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Title: WALNUT (*JUGLANS REGIA*) PHENOLS: I. CHANGES DURING RIPENING. II. ANTAGONISMS FOR CERTAIN MICROORGANISMS

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Phenols from defatted walnuts were extracted with absolute methanol. The phenols were divided into four groups on the basis of molecular size. The antagonism of each group towards *Escherichia coli*, *Staphylococcus aureus*, and *Aspergillus flavus* was determined. Small polymers were the best antagonists of *E. coli* and *Staph. aureus* followed by large polymers and oligomers. A fifth group of unknown composition was also an effective antagonist. *Staph. aureus* was always more affected than *E. coli*. *A. flavus* was not antagonized by any of the phenolic extracts.

During maturation the amount of phenols extracted in absolute methanol showed three distinct changes. Phenolic content increased in both the early and late stages of maturity and decreased in the period in between. Degree of polymerization was found to decrease during maturation.

Walnut (*Juglans regia*) Phenols: I. Changes During Ripening. II. Antagonisms for Certain Microorganisms

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WALNUT (*JUGLANS REGIA*) PHENOLS: I. CHANGES DURING RIPENING. II. ANTAGONISMS FOR CERTAIN MICROORGANISMS

INTRODUCTION

Tannin chemistry began with the discovery of gallic acid by Scheele in 1786 and the isolation of pure phenols 12 years later by Proust. Despite these early discoveries progress has been meager. Proust's early method of isolation is still one of the best available today.

The primary obstacles facing the chemists who study phenolic compounds are the multitude of polymeric compounds classed as tannins and the difficulty of structural determinations. Consequently research has been concentrated on the chemistry of the monomeric phenols which compose them. Attempts to determine tannin structures have been largely unsuccessful, although some progress was made by Emil Fischer and Karl Freudenberg. The ability of the tannins to antagonize microbes has also been recognized. Old wive's tales relate the usefulness of certain barks and leaves (which contain tannins and their monomeric phenols) in retarding infections and promoting healing of wounds.

Karl Freudenberg suggested tannins be classified into one of two groups: Hydrolyzable tannins and condensed tannins. This method of classification has generally been accepted. The hydrolyzable tannins

are themselves divided into two groups. They are the gallotannins which yield gallic acid upon hydrolysis with a dilute mineral acid and ellagitannins which yield ellagic acid under the same conditions. The condensed tannins have been widely studied because most commerically important tanning extracts fall in this category. These tannins all form an insoluble phlobaphene upon heating with dilute mineral acid and they give positive tests for phenolic hydroxyl groups. Flavan-3-ols and flavan-3, 4-diols are generally considered to be the monomers for the condensed tannins. Condensed tannins and their phenolic monomers are present in almost all tree leaves and bark.

The tannins are amorphous, rarely crystalline substances which are widely distributed in the plant kingdom. Their ability to combine with proteins is the basis of the process known as vegetable tannage, by which animal fiber is converted into leather. This important function gave scientists over many centuries the incentive to study the tree barks involved in tannage and ultimately the tannins themselves.

For over 3000 years, man has recognized the presence of astringents (tannins) in plants and indeed could even quantitate them by taste. Very few ancient writings on medicine are without mention of tannin-containing drugs. Unknowingly tannins were used to detect iron ions in solutions prior to the birth of Christ and the first quantitative method developed for the tannins was their precipitation by iron salts. Prior to 1850 many standard tests for tannins had been

recognized and by 1925 developed sufficiently to accurately classify tannins.

The walnut (Juglans regia), a fruit containing tannins, was introduced into England by the Romans during their conquest of that island. It then spread to the United States during the colonization period. Although California now produces nearly all of the United States' yield, Oregon has considerable acreage. The Oregon Franquette walnut is noted for its light color and large kernels. It also has a tightly sealed shell (which easily withstands handling), gives high yields per tree, and leafs out and blooms in the late Spring so it almost never contracts walnut blight. The disadvantages encountered with this variety are the following: It matures too late to meet early market demands (a time when the walnut can command a high price). The kernels are inclined to be astringent. And finally, the tree is susceptible to cold injury because it becomes dormant so late in the Autumn. A problem encountered by all Oregon walnut growers is the heavy rainfall which normally falls during the harvesting season. This results in muddy fields, making mechanical picking impossible. Despite these problems the Oregon Franquette is highly desired on the California market. Here it is combined with the darker California Franquette to give a lighter color mixture to comply with USDA standards. Since the advantages for the Oregon Franquette far outweigh the disadvantages, most of Oregon's acreage is planted with this

variety.

During the 1967 season, the Food and Drug Administration made seizures of some of the Oregon crop because it was contaminated with *Escherichia coli*, an indicator of fecal contamination. Since the antagonistic ability of tannins towards microbes has been shown, at least some stress would be placed on any organism present on the nut. The intent of this study is to determine whether walnut tannins can antagonize the growth of *Escherichia coli*, *Staphylococcus aureus* and *Aspergillus flavus* (all microorganisms with public health significance). Also of concern is the change in concentration of the tannins as the walnut ripens.

REVIEW OF LITERATURE

> The vegetable tannins occupy a unique part of the borderland between chemistry and botany and their use in the art of leather manufacture has led to an association in the original literature not only with these scientific disciplines but also with the various skills, practices and trades employed by man since early times. The chemistry of the vegetable tannins has remained something of an enigma; although Emil Fischer was able to make outstanding contributions to the study of the hydrolyzable tannins in the early years of this century, later work, particularly with the condensed or non-hydrolyzable tannins, mainly served to illustrate the complexity of the problems in this field and until comparatively recently the subject was in a very disorderly state. The advent of new techniques for the analysis and separation of water soluble materials has led within a very short period of time (as in related fields of organic chemistry) to substantial progress in the chemistry of these substances. Whilst detailed knowledge of the diverse structures of many of the hydrolyzable tannins has been realized the same cannot yet be said for the condensed tannins. Advances have never-the less been made toward the identification of their precursors and the probable pathways for their formation in plant tissues... (Haslam, 1966).

Haslam implies above that the chemistry of the tannins (despite their importance for at least 3000 years) has been advanced suprisingly little. Review of additional literature has shown this is indeed the case.

Tannins as Microbe Antagonists

Kokal (1965) determined the effect of walnut tannin extract on *E. coli*. The effect was greater in saline than on cells suspended in 0.1% peptone water. In 0.9% saline, 1×10^7 cells (100%) were killed at 0.62% walnut tannin concentration and 1×10^5 cells (100%) at 0.16%

both within one hour; whereas, 2.5% tannin concentration was required to kill 1 x 10^7 cells (100%) suspended in 0.1% peptone water within two hours.

Martin and Fowler (1934) found that application of 10% tannic acid solutions resulted in the complete kill of E. coli, Staph. aureus, and other organisms within 24 hours.

Schrauffstätter (1948) and Schrauffstätter and Bernt (1949) demonstrated the bacteriostatic ability of chalcone, flavone (and its derivatives), and flavanone toward Staph. aureus. While these flavanoids were very active for the gram positive Staphylococcus they were only marginally active at the same concentrations for gram negative Salmonella paratyphi. Schrauffstätter attributed the antagonism to α, β-unsaturated ketone groups, -C=C-C=O.

Tsuchihira and Ito (1951) demonstrated bacteriostasis of a 0.03% tannic acid solution toward three strains of E. coli.

Floch (1949) noted that 0.1% solutions of pharmactueical grade tannic acid were bactericidal for many pathogens, most notably Salmonella.

Krumperman (1968) found Aspergillus flavus would grow on mature dry walnuts, but the mold would not grow on nuts in the early stages of ripening. A. flavus is known to produce an enzyme capable of hydrolyzing the hydrolyzable tannins. This enzyme, referred to as tannase in the literature, has been widely characterized (Westlake,

Talbot, Blakely and Simpson, 1959; Nishira and Mugibayashi, 1960; Adachi, Watanabe and Yamada, 1968; Yamada, Adachi, Watanabe and Sato, 1968). Adachi *et al.* (1968) determined the molecular weight of the enzyme to be approximately 193,000 and to have a pI of 4.0. The molecule was found to consist of two chains of nearly equal length. Yamada *et al.* (1968) found the enzyme could hydrolyze the ester linkages of tannic acid. Optimal conditions for methyl gallate hydrolysis were determined to be pH 5.0 to 5.5 and a temperature in the range of 50-60°C.

Rice (1969) found hydrolyzable tannins extracted from *Euphorbia supina* seeds inhibited nitrogen fixing and nitrifying bacteria (*Azotobacter*, *Nitrobacter*, and especially *Rhizobium*). Blum and Rice (1969) found plants which produce large amounts of gallotannins would decrease the rate of addition of nitrogen to old fields by inhibiting the organisms responsible for symbiotic nitrogen fixation.

Chigrin, Bessmel'tseva and Rozum (1969) showed gallic acid, vanillic acid, *p*-hydroxybenzoic acid, syringic acid, catechol and chlorogenic acid had no inhibitory effect on *Puccinia graminis f tritici*, while coniferol, ferulic acid, quercetin, coumarin, scopoletin, rutin and vanillin (along with caffeic acid and esculetin at high concentration) did show inhibition.

Tannins in Walnuts and Other Food

Tannins in foods have been studied, but not extensively. Batesmith (1954a) reported the occurrence of tannins in some foods

and also gave an excellent chemical review of the phenolic monomers important in foods. Goldstein and Swain (1963) determined the changes in tannins in a number of ripening fruits and investigated tests that would discriminate for tannins as opposed to other phenols. They defined a tannin as a phenolic compound of sufficient molecular weight (above 500) to form reasonably strong complexes with proteins and other polymers under suitable conditions and pH. The study attempted to roughly quantitate the degree of polymerization. Polymer size increased during ripening, especially in persimmons and bananas with less change noted in peaches and plums.

Polishchuk (1962) studied the tannins from Moldavian walnut trees and found the trees to vary with region, morphological state, climate and frost resistance. This indicated the tannins are dynamic parts of the tree's metabolism and not just a useless end product. In an early study Polishchuk (1958) indicated the tannin content of the walnut leaves was higher than the bark which was higher than the ligneous fraction. Also young branches had a higher tannin concentration than old ones. Speranskii and Strakhova (1965) showed the tannins of _Juglans regia_ (grown in Moldavia, SSR) increased during the initial stage of ripening, but decreased with maturation and storage. In a study on the methanol extract of the bark of _Juglans regia_ var. _orientalis_ Sasaki (1965) found quercetin and quercitrin present. Batesmith (1954b) found _Juglans regia_ trees to have leucoanthocyanins which when digested with HCl yielded cyanidin. Batesmith and Lerner

(1954) concluded that plants high in leucoanthocyanins are also high in tannins. They discovered the leaves of J. regia to be high in leucoanthocyanins. Batesmith and Swain (1953) presented an argument which indicates leucoanthocyanins are or become the true tannins in foods. Jurd (1956) isolated two crude tannin fractions from the pellicles of J. regia as well as some monomeric phenols (gallic and ellagic acids). An attempt by Jurd (1958) to isolate pure compounds was relatively unsuccessful. He did accomplish further separation but did not obtain pure compounds. In these two articles Jurd notes the tannins of the pellicle are ellagitannins. He makes no mention of any condensed tannins. Working with the peanut testa Stansbury, Field and Guthrie (1950) identified the red color as a phlobaphene derived from catechol.

Tannin changes in various fruits during ripening have also been studied. Lakshiminarayana, Mathew and Parpia (1969) noted the phenols of sapota fruit (Achras zapota L) increased during ripening. During this period the flavans/total phenols ratio remained constant while the flavans/leucoanthocyanins ratio decreased, indicating an increase in the concentration of phenolic polymers. Ryugo (1969) with oriental pear fruit and Reeve (1959) with peaches both demonstrated that phenol content decreased during ripening. Reeve also revealed that in the final stages of maturity the tannins accumulate in the mesocarp parenchymal cells of the peach. Using paper

chromotography Durkee and Poapst (1965) identified the two major phenolic constituents of apple core tissue as chlorogenic acid and phloridzin. Chlorogenic acid was also found to be a major phenolic constituent of pear and apple fruits (both mature and immature) by Bradfield, Flood, Hulme and Williams (1952). Johnson, Meyer and Johnson (1951), using an anion exchange resin, showed the presence of catechol, tannic acid and chlorogenic acid in peaches. Joslyn, Nishira and Ito (1968) demonstrated that hydrolyzable tannins in carob pods decreased with ripening and that gallic acid content increased during ripening.

Swain and Hillis (1959) devised a scheme of analysis for phenols that determines not only total content but also degree of polymerization. Although the tests are best applied to model systems they can be used on plant extracts. Goldstein and Swain (1963) demonstrated the problems involved in the analysis of plant extracts, but still concluded that the method is the best available.

Jurd (1956) has shown that the walnut contains hydrolyzable tannins and Batesmith (1954b) has shown the presence of leucoanthocyanins in the leaves and bark. Toxicity to microbes has been shown for the hydrolyzable tannins. The purpose of this work is to make a preliminary determination of the changes in the phenol content during ripening (both changes in molecular concentration and degree of polymerization) and how these changes might relate to microbial

antagonisms. Walnut phenols were partitioned into groups on the basis of polymeric size and the content of each fraction determined. The microbial antagonistic ability of each fraction was then determined.

MATERIALS AND METHODS

Picking and Handling of Raw Product

Samples of Oregon Franquette walnuts were taken (from a single tree) throughout the growing season. The samples were transported immediately to the lab where weight range and average weight were determined. The samples were placed in freezer bags, quick frozen at -40°C, then transferred to a -10°C freezer where held until analysis.

Extraction of the Walnut Phenols

Fresh walnuts were removed from the -10° freezer and immediately pulverized in a waring blender containing petroleum ether (boiling range 30-60). The pulverized sample and the petroleum ether were then transferred to a 4000 ml erlenmeyer flask. The procedure of Jurd (1956) was then followed until the tannin gum had been extracted with ether, acetone, methanol, water, and aqueous pyridine (70% v/v) (Chart 1). The extracts were held in a refrigerator until analyzed. The pyridine was diluted to a final concentration of 30% (v/v) with distilled water.

Methods of Analysis

I. Total phenols: Total phenols were analyzed by the

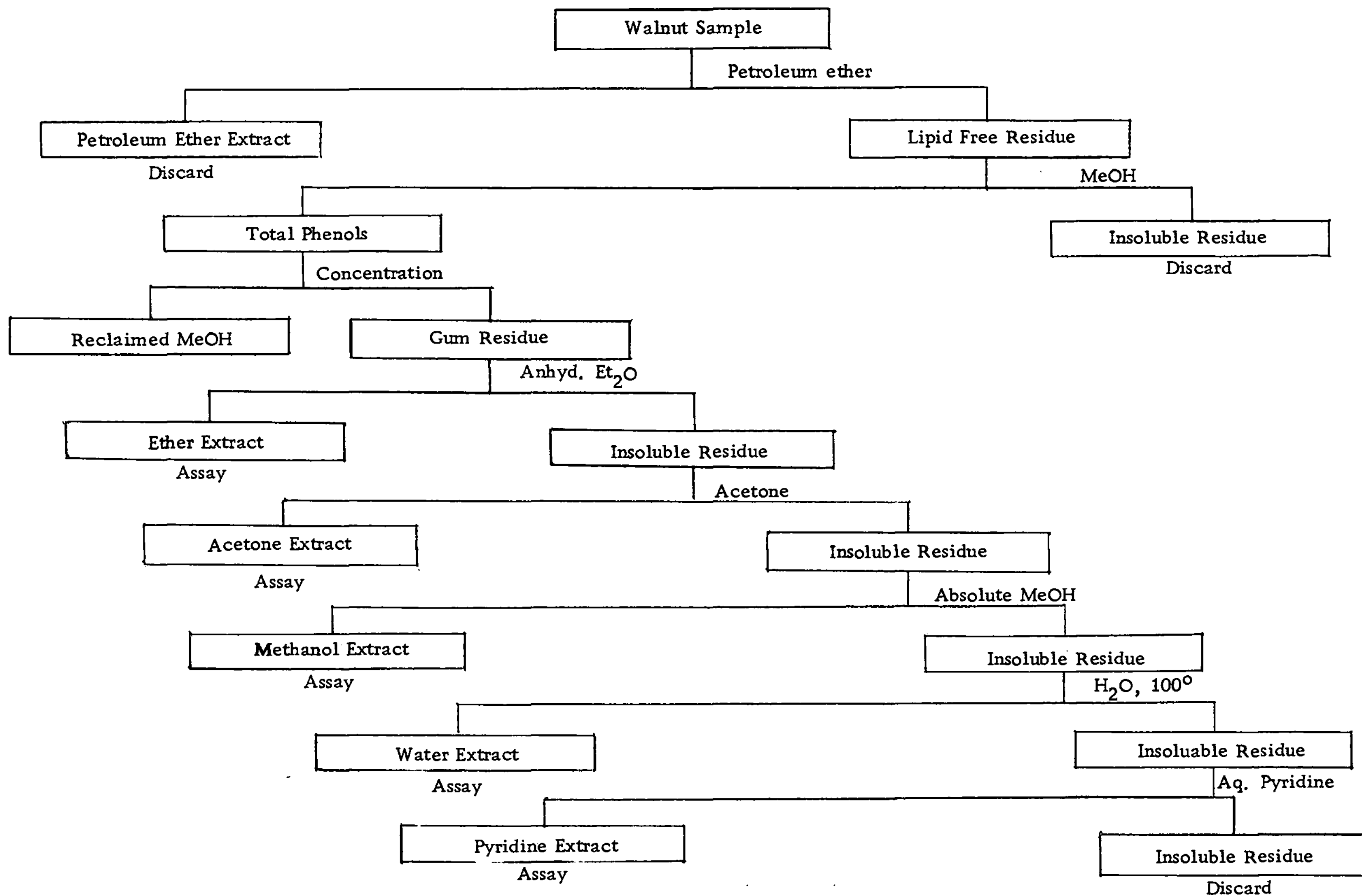

Chart 1. Extraction procedure.

Folin-Denis method as given in the AOAC (A. O. A. C. 1955, p. 144). Results were calculated as milligrams of tannic acid per 100 grams of fresh walnuts extracted.

II. Flavanoids: Flavanoids were analyzed by the vanillin procedure given by Swain and Hillis (1959). Large test tubes were used in place of 25 ml conical flasks. Results were expressed as milligrams of catechin per 100 grams of fresh walnuts extracted.

III. Leucoanthocyanins: Leucoanthocyanins were analyzed by the method of Swain and Hillis (1959) with the following modifications (modifications made to facilitate equipment available): A constant temperature oil bath was set at $97^{\circ} \pm 1$. The bath was placed in a room where light could be completely excluded. Test tubes (15 x 1.4 cm) using metal closures were substituted for glass stoppered ones described in the method. Data obtained indicated that the modifications did not invalidate the results.

Microbial Antagonism Studies

Organisms

A gram negative bacterium, *Escherichia coli*, wild strain (IMViC ++--) isolated from human feces; a gram positive bacterium, *Staphylococcus aureus*, coagulase positive isolated from a human source and a fungus, *Aspergillus flavus* (strain 15517), all microorganisms of public health significance, were the test microorganisms

used.

E. coli and *Staph. aureus* cultures were maintained on nutrient agar slants and transferred weekly throughout the study. The *A. flavus* was maintained on Czapek solution agar.

Media

Antagonism studies were performed on solid agar media. Nutrient agar (nine grams of Colab ionagar #2 and eight grams of Difco nutrient broth per liter of water) was used for the studies with *E. coli* and *Staph. aureus*, while Difco Czapek solution agar was used for studies on *A. flavus*.

Twenty-four hour bacterial cultures grown in Difco nutrient broth were used as inoculum for the antagonism study.

All media was prepared by the methods given in the Difco manual (1960).

Preparation of Cultures

Cultures of *E. coli* and *Staph. Aureus* were prepared by inoculating organisms from the stock cultures into separate flasks containing 75 ml of sterile nutrient broth. The cells were incubated 24 hours at 27°C. After 24 hours the broth cultures were poured into separate flasks of melted nutrient agar (at a temperature that the cheek could tolerate) in the proportion of 50 ml of broth culture per

500 ml of agar. *A. flavus* was transferred directly from seven day agar slants to the melted Czapek solution agar. One slant was used per 500 ml of agar. The transfer of the culture from the slant was accomplished by adding ten ml of sterile water to the slant then briskly shaking. The water containing the spores and some mycelia was added to the melted agar.

Antagonism Studies

Filter paper discs (12.4 mm diameter Whatman #1) were dipped into methanol solutions of the phenols from the various extracts. The saturated discs were then held in front of an air heat gun and the methanol evaporated. The dried discs were placed on the inoculated agar and incubated at 27°C. After 24 to 48 hours the antagonisms of the various extracts on the bacteria plates could be measured. The *A. flavus* plates required about four days incubation before readings could be made.

Antagonism was measured by determining the diameter of the clear zones occurring around the treated discs. The measurements were made using calipers and a ruler graduated in millimeters.

RESULTS AND DISCUSSION

Growth and Maturation

Walnut maturation consisted of three distinct phases as indicated in Table 1 and illustrated in Figure 1. The period of mitosis, which is illustrated as phase I in Figure 1, showed rapid weight gain for a period of about 30 days. The second period, phase II, is characterized by meiotic growth. During this phase weight was constant but the cells underwent development and differentiation. This period lasted about 75 days. Phase III, the death phase of the walnut fruit, showed a rapid decrease in weight and the seed (what one would normally consider to be the walnut and shell) was ejected from the fruit. The separation of the fruit from the seed complicated the analysis of phenolic content since analyses could now be performed only on the seed.

Changes in Tannin Content During Ripening

The walnut contains both hydrolyzable ellagitannins (Jurd, 1956) and condensed tannin monomers (Batesmith, 1954b; Batesmith and Lerner, 1954). The behavior of these phenolic compounds has been studied during ripening in other fruits. Predictions for the walnut could be made on the basis of these earlier studies. Joslyn *et al.* (1968) showed that the hydrolyzable gallotannins of the carob pod increased in the early stages of ripening then decreased in the final

Table 1. Weight changes for the Oregon Franquette walnut during ripening.

Date (1969)	Sample number	Average weight (grams)	Weight range (grams)
June 16	1	9.36	0.90 - 14.20
June 24	2	22.11	12.50 - 35.00
June 30	3	26.32	7.70 - 35.40
July 7	4	36.32	23.60 - 49.90
July 14	5	43.91	19.40 - 61.50
Aug. 4	6	46.43	32.20 - 61.10
Aug. 11	7	41.93	24.30 - 57.00
Aug. 25	8	47.54	20.80 - 61.20
Sept. 2	9	46.18	30.70 - 58.80
Sept. 8	10	45.17	14.90 - 62.10
Sept. 15	11	49.11	18.15 - 64.40
Sept. 22	12	47.84	9.00 - 66.10
Sept. 29	13	42.26	14.50 - 57.60
Oct. 6	14	19.23	5.30 - 63.80
Oct. 13	15	11.77	-- - --
Oct. 19	16	14.46	-- - --

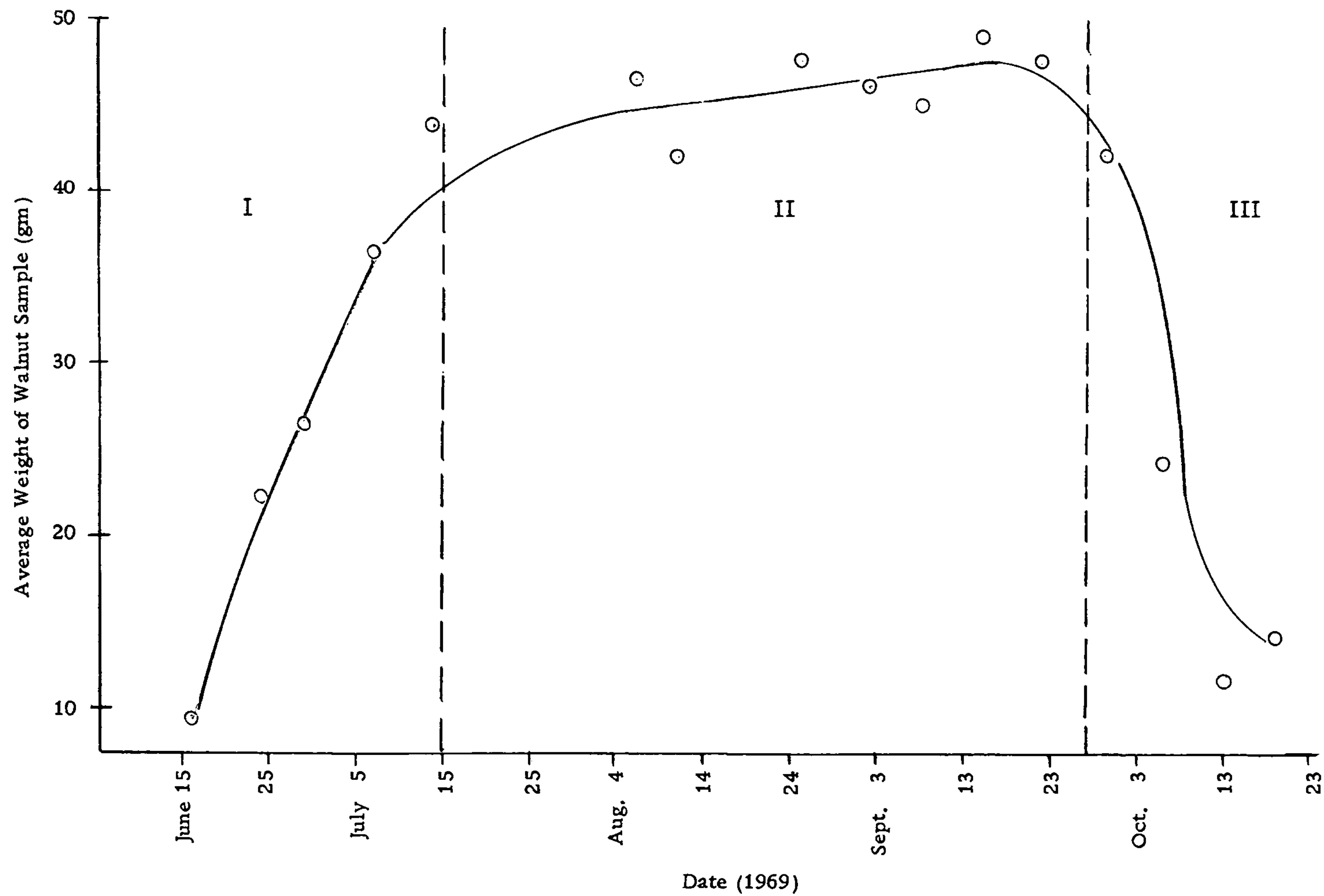

Figure 1. Growth curve for Oregon Franquette walnuts.

stages. The monomer for these tannins, gallic acid, decreased early in the ripening scheme, but increased during the latter stages. This indicates the tannins undergo hydrolysis during the final stages of ripening. Since the walnut contains hydrolyzable tannins this would be expected to happen with them also. Flavans (condensed tannins), on the other hand, continue to polymerize throughout maturation (Goldstein and Swain, 1963; Lakshiminarayana _et al._, 1969). At molecular weights of greater than 500 the flavans are very reactive, hence their usefulness to the leather industry. This reactivity could either contribute to or detract from the ability of these compounds to antagonize microbes. On the one hand their reactivity makes reaction with the microbial cell much simpler. Reaction is both kinetically and thermodynamically favorable. Unfortunately, these compounds are also reactive towards the constituents of the walnut. Reaction is again kinetically and thermodynamically favorable. Compounds in the walnut which could react with the available phenols are proteins, divalent cations, cell walls, carbohydrates, lignan and oxidizing substances. The monomeric units have also been shown to be anti-oxidants (Cruess and Armstrong, 1947; Janicki, Rutkowski, Furyk, Kulik and Olszewski, 1957). Phenolic compounds which react with the other constituents of the walnut will be removed from the pool of phenolic compounds free to antagonize microbes.

On the basis of these observations the following predictions can

be made: The hydrolyzable phenols should undergo polymerization during the initial stages of ripening then begin to hydrolyze in the latter stages of ripening. The flavans should polymerize throughout the ripening period of the walnut. The phenolic compounds should react throughout the maturation period with other components of the walnut leaving them unextractable and incapable of microbial antagonism.

Extraction of the walnut phenols was done with absolute methanol. This extraction removed only phenols which were not tightly bound to other components of the walnut. Once the phenols had been concentrated by evaporation they were partitioned into solvents of increasing dielectric constant. These solvents are noted in Table 2.

Table 2. Dielectric constants* of solvents used to extract phenols on the basis of molecular size.

Solvent	Temperature (°C)	Dielectric constant
Petroleum ether (B.R. 30-60)	20	1.86
Anhydrous diethyl ether	20	4.34
Acetone	25	20.7
Absolute methanol	25	33.63
Distilled water	25	78.54

*Source: Chemical Rubber Company, 1964. p. E30-34.

This resulted in the division of the phenols into groups on the basis of molecular size. The ether fraction contained monomers (shown by Jurd, 1956). The acetone fraction contained oligomers. The methanol

fraction contained small polymers while the water fraction contained larger ones. The pyridine fraction dissolved the remaining phenolics. The percent vanillin/leucoanthocyanin (%V/LA) ratio for the acetone, methanol, and water extracts indicated that the phenols were separated on the basis of molecular size (Table 3). The %V/LA ratio was found by Swain and Hillis (1959) to be an indicator of degree of polymerization. As the ratio decreases the degree of polymerization increases. Consequently, the figures showing changes in degree of polymerization during ripening will have negative slopes when polymerization is increasing.

Jurd (1956) indicated the ether and pyridine fractions both contained monomers. For that reason the extracts were considered together throughout this study (Table 4 gives the changes in these fractions combined). These two fractions showed parallel changes in phenol content during maturation (Figure 2). Following synthesis of the monomers in phase I there was a decrease in content of the monomers until nearly depleted at the end of phase II. Upon death of the fruit the content of the monomeric phenols increased. This behavior corresponded with predicted results.

The acetone fraction contained oligomers and some monomers (as indicated from the high values listed for %V/LA in Table 3). Like the ether and pyridine extracts, this fraction showed a decrease in phenolic content until the end of phase II (Figure 3). At this point the

Table 3. Changes in phenolic content of Oregon Franquette walnuts during ripening.

Sample number	Extract	Folin-Denis mg/100 gm	Vanillin mg/100 gm (V)	LA mg/100 gm	%V/LA
2	ether	13	0.51	1.04	49
	acetone	18	3.23	0	(1000)
	methanol	20	5.30	3.00	177
	water	20	1.39	10.23	14
	pyridine	31	1.09	0.29	376
6	ether	3	0.32	0.12	267
	acetone	6	3.69	0.43	858
	methanol	11	2.29	2.90	79
	water	7	3.45	10.37	33
	pyridine	17	0.96	1.16	83
10	ether	< 1	0.79	0	(1000)
	acetone	1	2.99	0.44	680
	methanol	15	3.51	2.24	157
	water	7	3.25	6.25	52
	pyridine	< 1	0.33	0	(1000)
14	ether	9	0.96	0	(1000)
	acetone	34	3.25	1.02	319
	methanol	21	3.72	3.56	104
	water	8	1.54	3.81	40
	pyridine	18	0.47	0	(1000)

Folin-Denis results expressed as milligrams of tannic acid per 100 grams of fresh walnuts extracted.

Vanillin results expressed as milligrams of catechin per 100 grams of fresh walnuts extracted.

Leucoanthocyanin (LA) results expressed as milligrams of cyanidin per 100 grams of fresh walnuts extracted.

Values of %V/LA when the LA result equaled zero were arbitrarily assigned a value of 1000.

Table 3A. Changes in total phenol content during ripening.

Sample number	Folin-Denis* (FD)	Vanillin* (V)	Leucoanthocyanin* (LA)	%V / LA
1	6	-	-	-
2	102	11.51	14.56	79
3	160	-	-	-
6	44	10.71	14.98	71
10	24	10.88	8.93	122
11	24	-	-	-
14	90	9.94	8.39	118

*Results expressed as in Table 3.

Table 4. Combined changes of the phenols in the ether and pyridine fractions.

Sample number	FD	V	LA	%V /LA
2	44	1.60	1.33	120
6	20	1.28	1.28	100
10	< 1	1.12	0	(1000)
14	27	1.43	0	(1000)

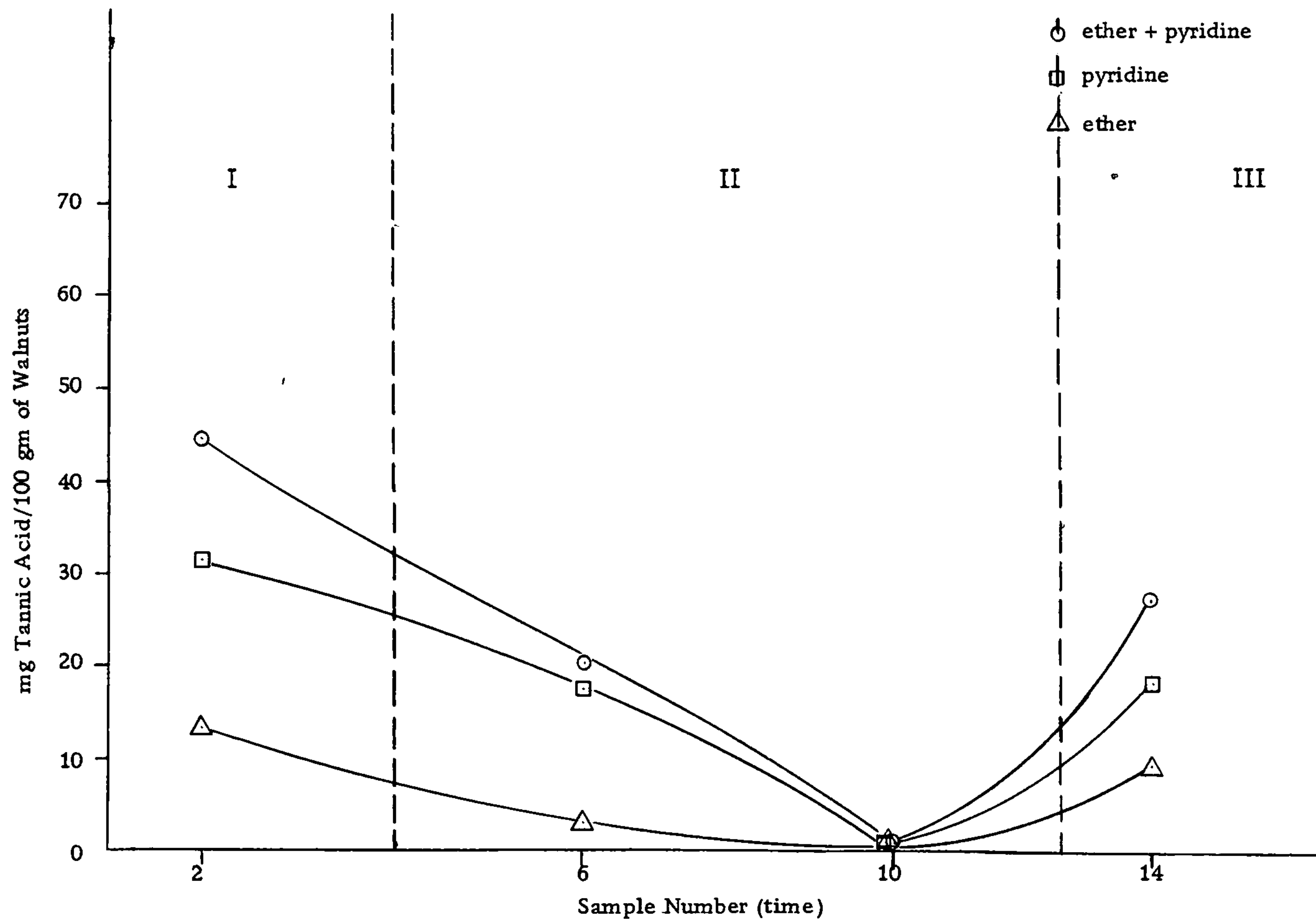

Figure 2. Changes in walnut phenolic content of the ether, pyridine, and combined extracts.

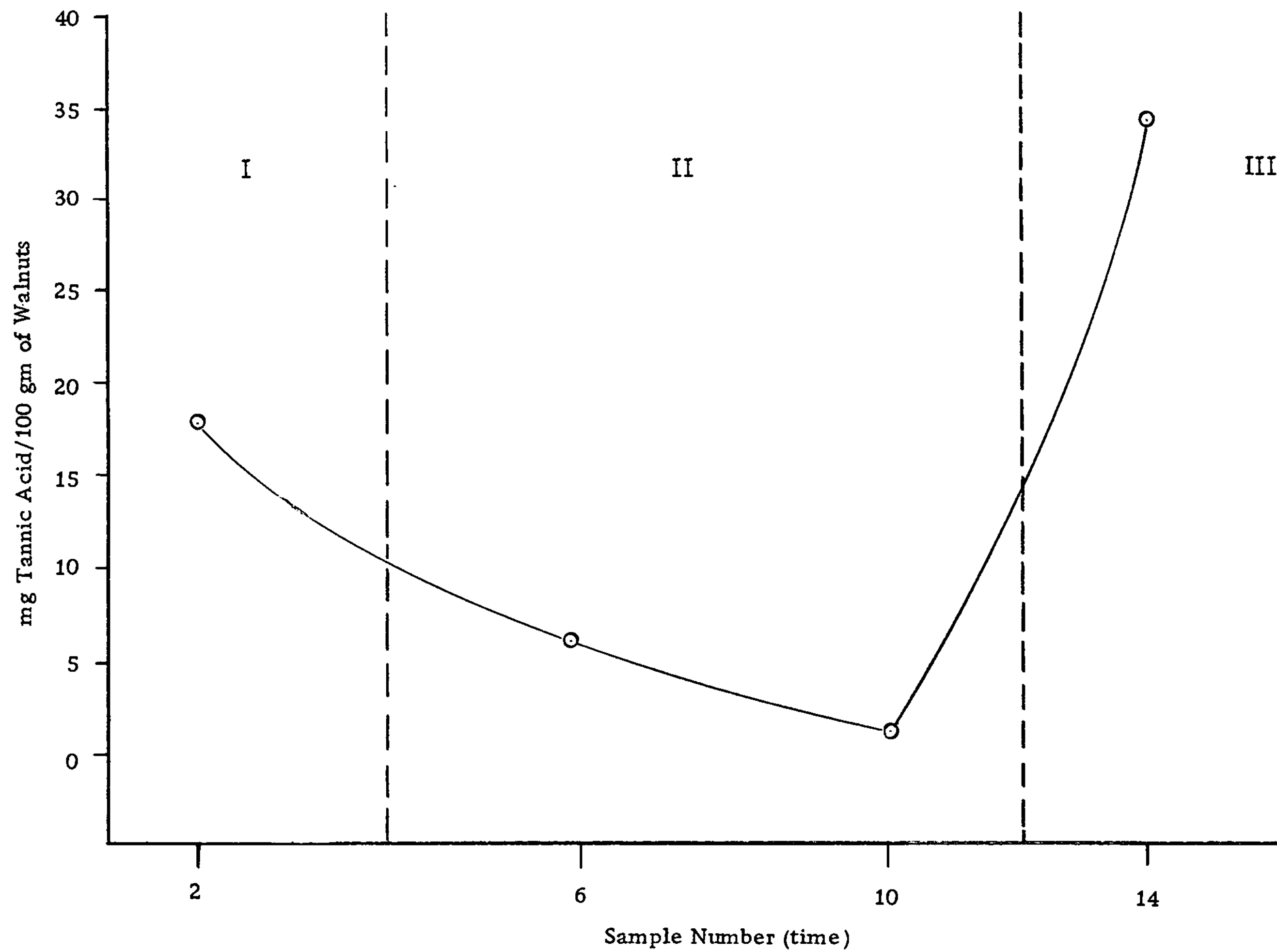

Figure 3. Changes in walnut phenolic content of the acetone extract.

phenolic content of the acetone extract increased precipitiously.

The methanol extract was the first in which polymers were present in large concentration (note %V/LA in Table 3). Unlike the previous extracts this fraction showed a decrease only during the final period of phase I (Figure 4). During phases II and III the phenolic content gradually increased.

The water extract also showed a decrease at the end of phase I (Figure 5), but during phases II and III the phenolic content was constant. Since this extract contained the largest polymers (extractable from the walnut with absolute methanol) there were three possible pathways these phenolics could follow. First they could polymerize further to become unextractable or they could be hydrolyzed and appear in the methanol and acetone fractions. Finally they could react with other components of the walnut and be rendered unextractable.

Total phenols (Table 3A) extracted by absolute methanol decreased during the latter part of phase I and all of phase II (Figure 6). Total phenol content increased in the early stages of phase I and all of phase III. The increase during phase I was due to net synthesis during mitosis and the increase during phase III was due to the hydrolysis of some of the large polymers which during phase II were unextractable with absolute methanol.

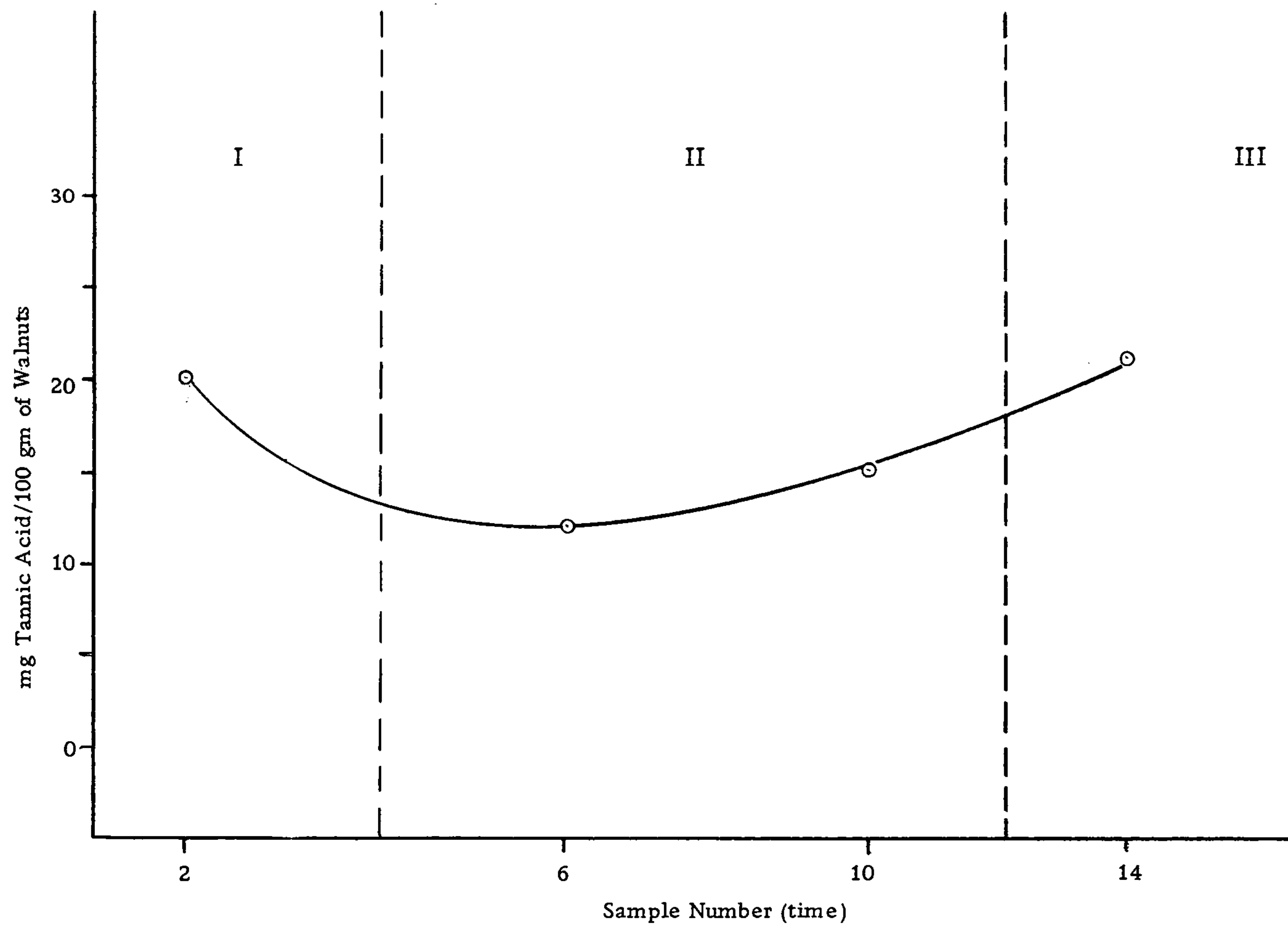

Figure 4. Changes in walnut phenolic content of the methanol extract.

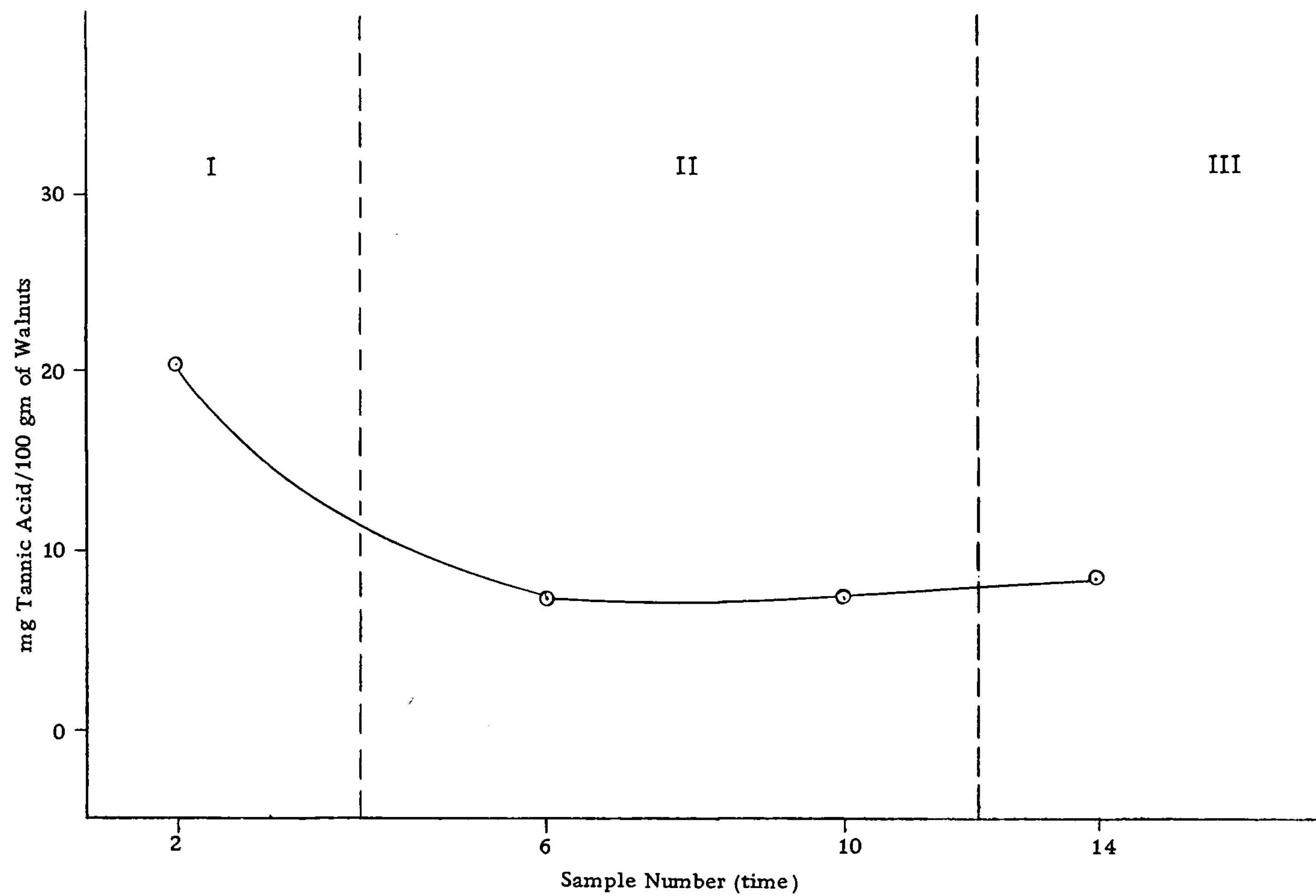

Figure 5. Changes in the walnut phenolic content of the water extract.

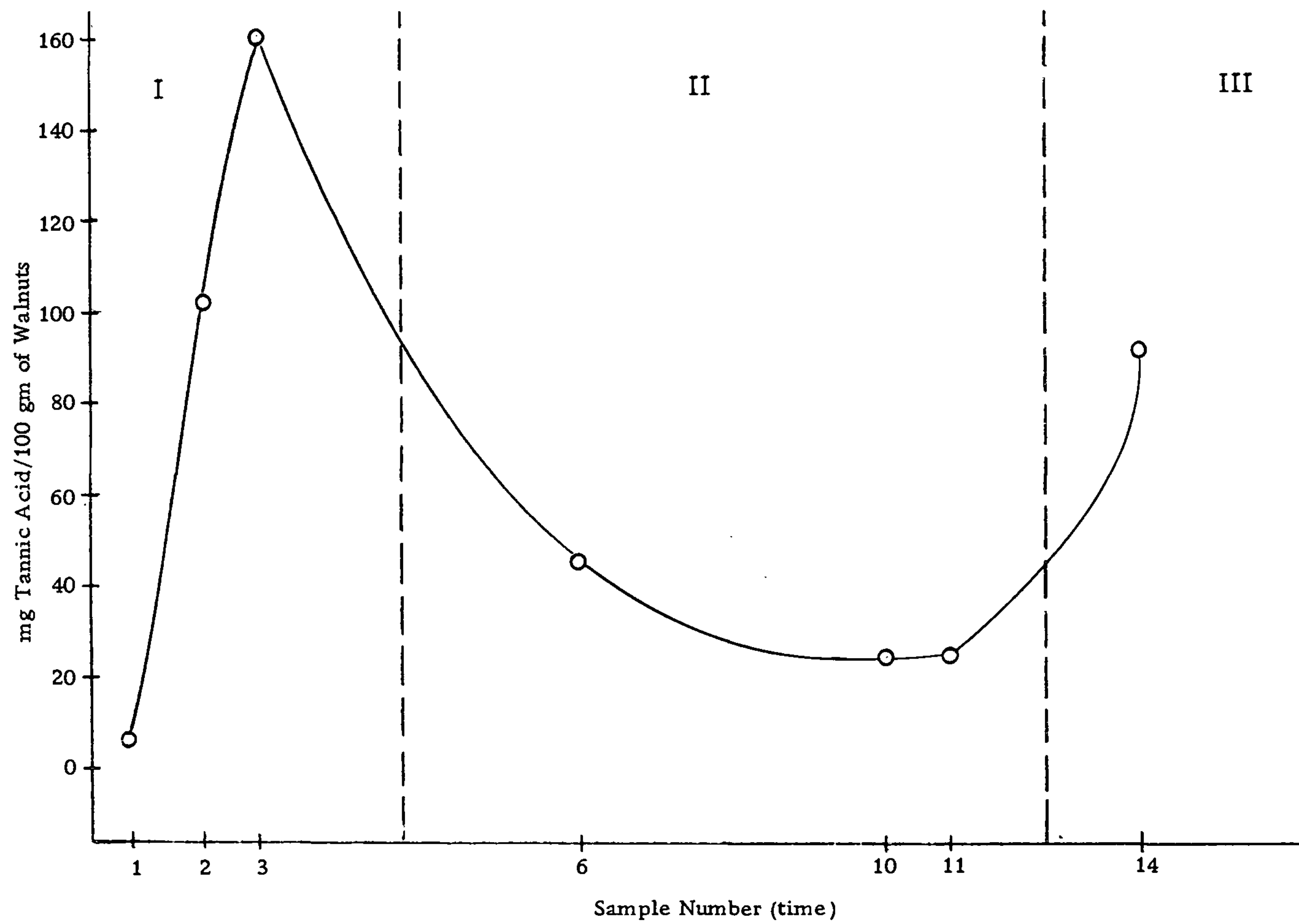

Figure 6. Changes in total phenols of the walnut during ripening.

Changes in Degree of Polymerization During Ripening

Of further interest were the changes in degree of polymerization of the phenolic compounds during ripening. Samples taken from the tree approximately 30 days (sample 2) and 85 days (sample 6) after fruit set exhibited a higher degree of polymerization than 115 day (sample 10) or 145 day (sample 14) samples. Figure 7 illustrates the changes in degree of polymerization of the total phenols extracted by absolute methanol. The net result was thus a decrease in degree of polymerization during maturation. The ether-pyridine (Figure 8) and the water (Figure 9) extracts paralleled this decrease. The acetone extract was the only one to undergo an increase in degree of polymerization throughout maturation (Figure 10). This was probably due to polymers breaking down to oligomers before hydrolyzing further. The changes in the methanol extract fluctuate so much that they are difficult to interpret (Figure 11). The author feels this fraction was experiencing polymerization and hydrolysis simultaneously. The small groups in the extract were tending to polymerize while the larger ones were undergoing hydrolysis.

Antagonism of Microbes

Inhibition of *Staph. aureus* (Table 5) by the phenolic constituents of walnuts was more pronounced for this organism than for the other two tested. The organism was inhibited by five percent phenolic

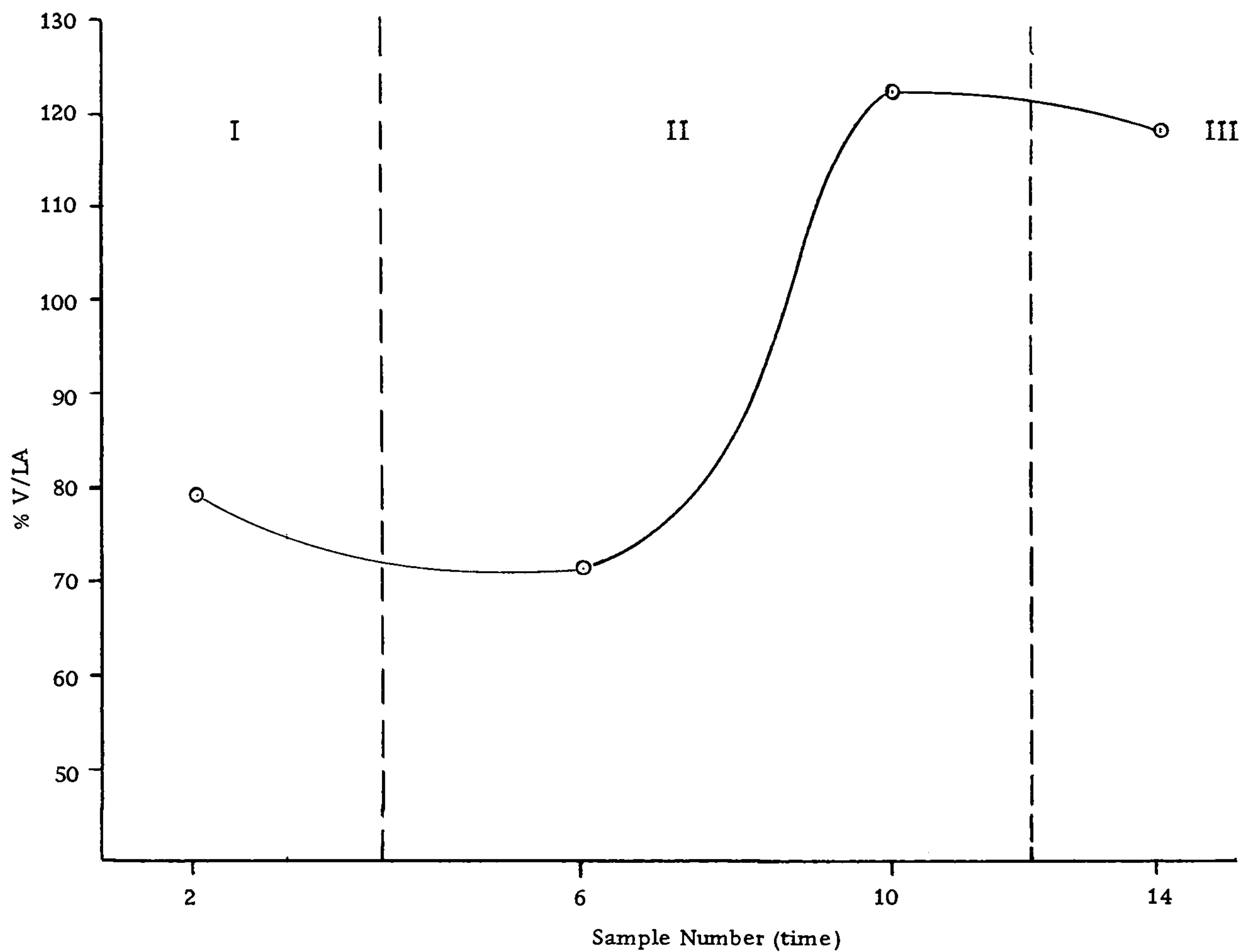

Figure 7. Changes in degree of polymerization of the total phenols in walnuts extracted with absolute methanol.

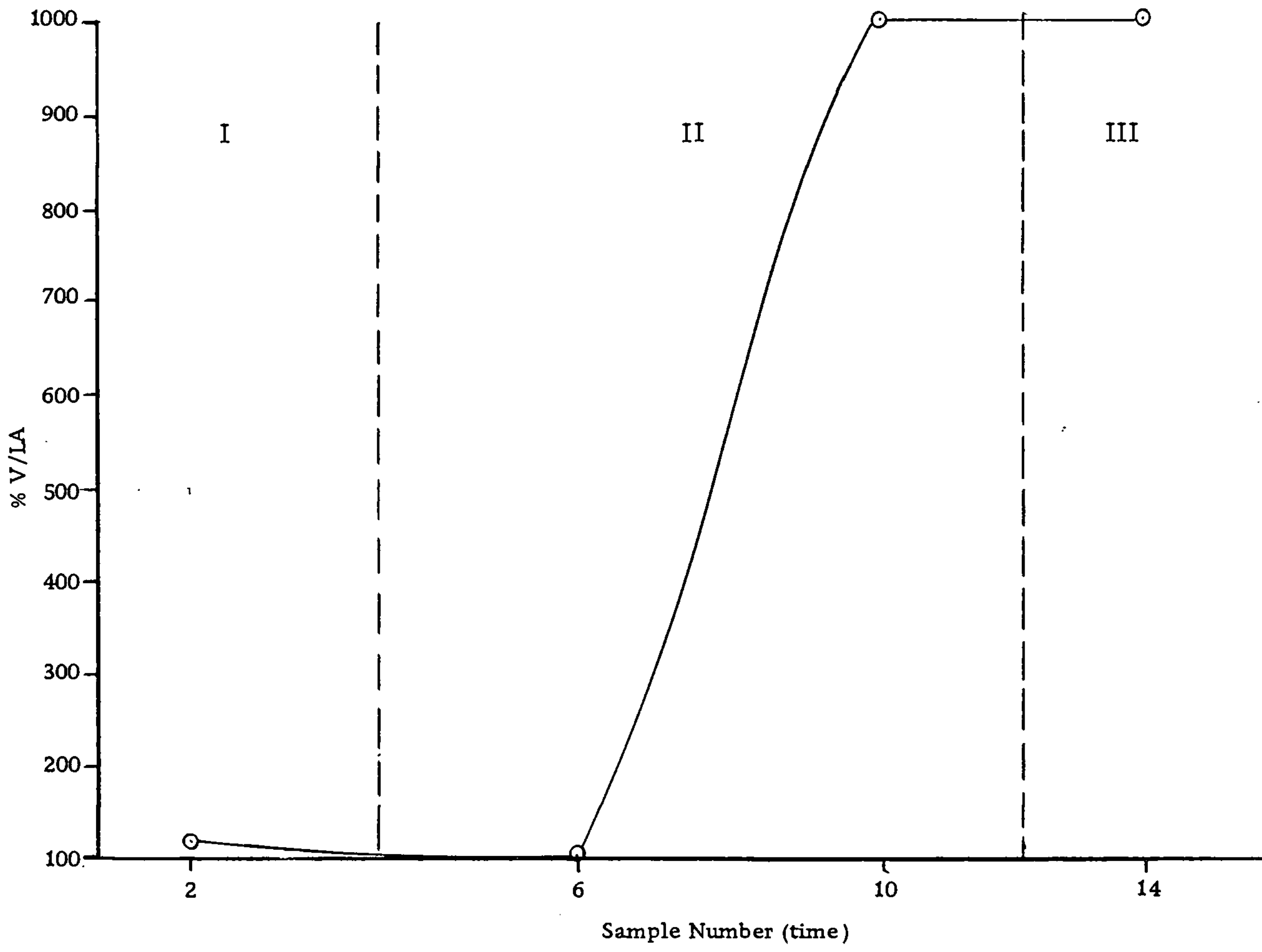

Figure 8. Changes in degree of polymerization of the phenols in the ether-pyridine extract of walnuts during ripening.

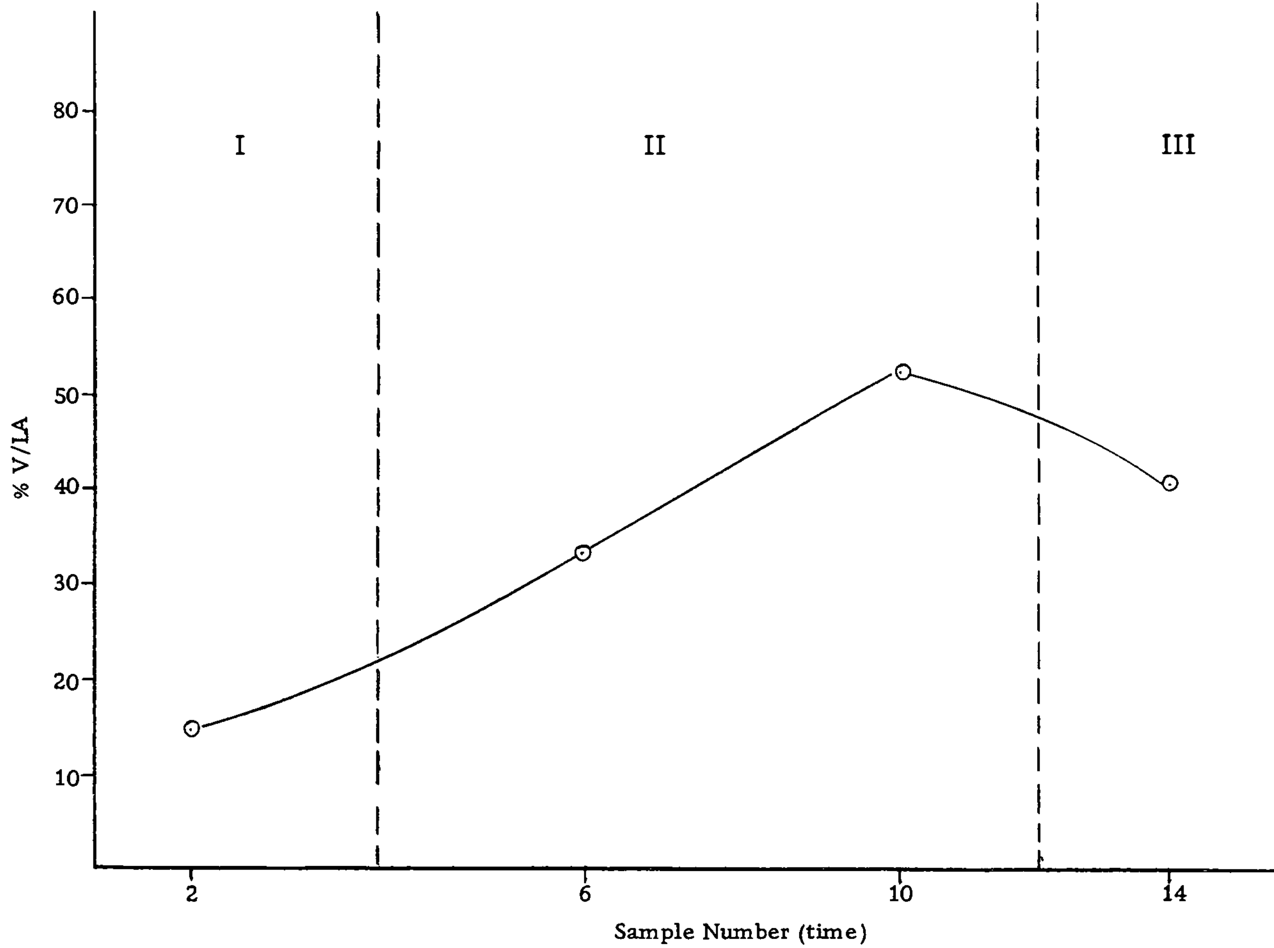

Figure 9. Changes in degree of polymerization of phenols in the water extract of walnuts during ripening.

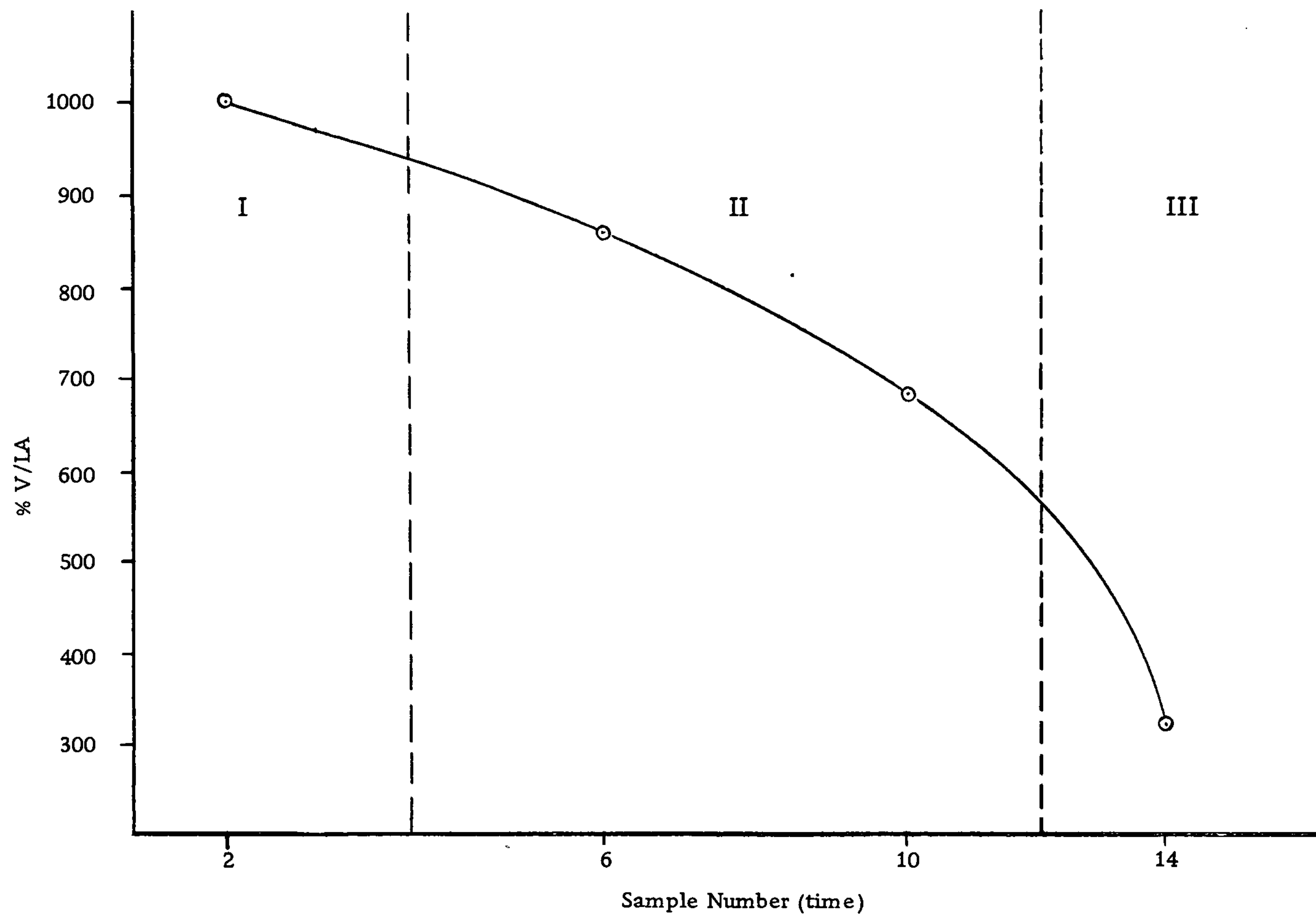

Figure 10. Changes in degree of polymerization of the phenols in the acetone extract of walnuts during ripening.

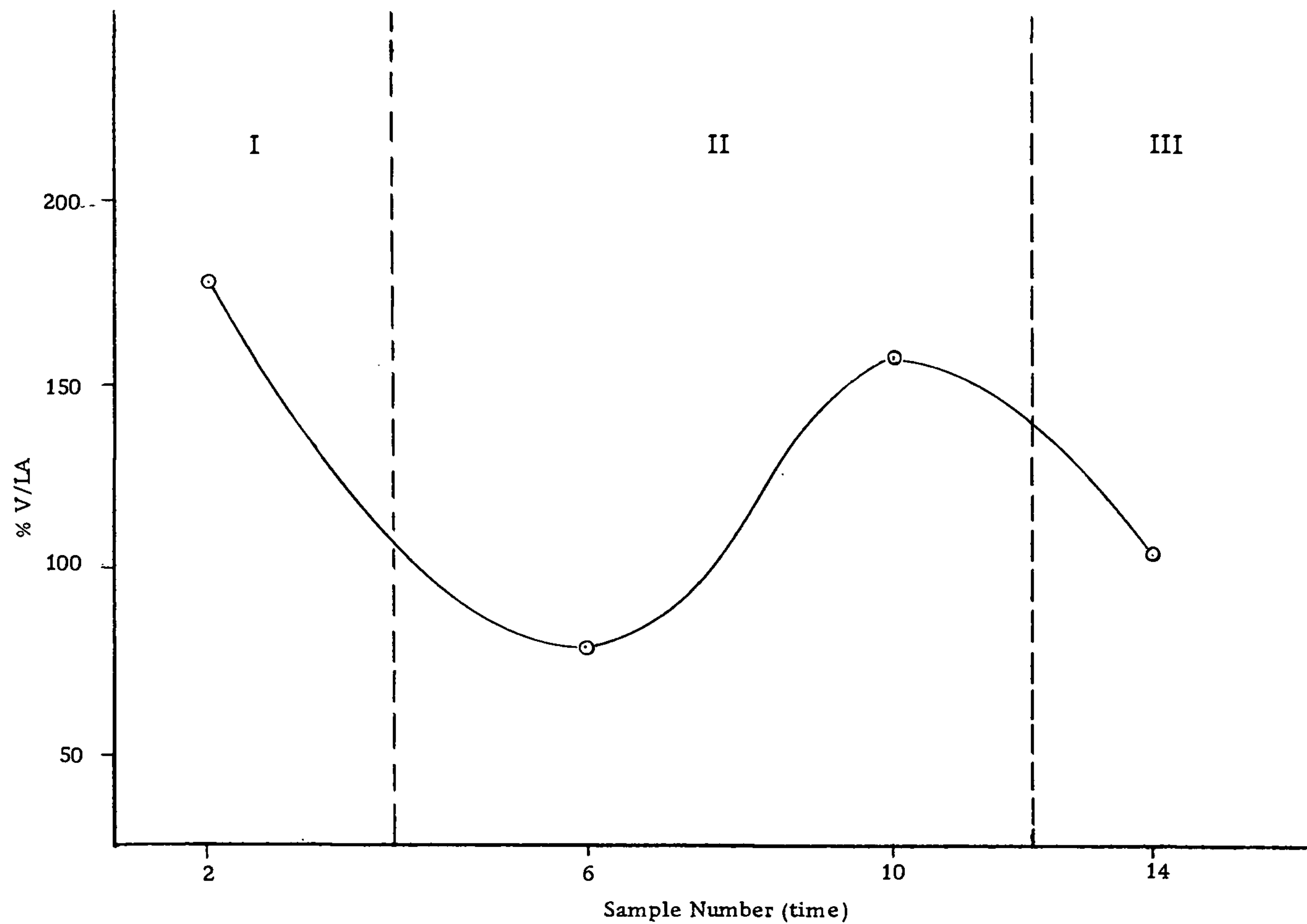

Figure 11. Changes in degree of polymerization of the phenols in the methanol extract of walnuts during ripening.

solutions of the acetone, water, and methanol fractions. At the one percent level the organism was inhibited by the methanol, water, and pyridine extracts (due to severe conditions required for evaporation the pyridine extracts could not be concentrated sufficiently to get a five percent solution). At lower levels the inhibition was seen only in the methanol extract (not reproducible). Degree of inhibition was greatest in the methanol fraction followed in order by the pyridine, water, and acetone extracts.

E. coli was not antagonized to the degree of *Staph. aureus* (Table 6). The methanol and acetone fractions inhibited the organism at a five percent level. The pyridine and methanol extracts inhibited the organism at the one percent level. No other extract inhibited this bacterium reproducibly.

Aspergillus flavus was not inhibited by any of the phenols or extracts tested (Table 7). In fact, the mold appeared to thrive on the acetone extract. This organism is known to elaborate tannase enzymes and therefore has the capability of utilizing the hydrolyzable tannins as an energy source.

Degree of polymerization is indicated as an important factor in the ability of the walnut phenolics to antagonize bacteria (Tables 5 and 6). The most effective phenols were those in the methanol extract. Phenols in this polymer range were more effective and more potent antagonists than either the tannins in the water extract or the oligomers

Table 5. Antagonism of Staphylococcus aureus by walnut phenols of different polymeric size at different concentration levels.

Extract	Diameter of zone of antagonism (mm)			
	5% level	1% level	0.05% level	0.05% level
ether	trace	0	-	0
acetone	17	trace	-	0
methanol	21 1/2	19	trace	0
water	18	trace	0	0
pyridine	-	18	-	0

Table 6. Antagonism of E. coli by walnut phenols of different polymeric size at different concentration levels.

Extract	Diameter of zone of antagonism (mm)			
	5% level	1% level	0.05% level	0.05% level
ether	0	0	-	0
acetone	trace	0	-	0
methanol	16 1/2	14 1/2	0	0
water	0	0	0	0
pyridine	-	13	-	0

Table 7. Antagonism of <u>Aspergillus</u> <u>flavus</u> by walnut phenols of different polymeric size at different concentration levels.

Extract	Diameter of zone of antagonism (mm)			
	5% level	1% level	0.05% level	0.05% level
ether	0	0	-	0
acetone	0	0	-	0
methanol	0	0	0	0
water	0	0	0	0
pyridine	-	0	-	0

in the acetone extract. The ability of the pyridine extract to antagonize microbes is difficult to interpret because the composition of the extract is unknown. Jurd (1956) indicated that this fraction consists of ellagic acid, but antagonism studies with ellagic acid were negative. Ellagic acid can occur in at least two forms. Perhaps one is an antagonist and the other passive (and the one tested was passive while the one extracted by the pyridine was active). It is the author's opinion that there are many more compounds present in this extract than just ellagic acid. It is felt that during the period of evaporation the reactivity of the phenols extracted from the walnut by absolute methanol increased. The reactivity is most likely channeled into reactions among the phenols (i.e., polymerization) but reaction with the non-phenolic substances extracted by the absolute methanol is also highly likely. Once a phenol reacts with a non-phenolic compound it can no longer be extracted from the gum by ether, acetone, methanol, or water. Since the ability of bases to dissolve ellagic acid has been

shown (Press and Hardcastle, 1969) and was observed in the process of this study for other phenols as well, then it seems highly likely that the phenols previously rendered unextractable are capable of being dissolved by a strong base. Once dissolved, their antagonistic ability is apparently increased.

SUMMARY AND CONCLUSIONS

The substance in the walnut which inhibited the growth of *A. flavus* in the study of Krumperman (1968) has been shown to be absent in the absolute methanol extract of fat extracted walnuts. One can conclude from this result that the antagonizing compound was therefore not a phenolic compound (since they were the compounds extracted in this study). Two results in particular supported this conclusion. First, the antagonism studies were all negative. Not one single phenolic compound or extract tested showed any antagonism for *A. flavus*. The only obvious result in these studies was a stimulation of growth in many cases. Second, the phenolic composition of the ripe walnuts was nearly the same as the phenolic composition of immature ones (i.e., the composition early in phase I was about the same as the composition late in phase III). The only net change during the maturation period was the reduction in the amount of phenolic compounds which could be extracted with absolute methanol.

This study did show that the walnut phenols can be bacterial antagonists and this is of potential importance. While these compounds were much more potent against the gram positive *Staph. aureus*, at high concentrations they also inhibited the gram negative *E. coli*. This could have two possible consequences. The walnut has a collection of compounds present throughout its lifetime which could protect the nut meat from microbial disease and later contamination

(especially by gram positive bacteria). On the other hand, if the walnut were subjected to fecal contamination during and after harvest the phenolic compounds present could sufficiently suppress E. coli so contamination might pass unnoticed leaving viable human pathogens sometimes associated with fecal contamination of food.

These compounds have interest from another standpoint. Among them might be compounds with high order toxicity toward bacteria yet harmless to humans. Such compounds might have value in certain applications to food preservation and food plant sanitation.

For the phenols extracted with absolute methanol from fat extracted walnuts the following net changes were shown: The amount of phenolic compounds decreased (this does not necessarily mean that the total content within the walnut decreased; it only means the amount within the absolute methanol extract decreased) and the degree of polymerization decreased. Further the data indicated that hydrolysis equilibrium (in phase III) lies in the acetone fraction. This was thought to be the case because the product formed that was extractable into this fraction increased much more markedly than the other fractions (i.e., polymers were broken down to oligomers rather than to monomers).

Comparison of samples in non-model systems are difficult and wrought with assumptions. The Folin-Denis determination measures substances which can reduce the phosphomolybdate-

phosphotungstate reagent used. Consequently, non-phenolic reducing substances which are extracted will react as phenols and raise the value of any result obtained. Furthermore, the reagent does not react stoichiometrically with different phenols (Goldstein and Swain, 1963) even when the number of phenolic hydroxyl groups are taken into account. One must therefore assume when using this procedure in a non-model system that interfering substances and that the stoichiometry will not change drastically during maturation. The limits of the leucoanthocyanin determination were also investigated by Goldstein and Swain (1963). The elimination reaction converting leucoanthocyanins to anthocyanidins is not the major reaction. Competing reactions and their products dominate by a ratio of at least three to one (when conditions for the elimination reaction are optimal). One must then assume that in this determination the conditions do not change drastically between samples. With these assumptions in mind one can see comparisons between samples are potentially tenuous. Comparison of extracts within any one sample should, however, be acceptable because conditions are essentially constant. Comparison of extracts between samples is subject to the problems mentioned and affects any conclusions which can be drawn. The value of this study is in its observation and not in its conclusive merit. Future workers will find value in what has been established in this study. The objective of this thesis was not intended to solve the "unsolvable" but rather construct a foundation for work which will follow.

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