THE BIO-GENESIS OF MAHUA OIL

By Gilbert J. Fowler and Talwar Dinanath.

INTRODUCTION.

The investigation described in the following paper was in continuation of former researches on the bio-chemistry of the flowers and fruit of *Bassia longifolia.*[2] The technical importance of these products and their general characteristics are outlined in the papers in question.

Much work has been done on the development of oil in seeds, notably by Hills and Hollingshead,[3] Hartwich and Uhlmann,[4] and others, chiefly in connection with the production of oil in the olive. The general theory of oil-formation arising from these researches is that carbohydrates break down in such a way as to yield glycerides, though it has been suggested also that tannins are the immediate precursors of fats and oils.

The trees from which the material was obtained for the present investigation being situated in convenient proximity to the laboratories of the Indian Institute of Science, samples of the fruit could be examined at intervals more frequent than were possible to other workers and characteristic changes which might otherwise be overlooked were brought under observation. Moreover, chemical examination by ordinary methods of analysis was correlated with micro-chemical examination of fresh and prepared sections.

The following programme was carried out :—

1. Weekly micro-chemical examination of the fresh sections from (*a*) seed (*b*) pulp or husk (*c*) stalk and (*d*) stem, for (i) starch (ii) tannins (iii) proteins (iv) lignins, and (v) cellulose.

2. Weekly enzyme analysis in fresh samples of (*a*) seed and (*b*) husk for oxidases, catalase and amylase.

3. Chemical analysis of the killed and fixed weekly samples of (*a*) seed and (*b*) husk for (i) oil-content (ii) sugars, mono and di-saccharides (iii) tannins, and (iv) starch.

[1] An expanded account of the work has been submitted by one of us (T. D.) as a thesis for the M.Sc. degree of the University of Bombay, and complete analytical details will be found in that dissertation.

[2] Fowler and others, This *Journal*, 1920, 3, 81. Fowler and Dinanath, *ibid.*, 1923, 6, 131.

[3] *Bull.* 803, U. S. A. *Dept. Ag.*

[4] *Arch. Pharm.*, 1902, 240, 471,

4. Determination of proteins in samples collected every week and dried immediately at 90–95°.

5. Examination of the oil obtained every week (3, i, above) for (i) refractive index (ii) acid value (iii) iodine value.

6. Microchemical examination of the fixed and killed weekly samples after embedding them in paraffin and staining the sections for (i) starch, cell walls and proteins (ii) tannins.

EXPERIMENTAL.

The following are the more important details observed in the various methods employed :—

General.—Samples were taken regularly at intervals of a week from June 21, to August 23, 1922, when no fruit remained on the tree. All the samples were taken from the same side of the same tree, from about the same height, and at about the same time of day throughout. About fifty to sixty fruits were brought in every Wednesday at about 9 a.m., with accompanying leaves, stem and stalk. Twenty to twenty-five fruits of all sizes were taken from all over the branches, removed at once from their stalks, packed in a wide-mouthed bottle and covered with 70 per cent. alcohol, containing 6 per cent. of its volume of formalin (10 per cent.) the bottle being then shaken at short intervals for 2–3 hours. The samples thus preserved were labelled and set apart for analysis.

An equal number were dried in a steam oven at 90–95° and separated into husks and seeds, which were powdered separately, and kept in tight-stoppered bottles for the estimation of nitrogen. About ten more fresh fruits were separated with a clean knife into seed and husk, each of which was then ground with pure washed sand and a little thymol, and examined for enzymes, after soaking in 50 c.c. of water for about two hours.

Hand sections of the seeds, husk (or pulp) and stalk were taken from the remaining fruits and examined under the microscope after appropriate staining. The sections were then fixed in glycerine jelly, sealed with shellac varnish, and kept for reference. Further, as described later, permanent microtome sections were made from the fruits preserved in alcohol and formalin.

In addition to the above routine samples, one sample (X) was taken from a second tree, and two (XI and XII) from a third in order to note whether any important differences exist between the fruit of separate trees. Another sample (XIII) was taken in May, 1923, when the fruits were very young. All chemical determinations were made in duplicate.

Micro-chemical Analysis.—The hand sections were examined systematically by staining for starch, tannins, cellular tissue and lignin the usual stains being used as given in the text-books.

To determine the presence of oils, satisfactory staining agents were not available. By addition, first of ether, and then of a drop of water to the dried section, droplets of oil could be clearly seen under the microscope.

No satisfactory results were obtained by any of the methods recommended in the literature for the micro-chemical detection of sugars.

The preliminary hand-section methods above described give results which are only very roughly quantitative but they serve the useful purpose of indicating the general variation and locality of the different constituents, thus providing an introduction to complete analysis. The stains, however, are many of them very fugitive and consequently are unsuitable for sustained comparison of sections over a prolonged period. Further, in the riper samples, the hardness of the seed-coat as compared with the rest of the specimen, makes the freehand cutting of a complete section almost impossible.

In consequence, the middle portion of a fruit from each weekly bottle of fruits, fixed in alcohol and formalin as described, was embedded in paraffin, and 30 μ sections cut with a microtome. By staining all the weekly sections at once, the stained constituents can be compared before the stain begins to fade. For the production of uniformly faultless sections a careful routine is necessary, and was reached after some failures. The following are the details of the process finally employed :—Sections about 1·5 cm. thick were cut from the middle of fruits taken from the fixing liquid, transferred at once to small cylindrical stoppered weighing-bottles, and covered with 92 per cent. alcohol. After twenty-four hours this alcohol was decanted and replaced by fresh 92 per cent. alcohol. Next day, at the same time, this was replaced by 98 per cent. alcohol. The next stages may be followed from the actual notes of one sample, all dates referring to February 1923 :—12th–13th, 98 per cent. alcohol ; 14th–15th, absolute alcohol ; 16th, xylol plus absolute alcohol (1 : 1) ; 17th, xylol ; 19th–21st, xylol plus increasing quantities of paraffin till a semi-solid consistency is reached ; 22nd–27th, kept in embedding bath at 60° with stoppers removed, with daily changes of paraffin. The embedded sections were fixed to dry warm slides with Meyer's albumin, and passed through the usual stages of xylol, xylol plus absolute alcohol, and dilute alcohol, to the aqueous stain and back again through the same stages, to xylol, after clearing in which they were fixed with Canada Balsam under a cover glass.

Attempts were made to photograph the sections thus obtained, but the image of the complete section was not large enough for satisfactory definition, quarter-plate being the largest size available in the laboratory for micro-photography.

The sections, however, gave perfect definition under the microscope, and many of the stained specimens had a very beautiful appearance. The results of micro-chemical examination, including freehand and microtome sections, may be summarised as follows :—

Starch, tannins, cellulose and lignin were sought for in the seed, the husk, the stalk and the stem.

Starch was found to be practically absent from the seed at all stages. The husk contained far more, the blue iodine reaction being obtained at all stages, a maximum being evident about the middle of the series. In the stalk the amount was much less, and was roughly inversely proportional to that present in the husk. It is practically absent from the stem.

Tannins are present in the seed at all stages, but in small amount. In the husk tannins are present in large quantity—throughout tending to concentrate at the outer edge of the section and showing a maximum at the middle of the series. Tannins are present both in the stalk and stem tending to an increase in quantity and a more general distribution over the section with the successive stages.

Cellulose (including 'hemi-cellulose') is present initially in the seed but tends to decrease throughout the series. In the husk it is found in greatest quantity in the samples approaching the middle of the series. It is present in moderate quantities both in stalk and stem.

Lignin is practically absent from the seed at all stages. In the husk it is concentrated mainly at the edge of the section and at 'eyes', which are evidently sections of fibre. It is present in definite rings in the stalk and stem.

Examination for Enzymes.—As already mentioned, fresh fruits were used; they were brought into the laboratory every Thursday, there not being time for their examination when the bulk of the samples were handled on the Wednesday. The fruits plucked from their stalks were separated into husks and seeds, ten grams of each being washed with distilled water, and ground separately with an ample amount of purified sand, together with five grams of hide-powder to remove tannins. 50 c.c. of distilled water were added to each, together with a fragment of thymol, and the whole allowed to remain in the mortar for about 1½ hours, after which the extract was

filtered through coarse muslin. In view of the general composition of the fruit, as revealed by preliminary examination, it was considered sufficient to examine for oxidases and for amylase.

Oxidases and peroxidases were tested for by means of a freshly prepared 1 per cent. solution of guaiacum resin, together with hydrogen peroxide.

The method chosen after some preliminary trials, for the detection of amylase, was the following modification by Wohlgemuth[1] of one proposed by Roberts.[2] 5 c.c. of a 1 per cent. solution of soluble starch was placed in each of 3 test-tubes, and 1 c.c., 0·5 c.c. and 1 c.c. of the enzyme extract added to each respectively, the extract being boiled before it was added to the third tube which thus served for control. The three tubes for the seed and the three for the husk were then placed in a water bath at 40° for half-an-hour, after which they were filled with distilled water, and a drop of N/10 iodine solution added. The tubes were well shaken and the depth of colour produced in each noted. The tube with a faint violet colour represented complete hydrolysis of the 5 c.c. of starch. Thus by using the same weight of the material and the same concentration of starch and iodine solutions, and by keeping the other conditions approximately the same throughout, rise and fall of the enzyme could be roughly estimated week by week.

Practically no evidence was obtained of the presence of an oxidase at any stage either in seed or husk. Amylase was present at almost all stages in the seed, increasing to a maximum at sample No. VIII; it was also found in the husk, the maximum occurring at a stage earlier than in the seed.

Chemical Analysis.

General.—The fruits, killed and fixed week by week as described, were separated by a clean sharp knife into seed, husk and shell, which were dried at 70° for seventy-two hours in a closed incubator. The seeds were cut into small pieces before drying, and the husks and shells powdered when dry. Preliminary experiments showed that there was no appreciable further loss of weight after three days at 72°.

The seeds were extracted by solvents separately from the husks and shells, after it had been found by experiment that direct extraction of the undried substance was unsatisfactory.

[1] *Biochem. Zeit.*, 1908, 9, 1.
[2] *Proc. Roy. Soc.*, 1881, 32, 145.

The general plan of analysis was based on that devised by Prescott,[1] and consisted briefly in extracting the thoroughly dry material (about five to eight grams) obtained as above, first with chloroform, and then with alcohol (s.g. 0·848) and examining the two extracts obtained, the first (containing the fatty bodies) for acid value, iodine value and refractive index, and the second for sugars and tannins. The residue from the two extractions was used for the estimation of starch. All determinations were made in duplicate.

Examination of the Chloroform Extract.—Preliminary experiment showed that, when the residue from the chloroform extract was taken up with alcohol, a few greenish yellow oily drops always appeared after evaporation of the alcohol, even though the extraction with chloroform was allowed to proceed for as long a period as twenty-four hours. Consequently the chloroform extraction was stopped after eight hours, when the siphoning liquid was colourless. The small percentage of oily matter, afterwards appearing in the alcohol extract, was determined by taking 20 c.c., drying in a small weighing-bottle, taking up the residue with chloroform, filtering, evaporating the chloroform, and weighing the residue, from which the amount to be added to the weight of the original chloroform extract could be calculated.

The extracts obtained as above were weighed and transferred to small sample-tubes, and kept till all could be examined together. This procedure was convenient and less liable to error due to differences in manipulative detail and general conditions of atmospheric temperature, moisture, etc.

In each case (12 samples and 12 duplicates) the iodine value, acid value and refractive index were determined. For the iodine value, the modification of Mossler's process, proposed by Winkler was used, following the details given by Lakhani and Sudborough.[2] The acid value was determined by the method of Fryer and Weston.[3] The refractive index observations were made with an Abbé refractometer at about 60°, and calculated exactly to that temperature. Since the presence of traces of chloroform might have an appreciable effect on the refractive index, a few samples of the oily extract were dried a second time in the steam-oven for about twenty-four hours, cooled in the desiccator and the constant again determined; the readings showed that this source of error was negligible.

The husks were extracted by chloroform in a similar manner to the seeds. The extracts, however, were found to be dark green

[1] Allen's *Commercial Organic Analysis*, vol. i, 445.
[2] This *Journal*, 1916, 1, 173.
[3] *Technical Analysis of Oils, Fats and Waxes*, vol. ii.

resinous masses, which stuck to the flasks tenaciously. No detailed examination was made of this material.

Alcohol Extract.—The residues in the thimbles, left after the first extraction with chloroform, were dried in a steam-oven, and extracted *in situ* with alcohol of s.g. 0·848. The siphoning solvent was colourless in twelve hours. The solution was then transferred, when cold, to the lower boiling-vessel of the extraction apparatus, the residue in the thimble repeatedly washed with fresh alcohol and the washings added to the flask, from which the major portion of the alcohol was then distilled. The residue was made up to 200 c.c. with distilled water, ammonia added to slight alkalinity, and a small piece of thymol added to prevent decomposition pending analysis. Thymol has been found to be equally effective an antiseptic with toluene, and does not interfere with titrations by forming a thin film on the surface of the liquid.

A small amount of green insoluble sediment was observed in all these extracts, consisting probably of substances such as resins and colouring matters soluble in alcohol but insoluble in water; the quantity was negligible.

Portions of the well-shaken extracts, obtained as above, were used to determine (*a*) the chloroform-soluble matter that invariably appeared in the seed extract as explained above, (*b*) total alcohol-soluble matter, (*c*) tannins, (*d*) sugars, (i) reducing and (ii) hydrolysable.

These were examined as follows:—(*a*) See chloroform extract p. 278 (*b*) 20 c.c. were transferred to clean, dry, weighed flasks, which were kept in the steam-oven for about six hours after the liquid had evaporated. The weight of the residue multiplied by ten gave the total alcohol extract.

(*c*) *Tannins.*—The gravimetric method adopted by the Association of Official Agricultural Chemists (A.O.A.C.) was found to be the most satisfactory for our work. The difference between the total alcohol extract, before and after detannisation, give the amount of tannins in the extract.

(*d*) *Sugars.*—A preliminary qualitative examination revealed only sucrose and glucose or laevulose. The quantitative examination, therefore, was confined to determining the proportion of reducing sugar and of sucrose. Cupric-reducing power and rotation were determined before and after inversion with hydrochloric acid of 1·19 per cent.

Starch.—After the chloroform and alcohol extractions, the residues left in the thimbles were ground as finely as possible, and after thoroughly drying in the steam-oven were used for the estimation of starch, by the A.O.A.C. method.

Proteins.—These were determined in the dried and powdered material by estimating the total nitrogen by the Kjeldahl-Gunning method, as adopted by the A.O.A.C. Considerable frothing occurred during the heating with sulphuric acid, particularly in the case of the seed (due probably to saponin), but no loss of material occurred if addition of potassium sulphate was deferred until frothing ceased.

Discussion of Results and Conclusions.[1]

1. The general shape of the curves shows, in the first place, that the changes in the different constituents at different stages do not take place continuously in the same direction. This observation is borne out by the curves and figures which Hills and Hollingshead (*loc. cit.*) obtained in their chemical study of the ripening of olives, and shows the importance, it may be repeated, of taking samples at close intervals. The smaller irregularities in the curves are probably of little significance.

2. The general variation of the figures and the general shapes of the curves obtained by us are, however, in accordance with the figures obtained by previous workers. In the case of the chloroform extract of the seeds, we notice, e.g., a decrease from sample II to III, rapid increase from III to V (from 29·5 per cent. to 36·3 per cent. in two weeks), and a slow increase from V to IX (36·8 to about 43 per cent. in four weeks). Most of the workers have also distinguished three similar stages in the development of fat in their materials. Rousille, e.g., in the case of olives obtained the following values:—

Date of gathering.	*Fat (per cent.)*
30th June	1·4
30th July	5·5
30th August	29·2
30th September	62·3
30th October	67·2
25th November	68·6

Leclerc du Sablon obtained with walnuts and almonds the following:—

Walnuts.		Almonds.	
Date.	*Fat (per cent.)*	*Date.*	*Fat (per cent.)*
6th July	3	9th July	2
1st August	16	4th July	10
15th August	42	1st August	37
1st September	59	1st September	44
4th October	62	4th October	46

[1] See figures I, II and III at end of paper.

Hartwich and Uhlmann also recognise three distinct periods in the growth of olives, and give tables to show that in the first and third periods the oil develops gradually and in small quantities, whilst in the second the increase of oil is rapid. In the experiments of Ivanow with *Linum* and *Brassica Napus*, the fat-content rapidly rose in the first month from 5–10 to 30–45 per cent. and later increased only by small percentages.

These periods in the metabolism of ripening seeds have also been shown externally (Gerber, quoted by Czapek and by Hartwich and Uhlmann, *loc. cit.*) by a change of respiratory coefficient. The ratio CO_2 / O_2 is less than unity in the first stage, exceeds unity in the second, and is again below unity when the seed is completely ripe.

3. From the curves there is seen to be a sharp change at sample III, which is the more remarkable as it occurs in all constituents of both husks and seeds. This cannot be accidental, or due to some mistake in the analysis since it is observed in materials which were analysed quite independently, such as starch and tannins, chloroform extract and alcohol extract, as well as total nitrogen, and in substances analysed separately and at different dates, such as husks and seeds. It is quite evident, therefore, that sample III marks the end of one stage in the development of fruit and seeds. The trees begin to fruit early in May (see p. 274) but the first sample was gathered only on June 21st; it is therefore quite probable that had earlier samples been obtained, their figures would have fallen in line with those of samples II and III. The reason for such a sharp change at a particular stage is not at once evident, but a similar phenomenon has been observed in other cases.[1]

4. As stated above (p. 274) samples X and XI were taken separately from trees other than the one furnishing the bulk of the samples. Analyses of these special samples are shown in the dotted portions of the curve and fall out from the rest. Sample XIII is also not strictly comparable with the majority (see p. 274), and together they emphasise the importance, in comparative studies, of taking samples from one tree only.

5. The individual constituents of the fruit and seeds may now be considered in more detail.

(*a*) *Starch.*—Micro-chemical analysis failed to reveal the presence of starch in the seeds at any stage; whereas from the chemical analysis and starch curve Fig. 2 it might be assumed to be present in quantity. Evidently the figures in the latter case do not represent

[1] Cf. Hills and Hollingshead and Hartwich and Uhlmann (*loc. cit.*) and also De Luca and Korsakow, *Compt. rend.*, 1912, 155, 1162.

starch but hemi-, pseudo-, or reserve-celluloses; these occur freely in many seeds, are easily hydrolysable, and are estimated in a manner which fails to differentiate them from starch. The advantage of employing both chemical and microscopical methods is thus obvious.

(*b*) *Tannins.*—In the seeds and husks the starch and tannins rise and fall together. In the husks both reach a maximum at sample V, whereas in the seeds they are at a minimum at sample VI. Before sample VII, moreover, the quantity of each in the husks is inversely proportional to the amount present in the seeds. After sample VII, however, till sample IX, the quantities in each case diminish. These observations are borne out by the micro-chemical analysis, especially in the case of tannins in the husks.

(*c*) *Chloroform Extract.*—The curves for iodine value and refractive index also change their direction completely at sample VII. It is very probable that the changes in the seed after this stage begin to occur independently of those in the husk or pulp, whereas, before that stage, there existed an intimate connection between the two.

(*d*) *Alcohol Extract.*—The total alcohol extract in the case of seeds falls regularly (with the exception of sample VII) after sample III till the end; the direction of the curve, however, is the same as those for tannins and starch. The curve for chloroform extract on the contrary rises when the total alcohol extract, tannins and starch decrease. This leads to the conclusion that oil is formed in the seeds at the expense of carbohydrates and possibly of tannins. Since, moreover, the starch and tannins in the husk rise and fall in a direction opposite to those in the seed before sample VII, and in the same direction after that stage, there is reason to believe that the change of carbohydrates (and possibly of tannins) into fats occurs *in situ* in the seeds after sample VII, but is dependent on the constituents of the husk before that stage. This in part bears out Pfeffer's original hypothesis,[1] maintaining that at first sugars enter the kernel from outside, and then fat-formation begins.

(*e*) *Sugars in Husk.*—The sugar-content of the husks is high in the beginning when starch and tannin, and also chloroform extract are low. After sample VIII the starch-content increases, while the chloroform extract and tannins diminish. This would support the conclusion that sugars are first formed, and then give rise to starch and tannin substances, these latter possibly acting as preservatives. The conversion of sugar into starch has been frequently observed in plant metabolism, and Onslow[2] describes experiments where leaves,

[1] Czapek, *Bio-Chemie der Pflanzen*, 743.
[2] *Plant Bio-Chemistry*, p. 7. *Cf.* Lecler c de Sablon.

floated in a solution of sugar, showed an increase of tannin derivatives in the tissues. The increase of starch during ripening has also been observed by previous workers.[1]

(*f*) *Proteins.*—Our analyses do not show much variation in the proportion of proteins in the seeds at different stages. In the case of the husks, however, there is an increase up to sample III, i.e., in the first stage of development of the fruit, but a steady decrease is afterwards observed, except in sample V, where, as already mentioned, a number of constituents change their proportion abruptly. In order properly to appreciate the significance of the protein content at various stages, it is evident that more detailed investigation is necessary, on the lines, e.g., of Kleberger, who has determined the proportions of amide as well as total nitrogen at each stage of development of five kinds of oil-seeds for four years.

(*g*) *Transformation of Fatty Acids.*—It is evident from the curves in figure 3 that the free fatty acids are at a maximum at the beginning, and then decrease rapidly till sample VII. This is in accordance with the results obtained by Rachenberg [2] and Ivanow,[3] and follows the commonly accepted theory that the carbohydrates give rise first to free fatty acids which are then esterified to glycerides.

The refractive index curve follows the acid-value curve closely and has its maxima and minima at exactly the same stages. The values for samples X and XI are abnormal for reasons already explained.

The figures for iodine absorption are roughly reciprocal to those for acid-value and refractive index, rising regularly to a maximum at sample V and again falling regularly to IX. The maximum, it will be observed, coincides with the maxima attained by the starch and tannin in the husks. The chloroform extract, the total alcohol extract, the tannin and the starch in the seeds also show a marked change in their proportions at about this stage.

According to the observations of Ivanow, the saturated acids are first formed from the sugars and these are converted into unsaturated acids which combine with glycerol to form fat. In our samples Nos. I–V also, the percentage of unsaturated glycerides and esters increases although the free fatty acids diminish. In samples subsequent to No. V the iodine value falls while the percentage of free fatty acids increases after sample VII. It is possible that the stearic acid first formed is transformed into the highly unsaturated acid

[1] *Chem. Umschau*, 28, 2 ; *cf.* also *Amer. Chem. Abst.*, 1921, 1151.
[2] *Ber.*, 1881, 14, 2216.
[3] *Ber. Bot. Ges.*, 29, 594–602.

linolenic acid which is then reduced back to oleic and perhaps a little stearic acid. All these acids are gradually converted into glycerides, the highest proportion of which is reached at the seventh stage. After that the lipase formed in the seeds may effect hydrolysis producing small amounts of free fatty acids which would account for the slight increase in the fatty acids in the later stages of ripening.

Ivanow [1] in a study of the transformation of fats during germination, concluded that 'the saturated fatty acids not uncommonly exist in a free state whilst the unsaturated acids occur in the form of glycerides.' This confirms the above explanation of simultaneous decrease of iodine value and increase of acid-value after sample VII, i.e., in the third stage of seed development.

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[1] *Jahr. Wiss. Bot.*, 1912, 50, 375; *cf.* also Haas and Hill, Vol. I, p. 39.

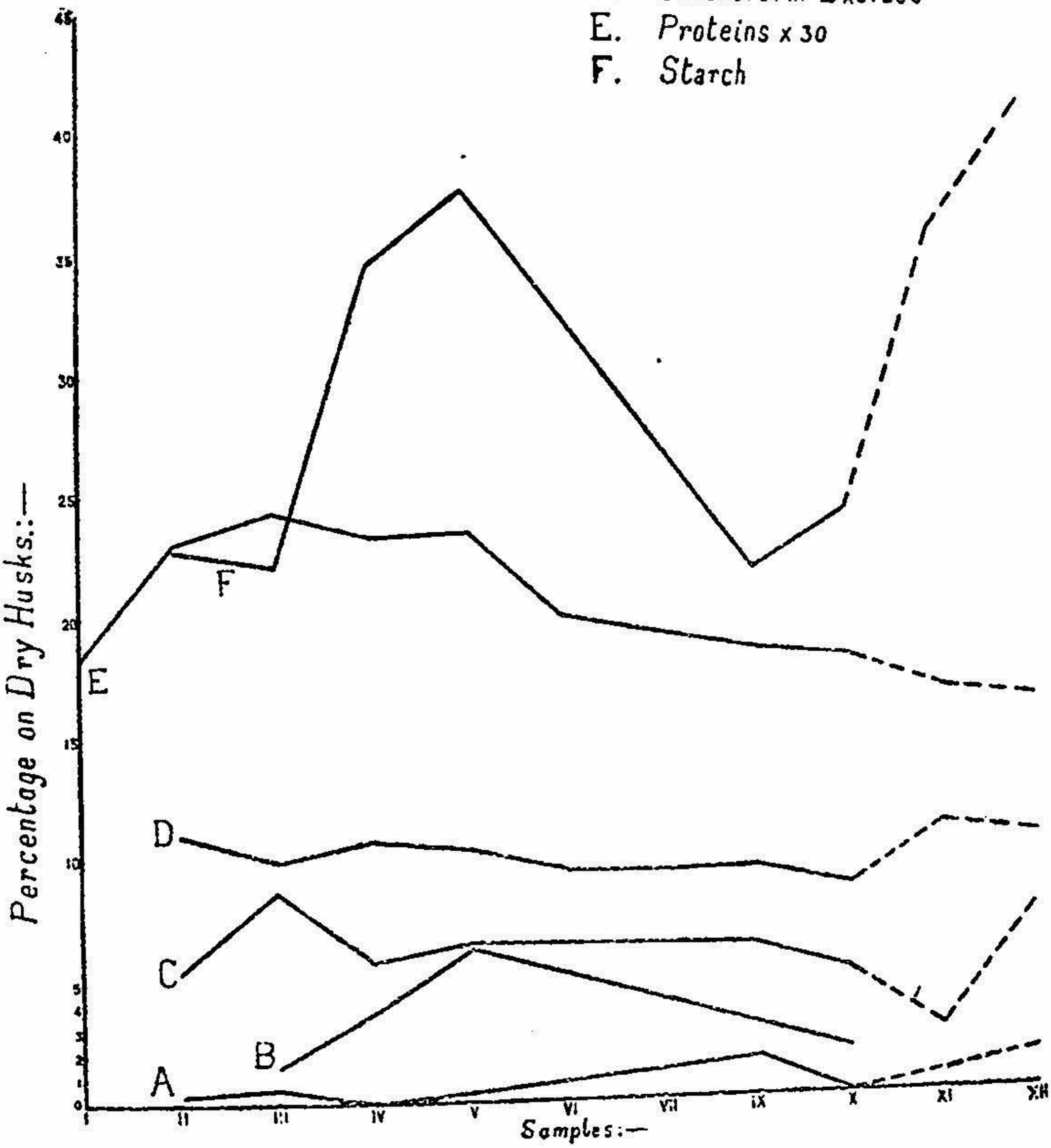

Figure I.
Husks.

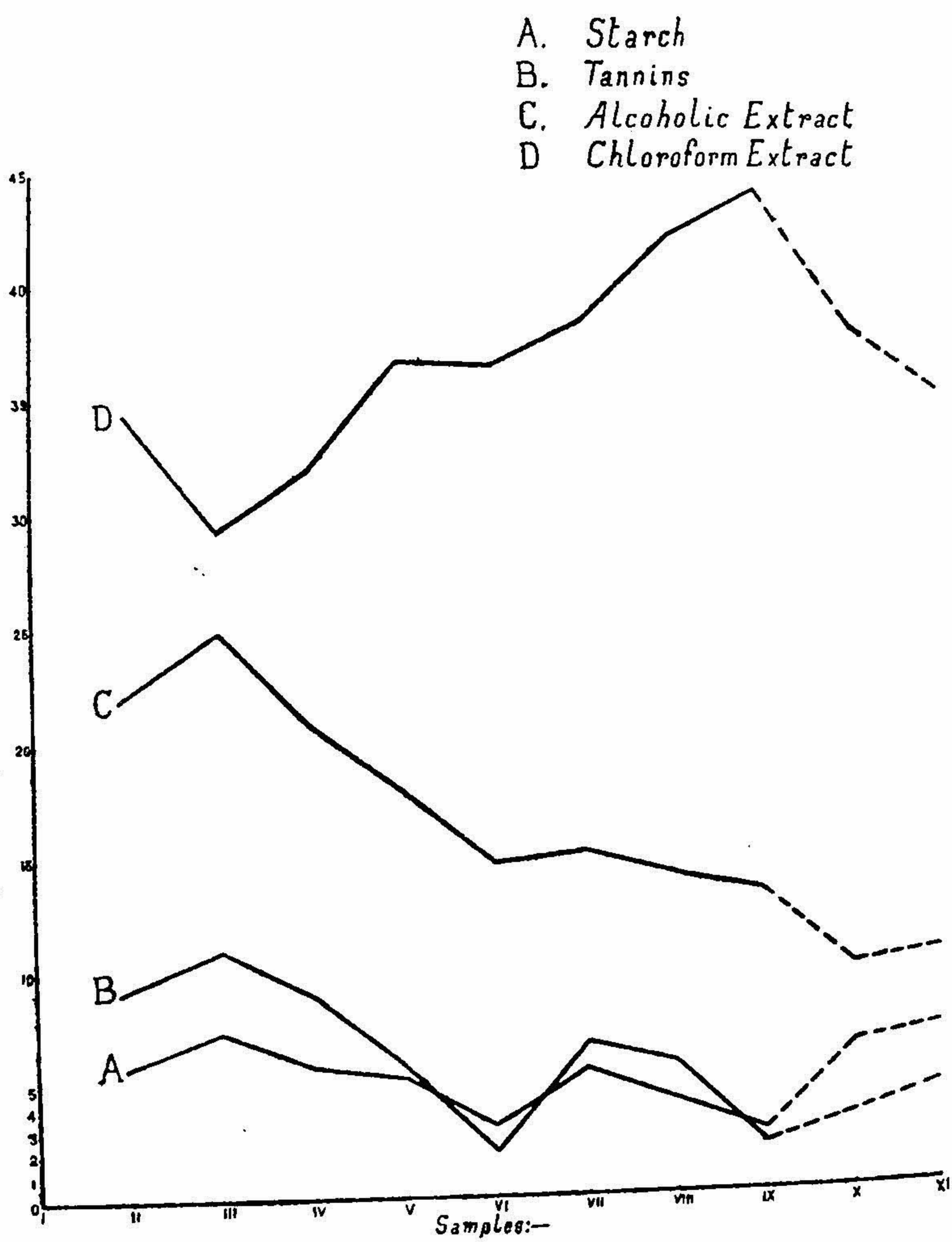

Figure II

Seeds.

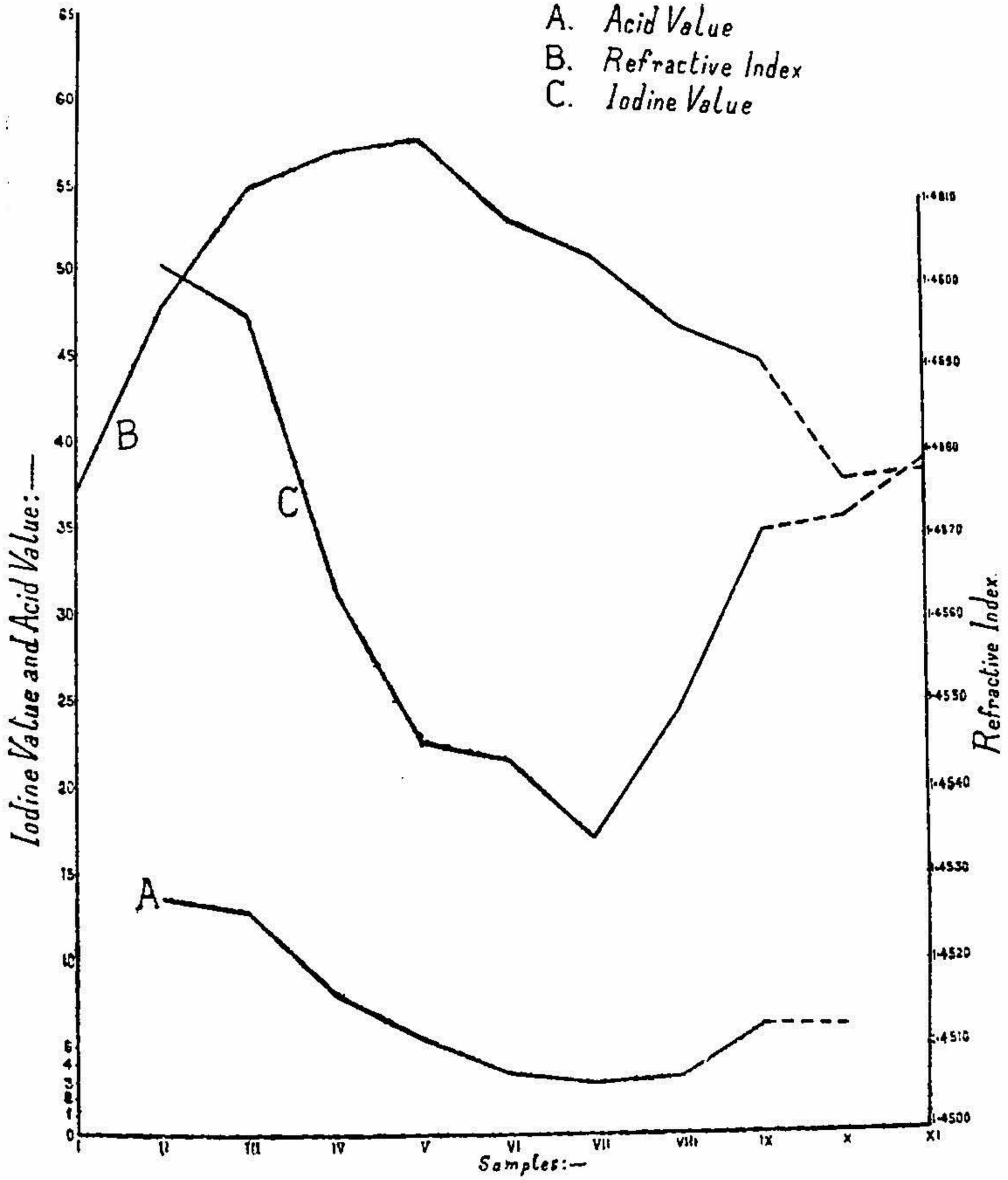

Figure III.

Iodine Value, Acid Value and Refractive Index.

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