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Development of a Simple Method to Detect and Quantify Benzoylecgonine, a Cocaine Metabolite, in Urine

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Abstract

Benzoylecgonine (BE) is the most abundant metabolite of cocaine found in the human body. Analysis of BE in urine by gas chromatography/mass spectrometry is the most common method currently used to detect cocaine abuse. This current method is costly and time consuming, so the investigation of an easier and more cost-effective approach is the goal of this research. Because BE is a zwitterion, it is highly soluble in water and very difficult to extract from urine. Once the pK_a values of BE are determined, either the cation or anion form of BE can be formed from the zwitterion by adjusting the pH appropriately. When BE is in a cationic or anionic state, it can be ion-paired with an appropriate counter-ion to form a neutral ion pair. This ion pair can then be extracted into a non-polar solvent, concentrated, and quantitatively determined by UV-Vis spectroscopy.

The following portions of this project have been completed: BE was synthesized, characterized, and purity was verified. Preliminary UV-Vis spectra of BE were measured to determine absorption bands of BE in various solvents. Based on these preliminary UV-Vis spectra, dichloromethane was determined to be the best organic extracting agent. Next, pK_a values were experimentally determined, with a pK_{a1} of 2.15 +/- .01 and a pK_{a2} of 11.41 +/- .01. The pH of the BE zwitterion solution was adjusted according to these values, and ion pairings and extractions were performed using various ions. Many ion pairs failed to give clear results. There has been some initial success, however, with the ion pair formed between BE and Dragendorf reagent, BiI_4^-. This ion pair was tested and data gathered in order to generate a clear calibration curve.

Introduction

Benzoylmethylecgonine, commonly known as cocaine, is the major alkaloid of *Erythroxylum coca*. This psychotropic drug has a long history of human use and, unfortunately, human abuse. Over 4000 years ago, people chewed coca leaves. In the early 1900s, cocaine was used as an ingredient in tonics and sodas[1,2,3]. Around this time, cocaine use soared and was considered safe for use by most clinicians, including Sigmund Freud. After its dangers became known more clearly, the use declined. Cocaine abuse seems to have a cyclic pattern with increasing use followed by decreasing use once dangers are recognized[4].

This cyclic pattern was repeated in the 1950s, 1960s, and again in the 1980s. In the 1980s though, cocaine was once again considered safe by abusers; it was a harmless, nonaddicting euphoriant. In this era, it was considered similar to marijuana and addiction to cocaine was dismissed. The rate of cocaine use at this time exploded. It has continued to be one of the more heavily used drugs up to this time. Currently the United States, as well as the world as a whole, is in its largest cocaine epidemic in history. One to three million people are estimated to be chronic cocaine abusers in this country alone[4]. It is thought that over 23 million people in America have tried cocaine[5].

Due to the large availability of cocaine across the country, the economic cost of its abuse is climbing. According to the National Clearinghouse for Alcohol and Drug Abuse, drug abuse costs for all drugs (resulting from health, crime, and other problems) totaled $97.7 billion dollars in 1992, and it rose to $109.8 billion in 1995. This was half of the Department of Defense budget in that year. These numbers emphasize the magnitude of the drug abuse problem. There are also many noneconomic consequences

of serious drug abuse. Illness, crime, domestic violence, reduced productivity, and a lost opportunity result often from drug abuse. Cocaine related emergency episodes totaled over 142,000 cases in 1995[6].

Cocaine is a serious problem because of its easy availability and easy use. Cocaine is introduced into the body in various ways, many of which have arisen in the last 30 years. It can be introduced by intranasal entry ("snorting"), by intravenous entry (injecting), oral ingestion, or smoking the free base form commonly referred to as "crack." These methods are all equally addictive. Smoking and snorting have the added "bonus" of the same effects without the danger of infection from intravenous injection. Cocaine has instantaneous and overpowering effects, which enhances its popularity. From a study of cocaine abusers, it was reported that it generally takes about 2 to 4 years from the first use for a serious addiction to develop. This extended period of time before chronic addiction often encourages the old idea that cocaine is harmless[1,2,3,4].

Cocaine's fundamental effect is the magnification of intensity of many of the body's normal pleasures. The surrounding environment becomes intensified without becoming distorted. Cocaine use enhances emotional feelings and self-confidence. Self-perception of mastery is also enhanced, while at the same time, anxiety is initially decreased. Feelings of social inhibition are reduced and feelings of interpersonal communication are aided. Appetite is suppressed so feelings associated with eating are not enhanced[4].

Cocaine addiction is believed to be a physiologic disorder. There is a need for some form of medical intervention to treat the abuse, dependence and addiction. These disease states are used to classify the person's need for the drug. Abuse is a level where

the drug use begins creating problems for the individual but control is still maintained. In addiction, the individual loses control. Drug dependence is a chronic disease in which relapse occurs frequently in abusers, which occurs after addiction begins[6]. In order to understand the problems associated with cocaine and its abuse, it is necessary to consider its biological basis.

Neurochemically, cocaine acts differently than other drugs of abuse such as heroin, nicotine, THC(marijuana), and PCP. Unlike the others, it does not act as a neurotransmitter receptor. Instead, cocaine, when abused, acts by blocking the re-uptake of norepinephrine, dopamine, and serotonin, which are monoamines associated with memory function in the brain. The dopamine transporter is a key target or receptor for the effects of cocaine, which is thought to result in the reinforcing effects of the drug. It is thought that the cocaine blocks the dopamine transporter stopping the reuptake of dopamine. Cocaine also acts as an indirect dopamine agonist. This results in build-up of dopamine and other monoamines in the synapses. The high concentrations of monoamines, especially dopamine, in the synapses result in the sought after effects: an increased sense of alertness, well-being, and euphoria. Unfortunately for the abuser, when cocaine is passed from the system, the concentration of monoamines in the synapses drops dramatically. Monoamine depletion in the presynapses results in what is referred to as a "crash" syndrome, or feelings of depression and physical discomfort. The only way to escape the "crash" feelings is to ingest more cocaine. This continues a vicious cycle of addiction: as abusers begin to "crash," they intake more and more cocaine[2,6].

Once a user decides to stop abusing cocaine, a triphasic pattern of symptoms from abstinence results. Phase one is the crash phase. It is when the euphoric mood and energy vanish. Depression, anxiety, agitation, and a craving for cocaine intensify. Some people experience suspicion and paranoia. A craving for sleep next occurs. After sleep commences, the person experiences intense hypersomnolence, with electroencephalographic changes characteristic of sleep deprivation. Hyperphagia can occur in brief periods of awakenings.

Phase two is the withdrawal stage. When a person becomes addicted to cocaine, a protracted dysphoric syndrome, which includes decreased activity, causes its continued abuse, anxiety, lack of motivation, and boredom. There is markedly decreased intensity of normal pleasurable experiences, or anhedonia that emerges after the crash in the withdrawal phase. Severe cocaine cravings occur from the memories of cocaine euphoria[4].

Cocaine is one of the most powerful reinforcing agents because it produces intense classical and operant conditioning. Events previously paired with cocaine use induce cravings. These events are experienced as partial memories of cocaine euphoria. Many things can cause this triggering. They include, but are not limited to, mood states (both positive and negative), specific people, locations, events, times of the year, alcohol use, personal problems previously eased by cocaine abuse, and also tangible items previously associated with the abuse such as syringes, money, and pipes. Finally, stage three is extinction of constant cravings, but the individual is not free from addiction. Cravings can still emerge years after the last abuse[4].

Along with this crash come other dangers. There are many commonly observed toxic side effects of cocaine. Cardiotoxicity is the most frequently observed, but central nervous system sensory disorders, pulmonary toxicity, and hepatotoxicity can also arise[3]. Cocaine abuse is also currently associated with the increasing AIDS epidemic in the country, due to needle sharing and sexual promiscuity associated with drugs of abuse[5]. Cocaine is similar to amphetamines in that it is distributed throughout the entire body quickly and is then cleared very rapidly. This rapid clearing had led many researchers to feel that one of the metabolites of cocaine may be causing the toxic effects and research is being conducted to establish which one exactly[3].

The horrible epidemic of cocaine abuse is spreading through the country. Although there has been extensive research in this area, there have been no developments of effective therapies for the craving associated with cocaine addiction. To begin isolating the problems of cocaine abuse, it is first necessary to find a way to detect the presence of cocaine in human abusers[5]. The detection of drugs, in this case cocaine and its metabolites, in biological tissues and fluids or media is an important component in clinical diagnosis, forensic testing, pharmacological research, and drug discovery[7]. To begin work with extracting cocaine or its metabolites from any bodily fluid or media, many factors need to be considered.

There are many metabolites of cocaine (Fig.1). In 1990, Zhang *et al.* reported the finding of 11 metabolites, including four previously unreported[8]. Four major metabolites of cocaine are easily recognizable and detectable and are formed in two distinct biochemical processes: hydrolysis and oxidation. Enzymes within the body play important roles in degrading cocaine to the metabolites. Cocaine (**A**) in Figure 1 is

oxidized by specific cytochromes P450 (CYPs) and N-demethylated to form norcocaine (**B**). It can be further oxidized to N-hydroxy cocaine (**C**), a low probability metabolite, by oxidative metabolism. Benzoylecgonine (**D**) and ecgonine methyl ester (**E**) are formed from the ester cleavage/hydrolysis of cocaine by serum cholinesterases and liver (hepatic) carboxylesterases. Further hydrolysis of these leads to the formation of ecgonine (**F**), another major metabolite. Around 86-90% of an administered dose of cocaine is recovered in urine as cocaine or one of its metabolites. Only about 1-5% of this is actually cocaine. Toxicological and analytical interest in benzoylecgonine and ecgonine methyl ester arises from their long half-lives; in the case of benzoylecgonine, it is six-times longer than that of cocaine, or around 5-8 hours. Benzoylecgonine is one of the main degradation products, reported at 46% of the excreted metabolites, making it of great toxicological and analytical interest[2,3].

The study of benzoylecgonine is complicated by its physical properties. Its hydrophilic nature renders it very difficult to extract from urine into organic solvents. The total charge of BE, controlled by pH, allows for some control over the form of BE that is present in the solution. Figure 2 illustrates the various forms of benzoylecgonine that occur when pH is changed.

The current method of detection used by laboratories that provide drug-testing services to employers is often a two level approach. The urine of test subjects is screened with an immunoassay to determine the difference between negative and positive samples. The initial immunoassays do have a significant false positive rate, so positive samples are then analyzed by gas chromatography- mass spectrometry, or GC-MS[9]. This method provides scientists with a qualitative and quantitative result.

A problem with quantitative GC-MS methods is the necessity of sample cleanup, because the urine must be purged of excess ions and contaminants that would alter or influence detection of cocaine and its metabolites. Cleanup is then followed by a quantitative derivatization to make the sample more volatile. All of this must occur before the sample is introduced into the GC-MS. This is necessary due to the fact that GC-MS utilizes a sample's volatility for separation and benzoylecgonine is virtually non-volatile. In an effort to avoid the problems of this expensive and time consuming derivatization, researchers have been attempting to find more cost-effective and time saving ways to detect and quantify benzoylecgonine in urine. Other available methods of detection include, but are not limited to, high-performance liquid chromatography with ultraviolet (UV) or fluorescence detection, time-of flight (TOF), MS and liquid chromatography(LC)/MS. There are other analytical methods used for semi-quantitative screening processes such as enzyme-multiplied immunoassay, radioimmunoassay, thin-layer chromatography (TLC), and fluorescence-polarization immunoassay[3].

Because urine analysis is the most effective means in the routine detection of cocaine, Wallace *et al.* (1975) used thin-layer chromatography (TLC) to provide a rapid and inexpensive method to screen for cocaine in urine. The report described a screening technique that could separate users from non-users and that provided a sensitivity level of approximately 0.1 μg/ml for cocaine and 0.25 μg/ml for benzoylecgonine in the analysis of a 5ml urine sample. A simple extraction was performed with a mixed organic solvent to isolate the cocaine and metabolites from the biological medium, urine. It was reported that unchanged cocaine could be extracted from urine with simple organic solvents such as diethyl ether or chloroform. Cocaine and benzoylecgonine can also be extracted with

mixed solvents such as chloroform-isopropanol. Extractions were performed at moderate and at very alkaline pH, where BE is in its anionic form. For the series of extractions, a 20% ethanol in chloroform mixture was utilized for both cocaine and benzoylecgonine. The extraction of BE was enhanced with increasing amounts of ethanol. Wallace *et al.* also found that acid hydrolysis destroys both cocaine and its principle metabolites. They analyzed their extraction efficiency with gas chromatography and found that the recovery was 90-95% for cocaine, and 65-70% for benzoylecgonine. Because the benzoylecgonine metabolite predominates in *in vivo* specimens, they developed optimal chromatographic conditions for its separation and determination. The method of TLC that they used seemed to increase sensitivity of detection well above previous methods[10].

Singh *et al.* (1999) validated a method in which cocaine and all of its metabolites could be detected and quantified in rat plasma. The method used stable isotope dilution liquid chromatography/tandem mass spectrometry, which appeared to have yielded high sensitivity. The method was sensitive enough to allow, for the first time, a detailed *in vivo* study of the pharmacokinetics of cocaine and its metabolites after administration to rats. This method was used to overcome previous problems reported with detection of cocaine and its metabolites in good sensitivity. An initial relationship between cocaine metabolism and toxicity is being established utilizing the results of this study. This technique allowed the separation of ecgonine methyl ester, benzoylecgonine, cocaine, and norcocaine. This was the first experiment where ecgonine methyl ester, a very polar metabolite, was detected in the presence of norcocaine, a relatively nonpolar metabolite. The results of retention times and separation were highly reproducible. With this method, minimal sample workup was needed, and no derivatization was required[3].

Clauwaert *et al.* (1996) used solid phase extractions (utilizing solid sorbents such as octadecylsilica), citing that due to the high hydrophilicity of benzoylecgonine, liquid-liquid extraction solvents required an alcohol to improve extraction efficiency. These alcohols increase the recovery of other metabolites besides benzoylecgonine that are also typically present in urine. This increases chemical background noise causing interfering signals, and reducing the column lifetime. The solid phase extractions this group performed yielded satisfactory results, again with high sensitivity, specificity, and accuracy. Hydrolysis of cocaine and its metabolites during extraction analysis was less than 0.5%. This method can be applied to corroborate cocaine use, establish cocaine overdoses, and to study pharmacological effects of cocaine and its metabolites. Solid phase extractions (SPEs) have become a popular way to analyze cocaine in urine since they give high recovery and clean extracts. SPE, however, shows poor batch-to-batch reproducibility. Internal standards are needed to maintain quality of quantitation[2].

Unfortunately, these methods are still either not quantitative enough, or still very expensive and time consuming. Similar to GC/MS, methodology that is based on HPLC and LC/MS also require heavy sample cleanup in order to obtain reasonable detection limits. Others, such as TOF/MS, are relatively insensitive and subject to heavy background noise from the biological matrix.

In a close study of the previous literature, there is no evidence of the use of ultra-violet/visible spectrophotometry and ion pairing for the detection and quantification of benzoylecgonine or cocaine metabolites. Some evidence of the use of ion pairing is given in Eisman *et al.* (1992). This paper reports the use of ion pairing and an automatic continuous flow method for atomic absorption of the ion-paired metal ion to detect

cocaine and its metabolites. This method permitted the determination of cocaine over a large concentration range. Metal complexes, such as Reinecke's Salt, $\{NH_4[Cr(SCN)_4(NH_3)_2]\}$ and Dragendorf reagent. $[NH_4BiI_4]$, paired with cocaine, and were then extracted into nitrobenzene or 1,2 dichloroethane. One problem with this method is that it does not allow for the identification of the metabolites individually, but only the determination of total cocaine and cocaine metabolites, because they all retain the same basic structure of cocaine. However, this method does allow for the determination of the use of cocaine, with no pretreatment of the sample[1].

It is the purpose of this study to find a new way to detect and quantify the presence of cocaine and its metabolites, particularly benzoylecgonine, in urine using ultra-violet/visible spectrophotometry (UV-Vis). By pairing benzoylecgonine with a metal containing complex ion[1], it would become possible to extract the hydrophilic, polar benzoylecgonine into a non-polar solvent. Initially the wavelength of maximum absorption for the benzoylecgonine ion pair would need to be determined. Using UV-Vis to compare absorption peaks in the solvent before and after extraction, the presence of benzoylecgonine could be detected, which would identify cocaine abuse. In order to quantify the BE in urine, calibrations curves could be generated with known concentrations of benzoylecgonine. The non-polar solvent of choice in this case is dichloromethane, due to cost effectiveness, and an acceptable cut-off wavelength. Multiple ions were tried to determine which would provide the clearest calibration curve with the largest amount of reproducibility.

Experimental

Data Analysis Benzoylecgonine is a zwitterion, so the molecule is polar and therefore hydrophilic. This makes it very difficult to extract from the aqueous urine solution that is used in these studies. By altering the pH of the solution, the benzoylecgonine can be converted to either its anionic or cationic form, depending on the pH to which it is adjusted. There are two pK_a values for benzoylecgonine and titrations were performed to determine both. The pK_a of importance in this study is that which leads to the cationic form, where the amine group is protonated. The pK_a values for BE were determined from the data obtained from the titrations.

By measuring the pH of the benzoylecgonine at every step of the titration, it was possible to know the proton concentration of the solution at each point. It was also necessary to add a specific amount of acid (in this case HCl) to calculate the C_a, or analytical concentration of benzoylecgonine in the acid form and C_b, the analytical concentration of benzoylecgonine in the basic form in the solution. In order to determine the two pK_a values, a series of calculations requiring these values need to be performed. The zwitterion form of BE will be referred to as $[BE\text{-}NH^+\text{-}O^-]$(Figure 2, Structure (B)). The deprotonated form of BE (Figure 2, Structure C) will be referred to as $[BE\text{-}N\text{-}O^-]^-$, and the fully protonated form will be referred to as $[BE\text{-}NH^+\text{-}OH]^+$ (Figure 2, Structure D). When an acid titration is performed, C_b is $[BE\text{-}NH^+\text{-}O^-]$, and C_a is $[BE\text{-}NH^+\text{-}OH]^+$. When a base titration is performed, the C_b is $[BE\text{-}N\text{-}O^-]^-$, while the C_a is $[BE\text{-}NH^+\text{-}O^-]$. From these values, the K_a and also pK_a of the solution could be determined, using the following method.

It is necessary to proceed through a series of equations to eliminate variables. For benzoylecgonine, there is a proton that can be displaced, giving it the acidic form:

$$HBE \rightleftharpoons H^+ + BE^-_{HBE} \qquad \text{(eq.1)}$$

In a buffered BE solution, there is also the presence of another source of BE, which proceeds all the way to completion.

$$NaBE \rightarrow Na^+ + BE^- \qquad \text{(eq. 2)}$$

Water also plays a role in this reaction:

$$H_2O \rightleftharpoons H^+ + OH^- \qquad \text{(eq. 3)}$$

The equilibrium expressions of these reactions are:

$$K_a = \frac{[H^+][BE^-]}{[HBE]} \qquad \text{(eq.4)}$$

$$K_w = [H^+][OH^-] \qquad \text{(eq.5)}$$

The proton balance of this reaction is:

$$[H^+] = [BE^-]_{HBE} + [OH^-] \qquad \text{(eq. 6)}$$

To determine the concentrations of the acidic and basic forms of benzoylecgonine, material balance equations can be written:

$$C_a = [HBE] + [BE^-]_{HBE} \qquad \text{(eq. 7)}$$

$$C_b = [BE^-] - [BE^-]_{HBE} \qquad \text{(eq. 8)}$$

By solving equation 6 for $[BE^-]_{HBE}$, and substituting into equations 7 and 8, the following equations are obtained:

$$C_a = [HBE] + [H^+] - [OH^-] \qquad \text{(eq. 9)}$$

$$C_b = [BE^-] - [H^+] + [OH^-] \qquad \text{(eq. 10)}$$

By rearranging equation 5, and substituting it into equations 9 and 10, one obtains:

$$[HBE] = C_a - [H^+] + K_w/[H^+] \qquad \text{(eq. 11)}$$

$$[BE^-] = C_b + [H^+] - K_w/[H^+] \qquad \text{(eq. 12)}$$

These two equations are then substituted into equation 4 and simplified to yield:

$$K_a = \frac{[H^+](C_b[H^+] + [H^+]^2 - K_w)}{(C_a[H^+] - [H^+]^2 + K_w)} \qquad \text{(eq. 13)}$$

In this equation, the $[H^+]$ can be determined by the pH and the – log of the K_a can be calculated to determine the pK_a needed for the experiment. This gives a pKa for a range of points and the average was taken for the actual pK_a to use[11].

Chemicals and Reagents

Ecgonine methyl ester benzoate (cocaine) hydrochloride and Anhydrous (99.9%) dichloromethane were purchased from Acros Chemical Company.

The following reagents were made following the procedures given in Eisman *et al.* (1992)[1]:

1. Dragendorf reagent: A $Bi(NO_3)_3 \cdot 5H_2O$ (Aldrich) solution was prepared by dissolving 5.0 g in 10 ml of 6 M HNO_3. This was brought to 100 ml with deionized H_2O, the final concentration being 0.103M. A standard KI solution (3.0M) was prepared by dissolving 50 g of KI (Sigma) into 100 ml of deionized H_2O. The carrier solution was prepared by mixing 0.4 ml of the Bi(III) solution, 6.0 ml of standard KI solution, and 10 ml of 0.1M HCl, and bringing to volume with deionized water in a 100 ml volumetric flask. This yielded a solution of 4.0 x 10^{-4}M tetraiodobismuthate(III), BiI_4^-, with a pH of 2.0.
2. Tetrakis(thiocyanato)cobalt(II) reagent: A standard solution of 1M was prepared by dissolving 29.1 g of $Co(NO_3)_2 \cdot 6H_2O$ (Aldrich) and 62 g of NH_4SCN (Aldrich) in 100 ml of deionized water. The carrier solution was made by diluting 50 ml of the 1M standard to 100 ml with deionized water.

3. Hexakis(thiocyanato)iron(III) Solution: A 0.05M carrier solution was prepared by dissolving 9.2 g of NH_4SCN (Aldrich) and 2.1 g of $Fe(NO_3)_3 \cdot 9H_2O$ (Aldrich) in 100 ml of deionized water. The pH was lowered to 1.8 drop wise with concentrated HCl.
4. Reinecke Salt (Ammonium Tetrakis(thiocyanato)diamminechromium(III), $NH_4[Cr(SCN)_4NH_3)_2$.): A 1% w/v solution of Reineke salt (Acros) was made in deionized water.

Synthesis

Benzoylecgonine was synthesized using a variation of the method of Stephen Findlay[12]. Cocaine hydrochloride (4.952g) was combined with excess $NaHCO_3$ solution (250ml). This solution was heated to boiling for approximately 30 minutes and stirred frequently. This formed free base cocaine. The cocaine solution was then combined with equal parts by volume of ether in a large separatory funnel to extract the cocaine into the organic layer. The aqueous layer was then separated and re-extracted a total of 5 times. The organic layer was saved during this time, and then placed on a roto-evaporator until it was dry. To convert cocaine to benzoylecgonine (hydrolysis reaction), the precipitate was dissolved in H_2O and the placed on the rotovap until it was partially dry. The benzoylecgonine precipitated, was filtered, and was further dried over P_2O_5 (3.967g, 80.1%).

Procedure

pK_a determination for benzoylecgonine:

An approximate 0.01M HCl solution was prepared and standardized by titration using phenolphthalein as an indicator. It was titrated against a KHP standardized 0.0135M NaOH solution. The titration was repeated in triplicate and the average of the three trials was used.

The potentiometric (pH) titrations for BE were performed using a 0.003521M benzoylecgonine solution. The BE solution (10ml) was titrated with the standardized HCl using a pH meter placed in the solution. A micropipetter was used to deliver 0.100 ml of HCl and the pH meter was read after each addition. The titrations with HCl were repeated and following accurate replication, the process was repeated with standardized NaOH.

Extraction of benzoylecgonine/ion complex with dichloromethane:

Before calibration curves were generated, many ions pairs were tested to determine if they did indeed pair well with BE, making it easier to be extracted into an organic phase.

A stock solution of benzoylecgonine was prepared by adding 0.4971g of benzoylecgonine to 100ml of deionized water (0.0017M). Standard BE solutions were prepared by serial dilutions. Solutions of 9.942ppm(~10ppm), 0.9942ppm (~1ppm), 29.826ppm(~30ppm), and 2.9826ppm(~3ppm) were prepared.

Initial extractions were performed by the following protocol: 5ml of a BE stock solution was mixed with 5ml of the carrier solution. The pH was lowered to ~2, where BE would be in the cationic form. The BE/ion complex was extracted with 5ml of

dichloromethane. The dichloromethane containing the neutral BE/ion complex was then analyzed using UV-Vis spectrophotometry. Dichloromethane blanks and deionized water blanks were analyzed for comparison with every ion. The aqueous layer was run for one trial before and after extraction to ensure that BE was being extracted. The solution was diluted until readable values were obtained within detection limits of the instrument. The cutoff wavelength of 235nm was used.

An alteration of the previous method was used for Dragendorf reagent. For BiI_4^-, the sample pH of BE was lowered to be 3.0. The carrier, or metal complex ion, pH was adjusted to 2.0. A $4x10^{-4}$M solution of Dragendorf reagent was used[1]. The initial protocol was altered so that only 2 ml of carrier solution was used for ion pairing. The yellow carrier solution was mixed with the clear BE solution and then the extraction was performed. Upon addition of dichloromethane, the solution turned pink. With higher concentrations of BE, the solution appeared more orange.

Results and Discussion

Using IR, NMR, GC/MS, and melting point determination, the synthesized benzoylecgonine was analyzed for purity. The melting point was determined at 140-158°C. No melting point data are available in the literature. The percent yield of benzoylecgonine (3.967g) calculated from the mass of cocaine hydrochloride (4.952g) used was 80.1%.

Infrared spectra obtained were compared to literature spectra[12]. There are similar peaks between the spectra. In Figure 3, the presence of a large carbonyl peak (at1727 cm^{-1} spectrum resembles the data in the literature[12]. Many of the peaks are very close to what are expected in the structure. The peak at 3426 cm^{-1} may result from impurities. It could correspond to a peak from various other metabolites, including N-hydroxy cocaine, which would show an N-OH peak, or norcocaine, which would show an N-H tertiary amine peak. (See Figure 1 for structures). This peak is not obvious in the literature structure, suggesting that there were some impurities in the BE synthesized. This is further supported by the GC data.

Following IR, ^1H NMR spectra were obtained. Figure 4 has peaks for each of the protons in the structure of BE. The two hydrogen peaks from the aromatic ring are located at 7.3 and 7.9 ppm. The large peak at 2.4 ppm corresponds to the three protons located in the $N\text{-}CH_3$ group. The remainder of the protons from the nitrogen-containing ring are located to the right of the large peak at 2.4 ppm in the mass of peaks. The peak labeled H_B has more than one proton, lending more support to impurities in the product. Even with an extensive literature search, no literature spectra have been found. This

makes it much more difficult to analyze correctly and compare the spectra, but provides basis for new explorations in benzoylecgonine.

The ^{13}C NMR, Figure 5, has characteristic carbon peaks for each of the two carbonyls, located at 166 and 173 ppm. It also has peaks at 128-133 ppm (corresponding to aromatic carbons). The alkane peaks occur at 24, 26, 34, 38, 48, and 61 ppm. These are the carbons located in the amine ring. The two peaks at 64 and 65 ppm are the peaks for the N-C and O-C carbons. To identify which of these carbon shifts pertains to specifically which carbon, a coupled ^{13}C NMR would need to be run. In the coupled spectra, the carbon bonded to the nitrogen would show up as a quartet, while the C-O-C would show up as a doublet. This could allow assignment of the exact carbon peak assignments.

GC/MS was used to further verify that the product synthesized was actually BE and not one of the other major metabolites. These spectra identified impurities in the product to be unreacted cocaine, which would not give the peak identified in the IR spectra, but another metabolite would. As visualized by the chromatogram, BE was the primary product in the reaction. The peaks of the mass spectrum can be identified as component parts of BE (Figure 6), with the largest mass-to-charge peak matching that of BE itself. Purity of benzoylecgonine was determined by gas chromatography to be 94.3%. This was an acceptable purity because cocaine metabolites in natural systems are always a mixture of benzoylecgonine and other metabolites. This product resembles that of natural systems more closely due to the impurities. The goal of this study is detection of cocaine and its metabolites; whichever one we detect is still a positive test. No

attempts to purify further were made. Additional purification would have been more helpful for NMR and IR characterization.

Once the BE was correctly synthesized and characterized, the pK_a values were determined by titration. The standardized HCl was 0.0112M as determined by titrations with KHP and standardized NaOH (0.01351M). To isolate BE in an environment where it is a charged molecule, the pK_a values were obtained experimentally by titration with both HCl and NaOH. Other laboratory members performed the series of NaOH titrations. Figure 7, plotted as pH versus equivalents of BE shows the results of those titrations. Equation 13 was used to determine the pK_a. Final values obtained were a pK_{a1}=2.15±0.01 and pK_{a2} =11.41±0.01. The lower pK_a value is utilized in this study, for the reason that below that pK_a value, BE is in its cationic form where the amine group is protonated. This positive ion can then be paired with a negative carrier ion to form the neutral ion pair, which can further be extracted into an organic, non-polar solvent.

In order to determine the solvent that worked best under the conditions of this study, preliminary UV-Visible spectra were obtained to determine absorption bands of BE in various solvents. Based on these spectra, dichloromethane was determined to be the best organic extracting agent. The molar absorptivity of benzoylecgonine in dichloromethane was 0.0796 at 217.00nm. Another key aspect of dichloromethane was that BE was readily soluble in it, in comparison to some of the other attempted solvents, such as benzene.

Once the solvent was chosen, it was necessary to find a carrier solution that could quickly and accurately pair with the BE. A high sensitivity was the goal. Selectivity of BE over other metabolites was not considered an issue. After initial trials with various

metal complexes by previous group members, the ion pairs of Eisman *et al.* (1992)[1] were utilized for this study. The ability of the benzoylecgonine to form ion pairs with the ligand complexes (in this case Co, Fe, Cr, or Bi) occurred by the action of the nitrogen atom. The nitrogen of the benzoylecgonine was protonated in the acidic media, which would aid in the formation of the ion pair with the negative metal complex[1].

Initial tests with $Co(SCN)_4^{2-}$ showed that it was not ideal for detecting BE in solution effectively. Variable results were obtained, with little or no repeatability. Because it is a divalent anion, $Co(SCN)_4^{2-}$ forms a 2:1 complex with cocaine and its metabolites, which may pose a problem with forming as many neutral complexes as might be possible with a 1:1 complex. This lack of forming neutral complexes could have hindered the efficient and repeatable extractions of BE into an organic layer.

Absorption of unpaired tetrakis(thiocyanato)cobalt(II) extracted with dichloromethane is 0.9221 absorption units at 243.00 nm. Absorption of the ion pair between tetrakis(thiocyanato)cobalt(II) and BE is 0.7147 absorption units at 242.00nm. This shows a decreasing absorbance with addition of BE. In this case, the unpaired carrier ion was doing the absorbing, and upon complexation with BE, it no longer absorbed, which led to the decrease in absorbance when BE was added. Even with alterations of extractions, the absorbance continued to decrease. A linear, increasing relationship between the formation of the ion pair and the concentration of BE present was the goal.

Reinecke Salt as an ion pair was also lacking in repeatability and a smooth calibration curve. Previous trials of other laboratory members offered more satisfactory results than this study. Reinecke Salt had problems associated with its study in that it

was difficult to keep in solution. The stock solution had to be made fresh daily due to settling problems when it was allowed to sit, and cost effectiveness was very important in this study. It did not appear to be completely soluble at the concentrations utilized by previous studies[1]. Preliminary tests ruled it out as a possibility after multiple trials and pH tests were performed. The pH of both the ion and the benzoylecgonine solutions were raised and lowered in various trials, but nothing improved the repeatability of the ion and extractions. The best circumstances for extraction with Reinecke Salt were obtained from the literature[1], with a benzoylecgonine sample pH of around 1.0 in the range of 0.3-2.5, and a carrier solution pH of 4.7 in the range of 2.8-6.2. Even with the optimal pH used in the study, the closest to a smooth linear curve is shown in Figure 8. There were problems in the lower concentrations of benzoylecgonine in that the ion often did not extract efficiently. Reinecke Salt was found in the literature to be one of the least sensitive ions to pair with cocaine or any of its metabolites, which corresponds to the results found in this study[1]. Molar absorptivity values of the ion pair between Reinecke Salt and BE are located in Table 1.

After trials with Reinecke salt, investigation of the $Fe(SCN)_6^{3-}$complex began. In many trials, the pH of both the benzoylecgonine and the $Fe(SCN)_6^{3-}$ ion were altered to attempt to find an ideal environment for the extractions. This complex forms a 3:1 complex with cocaine and its metabolites. This decreases its sensitivity to cocaine or its metabolites due to the fact that three BE ions need to bind to each metal complex ion[1]. It also had decreasing absorbance peaks with increasing concentrations. The decrease was not linear, so the calibration curve generated was not usable. An attempt at a calibration curve was made, after initial tests seemed to have positive results. Unfortunately, as

more and more tests were performed, the results became increasingly inconclusive. Figure 9 portrays the three best trial runs with the $Fe(SCN)_6^{3-}$ ion. As visualized by the figure, these tests were not repeatable enough to be useful.

For Dragendorf reagent, or BiI_4^-, variable concentrations of BE were used. In literature studies, 10μg/ml was used, which is equivalent to the 10ppm sample in this study. In previous studies, Dragendorf reagent was shown to have the highest sensitivity to cocaine and its metabolites. It forms an ion pair of 1:1 with BE, cocaine, or other metabolites. The ion pair between benzoylecgonine and Dragendorf reagent is shown in Figure 10. It does have a lower selectivity in that other alkaloids present would bind as well as cocaine or BE. It is optimal though because a lower inorganic layer concentration provides for better formation of ion pairs and improved extraction[1]. In this study, the carrier ion was always in excess of the BE to ensure that all possible BE was paired and then extracted. For Dragendorf reagent, when extracted with dichloromethane, it turns the dichloromethane from colorless to pink. In the presence of BE, it turns slightly orange, more so with increasing concentrations.

Figure 11 shows initial UV-Vis trials. As seen in the figure, the peak at 367 nm increases with increasing BE concentrations. There is also a characteristic orange color of the solution that increases with the increase of this peak. It is thought to be due to the increasing presence of the ion pair in the dichloromethane. In addition to the peak at 367 nm, there are two other peaks in the UV-Vis spectra. The peak at 260 nm is characteristic of benzene containing compounds, such as benzoylecgonine. It increased drastically when BE was not fully paired with Dragendorf reagent. The other peak present on the spectra is the one at around 500 nm. It remains constant with increasing

concentrations of BE, leading to the conclusion that it is caused by Dragendorf reagent, whose concentration was held constant throughout trials.

The linear increase between BE concentrations and absorbance occurs at the peak of 367 nm in Figure 11. After many trials and varying pH of solutions, the protocol for this ion was generated that allowed for optimal extraction and optimal sensitivity of Dragendorf reagent to BE. A calibration curve was generated with a fairly linear increase. In figure 12, the beginning of a calibration curve is portrayed. It shows each of the trials individually. These trials were then averaged to give a final calibration curve for the ion pair between Dragendorf reagent and benzoylecgonine.

Figure 13 shows the final calibration curve with Dragendorf reagent. The blue line on the graph represents the average calibration curve for all points on the graph. There is a source of error in that the final point seems nonlinear with the initial 4 points. The standard error of the slope is 6.91%. The red line on the graph is the calibration curve if the final point is removed. The standard error decreases to 3.71% and the points all lie close to the final line. The R^2 value for this graph is 0.9972. There are many reasons to feel that the final point is too variable for the calibration curve. The point is reaching the absorbance limits of the instrument at almost 2. There seems to be a limitation in this method in that it cannot detect above ~20ppm. Further trials need to be performed to test the range between 10 and 30 ppm. The low end of the concentration range also needs to be tested in order to develop or measure the limit of detection.

A large benefit of Dragendorf reagent is its sensitivity. In comparing data obtained of the other ions tried and for Dragendorf reagent, Dragendorf reagent had molar absorptivity values an order of magnitude greater than the other ions (Table 1).

This is obvious in comparison of the two calibration curves generated, that of hexakis (thiocyanato)iron III/ BE ion pair and that of the Dragendorf reagent/BE ion pair. The calibration curve of hexakis (thiocyanato)iron III/ BE ion pair had a molar absorptivity of 0.0027 M/cm in comparison to the molar absorptivity of the Dragendorf reagent/BE ion pair was almost 10 times that at 0.07838 M/cm (Table 1). This is very important for sensitivity, especially in the lower concentrations of BE. The lower concentrations of BE most resemble those that could be found in urine of test subjects, so sensitivity at these levels is extremely important.

This study successfully generated a new possible method to detect and quantify the presence of BE in urine. Dragendorf reagent has provided a sensitive and cost efficient method to replace or, at the very least, supplement current tests. It provides a very simplistic approach to detecting cocaine and its metabolites in urine. Although there are slight limitations in its upper range, it remains a qualitative test at these higher values. The major area of sensitivity though, that of the lower concentrations of 1-10 ppm BE, is the most important, especially because it is very quantitative at these values. This is the range where most concentrations of BE in urine will fall, and this is the area where Dragendorf reagent performs the best. Further experimentation does need to occur to solidify this data, but this study shows a start towards finding a more cost efficient method of detecting and quantifying benzoylecgonine in urine.

Acknowledgement

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Table 1

Carrier Ion	Molar Absorptivity,ε, (M/cm)
Dragendorf Reagent	0.07838
Reinecke Salt	0.0061
Hexakis (Thioncyanato)iron(III)	0.0027

Figure 1. Cocaine and its primary metabolites

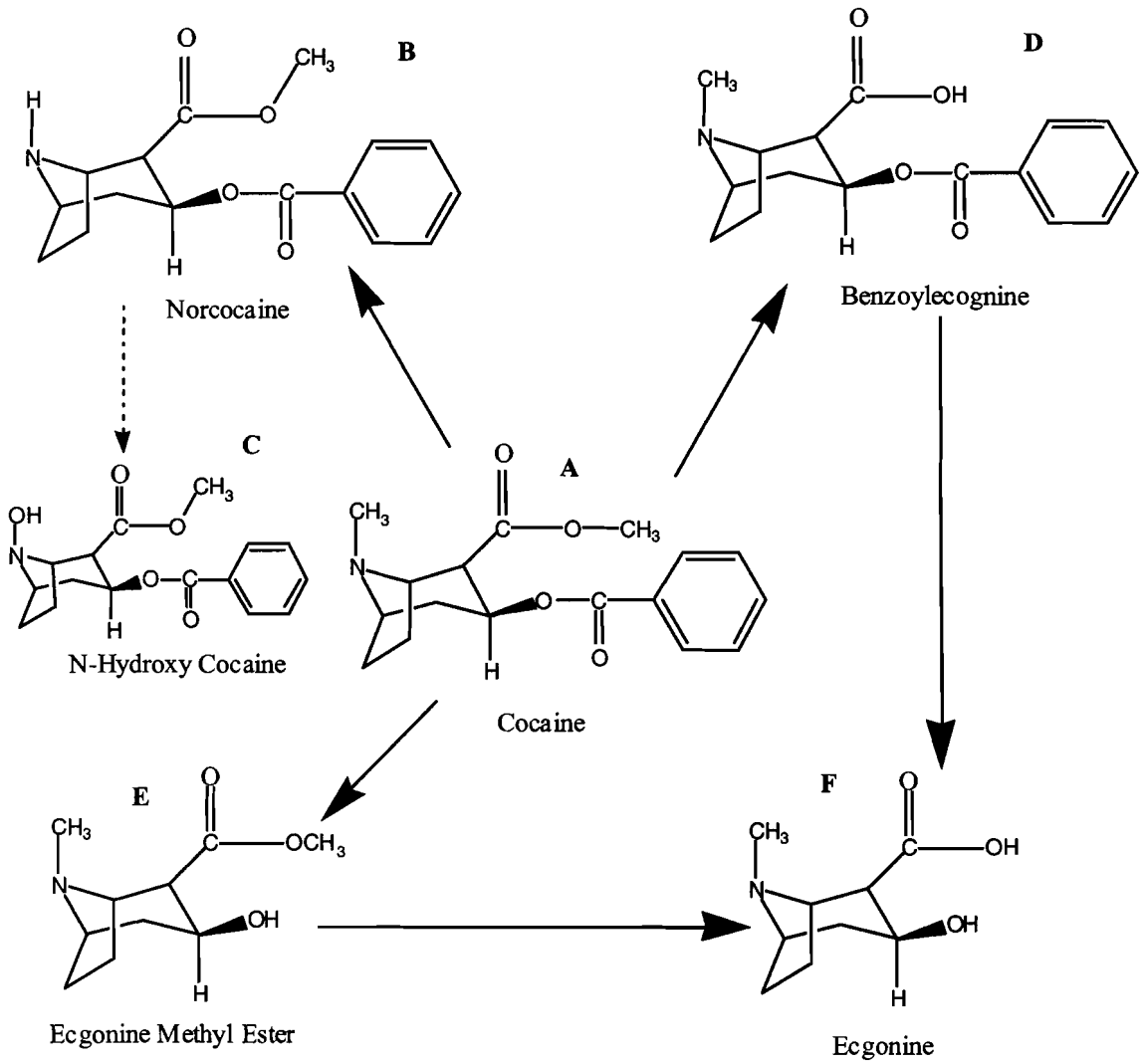


Figure 2. Different forms of benzoylecgonine

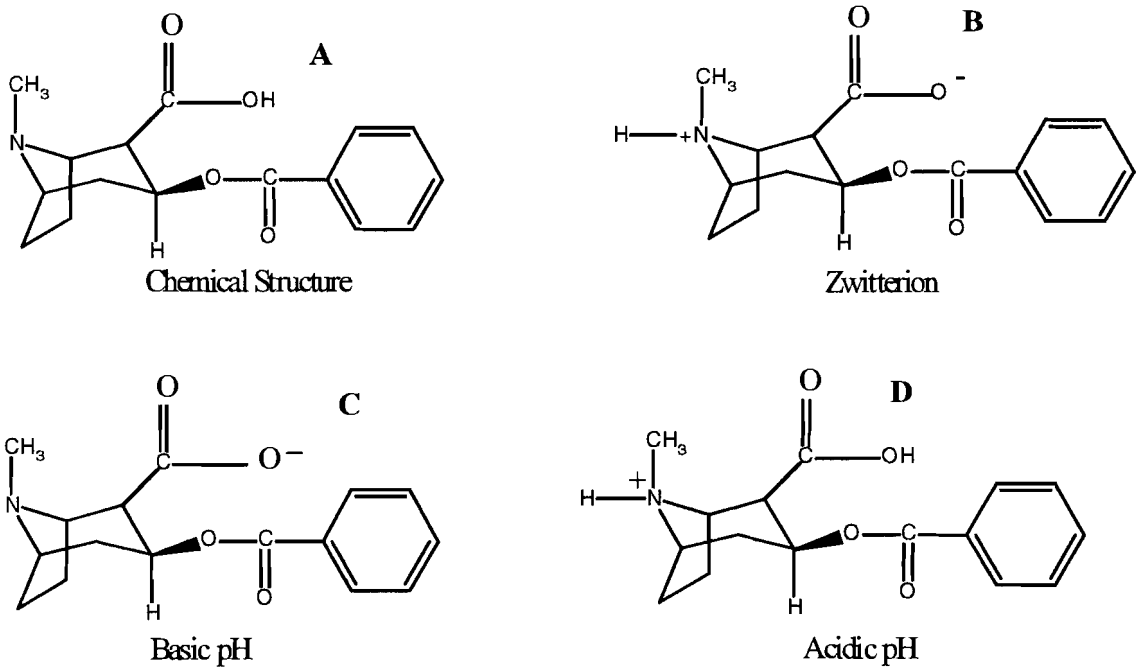
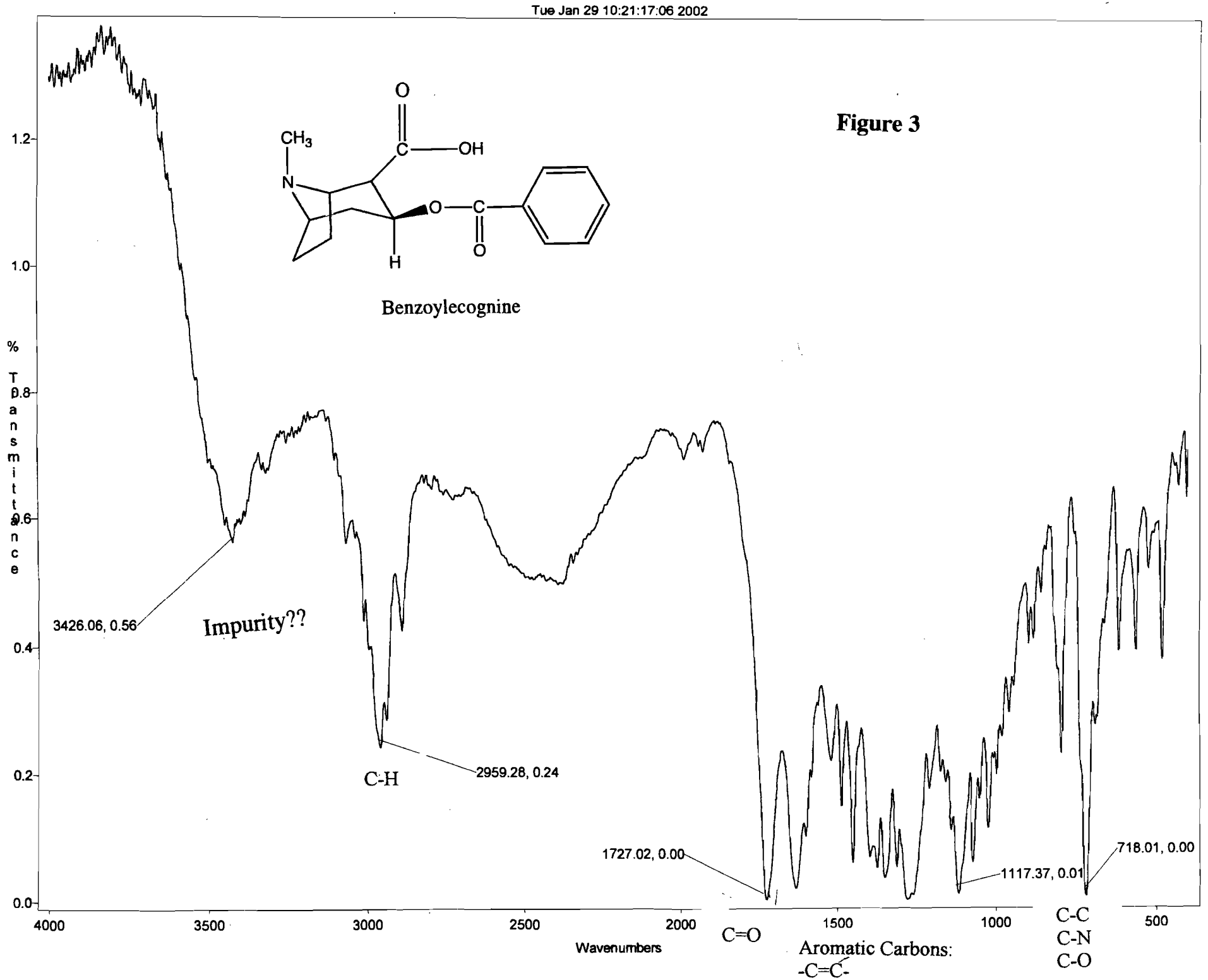
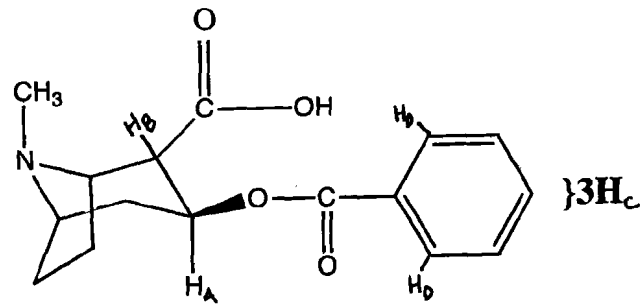


Figure 2: BE (A) exists in a neutral solution as zwitterion (B). In basic solution, the tertiary amine is deprotonated, forming the anionic form (C). In acidic solution, the carboxylic acid is protonated, forming the cationic form (D).


Tue Jan 29 10:21:17:06 2002
Figure 3
Benzoylecognine
% Transmittance
Wavenumbers
3426.06, 0.56
Impurity??
C-H
2959.28, 0.24
1727.02, 0.00
C=O
Aromatic Carbons:
-C=C-
1117.37, 0.01
718.01, 0.00
C-C
C-N
C-O

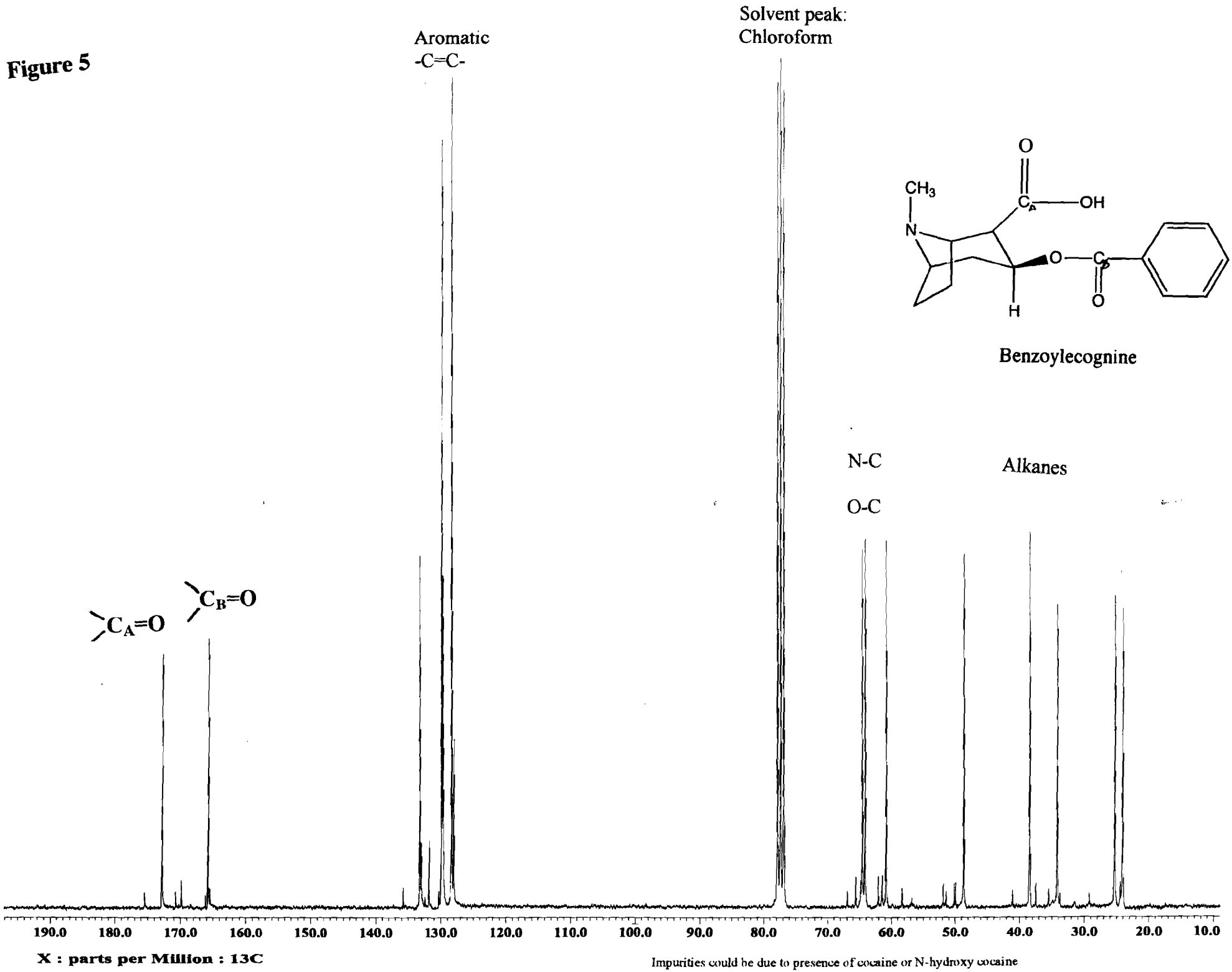
kyleebe2.3

Figure 4



Benzoylecognine

Figure 5



Benzoylecognine

Impurities could be due to presence of cocaine or N-hydroxy cocaine

Figure 6

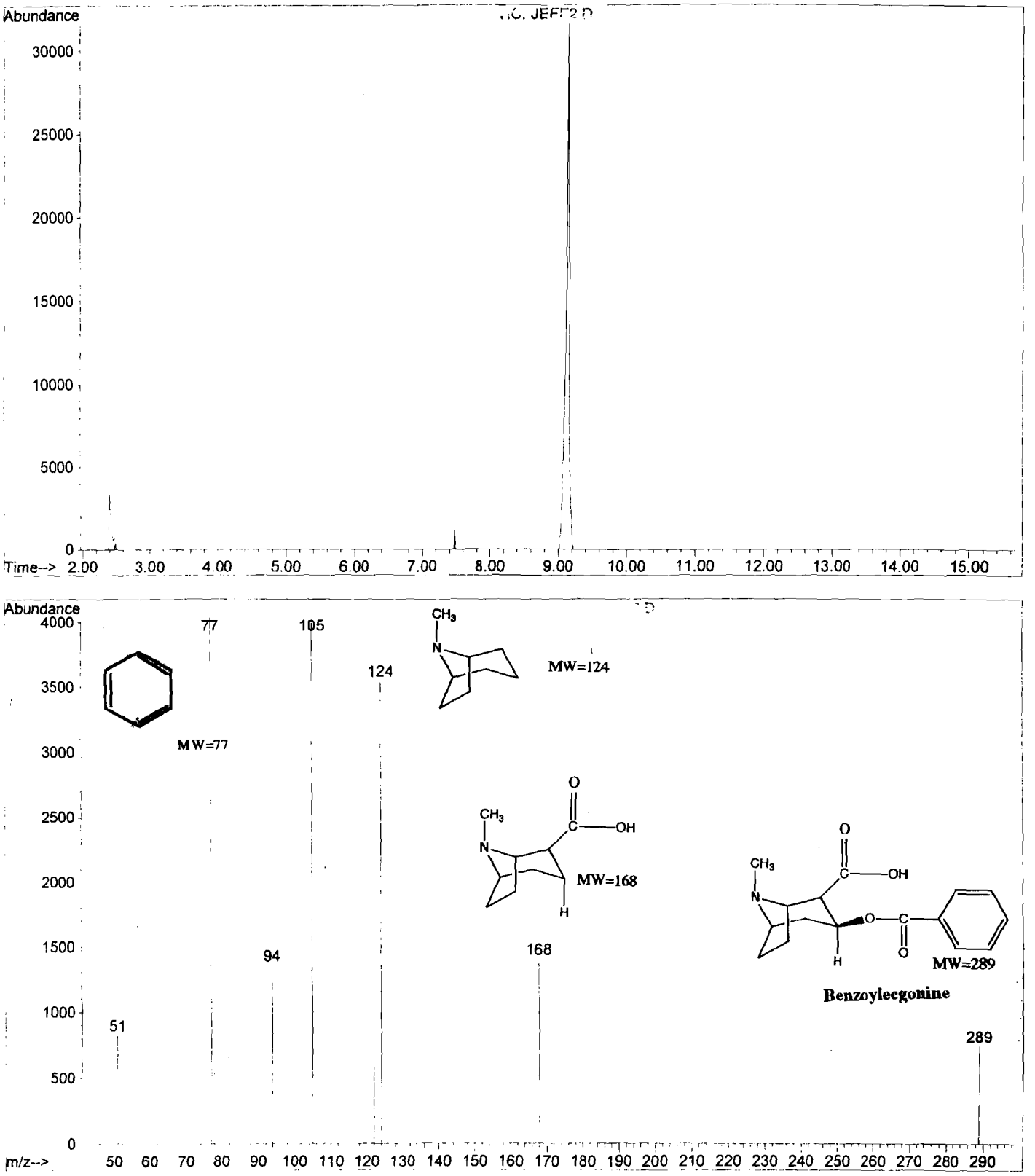

Abundance
30000
25000
20000
15000
10000
5000
0
Time--> 2.00 3.00 4.00 5.00 6.00 7.00 8.00 9.00 10.00 11.00 12.00 13.00 14.00 15.00
Abundance
4000
3500
3000
2500
2000
1500
1000
500
0
77
105
124
94
51
168
289
MW=77
MW=124
MW=168
MW=289
Benzoylecgonine
m/z--> 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290

Figure 7

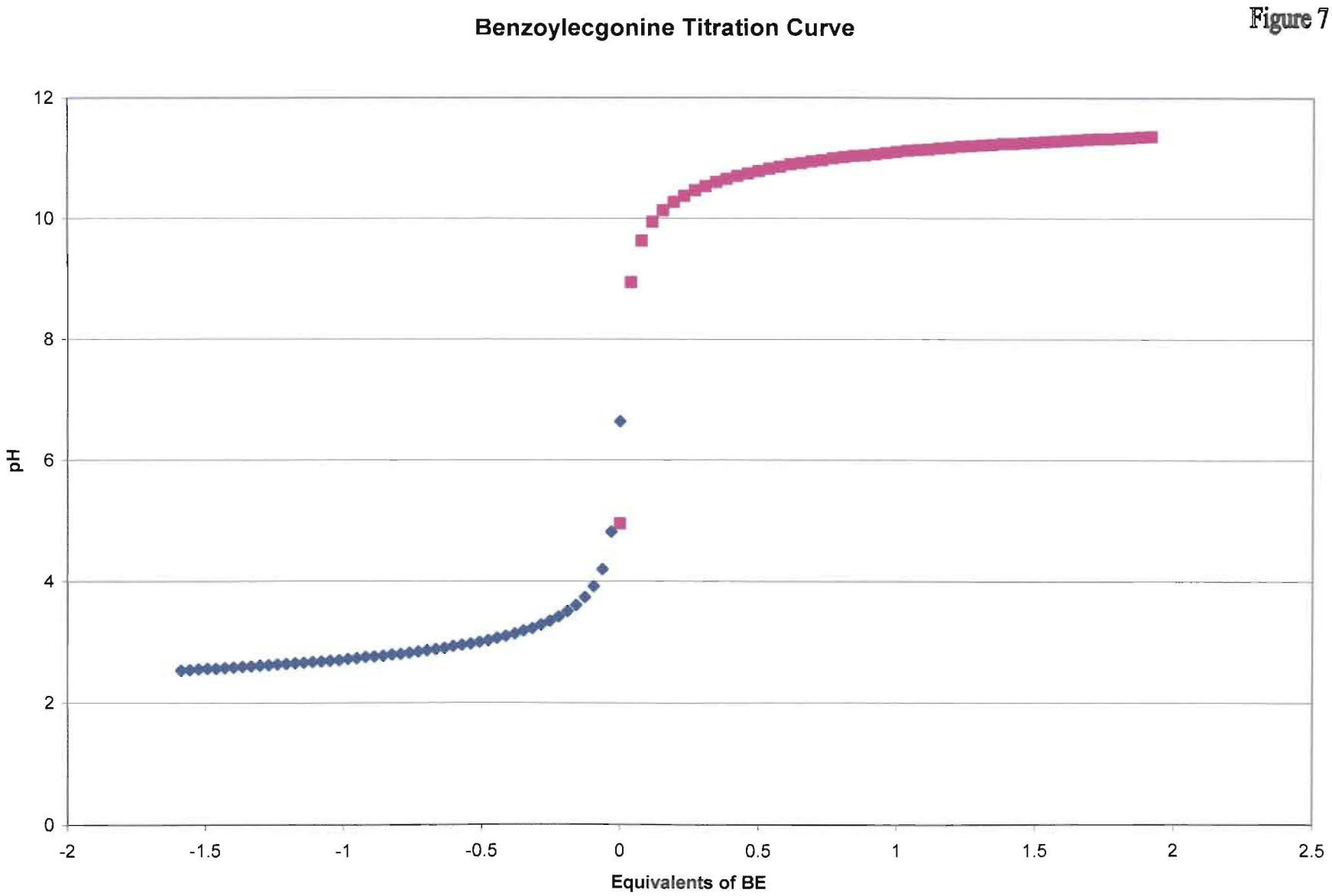

Benzoylecgonine Titration Curve
pH
12
10
8
6
4
2
0
-2
-1.5
-1
-0.5
0
0.5
1
1.5
2
2.5
Equivalents of BE

Figure 8

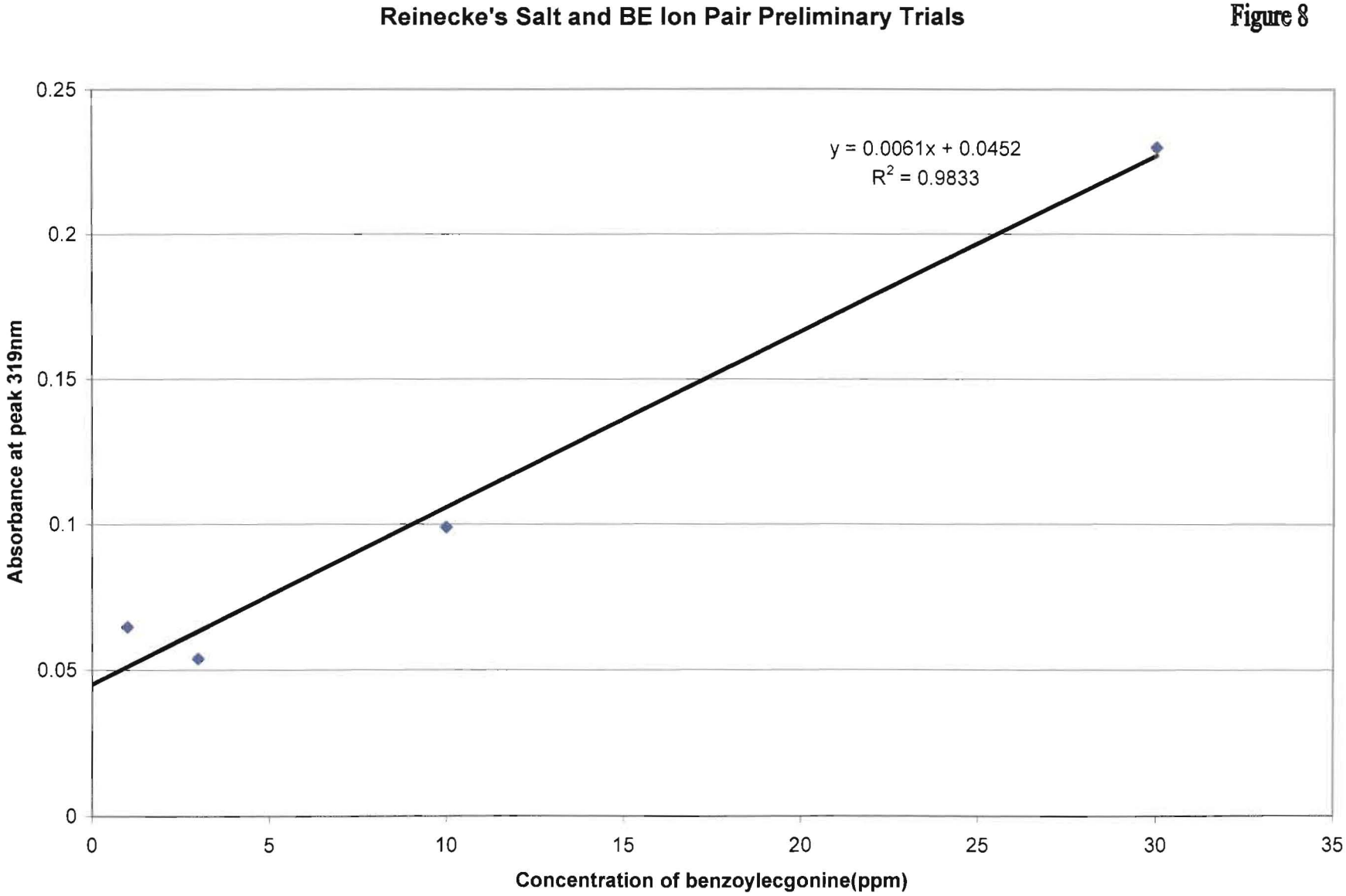

Reinecke's Salt and BE Ion Pair Preliminary Trials
y = 0.0061x + 0.0452
R2 = 0.9833
Absorbance at peak 319nm
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
25
30
35
Concentration of benzoylecgonine(ppm)

Figure 9

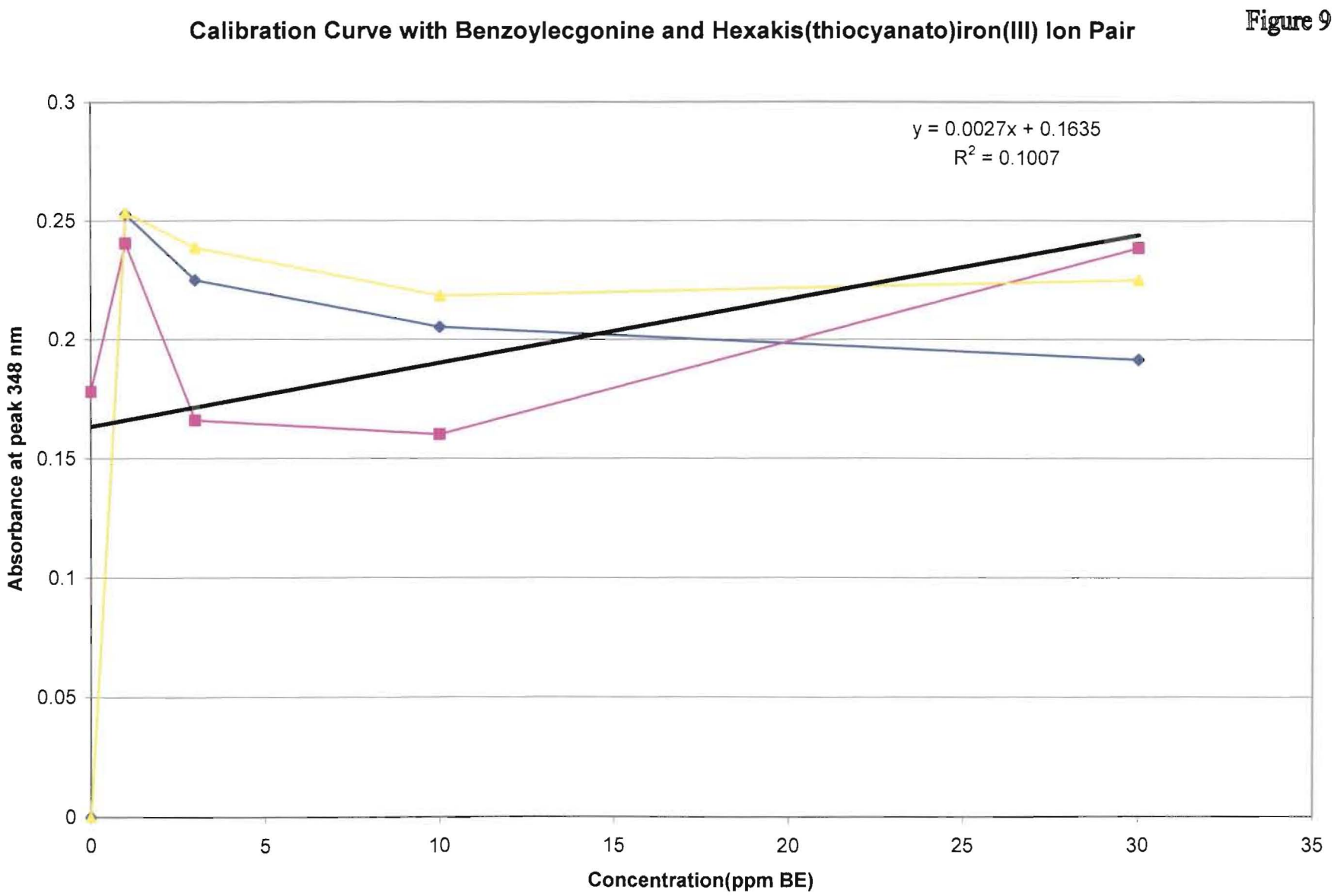
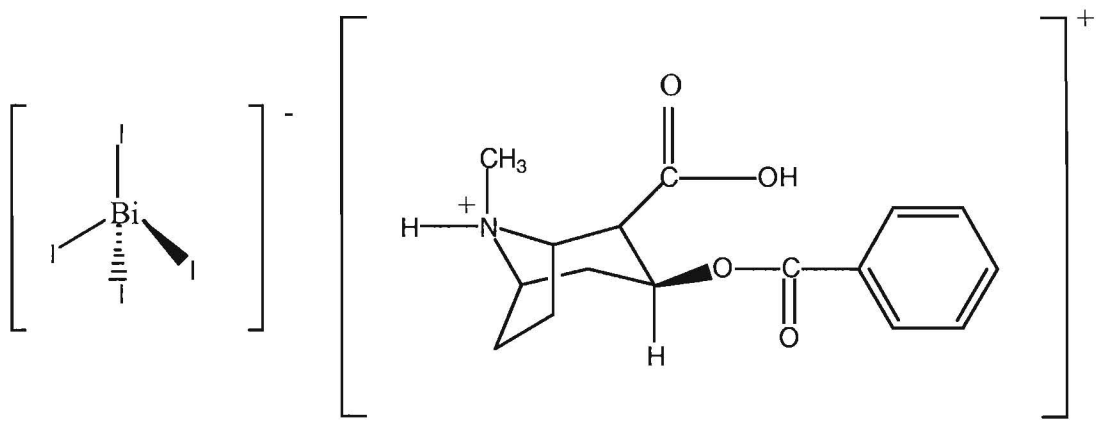

Calibration Curve with Benzoylecgonine and Hexakis(thiocyanato)iron(III) Ion Pair
y = 0.0027x + 0.1635
R2 = 0.1007
Absorbance at peak 348 nm
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
25
30
35
Concentration(ppm BE)

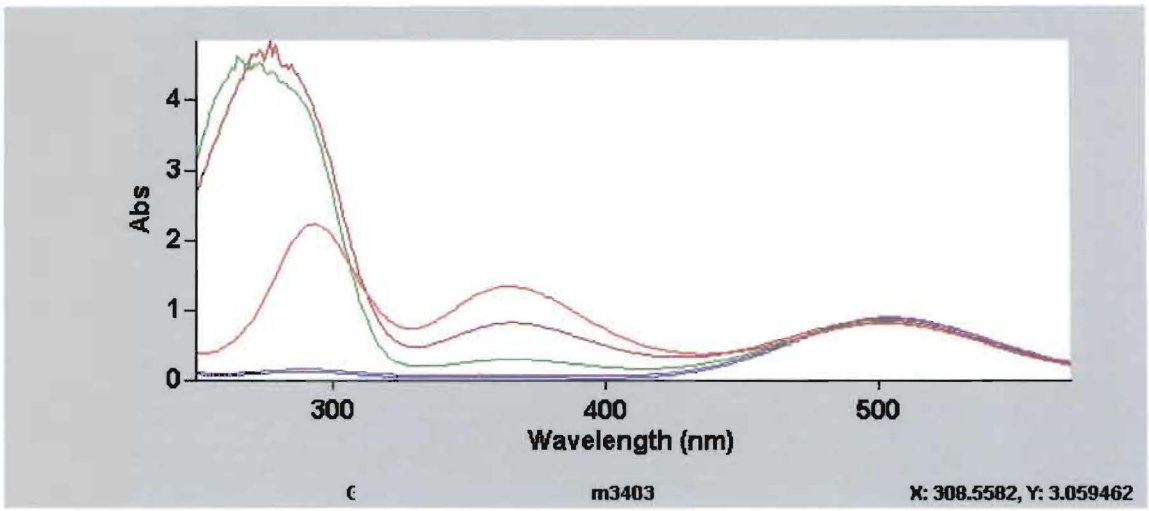
Figure 10



Dragendorf Reagent/Benzoylecgonine Ion Pair

Figure 11

UV-VIS of various concentrations of benzoylecgonine extracted with Dragendorf's Reagent



Red- 30 ppm benzoylecgonine
Brown- 10ppm benzoylecgonine
Green- 3ppm benzoylecgonine
Purple- 1ppm benzoylecgonine
Blue- blank, 0ppm benzoylecgonine

Figure 12

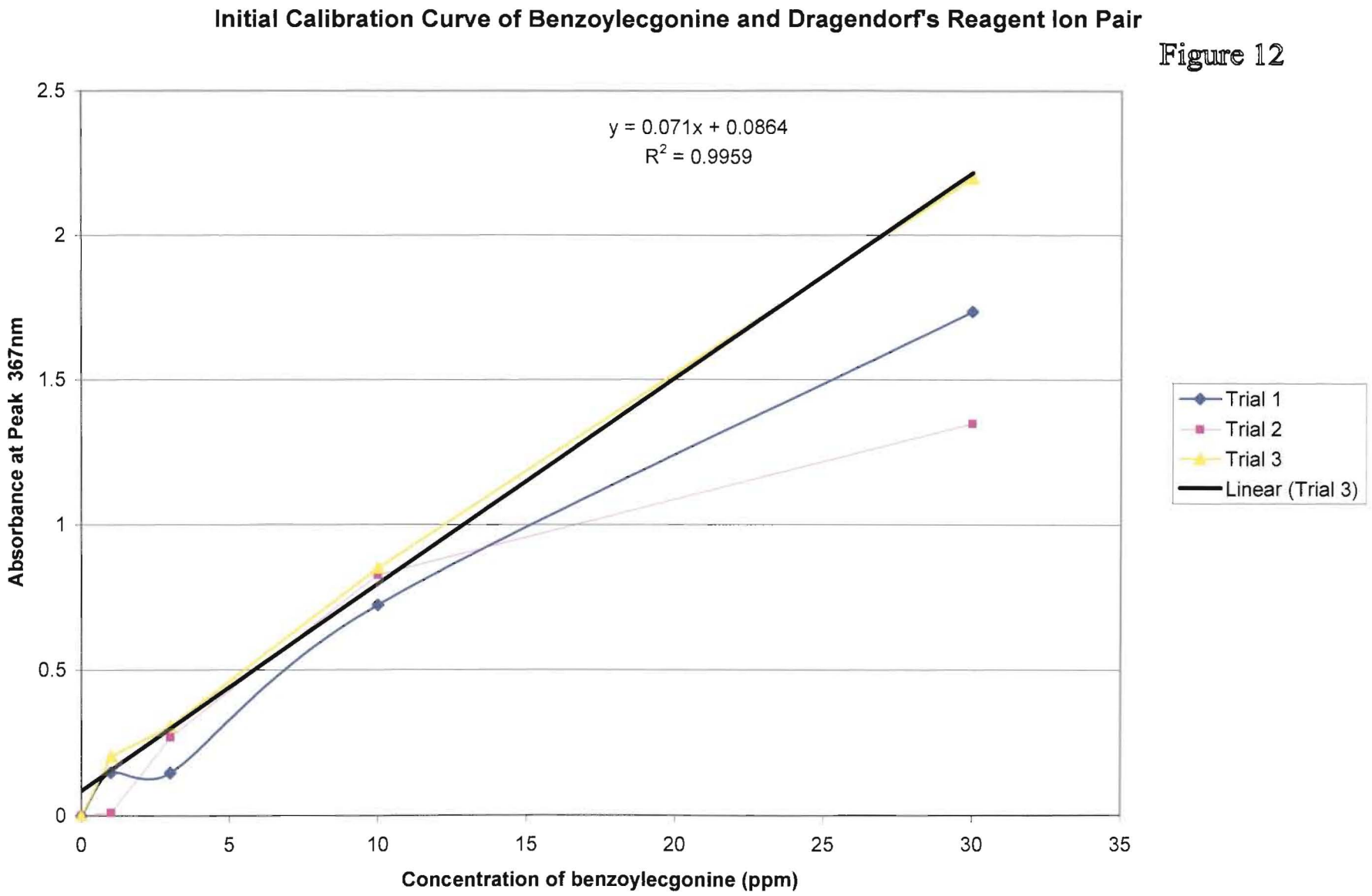
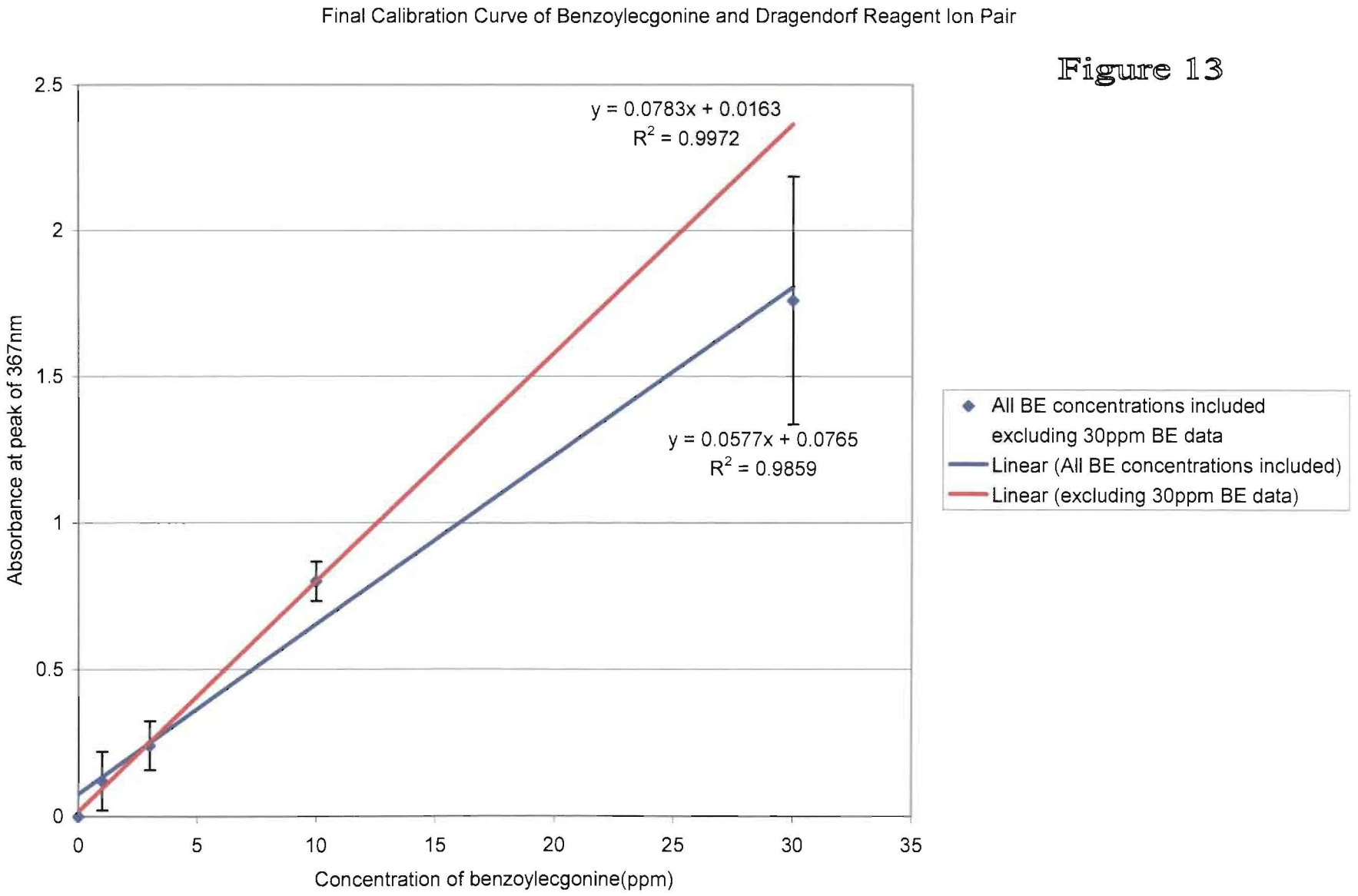

Initial Calibration Curve of Benzoylecgonine and Dragendorf's Reagent Ion Pair
y = 0.071x + 0.0864
R2 = 0.9959
Absorbance at Peak 367nm
Concentration of benzoylecgonine (ppm)
0
0.5
1
1.5
2
2.5
0
5
10
15
20
25
30
35
Trial 1
Trial 2
Trial 3
Linear (Trial 3)

Figure 13


Final Calibration Curve of Benzoylecgonine and Dragendorf Reagent Ion Pair
y = 0.0783x + 0.0163
R2 = 0.9972
y = 0.0577x + 0.0765
R2 = 0.9859
Absorbance at peak of 367nm
Concentration of benzoylecgonine(ppm)
0
0.5
1
1.5
2
2.5
0
5
10
15
20
25
30
35
All BE concentrations included
excluding 30ppm BE data
Linear (All BE concentrations included)
Linear (excluding 30ppm BE data)