IDENTIFICATION AND QUANTIFICATION USING HPLC-ES/MS OF FOUR PENTACYCLIC OXINDOLE ALKALOIDS IN *UNCARIA TOMENTOSA* WILD TYPE

Presented to the Graduate Council of
Texas State University-San Marcos
in Partial Fulfillment
of the Requirements

for the Degree

Master of SCIENCE

by

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San Marcos, Texas
April 2010

ACKNOWLEDGEMENTS

First I would like to thank the Holy Spirit for allowing me the opportunity to pursue a higher education and improve success in life. I would like to thank all people who have supported and influenced me over the last two years. I especially want to thank my advisor, Dr. Rachell Booth, for allowing me to take on this project and study at Texas State. Her encouragement, patience, and guidance made this project possible. I would also like to thank my committee members Dr. Walter Rudzinski and Dr. Wendi David. I would also like to thank Dr. Medhi Moini for his analytical knowledge and insight of mass spectrometry. His coaching and advice allowed me to acquire the knowledge to operate the electrospray ionization mass spectrometry. Dr. James Irvin deserves special mention for helping me build and put together my first HPLC protocol and analytical instrument. I would also like to acknowledge Dr. Cecilia Chi-ham and graduate student Barbara Blanco for cultivating and harvesting the *Uncaria tomentosa* plant material for me to work with. They were an essential part of this project and I greatly appreciated their hard work and patience. Additionally, I would like to thank the entire Chemistry & Biochemistry faculty and staff at Texas State for creating a learning environment where I could not only re-learn fundamentals and academia of biochemistry, but exceed my level of knowledge. I always appreciated how everyone was so willing to teach and support all students that walked through their doors, and build a companionship between faculty and student allowing science to be understandable and memorable

I would also like to express my gratitude to my lab partners; their company and support made the transition from military life to civilian life a lot easier. I would like to especially thank Jenny Browning, Raquel Ybanez, and Chad Gommert for always being so supportive and willing to lend a helping hand as well as their knowledge and advice. I have truly enjoyed working with you all. Additionally, I would like to thank the other lab members assigned later to our lab and different labs for their social welcomes and laughs. Their cheerful personalities made long hours in the lab fly by.

Lastly, I would like to thank my parents who have supported my decision to join the Military Services and pursue my career the way I see fit. Words cannot express how much your love, support, patience and encouragement has meant to me. Thank you so very much for being the parents whom motivate my dreams.

This manuscript was submitted on April 13, 2010.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

ABSTRACT

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SUPERVISING PROFESSOR: RACHELL E. BOOTH

Uncaria tomentosa of the *Rubiaceae* family is a liana or woody vine that is found in Central and South America, which derives its nickname **"Uña de Gato"** or translated **"Cats Claw"** from its claw shaped thorns that resemble the claws of a cat. These thorns are used for the plant vine to climb and stretch to a height of 30 m. *U. tomentosa* naturally produces a range of secondary metabolites such as alkaloids, triterpenes,

flavonoids, glycosides, and procyanidins. The metabolites in *U. tomentosa* are valuable in the treatment a number of diseases like bursitis, which is the inflammation of bursae (small sacs) of synovial fluid in the body, genital herpes, cancer, allergies, and those people infected with the HIV virus (4, 5). In the current research study we examine the concentrations of metabolites in the roots, leaves, and stems of *U. tomentosa* by establishing a method to accurately quantitate the concentrations of metabolites. In particular, we are interested in identifying and quantifying four principal pharmacologically active pentacyclic oxindole alkaloids: *Mitraphylline, Isomitraphylline, Uncaria C (pteropodine),* and *Uncaria E (isopteropodine.* In order to optimize growth conditions, media with the essential composition of nutrients were established for growth of the wild type. *U. tomentosa* wild type plants were grown in different media including Murashige and Skoog (MSO), ½ Murashige and Skoog (½ MS), Driver and Kuniyuki Walnut (DKW), Woody Plant Medium (WPM), Gamborg B5 medium (B5), Nitsch & Nitsch (N/N), Nitsch & Nitsch Charcoal, Schenk and Hilderbrant (SH), WHITE's, and Homeostatic Soil (HSO) to observe optimal growth conditions. The plants were harvested and sent for side by side analysis of pentacyclic oxindole alkaloid (POA) content. The coupling of High Performance Liquid Chromatography (HPLC) and Electrospray Ionization Mass Spectrometry (ESI/MS) was the analytical method of choice because of its selectivity and sensitivity toward these POA's (12, 13). We were able to compare HPLC-ESI/MS quantitative data on the cultivating properties of each medium used for micropropagation of *U. tomentosa* and found that the ½ MS was

proficient enough to produce efficient plant growth and alkaloid production. Side by side comparison of different growth media has never been reported, thus this work may improve other cultivating procedures in the case of optimizing raw material for further research.

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Uncaria tomentosa of the *Rubiaceae* family (Table 1) is a liana or woody vine that is found in Central and South America, which derives its nickname "Uña de Gato" or translated "Cats Claw" from its claw shaped thorns that resemble the claws of a cat (Figure 1). These thorns are used for the plant vine to climb and stretch to a height of 30 m.

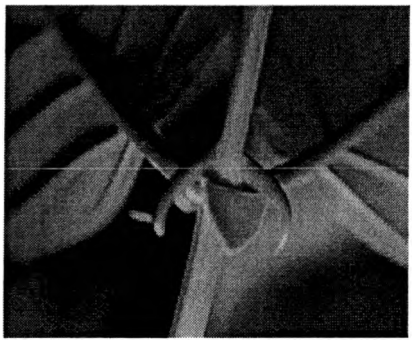


Figure 1: A picture of *Uncaria tomentosa* "Cats Claw"

Table 1: Scientific classification of *U. tomentosa*

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Gentianales
Family:	Rubiaceae
Genus:	*Uncaria*
Species:	U. tomentosa

Cat's Claw or *Uncaria tomentosa* thrives mainly in tropical areas such as the Rain Forest in South America. Its extracts have been used for hundreds, maybe even thousands of years as a medicinal treatment for spiritual and physical ailments by indigenous tribes such as the Ashaninka Indians in the Peruvian rain forest. Our main

focus is on the alkaloids produced by this woody vine. Alkaloids are naturally occurring chemical compounds that are produced by a variety of organisms such as plants, fungi, bacteria, and animals and are referred to as secondary metabolites because they are not directly involved in the growth or development of the plant and are often created by primary metabolite synthases (1, 2, 3).

The Rubiaceae family is commonly called "The Coffee botanical family," and consists of 650 genera and 13,000 species of trees, bushes and woody vines that contain adenine and guanine derived purine alkaloids. Adenine and guanine are two major purines that can be found in the Rubiaceae family. In the plant genus *Uncaria* there are two species of this plant that have provoked interest in pharmaceutical research, *U. guianensis* and the species of interest *U. tomentosa.* Each plant has different properties and medicinal uses that help distinguish between them at the chemical and molecular level. *U. tomentosa's* secondary metabolites are is generally used for immune modulation research and *U. guianensis* for osteoarthritis (4). Overall, *U. tomentosa* displays a diverse range of these bioactive secondary metabolites, including pentacyclic oxindole alkaloids (POA), tetracyclic oxindole alkaloids, triterpenes, flavonoids, glycosides, and procyanidins (1, 2). The largest number of compounds particularly alkaloids and triterpenes have been identified within *Uncaria* species. More than 50 different alkaloid compounds have been characterized and determined from the *Uncaria* genus; 35 of which have been identified in only a couple of other species (4). The leaves, stem, and bark of *U. tomentosa* were found to contain multiple alkaloidal constituents used for medicinal purposes (4). The most commonly known alkaloid is morphine (Fig. 2) an opiate analgesic first isolated in 1805 (3). Caffeine, quinine and febrifuge are also

alkaloid compounds. Analytical capillary electrophoresis and high performance liquid chromatography methods were developed to establish the presence of pentacyclic and tetracyclic oxindole alkaloids in the roots and other parts of the plants.

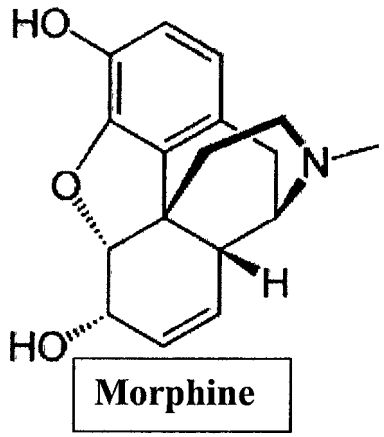
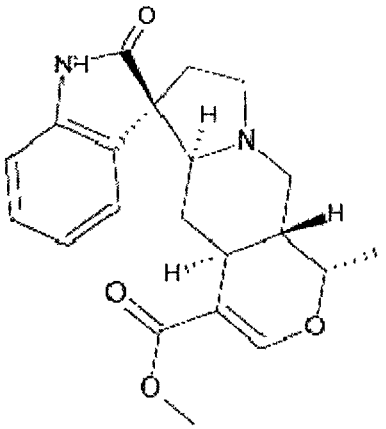
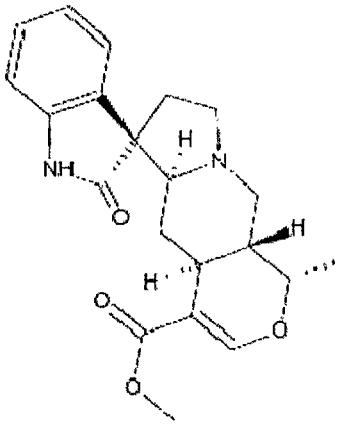


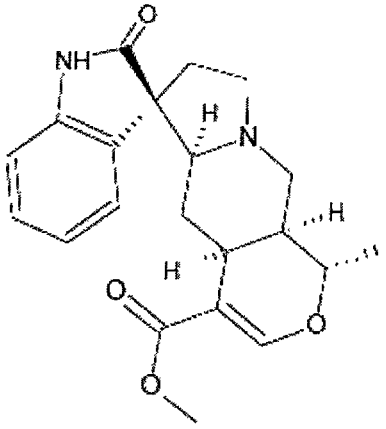
Figure 2: A structural view of morphine.



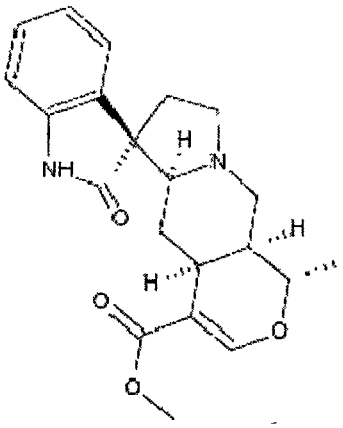
Mitraphylline



Isomitraphylline



Uncarine C
(Pteropodine)



Uncarine E
(Isopteropodine)

Figure 3: Structures of each of the four Pentacyclic Oxindole Alkaloids (POA).

Structures of each of the four pentacyclic oxindole alkaloids (POA) of interest in our study are shown in figure 3. These POAs are considered to be biochemical markers of the plant Cat's Claw and are reliable sources for cytotoxic, immuno-modulatory, anti-

amyloidosis (Alzheimer's disease) and anti-AIDS activities (2, 3, 5, 6, 7). It has been reported that the POA's found in the root of *U. tomentosa* act on the cellular immune system and increase white blood cells, granulocytes, and thus increase the rate of phagocytosis (7, 8) and, in part, help induce the release of a factor from endothelial cells that regulates the proliferation of lymphocytes (7, 9). Table 2 is a breakdown of the pharmacological activity that each POA exhibits on the immune system. Mitraphylline, Isomitraphylline, Uncarine C (pteropodine) and uncarine E (isopteropodine) have all showed immunostimulation by increases the production of normal human resting B and T lymphocytes (5). Uncarine C (pteropodine) and uncarine E (isopteropodine) have shown weak cytotoxicity towards certain cancerous cells as reported by Muhammad (1).

Table 2: Principal Pharmacological Activity POAs

Compound	*Pharmacological Activity*	*References*
Mitraphylline	Immunostimulation, increases the production of normal human resting B and T lymphocytes	Keplinger (5)
Isomitraphylline	Immunostimulation, increases the production of normal human resting B and T lymphocytes	Keplinger (5)
	Immunostimulation, causes a prominent enhancement on phagocytosis	Wagner (8)
Pteropodine (Uncarine C)	Cytoxicity	Muhammad (1)
	Immunostimulation, increases the production of normal human resting B and T lymphocytes	Keplinger (5)
	Immunostimulation, causes a prominent enhancement on phagocytosis	Wagner (8)
Pteropodine (Uncarine E)	Cytoxicity	Muhammad (1)
	Immunostimulation, increases the production of normal human resting B and T lymphocytes	Keplinger (5)
	Immunostimulation, causes a prominent enhancement on phagocytosis	Wagner (8)

Deforestation of the Amazon rain forest is leading to a strain on the availability of alkaloids from plant species such as *Uncaria tomentosa.* Thus, new sub-culture methods that increase the growth rate of root tissue and conserve the germoplasm are desirable for mass production of pharmacologically active plant-derived alkaloids. Transformation to

increase the synthesis of secondary metabolites by hairy roots has been reported (10) and carried out in *U. tomentosa* by Cecilia Chi-Ham and Barbara Blanco of UC Davis. In this process of transformation the bacterium *Agrobacterium* rhizogenes is the vector of choice. This bacterium causes hairy root disease in plants. *U. tomentosa* explants, usually leaves, are wounded using ethanol, surface sterilized with sodium hypochlorite containing Tween, and then inoculated with the bacterium to induce the hairy root disease. When the bacterium infects the plant, the [Transferred] T-DNA between the [Transferred Right] TR and [Transferred Left] TL regions of the [Root inducing] Ri-plasmid in the bacterium is transferred and integrated into the host plant's nuclear genome. The transformation process creates a by-product, hairy root, which will form at the site of infection (10).

There have been a number of *in vitro* studies that have been performed, in plants similar in alkaloid production to 'Cat's Claw', which have been *Agrobacterium rhizogenes*-transformed into hairy root sub-cultures. These sub-cultures have been found to be suitable for the high production of multiple secondary metabolites because of their high productivity and stability in cultural conditions without plant growth regulators (11). The transformed root can increase the production of secondary metabolites and maintain their stability, whereas other plant cell cultures are genetically and biochemically unstable and synthesis often low levels of secondary metabolites (10). Plasmid containing markers are then added in order to label and view the metabolites using fluorescence spectroscopy.

Auxin is a plant growth hormone or phytohormone that increases root growth. The hormone is added at different concentrations in order to optimize the induced growth

of the root without destruction of metabolites and root itself, but to increase the transformation yields and hairy roots biomass. A critical aspect for high production of secondary metabolites in hairy root cultures is optimizing the composition of nutrients for culturing. Addition of auxin and elicitors increases the synthesis of secondary metabolites (10), but an optimal medium has not been established. Below are some photos of the rapid growth effects of auxin induced hairy root *U. tomentosa* leaf explants completed by Cecilia Chi-Ham and Barbara Blanco of UC Davis (Fig 4).

+ Auxin **-Auxin**

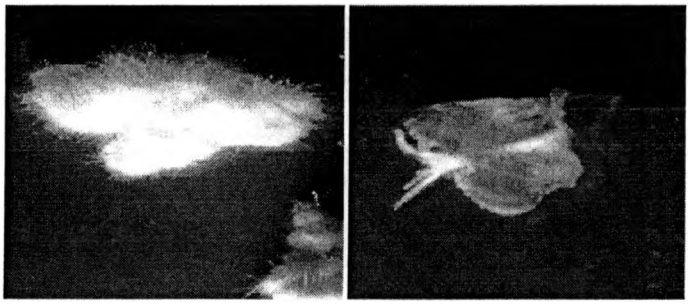


Figure 4: (Left) A *U. tomentosa* leaf explant with auxin, (Right) without auxin.

The healthy transformed roots are then selected, treated and sub-cultured in a media containing an antibiotic to eliminate the bacteria from causing any prolonged harm to the metabolic pathways or plant itself. By increasing the transformation yields hairy roots biomass extracts, from transformed and non-transformed *Uncaria tomentosa* roots can be generated with measurable alkaloid content. In order to optimize growth conditions a medium with the essential composition of nutrients must be established for growth of the wild type. There has not been side by side comparison on what medium optimizes the growth of the *U. tomentosa* wild type. The metabolites in *U. tomentosa* are valuable in the treatment a number of diseases like bursitis, which is the inflammation of

bursae (small sacs) of synovial fluid in the body, genital herpes, cancer, allergies, and those people infected with the HIV virus (4, 5).

U. tomentosa wild type plants were grown in different mediums including Murashige and Skoog (MSO), ½ Murashige and Skoog (½ MS), Driver and Kuniyuki Walnut (DKW), Woody Plant Medium (WPM), Gamborg B5 medium (B5), Nitsch & Nitsch (N/N), Nitsch & Nitsch Charcoal, Schenk and Hilderbrant (SH), WHITE's, and Homeostatic Soil (HSO, harvested and sent for analysis of pentacyclic oxindole alkaloid content. The coupling of High Performance Liquid Chromatography (HPLC) and Electrospray Ionization Mass Spectrometry (ESI/MS) is the analytical method of choice because of its selectivity and sensitivity toward these POA's (12, 13).

One important question is will these new roots maintain the same metabolite properties when nutrients provided in different medias are altered and the plant itself is grown out of its natural environment? The coupled technique (9) HPLC-ESI/MS will be used to separate, detect and quantitate each of the oxindole alkaloids in the *Uncaria* tomentosa extracts. Electrospray ionization mass spectrometry (ESI-MS) can differentiate and identify the pentacyclic oxindole alkaloids. We plan on quantifying and comparing the total alkaloid content in extracts from wild type *Uncaria tomentosa* roots, leaves, and stems, and soon after *U. tomentosa* plants harvested from hydroponic systems and transformed roots with *Agrobacterium rhizogenes,* in the hope to obtain secondary metabolites from each sample.

In the current research study we examine the concentrations of metabolites in the roots, leaves, and stems of *U. tomentosa* by establishing a method to accurately quantitate

the concentrations of metabolites. In particular, we are interested in identifying and quantifying four principal pharmacologically active pentacyclic oxindole alkaloids: *Mitraphylline, Isomitraphylline, Uncaria C (pteropodine),* and *Uncaria E (isopteropodine)* (Fig 3).

CHAPTER II

MATERIALS AND METHODS

HCL, HPLC grade acetonitrile and methanol, ammonium acetate, potassium phosphate-monobasic, potassium hydroxide, and ether were ACS Reagent Grade and were purchased from EMD chemicals (Darmstadt, Germany). HPLC grade water was purified using the MegaPure still system. Polyamide 6, for the removal of tannins in the extraction process was purchased from Sigma –Aldrich (St. Louis, MO). Centrifugation was carried out on a Beckman Coulter J2-21 centrifuge and Savant Speed Vac SC110 was used for concentrating the samples.

Plant Material.

Explants of *U. tomentosa* were provided and prepared by Cecilia Chi-Ham and Barbara Blanco of UC Davis. *U. tomentosa* was micropropagated for three months in different media- Murashige and Skoog (MSO), ½ Murashige and Skoog (½ MS), Driver and Kuniyuki Walnut (DKW), Woody Plant Medium (WPM), Gamborg B5 medium (B5), Nitsch & Nitsch (N/N), Nitsch & Nitsch Charcoal, Schenk and Hilderbrant (SH), WHITE's, and Homeostatic Soil (HSO)-, harvested and freeze dried. Plant specimens

including leaves/stems were harvested on 2 separate occasions, 4 April 2009 and 20 May 2009. Additional leaves and roots were harvested on 28 September 2009.

Pentacyclic Oxindole Alkaloids.

Standards for the four pentacyclic oxindole alkaloids or POA's of interest were purchased in a Cat's Claw Standard kit from Chromadex (Irvine, CA). The kit consisted of 5 mg of each standard including Uncarine C, Uncarine E, Isomitraphylline, and Mitraphylline. The standards were prepared following the analytical test method protocol provided by Chromadex. A standard stock solution containing a mixture of all four alkaloids with a concentration, each approximately 0.05 mg/ml was prepared by dissolving each compound in methanol via sonication in a 100 ml low-actinic glass (LAG) volumetric flask because of light sensitivity. Two standard solutions were then prepared in 10 ml volumetric flasks with concentrations of 25 µg/ml and 5 µg/ml. Two hundred milliliters of diluent was prepared with Methanol: Water (60:40) v/v.

Alkaloid Extraction.

POA extractions were done using a previously reported sample preparation method (2). Briefly, *U. tomentosa* dried plant mass ranged from ~0.10 – 3.0 g depending on growth medium: roots (~0.10-0.20 g), leaves (~0.10-0.70 g), and leaves/stems material (~0.10-3.0 g). Plant material was extracted four times with 3-5 ml of methanol by sonication for 15 min. After each sonication the extracts were centrifuged at ~14,000 x g, supernatant liquid decanted and combined into a 50 ml conical tube, and adjusted to final volume of choice with methanol. One hundred milligrams of polyamide powder was added to the tube for every 1.0 ml of extract. The liquid was then shaken for 2 min,

centrifuged at ~11,000 x g for 5 min, and filtered through a PALL Life Sciences (Ann Arbor, MI) FP-Vericel 0.45 μm membrane filter to remove the polyamide powder.

Chlorophyll Extraction.

Chlorophyll was removed from samples using a method reported by Govindachari et al (14). Plant extracts (200 μl) were mixed with 0.5 M of HCl (200 μl), vortexed for 1 min, and allowed to settle for ~2 min. Ether (400 μl) was added, vortexed for 1 min, and allowed to settle for ~2 min. The ether along with chlorophyll was decanted. Excess ether was removed and sample concentrated in a speed vacuum on high heat for 45 min to ~ 200 μl. Volume was adjusted to ~ 200 μl with methanol if needed.

HPLC-ES/MS.

Extracts were analyzed using an Agilent Technologies (Santa Clara, Ca) 1120 Compact LC consisting of a gradient pump with integrated degasser, Rheodyne 2-position, 6-port sample injection valve (20 μl loop), variable wavelength detector, and EZChrom Elite Compact Software. HPLC was in-line with a Finnigan (Thermo Fischer San Jose, CA) LCQ Classic MS ion trap LC-MS with electrospray (ESI). Alkaloids were separated using a Supelguard guard column SUPELCO (Bellefonte, PA), SUPELCOSIL LC-PAH RP-C18 column (15 cm x 4.6 mm; particle size 5μm) and a mobile phase of methanol:acetonitrile (1:1) as eluent A and 30 mM ammonium acetate (pH 6.6) as eluent B. Note all mobile phase solutions were filtered through a 0.45 μm filter and degassed for 15 min. Elution was performed by means of a linear gradient from 40:60 (A: B) to 70:30 over 60 min at a flow rate of 0.5 ml/min. Samples were monitored at 0.50 AUFS at 245 nm. The HPLC outlet was in-line with the ESI/MS. For analysis of the alkaloids the MS

was operated in positive ion mode, capillary temperature 200 °C, spray voltage 5 kV, and data acquired in MS and MS/MS scanning modes. MS spectrometric and chromatographic total ion current (TIC) range was m/z = 369.0-370.0.

Calibration.

Calibration curves for each of the alkaloids in the stock solution were created over the concentration range of 5-50 µg/ml with three different linearity solution levels and triplicate injections at each level. The average peak heights, anexample of the standard mix at 50 µg/ml (Fig. 5), were then plotted as (Peak height vs. concentration) with a linear fit. The calibration data obtained is shown in Table 3.

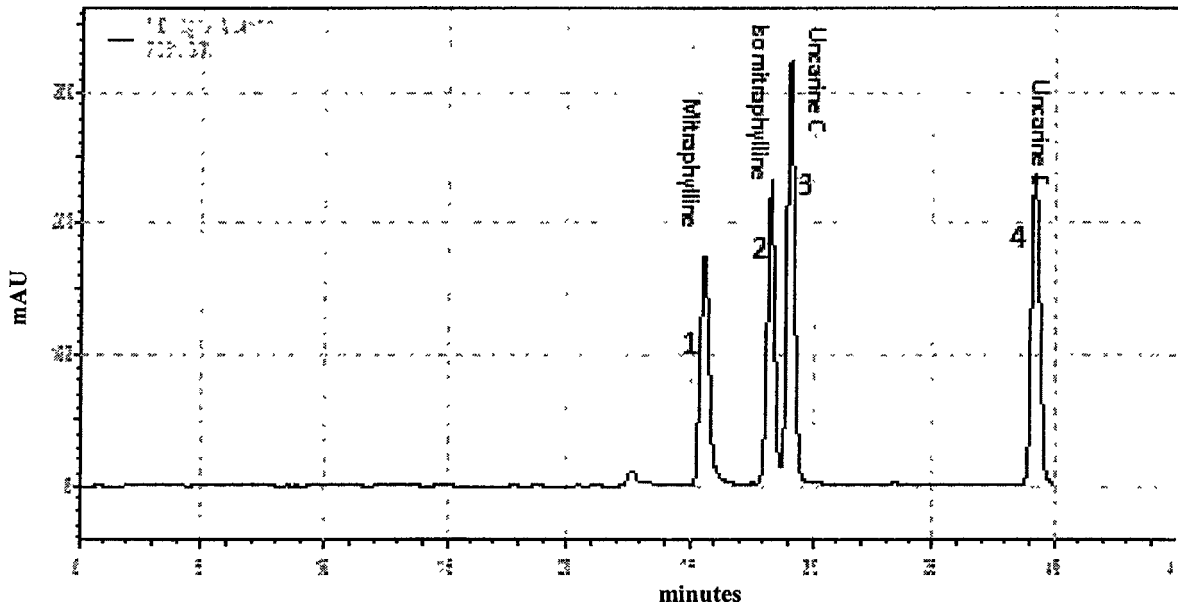


Figure 5 (same as 7a): HPLC Chromatograph of pentacyclic oxindole alkaloid stock standard solution (50 µg/ml conc.) mAU vs. minutes.

Table 3: Calibration data for the four standard Pentacyclic Oxindole Alkaloid compounds.

Calibration data for the four standard Pentacyclic Oxindole Alkaloid compounds		
Compound	**Regression Equation**	**Correlation Coefficient**
Mitraphylline	y = 1400970x	R^2 = 0.997787
Isomitraphylline	y = 1580510x	R^2 = 0.994601
Uncarine C	y = 2104880x	R^2 = 0.994376
Uncarine E	y = 1795090x	R^2 = 0.996738

CHAPTER III

RESULTS AND DISCUSSION

The purpose of the current study was to create a preliminary HPLC-ESI/MS protocol for identifying and quantitating four pentacyclic oxindole alkaloids, mitraphylline, isomitraphylline, uncarine C (pteropodine), uncarine E (isopteropodine) - produced from three month micropropagated *U. tomentosa* progenies. These progenies were grown in various media -- (Murashige and Skoog (MSO), ½ Murashige and Skoog (½ MS), Driver and Kuniyuki Walnut (DKW), Woody Plant Medium (WPM), Gamborg B5 medium (B5), Nitsch & Nitsch (N/N), Nitsch & Nitsch Charcoal, Schenk and Hilderbrant (SH), WHITE's, and Homeostatic Soil (HSO), each containing different plant nutrient compositions. As shown in (Fig. 6) some media produce good root growth and others leaf growth. There has not been a side by side quantitative comparison of alkaloid content produced between the basal media used to cultivate and optimize plant growth of *U. tomentosa.*

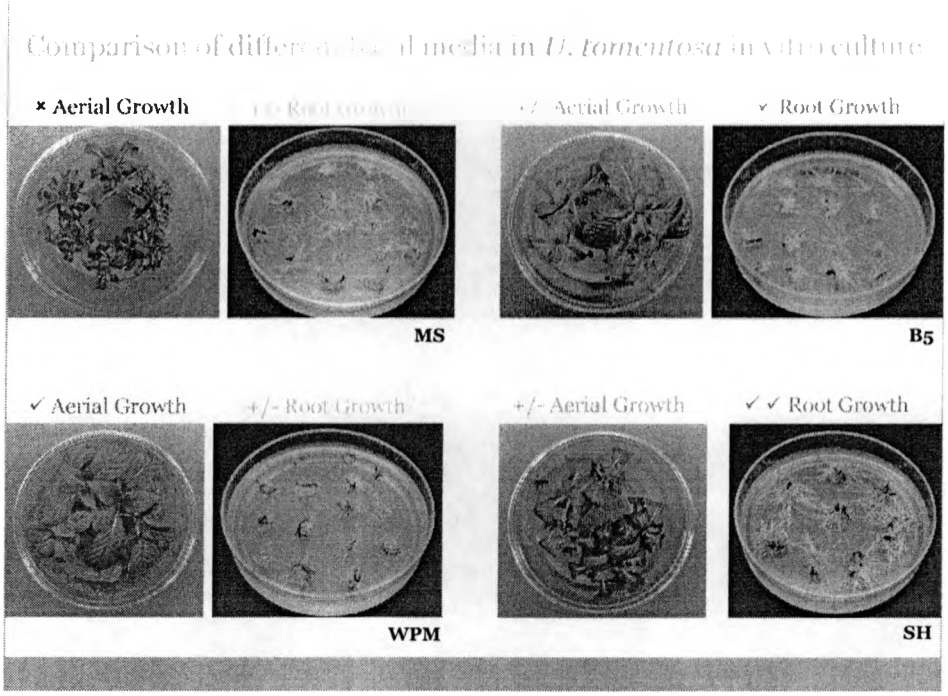


Figure 6: Example of plant growth with different media. Some produce quality root growth and others leaf and stem growth.

Standards for each of the four POA of interest were dissolved into a stock solution with the final concentration of each alkaloid standard approximately 50 µg/ml. Linearity solutions of 25 and 5 µg/ml were used along with the original stock solution for HPLC calibration. Retention times from each POA were used for alkaloid identification. The reason for coupling the HPLC with ESI/MS was for the sensitivity and specificity for the pentacyclic oxindole alkaloids as reported by Verpoorte R, and Niessen WMA. 1994 (13) and Montoro (12). The HPLC-ESI/MS offers the ability to use two parameters of data, retention time and MS information, for support in identificarion and quantification purposes. Quantitative information was obtained using the ratio of the normalization (NL) value - data analysis in which all peaks are reported with heights relative to the

highest peak height of our standards in the ESI/MS chromatograph (Uncarine C) - compared to each alkaloid found in the crude extracts by the NL compared to each alkaloid in the known standard (Table 10).

HPLC-ESI/MS analysis of alkaloids standards.

The HPLC-ESI/MS analysis from Motoro (12) was the basis for our protocol and was altered to fit our system. We found that a pH of 6.6 instead of pH 5 allowed for better separation of our standards. Flow rate was changed from 1 ml/min over a linear gradient of 30 min to 0.5 ml/min with a linear gradient of 60 min for better resolution. HPLC-ESI/MS standard retention times and comparison with literature data was used for the designation of each alkaloid. The four POA's, 50 μg each dissolved in methanol, were injected into the HPLC C18 column. Peaks for each POA including 1.) Mitraphylline, 2.) Isomitraphylline, 3.) Uncarine C (Pteropodine) and 4.) Uncarine E (Isopteropodine) were seen at retention times of 1) ~26.00 min, 2) ~28.00 min , 3) ~30.00 min and 4)~39.00 min respectively (Figure 7A & 7B). ESI/MS was operated in positive ion mode, with each ion identified as $[M + H]^+$ (Fig. 8A & 8B). An *m/z* scan 369-370 was used to exclude all non-relevant peaks. Twenty microliters of each POA at various concentrations were analyzed and used to identify peaks for future experiments (Fig. 7, 8, 10A-B).

Detection of the 5 μg/ml standard dilution alkaloid chromatograph was best fit for designating the alkaloids in the crude extracts because low concentration provided a factual reference of detection comparable to the minimal amount of POA concentration in

the actual injected extract (Fig. 8). Efficient separation of the alkaloid standards using gradient elution is evident in the chromatographs.

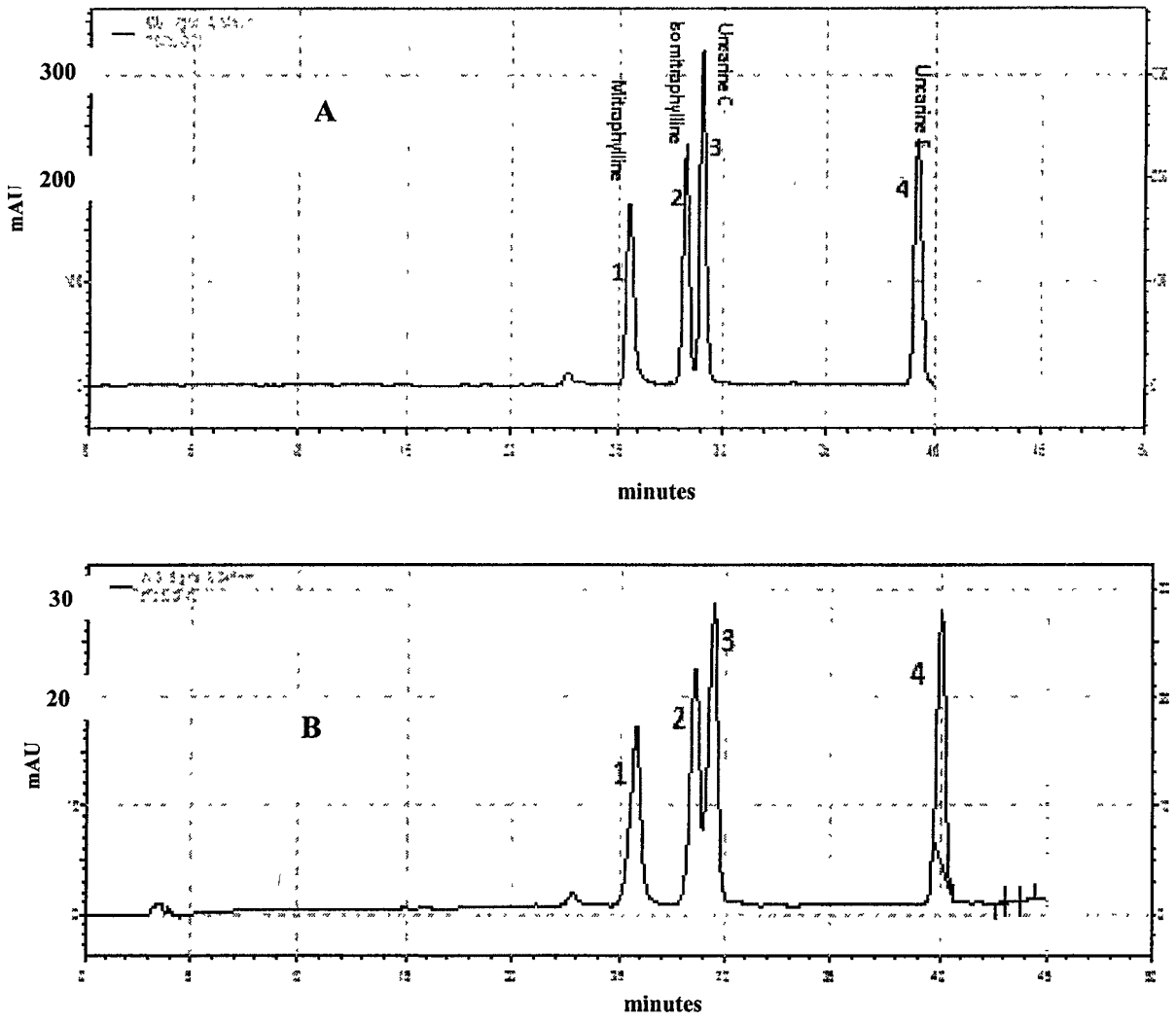


Figure 7: HPLC chromatographs of 50 and 5 µg/ml standard mix. Flowrate 0.5 ml/min. Figure 7A is 50 µg/ml and 7B is 5 µg/ml. Figure 7B is used for peak identification for HPLC extract analysis . From left to right compounds elute in this order: 1.) Mitraphylline, 2.) Isomitraphylline, 3.) Uncarine C (Pteropodine), 4.) Uncarine E (Isopteropodine)) were seen at retention times of 1) ~26.00 min, 2) ~28.00 min , 3) ~30.00 min and 4)~39.00 min respectively. The Uncarine D (Speciophylline) peak could not be accounted for in our data because the standard was not included in the kit. As reported in the literature uncarine D would elute ~ 1 min before mitraphylline.

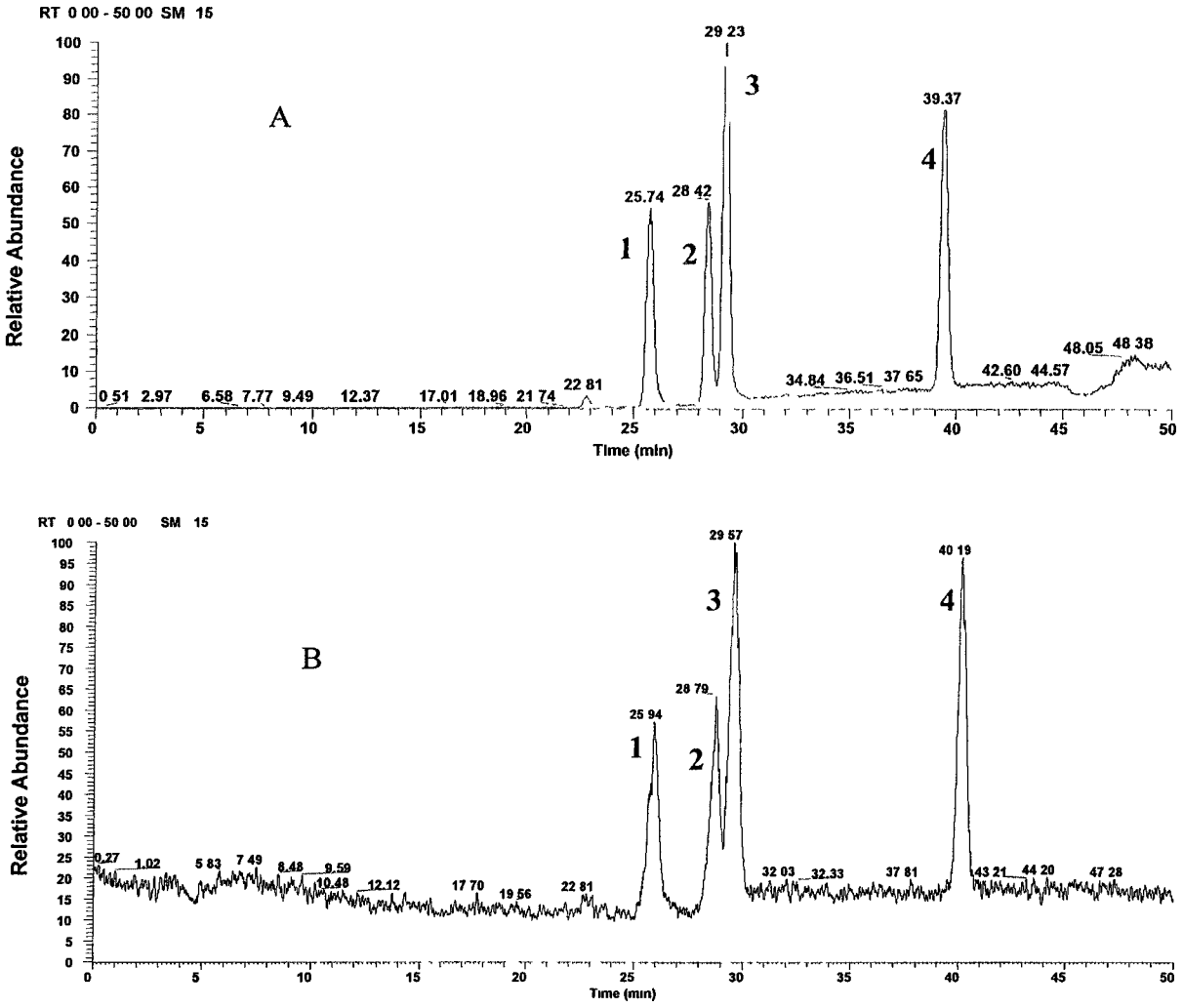


Figure 8: ESI/MS chromatographs of 50 and 5 µg/ml standard mix. Figure 8A is 50 µg/ml and Figure 8B is 5 µg/ml. Figure 8B is used for peak identification for ES/MS extract analysis. From left to right compounds elute in this order: 1.) Mitraphylline, 2.) Isomitraphylline, 3.) Uncarine C (Pteropodine), 4.) Uncarine E (Isopteropodine)) were seen at retention times of 1) ~25.00 min, 2) ~28.00 min , 3) ~30.00 min and 4)~39.00 min respectively. Normalization values for the ESI/MS A) 3.21E+07 and B) 3.01E+06. (Table 4).

Effects of Chlorophyll Extraction Step

The addition of a chlorophyll extraction step was to help optimize the extraction process further, and isolate the pentacyclic oxindole alkaloids, clean up background noise

without the aid of excessive dilution, and help prolong the life of the guard column in addition to the C-18 column. In previous runs 100 µl of the crude extract had to be concentrated down to a 20 µl sample because the amount of POA content present was minimal (Fig. 9A). The smaller dilutions were harder to see because there was too much background to distinguish peaks (data not shown). The series of extract runs, without the addition of the guard column and after an hour of washing, dirtied the C18 column preventing good resolution and detection of the POA standards and shortening the life of the column (Fig. 9B).

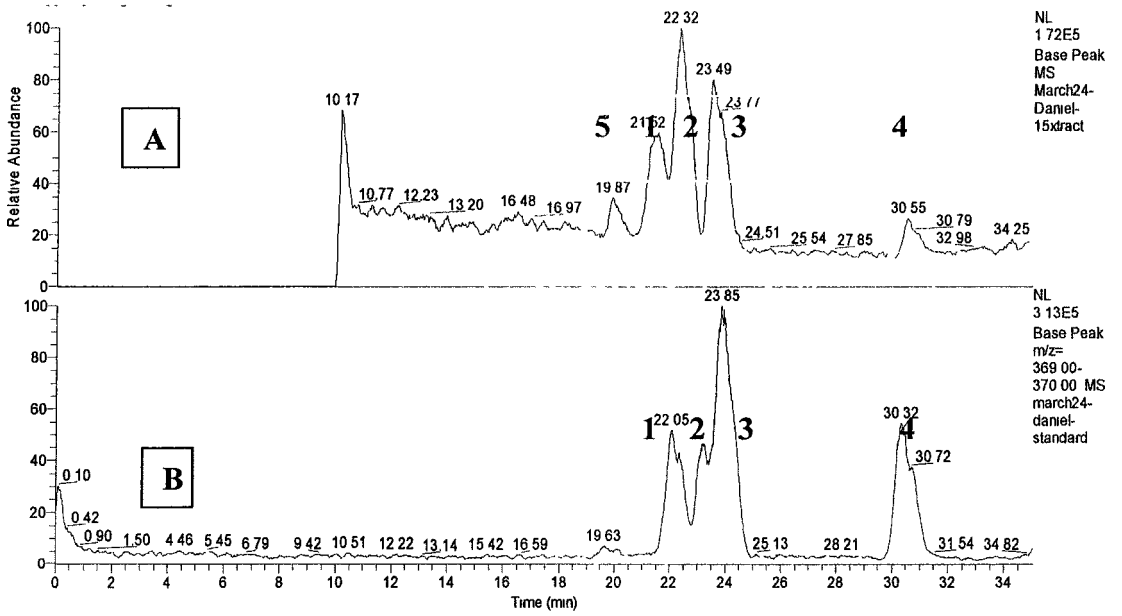


Figure 9: Preliminary chromatographs from previous runs for optimizing HPLC-ESI/MS. 100 µl of crude extract was concentrated down to increase alkaloid content for detection in a 20 µl injection of extract (A) and run against the 5 µg/ml stock standard solution on the same column after an hour of washing (B). From left to right compounds elute in this order: 1.) Mitraphylline, 2.) Isomitraphylline, 3.) Uncarine C (Pteropodine), 4.) Uncarine E (Isopteropodine).

Again the use of coupled HPLC-ESI/MS was chosen for this overall study because of the sensitivity and specificity for the pentacyclic oxindole alkaloids. The column, once dirtied, decreased the sensitivity and specificity of the system, thus, the

addition of a guard column and chlorophyll extraction. The chlorophyll extraction step was of concern, because it had not been used on alkaloids from Cat's Claw. To ensure that the addition and removal of HCL and ether in this step would not disrupt the alkaloids in the process 20 µl of, 5, 25, 50 µg/ml stock STDs were injected and analyzed for reference and sensitivity purposes. Then 200 µl of 50 µg/ml stock STD was put through the chlorophyll extraction process and marked as CONTROL. Reviewing the chromatographs (Fig. 10A) of the standards the alkaloids elute as mentioned before- From left to right 1.) Mitraphylline, 2.) Isomitraphylline, 3.) Uncarine C (Pteropodine), 4.) Uncarine E (Isopteropodine). As seen, the chromatograph of the 20 µl injection of the CONTROL (Fig. 10B), the chlorophyll process doesn't affect the pure stock STD and good peak resolution in both the HPLC and ES/MS chromatographs was maintained with elution of all four POAs. The peak height and normalization (NL) value of each POA standard in both HPLC-ESI/MS chromatographs (Fig. 10a) has decreased relative to the concentration of the dilution compared to the 50 µg/ml chromatograph (Fig. 10C and Table 4). The peaks and normalization (NL) value of the CONTROL was reduced by ~20 % due to the dilution by the chlorophyll process. A sample of WPM plant extract was spiked with 100 µl of 50 µg/ml STD and then subjected to the chlorophyll extraction process and marked as SPCONTROL to verify that neither the chlorophyll extraction process, nor components of the crude extract, would prohibit the detection of the alkaloids after chlorophyll extraction of the spiked extract (SPCONTROL). The HPLC chromatograph displayed decent peak resolution of all four POAs (Fig. 10B). However, the HPLC standard peak heights and ESI/MS normalization (NL) values decreased by

~75 percent when compared to the pure 50 μg/ml standard and less than half when compared to the CONTROL except for uncarine C (pteropodine) (Fig. 10C).

The uncarine C has a more intense peak in the HPLC chromatograph of SPCONTROL when compared to its' ESI/MS chromatograph. This could be caused by an unknown compound in the crude plant extract absorbing at 245 nm at the same retention time as uncarine C around 30.00 min. This compound must have a different molecular weight to not be detected by ESI/MS scan of *m/z* ratio 369 -370 because the normalization (NL) of 3) uncarine C doesn't correspond with the intensity of its HPLC peak. Overall the chlorophyll extraction process diluted the alkaloids but failed to impart any real harm to the alkaloids or their detections. Also there was no need to concentrate 100 μl of crude extract, as in the preliminary runs (Fig 9A), after the chlorophyll extraction process to detect alkaloid peaks beyond the background noise. Twenty microliters were taken directly from the crude extract and put through the process for a cleaner sample and injected for analysis.

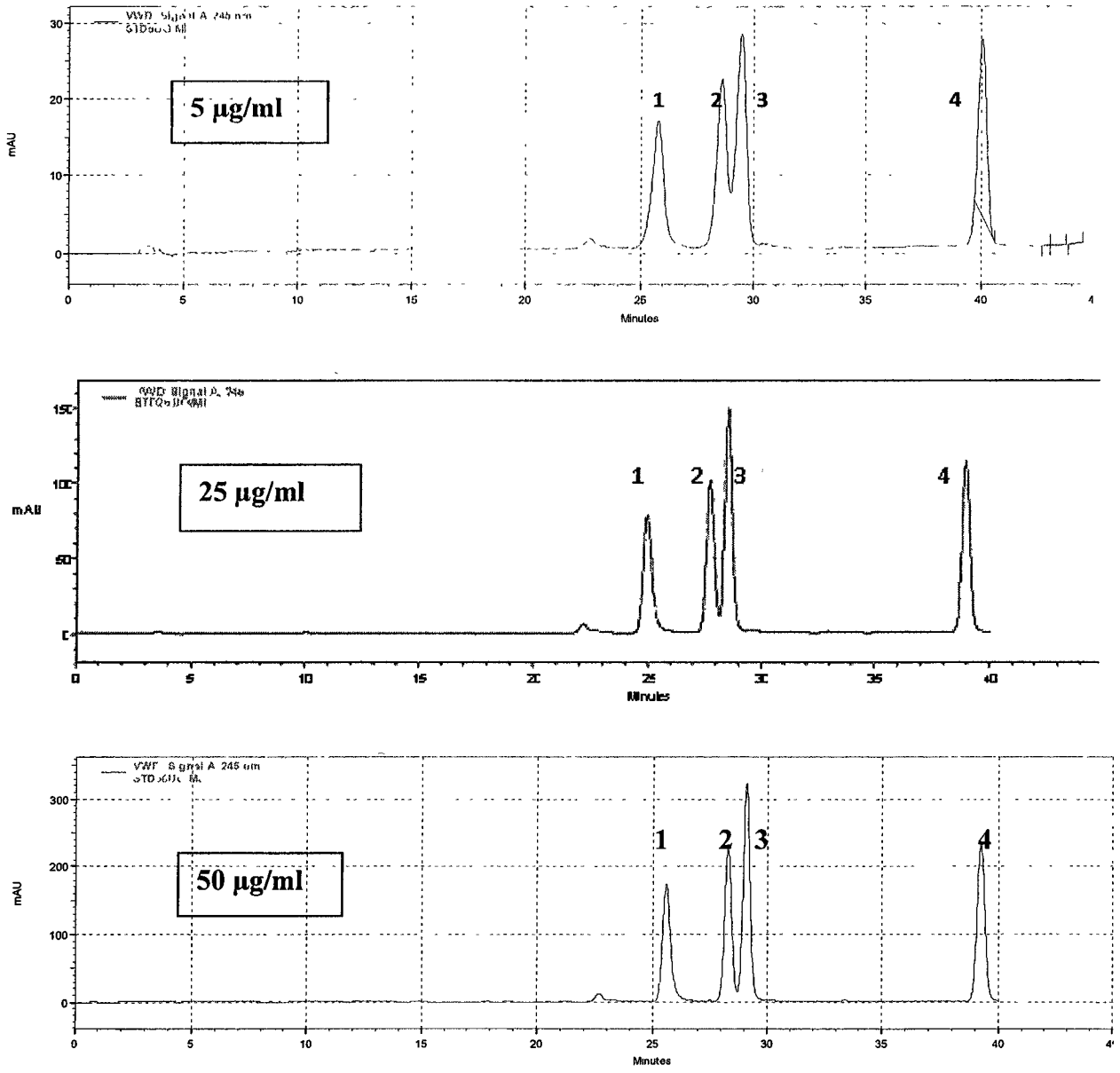


Figure 10A: HPLC chromatographs of stock standard containing all POAs. The stock STD in the dilutions of 5, 25, & 50 µg/ml were ran first to obtain references for detection and peak identification purposes. From left to right compounds elute in this order: 1.) Mitraphylline, 2.) Isomitraphylline, 3.) Uncarine C (Pteropodine), 4.) Uncarine E (Isopteropodine) were seen at retention times of 1) ~26.00 min, 2) ~28.00 min , 3) ~30.00 min and 4)~39.00 min respectively.

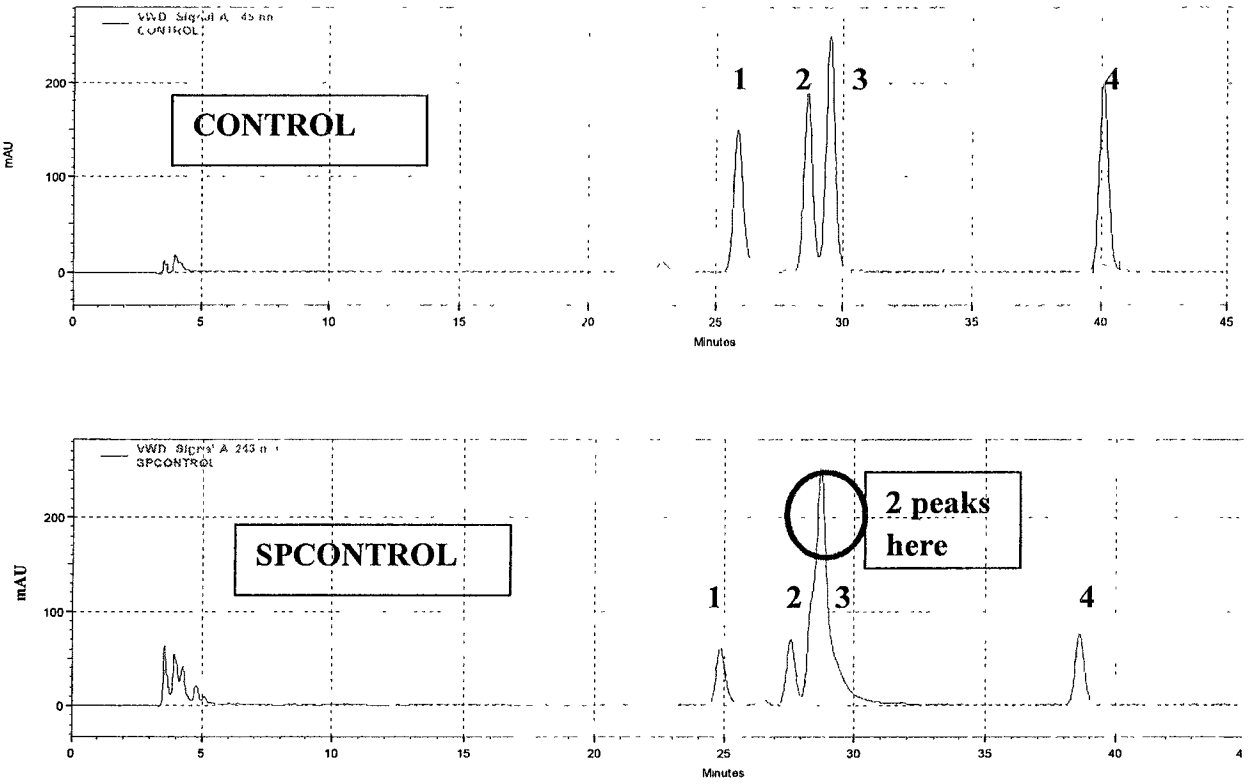


Figure 10B: HPLC chromatograh of chlorophyll extraction collaboration. 50 µg/ml stock STD was put through the chlorophyll extraction and marked as CONTROL. A sample of WPM plant extract was spiked with 100 µl of 50 µg/ml STD, and also put through the chlorophyll process and marked as SPCONTROL. The CONTROL chromatograph, chlorophyll process doesn't affect the pure stock STD and good peak resolutions were still obtained. The SPCONTROL, contents in the sample, along with the chlorophyll step, did not prevent the detection of the alkaloids. Peaks were smaller ~ 50 % in SPCONTROL compared to CONTROL due to sample matrix effects in the solution. The increase uncarine C peak in the HPLC chromatograph is due to some unknown in the crude extract also absorbing at 245 nm.

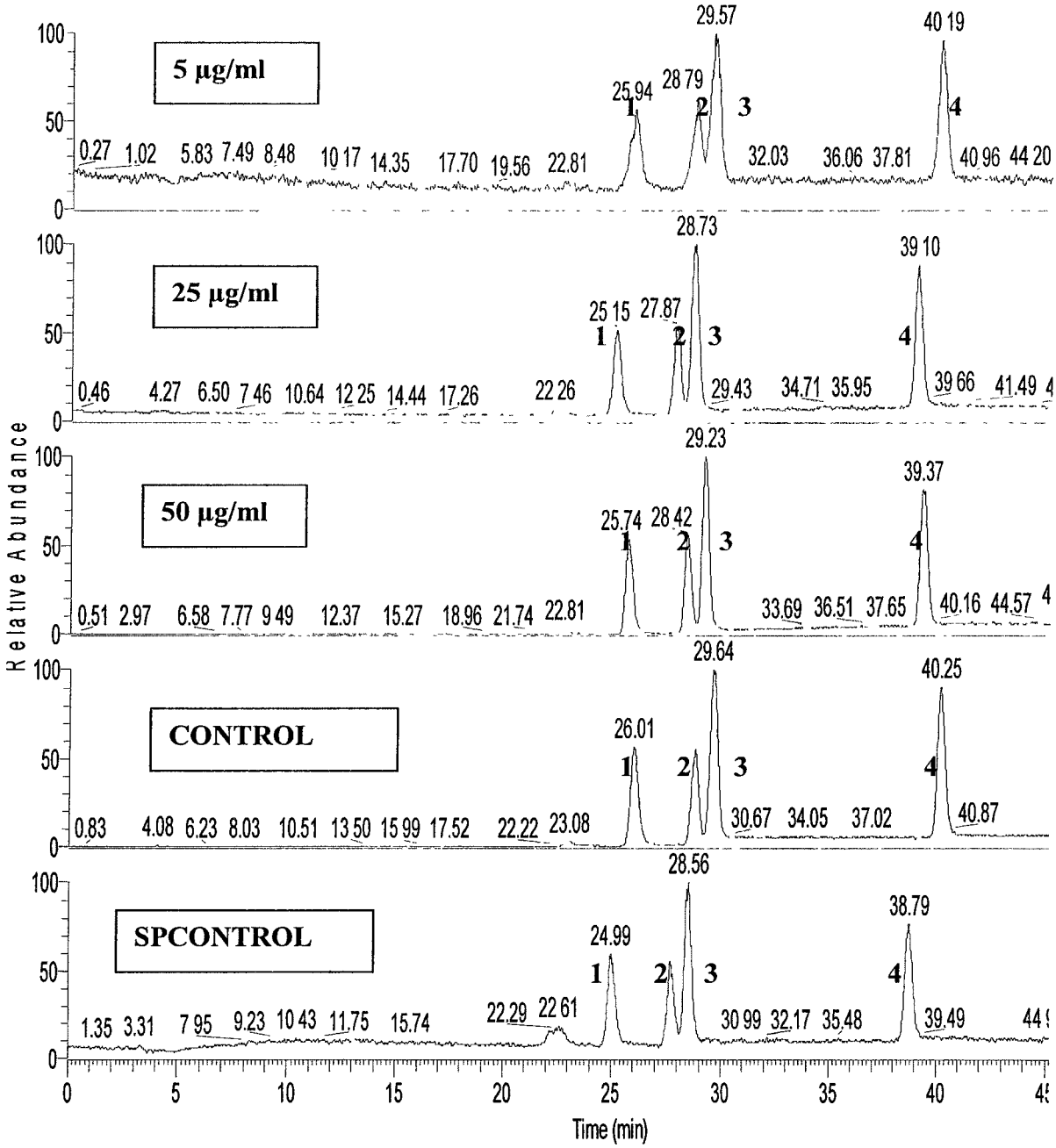


Figure 10C: ESI/MS chromatographs of stock standard containing all POAs, CONTROL, and SPCONTROL. The stock STD in the dilutions of 5, 25, & 50 µg/ml were run first to obtain references for detection and peak identification purposes. From left to right compounds elute in this order: 1.) Mitraphylline, 2.) Isomitraphylline, 3.) Uncarine C (Pteropodine), 4.) Uncarine E (Isopteropodine) were seen at retention times of 1) ~25.00 min, 2) ~28.00 min , 3) ~29.00 min and 4) ~39.00 min respectively. CONTROL - 50 µg/ml stock STD was put through the chlorophyll extraction. SPCONTROL - WPM plant extract was spiked with 100 µl of 50 µg/ml STD, and also put through the chlorophyll process.

Table 4: Normalization (NL) values from ESI/MS chlorophyll extraction process

Chlorophyll Extraction Process	
	Normalization (NL)
5 µg/ml	3.01E+06
25 µg/ml	1.46E+07
50 µg/ml	3.21E+07
CONTROL	2.54E+07
SPCONTROL	1.05E+07

Analysis of *U. tomentosa* Leaves/Stems

Crude extracts made from a combination of leaves and stems grown in different media - including B5, DKW, HSO, MSO, N/N, N/N Charcoal and WPM were analyzed using HPLC-ESI/MS. Each extract was treated to remove chlorophyll and then 20 µl was injected into the HPLC-ESI/MS system for analysis. The detection wavelength for the HPLC was set at 245 nm and ESI/MS mass was set at *m/z* =369. Extracts corresponding to the different media, generated peaks similar to each other on the HPLC –ESI/MS chromatographs. The difference in peak intensity in the HPLC chromatographs is determined by the amount of plant material produced by the growth media. A better development and yield of plant material naturally would allow for a better chance of alkaloid and secondary metabolite production. The plant extract yield can be reviewed further in Table 10 of the quantitative analysis section but is as follows in Table 5.

Table 5: The weight of plant material for leaves and stems.

4-April-2009 Extracts (plant material) 6-Dec-09 run	
Name	***WT of plant material***
WPM	~0.09g
MSO	~0.5g
HSO	~0.10g
B5	~0.14g
N/N	~0.14g
DKW	~0.05g
5-20-2009 Extracts (plant material) 20-Dec-09 run	
Name	***WT of plant material***
N/N charcoal	~0.11g
MSO	~3.26g
WPM	~0.16g
B5	~0.10g

Alkaloid content was later quantified using ESI/MS (Table 10-12). The chromatograph of each extract analyzed (Fig. 11A & 12A) generated small, less intense peaks within the time range of ~ 20- 27 min where some of the standard POAs also elute. However, alkaloid content in this range can be considered minimal because the peaks are low. The HPLC chromatographs of the standards were used to identify the large peak in the retention time range of ~ 28- 31 min in the HPLC chromatographs for each media as uncarine C or a combination of isomitraphylline and uncarine C. An additional peak in the retention time of ~ 28-30 min was detected in several samples including B5, MSO, DKW, N/N Charcoal, N/N leaves, WPM of both April and May harvest of leaves and stems (Fig. 11A & 12A).

Since this identification was based only on retention time collaborating ESI/MS results were obtained to confirm identification. The ESI/MS chromatographs of each

sample revealed POAs with increased sensitivity and resolution, which we used to designate and quantify the peaks. The ESI/MS scan parameters of *m/z* 369-370 detected no large peak correlating with the retention time of ~ 28 -30 min on the HPLC chromatograph in any of the extracts produced from the separate media. As seen in the previous section explaining the effects of the chlorophyll extraction process, there is still separation in the spiked control of the alkaloids but an increased peak in uncarine C in the HPLC chromatograph. Using the data from the chlorophyll extraction process analysis (Fig. 10B & 10C) we can assume that there is a compound in the crude extract interfering with the absorption at the retention time ~ 28 -30 min. The ESI/MS chromatograph can be used now to further examine this peak.

The ESI/MS standard chromatographs (Fig. 11B & 12B) were reliable enough to distinguish and identify peaks from individual POAS. Setting the *m/z* = 369-370 focuses the range of detection and removes compound and noise not within the mass range. All peaks, within the time range that correlates with small peaks observed in the HPLC chromatographs ~20 -27 min, are seen in each plant extract analyzed by the ESI/MS chromatographs. Peaks not of the mass range of *m/z* = 369 are removed suggesting the amount of alkaloid present was too small for the HPLC to accurately detect. In addition, a fairly stronger peak of an unknown alkaloid eluted before the retention time of mitraphylline. This can be identified as 5) uncarine D (speciophylline), which has a molecular weight of ~ 368 and absorbs at 245 nm, as reported previously (2, 9, 11). Reviewing the normalization (NL) values from the ESI/MS (Table 6) the MSO May harvest increased ~50 folds compared to April's harvest. Reason for this increase is

understood because the yield of MSO plant material has also increased (Table 5) providing more alkaloid content.

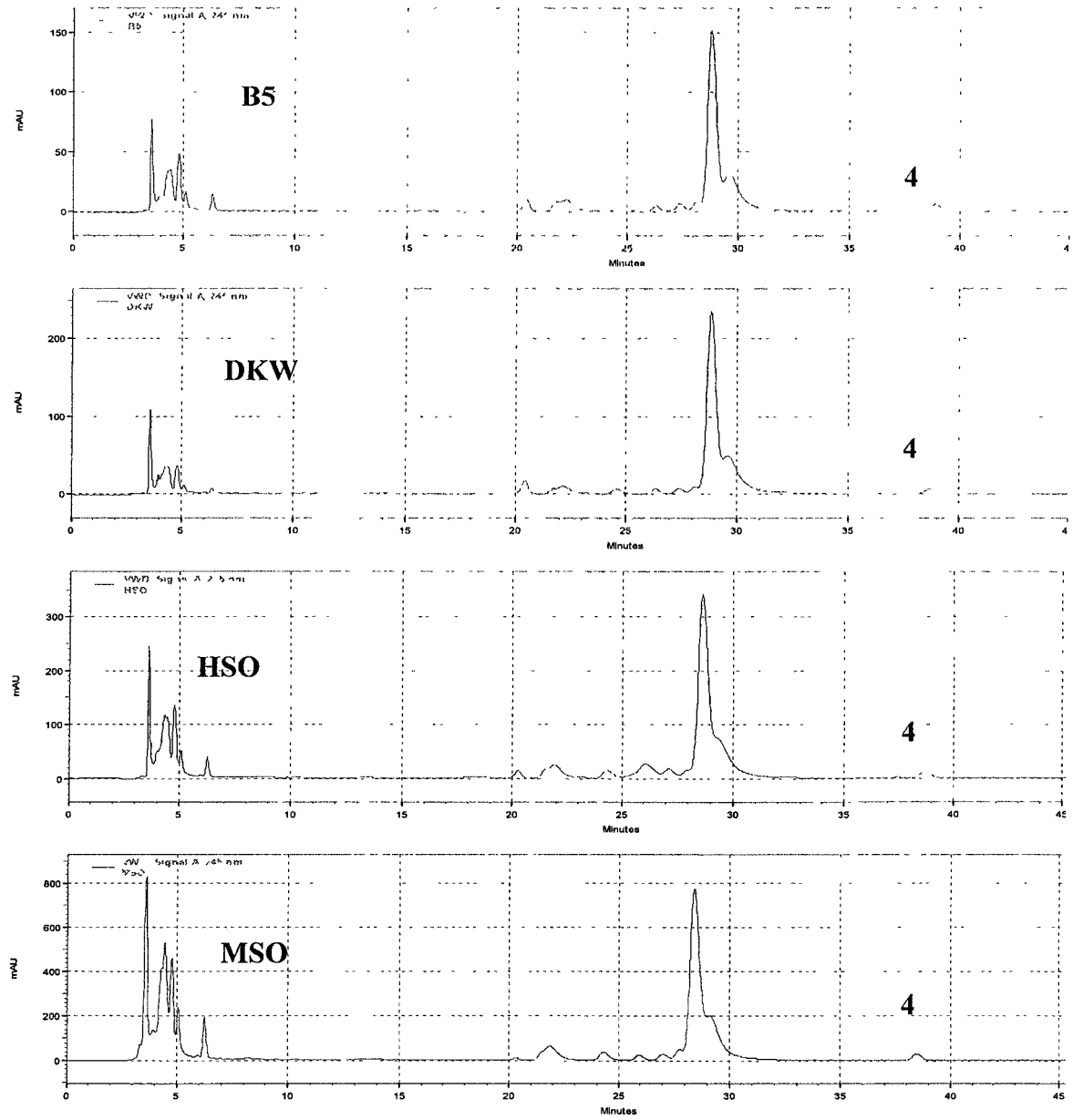


Figure 11A: HPLC chromatographs of crude extracts from April 2009 combination of *U. tomentosa* leaves and stems

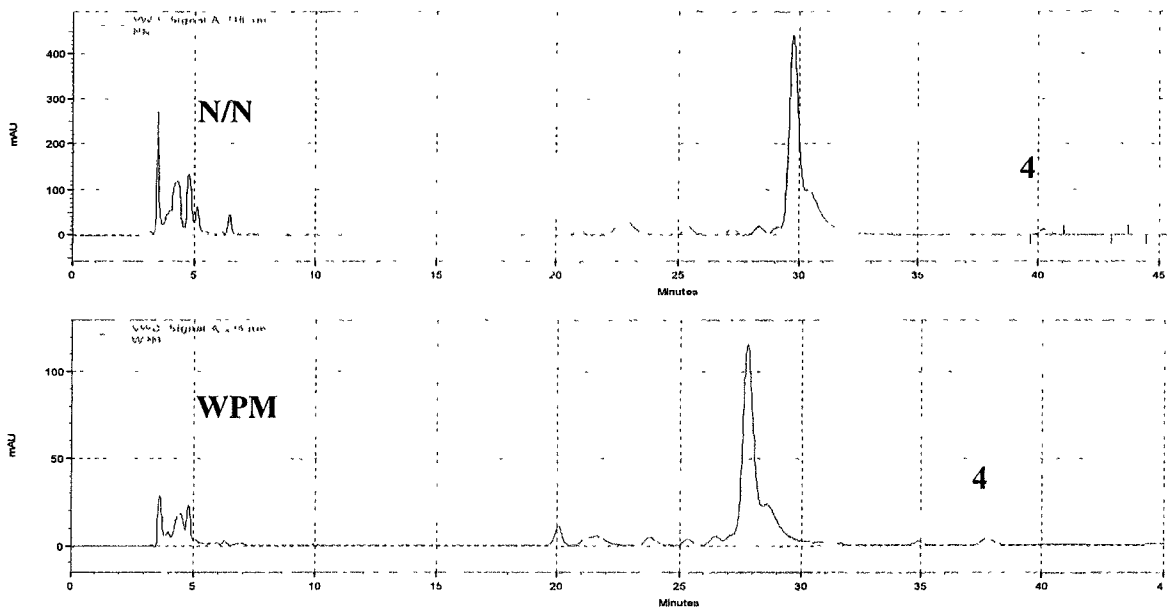


Figure 11A Continued: HPLC chromatographs of crude extracts from April 2009 combination of *U. tomentosa* leaves and stems. Plant was grown in media: B5, DKW, HSO, MSO, N/N, and WPM. Time shift ~ 1 min in some chromatographs: crude extract WPM is due to pressure interference from ESI/MS. Similar small peaks are noticeable in the time range of ~ 20- 27 min and ~39 - 40 min but concentration of compounds are too low for peak confirmation as alkaloids. Large peak at ~28-29 min is conjoined two peaks Data from the chlorophyll extraction process analysis (Fig. 10) can be used to assume that there is an unknown compound also absorbing at 245 nm; Small peak in the region of ~39 - 40 min maybe 4) uncarine E (isopteropodine) but concentration of compounds is too low for peak confirmation of alkaloids.

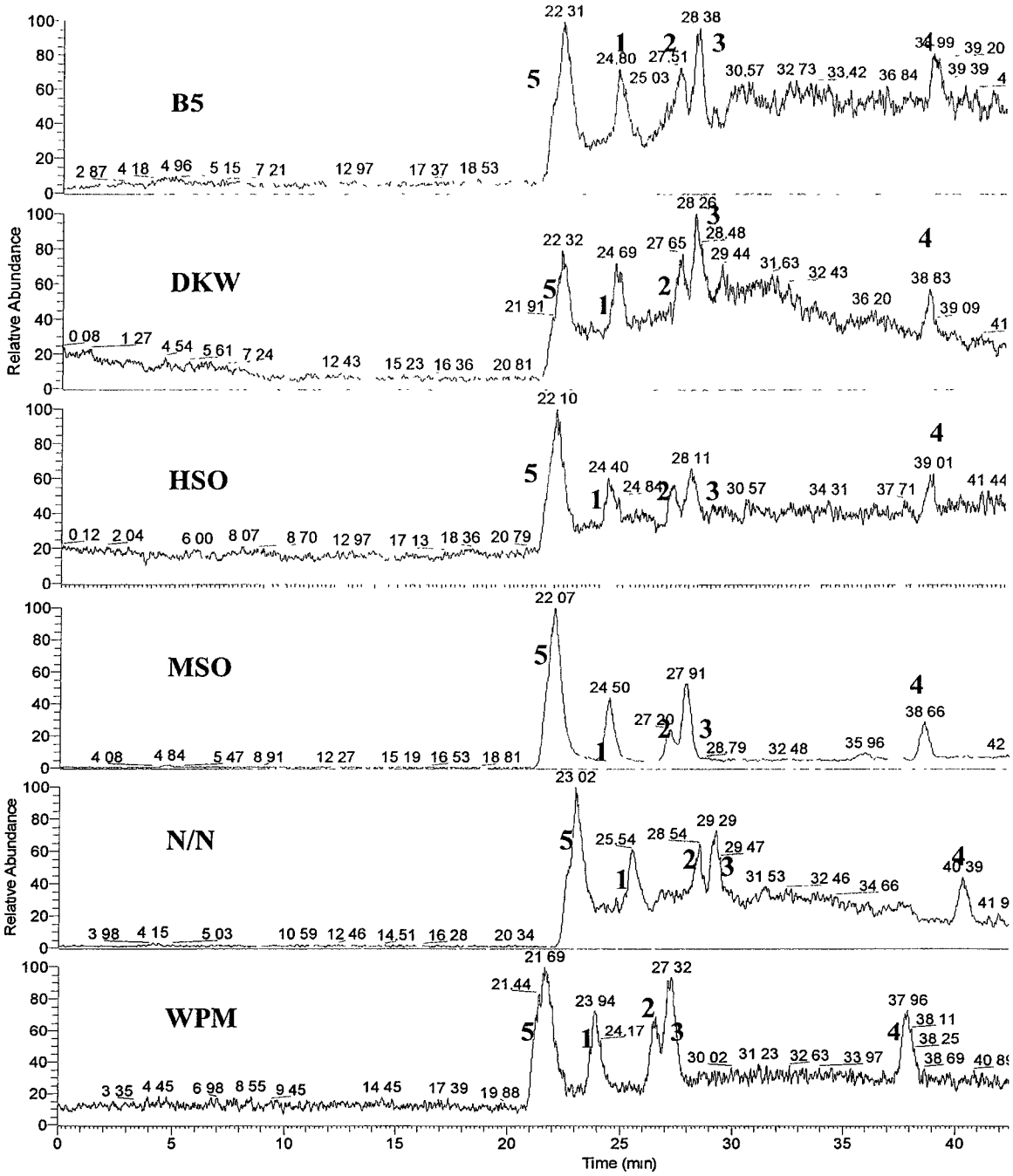


Figure 11B: ESI/MS chromatographs of extracts from April 2009 combination of *U. tomentosa* leaves and stems. Plant was grown in media: B5, DKW, HSO, MSO, N/N, and WPM. The molecular weight of the POA's is ~ 368. The peak sensitivity is enhanced through the ESI/MS ion trap $[M+H]^+$ $m/z = 369$ and reveals the smaller peaks to contain the four POA's 1.) Mitraphylline, 2.) Isomitraphylline, 3.) Uncarine C(Pteropodine), 4.) Uncarine E (Isopteropodine) as defined in the ESI/MS chromatographs of the alkaloid standards and 5.) Uncarine D (Speciophylline) as defined in the literature. The ESI/MS uncovers no large peak correlating with the retention time of ~ 28 -31 min on the HPLC chromatograph suggesting an unknown in the crude extract that is also absorbing at 245 nm in the retention time of uncarine C (pteropodine).

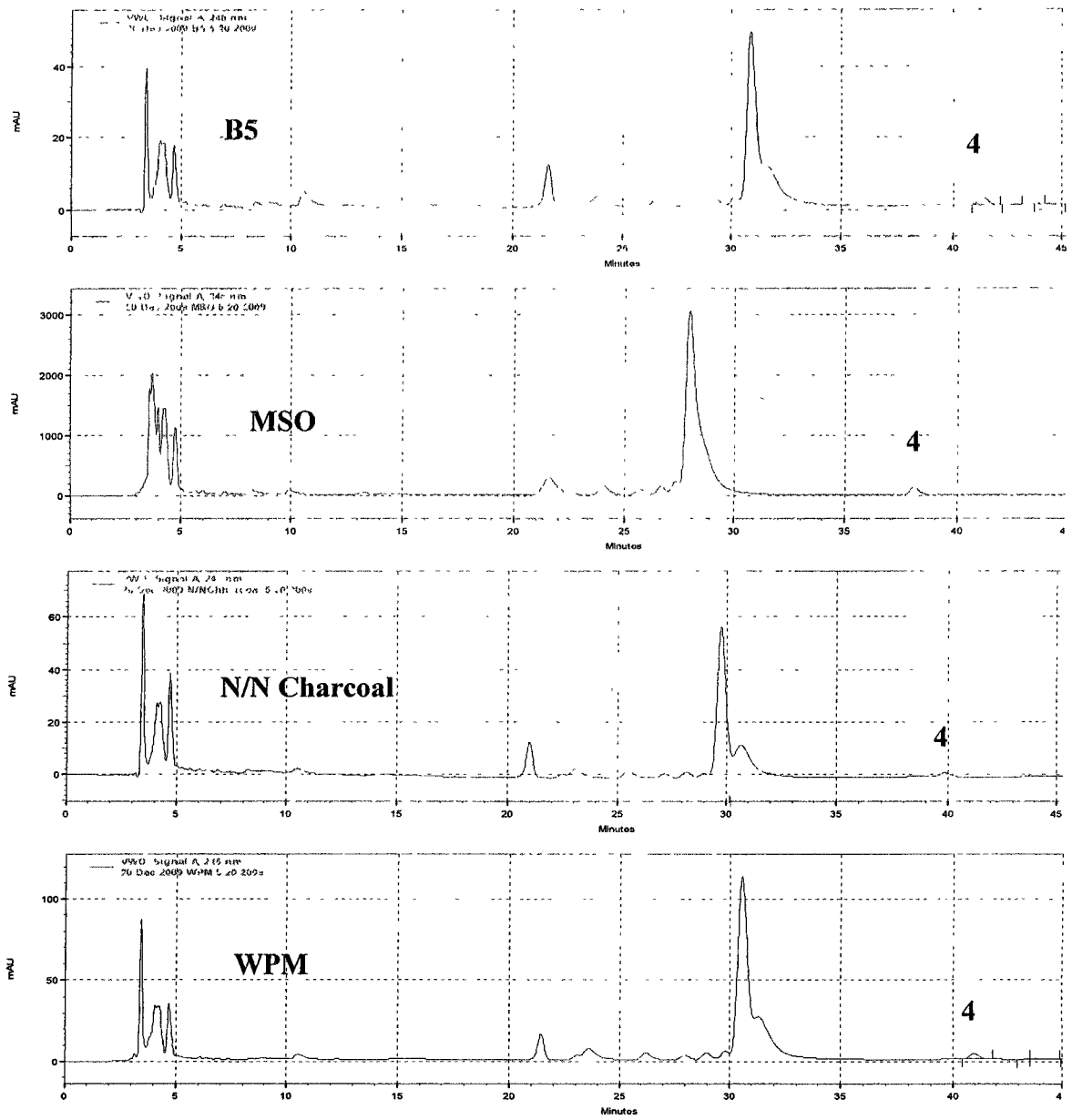


Figure 12A: HPLC chromatographs of crude extracts harvested in May 2009 from combination of *U. tomentosa* leaves and stems. Plant was grown in media: B5, MSO, N/N Charcoal, and WPM. Time shift ~ 1 min in some chromatographs: crude extract WPM and MSO is due to pressure interference from ESI/MS. Similar small peaks are noticeable in the time range of ~ 20- 27 min and ~ 39 -40 min in each crude extract but the concentration of compounds are too low for peak confirmation of alkaloids. Large peak at ~28-29 min is conjoined two peaks Data from the chlorophyll extraction process analysis (Fig. 10) can be used to assume that there is an unknown compound also absorbing at 245 nm; Small peak in the region of ~39 - 40 min maybe 4) uncarine E (isopteropodine) but concentration of compounds is too low for peak confirmation of alkaloids

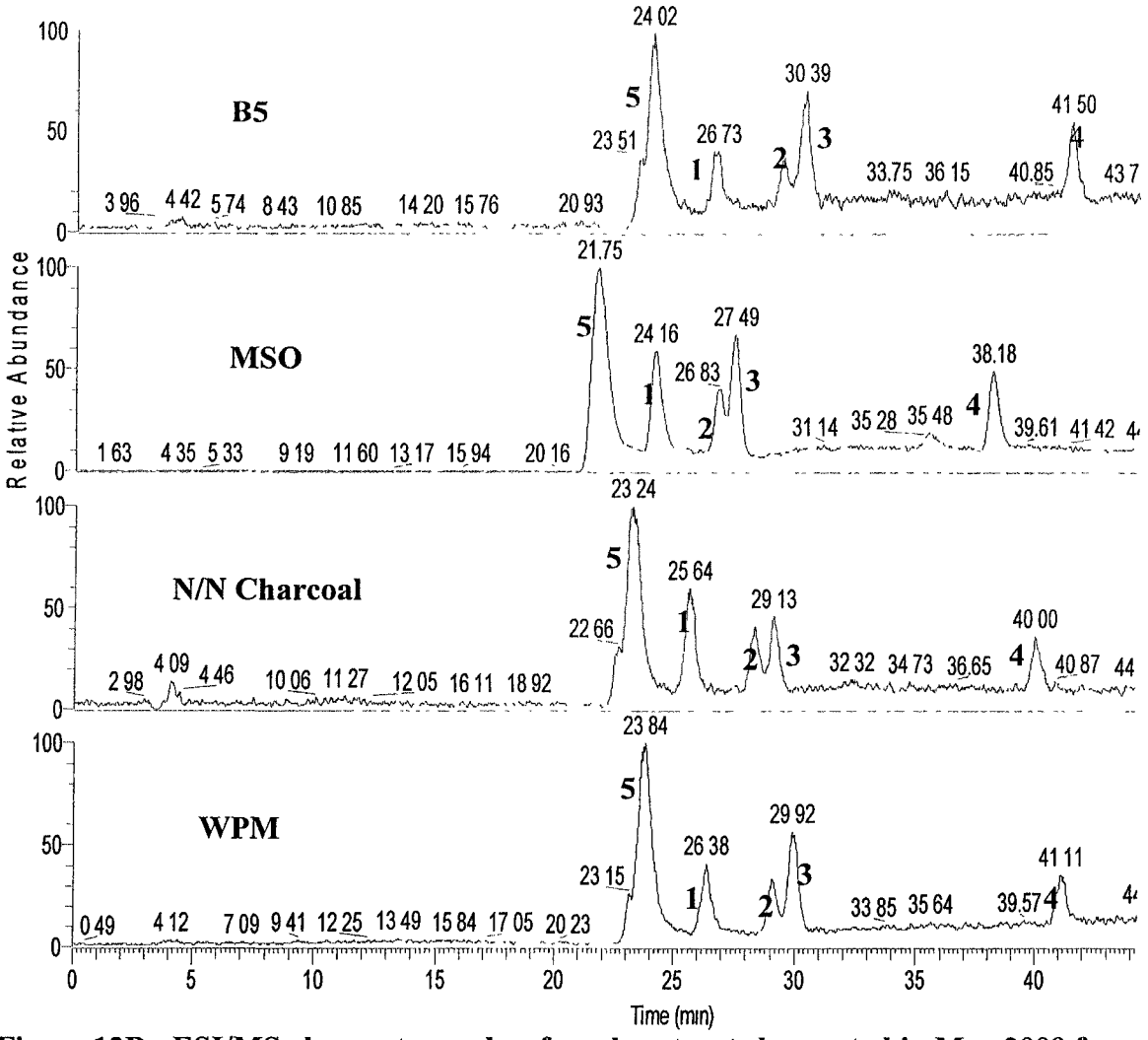


Figure 12B: ESI/MS chromatographs of crude extracts harvested in May 2009 from combination of *U. tomentosa* leaves and stems. Plants were grown in media: B5, MSO, N/N Charcoal, and WPM. Again, the molecular weight of the POA's is ~ 368. The peak sensitivity is enhanced through the ESI/MS ion trap $[M+H]^+$ $m/z = 369$ and reveals the smaller peaks to contain the four POA's 1.) Mitraphylline, 2.) Isomitraphylline, 3.) Uncarine C(Pteropodine), 4.) Uncarine E (Isopteropodine) as defined in the ESI/MS chromatographs of the alkaloid standards and 5.) Uncarine D (Speciophylline) as defined in the literature. The ES/MS uncovers no large peak correlating with the retention time of ~ 28 -31 min on the HPLC chromatograph suggesting an unknown in the crude extract that is also absorbing at 245 nm in the retention time of uncarine C (pteropodine).

Table 6: ESI/MS Normalization (NL) values for Leaves and Stems

4 April 2009 Leaves & Stems	
	Normalization (NL)
WPM	6.85E+05
MSO	5.61E+05
HSO	1.46E+06
B5	8.04E+05
N/N	1.93E+06
DKW	1.11E+06
5 May 2009 Leaves & Stems	
	Normalization (NL)
N/N charcoal	2.13E+05
MSO	3.09E+07
WPM	6.60E+05
B5	3.01E+05

HPLC – ES/MS Analysis of *U. tomentosa* Leaves

Crude extracts of *U. tomentosa* leaves grown in medias - MSO, SH, ½ MS, B5, DKW, N/N, N/N Charcoal, WPM, WHITE were analyzed using HPLC-ESI/MS. A sample of each extract was treated to remove chlorophyll and 20 µl was injected into the HPLC-ESI/MS for analysis. Alkaloid detection was monitored at 245 nm and ESI/MS mass was set to 369. The HPLC chromatograph for each crude extract of leaves (Fig. 13A) produced from different media display similar peaks as the chromatographs of the combination of leaves and stems seen in Figures 11-12. Small less intense peaks were seen within the time range of ~20 – 27 min where the specific pentacyclic oxindole alkaloids of interest along with other unknown compounds reside. Since the peaks are small, we can speculate that the amount of alkaloid or unknown present is minimal. A

large intense peak at ~28 – 30 min was also seen in the HPLC chromatographs extracts produced from leaves only (Fig. 13A). The unknown peak at ~28 - 30 min intensity was directly related to the amount of plant material provided in the crude extract which can be reviewed in Table 7 and further in Table 10 of the quantitative analysis section of the results.

Table 7: The weight of plant material for Leaves.

9-28-2009 Extracts (Leaves)22-Dec-09 run	
Name	***WT of plant mat.***
DKW	~0.37g
B5	~0.51g
WHITE	~0.12g
1/2MS	~0.29g
N/N	~0.32g
WPM	~0.49g
N/N Charcoal	~0.15g
MSO	~0.72g
SH	~0.22g

The larger peak in the range of ~28 – 30 min was identified as either uncarine C (pteropodine) or a combination of isomitraphylline and uncarine C. If correlated with the retention times of just the HPLC chromatographs of the stock standard the POA separation was apparent with an increase in the intensity of uncarine C. The ESI/MS data (Fig 13B) for all extracts revealed no large peak that correlated with the retention time of ~ 28 -31 min on the HPLC chromatograph. In the extracts from the media ½ MS, DKW, N/N Charcoal, N/N leaves, WPM, and whites, (Fig. 13A) another unknown compound eluted immediately following the larger peak at a retention time of ~ 28-30 min. These two conjoined peaks can probably be seen in the HPLC because the intensity, due to the

higher amount of one compound, is not overwhelming the peak detection of the other compound. Also, the amount of these compounds was slightly lower in leaves compared to both leaves and stems. Again, it was difficult to discern the alkaloids of interest from the HPLC chromatographs produced by the crude extracts just by correlating the retention times of the HPLC stock standard chromatographs. Thus, the ESI/MS chromatographs were used to further analyze the samples.

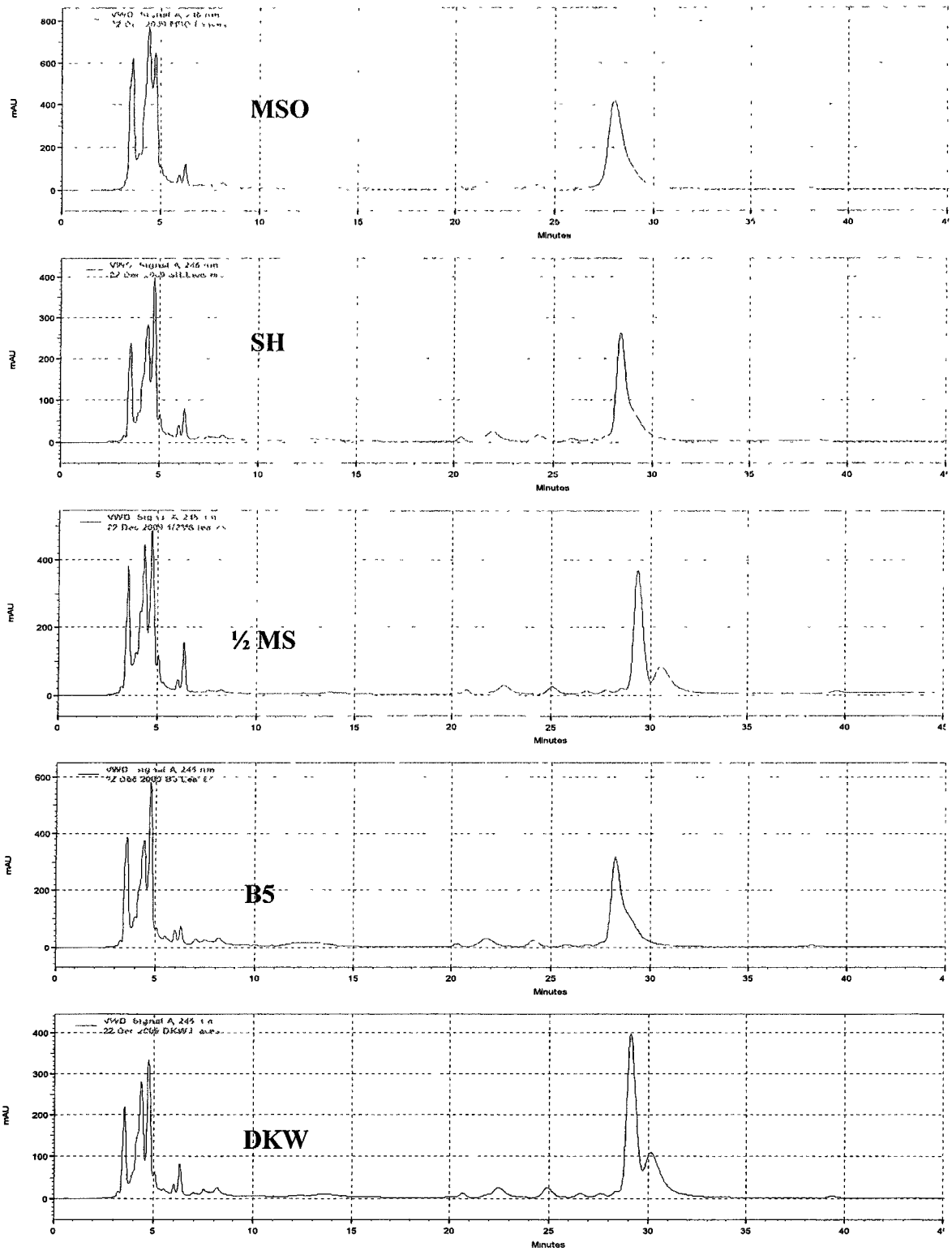


Figure 13A: HPLC chromatographs of crude extracts from *U. tomentosa* leaves.

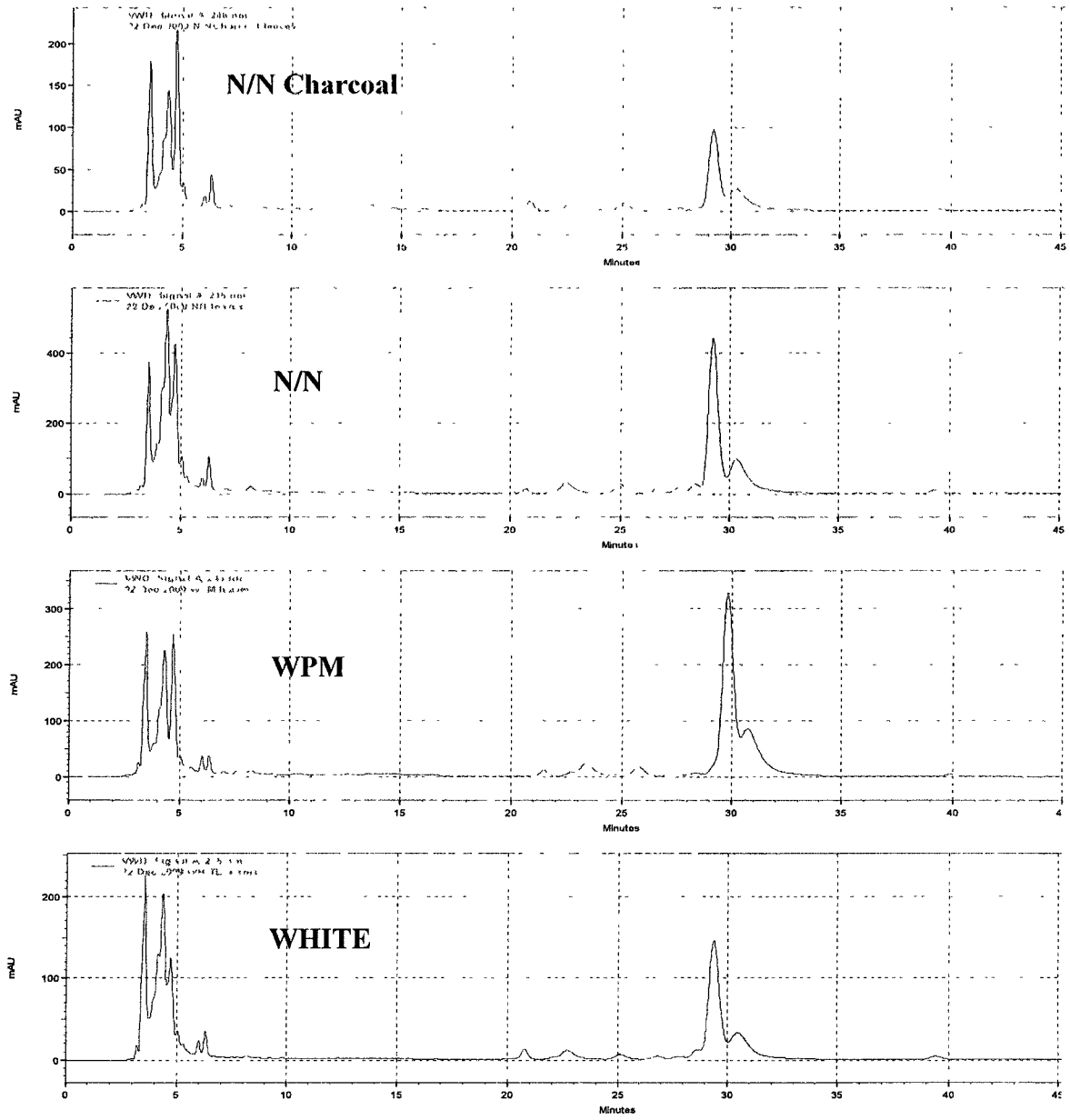


Figure 13A Continued: HPLC chromatographs of crude extracts from *U. tomentosa* leaves. Plants were grown in mediums: MSO, SH, ½ MS, B5, DKW, N/N, N/N Charcoal, WPM, WHITE. Again small peaks of low concentration are seen in the areas designated for the POA's by the standard chromatograph ~ 20 -29 min and ~ 38 – 40. Similar intense peak is seen ranging again from ~28 – 31 min. As observed before, the intense peak ranging from ~27 – 30 min could be considered as uncarine C, but as previously reviewed with the data regarding the effects of the chlorophyll process, this peak could be the accumulation of uncarine C and an unknown in the crude extract that is also absorbing at 245 nm.

The ESI/MS standard chromatographs (Fig. 13B) were used to distinguish and identify peaks of the *U. tomentosa* extracts from media, MSO, SH, ½ MS, B5, DKW, N/N, N/N Charcoal, WPM, WHITE. Setting the *m/z* = 369-370 enhances the sensitivity of the detection and removes all compounds that were not within the desired range. All peaks, within the time range correlating with the small peaks observed in the HPLC chromatographs ~20 -27 min, were seen in each plant extract analyzed by the ESI/MS.. Peaks not of the mass range of *m/z* = 369 were removed, suggesting the amount of alkaloid present was either too small for the HPLC to thoroughly detect or did not have the molecular weight of the pentacyclic oxindole alkaloids of interest. The isomitraphylline in the extracted sample from medium MSO could not be determined because there was no adequate peak resolution to determine the alkaloid. In addition, a fairly stronger peak of an unknown alkaloid eluted before the retention time of mitraphylline. This can be identified as uncarine D (speciophylline), which has a molecular weight of ~ 368 and absorbs at 245 nm, as reported (2, 12, 15). Reviewing the normalization (NL) values from the ESI/MS (Table 8) Nitsch and Nitsch (N/N) medium had the highest NL compared to MSO which produced a higher yield of plant material for extraction. Considering this collaboration provokes the idea that MSO is good for plant cultivation but not alkaloid production.

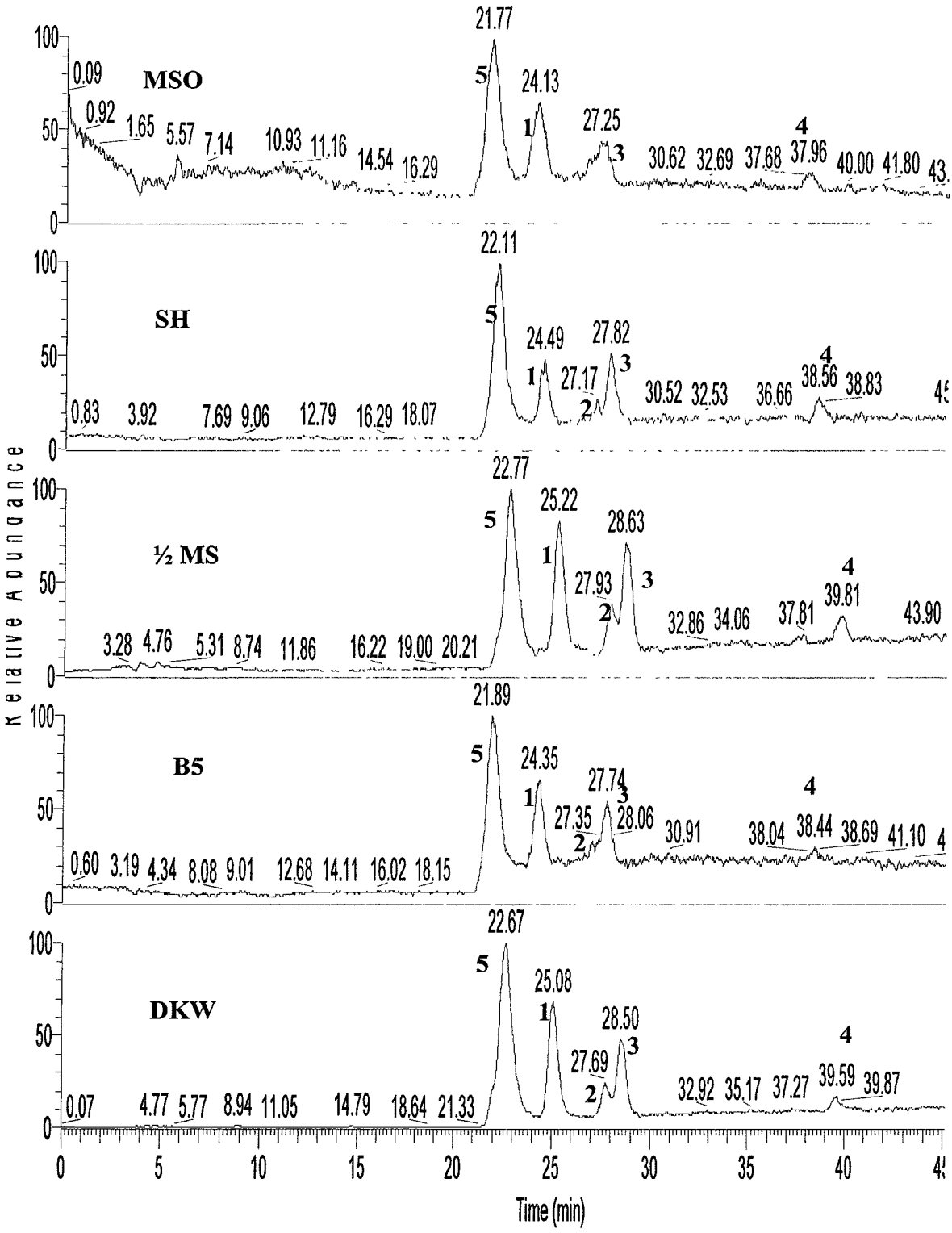


Figure 13B: ESI/MS chromatographs of crude extracts from *U. tomentosa* leaves.

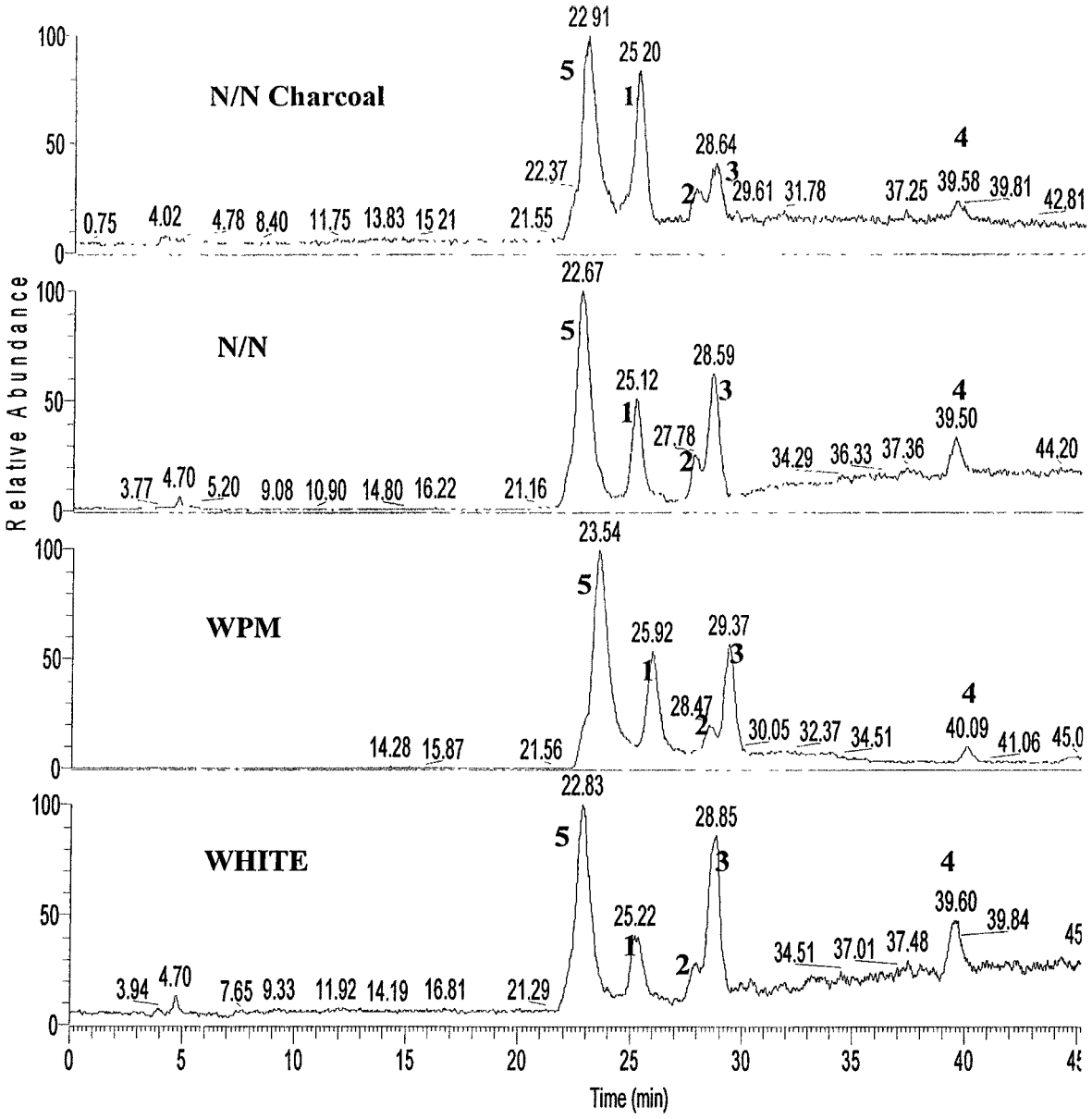


Figure 13B Continued: ESI/MS chromatographs of crude extracts from *U. tomentosa* leaves. Plants were grown in mediums: MSO, SH, ½ MS, B5, DKW, N/N, N/N Charcoal, WPM, WHITE. Again the molecular weight of the POA's is 368. The peak sensitivity is enhanced through the ESI/MS ion trap $[M+H]^+$ *m/z* = 369 and reveals the smaller peaks to be the four POA's 1.) Mitraphylline, 2.) Isomitraphylline, 3.) Uncarine C (Pteropodine), 4.) Uncarine E (Isopteropodine) as defined in the ESI/MS chromatographs of the alkaloid standards and 5.) Uncarine D (Speciophylline) as defined in the literature. Isomitraphylline in the extract sample from medium MSO could not be determine because there was no adequate peak resolution to determine the alkaloid.

Table 8: ESI/MS Normalization (NL) values for Leaves

28 september 2009 Leaves	
	Normalization (NL)
DKW	3.65E+06
B5	2.81E+06
WHITE	2.20E+06
1/2MS	4.75E+06
N/N	5.06E+06
WPM	3.78E+06
N/N Charcoal	8.87E+05
MSO	3.62E+06
SH	1.93E+06

Analysis of *U. tomentosa* Roots

Crude extracts of *U. tomentosa* roots grown in MSO, DKW, ½ MS, N/N, N/N Charcoal, B5, WPM, SH, and WHITE's media were analyzed using HPLC-ESI/MS. A sample of each extract was treated to remove chlorophyll and then 20 µl was injected into the system for analysis. Detection for HPLC was set at 245 nm and ESI/MS mass was set to 369. The HPLC – ESI/MS chromatographs (Fig. 14A & 14B) of the crude extracts from *U. tomentosa* roots grown in MSO, DKW, ½ MS, N/N, N/N Charcoal, B5, WPM, SH, and WHITE's media, doesn't display similar peaks as in the chromatographs of the combination of leaves and stems or leaves by themselves. There is an unknown peak identified in the time range of ~20 – 21 min (Fig 14A) that was seen only in DKW, N/N Charcoal, B5, WPM, SH crude extracts. A less intense peak was seen in MSO and ½ MS extracts. The large intense peak at ~ 27 – 30 min seen in the previous HPLC chromatographs containing the combination of leaves and stems and leaves alone did not appear in the roots, suggesting the unknown compound along with uncarine C found in

leaves and stems and leaves alone was not at a detectable level or not present. Again the sensitivity to low concentrations on the HPLC was fair but not exceptional unless there was a pure and fairly large amount of alkaloid. Small bulges in the baseline that could be considered as peaks appear in the specific areas expected from the standards' but cannot be considered as the alkaloids of interest for their levels are too low for identification.

Analyzing the ESI/MS chromatographs of the extracts from *U. tomentosa* roots, alkaloid detection was seen only in the WHITE's medium (Fig. 14B), but at a very minimal amount as will be shown in the quantitative section (Table 11). The WHITE's medium produced the four POA's. 1.) Mitraphylline, 2.) Isomitraphylline, 3.) Uncarine C (Pteropodine), 4.) Uncarine E (Isopteropodine) as defined in the ESI/MS chromatographs of the alkaloid standards and 5.) Uncarine D (Speciophylline) as defined in the literature. The N/N medium appeared to contain peaks in the designated time range of the POA standards, but too much background noise and a low amount of alkaloid inhibited the identification (Fig. 14B). The peak identified on the HPLC chromatograph ~ 20-21 min was not detected by the ESI/MS, indicating that its mass range does not coincide with the particular POA's of interest. The deficient detection of alkaloids in the other extract root samples might be due to the maturity of the plant. These young micropropagated *U. tomentosa* plants may lack maturity to produce the POA's of interest in the roots as reported (2, 5, 12, 16). Considering the detection of alkaloids in the WHITE's medium, production may not be due to maturity of the plant but the lack of nutrients provided in the specific growth medium. Reviewing the normalization (NL) values from the ESI/MS for roots (Table 9) we see values are relatively lower, ~ 2 exponentials, compared the combination of leaves and stems and isolated leave extracts.

The ESI/MS normalization for the WHITE medium is one exponential higher, ~10^5, compared to all the (NL) values from the other media. A limit of detection was not prepared for the alkaloids in this study but is in the process.

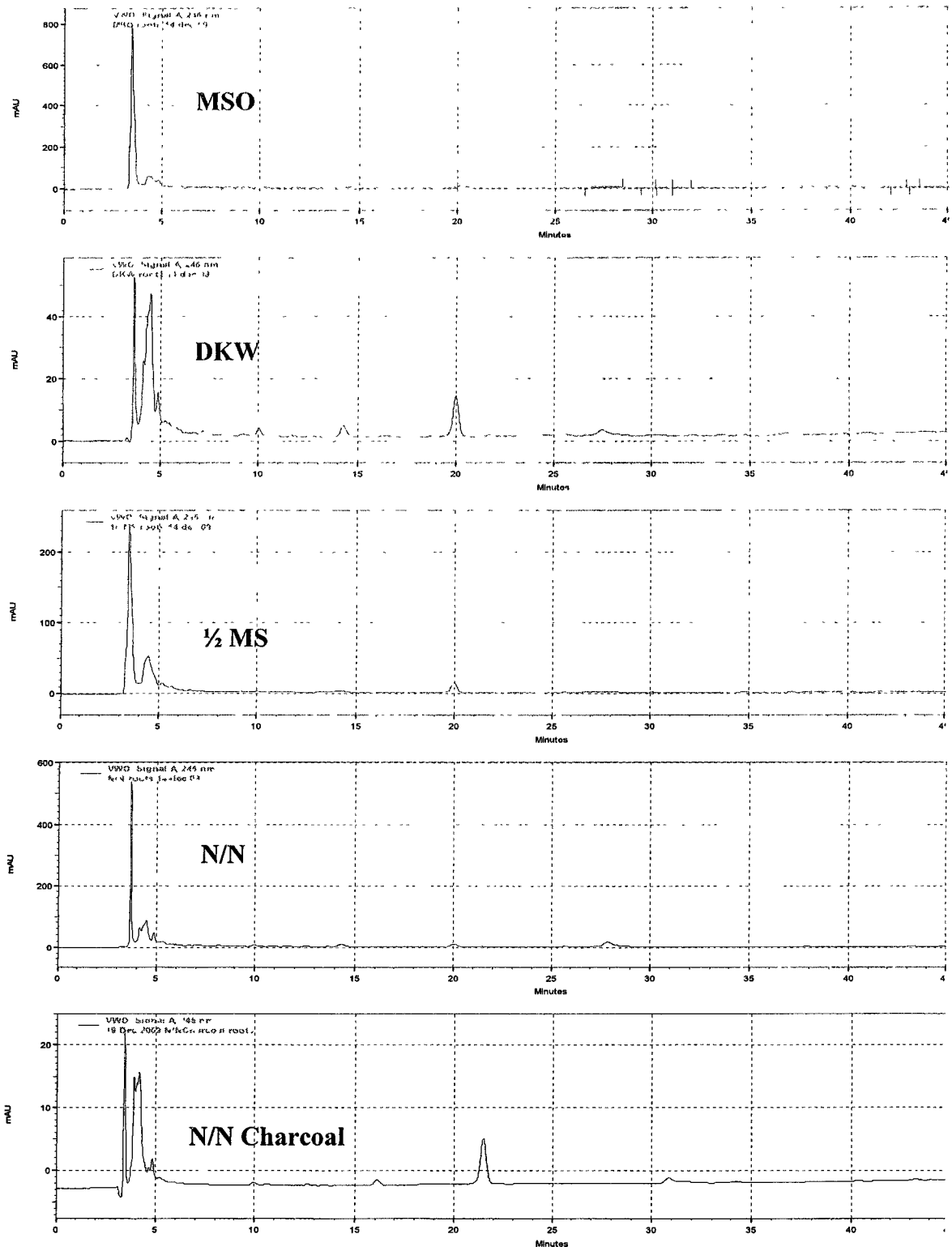


Figure 14A: HPLC chromatographs of crude extracts from *U. tomentosa* roots.

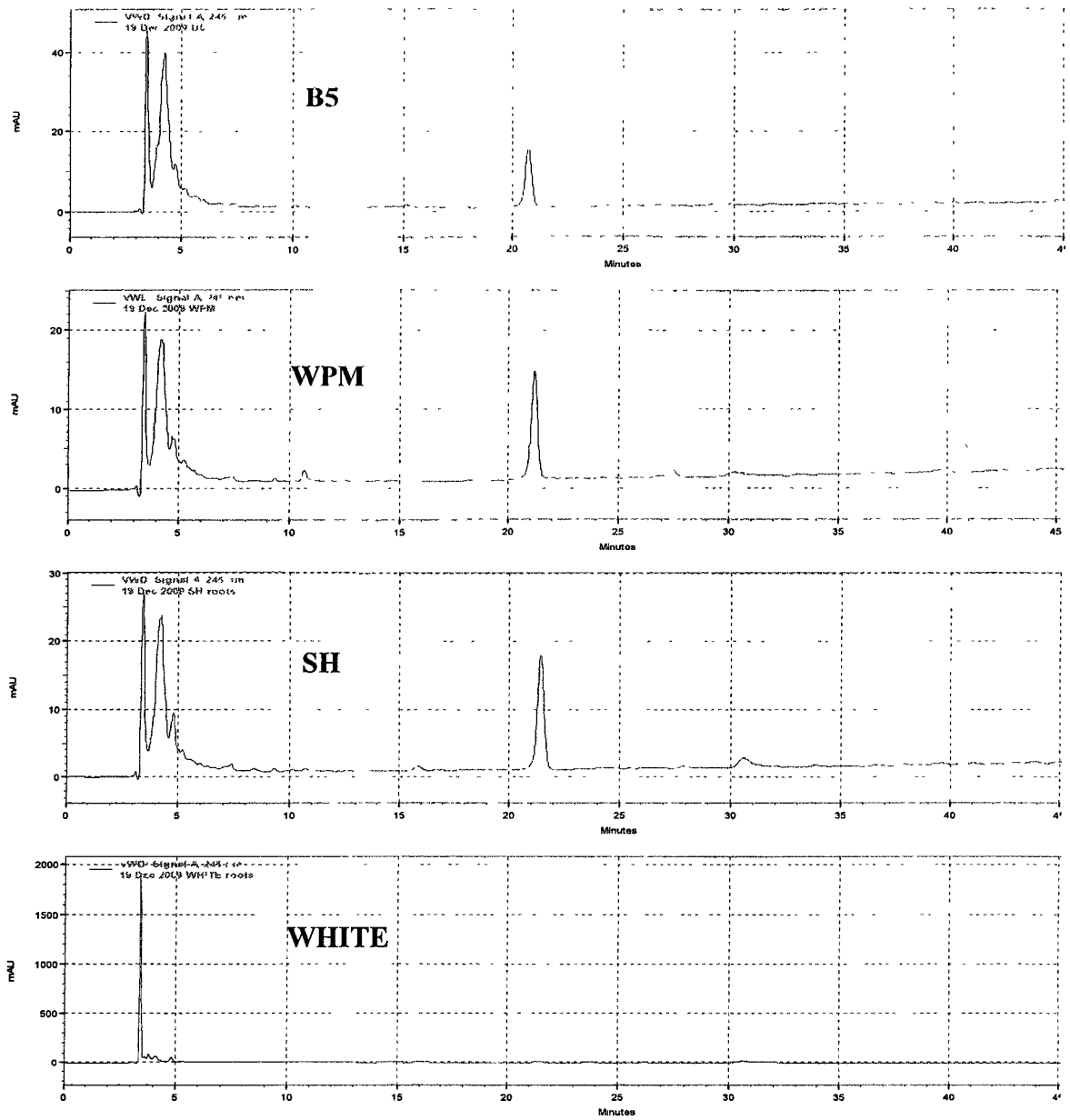


Figure 14A Continued: HPLC chromatographs of crude extracts from *U. tomentosa* roots. Plants were grown in mediums: MSO, DKW, ½ MS, N/N, N/N Charcoal, B5, WPM, SH, and WHITE. Hardly any peaks correlating with alkaloids are detected in the root extracts. An unknown was detected with a peak residing in the time range of ~20 – 21 min, and seen only in DKW, N/N Charcoal, B5, WPM, SH crude extracts. The peak is slightly seen in MSO and ½ MS extract. The large intense peak at ~ 27 – 30 min seen in the previous HPLC chromatographs containing leaves does not appear in the roots chromatograph. Again the sensitivity to low concentrations on the HPLC is fair but not exceptional unless there is a pure and fairly large amount of alkaloid. Small bulges in the baseline that could be considered as peaks are also seen but could not be identified as POA's.

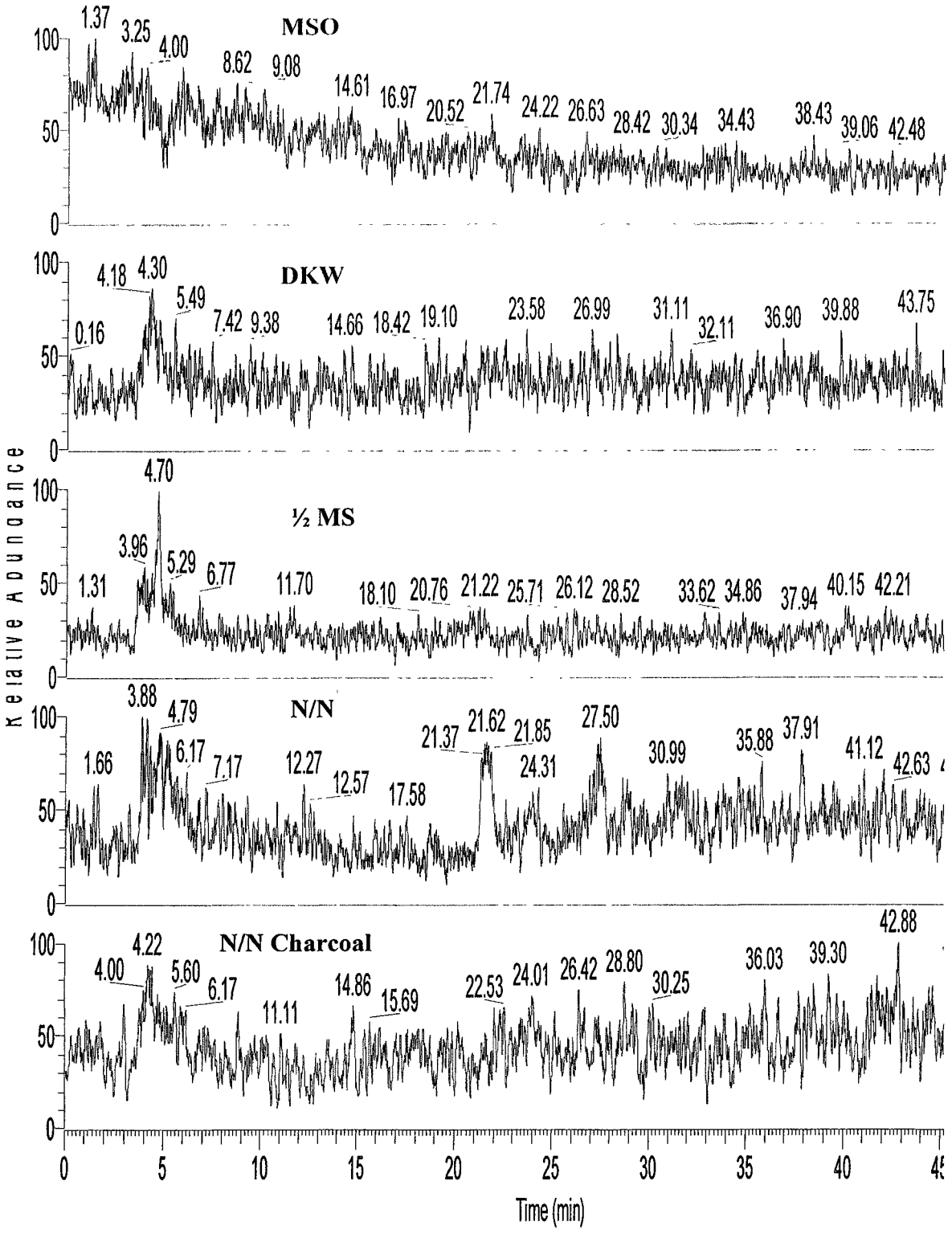


Figure 14B: ESI/MS chromatographs of crude extracts from *U. tomentosa* roots.

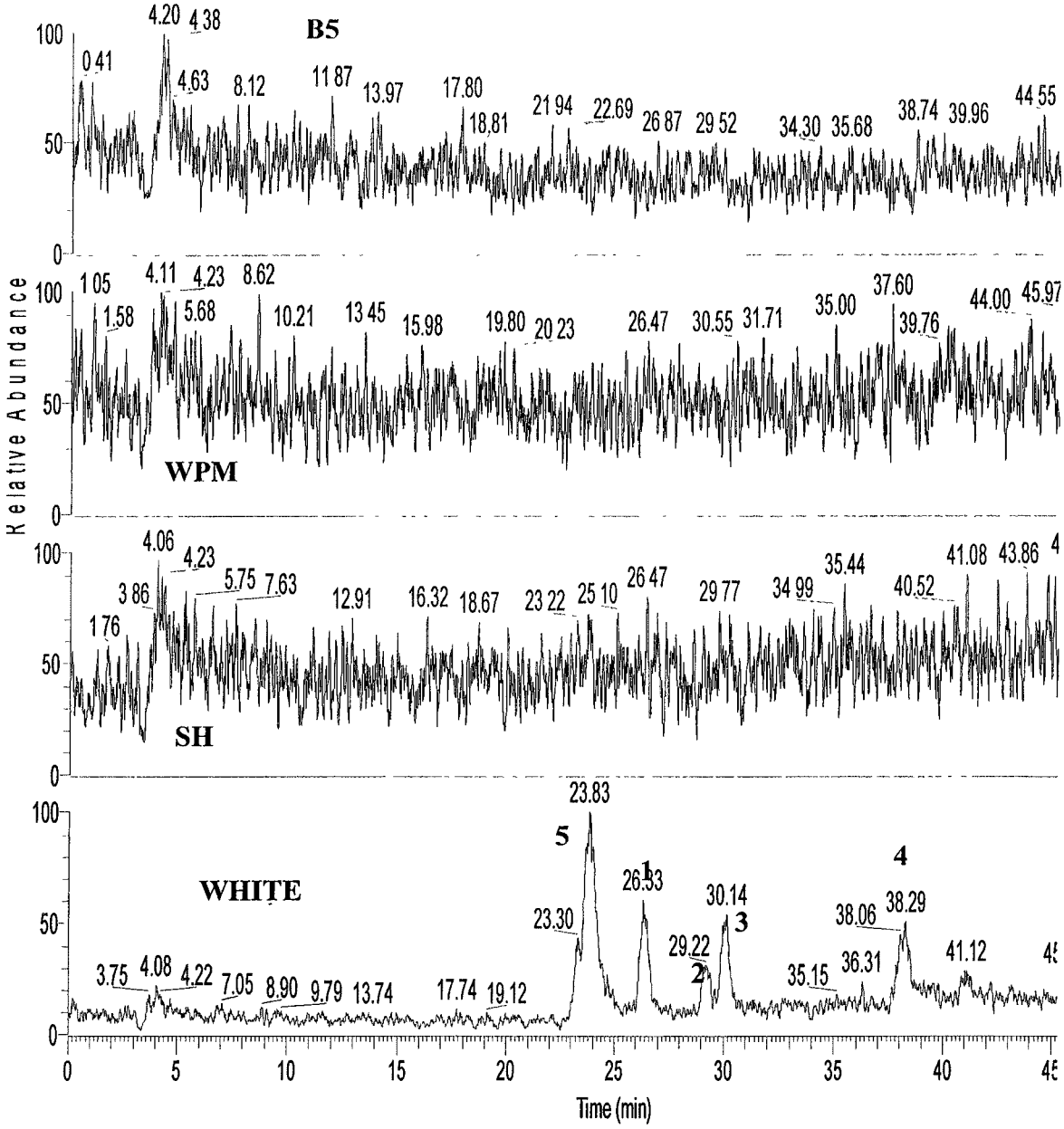


Figure 14B Continued: ESI/MS chromatographs of crude extracts from *U. tomentosa* roots. Plants were grown in mediums: MSO, DKW, ½ MS, N/N, N/N Charcoal, B5, WPM, SH, and WHITE. Again the molecular weight of the POA's is ~ 368. The peak sensitivity is enhanced through the ESI/MS on trap $[M+H]^+$ $m/z = 369$ and reveals smaller peaks only in the WHITE medium to contain the four POA's. 1.) Mitraphylline, 2.) Isomitraphylline, 3.) Uncarine C (Pteropodine), 4.) Uncarine E (Isopteropodine) as defined in the ESI/MS chromatographs of the alkaloid standards and 5.) Uncarine D (Speciophylline) as defined in the literature. The N/N medium appear to contain peaks in the designated time range of the justified POA standards, but too much background noise or low concentration of alkaloid inhibits the assumption. The peak identified on the HPLC chromatograph ~ 20-21 min is not detected by the ESI/MS.

Table 9: ESI/MS Normalization (NL) values for Roots.

	28 september 2009 Roots
	Normalization (NL)
MSO	8.11E+04
DKW	5.12E+04
1/2MS	4.38E+04
N/N	5.27E+04
B5	5.06E+04
WPM	4.33E+04
SH	4.83E+04
WHITES	1.21E+05
N/N Charcoal	3.24E+04

Quantitative analysis of pentacyclic oxindole alkaloids in cultivated *U. tomentosa* micropropagated in different mediums.

The HPLC – ESI/MS as described earlier was the method of choice because of its sensitivity and selectivity toward the alkaloids. The method was utilized to study production of alkaloid content in different media. Calibration graphs were obtained from plotting peak height vs. the known concentration of each alkaloid standard. These plots were found linear as reported in Table 3 of the calibration section of materials and methods. Again, quantitative information was obtained using the quotient between the product of the height ratio of each POA peak found in the crude extracts multiplied by the normalization (NL) value compared to the product of the height ratio of each POA of the known of three diluted concentrations of standard multiplied by its (NL) value. The mean of the three results is reported in *n*g, (Table 10). The standard deviation was calculated and compared to a 95 % confidence limit. The quantitative data identified the medium MSO as the optimal plant growth medium for plant material (Table 10), but the overall percent of pentacyclic oxindole alkaloids produced compared to plant material

grown were lower than other growth media (Table 12). MSO was identified as the optimal alkaloid per plant material based on samples provided in May 2009, combination of leaves and stems, but can't be confirmed by its data from leaves only or root extracts. From the combination of leaves and stem from April 2009 extracts DKW, HSO, and N/N these provide optimal alkaloid production per plant material grown compared to B5, WPM, N/N Charcoal, from May 2009 leaves and stems.

The root material extracts only gave quantitative data on the WHITE's media in low amounts, but as explained earlier the N/N media may have produced POA content but at a concentration too low to produce quantitative information. The rest of the other growth media produced very minute amounts for detection or there were no POA's produced.

The leave extracts contained most of the alkaloid produced according to the data. Reviewing each specific POA, a good yield (*n*g/µg) of mitraphylline per plant material, was produced from medias DKW, ½ MS, N/N, N/N Charcoal. The isomitraphylline yield of alkaloid per plant material was better produced in ½ MS, DKW and possibly N/N. Uncarine C (pteropodine) along with uncarine E (isopteropodine) had a higher yield of alkaloid produced per plant material in WHITE, ½ MS, and N/N media. ½ MS medium produced the most adequate amounts of mitraphylline and isomitraphylline with a POA content per plant material of 0.908 and 0.316 (*n*g/µg) respectively, as WHITE's medium produced the most adequate amount of uncarine C (pteropodine) and uncarine E (isopteropodine) of 0.519 and 0.275 (*n*g/µg) respectively. Overall, ½ MS medium produce the most sufficient amount of total pentacyclic oxindole alkaloids compared to

all other growth media and with ~ 9 hundredths lower production of uncarine C and uncarine E from the WHITE medium (Table 12).

Table 10: Yield of plant material harvested. Calculated amount of actual plant material injected per 20 µl injection of crude extract.

4-April-2009 Extracts (plant material)6-Dec-09 run				
Name	***WT of plant mat.***	***vol. plt. Extract***	***%of plt Extract***	***Plt. Mat. Injected (µg)***
WPM	~0.09g	10ml	0.2%	180
MSO	~0.5g	10ml	0.2%	1000
HSO	~0.10g	10ml	0.2%	200
B5	~0.14g	10ml	0.2%	280
N/N	~0.14g	10ml	0.2%	280
DKW	~0.05g	10ml	0.2%	100
5-20-2009 Extracts (plant material)20-Dec-09 run				
Name	***WT of plant mat.***	***vol. plt. Extract***	***%of plt Extract***	***Plt. Mat. Injected (µg)***
N/N charcoal	~0.11g	25ml	0.08%	88
MSO	~3.26g	25ml	0.08%	2608
WPM	~0.16g	25ml	0.08%	128
B5	~0.10g	25ml	0.08%	80
9-28-2009 Extracts (Roots)14&19-Dec-09 run				
Name	***WT of plant mat.***	***vol. plt. Extract***	***%of plt Extract***	***Plt. Mat. Injected(µg)***
MSO	~0.19g	15ml	0.13%	247
DKW	~0.11g	15ml	0.13%	143
1/2MS	~0.11g	15ml	0.13%	143
N/N	~0.16g	15ml	0.13%	208
B5	~0.12g	15ml	0.13%	156
WPM	~0.11g	15ml	0.13%	143
SH	~0.13g	15ml	0.13%	169
WHITES	~0.15g	15ml	0.13%	195
N/N Charcoal	~0.02g	15ml	0.13%	26
9-28-2009 Extracts (Leaves)22-Dec-09 run				
Name	***WT of plant mat.***	***vol. plt. Extract***	***%of plt Extract***	***Plt. Mat. Injected(µg)***
DKW	~0.37g	20ml	0.10%	370
B5	~0.51g	20ml	0.10%	510
WHITE	~0.12g	20ml	0.10%	120
1/2MS	~0.29g	20ml	0.10%	290
N/N	~0.32g	20ml	0.10%	320
WPM	~0.49g	20ml	0.10%	490
N/N Charcoal	~0.15g	20ml	0.10%	150
MSO	~0.72g	20ml	0.10%	720
SH	~0.22g	20ml	0.10%	220

Table 11: Average amount of Pentacyclic Oxindole Alkaloid content Quantitated. Quantitated from the ratio of the normalization (NL) value reported on the ESI/MS chromatographs compared to each alkaloid found in the crude extracts by the NL compared to each alkaloid in the known standard of three diluted concentrations. Standard deviation along with a 95% confidence limit is reported for error. N/D refers to 'No Data' obtained.

4 April 2009 Leaves & Stems - Average Alkaloid content in ng *(n=3)																
	Mitraphylline		*S.D*	*95% confidence limit*	*Isomitraphylline*		*S.D*	*95% confidence limit*	*Uncarine C*		*S.D*	*95% confidence limit*	*Uncarine E*		*S.D*	*95% confidence limit*
WPM	21.5	±	0.8	2.0	18.7	±	1.1	2.0	16.9	±	0.9	2.0	12.8	±	0.6	1.0
MSO	26.0	±	1.0	2.0	13.1	±	0.7	1.0	17.8	±	1.0	2.0	10.1	±	0.4	1.0
HSO	51.0	±	2.0	4.0	43.1	±	2.5	6.0	32.1	±	1.8	4.0	32.0	±	1.4	3.0
B5	19.4	±	0.8	1.0	19.2	±	1.1	2.0	17.7	±	1.0	2.0	14.7	±	0.6	1.0
N/N	58.2	±	2.3	5.0	61.3	±	3.5	8.0	42.4	±	2.4	5.0	19.1	±	0.8	2.0
DKW	25.4	±	1.0	2.0	27.7	±	1.6	3.0	24.4	±	1.4	3.0	9.7	±	0.4	1.0
20 May 2009 Leaves & Stems - Average Alkaloid content in ng *(n=3)																
	Mitraphylline		*S.D*	*95% confidence limit*	*Isomitraphylline*		*S.D*	*95% confidence limit*	*Uncarine C*		*S.D*	*95% confidence limit*	*Uncarine E*		*S.D*	*95% confidence limit*
N/N charcoal	12.8	±	0.5	1.0	7.5	±	0.4	1.0	5.7	±	0.3	0.0	4.6	±	0.2	0.0
MSO	1359.7	±	52.9	131.0	789.1	±	45.3	112.0	875.2	±	49.0	121.0	666.9	±	29.2	72.0
WPM	24.7	±	1.0	2.0	18.7	±	1.1	2.0	19.8	±	1.1	2.0	13.0	±	0.6	1.0
B5	7.6	±	0.3	0.0	6.3	±	0.4	0.0	8.5	±	0.5	1.0	6.9	±	0.3	0.0

Table 11 Continued: Average amount of Pentacyclic Oxindole Alkaloid content Quantitated. Quantitated from the ratio of the normalization (NL) value reported on the ESI/MS chromatographs compared to each alkaloid found in the crude extracts by the NL compared to each alkaloid in the known standard of three diluted concentrations. Standard deviation along with a 95% confidence limit is reported for error. N/D refers to 'No Data' obtained

28 september 2009 Roots - Average Alkaloid content in ng *(n=3)																
	Mitraphylline		*S.D*	*95% confidence limit*	*Isomitraphylline*		*S.D*	*95% confidence limit*	*Uncarine C*		*S.D*	*95% confidence limit*	*Uncarine E*		*S.D*	*95% confidence limit*
MSO	N/D	±			N/D	±			N/D	±			N/D	±		
DKW	N/D	±			N/D	±			N/D	±			N/D	±		
1/2MS	N/D	±			N/D	±			N/D	±			N/D	±		
N/N	N/D	±			N/D	±			N/D	±			N/D	±		
B5	N/D	±			N/D	±			N/D	±			N/D	±		
WPM	N/D	±			N/D	±			N/D	±			N/D	±		
SH	N/D	±			N/D	±			N/D	±			N/D	±		
WHITE	7.3	±	0.3	0.0	3.0	±	0.2	0.0	3.6	±	0.2	0.0	3.3	±	0.1	0.0
N/N Charcoal	N/D	±			N/D	±			N/D	±			N/D	±		
28 september 2009 Leaves - Average Alkaloid content in ng *(n=3)																
	Mitraphylline		*S.D*	*95% confidence limit*	*Isomitraphylline*		*S.D*	*95% confidence limit*	*Uncarine C*		*S.D*	*95% confidence limit*	*Uncarine E*		*S.D*	*95% confidence limit*
DKW	259.6	±	10.1	25.0	84.9	±	4.9	12.0	109.5	±	6.1	15.0	34.7	±	1.5	3.0
B5	149.1	±	5.8	14.0	51.0	±	2.9	7.0	65.5	±	3.7	9.0	25.7	±	1.1	2.0
WHITE	43.8	±	1.7	4.0	23.7	±	1.4	3.0	62.3	±	3.5	8.0	33.0	±	1.4	3.0
1/2MS	263.4	±	10.3	25.0	91.6	±	5.3	13.0	126.6	±	7.1	17.0	43.4	±	1.9	4.0
N/N	219.6	±	8.5	21.0	91.9	±	5.3	13.0	151.8	±	8.5	21.0	81.4	±	3.6	8.0
WPM	195.9	±	7.6	18.0	57.9	±	3.3	8.0	115.9	±	6.5	16.0	13.8	±	0.6	1.0
N/N Charcoal	6.2	±	0.2	0.0	19.1	±	1.1	2.0	19.5	±	1.1	2.0	7.8	±	0.3	0.0
MSO	213.8	±	8.3	20.0	N/D	±	0.0	0.0	79.6	±	4.5	11.0	37.1	±	1.6	4.0
SH	76.8	±	3.0	7.0	26.3	±	1.5	3.0	45.0	±	2.5	6.0	16.9	±	0.7	1.0

Table 12: The amount of average alkaloid content compared to the amount of plant material injected reported in *ng*/µg. DKW, N/N, HSO produced the most adequate amount of total POAs for April 2009 and MSO medium for May 2009. WHITE medium was the only root extract to yield enough alkaloid content for detection and produce data. ½ MS medium produced the most adequate amount of total POAs with ~ 8 % lower production of uncarine C and uncarine E (content/plt. material injected) in WHITE medium.

Leaves &Stems 4-April-2009 (content/plant material injected) in *ng*/µg				
	Mitraphylline	***Isomitraphylline***	***Uncarine C***	***Uncarine E***
WPM	0.119	0.104	0.094	0.071
MSO	0.026	0.013	0.018	0.010
HSO	0.255	0.215	0.161	0.160
B5	0.069	0.068	0.063	0.053
N/N	0.208	0.219	0.152	0.068
DKW	0.254	0.277	0.244	0.097
Leaves & Stems 20-May-2009 (content/plant material injected) in *ng*/µg				
	Mitraphylline	***Isomitraphylline***	***Uncarine C***	***Uncarine E***
N/N charcoal	0.146	0.085	0.065	0.052
MSO	0.521	0.303	0.336	0.256
WPM	0.193	0.146	0.155	0.102
B5	0.095	0.079	0.107	0.087
Roots (content/plant material injected) in *ng*/µg				
	Mitraphylline	***Isomitraphylline***	***Uncarine C***	***Uncarine E***
MSO	--	--	--	--
DKW	--	--	--	--
1/2MS	--	--	--	--
N/N	--	--	--	--
B5	--	--	--	--
WPM	--	--	--	--
SH	--	--	--	--
WHITE	0.037	0.015	0.018	0.017
N/N Charcoal	--	--	--	--
Leaves (content/plant material injected) in *ng*/µg				
	Mitraphylline	***Isomitraphylline***	***Uncarine C***	***Uncarine E***
DKW	0.702	0.230	0.296	0.094
B5	0.292	0.100	0.129	0.050
WHITE	0.365	0.198	0.519	0.275
1/2MS	0.908	0.316	0.437	0.150
N/N	0.686	0.287	0.474	0.255
WPM	0.400	0.118	0.237	0.028
N/N Charcoal	0.041	0.128	0.130	0.052
MSO	0.297	N/D	0.111	0.051
SH	0.349	0.119	0.205	0.077

CHAPTER IV

CONCLUSION

Our aim was to provide side by side comparative quantitative data on POA levels n *Uncaria tomentosa* micropropagated in different growth media -- Murashige and Skoog (MSO), ½ Murashige and Skoog (½ MS), Driver and Kuniyuki Walnut (DKW), Woody Plant Medium (WPM), Gamborg B5 medium (B5), Nitsch & Nitsch (N/N), Nitsch & Nitsch Charcoal, Schenk and Hilderbrant (SH), WHITE's, and Homeostatic Soil (HSO) -- for three month micropropagated *U. tomentosa* plant cultures. Transformation protocols using Agrobacterium rhizogenes to produce hairy roots, and increase root growth and alkaloid production have statistically failed or produced weak results on the basis that there is no set growth protocol for the wild type plant culturing. Reports of oxidative and hydrodynamic stress on *Uncaria tomentosa* cell cultures have been successful in alkaloid production, while using the medium of choice, Murashige and Skoog (MS) (12, 13). Overall our data points to the ½ MS as the ideal growth medium for micropropagating *U. tomentosa* plants and alkaloid production (Table 11, 12). Since these are just progenies of *U. tomentosa* the lack of alkaloid production in roots could be caused by the plant's maturity or lack of nutrients in the specific medium. Our method of coupling HPLC with ESI/MS was proposed as the technique of choice by Verpoorte R, Niessen WMA. 1994. (13) and Montoro P, Carbone V. 2004 (12) for the analysis of these

pentacyclic oxindole alkaloids and was revised to fit our system. The chlorophyll extraction protocol along with the addition of a guard column was added to remove background interference and noise for cleaner, more isolated mix of alkaloids while prolonging the use of our C-18 column.

Other peaks and compounds observed in the HPLC chromatographs maybe considered as other secondary metabolites that reside in the plants themselves, such as quinovic acid glycosides, polyhydroxylated triterpenes, and tetracyclic oxindole alkaloids (2, 5). The large peak observed in the HPLC analysis of both leaves and stems and leaves by themselves in the retention time range of ~ 28 -30 if investigated further could be the combination of the two compounds poorly separated. This weak separation can be observed in the extracts from medias B5, MSO, DKW, N/N Charcoal, N/N leaves, WPM, and, (Fig. 11A & 12A) of both leaves and stems and ½ MS, DKW, N/N Charcoal, N/N leaves, WPM, and whites, (Fig. 13A) of the leaves analysis. These unknown peaks can be further examined by changing wavelength and mass range of the HPLC and MS for better sensitivity towards the corresponding metabolites of interest, or the use of another analytical instrument such as NMR (12). As reported by Montoro et al. (12) identification of the alkaloids could not be obtained using the parameter MS/MS because each compound is isomeric and produces the same spectrum with the same fragmentation pattern. Out of the six major pentacyclic oxindole alkaloids, uncarine C (pteropodine), uncarine E (isopteropodine), uncarine D (speciophylline), uncarine F, mitraphylline and isomitraphylline we were able to observe and retrieve quantitative data on the four POA's of interest and also detect a fifth, speciophylline.

In conclusion we were able to compare HPLC-ESI/MS quantitative data on the cultivating properties of each medium used for micropropagation of *U. tomentosa* and found that the ½ MS was proficient enough to produce efficient plant growth and alkaloid production. Side by side comparison of different growth media has never been reported, thus this work may improve other cultivating procedures in the case of optimizing raw material for further research. The use of hydroponic systems for micropropagation may be a key for cultivating Cat's Claw roots. This data can help with regulation of nutrient supply for establishing optimal hydroponic *U. tomentosa* culturing conditions. Mass production of healthy *U. tomentosa* progenies can raise multiple opportunities for pharmaceutical research and environmental conservation. This study can provide a basis for plant enhancements and transformations to increase growth and alkaloid content in a short amount of time for further drug research. *U. tomentosa's* metabolic pathways have yet to be determined but by optimizing a reproducible growth system and preparing reliable plant samples similar to the wild type, discerning the pathway of alkaloid production is only a few steps away. With the deforestation of the rainforest, where this plant originates, a proficient micropropagation protocol would help Cat's Claws germoplasm conservation. Increasing the production of high quality raw material for *U. tomentosa* products – tea, tablets, capsules, and even candies containing dried plant material, or extracts – created by small–farm holders and women-based associations in Costa Rica, can help increase revenue for these associations. The HPLC-ES/MS techniques have proven to be efficient in terms of specificity and sensitivity of alkaloids in the extracts and may have been improved with the addition of chlorophyll extraction. In addition this analytical technique provides two data parameters, retention time and MS

information, to help support each POA's identification and quantification. This technique will provide quality data to help resolve issues concerning the next step mentioned in the Introduction section about transforming Cat's Claw with *A. rhizogenes* Does hairy root culture alter production of other metabolites by increasing non-targeted metabolites? Could hairy root culture or auxin itself divert certain plant metabolic pathways that would up or down regulate production of certain proteins or enzymes? Would this introduce production of foreign proteins or enzymes? It has been reported (10) that in some transformed hairy root cultures, not necessarily *U. tomentosa,* that over-expression of targeted enzymes doesn't always improve production of secondary metabolites. Also reduction of actual genetic material like chromosomes has been seen to occur during subculture along with alterations in morphology. These are all potential issues with the application of the hairy root sub-culture that can be taken into consideration and further tested with the use of this technique. Overall, this experiment provides significant information for producing, optimizing and further pursuing a basic micropropagation protocol for cultivating Cat's Claw wild type.

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VITA

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