AN ABSTRACT OF THE THESIS OF

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(Name) (Degree) (Major)

Date thesis is presented 5-11-65

Title A PHARMACOGNOSTIC STUDY OF THE ROOT OF HERACLEUM MANTEGAZZIANUM SOMIER ET LEVIER

Abstract approved [redacted]
(Major Professor)

A pharmacognostic study was made of the root of Heracleum mantegazzianum, an umbelliferous species which is of value as a source of natural coumarins.

Three groups of seeds were tested to determine the presence of a germination requirement for moist cold and the possible value of gibberellic acid in obviating this requirement. The first group of seeds was cold-treated for 74 days at a range of from 2 to 5°C. The second group of seeds was soaked for 20 hours in a solution containing 100 parts per million of gibberellic acid. The third group of seeds, used as controls, was soaked in water. When planted and maintained under greenhouse conditions only the cold-treated seeds germinated. The germination rate was 10.3%. Both seeds treated with gibberellic acid and the controls failed to germinate, showing that a cold requirement does exist and that treatment with gibberellic acid will not substitute for the cold requirement. A fourth

group of seeds, cold-treated for 294 days, germinated at a rate of approximately 55%, showing that germination is proportional to total days of cold treatment.

Sequential selective solvent extraction with a series of increasing polarity gave the following fractions as average percentages of dry weight: petroleum ether, 2.4; ether, anhydrous, 2.5; ether U.S.P., 2.0 alcohol 95%, 8.5; water, 19.4. Copious foaming of the aqueous fraction showed the possible presence of saponins.

The description and histology of the whole root as well as the character of the powdered root was recorded. Among diagnostic characters of the powdered root are its blue coloration when moistened with iodine water, a slight yellow fluorescence under ultraviolet light turning to luminescence for several seconds following extinction of the light, and the presence of numerous clustered starch grains which gelatinize rapidly in chloral hydrate solution.

Thin-layer chromatography of the ether extract of the air-dried root on Silica Gel G showed seven principal spots fluorescing under ultraviolet light. R_f values and colors corresponded to those of six standard coumarins. The following coumarins are presumed to be present: isobergapten, pimpinellin, bergapten, isopimpinellin, sphondin and umbelliferone. The seventh spot was not identified. The R_f values in two solvent systems and the fluorescent colors under ultraviolet light are reported for the extract spots as well as

for six coumarins.

Studies were also made to determine the relative stability of coumarins to heat. Six standard coumarins as well as the extract of the air-dried root were spotted individually on Silica Gel G plates and developed two-dimensionally. Following development in the first direction and prior to development in the second direction the plates were exposed to a temperature of 65°C. for 30 minutes in an oven. With the exception of sphondin the chromatograms of the individual coumarins showed from one to two additional spots. This indicates that coumarins as a group are subject to modification at a temperature of 65°C.

Regular two-dimensional chromatography of the ether extract of the root material which had been dried at 38°C. gave spots for only five coumarins. It is concluded that the roots should be dried only at the normal air temperatures.

A PHARMACOGNOSTIC STUDY OF THE ROOT OF *HERACLEUM MANTEGAZZIANUM* SOMIER ET LEVIER

by

EUGENE CARLTON LEE

A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

June 1965

APPROVED:

[redacted]

Professor of Pharmacognosy

In Charge of Major

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Dean of Graduate School

Date thesis is presented 5-11-65

Typed by Eula Weathers

ACKNOWLEDGEMENT

The author expresses his appreciation to Dr. Leo A. Sciuchetti, Professor of Pharmacognosy, for his counsel and guidance during the course of the research and the preparation of this paper.

The author also acknowledges the valued advice and interest of Dr. Philip Catalfomo, Assistant Professor of Pharmacognosy.

Thanks go also to Dr. David French of Reed College, Portland, Oregon, whose original interest in this plant and whose generous provision of seeds initiated this research; to Dr. Stewart A. Brown of Trent University, Peterborough, Ontario, Canada; and to Dr. Michiichi Fujita of the Tokyo College of Pharmacy, Tokyo, Japan, who freely provided coumarins for use as standards.

Special thanks are extended to my wife, Martine, whose assistance and encouragement made this work possible.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

A PHARMACOGNOSTIC STUDY OF THE ROOT OF HERACLEUM MANTEGAZZIANUM SOMIER ET LEVIER

I. INTRODUCTION

Renewed interest in the plant kingdom as a source of new drugs has led to various studies among genera of the Umbelliferae. Considerable attention has recently been focused upon species of the genus Heracleum.

Heracleum is a boreal genus of wide distribution consisting of about 60 species. The greatest distribution is to be found in China, Siberia and montane India, with the range extending southward to Ethiopia and eastward to Japan. Elsewhere the species are found chiefly throughout the mountains of southern Europe, in Spain, Greece, Sicily, throughout the Caucasus and parts of Asia Minor (9, p. 239).

The most common European species is Heracleum sphondylium L., extending from Spain through most of central and northern Europe to the western boundaries of the Orient and through Siberia to Kamchatka (9, p. 240). Heracleum sibiricum L. occupies a range in Europe and northern Asia (22, p. 1131). Heracleum mantegazzianum[1] Som. et Lev. was originally a species in the region of the Caucasus.

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[1] Named after Paolo Mantegazzi, an Italian natural scientist and ethnographer (1848-1922) (21, p. 1422).

It has since been introduced into England and become naturalized in waste areas, particularly near rivers (7, p. 674).

Only one species, Heracleum lanatum Michx., is native to North America. It is also found in Siberia and the Kurile Islands (21, p. 535). Hultén (24, p. 252) found it common on all islands of the Aleutian chain, agreeing completely with the Kamchatka specimens except that the Aleutian plants were of a more depressed growth.

The Heracleums[2] are large, sturdy, pubescent biennials or perennials having large, ternately or pinnately-compound leaves with broad, sheathing petioles generally conspicuously inflated (31, p. 268). The inflorescence is a large, flat-topped compound umbel with small flowers which are white (yellow) or tinged with red or green. The outer flowers of at least the marginal umbellets are usually irregular and larger than the inner and often bilobate (22, p. 535). The involucre may be present or lacking, with the involucel composed of numerous linear bractlets.

The fruits are flattened dorsally, and broadly ovate, ovate or orbicular, with thin lateral wings (37, p. 580). These characteristic dicarpellate fruits are termed "cremocarps." At maturity they separate into two halves, each of which is known as a

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[2]From the name for Hercules (31, p. 268).

"mericarp." The plane of separation is referred to as the "commissure" (43, p. 111).

Heracleum sphondylium L. (European Cow Parsnip, Hogweed, Eltrot) has been used in Europe as a vegetable. The young shoots and leaves were boiled and eaten while newly-sprouted shoots were considered to rival asparagus in flavor. The stalks stripped of their rind have been used as food in some parts of Asiatic Russia (11, p. 300).

In North America Heracleum lanatum L. (Cow Parsnip, Masterwort) has not generally been recognized by caucasians as a food plant although it has been used by the Indians. The northern Indians ate the peeled stalks, raw or cooked, while the Meswaki ate the cooked root (11, p. 298). Gunther (17, p. 42) states that the plants were also eaten by the Makah, the Chinook and the Quinault. Haskin (20, p. 235) states that all tribes from Alaska to California used the fresh stems.

Medicinally, Stuhr (52, p. 137) indicates that the roots and leaves are acrid, irritant and poisonous, yet reputed to have carminative, stimulant and antidyspeptic properties. The National Standard Dispensatory of 1908 (18, p. 1664) stated that the plant has been used with alleged value in epilepsy, apparently depending on gastro-intestinal irritation, and that it was thought to correct dyspeptic disorders. Train et al. (1, p. 57) enumerated various

medicinal uses by the Indian tribes of Nevada. Small pieces of the root were inserted into cavities to stop toothache, while preparations of the root were used as a gargle, poultice and a treatment for coughs and chest colds.

The National Standard Dispensatory (18, p. 1664) stated that in their fresh state the leaves and roots might cause vesication following contact with the skin and that the sap of some European species has been used as an escharotic for warts.

Uphof (59, p. 184), in his Dictionary of Economic Plants, lists only two species which have a present-day utility: Heracleum persicum Disf. of Iran, whose seeds are used as a condiment in pickles, and Heracleum sphondylium, whose boiled leaves and fruits are used to prepare an alcoholic beverage by the poorer classes in Slavic countries, and is used in France in liqueurs.

Attention has been focused upon the Heracleum species as a source of natural coumarins. The simple hydroxy and methoxy compounds, as well as coumarin itself, occur widely in many different families, but increasing complexity of the compounds is apparently accompanied by greater restriction in respect to familial distribution (47, p. 232). The coumarins are found principally in the Gramineae, Orchidaceae, Leguminosae, Labiatae, Rutaceae and Umbelliferae, and to a lesser extent in some other families.

The coumarins collectively constitute a somewhat diverse

group and may be classified according to several systems. A practical classification is outlined in Table I. In the following text the term "coumarins" will be used in a broad sense and will be inclusive of all categories unless qualified.

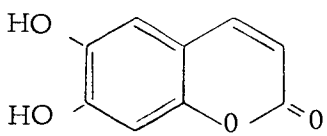
Although the coumarins constitute a large and significant class of compounds, their pharmacology has not yet been fully determined. Bose (5) has pointed out that they possess numerous and often unique physiological actions. Recognition of the anticoagulant character of the coumarin moiety has resulted in the development of such useful and effective drugs such as bishydroxycoumarin, warfarin, coumachlor, acenocoumarol and cyclocoumarol (47, p. 247).

The estrogenic properties of compounds such as coumestrol (VII) are well known and numerous related compounds have been studied to determine their comparative estrogenic activity. The unique dermal-photosensitizing action of the furanocoumarins has been utilized in the treatment of vitiligo and leucoderma (47, p. 254-257).

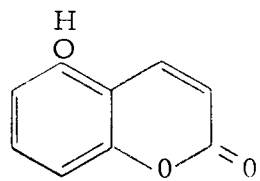
Other coumarins have demonstrated some degree of antibiotic activity. Novobiocin is a fungal metabolite of Streptomyces niveus. Chartreusin (VIII), another antibiotic, has been isolated as a metabolite of Streptomyces chartreusis (47, p. 258-259). Other important properties of the coumarins are curare-like, sedative, narcotic, analgesic and anthelmintic actions. These, as well as

TABLE I. REPRESENTATIVE COUMARINS

SUBSTITUTED COUMARINS

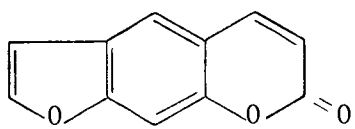


(I) Esculetin

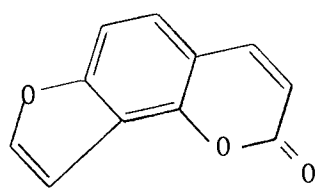


(II) 4-Hydroxy coumarin

FURANOCOUMARINS



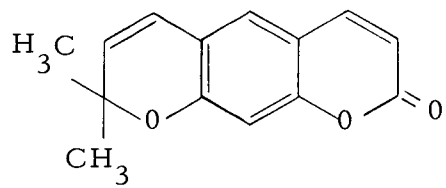
(III) Psoralen



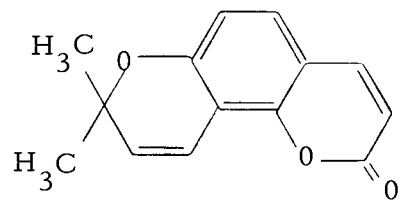
(IV) Angelicin

TABLE I. REPRESENTATIVE COUMARINS

PYRANOCOUMARINS

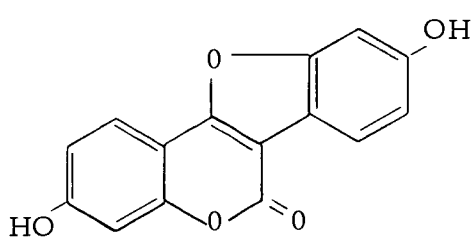


(V) Xanthoxyletin

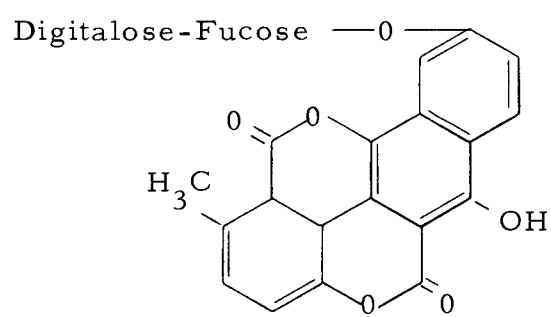


(VI) Seselin

COMPLEX COUMARINS



(VII) Coumestrol



(VIII) Chartruesin

other active properties, are summarized by Soine (47, p. 246).

Previous phytochemical studies have demonstrated the presence of coumarins in a number of Heracleum species. The distribution of coumarins throughout the species studied is presented in Table II. A survey of the literature revealed that no work had been done to show the presence of coumarins in Heracleum mantegazzianum although other chemical aspects have been reported.

Piguleoskii and Kovaleva (38) have determined that the essential oil distilled with steam from the fruit contained no aldehydes but unsaturated compounds which proved to be largely esters of octyl alcohol and, to some extent, hexyl alcohol with acetic, butyric and optically-inactive isovaleric acid. Piguleoskii (39) established that the volatile oil of the leaves, studied by the Raman method, contained ocimene (2, 6-dimethyl-1, 5, 7-octatriene). Ziegler and Mittel (66) found that sucrose was the only sugar present in the seive tube sap and Ziegler (64) showed the presence of uridine disphosphate-glucose in the sap of the phloem. Ziegler (65) also had studied the respiration and transport of substances in the isolated conductive vessels of the petiole.

In anatomical studies, Troll (58) had isolated from the pit cavity of the petioles folded vascular bundles which might be as long as one meter when stretched out.

Seeds of the Umbelliferae have been noted for germination

TABLE II. DISTRIBUTION OF COUMARINS IN HERACLEUM SPECIES.

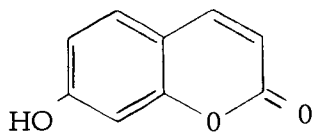
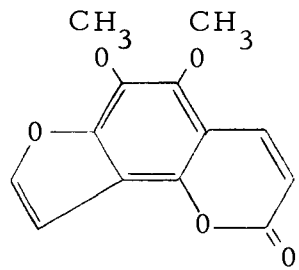
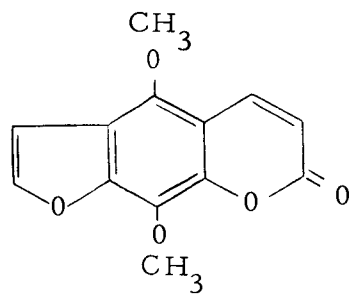
Name	Structure	Species	References
Umbelliferone		panaces	(56) (54)
		sibiricum	(55) (54) (27, p. 534)
		lanatum	
		var. nipponicum	(15)
Pimpinellin		sphondylium	(48) (25) (27, p. 560)
		lanatum	(14) (27, p. 560)
		var. asiaticum	(34)
		var. nipponicum	(54) (15)
		panaces	(54) (56)
		sibiricum	(55) (54) (28)
Isopimpinellin		sphondylium	(48) (25) (27, p. 555)
		concanense	(2)
		lanatum	(14) (27, p. 556)
		var. asiaticum	(34)
		var. nipponicum	(53) (15)
		panaces	(54) (56)
		sibiricum	(54) (55) (28)

TABLE II (Continued)

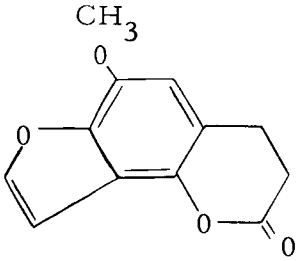
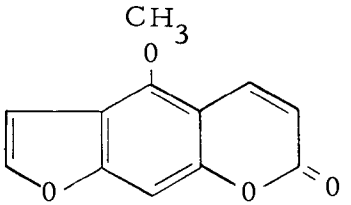
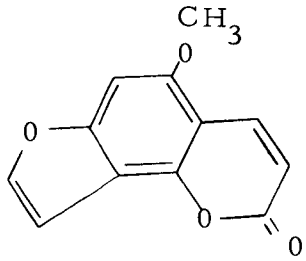
Name	Structure	Species	References
Sphondin		sphondylium	(48) (25) (27, p. 560)
		panaces	(56)
		sibiricum	(55) (28)
		lanatum	(14) (27, p. 560)
		var. nipponicum	(15)
Bergapten (Heraclin) (Majudin)		sphondylium	(25) (49) (27, p. 551)
		giganteum	(54) (27, p. 551)
		nepalense	(27, p. 552) (3)
		panaces	(54) (56)
		sibiricum	(55) (54) (28)
		concanense	(2)
		lanatum	(27, p. 552)
		var. asiaticum	(34)
		var. nipponicum	(15)
Isobergapten		sphondylium	(48) (27, p. 560)
		panaces	(54) (56)
		sibiricum	(55) (54) (28)
		lanatum	(14) (27, p. 560)
		var. asiaticum	(34)
		var. nipponicum	(15)

TABLE II. (Continued)

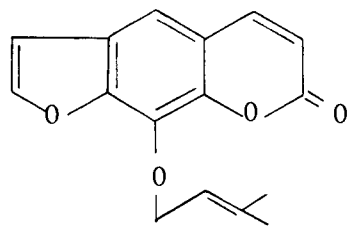
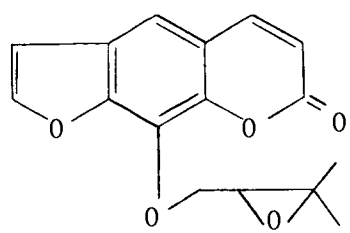
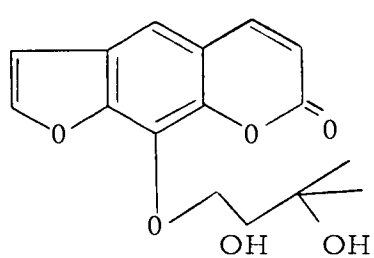
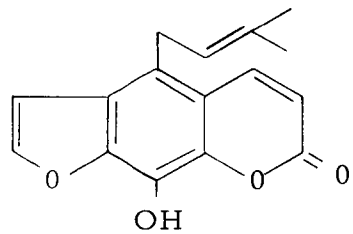
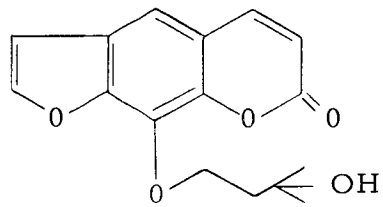
Name	Structure	Species	References
Imperatorin		lanatum var. asiaticum	(34)
Heraclenin		candicans	(45)
Heraclenol		candicans	(46)

TABLE II. (Continued

Name	Structure	Species	References
Alloimperatorin		nepalense	(3)
Byakangelican[3]		nepalense	(3)
Sphondylin[4]	$C_{12}H_8O_4$	sphondylium	(48)

[3] Probable identification (3).

[4] A monomethoxy furanocoumarin of uncertain structure, isometric with isobergapten and sphondin (47, p. 240). Wessely and Koltan reported in 1955 that sphondylin was resolved by paper chromatography into spots for sphondin and isobergapten (63).

difficulties. Germination standards for the cultivated Umbelliferae have been set much lower than those for the majority of other plants. In some cases this is due to a lack of embryos in apparently viable seeds. This problem appears to be universal, occurring in all parts of the world and in all members of the Umbelliferae and, moreover, appears to be correlated with the season of the year. There is strong evidence that infestation by the Lygus bug, as well as possibly other members of the Miridae, is a prime factor in the destruction of the embryo in numerous species of the Umbelliferae (41, p. 531-538).

Certain unsaturated lactones appear to be widely distributed in plants and to possess the power of inhibiting seed germination. Among these compounds are coumarin and parasorbic acid (33, p. 520). In spite of the frequent occurrence of other germination inhibitors the compounds have been identified only in comparatively few cases (60, p. 25).

The seeds of some species have a requirement for after-ripening in moist cold. Stokes has shown that in the case of Heracleum sphondylium there is an after-ripening requirement of 8 to 12 weeks of moist cold in order for the seeds to germinate (50). There have been no reports in the literature for such a requirement in the case of Heracleum mantegazzianum.

Attempts have been made to obviate the cold requirement in

the dormancy of certain seeds by chemical means. These efforts have not been rewarded by signal success (8, p. 104). Considerable interest has focused upon growth substances such as the gibberellins in these attempts. Gibberellin treatment substituted completely for the light requirement in the seeds of lettuce and several other species, but it only partially substituted for the cold requirement in experiments with the sweet cherry and peach. It did not totally replace the cold requirement (53, p. 380-381). No reports were found concerning the effect of gibberellins on the seed of Heracleum mantegazzianum.

In view of the foregoing information it was decided to pursue the following research objectives:

1. To conduct germination studies to ascertain the presence of a cold requirement in the seeds and, further, to determine if gibberellic acid treatment would substitute for such a cold requirement.

2. To propagate plants under greenhouse conditions for the purpose of harvesting root material for further pharmacognostic study.

3. To study the histological and diagnostic characters of the root.

4. To determine the type of components present in the roots by performing a selective solvent extraction.

5. To test for the presence of coumarins in the root by the use of thin-layer chromatography.

6. To determine if heat should be avoided in the drying of the root.

Figure 1. Heracleum mantegazzianum growing in garden of Dr. David French, Portland, Oregon. Umbellets are not fully expanded.

II. EXPERIMENTAL

All seeds used in this study were received in the fall of 1962 from Dr. David French, Professor of Anthropology at Reed College, Portland, Oregon. Dr. French had originally received seeds provided by Dr. C. Leo Hitchcock from his Seattle garden in 1958 and which Dr. Hitchcock had stated to be those of Heracleum mantegazzianum. These seeds had been planted by Dr. French in his garden at 3549 S. E. Woodstock, Portland, Oregon, in 1959. In 1960 the plants produced basal leaves only, but the following year they produced stalks which flowered and fruited. The plants were about ten feet tall. The seeds used in the research were harvested from one of these plants and are represented by lot no. 2122D.

In 1962 Dr. French submitted some of these plant materials to Dr. Lincoln Constance, a specialist in the Umbelliferae, at the University of California at Berkeley for confirmation. Documentary evidence of his verification is in the form of a letter from Dr. French to the author in which he states:

> Lincoln Constance at Berkeley, one of the world's foremost authorities on the Umbelliferae. . . . . has now positively identified 2122C (in flower) as Heracleum mantegazzianum Somm. & Lev. Specimens from such numbers as 2122D are from the same plants, and the identification can be extended to them as duplicates.

Dr. French has deposited a voucher specimen of this plant in the Oregon State University herbarium as well as vouchers for the

chromosome count (n=11) in the herbarium of the University of California at Berkeley.

Germination of Seeds

Group 1 (Cold Treatment)

On March 29, 1963 a total of 60 seeds were wrapped in cloth, moistened with tap water and placed in a refrigerator at a temperature range of from 0 to 2°C. Four days later, since ice crystals formed on top of the cloth, the temperature was raised to a range of from 2 to 5°C. On June 11, after 74 days of cold, they were removed and soaked in several changes of distilled water. Small and depauperate seeds were removed and the remaining 39 seeds were designated as Group 1.

Group 2 (G. A. Treatment)

Thirty-nine dry seeds, selected for size and ample endosperm, were placed in a beaker and soaked for 20 hours in a solution containing 100 p. p. m. of gibberellic acid[5] in distilled water. These seeds were designated as Group 2.

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[5] Gibberellic acid, 88. 9% pure. Supplied through the courtesy of Dr. Edwin F. Alder, Agricultural Research Center, Eli Lilly & Co., Greenfield, Ind.

Group 3 (Controls)

Thirty-nine dry seeds, selected for size and ample endosperm, were placed in a beaker and soaked for 20 hours in several changes of distilled water. These seeds were designated as Group 3 and intended for use as controls.

Germination

Each group of seeds was individually planted in a flat containing a mixture of one part sand and two parts sandy loam with 50 grams of complete fertilizer.[6] The seeds were planted by being placed in rows on top of the soil mixture and being covered lightly with a thin layer of vermiculite.

The seeds were maintained under normal greenhouse care and allowed to germinate at a temperature range of from 18 to 27°C. for a period of 38 days. At the end of that time the results were recorded.

In Group 1 (the cold-treated seeds) there was a total of three seedlings. Four seeds had actually germinated, but one seedling had failed on the 29th day, possibly due to "damping off." Germination did not occur in Group 2 (gibberellic acid-treated) or in Group 3 (controls).

[6]Organic Morcrop, Chas. Lilly Co., Seattle, Washington. (Analysis: total nitrogen, 5%; available phosphate, 3%; available potash, 2%.

Extended Cold-Treatment

On November 5, 1963, 1000 seeds, selected for good size and ample endosperm, were placed in a litre of tap water and soaked for a period of 36 hours. They were then removed and placed upon a towel which was then formed into a roll, moistened with water, and placed in a refrigerator at a temperature range of from 2 to 5°C. After a total of 294 days the cloth roll was removed from the refrigerator and examined. Extensive germination had occurred and many of the primary rootlets had penetrated the fabric. This resulted in considerable damage to the seedlings during the opening of the roll and made it impossible to determine exactly the total number of germinated seeds. However, it was estimated that at least 550 seeds had germinated.

Propagation of Plants

On July 26, 1963 the three seedlings obtained from the germination experiment of seed Group 1 were transplanted to ten inch peat pots containing a mixture of one part of sand, and two parts of sandy loam with ten grams of Organic Morcrop. These plants were respectively designated as SF-1, SF-2 and SF-3.

Earlier, on June 28, six seedlings, obtained by cold-treating and germinating seeds on moist blotting paper in Petri dishes, had

been transplanted to four inch peat pots with one part of sand and two parts of sandy loam with 2.5 grams of Organic Morcrop per pot. Two of the seedlings had failed. On July 26, the sides of the pots containing the four surviving seedlings were slashed and the pots were embedded in ten inch peat pots with the same soil mixture as Group 1. These plants were designated respectively as PD-1, PD-2, PD-3 and PD-4.

On June 15, a number of volunteer seedlings were obtained from the Portland garden of Dr. David French. These were all descendants of a single plant which had fruited in 1962 and had been designated as French 2122D. At the time of collection the seedlings varied from 13 cm. to 25 cm. in height. The seedlings were dug from the soil, their roots moistened, and were then transported by automobile to Corvallis. On the following day they were placed in ten inch peat pots with a soil mixture identical to that previously described and watered. On the next day it was observed that four of the plants were very badly wilted so their leaves were removed as well as part of the leaves of several other plants. The plants were maintained in the greenhouse where the temperatures ranged from 15°C. at night to 35°C. during the day. In order to discourage infestation by "white fly" the under surfaces of the leaves were

A B

Figure 2. Germinating seedling, showing: A. early stage; B. later stage with linear cotyledons emerging from "husk."

sprayed twice weekly with Miller's Tetradane.[7]

On August 15, it was observed that some foliar damage had occurred. On some of the younger leaves the areas at the endings of the veins in the apices and notches of the leaves displayed a drawn-together, shriveled appearance. These areas later became desiccated and devitalized. It was also observed that a number of young, emergent leaf buds had withered and died.

On the assumption that damage might have been caused by the spray in combination with high daytime temperatures the greenhouse temperatures were reduced to a range from 18°C. at night to 21°C. during the day, and from August 16, "white fly" was controlled by application of Ortho Rose Dust[8] to the tops and bottoms of the leaves with a dust gun. Although damage to tender leaves and buds was reduced it was not totally eliminated.

On October 20, at time of harvest, the heights of the plants were recorded. These data as well as the average height of each plant group are shown in Table III.

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[7]Miller's Tetradane (Insect Concentrate Spray) Ornamental and Rose Spray, Miller Products Co., Portland, Oregon. (Containing Kelthane, Diazinon, Lindane and DDT)

[8]Ortho Rose Dust, California Spray-Chemical Corp., Richmond, California. (Ingredients: Lindane 1%, Phaltan 7.5%, DDT 5%, Sulfur 30%, Inert ingredients 56.5%.)

TABLE III. GROWTH OF PLANTS AT HARVEST

Plant No.	Days From Transplant	Height Per Plant (cm.)	Average Height Per Group (cm.)
PD-1	92	36.3	33.5
PD-2	92	32.3	
PD-3	92	31.6	
PD-4	92	33.7	
SF-1	92	30.0	34.6
SF-2	92	33.7	
SF-3	92	40.0	
TP-1	132	13.2	19.4
TP-2	132	24.2	
TP-3	132	16.0	
TP-4	132	16.7	
TP-5	132	19.3	
TP-6	132	31.0	
TP-7	132	27.8	
TP-8	132	21.0	
TP-9	132	10.7	
TP-10	132	22.5	
TP-11	132	26.0	
TP-12	132	16.0	
TP-13	132	14.5	
TP-14	132	12.4	

Plant PD-1 Height: 33 cm.

Plant TP-8 Height: 20 cm.

Figure 3. Contrast in growth attained by two plants of different groups at harvest.

Harvest

On October 20, plants PD-1 and PD-4 were set aside for further study and the remainder of the plants of the three groups were harvested. The petioles were first severed several inches above the crowns and discarded together with the leaves. The roots were removed from the pots by washing the soil away with water from a hose in such a way as not to detach any small rootlets and to remove last traces of soil. Extraneous matter such as twigs and small bark fragments were removed by garbling. The remaining portions of the petioles were cut away from the crowns and excess moisture removed by blotting with a cloth towel. The roots were then cut into small pieces and the larger segments split to facilitate drying. The roots were immediately weighed on a Welch balance to determine fresh weight.

The roots were then spread out on mesh screen drying racks and placed in a forced-air drying oven at 38°C. for 50-1/2 hours. The roots were then removed from the drying oven, weighed to determine their dry weight, placed in plastic bags with closures and stored in clean, dry cans which were sealed with tape.

Histological Study

Preparation of Slides

On February 17, 1964 plants PD-1 and PD-4, previously reserved, were harvested. These plants had been maintained outside of the greenhouse under normal environmental conditions. A portion of the root from one of the plants was prepared for killing and fixing by being washed thoroughly with water and then sectioned into short segments. The segments were then immersed in formalin-aceto-alcohol solution[9] of the following formula:

Formaldehyde	5 ml.
Glacial acetic acid	5 ml.
Ethanol 95%	90 ml.

The vial containing the root segments in the killing solution was transferred to a refrigerator where it was stored for several weeks.

Upon removal from the killing solution the segments were washed with two changes of 50% ethanol and then run through the tertiary-butanol dehydration process according to the schedule of Table IV and as outlined by Johansen (26, p. 130-131) with modifications in regard to time.

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[9] This killing reagent may be used with almost any plant material intended for anatomical study. Material may be kept in it almost indefinitely without appreciable damage (26, p. 41).

TABLE IV. DEHYDRATION SCHEDULE

Steps	1	2	3	4	5	6[10]
Fluids in mls.						
Distilled water	50	30	15	---	---	---
Ethanol 95%	40	50	50	45	---	---
Ethanol 100%	---	---	---	---	25	---
Tertiary-butanol	10	20	35	55	75	100

Following dehydration the material was prepared for embedding by impregnation with 50% paraffin oil in tertiary-butanol at room temperature. It was then placed on solid Parowax in a glass vial, covered with the paraffin oil and tertiary-butanol mixture and heated in an oven at 75°C. for 12 hours. The segments were then removed and infiltrated successively with two lots of melted Parowax and two lots of melted tissue mat in an oven at 75°C. The vial was removed from the oven and the contents poured into a prepared embedding tray made from stiff, folded paper; the segments were positioned, and the tray was rapidly cooled in cold water and refrigerated. The finished blocks were prepared for mounting and microtoming by cutting and trimming.

[10] At this stage the alcohol was tinted with erythrocin in order to impart a light stain to the root material. This clarifies its orientation in the embedding material.

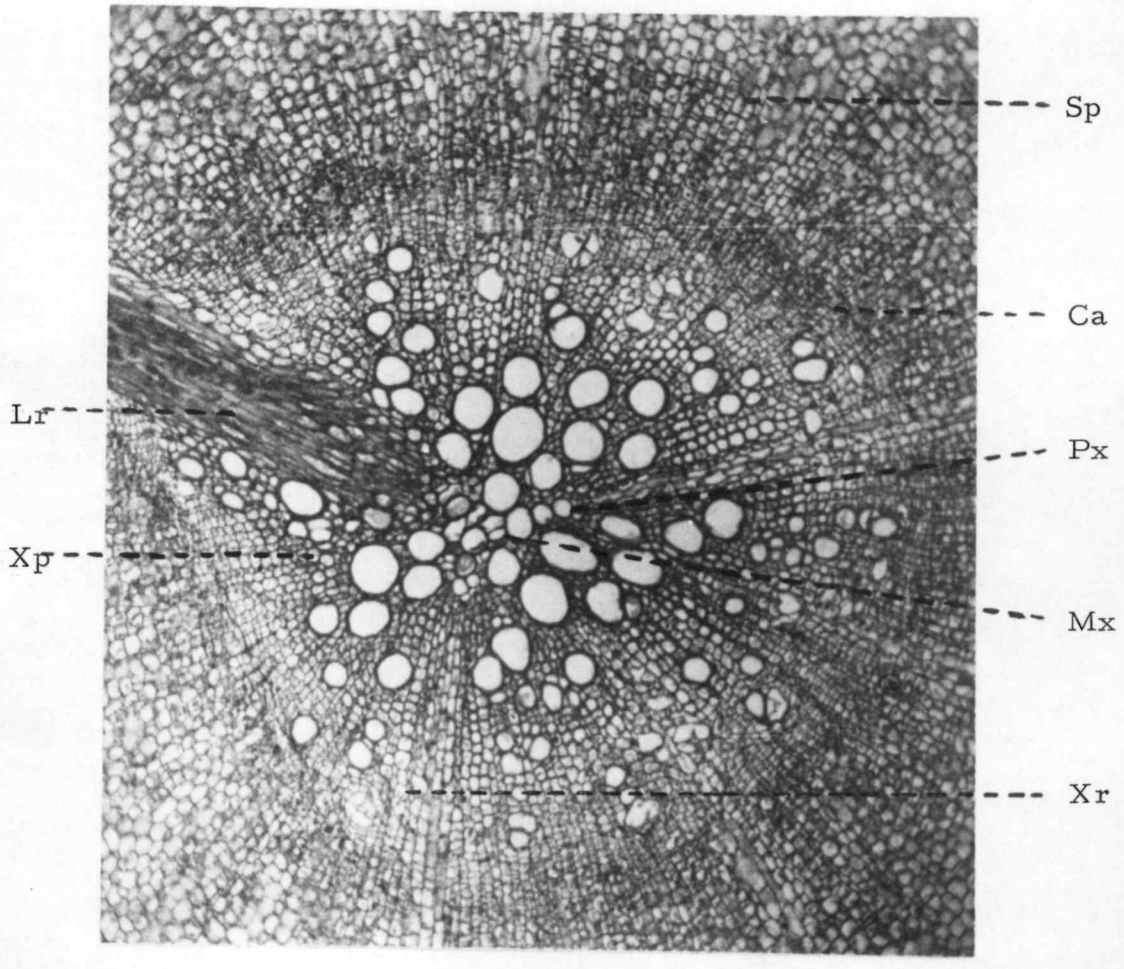
The microtoming, staining and completion of the slides was done by Mr. Gerald Bogar, a recent graduate student in the botany department, as hereafter briefly described. The trimmed blocks were sectioned at ten microns on a rotary microtome, the ribbons affixed to slides with Haupt's adhesive, and the sections stained with 1% safranin in water, destained with acid alcohol, and counterstained with aniline-blue in clove oil and 100% alcohol. The stained sections were cleared with clove oil, rinsed and washed with xylene and then mounted in 60% H. S. R. mounting resin in toluene.

Photomicrography

Photomicrographs were prepared as follows: The slides were placed upon the stage of a Bausch and Lomb binocular microscope using an independent light source[11] coupled with a Jefferson transformer. A 5X ocular was used in combination with 10X and 43X objectives. The camera was a 35 mm. model XV Exacta with the lens removed and equipped with an improvised adapter. No filters were used.

Eastman Plus-X film was used and the optimum exposures determined by experimentation. The negatives were developed with Ethol, single mix, ultra fine grain developer and were fixed with Eastman Kodak fixer. Enlargements were made on Eastman Kodabromide paper with an Omega type A2 enlarger. Interpretations of the photomicrographs are shown in Figures 4 and 5.

[11] Bausch and Lomb lamp, type 31-33-77.



85X

Figure 4. Transverse section of stele of root.
Explanation: Sp, secondary phloem with phloem parenchyma; Ca, cambium; Mx, metaxylem; Px, protoxylem; Xr, xylem ray; Lr, lateral root; Xp, xylem parenchyma.

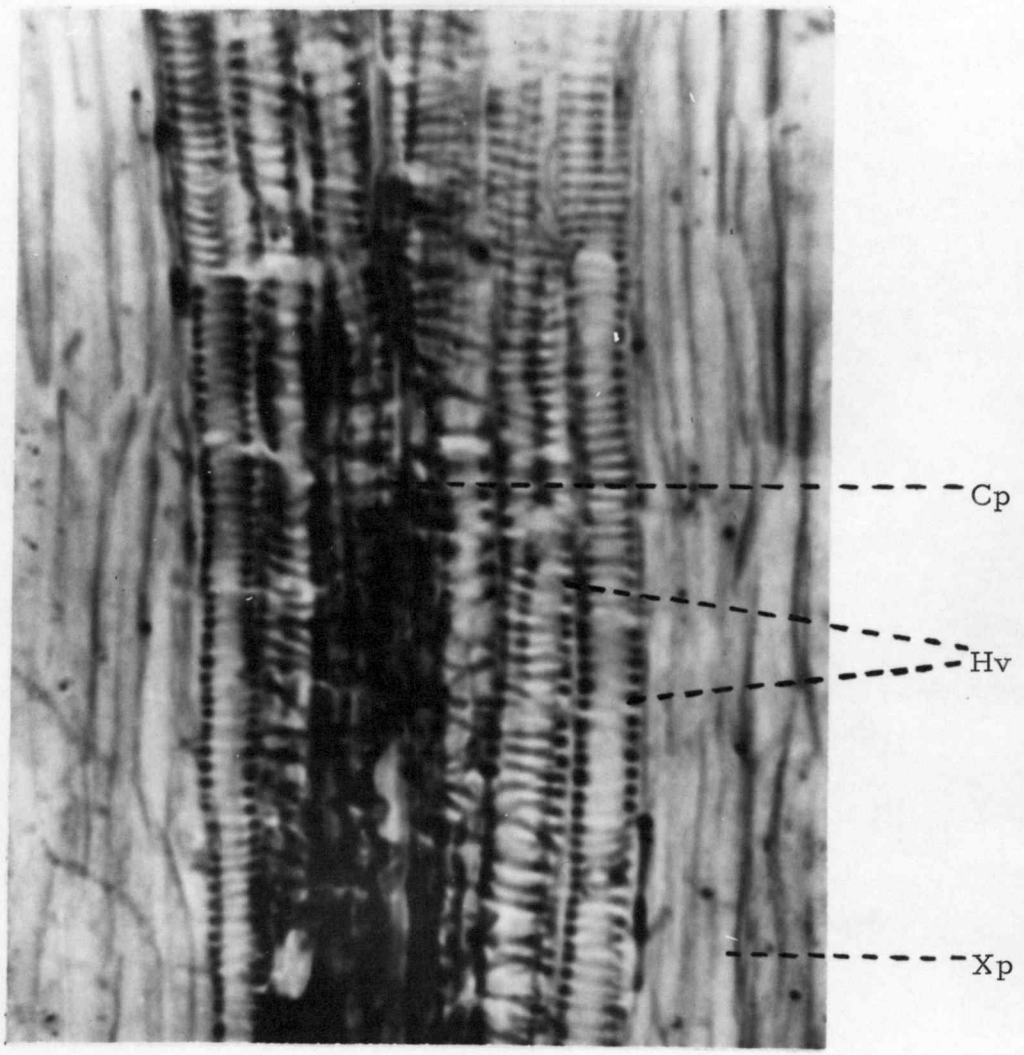


Figure 5. Longitudinal section of stele of root.
Explanation: Cp, collapsed protoxylem;
Hv, helically plated vessel elements;
Xp, xylem parenchyma.

Histology

The primary tissues of the root originally constituted a triarch protostele. The material under study shows substantial secondary growth with subsequent loss of the cortex. Peripheral tissues consist of four to seven layers of thin-walled, tabular cork cells (phellem) and a cork cambium (phellogen). These are subtended by a zone of pericycle from three to five cells in depth, contiguous to a broad zone of secondary phloem which is radially traversed opposite the protoxylem ridges by vascular rays from four to six cells wide. Sieve tube strands of the secondary phloem are separated from each other by parenchyma. A functional primary phloem is not discernible. A cambial zone separates the mature xylem from the secondary phloem. Lateral roots are very numerous and in different stages of development. The presence of secretory canals is observed in the secondary phloem. According to Metcalf and Chalk such canals are characteristic of the Umbelliferae (32, p. 717).

Description of the Unground Root

The root after being cut into segments and dried appears macroscopically as follows: split sections from larger diameters of the root range from 1 to 4 cm. in length and from 0.6 to 1.4 cm. across. The unsplit root segments range from 2 to 3.5 cm.

in length and from one to about five mm. in diameter. The external surface is weak brown to moderately yellowish-brown, slightly annulated and somewhat wrinkled longitudinally. Hair-like secondary rootlets are frequently present. The ends of the segments often show a spotty, orange-yellow coloration. The inner surfaces of the segments are dull white with flecks of orange-yellow. The fracture is short and the fractured surfaces are slightly irregular and porous. The odor is characteristic and light, the taste insipid and bland.

Description of the Powdered Root

The root reduced to a number 40 powder is a light yellowish-tan in color and slightly grainy when rubbed between the fingers. The characteristic odor is more pronounced than in the unground root and slightly acrid. When a small portion of the powder is moistened with Wallis' iodine[12] it turns blue.

Microscopic examination of the powder in a water mount reveals the presence of numerous single and clustered starch grains in such quantity as to obscure other features. The starch grains are ovoid to polyhedral, non-striated, with slight erosions radiating from the hilum or ridge. The grains vary in size from

[12]One volume of weak solution of iodine B. P. mixed with nine volumes of water (61, p. 222).

1.8 to 8.7 microns in diameter and the larger clusters from 70 to 126 microns. The starch gelatinizes very slowly or hardly at all in water. Water mounts examined under polarized light show the starch grains to have a bright cross at total extinction.

Examination of mounts in chloral hydrate solution[13] reveals rapid gelatinization of the starch with rupture of parenchyma cells containing large starch clusters. It would appear that many parenchyma cells are totally filled with starch. Parenchyma cells were observed to range in length from 70 to 140 microns.

Clearing with chloral hydrate shows the presence of fragmented vessel elements with helical to helical-scalariform pitting. When treated successively with phloroglucinol test solution[14] and concentrated hydrochloric acid the vessel elements are colored red - a positive test for lignified tissue. Tests with Sudan III did not reveal the presence of any oil. No crystals were observed. Line drawings of the microscopic characters are shown in Figure 6.

Ultraviolet Tests

Many drugs exhibit fluorescence when the cut surface or the

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[13] Fifty grams of chloral hydrate dissolved in 20 ml. of purified water (61, p. 220).

[14] One gram of phloroglucinol dissolved in 100 ml. of 90% alcohol (61, p. 222).

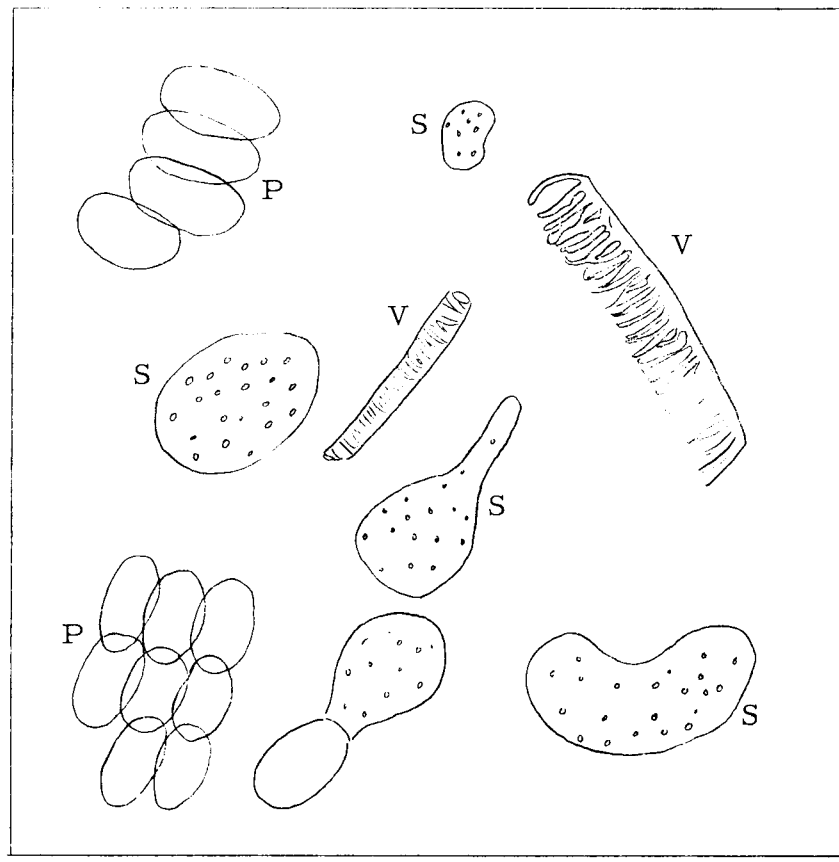

Figure 6. Histological features of powdered root, magnified about 100 times in chloral hydrate mount. Showing: P, parenchyma cells; S, gelatinizing starch grain clusters; V, vascular element gragments.

powder is exposed to ultraviolet radiation. This property provides a useful tool for the routine investigation of crude drugs. In some cases this method of examination will provide information which cannot be obtained by any other means (62, p. 553). In order to characterize the material under study, its reaction to irradiation from an ultraviolet lamp[15] was observed in a darkroom.

1. The intact external cork of split or cylindrical sections showed no fluorescence. The inner portions displayed a light-yellow fluorescence which was more intense and slightly tinged with blue on the surfaces of fresh cuts and fractures. In addition, these inner surfaces showed a definite luminescence which persisted for five seconds following extinction of the light.

2. The powdered dried root showed a slight yellow fluorescence which luminesced for several seconds following extinction of the light.

3. The powdered dried root, previously exhausted by ether extraction in a Soxhlet extractor, showed a somewhat blue and slightly more intense fluorescence while under irradiation but displayed no luminescence following extinction of the light.

4. A two-gram sample of powdered dried root was macerated with 20 ml. of 95% ethanol for one hour and filtered. Five ml. of

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[15]Mineralight, long-wave ultraviolet, model SL 3660 Ultraviolet Products, Inc., South Pasadena, California.

the filtrate was placed in each of two test tubes and to one of these one ml. of 28% ammonia was added. Both filtrates were then observed under ultraviolet light. The filtrate with ammonia gave a dark green fluorescence. A weak dark-blue fluorescence was noted with the other filtrate. No luminescence occurred.

Selective Solvent Extraction

The root material, previously dried at 30°C., was ground to a no. 40 powder in a Wiley mill. Fibrous rootlets were excluded. Five-gram and two-gram samples were extracted successively with solvents of an increasing order of polarity. The technique employed was a modification of the general method of Rosenthaler (42, p. 35-39). A Soxhlet extractor was used in performing the extractions with the five-gram samples and a micro-Soxhlet extractor was employed with the two-gram samples.

Each thimble with its contents was oven-dried following extraction with a solvent and the amount of extractive was determined by loss of weight. An oven temperature of 65°C. was used to remove the petroleum ether from the thimble and a temperature of 90°C. was employed for the other solvents.

In order to insure complete extraction with water following treatment with that solvent on the Soxhlet, each thimble with its contents was opened and macerated with distilled water in a beaker

for 14 hours. This was then reduced to a slurry in a Waring blender, filtered on a Büchner funnel with the aid of suction, and the residue washed and then dried at 90°C.

The period of time assigned to extractions with the sequential solvents are shown in Table V and the results are shown in Table VI.

TABLE V. EXTRACTION PERIODS USED WITH SOLVENTS

	Two-gram Samples	Five-gram Samples
	Hours	Hours
1. Petroleum ether	12	4
2. Ether U.S.P.	18	---
3. Ether, anhydrous	---	12
4. Alcohol 95%	12	22-1/2
5. Distilled water	---	20-1/2

The following studies made on the various fractions obtained are reported for those from the five-gram samples only.

Petroleum Ether Fraction

The petroleum ether fraction was colorless with a slight amount of white sediment. The extract was filtered and evaporated to near dryness on a steam bath. Upon evaporation to dryness at

TABLE VI. SELECTIVE SOLVENT EXTRACTION[16]

No.	Weight (grams)	Petroleum[17] Ether	Ether U. S. P.	Ether Anhyd.	Alcohol 95%	Water
1	1.914	2.2	2.1	---	7.8	---
2	1.691	2.5	2.0	---	8.7	---
3	1.927	2.4	2.0	---	8.1	---
4	5.078	2.4	---	2.5	9.1	20.7
5	5.087	2.6	---	2.5	8.5	18.5
6	5.085	2.5	---	2.5	8.4	18.9
7	5.094	2.5	---	2.5	8.9	20.1
	Averages	2.4	2.0	2.5	8.5	19.4

[16] Extractive fractions expressed as a percentage of the oven-dried weight.

[17] Petroleum ether (hexane) B. P. 68 to 69°C.

room temperature a brown varnish-like residue remained. Microscopic examination revealed the presence of numerous crystal clusters encased in droplets of a brownish liquid.

Moisture Content

Moisture content of the fresh root is represented by loss of weight on drying in a forced-air oven at a temperature of 38°C. for 50-1/2 hours following harvest.

Weight of fresh root	1,030.3 grams
Weight of dried root	268.9 grams
Loss of weight	761.4 grams
Percentage of moisture	73.9

Anhydrous Ether Fraction

The anhydrous ether fraction was almost imperceptibly light-yellow in color with a small amount of fine, white residue. Evaporation to dryness on a steam bath resulted in a brownish residue with a pleasing, aromatic odor. The residue was redissolved in anhydrous ether and allowed to evaporate to dryness at room temperature. Microscopic examination showed clumps of amorphous matter as well as numerous massed droplets of brown liquid.

Alcohol Fraction

The alcohol fraction appeared light-yellow in transmitted light and displayed a bluish opalescence with reflected light. A small amount of light-brown sediment was present. Upon evaporation to near-dryness on a steam bath a thick, brown, syrupy liquid remained.

Aqueous Fraction

The aqueous fraction was brown in color with a small amount of flocculent sediment. The copious foam produced by shaking the liquid in a flask indicated the possible presence of saponins.

Thin-Layer Chromatography

In order to determine the presence of coumarins in the root and to ascertain their identity a thin-layer chromatographic study was made.

Standards

The following coumarins and furanocoumarins were employed

as standards.[18]

1. Umbelliferone
2. Isobergapten
3. Pimpinellin
4. Isopimpinellin
5. Sphondin
6. Bergapten
7. Imperatorin

Several of these compounds contained traces of impurities. This, however, did not affect their value as standards.

The following amounts of the above materials were weighed on a Mettler balance: umbelliferone, 1.5 mg.; isobergapten, 3.5 mg.; pimpinellin, 6.9 mg.; isopimpinellin, 6.3 mg.; sphondin, 5.7 mg.; bergapten, 2.5 mg. and imperatorin, about 0.25 mg.

These quantities were transferred to individual 10 ml. volumetric flasks and, with the exception of the umbelliferone and the imperatorin, were filled to the mark with spectro grade chloroform. To the flask containing the imperatorin only 2 ml. of chloroform were added. The flask containing the umbelliferone was filled to the mark with 95% ethenol due to the limited solubility of umbelliferone

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[18] Obtained from the following sources: Comp. no. 1: British Drug Houses, the Ealing Corp., 33 University Road, Cambridge, Mass. Comp no. 2: Dr. Michiichi Fujita, Tokyo College of Pharmacy, Institute of Pharmacognosy and Plant Chemistry, 4-600 Kasiwagi, Shinjuku-ku, Tokyo, Japan. Comp. nos. 3, 4 and 5: Dr. Stewart A. Brown, Trent Univ., Peterborough, Ontario, Canada (originally from Dr. Baerheim Svendsen, School of Pharmacy, Oslo-Blindern, Norway). Comp. nos. 6 and 7: Dr. Stewart A. Brown.

in chloroform.

Equipment

The Desaga apparatus[19] was used for the preparation of the thin-layer plates. Spotting was done with a 5-lambda micropipette with the spot restricted to a maximum diameter of 5 mm. A current of cool air from an air gun was used to facilitate evaporation of the solvent at the site of application. Development was done in 8-1/2 x 8-1/2 x 4 inch glass chambers with ground glass tops and covers. The chambers were equilibrated for at least two hours prior to use. Long-wave ultraviolet light generated by a Mineralight lamp, model SL 3600, was used to visualize the spots on the developed chromatograms.

Solvents

The chromatograms were developed in two different solvent[20] systems. The compositions of these two systems were as follows:

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[19] C. Desaga, G.m.b.H., Hauptstrasse 60, Heidelberg, Germany. U.S. Distributor: C. A. Brinkmann and Co., Inc., 115 Cutter Mill Rd., Great Neck, New York.

[20] Ethyl acetate, A.C.S. Analyzed Reagent.
Hexane 98+%, B.P. 68° - 69°C.
Xylene, A.C.S. Reagent.

1. Ethyl acetate : Xylene 1:1

2. Hexane : Ethyl acetate 2:1

Preparation of Plates

Prior to use the glass plates were cleaned with Ajax scouring powder, rinsed successively with tap water and distilled water, and dried with a clean towel.

Thirty grams of Silica-Gel G (Acc. to Stahl) were placed in a 4-inch glass mortar and 60 ml. of distilled water were added in one portion. The mixture was carefully stirred with a stainless steel spatula for 60 seconds and then triturated with a glass pestle for 30 seconds. The slurry was immediately poured into the cylinder of the applicator positioned on the initial plate and set to produce a layer 250 microns thick. The cylinder was rotated and as soon as the slurry was seen to flow out the applicator was drawn smoothly and rapidly from left to right across the glass plates.

The layer was allowed to set for ten minutes before the plates were removed and placed in the drying rack. After approximately an additional five minutes the drying rack was placed in an oven preheated to 105^{o}C. and the plates were dried for one hour. After activation the plates were placed in a desiccator over Drierite until cool and used at once or as required.

Preparation of the Aid-Dried Root Extract

On February 17, 1964 the remainder of the roots of the two plants harvested as previously described on page 27 were thoroughly washed, cut into segments, placed upon absorbent paper in wooden flats and allowed to dry for several months in the air at normal laboratory temperatures. The material was then reduced to a no. 20 powder in a Wiley mill. A 13.35-gram portion of the powder was extracted with 100 ml. of ethyl ether in a Soxhlet apparatus. The ether solution was light-yellow in color with a small amount of fine, white sediment. The solution was filtered and the filtrate was evaporated to near-dryness on a steam bath. It was then redissolved with ether and transferred to a 10 ml. beaker. The solution was again evaporated to near-dryness on a steam bath and the remaining ether was allowed to evaporate at room temperature.

The residue consisted of a thick, brown liquid, somewhat fluid when warm, almost immobile when cold, and had a light, aromatic odor characteristic of the seeds. The residue was washed with 6 ml. of petroleum ether, in divided portions, to remove possible lipid material and the remaining residue redissolved in a mixture of ether and alcohol to a total volume of 10 ml. in a volumetric flask.

Preparation of Oven-Dried Extract

An extract from 13.35 grams of powdered, over-dried root was prepared as described above and the extract redissolved in a mixture of ether and alcohol to a volume of 10 ml.

Technique

Activated plates were removed from the desiccator as required. Lateral and bottom edges of all thin layers were trimmed with a plastic rule. Both square and rectangular plates were used for the determination of R_f values of the standards.

Due to confluescence of the spots when the root extracts were developed in one direction only these plates were developed in a second direction at 90 degrees to the first before R_f values were determined.

In development, the solvent was allowed to rise until all parts of the front had reached the line. Plates in which the solvent front was not substantially horizontal were rejected. Developed plates were dried in a current of warm air from an air gun and immediately examined under ultraviolet light. This is essential as the intensity of the fluorescent spots decreases rapidly.

Prior to being developed in the second direction the plates were dried for 15 minutes in a current of warm air from an air gun

and then cooled to room temperature.

Chromatography of Standards and Root Extracts

Effect of Load Upon R_f Values. In order to determine the effect of variations in load upon the R_f values 5-, 10-, and 15-lambda loads were spotted respectively from each of the following solutions:

1. Sphondin
2. Isopimpinellin
3. Isobergapten
4. Umbelliferone

The behaviour of these compounds may be considered to be characteristic of the coumarins as a group. The progressive loads of each individual solution were spotted onto the same plate. The chromatograms were developed with Ethyl acetate : Xylene 1:1 and the migrated spots were visualized with ultraviolet light. The results shown in Figures 7 and 8 demonstrate that within practical limits the size of the load has no significant effect upon R_f values. Slight variations may be considered inconsequential.

Fluorescence to Ultraviolet Light. Long-wave ultraviolet light was found to be an excellent tool for the visualization of developed chromatograms. Color values of the fluorescent spots of the respective coumarins are shown in Table VII. Due to the

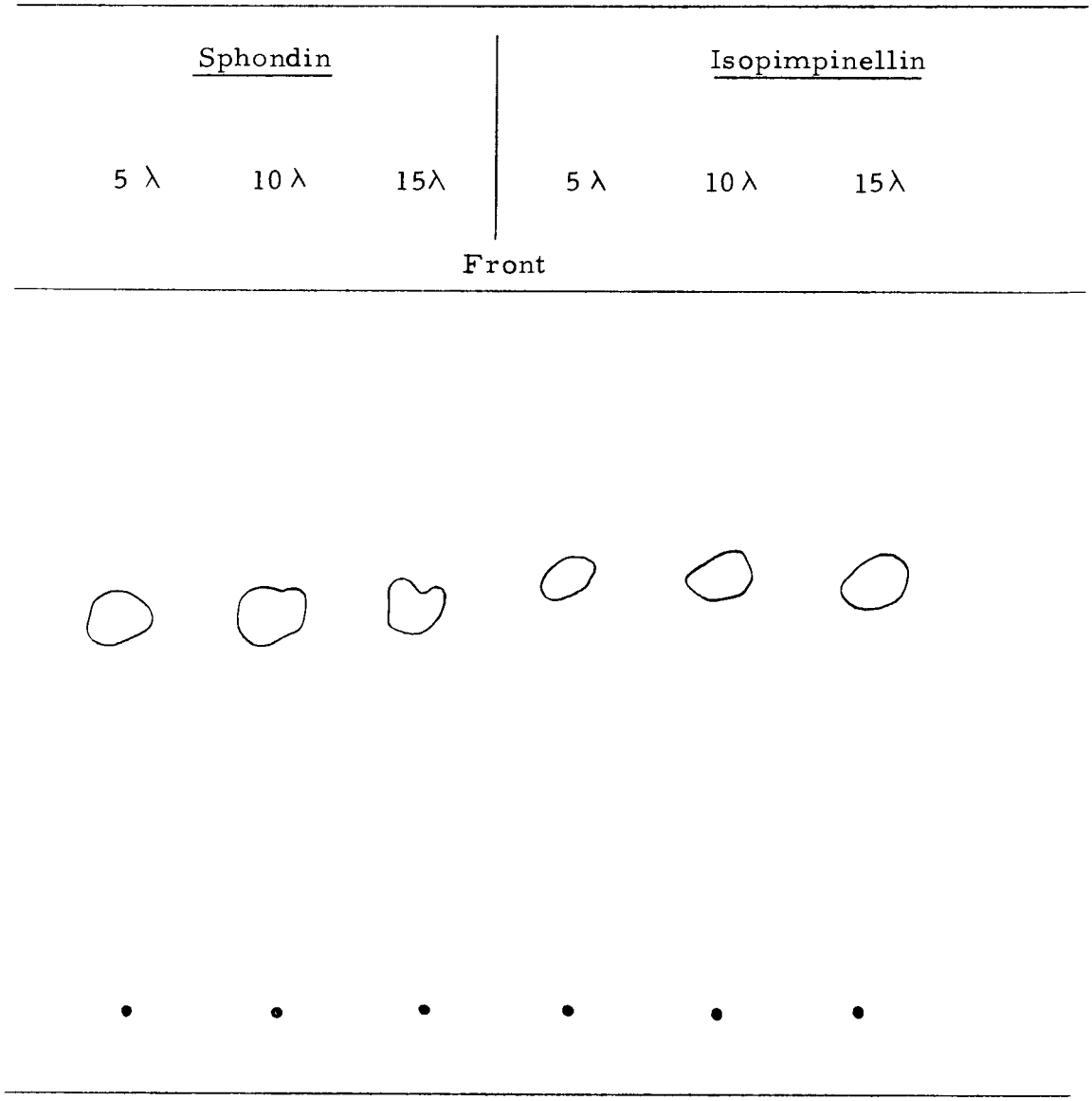


Figure 7. Effect of load on R_f values of sphondin and isopimpinellin.

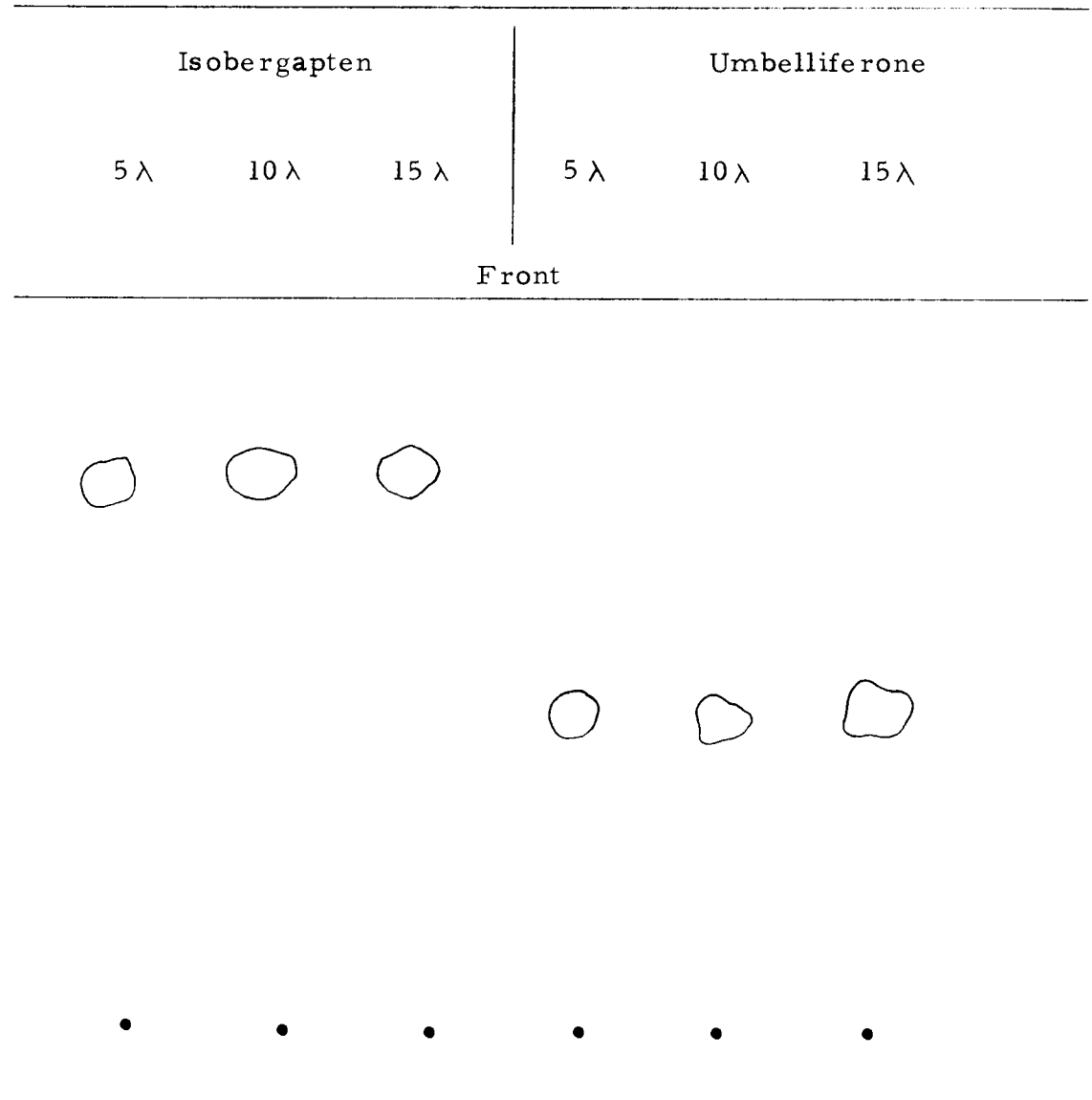


Figure 8. Effect of load on R_f values of isobergapten and umbelliferone.

difficulty of circumscribing colors effectively the table presents this author's evaluation together with their closest identity on the plates of the standard color reference, Maerz and Paul's Dictionary of Color (30).

TABLE VII. FLUORESCENT COLORS OF THE STANDARD COUMARINS TO LONG-WAVE ULTRAVIOLET LIGHT.

Coumarin	Color	Reference	Plate
1. Isobergapten	Yellow	L-1	18
2. Pimpinellin	Brown	I-7	14
3. Imperatorin	Yellow	L-1	18
4. Bergapten	Yellow	L-1	18
5. Isopimpinellin	Brown	I-7	14
6. Sphondin	Greenish-blue	K-7	34
7. Umbelliferone[21]	Bright blue	Variable	

[21] It is to be noted that when umbelliferone was spotted alone in 5-lambda amounts the color was bright blue. When it was spotted mixed with the other coumarins or with root extract the color appeared dark blue (equivalent to L-12 on plate 39). This darker color was also observed when a substantially attenuated dilution was spotted.

R_f Values of the Standard Coumarins. The R_f values of the standard coumarins as well as those of the principal spots of the root extracts were determined. R_f values shown in Table VIII represent an average of not less than three individual spots.

Chromatography of Air-Dried Extract. Two-dimensional chromatography of the extract of air-dried root revealed seven principal spots in the order of their decreasing R_f values. The relative positions of these spots as well as their colors to ultraviolet light are shown in Figure 9.

The standard coumarins, isobergapten, pimpinellin, bergapten, isopimpinellin, sphondin and umbelliferone were spotted together. When developed two-dimensionally the chromatograms show a pattern almost identical to that of the root extract with the exception of spot no. 7. Colors of the spots to ultraviolet light corresponded exactly. This is shown in Figure 10.

The standard coumarins and the air-dried root extract were spotted together and developed as described above. The chromatogram showed no significant divergence from the patterns of the standards when spotted together or from the root extract. This chromatogram is shown in Figure 11.

Chromatography of the Oven-Dried Extract. Two-dimensional chromatography of the extract of oven-dried root revealed five principal spots. This chromatogram is shown in Figure 12.

TABLE VIII. R_f VALUES OF STANDARD COUMARINS AND PRINCIPAL SPOTS FROM ROOT EXTRACT

	Solvent System 1	Solvent System 2
Spot no. 1	.73	.49
Isobergapten	.74	.53
Spot no. 2	.69	.43
Pimpinellin	.72	.47
Spot no. 3	.63	.33
Bergapten	.63	.38
Spot no. 4	.59	.26
Isopimpinellin	.60	.28
Spot no. 5	.53	.23
Sphondin	.55	.27
Spot no. 6	.43	.17
Umbelliferone	.43	.17
Spot no. 7	.20	.06

Solvent systems

(1) Ethyl acetate : Xylene 1:1

(2) Hexane : Ethyl acetate 2:1

Quantity spotted: 20λ

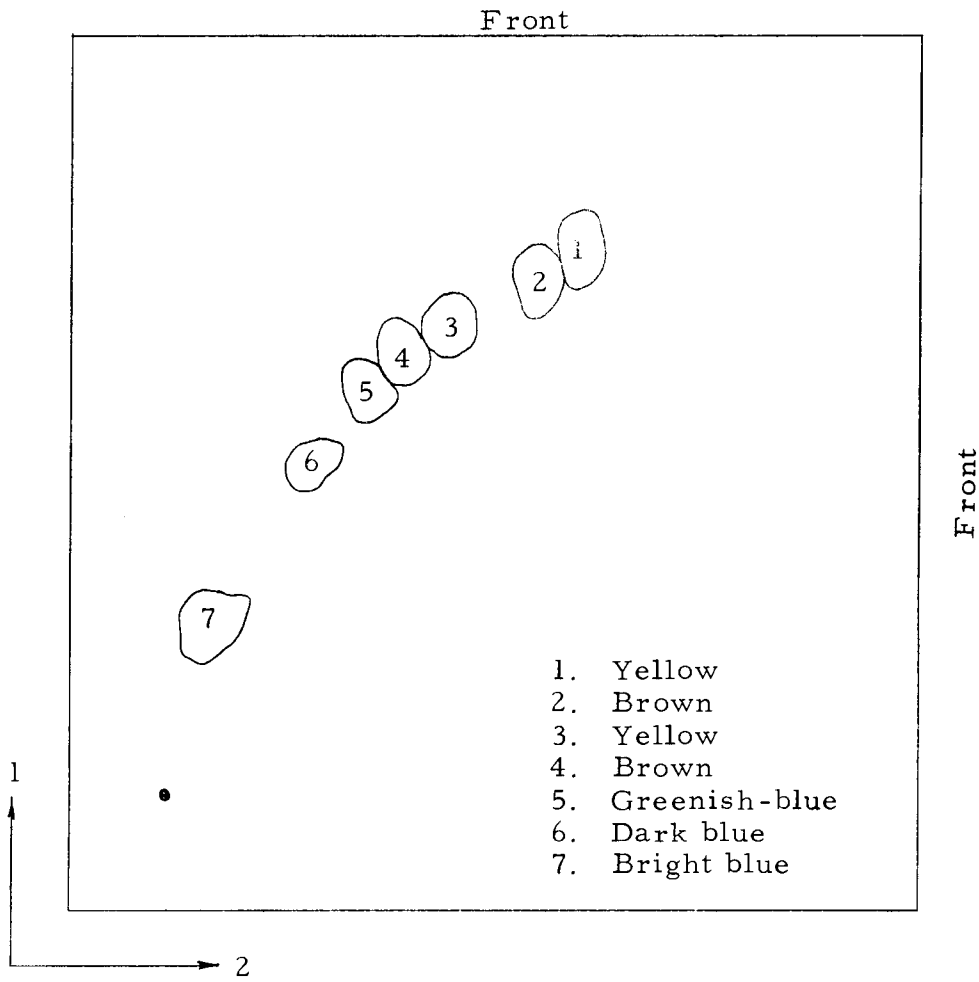


Figure 9. Chromatogram of extract of air-dried root.

Spot no.	Standard
1	5 λ Isobergapten
2	15 λ Pimpinellin
3	10 λ Bergapten
4	5 λ Isopimpinellin
5	5 λ Sphondin
6	5 λ Umbelliferone

Solvent systems

(1) Ethyl acetate : Xylene 1:1

(2) Hexane : Ethyl acetate 2:1

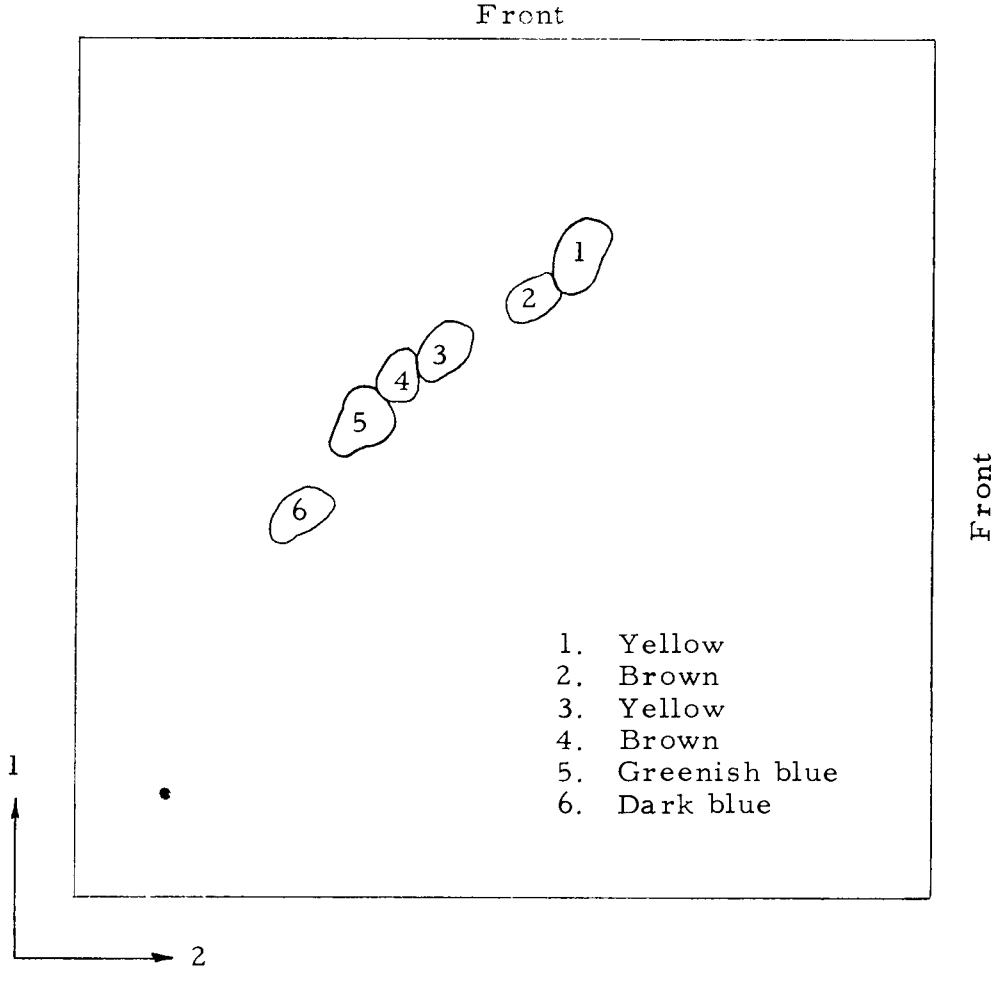


Figure 10. Chromatogram of mixed standard coumarins.

Quantities spotted

5 λ	Isobergapten	15 λ	Pimpinellin
10 λ	Bergapten	5 λ	Isopimpinellin
5 λ	Sphondin	20 λ	Root extract
5 λ	Umbelliferone		

Solvent systems

(1) Ethyl acetate : Xylene 1:1

(2) Hexane : Ethyl acetate 2:1

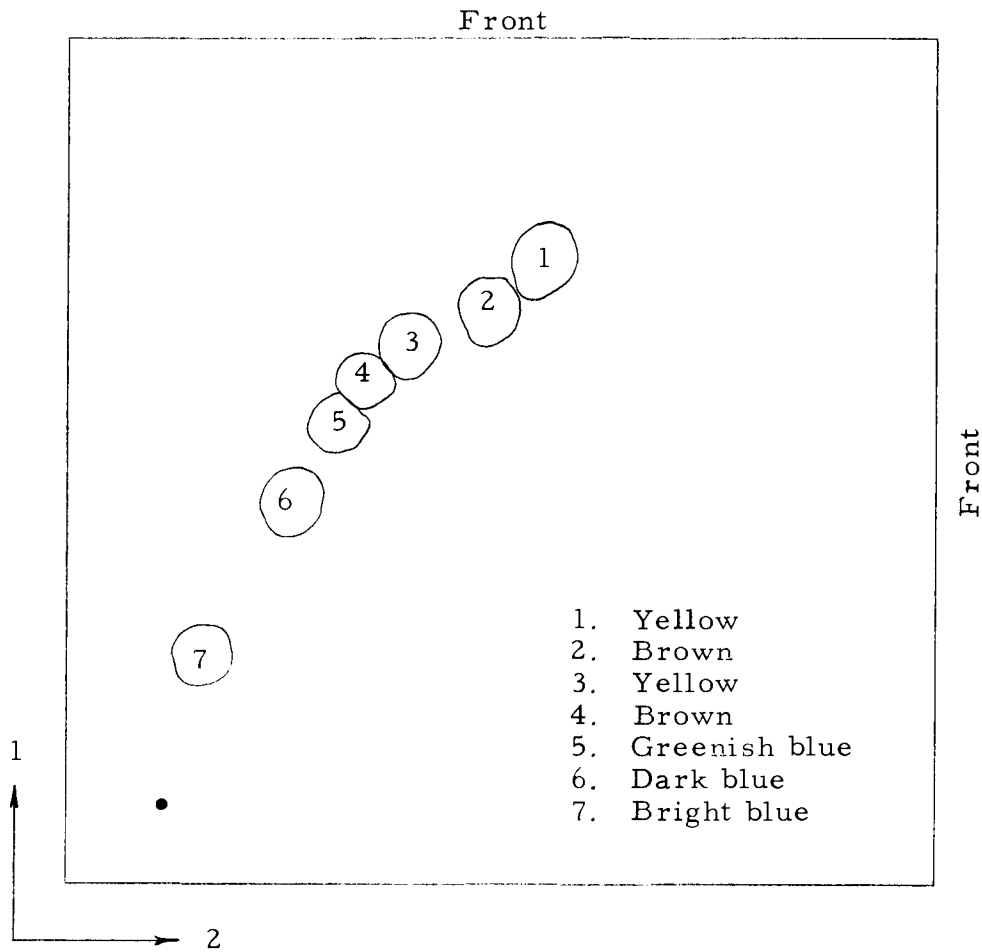


Figure 11. Chromatogram of mixed standard coumarins and root extract.

Solvent systems

(1) Ethyl acetate : Xylene 1:1

(2) Hexane : Ethyl acetate 2:1

Quantity spotted : 20 λ

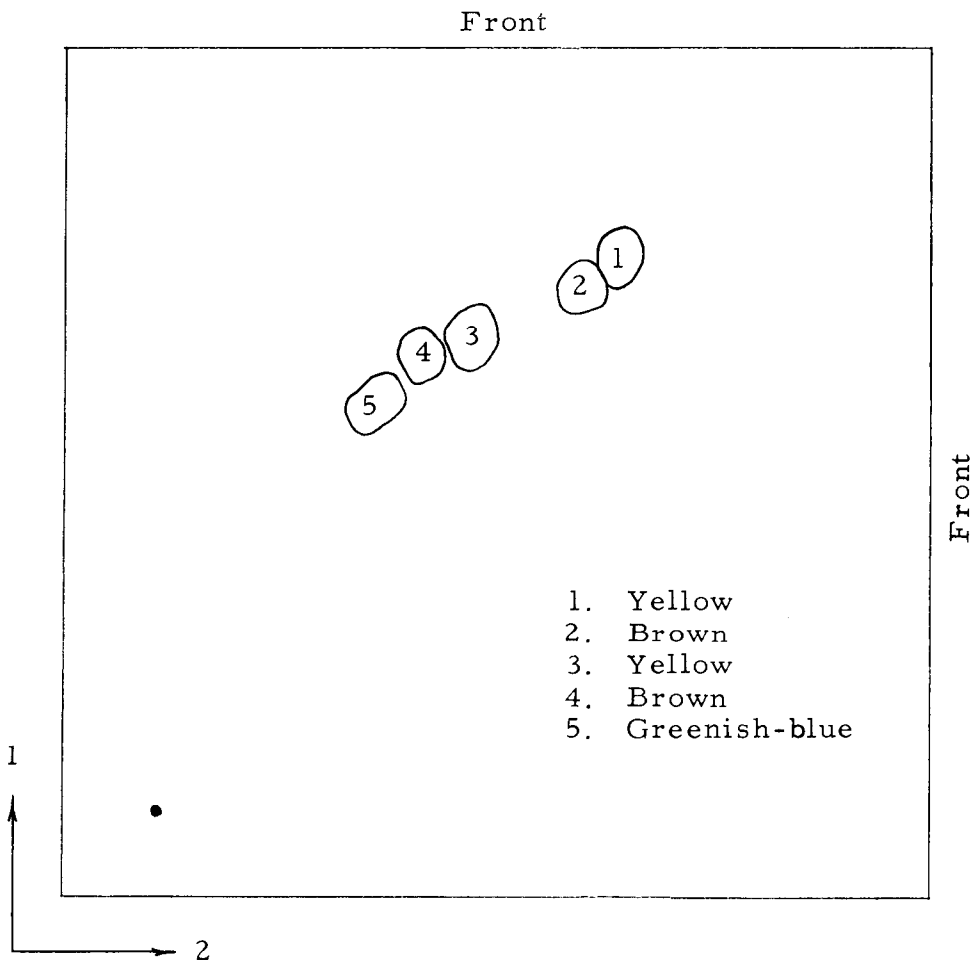


Figure 12. Chromatogram of extract of oven-dried root.

Effect of Heat Upon Coumarins on Silica Gel G

In order to determine what effect heat would have upon coumarins the following experiments were conducted on coumarins *in situ* in the silica gel of thin-layer plates. The standard coumarins, isobergapten, bergapten, sphondin, umbelliferone, pimpinellin and isopimpinellin were spotted individually onto thin-layer plates and the plates then developed in the first direction. The plates were then removed from the chamber and dried in a current of cool air from an air gun. The plates were then placed in an oven and exposed to a temperature of 65°C. for 30 minutes. The plates were cooled to room temperature and immediately developed in the second direction.

Examination showed that the umbelliferone was very slightly affected and that sphondin was not affected at all by this temperature. A study of other plates showed the presence of additional spots as follows:

1. Figure 13. Isopimpinellin. One additional spot, stationary and fluorescing yellow under ultraviolet light.
2. Figure 14. Isobergapten. One additional spot, stationary and fluorescing bright blue to ultraviolet light.
3. Figure 15. Bergapten. Two additional spots, one stationary and fluorescing bright blue, the other mobile and fluorescing yellow to ultraviolet light.

ISOPIMPINELLIN

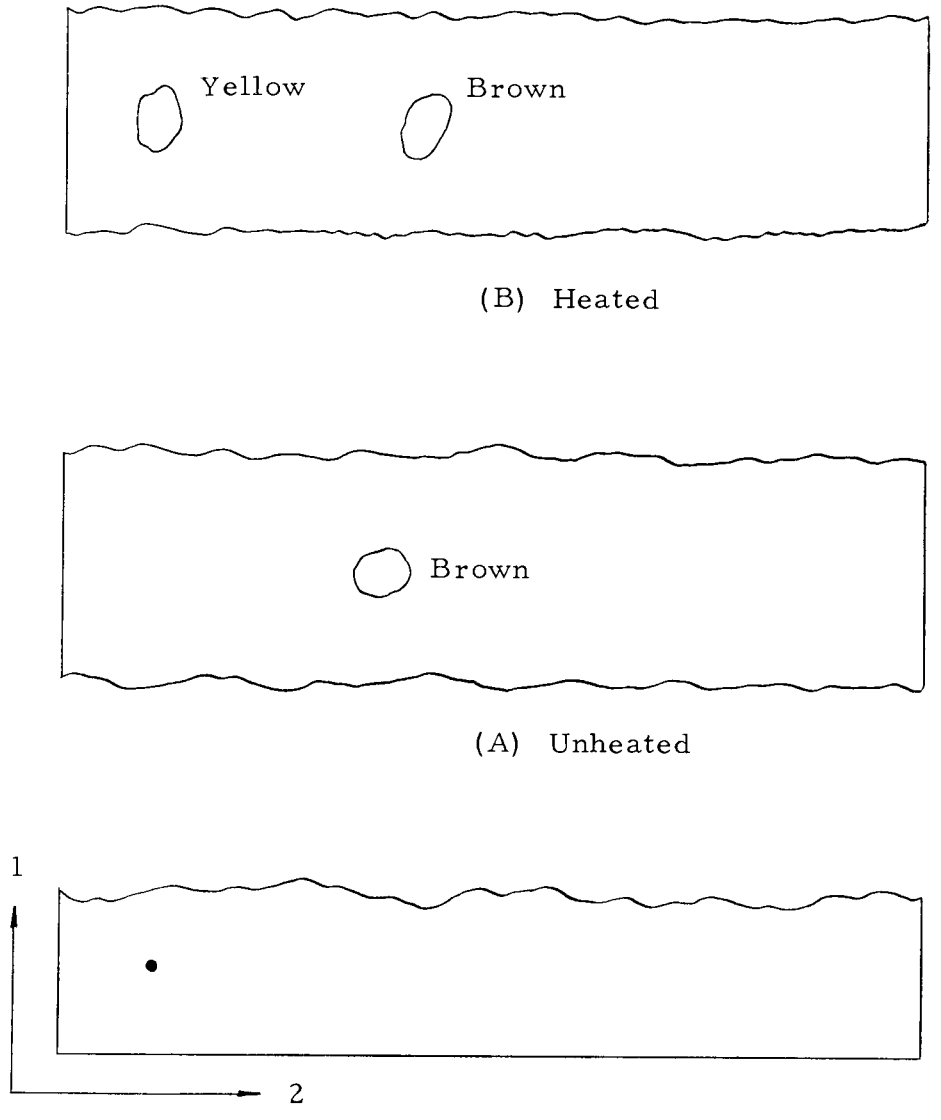


Figure 13. Artifacts noted from heating of isopimpinellin on a Silica Gel G plate.

ISOBERGAPTEN

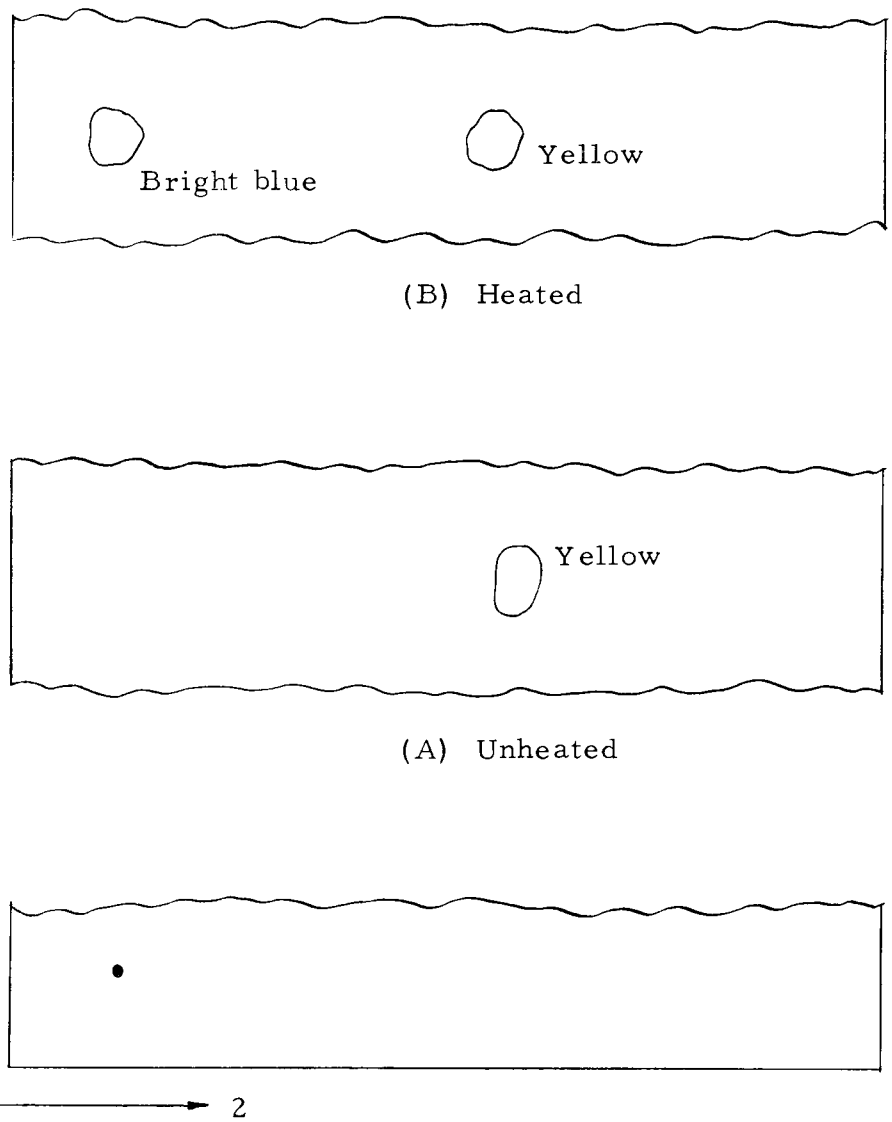
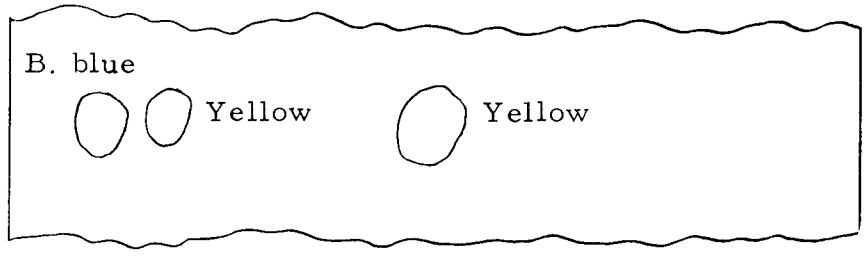
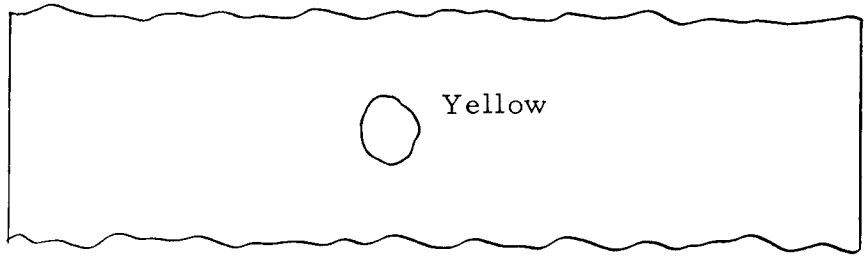


Figure 14. Artifacts noted from heating of isobergapten on a Silica Gel G plate.

BERGAPTEN



(B) Heated



(A) Unheated

Figure 15. Artifacts noted from heating of bergapten on a Silica Gel G plate.

4. Figure 16. Pimpinellin. One additional spot, stationary and fluorescing bright blue under ultraviolet light.

5. Figure 17. Sphondin. Sphondin itself was not affected. The additional bright blue spot was due to the conversion of a mobile, brown-fluorescent secondary spot to a stationary spot to a stationary spot fluorescing bright blue under ultraviolet light.

6. Figure 18. Imperatorin. Two additional spots, one stationary and fluorescing light green, the other mobile and fluorescing yellow under ultraviolet light.

Effect of Heat Upon Air-Dried Root Extract

Twenty lambdas of air-dried root extract were spotted onto a thin-layer plate and the plate was treated and developed as described above. The chromatogram is shown in Figure 19. Five new spots, fluorescent, under ultraviolet light were observed.

PIMPINELLIN

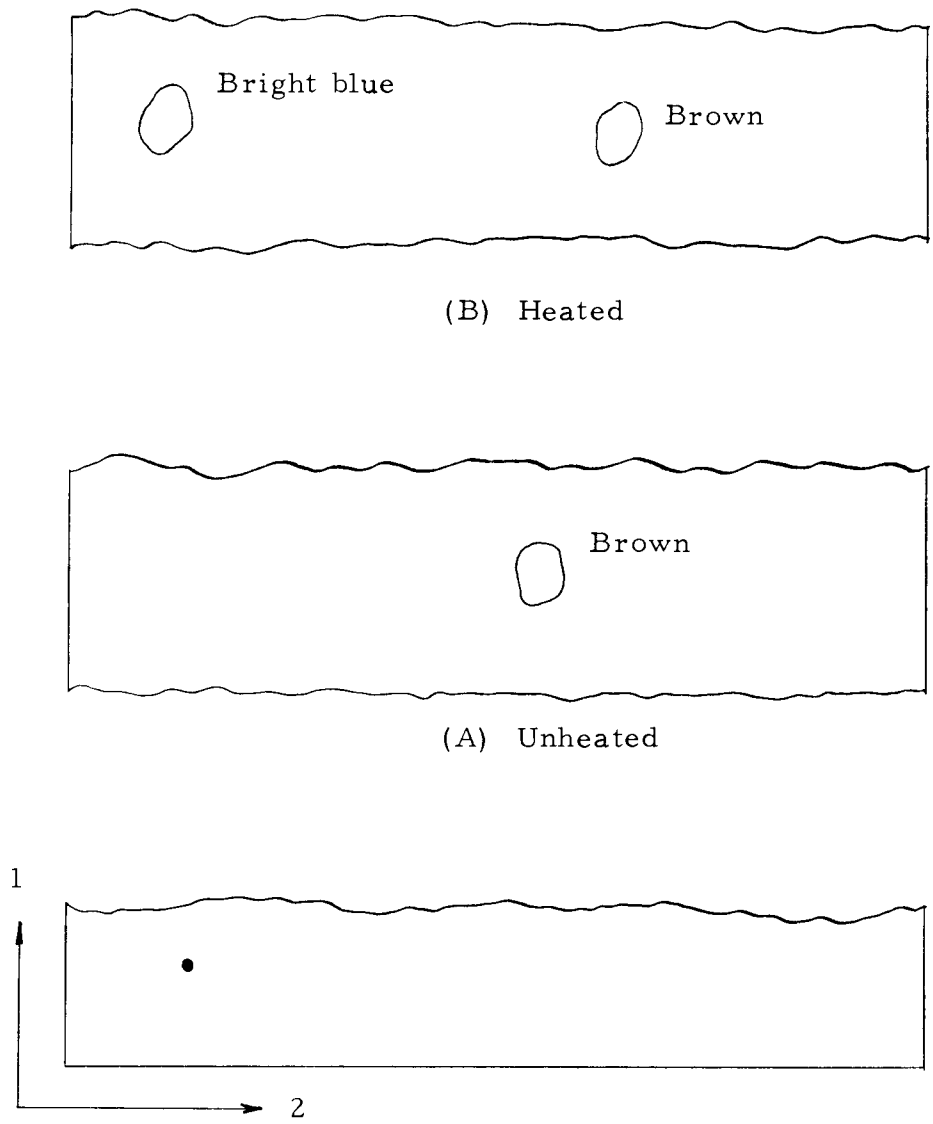
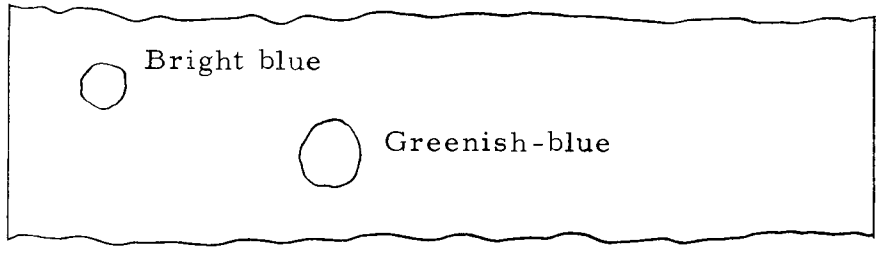
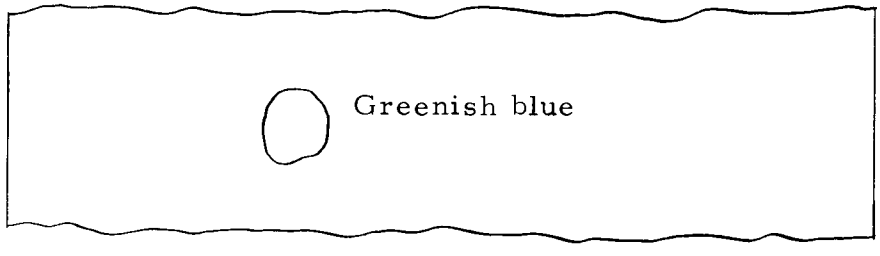


Figure 16. Artifacts noted from heating of pimpinellin on a silica-gel plate.

SPHONDIN



(B) Heated



(B) Unheated

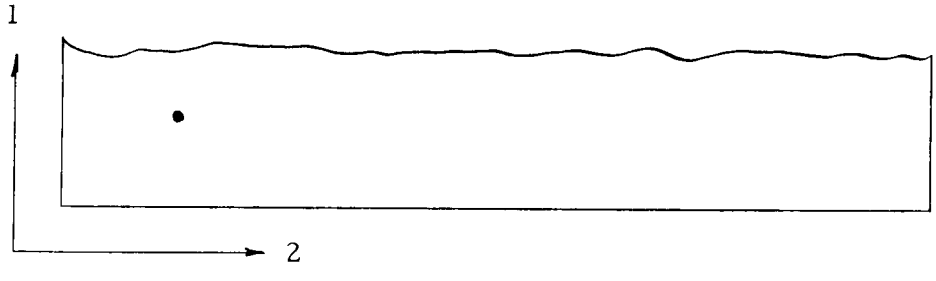


Figure 17. Artifacts noted from heating of sphondin on a Silica Gel G plate.

IMPERATORIN

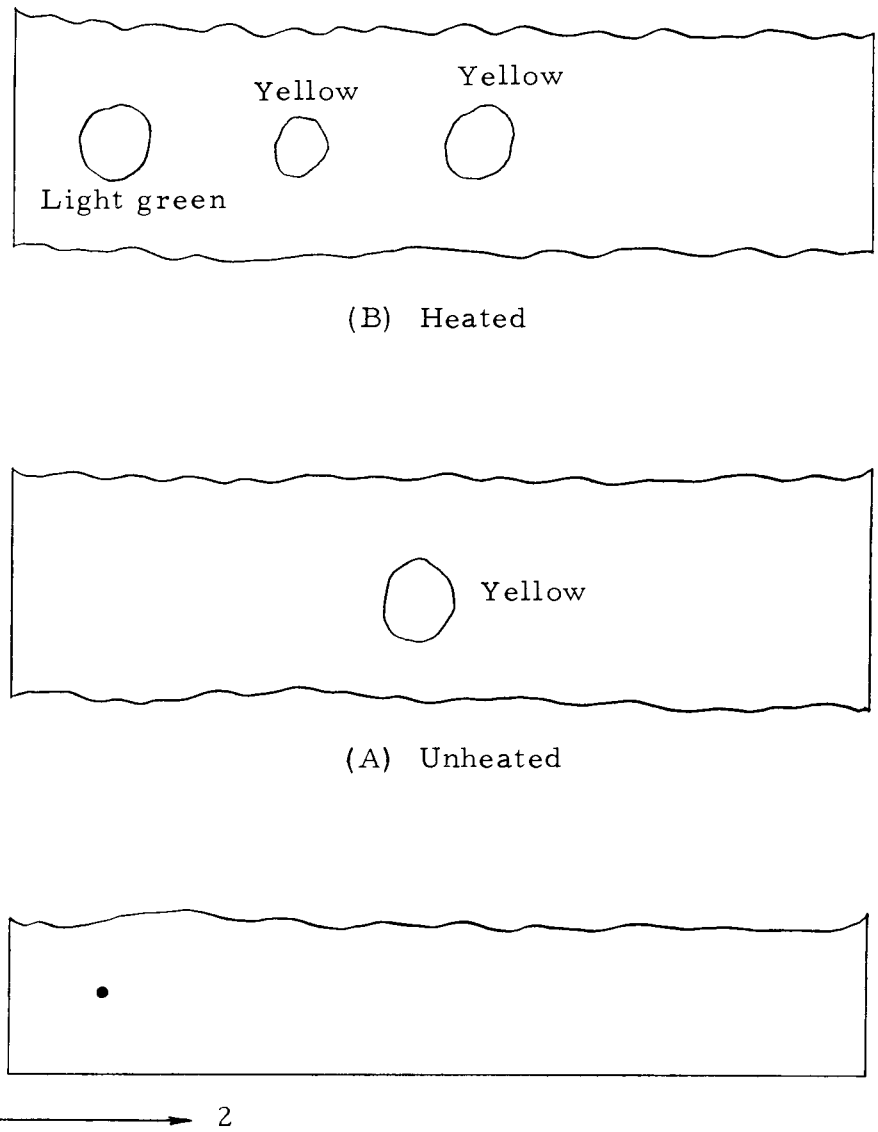


Figure 18. Artifacts noted from heating of imperatorin on Silica Gel G plate.

Solvent systems

(1) Ethyl acetate : Xylene 1:1

(2) Hexane : Ethyl acetate 2:1

Quantity spotted: 20 λ

Between first and second developments the plate was heated in an oven at 65° C. for 30 minutes.

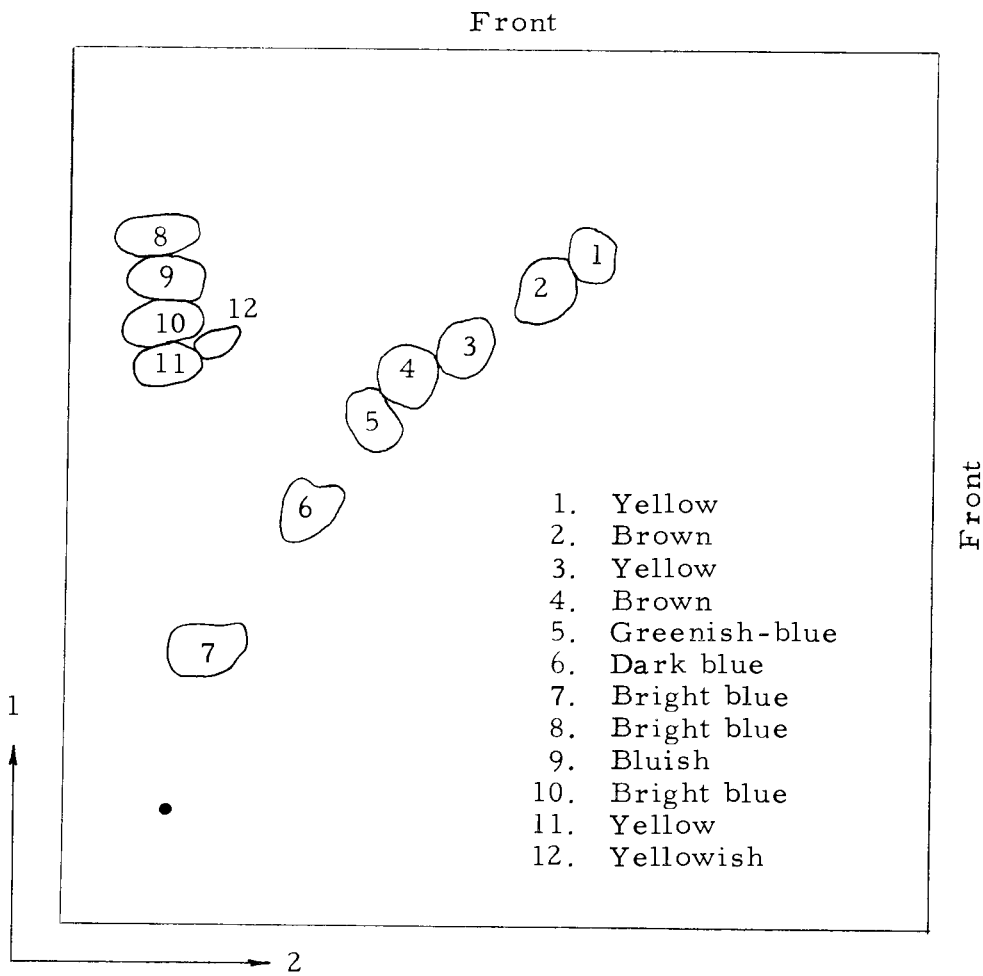


Figure 19. Artifacts noted from heating of air-dried root extract on a Silica Gel G plate.

III. SUMMARY AND CONCLUSIONS

1. Germination studies indicated that the group of seeds which served as controls failed to show any germination. On the other hand, the group of seeds which had been exposed to moist cold for a period of 74 days showed a germination rate of 10.3%. The fact that one of the four germinated seeds failed shortly after germination may possibly be ascribed to "damping off" or to an injury sustained in watering. The remaining three seeds of the group continued to develop in a satisfactory manner. This shows conclusively that the seeds of Heracleum mantegazzianum do have a requirement for moist cold.

2. The group of seeds treated with a solution of 100 p. p. m. of gibberellic acid failed to show any degree of germination. This indicates that gibberellic acid, in the strength employed, will not substitute for the cold requirement.

3. The group of seeds which was subjected to an extended period of moist cold (294 days) had an estimated germination rate of 55%. It was not possible to determine the rate exactly since many roots had grown through the fabric and were broken when the roll was opened. However, the considerable increase in the germination clearly demonstrates that in this species the germination rate is proportional to the total days of cold treatment. It is of interest to

note that in this group not only was the cold requirement satisfied but the seeds actually germinated and maintained some growth at a temperature range of from 2 to 5°C. This might be expected considering the original geographic distribution of the species.

4. The histology and diagnostic characters of the unground as well as the powdered root has been described on pages 32 to 37.

5. A selective solvent extraction was done by a modification of the general method of Rosenthaler, as previously described. The results are recorded on pages 38 to 41.

6. Thin-layer chromatographic studies of the ether extract of the air-dried root revealed seven principal spots under ultraviolet light. The R_f values and fluorescence of these spots corresponded to six of the standard coumarins. On this basis it was concluded that isobergapten, pimpinellin, bergapten, isopimpinellin, sphondin and umbelliferone are present. Imperatorin was not found to be present. The nature of spot no. 7 was not determined. The R_f values and fluorescence of six standard coumarins as well as the seven principal spots of the root extract were reported for the solvent systems used.

7. The effect of heat upon coumarins was investigated. The effect of exposure to 65°C. for 30 minutes was observed on six standard coumarins spotted individually and on air-dried root extract spotted on Silica Gel G plates. In the case of the individual

coumarins tested all chromatograms with the exception of that for sphondin showed at least one additional spot. The chromatogram of the root extract showed five additional spots.

8. The ether extract of the oven-dried root regularly chromatogramed on Silica Gel G gave spots for only five of the coumarins. The spot for umbelliferone was absent. The oven temperature employed in drying the roots had been 38°C. It was concluded that in order to preserve the character of the coumarins contained in the original plant material only the normal air temperatures should be employed in the drying process and that the use of supplemental heat should be avoided.

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