CHEMICAL AND PHYSIOLOGICAL INVESTIGATIONS ON GUAYULE ESSENTIAL OIL

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CHEMICAL AND PHYSIOLOGICAL INVESTIGATIONS ON GUAYULE ESSENTIAL OIL

Relatively little study has been made of the role which essential oils play in the metabolism of the plant. The prevalent view today is that essential oil compounds are by-products or end products of plant metabolism.

The analysis of the oils according to their locations in different tissues of plant organs is a necessary technique in the thorough study of essential oil relations. Appropriate methods have, therefore, been developed for this purpose. The formation of essential oils during the growth of the guayule leaf has been studied with these methods.

A large-scale study has also been made on the compositions of oils from different organs and the changes in composition of these oils with age.

Finally the construction and use of a precision fractionation apparatus has permitted the detection in and partial isolation from guayule essential oil of additional compounds.

The biochemical significance of the results will be discussed.

I. MICRO-ANALYTICAL METHODS FOR THE DETERMINATION OF THE ESSENTIAL OIL CONTENTS OF DIFFERENT TISSUES OF INTACT PLANT ORGANS

CONTENTS

I. MICRO-ANALYTICAL METHODS FOR THE DETERMINATION OF THE ESSENTIAL OIL CONTENTS OF DIFFERENT TISSUES OF INTACT PLANT ORGANS

Procedures will be described which permit the rapid micro-determination of the essential oil contents of small plant samples and which provide for a separation of the oil according to its location in different tissues of intact plant organs. The present method is based on a simple principle suggested by Haagen-Smit, namely, the direct volatilization of the small plant sample - a process which is essentially a micro-steam distillation in which the tissue water itself initially serves as the source of steam. The volatile oil is subsequently combusted and determined as carbon dioxide. A special feature is the separate and simultaneous absorption and determination of volatilized carbon dioxide (evolved directly from the tissue).

It is possible to take a small guayule leaf of 20 mg. (or less) dry weight and, within an average time of about 30 minutes, when a routine series of determinations is being carried out by one analyst, to obtain the following information: fresh weight, dry weight, a value for "loosely-bound" oil content, a value for "total" oil content, and the amount of "volatilized carbon dioxide" (carbon dioxide which is directly evolved from the tissue by heat). At the end of the run there is available a dry sample, ready for any subsequent analysis that may be desired. The time can be shortened to as little as 10 or 15

minutes if the fresh weight and total oil content are the only information desired. For a guayule leaf of 20 mg. dry weight, with a "loosely-bound" oil content of about 1.0% of dry weight (or about 200 of oil), the precision is within 15%.

In order to realize the full potentialities of the new technique, more work must proceed on the micro-determination of individual essential oil components. This second phase is now ripe for progress in view of the many color reactions available for essential oil compounds on one hand, and the current rapid development of colorimetry, spectro-photometry and related techniques on the other hand. With a vigorously supported effort along this channel, results are assured.

The present methods may also be of interest to the essential oil industry since they provide a rapid and convenient tool for keeping a running account of the crop yields of essential oil plants.

Applications may also be developed for the rapid evaluation of the effects on cell structure of various freezing procedures - an important consideration in the work of the food technologist. By determining the amount by which the rate of oil volatilization is increased by a preliminaty freezing procedure, the technologist can evaluate the degree of cell breakdown that is caused by this particular procedure.

Background.

Various procedures are used to obtain essential oils from plants, including solvent extraction, absorption in hot or cold fats, pressing, and steam distillation. The constituents of the oil from any given plant material will vary according to the method used. Therefore, for the present work, the essential oils have been defined as those oils obtained by steam distillation.

As steam distillation is ordinarily practiced on the macro-scale for the extraction and determination of essential oil, the oil is collected in a receiver as a separate layer usually floating on the surface of the water. But this oil contains more or less water, portions of it have been lost to the atmosphere, other portions are emulsified with the water, and still more will be lost during the subsequent transfer and drying of the oil prior to final weighing. In the micro-modification described in this paper, these difficulties could be avoided.

The present procedure makes use of a train for carbon micro-determination, as illustrated in Fig.15. The sample, contained in a small tube (Fig.16B), is volatilized at a suitable temperature and the volatile oil combusted to carbon dioxide which is then absorbed in standard barium hydroxide for volumetric determination. An accessory is the small absorption tube (Fig.16A) which, attached to the sample tube, catches all the volatilized carbon dioxide, while allowing the oil to pass. Subsequently this volatilized carbon dioxide may also be determined by

acidification and aeration of the absorption tube in the train of Fig. 18. For complete details, see the experimental section.

Objectives.

It is well known that the different organs of a plant (as roots, stems, and leaves) produce essential oils of different chemical compositions (Wulff,1923; Morrens,1845); furthermore, it is almost certainly true that different tissues of a plant tussue (as pith, parenchyma, and epidermis) also produce different oils. Therefore, in first class essential oil work, an adequate means must be made available for separately studying these different oils.

Some oil separations, as of petiole oil from mesophyll oil, can be readily accomplished by prelimary dissection of the organ. But if it is desired to make a separation of vein oil from parenchyma oil, the task is nearly impossible by simple dissection; and the loss of oil during manipulations is an ever present factor.

The sectioning of the tissue according to methods of microscopic technique and treatments of the section with specific reagents can yield information of considerable value from the qualitative standpoint. Moshkina (1940,1940a) has made such studies on guayule tissue. This procedure may well accompany other methods; but, by itself, the procedure is

inadequate for quantitative work. Let us now consider the basis for the new volatilization method.

Preliminary Experimental Observations.

A turgid guayule leaf, freshly picked from the plant, has a mild odor. As soon as it is crushed or even slightly torn, however, the odor is greatly intensified. This is a common observation, and it indicates the important fact that the intact tissue structure of a leaf serves as an effective brake on essential oil escape.

For convenience, let that oil which does escape at the relatively slow rate be called "free" oil. (Haagen-Smit has made quantitative measurements of the "free" oil of guayule, oil which is liberatedthroughout the life of the plant).

A fresh turgid guayule leaf, boiled in water for about one hour, removed, and the excess water absorbedoff, emits no essential oil odor. When, however, the leaf is crushed, the odor is released. It is noteworthy that, although such a thin tissue as a leaf is subjected to this extreme treatment (in fact, the leaf structure is so degraded that even its petiole is readily ground to a smooth paste between the fingers), it still contains essential oil in its more interior portions.

For convenience, let that oil which is tightly retained be called "tightly-bound" oil; let the postulated fraction intervening between it and "free" oil be called "loosely-bound" oil.

A detached guayule leaf that has dried to brittleness in the air gives off no odor, nor does it do so when soaked in water and cautiously heated to dryness again. (it has lost its

"free" oil and its "loosely-bound" oil). But let the initial dry leaf or the moist boiled leaf be crushed and the typical guayule oil odor (of its "tightly-bound" oil) is apparent.

Similar results have been reported for other organs. "Schimmel (Durrans, 1922) found that uncrushed Ajowan seeds yielded 20 per cent less oil than crushed seeds although the former were distilled twice as long as the latter," and further "found that whereas a certain batch of cut vertivert root yielded 1.09 per cent of essential oil on distillation, a similar batch of uncut root yielded only 0.3 per cent."

Quantitative Experimental Evidence; Tissue Lag and the Diffusion Process.

Presented in Fig.(1) are a set of curves for the steam distillation, on an approximate 25 kg. scale (fresh weight), of several lots of similar four year guayule stems (the important details of procedure being given with the figure). Consider the distillation of the unchopped stems. The great bulk of the oil comes over during the first 30 minutes of distillation; after about two hours, the oil distillation has essentially ceased. Yet when we chop these predistilled stems and begin redistillation, there is an immediate and rapid distillation of large quantities of oil. From the initial steepness of the latter curve, it is, furthermore, apparent that the oil now being distilled is at least as volatile as the oil which came over first. Due entirely to the structure of the stem, it is feasible to make a more or less sharp separation of the oil present in different tissues.

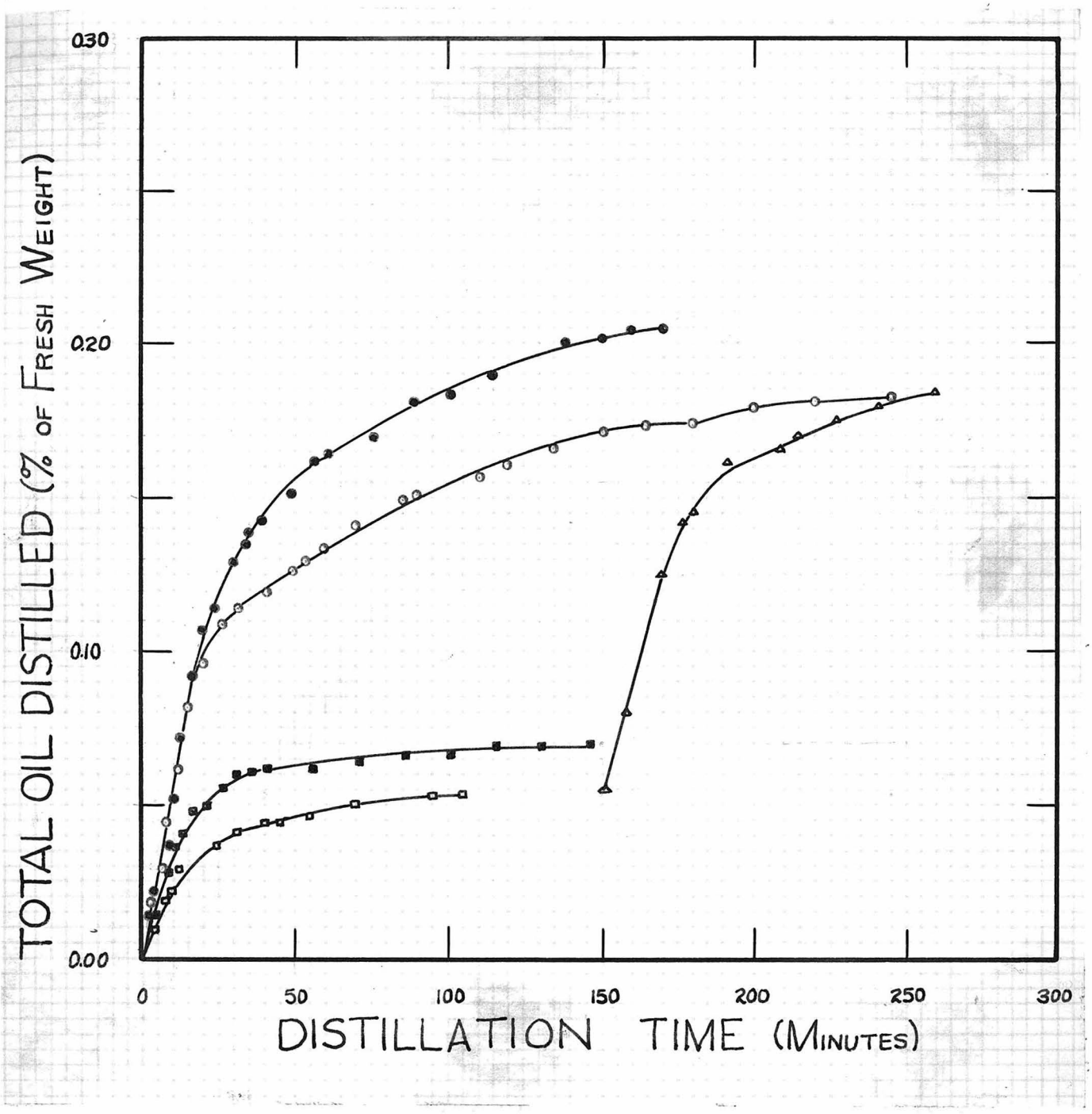


Fig. (1). Sharp Morphological Fractionation; Steam Distillation of Four Year Guayule Stems.

- ○ ● Two duplicate lots of stems, chopped into small pieces with a Wiley mill (See Fig. 26) and separately distilled.
- □ ■ Two other duplicate lots of stems, distilled without chopping.
- △ One lot of predistilled stems (□), chopped and distilled again.

As a check it is of importance that the total amounts of oil obtained, either by chopping at the outset or after a predistillation, are identical (within the variability range of the material).

The curves for similar large scale steam distillations of four year guayule leaves are presented in Fig.(2). The gentle slopes of the two curves suggest a gradual depletion of the oil content in the tissues by a slow process of diffusion brought into play by the leaf structure. The situation is basically the same in the stems, except that the oil separation is sharper, apparently due to the presence within the stem of a dense-walled structure separating the different tissues. Even where such a special separating structure is absent, it is possible that the oil collected in the earlier volatilization periods is the oil initially present in the more exterior cells, while the oil collected in later distillation periods comes from the more interior cells.

To obtain a quantitative picture of the real significance of this lag effect, the two very steep curves to the left of the leaf curves are included. These curves were obtained by adding to the still samples of free oil (which had previously been obtained by the steam distillation of three year leaves). These distillations of free oils illustrate the maximum limitation that can be imposed on oil distillation rate by a limited capacity of the steam to carry over oil with it (expressed in the theoretical water:oil ratio). A comparison of these curves with the leaf curves gives clear evidence that the oil:water

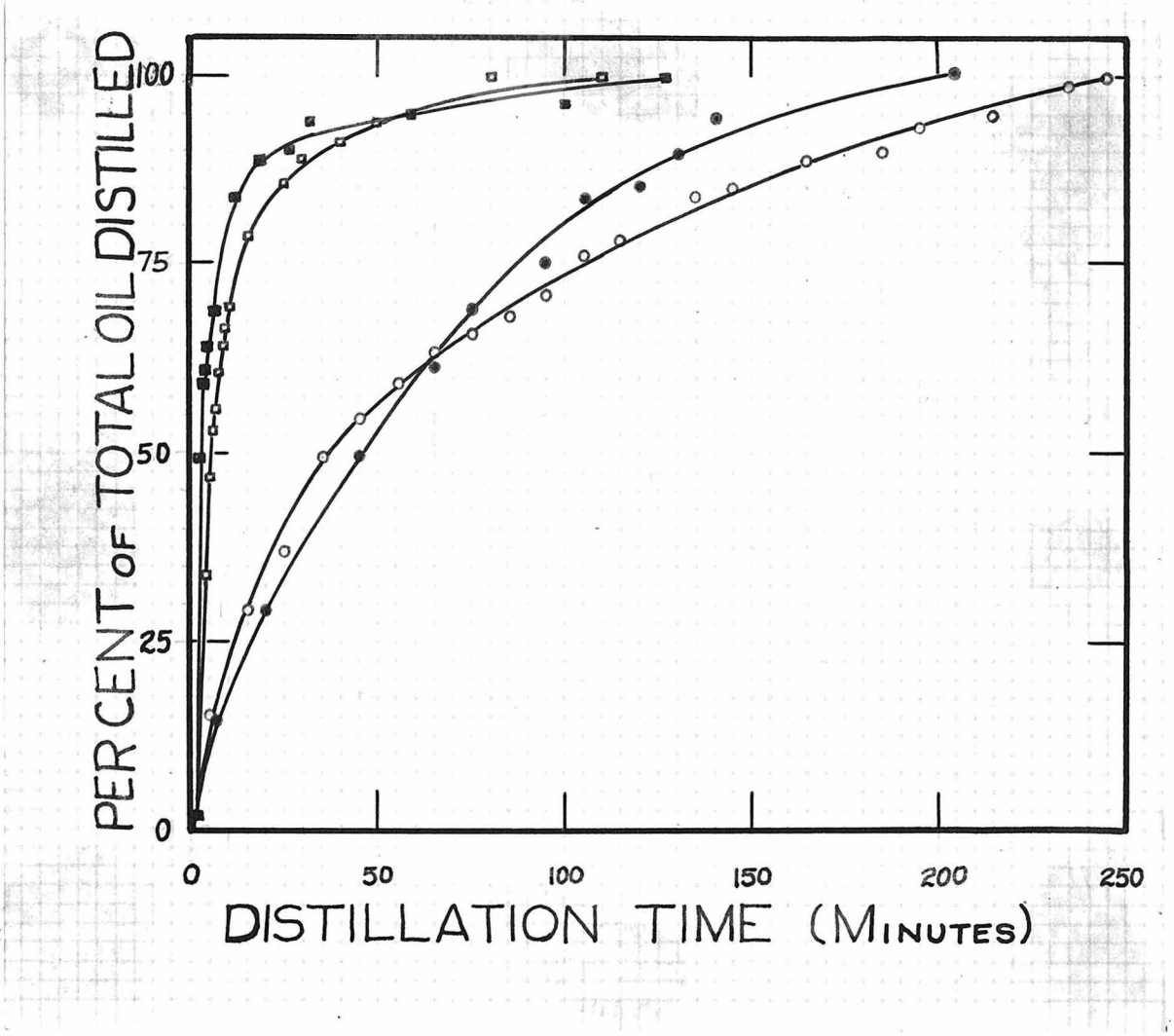


Fig.(2). Tissue Lag; Steam Distillations of Guayule Leaves and Free Guayule Leaf Oil.

○● Two duplicate lots of four year guayule leaves.

□■ Two duplicate samples of three year guayule leaf oil.

ratio, as a limiting factor in the steamdistillation of essential oils from plant tissues (even the very permeable thin leaf tissues) is entirely inconsequential !

Fig. (3) and (4) show the effects on oil volatilization of different preliminary treatments of the plant tissue. We observe that the volatilization of Sample 1, which was given no treatment that would effectively break down its structure, lags well behind the other three comparable samples, on all of which preliminary degrading treatments were employed (Fig. 3).

Another significant effect is noted in the case of Sample 1. First, let it be mentioned that a typical and natural effect of the volatilization procedure is the cessation of oil volatilization when the water is completely depleted from the tissue; more water may then be added, about 10 minutes allowed for it to be well absorbed, and the volatilization continued. When this treatment is applied to Sample 1, increments of oil are obtained, but when, after this treatment, the tissue is also ground and water added, the oil increment resulting on continued volatilization is significantly greater - again clear evidence that the intact tissue structure is a good barrier against oil escape.

Fig. (4), less informative in the present connection, shows the added effect of initial slow freezings at -7°C or at -1.5°C. The present results are inconclusive, it appearing, however, that -7°C treatment may help to "open up" the tissue. It is well

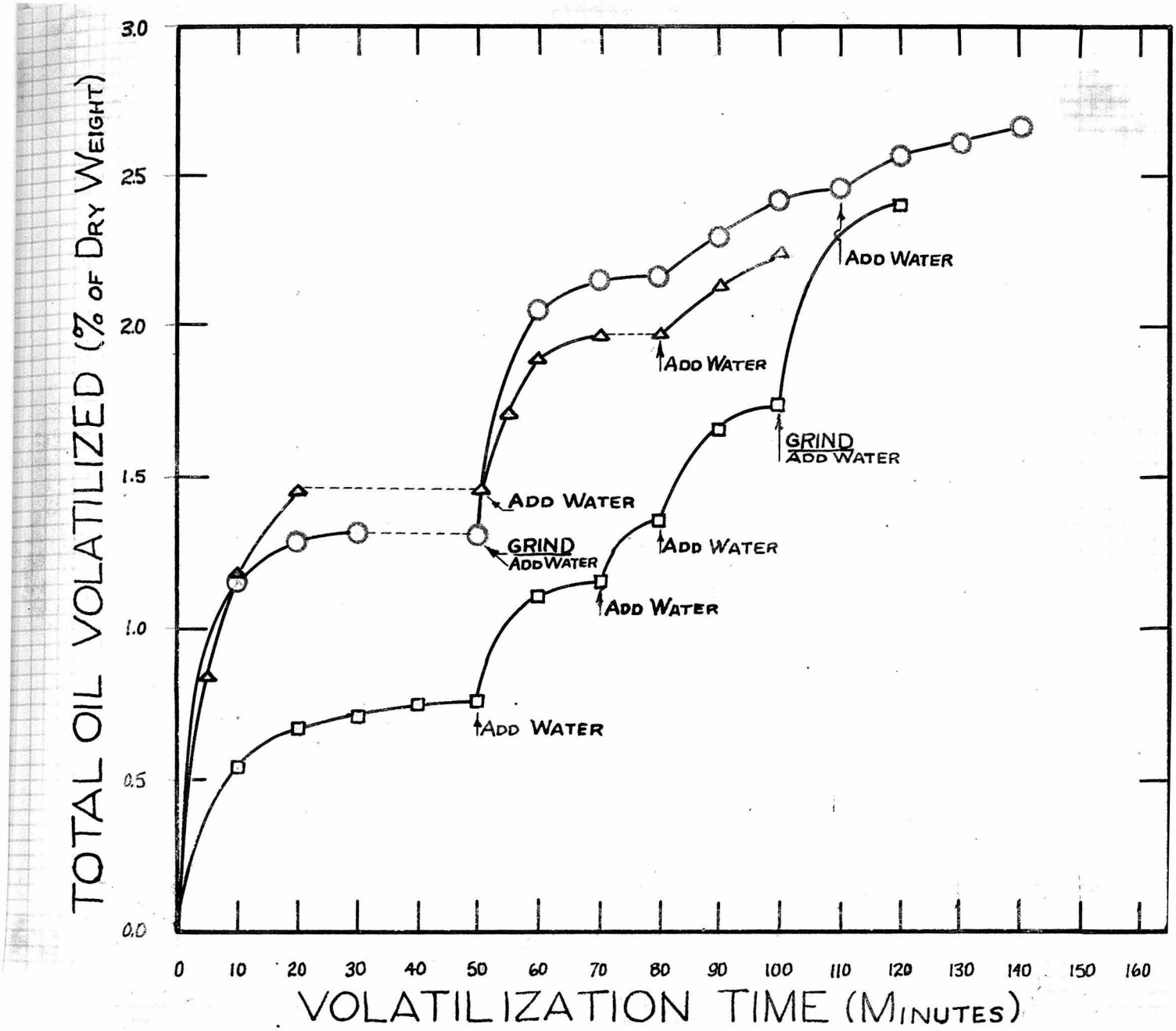


Fig. (3). Effects on Oil Volatilization of Treatments Affecting Tissue Structure.

□ Sample 1. Mild treatment (a single dry ice freezing and storage for less than one day).

○ Sample 2. Alternate dry ice freezing and thawing every 3 to 5 days for a period of about 60 days.

△ Sample 9. Dry ice freezing and pulverizing; storage for one day only.

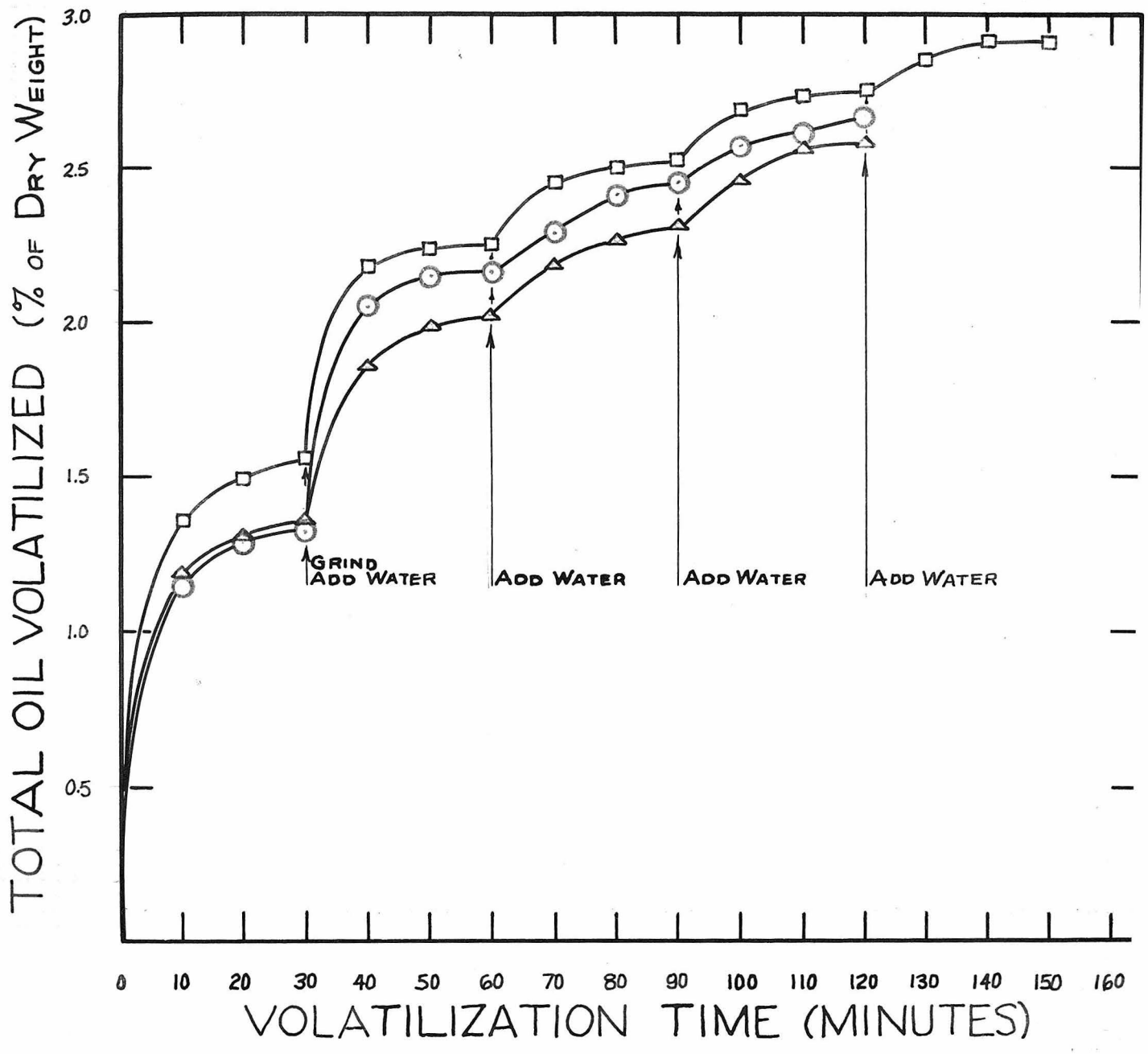


Fig. (4). Effects on Oil Volatilization of Treatments Affecting Tissue Structure.

○ Sample 2. Alternate dry ice freezing and thawing every 3 to 5 days for a period of about 60 days.

□ Sample 6. Initial slow freezing at -7°C, followed by treatment of Sample 2.

△ Sample 7. Initial slow freezing at -1.5°C, followed by treatment of Sample 2.

known that slow freezing techniques lead to large ice crystal formation and consequent internal crushing of the tissue.

"If a peach is cut in half, and one half frozen at -100°F (-73°C) and the other half at 0°F (-18°C), examination after thawing showsthat the former can hardly be distinguished from a fresh peach and that the latter is greatly damaged." (Jacobs, 1944).

As carried out here, the technique may not be optimum, and the result is obscured by the alternate freezings and thawings over the period of dry ice storage. The figure is most valuable as an indication of the reproducibility that can be achieved by the present method.

Finally Fig.(5) presents the effects of different volatilization temperatures. An important result to note is that the rate of oil volatilization during an initial volatilization to dryness is quite independent of the rate of water volatilization (again the insignificance of the oil: water ratio factor !). If the water is removed rapidly as at 120° or 110° (see Fig.(6) also), the diffusion process has a relatively short time in which to proceed and the oil obtained is relatively small in amount. On the other hand, if the water is removed more slowly as at 100° or especially 90°, more time is allowed for diffusion and amount of oil obtained is considerably greater.

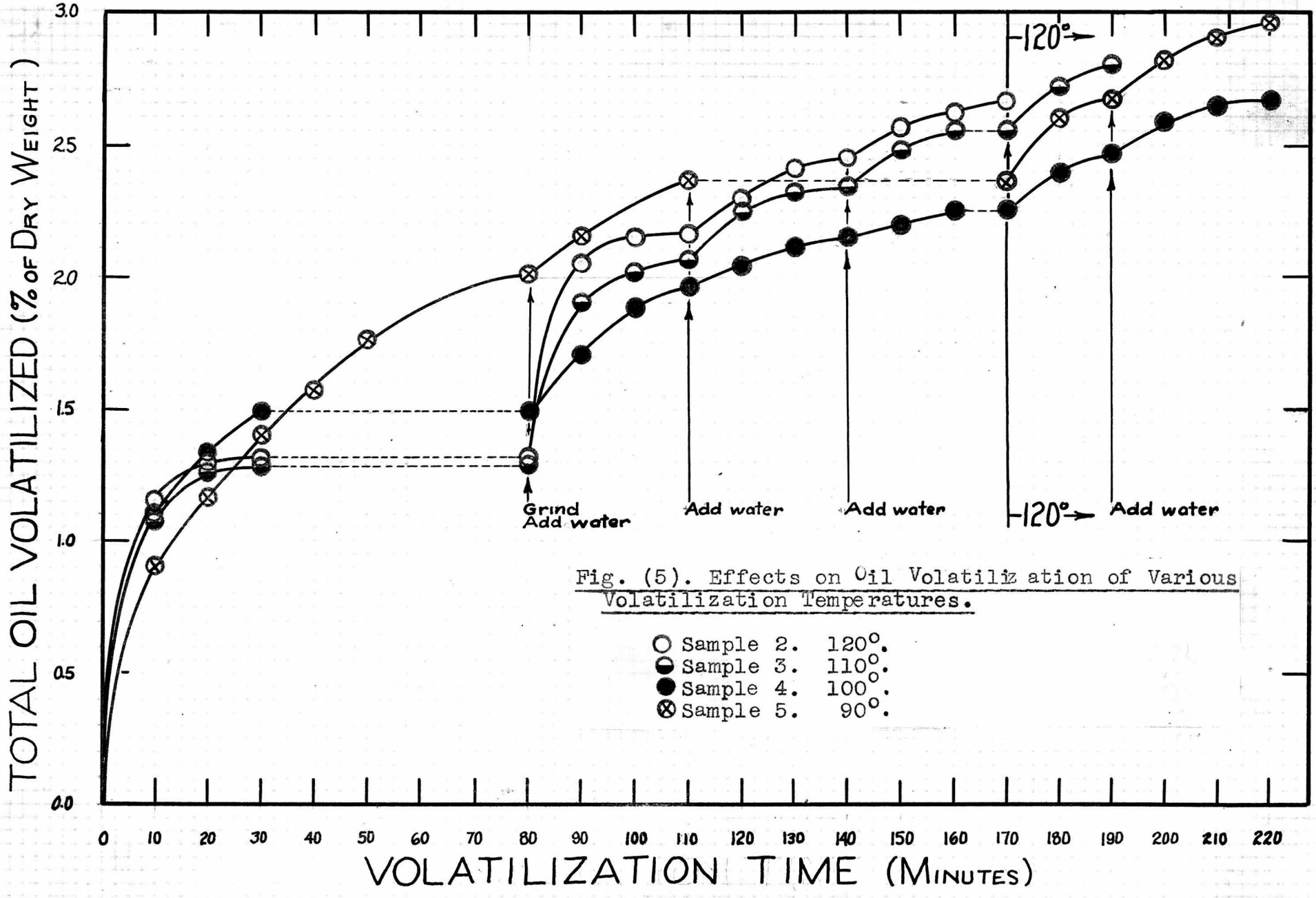


Fig. (5). Effects on Oil Volatilization of Various Volatilization Temperatures.

- ○ Sample 2. 120°.
- ◒ Sample 3. 110°.
- ● Sample 4. 100°.
- ⊗ Sample 5. 90°.

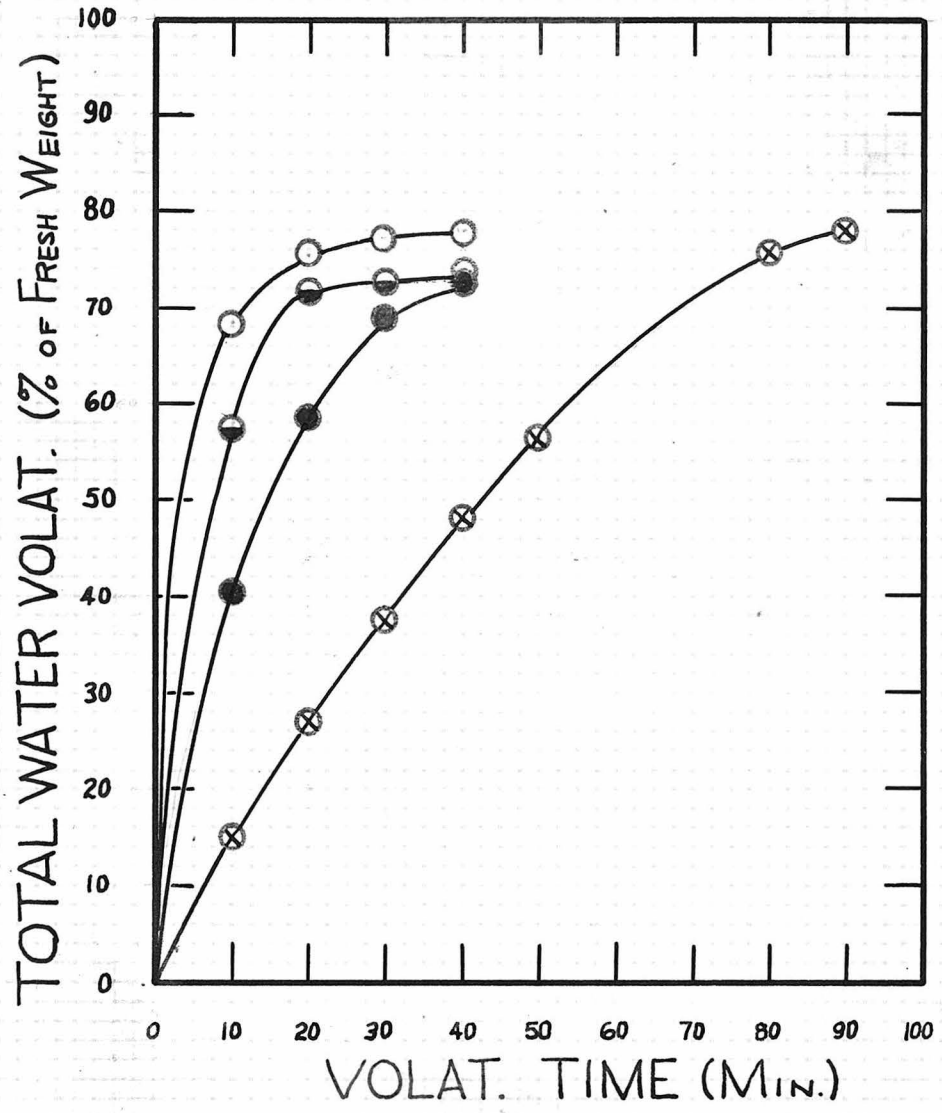


Fig. (6). Effects on Water Volatiliz ation of Various Volatiliz ation Temperatures.

Sample 2. 120°.
Sample 3. 110°.
Sample 4. 100°.
Sample 5. 90°.

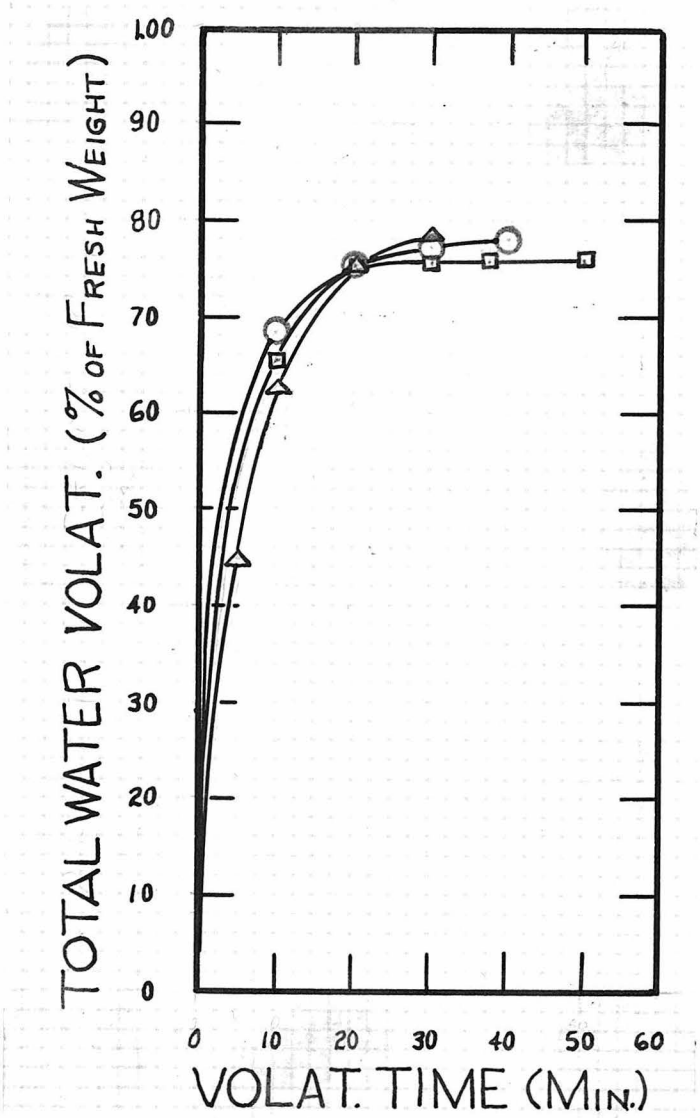


Fig. (7). Effects on Water Volatilization of Treatments Affecting Tissue Structure.

□ Sample 1. Mild treatment (a single dry ice freezing and storage for less than one day).

○ Sample 2. Alternate dry ice freezing and thawing every 3 to 5 days for a period of about 60 days.

△ Sample 9. Dry ice freezing and pulverizing; storage for one day only.

(It will be noted that in the studies just presented ~~now~~, no distinctions were made between the volatilized carbon dioxide arising directly from the tissue and that resulting from the actual combustion of oil, the procedure for separating the two having not been brought into use in time; and, therefore, the term "oil" includes both quantities. It is shown later, however, that the curves for volatilized carbon dioxide alone (Fig. 11 to 14) are strikingly similar in form to the present "oil" curves and quite reproducible in magnitude for all the plant material used in the present work. Therefore, if a correction were applied, the "oil" curves would be merely shifted lower along the ordinate, but would remain essentially unchanged in typical form and in relation to each other).

TABLE I.

A. General Data Common to All Samples

Plant Material. Parthenium argentatum Gray, Variety 593.

Date of Sowing. February 12, 1945

Place. Orlando Road Nursery of the California Institute, Pasadena.

Conditions. Some time after seed germination in sand flats, these flats were placed outside, and growth proceeded under weather exposure until transfer, at which time the plants, although vigorous in appearance, were much smaller (12 to 15 cm. height) than uncrowded plants of similar age; almost all still had single stems.

Date of Transfer. December 14, 1945

Place. One flat was transferred to Room 1 of the controlled condition greenhouses of the California Institute (Went, 1944). This room has a slanting glass roof and aluminum painted walls.

Conditions. 18°C (65°F) day, 13°C (55°F)night. Well lighted position, although only upper leaves of plants received direct sunlight.

Tissues Analyzed. Large, mature well-developed leaves from the largest plants.

B. Harvesting Data

Harvest No.	Date	Time	Light Conditions	Age (days)	Time in Room 1 (days)	Ave. No. Leaves per Sample(1
I	12/17/45	2:00 P.M.	Sunny	308	3	1
II	12/18/45	11:00 A.M.	Overcast	309	4	3
III	3/20/46	11:00 A.M.	Overcast, rainy	401	96	≥1
IV	3/25/46	11:00 A.M.	Sunny	406	101	≥1
V	4/1/46	11:00 A.M.	Alt. sunny & overcast	413	108	-
VI	4/6/46	8:00 A.M.	Sunny	418	113	≥1

(1) ≥1 signifies slightly less than 1.

TABLE II. CONDENSED DATA CONCERNING ESSENTIAL OIL(3)DETERMINATIONS AS ILLUSTRATED IN FIG. 3 TO7 .

Sample No.	Harvest No.(1)	Preliminary Treatment and Storage(2)	Vol.Temp. (°C)	Dry Wt. (mg.)	% Oil(3) (% Dry Wt.)	% Water (% Fresh Wt.)	Figures
1	II	Sterilize(2a), freeze in dry ice 7 hrs.	120	45.5	0.77	75.8	3,7
2	"	" " " " " 56 d.(2b)	120	34.8	1.32	78.0	3,4,5,6,7
3	"	" " " " " 43 d.	110	29.2	1.28	73.8	5,6
4	"	" " " " " 51 d.	100	32.6	1.48	72.2	5,6
5	"	Freeze in dry ice 55 d.	90	23.3	2.01	77.8	5,6
6	"	Freeze at -7°C(2c) & dry ice 62 d.	120	41.1	1.56	72.9	4
7	"	Freeze at -1.5°C & dry ice 63 d.	120	36.8	1.36	74.5	4
8	"	3°C 51 d., & dry ice 14 d.	120	23.0	1.63	75.9	17
9	III	Freeze in dry ice, pulv.(2d); plus 3°C 1 d.	120	20.0	1.46	78.9	3,7,11

Notes to Table II

(1) See Table I.

(2a) "Sterilization" is a rapid heat treatment above 100° (see p. 57)

(2b) These long storage periods in dry ice actually involved alternate freezings and thawings about every three days.

(2c) See p. 58 for the freezing procedure used here.

(2d) See p. 58 for the pulverizing technique used here.

(3) The term "oil" as used here was determined from the total amount of carbon dioxide found after combustion although a portion thereof was carbon dioxide directly evolved from the tissue during volatilization. See text for discussion. The conversion factor for converting cc. of N/10 barium hydroxide used to mg. oil is 0.34, on the basis of an oil composition of C_5H_8.

The values given are for amounts of oil obtained during initial periods of volatilization to dryness, and are, therefore, measures of the effects of the various treatments. More comprehensive pictures of the effects are given in the running accounts of the distillations, as provided by the figures referred to in the last column.

TABLE III. CONDENSED DATA CONCERNING VOLATILIZED (2) CARBON DIOXIDE DETERMINATIONS AS ILLUSTRATED IN FIGURES 11 to 14.

Sample No.	Harvest No. (1)	Preliminary Treatment and Storage	Vol. Temp. (°C)	Dry Wt. (mg.)	% CO_2 (2) (% Dry Wt)	% Water (% Fresh Wt.)	Figures
10	II	3°C 51 d., & dry ice 20 d.	120	32.4	0.61	77.7	14
11	"	3°C 51 d., & dry ice 25 d.	120	33.0	0.71	79.0	13, 14
12	"	Freeze at -4°C, & dry ice 79 d.	90	28.9	0.88	76.8	13
13	"	Sterilize, & dry ice 85 d.	23 to 120	38.8	0.25	76.3	12
14	III	Freeze in dry ice, pulv., & 3° ½ hr.	120	20.9	0.66	80.0	11
15	"	Freeze in dry ice, pulv., Sterilize & 3° sev.hrs.	120	23.9	0.65	76.4	11
16	IV	Freeze in dry ice, & 3° 5 d.	120	46.9	0.72	78.5	11, 14

(1) See Table I.

(2) Volatilized carbon dioxide ("CO_2" in the table) is the carbon dioxide which is released directly from the tissue by volatilization. It is determined here by omitting the combustion process. For quantitative comparisons with the oil values, the amounts are expressed on the C_5H_8 basis.

Typical Oil-Bearing Tissue Structures.

It is desirable to get a clear conception of the actual physical structures of various oil-bearing plant tissues with special regard to the location of the oil therein. The structures and oil distributions are quite variable from tissue to tissue and from plant to plant, and it is convenient for practical purposes to make a simple classification of the principle types by means of the much simplified diagrams (in longitudinal section) of Fig.(8).

Type A represents the case of an essential oil tissue from which oil is given off to the atmosphere as soon as it is formed; in other words, there is no storage. This oil may be considered as one type of the so-called "free" oil.

Type B represents a case where the oil is distributed throughout the cytoplasm of the living cells in the form of many tiny globules. Type C is a similar case where the globules are fewer and larger (Wulff, 1933; Moshkina, 1940). A portion of this oil may find its way to the atmosphere as "free" oil, while the rest would be called "loosely-bound" oil.

Type D represents the case where specially differentiated epidermal cells or hairs collect and store oil at the very surface of the tissue. This type of structure is perhaps the most prolific source of "free" oil.

Type E represents the case where internal pockets of oil are formed. The oil may be inside special cells, or the gland may be schizogenic, storing oil in a cavity between cells. This oil can be called "tightly-bound" oil.

Type F represents the case where certain columns of cells have degenerated and become filled with oil - resin ducts

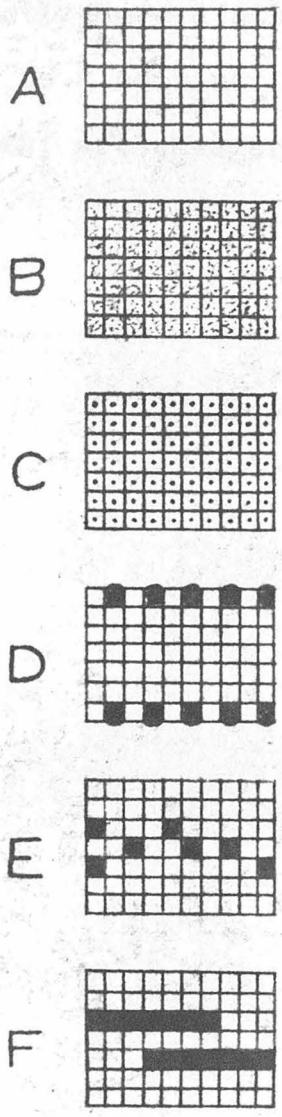


Fig. 8. Simplified Diagram Representing Typical Oil-Bearing Tissue Structures.

(lysogenic structure). The oil can also be called a "tightly-bound" oil.

Guayule leaves apparently have a structure and oil distribution corresponding to a combination of Types B and C and Type F (Moshkina, 1940; Artschwager, 1943).

Discussion.

The rate with which the oil is volatilized is determined by the rate at which the oil can diffuse out of the tissues. This diffusion is greatly influenced by particular structural elements between the tissues. These barriers of free diffusion can be broken down by grinding or freezing procedures.

It is a law of diffusion that the amount of material which will diffuse across a given medium in a given period of time is directly proportional to the concentration gradient across the medium. In the present case, whether the medium be cell membranes and walls or a structureless tissue mass, the external concentration is zero, and the amount of oil diffusing out during a given volatilization period is a direct measure of the oil concentration or content in that portion of the tissue from which the tissue structure permits diffusion to take place.

Accordingly if we submit a reasonably intact guayule leaf to a short period of volatilization, we may say, from a knowledge of the guayule leaf structure, that the value is a direct measure of the oil content of mesophyllic cells, it being postulated that diffusion from the resin ducts of the petiole and veins is negligibly slow. (This mesophyllic oil content is a specific type, thus, of "loosely-bound" oil).

Now if we initially pulverize a tissue and thoroughly homogenize it, all the oil from all parts of the tissue will

be thoroughly mixed and distributed uniformly throughout the mass. The result of a volatilization in this case will accordingly give a value that is a direct measure of total oil content. In a steam distillation of free essential oil, the composition of the oil which first comes over is by no means similar to that initially placed in the still; it predominates in lower boiling constituents. This effect is the result of a complex oil-water distilling composition, in which each constituent is represented in proportion to its particular vapor pressure at the temperature of distillation. Of course, the low boiling constituents are poorly represented, and the magnitude of the effect is illustrated in Fig.(9). Whereasthe water:oil ratio over the terpene range is less than 2, it is of the order of well over 100 for most of the sesquiterpene range. In other words, we may say the amount of terpenes initially coming over will be roughly 50 timesthe amount of sesquiterpenes, regardless of how much sesquiterpene may be present in the initial oil.

Now consider the situation for the steam distillation of oils from plant materials, where another distinct physical process, diffusion, is in control. The rate of oil volatilization will depend on the rates at which the various oil constituents diffuse from their respective locations within the tissue to the surface of the tissue. It is another law of diffusion that compounds diffuse at rates inversely proportional to the square roots of their molecular weights. Therefore, the relative rates at which terpenes ($C_{10}H_{16}$, mol.wt.136)

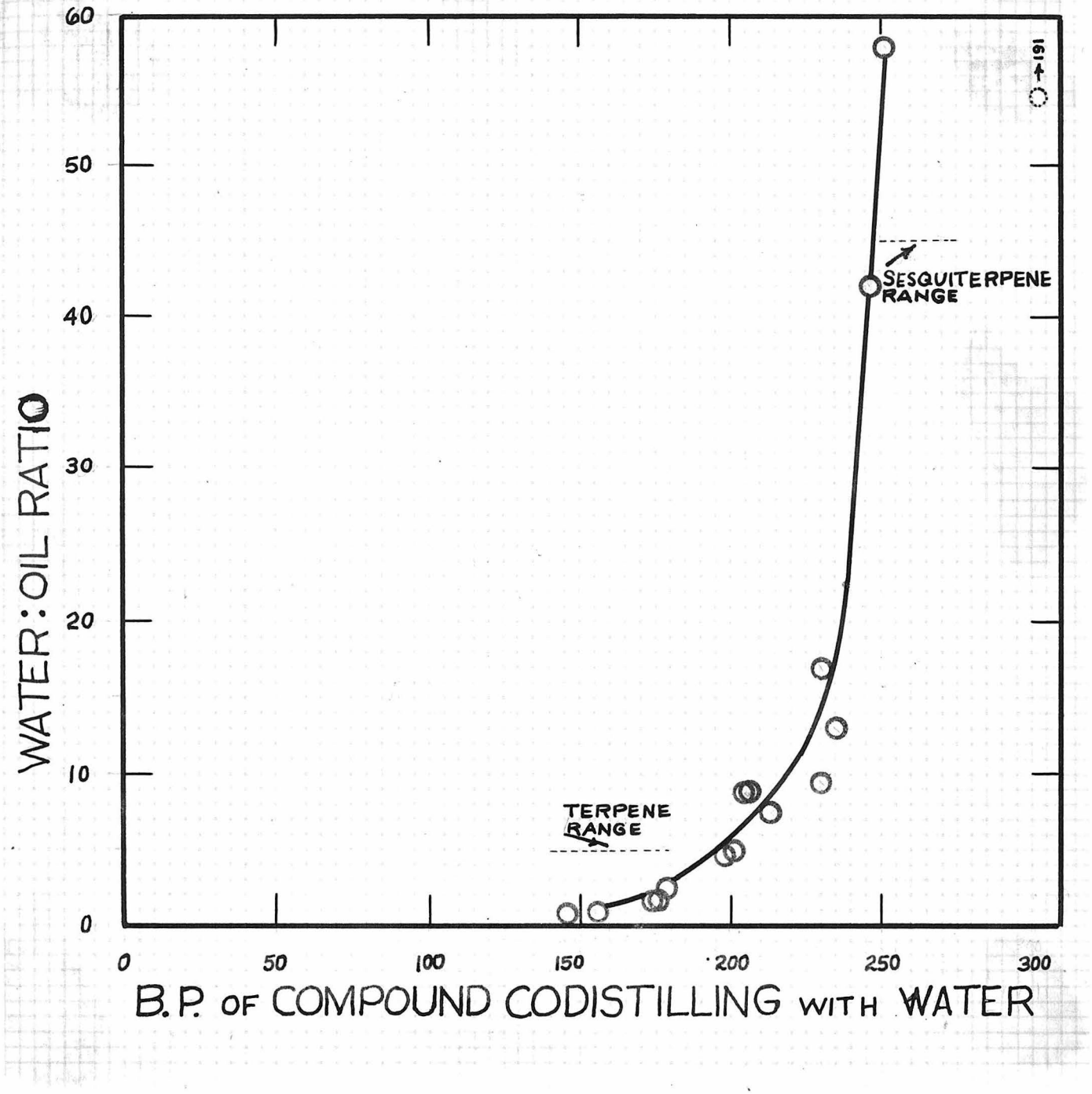


Fig.(9). The Compositions (Water:Oil Ratios) of Various Codistilling Systems of Water and an Immiscible Organic Compound as a Function of the Boiling Points of the Organic Compound.

(From data calculated from original data in Young, 1922).

and sesquiterpenes $C_{15}H_{24}$) mol.wt.204) diffuse out will be 204:136 = 1.2:1. In other words, the terpenes diffuse out but little faster than the sesquiterpenes. This means that as the various oils, located deeper and deeper within the tissue initially, begin to diffuse out, there will be a minimum of overlapping due tothe more volatile (smaller molecular weight) components of a more interior oil overtaking the less volatile (higher molecular weight) components of a more exterior oil; and as soon as each individual oil reaches the tissue surface, it is immediately whisked away in its entirety by the steam. This last statement is true since the amount of oil liberated during a given moment, even considering its content of compounds of relatively low volatility, is typically insufficient to saturate the capacity of the water that is available to carry it away immediately (even in the micro-volatilization method, where water is relatively scarce).

Summary: Tentative Scheme of Analysis.

A tentative scheme of analysis is suggested in Fig. (10). It will be noted that those most valuable possibilities, involving a study of the oil compositions of the fractions, are not at the moment experimentally realized. In order that this type of analysis may achieve its proper place as a valuable tool, analytical methods for the determination of specific classes of oil components or, better yet, specific individual oil components must be developed on the micro scale.

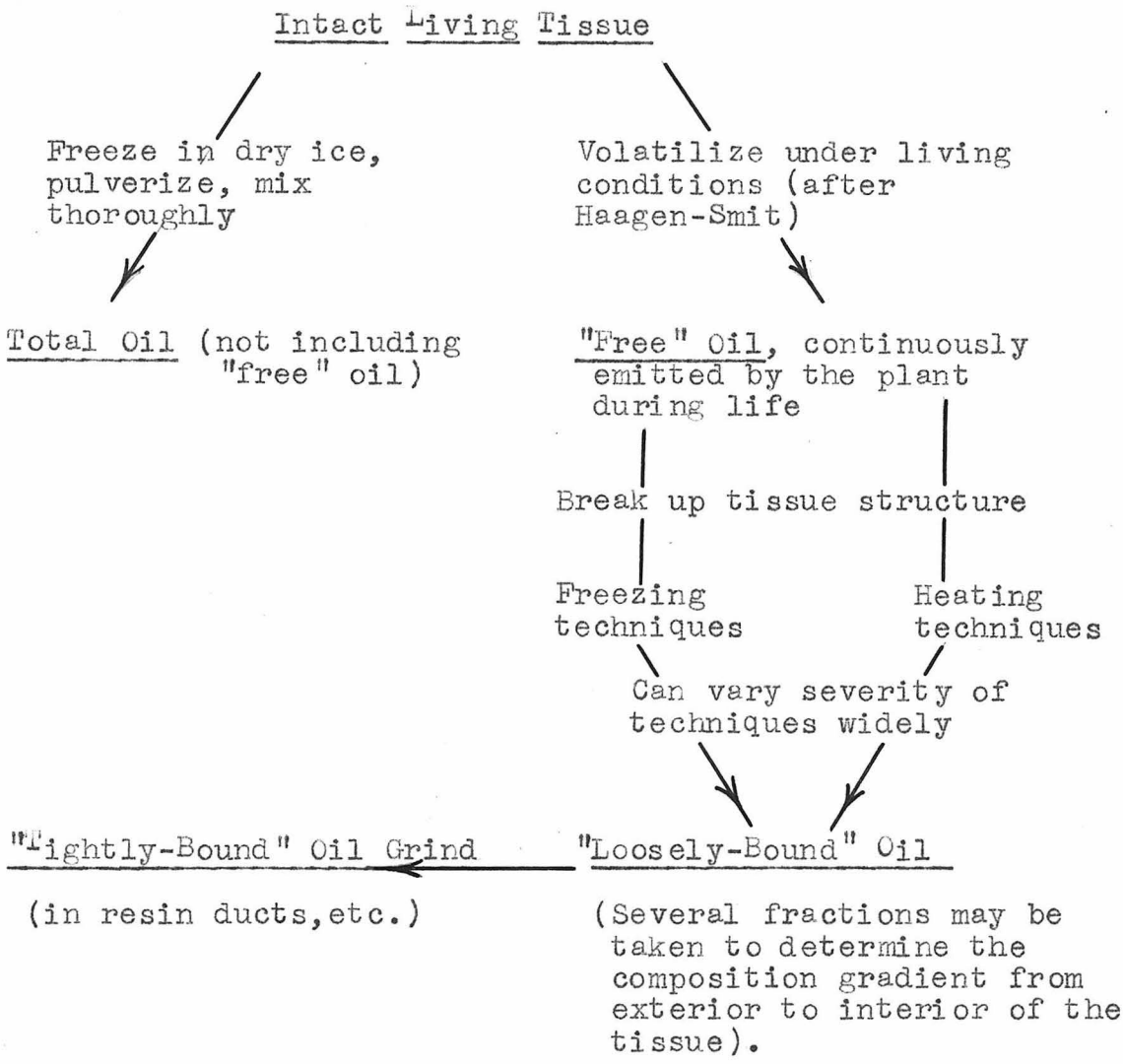


Fig.(10). Tentative Scheme of Micro-Morphological Fractionation by Volatilization.

This is a phase of work that is ripe for advancement in view of the great number of highly sensitive color reactions published in the literature, and the present-day rapid strudes in such techniques as colorimetry and spectro-photometry.

Volatilized Carbon Dioxide.

In the course of this work it was observed that considerable amounts of acidic material are evolved directly from the volatilized tissue. This material shall be called "volatilized carbon dioxide", to distinguish it from the carbon dioxide formed by combustion of the oil. The amounts obtained from different leaves are somewhat variable, but usually lie within a rather limited range of 0.5 to 0.9% of dry weight (calculated to a C_5H_8 basis for quantitative comparison with oil values). The determinations are represented in a series of curves (Fig. (11) to (14)) and by the data collected in Table III (in which and appended footnotes all immediately desirable information and experimental details are condensed). The full details of experimental procedure can be found in the experimental section.

The evolution of this material was originally a source of considerable concern. The apparent necessity of making separate runs on duplicate samples in order to determine the true oil content by difference was a seriousthreat to the method at that time. Since the work presented here, however, the simple means of quantitatively separating this volatilized carbon dioxide from the oil before combustion was devised.

The question is of interest where this carbon dioxide finds its origin. A few experiments were carried out to determine whether this carbon dioxide arises simply by the evolution of

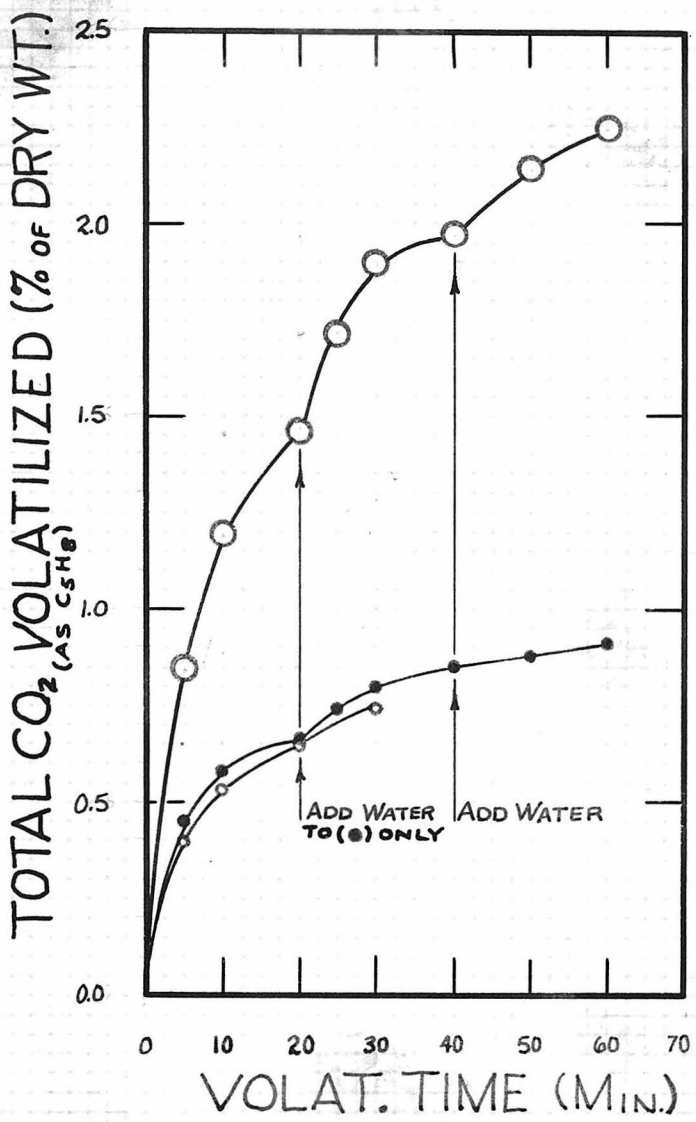


Fig. (11). Comparison of the Relative Amounts of Volatilized Carbon Dioxide, and Oil Plus Volatilized Carbon Dioxide.

○ Sample 9. Volatilized carbon dioxide plus oil.

• Sample 14. Volatilized carbon dioxide alone.

◦ Sample 15. Volatilized carbon dioxide alone ("blanched").

carbon dioxide held by buffers in the tissue sap, or whether it may arise from the decomposition of organic material.

The possibility existed that, during a gradual rise of the volatilization temperature by reasonably small increments from room temperature, the evolution of carbon dioxide in some particular range might suddenly increase more than would, for example, be accounted for by dissociation of buffer-bound carbon dioxide. However, no such marked evolution could be found as shown in Fig. (12).

However, the possibility of sudden decomposition is not at all disproved, for it is very plausible to believe that the old tissue lag factor thoroughly discussed elsewhere, and the consequent necessity of slow diffusion may effectively mask and distribute any sudden release of carbon dioxide within the tissue. (Fig.(13) suggests such a diffusion effect, slower but greater final evolution at 90° than at 120°). Carbon dioxide within the tissue is completely surrounded by water and can only escape by solution in and diffusion through the water. Presumably when the tissue becomes dry, it "closes up" and becomes impervious even to carbon dioxide gas which cannot leave until added moisture reopens a water path to it. (This view is supported by the accelerated evolution after fresh additions of water to the tissue).

Our experiments do not permit us to draw any definite conclusions regarding the specific form in which this volatilized

carbon dioxide is present. The views of Smith (1940), who attributed the carbon dioxide content of plant tissues to buffer systems, may satisfactorily apply in the present case.

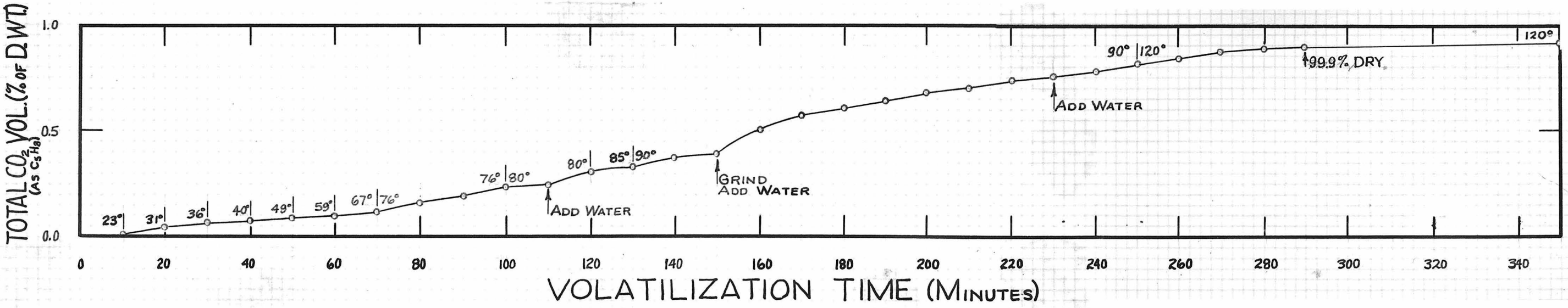


Fig.(12). Effects on Carbon Dioxide Volatilization of a Gradual Rise of Volatilization Temperature.

Sample 13.

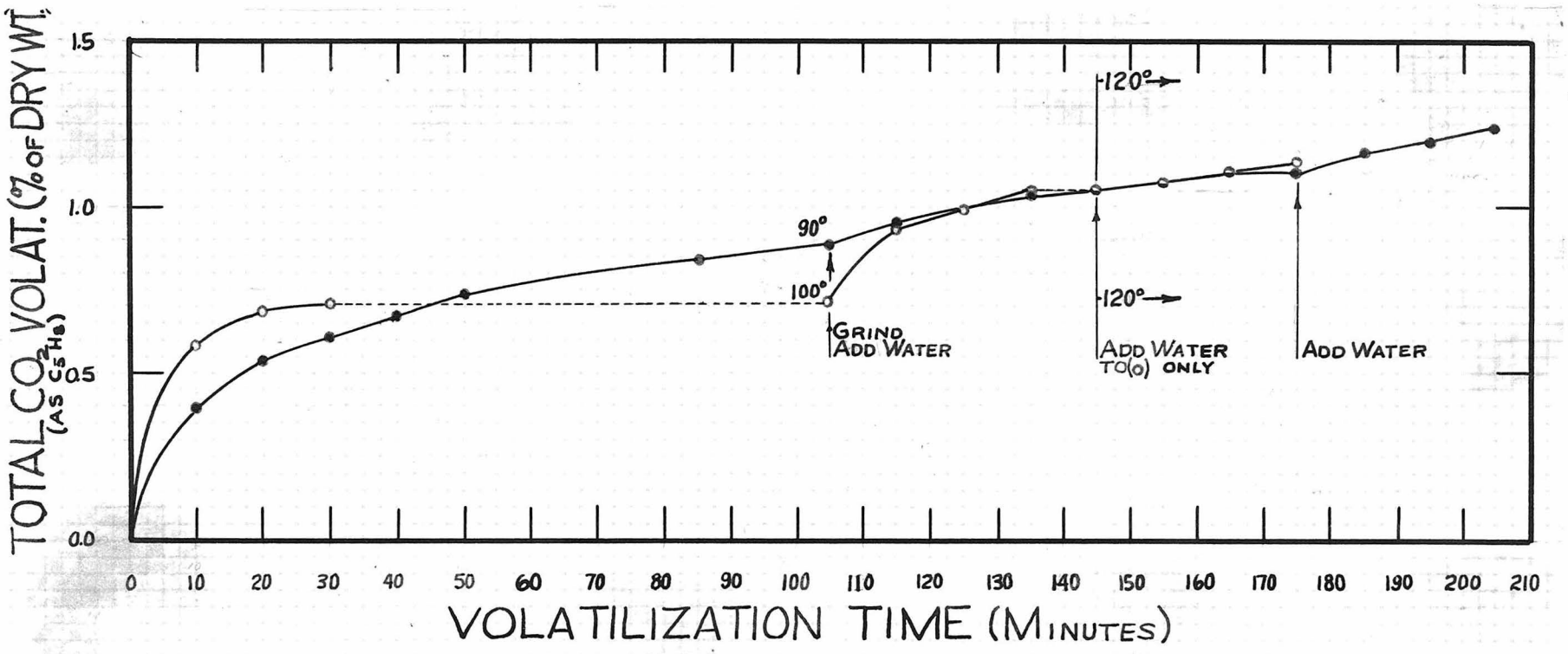


Fig.(13) Effects on Carbon Dioxide Volatilization of Two Volatilization Temperatures.

- o Sample 11. 120°.
- • Sample 12. 90°.

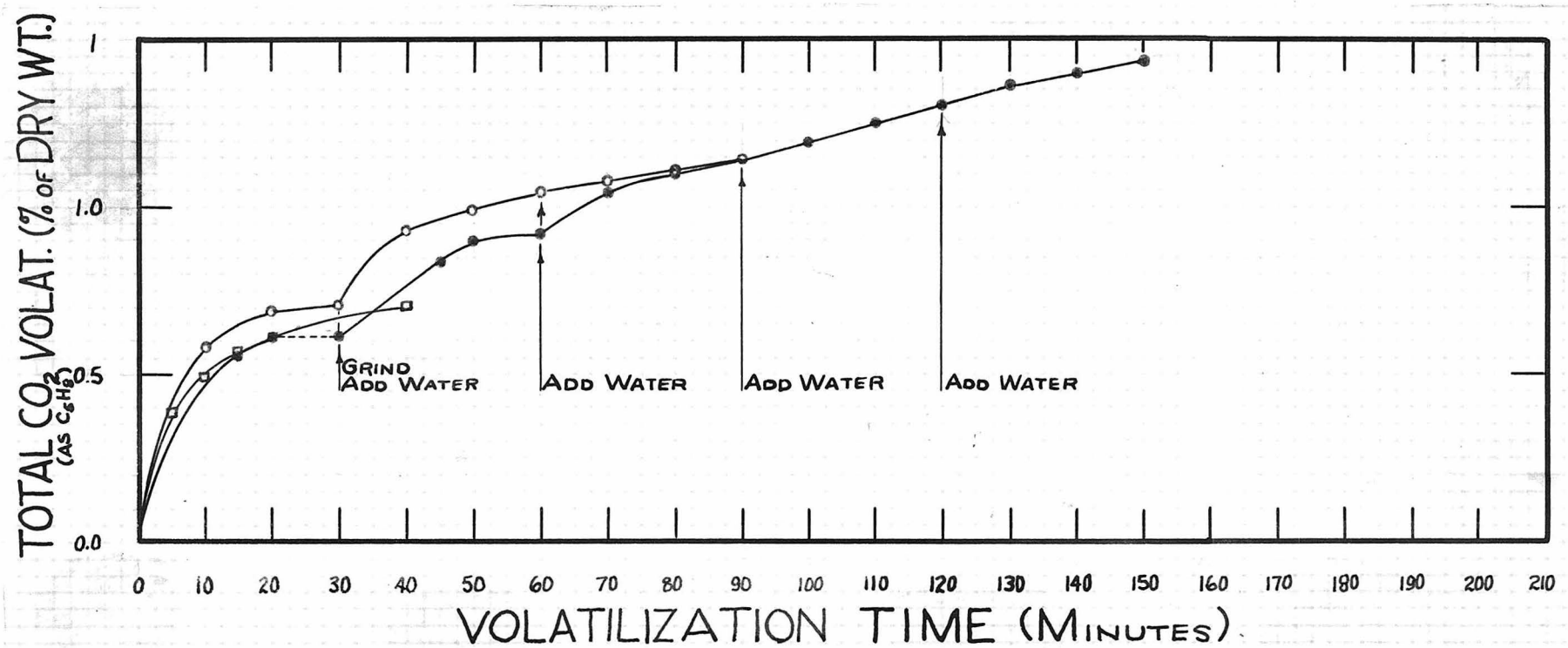


Fig.(14). Reproducibility of Volatilized Carbon Dioxide Determination.

- ○ ● Samples 11 and 10. Both stored at 3°C for 51 days and in dry ice for about 20 days. (Harvest II).
- □ Sample 16. Stored at 3°C for 3 days. (Harvest IV).

A short investigation was made to assure that this acidic material was actually carbon dioxide and did not contain appreciable quantities of volatile acids native to the oil itself (nor volatile acids arising from the tissue as a consequence of thermal decomposition); and, in addition, to assure that appreciable quantities of ammonia (which might arise from certain sensitive amides possibly in the tissue) were not produced to depress the actual acidity.

A brief description of the procedures used and the results are given in Table IV and footnotes. The essential results are collected in Table IV-B. We can draw these conclusions therefrom:

1. The amount of volatile basic material is negligible.

2. The amount of volatile acids is probably very small. (More data are needed, but on the basis of the data available, the error involved by neglecting the amount of volatile acids is 20% at worst). More evidence on this point is supplied by numerous acid analyses on a wide variety of guayule oils; the amounts of acid are consistently negligible.

Therefore, with a maximum errror that is probably well below 20%, we can justifiably call the evolved acidic material "Volatilized Carbon Dioxide".

TABLE IV. EVIDENCE THAT CARBON DIOXIDE IS THE ONLY ACIDIC (OR BASIC) MATERIAL DIRECTLY VOLATILIZED FROM GUAYULE LEAVES IN APPRECIABLE AMOUNTS (1).

A. Detailed Data.

Sample No.[4]	Arrangement of Receivers[2]	Dry Wt.(mg.)	Basic Material		Acidic Material			% Water
			Absorbed	Boiled Out	Absorbed	Boiled Out	Aver.	
17	Acid receiver first	21.9	0.03(2a)	0.01(2a)	0.28(2b)	0.36(2b)	0.32	75.6
18	" " "	21.9	0.01	0.09	0.40	0.42	0.41	77.6
19	Basic " "	28.3	0.01(3)	0.01	--	--	--	77.1
20	" " "	32.7	0.01	--	--	--	--	75.2

B. Summarized Data.

Sample No.	% NH_3	% Vol.Acids	% CO_2
17	0.01	-0.02	0.28,0.36
18	0.09	0.08	0.40,0.42
19	0.01,0.01	-	-
20	0.01	-	-

(1) All figures given in both parts of this table have been converted into terms of % oil (on a dry weight basis). This places all figures in the table on a comparable basis among themselves and the figures of other tables.

(2) The volatilized material was passed through two successive receivers, with the acid receiver first in two runs, the basic receiver first in the other two.

With the acid receiver first:

(a) The apparent basic material absorbed by the acid receiver is equal to the actual basic material absorbed (ammonia), minus the acidic material absorbed (volatile acids soluble in the dilute H Cl). (When made basic, the material boiled out is ammonia alone).

(b) The acidic material absorbed by the basic receiver must be carbon dioxide alone. (When made acid, the material boiled out can likewise be only carbon dioxide).
(The excess of the "boiled out" value over the "absorbed" value in the case of Sample 17 may have been due to contamination of the receiver by some carbonate carried over from a prior determination).

(3) With the basic receiver first, the basic material absorbed by the acid receiver can only be ammonia.

(4) All samples are from Harvest IV.

EXPERIMENTAL SECTION

EXPERIMENTAL SECTION

The full details of procedure will be given in this experimental section.

PRELIMINARY TREATMENT AND STORAGE PROCEDURES

The following procedures have been used for the preliminary treatment and storage of tissue samples:

Procedure I.(Recommended for Determination of Oil Contents of Different Tissues in an Intact Organ.

1. Number and weigh an empty sample tube (Fig.16B).
2. Harvest the sample and immediately transfer it to the sample tube.
3. Sterilize the sample promptly as follows:
   a. Insert the entire length of the sample tube into the close-fitting well of a heating block; temperature, 140 - 150°C; 20 seconds.
   b. Withdraw the sample tube and immediately immerse the greater portion of its length in ice water for 10 seconds. Remove and wipe dry.
4. Reweigh the sample tube.
5. Place the sample tube in a screw cap bottle (capacity of 30 or more tubes) and store either in:

a. Dry ice, or

b. Refrigerator

Procedure II. (Pulverizing; Recommended for Determination of Total Oil.

1a. (Replace Step 2, P.I). Wrap the sample in metal foil, freeze in dry ice, remove and pulverize thoroughly with a hammer; repeat one or two times.

1b. Thoroughly mix the pulverized mass and transfer it to a sample tube promptly.

Procedure III. (Slow Freezing).

4a. (Insert after Step 4, P.I). Place the sample tube in a small screw cap vial, which then immerse in an ice-salt-water bath of appropriate temperature (as -2° to -10°) and leave overnight in refrigerator.

Discussion and Notes.

(Note: Figures in parentheses refer to particular steps).

In Procedures I and III, the sample is initially stored in the same tube which will later be directly introduced into the volatilization apparatus. Thus there is no transfer and attendant losses at any time between harvest and volatilization - a marked advantage.

Sterilization is desirable if analyses for physiologically labile substances are to be carried out on the sample, subsequent to volatilization, a procedure that will become especially profitable in the future when the present volatilization method is converted to low temperature, low pressure operation. Otherwise this step may properly be omitted.

The step is similar to the process of blanching used in the food industry. Blanching, or immersion of the tissue in boiling water for a few moments, effectively inactivates the enzymes which would otherwise cause spoilage (chemical changes) upon storage; as attested, for example, by the increased stability of certain sensitive vitamins in tissues so treated. The brief blanching treatment of itself causes only negligible chemical change. The step is inserted here as a simple precaution, since there will often be delays between the time of harvest and the time the sample is frozen, or between the time the sample is removed from cold storage and the time volatilization is begun.

Data given in Table V show that the simple sterilization procedure, as described, effectively prevents mold growth which attacks untreated leaves repidly, especially when their cellular structure is first broken down by a prolonged series of dry ice freezings and thawings. The process presumably inactivates enzymes with similar efficiency.

(3b). The hot sample tube is promptly immersed in ice-water in order to condense all vaporized water (within the tube) on the walls of the tube and thereby avoid condensation on the cork; this condensed water is subsequently reabsorbed by the leaves. Data given in Table V show that loss of water from the sample tube due to "blanching" is negligible. Loss of material during periods of prolonged dry ice storage is also negligible, even after two months.

(1 ab). Until such time as a satisfactory means can be found for pulverizing the fresh sample in the same tube as used for volatilization, the pulverization method will suffer from a considerable disadvantage. Otherwise this method has particular merit in those cases where it is desired to obtain, with maximum speed, values representative of the total oil content.

(4a). Work in the frozen food industry has shown that, whereas rapid freezing (for example, with dry ice) causes the formation of many small ice crystals within the tissue, slow freezing permits the slow growth of ice crystals to relatively large size. These large crystals can be effective in breaking up tissue structure.

TABLE V. SOME EFFECTS OF STERILIZATION AND PROLONGED STORAGE.

Sample No.	Treatment	Storage	Fresh Wt. (mg.)	Change of Wt. After Sterilization (mg.)	After 51 d. Storage (mg.)	Mold Growth
21	none	23°	63.2	-	-22.8	Fair growth after 19 d.
22	"	"	80.9	-	-17.5	
23	"	dry ice 34 d. then 23°	-	-	-	Thick growth 6 d. after removal from dry ice
24	"	" " "	-	-	-	
25	Sterilized	23°	116.9	- 0.3	-22.7	
26	"	"	77.7	± 0.0	-22.2	
27	"	dry ice	147.0	- 0.2	- 0.2	No growth after four months
28	"	" "	74.0	- 0.2	+ 0.9	
29	none	" "	132.0	-	+ 0.3	
30	"	" "	90.3	-	+ 0.3	

THE VOLATILIZATION METHOD

Apparatus and Reagents

The apparatus, as shown in Fig.15, is a conventional train for the micro-determination of carbon. Placed immediately before the combustion zone is a small heating block, surrounding the volatilization zone in which the sample tube is placed. It is thereby possible to volatilize the water and oil from the sample at any suitable temperature, held constant within a range of two or three degrees; this volatilization step is the essence of the method. The oil is then combusted to carbon dioxide which is finally absorbed and determined by back titration in a receiver of standard barium hydroxide solution.

Appreciable amounts of volatilized carbon dioxide, evolved directly from the tissue, are quantitatively absorbed prior to combustion by a special absorption tube, attached directly to the sample tube (Fig.16A), andthrough which all material volatilized from the sample tube must pass. (The method for quantitatively determining the amount of volatilized carbon dioxide is described later).

Necessary Reagents are:

Standard 0.01 N $Ba(OH)_2$, 5% in $BaCl_2$ $2H_2O$.

Standard 0.05 N HCl.

Approximately 1 N $Ba(OH)_2$ (carbonate-free if the amount of volatilized carbon dioxide is to be determined subsequently).

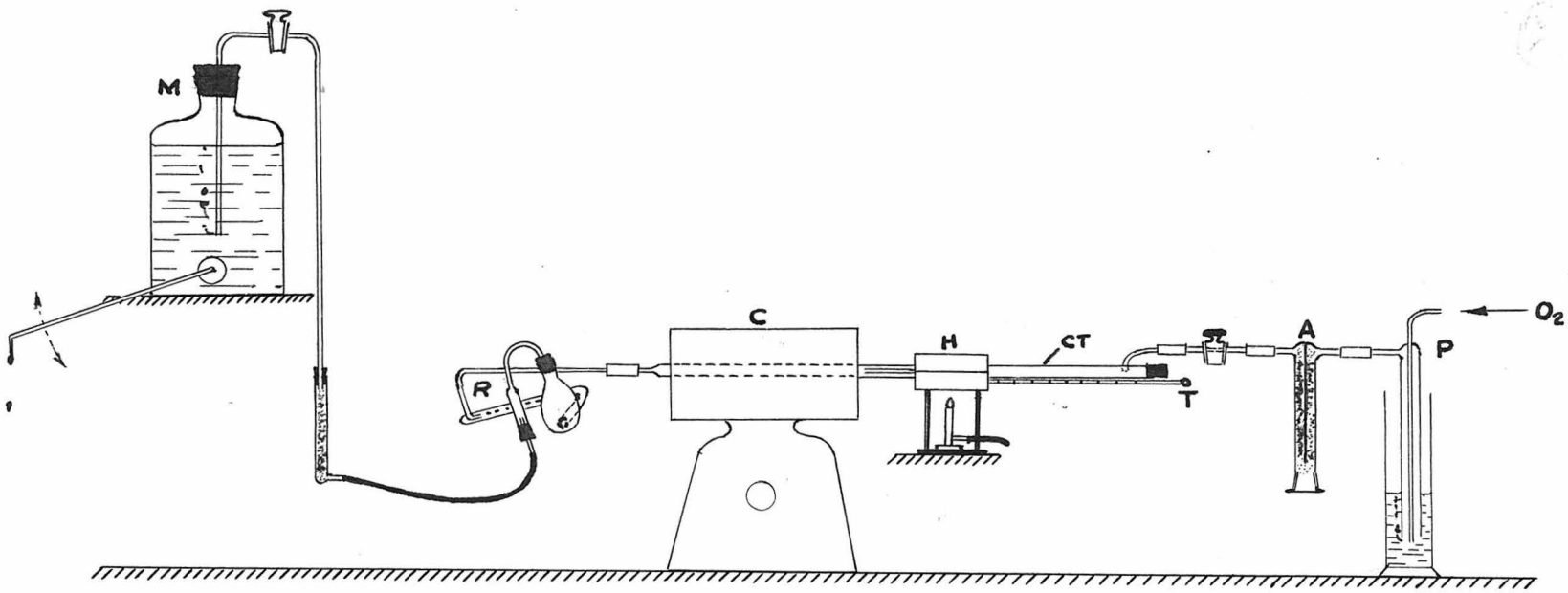


Fig.15. Complete Train as Used for the Micro-Determination of Essential Oil in Plant Tissues.

P. Pressure regulator.
A. Ascarite tube for purifying oxygen of carbon dioxide.
T. Thermometer.
CT. Combustion tube.
H. Heating block.
C. Combustion furnace.
R. Receiver for absorbing carbon dioxide.
M. Mariotte bottle.

(Approximate Scale, 1:8).

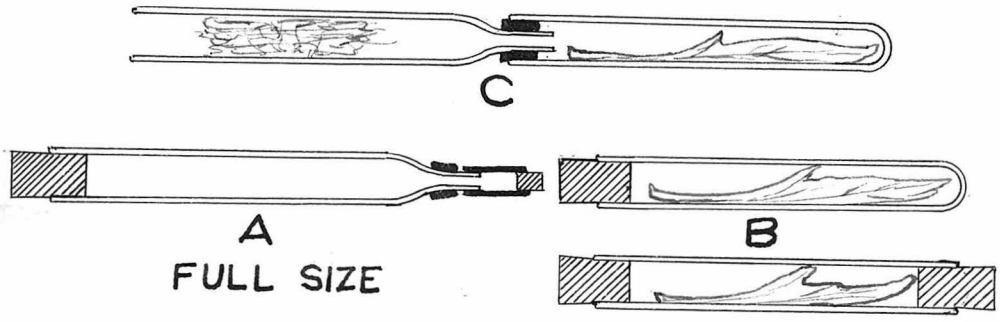


Fig. 16. Sample and Absorption Tubes.

A. Absorption Tube for absorbing volatilized carbon dioxide (evolved from tissue).

B. Sample tubes (closed end type preferred).

C. Prepared absorption tube attached to sample tube, assembly ready for volatilization.

General Procedure.

Instructions for the general procedure are as follows:

1. Adjust the combustion furnace to the proper temperature.

2. Adjust the heating block flame to give the proper volatilization temperature (usually 120° or 110°).

3. Aerate the entire train with a current of oxygen.

4. Run the proper known amount of standard barium hydroxide solution (8 to 10 cc.) into the receiver and attach it to the apparatus; attach the Mariotte bottle to the outlet tube of the receiver.

5. Open up the stopcock to the Mariotte bottle to draw a small current of oxygen through the receiver. Adjust the pressure regulator to give a pressure head of about 5 cm. of water. Adjust the arm of the Mariotte bottle to secure a proper rate of flow (3 to 5 cc. / min.).

6. Prepare the assembly shown in Fig.16C as follows: Rinse the absorption tube, shaking out excess water, push in a moistened wad of glass wool, to which then add about 3 drops of 1 N barium hydroxide solution. Finally attach this tube to the sample tube.

7. Push this assembly along the inside of the combustion tube until the sample tube is into position within the volatilization zone, thus beginning the volatilization.

8. Continue the volatilization for the proper period. Observe the following precautions:

a. All water, which may condense ahead of the combustion zone during the course of a run, must be finally driven into the combustion zone with the help of a burner.

b. It will also be found necessary periodically to heat the absorption tube to prevent the condensation of too much water therein. Heat the absorption tube to dryness by the end of the run.

c. The tubes following the combustion zone will also require occasional heating to clear them of condensing water.

9. Remove the assembly (a glass rod carrying a hooked wire which may pass alongside the small tubes and engage the lip of the open end is convenient), detach the sample tube, cork up, and weigh when cool.

10. Remove the receiver. Titrate the excess barium hydroxide with standard acid while bubbling through a slow current of carbon dioxide - free nitrogen.

11. Store the sample tube and its contents in a cool dark place to await possible additional analyses.

12. Determine the weight of oil by multiplying the volume of standard barium hydroxide by an appropriate factor, characteristic of the oil concerned.

In an efficient procedure for running a routine series of analyses, each carbon dioxide receiver, upon removal, should be immediately replaced by a fresh receiver; a new sample should be promptly introduced into the apparatus, and the next run begun. Ample time for titration and weighing is available while the volatilization of the next sample is under way.

Discussion and Notes on the General Procedure

This discussion will concern itself particularly with experimental details. A more comprehensive discussion, covering this procedure and its sub-procedures from the viewpoint of reproducibility is presented later.

(2). The temperature of the block, as read on the thermometer inserted into a well in the block, will be higher than the actual internal volatilization temperature by an amount readily determined from a calibration curve. (Such a curve is easily prepared by inserting a second thermometer into the volatilization zone and taking simultaneous readings of the two thermometers over the working range). The correction increases with temperature and depends on the block, it may amount to as much as 12°, for example, for an internal temperature of 120°. The correction is negligibly affected by the normal variation in oxygen rate of flow.

(5). Rate of flow is not a critical factor in the volatilization process and need not be closely controlled.

(6). The absorption tube and glass wool are necessarily moistened in order that the added barium hydroxide solution will readily distribute itself uniformly.

(8). It is believed that the procedure would be simplified if the absorption tube were also enclosed by a heating block kept at a temperature somewhat above 100°C.

(10). It is unnecessary to wash out the receiver after each titration; it is sufficient simply to pour out the titrated solution and immediately run in the next portion of standard base.

(12). For guayule oil of composition corresponding very nearly to C_5H_8: Volume of 0.05 N $Ba(OH)_2$(cc.) x 0.34 = weight of oil (mg.).

Sub-Procedure A: Total Oil Content and Oil Contents of Different Tissues of an Intact Organ.

1. Volatilize the sample at 120° or 110° until dried to brittleness . (Value for "loosely-bound" oil).

2. Grind the brittle sample directly in the sample tube, using a slender round-end glass rod as pestle.

3. Add to the ground sample several drops of water (as much or somewhat more than was in the fresh sample), and allow about 10 minutes for this water to be thoroughly absorbed.

4. Volatilize 5 minutes.

5. Again add water, let absorb 10 minutes, and volatilize 5 more minutes.

Repeat as needed or desired.

Discussion and Notes on Sub-Procedure A.

The law of diminishing returns obviously plays an important role in those volatilizations that have been discussed earlier (Fig. 3 to 5). For example, after only 10 minutes of treatment, there may be volatilized from a sample up to 90% or more of the oil that can be finally obtained during that volatilization period, regardless of how long it may be continued.(A volatilization period is defined as that period of volatilization during which no more water is added to the sample). Such an extended process involves an unnecessary waste of time and subjects the sample to an unnecessarily severe heat treatment. Therefore, one of the objects of the procedure just outlined is to continue each individual period only as long as it efficiently volatilizes oil. The second objective is to permit a rather elementary morphological fractionation; to obtain values for "loosely-bound" oil and total oil.

(1). The initial period must be continued until the tissue is dry and brittle enough to be readily ground. Practice has shown that this requires about 95% or more of the total tissue water to be removed. This requires about 15 minutes at 120°C for an average guayule leaf sample of 100 mg. fresh weight. As indicated in Fig.7, the various treatments affecting tissue structure have little effect on the rate of water removal (in contrast to theeffect on oil removal). The effect of temperature is illustrated in Fig.6.

It is particularly noteworthy that a temperature of 110°C is apparently as effective as 120°C, which suggests the use of 110°C in preference to 120°C in future work.

(2) The error due to loss of tissue particles clinging to the rod when it is removed from the sample tube has been demonstrated to be consistently negligible (by before and after weighings).

Fig. 17 compares the results of this procedure with the longer procedure used earlier. It is clear what a great savings in time is accomplished: Four short periods comprising only 27 minutes total time (and volatilizing 2.97% oil) are essentially equivalent to four long periods comprising 120 minutes total time (and volatilizing 2.63% oil).

It is noted that Sample 8, submitted to the more repid procedure has a greater oil content than the other sample; this is annoying for purposes of the present nature. It is interesting to note in passing, however, that Table II lists for Harvest II two samples with especially lower weights than all the rest (therefore, smaller leaves), Samples 5 and 8; and, furthermore, that they are the two samples which show the highest oil contents of the harvest. This observation fits in nicely with other facts to be presented on another occasion. The point in the present connection is that the "bad behavior" of Sample 8 is probably inherent in Sample 8, not a fault of the volatilization procedure !

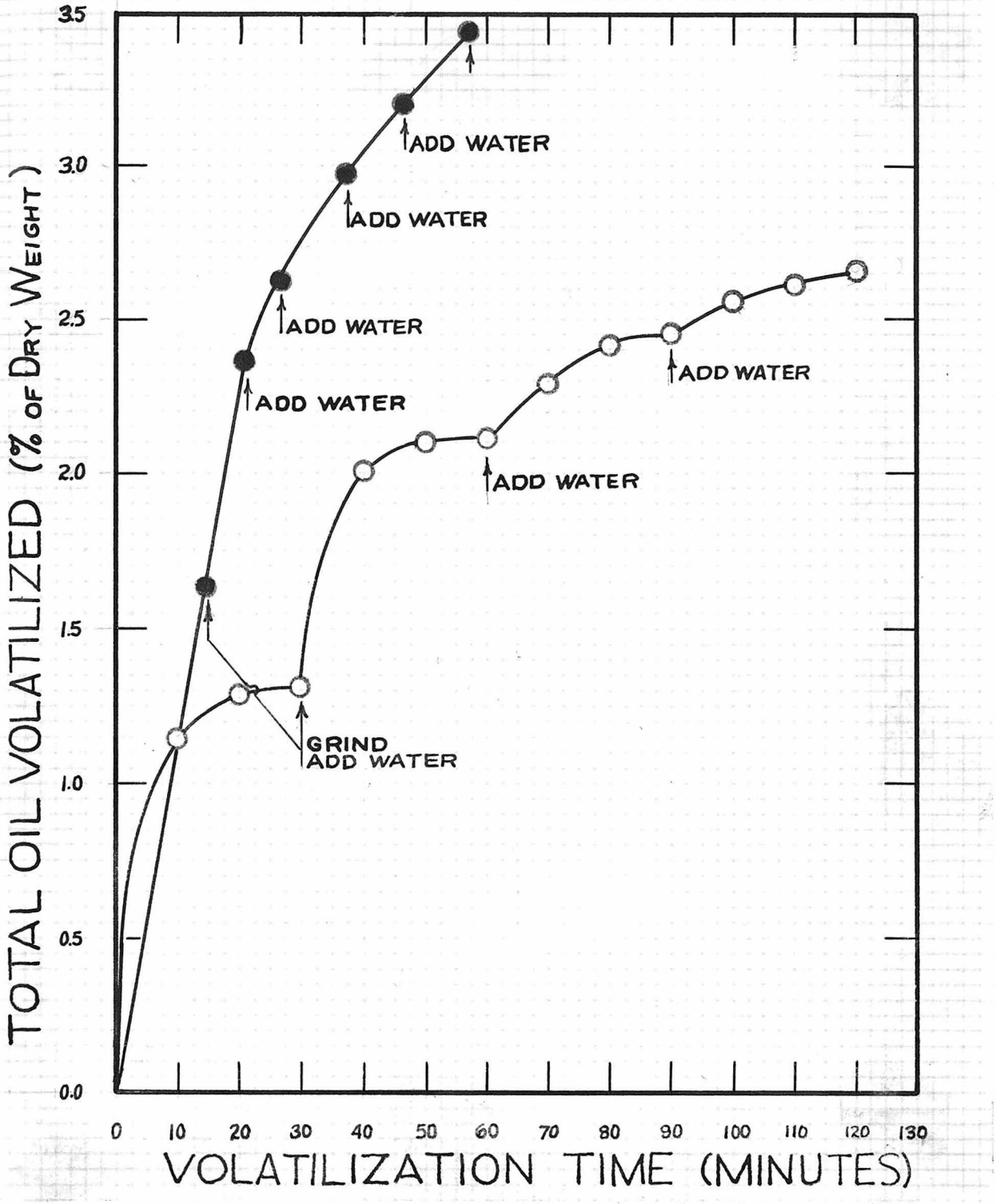


Fig. 17. Reduction of the Total Time Required for Exhaustive Volatilization.

○ Sample 2. Successive volatilization periods of 30 minutes each.

● Sample 8. Successive volatilization periods of 15, 6, 6, 10, 10, and 10 minutes.

Sub-Procedure B. The Rapid Determination of Total Oil Content.

Take the pulverized tissue mass which has been prepared according to the instructions of Procedure II, and simply volatilize it under standard conditions for a single suitable period: 5, 10, 15 or 20 minutes. In any case, as was clearly demonstrated on p.39, the value obtained will be a direct measure of the total oil content.

This method is valuable since it obviously gives a very rapid technique for comparing a series of more or less similar samples (as a series of leaves). If all determinations are carried out under standard conditions and for a specified time interval, the relative values obtained for the different samples will directly measure their relative total oil contents.

The principal disadvantage of the present procedure, referred to previously (p.59), is the increased handling and exposure of the samples, occasioning losses and some annoyance to the analyst. It is believed that in the future this disadvantage can be overcome by improved technique, specifically by using sample tubes made of a material that is flexible and tough enough at very low temperatures to permit freezing and pulverizing of the sample directly therein, and a material that at the same time is capable of withstanding temperatures above 100°C

without softening or deteriorating, and which is preferably chemically inert; and actually a tailor-made material would now seem to be in the offing - a silicone plastic ! (Collings, 1945).

Procedure for the Determination of Volatilized Carbon Dioxide.

As an integral part of the general procedure for the determination of essential oil, the volatilized carbon dioxide is directly absorbed, prior to combustion, by a special absorption tube. If the precaution has been taken of using carbonate-free barium hydroxide, it should seem possible to determine the volatilized carbon dioxide simply by acidification and aeration of the contents of the absorption tube after the run, absorbing the released carbon dioxide in standard barium hydroxide solution in the usual manner. Actually this is done in the apparatus shown in Fig.18. The procedure is as follows:

1. Attach a barium hydroxide receiver to the outlet tube of the apparatus.

2. Add 1 to 2 cc. of 6 N hydrocloric acid to the U-tube (U).

3. Insert the absorption tube (A) as shown.

4. Pass a gentle current of carbon-dioxide free nitrogen through the apparatus for 5 or 10 minutes. (The acid will be forced up into the absorption tube as a liquid column, alternately rising, collapsing in the expanded tube (E), and percolating back into the absorption tube).

5. Turn off the nitrogen flow, disconnect and titrate the excess standard base; ready the absorption tube for a fresh charge.

The completeness of the aeration may be judged by the data of Table VI (and also that of Table VII). The procedure is quite satisfactory for the present purposes.

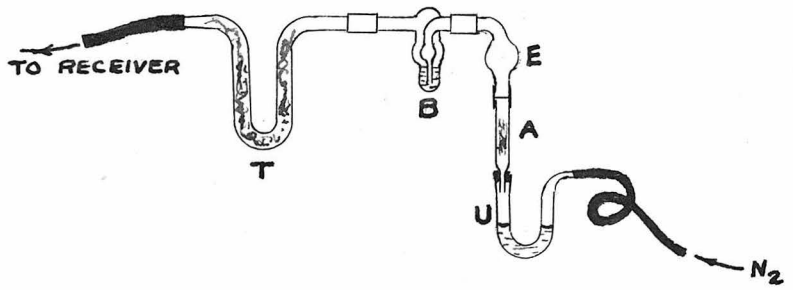


Fig. 18. Apparatus for Aeration of Carbon Dioxide from the Absorption Tube.

A. Absorption Tube.

B. Bubbler tube (to collect any acid thrown over from E).

E. Expanded tube (to assure collapse of rising liquid column).

T. Trap (containing glass wool (to catch any acid spray).

U. U-tube containing 6 N H_2SO_4 initially.

(Approx. Scale, 1:5)

Table VI. Evidence on the Reliability of the Aeration Procedure in the Determination of Volatilized Carbon Dioxide.

Sample No.	Amount of CO_2 Aerated (ml. N/20 $Ba(OH)_2$)		% Aerated during First 5 min.
	During First 5 Min.	During Second 5 Min.	
31	0.14	0.02	88%
32	0.32	0.02	94%
33	0.24	0.03	89%
34	0.52	0.11	83%

General Discussion of the New Method.

The reproducibility or precision attainable by the new method has been rather clearly evidenced by the studies previously presented in graph form. Despite initial differences in apparent oil contents, reflecting different initial treatments that modify cell structure, the curves in general show a strong tendency to converge toward a common ultimate value. In fact, as shown in TableVII, after a process of relatively exhaustive volatilization, the precision seems to be well within 15%.

In addition, analyses have been made on samples that are completely comparable from the outset, these samples being aliquots of a common well-mixed, and minced leaf mass. The results, give in TableVIII, show a standard deviation of 5% among 5 samples, a maximum difference or range of 10%.

Other evidences of reproducibility are given by the curves for volatilized carbon dioxide.

TABLE VII. REPRODUCIBILITY (PRECISION)(1)

Volatilized Material (% Dry Wt.).

Fig.	No.of(2) Vol.Per.	No. of Samples Taken	Min. Value	Max. Value	Max. Difference (Range)	% Difference	Initial Difference(1 in Tissues or Conditions
A. Essential Oil.							
3	4	3	2.41	2.67	0.26	10	Tissue Structure
5	5	4	2.67	2.96 +0.20	0.49	15	Vol. Temp.
4	4	3	2.58	2.75	0.17	6	Tissue Structure
17	4	2	2.63	2.97	0.34	11	Length of Individual Vol.Periods
B. Volatilized Carbon Dioxide.							
13	3	2	1.11	1.14	0.03	3	Vol. Temp.
14	3	3	1.11	1.14	0.03	3	No difference
	1	2	0.65	0.66	0.01	1	One sample "blanched"

(1) It is well to note that the various sets of results given in this table included samples for which there existed initial differences in some factor, affecting initial rate of volatilization, it being evident, however, that the initial rate effect had been overcome after several volatilization periods. It is also well to note that these samples consisted of separate leaves, subject to some normal variability.

(2) A volatilization period is defined as that period of volatilization during which nomore water is added to the sample.

Table VIII. Reproducibility (Precision).

Sample No.	Treatment	Storage	% water (% fresh wt.)	% oil (% dry wt.)
35	Untreated	25° 2 hrs.	71.1	1.00
36	"	3 hrs.	70.0	0.96
37	"	Dry ice 1 d.	70.6	0.90
38	"Blanched"	none	71.2	0.91
39	"	Dry ice 1 d.	71.0	1.01
Mean ± standard deviation			70.8 ± 0.5	0.96 ± 0.05
% Difference between extreme values			1.7%	10%

Description Top leaves were minced into very small pieces with a razor blade, the mass mixed, then divided into similar samples. Volatilization temperature: 120°; volatilization time, 20 minutes.

It will be noted that the previous results were obtained before there was introduced the technique of simultaneously determining oil and volatilized carbon dioxide, by the use of a special absorption tube. It is, therefore, now necessary to present some specific evidence as to the reproducitility of the final procedure. The results of this work are presented in Table IX. It is clear that the determination of oil is quite precise (10% difference between samples). The precision of the volatilized carbon dioxide determinations is considerably less on a percentage basis (maximum difference of 33%), but this is attributable to the smaller amounts of this material. The results are very pleasing.

Finally let us give some clean-cut answers to certain questions of significance. These answers are found in Table X.

1. Does the rubber tubing which is used to connect the sample and absorption tubes evolve any appreciable amount of carbon dioxide on heating? No, not even when the rubber is heated to slight tackiness, is measurable carbon dioxide obtained.

2. Does the carbon dioxide retained by the absorption tube filling evolve again when the tube is heated? No, even when the tube has absorbed a full quota of carbon dioxide, and is then heated so hot that the glass wobl

therein turns somewhat brown, the carbon dioxide evolved is negligible.

3. Does the essential oil pass quantitatively through the absorption tube? Yes, the values obtained for "% oil & % CO_2" are essentially the same whether the absorption tube is used or not.

TABLE IX. EVIDENCE SUPPORTING THE REPRODUCIBILITY OF THE SIMULTANEOUS DETERMINATIONS OF ESSENTIAL OIL AND VOLATILIZED CARBON DIOXIDE.

Samp.No.	Conditions(1) Abs.Tube	Comb.	Dry Wt. (mg.)	% Oil (% Dry Wt.)	% CO_2 (% Dry Wt)	% Oil + % CO_2 (% Dry Wt.)	% Water (% Fresh Wt.)
40	+	+	28.3	0.84	0.27	1.11	72.1
41	+	+	30.9	0.93	0.30	1.22	72.2
42	-	+	25.5	-	-	1.19	71.7
43	+	-	30.0	-	0.33	-	73.5
44	+	-	29.0	-	0.40	-	71.8
45	-	-	35.1	-	0.36	-	73.1
46	-	-	18.8	-	0.40	-	75.7
47	-	-	28.5	-	0.30	-	73.6
Mean ± standard deviation----------				0.89 ±0.06	0.34 ±0.05	1.17 ±0.06	73.0 ±1.3
% Difference between extreme values				10%	33%	9%	6%

(1) The plus and minus signs indicate whether or not the absorption tube was used, or whether or not combustion was employed. Thus all combinations were tried, some in duplicate.

All samples were of Harvest VI ; aliquots of a common pulverized leaf mass. Volatilization temperature: 120°; volatilization time: 20 minutes.

Table X. Evidence Supporting the Reliability of the Simultaneous Determinations of Essential Oil and Volatilized Carbon Dioxide.

Run	Volatilization Material & Conditions	Vol. Time (min.)	Volume of N/20 Base Used (ml.)
1	8 mm. section of No. 2 black rubber tubing (material used to connect sample and absorption tubes); heat same.	10 +15	0.00 0.00
2	a. Guayule leaf (1) volatilized; absorption tube (containing glass wool saturated with barium hydroxide solution) attached to sample tube	10	0.00
	b. Heat absorption tube to dryness	+10	0.00
	c. Heat absorption tube more strongly	+10	0.00
	d. Heat absorption tube so strongly that the glass wool browns and the rubber connection becomes slightly tacky	+15	<0.01
3	a. Same as 2; heat absorption tube strongly.	10	0.02
	b. Continue heat on absorption tube	+15	0.00
	c. Add water to the dried leaf and repeat	+10	0.00

(1) This guayule leaf would ordinarily have given off enough volatilized carbon dioxide to consume about 1.00 ml. of the standard base.

Several remaining potential sources of error in the new method require but brief mention. These are the following:

1. Leakage of carbon dioxide into the train.
2. Completeness of carbon dioxide absorption.
3. The accuracy of the carbon dioxide determination by titration.

Adequate determinations have been made to ascertain the errors possibly involved in the above cases, leading to the conclusion that they are entirely negligible.

SUMMARY

1. Procedures have been developed which permit the rapid and precise micro-determination of the essential oil content of plant organs.

2. Procedures have been developed which permit the semi-quantitative determination of the essential oil content in different tissues of an intact organ.

3. It has been demonstrated that the major process in oil volatilization from plant tissues is diffusion. Especially sharp oil separations are possible when a dense-walled structure separates different tissues.

4. It is possible to determine for a 20 mg. (dry weight) guayule leaf in an average time of about 30 minutes (in routine analyses) the following information:

a. Fresh weight.
b. Dry weight
c. A value for "loosely-bound" oil (precision of 15% or better).
d. A value for total oil (precision of 15% or better).
e. The amount of volatilized carbon dioxide (evolved directly from the tissue by heat).

5. The procedure finally yields a dry sample suitable for further analyses.

6. It is possible to determine a value for total oil content alone in 10 or 15 minutes from the time volatilization is begun.

7. The potential value of the new methods is suggested to growers of essential oil materials and to food technologists, as well as to those who are interested in the problems connected with essential oil metabolism.

8. Recommendations for further improvements of technique have been given.

9. It is recommended that research be actively carried out on methodsfor the quantitative microchemical analysis of specific essential oil components.

II. ESSENTIAL OIL RELATIONS DURING THE GROWTH OF THE GUAYULE LEAF

II. ESSENTIAL OIL RELATIONS DURING THE GROWTH OF THE GUAYULE LEAF

The course of essential oil formation during the growth of the guayule leaf has been studied. It has been shown possible to segregate this process into three rather distinct phases, coincident with three phases in the morphological development of the leaf. These results have been confirmed for a series of leaves on each of four different guayule plants, each plant growing, for a period of four months, under an individual temperature combination. Combinations employed were: 27°C (80°F) day and night; 27°C day and 16°C (60°F) night; 27°C day and 7°C (45°F) night; and 18°C (65°F) day and 13°C (55°F) night.

Procedure.

The plant material used was taken from the lot of guayule plants described in Table I (p. 31). These plants were sown from seed on February 12, 1945. On December 15, 1945, small groups of about 10 plants each were carefully transplanted, the roots being disturbed as little as possible, into 4-inch pots of sand. Thereafter each pot of plants grew under its own temperature combination in the controlled-temperature greenhouse of the California Institute (Went, 1944). They were watered daily with nutrient solution. (A very complete record of the growth of these plants was kept over a period of more than three months; these data shall be presented on another occasion).

Over the three day period, April 9, 1946 to April 11, 1946, a representative series of leaves, beginning with young leaves less than 2 cm. long, were harvested from a typical plant of each group. Values for "loosely-bound", mesophyllic oil content (see page 39) were determined for each leaf by means of the new micro-method that has been described in full detail in the previous section.

Results.

The principal results of these investigations are concisely presented in Fig. 19 . The full line curves show the values for mesophyllic oil content for leaves of increasing maturity (dry weight). The dotted line curves give the corresponding average elongation rates of the respective leaves over a period of 12 to 15 days prior to harvest.

During the period immediately prior to rapid elongation, there is an extra large production of essential oil (first phase). This large initial oil production is apparent in the case of every temperature combination. These values are real since even the smallest initial oil value of 0.04 mg. corresponds to 0.12 cc. of standard 0.05 N base in the oil determination procedure, whereas blanks are consistently less than 0.02 cc. (p. 85).

During the subsequent elongation period, however, oil formation is suppressed (second phase). Then as elongation subsides, the rate of oil formation gradually increases again (third phase). It is noted that this oil increase of

of the third phase occurs earlier in those cases where the elongation subsides earlier (27°C day); and occurs later or more gradually when the elongation subsides later (18° day). These observations suggest a connection between the elongation processes and the suppression of oil production.

It is well to note that the growth rate curves given are for a period prior to harvest of about 15 days. The curves for the two or three day prior period, which may be more pertinent in the present connection, would have their peaks shifted somewhat to the left.

These results agree with the findings of Frey-Wyssling and Blank (1940) with Salvia seedlings. They also found that a seedling accumulates the maximum amount of oil just prior to the maximum elongation period (Fig. 19A); thereafter the oil content actually decreases (presumably by loss to the atmosphere). Whereas in the guayule leaf, essential oil formation is only suppressed during elongation, it apparently ceases altogether in the Salvia seedling).

Another observation is that thermoperiodicy apparently causes no marked differences in mesophyllic oil contents among the various samples. This contrasts with the case of rubber in guayule; Bonner (1943) has shown that rubber accumulates rapidly in guayule stems and roots under a temperature combination of 27°C day and 7°C night, while failing to accumulate at all, for example, at constant 27°C temperature. However, this is not surprising in view

of the fact that mesophyllic oil is apparently not a storage oil: it gradually disappears from older leaves in which oil production apparently diminishes or ceases, the oil originally contained in the mesophyll therein being lost to the atmosphere (or possibly solubilized as glucosides or the like and transported to more interior portions of the plant for storage).

Perhaps the process of essential oil formation in the leaf may be simply explained as follows: There are in the leaf at least three semi-independent biochemical systems affecting oil production: The primary system is perhaps tied in with the laying down of dry weight material and is in operation throughout the course of leaf development. The second system is concerned with active cell division and is in intensive operation for only a relatively short period prior to cell elongation. The third system is concerned with cell elongation and somehow draws upon or depletes the oil-producing systems. That we may be dealing with several different biochemical sources of essential oil in a single tissue is in agreement with the view that different sets of metabolic reactions give rise to different sets of essential oils (p. 3).

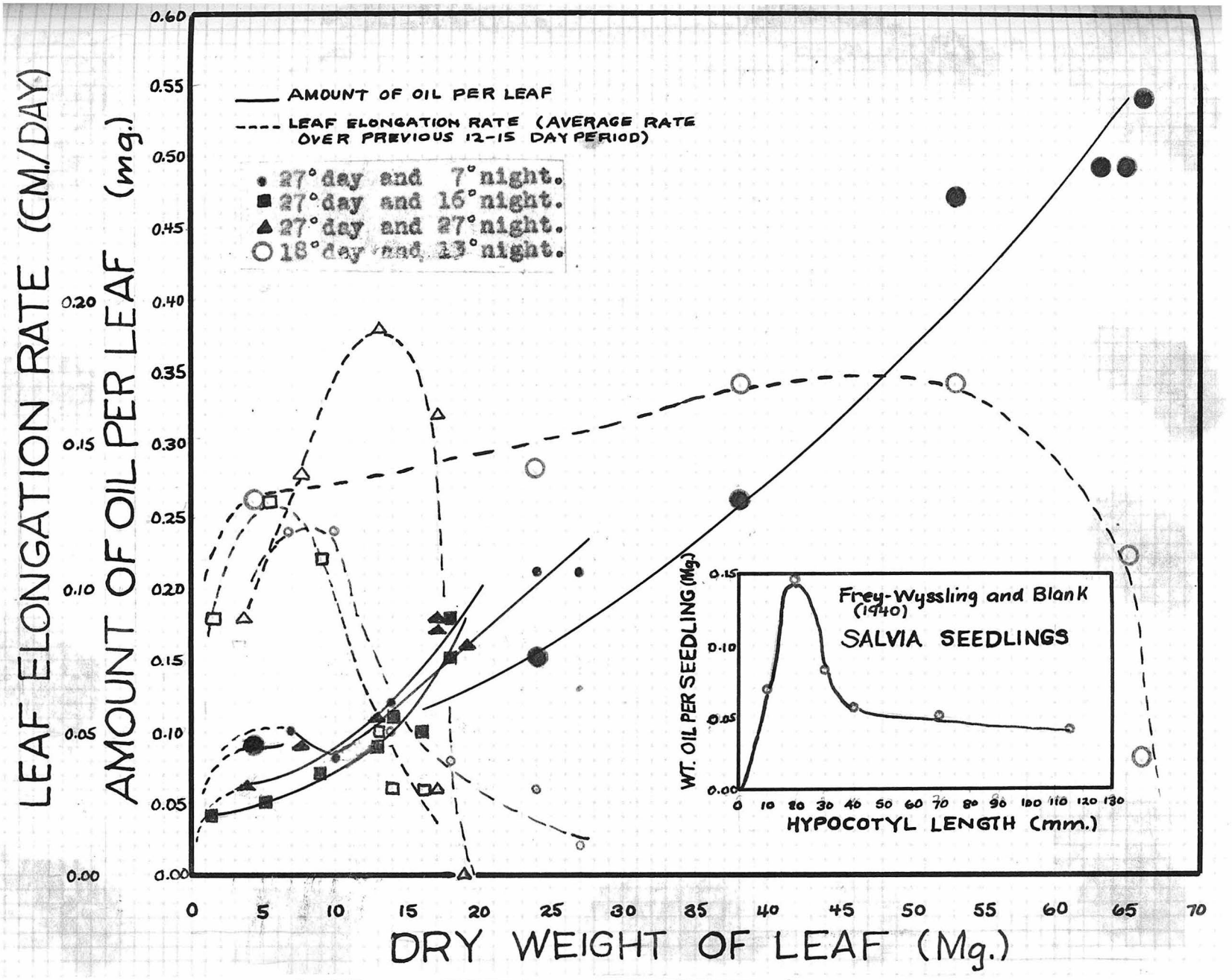


Fig. 19. Three Phase Essential Oil Production in the Guayule Leaf.

III. LARGE SCALE INVESTIGATIONS ON THE VARIATION OF GUAYULE OIL COMPOSITION WITH THE AGE AND ORGAN OF THE PLANT

CONTENTS

III. LARGE SCALE INVESTIGATIONS ON THE VARIATION OF GUAYULE OIL COMPOSITION WITH THE AGE AND ORGAN OF THE PLANT

The following investigations were initiated in order to determine the chemical compositions of guayule essential oils from different organs of the plant, and also to determine how these compositions change with the age of the plant. The present investigations have concerned the oils of leaves, stems, and roots (and some sub-fractions thereof) from plants of approximate ages of one, two, three and four years.

These investigations have been carried out on a relatively large scale. Somewhat over 1500 kg. (or about 3000 lbs.) by fresh weight of plant material have been sectioned and steam distilled, the oils being collected, dried, and stored for subsequent analyses. Over 70 individual steam distillations in 50 gallon stills were involved in this phase of the work.

The physical constants (density, refractive index, dispersion, and optical rotation) as well as ester contents have been measured for all oils. The primary method of attack has been the technique of fractional distillation. Fractional distillation permitsthe separation of the individual constituents of an oil to a greater or less degree, thus providing a picture of oil compositions, and also yields fractions that may be the subjects of further study.

After the plant material and the treatments to which it has been subjected (sectioning and steam distillation) have

been described, the work on the various resulting oils shall be taken up in two phases: First, the work on the unfractionated oils; and second, the fractionations and the study of the resulting fractions.

A substantial improvement in procedure has been the construction and application of a precision fractionating column, which has been of value, not only in the present work, but which has contributed significantly to our chemical knowledge of guayule essential oil (p.158).

TREATMENT OF THE PLANT MATERIAL

The history of the plant material, which was furnished through the courtesy of Dr.Hamilton Traub of the United States Department of Agriculture, is given in Table XI. When received at the California Institute, the material was promptly sectioned and steam distilled.

Sectioning Procedure

In view of the great quantity of material, a very careful separation of the plants into their parts was impractical. Sectioning was accomplished simply by use of a cleaver, each plant being chopped into the three portions, leaves, stems, and roots, in the manner shown in Fig. 20.

Fig. 20. Typical Manner of Sectioning.

It is to be noted that the portion called "leaves" actually includes a proportion of twigs and small stems. On the other hand, the stem portions were picked essentially free of the relatively few leaves they possessed initially. Typical examples of the material dealt with here are pictured in Fig. 21 to 25.

About one-half of the stems and roots were ground (more correctly, chopped) in a small Wiley mill before distillation. The typical nature of the ground material is shown in Fig. 26.

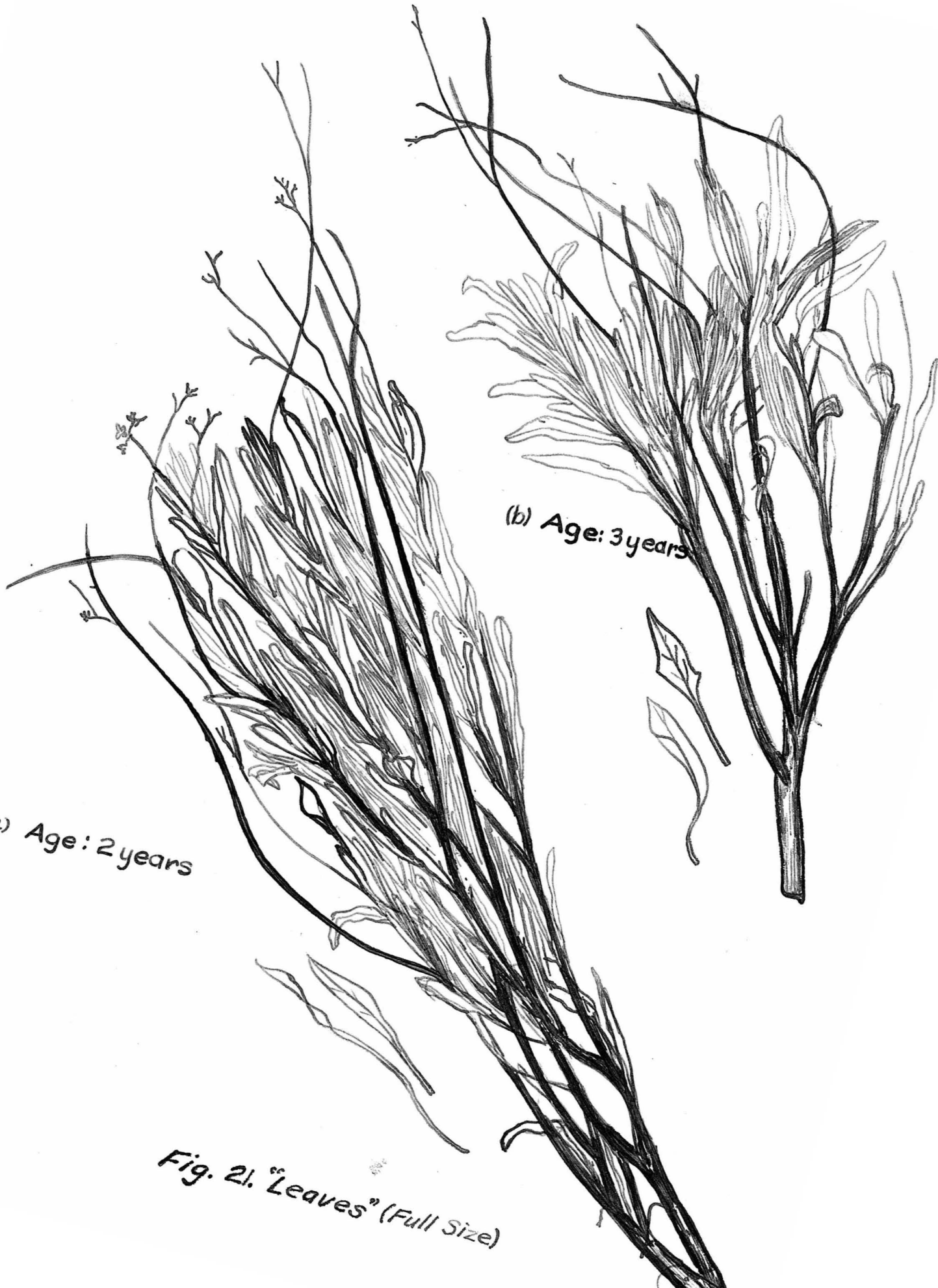


Fig. 21. "Leaves" (Full Size)

Fig. 22. "Stem", Age 3 years (Full Size)

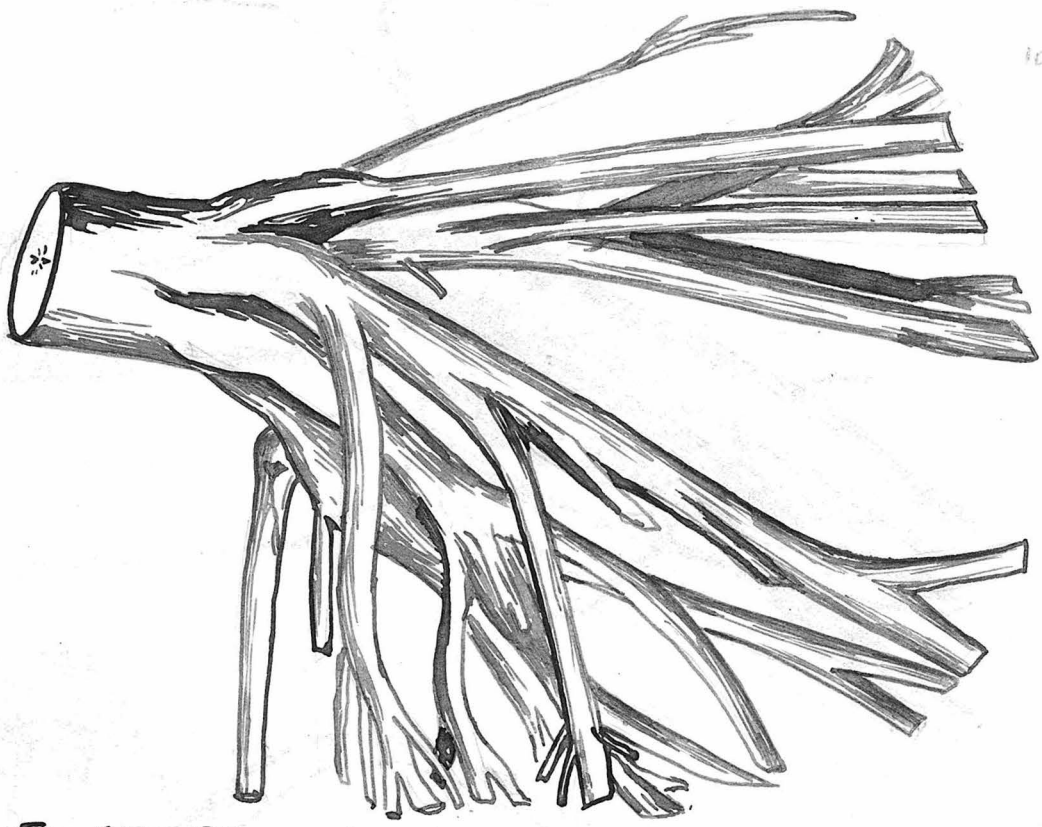


Fig. 23. Stem, Age 2 years (Full Size)

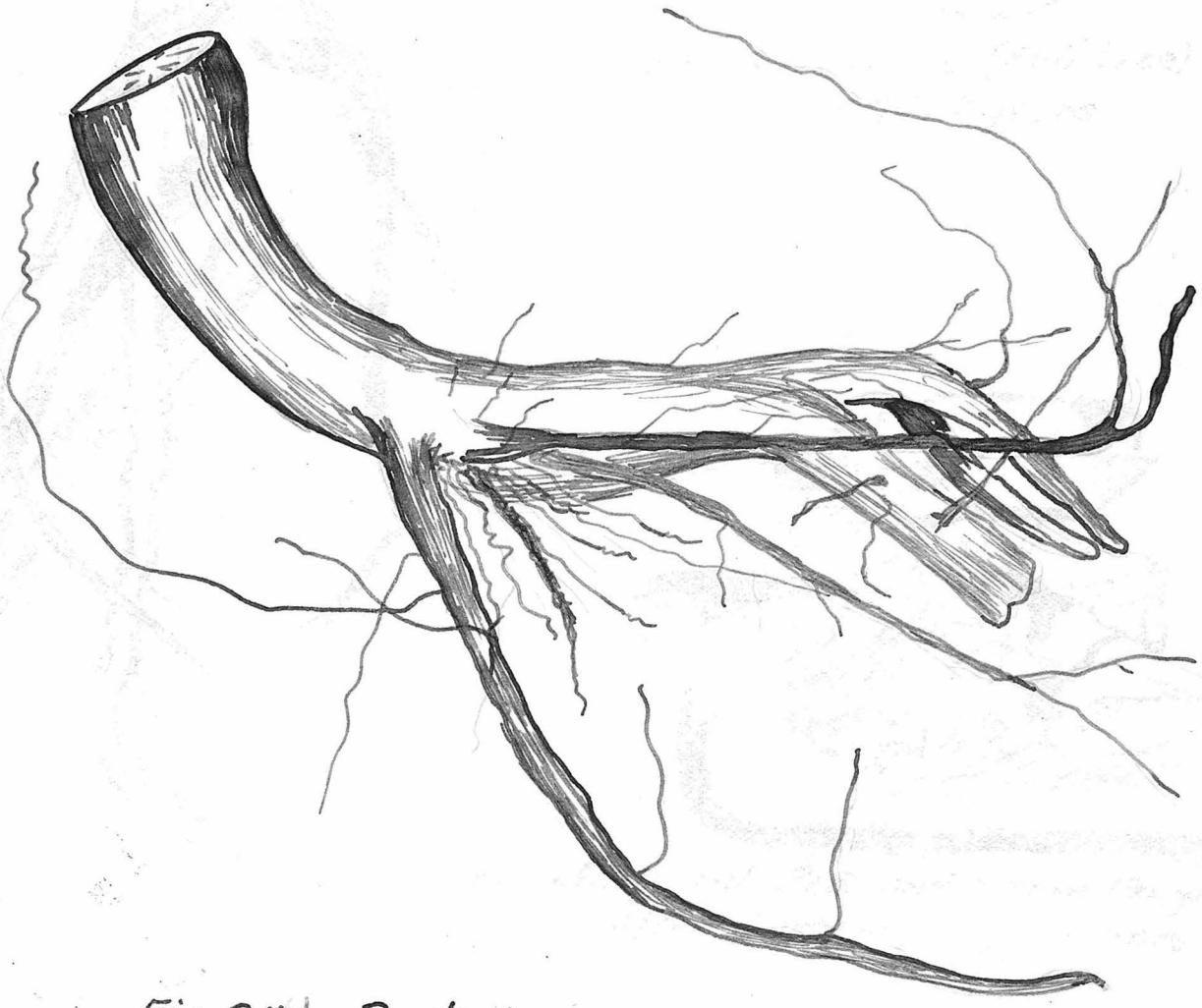


Fig. 24. Root, Age 3 years (Full Size)

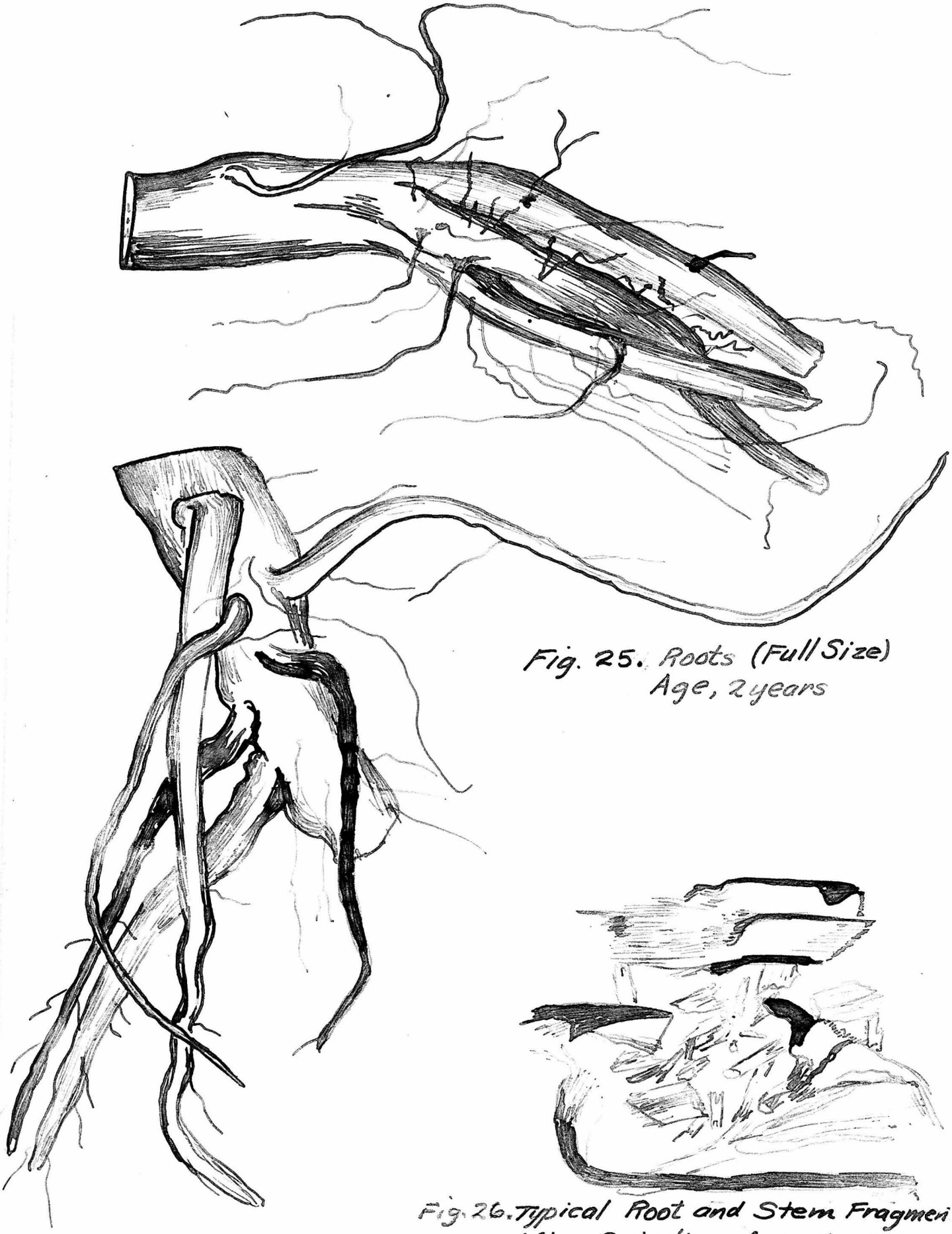


Fig. 25. Roots (Full Size)
Age, 2 years

Fig. 26. Typical Root and Stem Fragment
After Grinding. Age, 4 years

Table XI. History of the Plant Material (1).

Guayule, Parthenium Argentatum Gray

Plants	Variety	Field	Date Planted	Date Harvested(3)	Date Baled	Date Distilled	Age (Mo.)	Period from Harv. to Dist. (days)
I M(2)	593	Hanson & Lee Block 7	3/9/44	11/1/44	11/9/44	11/24-25/44	8	23-24
I	593	Spence	3/9/44	12/18/44	-	12/26-27/44	9	8-9
II	406F	Spence	11/-/42	10/7/44	10/13/44	10/21-25/44	23	14-15
III	593	Bardin	3-4/-/42	10/9/44	10/12/44	10/19-25/44	30	10-16
IV	593	Spence Block I	4/20/41	11/1/44	11/9/44	11/22-30/44	42	21-29

(1). All plants are from non-irrigated plots at Salinas, California.

(2). These plants were subjected to rain during transit and arrived at the Institute warm, and overgrown with mold.

(3). All harvests were within the period, October to December, which is during the period of dormancy for guayule.

Steam Distillation Procedure.

Some of the important aspects of steam distillation have been discussed in another portion of this thesis, where typical steam distillation results, obtained at this time, were presented. Fig. 1 and 2 give typical distillation curves for leaves and stems.

Apparatus. Each 50 gallon still is provided with a wire mesh false bottom in order to keep the plant material from direct contact with the hot outside bottom of the still which is heated directly by air blast burners. Each still is so supported that it can be readily tipped for convenient removal of spent plant material. The flanged still head engages the top of the still with a tight friction fit which, however, is readily disengaged when desired. The still head connects with a large water-cooled metal condenser. The condensed material is received in a special flask which separates out and collects the oil in a graduated neck, permitting a continuous record of the amount of oil distilled; meanwhile the distillation water passes directly back into the still (cohobation).

Procedure. Initially several gallons of water are added to each still and, while the water comes to a boil, plant material is weighed into the still by small box loads, the still head swung into position, and soon the distillation

may begin. Additional steam may also be supplied from outside steam jets connecting with inlet tubes passing into and almost reaching the bottom of the still. Between each run it is necessary merely to turn down the flame, remove the still head, tip the still, rake out the spent contents, and prepare for the next run. Since the cohobation process, which recirculates the distillation water, is used, additional water need be added only infrequently.

Time Analysis.

The following rough estimates of the times required for the various operations can be given:

Sectioning: 60 kg.(120 lbs.) of plant per hour per person.

Grinding: 20 kg. (40 lbs.) of plant per hour per mill. (Small Wiley mill used).

Steam Distillation: 25 kg. (50 lbs.) of plant per hour per still. This figure assumes use of the still at maximum capacity, about 50 kg.(100 lbs.), and with a two hour distillation period.

Fresh Weight Distribution; Oil Yields.

The original data collected in the present work have been:

(1) The fresh weights of the three fractions; leaves, stems, and roots.

(2). The fresh weights of single plants.

(3). The amounts of oil obtained from each fraction.

These and derived data are collected in Tables XII to XV.

Table XII .

Fresh Weight Distribution of Leaves, Stems, and Roots For All Plants Massed.

Portion	Total Fresh Weight (kg.)				
	1 yr. Moldy	1 yr.	2 yr.	3 yr.	4 yr.
Leaves	125	70	248	398	103
Stems	27	22	70	276	106
Roots	23	16	28	95	53
Total	175	108	346	769	262
Grand Total					1660

Portion	Percent of Fresh Weight				
	1 yr. Moldy	1 yr.	2 yr.	3 yr.	4 yr.
Leaves	71	65	72	52	39
Stems	15	20	20	36	40
Roots	14	15	8	12	21
Total	100	100	100	100	100

Table XIII .

Fresh Weight Distribution of Leaves, Stems, and Roots For Individual Plants.

Portion	Fresh Weight per Plant (gr.)				
	1 yr. Moldy	1 yr.	2 yr.	3 yr.	4 yr.
Leaves (1)	43	39	108	130	390
Stems (1)	9	12	30	90	400
Roots (1)	8	9	12	30	210
Total (2)	60	60	150	250	1000
Approximate No. of plants used (3)	2920	1800	2310	3076	262

(1). These values were derived by taking the proper percentage (as found in Table) of the weight of a corresponding total plant.

(2). In lieu of a count of the total number of plants used (which would have been preferable, although many plants were mutilated and incomplete), a few plants deemed typical by rough observation were weighed and the above figures derived as the best possible values; allow a rough deviation of 30%.

(3). No. of plants = $\frac{\text{Total fresh weight}}{\text{Fresh weight per plant}}$.

TABLE XIV.

ESSENTIAL OIL OBTAINED FROM ALL PLANTS.

Portion	Weight of Oil (gr.)				
	1 yr.Moldy	1 yr.	2 yr.	3 yr.	4 yr.
Leaves	173	236	291	895	226
Stems	46	33	53	247	174
Roots	30	34	26	110	127
Total	249	303	370	1252	527
Grand Total					2701

Percent Yield of Oil (% of Fresh Wt.).

Portion	1 yr.Moldy	1 yr.	2 yr.	3 yr.	4 yr.
Leaves	0.14	0.34	0.12	0.23	0.22
Stems	0.17	0.15	0.08	0.09	0.16
Roots	0.13	0.21	0.09	0.12	0.24
Average	0.14	0.28	0.11	0.16	0.20

TABLE XV.

Essential Oil Per Plant (Derived Data).

Portions	Weight of oil per plant (gr.)				
	1 Yr. Moldy	1 Yr.	2 Yr.	3 Yr.	4 Yr.
Leaves	0.04	0.13	0.13	0.29	0.86
Stems	0.01	0.02	0.02	0.08	0.66
Roots	0.01	0.02	0.01	0.04	0.49
Total	0.06	0.17	0.16	0.41	2.01

(1) The values for this table were derived from values found in previous tables.

Physical Constants and Quantitative Chemical Analyses of the Unfractionated Oils

The physical constants, including densities, refractive indices, dispersions, and optical rotations, and ester contents have been measured for 27 unfractionated oils. The system of designating these oils is explained in Table XVI.

Results.

Table XVII presents the complete data. To facilitate the comparison of these data and aid the possible detection of correlations, the data are repeated in different manners in Tables XIX and XX. Table XIX-A lists the oils in decreasing order for each type of measurement. Table XIX-B repeats Table XIX-A, but includes only those oils of primary interest. Table XX lists the measurements separately for each of the groups: leaves, stems, roots; one, two, three, and four year old plants.

No marked correlations are apparent in the cases of dispersions, refractive indices and densities. In the case of optical rotations, some tendency is noted for the rotations to become less negative going from one to four year old plants. There is a decided tendency for the rotations to become more negative going from leaves to stems and roots. All root oils (with one exception) exhibit far stronger negative rotations than any other oils.

Correlations are evident with the ester contents. Ester content decreases steadily going from one to two to three to four year old plants in all cases. Moreover, ester content also decreases (with one exception) going from leaves to stems to roots.

Table XVIII shows the effect of mold. The oil from moldy one year plants (plants which arrived warm and overgrown with mold) is compared with oil from fresher one year plants. In all cases the mold apparently caused decreased ester content.

Some favorable information on the reliability of the steam distillation procedure is given by the comparison of oils III L, III Lb and III Lr (III Lb was obtained from a particular run of three year leaves, during which the still was accidentally allowed to go dry, allowing charring of the bottom portions of the charge. The oil is very dark and has a strong burnt odor. III Lr is some of oil III L that was poured back into the still and re-steam distilled). The measurements for these oils are quite similar (Table XIX), suggesting that a treatment more severe than normal steam distillation has little effect on the oil compositions.

Further favorable evidence is given in regard to the reproducibility of the steam distillation procedure, that is, the similarity of oils from different runs on the same plant material. Oils IIIS and IIIS' ~~31-39~~, which are duplicates, show quite similar measurements.

Procedures.

The densities were determined with a one cc. pycnometer. The refractive indices and dispersions (drum readings or "Z" values) were measured on a Zeiss Abbe refractometer. The ester contents were determined by hydrolyzing approximately one gram samples in standard alcoholic sodium hydroxide under reflux for about one hour, then back titrating the excess base. The satisfactory precision obtained in the ester determinations is shown by the duplicate values reported in Table ~~XVII~~ .

Conclusions.

1. Ester content decreases steadily with increased age of plant; decreases steadily going from leaves to stems to roots.

2. Root oils have far stronger negative optical rotations than any other oils.

3. Mold growth on guayule plant sharply decreases the ester content of the oil obtained therefrom.

4. Some favorable evidence is given that the steam distillation procedure gives fairly reproducible results and causes only small changes in the oil composition.

TABLE XVI.

The System of Designating Oils

I, II, III, IV ----------Approx.(1) age of plants (years).

L ----------Leaf oil.

T ----------Twig oil (2).

S ----------Stem oil.

R ----------Root oil.

u (3)----------"Loosely-bound" oil (bark oil).

p (3)----------"Tightly-bound" oil (wood oil).

M ----------Oil from moldy plants (I only).

Example:

IRMp ----------Wood root oil of moldy one year plants.

(1) For the exact ages, see Table XI .

(2) The "twigs" consisted chiefly of very small stems and twigs plus some other material (IV only).

(3) The letter "u" designates oil from unground material. In accordance with nomenclature introduced elsewhere (p. 18), this oil may, therefore, be called "loosely-bound" oil. The letter "p" designates oil obtained after grinding and redistilling material that had already been predistilled unground, and may be called "tightly-bound" oil. The letter "g" designates oil from material

ground at the outset, or total oil, u + p. Since after a distillation, the bark of stems and roots is apparently well-steamed and can be readily stripped from the wood, it is probably that the "loosely-bound" oil represents chiefly bark oil and the "tightly-bound" oil represents chiefly wood oil. (See Fig. 1).

Table XVII. Physical Constants and Ester Contents of Oils from Different Ages and Portions of Plants.

Oil	d_{25}^{25}	n_D^{25}	$[\alpha]_D^{25}$	Z^{25}	Per Cent Ester(1) Ave. of 2 Detns.	
IL	0.880	1.4809	- 0.0	19.9	4.9	(4.5, 5.3)
IL3a	0.885	1.4840	-0.40	20.8	4.9	(3.9, 4.1)
IL3b	0.905	1.4922	-0.77	20.5	2.9	(2.5, 3.2)
ILM	0.894	1.4869	-7.62	19.8	2.3	(2.3, 2.3)
ISu	0.883	1.4805	-5.72	20.0	3.9	(3.7, 4.1)
ISp	0.906	1.4840	-7.06	20.0	5.9	(5.4, 6.4)
ISMg	0.879	1.4792	-7.65	20.2	2.9	(2.7, 3.0)
IRg	0.885	1.4827	-21.30	19.8	2.6	(2.5. 2.7)
IRMu	0.900	1.4893	-21.05	19.5	1.6	(1.4, 1.7)
IRMp	0.983	1.4913	-14.18	19.7	2.4	(2.3, 2.4)
IIL	0.891	1.4868	-4.71	20.0	4.5	(4.2, 4.5. 4.7)
IILf	0.874	1.4808	-0.14	20.2	3.9	(3.8, 3.9)
IIS	0.890	1.4822	-5.00	20.2	3.4	(3.2. 3.6)
IIR	0.883	1.4840	-10.15	20.0	2.4	(2.3, 2.4)
IIIL	0.873	1.4800	-3.04	20.3	4.5	(4.5. 4.5)
IIILb	0.879	1.4790	-2.45	20.0	4.1	(4.1, 4.1)
IIILr	0.872	1.4805	-1.34	20.2	4.1	(3.9, 4.3)
IIIS	0.874	1.4742	-5.61	20.2	2.7	(2.5, 2.9)
IIIS'	0.876	1.4782	-5.25	19.9	3.0	(2.7, 3.2)
IIIR	0.884	1.4821	-10.50	20.0	2.2	(1.9, 2.0, 2.2,2.5
IVL	0.881	1.4829	-4.67	21.0	3.7	(3.6, 3.8)
IVT	0.887	1.4829	-9.46	19.9	2.8	(2.7, 2.8)
IVSg	0.872	1.4795	-3.07	19.5	2,1	(2.0, 2.2)
IVSu	0.897	1.4840	-5.64	20.0	4.1	(3.9, 4.2)
IVSp	0.877	1,4827	-3.04	20.0	2.2	(2.0, 2.3)
IVRu	0.880	1.4805	-9.00	19.9	1.3	(1.3, 1.3)
IVRp	0.883	1.4830	-4.59	20.0	2.0	(1.8, 2.2)
(2)	0.865	1.4744	-2.70		3.5	

(1). Ester content is calculated on the basis of bornyl acetate (molecular weight= 198).

(2). Total 2 Year Oil of Haagen-Smit and Siu (1944).

TABLE XVIII. EFFECT OF MOLD ON ESTER CONTENT.

	OIL		PER CENT ESTER			
			S			
Non-Moldy	IL	4.9	ISp ISu	5.9 3.9	IRg	2.6
Moldy	ILM	2.3	ISMg	2.9	IRMp IRMu	2.4 1.6

TABLE XIX. PHYSICAL CONSTANTS AND ESTER CONTENTS OF OILS LISTED IN DECREASING ORDER FOR EACH TYPE OF MEASUREMENT.

% Ester		Z^{25}		n_D^{25}		$[\alpha]_D^{25}$		d_{25}^{25}	
ISp	5.9	IVL	21.0	IL3b	1.4922	IRg	-21.30	ISp	0.906
IL	4.9	IL3a	20.8	IRMp	1.4913	IRMu	-21.05	IL3b	0.905
IIL	4.5	IL3b	20.5	IRMu	1.4893	IRMp	-14.18	IRMp	0.903
IIIL	4.5	IIIL	20.3	ILM	1.4869	IIIR	-10.50	IRMu	0.900
IIILb	4.1	ISMg	20.2	IIL	1.4868	IIR	-10.15	IVSu	0.897
IIILr	4.1	IILf	20.2	IL3a	1.4840	IVT	- 9.46	ILM	0.894
IVSu	4.1	IIS	20.2	ISp	1.4840	IVRu	- 9.00	IIL	0.891
IL3a	4.0	IIILr	20.2	IIR	1.4840	ISMg	- 7.65	IIS	0.890
ISu	3.9	IIIS	20.2	IVSu	1.4840	ILM	- 7.62	IVT	0.887
IILf	3.9	ISu	20.0	IVRp	1.4830	ISp	- 7.06	IL3a	0.885
IVL	3.7	ISp	20.0	IVL	1.4829	ISu	- 5.72	IRg	0.885
IIS	3.4	IIL	20.0	IVT	1.4829	IVSu	- 5.64	IIIR	0.884
IIIS'	3.0	IIR	20.0	IRg	1.4827	IIIS	- 5.61	IIR	0.883
IL3b	2.9	IIILb	20.0	IVSp	1.4827	IIIS	- 5.25	ISu	0.883
ISMg	2.9	IIIR	20.0	IIS	1.4822	IIS	- 5.00	IVRp	0.883
IVT	2.8	IVSu	20.0	IIIR	1.4821	IIL	- 4.71	IVL	0.881
IIIS	2.7	IVSp	20.0	IL	1.4809	IVL	- 4.67	IL	0.880
IRg	2.6	IVRp	20.0	IILf	1.4808	IVRp	- 4.59	IVRu	0.880
IRMp	2.4	IL	19.9	ISu	1.4805	IVSg	- 3.07	ISMg	0.879
IIR	2.4	IIIS'	19.9	IIILr	1.4805	IIIL	- 3.04	IIILb	0.879
ILM	2.3	IVT	19.9	IVRu	1.4805	IVSp	- 3.04	IVSp	0.877
IIIR	2.2	IVRu	19.9	IIIL	1.4800	IIILb	- 2.45	IIIS	0.876
IVSp	2.2	ILM	19.8	IVSg	1.4795	IIILr	- 1.34	IILf	0.874
IVSg	2.1	IRg	19.8	ISMg	1.4792	IL3b	- 0.77	IIIS	0.874
IVRp	2.0	IRMp	19.7	IIILb	1.4790	IL3a	- 0.40	IIIL	0.873
IRMu	1.6	IRMu	19.5	IIIS	1.4782	IILf	- 0.14	IIILr	0.872
IVRu	1.3	IVSg	19.5	IIIS	1.4742	IL	- 0.0	IVSg	0.872

B

B

ISP	5.9	IVL	21.0	IIL	1.4868	IRg	-21.30	ISP	0.906
IL	4.9	IIIL	20.3	ISp	1.4840	IIIR	-10.50	IIL	0.891
IIL	4.5	IIS	20.2	IIR	1.4840	IIR	-10.15	IIS	0.890
~~IVRu~~	~~1.3~~	IVSg	19.5	IVRp	1.4830				
IIIL	4.5	IIIS	20.2	IVL	1.4829	IVT	- 9.46	IVT	0.887
ISU	3.9	ISu	20.0	IVT	1.4829	IVRu	- 9.00	IRg	0.885
IVL	3.7	ISp	20.0	IRg	1.4827	ISp	- 7.06	IIIR	0.884
IIS	3.4	IIL	20.0	IIS	1.4822	ISu	- 5.72	IIR	0.883
IVT	2.8	IIR	20.0	IIIR	1.4821	IIIS	- 5.61	ISu	0.883
IIIS	2.7	IIIR	20.0	IL	1.4809	IIS	- 5.00	IVRp	0.883
IRg	2.6	IVRp	20.0	ISu	1.4805	IIL	- 4.71	IVL	0.881
IIR	2.4	IL	19.9	IVRu	1.4805	IVL	- 4.67	IL	0.880
IIIR	2.2	IVT	19.9	IIIL	1.4800	IVRp	- 4.59	IVRu	0.880
IVSg	2.1	IVRu	19.9	IVSg	1.4795	IVSg	- 3.07	IIIS	0.874
IVRp	2.0	IRg	19.8	IIIS	1.4742	IIIL	- 3.04	IIIL	0.873
IVRu	1.3					IL	- 0.0	IVSg	0.872

TABLE XX. PHYSICAL CONSTANTS AND ESTER CONTENTS OF OILS LISTING THE MEASUREMENTS SEPARATELY FOR EACH OF THE GROUP: LEAVES, STEMS, ROOTS; I,II,III,& IV YEAR PLANTS.

PERCENT ESTER

L		S		R		I		II		III		IV	
IL	4.9	ISp	5.9	IRg	2.6	IL	4.9	IIL	4.5	IIIL	4.5	IVL	3.7
IIL	4.5	ISu	3.9	IIR	2.4	ISp	5.9	IIS	3.4	IIIS	2.7	IVT	2.8
IIIL	4.5	IIS	3.4	IIIR	2.2	ISu	3.9	IIR	2.4	IIIR	2.2	IVSg	2.2
IVL	3.7	IIIS	2.7	IVRp	2.0	IRa	2.6					IVRp	2.0
		IVS	2.1	IVRu	1.3							IVRu	1.3

DISPERSION (DRUM READINGS)

L		S		R		I		II		III		IV	
IL	19.9	ISp	20.0	IRg	19.8	IL	19.9	IIL	20.0	IIIL	20.3	IVL	21.0
IIL	20.0	ISu	20.0	IIR	20.0	ISp	20.0	IIS	20.2	IIIS	20.2	IVT	19.9
IIIL	20.3	IIS	20.2	IIIR	20.8	ISu	19.9	IIR	20.0	IIIR	20.0	IVSg	19.5
IVL	21.0	IIIS	20.2	IVRp	20.0	IRg	19.8					IVRp	20.0
		IVS	19.5	IVRu	19.9							IVRu	19.9

REFRACTIVE INDICES

L		S		R		I		II		III		IV	
IL	1.4809	ISp	1.4840	IRg	1.4827	IL	1.4809	IIL	1.4868	IIIL	1.4800	IVL	1.4829
IIL	1.4868	ISu	1.4805	IIR	1.4840	ISp	1.4840	IIS	1.4822	IIIS	1.4742	IVT	1.4829
IIIL	1.4800	IIS	1.4822	IIIR	1.4821	ISu	1.4805	IIR	1.4840	IIIR	1.4821	IVSg	1.4795
IVL	1.4829	IIIS	1.4742	IVRp	1.4830	IRg	1.4827					IVRp	1.4830
		IVS	1.4795	IVRu	1.4805							IVRu	1.4805

SPECIFIC ROTATIONS

L		S		R		I		II		III		IV	
IL	-0.0	ISp	-7.06	IRg	-21.30	IL	-0.0	IIL	-4.71	IIIL	-3.04	IVL	-4.67
IIL	-4.71	ISu	-5.72	IIR	-10.15	ISp	-7.06	IIS	-5.00	IIIS	-5.61	IVT	-9.46
IIIL	-3.04	IIS	-5.00	IIIR	-10.50	ISu	-5.72	IIR	-10.15	IIIR	-10.50	IVSg	-3.07
IVL	-4.67	IIIS	-5.61	IVRp	- 4.59	IRg	-21.30					IVRp	-4.59
		IVS	-3.07	IVRu	- 9.00							IVRu	-9.00

DENSITIES

L		S		R		I		II		III		IV	
IL	0.880	ISp	0.906	IRg	0.885	IL	0.880	IIL	0.891	IIIL	0.873	IVL	0.881
IIL	0.891	ISu	0.883	IIR	0.883	ISp	0.906	IIS	0.890	IIIS	0.874	IVT	0.887
IIIL	0.873	IIS	0.890	IIIR	0.884	ISu	0.883	IIR	0.883	IIIR	0.884	IVSg	0.872
IVL	0.881	IIIS	0.874	IVR	0.883	IRg	0.885					IVRp	0.883
		IVS	0.872	IVRu	0.880							IVRu	0.880

FRACTIONATION AND INVESTIGATIONS OF THE FRACTIONS

In line with the general purpose of determining the quantitative chemical compositions of each oil, the followingoils have been fractionally distilled: one, two, three, and four year leaf oils; one year moldy leaf oil; and three year stem oil. The refractive indices and dispersions of all the resulting fractions have been measured. The optical rotations of the fractions of three year leaf oil have been measured. In addition, ester determinations have been carried out on those fractions comprising rich ester regions.

For each oil the data have been plotted on a graph (Fig. 28 to 33). The data for the previous distillation of Haagen-Smit and Siu (1944) have been replotted on a similar graph (Fig. 27) to facilitate comparisons.

The Problem of Interpreting the Distillation Data. The fractional distillation of a mixture yields a series of fractions, boiling and distilling over at a successively higher temperatures. It is then possible to obtain a quantitative distribution curve by plotting, as ordinate, the weight of each fraction, or a more convenient equivalent as ~~to~~ the weight per 1000 grams per degree Centigrade, against, as abscissa, the temperature range over which the fraction distills.

With a perfect fractionation instrument, each fraction would be a pure compound (or a constant-boiling mixture of quantitative composition fixed by the components, or (which is very unlikely) a mixture of several compounds of identical boiling points), the entirety of which would distill over at its one characteristic boiling point. The corresponding distribution curve would consist of a series of completely separated vertical lines located at the respective boiling points of the various constituents. Such an ideal situation of complete resolution of components would obviously present relatively little difficulty of interpretation from the standpoint of quantitative analysis.

Actually with a finite apparatus, the degree of separation cannot be this sharp. The compound of one fraction will be contaminated by compounds from adjacent fractions to a greater or less degree; briefly, there will be more or less overlapping.

The actual extent of overlapping will be conditioned by many factors; On one hand, an increased efficiency or resolving power of the apparatus will minimize overlapping; on the other hand, many factors, but especially the closeness of boiling points of adjacent compounds, will increase overlapping. The corresponding distribution curve will consist of a series of peaks of varying heights, widths, and shapes; some peaks will be well separated and distinct, some less distinct, others almost merged. It is such curves as these that are presented by the present distillations of guayule essential oil. Let these latter distribution curves now be considered more closely.

The original work of Haagen-Smit and Sun (1944) on two year total oil gave a distribution curve (Fig. 27) composed of four well-defined groups, identified respectively as terpenes, oxygenated terpenes, sesquiterpenes, and sesquiterpene alcohols. The four groups contained ten peaks, in each of which the presence of a different compound was established, with the probability that this compound was the major component of that peak. This initially suggested the possibility that any guayule essential oil would yield, upon fractionation, a distribution curve of similar qualitative character: four major groups, ten definite peaks. In such a case, the method of interpreting the curve would be fairly simple and direct; the very nature of the distribution curve would serve as a guide

for dividing up the fractions into ten divisions, representing the amounts of the ten major compounds present. An additional guide, if necessary, would be the temperature of distillation.

One year moldy, two, three, and four year leaf oils, and three year stem oil were distilled with the apparatus used in the original work, a helices fractionation column with the total condensation, variable take-off still head of Whitmore and Lux (1932). (However, on account of the time factor as well as to minimize losses, each oil was fractionated only once, and not twice as in the original work). The distribution curves obtained, however, are not in accordance with the simple situation that has been anticipated above. Indeed, the curves are all quite erratic and show little apparent correlation with one another, either as to general form or as to distillation temperature. There is no separation into four major groups, much less into ten definite peaks. In short, any immediate interpretation of the curves in terms of quantitative analysis is quite impossible.

Much better results were obtained when there was finally brought into use an improved apparatus, featuring a silvered vacuum-jacketed meter long fractionating column, six mm. inside diameter, with a nichrome spiral as packing (p. 153). One year leaf oil was fractionated in duplicate with this apparatus, giving the distribution curves drawn on Fig. 28 .

Of particular significance are the very good results obtained in fractionating the terpenes, the forms of the distribution curves alone permitting immediate quantitative division of the terpenes into four groups. However, there again is no evidence of four major groups or a specific number of peaks for the whole distillation range.

It was such results as those which have just been recounted, which suggested that the increased fractionation power available in the new apparatus be applied to about 100 cc. of one of the higher boiling fractions previously obtained in the original investigation; and, indeed, the results were such as to warrant the extension of the refractionation procedure to a number of additional such fractions --- the overall result, a much fuller, more comprehensive picture of the chemical composition of guayule essential oil, and clear evidence that guayule oil is really very complex. This complexity can account for much of the difficulty experienced in the fractional distillation of the various oils.

Despite the difficulties which have been pointed out, it has been possible to obtain some fairly reasonable results from the distillation data. The method which was chosen to accomplish this will now be considered.

The Method of Interpreting the Distillation Data.

First this question, in regard to the limitations of the method, should be considered; Shall it be possible to determine

the amount of an individual compound, or shall it only be possible to determine the aggregate amount of several compounds massed together in a group (?). An examination of the present graphs indicates that it should be reasonably possible to determine the amounts for the following divisions, but no subdivision, without, at least, supplementary chemical analyses, is possible.

Division No.	Probable Chief Constituents
I	α - pinene
II	β - pinene, unknown terpene
III	limonene, "easily oxidizable terpene"
IV	- -
V	carbonyl compounds, ester
VI	sesquiterpenes
VII	sesquiterpene alcohols
VIII (residue)	(resins)
IX (trap)	- -

This summarizes the limitations of the present method. It is rather fortunate and important that each of the various divisions is probably comprised chiefly of compounds of similar chemical character; and, therefore, a knowledge of the amount of material within each division has real chemical and biological significance !

The actual method of dividing up the fractions into the aforementioned divisions may now be outlined as follows:

1. The original distribution curve of Haagen-Smit and Sui (1944) has been chosen as the working pattern. It has been divided up into the appropriate divisions on the basis of its characteristic shape in conjunction with the knowledge of where the various compounds are located. Vertical lines at the base of the graph have been drawn (as in all subsequent graphs) to mark off the divisions.

2. The refractive index ranges for the various divisions have then been taken and recorded in Table XXI.

3. These refractive index values have been chosen as the primary criteria by which all the other curves have been divided. The exact places of division have been modified in those cases where the forms of the distribution curves indicated a slight shift. Also two secondary criteria for certain dividing lines are mentioned in notes to Table XXI. Refractive index values have been chosen as criteria because they are readily and accurately obtained.

4. Several checks on the correct placing of any division line may be had by reference to the region in which each of the characteristic physical constant and ester curves is intersected by this line.

Note: The curves for the terpene ranges of IL and IL'; obtained with the improved apparatus, can be divided directly on the basis of their very characteristic and reproducible form alone.

TABLE XXI. Refractive Index Ranges for the Various Divisions.

Division No.	Refractive Index Range
I	1.466 - - 1.470
II	1.470 - - 1.475
III	1.475 - - 1.480
IV	1.480 - - 1.479[1]
V	1.479[1] - 1.496
VI	1.496 - - 1.502[2]
VII	1.502[2] - - -
VIII	(residue)
IX	(trap)

(1). A more reliable criterion for the division between groups IV and V is the ester value; the ester content rises sharply going into Division V.

(2). A more reliable criterion for the division between groups VI and VII is a point about one-half way up the side of the first high peak of the refractive index curve.

Results.

The consideration of the results will be in two phases: First, the forms of the physical constant and ester curves will be examined. Second, the actual quantitative chemical compositions of the oils, according to the divisions, will be tabulated and discussed.

The Forms of the Physical Constant and Ester Curves. Two purposes can be achieved by the examination of the forms of the physical constant and ester curves: First, any unusual curve, presumably suggesting an unusual oil, can be singled out for special attention. Second, the reliability of the method of division, as already stated, can be judged by noting if all the curves are intersected by the division lines in the proper regions.

The following points may be stated in regard to the interpretation of the physical constant curves:

In the case of a perfect fractionation, the form of a curve would depend solely upon the identity of the compounds present and would be entirely independent of amounts of these compounds.

In the case of an actual fractionation, however, the form of a curve will depend on both the identity and the amounts of the compounds present and will be further modified by such factors as where the cuts between fractions happen to be made.

For example, let us say that in the case of one oil we obtain a curve with a certain peak, while in the curve for another oil, this peak is absent. This difference may be explained in either of several ways:

1. The compound responsible for the peak may be missing from the second oil.

2. The amount of a neighboring compound in the second oil may be so large that an amount of it overlaps and distills with the compound responsible for the peak, an amount sufficiently large to completely mask the peak.

3. The arbitrary cut between fractions in the second case may have been so made as to divide and distribute the responsible compound to its neighbors on either side, thus eliminating the peak.

It should also be mentioned that the temperature measurements are relatively inaccurate, so that displacements of portions of the curves from right to left have little if any significance. To compare corresponding regions of curves, use the division lines as the proper primary guides.

It would seem to follow from these past considerations, that the primary value of the physical constant curves, from the viewpoint of analysis, lies in their ability to detect the broader, more fundamental changes in oil composition.

Consider now the curves of Fig. 27 to 33.

All the refractive index curves have the same general form, suggesting that these oils are roughly similar in composition. The curves for III S and ILM may show enough modifications to suggest somewhat different compositions. The regions where the curves are intersected by the dividing lines (marked ↓ ↑) are essentially the same in all the curves, thus checking, for these oils, the method of division used. However, in the cases of ILM and, especially, III S, the changes in forms of the curves are sufficient to arouse some doubts, for example, as

to the correct placing of the dividing lines that bound Division VI in the III S curve.

All the dispersion curves have the same general form. The intersection regions check quite well again, although there is again some doubt in the cases of III S and ILM. With III S, a number of peaks are missing or merged (?); with ILM, the latter portion of the curve is strangely depressed. Minor variations in form are noted for IL and IL^1 (in which cases the two peaks of Division VII are seemingly missing).

The optical rotations have been determined for the fractions of only one of the oils, III L. It is interesting that in this case the distribution of optical activity is precisely the same as was observed in the original work of Haagen-Smit and Siu on two year total plant oil: excesses of l-α-pinene, d-limonene, l-phellandral, l-sesquiterpenes, and d-sesquiterpene alcohols. It does seem desirable to determine the optical rotations for the other oils also.

All the ester content curves exhibit the same form, a high peak located in Division V in all cases (a further favorable check on the division method). In addition, some curves show a tiny peak immediately after the major peak and located in Division VI. Where investigated, smaller percentages of esters have been found in other regions also. The major peaks of ILM and IIIS are noticeably lower than those of the other oils.

In the foregoing discussion, it has been shown quite clearly, among other things, that the method of division is quite satisfactory for oils IL, IIL, and IIIL, perhaps somewhat less satisfactory for ILM and IIIS. These evaluations may be held in mind while considering the resulting data which will now be presented.

The Quantitative Chemical Compositions of the Oils.

Table XXII gives the results for one year leaves for which the improved apparatus was used. These results are better than those for any other oil and illustrate the reproducibility possible for the terpene range when using this apparatus. Table XXIII collects the quantitative chemical compositions of all the oils. Finally, Table XXIV is a presentation, unfortunately imcomplete, of the quantitative distributions of esters in the various oils.

After the data were tabulated, an effort was made to evaluate the reliability of each figure, reference being made to the original data and to the various graphs, and consideration being given to the points of the previous discussion. An asterick (*) indicates that a figure is reliable. A question mark (?) indicates that a figure is doubtful. In the case of esters, parentheses indicate that the figure is a minimum value only, saponification values for all the concerned fractions not being available.

TABLE XXII .

Quantitative Chemical Composition of One Year Leaf Oil Expressed as Weight Percentages of Total Oil Illustrating Reproducibility of Results with Improved Apparatus.

Division	Important Components	Wt.Percentage		Aprox.Wt.Percentag		
		IL	IL[1]	IL	IL[1]	Ave.
I	a-pinene	20.2	19.7	20	20	20
II Σ		27.0	23.2	27	23	25
IIa	β-pinene	15.3	14.0	15	14	15
IIb	(unknown terpene)	11.7	9.2	12	9	11
III	limonene	15.4	15.6	15	16	16
ΣI,II,III	terpenes	62.6	58.5	63	59	61
IV	- -	1.3	1.0	1	1	1
V	carbonyls,ester	5.8	7.6	6	8	7
VI	sesquiterpenes	8.6	8.0	9	8	9
VII	sesquit.-alcohols	14.1	12.1	14	12	13
VIII(residue)	(resins)	5.5	-	6	-	
IX (trap)	-	1.3	0.5	1	1	1
Σ		99.2				

TABLE XXIII.

Quantitative Chemical Compositions of Oils Expressed as Weight Percentages of Total Oil.

Division	IL	II L	III L	IV L	I LM	III S
I	20%	52(?)	23%	20%		
II	25%		33%	36%		
III	16%	5(?)	13%			
Σ I,II,III	61%	57%	69%	56%	23(?)	55%
IV	1%	1%	1%	0%	5%	4%
V	7%	7%	7%	6%	12(?)	16%
ester	3%	3%	4%	2%	2%	2%
non-ester	4%	4%	3%	4%	10(?)	14%
VI	9%	10%	9%	16(?)	21(?)	4(?)
VII	13%	13%	10%	6(?)	9(?)	3(?)
VIII	6	4	3	4	3	9
IX	1%	6%	0%	8%	20(?)	2%
Σ	98	98	99	96	93	93

TABLE XXIV.

Quantitative Distribution of Esters

Expressed as Weight Percentages of Total Oil.

Division	I L	II L	III L	IV L	I LM	III S
I	0.7%		(0.3)			
II	1.3%		(0.1)	(0.4)		
III	0.9%	-0%	(0.1)			
Σ I,II,III	2.9%		(0.5)			2.7(?)
IV	0.3%	0.1%	0.2%	0.1%	0%	0.1%
V	2.7%	2.6%	3.6%	2.1%	1.7%	1.9%
VI	0.9%	(0.7)	(0.2)	(0.2)	(0.9)	0.1%
VII			(0.1)			(-0)
VIII						
IX	0.3					-0%
Σ	(7.1)	(3.4)	(4.6)	(2.8)	(2.6)	4.8(?)

The following fairly reliable points of interest can be extracted from the tables:

1. The terpene contents of all the oils are substantially the same, with the exception of I LM, which has a much lower terpene content.

Ostensibly, the terpene contents (excepting I LM) range from 55% to 69%. However, in different distillations, varying amounts of material pass over into the trap (Div. IX), thus introducing another complication (which, fortunately, is largely eliminated with the improved apparatus). From experience, the author would judge offhand that this material in the trap consists largely of terpenes (pinene and limonene), plus further material that belongs in Div. III. Further, I suspect that the material in Div. IV consists largely of another terpene. If the amounts of these divisions (I, II, III, IV and IX) are combined, there are obtained the following values for "terpene content", with a range (exclusive of I LM) of 61 - 70%:

I L	II L	III L	IV L	I LM	III S
63	64	70	64	48	61

2. The relative proportions of the individual terpenes are substantially the same, on the basis of the reliable results available for I L, III L, and IV L.

If the amounts of the various divisions are converted into the percentages of total terpenes, these figures arise:

Division	I L	III L	IV L
I	33	33	36
II	41	48	-
III	26	19	-
Σ II, III	67	67	64
Σ I, II, III	100	100	100

3. The non-ester "carbonyl" content of Div. V is the same for all leaf oils (3-4%), is much higher for III S (14%), and is probably higher for I LM (10%?).

4. The ester block of Div. IV to Div. VI (but contained primarily in Div. V) is substantially the same for oils I L (3.9%), II L (3.4%), and III L (4.0%), but significantly less for oil IV L (2.4%). It is also significantly less for I LM (2.6%) and III S (2.0%). (These values, it is reminded, do not represent the total ester contents for these oils).

5. The sesquiterpane content (Div. VI) is the same for oils I L, II L, III L (9-10%). It is probably higher for IV L and I LM. It is definitely lower for III S.

6. The sesquiterpene alcohol content (Div. VII) is also the same for oils I L, II L, and III L (10-13%). It is definitely lower for III S.

7. The combined sesquiterpene and sesquiterpene alcohol content is substantially the same for all leaf oils (19-23%), greater for I LM (30%), and much less for III S (7%).

Discussion.

It has sometimes been proposed that the sesquiterpenes, diterpenes, triterpenes, tetraterpenes, and polyterpenes (rubber) are perhaps formed by simple polymerization of the terpenes. If this viewpoint were correct, it would be expected that, as a plant grew older and the polymerization proceeded, its oil would contain an increasing percentage of the higher molecular weight compounds.

A second viewpoint is that terpenes are not intermediates in the formation of the higher compounds (Prokofieff, 1940), but that these higher compounds may arise directly from more or less specific precursors. Accordingly, Haagen-Smit and Siu (1944) suggested that rubber may have "its origin in polyoses like starch and inulin, while the terpenes are derived in a similar way from simpler sugars as postulated by Emde" (1931). Haagen-Smit and Siu reasoned that, since the terpenes of guayule essential oil are at least 90% cyclic, it seems unlikely that the stable six-carbon rings would open up, a necessary step if they were to serve as precursors of the aliphatic rubber molecule.

Our present work gives additional support to the latter viewpoint. The content of sesquiterpene compounds, as well as that of terpenes, is essentially constant for the one, two, three, and four year leaf oils. Since after four years no noticeable accumulation of sesquiterpene compounds occurs, the polymerization of terpenes to sesquiterpenes is unlikely.

Notes on Future Procedure.

If work of the present nature is to be prosecuted most effectively, better analytical methods must be applied. The use of a precision fractionating column, as in the later stages of the present work, is a great aid, but is far from enough. Specifically we need:

1. Methods that are capable of handling smaller amounts of oil satisfactorily.

   a. Less plant material is thereby required.

   b. More select plant material can be chosen.

   c. The present high expenditure of time and effort can be reduced.

2. Methods that are capable of directly analyzing oil mixtures without preliminary separation.

   Spectrophotometry is perhaps the most valuable method of this kind and should be applied. The author has carried out considerable work and study on quantitative ozonization and predicts that this method may also be a future tool in this field.

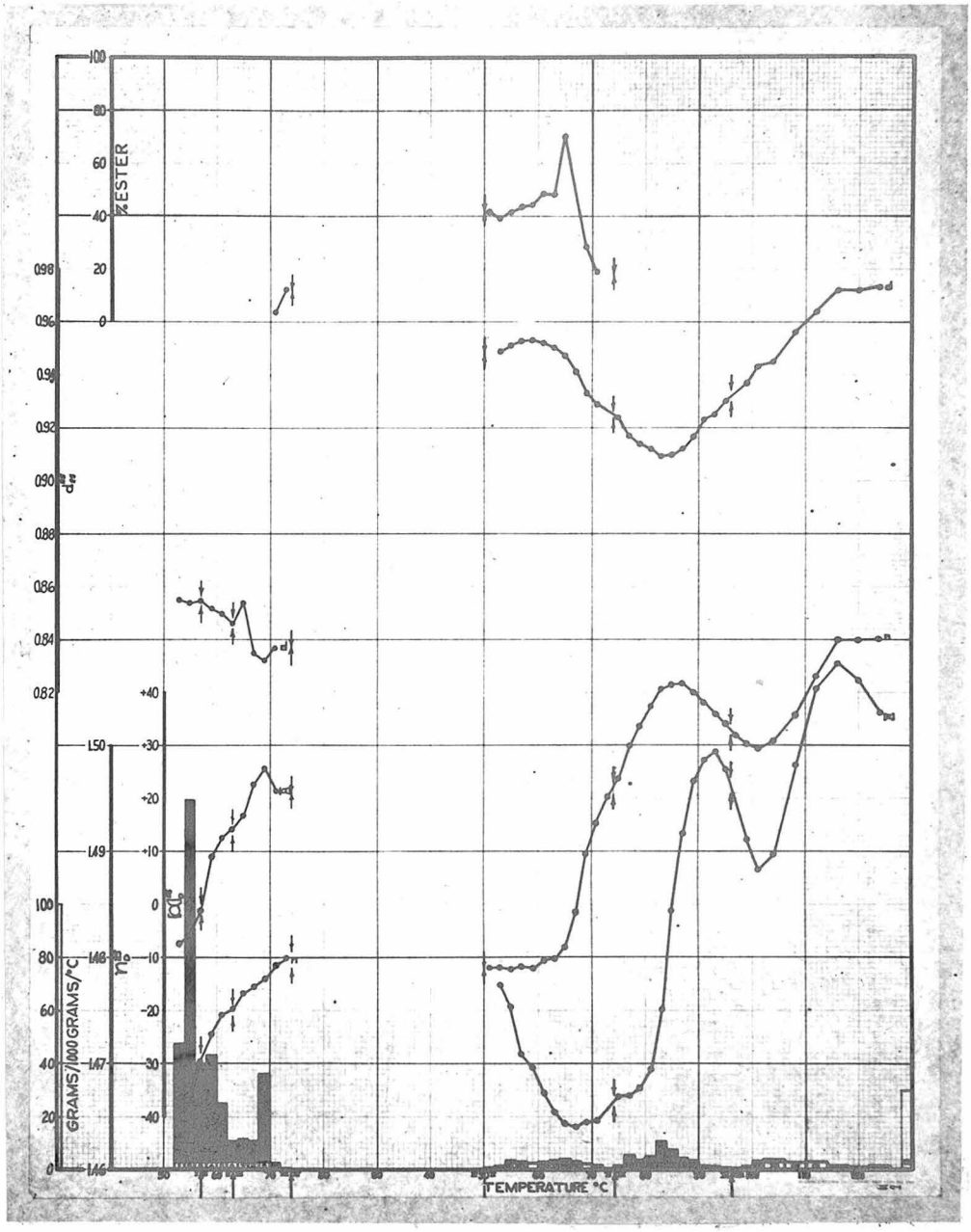


Fig. 27. Quantitative Distribution, Physical Constant, and Ester Content Curves for the Fractionation of Two Year Total Guayule Plant (Haagen-Smit and Siu, 1944).

IL, IL'

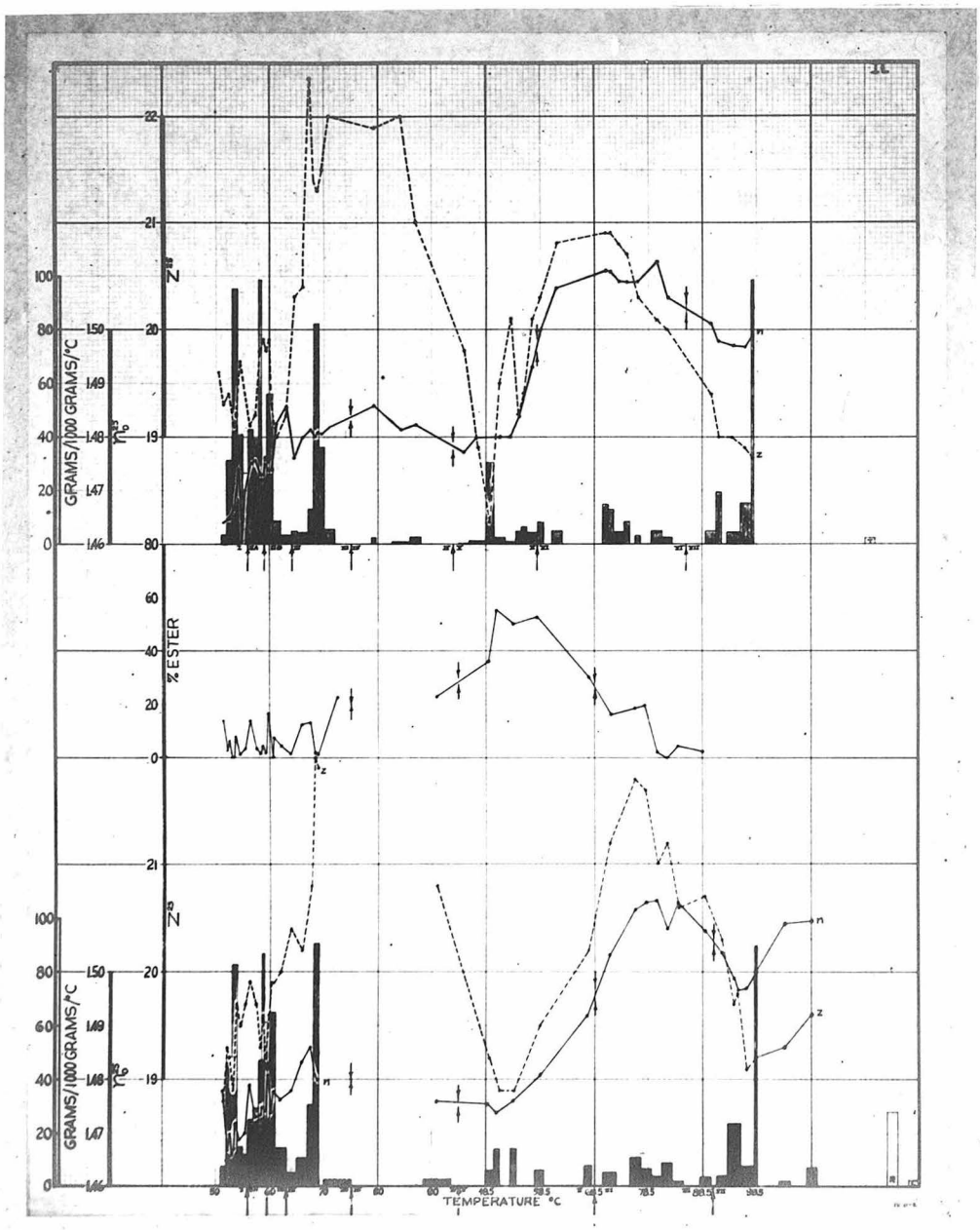


Fig 28. Duplicate Quantitative Distribution, Physical Constant, and Ester Content Curves for the Fractionation of ONE YEAR GUAYULE LEAVES.

II L

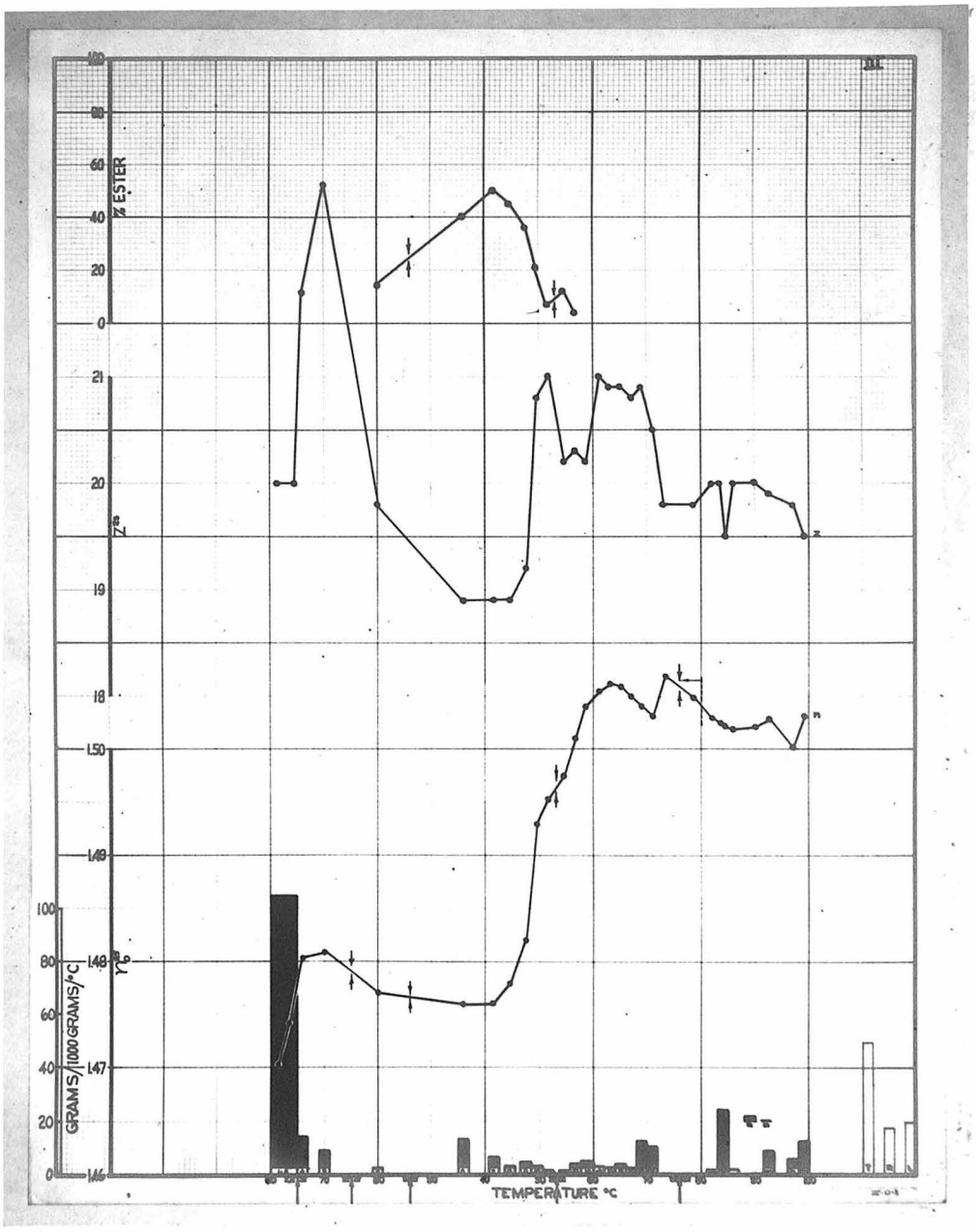


Fig. 28. Quantitative Distribution, Physical Constant, and Ester Content Curves for the Fractionation of TWO YEAR GUAYULE LEAVES.

III L

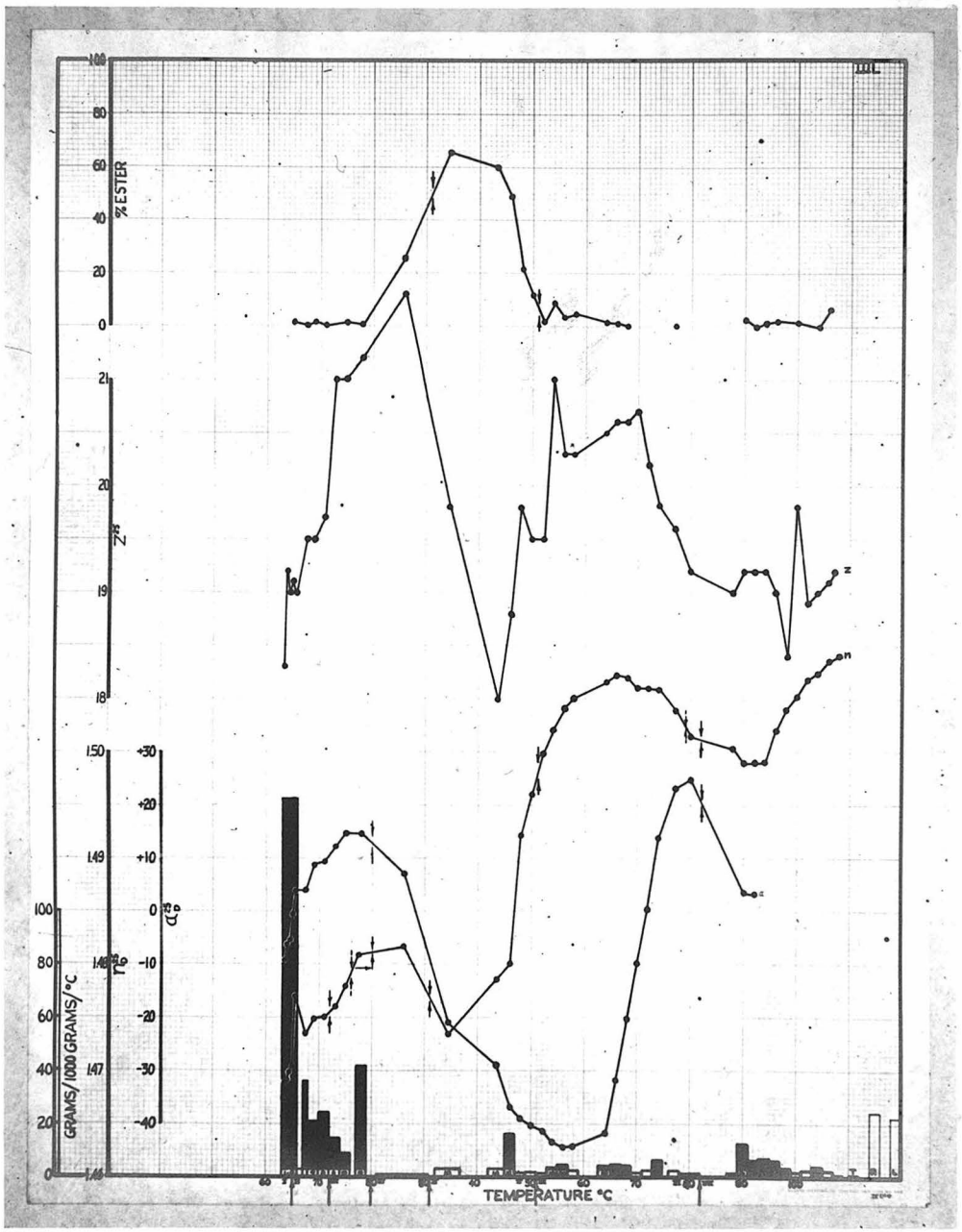


Fig. 30. Quantitative Distribution, Physical Constant, and Ester Content Curves for the Fractionation of THREE YEAR GUAYULE LEAVES.

IV L

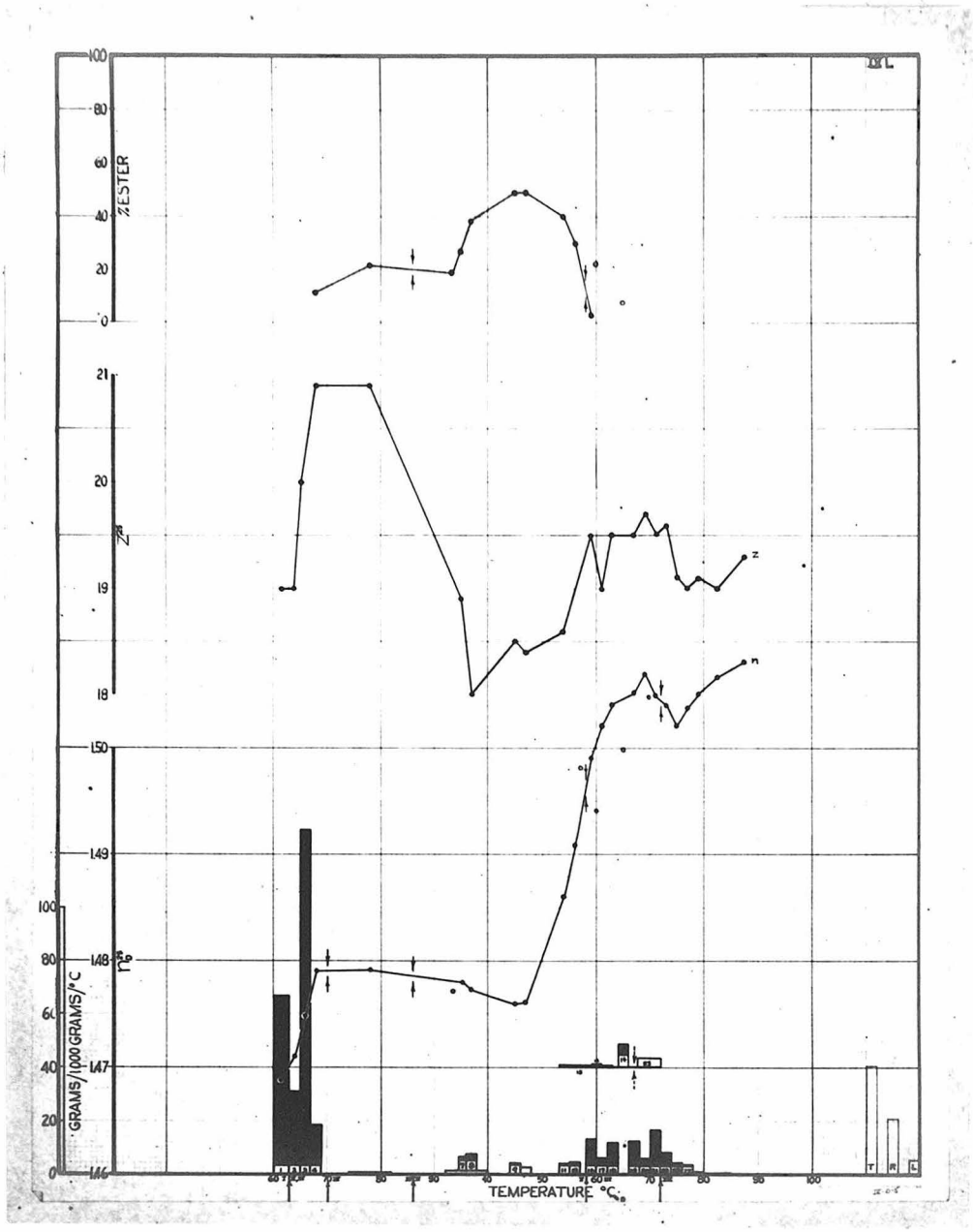


Fig. 34. Quantitative Distribution, Physical Constant, and Ester Content Curves for the Fractionation of FOUR YEAR GUAYULE LEAVES.

ILM

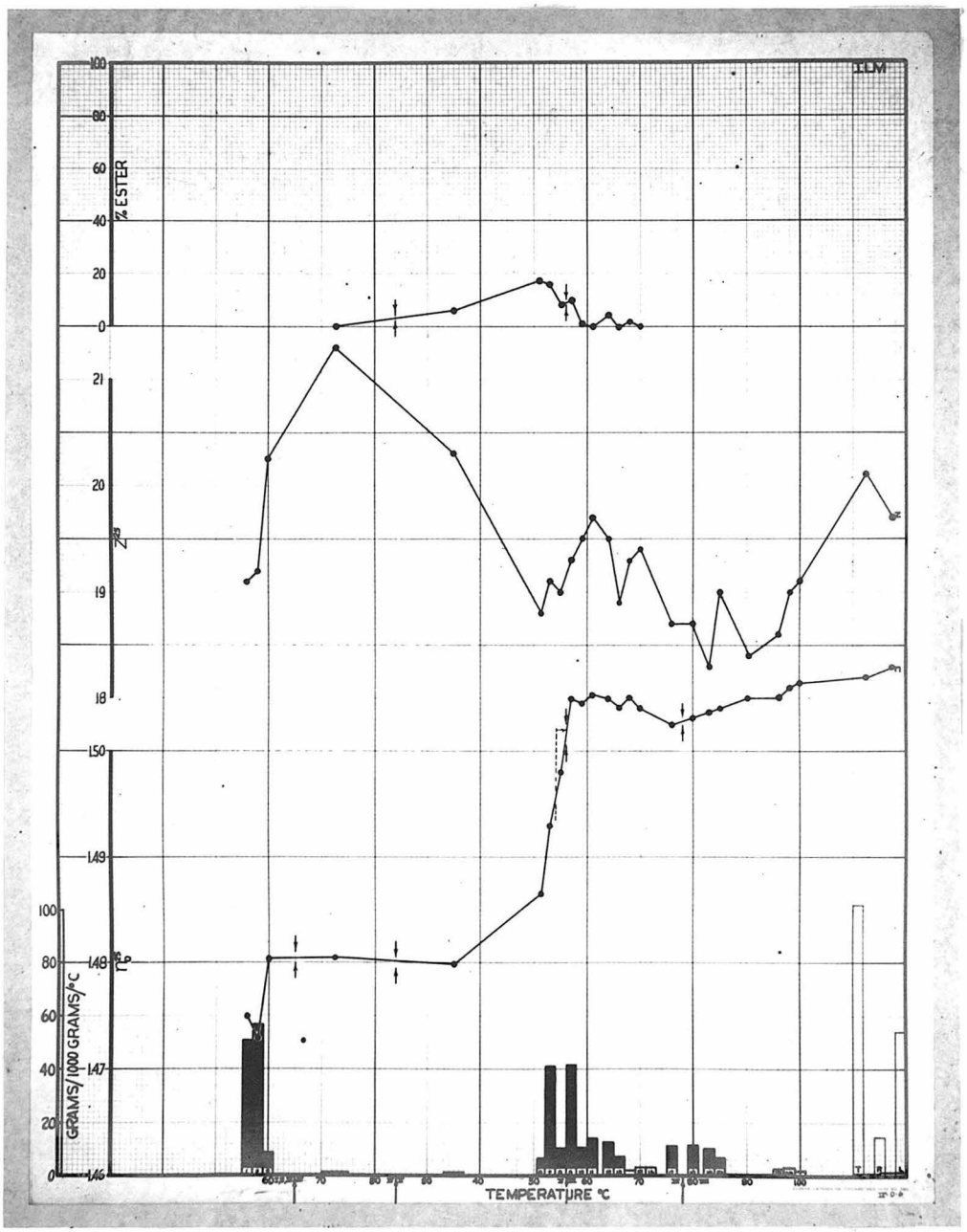


Fig.32. Quantitative Distribution, Physical Constant, and Ester Content Curves for the Fractionation of MOLDY ONE YEAR GUAYULE LEAVES.

III S

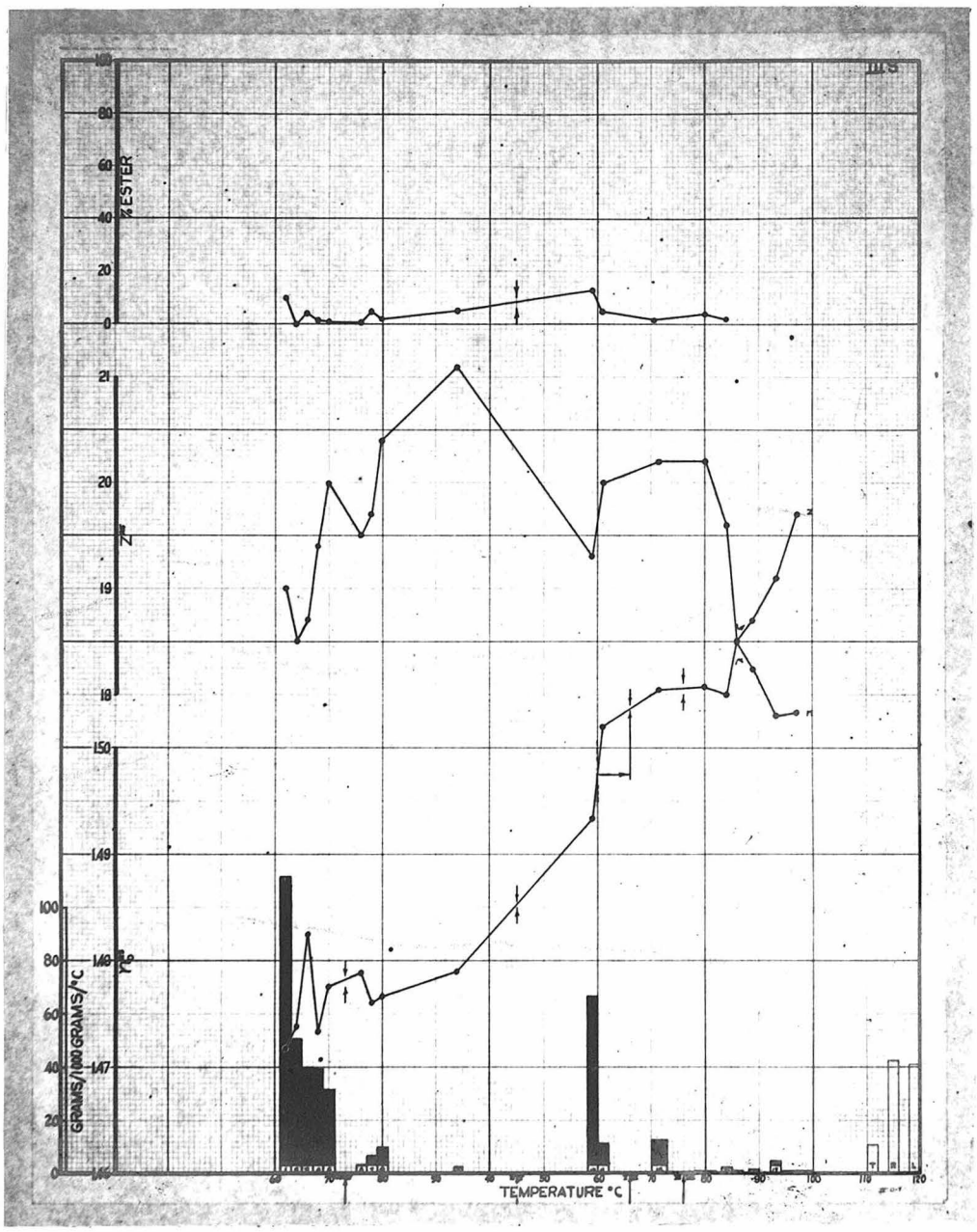


Fig. 38. Quantitative Distribution, Physical Constant, and Ester Content Curves for the Fractionation of THREE YEAR GUAYULE STEMS.

Summary.

1. Investigations on the chemical compositions of the essential oils of leaves, stems, and roots from guayule plants of approximate ages of one, two, three, and four years, have been carried out. Over 1500 kg. of fresh plant material have been handled by steam distillation.

2. Physical constants and ester contents have been determined for all the oils:

a. Ester content decreases steadily with increased age of the plant.

b. Ester content decreases steadily going from leaves to stems to roots.

c. Root oils have far stronger negative optical rotations than any other oils.

3. One, two, three, and four year leaf oils, one year moldy leaf oil, and three year stem oil have been fractionally distilled. The results thus derived on the quantitative chemical compositions of the oils have been carefully evaluated.

a. One, two, and three year leaf oils are substantially similar in the following respects:

1. Terpene content (65%).
2. Division V "carbonyl" content (4%).
3. Division IV, V, and VI ester block content (3.8%).
4. Sesquiterpene content (10%).
5. Sesquiterpene alcohol content (12%).

b. Four year leaf oil is similar to the one, two, and three year leaf oils in some respects:

1. Terpene content.
2. Division V "carbonyl" content.
3. Total sesquiterpene and sesquiterpene alcohol content.

B. (cont.) Four year leaf oil is different from one, two, and three year leaf oils in other respects:

1. Division IV, V, and VI ester block content less (2.4%).
2. Sesquiterpene content perhaps greater.

c. Three year stem oil is similar to the one, two, and three year leaf oils in only one respect, terpene content. It is different from them in these respects:

1. Division V "carbonyl" content much greater (14%).
2. Division IV, V, and VI ester block content less (2.0%).
3. Sesquiterpene content much less.
4. Sesquiterpene alcohol content much less. (Total sesquiterpene and sesquiterpene alcohol content is 7%).

d. One year moldy leaf oil is different from the one, two, and three year leaf oils in all observed respects:

1. Terpene content much lower (50%).
2. Division V "carbonyl" content probably greater.
3. Division IV, V, and VI ester blockcontent less (2.6%).
4. Total sesquiterpene and sesquiterpene alcohol content greater (30%).

4. The significance of some of the results in connection with essential oil relations in the guayule plant has been discussed.

IV. APPENDIX

IV. APPENDIX

DESCRIPTION OF THE PRECISION FRACTIONATION APPARATUS

The present apparatus is similar to that described by Podbielniak (1933). It is shown in Fig. 33 . A performance curve is given in Fig. 34.

Column. The column consists of a meter-long fractionating tube, 6 mm. inside diameter, packed with a single nichrome helix, 2 turns per cm. Heat insulation is secured by a jacket; the inside of the jacket and the outside of the fractionating tube are silvered, several small windows being provided at appropriate places to allow observation of the packing; the jacket is kept evacuated by permanent attachment to a mercury diffusion pump during the course of a fractionation. To avoid breakage due to differential temperature elongation (of the hot fractionating tube over the cold jacket), the jacket is separated at one place and recoupled with a rubber sleeve.

Still Head. The still head is made small and compact to minimize hold-up. A tiny thermo-couple well replaces the usual thermometer. Means for changing the receivers without interrupting the fractionation are provided by a 3 way take-off stop-cock.

Pressure Control and Measuring System. The vacuum source for all fractionations is the mercury diffusion pump, backed by a Hyvae oil pump. In the case of 20 mm. pressure, a simple mercury pressure regulator is interposed in the system; while, in the case of very low pressures, the same regulator, but with

butyl sebacate substituted for mercury, is used. Instead of by the troublesome bubbling of nitrogen through the boiler contents, bumping is avoided by sealing a platinum wire into the bottom of the distillation flask. The pressure is measured with a suitable manometer or McLeod gauge attached as near as practical to the top of the fractionating column.

Temperature Control and Measuring System. Instead of a troublesome oil bath, a Glas - Col heating mantle, regulated by a Varitran, is employed. The temperature of the boiling liquid (instead of the less important bath temperature) is measured by a thermometer inserted into an appropriate well. The heat insulation of the column has already been mentioned. The temperature of distillation is measured by means of a thermo-couple system. One junction is inserted in the well at the top of the fractionating column, the other junction is attached to a thermometer bulb, which is also wrapped with glass cord and a heating wire. When the galvanometer included in the circuit registers zero current, the temperature of the thermometer, as readily regulated by the heating wire and an air jet (for cooling), records directly the distillation temperature at the top of the column (a suggestion of Dr. W.B. Dandliker, communicated through Dr. J.G. Kirchner).

Miscellaneous Apparatus. A dry ice trap is provided to catch any uncondensed vapors passing over. Of particular note

are the vacuum-jacketed standard taper joints (Podbielniak, 1941), located at points exposed to high temperature, which, lubricated with silicone grease, are highly leak-proof and otherwise commendable. The oil fractions are directly received in small screw cap vials in which they are stored.

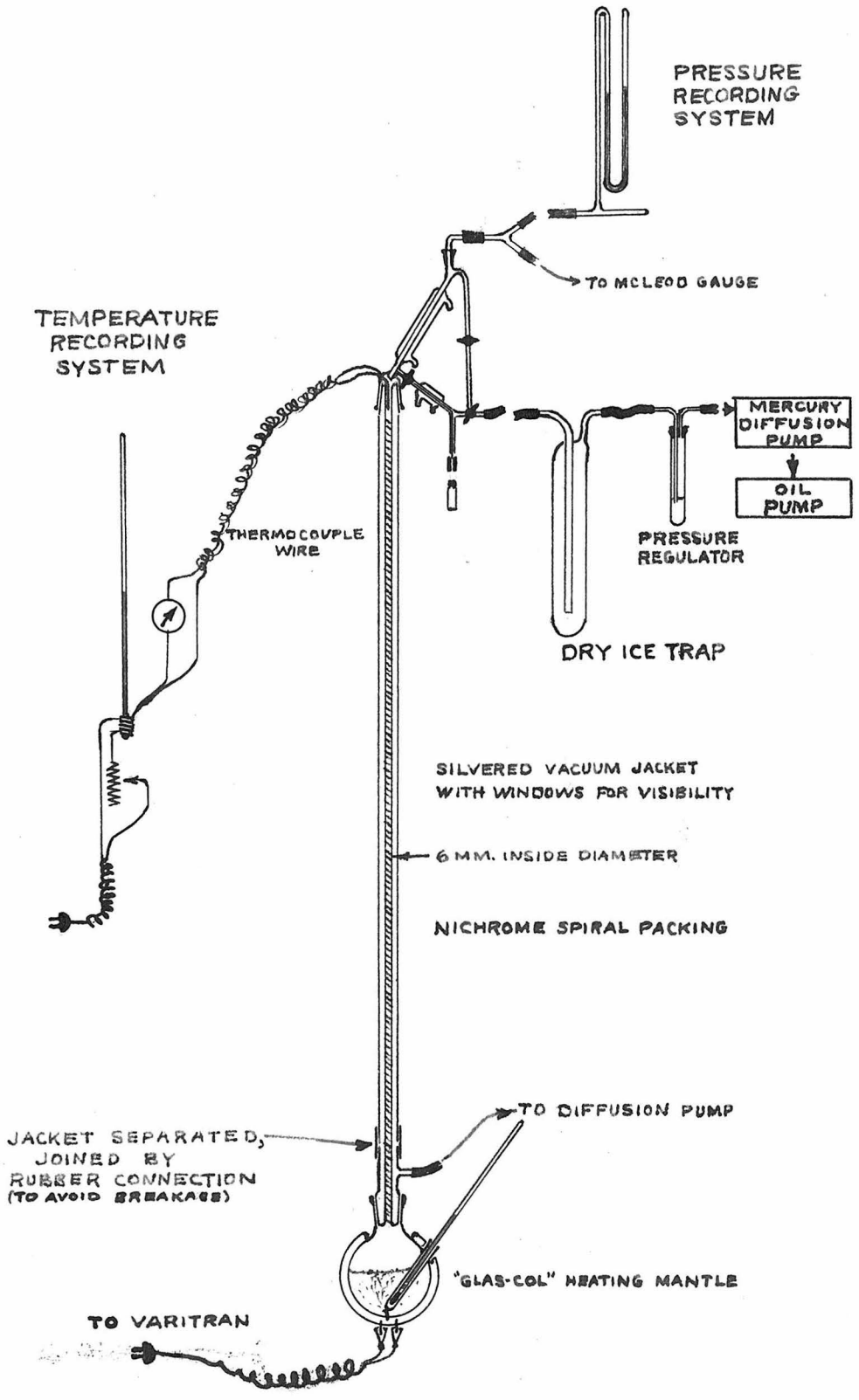


Fig. 33. High Precision Fractionation Apparatus.

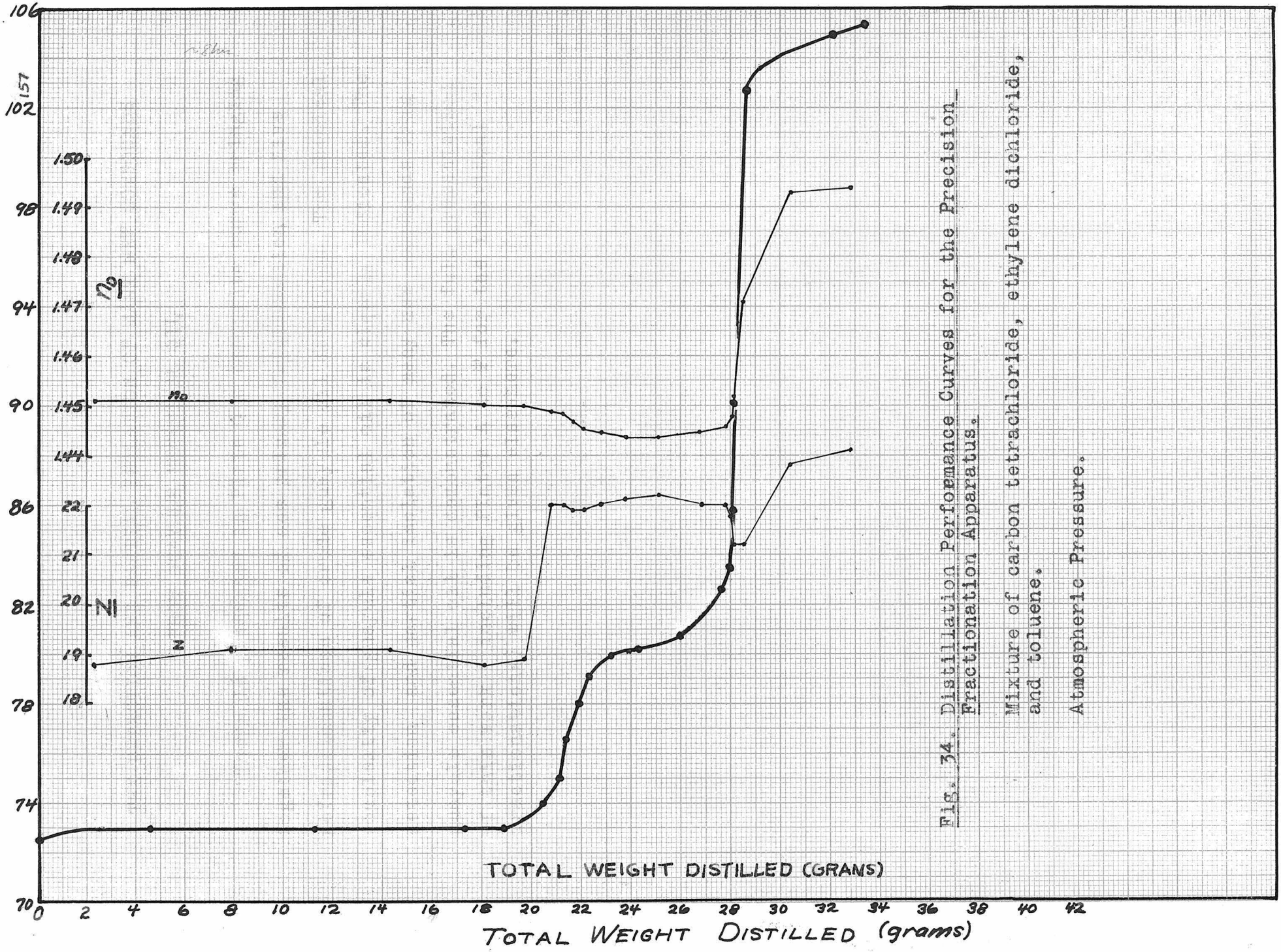


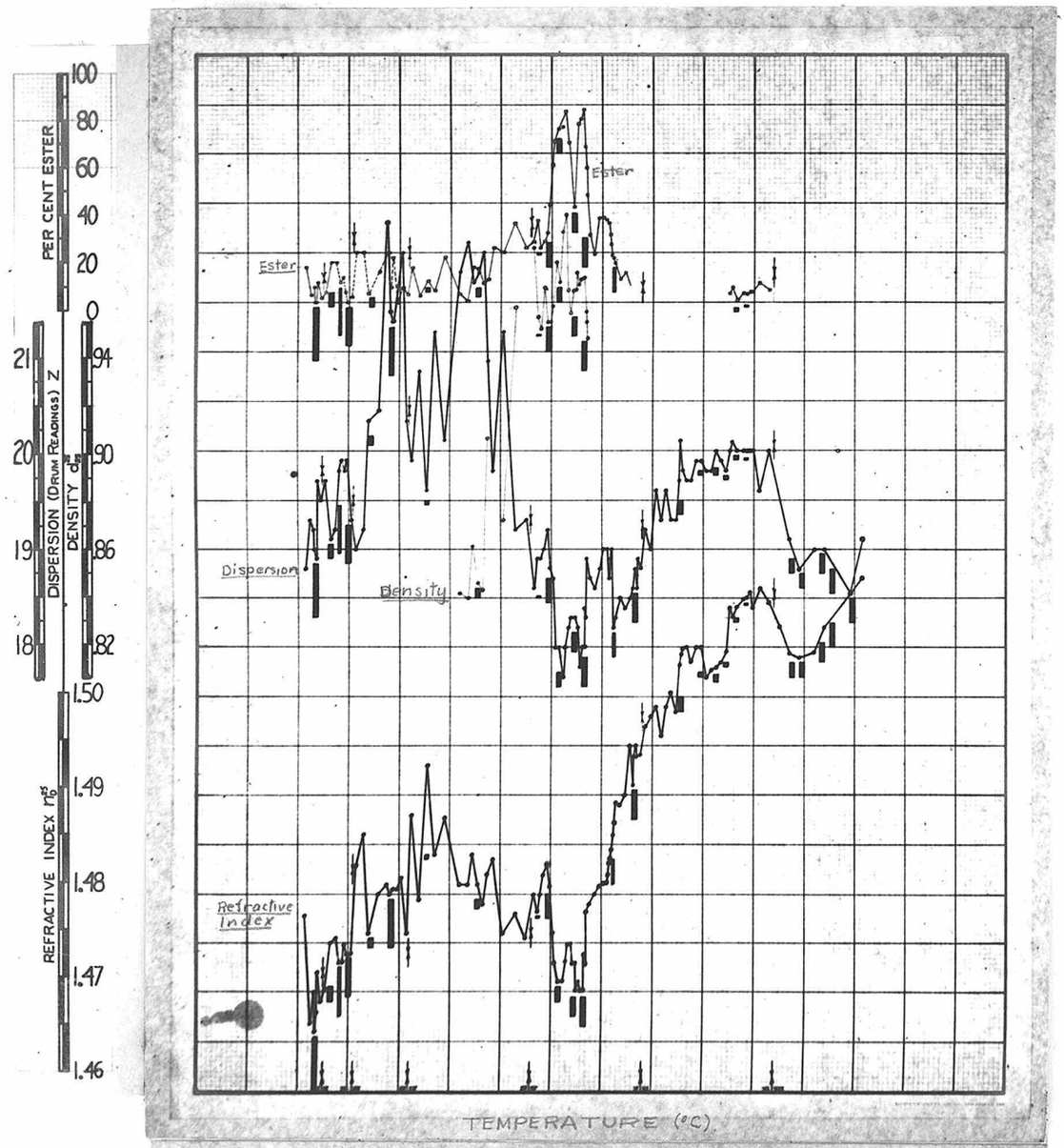
Fig. 34. Distillation Performance Curves for the Precision Fractionation Apparatus.

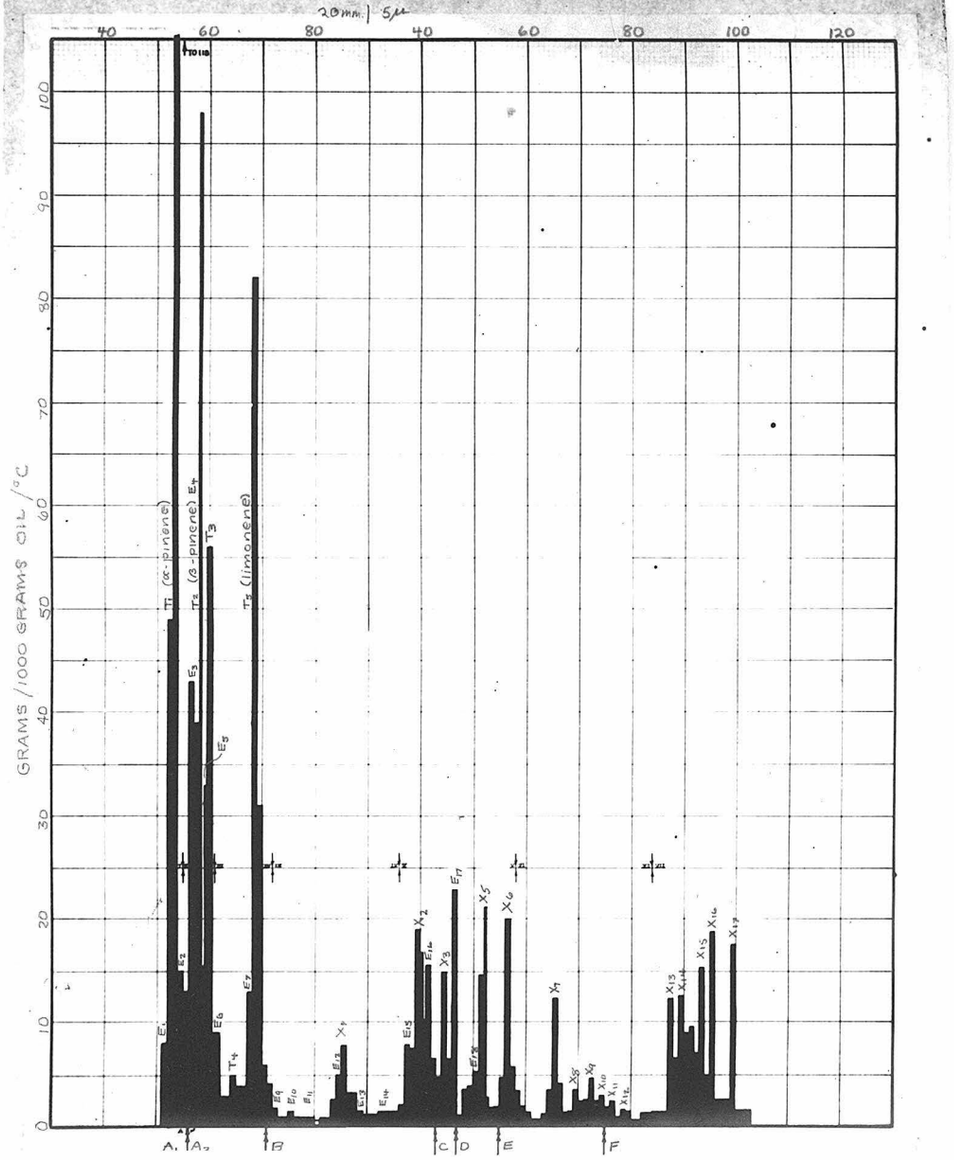
Mixture of carbon tetrachloride, ethylene dichloride, and toluene.

Atmospheric Pressure.

THE DETECTION AND PARTIAL ISOLATION OF MANY NEW COMPOUNDS FROM GUAYULE ESSENTIAL OIL

There is presented in Plate G a composite curve bringing together the results of one primary fractionation and five refractionations of crude guayule oil fractions. The chemical complexity of guayule essential oil is clearly indicated. The detection and partial isolation of many new compounds from guayule essential oil is demonstrated. The full presentation of this work (including Plates A to F for the individual fractionations) will be given elsewhere.


PER CENT ESTER
100
80
60
40
20
0
DISPERSION (Drum Readings) Z
21
20
19
18
DENSITY D_{4}^{20}
94
90
86
82
REFRACTIVE INDEX n_D^{25}
1.50
1.49
1.48
1.47
1.46
Ester
Ester
Dispersion
Density
Refractive Index
TEMPERATURE (°C)


20mm. | 5μ
40
60
80
40
60
80
100
120
GRAMS / 1000 GRAMS OIL / °C
0
10
20
30
40
50
60
70
80
90
100
T1 (α-pinene)
T2 (α-pinene)
T3
T5 (limonene)
A1
A2
B
C
D
E
F

GENERAL SUMMARY

GENERAL SUMMARY

Several more detailed summaries will be found appended to the more lengthy individual sections (p.87,150). The principal results of the thesis as a whole are collected here.

1. Procedures have been developed which permit the rapid and precise micro-determination of the essential oil contents of plant organs and the oil contents of different tissues therein.

2. It has been recommended that work be carried out on the next phase of the program, namely, the micro-chemical determination of specific oil components, so that the compositions of the oils from different tissues can be studied.

3. It has been shown possible to segregate the course of essential oil formation in the young growing guayule leaf into three phases, corresponding to three phases in the morphological development of the leaf.

4. Large scale investigations have been carried out on the chemical compositions of the essential oils of leaves, stems, and roots from guayule plants of one, two, three, and four years.

   a. Ester contents decrease steadily with increased age of the plant; decrease steadily going from leaves to stems to roots.

   b. Root oils have far stronger negative optical rotations than any other oils.

   c. One, two, three, and four year leaf oils are of rather similar composition, the four year oil showing perhaps some differences.

   d. Three year stem oil has a much greater "carbonyl" content and a much smaller content of sesquiterpenes and derivatives than the leaf oils.

   e. The biochemical significance of some of these results has been discussed.

5. A graphical communication of the detection and partial isolation of many new compounds from guayule essential oil is appended

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