## CHAPTER THREE

### Identification and Quantification of Salvinorin A

Thin Layer Chromatography Assays

Salvinorin A can be rapidly identified by thin-layer chromatography (TLC) on silica plates with a variety of different solvent systems. Effective separation has been reported using a developing solvent mixture of chloroform, methanol and water ($CHCl_3$:MeOH:$H_2O$, 100:10:1) (Valdés et al., 1984) or a solvent mixture of hexane and ethyl acetate (hexane:EtOAc 65:45) (Ortega et al., 1982). Visualization of salvinorin spots can be accomplished by spraying the developed plates with 10% phosphomolybdic acid in 2-propanol followed by heating (Ortega et al., 1982) or by spraying with vanillin-sulfuric acid or anisaldehyde-sulfuric acid spray reagents, followed by heating (Siebert, 1995). Thin-layer chromatography allows rapid, simultaneous analysis of multiple fractions from extracts subjected to column chromatography for purification. The sensitivity of the TLC assay, however, does not permit detection of salvinorin A at the sub-microgram levels that can be observed in the HPLC assay described below. Accurate quantification of the salvinorin A contained in a sample is also difficult with TLC methods.

Typical TLC analyses carried out during the extraction of *S. divinorum* samples and purification by column chromatography used 20 cm x 20 cm silica gel G-$F_{254}$ plates [Analtech, 75 Blue Hen Dr., Newark DE 19713]. Plates were developed with a mixture of hexane: EtOAc, 1:1, and salvinorin spots visualized using either the vanillin-sulfuric acid or anisaldehyde sulfuric acid spray reagent, followed by heating in an oven at 100 °C for 10 minutes. All solvents used for TLC analysis of salvinorin A and *S. divinorum* extracts

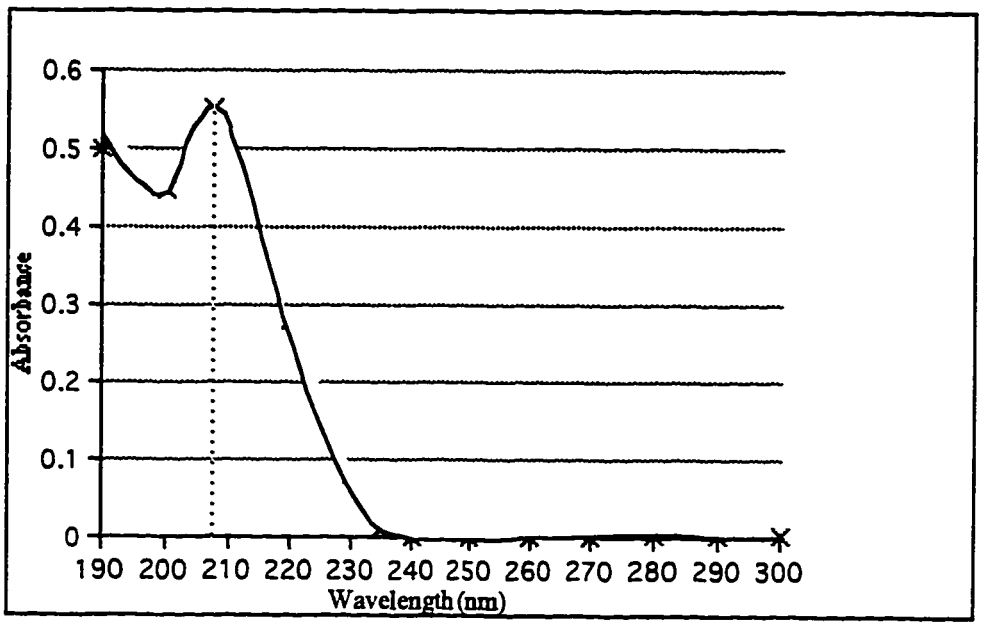
were ACS reagent grade or better. Authentic salvinorin A, provided by Daniel Siebert, appeared as a violet-blue spot with an Rf of 0.45 - 0.53.

Reverse Phase HPLC Assay

To analyze salvinorin A in extracts of *S. divinorum* tissues, a reverse phase isocratic HPLC method was developed using a Zorbax SB-300 diisobutyl *n*-octadecyl (C-18) column (4.6 mm i.d. x 250 mm, 5 μm particle diameter, 300 Å average pore size). This column is designed to achieve separations of high molecular weight compounds while providing more rapid elution of strongly retained organic compounds. The larger pore size typically produces shorter retention times than a comparable C-18 column with 80 Å pores. This column is particularly appropriate for separating the wide variety of components that may be encountered in a crude plant tissue extract and provides efficient separation of the analyte of interest in a rapid analysis. To protect the integrity of the analytical column, all analyses were performed with a C-18 guard column coupled to the main column.

The HPLC separations were performed using a Milton Roy Constametric 3000 or IIIG metering pump with a flow rate of 1.00 mL / min, achieving a typical column pressure of 1900 to 2000 p.s.i.. The reported $\lambda_{max}$ for salvinorin A is 211 nm in MeOH with an extinction coefficient $\varepsilon = 5{,}260$ (Valdés et al., 1984). To confirm this value and test for any difference in $\lambda_{max}$ using the solvents employed for HPLC, an ultraviolet spectrum was obtained using a reference standard of salvinorin A dissolved in a mixture of 45% acetonitrile / 55% $H_2O$. The concentration of salvinorin A in the solution used for ultraviolet absorbance was 0.048 mg/mL ($1.11 \times 10^{-4}$ M). The $\lambda_{max}$ for salvinorin A in the acetonitrile/water mixture was 207.6 nm, with an

absorbance value of 0.551 AU. The spectrum obtained is shown in Figure 9. The molar absorptivity was calculated to be 4,960 L $mol^{-1}$ $cm^{-1}$ as shown below. To approximate the $\lambda_{max}$ for salvinorin A in the acetonitrile/water mobile phase, ultraviolet detection was accomplished at a wavelength of 208 nm using a Milton Roy SpectroMonitor 3100 variable wavelength detector. The analog signal collected from the detector was converted to a digital signal by a Dionex Advanced Computer Interface and was processed by the Dionex AI-450 software to produce chromatograms and perform integration of peaks.




λ-max = 207.6 nm, Abs = 0.551

Figure 9. Ultraviolet spectrum of 0.048 mg/mL salvinorin A in 45% acetonitrile / 55% $H_2O$.

$$Abs = \varepsilon\ b\ c$$

$$\varepsilon_{207.6} = A_{207.6} / b\ c$$

$$\varepsilon_{207.6} = 0.551 / (1.00\ cm)(1.11 \times 10\text{-}4\ mol/\ L)$$

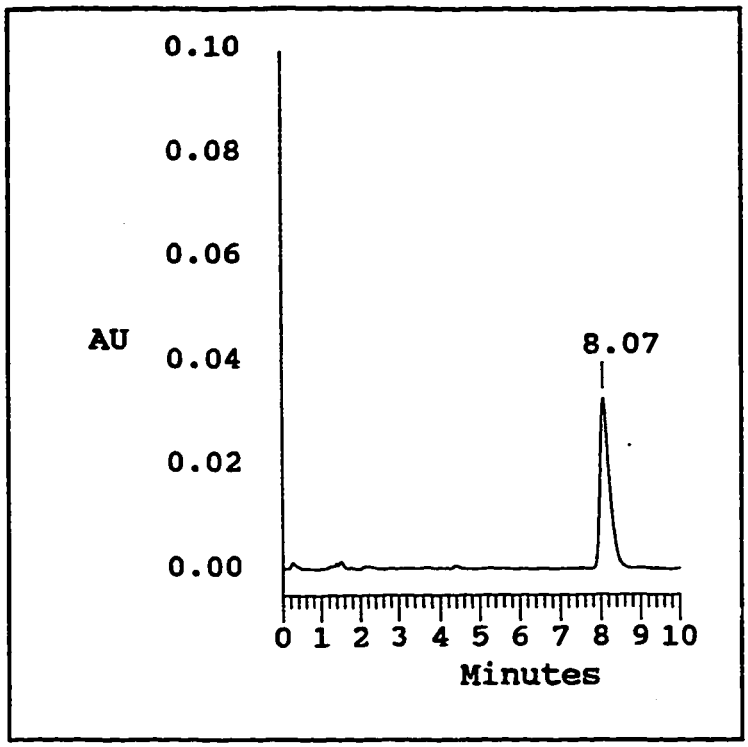
$$\varepsilon_{207.6} = 4{,}960\ L\ mol^{-1}\ cm^{-1}$$

All acetonitrile used for mixing mobile phase was Fisher HPLC grade acetonitrile; water used in mobile phase mixtures was distilled water filtered through a 0.22 μm filter membrane. All mobile phase mixtures were then vacuum-filtered through a 0.45 μm filter before bottling for HPLC use. Several mobile phase mixtures were tested beginning with 100% acetonitrile and then decreasing the percentage of acetonitrile by mixing with water to increase the polarity of the solvent. The final isocratic mobile phase mixture was chosen to balance sufficient separation of components in tissue extracts with rapid analysis time and preservation of a more desirable, narrower peak shape for the analyte of interest. A mobile phase mixture of 45% acetonitrile / 55% $H_2O$ was selected as the optimal mixture in this solvent system. Salvinorin A elutes from the column in approximately 8.1 minutes with this mobile phase at a flow rate of 1.0 mL / min (Figure 10). This retention time for salvinorin A achieves good separation from other components in a leaf tissue extract while preserving a rapid overall analysis. A second mobile phase was used to confirm the singularity of the salvinorin A peak when given longer separation times on the column. A mixture of 35% acetonitrile / 65 % $H_2O$ produces an elution time of approximately 22 minutes for salvinorin A at a flow rate of 1.0 mL/min (Figure 10). Standard solutions of authenticated salvinorin A dissolved in mobile phase as well as tissue extracts of *S. divinorum* leaves were used in the development of the isocratic HPLC method. The peak identified as salvinorin A from authenticated standard solutions was observed to occur with the same retention time in extracts of leaves from *S. divinorum* and was designated the salvinorin A peak (Figure 11). To further demonstrate the reproducibility of the separation, the

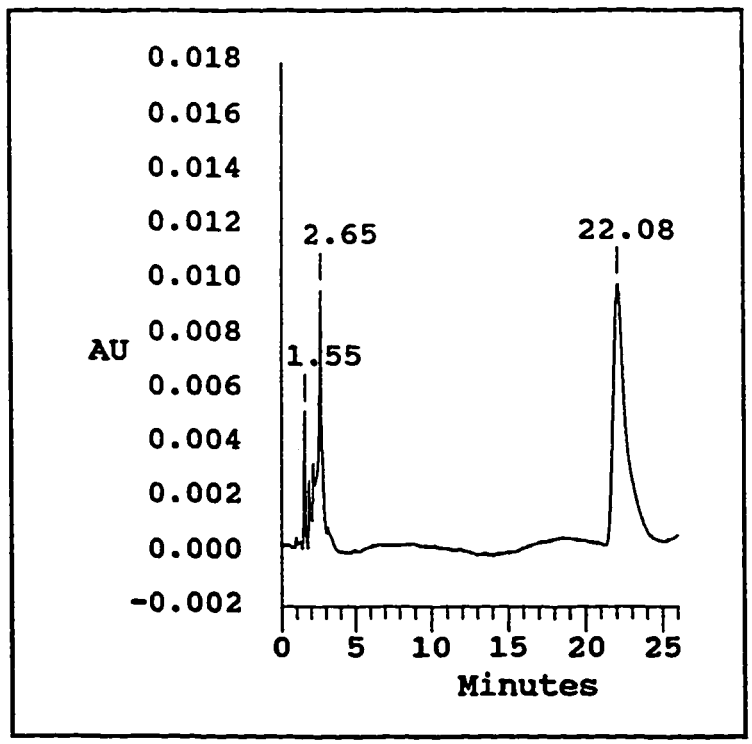
salvinorin A peak was on two separate occasions collected manually or with an automated fraction collector. The collected peak in mobile phase was then reinjected onto the column and its retention time was verified for consistency (Figure 12).

Development of Calibration Curves for Salvinorin A

To establish a relationship between the mass of salvinorin A injected into the HPLC and the resultant peak area at 208 nm, a series of calibration curves were derived from multiple injections of a salvinorin A standard solution. Aliquots of salvinorin A dissolved in mobile phase were prepared by dissolving 0.73 mg of salvinorin A in 1.200 mL of the HPLC mobile phase, 45 % acetonitrile and 55% water. The initial solution of salvinorin A was then divided into eight 100 $\mu$L portions in 1.5 mL Eppendorf microtubes, and diluted to a total final volume of 1.200 mL with HPLC mobile phase. The resulting aliquots of standard solution had a concentration of 0.051 mg/mL salvinorin A. These solutions were stored at 4 °C in the microtubes. Calibration curves were produced by injecting six different volumes of this solution into the HPLC and recording the integrated areas for the salvinorin A peak. Each individual calibration curve was produced from a fresh aliquot of the standard solution. Using a 50 $\mu$L syringe, individual HPLC analyses were performed with 5 $\mu$L, 10 $\mu$L, 15 $\mu$L, 20 $\mu$L, 25 $\mu$L, and 30 $\mu$L of standard solution. Each solution volume injection was repeated three times, and the peak areas obtained were averaged to establish a single value for the replicates. The relationship between mass of salvinorin A injected and average peak area was then plotted with a regression analysis to assess the linearity of the relationship. Three separate calibration curves were produced

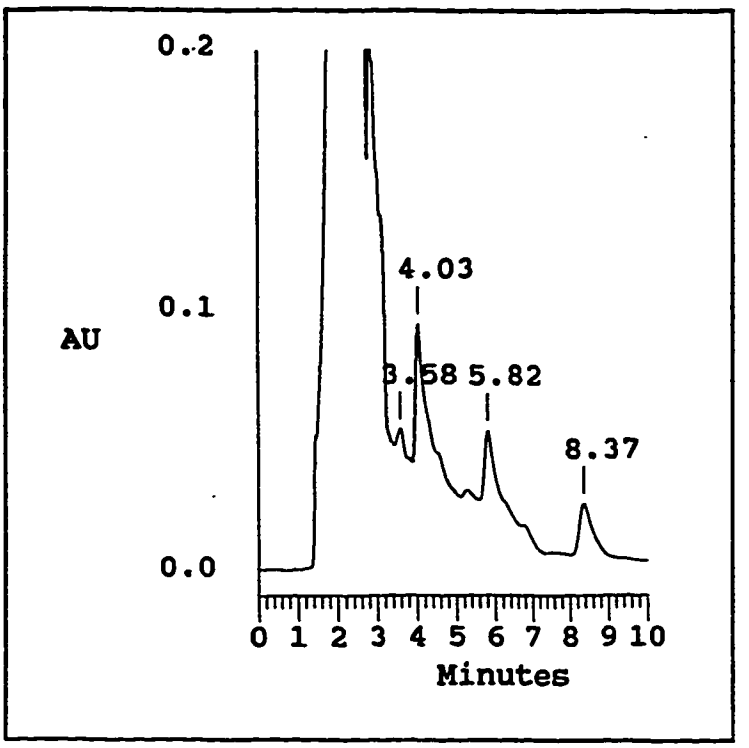



A. 20 μL of salvinorin A standard solution (0.051 mg/mL) in mobile phase of 45% acetonitrile and 55% water. Flow rate of 1.00 mL/min.

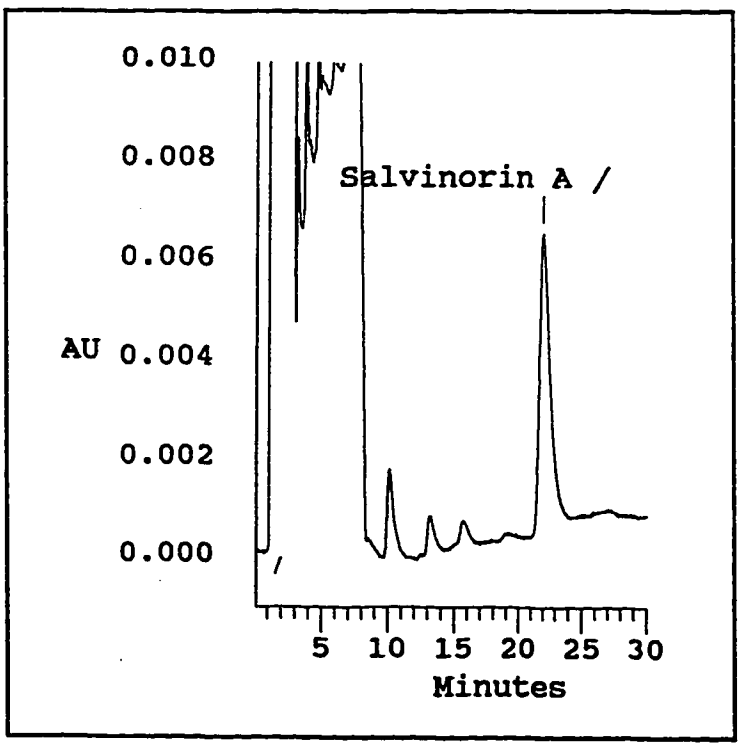



B. 20 μL of salvinorin A standard solution (0.051 mg/mL) in mobile phase of 35% acetonitrile and 65% water. Flow rate of 1.00 mL/min.

**Figure 10. Elution of Salvinorin A standard solution in two different mobile phases.**

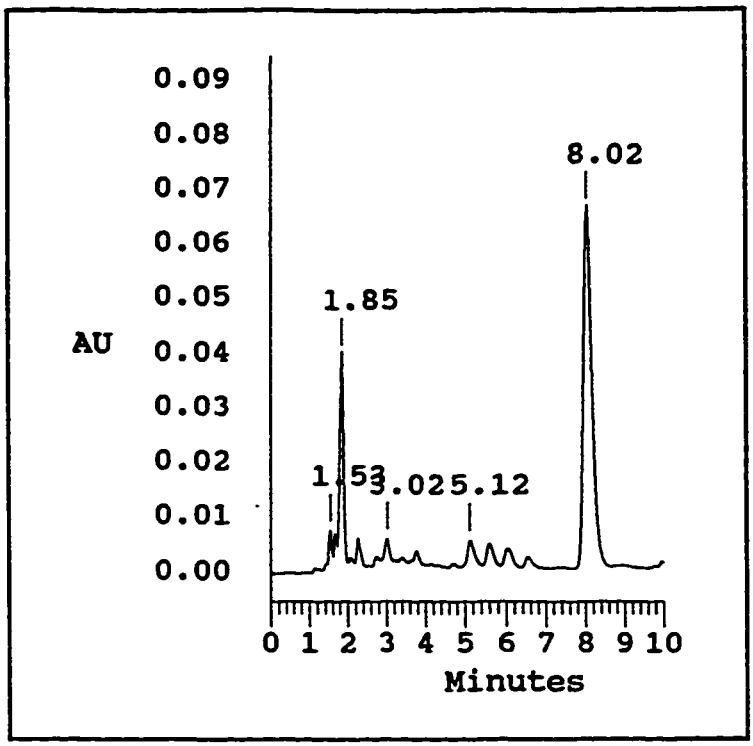



**A.** 20 μL of *S. divinorum* methanolic leaf extract in a mobile phase of 45% acetonitrile and 55% water. Flow rate of 1.00 mL/min. Ultraviolet detection at 208 nm.

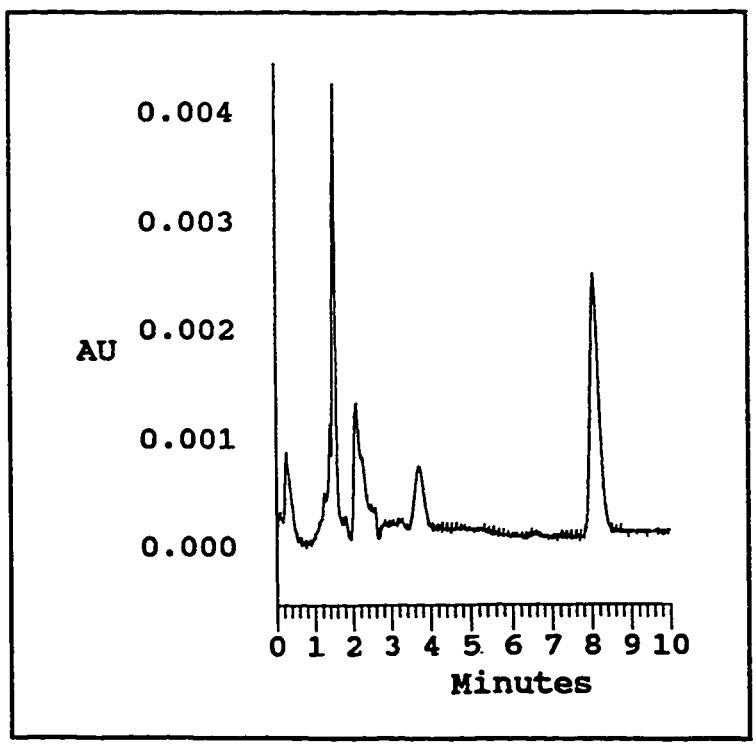



**B.** 10 μL of *S. divinorum* methanolic leaf extract in a mobile phase of 35% acetonitrile and 65% water. Flow rate of 1.00 mL/min. Ultraviolet detection at 208 nm.

**Figure 11. Identification of salvinorin A peak from leaf extracts analyzed in two different mobile phases.**




**A.** 20 μL of diluted *Salvia divinorum* leaf extract (partially purified on Si-gel column) in mobile phase of 45% acetonitrile and 55% water. Flow rate of 1.00 mL/min. Ultraviolet detection at 208 nm. A 0.7 mL fraction was collected at 8 minutes.



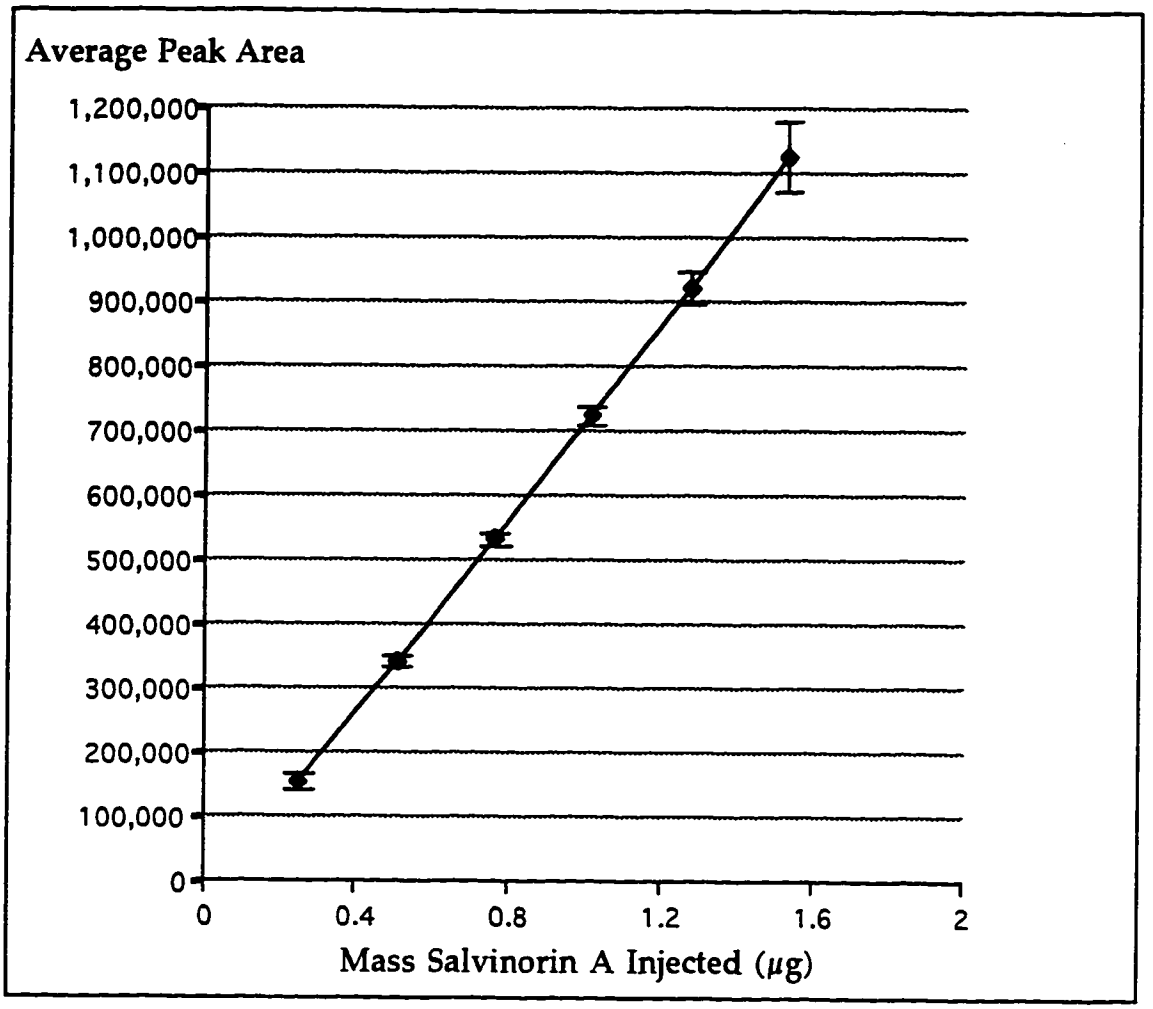

**B.** Reinjection of 20 μL of the fraction collected from analysis in (A) above. The salvinorin A peak is present in this fraction at a much lower concetration due to the dilution in a relatively large volume of mobile phase.

**Figure 12. Collection of salvinorin A peak from leaf extract and reinjection of collected material.**

to validate the HPLC method with the above described standard solutions between January 18, 1996 and May 18, 1996. Figure 13 shows the calibration curve generated from the average of these three determinations together with the results from the regression analysis for these data points. The coefficients of variation for each of the six injected standard solutions are presented in Table 3.

Table 3. Standard Deviations and Coefficients of Variation for Standard Calibration Curve

| Mass ($\mu$g) | 0.255 | 0.51 | 0.765 | 1.02 | 1.275 | 1.53 |
|---|---|---|---|---|---|---|
| Average area | 155802 | 342482 | 532713 | 723682 | 922384 | 1125336 |
| Standard Deviation | 8909 | 7051 | 8811 | 15484 | 28128 | 52656 |
| Coefficient of Variation | 5.7% | 2.1% | 1.7% | 2.1% | 3.0% | 4.7% |




I Error Bars show ± 1 Standard Deviation

Regression Analysis Result:

$y = 759{,}479\,x - 44{,}101$

$r^2 = 0.9999$

Figure 13. Calibration curve for salvinorin A standard solution injections.

Average of three separate determinations of peak area vs. mass injected. Mobile phase of 45% acetonitrile / 55% water. Flow rate of 1.00 mL/min. Ultraviolet detection at 208 nm.

Isolation of Salvinorin A for reference material:

To produce pure salvinorin A crystals for use in quantification studies, a batch of 30.00 grams of ground, lyophilized *S. divinorum* leaf was extracted in a 60 mm cellulose thimble with a Soxhlet extractor in November, 1995. The leaf material was extracted for 3 hours in a total of 500 mL of Fisher HPLC grade chloroform (pentene-stabilized). The chloroform solvent was evaporated *in vacuo*, leaving 1.8 g of a dark green residue. This material was partitioned between hexane and 90% MeOH in a separatory funnel and the methanolic fraction was retained and evaporated to dryness. The residue was taken up again in chloroform and divided roughly in half. The first half of the extract was then applied to a silica gel column for further purification. The column was prepared using 33.2 grams of Baker analyzed silica gel (60 - 200 mesh), mixed as a slurry in hexane and poured to a bed height of 24 cm in a 24 mm i.d. glass column. The *Salvia* leaf extract in chloroform was mixed with clean dry silica by applying dropwise with a Pasteur pipette. The silica containing the extract was allowed to dry until it was free-running, and was then applied to the top of the silica gel column and covered with an additional layer of clean, dry silica gel. The column was then eluted with 10 mL solvent portions in the following sequence: 10 portions 100% hexane, 5 portions 90% hexane / 10 % ethyl acetate, 5 portions 80% hexane / 20 % ethyl acetate, 10 portions 70% hexane / 30 % ethyl acetate, and 27 portions 60% hexane / 40 % ethyl acetate. As each solvent portion was applied at the top of the column, a 10 mL fraction was also collected from the end of the column. A total of 52 fractions were collected in all. These were screened by TLC analysis for salvinorin A content, and the six fractions (#37 - #42) containing

the highest content of salvinorin A were combined and evaporated. The remaining residue was stored under refrigeration. In June, 1996, the second half of the methanolic fraction of extract was purified by column chromatography in similar fashion to the first. Prior to its application to the silica gel column, this extract was first taken up in 100% acetonitrile and cleaned by passing it through a Sep-Pak C-18 solid phase extraction cartridge [Water Assoc., Milford, MA]. The cartridge retained a quantity of chlorophyll while allowing the majority of the salvinorin A present to pass through. The acetonitrile was then evaporated *in vacuo* in a rotary evaporator, and the extract was taken up again in chloroform. This chloroform extract was applied to a silica gel column in the manner described above. The column was eluted with the same solvent sequence as previously described. The solvent sequence added was as follows: 100 mL of 100% hexane, 50 mL of 90% hexane / 10% ethyl acetate, 50 mL of 80% hexane / 20% ethyl acetate, 100 mL of 70% hexane / 30% ethyl acetate, and 300 mL of 60% hexane / 40% ethyl acetate. Numbered fractions were collected in 10 mL portions beginning with the 60% hexane / 40% ethyl acetate solvent addition. As a result, fraction #1 from this June, 1996 column corresponds to fraction #31 from the column run in November, 1995. The collected fractions were analyzed by TLC for salvinorin A content. The first appearance of salvinorin A was in fraction # 12, while the highest concentration with the least amount of impurities appeared in fraction #14. To isolate clean salvinorin A, fractions # 13 - 21 were combined and the solvent evaporated with a rotary evaporator. These fractions would correspond to fractions 43 - 51 from the November 1995 column. The impure salvinorin A obtained in fractions 13 - 21 was purified by recrystallization

from methanol and filtered out of the solvent. The resulting crystals were needle-shaped, with a greenish cast, presumably from residual traces of chlorophyll. A portion of these crystals were further washed with cold methanol, producing 4 mg of white salvinorin A crystals. The remainder of the crystals (18 mg) were subjected to further analysis. These two samples of crystals were stored separately in 1.5 mL Eppendorf microtubes over $CaSO_4$ dessicant in a freezer. The 4 mg of white crystals were subjected to melting point analysis, and analyzed by GC-MS and FTIR. The 18 mg sample was analyzed by GC-MS and was subjected to $^{1}H$-NMR and $^{13}C$-NMR to verify the structure of salvinorin A. The results of these analyses are summarized below with the corresponding spectra. The melting point was taken with a Mel-Temp electric melting point apparatus, and a melting temperature of 234 - 235 °C (uncorrected) was obtained. The literature values reported for salvinorin A are 238 - 240 °C (Ortega et al., 1982) and 242 - 244 °C (Valdés et al., 1984). The identity of the crystals was verified by GC/MS analysis [Hewlett Packard 5890 Series II gas chromatograph with HP5971 A mass selective detector], Fourier transform infrared spectrum, and NMR spectra. Figure 14 shows the GC/MS results for a sample of the 4 mg salvinorin A crystals dissolved in acetone. Table 4 summarizes the mass spectral peaks reported in the literature in earlier analyses together with those determined by GC-MS analysis of material purified from *S. divinorum* leaf in this investigation.

File: D:\DATA\JWGSAL2.D
Operator:
Date Acquired: 7 Jun 96 2:07 pm
Method File Name: JWG1.M
Sample Name:
Misc Info:
Bottle Number: 1

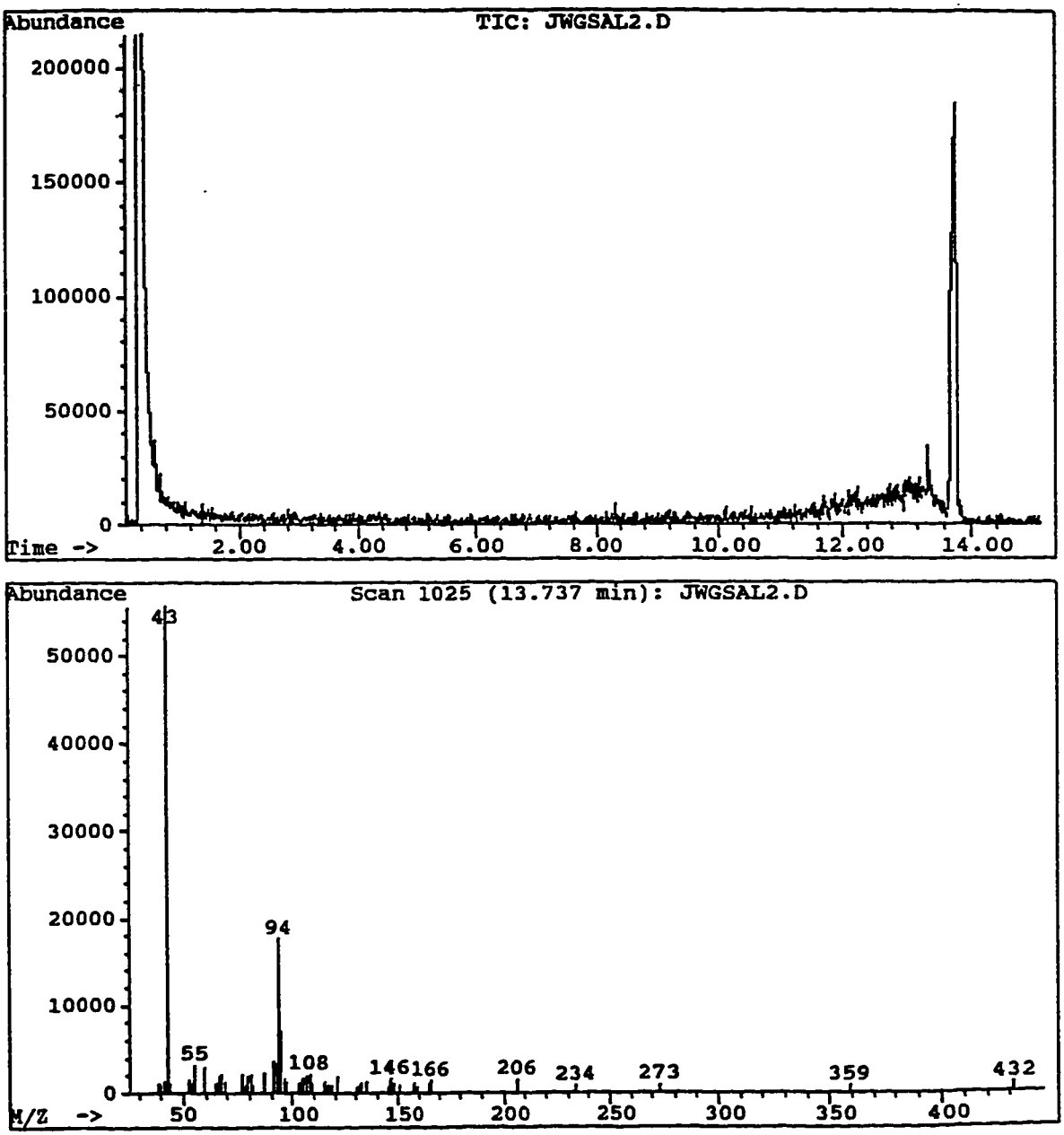



Figure 14. Gas chromatogram of salvinorin A sample and accompanying mass spectrum for predominant peak (13.7 minutes). Table 3 provides a summary of the mass peaks and relative abundances.

| Table 4. Mass Spectrum Peaks and Abundances | | | |
|---|---|---|---|
| | Normalized Abundances (Parent Peak = 94) | | |
| | Valdés et al. | Ortega et al. | |
| m/z peak | (1984) | (1982) | This paper |
| 432 | 1.5 | 20 | 5.6 |
| 404 | n.r.† | 15 | n.r. |
| 359 | n.r. | 5 | 4.3 |
| 318 | n.r. | 20 | n.r. |
| 273 | 6.5 | 30 | 4.5 |
| 166 | 8.6 | n.r. | 7.8 |
| 121 | 13 | n.r. | 10.8 |
| 108 | 8 | n.r. | 11.7 |
| 107 | 9.7 | n.r. | 10.7 |
| 95 | 17.9 | n.r. | 39.6 |
| 94 | 100 | 100 | 100 |
| 93 | 9.9 | n.r. | 18.6 |
| 91 | 6.9 | n.r. | 20.4 |
| 81 | 11.2 | n.r. | 12.1 |
| 79 | 5.5 | n.r. | 10.1 |
| 55 | 13.7 | n.r. | 18.0 |
| | † n.r. = not reported in spectrum | | |

The correlation between the mass spectrum obtained for the sample of salvinorin A and those reported in the literature is satisfactory. The peak at m/z of 404 that was reported by Ortega et al. (1982) but was not seen by Valdés et al. (1984) was observed in a mass spectrum for a second sample of salvinorin A taken from the 18 mg of recrystallized material described above. The most revealing analytical spectra, however, were the $^{1}$H-NMR and $^{13}$C-NMR spectra, which corresponded nearly perfectly with the literature values for salvinorin A reported by Valdés et al. (1984). NMR spectra were obtained with a Varian XL-200 200 MHz NMR instrument at Villanova University. Table 5 summarizes the $^{13}$C-NMR $\delta$ values obtained in comparison to those

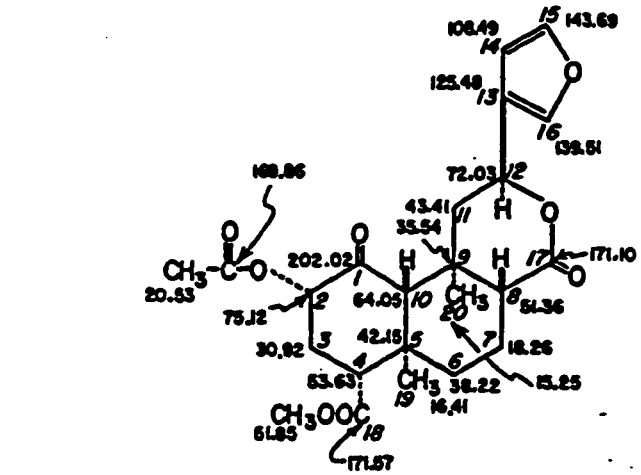
reported in the literature by Valdés et al. (1984). Distinguishing peaks observed in the NMR spectrum include the ketone at C-1 with a $\delta$ value of 202 , and the aromatic carbons of the furan ring (C-13, C-14, C-15 and C-16) with $\delta$ values of approximately 108 to 144.

Table 5. $^{13}$C-NMR $\delta$ values from $(CH_3)_4Si$

| Valdés et al. (1984) | Observed (diff.) |
|---|---|
| (carbon assignment) | |
| 15.25 (C-20) | 15.08 (-0.17) |
| 16.41 (C-19) | 16.28 (-0.13) |
| 18.26 (C-7) | 18.20 (-0.06) |
| 20.53 ($CH_3$ acetyl) | 20.46 (-0.07) |
| 30.92 (C-3) | 30.64 (-0.28) |
| 35.54 (C-9) | 35.33 (-0.21) |
| 38.22 (C-6) | 38.03 (-0.19) |
| 42.15 (C-5) | 41.96 (-0.19) |
| 43.41 (C-11) | 43.24 (-0.17) |
| 51.36 (C-8) | 51.26 (-0.10) |
| 51.85 ($CH_3$ methyl ester) | 51.85 (-0.00) |
| 53.63 (C-4) | 53.44 (-0.19) |
| 64.05 (C-10) | 63.92 (-0.13) |
| 72.03 (C-12) | 71.91 (-0.12) |
| 75.12 (C-2) | 74.90 (-0.22) |
| 108.49 (C-14) | 108.26 (-0.23) |
| 125.48 (C-13) | 125.07 (-0.41) |
| 139.51 (C-16) | 139.31 (-0.20) |
| 143.69 (C-15) | 143.59 (-0.10) |
| 169.86 (C=O acetyl) | n.r. ‡ |
| 171.10 (C-17) | n.r. ‡ |
| 171.57 (C-18) | n.r. ‡ |
| 202.02 (C-1) | 201.91 (-0.11) |

‡ peak not resolved sufficiently for inclusion in spectrum

Figure 15. $^{13}$C-NMR (top) and $^{1}$H-NMR (bottom) Assignments of Salvinorin A from Valdés et al., 1984.



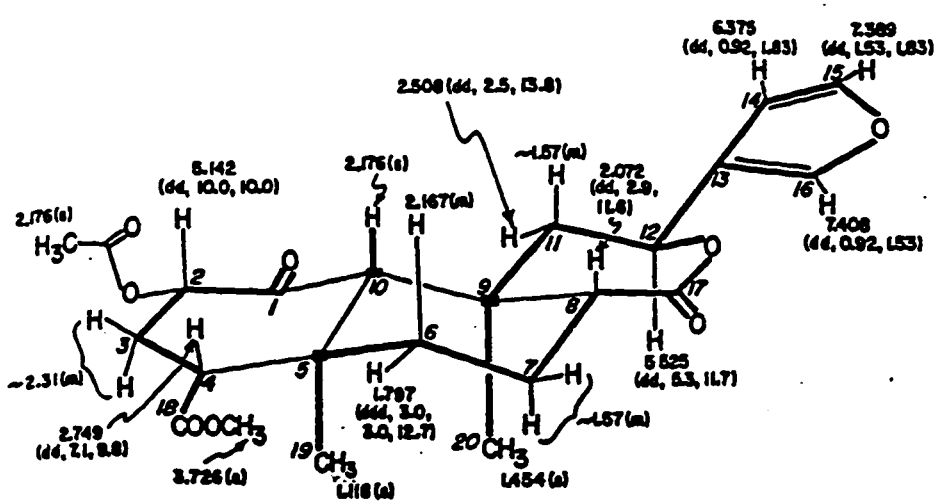


Figure 16. $^{13}$C-NMR Spectrum of Salvinorin A

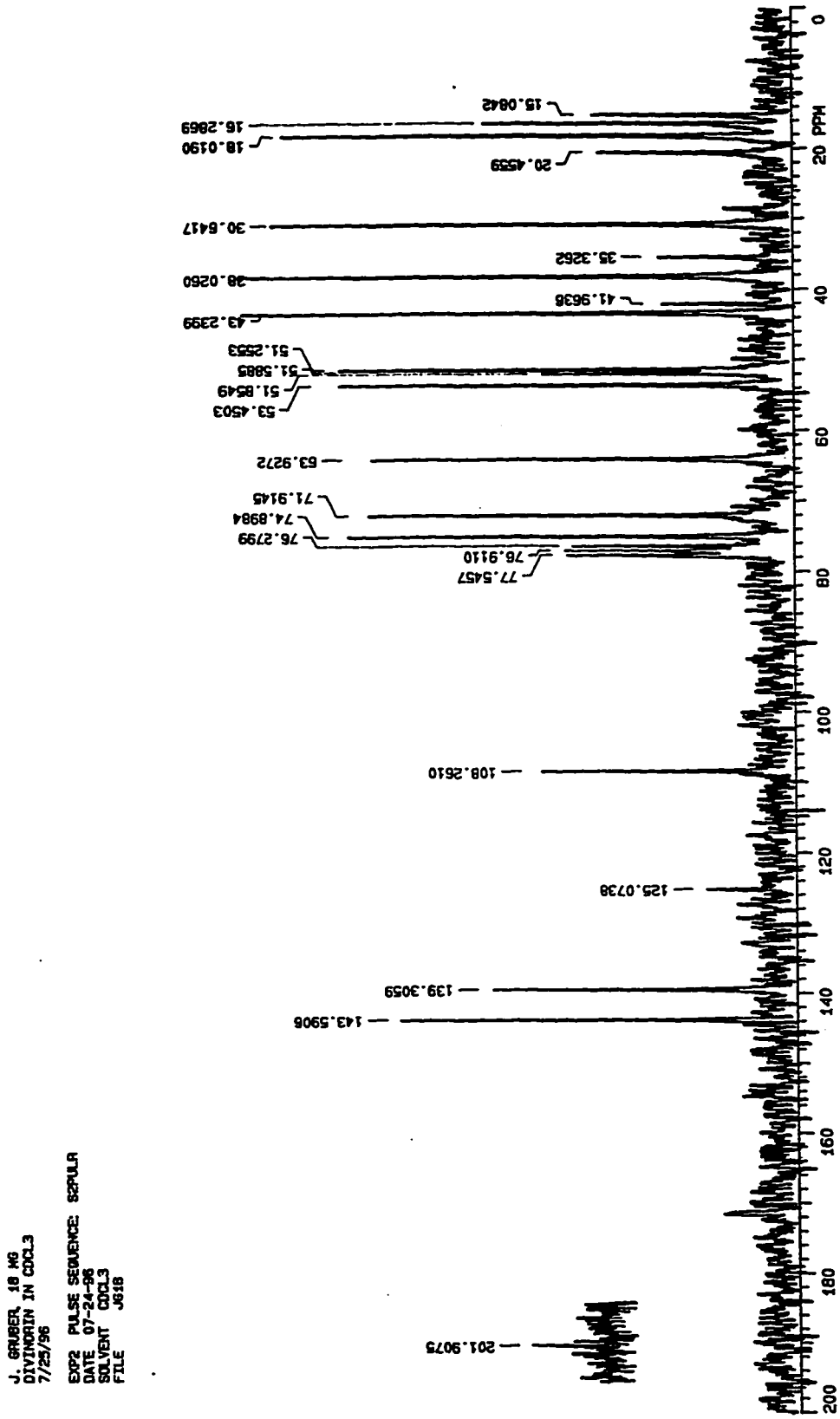


Figure 17. $^{1}H$-NMR Spectrum of Salvinorin A

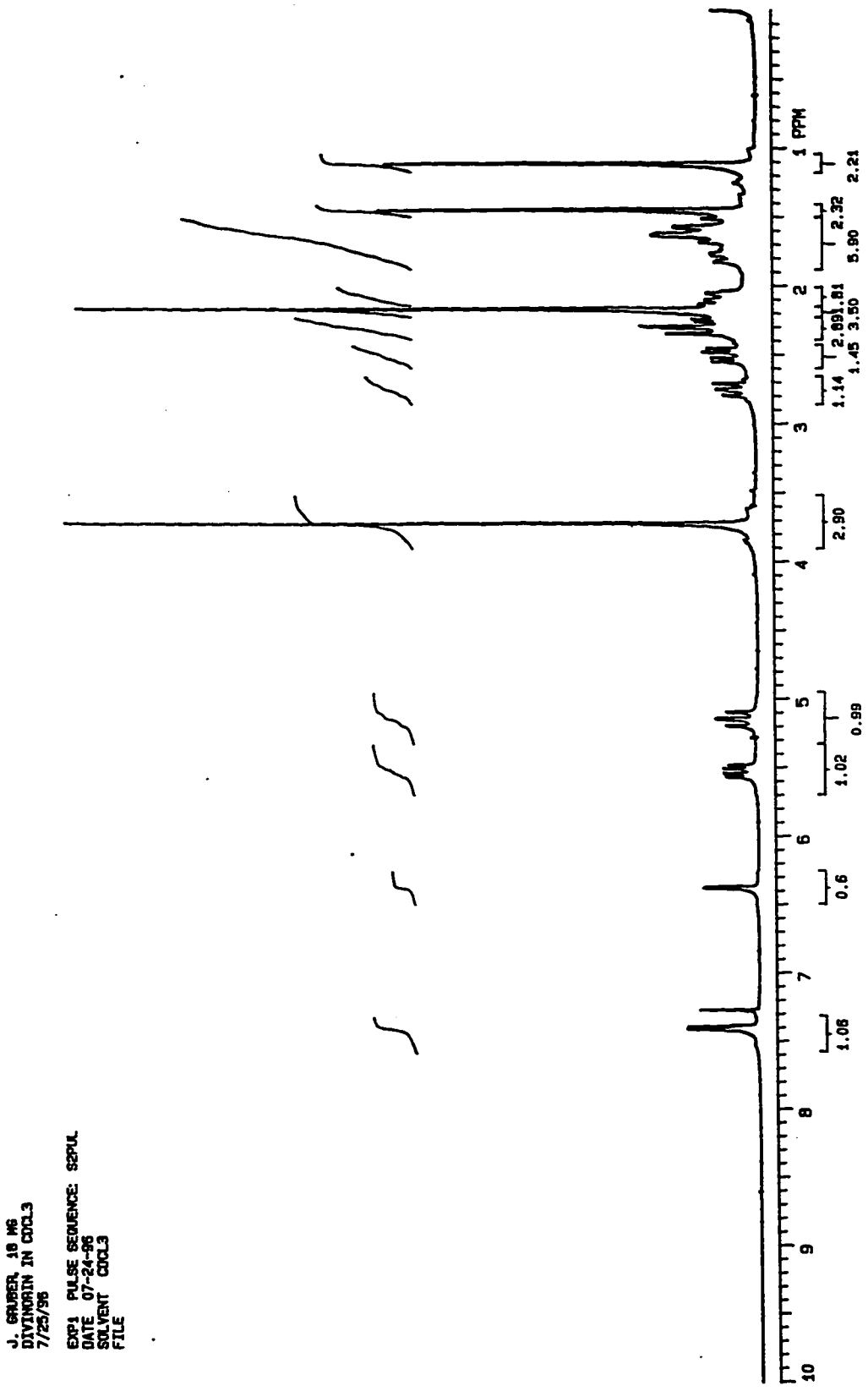
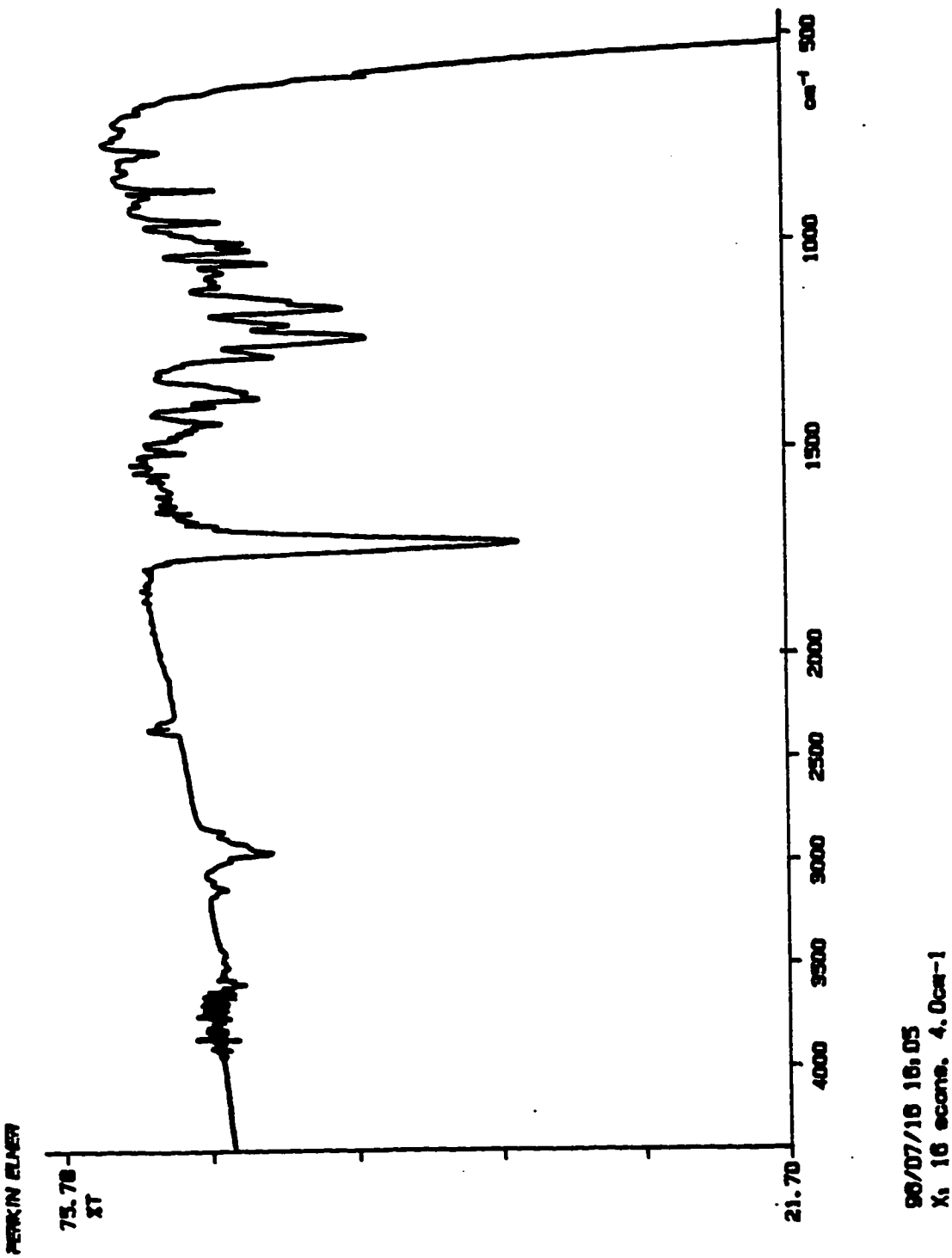


Figure 18. FTIR Spectrum of Salvinorin A



Ultraviolet Wavelength Ratio Experiment

For standardized HPLC analyses, 208 nm was chosen as the standard ultraviolet detection wavelength to approximate the $\lambda_{max}$ for salvinorin A in the mobile phase solvents. Other wavelengths were used in a series of tests with salvinorin A standard solution to establish an approximate ratio of absorbance and peak areas between the standard base wavelength (208 nm) and other wavelengths. Using the standard salvinorin A solution of 0.051 mg/mL, a series of 20 µL injections was performed at 208 nm, 215 nm, 220 nm, and 230 nm. The ratios of peak area for the salvinorin A peak were also determined in a *S. divinorum* leaf extract at 220 nm and 230 nm to compare to the base peak at 208 nm. A separate leaf extract of *S. divinorum* was used to determine the ratio of peak area at 215 nm to the base peak at 208 nm. A summary of the peak areas and the resultant ratios is presented in Table 6 below.

Table 6. Peak Ratios at 4 Different Analytical Wavelengths

| | Salvinorin A Standard Solution | | *S. divinorum* Leaf Extract | | *S. divinorum* Leaf Extract | |
|---|---|---|---|---|---|---|
| Wavelength | Avg. Area | Percent of Base Peak | Avg. Area | Percent of Base Peak | Avg. Area | Percent of Base Peak |
| 208 nm | 624,191 | 100% | 551,564 | 100% | 413,640 | 100% |
| 215 nm | 541,402 | 87% | n.a. | n.a. | 356,483 | 86% |
| 220 nm | 366,818 | 59% | 337,635 | 61% | | |
| 230 nm | 72,771 | 12% | 57,324 | 10% | | |

The peak ratios between authentic salvinorin A standard solution and the salvinorin A peak in leaf extract correspond favorably at 220 and 230 nm. A similar analysis could be undertaken with a peak of matching retention time in other samples to build evidence for its identity as salvinorin A.

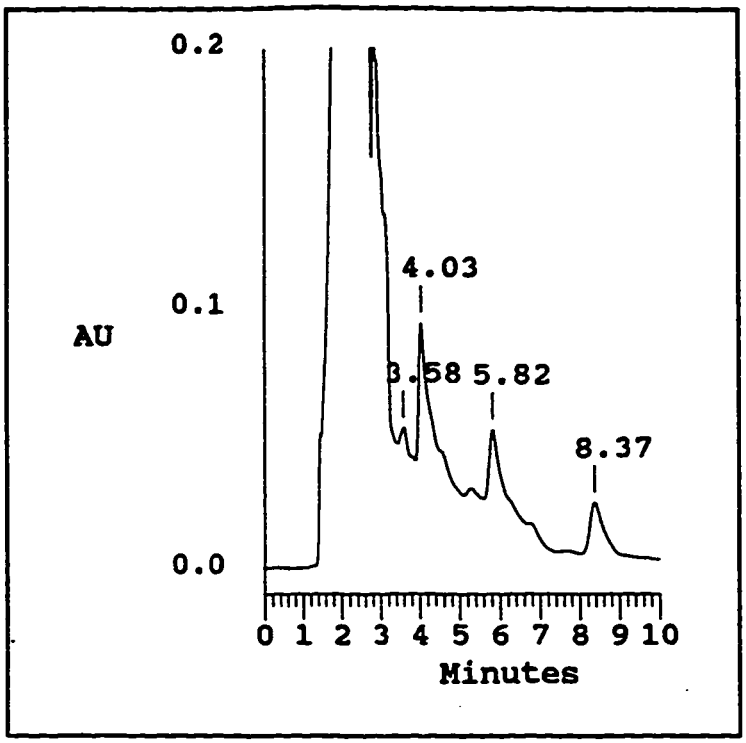
Optimization of Salvinorin A Extraction Procedures

Several methods were examined for optimal extraction of salvinorin A from dried leaf tissues of *Salvia* species. Valdés (1994) reported that an extraction in ethyl ether with a Soxhlet apparatus followed by partitioning of the extract between hexane and 90% methanol yielded a methanolic fraction that was up to 10% salvinorin A by weight. This extraction method thus has the advantage of providing an extract that is substantially reduced in organic impurities and well suited for further purification and isolation of salvinorin A. Initial assays for salvinorin A quantification were based on this method, using chloroform for the primary extraction rather than ethyl ether. Results from these extractions, however, showed that with a constant volume of solvents, the salvinorin A carried into the 90% methanol layer and then quantified by HPLC was dependent on the initial size of the leaf batch. For example, a 3.00 gram sample of dried leaves yielded an estimate of 2.14 mg salvinorin A per gram of leaf, while an identical treatment of a 0.510 g sample of leaves produced an estimate of only 1.35 mg salvinorin A per gram of leaf. This difference most likely resulted from the quantity of salvinorin A required to saturate the hexane layer in the liquid-liquid partitioning between hexane and 90% methanol. Since a constant mass of salvinorin A will remain behind in the hexane layer, the percent of salvinorin A lost to the hexane will be dependent on the total amount present and thus upon the size of the leaf batch being extracted (assuming all solvent volumes remain constant). Based on these findings, it was determined that the extraction process for quantification of salvinorin A should minimize the steps between initial extraction and injection into the HPLC for quantification.

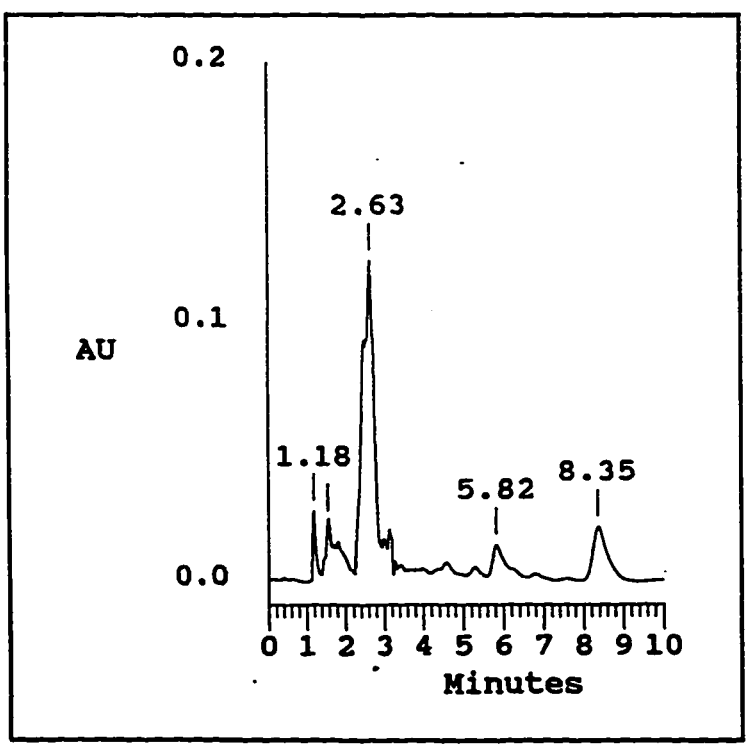
In an attempt to develop a direct one-step or two-step extraction procedure to remove the maximum percentage of salvinorin A, several different approaches were investigated. Using matched 1.00 gram samples of *S. divinorum* leaf, extractions were performed by refluxing in 50.0 mL MeOH for 100 min., refluxing in 50.0 mL chloroform for 100 min, steeping in MeOH at room temperature for 96 h, and steeping in 50.0 mL chloroform at room temperature for 96 h. All solvents used were Fisher HPLC grade or Optima grade (for methanol). The Fisher HPLC grade chloroform is pentene-stabilized. The extracts in chloroform were filtered, evaporated to dryness *in vacuo*, and reconstituted in 50.0 mL of methanol. The profile of the methanol extractions compared to the chloroform extractions was markedly different owing to the different polarities of the extracting solvents (Figure 19). Initial extraction in chloroform afforded an extract that was higher in salvinorin A with lower levels of other impurities, most of which are more polar than salvinorin A and elute in advance of it on the reverse phase HPLC column. Table 7 summarizes the results of these extractions.

Table 7. Comparison of salvinorin A estimates by four different extraction methods.

| Extraction Treatment | Peak Area (20 uL injection) | Estimated salvinorin A (mg/g) |
|---|---|---|
| Steep 96 h in methanol | 439000 | 1.56 |
| Steep 96 h in chloroform | 500000 | 1.75 |
| Reflux 100 min in chloroform | 391500 | 1.41 |
| Reflux 100 min in methanol | 71933 | 0.44 |




**A.** 20 μL of *S. divinorum* methanolic leaf extract in a mobile phase of 45% acetonitrile and 55% water. Flow rate of 1.00 mL/min. Ultraviolet detection at 208 nm. Salvinorin A peak estimate 1.56 mg/g.




**B.** 20 μL of *S. divinorum* chloroform leaf extract in a mobile phase of 45% acetonitrile and 55% water. Flow rate of 1.00 mL/min. Ultraviolet detection at 208 nm. Salvinorin A peak estimate 1.75 mg/g.

**Figure 19. Comparison of methanol vs. chloroform extraction of *S. divinorum* leaf**

None of the four methods tested matched the level of salvinorin determined for the earlier Soxhlet extraction of a 3.00 gram leaf batch (2.14 mg/g), and a 1.00 gram sample left for four weeks in chloroform showed substantially lower levels of salvinorin A (0.78 mg/g) than an equivalent sample extracted for 4 days (1.75 mg/g). This observation led to a series of experiments to investigate the effect of time in chloroform on salvinorin A available in the extract for quantification. Matched samples of 1.00 grams of lyophilized, powdered *S. divinorum* leaf were steeped in 50.0 mL chloroform for 15 minutes, 30 minutes, 45 minutes, 1 hour, 7 hours, 24 hours, and 52 hours. After the appointed time in chloroform, the extract was filtered, the leaf material and flask were rinsed with approximately 30 mL of additional fresh chloroform, and the chloroform extract was evaporated to dryness *in vacuo*. To assist in redissolving all of the extracted material, the extract was taken up in 10.0 mL of acetone and 40.0 mL of methanol, rinsing the extract from the flask with alternating portions of acetone and methanol until they were completely transferred into two ounce amber sample bottles. Results from HPLC analyses of these samples showed a time dependence of measurable salvinorin A in the extract, with levels falling off from an average estimate of 2.31 mg/g after 1 hour or less in chloroform to 1.44 mg/g after 52 hours in chloroform. Figure 20 shows the estimated salvinorin A values for 1.00 g leaf samples with extraction times of 15 minutes to 52 hours in chloroform. The decrease in salvinorin A concentration with longer extractions may be the result of precipitation of salvinorin A out of solution as increasing amounts of other leaf components are extracted. Based on these results, an extraction time of 30 minutes was chosen as the standard extraction time.

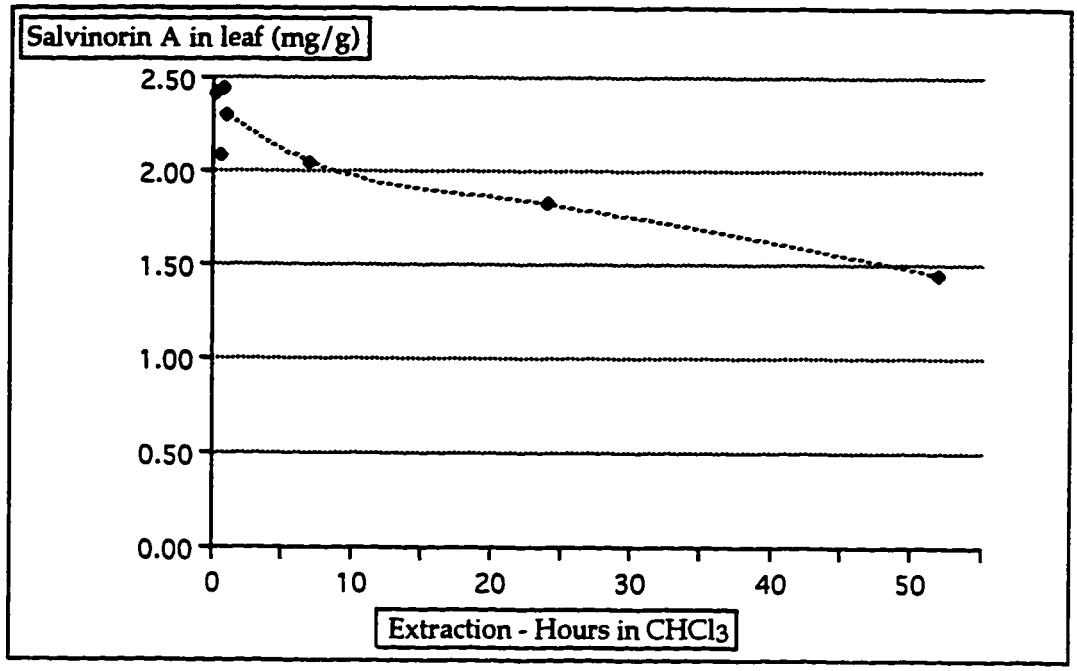



Figure 20. Salvinorin A estimates vs. extraction time in chloroform.

Percent Recovery experiments:

To validate the extraction procedure and quantify the recovery of salvinorin A from leaf tissues, several experiments were undertaken to estimate a percent recovery value. This value is intended to quantify the extraction yield and substantiate the values reported for salvinorin A in leaf tissue. As an initial characterization of the efficiency of extraction, a residual re-extraction experiment was conducted to test for salvinorin A left behind in filtered solids. A batch of 1.00 g of *S. divinorum* leaf was extracted in chloroform for 7 hours. The extract was treated according to standard methods described in Chapter 3 above, and a quantitative estimate of 1.83 mg salvinorin A per gram of leaf was determined. The filtered solid leaf material and filter paper were then subjected to a secondary extraction in a fresh 50 mL of chloroform for 1 hour, and were then rinsed, with the resulting extract

evaporated and reconstituted in methanol/acetone in the same manner as the previous extraction. The residuals extraction yielded trace quantities of salvinorin A that were not integrated by the HPLC method. When the integration threshold was lowered to force integration of this quantity, the resulting peak area was below the lowest level measured on the established calibration curve, and therefore could not be reliably quantified. However, if the area was considered as a fraction of the total area for the 7-hour extraction, the residual quantity 14,000/529,000 represents approximately 2.6% of the original total, suggesting an extraction of better than 95% of the total salvinorin A.

To quantify the percent recovery more precisely, a 1.00 g batch of *S. divinorum* leaves was extracted according to standard methods with the addition of a weighed quantity of pure salvinorin A to the leaves prior to the extraction. Salvinorin A extracted from *S. divinorum* leaves, purified by recrystallization, and authenticated by NMR was weighed (2.12 mg) into an Eppendorf microtube and transferred onto the ground, dried leaf tissue by dissolving it in chloroform and pipetting the chloroform onto the leaves. The leaves with added salvinorin A were then extracted with HPLC grade chloroform for 30 minutes. The leaf solids and filter paper were rinsed with an additional 35 mL of fresh chloroform, and the resulting chloroform extract was evaporated to dryness with a rotary evaporator. After reconstituting in 50.0 mL methanol/acetone, this extract was analyzed by HPLC and the salvinorin A quantity compared to standard 30 minute extractions of 1.00 gram samples of leaf without added salvinorin A. Table 8 shows the results of these calculations with the overall percent recovery estimate of 89%.

| Table 8. Percent recovery estimate for salvinorin A from leaf | | | | | |
|---|---|---|---|---|---|
| Weighed Salvinorin A (mg) | Extract Volume (mL) | Conc. added Salv. (μg/μL) | Injection volume (μL) | Injected mass (μg) | Expected Area Increase |
| | | | | | |
| 2.12 | 50.0 | 0.0424 | 20.0 | 0.848 | 537,733 |
| | | | | | |
| | | | | | |
| | Expected Area Increase from Added Salvinorin A | | | | 537,733 |
| | Peak Area from Endogenous Salvinorin A in Leaves | | | | 526,654 |
| | Total Peak Area Expected for Leaves + Addition | | | | 1,064,387 |
| | Peak Area Observed for Leaves + Addition | | | | 1,005,000 |
| | Peak Area Attributed to Addition | | | | 478,346 |
| | Percent Recovery | | | | 89% |
| | | | | | |
| | | | | | |

While the percent recovery value obtained through the above described standard addition experiment seems slightly low (89%), it is believed to be an underestimate of the actual recovery of salvinorin A from the leaf samples. The salvinorin A sample weighed for standard addition was taken from the 18 mg of crystals purified through recrystallization. Several pieces of evidence indicate that these crystals were not 100% pure, and the weighed mass of 2.12 mg salvinorin A for standard addition actually represented a lesser mass of actual salvinorin A. A standard solution of this sample was prepared by weighing 0.50 mg of salvinorin A and dissolving in 1.00 mL of HPLC mobile phase. This solution then was diluted 1:10 in a volumetric flask, and the resultant solution with a concentration of 0.050 mg/mL salvinorin A was transferred into ten Eppendorf microtubes for storage. When 20 μL of this standard solution was evaluated by HPLC analysis, it was expected to produce a peak that approximated the same integrated area as the peak from the previous batch of salvinorin A standard solution, which had a

concentration of 0.051 mg/mL. The new standard solution, however, produced a peak with an area of 550,000 compared to the average value of 665,000 for the previous (0.051 mg/mL) standard solution. This represents only 85% of the expected area for this mass, and indicates that some other impurities may still be present in the salvinorin A used for the percent recovery study. In addition, an HPLC analysis of a sample of the same 18 mg of salvinorin A indicated only 82% - 88% purity by ultraviolet absorbance at 208 nm, with 6 other peaks making up most of the remaining total (Figure 21). This value (82% - 88 %) may not correlate to an equivalent mass percent purity, but it does give an indication of the extent to which traces of other compounds may be present. In conclusion, it seems fair to presume that the actual percent recovery is greater than the 89% calculated by the percent recovery experiment, and may in fact be greater than 97% as indicated by residuals extractions and consideration of impurities in the salvinorin A crystals used for standard addition.

Conclusions:

An effective, rapid separation of salvinorin A has been demonstrated by HPLC on a reverse phase C-18 column using a mobile phase of 45% acetonitrile and 55% water with a 1.00 mL/min flow rate and ultraviolet detection at 208 nm. Accurate quantification of salvinorin A in extracts has been validated through the development of calibration curves correlating HPLC peak area with the injected mass of salvinorin A. Extraction of salvinorin A for quantification can be accomplished for dried leaf tissue by steeping 1.00 g of the powdered leaves in 50 mL of chloroform for 30 minutes, followed by filtration, evaporation of the chloroform and reconstitution of

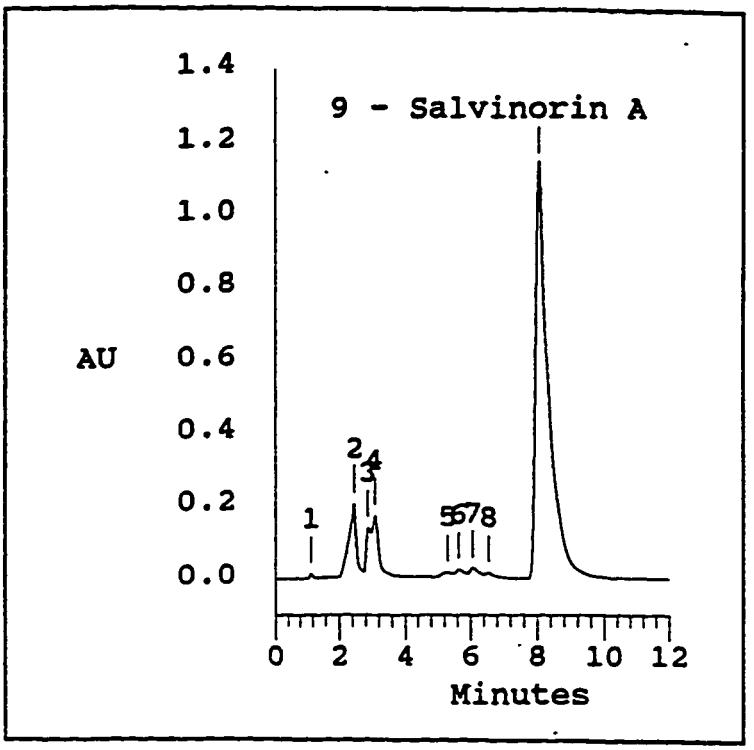



Figure 21. HPLC analysis of a sample of 18 mg of salvinorin A

The chromatogram shows several small peaks in addition to the predominant salvinorin A peak at 8.03 minutes. The peak at 2.87 minutes (peak #3 ) is derived from the acetone in which the salvinorin A was dissolved.

the extract in 50 mL of 80% methanol / 20% acetone. The percent recovery estimates to quantify the extraction of available salvinorin A indicate that greater than 89 % of the total salvinorin A is removed in this manner.