SENSITIVE DETECTION/SEPARATION OF NICOTINE AND ITS
METABOLITES BY LASER WAVE-MIXING SPECTROSCOPY FOR
ENVIRONMENTAL AND BIOMEDICAL APPLICATIONS

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Master of Science
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DEDICATION

I dedicate my thesis to my family for supporting and motivating me unconditionally every step of the way. I would not be the person I am today personally and professionally if it was not for their sacrifices and their constant support.
ABSTRACT OF THE THESIS

Sensitive Detection/Separation of Nicotine and its Metabolites by Laser Wave-Mixing Spectroscopy for Environmental and Biomedical Applications

by

Zarina Munshi
Master of Science in Chemistry
San Diego State University, 2016

Ultrasensitive detection methods for nicotine and its major metabolite, cotinine, are needed in order to provide reliable data that can help study environmental tobacco smoke and their effects on non-smokers especially children. Some of the studies on passive smoke have confirmed that the exposure of smoke jeopardizes children’s health even prior to their birth and is a primary cause for many abnormalities. To assess and understand the correlations of smoke exposure to smoke-related diseases, researchers have used nicotine and cotinine as the principle biomarkers. However, there is a lack of a sensitive analytical method that can monitor trace levels of nicotine in second hand smoke and third hand smoke in a compact portable detector design with minimal sample preparation and quick analysis time. Reliable and sensitive detection of nicotine is essential to track and understand numerous health and psychological effects caused by passive smoke.

We demonstrate laser wave mixing, as a highly sensitive nonlinear spectroscopic method that is suitable for field use for a wide range of environmental and biomedical applications. Wave mixing only requires a small amount of sample (nanogram). Hence, it can be conveniently interfaced to microarrays, microfluidics, chip-based capillary electrophoresis, and other flow systems to yield excellent chemical specificity and detection sensitivity levels (pico- or femto-mole). Two of the major factors that allow wave-mixing signals to yield high signal-to-noise ratio are cubic dependence on laser power and square dependence on analyte concentration.

Thus far, the results indicate that nicotine and cotinine can be detected and separated in their native form label-free using an ultraviolet laser as well as their complexes obtained by a two-reagent reaction. Current results show the detection limit of 2.7 ng /mL for nicotine (2.3 x 10^{-18} mol) and 544 ng /mL for cotinine (6.0 x 10^{-17} mol) complexes using a visible laser. The detection limit has a great chance to meet the requirement for real world applications. The method could potentially allow reliable real-time detection of nicotine in indoor environments to accurately monitor smoke exposure to children. Moreover, measurement of cotinine in biological fluids would assist in understanding smoke-related diseases.
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<th>Description</th>
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<tr>
<td>B-DFWM</td>
<td>Backward-Scattering Degenerate Four-Wave Mixing</td>
</tr>
<tr>
<td>C^4D</td>
<td>Capacity-Coupled Contactless Conductivity Detection</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary Electrophoresis</td>
</tr>
<tr>
<td>CGE</td>
<td>Capillary Get Electrophoresis</td>
</tr>
<tr>
<td>DFWM</td>
<td>Degenerate Four-Wave Mixing</td>
</tr>
<tr>
<td>EOF</td>
<td>Electroosmotic Flow</td>
</tr>
<tr>
<td>ETS</td>
<td>Environmental Tobacco Smoke</td>
</tr>
<tr>
<td>FD</td>
<td>Fluorescence Detector</td>
</tr>
<tr>
<td>F-DFWM</td>
<td>Forward-Scattering Degenerate Four-Wave Mixing</td>
</tr>
<tr>
<td>FHS</td>
<td>Firsthand Smoke</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HeNe</td>
<td>Helium Neon</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>LIF</td>
<td>Laser-Induced Fluorescence</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid Chromatography-Tandem Mass Spectrometry</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of Detection</td>
</tr>
<tr>
<td>MS</td>
<td>Mainstream Smoke</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative Standard Deviation</td>
</tr>
<tr>
<td>SHS</td>
<td>Secondhand Smoke</td>
</tr>
<tr>
<td>S/N</td>
<td>Signal-to-Noise</td>
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<tr>
<td>SPE</td>
<td>Solid Phase Extraction</td>
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<tr>
<td>THS</td>
<td>Thirdhand Smoke</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

1.1 DETECTION OF NICOTINE AND COTININE

1.1.1 Background on Tobacco Smoke

It is important to understand and acknowledge the difference between various types of tobacco smoke. Mainstream (MS) is the smoke inhaled by a smoker.\textsuperscript{1} The tobacco smoked by the nonsmokers often referred to as “passive smoking” or “involuntary smoking.”\textsuperscript{1} Second-hand smoke (SHS) or environmental tobacco smoke (ETS) is the smoke that nonsmokers inhale from either smoke that is exposed by a smoker’s exhaled smoke or smoke produced from burning tobacco that contaminates indoor spaces and outdoor environments.\textsuperscript{1–3} Third-hand smoke (THS) is the remaining airborne smoke contaminants mixed with dust particles that are absorbed by indoor surfaces many hours after smoking has ended.\textsuperscript{4} The short- and long-term effects of MS have been studied and well understood for quite some time. SHS has been fairly understood. Involuntary inhaling of tobacco smoke exposes a non-smoker to toxic chemicals. Consequently, this fact has been the motivating factor for laws restricting smoking in many public locations forcing smokers to smoke in outdoor environments distant from public places. Despite smokers taking sufficient measures to eliminate if not minimize SHS to keep non-smokers away from harm, they continue to subject their family members, especially children, to smoke.\textsuperscript{5} The homes, cars, hair, clothes and skin of smokers are very likely to have significant levels of THS. One may conclude that THS might be more harmful, especially to infants. In contrast to MS and SHS, there is a lack of studies done on THS. The risk factor worsens due to the chemicals found in THS that are not limited to the nicotine residues and toxic chemicals from cigarette smoke, but also include the substances that are byproducts of cigarette smoke toxins reacting with environmental substances resulting in more hazardous constituents, which are collected on
surfaces over time. Children and infants spend sizable amounts of time in contact with floors and other surfaces. Consequently, the likely chance of them being exposed to THS is much higher specifically because children frequently put their hands in their mouth without often washing their hands after touching contaminated surfaces. The constant growing studies on THS demonstrate its alarming toxicity that has yet to be vastly recognized.

It is well understood that the long-term effects of actively smoking expose smokers to risk factors for cardiovascular diseases, oral cancers and numerous pulmonary diseases. The studies on the effects of impassive smoke on non smokers have been constantly increasing. The tobacco smoke exposure on children can potentially start from prenatal growth. There is an association between children who are exposed to smoke and implications for the cause and development of having one of many disorders resulting from the oxidative stress. Specific to newborns, they can have growth deficiency, or breathing and lung problems (asthma), or due to the severity of complications, these can lead to death. Conclusively, there are doubts raised as to whether the current laws and restrictions are enough to eliminate impassive smoke exposure to children.

1.1.2 Motivation

In order to understand the health and psychological effects on people, specifically children, it is important to effectively measure and analyze the alkaloids involved. Nicotine is a naturally occurring alkaloid that is found in tobacco plants and a main known addictive component of tobacco smoke. Nicotine is significantly metabolized to numerous metabolites by the liver. Figure 1.1 shows nicotine’s main metabolic pathway with 70-80% of nicotine rapidly converted to its primary metabolite, cotinine. It has been numerous reported that cotinine has a fairly longer half-life of 16 hours as compared to 2 hours for nicotine. As a result, there is a higher concentration of cotinine than nicotine found in the blood of cigarette smokers. Subsequently, cotinine has been used as a biomarker to study the daily intake not only in cigarette smokers but also in those who are exposed to SHS and THS because the presence of cotinine found in biological fluids indicates exposure to nicotine.
There is a lack of reliable detection methods that can monitor tobacco-specific vapor such as nicotine in real time while accurately measuring the cotinine levels in biological fluids specific to nicotine exposure. As a result, the collected data can potentially be studied for correlations between the nicotine levels in environmental samples and cotinine levels found in biological fluid samples to better understand the effects of impassive smoking and
to assist in the development of policies and practices that can shield children and other non-smokers.4

1.1.3 Comparative Analytical Spectroscopic Methods

Some of the most commonly used methods include liquid chromatography (LC), gas chromatography (GC), capillary electrophoresis (CE), and mass spectrometry (MS). LC is a separation based on the interactions of target analyte with the mobile and stationary phase. High-performance liquid chromatography (HPLC) specifically requires a specialized injection system, a high-pressure pump, and a tightly packed column resulting from high pressure. GC is a separation method for volatile analytes in the gas phase. The analyte is dissolved in a solvent and vaporized in order to separate the analytes by distributing the sample between the mobile and stationary phase. In a mass spectrometer, analytes are ionized, sometimes fragmented, and then directed to a mass analyzer and separated according to their mass-to-charge ratio (m/z). CE is a separation method that requires voltage gradient as the driving force and molecules dissolved in an electrolyte. Resulting separations occur based on a mass, charge and charge density differences. All these methods have advantages and disadvantages as listed in Table 1.1.11

Table 1.1. Advantages and Disadvantages of Conventional Analytical Methods

<table>
<thead>
<tr>
<th>Methods</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td>Efficient (high speed, resolution, sensitivity, accuracy) Selective Widely applicable Require only small sample Applicable to nonvolatile samples</td>
<td>Expensive instrumentation/ supplies Poor separation efficiency</td>
</tr>
<tr>
<td>GC</td>
<td>Efficient Selective Inexpensive Widely applicable Requires only small sample size</td>
<td>Labor intensive Expensive instrumentation Samples must be volatile Not suitable for thermally liable sample</td>
</tr>
<tr>
<td>CE</td>
<td>Less expensive chiral analyses Widely applicable Small volume injection Short analysis time Low cost reagents</td>
<td>Poor sensitivity High voltage hazard Poor quantitative precision Poor migration time precision</td>
</tr>
<tr>
<td>MS</td>
<td>Small sample size Sensitive Fast Highly Compatible with other analytical methods</td>
<td>Expensive Instrumentation Requires fairly pure sample Specificity limitations</td>
</tr>
</tbody>
</table>
The most commonly used detection methods include fixed or variable UV wavelength absorption, fluorescence, mass spectrometer, and electrochemical methods. The combination of two or more methods and a specific detector efficiently elevate a qualitative and a quantitative analysis value as well as produces the best sensitivity (LOD). Table 1.2 summarizes some of the most conventional methods currently being used to detect and analyze numerous types of analytes including nicotine and cotinine.

### Table 1.2. Limits of Detection for Nicotine and Cotinine Using Comparative Analytical Methods

<table>
<thead>
<tr>
<th>Method</th>
<th>LOD of Nicotine (ng/mL)</th>
<th>LOD of Cotinine (ng/mL)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC-MS/MS</td>
<td>0.1</td>
<td>0.05</td>
<td>12</td>
</tr>
<tr>
<td>GC</td>
<td>0.005</td>
<td>0.01</td>
<td>13</td>
</tr>
<tr>
<td>GC-MS</td>
<td>0.2</td>
<td>0.2</td>
<td>14</td>
</tr>
<tr>
<td>HPLC-FD- UV-Photo Irradiation</td>
<td>7.5</td>
<td>1.5</td>
<td>15</td>
</tr>
<tr>
<td>HPLC-UV Absorption</td>
<td>10</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>CE-C4D/FD</td>
<td>200</td>
<td>388</td>
<td>10</td>
</tr>
<tr>
<td>CE-MS-SPE</td>
<td>0.55</td>
<td>11.25</td>
<td>17</td>
</tr>
<tr>
<td>CE</td>
<td>150</td>
<td>150</td>
<td>18</td>
</tr>
</tbody>
</table>

### 1.2 LASERS

The laser is a light source that is coherent, monochromatic, and has a highly directional nature that generates or amplifies coherent radiation at the frequencies from the infrared (IR) to the UV region of the electromagnetic spectrum. Moreover, the laser beam has a high spatial coherence and the spectral linewidth of a laser is smaller than those of the atomic and molecular emission allowing efficient and selective excitation of target analytes.\(^9,10\) The functions of coherent signal generation, amplification, modulation, information transmission and detection became possible at many wavelength ranges, pushing the upper limit for possibilities for laser-based devices.\(^19\) It encouraged engineers and scientists in the field of technology to test lasers for their applicability in many fields including medicine, engineering, and scientific research. Particularly in the field of analytical spectroscopy, lasers not only have enhanced but also have played a major role in the development for more effective methods for chemical analysis. Laser-based methods offer orders of magnitude improvement in spectral resolution and detection sensitivity compared to conventional spectroscopic methods.\(^20\)
1.2.1 Laser Based Analytical Spectroscopic Methods

The first laser was developed in 1960. Within a decade, lasers were used to develop many novel laser spectroscopic methods. The characteristics of the laser beam allow development of many effective absorption and emission-based optical methods. Absorption or emission spectroscopy is a measure of absorption or emission of radiation by the analyte of interest. The resulting optical spectrum assists in identifying the atomic and molecular composition of the samples. Some of the most common analytical optical methods include IR, UV-visible and fluorescence spectroscopic methods. These methods can further be categorized as linear laser-based methods if they involve a one-photon excitation step that results in a thermal and/or radiative process. Nonlinear laser spectroscopy involves multi-photon multi-step excitation processes and offers unique advantages in the studies of the interaction between radiation and matters using low and high intensity of lasers.

1.2.1.1 NONLINEAR LASER SPECTROSCOPY

With two or more electromagnetic fields involved, the relationship between the analyte and the signal intensity can be more complex with a nonlinear response. This nonlinear dependence actually offers some advantages including excellent detection sensitivity and more effective monitoring of small changes in analytes. Multi-photon four-wave mixing is a novel nonlinear laser method that offers significant advantages including the generation of a laser-like coherent signal beam, highly efficient signal detection with great signal-to-noise ratio, and excellent detection sensitivity levels for a broad range of potential real-world applications. Four-wave mixing is one of the most effective nonlinear methods and Chapter 2 describes the theory, experimental setups and advantages in more detail.

1.3 THESIS OUTLINE

Chapter 2 of the thesis describes theoretical concepts of degenerate four-wave mixing (DFWM) spectroscopic method, its effective use for detecting small molecules and proteins for environmental and biomedical applications, and the comparative advantages over conventional detection methods. It also details essential parameters for optimal alignment of
the detection system that allows ultrasensitive detection of various analytes with a minimal sample preparation and analysis time. Chapter 3 discusses the results of using forward-scattering degenerate four-wave mixing (F-DFWM) with a 266 nm UV laser source to detect and separate native and label-free forms of standard nicotine and cotinine samples after coupling with capillary electrophoresis. The chapter also discusses the effects of changing certain parameters of the capillary electrophoresis system and shows the results of the optimal condition used to achieve the highest signal-to-noise ratio (S/N). Chapter 4 discusses the results from experimenting with multiple reactions for indirect detection of the analytes in the visible wavelength range with potentially higher specificity levels.
CHAPTER 2

NONLINEAR DEGENERATE FOUR-WAVE MIXING LASER SPECTROSCOPY

2.1 DEGENERATE FOUR-WAVE MIXING SPECTROSCOPY

Degenerate four-wave mixing (DFWM) is a nonlinear absorption-based spectroscopic method that has been demonstrated to be one of the most effective, versatile methods used to identify and quantify various sample types. This laser method is defined as degenerate because all the input beams (waves) involved have the same frequency. The optical setup consists of pump and probe input beams mixing inside the nonlinear absorbing analyte in order to produce a third-order nonlinear polarization yielding to DFWM signal beam. The mixing of two input beams results in a pattern of destructive and constructive interferences. The process leads to the creation of dynamic gratings that interact with the absorbing target analyte to produce the coherent laser-like DFWM signal beam. There are two types of DFWM: Backward-Scattering (B-DFWM) and Forward-Scattering (F-DFWM).

2.1.1 Backward-Scattering Degenerate Four-Wave Mixing

The B-DFWM has unique characteristics that involve optical phase conjugation, a process of reversing a phase variation and the propagation direction of the input beams and forming a conjugate signal beam. In a B-DFWM setup shown in Figure 2.1, E₁ is the forward pump beam, E₂ is the backward pump beam, and E₃ is the probe beam. The two counter-propagating pump beams and the probe beam interact with the analyte and generate a signal beam, E₄, which retraces the path of the probe beam. The diffraction pattern created from the interaction produces a phase-conjugate signal beam. B-DFWM is more often utilized for detection of gas-phase analytes.
2.1.2 Forward-Scattering Degenerate Four-Wave Mixing

A typical F-DFWM setup is shown in Figure 2.2. A single laser beam is split into two input beams (E₁ and E₂). The two input beams (pumps) are directed in the same direction until focused and mixed at an absorbing analyte at a specific angle. The interference pattern created by the input beams interacts with the analyte producing two additional laser-like signal beams (E₃ and E₄) in a similar angle as the two pump beams. Unlike B-DFWM, there is no separate probe beam involved. However, one of the pump beams serves as the probe beam generating two signal beams. Even though F-DFWM produces two signal beams, only one of them is isolated and analyzed. The choice is made based on comparing each of the signal beam’s characteristics. The concept is further discussed later in the chapter. Since the principle objective of this thesis is to analyze liquid-phase analytes, the remaining chapter will primarily focus on the concepts involving F-DFWM.
2.2 INTERFERENCE PATTERN

The two input beams (E₁ and E₂) of the same wavelength interact at a small angle producing a pattern of constructive and destructive interferences shown in Figure 2.3. The pattern is further described using the light intensity modulation expression with respect to spatial coordination, x, spatial period of the interference, Λ, and fringe modulation, β:

\[ I(x) = I₀ [1 + β \cos\left(\frac{2πx}{Λ}\right)] \]  

(2.1)

The Fringe modulation, β, depends on the intensity of the two input beams. The relationship can be expressed by the following equation where I₀ is the total laser intensity (I₁+I₂).

\[ β = \frac{2\sqrt{I₁I₂}}{I₀} \]  

(2.2)

As a result, the spatial grating period can be described as:

\[ Λ = \frac{λ}{2 \sin\left(\frac{θ}{2}\right)} \]  

(2.3)

Equation 2.3 indicates that the spatial grating period of an interference pattern is directly proportional to the angle formed between the input beams and the laser wavelength.
As a result, the absorbing analyte inside the resulting interference mimics the spatial modulation of the interference pattern generating the laser-induced diffraction gratings.¹⁹,²²,²³

Figure 2.3. Interference pattern created by mixing two input beams (E₁ and E₂) inside an absorbing analyte.

### 2.3 Laser-Induced Diffraction Gratings

In liquid-phase analytes, the constructive and destructive interferences yield thermal gratings inside the liquid analyte. More specifically, when an absorbing analyte passes through the spatial interference patterns, the molecules are excited in the region of constructive interference and remain less excited in the region of deconstructive interference. The heat transfer within the absorbing analyte results in a refractive-index change, which then diffracts subsequent photons to generate a wave-mixing signal beam.²⁰,²²–²⁴,²⁶

### 2.4 Forward-Scattering Wave-Mixing Signal

In a F-DFWM setup, the interaction of the two pump beams (E₁ and E₂) generates an interference pattern, which then yields a thermal grating. Figure 2.4 illustrates optimal F-
DFWM geometry in condensed-phase studies. The two input beams work under a self-diffraction mode, i.e., they serve both as the pump beam (E₁ and E₂) and the probe beam (E₃), and as a result, diffract a portion of its photons to yield signal beams (E₄). Even though there are two input beams involved in the setup, this process is still defined as four-wave mixing because there are four photons involved in creating the wave-mixing signal. Since the wave-mixing signals are coherent laser-like beams, they can be isolated and directed away from incoherent noise levels generated from any stray light sources. Because the two input beams have different intensity levels (~70:30), one of the signal beams is much stronger than the other. Because most of the experiments require only one signal beam, only the signal beam with higher intensity is isolated for detection.

Figure 2.4. F-DFWM geometry with the intersection of the two pump beams (E₁ and E₂) within an absorbing analyte to generate wave-mixing signal (E₄) with a phase matching diagram for each signal beam.
Bragg conditions explain that both energy and momentum of input beams must be conserved. The energy conservation requires:

$$\omega_4 = \omega_1 + \omega_3 - \omega_2$$  \hspace{1cm} (2.4)

Where $\omega_1$, $\omega_2$ and $\omega_3$ are the frequencies of pump and probe beams producing $\omega_4$, the frequency of the signal beam. Since all the laser beams have the same frequency, the resulting signal beam must have the same frequency. The phase vectors associated with each beam (Figure 2.4) can be defined as:

$$\mathbf{k}_4 = \mathbf{k}_1 + \mathbf{k}_3 - \mathbf{k}_2$$  \hspace{1cm} (2.5)

The phase vectors satisfy the conservation of momentum in a similar fashion as the frequencies. However, even a minor difference in the phase changes can affect the resulting phase vector of the signal beam. Hence, to avoid phase mismatches, it is important to have the beams intersect at a small angle inside the absorbing analyte in order to increase the length of the interaction. In an ideal F-DFWM scheme, the resulting signal is optimized at $\Delta k = 0$ which is impossible to achieve. The phase mismatch $\Delta k$ can be expressed as:

$$\Delta k = \mathbf{k}_1 + \mathbf{k}_3 + \mathbf{k}_2 - \mathbf{k}_4$$  \hspace{1cm} (2.6)

The intensity of the wave-mixing signal can be described by the following equation:\textsuperscript{18-22}

$$I_3 = \frac{b}{8} \frac{I_1^2 I_2}{\sin^4 (\lambda / 2)} \frac{dn}{dT} \frac{\Delta n}{2}$$  \hspace{1cm} (2.7)

Equation 2.7 describes the signal intensity with respect to the following variables: $b$, the optical path length; $I_1$, and $I_2$, the two input (pump) beams; $\lambda$, excitation wavelength; $\theta$, the angle between the two input beams; $dn/dT$, change in the refractive index of the solvent with respect to temperature modulation; $\kappa$, thermal conductivity of the solvent; and $\alpha$, molar absorptivity of the analyte. The cubic dependence on laser power and the quadratic dependence on molar absorptivity (analyte concentration) play a significant role in validating the DFWM signal. The quadratic dependence on analyte concentration gives the DFWM method a unique advantage as compared to conventional absorption-based methods. Cubic
dependence on laser power allows the use of relatively low power (mW) laser sources. These advantages are further demonstrated and confirmed in the DFWM experiments described in this thesis.

### 2.5 Previous Work

Table 2.1 summarizes DFWM detection limits that have successfully been achieved for various types of biological and environmental analytes utilizing various types of sample cells.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration LOD (M)</th>
<th>Mass LOD (Mol)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV-Ab</td>
<td>$6.4 \times 10^{-14}$</td>
<td>$2.6 \times 10^{-17}$</td>
<td>27</td>
</tr>
<tr>
<td>BSA</td>
<td>$3.4 \times 10^{-19}$</td>
<td>$1.7 \times 10^{-22}$</td>
<td>27</td>
</tr>
<tr>
<td>Cellular Proteins</td>
<td>$3.5 \times 10^{-16}$</td>
<td></td>
<td>26</td>
</tr>
<tr>
<td>Cytokines</td>
<td>$6.0 \times 10^{-13}$</td>
<td>$6.0 \times 10^{-21}$</td>
<td>26</td>
</tr>
<tr>
<td>DNA</td>
<td>$8.7 \times 10^{-16}$</td>
<td>$1.0 \times 10^{-23}$</td>
<td>26</td>
</tr>
<tr>
<td>Cellular Proteins</td>
<td>$3.6 \times 10^{-16}$</td>
<td>$2.2 \times 10^{-23}$</td>
<td>23</td>
</tr>
<tr>
<td>Neurotransmitters</td>
<td>$3.7 \times 10^{-13}$</td>
<td>$2.0 \times 10^{-23}$</td>
<td>21</td>
</tr>
<tr>
<td>HIV Protein (p24)</td>
<td>$4.2 \times 10^{-10}$</td>
<td>$3.13 \times 10^{-20}$</td>
<td>21</td>
</tr>
<tr>
<td>CEA</td>
<td>$3.3 \times 10^{-12}$</td>
<td>N/A</td>
<td>28</td>
</tr>
<tr>
<td>Fe (II)</td>
<td>$1.7 \times 10^{-14}$</td>
<td></td>
<td>26</td>
</tr>
<tr>
<td>Cr (VI)</td>
<td>$1.15 \times 10^{-9}$</td>
<td>N/A</td>
<td>26</td>
</tr>
<tr>
<td>Ni (II)</td>
<td>$2.6 \times 10^{-9}$</td>
<td>$5.0 \times 10^{-20}$</td>
<td>24</td>
</tr>
<tr>
<td>Cu (II)</td>
<td>$8.5 \times 10^{-8}$</td>
<td>$1.6 \times 10^{-18}$</td>
<td>24</td>
</tr>
<tr>
<td>Crystal Violet</td>
<td>$6.9 \times 10^{-18}$</td>
<td>$2.5 \times 10^{-19}$</td>
<td>28</td>
</tr>
<tr>
<td>Malachite Green</td>
<td>$8.3 \times 10^{-11}$</td>
<td>$3.0 \times 10^{-20}$</td>
<td>28</td>
</tr>
<tr>
<td>TNT-complex</td>
<td>$2.3 \times 10^{-12}$</td>
<td>$2.1 \times 10^{-18}$</td>
<td>22</td>
</tr>
<tr>
<td>TATP-complex</td>
<td>$3.7$ (pg/mL)</td>
<td>$7.8 \times 10^{-18}$</td>
<td>22</td>
</tr>
<tr>
<td>Nitro-Aromatic Explosives</td>
<td>$4.4 \times 10^{-13}$</td>
<td>$2.4 \times 10^{-23}$</td>
<td>21</td>
</tr>
<tr>
<td>Ammonium Nitrate</td>
<td>$1.5 \times 10^{-9}$</td>
<td>$5.2 \times 10^{-18}$</td>
<td>28</td>
</tr>
</tbody>
</table>

### 2.6 DFWM Advantages

The DFWM method offers several advantages over conventional analytical methods because of the unique characteristics of a nonlinear laser method. Advantages include small sample size requirements, short path lengths, excellent detection sensitivity, and a good standoff detection capability. Even when using fluorophore and chromophore reagents for labeling an analyte, detection sensitive levels are comparable or better than those of other
fluorescence-based methods. Its excellent sensitivity levels (e.g., ppq) allow the reliable study of trace analytes in complex mixtures. The characteristics of a laser-like signal beam offer standoff detection using many target analytes for biological, environmental and security applications. Laser-based DFWM is demonstrated not only as a sensitive detector but also as a simple, inexpensive, compact, portable detector. It is one of the most adaptable methods. Chapters 3 and 4 will further explore DFWM for sensitive detection of nicotine and cotinine for second- and third-hand smoke studies.
CHAPTER 3
DETECTION AND SEPARATION OF LABEL-FREE NICOTINE AND COTININE BY DEGENERATE FOUR-WAVE MIXING COUPLED WITH CAPILLARY ELECTROPHORESIS

3.1 INTRODUCTION
The significance of detecting nicotine and cotinine (specifically in the biological fluids) for second- and third-hand smoke studies concerning the health and psychological effects of impassive smoke exposures on non-smokers has been discussed comprehensively in Chapter 1. Because nicotine is a tracer of tobacco smoke, detection of nicotine is utilized to monitor and evaluate the ETS exposure. The significance of detecting cotinine alongside nicotine over other nicotine-related metabolites rises because cotinine is known as the primary metabolite of nicotine. The transformation of nicotine into cotinine by the enzymatic process of cytochrome P450 and aldehyde oxidase is shown in Figure 3.1. Cotinine is found in higher abundance (70-80%) compared to other numerous metabolites. Moreover, cotinine has a longer half-life (16 hours) than nicotine (2 hours). Consequently, the detection of cotinine in biological fluids assists in analyzing nicotine exposure and related diseases.
Figure 3.1. The transformation of nicotine into cotinine with two-steps conversion by enzymatic process of cytochrome P450 and aldehyde oxidase.\textsuperscript{8,29}

Chapter 1 discusses comparative conventional methods and their limitations. Although GC, HPLC, GC-MS, and LC-MS are the most common and reliable analytical methods used, these methods are bulky, expensive, not portable, and too slow for “real-time” analysis. Conventional CE offers great separation efficiency, adaptability, and fast analysis time, however, CE by itself lacks detection sensitivity as well as portability due to the commonly used MS, FD, and UV-based detectors for CE. The limitations of current methods still leave a demand for a simple and sensitive, yet compact and portable device that can be used for a quick and/or “real-time” analysis.

As described in Chapter 2, DFWM proved to be one of the most versatile and sensitive absorption-based detection methods. The laser-like coherent signal beam is produced through a series of processes, which includes mixing two input beams in a sample cell to form an interference pattern. This pattern interacts with an absorbing analyte to temporarily create a thermal grating, which diffracts photons to create a laser-like wave-mixing signal beam. A beam splitter splits a single laser beam into two beams, pump/probe and pump beam with a 70:30 intensity ratio. The stronger signal beam is filtered and collected. The wave-mixing signal demonstrates a cubic dependence on laser power and a quadratic dependence on analyte molar absorptivity. These nonlinear dependencies permit a more effective way to monitor smaller changes in analyte concentrations. Furthermore, better detection sensitivity can be achieved as compared to other conventional methods even
when low power laser sources (mW) or analyte with a small extinction coefficient is involved, making F-DFWM an effective tool for chemical analysis.

Figure 3.1 shows structural similarities of nicotine and cotinine with merely a 14-g/mol molecular weight difference. Figure 3.2 displays the UV-visible spectra of standard nicotine and cotinine with water as their solvent. Both express a maximum absorption peak at 260 nm (extinction coefficients of nicotine and cotinine at 266 nm are determined to be 5,900 M\(^{-1}\)cm\(^{-1}\) and 3,700 M\(^{-1}\)cm\(^{-1}\), respectively). Despite similarities of nicotine and cotinine and their small extinction coefficients in the UV region, the wave-mixing method can distinguish them and overcome detection sensitivity limitations of some of the conventional absorption-based methods because of its enhanced sensitivity levels and inherently small probe volumes. The small probe volumes required in wave mixing allow the convenient interface to CE systems. This chapter will demonstrate F-DFWM as an effective tool for detecting trace amounts of nicotine and cotinine and separating them after coupling with a CE system.

![Figure 3.2. UV-visible spectra of nicotine (0.324 mM; 53 μg/mL) and cotinine (0.567 mM; 100 μg/mL) in water.](image-url)
3.2 EXPERIMENTAL SECTION

3.2.1 Chemicals

(-)-Nicotine tartrate (Figure 3.3) and (-)-cotinine standards were purchased from Thermo Fisher Scientific (Waltham, MA). Supplies needed for laser wave-mixing alignment and optimization steps, including methyl red dye, acetonitrile and methanol, were purchased from Thermo Fisher Scientific. Other chemicals including sodium hydroxide, sodium tetraborate and sodium phosphate for capillary conditioning were purchased from Sigma Aldrich (St. Louis, MO). Appropriate concentrations of sodium tetraborate and sodium phosphate buffers were prepared in distilled water. All the chemicals used were analytical grade standards.

![Chemical Structure](image)

**Figure 3.3.** Nicotine acid tartrate or (-)-nicotine hydrogen tartrate salt form \((C_{10}H_{14}N_2\cdot2C_4H_6O_6)\) with a molecular weight of 462.41 g/mol.

3.2.2 Forward-Scattering Degenerate Four-Wave Mixing Alignment

A typical F-DFWM optical alignment is presented in Figure 3.4. A single wavelength laser source is selected based on the absorption spectrum of the analyte collected by using a UV-visible spectrometer (Agilent, Santa Clara, CA). Figure 3.2 shows UV-visible spectra of nicotine and cotinine with \(\lambda_{\text{max}}\) in the UV wavelength region. As a result, a solid-state 266 nm UV laser (JDS Uniphase, 6 mW) is selected for wave-mixing analysis. The single laser beam is then split into two beams (70% transmission, 30% reflection) by a beam splitter. Multiple mirrors are utilized to direct the two beams in a “square box” placement,
aligning them in a close parallel arrangement. The mirrors with UV coating are incorporated (>90% reflectivity) for the 266 nm UV laser. The two closely parallel beams pass through a focusing lens (10 cm) allowing both beams to cross at a sample cell. The input beams excite the analytes to generate laser-induced thermal diffraction gratings, resulting in a change in refractive index and producing a wave-mixing laser-like signal. The one with stronger signal intensity and higher signal-to-noise ratio (S/N) is collected by a collimating lens and directed onto a photodetector (PDA25K 150-550 nm or PDA36A 350-1100 nm, ThorLabs, Newton, NJ). Optical alignment (S/N) of the F-DFWM signal is optimized using spatial apertures as shown in Figure 3.5. The alignment is initiated first using a relatively high concentration (10^-4 M) absorbing solution (e.g., dye) that can produce a bright signal spot that is visible to the naked eye. The visible signal assists in the initial alignment of the detector. Subsequently, one of the input beams (the weaker beam) is modulated by an optical chopper (Stanford Research Systems SR540) at 200 Hz or higher. The optical chopper is interfaced to a digital lock-in amplifier (Stanford Research Systems SR810DSP) in order to enhance the signal-to-noise ratio of the wave-mixing signal. The lock-in amplifier enables further improvement of the setup by relying on its real-time phase-locked feedback of the signal. Optical apertures and beam blockers are incorporated throughout the setup to minimize background optical noise levels. Ultimately, the lock-in amplifier output is digitized by our custom-built data acquisition software AIDA.
Figure 3.4. Typical forward-scattering degenerate four-wave mixing setup.

Figure 3.5. Predicted spatial orientation of the resulting signal beams based on the center-to-center distance (d) between the two input beams.
Laser wave mixing requires significantly smaller pathlengths (micrometer) for sensitive detection as compared to those used in many conventional absorption-based methods. Essentially, the small probe volume advantage expands the adaptability of DFWM to various types of sample cells (e.g., capillary cells with 20 -100 μm i.d.), Quartz plate microscope slides, and microfluidic chips. The type of sample cell is selected based on the type of target analytes.

3.2.2.1 Capillary Electrophoresis System

Due to the structural similarities of nicotine and cotinine, a CE system was chosen to enhance chemical specificity levels. A simple CE system was custom built and interfaced to our wave-mixing detector as shown in Figure 3.6. The CE system consists of a polyimide-coated fused-capillary (360 μm o.d., 75 μm i.d., Molex, Lisle, IL) with a small window to allow laser beams to pass through, a 30 kV modulated power supply (Glassman High Voltage Inc., MJ30P400, High Bridge, NJ), a custom-built control system to apply current across the cell, and two platinum electrodes connected to the cathode and anode ends of the capillary while placed into respective buffer vials. This customized CE system allows quick adjustment of many parameters including total capillary lengths, effective capillary lengths, applied voltage and current. A few external CE parameters were examined for optimal detection and separation of nicotine and cotinine as discussed below.
3.3 RESULTS AND DISCUSSION

Interfacing a CE system to the F-DFWM detector offers many advantages and it is important to understand the effects of a few major CE specific parameters in order to take full advantage of the enhancements. First, commonly used solvents were tested to compare their effects on nicotine optical absorbance in the UV-region, as shown in Figure 3.7. There were no drastic differences observed between each solvent. Similar observations were made for cotinine as shown in Figure 3.8, where absorbance of nicotine and cotinine were shown in each solvent at $\lambda_{\text{max}}$. Water, methanol and acetonitrile are most commonly used solvents in many conventional analytical methods, and any of these common solvents can be used in a CE system interfaced to an F-DFWM detector.

In our setup, sodium tetraborate and sodium phosphate were selected for CE analysis since these two sodium-based solvents are commonly used in CE (among many other sodium
and ammonium based solvents). There are numerous publications that reported the effects of CE parameters on separation efficiency of many small molecules including nicotine and cotinine.\textsuperscript{10,11,17,18}

Figure 3.7. UV-visible spectra of nicotine (~10\textsuperscript{-4} M) in various common solvents (Wcetonitrile: water, 70:30; Acetonitrile:Methanol, 50:50).
Capillary electrophoresis is a separation method commonly used for small analytes and biomolecules (e.g., proteins). The most basic CE mode, also known as capillary zone electrophoresis (CZE), uses a voltage gradient as the driving force for the separation (Figure 3.9). The charged analytes are dissolved in an electrolyte and subjected to a voltage gradient that causes them to move toward the electrode carrying the opposite charge. Electroosmotic flow (EOF) is a product of electrophoretic media in a silica-coated capillary that moves toward the cathode end. A separation occurs when cations and anions resulted in a move in opposite directions and/or when there is a distinction in the rates of migration of similarly charged ions (Figure 3.10). The CE separation is recorded in an electropherogram presenting signal intensity with respect to time. As shown in Figure 3.11, each peak represents an analyte with a unique migration time according to an elution order (small highly charged
cations first to small highly charged anions last). The signal intensity of an analyte is affected by the solvent used, applied voltage and analyte concentration.\textsuperscript{11}

![Diagram of capillary zone electrophoresis](image)

**Figure 3.9.** Schematic of capillary zone electrophoresis.

![Diagram showing CE separation](image)

**Figure 3.10.** CE separation of charged analytes with respect to their ion size and charge.
3.3.1 Selecting Background Electrolytes

As stated in the experimental section of this chapter, a polyimide fused-silica capillary (75 μm i.d., 30 cm long) was used. By utilizing a small-sized capillary, Joule’s heating at high current levels is minimized. The amount of heat released is proportional to the square of the current (also known as Ohmic heating) and it can be expressed as:

\[ H \propto I^2 R t \]  

(3.1)

\[ P = I \times V \]  

(3.2)

Where \( H \) is the Joule’s heating, \( I \) is the current, \( R \) is the resistance, \( t \) is the temperature, \( P \) is the energy, and \( V \) is the applied voltage. To avoid Joule’s heating, higher voltage and lower current levels (higher resistance) are used when possible. The Ohm’s plot shown in Figure 3.12 compares the stability of the running buffers with the appropriate concentration and applied voltage levels. The applied voltage affects the current level. For our custom built CE system, we use an applied voltage level that produces 40 μA or lower current levels. Higher current levels cause instability during CE runs, resulting in higher noise levels and even current drops. To minimize or avoid Joule’s heating, 50 mM sodium tetraborate was initially selected for our CE system.
3.3.2 Sample-Stacking CE Mode

Figure 3.13 examines the stability of each buffer at a higher concentration level as compared to that of a solvent concentration level for the analytes (Figure 3.7; 3.8). There is an additional factor of sample stacking one must consider in order to optimize the separation efficiency. Unlike in capillary zone electrophoresis (CZE), sample-stacking CE uses an analyte solvent with a lower ionic strength (lower concentration) than that of the running buffer. The resulting difference in the mobility of the charged ions and the running buffer (background electrolyte) forms a narrowly stacked region when a voltage is applied, as shown in Figure 3.13. As a result, the separation resolution and peak sensitivity are enhanced. 17
Figure 3.13. Schematic of sample-stacking CE separation.

The capillary is treated with 0.1 M sodium hydroxide, water and an appropriate running buffer for 10 minutes, in this respective order, using a peristaltic pump. Figure 3.14 were obtained using our DFWM-CE system. Figure 3.14 shows the electropherogram obtained (S/N of 6) using the following CE conditions: 25 mM sodium tetraborate buffer at pH 9 as the analyte solvent, 3 second injection time at +10 kV applied voltage, 75 μm i.d., 30 cm total capillary length, 15 cm effective capillary length, and 50 mM sodium tetraborate at pH 9 as the running buffer. Because the concentration of nicotine was too high, we observed
some saturation problems. S/N was determined using the peak height (signal) and the standard deviation of the baseline (noise).

![Electropherogram](image)

**Figure 3.14.** Electropherogram of 4.3 mM nicotine tartrate (corrected nicotine concentration of 0.702 mg/mL) in 25 mM sodium tetraborate buffer at pH 9. Injection time: 3 sec at +10 kV applied voltage. Capillary dimensions: 75 μm i.d., 30 cm total length and 15 cm effective length. Running buffer: 50 mM sodium tetraborate at pH 9.

Figure 3.15 shows the electropherogram (S/N of 4) collected after the applied voltage was changed from +10 kV to +16 kV while keeping the other CE parameters the same as described above. The elution time deviated only 5 seconds longer compared to Figure 3.14 because of the slight change in the current output. Figure 3.16 compares individual electropherograms of nicotine and cotinine with a migration time difference of 3 s. Despite a slightly higher S/N observed for cotinine, there was a lack of peak separation.
Figure 3.15. Electropherogram of 2 mM nicotine tartrate (corrected nicotine concentration of 0.39 mg/mL) in 25 mM sodium tetraborate buffer at pH 9. Injection time: 3 sec at +16 kV applied voltage. Capillary dimensions: 75 μm i.d., 30 cm total length and 15 cm effective length. Running buffer: 50 mM sodium tetraborate at pH 9 (1:2 sample stacking).
Figure 3.16. Electropherograms of 2 mM nicotine (bottom) and 7 mM cotinine (top) in 25 mM sodium tetraborate buffer at pH 9. Injection time: 3 sec at +16 kV applied voltage. Capillary dimensions: 75 μm i.d., 30 cm total length and 15 cm effective length. Running buffer: 50 mM sodium tetraborate at pH 9 (1:2 sample stacking).

Figure 3.17 shows a well resolved electropherogram (S/N of 1132) using the following CE conditions: mixture of 3 mM sodium tetraborate and 1 mM sodium phosphate at pH 9 as the analyte solvent, 5 second injection time with +6 kV applied voltage, capillary dimensions of 75 μm i.d., 30 cm total length and 15 cm effective length, and mixture of 30 mM sodium tetraborate and 10 mM sodium phosphate at pH 9 as the running buffer. The migration times almost doubled for both nicotine and cotinine (Figure 3.18) when using 1:10
stacking with the mixture of sodium tetraborate and sodium phosphate. However, the peaks are much better resolved with higher S/N.

Figure 3.17. An electropherogram of 3 mM nicotine tartrate (corrected nicotine concentration of 0.63 mg/mL) in a 3 mM sodium tetraborate + 1 mM sodium phosphate buffer at pH 9. Injection time: 5 sec at +6 kV applied voltage. Capillary dimensions: 75 μm I.D., 30 cm total length and 15 effective. Running buffer: 30 mM Sodium Tetraborate +10 mM Sodium Phosphate at pH 9 (1:10 sample stacking).

Figure 3.18 compares electropherograms for nicotine, cotinine and a mixture of nicotine and cotinine. The nicotine and cotinine individual electropherograms can be used as internal standards to verify those in the mixture. Figure 3.19 simply shows reproducibility of CE separation using optimal conditions. Table 3.1 summarizes and compares the recovery and relative standard deviation (RSD) % of each CE condition used.
Figure 3.18. *Top:* 3 mM nicotine tartrate; *Middle:* 7 mM cotinine; *Bottom:* Mixture - in a 3 mM sodium tetraborate + 1 mM sodium phosphate buffer at pH 9. Injection time: 5 sec at +6 kV applied voltage. Capillary dimensions: 75 μm i.d., 30 cm total length and 15 effective. *Running Buffer:* 30 mM Sodium Tetraborate +10 mM Sodium Phosphate at pH 9.
Figure 3.19. Separation of nicotine and cotinine with excellent S/N using optimal CE conditions.

Table 3.1 Sensitive Levels of Detection of (-)-Nicotine and (-)-Cotinine Using CE-DFWM

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Mole Sensitive Levels</th>
<th>Migration Time (min)</th>
<th>Peak Height</th>
<th>Peak Height RSD % (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-Nicotine</td>
<td>$1.56 \times 10^{-13}$ mol</td>
<td>2.08</td>
<td>3.7</td>
<td>3.4</td>
</tr>
<tr>
<td>(-)-Cotinine</td>
<td>$2.72 \times 10^{-13}$ mol</td>
<td>2.55</td>
<td>4.45</td>
<td>3.1</td>
</tr>
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</table>

The concentration sensitive levels for nicotine and cotinine were determined to be 3 mM (0.38 mg/mL) and 4.94 mM, respectively. Since 1.1 mm diameter of our 266 nm UV laser beam yields a laser probe volume of 55 pL, the following mass sensitive levels were
determined: 1.56 x 10^{-13} \text{ mol or 25 pg for nicotine, and 2.72 x 10^{-13} mol or 48 pg for cotinine.} Table 3.1 compares nicotine and cotinine detection and separation data using our DFWM detector interfaced to a sample stacking CE system.

**3.4 Conclusion**

The results obtained from our CE-DFWM system promise great potential for sensitive detection and high-resolution separation of nicotine and cotinine. UV laser-based wave mixing offers fast label-free native detection and separation with minimal sample preparation when interfaced to a sample-stacking CE system. CE-DFWM offers significant advantages including compact portable detection configurations, small probe volumes (pL), excellent detection sensitivity, high S/N, and coherent laser-like signal beams. Our custom CE system allows modular flexibility when interfaced to the wave-mixing setup and easy adjustment of CE parameters. Our detection sensitivity levels could still be further enhanced by using higher laser power levels.
CHAPTER 4

DETECTION OF NICOTINE AND COTININE COMPLEXES BY DEGENERATE FOUR-WAVE MIXING SPECTROSCOPY

4.1 INTRODUCTION

Chapter 3 discusses a label-free native detection and separation method for nicotine and cotinine. Low extinction coefficients make it challenging to generate a strong signal at trace-concentration levels. The alternative to utilizing a UV laser for native label-free detection is to use a two-reagent nicotine reaction with potassium permanganate in sodium hydroxide to shift the absorption peaks from the UV region to the visible region so that one could use a visible laser to probe the analytes. The reaction was further analyzed with standard cotinine due to its structural similarities to nicotine using a UV spectrophotometer. Similar to the referenced nicotine reaction, the effects of reagents’ concentration were examined when the reaction was applied to cotinine. Since the CE separation parameters were established as described in Chapter 3, this chapter focuses on improving the detection sensitivity level for each analyte using a capillary cell in the continuous-flow mode. The absorbance shift permits utilizing a simple visible laser source instead of a bigger UV laser. Furthermore, many small molecules absorb in the UV region, and the reagents enhance chemical specificity level even when using a continuous-flow detection mode instead of CE-based detection mode.

4.2 EXPERIMENTAL SECTION

4.2.1 Chemicals

(-)-Nicotine tartrate, (-)-cotinine standard, Coomassie Blue (for wave-mixing optical alignment), and methanol were purchased from Thermo Fisher Scientific (Waltham, MA).
Other chemicals including sodium hydroxide and potassium permanganate were purchased from Sigma-Aldrich (St. Louis, MO). All of the chemicals used were up to analytical grade standards.

### 4.2.2 Laser Wave-Mixing Continuous-Flow Detection

The F-DFWM configuration is the same as discussed in Chapter 3. A Helium-Neon laser (Uniphase, Milpitas, CA, Model 1125-P) was used as the light source (17 mW, 633 nm). The input beams were focused into a capillary cell as shown in Figure 4.1, using a 10 cm focusing lens. The capillary cell (100 μm i.d., 360 μm o.d.) was used to flow the analytes using a peristaltic pump (Rainin Instruments, Oakland, CA). Coomassie Brilliant Blue was used as an optical alignment dye for the DFWM setup due to its high extinction coefficient.

![Figure 4.1. Continuous-flow capillary DFWM detection.](image)

Figure 4.2 shows confirmation of continuous-flow wave-mixing detection using a 100 μm i.d., 360 μm o.d. capillary for Coomassie Blue dye at the sub μM level. The signal is
confirmed by blocking one of the input beams alternatively in front of the capillary cell. From 5-10 seconds, chopped and unchopped input beams are blocked; from 10-20 seconds, both input beams are unblocked; from 20-25 seconds, the unchopped beam is blocked; from 25-30 seconds, both input beams are unblocked; and finally from 35-40 seconds, the chopped input beam is blocked. Baseline noise levels of the chopped beam provide a reference for the baseline noise level for the unchopped beam (one of the input beams is chopped by an optical modulator at the desired frequency).

![Graph showing signal intensity changes over time](image)

**Figure 4.2.** Continuous-flow wave-mixing detection of $5.0 \times 10^{-7}$ M Coomassie Blue in methanol using a 100 μm i.d., 360 μm o.d. capillary.

The signal intensity is optimized by fine tuning the spatial alignment of the wave-mixing setup. For trace-concentration analytes, a photodetector and a lock-in amplifier are utilized to optimize the signal intensity while minimizing the noise levels by using apertures and beam blockers. The wave-mixing alignment is tested and optimized daily before analyzing target samples. As the wave-mixing signal equation (Eq. 2.4) indicates, the signal
intensity has a quadratic dependence on analyte concentration. The relationship was experimentally confirmed by graphing signal intensity vs. analyte concentration in a log-log plot with an expected slope of 2. Figure 4.3 confirmed this dependency ($y = 1.7593x + 5.2387$ and $R^2$ value of 0.99743) for Coomassie Blue dye for a range of concentration values (2.5 mM – 0.25 mM) tested. The experimental slope was only 1.76 (instead of the theoretical value of 2.0) due to some background noise level.

![Figure 4.3. Quadratic dependence of the wave-mixing signal on analyte concentration (molar absorptivity).](image)

### 4.3 Results and Discussion

#### 4.3.1 Reaction Analysis

A few reactions have been reported by others that showed optical absorption shift of nicotine and cotinine to the visible wavelength range. The nicotine reaction, utilizing leuco crystal violet and potassium iodide, lacked in clarity for color transformation specific to nicotine. The reaction involved bromination of nicotine to form dibromo nicotine which
reacted with potassium iodide, liberating iodine and lastly, selectively oxidizing leuco crystal violet to from crystal violet.\textsuperscript{31} The modified König reaction, using harmful reagents such as cyanogen bromide and aniline in acidic condition, lacks the stability of the nicotine and cotinine derivatives for more than an hour.\textsuperscript{10} These reactions also lack the ability to increase the extinction coefficients. Hence, we chose the reaction with potassium permanganate in sodium hydroxide since it offers quick sample preparation, increased extinction coefficients and better detection sensitivity levels.

The reaction involves oxidation of nicotine by potassium permanganate in an alkaline medium (sodium hydroxide) to form nicotinic acid complexing (green product). Zaafarany’s in depth kinetics results showed that the reaction is first-order dependence with respect to permanganate and fractional-first order dependence with respect to concentration of nicotine. Furthermore, the paper reported inverse first-order reaction rates with respect to hydrogen ion concentration.\textsuperscript{32} Thus, the oxidative rates increased as the hydrogen ion concentration decreased. Due to the high oxidative properties of permanganate, the reaction behaves similarly with other pyridine groups. The specificity of detecting nicotine can still be determined by coupling CE with DFWM to isolate nicotine from other possible impurities that potentially reacts similarly with the reagents.

Figure 4.4 shows a visible color change from a clear label-free nicotine/cotinine standard solution in water to a pink solution after adding a mixture of two reagents, and then to a green product after a few minutes of heating. Figure 4.5 displays the UV-visible absorption spectra of individual reagents and those after different reagents were added. The UV-visible spectra also display absorption shifts from the UV wavelength region to 500 nm visible region (specific for potassium permanganate oxidizing (-)-nicotine tartrate), and to 600 nm (specific for oxidizing cotinine with potassium permanganate in basic sodium hydroxide solution). Based on these results, we chose a 633 nm laser for the wave-mixing detection setup. Some minor absorption of the potassium permanganate and sodium hydroxide mixture at 633 nm can be subtracted from the total absorbance of the reaction to determine the net absorbance of nicotine.
Figure 4.4. Left: Label-free nicotine and cotinine in water; Middle: KMnO₄ + NaOH + nicotine/cotinine before heating; Right: KMnO₄ + NaOH + nicotine/cotinine after heating for 10 minutes at 100°C.

Figure 4.5. UV-visible spectra of different reacting solutions: a) KMnO₄, b) KMnO₄ + nicotine, c) KMnO₄ + NaOH, and d) KMnO₄ + NaOH + nicotine. The concentration levels used were: potassium permanganate (5 ✴ 10⁻⁴ M), sodium hydroxide (0.5 M) and nicotine tartrate (10 μM). Corrected nicotine concentration of 2.39 μg/ml after heating for 10 minutes at 100°C.

Figure 4.6 displays wavelength shifts that occur from heating for 10 minutes each of the combination (KMnO₄, KMnO₄+ NaOH, KMnO₄ + Nicotine, and KMnO₄ + NaOH +
Nicotine). The major UV-visible absorption shift is observed for KMnO₄ + Nicotine and KMnO₄ + NaOH + Nicotine. However, KMnO₄ + Nicotine solution only displays the decrease in absorbance in the 250-500 nm regions without any specific shift for nicotine. Hence, the UV-visible absorption studies clarified that the color change (absorbance shift) from the potassium permanganate and sodium hydroxide reaction is specific to nicotine (10 μM).

![Figure 4.6](image)

**Figure 4.6.** a) Potassium permanganate, b) potassium permanganate and sodium hydroxide; c) potassium permanganate and nicotine tartrate (10 μM), and d) potassium permanganate, sodium hydroxide and nicotine tartrate (10 μM) before and after heating for 10 minutes at 100°C.
When the reaction was tested with a much higher nicotine concentration level, the color change was obtained instantaneously without any heating. Figure 4.7 compares UV-visible absorption spectra for the reaction with 25 μg/mL of nicotine without heating and 2.1 μg/mL nicotine with 10 minutes of heating at 100 °C to reach approximately the same absorbance around 600 nm. However, the instantaneous reaction with a higher nicotine concentration level resulted in quick brown precipitation. As a result, we decided to utilize lower concentration levels (10 μg/ml or lower) of nicotine for the reaction.

![Figure 4.7. Nicotine concentration dependence on the reaction.](image)

### 4.3.1.1 Cotinine Reaction Similarity

The same reaction scheme was applied to (-)-cotinine standards and examined for similar reactivity as that described for nicotine above, and the effects of each reagent’s concentration were examined. Figure 4.8 shows the effect of sodium hydroxide from 0.5 M to 2 M while keeping the other factors constant. Similarly, the figure suggested that 0.5M of
sodium hydroxide is the best concentration level that can be used to obtain optimal absorbance for the reaction.

Figure 4.8. Sodium hydroxide concentration effect on the cotinine reaction using cotinine (11 μg/mL) and KMnO₄ (5 x 10⁻⁴ M), heating for 10 minutes at 100°C.

Figure 4.9 shows the effect of potassium permanganate concentration ranging from 5 x 10⁻⁵ to 6 x 10⁻⁴ M, indicating similar results with the best absorbance for 5x10⁻⁴ M of potassium permanganate without any saturation. Figure 4.10 shows overall change in absorbance for both complexes over a 4-day period. Prior to obtaining optical absorption spectra each day, the solutions were filtered to avoid any precipitation that could affect the measurements. After 4 days, the solutions precipitated to brown solutions. The linear increase in absorbance is observed for both complexes. As a result, it allowed using the solutions over a couple-days period without compromising in absorbance. However, the increase in absorbance can be further studied to understand the reasoning for what causes the linear increase instead of remaining constant.
Figure 4.9. Potassium hydroxide concentration effect on the cotinine reaction using cotinine (11 μg/mL) and NaOH (0.5M), heating for 10 minutes at 100°C.

Figure 4.10. Stability of the reaction over a 4-day period.
Figure 4.11 compares UV-visible absorption spectra of nicotine and cotinine standards and their complexes using $5 \times 10^{-4}$ M of potassium permanganate and 0.5 M of sodium hydroxide. Figure 4.11.a shows UV-visible absorption spectra of nicotine (0.324 mM) and cotinine (0.567 mM) in water, and Figure 4.11.b shows UV-visible absorption spectra after mixing KMnO$_4$, NaOH, and nicotine or cotinine. Using $2.011 \times 10^{-5}$ M of nicotine tartrate, an adjusted nicotine concentration of 3.26 μg/mL was determined, and similarly $6.58 \times 10^{-5}$ M (or 11.6 μg/mL) for cotinine, in the reaction after approximately heating for 10 minutes in a water bath (100° C). The figure not only shows the $\lambda_{\text{max}}$ shift from the UV region (260 nm) to the visible region (600 nm) but also the increase in absorbance when using complexing agents. Based on Beer's Law ($A = \varepsilon b C$), the extinction coefficients of standard nicotine and cotinine are determined to be $5,900 \text{ M}^{-1}\text{cm}^{-1}$ and $3700 \text{ M}^{-1}\text{cm}^{-1}$. The complexing agents enhanced the extinction coefficient of nicotine and cotinine to $39,780 \text{ cm}^{-1}\text{M}^{-1}$ and $12,370 \text{ cm}^{-1}\text{M}^{-1}$ at $\lambda_{\text{max}}$, accordingly. These significant enhancements in molar absorptivity for nicotine and cotinine complexes in the visible region offer great potential for sensitive detection limits for continuous-flow wave-mixing detection.

![Figure 4.11](image-url)
4.3.2 Continuous-Flow Wave-Mixing Detection

Initially, the wave-mixing signal was not successfully obtained using a 75 μm i.d. capillary because the capillary was too small for the probe laser beam, resulting in stray light and weak signal levels. The problem was resolved when using a 100 μm i.d. capillary. The capillary was treated first by rinsing it with methanol and then with water for 5 minutes each. Because the main solvent of the reaction was water with a trace amount of sodium hydroxide, water was used to rinse the capillary cells. Rinsing with methanol alone resulted in the precipitation inside the capillary, resulting in inconsistent signal levels.

Figure 4.12 shows excellent continuous-flow wave-mixing signal levels for nicotine (20 μM) using a 100 μm i.d. capillary and a 17 mW 633 nm He-Ne laser. The concentration detection sensitivity level for nicotine was determined to be 2.01 μM at S/N of 22. This corresponds to a mass detection sensitivity level of 2.83 x 10^{-16} mol or 46 fg (based on the 140 pL probe volume). Similarly, a concentration detection sensitivity level of 6.58 x 10^{-5} M was determined for cotinine, as shown in Figure 4.13, and a mass detection sensitivity level of 9.28 x 10^{-15} mol or 1.6 pg was determined for cotinine. Based on the nonlinear dependence of the concentration on a signal, at S/N of 2, the concentration detection limit of 16.7 nM (2.69 ng/ mL) and 0.544 μM (544 ng/ mL) were calculated for nicotine and cotinine, respectively. Mole /mass detection limits of 2.34 x 10^{-18} mol (0.38 fg and 7.6 x 10^{-17} mol (13 fg) were determined for nicotine and cotinine, accordingly.
Figure 4.12. Continuous-flow wave-mixing signal of nicotine (2.05 x 10^{-5} M, 3.26 μg/mL) using a 100 μm i.d. capillary cell.
4.4 CONCLUSION

This chapter describes a successful demonstration of detection sensitivity enhancement by utilizing a quick two-reagent reaction to elevate molar absorptivity levels in the visible wavelength range. Optimal conditions were determined using a UV-visible spectrometer for different complexing agents for both nicotine and cotinine. The calculated LOD for nicotine is 10 folds more sensitive than the value reported in the spectroscopic studies of referenced reaction as shown in Table 4.1. However, the detected concentration levels of 0.32 μg/mL for nicotine needed to improve by approximately 100 fold to be comparable to reported values for currently available analytical methods. Our excellent detection limits can be further enhanced by using higher laser power levels (e.g., compact and solid-state 635 nm laser) for nicotine and cotinine. These and other enhancement works are in progress.
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<tr>
<th>Analyte</th>
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<td>80</td>
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<td>(-) - Nicotine</td>
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<tr>
<td>(-) - Cotinine</td>
<td>544</td>
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REFERENCES


