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CHEMICAL INVESTIGATIONS OF THE TOBACCO PLANT

I. A PRELIMINARY STUDY OF THE NON-VOLATILE ORGANIC ACIDS OF TOBACCO LEAVES

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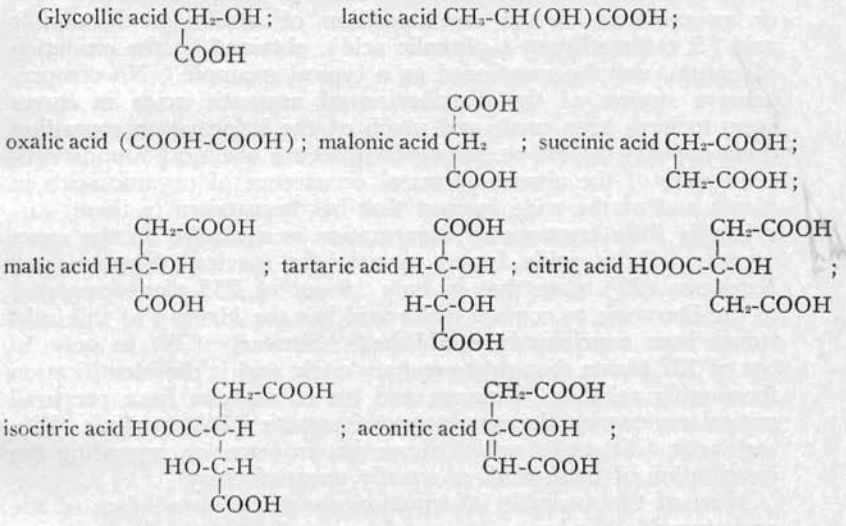
CONTENTS

1. THE ORIGIN OF THE ORGANIC ACIDS IN LEAF TISSUES

The term "organic acids" has generally been restricted, in the literature of plant chemistry, to a group of non-nitrogenous carbon compounds that contain one or more carboxyl groups and that can be extracted from acidified aqueous solution by means of ether and subsequently esterified. Some of these compounds are volatile, but most of them are not volatile either directly or on distillation with steam. The present discussion will deal only with non-volatile acids and is further restricted for the most part to the aliphatic acids of the type shown in the following list. These are the commonest and most widely distributed of the substances generally designated by the term "plant organic acids."



NOTE: The chemical investigations of tobacco herein described were carried out as part of a general project under the title "Cell Chemistry," by the Department of Biochemistry of the Connecticut Agricultural Experiment Station, New Haven, Conn. The Department has enjoyed the benefit of the close coöperation of the Tobacco Substation. The expenses were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

The presence of lactic, oxalic, malic, citric, succinic, and tartaric acids has been demonstrated in a large number of plants. Malonic, fumaric, glycollic, aconitic, and isocitric acids are, so far as is known, somewhat rare plant acids.

Many aromatic acids have been identified in various plant tissues, but usually these substances are found as esters in the essential oils, balsams, and resins, or as glucosides. Although the simplest aromatic acid, benzoic acid, C_6H_5-COOH, has been reported to occur in the leaves of *Pinguicula vulgaris* (46), most of the aromatic acids that have been found in leaves are unsaturated or phenolic substances. Cinnamic acid ($C_6H_5CH=CHCOOH$) (19), salicylic acid (o-hydroxybenzoic acid) (33), and protocatechuic acid (3, 4-dihydroxybenzoic acid) are among the simplest of these. More complex substances, such as caffeic acid (3, 4-dihydroxycinnamic acid) and quinic acid (either 2, 3, 4- or 3, 4, 6-trihydroxybenzoic acid) are perhaps more often observed. These two acids in particular have been stated to be present in tobacco leaves. A whole series of complex aromatic acids is found associated with or as products of the decomposition of alkaloids. Hemipinic acid (5, 6-dimethyoxy-o-phthalic acid), obtained by the oxidation of opium, may be mentioned as a typical example. No comprehensive studies of the distribution of aromatic acids in leaves seem to have been made and much of the information regarding them appears to rest on somewhat insecure chemical foundations.

In spite of the almost universal occurrence of organic acids in plants and of the wide interest that has been taken in them, surprisingly little trustworthy information is available on the exact identity of the acids found in a given species. Franzen and Keyssner (27) state that in only 15 out of 235 plants reported in the literature to contain malic acid has the identity of this substance been conclusively established. Similarly (25) in only 16 out of 137 plants thought to contain citric acid is the identification thoroughly reliable. Franzen and his co-workers have prepared critical reviews on the occurrence of tartaric (26), succinic (28), and lactic (30) acids, which show that information regarding the distribution of these acids is equally unsatisfactory.

Most of the available information about the metabolism of the organic acids has been obtained from studies of the lower forms of plant life, especially molds and yeasts. The conditions that have been found to influence the formation of organic acids by such organisms are the composition and hydrogen ion activity of the culture medium, the amount and type of nitrogenous substances in the medium, the strain as well as the species of organism, and the temperature. The age and growth intensity seem also to be important.

According to Butkewitsch (11) and Bernhauer (6, 7, 8) the citric acid metabolism of molds is related in some way to the nitrogen metabolism of the organism. Kostytschew and Tsches-

nokov (45) believe that the formation of citric acid is decreased if nitrogen assimilation is increased. Wetzel (90), however, does not believe that citric acid accumulation is necessarily related to a decrease of nitrogen assimilation, but thinks it is also related to other changes in metabolism and to the cessation of growth. Both authors agree that the organic acids are not intermediate products of carbohydrate metabolism during respiration, as was held by earlier investigators, but that, in all probability, these substances arise largely from the deamination of the amides and amino acids derived from protein katabolism.

Butkewitsch (9) obtained evidence that ammonia is formed, in peptone cultures of *Aspergillus niger,* from amino acids. Emmerling (18) showed that *Aspergillus niger* could deaminize α-amino acids but not β-amino acids. Raciborski (64) confirmed the results of Emmerling, and Abderhalden and Teruuchi (1) demonstrated that synthetic polypeptides could be deaminized by the same organism. In all of the above cases the formation of oxalic acid was observed. Emmerling observed no increase in oxalic acid formation by *Aspergillus* grown on sugar cultures and Wehmer (89) obtained only occasional increases of oxalic acid on this medium. Butkewitsch (10) finally showed that in peptone cultures of *Aspergillus niger,* 78 per cent of the soluble nitrogen was in the form of ammonia nitrogen, that this was accompanied by large amounts of oxalic acid, and that the ratio of ammonia to oxalic acid was very close to that for neutral ammonium oxalate. On the other hand, cultures of *Penicillium glaucum* contained only 25 per cent of their soluble nitrogen in the form of ammonia and 75 per cent as amino nitrogen. If the oxalic acid formed in the *Aspergillus niger* culture was continually neutralized with calcium carbonate, ammonia and acid formation were repressed and the organism behaved like the *Penicillium.* Similarly the *Penicillium* could be made to resemble the *Aspergillus* by acidification of the peptone culture with phosphoric acid. This parallelism between oxalic acid formation and ammonia production is evidence for a relationship between the deamination of amino acids and acid production.

According to Mayer (47) a few molds can hydrolytically deaminate amino acids with the formation of the corresponding oxyacid. The researches of Dakin (14), G. Oparin (56), A. Oparin (54), Neubauer and Fromherz (51), and Neuberg and Karczag (52) indicate, however, that, in yeast, deamination is an oxidative process that occurs according to the following scheme:

$$R\text{-}CH(NH_2)COOH + O \longrightarrow R\text{-}C(OH)(NH_2)COOH \longrightarrow R\text{-}CO\text{-}COOH + NH_3$$

$$R\text{-}CHO + CO_2 \longleftarrow R\text{-}CO\text{-}COOH \longrightarrow R\text{-}CHOH\text{-}COOH$$

(aldehydes) (keto-acids) (oxyacids)

$$R\text{-}CHO + O \longrightarrow R\text{-}COOH$$

This is the type of oxidative deamination that occurs almost exclusively in the higher animal organisms and in plants. The processes yield a variety of keto-acids which differ at various stages of development of the plant. By the action of carboxylase the ketoacids may be converted into compounds poorer in carbon; they may be further oxidized or they may, by reduction, be converted into hydroxyacids. The quantitative relationships of the organic acids in the plant at the various stages of development are undoubtedly regulated by the simultaneous occurrence of oxidizing, reducing, and hydrolytic reactions. The hydroxyacids may themselves undergo decomposition but, in general, they are less reactive than keto-acids and their decomposition might be expected to be slower. The probable end product of the decomposition of the hydroxyacids is oxalic acid. This substance can arise from a large number of precursors and, in view of this, it is almost impossible to establish its exact origin. Acids that contain longer carbon chains occasionally, however, retain enough similarity to their amino acid precursors to permit a logical speculation upon their origin. Thus Ehrlich (17) showed that, in yeast cultures, succinic acid may originate from glutamic acid. Similarly Kostytschew (44) was able to prove that malic acid may be, in yeast cultures, a deamination product of asparagine. Nevertheless it must be borne in mind that so few of the reactive breakdown products have been identified that a relationship between a given organic acid and an amino acid precursor is still, for the most part, purely speculative.

The synthesis and transformations of the organic acids in higher plants are even more obscure than is the case in the molds and yeasts. Schulow (76) and Petrow (58) showed that malic acid is formed during the germination of seeds rich in asparagine. Smirnow (77) cited evidence that germinating seeds can synthesize asparagine from ammonium malate. Smirnow's data suggest that organic acids may be utilized for the resynthesis of amino acids, but definite experimental evidence on this point is lacking. Szent-Györgyi (80) recently showed that the conversion of malic to succinic acid is a reversible process and that these acids may form an important oxidation-reduction system. Quastel and Woolf (63) deduce from their experiments on bacterial metabolism that there are two mechanisms for the deamination of aspartic acid, the one common to strict aërobes, anaërobes and facultative anaërobes, in which deamination is accompanied by reduction to succinic acid and is inhibited by certain antiseptics. The other mechanism is possessed by facultative anaërobes only and is a reversible process that continues in the presence of certain antiseptics.

Ruhland and Wetzel (67) showed that *Begonia semperflorens* produces large amounts of ammonia, but practically no amide

nitrogen; large amounts of oxalic acid are also formed. These authors believe that oxalic acid is produced as a reaction to the toxic effect of ammonia and that it therefore plays a role similar to that suggested by Prianischnikow (62) for asparagine. Some experimental data of Ullrich (81) also suggest that the oxalic, malic, and succinic acids in the full grown leaves of *Anemone nemorosa, Rubus idaeus, Begonia semperflorens* and *Lactuca sativa* are not related to carbohydrate metabolism.

Although a close relationship is indicated between deamination of amino acids and organic acid formation, it is not necessarily implied that this is the only mechanism by which these acids are formed. Indeed it is certain that, in many instances, organic acids can arise from the oxidation of carbohydrates. Thus Butkewitsch (11) demonstrated that *Aspergillus niger* can oxidize glucose to gluconic acid, which in turn may be converted into oxalic acid. This observation emphasizes the fact that an organism may produce one and the same end product in several different ways. These different mechanisms are revealed only by suitable change in the experimental conditions.

The few organic acids that have been studied represent those that are present in the largest quantity, and those that have the greatest chemical stability and are fairly easy to identify. It is undoubtedly equally as important to investigate the organic acids that occur in small amount and it is even more desirable to isolate those acids of greater chemical reactivity that behave as intermediates in the different reactions. That reactive substances do occur is suggested by the work of Schmalfuss and Barthmeyer (72) who obtained evidence for the presence of dihydroxymaleic acid in *Glaucium luteum*. In comparison with the commonly occurring plant acids this is a very reactive substance. Chlorogenic acid is another example of a highly reactive acid. G. Oparin (55, 56) and A. Oparin (54) showed that this substance has the property of acting as a deaminizing reagent and that, by the elimination of carbon dioxide and ammonia, it can convert a number of aliphatic and aromatic amino acids into the next lower homologous aldehyde.

2. THE ORGANIC ACIDS OF TOBACCO

It was pointed out in the reviews of Franzen and his co-workers that the older data on the identity of the organic acids of the tobacco leaf are very unsatisfactory. As late as 1928 the presence of malic and citric acid was only a probability; since then, however, the Russian workers at Krasnodar have obtained much valuable information on the organic acids of Oriental tobaccos. According

to the excellent review by Schmuck (74) the following acids are present:

1. Volatile fatty acids: formic, acetic, butyric, (propionic?).
2. Non-volatile fatty acids: oxalic, succinic, fumaric.
3. Non-volatile hydroxyacids: malic, citric.
4. Aromatic acids: chlorogenic, caffeic, (quinic?).

The observations upon which this summary is founded were made on samples of cured or fermented tobacco. Some of these acids may be products of the curing or fermentation process and hence are not necessarily those originally present in the green leaf.

The evidence for the presence of the several non-volatile acids may be briefly stated as follows.

OXALIC ACID

Behrens (5) observed small calcium oxalate crystals in certain cells of the mesophyll as well as in the rib parenchyma. The same salt was also seen in the hair cells. Ridgway (65) identified cryptocrystalline calcium oxalate in the cells of cured tobacco leaves. These observations were made on cured or fermented tobaccos and give the impression that oxalic acid must be present in considerable amounts. Schmuck (73) conclusively established the presence of oxalic acid in fermented tobacco of the Trebizond type. No quantitative figures are given, but the impression is conveyed that this acid occurs in appreciable quantity. The only reference to the occurrence of oxalic acid in the fresh leaf is that of Smirnow, Drboglow and Maschkowzen (79), who stated that oxalic acid is found in young tobacco plants (5 to 6 leaves), but that it disappears in the next developmental stage and is not found in old leaves.

SUCCINIC ACID

Behrens (5) mentioned, without literature references or experimental proof, that succinic acid occurs only after the fermentation process. Schmuck (73) confirmed the presence of succinic acid in fermented tobacco, but stated there is no evidence that it is a product of the fermentation process.

FUMARIC ACID

Schmuck (73) was apparently the first to isolate and identify fumaric acid in small quantity from fermented tobacco. Anschütz and Reitler (3) have pointed out, however, that fumaric acid is formed in small amounts during the esterification of malic acid

when this is conducted in the usual way. This observation therefore suggests the possibility that the trace of fumaric acid encountered among the acids of tobacco may have been an artifact.

MALIC ACID

Vauquelin (83), Goupil (32), and Schlösing (71) all mentioned the occurrence of calcium malate in tobacco but these investigators, with the possible exception of Goupil, did not, according to Franzen and Keyssner (27), adequately identify the acid. Oosthuizen and Shedd (53) stated that citric, malic, and oxalic acid are present in cured leaf, although in smaller quantities than in green leaf.

Schmuck (73) positively identified malic acid in fermented tobacco. Smirnow, Drboglow and Maschkowzen (79), by the use of the Denigès reaction, established the probability of its occurrence in fresh tobacco leaves but, up to the present, it has not been positively identified.

CITRIC ACID

The work of Kissling (42) and of Klütschareff (43) suggested that citric acid is present in tobacco. Goupil (32) had, much earlier, drawn the same conclusion because a crystalline substance he obtained from tobacco extracts, on distillation, yielded aconitic acid. Rundshagen (68), by distillation of the ethyl esters of the acids from tobacco, obtained a small fraction which he characterized from the refractive index as citric ester. Schmuck and Piatnitski (75) conclusively established the presence of citric acid in cured and fermented Oriental tobacco by isolation and analysis of the free acid, and Smirnow, Drboglow and Maschkowzen (79), by means of the Stahre reaction (pentabromoacetone), demonstrated the presence of citric acid in extracts of fresh tobacco leaves.

AROMATIC ACIDS

Very little is known of the aromatic acids of tobacco. Kissling (42) mentioned that quinic acid was present but Schmuck and Piatnitski (75) were unable to obtain any positive test for it. Savery (69) claimed that tobacco contains considerable quantities of tannin and tannic acid but, according to Schmuck (74), the presence of tannic acid is not firmly established.

Schmuck and Piatnitski (75) obtained color tests which they supposed indicated the presence of chlorogenic acid, but attempts at isolation failed. These authors also stated that caffeic acid had

been obtained from fermented tobacco in crystalline form by them and identified by elementary analysis. The melting point of their preparation was 205-209°. Beilstein gives the melting point as 195°. Meyer and Jacobson (48) state that caffeic acid is quantitatively decarboxylated at 200°. It seems desirable that pure caffeic acid should be prepared and investigated in order to be certain of Schmuck and Piatnitski's identification.

The information on the organic acids of tobacco may be summarized as follows.

TABLE 1. THE ORGANIC ACIDS OF TOBACCO

Acid	Fresh leaf	Cured or fermented leaf
Fumaric	No data	Isolated and identified
Succinic	No data	Probably isolated
Malic	Qualitative test	Isolated and identified
Oxalic	Qualitative test	Isolated and identified
Citric	Qualitative test	Isolated and identified
Tartaric	Doubtful	No data
Caffeic	No data	Possibly isolated
Quinic	No data	Doubtful
Chlorogenic	No data	Doubtful

3. METHODS FOR THE DETERMINATION OF ORGANIC ACIDS

Although many methods have been proposed for the determination of organic acids from plant extracts, critical examination shows that, for the most part, they yield only approximate separations of the acids and give little better than qualitative results. This is emphasized by the review of Bachmann (4). Kissling has described a method for the determination of oxalic, citric, and malic acids in tobacco that has been and is still widely employed (31). According to this method (41) 10 gm. of finely ground tobacco are mixed with an equal weight of 20 per cent sulfuric acid and pumice is added. The mixture is extracted with ether in a Soxhlet apparatus for 20 hours. Water is added to the ether extract which is evaporated to remove the ether; the solution is then made to a definite volume. Half of this solution is treated with calcium acetate in the usual way for the determination of oxalic acid; the other half is neutralized with barium hydroxide, and alcohol is added to make 20 per cent by volume. The precipitate that forms is filtered off at once and the alcohol concentration of the filtrate is raised to 70 per cent. The precipitate so produced is filtered after several hours. The two precipitates are ignited and the small amount of barium oxide formed is converted to carbonate by the addition of ammonium carbonate solution followed by gentle ignition. The weights of the residues,

assumed to be pure barium carbonate, are taken as the equivalents of the sum of the oxalic and citric acids for the first precipitate and as the equivalent of the malic acid for the second. This method, although it gave fairly satisfactory results on mixtures of the three pure acids, was not regarded by Kissling himself as very accurate. He recognized the inadequacy of the separation of the acids and also the difficulty of securing a quantitative extraction by ether. Furthermore it may be pointed out that no account is taken of the possibility that other substances may contaminate the precipitates and no tests for homogeneity are provided.

The method as described is a development of two previous methods (39, 40) which differed somewhat in detail. It is not always clear, in reading the literature of tobacco chemistry, which of these methods has been used. At best Kissling's method is only roughly approximate and, as will be shown later, may give grossly erroneous results.

The method designed by Fleischer in 1874 (21) for the determination of citric and tartaric acids in fruit juices has been extensively employed by the Russian workers for the analysis of tobacco. According to Fleischer's original description the juice is first treated with alcohol to precipitate gums, etc.; lead acetate is then added to the clear filtrate to precipitate tartaric, citric, malic, phosphoric, sulfuric, and oxalic acids. The precipitate, after washing with dilute alcohol, is treated with ammonia and filtered. The filtrate, which contains the tartaric, malic, and citric acids, is treated with ammonium sulfide and acetic acid, and lead sulfide is removed. Tartaric acid is then precipitated by means of potassium acetate and alcohol, and the acid potassium salt is filtered off and titrated with decinormal alkali. Calcium chloride, ammonia, and a "little" alcohol[1] are added to the filtrate from which the tartaric acid has been removed. The precipitate produced contains all of the citric acid and some of the malic acid. It is washed with hot calcium hydroxide solution, whereby all of the malic acid is removed, and the insoluble calcium citrate is left; this is dissolved in acetic acid and the citric acid is precipitated by neutral lead acetate. After washing with a 50 per cent alcohol-water mixture the precipitate is suspended in water and decomposed with hydrogen sulfide; the solution is then boiled to remove hydrogen sulfide. Titration with 0.5 N ammonia gives the content of citric acid (1 cc. 0.5 N ammonia = 35 mg. citric acid). The precipitate of lead salts that failed to dissolve in ammonia is treated with sodium hydroxide and ammonium sulfide and acidified with acetic

[1] Fleischer explains in the text of the paper that at least 1½ volumes of alcohol to one volume of water are necessary for the quantitative precipitation of calcium citrate with calcium chloride.

or hydrochloric acid. After boiling to remove the hydrogen sulfide the oxalic acid is titrated with potassium permanganate.

Fleischer's directions with regard to the concentration of the reagents are not explicit and no experimental data are presented to demonstrate that the various precipitations yield quantitative results. No directions are given for the determination of malic acid.

Schmuck (74) describes a somewhat modified form of the method of Fleischer to be applied to tobacco. The sample is extracted with dilute alcohol that contains about 2 per cent of hydrochloric acid. The extract is concentrated on a water bath to remove the alcohol, the aqueous solution is neutralized with sodium hydroxide and treated with lead acetate. The precipitated acids are washed with 20 per cent alcohol and are then treated with ammonium hydroxide and the filtrate is tested for tartaric acid as described by Fleischer. Schmuck states that no tartaric acid could be detected in the samples of tobacco he analyzed. Calcium chloride, ammonia, and alcohol are then added until maximal precipitation is attained. The precipitate consists of the calcium salts of citric and malic acids which are filtered, washed with alcohol, dried and weighed. The calcium malate is dissolved from the precipitate by extraction with hot calcium hydroxide solution and the residue of calcium citrate is dissolved in acetic acid; the citric acid is then precipitated with lead acetate. The lead salt is decomposed with hydrogen sulfide and the solution so obtained is titrated with 0-1 N alkali. Malic acid is evaluated by subtracting from the total weight of the calcium salts the amount of calcium citrate calculated from the titration data for citric acid. Oxalic acid is determined exactly as prescribed by Fleischer.

No experimental verification of the method is given and no data are presented that give any hint as to the purity or completeness of precipitation of the various fractions secured. Moreover, it will be observed that, in Schmuck's modification, the original precipitation of the acids with lead acetate is carried out in aqueous solution in contrast to the aqueous alcohol precipitation prescribed by Fleischer. This change may be of importance not only from the viewpoint of quantitative precipitation, but may also have considerable effect on the composition of the various fractions analyzed. Furthermore it is assumed that calcium chloride and alcohol precipitate only malic and citric acids and the accuracy of figures for malic acid rest upon this assumption. Fleischer states that all of the citric acid is precipitated, but only a part of the malic acid is carried down. This at once casts a doubt upon any figure obtained for malic acid by the method described by Schmuck. No evidence is presented either by Schmuck or by Fleischer that the calcium salts are pure and it is quite probable that they contain the calcium salts of other organic acids. If this is the case the

malic acid values are certainly erroneous and even the values for citric acid are questionable, since they rest upon the assumption that calcium citrate is the only calcium salt insoluble in hot lime water. The figures obtained by the Fleischer method cannot be interpreted until the purity of the precipitates obtained has been conclusively established.

Piatnitski (60) has recently described another modification of the Fleischer method proposed by Rosenthaler (66), and adapted it to the determination of the organic acids of tobacco.

A 20 gm. sample of tobacco is treated with boiling water, acidified with 15 to 20 cc. of 25 per cent hydrochloric acid, in a 500 cc. volumetric flask. After cooling, the solution is diluted to the mark, shaken, and allowed to settle; 250 cc. of the clear fluid, equivalent to 10 gm. of tobacco, are carefully neutralized with 10 per cent sodium carbonate, the end point being recognized by a change in color from cinnamon to roasted coffee. The reaction must be kept acid to avoid precipitation of calcium oxalate. The organic acids are then precipitated by means of basic lead acetate according to the directions of Demianoff (15). Since the lead salts of some of the organic acids are soluble in an excess of the reagent it is essential to detect the exact endpoint by adding the reagent gradually and noting when maximum precipitation is attained. The lead salts are filtered off, are suspended in a small quantity of water and triturated for 15 minutes with 30 cc. of 25 per cent ammonium hydroxide solution. During this period only the salts of malic and citric acids are dissolved. The insoluble lead salts are filtered off and washed with cold water. Lead is removed from the filtrate by means of hydrogen sulfide, the solution is concentrated to about 50 to 70 cc., 1 to 1.5 cc. of 14 per cent ammonium hydroxide are added and 96 per cent alcohol is then added until a concentration of 80 per cent is reached. The calcium salts of malic and citric acid are then precipitated by adding 10 cc. of calcium chloride solution. This precipitate is filtered after standing 8 to 10 hours and is washed with 50 per cent alcohol that contains a little ammonium hydroxide; calcium malate is then extracted from the precipitate on the filter by treatment with boiling calcium hydroxide solution. The precipitate of calcium citrate is dissolved from the filter with 20 per cent acetic acid. Two solutions are thus obtained, the one an acetic acid solution of citric acid, the other an alkaline solution of calcium malate. This is acidified with acetic acid. These solutions are then separately treated with boiling 20 per cent lead acetate dissolved in 20 to 30 per cent alcohol, which is added until a clear liquid layer is obtained. The precipitates are filtered, washed with 50 per cent alcohol, transferred to a beaker and the lead is removed with hydrogen sulfide. After filtration and boiling to remove hydrogen sulfide the quantities of the acids are determined by

titration with 0.1 N sodium hydroxide, the results being expressed in terms of anhydrous malic and citric acids. No attempts were made to estimate oxalic acid in the lead precipitate insoluble in ammonium hydroxide as this precipitate contained other substances which made the figures untrustworthy.

Piatnitski substituted basic lead acetate for the normal lead acetate used by Fleischer in the precipitation of the organic acids, a change that is unsatisfactory from a quantitative point of view, since great care must be employed to avoid the addition of an excess of the reagent. In other respects the method is similar in principle to that outlined by Fleischer, and by Schmuck. Instead of weighing the calcium salts of malic and citric acid, these are separated by extraction with calcium hydroxide solution, the two fractions are separately converted into lead salts and, after decomposition, titrated with standard alkali. It is impossible to evaluate the results, since no data are presented to demonstrate the purity of the fractions.

Piatnitski (60) employed this method to analyze a number of samples of Russian tobacco and obtained values of from 0.5 to 2.0 per cent for malic acid but found only traces of citric acid. The figures are of a much lower order of magnitude than those obtained by other Russian workers, such as Vitin and Krevsa (88). These investigators obtained results for malic acid varying from 2.96 to 7.32 per cent and for citric acid of from 0.78 to 3.17 per cent. According to Piatnitski (60) the method of analysis they used is not specified and it is therefore impossible to discuss the obvious discrepancies. The values of Vitin and Krevsa agree in order of magnitude, however, with many analyses reported by Kissling and by others who have employed his method.

Piatnitski (61) recognized that tobacco may contain other ether soluble organic acids than those determined by the Kissling or Fleischer methods and developed an empirical method by means of which the total acids soluble in ether could be titrated with standard alkali. The results were expressed in terms of anhydrous malic acid. The details of this method are as follows. A 0.25 gm. sample of dry material is placed in a dry stoppered flask together with 0.75 gm. of pumice, 1.25 cc. of 40 per cent sulfuric acid, and 100 cc. of dry ether. The mixture is shaken three times at 5 minute intervals and is then allowed to stand 12 hours. A 25 to 50 cc. sample of the ether is removed, 5 cc. of water are added, the ether is evaporated, and the residue is titrated with 0.025 N sodium hydroxide using phenolphthalein as an indicator. A blank determination is conducted on the reagents; the excess titration value represents the organic acid content. By means of this procedure values of from 9.6 to 16 per cent of organic acid in terms of malic acid were obtained. These figures are much higher than can be accounted for by the results of Piatnitski's

modification of the Fleischer method, as is shown by the data from Piatnitski's paper (60) given in Table 2.

TABLE 2. COMPARISON OF THE TOTAL ACIDITY WITH THE CONTENT OF MALIC AND CITRIC ACID IN DUBEK TOBACCO. (*Piatnitski*)

Malic acid	Citric acid	Total organic acids as malic acid
%	%	%
1.21	0	15.21
0.76	trace	12.84
0.58	0	12.18
0.14	trace	12.80
0.80	0	13.73

No figures for oxalic acid are given by Piatnitski, but if those of Vitin and Krevsa (2.08 per cent average) are accepted, it is clear that the sum of the known acids determined by the modified Fleischer method comprises only a small part of the total organic acids as determined by titration of an ether extract. Piatnitski made no attempt to determine the nature of the other organic acids; he merely suggested that part of the acidity may be due to aromatic acids. When the value obtained for the total ether soluble organic acid calculated as malic acid was compared with that calculated from the alkalinity of the ash of tobacco, Piatnitski found a very close agreement. This relationship is evidence of the desirability of an accurate method whereby the several acids of the tissue may be evaluated.

Piatnitski is the first, so far as we have found, to point out the discrepancy between the total titratable organic acids of tobacco and the sum of the individual acids determined by the customary analytical procedures. The data of Vitin and of Krevsa give for the sum of the oxalic, malic, and citric acids an average of 8.4 per cent, whereas Kissling (40) obtained an average of 12.5 per cent. The latter figure agrees fairly well with the total titration data obtained by Piatnitski. As will appear in what follows, Piatnitski's results are probably much nearer the truth than are those obtained by the original Kissling method or any of the other modifications of the Fleischer method. Nevertheless, his methods, both for the estimation of malic and of citric acids and for the determination of total acidity, are open to criticism. They are essentially empirical methods and the reproducibility of the results depends to an unusual degree upon control of the experimental conditions and upon subjective judgments. No satisfactory indirect method has yet been described by which reliable quantitative data for the organic acid content of tobacco can be secured. Figures, it is true, are obtained, but the results have little precise meaning and are seldom useful for the quantitative interpretation of metabolic or distributional changes.

4. FUNCTION AND METABOLISM OF THE ORGANIC ACIDS OF TOBACCO

Smirnow (78) studied the behavior of the organic acids during the fermentation of tobacco and reached the following conclusions: 1. Under anaërobic conditions malic and citric acids practically disappear; in the presence of oxygen there is scarcely any change. 2. Fresh tobacco leaves, on autolysis under anaërobic conditions, form malic acid. 3. Fermentation decreases the percentage of organic acids but the change in the citric acid content is greater than that of the malic acid. 4. Fermentation increases the organic acid content of cured tobacco. This increase persists after a second fermentation.

The variation in the proportions of the several organic acids of fresh tobacco leaves at various stages of development has been studied by Smirnow, Drboglow and Maschkowzen (79). The method of analysis used was that of Fleischer (21) and consequently the values obtained may be in error. Smirnow has pointed out that, owing to the lack of exact quantitative methods, it is impossible to gain a clear picture of the changes that occur in the organic acids during the vegetative period of the leaf and that the results merely present a general picture of their behavior. His discussion, however, is the only one available on the organic acid metabolism of fresh tobacco leaf. In tobacco plants that bear five or six pairs of leaves a small amount of oxalic acid is accompanied by citric and malic acids. The presence of oxalic acid during this period seems to be related to the more energetic nitrogen metabolism. The next developmental stage is associated with slower leaf growth and correspondingly diminished nitrogen metabolism. Oxalic acid is no longer found and the amounts of citric and malic acids are increased. This statement of Smirnow is very interesting in view of the observation that oxalic acid is present in cured and fermented tobacco. It leads to the conclusion that oxalic acid is formed at some stage in the curing or fermentation process.

Smirnow pointed out that, at the stage of technical ripeness, tobacco leaves contain more malic than citric acid; after ripening, and particularly after yellowing, malic acid decreases. This decrease seems to be connected with asparagine synthesis, since during this period there is an increase of amide nitrogen. The greatest increase of malic acid occurs in leaves at the seventh stage of development (over-ripe and beginning to yellow) at a time when the amide nitrogen has reached its maximum. On the other hand the maximum accumulation of citric acid occurs in the fifth stage (end of blossoming and formation of seed capsules) in leaves poorer in amide nitrogen and about one month younger. Smirnow found that the maximum citric acid production does

not occur in the sixth stage (technically ripe leaves) in which there is more protein nitrogen than in the previous or following stages. This result he regarded as indirect evidence against the views of Kostytschew (44) who held that citric acid may arise from isoleucine or related products of protein decomposition.

Smirnow also studied the decomposition of the organic acids in fresh leaf tissue by means of autolysis experiments under aërobic and anaërobic conditions. The changes in acid content were found to be dependent both upon the age and upon the amount of oxygen supplied. Full grown leaves showed an increase in organic acid both under aërobic and anaërobic conditions. The accumulation of malic acid under conditions of diminished oxygen supply was thought to be due to the deamination of aspartic acid. Table 3 presents a summary of his results.

TABLE 3. EFFECT OF AËROBIC AND ANAËROBIC AUTOLYSIS ON THE ORGANIC ACIDS OF TOBACCO LEAVES

Stage of development	Aërobic autolysis	Anaërobic autolysis	Starvation
Leaves of young plants of 5-6 leaves	Increase of oxalic acid of 6.65%	Loss of *all* organic acids	Increase of oxalic acid of 46.87%
Leaves of older plants (topped) up to technical ripeness	Malic acid decreased in 2 out of 3 cases, small increase in one case; citric acid decreased	Increase of malic and citric acids	Increase of acids not so great as under anaërobic conditions
Leaves of plants (topped) at technical ripeness	Malic acid decreased in 2 out of 3 cases, small increase in one case; citric acid decreased	Small loss of citric acid	Increase of acids not so great as under anaërobic conditions

A number of investigators, whose work is summarized by Haley, Nasset and Olson (35), have attributed to the organic acids and their alkali salts a function in the "burning qualities" of tobaccos. This work is qualitative in nature and the evidence presented does not allow of definite conclusions. So many factors are involved in the proper burning of tobacco that, at the present time, it is difficult to judge the effect of a single group of chemical compounds such as the organic acids, particularly when the proportion of organic acids is determined by unconvincing indirect methods. Haley and Olson (36) also note that iron has a catalytic effect on combustion and state that the tobacco leaf contains complex organic iron perhaps combined with the hydroxyacids. The work of Ridgway (65) on the grain of tobacco tends to support the view that organic acids have an effect upon combustion. The

so-called "grain" appears to consist largely of calcium and magnesium salts of organic acids which crystallize out in tiny nodules during curing. The grain is especially well marked in Connecticut wrapper tobacco. Combustion proceeds smoothly along the surface of the web of the leaf between the nodules. Flue cured tobacco in which no grain has developed has much poorer burning qualities.

Piatnitski (61) has attempted to correlate the quality of tobacco with the organic acid content. He believes that the poorer grades of tobacco contain a higher proportion of these acids.

5. THE PRECIPITATION OF THE ORGANIC ACIDS FROM TOBACCO EXTRACTS

The unsatisfactory state of the existing knowledge of the organic acids of green tobacco leaves is shown by Table 1. The data on cured and fermented tobaccos have, for the most part, been obtained from Oriental species, although Garner, Bacon and Foubert (31) have given estimations of the oxalic, malic, and citric acid content of green and cured leaves of several kinds of Connecticut tobacco. All of the quantitative data for the organic acids of tobacco leaves have been secured by indirect methods, usually the Kissling or the Fleischer method and, as has already been pointed out, neither of these is capable of giving trustworthy results.

We have therefore undertaken quantitative studies of the organic acids of Connecticut shade-grown tobacco leaves and, in order to secure data from which conclusions as to the metabolism of the acids can be drawn, hot water extracts were prepared from fat-free tobacco seed, from fresh leaves of young plants (5 to 6 leaves), from fresh leaves of mature plants, and from cured leaf that had been prepared commercially from the same lot of mature leaf.

The most satisfactory general procedure for the identification of organic acids of plant tissues hitherto described is that developed by Franzen and his associates (e. g., 29). The first step in their procedure is the precipitation of the acids from an aqueous extract of the tissue by means of lead acetate. The acids are then liberated from the lead salts and the acidified solution is thoroughly extracted with ether. The ether soluble acids are esterified and subjected to fractional distillation. In our investigations we have utilized the general principles of the above method, but have introduced changes and modifications designed to meet our specific demands and to render the method of value in gaining an idea of the quantitative as well as the qualitative distribution of the acids. Furthermore the experimental procedure has been rearranged so

that an approximately quantitative picture may also be obtained of the other acidic fractions present that do not belong to the group of substances usually classified as plant acids. Franzen and Helwert (24) implied that such acidic substances occurred in fruit extracts but did not investigate them nor give any data on the relative quantity present.

The first step in our investigation was the search for a reagent which would quantitatively precipitate the organic acids from the plant extract. The classical reagent, basic lead acetate, is unsatisfactory for many reasons. It is not highly selective; much carbohydrate material is found in the precipitates produced by it and Vickery and Vinson (87) found that basic lead acetate precipitates considerable proportions of widely diversified nitrogenous substances from extracts of alfalfa leaves. Furthermore the lead salt of succinic acid (4) redissolves if an excess of the reagent is added, and the directions in the literature for its use contain many empirical prescriptions on the adjustment of the reaction and on other points that suggest that much difficulty has been experienced in obtaining complete precipitation of the acids.

It was shown in this laboratory some years ago (84, 85) that barium hydroxide, when added in excess to extracts from alfalfa leaves or from yeast, on the further addition of an equal volume of alcohol, yielded precipitates that removed much organic material of a non-nitrogenous nature. The precipitates were found to contain large quantities of organic acids (unpublished observations), but only small amounts of nitrogen.

The use of alcohol to precipitate the barium or calcium salts of these organic acids is, of course, not new. In fact Scheele (70) discovered malic acid as a result of adding alcohol to an aqueous extract from apples the acidity of which had been neutralized by heating with calcium carbonate, and both calcium and barium salts have figured in many procedures that have been recommended since. Alcohol precipitation of barium malate was employed by Kissling (39, 40) and also by Muttelet (50).

Data given in Table 4 show that malic, maleic, succinic, oxalic, tartaric, and citric acids are completely precipitated by barium hydroxide in the presence of alcohol, that the precipitates can be extensively washed with a suitable alcohol-water mixture and that the acids can be subsequently recovered quantitatively from the precipitates by decomposition with sulfuric acid. A sample of a solution of the mixed acids was titrated between the limits pH 8.0 to 3.0 according to the method of Van Slyke and Palmer (82) (see Section 13) in order to ascertain its initial concentration. The precipitation was then conducted by adding an excess of barium hydroxide and 2 volumes of alcohol; the precipitate was centrifuged off, washed as shown in the table, and decomposed with sulfuric acid. Titration of the solution obtained after the

removal of barium sulfate revealed the quantity of organic acid recovered.

TABLE 4. PRECIPITATION OF ORGANIC ACIDS BY BARIUM HYDROXIDE AND ALCOHOL

	Total volume for precipitation	Volume of washings 60% alcohol	Acid recovered
	cc.	cc.	%
A mixture of equal amounts of 1% solutions of citric, oxalic, succinic,	100	120	100
malic, maleic, and tartaric acids that contained 0.100 gm. total acid	200	250	101.5
0.200 gm. total mixed acids	300	200	100
	400	400	99.5

That malic and citric acids are practically completely precipitated as their barium salts from tobacco leaf extracts by the addition of alcohol was shown by an investigation of the barium salts precipitate and of the filtrate from it. The precipitate was decomposed by sulfuric acid and the organic acids were extracted from the solution in a continuous ether extraction apparatus. The filtrates were likewise acidified and extracted with ether. Only about 77 per cent of the total acidity was found in the barium salts precipitate, but the filtrate contained no malic nor citric acids nor other acids that form silver salts insoluble at pH 7.0. To make certain that the malic acid had indeed been completely precipitated, a

TABLE 5. RECOVERY OF MALIC ACID AFTER PRECIPITATION OF THE BARIUM SALTS BY ALCOHOL

The designations refer to Diagram 1

	Filtrate A[1]		Filtrate B[1]		Barium salts 2	Recovery
Extract	Volume	Malic acid	Volume	Malic acid	Malic acid	
	l.	gm.	l.	gm.	gm.	%
Cured leaf 1.8 kilos	20	0.00	19	0.31	30.3	99
Fresh leaf 57.27 kilos ..	51	0.00	53	4.4	190.0	97.8

study was made of the distribution of malic acid in the different precipitates and filtrates obtained in the large scale operations (see Table 5) on both cured and fresh leaf material. In the absence of tartaric acid, malic acid can be determined by measurements of the optical activity of the acids extracted by ether (16). Careful investigation of the fractions in which tartaric acid should

[1] An ether extract contained no citric acid detectable by the pentabromoacetone method nor could insoluble silver salts be obtained at pH 7.0. This indicates the absence of appreciable quantities of succinic, oxalic, and fumaric acids.

occur showed that this acid was not present in our specimen of tobacco leaves, and, consequently, the polarimetric method for the estimation of malic acid could be applied. It was found that about 98 per cent of the malic acid of the leaf extract was precipitated by barium hydroxide and alcohol. As will later appear, citric acid is also completely precipitated from tobacco extracts by these reagents. Inasmuch as these two acids make up nearly 90 per cent of the organic acids of green tobacco leaves the esters of which can be distilled, and also because of the completeness with which a wide variety of pure acids were precipitated in the preliminary tests, it seemed safe to assume that most of the esterifiable acids of familiar types that occur in tobacco leaf extract could be precipitated as their barium salts from dilute alcohol. Precipitation in this way therefore appeared to offer an advantageous quantitative means to remove the simpler organic acids from extracts of tobacco leaves.

It must be pointed out, however, that by no means all of the ether soluble titrable acidity is precipitated from tobacco leaf extracts by the barium hydroxide alcohol method. About 22.4 per cent of the total ether soluble organic acids was contained in the filtrates from the barium salts precipitate. The aliphatic organic acids of the malic acid type are quantitatively precipitated by the reagent we have employed, but nothing is yet known about the solubility of the barium salts of the organic acids of other types that are present in tobacco. It will later be shown that organic acids of other types make up a very large part of the total acids of this plant.

6. THE ORGANIC ACIDS OF GREEN TOBACCO LEAF

The procedure by which the organic acids were isolated from a hot water extract of tobacco leaves can be most readily followed by reference to Diagram 1. The fresh leaves weighed 57.27 kilos and contained 6.65 kilos of total solids. The aqueous extract was prepared by dropping the leaves, in small quantities at a time, into a large volume of boiling water. After a convenient amount had been added, the leaves were boiled until the midrib was soft (½ hour) and were then removed and pressed at the hydraulic press. Meanwhile more leaves were added to the boiling liquor and similarly treated. In this way the entire quantity was extracted within 3 hours. The residues of extracted leaves in the press cakes were ground in a meat grinder and re-extracted with boiling water as before and this process was repeated a third time. The acidity of the hot water employed was maintained at about pH 4 to 5 by occasional additions of sulfuric acid. The three successive extracts were collected quantitatively, filtered, and concentrated *in vacuo* to a volume of 5 liters.

DIAGRAM 1

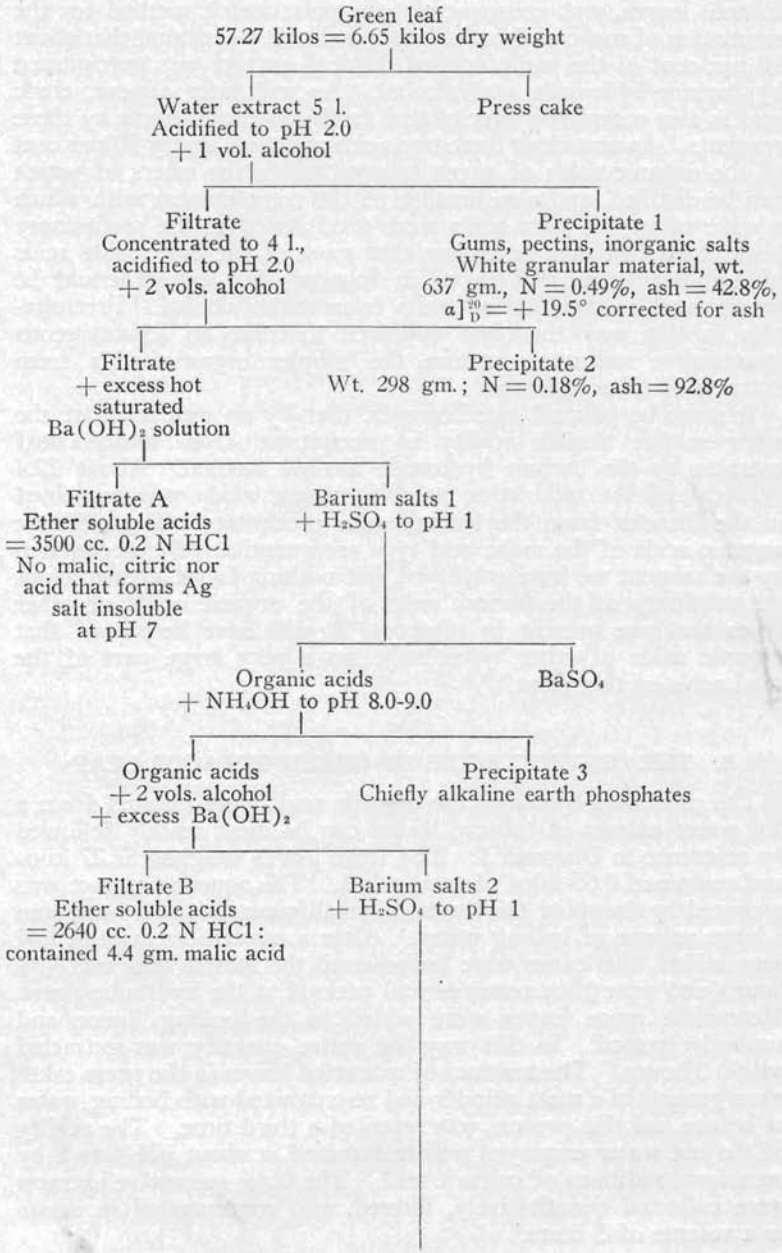
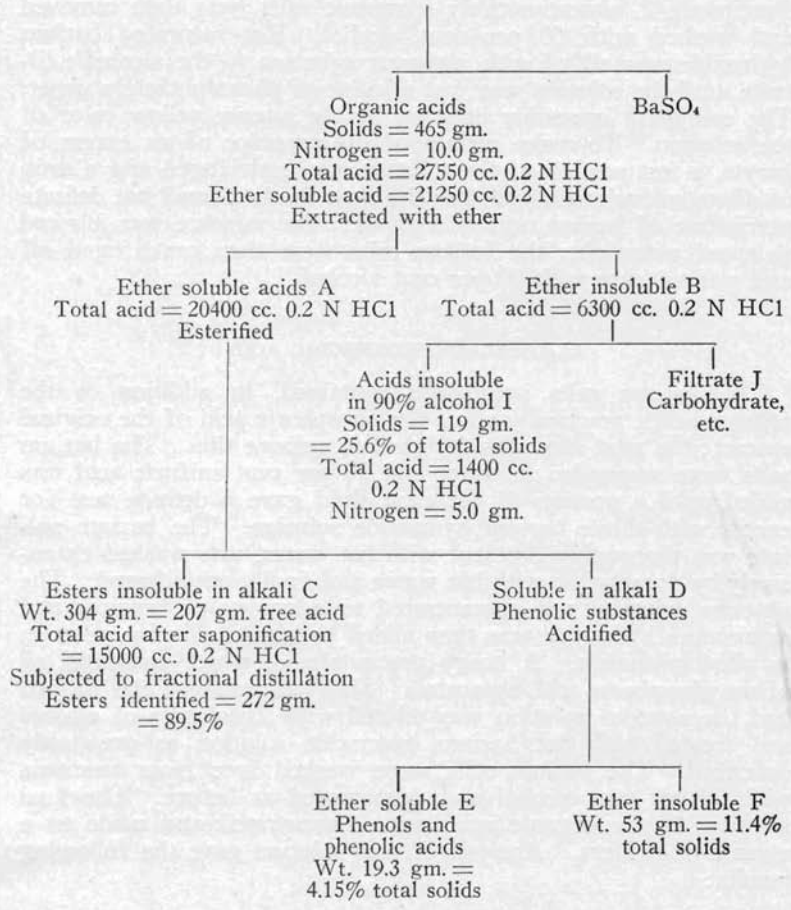

Green leaf
57.27 kilos = 6.65 kilos dry weight
Water extract 5 l.
Acidified to pH 2.0
+ 1 vol. alcohol
Press cake
Filtrate
Concentrated to 4 l.,
acidified to pH 2.0
+ 2 vols. alcohol
Precipitate 1
Gums, pectins, inorganic salts
White granular material, wt.
637 gm., N = 0.49%, ash = 42.8%,
α]D20 = + 19.5° corrected for ash
Filtrate
+ excess hot
saturated
Ba(OH)2 solution
Precipitate 2
Wt. 298 gm.; N = 0.18%, ash = 92.8%
Filtrate A
Ether soluble acids
= 3500 cc. 0.2 N HCl
No malic, citric nor
acid that forms Ag
salt insoluble
at pH 7
Barium salts 1
+ H2SO4 to pH 1
Organic acids
+ NH4OH to pH 8.0-9.0
BaSO4
Organic acids
+ 2 vols. alcohol
+ excess Ba(OH)2
Precipitate 3
Chiefly alkaline earth phosphates
Filtrate B
Ether soluble acids
= 2640 cc. 0.2 N HCl:
contained 4.4 gm. malic acid
Barium salts 2
+ H2SO4 to pH 1

DIAGRAM 1—*Continued*



PRECIPITATION OF BARIUM SALTS AND THEIR DECOMPOSITION

The extract (5 liters) was first adjusted to pH 2.0 with 1:1 sulfuric acid using thymol blue paper as an indicator; an equal volume of alcohol (92 per cent) was added and the precipitate was removed after about an hour. This material (Precipitate 1) was a mixture that contained 42.8 per cent of its dry weight as inorganic salts, the balance being organic material that is, in all probability, related to the pectins. The optical activity, corrected for the inorganic salts, was $\alpha]_D^{20} = +19.5°$. After washing the precipitate free from nicotine the filtrate was concentrated to

about 4 liters and 2 volumes of 92 per cent alcohol were added. Precipitate 2, almost entirely inorganic salts, was then removed and washed with 60 per cent alcohol. Hot saturated barium hydroxide was added with vigorous agitation to the alcoholic filtrate until the solution was just alkaline to phenolphthalein paper. The end point is readily detected by the intense yellow color of the solution. To make certain of the presence of an excess of baryta, a test portion of the solution was centrifuged and a drop of dilute sulfuric acid added to the clear fluid; a small but definite precipitate of barium sulfate formed. The mixture was allowed to stand overnight; the barium salts were then centrifuged off and washed once with 60 per cent alcohol.

REMOVAL OF PHOSPHORIC ACID

The barium salts precipitate contained, in addition to the organic acids, practically all of the phosphoric acid of the original extract; the next step was designed to remove this. The barium salts were suspended in water and 50 per cent sulfuric acid was added until a portion of the clear fluid gave a definite test for excess with dilute barium hydroxide solution. The barium sulfate was thoroughly digested with hot water, was washed extensively by decantation with hot water and finally centrifuged. The aqueous solution was concentrated to a convenient volume and ammonium hydroxide was then added to a faint alkaline reaction to phenolphthalein. A heavy precipitate, 3, which contained all of the phosphoric acid, separated. This was removed and washed and the aqueous solution was diluted with 2 volumes of alcohol and treated with hot barium hydroxide solution as previously described. The barium salts were washed free from ammonia with 60 per cent alcohol and decomposed as before. The final solution of the organic acids was concentrated and made to a volume of 6 liters. Analysis of this solution gave the following results:

Total nitrogen	10.06 gm. = 2.03% of total solids
Total solids	486.0 gm.
Organic solids	465.0 gm. = 95.5% of total solids
Inorganic solids	21.0 gm.
Total organic acid	27,550 cc. 0.2 N HCl
Ether soluble acid	21,250 cc. 0.2 N HCl

The ash consisted chiefly of magnesium salts. No calcium was present and only traces of phosphorus were revealed by the Fiske and Subbarow test (20).

The organic acid solution was then concentrated to about 3 liters and extracted with ether in a continuous extraction apparatus (see Section 11), in two batches of 1,500 cc., for about 320 hours each.

The efficiency of this large scale extraction was ascertained by comparison with data obtained in a small scale apparatus. A 25 cc. aliquot of the main organic acid fraction yielded ether soluble acids equivalent to 21,250 cc. of 0.2 N hydrochloric acid calculated on the whole solution. The solution of the ether soluble organic acids from the large extraction was found on titration to be equivalent to 20,400 cc. of 0.2 N hydrochloric acid, a recovery of 96 per cent. The main ether extracts were combined and, after evaporation of the ether, were concentrated *in vacuo* to a thick syrup, which was dehydrated by concentrating several times with absolute alcohol.

ESTERIFICATION OF ORGANIC ACID FRACTION

The thick syrup was esterified with ethyl alcohol and alcoholic hydrochloric acid at 115 to 120° by the alcohol vapor distillation method of Phelps and Phelps (59). This procedure is far more efficient than that employed by Franzen and his co-workers. The operation was continued until the distillate attained a constant specific gravity. The solution of the esters was concentrated *in vacuo* and dissolved in about 1,000 cc. of ether; this yielded a clear, dark red solution with only traces of tar. The ether solution was then shaken with the minimal volume of 30 to 40 per cent sodium hydroxide required to neutralize the free acid; a dark-colored sludge separated. The ether was decanted and the alkaline sludge was extracted three times with 200 cc. portions of ether.

TREATMENT OF ETHER SOLUBLE ESTERS OF THE ORGANIC ACID

The combined ether solution was shaken with successive 10 to 20 cc. portions of water until neutral to litmus (the aqueous fluid was added to the alkaline sludge) and was then dried over anhydrous sodium sulfate. The ether was distilled off and the esters were subjected to fractional distillation *in vacuo*. The boiling points of the more volatile esters secured from tobacco leaves are so far from that of the chief constituent, malic ester, that the low boiling esters can be entirely separated from malic ester by means of an appropriate fractionating column. A single distillation separated oxalic, succinic, and fumaric esters from the malic ester. The main malic ester fraction was, as judged from optical rotation data, uncontaminated by the lower or higher boiling fractions. The citric ester fraction could not be obtained pure by one distillation but it was possible to obtain fractions of maximum and minimum citric ester content.

The apparatus employed for this distillation consisted of a 500 cc. three-necked pyrex flask to the central neck of which a series of fractionating columns could be attached. The smaller necks

of the flask were furnished with capillary inlet tubes through which a small current of air was admitted during the distillation. As a further aid to smooth boiling a number of angular quartz pebbles were placed in the flask. The fractionating columns were each furnished with a rubber stopper which was slipped on the bottom of the column in an inverted position. The stopper was of a diameter slightly greater than the outside diameter of the ring neck of the flask. The upper surfaces of the necks of these flasks are ground flat and the flat surface of the rubber stopper is pressed down on the ground glass surface. When the vacuum pump is started the stopper adheres firmly and forms a joint that is tight enough for distillations at moderate vacua. This device facilitated the removal of the column when it became necessary to make a change and greatly reduced the area of rubber exposed to the vapors of the esters. It may be mentioned that the bottom portion of the distillation columns was made of glass tubing as wide as possible so as further to reduce the exposure of rubber.

The vapors from the columns passed through a small condenser and then into a receiver of the Fischer triangle type whereby fractions could be removed without interrupting the distillation. The esters were heated by means of a hot air bath and distillation was conducted at a uniform pressure of 7 to 8 mm. A rotary vacuum pump was employed.

In the first part of the distillation the small fractionating column described by Cooper and Fasce (12) was employed. By means of this the low boiling fractions were removed while, at the same time, all the malic ester was kept back. When the boiling point of malic ester was reached the small column could not carry the rush of vapor. Flooding occurred and it was necessary to interrupt the distillation and substitute a larger bore fractionating column without a dephlegmator. Pure malic ester then distilled and this was followed by fractions that contained citric ester uncontaminated by malic ester. When the boiling point of citric ester was passed the fractionating column was removed entirely and distillation was continued through a simple bent tube. A residue of less than 3 per cent remained at the completion of the process.

IDENTIFICATION OF THE ESTERS FROM MATURE GREEN LEAF

Table 6 presents the distillation data calculated to the equivalent of the original 57.27 kilos (6.65 kilos dry substance) of fresh leaf. It will be observed that a 98 per cent recovery was attained and that the malic ester is in two pure fractions. The original organic acid solution was analyzed before esterification for its malic and citric acid content. Malic acid was determined by the polarimetric method of Dunbar and Bacon (16) and citric acid

by the modified pentabromoacetone method of Hartman and Hillig (37, 38). From the results of this analysis 190 gm. of malic and 4.0 gm. of citric acid were expected. After the distillation the equivalent of 181 gm. of malic acid and 5.24 gm. of citric acid were found as esters. The agreement is excellent and indicates that the ester method may be used for the quantitative evaluation of malic and citric acids.

TABLE 6. DISTILLATION OF ESTERS OF ORGANIC ACID FROM LEAVES OF MATURE TOBACCO PLANT

57.27 kilos fresh leaf (6.65 kilos dry solids).
Weight of ether soluble acids before esterification = 386 gm.
Weight of ether soluble acids after esterification = 434 gm.
Weight of ether soluble alkali insoluble esters = 310 gm.
Pressure 7-8 mm.

Fraction	Boiling point °C	Ester gm.	Remarks
1	53-63	12.4	Impure mixture of hydrazides, no malic ester
2	88-90	52.2	Pure malic ester
3	92-96	200.0	Pure malic ester
3a	103-112	6.4	Malic acid 60%, citric acid 40%
4	138-140	9.4	No definite hydrazides
5	142-157	4.7	No definite hydrazides
6	165-175	9.5	No definite hydrazides
7 + 8	176-178	4.4	No definite hydrazides
Total distillate		299.1	
Residue		4.9	
		304.0 = 98% recovery	

Fraction 1 (b. p. 53-63°: sp. gr. 1.047 at 19°), on treatment of 0.5 gm. with excess of hydrazine hydrate (1 cc. of 42 per cent) and 10 cc. of absolute alcohol yielded no crystals until after 24 hours. After the addition of more alcohol amorphous white material separated which could not be induced to crystallize well. The melting point of this preparation after four recrystallizations was 138 to 141° (uncorr.). It was obviously a mixture. No oxalic acid dihydrazide could be detected. No additional information was secured from the benzylidene derivatives.

The esters remaining (8.15 gm.) were saponified with sodium hydroxide, and were then treated with a slight excess of sulfuric acid; 2 to 3 volumes of alcohol were then added to precipitate the sodium sulfate.[1] The alcohol was distilled off and the aqueous solution was exactly freed from sulfuric acid by means of barium hydroxide. The aqueous solution of the free acids was then subjected to fractional crystallization with results as shown

[1] This is an extremely convenient and practically quantitative method to remove sodium from a solution.

TABLE 7. FRACTIONAL CRYSTALLIZATION OF FRACTION 1

Total ester hydrolyzed = 8.15 gm.
Free acid formed = 5.10 gm., i.e., 62.7%

	gm.	%
Fumaric acid	0.42	8.2
Succinic acid	0.42	8.2
Unidentified acids	4.26	83.6

in Table 7. Fumaric acid was identified by its melting point of 283 to 284° (sealed tube); a mixed melting point with an authentic sample of fumaric acid showed no depression. Owing to its low solubility the amount of fumaric acid isolated is a fairly accurate measure of the total quantity present in this fraction. Succinic acid was identified by its melting point of 185 to 186° and by a mixed melting point with a pure specimen. The o-toluidene condensation product was also prepared; this product melted sharply at 254-255° and a mixture with a sample prepared from pure succinic acid showed no depression of the melting point. Mulliken (49) gives the melting point of this compound as 254.5 to 255.5°. Owing to its solubility the amount of succinic acid isolated does not at all represent the total amount present. It is interesting to note that the purest sample of succinic acid secured still contained some easily oxidizable impurity, for a solution of it decolorized a weak acid solution of permanganate in the cold. The proportion of impurity must have been exceedingly small, however, since the melting points were not affected.

The remainder of the acids in this fraction were very soluble in water and yielded, on concentration, either non-crystallizable syrups or an occasional crop of crystals, the melting points of which varied from 130 to 156°.

Fraction 1 contained no oxalic acid; a solution of the free acids gave no precipitate with calcium acetate. The fumaric acid amounted to about 8 per cent and the succinic acid to at least 8 per cent. The remaining 84 per cent consisted of very soluble and as yet unidentified acids.

Fractions 2 and 3 were practically pure l-diethyl malate as is shown by the following data:

Fraction	Dihydrazide m.p.	$\alpha]_D$ absolute alcohol
2	179-180°[1]	−10.34[2]
3	178.5-179.5°[1]	−10.88[3]

Curtius and von Hofe gave the melting point of l-malic acid dihydrazide as 177.5° (13).

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[1] Mixed melting point with pure specimen showed no depression.
[2] This fraction had a yellow tinge.
[3] Average of four determinations: 10.88, 10.89, 10.86, 10.90.

The optical rotations of l-diethyl malate here reported are slightly higher than those given in the literature. Frankland and Wharton (23) reported —10.44° and Vickery observed —10.46° on a sample secured from alfalfa leaves (unpublished). The free acid was obtained by hydrolysis of the esters. The melting point was 100 to 100.7° (uncorr.) and the neutralization equivalent was 68 (theory 67.1).

Fraction 3a was a small intermediate fraction that contained 63.6 per cent of malic acid, and 36.4 per cent of citric acid. The citric acid was determined by the pentabromoacetone method after hydrolysis of the esters. The malic acid was determined polarimetrically. Pure malic acid dihydrazide of melting point 179° was also isolated.

Fractions 4 to 8 did not yield solid dihydrazides. In view of this the citric acid content was quantitatively determined by the pentabromoacetone method. This is a specific reaction for citric acid and is consequently the best procedure for its identification. The data are given in Table 8.

TABLE 8. COMPOSITION OF END FRACTIONS

Fraction	Triethyl citrate[1] %	Unknown ester %
4	43.6	56.4
5	16.0	84.0
6	3.14	96.86
7 + 8	1.38	98.62

These fractions were mixtures of triethyl citrate with other esters of nearly the same boiling point. Fractions 7 and 8 were united, hydrolyzed, and the acids were fractionally crystallized; 0.56 gm. of pure fumaric acid was isolated. This acid was identified by its melting point (283 to 284°) and by analysis. Found C, 41.37; H, 3.67: calculated for $C_4H_4O_4$; C, 41.37; H, 3.45.

Fractions 4, 5, and 6 were also united and hydrolyzed. Fumaric acid to the extent of 2.6 gms. was obtained, making a total of 3.16 gm. from the high boiling fractions. Unsuccessful attempts were made to identify the other acids present. The acids did not reduce alkaline silver nitrate and were optically inactive. This indicates that isocitric and tartaric acids were absent.

So far as we are aware this is the first time that fumaric acid has been identified in a high boiling ester fraction secured from plant material. Its presence can, however, be readily accounted for. Although diethyl fumarate possesses a boiling point almost identical with that of diethyl succinate and therefore distils in the low boiling fraction, monoethyl fumarate, according to Anschütz and Drugman (2), melts at 66° and distils at 147° at a pressure

[1] In all cases the pentabromoacetone isolated melted at 70 to 72°, as did a sample prepared from pure citric acid.

of 16 mm. This boiling point is above that of diethyl malate and consequently monoethyl fumarate, if present, would be found in the citric ester fraction. Furthermore it is known that fumaric acid is difficult to esterify completely. It was noted that, in all our distillations, the oily triethyl citrate fractions always contained small flakes of solid material. This was undoubtedly monoethyl fumarate. It should be mentioned, however, that the isolation of fumaric acid from a mixture of esters that contains malic ester is not a proof that the fumaric acid was actually present in the original leaf tissue. Anschütz and Reitler (3) have pointed out that it is possible to convert malic acid in part to fumaric acid during the process of esterification if this is done at high temperature in the customary way. The small amount of fumaric acid observed in our experiments suggest, however, that this conversion is not extensive when the esterification is conducted by the Phelps and Phelps method and in any case the value of our quantitative results for malic acid is scarcely affected by this possibility.

It is clear from the above data that 57.27 kilos of fresh tobacco leaf yielded 304 gm. of organic acid esters about 90 per cent of which has been positively identified, 85 per cent as diethyl l-malate, 2.5 per cent as triethyl citrate, 1.6 per cent as monoethyl and diethyl fumarate, and about 0.2 per cent as diethyl succinate. The remaining 10.7 per cent of the esters has not as yet been identified, but there is little doubt that oxalic, malonic, tartaric, and isocitric acid were absent. Approximately 70 per cent of this unidentified group consisted of esters with boiling points close to, or above, that of triethyl citrate; the rest consisted of esters with boiling points below that of malic ester.

EXAMINATION OF PRESS CAKE FOR OXALIC ACID

Oxalic acid could not be detected in the organic acid fraction from the fresh leaf. The press cake from which the extract was prepared was therefore thoroughly digested with warm 2 N sulfuric acid and the acid extract was concentrated and extracted by ether. The ether extract yielded a small quantity of typical calcium oxalate crystals and the total amount of oxalic acid in it was determined by titration with permanganate. The results indicated that the 57.27 kilo lot of fresh leaf contained the equivalent of only 1.27 gm. of anhydrous oxalic acid.

The examination of the press cakes also presented an opportunity to test the efficiency of the initial hot water extraction. It has been claimed that malic and citric acid are present in tobacco as calcium salts. The data on the organic acids in an extract may therefore be incomplete since these substances may not have been completely soluble. The acid extract of the press cakes was found, however, to be free from both citric and malic acids, since no

pentabromoacetone could be obtained and the solution of the acids was optically inactive. It is evident that hot water extraction removed all of the organic acids with the exception of oxalic, which must therefore have been present in the leaves entirely as an insoluble salt, probably as calcium oxalate.

THE EXAMINATION OF THE BARIUM SALTS PRECIPITATE FOR OTHER ACIDIC SUBSTANCES

The barium salts precipitate from fresh tobacco leaf contained 465 gm. of organic solids. Of these only 207 gm. or 44.5 per cent was accounted for in terms of "organic acids" as usually defined. The remaining 55.5 per cent were, nevertheless, substances which, in the presence of alcohol, formed insoluble barium salts. They are therefore acidic in nature. Most of these substances are non-nitrogenous since the whole fraction contained only 10 gm. of nitrogen and an appreciable part of this nitrogen consisted of nitrate. Moreover when the barium salts fraction was titrated for organic acids by the method of Van Slyke and Palmer (82) the equivalent of 27,550 cc. of 0.2 N hydrochloric acid were required to carry the reaction from pH 8.0 to pH 3.0, whereas the total quantity of organic acids isolated as esters required only the equivalent of about 15,000 cc. of 0.2 N hydrochloric acid. The difference, 12,550 cc. or 46 per cent of the total acidity, represents organic compounds of acidic properties of unknown constitution, since special precautions were taken to remove phosphoric acid, the only inorganic acid likely to be present in plant extracts which titrates between these limits. This group of substances does not possess optical activity and consequently does not seem to be related to the acids which might be formed by the direct oxidation of carbohydrates or by the hydrolysis of the soluble pectins (e. g., uronic acids). The role that these substances play in the organic acid metabolism of the plant is at present unknown. It is safe to assume, however, that the group of substances usually referred to in the literature as plant organic acids forms less than half of the acidic organic substances present in the tobacco leaf.

It may be well to emphasize again at this point that our data refer only to the non-volatile organic acids of the extracts from tobacco leaves. We have not yet investigated the volatile acids and no titrations were carried out on the original extract because of the presence of interfering substances such as nicotine and other organic bases, and phosphoric acid. The total acidic substances in the barium salts fraction could be titrated by the Van Slyke and Palmer method because nicotine and phosphoric acid had been removed during the procedure by which this fraction was secured. We were forced to rely upon the titration of ether

extracts of the other fractions to obtain a measure of the acidity in them.

Reference to the diagram (page 174) will make clear the different steps employed in the fractionation of the acidic substances that do not form esters that can be distilled. The barium salts precipitate contained total organic acids the equivalent of 27,550 cc. of 0.2 N acid. Of this amount 21,250 cc. could be extracted by ether when tests were made on a small aliquot part. The large scale extractor employed actually removed the equivalent of 20,400 cc. and the equivalent of approximately 15,000 cc. was secured in the form of distillable esters.

The filtrates from the barium salts precipitates (A and B in the diagram) contained ether extractable acids equivalent to 6,140 cc. of 0.2 N acid, which is 22.4 per cent of the total *ether extractable* acids. Nothing has yet been learned of the nature of this acid except that a very small part of it consisted of malic acid that was not entirely thrown down when the barium salts were precipitated the second time. No appreciable part of it consisted of acids that form silver salts insoluble at pH 7.0.

Of the total acids in the barium salts precipitate the equivalent of 6,300 cc. of 0.2 N acid or 23 per cent was not extracted by ether (Ether insoluble B). This solution was concentrated to small volume and 9 volumes of absolute alcohol were added. The precipitate produced weighed 119 gm. or 25.6 per cent of the total solids of the barium salts fraction and contained the equivalent of 1,400 cc. 0.2 N acid. About half of the nitrogen of the barium salts fraction was present in this precipitate. The hygroscopic material was optically inactive; a solution of it was acid to Congo red, and did not reduce Benedict's solution.

The filtrate from this 90 per cent alcohol precipitate (Filtrate J) contained carbohydrate material. It reduced Benedict's solution and yielded a mixture of osazones. Glucose was probably present, but could not be positively identified. This filtrate contained organic acid equivalent to about 4,900 cc. of 0.2 N acid or 78 per cent of the total acidity of the fraction denoted Ether insoluble B. When a sample was treated with ferric chloride an intense green color developed and the material also produced an intense blue color with Folin and Marenzi's phosphotungstic acid reagent (22). Reducing substances were therefore present.

The ether soluble acids (A) were esterified and the esters were dissolved in ether. This solution was treated with sodium hydroxide solution but 71 per cent of the weight of the total esters remained in the ether (Fraction C). This part represents the substances ordinarily defined as plant organic acids; the identification of the acids in this fraction has already been discussed. The remaining 29 per cent of the ester fraction passed into the aqueous alkaline solution. After acidification this material was

shaken with ether, whereby it was divided into two groups, (E) and (F). The part that was readily soluble in ether yielded 19.3 gm. of a dark red oil (Fraction E) which solidified on long standing. This material was acid to Congo red and, on the addition of ferric chloride, gave an intense green color, which changed to a violet red on the addition of sodium hydroxide, a color test for caffeic acid described by Schmuck (74). Caffeic acid is dihydroxycinnamic acid and it, or its ester, should be concentrated in this fraction. An aqueous solution of the material in Fraction E reduced ammoniacal silver nitrate solution in the cold; on the addition of copper acetate a greenish precipitate was formed; bromine water gave a heavy flocculent yellow precipitate, a reaction that suggests the formation of brominated phenols. This extremely interesting fraction probably contains the aromatic hydroxyacids and phenols of the leaf. Very little information is available regarding these substances.

Fraction F contained 53 gm. of organic matter. The solution was freed from chlorides by the addition of silver oxide and dilute sulfuric acid. The filtrate was then concentrated and the sodium sulfate was precipitated by the addition of 2 to 3 volumes of alcohol. After evaporation of the alcohol the sulfuric acid was quantitatively removed by barium hydroxide. The aqueous solution was then concentrated to a syrup and dehydrated by repeated concentration with absolute alcohol. A thick viscous light yellow syrup remained, which did not solidify even after standing for several months in a calcium chloride desiccator. This oil was acid to Congo red and gave no test for sulfate or chloride; it was optically inactive, and gave no color reaction with ferric chloride. The material was therefore entirely free from the substances characteristic of Fraction E; it represents acidic substances that either failed to esterify completely or were non-esterifiable and consequently retained their solubility in aqueous alkali.*

These experiments show that the barium salts precipitate obtained from an extract of fresh tobacco leaf is a very complex mixture. It contains not only the organic acids that are generally recognized as such, but also an unexpectedly high proportion of acidic substances of unknown chemical constitution. The complexity of the barium salts precipitate can perhaps best be appreciated from the summary presented in Table 9, in which the

* Footnote Added to Proof. An examination of Fraction F has since revealed the presence of considerable malic acid. The quantity indicated by the determination of the optical activity in the presence of uranium acetate is equivalent to 18.1 gm. of this substance, but we are not yet certain that malic acid is the only optically active acid present. After saponification of the esters in this fraction a solution that contained 23.8 gm. of organic solids was obtained. The anilide of malic acid was isolated from this. It melted at 197-198° and a mixture with pure authentic malanilide of the same melting point melted at 198°.

weights of the various fractions are calculated as a function of the total organic solids of this fraction. The designations of these fractions refer to the diagram on page 174.

TABLE 9. ACIDS OF FRESH TOBACCO LEAVES EXPRESSED AS A FUNCTION OF THE TOTAL ORGANIC SOLIDS OF THE BARIUM SALTS PRECIPITATE

57.27 kilos fresh leaf (6.65 kilos dry solids).
Total organic solids of barium salts precipitate = 465 gm.

Ether extractable acids	gm.	%	
Organic acids identified from esters, Fraction C	186	40.0	44.5
Organic acids not identified from esters, Fraction C	21	4.5	
Phenolic acids and phenols, Fraction E	19.3	4.2	15.1
Non-phenolic acids, Fraction F	53.0	11.4	
Ether insoluble acids			
From Fraction I[1]	119.0	25.6	40.0
From Fraction J[2] (by difference)	66.7	14.4	

7. THE ORGANIC ACIDS OF FRESH LEAVES OF YOUNG TOBACCO PLANTS (4 TO 6 LEAVES)

A hot water extract was prepared from 10.55 kilos (802 gm. dry solids) of leaves stripped from young plants (4 to 6 leaves). The barium salts precipitate was prepared as described in the previous section. On account of the small amount of material special attention was paid only to the ordinary organic acid fraction; the weights of the other fractions were, however, recorded. In order to determine the oxalic acid, none of which was water soluble, acid extracts of the press cake were prepared. From these calcium oxalate was isolated and the quantity of oxalic acid was estimated by titration with permanganate; 1.27 gm. of anhydrous oxalic acid were found in 10.55 kilos of the fresh leaf. Because of the rather unusual crystalline form of the calcium oxalate obtained, the free acid was isolated. It separated in long white needles melting at 101° (corr.) which, when mixed with pure oxalic acid, melted at the same temperature. The calcium oxalate contained 27.5 per cent of calcium (theory 27.43 per cent). The acid extract of the press cake was found to be free from malic, citric, and tartaric acids, indicating that the hot water extraction removed all the other acids.

The data on the organic acid esters obtained from the barium salts fraction are presented in Table 10. The fractions were

[1] Contains about 50% of the total nitrogen of the barium salts precipitate.
[2] This fraction contains, in addition to acidic substances, other compounds, some of which are unquestionably carbohydrates and some of which are nitrogenous.

TABLE 10. DISTILLATION OF THE ORGANIC ACID ESTERS FROM LEAVES OF YOUNG TOBACCO PLANTS

10.55 kilos fresh leaf (802 gm. dry solids).
Total weight of esters before distillation = 41.5 gm.
Pressure 7-8 mm.

Fraction	Boiling point °C	Ester gm.	Remarks
1	56-62	1.23	Trace fumaric acid: rest unknown
2	92-93	31.0	Pure l-diethyl malate $\alpha]_D^{25} = -10.83°$
3	100-120	0.59	Malic ester with trace of citric ester
4	121-125	4.25	83.8% citric and 16.2% unknown esters, trace fumaric acid
Total distilled		37.07	
Residue		2.7	
		39.77 = 95% recovery	

treated by methods similar to those described for the fresh leaf of the adult plant. Fraction 2 was pure malic ester and yielded a dihydrazide melting at 179°. The rotation in absolute alcoholic solution was —10.83° in agreement with the determinations previously obtained and again slightly higher than the values recorded in the literature. On account of the small amounts of Fractions 1 and 3 no quantitative figures were obtained. From Fraction 1 as well as Fraction 4 small amounts of pure fumaric acid were isolated. Fraction 4 was chiefly citric acid, as shown by the weight and melting point of the pentabromoacetone obtained from it. Determinations of malic and citric acids on the original fraction before esterification showed that the esterification procedure yielded almost quantitative results.

	In Ba salts fraction gm.	Isolated through esters gm.
Malic acid	23.6	22.4
Citric acid	3.0	2.5

Young plants contain oxalic acid apparently entirely in the form of water insoluble salts. The distilled esters consisted to the extent of 79.5 per cent of l-diethyl malate, 9.0 per cent of triethyl citrate and about 12 per cent of esters of unknown acids, most of which distilled in the citric acid fraction. A small quantity of fumaric acid was found.

The total ether soluble acids in the barium salts precipitate were equivalent to 3,375 cc. 0.2 N hydrochloric acid. Of this about 1,960 cc., or 58 per cent, could be accounted for as ordinary organic acids. The remaining 42 per cent belongs to the groups of acidic compounds discussed more fully in the previous section. A phenolic acid fraction that weighed 2.1 gms. was obtained; this gave a strong test for caffeic acid with ferric chloride and sodium hydroxide. The non-phenolic acids residue weighed 17.4 gm. No attempts at further fractionation were made.

8. THE ORGANIC ACIDS OF CURED TOBACCO

An aqueous extract was prepared from 1,828 gm. (1,462 gm. dry solids) of cured tobacco that had been obtained from the crop upon which the mature fresh leaf studies were made. The barium salts fraction was secured exactly as described for the fresh leaf extract. It contained:

Total solids	163 gm.
Organic solids	160.3 gm.
Inorganic solids	2.7 gm.
Total nitrogen	7.04 gm.
Total acids titrating between pH 8.0-3.0	9,750 cc. 0.2 N HCl
Total acids extracted by ether	6,600 cc. 0.2 N HCl = 69%
Total acids not extracted by ether	3,150 cc. 0.2 N HCl = 31%

No oxalic acid could be detected in the ester fractions but the equivalent of 0.61 gm. of anhydrous oxalic acid was found in the press cake by permanganate titration of the calcium salt obtained as previously described. The acid extract of the press cake contained neither malic nor citric acid.

Table 11 shows the composition of the fractions secured from the distillation of the esters. About 70 per cent of the esters

TABLE 11. DISTILLATION OF THE ORGANIC ACID ESTERS FROM CURED TOBACCO LEAF[1]

1,828 gm. material (1,462 gm. dry solids).
Total weight of esters before distillation = 64 gm.
Pressure 7-8 mm.

Fraction	Boiling point °C	Ester gm.	Remarks
1	55-67	3.64	No oxalic, trace fumaric
2	90-93	43.0	Pure l-diethyl malate, $\alpha]_D^{25} = -10.53°$
3	106-116	0.64	Chiefly malic ester, trace citric ester
4	120-124	14.6	66.9% citric ester, 33.1% unknown of which a small part is fumaric acid
Total distilled		61.9	
Residue		1.03	
		62.93 = 96.7% recovery	

isolated was identified as l-diethyl malate as the dihydrazide; 15 per cent consisted of triethyl citrate (pentabromoacetone) while 15 per cent was not identified although a small part of this consisted of fumaric acid.

The barium salts fraction contained ether soluble acids equiva-

[1] The methods of identification were similar to those described under mature fresh leaf. Fumaric acid was isolated from both Fractions 1 and 4 in small amounts.

lent to 6,600 cc. of 0.2 N hydrochloric acid; 65 per cent (the equivalent of 4,300 cc.) was accounted for in the ester fraction. The remaining 35 per cent belonged to groups of acidic substances of unknown nature. The so-called phenol fraction contained 7.0 gm. of material. This differed in its qualitative reactions from that isolated from the fresh leaves of either young or old plants. It gave no color test for caffeic acid and only a trace of color with the Folin and Marenzi reagent, a behavior which suggests that, during curing, the highly reactive substances that were found in fresh leaves disappeared. The residue from the phenol fraction yielded 29.0 gm. of an optically inactive syrup that was acid to Congo red.

The data summarized in Table 12 show that 27.6 per cent of the organic solids of the barium salts precipitate was present as organic acids of the usual type, and 22.5 per cent of the solids

TABLE 12. ACIDS IN CURED TOBACCO LEAF EXPRESSED AS A FUNCTION OF THE TOTAL ORGANIC SOLIDS OF THE BARIUM SALTS PRECIPITATE

1,828 gm. material (1,462 gm. dry solids).
Total organic solids in barium salts precipitate = 160.3 gm.

	gm.	%
Organic acids calculated from esters, Fraction A[1]	44.0	27.6
Phenol fraction, Fraction E[1]	7.0	4.4
Non-phenolic acids, Fraction F[1]	29.0	18.1
Ether insoluble acids,[2] Fraction I[1]	45 0	28.1
Other substance not titrating between pH 8.0-3.0	25.0	15.6
Not accounted for	10.3	6.2

was acidic and extractable by ether, but belonged to a different class. About 28 per cent consisted of acidic substances not extractable by ether. The remaining 15 per cent represented substances that did not titrate between pH 8.0-3.0, but which were precipitable by barium and alcohol. These substances are not ordinary carbohydrates, since this fraction, in contrast to that from the fresh leaf, did not reduce Benedict's solution and it gave no well crystallized osazones.

9. THE ORGANIC ACIDS OF TOBACCO SEED

No previous attempts to investigate the organic acids present in tobacco seed have come to our attention. A hot water extract was prepared from a quantity of finely ground fat-free seed equivalent to 5.1 kilos of air dry seed. The barium salts fraction was

[1] Designations of the fractions refer to a procedure analogous to that given in Diagram 1, page 174.

[2] This is an approximation derived on the assumption that the cc. 0.2 N hydrochloric acid in the ether insoluble fraction is equivalent to malic acid.

prepared and analyzed in the manner already described. The data obtained on the organic acid ester fraction are given in Table 13.

TABLE 13. DISTILLATION OF THE ORGANIC ACID ESTERS FROM TOBACCO SEED

5 1 kilos seed (2.99 kilos fat-free seed).
Total ester = 11.8 gm.
Pressure 7-8 gm.

Fraction	Boiling point °C	Ester gm.	Remarks
1	73-91	1.66	Trace fumaric acid, 44% malic ester
2	101-126	0.40	Chiefly citric ester
3	128-130	7.95	88.5% triethyl citrate
Total distilled		10.01	
Residue		1.15	
		11.16 = 97% recovery	

Fraction 1, when treated with hydrazine hydrate, slowly deposited a hydrazide which was not very soluble in absolute alcohol. On recrystallization radiating masses of long needles were obtained, which, examined under high power, seemed to contain small dark nodules. The material, after another recrystallization, melted sharply at 176°; further recrystallization neither changed the melting point nor the crystalline habit. The solubility was about one part in 800 parts of absolute alcohol, identical with the solubility of l-malic acid dihydrazide given by Franzen and Helwert (24); furthermore, when mixed with pure l-malic acid dihydrazide no depression of the melting point occurred. These data indicate that Fraction 1 contains malic acid, although the behavior of the derivative on crystallization was quite different from that usually observed.

In order to ascertain the proportion of malic acid in Fraction 1, 1.12 gm. of the ester were hydrolyzed and subjected to fractional crystallization. A quantity of fumaric acid just sufficient for a melting point determination was obtained. The filtrate on further concentration yielded a viscous oil. The rotation of this material in the presence of uranium acetate corresponded to the presence of malic acid to the extent of 44 per cent of the weight of the fraction.

Fraction 3 contained 88.5 per cent of triethyl citrate on the basis of the pentabromoacetone isolated. This melted at 70 to 72° and showed no depression of melting point when mixed with pure material.

The above data show that the organic acids of tobacco seed are chiefly citric with some malic. Traces of fumaric acid were found. These acids are accompanied by small amounts of unidentified acids, the esters of which distil in the high boiling fractions.

A phenolic fraction which weighed 0.81 gm. was also obtained. It gave a strong test for caffeic acid with ferric chloride and sodium hydroxide and also an intense color with Folin and Marenzi's phosphotungstic acid reagent.

10. DISCUSSION

The data that have been presented are further summarized in Table 14. They constitute, as far as we are aware, the first attempt to express the composition of the organic acid fraction secured from green leaves in terms which are founded upon analyses conducted with quantitative rigor and which also involve the

TABLE 14. CONCENTRATION OF ORGANIC ACIDS IN CONNECTICUT SHADE-GROWN TOBACCO EXPRESSED AS A PERCENTAGE OF THE DRY WEIGHT OF THE TISSUES

Acid	Seed	Young plant	Mature plant	Cured leaf
	%	%	%	%
Oxalic (anhydrous)	0.012[1]	0.078	0.019	0.06
Succinic	.....	.....	0.01	.....
Fumaric[2]	+	+	0.057	+
l-malic	0.010	2.76	2.72*	2.11
Citric (anhydrous)	0.102	0.31	0.082	0.47
Unknown acids,[3] low boiling esters	0.012	0.103	0.103	0.174
Unknown acids,[4] high boiling esters	0.013	0.059	0.190	0.25
Total acids	0.149	3.31	3.18	3.06

isolation of the chief constituent substances in pure form. Although the absolute magnitude of the figures may be slightly altered by subsequent refinements of technique, the greater part of the organic acids of the tobacco leaf, as this term is ordinarily used, has been isolated in fractions whose chemical identity has been established and the relative proportions in which these acids occur has been ascertained. The experimental evidence which supports the quantitative interpretation of our data may be summarized as follows:

[1] This figure is probably low, inasmuch as the press cake was not examined.

[2] It is possible that the fumaric acid isolated in these experiments was an artifact derived from the malic acid as a result of loss of water during the esterification process.

[3] Calculated as fumaric acid.

[4] Calculated as citric acid. The above figure represents only the data calculated from the esters that were distilled. If the distillation residue were included, the percentage of unknown acids would be somewhat higher.

* FOOTNOTE ADDED TO PROOF. The discovery of an appreciable quantity of malic acid in Fraction F (Diagram 1) indicates that this figure is somewhat too low. If it be assumed that malic acid is the only optically active acid in Fraction F, the proportion of malic acid in the mature plant should be 2.99 per cent, and the proportion of total acids will therefore become 3.45 per cent.

1. Investigation of the residues of extracted leaves showed that citric, malic, and tartaric acids were absent. Furthermore, with the exception of oxalic acid, the press cakes contained no acids that form silver salts insoluble at pH 7.0 to 8.0. Oxalic acid could not be detected in the aqueous extracts. The hot water extracts examined therefore contained all of the organic acid the esters of which could be distilled, and all of the oxalic acid was retained in the press cakes as an insoluble salt.
2. Tables 4 and 5 show that organic acids of the usual type can be quantitatively precipitated both from pure solution as well as from tobacco extracts by means of barium hydroxide and alcohol.
3. Titration of the total organic acids by the method of Van Slyke and Palmer together with studies of the efficiency of the ether extraction apparatus used indicate that the ether soluble acids in the barium salts fraction were recovered from the barium salts to the extent of at least 90 per cent.
4. The sum of the malic and citric acids isolated as esters agrees with the quantity estimated by indirect methods before esterification, indicating that the esterification and subsequent fractional distillation were conducted without significant loss.

The concentration of oxalic acid in Connecticut shade-grown tobacco is much smaller than that reported in the Russian literature for Oriental varieties of tobacco or by Garner, Bacon and Foubert (31) for Connecticut tobacco. Schmuck (74) gave values ranging from 1.02 to 2.26 per cent for cured leaf, Garner gave figures from 1.47 to 2.55 per cent for dry green leaf. These results are in marked contrast to our value of 0.06 per cent. Comparison of the data in this way is, however, meaningless. The indirect methods employed by the Russian workers and by Garner, Bacon and Foubert furnish a measure of the substances that form insoluble calcium compounds under specific conditions. The weight of the calcium oxide obtained by the ignition of the precipitate is calculated in terms of oxalic acid, but no criteria for the purity or homogeneity of the calcium precipitate were applied. Smirnow has stated that oxalic acid occurs in the young plant, is absent in the mature leaf, but appears again in relatively large quantities in the cured sample. Our data show that oxalic acid is found in all stages of development, but the percentage concentration is maximal in the young plant, decreases in the mature plant and increases again during the curing process.

Malic acid attains a maximum concentration in the leaf of the young plant that is almost 400 times that found in the tobacco seed. Our data on Connecticut tobacco do not confirm those of Smirnow, Drboglow and Maschkowzen (79) who state that in older leaves there is an increase of malic acid. On the contrary an increase of malic acid occurs in the early life of the plant and this acid has already attained its maximum concentration in a young plant that bears only 5 to 6 leaves. After this it seems to

remain nearly constant until the leaf is subjected to the curing process.

It is interesting to note that the maximum malic acid concentration is attained long before the nicotine has reached its highest level. This suggests that the production of malic acid is independent of nicotine synthesis and that, in the young plant, malic acid must be combined to a large extent with bases other than nicotine. A consideration of the dissociation curves of nicotine (86) shows that nicotine is largely combined with acidic groups at the reaction of the leaf tissue, and it is usually assumed that, in the mature leaf, nicotine is present in the form of nicotine malate. It is evident that a study of the inorganic salts of malic acid and their behavior in the presence of nicotine might be of considerable interest.

Citric acid is the predominating acid of the tobacco seed and forms, as shown by Table 15, 68.5 per cent of the total acids obtained. There is a marked increase in the proportion of this acid in the young leaf, which is followed by a marked diminution

TABLE 15. DISTRIBUTION OF ORGANIC ACIDS IN CONNECTICUT SHADE-GROWN TOBACCO EXPRESSED AS A PERCENTAGE OF THE TOTAL ACIDS ISOLATED

Acid	Seed	Young plant	Mature plant*	Cured leaf
	%	%	%	%
Oxalic (anhydrous)	8.1[1]	2.4	0.6	1.9
Succinic	....	....	0.3	....
Fumaric	+	+	1.8	+
l-malic	6.7	83.4	85.5	69.0
Citric (anhydrous)	68.5	9.4	2.6	15.4
Unknown acids,[2] low boiling esters	8.0	3.1	3.2	5.5
Unknown acids,[3] high boiling esters	8.7	1.7	6.0	8.2

in the adult leaf. This again, as in the case of malic acid, suggests that valuable information on the metabolism of organic acids is to be obtained from the study of the early stages of growth of the plant. During curing of the leaf there is a marked increase of citric acid. These results are unlike those obtained by Smirnow on Oriental tobacco. He observed a considerable increase of citric acid in the leaf at the stage of technical ripeness.

Fumaric acid has been isolated in small amounts from all samples studied, but no interpretation of the results can be attempted at the present stage of this study. All that can be said is that fumaric acid was obtained in larger proportion from the

[1] Low due to losses in press cake.
[2] Calculated as fumaric acid.
[3] Calculated as citric acid. See note Table 14.

* FOOTNOTE ADDED TO PROOF. The small amount of malic acid found in Fraction F (Diagram 1) makes very little change in the figures in this table. The proportion of malic acid is increased to 86.6 per cent, citric acid is decreased to 2.4 per cent, and the proportions of unknown acids of low and high boiling esters become 3.0 and 5.5 per cent respectively.

young and adult fresh leaf than from the seed. The possibility that it may have been produced from malic acid during the esterification process renders a discussion of its metabolism futile. Fumaric acid has not hitherto been reported as being present in the fresh leaves of the tobacco plant. Appreciable quantities of unknown esterifiable acids are also present in tobacco, the greater part of these being found in the high boiling ester fractions along with citric acid. A marked increase in the proportion of these unknown acids occurs between the young leaf and mature leaf stage.

TABLE 16. FRACTIONS OBTAINED FROM THE BARIUM SALTS PRECIPITATE EXPRESSED AS PERCENTAGE OF THE ORGANIC SOLIDS OF THIS PRECIPITATE

	Fresh leaf %		Cured leaf %	
Organic acids from esters	44.5		27.6	
Phenol fraction	4.2		4.4	
Non-phenolic acids	11.4	55.6	18.1	66.2
Ether insoluble acids	25.6		28.1	
Substances not titrating between pH 8.0-3.0	14.4		15.6	

In addition to the organic acids of the familiar type a large group of acidic substances of unknown composition occurs in tobacco leaves at all stages of growth. Their relative proportions can be judged from Table 16, in which the distribution is expressed in terms of the total solids of the barium salts fraction. About 56 per cent of the substances in the barium salts fraction of fresh leaf do not belong to the group of substances ordinarily referred to as plant acids. In the cured leaf the percentage is even higher. It is clear that the barium salts fraction from tobacco leaves is an extremely complex mixture; it is our purpose to subject it to further detailed study.

11. ETHER EXTRACTION OF THE ORGANIC ACIDS

LARGE SCALE CONTINUOUS EXTRACTOR

The partition coefficients between ether and water of the organic acids that are usually found in plants are very high. The coefficient for citric acid is in the neighborhood of 200 and that for tartaric acid is about the same. It is essential therefore to have available an efficient continuous liquid extraction apparatus if these acids are to be removed quantitatively from aqueous solution. Palkin, Murray and Watkins (57) have shown that, for substances of high partition coefficient, the most efficient liquid extractor is one in which the solvent enters the aqueous fluid wholly or partly in the form of vapor, thereby producing vigorous agitation. A convenient modification of an apparatus originally described by Hagemann (34), which is capable of extracting 1.5 to 2 liters of fluid, is represented in Figure 11.

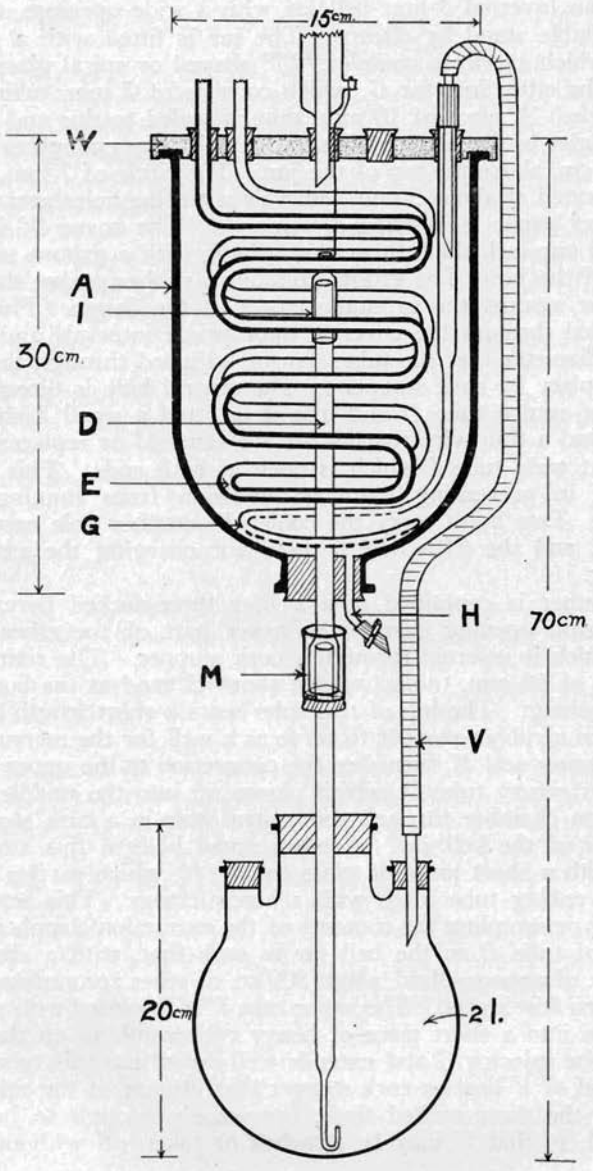

FIGURE 11. Continuous liquid extraction apparatus of the Hagemann type suitable for the extraction of 1.5 to 2 liters of aqueous fluid with ether.

A is an inverted 3 liter bell jar with a wide opening, attached to a suitable stand by clamps. The jar is fitted with a wooden cover, which carries a complex "U" shaped or spiral glass cooler *E* and the ether injector *G*, which consists of 7 mm. tubing bent in a circle. A piece of 10 mm. tube is sealed to this and bent at right angles to the plane of the circle; it is long enough to extend about 2 cm. above the top of the jar. The circle of 7 mm. tubing is perforated at about 5 mm. intervals with fine holes arranged so that ether vapor is injected downwards. The cover *W* is made of wood one inch thick turned on a lathe with a groove to fit the flange of the jar. The groove contains a rubber gasket that seals the cover against the ground surface of the flange. Five holes are drilled through the cover at appropriate intervals and are of such a diameter that the tubes can be adjusted through them and held in place by cork stoppers. The central hole is directly over the ether return tube *D* and into it is fitted a small Liebig condenser and a thin wire that assists the removal or replacement of the short wide tube *I* which is open at both ends. This tube is valuable in preventing foam or emulsion from running down tube *D*. Two holes carry the cooler *E*, another hole carries the injector, and the fifth hole is used for charging the extraction chamber.

The ether is contained in a 2 liter three-necked pyrex flask. The central opening carries the lower part of the ether return tube, which is inserted through a cork stopper. The return tube is made of 10 mm. tubing with a short U bend at the bottom of 4 mm. tubing. The top of this tube bears a short length of wide tubing on a rubber stopper to serve as a well for the mercury seal. The mercury seal *M* furnishes the connection to the upper part of the ether return tube *D*, which passes up into the middle of the extraction chamber through the central hole in a cork stopper in the neck of the bell jar. Another small hole in this stopper is fitted with a short piece of glass tubing *H*, which carries a stopcock or rubber tube fitted with a screw clamp. This serves for draining or sampling the contents of the extraction chamber. The height of tube *D* in the bell jar is such that, with a charge of 1,500 cc. of aqueous fluid, about 500 cc. of ether accumulate before the return flow starts. The vapor tube *V* is insulated with asbestos and slips into a short piece of heavy rubber tubing on the upper end of the injector *G* and extends well down into this tube. The other end of *V* bears a cork stopper that fits one of the side openings of the three-necked flask, but loosely enough to be easily removed, so that *V* may be attached or taken off without undue strain.

The three-necked flask is charged with about 1,000 cc. of ether and a few angular quartz pebbles; it is set in a wire basket immersed in an appropriate air or water bath, heated by an

electric hot plate, directly under the extraction chamber *A*, and adjusted to such a height that the lower end of return tube *D* just clears the top of the mercury well. The bell jar is lowered until *D* dips almost to the bottom of the mercury seal. Mercury is then placed in the annular space. The liquid to be extracted is poured into the jar through the appropriate opening in the top of the cover, ether is added until it overflows down tube *D* and the cap *I* is adjusted over the upper end of *D*. The lower end of the vapor tube *V* is placed loosely into the side opening of the flask and the upper end attached to the top of the injector tube *G*, the cork is then tightened into the neck of the flask. All corks may be sealed in place with a paste of litharge and glycerine, which is insoluble in ether but can be easily removed, when desired, by means of a thin bladed spatula. The water is then turned on and the heating is begun.

The extractor, when running properly, requires little attention; occasionally ether must be added. If the extract becomes too concentrated it may be removed without dismantling the apparatus through the third opening of the extraction flask by means of a siphon. When the extraction is completed, the stopper of the vapor tube *V* is loosened and the jar raised until *D* is out of the mercury seal. The contents of the jar are drained through *H;* after rinsing, the apparatus is ready for a fresh charge. The extraction apparatus can be set up permanently; it need only be dismantled for occasional cleaning, since charging and discharging are accomplished through suitable openings.

The efficiency of this apparatus can be judged from Table 17, which shows the rate of extraction of 1,500 cc. of an aqueous solution that contained 30 gm. of citric acid. The data were obtained by removing small samples through tube *H* and titrating.

TABLE 17. RATE OF EXTRACTION OF 30 GM. OF CITRIC ACID FROM 1,500 CC. OF SOLUTION

Time hrs.	Extracted %	Time hrs.	Extracted %
6	12	116	64
16	16	136	71
26	21	160	78
42	30	186	82
66	43	216	88
90	54	252	94

SMALL SCALE CONTINUOUS EXTRACTOR

Small volumes of fluid (10 to 50 cc.) may be quickly extracted with ether by converting an ordinary Soxhlet apparatus into an all-glass continuous liquid extractor by means of the attachment shown in Figure 12.

A is a short wide test tube with an opening blown in the side

about 2 cm. from the top. *B* is a Folin aeration tube, cut to fit the test tube, to the top of which is sealed a short length of 12 to 14 mm. glass tubing. This tube is held in place in the test tube by a thin slice of cork. The liquid to be extracted is placed in the test tube, which is lowered into the extraction chamber of a Soxhlet apparatus. The condenser is then adjusted so that the return stream of ether will fall into the flare on the aeration tube.

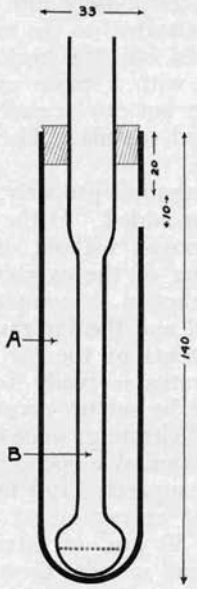

FIGURE 12. Device to be placed in Soxhlet extraction apparatus to convert this into a continuous liquid extraction apparatus suitable for small volume of fluid.

The extraction flask is charged with ether which, as it refluxes, drops through the aeration tube, rises through the liquid in the test tube, overflows through the opening near the top and fills the annular space around the test tube. When the ether has reached the level of the Soxhlet siphon it is returned in the ordinary way to the extraction flask. The efficiency of this apparatus is seen from the data recorded in Table 18.

The large continuous extractor was utilized for extracting the main organic acid fraction, and small extractors were utilized for obtaining data on the ether soluble acids in aliquot parts of the other fractions.

TABLE 18. EXTRACTION OF CITRIC ACID WITH THE SMALL LIQUID EXTRACTOR

Volume of solution	Citric acid	Time of extraction	Recovery
cc.	gm.	hrs.	%
20	0.2	43	99.2
20	0.2	43	99.0
25	1.0	49	94.0
25	1 0	49	97.0

12. THE ESTIMATION OF ORGANIC ACIDS BY TITRATION

The Van Slyke and Palmer (82) method for the titration of organic acids in urine was applied to the various acid fractions secured from tobacco extracts. An aliquot part of the solution to be investigated was diluted with water until the color was reduced to a light brown or yellow; usually a 1:5 or 1:10 dilution was sufficient. A preliminary titration was carried out on a 5 or 10 cc. portion of this diluted solution in order to ascertain the approximate acidity. From the result of this titration the size of the aliquot part which should contain organic acids equivalent to 10 to 15 cc. of 0.2 N hydrochloric acid was determined and the necessary amount was pipetted into 100 cc. centrifuge tubes graduated at 60 cc.; 5 cc. of alcohol and 0.5 cc. of a 1 per cent phenolphthalein solution were added. 2.0 N sodium hydroxide solution was then added from a pipette until a permanent red color was obtained. The reaction was then adjusted with 0.2 N hydrochloric acid until the pink color just disappeared. It is often convenient, at this point, to use a comparison tube that contains an equivalent volume of the unknown solution prepared as described, but to which no alkali has been added. Five drops of 0.04 per cent solution of brom phenol blue were introduced and the solution titrated rapidly with 0.2 N hydrochloric acid to the disappearance of the blue color; 5 cc. of 0.02 per cent aqueous solution of tropeolin 00 were then added and the titration continued exactly as described by Van Slyke and Palmer until a red color was attained equal in intensity to that of a comparison solution that contains 0.6 cc. 0.2 N hydrochloric acid, 5 cc. alcohol, 5 drops brom phenol blue, 5 cc. tropeolin 00 and water to a total volume of 60 cc. Duplicate titrations should agree within 0.2 cc. of 0.2 N hydrochloric acid.

13. SUMMARY

1. A review of the literature on the formation of organic acids in plants and a critique of their occurrence and estimation in tobacco have been presented.

2. A procedure for the precipitation of the barium salts of the

organic acids by means of alcohol has been devised and found to yield quantitative results with respect to the organic acids of which the esters can be distilled.

3. The barium salts precipitate is a complex mixture. The acids may be separated into a number of fractions of which, in the case of tobacco leaves, the organic acids capable of esterification and fractional distillation form less than half. Data are presented on the distribution and qualitative chemical behavior of the other acidic substances.

4. Experimental evidence is presented to show that the ether extraction and esterification method employed by Franzen and his co-workers may be used not only for qualitative but also for approximately quantitative evaluation of the organic acids in extracts from plant tissues.

5. The identity of the chief organic acids of Connecticut shade-grown tobacco has been established and their quantitative distribution ascertained. l-Malic is the predominating acid of fresh leaves and makes up about 85 per cent of the acids isolated as esters. A considerable part of the remainder is citric acid. Fumaric, succinic, and oxalic acids were found in small amounts. About 10 per cent of the organic acid esters isolated were derived from acids that have not as yet been identified.

6. Citric acid is the predominating acid of tobacco seed. It is accompanied by small amounts of malic and fumaric acids.

7. Oxalic acid decreases, but does not entirely disappear during the development of the plant, as was found by Smirnow in the case of Oriental tobacco. Malic acid attains its maximum concentration in the young plant and the proportion does not change appreciably in the mature leaf. The concentration decreases during curing. The concentration of citric acid is also maximal in the young plant, decreasing markedly in the mature fresh leaf and again increasing during the curing process.

8. Efficient and simple continuous ether-extraction apparatus for large and small quantities of fluid are described.

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