5-2011

Oxidative Derivatization of Folic Acid Monitored by Capillary Electrophoresis with UV Absorbance and Fluorescence Detection

Jeffrey P. Cunniff

Oxidative Derivatization of Folic Acid Monitored by Capillary Electrophoresis with UV Absorbance and Fluorescence Detection

by

Jeffrey P. Cunniff

An Undergraduate Honors Thesis under the direction of

Dr. S. Douglass Gilman

Department of Chemistry

Submitted to the LSU Honors College in partial fulfillment of

the Upper Division Honors Program

May 2011

Louisiana State University

& Agricultural and Mechanical College

Baton Rouge, Louisiana

Table of Contents

Abstract

Folic acid is an important biological compound required for numerous bodily functions involving cell growth and division. A lack of folic acid can give rise to folate deficiency, which can have severe effects on fetuses. Folic acid is commonly detected by UV absorbance spectroscopy, sometimes in conjunction with separations techniques. There are several reports in the literature that conclude that the oxidation of folic acid produces highly fluorescent products, which would provide lower detection limits relative to absorbance techniques. This work investigated five oxidants (sodium hypochlorite, hydrogen peroxide, potassium ferricyanide, sodium nitrite and potassium persulfate) and determined their effectiveness for oxidation of folic acid. The reactions were conducted off-line and the reaction components were subsequently separated using capillary electrophoresis. The reaction was monitored by both laser-induced fluorescence (LIF) to detect fluorescent products and UV absorbance to detect both folic acid consumption and reaction product formation. The folic acid concentration used was 100 μM and the samples were subjected to both low oxidant concentrations of 100 μM (1:1 molar ratio) as well as high concentrations of 10 mM (1:1000 molar ratio). An oxidizing agent was considered successful if a fluorescent product was produced as detected by LIF and if the area of the folic acid peak detected by UV absorbance decreased. Sodium hypochlorite was found to be the best oxidizing agent of the five studied. New fluorescent product peaks were observed along with a reduction in the folic acid peak area by 81% (UV absorbance) for low oxidant concentrations and a reduction of 100% under high oxidant concentrations. The determination of the most effective oxidizing reagent could lead to the development of more sensitive and accurate methods of folic acid detection.

Introduction

Folic Acid

Folic acid (FA) was discovered in 1931 by Lucy Wills as a component of yeast capable of curing what she called "pernicious anemia of pregnancy".[1] The true structure of folic acid was not determined for another 15 years due to the enormous diversity of folate structures with variations ranging from substitution differences in the length of the glutamyl side chain to the oxidation state of the pterin ring structure. The form shown below (Fig. 1) typically is referred to as folic acid or folate.[1,2] Reduced folates are synthesized by plants naturally, but folic acid does not occur in nature to any notable extent. Instead it is produced by commercial synthesis with chemically similar properties to the many folates and can be found in trace amounts in many vitamin supplements and in fortified foods such as cereals and flours.[1,3]

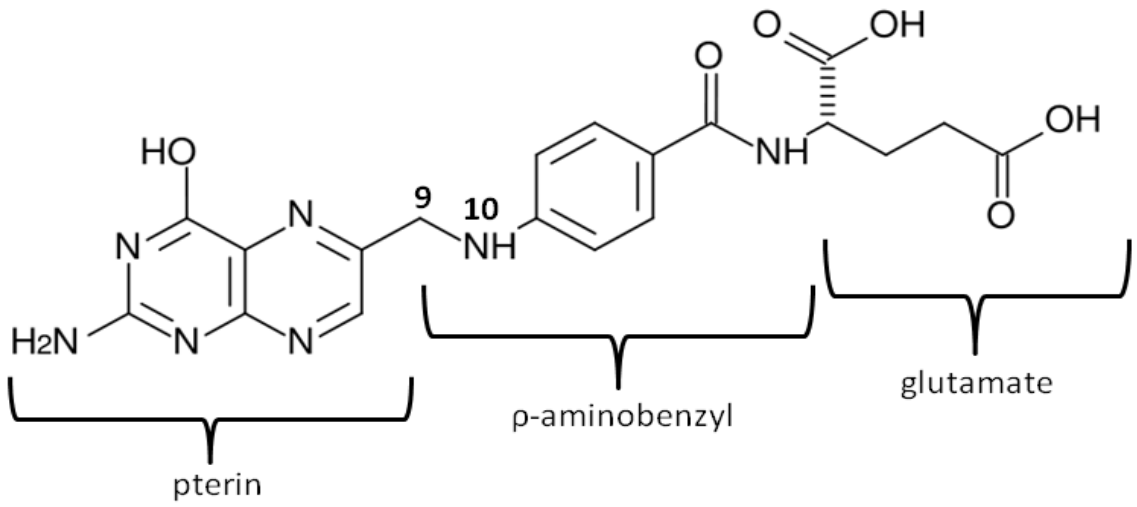

Figure 1. Chemical structure of folic acid. Three distinct groups make up the structure: pterin, ρ-aminobenyzl and glutamate residues. Oxidative cleavage of folic acid is believed to occur at the C9-N10 bond.

Biologically, folates are cofactors and substrates for numerous bodily functions centered on cell growth and division processes as well as metabolic regulation. Upon entering the body, folic acid is biologically inactive and needs to first be converted into an active form such as tetrahydrofolate (THF). The formation of THF from folic acid is carried out in the liver by the enzyme dihydrofolate reductase, which reduces another folate derivative, dihydrofolate (DHF), to THF.[2,3] This process is surprisingly slow even with an enzyme present and sets a limit for how much folic acid the body receives and can utilize at any time.

Once folic acid is converted into THF or DHF it participates in various biological pathways, most notably in DNA biosynthesis and the methylation cycle.[1,3] In DNA synthesis THF is one compound involved in the synthesis of purines and pyrimidines and acts as a substrate in single-carbon transfer reactions, such as remethylation, to produce complete viable DNA strands. In the methylation cycle THF helps to supply numerous methyltransferase enzymes with S-adenosylmethionine (SAM) which is an important methyl donor involved in the production of homocysteine.

Folic acid is vital to the proper functioning of the body, but the difficulty in obtaining it in a regular diet can lead to health problems. A shortage of folic acid in the body is clinically referred to as folate deficiency. A deficiency of folates in the diet can lead to a number of health problems such as anemia due to a shortage of viable red blood cells being produced in the bone marrow. Low amounts of folates result in dwindling amounts of THF throughout the body, which disrupts biosynthesis cycles such as the methylation cycle where depletion can

lead to an accumulation of homocysteine. The build-up of homocysteine and onset of anemia are thought to be linked to various forms of vascular disease such as coronary heart disease.[1,3]

Pregnant women are most susceptible to folate deficiency since they require enough folates to adequately sustain biological functions in themselves and in their developing fetus. Folate deficiency can have severe effects on a fetus as it severely hinders the rate of cell division and growth. This can result in neural tube defects, which involve malformations of the spine, skull and brain.[1]

Capillary Electrophoresis

Electrophoresis is a process that involves the differential movement or migration of ions in an applied electrical field. The driving force of the separation technique is the voltage gradient. Two electrodes are placed in an ionic solution, and when a potential is applied, cations and anions move in opposite directions at different migration speeds towards their oppositely charged electrodes. Capillary electrophoresis (CE) takes the principles of electrophoresis and utilizes it in buffer-filled capillaries ranging from 25−100 μm internal diameter.

The instrumentation for CE separations is fairly straightforward with each end of a capillary placed in a separate buffer reservoir containing an electrode connected to a power supply. Samples are injected by replacing one of the buffer reservoirs with the desired sample and applying a brief electric potential or pressure to introduce a short plug of the sample into the capillary. Once the sample is injected, the sample solution is replaced by the buffer reservoir and an electric potential is applied to perform the ion separation. Detectors can be

placed at a "window" created farther down the capillary that provides a gap in the capillary's outer polymer coating, allowing for analysis of the injected sample by various optical methods. Commonly, the detector is located near the cathodic end of the capillary, and samples are injected into the capillary at the anodic end.

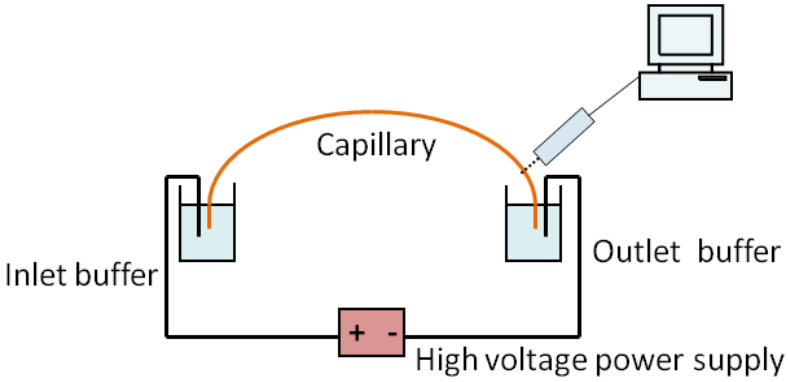

Figure 2. Capillary electrophoresis system. A high-voltage power supply is connected between the inlet and outlet buffer reservoirs, and a capillary is submerged in each buffer solution. When voltage is applied, the buffer flows from the inlet buffer to the outlet buffer and the solution can be detected optically at the window near the outlet.

The separation mechanism of CE depends on differences in migration velocity of the sample ions known as described by electrophoretic mobility. The equation for calculating electrophorectic mobility (μ_e) is:

$$\mu_e = \frac{q}{6\pi\eta r}$$

Where q represents the ion charge, η is the viscosity of solution and r is the ion hydrodyamic radius. In CE, ions are separated based on a ratio of their charge to hydrodynamic radius (size).[4] Highly charged, small ions have higher mobility than weakly charged, large ions, towards their respective electrodes. Electrophoretic mobility is a constant for a given ion in a given buffer system and is proportional to the speed of migration. Utilizing the electrophoretic mobility

equation and common ionic properties, additional conclusions can be formed such as that divalent cations will migrate twice as fast as univalent cations of the same size and that low viscosity solutions and high ion charge will result in increased mobilities and, thus, faster separations.

While electrophoretic mobility is the basis of CE separations, electroosmotic flow (EOF) is the driving force for the bulk flow of buffer solution through the capillary. Fused-silica capillaries are used in CE and are pretreated with a strong base to slightly hydrolyze the inner surface, clear away any contaminants inside the capillary and increase the number of silanol groups available on the inner surface. The silanol groups are deprotonated at neutral or basic pH resulting in a negatively charged capillary wall. The negatively charged wall attracts positive ions and solvated water molecules in the buffer solution, and they form an electrical double layer along the inner wall of the capillary. A potential difference is also formed at the electrical double layer known as zeta potential. Zeta potential is the electrical potential at any location in the double layer, and its strength decreases exponentially as the distance from the capillary wall increases. Once the double layer has formed, cations that are too far removed from the capillary wall possess a weak zeta potential. The electrical force on these cations from the double layer is not strong enough to overcome the electrical force from the negative cathode, and, as a result, they migrate towards the negatively charged cathode dragging the bulk solvent/buffer along, resulting in a bulk flow of solution down the capillary.[4]

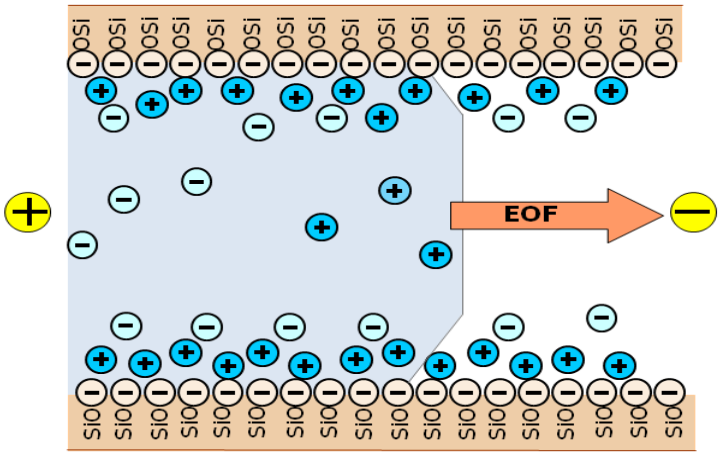

Figure 3. Electroosmotic flow (bulk fluid flow). Silanol groups on the inner surface of the capillary are deprotonated and cause the capillary wall to become negatively charged. A double layer is formed when positive charges become attracted to the negatively charged wall. Cations with a low zeta potential experience a greater force of attraction from the negative cathode than the negative capillary wall double layer, and ultimately migrate toward the cathode, dragging the bulk solvent along to generate a bulk flow of solution.

The EOF depends on the dielectric constant, the viscosity of the buffer and, most importantly, the pH of the buffer solution. If the buffer is more acidic or basic, the net charge of the capillary surface can dramatically change. For example, at basic pH values, there is a greater amount of negative charge on the capillary wall due to deprotonation of the silanol groups. This strong negative charge leads to a larger zeta potential and a larger EOF. At basic pH values, the EOF mobility is typically sufficient to ensure the net migration of most ions towards the negatively charged cathode regardless of their individual charge.[4]

When determining an ion's migration rate, it is important to take both EOF and electrophoretic mobility into consideration. Cations migrate the fastest and possess a positive net mobility. Neutral molecules migrate at a velocity equal to the EOF and experience zero net

mobility. Anions migrate the slowest since they oppose the bulk solvent flow towards the negative cathode, resulting in a negative net mobility.

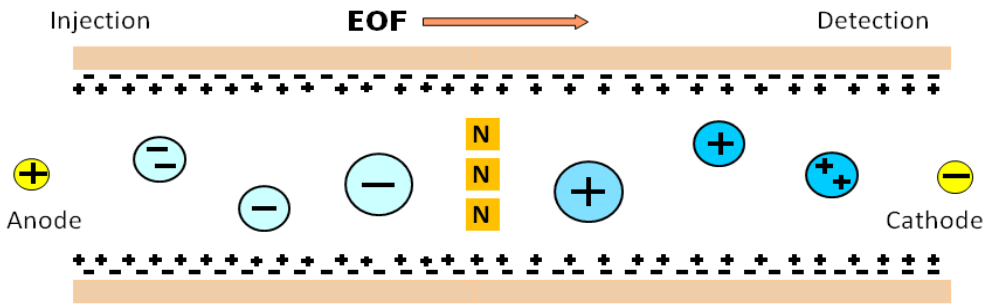

Figure 4. Electrophoretic mobility of different ions. Smaller, more positive cations migrate faster due to summation of EOF and electric gradient forces. Neutral molecules migrate at a rate equal to the EOF since they are uncharged. Small, negative anions experience a conflict between EOF migration and their electric gradient causing them to oppose the bulk flow of solution. As a result, they migrate slowly towards the detector.

A final, unique trait of the EOF is that it has a flat flow profile that is generated from the uniform distribution of charge along the capillary wall resulting from the electric double-layer and zeta potential. This results in an absence of pressure drops typically found in other separation methods, such as high-performance liquid chromatography (HPLC), that require an external pump to inject samples and force migration as well as a uniform flow velocity instead of a parabolic flow profile. Having a uniform flat flow profile increases the separation efficiency and peak capacity substantially.

Capillary Electrophoresis and Folic Acid Analysis

Many techniques have been developed with the goal of successfully separating and detecting folic acid , but many of them, such as thin-layer and column chromatography and immunoassays, require a substantial amount of time and have limited reproducibility.[5] Other methods including high-performance liquid chromatography (HPLC) require complicated instruments in terms of operation and maintenance and are also limited by sample consumption, waste production and expensive supplies.[6] In contrast, analytical methods involving capillary electrophoresis (CE) feature separations with high resolution, rapid separation and high efficiency while at the same time consuming minimal amounts of sample[6].

Folic acid is present in trace amounts in many natural foods and standard samples. As a result, sensitive techniques with low limits of detection are required.[7,8] Laser-induced fluorescence (LIF) is a commonly used CE detection technique with low, often nanomolar, detection limits. The success of LIF detection depends largely on the target analyte's ability to fluoresce when excited by a laser. By focusing the laser emission at a specific point on the capillary, referred to as the window, the target molecules are able to absorb the energy and enter an excited state. Once excited, the molecules give off photons, which are then detected by specialized light sensitive equipment that measures the intensity of the fluorescence. Detection with LIF is highly selective for samples that possess the ability to fluoresce. Selectivity for LIF can be further refined with the selection of excitation and emission wavelengths to obtain maximum sample fluorescence.

In order for LIF to be an effective method of detection, the target analytes must be fluorescent. Unfortunately, the fluorescent properties of folic acid are highly debated in the literature with various reports suggesting that it is nonfluorescent[7,9,10] and others claiming it is weakly fluorescent.[11-15] Thomas et al. studied the fluorescent properties of select folate compounds including folic acid and determined that folic acid had fluorescent quantum yields of less than 0.005.[11] This means that whatever fluorescence folic acid gives off, if any, will be overshadowed by background noise. It has been theorized that the aminobenzoyl substituent of folic acid may act as an "internal quencher", suppressing the fluorescence given off from the adjacent pterin substituent.[11-13,16]

Early methods for examining folic acid fluorescence were performed with bulk measurements of fluorescence with no prior separation of the samples. This meant that folic acid along with any contaminants in solution were excited, and the combined fluorescence measurement was misinterpreted as due to folic acid alone. As separation techniques have been increasingly incorporated into fluorescence measurements, the assumptions regarding the native fluorescence of folic acid have been challenged. Regmi et al. examined the differing fluorescent traits in three distinct samples of folic acid from two manufacturers. The fluorescence of each sample batch was measured with and without separation by CE, and the peak mobilities in both LIF and UV absorbance electropherograms were calculated and compared to determine if there was a significant difference in signal intensity. An excitation emission matrix (EEM) was also obtained in order to further characterize the fluorescence of these samples.[17]

Regmi et al. concluded that the fluorescence thought to originate from the excitation of folic acid could actually be attributed to trace contaminants in solution that were highly fluorescent. These findings were determined by analyzing and comparing migration times of folic acid from UV absorption spectroscopy data against migration times of the fluorescent peaks in associated LIF experiments. The electrophoretic mobility of folic acid did not match with any of the mobilities from the fluorescent peaks, confirming that folic acid was not responsible for the fluorescence.[17] These conclusions provided further evidence that folic acid is not natively fluorescent.

While folic acid does not possess native fluorescence and cannot be effectively detected in its commercial form with LIF, its presence in solution can be confirmed using UV absorption spectroscopy as mentioned above.[6,9,18-20] Absorption spectroscopy is able to confirm the presence and quantity of a given sample, but generally has higher detection limits than LIF. Absorbance detection is used more often than LIF since most compounds will absorb certain wavelengths of light, while very few compounds naturally fluoresce. In order for LIF to be a viable detection method for nonfluorescent compounds like folic acid, they must be derivatized to a fluorescent product. The derivatized product can then be measured with the lower detection limits of an LIF system.

Oxidation Agents

As discussed earlier, folic acid is not fluorescent in its native state. A possible solution to this limitation is to utilize a strong oxidizing agent to convert folic acid into a derivative that is fluorescent. As folic acid reacts with the oxidant, the fluorescent derivative can be detected and quantified to determine the original amount of folic acid present in a given sample.[7,12] Examining fluorescence after separation provides a better insight into whether folic acid or the contaminants in solution are responsible for the increased intensity. There are numerous oxidizing agents cited in the literature for the successful detection of folic acid using bulk fluorescence measurements without separation of the reaction products.[12,21,22] Five of the most common oxidants for folic acid derivatization are: potassium ferricyanide, sodium nitrite, potassium persulfate, hydrogen peroxide and sodium hypochlorite.

The proposed mechanism by which all of these oxidants are believed to react with folic acid involves the cleavage of the C9 – N10 bond that connects the aminobenzoyl and pterin substituents (See Figure 1). Cleaving this bond allows the fluorescence given off by the pterin group to be detectable instead of being quenched by the aminobenzoyl group.[11,13,16] The efficiency of this cleavage determines the degree of success in production and detection of the fluorescent product.

Controlling the oxidation reaction is crucial to ensuring the optimal detection of the fluorescent product. If the oxidant is too strong, the fluorescent derivative may be further oxidized to form nonfluorescent species, but if the oxidant isn't strong enough, the C9 – N10 bond will not be fully cleaved and only minimal fluorescent product will be detected.

Ferricyanide, potassium persulfate and sodium hypochlorite all are reported to provide mild, rapid and easily controlled oxidation reactions that give comparable results consistently and limit the amount of any undesired side reactions.[5,21,23]

Sodium nitrite is also an oxidant of interest since it is typically found in some food samples that contain folic acid. Interactions between sodium nitrite and folic acid in food samples have resulted in sodium nitrite being classified as a potential oxidant of folic acid.[22]

Hydrogen peroxide is a widely accepted oxidizing agent, but it is reported that it requires the presence of Cu (II) ions to successfully oxidize folic acid. Without copper ions, no folic acid derivatization occurs and no fluorescence is produced.[12] The exact mechanism by which the copper ions aid hydrogen peroxide in the oxidation reaction is currently unknown.

One of the common fluorescent products formed from the oxidation of folic acid is 6-carboxypterin. When the C9 – N10 bond is cleaved in folic acid, the aminobenzyl substituent is separated from the pterin substituent and is unable to act as an internal quencher (See Figure 1). If a strong oxidant is present in high enough concentrations, 6-carboxypterin can undergo further oxidation to a nonfluorescent product or be broken down entirely. Both instances would result in lower fluorescence intensities during LIF detection even though successful oxidation of folic acid occurs.

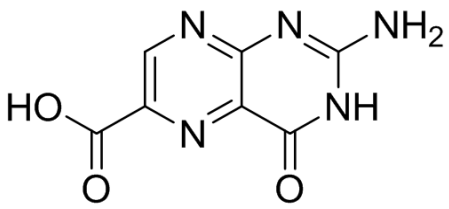

Figure 5. Chemical structure of 6-carboxypterin. During folic acid oxidation, the C9 – N10 bond between the pterin substituent and ρ-aminobenzyl substituent is cleaved. Without the quenching properties of the ρ-aminobenyzl substituent, 6-carboxypterin is highly fluorescent and easily detectable with LIF.

Project Goals

Folic acid is not natively fluorescent, and contaminants in commercial folic acid samples have been found to be responsible for the fluorescence detected during LIF experiments. As this eliminates fluorescence as a detection method for folic acid quantification, oxidative derivatization to a fluorescent product was explored. Five oxidants that have been reported to successfully oxidize folic acid to a fluorescent derivative in the literature were studied: potassium ferricyanide, hydrogen peroxide, sodium nitrite, potassium persulfate and sodium hypochlorite.

The goal of this research is to determine which of these reagents best oxidizes folic acid to produce a highly fluorescent derivative. An oxidant is considered successful if it fulfills two criteria. First, it must produce a fluorescent product as evidenced by a new fluorescent peak or a significant increase in a previously detected peak during LIF detection. Second, the reaction must show consumption of the folic acid sample, which is monitored by measuring the decrease in area of the folic acid control peak in UV absorbance detection and not a reduction in one of the solution contaminants.

Materials and Methods

Chemicals

Folic acid, potassium ferricyanide, potassium persulfate, hydrogen peroxide, sodium nitrite and copper (II) chloride were obtained from Sigma Aldrich (St. Louis, MO). Boric acid and sodium hydroxide were from Fisher Scientific (Pittsburgh, PA). Coumarin 460 was from Exciton (Dayton, OH), mesityl oxide was obtained from Alfa Aesar (Ward Hill, MA), and methanol was purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). Bleach (5% solution of sodium hypochlorite) was purchased from a local store.

Borate buffers were prepared at concentrations of 25 mM and 50 mM at pH 9.50 in ultrapure water (>18 MΩ cm) from a Modulab water purification system (United States Filter; Palm Desert, CA). Stock solutions of 2.5 mM folic acid were prepared in 25 mM borate buffer. Stock solutions of 7.5 mM coumarin 460 were prepared by dissolving the solid dye in a 50:50 solution of methanol and ultrapure water. Mesityl oxide neutral marker solutions were prepared by dissolving the 8.7 M stock solution obtained from the manufacturer into 25 mM borate buffer.

Sample Preparation

Solutions of the LIF neutral marker, coumarin 460, and UV absorbance neutral marker, mesityl oxide, were made using 1.0 µL of the purchased stock solutions with subsequent dilution in 1500 µL of borate buffer, leading to overall injection concentrations of 5.0 µM for coumarin 460 and 5.8 mM for mesityl oxide. Folic acid samples used for both controls and oxidation reactions were diluted from the 2.5 mM stock solution to 0.1 mM for injection.

Stock dilutions of each oxidant studied were prepared by dissolving the appropriate amounts of oxidant in ultrapure water. Stock oxidant solution concentrations were: 0.67 M bleach, 8.8 M hydrogen peroxide, 50 mM potassium persulfate, 50 mM sodium nitrite and 100 mM potassium ferricyanide. Dilutions of these stock concentrations were made as needed. Control and folic acid oxidation reaction mixtures of bleach, potassium persulfate and potassium ferricyanide were diluted in 25 mM borate buffer so that their concentrations were either 100 µM or 10 mM depending on the reaction conditions under study. All reaction mixtures had a folic acid concentration of 100 µM.

The sodium nitrite and hydrogen peroxide/Cu (II) oxidation reactions with folic acid were initially prepared in a 25 mM acetic acid buffer, pH 5.00 for sodium nitrite and in a 10 mM phosphate buffer, pH 7.60 for hydrogen peroxide and both were allowed to react for 15 min. The samples were then diluted by a factor of three in borate buffer for injection and separation. This variation in buffers is due to optimization of sodium nitrite and hydrogen peroxide/Cu (II) catalyzed oxidation at low pH values.[12,22,24]

Hydrogen peroxide can donate up to two oxygens to the oxidation reaction with folic acid. To account for this the concentrations used are half that of the previous oxidants, 5.0 mM and 0.05 mM. This variation was made to ensure that equal amounts of each oxidizing agent were available for the oxidation of folic acid in order to obtain comparable results.

Capillary Electrophoresis Instrumentation

Capillary electrophoresis experiments were performed using P/ACE MDQ CE systems with 32 Karat, version 5.0 software (Beckman Coulter; Fullerton, CA). For absorbance detection,

a P/ACE MDQ was used with a deuterium lamp light source and a photodiode array (PDA) detection module. The PDA wavelength range for detection was 190 − 600 nm. The deuterium lamp emitted a continuous spectrum from 180 − 370 nm.

A Coherent Innova 622 Argon Ion Laser, which emits several UV lines from 351 – 364 nm, was used for LIF excitation. The UV lines were isolated using a prism and a 355 ± 20 nm Semrock bandpass filter (Rochester, NY). A UV-grade plano convex lens with focal length of 25.0 mm from Newport (Sanford, CT) focused the lines onto a 200 µm, high-OH optical fiber from Ocean Optics (Dunedin, FL), which guided the laser light to the laser input module of the P/ACE MDQ. The excitation power at the detection window was 0.6 mW. The detection module used a 450 ± 40 nm emission filter and a neutral density filter from Andover (Salem, NH).

Fused-silica capillaries with an inner diameter of 52 µm and an outer diameter of 362 µm were used for all CE experiments and were obtained from Polymicro Technologies (Phoenix, AZ). The capillaries were 60.0 cm in total length and 50.0 cm to the detection window.

All sample solutions were injected by pressure for 5.0 seconds at 0.5 psi, and data points were acquired at a frequency of 16 Hz for the LIF system and 32 Hz for the UV absorbance system. Each reaction sample was run in duplicate and folic acid and oxidant control solutions were run prior to oxidation reaction mixtures. Data gathered from both LIF and UV absorbance detection systems were analyzed and plotted using OriginPro 7.5 from Origin Lab (Northampton, MA) and Microsoft Excel from Microsoft Corp (Redmond, WA).

Results and Discussion

To confirm an oxidant's successful reaction with folic acid, it must fulfill two criteria. First, a new fluorescent product must be observed by CE with laser-induced fluorescence detection signifying the production of a fluorescent derivative of folic acid such as 6-carboxypterin. Second, using CE with UV absorbance detection, thepeak due to folic acid should decrease to demonstrate that folic acid, and not one of the contaminant compounds in solution, is being consumed to produce the fluorescent derivative. If both requirements are met, then the oxidant can be considered as a viable reagent for quantitative fluorescent derivatization and detection of folic acid.

The amount of oxidant utilized in the reactions can significantly impact the amount of fluorescent products produced. For this reason, both 100 µM and 10 mM oxidant concentrations were used in order to study the products produced when the molar ratio of folic acid to oxidant was 1:1 and 1:100 respectively. The oxidants studied were: potassium persulfate, hydrogen peroxide, sodium hypochlorite, sodium nitrite and potassium ferricyanide. One common derivative of folic acid is 6-carboxypterin. This derivative has been reported to be a significant product from folic acid oxidation by hydrogen peroxide in the presence of copper (II) chloride and a minor product from sodium hydroxide oxidation[24]. A standard of this compound was analyzed to determine if 6-carboxypterin was a major product for oxidation of folic acid using the oxidants examined in this work.

A stock solution of 50 mM potassium persulfate and a 2.5 mM stock solution of folic acid were diluted in 25 mM borate buffer pH 9.50 to achieve injection concentrations of 100

μM or 10 mM for potassium persulfate and 100 μM for folic acid. The reaction took place in a glass MDQ injection vial and was allowed to react for 15 min before injection and separation. A diluted stock solution of hydrogen peroxide of 0.1 M was used in the preparations of the next sequence of reaction mixtures. Hydrogen peroxide, copper (II) chloride and folic acid were all combined in a 10 mM phosphate buffer, pH 7.60 and allowed to react for 15 min. After time had elapsed, the solution was diluted by a factor of three to reach the correct injection oxidant concentrations of 50 μM and 5 mM. A stock solution of 10 mM 0.67 M stock of sodium hypochlorite was used for the next oxidation study. The preparation and injection of 100 μM and 10 mM oxidation mixtures was identical to the potassium persulfate procedure described above. Many compounds including folic acid and potential reaction products display strong absorbance at 190 nm, so this wavelength was plotted in the electropherograms and used for data analysis.

Potassium Persulfate Oxidation - Laser-induced Fluorescence

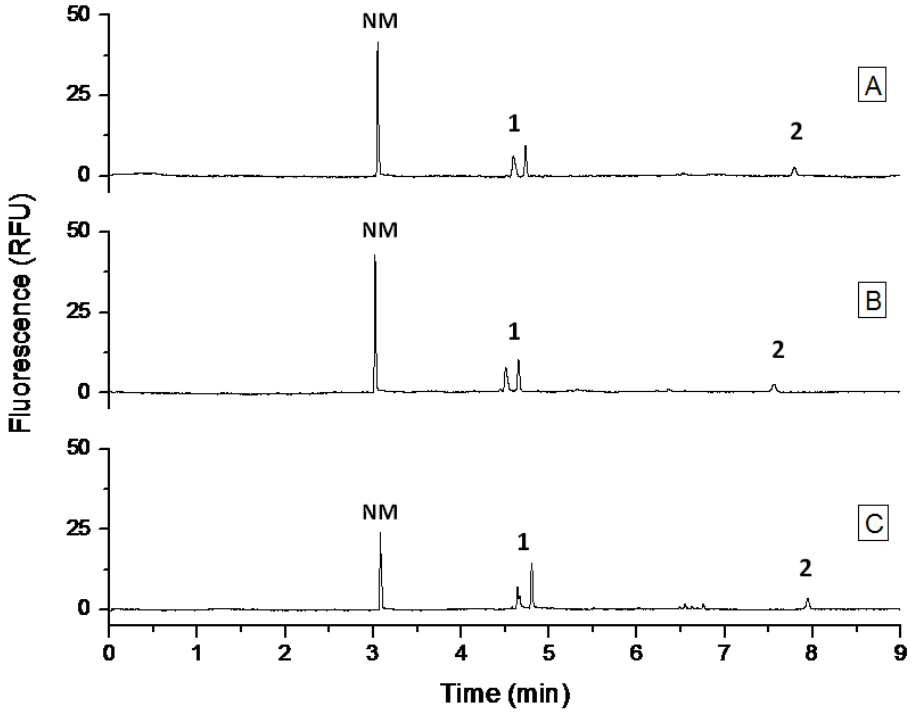

Figure 6. Electropherograms of 100 μM folic acid oxidation by potassium persulfate monitored by LIF detection (excitation by argon ion laser with lines at 351–364 nm filtered by a 350 ± 20 nm filter, emission: 450 ± 40 nm). Capillary length was 60 cm total with 50 cm to the detection window. The separation buffer was 25 mM borate buffer pH 9.50, which produced a 14.5 μA current at a separation voltage of 25 kV. Data points were acquired at a frequency of 16 Hz. Solutions of the neutral marker (coumarin 460, NM), folic acid and oxidant were injected for 5.0 s at 0.5 psi. The peaks produced at the 3 min mark are due to coumarin 460 while the other peaks are from compounds in the folic acid sample. (A) Control run with only NM and 100 μM FA. (B) NM, 100 μM potassium persulfate and 100 μM FA. (C) NM, 10 mM potassium persulfate and 100 μM FA. The peaks labeled 1 and 2 are described in the text.

The control run (Figure 6A) of coumarin 460 and 100 μM folic acid shows three distinct contaminant peaks, two peaks (Figure 6, group 1) at 4.5 min and a single peak at 8.0 min (Figure 6 – group 2), in addition to the neutral marker (NM) peak at 3.0 min. The 100 μM potassium persulfate oxidation (Figure 6B) shows the same three folic acid contaminant peaks present previously in the control run. The total fluorescence of the contaminant peaks from the control

run (Figure 6A) versus the same peaks in the 100 µM potassium persulfate sample (Figure 6B) shows a 0.8% decrease in fluorescence, which suggests that very little, if any, folic acid oxidation and subsequent fluorescent product formation occurred. No new fluorescent products were detected.

In the 10 mM potassium persulfate oxidation sample (Figure 6C), the same three folic acid contaminant peaks are present and show a 22% decrease in overall fluorescence intensity from the control run. Along with the decrease in total fluorescence, no significant new fluorescent products were detected, although numerous minor fluorescent peaks can be seen. The decrease in fluorescence and presence of trace peaks could indicate the breakdown of the fluorescent folic acid contaminants in solution due to such a high oxidant concentration.

Potassium Persulfate Oxidation – UV Absorbance

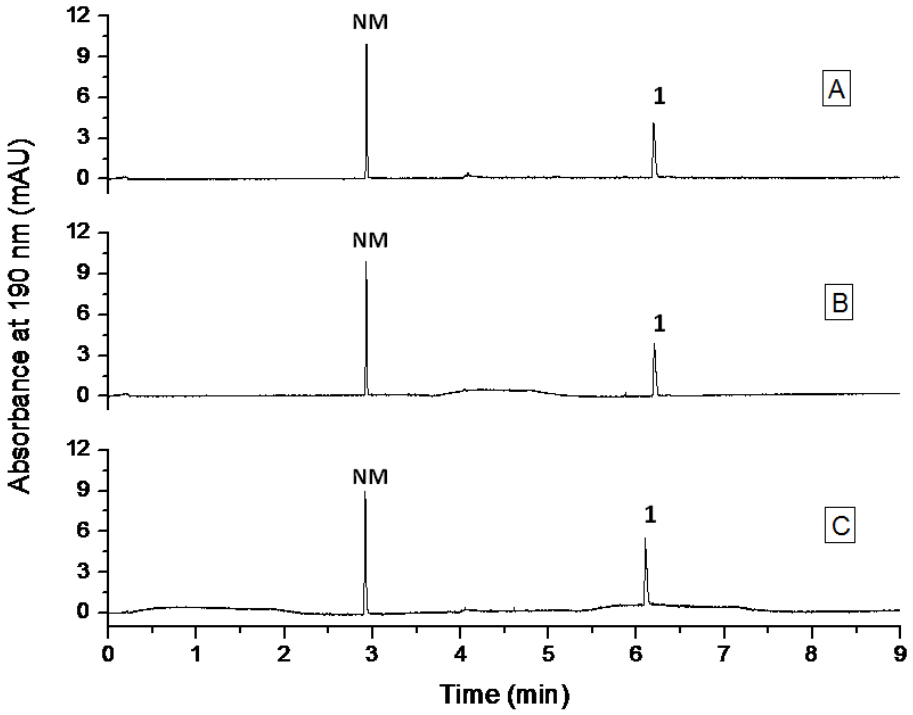

Figure 7. Electropherograms of 100 μM folic acid oxidation by potassium persulfate monitored by UV absorbance detection at 190 nm. See Figure 6 for specific injection and separation conditions. The peaks at 3 min are the neutral marker, mesityl oxide, and the additional peaks are due to folic acid in solution. (A) Control run with only NM and 100 μM FA. (B) NM, 100 μM potassium persulfate and 100 μM FA. (C) NM, 10 mM potassium persulfate and 100 μM FA.

All three electropherograms with UV absorbance detection from the potassium persulfate oxidation of folic acid show almost no changes in the amount of folic acid initially present in solution, and no additional reaction products are detected. The neutral marker, mesityl oxide, migrated at 3 min, and the folic acid peak eluted at 6.1 min (Figure 7 – Peak 1). The folic acid peak areas deviated from the control run (Figure 7A) by 0.8% in the 100 μM oxidation experiment (Figure 7B) and by 5.6% in the 10 mM oxidation experiment (Figure 7C). Potassium persulfate does not oxidize folic acid to a significant extent for these conditions.

The data obtained from the UV absorbance separations of the 100 μM and 10 mM potassium persulfate oxidation reactions confirmed that the absence of a new fluorescent product in the CE experiments with laser-induced fluorescence runs was due to a lack of folic acid oxidation. Potassium persulfate fails both of the proposed criteria for confirming the successful oxidation of folic acid for LIF detection.

Hydrogen Peroxide Oxidation – Laser-induced Fluorescence

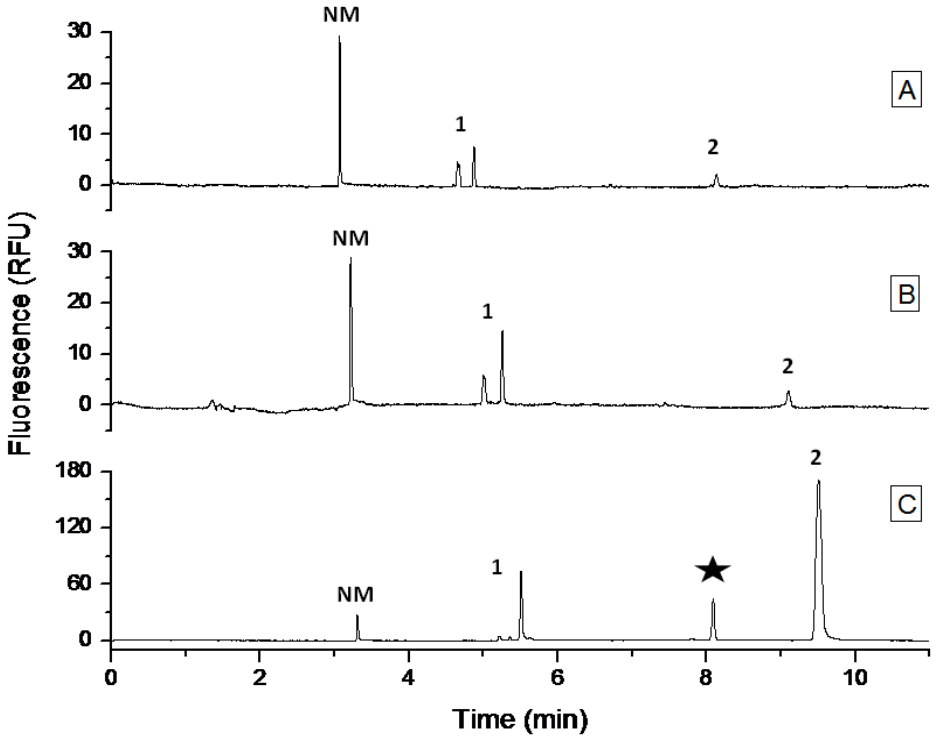

Figure 8. Electropherograms of 100 μM folic acid oxidation with hydrogen peroxide/Cu (II) monitored by CE with LIF detection. Injection and detection parameters are identical to those for potassium persulfate experiments except that the separation buffer was 50 mM borate buffer pH 9.50, which produced a 21.0 μA current at a separation voltage of 25 kV (Figure 6). (A) Control run with NM (coumarin 460) and 100 μM FA. (B) NM, 50 μM hydrogen peroxide and 100 μM FA. (C) NM, 5 mM hydrogen peroxide and 100 μM FA.

Slight fluorescent increases can be seen when comparing the control reaction (Figure 8A) to the 50 μM oxidation reaction (Figure 8B). Most of the fluorescence intensity fluctuations between these two experiments can be attributed to deviations in the injection procedure. In contrast, the 5 mM oxidation reaction displays a significant new oxidation product (starred peak) as well as noticeable increases in fluorescence intensity for the contaminant peaks (Figure 8 – Peaks 1 and Peak 2). According to the literature, 6-carboxypterin is a major product of hydrogen peroxide/Cu (II) oxidation of folic acid.[12,24] After analysis of electrophoretic

mobilities for each fluorescent contaminant peak, it was determined that the new fluorescent product detected at 8.0 min in the 5 mM oxidation reaction was not 6-carboxypterin. Instead 6-carboxypterin was identified to be the third contaminant peak from the control run and present in larger fluorescent quantities in the subsequent oxidation reactions (Figure 8 – Peak 2).

Fluorescence intensities in the 50 µM oxidation reaction showed very minor increases of 10% when compared to the control reaction. On the other hand, the intensity differences between the control reaction and the 5 mM oxidation reactions are significant with upwards of a 10,000% increase in fluorescence intensity for Peak 2 (Figure 8C). To confirm hydrogen peroxide's effectiveness at only saturated levels of oxidant, a third reaction was performed using 500 µM of oxidant. The results from this additional reaction were aligned closely with the data from the 50 µM oxidation reaction (Figure 8B). These results suggest that hydrogen peroxide/Cu (II) could be a successful oxidant for folic acid derivatization, but only at high concentrations.

Hydrogen Peroxide Oxidation – UV Absorbance

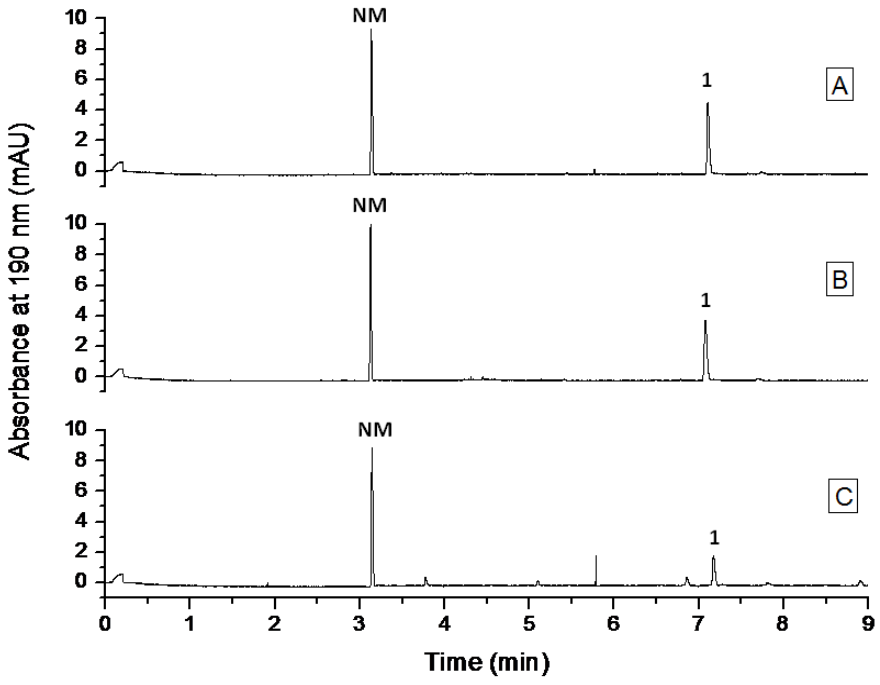

Figure 9. Electropherograms of 100 μM folic acid oxidation by hydrogen peroxide/Cu (II) monitored with UV absorbance detection at 190 nm. Similar detection and injection procedures were used as in the hydrogen peroxide LIF oxidation experiments (See Figure 8). (A) Control experiment with NM (mesityl oxide) and 100 μM FA. (B) NM, 50 μM hydrogen peroxide and 100 μM FA. (C) NM, 5 mM hydrogen peroxide and 100 μM FA.

The differences in folic acid peak area between the 50 μM oxidation reaction (Figure 9B) and the control reaction (Figure 9A) varied by 0.6% suggesting that folic acid either is not reacting with the low amounts of oxidant or that the reaction requires more than 15 min to initiate. With no clear signs of oxidation at low concentrations of hydrogen peroxide/Cu (II) after 15 min, it can be concluded that while the oxidant might eventually derivatize folic acid, it would not be able to do so in a reasonable amount of time. The oxidation reaction of folic acid with 5 mM hydrogen peroxide/Cu (II) (Figure 9C) was found to consume moderate amounts of folic acid with a 55% decrease in the folic acid peak area. A number of oxidation products are detected in the 5 mM oxidation reaction. These products could result from folic acid derivatization or from derivatization of the various folic acid contaminants present in the solution.

When applying the criteria for determining the success of an oxidant to derivatize folic acid, hydrogen peroxide/Cu (II) either succeeds or fails depending on which concentrations are examined. At low concentrations of 50 μM and 500 μM, the LIF and UV results show no new fluorescent products and no significant decrease in folic acid peak area. While some of the contaminants in the LIF electropherograms (Figure 8B) show strong increases in fluorescence intensity, without the associated decline in folic acid peak area, it appears that only the folic acid contaminants are reacting with hydrogen peroxide/Cu (II). In contrast, high concentrations

of 5 mM reveal a new fluorescent product in LIF (Figure 8C) and display a notable decrease in the folic acid peak area (Figure 9C). In conclusion, hydrogen peroxide/Cu (II) appears to react and possibly derivatize folic acid only at high concentrations.

Sodium Hypochlorite Oxidation- Laser-induced Fluorescence

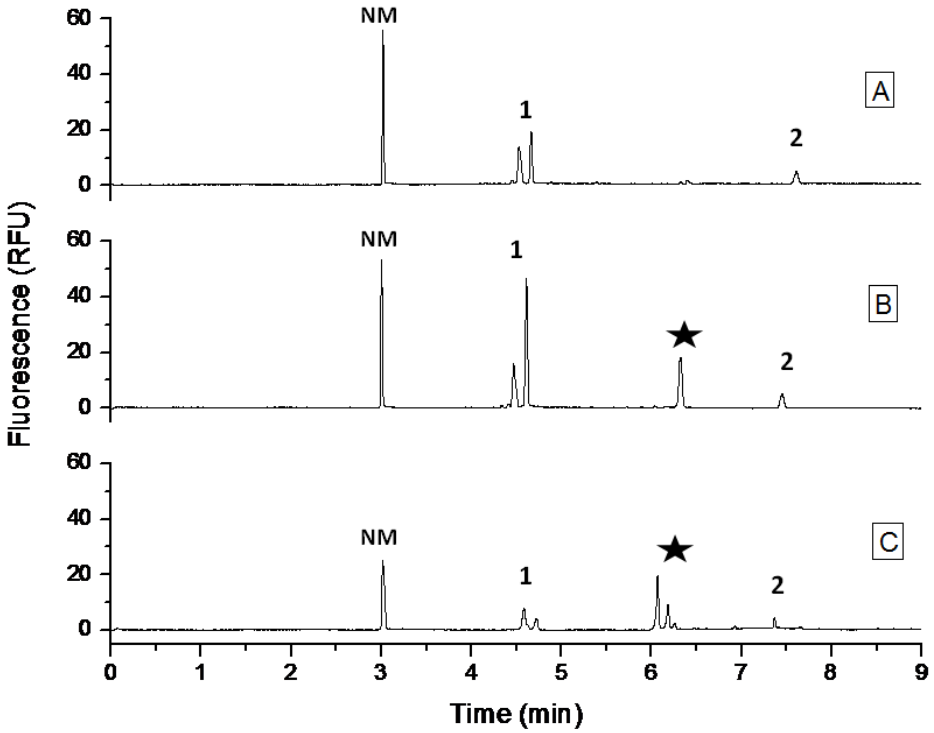

Figure 10. Electropherograms of 100 μM folic acid oxidation with sodium hypochlorite monitored by CE with LIF detection. Injection and detection procedures are identical to those for potassium persulfate experiments(Figure 6). (A) Control run with NM (coumarin 460) and 100 μM FA. (B) NM, 100 μM sodium hypochlorite and 100 μM FA. (C) NM, 10 mM sodium hypochlorite and 100 μM FA.

Both the 100 μM and 10 mM sodium hypochlorite reactions (Figure 10 - B and C) reveal a new fluorescent product that migrates at 6.1 min (starred peaks). Comparison of the electrophoretic mobility of this newly detected product to standards of 6-carboxypterin indicates that it is not 6-carboxypterin, a common fluorescent derivative of folic acid. 6-

carboxypterin was actually present as a minor peak at 7.5 min for both oxidation reactions. This finding agrees with the literature, which states that 6-carboxypterin is a minor oxidation product when sodium hypochlorite acts as an oxidizing agent with folic acid.[24]

In addition to the appearance of a new fluorescent product, the total fluorescence intensity of the control run contaminant peaks increased by 120% for the 100 µM sodium hypochlorite reaction. The fluorescent contaminant peaks in the 10 mM reaction instead decreased, presumably due to further oxidation of these fluorescent contaminants to form nonfluorescent compounds. For the reaction with 10 mM sodium hypochlorite, a small, new fluorescent product is observed near the new product peak at 6.1 min observed for the 100 µM sodium hypochlorite reaction.

Sodium Hypochlorite Oxidation – UV Absorbance

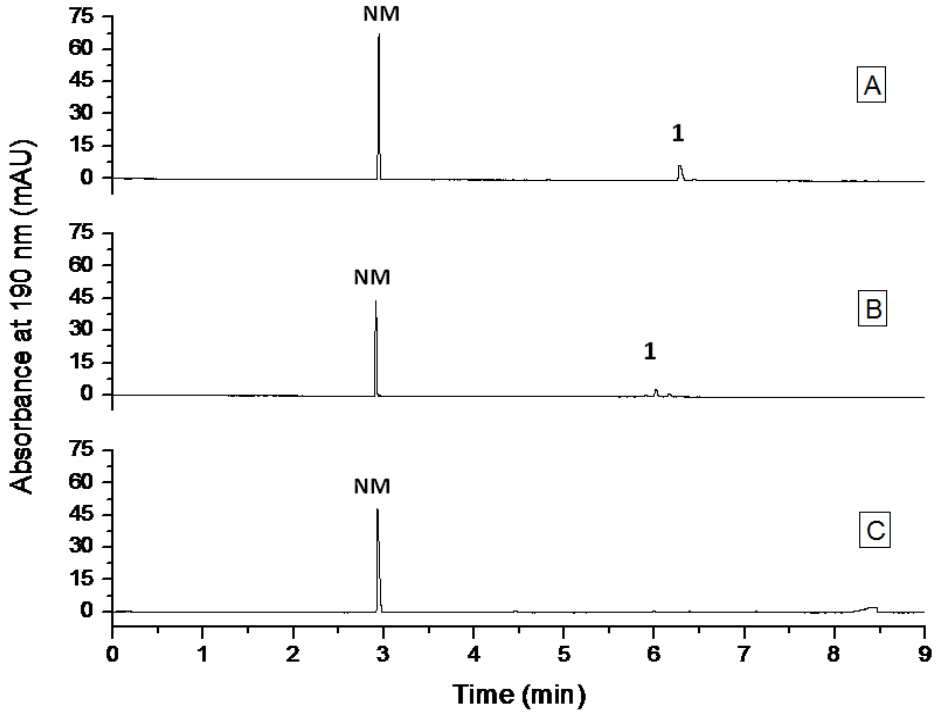

Figure 11. Electropherograms of 100 μM folic acid oxidation by sodium hypochlorite monitored with UV absorbance detection at 190 nm. Detection and injection procedures were identical to those in Figure 6. (A) Control experiment with NM (mesityl oxide) and 100 μM FA. (B) NM, 100 μM sodium hypochlorite and 100 μM FA. (C) NM, 10 mM sodium hypochlorite and 100 μM FA.

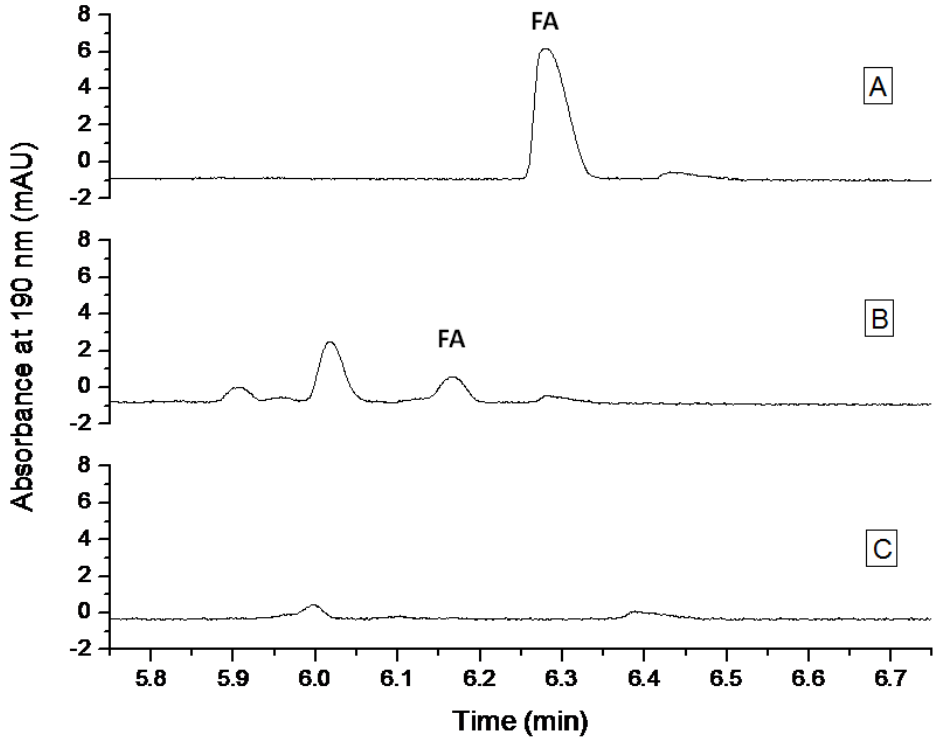

Figure 12. Enlarged electropherograms of folic acid peaks from sodium hypochlorite oxidation reactions. Analysis of peak mobilities reveals that the third peak in the 100 μM reaction (B) is folic acid. The 10 mM reaction (C) does not show any detectable folic acid.

The CE data from UV absorbance detection for the sodium hypochlorite oxidation reaction indicates that the oxidant reacted significantly with folic acid. The 1:1 ratio of folic acid to sodium hypochlorite at 100 μM results in an 81% decrease in the folic acid peak area while the 1:10 ratio in the 10 mM sodium hypochlorite reaction consumes 100% of the folic acid present in solution (Figure 11B and C, Figure 12 B and C). It is difficult to determine the identity

of the other peaks present in the 100 µM reaction. They were not present in an injection of solely oxidant, so they must be related to the folic acid reaction in some way.

Folic acid was determined to be the third peak (Figure 11 & 12 B) based on its electrophoretic mobility with a migration time of approximately 6.2 min. The other two peaks present at 5.9 and 6.0 min are likely products of FA oxidation but have not been identified. While the oxidation reaction was only partially complete in the 100 µM reaction, oxidation with 10 mM sodium hypochlorite leads to complete consumption of folic acid by the time the separation is carried out.

The substantial decrease in folic acid peak area from UV absorbance in connection with the presence of a new fluorescent product with LIF provides strong evidence that sodium hypochlorite successfully oxidizes folic acid to a fluorescent derivative. Both the LIF and UV criteria set forth to identify a successful oxidant are achieved by sodium hypochlorite, confirming it as an effective oxidant for folic acid derivatization and detection via LIF.

Sodium Nitrite & Potassium Ferricyanide Oxidation

Both the sodium nitrite and potassium ferricyanide oxidation of folic acid produced similar results to the data obtained from the potassium persulfate experiments. Minor fluctuations in the amount of folic acid as measured by CE with UV absorbance correlated with no significant new fluorescent products in CE experiments with laser-induced fluorescence detection for both oxidants. A trace contaminant peak found in the sodium nitrite controls was determined to be trace amounts of 6-carboxypterin, but the peak did not show any increase in fluorescence intensity for either the 100 µM or 10 mM oxidations. Potassium ferricyanide yielded a number of trace fluorescent product peaks in LIF, but the peak areas of folic acid in the UV absorbance experiments indicated that it did not experience any significant oxidation. The small fluorescent peaks were concluded to be oxidation products from additional contaminants in the folic acid solution.

Table 1. Summary of laser-induced fluorescence and UV absorbance data from the oxidation of 100 µM folic acid with 100 µM and 10 mM oxidant. Hydrogen peroxide oxidant concentrations of 50 µM and 5 mM were used to account for its donation of two oxygen ions to the reaction.

Oxidant	LIF Products (Yes/No)		% Folic Acid Decrease in UV	
	100 µM	10 mM	100 µM	10 mM
Potassium Persulfate	No	No	↑ 0.8	↑ 5.6
Hydrogen Peroxide	No	Yes	↑ 0.6	↓ 55
Sodium Hypochlorite	Yes	Yes	↓ 81	↓ 100
Sodium Nitrite	No	No	↓ 6.9	↓7.8
Potassium Ferricyanide	No	No	↑ 2.9	↑ 5.4

Conclusion

Folic acid does not exhibit native fluorescence and cannot be effectively detected with laser-induced fluorescence (LIF) alone. To enable successful LIF detection, folic acid can be oxidatively derivatized to a highly fluorescent product such as 6-carboxypterin. Five different oxidants were selected from the literature to test their effectiveness at converting folic acid to a detectable product. An oxidant was deemed successful if it both produced a new fluorescent product detected with LIF and indicated an associated decrease in folic acid with UV absorbance.

Five oxidizing agents were studied for folic acid derivatization: potassium persulfate, hydrogen peroxide, sodium hypochlorite, sodium nitrite and potassium ferricyanide. Of these five oxidants, only sodium hypochlorite succeeded in meeting both standards for effective oxidation of folic acid. The other four oxidants appeared to be reacting with folic acid only at significantly high concentrations in the case of hydrogen peroxide/Cu (II), or oxidizing trace contaminants in the folic acid solution to produce fluorescent products such as with potassium persulfate, sodium nitrite and potassium ferricyanide. While sodium hypochloride did provide the best oxidative derivatization results, it is still too early to classify it as a definitive method of folic acid detection. Further research needs to be performed to determine what degree of control can be had during the oxidation reaction with folic acid and to identify the specific fluorescent products that are being produced.

In many natural foods and standard samples folic acid is present only in trace amounts, requiring sensitive detection methods with low detection limits. By determining the impact

these five oxidants have on derivatizing folic acid, more accurate detection procedures can be developed with confidence that folic acid itself is being detected instead of a highly fluorescent contaminant in the same solution. Utilizing capillary electrophoresis coupled with fluorescence detection and oxidative derivatization, fast and sensitive separations can be refined to further research folic acid's influence in the human body.

Acknowledgements

I would sincerely like to thank Dr. Doug Gilman for all of his support and guidance as my Thesis Director and Research Advisor for the past three years that I've worked in his lab.

I would also like to express my gratitude to both Rachel Henken and Suresh Regmi for their valuable advice, insight and guidance in all the stages of my thesis project. I want to thank Funda Kizilkaya for her help in creating the capillary electrophoresis and electrophoretic mobility figures used in this paper.

Finally, I am truly thankful to the entire Gilman Research Group for their friendship and support the past three years, which has made my undergraduate research experience unforgettable.

References

(1) Quinlivan, E. P.; Hanson, A. D.; Gregory, J. F. *Anal. Biochem.* **2006**, *348*, 163.

(2) Smith, A. D.; Kim, Y.-I.; Refsum, H. *American Journal of Clinical Nutrition* **2008**, *87*, 517.

(3) Scott, J.; Rebeille, F.; Fletcher, J. *J. Sci. Food Agric.* **2000**, *80*, 795.

(4) Cruz, L.; Shippy, S. A.; Sweedler, J. V. In *High Performance Capillary Electrophoresis Theory, Techniques, and Applications*; Khaledi, M. G., Ed.; John Wiley & Sons, Inc.: New York, 1998, p 304.

(5) Hahn, A.; Stein, J.; Rump, U.; Rehner, G. *J. Chromatogr.* **1991**, *540*, 207.

(6) Zhao, S.; Yuan, H.; Xie, C.; Xiao, D. *Journal of Chromatography, A* **2006**, *1107*, 290.

(7) Zhang, G.-F.; Maudens, K. E.; Storozhenko, S.; Mortier, K. A.; Van, d. S. D.; Lambert, W. E. *J. Agric. Food Chem.* **2003**, *51*, 7872.

(8) Gregory, J. F., III; Sartain, D. B.; Day, B. P. F. *J. Nutr.* **1984**, *114*, 341.

(9) Gujska, E.; Kuncewicz, A. *Eur. Food Res. Technol.* **2005**, *221*, 208.

(10) Vahteristo, L. T.; Ollilainen, V.; Koivistoinen, P. E.; Varo, P. *J. Agric. Food Chem.* **1996**, *44*, 477.

(11) Thomas, A. H.; Lorente, C.; Capparelli, A. L.; Pokhrel, M. R.; Braun, A. M.; Oliveros, E. *Photochem. Photobiol. Sci.* **2002**, *1*, 421.

(12) Hirakawa, K. *Anal. Bioanal. Chem.* **2006**, *386*, 244.

(13) Off, M. K.; Steindal, A. E.; Porojnicu, A. C.; Juzeniene, A.; Vorobey, A.; Johnsson, A.; Moan, J. *J. Photochem. Photobiol., B* **2005**, *80*, 47.

(14) Huang, J.-C.; Li, D.-J.; Diao, J.-C.; Hou, J.; Yuan, J.-L.; Zou, G.-L. *Talanta* **2007**, *72*, 1283.

(15) Tyagi, A.; Penzkofer, A. *Chem. Phys.* **2010**, *367*, 83.

(16) Hirakawa, K.; Suzuki, H.; Oikawa, S.; Kawanishi, S. *Arch. Biochem. Biophys.* **2003**, *410*, 261.

(17) Regmi, S. C.; Challa, S.; Warner, I. M.; Gilman, S. D.; Louisiana State University: Baton Rouge, In preparation.

(18) Uysal, U. D.; Oncu-Kaya, E. M.; Tuncel, M. *Chromatographia* **2010**, *71*, 653.

(19) Flores, J. R.; Penalvo, G. C.; Mansilla, A. E.; Gomez, M. J. R. *Journal of Chromatography B* **2005**, 141.

(20) Poo-Prieto, R.; Haytowitz, D. B.; Holden, J. M.; Rogers, G.; Choumenkovitch, S. F.; Jacques, P. F.; Selhub, J. *J. Nutr.* **2006**, *136*, 3079.

(21) Chippel, D.; Scrimgeour, K. G. *Can. J. Biochem.* **1970**, *48*, 999.

(22) Reed, L. S.; Archer, M. C. *J. Agric. Food Chem.* **1979**, *27*, 995.

(23) Holt, D. L.; Wehling, R. L.; Zeece, M. G. *J. Chromatogr.* **1988**, *449*, 271.

(24) Regmi, S. C.; Phipps, A.; Gilman, S. D.; Louisiana State University: Baton Rouge, In preparation.