Refinement of a HPLC Assay of Lipid Hydroperoxides Caused by Free Radical-Mediated Oxidation of Membrane Lipids

An Honors Thesis (HONRS 499)

by

Laura Kenkel

Thesis Advisor: Dr. Scott Pattison

Ball State University
Muncie, Indiana

May 2004

Expected Date of Graduation: May 8, 2004

Table of Contents

Abstract

A sensitive and easy-to-use HPLC assay that measures the amount of free radical-mediated oxidation of membrane lipids is refined. A reaction that models the effects of free radical oxidation on cell membrane lipids is used. The rate of the reaction between diphenyl-1-pyrenylphosphine (DPPP) and tert-butylhydroperoxide (tBuOOH; a model compound for peroxidized lipids) is detected by measuring the amount of fluorescent DPPPO. The levels of two catalysts, pyridine and iron (III), are adjusted so that the reaction they catalyze is complete within approximately one minute, with a goal of extrapolating back to reaction conditions that would be conducive to completion within one second. Samples of the reacting solution are injected into an HPLC machine, bypassing the column in favor of a spectrofluorometer.

Key words: free radical-mediated oxidation, lipid hydroperoxide, diphenyl-1-pyrenylphosphine (DPPP), high pressure liquid chromatography (HPLC), membrane lipid, tert-butylhydroperoxide (tBuOOH), pyridine, iron (III).

Introduction

"You could be walking down the street when it hits you. An invisible molecule of, say, air pollution enters your body. It ricochets around inside of you, chewing up genes. Years later, seemingly out of nowhere, you're diagnosed with cancer." [1]

Quotes from popular magazine articles, like the one above, use analogies that compare free radicals to tiny molecular gremlins that run around a person's body while wreaking havoc at the cellular level. Articles personify antioxidants as heroes that by reacting with free radicals can help "reel in those nasty renegades," and battle "cellular terrorism." [1] Admittedly, this gives the general population a concept that can be grasped, and informs people about the usefulness of an antioxidant-rich diet. What these popular articles fail to do though is thoroughly describe why free radicals are so dangerous at a cellular level. Popular articles, instead of describing what happens on a molecular level, offer up solutions to the free radical problem ranging from eating unusual food products like whey [2] to eating an extreme low calorie diet (about one-third fewer calories than the average person [3]

From a scientific standpoint, it is not enough to know that free radicals steal electrons from cell membranes and cause a weakening of the membrane. In order to understand what free radicals do, it is necessary to go more in-depth into the chemistry of free radical-lipid reactions.

A free radical is an atom or molecule that has an unpaired electron. Free radicals react with molecules (like lipids) they can easily pull an extra electron from, which gives the free radical a more stable electron situation. However, this makes the molecule

vulnerable to attack by other molecules, such as oxygen, and a chain reaction begins that eventually leads to the breakdown of the membrane (see Figure 1).

Free Radical-Mediated Oxidation of a Polyunsaturated Fatty Acid

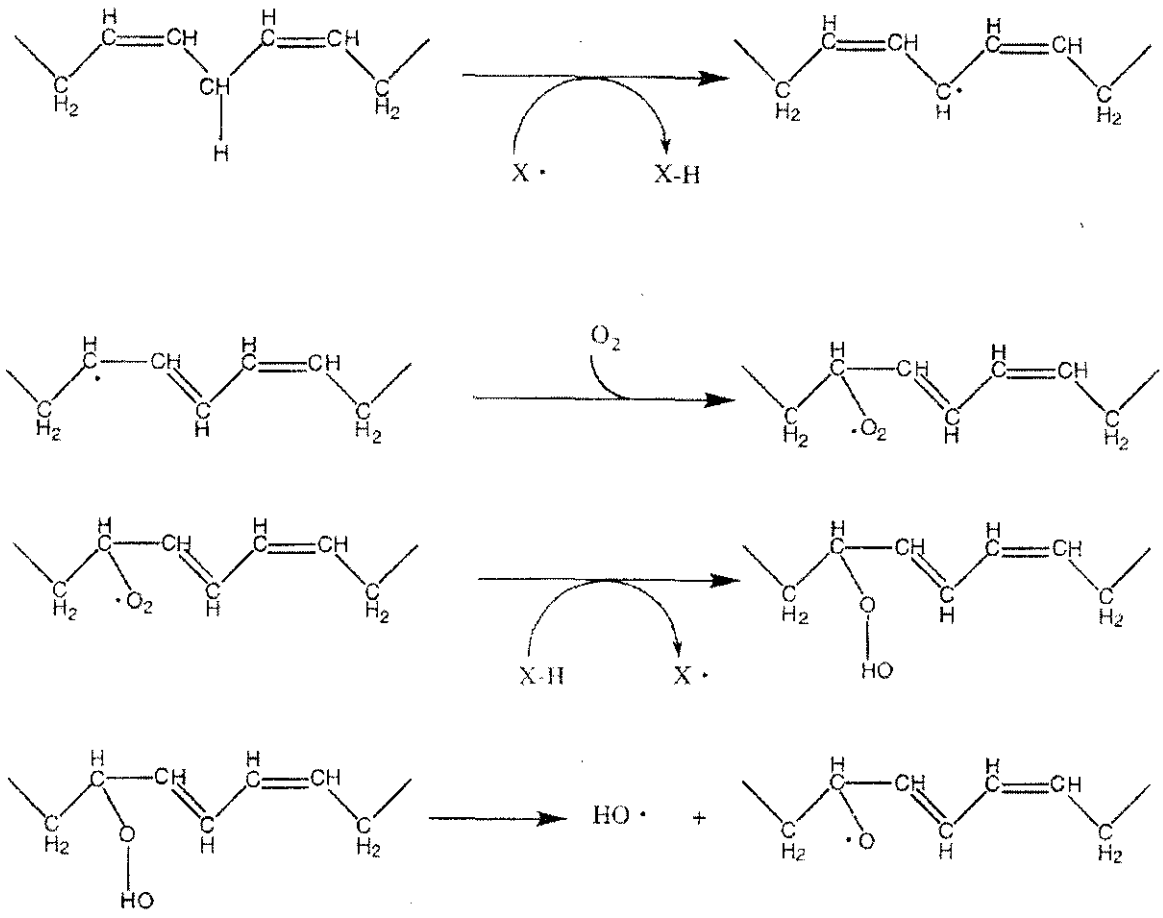


Figure 1 – When a free radical ($X^{\cdot}$) attacks a membrane lipid in the first reaction, it takes a hydrogen atom away from the lipid by binding with it, thus stabilizing the radical, but weakening the lipid's structure. In the second reaction, the lipid tries to stabilize itself by shifting its resonance structure, but it is still vulnerable to attack by oxygen. In the third reaction, a hydroperoxide forms and in the fourth reaction, the hydroxide group breaks away from the lipid, leaving the lipid vulnerable to further degeneration.

When free radicals attack the lipids in a cell membrane, the membrane becomes more susceptible to substances that the cell membrane would normally keep out. The fluidity of the cell membrane also decreases, which leads to a decrease in overall cell health. The effects of free radical-mediated oxidation of membrane lipids, as well as other biochemical molecules, have been a subject of study in many physiological and

biochemical studies. These studies point to the importance of understanding free radical-mediated oxidation and its role in membrane physiology and many types of pathology. Watanabe *et al.* found that oxygen-derived free radicals lead to a decrease in erythrocyte (red blood cell) membrane fluidity and morphology [4]. It is also noted that increased free radical levels are found accumulated in the myocardium during ischemia and reperfusion, supporting the hypothesis that the changes in membranes caused by free radical-mediated oxidation have a significant impact on cell injury [4]. Kaneko *et al* [5] suggest that free radical-mediated lipid peroxidation, by increasing membrane permeability and loss of membrane integrity, may influence the activities of enzymes bound in the membrane, which also compromise the health of the cell (in Kaneko *et al's* research [5], possibly depressing heart sarcolemmal Ca^{2+}-pump activity).

There is an obvious value to research dealing with free radical-mediated oxidation. If the mechanisms behind free radical-mediated lipid peroxidation can be elucidated, new methods to combat free radicals and their damaging effects can be developed. One of the challenges in such research is to find sensitive and easy-to-use methods that measure the oxidative damage done by free radicals.

Iodometric determination of hydroperoxides is one method used to determine the amount of damage done to a lipid by a free radical [6]. Hydroperoxides react with I^- according to this equation:

$$ROOH + 2\ H^+ + 2\ I^- \rightarrow ROH + H_2O + I_2 \qquad [6]$$

Since the exact stoichiometry of this reaction is well known, it can easily be used to determine the amount of hydroperoxide in a lipid, which indicates to what extent free radicals have damaged the lipid membrane [6]. In this method, the I_2 produced by the

reaction with the hydroperoxide, ROOH, can be measured by measuring the I_3^- which is formed in the presence of excess iodide, according to the following equation:

$$I^- + I_2 \leftrightarrow I_3^- \qquad [6]$$

This method has a sensitivity of approximately 0.2 – 1.0 nanomoles of hydroperoxide [6] which seems impressive, but is not as sensitive as other available methods. In addition, because of the sensitivity of acidified iodide solutions to oxygen, the iodometric assay has to be performed under anaerobic conditions, which makes it more complicated and less easy to use [6].

Another method used to determine the amount of hydroperoxides resulting from free radical-mediated oxidation is chemiluminescent flow injection. In this method, hydroperoxides are reacted with a luminescent reagent, resulting in chemiluminescence that is measured by a chemiluminescence detector [7]. The idea behind this method is similar to that of the iodometric assay [6]. The amount of chemiluminescence indicates the amount of hydroperoxide, which indicates the amount of oxidative damage done by free radicals. This method has high reproducibility and has a sensitivity level of as low as 10 picomoles of hydroperoxide [7]. However, this method requires lipid extraction before the assay can be performed, which is time consuming and complicated [7].

Yet another method for measuring free radical-mediated damage is by measuring cyclooxygenase (prostaglandin H synthase) activity stimulated by hydroperoxides [8]. The sensitivity of this method is such that there is an enzymatic response to peroxide levels as low as 10 picomoles of lipid hydroperoxides [8]. However, it is not necessarily easy to obtain cyclooxygenases [9].

While all of the methods just mentioned are able to measure levels of peroxidized lipids, each have their drawbacks. There is a need for a sensitive assay that uses high pressure liquid chromatography (HPLC) to determine the amount of lipid hydroperoxides generated by free radical-mediated oxidation. This research attempts to design and refine a new HPLC assay for lipid hydroperoxides that is both sensitive and easy to use.

The backbone of this assay is the reaction between the lipid hydroperoxides and a compound called diphenyl-1-pyrenylphosphine (DPPP). DPPP has been deemed best for reactions with lipid hydroperoxides because it has high sensitivity of hydroperoxide levels, high reactivity with hydroperoxides, stability against oxidation with atmospheric oxygen and is relatively easy to prepare [9]. DPPP is also useful in an assay of lipid hydroperoxides because it does not react with unoxidized lipids or antioxidation agents [9]. Additionally, DPPP can be used as a fluorescent probe to measure free radical-mediated lipid peroxidation in live cells [10]. Fluorescence is generated when DPPP reacts with peroxides to create DPPPO (see Figure 2), which allows for easy measurement.

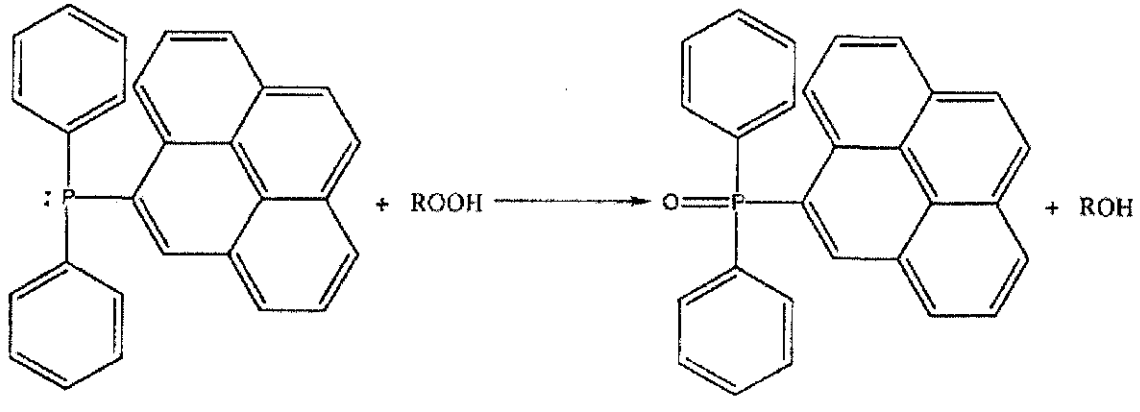
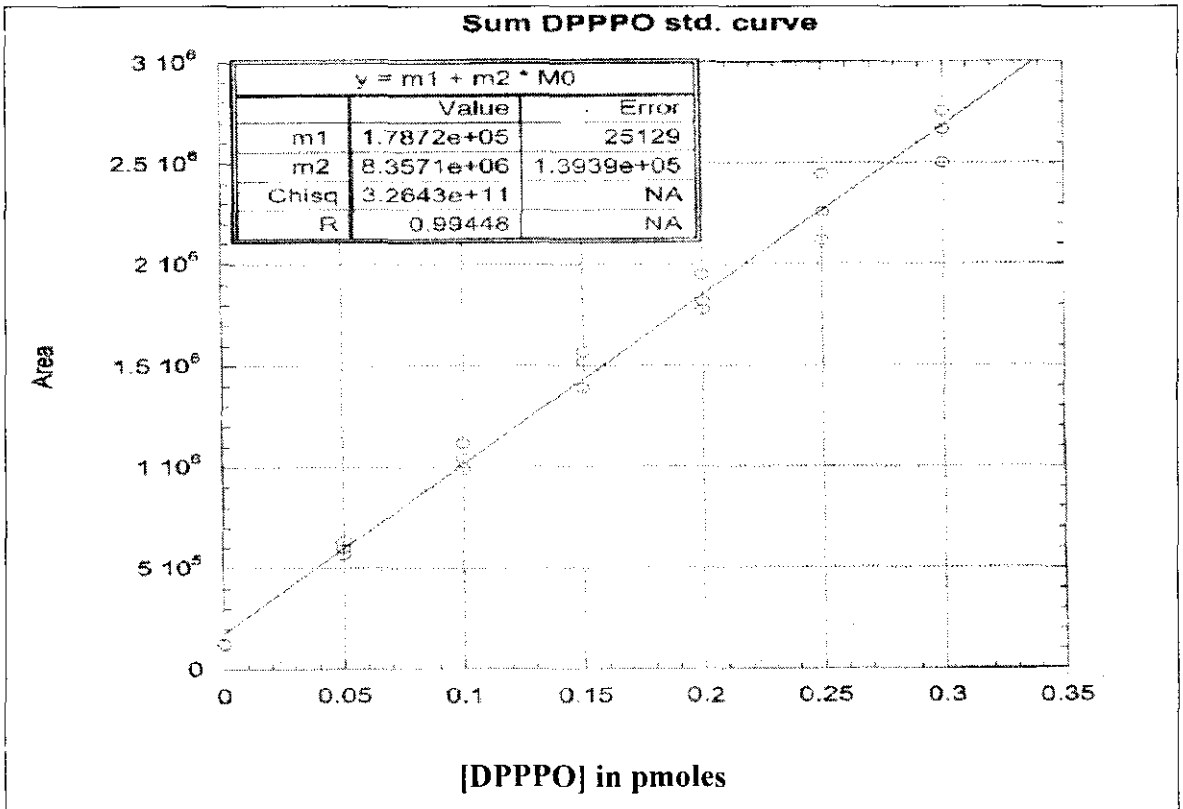


Figure 2 – The lone electron pair on the phosphorous quenches the fluorescence of the pyrene. When oxygen from the peroxide binds to the phosphorous, the lone pair (now in a double bond between oxygen and phosphorous) can no longer quench the pyrene, which results in fluorescence that can be detected by a spectrofluorometer.

The sensitivity of DPPP to hydroperoxides is below the picomole level. A standardized curve of DPPPO (Graph 1) shows that there are measurable fluorescent areas for just 0.05 picomoles of DPPPO, which indicates the high sensitivity of this assay.



Graph 1 – This standardized curve of DPPPO concentrations (in pmoles) demonstrates the high potential sensitivity of an assay that uses DPPP to measure hydroperoxide concentrations. There is a reliable, reproducible fluorescent area produced by less than 1 (0.05) picomole.

Again, the foundation of this assay is the knowledge that there is a stoichiometric relationship between the amount of lipid hydroperoxides and the amount of DPPPO [10]. This means that the amount of DPPPO measured (via fluorescence detection) is equal to the amount of lipid hydroperoxides, which is ultimately a quantifiable indication of the damage done by free radical-mediated oxidation.

Besides being sensitive to lipid hydroperoxides of less than 1 picomole, the assay has the advantage of being easy to use. As mentioned before, DPPP is easy to synthesize and is selectively reactive with only lipid hydroperoxides [9]. Also, the lipid hydroperoxide sample does not have to be extracted pre-assay and the solvent (chloroform methanol) and the catalysts (iron III and pyridine) are easily obtained and are not dangerous to use.

An analogous reaction modeling the reaction between a lipid hydroperoxide and DPPP can be used to refine this assay. In these experiments, DPPP is reacted with tert-butylhydroperoxide (tBuOOH) in a solvent of chloroform-methanol ($CHCl_3$:MeOH) with the help of catalysts iron III (Fe^{3+}) and pyridine.

The ultimate goal within this research is to adjust catalyst levels so that the reaction between DPPP and tBuOOH is complete within one second. One second is the timeframe needed for use in a HPLC assay. However, there is no possible way to mix the reaction solution and inject a sample into the HPLC apparatus within a one second timeframe. Thus, the current research is really an attempt to adjust catalyst levels so that the reaction between DPPP and tBuOOH will be complete within one minute. Once this objective is completed, an extrapolation can be performed to determine the conditions needed for the reaction to be complete within one second.

In terms of reaction rate, the reaction between DPPP and tBuOOH can be described as a second order type I reaction:

reaction rate = k [tBuOOH] [DPPP]

However, in the current research, the concentration of DPPP is in great excess, and creates a pseudo-first order reaction:

reaction rate = k [tBuOOH]

This rate equation simplifies the calculations needed to determine the conditions of the assay, adding to the ease of use of this assay.

One of the immediate goals of the experiments was to level out the fluorescent peaks. These peaks indicate. by fluorescent area, the amount of DPPPO is present in each injected sample. With an ideal reaction that completes within one minute, the fluorescent peaks seen after the addition of tBuOOH would be level (not increasing or decreasing in height). This could indicate that the reaction between tBuOOH and DPPP has completed by the time of the first injection (about one minute after the addition of tBuOOH) (see Figure 3). However, if fluorescent peaks increase after the addition of tBuOOH to the reaction solution, it could indicate that the reaction between DPPP and tBuOOH is still continuing and is producing DPPPO (see Figure 4).

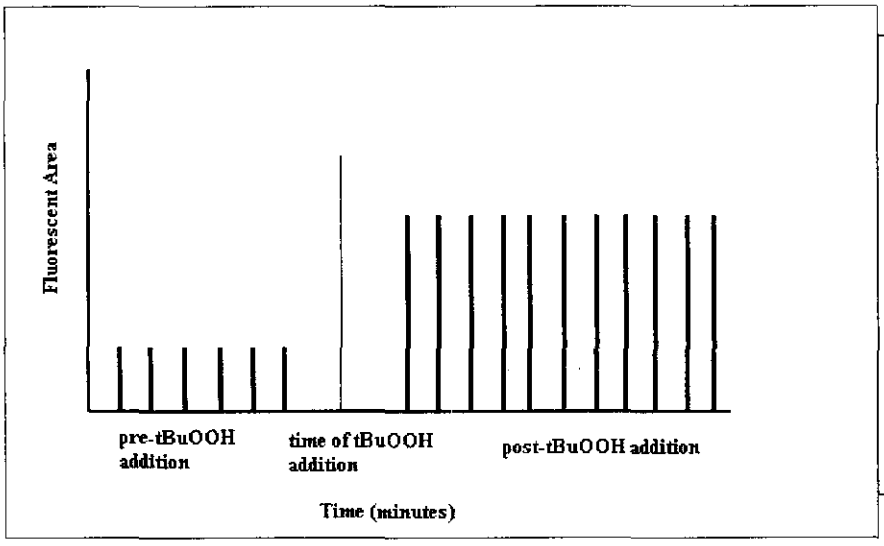


Figure 3 – This is an example of an ideal fluorescent readout. The fluorescent peaks after the addition of tBuOOH do not increase as time goes on, indicating that the reaction between DPPP and tBuOOH has completed by the time of the first injection.

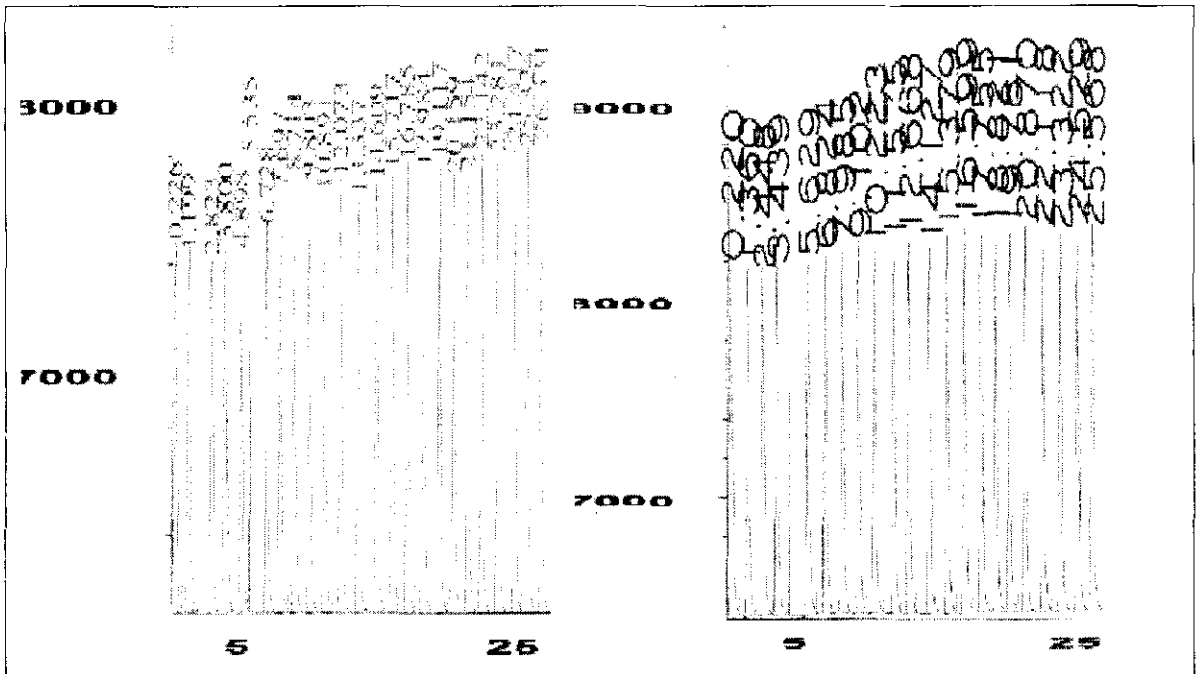
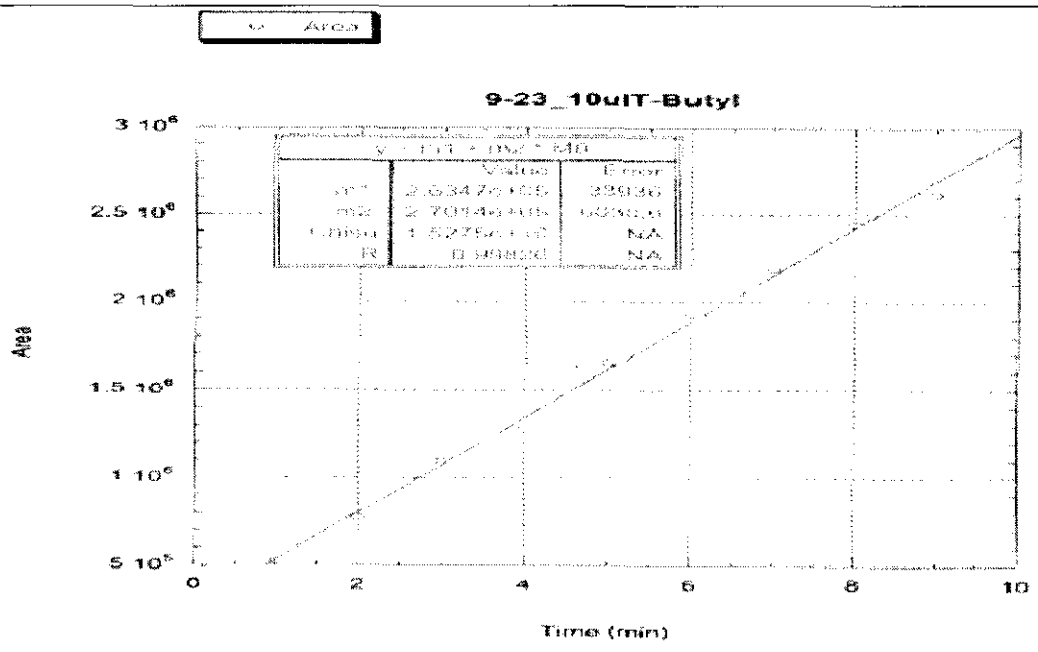


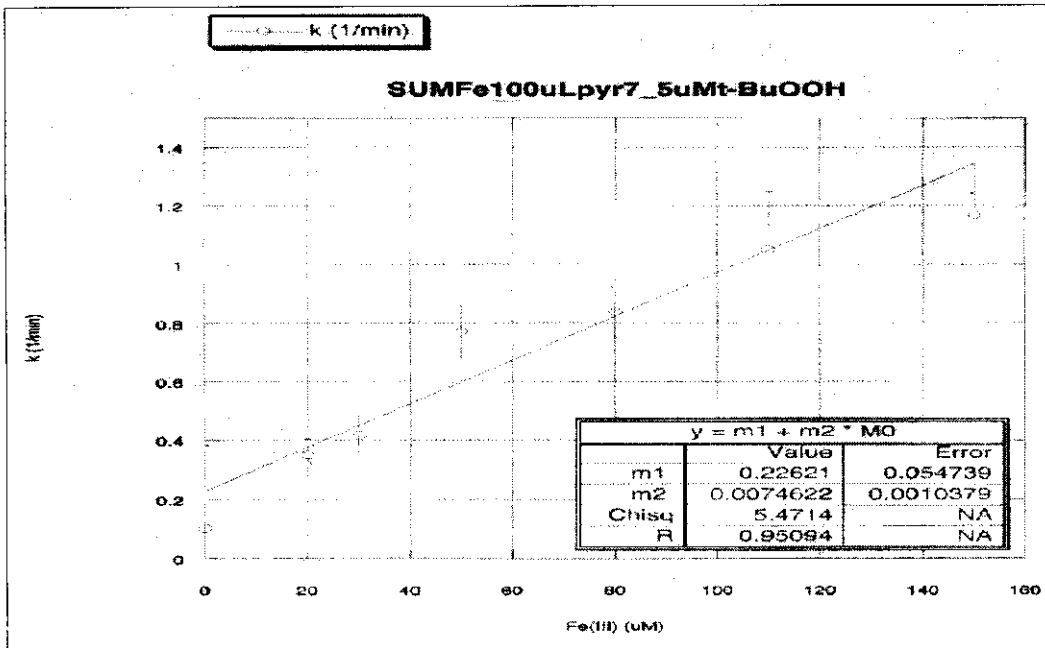
Figure 4 –These two actual fluorescent readouts help to illustrate one of the objectives of the experiment. On the left, the peaks keep increasing, which could indicate that the reaction between DPPP and tBuOOH is not yet finished. On the right, the fluorescent peaks seem to be leveling out, which could indicate that the catalysts have been adjusted so that by the first injection, the reaction between tBuOOH and DPPP is nearly complete.

In an attempt to get results similar to the ideal results in Figure 3, we have been varying the levels of the two catalysts, Fe^{3+} and pyridine. The reaction between tBuOOH and DPPP proceeds too slowly without catalysts for use in an HPLC assay (see Graph 2)

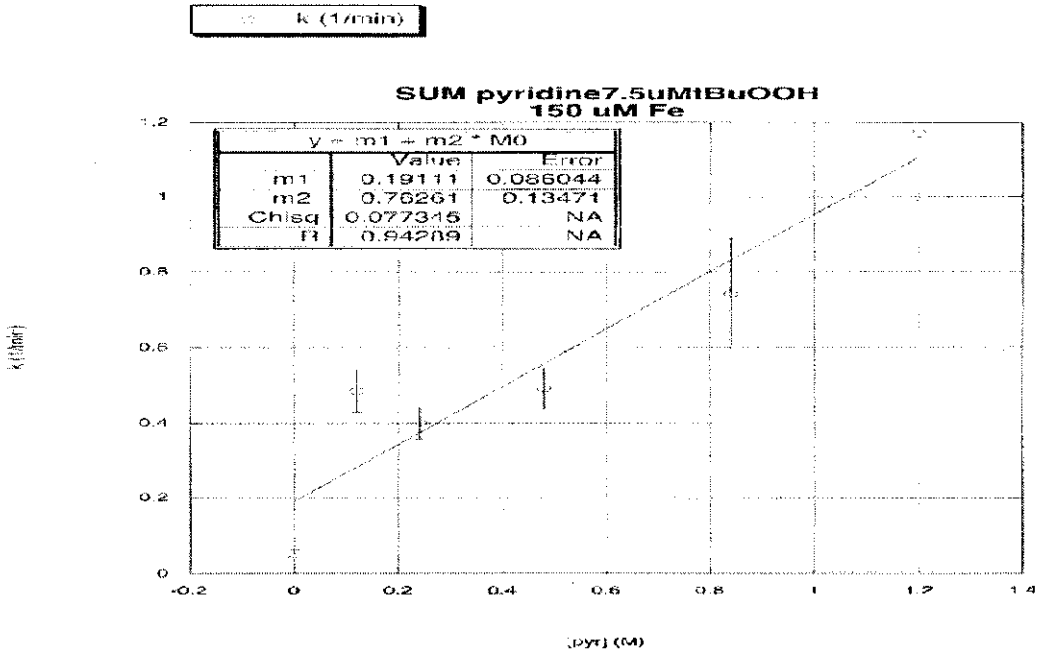


Graph 2 – This graph shows that a reaction between tBuOOH and DPPP without catalysts proceeds too slowly to be used in an HPLC assay – even after 10 minutes, the reaction is still progressing, as indicated by the increase in fluorescent area (indicating an increase in DPPPO).

Addition of Fe^{3+} and pyridine increases the rate of the reaction, as seen in Graphs 3 and 4 (next page).



Graph 3 – This graph shows that as the concentration of Fe^{3+} that is added to the reaction solution increases, the rate of reaction (k) increases as well, illustrating that Fe^{3+} is an effective catalyst for DPPP-tBuOOH reaction.



Graph 4 – This graph shows that as increasing concentrations of pyridine are added to a reaction solution containing 7.5μM tBuOOH and 150 μM Fe^{3+}, the reaction rate (k) increases, which shows that the addition of pyridine (in combination with Fe^{3+}) can effectively catalyze the reaction between DPPP and tBuOOH.

Materials and Methods

HPLC apparatus

A Waters-600 MS System Controller was used to monitor and control the flow of 1 mL/min $CHCl_3$:MeOH and to bypass the column in favor of sending the injected samples through a Shimadzu Fluorescence Spectromonitor RF-530. Emission wavelength was set at 380 nm and excitation wavelength was set at 352 nm, with a range of 16. The flow rate for all experiments was 1 mL/minute and for all experiments, $CHCl_3$:MeOH was used for the flow.

Varying amounts of Fe^{3+}

The effect on reaction rate caused by varying amounts and concentrations of Fe^{3+} was tested by using a reaction solution that consisted of 50 µL of 1.2M pyridine, 40 µL of 1mg/mL DPPP in $CHCl_3$:MeOH, and enough solvent ($CHCl_3$:MeOH) to make up a total solution volume of 1 mL, depending on the volume of Fe^{3+} used.

The DPPP solution was made each week, and stored in the freezer. A 1:10,000 dilution of 7M tBuOOH was made daily before the day's experiments. The Fe^{3+} solution was made by mixing $FeCl_3$ in MeOH (methanol), and could be kept at room temperature until the entire solution was used. The pyridine was also kept at room temperature.

The entire reaction solution was mixed immediately before the experiment, and 6 µL samples were injected at a rate of approximately one injection per minute into the HPLC unit, by which they were directed into the spectrofluorometer. After a baseline of the fluorescence of the reagents was established, 2 µL of the 1:10,000 diluted tBuOOH sample was added to the reaction solution and the solution was mixed via shaking. After

mixing, another 6 μL of the sample was injected into the HPLC unit, and this was continued for approximately 20 minutes at a rate of approximately one injection per minute, in order to give an accurate picture of the progress and rate of the reaction.

Varying concentrations of tBuOOH

To determine the optimum dilution of 7M tBuOOH, various dilution experiments were performed. Each experiment began by mixing a reaction solution of 50 μL 1.2M pyridine, 40 μL 1mg/mL DPPP in $CHCl_3$:MeOH, 150 μL of .01M Fe^{3+}, and 760 μL CHCl3:MeOH, totaling 1 mL of reaction solution. 6 μL samples of this solution were injected via the HPLC unit into the spectrofluorometer at a rate of approximately one injection per minute to achieve a baseline fluorescence. After a baseline was established, 10 μL of a diluted solution of tBuOOH in MeOH is added to the reaction solution and mixed. Then 6 μL samples of the reacting solution were injected at a rate of approximately one injection per minute.

Varying amounts of pyridine

The procedure for testing varying amounts of pyridine was similar to that used for testing varying amounts of Fe^{3+}. The reaction solution before the addition of 10 μL of 1:1million diluted 7M tBuOOH totaled 1 mL and consisted of 150 μL 0.01M Fe^{3+} (in MeOH), 40 μL of .01mg/mL DPPP (diluted in $CHCl_3$:MeOH), varying amounts of 1.2M pyridine and enough $CHCl_3$:MeOH to make up the rest of the 1 mL of solution.

The same experimental procedure used for testing varying amounts of Fe^{3+} and tBuOOH applies here as well.

There were also additional experiments that attempted to find the effect of pyridine levels on the reagent solution (without the addition of 10 μL 1:1million diluted 7M tBuOOH.) In these experiments, the reaction solution consisted of 150 μL .01M Fe^{3+} (in MeOH), 40 μL .01mg/mL DPPP (in $CHCl_3$:MeOH), the experimental amount of 1.2M pyridine, and enough $CHCl_3$:MeOH to create a total of 1mL of reaction solution. 6 μL samples of the reaction solution were injected and the fluorescent peaks of those samples were measured via a spectrofluorometer for a period of 20-30 minutes.

Results and Discussion

Determination of optimum Fe^{3+} concentration

Experimental results of the effects of varying volumes and concentrations of the catalyst Fe^{3+} are presented in Table 1.

Table 1 - Comparison of Slopes of Varying Volumes and Concentrations of Fe^{3+}

Fe^{3+} concentration and volume	Slope	Error
.8 mL .006 M	5.93E-05	1.21E-05
.9mL .006 M	7.32E-05	1.12E-05
.9 mL .0012 M	2.4E-05	3.94E-06
.06mL .01 M	4.33E-05	2.81E-06
150 μL .01 M	1.87E-05	5.13E-06

Testing of the effects of various concentrations and volumes of Fe^{3+} on the reaction between tBuOOH and DPPP suggests that (based on calculations of the slopes of the post-tBuOOH-addition peaks, see Table 1) 150 μL of .01M Fe^{3+} brings the reaction closer to completion by the time of the first injection of the solution after the addition of tBuOOH to the reaction mixture.

Determination of Optimum tBuOOH dilution

The goal of experiments to find an optimum tBuOOH dilution was to find the dilution of tBuOOH that, when reacted with DPPP, would give the smallest noticeable difference (distinguishable to the eye) between the fluorescent peaks pre-tBuOOH addition and post-tBuOOH addition. It was also important that the dilution be relatively simple.

A 1:1million dilution of tBuOOH satisfied these requirements well. The dilution was relatively easy to prepare, and there was a noticeable difference between the peaks

pre- and post-tBuOOH addition. The other dilution that was tested (1:1.5million) was more difficult to prepare, had an unreliable difference between the pre- and post-tBuOOH peaks and that difference was not nearly as noticeable as that found when the 1:1million dilution was used.

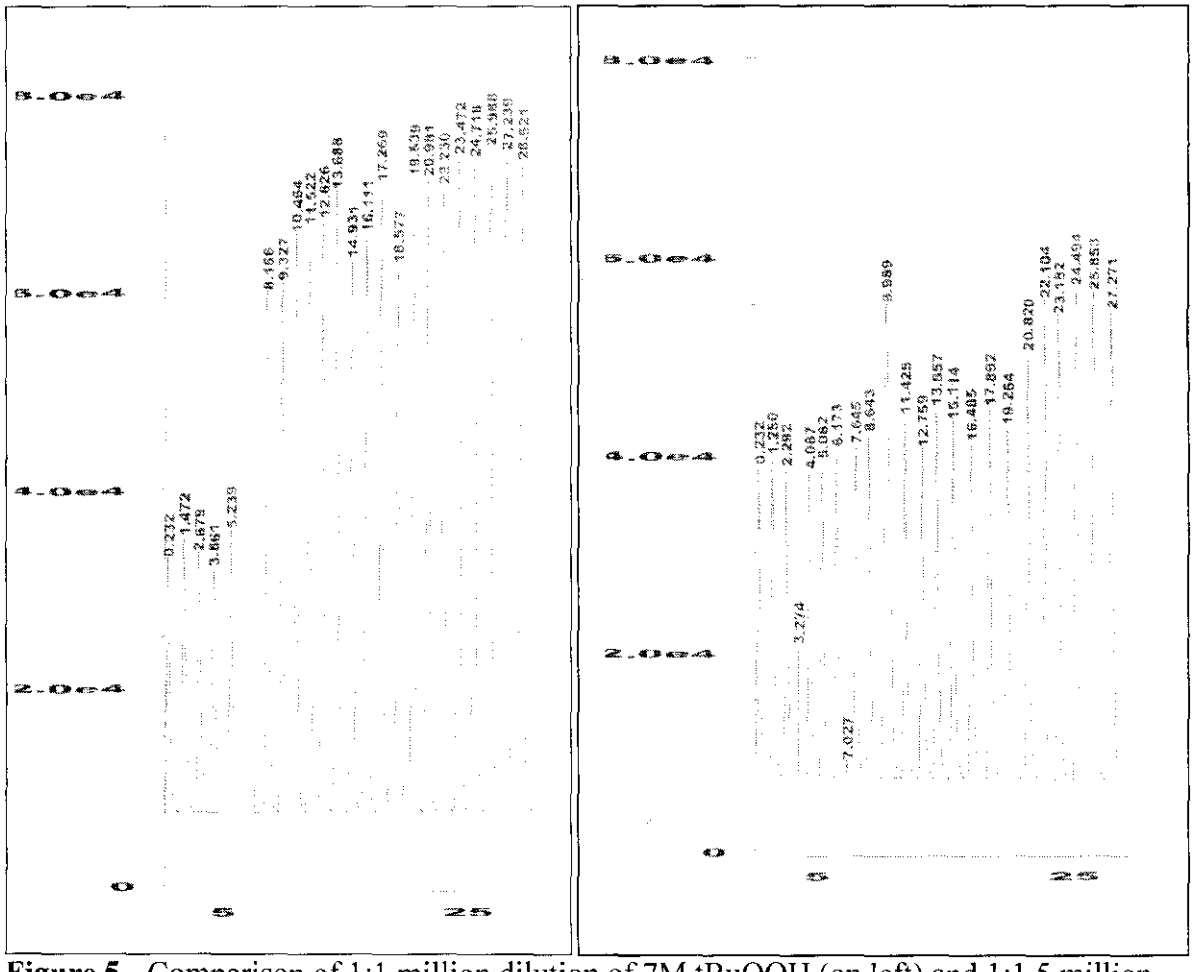
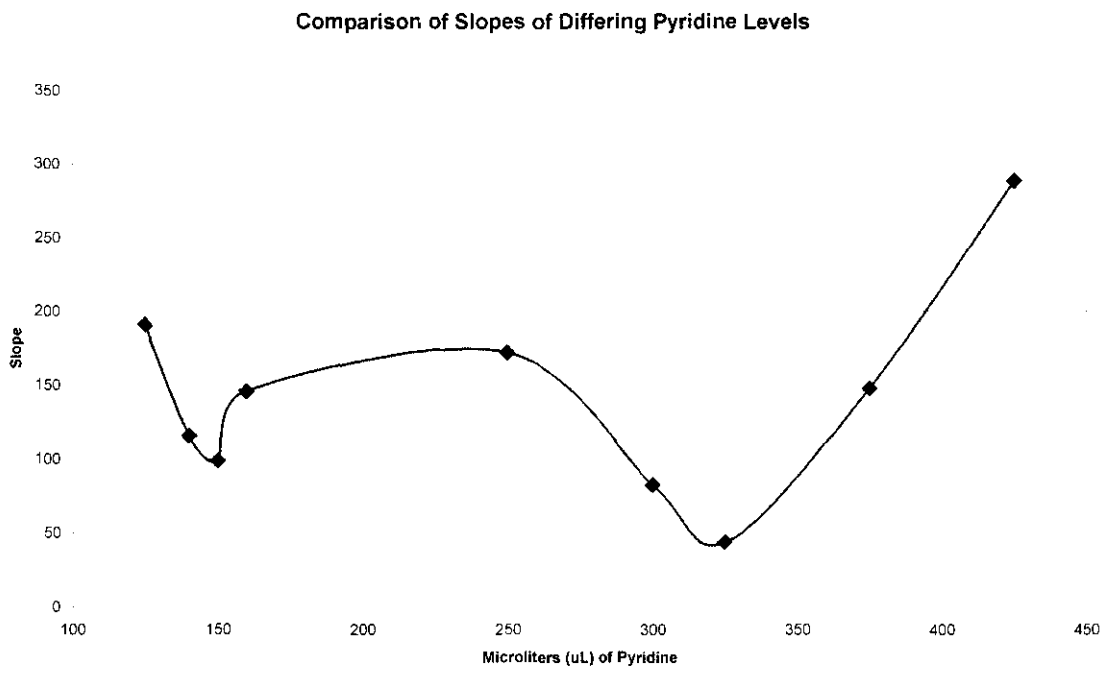


Figure 5 - Comparison of 1:1 million dilution of 7M tBuOOH (on left) and 1:1.5 million dilution of 7M tBuOOH on the right.

Determination of optimum 1.2M pyridine volume

125 μL, 140 μL, 150 μL, 160 μL, 250 μL, 300 μL, 325 μL, 375 μL and 425 μL amounts of 1.2 M pyridine were each utilized in separate experiments to find the effect of each on the post-baseline peaks. Graph 5 shows the comparison of the resulting slopes of the peaks.

Graph 5

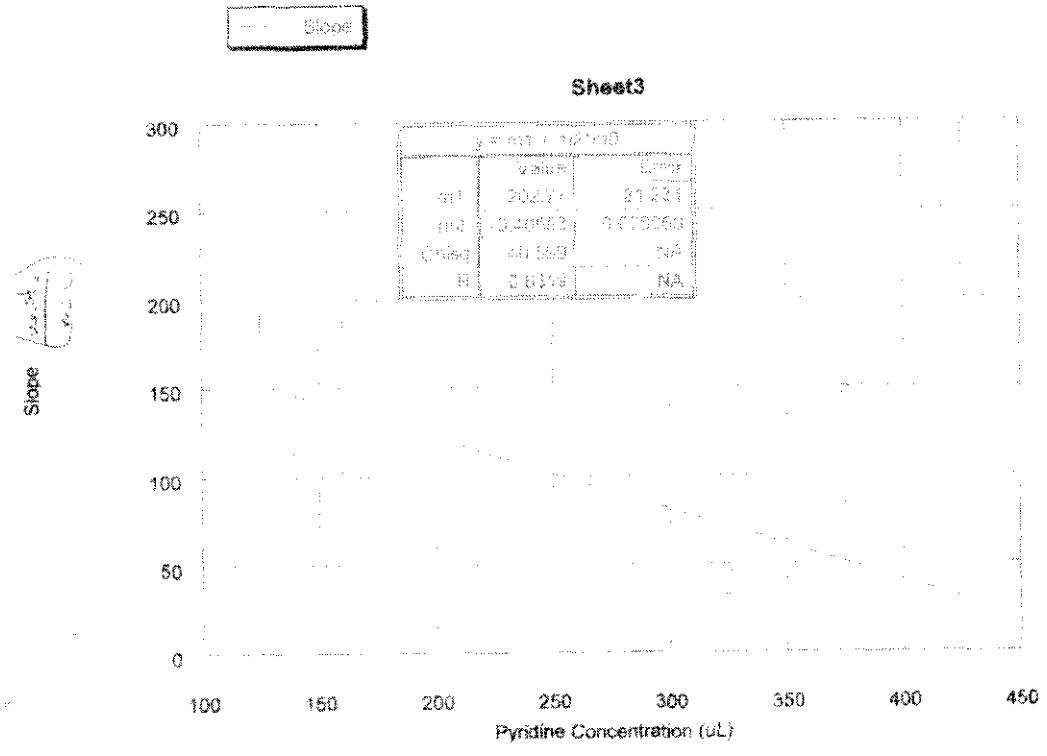


In order to gain a more descriptive graph of the results, the error was added into the graph (see Table 2 and Graph 6).

Table 2 - Comparison of pyridine concentrations, and the slope (and its error) of the resulting peaks

Pyridine Concentration (μL)	Slope	Error
125	190.63	21.11716
140	115.88	21.68603
150	99.017	19.60183
160	146.14	43.38857
250	172.11	17.83013
300	82.983	9.627248
325	43.825	11.43256
375	148.01	64.33961
425	288.68	98.96702

Graph 6 - Comparison of Pyridine Slopes with Error Bars included



Experimentation examining the pyridine's effect on DPPP's reaction with atmospheric oxygen yielded results shown in Table 3.

Table 3 – Comparison of pyridine's effect on the reaction between DPPP and atmospheric oxygen

Pyridine volume (µL)	Slope	Error
0	277.9309	36.9887
20	233.8531	23.4092
150	223.9687	10.7402
300	227.3611	10.48685

It was hypothesized before performing the first pyridine experiments that increasing the pyridine volume in the reaction solution would dampen the peak increase after the addition of tBuOOH. The idea behind this hypothesis was that more pyridine would bind up any free-floating Fe^{3+} atoms that might have been catalyzing a reaction between DPPP and atmospheric oxygen, thus increasing the peaks. However, as more experiments were performed it seemed that this hypothesis was not valid – the experimental data seemed to show that there was no consistent relationship between increasing pyridine amounts and dampening of post-tBuOOH peaks.

When considering this problem, it was initially hypothesized that perhaps as pyridine volume increases to a point where pyridine becomes more of a solvent than $CHCl_3$:MeOH, pyridine may dissolve more oxygen from the atmosphere, which would then react with DPPP to increase the concentrations of fluorescent DPPPO. If that hypothesis is valid, then it would suggest that the reaction between tBuOOH and DPPP is complete by the time of the first post-baseline injection, and any increasing fluorescent peak area is due to DPPP reaction with atmospheric oxygen. Although the increasing pyridine volumes did not initially seem to linearly decrease the slopes of the post-baseline fluorescent peaks (see Graph 5), data analysis that took the error of the data sets into consideration indicated that higher pyridine concentrations did have a higher negative effect on the slope of the fluorescent peaks (see Graph 6).

In order to try to find out whether pyridine is in fact dampening fluorescent peaks by either increasing the reaction between tBuOOH and DPPP, or by inhibiting a reaction between DPPP and atmospheric oxygen, a series of experiments were run using varying amounts of pyridine along with the other reagents, but without the addition of tBuOOH.

The results (shown in Table 3) seem to indicate that (at least according to initial experiments) increasing volumes of pyridine do not seem to have an increasingly inhibitory effect on the reaction between DPPP and atmospheric oxygen when the errors of the slopes are taken into consideration. Further research is needed here to help elucidate pyridine's effect on the reaction.

Overall, the current research tries to refine a new HPLC assay that is easy to use and is sensitive to lipid hydroperoxide levels of less than one picomole. The precise effects of the two catalysts (Fe^{3+} and pyridine) on the reaction between DPPP and tBuOOH (a reaction analogous to the reaction between DPPP and a lipid hydroperoxide) are still being researched. It is obvious, however, that the two catalysts increase the rate of the reaction, and hopefully can be adjusted to bring the reaction between tBuOOH and DPPP to completion within one second, so that the method can be an effective HPLC assay.

Acknowledgements:

Thanks to Dr. Scott Pattison for the opportunity to participate in this research, as well as for giving unlimited guidance and patience. Thanks also go to my research partner Erin Nablo and the Ball State Chemistry Department for the facilities provided and for the general support given to me by the faculty and staff. Additional thanks are owed to the Ball State Honors College for four years of expanded learning opportunities and the true impetus to complete this capstone project.

References:

[1] M. Downey, Better Nutrition. 65(9) (2003).

[2] USA Today Magazine. 132(2705) (2004).

[3] J. Achenbach. National Geographic. 203(3) (2003).

[4] H. Watanabe, A. Kobayashi, T. Yamamoto, S. Suzuki, H. Hayashi, N. Yamazaki, Free Radical Bio. & Med. 9 (1990) 507-514.

[5] M. Kaneko, R.E. Beamish, N.S. Dhalla. Am. J. Physiol. 256 (1989) H368-H374.

[6] W. Jessup, R.T. Dean, J.M. Gebicki, Methods in Enzymology. 233 (1994) 289-301.

[7] T. Miyazawa, T. Suzuki, K. Fujimoto, K. Yasuda, J. Lipid Research. 33 (1992) 1054-1059.

[8] P.J. Marshall, M.A. Warso, W.E.M. Lands, Anal. Biochem. 145 (1985) 192-199.

[9] K. Akasaka, H. Ohrui, J. Chrom. A. 881 (2000) 159-170.

[10] M. Takahashi, M. Shibata, E. Niki. Free Radical Bio. & Med. 31(2) (2001) 164-174.