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Capillary electrophoresis of pteridines in urine and plants and quantification of pharmaceutical and personal care products in Missouri waste water

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CAPILLARY ELECTROPHORESIS OF PTERIDINES IN URINE AND PLANTS AND QUANTIFICATION OF PHARMACEUTICAL AND PERSONAL CARE PRODUCTS IN MISSOURI WASTE WATER

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To my wife, Briana

And my children, Ariana & Tierney

Thank you for your love and support during this long journey

PUBLICATION DISSERTATION OPTION

This dissertation consists of the following three articles that have been published, submitted for publication, or will be submitted for publication as follows:

Pages 40-61 were published in ELECTROPHORESIS

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ABSTRACT

Capillary electrophoresis (CE) is a powerful technique that separates molecules based on their respective charge and hydrodynamic size. The major advantages of CE are high separation efficiency, small sample volume requirements, simple to no sample preparation and often organic solvents are not required for the separation.

The work presented in this dissertation utilized capillary electrophoresis for the analysis two different classes of compounds in different matrices. The first project investigated pteridines in urine and plants. The second project investigated pharmaceutical and personal care products (PPCP's) in Missouri waste water.

As cancer continues to be one of the top three causes of human fatalities, early cancer screening research continues to gain momentum. One specific category of compounds known as pteridines is a class of compounds with potential linkage to cancer progression and deserves further investigation. Therefore, we constructed and performed a systematic investigation and optimization of a method for urinary pteridine analysis using capillary electrophoresis with laser induced fluorescence. This method is believed to possess the potential for clinical diagnosis of the presence of cancer at earlier stages than current methodologies. In addition, the method was also extended to the investigation of pteridines in plants for nutritional research. Due to the matrix differences, a systematic study was also performed.

There is a growing concern with the amount of PPCP's in the environment. One major area is the levels of PPCP's in the waste water. As a result, the detection and quantification of PPCP's in waste water offers valuable insight to the amount of contaminations being introduced. Several methods for detecting and quantifying PPCP's have been developed using LC-MS/MS. However, an alternative technique is urgently needed to conduct PPCP analysis when LC/MS is not available laboratories. Therefore, we have developed a method using CE-UV for the simultaneous analysis of eight different PPCP compounds in untreated waste water. Capillary electrophoresis (CE) offers a simple, fast, and low cost alternative analytical technique to LC/MS for PPCP determination in waste water.

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The data collected during my research came from samples that were provided by Dr. Clay Anderson from the Ellis Fischel Cancer Research Center in Columbia, MO, Dr. Tahzeeba Hossain from The Donald Danforth Plant Science Center, St. Louis MO, and from the Missouri State waste water treatment facilities. Without their support, there would have been no data to collect.

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Without my faith in God and Jesus Christ, I would not have had the strength to complete this exhausting challenge. Daily renewal of strength, faith and confidence has allowed me to push through the toughest of times.

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1. INTRODUCTION

1.1. CAPILLARY ELECTROPHORESIS

1.1.1. Introduction. Capillary electrophoresis has been looked upon as a cornerstone analytical separation tool for proteins and peptides [1-8]. However, this technique has spread far beyond applications in biosciences. In the past few years capillary electrophoresis has been used for amino acid analysis [9], determination of cholesterol and thiols [10, 11], determination of pteridines for cancer screening [12, 13], drug analysis by chemiluminescence [14], lost cost drug quality control and counterfeit medicines [15], analysis of organic compounds in factory waste water [16] and determination of phenolic disinfectant in disinfectant products [17].

Capillary electrophoresis uses a polymer coated glass capillary, a buffered solution and high voltage to carry out separation of molecules and ions. Each end of the capillary is placed into separate buffered solution and connected to a high voltage power supply (Figure 1.1.). Under an applied voltage, positively charged cations will migrate towards the cathode and the negatively charged anions will migrate towards the anode. Additionally, in the presence of applied voltage, the buffered solution will flow through the capillary by electroosmosis. The resulting flow is called electroosmotic flow (EOF). Electroosmotic flow will cause both ionic and neutral species to flow from the anode toward the cathode. The rate at which species move is related to their mass to charge ratio and buffer ion strength. Therefore, smaller, highly charged species will move faster that larger less charged species. Detectors can be placed either on or off capillary to measure the response of the analytes. On-capillary detectors include UV and fluorescence [18-20].

Off-capillary include electrochemical, conductivity refractive index and mass spectrometry [18-20].

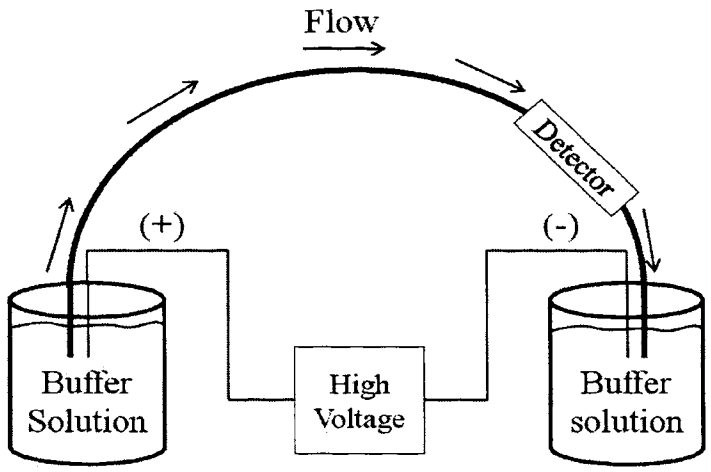

Figure 1.1. Capillary Electrophoresis Setup

1.1.2. Fundamentals of Capillary Electrophoresis. Separation of analytes is based on the difference in migrational velocities of individual species under an applied potential. The velocity of a specific analyte is given by eq. 1.

$$v = \mu_e E \tag{1}$$

where v is the velocity of the ion, μ_e is its electrophoretic mobility and E is the applied potential. Electrophoretic mobility (μ_e) is influenced by electric force and linear drag (frictional forces of the analyte moving through the buffered solution) [20].

$$\mu_e \propto \frac{Electric\ Force\ (F_E)}{Linear\ Drag\ (F_d)} \tag{2}$$

The forces experienced by an ion under an electric potential (F_E) and linear drag (F_d) are described in eq. 3 and 4 [20].

$$F_E = Eq \tag{3}$$

$$F_d = 6\pi\eta r v \tag{4}$$

where q is the charge of the ion, η is the viscosity of the fluid and r is the Stokes radius of the particle [20-22]. Furthermore, in capillary electrophoresis the electric force and linear drag achieve a steady state becoming equal in opposite directions resulting in Eq. 5.

$$F_E = Eq = 6\pi\eta r v \tag{5}$$

Eq. 5 can be re-written such that

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

where it is shown that smaller, higher charged species will move faster than larger, smaller charges species.

1.1.3. Electroosmotic / Electrophoretic Flow. When potential is applied across a capillary filled with electrolytic solution, the solution inside the capillary flows. This flow is called "electroosmotic flow" (EOF) [22]. Electroosmotic flow results from the formation of an electric double layer at the capillary wall and the attraction of solvated cations in the diffuse outer layer to the cathode (Figure 1.2.). The velocity at which the electoosmoitc flow moves is called the electroosmotic velocity (v_{eo}). The electoosmoitc flow is dependent on the pH and concentration of the buffer solution. Higher pH (above 9) will fully ionize the acidic silanols on the capillary surface increasing the EOF. More concentrated buffer solutions reduce the zeta-potential, reducing the electoosmoitc flow [21]. Electroosmotic velocity (v_{eo}) and mobility (μ_{eo}) are defined as

$$v_{eo} = \mu_{eo} E \tag{7}$$

$$\mu_{eo} = \frac{\in \zeta}{\eta} \tag{8}$$

where $\in$ is the dielectric constant, ζ is the zeta potential, which is the change in potential across the electric double layer, η is the viscosity of the buffer and E is the applied electric potential in volts cm^{-1} [19, 21, 22]. The zeta potential is proportional charge on the capillary wall and to the width of the double layer [22].

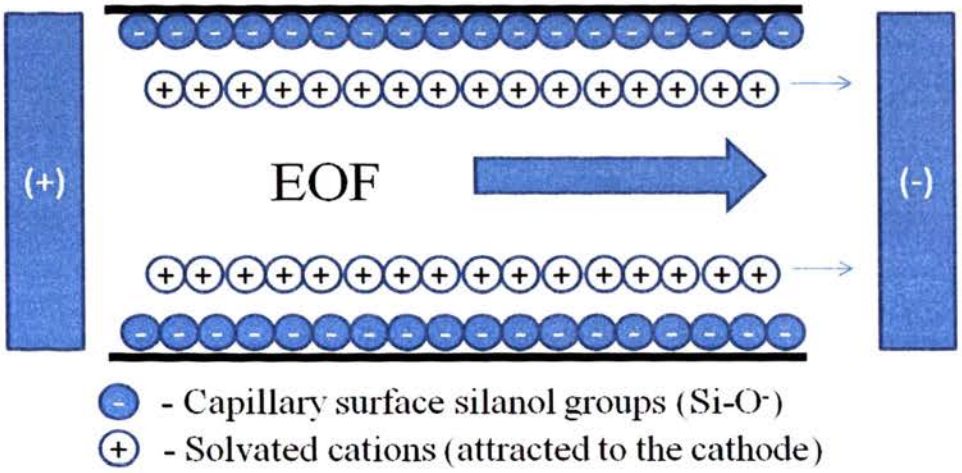

Figure 1.2. Electric double layer

Electrophoretic flow is the flow of ions toward an electrode with an applied potential. Cations are drawn towards cathodes, anions towards anodes and neutrals are unaffected. The mobility of ions (μ_e) increases as charge increases Figure 1.3., eq. 9 [19].

$$\nu_e = \mu_e E \tag{9}$$

The rate at which ions move under electrophoretic flow is called "electrophoretic velocity" (v_e).

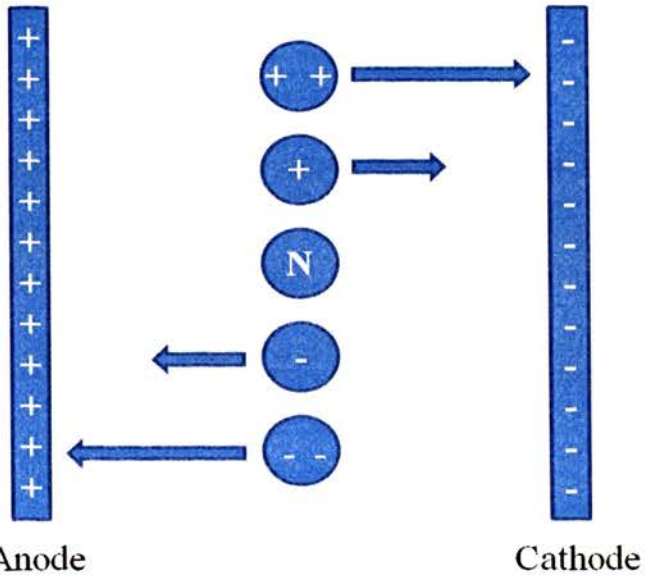

Figure 1.3. Electrophoretic flow [19]

Under the influence of electroosmosis, the net velocity of an ion is the sum of its migrational velocity and the velocity of the electroosmotic flow (eq.10) [19].

$$\nu_{Total} = \nu_e + \nu_{eo} \tag{10}$$

The rate at which the analytes migrate primarily depends on charge strength and size [21]. Since electroosmotic flow is usually sufficient to overcome electromigrational forces, all species will all be carried towards the cathode [19]. The order of elution will

be; cations, neutrals, then anions. The net velocity of the analytes is described in Figure 1.4..

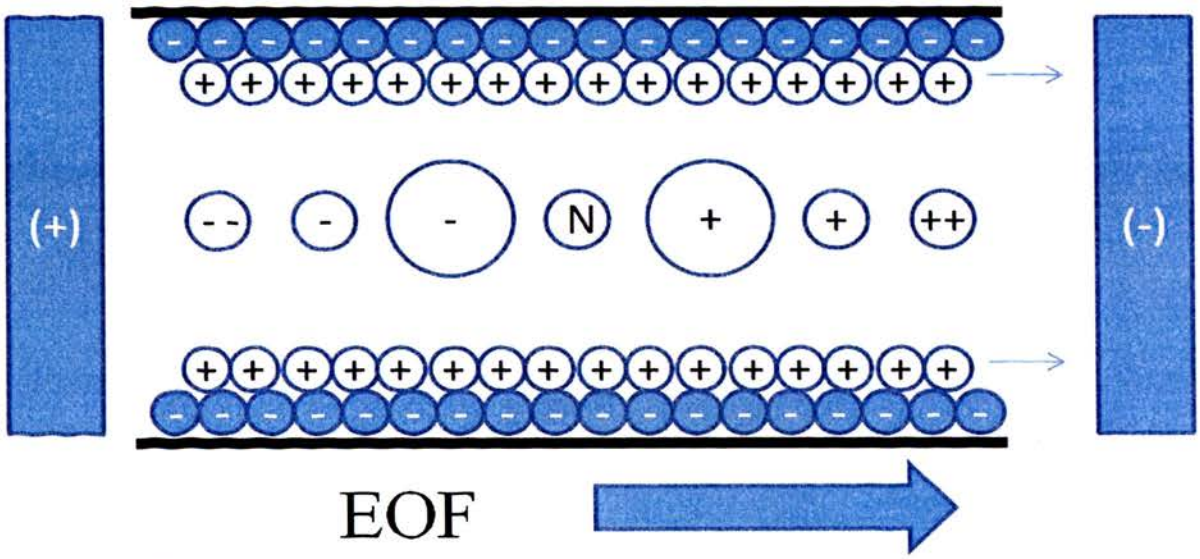

Figure 1.4. Superimposition of electrophoretic and electroosmotic flow

A unique feature of electroosmotic flow in capillary electrophoresis is the flat flow profile [19, 22]. This is a direct result of the laminar flow of the solvated cations along the electric double layer [22]. Since the flow is along the edge of the capillary, dispersion in the capillary is virtually non-existent resulting in elution of narrow peaks. This is uniquely different when compared to systems like high pressure liquid chromatography, where the flow of the solvent through the fluidic pathway is pressure driven [19]. In a pressure driven system, solvent drags alongside the interface of the fluidic pathway, inducing a center driven profile. This results in broadening of peaks and

reduced resolution [19]. Figure 1.5. shows electroosmotic and pressure induced flow profiles.

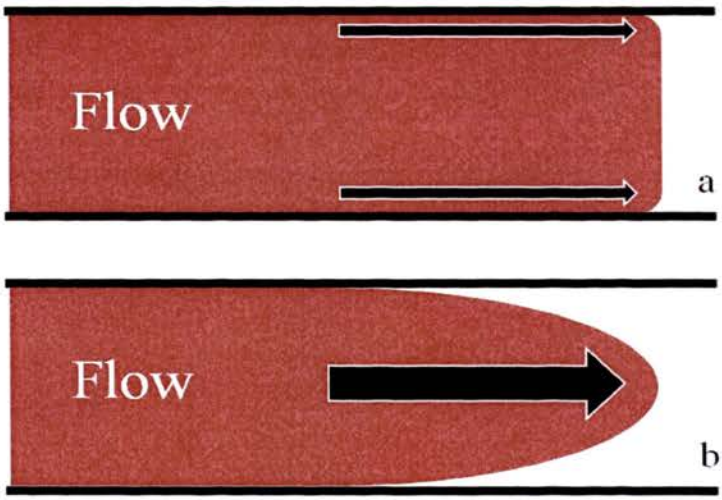

Figure 1.5. Flow profiles for liquids under a) electroosmotic flow and b) flow induced through pressure [19, 22].

1.1.4. Sample Introduction. Sample introduction is different for capillary electrophoresis than other analytical techniques such as HPLC or GC. The capillary is placed into the sample vial, introduced and returned to the buffer for separation [21]. There are three different injection techniques used; pressure, gravimetric (siphon) and electrokinetic [21]. In these three examples, samples are prepared in running buffer or sample buffer. Running buffer is the electrolytic solution used to carry out the separation. Sample buffers can differ from running buffers in several ways such as; pH, buffer strength, additives, or other properties to influence analyte migration [21, 22].

Pressure injections are performed by placing the capillary in to the sample vial, sealed and pressure is used to force the sample into the capillary [21]. The sample volume injected is related to the time and pressure applied during the injection and is calculated by eq. 11 [21].

$$volume = \frac{\Delta P d^4 \pi t}{128 \eta L} \tag{11}$$

where ΔP is the pressure differential, η is the buffer viscosity, d is the capillary diameter, L is the total capillary length and t is the injection duration in seconds [21]. The major advantage for pressure injections is its representative sampling. The major drawback is dilution of the sample since both sample and buffer are injected.

Gravimetric injections use gravity to inject the sample. The capillary is placed into the sample vial, but not sealed. The sample is injected though siphoning and / or capillary action [21]. The injection volume is dependent on difference between the height of the sample and the waste reservoir and the length of time the capillary remains in the sample reservoir. The injection volume is calculated by eq. 12 [21].

$$volume = \frac{\rho g \Delta H d^4 \pi t}{128 \eta L} \tag{12}$$

where ρ is the density of the sample, g is the gravitational constant, ΔH is the height differential, η is the buffer viscosity, d is the capillary diameter, L is the total capillary length and t is the injection duration in seconds [21]. As with pressure injections, the major advantage for pressure and gravimetric injections are its representative sampling. The major drawback is dilution of the sample since both sample and buffer are injected.

In electokinetic injections, the samples are prepared in buffered solution and the capillary and electrode are placed into the sample together. A potential is applied and ions selectively migrate from the sample into the capillary through electrophoretic migration and electroosmotic flow [21]. The advantage to this technique is that it functions as an on-line concentrator of the sample. The technique is also highly selective to ions that migrate during the injection process. Therefore electrokinetic injections can be employed to "bias" the sample during injection [21]. The amount of sample injected is calculated by eq. 13 [21].

$$Q = \frac{(\mu_e + \mu_{eo})V\pi r^2 Ct}{L} \tag{13}$$

where Q is the amount injected, μ_e is the electrophoretic mobility, μ_{eo} is the electroosmotic mobility, V is the applied voltage, r is the capillary diameter, C is the sample concentration, L is the total capillary length and t is the injection duration in seconds [21].

1.1.5. Mobility and Migration. As described above, the migration time of an analyte is the time it takes to get from the injector to the detector and is related to both the electroosmotic flow and the mobility of the analyte [23]. The apparent mobility (μ_A) is calculated from the migration time of the analyte and is the summation of the electrophoretic and electroosmotic mobilities eq 14 [23].

$$\mu_A = \mu_e + \mu_{eo} = \frac{lL}{tV} \tag{14}$$

where l is the length of the capillary from the injection end to the detector, L is the total length of the capillary, t is the migration time and V is the applied voltage. The effective mobility (μ_e) for a separation system is determined by measuring the electroosmotic flow with neutral markers such as acetone or benzyl alcohol [23].

1.1.6. Resolution. Resolution (R_S) is the degree two analytes have been separated. A basic equation for resolution is shown in eq. 15 [19].

$$R_S = \frac{\Delta Z}{W_A + W_B} \tag{15}$$

Where ΔZ is the difference in elution time between the two peaks, and W_A and W_B are the peak widths for peak A and B respectively [19]. Figure 1.6. shows a pictorial representation of resolution (R_S).

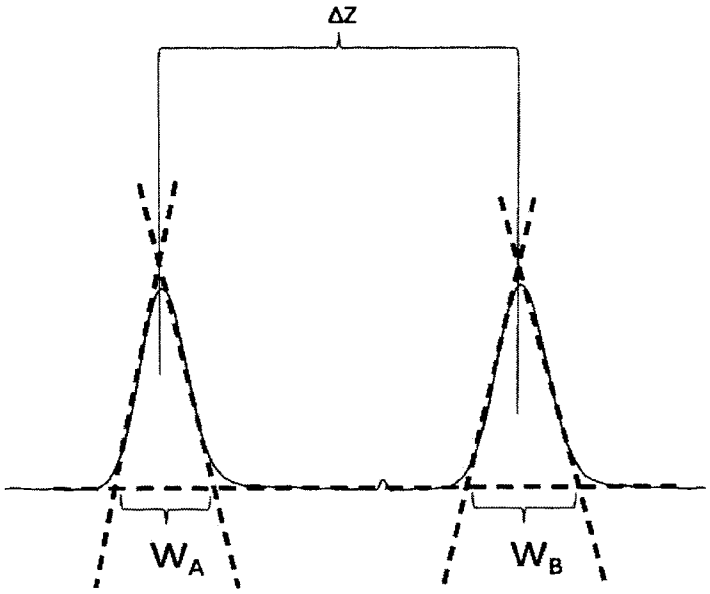

Figure 1.6. Pictorial representation for resolution (R_S) [19]

In capillary electrophoresis separation is influenced more by efficiency than selectivity [20]. Therefore, resolution can also be expressed with respect to efficiency (eq. 16) [20].

$$R_S = \frac{1}{4}\frac{\Delta\mu_e}{\overline{\mu_e}}\sqrt{N} \tag{16}$$

where $\Delta\mu_e$ is the difference in electrophoretic mobilities between the two analytes, $\overline{\mu_e}$ is the average electrophoretic mobility of the two analytes and N is the number of theoretical plates [19].

Longitudinal and mass-transfer resistance contribute to peak dispersion in chromatography [19, 22]. For capillary electrophoresis, only longitudinal diffusion needs

to accounted for [19]. Therefore, the number of theoretical plates (N) in capillary electrophoresis is determined by eq. 17 [19, 22]:

$$N = \frac{\mu_e V}{2D_m} \tag{17}$$

where μ_e is the electrophoretic mobility of the analyte, V is the applied voltage and D_m is the diffusion coefficient of the analyte [19, 22]. Since resolution increases as the number of theoretical plates increase (eq. 16), higher applied potentials are used to obtain high resolution separations [19]. However, Joule heating limits the use of increased voltage and will be described below [19, 21, 22, 24].

1.1.7. Factors that Affect Separation. Several different factors affect the separation during analysis. In this section the following are described; capillary diameter, Joule heating and temperature, voltage, pH, buffer strength and additives.

1.1.7.1. Capillary Diameter. Typical inner diameters of capillaries range from 10nm to 200nm [22]. Smaller inner diameters produce better resolution due to reduced radial temperature gradients [22, 24]. However, reducing the inner diameter reduces detector path lengths and sample loadability [22]. Another limitation of smaller capillaries (<50nm) is that they are more prone to clogging than larger ones, although this is minimized by filtering buffer solutions and samples [19, 22].

1.1.7.2. Joule Heating / Temperature. Resistance is generated when high voltage is applied to a buffered solution across a capillary [22]. This resistance generates heat. The amount of heat generated is proportional to the field strength [22]. Heat stems from the center of the capillary and diffuses outwards towards the capillary walls, creating radial temperature gradients and global changes in temperature [22, 24]. Two items influenced by temperature are viscosity of the buffer solution and mobility of the ions (reportedly ~2% per degree Kelvin) [22]. The decrease in viscosity and the change in the flow profile from electroosmotic to hydrodynamic result in band broadening [22]. The temperature generated can be controlled by capillary length, capillary diameter, buffer concentration, voltage and cooling systems [19, 21, 22, 24].

1.1.7.3. Voltage. The separation of the analytes results from differences in migration. Since electrophoretic and electroosmotic velocities are directly proportional to the field strength [22], an increase in voltage will result in shortened run times. Diffusion is the primary influence in band broadening [21, 22]. Therefore, short run times should generate the highest efficiencies. Unfortunately, Joule heating becomes the limiting factor for the voltage applied [18-22, 24].

1.1.7.4. pH. The primary function of pH is to control the degree of electroosmotic flow during the separation through the ionization of the acidic silanols on the capillary wall [21]. The reduction of the ionization results in a decrease of the zeta-potential and subsequently the electroosmotic flow [21]. At low pH vales ($pH < 4$) ionization is small and the effective electroosmotic flow is insignificant [21]. At high pH

levels (pH > 9) there is near complete ionization of the silanol groups resulting in a fast electroosmotic flow [21]. Therefore, by adjusting the pH of the buffer solution, the net velocity of the analytes is optimization through balancing the electrophoretic and electroosmotic velocities to maximize resolution [19, 21].

1.1.7.5. Buffer Type and Strength. Buffered solutions contain a weak acid and its conjugate base or vice versa. The ionic strength I is defined as [24]:

$$I = \frac{1}{2}\Sigma z_i^2 \cdot [i] \tag{18}$$

Where z_i is the charge of the ionic species and $[i]$ is the equilibrium concentration. To calculate the total strength of the buffer, this equation should be applied to all of the buffered components in the solution. Reijenga *et al.* [24] showed that there is strong effect on the effective mobility on ions as a result of changing the background electrolyte (buffer) strength. Electroosmotic flow is related to the zeta-potential [21, 22]. Zeta-potential is inversely related the square root of the buffer concentration, the number of valence electrons and the charge of the surface area [21, 22]. Therefore, under the same applied voltage, the higher the ionic strength of a buffered solution, the slower the resulting electroosmotic flow will be [21, 22, 24].

There are a wide range of buffers available. Buffers are most effective when used in a pH range within one or two units from its isoelectric point (pI) [22]. Additionally, Zwitterionic buffers can also be used to provide the added benefit of generating less Joule

heating when used near its pI [22, 24]. Table 1.1. shows some conventional buffer options.

Table 1.1. Buffers for Capillary Electrophoresis [22]

Buffer	Useful pH Range
Phosphate	1.14 - 3.14
Acetate	3.76 - 5.76
Phosphate	6.20 - 8.20
Borate	8.14 - 10.14
Zwitterion Buffer	
MES	5.15 - 7.15
PIPES	5.80 - 7.80
HPES	6.55 - 8.55
Tricine	7.15 - 9.15
Tris	7.30 - 9.30

1.1.7.6. Additives. There are times when two compounds have near identical electrophoretic mobilities. For a given buffer solution, changes in voltage, ions strength and pH seem to have no impact on their separation. Additives have been used to selectively change the effective mobility of different analytes [22]. This results in a net migrational change for each analyte. Some of the more common additives in capillary electrophoresis include; organic solvents, and anionic, cationic and neutral surfactants [22, 23]. Additionally, bile salts or cyclodextrins are used to achieve chiral selectivity [22, 23].

1.1.7.7. Detection. There are several different detection techniques used with capillary electrophoresis [19]. On-capillary techniques refer to detection that occurs while the analyte is still inside the capillary. The primary on-capillary techniques used are absorbance and fluorescence detection [19]. Off-capillary techniques refer to detection that occurs after the analyte leaves the capillary. The primary off-capillary techniques used are electrochemical (conductivity, potentiometry and amperometry) and mass spectrometry [19].

In UV absorbance detection, an ultraviolet beam is directed through the capillary and a detector measures the intensity of the light passing through the capillary [19]. As analytes migrate past the detector they absorb the light energy, causing a change the amount of light energy the sensor detects [19].

In fluorescence, the analytes are excited as they pass through an excitation beam. A detector (typically 90° from the excitation beam) collects the fluorescent emissions from fluorescing analytes.

The major advantage of fluorescence over UV detection is sensitivity [19]. General detection limits for UV absorption can reach 10^{-13} to 10^{-15}M, where fluorescence detection can range from 10^{-17} to 10^{-20}M [19].

1.2. Pteridines

1.2.1. Introduction. Researchers in bio-chemical and bio-analytical fields continue to look for simple, cost effective, non-invasive methods for detecting cancer in the human body. Over the past 20 to 30 years what began as detection viruses and diseases, has evolved into an approach involving the analysis of urine for cancer detection [12, 13, 25, 26]. It is theorized that as diseases or abnormalities attack the human immune system, pteridine levels will change in a predictable manner. Different approaches have been used to identify and analyze pteridines, such as thin layer chromatography [27], high performance liquid chromatography [25, 28, 29] and capillary electrophoresis [12, 13]. Each investigation researching pteridine levels to identify the existence and severity of diseases [12, 13, 25-38]. The focus of pteridine research has shifted to cancer over the past decade [12, 13, 26].

1.2.2. Signatures in Diseases. "A key challenge in cancer control and prevention is detection of the disease as early as possible..." [39]. Success depends on the ability to identify and detect biomarkers that communicate properties about malignancies [40]. Biomarkers contain the information about the physiological state of the disease and are analyzed to monitor the changes that occur in the disease as it progresses [40]. Pteridines have been shown to change in concentration for a variety of diseases [29, 32, 33, 35, 36, 41, 42].

Mazda et al. reviewed the usefulness of pteridines as clinical markers. Mazda analyzed the amount of neopterin and xanthopterin extracted from urine of liver disease patients [32]. The results showed that different liver diseases produced different ratios of

neopterin to xanthopterin [32]. Patients with chronic active hepatitis and chronic persistence hepatitis showed increased levels of urinary neopterin [32]. However, patients with alcohol induced liver disease and non-alcohol fatty liver diseases did not [32]. Mazda also observed that infectious diseases involving activated cell-mediated immunity also increased urinary neopterin and concluded that increased urinary xanthopterin signified liver-cell damage [32].

Altindag et al. investigated urinary neopterin levels as an indicator of disease activation in Behcet's disease [41]. Altindag discovered that patients with active Bechet's disease showed higher levels than both the inactive disease and the control group. Altingad went on to find no significant difference between the control group and the group with inactive Behcet's disease [41].

Granditsch et al. re-demonstrated that Crohn's disease activity can be directly correlated with urinary neopterin excretion levels [42].

Rokos investigated the mechanism behind increased urinary pteridine levels in patients with malignant diseases than those of the control groups analyzed [29]. He further reported that xanthopterin levels were increased when neopterin levels had increased and he suggested that "alterations in pteridine metabolims are common in malignant diseases." [29] Rokos also proved that changes in the pteridine levels of cancer patients was a result of the increased guanosine triphosphate (GTP) levels [36, 43]. Rokos further concluded that Crohn's disease caused increased levels of urinary neopterin levels along with sepsis, colitis ulcerous, myocardial infraction, heart failure, periarteritis nodosa, and Gardner Syndrome [35].

Nogales et al. performed a series of evaluations of children's infections using the neopterin factor, defined as the neopterin / creatinine ratio [33]. Thirty-one children with lymphocytic meningitis, mononucleosis infectious, hepatitis A, herpetic gingivostomatitis, viral urticaria, viral brochiolitis and bronchopneumonia, bacteriological pneumonia, mycoplasmal pneumonia, and meingococceal sepsis were tested [33]. Results showed that all of the infections generated an increased neopterin factor that was differentiable from the control group [33]. It was concluded that the neopterin factor was not selective for identification of infections, but could be used as an evolutionary monitor for diseases [33].

1.2.3. Cancer Detection. Fukushima, began investigating pteridine chemistry and exploring urinary pteridines for analysis in cancer research in 1980 [30]. The analytical techniques that have been used to investigate pteridines are thin layer chromatography, refractive index analysis, high performance liquid chromatography, and capillary electrophoresis [12, 13, 25, 26, 28-33, 35-38, 41, 42, 44, 45].

Pteridines have been identified in urine, serum, saliva and body tissues [12, 13, 25, 26, 28-33, 35-38, 41, 42, 44-54]. Nine specific compounds have been investigated to date; neopterin, biopterin, oncopterin, 6,7-dimethylpterin, pterin, pterin-6-carboxylic acid, xanthopterin, isoxanthopterin, and 6-hydroxymethylpterin [12, 13, 25, 26, 28-33, 35-38, 41, 42, 44-54]. Pteridines have been investigated as a biomarker for cancer for almost 40 years. Several finding show that pteridine levels are influenced by cancer formation. However, no correlations have been validated to date.

1.2.4. Mechanisms. Pteridines are a class of compound that contains 2-amino-4-hydroxypteridine (Figure 2.1.) and all of the forms of pteridines currently under investigation contain a form this structure. The pteridines actively being investigated in body fluid analysis are: neopterin, biopterin, oncopterin, 6,7-dimethylpterin, pterin, pterin-6-carboxylic acid, xanthopterin, isoxanthopterin, and 6-hydroxymethylpterin [12, 13, 25, 26, 28-33, 35-38, 41, 42, 44-54]. Many of these chemicals are detectable in their unconjugated form through serum or urine analysis [36].

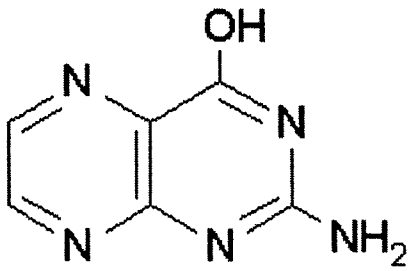

Figure 2.1. Pteridine bi-cyclic structure

These compounds play a role in human metabolic processes (in reduced forms) and can serve as the cofactor for conversion amino acids to the neurotransmitters [44, 55]. Figure 2.2. outlines a proposed biosynthetic pathway for the formation of pteridines. Additionally, these proposed reactions are the same that occur in the natural degradation of pteridines and furthermore are found as terminal metabolites in the medium of urine and cell cultures [31].

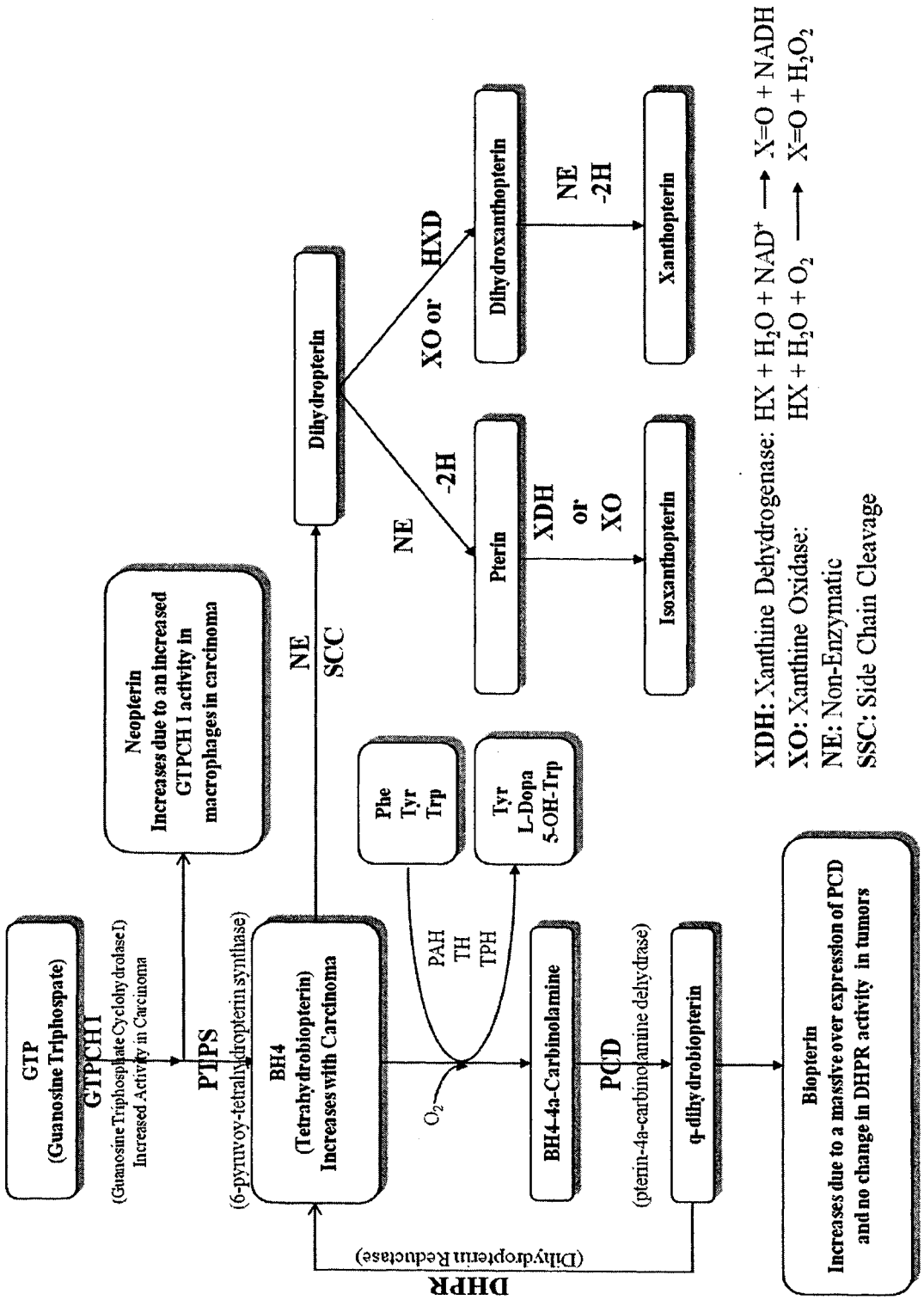

Figure 2.2. Proposed biosynthetic pathway for pteridine biomarkers [34, 56-65]

It has been postulated that pteridines also form from folic acid derivatives [36]. Although the exact mechanism is not fully understood Figure 2.3. shows a summary of the proposed pteridine – folic acid catabolism) it has been observed that male patients who have received injections of H-folic acid, produced urine with H-pteridines [66], supporting this postulate.

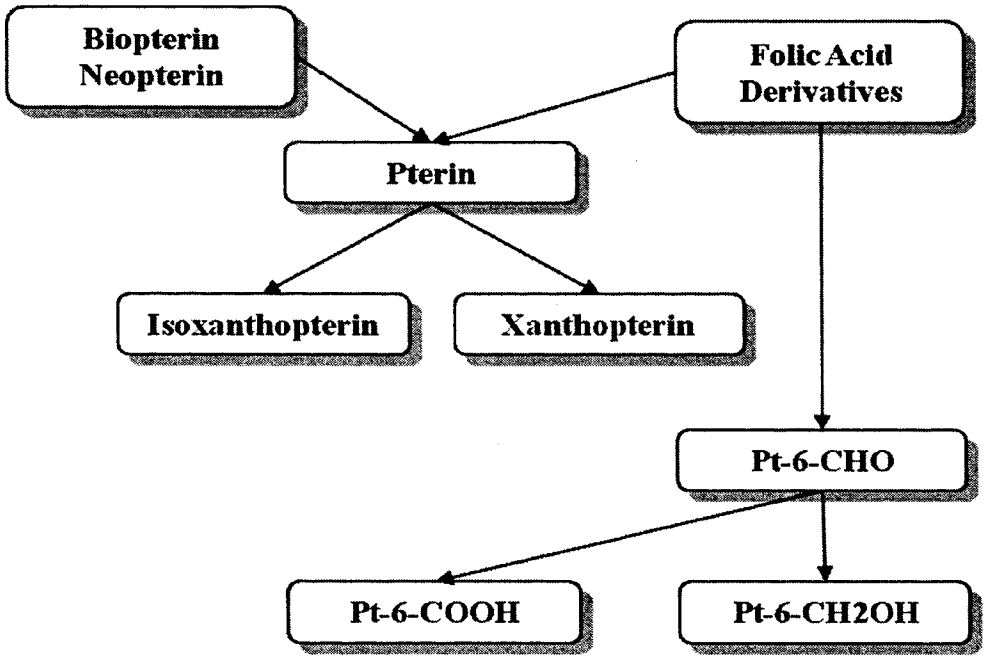

Figure 2.3. Proposed pteridine – folic acid catabolism [36, 66]

1.2.5. Pteridine Expression in Biofortification of Plants. Maintaining proper heath in humans requires a balanced diet [67-69]. Folates (folic acid or vitamin B_9) are essential in several function of the body through its transformation to tetrahydrofolate [69]. These functions include nucleotide biosynthesis to the remethylation of

homocysteine [70], DNA synthesis [70], repair and methylation [70], and production of healthy red blood cells [71].

Diets that lack insufficient folic acid intake can lead to folate deficiency [69]. Folate deficiency can subsequently lead to megaloblastic anemia, birth defects, impaired cognitive development and increased risk of vascular disease and cancer [68, 69].

The recommended daily allowances are approximately 400μg per day for adults and up to 600μg per day for pregnant women [67]. Folate deficiencies are overcome through supplements or food fortification [68]. However, these options maintain recurring costs and are not viable options for third world countries [69]. An alternative option investigated and successfully achieved is folate enhancement though bioengineering or metabolic engineering of plants on the gene level [68, 72, 73]. Basset *et al.* [74] investigated three possible approaches for engineering higher folate concentrations in plants; increased folate synthesis by over expressing enzymes, increased transport and stock piling of folates in metabolically inert regions of the plant and, decreasing the chemical and enzymatic breakdown through various means [74]. At the end of their investigation, the synthetic pathway of folates became largely understood and subsequently many laboratories have started biofortification trials of their own [74].

1.2.6. Biosynthesis of Folates in Plants. DellaPenna presented the structure and biosynthetic pathway in of folates in plants in a commentary in 2007 (Figure 2.4.). Hossain *et al.* investigated and validated that the expression of GTP cyclohydrolase-1 is a rate-determining step in the pterin and folate biosynthesis in plants [69]. The expression of GTP cyclohydrolase-1 results in increased pteridine and folate levels [69]. These

finding allowed for indirect measurement of folate fortification by monitoring neopterin, xanthopterin and isoxanthopterin levels [69]. Hossain also proposed a pathway and localization of key enzymes necessary for pteridine synthesis in plants (Figure 2.5.) [69].

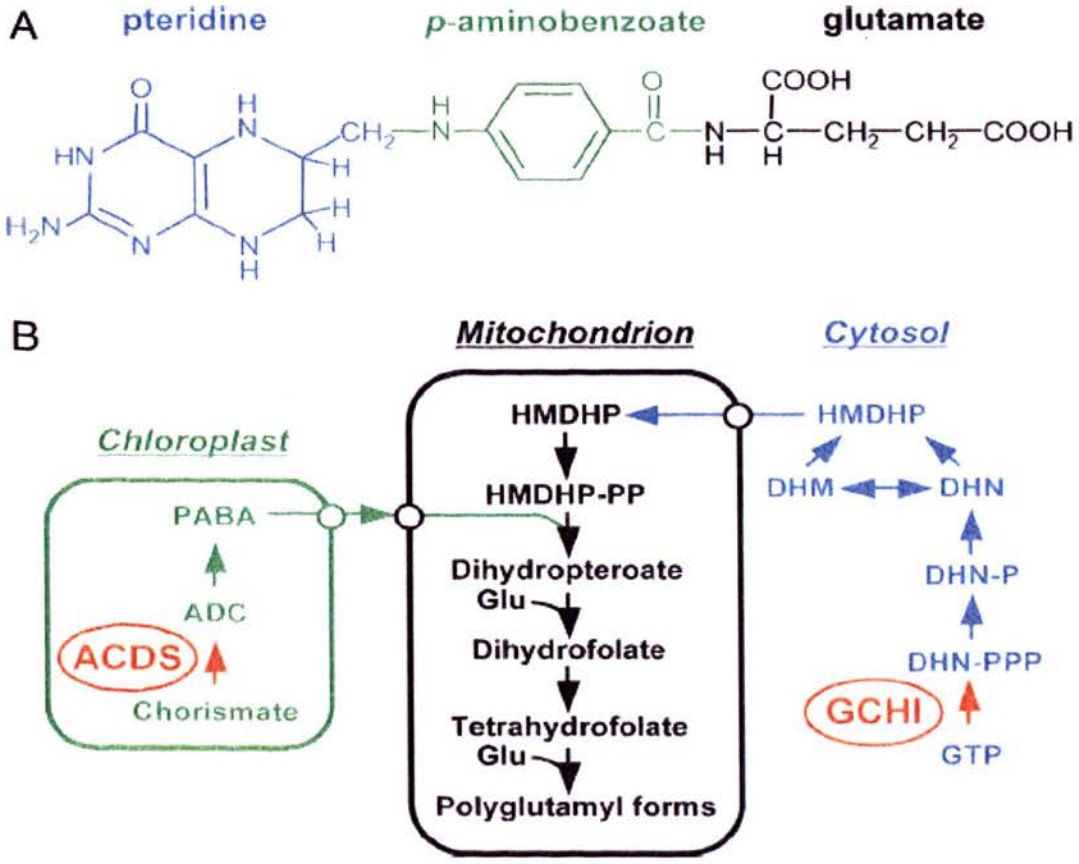

Figure 2.4. "Structure and biosynthesis of folates in plants. (A) The chemical structure of monoglutamyltetrahydrofolate is shown. The pteridine-, PABA-, and glutamate-derived moieties are color-coded. (B) The plant folate biosynthetic pathway is shown. The pteridine pathway leading to hydroxymethyldihydropterin(HMDHP) is shown in blue, the pathway leading to p-aminobenzoate is shown in green, andsteps localized in the mitochondria are in black. Open circles indicate possible transporters. Red arrows indicate the two enzymes (GCHI and ADCS) engineered by Diaz de la Garza et al. [68]. DHN, dihydroneopterin;-P, monophosphate; -PP, pyrophosphate; -PPP, triphosphate; DHM, dihydromonapterin." [67]

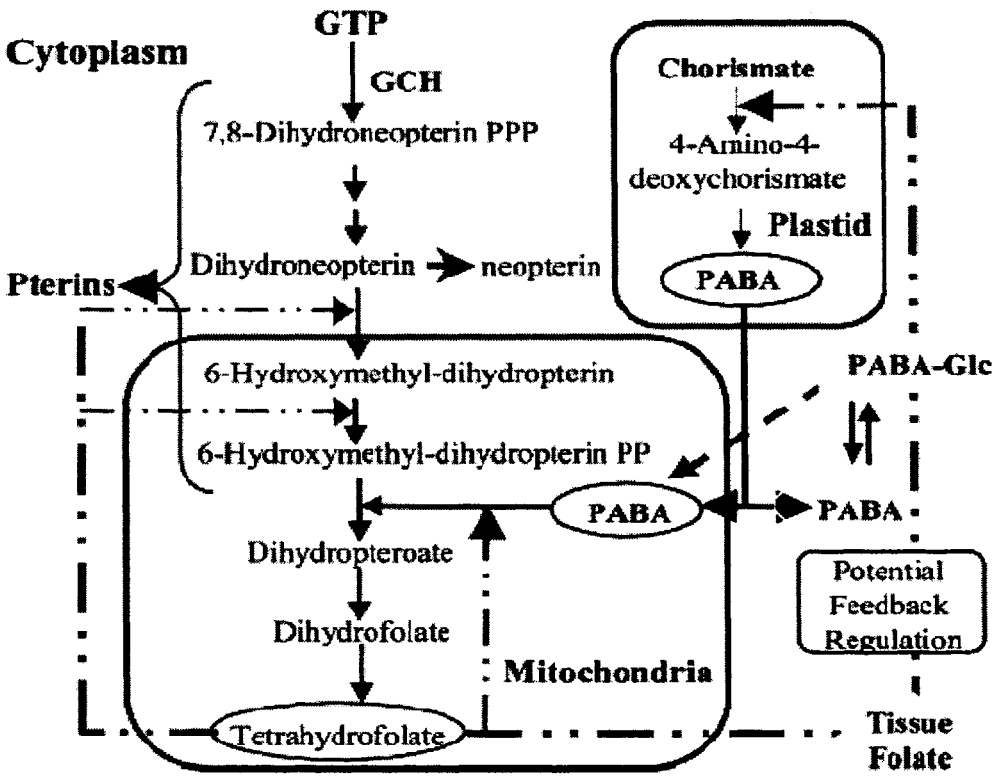

Figure 2.5. "Proposed synthetic pathway and localization of key enzymes for pteridines and folates in plant samples" [69].

1.3. Pharmaceutical and Personal Care Products (PPCP)

1.3.1. Introduction. Pharmaceutical and personal care products (PPCP's) compounds are defined by the U.S. Environmental Protection Agency (EPA) as: "...in general..., ...any product used by individuals for personal health or cosmetic reasons or used by agribusiness to enhance growth or health of livestock. PPCP's comprise a diverse collection of thousands of chemical substances, including prescription and over-the-counter therapeutic drugs, veterinary drugs, fragrances, and cosmetics." [75]

PPCP's include a variety of products that make life more enjoyable or convenient. Items such as antibiotics, anticonvulsants, mood stabilizers and pain relievers help with physical ailments. Cosmetics, aging creams, sun screens and other beautifiers help with psychological well being [76, 77]. As billions of people on earth use these products daily, a growing concern for their environmental impact has developed [78].

1.3.2. Introduction into the Environment. PPCP's can find their way into the environment and ultimately water systems through a variety of pathways [79]. The U.S. EPA in conjunction with the Office of Research and Development, The National Exposure Research Laboratory, the Environmental Sciences Division and the Environmental Chemistry Branch, put together a systematic flow from (Figure 12) sources to end fates of PPCP's as they migrate through their life span in the environment [79].

Figure 3.1. Origins and fate of PPCP's in the environment. Reproduced from the EPA website [79].

- "The drinking water for Washington, D.C., and surrounding areas tested positive for six pharmaceuticals."
- "Three medications, including an antibiotic, were found in drinking water supplied to Tucson, Ariz."

Water professionals are currently researching the effectiveness of treatment techniques for removal of PPCP's and other organic compounds from water systems [94]. The large disparity of structures and properties associated with PPCP's makes the removal process difficult [94]. Subsequently, removing all PPCP's detected with a single method is virtually impossible [94]. Membranes and granular activated carbon are under investigation to physically remove compounds [94]. Ozone and UV light is used to break the compounds down into non-toxic species [94].

1.3.4. Current Analysis Methods (Water Matrix). Liquid chromatography coupled with mass or tandem mass spectrometry has by used to analyze PPCP's in water matrices [95-100]. LC/MS and LC-MS/MS possesses the sensitivity, selectivity, and repeatability to perform trace analysis. Some work has been done using gas chromatography, but many of the PPCP's are polar, thermally labile and non-volatile, requiring derivatization prior to analysis [101]. Capillary electrophoresis has been used, but not extensively [102]. Additionally, there publications available that discuss the different analyses of PPCP's in water [103-106].

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PAPER

1. OPTIMIZATION OF URINARY PTERIDINE ANALYSIS CONDITIONS BY CE-LIF FOR CLINICAL USE IN EARLY CANCER DETECTION

Abstract

The second leading cause of death in the US is cancer and early discovery of the disease has translated into reduced fatality rates. We have identified and performed a systematic investigation of a method for urinary pteridine analysis by using CE-LIF, which is believed to possess the potential to diagnose the presence of cancer even earlier than existing methodologies. Through system enhancements, we have been able to improve the resolution of the two least resolved sets of peaks (6,7-dimethylpterin versus 6-biopterin and D-(+)-neopterin versus 6-hydroxymethylpterin) from 0.85 to 2.48 and 0/90 to 3.58, respectively. Additionally, we have discovered that the preparation of the urine samples in previous works was inadequate, and we have corrected the method to fully oxidize the pteridines in the urine, resulting in significantly less variability in quantification and greater ease of defining p-values for healthy versus cancer patients. Finally, we have performed validation steps of spike and recover and short-term aging studies to demonstrate the method's robustness. As a result, we present an optimized and validated method ready for transfer from discovery phase to clinical trial that can potentially act as a non-invasive pre-screening test for cancer.

Key Words

CE; conditional study; urinary pteridine analysis

Introduction

Today, cancer maintains its status as the second leading cause of death in the US. Organizations such as the American Cancer Society and The National Cancer Institute have multi-facetted goals for their research including the discovery of the causation, treatment and prevention of cancer. Until a cure for cancer is developed, hundreds of government, private and educational institutions will continue to put their focus on detection and treatment. Data has shown that the earlier cancers are discovered, the better the chance treatment will be successful. One of the concerns for methods used in cancer or pre-cancer screening is the validation of those methods to reduce "chance and bias [1,2]. Due to the complexity of biological sample matrices, method optimization and validation must be a primary concern when looking to transfer techniques from discovery to clinical trials in order to prevent possible controversies in the conclusions obtained from the data [3].

Biomarkers are compounds in the body that can be indicative of medical conditions or biological states. They have been used over the years as indicators for many diseases ranging from arthritis [4] to diseases forming during fetal development [5] to cancer [6]. One specific discovery that showed promise in early cancer screening through the identification of biomarker molecules was the analysis of urinary pteridines using CE-LIF [7]. Interest in this work stemmed from the findings that the onset of different viruses, cancers and other medical conditions could affect the levels of pteridines [7-12].

These molecules consist of a pterin base with a variety of different functional groups bonded to the C6 carbon of the bi-cyclic pterin molecule, forming the pteridines of interest [13]. Their structures are shown in Figure 1. As evidenced by the vital role they play in the catabolism of phenyalalanine through aromatic amino acid hydroxylases, pteridines are important in many function of the body [14-16].

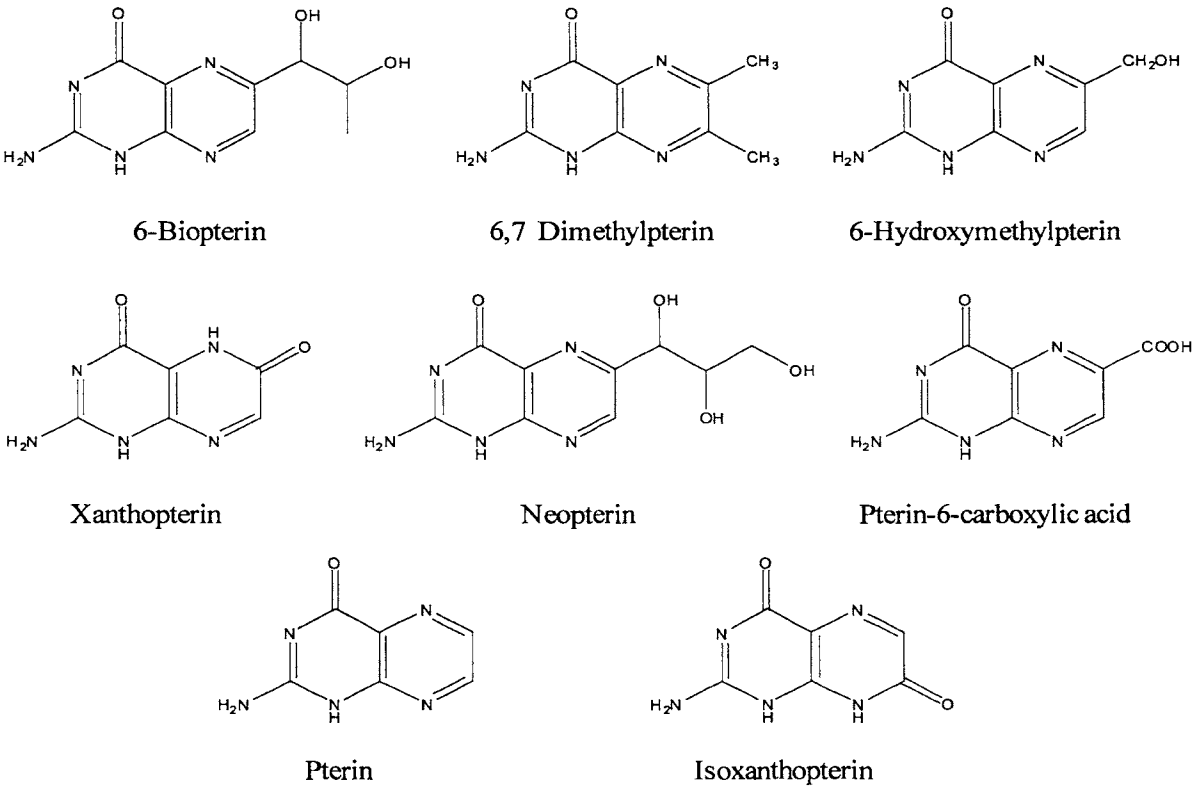

Figure 1. Structures of pterin bi-cyclic molecules

Even though CE-LIF in previous pteridine work has shown particular promise in its feasibility for clinical laboratories, only a limited number of parameters were

investigated [7]. In addition, because of the complexity of urine samples, the method encountered significant challenges in avoiding interferences from other compounds during the separation and quantification of pteridines in the real urine samples. Variables affecting quantification and reproducibility remained yet to be studied and validated, which were necessary to supply confidence in applying this method for clinical diagnosis.

To correct these limitations, a comprehensive conditional study of pteridine analysis by CE-LIF was conducted. Instrument setup, method parameters and sample preparation have been investigated and optimized to enhance the resolution, sensitivity and accuracy to the levels necessary for pre-cancer screening. Additionally, we have performed validation testing of the optimized method to include a short term again study (0-15 days) of unmodified urine samples under different storage conditions, short term (up to 6 hours) sample stability after preparation, spike and recover studies to determine the efficiency of our sample preparation and limits of detection and linear ranges used for quantification.

These conditional investigations have facilitated the enhancement and validations necessary to drive this technique towards its goal of use in a clinical environment.

Materials and methods

Chemicals

6-Biopterin, D-(+)-neopterin, pterin, isoxanthopterin and xanthopterin pteridine standards, boric acid and Tris (hydroxymethyl)aminomethane were purchased from Sigma (St. Louis, MO, USA). 6,7-Dimethylpterin and 6-hydroxymethylpterin were purchased from Schircks Laboratories (Jona, Switzerland). EDTA disodium salt, iodide, potassium iodine, sodium hydroxide and sodium phosphate dibasic were purchased from

Fisher Scientific (Fair Lawn, NJ, USA). Ultra pure water (18.2 MΩ) was prepared by a Millipore water purifier, model Simplicity 185.

Buffer preparation

The sample buffer, used to dilute both standards and samples, was a 50mM aqueous solution of sodium phosphate dibasic (Na_2HPO_4). The solution was prepared by dissolving approximately 1.77g of Na_2HPO_4 in 200mL of ultra pure water and adjusting the pH to 7.70 ± 0.02 using concentrated phosphoric acid. The solution was then vacuum filtered through a 0.45μm membrane filter, transferred into a 250mL volumetric flask and diluted to a final volume of 250mL using ultra pure water. The running buffer was an aqueous solution of 0.1M Tris-(hydroxymethyl)aminomethane (Tris), 0.1M boric acid and 2mM EDTA disodium salt. The solution was prepared by dissolving approximately 3.03g of Tris, 1.55g of boric acid and 0.19g of EDTA disodium salt in 150mL ultra pure water and adjusting the pH of the solution to 9.63 ± 0.02 using 2,0M sodium hydroxide. The solution was then vacuum filtered through a 0.45μm membrane filter, transferred into a 250mL volumetric flask and diluted to a final volume of 250mL using ultra pure water. All pH measurements were performed on an Accumet® Excel XL-15 pH meter and standardized using pH standard 4, 6, and 10 (Fisher Scientific, Fair Lawn, NJ).

Standard preparation

Pteridine standard solutions were prepared by dissolving 2mg of pteridine standard in a solution consisting of 0.3mL of 1.0M sodium hydroxide and 9.7mL of sample buffer. A standard mixture was prepared by combining equal-molar

concentrations of each standard and diluted to a final concentration of 5.0×10^{-5}M with sample buffer. This solution was then used, via serial dilutions, in the generation of the calibration curves used for quantification.

Oxidizing solution preparation

The oxidizing agent used to prepare the urine samples was a solution consisting of 4.0% potassium iodide and 2.0% iodine (w/v). The solution was prepared by dissolving 0.80g of potassium iodide in 20mL of ultra pure water. Once the potassium iodide was dissolved, 0.40g of iodine was added to the solution and the solution was stirred until the iodine was completely dissolved. The final solution was stored in a dark vial, the cap wrapped with Parafilm M® and the vial wrapped with aluminum foil to prevent photo degradation of the iodine.

Urine sample preparation

Urine samples were collected and stored either in the refrigerator or freezer, depending on the time from collection to analysis. Prior to analysis, the samples were removed from cold storage and brought to room temperature. A 1000µl aliquot was taken using a micropipette and placed into a 1.5mL yellow vial. Four-hundred microliters of the oxidizing solution and 100µl of 2.0M sodium hydroxide were then added to the sample and mixed thoroughly. The sample mixture was incubated at 4°C for 30 minutes and centrifuged at 5000rpm for 20 minutes at 4°C. The supernatant was diluted 1:1 (500µl :500µl) with sample buffer, mixed thoroughly and directly injected into the CE for analysis.

CE-LIF system

The CE-LIF system used in this study was a home-built instrument. Even though it was similar to that in our previous study, some modifications have been made to enhance sensitivity and reproducibility. A Milles Griot Omnichrome Series-74, 325nm laser (35mW power, Carlsbad, CA, USA) was used for excitation and the stray and scattered light from the non-polarized laser was removed using a 325nm band pass filter (Ealing, Holliston, MA, USA; model UG-11). The laser beam passed through an iris and was focused onto a capillary window with a 2.0cm focal length lens. A 43x microscope objective was positioned at 90° to collect and focus the fluorescence emission onto a R982 Hamamatsu photomultiplier tube (Bridgewater, NJ, USA). The fluorescence emission was filtered using a band-pass filter (400 – 539nm, Ealing model 35-532) to minimize the background noise during the analysis. The resulting output current form the photomultiplier tube was converted to a voltage signal through a home built current-to-voltage converter, the analog signal was digitalized through a Logger Pro analog-to-digital converter (Venier® software and technology, Beaverton, OR, USA) and was finally recorded using Logger Pro 3.1 data collection software. The instrumental design is shown in Figure 2.

Sample analysis using CE-LIF

A 50 μm i.d. x 70 cm fused silica capillary (Polymicro Techniques, Phoenix, AZ) was used for the separation. The capillary was pretreated with 1.0 M NaOH for 30 minutes followed by water rinsing to clean the capillary. A one centimeter section of the polymer coating was burned off at 35 cm from the cathodic end of the capillary to form

the detection window, providing an effective capillary length of 35 cm. Samples were injected into the separation capillary using the gravimetric method, which provides a better representation of the actual composition of the urine samples compared to electrokinetic injections (used in the previous study [7]). The injection was performed 17.5 cm from the top of the sample to the instrument table with an injection time of 10 seconds, resulting in an injection volume of 5-7nl. Electrophoresis was carried out at 26 kV (371 V/cm) for 20 minutes. The capillary was regenerated after each run by flushing with 0.2M sodium hydroxide for one minute followed by a 2-minute water rinse and a 2-minute running buffer rinse. The purpose of this procedure was to ensure the best separation and reproducibility. The identification of the individual pteridine peaks in the urine sample was performed by retention time comparison and standard addition. The experimental concentration of each pteridine (ECP) was determined using the peak area obtained from the Logger Pro software and calculated by linear regression against experimentally generated calibration curves.

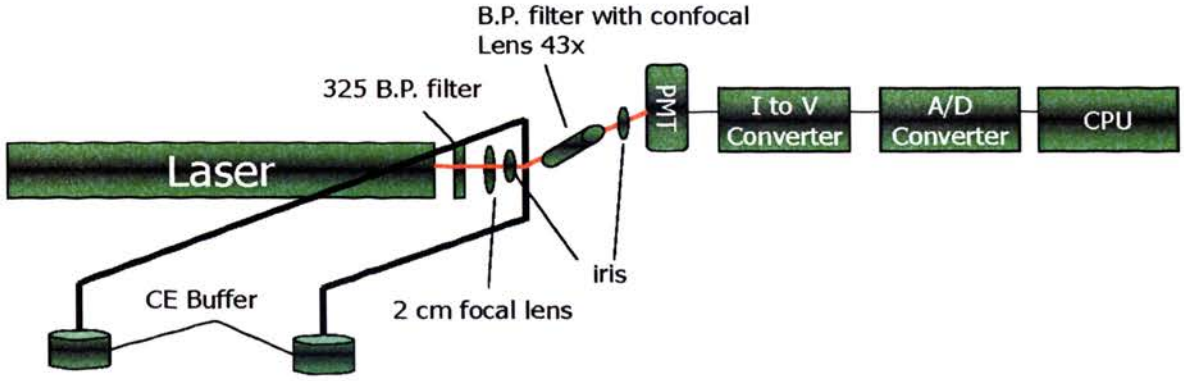

Figure 2. Instrumental design

Results and discussion

Instrumental enhancement

Characterizing the sample in an unbiased manner is critical in determining the actual concentrations of biomarkers for clinical studies. The previous research employed electrokinetic injections to introduce sample into the analysis system. Electrokinetic injections do have advantages, such as ease of use, good repeatability and online concentration of analytes. However, electrokinetic injections can also be highly selective due to differing electrophoretic mobilities among the analytes. In addition, the differing matrices of various urine samples can potentially cause further discrepancies in the consistency of the amount of individual urinary pteridines being introduced. Since the goal of this work was quantification of the total unbiased concentrations of pteridines present in the urine samples collected, gravimetric injection was used in this study because it is unbiased injection technique. Using this technique also opened a pathway to discovery of additional pteridine markers that may further help to distinguish between those individuals who have and have not developed cancer.

One of the drawbacks of gravimetric injections was that the concentration of the individual urinary pteridines was significantly reduced in each injection. To overcome this, it was necessary to upgrade from the previously used laser [7] to a more powerful model (35mW@ 325 nm), and thereby enhance pteridine excitation. Background noise arising from non-polarized light scattering off of the outer wall of the capillary was reduced by replacing a 10x microscope lens with a 43x microscope lens. Additionally, the higher magnification lens allowed for more precise focusing on the central portion of the capillary, which resulted in more efficient collection of the fluorescence emission

from the pteridines molecules. Finally, the photodiode detector was replaced with a photomultiplier tube for enhanced sensitivity and reproducibility. These combined modifications provided a stable baseline of less than 1 mV, which was integral in the system maintaining the sensitivity (in the range of 1.0×10^{-10} M) necessary for analysis of small molecules in CE-LIF [17] while the less sensitive gravimetric sample introduction was used.

Method optimization

Many factors in CE influence the separation, elution time and resolution of analytes. These factors include but are not limited to pH, buffer composition, coatings, gels, electrophoretic potential (EP) and additives, such as SDS. Imperative in a clinical method are robustness and simplicity, for the maximization of repeatability, reproducibility, resolution and capability while minimizing the instrument setup, cost and sample preparation. For these purposes, separations requiring capillary coatings or gels were not considered. As past work had optimized buffer composition for this system [7], the effects of certain additives upon the system's resolution and sensitivity were evaluated while maintaining the shortest assay time possible. Two additives were added; 18 Crown 6 (1,4,7,10,13,16-hexaoxacyclooctadecane) and sodium dodecyl sulfate (SDS) in various concentrations from 5 to 20mM in the running buffer matrix. The additive 18 Crown 6 showed improvement in resolving peaks 1 and 2 (6,7-dimethylpterin, 6-biopterin), but had a negative impact on peaks 3 and 4 (D-(+)- neopterin; (4), 6-hydroxymethylpterin). The surfactant SDS showed promise in improving the resolution of all of the pteridines with the exception of pterin-6-carboxylic acid. The SDS changed

the running buffer sufficiently to where it would no longer elute the pterin-6-carboxylic acid moiety (with collection times up to 60 minutes). Therefore, the focus was shifted to the optimization of pH and EP. However, rather than investigating pH and EP independently, a design-of-experiment was performed using the running buffer pH and the EP as parameters. The resolution of two pairs of peaks (6,7-dimethylpterin versus 6-biopterin) and (D-(1)-neopterin versus 6-hydroxymethylpterin) were evaluated, as these pairs proved to be the most difficult to resolve. Preliminary experimental study showed that the optimal running buffer pH would be at about 9.5 – 9.6; therefore a comprehensive investigation was conducted within a pH range of 9.37 to 9.90 with an increment of 0.1. At each pH, the sample was assayed at the following EP's: 22.5, 26 and 28 kV. It was observed that there was insufficient electroosmotic flow to elute the pterin-6-carboxylic acid when a potential of 22.5 kV was applied regardless of the pH of the running buffer. When a potential of 28 kV was utilized, separation for all of the standards was sufficient and the assay time was less than 15 min; however, there was significant current leakage, which due to the design of the system could not be overcome. The use of a 26 kV potential provided better separation of the pteridines than when a 28 kV was used, and it provided sufficient electroosmotic flow to facilitate the elution of the pterin-6-carboxylic acid standard without the side effects of current leakage. A pH of 9.63 was found to be the optimal pH to resolve all of the pteridine peaks and other matrix peaks while keeping the elution time under 16 min. At the optimized conditions of pH = 9.63 and 26 kV, a standard mixture of eight pteridines was baseline separated within 16 minutes, as is shown in Figure 3.

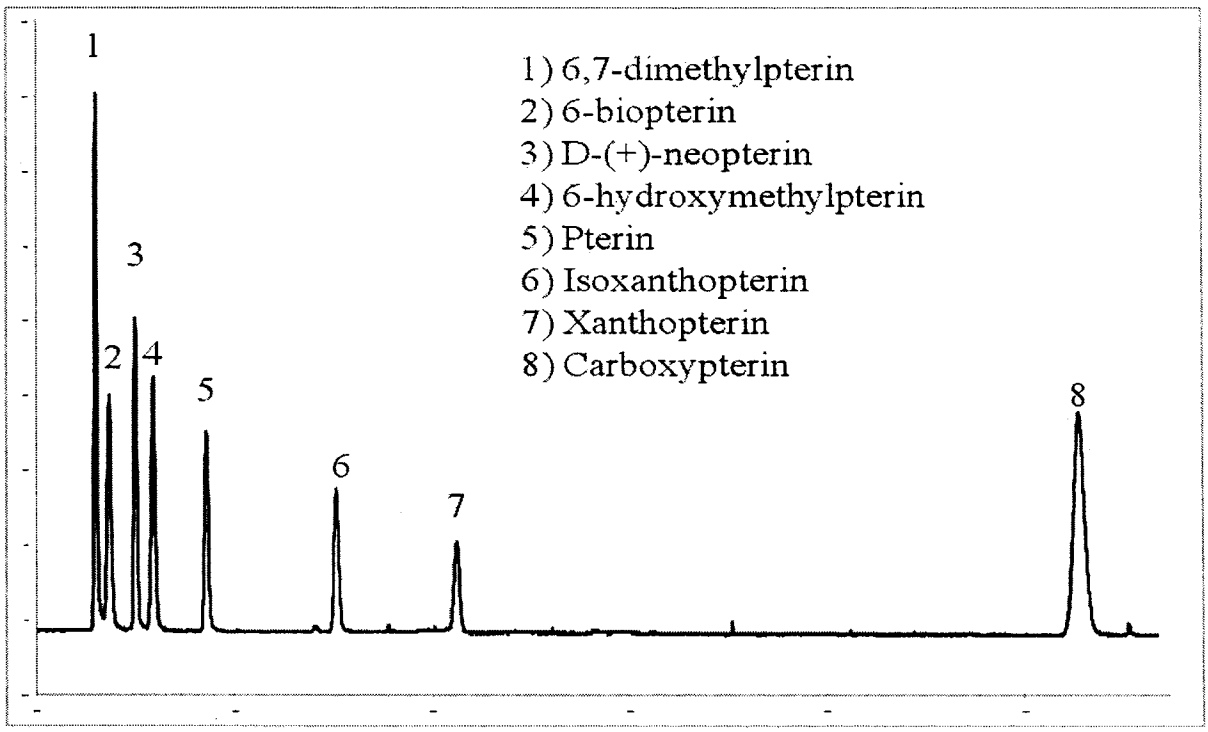

Figure 3. Representative electropherogram of the separation of 8 standard pteridines utilizing the optimized and validated method. Experimental conditions were as follows: Running buffer: 0.1 M Tris-0.1 M borate-2 mM EDTA, pH 9.63; Capillary: 50 μm i.d. x 70 cm (35 cm effective column length); Injection: gravimetric (17.5 cm from the end of the sample to the instrument table and the injection time was 10 seconds). Running voltage: 371 V/cm; LIF detection at 325 nm/445 nm (ex/em); Peak identification: (1), 6,7-dimethylpterin; (2), 6-biopterin; (3), D-(+)-neopterin; (4), 6-hydroxymethylpterin; (5), Pterin; (6), Isoxanthopterin; (7), Xanthopterin; (8), Carboxypterin. Concentration of each pteridine standard: 1.0 x 10-9 M. The detailed experimental conditions were described in the experimental section.

The resulting resolution of 6,7-dimethylpterin versus 6-biopterin improved from 0.85 to 2.48 and the resolution of D-(+)-neopterin versus 6-hydroxymethylpterin improved from 0.90 to 3.58 when compared with the previous study [7]. Calculations were performed based on Eq. (1) [18]. The pictorial definition of Eq. (1) is shown in Figure 4.

$$R_s = \frac{\Delta Z}{W_A + W_B} \quad (1)$$

This was a crucial improvement for real urine sample analysis because it significantly decreased the co-elution or overlap of other compounds in the urine with pteridine peaks as observed in the previous study [7], which greatly enhanced the accuracy of pteridine quantification. Figure 5 shows the separation of the pteridine peaks in a patient urine sample with no detectable interfering peaks.

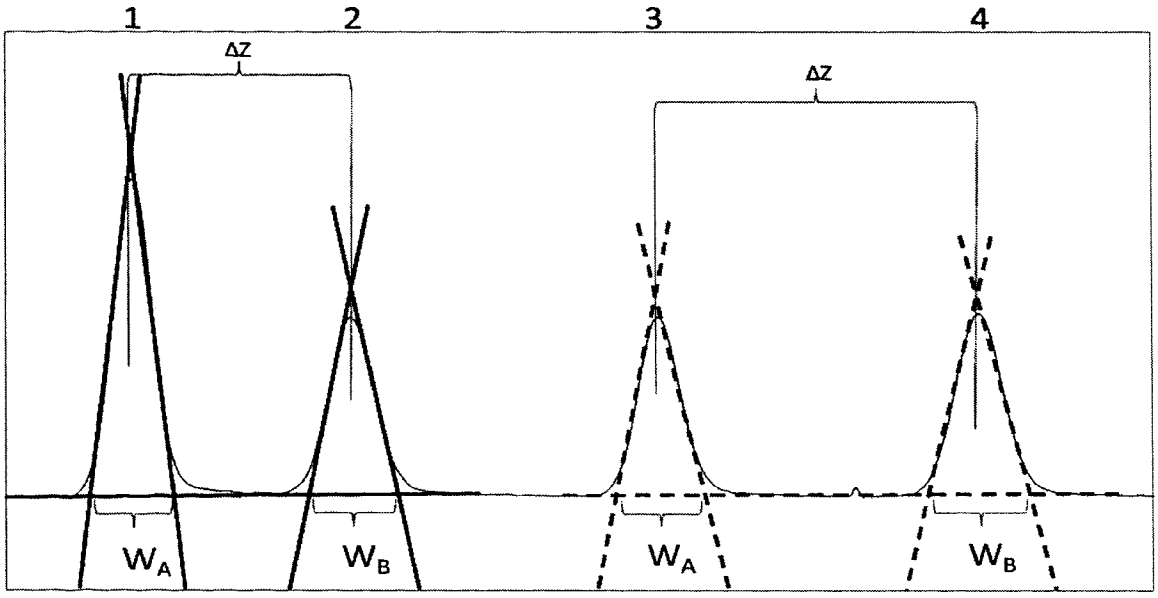

Figure 4. Pictorial explanation of the calculation of resolution between two peaks (Rs) defined by equation 1 [18]. Figure 4 is an expanded version of Figure 3 focusing on the first four pteridine standards.

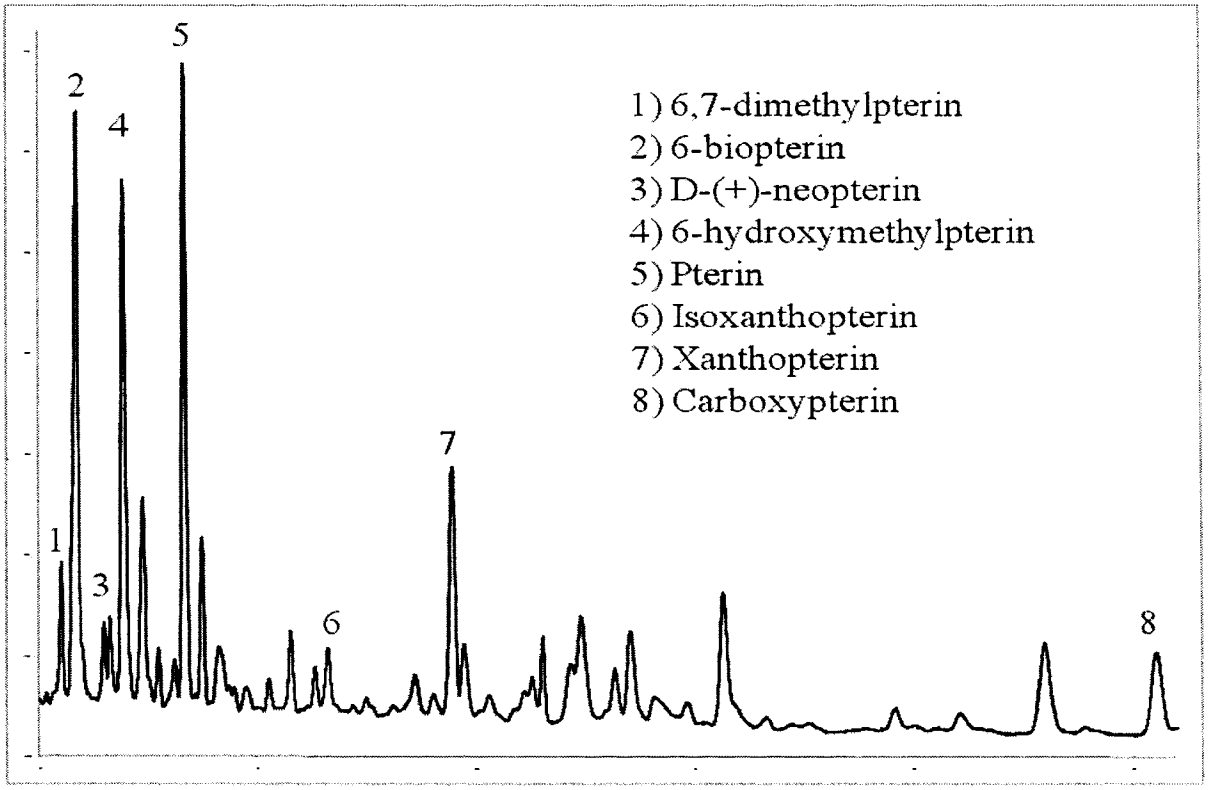

Figure 5. Electropherogram of the separation of urinary pteridines in a lung cancer patient urine sample utilizing the optimized and validated method. Experimental conditions were the same as those in Figure 3.

Oxidation study

Pteridines naturally exist in three oxidative states; tetrahydro-, dihydro-, and fully oxidized. As a result, accurate quantification of these compounds requires sample preparation that either fully oxidizes or reduces the pteridines. Both approaches were investigated, and full oxidization of the urine samples was selected due to ease of sample preparation and because it yielded a less complex sample matrix with less background interference. Meras et. al. reviewed Trehan's oxidation method [19] and concluded that the samples were not fully oxidized (as a result of a lower BIO/CREA ratio to Meras).

The oxidation method that was ultimately adopted from Han [7] and Trehan [19] utilized the KI–I_2 solution described previously since no interfering peaks were detected. As only fully oxidized samples would provide accurate results, a study was performed to determine the optimal sample preparation conditions to ensure that there was a sufficient amount of oxidant to fully oxidize the pteridines in the samples. Experiments tested varying oxidant volumes from 100 to 600 μL (2–4% KI–I_2) and reaction times from 30 to 180min reacting with 1000 μL of urine and 100mL of 2.0M NaOH. The results showed that 300 μL was insufficient, but 400 μL was able to fully oxidize the pteridines in the sample. Additionally, there was no detectable difference between the 400 and 600 μL aliquots (Figure 6).

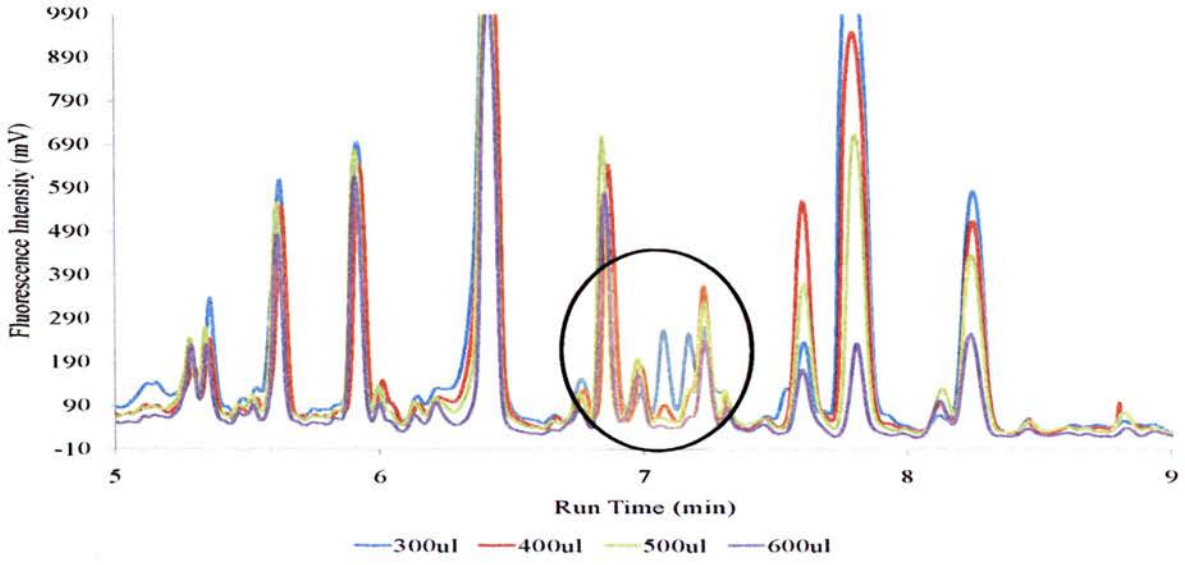

Figure 6. Overlay of varying concentrations (by volume) of oxidizing solution. The peaks in the area highlighted by the circle show the impact of adequate versus inadequate concentrations of oxidizing solution during the sample preparation.

It was also found that the concentration, not time, was the critical parameter to ensure complete oxidation of the pteridines in urine samples. The final parameters chosen for a 1000 mL aliquot of urine were 400 mL oxidizing solution followed by 30 minutes of incubation at 4°C, providing the necessary reaction conditions to fully oxidize all of the pteridines in the urine sample solution. Once the oxidization method was finalized, the stability of oxidized samples was tested by the injection and comparison of the same sample, three times, over a 6 hour time period (Figure 7). Under these optimized conditions, it was confirmed that the samples were stable for at least 6 h from the time they were diluted with sample buffer. This experiment was run in triplicate to ensure repeatability.

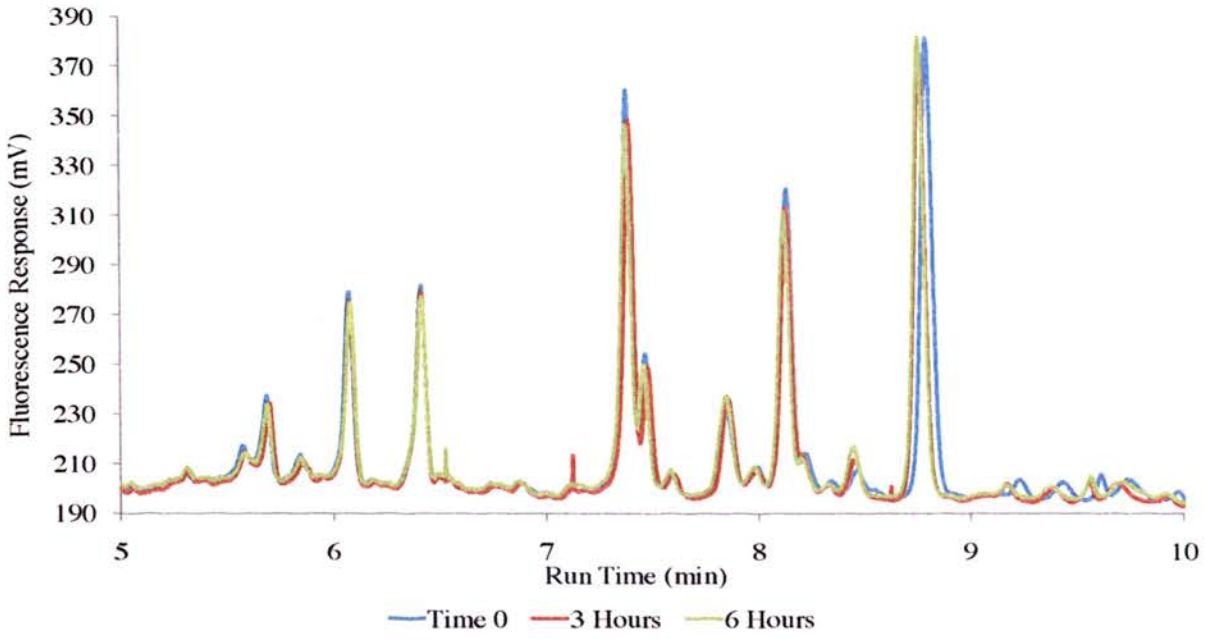

Figure 7. Overlay of triplicate injections performed on a post-oxidized sample over 6-hours. Experimental conditions were the same as those in Figure 3.

Short term aging study

Freshly collected urine samples were divided into 1.0mL aliquots and stored at 22°C (room temperature), 4°C (refrigerator) and -20°C (freezer). The samples were analyzed immediately (noted as day 0) and again on days 1, 3, 6 and 15. Samples from each of the three storage conditions were analyzed along with a standard control to ensure system stability from day to day. The data was evaluated and a cutoff of ±20.0% change in peak area from day zero was used as the criteria to define the period of sample stability. The results of the study showed that the neopterin and pterin were the most sensitive to the different storage conditions of all the pteridines tested (Figure 8). Therefore, based on these two compounds, it was concluded that freezer samples were stable up to 15 days, refrigerator samples were stable up to 6 days and room temperature samples were stable up to 24 hours.

Recovery Study

The determination of the percent recovery of the urinary pteridines provided a measure of the effectiveness of the sample preparation and subsequent analysis. In this experiment, unmodified urine samples were assayed and the ECP were determined by linear regression against prerun calibration curves. Then a different aliquot of the same urine sample was spiked with a known amount of each of the pteridine standards theoretical concentration of the spiked pteridines (TCSP), processed and assayed. Concentrations of the pteridines in the spiked urine samples were determined using the same technique as the unmodified urine sample (experimental concentration of the spiked pteridines (ECSP)).

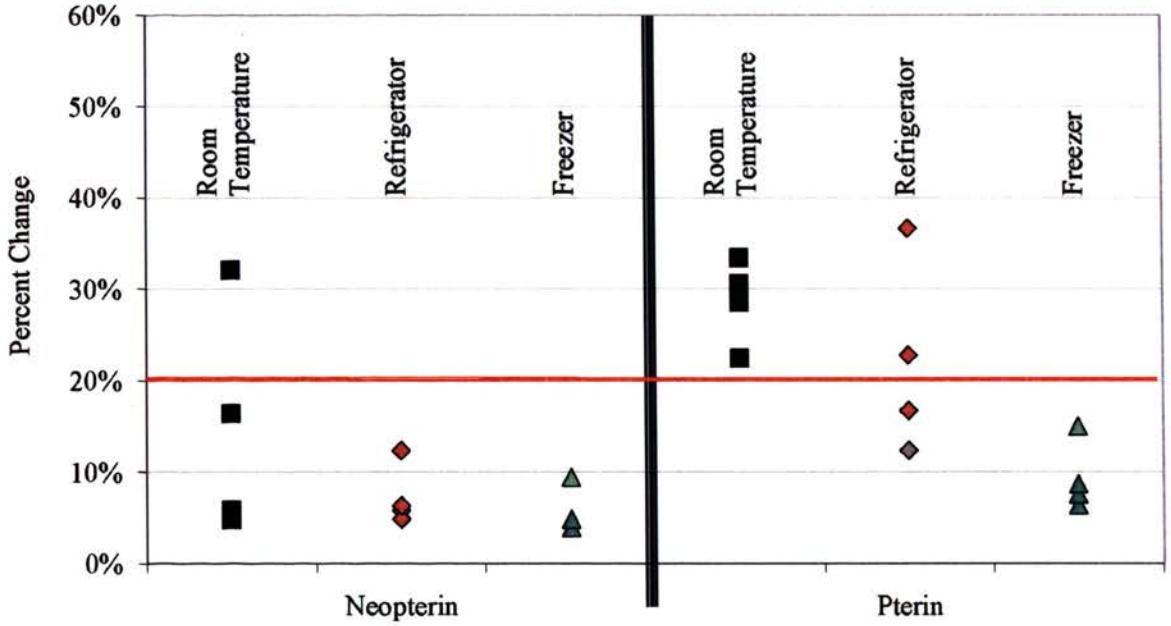

Figure 8. Graphical representation of the aging effects of different storage condtions. From left to right in each standard data set, the data represents room temperature (22°C), refrigerator (4°C) and freezer (-20°C) respectively. Neopterin and pterin were the most sensitive to this evaluation and the only two pteridines that exceeded 20% change for all conditions tested.

The percent recovery of each pteridine in the urine matrix was obtained by comparing the concentrations of the standard injections versus the spiked urine samples via Eq. (2).

$$\text{Percent Recovery} = \frac{\text{ECSP}}{\text{TCSP} + \text{ECP}} \times 100 \qquad (2)$$

The average pteridine recovery for this experiment was 99.9%, ranging from 69.8 to 110.7%. The results are shown in Table 1.

Table 1. Calculated average percentage recovery ± standard deviation of pteridines in urine sample (n=5)

Standard	Average % Recovery
6,7 Dimethylpterin	94.9 ± 3.5
6-biopterin	97.0 ± 8.5
D-(+)-neopterin	87.1 ± 3.8
6-hydroxymethylpterin	106.3 ± 11.4
Pterin	96.9 ± 5.2
Isoxanthopterin	91.3 ± 3.9
Xanthopterin	69.8 ± 4.9
6-Carboxypterin	110.7 ± 10.1

The experimental conditions were the same as those of Fig. 3.

Linear ranges, limits of detection and CV's

The modifications of the CE-LIF system have shown a significant improvement in the resolution and reproducibility of pteridine analysis. Standards of all eight pteridines were analyzed to determine the linear range and limits of detection for each. The linear ranges (peak area versus pteridine concentration in mg/L), (where the R^2 of a calibration curve was greater than 0.99) covered a span of greater than two orders of magnitude of concentrations. The limits of detection were determined by the 3s method where the baseline was determined by taking the standard deviation of a blank injection (sample buffer) and multiplying the average response by three. Both the linear ranges and limits of detection were comparable with the values determined in previous studies [7]; the comparisons of the limits of detection from our study to the previous are shown in Table 2. The intra-assay CV was determined by running one sample several times in the same day. The inter-assay CV was determined by using a single standard as a control and taking the data collected over two weeks. The results are summarized in Table 3.

Table 2. Limits of detection

Standard	Current Limit of Detection (M)	Previous Limit of Detection (M)
6,7 Dimethylpterin	2.50×10^{-10}	4.00×10^{-11}
6-biopterin	2.50×10^{-10}	1.30×10^{-10}
D-(+)-neopterin	2.50×10^{-10}	1.30×10^{-10}
6-hydroxymethylpterin	2.50×10^{-10}	9.80×10^{-11}
Pterin	2.50×10^{-10}	8.00×10^{-11}
Isoxanthopterin	2.50×10^{-10}	1.30×10^{-10}
Xanthopterin	2.50×10^{-10}	1.10×10^{-10}
6-Carboxypterin	4.72×10^{-10}	2.00×10^{-10}

Current study *versus* previous study.

Table 3. Intra- and inter-assay CV

Standard	µM ± µM	CV
Intra-assay		
6,7 Dimethylpterin	0.4878 ± 0.0099	2.04%
6-biopterin	0.7672 ± 0.0118	1.54%
D-(+)-neopterin	4.2505 ± 0.0092	0.22%
6-hydroxymethylpterin	1.9846 ± 0.0156	0.79%
Monapterin	38.0012 ± 0.1707	0.45%
Pterin	0.0901 ± 0.0033	3.65%
Isoxanthopterin	0.5546 ± 0.0060	1.08%
Xanthopterin	0.1914 ± 0.0205	10.71%
6-Carboxypterin	1.4146 ± 0.0403	2.85%
Inter-assay		
D-(+)-neopterin	2.8239 ± 0.1767	6.26%

Concluding Remarks

In this investigation, the necessary instrumental, systemic and preparative parameters for early cancer screening using urinary pteridine analysis by CE-LIF were identified and modified while the method was simultaneously validated and optimized. An improved resolution of the two least-resolved pteridines was investigated and shown to be nearly threefold. Additionally, while a less sensitive sample introduction technique was incorporated, system sensitivity was maintained. Furthermore, it was shown that the raw urine samples were stable in frozen conditions up to at least 15 days in the event that samples could not be assayed at the time of collection – a vital piece of information for clinical analysts. Finally, the investigation showed through preparation and recovery experiments that the technique employed in sample preparation was adequate in extracting all of the pteridines and in an isolated oxidized form. This study has played a vital role in the optimization and validation of a quantitative method for urinary pteridines analysis, which can be utilized as an early cancer screening method in clinical laboratories.

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2. DETERMINATION OF PTERIDINE LEVELS IN BIOFORTIFIED PLANT SAMPLES BY CAPILLARY ELECTROPHORESIS WITH LASER INDUCED FLUORESCENCE

Abstract

It has been observed through high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) that pteridines are naturally present in plants. Previous research results have shown that folate biofortification of plants through metabolic engineering increases the synthesis of pteridines. Subsequently, the ability to analyze these plant samples and quantify the pteridine levels is one way to determine the effectiveness of the folate biofortification. Although a variety of techniques have been used to measure the pteridine levels in plants, a high resolution separation technique is still needed to overcome the matrix effects in plant tissues. In this paper, we have developed a method to quantitatively determine the levels of nine pteridine compounds in plant samples of wild type non-transgenic and transgenic plants engineered for increased pteridine synthesis using capillary electrophoresis with laser induced fluorescence (CE-LIF).

Key words

Capillary electrophoresis; laser induced fluorescence, pteridine

Introduction

Megaloblastic anemia, birth defects, impaired cognitive development and increased risk of cardiovascular disease and cancer are all conditions that may result from folate deficiency [1,2]. Since humans lack the ability to synthesize folates de novo, The main source of folate intake for humans is through agricultural plants [1-3]. As a result there has been much interest in the fortification of food crops with folates. Hossain, Schubert and de la Garza have increased de novo pteridine biosynthesis in plants through metabolic engineering and the increase in pteridine biosynthesis resulted in a corresponding enhancement in total folates in these plants [2, 3]. Several pteridine compounds are known to be influential in the optimization of folate enhancement [3]. Therefore, we investigated nine specific pteridine compounds; 6,7-dimethylpterin, 6-biopterin, D-(+)-neopterin, 6-hydroxymethylpterin, pterin, monapterin, isoxanthopterin, xanthopterin, carboxypterin. It is important to identify and measure the levels of the pteridines that accumulate in these genetically modified plants in order to determine the effectiveness of the fortification and to understand how pteridine biosynthesis is regulated in plants. Even though both high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) have been used to measure the presence and accumulation of a number of pteridine compounds [4-8], a high resolution separation technique is still urgently needed due to the complicated matrices of plant samples. In this paper, a capillary electrophoresis with laser-induced fluorescence technique was developed specifically for quantitative analysis of pteridines in plant samples of wild type non-transgenic and transgenic plants metabolically engineered.

Experimental

Chemicals

6,7-dimethylpterin, 6-hydroxymethylpterin and monapterin were purchased from Schircks Laboratories (Jona, Switzerland). 6-biopterin, D-(+)-neopterin, pterin, isoxanthopterin and xanthopterin pteridine standards, boric acid and Tris(hydroxymethyl)aminomethane were purchased from Sigma (St. Louis, MO). EDTA disodium salt, iodide, potassium iodine, sodium hydroxide and sodium phosphate dibasic were purchased from Fisher (Fair Lawn, NJ). Ultra pure water (18.2 MΩ) was prepared by a Millipore, model Simplicity 185, water purifier.

Buffer solutions

The sample buffer, used in the dilution of standards and samples, was a 50mM aqueous solution of sodium phosphate dibasic (Na_2HPO_4), pH 7.70. The running buffer used was an aqueous solution of 0.1M Tris-hydroxymethyl)aminomethane (Tris), 0.1M boric acid and 2mM EDTA disodium salt. Running buffers with two different pH values (8.75 and 9.33) were employed. All of the buffer solutions were prepared as described in reference [9].

Standard Preparation

Individual standards were prepared by diluting approximately 2.0mg of pteridine standard in 10ml of solution (9.7ml sample buffer and 0.3ml 1.0M sodium hydroxide). Each of the standard solutions was then combined such that the final concentration of the

individual pteridines in the mixture was $5.0x10^{-5}$ M. The standard mixture was then used to generate calibration curves through serial dilution.

Sample Preparation

Transgenic *Arabidopsis* plants were grown from T3 transgenic seeds [2] in a Conviron growth chamber (22°C, 50% relative humidity, 200 micromoles m^{-2} s^{-1} of light, 10-h photoperiod) for pterin analysis. The T3 plants were BASTA sprayed for selection at four-leaf stage. Leaves were harvested from wild-type Columbia and transgenic T3 plants before onset of flowering. Presence of *E. coli* folE gene and EcGCH1 protein were confirmed in leaf tissue via PCR and western blot respectively according to the method described in Hossain et al [2]. Leaf tissue (approximately 0.5g / 2.5 mL H_2O) from transgenic and non-transgenic *Arabidopsis* was triturated in liquid nitrogen and re-suspended in deionized water. Re-suspended extract was filtered through two layers of Miracloth™ and centrifuged at 10,000 x g for 15 min. The supernatant (crude extract) was used for analysis of pterins. The prepared samples arrived frozen, packed in dry ice. The samples were thawed out and centrifuged at 4°C for 10 minutes at 6000rpm then the supernatant was diluted 1:1 in the sample buffer and injected for analysis. If the pteridine concentration was above the linear range of the calibration curve, further dilutions were made to analyze the sample in the linear range.

Instrumentation

The CE analyses performed in this experiment were carried out using a similar system as designed for the analysis of urinary pteridines [9]. Pteridines have an excitation

wavelength near 360 nm and an emission wavelength around 450nm. A He-Cd laser with 325nm wavelength was selected for the excitation source as it has been proven effective [9,10]. The separation of the samples was carried out using 75μm and 50μm id polymer coated silica capillaries (Polymicro Techniques, Phoenix, AZ) 65cm in length. The capillaries were pre-treated with 0.10 M hydrochloric acid, 0.10 M sodium hydroxide and deionized water. A one cm detection window was burned into the capillary at 35cm from the injection end of the capillary. A voltage of 25kV was utilized providing an effective voltage of approximately 357 Vcm^{-1}. Samples were injected gravimetrically with volumes of about 5nl. After each analysis the capillary was washed with 0.20 M sodium hydroxide, rinsed with filtered DI water and followed with running buffer, each for two minutes to ensure injection to injection repeatability.

Statistics

Each of the samples was run in triplicate to facilitate generation of standard deviations. Inter-assay CV was determined by injecting one sample over multiple days and are summarized in **Table 1**. Since three samples were analyzed several times each for our standard analysis of the plant samples, the intra-assay CV was accounted for and reported as percent relative standard deviation (%RSD) in our results for specific samples **(Table 2)**.

Table 1. Inter assay CV's

Standard	Inter-assay		
	Avg (μM) ±	Stdev (μM)	CV
6,7-dimethyl pterin	0.250 ±	0.005	2.06%
6-biopterin	0.568 ±	0.006	1.11%
D-(+)-neopterin	2.629 ±	0.048	1.83%
6-hydroxy methyl pterin	1.867 ±	0.020	1.07%
Pterin	0.120 ±	0.002	1.67%
Isoxanthopterin	0.409 ±	0.013	3.22%
Xanthopterin	0.107 ±	0.018	16.73%
Monapterin	0.540 ±	0.014	2.54%
Carboxypterin	0.475 ±	0.025	5.34%

Table 2. Pteridine levels in plant samples by CE-LIF

Lot	Sample	6,7-dimethyl pterin µM	6-biopterin µM	D-(+)-neopterin µM	6-hydroxy methyl pterin µM	Pterin µM	Isoxanthopterin µM	Xanthopterin µM	Monapterin µM	Carboxypterin µM
		Method 'A'								Method 'B'
1	1-TH	*CoE*	0.234	8.236	2.357	0.644	1.604	2.049		
	3-TH	*CoE*	0.222	13.267	3.563	*LoQ*	1.018	5.208		
	4-TH	*LoQ*	0.461	0.055	*LoQ*	*LoQ*	0.095	0.096		
	5-TH	*LoQ*	0.880	17.196	2.331	0.086	0.697	0.698		
	6-TH	*CoE*	0.357	14.062	4.528	0.607	1.916	16.541		
2A	S1	*CoE*	0.662	>10	7.234	0.095	1.170	3.153		
	S2	*LoQ*	0.136	>10	3.621	0.149	0.505	0.518		
	S3-C	*LoQ*	0.704	0.032	0.027	0.190	0.337	0.073		
	S4-C	*LoQ*	0.014	0.034	*LoQ*	0.039	0.041	0.008		
3A	S5	*LoQ*	*CoE*	0.207	*LoQ*	*LoQ*	0.203	*LoQ*		
	S6	*LoQ*	0.030	*LoQ*	*LoQ*	*LoQ*	0.047	*LoQ*		
	S7	*LoQ*	*CoE*	0.100	0.021	0.016	0.284	*LoQ*		
	S8	*LoQ*	*CoE*	*LoQ*	*LoQ*	*LoQ*	*LoQ*	*LoQ*		
3	M9	0.012	0.368	0.411	1.748	0.150	0.262	0.023	0.695	0.645
	M48	*CoE*	0.033	0.345	1.103	0.076	0.259	*LoQ*	0.254	0.435
	215	0.488	0.767	4.847	1.985	0.090	0.555	0.191	38.001	1.415
Avg % RDS		**2.7%**	**4.0%**	**1.3%**	**2.3%**	**4.7%**	**4.4%**	**8.4%**	**5.5%**	**2.5%**

CoE Peak of interest is co-eluting with an unidentified peak
LoQ Peak are of the peak of interest is below the range of the calibration curve

Results and Discussions

Pteridines have been found to natively fluoresce around 450nm when excited. Therefore, we employed the same capillary electrophoresis system designed for urinary pteridine analysis [9], to evaluate pteridines in our metabolically engineered plant samples. Initially, we started investigating eight of the nine pteridines while we were waiting on monapterin to arrive; 6,7-hydroxymethylpterin, 6-biopterin, D-(+)-neopterin, 6-hydroxymethylpterin, pterin, isoxanthopterin, xanthopterin and carboxypterin. Initial separation parameters were modeled on the parameters outlined by Han [10] with some minor adjustments; 7.70 pH sample buffer, 8.75 pH running buffer, 75μm capillary, 25kV electrophoretic potential, denoted as method 'A'. **Figure 1** shows the standard separation of the initial investigation.

Seven of the eight standards eluted and the resolution was excellent, but we were unable to elute the carboxypterin. As this method was sufficient to determine the concentrations of the majority of the pteridines, we maintained this method for the evaluation of the plant samples for 6,7-hydroxymethylpterin, 6-biopterin, D-(+)-neopterin, 6-hydroxymethylpterin, pterin, isoxanthopterin and xanthopterin. Residual pteridines and other compounds in the plants that may have not eluted were washed out with 0.20 M sodium hydroxide, filtered DI water and running buffer, each for two minutes.

Limits of detection (LOD) were determined the using standard responses that were three times the standard deviation of the baseline signal. **Table 2** summarizes the LoD for this method. Limits of quantification (LoQ) were determined through the evaluation of the coefficient of determination, R^2, utilizing the linear range of the

calibration curve [11]. Calibration curves were typically taken from $1.0x10^{-6}$ M to $1.0x10^{-8}$ M with resulting R^2 values of at least 0.990.

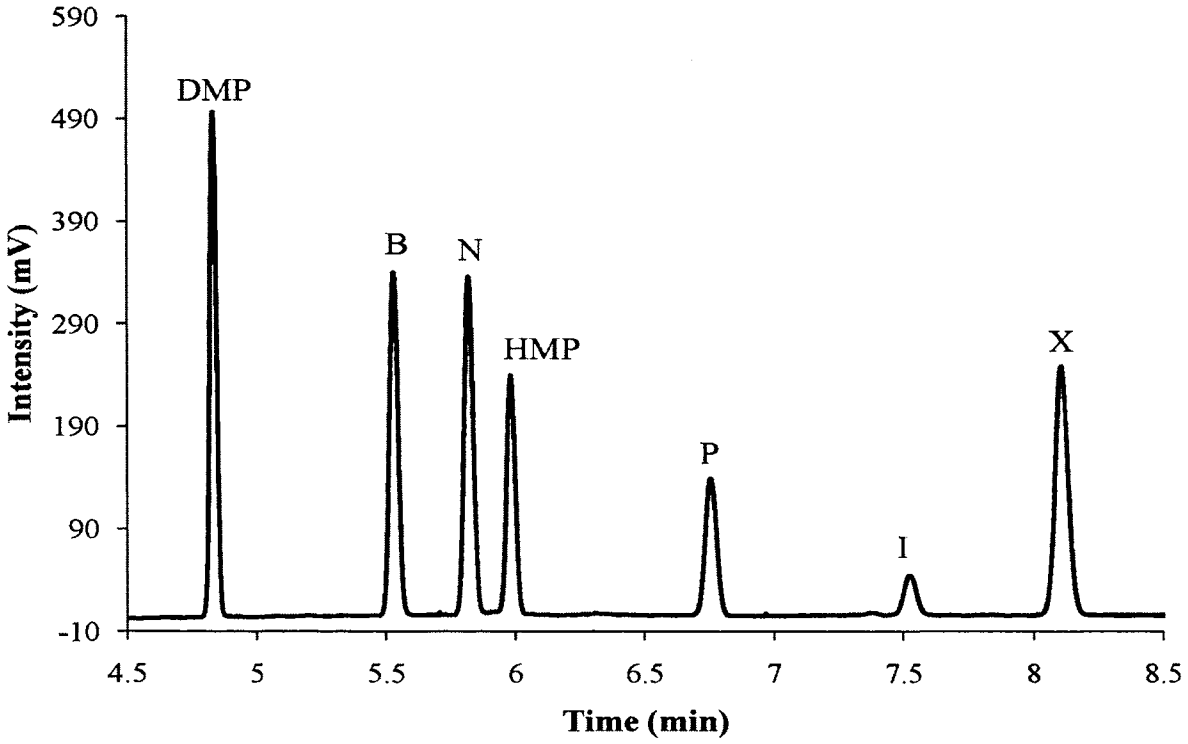

Figure 1. Method 'A' standard injection. Peak identification: (DMP) 6,7-dimethylpterin , (B) 6-biopterin , (N) D-(+)-neopterin, (HMP) 6-hydroxymethylpterin, (P) pterin, (I) isoxanthopterin and (X) xanthopterin.

Four lots (1, 2A, 2B and 3) containing 17 samples were provided by the Donald Dansforth Plant Science Center for evaluation using method 'A'. Peak identification was performed though both elution time matching and standard addition for each sample analyzed. **Figure 2** shows a representation of the peak identification method employed.

Three different assays for each sample were performed to allow for statistical analysis of the results.

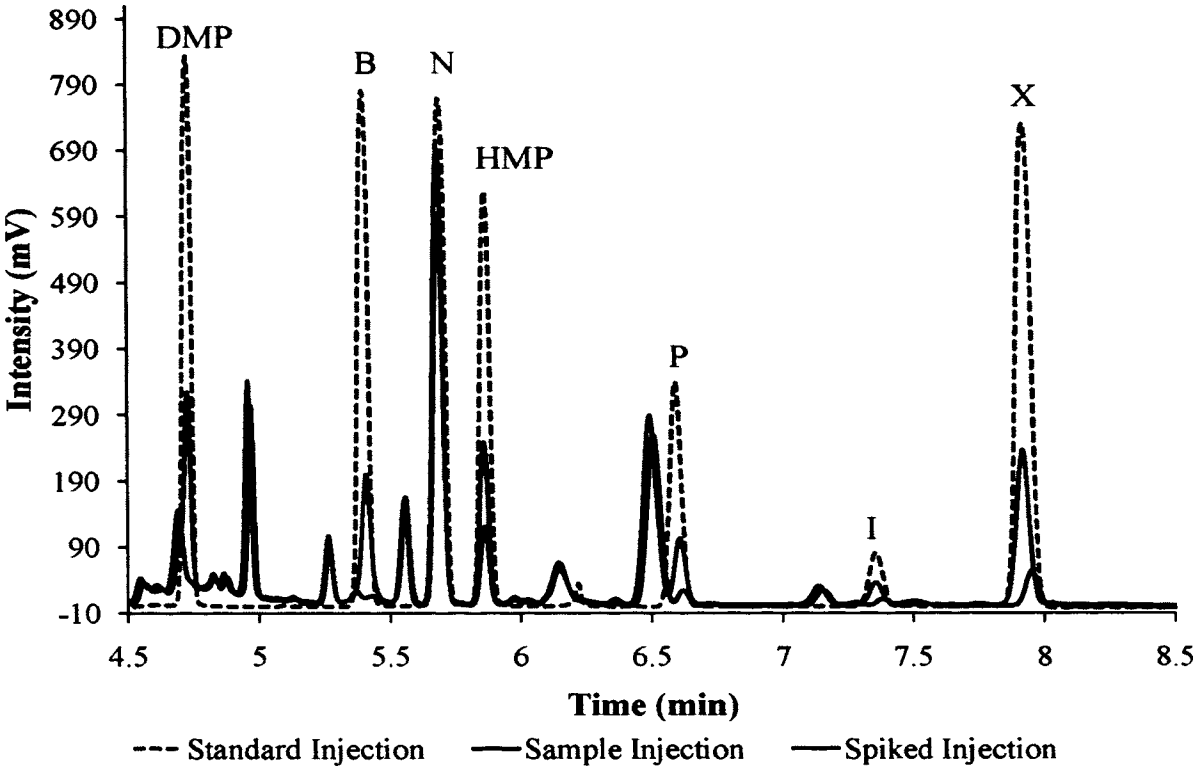

Figure 2. Overlay of standard injection, plant sample and spiked plant sample using method 'A'. Peak identification: (DMP) 6,7-dimethylpterin , (B) 6-biopterin , (N) D-(+)-neopterin, (HMP) 6-hydroxymethylpterin, (P) pterin, (I) isoxanthopterin and (X) xanthopterin.

Figure 3 (one sample from each lot provided) clearly shows that each of the seven pteridines that we sought to quantify was well separated, with the exception of 6,7-dimethylpterin. It was observed that for all except two samples (M9 and 215), the

concentration of 6,7-dimethylpterin was either below the LoQ or LoD and could not be resolved from the sample matrix. Additionally, 79% of the pteridines evaluated for the samples in lot 2A, were either below the LoQ or (in the case of 6-biopterin) co-eluted with an unknown interfering peak. **Table 2**, shows the results of the pteridine concentrations for all four lots tested.

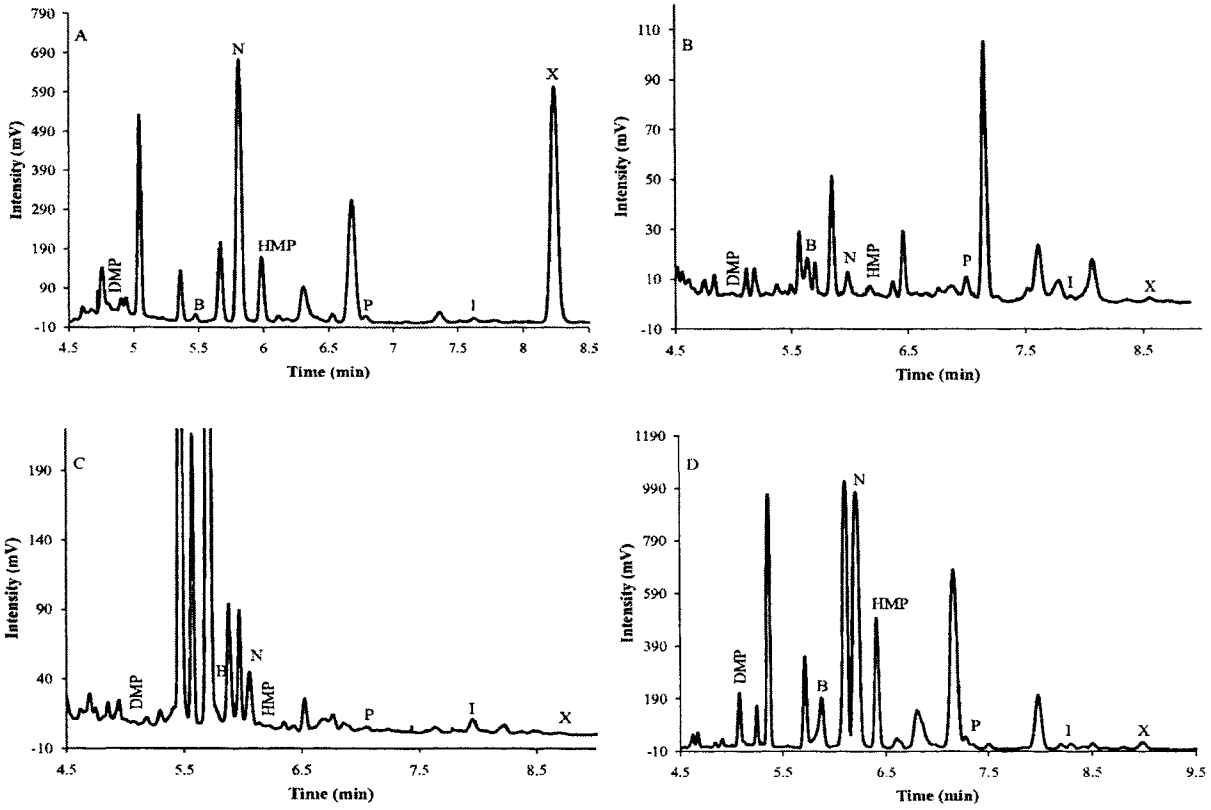

Figure 3. Sample injections from each lot using method 'A'. A-D reflects lots 1, 2A, 2B and 3 respectively. Peak identification: (DMP) 6,7-dimethylpterin , (B) 6-biopterin , (N) D-(+)-neopterin, (HMP) 6-hydroxymethylpterin, (P) pterin, (I) isoxanthopterin and (X) xanthopterin.

The determination of carboxypterin proved to be a bit more challenging. Since the current method was not able to elute the carboxypterin compound as previously shown [10], different parameters were evaluated to derive a new method to facilitate elution of the carboxypterin. As only carboxypterin was of interest for this round of analysis, the separation and sensitivity of the pteridines testing in the initial analysis was not important. Several parameters can influence the elution of compounds in electrophoresis such as; pH, buffer composition, coating, gels, electrophoretic potential (EP) and additives [9]. In order to minimize time and cost, coatings and gels were not evaluated. The pH and composition of the sample buffer had been previously optimized for pteridine compounds [10], which left pH and buffer composition of the running buffer, EP and additives. Two different additives extensively utilized in capillary electrophoresis, SDS and 18 Crown 6 were evaluated at several different concentrations up to 20mM as in previous studies [9], but without the constraints of having to separate all of the other pteridines. Unfortunately, nether of additives had any impact on the elution of the carboxypterin. A hypothesis was formed suggesting that the carboxypterin was interacting too strongly with the hydrogen on the capillary and a more aggressive electroosmotic flow to "push" the carboxypterin through the capillary was required. Since pH of the running buffer would influence both factors it was the next parameter selected for evaluation. A running buffer with a more alkaline pH will not only increase the electroosmotic flow of the system, due to increased ionization of the silanols [12], but also decrease the interaction between the carboxypterin with the capillary wall. The pH was systematically increased stepwise from 8.75 to 9.50. It was observed for the plant samples, that a pH of approximately 9.30 (denoted as method 'B') provided excellent

elution of the carboxypterin (between 9 and 10 min), validating the hypotheses. Additionally no interferences from any of the compounds previously observed in the samples analyzed were introduced. There were only three of the samples remaining (M9, M48, 215) at the time method 'B' was ready for analysis. **Figure 4** shows the carboxypterin peaks from the sample and a sample spiked with standard carboxypterin for peak identification. **Table 2** summarizes the carboxypterin levels for the samples tested.

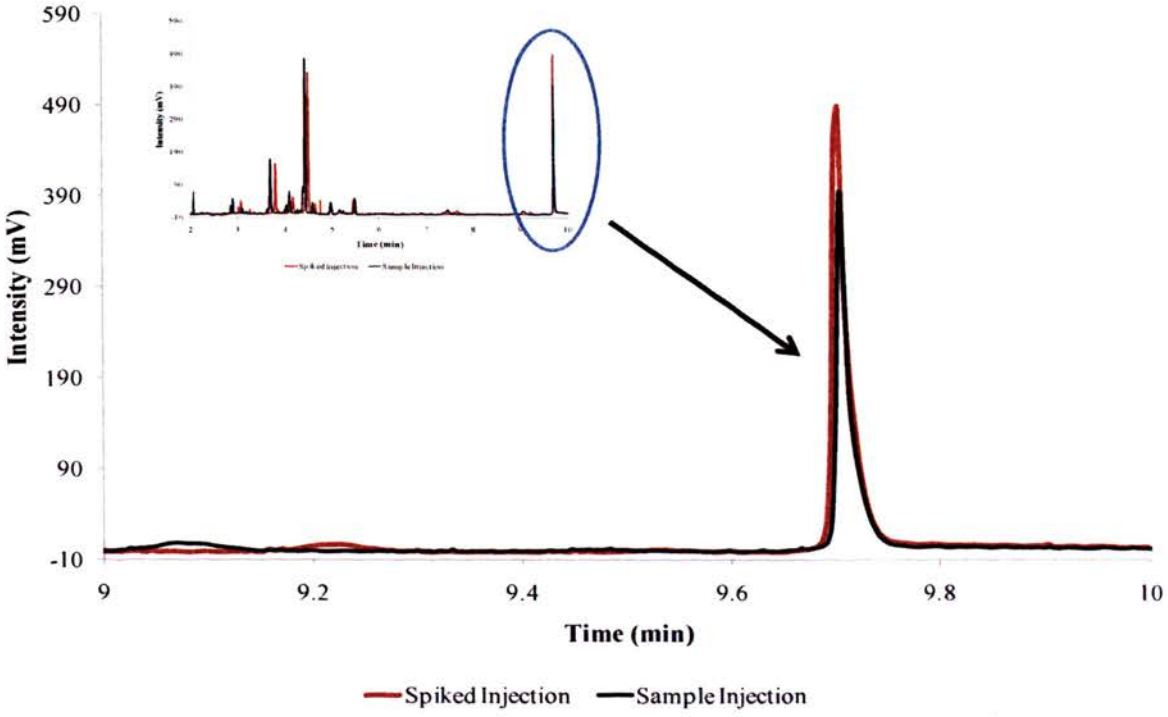

Figure 4. Overlay of plant sample and spiked plant sample for carboxypterin analysis using method 'B'.

As with the carboxypterin analysis, only three samples remained for monapterin analysis; M9, M48 and 215. The samples were run, spiked with monapterin and run again. The electropherograms were overlayed along with a standard injection to determine which peak in the samples should be used for quantification (**Figure 6**). The results of the monapterin analysis are summarized in **Table 2**.

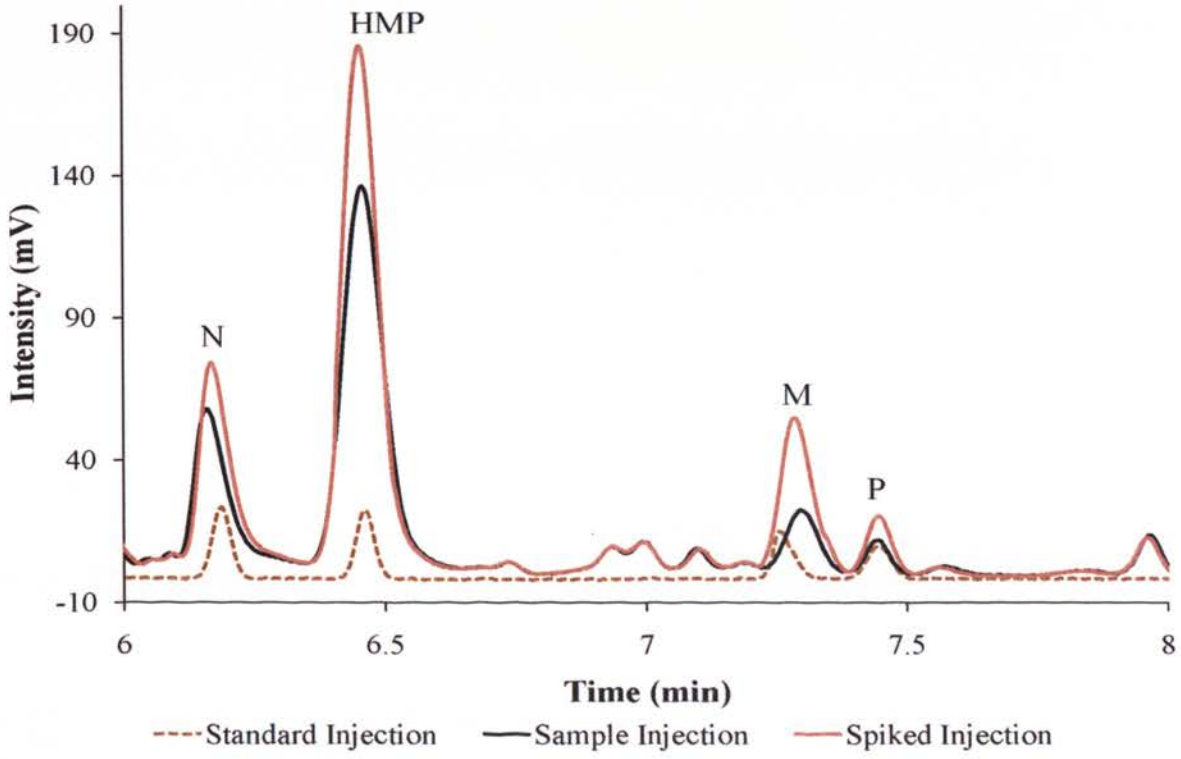

Figure 6. Overlay of standard, plant sample and spiked plant sample for monapterin determination using method 'A'. Peak identification: (N) D-(+)-neopterin, (HMP) 6-hydroxymethylpterin, (M) monapterin, (P) pterin.

Conclusions

We have demonstrated ability to quickly and effectively quantify nine pteridine compounds in transgenic and non-transgenic plant samples by capillary electrophoresis with laser induced fluorescence. This development provides a complementary technique to HLPC and TLC to measure the effectiveness of folate fortification of genetically modified plants. Additionally we have opened the door to identify additional compound of interest that may provide additional information to the folate or additional fortifications of genetically modified plants.

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3. DETERMINATION OF PHARMACEUTICAL AND PERSONAL CARE PRODUCTS IN WASTE WATER BY CAPILLARY ELECTROPHORESIS WITH UV DETECTION

Abstract

As the usage of pharmaceutical and personal care products (PPCP's) continues to increase over time, so does the risk of water contamination. Therefore, fast, sensitive, and accurate methods are needed to monitor the concentrations of PPCP's in different water matrices to ensure the safety of humans and other living animals. Methods for PPCP determination in water samples have been developed and employed using liquid chromatography - mass spectrometry (LC/MS). However, an alternative technique is urgently needed to conduct PPCP analysis when a LC/MS is not available in many laboratories. Capillary electrophoresis (CE) offers a simple, fast, and low cost alternative analytical technique to LC/MS for many PPCP compounds. In this study, we have developed a method that can simultaneously analyze eight different PPCP compounds in untreated waste water (ibuprofen, triclosan, carbamazepine, caffeine, acetaminophen, sulfamethoxazole, trimethoprim, and lincomycin), using capillary electrophoresis with UV detection (CE-UV). The method detection limit (MDL) ranged from 1.6 to 68.7 ppb through solid phase extraction. The standard limit of quantification (LOQ) ranged from 0.63 – 7.72 ppm. Factors affecting separation and quantification of PPCP's, such as pH, electrophoretic potential, buffer strength, buffer type, and additives, were investigated and optimized. Water samples from two different waste water treatment plants were collected and analyzed. The results obtained were comparable with those of LC-MS/MS. The technique developed in this study provides a low cost, simple, fast, and relatively

sensitive method for determination of various PPCP's in waste water samples for PPCP screening.

Key Words

Capillary electrophoresis, pharmaceutical and personal care products, waste water

Introduction

Pharmaceutical and personal care products (PPCP's) are used and disposed of every day. Over time various PPCP compounds are transported into different water sources, including waste water [1-3]. Pharmaceuticals in natural and waste water can also result in their occurrence in drinking water with unknown impacts on human health. Even though the risk to humans of pharmaceuticals in waters is not fully understood, due to their biologically-active nature, it is important to know the concentrations of these compounds in natural and waste waters. In recent years, a number of PPCP compounds have been detected in many natural water systems globally, including rivers, lakes, and reservoirs [4-15]. Therefore, the U.S. EPA has heightened government awareness of the possible health hazards associated with PPCP's [5, 16]. The EPA has created a web site that lists the published literature relevant to PPCP's as potential environmental contaminants [17]. This web site also contains a specific listing of Drug Disposal and Environmental Stewardship Contamination topics, and now includes over 2,000 publications.

Determining the level of PPCP's in waste water, prior to treatment, is important for determining the effectiveness of the treatment. Several methods have been developed for identification and quantification of PPCP's in water samples by using liquid

chromatography – tandem mass spectrometry (LC-MS/MS) [15, 18-23]. However, an alternative technique is urgently needed to conduct PPCP analysis when a LC/MS is not available. Capillary electrophoresis (CE) has been demonstrated to be a versatile separation technique with high resolution for many different compounds [24, 25]. CE offers an attractive alternative to LC-MS/MS for the analysis of PPCP's in waste water. The advantages of capillary electrophoresis over LC-MS/MS are the cost and flexible selectivity through buffer concentration, additives, and pH tuning. Several CE methods have been developed to measure individual PPCP compounds in natural water [18], livestock feed [26], meat and groundwater [27], dietary products [28] and other matrices [29, 30]. However, no CE methods have been developed for determining PPCP in waste water. In this study, we developed a novel CE-UV method that can detect lower ppm levels (<10 ppm) of PPCP's for direct injection of standards and ppb levels (<70 ppb) of PPCP's in waste water through sample preparation. The method is capable of analyzing eight specific PPCP's in Missouri waste waters. The selection of these eight PPCP compounds was based on the occurrence of PPCP's in Missouri's surface water systems as delineated in our previous study.

Experimental Section

Chemicals

Acetaminophen, caffeine, trimethoprim, carbamazepine, ibuprofen, sulfamethoxazole, 18 Crown 6, boric acid (H_3BO_3), and tetrasodium ethylenediamine tetraacetate hydrate ($Na_4EDTA{\cdot}2H_2O$) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium phosphate dibasic (Na_2HPO_4), phosphoric acid (85%), methanol (Optima

grade) and sodium hydroxide were purchased from Fisher Scientific (Fair Lawn, NJ). Lincomycin was purchased from MP Biomedicals (Aurora, OH). Triclosan (Irgasan) was purchased from Fluka (Switzerland). Ultra pure water (18.2 MΩ) was prepared by a Millipore Advantage A10 Milli-Q system.

Running Buffer Solutions

A 50 mM borate buffer solution was prepared using ultra pure water and boric acid. The buffer was prepared by dissolving approximately 1.55 g of boric acid in 400 mL of ultra pure water. The solution was transferred to a 500 mL volumetric flask and diluted to volume with ultra pure water. The resulting mixture was filtered via vacuum filtration through a 0.22 μm Nylon membrane filter (Osmonics, Inc, Minnetonka, MN). The borate buffer was split into two aliquots and the pH of the aliquots was adjusted using 1N sodium hydroxide to 8.52 and 9.51.

Phosphate buffer solutions (5, 10, 50, 10 0mM) were prepared using ultra pure water and sodium phosphate dibasic. The buffer was prepared by dissolving the sodium phosphate dibasic in ultra pure water (to about 80% of the total volume). The solutions were transferred to a volumetric flask and diluted to volume with ultra pure water. The resulting solution was filtered via vacuum filtration through a 0.22 μm Nylon membrane filter (Osmonics, Inc, Minnetonka, MN) for the first experiment and 0.45 μm Nylon membrane filters (Pall, Inc, Ann Arbor, MI) for all subsequent experiments. The pH of the phosphate buffers was adjusted using concentrated phosphoric acid (85%) and an Accumet® Excel XL15 pH meter from Fisher Scientific (Fair Lawn, NJ).

To improve separation of the selected PPCP compounds, 18 Crown 6 was added to the buffer solution [31, 32].

Standard Preparation

Most PPCP compounds have minimal solubility in pure water. Therefore, standards were first prepared in Optima grade methanol. A mixture of the eight compounds was prepared by combining 1 mL of each individually prepared standard into one solution. The final dilution was prepared by diluting 100 μL of the standard mixture to 1500 μL of running buffer.

Waste Water Sample Collection and Preparation

Waste water samples were collected from two independent waste water treatment facilities in Mid-Missouri. Each sample was collected in a 4-liter amber glass bottle (rinsed thoroughly with ultra-pure water) to minimize contamination and to prevent photo degradation. The waste water samples were refrigerated (~4°C) until analyzed. Step wise filtration using filter paper and 0.45 μm Nylon membrane filters was performed on each sample to remove as much of the physical particulate prior to solid phase extraction (SPE) of the PPCP's. After filtration, the pH of the samples was adjusted to 2.0 ± 0.2 with concentrated hydrochloric acid [15, 19].

Samples were separated into two groups with 1-liter aliquots for each waste water source. Five-hundred mg of $Na_4EDTA{\cdot}2H_2O$ were added to each of the samples as a stabilizing agent during the SPE process [15, 19]. Solid phase extraction was performed by following EPA method 1694. Briefly, the SPE was accomplished using Waters Corp.

Oasis HLB 20 cc cartridges. The cartridges were pre-conditioned with 20 mL of methanol, 20 mL of ultra pure water adjusted to pH 2.0, and 20 mL of unbuffered ultra pure water [15, 19]. The waste water was passed through the SPE cartridges at a rate of one drop every 2-to-3 seconds. After the extraction was complete, 20 mL of ultra pure water wer passed through the SPE membrane to remove the EDTA stabilizer [15, 19]. The retained PPCP's were eluted into 50mL glass centrifuge tubes using 20 mL of methanol, followed by 20 mL of methanol and acetone (1:1 v/v) at a rate of about one drop every 2-to-3 seconds [15, 19]. The eluent was evaporated to about 100 μL using a Turbovap LV operating at 50°C ± 5°C [15, 19]. The samples were re-constituted by adding 400 μL of methanol to the eluent and transferred to a 1.5 mL CE vial, followed by a 200 μL final rinse to ensure that all of the eluent was reconstituted. Finally, two dilutions were prepared for each sample, prior to analysis.

Instrumentation

A CARY 50 Bio UV-Visible Spectrophotometer (Agilent Technologies) was used to measure the absorption properties of each compound to ensure that the best UV wavelength was selected to detect the analytes.

The capillary electrophoresis instrumentation was a Beckman Coulter P/ACETM MDQ Capillary Electrophoresis System with UV detection. The capillary used was from Polymicro Technologies (Phoenix, AZ) 50 μm i.d. x 55 cm (45 cm to the detecion window). The capillary was pretreated with sodium hydroxide (1.0 N) for 30 minutes, ultra-pure water rinse for 15 minutes, hydrochloric acid (1.0 N) for 10 minutes, and a

final rinse using ultra-pure water for 15 minutes. Finally, the capillary was rinsed and conditioned with running buffer. Samples were injected using pressure injection (0.5 psi).

An algorithm was written using Microsoft Excel to remove the baseline noise. For the standard injections, the algorithm was applied to any other noise not associated with the standard peak to aid in peak area integration for quantification purposes. For the samples, the algorithm was applied only to clean up the baseline noise to assist in peak area determination.

Results and Discussions

UV-Absorbance Evaluation

The CE-UV instrument is configured to run one of four different wavelengths, 200, 214, 254 and 280 nm. Therefore, UV absorbance scans were performed on the PPCP standards to determine the best wavelength for analysis. Based on the UV scans obtained, 214 nm was selected as the detection wavelength. The concentrations of the standards during this experiment were approximately 1mg/mL in methanol (data not shown).

Buffer Selection

Several methods have been published for quantifying individual PPCP's unrelated matrices by capillary electrophoresis [26, 29, 33]. The buffer selection varied from phosphate to borate, ammonium acetate, lactic acid, Tris, and sodium carbonate [26, 30, 33-35]. Several additives were also used to aid separation including SDS, methanol, acetonitrile, and cyclodextrine [29, 30, 35]. Initial investigations began with alkaline phosphate and borate buffer solutions. Based on the literature, useful pHs for phosphate buffers range from

1.14 - 3.14 and 6.20 - 8.20 and for borate buffers 8.14 - 10.14 [36]. Some of the compounds under investigation were not soluble in acid conditions. Therefore, acidic pHs were excluded. A study was performed to compare borate and phosphate buffers with pHs that encompassed the recommended alkaline range. Starting buffer strength of 50 mM was chosen and standards were injected individually for screening. Additionally, the electrophoretic potential was evaluated for each pH. The voltage ranged from 10 to 30kV. Results demonstrated that phosphate buffer, pH 7.2, and 20 kV showed the most promise. One reason for phosphate buffer performing better than the borate buffer is that many of our compounds have hydroxyl functional groups, which can complex with boric-based buffers and affect separation of the compounds [37].

Buffer Strength

Preliminary experimental data showed that simple selection of buffer type and pH was insufficient to adequately separate all of the PPCP compounds. Since buffer strength can affect the electroosmotic flow of the system and the separation [38], buffer strengths of 10, 50, and 100 mM were compared using electrophoretic potentials of 10, 20, and 30 kV. Due to increased zeta-potentials at buffer concentrations of 10 and 50 mM, the electroosmotic flow that was generated was too strong to allow for separation of the PPCP compounds [38]. At 100 mM, there was still room for improvement, but the current was approaching 150 μA and Joule heating was becoming a concern [36, 38]. Buffer strength of 100 mM and 20 kV was selected.

Buffer pH Study

It is well known that buffer pH has a significant effect on CE separations due its impact on the magnitude of EOF [36, 38]. For the pH study, a standard mixture was used to perform all testing. This removed injection to injection variability that could mask potential co-elution of individual standards. The characterization of the pH was centered on pH 7.2. Buffer solutions with pHs of 6.4, 6.8, 7.2, 7.6, and 8.0 were prepared. The same buffer was used for both sample dilution and running buffer. At each pH, electrophoretic potentials (EP) of 5, 10, 15, and 20 kV were investigated to evaluate the interaction between pH and EP. **Figure 1** shows the effect of pH on the separation of PPCP's at 15 kV, the EP that showed the most promise.

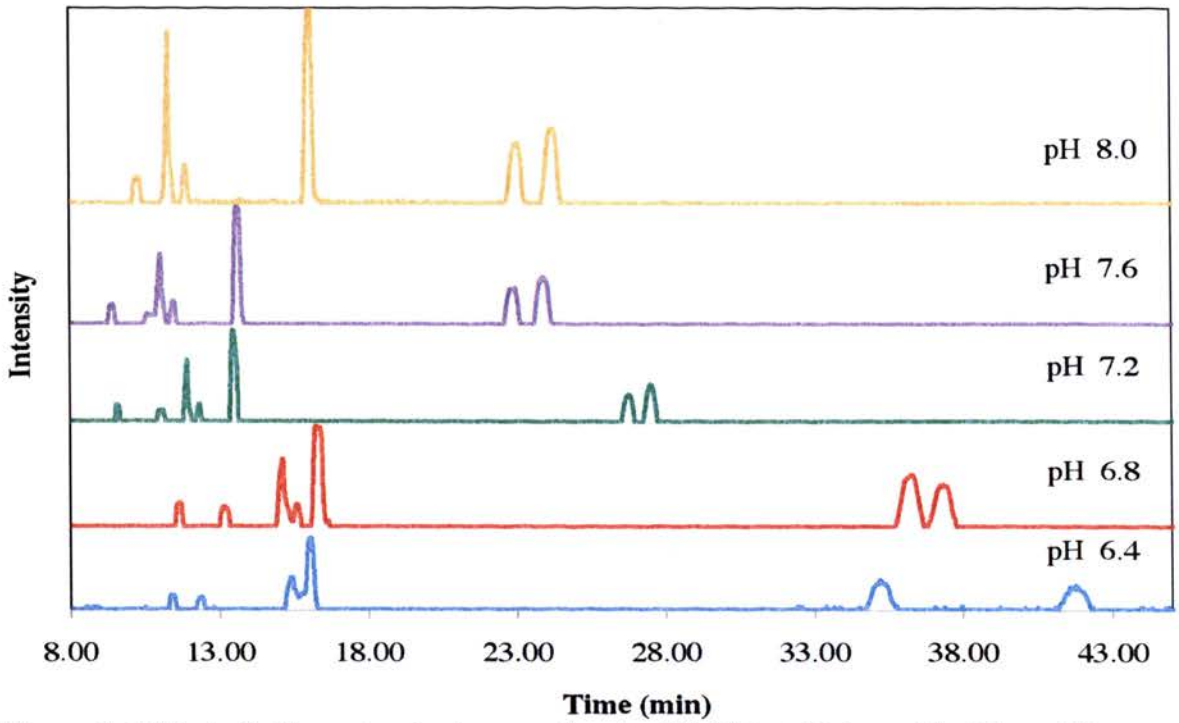

Figure 1. Effect of pH on standard separation by CE-UV at 214 nm. Capillary, 50 μm (i.d.) x 55 cm (45 cm to the detection window); applied voltage 15 kV; temperature, 25°C; sample injection, 0.5 psi for 10 s; running buffer, 100 mM Na_2HPO_4.

At pH 7.2 (shaded trace), the separation of the compounds and the overall runtime provided an optimum solution. It was observed that two important compounds, acetaminophen and caffeine, could not be resolved.

Additives

Additives in the buffer matrix can selectively affect the electrophoretic mobility of ions [36, 39]. The approach to separate our eight compounds has worked well except for the separation of caffeine and acetaminophen. In order to separate these two compounds, 18 Crown 6 was added to the running buffer as an additive to improve the separation. Initial investigations included 5, 10, 20, 50, and 100 mM of 18 Crown 6 diluted in the running buffer (100 mM phosphate, pH 7.20), while keeping the sample dilution buffer unchanged. **Figure 2** shows the separation at 15kV, the most promising EP for this investigation. The concentrations of 18 Crown 6 *versus* electrophoretic potentials of 20, 25, and 30 kV were also investigated (data not shown).

It can be clearly seen from **Figure 2** that separation of all of the compounds used was achieved after 100 mM of 18 Crown 6 were added to the running buffer. However, the last two compounds (sulfamethoxazole and ibuprofen) eluted well beyond 60 minutes. Therefore, using data previously collected, it was determined that a lower voltage (15kV) could be used to elute the first six compounds then stepped up to a higher voltage (30kV) to speed up the elution of the last two. Additionally, concentrations of 60 and 80 mM 18 Crown 6 were compared with the initial concentration study (data not shown). The final separation parameters chosen were: applied voltage 15 kV for 18

minutes and then 30 kV for 22 minutes; temperature, 25°C; sample injection, 0.5 psi for 10 seconds; running buffer, 100 mM of Na_2HPO_4, pH 7.2, 80 mM of 18 Crown 6.

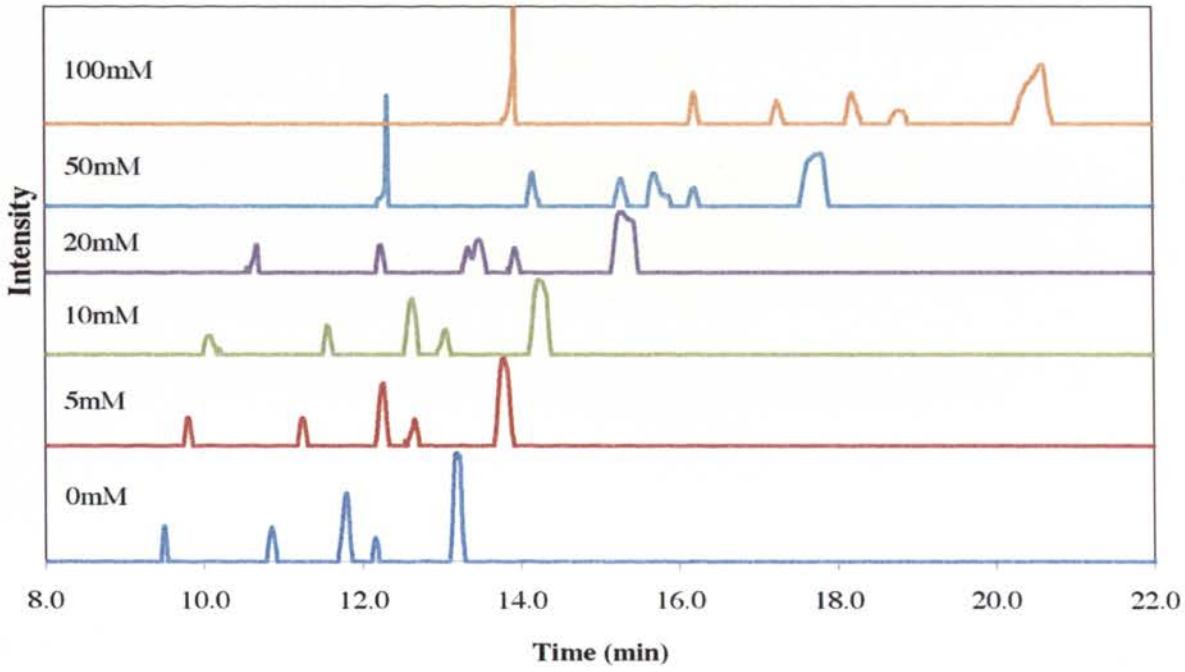

Figure 2. Separation of PPCP standards at different 18 Crown 6 concentrations. Other electrophoretic conditions were the same as those in **Figure 1**. Sulfamethoxazole and ibuprofen not shown due to scale.

Method Validation

Validation of the optimized method included linearity range, instrumental detection limit (IDL), instrument quantification limit (IQL), method detection limit (MDL), and selectivity [40]. The same extraction technique was used as our previous study, therefore recovery was not investigated during validation [15].

Linearity, IDL, IQL, and MDL were determined through serial dilutions of the standard mixture. Instrument detection limits were based on the 3σ criterion. Here, peak heights less than three times the standard deviation were considered noise. Instrument quantification limits were similarly determined using 10σ of the baseline [41]. For standards that have the same IDL and IQL, the peaks that were detected had intensities greater than 10σ, but the next dilution injected fell below 3σ of the baseline. MDL was determined by taking the standard peak area from the IDL and back calculating the detection limit based on the sample concentration factor. The results are summarized in **Table 1**. Selectivity was performed through elution matching and standard additions of standards to the standard mixture and waste water samples to assure proper identification of the peaks in the water matrix.

Table 1. Summary of linear range, IDL, IQL and MDL

Standard	Instrument Linear Range (ppm)			IQL (ppm) 10σ	IDL (ppm) 3σ	MDL (ppb)
lincomycin	3.86	-	123.5	7.72	3.86	1.6
trimethoprim	0.95	-	3.79	0.95	0.95	7.9
carbamazepine	0.69	-	5.54	1.38	1.38	17.2
caffeine	0.63	-	10.1	0.63	0.63	7.7
acetaminophen	0.71	-	11.4	1.42	0.71	4.6
triclosan	2.39	-	38.3	2.39	1.20	68.7
sulfamethoxazole	1.66	-	26.5	1.66	0.83	55.4
ibuprofen	2.00	-	32.0	2.00	1.00	36.3

Waste Water Analysis

Two different waste water samples were collected from two Missouri waste water treatment facilities (denoted R and C), and then concentrated and diluted with running buffer. Each sample was prepared twice and run in triplicate by the optimized CE method previously described. **Figure 3** shows the electropherograms of the two samples *versus* the standard.

It can be seen in **Figure 3** that five compounds, lincomycin, acetaminophen, caffeine, sulfamethoxazole and ibuprofen can be detected in sample 'C'. Only four compounds were detected in sample 'R' (ibuprofen not detected). Peaks were identified using both eulition time matching and standard addition. The remaining three compounds, triclosan, trimethoprim and carbamazepine were not detected in the waste water samples collected. To confirm our findings, the samples were independently analyzed using LC-MS/MS, as outlined by Chuan *et al.* [15]. **Table 2** summarizes the results of all analyses.

The data in **Table 2** shows that capillary electrophoresis with UV detection is capable of measuring PPCP's in Missouri waste water samples. Differences between the two methods can be attributed by several factors, including the waste water matrix and sample preparation. In **Figure 3** the water matrix has clearly caused some interference with the caffeine and acetaminophen peaks. This interference can be overcome through further optimization for the specific waste water matrix.

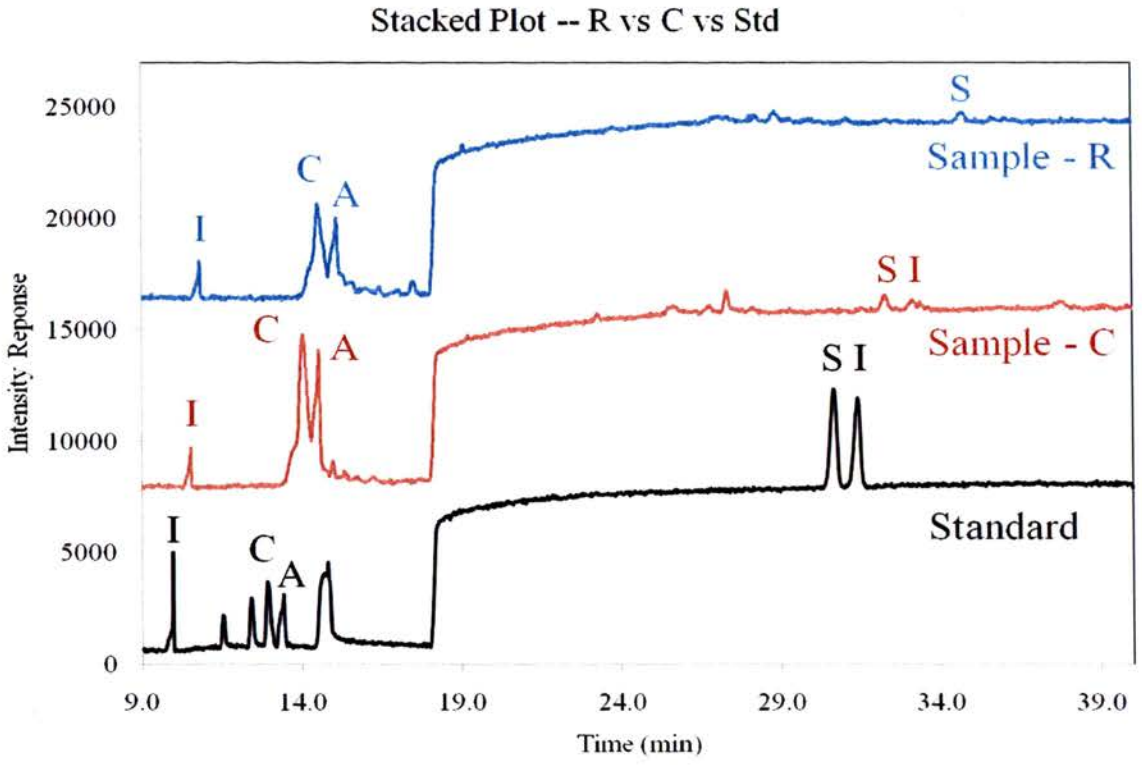

Figure 3. Representative electropherogram of both waste water samples (R and C) and a standard injection. Capillary, 50μm (i.d.) x 55 cm (45 cm to the detection window); applied voltage 15 kV for 18 m / 30 kV for 22 m; temperature, 25°C; sample injection, 0.5 psi for 10 s; running buffer, 100 mM Na_2HPO_4, pH 7.2, 80 mM 18 Crown 6. Only the peaks found in the samples were labeled. (L) lincomycin, (C) caffeine, (A) acetaminophen, (S) sulfamethoxazole, and (I) ibuprofen.

The sample matrices have also caused subtle shifts in the elution time. In **Figure 3,** this shift can be observed from the standard injection to sample 'C' and to sample 'R' (order of analysis). This is contributed to differences in the matrix as well as a change in the double layer over time. This is minimized using a NaOH (0.1 N) rinse between injections. However, when samples are especially complex, changes are difficult to control. Due to this phenomenon, standard addition is required for peak identification.

Additionally, due to the low concentrations necessary for LC-MS/MS analysis, high dilutions are required. Propagation of error through these dilutions also account for some of the discrepancy between the two techniques [40].

Table 2. Summary of PPCP data from two waste water samples collected from waste water treatment facilities. All concentrations listed in ppb.

	CE			MS		
Compounds	Location 'R' Average (ppb)		Location 'R' Stdev (ppb)	Location 'R' Average (ppb)		Location 'R' Stdev (ppb)
lincomycin	3.5	±	0.6	5.2	±	2.0
caffeine	380	±	17.8	325	±	26.4
acetaminophen	930	±	38.3	910	±	86.0
sulfamethoxazole	106	±	12.0	107	±	4.8

Compounds	Location 'C' Average (ppb)		Location 'C' Stdev (ppb)	Location 'C' Average (ppb)		Location 'C' Stdev (ppb)
lincomycin	20.0	±	0.9	19.8	±	3.2
caffeine	760	±	36.3	710	±	81.9
acetaminophen	2080	±	544.9	1740	±	206.5
sulfamethoxazole	87	±	9.1	116	±	5.5
ibuprofen	230	±	46.9	190	±	12.3

Conclusions

A fast, simple, low-cost, sensitive method was developed and validated to separate and quantify select PPCP compounds in Missouri waste water by capillary electrophoresis with UV detection. Although more data is required to determine its applicability, the method offers an alternative to LC/MS and LC-MS/MS for PPCP determination in waste water.

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CONCLUSIONS

Capillary electrophoresis has spread its roots into many fields of scientific work and has established itself as an analytical technique with great potential and applicability for years to come. In our studies we have used capillary electrophoresis for three specific applications; 1) biomarker analysis in urine samples, 2) monitor folate enhancement for plant samples and 3) PPCP analysis in Missouri waste water.

In the biomarker project, we investigated instrumental, systemic and preparative parameters for early cancer screening through the analysis of urinary pteridine. At the same time optimization and validation was performed. The final method showed a threefold increase in resolution over previous work. Additionally, sensitivity was maintained when sample introduction was switched from electrokinetic to gravimetric. Aging data showed urine samples were stable at -2°C for up to 15 days.

A method for the quick and effective quantification of nine pteridine compounds in transgenic and non-transgenic plant samples has been developed for CE-LIF. This method provides a complementary technique to HLPC and TLC to measure the effectiveness of folate fortification of genetically modified plants. Further investigations into complementary compounds may now be explored due to the high resolution of CE-LIF.

A fast, simple, sensitive method was developed and validated to separate and quantify select PPCP compounds in Missouri waste water by capillary electrophoresis with UV detection. Although more data is required to determine its applicability, the method offers an alternative to LC/MS and LC-MS/MS for PPCP determination in waste water.

VITA

Stephen Eric Gibbons was born on August 29, 1972 in the state of Pennsylvania. He graduated with a Bachelor of Science in Mathematics from Texas Tech University in Lubbock, Texas in 1995. From 1995 to 1998, Stephen enlisted in the United States Army as a Calvary Scout. After completing his tour of duty in November of 1998, Stephen began working in the private sector for Rent-A-Center. In June of 2000, Stephen made a career change by joining Brewer Science as a Laboratory Analyst. In 2003, while maintaining full time employment at Brewer Science, Stephen began his Ph.D. program at Missouri University of Science and Technology (formerly the University of Missouri, Rolla). Stephen maintained his employment status until he received his Ph.D. in Chemistry in December of 2010.

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