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Thesis

CHARACTERIZATION AND COLORIMETRIC ANALYSIS OF SEMI-SYNTHETIC SALVIA DIVINORUM ANALOGUES

by

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"If I have seen further it is by standing on the shoulders of giants."

- Isaac Newton, 1676

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CHARACTERIZATION AND COLORIMETRIC ANALYSIS OF SEMI-SYNTHETIC SALVIA DIVINORUM ANALOGUES

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Boston University School of Medicine, 2014

ABSTRACT

Salvia divinorum is a hallucinogenic herb from the mint family, *Lamiaceae*. An estimated 1.8 million people over the age of 12 have used *S. divinorum* in their lifetime as of 2008. The abuse of *S. divinorum* is attractive to teens and young adults who wish to experiment with psychoactive materials. The plant material and extracts are widely available via the Internet, and it is known that *S. divinorum* will not show up on common drug screens.

The active component in *S. divinorum* is salvinorin A, which is a non-nitrogenous diterpene that is a highly selective kappa opioid receptor (KOR) agonist, reported to be the most potent naturally occurring hallucinogen. Since salvinorin A is such a selective and potent agonist of the KOR, there is interest in researching analogues in efforts to develop and understand therapeutic drugs for depression, schizophrenia, and other mental illnesses, resulting in the discovery of analogues with increased potency. These semi-synthetic salvinorin analogues have been abused by spraying the drug on innocuous plant material or on cigarette papers as a substrate for smoking. This practice poses a significant

health risk, as most new analogues will have little safety and toxicity data associated with common abuse routes.

Chemical characterization of the potent analogue, salvinorin B ethoxymethyl ether (SB-EME) was performed in order to develop methods of differentiation from *Salvia divinorum* and salvinorin A. These characterization techniques include HPLC, UV/Vis, NMR, and a colorimetric assay with Ehrlich's reagent. Adulteration of other plant materials with salvinorin A and analogues was performed and analyzed to determine if fortified materials can be detected by colorimetric assay.

The validation studies of the HPLC method for SB-EME were found to be accurate (%RE < 12%), precise (RSD = 12%), and linear (R^2 = 0.9993) over the mass range of 0.038 µg – 4.8 µg. The LOD was determined to be 0.038 µg, and the LOQ was determined to be 0.113 µg. Significant matrix effects were observed when using *Salvia officinalis* as a blank matrix, affecting the accuracy and selectivity of the method. However, the purified solutions of SB-EME had baseline resolution from salvinorin A and salvinorin B, which allows for easy qualitative distinction if adulterated samples are suspected.

UV/Vis analysis provided a rapid and facile SB-EME characterization method. The UV/Vis trace for SB-EME was distinguishable from both salvinorin A and salvinorin B. NMR analysis confirmed the structures of salvinorin A, salvinorin B and SB-EME, with resonances specific to each compound.

The colorimetric assay with Ehrlich's reagent provided a red-orange result with salvinorin B and SB-EME, similar to salvinorin A. While this does not provide differentiation in the field, it does allow all materials related to *Salvia divinorum* to be identified and collected for further analysis in the lab, as this colorimetric analysis allows easy distinction from common kitchen herbs such as mint, basil, and sage. Characterization of the colored species in the assay with Ehrlich's reagent was performed with UV/Vis, HPLC, and NMR. The UV/Vis analysis showed a new peak at 500 nm in the aqueous layer, which would correspond to a red-orange color. HPLC analysis revealed a new, highly retained peak from the DCM layer of the assay. ^{1}H NMR analysis indicated that the backbone of the salvinorins was not stable in acid, and the molecule that creates the color was likely a degraded analogue.

The analysis of the adulterated plant materials by colorimetric assay was inconclusive, as color intensity decreased as concentration of spiked standard increased. HPLC analysis of the vial remnants after the colorimetric assay confirm recoveries of the spiking compounds up to an average of 22% for salvinorin A, 96% for salvinorin B, and 41% for SB-EME over all matrices, indicating incomplete deposition of standard material onto the plant material.

In conclusion, salvinorin B ethoxymethyl ether can be detected in the field through the use of Ehrlich's reagent as a colorimetric assay. Further laboratory tests, including HPLC and UV/Vis, were shown to easily distinguish the ether derivative from salvinorin A and B.

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LIST OF ABBREVIATIONS

=O	Carbonyl group
^{1}H	Proton
2D	Two Dimensional
Ac	Acetyl group
ACN	Acetonitrile
ACS	American Chemical Society
Ave	Average
BC	British Columbia
C	Celsius
C17	Carbon at Position-17
C18	Octadecyl carbon chain
C2	Carbon at Position-2
C4	Carbon at Position-4
$CDCl_3$	Deuterated chloroform
CH_2Cl_2	Dichloromethane
cm	Centimeters
Conc	Concentrated
COSY	Correlation Spectroscopy
d	Doublet
DCM	Dichloromethane
dd	Doublet of Doublets

ddd	Doublet of Doublet of Doublets
DEA	Drug Enforcement Administration
DESI-MS	Desorption Electrospray Ionization-Mass Spectrometry
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
dt	Doublet of Triplets
EC_{50}	Half Maximal Effective Concentration
EOM-Cl	Chloromethyl Ethyl Ether
et al.	*et alia*
EtOAc	Ethyl acetate
EtOH	Ethanol
g	Grams
GC/MS	Gas Chromatography/Mass Spectrometry
GI	Gastrointestinal
H	Hydrogen
H_2O	Water
H_2SO_4	Sulfuric Acid
HCl	Hydrochloric Acid
HPLC	High Pressure Liquid Chromatography
Hz	Hertz
iPr_2NEt	N,N-Diisoproylethylamine, Hünig's Base
IR	Infrared Spectroscopy

J	J-coupling, indirect dipole dipole coupling
KOR	Kappa Opioid Receptor
L	Liter
LC/MS	Liquid Chromatography/Mass Spectrometry
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantitation
LSD	Lysergic Acid Diethylamide
M	Molar
m	Multiplet
mAU*s	milliAbsorbance Units times seconds
Me	Methyl group
MeOH	Methanol
mg	Milligram
MHz	Megahertz
min	Minute
mL	Milliliters
mm	Millimeters
mmol	Millimoles
mol	Moles
MOM-Cl	Chloromethyl Methyl Ether
MW	Molecular Weight

N	Normal
n	Number
Na_2CO_3	Sodium Carbonate
Na_2SO_4	Sodium Sulfate
$NaHCO_3$	Sodium Bicarbonate
ND	Not Detected
nM	Nanomolar
nm	Nanometers
NMR	Nuclear Magnetic Resonance Spectroscopy
OAc	Acetate Group
OH	Hydroxyl Group
OMe	Methoxy Group
pDMAB	*para*-Dimethylaminobenzaldehyde
ppm	Parts-Per-Million
PTFE	Polytetrafluoroethylene
RE	Relative Error
R_f	Retardation Factor
RSD	Relative Standard Deviation
RT	Room Temperature
s	Singlet
S. divinorum	*Salvia divinorum*
S. officinalis	*Salvia officinalis*

Sec	Seconds
S/N	Signal-to-Noise Ratio
Sal A	Salvinorin A
Sal B	Salvinorin B
SAR	Structure-Activity Relationship
SB-EME	Salvinorin B Ethoxymethyl Ether
SB-MME	Salvinorin B Methoxymethyl Ether
SPE	Solid Phase Extraction
StdDev	Standard Deviation
SWGDRUG	Scientific Working Group for the Analysis of Seized Drugs
t	Triplet
THC	Δ^9-tetrahydrocannabinol
TLC	Thin Layer Chromatography
US	United States
UV/Vis	Ultraviolet/Visible Light Spectroscopy
δ	Chemical Shift
λ_{max}	Wavelength at Maximum Absorbance
µL	Microliters
µm	Microns
µmol	Micromoles

1. INTRODUCTION

1.1. Background of *Salvia divinorum*

Salvia divinorum is an herb from the mint family, *Lamiaceae* (1). The *Salvia* genus contains a variety of herbs that are commonly known as sage. *S. divinorum* originates from Oaxaca, Mexico and is known to play a role in spiritual ceremonies of the Mazatecs due to the herb's psychoactive properties (2). The plant is typically chewed or brewed into a tea for these ceremonies (1). Consumption of *S. divinorum* produces vivid hallucinations and has been traditionally used to treat anemia, headache, rheumatism, and diarrhea, among other ailments (3).

S. divinorum is a green leafy plant, with large, spade-shaped leaves, hollow square stems and white flowers with purple calyces (see **Figure 1**) (4). The plant rarely flowers or sets seed, therefore it is mainly propagated through stem cuttings (2).

Figure 1. *Salvia divinorum*. Image source: Babu *et al*. (5).

1.2. Abuse Potential

Due to its hallucinogenic properties, this herb has a high potential for abuse. It is commonly known as “salvia,” “diviner’s sage,” “Maria pastora,” “Sally-D,” “purple sticky,” and “magic mint” (4). The effects are reported to include hallucinations of bright lights, vivid colors, and object distortions. Uncontrollable laughter, fear, and other hallucinations have also been described (4). An estimated 1.8 million people over the age of 12 have used *S. divinorum* in their lifetime as of 2008 (6). In 2011, the “Monitoring the Future” survey reported 5.9% of 12th graders had used *S. divinorum* in the past year (6).

There are many factors that make *S. divinorum* attractive as a drug of abuse. The plant material and extracts are widely available via the Internet, “head shops,” and some retail stores. Many websites will provide tips for cultivation, extraction of the psychoactive components, and methods of use (7). It is widely known that *S. divinorum* is not tested for on common drug screens, such as the National Institute on Drug Abuse 5 panel, which tests for cocaine, marijuana, amphetamines, opiates and phencyclidine (5, 8). The regulation of *S. divinorum* is not consistent; for example, it is handled on a state-by-state basis in the US (9). Finally, there is a plethora of anecdotal information on the Internet that indicates that *S. divinorum* is safe to abuse recreationally (5). All of these factors make the use of *S. divinorum* attractive to teens and young adults who wish to experiment with psychoactive materials.

The abuse of *S. divinorum* has also been popularized through social media outlets and news reports of celebrity use. The Internet site, YouTube, allows users to post videos of any topic for public viewing. A search of “Salvia” on this site shows thousands of users posting videos of their experiences taking the drug (10). This popular outlet mischaracterizes the use of hallucinogens as safe and encourages their use.

1.3. Legal Status

S. divinorum is not federally scheduled but has been listed as a “Drug of Concern” by the DEA in 2004 (4, 5). Some states and countries have enacted various regulatory controls on *Salvia divinorum* and/or its active component, salvinorin A, ranging from the strictest Schedule I classification to simple town ordinances regulating possession or sale. In the United States, Alabama, California, Delaware, Florida, Hawaii, Illinois, Kansas, Kentucky, Louisiana, Maine, Maryland, Minnesota, Missouri, Nebraska, North Carolina, North Dakota, Ohio, Oklahoma, Tennessee, Virginia, and Wisconsin have enacted legislation as of July 2012 (6). For example, Brett’s Law, SB259, was enacted in 2006 in Delaware and lists *Salvia divinorum* as a Schedule I controlled substance, making possession, use, or consumption illegal (11). Regulatory controls have been enacted in other countries, such as Australia, Belgium, Denmark, Estonia, Finland, Italy, Japan, Spain, and Sweden (6). The synthetic analogues and by-

products of *Salvia divinorum*, however, are not scheduled or specifically controlled by the states in the US or other countries.

1.4. Salvinorin A

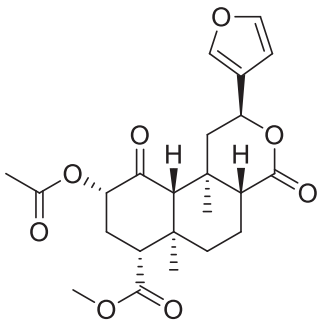


Figure 2. Chemical structure of salvinorin A.

The active component in S. divinorum, salvinorin A (**Figure 2**), was discovered by Ortega *et al.* in 1982 (12). Salvinorin A is a non-nitrogenous diterpene that is a highly selective kappa opioid receptor (KOR) agonist, reported to be the most potent naturally occurring hallucinogen (13). Salvinorin A is found in the peltate glandular trichomes underneath the leaves in concentrations ranging from 0.089 - 0.37% of leaf weight (3, 14). *S. divinorum* is frequently compared to LSD, however the active component is structurally dissimilar to LSD (**Figure 3**) and does not have any effect at the $5HT_{2A}$ receptor, which is the receptor responsible for the hallucinogenic activity of LSD and other drugs (15). Compared to typical KOR ligands, salvinorin A is unusual in that it is not an alkaloid and does not contain any nitrogen atoms (16). It was previously thought that the binding of opioids at the KOR required an interaction between an

aspartate residue of the receptor and an amino group of the opioid (3). There has since been much work to understand if salvinorin A interacts with the receptor site in a different manner, or at a different site of the KOR altogether (3). Salvinorin A is consistent with other KOR agonists, such as dynorphin (3), which produce effects such as sedation, analgesia, inhibition of GI effects, aversion, and depression (1).

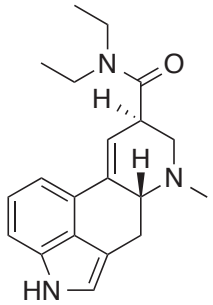


Figure 3. Chemical structure of LSD.

The absorption of salvinorin A occurs primarily through buccal or pulmonary routes. Chewing the leaves produces a hallucinogenic effect with an onset of seconds to minutes and the effects will last up to an hour. Smoking the dried leaves or inhaling vaporized salvinorin A will produce hallucinogenic effects within seconds and last for 20 - 30 min (1). However, the analysis of the thermal degradation products in the smoke of salvinorin A has shown the molecule undergoes deacetylation and conversion to products that are not as potent at the KOR (17).

1.5. Current Presumptive Characterization Techniques

Characterization and analytical detection methods have been described for *Salvia divinorum* and salvinorin A in drug products to include HPLC, LC/MS, GC/MS, TLC/DESI-MS, IR, X-ray crystallography, and NMR (18-27). Salvinorin A can also be detected in biological specimens, such as human plasma, urine, sweat, saliva, blood, pericardial fluid, and vitreous humor by LLE or SPE coupled with various forms of LC/MS or GC/MS (28). Most of these techniques are important to satisfy the Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) guidelines for confirmatory testing, however many of the techniques are very time consuming or require expensive equipment not available to most forensic labs (29). It is important to explore all available analytical techniques to include presumptive, or screening, techniques as well. Proper field screening of drug materials is important to eliminate costly lab testing for confirmatory analysis of unrelated materials.

The characteristics of *Salvia divinorum* plant materials are not distinctive enough to allow differentiation by anyone other than a skilled botanist. One method that would be useful as a screening tool is thin layer chromatography (TLC). This technique has been used to characterize salvinorin A and other compounds extracted from *S. divinorum* by utilizing a vanillin stain to visualize the spots produced (20, 30). It has been reported that salvinorin A can be distinguished from THC and 13 other species of *Salvia* based on TLC (20). Another screening method that has been investigated by our lab for salvinorin A

is UV/Vis spectroscopy. Salvinorin A was shown to absorb at 210 nm in acetone, and was distinguishable from co-extracted plant pigments (31). However, both TLC and UV/Vis analysis require extraction of the *Salvia divinorum* leaves prior to analysis, which will add time and complexity to the screening method.

Our lab has previously developed another screening test that utilizes Ehrlich's reagent as a colorimetric assay for *S. divinorum* (32, 33). Ehrlich's reagent consists of *para*-dimethylaminobenzaldehyde (pDMAB, **Figure 4**) as a 2% solution in ethanol:HCl (1:1). Ehrlich's reagent is typically used as a colorimetric assay for LSD, which results in a deep purple color due to the reaction of pDMAB with the indole ring of the LSD molecule (34). The general procedure was modified to include the addition of dichloromethane to extract salvinorin A from the trichomes of the leaves first, and then three drops of Ehrlich's reagent is added (33). The result is a medium to dark shade of red-orange, which is produced in 0.5 - 2 min. This presumptive test is fairly sensitive, with a distinctive color result from as little as 1 mg of dried leaf material. A screen of other common herbs and plants from the *Salvia* genus showed good selectivity, with cross reactivity only with *Salvia farinacea* (mealy sage) (33). This colorimetric assay has the advantage that it is very quick and eliminates the need for an extraction step prior to analysis.

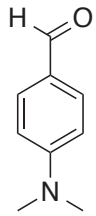


Figure 4. Chemical structure of *para*-dimethylaminobenzaldehyde.

1.6. Other Compounds Isolated from *Salvia divinorum*

Numerous other compounds have been isolated from *Salvia divinorum* (35). Most notably, salvinorin B, which is the deacetylated version of salvinorin A, was found to be pharmacologically inactive at the KOR (3). The chemical structure of salvinorin B and over 20 other compounds isolated from *Salvia divinorum* are illustrated in **Figure 5** and described in **Table 1** (18, 35-40). There are nine major structural classes for the isolated compounds, with hardwickiic acid being the simplest compound with the neoclerodane backbone (structure 2, **Figure 5**). The rest of the structures can be divided into: salvinorins, which contain a lactone ring (structures 1 and 3, **Figure 5**); divinatorins, which lack a lactone ring (structure 2, **Figure 5**); salvidivins, containing an oxidized furan ring, γ-hydroxybutenolide (structures 4 and 5, **Figure 5**); and salvinicins, with a more extensively oxidized furan ring, dihydroxydimethoxy-tetrahydrofuran (structure 6, **Figure 5**). The biosynthetic sequence can then be hypothesized to go from hardwickiic acid to the divinatorins, to the salvinorins, and then to the salvidivins and the salvinicins (35). Other natural products have been isolated, including the anti-oxidant nepetoidin B (structure 7, **Figure 5**), the ant-repellant loliolide

(structure 9, **Figure 5**), and dehydrovomifoliol (structure 8, **Figure 5**), which is involved in the synthesis of plant growth inhibitors (35, 41).

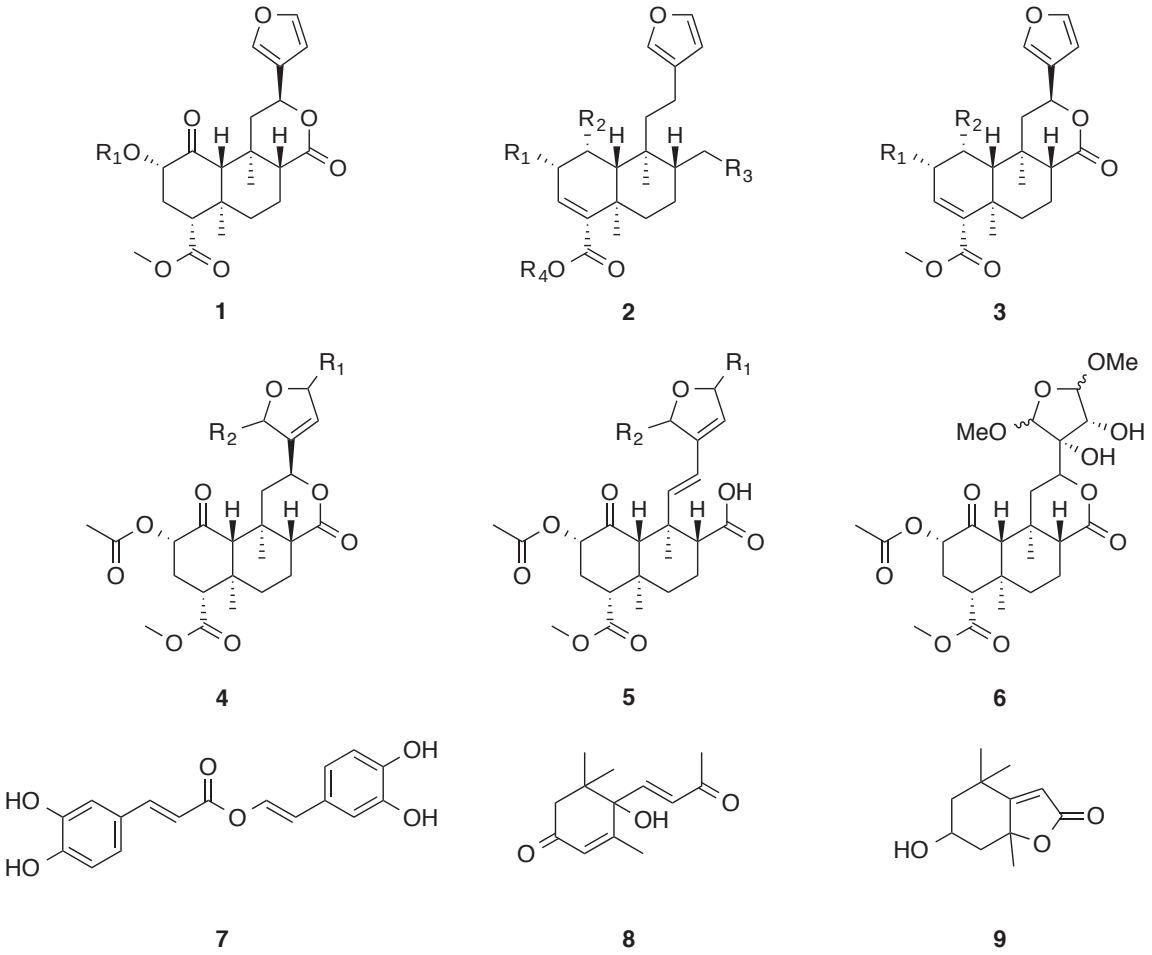


Figure 5. Structures of compounds isolated from *Salvia divinorum*. Salvinorin A and B are indicated by structure **1**. Descriptions following in **Table 1** (35)**.**

Table 1. Compounds isolated from *Salvia divinorum*. R-groups correspond to structures preceding in **Figure 5** (35).

Compound	Parent Structure	R_1	R_2	R_3	R_4
Salvinorin A	1	Ac			
Salvinorin B	1	H			
Hardwickiic acid	2	H	H	H	H
Divinatorin A	2	H	OH	H	H
Divinatorin B	2	H	OH	OH	Me
Divinatorin C	2	H	H	OAc	H
Divinatorin D	2	H	OH	OAc	Me
Divinatorin E	2	H	OH	=O	Me
Divinatorin F	2	OH	OH	OH	Me
Salvinorin C	3	OAc	OAc		
Salvinorin D	3	OH	OAc		
Salvinorin E	3	OAc	OH		
Salvinorin F	3	H	OH		
Salvinorin G	3	=O	OAc		
Salvinorin H	3	OH	OH		
Salvinorin I	3	OH	OH	(δ-lactol)	
Salvidivin A	4	=O	OH		
Salvidivin B	4	OH	=O		
Salvidivin C	5	=O	OH		
Salvidivin D	5	OH	=O		
Salvinicin A	6	β-OMe	β-OMe		
Salvinicin B	6	α-OMe	α-OMe		
Nepetoidin B	7				
Dehydrovomifoliol	8				
Loliolide	9				

1.7. Semi-Synthetic Derivatives

Since salvinorin A is a selective and potent agonist of the KOR, there is interest in researching analogues in efforts to develop and understand therapeutic drugs for depression, schizophrenia, and other mental illnesses (1). Significant SAR experiments have been undertaken on salvinorin A, focusing on four main areas: the C2 acetyl group, the C4 methyl-ester, the C17 carbonyl, and

the furan ring (**Figure 6**) (42). Salvinorin A can be synthesized, but it is currently more efficient to extract and purify the active compound from the leaves of *S. divinorum* and perform modifications afterwards (30, 43). Most SAR efforts have focused on the C2 position, as a simple hydrolysis to salvinorin B loses activity at the KOR (42). In addition, this location provides the easiest access to modification. It was found that small alkyl groups at C2 favored KOR activity, with methoxymethyl ether and ethoxymethyl ether groups providing potency up to 13 times more potent than salvinorin A (**Figure 7**) (44). Salvinorin A has an EC_{50} of 1.8 nM, salvinorin B methoxymethyl ether (SB-MME) has an EC_{50} of 0.40 nM, and salvinorin B ethoxymethyl ether (SB-EME) has an EC_{50} of 0.14 nM (44).

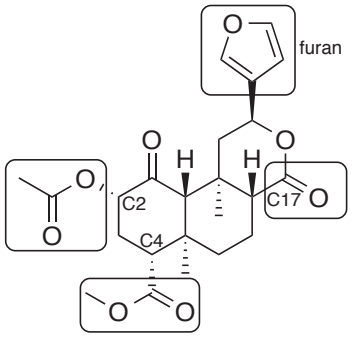
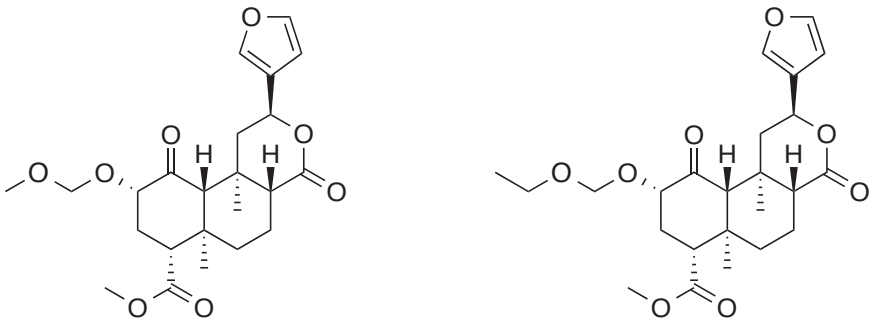


Figure 6. SAR Locations on salvinorin A for probing KOR activity. Modifications focused at the C2 acetyl group, C4 methyl ester, C17 carbonyl and the furan ring (42).

Abuse of the salvinorin analogues has not yet been reported in the literature, but accounts of use can be found on the Internet, in which SB-EME has been nicknamed “Symmetry” (45). The semi-synthetic salvinorins can be sprayed on innocuous plant material or on cigarette papers as a substrate for smoking. Salvinorin A has been known to be extracted from the leaves of *S.*

divinorum and sprayed back onto the original drug plant material, known as “fortifying” or “enhancing” (46). Applying this practice with semi-synthetic salvinorins poses a significant health risk, as most new analogues will have very little safety and toxicity data associated with common abuse routes. In addition, clandestinely made synthetic drugs pose a risk in introducing unknown impurities during the synthesis of the desired compound (47). With the ether analogues, the chloroalkyl ethers used as starting materials to make the ether functionality are fairly toxic and known carcinogens (48).



Salvinorin B Methoxymethyl Ether Salvinorin B Ethoxymethyl Ether

Figure 7. Semi-synthetic analogues: salvinorin B methoxymethyl ether (SB-MME) and salvinorin B ethoxymethyl ether (SB-EME).

1.8. Scope of Thesis

Given the public health risk involved with semi-synthetic analogues of salvinorin A, it is important to develop proper field and lab analytical techniques to identify and characterize the semi-synthetic salvinorins in forensically relevant media. First, salvinorin A was extracted from the dried leaves of *Salvia*

divinorum. This material was then deacetylated to salvinorin B and converted to the ether analogues (see **Figure 8**).

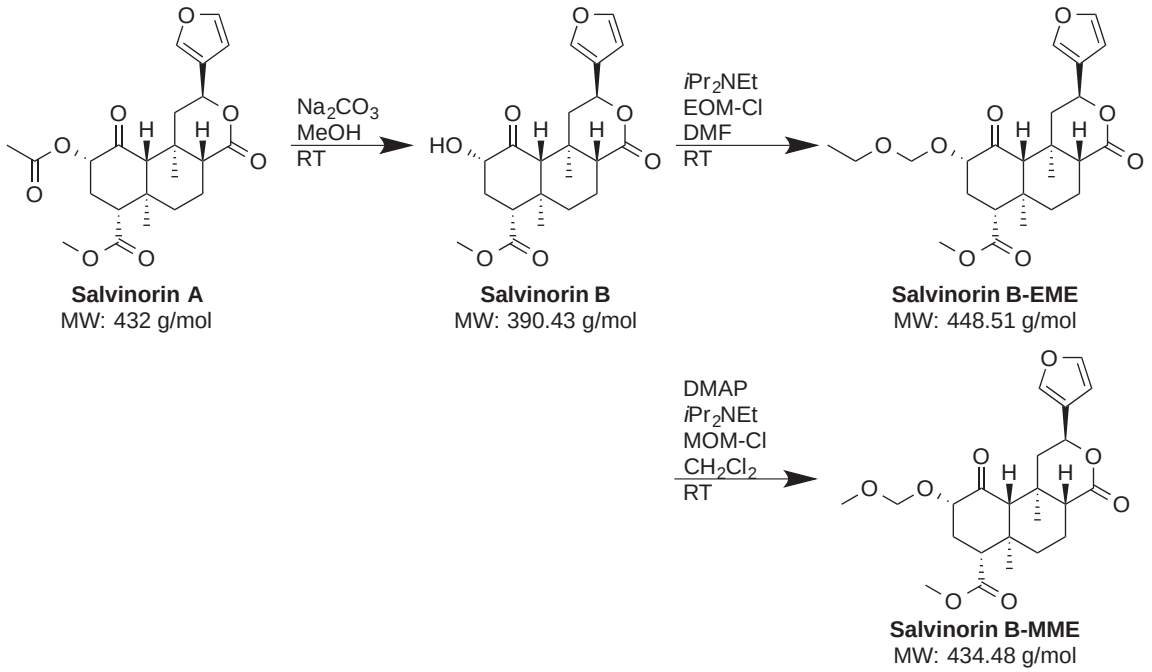


Figure 8. Synthetic scheme of salvinorin B methoxymethyl ether (SB-MME) and salvinorin B ethoxymethyl ether (SB-EME) from salvinorin A. Salvinorin A is converted to salvinorin B by hydrolysis at the C2 position. Condensation with various chloroalkyl ethers provides the desired analogues.

Chemical characterization of prepared analogues was performed in order to develop methods of differentiation from *Salvia divinorum* and salvinorin A. Characterization techniques include HPLC, UV/Vis, NMR, and a colorimetric assay with Ehrlich's reagent. Finally, adulteration of other plant materials with salvinorin A and analogues was performed and analyzed to determine if fortified materials can be detected by colorimetric assay.

2. METHODS

2.1. Analytical Methods

2.1.1. TLC

Using vanillin as a developing stain, extractions and reactions were monitored by TLC. The stain was made by dissolving 6 g of vanillin (Alfa Aesar, 99%) into 95 mL of EtOH (EMD, OmniSolv), followed by 1.5 mL of H_2SO_4 (Sigma-Aldrich, reagent grade). The solution was stored at 5ºC and shielded from light until use. To develop the glass TLC plates, approximately 3 µL of a 1 mg/mL solution was spotted onto the plate and ran in 10% acetone/DCM (EMD, HPLC grade/Alfa Aesar, ACS grade). Once the solvent was evaporated, the plate was dipped into the vanillin stain and the excess was wiped off. The plate was then heated at 60 ºC until a color developed. The compounds of interest were stained red with this procedure, with a yellow-green background (see **Appendix A.1**).

2.1.2. UV/Vis

Analysis by UV/Vis was performed on a Thermo Scientific Multiskan GO with SkanIT Software (Thermo Scientific, V. 3.2.0.35). Analysis was performed in a quartz 2 mL cuvette. The test parameters were set for fast measurement mode

with 1 scan per nm, spanning 200 to 1000 nm. All measurements were evaluated after a subtraction from the solvent blank.

2.1.3. HPLC

Based on a method developed by Hock *et al.* (25) HPLC was performed using an Agilent 1200 series HPLC equipped with an ultraviolet diode array detector, an autosampler, and Chemstation software (Agilent, V. C.01.04). The HPLC column was an Agilent Zorbax SB-C18 column (4.6 x 75 mm, 3.5 µm particle size, C18 guard column). The mobile phase was an isocratic elution of acetonitrile:water (45:55) at 1.0 mL/min, with a 10 min runtime. The injection size was 75 µL and the column temperature was set at 30ºC. Detection was performed at 208 nm (4 nm bandwidth, 700 nm reference). Salvinorin A eluted at 5.76 min, salvinorin B eluted at 2.33 min, and SB-EME eluted at 6.44 min.

2.1.4. NMR

^{1}H NMR spectroscopy was performed on a Bruker AVANCE-400 NMR Spectrometer (400 MHz) and a Bruker AVANCE-600 NMR Spectrometer (600 MHz), located at the Massachusetts Institute of Technology Department of Chemistry Instrumentation Facility. Both instruments were equipped with a Magnex Scientific superconducting magnet, and the data was collected on TopSpin 3.1 software. The chemical shifts were referenced to the residual

solvent peaks of $CDCl_3$ at 7.26 ppm. Data processing was performed on MestReNova (V. 8.0.2-11021).

2.2. Extraction

The extraction proceeded based on a method developed by Smith *et al.* (49). Approximately 50 g of dried *Salvia divinorum* leaves (Botanical Spirit Shop, Surrey, BC, Canada) (50) were placed into a 2 L Erlenmeyer flask, followed by 1 L of DCM (Alfa Aesar, ACS grade). An overhead stirrer (Heidolph Electronic Overhead Stirrer, RZR 2102) with a PTFE stirrer blade was used to vigorously stir the solution for 5 minutes. The DCM was decanted over packed celite (Fisher Science Education) in a sintered glass funnel (VWR, 40M). Additional DCM (500 mL) was added, and the leaves were extracted for 5 minutes and filtered as above. A final addition of DCM (500 mL) was added to total three extractions and filtrations for the portion of leaves. The extracts were combined and reduced under rotary evaporation (Büchi Rotavapor R-205 with heating bath) to a dark green residue (0.84 g crude weight). Approximately 10 mL of EtOH (EMD, OmniSolv) were added to the residue and allowed to stir for 24 hours with a stir bar. A light tan solid was filtered (Whatman, qualitative 125 mm) from the green solution, rinsing with EtOH, and washed with hexanes to yield 173 mg of salvinorin A (89% pure by HPLC). The material was brought forward with no further purification. R_f = 0.54 (10% acetone/DCM, developed with vanillin); ^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.41 (s, 1H), 7.39 (s, 1H), 6.37 (s, 1H), 5.53

(dd, *J* = 11.7, 4.9 Hz, 1H), 5.17 – 5.11 (m, 1H), 3.73 (s, 3H), 2.75 (dd, *J* = 10.6, 6.2 Hz, 1H), 2.51 (dd, *J* = 13.4, 5.1 Hz, 1H), 2.36 – 2.27 (m, 2H), 2.20 – 2.13 (m, 5H), 2.07 (dd, *J* = 11.7, 2.6 Hz, 1H), 1.79 (d, 1H), 1.62 – 1.56 (m, 3H), 1.45 (s, 3H), 1.12 (s, 3H).

2.3. Synthesis

2.3.1. Salvinorin B

The synthesis of salvinorin B was performed according to Munro's dissertation (30). Salvinorin A (150 mg, 0.347 mmol) and sodium carbonate (ACS grade, 131 mg, 1.23 mmol) were added to a vial, followed by MeOH (EMD, Omnisolv, 3.1 mL). The solution was stirred for 24 hours at room temperature. The solvent was reduced under rotary evaporation, dissolved in DCM (12 mL), and washed with 2 N HCl (3 x 10 mL) in a separatory funnel. The aqueous layer was back-extracted with DCM (1 x 5 mL). The combined organic extracts were washed with brine (1 x 10 mL) and dried over Na_2SO_4. The yellow solution was filtered over celite and reduced under rotary evaporation to a light yellow powder. The solid was triturated with MeOH (5 mL) and filtered to yield 82 mg of salvinorin B as a white crystalline powder (93% pure by HPLC). R_f = 0.38 (10% acetone/DCM, developed with vanillin); ^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.42 (s, 1H), 7.40 (s, 1H), 6.38 (s, 1H), 5.58 (dd, *J* = 5.1 Hz, 1H), 4.08 (dd, 1H), 3.72 (s, 3H), 3.60 (d, *J* = 3.2 Hz, 1H), 2.71 (dd, *J* = 13.5, 3.0 Hz, 1H), 2.54 (dd, *J* = 13.3, 5.1 Hz, 1H), 2.48 (ddd, *J* = 13.3, 7.6, 3.1 Hz, 1H), 2.22 – 2.12 (m, 2H), 2.11

– 1.98 (m, 2H), 1.79 (dt, 1H), 1.72 – 1.61 (m, 1H), 1.56 (d, *J* = 12.6 Hz, 2H), 1.48 (s, 3H), 1.10 (s, 3H).

2.3.2. Salvinorin B Methoxymethyl Ether

Salvinorin B (25 mg, 57.0 µmol) was dissolved in 450 µL of DMF (J.T. Baker, ACS grade), followed by Hünig's base (Acros Organics, 99.5%, 50 µL, 287 µmol) and chloromethyl methyl ether (technical grade, 28 µL, 291 µmol), following the procedure from Munro (44). The tan slurry was stirred at room temperature for 72 hours. TLC indicated only 40% conversion. The solution was diluted with EtOAc (3 mL) and washed with 2 N HCl (3 x 3 mL), water (2 x 3 mL), saturated $NaHCO_3$ (2 x 3 mL), and brine (1 x 3 mL), then dried over Na_2SO_4. The solution was filtered and reduced under rotary evaporation to a yellow residue. Approximately 43% starting material remained by HPLC; therefore, final characterization was abandoned due to the limited amount of desired product and significant effort required for purification.

2.3.3. Salvinorin B Ethoxymethyl Ether

As outlined by Munro (44), salvinorin B (24.1 mg, 57.4 µmol) was dissolved in 450 µL of DMF (J.T. Baker, ACS grade), followed by Hünig's base (Acros Organics, 99.5%, 50 µL, 287 µmol) and chloromethyl ethyl ether (technical grade, 34 µL, 293 µmol). The white slurry was stirred at room temperature for 24 hours and deemed approximately 90% complete by TLC. The

solution was diluted with EtOAc (3 mL) and washed with 2 N HCl (3 x 3 mL), water (2 x 3 mL), saturated $NaHCO_3$ (2 x 3 mL), and brine (1 x 3 mL), then dried over Na_2SO_4. The solution was filtered and reduced under rotary evaporation to a white residue. The crude solid contained approximately 10% salvinorin B and was purified by flash column chromatography (25-50% EtOAc/hexanes, then 20% MeOH/DCM) to give an amorphous white solid (87% pure by HPLC). R_f = 0.58 (10% acetone/DCM, developed with vanillin); ^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.41 (s, 1H), 7.40 (s, 1H), 6.39 – 6.35 (m, 1H), 5.55 (dd, *J* = 11.7, 5.8 Hz, 1H), 4.78 (d, *J* = 7.2 Hz, 1H), 4.75 (d, *J* = 7.2 Hz, 1H), 4.17 (dd, *J* = 12.2, 7.3 Hz, 1H), 3.77 – 3.65 (m, 4H), 3.65 – 3.53 (m, 1H), 2.69 (dd, *J* = 13.5, 3.4 Hz, 1H), 2.53 (dd, *J* = 13.3, 5.2 Hz, 1H), 2.34 (ddd, *J* = 13.6, 7.4, 3.4 Hz, 1H), 2.24 – 2.10 (m, 2H), 2.09 – 2.01 (m, 2H), 1.82 – 1.74 (m, 1H), 1.72 – 1.49 (m, 3H), 1.47 (s, 3H), 1.24 (s, 3H), 1.11 (s, 3H).

2.4. HPLC Method Validation of Salvinorin B Ethoxymethyl Ether

2.4.1. Standard Preparation

A 1 mg/mL solution of salvinorin B ethoxymethyl ether was diluted into 45% acetonitrile/H_2O according to **Table 2**. Samples were prepared in 2 mL vials and mixed thoroughly prior to analysis.

Table 2. **Dilution of salvinorin B ethoxymethyl ether standards.**

Standard	Volume 1 mg/mL Stock (µL)	Volume 45% ACN/H_2O (µL)	Concentration (mg/mL)
1	0.500	1000	0.0005
2	1.000	999	0.0010
3	1.500	999	0.0015
4	2.00	998	0.0020
5	4.00	996	0.0040
6	8.00	992	0.0080
7	16.00	984	0.0160
8	32.0	968	0.0320
9	64.0	936	0.0640

2.4.2. Accuracy

The accuracy of the HPLC method was evaluated by determining the agreement of the test concentration to the theoretical concentration, using both salvinorin B and SB-EME. *Salvia officinalis* (common sage) was used as a matrix blank and the standard of interest was spiked as a 2 mg/mL solution in acetone onto 75 mg of the dried herb to total 15 mg/g. The herb was extracted in triplicate with 2.0 mL aliquots of DCM for 5 min. The solution was filtered through a 0.45 µm nylon syringe filter and rinsed with 1.0 mL of DCM. The filtered solutions were evaporated to dryness and reconstituted in 1 mL of acetone. The acetone solution was sonicated and heated to 50 ºC until a clear solution resulted. The solution was diluted 160 µL into 840 µL of mobile phase (45% ACN/H_2O) for a final theoretical concentration of 0.18 mg/mL and mixed thoroughly via vortex before HPLC analysis.

2.4.3. Precision

The precision of the HPLC method was evaluated by the analytical repeatability and intermediate precision of a set of standards. The standards were prepared in triplicate and injected on the previously described HPLC method with triplicate injections.

2.4.4. Linearity and Range

The linearity and range of the method was evaluated by preparing a standard curve for the standards prepared in section **2.4.1**. The linearity was assessed by a best fit of the calibration response versus the concentration of the analyte to a linear function. The range was the interval between the upper and lower concentration for which precision, accuracy, and linearity was maintained.

2.4.5. Selectivity

Selectivity, or specificity, was evaluated by the presence or absence of interfering peaks in the blank matrix. The diluent and standards are also screened to verify matrix interference, peak carryover, or contamination of test solutions.

2.4.6. Sensitivity

Sensitivity was determined by establishing the limit of detection (LOD) and limit of quantitation (LOQ) for the HPLC method. The LOD is the concentration at which the signal-to-noise ratio (S/N) is equal to 3, and the LOQ is the

concentration at which the S/N is equal to 10. An average of 6 injections was used to calculate the S/N.

2.5. UV/Vis Analysis of Salvinorin Derivatives

Solutions of salvinorin A, salvinorin B, and SB-EME were made in DCM, each at 1 mg/mL. Each standard was diluted into seven equivalent molar concentrations (see **Table 3**) and analyzed in a 2 mL quartz cuvette on an UV/Vis spectrophotometer as previously described. The molar absorptivity of each solution and the UV/Vis spectra were gathered for each concentration.

Table 3. Molar concentrations of salvinorin A, salvinorin B, and SB-EME.

Sample	Concentration (mmol/L)	Salvinorin A (mg/mL)	Salvinorin B (mg/mL)	SB-EME (mg/mL)
1	2.30	1.00	0.90	1.03
2	1.50	0.65	0.59	0.67
3	1.00	0.43	0.39	0.45
4	0.50	0.22	0.20	0.22
5	0.25	0.10	0.10	0.11
6	0.05	0.02	0.02	0.02
7	0.01	0.004	0.004	0.004

2.6. Colorimetric Assay with Ehrlich's Reagent

2.6.1. Standard Procedure with Ehrlich's Reagent

The standard colorimetric assay on plant material consisted of 1 – 3 mg of dried leaves in a spot well, followed by the addition of 50 µL of DCM. Three drops of Ehrlich's reagent was then added. Color development was present within 30 sec, with full color development in 2 min. The assay was biphasic, with a red-orange positive result in the top aqueous layer and a green color in the bottom

DCM layer. The colorimetric assay was performed on standard solutions of DCM at a concentration of 1 mg/mL of sample followed by 0.2 mL of Ehrlich's reagent.

2.6.2. UV/Vis Characterization of Colorimetric Assay

The standard colorimetric assay was performed in a 2 mL cuvette on a DCM solution of salvinorin B. The path of the light for the instrument passed through the bottom DCM layer of the biphasic solution. UV/Vis analysis on the bottom DCM layer was performed by taking scans at multiple different time points occurring over a 30 min period. Agitation was performed by a pipette of the biphasic solution after 4 minutes. After 30 min the top, aqueous red-orange layer was removed, and UV/Vis analysis was performed on the solution.

To determine if there was any contribution to the spectrum from just Ehrlich's reagent, analysis was performed on the reagent itself. A 1:10 dilution of Ehrlich's reagent was performed, diluting in EtOH, and an UV/Vis spectrum was taken.

2.6.3. HPLC Characterization of Colorimetric Assay

The DCM layer of the above colorimetric assay on a standard of salvinorin B and an extract of *Salvia divinorum* was evaporated and reconstituted in acetone. The acetone solution was diluted into 45% ACN/H_2O and analyzed on a gradient HPLC method. Any attempts at working up the top, aqueous layer for HPLC studies quenched the color.

HPLC was performed using an Agilent 1200 series HPLC equipped with an ultraviolet diode array detector, an autosampler, and Chemstation software (Agilent, V. C.01.04). The HPLC column was an Agilent Zorbax SB-C18 column (4.6 x 75 mm, 3.5 µm particle size, C18 guard column). The mobile phase was a gradient elution of 35% ACN/H_2O to 45% in 15 min, increase to 95% in 5 min, and hold for 10 min. The method was run at 1.0 mL/min, with a 30 min runtime. The injection size was 100 µL and the column temperature was set at 30ºC. Detection was performed at 208 nm (4 nm bandwidth, 700 nm reference) and 380 nm (4 nm bandwidth, no reference). Salvinorin A eluted at 11.79 min, salvinorin B eluted at 4.46 min, and SB-EME eluted at 12.78 min.

2.6.4. ^{1}H NMR Characterization of Colorimetric Assay

The DCM layer of a colorimetric assay on a standard of salvinorin B was evaporated and reconstituted in $CDCl_3$. ^{1}H NMR spectroscopy was performed to analyze the final product of the reaction in the organic layer. Any attempts at working up the top, aqueous layer for NMR studies quenched the color. A control of salvinorin B in DCM, washed with a 6N HCl and ethanol solution, was also analyzed to determine the effects of the presence of strong acid in Ehrlich's reagent on the compounds of interest.

2.7. Adulteration of Plant Materials

2.7.1. Spiking of Plant Material

Salvinorin A, salvinorin B, and SB-EME were spiked onto dried leaves of *Salvia divinorum*, *Salvia officinalis*, *Mitragyna speciosa* (Kratom), and *Calea zacatechichi* (Dream Herb) at concentrations of 3.4 mg/g, 15 mg/g, and 40 mg/g in acetone. The solvent was allowed to evaporate at room temperature and occasionally agitated to ensure even coverage. *Salvia divinorum* was not spiked with salvinorin A, as the analysis of salvinorin A spiked *Salvia divinorum* has been previously reported (51).

2.7.2. Colorimetric Assay of Adulterated Plant Materials

To analyze the adulterated plant materials with Ehrlich's reagent, 3 mg of each spike level from section **2.7.1** were placed on a spot well plate. Each plate consisted of the analysis of one type of plant material (see **Table 4** as an example). Dichloromethane (50 µL) was added to each well, followed by 3 drops of Ehrlich's reagent. Photographs were taken at 2 min.

Table 4. Example spot well plate setup for spiking *Salvia officinalis*. Three replicates of unadulterated *S. divinorum* are located in the top row as a control. Salvinorin A, salvinorin B, and SB-EME are spiked in each of the following rows at concentrations of 3.4 mg/g, 15 mg/g, and 40 mg/g.

Spiking Solution	3.4 mg/g	15 mg/g	40 mg/g
None	Control *S. divinorum*	Control *S. divinorum*	Control *S. divinorum*
Salvinorin A	Sal. A Spiked *S. officinalis*	Sal. A Spiked *S. officinalis*	Sal. A Spiked *S. officinalis*
Salvinorin B	Sal. B Spiked *S. officinalis*	Sal. B Spiked *S. officinalis*	Sal. B Spiked *S. officinalis*
SB-EME	SB-EME Spiked *S. officinalis*	SB-EME Spiked *S. officinalis*	SB-EME Spiked *S. officinalis*

2.7.3. HPLC Analysis of Adulterated Plant Materials

The vials left over from the colorimetric analysis were analyzed for residual active components. Acetone (1 mL) was used to rinse the vials and was diluted by pipetting 400 µL into 600 µL of mobile phase diluent (45% ACN/H_2O).

3. RESULTS AND DISCUSSION

3.1. Extraction and Synthesis

3.1.1. Extraction and Characterization of Salvinorin A

The extraction of salvinorin A proceeded as detailed in the literature (25, 30, 49). Dichloromethane was chosen as the extraction solvent of choice to reduce the amount of coextracted compounds and plant pigments that may interfere with long-term stability (49). Salvinorin A was shown to be most stable in dichloromethane (49), so extracts were stored at -20ºC in DCM until analysis. Degradation of salvinorin A was not seen by HPLC or NMR upon approximately 6 months of storage. The approximate yield of salvinorin A from this source (50) of *Salvia divinorum* was 0.346%, which was consistent with reported values (3, 30). The adapted HPLC method (25) showed a symmetrical peak, well resolved from other impurities present in the extract. **Figure 9** shows a salvinorin A standard of 0.032 mg/mL, or 2.4 µg. Even in the commercial standard of salvinorin A, some salvinorin B is present. The acetone peak was introduced from the dilution of the standard. Regardless of the impurities present, characterization by ^{1}H NMR was straightforward. A minor amount of salvinorin B is naturally present and was seen in both the HPLC trace and in the NMR based on the –OH peak. See the **Appendix A.2** for the detailed ^{1}H NMR spectra; the assignments match previously reported values (52).

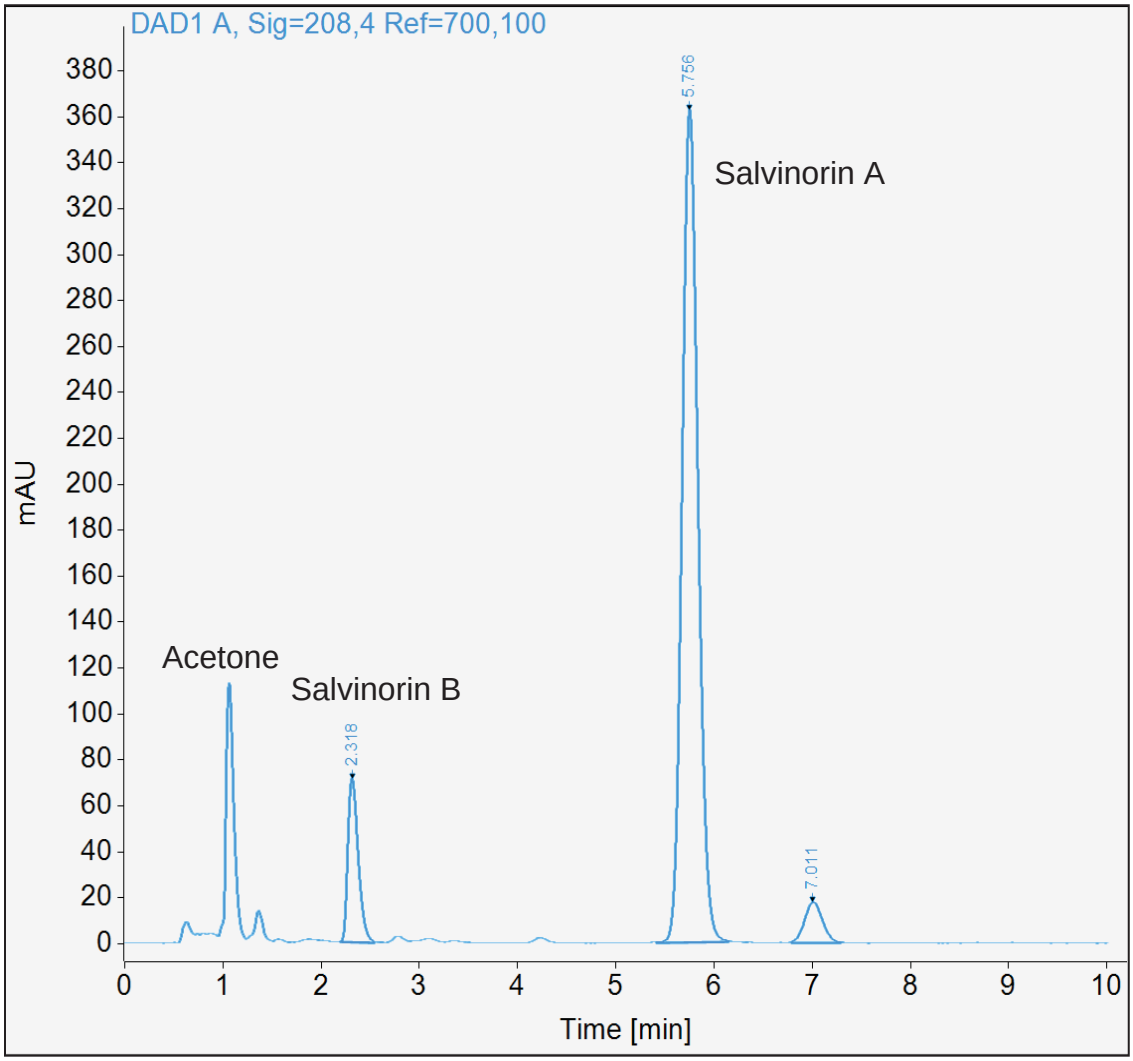


Figure 9. HPLC trace of salvinorin A standard at 2.4 µg. Some amount of salvinorin B was present in the commercial standard; along with an unidentified impurity at 7 min. Acetone was present due to the dilution procedure.

3.1.2. Synthesis and Characterization of Salvinorin B

Salvinorin A was deacetylated as previously described (53) with sodium carbonate in methanol. This reaction proceeded at room temperature with cheap, common reagents. This reaction could be easily performed outside of a laboratory environment. In addition, the trituration in methanol after work-up gave

an increase in purity, allowing some of the contaminants from the extraction to be dissolved away. The HPLC peak of salvinorin B is located in a region where many other compounds from the extraction elute. Salvinorin B was not quantifiable from the extract due to the coelution, however the final purity after deacetylation allows for resolution from the remaining impurities (**Figure 10**). The ^{1}H NMR spectrum shows an absence of the methyl group from the acetate functionality and a new peak from the resulting hydroxyl group (see **Appendix A.3**). The assignments match previously reported values (18).

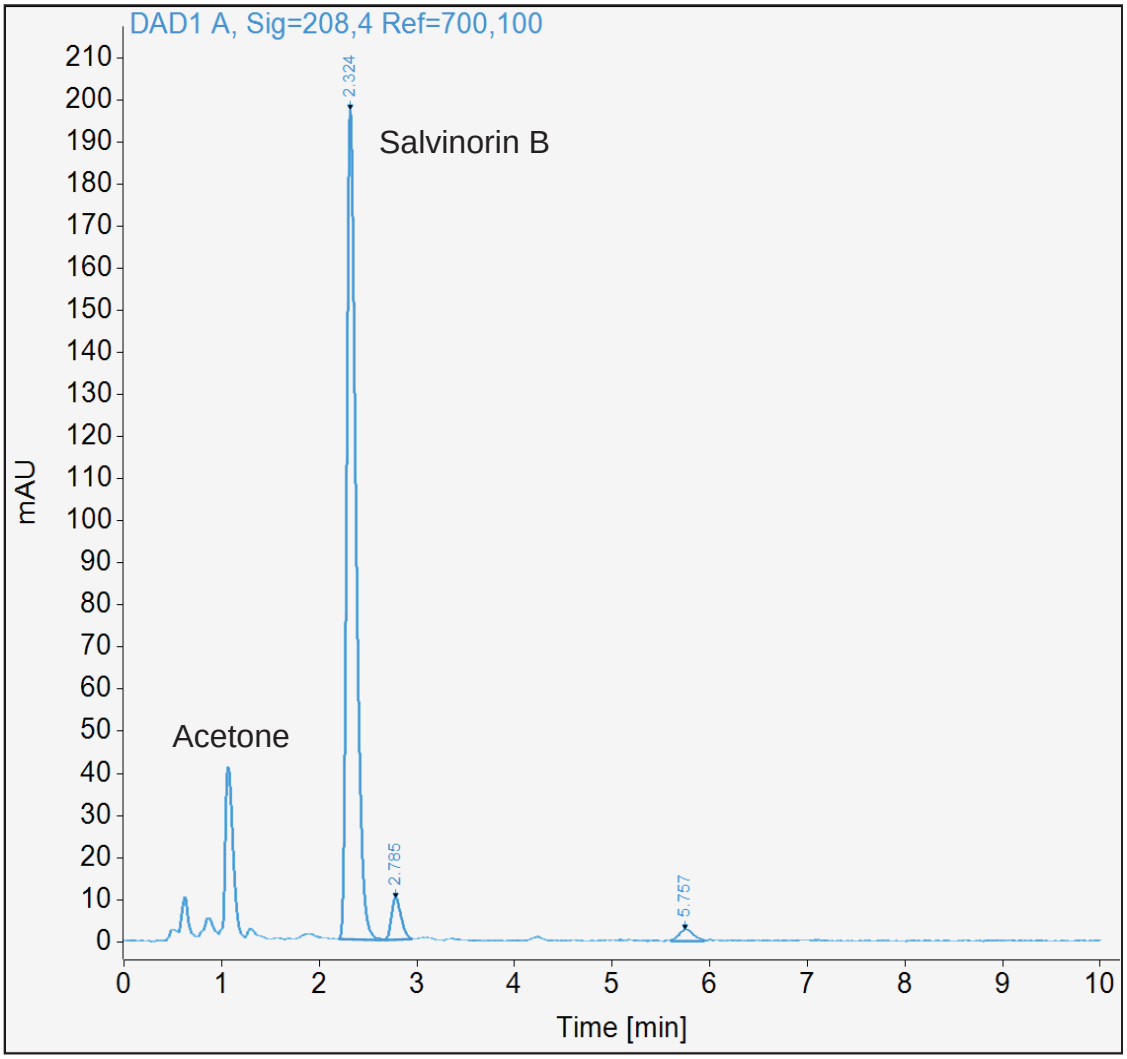


Figure 10. HPLC trace of salvinorin B at 1.8 µg. Residual amounts of salvinorin A remained after deacetylation. Acetone was introduced due to the dilution procedure.

3.1.3. Synthesis of Salvinorin B Methoxymethyl Ether

Two procedures were attempted for the synthesis of salvinorin B methoxymethyl ether (44, 54). The reported synthesis used a catalytic amount of dimethylaminopyridine (DMAP) with Hünig's base and MOM-Cl in DCM. This procedure was reported to be complete after 48 hours, however no conversion of

the starting material was seen by TLC. The second procedure mimicked the conditions used for SB-EME, where the solvent was DMF and no catalyst was used. This procedure did show some conversion but stalled after 24 hours by TLC. Likely the DMF was a better solvent for the reaction, but the solvent was not dried or distilled prior to use. Chloroalkyl ethers are relatively unstable and will hydrolyze readily, which is why they are used in such excess (5–fold) in these reactions. The presence of water would exacerbate the hydrolysis of the reagent, potentially consuming enough to unbalance the stoichiometry of the reaction. Removal of the water in the system would help drive it towards completion. Given clandestine chemists do not typically work with pure reagents and dry solvents, it was presumed more forensically relevant to proceed with the reaction with the reagents as is. The very low conversion of this reaction will give some insight into the limited likelihood this synthesis will find popularity in the field.

The HPLC trace was difficult to analyze given more than one new peak appeared after the synthesis (see **Figure 11**). Due to the complexity of purification needed, further characterization of the compound was abandoned. Given its high potency of 0.4 nM EC_{50}, compared to salvinorin A at 1.8 nM (44), it is plausible clandestine chemists would accept a mixture for recreational use. However, for the purposes of characterizing and identifying differentiating analytical techniques, material that is relatively pure would be needed to continue in this study.

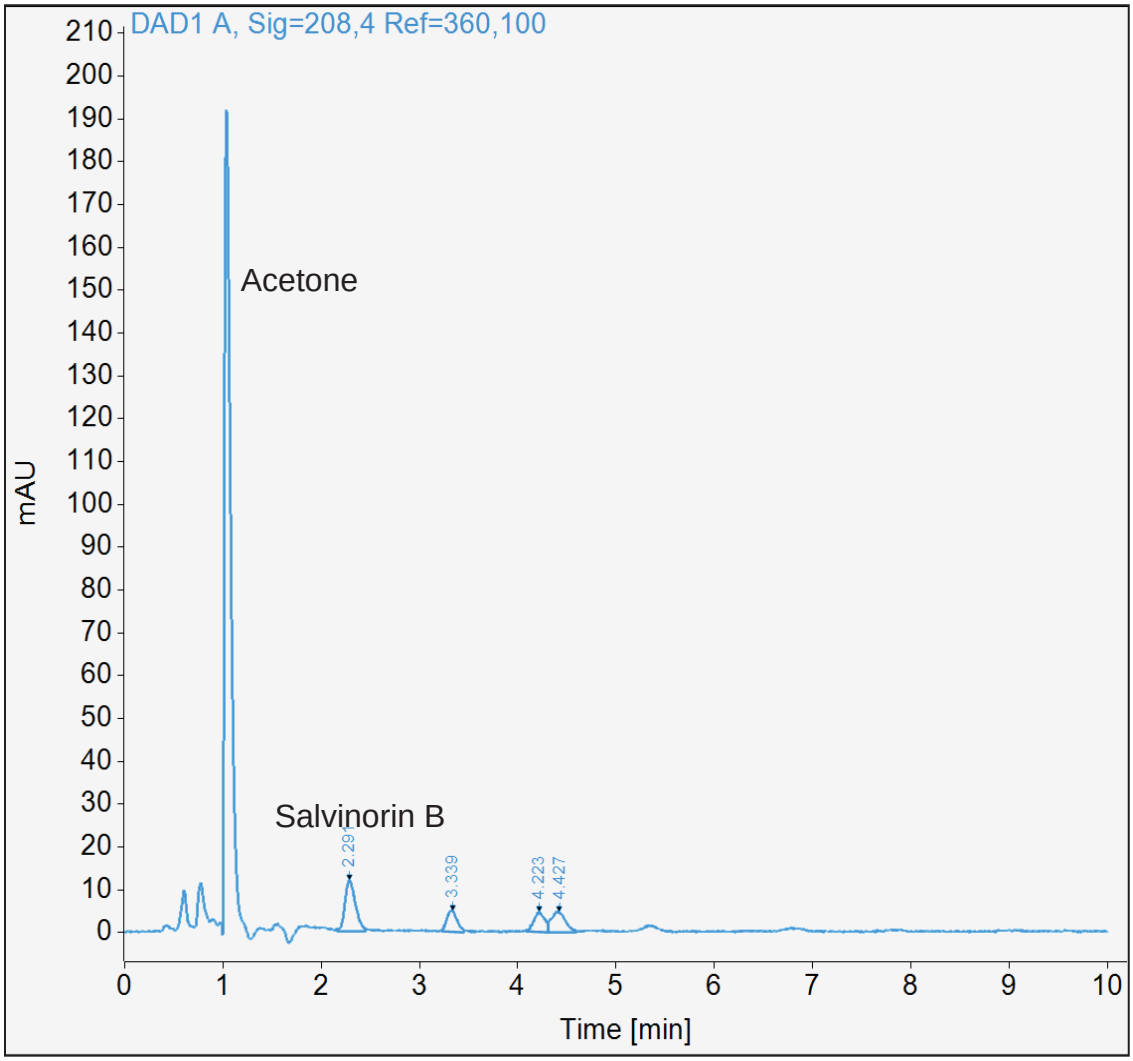


Figure 11. HPLC trace of salvinorin B methoxymethyl ether. Approximately 43% of salvinorin B starting material is present, along with three new minor peaks. The specific peak to SB-MME was not identified. Acetone was introduced during sample preparation.

3.1.4. Synthesis and Characterization of Salvinorin B Ethoxymethyl Ether

The synthesis of SB-EME proceeded more efficiently than the methoxy-derivative, however the obtained yield was much lower than reported (44). This is also likely due to the instability of the chloroalkyl ethers (refer to section **3.1.3**). It is hypothesized the presence of the initial water in the solvent will degrade more

of the chloroalkyl ether and additional equivalents should be added to compensate.

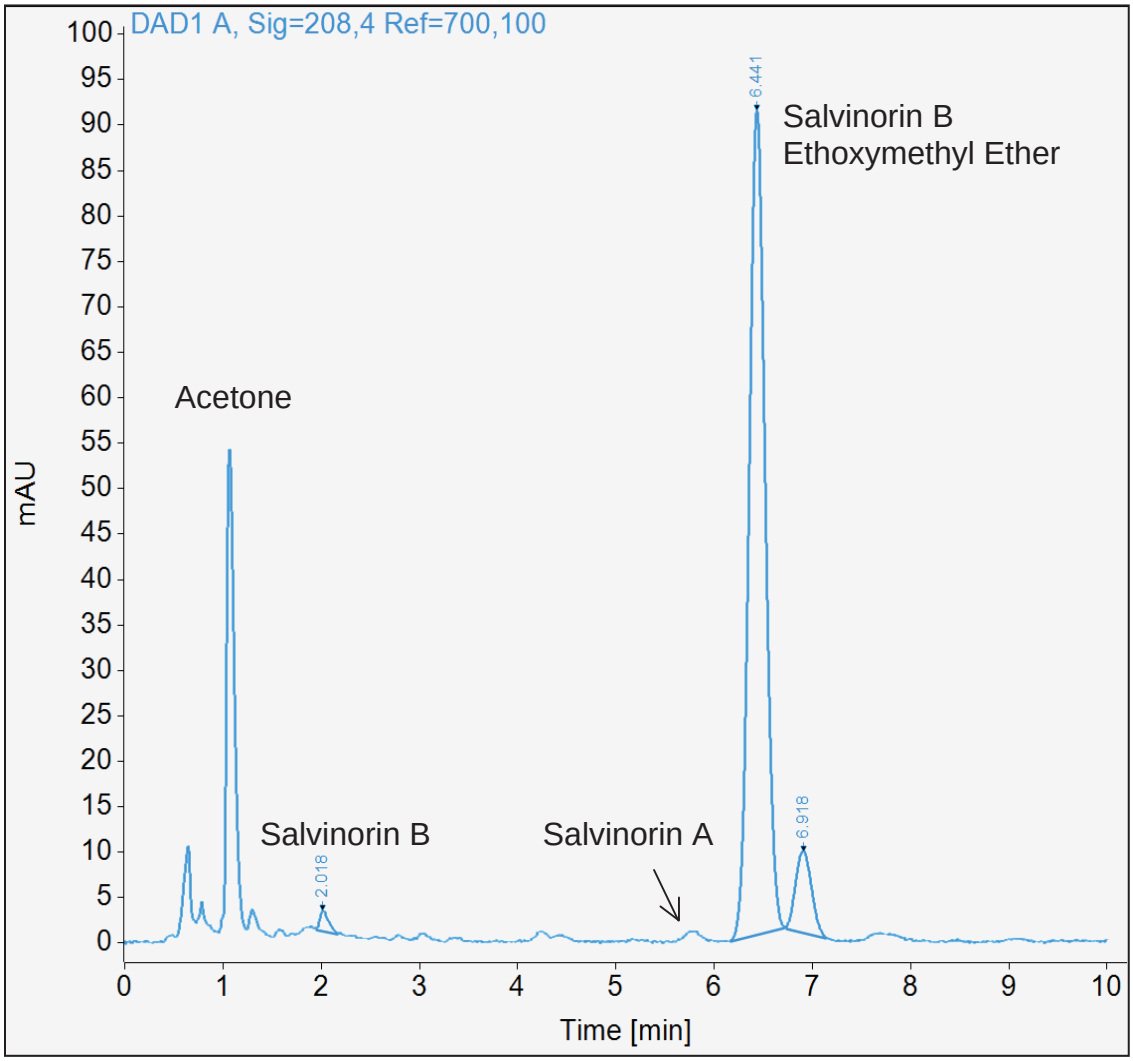


Figure 12. HPLC trace of SB-EME at 4.8 µg. The RT of SB-EME is 6.44 min, achieving baseline resolution from salvinorin A, salvinorin B, and other impurities.

After column chromatography, SB-EME was 87% pure by HPLC (see **Figure 12**). The resulting peak has a close retention time to salvinorin A (salvinorin A @ 5.76 min; salvinorin B EME @ 6.44 min); however, baseline

resolution is achieved. The resolution on TLC (10% acetone/DCM) was more difficult to establish from salvinorin A. Resolution from salvinorin B was evident.

^{1}H NMR analysis matched previously reported values (44). In particular, the disappearance of the hydroxyl peak and presence of three new sets of peaks corresponding to the ether methyl and two methylene groups was evident (see **Appendix A.4**).

3.2. HPLC Method Validation Salvinorin B Ethoxymethyl Ether

3.2.1. Accuracy

The accuracy of the method was evaluated based on the degree of agreement of the test concentration to the theoretical concentration by spiking salvinorin B and salvinorin B ethoxymethyl ether separately onto *Salvia officinalis*. However, matrix effects from the extraction of the *Salvia officinalis* plant material in DCM limited the quantitative ability of this experiment. See section **3.2.4** for more discussion on the selectivity of the method.

The spiking experiment was performed in triplicate. The results show the recovery of salvinorin B was approximately 88%, and the recovery of SB-EME was approximately 100% using this method (see **Appendix A.5.1** for data table). However, given the known matrix issues, the error in these values is not well defined. Therefore, spiked material should be evaluated qualitatively until the effect by the matrix can be mitigated.

The accuracy in the prepared standards was evaluated by the percent relative error calculated against the generated calibration curve in **Figure 13**. Three sets of standards of SB-EME were prepared and combined into a single curve. The equation for the best-fit line was then used to back calculate the concentrations of the solutions and the error was evaluated. The raw data tables are located in **Appendix A.5.2**. The first set of standards had an absolute range of error from 0.03 – 11.79%, which is indicative of a fairly accurate method. However, the subsequent second and third sets of standards increased in relative error from 1.98 – 22.89% to 6.24 – 19.47%, respectively. The trend towards increasing error for the samples analyzed later indicates there may be a stability consideration for SB-EME. The analysis of the three sets of standards took approximately 36 hours to complete by HPLC. Salvinorin A has shown instability in solvents such as ACN previously (31, 49).

3.2.2. Precision

The precision of the HPLC method was evaluated by the analytical repeatability and intermediate precision of a set of standards. The standards were prepared in triplicate and injected on the previously described HPLC method with triplicate injections. Refer to **Appendix A.5** for the raw data tables. The average areas of the triplicate preparations were graphed versus the concentration of the solutions in **Figure 13.**

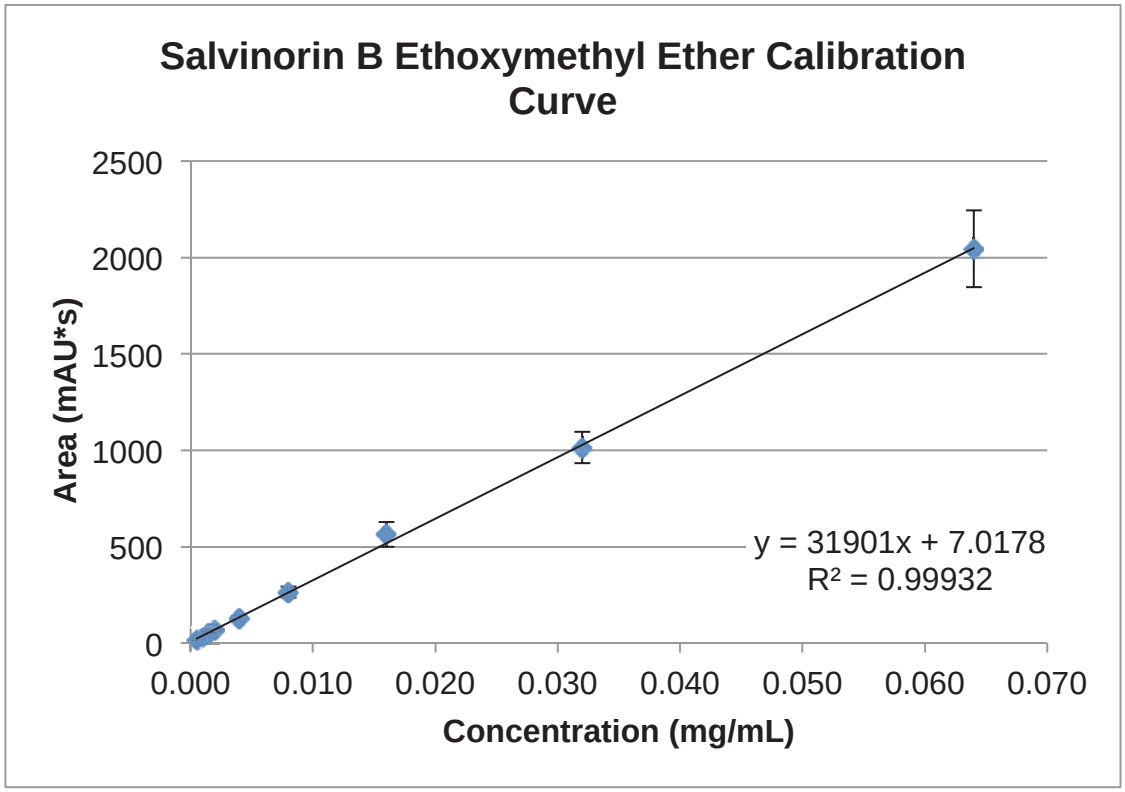


Figure 13. SB-EME calibration curve. This data was used to determine the accuracy, precision, linearity, and range of the HPLC method. Error bars indicate standard deviations.

The repeatability within each set of standards was below 9% RSD for triplicate injections. The intermediate precision between the three sets of standards was <12% RSD. An acceptable % RSD for method validation is <10%. The inherent error in the analytical balance was about 10% when weighing 1 mg of material. Given the material was very limited, weighing out 1 mg in triplicate will introduce a large amount of error. With this error in mind in addition to stability concerns, a % RSD of 12% is acceptable for this method.

3.2.3. Linearity and Range

The linearity and range were assessed based on the calibration curve in **Figure 13.** The linearity was determined by the best fit of the calibration curve, which is 0.9993, indicating good linearity over the range of the standards. The range covers two orders of magnitude from 0.0005 – 0.064 mg/mL, or a mass of 0.038 µg – 4.8 µg. Loss of linearity was not observed so this method could be useful for a wider range than presented here.

3.2.4. Selectivity

Selectivity, or specificity, of the HPLC method was evaluated by the presence or absence of interfering peaks in a blank matrix. *Salvia officinalis* was chosen as the matrix to use, as it does not contain any inherent salvinorin-type compounds found in *Salvia divinorum*. A spectrum of salvinorin B (15 mg/g) spiked onto *Salvia officinalis* is shown in **Figure 14**. Salvinorin B is apparent in the spectrum at 2.3 min, but does not achieve baseline resolution from the previous peak. Baseline resolution is the difference in retention time between two adjacent peaks, divided by the sum of the peak widths; a resolution greater than 1 allows for identification of separate peaks, but a value of 1.5 or greater is needed for resolution down to the baseline. The resolution of the salvinorin B peak from the previous peak is 1.11 and is coeluting on the shoulder of the previous peak. This will affect the integration value of the peak, impacting the accuracy of the quantitation.

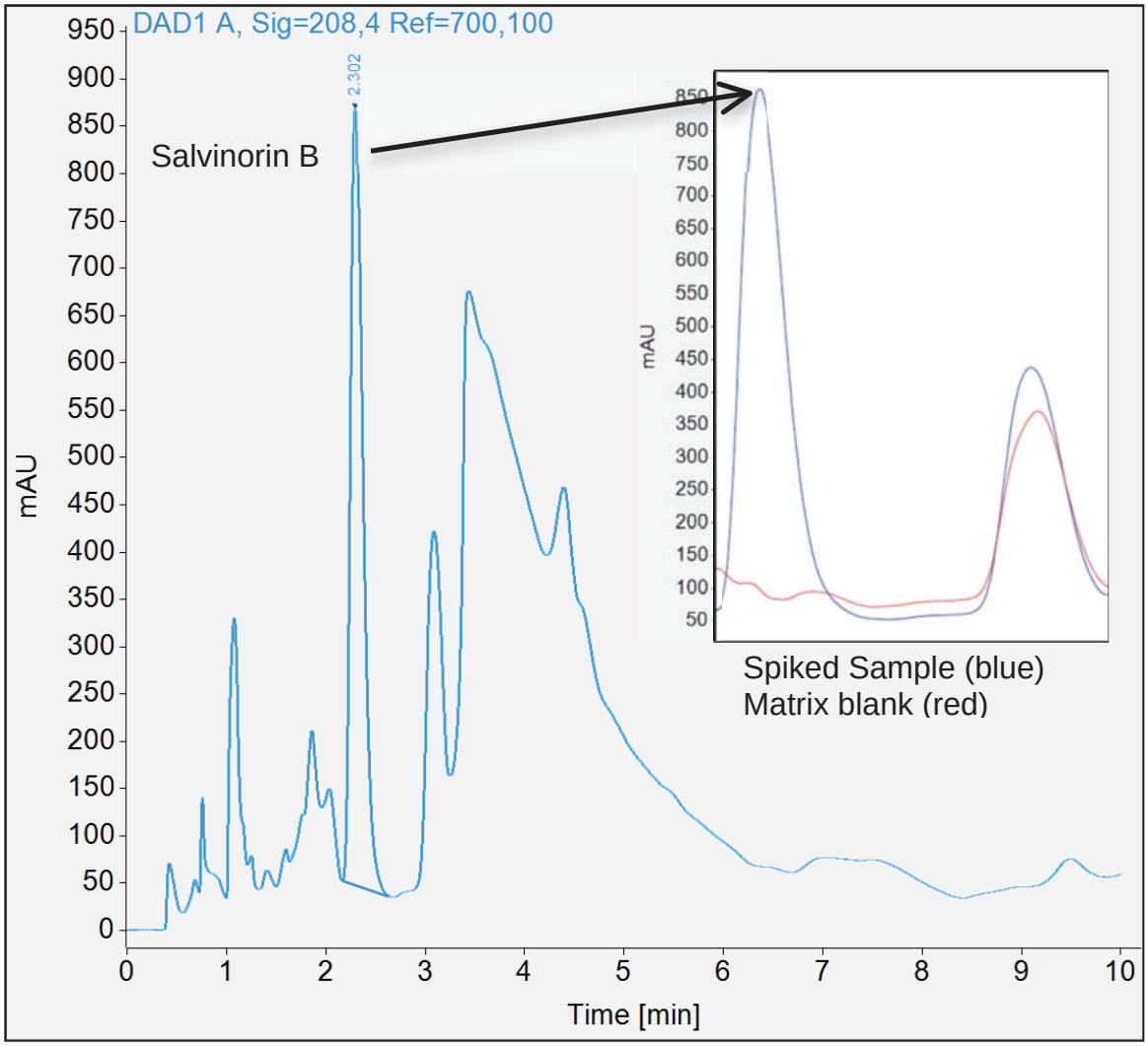


Figure 14. HPLC of salvinorin B spiked *Salvia officinalis* at 15 mg/g. Inset shows spiked sample in blue overlaid with a matrix blank in red.

The *Salvia officinalis* extract contains many peaks, including a significant, broad, tailing peak starting at 3.5 min. *Salvia officinalis* has been used as a successful matrix blank in GC/MS analyses (51), but it might not be suitable for HPLC analysis due to the solubility of compounds from the leaf matrix in DCM. Salvinorin B ethoxymethyl ether was also spiked onto *Salvia officinalis* at 15 mg/g. The spectrum is shown in **Figure 15**. In this case, the SB-EME peak elutes

on the tail of the broad *Salvia officinalis* peak, having no resolution from the matrix. The presence of the SB-EME peak can be determined easily, but quantitation will be significantly affected. Integration was performed by drawing a flat baseline across the spectrum and dropping a line down on either side of the peak. These resolution issues will also affect the LOD these compounds, as trace amounts would be lost in the coeluting matrix peaks.

Evaluation of standards on this HPLC method shows good resolution of salvinorin A, salvinorin B, and salvinorin B ethoxymethyl ether from each other and from other impurities (see **Figures 9**, **10**, **12**, respectively). However, it will be important to fully evaluate any matrix effects prior to quantitative analysis on any plant materials. A method to clean up the plant extracts could be developed in order to achieve better selectivity.

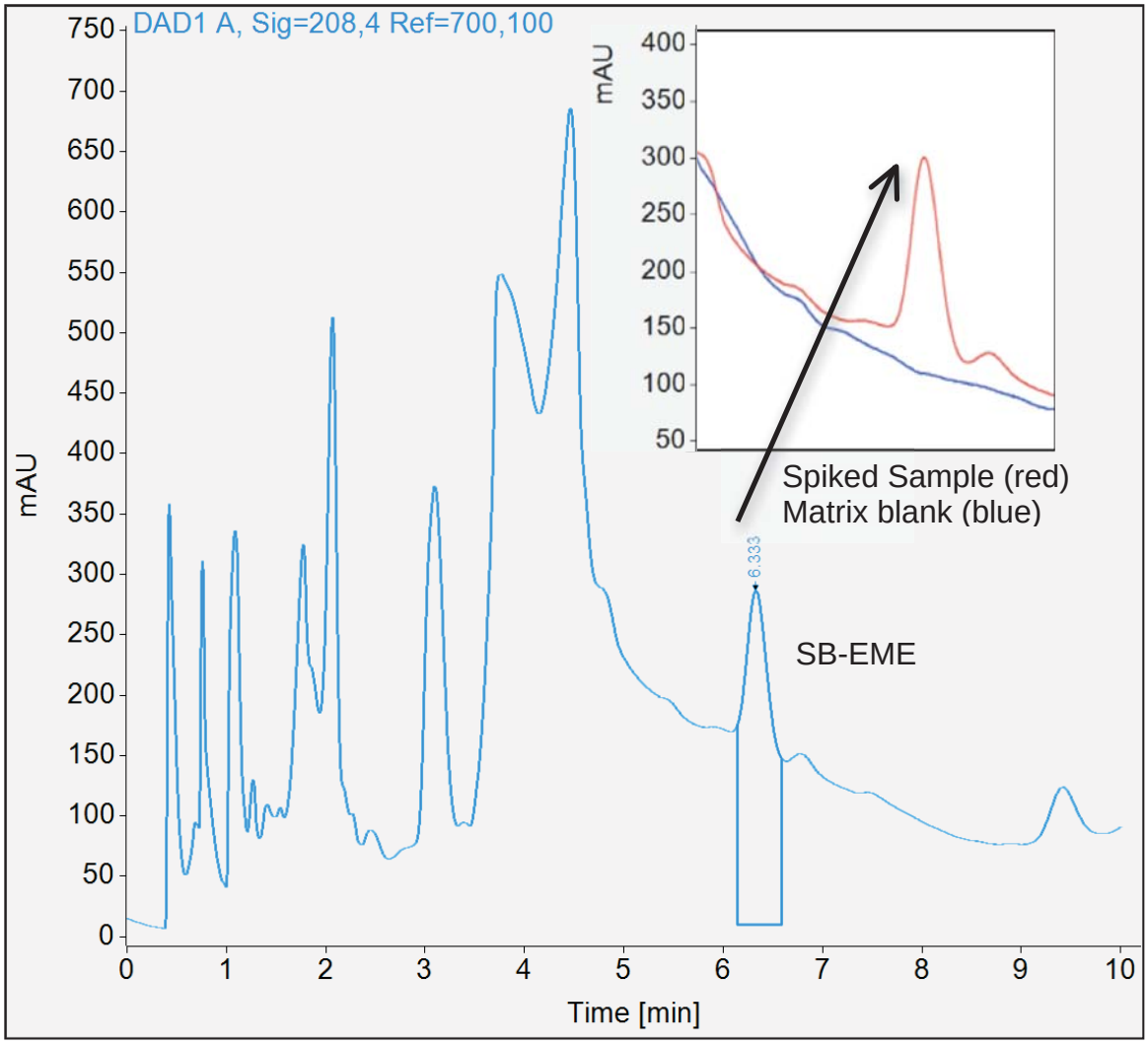


Figure 15. HPLC of SB-EME spiked *Salvia officinalis* at 15 mg/g. Inset shows spiked sample in red overlaid with a matrix blank in blue.

3.2.5. Sensitivity

The LOD was determined to be 0.0005 mg/mL, or 0.038 µg of SB-EME. The LOQ was determined to be 0.0015 mg/mL, or 0.113 µg. The S/N values are reported in **Table 5** and the S/N values for the entire calibration curve is found in **Appendix A.6**.

Table 5. LOD and LOQ determination for SB-EME. The LOD is at S/N of 3 and the LOQ is at a S/N of 10.

Concentration (mg/mL)	Set 1 S/N	Set 2 S/N	Set 3 S/N	LOD S/N	LOQ S/N
0.0005	3.8	4.8	3.5	4.3	
	4.4	4.2	3.6		
	4.3	5.9	3.9		
0.0015	10.2	10.9	9.4		10.6
	12.3	10.8	9.7		
	12.0	12.2	7.9		

3.3. UV/Vis Analysis of Salvinorin Derivatives

The resulting spectra of the UV/Vis analysis on the concentrations of salvinorin A, salvinorin B, and SB-EME standards in DCM from **Table 3** are presented in **Appendix 7**, and an overlay of the highest concentration of each is located in **Figure 16**. As the extraction is performed in DCM, the solutions of standards are analyzed in the same solvent. This would allow for screening of the extracted solutions by UV/Vis prior to confirmatory analysis. Salvinorin A shows slight absorbance of impurities at 400 nm and 660 nm, which have been previously characterized (31). The remainder of the spectrum is similar to salvinorin B; both have absorbance maxima at 279 nm. The SB-EME spectrum displays a diminished absorbance at 280 nm and new absorbance at 337 nm relative to salvinorin A and salvinorin B; therefore, the overall trace is distinguishable from both salvinorin A and salvinorin B.

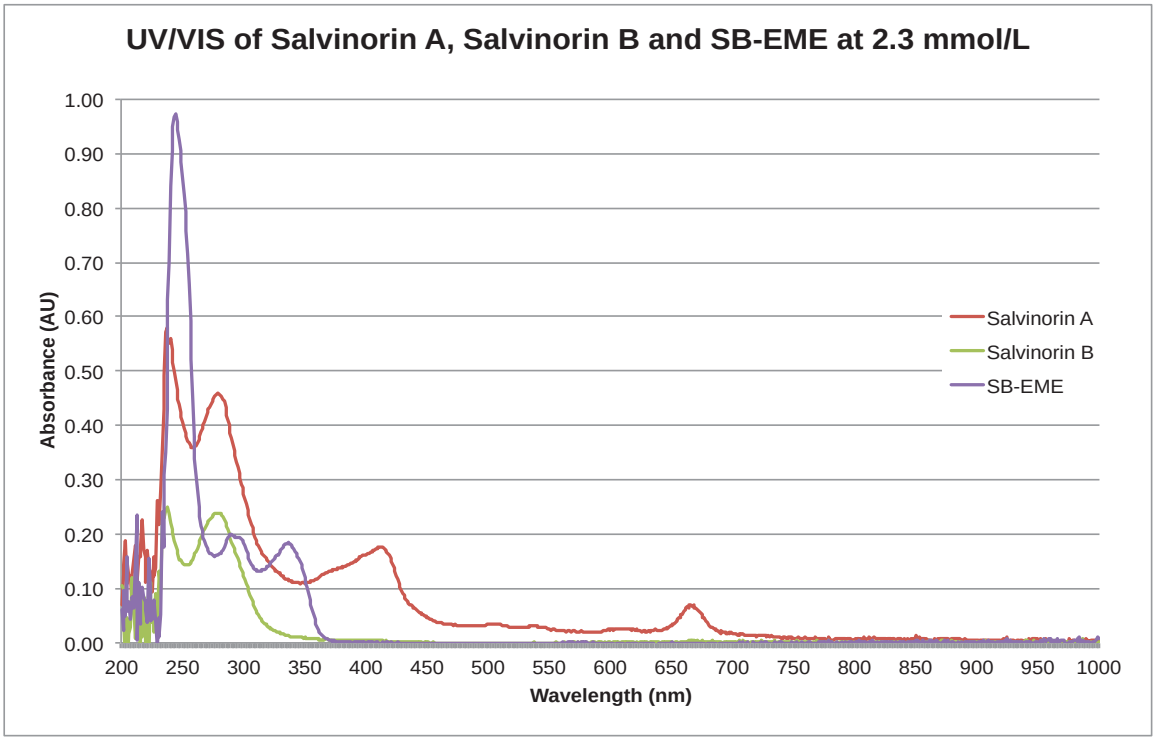


Figure 16. UV/Vis of salvinorin A, salvinorin B, and SB-EME at 2.3 mmol/L. Salvinorin A (red) and salvinorin B (green) have similar maxima, where SB-EME (purple) shows a new maxima at 337 nm.

The molar absorptivities, or the strength of absorbance at a given wavelength, of each standard was determined by plotting the maximum absorbance (λ_{max}) versus the concentration. The molar absorptivity is the value of the slope of the curve in units of $M^{-1}cm^{-1}$ and were calculated from using 5 of the standards in which the linearity was maintained. The results are presented in **Figures 17**, **18**, and **19**. The λ_{max} of salvinorin A and B was chosen at 279 nm to avoid the interference caused by the subtraction of DCM. The UV/Vis cuttoff of DCM is 230 nm, below which quantitative analysis is hindered by the absorbance of the solvent. The molar absorptivity of salvinorin A (@279 nm) is 227.04 $M^{-1}cm^{-1}$,

of salvinorin B (@279 nm) is 162.33 $M^{-1}cm^{-1}$, and SB-EME (@244 nm) is 199.63 $M^{-1}cm^{-1}$.

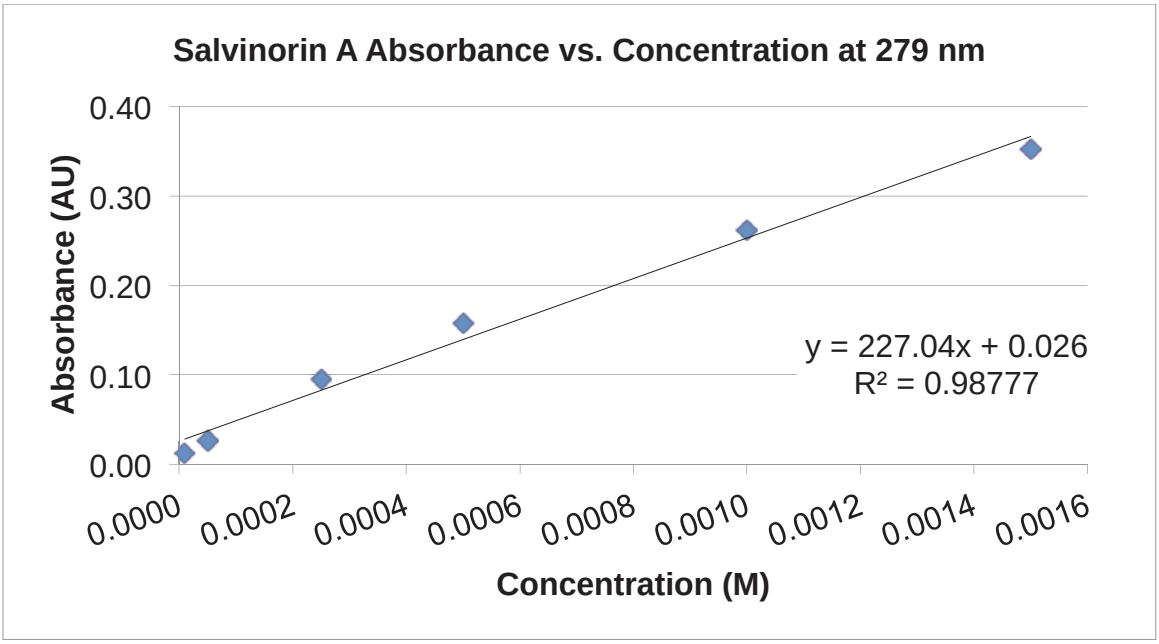


Figure 17. Salvinorin A absorbance versus concentration at 279 nm. The molar absorptivity at 279 nm is 227.04 $M^{-1}cm^{-1}$.

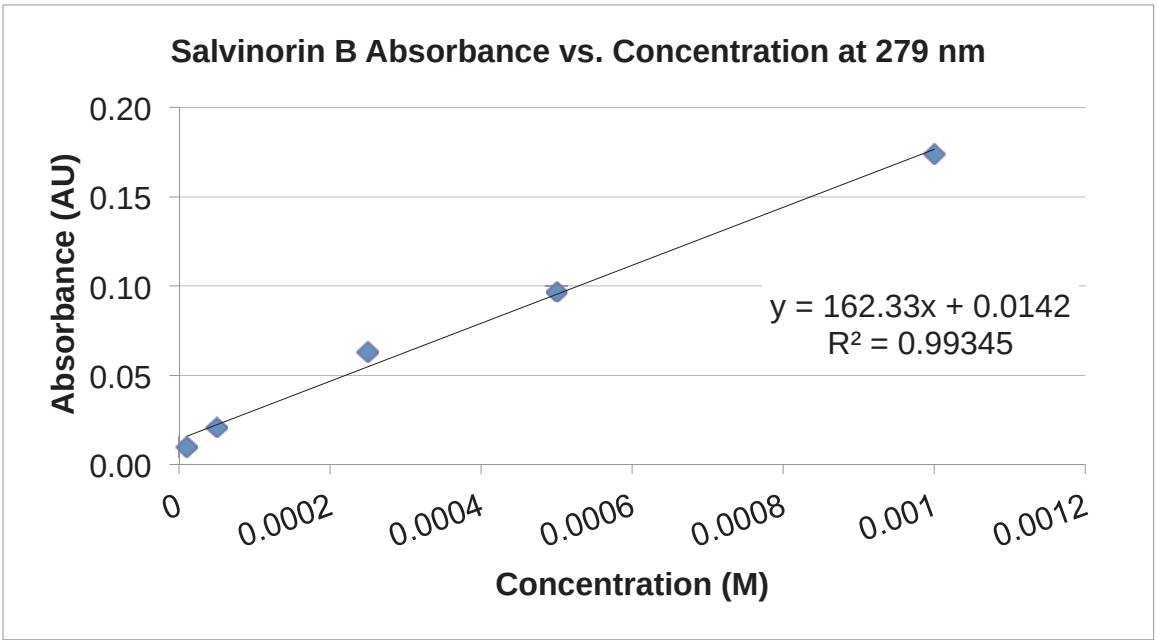


Figure 18. Salvinorin B absorbance versus concentration at 279 nm. The molar absorptivity at 279 nm is 162.33 $M^{-1}cm^{-1}$.

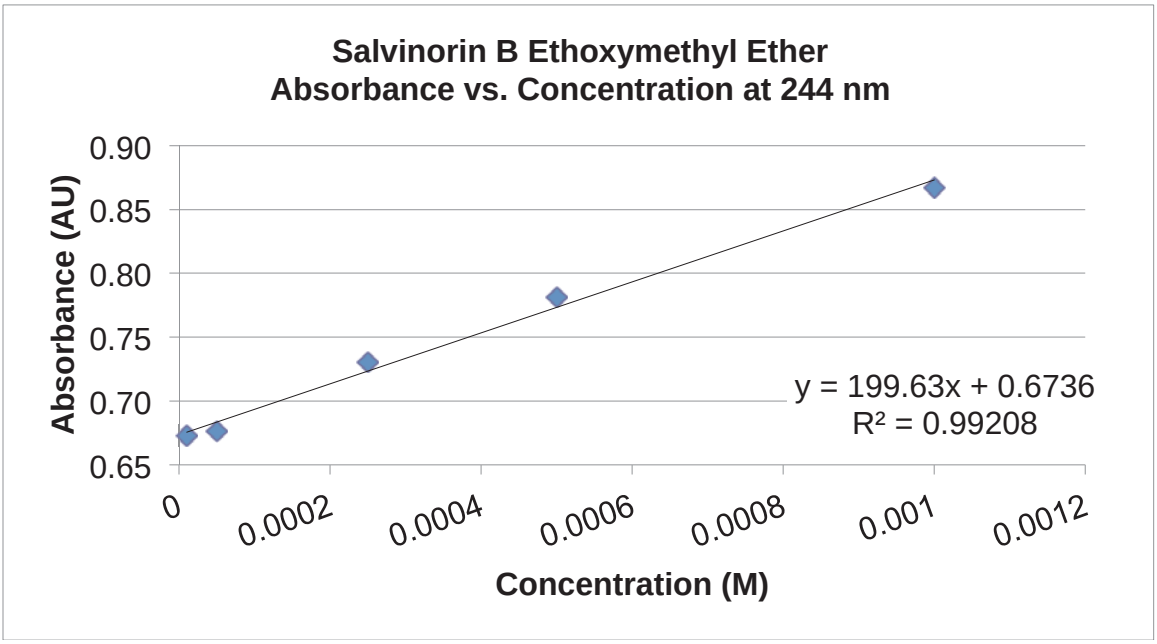


Figure 19. SB-EME absorbance versus concentration at 244 nm. The molar absorptivity at 244 nm is 199.63 $M^{-1}cm^{-1}$.

3.4. Colorimetric Assay and Mechanistic Studies

3.4.1. Standard Ehrlich's Procedure and Mechanistic Discussion

The reaction of *Salvia divinorum* with Ehrlich's reagent performed as previously reported (32, 33). A red-orange color developed within 30 seconds and was fully developed in 2 minutes (see **Figure 20**).

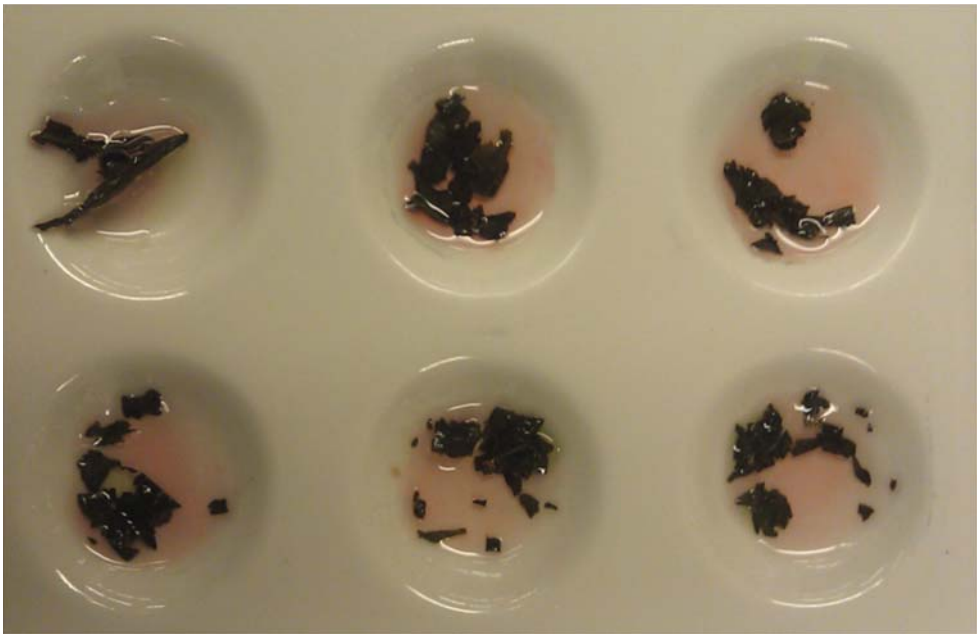


Figure 20. *Salvia divinorum* after addition of Ehrlich's reagent. Photograph was taken after 2 minutes of reaction (n=6).

To understand the mechanism behind the red-orange colored result of Ehrlich's reagent with *Salvia divinorum*, the reported mechanism of Ehrlich's reagent with LSD was first examined (see scheme represented in **Figure 21**). The mechanism is reported to proceed by an electrophilic aromatic substitution of the indole ring from LSD with the aldehyde from pDMAB (55). The reaction goes through a cationic intermediate to yield a final 2:1 adduct of 2 molecules of LSD for every one of pDMAB. The final adduct is described as a cyanine dye (55). The furan ring will perform the same type of reaction with Ehrlich's reagent as an indole ring since analogous heterocycles perform similar reactions (56). **Figure 22** illustrates the relative reactivities of a pyrrole ring, which is a part of an indole ring, to a furan ring and to a thiophene ring. These heterocycles only differ in the

electron density around the heteroatom and therefore will differ only with the rate of reaction.

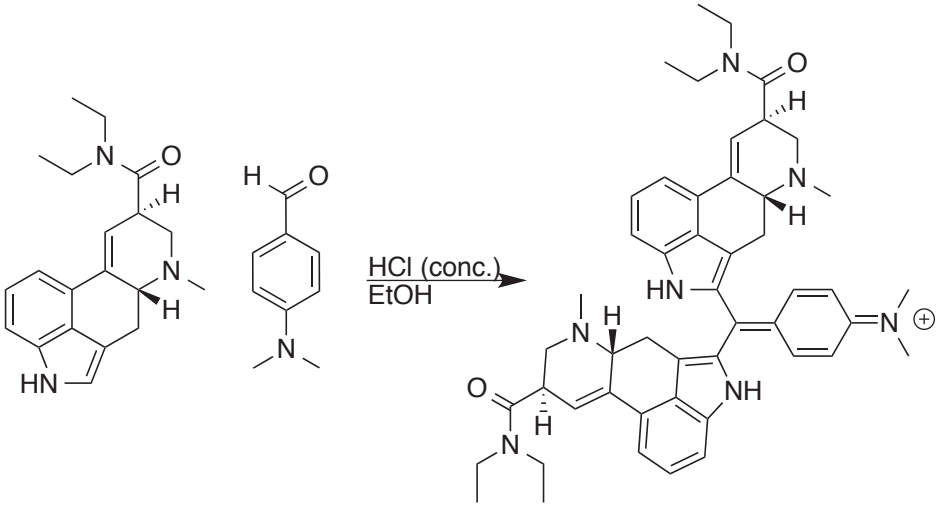


Figure 21. Reported reaction scheme of Ehrlich's reagent with LSD. The aldehyde of pDMAB performs an electrophilic aromatic substitution on the indole ring of LSD, forming a 2:1 final adduct (55).

It has been reported in the literature by Kuroda *et al.* that Ehrlich's reagent can be used to detect furan-type compounds in natural products (57). The resulting substituents around the heterocycle will dictate the color of the reaction (58). However, these authors report the colored component of the reaction is the cationic intermediate (**Figure 23**, in brackets), and not the final 2:1 adduct (57). The intermediate could not be isolated, and the final 2:1 adduct was not colored, so the authors attempted to quench the reaction to trap the intermediate. **Figure 23** illustrates the scheme the authors used to characterize the intermediate. They were able to isolate about 1% of the ethanol adduct for characterization by NMR. The rest of the reaction yielded the 2:1 adduct or decomposition products (57).

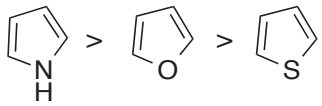


Figure 22. Relative heterocyclic reactivity of pyrrole, furan, and thiophene.

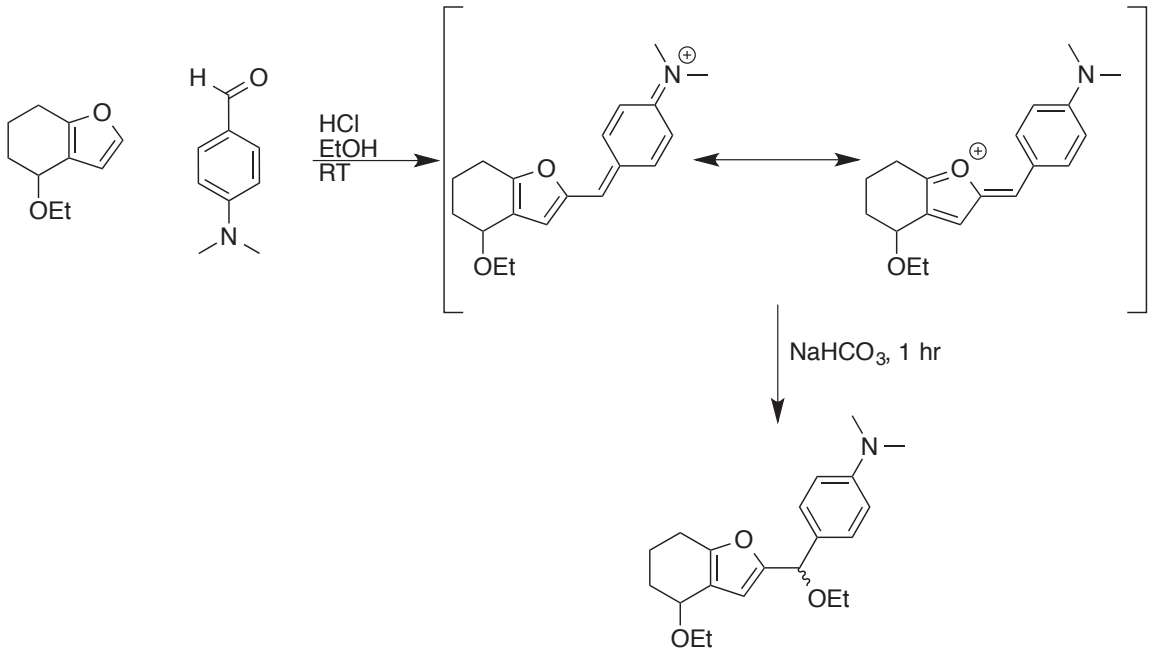


Figure 23. Isolation of colored cationic intermediate from reaction of furanoeremophilanes with Ehrlich's reagent. A model compound was reacted with pDMAB and quenched with ethanol to trap the colored intermediate. Characterization by Kuroda *et al.* (57).

It is hypothesized that the salvinorins and divinatorins (**Figure 5** and **Table 1**) in *Salvia divinorum* will react in the same way with an electrophilic aromatic substitution of the furan ring with pDMAB (see **Figure 24**). This hypothesis is supported by secondary experimental observations. The aqueous layer of the colorimetric assay is colored, in which a small cationic molecule will be soluble. The color is not stable and will turn to a green-blue over time, also suggesting a temporary intermediate is creating the color. Finally, any attempts at isolating or analyzing the aqueous layer quickly quench the color, which does not allow for

direct analysis. Since the intermediate cannot be directly characterized, efforts were switched to characterizing the 2:1 adduct of salvinorin A with pDMAB. If this adduct exists in the DCM layer of the reaction, it could be easily isolated and the structure determined. This could give indirect evidence that the mechanism proceeds through the cationic intermediate to yield the final 2:1 adduct. The following studies with UV/Vis, HPLC, and NMR will focus on the characterization of the 2:1 adduct.

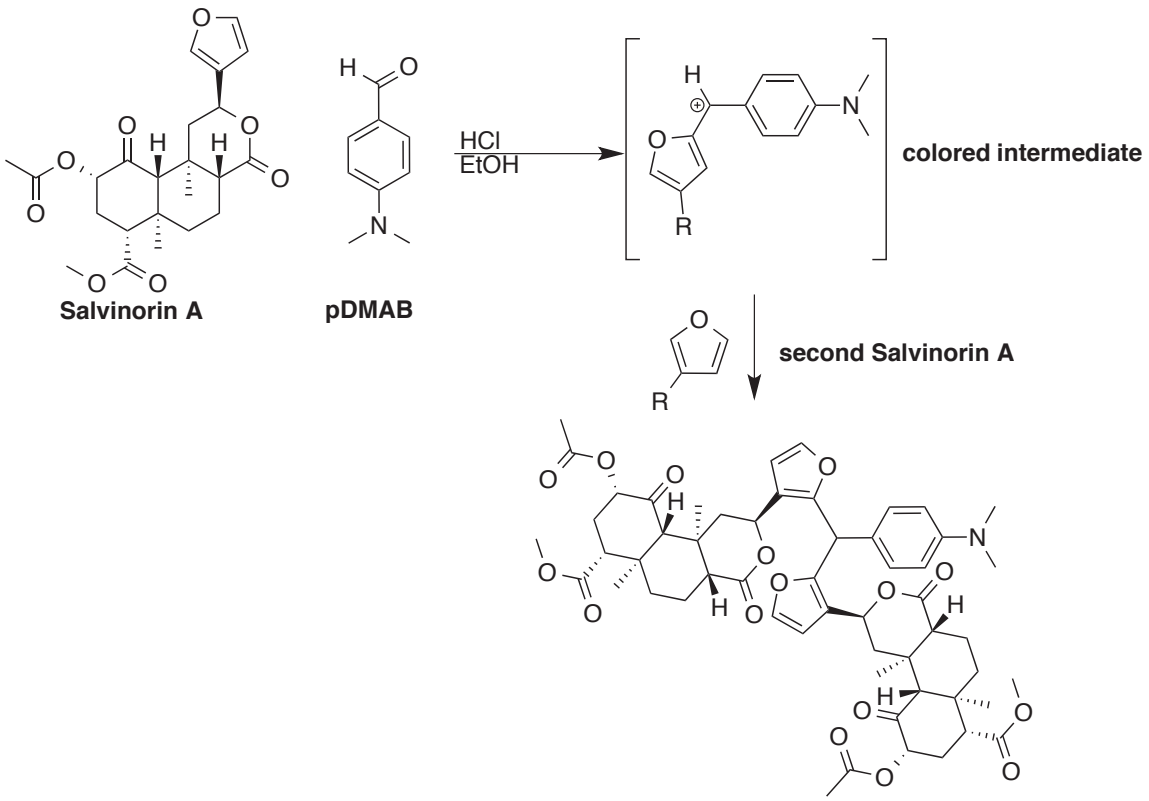
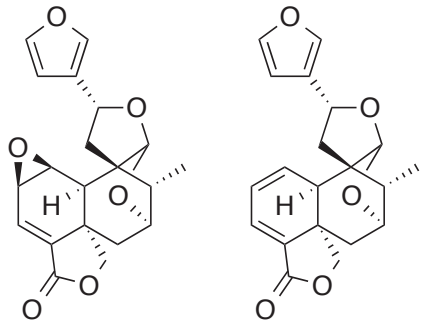


Figure 24. Proposed reaction scheme of salvinorin A with Ehrlich's reagent. Salvinorin A undergoes an electrophilic aromatic substitution of the furan ring with the aldehyde on pDMAB. The intermediate of the reaction creates a red-orange color and the final product is a 2:1 adduct.

Since many compounds isolated from *S. divinorum* contain a furan ring, all of the analogous structures from **Figure 5** and **Table 1** will react with Ehrlich's reagent. This might be the reason as to why the results on 50 µg standards of salvinorin A produces a weaker color than 1 mg of *Salvia divinorum* leaves, which contains only 3.4 µg of salvinorin A, as shown in previous work (33). In addition, the cross-reactivity with *Salvia farinacea* (mealy sage) (33) can be explained by examining the known compounds isolated from that plant (see **Figure 25**). While not considered salvinorins, the compounds salvifarin and salvifaricin both contain a furan ring, which should perform the same chemistry with Ehrlich's reagent.



Salvifarin Salvifaricin

Figure 25. Compounds isolated from *Salvia farinacea*. Salvifarin and salvifaricin both contain a furan ring, which reacts with Ehrlich's reagent.

3.4.2. UV/Vis Characterization of Colorimetric Assay

The top aqueous layer and bottom organic layer of the reaction with Ehrlich's reagent were characterized by UV/Vis, using a standard of salvinorin B in DCM as a blank subtraction. For analyzing the bottom organic layer, the

solutions were placed in a narrow 2 mL cuvette and agitated via pipette after 4 min. There was no appearance of color within the first 4 minutes, and it is hypothesized that the reduced surface area of the cuvette accounts for the slower reaction rate when compared to a spot plate. When performing the assay in a spot well plate, the leaves noticeably swirl around the well, providing some agitation. On a standard solution in a cuvette, very little natural agitation occurs until the solution is manually mixed. After agitation at 4 min, the red-orange color developed. In **Figure 26**, the UV/Vis spectra of the bottom, DCM layer is shown after multiple measurements over a 30 min time period. The spectra for the first 4 minutes have minimal absorbance as shown in the zoomed image in **Figure 27**. The flat baseline indicates there is no change in absorbance from the reference standard of salvinorin B that was used as a subtraction blank. Once the solution is agitated, there is an immediate presence of a new absorbance profile (blue spectra in **Figure 26** and **27**). This major profile does not absorb into the visible region, however, indicating it is not colored. There is a slight absorbance at 500 nm, which is the red-orange range that increases after mixing; however, the DCM layer is not the layer that is strongly colored.

The drastic change in the spectra after mixing could be hypothesized to be the formation of the 2:1 adduct, absorbance from Ehrlich's reagent (**Figure 28**), or formation of degradants from interaction with the HCl. In general, the 2:1 adduct would have a combination of absorbance features from both salvinorin B and Ehrlich's reagent due to the functional groups contributing to the spectrum.

The presence of a broader peak spanning 250 – 300 nm could encompass these features shown in **Appendix 7.2** and **Figure 28**. It is also plausible that the spectral change could be due to the absorbance of Ehrlich's reagent alone. The reaction is traditionally performed with an excess of Ehrlich's reagent, and the high concentration could account for the saturation of the detector above 2.5 AU as seen in **Figure 26**. Finally, the interaction of salvinorin B with 6N HCl could have affected the structural integrity of the molecule. Future studies should include UV/Vis analysis on the salvinorin B standard with 6N HCl in ethanol. This would help eliminate any contribution from the high concentration of pDMAB and highlight the affects of the acid on the molecule.

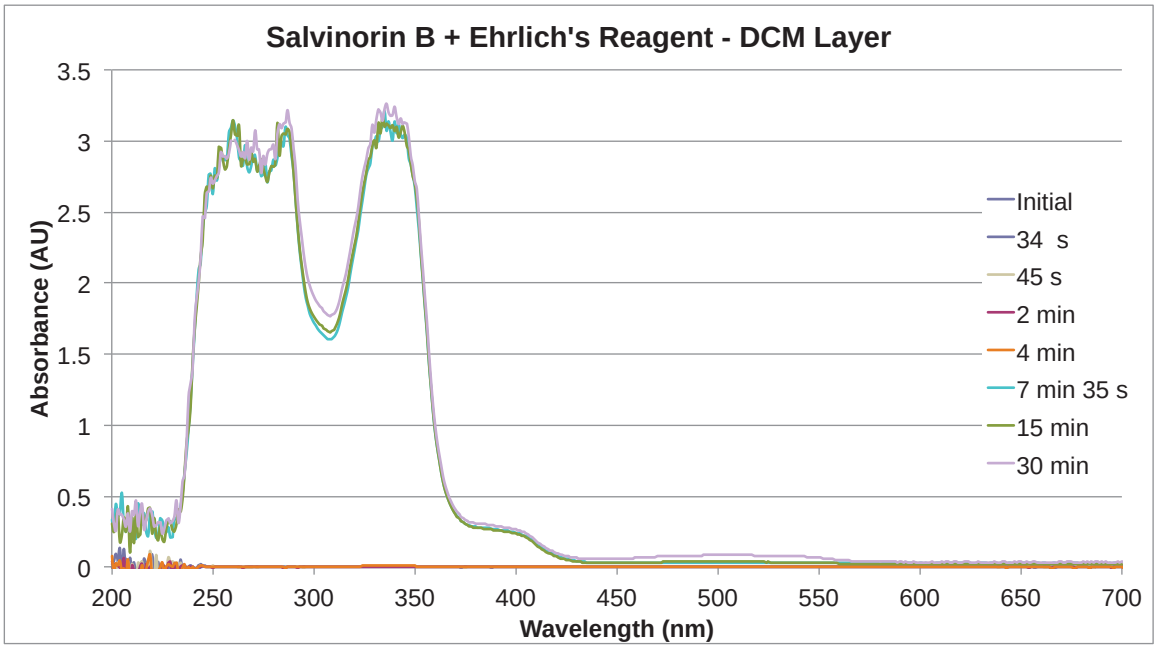


Figure 26. UV/Vis of salvinorin B in DCM layer during reaction with Ehrlich's reagent. Spectra were measured multiple times over 30 min. Agitation of the reaction occurred after 4 min.

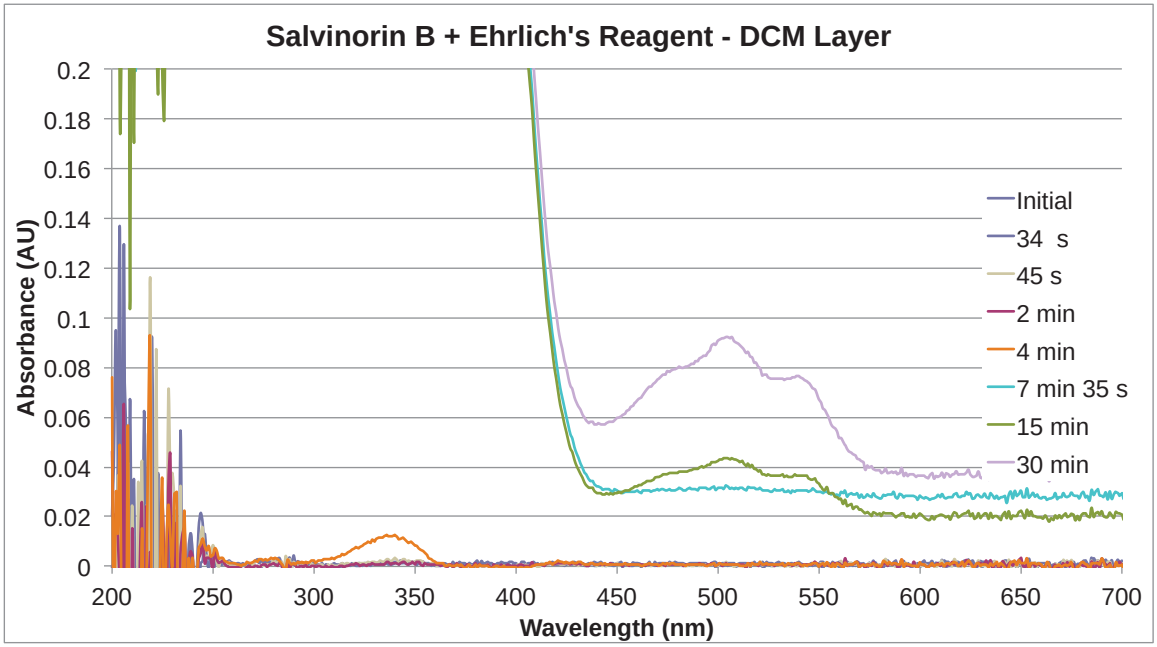


Figure 27. Zoomed baseline of UV/Vis of salvinorin B in DCM layer during reaction with Ehrlich's reagent. Spectra were measured multiple times over 30 min. Agitation of the reaction occurred after 4 min.

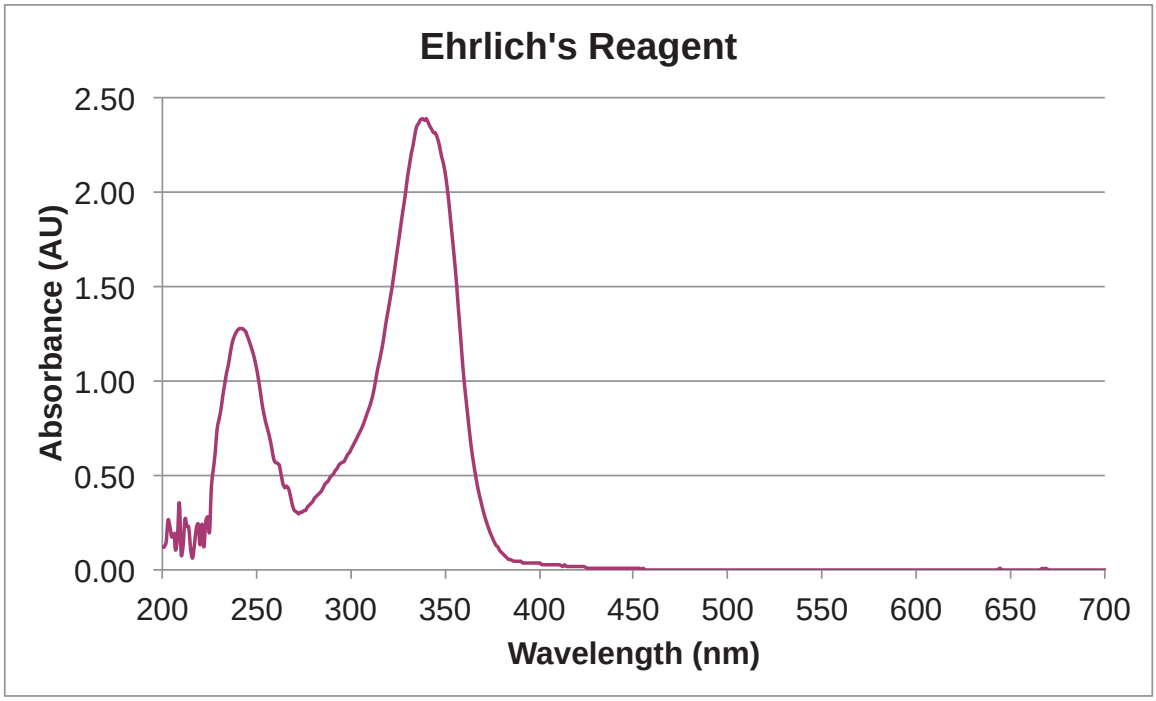


Figure 28. UV/Vis of Ehrlich's reagent. 1:10 dilution in ethanol.

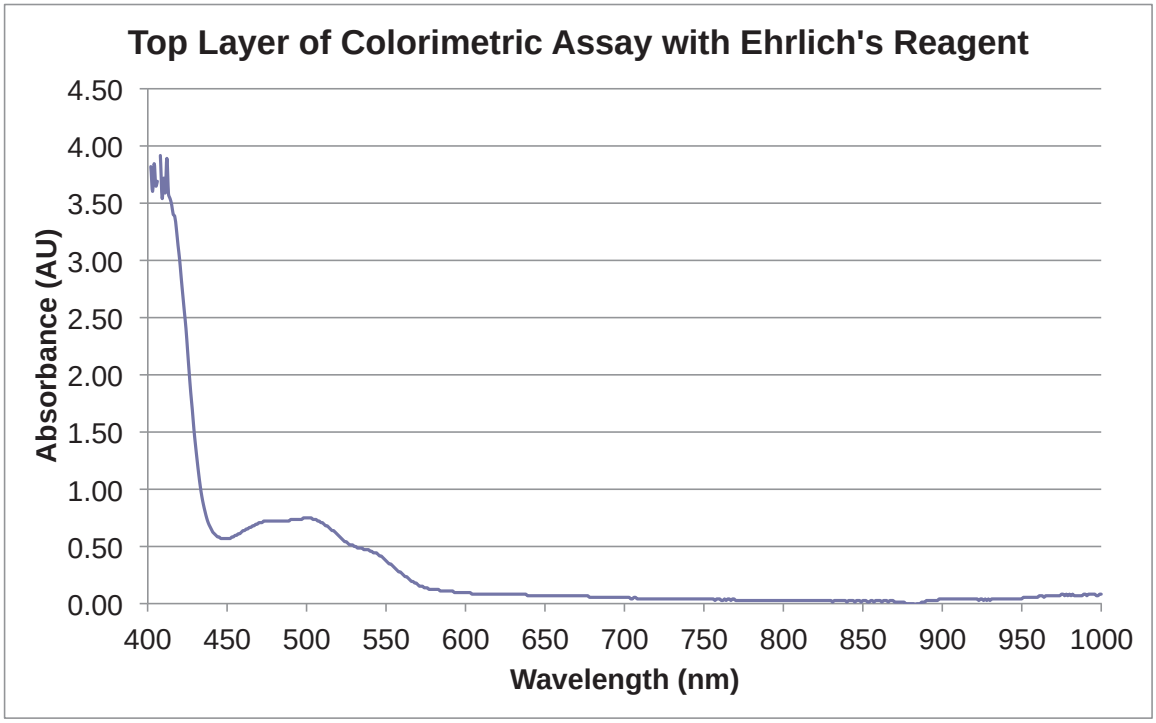


Figure 29. **UV/Vis of the aqueous layer of colorimetric assay with Ehrlich's reagent.** Solution was reacted for 30 min with salvinorin B.

Figure 29 shows the UV/Vis trace of the top, aqueous layer after reaction of 30 min with salvinorin B. There is an increase in absorbance at 500 nm that is not present in the original reagent, as seen in **Figure 28**, which shows fresh Ehrlich's reagent, diluted 1:10. Absorbance in the 500 nm region translates to a red-orange color. The small amount of absorbance at 500 nm seen in the DCM layer in **Figure 27** could be due to incomplete separation of the layers during the analysis. The peak has the same profile with a maximum at 500 nm, and a shoulder at 550 nm.

3.4.3. HPLC Characterization of Colorimetric Assay

The analysis of the DCM layer on HPLC is shown in **Figure 30**. The method was changed to a gradient method to allow for a highly retained new peak to elute off of the column. This compound is very non-polar and has local maxima at 225 nm and 275 nm. The negative peaks seen in the trace indicate the compound eluting off has less absorbance than the mobile phase at that wavelength. The presence of the new peak and the negative peaks indicates that some new chemistry has occurred with salvinorin B. The new peak could be the final 2:1 adduct, and/or the negative peaks could be quenched molecules of the cationic intermediate or some other byproducts. The trace does not provide any additional differentiation, however.

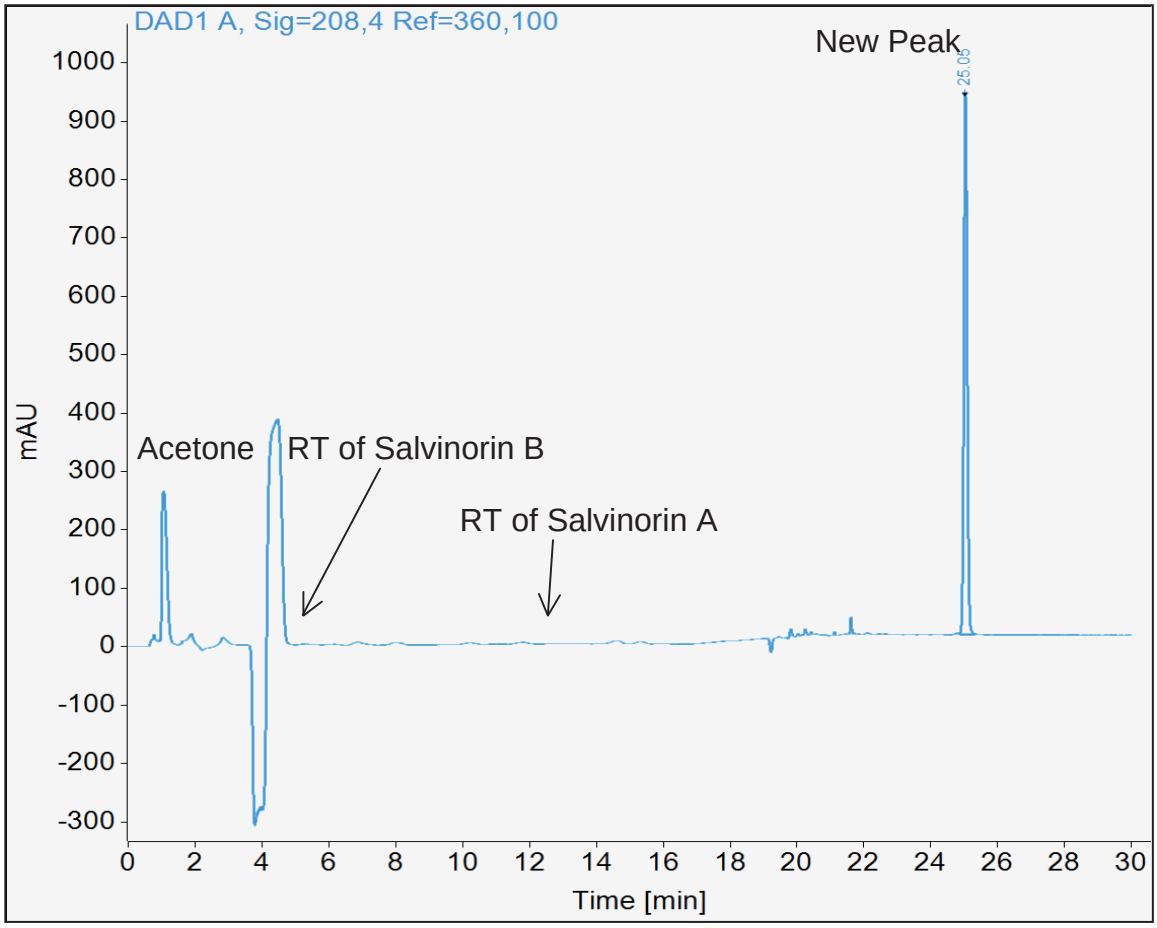


Figure 30. HPLC trace of DCM layer after 30 min reaction with Ehrlich's reagent. Arrows indicate positions of salvinorin A and salvinorin B.

3.4.4. ^{1}H NMR Characterization of Colorimetric Assay

Presuming that the colored species is located in the top aqueous layer, the DCM layer was analyzed by ^{1}H NMR. The layer was separated from the acid, washed with $NaHCO_3$ to neutralize the pH, dried over Na_2SO_4, filtered, and evaporated down. The residue was reconstituted in $CDCl_3$ for ^{1}H NMR analysis. A separate sample was analyzed in which salvinorin B in DCM was washed with 6N HCl in ethanol for 30 min and worked up as before to determine how the acid affects the structural integrity. **Figure 31** shows the NMR spectra of salvinorin B

standard (top, blue), the DCM layer (middle, red), and the acid-washed salvinorin B (bottom, green) (see **Appendix A.8** for larger versions). Of note is the area from 6.0 ppm to 10 ppm, which contains the aromatic protons. In the salvinorin B standard (top, blue), three singlet peaks are seen, indicating the three protons of the furan ring (excluding 7.26 ppm, which is residual $CHCl_3$). The structural characterization of the salvinorins has been well established in the literature, and the analysis here of salvinorin B was consistent with the reported results (52). No measurable coupling of the protons on the furan ring was seen in the standard solutions. In the DCM layer (middle, red) that has undergone reaction with pDMAB in Ehrlich's reagent, these peaks have changed, and new peaks are present. There are only two peaks corresponding to the aromatic furan protons, and three peaks corresponding to pDMAB are now present. This is encouraging, since it is expected that one furan peak will disappear once reacted, and the pDMAB peaks will appear in the DCM layer. However, the spectrum is more complex than expected due to the interaction with the HCl present in Ehrlich's reagent. As seen in the acid-washed spectrum (bottom, green), the same pattern for the two furan protons is present, indicating this is due to the interaction with the acid and not with reaction with pDMAB. Upon examination of the rest of the spectra, from 2.0 to 3.0 ppm for example, most of the other protons from the salvinorin B standard (top, blue) have disappeared in the DCM layer (middle, red), and mimic the pattern seen in the acid washed standard (bottom, green). These proton signals are not expected to change based on the proposed

mechanism. This indicates the acid has likely broken the molecule apart, which makes characterization more complex.

What can be elucidated from this data is that there are still aromatic protons present. Of importance is that there are only two furan protons, as the rest are attributed to chloroform-d, or pDMAB. Since the third proton is missing, it likely has reacted, which supports the proposed mechanism for salvinorin A and related compounds. While the strong acid in Ehrlich's reagent has destroyed the molecule, it is proposed that the interaction with pDMAB is still significant enough to still create a colored intermediate.

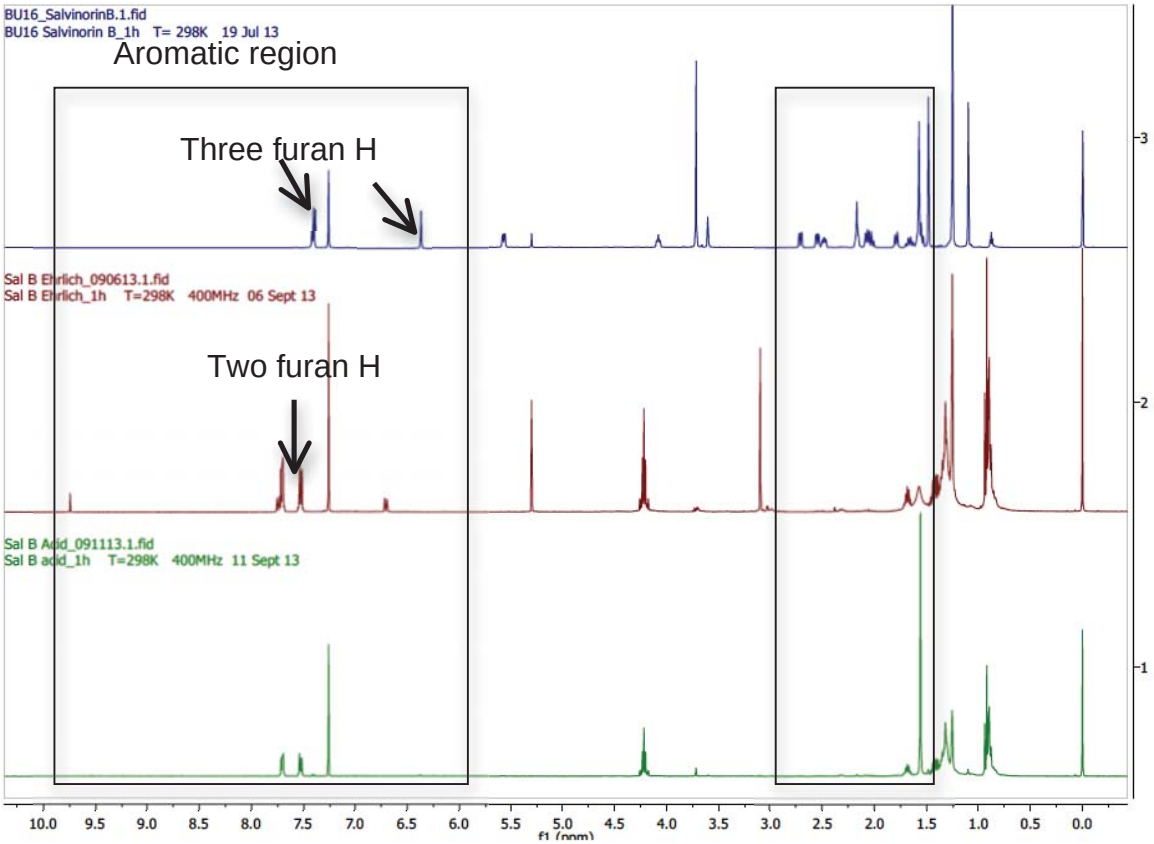


Figure 31. ^{1}H NMR analysis of colorimetric assay with Ehrlich's reagent. Salvinorin B (blue), DCM layer assay with Ehrlich's reagent (red), and acid washed salvinorin B (green) spectra are shown. Left box highlights the aromatic region with the furan protons. Right box highlights the change of protons from the rest of the molecule after reaction with HCl.

To determine which proton of the furan ring reacted in this reaction, a 2D ^{1}H correlation spectroscopy (COSY) NMR experiment was performed, using salvinorin B as the model compound. This experiment shows the J-coupling, or the interaction the magnetic spin of two adjacent protons have with each other (59). The COSY spectrum is shown in **Figure 32** (larger version in **Appendix A.8.4)**, and the indicated coupling is highlighted by a box. Symmetrical signals are called crosspeaks and indicate the coupling within the molecule. This

interaction of the two aromatic protons indicates they must be adjacent to each other, and located on the same side of the furan ring. Therefore, the final proposed mechanism is thought to react on the side of the furan ring with the single α-proton as indicated in the reaction scheme in **Figure 24**.

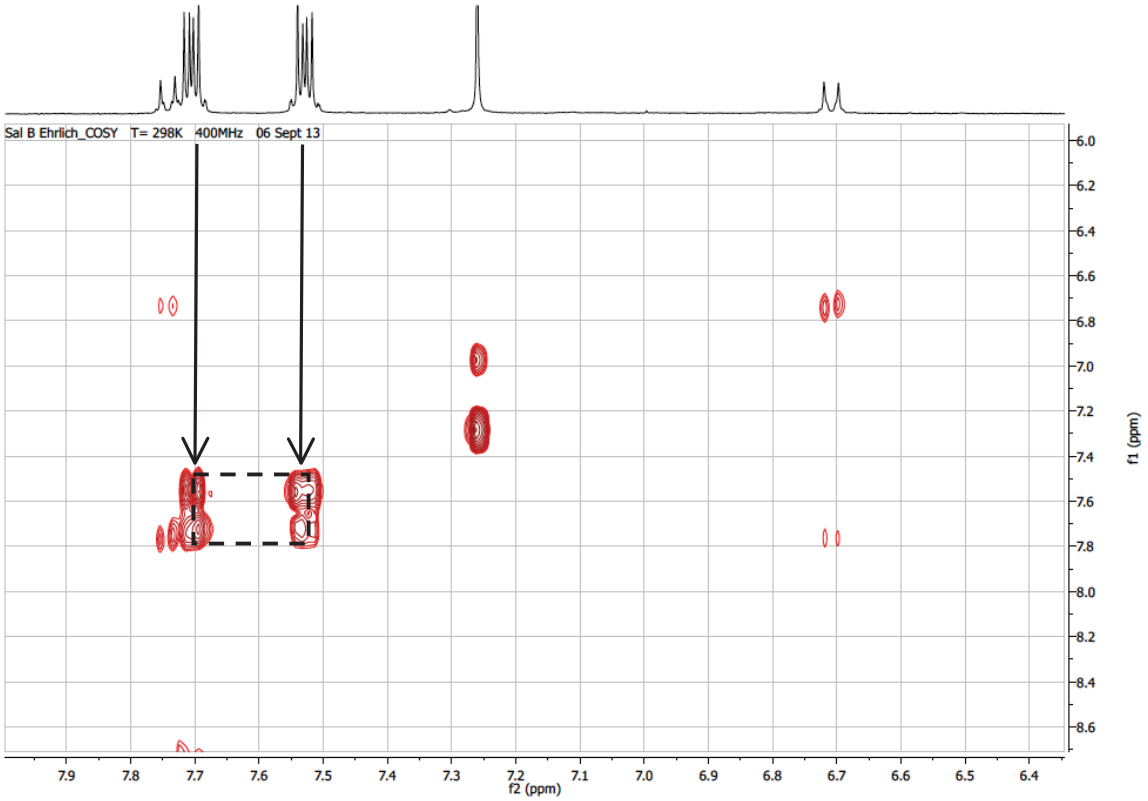


Figure 32. COSY NMR of salvinorin B reacted with Ehrlich's reagent. The aromatic region is expanded from 6.4 ppm to 7.9 ppm, and the interaction between the two furan protons is highlighted by a dotted box.

3.5. Adulteration of Plant Materials

3.5.1 Colorimetric Assay of Adulterated Plant Materials

The colorimetric assay with Ehrlich's reagent was used to determine if salvinorin A, salvinorin B, or salvinorin B ethoxymethyl ether could be detectable

on adulterated plant materials. First, a screen on the standards alone was performed, as seen in **Figure 33**. The resulting color is lighter than what was visualized on dried plant material as previously discussed in section **3.4.1**, but all three produced a red-orange color.

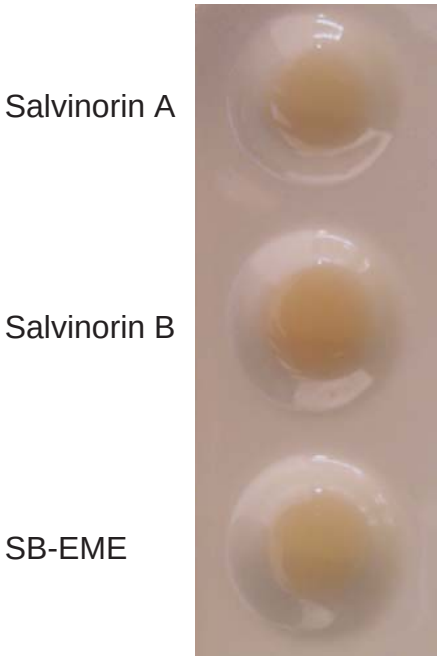


Figure 33. Colorimetric analysis of salvinorin A, salvinorin B, and SB-EME standards. Standards of 1 mmol/L were analyzed and allowed to react for 2 min prior to photograph.

The lowest level of spike was 3.4 mg/g of dried leaf material, as that is the concentration reported in the literature of the natural *Salvia divinorum* plant (30). The next two levels, 15 mg/g and 40 mg/g, were chosen to represent an “enhanced regular-strength” and an “enhanced extra-strength” dose (46). The standards were spiked in an acetone solution and allowed to air dry on the leaves. *Salvia officinalis* was chosen to represent a matrix blank, as it has the same genus as *Salvia divinorum* and no salvinorin type compounds have been

previously isolated from this plant (51). Kratom and Dream Herb were chosen to represent a range of plant materials that were inexpensive, easily accessible, and known for their abuse potential (1). These two plant materials also represented a range of phenotypes, where Kratom was a uniform sample of small, dark brown broken leaves, and Dream Herb was an inhomogeneous sample of larger pieces of green leaves and flower buds.

First, the colorimetric assay was performed in triplicate on all of the unadulterated plant materials prior to spiking to determine if any interfering colors or side reactions resulted. The plain leaves are pictured in **Figure 34,** and the assay with Ehrlich's reagent on the leaves after 2 min are pictured in **Figure 35**. *Salvia divinorum* turns a red-orange color, as expected in the presence of Ehrlich's reagent. *Salvia officinalis* and Kratom have no color change, with the solution staying yellow after addition of Ehrlich's reagent. Dream Herb appears to have a more intense yellow color after the reaction. While no furan compounds have been isolated from Dream Herb, furanone type molecules have been identified (60). These structures are more complex, and therefore more empirical data is needed to determine if they will produce a color result with Ehrlich's reagent. See **Figure 36** for a representative structure from Dream Herb.

S. divinorum

S. officinalis

Kratom

Dream Herb

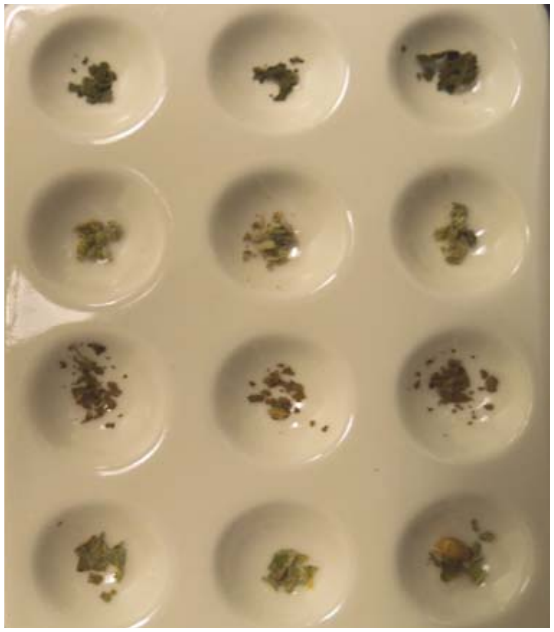


Figure 34. Samples of *S. divinorum*, *S. officinalis*, Kratom, and Dream Herb. Unadulterated leaves, prior to assay with Ehrlich's reagent (n = 3).

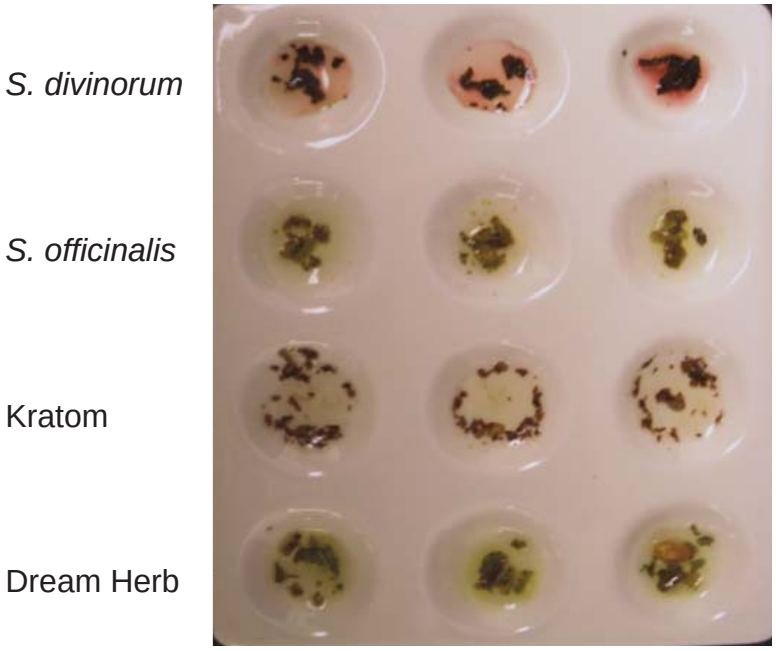


Figure 35. Samples of unadulterated *S. divinorum*, *S. officinalis*, Kratom, and Dream Herb after reaction with Ehrlich's reagent. Samples reacted for 2 min (n = 3).

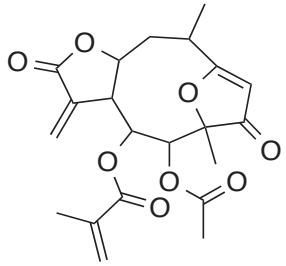


Figure 36. Structure of 9α-acetoxyzexbrevin from Dream Herb. Stereochemistry not indicated.

The reaction of adulterated *Salvia divinorum* is seen in **Figure 37**. The unadulterated leaves are analyzed in triplicate in the top row. Only salvinorin B and salvinorin B ethoxymethyl ether were spiked onto dried *Salvia divinorum*, however the color result was opposite of what was expected. With the addition of more furan containing compounds, the red-orange color was expected to

intensify. Instead, the color result decreased as the concentration of spiked standard increased.

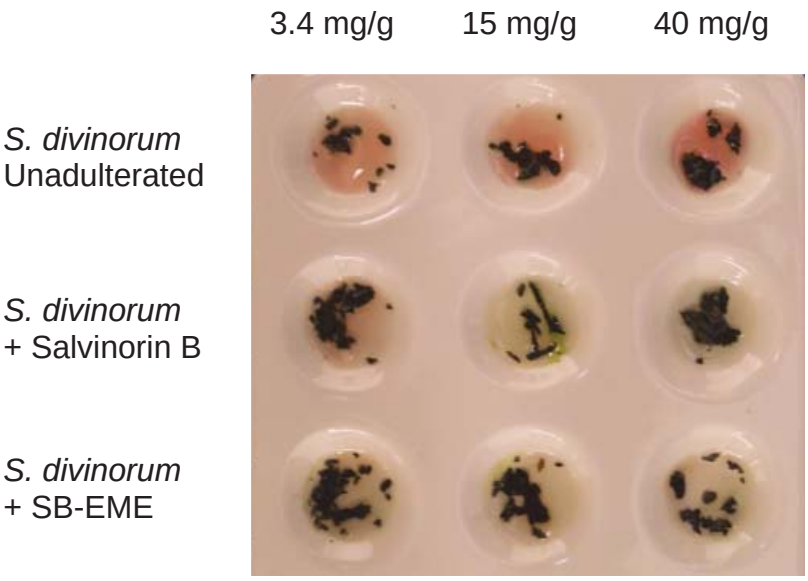


Figure 37. Adulterated *Salvia divinorum* with salvinorin B and SB-EME after reaction with Ehrlich's reagent. After 2 min, the adulterated samples showed a less intense or negative result. The top row contained 3 replicates of unadulterated leaves as a control. Each column contains 3.4, 15, or 40 mg/g of spiking standard, respectively.

It is hypothesized that this result is due to the spiking procedure used. Stock solutions of the standards were made in acetone, and the volume of the stock solution was adjusted to achieve the desired amount of additional compound added. This resulted in very small volumes added to the leaves for the 3.4 mg/g spike and larger volumes added to the leaves for the 40 mg/g spike. A visible residue was seen in the vials of the 40 mg/g spike, indicating the extra solvent potentially extracted out the inherent salvinorin A in the leaves and deposited both salvinorin A and the spiking standard onto the glass of the vial.

Efforts were made to scrape out all of the contents of the vial, but the transfer was not quantitative.

The spiking study was modeled after a similar study performed by Willard *et al.*, where the authors spiked samples of *Salvia divinorum* with salvinorin A and analyzed by GC/MS (51). After the spiking of the samples, the vials from Willard were directly analyzed and extracted for GC/MS analysis, whereas the samples in this study were analyzed with Ehrlich's reagent on a separate spot well plate. This resulted in analysis on only the plant material for this study, and did not include the vial or original container used to deposit the spiking standard. In addition, the authors only looked for the presence of salvinorin A by GC/MS and did not present any quantitative data on the recoveries of the spiking experiment. This information would be helpful to compare the point of difference between the two experiments. In order to evaluate this hypothesis that the spiking standard remained in the original vial and did not transfer to the plant material, the empty vials were extracted with acetone and subsequently analyzed. See discussion in Section **3.5.2**.

This result was also seen in all of the other spiked plant materials. *Salvia officinalis* spiked with salvinorin A, salvinorin B, and SB-EME is presented in **Figure 38**. Kratom and Dream Herb spiked samples are presented in **Figures 39** and **40**, respectively. No color change was seen on any of the spiked plant materials after 2 minutes. If left for 10 minutes, a very slight change was seen at the 3.4 mg/g spike level. However, at this time all of the DCM had evaporated

from the S. divinorum control wells, and no red-orange color remained. Therefore, the slight color change cannot be read as a positive result.

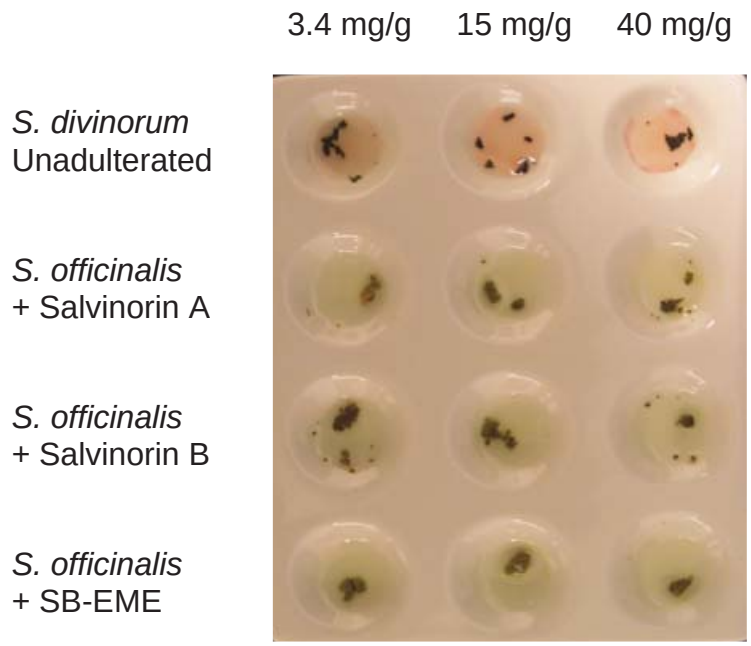


Figure 38. Adulterated *Salvia officinalis* with salvinorin A, salvinorin B, and SB-EME after reaction with Ehrlich's reagent. After 2 min, the adulterated samples showed a negative result. The top row contained 3 replicates of unadulterated *S. divinorum* as a control. Each column contains 3.4, 15, or 40 mg/g of spiking standard, respectively.

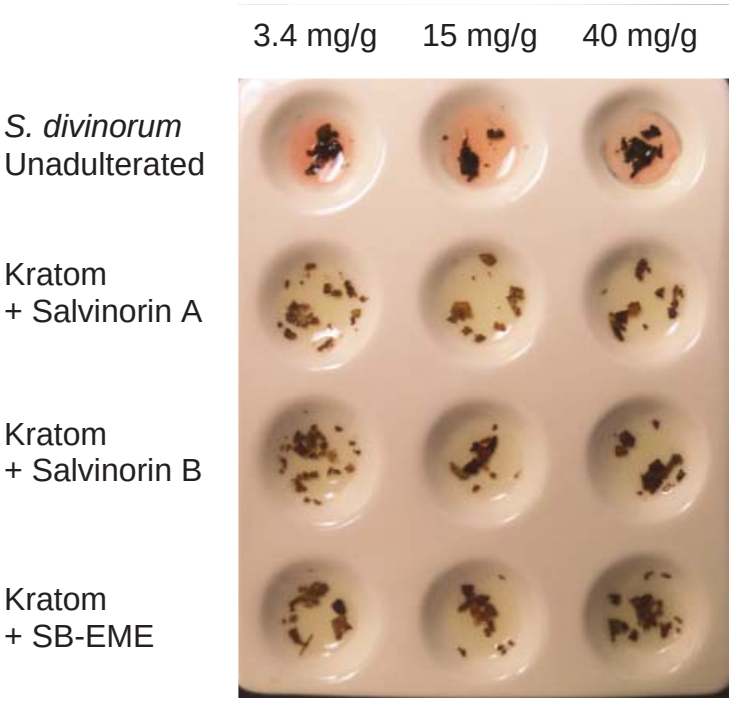


Figure 39. Adulterated Kratom with salvinorin A, salvinorin B, and SB-EME after reaction with Ehrlich's reagent. After 2 min, the adulterated samples showed a negative result. The top row contained 3 replicates of unadulterated *S. divinorum* as a control. Each column contains 3.4, 15, or 40 mg/g of spiking standard, respectively.

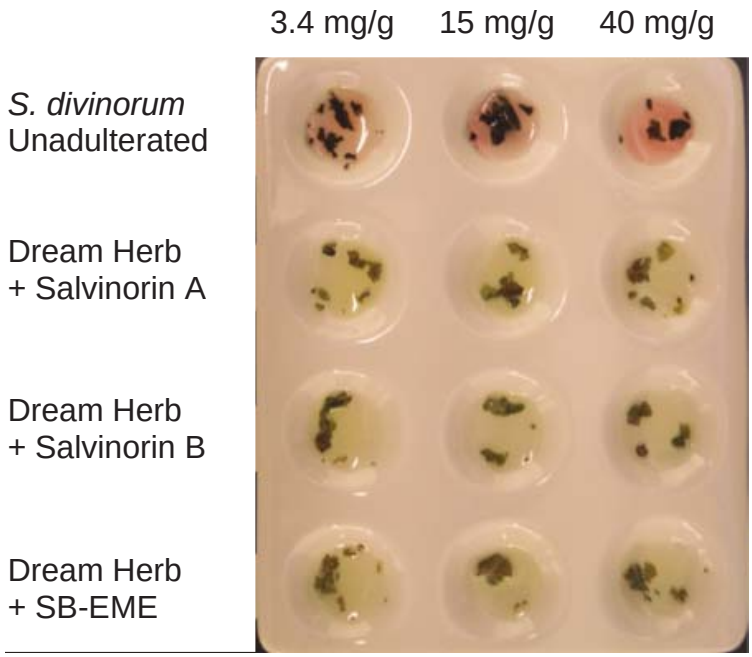


Figure 40. Adulterated Dream Herb with salvinorin A, salvinorin B, and SB-EME after reaction with Ehrlich's reagent. After 2 min, the adulterated samples showed a negative result. The top row contained 3 replicates of unadulterated *S. divinorum* as a control. Each column contains 3.4, 15, or 40 mg/g of spiking standard, respectively.

3.5.2. HPLC Analysis of Adulterated Plant Materials

To evaluate the hypothesis that the spiking standard preferentially deposited onto the glass vials and not onto the plant material, the vials used to make the adulterated materials were analyzed by HPLC for the residual standards. The resulting amounts of material recovered from this analysis are summarized in **Table 6**. Salvinorin A was not spiked onto *Salvia divinorum* as it has been previously performed in the literature (51). The percent recovery of salvinorin A from the empty vials was approximately 0%, 12%, and 22% at each

respective concentration level on the three different matrices of *S. officinalis*, Kratom, and Dream Herb. This result supports the hypothesis that some of the spiking standard was left behind in the vials used to make the adulterated materials.

Table 6. HPLC recoveries of vials after spiking plant materials with salvinorin A, salvinorin B, and SB-EME. ND = not detected.

Matrix	Spiked Conc. (mg/g)	Sal A (µg)	Sal A % Yield	Sal B (µg)	Sal B% Yield	SB-EME (µg)	SB-EME % Yield
Salvia officinalis	3.4	ND	0.0	5.9	57.7	ND	0.0
	15.0	3.3	7.4	9.0	20.0	ND	0.0
	40.0	20.6	17.1	129.6	108.0	25.6	21.3
Salvia divinorum	3.4	-	-	2.9	28.0	ND	0.0
	15.0	-	-	1.4	3.2	ND	0.0
	40.0	-	-	94.7	79.0	36.7	30.6
Kratom	3.4	ND	0.0	3.9	37.9	2.0	19.4
	15.0	8.4	18.7	3.3	7.4	ND	0.0
	40.0	28.1	23.5	121.1	100.9	58.8	49.0
Dream Herb	3.4	ND	0.0	5.2	51.0	1.7	17.1
	15.0	3.9	8.7	ND	0.0	ND	0.0
	40.0	31.1	25.9	116.1	96.7	76.3	63.6
Averages	3.4	ND	**0.0**	4.5	**43.7**	0.9	**9.1**
	15.0	5.2	**11.6**	3.4	**7.7**	ND	**0.0**
	40.0	26.6	**22.2**	115.4	**96.2**	49.4	**41.1**

The results for salvinorin B and SB-EME showed less of a consistent trend of increasing spiking concentration leading to increased recovery from the empty vials. These two standards showed little to no recovery at the 15 mg/g level compared to the 3.4 mg/g and 40 mg/g levels. This is because salvinorin B and SB-EME were originally spiked onto a bulk portion of leaves at the 15 mg/g level and then portioned out for analysis into different, smaller vials. These samples were made in bulk at the 15 mg/g concentration level for other experiments that

fell outside the scope of this thesis. This resulted in the extraction of the vials that were not the original vial used to make the adulterated samples. The other two concentration levels were made similarly to salvinorin A, extracting the original vial used for the adulteration. This accidental inconsistency still supports the hypothesis that a portion of the material is being deposited in the vials, as the vials that were not used to make the adulterated plant materials ended up with lower recoveries. The recovery at the 3.4 mg/g level was an average of 44% for salvinorin B and an average of 9% for SB-EME. The recovery at the 15 mg/g level was an average of 7% for salvinorin B and 0% for SB-EME. At the 40 mg/g level, the recovery for salvinorin B was an average of 96% and for SB-EME was an average of 41%.

Therefore, it can be concluded that major portions of the spiking standards were recovered from the glass vials after the colorimetric assay with Ehrlich's reagent. This could be the reason as to why very little color was seen during the reaction, even with the *Salvia divinorum* samples, as the inherent salvinorin compounds could have been extracted from the leaves and deposited in the vials.

4. CONCLUSIONS

The extraction of salvinorin A from *Salvia divinorum* was a straightforward process, which makes it easy to see why many “Salvia” products on the Internet are extracts or fortified leaves (46). The synthesis to salvinorin B also proceeded simply, at room temperature, with cheap, common reagents. This reaction could be easily performed outside of a laboratory environment. In addition, the work-up procedure gave an increase in purity, allowing some of the contaminants from the extraction to be filtered away. The potential for clandestine chemists to experiment with salvinorin B to make other derivatives is a real risk, given the current environment for synthetic cannabinoids and bath salts flooding the drug market (1).

However, the synthesis of the ether derivatives SB-MME and SB-EME are less likely to become popular for several reasons. First, the chloroalkyl ethers necessary to make the ethers are specialty chemicals, with no consumer equivalent that would allow for easy access. The chemicals would have to be diverted from the chemical or academic industries, which would not allow for large-scale synthesis. In addition, the chloroalkyl ethers used in this study are known carcinogens, which require strict safe handling procedures. The resulting condensation reactions have low yields when not using dry or freshly distilled solvents. However, given the higher potency of the ethers compared to salvinorin A, it is plausible that clandestine chemists would still accept a mixture for recreational use.

The accuracy of the HPLC method was found to be approximately 88% for salvinorin B, and was approximately 100% for the recovery of SB-EME. However, given the known matrix issues, the error in these values is not well defined. Using the first set of SB-EME calibration standards, the method had an absolute range of error from 0.03 – 11.79%. The analytical repeatability within each set of SB-EME standards was below 9% RSD for triplicate injections. The intermediate precision between the three sets of SB-EME standards was <12% RSD. The linearity was 0.9993, indicating good linearity over the range of the SB-EME standards. The range covered two orders of magnitude from 0.0005 – 0.064 mg/mL, or a mass of 0.038 µg – 4.8 µg. The LOD was determined to be 0.0005 mg/mL, or 0.038 µg of SB-EME, and the LOQ was determined to be 0.0015 mg/mL, or 0.113 µg. The selectivity of the method was more challenging, as significant matrix effects were seen when using *Salvia officinalis* as the blank matrix. However, the purified solutions of SB-EME had baseline resolution from salvinorin A and salvinorin B, which allows for easy qualitative distinction if adulterated samples are suspected. It will be important to evaluate the contributions from the matrix with this HPLC method.

UV/Vis analysis was an easy, quick method of characterization. The UV/Vis spectrum for SB-EME was distinguishable from both salvinorin A and salvinorin B.

The colorimetric assay with Ehrlich's reagent also provided a red-orange result with salvinorin B and SB-EME. While this does not provide differentiation in

the field, it does allow all materials related to *Salvia divinorum* to be identified and collected for further analysis in the lab. This colorimetric analysis will allow easy separation from common kitchen herbs, such as mint, basil, and sage. Since a confirmatory test will have to be performed in the lab according to SWGDRUG guidelines, the use of Ehrlich's reagent as a screening test will help the analyst choose the appropriate confirmatory method.

Characterization of the colored species in the assay with Ehrlich's reagent was performed with UV/Vis, HPLC, and NMR. The UV/Vis analysis showed a new peak at 500 nm in the aqueous layer, which would correspond to a red-orange color. HPLC analysis revealed a new, highly retained peak from the DCM layer of the assay. ^{1}H NMR analysis revealed that the backbone of the salvinorins was not stable in acid, and the molecule that creates the color was likely a decomposition product. There were still two aromatic protons present, however, which leads to the hypothesis that the third proton has reacted with the pDMAB in Ehrlich's reagent. This supports the proposed mechanism for salvinorin A and related compounds proceeding by an electrophilic aromatic substitution of the furan ring.

The spiking experiment to create adulterated samples gave the opposite color result from what was expected. With the addition of more furan containing compounds, the red-orange color was expected to intensify. However, the color result decreased as the concentration of spiked standard increased. It is hypothesized that this result is due to the spiking procedure used, as the solvent

added to the leaves potentially extracted out the inherent salvinorin A in the leaves and deposited both salvinorin A and the spiking standard onto the glass of the vial. HPLC analysis of the leftover vials after the colorimetric assay confirmed recoveries of the spiking compounds up to an average of 22% for salvinorin A, 96% for salvinorin B, and 41% for SB-EME over all matrices.

In conclusion, salvinorin B ethoxymethyl ether can be detected in the field through the use of Ehrlich's reagent as a colorimetric assay. Further laboratory tests, including HPLC and UV/Vis, were shown to easily distinguish the ether derivative from salvinorin A and B. If clandestine chemists attempt synthetic derivatives of salvinorin A, they should be easily detectable by the methods described.

5. FUTURE DIRECTIONS

Given the new insights into the mechanism of salvinorin A with Ehrlich's reagent, it would be interesting to return to the original proposal of the LSD mechanism. For one, the acid stability of LSD should be assessed. It is likely that the LSD molecule will also be broken apart by the strong acid in the solution of Ehrlich's reagent. Therefore, the final 2:1 adduct would have a different structure than what was originally proposed. In addition, the literature behind the mechanism dates back to 1989, and contains very little data to support the mechanism (55). It would be worthwhile to update this proposed mechanism to provide more understanding to a colorimetric assay in frequent use in forensic labs.

Another colorimetric assay that would be interesting to pursue for the analysis of salvinorin A and derivatives is the Duquenois-Levine test. This test uses vanillin as a reagent to react with the THC in marijuana. Vanillin is used frequently in TLC, and was used in this research to color the salvinorin derivatives red. It has been reported that analysis of *Salvia divinorum* with the Duquenois-Levine Test turns the top aqueous layer a wine-red color (61). It would be useful to validate this assay for use with *Salvia divinorum* to add as a complementary test. Given that the Duquenois-Levine test is already in use by forensic labs, there should be little barrier to implementation.

The result with Dream Herb, where the Ehrlich's reagent turned a brighter yellow, requires further investigation. It is possible that 9α-acetoxyzexbrevin

reacts with Ehrlich's reagent or potentially another compound from Dream Herb not yet identified. UV/Vis analysis would be useful to use in this case, as the distinction between the light yellow Ehrlich's reagent and the brighter yellow from Dream herb might be difficult to distinguish visually.

Additional UV/Vis experiments on the colorimetric assay with Ehrlich's reagent would be useful. The molar absorptivity of the colored intermediate would be useful characterization data to have. However, one important consideration would be determining the concentration of the colored intermediate in solution first. This is necessary to calculate the molar absorptivity using Beer's Law. Insights into the kinetics of the reaction of salvinorin A and derivatives with Ehrlich's reagent using UV/Vis or NMR would be interesting to investigate. In addition, UV/Vis could also be used to attempt to distinguish mixtures of salvinorin derivatives or plant materials adulterated with salvinorin derivatives. This would have the advantage of an analysis that is quicker and easier to use than HPLC.

The colorimetric assay on adulterated materials has some opportunities for improvement. Initially, performing the reaction with Ehrlich's reagent in the vial in which the plant materials were spiked would serve as a good control, as this would serve to show any influences by the plant matrices while ensuring all of the standard will be analyzed. Investigating the addition of an internal standard would help determine a more accurate percent recovery by HPLC. Finally, the method of spiking could also be optimized to avoid the influence of the deposition on the

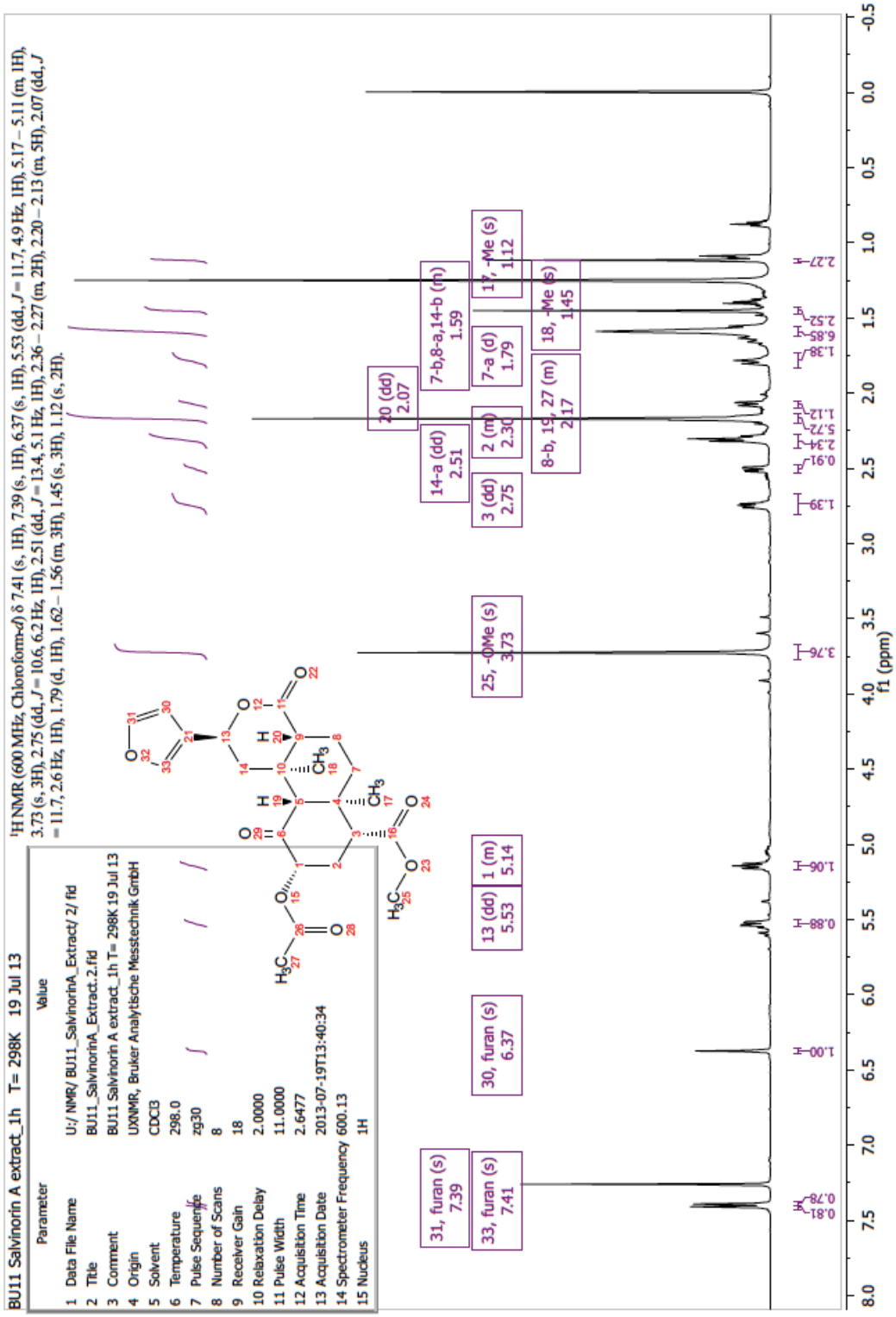
glass vials. Perhaps a different type or volume of solvent, or different containers, such as plastic vials could avoid the loss of material onto the vial walls.

6. APPENDICES

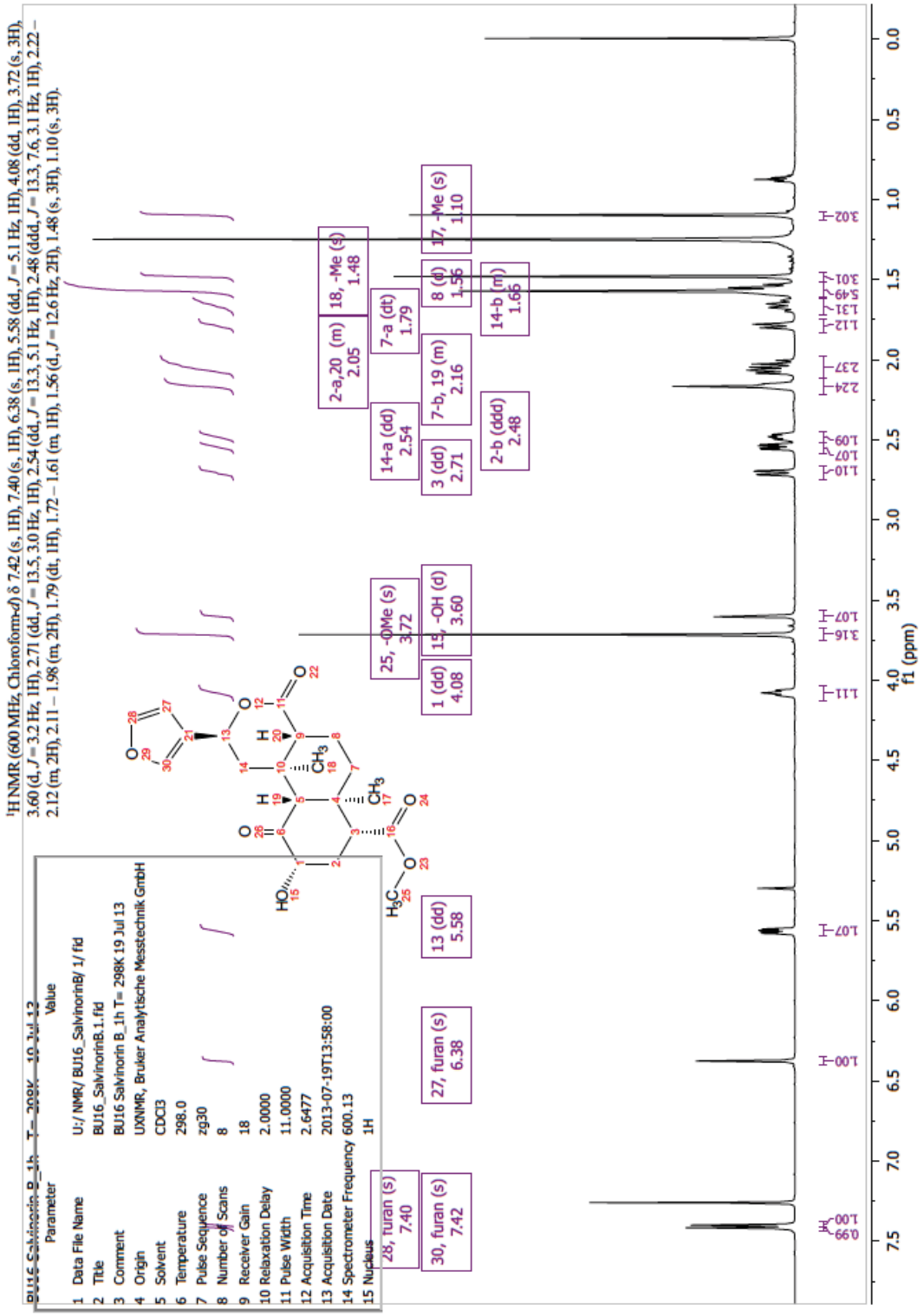
A.1. TLC of Salvinorin A, Salvinorin B, and SB-EME

SA = salvinorin A, SB = salvinorin B. TLC conditions were 10% acetone/DCM, and visualized with a vanillin stain.

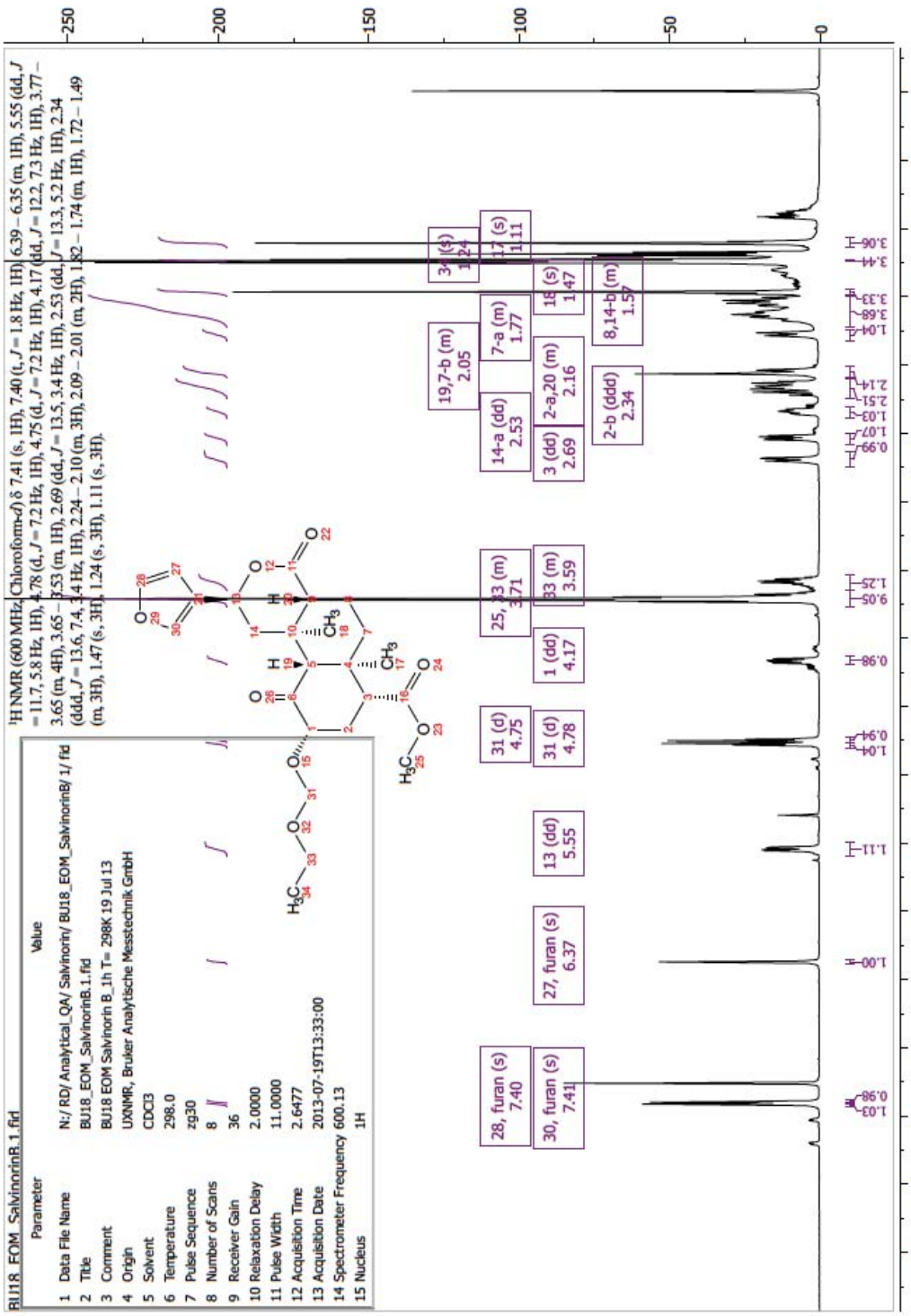
A.2. Salvinorin A NMR



A.3. Salvinorin B NMR



A.4. Salvinorin B Ethoxymethyl Ether NMR



A.5. HPLC Validation Raw Data Tables

A.5.1. Accuracy Data Table

Sample	Ave Area (mAU*s)	Std Dev	% RSD	Conc. (mg/mL)	Total (mg)	Ave (mg)	Std Dev	% RSD	% Recovery	Ave % Recovery
S. officinalis/ Sal B 1	8481.2	69.47	0.82	0.154	0.96	0.99	0.02	2.12	85.69	87.83
S. officinalis/ Sal B 2	8794.7	74.34	0.85	0.160	1.00				88.86	
S. officinalis/ Sal B 3	8804.7	117.47	1.33	0.160	1.00				88.96	
S. officinalis/ SB-EME 1	5789.7	290.35	5.02	0.181	1.13	1.13	0.01	0.91	100.70	100.04
S. officinalis/ SB-EME 2	5773.4	651.63	11.29	0.181	1.13				100.42	
S. officinalis/ SB-EME 3	5692.1	580.84	10.20	0.178	1.11				99.01	

A.5.2. SB-EME Standards Set 1

Concentration (mg/mL)	Set 1 Area (mAU*s)	Ave Area (mAU*s)	StdDev	%RSD	Back Calculation	% RE
0.0005	17.5	17.13	1.19	6.96	0.0003	-34.25
	18.1				0.0003	-30.49
	15.8				0.0003	-44.91
0.0010	33.8	33.27	1.57	4.72	0.0008	-16.05
	34.5				0.0009	-13.85
	31.5				0.0008	-23.26
0.0015	52.1	53.33	1.88	3.53	0.0014	-5.74
	55.5				0.0015	1.37
	52.4				0.0014	-5.11
0.0020	65.1	65.53	2.48	3.78	0.0018	-8.97
	68.2				0.0019	-4.11
	63.3				0.0018	-11.79
0.0040	140.1	139.50	1.22	0.87	0.0042	4.29
	138.1				0.0041	2.72
	140.3				0.0042	4.45
0.0080	270.4	272.30	1.65	0.60	0.0083	3.20
	273.2				0.0083	4.30
	273.3				0.0083	4.34
0.0160	573.7	573.30	1.25	0.22	0.0178	11.02
	574.3				0.0178	11.14
	571.9				0.0177	10.67
0.0320	1028.6	1026.80	2.31	0.22	0.0320	0.07
	1027.6				0.0320	-0.03
	1024.2				0.0319	-0.36
0.0640	2102.3	2093.30	7.80	0.37	0.0657	2.63
	2089.2				0.0653	1.98
	2088.4				0.0652	1.94

A.5.3. SB-EME Standards Set 2

Concentration (mg/mL)	Set 2 Area (mAU*s)	Ave Area (mAU*s)	StdDev	%RSD	Back Calculation	% RE
0.0005	19.4	19.63	0.25	1.28	0.0004	-22.33
	19.6				0.0004	-21.08
	19.9				0.0004	-19.20
0.0010	30.7	29.53	2.02	6.84	0.0007	-25.76
	27.2				0.0006	-36.74
	30.7				0.0007	-25.76
0.0015	53.2	55.27	2.76	4.99	0.0014	-3.44
	58.4				0.0016	7.43
	54.2				0.0015	-1.35
0.0020	73.7	74.17	0.45	0.61	0.0021	4.51
	74.2				0.0021	5.30
	74.6				0.0021	5.92
0.0040	113.1	127.47	12.70	9.96	0.0033	-16.87
	137.2				0.0041	2.02
	132.1				0.0039	-1.98
0.0080	292.2	289.97	2.04	0.70	0.0089	11.74
	289.5				0.0089	10.69
	288.2				0.0088	10.18
0.0160	628	631.50	3.21	0.51	0.0195	21.66
	634.3				0.0197	22.89
	632.2				0.0196	22.48
0.0320	1102.9	1099.57	3.30	0.30	0.0344	7.35
	1099.5				0.0342	7.02
	1096.3				0.0341	6.70
0.0640	2254.1	2248.47	5.50	0.24	0.0704	10.06
	2243.1				0.0701	9.52
	2248.2				0.0703	9.77

A.5.4. SB-EME Standards Set 3

Concentration (mg/mL)	Set 3 Area (mAU*s)	Ave Area (mAU*s)	StdDev	%RSD	Back Calculation	% RE
0.0005	15.8	15.47	0.95	6.11	0.0003	-44.91
	14.4				0.0002	-53.70
	16.2				0.0003	-42.41
0.0010	30.6	29.17	1.56	5.36	0.0007	-26.08
	29.4				0.0007	-29.84
	27.5				0.0006	-35.80
0.0015	44.8	43.63	3.88	8.90	0.0012	-21.00
	46.8				0.0012	-16.82
	39.3				0.0010	-32.50
0.0020	58.4	60.13	1.58	2.63	0.0016	-19.47
	60.5				0.0017	-16.18
	61.5				0.0017	-14.61
0.0040	114.5	115.67	1.04	0.90	0.0034	-15.77
	116.5				0.0034	-14.20
	116				0.0034	-14.59
0.0080	226.5	228.80	3.40	1.48	0.0069	-14.00
	227.2				0.0069	-13.73
	232.7				0.0071	-11.57
0.0160	485.6	484.97	0.71	0.15	0.0150	-6.24
	485.1				0.0150	-6.34
	484.2				0.0150	-6.51
0.0320	913	913.53	1.19	0.13	0.0284	-11.25
	914.9				0.0285	-11.07
	912.7				0.0284	-11.28
0.0640	1796.6	1793.80	3.94	0.22	0.0561	-12.35
	1795.5				0.0561	-12.40
	1789.3				0.0559	-12.71

A.5.5. SB-EME Standards Averages

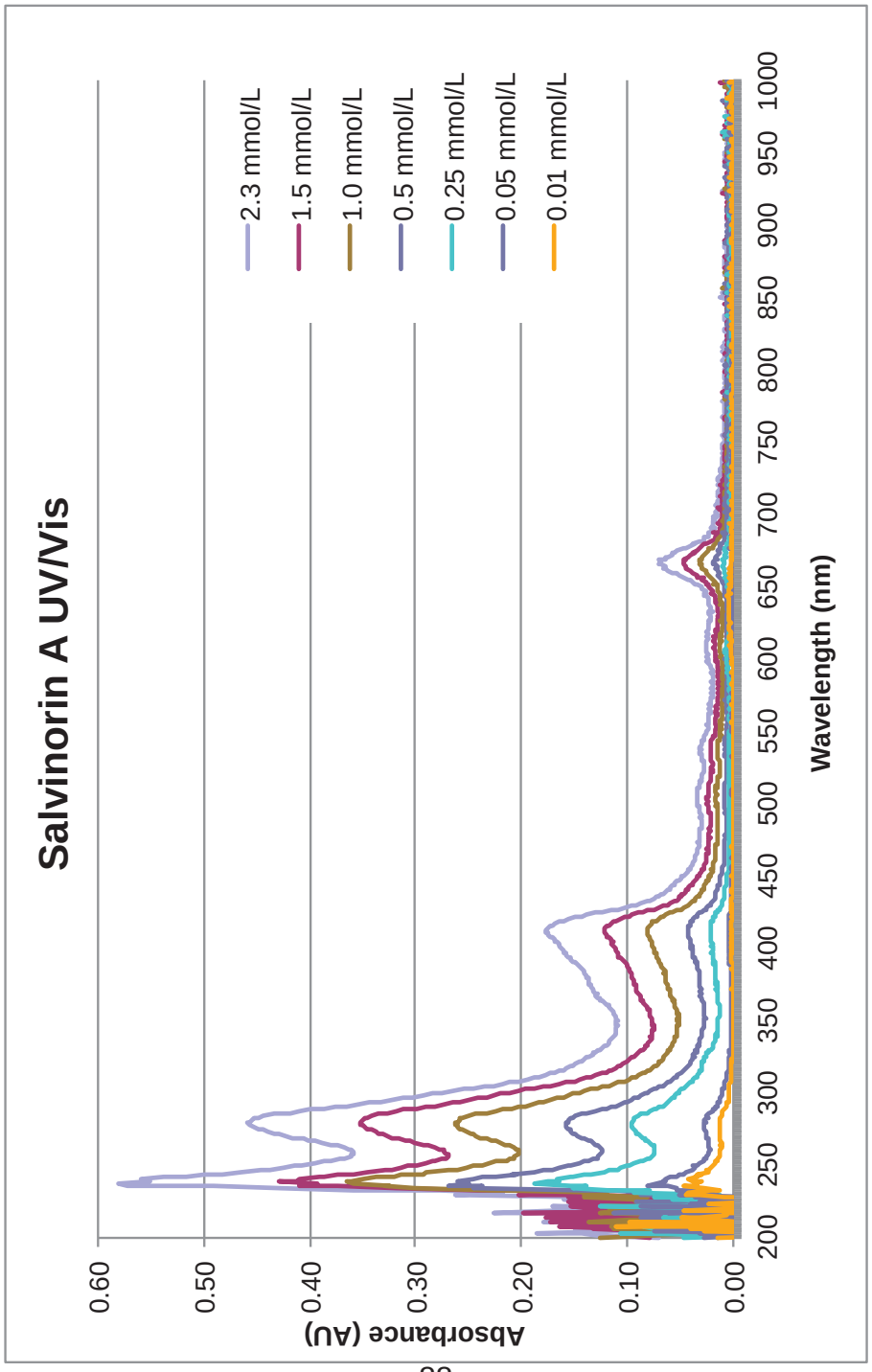
Concentration (mg/mL)	Total Ave Area (mAU*s)	Total StdDev	Total % RSD
0.0005	17.41	1.97	11.33
0.0010	30.66	2.47	8.06
0.0015	50.74	5.98	11.78
0.0020	66.61	6.31	9.47
0.0040	127.54	12.14	9.52
0.0080	263.69	27.35	10.37
0.0160	563.26	63.92	11.35
0.0320	1013.30	81.22	8.01
0.0640	2045.19	200.22	9.79

A.6. LOD/LOQ Raw Data Table

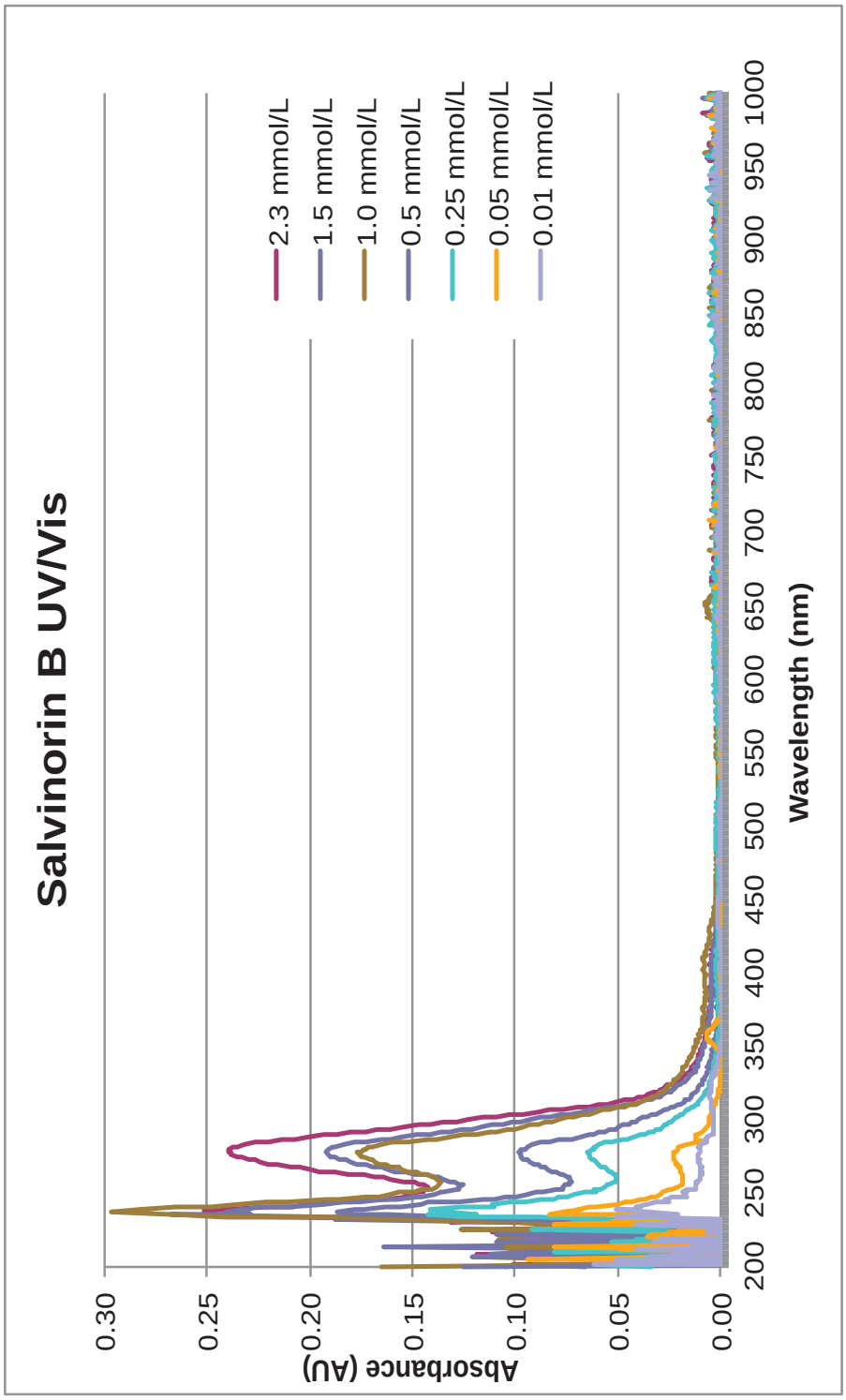
Concentration (mg/mL)	Set 1 Area (mAU*s)	S/N	Set 2 Area (mAU*s)	S/N	Set 3 Area (mAU*s)	S/N
0.0005	17.5	3.8	19.4	4.8	15.8	3.5
	18.1	4.4	19.6	4.2	14.4	3.6
	15.8	4.3	19.9	5.9	16.2	3.9
0.0010	33.8	6.7	30.7	6.7	30.6	7.0
	34.5	7.5	27.2	6.1	29.4	5.3
	31.5	6.4	30.7	6.7	27.5	6.0
0.0015	52.1	10.2	53.2	10.9	44.8	9.4
	55.5	12.3	58.4	10.8	46.8	9.7
	52.4	12.0	54.2	12.2	39.3	7.9
0.0020	65.1	12.8	73.7	14.7	58.4	12.4
	68.2	12.8	74.2	13.7	60.5	11.9
	63.3	12.9	74.6	15.7	61.5	14.2
0.0040	140.1	26.4	113.1	31.7	114.5	23.3
	138.1	30.4	137.2	33.2	116.5	26.1
	140.3	30.7	132.1	29.4	116.0	23.7
0.0080	270.4	58.8	292.2	56.2	226.5	48.1
	273.2	50.1	289.5	56.4	227.2	51.1
	273.3	55.2	288.2	62.0	232.7	47.3
0.0160	573.7	93.6	628.0	111.7	485.6	91.5
	574.3	106.1	634.3	123.9	485.1	84.5
	571.9	118.8	632.2	112.9	484.2	100.6
0.0320	1028.6	153.5	1102.9	167.7	913.0	157.1
	1027.6	151.6	1099.5	196.2	914.9	135.1
	1024.2	185.7	1096.3	183.4	912.7	133.8
0.0640	2102.3	307.5	2254.1	252	1796.6	212.1
	2089.2	231.6	2243.1	233.5	1795.5	193.6
	2088.4	267.2	2248.2	236.2	1789.3	210.3

A.7. UV/Vis Spectra

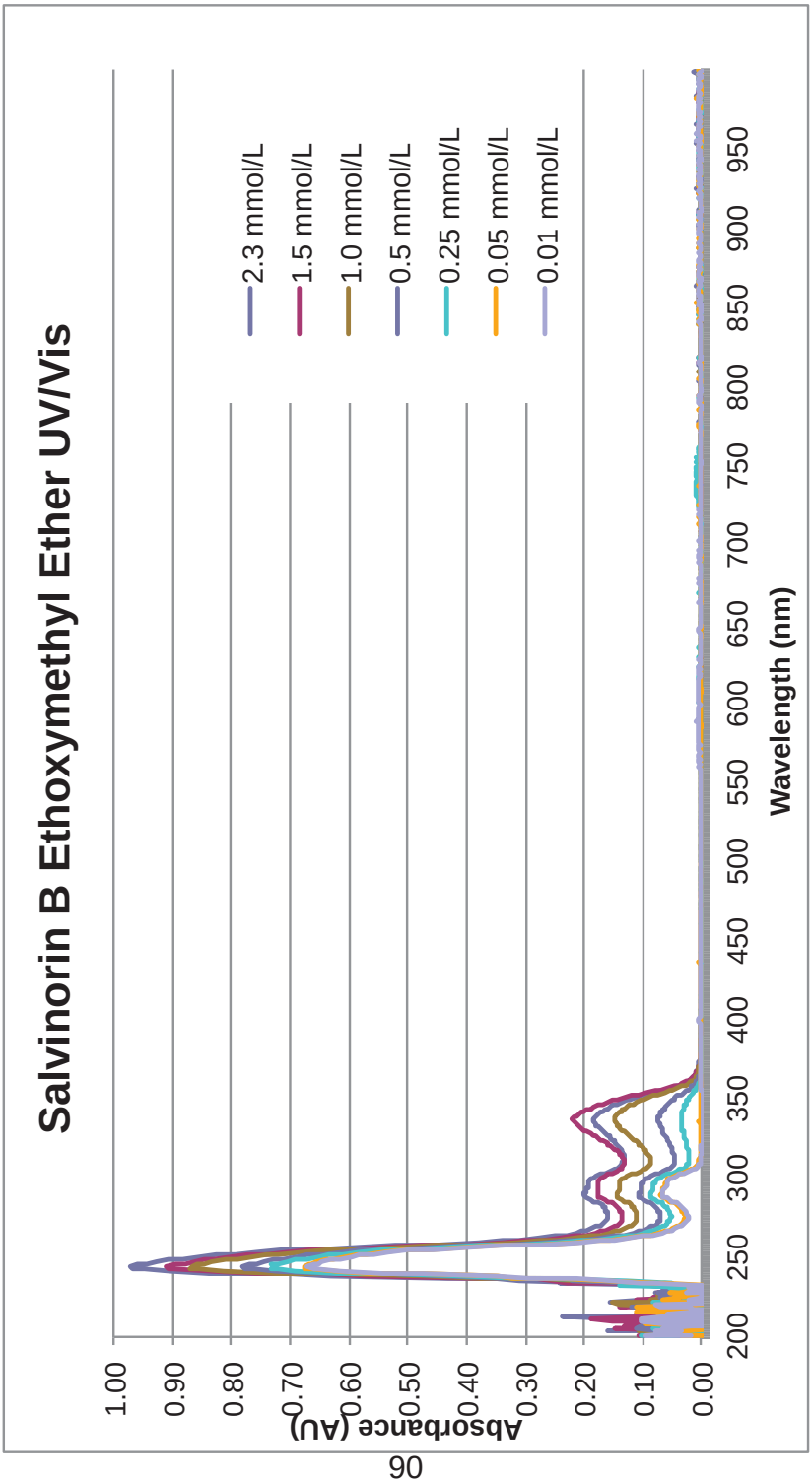
A.7.1. Salvinorin A UV/Vis Spectrum



A.7.2. Salvinorin B UV/Vis Spectrum



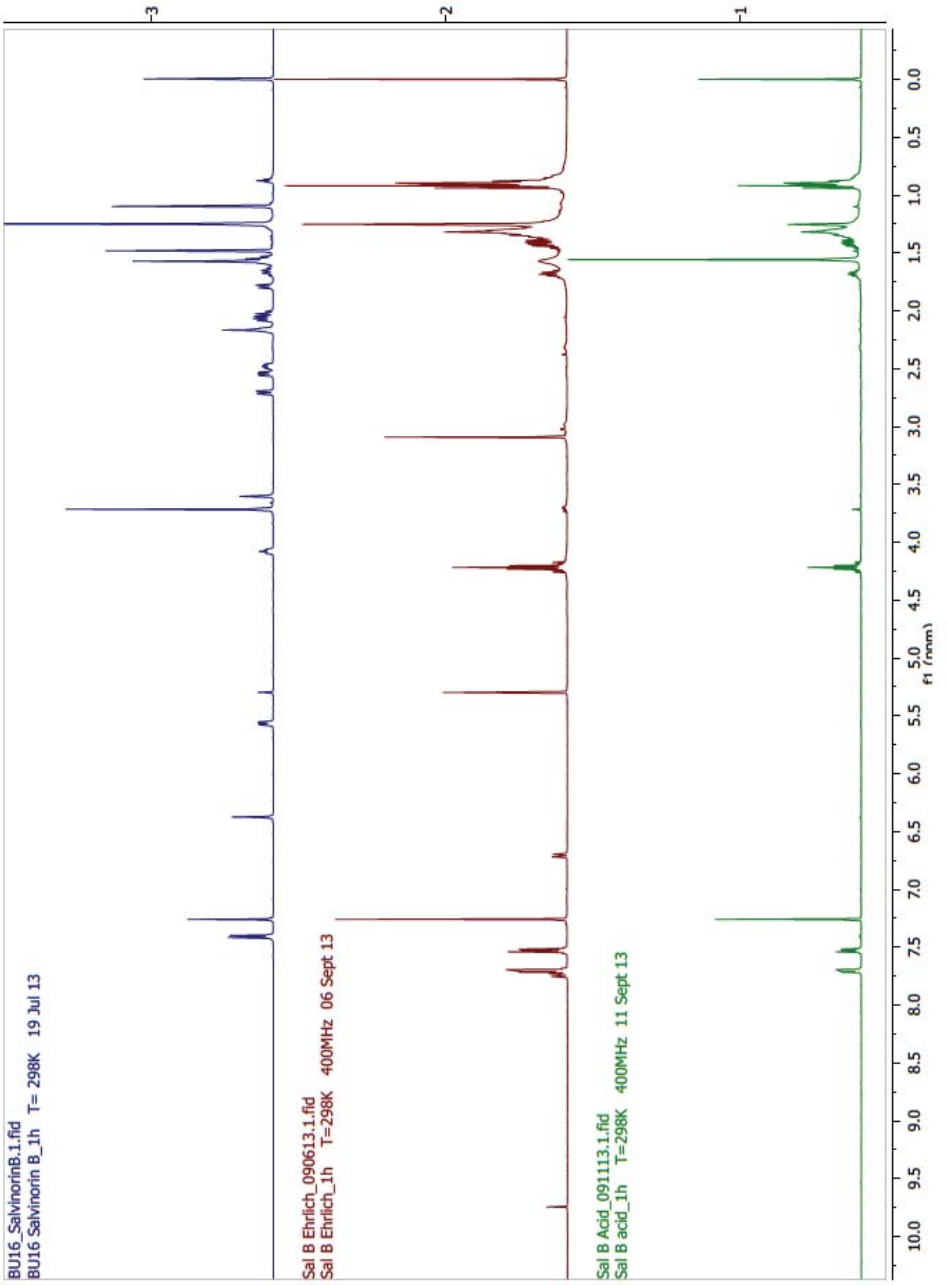
A.7.3. SB-EME UV/Vis Spectrum



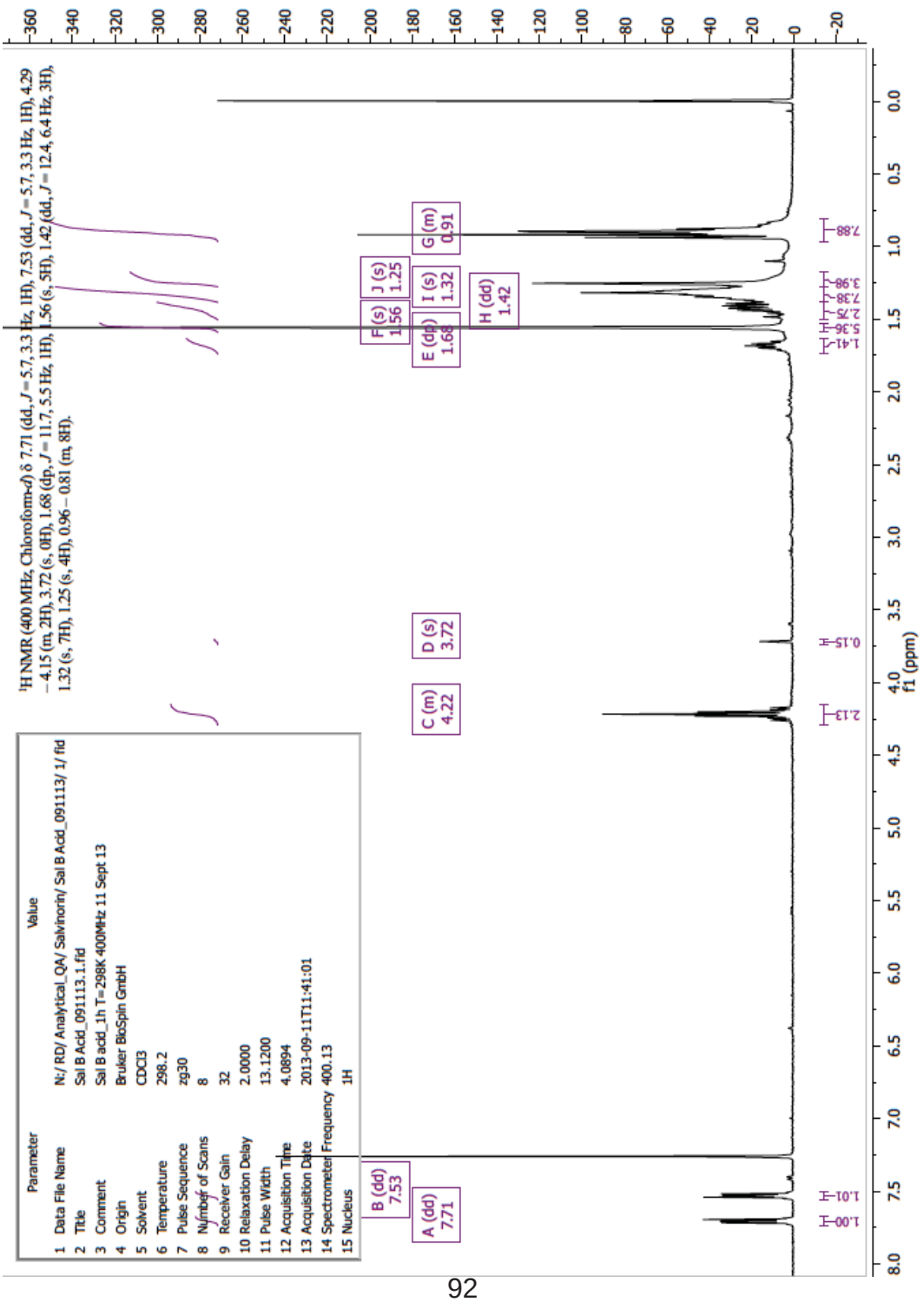
A.8. ^{1}H NMR of Colorimetric Assay with Ehrlich's Reagent

A.8.1. ^{1}H NMR Stack

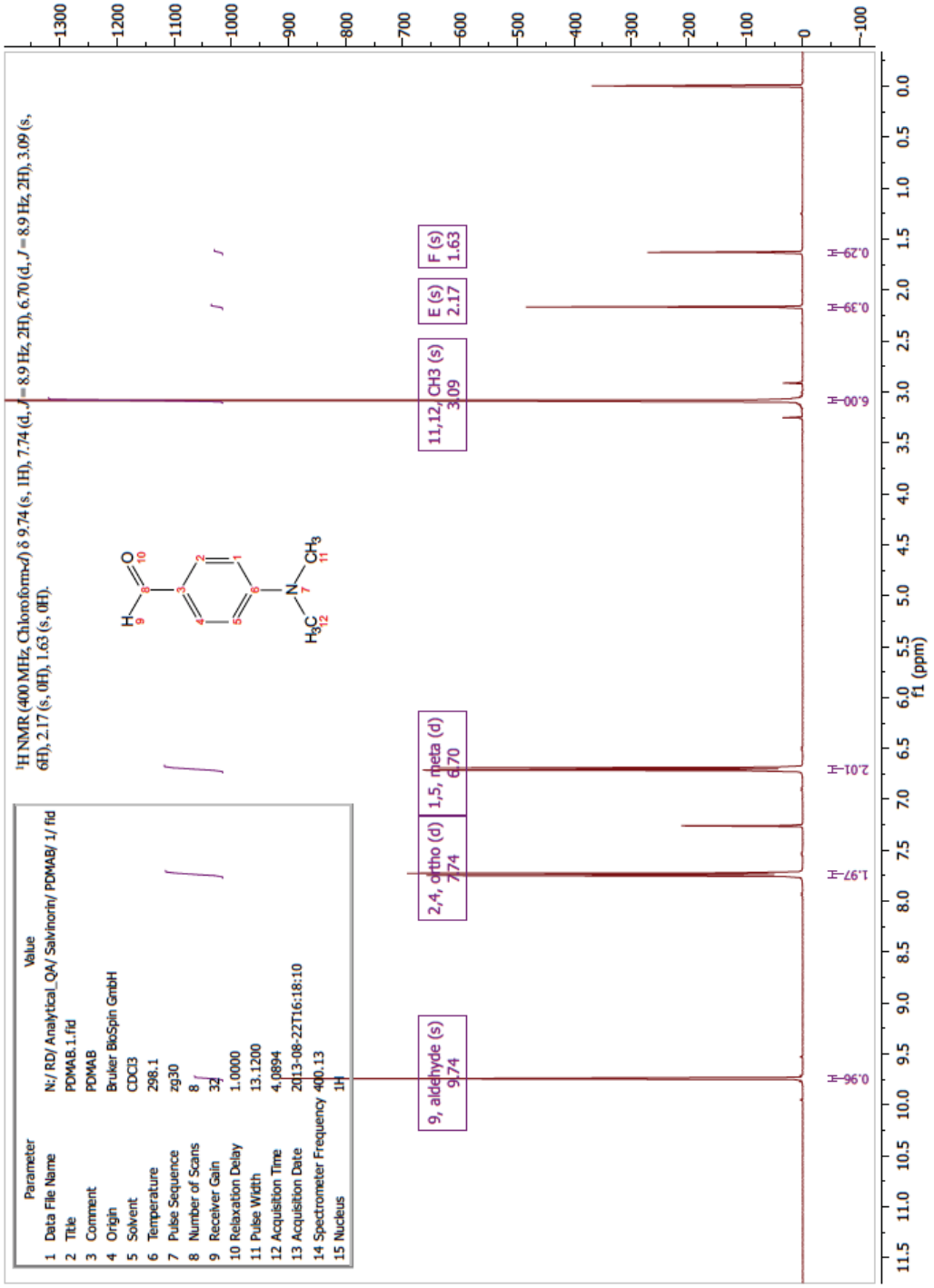
Salvinorin B (blue), DCM Layer of Salvinorin B after Reaction with Ehrlich's Reagent (red), and Salvinorin B after Acid Wash (green).



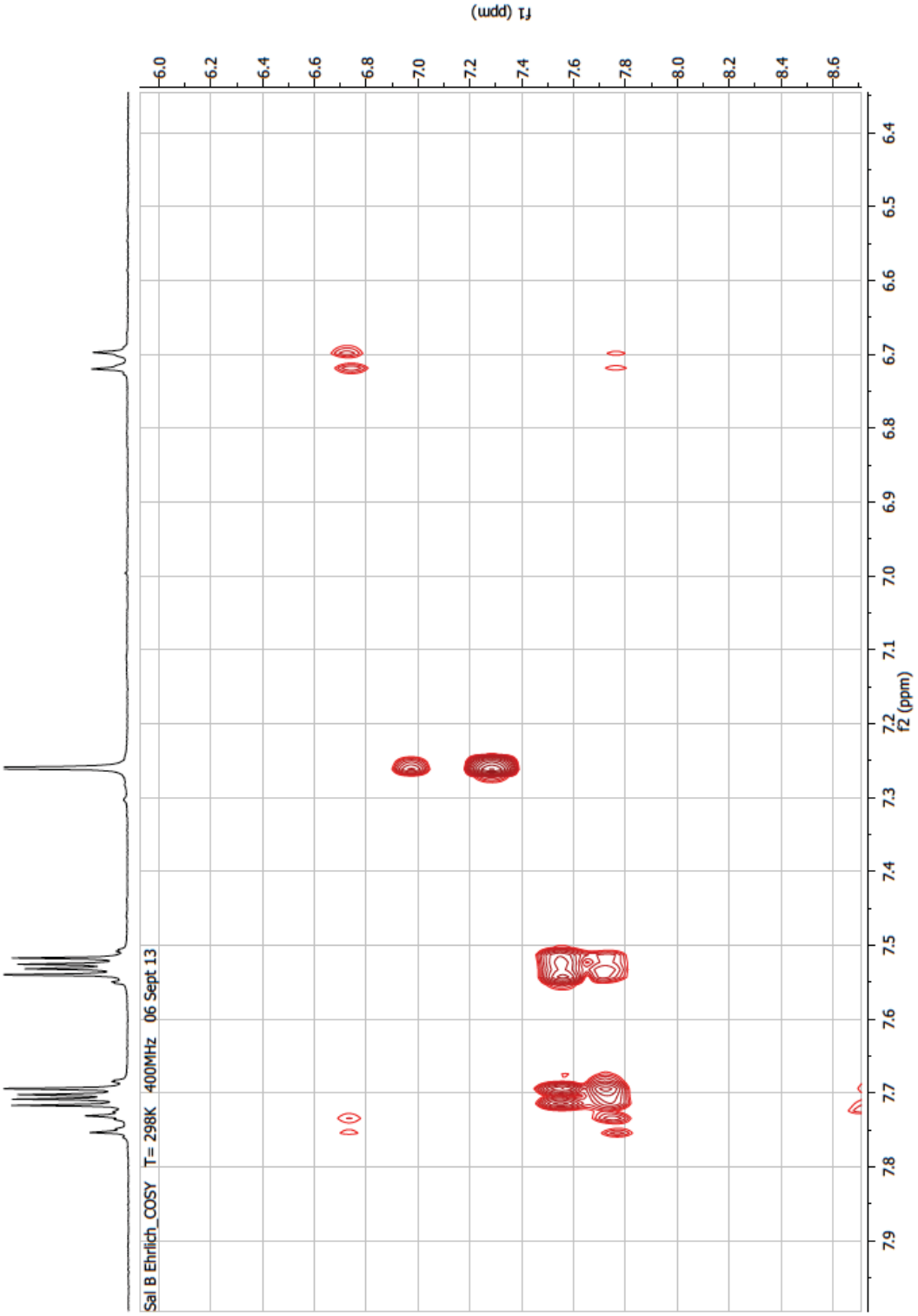
A.8.2. ^{1}H NMR of Salvinorin B after Acid Wash



A.8.3. pDMAB ^{1}H NMR



A.8.4. 2D COSY ^{1}H NMR of Salvinorin B after Reaction with Ehrlich's Reagent



LIST OF JOURNAL ABBREVIATIONS

Bioorg Med Chem Lett.	Bioorganic & Medicinal Chemistry Letters
Bioorg Med Chem.	Bioorganic & Medicinal Chemistry
Bull Chem Soc Jpn	Bulletin of the Chemical Society of Japan
Chem Rev.	Chemical Reviews
Drug Alcohol Depend.	Drug and Alcohol Dependence
Forensic Sci Int.	Forensic Science International
J Am Chem Soc.	Journal of the American Chemical Society
J Chem Soc , Perkin Trans 1.	Journal of the Chemical Society. Perkin Transactions 1
J Med Toxicol.	Journal of Medical Toxicology
J Nat Prod.	Journal of Natural Products
J Org Chem.	The Journal of Organic Chemistry
J Forensic Sci.	Journal of Forensic Sciences
J Plant Res.	Journal of Plant Research
J Psychoactive Drugs	Journal of Psychoactive Drugs
Magn Reson Chem.	Magnetic Resonance in Chemistry
Org Lett.	Organic Letters
Phytochem Anal.	Phytochemical Analysis
Sci Prog.	Science Progress
Tetrahedron Lett.	Tetrahedron Letters
Z Lebensm Unters Forsch	European Food Research and Technology

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CURRICULUM VITAE

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1984

EDUCATION

Boston University School of Medicine, Boston, MA
Masters of Science in Biomedical Forensic Science, 2014 (anticipated)

University of Wisconsin, Madison, WI
Masters of Science in Organic Chemistry, August 2007
Graduate GPA 3.4/4.0

College of Charleston, Charleston, SC
Bachelor of Science in Chemistry and Biochemistry, May 2006
Minor: Biology
Undergraduate GPA 3.4/4.0

CERTIFICATIONS

Certified Massachusetts Wastewater Treatment Plant Operator
Certified Operator Grade 2-I, Full
Registration Number: 16264, expiration 12/31/2013

Department Of Transportation (DOT) Certified
DOT Awareness
January 4th, 2011

Resource Conservation and Recovery Act (RCRA) Certified
Hazardous Waste Management
June, 2012

PROFESSIONAL AFFILIATIONS

American Chemical Society
Alpha Chi Sigma – Gamma Delta
Association for Women in Science

EMPLOYMENT

Living Proof, Cambridge, MA
Research Associate IV **Jan 2012 to present**
Research Associate III **Dec 2010 to Jan 2012**

Analytical chemist providing full QA/QC support to production batches and RMs
Develops and validates analytical methods for existing and new materials
Implements new analytical technologies, such as charged aerosol detection
Implemented and maintains a stability program for all formulations
Troubleshoots and assists in characterizations of impurities
Designs analytical processes to model GLP requirements
Instrumentation utilized: HPLC, GPC, UV/VIS Spectrophotometer, IR, NMR, GC/MS, LC/MS, NIR

Chemical Hygiene Officer: performs laboratory safety checks, coordinates waste disposal, manages EH&S program

Merck & Co., Inc., Boston, MA **April 2008 to Nov 2010**
Staff Chemist

Process chemist in the Basic Pharmaceutical Sciences group
Synthesized intermediates and APIs to support Medicinal Chemistry efforts on milligram to kilogram scale
Developed key technology (synthetic steps, separations, isolations, crystallizations, or salt selections) that contributed to development of a viable process
Focused on route and method improvements to create efficient and scalable processes

Pharmaceutical Product Development, Inc., Middleton, WI **Oct 2007 to Mar 2008**
Associate Scientist

Analytical chemist in the Biopharmaceutical Services group, a cGMP laboratory
Quantitatively measured properties of pharmaceutical compounds in a variety of formulations using a variety of complex sample preparations and analytical procedures

University of Wisconsin, Madison, WI **Aug 2006 to Jun 2007**
Teaching Assistant

Taught two discussion sections and two general chemistry labs for two semesters
Led students through course material in an informal lecture/discussion section, graded materials, and prepared practice problems/handouts/examples

College of Charleston, Charleston, SC **May 2005 to Aug 2006**
Undergraduate Researcher

Synthesized derivations of a weak antibiotic, Cytosporone E, to study SAR and increase potency
Optimized the Suzuki-Miyaura cross-coupling reaction to work for sterically hindered aryl bromide substrates
Lab work included small molecule organic synthesis using air-sensitive techniques on a Schlenk line, extractions, recrystallizations, flash chromatography, and radial chromatography

USDA ARS Vegetable Lab, Charleston, SC **Jan 2005 to May 2005**
Lab Assistant

Laboratory assistant in a plant pathology lab
Isolated bacterial colonies, prepared agar and nutrient media, plated and maintained bacterial colonies, isolated bacterial DNA, performed gel electrophoresis and PCR

College of Charleston, Charleston, SC **May 2004 to Aug 2004**
Undergraduate Researcher

Performed basic organometallic chemistry reactions to model the active sites of metalloenzymes
Reactions included strong-base chemistry and inorganic chemistry
Performed air-sensitive techniques on a Schlenk line or in a glove box, extractions, and recrystallizations

Clemson University, Clemson, SC **May 2001 to Aug 2001**
Research Intern

Chemically modified bioprosthetic heart valves to prevent calcification *in vivo*
Extracted heart valves from porcine hearts, assisted in implantation of heart valves in mice, performed assays on modified heart valves, and conducted histology studies

PUBLICATIONS

Maddess, Matthew L.; **Carter, Rhiannon R.**; S_nAr Reactions of 2-Methylthio-4-pyrimidinones in Pivalic Acid: Access to Functionalized Pyrimidinones and Pyrimidines. *Synthesis*. **2012**, *7*, 1109-1118.

Brooker, Matthew D.; Cooper Jr., Stefan M.; Hodges, Dena R.; **Carter, Rhiannon R.**; Wyatt, Justin K. Studies of microwave-enhanced Suzuki-Miyaura vinylation of electron-rich sterically hindered substrates utilizing potassium vinyltrifluoroborate. *Tetrahedron Letters.* **2010**, *51*, 6748-6752.

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Carter, Rhiannon R.; Overby, Jason S. Synthetic Studies of Models for Metalloenzyme Active Sites. *The 56th Southeastern Regional Meeting of the American Chemical Society Bulletin.* **2004** Abstract.

PRESENTATIONS

Carter, Rhiannon R.; LaBelle, Keri; Tozier, Lisa; Cawrse, Brian; Hall, Adam B. Colorimetric Analysis of *Salvia divinorum* Utilizing Ehrlich's Reagent. *39th Annual Meeting of the Northeaster Association of Forensic Scientists*, **2013**. Oral presentation.

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Carter, Rhiannon R.; Wyatt, Justin K. Suzuki Cross-Coupling Reaction of Potassium Vinyltrifluoroborate with a Sterically Hindered System: Progress Toward New Derivatives of the Antibiotic Cytosporone E. *College of Charleston Summer Undergraduate Research with Faculty Student Seminar*, **2005**. Oral presentation.

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Carter, Rhiannon R.; Overby, Jason S. Synthetic Studies of Models for Metalloenzyme Active Sites. *The 56th Southeastern Regional Meeting of the American Chemical Society,* **2004**. Poster presentation.

Carter, Rhiannon R.; Overby, Jason S. Synthetic Studies of Models for Metalloenzyme Active Sites*. College of Charleston Summer Undergraduate Research with Faculty Poster Session*, **2004**. Poster presentation.