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AN INVESTIGATION INTO THE TOXICOLOGIC PROPERTIES OF BLACK WALNUT (JUGLANS NIGRA) IN RELATION TO EQUINE LAMINITIS

presented by

PAUL DAVID MINNICK

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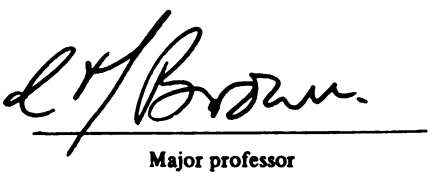

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AN INVESTIGATION INTO THE TOXICOLOGIC PROPERTIES OF BLACK WALNUT (JUGLANS NIGRA) IN RELATION TO EQUINE LAMINITIS

BY

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ABSTRACT

AN INVESTIGATION INTO THE TOXICOLOGIC PROPERTIES OF BLACK WALNUT (JUGLANS NIGRA) IN RELATION TO EQUINE LAMINITIS

By

Paul David Minnick

In order to determine if extracts of black walnut, Juglans nigra, would produce laminitis in the equine and to determine if the laminogenic activity was associated with juglone, ten horses were administered oral extracts of black walnut heartwood in a clinical trial and samples of bark, nuts, and heartwood were extracted by column chromatography, fractionated by gel permeation chromatography and juglone identified with gas chromatography/mass spectrometry (GC/MS). It was determined that severe laminitis could be produced by oral administration of an aqueous extract at a dose of 2 gm/kg of heartwood. Juglone was detected by GC/MS at a sensitivity of 150 ug/ul in nuts and bark but not in heartwood. These results indicate that laminitis was produced by water soluble agents other than juglone.

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INTRODUCTION

As early as the first century A.D. the toxicology of Juglans nigra (black walnut) was recognized. When in close association with the roots of black walnut the incompatibility between walnut trees and tomato plants was shown by the wilting and death of the intruding plant.[7]

Animal intoxication from exposure to Juglans sp. was first reported by early settlers. They used extracts from nuts and bark in their folk medicine, and indians poisoned fish in small impoundments and streams by throwing in walnut hulls.

In 1981, in the State of Michigan, approximately 15 incidents of suspected walnut toxicosis were reported after horses came in contact with walnut shavings used in their bedding.

The purpose of this project was twofold: first, to determine if oral administration of an extract of walnut shavings will produce laminitis in horses, and secondly, to attempt isolation of the suspected causative agent juglone from the shavings.

LITERATURE REVIEW

Walnut Toxicology

Plinius Secundus in 37 A.D. wrote "the shadow of the walnut was poisonous to other vegetation."[11] The exact species of walnut referred to is unrecorded, presumably it was Persian walnut (*Juglans regia*) common to the Mediterranean area.

Scientific literature is very sparse on the subject of walnut toxicity. Folklore and old wives tales tell of using walnut hulls for treatment of ringworm, as a sun-screen, as a clothes dye and as an agent to kill fish.[11] Research by Massey in 1940 reported tomatoes were killed or stunted when exposed to the roots of walnut trees.[17] In 1942 Brown showed inhibition of growth of tomato and alfalfa seedlings utilizing strips of fresh bark from live walnut roots.[3]

A complete analysis of the plant to find the active ingredient has, to the author's knowledge, not been performed. Juglone has been isolated from nuts, roots, and bark of black walnut but has not been reported in the wood.[31] Massey in 1943 proposed the toxic agent in walnut was juglone, a naphthoquinone.[17] Juglone had been extracted earlier[25] and reported along with other

naturally occurring quinones. Studies on the extraction of juglone from walnut trees indicated the compound occurs as a glycoside in vivo and was easily oxidated and hydrolyzed.[25] Pharmaceutically, juglone has been shown to be a potent uncoupler of oxidative phosphorylation,[13] to have fungicidal,[15] antimicrobial, viracidal[1,2,29] activities and to inhibit growth in certain species of plants.[14] Cancer research has found it to have anti-tumor activities[2,30] as well as tumor promotion abilities,[1,2] depending on the tumor group.

The naphthoquinones are naturally occurring protectors for plant life. They serve as feeding deterrents for the bark beetle (Scolytus multistriatus) and the cockroach (Periplaneta americana).[4] These compounds (1,4-naphthoquinones) form complexes with sulfhydryl groups of the sensory receptor proteins of the animals. This is considered the mechanism by which the compounds are detected by the animal.[4]

McDaniels et al., summarized the opinions of several authors as to the toxicology of walnut trees as follows:[16]

1. The antagonism between walnut and other plants has been recognized for centuries.
2. Juglone, a naphthoquinone, has been isolated from many plants in the walnut family (Juglandacea) including Persian walnut (J. regia); black walnut (J. nigra); butternut (J. cinera, J. suboldina, and J. mandshurica). The hickories, Carya ovata,

C. alba; C. olivaeformis, and Pterocarya caucasica.

3. Walnut toxicity is found only when roots of affected plants are in contact with or very close to live walnut roots. Although juglone has been extracted from leaves, bark, and husks of several species of walnut and butternut, the toxic effects on plants seem to be with root contact only.
4. Leachate from walnut husks poured on the ground had an adverse effect on earthworms. In fact earthworm populations are apparently obliterated in areas where there are walnut trees, and "this suggests uncertainty as to whether or not the substance juglone, by itself, is responsible for the toxic reaction or whether there may be a synergistic reaction with other materials."
5. Tomatoes and alfalfa (two susceptible plants to the toxicity of walnut) have been observed growing close to young trees and the fact that many shallow-rooted plants go unaffected by growing under walnut trees may suggest that not all walnut trees form the toxic substance.
6. Where tomatoes have failed in a garden next to a walnut tree; a year after removal of the tree, tomatoes had no problem when planted in the same site. Therefore, one could surmise the toxic principle did not last in the soil for a long period of time.

The toxic affects have also been reported in certain species of animals. As stated earlier walnut extracts from crushed hulls have been used for generations as various forms of folk medicine. Westfall et al., studied the effects of walnut extracts on the primitive organism Daphnia magna, and on higher forms, leopard frogs (Rana pipiens); yellow perch (Perca flavescens); channel catfish (Ictalurus punctatus); and goldfish (Carassius auratus).[30] They prepared various types of extracts from walnut hulls, using water, acetone, ethyl alcohol, chloroform, diethyl oxide (ether U.S.P.), and petroleum ether. They found the petroleum ether and ether U.S.P. proved to be the best solvents for extracting the active principle. After extraction the solvent was allowed to evaporate at room temperature leaving a residue they described as "dark and scaly". This residue was found to sedate fish, with perch being most sensitive and goldfish least reactive of the species tested. A water extract of the residue was also found to have a markedly sedative action on the fish. The water extract of the residue was injected into leopard frogs, albino mice and rats and had the same sedative action. Recovery was noted by all the animals provided the dose did not exceed 1 mg/g of body weight. In attempts to identify the compounds present in the crude ether extracts, Juglone (5-hydroxy-1,4-naphthoquinone) was found. One gram of crude ether extract yielded 0.156 of juglone. Purified juglone given to goldfish, rabbits, and rats produced

sedation. However the sedation was less profound with purified juglone than with the crude material. They concluded there were other active compounds in the extract other than juglone.[30]

Domestic animals have been reported to have adverse reactions to walnut tree products. One incident occurred when a dog chewed on a black walnut statue, fell into a deep sleep for 48 hours then awoke with no apparent ill effects.[22]

Attacks of acute laminitis from bedding horses on walnut shavings are commonplace. At a Wisconsin horse show, six horses out of 30 developed laminitis after a night on fresh bedding containing black walnut.[24] They all eventually recovered. At a Michigan horse show, 11 horses developed acute laminitis after being bedded on shavings containing walnut heartwood. Of these, eight recovered, one died, and two had to be euthanatized for humane reasons due to irreversible laminitis.[24]

True et al. performed an epizootiologic study to determine the incidence of horses developing acute laminitis after being bedded on shavings containing fresh walnut.[24] On six farms surveyed, 116 out of 212 (about 55%) horses developed acute laminitis after being stalled on shavings containing walnut.[26] In an attempt to reproduce the disease, ponies were bedded on shavings and also given 100 gm of shavings *per os* with water from a farm where 28 of 32 horses had developed the disease two weeks earlier. No

ponies developed laminitis. In another attempt shavings from a more recent outbreak (four days earlier) were used to bed two ponies, minimal signs occurred the next day including increased hoof temperature and digital pulse, but no lameness. The animals were normal within 24 hours. The same two ponies were utilized in further experiments. One was given 25 g of finely ground shavings in water via stomach pump. There was no effect. Then they were both fed a mixture of molasses, grain and shavings, again with no results. Then two weeks later both ponies had shavings packed around their lower legs under a plastic sheet covered by a cotton and flannel wrap. No effect was seen after several days. The investigators concluded: 1) Foals were not affected and yearlings were acutely affected but recovered quickly therefore age could be a factor; 2) Ponies were only mildly affected and their experimental attempts with ponies were unrewarding, therefore ponies appear more resistant than adult horses.

Pursuing the theory of juglone being the causative agent in walnut toxicosis True and Lowe performed a series of trials administering juglone in large quantities either intravenously, orally or topically to horses.[27] The animals were divided into four groups. Group 1 was given juglone-ethanol solutions _per os_ at dosages of juglone ranging from 250 mg to 1000 mg mixed in solution with 100 ml of ethanol. Four of the ponies showed no clinical response to 750 mg juglone in 100 ml ethanol _per os_. Two ponies

given 250 mg of juglone in 100 ml of ethanol developed an increased digital pulse and temperature in both forefeet along with increased borborygmi five hours post treatment. The ponies returned to normal within 18 hours. The treatment was repeated in these two ponies two weeks later using 1000 mg juglone in 100 ml ethanol. This treatment did produce mild laminitis in both ponies along with mild colic in one pony. Both ponies were clinically normal within 24 hours.

Another group of two ponies were given 750 mg of juglone orally in a gelatin capsule with no clinical effects.

The third group of three ponies had a solution of 100 mg juglone in 40 ml ethanol applied topically to the forelimbs. All the horses showed signs of increased digital pulses but had clinically returned to normal by 10 hours.

The fourth group of four ponies were given juglone intravenously with varied results. One pony given 750 mg I.V. in ethanol and lactated Ringer's solution died of pulmonary edema 4.5 hours after injection. Two ponies showed no results to three separate injections of 12.5 mg juglone in 5 ml ethanol. One pony showed mild pulmonary edema followed by recovery after a second injection. It was suggested that juglone may have acted as a hapten, causing some of the ponies to react to the second exposure when non-reactive to the first.[28]

The toxic effects of walnut trees can be summarized as follows: the walnut tree does indeed seem to be an allopathic organism. The exact mechanism and degree of allopathy has not been explained. In most species of animals tested some form of pharmacological reaction occurred. Sedation was the most common clinical sign in fish, amphibians, mice, rabbits and canine.

The laminogenic properties of the walnut tree remain a mystery. In field cases certain epidemiological and clinical findings appear consistently. Those being: 1) the bedding of horses on walnut shavings is likely to result in acute laminitis; 2) older, overweight, pampered animals appear more susceptible to the condition and younger animals appear more resistant. Even though some of the toxic properties have been known as early as 37 A.D. they for the most part remain a mystery.

EQUINE LAMINITIS

Clinical Factors

Laminitis is a disease of the equine foot, leading in severe cases to a permanent dislocation of the os pedis, the sensitive laminae and of the deepest layer of the epidermis.[19] The dislocation is due partly to a sinking and partly to a rotation of the os pedis.[19] The cause of this dislocation was suggested to be due to a weakening of the junction between the stratum germinativum and the horny laminae, to the point of inability to support the animal's weight.

Obel developed a grading system to record the degree of severity in an affected animal:

Grade 1--In a standing position the horse lifts the feet incessantly, often at intervals of a few seconds (paddling). At a walk it does not show any lameness but the trot is short and stiff.

Grade 2--The horse moves quite willingly at a walk but the gait is characteristic for laminitis, a forefoot may be lifted without difficulty.

Grade 3--The horse moves most reluctantly. Vigorously resists attempts to life a forefoot.

Grade 4--At this stage the horse does not move without being forced.

The Obel grading system is still widely used by veterinarians for grading severity and for making a prognosis in clinical cases of laminitis.

A comprehensive review by Garner[8] on equine laminitis lists previous research under three categories; descriptive, epidemiologic and mechanistic. Descriptive investigations have dealt with "the known facts" about laminitis including: 1) the crippling effects of abnormal growth rates of quarter and toe as well as rotation of the coffin bone; 2) causes of laminitis such as carbohydrate overload, excessive cold water ingestion, retained placenta, and "road founder"; 3) the pathophysiologic effects including hypertension, digital ischemia coupled with regional digital hyperemia, alterations in blood electrolytes, packed cell volume (PCV), total protein (TP), and blood lactate.

Risk Factors

Garner discussed the relative risk factors for developing laminitis. Ponies posed a higher risk factor for laminitis as far as case numbers were concerned, but horses

effects. It was determined that this was due to the ratio of body mass to the weight-bearing surface of the foot,[8,9,10] with ponies at high risk on the opposite end of the scale from Thoroughbreds and Arabians. The high risk period for ponies seemed to be during the lush growing season of fall and spring, whereas the high risk period for horses seemed to be during the summer and fall; the peak show season. The stress of showing and simultaneous increase of carbohydrate diet imposed on show horses may be the cause for the higher risk in this latter group.

The risk factors related to sex are also related to age. The higher risk for females is between the ages of four and seven and for males it is between seven and ten years.[8,9,10] It is apparent that management, dietary, genetic, and stress factors may be involved in the etiology of equine laminitis.[8,9,10]

Mechanics of the Hoof Lesions in Laminitis

Mechanical integrity of the hoof is based on the density of disulfide bonds. It was found that the decreased incorporation of cystine into keratogenous cells is apparently responsible for the loss of mechanical integrity of the hoof.[5] Precursor cell maturation is though to be methionine-dependent. Depletion of methionine has been hypothesized as an ancillary factor in the pathogenesis of laminitis through the sulfate conjugation of indole and phenol associated with digestive problems.[23]

It is fairly widely agreed that the mechanics of laminitis involves a loss of the onychogenic substance with a decrease in the integrity of epidermal tissue which then separates at the junction of the primary and secondary laminae. This allows a separation of the dermal and epidermal laminae; resulting in rotation of the third phalanx.

PATHOPHYSIOLOGY

Hemodynamics

There are basically two schools of though regarding the hemodynamics of equine laminitis.[12] One group suggests the principle alteration is vasodilation,[20,21] this is based on their findings in isolated perfused feed. During the acute phase of the disease blood flow to the distal leg was increased, vascular resistance was lowered, and the vessels were responsive to vasoactive compounds. There was little variation in either the rate of lymph flow or in lymph protein concentration.

The second school of thought is based on the results of angiographic techniques. These studies indicate a decrease in the terminal vessel size during the acute phase of laminitis. This was interpreted as vasoconstriction and reduced blood flow to the foot.[6,12]

Coffman and Obel have both reported the presence of edema in the coronary band area during the early disease stages; however, there is no true inflammation until 30 to 36 hours after clinical signs appear.[6,19]

Radioisotopic techniques performed by Hood indicated a decrease in perfusion of capillaries occurred along with a

significant amount of arteriovenous shunting in laminitic horse. The changes occurred proportionately with the degree of severity of laminitis. Hood's findings are consistent with studies that indicated an increased flow and a decreased vessel size.[12] This indicates that both schools of thought could be correct. Robinson and workers measuring arterial flow did see an increased blood flow.[20] However, due to arterial-venous shunting there was a decrease in the capillary perfusion measured by Coffman.[6]

Hematology

In laminitis caused by carbohydrate overload there were marked hematologic changes during the acute phase (8-13 hours). Plasma proteins were increased throughout the experiments. The packed cell volume started showing an increase sixteen hours before onset of Obel grade three laminitis. Red blood count and total leukocyte counts remained unchanged, however, a shift in the differential blood count occurred with an increase in both nonsegmented and segmented neutrophils, whole lymphocytes and eosinophils decreased. A significant decrease occurred in platelets only eight hours prior to Obel grade three laminitis.[18] The hematologic changes occurring in the acute phases of laminitis are suggestive of endotoxemia.[18] Platelet

decease and thrombocytopenia can be explained because of increased destruction, and sequestration of platelets.[18]

Histopathology

A histological description of laminitis described in Obel's work is in brief: "The interstices between the original horny laminae are partly empty, partly filled with exudate. The dislocated primary sensitive laminae are, particularly distally, of varying width but otherwise display remarkably mild morphological changes. The connective tissue appears somewhat richer in cells than normal and near the free edge of the laminae decomposing leukocytes are visible. The secondary laminae vary greatly in form and direction. At the base of the primary laminae they are very low or entirely missing. Intergrowth of the secondary laminae belonging to two adjacent primary laminae is common. The sensitive laminae are covered by a heavy layer of non-differentiated epithelial cells, which, however are flattened, partly peripherally towards the wall, partly in the center of the cell masses filling the interstices between the sensitive laminae, and in these places they display a changed affinity for dyes, a sign of beginning keratinization. The space between the original horny laminae and the epithelial covering to the sensitive laminae is filled with a layer of exudate adhering to the latter and having in certain places a thickness of more than 2 mm. The

peripheral part of this layer mainly consists of fibrin while in the deeper part there are diffuse and isolated hemorrhages and numerous decomposing leukocytes filling the meshes of the fibrinous network. In the distal part of the toe there are numerous isolated hemorrhagic areas adjacent to the free edge of the sensitive laminae and sometimes directly connected with these (epithelial covering missing)."[19]

Models of Equine Laminitis

A reliable model for the study of equine laminitis is not available. This has hampered research in this area and in part accounts for the poor understanding of the pathophysiology of this disease. A limited amount of work has been done on developing a model for the study of laminitis.

To produce laminitis Obel fasted the animals 48 hours. Then via a stomach tube administered 1.5 liters of 0.4% sodium bicarbonate and 1.0 liter of colibacilli culture that had been incubated for 6-8 hours followed by 0.5 liters more of the bicarbonate.[19] The horses were then fed rye grain ad libitum. Eight of nine horses developed typical laminitis. In all cases hoof symptoms were preceded by violent diarrhea and a considerable rise in body temperature and pulse rate.[19]

A starch model for experimentally inducing laminitis is available. Garner and co-workers compared vital signs of starch induced laminitis with the naturally occurring disease. Their model consisted of tube feeding horses of gruel of 85% cornstarch and 15% wood cellulose flour (Teracon Inc., Topeka, KS). They noted a simularity in changes in arterial pressure, heart rate, central venous pressure, rectal temperature, packed cell volume, leukocyte counts and total protein between the natural disease and the starch model.[10] Cardiovascular collapse, cerebral edema, anorexia, diarrhea, endotoxic shock, death are all complications associated with the starch model making study of the equine foot difficult at best.

The objectives of this study are to determine if walnut shavings can cause clinical laminitis in the equine and to gain information for the following questions: 1) Is juglone the sole causative agent? 2) Can an aqueous extract of walnut shavings be used as a model for future laminitis research?

The plan was to produce laminitis in horses using an aqueous extract of black walnut heartwood. Water was chosen as the solvent because of juglones aqueous insolubility. This was to aid in the determination of the possibility that other compound(s) are present as the laminogenic agent and would suggest the hypothesis that an agent/agents other than juglone are the factor(s). Laboratory analysis of the

aqueous extract for the presence of juglone will give further credence to the hypothesis that a substance(s) other than juglone is/are responsible for causing laminitis in horses stalled on fresh walnut shavings.

MATERIALS AND METHODS

Animal Experiments

Four mares and six geldings between five and fifteen years of age and weighing between 425 and 550 kg were used in this experiment. Each horse was given an examination prior to inclusion in the study and a blood count, total protein, packed cell volume and stool examination for parasite ova was performed.

A soundness examination was also performed prior to experimentation to determine the health status of the animals feet. If any foot problems were present the animal was rejected from the experiment.

The animals were held on a 40 acre pasture with adequate water and shelter until used. Prior to experimentation the horses were transferred into the Veterinary Clinical Center and housed in 10 x 10 foot stalls with straw bedding over a concrete floor. Water and good quality alfalfa hay fed free choice and one kilogram of oats was fed twice daily.

Shavings Preparation and Administration

In a pilot study utilizing three horses it was determined that whole ground shavings were impractical to administer. The pilot study had also indicated that a water extract of shavings was effective in producing laminitis. A walnut branch (Juglans nigra) approximately 25 cm in diameter and 60 cm in length was obtained from a living tree in the fall of the year. After removal of the bark and sapwood the heartwood was passed through a power planer (Sears, Roebuck and Co.) to create large (approximately 1 mm thick) shavings. The shavings were then weighed into 500 gm portions, sealed with twist ties in clear plastic bags (8x4x34 inches .006 PAK-SAK Industries Inc., 122 A. Aspen Street, Sparta, MI 49345) and frozen at -4^{o}C until ready to use. Prior to use the frozen shavings were weighed to the appropriate dosage (2 g/kg body weight) and passed through a Wiley^{R} mill (screen size 2 mm) (Arthur H. Thomas Co., Philadelphia, PA) to increase the surface area. The horses were dosed at 2 g of shavings per kg of body weight. After the shavings were weighed, approximately 7 to 8 liters of distilled water were added to each batch by adding water to the original plastic bag. The bag was placed in a two and a half gallon bucket for support, then sealed with a twist tie and placed on a shaker (Eberbach Corp., Ann Arbor, MI 48106) set on low overnight at room

temperature. The mixture was filtered through cheesecloth and glass wool to remove particulate material. The filtrate was then administered via stomach tube. Vital signs (temperature, pulse, and respiration) were monitored at two-hour intervals for 24 hours. For humane reasons animals were treated for laminitis within 24 hours of clinically displaying laminitis. The degree of clinical laminitis was assessed using the Obel grading method.[19]

Preparation of Heartwood, Bark, and Nuts for Extraction

One hundred grams each of heartwood, bark, and nuts were obtained from the same tree used in the clinical phase of the experiment and passed through a Wiley^{R} mill. The mill was thoroughly cleaned between each grinding.

The 100 g sample was added to 2000 ml of distilled water, covered, and placed on a shaker overnight at room temperature. The solution was then filtered using Whatman #4 qualitative paper with suction (Fischer Scientific Product, Livonia, MI 48150).

XAD-2 Column Chromatography

Active compounds were extracted by passing 500 ml of filtrate through a column containing XAD_2 resin (Supleco, Bellefonk, PA 16823). The XAD column was prepared as follows: XAD_2 resin was equilibrated in water with 0.1% sodium azide until ready for use. Glass wool was

placed at the bottom of a 25 ml Pyrex buret (Fischer Scientific), filled with resin, and capped with glass wool at the top. The XAD column was rinsed with 100 ml of methanol; 50 ml 6:1 chloroform-isopropanol; 50 ml methanol; and 200 ml of milli-Q water (18 meg-ohm resistance, 4 bowl milli-Q system, Millipore Corp., Bedford, MA 01730). After rinsing, 240 ml of the sample filtrate was passed through the XAD column followed by 5 ml milli-Q water. Next the column was eluted with 60 ml of chloroform-isopropanol (6:1) and the eluate was dried by passing through anhydrous sodium sulfate. After elution the sample volume was reduced under vacuum on a rotary evaporator to approximately 5 ml. The sample was then ready for fractionation in preparation of gas chromatography-mass spectrometry.

Gel Permeation Column Preparation

Seventeen grams of Biobeads SX-3^{R} (Biorad Laboratories, Richmond, CA 94804) were placed in a beaker and covered with toluene:metylene chloride (85:15). The biobead particles were allowed to swell overnight. After 24 hours the solvent was decanted and fresh solvent added. This procedure was repeated two or three times in order to remove the fine particles.

A glass wool plug was placed in the bottom of a 100 ml buret. The gel was stirred into a slurry and poured into the column. The teflon stopcock was opened and the solvent

was allowed to drain while fresh solvent was continually added. This procedure was continued for two to three hours in order to allow the column to pack as much as possible. This formed about a 72-cm bed of SX-3 biobeads.

The sample was placed on the column and the first 50 ml was collected to be used as a blank. Five milliliter fractions were then collected up to 100 ml and another 50 ml was collected to ensure column cleanup. The fractions were then taken to dryness under a stream of nitrogen in preparation for gas chromatography/mass spectrometry (GC/MS).

Gas Chromatography/Mass Spectrometry

GC/MS was carried out on a Finnigan 3200 GC/MS with a Riber SADR data system. The column was 1.5 m x 2 mm I.D. packed with 1% OV-17 on Ultrabond 630. Helium carrier flow was 30 ml/min, and oven temperature was 150^{o}C. Spectra were taken at an electron energy of 70 eV.

The fractions from gel permeation chromatography were redissolved in 50 ul of chloroform, and 1-2 ul of sample were injected. To prevent column contamination throughout all procedures the samples were prepared and run in the following order; a blank, shavings, nuts, bark and juglone standard. The wood was run first in order to minimize possible column contamination, since juglone has been reported in the nuts and bark but not in the heart of juglandaceae wood.[22]

Quantification of Standard

Quantification of juglone was accomplished by interpolation from a standard curve of GC/MS peak area versus concentration. Reagent grade juglone was obtained from Sigma Chemical Co. (St. Louis, MO 63178). Fifty milligrams of juglone were dissolved in 50 ml of chloroform-isopropanol (6:1). This provided a standard juglone solution of one ug/ml. From the standard solution a serial dilution was made to give working solutions of 500 ng/ul, 250 ng/ul, 125 ng/ul, and 62.5 ng/ul. From these solutions 0.8 ul, lul, 1.2 ul, 1.5 ul, 1.7 ul, of the 50 ng/ul solution, 1 ul, 1.3 ul, and 1.7 ul of the 250 ng/ul solution, and 1.7 ul, 1.0 ul of the 125 g/ul solution were injected into the GC/MS using a ten microliter syringe (701-N Hamilton Co., Reno, NV 89510). A blank was run between each sample to ensure a clean column.

Interpretation of Data

The retention time of reference juglone samples was compared with retention times of the samples comparing total and specific ion (118, 173, 174) chromatograms. Peak identity was confirmed through the use of the Riber SADR data system of the GC/MS.

RESULTS

A. Animal Experiment

Clinical Trial

Eight of the ten horses receiving the aqueous extract of walnut shavings developed clinical laminitis of Obel grade 3 or greater. Of the eight affected horses, one was euthanatized for humane reasons. The remaining seven recovered uneventfully with treatment. Treatment consisted of short-term phenylbutazone therapy (4 mg/kg every 12 hours for 48 hours) and moving the animal to a marshy area of soft ground.

Of the two animals that failed to show clinical laminitis, one of the horses, a mare, was in heat. Her feet appeared warmer than expected and an increase in rectal temperature occurred from $99^{\circ}F$ to $101^{\circ}F$. Within 24 hours the temperature returned to $99^{\circ}F$. The other animal, a gelding, leaned against a wall exhibiting penile relaxation and was stuporous as if tranquilized. The gelding was normal within 24 hours.

Clinical Parameters

Within eight to twelve hours post-dosing the temperature, pulse, and respiration of the affected animal showed a noticeable increase, returning to base line values in approximately 48 hours. The temperature ranges of the pretreatment animals varied from 99°F to 100°F and during the acute phase ranged from 101°F to 103°F, an increase of 2-4°F.

Pulse rates of the pretreatment animals ranged from 30 to 40 beats per minute and during the acute phase varied from 40 to 60 beats per minute. During the acute phase of the clinical disease the normal breathing pattern was altered. Flaring of the nostrils and rapid shallow breathing was exhibited by all affected animals.

Edema was prominent in the forelimbs of all affected horses from the coronary band to the carpus. In four animals a marked pitting edema was noted from the stifle area to the coronary band and from the neck to the chest. Occasionally it was noted that edema was more severe in an individual limb.

B. Laboratory Analysis

XAD-2-Column Chromatography Results

Results of the extraction of reference juglone from an aqueous solution using XAD column chromatography demonstrated that greater than 85% recovery was obtained. The method was sufficient for recovery of juglone from aqueous heartwood, bark, and nut extracts. In addition, the method afforded some measure of clean-up from aqueous solution. A large amount of material was left on the column after juglone was eluted.

Gel Permeation Chromatography (GPC)

The results of reference juglone chromatography on a permeation column and GC/MS analysis of the eluent indicated that juglone eluted at highest concentration in the 65-70 ml fraction and in lower concentrations in the 60-65 and 70-75 fractions. Juglone was not detected in any other fractions. Following chromatography of the XAD extracts of heartwood, bark and nut sample, a yellow color was present in the fractions containing juglone, deepest in the 65-70 ml fraction which contained the highest concentration.

Gas Chromatography/Mass Spectrometry

Reference juglone was chromatographed at various oven temperatures to obtain the optimum temperature for elution (140°C, 150°C, 160°C). From these preliminary runs it was found that the optimum temperature for chromatography was 150°C. Juglone eluted with a retention time (RT) of 2.1 minutes under these conditions (Figure 1).

The mass spectrum of reference juglone contained major fragment ions at M^+=174, M/e 173 and 118. These ions were therefore chosen to identify juglone by selective ion monitoring.

Both total ion and specific ion chromatograms of the nut and bark revealed peaks with the same retention time (RT=2.1 minutes) as juglone (Figures 2,3,4,5). The sample of heartwood shavings exhibited a large distinct peak at approximately 3.30 minutes, but ions of 174, 173 and 118 were not present in the specific ion chromatograms (Figures 6 and 7), and no evidence of juglone at RT=2.1 minutes was apparent.

Mass spectra of the nut and bark samples corresponded to that of the reference standard indicating the presence of juglone (Figure 8). None of the major ions of juglone 174, 173 and 118 were

present in the shavings at RT=2.1 minutes indicating the absence of this compound at the sensitivity of the GC/MS (150 ng) (Figure 8). Furthermore, the large peaks which eluted at 3.3 and 5.0 minutes did not contain the molecular ion or major fragment ions of juglone.

Quantification of the juglone present in the bark and nut extracts revealed the highest concentration was present in the bark, 365.11 ug, while the nut extract contained 192.66 ug. The values were based on a 100 g sample with no correction for extraction loss.

Figure 1. Selected ion chromatograms of juglone. Scan 23, retention time (RT) = 2.0 minutes. Selected ions at M/e 174, 173, 118, and total ion scan.

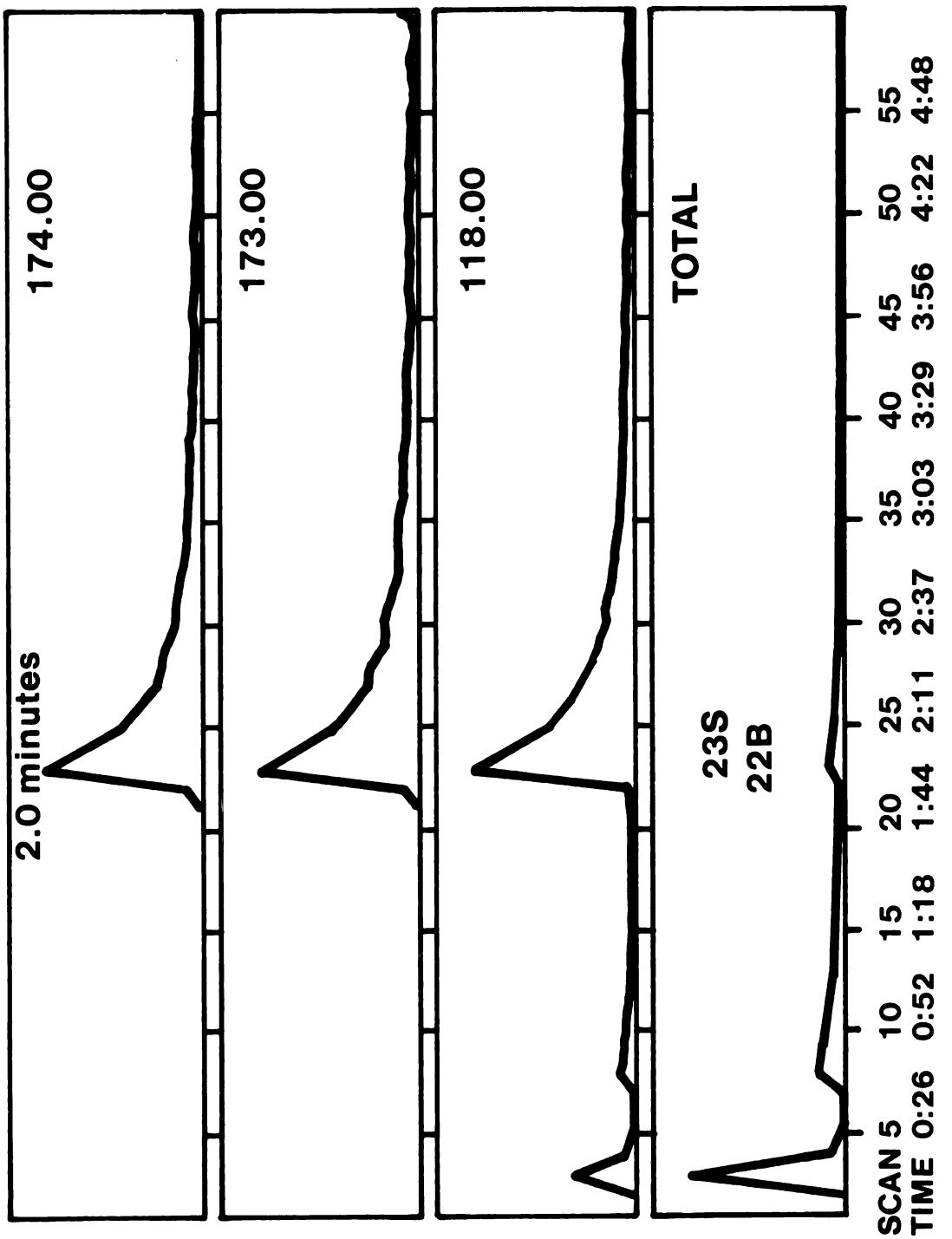

174.00
173.00
118.00
TOTAL
2.0 minutes
23S
22B
SCAN 5 10 15 20 25 30 35 40 45 50 55
TIME 0:26 0:52 1:18 1:44 2:11 2:37 3:03 3:29 3:56 4:22 4:48

Figure 1.

Figure 2. Total ion chromatogram of 65-70 ml fraction of bark following gel permeation chromatography. Note peak at scan 23, RT=2.1 minutes.

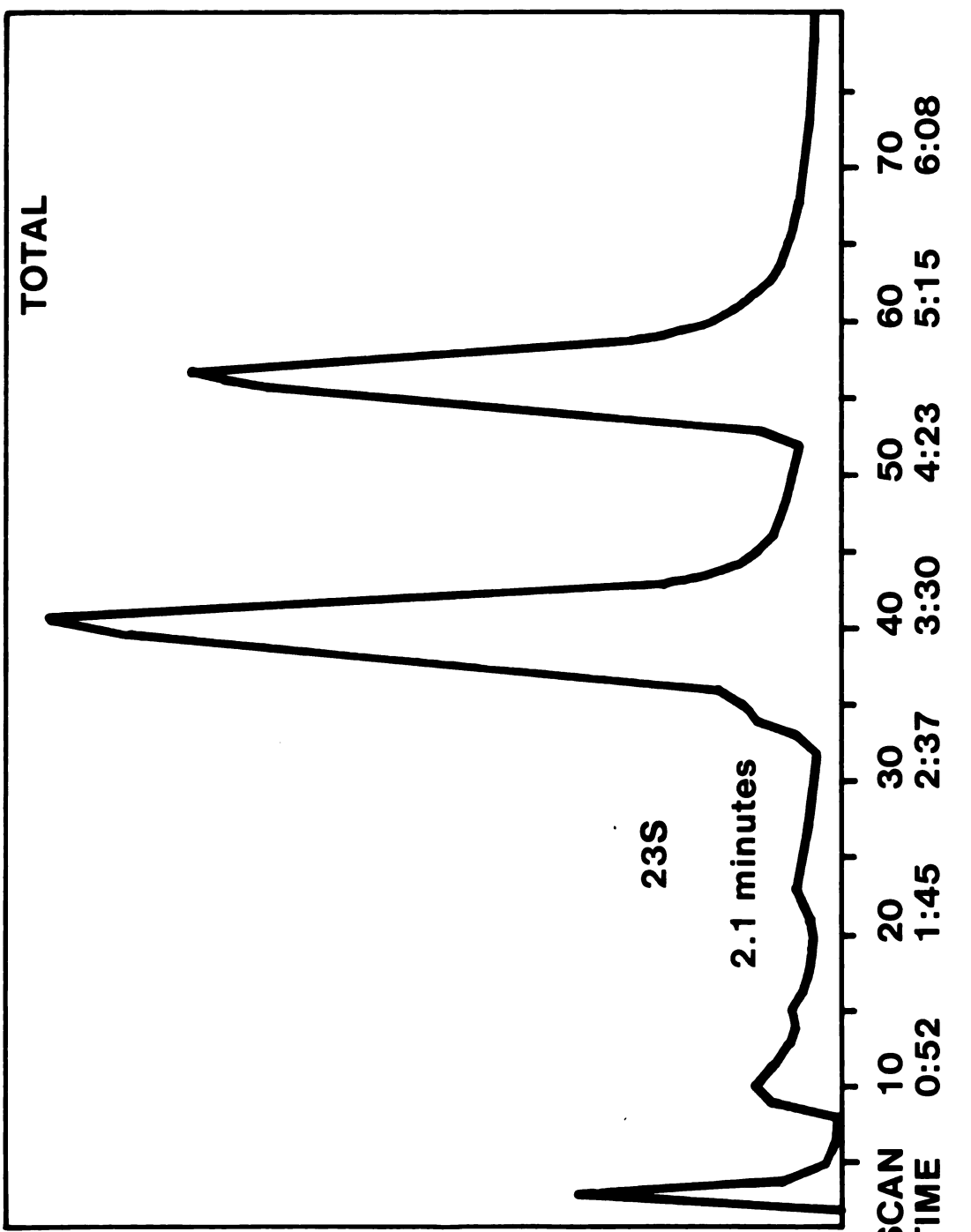

TOTAL
23S
2.1 minutes
SCAN 10 20 30 40 50 60 70
TIME 0:52 1:45 2:37 3:30 4:23 5:15 6:08

Figure 2.

Figure 3. Selected ion chromatogram of 65-70 ml fraction of bark following gel permeation chromatography. Selected M/e 118, 174, 173, and total ion scan, scan 23, RT=2.1 minutes.

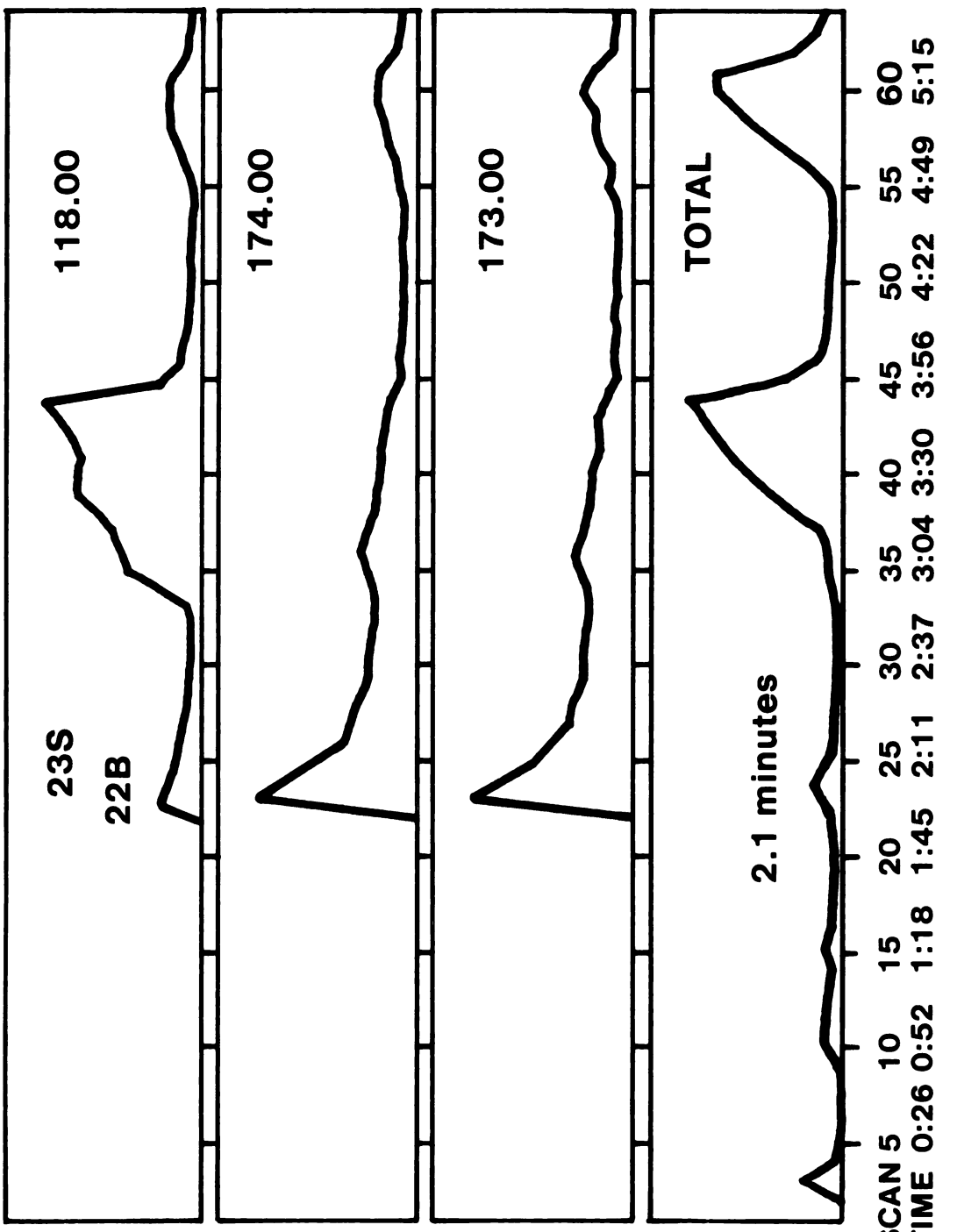

23S
22B
118.00
174.00
173.00
TOTAL
2.1 minutes
SCAN 5 10 15 20 25 30 35 40 45 50 55 60
TIME 0:26 0:52 1:18 1:45 2:11 2:37 3:04 3:30 3:56 4:22 4:49 5:15

Figure 3.

Figure 4. Total ion chromatogram of 65-70 ml fraction of nuts following gel permeation chromatography. Note peak at scan 23, RT=2.1 minutes.

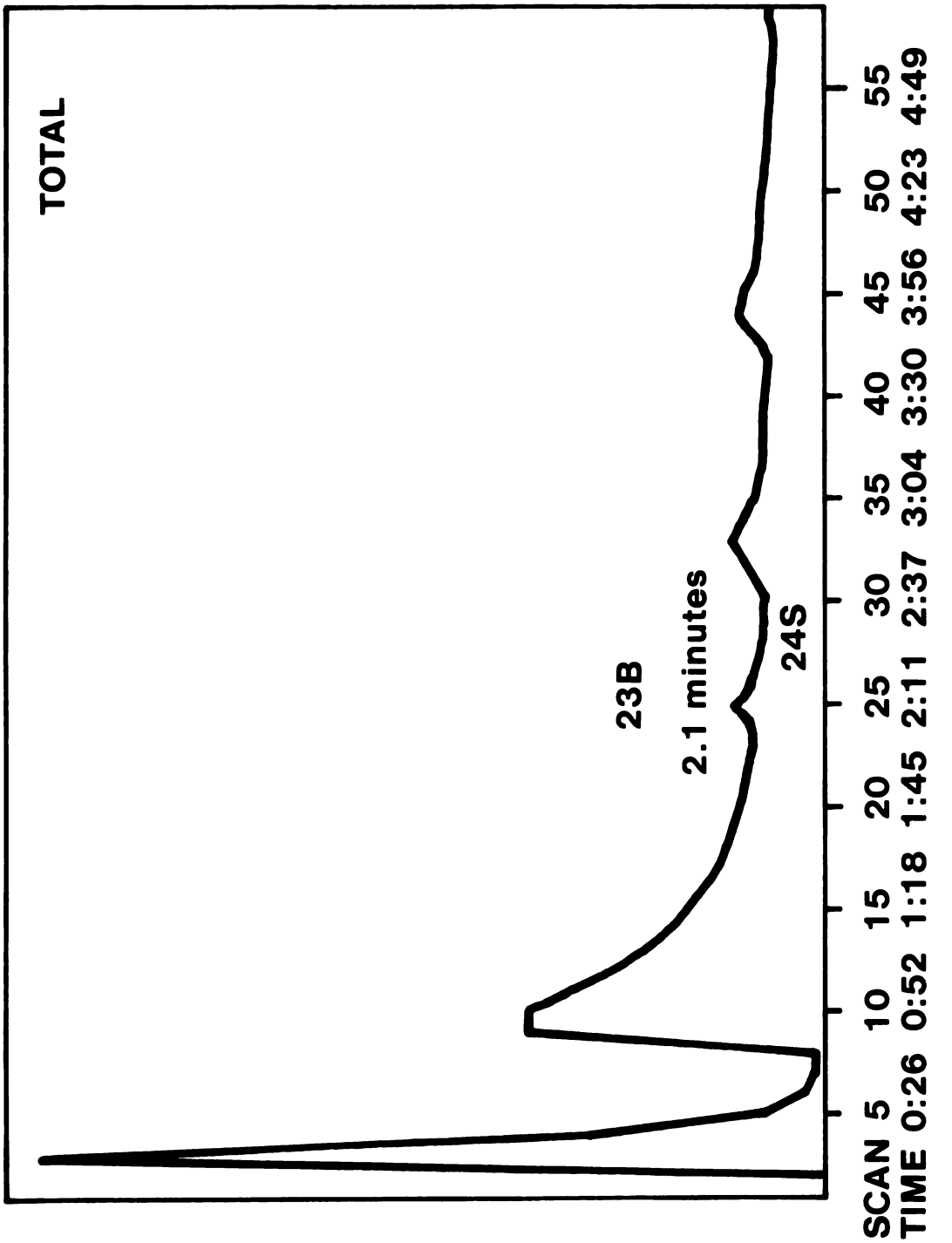

TOTAL
23B
2.1 minutes
24S
SCAN 5 10 15 20 25 30 35 40 45 50 55
TIME 0:26 0:52 1:18 1:45 2:11 2:37 3:04 3:30 3:56 4:23 4:49

Figure 4.

Figure 5. Selected ion chromatogram of 65-70 ml fraction of nuts following gel permeation chromatography. Selected ions at M/e 174, 173, 118 and total ion scan. RT=2.1 minutes.

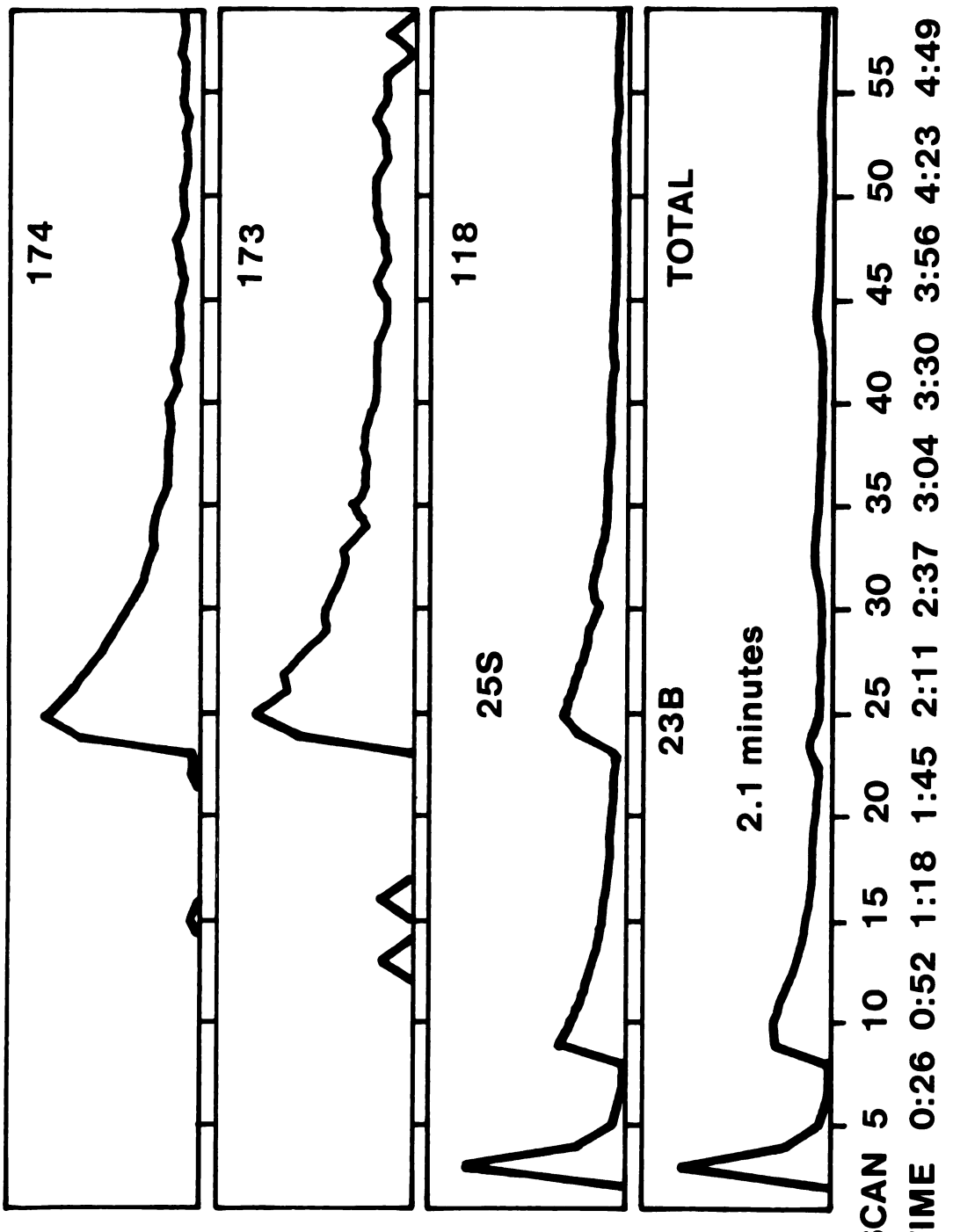

174
173
118
25S
TOTAL
23B
2.1 minutes
SCAN 5 10 15 20 25 30 35 40 45 50 55
TIME 0:26 0:52 1:18 1:45 2:11 2:37 3:04 3:30 3:56 4:23 4:49

Figure 5.

Figure 6. Total ion chromatogram of 65-70 ml fraction of shavings following gel permeation chromatography. Note: absence of peak at RT=2.1 minutes.

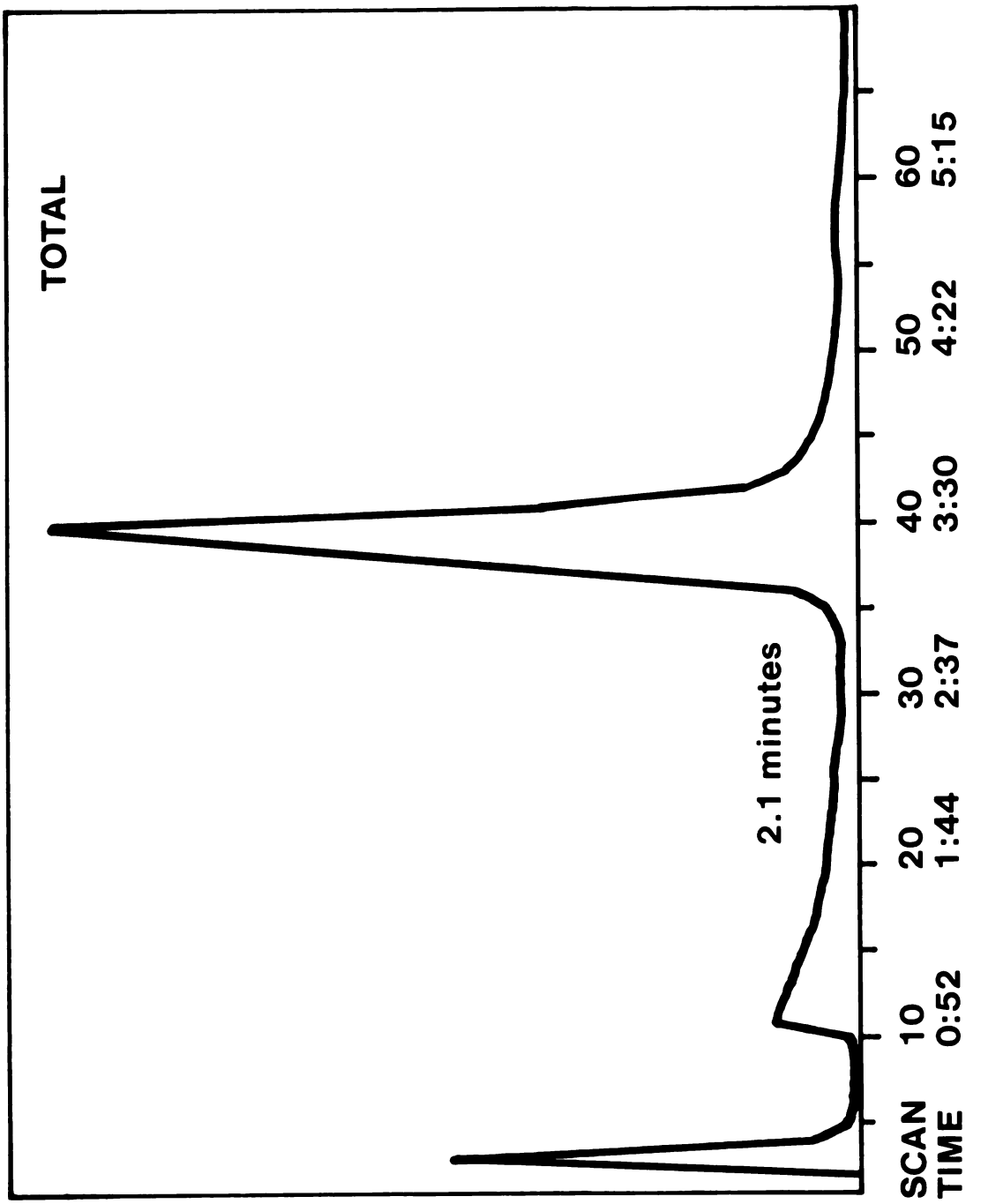

TOTAL
2.1 minutes
SCAN 10 20 30 40 50 60
TIME 0:52 1:44 2:37 3:30 4:22 5:15

Figure 6.

Figure 7. Selected ion chromatogram of 65-70 ml fraction of shavings following gel permeation chromatography. Lack of ions 118, 174, 175 at RT=2.1 minutes indicates absence of juglone in detectable quantities.

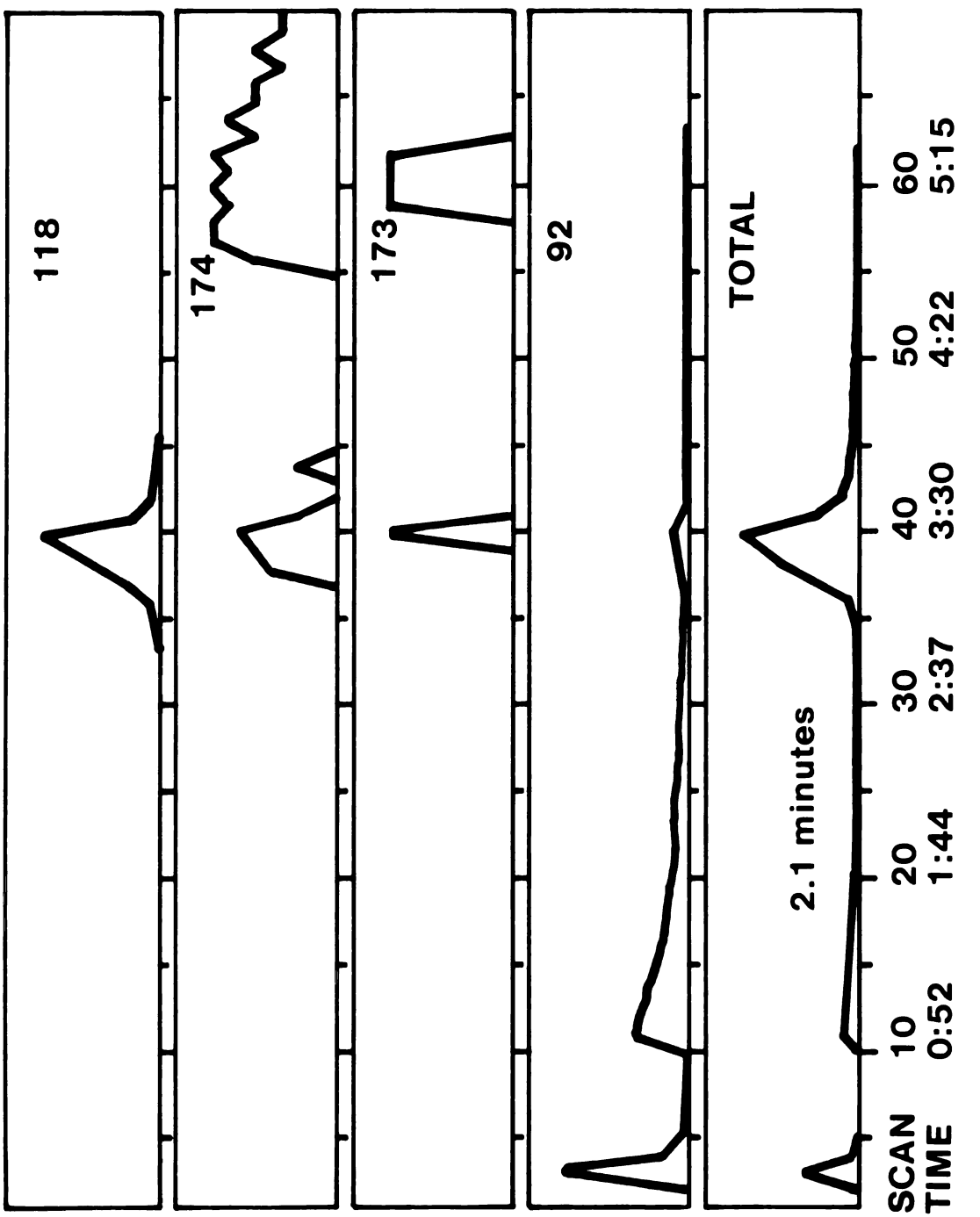
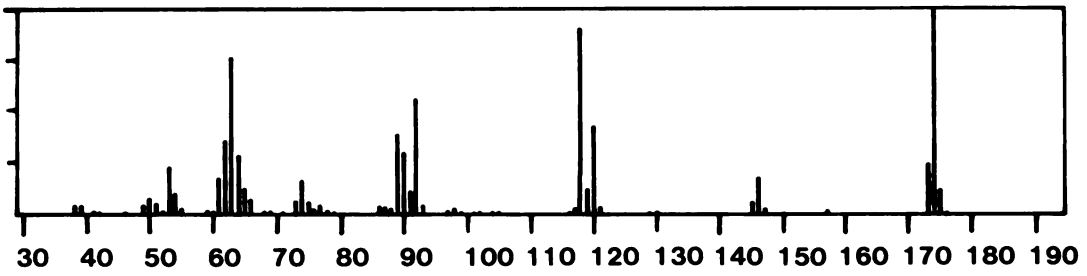

118
174
173
92
TOTAL
2.1 minutes
SCAN 10 20 30 40 50 60
TIME 0:52 1:44 2:37 3:30 4:22 5:15

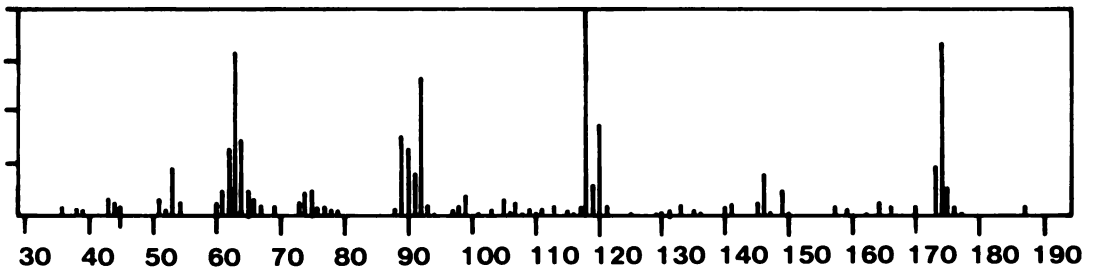
Figure 7.

Figure 8. Mass spectra of juglone, nuts, and bark of 65-70 ml fractions after gel permeation chromatography. Confirming presence of ions M/e of 118, 173, 174, in nuts and bark.

STANDARD AFTER GPC JUGLONE 1 UL



NUTS 65-70 ML
Scan 25 RT=2:1



BARK 65-70 ML
SCAN 23 RT=2:1

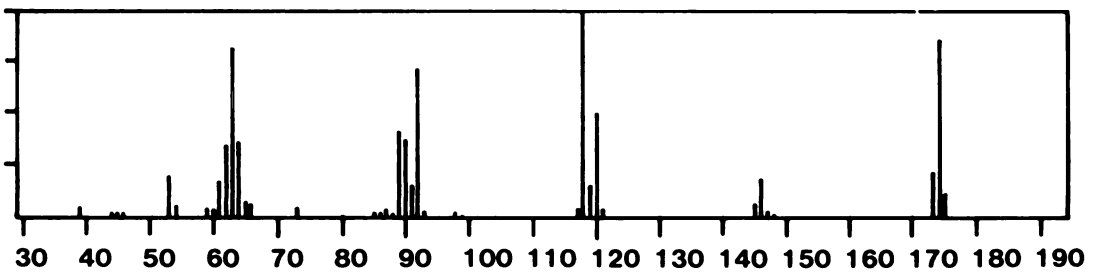


Figure 8.

DISCUSSION

Walnut

It is widely agreed that the black walnut tree has noxious as well as therapeutic properties.[7,11,16,17] The results of the present experiments are in concurrence with previous pharmacologic findings. Early work by Massey in 1940 and Brown in 1942 demonstrated allopathic affects of walnut trees on tomato and alfalfa plants. The isolation of juglone, a 1,4-naphthoquinone, by Thomson brought this compound into the spotlight for further work. Since juglone was shown to be pharmaceutically active[1,2,13,14,15] and functioned in nature as a deterrent by forming complexes with sulfhydryl groups[4] it became the prime suspect for the causative agent in equine laminitis associated with walnut shavings. True's unsuccessful attempts to produce laminitis with purified juglone administered intramuscularly, intravenously, or *per os* questioned its involvement and agrees with the hypothesis of this paper that some other agent or agents are responsible for the laminogenic properties of walnut shavings.

Westfall's work indicated the presence of other active compounds by the production of a more profound sedation in perch, leopard frogs, mice, rats, and rabbits in aqueous and ether extracts of walnut hulls than with purified juglone.[30] Rowe also reported sedation in a dog that chewed on a walnut statue.[22] These findings correlate with the results of this experiment in that sedation was noted in one of the two horses that did not develop laminitis from the aqueous extract of heartwood.

Conformation of walnut shavings having laminogenic qualities was achieved when eight of the ten horses studied developed clinical laminitis after oral dosing of an aqueous heartwood extract. These results agree with the reported natural outbreaks, when horses developed laminitis after being bedded on fresh shavings containing black walnut.

Laboratory

This experiment utilized gas chromatography/mass spectroscopy (GC/MS) to detect the presence of juglone in an aqueous extract of walnut heartwood, bark, and nuts. The results agree with Woods findings that juglone is present in nuts, and bark but not reported to be found in heartwood suggesting that juglone is not the causative agent.[31]

The lack of juglones water solubility further decreases the likelihood of it being a laminogenic agent. Using water as a solvent, increases the risk of not detecting its presence. In these experiments, that possibility is discounted since juglone was detected in bark and nuts after aqueous extraction. If it was present in the heartwood in quantities less than the detection limits of the GC/MS it would be in quantities many times less than those administered unsuccessfully in True's experiments.[26,27] Both the experiments reported by True and this study suggest that juglone is not a major laminogenic agent. Further analytical studies are indicated to characterize compounds present in the aqueous extract for their possible laminogenic qualities.

The total ion chromatogram in Figure 2 contains two distinct peaks at about 3.3 minutes and five minutes. Identification of the compounds creating these peaks could be of interest for further studies. Another possibility for the active substance is a precursor of juglone, dihydrojuglone. True may have unknowingly chemically transformed juglone into dihydrojuglone in an experimental group in which ethyl alcohol was used as a diluent for oral administration of juglone. It is worth noting that this is the only group in which two ponies out of six reacted mildly with an Obel grade 1 laminitis.[27] A commercial source of dihydrojuglone has not been identified.

Equine Laminitis

Equine laminitis is an extensively researched and poorly understood disease. Its severity ranges from mild discomfort to the horses foot to a severely debilitating and life threatening condition. The mechanics and epidemiology of laminitis is for the most part widely agreed on by researchers. However, the pathophysiology of laminitis remains unclear and the concepts are continually being challenged. Presently there are two schools of thought regarding the distribution and flow of blood to the distal laminitic leg. Robinson, using perfusion techniques reported vasodilation, with increased blood flow to the distal leg along with lowered vascular resistance. Coffman utilized angiographic techniques to show reduced filling of laminar vessels. This was interpreted as vasoconstriction and reduced blood flow to the foot. Perhaps both schools of thought are correct.[6] The techniques of measurement could be the primary difference and arterio-venus shunting being the physiological occurrence being measured by both groups.

This experiment was not designed to be a detailed study of equine laminitis but rather a means to hopefully improve the "tools" available for expanding the knowledge of equine laminitis. A significant result of this research was the development of an alternate method of inducing

laminitis. With starch administration, the present method of induction, the study of laminitis is unpredictable and clinical complications are a significant problem. Starch induction is accompanied by a variety of clinical manifestations resembling endotoxemia, neutropenia, colic, diarrhea, cerebral edema and sporadic death. On the contrary, laminitis observed in this experiment with walnut induction, was uncomplicated. Animals, after six to eight hours post-induction, were clinically normal except for laminitis and edema of the lower legs. The horses maintained normal appetites and bowel movements throughout the experiment. Also, since eight of ten horses reacted to the aqueous walnut extract with laminitis, the method may evolve into one which will predictably produce laminitis.

Even though this study showed laminitis could be produced using an aqueous extract o walnut heartwood its potential for an improved model for laminitis study requires further research.

This investigation dealt only with the clinical production of laminitis with an aqueous extract. Physiologic parameters other than temperature, pulse and respiration rates were not determined; therefore, physiological parameters between other models and this model cannot be compared. Due to the small number of animals used in the experiment the frequency of laminitis production is not firmly established. Factors, such as the

stage of growth, age, and geographical location of the black walnut tree were uncontrolled and could be important aspects of the toxicity. Finally, further chemical characteristics of the aqueous extract is important since the actual compound or compounds responsible for laminitis in the equine remain unknown.

SUMMARY

The following conclusions can be drawn from this experiment.

Clinical Results

1. Shavings of heartwood from a black walnut tree (Juglans nigra) does contain substances laminogenic to horses.
2. The causative agent or agents can be separated from the shavings by an aqueous extraction procedure.
3. The aqueous extract appears to be a predictable model in that it produced clinical laminitis in eight out of ten horses within 8 to 12 hours of oral administration.
4. The aqueous extract appears to be an improvement over available models now used to study equine laminitis because the horses appear clinically normal except for their laminitic feet. This will greatly simplify the study of the pathophysiology of equine laminitis.

Chemistry Results

Juglone, the previously incriminated compound was not found in the aqueous extract of walnut heartwood, but was detected via GC/MS in aqueous extracts of nuts and bark of the walnut tree.

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