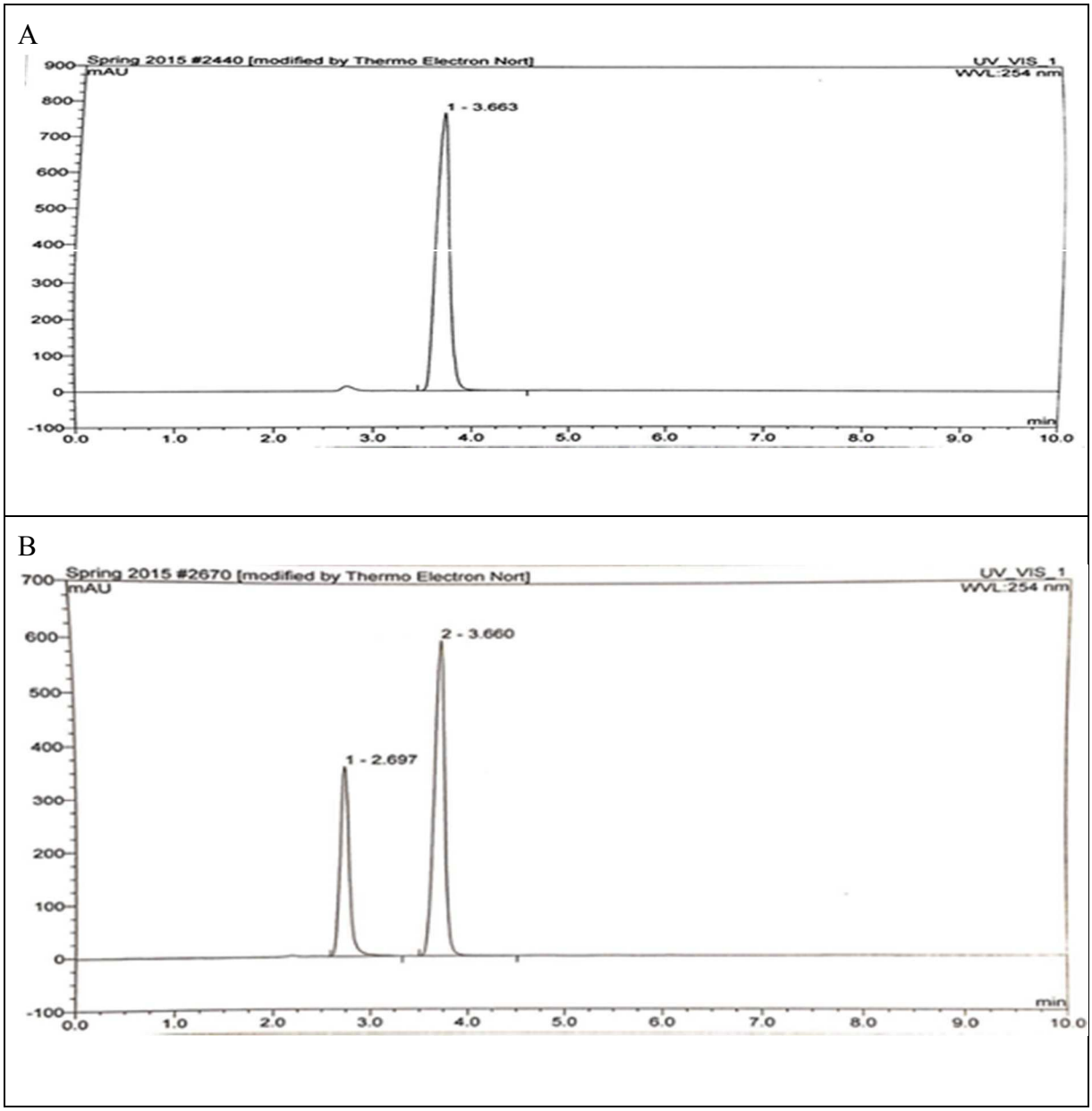


Figure 18: Uridine to uracil conversion catalyzed by uridine nucleosidase in okra. The reaction mixture included 1 mM uridine 1 mL and okra extract (100 µL) in 10 mM Tris buffer pH 7.2. (A) Reaction mixture before okra extract addition. It shows a uridine peak at 3.663 minutes. (B) Reaction mixture after okra extract addition. Uridine peak appeared at 3.660 minutes. A second peak, with a retention time expected for uracil (2.697 minutes) appeared two and half hours later.

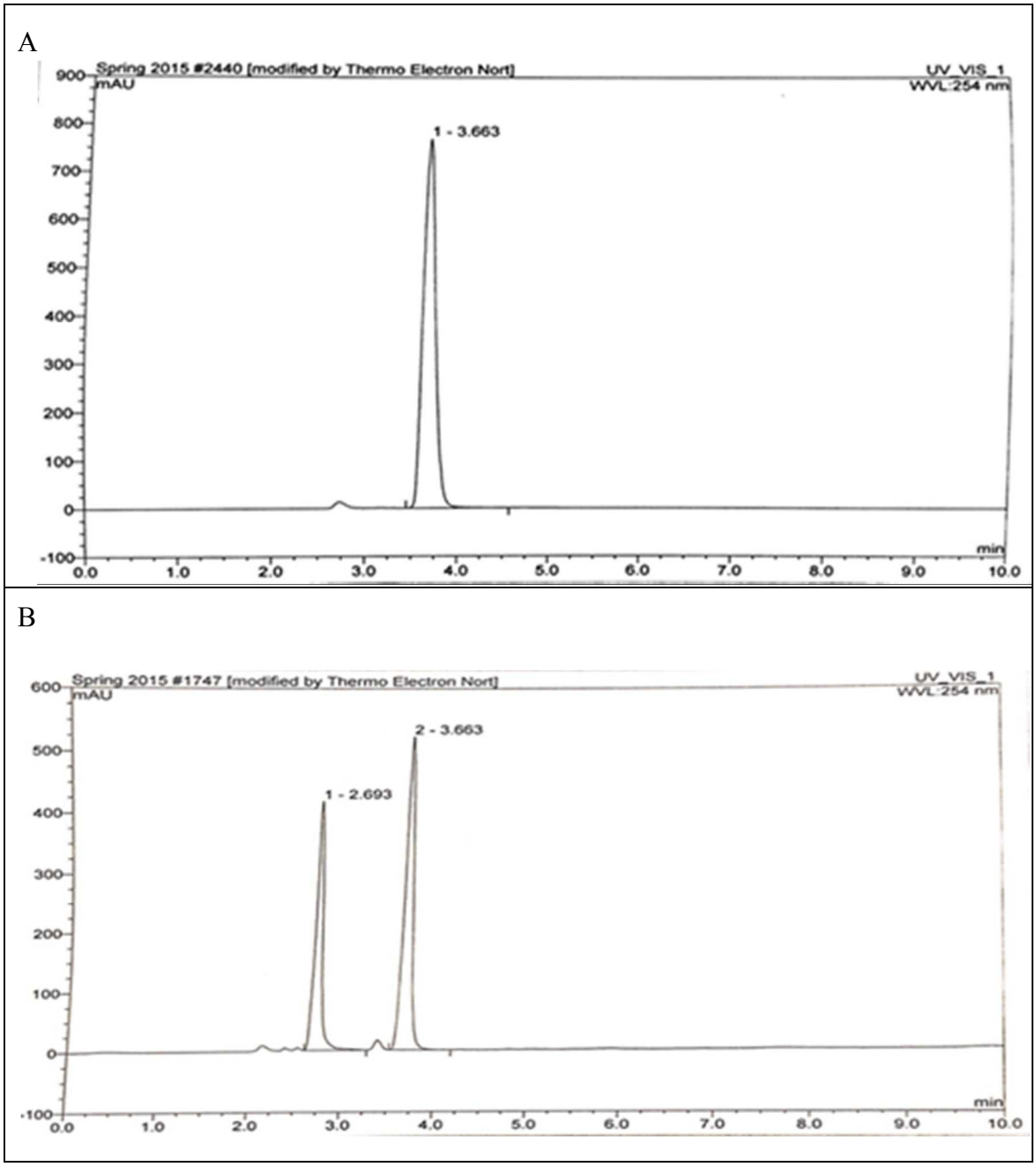


Figure 19: Uridine to uracil conversion catalyzed by uridine nucleosidase in organic okra. The reaction mixture included 1 mM uridine 1 mL and organic okra extract (100 µL) in 10 mM Tris buffer pH 7.2. (A) Reaction mixture before organic okra extract addition. It shows a uridine peak at 3.663 minutes. (B) Reaction mixture after organic okra extract addition. Uridine peak still appeared at 3.663 minutes. A second peak, with a retention time expected for uracil at 2.693 minutes appeared five hours later.

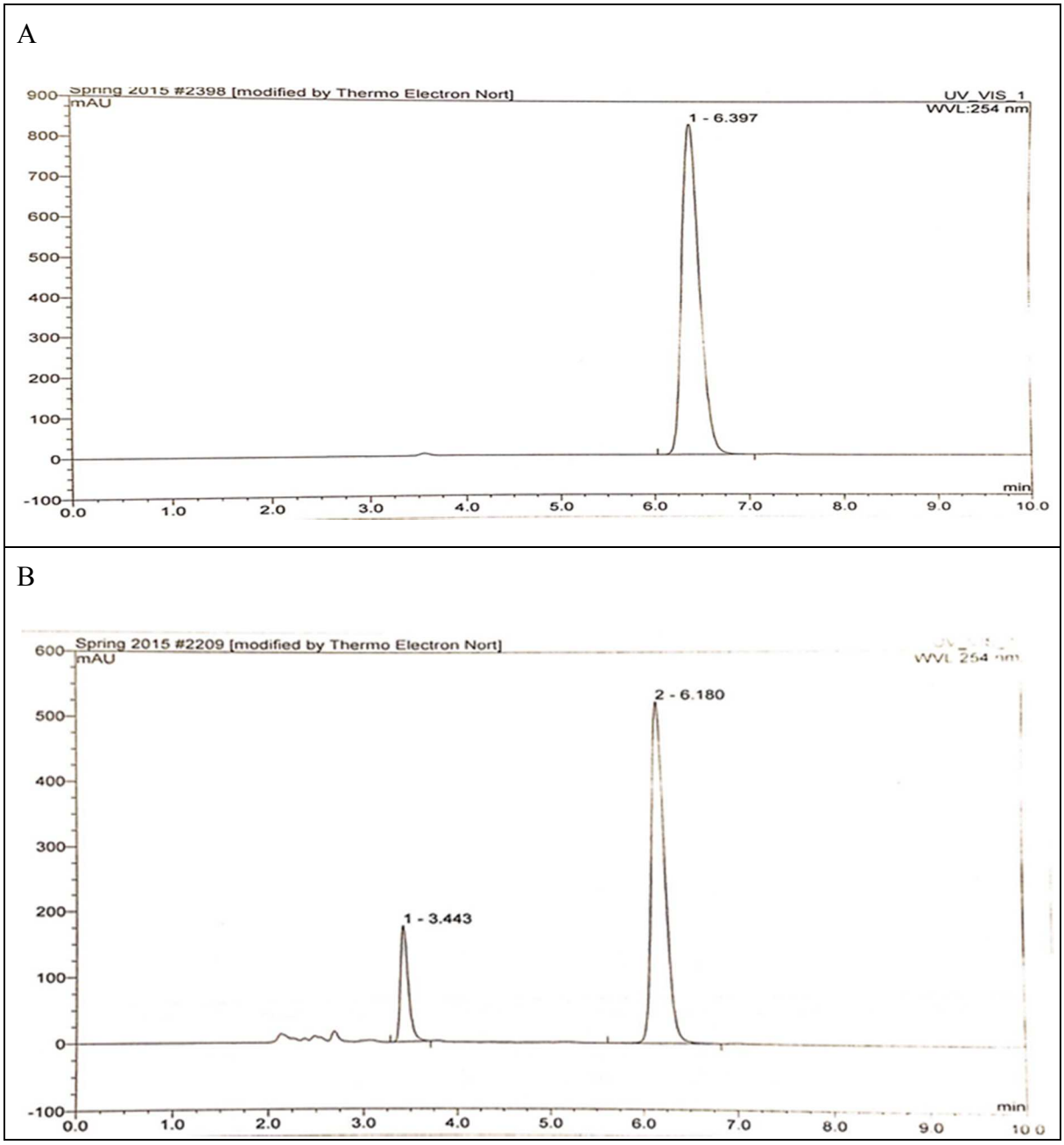


Figure 20: Inosine to hypoxanthine conversion catalyzed by inosine nucleosidase in okra. The reaction mixture included 1 mM inosine 1 mL and okra extract (100 µL) in 10 mM Tris buffer pH 7.2. (A) Reaction mixture before okra extract addition. It shows inosine peak at 6.397 minutes. (B) Reaction mixture after okra extract addition. Inosine appeared at 6.180 minutes. A second peak, with a retention time expected for hypoxanthine, appeared four hours later at 3.443 minutes.

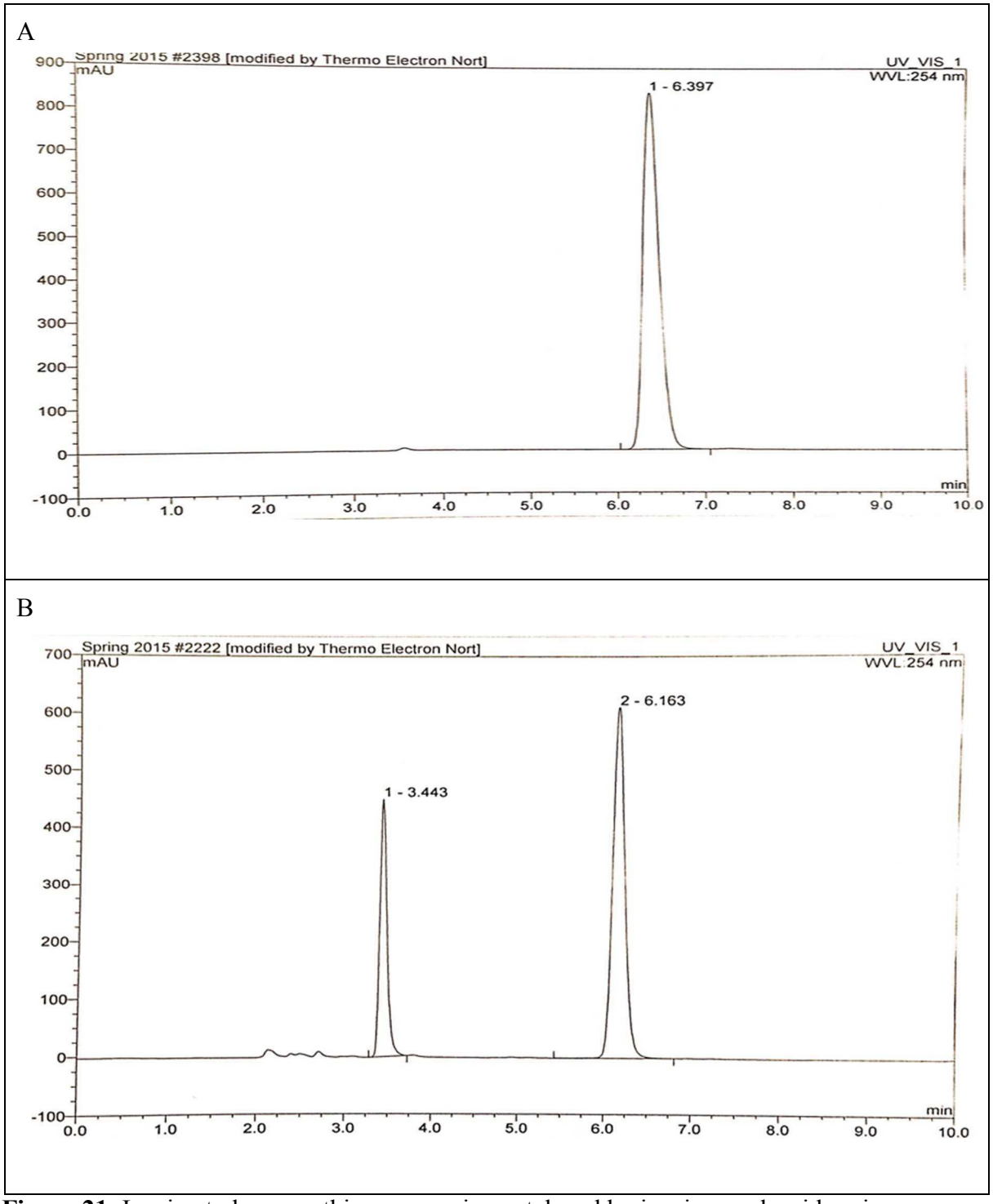


Figure 21: Inosine to hypoxanthine conversion catalyzed by inosine nucleosidase in organic okra. The reaction mixture included 1 mM inosine 1 mL and organic okra extract (100 µL) in 10 mM Tris buffer pH 7.2 (1 mL). (A) Reaction mixture before organic okra extract addition. It shows inosine peak at 6.397 minutes. (B) Reaction mixture after organic okra extract addition. Inosine appeared at 6.163 minutes. A second peak, with a retention time expected for hypoxanthine, appeared four hours later at 3.443 minutes.

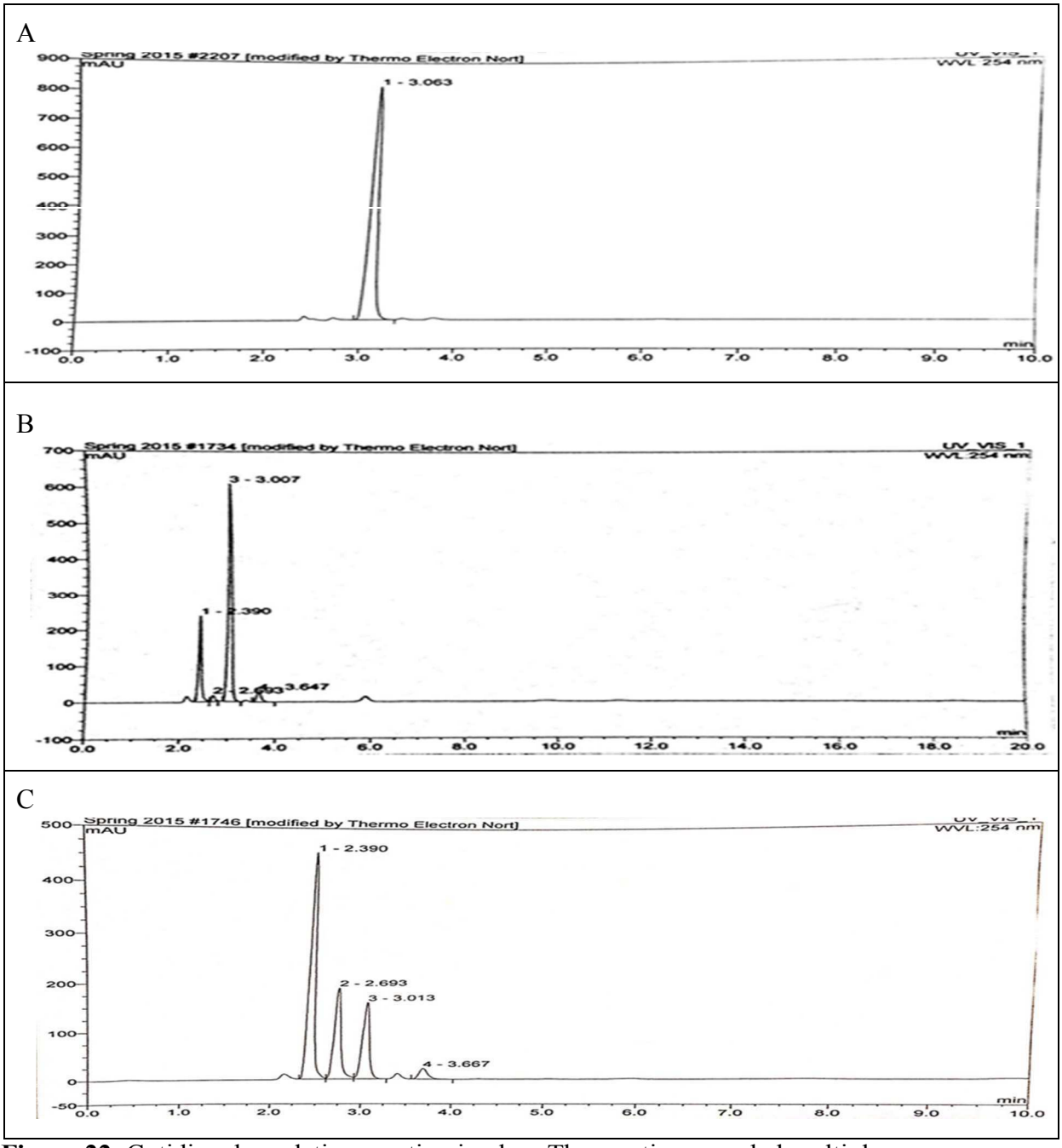


Figure 22: Cytidine degradation reaction in okra. The reaction revealed multiple activities: cytidine nucleosidase, cytidine deaminase and uridine nucleosidase. (A) Reaction mixture before okra extract (100 µL) added to 1 mL of 1 mM cytidine. Cytidine appeared at 3.063 minutes (B) Reaction mixture after okra extract addition. Product formation was rapid and after just two hours. Cytidine peak appeared at 3.007 minutes, cytosine can be seen at 2.390 minutes uridine at 3.647 minutes, uracil can be seen at 2.693 minutes (C) Significant product formation is observed after seven hours from reaction initiation. Cytidine appeared at 3.013 minutes, cytosine can be seen at 2.390 minutes, uridine at 3.667 minutes and uracil at 2.693 minutes.

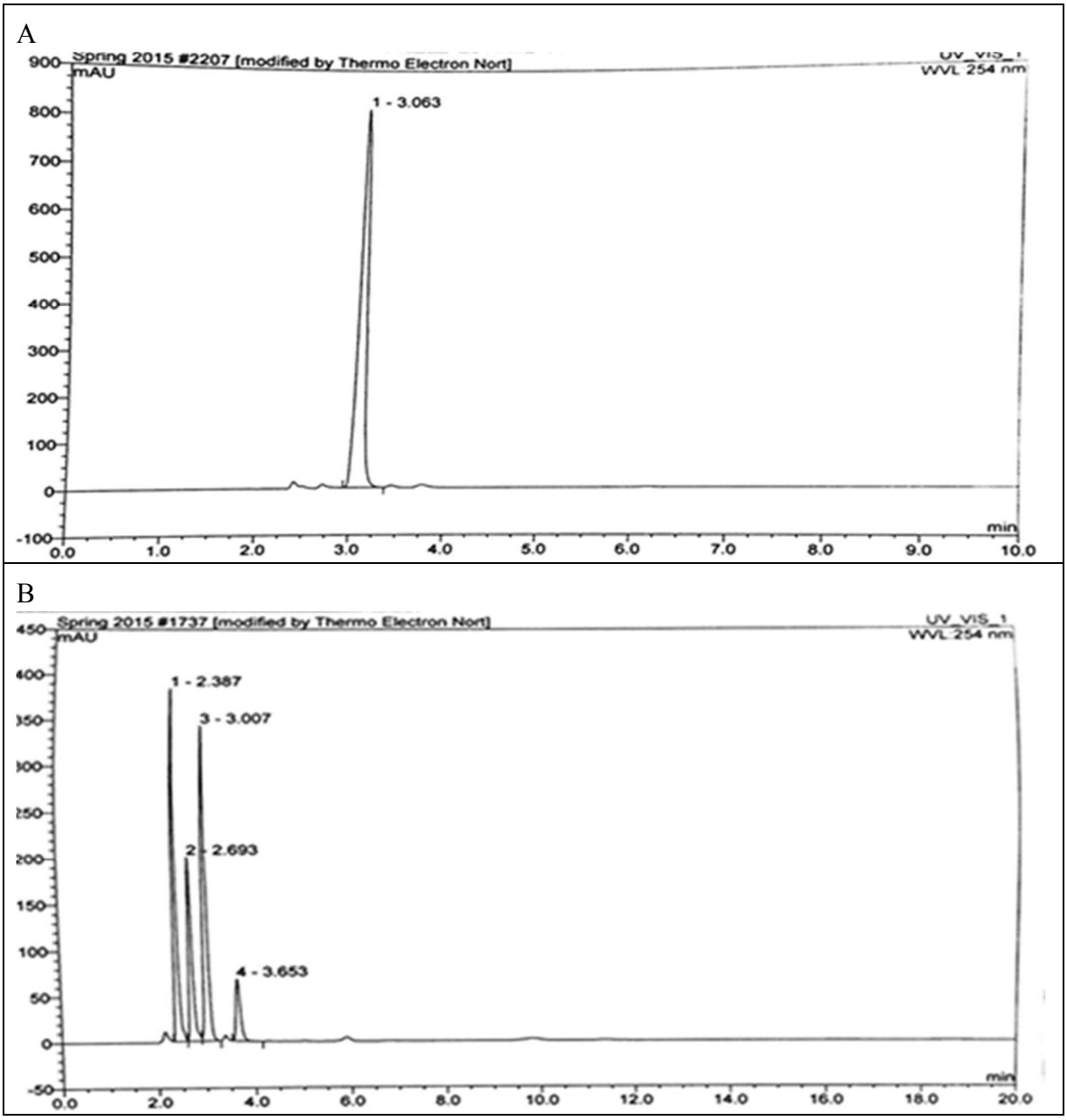


Figure 23: Cytidine degradation reaction in organic okra. The reaction revealed multiple activities: cytidine nucleosidase, cytidine deaminase and uridine nucleosidase. (A) Reaction mixture before organic okra extract (100 µL) added to 1 mL of 1 mM cytidine. Cytidine appeared at 3.063 minutes. (B) Reaction mixture after organic okra extract addition. After four hours cytidine appeared at 3.007 minutes, cytosine can be seen at 2.387 minutes, uridine at 3.653 minutes and uracil at 2.693 minutes.

Adenosine and 2'-deoxyadenosine were also used as substrates to assess the presence of hydrolyzing activity associated with a nucleosidase (Figures 24-25 and 2627). Adenosine and 2'-deoxyadenosine nucleosidase were active in okra and organic okra. The activity level for adenosine nucleosidase in okra were higher than in organic okra. The specific activities were 0.0158 μmol/(min mg) in okra and 0.0062 μmol/(min mg) in organic okra for adenosine. The activities were similar for 2'-deoxyadenosine. They were 0.00041 μmol/(min mg) and 0.00193 μmol/(min mg), respectively (Table 2 and Table 3). In organic okra it took well over six hours before adenosine completely disappeared, while in okra it took five hours.

The degradation reaction in organic okra for adenosine and 2'-deoxyadenosine showed multiple peaks: adenine, inosine, hypoxanthine, and two others peaks. The degradation reaction in okra for 2'-deoxyadenosine showed multiple peaks: adenine, inosine, hypoxanthine, and two others peaks. Those two peaks could be identified as xanthine being produced by hypoxanthine oxidase and inosine could be converted to xanthosine. An adenine peak does not appear with adenosine in okra.

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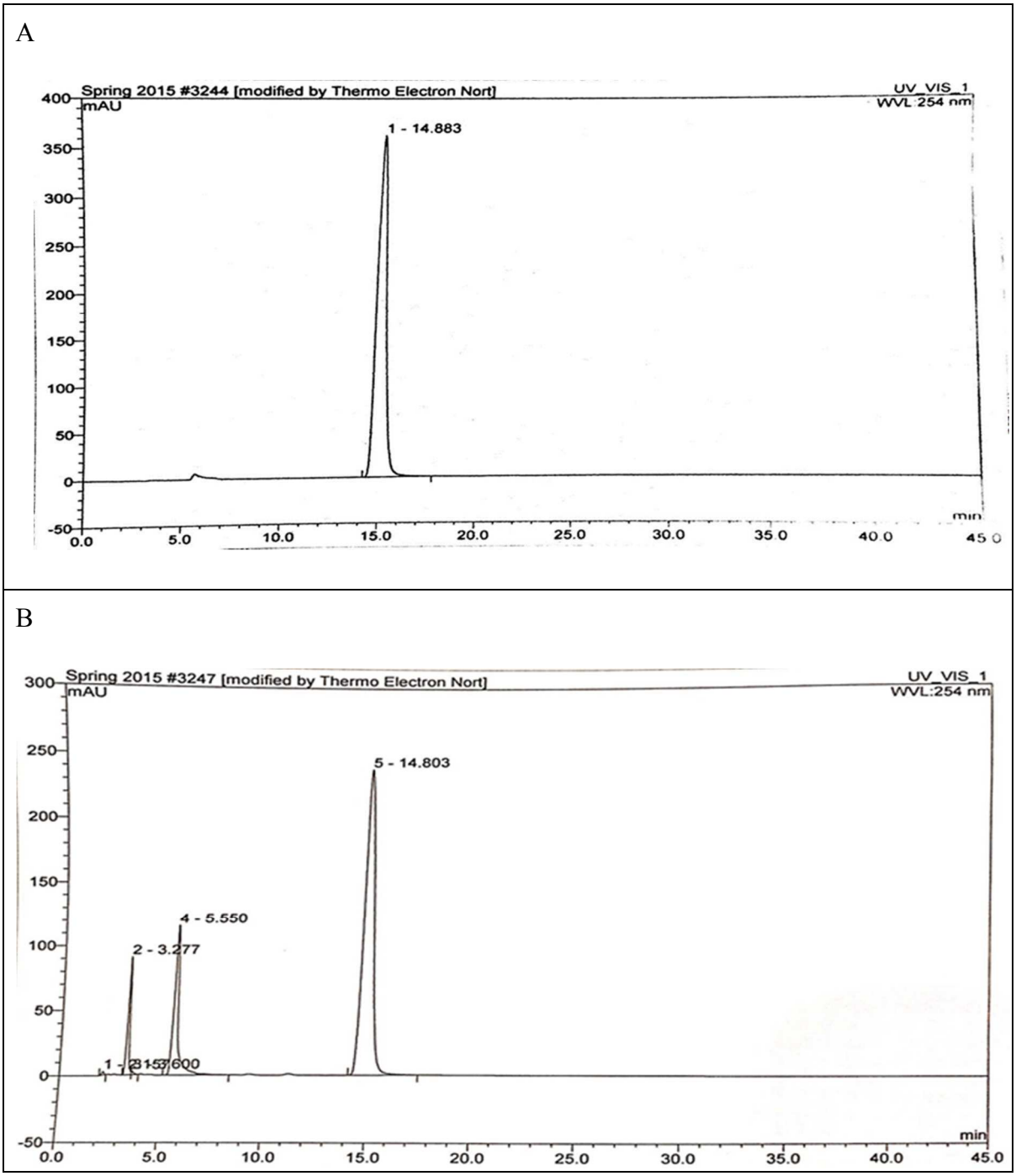

Figure 24: Adenosine breakdown reaction in okra. The reaction revealed multiple peaks: Inosine, hypoxanthine, and fourth peak identified as xanthine. Okra extract (100 µL) was added to 1 mL of 1 mM adenosine solution. (A) Reaction mixture before okra extract addition is shown, adenosine at 14.883 minutes. (B) Reaction mixture after okra extract addition. Three hours following reaction initiation adenosine is present at 14.803 minutes. Inosine was clearly visible at 5.550 minutes. Small peak appeared for hypoxanthine at 3.600 minutes. Xanthine peak appeared at 3.277 minutes. An unidentified peak appeared at 2.160 minutes. An adenine peak does not appear.

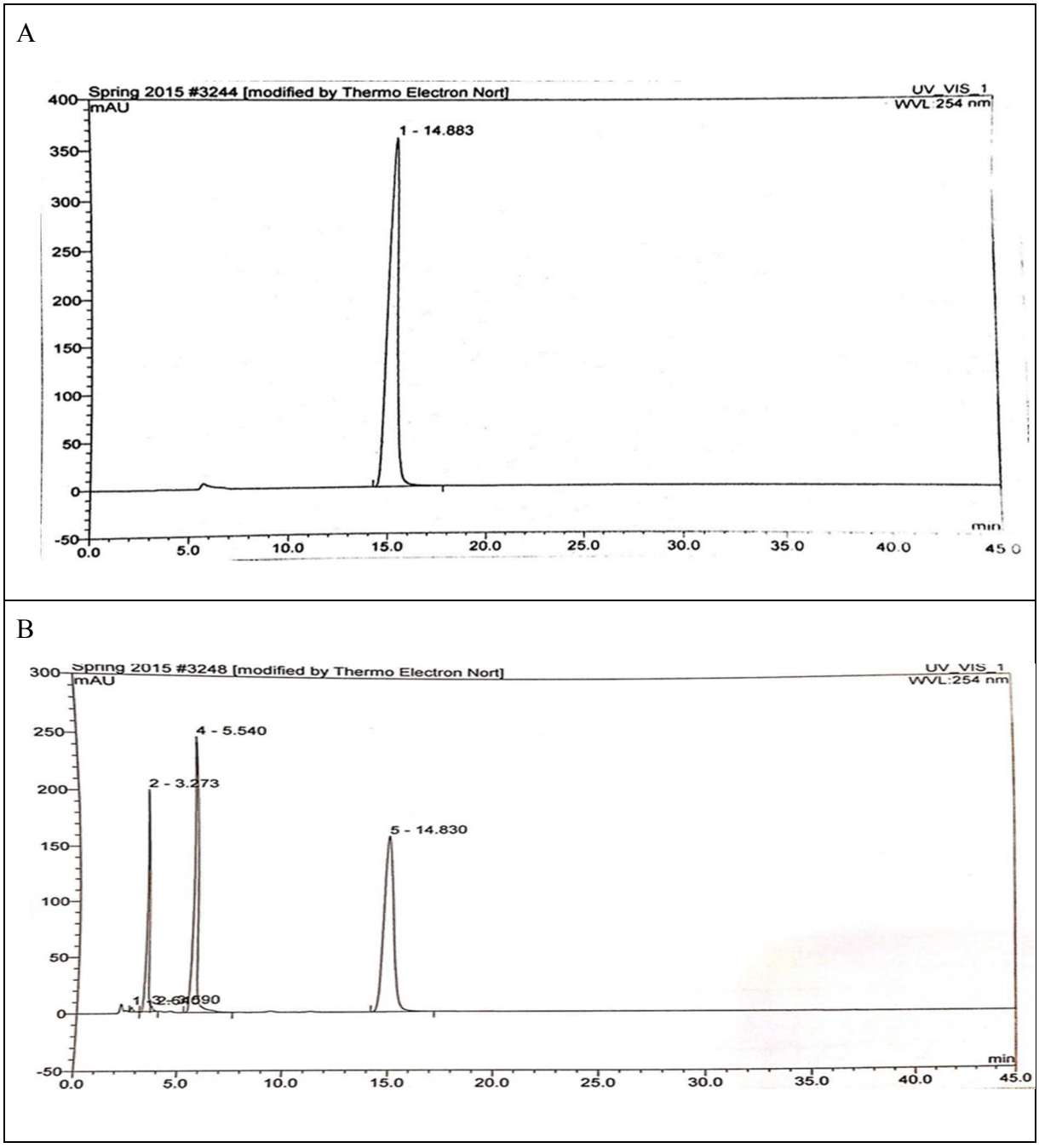

Figure 25: Adenosine breakdown reaction in organic okra. The reaction revealed multiple peaks: adenine, inosine, hypoxanthine, and fourth peak identified as xanthine. Organic okra extract (100 µL) was added to 1 mL of 1 mM adenosine solution. (A) Reaction mixture before organic okra extract addition is shown, adenosine at 14.883 minutes. (B) Reaction mixture after organic okra extract addition. Six hours following reaction initiation adenosine present at 14.830 minutes. Inosine is clearly visible at 5.540 minutes. Small peaks appeared for both adenine and hypoxanthine. Adenine can be seen at 2.640 minutes while hypoxanthine appeared at 3.590 minutes. Xanthine peak appeared at 3.273 minutes.

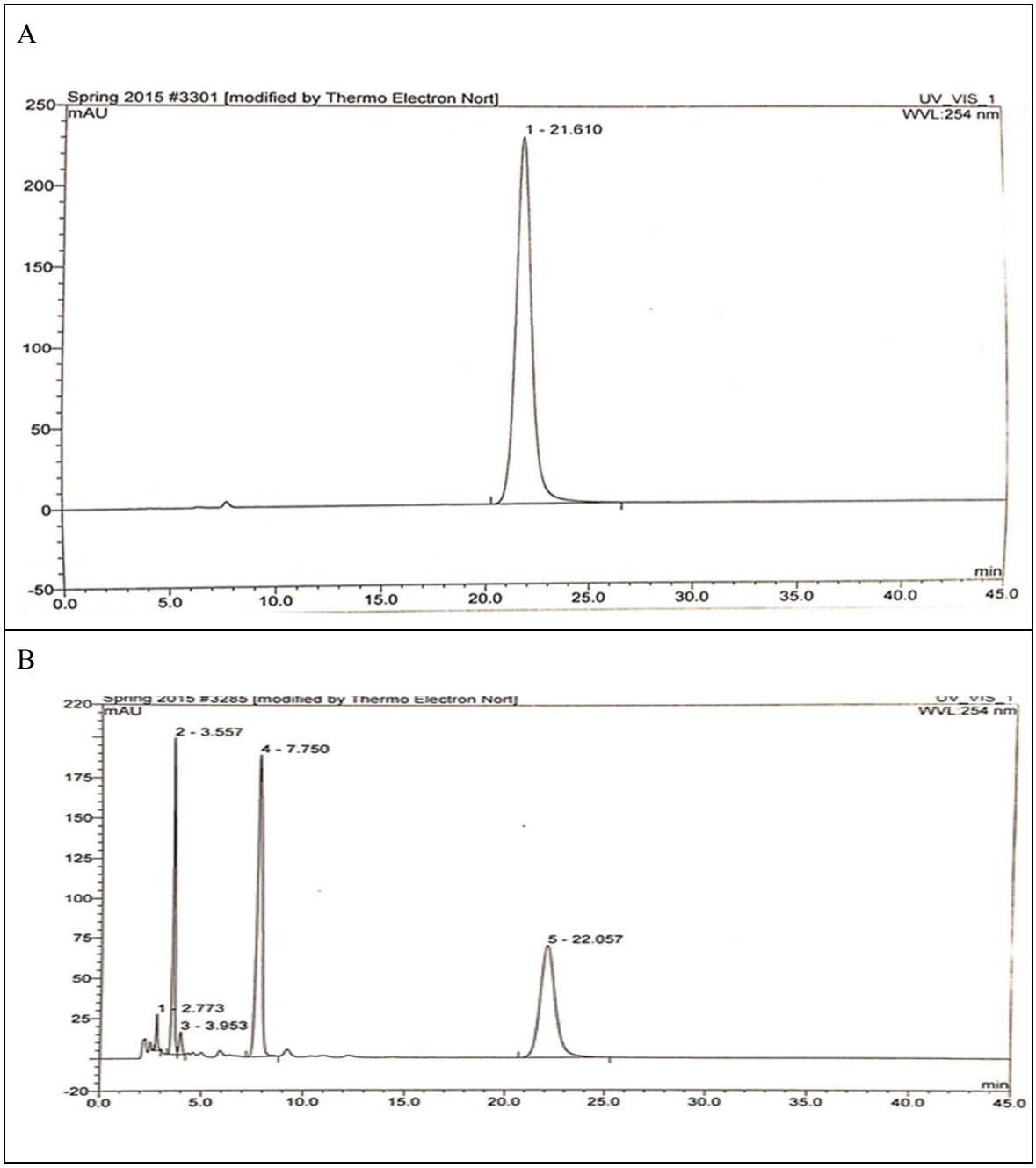

Figure 26: 2'-Deoxyadenosine degrading reaction in okra. The reaction yielded adenine, 2'-deoxyinosine, hypoxanthine and xanthine. Okra extract (100 µL) was added to 1 mL of 1 mM 2'-deoxyadenosine solution. (A) Reaction mixture before okra extract addition. 2'Deoxyadenosine appeared at 21.610 minutes. (B) Five hours following reaction initiation, 2'-deoxyadenosine appeared at 22.057 minutes. 2'-deoxyinosine is clearly visible at 7.750 minutes. A peak can be seen for adenine at 2.773 minutes and hypoxanthine also appeared at 3.557 minutes. Xanthine appeared at 3.953 minutes.

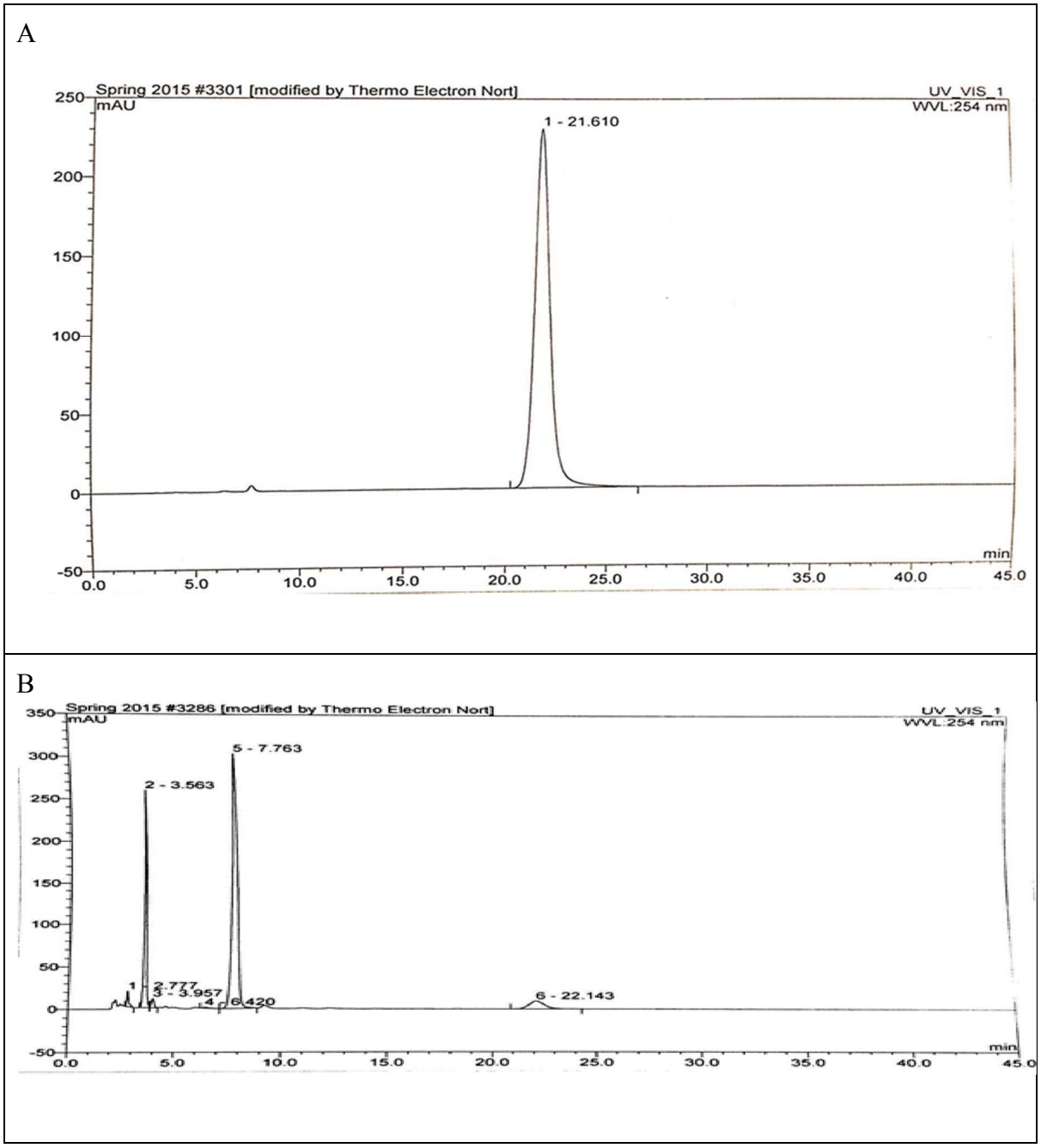

Figure 27: 2'-Deoxyadenosine degradation reaction in organic okra. The reaction yielded adenine, 2'- deoxyinosine, hypoxanthine and two others peaks identified as xanthosine and xanthine. Organic okra extract (100 µL) was added to 1 mL of 1 mM 2'deoxyadenosine solution. (A) Reaction mixture before organic okra extract addition. 2'Deoxyadenosine appeared at 21.610 minutes. (B) Six hours following reaction initiation, 2'-deoxyadenosine appeared at 22.143 minutes. 2'-deoxyinosine is clearly visible at 7.763 minutes. A peak can be seen for adenine at 2.777 minutes and hypoxanthine appeared at 3.563 minutes. Xanthosine appeared at 6.420 while xanthine appeared at 3.957 minutes.

Table 2: Nucleosidase and deaminase activities in okra. Nucleosidase specific activities found in okra 5-9 day old seedlings. The values reported are an average of at least three measurements.

Enzyme	Presence	Specific Activities μmol/(min mg)
Uridine Nucleosidase	Y	0.017
Inosine Nucleosidase	Y	0.0096
Cytidine Nucleosidase/Deaminase	Y	0.0036
Adenosine Nucleosidase/ Deaminase	Y	0.0158
2'-Deoxyadenosine Nucleosidase	Y	0.00041

Table 3: Nucleosidase and deaminase activities in organic okra. Nucleosidase specific activities found in organic okra 5-9 day old seedlings. The values reported are an average of at least three measurements.

Enzyme	Presence	Specific Activities μmol/(min mg)
Uridine Nucleosidase	Y	0.025
Inosine Nucleosidase	Y	0.0043
Cytidine Nucleosidase/Deaminase	Y	0.0052
Adenosine Nucleosidase/ Deaminase	Y	0.0062
2'-Deoxyadenosine Nucleosidase	Y	0.00193

Enzyme Activities in Bean (Blue Lake 274 Bush), and Organic Bean (Blue Lake 274 Bush).

Bean (Blue Lake 274 Bush), and organic bean (Blue Lake 274 Bush) are plants that have great nutritional value, especially its seeds which are a good source of protein.[42] Nucleosidase and deaminase activities were evaluated in 5-9 day old bean and organic bean seedlings. The level of activity observed was lower when compared to okra and organic okra across all substrates tested. All reactions took a substantially longer time to initiate.

Uridine was converted to uracil (Figures 28-29). The specific activity in both plants was 0.0015 μmol/(min mg) in bean and 0.0046 μmol/(min mg) in organic bean. An unidentified peak appeared in both bean and organic bean at 2.113 minutes and 2.123 minutes, respectively. This was not evaluated in this study but probably represents a product further along the degradation pathway. Identification of this third peak remains to be determined in the future.

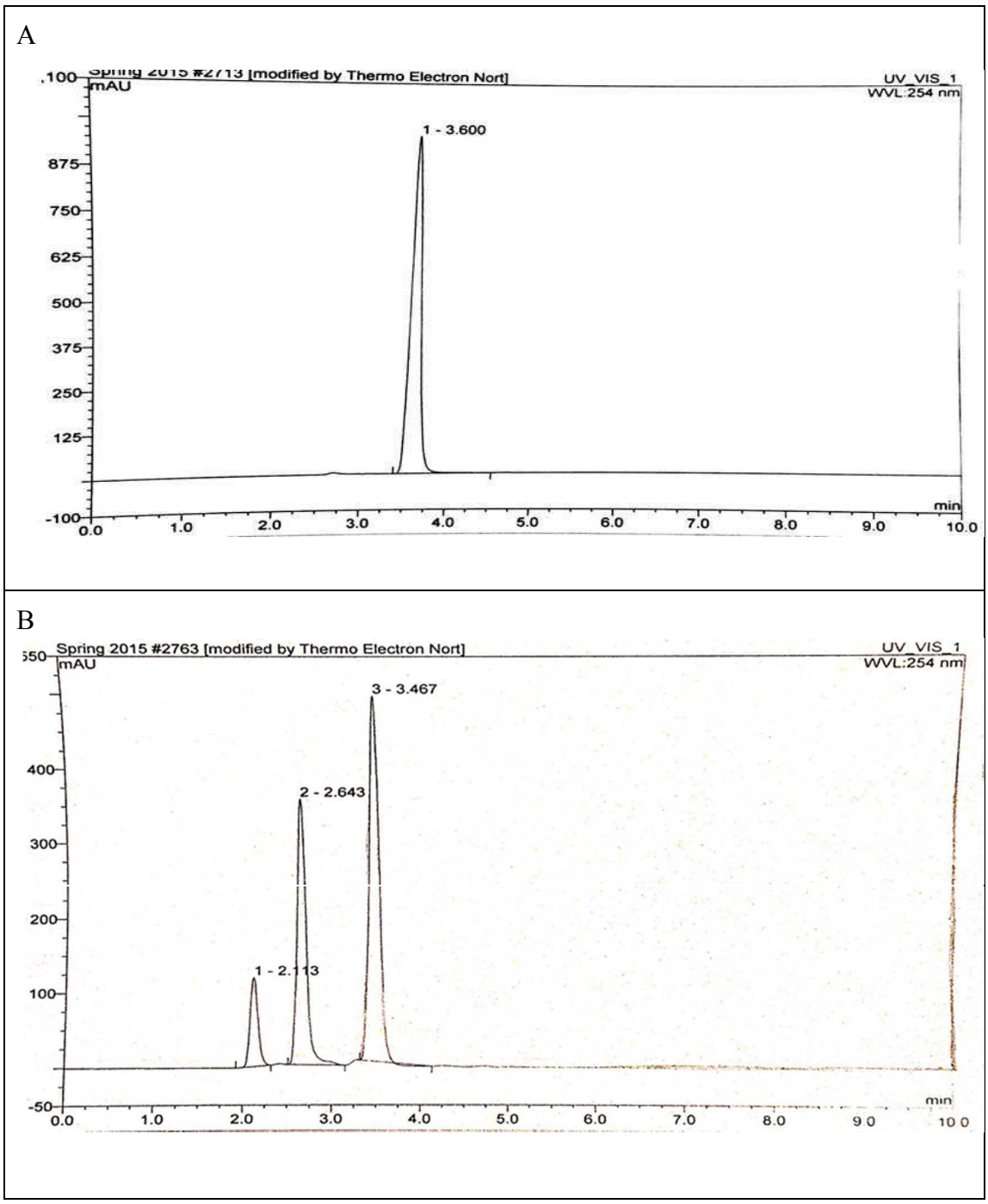

Figure 28: Uridine to uracil conversion catalyzed by uridine nucleosidase in bean. The reaction mixture included 1 mM uridine (1 mL) and bean extract (100 µL) in 10 mM Tris buffer pH 7.2. (A) Reaction mixture before bean extract addition. It shows a uridine peak at 3.600 minutes. (B) Reaction mixture after bean extract addition. Uridine appeared at 3.467 minutes. A second peak, with the retention time expected for uracil (2.643 minutes) appeared one and half hours later. An unidentified peak appeared at 2.113 minutes.

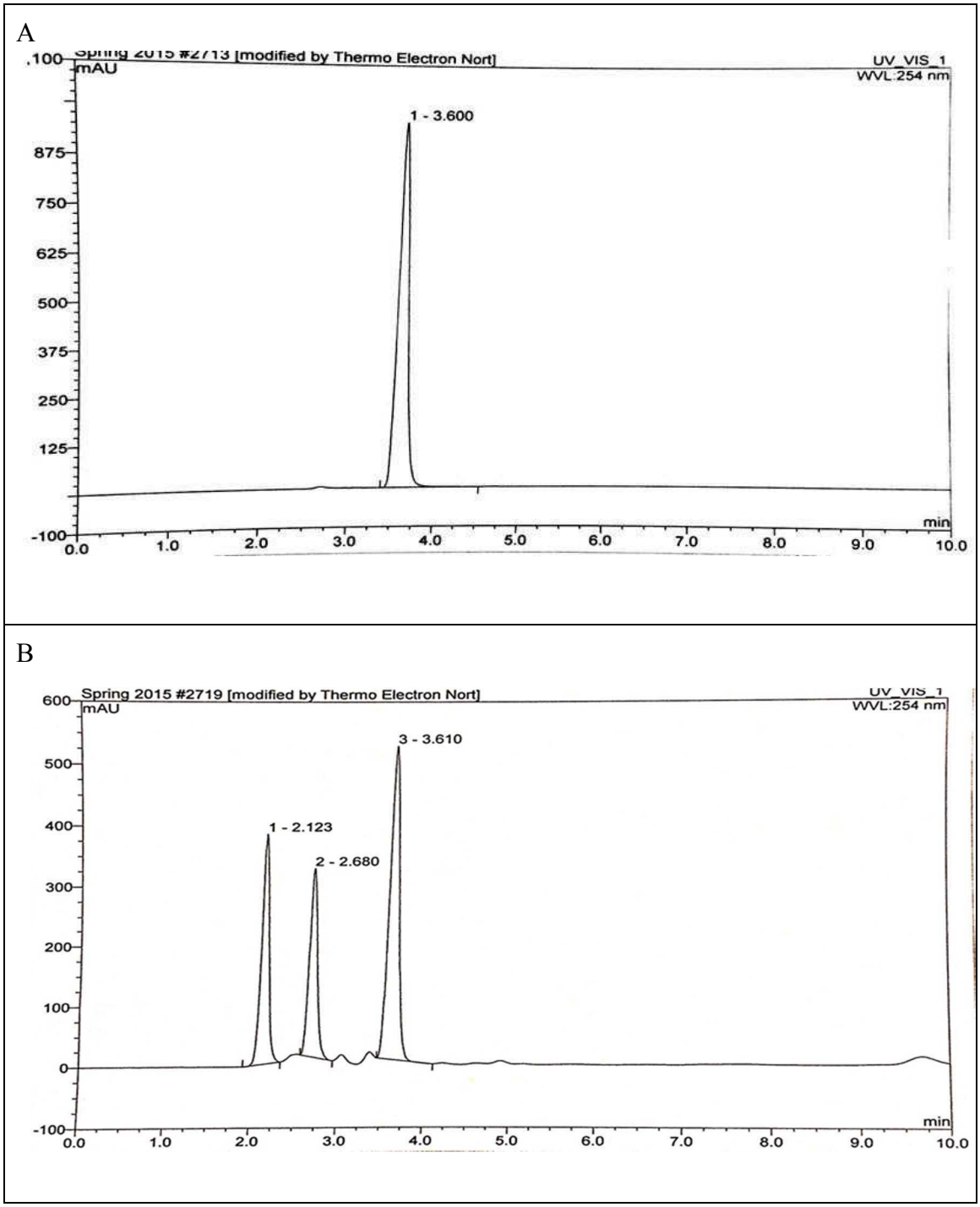

Figure 29: Uridine to uracil conversion catalyzed by uridine nucleosidase in organic bean. The reaction mixture included 1 mM uridine (1 mL) and organic bean extract (100 µL) in 10 mM Tris buffer pH 7.2. (A) Reaction mixture before organic bean extract addition. It shows a uridine peak at 3.600 minutes. (B) Reaction mixture after organic bean extract addition. Uridine appeared at 3.610 minutes. A second peak, with a retention time expected for uracil (2.680 minutes) appeared three hours later. An unidentified peak appeared at 2.123 minutes.

Adenosine and 2'-deoxyadenosine nucleosidase and deaminase were active in bean and organic bean. The specific activity observed for 2'-deoxyadenosine is very similar to that for adenosine in both okra and organic okra. The specific activity for adenosine in bean was 0.0094 μmol/(min mg) and 0.0044 μmol/(min mg) in organic bean. In bean and organic bean adenosine took five hours to completely disappear while 2'deoxyadenosine took six hours.

The specific activity for 2'-deoxyadenosine nucleosidase and deaminase in bean and organic bean were 0.0027 μmol/(min mg) and 0.0054 μmol/(min mg). The three products formed during the course of the reaction, when adenosine and 2'deoxyadenosine were used as a substrate are adenine, inosine or 2'-deoxyinosine and hypoxanthine. Also, there are two additional peaks appeared during the course of the reaction with adenosine or 2'-deoxyadenosine. One of the two peaks could be xanthine based on its retention time. The second peak, which was not identified, occurred at 2.1 minutes (Figures 30-31 and 33). In bean 2'-deoxyinosine peak does not appear (Figure 32).The reaction in both okra and organic okra took about four hours before adenosine completely disappeared and only hypoxanthine remained. However it took about six hours for 2'-deoxyadenosine to completely disappear.

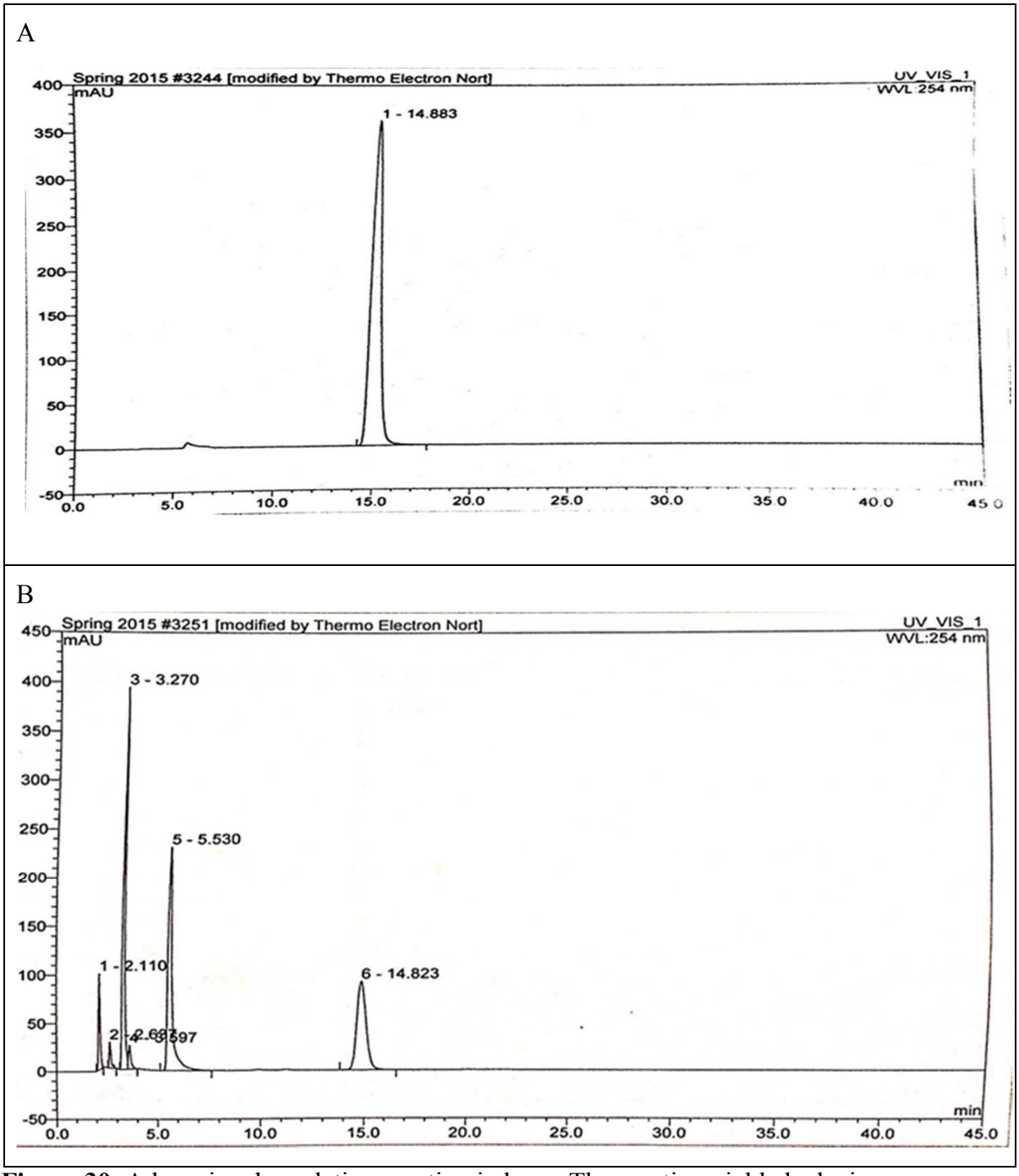

Figure 30: Adenosine degradation reaction in bean. The reaction yielded adenine, inosine, hypoxanthine and two others peaks. Bean extract (100 µL) was added to 1 mL of 1 mM adenosine solution. (A) Reaction mixture before bean extract addition is shown. Adenosine appeared at 14.883 minutes. (B) Reaction mixture after bean extract addition. Five hours following reaction initiation, adenosine present at 14.823 minutes. Inosine is clearly visible at 5.530 minutes. Adenine can be seen at 2.637 minutes while hypoxanthine appeared at 3.597 minutes. Xanthine appeared at 3.270 minutes. An unidentified peak appeared at 2.110 minutes.

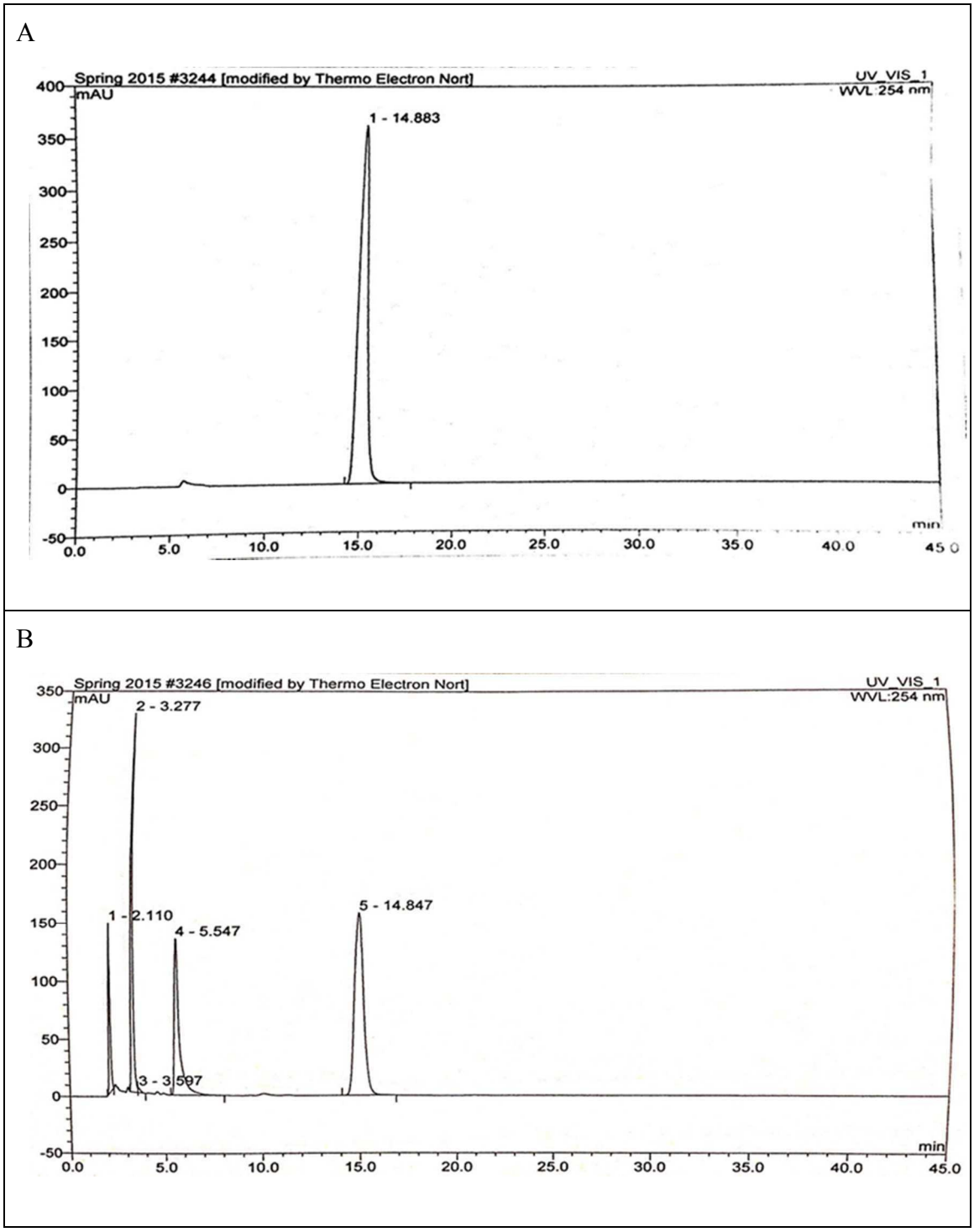

Figure 31: Adenosine degradation reaction in organic bean. The reaction yielded adenine, inosine, and hypoxanthine. Organic bean extract (100 µL) was added to 1 mL of 1 mM adenosine solution. (A) Reaction mixture before organic bean extract addition. Adenosine appeared at 14.883 minutes. (B) Four hours following reaction initiation, adenosine is present at 14.847 minutes. Inosine is visible at 5.547 minutes. An adenine peak does not appear while hypoxanthine appeared at 3.597 minutes. Xanthine appeared at 3.277 minutes. An unidentified peak appeared at 2.110 minutes.

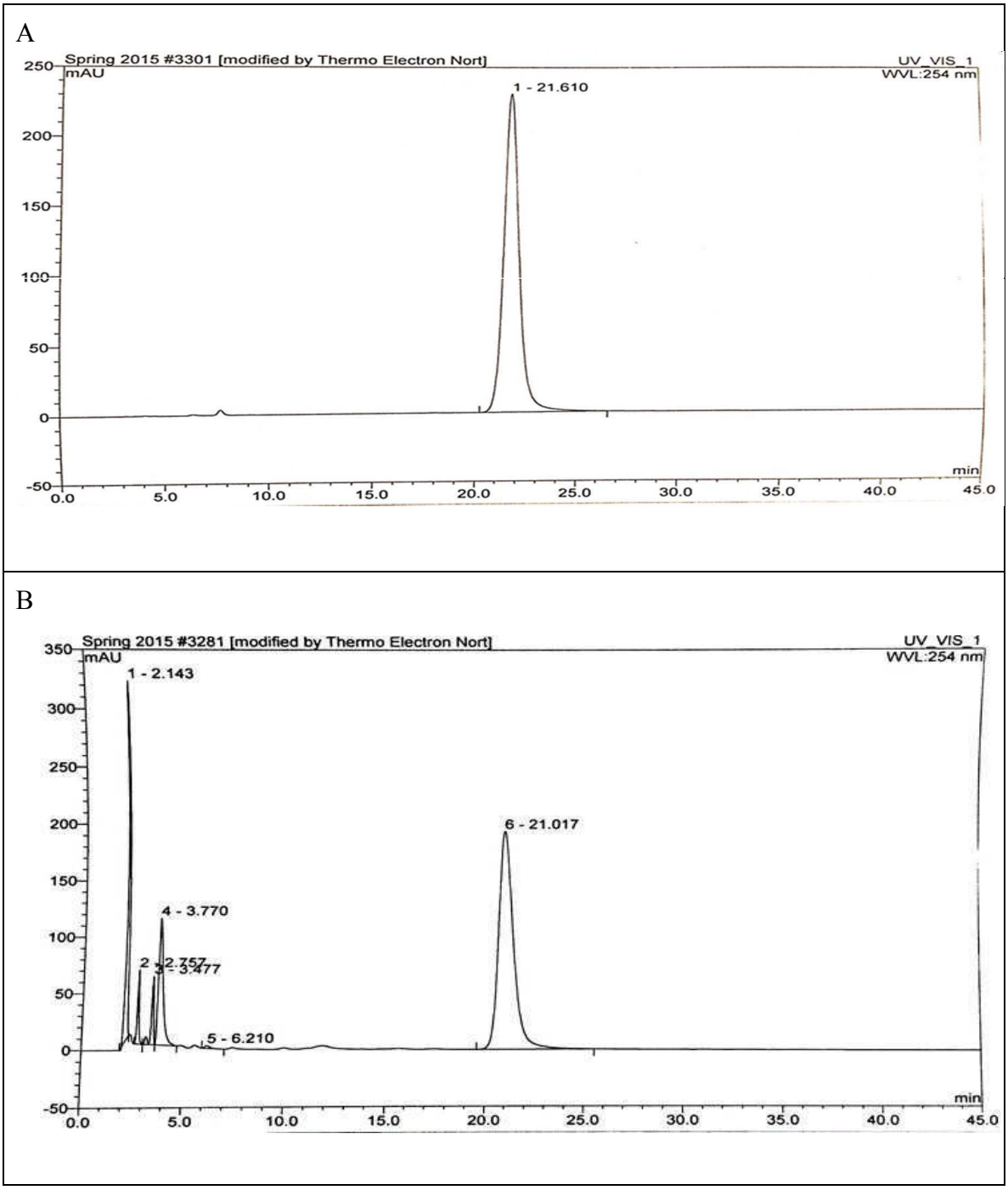

Figure 32: 2'-Deoxyadenosine breakdown reaction in bean. The reaction yielded adenine, hypoxanthine, xanthine and xanthosine. Bean extract (100 µL) was added to 1 mL of 1 mM 2'-deoxyadenosine solution. (A) Reaction mixture before bean extract addition. 2'deoxyadenosine appeared at 21.610 minutes. (B) Six hours following reaction initiation, 2'-deoxyadenosine appeared at 21.017 minutes. A peak can be seen for adenine at 2.757 minutes and hypoxanthine also appeared at 3.477 minutes. Xanthine appeared at 3.770 minutes and xanthosine appeared at 6.210 minutes. An unidentified peak appeared at 2.143 minutes. 2'-Deoxyinosine peak does not appear.

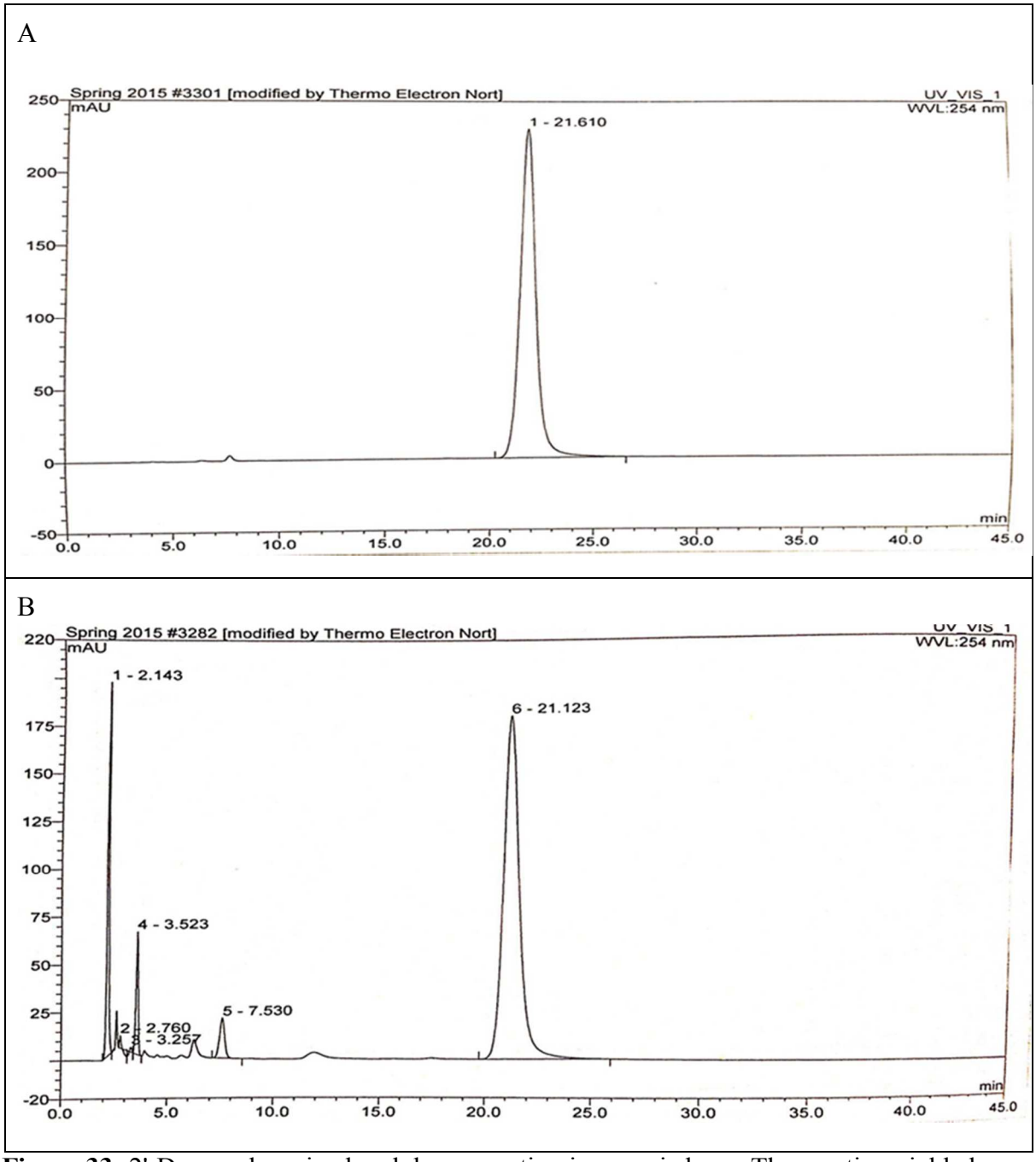

Figure 33: 2'-Deoxyadenosine breakdown reaction in organic bean. The reaction yielded adenine, 2'-deoxy inosine, hypoxanthine and xanthine. Organic bean (100 µL) extract was added to 1 mL of 1 mM 2'-deoxyadenosine solution. (A) Reaction mixture before organic bean extract addition. 2'-deoxyadenosine appeared at 21.610 minutes. (B) Reaction mixture after organic bean extract addition. Six hours following reaction initiation, 2'deoxyadenosine appeared at 7.530 minutes. 2'-Deoxyinosine is clearly visible at 7.530 minutes. A peak can be seen for adenine at 2.760 minutes and hypoxanthine appeared at 3.523 minutes. Xanthine appeared at 3.257 minutes. An unidentified peak appeared at 2.143 minutes.

Inosine was also used as a nucleosidase substrate in bean and organic bean. The specific activity observed for inosine in bean was very similar to that in organic bean. Inosine nucleosidase was active at 0.0066 μmol/(min mg) in bean and 0.0068 μmol/(min mg) in organic bean. Similar to okra and organic okra, it appears that inosine nucleosidase was active in bean and organic bean as well (Figures 34-35).

Cytidine nucleosidase was active in bean and organic bean. Based on the HPLC chromatogram cytidine deaminase activity was also present. Cytidine with bean and organic bean extracts generated multiple products (Figures 36-37). Cytosine, uridine and uracil eluted along with cytidine during the course of the reaction. The specific activity for enzymes catalyzing degradation of cytidine in organic bean was 0.014 μmol/(min mg). The specific activity in bean was somewhat lower at 0.0078 μmol/(min mg) (Table 4 and Table 5). The reaction was faster in organic bean than bean. The reaction took about nine hours in bean and just two hours in organic bean.

Similar to uridine, an additional product were observed during the course of the reaction with both inosine and cytidine in bean and organic bean. Identification of this peak at 2.143 min remains to be accomplished in the future.

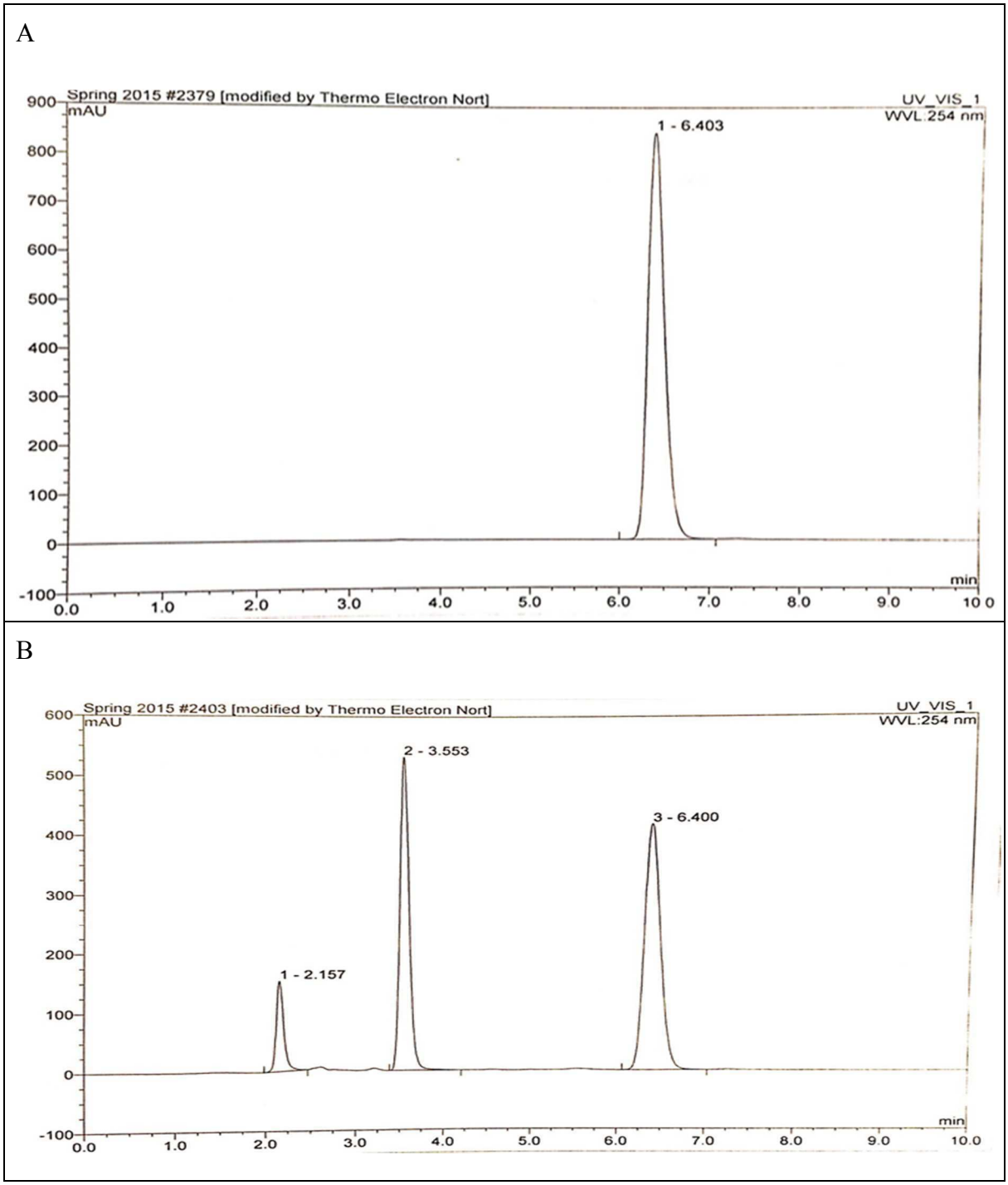

Figure 34: Inosine to hypoxanthine conversion catalyzed by inosine nucleosidase in bean. The reaction mixture included 1 mM inosine (1 mL) and bean extract (100 µL) in 10 mM Tris buffer pH 7.2. (A) Reaction mixture before bean extract addition. It shows an inosine peak at 6.403 minutes. (B) Reaction mixture after bean extract addition. Inosine appeared at 6.400 minutes. A second peak, with a retention time expected for hypoxanthine, appeared at 3.553 minutes. An unidentified peak appeared at 2.157 minutes.

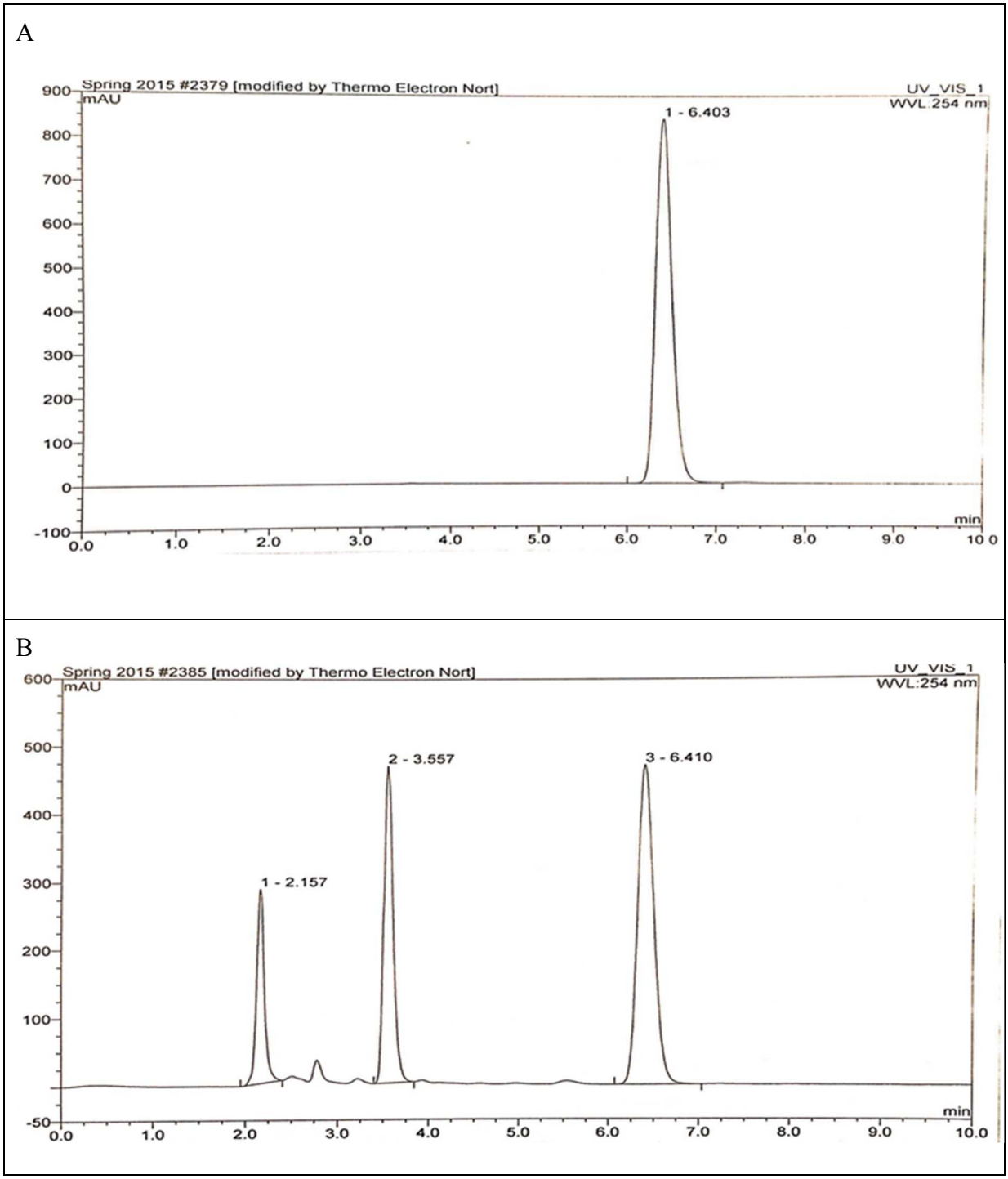

Figure 35: Inosine to hypoxanthine conversion catalyzed by inosine nucleosidase in organic bean. The reaction mixture included 1 mM inosine (1 mL) and organic bean extract (100 µL) in 10 mM Tris buffer pH 7.2. (A) Reaction mixture before organic bean extract addition. It shows an inosine peak at 6.403 minutes. (B) Reaction mixture after organic bean extract addition. Inosine appeared at 6.410 minutes. A second peak, with a retention time expected for hypoxanthine, appeared at 3.557 minutes. An unidentified peak appeared at 2.157 minutes.

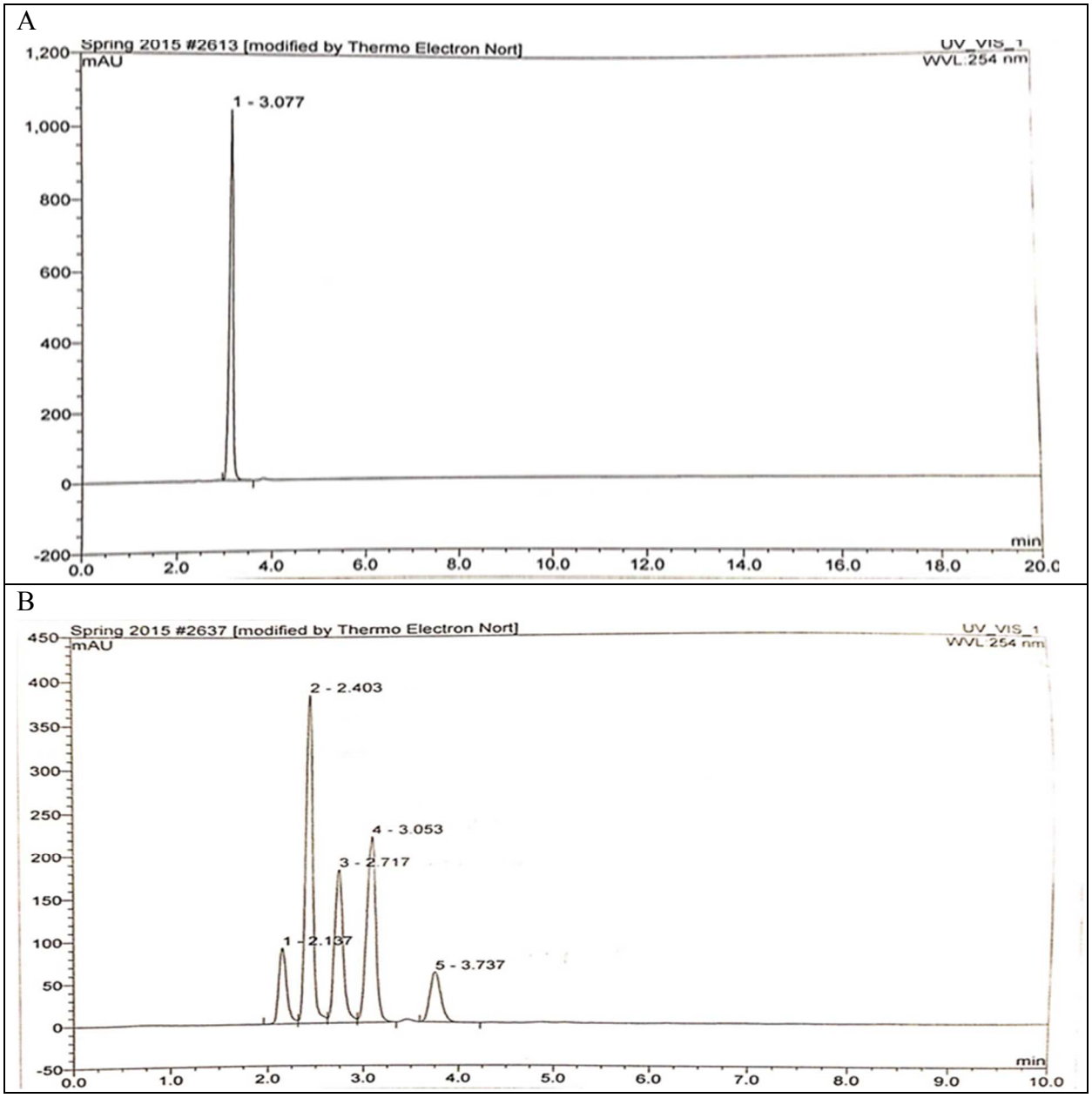

Figure 36: Cytidine degradation reaction in bean. The reaction revealed multiple activities: cytidine nucleosidase, cytidine deaminase and uridine nucleosidase. (A) Reaction mixture before bean extract (100 µL) added to 1 mL of 1 mM cytidine. Cytidine appeared at 3.077 minutes. (B) Reaction mixture after bean extract addition. Cytidine appeared at 3.053 minutes. Cytosine can be seen at 2.403 minutes, uridine at 3.737 minutes and uracil at 2.717 minutes. An unidentified peak appeared at 2.137 minutes.

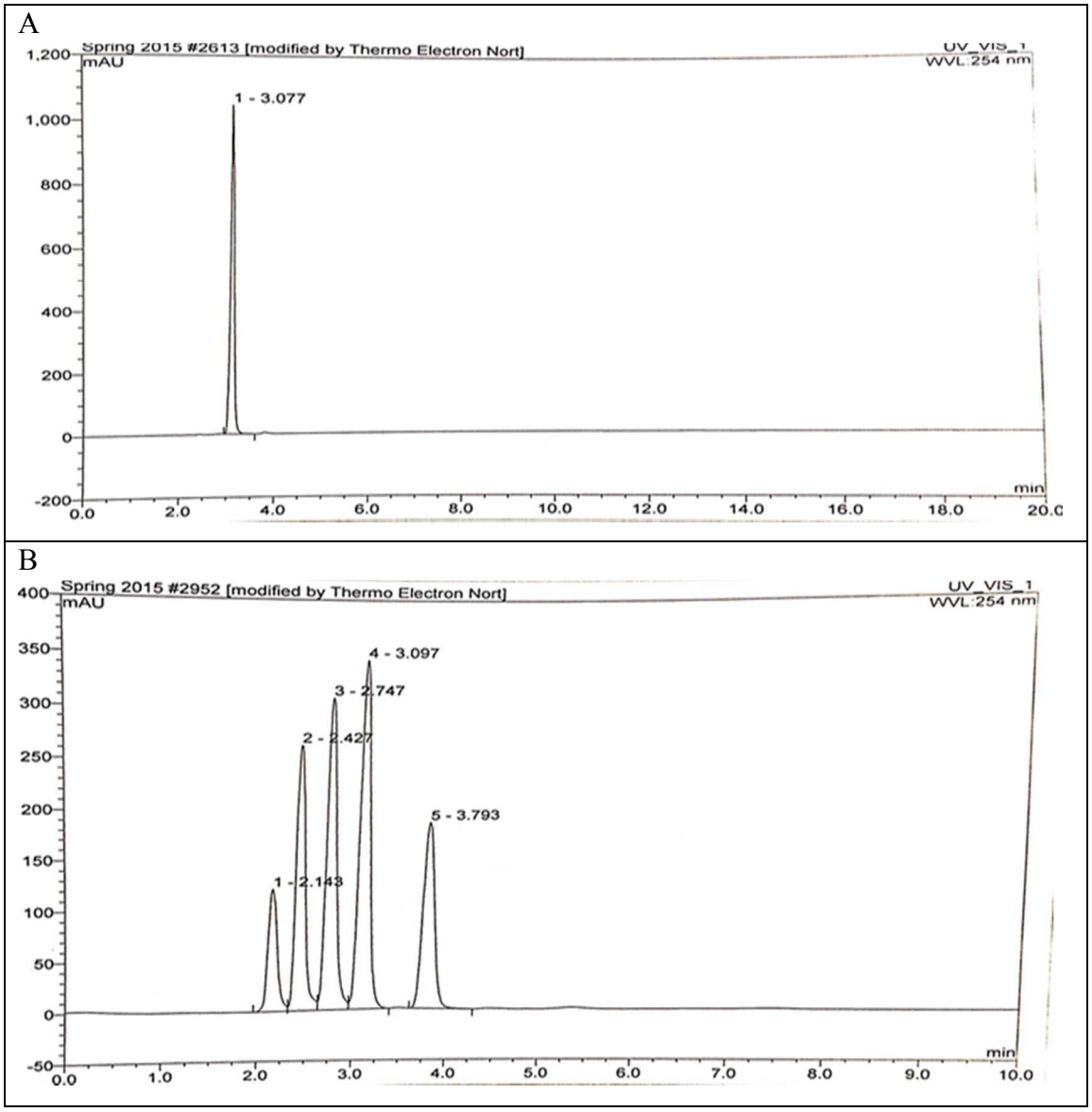

Figure 37: Cytidine degradation reaction in organic bean. The reaction revealed multiple activities: cytidine nucleosidase, cytidine deaminase and uridine nucleosidase. (A) Reaction mixture before organic bean extract (100 µL) added to 1 mL of 1 mM cytidine. Cytidine appeared at 3.077 minutes. (B) Reaction mixture after organic bean extract addition. Cytidine appeared at 3.097 minutes. Cytosine can be seen at 2.427 minutes, uridine at 3.793 minutes and uracil at 2.747 minutes. An unidentified peak appeared at
2.143 minutes.

Table 4: Nucleosidase and deaminase activities in bean. Nucleosidase specific activities found in bean 5-9 day old seedlings. The values reported are an average of at least three measurements

Enzyme	Presence	Specific Activity μmol/(min mg)
Uridine Nucleosidase	Y	0.0015
Inosine Nucleosidase	Y	0.0043
Cytidine Nucleosidase/Deaminase	Y	0.00076
Adenosine Nucleosidase/ Deaminase	Y	0.0094
2'-Deoxyadenosine Nucleosidase	Y	0.00027

Table 5: Nucleosidase and deaminase activities in organic bean. Nucleosidase specific activities found in organic bean 5-9 day old seedlings. The values reported are an average of at least three measurements.

Enzyme	Presence	Specific Activity μmol/(min mg)
Uridine Nucleosidase	Y	0.0046
Inosine Nucleosidase	Y	0.0041
Cytidine Nucleosidase/Deaminase	Y	0.014
Adenosine Nucleosidase /Deaminase	Y	0.0044
2'-Deoxyadenosine Nucleosidase	Y	0.0054

Enzyme Activities in Squash (Early Summer Crookneck) and Organic Squash (Early Summer Crookneck).

Squash (Early Summer Crookneck) and organic squash (Early Summer Crookneck) originated in the Americas and is one of the major and earliest plants popular in Mexico and North America. Squash is a plant that has nutritional value and exists in many varieties.[44]

Adenosine nucleosidases are active in squash and organic squash. Adenosine was degraded quickly to adenine initially via a purine nucleosidase. Inosine was formed rapidly within the first two hours of the reaction and was clearly converted to hypoxanthine by the action of inosine nucleosidase (Figures 38-39). In organic squash a hypoxanthine peak does not appear. The specific activities for enzymes catalyzing degradation of adenosine were 0.012 μmol/(min mg) in squash and 0.010 μmol/(min mg), in organic squash.

Also, reaction of 2'-deoxyadenosine with the initial extract from squash and organic squash were degraded to multiple peaks. The degradation reaction in squash and organic squash yielded adenine, 2'-deoxyinosine, hypoxanthine and third peak identified as xanthine (Figures 40-41). The 2'-deoxyadenosine degradation reaction in squash took Seven hours. However it took about nine hours in organic squash. The specific activities for enzymes catalyzing degradation of 2'-deoxyadenosine was 0.0047 μmol/(min mg) in squash and 0.0070 μmol/(min mg) in organic squash.

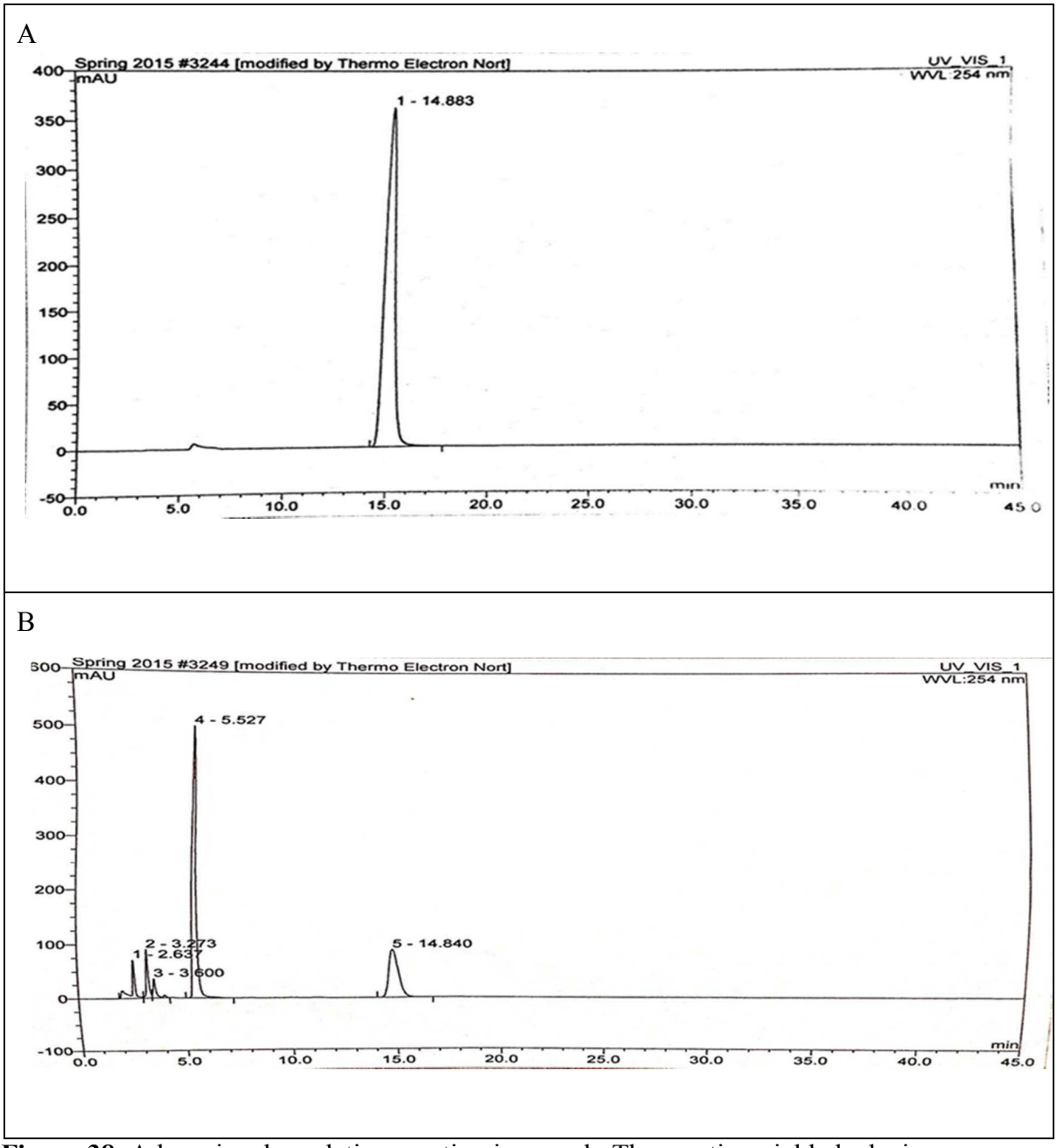

Figure 38: Adenosine degradation reaction in squash. The reaction yielded adenine, inosine, and hypoxanthine. Squash extract (100 µL) was added to 1 mL of 1 mM adenosine solution. (A) Reaction mixture before squash extract addition. Adenosine appeared at 14.883 minutes. (B) Reaction mixture after squash extract addition. Two hours following reaction initiation, adenosine appeared at 14.840 minutes. Inosine is clearly visible at 5.527 minutes. A peak can be seen for adenine at 2.637 minutes and hypoxanthine at 3.600 minutes. Xanthine appeared at 3.273 minutes.

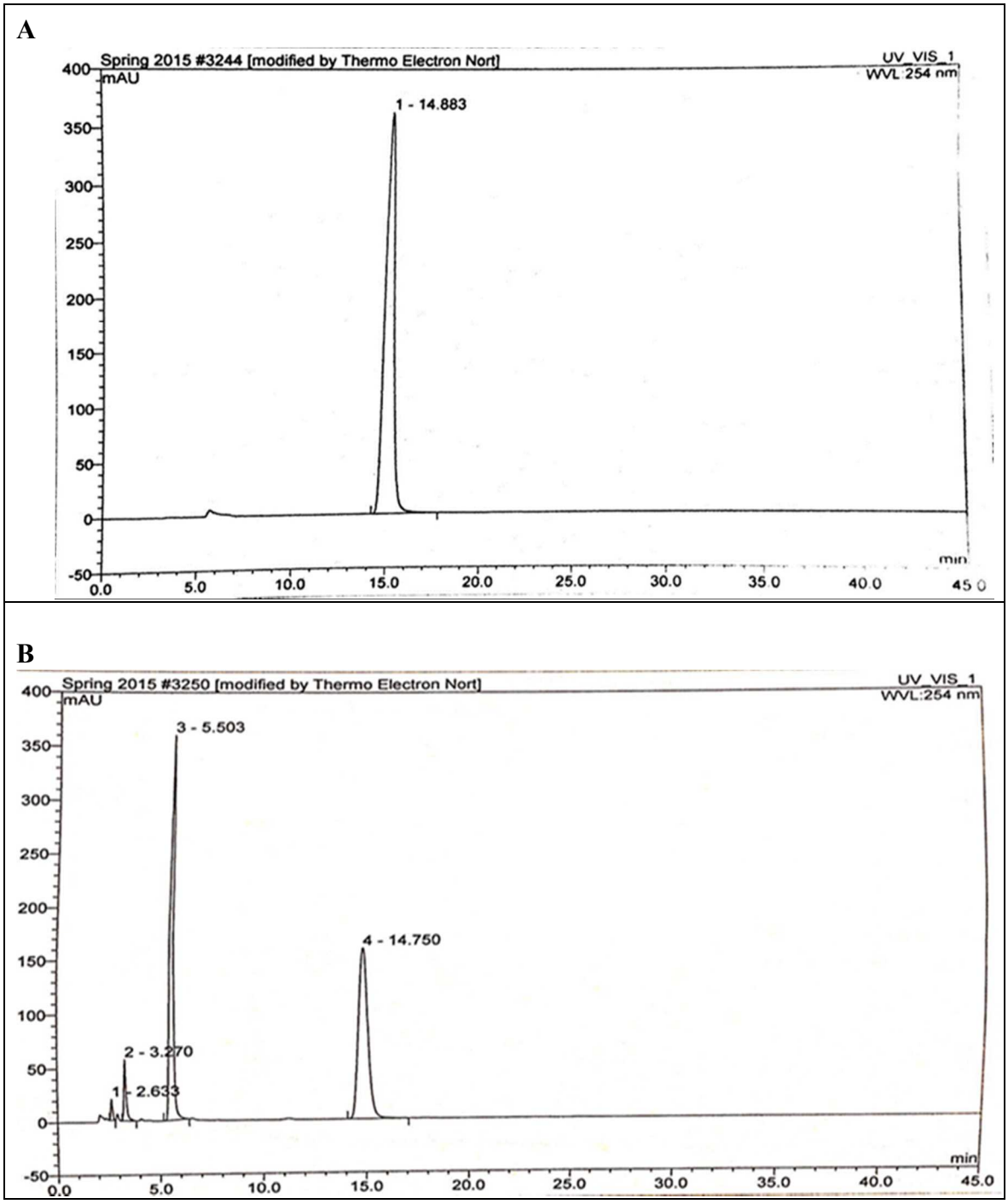

Figure 39: Adenosine degradation reaction in organic squash. The reaction yielded adenine, inosine, and Xanthine. Organic squash extract (100 µL) was added to 1 mL of 1 mM adenosine solution. (A) Reaction mixture before organic squash extract addition. Adenosine appeared at 14.883 minutes. (B) Reaction mixture after organic squash extract addition. Four hours following reaction initiation, adenosine appeared at 14.750 minutes. Inosine is clearly visible at 5.503 minutes. A peak can be seen for adenine at 2.633 minutes. Xanthine appeared at 3.270 minutes. A hypoxanthine peak does not appear.

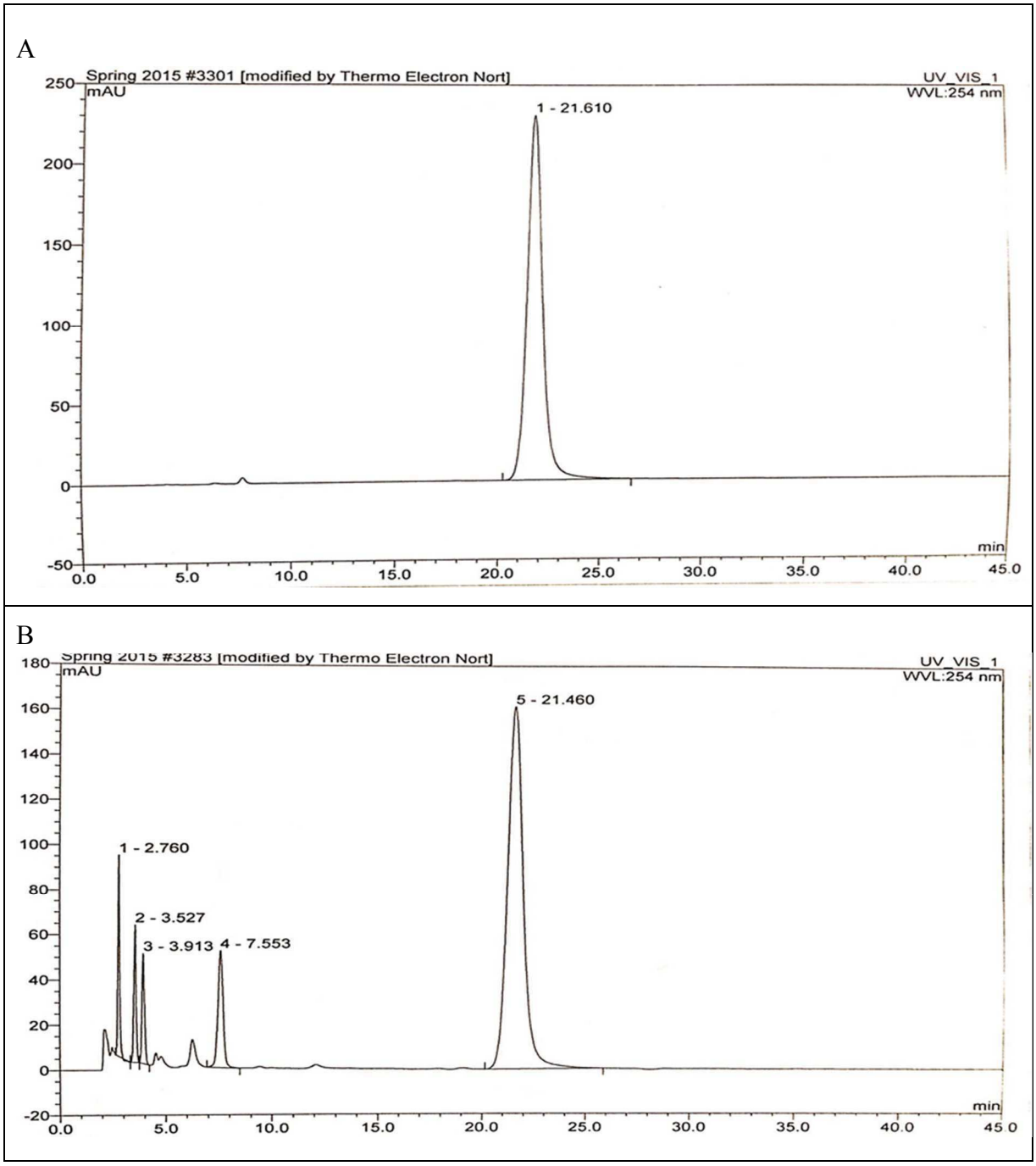

Figure 40: 2'-Deoxyadenosine degradation reaction in squash. The reaction yielded adenine, 2'-deoxyinosine, and hypoxanthine. Squash (100 µL) extract was added to 1 mL of 1 mM. 2'-deoxyadenosine solution. (A) Reaction mixture before squash extract addition. 2'-deoxyadenosine appeared at 21.610 minutes. (B) Reaction mixture after squash extract addition. Seven hours following reaction initiation, 2'-deoxyadenosine appeared at 21.460 minutes. 2'-Deoxyinosine is clearly visible at 7.553 minutes. A peak can be seen for adenine at 2.760 minutes and hypoxanthine appeared at 3.527 minutes. Xanthine appeared at 3.913 minutes.

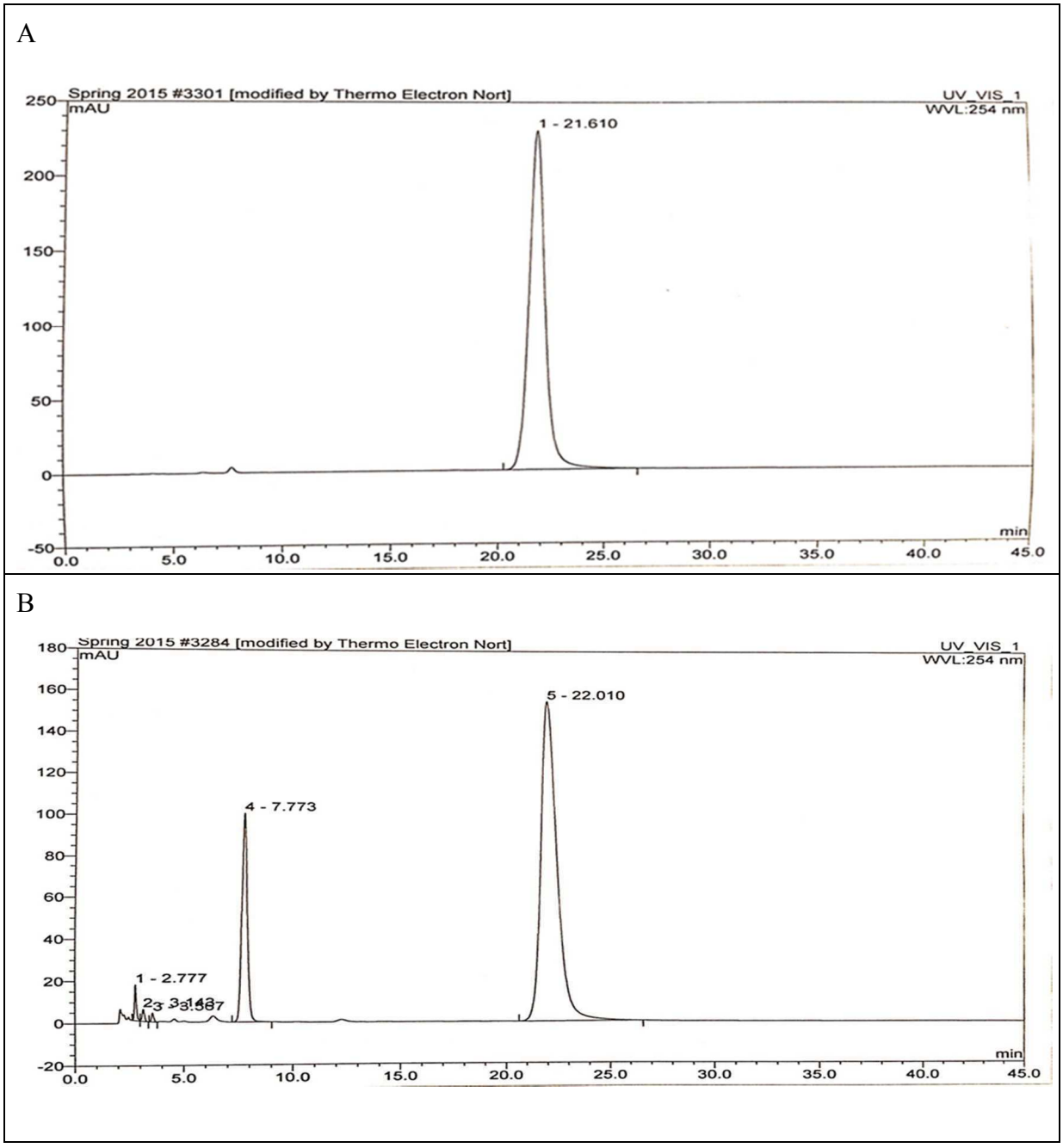

Figure 41: 2'-Deoxyadenosine degradation reaction in organic squash. The reaction yielded adenine, 2'-deoxyinosine, hypoxanthine and xanthine. Organic squash (100 µL) extract was added to 1 mL of 1 mM 2'-deoxyadenosine solution in 10 mM Tris buffer pH 7.2. (A) Reaction mixture before squash extract addition. 2'-deoxyadenosine appeared at 21.610 minutes. (B) Reaction mixture after organic squash extract addition. Nine hours following reaction initiation, 2'-deoxyadenosine appeared at 22.010 minutes. 2'Deoxyinosine is clearly visible at 7.773 minutes A Peak can be seen for adenine at 2.777 minutes and hypoxanthine appeared at 3.567 minutes. Xanthine appeared at 3.143 minutes.

Inosine to hypoxanthine conversion is mediated by inosine nucleosidase. The level of activity observed was similar for inosine when compared to okra, organic okra, bean, and organic bean across all inosine nucleosidases tested (Figures 42-43). Inosine nucleosidase appears to be active in squash at similar level to that found in organic squash. The reaction lasted approximately seven hours for both squash and organic squash with specific activity 0.0086 and 0.0068 μmol/(min mg), respectively.

Uridine nucleosidase appears to be active in squash and organic squash. Uridine was degraded to uracil (Figures 44-45). No other products were observed. Uridine nucleosidase was less active in squash than it was in organic squash. Specific activities were 0.0089 μmol/(min mg), in squash and 0.0129 μmol/(min mg), in organic squash.

Cytidine was also evaluated as a substrate for nucleosidases and deaminases that may be present in squash and organic squash seedlings (Figures 46-47). The enzymes involved in cytidine processing in squash and organic squash had a total specific activity of 0.0197 μmol/(min mg) and 0.0055 μmol/(min mg), respectively. Uridine, cytosine and uracil peaks were observed after addition of squash and organic squash initial extract. A summary of the results can be found below in Table 6 and Table 7.

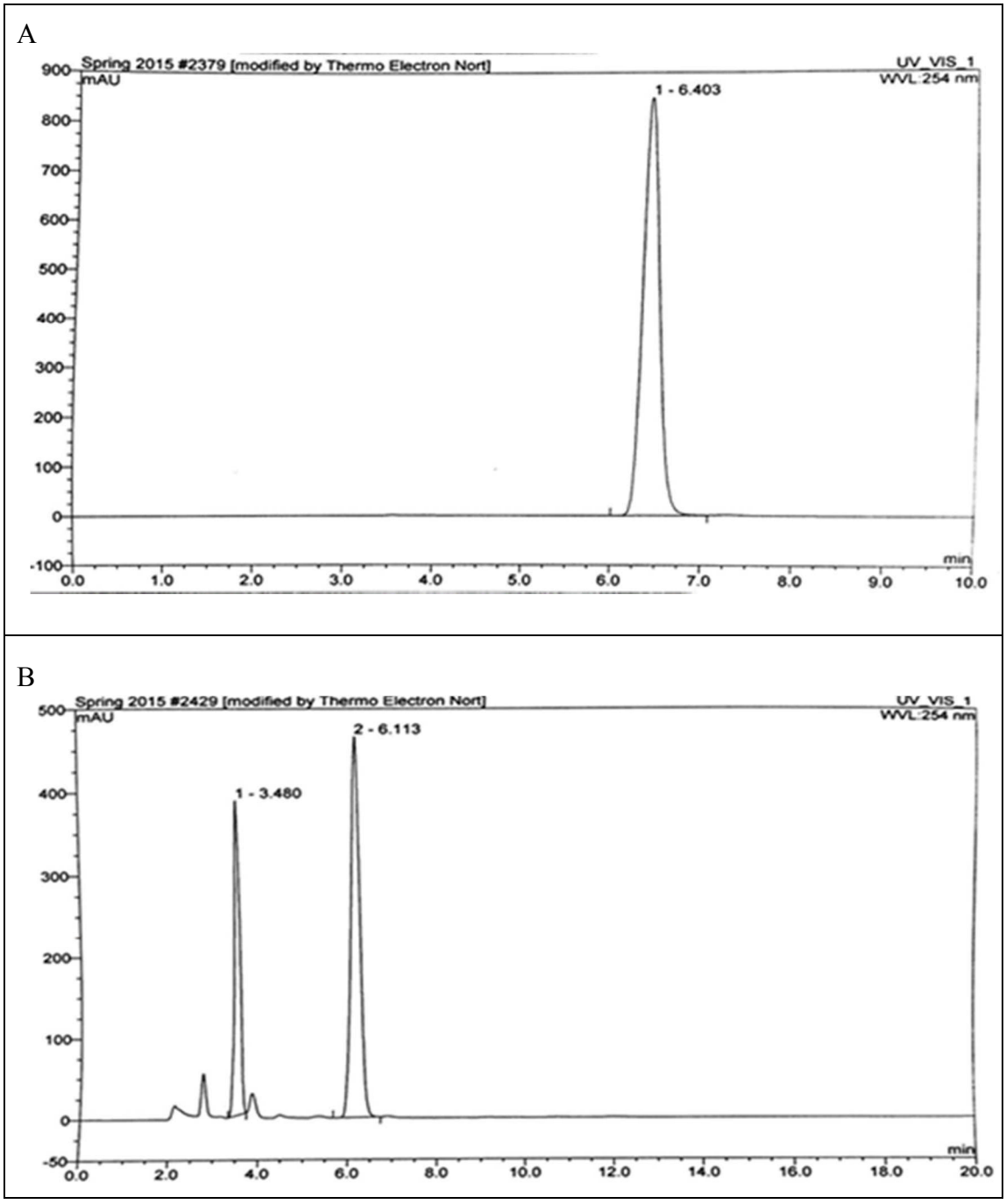

Figure 42: Inosine to hypoxanthine conversion catalyzed by inosine nucleosidase in squash. The reaction mixture included 1 mM inosine (1 mL) and squash extract (100 µL) in 10 mM Tris buffer pH 7.2. (A) Reaction mixture before squash extract addition. It shows an inosine peak at 6.403 minutes. (B) Reaction mixture after squash extract addition. An inosine peak appeared at 6.113 minutes. A second peak, with a retention time expected for hypoxanthine, appeared seven hours later at 3.480 minutes.

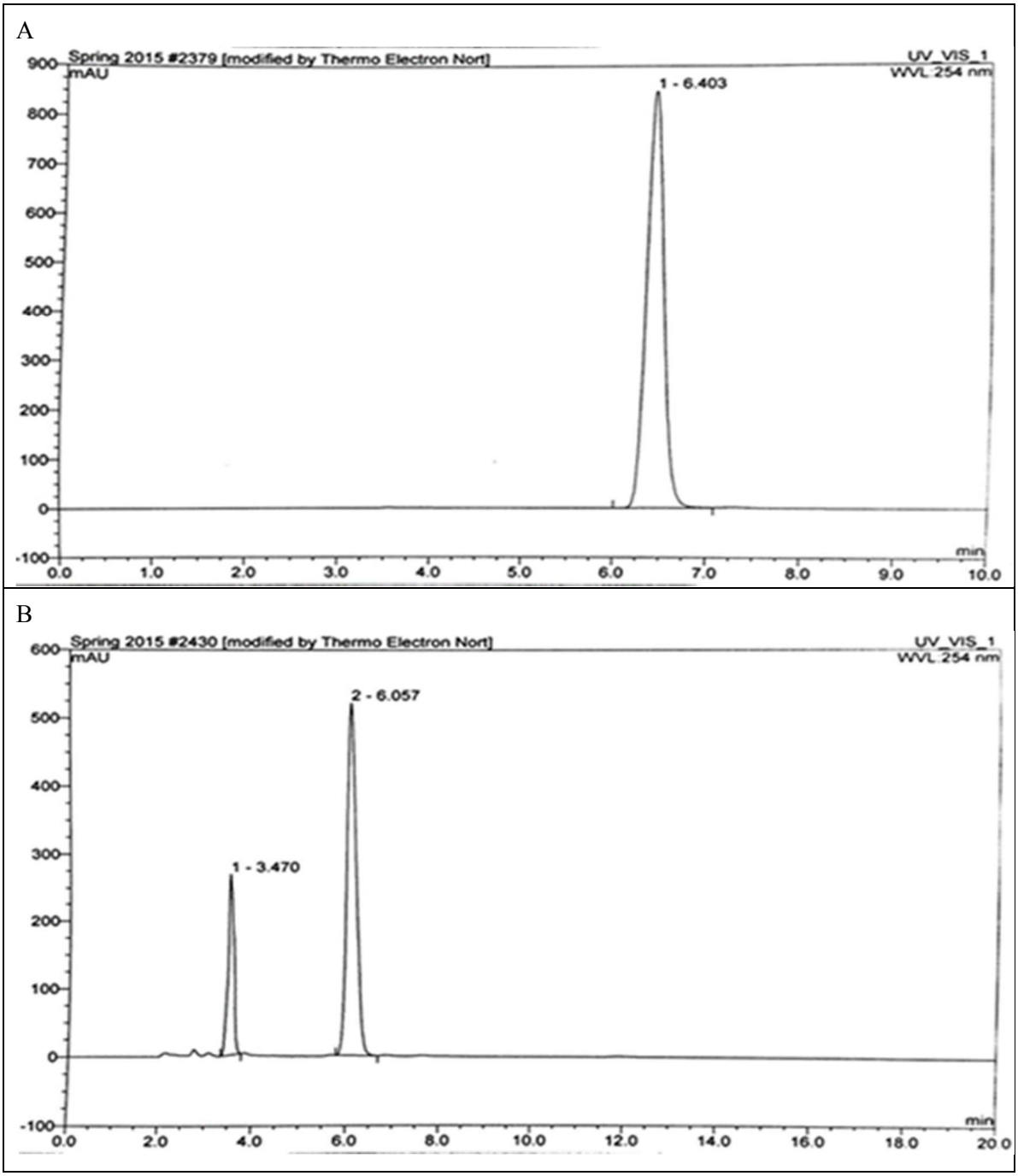

Figure 43: Inosine to hypoxanthine conversion catalyzed by inosine nucleosidase in organic squash. The reaction mixture included 1 mM inosine (1 mL) and organic squash extract (100 µL) in 10 mM Tris buffer pH 7.2. (A) Reaction mixture before organic squash extract addition. It shows an inosine at 6.403 minutes. (B) Reaction mixture after organic squash extract addition. The inosine peak appeared at 6.057. A second peak, with a retention time expected for hypoxanthine, appeared seven hours later at 3.470 minutes.

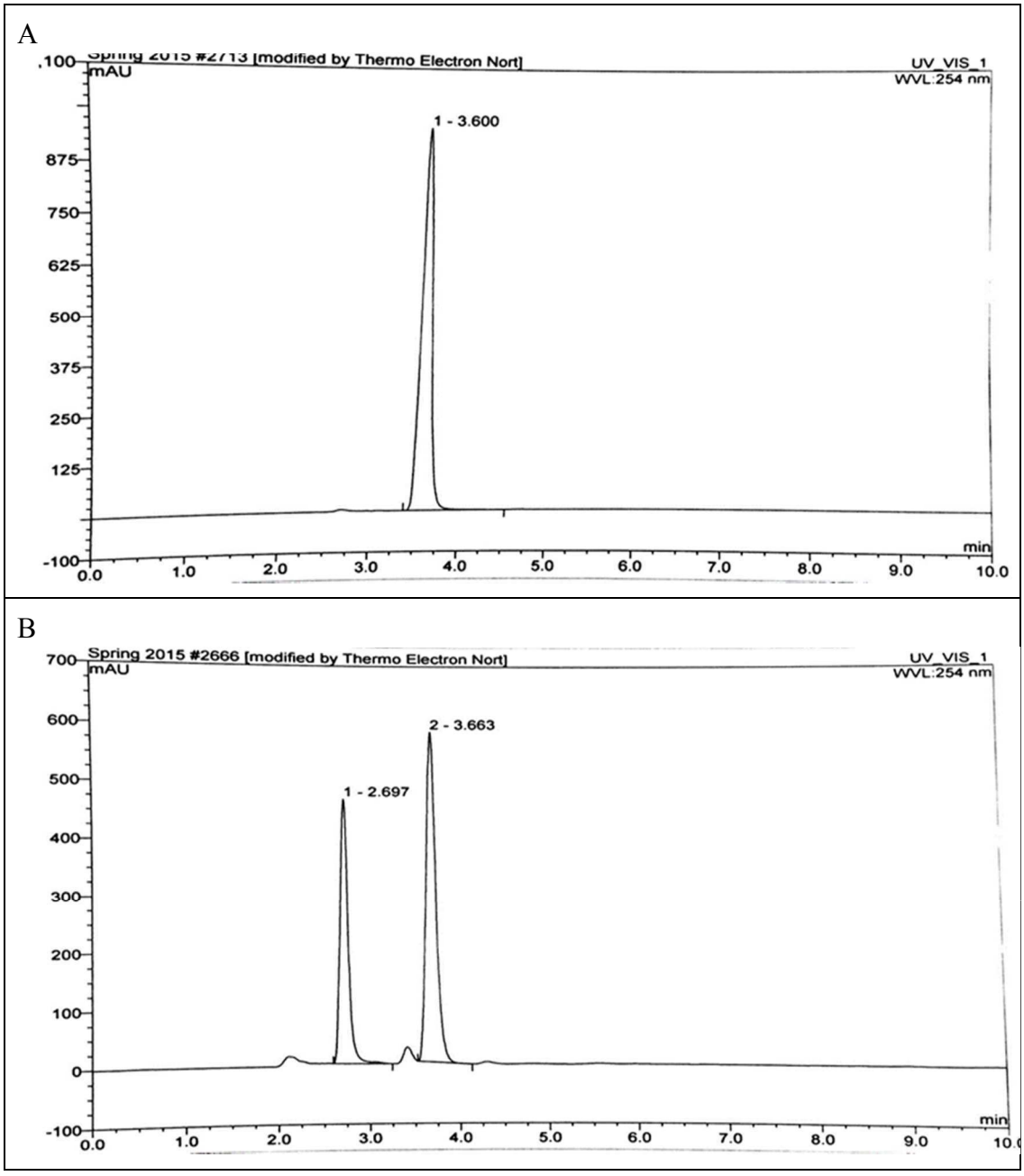

Figure 44: Uridine to uracil conversion catalyzed by uridine nucleosidase in squash. The reaction mixture included 1 mM uridine (1 mL) and squash extract (100 µL) in 10 mM Tris buffer pH 7.2. (A) Reaction mixture before squash extract addition. It shows a uridine peak at 3.600 minutes. (B) Reaction mixture after squash extract addition. Uridine appeared at 3.663 minutes. A second peak, with a retention time expected for uracil (2.697 minutes) appeared seven hours later.

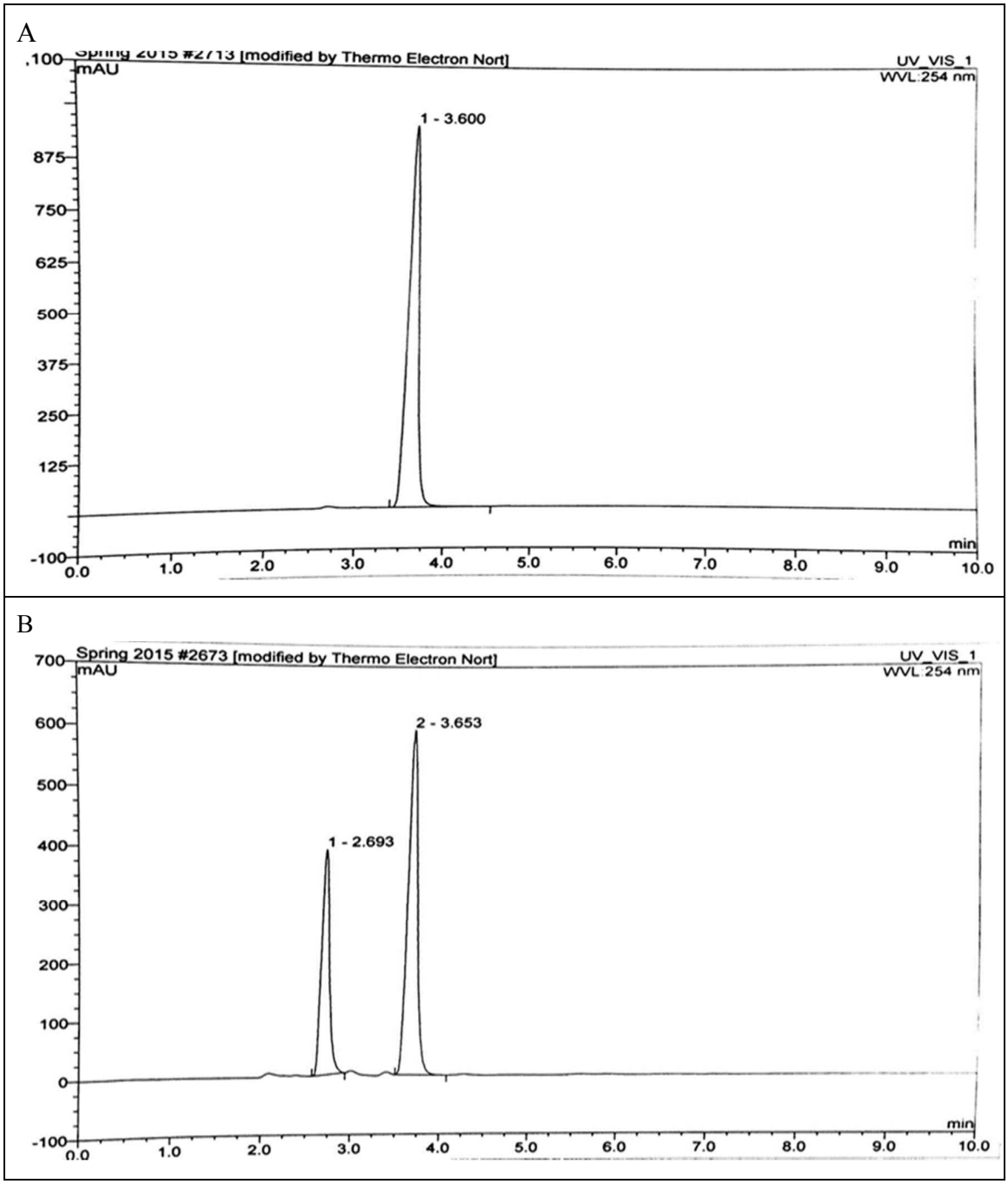

Figure 45: Uridine to uracil conversion catalyzed by uridine nucleosidase in organic squash. The reaction mixture included 1 mM uridine (1 mL) and organic squash extract (100 μL) in 10 mM Tris buffer pH 7.2. (A) Reaction mixture before organic squash extract addition. It shows a uridine peak at 3.600 minutes. (B) Reaction mixture after organic squash extract addition. Uridine appeared at 3.653 minutes. A second peak, with a retention time expected for uracil (2.693 minutes) appeared seven hours later.

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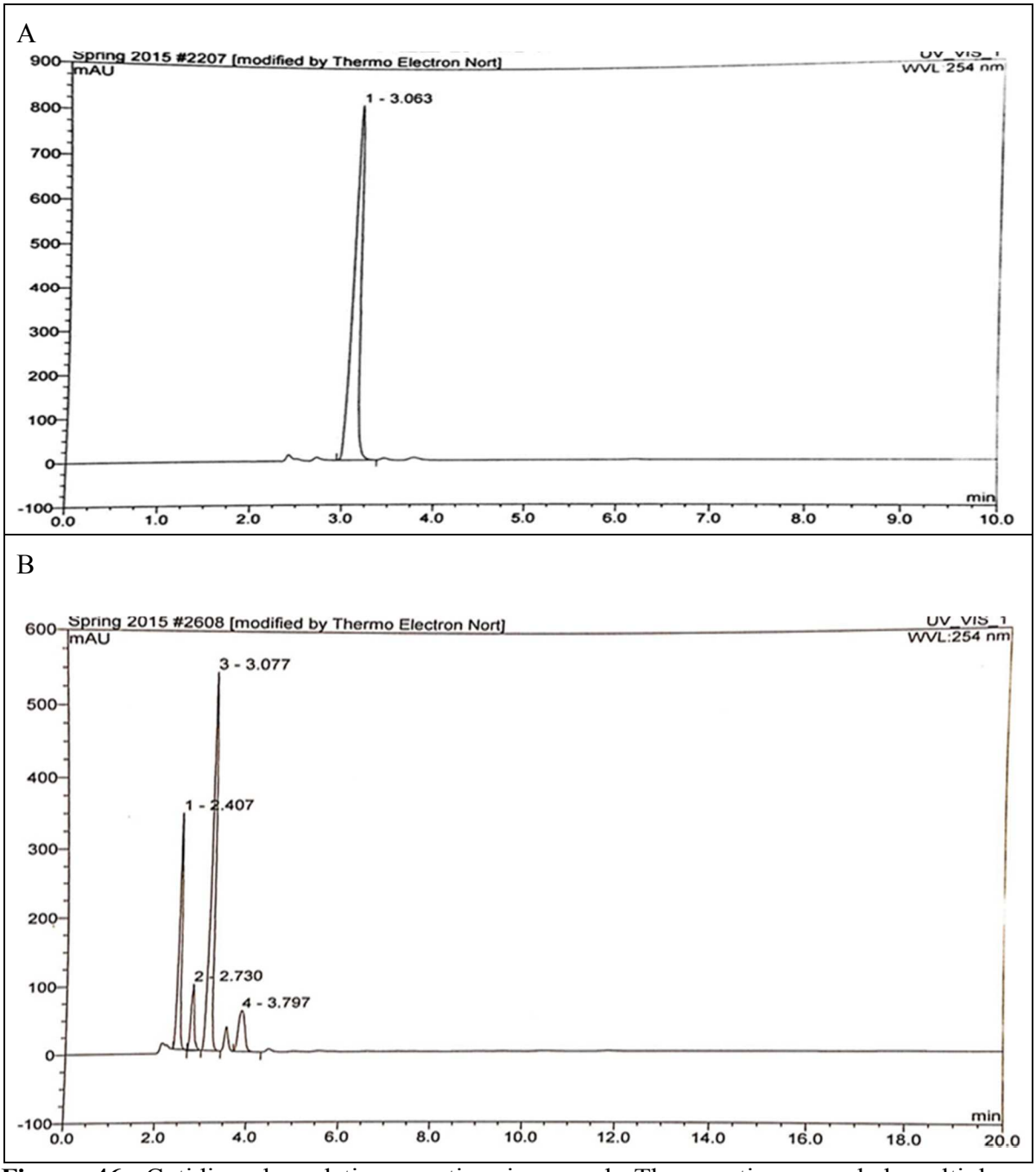

Figure 46: Cytidine degradation reaction in squash The reaction revealed multiple activities: cytidine nucleosidase, cytidine deaminase and uridine nucleosidase. (A) Reaction mixture before squash extract (100 µL) added to 1 mL of 1 mM cytidine. Cytidine appeared at 3.063 minutes. (B) Reaction mixture after squash extract addition. Cytosine can be seen at 2.407 minutes, uridine at 3.797 minutes and uracil at 2.730 minutes.

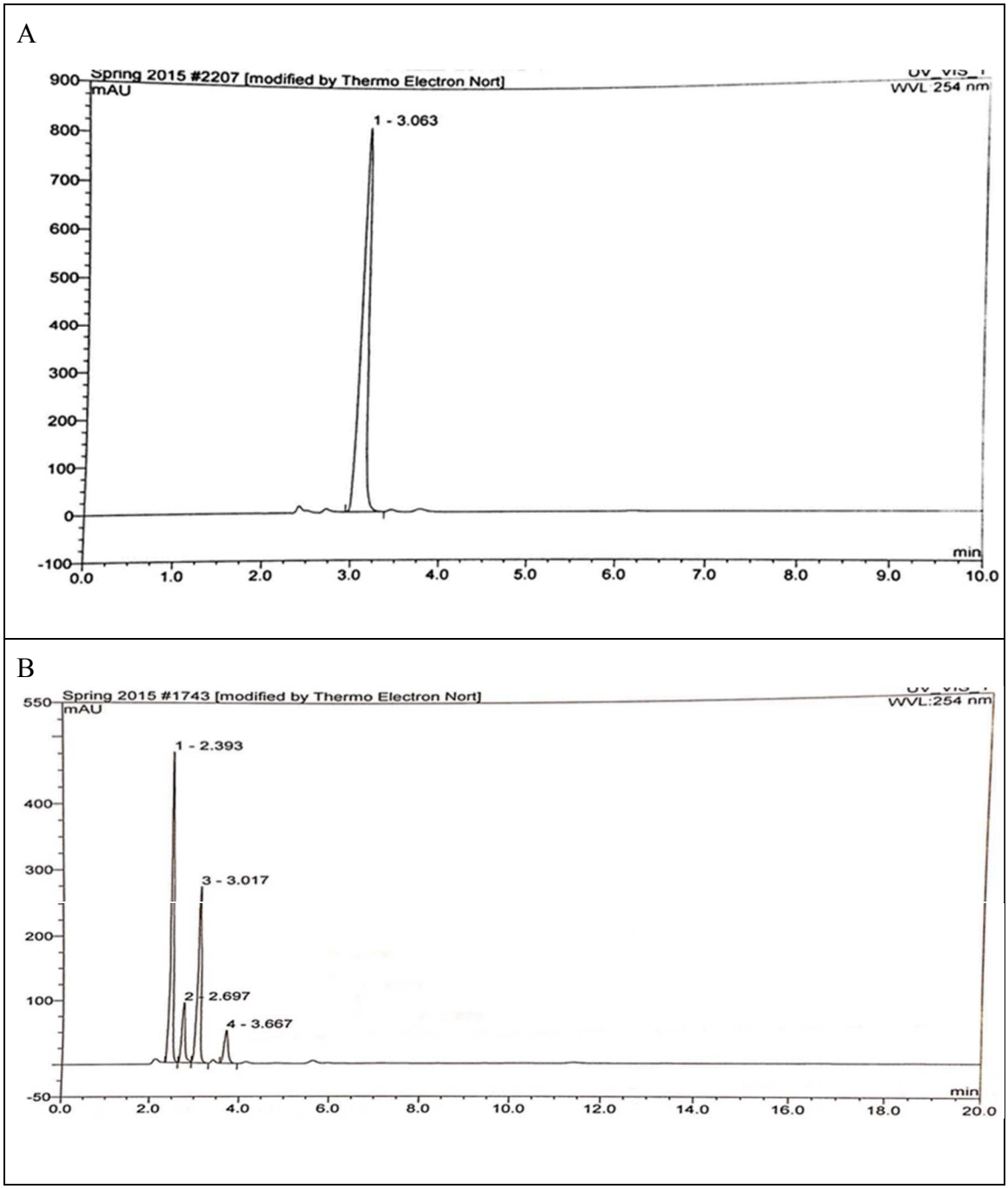

Figure 47: Cytidine degradation reaction in organic squash. The reaction revealed multiple activities: cytidine nucleosidase, cytidine deaminase and uridine nucleosidase. (A) Reaction mixture before organic squash extract (100 µL) added to 1 mL of 1 mM cytidine. Cytidine appeared at 3.063 minutes. (B) Reaction mixture after organic squash extract addition. Cytidine appeared at 3.017 minutes Cytosine can be seen at 2.393 minutes, uridine at 3.667 minutes and uracil at 2.697 minutes.

Table 6: Nucleosidase and deaminase activities in squash. Nucleosidase specific activities found in squash 5-9 day old seedlings. The values reported are an average of at least three measurements

Enzyme	Presence	Specific Activity μmol/(min mg)
Uridine Nucleosidase	Y	0.0086
Inosine Nucleosidase	Y	0.0089
Cytidine Nucleosidase/Deaminase	Y	0.0197
Adenosine Nucleosidase/ Deaminase	Y	0.012
2'-Deoxyadenosine Nucleosidase	Y	0.0047

Table 7: Nucleosidase and deaminase activities in organic squash. Nucleosidase specific activities found in organic squash 5-9 day old seedlings. The values reported are an average of at least three measurements.

Enzyme	Presence	Specific Activity μmol/(min mg)
Uridine Nucleosidase	Y	0.019
Inosine Nucleosidase	Y	0.0068
Cytidine Nucleosidase/Deaminase	Y	0.0055
Adenosine Nucleosidase/ Deaminase	Y	0.010
2'-Deoxyadenosine Nucleosidase	Y	0.0070

CHAPTER IV

CONCLUSIONS

Six varieties of seeds were tested for the presence of a number of nucleoside metabolizing enzymes. Two groups of enzymes were tested, nucleosidases, and deaminases. The level of enzyme activity varied from one seed type to another. The enzymes examined in this research included inosine, uridine, cytidine and adenosine nucleosidases. 2'-Deoxyadenosine nucleosidase is another enzyme that was investigated in this research. The activities of the enzyme were determined on a Dionex Ultimate 3000 High Performance Liquid chromatography system (HPLC).The disappearance of parent nucleoside was used to calculate enzyme activity.

All five nucleosides tested were hydrolyzed in the presence of the crude protein extract from 5-9 day old okra, organic okra, bean, organic bean, squash and organic squash seedling. In okra, based on the specific activity, the highest activity was recorded for uridine and adenosine compared to inosine, cytidine and 2`-deoxyadenosine. In organic okra, the highest activity was recorded for uridine. This result was consistent with the previous result for soybean reported by others. The other four nucleosidases were similar in nucleoside break down rate.

In organic bean, cytidine has the highest activity compared to uridine, inosine, adenosine and 2`-deoxyadenosine. This is the same result reported for Alaska pea seeds. In squash, cytidine and adenosine were the highest in activity compared to the three other nucleosides. Nucleosidase activities in organic squash were also present. Uridine and

adenosine were higher in activity than inosine, cytidine and 2`-deoxyadenosine. Bean had the lowest level of nucleosidase activity with all five nucleosidase compared to nucleosidase metabolizing activities in okra, organic okra, organic bean, squash and organic squash.

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