THE FRACTIONATION AND STUDY OF SUNFLOWER SEED PROTEINS

by

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ABSTRACT

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Filter paper electrophoresis was used to fractionate the prolamines, albumins, globulins and glutelins extracted from oil-free sunflower meal of five varieties by their respective solvents. At least four different protein fractions were found in each group. The prolamine group, as extracted by a 50% ethanol-water solution, contained as many as ten different fractions.

The globulin group was further investigated using precipitation techniques. It was found that the addition of 95% ethanol has a dehydrating effect on the colloidal micelle, gradually precipitating all proteins. The addition of increasing amounts of ammonium sulphate, on the other hand, preferentially precipitates the proteins of high molecular weight.

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INTRODUCTION

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Proteins are a prime requirement of life. Indeed they owe their name to this, since the word protein is derived from the Greek πρωτος "first". The living organism can use proteins in a variety of ways: as inert material for protection in forms like hair and horn; as a contractile substance to transform chemical energy into mechanical energy (muscle contractions are the contractions of proteins); as enzymes to catalyze biochemical reactions. For such a highly diversified number of functions only a detailed investigation of many different proteins will eventually lead to an explanation of them on the basis of molecular structure and composition. An investigation of the proteins of sunflower seed has been made in this laboratory as a partial contribution to this understanding.

Sunflower seed was chosen for two reasons. In the first place, a large crop of sunflowers is grown in certain parts of Manitoba. The residue after oil extraction is used as animal feed and its protein content is therefore of considerable importance. In the second place, seeds are good sources of proteins. The capacity of the living organism for storing proteins is relatively small; however under special conditions proteins can be stored (as in eggs and seeds) for

use by a developing organism until it can obtain food from its own environment.

It is logical to expect that protein extracted from a living cell (containing enzymes, hormones, structural proteins, and proteins in synthesis and metabolism) may be a highly complex mixture, while protein extracted from seeds may be a simple mixture, since it functions largely as food storage and may possibly contain only one or a small number of proteins in high concentration.

Early investigators used elementary analysis as a criterion to establish the identity of proteins. In the light of modern knowledge this procedure can only be described as totally unsatisfactory; firstly, because the amino acids of which proteins are composed are themselves much alike in elementary composition, so that totally different proteins may show similar analytical results; secondly, because proteins of identical amino acid composition are only identical when their amino acids are arranged in the same sequence.

Proteins are extracted from their natural sources by a variety of solvents, in which they show varying solubilities. These differences in solubility have been used as the basis of a protein classification. Those proteins which are soluble in water and coagulate on heating are called albumins.

Those which are not soluble in water but dissolve in neutral salt solution (a 10% solution of sodium chloride in water is the accepted medium) are called globulins. There are five other groups.

Differences between proteins of different classes are not as great as the differences in solubility might lead us to believe. They can be explained in part by differences in chemical nature. Thus, an albumin has a high ratio of hydrophilic side chain groups to molecular weight, while a scleroprotein, one which is insoluble in any common laboratory solvent, has a very low ratio.

Among the hydrophilic groups that facilitate protein solution are acidic and basic groups. These are ionized in solution, making the protein a charged particle. Therefore, if a potential is applied across the solution, these particles will travel towards one or the other of the electrodes, depending on whether they are positively or negatively charged. The size and polarity of the charge are determined by the pH of the medium. For each protein particle there is a pH of the medium at which the number of negative charges equals the number of positive charges and this is called its iso-electric point. At this pH the protein particle does not travel under an applied field. Accordingly, if a potential is applied across a solution containing a mixture of

proteins, the pH will determine whether or not this mixture will separate into its components.

The phenomenon of a protein travelling under an applied electric field is called electrophoresis. Electrophoresis has provided a powerful tool in the separation of proteins. Sometimes this is done on strips of filter paper and is referred to as paper electrophoresis.

In the investigation reported herein, an attempt was made to characterize the proteins of sunflower seed by paper electrophoresis.

REVIEW OF THE LITERATURE

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Sunflowers have been grown for many years. The seeds are edible and highly nutritious. For this reason sunflowers were observed in many gardens in the occupied countries during the war years. The oil content of the seeds is high (50%) and sunflower crops are grown commercially for oil production. The high protein content makes them desirable as animal feed.

The proteins of sunflower seeds have not been as widely investigated as those from many other sources. For instance, many a paper has been published on the poisonous castor bean protein. This is in keeping with the trend that research often stresses things potentially dangerous to human beings. However the results of such research can usually be applied to other fields, thus enhancing its value. The results obtained from research on lupin protein have herein been applied to the study of sunflower seed protein.

The first records of investigations into the proteins of sunflowers are those of Ritthausen (1880) and Vines (1893) who reported results of elementary analysis on proteins obtained by extraction and precipitation techniques. Osborne and Campbell (1897) investigated a series of seed proteins and compared the results of elementary analysis. They concluded that the principal protein of sunflower seed is a

globulin , identical with the edestin of other oil-bearing seeds. They extracted ten samples of fat-free meal with 10% sodium chloride solution and obtained the protein from the extract either by dilution, dialysis, or saturation with sodium chloride. In comparing the results of their elementary analysis with those of edestin, they found small discrepancies. However, their preparation was always coloured due to helianthotannic acid (chlorogenic acid). By preliminary extraction of the seeds with ethyl alcohol, this acid could be removed in large part and the results approached more closely those of edestin.

Smith and Johnson (1948) studied the isolation of the protein by the method of alkali extraction and acid precipitation. They took the total protein content to be directly related to the total nitrogen content. Their results show that one hundred per cent nitrogen could be extracted with an alkali solution of pH 11. The acid precipitation curve shows that approximately eighty per cent of the total nitrogen is precipitated from pH 3 to pH 7.

Joubert (1955[a]) investigated a globulin extract of sunflower seed by ultracentrifugation. Before extraction with 10% sodium chloride, he subjected the oil-free meal to preliminary extractions with 50% ethanol-water, followed by an extraction with cold water using a Waring Blendor. The

meal thus obtained was free of chlorogenic acid as judged by Hoepfner's (1932) test. In the sodium chloride extract, containing eighty per cent of the protein, he found four components, two major components of sedimentation constants 11.9 and 1.7 S.U., and minor quantities of components 7.8 and 18.1 S.U.

Joubert (1955[b]) also investigated lupin seed proteins. In this case he used the ultracentrifuge and electrophoresis. He found that without removal of coloured components, electrophoresis showed four components and the ultracentrifuge three. To remove colour he followed the extraction procedure described under his investigation of sunflower seeds. After removal of colour he found that in electrophoresis the peaks were now easily resolved and in accord with the number obtained by the sedimentation process.

There is in the literature no mention of an investigation of the sunflower seed proteins by electrophoresis, the method used here. The method of protein separation by electrophoresis has been known since 1937 and was first used to separate protein mixtures by Köning (1939) in an investigation of the yellow pigment from a snake venom. The method, however, came into wide use only after the development of paper chromatography. It was seized upon by the medical profession because it provided the best method

to date for separating serum proteins [Cremer and Tiselius (1950), Grassmann and Hannig (1950), Hartmann and Schumacher (1950)].

The most troublesome factor in paper electrophoresis is that of heating and evaporation. To overcome this, the electrophoresis is carried out in a cold room or use is made of low currents for longer periods of time. Cremer and Tiselius (1950) describe a method by which the paper strip is placed between glass plates and submerged in a coolant (chlorobenzene).

Since proteins are colourless and invisible to the naked eye, the strips are dyed after electrophoresis to bring out the pattern of separation. In this investigation the paper strips were stained with either ninhydrin or azo-carmine-B.

THEORY AND PRINCIPLE OF THE METHOD

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Substances which may be separated by paper chromatography are defined as having different Rf values. The Rf value is the ratio of the distance travelled by the substance to the distance travelled by the solvent front. Electrophoretic mobilities are seldom defined in the literature. This is due to the difficulty of reproducing identical circumstances of electrophoresis.

A protein particle in solution can be considered as a colloidal micelle and its migration will be influenced by the electrokinetic properties of the solid-liquid interface. Helmholtz (1879) developed the concept of the electrical double layer at the solid-liquid interface of a colloidal particle. He regarded the electrical interface between the solid and the liquid as a condenser with parallel plates. Then according to electrostatic theory

$$\zeta = \frac{4\pi\sigma d}{D}$$

where ζ = potential across the solid-liquid interface

d = distance between the centres of gravity of the charges of the particle and the surrounding liquid

σ = charge density on unit area of the layers

D = dielectric constant of the medium between the layers

It can be shown [Smoluchowski (1903)] that the electrophoretic mobility of the colloidal micelle is given by the expression

$$u = \frac{\zeta D E}{4 \pi \eta}$$

where ζ = Helmholtz or zeta potential

D = dielectric constant

E = electrostatic gradient in electrostatic volts/cm.

η = coefficient of viscosity in poise

u = mobility in cm./sec.

However when electrophoresis is carried out on filter paper strips, the electrophoretic mobility cannot be determined solely from the above equation, as it is also greatly influenced by electroosmosis and by the fact that the paper at most pH's carries a negative charge. The phenomenon of electroosmosis occurs when electrophoresis of a suspensoid in an applied electric field is prevented by some suitable means. In this case the medium can be made to move under the influence of the applied potential. In a paper strip there are many capillaries which retard the colloidal micelle and thus give rise to an electroosmotic effect. Since the protein is soluble in the buffer, it will be carried back by the flow of the medium. The negative charge of the filter paper at most pH's gives rise to a flow of electro-

lyte toward the cathode, which again interferes with the electrophoretic mobility. Thus in reporting an electrophoretic mobility the following conditions must be stated: pH, buffer, ionic strength, type of paper used, treatment of paper before use, type of extract used, amount of evaporation taking place, amperage per width of paper.

EXPERIMENTAL

EXPERIMENTAL

Apparatus

The apparatus used is shown in Figures 1 and 2. Both use the open strip technique of paper electrophoresis. In this technique, the paper strip hangs free in the surrounding saturated gaseous phase enclosed in a chamber. In Figure 1 the paper strip was pulled horizontally taut, while the ends of the strips were secured in place with glass stoppers. This apparatus was used in the cold room so as to minimize possible heating effects. In Figure 2 the paper strip was supported at the apex to reduce diffusion of electrolyte from the electrode compartments. A cooling system is here provided by a continuous flow of water through a spiral column in the base directly underneath the paper.

Electrical supply

The source of potential was provided by a regulated power supply able to deliver any constant voltage between 0 and 500 volts. The voltage applied during electrophoresis was one at which no condensed water appeared on the chamber sides. The appearance of condensed water shows excessive heating effects and increases the danger of denaturation. The value of this voltage depends also on the type of filter paper used.

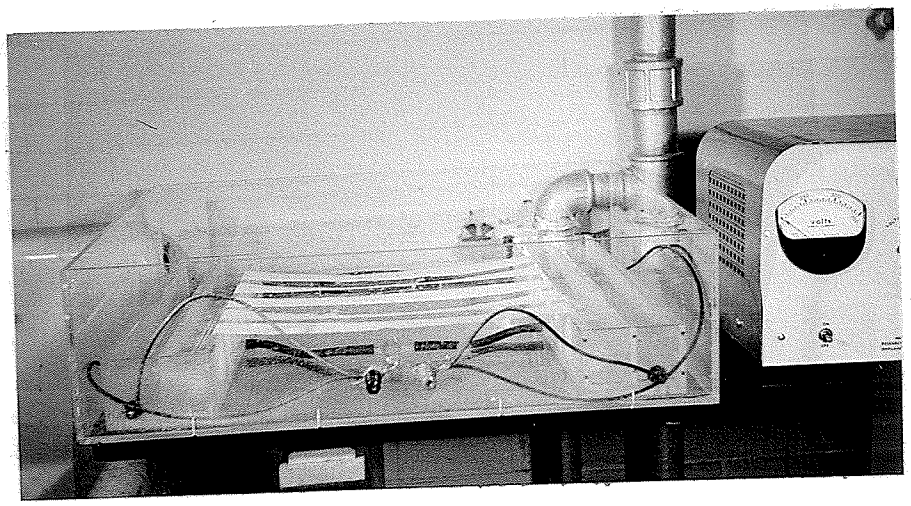

FIGURE 1

ELECTROPHORESIS APPARATUS USING
OPEN STRIP TECHNIQUE (PAPER
PULLED HORIZONTALLY TAUT)

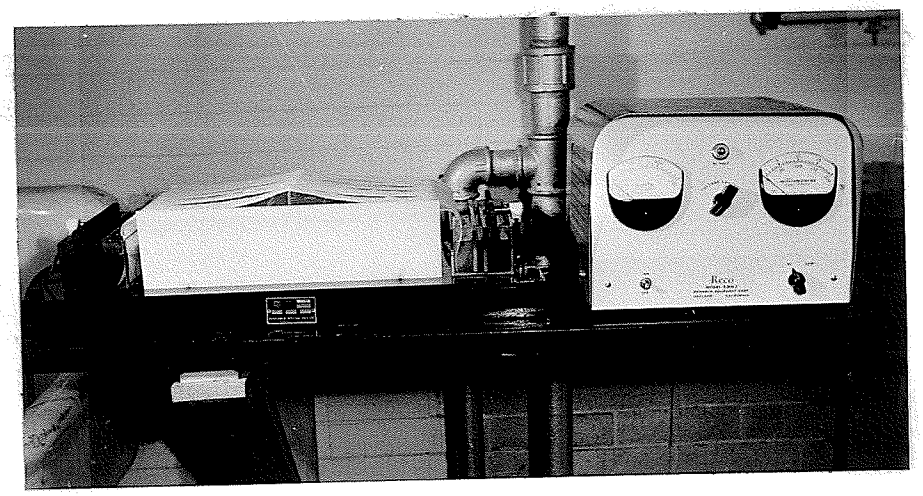

FIGURE 2

ELECTROPHORESIS APPARATUS USING
OPEN STRIP TECHNIQUE (PAPER
SUPPORTED AT THE APEX)

Selection of filter paper

Four types of filter paper were tested, Whatman #1 and #100 and Schleicher and Schüll #595 and #598g. The Schleicher and Schüll papers were considered more satisfactory for two reasons: firstly, they are thicker and more protein extract can be applied on the strips; secondly, they permit the passage of a larger current without the appearance of excessive heating effects.

Procedure of electrophoresis

The filter paper strips were placed in the electrophoresis apparatus and the electrolyte from the electrode baths was allowed to diffuse towards the centre. When only a small strip of dry paper was left in the centre, the protein extract was quickly applied there with the use of a small syringe [Common, McKinley, and Maw (1953)] . Strips prepared in this manner show a fine starting line, since the electrolyte diffusing to the centre carries protein particles with it. An added advantage is that the protein extract on the paper never becomes dry and the possibility of denaturation is minimized. After sufficient time for equilibration elapsed the desired potential was applied and electrophoresis begun. When the electrophoresis was completed the strips were removed and dried in an oven at 100° C.

for fifteen minutes. Denaturation of the protein was now allowed to take place so that the protein would be fixed and not removed in subsequent dyeing procedures.

Dyeing procedure

Two dyeing methods were used. The first was similar to that of Turba and Enenkel (1950). The dyeing solution is a saturated solution of azocarmine-B in a mixture of 50% ethanol-water to which acetic acid was added to make a final concentration of 10% acetic acid. The filter paper strips were immersed in this solution for approximately ten minutes and then washed in a 10% acetic acid bath for twenty minutes. The second method was staining with ninhydrin. The paper strips were sprayed with a 0.2% solution of ninhydrin in 95% ethanol and were then left in the dark to dry and for the colour to develop.

Selection of buffer

Electrophoresis is always performed in adequately buffered solutions, since the mobility of the particle depends on the pH and ionic strength of the solution. A phosphate buffer (0.1 M KH_2PO_4 and 0.1 M Na_2HPO_4) was selected as the most suitable for this investigation on the basis of results obtained with a series of buffers. These results

are shown in Table I. The pH values of the buffers were measured with a Beckman pH Meter. The phosphate buffer shows good separation over a range of pH values and, for easier manipulation, was made up according to concentration rather than pH.

Preparation of seeds for protein extraction

Sunflower seeds of five varieties, Beacon, Sunrise, Striped Mennonite, Black Mennonite, and Advance were used. The seeds were dehulled and separated from the hulls in a water bath. The hulls and bad seeds floated and could be scooped off. The water was then immediately drained off and the seeds were washed twice with 95% ethanol and then with ether. The high oil content of the seeds protected the proteins against denaturation during this time. A fat extraction with ether in a Soxhlet apparatus followed. After fat extraction the seeds were ground in a Wiley Mill. The meal was once again extracted with cold ether to remove possible residual oil. The meal finally obtained was of fine texture and had a slight yellowish tint.

TABLE I

EFFECT OF BUFFER ON PROTEIN FRACTIONATION

Buffer	pH values					
	4	5	6	7	8	9
citrate- HCl	slight	slight				
KH_2PO_4- Na_2HPO_4			good	good		
KH_2PO_4-NaOH		medium	medium	medium	slight	
boric acid-NaOH					slight	slight

Extraction of protein

The meals of all five varieties were analyzed for total nitrogen by the Kjeldahl method. The amounts of nitrogen extracted by 50% ethanol-water, 10% sodium chloride, and 0.1 M sodium hydroxide respectively, were determined by shaking 1 g. portions of meal with 35 ml. of solvent and determining the amount of nitrogen in the residue or aliquots of the filtrate. The results are shown in Table II. These tests were performed to give some indication of the amounts of different kinds of proteins that are present in sunflower meal.

The extracts to be investigated by electrophoresis were prepared from the meal as follows:

1. The meal was repeatedly extracted with 50% ethanol-water until the solution was practically colourless.
2. This was followed by an extraction with cold water using a Waring Blendor.
3. The meal obtained after filtration was then extracted with a 10% sodium chloride solution.
4. The final residue was extracted with 0.1 M sodium hydroxide solution.

TABLE II

AMOUNTS OF NITROGEN EXTRACTED FROM OIL-FREE MEAL USING VARIOUS SOLVENTS

Variety	Total nitrogen content %	Percentage extraction		
		50% alcohol-water	10% sodium chloride	0.1 M sodium hydroxide
Beacon	7.46	11.2	18.9	100
Sunrise	8.05	9.7	20.3	100
Advance	7.22	13.4	23.8	100
Black Mennonite	6.97	10.3	24.1	100
Striped Mennonite	7.12	14.6	26.5	100

It was found that the meal after the 50% ethanol-water extraction could be dried without any visible denaturation of protein. However, if the meal was dried after the water extraction or sodium chloride extraction, visible denaturation took place. Therefore the meal was never allowed to dry during the extraction procedure. All extractions were done continuously, the residues being washed several times with the solvent used.

Electrophoresis of the protein extracts

The apparatus shown in Figure 2 can hold a paper strip 8 inches wide. To be able to investigate the extracts of the five varieties simultaneously, it was decided to make use of paper strips 1.5 inches wide. Thus fifteen strips of 1.5 x 22 inches were cut out of a sheet of Schleicher and Schüll filter paper of 22 x 23 inches.

To obtain a permanent record of the electrophoretic results, the strips were photographed with high contrast film after dyeing. In the pictures, unless otherwise stated, the five varieties were placed in the following manner: on top Beacon, then in order Sunrise, Advance, Black Mennonite, Striped Mennonite. The results are shown in the Figures 3 to 14 as follows:

Figure 3. Ten per cent sodium chloride extract of Beacon meal. Top and bottom strips show the direct sodium chloride extract not previously extracted with 50% ethanol-water. The second strip is the supernatant after protein has been precipitated with 95% ethanol, to a final concentration of 50%. The fourth strip is that of the precipitate redissolved in 10% sodium chloride, while the third is that of the supernatant after a second precipitation with 95% ethanol.

Figure 4. A fifty per cent ethanol-water extract of the variety Beacon. For the strips from top to bottom electrophoresis was carried out for 1; 2; 4; 20 hours respectively, each strip drawing 3 milliamp. per inch

Figure 5. A fifty per cent ethanol-water extract of all five varieties. Conditions: amperage as before, time 1.5 hours.

Figure 6. A fifty per cent ethanol-water extract of all five varieties. Conditions: amperage as before, time 20 hours.

Figure 7. Water extract of all five varieties. Conditions: amperage as before, time 1.5 hours.

Figure 8. Ten per cent sodium chloride extract of all five

varieties. Conditions: amperage as before, time 1.5 hours.

Figure 9. A 0.1 M sodium hydroxide extract of all five varieties. Conditions: amperage as before, time 2 hours.

Figure 10. Four extracts of the variety Beacon. Simultaneous electrophoresis. Conditions: amperage 4 millamp./ inch, time 1 hour.

Figure 11. Ten per cent sodium chloride extract of the Beacon variety. In this experiment varying amounts of ethanol added were, from top to bottom respectively, 2.5; 5; 10; 20; and 40 ml. of 95% ethanol per 10 ml. of extract.

Figure 12. Ten per cent sodium chloride extract of the Beacon variety. This time varying amounts of ammonium sulfate were added to the sodium chloride extract and the supernatants again subjected to electrophoresis. The amounts of ammonium sulfate added were, from top to bottom respectively, 1; 2; 3; 4; and 5 g. per 10 ml. of extract.

Figure 13. Ten per cent sodium chloride extracts of all five varieties. Conditions: amperage 2 milliamp./inch of paper width, time 20 hours.

Figure 14. Ten per cent sodium chloride extracts of all five varieties. Conditions: amperage 5 milliamp./inch of paper width, time 20 hours.

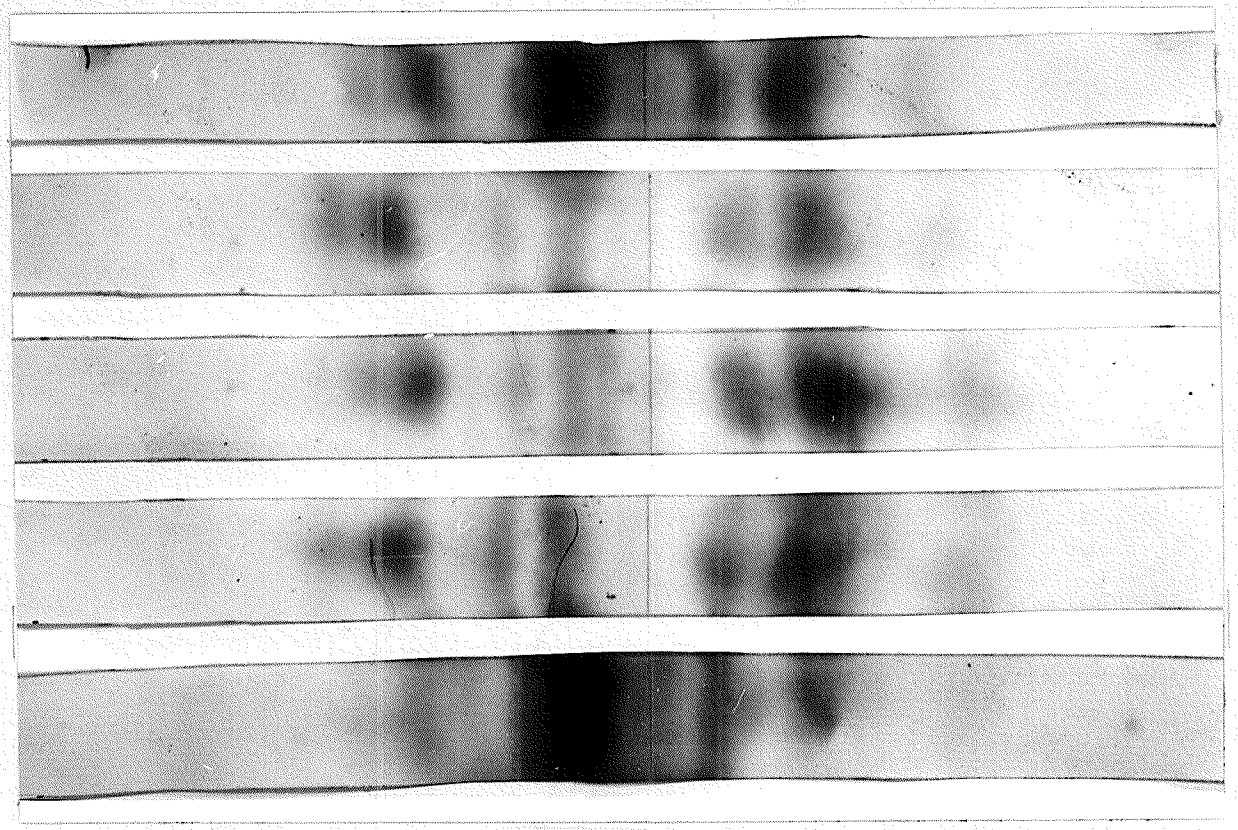

FIGURE 3

TEN PER CENT SODIUM CHLORIDE EXTRACT OF BEACON MEAL

FIGURE 4

A FIFTY PER CENT ETHANOL-WATER EXTRACT OF THE VARIETY BEACON

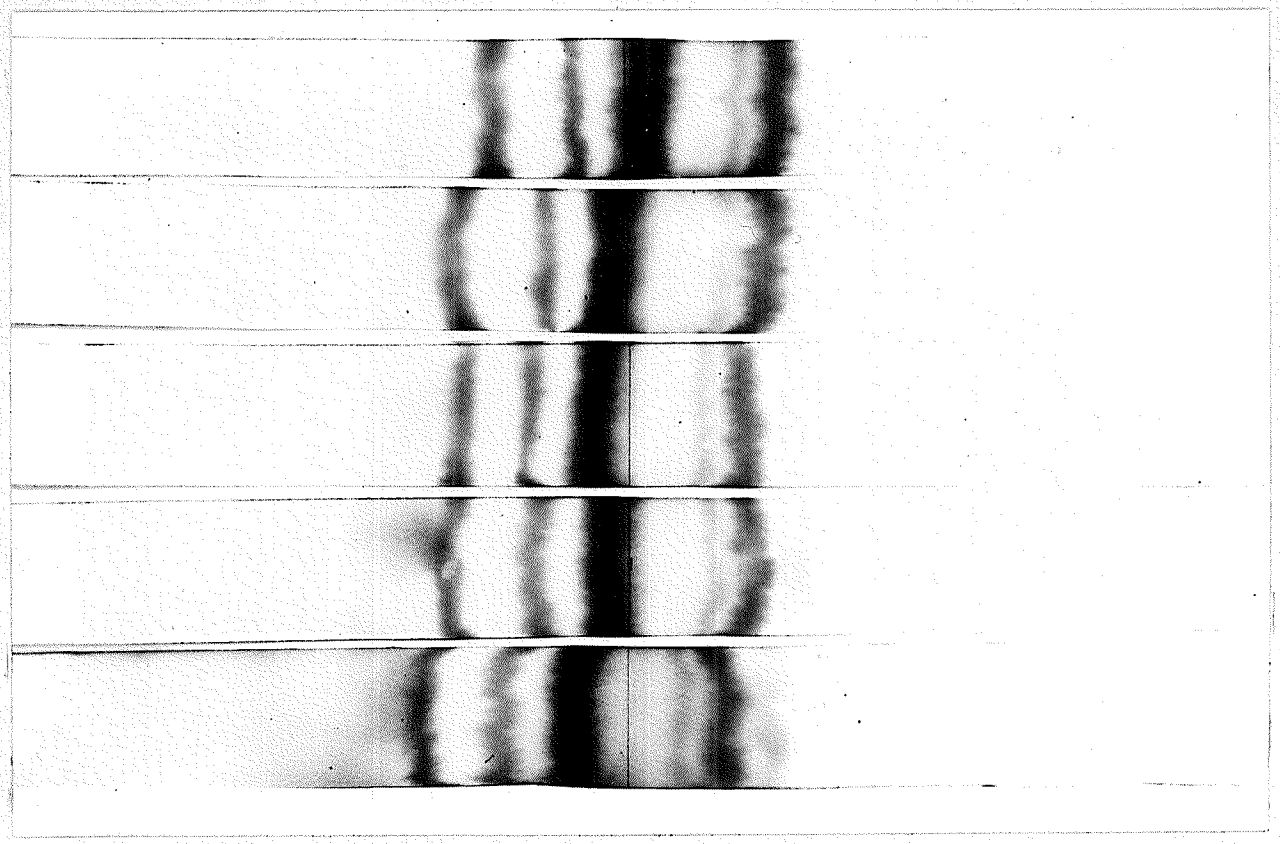

FIGURE 5

A FIFTY PER CENT ETHANOL-WATER EXTRACT OF ALL FIVE VARIETIES

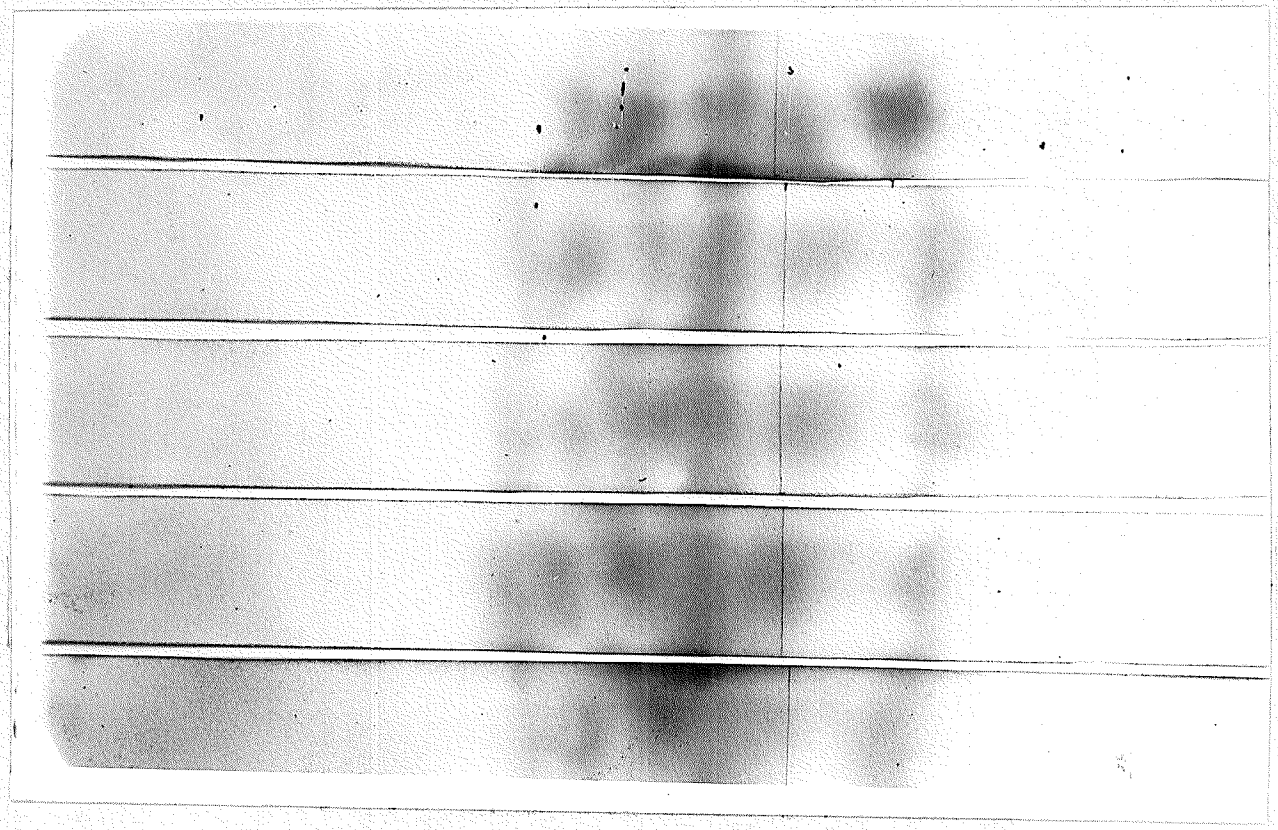

FIGURE 6

A FIFTY PER CENT ETHANOL-WATER EXTRACT OF ALL FIVE VARIETIES

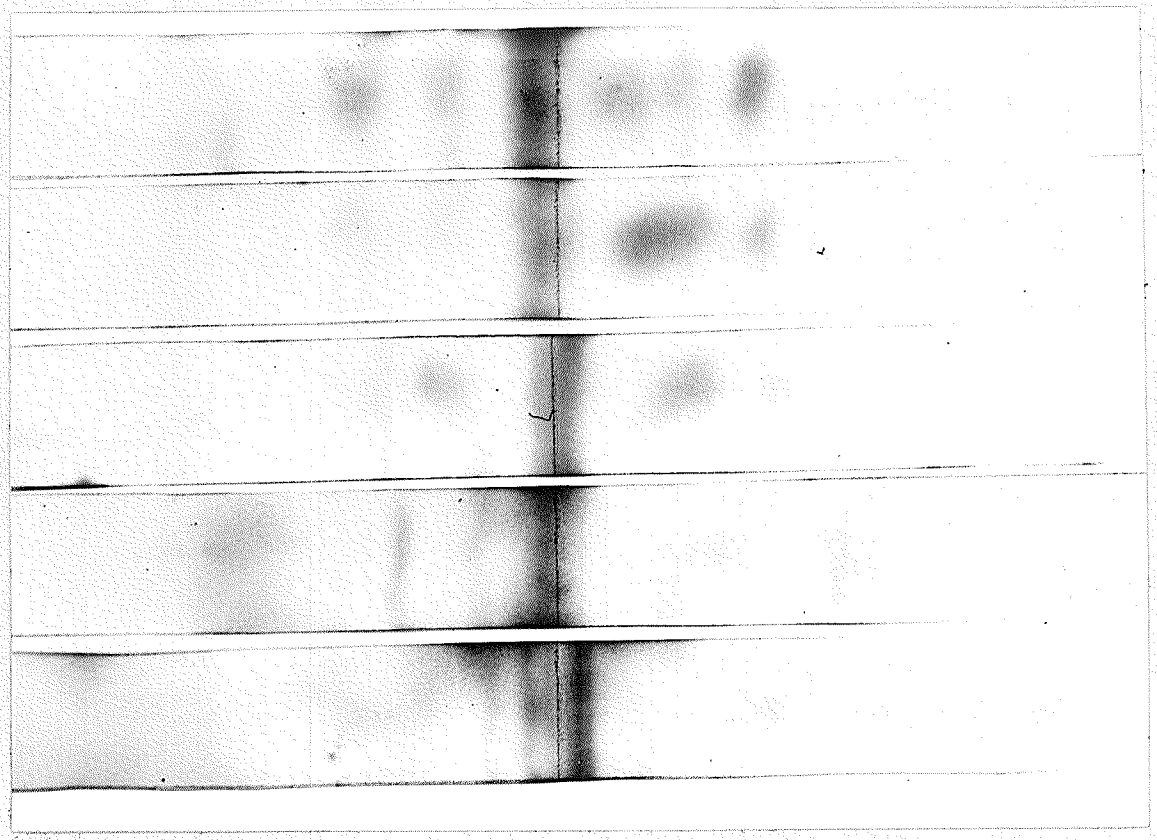

FIGURE 7

WATER EXTRACT OF ALL FIVE VARIETIES

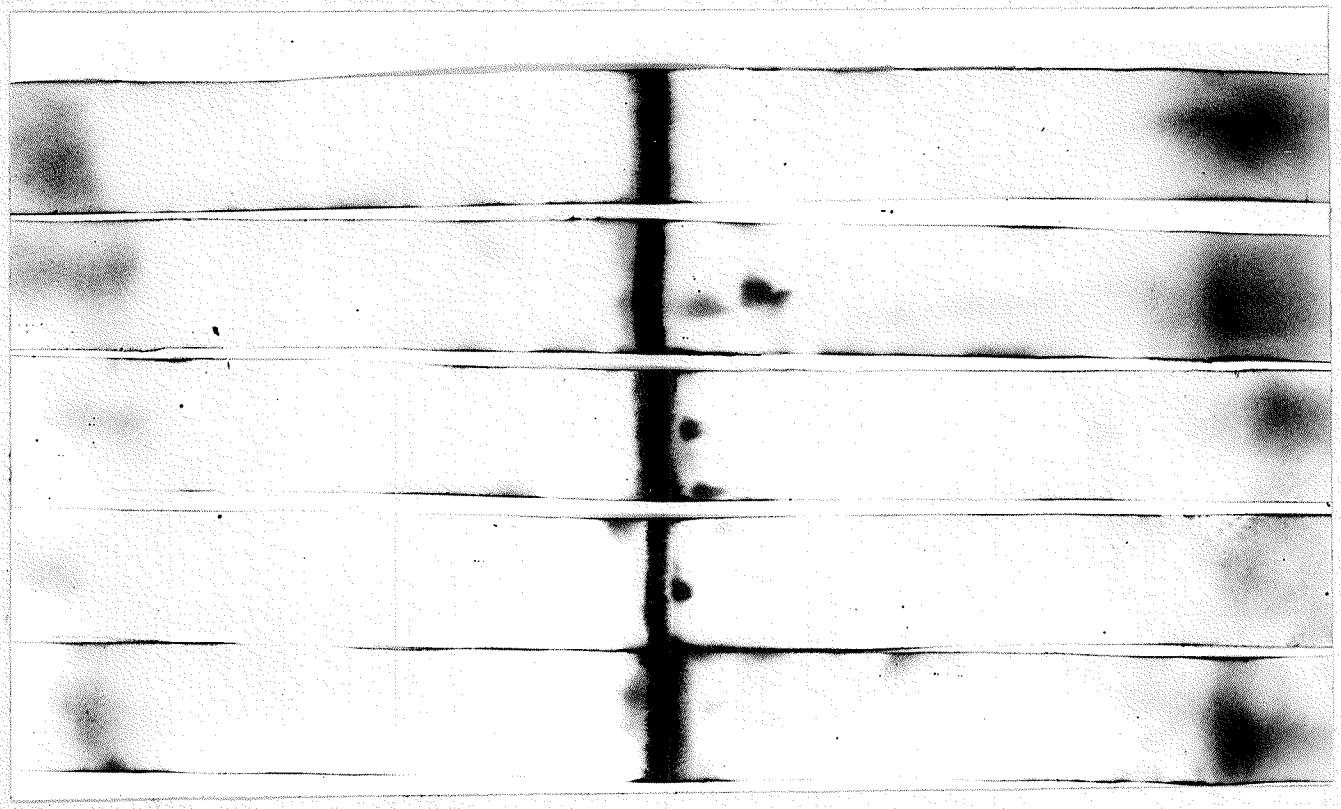

FIGURE 8

TEN PER CENT SODIUM CHLORIDE EXTRACT OF ALL FIVE VARIETIES

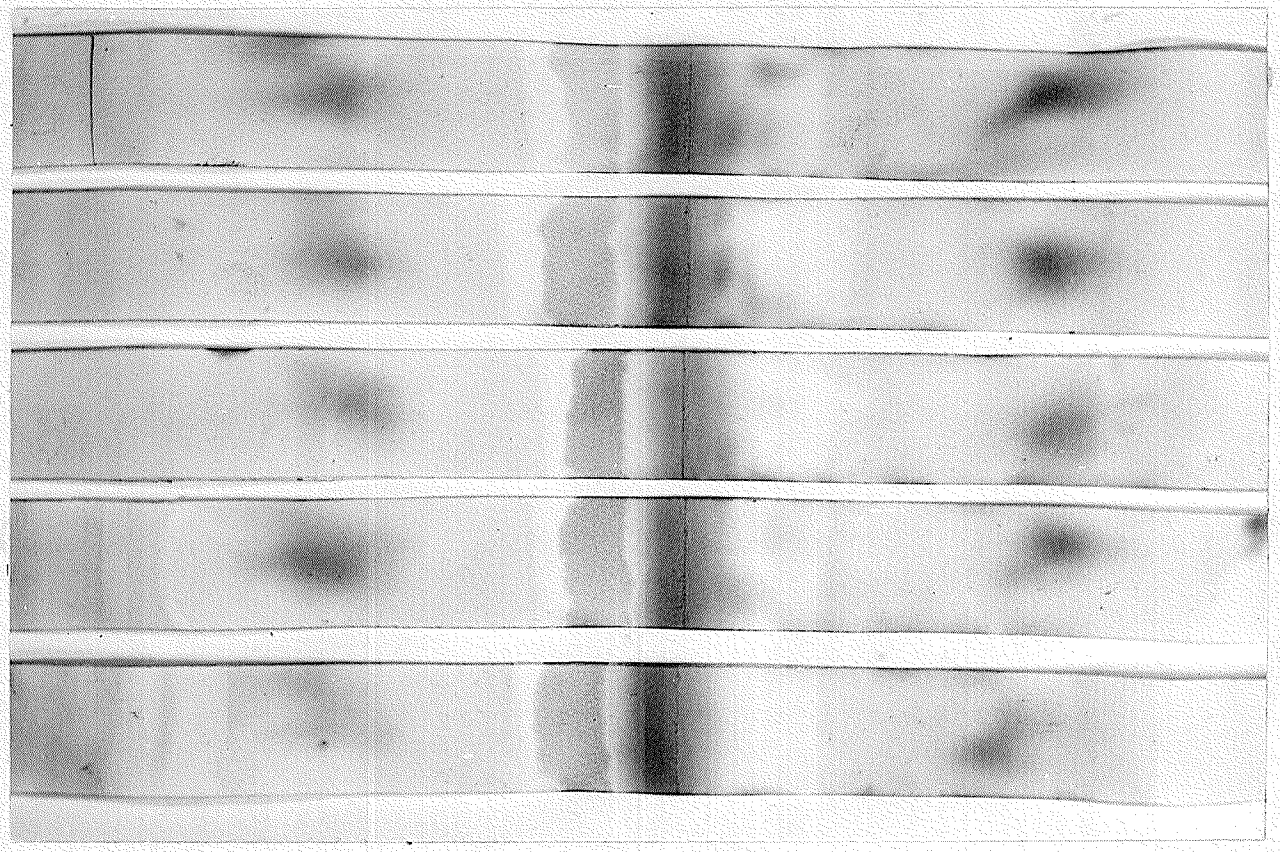

FIGURE 9

A 0.1 M SODIUM HYDROXIDE EXTRACT OF ALL FIVE VARIETIES

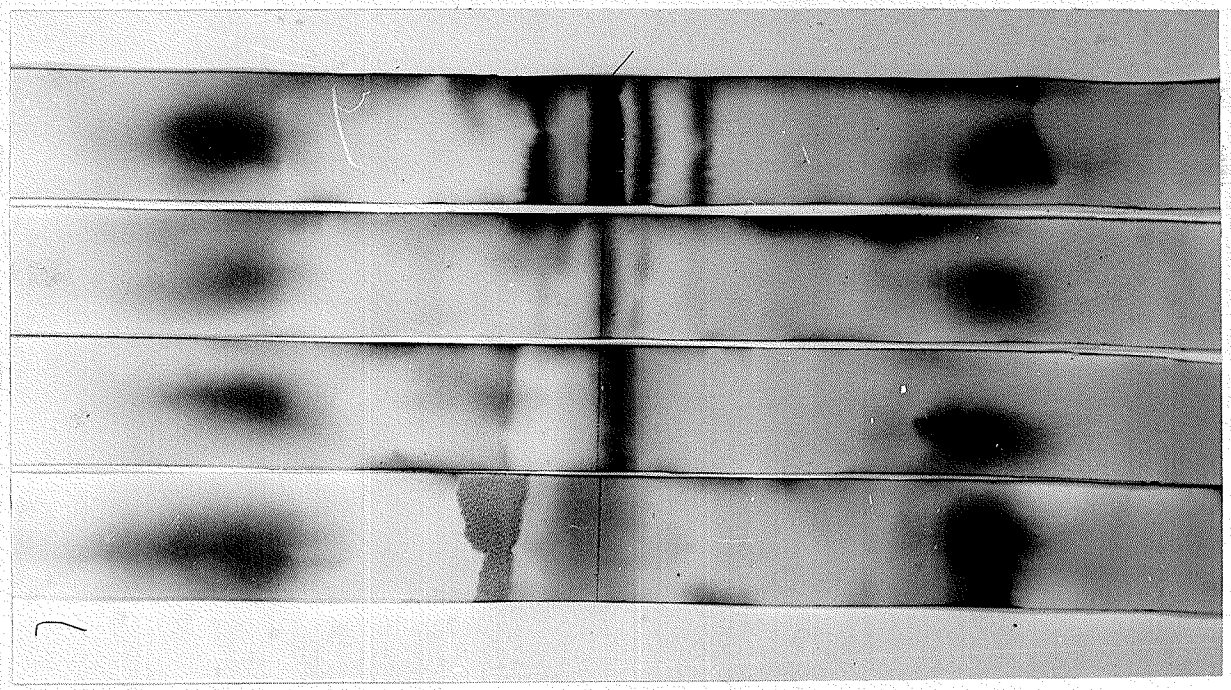

FIGURE 10

FOUR EXTRACTS OF THE VARIETY BEACON

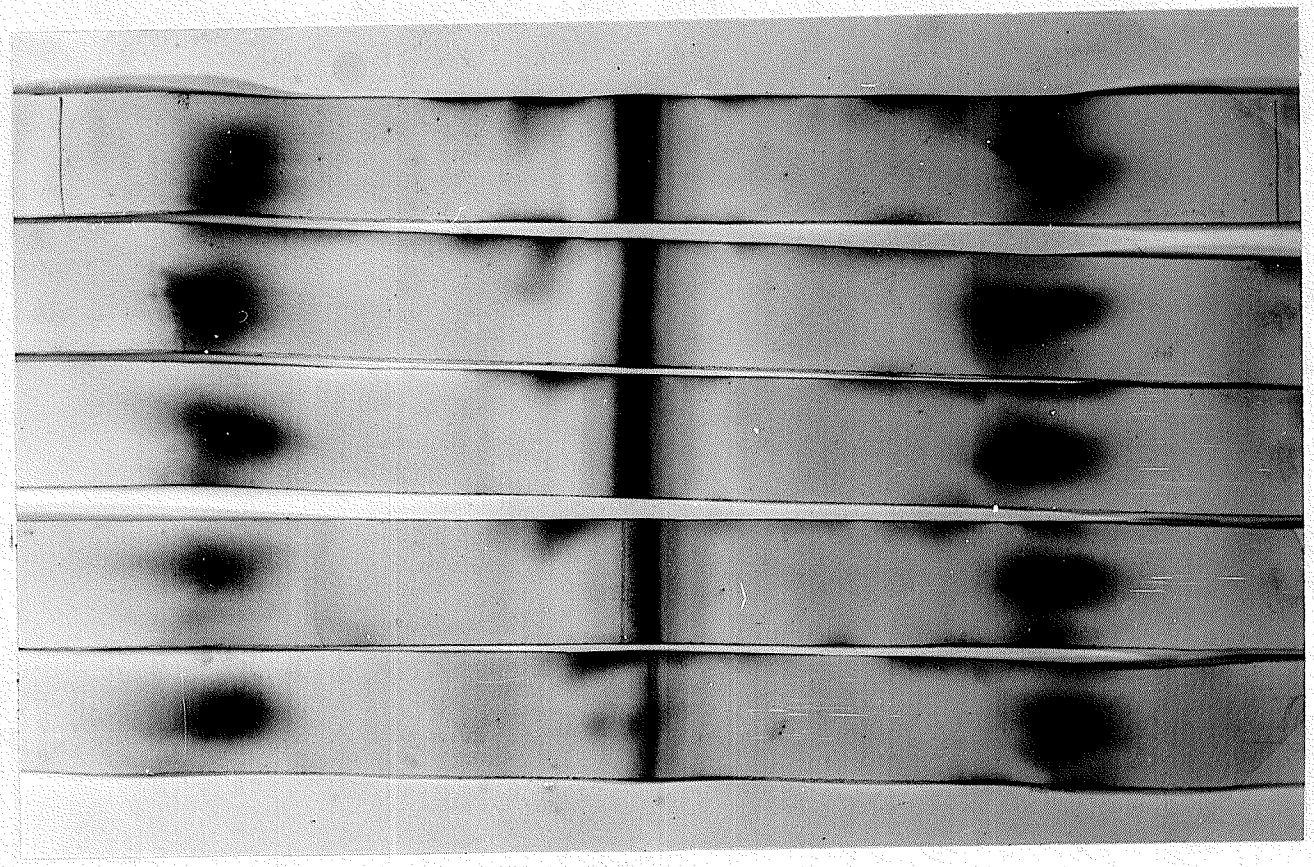

FIGURE 11

TEN PER CENT SODIUM CHLORIDE EXTRACT OF THE BEACON VARIETY

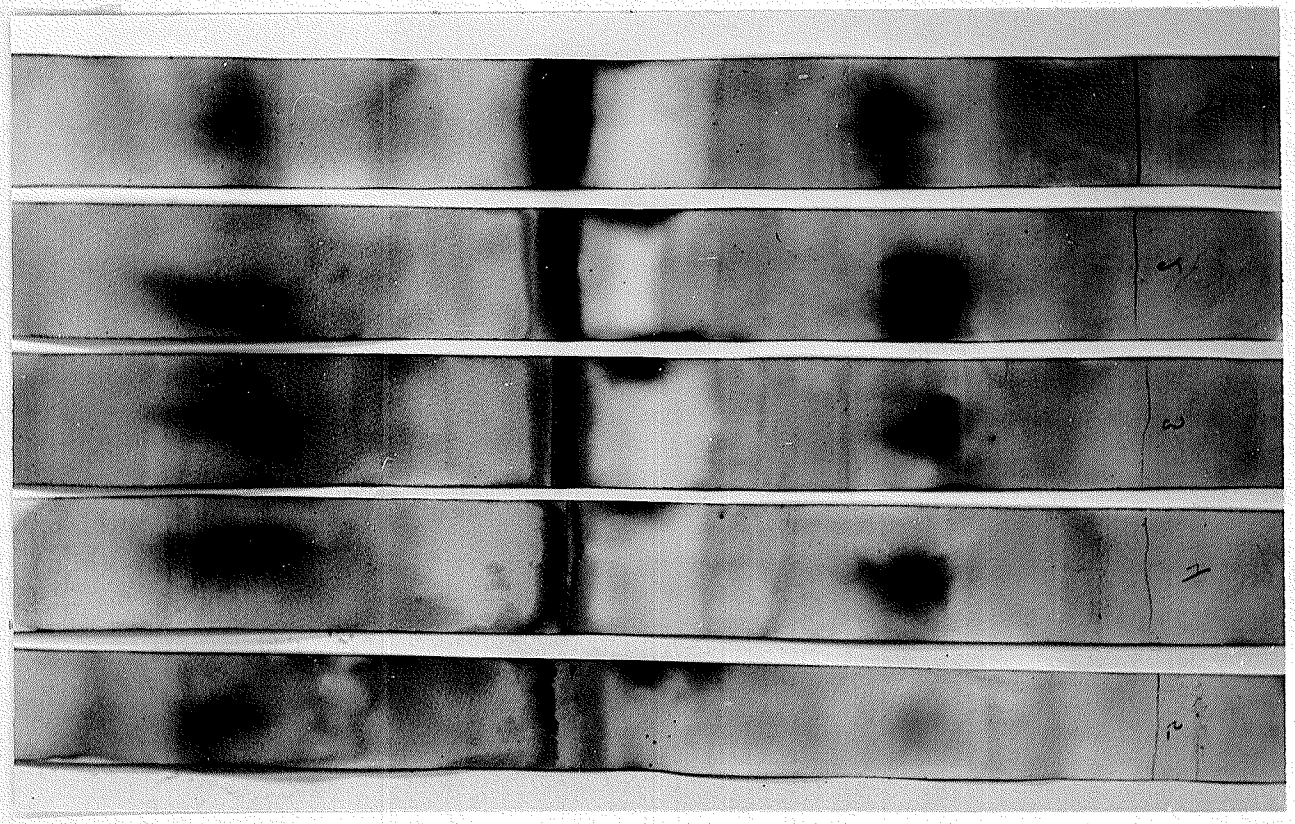

FIGURE 12

TEN PER CENT SODIUM CHLORIDE EXTRACT OF THE BEACON VARIETY

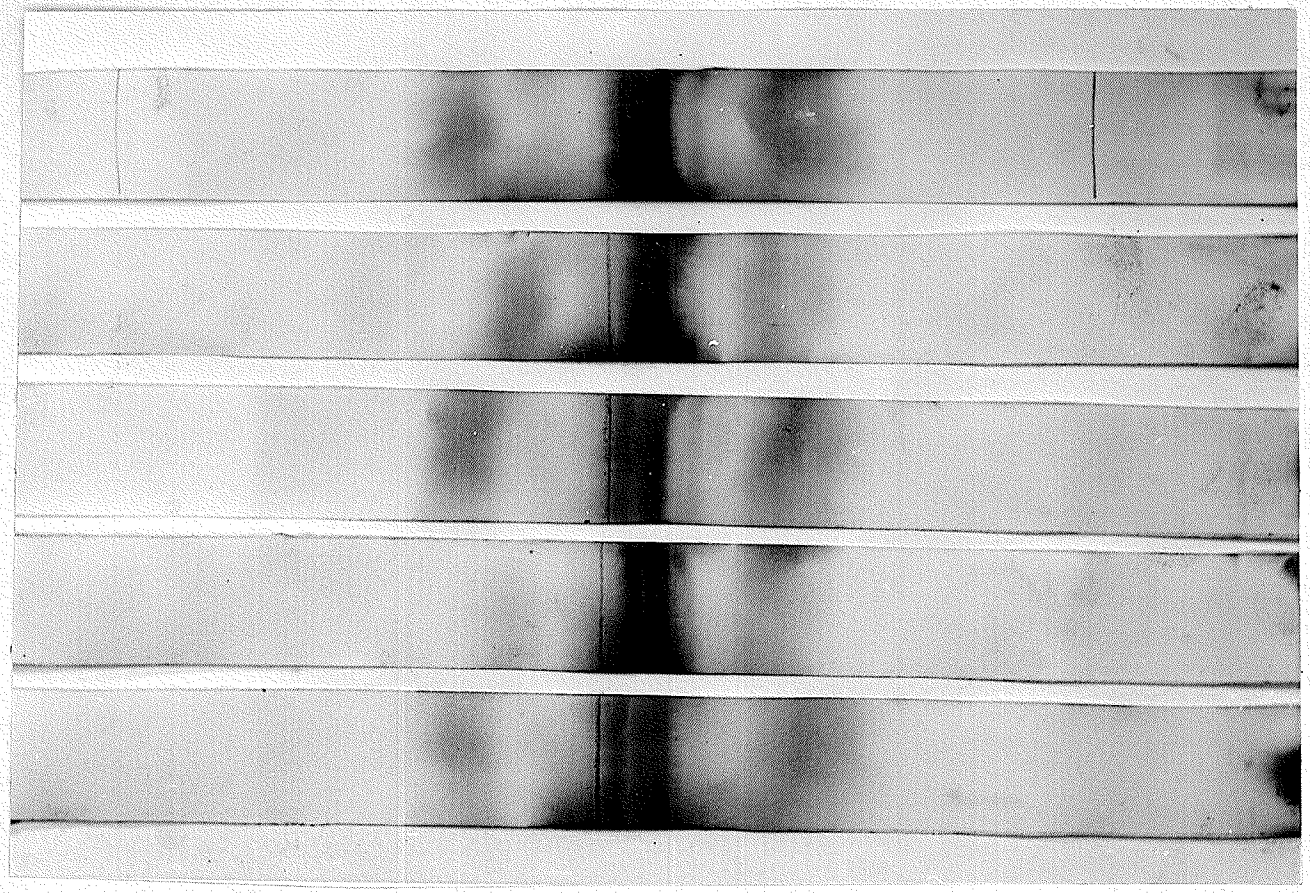

FIGURE 13

TEN PER CENT SODIUM CHLORIDE EXTRACTS OF ALL FIVE VARIETIES

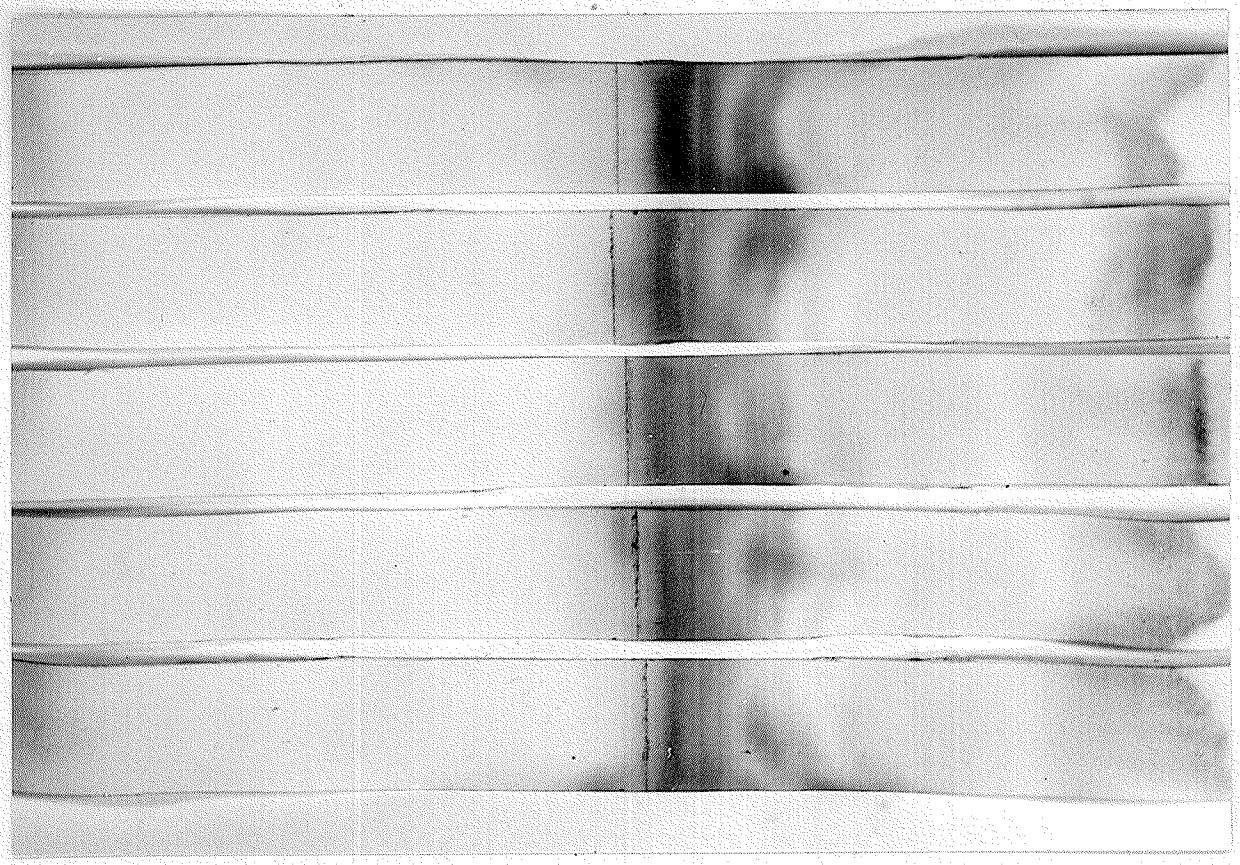

FIGURE 14

TEN PER CENT SODIUM CHLORIDE EXTRACTS OF ALL FIVE VARIETIES

DISCUSSION

DISCUSSION

It has been pointed out before that one of the reasons for choosing sunflower seeds for this investigation was that they might provide a protein extract of a comparatively simple nature. The results of this investigation show that this is not the case. Four different protein extracts were obtained from the sunflower seeds and each showed several fractions.

Joubert (1955[b]) reported that chlorogenic acid, present in sunflowers, interfered with protein electrophoresis. That this is indeed the case is shown in Figure 3. The dark band just left of centre (top and bottom strip) is a complex of at least two proteins with chlorogenic acid. Before staining, this band had a yellow colour showing the presence of chlorogenic acid. The addition of ethanol destroyed this complex and separation of proteins is shown in the other three strips.

The main reason for extracting with a 50% ethanol-water solution was, therefore, the removal of chlorogenic acid. However, on investigating the protein content of this extract, electrophoresis showed that there was a variety of proteins present. Figure 5 shows three major and one minor fast travelling components, plus extra ones in the Mennonite varieties. The heavy centre band contains six slow travel-

ling components as shown in Figure 6. The top strip in Figure 6 is the same as the last strip in Figure 4, which shows that the concentration of slow moving components is only small. The detail in Figure 6 is brought out by a longer exposure time, which could only be obtained when every strip in one set was of equal colour intensity.

While the 50% ethanol-water extracts show nearly identical composition for all sunflower varieties, the water extracts show some differences. Figure 7 shows the conformity of the water extracts. The Mennonite varieties show the greatest differences. If differences in protein content were present, they were expected to show predominantly in these varieties, since the meals obtained from them are of slightly different textures than those of the other three, being not as fluffy.

The 10% sodium chloride extract shown in Figure 8 has two fast travelling components, of slightly greater concentration in the Mennonite varieties. After longer electrophoresis two medium-fast components separate from the centre band (Figure 13), while the final separation of this band (Figure 14) shows one major and three minor components, probably identical with those found by Joubert (1955[a]).

In Figure 9 the results of electrophoresis on the 0.1 M sodium hydroxide extract are shown. Again two fast

travelling components are found and just to the right of centre there is a band, which when stained with ninhydrin shows a bluish colour. This is in contrast to all the other protein fractions which always show a purple to red colour.

The 10% sodium chloride extract was investigated further using protein precipitation techniques. Figure 11 shows what happens when protein is precipitated from the 10% sodium chloride extract by ethanol. The colour intensity of the centre strip gradually decreases, indicating that ethanol has only a dehydrating effect on the colloidal micelle and that, with the possible exception of the small fast travelling ones, all the proteins present are similarly affected. This was also shown in Figure 3, where the precipitate obtained with ethanol showed the same fractions as the supernatant liquid. Figure 12 shows the results of electrophoresis of the supernatants left after precipitation of some of the protein with ammonium sulfate. The effect here is slightly different since the most slowly travelling components are preferentially precipitated. These might be presumed to be those with the highest molecular weight.

When electrophoresis was done on all four extracts simultaneously (Figure 10) it was observed that the more rapidly moving components of all extracts were the same. This suggests that these might be protein building blocks

which are formed through possible hydrolysis taking place during the extraction procedure. Otherwise there seems to be no reason why these components should not be completely removed with the first ethanol-water extraction. This suggests also that two of the slower travelling components found in the sodium chloride extract could be derivatives and might not necessarily be true protein fractions. However only an investigation of the amino acid composition and sequence could show whether or not this is the case.

SUMMARY

SUMMARY

The investigation made of the proteins contained in five varieties of sunflower, Beacon, Sunrise, Advance, Black Mennonite and Striped Mennonite, leads to the following conclusions:

1. Chlorogenic acid present in sunflower seeds interferes with the fractionation of proteins by electrophoresis.
2. Chlorogenic acid may be removed from the oil-free sunflower meal by an extraction with a 50% ethanol-water solution, followed by an extraction with water using a Waring Blendor.
3. The extracts obtained when the chlorogenic acid was removed were investigated by electrophoresis. The 50% ethanol-water extract showed ten protein fractions which were nearly identical for all five varieties. The water extract, on the other hand, showed many dissimilarities among the five varieties.
4. A 10% sodium chloride extract was made of the meal from which chlorogenic acid was removed. This extract was shown to contain eight protein fractions.

5. A final extract was made with 0.1 M sodium hydroxide and showed four protein fractions.

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