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Monitoring the Chemical Hydroxylation of Complex Phenolic Compounds

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MONITORING THE CHEMICAL HYDROXYLATION OF COMPLEX PHENOLIC COMPOUNDS

By

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ABSTRACT

It might be industrially or bio-medically important to confirm and monitor the hydroxylation of phenolic amine substrates via mass spectrometry. Phenolic amines may be assayed by colorimetric reactions, liquid chromatography (LC) or thin layer chromatography (TLC), spectrophotometry (UV VIS) or other methods that may not confirm the product molecule with reasonable specificity. Phenolic amine compounds may easily enter the gas phase by electrospray ionization (ESI) and the compounds parent and subsequent fragment ions examined by tandem mass spectrometry (MS/MS). Thus a number of phenolic amine or other reaction products might be monitored and confirmed by liquid chromatography with electrospray ionization and tandem mass spectrometry (LC-ESI-MS/MS). L-tyrosine was reacted with dihydroxyfumaric acid (DHFA) at 0 °C in the presence of bubbling O_2 in 400 mL flask respectively or ≥ 100 µL volume in a 96 well plate in an oxygen atmosphere resulting in the product L-DOPA (L-3,4 dihydroxyphenylalanine). The production of L-DOPA was examined with nitrite-molybdate in 0.5 M HCl followed the addition of 1 M of NaOH to form a red color quantified by absorbance at 510 nm. Thin layer chromatography with staining for amines by ninhydrin was used to detect the production of L-DOPA. LC-ESI-MS/MS confirmed the molecular identity of the L-DOPA product with a parent ion predominately observed at an m/z value of 198 [M+1H] and the major fragment ions at 181m/z and 151m/z. Monitoring the 181 m/z fragment ion permitted the quantification of L-DOPA over time to ≤1 pM in the reaction vessel with respect to external standards. The hydroxylation of tyrosine was observed to require O_2 and DHFA and produced a strong yield at pH 2 but was not dependent on Horseradish peroxidase (HRP) and proceeded in the presence of EDTA. The hydroxylation reaction of tyrosine was depending on DHFA, oxygen and acid (DOA).We conclude that DOA hydroxylation by LC-ESI-MS/MS may be directly applicable to monitoring the industrial modification of a wide class of phenolic amines.

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LIST OF SELECTED ABBREVIATIONS

4IPBA	4-iodophenylboronic acid
AP	Alkaline phosphatase
BH_4	Tetrahydrobiopterin
CYP-450	Cytochrome P-450
DHFA	Dihydroxyfumaric acid
DMB	Diaminobenzidine
DHQ	Dihydroquinine
DOPA	3,4-Dihydroxy-L-phenylalanine
ECL	Enhanced Chemiluminescence
ESI	Electrospray ionization
FT	Fourier transform ion cyclotron
FDA	Food and Drugs Administrations
H_2O_2	Hydrogen peroxide
HAC	Acidic Acid
HCL	Hydrochloric acid
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IR	Infrared
LC	Liquid chromatography
MALDI	Matrix-assisted laser desorption/ ionization

LIST OF SELECTED ABBREVIATIONS (Continued)

MS	Mass spectrometers
MYP	Myeloperoxidase
m/z	Mass to charge ratio
NaOH	Sodium hydroxide
NH_4HCO_3	Ammonium bicarbonate
PA	Phenylalanine
PBS	Phosphate buffered saline
PD	Parkinson's disease
PVDF	Polyvinylidene fluoride
TLC	Thin layer chromatography techniques
TMB	Tetramethyl benzidene
TOF	Time-of-flight
Tyr	Tyrosine
UV/VIS	Ultra violet visible

INTRODUCTION

1. Biological Significance of DOPA

L-DOPA (L-3,4-dihydroxyphenylalanine) is an aromatic compound, which may act as a precursor for dopamine, norepinephrine and epinephrine that are important neurotransmitters. L-DOPA is a non-protein amino acid that can be synthesized biologically in the human body from L-tyrosine by tyrosine hydroxylase (TH) enzyme using O_2, as well as Fe^{2+} and tetrahydrobiopterin (BH_4) as cofactors. L-DOPA may act as an efficient therapeutic agent. Thus, DOPA is on the metabolic pathway to the biosynthesis of the catecholamine neurotransmitters and is thus one of the most effective drugs in the treatment of Parkinson's disease (PD) *(Hornykiewicz, 2002)*. L-DOPA is converted to dopamine by another enzyme (DOPA decarboxylase) (*Kaufman, 1995)*. In addition, L-DOPA has been shown to be effective in the treatment of dopamine-responsive dystonia *(Segawa, 2000)*. The convenient hydroxylation of tyrosine to yield L-DOPA has been reported to occur *in vitro* via catalysis by HRP and other enzymes with the optimal reaction conditions *(Mason, Onopryenko, et al. 1957; Buhler and Mason 1961; Halliwell, and Ahluwalia, 1976; Nordblom, White, et al. 1976; Halliwell, 1977; Dordick, Klibanov, et al. 1986)*

2. Mass Spectrometry

Mass spectrometry (MS) is an analytical tool that may be used to measure the mass-to-charge ratio and intensities of charged particles (ions). Of particular interest to the determination of masses of particles and the elemental composition of a molecule is MS,

which is also used for identifying the chemical structures of molecules such as peptides and other chemical compounds. The analytes are ionized in the gas phase and then detected by MS (*Sparkman, 2000)*. Hence mass spectrometry may be used directly to measure the hydroxylation of purified compounds.

Mass spectrometers play an important role in industry towards applications for routine measurements and in academia for research purposes. MS techniques may be used in proteins, peptides, or oligonucleotides analysis (Biotechnology) or small molecule, drug discovery, metabolism, and pharmacokinetics (Pharmaceutical). In addition MS finds applications in environmental and Geological measurements such as elemental analysis, PAHs, PCBs, water quality, and food contamination. Thus MS seems to be one of the most promising analytical techniques to examine a number of very small quantity samples simultaneously quickly, precisely, and accurately *(Kostiainen, Kotiaho, et al., 2003)*.

2.1. The Components of general MS systems

A mass spectrometer is composed of three main parts including an ion source, a mass analyser that measures the mass to charge ratio (m/z) of the ionized analytes, and a detector that records the total number of ions at each *m/z* value (Figure 1). Electrospray ionization (ESI) and matrix-assisted laser desorption/ ionization (MALDI) are the two most commonly used ion sources for mass spectrometric analysis *(Fenn, Mann, et al., 1989)*. ESI ionizes the analytes out of a solution and is thus directly coupled to liquid-based separations *(Fenn, Mann, et al., 1989)*. MALDI ionizes the samples out of a dry, crystalline matrix via laser beams. The ionization of the MALDI matrix may interfere with the detection of some small molecules *(Karas, & Hillenkamp, 1988)*.

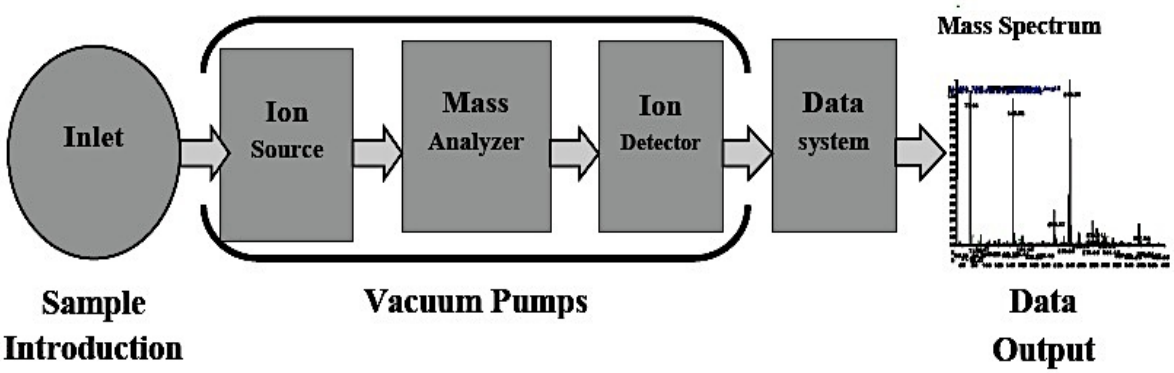

Figure 1: Components of mass spectrometry

1) Introduction of a sample for the ionization steps, 2) Production of ions from the sample, 3) Separation of ions with different masses, 4) Detection of the number of ions of each mass that is produced, 5) Collection of data to generate the mass spectrum.

The second part of the MS is the mass analyser, which is considered the main functional part of the instrument that separates parent or fragment ions according to their mass to charge ratio *(Pandey, & Mann 2000; Aebersold & Goodlett, 2001; Mann, Hendrickson, et al., 2001)*. There are four elemental types of mass analysers mostly used. These are the ion trap, time-of-flight (TOF), quadrupole and Fourier transform ion cyclotron (FT-MS) analyzers. Each one of them has its own strength and weakness although their design and performance are varied and they can also be combined together in tandem to create hybrid instruments *(Mann, Hendrickson, et al., 2001)*. For example, the quadrupole-TOF instruments have collision cells, which are located between a quadrupole mass filter and a TOF analyzer *(Loboda, Krutchinsky, et al., 2000)*. In contrast to hybrid instruments, ion traps separate the resolution of parent ions, and their fragments, a few milli seconds apart in the same mass analyzer, and so are relatively inexpensive. Ions of a specific mass to charge ratios are selected in a first mass analysis, fragmented with a collision gas and then the fragment-ion masses are analyzed by a second mass analyser. Thus, ion trap analysers are characterized by high sensitivity, low resolution and useful mass accuracy. Furthermore, the generated fragment ion spectra are often more widespread and informative than those that formed in trapping instruments. The FT-MS instruments

will be promising in the future although proteomics are controlled currently by instruments like TOF, ion-trap and hybrid TOF *(Aebersold, & Mann, 2003)*. MALDI is often coupled to TOF analysers that quantify the mass of intact peptides, while ion traps and triple quadrupole instruments are compatible with ESI and used to form fragment ion spectra or collision-induced spectra (CID) of selected precursor ions *(Aebersold, & Goodlett, 2001)*. Liquid chromatography/tandem mass spectrometry (LC/MS/MS) coupled to ESI ion source is the most common method in analytical chemistry. Currently, liquid chromatography-mass spectrometry (LC/MS) is considered the first determination method of trace level compounds that are essential to demonstrate their biological aspects and functions in living systems. In addition, LC-ESI-MS/MS utilizes ionization at lower temperatures compared to the other methods. Moreover, LC-ESI-MS/MS permits highly sensitive detection of desired compounds since the level of its background noise is very low *(Nordström, Tarkowski et al., 2004)*.

Since polyphenolic compounds are normally found as a complex mixture, they may require techniques such as LC-MS for better detection. LC-MS is a rapid and reliable technique with different ionization sources (ESI or MALDI) for identification of phenolic substances *(Lin, & Harnly, 2008)*. In addition, MS has become one of the methods for the rapid detection of proteins and the characterization of post-translational modification of amino acids *(Blackstock, & Weir, 1999)*. Thus if MS has the capacity to detect the substrates and reaction products, it might be used to read reactions and standards to allow absolute quantification. Many types of phenolic amines may be detected by ESI-MS *(Palumbo, Napolitano et al. 1999)*. The large changes in mass that results from hydroxylation should be easily resolved even on a simple ion trap instrument. Thus if LC-ESI-MS/MS is able to detect the substrates and reaction products, it can be used to read reactions and standards to allow absolute quantification.

3. Chromatographic systems

Chromatographic systems consist of a mobile phase that carries the sample through a stationary phase. Separation of a complex mixture occurs when the sample molecules have different affinities for the stationary media (*Braithwaite, & Smith, 1996).* As a result, the retention time for a particular molecule to travel through the chromatographic medium will depend on its physicochemical properties. The two main types of chromatography are liquid chromatography, where the mobile phase is a liquid, and gas chromatography, where gas is used as the moving phase *(Braithwaite, & Smith, 1996).*

3.1. Liquid chromatography- mass spectrometry

In the early 1900s, liquid chromatography was discovered, which was known at beginning as ‘‘classical column chromatography’’. The Nobel Prize for partition chromatography was awarded in 1952 to Martin & Synge. A sample was added to the top of glass cylinder after it was filled out with a powder such as chalk, and then a solvent was poured onto the column. The components of the sample start to elute while the solvent flows down the column by gravity, through the column at different speeds and become separated *(Snyder, Kirkland, et al., 2010).* In liquid chromatography (LC) the stationary phase typically consists of porous particles with a large surface area, for example, silica beads coated with different functional groups *(Lough & Wainer, 1995)*. Sample molecules travel through the chromatographic system and interact with the surface of the stationary phase. Sample molecules that interact strongly with the stationary phase will have longer retention times.

High pressure or high performance liquid chromatography (HPLC) utilizes smaller diameter packing materials with increased flow pressure to yield higher resolving power and faster analyses *(Lough & Wainer, 1995)*. As a consequence, HPLC is one of the most useful and powerfully applied analytical techniques that has the ability to separate and analyze chemical mixtures, compared to other chromatographic methods. HPLC has many other characteristics in addition to being almost universally applicable and having a wide variety of tools, columns and other materials that are commercially available. These characteristics allow the utilization of this machine in nearly every field and most likely analytical chemistry laboratories *(Snyder, Kirkland, et al., 2010)*. Many different separation methods have been used in conjunction with mass spectrometry, which is a commonly used method to identify and quantify chemical mixtures *(Petrovic, Eljarrat, et al., 2002)*. There are many advantages to combining MS and separation methods, including increased sensitivity, dynamic range, and selectivity. On the other hand, there are a few disadvantages including the difficulty of sample cleaning before use, which is very time-consuming and costly and fouling of the source is very common. As a result, the sample throughput is normally low and there is always a somewhat long period between sample collection and result analysis *(Wang, Pan, et al., 2009)*. Frequent exchange of the electrospray emitter and extensive clean up of the source and instrument is frequently required to control the background *(Farré, Kantiani, et al., 2007)*.

Since polyphenolic compounds are normally found as a complex mixture, they may require techniques such as LC-MS for sensitive detection. LC-MS exhibits a rapid and reliable technique with different ionization methods for identification of phenolic substances *(Lin, & Harnly, 2008)*. In addition, MS has become one of the prefered methods for the rapid detection and analysis of amino acids metabolism *(Blackstock, & Weir, 1999)*. These same types of phenolic amines are known to be detectable by ESI-MS *(Palumbo, Napolitano et al. 1999)*. The large changes in mass anticipated might permit the monitoring of the reactions progress by LC-ESI-MS/MS. Thus if LC-ESI-MS/MS is able to detect the substrates and reaction products, it can be used to read reactions and standards to allow absolute quantification.

3.2. Thin layer chromatography (TLC)

The term Thin layer Chromatography is used to describe a technique in which mixtures of different compounds are separated into individual substances using a dry stationary phase and a mobile phase that is drawn by capillary action. The individual substances interact differently with both the stationary and mobile phases since they have various molecular structures; thus they are transferred at different rates by the mobile phase *(Reich, & Schibli, 2007)*. In the 1960s and 1970s, the thin layer chromatography (TLC) systems were developed and used commonly in almost all chemical and analytical laboratories *(Pestka, 1986)*. TLC utilizes a thin layer of adsorbent material as stationary phase that is bound to a solid support such as glass, plastic or aluminum sheet. Silica gel for instance, is the most common stationary phase, which is a form of silicon oxide (SiO_2) that has hydrated surfaces. Since silica gel is an adsorptive material and it has a large surface area that allows strong interactions with molecules (*D'Aniello, D'Onofrio, et al., 1985).*

A solution containing the analyte of interest or mixture is spotted and dried a few centimeters from one end of the plate. The TLC plates are transferred to a sealed container with an appropriate mobile phase no more than a centimeter deep or so. As a consequence of the capillary action, the mobile phase will rise and reach the sample mixture that will be dissolved and carried by the rising solvent. Each compound in the mixture will travel a distance along the TLC plate yielding different spots depending on the fraction of time spent in the mobile phase. These spots can be analyzed either directly, if the compounds are colored, or indirectly utilizing an indicator such as UV-light. On the other hand, amino acids are coluorless compounds and can be determined by ninhydrin solution, which is a widely used chemical for this purpose. In this case, an aldehyde and carbon dioxide are produced when the ninhydrin solution reacts with free alpha-amino groups (*D'Aniello, D'Onofrio, et al., 1985).*

4. Detection of phenolic compounds by Ultra violet visible (UV/VIS) spectroscopy

Ultraviolet/visible (UV/VIS) spectrophotometry has wide application in analytical laboratories to measure both the endogenous absorption of light from biochemical compounds and those of specific coluorimetric reactions. UV/VIS provides high sample throughput and many specific reagents and protocols have been developed for the rapid and convenient measurement of a wide range of analytes *(Upstone, 2000).* UV (250-400 nm) and VIS (400-700 nm) radiation consist of only a narrow part of the electromagnetic spectrum, and does not address other frequencies of radiation such as radio, infrared (IR) and X rays *(Islam, Singh, et al., 2003).*. UV/VIS light is usually applied to molecules or inorganic complexes in solution where it is absorbed and thereby promotes outer electrons to higher energy levels. The concentrations of the analytes can be determined by measuring the absorbance at some wavelength using the Beer-Lambert Law based on known extinction coefficients or alongside a standard concentration series *(Upstone, 2000)*. UV/VIS spectroscopy is an essential detection method of phenolic compounds *(Sherma, 2010)*. Due to the presence of conjugated double and aromatic bonds, the absorption phenolic compounds can vary in UV/VIS *(Sherma, 2010)*. These kinds of compounds are strong chromophores with high UV absorptions due to the presence of several conjunction bonds *(Harnly, Bhagwat, et al., 2007).*

5. Phenolic compounds and derivatives

It is commonly cited that a large number of different substrate molecules including phenols, polyphenols, amino acids *(Patel, and Okun, 1977),* flavenoids, diaminobenzidine, o-phenyldiamine, dihydroquinone, alpha napthol and dopamine

(Palumbo, Napolitano, et al. 1999) may be polymerized, hydroxylated or otherwise modified by horse radish peroxidase (HRP). Of importance to the present study, it has been reported that HRP may hydroxylate phenolic amines at pH 5.0 with a high enzymatic reaction rate using the co-factor DHFA in the presence of O_2 at low temperature *(Mason Onopryenko, et al. 1957; Buhler and Mason 1961; Halliwell, and Ahluwalia, 1976; Halliwell, 1977; Dordick, Klibanov, et al. 1986, Klibanov, Berman, et al. 1981)*. The reported hydroxylation of phenolic substrates by HRP under previously defined reaction conditions would thus modify the mass of substrates by 16 Daltons *(Halliwell, and Ahluwalia, 1976; Dordick, Klibanov, et al. 1986)* and even larger modifications in mass can be observed for polymerizations *(Koduri, & Tien, 1995)*. It has been suggested that the relative concentrations of substrates such as hydrogen peroxidase, DHFA and cofactors play a significant role in controlling the direction of hydroxylation or other modification reactions. Under optimal conditions the hydroxylation reaction has been reported to be highly efficient and proceed to completion with no appreciable side reactions and where all the substrates and products can be accounted for *(Dordick, Klibanov, et al. 1986)*. More recently it has been suggested that while the iron-containing enzyme HRP is sufficient to catalyze the hydroxylation of phenolic amines, the reaction has also been reported to proceed where iron ions, Fe^{2+} or Fe^{3+}, act as the required catalyst (*Durliat, Courteix, et al., 1992)*. Even more recent papers seem to suggest that other proteins/or compounds may also act as a catalyst for the hydroxylation of phenolic amines. In contrast, in the present work we have observed that the hydroxylation of tyrosine by DHFA in the presence of O_2 proceeds without HRP, Fe ions or any other protein catalyst (*Durliat, Courteix, et al., 1992; Dordick, Klibanov, et al., 1986*; *Klibanov, Berman*, *et al. 1981; Klapper, and Hackett, 1963; Halliwell, and Ahluwalia, 1976; Halliwell, 1977; Dordick, Klibanov, et al. 1986)*.

6. Peroxidase

Heme is a well-known prosthetic group and the hemoproteins are very common in nature and play important biological roles. Proteins that contain protoporphyrin IX-heme as the prosthetic group include hemoglobin, cytochromes P-450 (CYP-450), and

peroxidase that are quite different in their functions and reactivities, especially with respect to molecular oxygen. Hemoglobin for instance, is the oxygen transporting protein in mammals; while the CYP-450 enzymes associated with hydroxylation of aromatic amines and phenols and catalyze the peroxidative substrates (*Dordick, Klibanov, et al., 1986).* Although these proteins have the same heme and are similar in structures, the type of chemistry carried out in each case is unique (*Dordick, Klibanov, et al. 1986).* In addition to peroxidase, hemoglobin (*Keilin, & Hartree, 1935),* and CYP-450 *(Nordblom, White, et al., 1976)* have been reported to exhibit peroxidase activity. Indeed, peroxidase activity is more common in proteins containing heme and it is associated with some other reactions such as N-demethylations *(Kedderis, & Hollenberg, 1983).* In addition to HRP other heme containing proteins like CYP-450, and Myelo-peroxidase (MYP) may hydroxylate substrates under known reaction conditions *(Mason, Onopryenko, et al. 1957; Buhler and Mason 1961; Halliwell, and Ahluwalia, 1976; Nordblom, White, et al. 1976; Halliwell, 1977; Dordick, Klibanov, et al. 1986).*

6.1 The biochemical significance of HRP

HRP is one of the heme peroxidases, which catalyzes different of oxidative transformations of both organic and inorganic substrates in the presence of a reducing compound by hydrogen peroxide *(Van Haandel, Claassens, et al., 1999),* or alkyl peroxides *(Bodtke, Pfeiffer, et al., 2005).* HRP also has the capacity to polymerize amines and phenols resulting in polyphenols. For example, the amber colored product produced from the polymerization of guaiacol in the presence of H_2O_2 has been reported in the past as tetraguaiacol *(Whitaker, 1972; Korduri, and Tien, 1995).* The sensitivity of colorimetric and chemiluminescent substrates that are commercially available makes HRP a very useful tool in biological research *(Bronstein, Voyta, et al.,1989)*. The hydroxylation reaction reportedly catalyzed by HRP requires the presence of a strong reducing agent, such as dihydroxyfumaric acid (DHFA) (*Durliat, Courteix, et al., 1992).* Yamazaki & Piette (1963) proposed also that O_2 was involved in a number of reactions catalyzed by HRP

(*Yamazaki, & Piette, 1963)*. Many approaches for hydroxylation may have significant problems since they are time consuming, and providing low yields *(Sih, Foss, et al., 1969)*. Phenols and hydroxylated phenols or their derivatives, especially amines could enter the gas phase and thus be analyzed by MS *(Okuda, Ohara, et al. 2008)*. Hence LC-ESI-MS/MS might be used to examine new chemical or biochemical strategies for hydroxylation reactions.

RATIONALE

Many heme-containing proteins such as HRP, CYP 450, and MYP, reportedly have the capacity to oxidize, hydroxylate or polymerize different substrates under known reaction conditions *(Mason, Onopryenko, et al. 1957; Buhler and Mason 1961; Halliwell, and Ahluwalia, 1976; Nordblom, White, et al. 1976; Halliwell, 1977; Dordick, Klibanov, et al. 1986; Whitaker, 1972; Koduri, & Tien, 1995)*. HRP and CYP 450 in particular share the capacity to act as hydroxylating agents *(Klapper, and Hackett, 1963; Halliwell, and Ahluwalia, 1976; Halliwell, 1977; Dordick, Klibanov, et al. 1986)*. In addition, there is considerable evidence that hydroxylation reactions require the presence of strong reducing agents, such as DHFA as hydrogen donors and the presence of molecular oxygen as well as requiring the presence of catalysts for the progression of the reaction (*Durliat, Courteix, et al., 1992; Klibanov, Berman*, *et al. 1981)*. It will be important to create analytical systems that can be used to determine the best methods for the hydroxylation of phenolic amines. Moreover, similar analytical strategies may be applied to the products with colorimetric dyes such as indole derivatives *(Bronstein, Voyta, et al. 1989),* TMB and DMB *(Liem, Cardenas, et al. 1979)* that can be detected by MS. The application of LC-ESI-MS/MS may a useful tool for the analysis of the products of hydroxylation reactions that will provide greater certainty of the molecular species by the estimation of the mass to charge ratio (m/z) of their parent and fragment ions.

CENTRAL HYPOTHESIS

The present thesis regards analytical biochemistry and is concerned with the identification and measurement of biomolecules. The central hypothesis of this research is that LC-ESI-MS/MS may be used to confirm the molecular identity of the reaction products of the well-cited hydroxylation of tyrosine by horseradish peroxidase with reasonable certainty. A corollary of this hypothesis is that LC-ESI-MS/MS may then be used to establish the reaction conditions required for the hydroxylation of phenolic amines such as tyrosine. A further corollary of the central hypothesis is that LC-ESI-MS/MS should agree qualitatively and quantitatively with the results of other methods of analysis of tyrosine hydroxylation reactions. A final novel hypothesis that emerged from the above lines of enquiry is the proposition that the well described hydroxylation of tyrosine, and perhaps many other phenolic amines, does not require a catalyst in contrast to the widely reported to requirement for iron (Fe) or heme containing proteins or other proteins as catalysts.

MATERIALS AND METHODS

1. Materials

L-tyrosine (L-Tyr), Guaiacol, p-nitrophenyl phosphate, CSPD, Luminol, TMB, P-coumaric acid, Phenol, L-Tyrosine, L-Proline, MTT Formazan, Indigo, X-Gal, Melanin, Adenine, Guanine, 4-Aminopyrazolo[3,4-d]pyrimidine, D-Phenylalanine, 3-Aminopyrazole-4- carbonitrile, 3,3′-Diaminobenzidine, Hydroquinine (Dihydroquinine), Hydroquinone, L-Ascorbic acid, 3,4-Dihydroxy-L phenylalanine (L-DOPA), Pyrocatechol (Catechol), 3,3'-Diaminobenzidine, β-Alanineand, DHFA, sodium molybdate (Sigma, 98+%) were purchased from Sigma Aldrich (Oakville, ON, Canada). Solvents and organic modifiers such as Acetic acid (CALEDON, George town, ON, Canada) and Formic acid (Sigma Aldrich, Oakville, ON, Canada) were HPLC grade. Acetate buffer was obtained from Bioshop (Burlington, ON, Canada). Compressed Oxygen gas was obtained from Linde (Brampton, ON, Canada). A TLC and developing chamber was purchased from Sigma Aldrich (Oakville, ON Canada). TLC plates (Silica Gel 60 F254 200 μm) were purchased from VWR (Mississauga, ON, Canada). Unless stated all salts, buffers, dyes reagents and phenolic amines were obtained from the SIGMA chemical company (St Louis MO, USA) and were of the highest quality available.

2. Direct infusion ESI-MS/MS

As a first step a variety of phenolic amines or other amino compounds and or some of their predicted hydroxylation products were obtained based on their structural similarity to compounds that were known to ionize by ESI from the literature (Table 2). The compounds were analyzed by direct infusion with a Hamilton gas tight syringe via a metal needle the electrospray ionization where the voltage was connect via a liquid junction with a flow rate of 2 μL per minute. The detection of the analytes was compared

with an LTQ linear ion trap instrument (Thermo Fisher Scientific). The ionization and transmission conditions was tuned separately for each potential substrate or product using a 100 μM solution in 0.1% formic acid to determine the optimal ionization voltages, nitrogen flow, transfer capillary temperature and lens voltages.

3. Hydroxylation reactions

For large-scale reactions the hydroxylation system proposed by Klibanov and coworkers (1981) was used in this experiment without modifications. L-tyrosine was reacted with dihydroxyfumaric acid (DHFA) at 0 °C in the presence of bubbling O_2 in 1L Erlenmeyer flask on ice. Alternatively the total volume of the reaction was scaled down to ≥ 100 μL for use in a 96 well ELISA plate in an oxygen atmosphere where the layer of the liquid reaction mixture was sufficiently thin (≤ 2 mm) so that oxygen could be supplied to the reaction by simple diffusion. LC-ESI-MS/MS (see below) was used to confirm that both the large scale and small scale reactions resulted in the product L-DOPA (L-3,4 dihydroxyphenylalanine). The reagents were placed first in eppendorf ® reaction tubes where 200μL of 10 mM stock solution of Tyr (2mM, 18.1mg/10ml in buffer) was added to 60 mM acetate buffer (pH 5.0) to make 1mL reaction and the tube was cooled down to 0 ^{o}C before adding 400μL of 10mM stock solution of DHFA (4mM, 14.2mg/10ml in buffer) followed immediately by 2.8μL (0.5mg/L) of 4.42mg/mL HRP stock solution was added to the reaction tube and the stopwatch was set up (however in the absence of HRP the reaction was begun after adding DHFA). Then, ≥ 100 μL of that reaction tube was placed in different wells labeled (0-3hr). After 1hr, an additional amount of 4mM of DHFA were added to the wells that had 2hr and 3hr samples. And the same amount again was added after another hour to 3hr sample only. The samples were measured immediately at each sampling time with the various techniques including (TLC, coluorimetric and LC-ESI-MS/MS).

4. Thin Layer Chromatography (TLC)

Standards of L-Dopa and L-tyrosine solutions were dissolved in acetate-buffer (pH 5.0). L-Dopa (0.276 g/L) and L-Tyr (0.362 g/L) standards were spotted on labeled TLC plates 4x and 3x, respectively; 1 cm apart. Time course samples (0, 15, 30, 1hr, 2hr, 3hr) containing mixture of L-tyrosine and DHFA in acetate-buffer (pH 5.0), with and without HRP were spotted ~ 5-6 x with a glass pipette. Enough sample was drawn up at different time courses (0-3hr) with the glass pipette to form a spot with ~ 3-4 mm diameter. Alternatively the reaction was performed in pH 2 or pH 3 HCl and spotted. The plate was placed on a heater in order to allow the spots to completely dry between consecutive spotting. Then the plates were developed in a chamber containing 75% phenol in H_2O with the lid covering the top. After the solvent in the chamber had travelled ¾ of the way to the top of the plate (~40-45 min), the plates were dried with a heat gun, and then sprayed with Ninhydrin reagent (200mg of Ninhydrin was added to 100 mL acetic acid) until spots were visible. The plates were allowed to dry prior to be read and compared.

5. Colorimetric Assay

The assay of Arnow for the determination of L-DOPA was employed with some modifications *(Arnow, 1937a)*. Nitrite-Molybdate reagent (10g of sodium nitrite and 10 g of sodium molybdate (Sigma, 98+%) each was dissolved in 100 mL of distilled water). Number of different concentrations of 10 mM stock of DOPA standard arrange from 0.025 to 0.2 mM (0.005-0.039mg/ml) were analyzed by (Lambda 20/40) UV/VIS spectrophotometer at 510 nm wavelength to make a standard curve. After that, the reaction of Tyr to create DOPA was set up with known reaction conditions (2mM Tyr, 4mM DHFA at 0^oC) for 3hr with and without the presence of HRP in 96 well plates 3 replications each. Enough samples (125 μL) were taken from wells at various time courses (0min, 15min, 30min, 1hr, 2hr, 3hr) to make 1mM dilution and placed in a test-tube containing 125 μL of the buffer. Then, 250 μL of both 0.5 M of HCl (CALEDON Georgetown, ON, Canada) and nitrite-

molybdate reagent were placed respectively in the same test tube. After which another 250 µL of 1 M of sodium hydroxide (NaOH) was added to each test-tube to make a total volume of 1 mL and mixed well to form a red color. The system was blanked with a mixture containing 1mM of Tyrosine and DHFA in addition to the other reagents used in the assay. Then, the time course samples were analysed using quartz cuvettes at 510 nm by visible light (UV/VIS, lambda 20).

6. UV/VIS Spectrophotometry

The absorbance of DOPA standards and hydroxylation reactions was measured on a Perkin-Elmer Lambda 20 UV/VIS (Toronto, ON, Canada). Different concentrations of L-DOPA standards (0.025mM, 0.050mM, 0.075mM, 0.1mM and 0.15mM, 0.20mM) were quantified by the UV/VIS using quartz cuvettes (Aldrich Chemical, Oakville, ON). The system was blanked with acetate buffer and the wavelength scan range was from 200 to 400 nm. After that, samples of 0min, 15min, 30min, 60min, 120min and 180min from the main reactions in the presence or absence of HRP were measured.

7. Liquid Chromatography-Electro Spray Ionization-tandem Mass Spectrometry (LC-ESI-MS/MS)

Hydroxylation reactions were separated by HPLC coupled to ESI-MS to resolve the substrate, cofactors, and buffer components from the hydroxylated product and thus permit efficient ionization and sensitive detection. The DOPA standard (0.000197 g/tube) was aliquoted, dried and stored at -80 ºC. Of each experiment a standard HRP aliquot was dissolved in 985µL of HPLC water. A stock of 200 ng/µL was prepared in order to make standard series in 5% FA according to (Table I). Prior to start injection of the standard or

sample series, the LC-ESI-MS/MS system was cleaned at 10µL per minute with 50% acetonitrile in the mobile phase which was run off line through the column and the sample loop cleaned several times with 5% FA in the load and inject positions. The baseline was established and the blank injected three times at 2-5 minutes apart. Based on the peak size and how much time was required to return to baseline, the injection period was varied between 5-15 minutes apart with three technical replicates.

Table 1: Stock of L-DOPA and the final concentrations to a standard curve

Stock 200 (ng/µL)	5% formic Acid (µL)	Final conc. (mg/mL)
25 **µL**	975	0.005
12.5 **µL**	987.5	0.0025
5.0 **µL**	995	0.001
3.75 **µL**	996	0.00075
2.5 **µL**	997	0.0005
1.25 **µL**	998	0.00025
0.5 **µL**	999	0.0001

The crude products that were obtained from the reaction at various time-course were simply diluted (1/100) in 5% formic acid prior to isocratic separation over C-18 (300 micron ID x 15 cm column) (Vydac, Hesperia, CA, USA) at 10 µL per minute with an Agilent 1100 HPLC connected via an electrospray ionization source to a linear ion trap (LTQ Thermo Fisher). The effect of varying tyrosine in the presence of O_2 versus DHFA concentration or HRP on the reaction progress was directly monitored by LC-ESI-MS/MS. The mobile phase was 80/20/0.05 of H_2O/acetonitrile/formic acid (HPLC grade). The source and instrument was cleaned between sample determinations.

8. HRP Enzyme activity assays

The vigorous activity of the HRP enzyme used in these experiments was confirmed with ECL and colorimetric enzyme assays.

8.1. Chemiluminescent enzymatic assay (Dot Blot)

A small piece of PVDF membrane was labeled using a pencil prior to being placed in the dish-containing methanol. The filter paper was soaked in methanol in a small plastic dish and excess was allowed to evaporate by placing the dish in the fume hood for a couple minutes. A sample of the HRP enzyme was spotted at different volumes including 1µL, 2µL, 3µL and 4µL on top of the damp PVDF membrane then allowed to dry. The HRP bound membrane was then washed with distilled water. ECL solution was prepared by adding 1mL of Tris/HCl (1 M, pH 8.8), 9 mL distilled water, 50µL Luminol (250 mM in DMSO), and 222µL 4IPBA (90 mM in DMSO) in this precise order. The ECL solution was then poured on the surface of the membrane and shaken for ~1 min. The membrane was then drained of excess reaction buffer and immediately wrapped in saran wrap and placed inside the film cassette. The membrane was then exposed to a piece of film corresponding to the size of the membrane in a dark place for ~3 min. The film was then developed by placing in a developer solution until spots became visible then rinsed with cold water and placed in fixer solution for ~ 2min. Kodak Professional T-max (4:1 parts water) and Kodafix Solution (3:1 parts water) were used as developer and fixer respectively. The developed film was then allowed to air dry before being scanned with an EPSON EXPRESSION 1680 scanner. The exposure of the film confirmed the activity of the enzyme.

8.2. Colorimetric assay of the Polymerization of guaiacol

According to Danelle Furlong (2007), the HRP enzymatic activity measurement corresponds to the rate of oxidation of guaiacol by hydrogen peroxide. 100 mM of sodium acetate buffer (PH 5.0) was prepared and stored at room temperature. Both guaiacol and 30% (w/w) hydrogen peroxidase (H_2O_2) were purchased from Sigma-Aldrich (Oakville, ON, Canada). 5µL of guaiacol (0.306mM) was placed in test tube filled with 233µL of sodium acetate buffer (100mM, pH 5). After that, 7µL and 5µL of HRP (3.18µM) and H_2O_2 (14.69mM) were added respectively to the same test tube *(Furlong, 2007)*. The generation of an amber color confirmed the activity of the enzyme.

Table 2: List of substrates and products, sources and molecular weights.

Substrate/Product	Source	Mol. Weight
Guaiacol	Sigma Aldrich	124.14 g/mol
p-nitrophenyl phosphate	Sigma Aldrich	144.2 g/mol
CSPD	Roche	460 g/mol
Luminol	Sigma Aldrich	199.15 g/mol
TMB	Sigma Aldrich	240.34 g/mol
P-coumaric acid	Sigma Aldrich	164.16 g/mol
Phenol	Sigma Aldrich	94.11 g/mol
L-Tyrosine	Sigma Aldrich	181.19 g/mol
L-Proline	Sigma Aldrich	115.13g/mol
MTT-Formazan	Sigma Aldrich	335.43 g/mol

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Indigo	Sigma Aldrich	262.26 g/mol
X-Gal	Sigma Aldrich	408.64 g/mol
Melanin	Sigma Aldrich	126.22 g/mol
Adenine	Sigma Aldrich	135.18 g/mol
Guanine	Sigma Aldrich	151.13 g/mol
4-Aminopyrazolo[3,4-*d*] pyrimidine	Sigma Aldrich	135.13 g/mol
D-Phenylalanine	Sigma Aldrich	165.19 g/mol
-Aminopyrazole-4-carbonitrile	Sigma Aldrich	180.1 g/mol
3,3′-Diaminobenzidine	Sigma Aldrich	168.14 g/mol
Hydroquinine (Dihydroquinine)	Sigma Aldrich	326.44 g/mol
Hydroquinone	Floka	94.11 g/mol
L-Ascorbic acid	Sigma Aldrich	176.12 g/mol
3,4-Dihydroxy-L-phenylalanine (L-DOPA)	Sigma Aldrich	197.16 g/mol
Pyrocatechol (Catechol)	Sigma Aldrich	110.1 g/mol
3,3'-Diaminobenzidine	Sigma Aldrich	214.27 g/mol
β-Alanine	Sigma Aldrich	89.09 g/mol

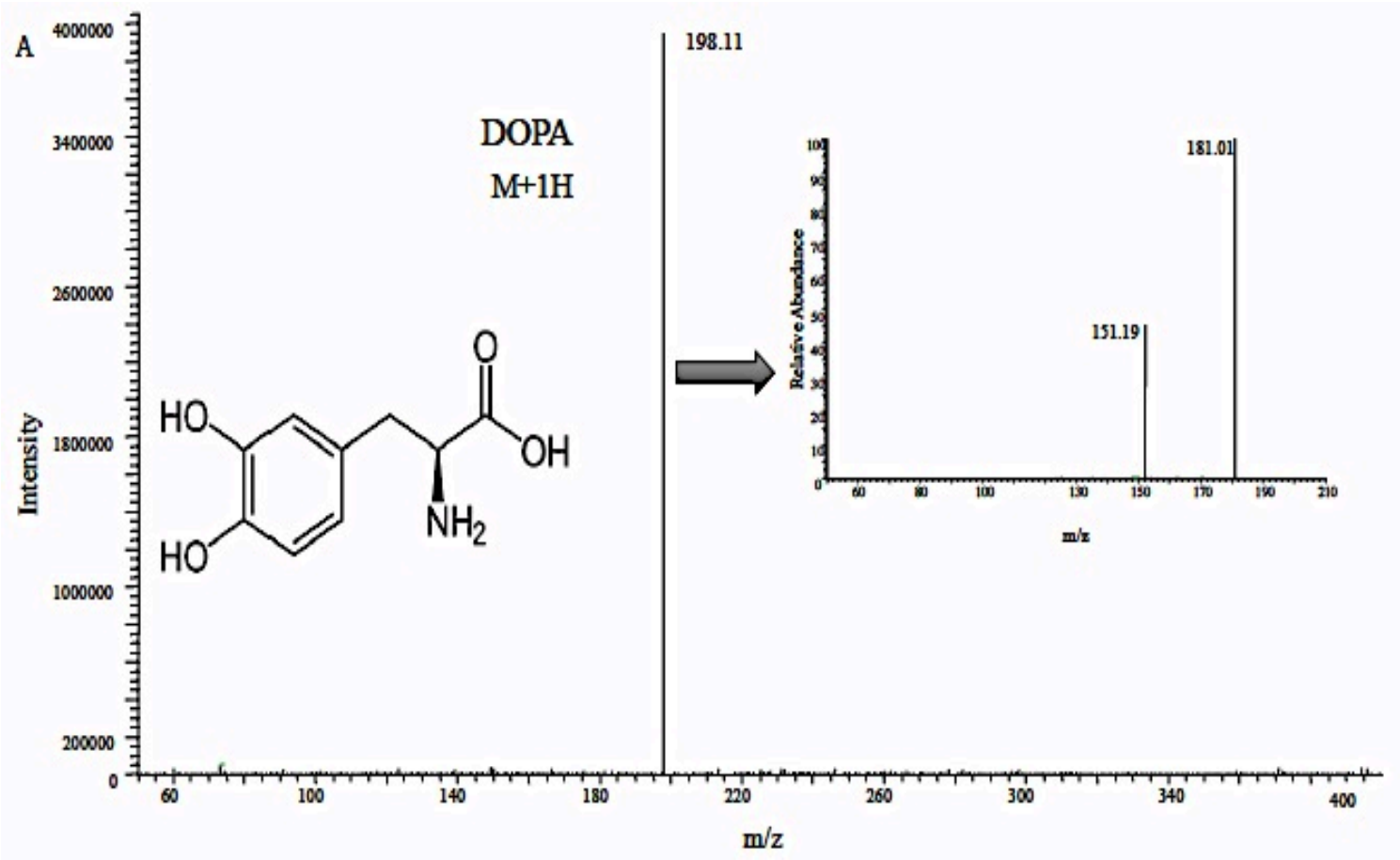
RESULTS

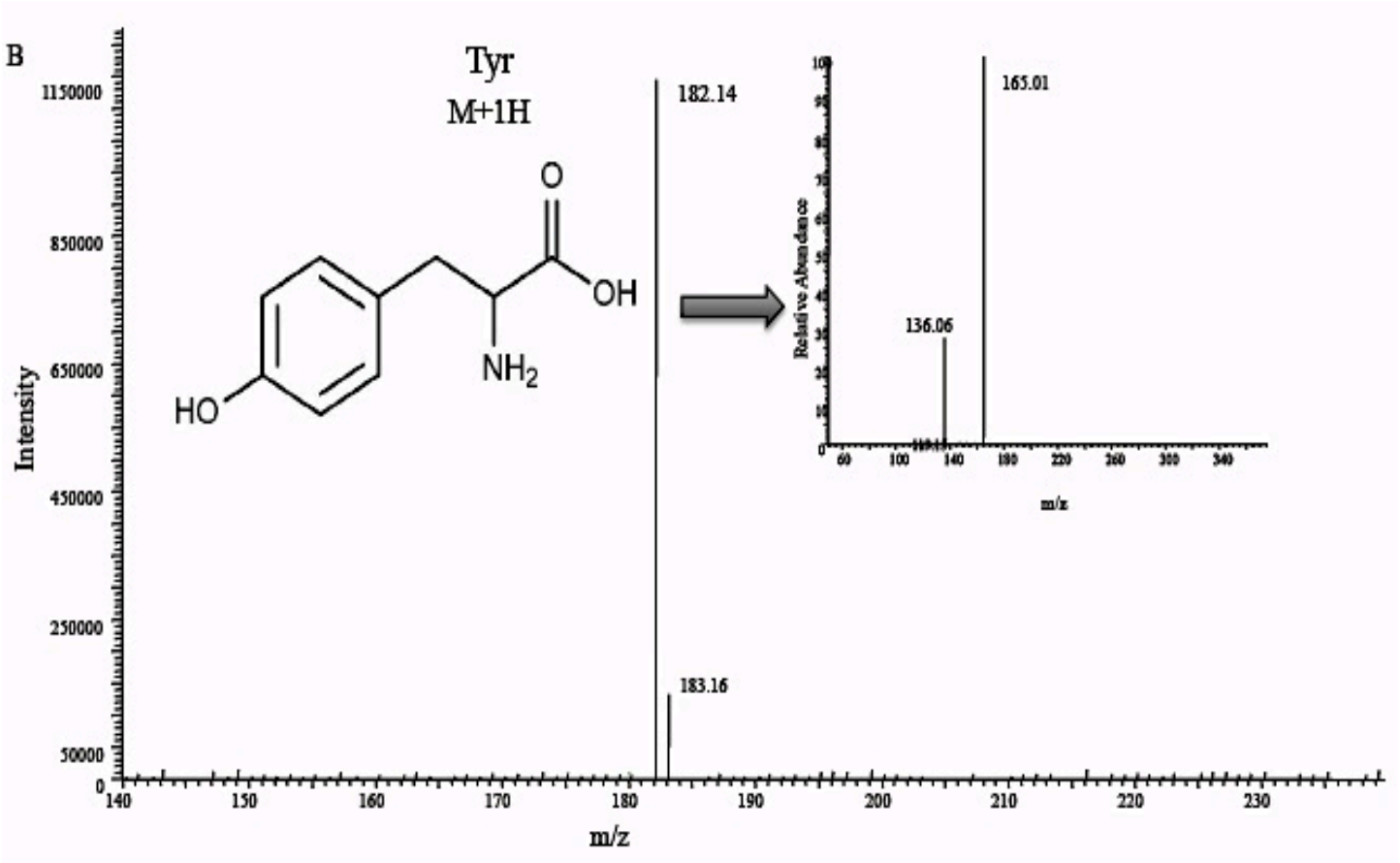
1. Screening the ionization of potential substrates and products

As a first step a variety of phenolic amines or other amino compounds and or some of their predicted hydroxylation products were obtained based on their structural similarity to compounds that were known to ionize by ESI from the literature (Table II). The electrospray ionization source and detection of these were compared by direct infusion with a linear ion trap. A small range of phenolic and other compounds including phenols, tyrosine, proline, phenyl-alanine, dopamine, other amino acids, polyphones, diaminobenzidine, o-phenyldiamine, dihydroquinone, alpha-naphthol and their hydroxylated products and many others, were run in different concentrations as low as femto M in 0.1% formic acid by direct infusion into the MS, in order to determine the sensitivity and limit of detection and to draw standard response curves. Additionally, ESI in positive and negative mode was used to examine the substrates and products *(Chu, Haffner et al. 2005)*. The substrates and products that presented great sensitivity and resolution from all others were utilized for subsequent experiments. We observed that both tyrosine and its predicted hydroxylation product L-DOPA ionized well.

A series of preliminary experiments were undertaken to determine the most compatible buffer for the hydroxylation reaction after the optimal substrates and products have been analyzed. In addition to an acetate buffer, other couple reaction buffers such as (Na acetate, citric acid and acetic acid at pH 5.0, NH_4HCO_3 at pH 8.8, & PBS pH 6 respectively) were examined. In addition, strong reducing agents such as DHFA and H_2O_2 were tested along with these compounds in the presence/absence of O_2 and HRP. Some direct and rapid reactions were performed. For instance, the polymerization reaction of guaiacol to form tetraguaiacol occurs in the presence of HRP and H_2O_2, however this compound have not shown well ionization by the direct infusion ESI-MS. The polymerization reaction therefore was excluded as the optimal reaction for the experiment.

After the ionization capacity of all the above listed substrates and products (Table II) were analysed and compared by direct infusion to the MS. Only five of the samples showed ionization with concentrations as low as Pico mole (E^{-12}) by the ESI-MS when dissolved them in a number of different buffer solutions with various pH. These compounds including 3,4-Dihydroxy-L-phenylalanine (DOPA), L-tyrosine (Tyr), L-phenylalanine (PA), hydroquinine (DHQ) and tetramethylbenzidine (TMB). In the case of using the mass spectrometry as a detector, some substrates and/or products are ionized and some of them are not. This was considered one of the challenging tasks in this project since some substrates known to be ionized while their products might not, and vise versa.




B
Tyr
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136.06
Relative Abundance
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HO
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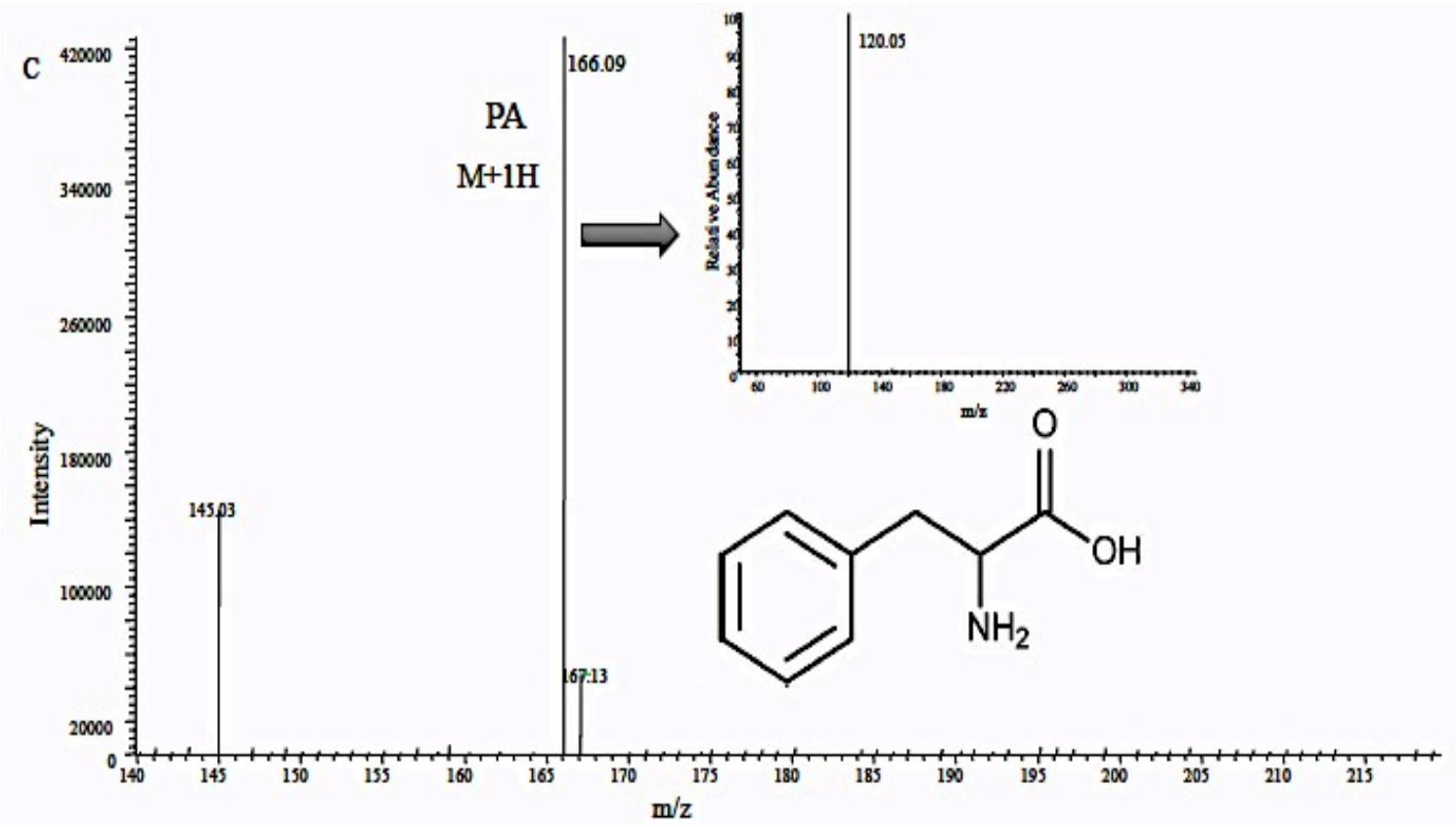

C
PA
M+1H
166.09
120.05
145.03
167.13
Relative Abundance
Intensity
m/z
NH2
OH
O

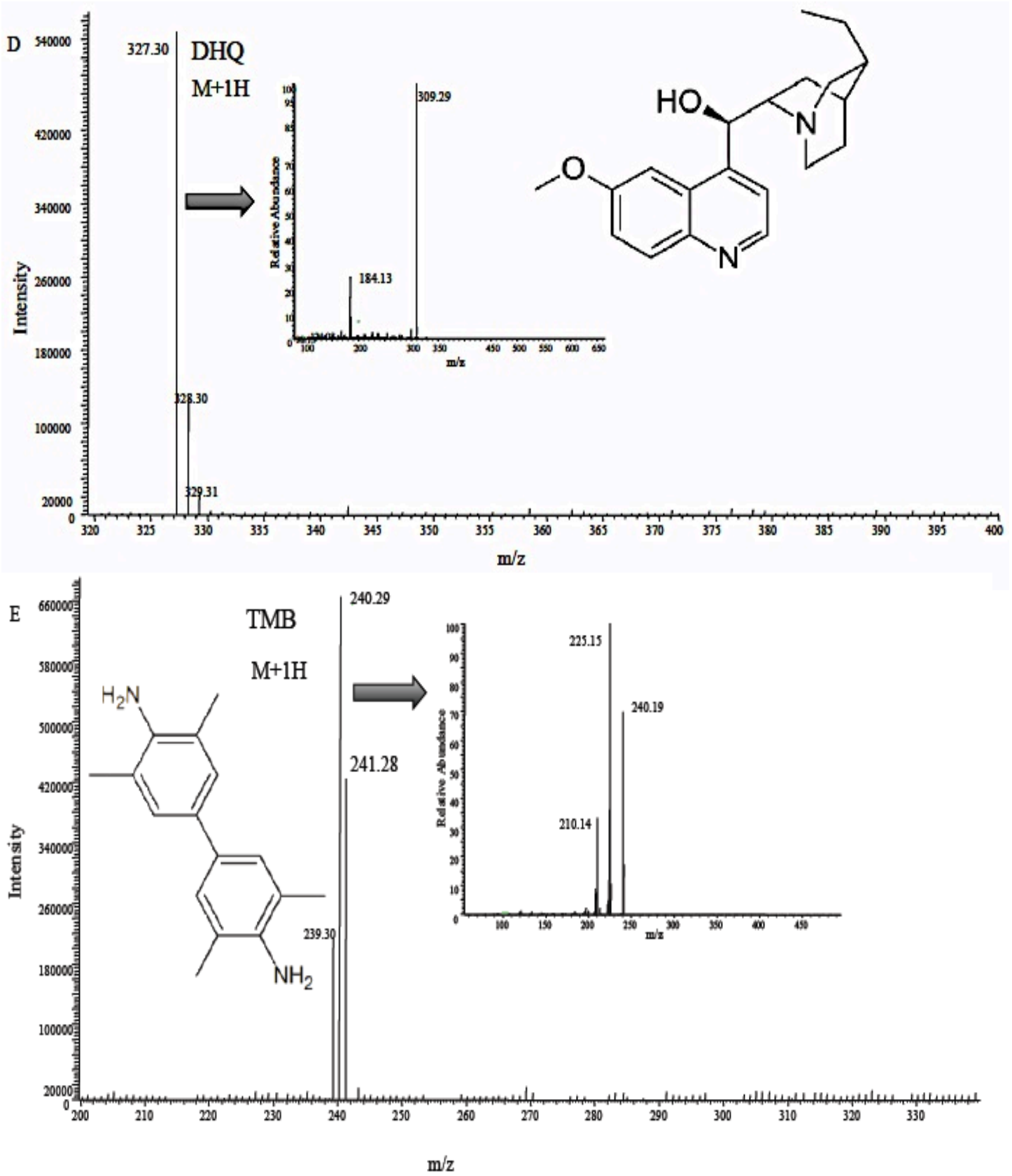


Figure 2: Substrates and products ionized by direct infusion ESI-MS.

Different substrates and products in various concentrations that were diluted with 1%

formic acid were measured by the direct infusion ESI-MS to examine their ionization solvent. Thus, the mass spectra highlight compounds that yield sharp peaks only at lower concentrations as listed in Table 2 as well as their MS/MS molecule fragments. These peaks consist of (A) 3,4-Dihydroxy-L-phenylalanine (L-DOPA) at mass 198 Da; (B) L-tyrosine (mass 182 Da); (C) L-phenylalanine (166 Da); (D) hydroquinine (DHQ) with very high peak at m/z=327 Da and finally (E) tetramethylbenzidine (TMB) is consisting of three peaks, the most abundant having a mass 240 Da then 241 Da and the minor peak at a m/z 239 Da. From the top five analytes, Tyr and its product DOPA were determined as the optimal substrates and products, in which both have the ability to be ionized by MS. The proposed reaction that converts Tyr to DOPA is known as a hydroxylation reaction (*Klibanov, Berman*, *et al. 1981*).

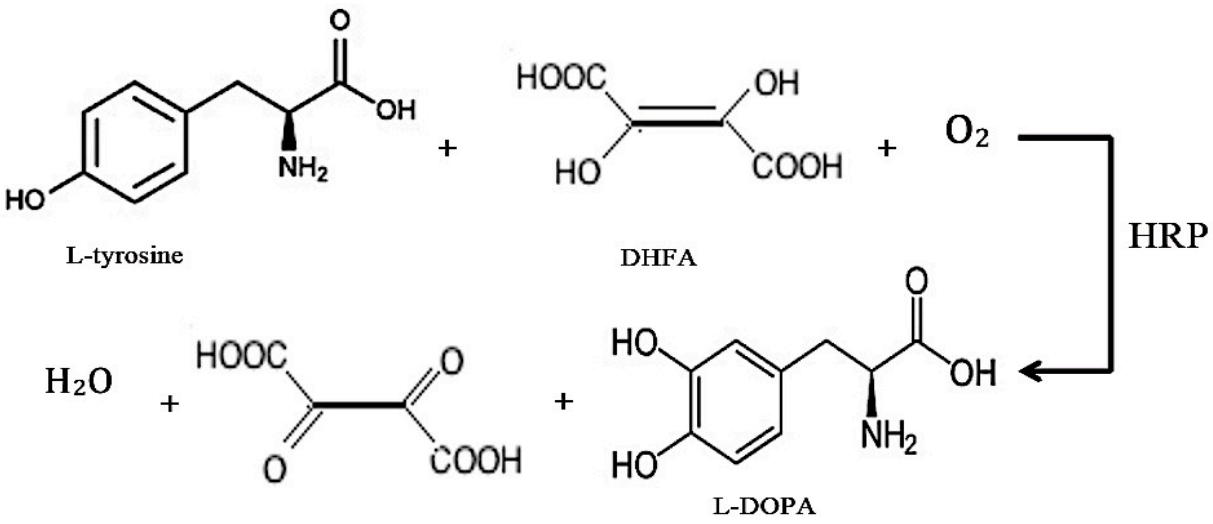


Figure 3: The synthesis of L-DOPA from the hydroxylation of L-tyrosine with known conditions (*Klibanov, Berman*, *et al. 1981)*. In the proposed mechanism, DHFA acts as a hydrogen donor in a reaction to make a hydroxyl group (-OH) and thus form L-DOPA and other compounds in the presence of O_2 and HRP. The product has the chemical formula $C_9H_{11}NO_4$ with a molar mass of 197.19 g/mole very close to the substrate which only one –OH group are differentiate between them.

2. Linearity with respect to concentration

The intensity of ESI signals is proportional to concentration of the analyte over a range of concentrations that depends in part on the design of the instrument. High concentrations of the analyte provide strong signals but also require extended periods of time to clean the ion source and instrument between samples. A 20 μL injection of a concentration of about a 0.1 mM to 1 mM concentration diluted 100 fold in injection buffer (final 1 μM to 10 μM) provided sufficient signal to noise. Higher concentrations resulted in fouling of the source and required extended cleaning.

3. Determining the time and enzyme linearity

After determining the minimally sufficient phenolic substrates, buffers and the cofactors, the linearity of the system regarding the concentration of pure HRP was determined. The linearity of the system regarding time of reactions was tested. It was observed in preliminary experiments the production of DOPA increased with time for at least 3h. However based on calculations from the standard curve the reaction did not seem to go to completion as anticipated. Finally, the working protocol was performed for the hydroxylation reaction of tyrosine with DHFA and H_2O_2 to determine the linearity with enzyme. However there seemed to be no relationship between HRP concentration and yield in the preliminary experiments ionized by MS direct infusion (not shown).

4. HRP Enzymatic assays

HRP was dissolved in water and aliquoted with (4.42mg) per tube before drying in a speed vac and storage at -80 °C. Given the poor yield of DOPA and lack of a clear linear

relationship between enzyme concentration and product by mass spectrometry the potency of the HRP was suspected. As a control an aliquot of HRP was re-dissolved in water and incubated at room temperature 5 days was compared to a fresh HRP by a colorimetric assay and Enhanced Chemiluminescent assay (ECL).

4.1. Polymerization of guaiacol

It has been previously demonstrated that an amber color was formed by guaiacol in the presence of H_2O_2 and HRP from the formation of tetraguaiacol *(Koduri, & Tien, 1995)*. The activity of the HRP used in preliminary reactions was confirmed by the formation of tetraguaiacol. An old HRP that was kept in the room temperature for about 5 days, and a new/fresh (fresh aliquoted enzyme) HRP were examined. An amber color was formed directly after adding the fresh enzyme. As shown in (figure 4) there is a difference in color between the old and new, which showed that the aliquot fresh HRP was working.

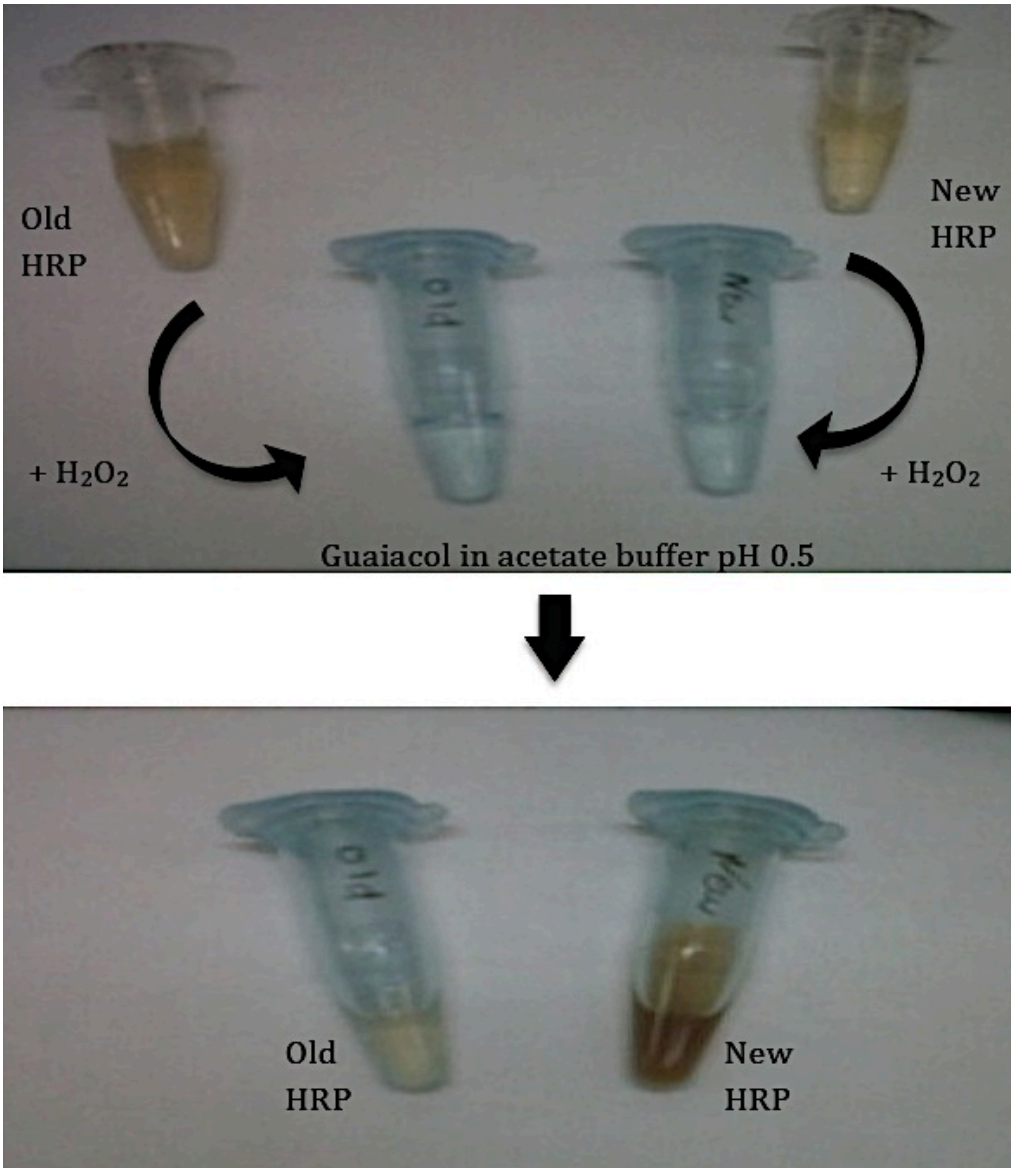


Figure 4: Colorimetric Enzyme assay (The polymerization reaction of Guaiacol). Where 0.306mM of Guaiacol was added to 1 ml test tube filled with 100mM sodium acetate buffer (pH 5.0) and mixed well. After which, H_2O_2 (14.69mM) and 3.18µM HRP enzymes were placed in the tubes respectively.

4.2. Dot Blot

The potency of the HRP enzyme was also confirmed by enhanced chemiluminescence using the protocol of Haan, & Behrmann, *(Haan, & Behrmann, 2007)*. The same two enzymes (old and new) that were used in the previous experiment (4.1) were also used to measure enzyme activity by dot blotting and exposure of x-ray film. The sensitive ECL method showed an increasing relationship between the amount of HRP spotted and the total enzymatic activity in the assay**.**

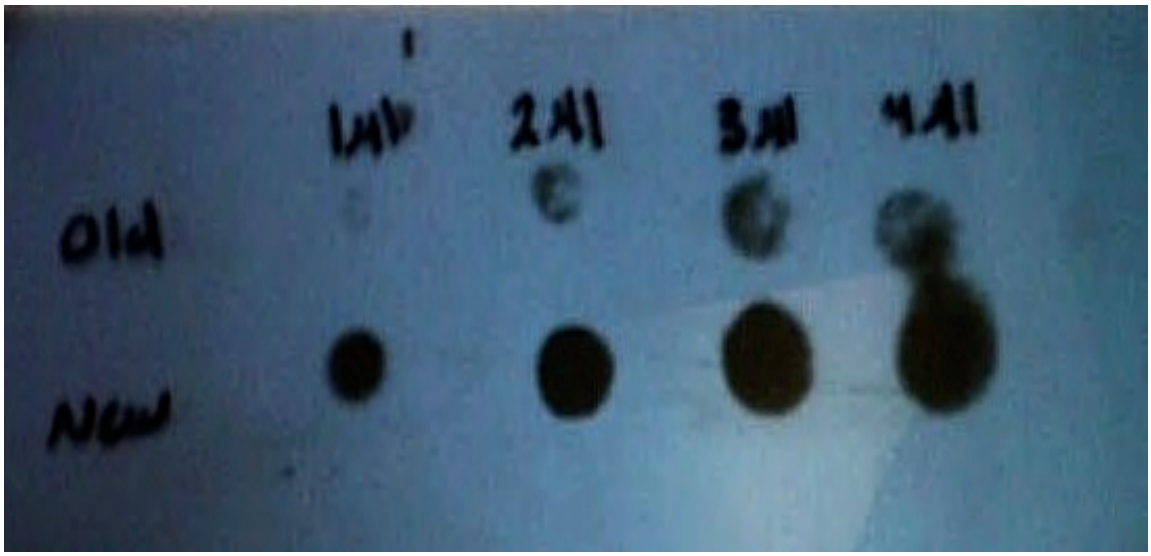


Figure 5: Comparing between two enzymes by Dot-Blot (ECL). An old HRP that was kept in the room temperature for about 5 day, and a new (fresh aliquot of enzyme. The HRP samples were spotted at different volume (1µL, 2µL, 3µL & 4µL) on a socked PVDF membrane in methanol which then placed in ECL solution and exposed to a piece of film for about 3 min in a dark place. As it is demonstrated the new (bottom) HRP spots look darker than the old spots (top).

5. Ultra violet/ visible (UV/VIS) spectrophotometry

In previous studies, UV/VIS spectroscopy was used to measure samples of hydroxylation reactions of phenol, to detect the absorptions of Catechol and thus calculate the concentration of the reaction products *(Dordick, Klibanov, et al. 1986)*. We attempted to reproduce these results by first creating a standard curve of DOPA concentration to absorption at 280 nm. A clear linear relationship was observed between absorption and DOPA concentration yielding an R^2 value of ~0.972 (Figure 6).

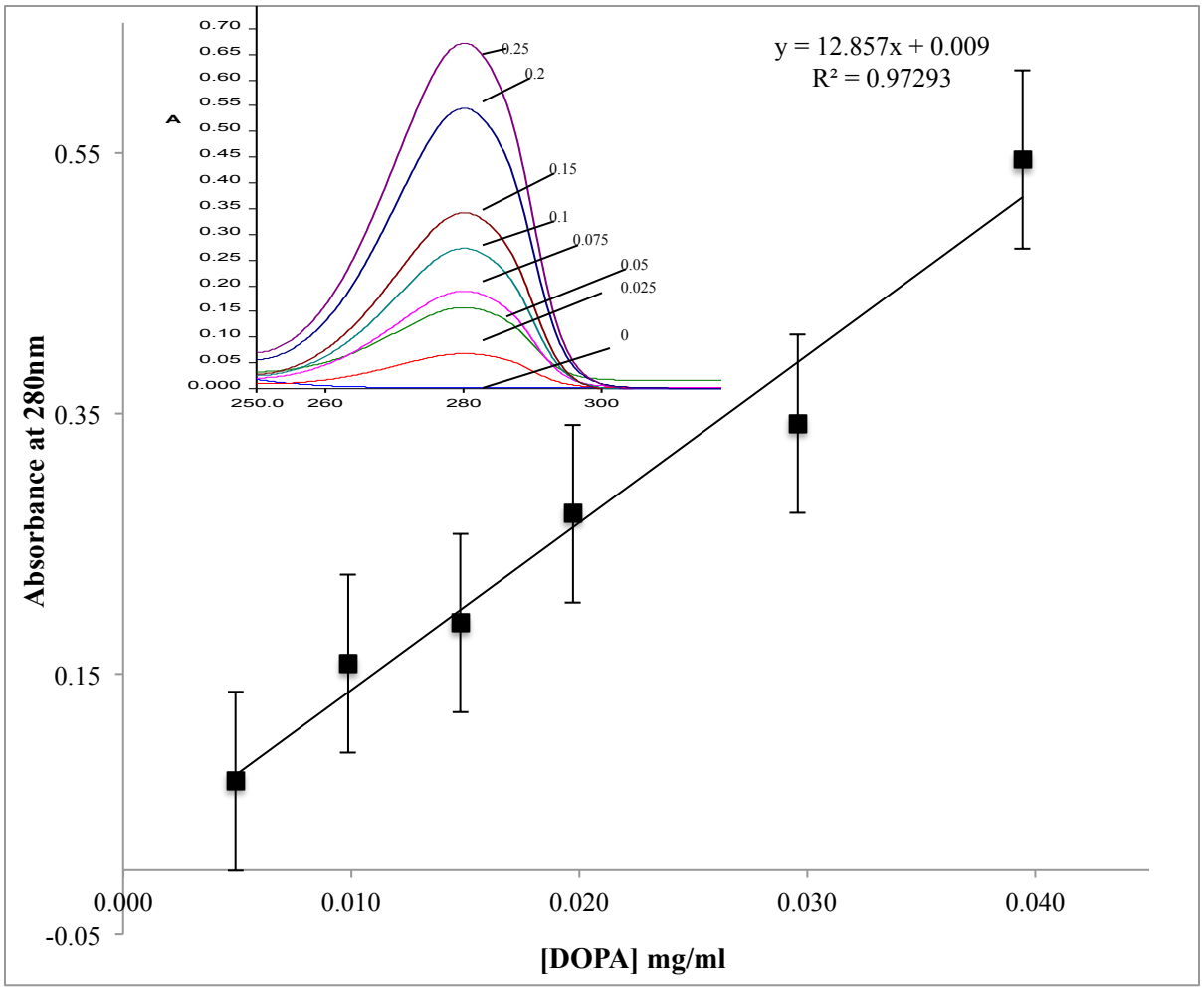


Figure 6: Standard concentration curve of DOPA versus absorption by UV/VIS. A stock containing 10 mM DOPA was diluted to various concentrations range between 0.005 and 0.039 mg/mL (0.025, 0.05, 0.075, 0.1, 0.15 and 0.2 mM) and thus the

absorptions at 280 nm were obtained. Then these concentrations of DOPA standard were plotted against absorptions. Thus the concentrations of DOPA over time can be calculated from the equation obtained from the standard concentration curve (Y = 12.857X + 0.009).

The time course of absorption at 280 nm was recorded by UV/VIS spectrophotometry over the course of a 3-hr reaction. The reaction containing a mixture of 2 mM Tyr and 4 mM DHFA was performed in the presence of 0.5 mg/L HRP and analyzed directly by the UV/VIS at 200-400nm. In contrast, the expected increase in absorption at 280 nm with time instead resulted in absorption values that decline precipitously until the addition of more DHFA after 60 minutes. Samples 0 to 60 minutes had only one dose of DHFA, while samples at 120 min and 180 min had two and three doses of DHFA respectively *(Klibanov, Berman, et al. 1981)*. There is a slight increase over the 1 hr absorption at 2 hr and 3 hr with the addition of further DHFA. Moreover the spectra observed were much broader than that expected for DOPA and the major peak extended far close to 300 nm. Therefore we were not able to attribute the observed absorption spectra to DOPA production.

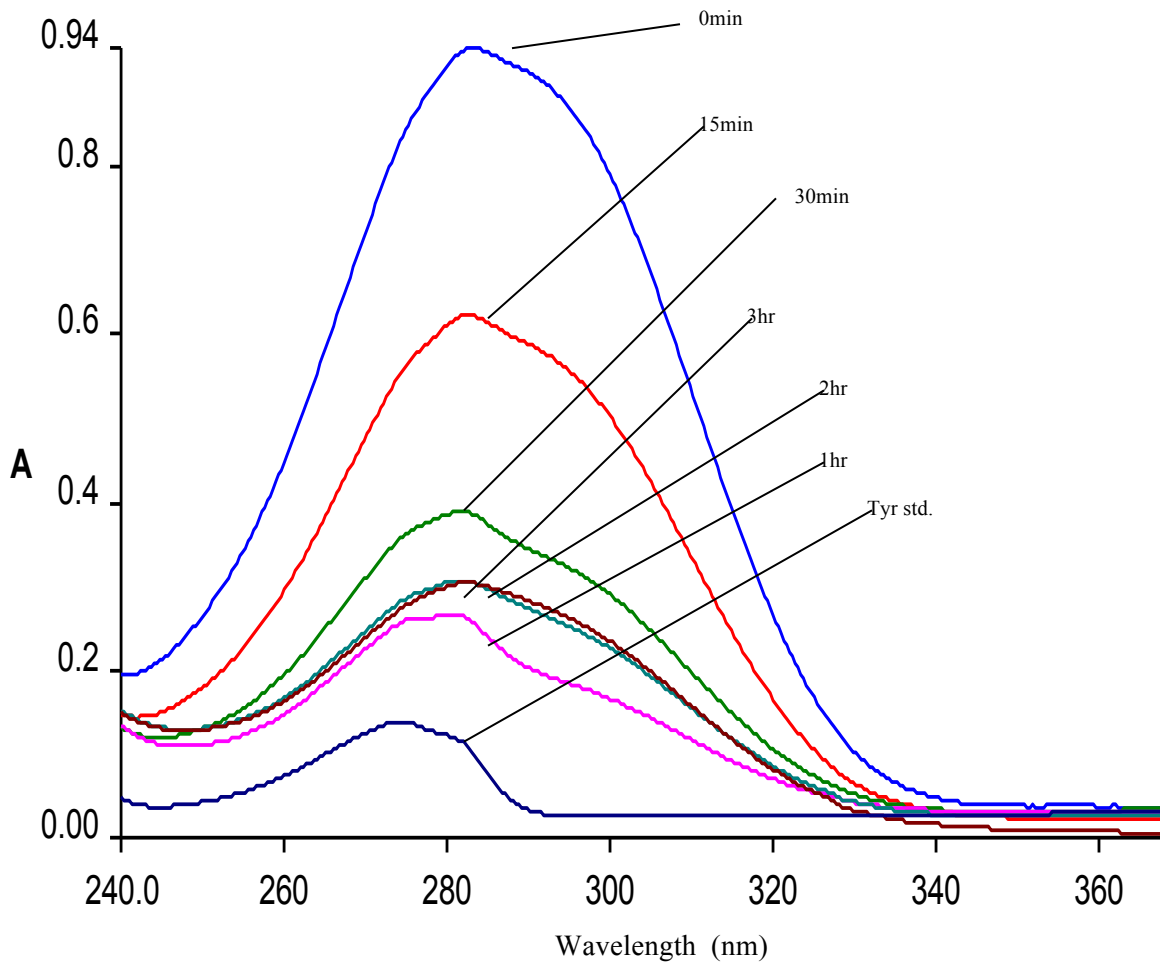


Figure 7: Detection of the absorptions using UV/VIS. The reaction was applied in 96 well dish including 2mM Tyr, 4mM DHFA (added hourly) and 0.5mg/L HRP for 3hr. The system was blanked with acetate buffer alone using a Quartz cuvette. At the indicated time, the samples were diluted to 0.1mM in acetate buffer (pH 5.0) and measured directly between (200-400 nm). The absorbance of samples was decreased over the time until 1hr and then was increased again at time 2 and 3hr.

In order to understand the puzzling results observed in the DOPA reactions by UV/VIS, we examined the spectra of DOPA, tyrosine and DHFA compared to that of the 3-hr reaction. The absorption wavelengths of the tyrosine substrate and the expected DOPA product ranged between 270 to 282 nm. Tyrosine and DOPA were observed to show overlapping spectra, which might be expected since there is only one hydroxyl group is separating between their chemical structures. The spectra of the reaction mixture were most similar to that of the co-factor DHFA (Figure 8).

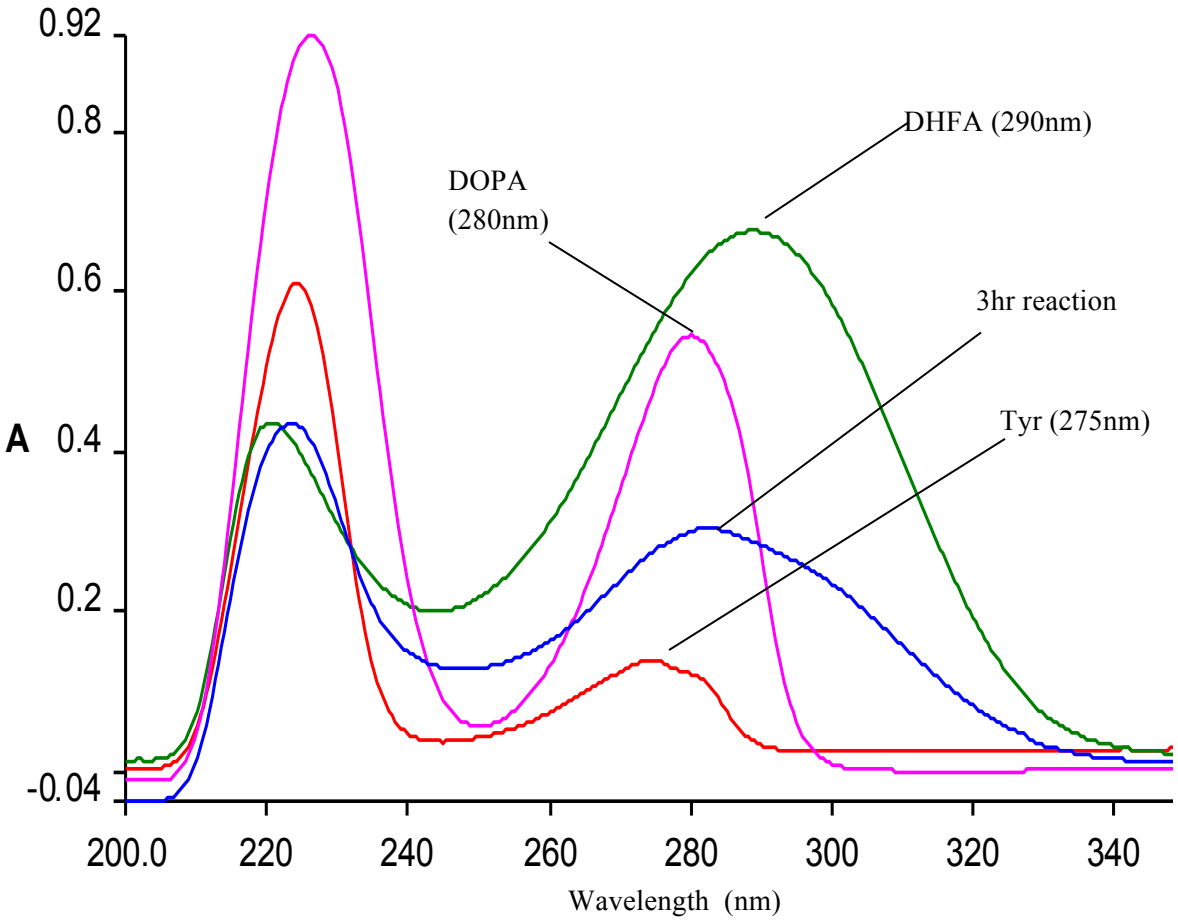


Figure 8: Tyrosine, DHFS and DOPA standards by UV light. A typical chromatogram UV/VIS (lambda 20), obtained at 274nm and 280 nm for the substrate (L-Tyrosine) and product (L-DOPA) standards respectively. DHFA standard was in addition detected at 290nm. The wavelengths of these peaks as shown were very close which result in overlapping the sample at 3hr reaction.

6. Determination of the product formation by a specific Colorimetric Assay

In order to avoid the cofounding effect of the overlapping spectra observed for the tyrosine substrate, DOPA product and DHFA cofactor we utilized a specific colorimetric reaction. The assay of Arnow for L-DOPA using Nitrite-Molybdate reagent was employed to detect DOPA without interference from other compounds *(Arnow, 1937a).* A stock of 10 mM of DOPA standard was diluted to different concentrations range from 0.025 to 0.2 mM. Then, they were analyzed by (Lambda 20/40) Ultra Violet-Visible (UV-VIS) spectrophotometer at 510 nm wavelength to make a standard curve. The system was blanked with a mixture containing the standard of DOPA and the other reagents of the colorimetric assay. The full-scale assay using a assay produced a standard curve with an R^2 value of 0.993.

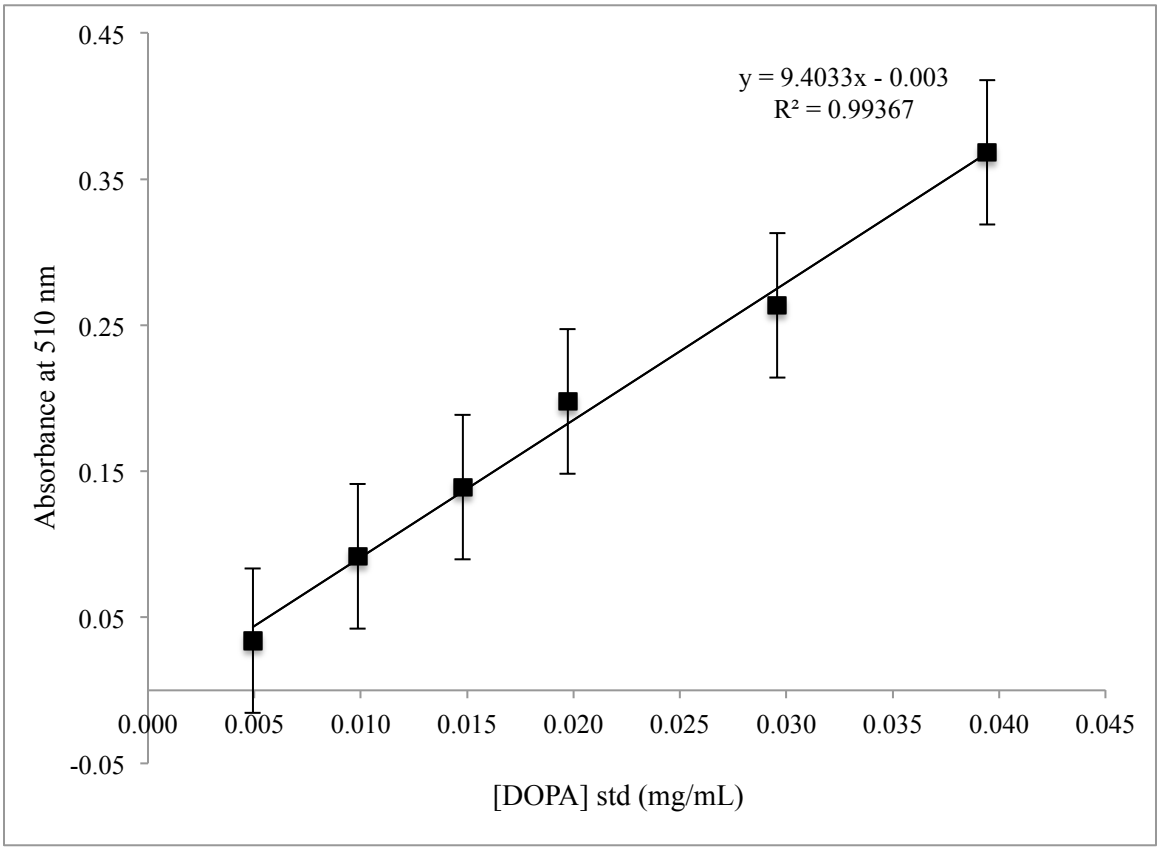


Figure 9: Colorimetric Analysis of DOPA Standard Curve Absorbance

versus concentration. Different concentrations of DOPA ranges from 0.005 to 0.039 mg/ml (0.025 to 0.2 mM) plotted against the absorbance that obtained from UV/VIS spectrophotometer at 510 nm was showed liner points with $R^2 = 0.993$.

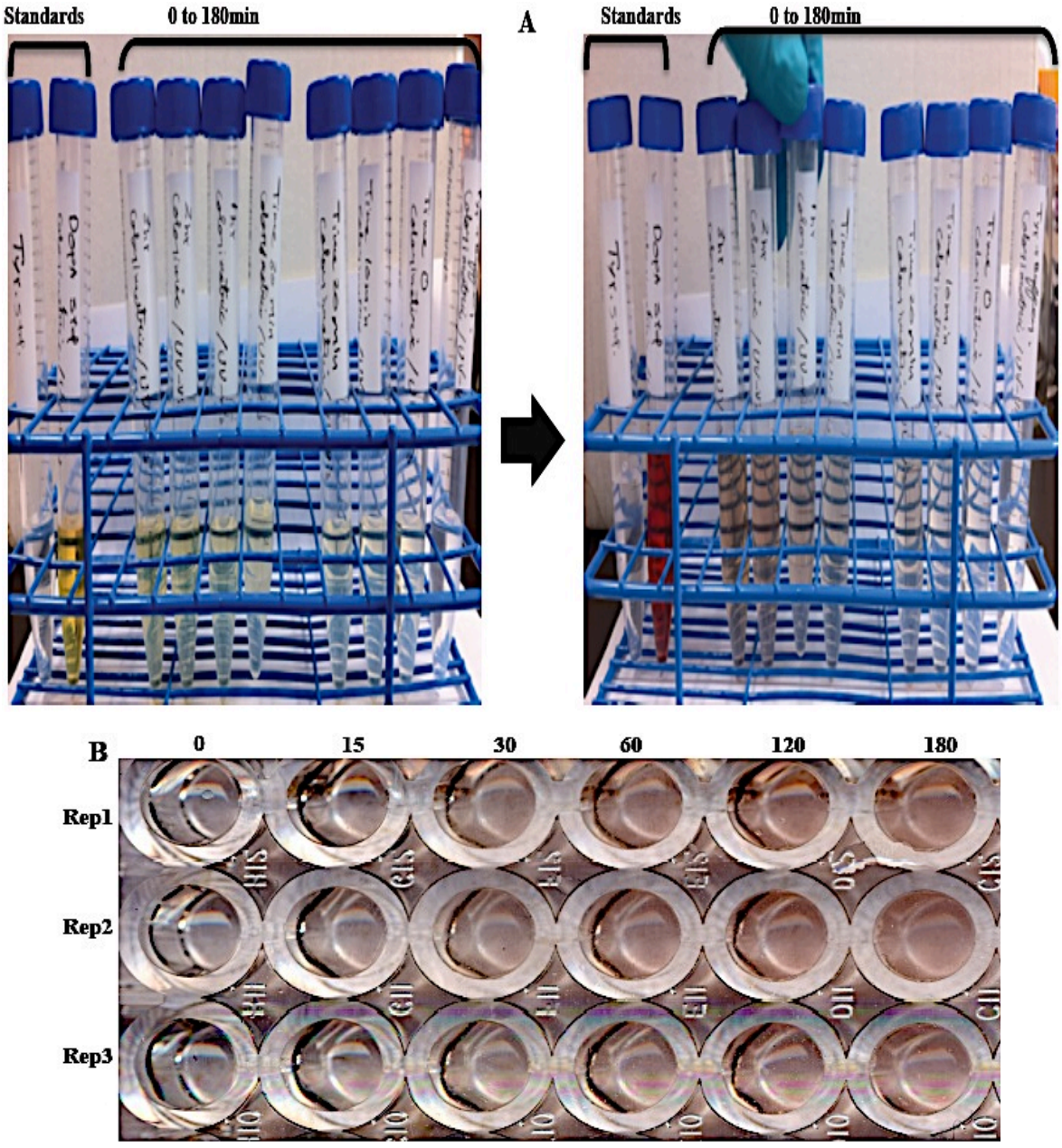


Figure 10: A colorimetric assay for determining of DOPA formation. 2mM tyrosine was dissolved in 60mM acetate buffer (pH 5.0) in the presence of DHFA for

3hr reaction in the absence and existence of HRP in 96 well plates (3 replications each). Samples were collected at various time courses (0min, 15min, 30min, 1hr, 2hr, 3hr) and placed in a test-tube containing acetate buffer to make 1 mM dilution. Then, 0.5 M of HCl and nitrite-molybdate reagent stocks were placed respectively in the same test tube and a yellow color was formed. After which another amount 1 M of NaOH stock was added to each test-tube to form a red color. (A) Tubes that containing Tyr without HRP or/and DHFA were looked clear; no color was formed, while the yellow color was started to form after about 15min in the samples containing the enzyme. These yellow colored samples were turned red after adding the strong base. (B) The same procedure was performed in 96 well dish but the amounts were scaled down to ≥ 100μL for both the presence and absence of the enzyme.

Examining the hydroxylation reaction of tyrosine by DHFA using the colorimetric assay seemed to indicate that DOPA was in fact produced by the reaction in agreement with previous results. However we observed that similar amounts of what was presumably DOPA was produced with or without the addition of HRP enzyme. The final yield of DOPA produced was ≈ 0.018 mg/mL as estimated by reading the absorbance obtained from the reaction from the standard curve.

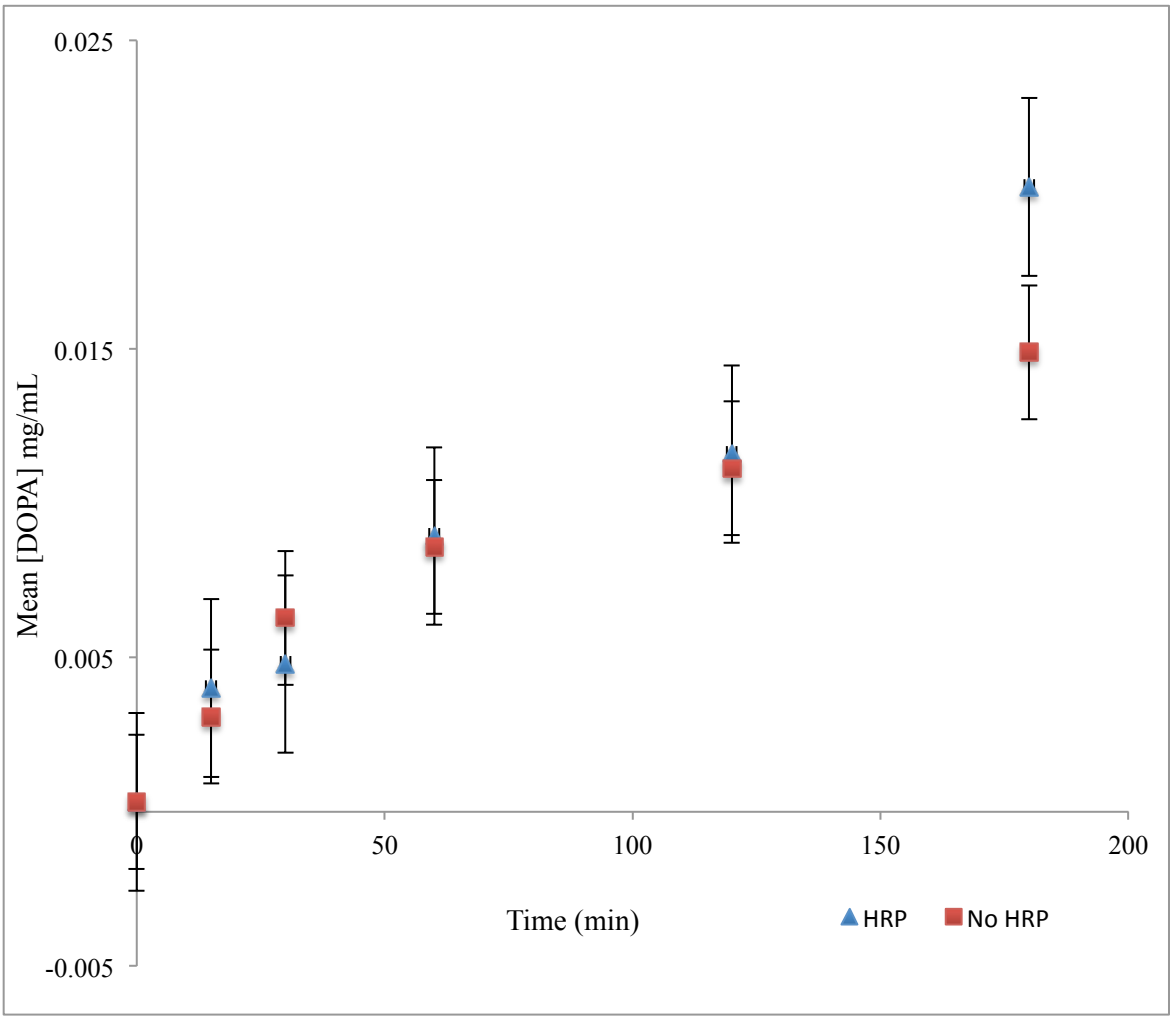


Figure 11: The mean concentrations of DOPA by colorimetric assay in the presence and absence of HRP using visible light (VIS). The absorbance of the samples of both reactions (absence/ presence of HRP) was detected by UV- VIS at 510 nm after blanking the system with buffer containing all the reagents used for this assay but the product. Then the concentrations of DOPA over the time courses were calculated for each replicate from an equation obtained from the standard concentration curve (Y= 9.4033x - 0.003). And thus, the mean of the concentrations of DOPA for both experiments over the time was plotted against time in minutes. A total of 3 sample replicates were performed with and without enzyme at each sampling time.

7. Thin layer chromatography (TLC)

We were unable to confirm with certainty the molecular identity of the given DOPA product detected by the colorimetric product. Since purified standards were available for the substrates and products in this study, the TLC method was used to analyze the samples of the hydroxylation reaction of tyrosine along side molecular standards. This TLC technique was used to separate compounds simply and without using complicated equipment. All samples were spotted at different time courses (0, 10min, 20min, 30min, 1hr, 2hr & 3hr). Tyr and DOPA standards were dissolved using acetate buffer and spotted on bottom of the silica gel plates. Thus in this method, the separations of samples are dependent on the hydrophobicity of the compounds. Since silica gel plates are polar, less polar compounds (Tyrosine) will migrate faster than the more polar (DOPA), which was retained in the plate. In preliminary experiments, the reactions were quenched with acid and were left in tubes with ice for 3-hr reaction prior to spotting that resulted in the detection of the DOPA product even in the time 0 samples (Figure 12).

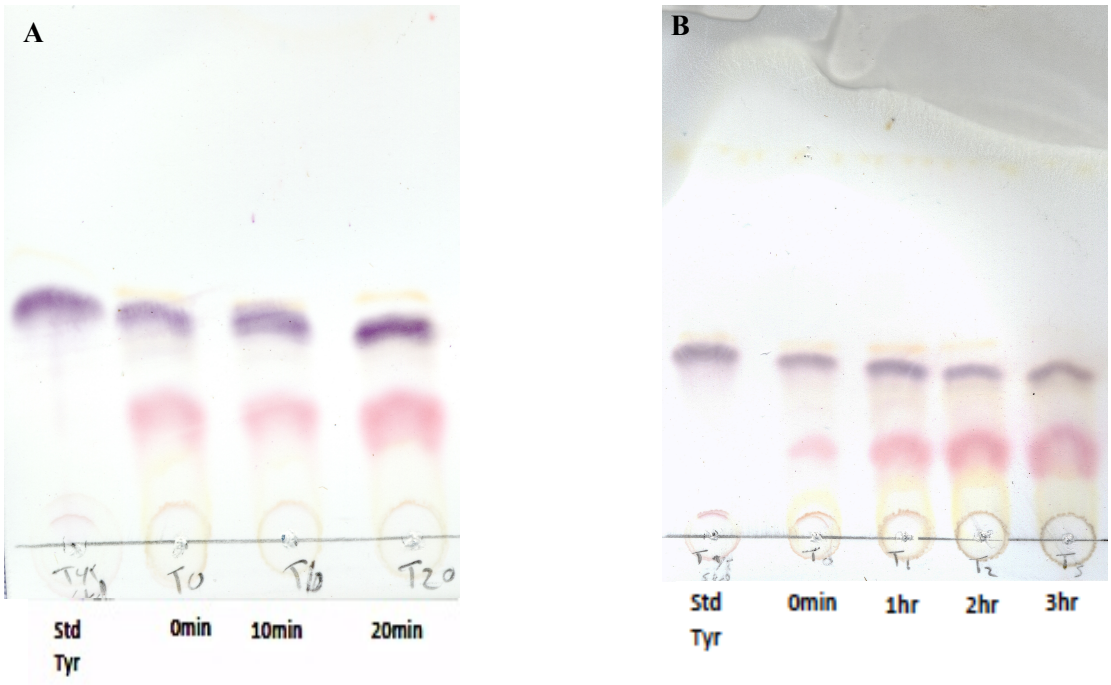


Figure 12: TLC plates of the reaction in the absence of HRP with acidification. The reaction was performed in 96 well dish containing 2mM Tyrosine and

4mM DHFA added hourly for 3hr at 0^0 C. The samples at different time were quenched with 1μL formic acid prior spotting on the labeled plates using a glass pipet. The plates were placed in a container containing 75% phenol. These plates were then stained with ninhydrin (A,B) and DOPA was formed at time 0-3hr from hydroxylation reaction as comparing to the purple color of starting material of only tyrosine standards.

Subsequently the protocol was modified such that the samples were spotted onto the plate at the time of sampling indicated. When the samples were directly spotted a product that showed greater retention on the plate and a color similar to that of DOPA was observed only after prolonged incubation and was not apparent at zero minutes (Figure13).

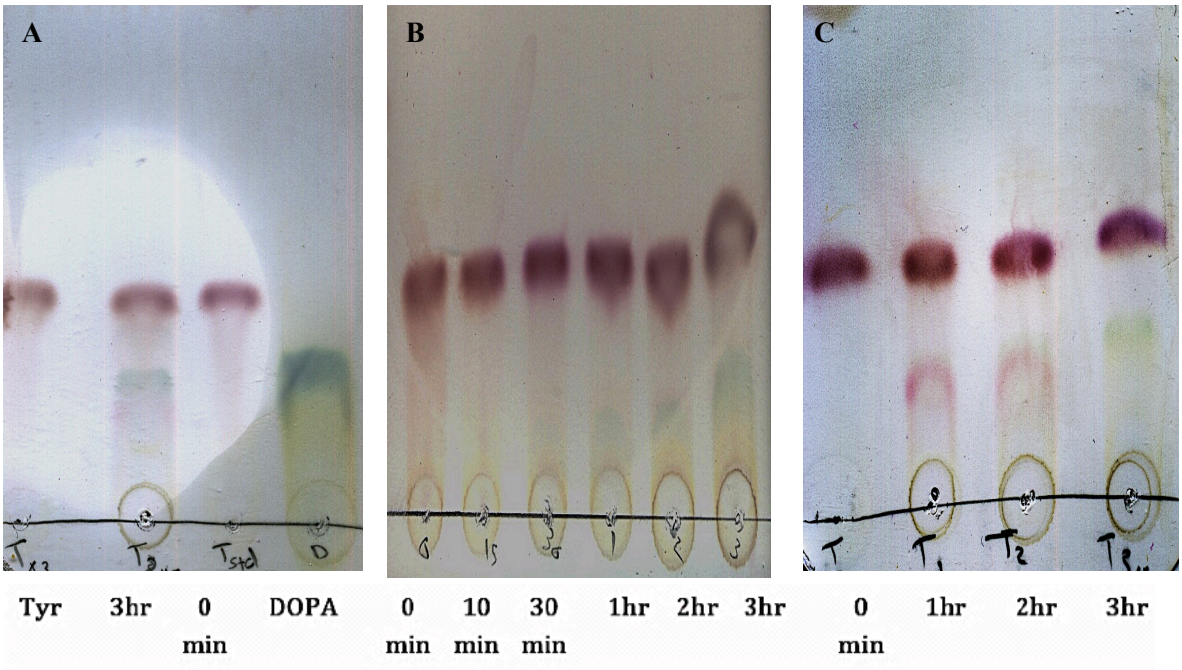


Figure 13: TLC plates of the hydroxylation reaction of Tyr to create DOPA in the presence of HRP. The hydroxylation reactions were prepared in 96 well dish containing 2mM Tyrosine and 4mM DHFA added hourly for 3hr at 0^0C in the presence of 0.5 mg/L of HRP (n=3). The samples were spotted on the labeled plates using a glass pipet directly without quenching the reactions in acid at the times indicated. The

plates were placed in a container containing the mobile phase. These plates were then stained with ninhydrin (A) DOPA was formed (blue) at 3hr samples from hydroxylation reaction as comparing to the starting material (0min) which showing purple color (only Tyr). (B,C) The samples of different time courses of the reaction (0-3hr) were visually compared which show that DOPA was formed gradually over the time.

8. LC-ESI-MS/MS

In order to characterize the product of the reaction of tyrosine with DHFA in the presence of O_2 the reaction products were analyzed by LC-ESI-MS/MS. The use of isocratic HPLC effected the separation of the reaction components is permitted less competition for ionization and thus better detection. The MS/MS analysis permitted the measurement of the parent ion charge to mass ratio and the determination of the fragment mass to charge spectra. Moreover, since a standard curve of DOPA concentration was made by collecting the parent ion at 198 [M+1H] and plotting the intensity of the major fragment at 181 [M+H], it was possible to unambiguously determine the nature and amount of the doubly hydroxylated product of tyrosine produced in the reaction. LC was used to allow better separation and purification of the mixture of the chemical reactions that eluted according to their hydrophobicity, which also increased in sensitivity and selectivity. After that, the mean intensity of the three trials was determined, and thus standard deviation was calculated. The mean intensity was read against the known concentrations of DOPA standard.

A similar amount of a doubly hydroxylated species with a parent ion of 198 [M+H] and major fragment ions at 181 [M+H] and 151[M+H] are consistent with L-DOPA. Moreover the doubly hydroxylated products were observed whether the HRP enzyme was added to the reaction or not. Thus in agreement with the colorimetric reaction, HRP did not seem to be required for the reaction to proceed and in agreement with TLC the reaction product was apparently DOPA.

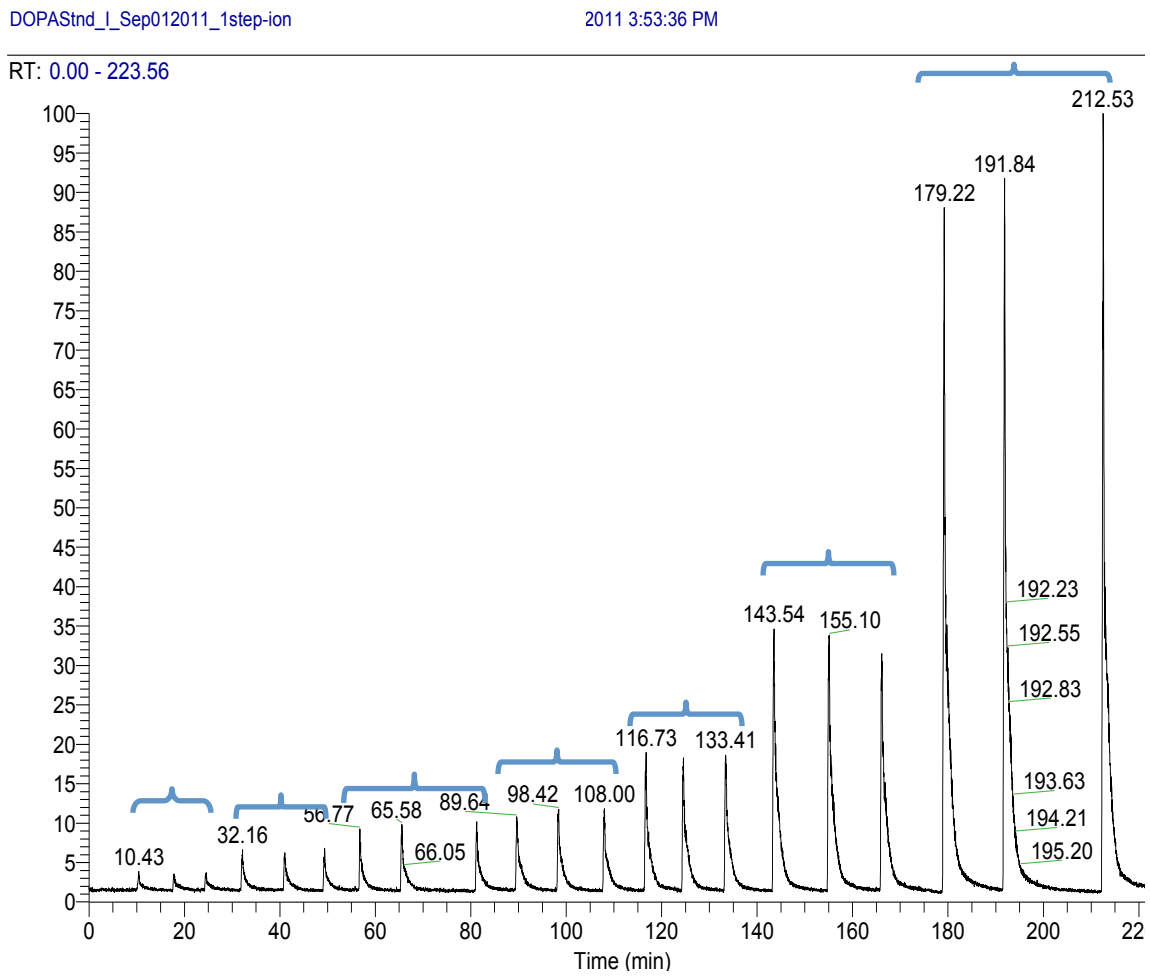


Figure 14: Total ion current (TIC) of DOPA standard. Different concentrations of L-DOPA standard (0.0001, 0.25, 0.5, 0.75, 1, 2.5, 5 mg/mL) were diluted in 5% formic acid according to Table 1 before to isocratic RP C18 LC-ESI-MS/MS at 10 µL/min using a 0.3mm ID x 15cm column to make the standard curve of DOPA. These samples were eluted with 80/20/0.05 of H_2O/ACN/FA (HPLC grade). Each mixture of these concentrations was injected 3 times 5-15min apart depending on the peak size and how fast it get back to the baseline.

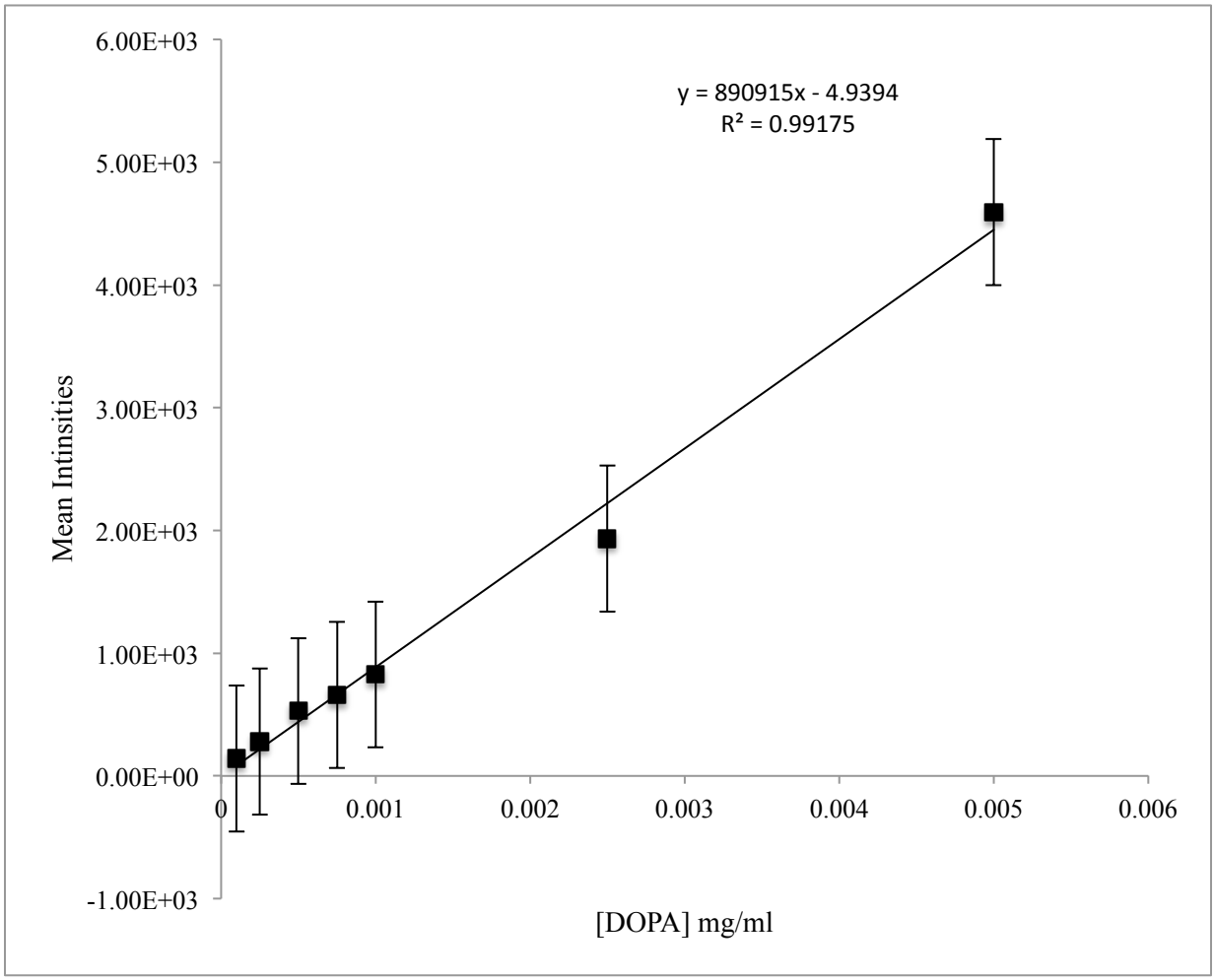


Figure 15: The standard curve of L-DOPA by LC-ESI-MS/MS. The intensities of three technical replicates of DOPA standard were obtained by liquid chromatography MS/MS. Then the mean of DOPA intensities was calculated and plotted against the known concentrations in (mg/ml) of DOPA, which are illustrated very good linear. After that, the concentrations of the products formed in the reactions can be determined by applying Y = 890915x - 4.9394 that obtained from the graph and then multiply the value by 100 to calculate the actual concentrations.

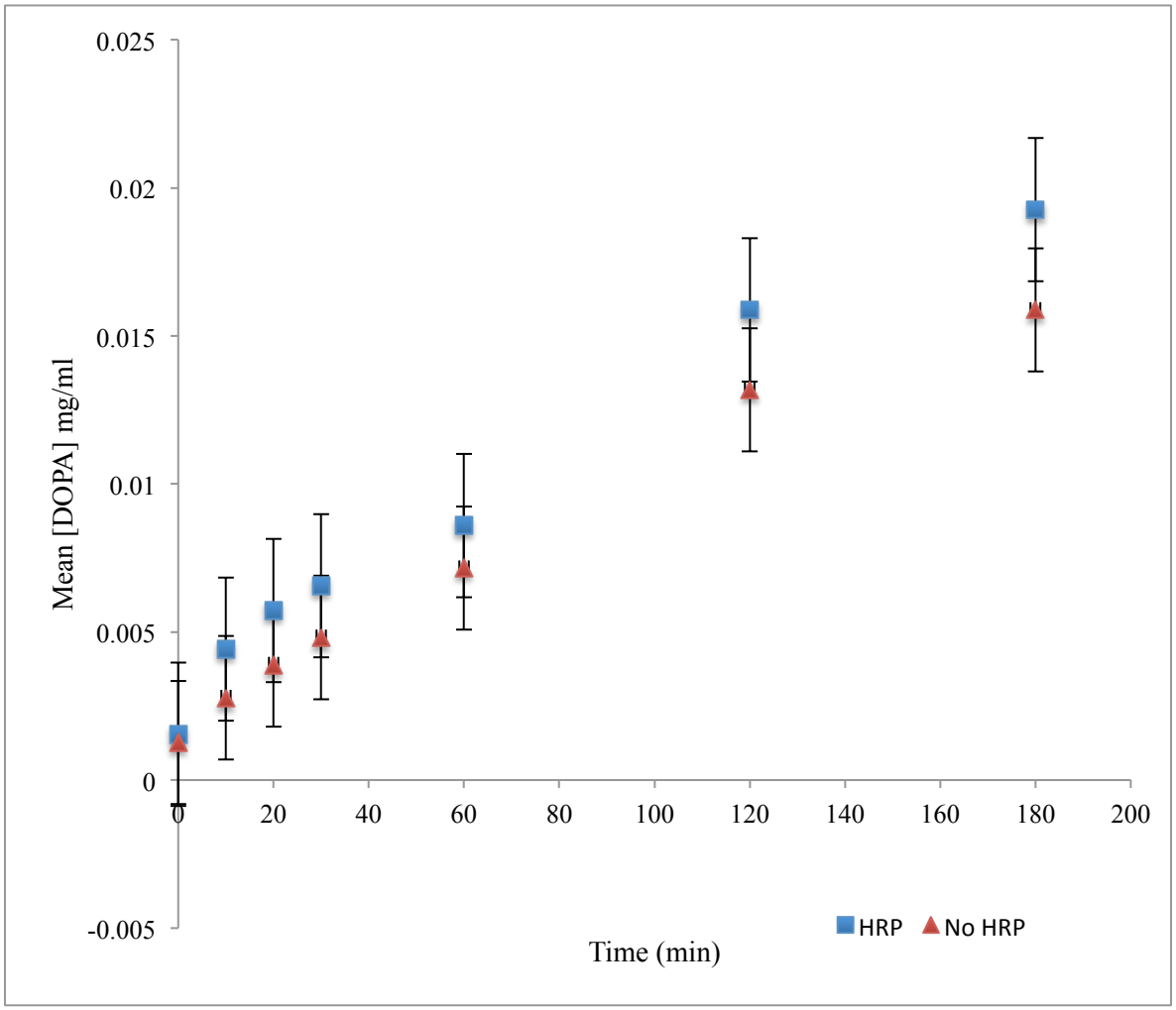


Figure 16: 181 intensities comparison of the parent in the presence/ absence of HRP. The reactions were also performed in 96 well plate including 2mM of Tyr and 4mM DHFA (added each hour for 3hr) in 60mM acetate buffer (pH 5.0). The mean concentration of 181 fragments of both reactions in the absence and presence of HRP (3 replications each) over time (0-180 min) was multiply by 100 (since we diluted the samples 1 in 100) were plotted against time in minutes that show similarity in concentrations of DOPA with time.

9. Reactions in the absence of HRP or Iron Catalyst

It has been suggested that the iron-containing enzyme, HRP, while sufficient to catalyze the formation of DOPA, is not required and in fact Fe^{2+} or Fe^{3+} ions can directly act as a catalyst *(Durliat, Courteix, et al., 1992)*. Alternatively it is be hypothesized that H^{+} ions from the media in the presence of DHFA and oxygen create the conditions for a chemical reaction that has the effect of hydroxylating tyrosine. Such a hypothesis would be consistent with the detection of time 0 incubation samples that were quenched with acid prior to spotting and yet still should the strong production of DOPA.

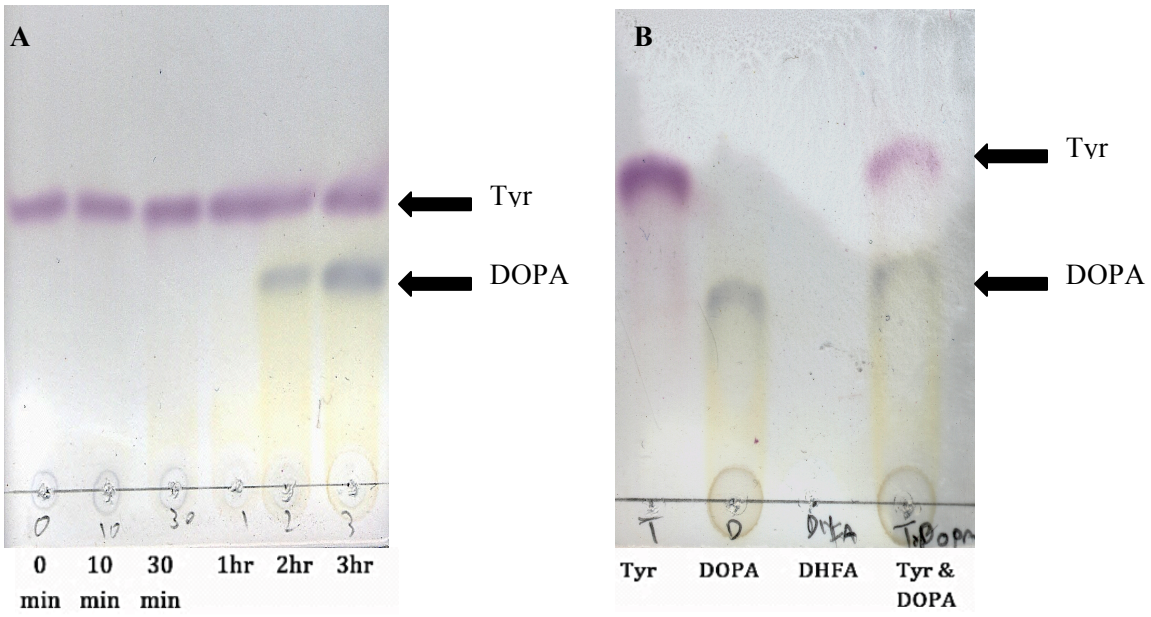


Figure 17: Formation of DOPA in the absence of the enzyme. The hydroxylation of tyrosine (2 mM) was performed at 0°C in the presence of DHFA (4mM) in HCL without HRP in a 96 well plates. The samples at various time courses were spotted several times on plates by a glass pipet (without quenching the reactions). The plates were placed in a container containing 75% phenol in H_2O. Plates were then stained with ninhydrin. (A) Samples of different time courses of the hydroxylation reaction in pH 2 (10 mM HCl) were spotted directly at various time courses (0, 10 min, 30 min, 1hr, 2hr & 3hr). In addition, the intensity and size of the dots is relatively proportional to the number of

times the samples were spotted. (B) Tyrosine and DOPA standards were used as control however DHFA in the same buffer was shown no effect by TLC.

Thus, it seems unlikely that the DOPA product formed was because of the presence of a trace of iron in the HPLC water that used in the experiment. In this regard the reaction was performed in 10 mM HCl (pH 2) or 1 mM HCl (pH 3) in HPLC grade water where the iron content is known to be less than 0.1 ppm and in the presence 2 mM EDTA, an effective iron chelating compound. It was observed that the reaction proceeded in the presence of HCl alone and that EDTA did not prevent the reaction. Hence, the reaction proceeded efficiently in acid and in the absence of any added catalyst.

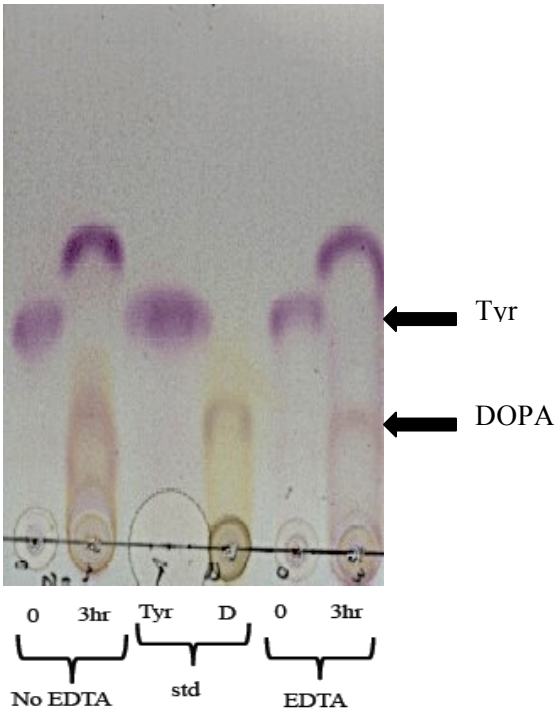


Figure 18: The reaction in the absence of expected $Fe2^{+}$ in the H_2O. The reactions of 2mM Tyr (3x rep) were applied in 1mM of HCl (pH3) in the absence of HRP in 1 mL test tubes. 2 mM of EDTA was placed in tubes initially before adding the DHFA. No products were formed at 0 min in the absence and presence of EDTA, while after 3 hr some DOPA products were formed in both reactions.

DISCUSSION

The selective hydroxylation of aromatic compounds in preparative organic chemistry has remained a challenging and unfavorable reaction *(Norman, & Taylor, 1965)*. The production and testing of hydroxylated products such as L-DOPA may have significant industrial and biomedical applications. In this study we have systematically examined the factors that contribute the successful hydroxylation of a model phenolic amine species and the satisfactory analysis of the resulting biochemical.

1. Reagents

The empirical comparison of reagents in terms of their ionization and detection by mass spectrometry indicated that many of the potential hydroxylation substrates and products failed to ionize efficiently. A subset of compound including L-tyrosine (mass 182Da); 3,4-Dihydroxy-L-phenylalanine (L-DOPA), L-phenylalanine (165Da); Hydroquinine (DHQ) and Tetramethylbenzidine (TMB) were all found to show sensitive detection. We concluded that tyrosine and DOPA were the best suited since both the substrate and product were both available standards and that this might prove to a significant advantage in the experiments.

2. Analytical Methods

The selection of tyrosine to DOPA was also attractive since there was a range of analytical options. A number of different analytical methods including auto absorption by UV/VIS, colorimetric assays, TLC and LC-ESI-MS/MS were compared that each showed advantages and disadvantages.

2.1. Full Scale versus 96 well dish scale reactions

Some analytical methods such as scanning UV/VIS spectrometry require quartz cuvettes and therefor are commonly preformed on analytical spectrophotometer with a 1 cm optical path and reference beam for analytical precision. However other techniques such as colorimetric reaction and LC-ESI-MS/MS only require small amounts of sample and so can utilize a 100µl reaction volume in a 96 well dish as the sample source. We found that little product was formed in the absence of bubbling oxygen in the 400 ml flask reaction or without an oxygen atmosphere in the 96 well dish reaction where the thin layer of reaction can be supplied with oxygen by diffusion. However both of these experimental systems were essentially in good agreement. The hydroxylation of tyrosine was replicated few times in a 96 well dish and Erlenmeyer flask under optimal reaction conditions to create DOPA. Nevertheless the thin layer reaction in a 96 well dish presented here is likely to find greater application in pharmaceutical or biomedical research based on its economy and convenience.

2.2. UV/VIS

The original and well cited studies that we attempted to reproduce were made in 1L reaction with a quartz cuvette using the venerable UV/VIS system that has been very commonly used over the years in analytical biochemistry *(Dordick, Klibanov, et al. 1986).* However in the present instance we observed that auto spectrophotometry could not easily discern and discriminate between the tyrosine substrate, the DOPA product and the DHFA cofactor. In contrast to previous reports we observed that the absorbance at 280 nm actually decreased, rather than increased, during the course of the hydroxylation reaction. The decline in the absorbance at 280 nm might be interpreted as the reaction of the relatively non-specific reagent DHFA with the acetate buffer. The rapid loss of DHFA from the system via a reaction with acetate buffer would not create a compensating production of DOPA with associated absorbance at 280 nm and might result in the observed decrease in absorbance at 280 nm.

2.3. Colorimetric reaction

The colorimetric reaction of DOPA with Nitrite-Molybdate reagent *(Arnow, 1937a)* was rapid simple and relatively sensitive and was found to be in good agreement with the other successful methods tried. The creation of specific reagent systems that lead to selective color reactions extended the utility of UV/VIS and resulted in robust assay for DOPA. However while such a reagent system has been demonstrated for DOPA there are not specific reagent systems for every product that might require identification and quantification. Moreover, the specificity of the colorimetric reaction is not unambiguously apparent and it remains possible that other doubly hydroxylated species might also react with Arnow's reagent.

2.4. TLC

The reaction of tyrosine to form DOPA could be rapidly visualized using TLC that provided some clues as the molecular identity of the product based on its migration relative to standard and the color of the product after reaction with ninhydrin. The TLC separation technique provided well-defined spots that agreed with previous results (*Guenendi, & Pamuk, 1999).* The spots were showing that the concentration of DOPA was increased also over time. Different mobile phases such as pure Methanol, 1-2% of either HAC or NH_4OH in methanol were examined. Among these solvents, 75% of phenol-water was showed the best separation of these phenolic amine compounds. Moreover quality of the TLC separation seems to be positively influence by the presence of HCl. However the method suffers from the semi quantitative results based on the intensity of the color reaction that varies with amount spotted and the use of heat to help develop the image and so it is somewhat difficult to reproduce.

2.5. LC-ESI-MS/MS

MS analysis consists of ionizing chemical compounds to generate charged molecules and measurement of their mass-to-charge ratios *(m/z) (*Ardrey, (1994). The ion trap MS/MS may be programmed to collect selected target ions and to produce quantify specific fragment ions. It has been well published and cited that with appropriate substrates, cofactors and reaction conditions *(Halliwell, and Ahluwalia, 1976)* that as change of 16 Da in the case of hydroxylation reactions *(Dordick, Klibanov, et al. 1986)*, or even larger modifications in mass for polymerizations *(Korduri, and Tien, 1995),* might be observed. Indeed, the ease of hydroxylation or other modifications depend on the presence of strong reducing agents or cofactors such as hydrogen peroxidase and DHFA. Okuda and others proposed that phenol and hydroxylated phenol or their derivatives; especially amines could enter the gas phase and thus be analyzed by MS *(Okuda, Ohara, et al. 2008).* It might be required to derivatize some small molecules with ionization group to permit the detection. However the LC-ESI-MS/MS method might also be limited by the range of biochemical that effectively ionize. Among the many phenolic amines or similar compounds tested here only several showed excellent ionization. Moreover many of the products ions also might not ionize, for example the polymerization reaction of guaiacol by HRP to create tetraguaiacol did not produce a product that was readily detected by LC-ESI-MS/MS *(Furlong, 2007)*. Despite these drawbacks, the measurements of DOPA productions using LC-ESI-MS/MS demonstrated greater specificity and sensitivity compared to TLC, UV/VIS or colorimetric reaction.

3. The production of DOPA from tyrosine in the absence of HRP

There is universal agreement that the presence of a strong reducing agent such DHFA that works as a hydrogen donor is essential to the hydroxylation reactions of phenolic compounds *(Durliat, Courteix, et al., 1992)*. In addition, Yamazaki & Piette (1963) and others proposed that an oxygen molecule was involved in a number of reactions and considered crucial *(Yamazaki, & Piette, 1963; Mason, Onopryenko, et al., 1957).*

Recently it has been suggested that while the iron (Fe) containing enzyme HRP is sufficient to catalyze the reaction, Fe itself may act as the catalyst *(Durliat, Courteix, et al., 1992)*. Durliat and coworkers hypothesized that hydroxylation occurs via the creation of hydroxyl radicals, which can also be formed by other iron compounds. Thus, they found that hydroxylation reactions in the presence of Fe were the same as that in the presence of HRP (*Durliat, Courteix, et al., 1992).* These observations suggest the reaction proceeds in the absence of any known catalyst, and help unify the observations of these different experiments. Here it was shown that the hydroxylation reactions of the phenolic compound proceeds efficiently in hydrochloric acid at pH consistent with the proposal the H^+ from the medium is required for the progression of the reaction mechanism. In addition, we attempted to chelate Fe with EDTA at low pH (acidic) *(Bugter, & Reichwein, 2005)*, this experiment indicated that the reaction proceeded spontaneously and did not require a catalyst (Fe). We concluded that the reaction is chemical and works independently in the absence of an enzyme.

Table III: Analytical techniques comparison

Techniques	Selectivity	Sensitivity	Accuracy	Cost	Simplicity
UV/VIS	**(--)**	**(--)**	**(+)**	**(+)**	**(++)**
Coluorimetric	**(++)**	**(++)**	**(+)**	**(+)**	**(+++)**
TLC	**(++)**	**(+)**	**(+)**	**(+++)**	**(+++)**
LC-ESI_MS/MS	**(++++)**	**(++++)**	**(++++)**	**(----)**	**(+/-)**

CONCLUSIONS

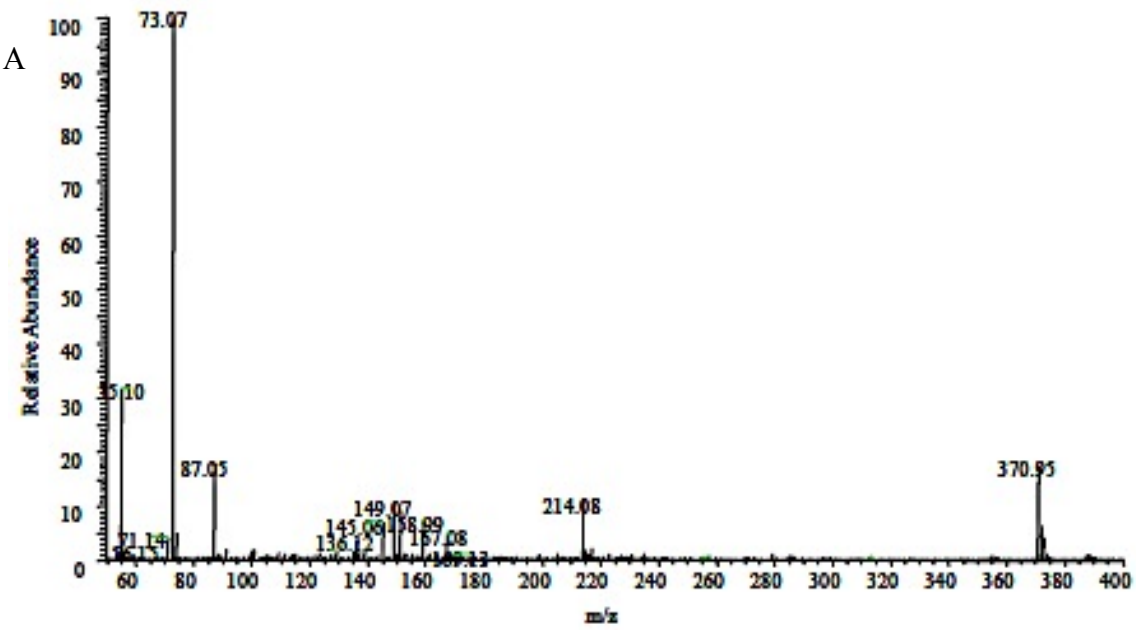
We conclude that in the case of DOPA, or other molecules, where a specific reagent system exists; the coluorimetric reaction is rapid, simple and sensitive enough to detect hydroxylation. Where purified standards and detection reagents are available the TLC system offer many obvious advantages in terms of simplicity. In the absence of specific reagent systems the LC-ESI-MS/MS method has the appeal of great sensitivity and broader, but not unlimited applicability. Regarding the mechanism of the reaction, we have shown that the chemical reaction of tyrosine to create L-DOPA proceeded independently without the need of HRP as detected and verified by three different methods (LC-ESI-MS/MS, TLC and Colorimetric assay using visible light). Additionally to that, many previous publications regarding similar hydroxylation reactions *(Mason, Onopryenko, et al. 1957; Halliwell, and Ahluwalia, 1976; Nordblom, White, et al. 1976; Halliwell, 1977; Dordick, Klibanov, et al. 1986)* incorrectly state that these hydroxylation reactions are HRP-dependent. We attempted to use a thin layer 96 well plate technique in combination with LC-ESI-MS/MS to expand the sensitivity and versatility of investigations in to hydroxylation reactions, a long standing problem in some areas of bioorganic chemistry. The application of derivatization reagents to increase the ionization level of some compounds that are not easily ionized by ESI/MS direct infusion may be required. It is possible that the simple and efficient hydroxylation reaction of tyrosine described herein may be beneficial for investigating the preparative transformations of various pharmaceuticals and fine chemicals.

APPENDIX

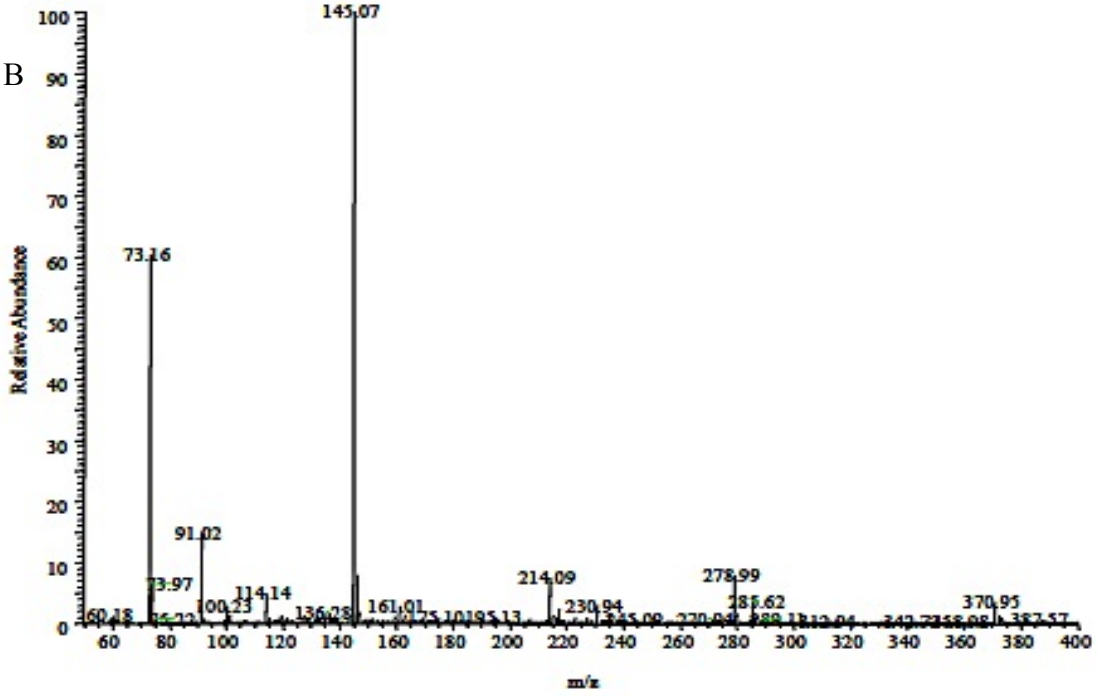
APPENDIX A- Examining the substrates and products by direct infusion by MS.

A) 3 APC, B) Adenine, C) Alanine, D) Alpha naphthol, E) CSPD, F) DMB, G) Guaiacol H) Hydroquinone, I) Indigo (negative mode), J), Luminol, K) Melanine, L) MMT_Farmazone, M) phenol, N) pNPP, O) Proline, P) XGal, Q) P-Coumaric Acid.

A



B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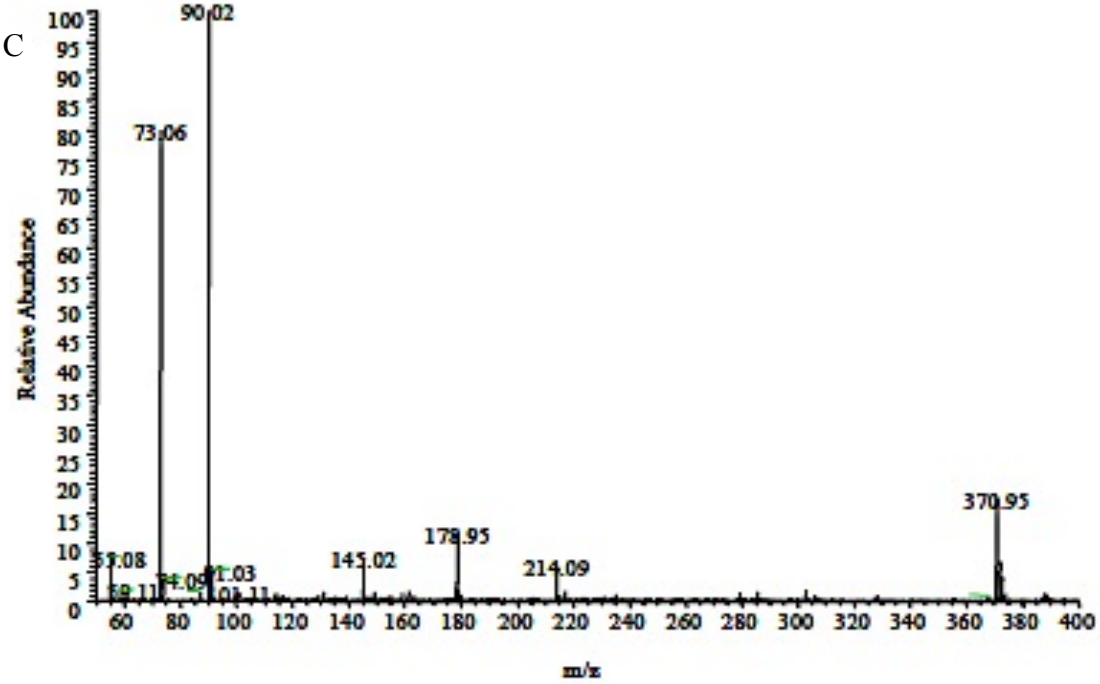
73.16
91.02
73.97
114.14
214.09
278.99
370.95
Relative Abundance
m/z

C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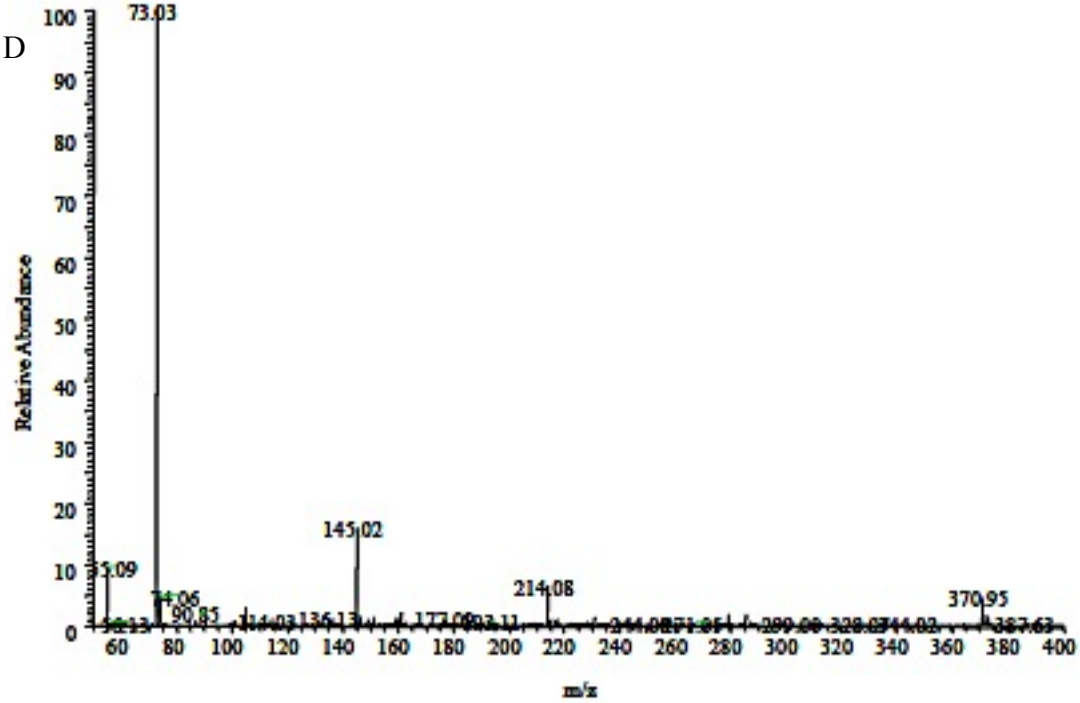
73.06
370.95
178.95
145.02
214.09
Relative Abundance
m/z

D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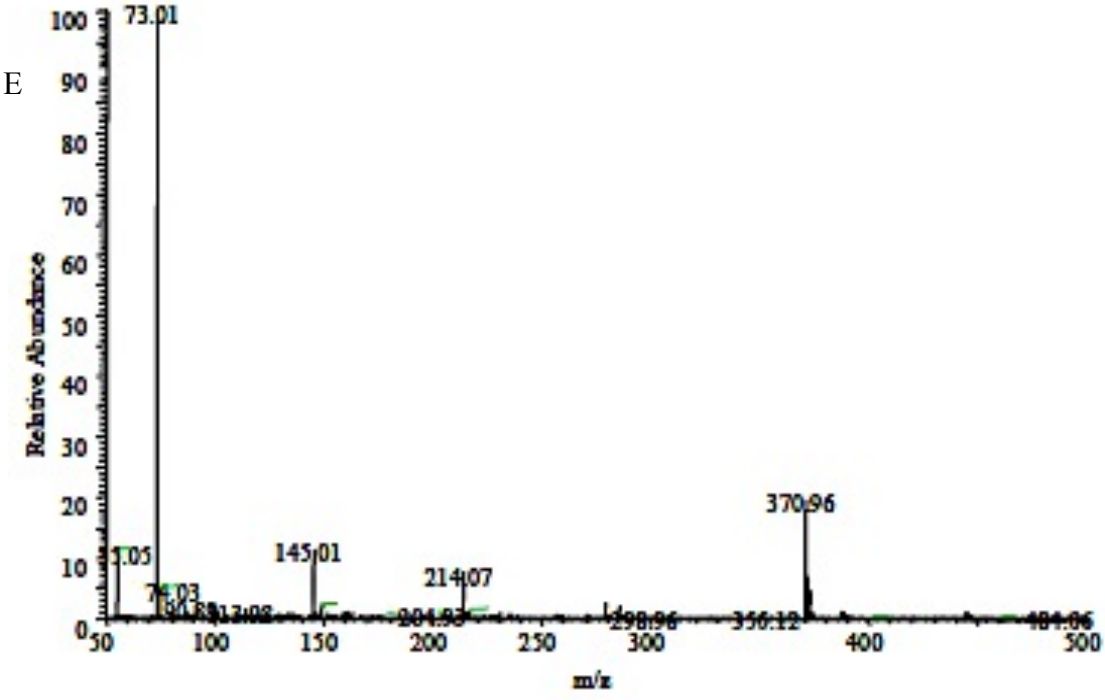
370.95
Relative Abundance
m/z

E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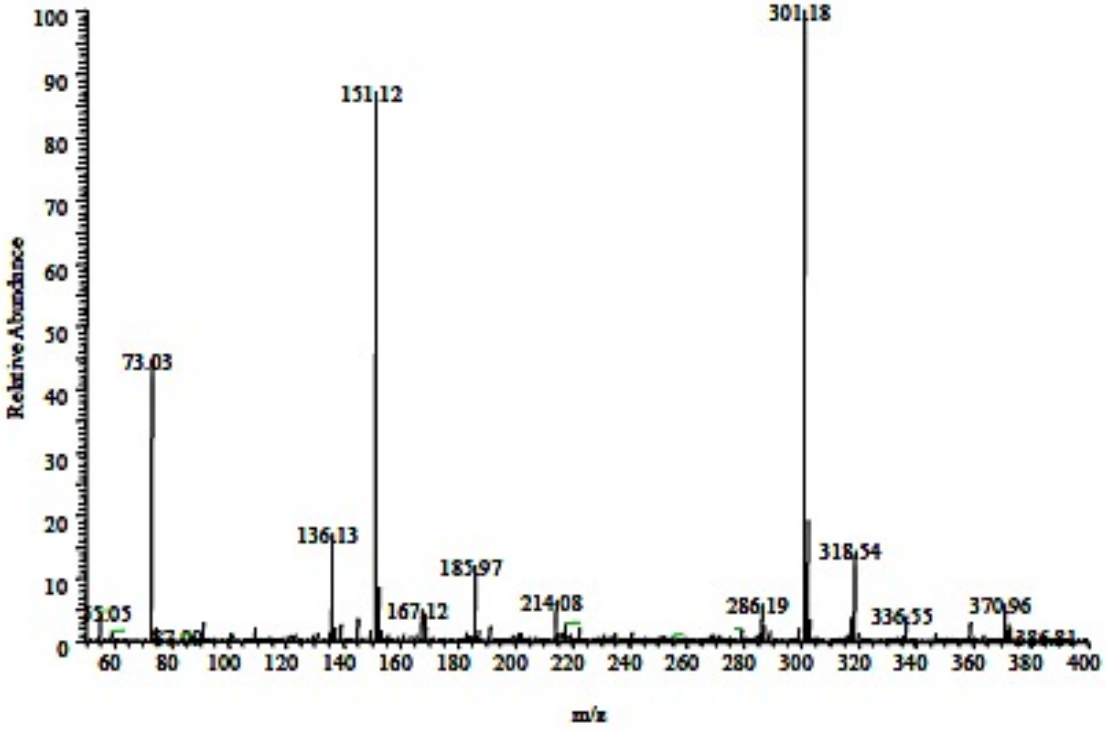
214.07
Relative Abundance
m/z

F


301.18
151.12
73.03
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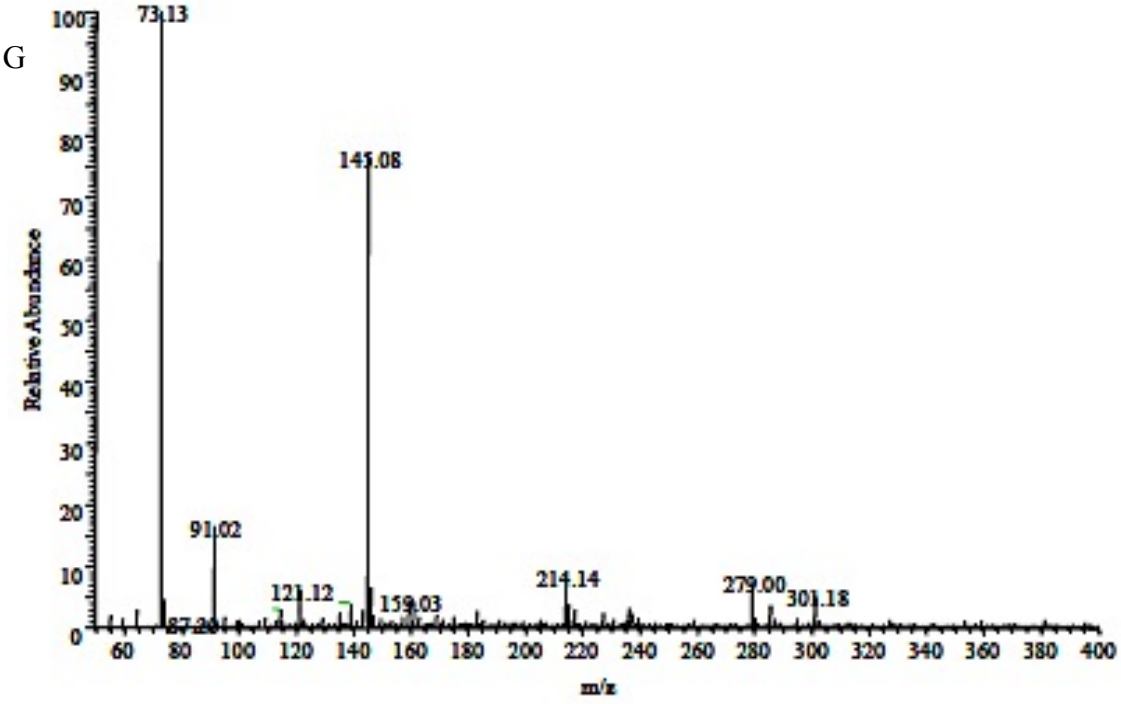
185.97
167.12
214.08
286.19
318.54
336.55
370.96
Relative Abundance
m/z

G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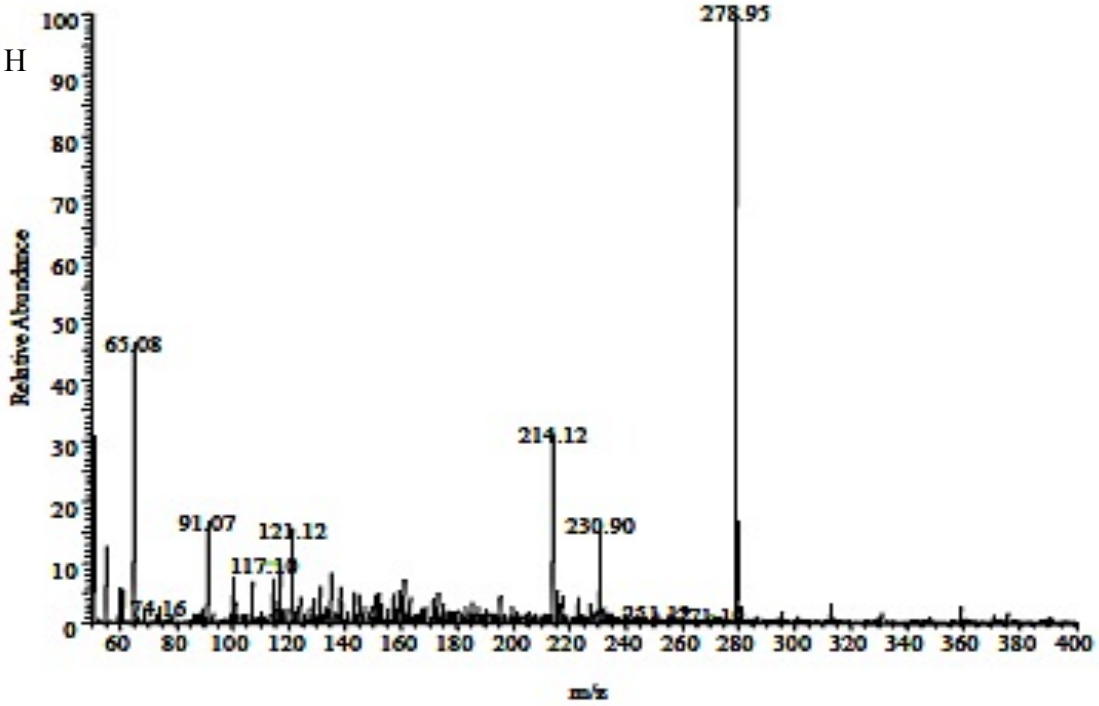
121.12
159.03
214.14
279.00
301.18
Relative Abundance
m/z

H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m/z
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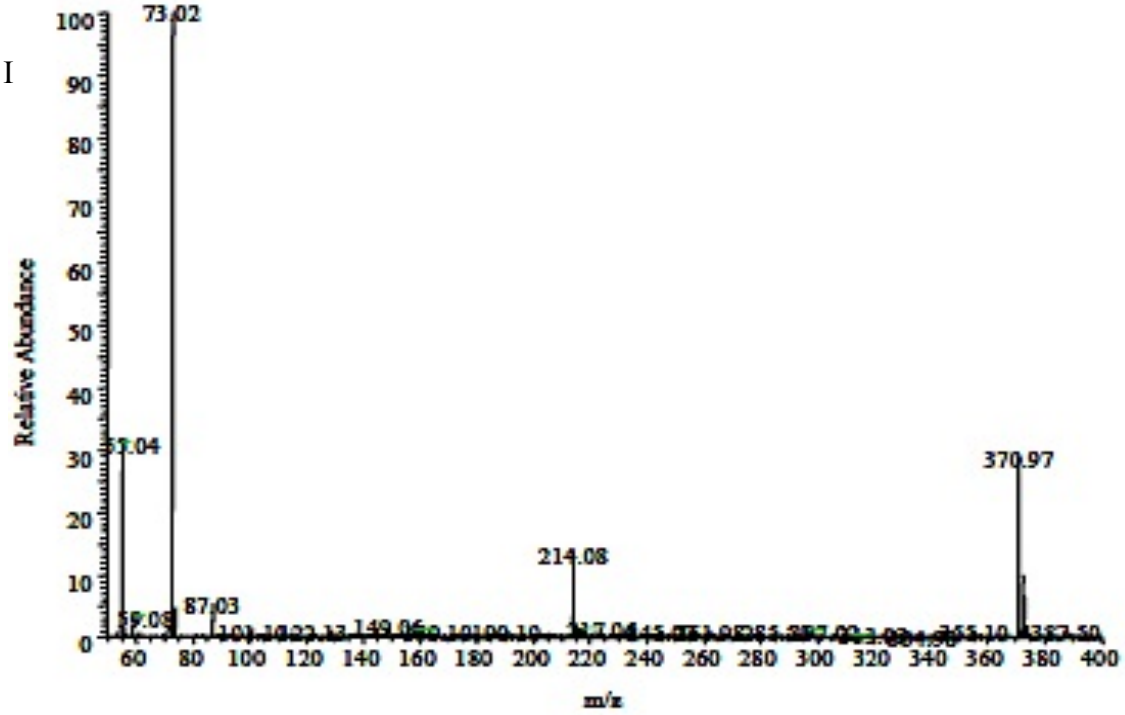
91.07
121.12
230.90
117.10
74.16

I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m/z
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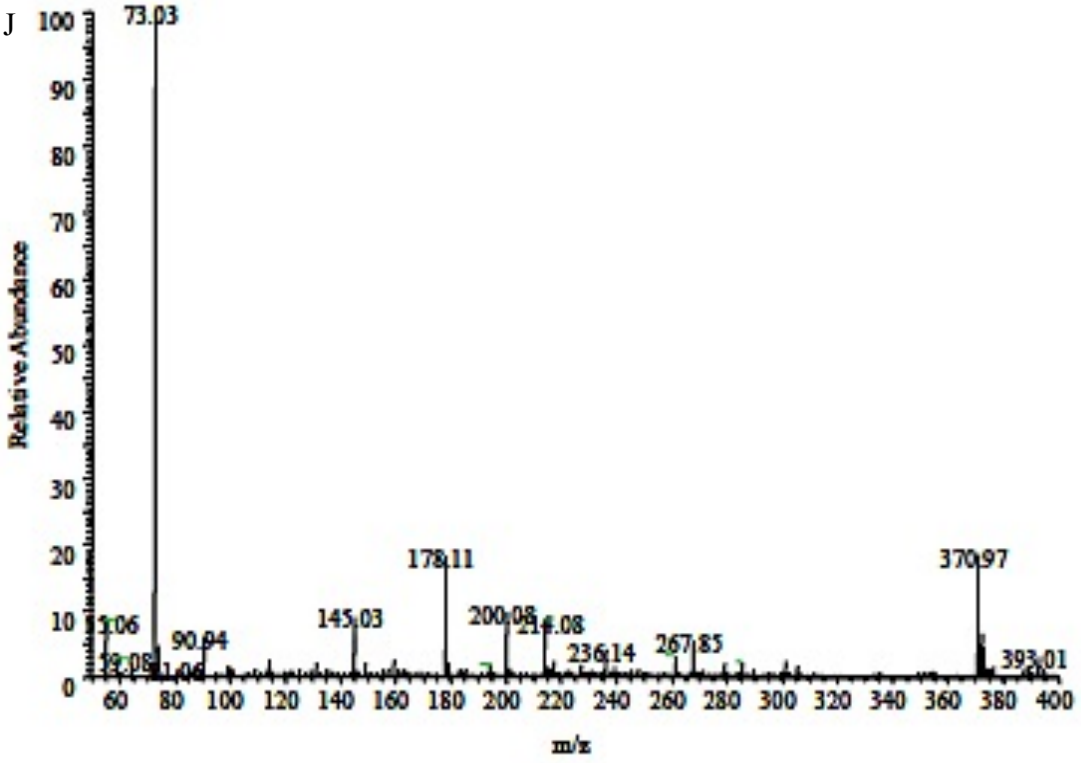
214.08
87.03

J


Relative Abundance
m/z
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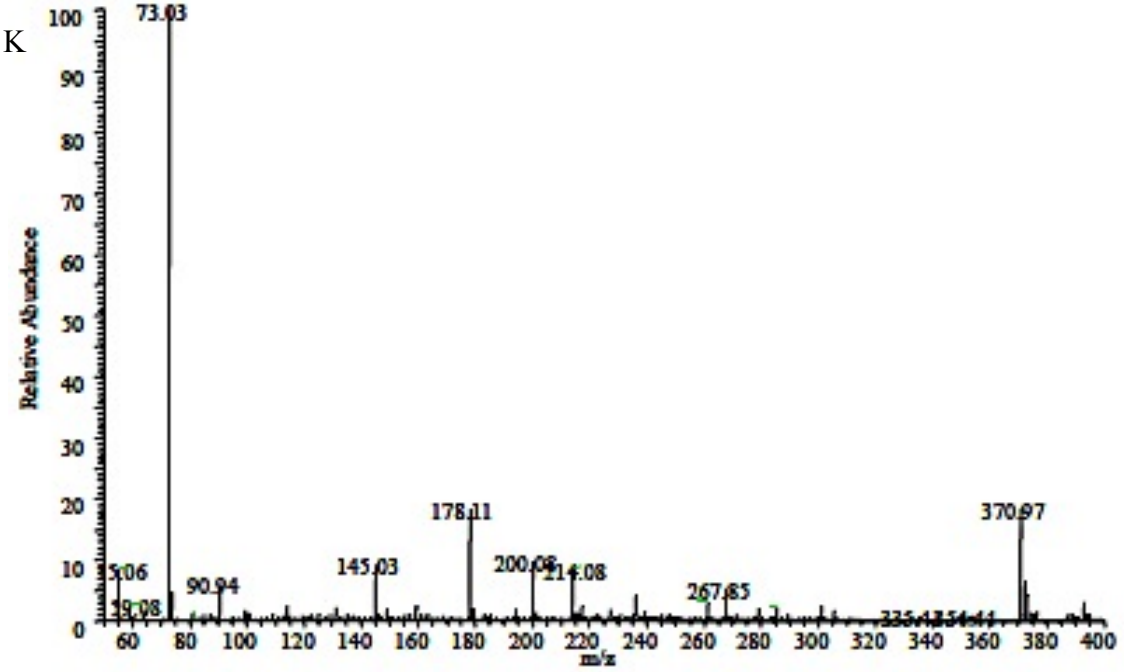
370.97
145.03
200.08
214.08
90.94
236.14
267.85
393.01

K


Relative Abundance
m/z
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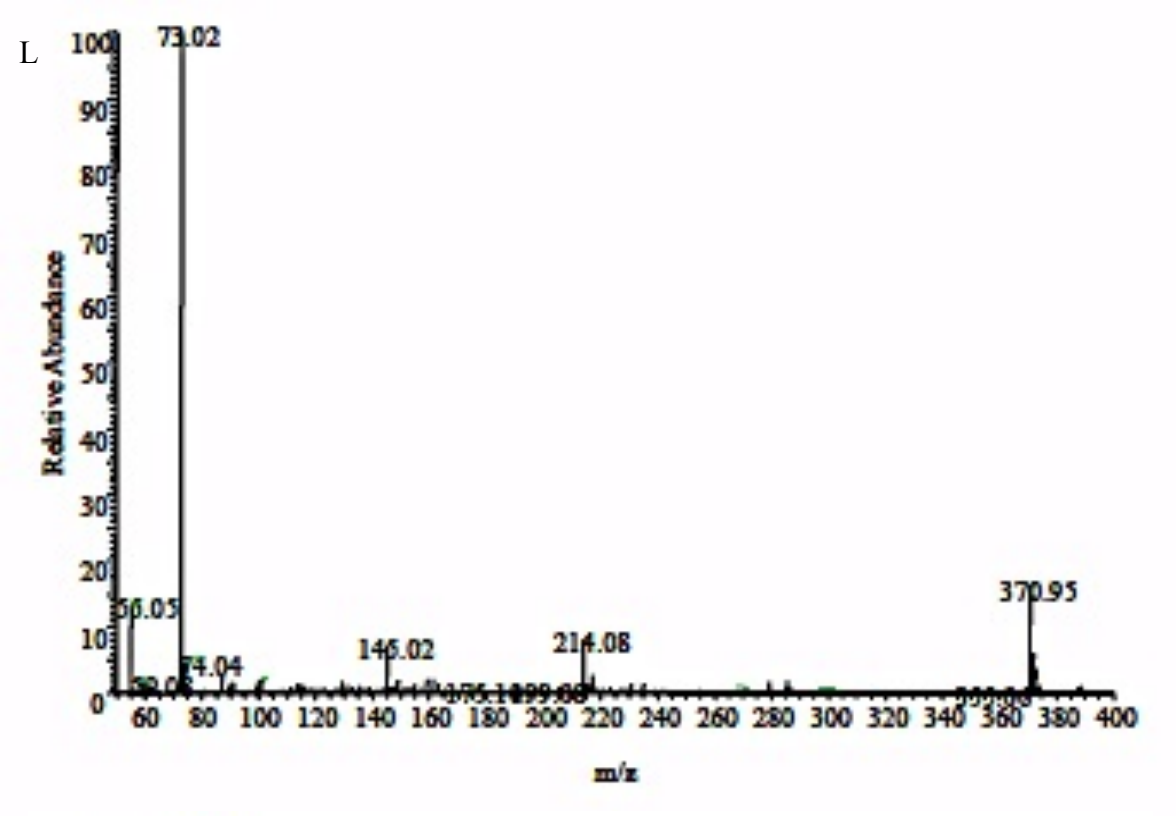
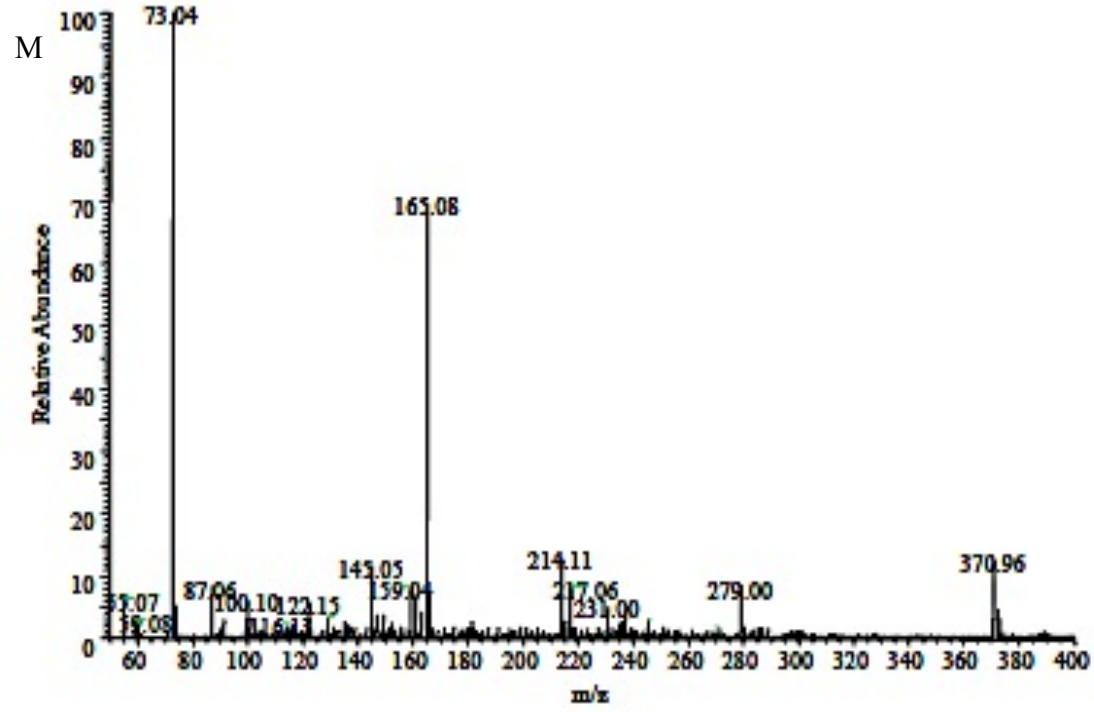
370.97
145.03
200.08
214.08
90.94
267.85

L


73.02
Relative Abundance
56.05
74.04
146.02
214.08
370.95
m/z

M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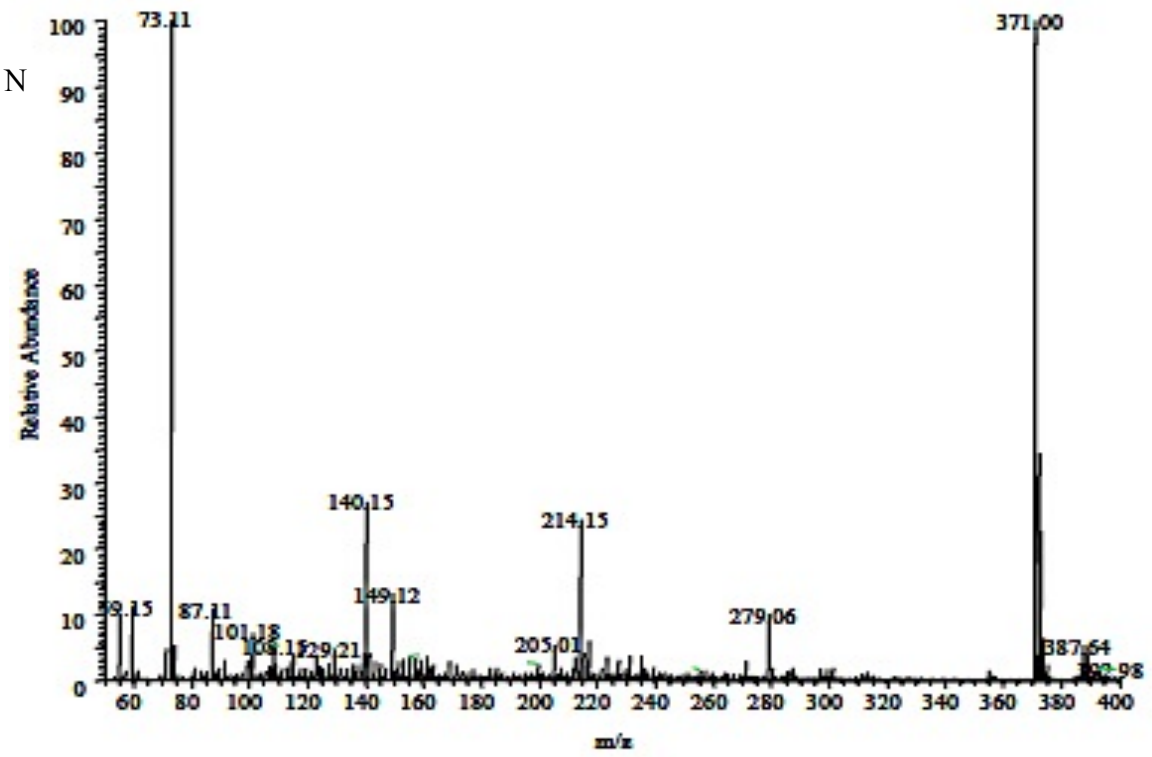
Relative Abundance
145.05
214.11
370.96
87.06
159.04
217.06
279.00
m/z

N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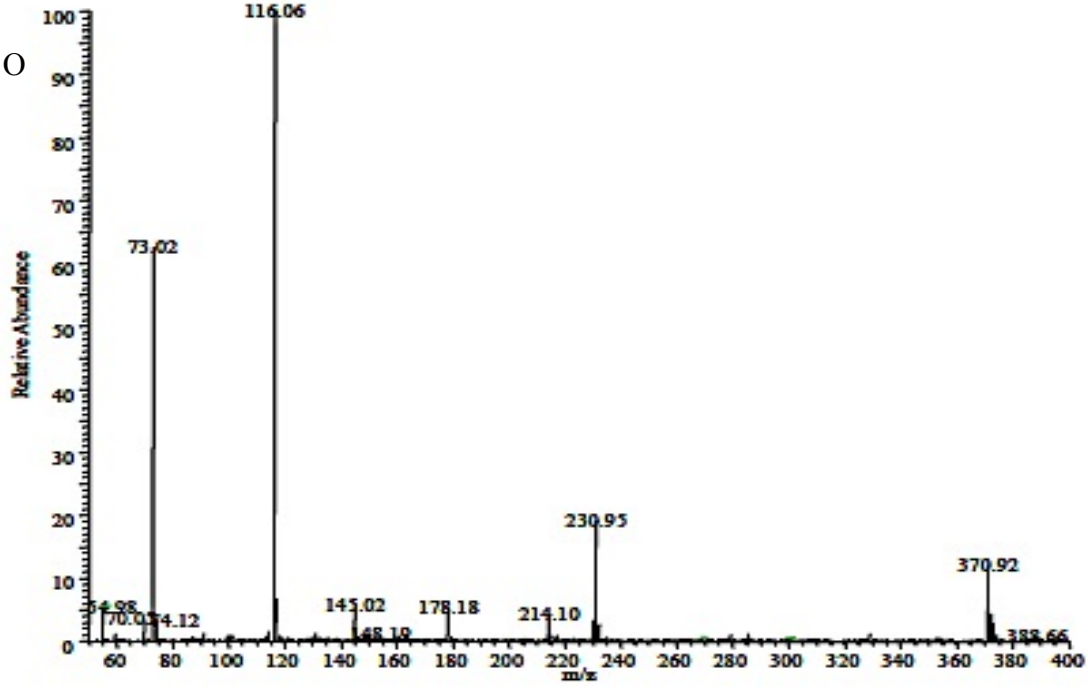
101.18
205.01
387.64
Relative Abundance
m/z

O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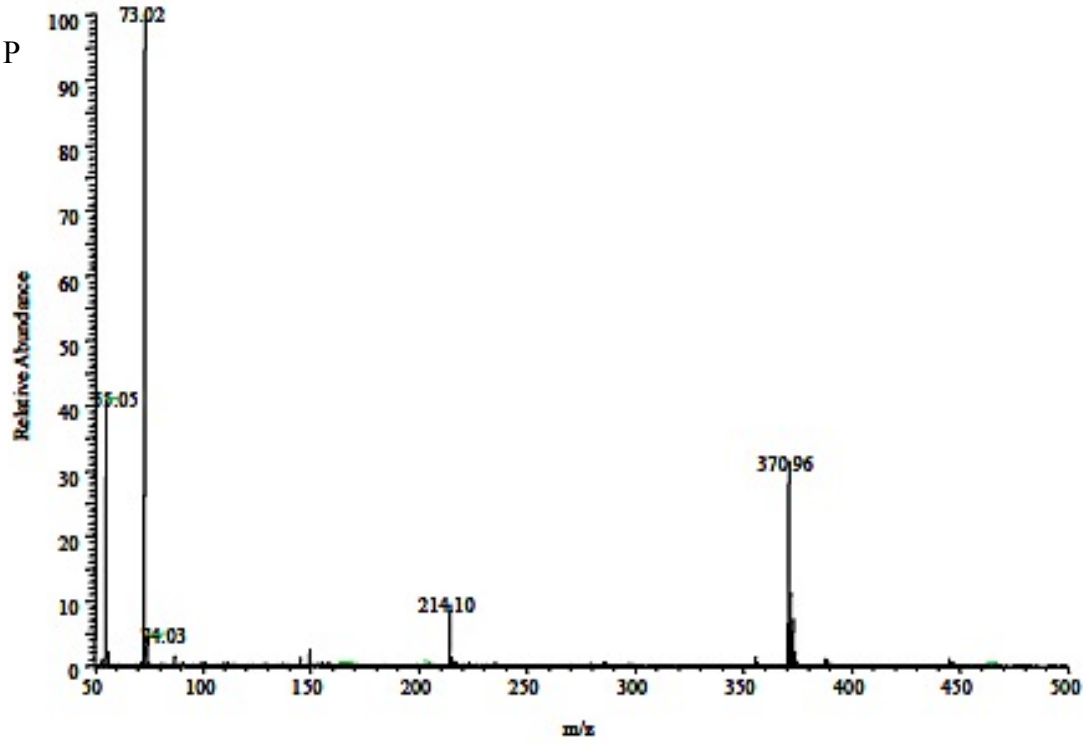
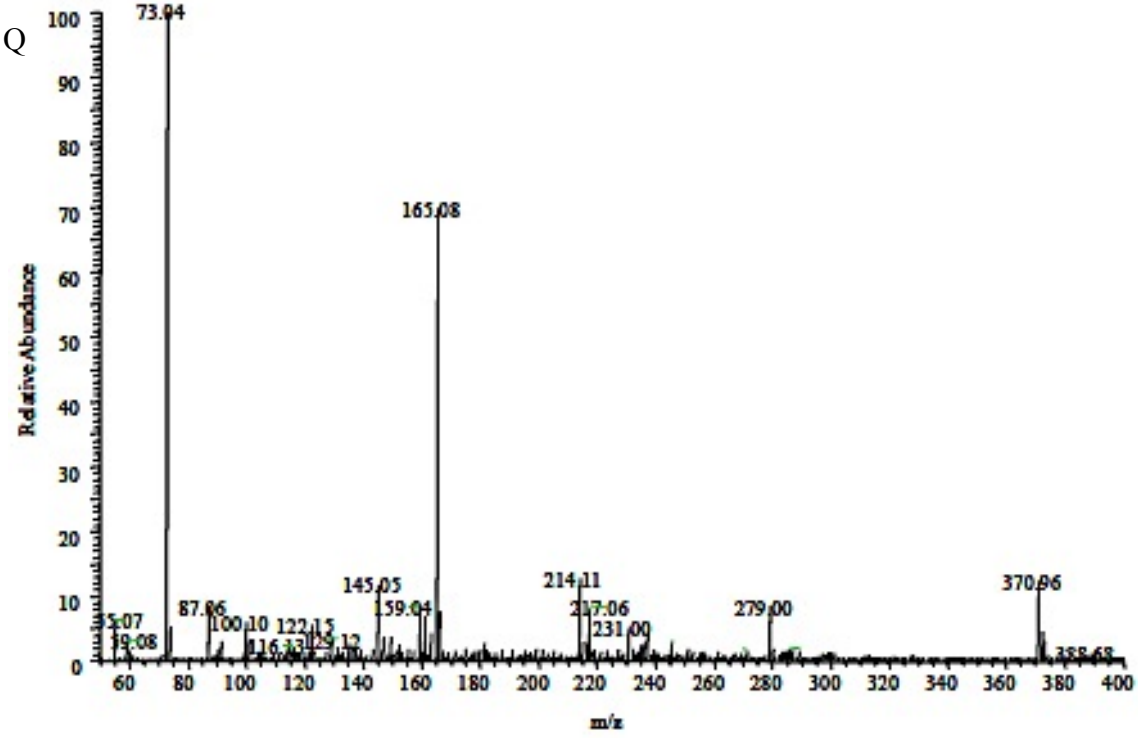
145.02
178.18
214.10
Relative Abundance
m/z

P

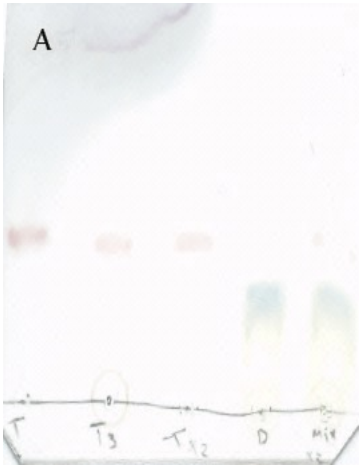

73.02
214.10
74.03
370.96
Relative Abundance
m/z

Q

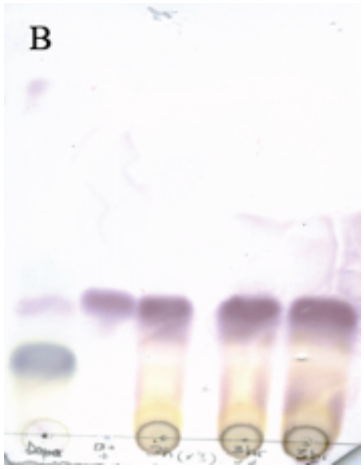

73.04
165.08
145.05
214.11
370.96
279.00
231.00
Relative Abundance
m/z

APPENDIX B- *Factors affecting the TLC plates*

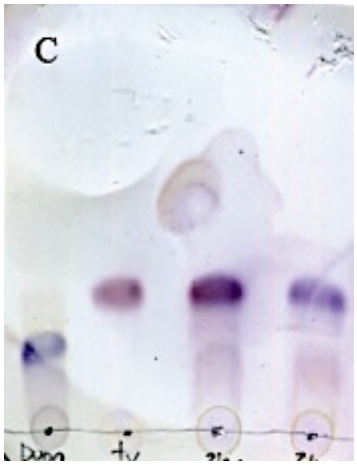
There are many factors were playing a significant role in TLC techniques A) the number of spotting less than 3 times, B) the number of spotting more than 6 times, C) the plate was over heated, D) pure methanol was used as solvent and E) 2% of HAC in Methanol.



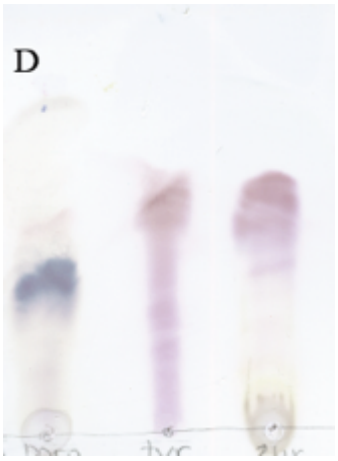
Tyr std | 3hr 2x | 3hr 3x | DOPA std | D&T std



DOPA std | Tyr std | 3hr 6x | 3hr 7x | 3hr 8x



DOPA std | Tyr std | 2hr | 3hr



DOPA std | Tyr std | 3hr

DOPA std | Tyr std | 3hr

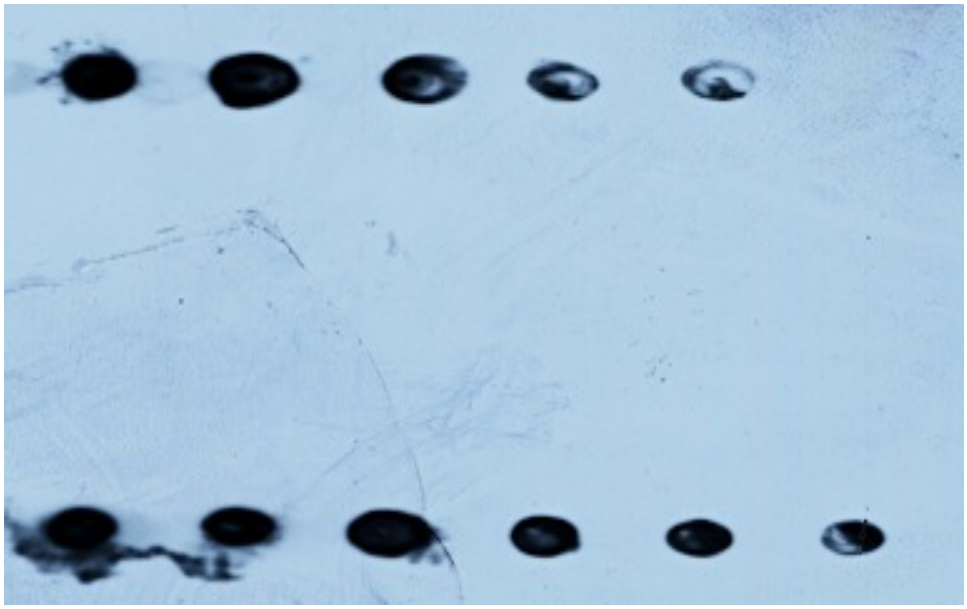
APPENDIX C- *Limit of detections of HRP on Dot Blot*

A small piece of PVDF membrane was labeled and placed on top of the methanol soaked filter paper. 2µL of Diluted samples of HRP type I, and Goat anti Mouse HRP secondary antibody were spotted on top of the marinated PVDF membrane. Meanwhile, the ECL solution was prepared by adding 1ml of Tris/HCl, 9 mL distilled water, 50µL Luminol, and 222µL 4IPBA in this precise order. The ECL solution was then poured on the surface of the membrane, which was then immediately wrapped and placed inside the film cassette. The membrane was then exposed to a piece of film corresponding to the size of the membrane for ~3 min in a dark room. The film was then developed by placing in a developer solution until spots became visible then rinsed with cold water and placed in fixer solution for ~ 2min. The developed film was then allowed to air dry before being scanned. The following dots of HRP and Goat anti Mouse HRP were diluted: (L➔R) 0.010x, 0.0075x, 0.0050x, 0.0025x, 0.0010x, and 0.00010 x.

.

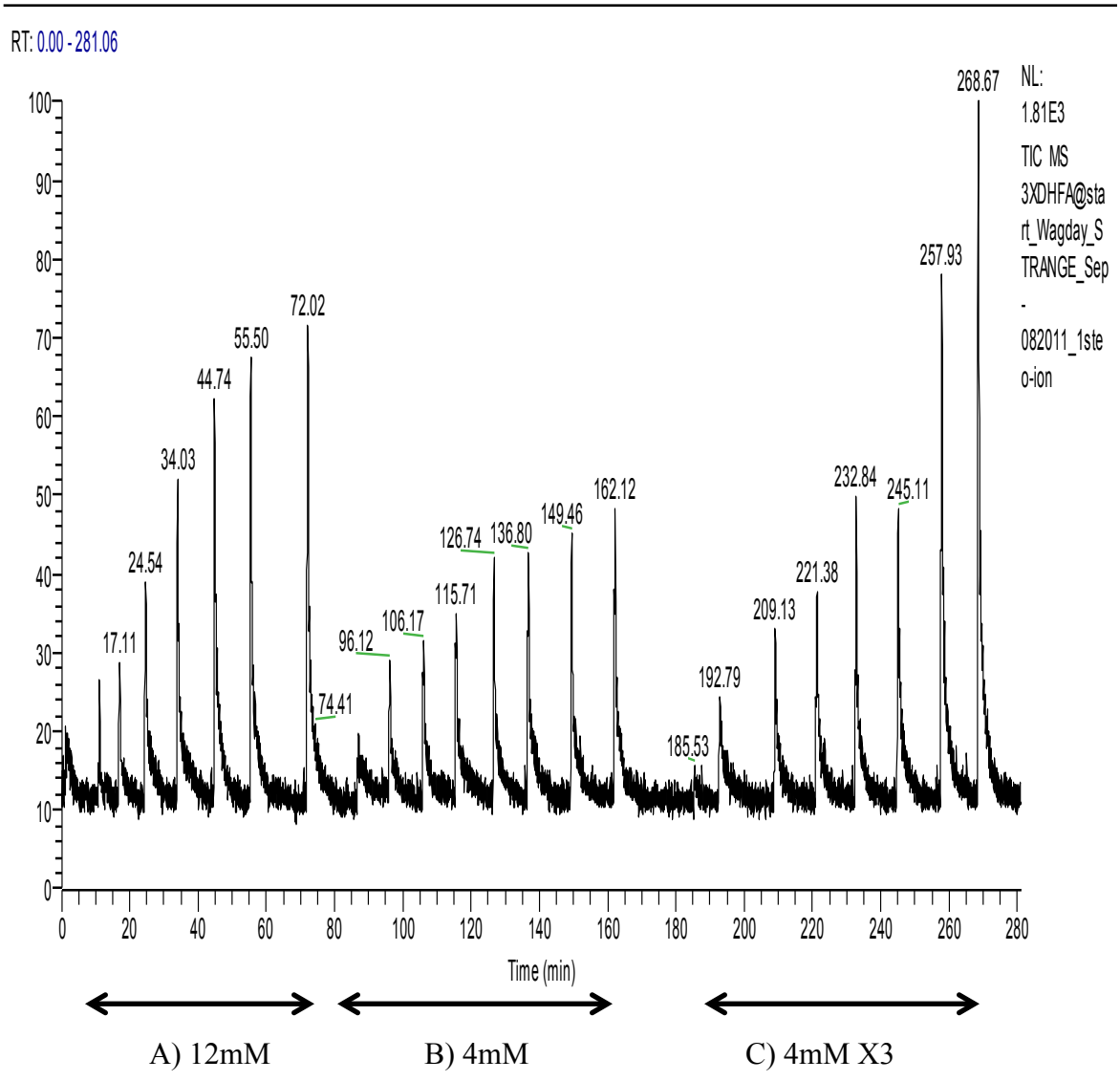
HRP I

Goat anti Mouse HRP



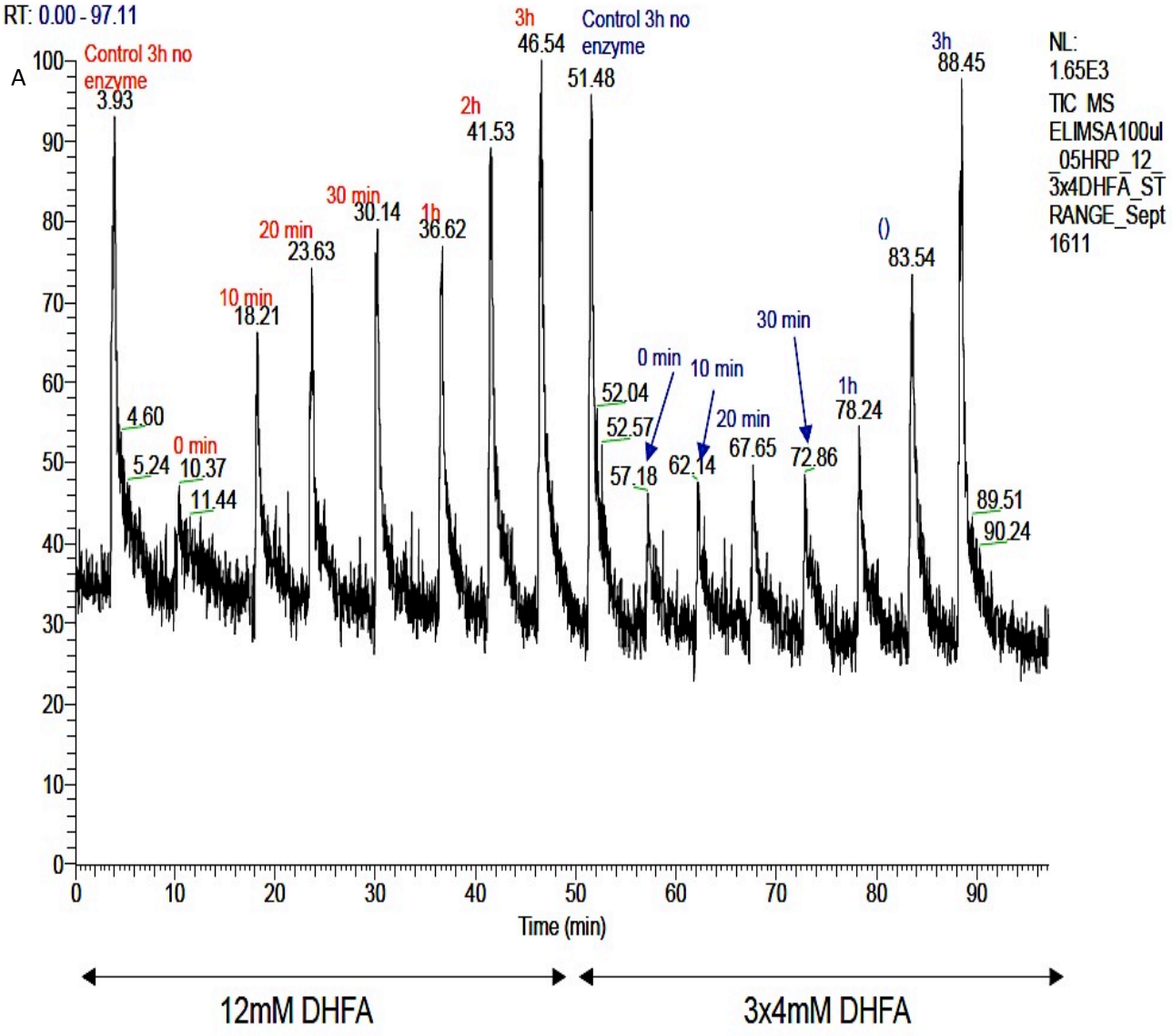
APPENDIX D- Total ion current (TIC) of 181 intensity (a fragment of DOPA) in 400 mL reactions

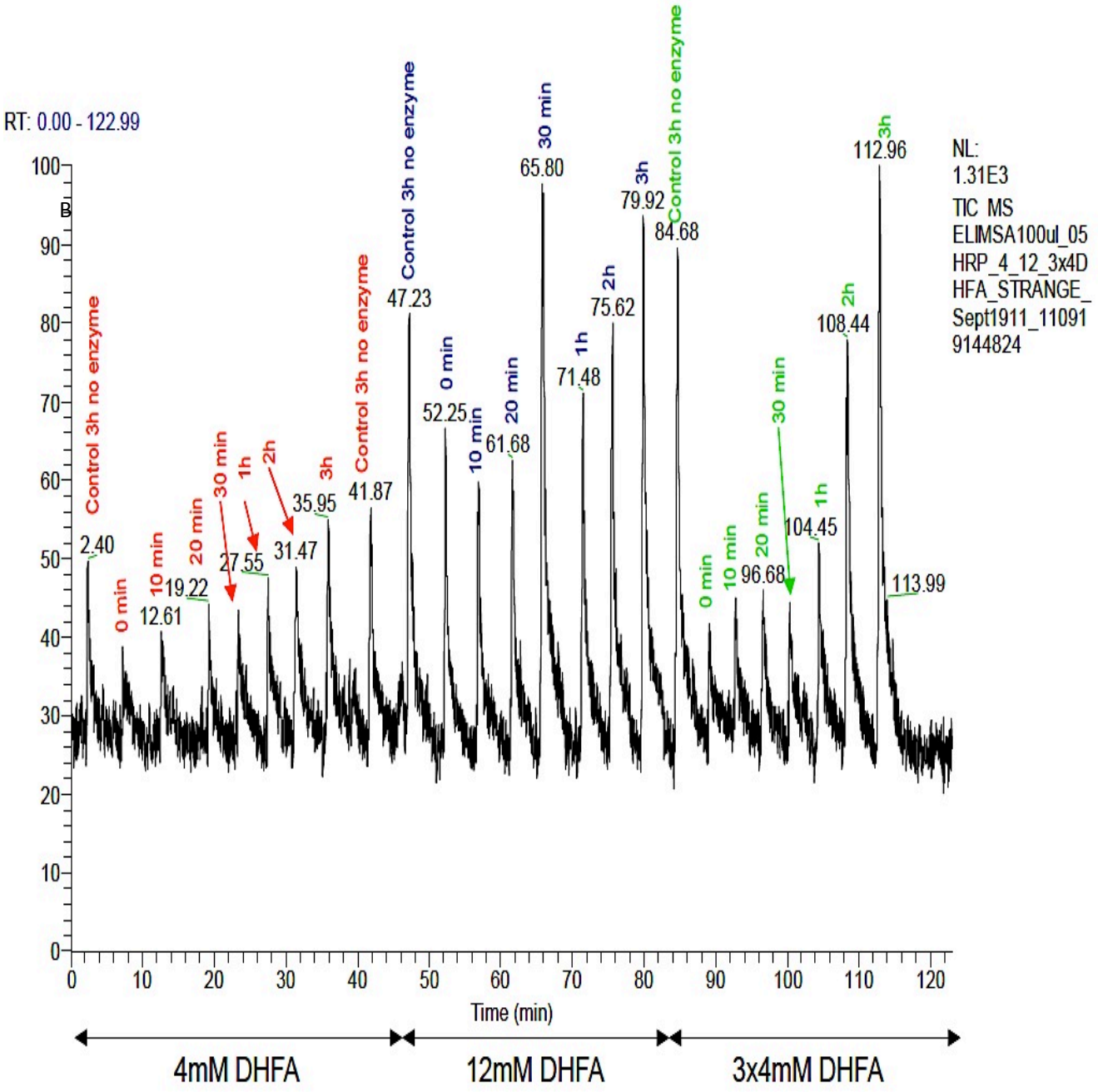
TIC of different concentrations of DHFA that were added to the reaction in 400 ml of 60 mM of acetate buffer A) 12mM at beginning, B) 4mM at beginning, C) 3X of 4mM hourly.



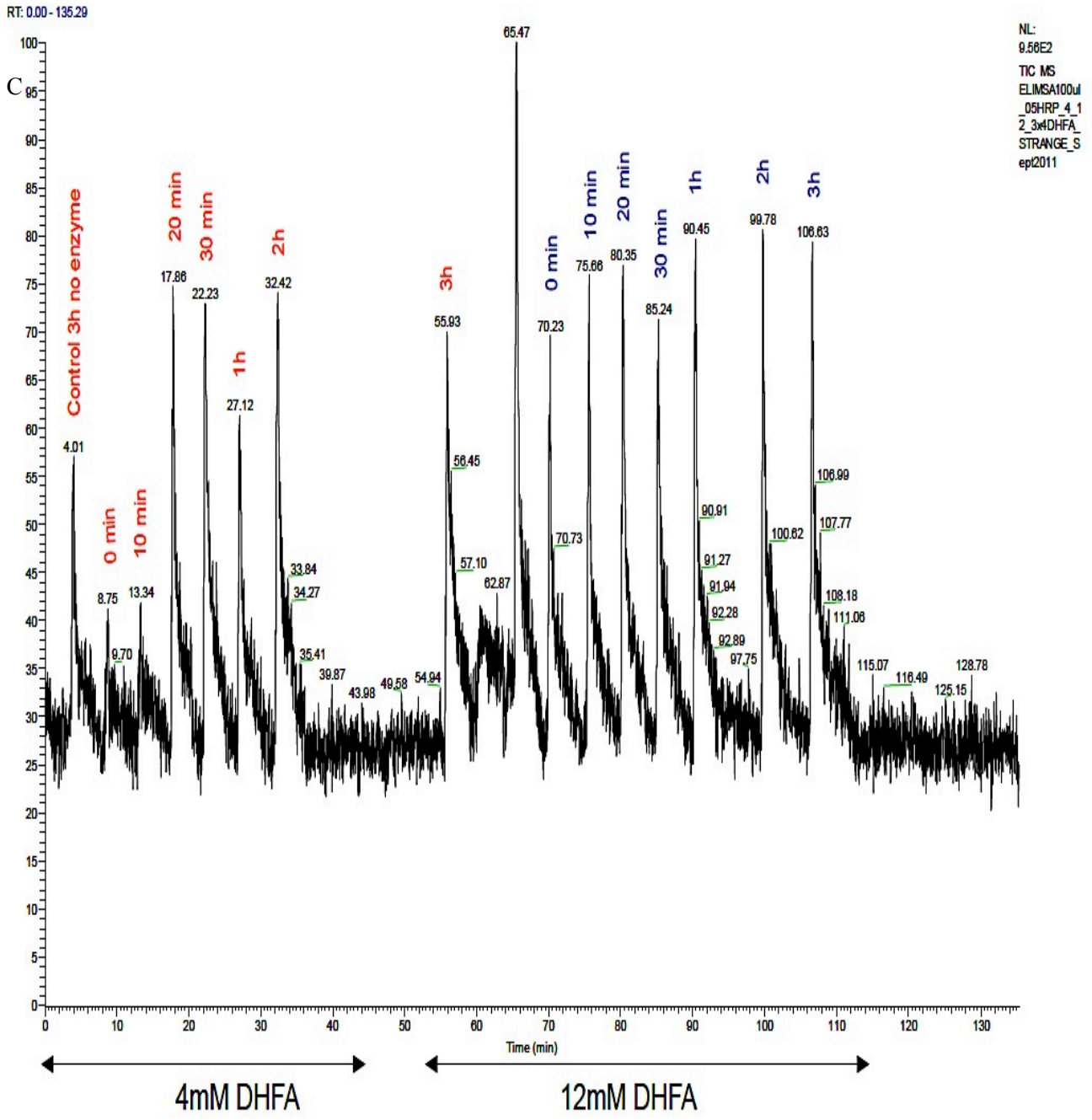
APPENDIX E- TIC of chemical reactions in the presence and absence of DHFA in 96 well plates

A) TIC of 12mM and 3x of 4mM DHFA (1st replication), B) TIC of 4mM, (1st replication) 12mM and 3x of 4mM DHFA (2nd replication), C) TIC of 4mM (2nd replication).



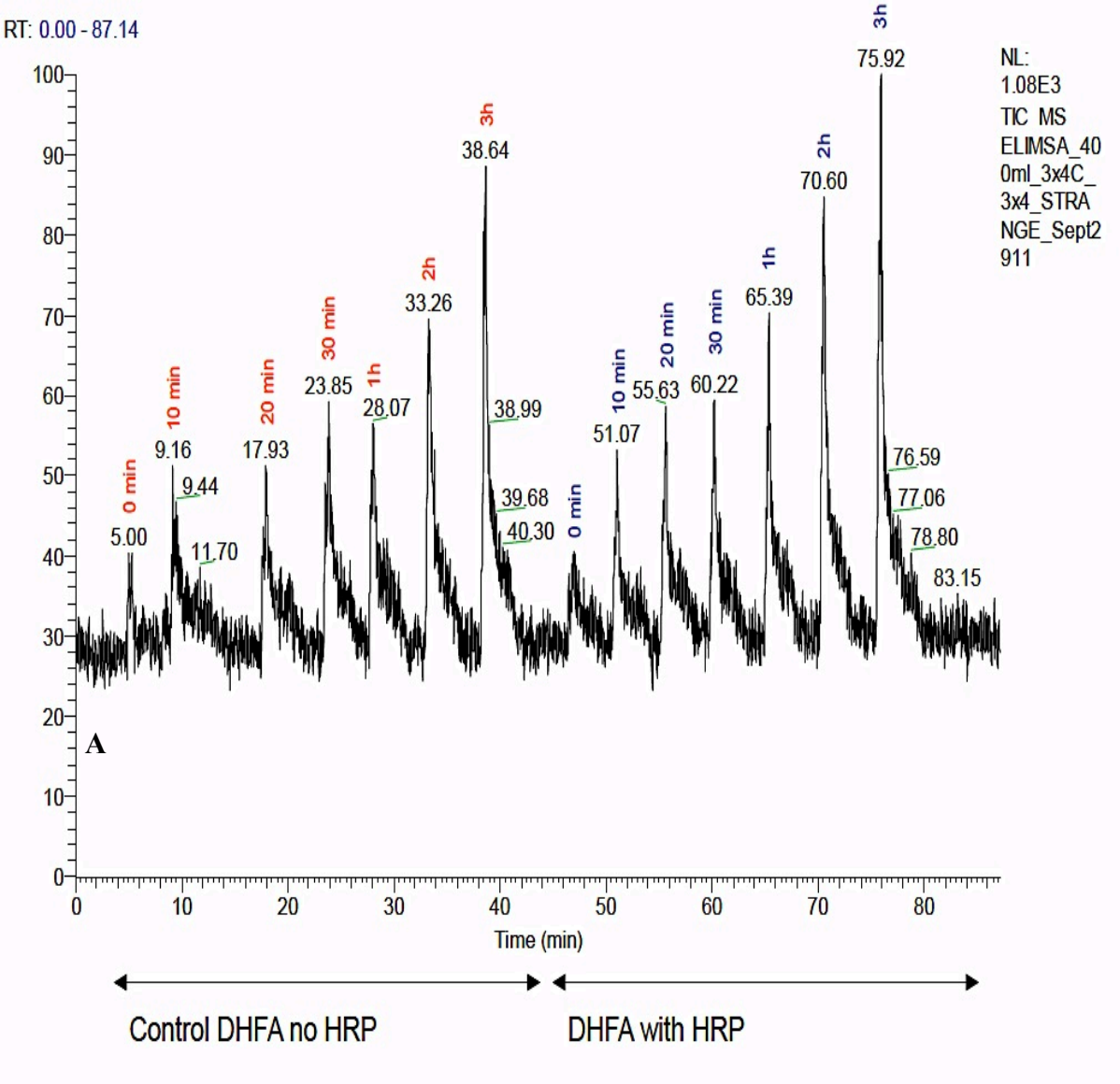

RT: 0.00 - 122.99
NL:
1.31E3
TIC MS
ELIMSA100ul_05
HRP_4_12_3x4D
HFA_STRANGE_
Sept1911_11091
9144824
Control 3h no enzyme
0 min
10 min
20 min
30 min
1h
2h
3h
2.40
12.61
19.22
27.55
31.47
35.95
41.87
47.23
52.25
61.68
65.80
71.48
75.62
79.92
84.68
96.68
104.45
108.44
112.96
113.99
Time (min)
4mM DHFA
12mM DHFA
3x4mM DHFA

C


RT: 0.00 - 135.29
NL:
9.56E2
TIC MS
ELIMSA100ul
_05HRP_4_1
2_3x4DHFA_
STRANGE_S
ept2011
Control 3h no enzyme
0 min
10 min
20 min
30 min
1h
2h
3h
0 min
10 min
20 min
30 min
1h
2h
3h
4.01
8.75
9.70
13.34
17.86
22.23
27.12
32.42
33.84
34.27
35.41
39.87
43.98
49.58
54.94
55.93
56.45
57.10
62.87
65.47
70.23
70.73
75.66
80.35
85.24
90.45
90.91
91.27
91.94
92.28
92.89
97.75
99.78
100.62
106.63
106.99
107.77
108.18
111.06
115.07
116.49
125.15
128.78
Time (min)
4mM DHFA
12mM DHFA

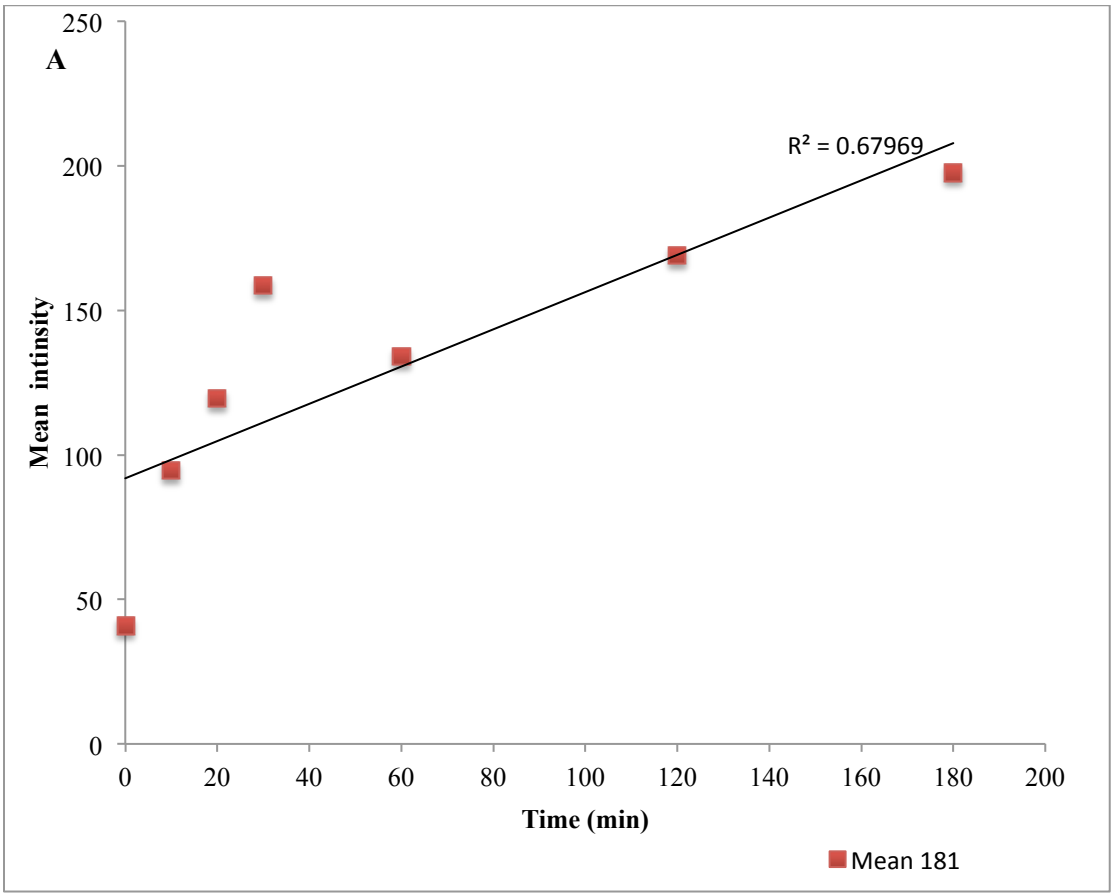
APPENDIX F- TIC of DHFA standards

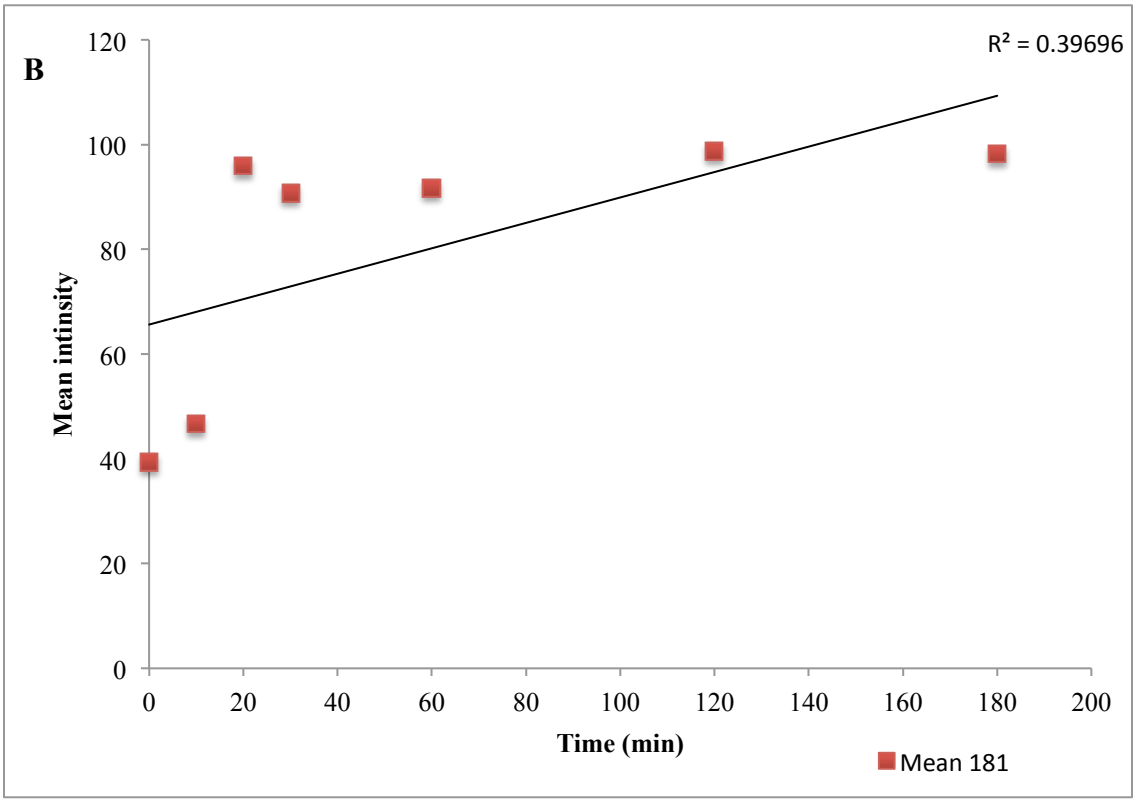
DHFA standard was analysed with direct infusion MS A) TIC of DHFA in the absence of HRP, B) TIC of DHFA in the presence of HRP

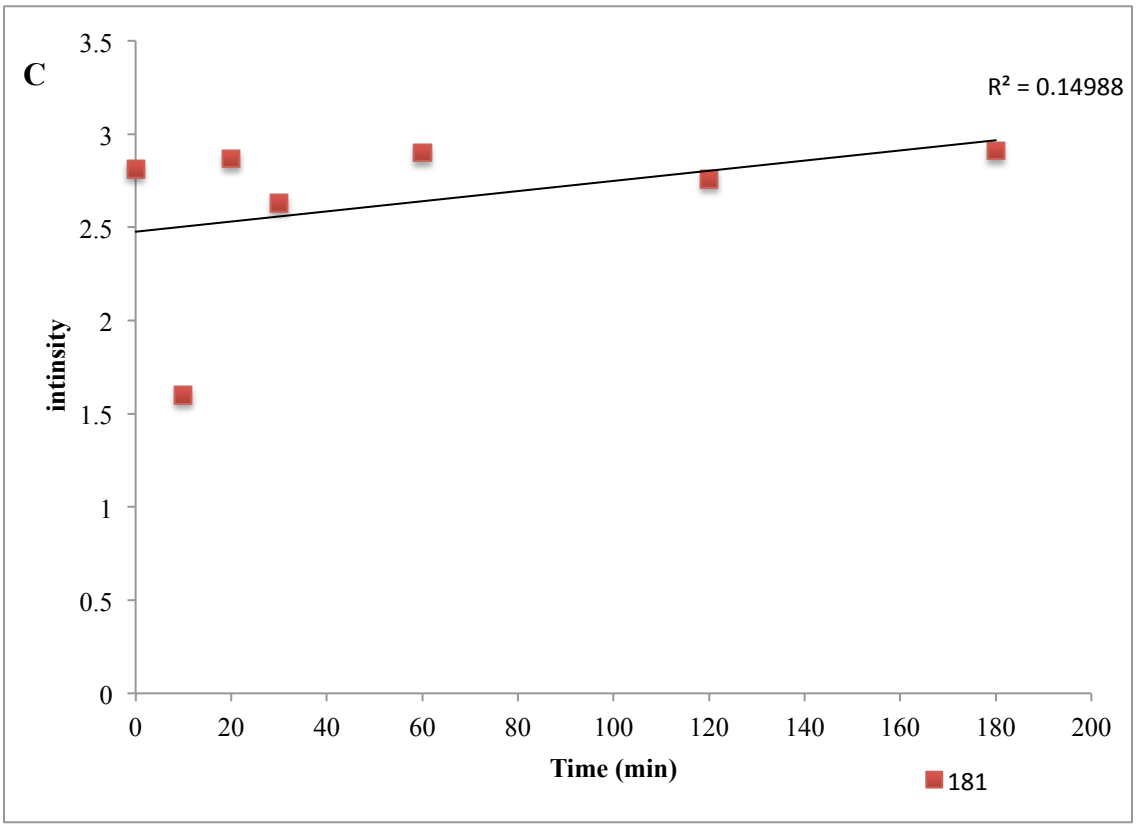


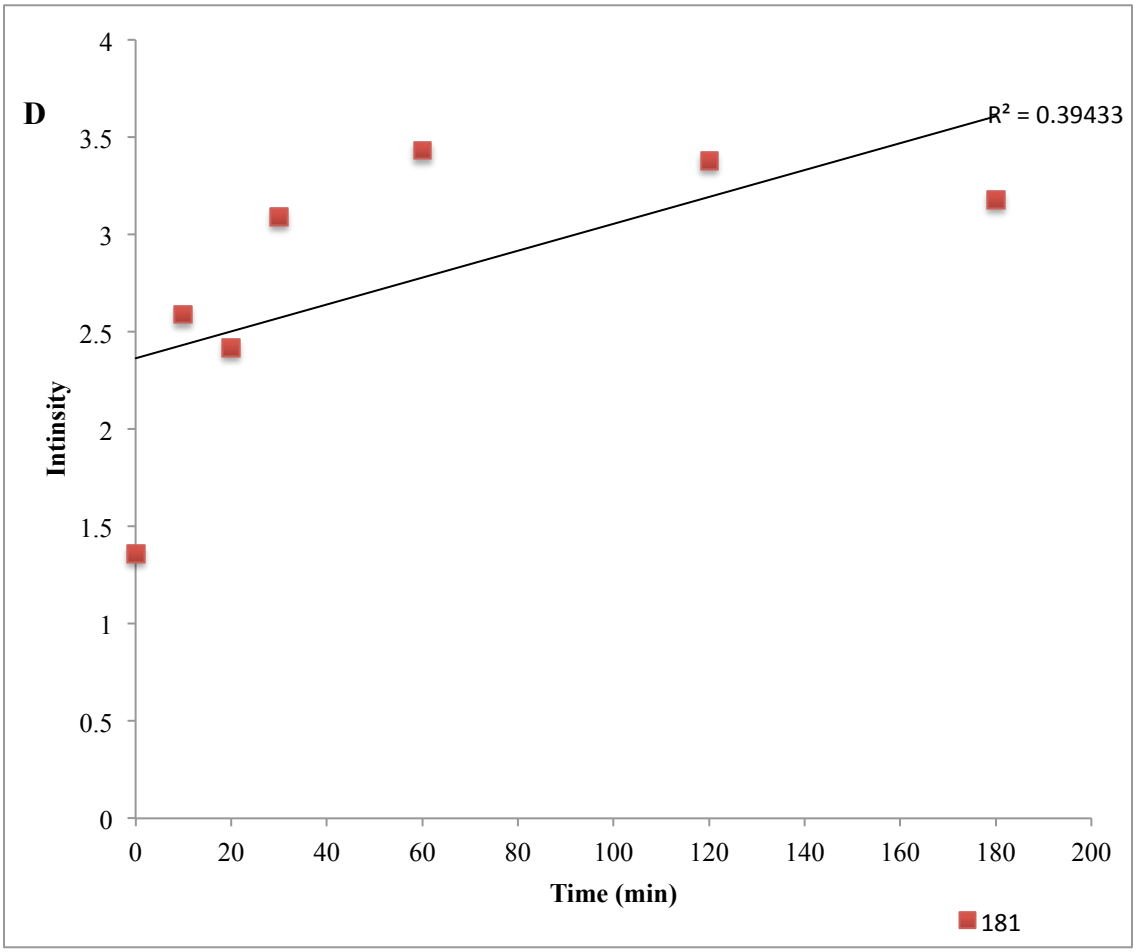
APPENDIX G- DHFA reactions in 100 µl (96 well plate)

The chemical reactions were had similar reaction conditions, but they were varied in the concentrations and the presence/absence of DHFA. A) 12mM of DHFA at begging, B) 4mM of DHFA at time 0 min, C) Reaction with no DHFA and HRP, D) reaction with HRP and no DHFA.




B
R² = 0.39696
120
100
80
60
40
20
0
Mean intinsity
0
20
40
60
80
100
120
140
160
180
200
Time (min)
Mean 181


C
R² = 0.14988
3.5
3
2.5
2
1.5
1
0.5
0
intinsity
0
20
40
60
80
100
120
140
160
180
200
Time (min)
181


D
R² = 0.39433
Intinsity
Time (min)
181
0
0.5
1
1.5
2
2.5
3
3.5
4
0
20
40
60
80
100
120
140
160
180
200

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