Cocaethylene as a Biomarker in Human Hair of Concomitant Alcohol and Cocaine Use in a High-Risk Population.

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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ABSTRACT

COCAETHYLENE AS A BIOMARKER IN HUMAN HAIR OF CONCOMITANT ALCOHOL AND COCAINE USE IN A HIGH-RISK POPULATION

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Cocaethylene (CE) is a cocaine metabolite formed during alcohol and cocaine co-consumption. To our knowledge, no previous studies were conducted assessing CE as a biomarker indicating chronic excessive alcohol consumption in a suspected high-risk population. In this study, we hypothesized that hair CE can be an effective marker for alcohol consumption in a high-risk population. We recorded cocaine, benzoylecgonine, and CE levels in hair samples from individuals, establishing the predictive value of CE by comparing it to hair levels of the widely used hair fatty acid ethyl esters (FAEE), direct markers of chronic excessive alcohol consumption. CE had 14.04% sensitivity and 95.18% specificity in samples separating FAEE positive/negative results. The positive predictive value was 0.66, showing that the results for individuals with CE positive results were more than likely to be FAEE positive, but not conclusively. Thus, CE cannot be used as a definitive marker, indicating chronic excessive alcohol consumption.
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To all of my friends and family, thank you for standing by me and being there for me. To Mama, Baba, and Sneha, I am blessed to have all of your unconditional love and support since the day I arrived into this world. I will never forget it, and will cherish my time with you every day.
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<tr>
<td>CE</td>
<td>Cocaethylene</td>
</tr>
<tr>
<td>BE</td>
<td>Benzoylecgonine</td>
</tr>
<tr>
<td>FAEE</td>
<td>Fatty Acid Ethyl Esters</td>
</tr>
<tr>
<td>CES 1</td>
<td>Human Carboxylesterase-1</td>
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<tr>
<td>EME</td>
<td>Ecgonine Methyl Ester</td>
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<tr>
<td>EEE</td>
<td>Ecgonine Ethyl Ester</td>
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<tr>
<td>BAC</td>
<td>Blood Alcohol Concentration</td>
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<tr>
<td>MDTL</td>
<td>Motherisk Drug Testing Laboratory</td>
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<tr>
<td>SPME</td>
<td>Solid-Phase Microextraction</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas Chromatography-Mass Spectrometry</td>
</tr>
<tr>
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<td>Automated Solid-Phase Extraction</td>
</tr>
<tr>
<td>PPV</td>
<td>Positive Predictive Value</td>
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<tr>
<td>NPV</td>
<td>Negative Predictive Value</td>
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<tr>
<td>FASD</td>
<td>Fetal Alcohol Spectrum Disorder</td>
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CHAPTER 1: INTRODUCTION

1.1 Statement of Current Knowledge

In the United States, it is estimated that about 3 million people are regular cocaine users. In Canada, approximately 238,000 individuals use cocaine. While these statistics are alarming, even more surprising is that approximately 60-90% of cocaine users also abuse alcohol concomitantly. It is said that alcohol helps to prolong cocaine-mediated effects, as well as reduce the negative effects of cocaine use, such as anxiety and uneasiness. While the combination is popular, it has been shown to be more dangerous than either drug alone. It has been demonstrated that concomitant alcohol and cocaine use can increase the risk of heart attack and stroke, and also increase the risk of homicidal thoughts in special populations.

Cocaethylene (CE) is a cocaine metabolite that forms in the presence of ethanol. Normally, cocaine is hydrolyzed to benzoylecgonine (BE) by human carboxylesterase 1 in the liver. When ethanol is consumed, about 17% of cocaine is transesterified to CE. Being pharmacologically active, CE is thought to prolong cocaine-mediated effects, as it acts on the same receptors and has a longer elimination half-life than cocaine.

Cocaine and BE have been found in scalp hair of cocaine users, and are used as markers to indicate cocaine use or exposure. Fatty acid ethyl esters (FAEE) are the gold standard in hair testing to indicate chronic excessive alcohol consumption. CE has been found in scalp hair and there have been indications for its use as a marker in drug testing to indicate concomitant cocaine and alcohol use. While CE has been studied in the hair of known cocaine users, the prevalence of CE in a population whose cocaine and alcohol consumption patterns are unknown is still largely unstudied. Thus, more knowledge needs to be gathered before CE can be fully used in practice.
By understanding the effectiveness of using CE in human hair as a marker to indicate cocaine and alcohol consumption in a high-risk population, more targeted approaches to drug abuse treatment and other medical and social strategies can be pursued. With cocaine and alcohol use still prominent in North America, having the knowledge about CE in hair testing could possibly aid Children’s Aid societies and legal professionals to make more individualized decisions regarding the suspected cocaine and alcohol abusers involved. In turn, children living in such at-risk environments would be protected from harm caused by cocaine and alcohol abusing parents.
1.2 Purpose and Study Objectives

The purpose of the study was to determine if CE in scalp hair could be used to indicate alcohol consumption in a suspected cocaine-user population. The following two objectives were employed for this investigation:

**Objective 1:** To determine if positive FAEE values could be used to predict positive CE values in samples identified positive for cocaine use.

**Objective 2:** To determine significant relationships between cocaine and metabolite concentrations in relation to chronic excessive alcohol consumption by testing FAEE concentrations in scalp hair.
1.3 Research Hypothesis and Rationale

The following hypothesis for the present study is below:

**Hypothesis 1**: A previous study found that CE and FAEE concentrations were related in the scalp hair of known cocaine users. However, there is no literature on how these relationships are distributed in a large population for hair testing in a clinical setting. We hypothesized that positive CE levels could predict positive FAEE levels in people whose cocaine and alcohol use habits were unknown to us, but were only suspected.
CHAPTER 2: REVIEW OF THE LITERATURE

2.1 Cocaine and Alcohol Abuse

2.1.1 Prevalence and Description

Cocaine and alcohol are widely abused in Canada and the United States. The prevalence of cocaine users in Canada, from 15 years of age and older, appears to have decreased from 1.9% to 0.7% from 2004 to 2010. Also, lifetime alcohol use significantly decreased from 92.8% to 88.9%, while alcohol use within the last 12 months slightly decreased from 79.3% to 77%. The proportion of heavy frequent drinkers decreased from 7.1% to 4.3%, while the proportion of light frequent drinkers increased from 27.7% to 32.2%. In the United States, the most recent statistics on cocaine and alcohol abuse are on adolescents in grades 9-12. The estimated prevalence of students from grades 9 to 12 who used any form of cocaine one or more times significantly decreased from 9.5% to 6.4% between 1999 and 2009, while 2.8% of students surveyed in 2009 admitted to using some form of cocaine within 30 days of the survey. In the 2009 National Survey on Drug Use and Health, approximately 30.2 million (12%) people aged 12 or older admitted to driving under the influence of alcohol. In North America, an estimated 5.69 million people were using cocaine in 2009, and approximately 14.25 million to 20.52 million people worldwide were using cocaine. This is important because it has been estimated that 50% to 90% of cocaine users also co-abuse alcohol. That would mean that approximately 7.125 million to 18.468 million people co-abuse cocaine and alcohol, making it a significant global issue.
2.2.1 Risk Factors of Concomitant Cocaine and Alcohol Use

Some reasons for co-abusing cocaine with alcohol is that alcohol prolongs cocaine-mediated effects, and reduce anxiety, uneasiness, and other psychological adverse effects associated with the cocaine “crash”\textsuperscript{10-12}. While the combination of cocaine and alcohol use is very common, it is also very harmful to the body. In dogs, cocaine and alcohol together were found to decrease the heart stroke volume by 34% and increase the heart rate by 89%, demonstrating that there is a synergistic depression of ventricular contraction and relaxation that exceeds the sum of the depressive effects of cocaine or alcohol alone\textsuperscript{13}. In humans, the combination of cocaine and alcohol may increase the risk of cardiologic and neurological complications, such as stroke and torsades\textsuperscript{14}. Cocaine and alcohol together demonstrate a trend towards greater-than-additive effects on heart rate\textsuperscript{6,10,15-19}. As cocaine alone can cause cardiovascular complications, concomitant use of cocaine and alcohol may put the user at serious clinical risk for cardiotoxicity\textsuperscript{20}. The combination of cocaine and alcohol was found to increase the heart rate of volunteers by 41 beats per minute compared to placebo\textsuperscript{21}. Additionally, there seems to be a cocaine dose-dependent effect on heart rate when in combination with alcohol, as volunteers had a significantly higher heart rate at a high cocaine dose (1.2 mg/kg) vs. a low cocaine dose (0.3 mg/kg)\textsuperscript{22}. Although the volunteers were experienced concomitant cocaine and alcohol users, four participants felt “nauseous” in almost all of the treatment sessions, and mild headaches were reported several hours after dosing\textsuperscript{22}.

In addition to the physical effects of cocaine and alcohol together, there are also psychological adverse effects. A study conducted on 320 male veterans in treatment for cocaine abuse found that those who used the combination of cocaine and alcohol rather than cocaine alone were more likely to attack people physically and more likely to threaten people with a weapon\textsuperscript{23}. Another
study showed that those who used the combination were three times more likely to have homicidal thoughts or plans than those who drank alcohol only, and five times more likely than those who used cocaine only\textsuperscript{24}. In both cases, however, there are other factors that should be considered before such associations are taken at face-value. For example, individuals in the military may already have a propensity toward violence, thus a significant relationship between cocaine and alcohol abuse vs. violence should be expected. The same theory could be applied to the second study, where the individuals may have already had a tendency toward violent action prior to drug and alcohol abuse. Additionally, veterans of war may have encountered traumatic experiences, thus current environmental “triggers” could provoke these individuals to act violently irrespective of their cocaine and alcohol abuse.

2.2 Cocaine

2.2.1 Routes of Administration

Cocaine is harvested from the coca shrubs \textit{Erythroxylum coca} and \textit{Erythroxylum novogranatense}\textsuperscript{25}. Once harvested, cocaine is prepared into different forms that are meant for specific routes of administration. The most common mode of cocaine consumption is through the intranasal route by sniffing cocaine hydrochloride\textsuperscript{26}. This route has been demonstrated to take approximately 14.6±8 min to reach peak subjective “high”\textsuperscript{27}. “Freebase” cocaine, also known as “crack” is produced by mixing cocaine with a base, and is typically smoked\textsuperscript{28}. This has become the preferred method of cocaine consumption\textsuperscript{26} since it creates an intense and immediate “high” within 10-15 sec\textsuperscript{29}, and the peak subjective “high” was reached within 1.4±0.5 min\textsuperscript{27}. Cocaine can also be injected\textsuperscript{30}, which allows for the highest blood concentration in the shortest amount of time, and the time to peak subjective effects is 3.1±0.9 min\textsuperscript{27}. Cocaine can also be swallowed, usually through the chewing and swallowing of coca leaves\textsuperscript{31}. 
2.2.2 Receptor Interactions and Effects

Cocaine mediates its effects for monoamine neurotransmitters in the brain, including serotonin, dopamine, and norepinephrine\textsuperscript{32-37} by blocking neurotransmitter re-uptake\textsuperscript{38}. Short term effects include increased energy, mental alertness, euphoria, and decreased appetite. Acute cocaine exposure may induce cardiac arrest, seizures, and/or respiratory failure. Long-term cocaine abuse can lead to paranoia, auditory hallucinations, irritability, fever, heart attack, gastrointestinal problems and muscle spams\textsuperscript{39-41}.

2.2.3 Cocaine Pharmacokinetics

Once cocaine has entered the blood stream, it is metabolized mostly by the liver, but also by the heart, stomach, and kidney\textsuperscript{42,43}. In humans, cocaine is metabolized by a family of carboxylesterases, particularly carboxylesterase 1 (CES 1) in the liver and carboxylesterase 2 (CES 2) in the intestines\textsuperscript{43,45,46}. Cocaine is primarily hydrolyzed to benzoylecgonine (BE) by CES 1 and ecgonine methyl ester (EME) by CES 2\textsuperscript{47}, but is also metabolized to norcocaine\textsuperscript{48} and several other metabolites\textsuperscript{49}. Cocaine’s breakdown into various products is depicted in Figure 1.
Figure 1. Cocaine and its various metabolites that are formed in the body (Adapted from Cone et al., 2003)

Cocaine’s bioavailability through the nasal insufflation route averages about 80±13% with an average elimination half-life of 80 min\textsuperscript{50}. Bioavailability through smoking averages 57±19% with an average elimination half-life of 56 min. Simulated studies found that only 44% of what was inhaled was actually cocaine\textsuperscript{51}, suggesting that most of the un-decomposed cocaine reaches systemic circulation as its parent form\textsuperscript{50}. The lower bioavailability is most likely due to decomposition before inhalation\textsuperscript{50}. When given intravenously, cocaine has an average elimination half-life of 78-90 min\textsuperscript{50,52}. A study found that the elimination half-life of cocaine through the intranasal and gastrointestinal routes was as high as 5 h\textsuperscript{53}. While BE and EME have no bioavailabilities, their elimination half-lives are 7.5 and 3.6 hours respectively\textsuperscript{48,53}.

Cocaine and its metabolites are excreted in urine and can be detectable for several days depending on the dose used\textsuperscript{26,54}. In addition, the same products can also be found in oral fluids\textsuperscript{55}, sweat\textsuperscript{56,57}, and hair\textsuperscript{58}.

### 2.3 Fatty Acid Ethyl Esters

Fatty Acid Ethyl Esters (FAEEs) are products of non-oxidative ethanol metabolism by the conjugation of ethanol to endogenous free fatty acids and fatty acyl-CoA. FAEE can be spontaneously formed, but is usually catalyzed by microsomal acyl-coA:ethanol O-acyltransferase\textsuperscript{59}. Also, enzymes with FAEE synthetic activity, such as carboxylesterases, carboxylester lipases, and lipoprotein lipases, in various organs also use ethanol and free fatty acids as substrates\textsuperscript{60-63}. For example, FAEE can be found in the liver\textsuperscript{60}, pancreas\textsuperscript{62}, plasma\textsuperscript{64}, skeletal muscles, and the heart\textsuperscript{65}. It can be presumed that because alcohol can have effects on each of the organs mentioned, FAEE could form in the respective organs as well.
Although FAEE is produced naturally, they are known to be toxic to the body. FAEE have been demonstrated to induce apoptosis and necrosis in human peripheral blood mononuclear cells\textsuperscript{66}. Additionally, FAEE have been implicated in hepatocellular and pancreatitis-like injury\textsuperscript{67-69}. FAEE is normally excreted through the urine\textsuperscript{70}.

2.4 Cocaethylene

2.4.1 Formation and Properties

Normally, cocaine is hydrolyzed into BE by CES 1. However, when ethanol is consumed at the same time as cocaine, a portion of cocaine hydrolysis is shifted towards transesterification to produce cocaethylene (CE)\textsuperscript{71,72}. Cocaine metabolism by CES 1 and CES 2 to its respective metabolites is summarized in Fig 2.
In the presence of ethanol, a portion of the metabolism shifts from hydrolysis to transesterification by CES 1 to produce CE. If enough ethanol remains, then CE can then remain unmetabolized. CE can be hydrolyzed to BE, or form ecgonine ethyl ester (EEE). Reprinted from Drug Metabolism and Disposition, Vol. 31, Laizure et al. Carboxylesterase metabolism of cocaine. Pg. 16-20. Copyright 2003, with permission from American Society for Pharmacology and Experimental Therapeutics.
A study conducted in volunteers given varying doses of IV cocaine with 1 g/kg ethanol found that plasma CE concentrations reached about 17% of cocaine concentrations, indicating that only partial transesterification occurred\(^{22}\). Unlike BE, which is inactive\(^{44,45,71}\), CE only differs structurally from cocaine by the substitution of an ethyl instead of a methyl ester\(^{47}\). As such, CE has similar pharmacological effects as cocaine at the monoamine transport binding sites in the brain, and is equipotent in binding to dopamine receptors in the brain as well\(^{14,73}\). Blocking the re-uptake of dopamine likely contributes to the increased euphoria\(^{73}\), thus contributing to the reinforcement of subjective responses and acute physiological responses of combined alcohol and cocaine use\(^{22,74}\). Consequently, the prolonging of cocaine-mediated effects has been attributed to CE, and this provides a plausible explanation why many cocaine users co-abuse alcohol\(^{75,76}\).

It is also important to note that CE can be produced spontaneously in the presence of alcohol for prolonged periods of time. A study conducted found that cocaine was dissolved in liquor bottles and smuggled into Canada and was then extracted and converted into its hydrochloride salt. Analysis of this product found that approximately 20% of the cocaine had undergone transesterification into CE. Consequently, CE appeared as a major component of the final cocaine hydrochloride salt product\(^ {77}\). Additionally, the DEA found that the Peruvian method of processing uses ethanol to purify the cocaine base. Upon analysis of these products, CE was found in all the samples with an average concentration of 0.12% relative to cocaine, with the highest CE concentration to be 0.93%. CE reached average concentrations of 0.013% relative to cocaine hydrochloride, with the highest concentration being 0.084%. Colombian methods convert cocaine base to cocaine hydrochloride using ethanolic hydrochloride, thus it is expected to form CE. CE was found to be 2% of cocaine concentrations in Colombian-processed
samples. As well, CE was found to range from 0.08% to 1.16% of pharmaceutical cocaine produced for research purposes. Thus, the exposure to ethanol for a certain period of time might be enough to produce CE, and metabolism might not be required. As seen in Fig 2, if CE is already entered circulation, then the transition from cocaine to CE is bypassed, and only the second half of the figure would be applicable. With ethanol present, then CE would remain as itself, and if CE was present in the original cocaine dose, then theoretically the pharmacological properties of CE could be prolonged.

2.4.2 Cocaethylene Toxicity

In dogs, CE caused hypertension by increased systemic vascular resistance. When CE was present in high concentrations, it decreased myocardial function, slowed cardiac conduction, and was arrhythmogenic. In humans, CE was less potent in elevating heart rate compared to equivalent cocaine doses. Also, similar differences were found for subjective measures of "cocaine high", "rush", "stimulated", and "good drug effects". There was also a significant increase in systolic blood pressure compared to placebo, but not significant for diastolic blood pressure. Another human study also found that the effect of CE on heart rate was similar to equivalent doses of cocaine, but may be less potent since higher plasma concentrations were achieved for CE than cocaine. The authors also found that CE is at least as potent as cocaine with respect to increasing systolic blood pressure.

CE has been reported to be more toxic than cocaine in rats. CE had an LD₅₀ of 60.7 mg/kg in females and 63.8 mg/kg in males, while cocaine had an LD₅₀ of 92.4 mg/kg in females and 93.0 mg/kg in males. Another study also found CE given through intraperitoneal injection was significantly more potent than cocaine in causing death (201.5 µmol/kg vs. 237.7 µmol/kg). Postmortem studies in humans found high CE levels in overdose victims who had recently
ingested cocaine and alcohol\textsuperscript{73,85,86}. One study of post-mortem samples found that CE was not found when cocaine was absent. As well, even when ethanol and BE were present but CE was not, cocaine was only in trace amounts, thus CE might have been eliminated\textsuperscript{85}. In another study, many had detectable plasma CE concentrations. Cocaine concentrations showed a significant correlation with CE levels, and CE concentrations were as high 249 µg/L, while cocaine concentrations reached as high as 441µg/L\textsuperscript{86}.

### 2.4.3 CE Pharmacokinetics

In dogs, cocaine and CE had similar clearance values of 0.91±0.22 and 0.79±0.16 L/min and similar volumes of distribution of 2.6±0.82 and 2.7±0.47 L/kg respectively. In the presence of ethanol, both cocaine and CE clearance values decreased by approximately 20\%, indicating that both compounds remained in the body for a longer period of time\textsuperscript{47}. In humans, when cocaine and CE are given intranasally, CE at low and high doses (0.48 and 0.95 mg/kg) had lower clearance values than cocaine at a high dose (0.92 mg/kg). With 0.015 and 0.017 L/min/kg for the low and high CE doses vs. 0.0333 L/min/kg for cocaine, this would explain why the elimination half life for CE was about 24\% longer than cocaine at almost equivalent doses (138 min vs. 111 min respectively)\textsuperscript{82}. Also, CE $C_{\text{max}}$ was found to be much higher than cocaine (251 vs. 144 ng/ml respectively) with a much shorter absorption half-life (13 vs. 17 min respectively)\textsuperscript{82}. These results suggest that CE is more readily absorbed and remains in the body for a longer period of time in humans like it did in dogs.

While CE acts on the same receptors as cocaine to prolong cocaine-mediated effects, it is also metabolized by the same enzymes as cocaine. As shown in Fig 2, if enough cocaine and ethanol are around, then CE production continues. Also, ethanol can continue to interact with CES 1, thus preventing CE hydrolysis to BE. Otherwise, it can be metabolized by CES 2 into ecgonine
ethyl ester (EEE) or by CES 1 to BE. In dogs, when CE was administered alone, BE achieved concentrations similar to when cocaine was administered, indicating that BE is a major CE metabolite. A steady dose of ethanol (1 g/kg) with different IV cocaine doses (0.3, 0.6 or 1.2 mg/kg) in volunteers exhibited a dose-dependent increase in CE concentrations. In fact, for every 10 mg of cocaine given, an average of 1.8 mg CE was produced (range 1-4 mg), with approximately 17±6% of cocaine being converted to CE. Even more important was the fact that the fractional conversion from cocaine to CE did not differ significantly among cocaine doses.

Within post-mortem samples of one study, all seven subjects had different plasma ethanol concentrations, ranging from 20-240 mg/dl. Plasma cocaine concentrations ranged from 91-4370 mg/ml and plasma CE concentrations ranged from 142-1447 ng/ml. There was no discernible pattern between increasing CE concentrations with ethanol concentrations. It has been postulated that CE formation may not be proportional with low or high ethanol doses. This is further supported by a study conducted in volunteers given cocaine, but no ethanol. The authors tested the hair of volunteers, and found about 2.33% of the low dose cocaine and 2.22% of the high dose cocaine was converted to CE. It was suspected that soft drinks and other beverages provided small amounts of alcohol. This provides further support to the hypothesis that CE is not dependent on the amount of ethanol consumed. It is also possible that CE is rapidly metabolized to BE and EEE hence providing an alternate explanation for the lack of correlation between alcohol dose and CE production. This theory has not been studied to date, so no conclusions can be made at this point.
2.5 Hair Testing

2.5.1 Conventional Drug Testing Matrices

Cocaine and its metabolites have been detected in urine\textsuperscript{89,90}, blood\textsuperscript{91}, and oral fluid\textsuperscript{92,93} as a part of substance abuse monitoring. While these drug testing methods are used regularly, they have disadvantages. For example, cocaine and its metabolites can be detected in urine, but only for a short time period after use. Intravenous cocaine has been reported to no longer be detectable after 13.5-45 h. BE concentrations (ng/ml) reached single digits after 72.3 h, and EME was almost 0 at about 60 h\textsuperscript{26}. When cocaine was smoked, it was no longer detectable after 3.7-37.2 h, while BE and EME reached single digits after 70 and 69 h respectively. Cocaine taken through the intranasal route was no longer detectable after 10.5-49.7 h, and BE and EME were almost 0 after 70.8 h for both\textsuperscript{26}. Although there is a wide range depending on the route of administration, cocaine will be positive for only 40-120 h in urinalysis\textsuperscript{94,95}. Using plasma levels is even more challenging because of the short elimination half-lives of cocaine than in urine\textsuperscript{96}. This means that in order to use plasma levels to monitor cocaine use, sampling has to occur within a short time frame. As CE has a longer elimination half-life and larger volume of distribution than cocaine\textsuperscript{74}, it can be presumed that CE can be detected for longer periods of time than cocaine and its other metabolites, but not much longer.

Saliva can be used for substance monitoring, and it is less invasive than plasma collection. One study found that saliva to plasma concentration ratios varied from 0.5-2.9 at different time points, but had significant correlations of saliva to plasma cocaine levels for three doses of IV administered cocaine\textsuperscript{97}. Another study found cocaine to be the predominant analyte found in saliva, but BE and EME were also found. When volunteers were stimulated to produce saliva by chewing on candy, cocaine concentrations in saliva decreased. This was likely due to an increase
in saliva pH associated with increased saliva flow. Another possible hindrance to using saliva to screen for cocaine use is that an increase in saliva pH levels impedes the amount of cocaine that can enter the fluid\textsuperscript{98}. A study looking at saliva cocaine and metabolite levels in chronic cocaine users seeking treatment found that unmetabolized cocaine could be found 24 h entering treatment, and up to 5-10 days during abstinence\textsuperscript{99}. However, the detection of unmetabolized cocaine days after the last administration suggests that multiple doses and high cocaine exposures could lead to accumulation in deep body compartments, and be released slowly back into circulation and then excretion\textsuperscript{99}. Having cocaine detectable for up to 5-10 days is beneficial, but only for a subset of cocaine users. This can affect cocaine use monitoring, especially in treatment programs\textsuperscript{99}. CE has also been found in oral fluids, including saliva, and because CE has similar properties to cocaine, it can be inferred that CE will be found in saliva for similar periods of time\textsuperscript{100}.

Another body fluid that can be used for cocaine use monitoring is sweat. Sweat levels can be monitored through the use of adhesive dermal patches that are placed in areas where sweat is released\textsuperscript{101,102}. Drugs can be incorporated into sweat through several different mechanisms, including passive diffusion from blood into sweat glands, as well as trans-dermal drug passage across the dermis of the skin\textsuperscript{103,104}. As such, sweat can be used as a non-invasive method to detect cocaine use due to the relative ease of sample collection. Additionally, the time frame of detection of cocaine can range from 24 hours after the last cocaine administration and up to 7 days\textsuperscript{103,105,106}. CE can also be found in sweat of individuals who used cocaine with good sensitivity while BE was not found with good sensitivity\textsuperscript{104}, indicating that CE has similar properties to cocaine with respect to its incorporation into sweat glands.
Normally, testing for alcohol consumption is conducted with the traditional matrices such as blood, urine, saliva, and expired air for drug licensing and substance abuse monitoring programs\textsuperscript{107-110}. However, there are some disadvantages to using these matrices for alcohol testing. Firstly, ethanol is eliminated rapidly, so multiple samples are required to determine amount and timing of exposure. Secondly, the body metabolizes about 7 g of alcohol per hour, equivalent to one drink per hour\textsuperscript{107}. This equates to a rate of 0.015% of blood alcohol concentration (BAC) per hour. A person with a BAC of 0.08% (the previous legal limit for driving in Canada) will not have any measurable alcohol within 5.5 hours of the last drink, and this applies to breathe testing as well\textsuperscript{111}. As mentioned previously, measuring ethanol in the traditional matrices is only beneficial if alcohol is consumed within hours of sampling. FAEE on the other hand, were found in the serum of volunteers anywhere from 13-24h after ethanol administration\textsuperscript{112,113}, and FAEE increased rapidly after ethanol consumption and persisted even after ethanol was no longer detectable\textsuperscript{112}. Serum FAEE levels can be used as a marker to indicate recent, especially binge-type, ethanol consumption\textsuperscript{113}. Other unique testing methods for FAEE include human pancreas, liver, heart, brain, and white blood cells\textsuperscript{114-116}. Additionally, FAEE have been found in post-mortem liver and adipose tissue\textsuperscript{117}. FAEE has also been found in meconium, a matrix that forms during the last two trimesters of pregnancy\textsuperscript{118}.

\textbf{2.5.2 Advantages of Hair Testing}

While urine, blood, oral fluids, and tissues can be used to detect cocaine and alcohol consumption, these matrices are useful to detect use within a few hours to a few days of sampling. Adult scalp hair grows on average 1 cm per month, and can be segmented, which can reflect both short-term and chronic use and/or exposure\textsuperscript{119}. This is due in part to the fact that the hair matrix remains relatively stable for many months\textsuperscript{120}. Such a phenomenon occurs because
hair integrates xenobiotics during its growth, such as drugs and products of ethanol metabolism, which are present in the blood. Compounds that are trapped within the hair shaft are thus protected from environmental degradation\textsuperscript{121}. 

![Diagram of the simple diffusion model for drug incorporation into hair showing drugs transferring only from blood to the growing hair cells (Adapted from Henderson, 1993)](image)

Figure 3. Diagram of the simple diffusion model for drug incorporation into hair showing drugs transferring only from blood to the growing hair cells (Adapted from Henderson, 1993)

With cocaine and its metabolites, hair provides a unique matrix to determine patterns of use over a long period of time. For example, when compared with urine screening, hair testing had 3.8-fold higher rates of positivity for cocaine\textsuperscript{123}. Such a higher rate of positivity is due to the longer cocaine half-life in hair, which was found to be at a median of 1.5 months for males and 1.5 months for females at an average growth rate of 1 cm/month. BE was found to have a median half life of 1.46 months for males and 1.48 months for females with the same average growth rate\textsuperscript{124}. In fact, it was reported that at least 3 months need to pass before someone who is abstinent is considered negative for cocaine in the segment proximal to the scalp\textsuperscript{125}, and this result was confirmed\textsuperscript{124}. With such a long period of time before someone will have a negative result, this indicates that hair is a viable matrix to use for cocaine testing.

An important aspect of hair testing is understanding the possible routes of cocaine and metabolites incorporation into hair. As stated before, hair incorporates cocaine and its metabolites from the blood stream that surrounds the follicle\textsuperscript{121}, but there are also other routes for cocaine and its metabolites to incorporate into hair.
A more accurate representation of how cocaine and its metabolites are incorporated into adult scalp hair. External contamination, sweat glands, apocrine and sebaceous glands, as well as skin also contribute to total cocaine and metabolite incorporation. Reprinted from Forensic Science International, Vol. 63, Henderson. Mechanisms of drug incorporation into hair. Pg 19-29. Copyright 1993, with permission from Elsevier Limited.
External contamination, usually through contact with cocaine residues or crack smoke in the environment, usually can contribute to positive cocaine and BE results since cocaine and its metabolites remain mostly on the exterior of the hair shaft, but this can be prevented for the most part through proper decontamination procedures\textsuperscript{126}. As mentioned previously, cocaine, BE, and CE can be found in sweat which deposits to the external hair shaft, from which drugs and metabolites can enter into the hair\textsuperscript{122}. No clear correlation was found between dose and concentration in sweat, but the amount of cocaine incorporated into hair appeared to be dose-dependent\textsuperscript{127}. Additionally, cocaine and BE found in sebum can be incorporated into the hair shaft\textsuperscript{128}. Cocaine was also found within skin as well, but BE was not detected, indicating that skin may not act as a cocaine resevoir, but rather cocaine is distributed rapidly to the skin and then eliminated\textsuperscript{128}. Although it is not known how much cocaine is incorporated into hair from each possibility, hair testing provides a good estimation of cocaine use or exposure within a period of months depending on the segment length. The International Society for Hair Testing determined that BE/cocaine ratio must be at least 0.05 or greater to differentiate between use and external contamination\textsuperscript{129}.

In our laboratory, the sum of four FAEEs, specifically ethyl myristate, ethyl palmitate, ethyl oleate and ethyl stearate, are used to determine the amount of alcohol consumption. The four FAEE have been measured in the hair of alcoholics and used successfully to differentiate them from social and non-drinkers\textsuperscript{129,130}. Furthermore, the use of FAEE hair testing has proven to be a useful tool in substance abuse monitoring to confirm abstinence\textsuperscript{119,131}. Because the human body does produce low levels of FAEE from endogenously-circulating ethanol\textsuperscript{108}, a cutoff was established by the Society of Hair Testing in 2009. The sum of the four FAEEs must be at least 0.5 ng/mg to distinguish between social drinking and chronic excessive alcohol consumption\textsuperscript{132}. 
An FAEE level above 0.5 ng/mg scalp hair to has high sensitivity and specificity (90% for each) for chronic excessive alcohol consumption\textsuperscript{129}. Generally, the FAEE level of 0.5 ng/mg corresponds to chronic excessive alcohol consumption, which is defined by the World Health Organization as consuming more than 60 g of pure ethanol per day for several months\textsuperscript{109}. This is the same definition that is used by the consensus of the Society of Hair Testing as well as by most laboratories today in hair testing for chronic excessive alcohol consumption. FAEE levels between 0.2 and 0.5 ng/mg are usually indicative of individuals who consume alcohol socially\textsuperscript{108}, defined as one to two standard drinks per day\textsuperscript{109}. As such, the cutoff of 0.5ng/mg is used clinically as an indicator to identify a positive result for chronic excessive alcohol consumption\textsuperscript{133-135}.

2.5.3 Cocaethylene in Hair

CE, while studied mostly in serum, has also been found in hair of individuals who were known cocaine users and positive for markers of alcohol consumption\textsuperscript{136-141}. CE hair concentrations have been reported to range between 2.5 and 30 ng/mg\textsuperscript{142,143}, but an acceptable range of detected CE concentrations has not been established. Recommendations have been made by the Society of Hair Testing, among other entities, that CE can be used as a marker in hair testing to indicate simultaneous alcohol and cocaine consumption\textsuperscript{144}. To our knowledge, there have been no previous attempts to look at the utilization of CE as a marker of ethanol consumption in a large high-risk population. Such a biomarker may be practically important, as very few laboratories worldwide measure FAEE, while numerous laboratories can measure CE.
CHAPTER 3: MATERIALS & METHODS

3.1 Subject Recruitment

3.1.1 Hair Samples

Many of the hair samples were from individuals involved with Children’s Aid societies for suspected drug and/or alcohol abuse in the context of cases concerning child safety. Others were required to have hair testing for drugs and/or alcohol by the legal system. Individuals consented to providing a hair sample for testing at the Motherisk Clinic at the Hospital for Sick Children, Toronto, Canada, on the advice of Children’s Aid societies or legal professionals. If the clients were unable to visit the clinic, their hair was cut at appropriate sites and mailed to the Motherisk Laboratory, ensuring that geographic location would not be a limitation for hair testing. The use of clinical hair testing results available at the Motherisk Laboratory database was approved by the Research Ethics Committee at the Hospital for Sick Children.

3.1.2 Inclusion and exclusion criteria

Only data from samples which were requested for both cocaine and alcohol testing were used for this study. All data were collected from adult male and female samples, defined as anyone 16 years or older. Sex, ethnicity and hair colour were not required from social workers or legal professionals, thus were not recorded. Sex was found by conversing with social workers and legal professionals, and also based on the name. Samples were included even if they had unmeasurable values for either cocaine and/or alcohol. Additional segments for each person were given different notations and were counted as separate samples to be included in the study.
3.1.3 Sample Collection

Using scissors, a section of hair was lifted from the rear “crown” of the scalp to clear an area for hair sampling. While firmly holding approximately 75 strands (30mg), hair was cut on the vertex posterior (“crown”) as close the scalp as possible using disposable gloves. Two separate samples were required to ensure there was enough hair to test for alcohol and drugs. The root ends were kept aligned and taped to a blank sheet of paper. The paper was folded and then placed in an envelope and sealed. The samples were submitted to the Motherisk Drug Testing Laboratory (MDTL) at the Hospital for Sick Children, Toronto, Canada, along with the Motherisk Chain of Custody Requisition form (Form MDTL 7030A). The form required the following information: the sample collector’s statement and signature to identify the collector, certifying that the collected hair was obtained with informed consent from the donor and photo identification was provided; statement from the donor certifying it is their hair, was given with their consent, and sealed in their presence; drugs to be tested; name and address for results recipient and billing address. For cocaine, cocaethylene, and chronic excessive alcohol consumption using established methods\textsuperscript{145,146}. Results from a total of 588 tests were used for the study dating from September 1, 2010 to May 24, 2011.

3.2 Cocaine, Benzoylecgonine, and CE preparation

3.2.1 Solute Extraction

For cocaine, CE, and BE testing, 10 mg of scalp hair was measured and weighed in a scintillation vial (Kimble Chase, Vineland, NJ) washed with 1 mL dichloromethane for 1 min. The dichloromethane was aspirated and the step was repeated. The hair dried for 10 min, and 1 mL methanol was added. The hair was cut into 1-3 mm sections before the vial was capped and wrapped with parafilm. After intubation for 18 h at 56°C, the methanol was transferred to clean
12x75mm glass test tubes (Fisher Scientific, Ottawa, ON) and dried with 15psi nitrogen on a 35°C pre-heated heating block. The residue was re-suspended in 400µL 1xPBS (pH 7.4). Half the solution was screened for cocaine using the Cocaine Direct Elisa Kit (Immunalysis, Pomona, CA). The technique utilizes a competitive binding receptor assay to verify the presence of cocaine in solution. The method is used qualitatively to identify the presence of cocaine. If positive for cocaine, results were confirmed by headspace solid-phase microextraction (HS-SPME) and GC-MS (Mandel Shimadzu GC-MS QP2010 Plus, Guelph, ON) using a method established in our laboratory.145

3.2.2 Automated Solid-Phase Extraction (ASPEC)

If the samples were confirmed positive with ELISA, 200 µL of solution was transferred to 13x100mm glass test tubes and 20µL of deuterated standard and 20 µL of 20% methoxiamine solution were added to each tube. After vortexing and a 1 h incubation, 2mL of 0.1M phosphate solution were added to the solutions, vortexed, and incubated for 15 min. During the incubation, 100mL of 0.1M HCL and 200mL of elution solution: dichloromethane: isopropanol: ammonium hydroxide (80:20:2) solutions were prepared for automated solid-phase extraction.

The ASPEC (Gilson, Middleton, WI) was turned on and primed for the sample run. Then appropriately labelled 12x75mm glass collection tubes were placed in slots corresponding to the tubes in the collection rack. The test tubes were then placed in their slots and four 700mL ASPEC reservoir bottles were filled with the following solutions were prepared and placed in the machine in the following order: (1) methanol; (2) 0.1M phosphate solution; (3) 0.1M HCl; (4) Elution Solution. The ASPEC program was started and allowed to complete. Once completed, the solutions in collection tubes were transferred to appropriately labelled solid-phase microextraction (SPME) vials (Sigma-Aldrich, Oakville, ON) and dried with N₂ at 35°C. Once the
vials were completely dry, a mixture of BSTFA and MSTFA (3:1) was prepared. Once the heating block cooled, 10µL of derivatizing mixture was added to each vial under N₂ and then placed on the GC-MS rack.

3.2.3 Gas Chromatography/Mass-Spectrometry (GC-MS)

All the GC-MS parameters required to run the samples were completed before the samples were run. The samples were run using an automated SPME injector and were analyzed using a pre-programmed method using GCMS Real-Time Analysis (Shimadzu, Laval, QC). Cocaine ions were identified with m/z ratios of 182, 82, and 303, while CE ions had m/z ratios of 196, 82, and 317. BE ions had m/z ratios of 240, 82, and 361. Cocaine was also identified using the retention time of 15.602 compared with the standard retention time of 15.583. CE had a retention time of 15.583 and its standard had a retention time of 16.212, while BE was 16.24 and its standard had a retention time of 16.193. Because BE and CE have similar retention times, if there was a large BE detected and CE was positive, then the assumption was made that the positive CE could have been due to the high BE concentration, and thus was considered negative. The lower Limit of Quantification (LOQ) was 0.4 ng/mg and the Limit of Detection (LOD) was one-third of the LOQ. Trace CE results were concentrations between the LOD and LOQ and were not quantifiable. Trace CE values were given a value of 0.13, representing the lowest possible concentration that can be detected by GC-MS.
3.3 Fatty Acid Ethyl Esters Sample Analysis

3.3.1 FAEE Extraction

For alcohol testing, the samples were pre-washed with heptanes (approximately 30 ml) in a 50 mL beaker by stirring with a spatula every 5-10 min for 30 min to remove external contamination. The solvent was decanted and the hair was allowed to dry for approximately 30 min. Once dry, the samples were cut into 1-3 mm long pieces using scissors and 20 mg were weighed out for extraction into a 4 ml amber vial and stored in a dry place at room temperature until testing. When ready for testing, 20 µl IS was added to each vial along with 500 µl DMSO and 2 ml heptanes. The samples were capped and placed in the shaker at 975 rpm for 15 h overnight at room temperature. The samples were removed and placed in a cold room (2-8°C) for 60 min until the DMSO became solid, and then the heptane was decanted into 10 mL clear SPME vials (Sigma-Aldrich, Oakville, ON) in the fume hood. The heptane layer was evaporated at 40°C under nitrogen flow until the samples were completely dry. Then 1 ml of 0.1M phosphate buffer, pH 7.6 was added to each sample and they were capped and sent off for the GC-MS for analysis. Extraction and analysis of four FAEE species (ethyl oleate, ethyl myristate, ethyl palmitate, ethyl stearate) was conducted by liquid-liquid extraction with a heptane and dimethyl sulfoxide mixture followed by automated headspace solid-phase microextraction and GC-MS/EI (Mandel Shimadzu GC-MS QP2010 Plus, Guelph, ON) analysis as previously reported\(^{146}\).
3.3.2 Gas Chromatography/Mass-Spectrometry

All the GC-MS parameters required to run the samples were completed before the samples were run. The samples were run using an automated SPME injector and was analyzed using a pre-programmed method using GCMS Real-Time Analysis (Mandel, Guelph, ON). Ethyl myristate had qualifier m/z ratios of 88, 101, and 157 and a quantifying m/z ratio of 256. Ethyl palmitate had the same qualifier m/z ratios but a quantifying m/z ratio of 284. Ethyl oleate had qualifier m/z ratios of 88 and 101 and a quantifying m/z ratio of 310. Ethyl stearate had the same qualifier m/z ratios as ethyl myristate, but a quantifying m/z ratio of 312. Samples were reported as NSQ if there was not enough hair to run the samples. If the sum of the four did not amount to ≥0.2 ng/mg, then it was reported as negative ≤0.2 ng/mg. If the samples were between 0.2-0.49 ng/mg, then it was reported as negative for chronic excessive alcohol consumption and an explanation is provided below. However, if the samples ≥0.5 ng/mg, then the results were reported as positive for FAEE, indicating chronic excessive alcohol consumption. FAEE between 0.2-0.49 ng/mg can be indicative of low/moderate alcohol consumption, but there are many confounding factors that prevent the identification of concentrations within this range to be considered low/moderate alcohol consumption conclusively.

3.4 Statistical Analysis

3.4.1 Data Collection

We identified cases where cocaine, BE, CE, and FAEE were measured, and the concentrations were collected into an Excel file and organized these based on case sample number. Only individuals who were tested for both cocaine and alcohol were used in the study. Both positive and negative results were recorded.
3.4.2 Statistical Analysis

Statistical analyses were performed using SPSS Package Software (SPSS, Version 17, Chicago, Illinois) and GraphPad Prism Software (GraphPad Prism, Version 5, La Jolla, California). The total population was stratified into two subgroups, one representing individuals who were chronic alcohol abusers (FAEE ≥0.5 ng/mg of hair), and the other being social/non-drinkers (FAEE 0.2-0.49 ng/mg of hair). For the purpose of the study, social drinkers were identified with FAEE concentrations between 0.2-0.49 ng/mg, but this was only used for the study and was not used when reporting results. Within the alcohol abuse cohort, individuals were stratified into cocaine users (BE≥5% of cocaine) versus just cocaine-exposed (BE<5% of cocaine). To assess whether cocaine, BE, and CE concentrations increased with FAEE concentrations indicating chronic excessive alcohol consumption, we used the Mann-Whitney Test. A Chi-Squared Test was used to compare proportions of cocaine users who were identified as chronic excessive alcohol abusers within the study population. Logistic regression was used to identify if being positive for FAEE and cocaine use could predict a positive CE result. Lastly the sensitivity and specificity along with positive and negative predictive values of using CE to indicate chronic excessive alcohol consumption were calculated, using the following formulae:

\[
\text{Sensitivity} = \frac{TP}{TP + FN}, \quad \text{Specificity} = \frac{TN}{TN + FP}
\]

\[
PPV = \frac{TP}{TP + FP}, \quad NPV = \frac{TN}{TN + FN}
\]

TP=True Positive \quad TN= True Negative \quad FN= False Negative\quad FP= False Positive \quad PPV= Positive Predictive Value \quad NPV= Negative Predictive Value
CHAPTER 4: RESULTS

4.1 Demographics

A total of 539 individuals were tested during the study time frame, for which a total of 588 samples were tested. Of the 588 samples, 288 were from females and 127 were from males. The remaining 173 were unknown because the sexes of the individuals were kept confidential by Children’s Aid societies and legal professionals. The ages ranged from 17-64, with the median age being 30 years. While many individuals were from Ontario, samples were received from all across Canada. No information was available on education, socioeconomic background, and living situation as those were kept confidential.

4.2 Comparison of Cocaine, BE, and CE Concentrations with FAEE Concentrations

Out of 588 samples, a total of 353 non/social drinking and 235 chronic excessive alcohol abuse samples (≥0.5 ng/mg) were identified based on the accepted cut-offs of hair FAEE. No comparisons were made between FAEE <0.2 ng/mg and FAEE between 0.2-0.49 ng/mg because there were too few samples compared to the other groups used for statistical analysis. Overall, 44 samples were found to have quantifiable CE concentrations and 6 had trace CE concentrations. The number of individuals in each classification of cocaine use (BE≥5% of cocaine concentration) is listed in Table 1. CE concentrations were found to range from 3.5% to 104% of cocaine concentrations.
Table 1. Number of positive cocaine use samples in each range identified by the concentration of cocaine in hair.

The ranges represent the percentile concentrations of cocaine associated with specific patterns of use. The low range indicates isolated or sporadic use, the medium range indicates repeated cocaine use, while the high range indicates frequent cocaine use on more than one occasion.

The mean cocaine levels in the social drinking vs. chronic alcohol abuse population was significantly different (Mann-Whitney U Test: 273.02 and 326.76, \( P<0.001 \)) respectively. For BE, the mean levels in the same groups were also significantly different (Mean Ranks: 277.31 and 320.33, \( P<0.001 \)). For CE, the mean levels were similar (Mean Ranks: 294.23 and 294.91, \( P=0.962 \)). The mean levels for cocaine use in social drinking vs. chronic alcohol abuse populations were significantly different (Mean Ranks: 280.05 and 315.60, \( P=0.001 \)).

4.3 Cocaine Abusers who also Abused Alcohol Chronically

Analysis of the social drinking/non-drinking population, consisting of 353 positive samples, found that 250 were only cocaine-exposed, while 103 were identified as positive cocaine use. Of the 235 samples in the chronic alcohol abuse population, 136 were identified as only cocaine-exposed, while 99 were identified as positive cocaine users. Of the 353 social/non-drinking individuals, 250 were either exposed to cocaine or negative, comprising 70.8% of the subset of
the study population. This subset consisted of samples having FAEE concentrations indicating social drinking, as well as abstinence from alcohol. The remaining 103 were identified as cocaine use, comprising 29.2% of the same population. Of the 235 chronic alcohol abuse samples (FAEE ≥ 0.5 ng/mg), 136 were not considered as cocaine use, about 57.9% of the subset. The remaining 99 FAEE positive samples were also positive for cocaine use, comprising 42.1% of the subset of the alcohol abuse group. The proportions of cocaine use samples also exposed to chronic alcohol abuse or social/non-drinking were found to be significantly different from the proportion of cocaine-exposed or cocaine negative samples (OR 1.767, 95% CI 1.25-2.497, P=0.002).

4.4 Predicting Positive CE Results

Conducting a logistic regression, cocaine use (BE ≥ 5% of cocaine concentration) was associated with a positive CE result (OR = 15.56, 95% CI 5.95-40.67, P<0.001). A positive FAEE result (≥ 0.5 ng/mg) was also found to be associated with a positive CE result (OR = 2.44, 95% CI 1.22-4.87, P=0.012). For the overall cocaine group, which included cocaine users, cocaine-exposed, and negative cocaine results, no association was found with a positive CE level (OR = 1.053, 95% CI 0.98-1.13, P=0.134).

4.5 Sensitivity and Specificity of CE as a Biomarker of Chronic Alcohol Abuse

The values for the CE and FAEE positive and negative results are listed in Tables 2-4. Table 2 included all samples, but considered trace CE results as negative. Table 3 included all the values for all of the samples tested, while Table 4 was selective to the samples positive for cocaine use. The values for CE positive and negative with FAEE < 0.2 ng/mg and FAEE ≥ 0.2 ng/mg are listed in Table 5, Table 6 and Table 7. Table 5 has the same conditions as Table 2, Table 6 the same conditions for samples as Table 3, and Table 7 the same as Table 4.
<table>
<thead>
<tr>
<th></th>
<th>FAEE Positive (≥0.5 ng/mg)</th>
<th>FAEE Negative (&lt;0.5 ng/mg)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE Positive</td>
<td>27</td>
<td>15</td>
<td>42</td>
</tr>
<tr>
<td>CE Negative</td>
<td>208</td>
<td>338</td>
<td>546</td>
</tr>
<tr>
<td>Total</td>
<td>235</td>
<td>353</td>
<td>588</td>
</tr>
</tbody>
</table>

**Table 2.** Distribution of results for each condition of FAEE positive/negative with CE positive/negative values for all the tested samples with trace CE considered as negative.

<table>
<thead>
<tr>
<th></th>
<th>FAEE Positive (≥0.5 ng/mg)</th>
<th>FAEE Negative (&lt;0.5 ng/mg)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE Positive</td>
<td>33</td>
<td>17</td>
<td>50</td>
</tr>
<tr>
<td>CE Negative</td>
<td>202</td>
<td>336</td>
<td>538</td>
</tr>
<tr>
<td>Total</td>
<td>235</td>
<td>353</td>
<td>588</td>
</tr>
</tbody>
</table>

**Table 3.** Distribution of results for each condition of FAEE positive/negative with CE positive/negative values for all the tested samples. Trace CE values were considered as positive.

<table>
<thead>
<tr>
<th></th>
<th>FAEE Positive (≥0.5 ng/mg)</th>
<th>FAEE Negative (&lt;0.5 ng/mg)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE Positive</td>
<td>33</td>
<td>17</td>
<td>50</td>
</tr>
<tr>
<td>CE Negative</td>
<td>67</td>
<td>87</td>
<td>154</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>104</td>
<td>204</td>
</tr>
</tbody>
</table>

**Table 4.** Distribution of results for each condition of FAEE positive/negative with CE positive/negative values for samples positive for cocaine use.
<table>
<thead>
<tr>
<th></th>
<th>FAEE ≥0.2 ng/mg</th>
<th>FAEE &lt;0.2 ng/mg</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE Positive</td>
<td>38</td>
<td>4</td>
<td>42</td>
</tr>
<tr>
<td>CE Negative</td>
<td>403</td>
<td>143</td>
<td>546</td>
</tr>
<tr>
<td>Total</td>
<td>441</td>
<td>147</td>
<td>588</td>
</tr>
</tbody>
</table>

Table 5. Distribution of results for each condition of FAEE <0.2 ng/mg and ≥0.2 ng/mg with CE positive/negative values for all the tested samples with trace CE considered as negative.

<table>
<thead>
<tr>
<th></th>
<th>FAEE ≥0.2 ng/mg</th>
<th>FAEE &lt;0.2 ng/mg</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE Positive</td>
<td>46</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>CE Negative</td>
<td>395</td>
<td>143</td>
<td>538</td>
</tr>
<tr>
<td>Total</td>
<td>441</td>
<td>147</td>
<td>588</td>
</tr>
</tbody>
</table>

Table 6. Distribution of results for each condition of FAEE <0.2 ng/mg and ≥0.2 ng/mg with CE positive/negative values for all the tested samples.

<table>
<thead>
<tr>
<th></th>
<th>FAEE ≥0.2 ng/mg</th>
<th>FAEE &lt;0.2 ng/mg</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE Positive</td>
<td>46</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>CE Negative</td>
<td>116</td>
<td>38</td>
<td>154</td>
</tr>
<tr>
<td>Total</td>
<td>162</td>
<td>42</td>
<td>204</td>
</tr>
</tbody>
</table>

Table 7. Distribution of results for each condition of FAEE <0.2 ng/mg and ≥0.2 ng/mg with CE positive/negative values for all the samples positive for cocaine use.

The sensitivity and specificity of FAEE to indicate positive/negative CE results, along with the PPV and NPV of CE to identify positive FAEE results are listed in Tables 8-10. Table 8 included all the samples and trace CE results were considered negative. Table 9 included all the samples, where trace values were considered positive. Table 10 was restricted to samples positive for cocaine use. The distinctions are made between chronic alcohol abuse (FAEE ≥0.5 ng/mg) and
social/non-drinking (FAEE <0.5 ng/mg). Also, another comparison is made between non-drinking (FAEE<0.2 ng/mg) and any amount of alcohol consumption (FAEE ≥0.2 ng/mg).

<table>
<thead>
<tr>
<th>CE Value</th>
<th>FAEE (ng/mg)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>AUC</th>
<th>Sig (P)</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;0.200</td>
<td>&lt;0.5 or ≥0.5</td>
<td>11.49</td>
<td>95.73</td>
<td>0.5457</td>
<td>0.06012</td>
<td>0.6428</td>
<td>0.6190</td>
</tr>
<tr>
<td>&gt;0.065</td>
<td>&lt;0.200 or ≥0.200</td>
<td>4.36</td>
<td>97.28</td>
<td>0.5080</td>
<td>0.7739</td>
<td>0.904</td>
<td>0.2619</td>
</tr>
</tbody>
</table>

Table 8. The sensitivity and specificity of FAEE for CE was assessed in chronic excessive alcohol abuse population (FAEE >=0.5 ng/mg) vs. a social/non-drinking population (FAEE <0.5 ng/mg). The sensitivity and specificity of FAEE for CE was also assessed in the non-drinking (<0.2 ng/mg FAEE) versus any amount of alcohol consumption (≥0.2 ng/mg FAEE). The positive and negative predictive values for each condition were also calculated. Trace CE results were considered negative.

<table>
<thead>
<tr>
<th>CE Value</th>
<th>FAEE (ng/mg)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>AUC</th>
<th>Sig (P)</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;0.065</td>
<td>&lt;0.5 or ≥0.5</td>
<td>14.04</td>
<td>95.18</td>
<td>0.5457</td>
<td>0.06012</td>
<td>0.66</td>
<td>0.6245</td>
</tr>
<tr>
<td>&gt;0.065</td>
<td>&lt;0.200 or ≥0.200</td>
<td>10.43</td>
<td>97.28</td>
<td>0.5385</td>
<td>0.1618</td>
<td>0.9200</td>
<td>0.2657</td>
</tr>
</tbody>
</table>

Table 9. The sensitivity and specificity of FAEE for CE was assessed in chronic excessive alcohol abuse population (FAEE >=0.5 ng/mg) vs. a social/non-drinking population (FAEE <0.5 ng/mg). The sensitivity and specificity of FAEE for CE was also assessed in the non-drinking (<0.2 ng/mg FAEE) versus any amount of alcohol consumption (≥0.2 ng/mg FAEE). The positive and negative predictive values for each condition were also calculated. Trace CE values were considered positive.
Table 10. The sensitivity and specificity of FAEE for CE was assessed in chronic excessive alcohol abuse population (FAEE ≥0.5 ng/mg) vs. a social/non-drinking population (FAEE <0.5 ng/mg). The sensitivity and specificity of FAEE for CE was also assessed in the non-drinking (<0.2 ng/mg FAEE) versus any amount of alcohol consumption (≥0.2 ng/mg FAEE). The positive and negative predictive values for each condition were also calculated. Only positive cocaine use samples were used.

If trace CE results were considered negative, FAEE≥0.5 ng/mg was found to have 11.49% sensitivity for positive CE and FAEE<0.5 ng/mg had 95.73% specificity for negative CE for all the samples. However, when trace CE results were considered positive, the sensitivity of FAEE≥0.5 ng/mg to detect positive CE increased to 14.04%, while the sensitivity of FAEE<0.5 ng/mg to detect negative CE results decreased very little to 95.18%. When restricted to the cocaine use samples, the sensitivity increased tremendously to 33.00% and specificity decreased to 83.65%, but the relationship was found to be significant. The PPV was higher in the condition where trace CE results were considered positive, thus improving the proportion of positive CE results that are expected to be FAEE positive. Also, the NPV increased, increasing the proportion of negative CE results that are expected to be FAEE negative. It did not matter if only cocaine use samples were considered, as the PPV between all samples and cocaine use samples remained the same. Unexpectedly, the NPV decreased in the cocaine use condition compared to when all samples were considered.
When comparing FAEE $\geq 0.2$ ng/mg against FAEE $<0.2$ ng/mg, the results were similar. When trace CE results were considered positive, the sensitivity of FAEE $\geq 0.2$ ng/mg for positive CE results went from 4.36% to 10.43% and the specificity of FAEE $<0.2$ ng/mg for negative CE results remained the same. The PPV and NPV also increased, although not by a large amount. In the cocaine use samples, sensitivity increased to 27.95% and specificity decreased to 90.48%. The PPV decreased compared to all the samples where trace CE results were considered positive, but were very similar. The same statement can be made for the NPV when comparing the same conditions. The relationships of sensitivity and specificity were not significant in all three conditions.
CHAPTER 5: DISCUSSION

5.1 Effectiveness of CE as a Biomarker of Chronic Alcohol Consumption

5.1.1 Explanation of Demographics

Of 588 tests during the time period, about 49% of the samples, for which the sexes were known, were provided by female individuals, the clear majority of the total sampling population. With 173 samples belonging to people of unknown sex, there is a possibility the number would increase. This seems contrary to what is generally understood about drug use between sexes, where men usually are more likely to use illegal drugs and consume alcohol than women. This could be due to several possible reasons. Motherisk is an organization that is primarily associated with drug use and safety during pregnancy, which caters primarily to a female audience. As well, the Motherisk Laboratory conducts adult, child and neonatal hair tests, as well as meconium tests which cater to a specialized population. Since our laboratory deals mainly with many children’s aid societies, the need to test pregnant women and women with children is high. As such, this leads to a majority of hair donors being female.

Additionally, the age range of our population fell within expected ranges as Canadians as young as 15 have admitted to using cocaine and consuming alcohol. Equally important is that an upper age limit has not been established for cocaine and alcohol abuse. Only individuals 16 years of age or older were selected for the study due to their ability to give independent consent for hair testing, and are considered to be adults with respect to hair testing. Another important fact is that many statistics about patterns of use exist for individuals 15 years of age or older, so it was a logical decision to select a similar age group.
5.1.2 Cocaine and Metabolite Concentrations Compared with FAEE Concentrations

Cocaine and BE concentrations were significantly higher in the FAEE positive (≥0.5 ng/mg) group than in the FAEE negative (<0.5 ng/mg) group. Cocaine and BE concentrations in the cocaine use group were also significantly higher in the FAEE positive group than the FAEE negative group. While there was no obvious dose-response that was detected, the result is still an important finding regarding cocaine and alcohol use. It was found that cocaine powder users consumed approximately 108g of ethanol in a drinking episode when not using cocaine, but that number increased to approximately 147.2g when ethanol was consumed with cocaine. Furthermore, there was a statistically significant increase in the mean amount of cocaine consumed during an episode of concomitant cocaine and alcohol use. This increase was in the amount of cocaine powder consumed during an episode, and for total amount of cocaine consumed. Our results also indicate that there was an increased amount of cocaine detected in FAEE positive samples. Additionally, there was also an increase in BE concentrations in the same samples, suggesting the possibility that there was increased cocaine consumption, either in the amount or total consumption, with ethanol use. This is important because our study assessed cocaine, BE, CE and FAEE concentrations in a high-risk population, without knowing use patterns conclusively before testing. Our results demonstrate that a high-risk population may have fewer numbers of positive cocaine and alcohol users, but there are still significant patterns of cocaine and alcohol co-abuse. For example, as seen in Table 1, majority of the cocaine users were classified as repeat or frequent users, indicating the possibility of addiction and the increased likelihood of other risk-taking behaviours. These results are disturbing because the combined use of cocaine and ethanol can be highly toxic. With long-term concomitant cocaine and alcohol use being damaging, the combination is potentially lethal.
CE concentrations were not found to be significantly higher in FAEE positive samples compared to FAEE negative samples in the present study. In fact, CE concentrations ranged from 3.5% to 71.44% of cocaine concentrations, suggesting that the rate of CE incorporation into hair does not reflect serum concentrations\textsuperscript{17,22}. Another study assessing the concentrations of CE in hair found CE levels to be between 0%-18.98%, and made the assumption that it relates to heavy alcohol use\textsuperscript{150}, as 17±6% was reported in the blood\textsuperscript{22}. Although this is possible, there are no studies that have demonstrated the incorporation ratio of cocaine from all the possible sources into hair, thus this assumption cannot be made. With respect to the CE concentrations found in our study, there are a few possibilities as to why our results did not match concentrations found in literature. Firstly, there were approximately 73 CE positive samples that were not tested for FAEE as alcohol testing was not requested. It is possible that many of the CE positive results would have also been FAEE positive, thus affecting the significance of CE concentrations in relation to FAEE positive or negative results. Due to ethical reasons surrounding our testing protocol, these samples could not be tested for at least another year from the date when the samples were collected. Secondly, in some cases, CE was classified as inconclusive, which was counted as negative, but the sample could have potentially been positive for CE. The inconclusive result could be due to experimental or mechanical error, and many of the samples were not re-tested due to lack of sufficient hair. Another possible explanation might seem more plausible based on what has been published in the literature. A theory has been proposed that CE formation is not affected by the amount of alcohol consumed\textsuperscript{87}. However, trace amounts of ethanol in soft drinks can induce CE formation\textsuperscript{88}; hence it is unknown how much ethanol is actually required for the transesterification of cocaine to CE to occur. It is important to note that while CE was found in hair of volunteers who did not consume alcoholic beverages; CE was measured at the pg level\textsuperscript{87},
while our laboratory measures CE at the ng level, which is 1000-fold higher than in the study. Thus, any CE formed through the consumption of soft drinks may be inconsequential. Although the measuring at the pg level may not have clinical significance, there is also the issue of cocaine spontaneously transesterifying to CE in ethanol bottles. If individuals identified as positive for CE took the contaminated cocaine, then that could have contributed to such high CE concentrations in hair.

Another possibility is the order in which alcohol and cocaine were consumed. Most studies were conducted with alcohol being given before cocaine, but one study where alcohol was given after cocaine found CE concentrations to range between 4.3%–20.1% of cocaine concentrations\textsuperscript{151}, thus lower than when alcohol was given before cocaine. This finding raises the possibility that the order of administration is important with regards to the formation of CE. If our subjects consumed alcohol at time points after 80 min, this could lead to a lower concentration of CE being formed since it was after cocaine’s half-life. Although this theory is plausible, there is no published literature on the subject, thus no conclusions about the order of administration can be made from our results.

5.1.3 Proportion of Cocaine Use within the FAEE Positive/Negative Groups

Our results demonstrate that a greater proportion of alcohol abusers did also use cocaine compared to the proportion of social/non-drinkers that used cocaine, and this was likely to be concomitant use. When considering it from the perspective of the cocaine use population, the 99 samples represented 49% of the cocaine use samples. While this proportion was closer to 50%, it was still below the expected range of 50–90%\textsuperscript{6–9}. This could be due to the fact that the study population were only suspected of cocaine use, and not actually confirmed cocaine users. In fact, in the majority of the results, either the individuals were negative for cocaine, or were only
exposed to cocaine. It is possible that those individuals were recreational or sporadic cocaine users, and that their use was not detected due to differences in incorporation, amount of cocaine consumed, or consumption that occurred prior to the tested time frame. Another fact is that it is expected that majority of alcohol abusers do not abuse cocaine\textsuperscript{152}, thus our population may reflect the accepted statistics regarding alcohol abusers.

Despite the possibilities mentioned above, the results are important to consider on a population perspective. The sample population represents only a 9 month period from which data were included only where both cocaine and alcohol testing was requested. There were many more samples that were positive for cocaine use but not tested for alcohol abuse, so how those values could affect our proportions is unknown at this time. Even within a suspected population, these results demonstrate possible high-risk behaviour by many individuals, and how this behaviour impacts their lifestyle is yet to be determined. While social workers and legal professionals are aware of cocaine and alcohol abuse trends, these results might suggest the need to emphasize the benefits of hair testing for both in individuals. With more knowledge of hair testing, we should see an increase in testing for both cocaine and alcohol.

5.2 Use of CE in General Hair Testing for Drugs of Abuse

5.2.1 Do Cocaine and Alcohol Use Predict Positive CE Results?

We first looked to see if just having a cocaine positive result would predict the ability to achieve a CE positive result. The group called “cocaine” included cocaine use as well as cocaine exposure. As majority of the results were only cocaine exposure, it was expected that there would not be a significant relationship between “cocaine” and CE results. In the context of how a sample could be considered cocaine exposure, there are a few possibilities. Firstly, there could have been use, but it was not determined from the hair test. In addition, there may have been use
prior to the tested time frame, and the cocaine concentration remaining would be residual amounts. Also, if an individual touches a contaminated surface, or handles cocaine\textsuperscript{153}, then the residues remaining on their hands can be rubbed into hair and enters the hair shaft. As well, if an individual is in direct, frequent, intimate, physical contact with a cocaine user, then residues from the user can transfer to the other individual. Lastly, an individual can be positive for cocaine exposure if they are in an environment where cocaine “crack” is being smoked\textsuperscript{153}. As expected, the overall cocaine group positive result does not indicate the likelihood of achieving a positive CE result. As mentioned previously, CE is formed endogenously in the liver by CES 1 in the presence of ethanol. If individuals are only exposed to cocaine, meaning that cocaine was not ingested, then CE should not be detected. Even while CE can be found in the cocaine product used by individuals, there were not many results that had CE, thus suggesting that CE being part of the cocaine “formulation” may not have influenced the results.

When only the cocaine use (BE\textgeq5\% of cocaine concentrations) results were considered, positive results were 15.56 times more likely to achieve a positive CE result. This is in line what was expected since CE is thought to be produced when cocaine has been ingested into the body and undergone transesterification in the liver. CE was not seen if cocaine was not present, and in all cases, CE was only present if BE was present in at least trace concentrations. This provides further support to the expectation that CE was only present when cocaine was consumed, and that CE was not a part of the original product. Six samples were CE positive but had FAEE levels below our limit of quantification, equivalent to a teetotaller or someone who drinks on rare occasions. Those results also provide support to the theory that CE production may not be dependent on the amount of alcohol consumed, but this has yet to be confirmed. The trace CE
results could also be due to the possibility that CE was a part of the original cocaine product that was used.

FAEE positive results were only 2.44 times more likely to achieve a positive CE result. While this relationship was significant, FAEE’s ability to predict a positive CE result was much less than the ability of the cocaine use group to predict positive CE levels. This could be due to a few possible reasons. The simplest explanation is that many people may consume excess amounts of alcohol at different times than when they use cocaine. This conclusion was made previously, among others regarding cocaine and alcohol consumption\textsuperscript{150}. While this may be true in some cases, this cannot be assumed for all cases, as cocaine and alcohol use patterns were not reported by the individuals who provided the samples for testing. Additionally, there are other concerns that need to be addressed before making such conclusions, and this was discussed in a commentary\textsuperscript{154}. Another explanation is that because majority of the samples were FAEE positive, this indicates that many individuals were chronic excessive alcohol abusers. As already mentioned, majority of alcohol abusers do not abuse cocaine\textsuperscript{152}, so it is expected that FAEE positive results will have a low predictability of CE positive results.

CE positive results where FAEE concentrations were <0.5 ng/mg may have also contributed to the lower likelihood of predicting positive CE results. This may have occurred due to a few reasons. For instance, cocaine users may not have consumed alcohol in excess when using cocaine, and may not have consumed excess amounts of alcohol chronically outside of using cocaine as well. Also, it might be possible that on the rare occasions when cocaine was consumed, that alcohol was also abused, and that specific pattern of alcohol consumption would not be detected. This, however, is only a theory and thus should not be interpreted as a conclusion. Lastly, the possibility that CE was a part of the final product cannot be ruled out.
5.2.2 Ability of Positive FAEE to Predict Positive CE Results

As seen in the results, Tables 2-7 list the number of samples that were positive and negative for the combination of FAEE and CE concentrations. Table 2 has fewer positive values than Table 3 for the combination of FAEE≥0.5 ng/mg and CE positive results. This is due to the fact that trace values were considered negative results in Table 2. Our laboratory defines trace concentrations as between the LOD and the LOQ. Since trace concentrations cannot be quantified, the results may be interpreted as negative. Tables 8-10 list the sensitivity and specificity of the proportion of FAEE positive/negative and FAEE <0.2/≥0.2 ng/mg that were positive or negative for CE. The PPV and NPV in each condition are listed, along with the concentration cutoffs at which the results apply. In this situation, we demonstrated that 11.49% of FAEE positive (≥0.5 ng/mg) results tested positive for CE, and 95.73% of FAEE negative (<0.5 ng/mg) results tested negative for CE. However, because there is no established dose-response relationship between ethanol and CE, even trace CE concentrations may indicate alcohol and cocaine co-consumption. As such, we decided to see if there would be any improvement in the sensitivity and specificity if trace concentrations were considered as CE positive. As expected, sensitivity increased to 14.04% of FAEE positive samples tested positive for CE. Also, there was very little change in specificity, with 95.18% of FAEE negative samples testing negative for CE. This demonstrated that including trace CE results as positive improves the sensitivity, thus increasing the proportion of FAEE positive results that tested positive for CE. It should be noted that trace CE values should be used with caution to predict FAEE positive results, as their concentrations could not be quantified. Another concern is that CE could be incorporated from its presence in the original cocaine product that was used by individuals. Rather, trace CE concentrations could possibly be
used to indicate cocaine use, and possibly some concomitant alcohol consumption, which could still be dangerous to the cocaine user.

When the results were restricted to only samples positive for cocaine use, the sensitivity increased to 33.00%, thus further increasing the proportion of FAEE positive results that tested positive for CE. The specificity decreased to 83.65%, but this was expected as many of the results with no detectable cocaine, BE, and CE concentrations were excluded. Even with an approximately 14% decrease, many of the FAEE negative results tested negative for CE. It was expected that majority of samples positive for cocaine use would also be positive for FAEE, as majority of cocaine users also abuse alcohol. However, this was not the case, as only 99 samples were positive for cocaine and FAEE. This could be due to the fact that the samples were collected from suspected cocaine and alcohol abusers, so our study population is not representative of a known cocaine use population.

Among the three conditions, sensitivity increased, but never reached a level that would indicate that majority of FAEE positive results tested positive for CE. This can make it difficult to be used as a screening tool to confirm co-abuse, because there is a high rate of false negative results. In other words, many individuals who are suspected of concomitant cocaine and alcohol use would not be positive for co-abuse. This is in spite of CE being found in cocaine products before consumption. As discussed in previous sections, the majority of alcohol abusers do not abuse cocaine\textsuperscript{152}, thus it is expected that majority of the results would be negative for CE. On the other hand, the specificity demonstrated the opposite trend, decreasing as the conditions of samples considered for testing changed. Although there was a decrease, the specificity of negative FAEE results testing negative for CE remained high. As a screening tool, this is beneficial because it reduces the chance of false positives that might result if the specificity was
low. In a clinical setting, this can be interpreted that if individuals were negative for chronic excessive alcohol consumption, then it is possible that they would not be positive for CE.

We then wanted to assess the sensitivity and specificity of positive FAEE≥0.2 ng/mg to detect positive CE results. If trace CE results were considered negative, then FAEE≥0.2 ng/mg were only positive for CE 4.36% of the time, whereas FAEE<0.2 ng/mg were negative for CE 97.28% of the time. When trace CE results were considered positive, FAEE≥0.2 ng/mg were positive for CE 10.43% of the time, while the specificity remained the same. Once again, the increase in sensitivity demonstrated the benefit of including trace amounts as positive results. Within the cocaine use samples, the proportion of FAEE≥0.2 ng/mg results that were positive for CE increased to 27.95%, while the proportion of FAEE<0.2 ng/mg that were negative for CE decreased to 90.48%. Just as in the separation between FAEE≥0.5 ng/mg and FAEE<0.5 ng/mg, the sensitivity increased. This was expected, as CE should not form if cocaine was not consumed. Even if the original cocaine product was contaminated with CE, this did not seem to influence the results; otherwise we would have seen many more CE positive results. Although the specificity decreased, FAEE<0.2 ng/mg results indicate that CE was not detected in almost all the cases, decreasing the rate of false positivity tremendously. This is also beneficial as a screening tool, because if any amount of alcohol was consumed, it was not consumed with cocaine. Additionally, this indicates the possibility that cocaine contaminated with CE was not consumed, reducing the potential for health risks in these individuals.

5.2.3 Ability of Positive CE to Predict Positive FAEE Results

The PPV and NPV were calculated to find the proportion of CE positive results that were also FAEE positive and negative in all three conditions. When trace CE concentrations were considered negative, 64.28% of positive CE results were also positive for FAEE (≥0.5 ng/mg),
and 61.9% of negative CE results were also negative for FAEE (<0.5 ng/mg). As a result, majority of CE positive results indicated that alcohol was consumed chronically in excessive amounts. When trace CE concentrations were considered positive, 66% of positive CE results were also positive for FAEE, and 62.45% of negative CE results were negative for FAEE. Including the trace concentrations in the samples increases the proportion of FAEE positive results, as well as the proportion of FAEE negative results. In the cocaine use group, the proportion of positive CE results that were popular for FAEE remained the same, while the proportion of negative CE results that tested negative for FAEE decreased to 56.49%. The decrease in the NPV was due to the reduction of negative values when only selecting for cocaine use. The results indicate that if positive CE is detected in a particular hair sample for an individual, then it is more than likely that the same individual could be classified as a chronic excessive alcohol abuser. From a clinical stand point, the result of 0.66 is not enough to completely confirm a diagnosis of chronic excessive alcohol consumption. This can be an issue in alcohol abuse treatment, as other routes of identification are already established. Instead, it is possible that using CE in combination with other identifiers, such as FAEE, could be of some use in a clinical setting. If both CE and FAEE (≥0.5 ng/mg) were positive, then this could suggest that the individual(s) in question are possible chronic excessive alcohol consumers. With NPV values ranging between 56.49%-62.45%, this indicates that an individual with a negative CE result would also be expected to be negative for FAEE, but this is not proven conclusively. It would be recommended to assess the individual’s behaviour and see if there is any cause to believe that alcohol may be abused by the individual before determining if further testing for FAEE is required.
Although the PPV does not prove definitively that positive CE results were all FAEE positive, with a proportion of 66%, it is more than reasonable to suspect chronic excessive alcohol consumption in addition to the cocaine use. Consequently, a positive CE test would suggest that individual(s) in question are indeed using cocaine, and possibly are using cocaine that is contaminated with CE. Thus, they should be assessed for additional health risks, as CE is known to be toxic. Social workers and legal professionals involved with child custody issues could use the same information provided by the test to their benefit. If CE is positive in hair testing, then this indicates that parents were possibly consuming alcohol in addition to cocaine use. Equally important, it could suggest that the parents were using contaminated cocaine. Either way, the children are at risk for cocaine and/or alcohol exposure. Due to the fact that cocaine is requested far more frequently than alcohol testing, the use of CE as a marker to indicate possible chronic excessive alcohol consumption is only a benefit for a population screening of cocaine and alcohol use. Other than indicating possible co-consumption, positive CE results also indicate the risky behaviour associated with cocaine use, putting children at even greater danger due to other factors that are also involved with cocaine use, such as the physical and psychological side effects.

We also wished to understand the effectiveness of positive CE results to predict any amount of alcohol consumption, so we calculated the PPV and NPV of positive CE to indicate FAEE≥0.2 ng/mg and FAEE<0.2 ng/mg. When trace CE results were considered negative, the proportion of positive CE results that tested positive for FAEE≥0.2 ng/mg was 90.4%, and the proportion of CE negative results that tested negative for FAEE<0.2 ng/mg was 26.19%. When CE trace results were considered positive, the proportion of positive results that tested positive for FAEE≥0.2 ng/mg increased to 92% and the proportion of negative CE results that tested negative
for FAEE<0.2 ng/mg increased to 26.57%. When the results were restricted to only cocaine use samples, both the PPV and NPV decreased to 91.8% and 24.7% respectively. The low NPV values for all three conditions were expected, as only 4 positive CE values had FAEE<0.2 ng/mg. This is important because it suggests that most positive CE results will indicate that either alcohol was consumed with cocaine at levels that would be considered social drinking, or cocaine, that was in alcohol for extended periods of time, was used. This is supported by the fact that the PPV values for all the conditions were at least 90%, implying that alcohol may have been consumed on more than one occasion, which includes social consumption as well as chronic excessive alcohol consumption. Further support is also provided to the assumption that contaminated cocaine was used by the same PPV value. Because it looks at FAEE≥0.2 ng/mg, it is possible that some alcohol was consumed with cocaine, as almost all of our CE positive results had quantifiable FAEE levels.

5.3 Study Limitations

There were limitations to the applicability of our results concerning cocaine and alcohol use to the general population. There is a potential ethnic bias with respect to cocaine incorporation into hair between Caucasians and non-Caucasians. It was found that nine non-Caucasians incorporated on average 2.7 times more cocaine into their hair than Caucasians who were given the same cocaine dose. In particular, it is suggested that African-Americans, Asians, and Hispanics incorporate more cocaine than Caucasians, therefore representing a potential bias in drug testing using hair. It is suspected that darker and thicker hair have higher rates of cocaine incorporation compared to lighter and thinner hair. Published studies have found greater amounts of cocaine in pigmented versus non-pigmented hair within animal and human subjects. Because a greater proportion of minority groups have darker and thicker hair, this may lead in
outcomes that demonstrate a “bias” toward people with dark hair\textsuperscript{161-163}. However, a study which looked at other studies that considered ethnicity as a factor found some issues with study design and some of the conclusions made from results\textsuperscript{160}. For example, many of the studies, there was a small sample size which in itself does not allow for anyone to make conclusions based on their results. Other studies soak the hair in cocaine solutions for an extended period of time, which does not represent conditions seen in vivo. The authors found that black and brown hair had significantly higher rates of cocaine positivity than blond, light brown and medium brown hair. Additionally, grey and red hairs had much less cocaine positivity. Hair and urine analysis for cocaine were similar, but this could be due the drug prevalence in different ethnic/racial groups, as found in epidemiological data\textsuperscript{164,165}. Even though race/ethnicity and hair colour might be a limitation in the generalization of results, there is conflicting evidence that might prove otherwise. Thus, the amount of incorporation, or lack thereof, of cocaine and BE could be due to a person’s ethnicity, but this limitation is taken with caution until the issue of hair colour and ethnicity affecting hair testing results is resolved.

Another limitation was with regard to the sex of our sampling population. Almost majority of our study population were female, thus affecting the applicability of our results to the rest of the population. It is understood that more men use cocaine\textsuperscript{147} and consume alcohol\textsuperscript{1} than women, thus our results might be underreporting the prevalence of cocaine and alcohol abuse in a high-risk population. Another fact is that cosmetic treatment to hair can affect the amount of cocaine detected in hair. For example, in the case of bleaching treatments to hair, the melanin content in hair is reduced\textsuperscript{165}. As cocaine does interact with melanin molecules\textsuperscript{156,159}, it is possible this can lead to reduced cocaine concentrations in hair, and even false negative results. It has been reported that cocaine and BE concentrations decreased to average concentrations of 10% of
starting concentrations\textsuperscript{166}. However, permed and bleached hair seems to have increased absorption capabilities for drugs from sweat, sebum, and external sources; this could lead to an increased rate of false positives\textsuperscript{166}. As it is assumed that women generally cosmetically treat their hair compared to men, this could have lead to an over-reporting of cocaine exposure and/or use, but this has yet to be confirmed.

There is also a potentially important limitation that must be considered with regards to FAEE concentrations in hair. FAEE have been detected in hair care products, most likely formed during production or storage from ethanol and lipid traces. These FAEE may contribute to the FAEE detected in hair, but the concentrations were very low. Permanent wave hair treatment reduced FAEE concentrations by approximately 12%, hair shading reduced $\sum$FAEE in hair matrix up to 11%, and $\sum$FAEE from the hair surface by up to 50%. Ethanol-containing hair spray was found to cause a false-positive result, where the $\sum$FAEE concentrations resembled what occurs after ethanol consumption. In summary, hair lotion and hair spray were found to elevate $\sum$FAEE concentrations and lead to false-positive results\textsuperscript{167}. These results were supported by another study that found hair care products containing as little as 10% ethanol could potentially elevate FAEE concentrations beyond the 0.5 ng/mg cutoff, misidentifying a social drinker or teetotaller as an individual who chronically consumes alcohol in excessive amounts\textsuperscript{168}. The use of specific hair care products was not recorded when samples were collected, and no attempts were made to find out due to the possible impact it could have in many cases, as individuals would buy those products and pretend to use them. It can be assumed that women tend to use more hair care products than men, and as majority of our population was female, this could be a confounding factor that affects the results. Further analysis between sexes is required to assess how this could influence the results.
With respect to CE, there are a few limitations to using CE as a biomarker in hair testing to predict cocaine and alcohol co-consumption. Firstly, the potential ethnic bias that occurs for cocaine incorporation into hair may also exist for CE. This is plausible due to its similar chemical and physical properties\(^4\), meaning that CE could interact with melanin molecules in a similar manner as cocaine. If so, then higher hair CE concentrations should be seen in ethnicities that have darker and thicker hair (such as Hispanics, Asians, and African Americans). However, this requires further study before any conclusions can be made.

Another limitation is the sex bias that occurred within the sample population. Because more women were tested than men, this could influence the presence of CE within the hair samples. As women are more likely to undergo cosmetic treatment on their hair, this permed and bleached hair seem to increase absorption capabilities of drugs from sweat, sebum, and external sources\(^1\), this could lead to a high rate of false positives of CE. A conflicting study found that bleaching hair reduced melanin content, thus cocaine and BE decreased to an average of 10% of their original concentrations\(^1\). Either way, cosmetic treatment affects the rate of cocaine incorporation, so the same can be expected for CE. In addition to the cosmetic treatment, women consume less cocaine\(^1\) and alcohol\(^1\) than men, resulting in a decreased proportion of positive CE results. Therefore, the results achieved are not equally representative of both sexes in a high-risk population. Our belief is that Motherisk, as an organization, is associated with mainly assisting the female population, hence a strong reason for the sex bias. There is also a lack of awareness about hair drug testing within Canada. If more information was provided, our sample population would have been more representative of the general high-risk population.

Lastly, the theory that CE is formed exclusively through cocaine and alcohol co-consumption needs to be explored further before it is accepted. The fact that CE can form from street cocaine...
manufacturing and smuggling practices\textsuperscript{77,78}, as well as in pharmaceutical grade research\textsuperscript{79}, is concerning if CE is to be used as a biomarker in hair testing. With respect to studying cocaine production, the issue of CE production through techniques seems to have been solved\textsuperscript{85}, but it is still possible that some CE could be produced. In regards to illicit cocaine, it is crucial to note that there is no published literature about how much of an effect the production of cocaine or the smuggling techniques have on the concentration of CE found in scalp hair. This must be considered when interpreting the results.

**CHAPTER 6: CONCLUSIONS & FUTURE DIRECTIONS**

To our knowledge, our study is the first to investigate using CE as a biomarker of chronic excessive alcohol consumption in a suspected cocaine-user population. While there was great variability between CE concentrations compared to cocaine concentrations, important trends were identified. Our study demonstrated that expected trends in cocaine and alcohol consumption can be seen in a large sampling population of high-risk individuals. Increased cocaine and BE concentrations within the alcohol abuse group compared to the social/non-drinking revealed that cocaine and alcohol co-consumption is still an important issue in Canada, as it is in the United States\textsuperscript{6-9}. However, CE concentrations were not higher in the alcohol abuse samples, probably due to the few CE positive results compared to all of the samples tested. If more male samples were tested, we might expect to see higher proportions of concomitant users, but such a hypothesis should be taken cautiously considering the study limitations. Either way, cocaine use is still prominent in our society despite countless efforts to educate the public about the dangers of cocaine and alcohol abuse. Clearly, drug prevention efforts are not reaching everyone, and there are at-risk individuals who need to be identified from an early stage.
We also had a greater proportion of positive cocaine use samples in the alcohol abuse group than the proportion of positive cocaine use samples in the social/non-drinking group. While 42.1% is not majority of the sampling population, it provided further evidence that most alcohol abusers do not abuse other drugs. When looking from the cocaine use perspective, approximately 49% of positive cocaine use samples were also positive for alcohol abuse, supporting the accepted standard of 50-90% of cocaine users abusing alcohol. The sex bias that we experienced could have contributed to lower proportions of cocaine and alcohol abusers, which again raises the question of how the proportions would be influenced with more male subjects.

When we considered if cocaine and FAEE concentrations would be associated with positive CE results, we were amazed by how similar our results were to our expectations. While these were common expectations, this is the first study to take into account such associations on a large scale. When all cocaine samples were considered, including cocaine-exposed and cocaine negative, there was no association with positive CE results. This was despite the fact that cocaine can be contaminated with CE before it is used. When only cocaine use samples were considered, positive samples were more likely to be CE positive as well. Even though positive FAEE results were also likely to be CE positive, there was not as strong an association as there was with cocaine use. This was expected because CE is a metabolite of cocaine, and not of alcohol. Despite that fact, the weaker association between FAEE and CE could be due to the fact individuals may have been consuming alcohol at different times than when they were using cocaine. It could also be due to lack of chronic excessive alcohol consumption in our population, thus more people are unlikely to use cocaine as well. Although CE can be produced spontaneously in the presence of ethanol, many of our samples still had some amount of alcohol consumption. Also, with all the CE positive samples, BE was also present at concentrations that
were at least 5% of cocaine. Hence, each of the samples was considered positive for cocaine use as well. For the CE positive samples that had FAEE<0.2 ng/mg, those individuals may have consumed some alcohol with cocaine, but not in excess amounts chronically. Also, the CE results could have come from contaminated cocaine. Still, the amount of alcohol required to produce CE still needs to be studied before any further conclusions can be made regarding dose-concentration relationships.

Lastly, we calculated the sensitivity and specificity of CE positive values to predict chronic excessive alcohol consumption, and also social drinking. When trace values were included as positive results, sensitivity increased, demonstrating that the CE trace levels will positively impact the effectiveness of its use a biomarker. After isolating for only cocaine use samples, the sensitivity further increased, demonstrating that cocaine use impacts the presence of CE in hair. Although the specificity decreased, majority of FAEE negative results were still CE negative as well. An equally important point was that the PPV increased when trace CE concentrations were considered positive results. This means that the proportion of CE positive results that were FAEE positive increased. If an individual has a positive CE result, then it’s more than likely that FAEE would be positive as well. Thus, the possibility of this occurring due to chance is not a valid argument against FAEE testing when CE is positive. With no change in the PPV and a slight decrease in the NPV in cocaine use samples, it can be implied that the PPV value can be applied to our entire population. However, it must be taken into consideration that the PPV does not provide a definite association, so it should not be used as the sole marker to indicate chronic excessive alcohol consumption. Rather, CE could be used as an indicator to test for FAEE in a positive CE sample. Before this can occur, studies need to be conducted that assess how cocaine contaminated with CE affects the results attained in a high-risk population.
When only considering the sensitivity and specificity of FAEE<0.2 ng/mg vs. FAEE≥0.2 ng/mg, it was understood that sensitivity would decrease because of the larger number of FAEE≥0.2 ng/mg/CE negative results. However, the sensitivity increased when CE trace concentrations were considered positive. Similarly, sensitivity increased in samples indicating cocaine use. The PPV increased when CE trace values were positive, but decreased very little in cocaine use samples. This showed that a large proportion of CE positive values were also having concentrations of FAEE≥0.2 ng/mg. While this result does not indicate chronic excessive alcohol consumption, it does indicate that possibly some alcohol may have been consumed with cocaine use. Once the issue of contaminated cocaine and CE is resolved, there is a potential application for the use of CE in substance abuse treatment programs, but this is many years away. Of course, CE would be used with a multitude of other treatment options, as substance abuse is a very complex issue.

In conclusion, this study provides critical information on the utility of CE as an indicator to prompt FAEE testing for possible concomitant cocaine and alcohol abuse. The issue of contaminated cocaine with CE needs to be taken into consideration when conducting interpretations of results. Our results demonstrate that cocaine and alcohol co-consumption is an important public health issue that needs to be discussed more openly in society. Further studies are required to comprehend the effect of varying amounts of alcohol on CE formation and incorporation into hair.

6.1 Future Directions

Although our study demonstrated that positive CE levels were more than likely to indicate chronic excessive alcohol consumption, there are certain factors that prevent us from being able
to estimate the amount of cocaine and alcohol co-consumption required to produce various CE concentrations. Further investigations would be able to examine this issue in greater detail.

6.1.1 Additional CE Incorporation Investigations

Until accurate incorporation rates of CE from blood, sweat, sebum and other routes into hair are established, no definitive conclusions can be made regarding levels of cocaine and alcohol consumption. This can be done by conducting similar studies as done previously\(^{22,129}\) using human volunteers and providing them with cocaine and alcohol. Using radioactively-labelled cocaine can help determine the cocaine pharmacokinetics in those individuals, and help determine how much CE was incorporated into each body fluid. Each concentration from every body fluid must be compared to CE concentrations measured in adult scalp hair. Having this information will provide estimates of how much CE from each fluid contributed to the concentrations found in hair.

Also, the issue of ethnicity and hair colour needs to be studied further with respect to CE incorporation. Conducting the same study as above, but in individuals from different ethnic backgrounds and having different hair colour and thickness will help to potentially resolve the issue of ethnicity. It is important to ensure that enough participants are selected to achieve significant statistical power. As well, there should be equal numbers of men and women in each group matched for age, socioeconomic background, and education level, as well as other confounding factors that may influence the results.

6.1.2 Additional CE Formation Investigations

While the theory has been proposed that the amount of alcohol consumed does not affect CE formation, there is not sufficient support for this theory to be accepted by the scientific
community. CE was previously found in hair of volunteers not given alcohol at 2% of cocaine concentrations, and its levels were attributed to possible trace amounts found in ethanol. Even though this may be a possibility, endogenous levels of ethanol remaining in the body may also have produced the CE detected. An important note is that the CE concentrations were detected at the pg level, which is 1000-fold less than our detected concentrations, which was measured in ng.

A proposed method would be to conduct clinical trials in healthy human cocaine users. The male and female participants would be from different ethnicities and matched for age, socioeconomic background, educational level, and other possible confounding factors that would affect the validity of the study. The participants would be housed in a facility, so their cocaine and alcohol consumption can be monitored. Each subject would be given different concentrations of cocaine and ethanol, and the order of both would be different. Cocaine, BE, CE, and ethanol concentrations would be measured in blood, urine, and other body fluids over periods of time to track the pharmacokinetics of cocaine and ethanol. Hair concentrations would also be measured to understand the incorporation of CE from the various sources when different cocaine and ethanol concentrations were administered. The goals of this proposed study would be to understand how much CE was formed when different ethanol concentrations were consumed. A second goal would be to understand if a possible estimate could be made about how much cocaine and ethanol was consumed based on hair CE concentrations.

Another technique that could be used would be conducting dose-concentration relationship analyses using enzymatic assays. CES 1 and CES 2 could be exposed to different cocaine and ethanol concentrations to assess the amount of cocaine hydrolysis that is transferred to transesterification to form CE. Conducting such a study would be less expensive and less
invasive. Additionally, enzymatic assays should be conducted to propose specific concentrations which can then be tested in animals and humans. On the other hand, in-vitro studies of CE formation are not representative of what occurs in vivo, where there are many other factors that could affect cocaine and ethanol metabolism. Thus, the best solution would be to first perform enzymatic assays, and then move onto clinical trials with human volunteers.

Also, cocaine can be dissolved in different ethanol concentrations for different periods of time to understand how much ethanol exposure is required for CE to form without enzymatic activity. Once CE forms in the different solutions, the cocaine/CE compound would be extracted and provided to human volunteers to assess how much of the CE from the contaminated cocaine product enters the various compartments, including the scalp hair. This will help to find a possible answer to the question of how much CE in street cocaine is incorporated in human scalp hair.

6.1.3 CE Scalp Hair Elimination Rates

Cocaine was found to be detectable in adult scalp hair for a minimum of 3 months until it was no longer detectable in an individual abstaining from cocaine use or exposure\textsuperscript{125}. To our knowledge, there are no published studies about the elimination rate of CE from hair, so there is a possibility that it stays in hair longer than cocaine. It would be recommended to collect scalp hair samples of individuals undergoing successful treatment of cocaine and alcohol abuse. Scalp hair samples would be collected at specific time points during their treatment, and concentrations would be measured. Samples would also be collected for a period of time after completing treatment to determine how long CE is detectable in human hair. This would help to prevent false positives in drug testing; particularly in special cases where individuals claim to be drug and alcohol free for months, yet concentrations are still detected in their hair. By understanding the rate of hair CE
elimination, an estimated period of time between abstinence and hair testing could be provided to best prevent a false positive result. Such knowledge would not only save time in the laboratory, but would also save clients large amounts of money.

6.1.4 Possible Clinical Applications of Hair CE Testing

Hair testing is routinely done in many labs for cocaine and alcohol consumption. In many cases, individuals are suspected of using cocaine and alcohol, and hair testing is useful as a screening process to indicate several months’ worth of consumption or exposure. Since cocaine is already identified using GC-MS methods, the addition of CE to standard cocaine testing is not a complicated process. The methods are similar, with only adding the ability of the analytical machine to identify CE correctly. In addition to testing parents involved with child custody cases, the proposal of using hair testing for CE in a medical setting would aid physicians identify individuals at greater risk for physiological and psychological side effects. By identifying these risks early on in a person’s life, physicians and medical professionals can implement appropriate measures to ensure that people attain the correct substance abuse treatment. The complexity of substance abuse makes it so that CE can be used as a measure alone, but can be a small part of a large network of treatment and intervention options.

Another possible application of hair CE testing would be considered both medical and social. Pregnant women represent a special subsection of the general population because their drug and alcohol use not only affects them, but also their unborn child. Accordingly, hair testing on a pregnant woman would provide evidence of drug and alcohol use during her pregnancy and also provide information on what the fetus was exposed in-utero. Since cocaine is requested much more frequently than alcohol in the Motherisk Laboratory, the presence of CE in scalp hair from the woman could indicate possible alcohol consumption in addition to cocaine use. In-utero
cocaine exposure can affect mother-child interactions\textsuperscript{170}, spontaneous abortion, preterm births, placental abruption and congenital anomalies\textsuperscript{171}. In-utero alcohol exposure is associated with fetal alcohol spectrum disorder (FASD)\textsuperscript{172}, which causes several physical and mental deficiencies\textsuperscript{173}. As there is still no safe level of alcohol consumption that has been established in pregnancy\textsuperscript{173}, any amount of alcohol could pose a danger to the fetus. If CE is detected later in pregnancy, the appropriate measures could be taken. This will enable medical professionals to follow up with the baby for FASD and provide necessary assessments and interventions.

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LIST OF PUBLICATIONS & ABSTRACTS

PUBLICATIONS


ABSTRACTS

