University of Cincinnati

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I, Dusten T Dussex, hereby submit this original work as part of the requirements for the degree of Master of Science in Industrial Hygiene (Environmental Health).

It is entitled:

The Utilization of Fluorescent Products to Detect the Inhibition of Acetylcholinesterase by Carbamates and Organophosphate Pesticides: The Groundwork For a New Assay

Student's name: **Dusten T Dussex**

This work and its defense approved by:

Committee chair: Mary Beth Genter, Ph.D.

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The Utilization of Fluorescent Products to Detect the Inhibition of Acetylcholinesterase by Carbamates and Organophosphate Pesticides: The Groundwork for a New Assay

A thesis submitted to the Graduate School of the University of Cincinnati in partial fulfillment of the requirements for the degree of:

Master of Science in Industrial Hygiene

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Committee Chair: Dr. Mary Beth Genter

Abstract:

Acetylcholinesterase (AChE) inhibitors such as organophosphate insecticides and carbamate insecticides have a severe effect on the human nervous system and other functions of the body. It is important to be able to quickly and accurately monitor water sources near agriculture for contamination by these pesticides. This thesis details the early stages of assay development for the detection of AChE inhibitors with a method that utilizes the formation of fluorescent products from the activity of the enzyme. AChE converts 2-naphthyl acetate to form 2-naphthol, a highly fluorescent product, which was detected via high performance liquid chromatography and fluorescence spectrometry (HPLC/FS). The formation of this product is limited as the enzyme activity is inhibited. This inhibition is quantified through the quantification of 2-naphthol formed. Though statistical significance was not analyzed, inhibition percentages of 5.5-70.9% were found for levels of aldicarb, a carbamate pesticide and potent inhibitor of AChE, from 0.0165 to 3.3 ppm. This assay is within an order of magnitude of target sensitivity based on EPA drinking standards, and has room for improvement for sensitivity as well as precision.

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Introduction:

Acetylcholine is the most abundant neurotransmitter in the body, playing important roles in both the peripheral and central nervous systems. Acetylcholine is released into the synaptic cleft of motor neurons in order to activate muscles, and can also be used as a neuromodulator within the brain in order to regulate groups of neurons at a time. After acetylcholine has served its purpose within the synaptic cleft, it is rapidly degraded into choline and acetyl CoA through hydrolysis by the enzyme acetylcholinesterase (AChE), and reabsorbed by the neuron to be recycled and used again. When AChE is interfered with by enzyme inhibitors, acetylcholine is not degraded and reabsorbed and its action is prolonged. This can then lead to incessant stimulation of muscles, glands, and the central nervous system, and depending on the dose, can be fatal[1].

Once these effects were noted in synthesized compounds, they were used to develop nerve agents as weapons of war during the 1930's, and later to develop a new form of pesticides. Carbamates and organophosphate compounds, AChE inhibitors, became the dominant form of pesticides used in the U.S. by the 1970's due to their relatively short persistence in the environment. Though pyrethrin compounds have since largely replaced them in the U.S., both carbamates and organophosphates are still readily available and have significant use across the globe[2].

Exposure to these compounds can take place directly through the application via inhalation and dermal absorption, or indirectly through the ingestion of contaminated foods and water sources. For this reason it is important to be able to monitor both exposure after it has taken place within the biological system, and contamination of food and water sources. From the time that these compounds began being used in agriculture, research on methods of detection have continued. When the specific contamination components are known, traditional detection

and quantification methods utilizing mass spectrometry, gas chromatography, high-performance liquid chromatography, and different couplings of these procedures can be employed. The list of organophosphate and carbamate pesticides that are used is innumerous, however, and they are often used in combination. Thus, it is generally not possible to determine what specific compounds may be contaminating water sources or the blood of an agricultural worker. For this reason, methods that focus instead on the detection of the inhibition of enzyme have been developed in order to detect comprehensive contamination from these types of pesticides. This approach is not new, with inhibition of AChE being utilized for pesticide detection predating the period when organophosphates and carbamates became the dominant form of pesticides used in the U.S.

In the 1930's, methods of quantification for AChE activity were being developed before organophosphates or carbamates had been developed for use as pesticides or nerve agents. In 1933, R. Ammon detailed a method for detecting AChE inhibition that utilized human blood serum, acetylcholinechloride, sodium bicarbonate, and a carbonic acid buffer[3]. The blood serum contained butyrlcholinesterase (a surrogate for AChE in the brain), which cleaved acetic acid from acetylcholinechloride. This acetic acid then reacted with sodium bicarbonate to produce carbon dioxide gas. This gas was then measured as the indicator of reaction completion. Ammon utilized physostigmine, a naturally occurring product of the poisonous calabar bean of West Africa, for AChE inhibition. He was able to successfully demonstrate inhibition by the slowed, yet linear, production of CO_2 over time as the reaction proceeded toward completion (in comparison to the uninhibited system)[3].

By the 1940's, the mechanism of action for organophosphate and carbamate pesticides began to become linked to AChE inhibition, as workers in Britain noted similar symptoms

coming from exposure to the pesticides and what was described for physostigmine poisoning[4]. In 1946, A. Mazur used the detection method described by Ammon to examine the AChE inhibition produced by both physostigmine and diisopropyl fluorophosphate, an organophosphate pesticide[5]. Mazur performed a variety of in vitro and in vivo experiments involving rabbits, rhesus monkeys, and humans in order to investigate both the effect of diisopropyl fluorophosphates on AChE activity and the recovery process after exposure. Through his studies he was able to confirm that this organophosphate pesticide had an effect on the enzyme activity both in vitro and in vivo, and that this effect had a similar potency to that of physostigmine. From his in vitro studies he found that the severity of the effect varied with the type of sample, between red blood cells, serum, brain tissue, and muscle tissue. Through his in vivo studies he demonstrated that for an acute exposure, enzyme activity levels took weeks to return to normal for blood serum in humans, and up to two months for brain tissue in rabbits[5].

In 1949, Michel demonstrated a new and simple method of analyzing cholinesterase activity through the use of pH meters[6]. This method required blood or homogenized tissue as a source for enzyme, and incubation at 37°C for twenty minutes with a barbital buffer and either acetylcholine iodide or butyrylcholine iodide as substrate. The change in pH due to the production of acetic acid from substrate was then used to define the level of enzyme activity, where the blank without the addition of substrate was defined as the baseline. Being a simple and reproducible method, the Michel assay went on to be used and adapted for decades to come[6].

Weiss, in 1958, demonstrated another method for detecting change in AChE following pesticide exposure using fish for exposure subjects[7]. Weiss exposed fish by maintaining their tank water at a controlled contamination level of pesticide (Sarin), then removing and homogenizing the brain tissue. He then mixed brain tissue with acetylcholinechloride, and

residual ACh was reacted with alkaline hydroxylamine in order to provide a color change that could be measured using a photometer. AChE activity was then reported in terms of μmoles ACh split/mg brain/hour. In 1964, Weiss published an additional paper detailing an experiment that utilized the same method for inhibition quantification, but using a multitude of different organophosphate inhibitors with different exposure times, up to 30 days[8]. He found different potencies for the many different inhibitors used, and also investigated the possibility for synergistic effects for compounded exposures. Ultimately, the effect of compounded exposures seemed to indicate an additive interaction.

In 1961, George Ellman and his associates at the University of California San Francisco published a paper detailing a new photometric method for quantifying cholinesterase activity[9]. This method also used blood or homogenized tissue as the source for ChE, but used acetylthiocholine iodide as the substrate within a phosphate buffer. The enzyme hydrolyzed acetylthiocholine into thiocholine and acetate. Thiocholine then reacted with dithiobisnitrobenzoate, a highly rapid reaction, to produce an increase in yellow color that could be detected by a spectrophotometer as an increase in absorbance at 412 nm. The enzyme activity was defined as the increase in the rate of absorbance/min, with a sample containing no substrate serving as the blank. This method became very popular due to its sensitivity, as only 10 μl of blood were required to detect enzyme activity[9].

By the late 1960's, the approach of analyzing comprehensive AChE inhibition in water samples began as a means of evaluating water contamination. In 1967, Malaney and Davis published one of the first papers using this approach[10]. They used an activated carbon column to collect micropollutants from tap water passing through the municipal distribution system, then chloroform gas to remove the adsorbed materials. This carbon chloroform extract (CCE) was

then analyzed for AChE inhibition using a method detailed by Rappaport in 1959[11]. The Rappaport assay involved the use of human blood serum, acetylcholinechloride, m-nitrophenol, and sodium phosphate buffer. Similar to the Ammon method, the enzyme cleaves acetic acid from acetylcholinechloride while the mixture is allowed to incubate for 30 minutes. This acetic acid then lowers the pH in the m-nitrophenol containing solution, which causes a color change that is then quantified by a colorimeter. Malaney and Davis used 1 mg of CCE combined with 1 mL of blood serum to carry out this procedure and were able to demonstrate that AChE inhibiting components were clearly present in the water source. There were 17 different samples, each taken over a few days' time from the passing of 800 gallons of water. On average, for 800 gallons of water there were 334 mg of CCE, with the mean inhibition of enzyme activity (1ml of blood) by 1 mg of CCE being 45%. The authors noted that the biological significance of this effect is low, as one would have to drink about 2 gallons of water to ingest 1 mg of CCE and there are roughly 5,000 ml of blood in the average adult body. However, the paper both showed that the contamination did exist in the water source and demonstrated a new approach to quantifying that contamination.

As decades have passed, the concept of using inhibition as a surrogate for contamination from Malaney (1967) is still being used, but the available technology has remolded the approach. The colorimeters and photometers of the 1950's and 1960's don't stack up against high-performance liquid chromatography, fluorescence spectrometry, gas chromatography mass spectrometry, and other more modern equipment. Most detection methods still revolve around either the Michel or Ellman assays, but methods with different approaches are still being developed. In 1997, Diaz et al. worked toward a new method that utilized fluorescence

spectrometry, and the formation of fluorescent by-products as a means of quantifying inhibition[12].

Diaz et al. used the orphanophosphate fenitrothion as an inhibitor, and worked with different combinations of two enzymes and two substrates. The two enzymes analyzed were acetylcholinesterase and "cholinesterase", while the two substrates used were indoxyl acetate and 2-naphthyl acetate. When reacting with the enzyme, indoxyl acetate is hydrolyzed into 3-hydroxy-indole and acetic acid while 2-nathphyl acetate forms 2-naphthol and acetic acid. These two byproducts, 3-hydroxy-indole and 2-naphthol are each highly fluorescent, thus can be detected at very low concentrations. Diaz et al. looked at how the rate of formation (change in fluorescence with increased reaction time) changed with different concentrations of substrate with and without a constant concentration of inhibitor. They were able to produce kinetic curves for each of the four substrate/enzyme systems, with and without inhibitor present. From these data, inhibition percentages were calculated for the systems at the different concentrations of substrate. It is noted by the authors that the optimal substrate concentration comes from the linear zone of the kinetic curve, or the point where the rate of reaction is optimized, yet the best detection limit comes from using the lowest substrate concentration possible. The less substrate that can be used, the less enzyme is necessary, and the more sensitive to inhibitor the system is. The limiting factor is at what concentration of substrate can the product still be accurately detected by the fluorescence spectrometer? At low concentrations, 2-napthol (the product of the 2-napthyl acetate reaction with enzyme), has a stronger fluorescence than that of 3-hydroxy-indole. Thus, the 2-napthyl acetate system is better suited for detecting lower levels of inhibition[12].

Diaz et al. also looked at the type of inhibition in each system, using Lineweaver Burk plots, where 1/V is plotted against 1/[S], V being reaction velocity and [S] being substrate concentration. The Lineweaver Burk plots demonstrated an intersection of the lines for both the uninhibited and inhibited systems using acetylcholinesterase for the enzyme, indicating non-competitive inhibition. These plots produced for the cholinesterase systems were less indicative; uncompetitive for the system using 2-naphthyl acetate and not determined for the system using indoxyl acetate[12]. Uncompetitive inhibition is less favorable, as it requires the substrate enzyme complex to form to bind and inhibit, thus is better suited for a system with high substrate concentration.

The Talaska lab at the University of Cincinnati is working toward developing a method that combines the experimental methods of Diaz et al. (1997), with the aims of Malaney, Davis (1967). The goal is to develop a detection method for comprehensive AChE inhibition that utilizes the formation of fluorescent products from non-fluorescent substrates. This is to be accomplished using high powered liquid chromatography in tandem with fluorescence detection (HPLC/FS) in order to detect the fluorescent product of the reaction between enzyme and substrate. The substrates used were 1-naphthyl acetate and 2-naphthyl acetate, which form 1-naphthol and 2-naphthol, respectively when interacting with the enzyme. Before this thesis work had begun, the Talaska lab had come to a point in which various details for an assay had been sorted out, such as the use of a sodium phosphate buffer (pH 7, 0.8 M), the use of methanol to "freeze" the reaction at different time points, and the details for HPLC and fluorescence detection. This thesis has revolved around further exploring this assay as an option for fluorescence detection and to determine its reliability and sensitivity.

Methods:

Buffer Preparation/storage

In order to prepare a 0.8M sodium phosphate buffer with pH of 7, first two 1 L solutions of 0.8M monosodium phosphate and 0.8M disodium phosphate were prepared. Using a milligram balance, 95.9 grams of monosodium phosphate (NaH_2PO_4, M.W. 119.98 g/mol) were weighed and then added to approximately 900 mL of Millipore water in a 1 L beaker. This solution was heated and magnetically stirred until solute was dissolved. This solution was then transferred into a 1 L volumetric flask using a glass funnel and brought to volume. For preparation of 0.8M disodium phosphate (Na_2HPO_4, M.W. 141.96 g/mol), the steps were identical, except that 113.6 grams of solute were used.

To create the buffer, approximately 500 mL of 0.8M disodium phosphate solution were added into a 1 L beaker with a large magnetic stir bar. A pH meter probe was situated on one side of the beaker, about halfway down into the solution. 0.8 M Monosodium phosphate solution was slowly added into the beaker, letting the pH reading settle between each addition. Once the solution had a stable pH reading of 7.00 it was transferred into an appropriately labeled 1 L glass bottle and stored in a refrigerator at 8°C, along with the sodium phosphate and disodium phosphate solutions.

At this storage temperature, bacterial growth is prevented but the phosphate solutions form a crystalline precipitate that constitutes approximately 30% of the solution volume. Thus, the buffer solution must be heated, shaken, and stirred as necessary to dissolve this precipitate before use.

50 µM 2-Napthyl Acetate/2-Napthol Standard Solution Preparation/storage

Using a microbalance, 0.0186 grams of 2-naphthyl acetate ($C_{12}H_{10}O_2$, M.W. 186.21 g/mol) were weighed and dissolved in approximately 20 mL of methanol. This methanol was then added to a 2 L volumetric flask and brought to volume with Millipore water. The solution, covered with parafilm, was allowed to stir overnight using a large magnetic stir bar and a hot plate set to low. The 50 µM 2-naphthol ($C_{10}H_8O$, M.W. 144.17 g/mol) solution was prepared using the same methods, but using 0.0144 grams instead. HPLC analysis found that refrigeration drove solute out of solution, so solutions were stored at room temperature and prepared every two weeks.

HPLC/Fluorescence detection

A Waters 2595 HPLC was used in combination with a Waters 2695 fluorescence detector and the Empower 2 software. An isocratic solvent method of 48% methanol and 52% water was used with a run time of 12 minutes, a flow rate of 0.8 mL/min, and an injection volume of 50 µL. An excitation wavelength of 227 nm and emission wavelength of 355 nm were used for the fluorescence detection of 2-naphthol. The 2-naphthol peak formed after about 3½ minutes while the 2-naphthyl acetate peak formed after about 7½ minutes. Both peak height and area under the curve (AUC) were recorded.

Standard Analysis

2-Naphthol standards of 1 µM, 0.5 µM, 0.1 µM, and 0.01 µM were prepared by bringing 10 mL, 5 mL, 1 mL, and 100 µL of 50 µM 2-naphthol standard to volumes of 500 mL. Two samples

from each standard were then analyzed three times each by HPLC and fluorescence spectrometry in order to inform results from reaction analysis.

Enzyme Solution Preparation/storage

Approximately 50 units of acetylcholinesterase enzyme from Sigma-Aldrich from human erythrocytes was used for this experiment, containing ≥500 units/mg enzyme. Using a 1000P micropipette, it was determined that the total volume of concentrated enzyme solution was approximately 230 µL. The end goal was to have a 5 mL enzyme solution with approximately 0.01 U/µL. In order to accomplish this, 0.8 M sodium phosphate buffer solution (pH 7.0) was repeatedly added to the original enzyme containing vial, mixed, and then transferred into the final enzyme solution vial. This was done by adding 477 µL of buffer to the first vial, mixing by recapping/shaking/centrifuging, then setting the micropipette to 490 µL and transferring to the second vial. This was done ten times, until all of the enzyme solution in the first vial was gone and the new solution made up 5 mL. This solution, consisting mostly of sodium phosphate buffer, formed a crystalline precipitate as well while stored in refrigeration. This was combatted by simply warming in the hand and gently shaking. Enzyme solution was split into 1 mL aliquots, to reduce the number of times the solution would have to be brought to room temperature.

Inhibitor Solution Preparation/storage

Being handled particularly carefully, 9.9 mg of Aldicarb was weighed using a microbalance and dissolved in 1 L of Millipore water using a large magnetic stir bar.

Reaction Methods

Five 1.5 mL centrifuge tubes were prepared with labels “0”, “2”, “5”, “10”, and “15”, along with a 7 mL scintillation vial labeled “Rx”. The numbers represented the minute at which that portion of the reaction was stopped and the vial labeled “Rx” contained the reaction mixture. A half mL of methanol was added to each numbered vial, 50 µL of 2-naphthyl acetate solution and a combination of buffer and Aldicarb solution (buffer vol + inhibitor vol = 2.75 mL) were added to the reaction vial. All centrifuge tubes remained capped when not being used. The reaction vial was vortexed, and then a 0.5i mL was removed and added to the “0” tube and vortexed. Then, 200 µL of enzyme solution, or approximately 2 units, were added to the reaction vial and this vial was vortexed. After vortexing, a timer was started and at every time point, a half mL was removed from the reaction vial, added to the respective time point tube, capped, and then vortexed. After these steps were completed, each fraction was prepared for HPLC. Since, upon the meeting of the sodium phosphate buffer solution and methanol, a precipitate was formed, centrifugation was required all samples before HPLC (filtration was initially used to remove precipitate, but was found to interfere with consistency of 2-naphthol detection). After centrifugation, 0.75 mL of the supernatant was removed and added to a 1.5 mL HPLC vial for analysis. For six different inhibited reactions, 5 µL, 50 µL, 100 µL, 250 µL, 500 µL, and 1 mL of 9.9 mg/L aldicarb were used, corresponding to 0.0165 ppm, 0.165 ppm, 0.33 ppm, 0.825 ppm, 1.65 ppm, and 3.3 ppm levels of inhibitor within the 3 mL of total reaction volume.

Results:

Standard analysis

For a full list of the HPLC/FS results of standards, please refer to table 5 in the Appendix. The coefficients of variation (CV) for area under the curve (AUC) and peak height were calculated for each sample tested (8 samples, 3 measurements each) and for each standard (4 standards, 6 measurements each). The results from the latter, which are consistent with those seen from the sample CV analysis, can be seen below in Table 1.

Table 1. Comparison of coefficients of variation for area under the curve and peak height for standards

Standard (µM)	AUC CV	> or <	Height CV
0.01	0.638	>	0.215
0.1	0.996	>	0.287
0.5	1.069	>	0.804
1	9.204	>	2.968

From Table 1 it can be seen that the CV values for peak height measurements were consistently and considerably lower than those of AUC measurements. For this reason, peak height will be the predominant parameter analyzed for this assay. Figure 1 demonstrates that there is a highly linear relationship between peak height and standard concentration. Table 2 shows the results of the p value analysis for t tests (two tailed, type 2) comparing the peak height measurements for the two samples taken from each standard and demonstrates that for each standard, the sample peak height values are not statistically different.

Figure 1. Peak height results from HPLC/FS analysis are plotted against the concentration of the 2-naphthol standard.

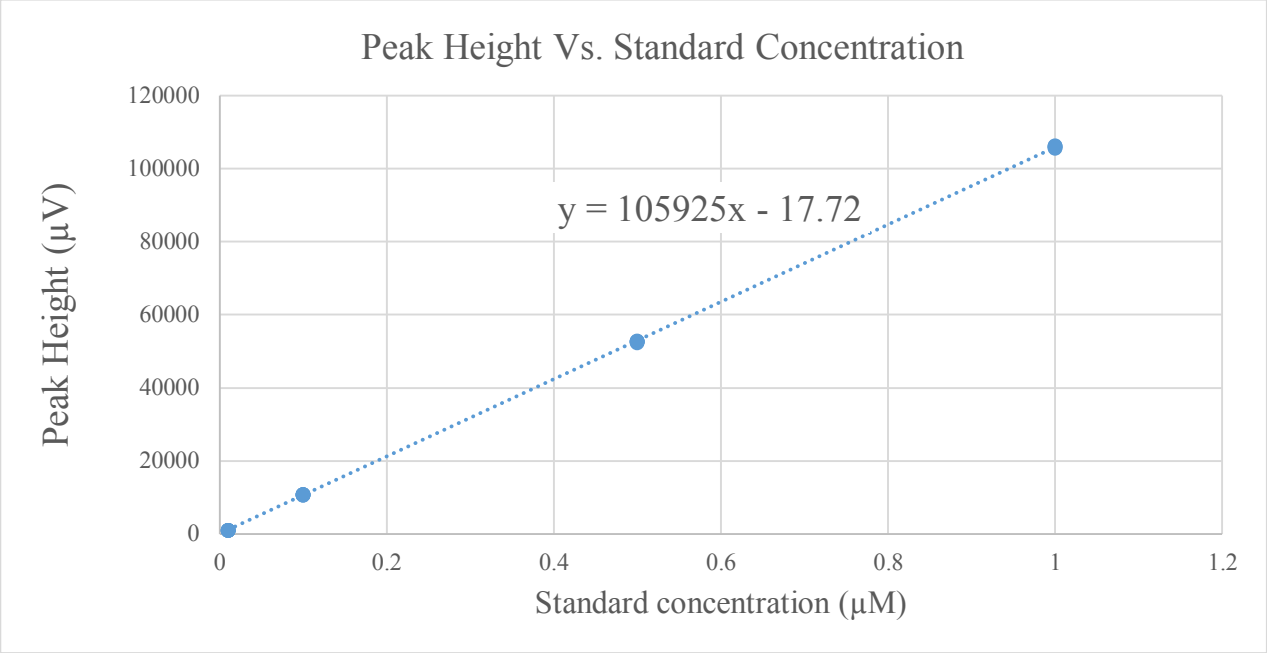


Table 2. P-values from two tailed/type 2 t tests comparing peak height results from the two samples from each standard

Standard (µM)	P-value
0.01	0.568
0.1	0.083
0.5	0.622
1	0.499

Uninhibited System

Two reactions were run with no inhibitor present in order to demonstrate the completion of the reaction. The peak height results for both 2-napthol and 2-naphthyl acetate can be seen in Table 3.

Table 3. HPLC/FS results from uninhibited systems #1 and #2 showing the height increase for the 2-naphthol peak and height decrease for the 2-naphthyl acetate peak.

	Uninhibited Reaction #1		Uninhibited Reaction #2	
Rx Time (min)	2-Nap Height (μV)	2-NA Height	2-Nap Height	2-NA Height
0	3315	1851	4523	1678
2	19107	882	20701	880
5	31008	415	31532	383
10	38673	ND	39642	ND
15	41075	ND	42441	ND

Using the trend line equation from figure 1 (y = 105925x – 17.72), we can calculate the final 2-naphthol concentrations from the uninhibited reactions, where y is the peak height and x is the concentration. Peak heights of 41,075 and 42,441 μV correspond to 2-naphthol concentrations of 0.39 μM and 0.40 μM, respectively

Inhibition Results

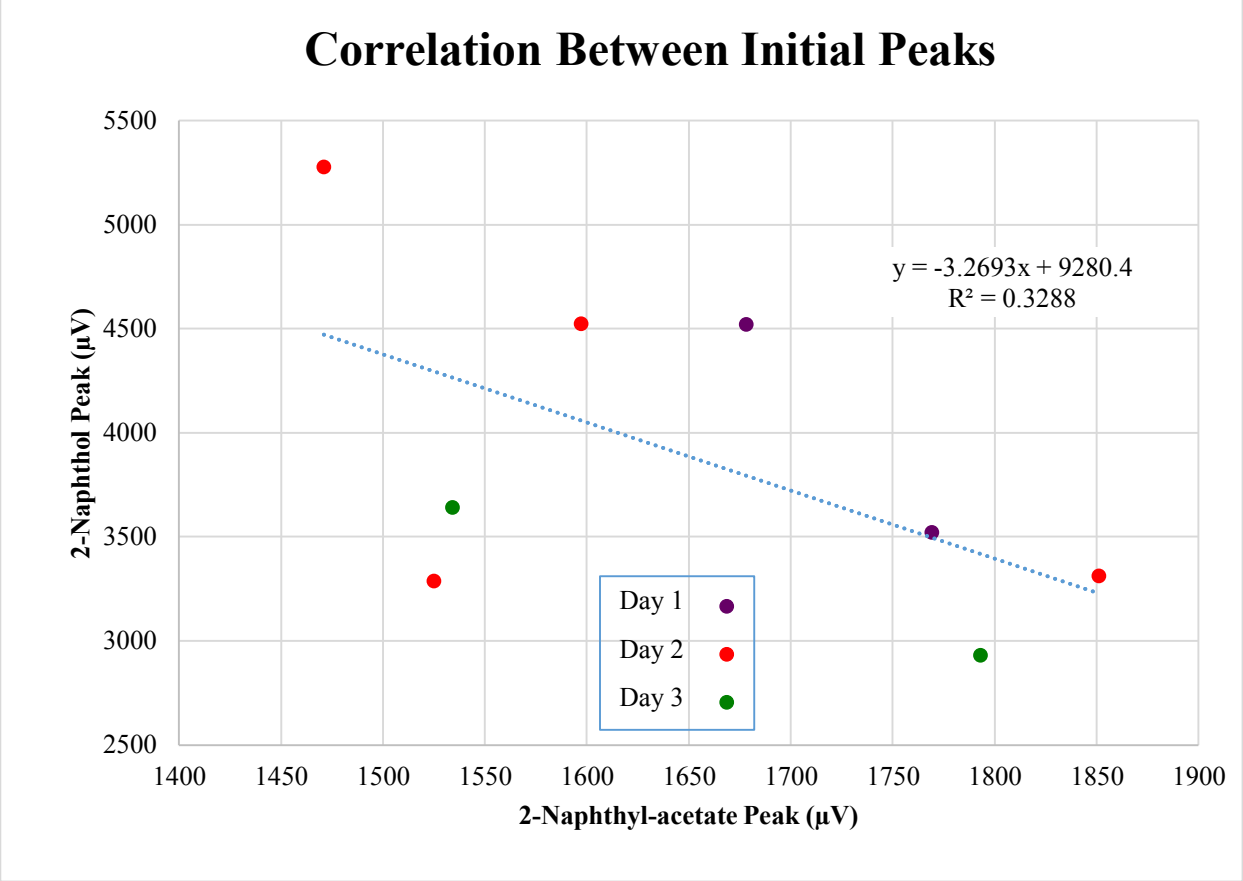
For the full table of HPLC/FS results of the inhibited reactions, refer o Table 6 in the Appendix.

Tables 4 demonstrates the variability in initial peak height for each reaction.

Reaction	Initial Peak Height 2-Naphthol (μV)	Mean	3878.8
Uninhibited 1	3315	Standard Deviation	805.5
Uninhibited 2	4523	Coefficient of Variation	20.8
0.0165 ppm	5280		
0.165 ppm	3288		
0.33 ppm	3523		
0.825 ppm	2933		
1.65 ppm	4526		
3.3 ppm	3642		

Figure 2 shows the values for the initial peak heights for both 2-naphthol and 2-naphthyl acetate plotted against one another.

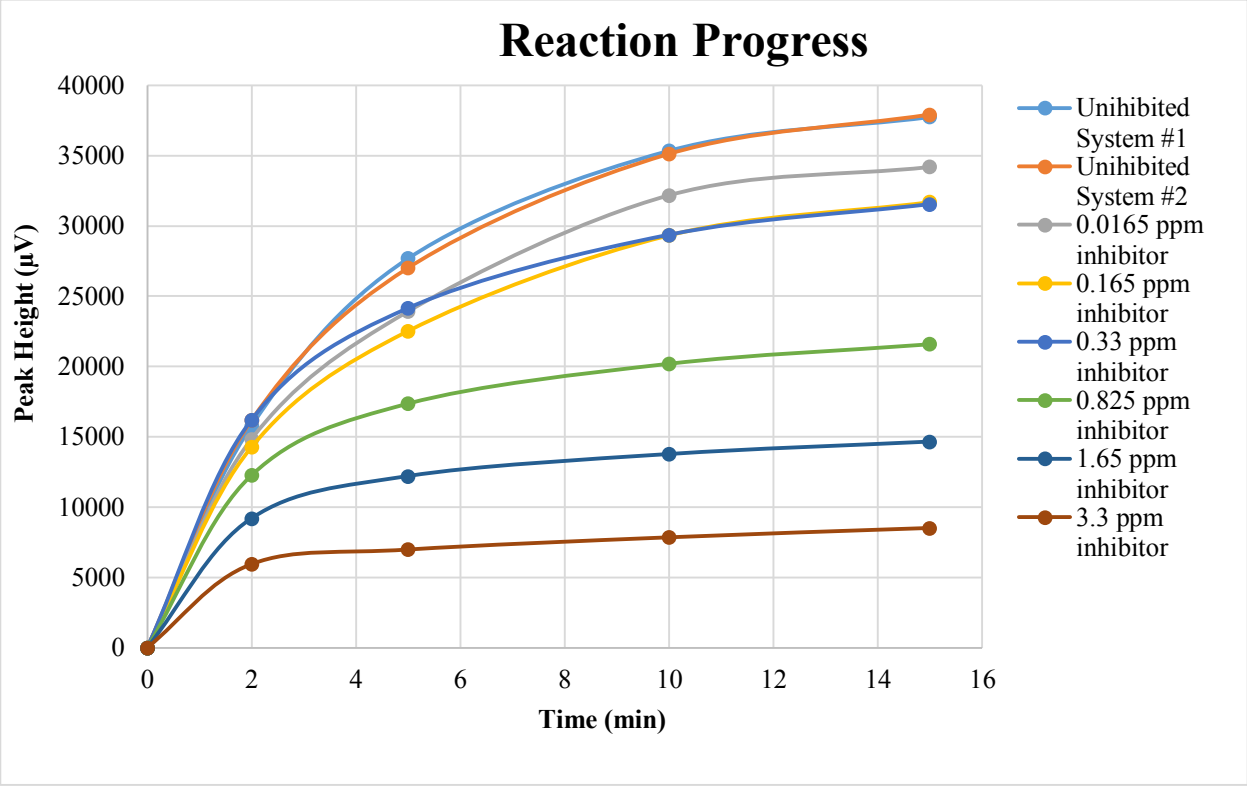
Figure 2. Initial peak heights for 2-naphthol vs. 2-naphthyl acetate, with day of sampling indicated.



See Figures 6-10 in the Appendix for examples of chromatographs, where both 2-naphthol and 2-napthyl acetate peaks can be seen. The example chromatographs come from the first uninhibited reaction.

Figure 3 shows the progress of each reaction, inhibited and uninhibited, via 2-naphthol peak height with initial peaks subtracted from each time point.

Figure 3. Peak height plotted against reaction time point to demonstrate reaction progression for all 8 reactions. Zero minute peaks have been subtracted from each time point height.



In order to calculate % inhibition, the rate of the reaction of the inhibited system is compared to the rate of reaction of the uninhibited system, with rate of reaction being the change in fluorescence divided by the change in time.

$$Inhibition\ \% = \frac{\Delta F^*/\Delta t - \Delta F/\Delta t}{\Delta F^*/\Delta t} \times 100$$

-Where ΔF*/Δt is equal to the average change in fluorescence over time between the two uninhibited systems, which is 2,522 µV/min.

For the inhibited systems containing 0.0165 ppm, 0.165 ppm, 0.33 ppm, 0.825 ppm, 1.65 ppm, and 3.3 ppm, inhibition percentages of 5.5%, 16.2%, 16.0, 41.3%, 54.0%, and 71.9% were

calculated respectively. See Figure 4 for graphical representation of this data. Figure 5 shows the inhibition percentages if inhibition is calculated using the 5-15 minute peaks.

Figure 4. Concentration of Aldicarb vs. Inhibition % from 0 to 15 minutes.

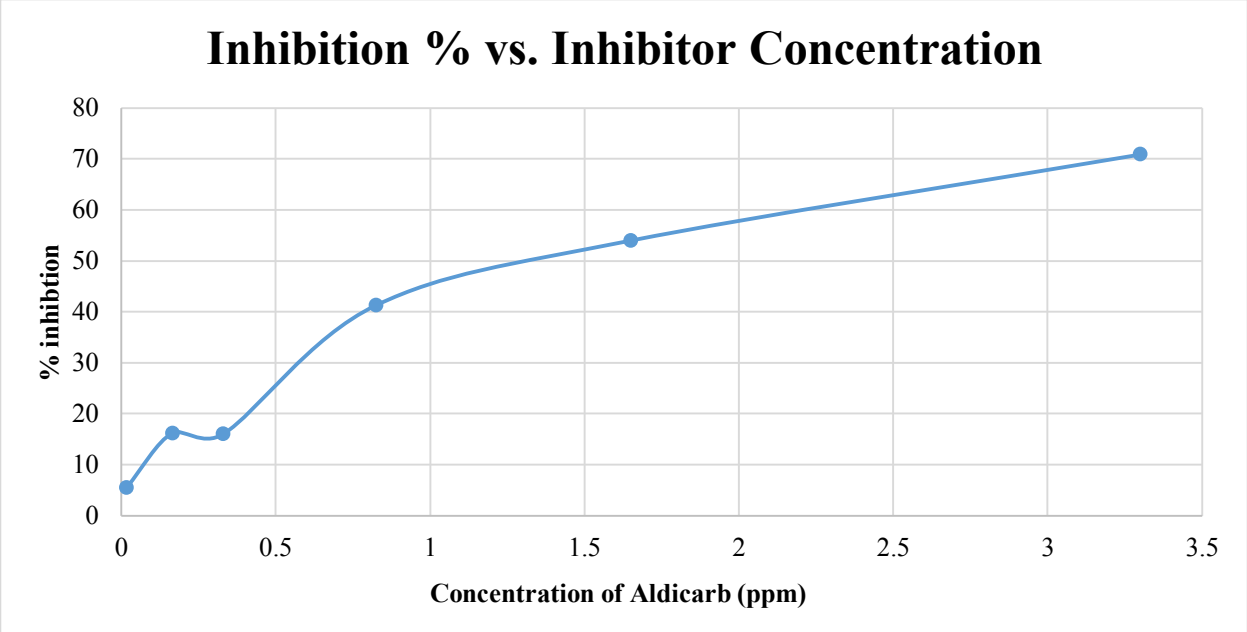
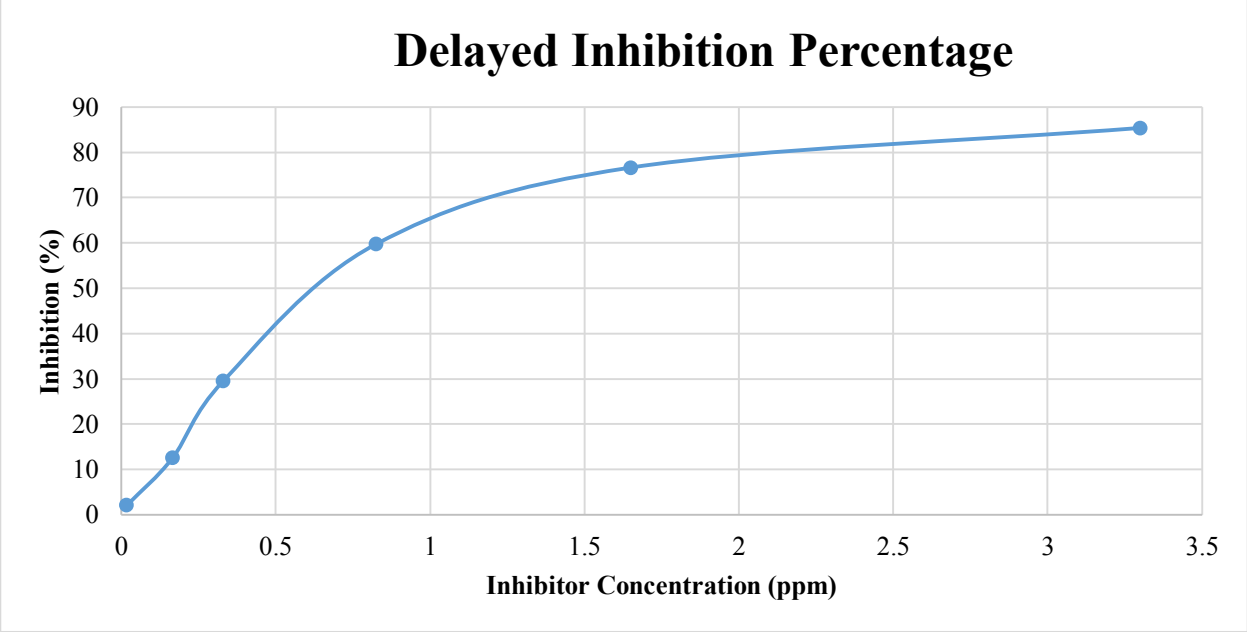


Figure 5. Inhibition % calculated from velocity of reaction from time 5 minutes to 15 minutes.



Discussion:

The results for both area under the curve measurements and peak height measurements from the standard analysis demonstrate a highly linear relationship between standard concentration and HPLC/FS results. This linear relationship can be observed in Figure 1. The trend line from Figure 1 also provides the equation for converting from peak height to 2-naphthol concentration (μM). The coefficients of variation, seen in Table 1, show more consistency for peak height results taken from samples within the same standard compared to that of area under the curve measurements. Area under the curve measurements may be more susceptible to inconsistencies within the HPLC/FS system. A slight change in retention time and peak width can have an affect on the area under the curve measurement. Due to the superior CV values for the peak height measurements from the standard analysis, peak height was selected as the primary variable for analysis going forward. The relatively high p-values from table 2 show that samples taken from the same standard produce results that are not statistically different. Thus, the variability in solute of well-mixed solutions should not be a major contributing factor toward error in this assay.

Table 3 demonstrates the completion of the two uninhibited reactions via both the formation of 2-naphthol and the disappearance of 2-naphthyl acetate. At the excitation and emission wavelengths used, 2-naphthol is roughly twenty times as fluorescent as 2-naphthyl acetate. However, an observable 2-naphthyl acetate peak is still formed from the HPLC/FS analysis, and its disappearance can be monitored as a second manner for monitoring reaction progress. Both reactions follow a similar curve, and the variability between them is accounted for when an adjustment based on the 0-minute peak is made, where the difference in the total change in fluorescence (peak height) between the two reactions is only 158 μV (<1% of the

mean). The ending peaks were calculated to represent 2-naphthol concentrations of 0.39 and 0.40 μM, which would correlate to estimate reaction completions of 94% and 96% (50 μL of 50 μM 2-naphthyl acetate, 3 mL of reaction, each ½ mL of reaction component dissolved in ½ mL of methanol).

There was a wide variability in initial peak height for 2-naphthol, where standards at lower concentrations were far more consistent. 2-Naphthol spontaneously forms from 2-naphthyl acetate at a slow rate, so the initial 2-naphthol peak is inevitable. This variation, as can be seen from table 4 is considerably large, with a CV of 20.8, and must be accounted for when analyzing the results for the progress of the reaction and comparing reaction curves. Figure 2 shows that there is a very weak negative correlation between the initial concentration of 2-naphthol and 2-naphthyl acetate (Slope = -3.27, $R^2 = 0.329$). If the correlation were strongly positive, than this would imply an overall variability of substrate within solution, and if it were more strongly negative, than it would imply that the increased formation of 2-naphthol from 2-naphthyl acetate with time was the culprit. It is likely, however, that both of these factors are at play here to an extent. All reactions were carried out within the span of 3 days, and as can also be seen from figure 2, there does not appear to be a pattern between day of experiment and 2-naphthol/2-naphthyl acetate initial peak heights. Daily heating/stirring of substrate solution may have a positive impact on the variability of solute within solution. However, given the uncertainty behind this variability, it is best to simply subtract the initial peak from each data point when plotting curves and to do no further complex adjustments.

The adjusted reaction curves in Figure 3 show that as the reactions progress, the effect of inhibition becomes starker. Figures 3 and 4 show an irregularity within an otherwise desirable dose response relationship. From Figure 3, the rate of reaction for the first two minutes of the

0.33 ppm inhibited system is higher than even that of the uninhibited systems, but ultimately the curve settles just below that of the 0.165 ppm reaction. The % inhibition for the 0.33 ppm aldicarb reaction lies well below the rest of the curve line. There are a few possible explanations for this. This could be due to the variability in substrate between the reactions, or it could be due to variance in enzyme quality. The enzyme was separated into aliquots that were ultimately larger than the amount used for a reaction. Thus, it is possible that the enzyme used from a fresh aliquot would lead to a reaction that would complete faster than enzyme from an aliquot that had been taken from the refrigerator. Regardless, if the first 2 minutes of the reaction are discounted, and the only the velocity of the reaction from 5-15 minutes is considered, the curve relating inhibition % to concentration of inhibitor becomes a bit more like what would be expected. This is demonstrated in Figure 5.

This thesis work demonstrated that the assay is a viable method for detecting inhibition of AChE. Although with imperfection, a clear dose response relationship was established and the groundwork for a more competitive assay has been laid out. Though not proven to be statistically significant, inhibition was detected at 16 ppb levels of a potent inhibitor, aldicarb. To determine the usefulness of an assay of this nature, the level of inhibitor detected must be compared to drinking water standards for that inhibitor. As drinking water standards for AChE inhibitors are based on their inhibition capability, it can be inferred that an assay's ability to detect toxicologically relevant levels for one inhibitor would reflect it's ability to detect toxicologically relevant levels of AChE inhibitors in general. The MCL_G set by the EPA for aldicarb in drinking water, or the lowest level for which there is no known or expected risk to human health, is 0.001 mg/L (ppm). The MCL set by the EPA for aldicarb, or the highest level of contaminant allowed in drinking water is 0.003 mg/L (ppm)[13]. For an assay to be truly useful

in the detection of AChE inhibition, it must have a limit of detection at or below the MCL, or even better, the MCL_G. At this point, the assay is not sensitive enough to detect inhibition at these low ppb levels, and is short by at least one order of magnitude. The assay needs to be improved both in terms of precision, so that there can be a high level of confidence in results, and sensitivity, so that more toxicologically relevant levels can be detected. Luckily, however, there is much room for improvement on this assay.

The concentration of sodium phosphate buffer previously used by the lab is notably high (0.8 M) and worth reconsideration. If the buffer concentration were lower, there may not be the precipitation that happens for the refrigerated storage of both the stock buffer and the enzyme solution prepared in buffer. Mild warming and shaking of the enzyme solution is necessary to dissolve the sodium phosphate precipitate, which may impact the integrity of the enzyme and opens the door for variability. Also, as groups of reaction components are HPLC/FS analyzed at a time for efficiency, further precipitate forms between methanol and buffer over the timespan of 1-2 hours. These solids pose a risk to the HPLC system, so after they have formed, further analysis of samples is unwise. Thus, repeated measurements of individual reaction components are difficult to obtain and statistical significance through the use of t-tests is not possible. With a lower concentration of buffer, this precipitate formed between the buffer and methanol may be less of a problem and repeated measurements may be more easily obtained. The aliquots used for enzyme solution storage must be the same amount that is used for an individual reaction, in this case, 200 µL. This will limit the number of times enzyme is removed from/placed back into refrigeration and will reduce the variability of enzyme performance. Temperature should also be controlled when carrying out these reactions, as variability in temperature will contribute to variability of results. These reactions were run at room temperature, which varied (but was not

recorded) from day to day. If money was no object, higher levels of substrate and enzyme could be used, the latter of which being the limiting factor with regards to cost. Carrying the reaction out at a more biologically relevant temperature as opposed to room temperature may allow for more efficient enzyme activity, allowing more substrate to be used while still completing the reaction in a reasonable time frame. These are all factors that could greatly contribute to improving the precision of the assay.

In terms of enhancing the sensitivity of this assay, the single greatest factor may be the point at which the enzyme and inhibitor are introduced. As can be seen by the slopes from figure 3, the effects of the inhibition are more prevalent as the reaction proceeds. This may be due to the lack of interaction between inhibitor and enzyme before the reaction is began. The current assay involves the addition of enzyme as the final step, but if substrate were added as the final step after enzyme and inhibitor were allowed some prior time to interact, then inhibition may be possible to detect at lower levels of inhibitor.

In order to apply this method of detection of laboratory prepared standards to the detection of real world water samples, some minor adjustments to the protocol would need to be made to maintain the level of sensitivity. It should also be noted that many of organophosphate pesticides are not toxic until, or become more toxic after, they have been bioactivated through metabolism. Diazinon is a known example of one of these compounds, as it has been shown to become a more potent inhibitor of AChE after bioactivation through the activity of the P450 enzyme system[14]. Thus, further research should be done on how this can be accounted for by an assay using inhibition of AChE as the primary marker for contamination.

Conclusion:

A pilot assay for the detection of AChE inhibition has been put forth with considerable success. The compound of focus for HPLC/FS detection, 2-naphthol proved to be a highly sensitive and precise target. The overall assay showed a dose-response relationship, with inhibition percentages ranging from 5.5-70.9% for aldicarb concentrations of 0.0165-3.3 ppm, though statistical significance of inhibition was not able to be determined. The lowest level of inhibitor detected was an order of magnitude greater than toxicologically relevant levels, but many improvements can be made to the assay to move it closer toward this degree of sensitivity and precision. This method should be considered and improved upon for use in detecting organophosphate and carbamate pesticide contamination of water sources.

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Appendix

Figure 6. Chromatograph produced by Empower software. Uninhibited reaction 1, time 0.

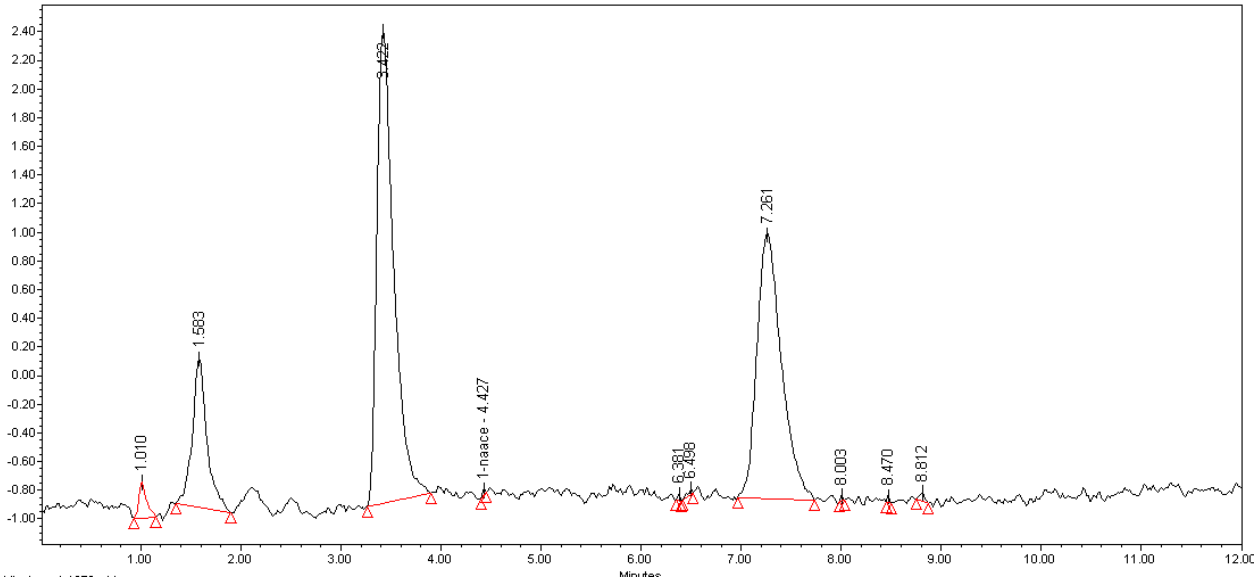

Figure 7. Chromatograph produced by Empower software. Uninhibited reaction 1, time 2.

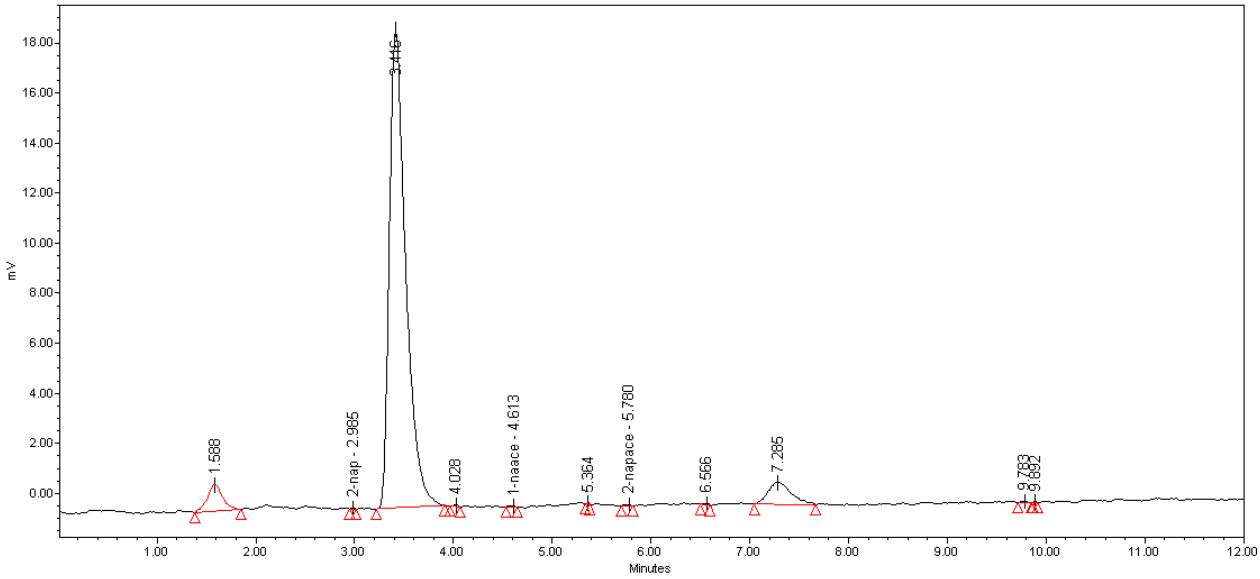

Figure 8. Chromatograph produced by Empower software. Uninhibited reaction 1, time 5.

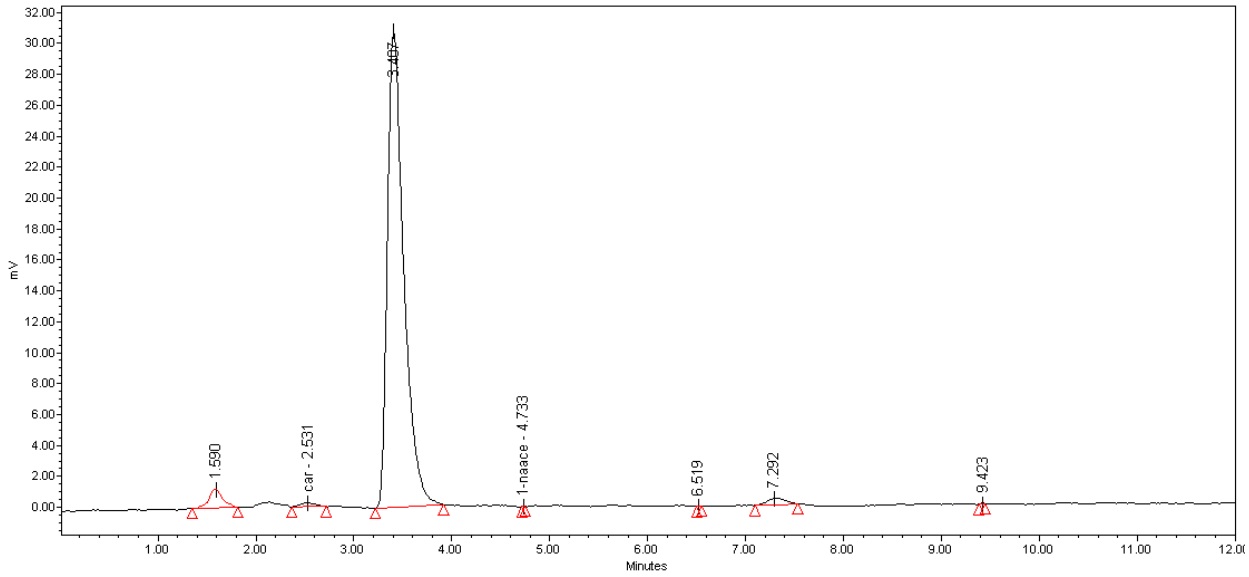

Figure 9. Chromatograph produced by Empower software. Uninhibited reaction 1, time 10.

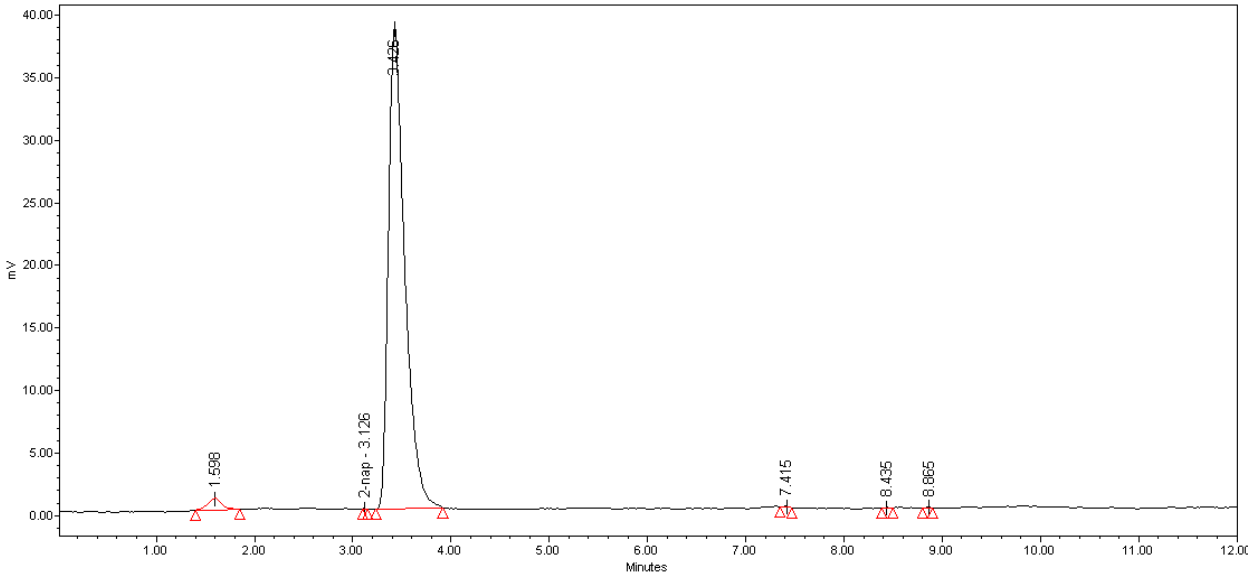

Figure 10. Chromatograph produced by Empower software. Uninhibited reaction 1, time 15.

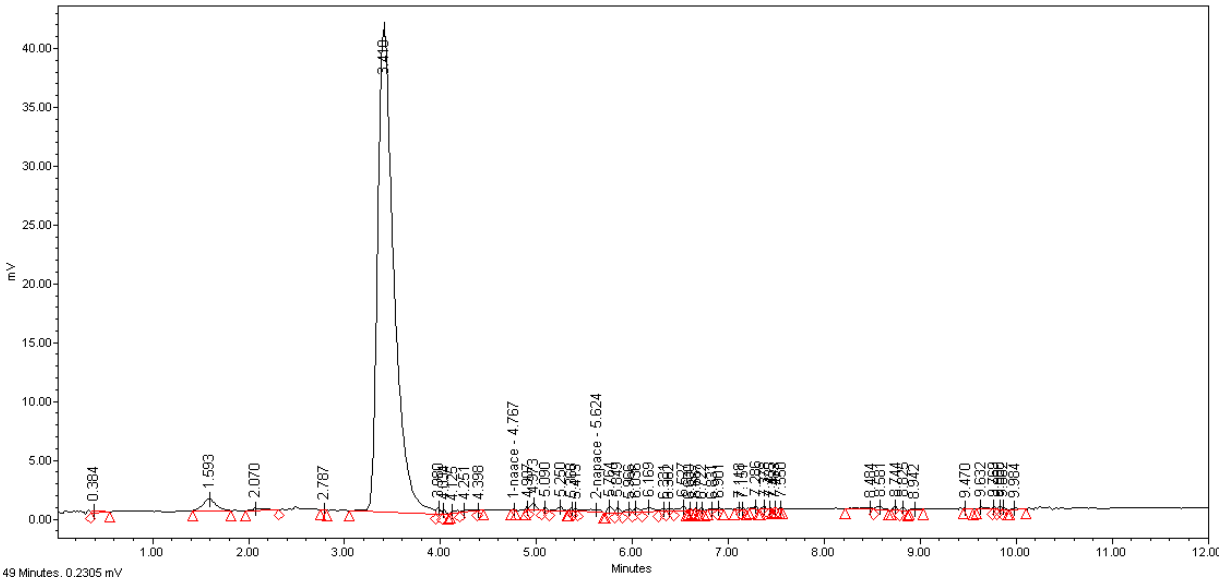

Table 5. Full results from standard analysis. Peaks from 50 µM standards plateaued and should not be compared to other results.

Standard concentration (µM)	sample	inj #	AUC (µV*sec)	Peak Height (µV)
50	1	1	31501737	1089377
50	2	1	32408877	1096703
1	1	1	1750955	105722
1	1	2	1762710	106128
1	2	1	1770007	106138
1	2	2	1777654	106281
1	2	3	1777662	105861
0.5	1	1	884856	52798
0.5	1	2	887938	52701
0.5	1	3	894349	52766
0.5	2	1	899196	52426
0.5	2	2	903874	52727
0.5	2	3	907621	52860
0.1	1	1	186497	10797
0.1	1	2	187175	10809
0.1	1	3	190640	10841
0.1	2	1	187271	10599
0.1	2	2	190375	10744
0.1	2	3	190858	10726
0.01	1	1	11868	1000
0.01	1	2	12309	985
0.01	1	3	14607	1041
0.01	2	1	13546	995
0.01	2	2	13505	1010
0.01	2	3	15046	951

Table 6. Complete results from inhibition reactions.

Inh ppm	Vol	Rx Time	AUC	Height	Height - Initial
0.0165	5	0	63109	5280	0
0.0165	5	2	246227	20129	14849
0.0165	5	5	362901	29205	23925
0.0165	5	10	461790	37463	32183
0.0165	5	15	496135	39478	34198
0.165	50	0	40121	3288	0
0.165	50	2	210046	17566	14278
0.165	50	5	308999	25823	22535
0.165	50	10	391658	32633	29345
0.165	50	15	421033	34996	31708
0.33	100	0	43877	3523	0
0.33	100	2	248199	19730	16207
0.33	100	5	350481	27672	24149
0.33	100	10	419476	32886	29363
0.33	100	15	444481	35060	31537
0.825	250	0	41506	2933	0
0.825	250	2	218523	15202	12269
0.825	250	5	292480	20300	17367
0.825	250	10	334447	23134	20201
0.825	250	15	356599	24522	21589
1.65	500	0	57093	4526	0
1.65	500	2	177777	13737	9211
1.65	500	5	216329	16741	12215
1.65	500	10	233673	18308	13782
1.65	500	15	250598	19194	14668
3.3	1000	0	49669	3642	0
3.3	1000	2	141075	9591	5949
3.3	1000	5	58439	10627	6985
3.3	1000	10	172842	11498	7856
3.3	1000	15	179640	12164	8522