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SEASONAL VARIATION IN THE COMPOSITION OF BLACK LOCUST (Robinia pseudoacacia L.) SAPWOOD EXTRACTS

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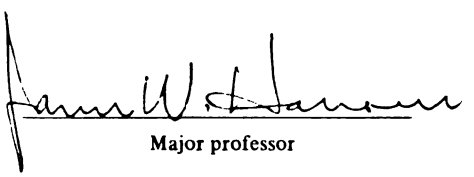

Major professor

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# SEASONAL VARIATION IN THE COMPOSITION OF BLACK LOCUST (Robinia pseudoacacia L.) SAPWOOD EXTRACTS

By

Shyong, Bao-Jen

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## ABSTRACT

SEASONAL VARIATION IN THE COMPOSITION OF BLACK LOCUST (Robinia pseudoacacia L.) SAPWOOD EXTRACTS

By

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The heartwood of black locust is not only highly resistant to decay but it also contains a high level of flavonoids among its extractable chemicals. The overall objective of this study was to determine where, and when flavonoid biosynthesis takes place in the wood of black locust (Robinia pseudoacacia L.).

In this study, the level of flavonoids and/or flavonoid precursors in the cambium, sapwood, transition zone and heartwood were investigated seasonally by quantitative and qualitative analysis. The results show that the flavonoids exist only in the heartwood and transition zone of black locust while the simple phenolics are found in the sapwood of black locust. Thus, biosynthesis of these flavonoids must occur during the transition from sapwood to heartwood. There are 3 possible precursors (compound a, b, d) of flavonoid biosynthesis found in the sapwood of black locust. It is necessary to accumlate more data and prove the hypothesis.

This is interesting because there are relatively few cells in sapwood which are physiologically active. The quantity of starch and soluble sugars in a series of locations from cambium to heartwood was also determined in an attempt to understand the relationship between primary (starch,

soluble sugars) and secondary (flavonoids) metabolism. The results suggest that primary metabolities could be an indirect source of carbon for flavonoids biosynthesis.

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# INTRODUCTION

## (A) BACKGROUND AND PROPOSED WORK

Due to its valuable characteristics and many uses, black locust has been extensively planted in the United States and introduced into Australia and many countries in Europe and Asia since the early seventeenth century. Black locust is one of the most widely planted boardleaf species in the world (Keresztesi,1981). The reasons for this widespread planting of black locust are: a wide site tolerance, the ability to fix nitrogen, abundant and frequent seed crops, fast growth, excellent sprouting ability, high timber yield, short rotation age, and relatively few damaging pests and diseases (Keresztesi, 1988).

The results of *in vitro* and *in vivo* durability studies have suggested that the extractable chemicals from black locust heartwood play an important role in the decay resistance of wood. The methanol extracts from black locust heartwood consist mainly of flavonoids (dihydrorobinetin and robinetin) and a minor amount of tannins (Roux and Paulus, 1962; Shain, 1977; Smith *et al.*, 1988). Preliminary qualitative analysis indicates that the sapwood of black locust does not contain the major flavonoids or flavonoid glycosides found in the heartwood.

The durability of black locust has received much attention and has been compared with that of poplars,

pedunculated oak and Norway spruce (Koloc, 1953). According to reports, the decay resistance of black locust is found only in the heartwood, but not in the sapwood. The durability of black locust has been examined in this laboratory. These studies indicated that the heartwood extracts are at least partially responsible for the durability of the wood (Smith et al.,1988). Additionally, Shain (1977), Freudenberg and Hartmann (1954) and Erdtman (1953) studied the inhibition of several types of fungus by flavonoids in vitro and suggested that flavonoids, especially robinetin and dihydrorobinetin, found in the heartwood extracts are able to inhibit fungal growth.

These facts provide an interesting question about how and where flavonoid biosynthesis takes place in black locust. The objective of this study is to investigate this process as follows:

1) To determine, by quantitative analysis, the levels of flavonoids or flavonoid precursors in the cambium, sapwood, transition zone and heartwood of black locust ;

2) To investigate the quantity of starch in a series of locations from cambium to pith to help in understanding the relationship between primary (starch) and secondary (flavonoid) metabolism;

3) To perform the above analyses seasonally on samples which are collected at 1-2 month intervals over one year in

order to correlate flavonoid formation with the seasonal changes.

## (B) CHARACTERISTICS OF BLACK LOCUST

### (1) Species Characteristics

The growth season of Black locust is from the beginning of April to the end of July. The heartwood formation of black locust is thought to start in July and end by the end of the following March (Nobachi,T. et al., 1984b). Black locust flowers in May and early June, about one month after flushing.

Black locust has less sensitivity to poor sites than other species in the same family, but it requires strong light for growth. Suitable drainage and soil will help the tree grow and improve the quality of timber; however, black locust is able to grow under conditions of low precipitation.

Black locust, like the other legumes, is able to improve the soil through a symbiotic process with nitrogen-fixing bacteria living in the nodules of the roots (Keresztesi, 1988).

Black locust is one of fastest growing species, especially in youth; its growth averages 2 to 4 ft in height per year on the better sites (Keresztesi, 1988). In closed forest stands, it has a straight bole free of branches, but in free growing areas it often develops crooked and twisted stems with thick branches. In spite of the fact that black

locust seed hard germinates, the species is spread over a wide range of the world because of the excellent rooting ability of the seedling (Keresztesi, 1988).

(2)Wood Characteristics

Black locust is a ring porous hardwood with "shining" annual rings and rays. The sapwood and the heartwood of black locust can be distinguished by the difference in wood color and by the fluorescence produced by illuminating the wood under UV light (254nm). The normal color of heartwood is brown to dark-brown and sapwood is light-yellow. The heartwood fluoresces yellow and the sapwood blue. The wood of black locust is hard and stiff (Koloc, 1953). Its density is 0.73 g/cc and is classed as a medium-heavy wood by Koloc (1953).

An outstanding property of black locust is its durability. The durability of black locust has been examined by several authors (Shain, 1977; Hart et al., 1977; Smith et al.; 1988). Their reports have shown that the extractable chemicals (primarily flavonoids) of black locust are at least partially responsible for the durability of the wood.

(3)Utilization of Black Locust

Traditional utilization of black locust wood has emphasized timber products such as firewood and fencepost due to the strength and durability of its heartwood (Funk, 1961; Scheffer, 1966). The current direction is to develop multiple

utilization of black locust due to its wide distribution and potential. Potentially valuable chemicals could be extracted from wood and used as anti-fungal treatments, dyes, instead for dihydrrobinetin having partaial resistance for fungi growth in aspen wood, and robinetin having golden yellow color. Although black locust has a high level of extractable chemicals in the heartwood, the pulp of this species also has been tested with favorable results and considered for use in the pulp industry (Menasha Corp., Otsego, MI). The foliage of black locust is thought to have potential as a fodder due to a high level of protein, although it contains a high concentration of glycoside flavonoids in leaves (Oehme, 1978). Baertsche, Yokoyama, and Hanover (1986) showed that the yield of protein from the foliage of black locust was 30% of dry weight for primary growth and 20% for regrowth, which was significantly higher than other species compared.

(C) DEFINITION OF TECHNICAL TERMS

(1) Sapwood and Heartwood

Figure 1 shows a cross section of a tree. The phloem of a tree is the tissue principally concerned with the distribution of organic substances. The cambial zone is a very thin meristematic layer between the xylem and the inner bark; it is a lateral meristem responsible for the formation of xylem and phloem. The xylem of a tree is organized into concentric annual growth rings which function as the principal

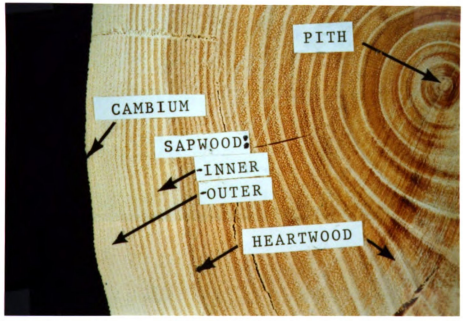



Figure 1: The cross section of black locust stem.

strengthening and water-conducting tissue of the stem, roots and leaves. Newly formed xylem cells not only serve in the support and water-conduction function of the tree but also provide a place for storage of the food reserve. The storage activity in the xylem is carried out in the living parenchyma cells which run through the xylem in both horizontal and vertical directions. Sapwood is that portion of the xylem which has parenchyma cells which are physiologically active. After a period of years, the living cells in the xylem die. This is accompanied by many secondary changes such as wood coloration, metabolism rate, and structure feature, and then the formation of the physiologically dead part of the xylem called heartwood (Panshin and de Zeeuw, 1980).

(2)Sapwood/heartwood Boundary

The sapwood/heartwood boundary (intermediate zone) occurs as a zone between the inner sapwood and the outer heartwood (Frey-Wyssling, 1959; Nobuchi et al., 1982). In a number of species, an intermediate zone, a narrow zone surrounding the outer heartwood, is called a transition zone. It has been reported that the transition zone of Pinus Radiata contains living parenchyma cells (Sandermann et al., 1967). Hillis (1977) found that a transition zone usually contained a smaller amount of extractives and lower moisture content than the surrounding tissues. It was suggested that the formation of heartwood extractives is initiated in this zone.

The apparent changes in the transition zone are not always obvious. For example, the color change of the transition zone in Acer saccharum was very gradual preventing the destinction of heartwood and sapwood. However, the transition zone of sugi (CrytomeriaJaponica) is easily observed because it appears paler than either sapwood or heartwood (Nobuchi et al., 1982; Sameshima et al., 1967). Thus, this boundary is frequently combined with either heartwood or sapwood when the zone is not distinctly colored.

(3)Flavonoids

Flavonoids are one type of secondary metabolites which are extractable from a variety of tissues. A $C_6$-$C_3$-$C_6$ unit provides the basic building block of the flavonoids and this basic unit is modified by hydroxylation and methylation to form the different flavonoid compounds. Large numbers of flavonoids have been found in the heartwood of many species. Most flavonoids are hydroxylated at the $C_5$ position; however, flavonoids in the heartwood of the Anacardiaceae family and some of the Leguminoseae lack a $C_5$ hydroxyl. The $C_5$ deoxy pattern of flavonoids is unique by fluorescence under ultraviolet (UV) light. This fluorescence allows for the detection of heartwood.

## LITERATURE REVIEW

### (A) PREVIOUS STUDIES RELATED TO THE EXTRACTABLE MATERIAL OF BLACK LOCUST

Roux and Paulus (1961) have isolated 10 flavonoids from the methanol extract of the heartwood and sapwood of black locust (Robinia pseudoacacia L.); they identified the flavonoids using two-dimensional paper chromatography. Their report indicated that the wax-free methanol extracts from the sapwood of black locust contained 1% of flavonoids 60% of which was (+)dihydrorobinetin, 22% robinetin and 10% (+)7,3',4',5'-tetrahydroxyflavan -3,4-diol. The heartwood contained 6% flavonoids of which 52% was (+)dihydrobinetin, 20% robinetin, and 13% (+)7,3',4',5'-tetrahydroxyflavan-3,4-diol. The flavonoids of the heartwood and sapwood of Robinia pseudoacacia L. are shown in Table 1. Smith et al. (1988) obtained similar results from the heartwood, they found that the methanol extract were 5.8% of the dry weight of the sample and that it contained about 90% flavonoids. The flavonoids and flavonoid glycosides of the leaves of black locust have also been investigated (Farkas et al., 1976; Kubota and Hase, 1966; Ebel et al., 1970; and Freudenberg and Hartmann, 1954). Flavonoid aglycones identified in leaves included kaempferol, apigenin, acacetin, and quercetin. These authors also determined that the flavonoid glycosides were not present in

Table 1: The concentration of flavonoids in MeOH extracts of Black Locust (Roux and Paulus, 1962)

| Flavonoid | Sapwood[1] | Heartwood[1] |
|---|---|---|
| (+)dihydrorobinetin | 2.3 | 20.0 |
| Robinetin | 0.8 | 8.0 |
| (+)7,3',4',5'-tetrahydroxy-flavan-3,4-diol | 0.4 | 6.2 |
| Leuco-robinetinidin | 0.2* | 1.0* |
| Robtin | 0.05 | 1.5 |
| Robtein | absent | 0.9 |
| (+)7,3',4'-trihydroxy-Flavan-3,4-diol | absent | ___ |
| Fustin | absent | 0.5 |
| Butin | absent | 0.5 |
| Butein | absent | 0.4 |
| Fisetin | traces | ___ |
| 2',4',4-trihydroxychalcone | absent | 0.01 |

1. The concentration is calculated based on total dry weight of extracted material.

*: Values are with reference to (+)7,3',4',5'-tetrahydroxyflavan-3,4-diol.

the heartwood of black locust (Freudenberg and Hartmann, 1953).

Holl and Poschenrieder (1975) studied the radial distribution of lipid extracts in the xylem of black locust wood from cambium to heartwood. They divided tissues into 5 areas by annual rings and titrated the quantity of lipids in the extracts by measuring acid value, unsaponifiable material, ester value, and iodine value and characterized the lipids with paper chromatography and thin layer chromatography. It was suggested that heartwood formation might be accompanied by fatty acid turnover because the ester value, iodine value, and unsaponifiable material were highest in the extracts from the innermost heartwood. The lipid extracts from the innermost sapwood of black locust had the maximum acid value, therefore, the lipid extracts from this region consist of more free fatty acids than extracts from other regions. According to TLC results, the higher lipid concentration in the older part of the trunk of black locust came partly from an accumulation of more polar lipid classes but did not include phospholipids. In general, energy rich triglycerides should be deposited in a tissue which exhibited only dead cells. However, Holl and Poschenrieder's study (1975) indicated that the increase in lipid content of the interior part of black locust is not due to an accumulation of triglycerides.

Tchorbajiev, Ivanov and Stefanov (1969) characterized the composition of the hydrocarbons of Bulgarian black locust

flowers. Their work showed that the concrete of black locust consisted of normal paraffins from $C_{17}H_{36}$ to $C_{33}H_{68}$ and the amount of odd hydrocarbons (89.16% of the hydrocarbons) are significantly higher than that of the even hydrocarbons (10.97% of the hydrocarbons). The major hydrocarbons present in the concrete are $C_{27}$, $C_{29}$, and $C_{25}$ hydrocarbons.

## (B)GENERAL CONCEPTS IN FLAVONOIDS BIOSYNTHESIS

It is recognized that the flavonoid biosynthetic pathway incorporates precursors from both the "shikimate" and "acetate-malonate" pathway (Hahlbrock and Grisebach, 1975; Wong, 1976). Tracer experiments indicate that the B-ring and the carbons at positions 2, 3, and 4 on the C-ring of flavonoids come from a phenylpropanoid ($C_6$-$C_3$) unit while the A-ring is made from three acetate ($C_2$) units. The labeling pattern obtained is shown as Figure 2. (Grisebach, 1952). Hillis et al., (1963) pointed out that cultured cambial tissues can utilize glucose to synthesize shikimic acid and lignin. The shikimate pathway also leads to synthesis of the phenylpropanoid compounds via L-phenylalanine. Cinnamic acid, the starting compound for the biosynthesis of flavonoids, is derived from phenylalanine via the phenylalanine cinnamic acid pathway (Gross, 1985). Hence, shikimic acid is the common precursor in the synthesis of lignin and flavonoids. An outline of the relationship between different primary

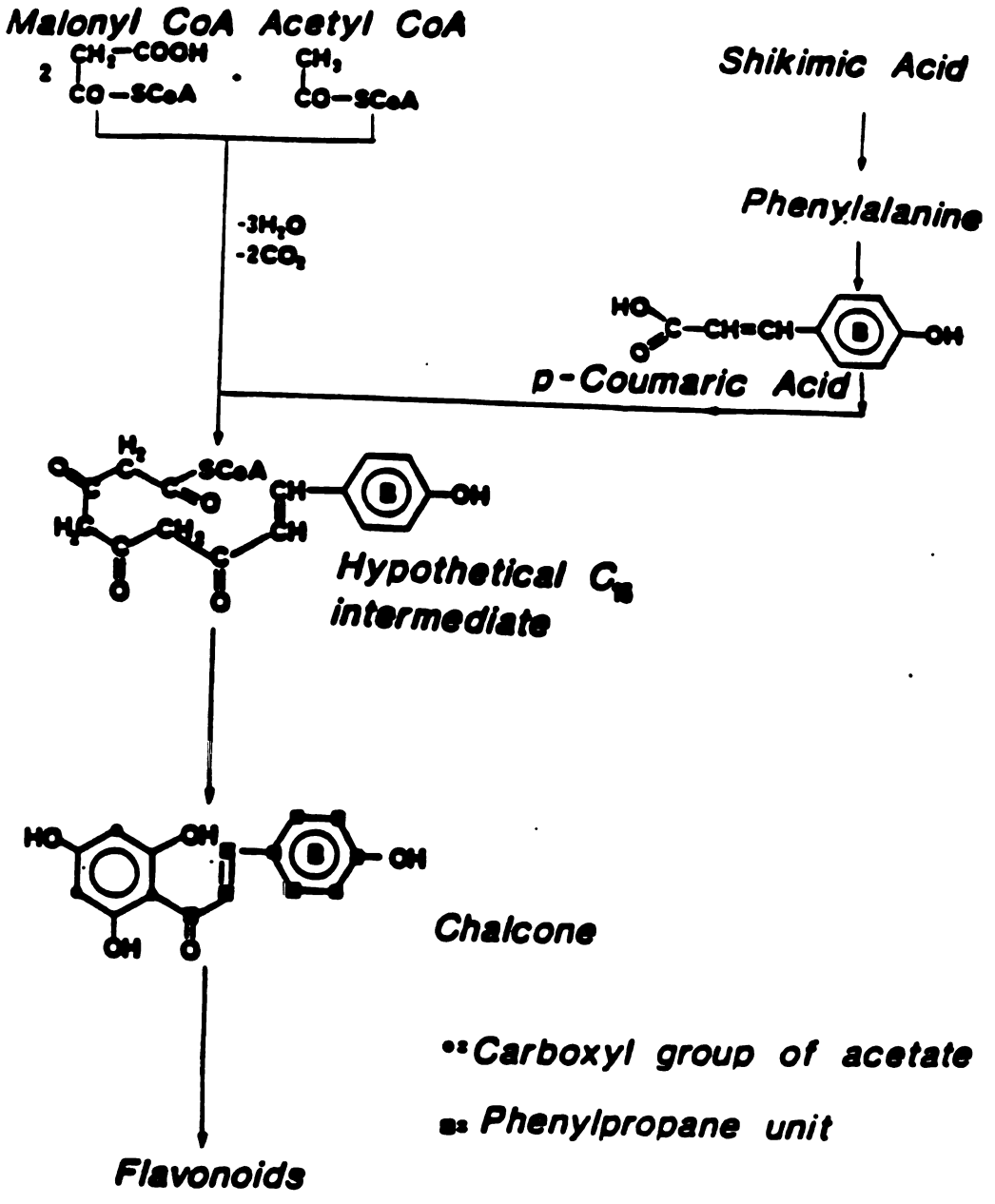



Figure 2: The labeling pattern of chalcone (Walker, 1975)

metabolites with flavonoid biosynthetic pathway is drawn in Figure 3. (Hillis, 1986).

The first flavonoid produced after the confluence of the acetate and shikimic acid pathways is thought to be the chalcone- 2',4,4',6'-tetrahydroxy-1,3-diphenyl-2-propen-1-one. All other flavonoid are derived from this compound (Light and Hahlbrock, 1980; Heller and Hahlbrock, 1980). Tracer experiments and enzymatic studies have confirmed this (Wong, 1976). The key enzyme for synthesis of this chalcone intermediate of flavonoid is chalcone synthase. Much evidence from in vitro and in vivo experiments have suggested that 4-hydroxy-cinnamoyl CoA ester is the most efficient substrate of chalcone synthase (Grisebach, 1985; Hrazdina et al., 1976; Stich and Forkmann, 1987). Chalcone synthase can also accept another phenylpropanoid thioester, caffeic CoA, as a substrate having an optimum activity at pH =6.8 while 4-hydroxy-cinnamoyl CoA having an optimum activity at pH =8.0 (Krenzaler et al., 1979; Welle and Grisebach, 1987). However, Stich and Forkmann (1987) found that the predominant product came from the 4-hydroxy-cinnamoyl CoA when the two precursors of chalcone synthase were incubated with the enzyme at pH =6.8. Evidence indicates that chalcone synthase has a high substrate specificity for 4-hydroxy-cinnamoyl-CoA ester and that the further hydroxylation of the flavonoid B-ring takes place at the $C_{15}$ level (Grisebach, 1985).

It is not easy to isolate and incubate the chalcone

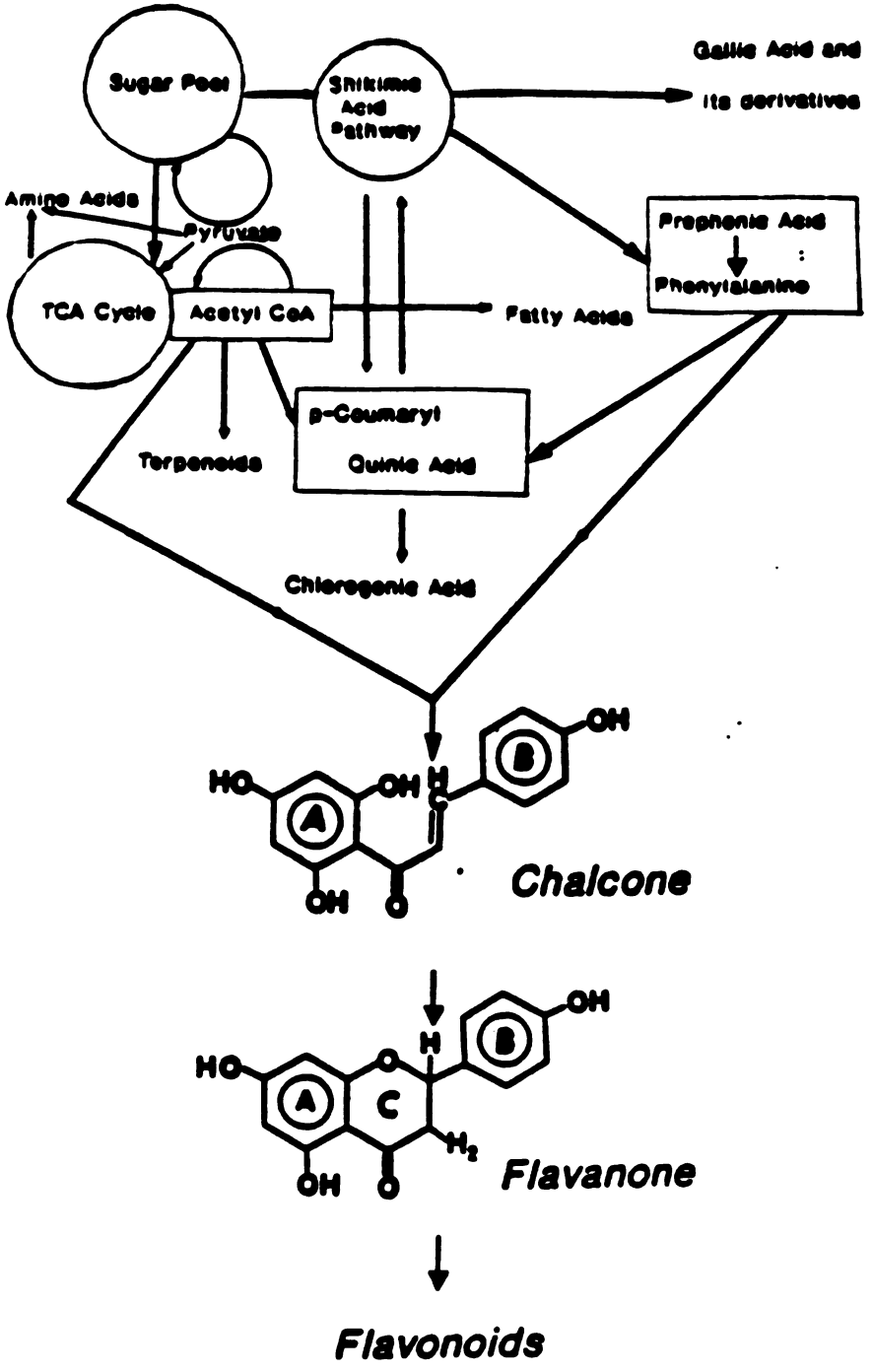



Figure 3: Biosynthetic pathway of flavonoids from primary metabolities. (Hillis, 1987).

intermediate in flavonoid biosynthesis pathway because the isomers (flavanones) are synthesized more rapidly by a very active enzyme (chalcone isomerase) (Heller, 1986). After flavanones are formed, the flavonoid biosynthesis pathway goes in several directions and toward final products. These final products include flavones, dihydroflavonols, flavanols, anthocyanidins and condensed tannins. The relationship between the chalcone intermediate and final products is shown in Figure 4. These products are classified by different oxidation pattern and stereochemistry at the C-ring. Numerous enzymes are involved in the sequential procedures.

The flavonoids from black locust heartwood are 5-deoxy, which have no hydroxyl group at C-5 and show yellow fluorescence under UV light (254 nm). Although the biosynthesis of 5-deoxy flavonoids has not been completely described, Grisebach's studies (1988) found that the chalone synthase and an "extra protein" are required for the formation of 5-deoxy chalone. Holl and Lendzian (1973) have shown that in black locust the maximum oxygen consumption is in the oldest sapwood ring (perhaps at the sapwood/heartwood boundary).

## (C) SAPWOOD-HEARTWOOD TRANSFORMATION

It has been suggested by Hart (1968) that the transformation of sapwood to heartwood is associated with a) the death of the living cells in the sapwood, b) an abrupt

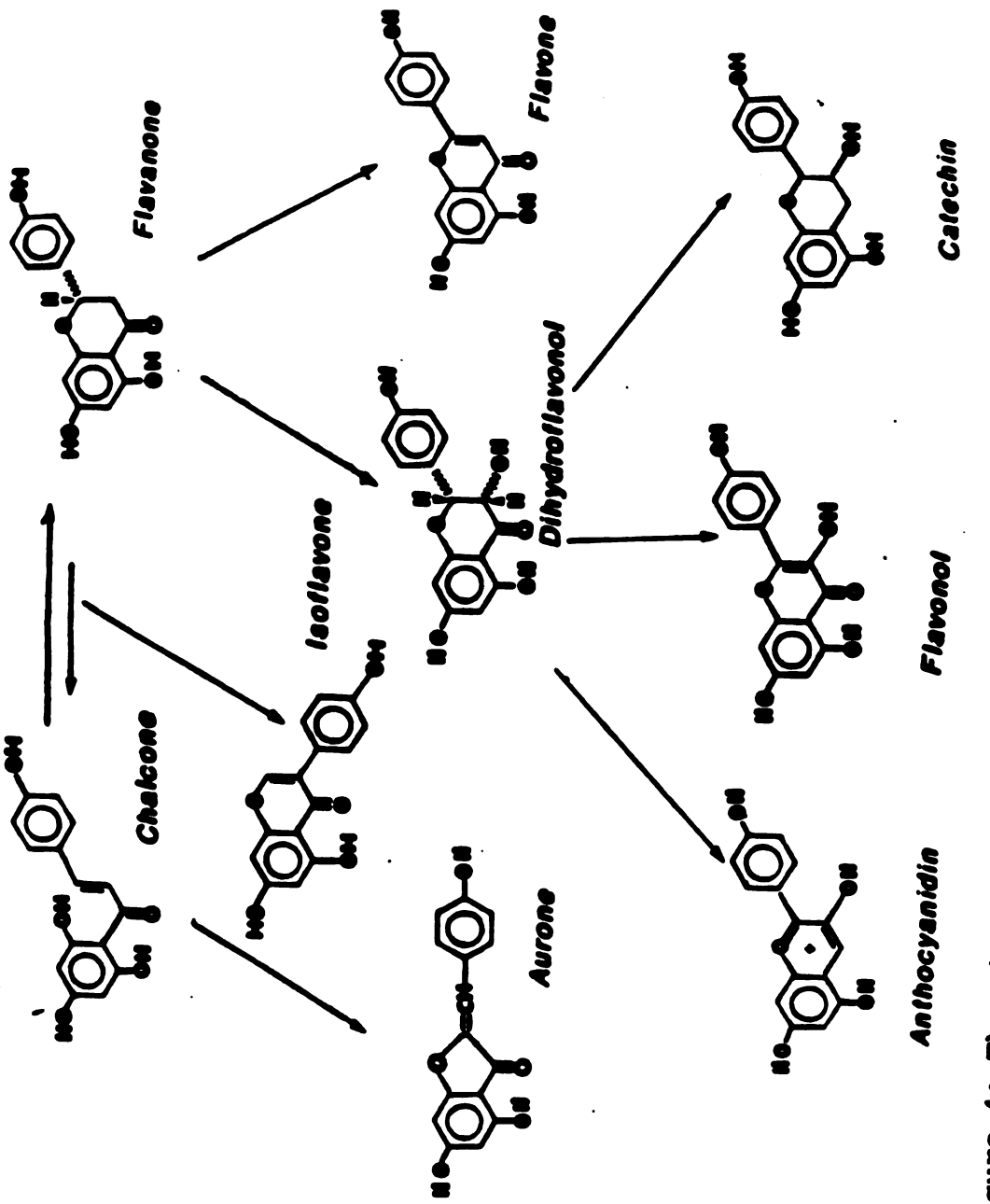



Figure 4: The structure of flavonoid products from chalcone intermediate.
(Griseback, 1985)

decrease in the amount of starch and non-cell wall carbohydrates, and c) an abrupt increase in the amount of polyphenolics (tannin, lignin, flavonoid). Data from Hart's study indicated that starch granules of black locust were numerous in the ray cells of the sapwood but none were found in the heartwood or discolored sapwood.

The seasonal variation in the reversable carbohydrates of living bark of black locust and the relation to its frost hardiness have been studied by Siminovitch et al. (1953). They suggeated that the carbohydrate fluctuation was related to a precise temperature control mechanism. The storage carbohydrates, sucrose and starch, are used in the late spring by the flush of new growth. The starch and sucrose level decreased rapidly in the growth season. The sucrose level, however, increased again after a short time and reached a maximum in September and maintained that level during the cold weather. In contrast, the starch level accumulated until September, then decreased again during the cold weather. In the winter season, the reducing sugars reached a maximum level of about 3% of dry weight. The levels of reducing sugars were constant (0.8% to 1.1% of dry weight) in the other seasons of a year.

Nobuchi et al. (1984b) have observed tissues from the cambium to the heartwood of black locust from a cytological point of view to investigate the season of heartwood formation. The activity of ray parenchyma cells near sapwood/

heartwood boundary was determined by observing the changes in the cell contents. Their report indicated that the nuclei of parenchyma cells, starch granules, and lipid droplets of the boundary appeared to change in the summer season. After starch granules disappeared from the innermost annual ring of the sapwood in late summer, the formation of colored compounds associated with the heartwood formed. During heartwood formation, the size of lipid droplets was shown to be rapidly increasing and starch granules and nuclei of the ray parenchyma cells were present in the heartwood. These results observed in the heartwood conflict with the definition of International Association of Wood Anatomy (IAWA) for heartwood which states that there are no living cells and starch granules. It was suggested that the boundary zone has significant changes during heartwood formation and that the living cells in the heartwood gradually produce the heartwood substances and become dead cells before the next onset of cambial activity. Frey-Wyssling and Bosshard (1959) got similar results with several species of gymnosperms and angiosperms. In this experiment, the activity of mitochondria was determined an indicator of normal respiration. Mitochondria in the living cells can accumulate the stain of a dye (Janus green B) and transform it into colorless leucodye because reduction of Janus green B provides a proton which enters the TCA cycle. The ability of a cell to reduce this dye indicates active mitochondria. Frey-Wyssling and Bosshard

determined that mitochondria in wood lose their activity at a very early age (a few rings inward from the cambium). The heartwood phenolics of Sugi (Cryptomeria japonica D. Don) have been examined by Nobuchi (1984a). They found three main heartwood phenolics and that each compound occurred in a different radial position and in different types of ray parenchyma cells. One exists only in the transition zone to heartwood. The others are located in the outer heartwood.

Holl (1972 and 1973) has determined the radial changes of enzymatic activity in black locust wood. There were two active amylases in the outer sapwood, 4-glucano-hydrolase and 4-glucano-maltohydrolase while only 4-glucano-hydrolase was found in the inner sapwood. The total amylase activity in black locust was shown to increase toward the center of the wood and the sapwood/heartwood boundary had the most intensive activity of starch-decomposing enzymes. In contrast, the enzymes involved in starch synthesis decreased in activity from peripheral to central tissues. The amyloplast at the boundary zone had an inhibitor compound which suppressed starch synthesis in the peripheral annual rings of black locust trunk.

Radioactivity studies aimed at identifying the fluctuation of phenolic compounds found in the boundary zone of Prunus yedoensis were undertaken by Hasegawa et al. (1966). They assumed that the sucrose or other sugars were

translocated from leaves, and then the phenolic compounds were synthesized in situ. $^{14}C$-labeled sucrose was administered to the cambium of Prunus yedoensis for 3 hours in November and the labeled tree cut down after 12 days. They found that metabolism varied in the different tissues. The labeled compounds were translocated into the sapwood/heartwood boundary via ray cells after the labeled sucrose was converted. The labeled shikimic acid in the outer sapwood had a higher density than the inner sapwood and transition zone. The labeled quinic acid and citric acid, however, were present in higher radioactivity in the inner sapwood and transition zone. The radioactivity of essential amino acids was also determined and appeared that tyrosine had a higher density in the inner sapwood and transition zone. The radioactivity of basic components decreased at the sapwood/heartwood boundary. Simple sugars converted from the labeled sucrose were also examined. The radioactivity of glucose and fructose decreased in the boundary when the radiation of those sugars increased in the outer sapwood.

## MATERIALS AND METHODS

### (A) SAMPLES DESCRIPTION AND PREPARATION

Wood disks (2-3 inch thick) were collected from black locust trees (Robinia pseudoacacia L.) at 1-2 month intervals for one year. One tree per month was sampled unless noted otherwise. The trees (average age 15-20 years old) were from one of two Michigan State University locations: the Water Quality research area and Kellogg Forest. Two disks were taken from each tree at breast height. One was used to measure the moisture content and the other for the qualitative and quantitative analyses. Detailed information about individual trees is listed in Table 2.

After the disks were cut, the samples were immediately placed into a cooler with ice to inhibit the activity of amylase and brought back to the laboratory. One of two disks was immediately dried in an oven at 100°C for 2 hours and then dried to constant weight at 70°C to destroy the activity of amylase. Each disk was separated into 5 parts using a chisel according to color differences in the fluorescence under UV light at 254 nm and in the wood coloration as described previously. The first section, cambium, was removed from the inside of the bark. Next, the sapwood portion of each section was removed. The intermediate zone included the edge of the heartwood/sapwood boundary as determined by fluorescence and

Table 2: The harvest date, sample number, and moisture content of black locust

| Harvest Date | Samples Location | Number Samples | Moisture Content (% of Dry Weight) Cambium | Sapwood | Transition | Heartwood |
|---|---|---|---|---|---|---|
| September 26, 88 | W[1] | 1 | 84.98 | 52.33 | 44.82 | 33.64 |
| December 12, 88 | W | 1 | 86.67 | 40.54 | 37.97 | 30.63 |
| January 14, 89 | W | 1 | 70.12 | 37.34 | 31.65 | 30.44 |
| February 20, 89 | W | 1 | 68.56 | 34.64 | 22.52 | 20.03 |
| March 17, 89 | K[2] | 4 | 58.57 | 39.97 | 29.35 | 30.87 |
| April 15, 89 | W | 1 | 98.71 | 46.43 | 40.38 | 32.00 |
| May 15, 89* | W | 1 | 78.59 | 44.92 | 36.53 | 22.33 |
| June 15, 89 | W | 1 | 156.51 | 74.95 | 39.39 | 33.49 |
| July 12, 89 | W | 1 | 104.94 | 51.60 | 38.87 | 37.55 |
| August 10, 89 | W | 1 | 136.37 | 69.83 | 45.95 | 41.18 |

* The moisture content measurements were done 2 weeks after the sample was harvested.
1= Water Quality
2= Kellogg Forest

visual inspection and 0.1-0.3 cm of sapwood. After the intermediate zone was removed, the heartwood was collected. For each type of sample, the small pieces obtained were ground in a Wiley mill to pass through a 40 mesh screen. The ground samples were stored in glass bottles at room temperature.

## (B) FLAVONOIDS OR FLAVONOID PRECURSORS

### (1) Qualitative Analysis

#### (a) Equipment

The gas chromatograph-mass spectrophotometer (GC-MS) utilized in this study was a HEWLET PACKARD (HP) 5970B mass selective detector with a 5890A gas chromatograph. The column used was a J&W SCIENTIFIC fused silica capillary column (film thickness: 0.25 micrometer) with nonpolar DB-1 (methylsilicone) phase. The temperature program used was an initial temperature of 200°C and held for 5 minutes, an increased rate of temperature of 5°C/min, and the final temperature of 280°C for 30 minutes.

The gas chromatograph-Fourier transform infrared spectrophotometer (GC-FTIR) utilized in this study was a BIO-RAD Digilab Division FTS-40 Fourier transform infrared spectroscopy system with a HEWLET PACKARD (HP) 5890A gas chromatograph. The column used was a J&W SCIENTIFIC fused silica capillary column (film thickness: 0.25 micrometer) with nonpolar DB-1 (methylsilicone) phase.

(b) Chemicals

The solvents (analyzed grade) used for extraction were purchased from J.T. Baker and Aldrich. The silylation reagent, bis(trimethylsilyl)trifluoroacetamide, BSTFA was purchased from SUPELCO.

(c) Experimental Procedure

The ground wood meal was extracted with 100% methanol followed by 50% aqueous acetone at room temperature for one day. The extracts were filtered, concentrated to dryness by rotary-evaporation, lyophilized and then weighed. The yield of extractives is listed in Table 3. A sample of the crude extract (generally 100 milligrams) was redissolved in 10 ml $H_2O$ and extracted with 10 ml hexane (2x) to remove wax from the extracts followed by 10 ml ethyl acetate (EtOAc)(2x) to isolate the neutral flavonoids. The residual aqueous solution was hydrolyzed with cellulase for 24 hours to break sugar-flavonoid bonds. The acid treatment of the residual aqueous solution were also taken to determine whether both hydydrolysis methods occurred properly. The flavonoid aglycones were extracted from the aqueous hydrolysis mixture with EtOAc (2x). Both ethyl acetate extracts were taken to dryness by rotary-evaporation and redissolved into 400 microliters of acetonitrile. An aliquot (100 microliter) of each sample was analyzed by thin-layer chromatography (silica gel with fluorescent indicator) using chloroform: ethyl

Table 3: The Yield of Extractives in Black Locust[1]

| Heavest | Cambium | | Sapwood | | Transiton | |
|---|---|---|---|---|---|---|
| Month | MeOH | 50% Acetone | MeOH | 50% Acetone | MeOH | 50% Acetone |
| September | 3.83 | 0.94 | 1.00 | 0.54 | 2.46 | 1.72 |
| December | 8.61 | 1.59 | 1.74 | 0.80 | 2.94 | 1.88 |
| January | 5.32 | 3.01 | 2.74 | 0.60 | 1.08 | 1.56 |
| February | 8.95 | 3.57 | 3.04 | 0.34 | 2.33 | 1.67 |
| March | 7.63 | 2.01 | 2.48 | 0.52 | 2.99 | 1.72 |
| April | 3.71 | 1.12 | 1.91 | 0.57 | 2.24 | 1.54 |
| May | 8.65 | 3.04 | 2.55 | 0.62 | 2.94 | 1.86 |
| June | 2.72 | 0.54 | 2.31 | 0.46 | 1.55 | 1.37 |
| July | 3.96 | 0.79 | 1.40 | 0.52 | 1.77 | 1.55 |
| August | | | | | | |

1. The yield of extractives is calculated as the percentage of the dry weight of samples.

acetate: formic acid (5:5:1) as the mobile phase and shortwave UV light (254nm) for detection of spots. A 50 microliter aliquot of the acetonitrile sample was silylated with bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 280°C for 1 hour. The silylated compounds were then analyzed by gas chromatography-mass spectrometry (GC-MS) and gas chromatography-Fourier transform infrared spectroscopy (GC-FTIR).

(2) Quantitative Analysis

(a) Equipment

All absorption spectra were determined with a PERKIN-ELMER lambda 4B uv/vis spectrophotometer with tungsten-bromide and deuterium lamps using 10 mm light-path absorption cells.

(b) Chemicals

For colormetric tests, the standard compounds (D-(+)-glucose, starch, and gallic acid) were purchased from Sigma. The Folin-Ciocalteu (FC) reagent of phenolic test was also purchased from Sigma.

(c) Preparation of Standard Phenolics Curve

A series of standard gallic acid solution ( $1x10^{-7}$, $2x10^{-7}$, $5x10^{-7}$, $1x10^{-6}$, $2x10^{-6}$, $5x10^{-6}$, $6x10^{-6}$, $8x10^{-6}$, $1x10^{-5}$, and $5x10^{-5}$ g/ml) were prepared by dilution a 0.1g/100ml solution freshly prepared, and analyzed using a modification of the phosphomolybdic-phosphotungstic acid method (Singleton and

Joseph A.Rossi, 1965). A 1 ml of Folin-Ciocalteu (FC) reagent was added and mixed with a 10 ml standard gallic acid solution followed by adding 3 ml of 20% (w/v) sodium carbonate aqueous solution; the procedure was completely finished within 8 mins. Then, color was allowed to develop for 2 hours to obtain the maximum absorption. The absorbance was determined at 765 nm. A blank was also prepared. The standard curve of phenolics was generated by plotted the absorption vs. concentration of standard phenolic solutions. The linear range of standard curve was determined by the correlation coefficient of the linear regression.

(d)Analysis of Black Locust Samples

For each ground sample ten replications (0.5g) were placed into a 50 ml graduated test tube and extracted with hexane (5 mL) for 30 mins. in a sonicator followed by centrifugation at 500 rpm for 3 mins. The hexane extracts containing wax, were discarded. The residue was extracted with 80% aqueous ethanol (15 mL) for 12 hours then centrifuged at 500 rpm for 3 mins. It was determined that the amount of total phenolics and soluble sugars extracted did not significantly increase after 12 hours extraction. The supernatant was removed from the tube and used in the analyses of total phenolics and soluble sugars. The residue was dried overnight and then used for starch analysis since starch was not extractable in 80% aqueous ethanol. In order to fall into

the linear range of the standard curve, the ethanol extract was diluted to 50 ml volume (solution I) with double distilled water. A 10 ml aliquot from solution I was diluted to 50 ml (solution II). In the transition zone and heartwood portion, solution II was used for the phenolic test while solution I was used in the cambium zone and sapwood samples. The total phenolics in a portion of black locust was expressed as equivalents of gallic acid by comparison with the standard curve. The procedure for the quantitative determination of total phenolics from black locust was the same as that described for preparing the standard curve (Singleton and Joseph A.Rossi, 1965).

(C)CARBOHYDRATES

(1)Quantitative Analysis

(a)Soluble sugars

(i)Preparation of Standard Curves: The sugar standard used was D-(+)-glucose. The glucose standard solutions were prepared by dilution of a 10mg/ml solution. The absorption of a series ($1x10^{-6}$, $2x10^{-5}$, $4x10^{-5}$, $5x10^{-5}$, $1x10^{-4}$, $2x10^{-4}$, $4x10^{-4}$, $5x10^{-4}$, and $1x10^{-3}$ g/ml) of D-(+)-glucose solutions were analyzed to construct a standard curve using the phenol-sulfuric acid colorimetric method (Hodge and Hofreiter, 1962). 1 ml aliquots of the solutions were placed into a 25 ml glass test tube with 16 to 20 mm diameter, 1ml

of 5% (w/v) aqueous phenol was added. Then, 5 ml of concentrated sulfuric acid was added rapidly. The tubes were allowed to stand 10 minutes, then they were shaken and placed for 20 mins. in an ice bucket before the absorbance was taken. Absorbance were measured at 490 nm. Water was used as a reference. The standard curve of absorbance vs concentration for glucose was generated by the linear regression. The linear range of the curve was determinated with the correlation coefficient.

(ii)Analysis of Black Locust Samples: Each ground sample (0.5g) was extracted with hexane and followed with 80% ethanol in which the detail of this procedure was as described for the total phenolics analysis in black locust samples. The ethanol extract was diluted to 50 ml (solution I). A 10 ml aliquot of solution I was diluted to 50 ml (solution II). A 1 ml aliquot from solution II was the sample used to determine the concentration of soluble sugars. The procedure for the quantitative determination of soluble sugars from black locust was the same as that described for preparing the standard curve (Hodge and Hofreiter, 1962). Each portion in this experiment was replicated ten times.

(b)Starch

(i)Preparation of Standard Curve: The absorption of a series of starch standard solutions ($1.6x10^{-3}$, $2.2x10^{-3}$, $5.5x10^{-3}$, $9.1x10^{-3}$, $1.06x10^{-2}$, $2.25x10^{-2}$, $5.5x10^{-2}$, $8.09x10^{-2}$,

$1.065x10^{-1}$ g/50 ml) were prepared and analyzed by a modified perchloric acid colorimetric method (Humphreys F.R. and Kelly J., 1961). A weighed starch sample was placed into a 50ml plastic graduated test tube, dispersed with 5 ml of 7.2M perchloric acid and allowed to react for 20 mins. with occasional stirring. The volume was then brought to 50 ml (solution I) with double distilled water. The sample was centrifuged at 500 rpm for 3 mins. A 10 ml aliquot from solution I was placed into a 50 ml graduated test tube with 1 drop of phenolphthalein and made alkaline with 2N sodium hydroxide. 2N acetic acid was added until the color disappeared, 2.5 ml more acetic acid was added, followed by 20 drops of 10% (w/v) potassium iodide and 5 ml 0.01N potassium iodate. The color was allowed to develop for 20 mins followed by dilution of the sample to 50 ml. The absorbance was measured at 650nm. Water was used for reference. The standard curve of starch was generated by plotted the absorption vs. concentration of standard starch solutions. The linear range of the curve was determinated by the correlation coefficient of the linear regression.

(ii)Analysis of Black Locust Samples: The starch content in 0.5 gram of ground wood meal was determined by the procedure used for the standard curve of starch preparation. After hydrolysis with procholoric acid, the samples were diluted to 50 ml (solution I). Black locust contained higher

levels of starch than the standard starch samples so that black locust samples were diluted and fell into the linear range of the standard curve. A 10 ml aliquot from solution I was diluted to 50 ml (solution II). A 10 ml aliquot from solution II was used to determine the starch content of wood. Ten replication of each portion in this experiment were examined.

## RESULTS AND DISCUSSION

All woody stems original from the cambial zone and through aging processes for several years. The cambial zone, parenchyma cells and ray cells are living tissues in a wood stem. The cambial activity, cell division, enlargement, and differentiation, is modified by environmental conditions such as temperature, soil, light, or moisture and mediated by internal processes such as the rate of photosynthesis and hormone production. However, the factors affecting wood structure do not influence the distribution of extractable chemicals in the cell lumen. Individual cell types within a species may differ in the distribution of extractable chemicals. Multiple samples were collected in March from one location and the three quantitative procedures were tested for tree to tree variability. The detailed information of variation for each analysis is listed in Table 4. It appears that the proportional variation from multiple samples is much greater than the variation between trees even though both sources of both variations are significant. In other words, the tree variation in this study plays a minor part indetermining the relative level of metabolic compounds.

If it is true that the flavonoids (dihydrorobinetin and robinetin) found in the black locust heartwood are responsible for the durability of heartwood, it would be interesting to

Table 4: (a) ANOVA of total phenolics test for multiple samples in March. (b) ANOVA of soluble sugars test for multiple samples in March. (c) ANOVA of starch test for multiple samples in March.

(a)

| SOURCE | DF | $(SUM)^2$ | $(MEAN)^2$ | F-RATIO |
|---|---|---|---|---|
| TREE(A) | 3 | 1.151744 | .3839146 | 75.49 |
| LOCATION(B) | 3 | 57.66125 | 19.22042 | 3779.52 |
| A X B | 9 | 2.657956 | .2953285 | 58.07 |
| ERROR | 144 | .7322992 | 5.085E-03 | |
| TOTAL | 159 | 62.20324 | | |

(b)

| SOURCE | DF | $(SUM)^2$ | $(MEAN)^2$ | F-RATIO |
|---|---|---|---|---|
| TREE(A) | 3 | 1.268E-03 | 4.228E-04 | 176.40 |
| LOCATION(B) | 3 | 1.264E-02 | 4.214E-03 | 1757.88 |
| A X B | 9 | 8.003E-04 | 8.893E-05 | 37.10 |
| ERROR | 144 | 3.452E-04 | 2.397E-06 | |
| TOTAL | 159 | .0150568 | | |

(c)

| SOURCE | DF | $(SUM)^2$ | $(MEAN)^2$ | F-RATIO |
|---|---|---|---|---|
| TREE(A) | 3 | 3.603E-04 | 1.201E-04 | 153.40 |
| LOCATION(B) | 3 | 2.286E-03 | 7.620E-04 | 973.11 |
| A X B | 9 | 2.467E-04 | 2.741E-05 | 35.01 |
| ERROR | 144 | 1.127E-04 | 7.831E-07 | |
| TOTAL | 159 | 3.006E-03 | | |

determine the location of flavonoid biosynthesis. The heartwood of black locust contains a high level of flavonoids, about 6% of dry weight. The sapwood, however, has a very low level of flavonoids less than 1% of dry weight (Roux and Paulus, 1962). It has been suggested that flavonoids in heartwood may be translocated from the cambial zone and/or the sapwood into the heartwood with the amount increasing and transformed in specific tissues such as the transition zone. Therefore, in this study, the flavonoids in the heartwood of black locust were analyzed qualitatively to confirm the location of the flavonoids of black locust using the procedure described previously. The total phenolics as gallic acid equivalents, starch, and soluble sugars, from cambium to heartwood, were analyzed quantitatively over a period of one year to look for a flux of flavonoids into the heartwood.

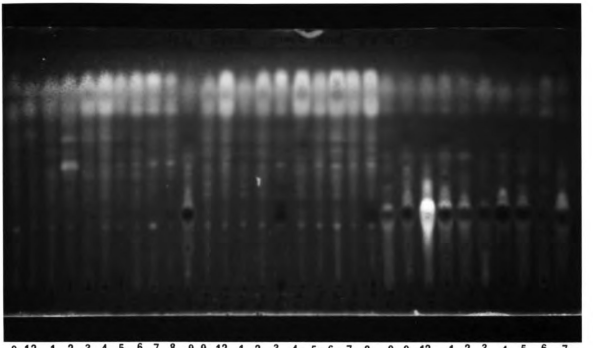
## (A) FLAVONOIDS

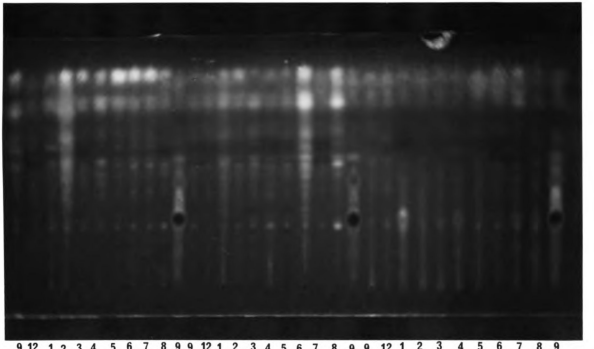
### (1) Qualitative Analysis

#### (a) TLC Results

An ethyl acetate extract of the unhydrolyzed heartwood was used as a reference. The EtOAc extracts of samples, obtained as described previously, were separated by TLC. Samples from cambial zone, sapwood, and transition zone were separated both before and after hydrolysis with cellulase. The results are presented in Figure 5. The TLC

Figure 5: (Top) The TLC results of EtOAc extractives before hydrolysis with cellulase. (Bottom) The TLC result of EtoAc extractives after hydrolysis with cellulase.
(C= Cambial zone, S= Sapwood, T= Transition zone, H= Heartwood, and the numbers are presented the month)


9 12 1 2 3 4 5 6 7 8 9 9 12 1 2 3 4 5 6 7 8 9 9 12 1 2 3 4 5 6 7
S S S S S S S S S H C C C C C C C C C T H T T T T T T T T


9 12 1 2 3 4 5 6 7 8 9 9 12 1 2 3 4 5 6 7 8 9 12 1 2 3 4 5 6 7 8 9
C C C C C C C C C H S S S S S S S S S H T T T T T T T T T H

separations showed that the EtOAc extracts of the heartwood and transition zone before hydrolysis contained neutral flavonoids (dihydrorobinetin and robinetin) while the sapwood and cambium contained only simple phenolic compounds. However, the TLC results from EtOAc extracts of the transition zone after hydrolysis suggested that the flavonoids in the transition zone did not exist as glycosides or that glycosides were present at a low level. It was necessary to use the more precise analysis technique to monitor the particular flavonoids (dihydrorobinetin and robinetin). The TLC separation pattern from the EtoAc extracts of the sapwood and cambial zone before hydrolysis were similar to that obtained after hydrolysis. The results suggested that the compounds form sapwood extracts had some compounds in common with the cambial zone. The TLC comparison of sapwood before hydrolysis and after hydrolysis with cellulase and 2N HCl:MeOH (1:1) appears in Figure 6. The chromatography demonstrated that the two hydrolysis methods lead to the formation of two different set compounds.

(b) Instrumental Analyses

The EtOAc extractives before and after hydrolysis with cellulase were analyzed with GC-MS in order to whether the minor amounts of robinetin, dihydrorobinetin and/or other flavonoids were present but not detectable by TLC. Samples from December and February sapwood were also selected and

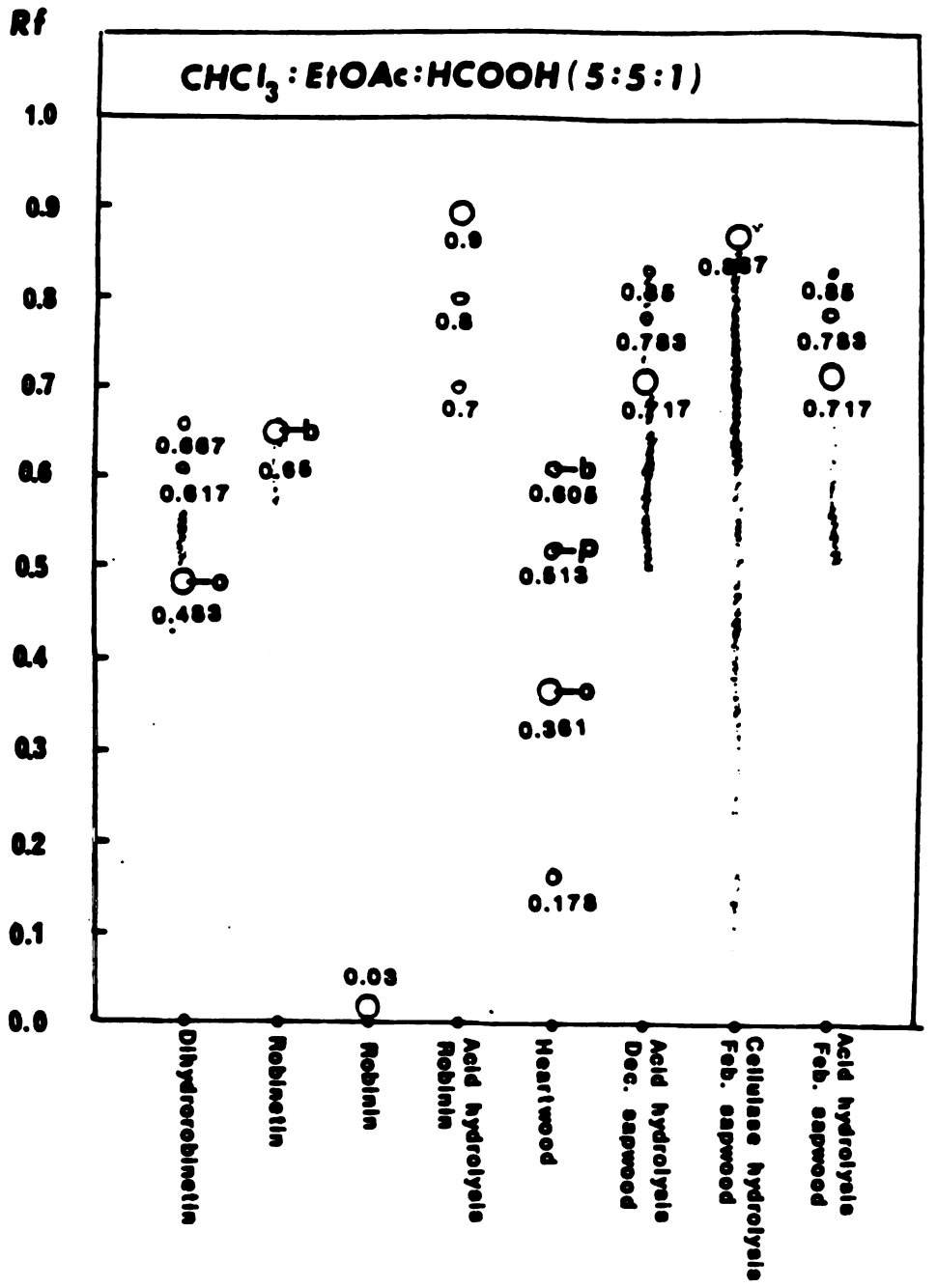



Figure 6: The TLC comparison of sapwood before and after hydrolysis with cellulase and 2N HCl:MeOH (1:1).

hydyolyzed 2N HCl:MeOH (1:1). The GC-MS comparison of sapwood before hydrolysis and after hydrolysis with cellulase and 2N HCl:MeOH (1:1) is presented in Figure 7. Both results from the total ion chromatography and ion current profile suggested that dihydrorobinetin and robinetin were not present in the sapwood or the cambial zone of black locust. The chromatograms, samples from each portion of black locust wood hydrolyzed with cellulase, are presented in Figure 8, 9, and 10, respectively. Fatty acids, hexadecanoic acids ($C_{16}$), and octadecanoic acid ($C_{18}$), were the major fatty acid found in the cambial zone, sapwood, and transition zone. Only dihydrorobinetin was found in the transition zone after samples were hydrolyzed with cellulase. From biosynthesis pointview, this result suggested that dihydrorobinetin was first synthesized in the pathway. Some monosaccharides (D-glucose and D-mannose) were found in the cambial zone and the sapwood. The flavonoids were not found in the sapwood through a whole year. The compounds (a) and (b) were presented in every tissue sample from the cambial zone to the heartwood. The compound (a) had a retention time 25.390 min., the base peak 239, and the molecular ion 372. The compound (b) had a retention time 27.063 min., the base peak 387, and the molecular ion 402. Beside compound (a), and (b), simple phenolic compound, benzoic acid, was found in the extracts of sapwood and cambial zone after acid hydrolysis.

The chromatograms of unhydrolysis samples from the

Figure 7: The Total ion chromatograph (TIC) comparison of GC-MS in sapwood before and after hydrolysis with cellulase and 2N HCl:MeOH (1:1)

(1) December sample before hydrolysis with cellulase.

(2) December sample after hydrolysis with acid.

(3) December sample after hydrolysis with cellulase.

(1') February sample before hydrolysis with cellulase.

(2') February sample after hydrolysis with acid.

(3') February sample after hydrolysis with cellulase.

(a, and b are presented the unidentified compounds which are the possible precursors of flavonoids)

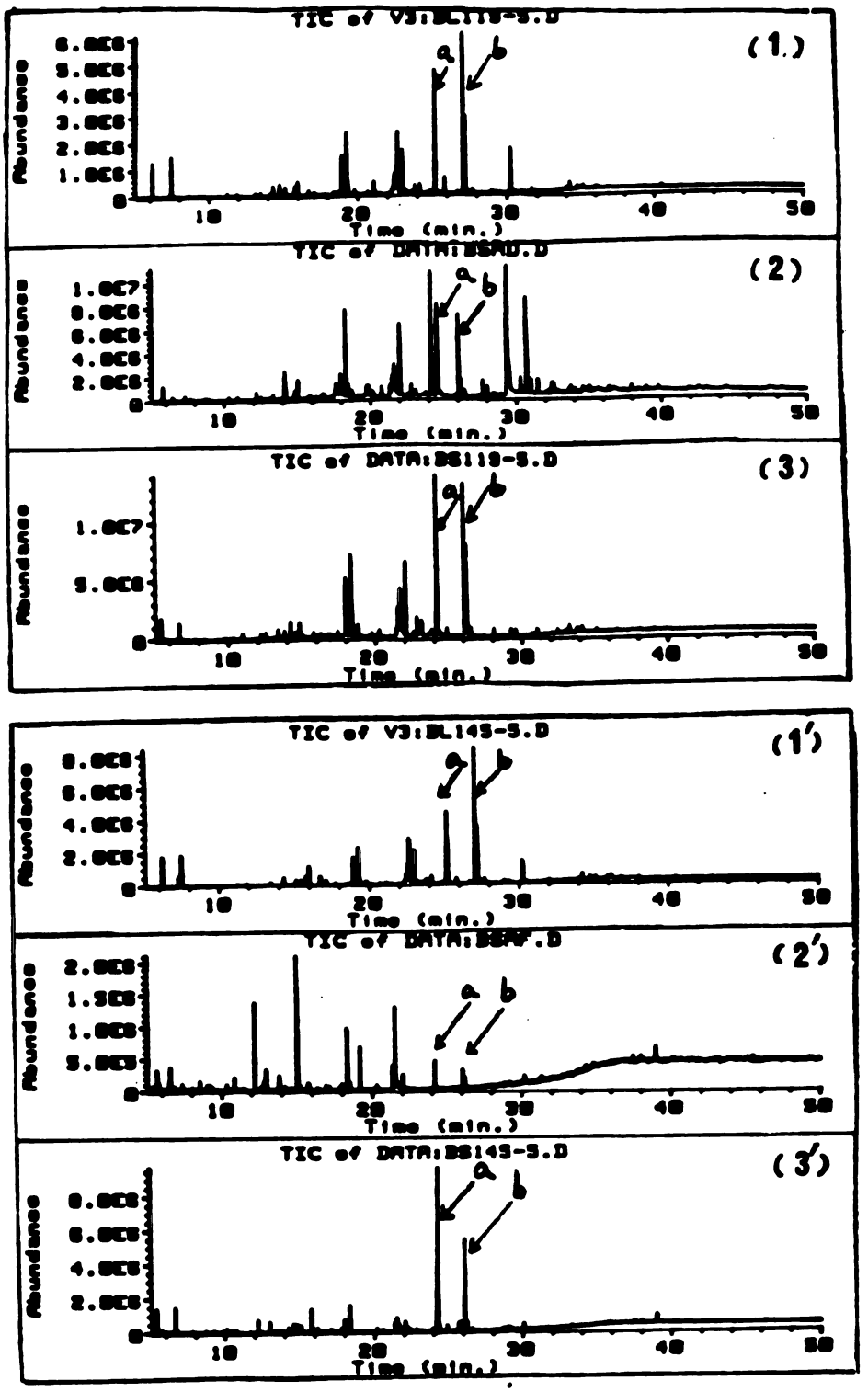
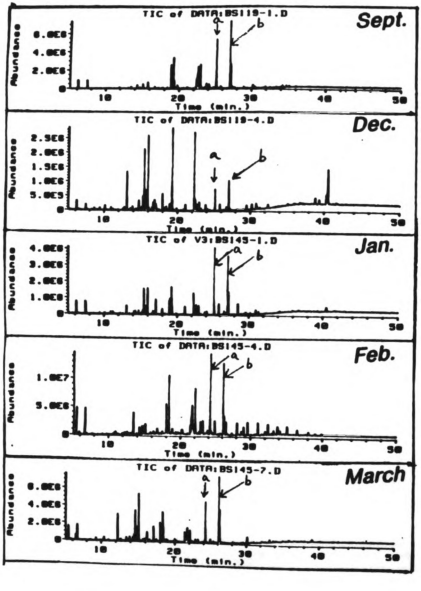

TIC of V3:BL118-S.D
(1.)
TIC of DATA:BSAD.D
(2)
TIC of DATA:BS118-S.D
(3)
TIC of V3:BL145-S.D
(1')
TIC of DATA:BSAF.D
(2')
TIC of DATA:BS145-S.D
(3')
Abundance
Time (min.)
a
b

Figure 8: Total ion chromatograph (TIC) of GC-MS in the cambial zone of black locust after samples hydrolyzed with cellulase.
(a, and b are presented the unidentified compounds which are the possible precursors of flavonoids)


TIC of DATA:BS119-1.D
Sept.
TIC of DATA:BS119-4.D
Dec.
TIC of V3:BS145-1.D
Jan.
TIC of DATA:BS145-4.D
Feb.
TIC of DATA:BS145-7.D
March
Abundance
Time (min.)
a
b

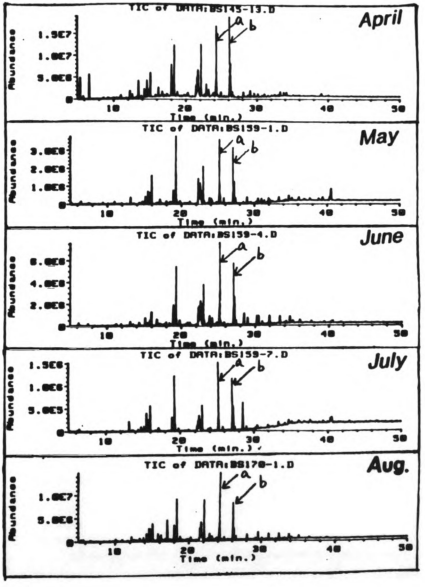
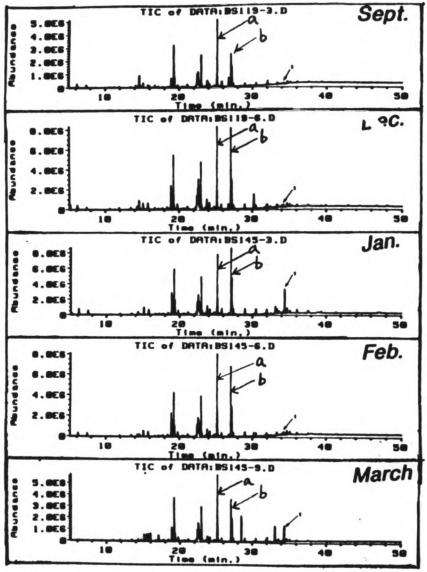
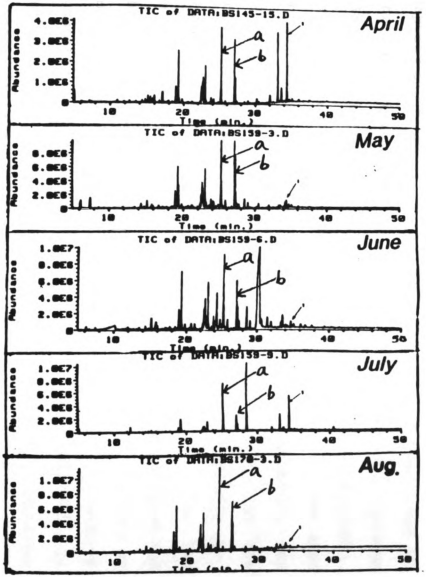

TIC of DATA:BS145-13.D
April
TIC of DATA:BS159-1.D
May
TIC of DATA:BS159-4.D
June
TIC of DATA:BS159-7.D
July
TIC of DATA:BS170-1.D
Aug.
Abundance
Time (min.)
a
b

Figure 9: Total ion chromatograph (TIC) of GC-MS in the sapwood of black locust after samples hydrolyzed with cellulase.
(a, and b are presented the unidentified compounds which are the possible precursors of flavonoids)

Sept.
Dec.
Jan.
Feb.
March
April
May
June
July
Aug.
Abundance
Time (min.)

Figure 10: Total ion chromatograph (TIC) of GC-MS in the transition zone of black locust after samples hydrolyzed with cellulase.
(a, and b are presented the unidentified compounds which are the possible precursors of flavonoids;
1= dihydrorobinetin, 2= robinetin)


TIC of DATA:BS119-3.D
Sept.
TIC of DATA:BS119-6.D
Dec.
TIC of DATA:BS145-3.D
Jan.
TIC of DATA:BS145-6.D
Feb.
TIC of DATA:BS145-9.D
March
Abundance
Time (min.)
a
b


TIC of DATA:BS145-15.D
April
TIC of DATA:BS158-3.D
May
TIC of DATA:BS158-6.D
June
TIC of DATA:BS158-9.D
July
TIC of DATA:BS170-3.D
Aug.
Abundance
Time (min.)
a
b

sapwood and transition zone are presented in Figure 11 and 12. The compositions of samples in the transition included flavonoids (dihydrorobinetin, robinetin, and robinetinidol), saturated and unsaturated fatty acids ($C_{16}$, $C_{18}$) and some unidentified compounds (a) and (b). In comparison, the composition of sapwood were only fatty acids and the unidentified compounds (a), (b) and (d). The compound (d), having a retention time 31.188 min., base paek 398, and molecular ion 590 (?) was only presented in August sapwood and the transition zone through entire year. The mass spectra of compound (a), (b) and (d) apppears in Figure 13. The results suggested that the flavonoids found in the heartwood began to synthesize in the transition zone without the seasonal changes. According to the current data, compound (a), (b) and (d) are possible precursors of flavonoid biosynthesis. However, more evidences have to be accumulated to illustrate the hypothesis.

(2)Quantitative Analysis

(a)Standard Reference Curve of Phenolics

A standard reference curve for phenolics was created using gallic acid. Gallic acid solutions containing from $10^{-7}$g/ml to $10^{-4}$g/ml were prepared by dilution from a freshly prepared 0.100g/100ml solution. Total phenolic concentration, as gallic acid equivalents, was determined using the procedure described previously. The concentration of the standard

Figure 11: Total ion chromatograph (TIC) of GC-MS in the sapwood of black locust before samples hydrolyzed with cellulase.
(a, b, and d are presented the unidentified compounds which are the possible precursors of flavonoids)

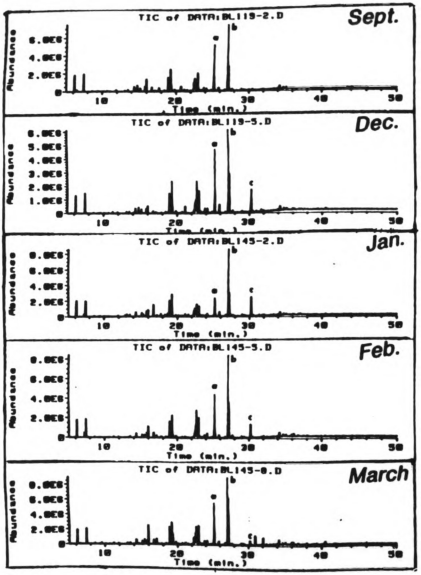
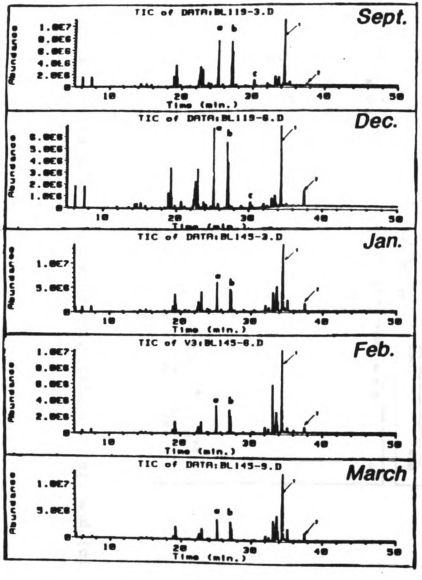
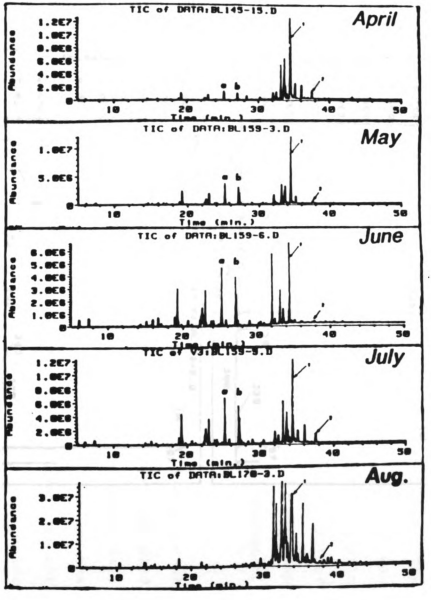

Sept.
Dec.
Jan.
Feb.
March
April
May
June
July
Aug.
Abundance
Time (min.)

Figure 12: Total ion chromatograph (TIC) of GC-MS in the transition zone of black locust before samples hydrolyzed with cellulase.

(a, and b are presented the unidentified compounds which are the possible precursors of flavonoids; 1= dihydrorobinetin, 2= robinetin)


TIC of DATA:BL119-3.D
Sept.
TIC of DATA:BL119-6.D
Dec.
TIC of DATA:BL145-3.D
Jan.
TIC of V3:BL145-6.D
Feb.
TIC of DATA:BL145-9.D
March
Abundance
Time (min.)


TIC of DATA:BL145-15.D
April
TIC of DATA:BL159-3.D
May
TIC of DATA:BL159-6.D
June
TIC of V3:BL159-9.D
July
TIC of DATA:BL170-3.D
Aug.
Abundance
Time (min.)

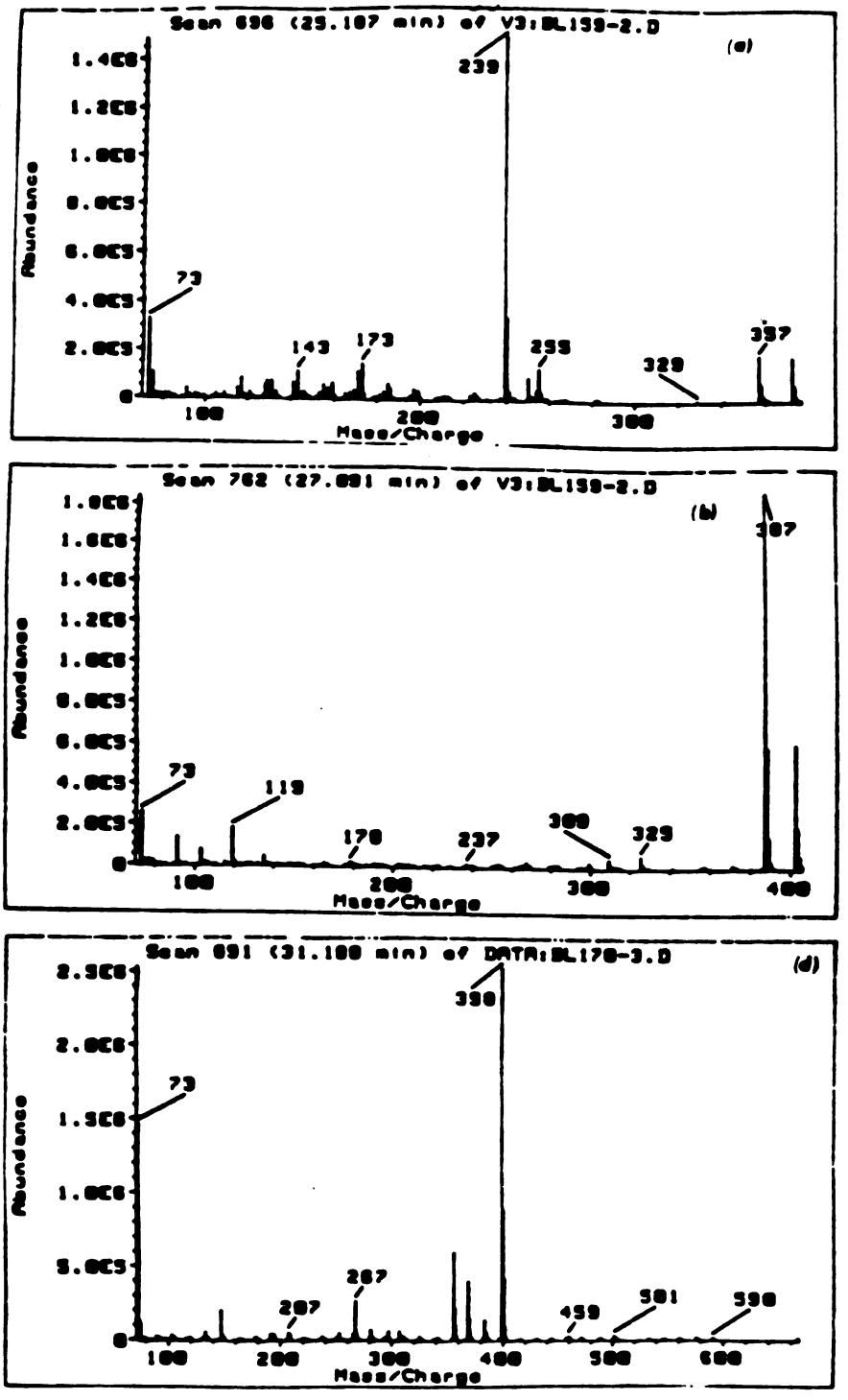



Figure 13: The mass spectra of compound (a), (b), and (c).

solutions was plotted against the absorption at 750nm. The formulate of a reference curve obtained from the data was **Y=1.353355E-06 + 1.290269E-05 * X** (Y= concentration of gallic acid, X= Absorption), which had a correlation coefficient of 0.998. When the concentration of gallic acid was higher than $2x10^{-5}$g, the absorption was out of the range of the instrument.

(b)Variation of Total Phenolics

The seasonal variation of phenolics in black locust is presented in Figure 14. The phenolic content was also calculated based on the dry weight of tissues. These data showed that the major location of phenolics is in the transition zone and heartwood of black locust. The seasonal variation of phenolics in the heartwood was roughly parallel with that in the transition zone. The level of phenolics in the heartwood was relatively constant during the whole year except the heartwood formation season (from July to late September). The phenolic concentration of July heartwood was the highest and the concentration variation of phenolics in heartwood formation season could result from which the activity of enzymes in the outermost heartwood rapidly increased. The phenolic level in the transition zone was variable and may be a function of the size of the sapwood portion of the sampling. The extractable flavonoids in the heartwood of black locust were about 6% of dry weight (Roux and Paulus, 1962; Smith, A.L. et al., 1987).

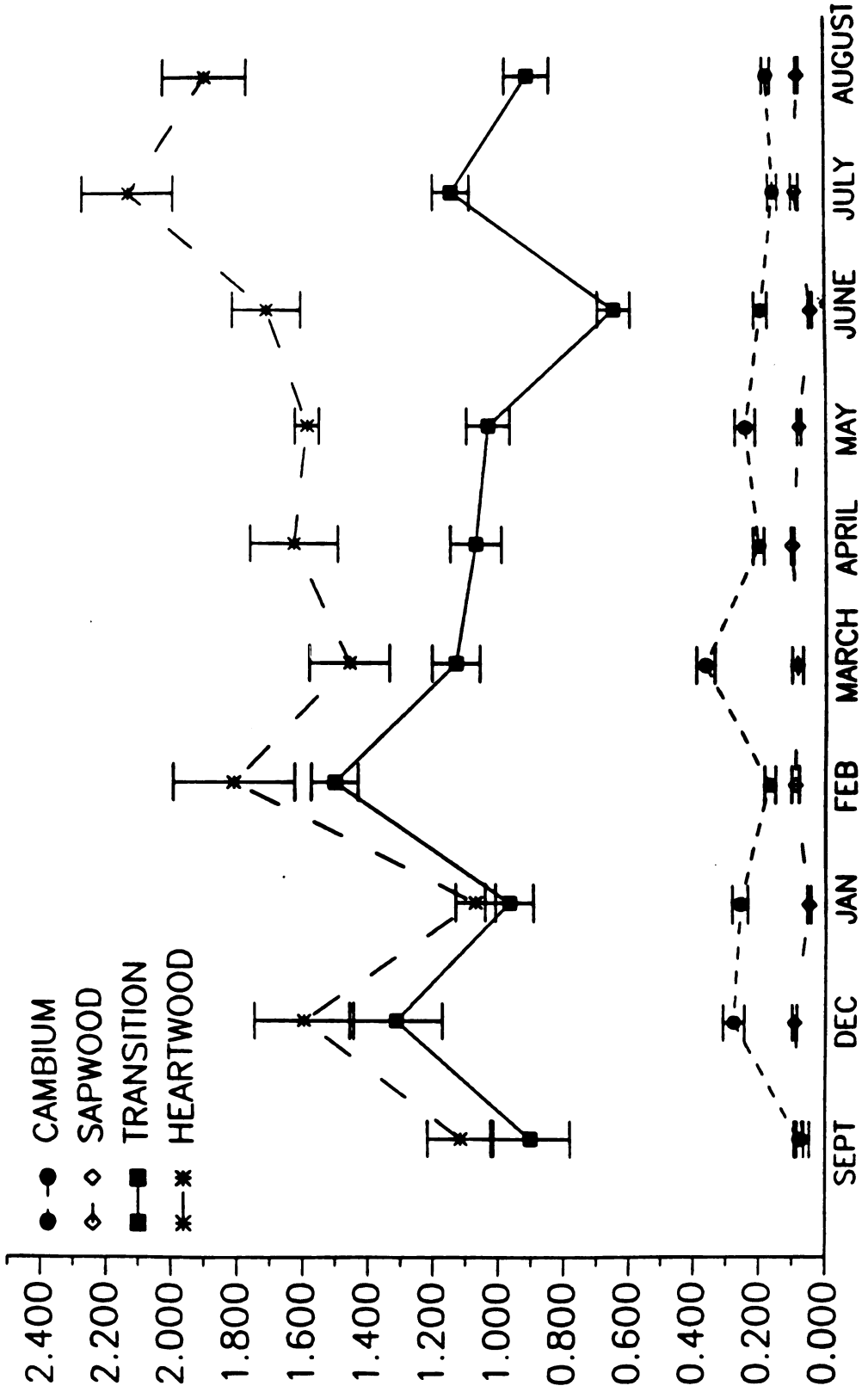



**Figure 14: The seasonal variation of phenolics in black locust.**

The phenolic content of the cambial zone and sapwood was relatively low compared with the heartwood and transition zone. The level of phenolics detected in the cambial zone of black locust in this study was higher than in the sapwood. The fluctuation of phenolic in the cambial zone declined to a valley (0.1 mg/g) on September, increased to a maximum value (0.3 mg/g) in late fall, and remained at a constant level (0.3 mg/g) over the other months; until the next growth season the phenolic level started to build up again to synthesize lignin. The result from the cambial zone could be explained by Nobuchi's study (1984b). Nobuchi (1984b) indicated that the most part of heartwood formation in black locust occurred from July to late September. In addition, the GC-MS result demonstrateed that the flavonoids were not found in th cambial zone. The level of phenolics in sapwood was a constant ( less than 0.1 mg/g) through one year.

It is generally accepted that heartwood formation is related to the deposition of the phenolic substances into the ray parenchyma cells of heartwood, which leads to the death of ray parenchyma cells. However, the quantitative changes of phenolics in heartwood formation varied among species (Hillis, 1987). The relationship between heartwood formation and cytological structure has been widely studied by Frey-Wyssling and Bosshard (1959), Fahn and Arnon (1963), and Nobuchi (1984 a,b). It was thought that the living ray parenchyma cells in the transition zone and the heartwood are

concerned with the formation of heartwood substances. Nobuchi's study indicated that there were no living ray parenchyma cells in the heartwood of black locust from April to July, but the nuclei of the ray parenchyma cells existed in the heartwood after July and through next spring. Additionally, the nuclei of the living ray parenchyma cells existed in the sapwood of black locust in the spring; the transition zone had nuclei after July and extended into the heartwood.

Frey-Wyssling et al. (1959) suggested that heartwood formation occurred by a semi-anaerobic mechanism, in which the oxidation of the phenols was stimulated by the hydrolysis of starch in the transition zone under low concentration of oxygen (Figure 15). However, Hillis (1972) and Holl (1973) obtained results that indicated respiration in the transition zone of Eucalyptus and black locust studies both in vivo and in vitro had a higher respiration than sapwood.

It is known that the proportions of oxygen and carbon dioxide inside the wood are very different from those in the atmosphere. It might be expected that a higher concentration of carbon dioxide within the wood would result in an increase in the production of malonyl-CoA and phosphoenol pyruvate from increasing respiration. These compounds are known to be precursors of polyphenolics and flavonoids. If heartwood formation and the death of ray parenchyma cells were due to a reduction in the level of oxygen and/or an increase in the

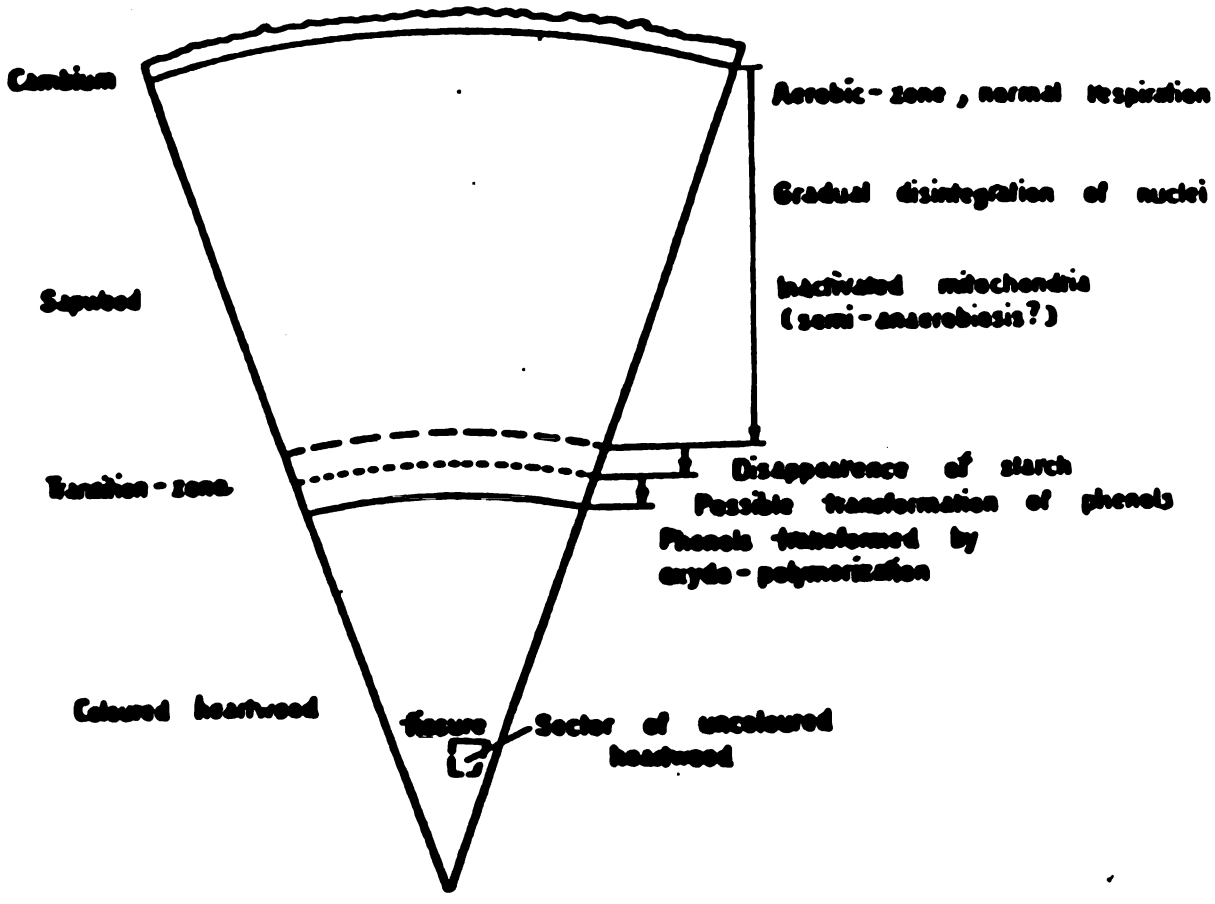



Figure 15: Sector of a tree with heartwood showing diagrammatically the different zones between cambium and pith with their various cytological and physiological characteristics.
(Frey-wyssling, 1959)

level of carbon dioxide within the wood, one would expect that the production of heartwood substances (polyphenolics and flavonoids) could be detected *in vitro* under a high concentration of carbon dioxide.

Carrodus (1971) examined the effect of high concentrations of carbon dioxide on the formation of flavonoids in the heartwood of *Acacia mearnsii*. Production of three out of four flavonoids in the heartwood were stimulated by carbon dioxide when the sapwood and transition zone contained no heartwood portion. In this experiment, those flavonoids were absent when the sapwood and middle transition zone were exposed to the presence of nitrogen. Thus, confirming the response was due to the presence of carbon dioxide, not the lack of oxygen. Seasonal variation of this reaction to the presence of carbon dioxide in the wood tissue paralleled the formation flavonoids (late spring and summer). It is suggested that polyphenolic formation in the heartwood of *A. mearnsii* might be stimulated from June to September due to a high level of carbon dioxide produced in the wood tissues.

The results from quantitative and qualitative analysis here both indicted that the flavonoids in the heartwood of black locust might be transformed in the transition zone (sapwood/heartwood boundary) in a very short period of time. Thus, the seasonal variation of primary metabolites will be one of possible methods to monitor a flux between secondary

and primary metabolites. The carbon source of flavonoids could be from several directions: small phenolics, starch, soluble sugars and degraded cellulose. In addition, carbohydrates are essential compounds for flavonoids biosynthesis. The seasonal variation of soluble sugars and starch was examined to determine the fluctuation.

(B) CARBOHYDRATES

(1) Quantitative Analysis

(a) Soluble Sugars

(i) Standard Reference Curve of Soluble Sugars: A standard reference curve of soluble sugars was established using a glucose standard solution purchased from Sigma chemical Co. The standard glucose solutions ranged from $1x10^{-6}$ g/ml to $1x10^{-2}$ g/ml and were prepared by dilution. The absorption of standard glucose solutions was determined using the "phenol-sulfuric acid" method described above. The concentration of the standard solution was plotted against the absorption at 490nm. The formulate of a reference curve obtained from the data was **Y=-2.92348E-05 + 1.913707E-04 * X** (Y= concentration of glucose, X=absorption), the linear regression revealed a correlation coefficient of 0.932. The correlation coefficient of linear regression became to 0.989 when based on a concentration range of glucose standard solutions from $1x10^{-6}$ g/ml to $5x10^{-4}$ g/ml. Therefore, it was

suggested that a linear relationship might not exist between the absorption and glucose solutions at low and very high concentrations. Black locust samples were prepared by dilution so that their absorption fell into the linear range.

(ii)Variation of Soluble Sugars: The seasonal variation of soluble sugars in black locust is depicted in Figure 16. The amount of soluble sugars was calculated based on the dry weight of the tissues and expressed as g/g. The data from the cambial zone shows that the amount of soluble sugars was higher than in the other tissues. The concentration of soluble sugars in the cambial zone increased during the cold weather seasons and decreased during the growth season. This result agrees with the results of previous investigations. Jones and Bradlee (1933) studied the seasonal changes of hexose sugars in the inner bark of sugar maple. It was observed that the hexose sugars content increased in the mid-autumn, reached a maximum point in January, then decreased from this point during spring, and remained a low concentration in the summer. Siminovitch et al. (1953) examined carbohydrate fluctuation using Hassid's oxidation with ferricyanide and ceric sulfate titration (1936) and the results were expressed in Figure 17. Siminovitch's study found that the reducing sugars of the living bark of black locust stayed at a relatively constant, low amount (0.8% to 1.1% of dry weight of tissues) over the year, reaching a

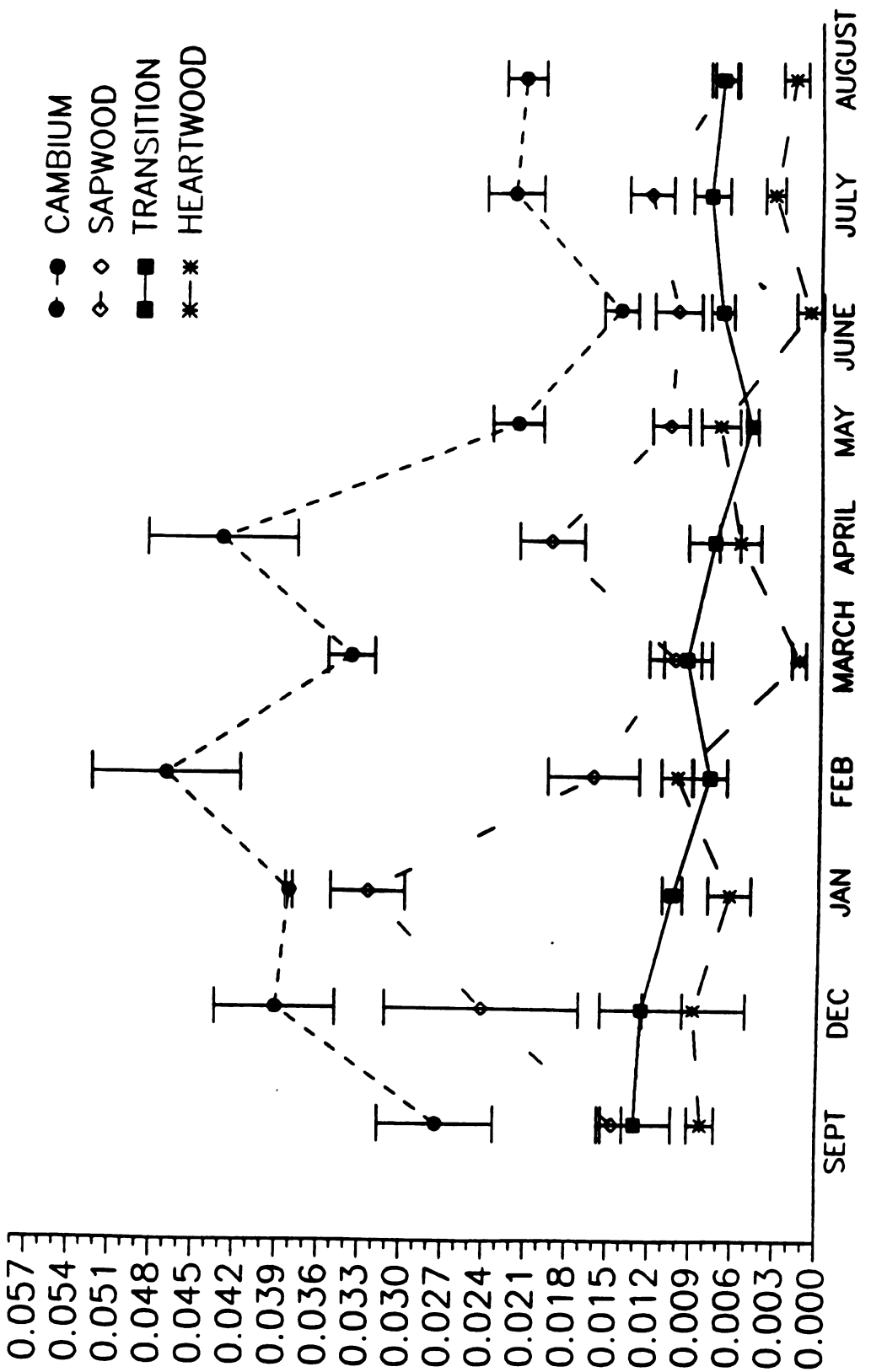



**Figure 16: The seasonal variation of soluble sugars in black locust.**

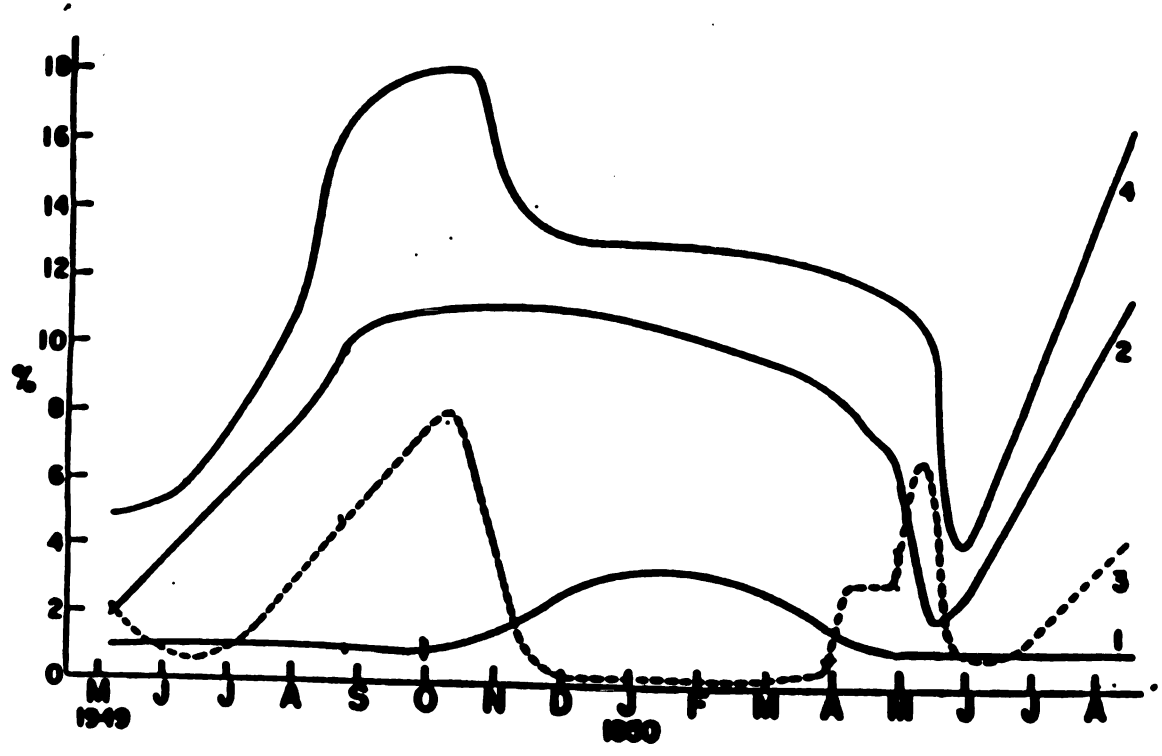



Figure 17: Average seasonal variation as per cent. of dry weight of tissue, in reducing sugars (curve 1), sucrose (curve 2), starch (curve 3), and total reserve carbohydrates (curve 4) in the living bark tissue of 12 Robinia trees during the period from May 1949 to August 1950. (Siminovitch, 1952)

maximum (3% of dry weight) in late winter. The results from this study had a higher level of soluble sugars in the cambial zone than the previous studies.

Generally speaking, the level of soluble sugars in the sapwood was lower than that of cambial zone in this study. The level of soluble sugars in the sapwood continuously increased from September until January, reaching a maximum of 3.2% of dry weight, then decreased to 1.0% in February and March. The soluble sugars content of the sapwood increased to 2.1% of dry weight in April and then decreased to 1.0% and maintained the level through the growth season. Jones and Bradlee's (1933) study also determined the seasonal variation in hexose sugars of the outer wood (outer sapwood) of sugar maple in northern Vermont. Their results suggested that the content of hexose sugars reached a maximum in early winter, began to decrease from there, and decreased rapidly to a low content (less than 0.1% of dry weight) in spring season. The changes of soluble sugars in sapwood of black locust were similar to that of sugar maple in the fall and winter. During the growth season the changes of soluble sugars in the sapwood were different from those of sugar maple. In Hansen and Grauslund's (1973) study, seasonal variation in the concentrations of soluble sugars of wood from the trunk of young apple trees showed similar results. The recent study of Holl (1985) in the trunkwood of spruce (Picea abies (L) Karst) indicated that the concentration of soluble sugars was

higher during the cold period (from December until early March) than in the spring and summer months.

The results from this study substantiated the physiological roles of the various tissues. The level of soluble sugars in the cambial zone is higher than that of sapwood since the function of a cambial zone is to transport nourish from leaves to roots. The level of soluble sugars in the transition zone and heartwood remained a constant low level, 1% and 0.78% of dry weight, respectively. The data obtained in March were lower than an expected perhaps due to site variations. Few attempts have been made to study the soluble sugars content in transition zone and heartwood because the two positions were thought to the physiologically inactive. However, cytological differences in transition zone and heartwood have been widely investigated in various species. It is generally accepted that the outer most rings of heartwood may have physiological living cells while the inner heartwood is a completely dead tissue. In addition, the function of soluble carbohydrates is to increase the osmotic pressure of the cell sap. Thus, the level of soluble sugars in heartwood and transition remained a constant and low level.

(b)Starch

(i)<u>Standard Reference Curve of Starch</u>: A standard reference curve for starch was prepared using a Chem Service analytical starch standard. Starch solutions ranging from

$1.4x10^{-3}$g/50ml to $1.196x10^{-1}$g/50ml were obtained by dilution and analyzed using the procedure described above. The concentration of the standard solution was plotted against the absorption at 650nm. The formula of a reference curve obtained from the data was **Y=-8.0637396E-04 + 3.633665E-02 * X** (Y=concentration of starch, X=absorption). A correlation coefficient of 0.962 was determined by linear regression. The correlation coefficient of linear regression became to 0.991 when the concentration range of starch standard solutions was reduced to a range of $1.4x10^{-3}$g/50ml to $5.51x10^{-2}$g/50ml. Black locust samples were prepared by dilution such that they fell into the linear range.

(ii)Variation of Starch: The seasonal variation of starch in black locust sections is shown in Figure 18. The starch content was calculated based on the dry weight of the tissues. It is clear that the highest concentration of the starch is in the sapwood. This is consistent with a storage function for sapwood. The starch content of sapwood decreased from fall until February, reaching a minimum point of 0.7% of dry weight, then began to increase in late winter and early spring. The starch level of the sapwood dropped gradually from early spring to late spring, reaching the other minimum point of 0.7% of dry weight, then increased again after the growth season. The starch concentration of the sapwood in black locust varied in a manner similar to that of the outer

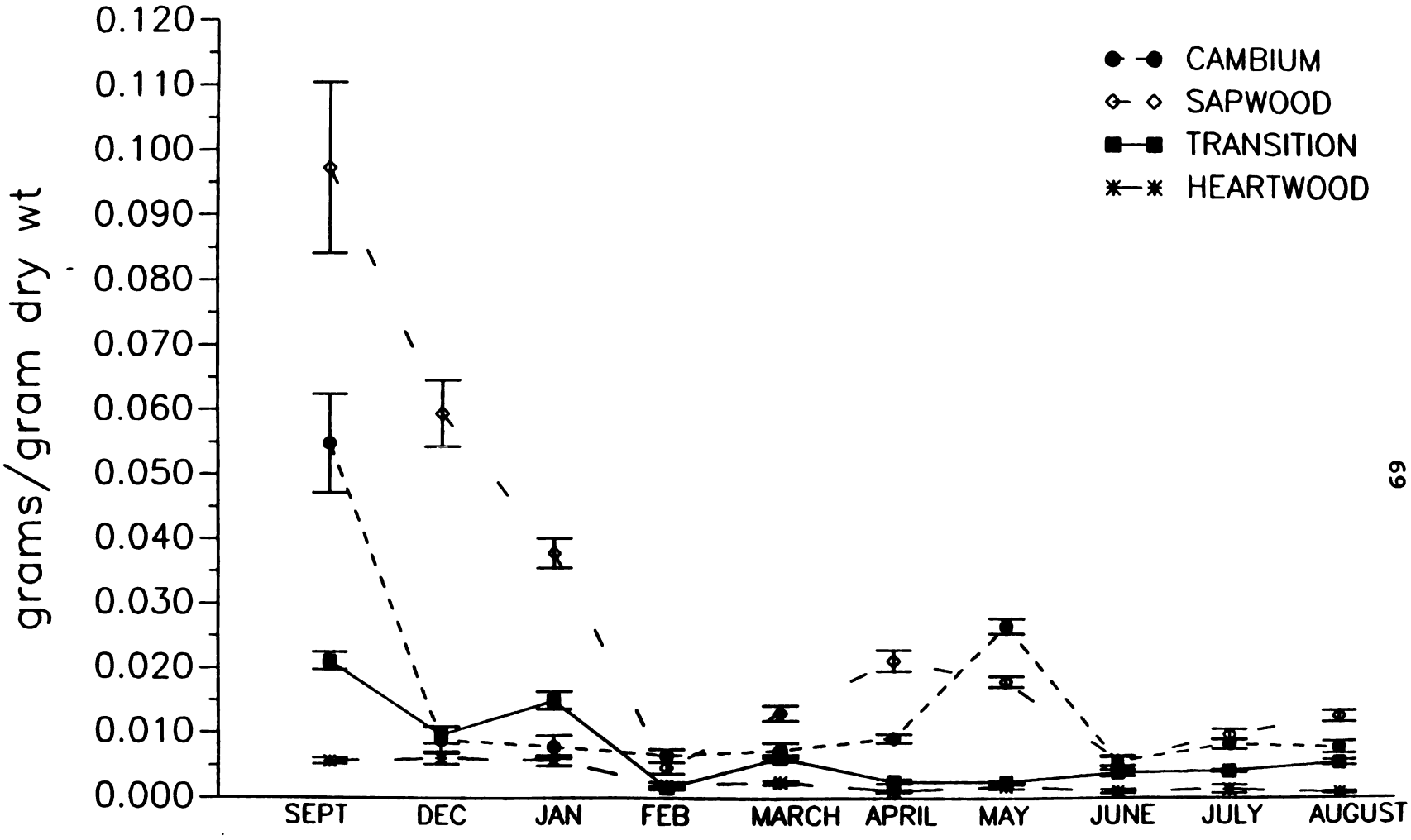



**Figure 18: The seasonal variation of starch in black locust.**

sapwood of sugar maple in the growth season from the end of march to August (Jones and Bradlee, 1933). Black locust sapwood, however, had a higher overall maximum starch content (about 9.8% of dry weight) than did sugar maple (about 3% of dry weight). Investigation of starch-glucose in the outermost 5-7 xylem rings of spruce (Holl, 1985) revealed that the starch contents were relatively constant from early autumn to late winter, and increased in the spring and decreased in the summer. In comparison, the amount of starch in the sapwood of black locust rapidly dropped from fall to the mid-winter, and then slowly increased in the growth season.

The starch level in the cambial zone decreased from a maximum value (5.5%) in September to a minimum level (0.78%) in February, then increased from early spring to May and dropped on June and slightly increased again. The fluctuation of starch in the cambial zone was cooperated with the sucrose level in this tissue and the transformation of photosynthesis products. The starch analysis of the cambial zone showed no significant differences during winter and early spring (average= 0.8% of dry weight). Siminovitch et al. (1953) also found that the starch concentration of living bark in black locust rapidly decreased from a maximum point (8% of dry weight) in early autumn to reached a minimum value (0.1%) in February, and remained at this level during June and July in Figure 15. Their data also demonstrated that after the sucrose level reached 5% or 6% of dry weight in early fall,

the starch began to accumulate. In the growing season an increase in starch was coupled with a decrease in sucrose. Thus, they suggested that the seasonal variation in the carbohydrates of black locust was related to temperature and reversible enzymes in the bark.

The seasonal changes of starch concentration in the transition zone of black locust were similar to the trend shown for the sapwood. The transition zone, however, had a low level of starch (the maximum about 2% and the minimum less than 0.1%). The concentration of starch in the heartwood of black locust remained at a constant low level (less than 0.5% of dry weight). Jones and Bradlee (1933) found that the inner wood of sugar maple had a lower starch concentration than the outer wood.

In most cases, cytological studies have show that starch granules will not be present in transition zone and heartwood. However, a few starch granules were found in the transition zone and heartwood of black locust. These granules existed in the ray parenchyma cells from late summer to late winter (Nobuchi et al., 1984a).

The results from this study of total phenolics, starch, and soluble sugars indicate that the phenol level of the heartwood increases in the fall and winter while the starch level of the sapwood decreases during these seasons. On the other hand, the level of soluble sugars increases in these seasons. It has been agreed that soluble sugars

increase the osmotic pressure of the cell sap which is considered to the tolerance of cold temperature. They also serve as an important energy source for the numerous metabolic activities (Smith, 1968; Alden et al., 1971; Krasnuk et al., 1975). Starch is a storage form of carbohydrates. Starch hydrolysis is precisely regulated by low temperature (Krasnuk et al., 1976; Dear et al.,1972; Marvin and Morselli, 1971; Siminovitch et al., 1953). The soluble sugars produced from starch hydrolysis might cause a rise in the level of energy-releasing compounds or pentose sugars for nucleic acid synthesis. In addition, flavonoid synthesis requires ATP as energy and cofactor. Thus, it is suggested that soluble sugars and starch could be utilized as indirect or direct sources for flavonoids biosynthesis in the heartwood of black locust.

## CONCLUSIONS

The major flavonoids (dihydrorobinetin and robinetin) found in the heartwood of black locust are thought to be responsible for its durability. The results obtained in this study suggest that flavonoid biosynthesis occurs at the transition zone. The apparent site of synthesis does not change with the season. No major flavonoids could be detected by TLC and GC-MS in the sapwood portion even though the samples were hydrolyzed with cellulase and 2N HCl:MeOH (1:1). Three possible processors of flavonoids (compound (a),(b), and (d)) showed a fluorescence with varied Rf value under UV light (254nm) have been detected. The unhydrolyzed samples from the transition zone contained both major flavonoids while the hydrolyzed samples showed only dihydrorobinetin. The starch level of the transition zone was much less than that of the sapwood while the total phenolics content of the transition zone was greater than that of the sapwood. The level of extractable chemicals found in the transition zone was one third to one half of the amount found in the heartwood. Flavonoids were the major extractable compounds in both the transition zone and heartwood.

Quantitative analysis of the phenolic content of sapwood from this study was inconsistent with the results of previous studies (Roux and Paulus, 1962). The investigators found that both dihydrorobinetin and robinetin were detected in the sapwood and heartwood while we found that the

flavonoids were only detected in the heartwood and transition zone. This discrepancy might be due to a difference in the definition, in terms of wood coloration and physiological activity, of transition zone and heartwood. The range of the transition zone in this study included the edge of the heartwood and 0.1-0.3 cm of sapwood. The data from TLC and GC-MS analysis confirmed that the major flavonoids found in the heartwood of black locust did not exist in the cambial zone. However, an intermediate precursor (cinnamic acid) has been detected in 50% acetone extractives of sapwood. Cinnamic acid is also a lignin precursor.

The variation in total phenolics of the wood from sapwood to heartwood is the reverse of the starch variation. Starch is hydrolyzed and then transformed to soluble sugars so that the level of soluble sugars increases. It has been suggested that the activity of starch hydrolysis increases in the transition zone. After starch hydrolysis occurs, the activity of enzymes in the parenchyma cells of the sapwood will be changed. This change in activity may be transmitted through the various zones by ray cells so that flavonoids are rapidly synthesized in the sapwood/heartwood boundary. The results from this study show that the level of starch in the transition zone is less than that of the sapwood. The results of these experiments, combined with the qualitative results, suggest that the carbohydrate flux (soluble sugars, starch)

may be an indirect carbon source for flavonoids or may be the energy source of flavonoid biosynthesis.

## RECOMMENDATIONS FOR FURTHER STUDY

The recommendations for further work in this study are as follows:

1) To look for the biochemical changes across the stem by growth rings of stand trees using labeled compounds (sucrose, glucose, or cinnamate),

2) Continue to identify the possible precursors (compound a, b, and d) of flavonoids,

3) Isolate and characterize the enzymes which are related with flavonoid biosynthesis in the sapwood or transition zone of black locust,

4) To investigate the factors affecting flavonoid biosynthesis in the parenchyma cells of the transition zone such as the ratio of carbon dioxide/oxygen, pH value.

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