INVESTIGATION OF PROTEOLYSIS BROUGHT ABOUT BY EXTRACTS FROM FILBERT NUTS

by

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CHAPTER I

INTRODUCTION

During the period from 1927 to 1949, the production of filbert nuts in Oregon increased from 60 to 9,700 tons (13) and this state now produces about 90 per cent of the filberts grown in this country. Many new orchards are coming into bearing and over-production may result unless new markets and uses can be found for this commodity.

Processing, such as roasting and filbert butter manufacture (44), as a means of utilizing the crop, has been investigated. Filberts are high in food value, especially in lipids and proteins (27, 71) and, therefore, offer opportunities for valuable food products.

No work has been reported on the enzyme content of filberts. This provides fruitful opportunities for investigation. This thesis reports the results of the study of proteolytic activity of extracts from filbert nuts. Preliminary application of these extracts in accelerating the proteolysis during the ripening of cheddar cheese has shown some promise.

CHAPTER II

LITERATURE REVIEW

A. The Filbert Nut

The filbert is among the oldest, yet perhaps the least publicized and least investigated, of the nuts. It is reported to have been cultivated by the early Romans and was called Abellinae after their native place, Abellina (51, 52), from which is derived the species name avellana. The term hazel nut is applied to native American species. According to some investigators, the term filbert is supposed to have been derived from St. Philibert, as August 22 is dedicated to him, a date that corresponds to the ripening period of the earliest filberts in England.

The two common species in North America are C. americana and C. cornuta.

Varieties of Filbert

Varieties most commonly grown in the States of Oregon and Washington are Barcelona and Du Chilly. The former predominates in Oregon. There are some other varieties such as Daviana, White Aveline, Bolioller, Du Provence, Montello and Nottingham, which are primarily grown for the purpose of pollination (53).

B. Proteolytic Activity of Seeds

A search of literature failed to bring to light any published work on the proteolytic properties of filberts or any other kind of nuts. It is necessary, therefore, to draw on the literature in related fields. Some published material is available on other seeds, especially cereal grains.

It has been reported that one of the earliest observations of proteolysis was made by workers handling certain fruits, such as figs, cantaloupes, pineapples and green papayas, when sometimes their hands became sore, particularly around the fingernails. According to Balls (4), the presence of a protein-digesting substance in the leaves and green fruit of the papaya (Carica Papaya) has long been known. The presence in flour of an enzyme capable of digesting protein was reported by Ballard in 1894 (33, p.225). The existence of a similar meat-digesting enzyme in the juice of pineapple was first noted by Marcano of Venezuela (4). Balls (5) has also pointed out the presence of demonstrable quantities of proteolytic enzymes in many other plants.

Proteinases in figs, milkweed, seeds of lima beans and the kernels of wheat have been found to be related to papain and bromelin (5, 74). Walti (72) has claimed that

ficin is a papainase and is the first proteolytic enzyme to be obtained from plant sources.

Factors Affecting Enzymes in Seeds

One of the first factors which affects the amount and the type of enzymes present in the seeds is the type of plant itself (3). In addition, many other variables such as fertilizers and their composition, climatic conditions, temperature, presence of inhibitors, pH, effect of other constituents of the seeds, conditions of storage and perhaps a few unknown factors influence enzyme activity.

Alten and Schutle (2) found that germination of cereal grains which involves the action of enzymes is influenced by the fertilizer treatment of the mother plant (rather than the nitrogen, phosphorus or potassium content of the seed). They concluded that different fertilizers can impart different physiological characteristics to the genetically similar seeds.

Gaumann (22) germinated seedlings at temperatures varying between 3° and 33° C. to study the effect of temperature on the composition (and hence activity of the enzymes). Lipid material and ash content showed the greatest differences; cell wall components less; and total carbohydrates as well as total nitrogen containing

compounds showed only very small differences in the quantities present in the seedlings grown at different temperatures. Residual nitrogen showed noteworthy difference.

Climatic conditions seem to have a considerable bearing upon the enzyme activity of seeds. It has been reported by Melloy (41) that any time during the growing season of cotton, if the moisture decreases due to excessive heat or other factors, the density of the juices in the seeds increases and the activity of enzymes decreases. Ivanov (28) reported that cereals such as barley and wheat have a higher catalase content when grown in mountainous or northern regions, but peas and soybeans show no such differences. This indicates that certain seeds are affected more than others by varying climatic conditions. The same holds true as far as the relationship between the amounts of different enzymes in the seeds is concerned. Davidson (14) found that barley and rye with relatively high amylase content had low proteinase content, while oats with relatively low amylase content had the highest proteinase content of all the cereals. On the other hand, soybeans with a high amylase content had the highest proteinase content. Wheat and bran had a lower total amylase content and a higher proteinase content. This shows that individuality of seeds is an important factor.

Borchers, Ackerson and Kimmet (10) found a natural trypsin inhibitor in the seeds of peanuts, blackeye and chick pea, soybean, mung, as well as in scarlet runner, lima, garden and velvet beans. It is interesting to note that all these seeds belong to the Leguminosae family. The inhibitor was not found in other legumes including the jack and horse bean, guar, lintil, garden pea, or in any non-legumes studied. Bamann and Schmike (8, 9) observed that the products of hydrolysis tended to inhibit the proteolysis during germination. They also studied the effect of some added activators and found that manganese and cysteine together stimulated the activity more than manganese alone. L-leucine completely inhibited the activity of d-peptidase.

C. Review of Enzyme Extraction Methods

One of the earliest methods for the extraction of soybean urease was developed by Van Slyke and Cullen (65) in 1914. According to this method, one part of fat-free soybean meal is extracted for one hour with five parts of water and centrifuged. The supernatant is poured with stirring into 10 times its volume of acetone. The precipitate is centrifuged off and dried in vacuo over sulfuric acid. They described also a method for extraction of

enzymes from yeast.

Protease or proteinase was isolated in the form of a safranine complex from flour by Baker and Hulton, Swanson and Tague, and Sharp and Elmer in 1928 (33, p.225). The enzyme was isolated in purer form by Balls and Hale (5) by precipitation from bran extract using dilute ammonium sulfate solution containing a trace of cysteine for the protection of the enzyme. The enzyme was then concentrated by fractional precipitation with ammonium sulfate. The protein fraction which was precipitated between 0.4 and 0.8 saturation contained the enzyme. It was dried in the cold and constituted a fairly good material for further purification. The precipitate was redissolved in water and dialysed against 25% glycerine, containing a trace of cysteine; this resulted in heavy precipitation of globulin. The resulting solution of the enzyme was made more stable by increasing the concentration of glycerine to 50%. They showed that purification is also possible by precipitation with acetone. The enzyme was precipitated completely with concentrations of acetone between 50 and 75 per cent.

Davis (15) used very similar methods for the extraction of proteolytic enzymes from lima beans.

Sumner and Somers (60, pp.43-53) described methods

for extracting enzymes from finely ground materials with water, dilute alcohol or dilute acetone. It is often necessary to defat the meal after grinding and to discard the hull. They also found that sometimes, as in the extraction of emulsin from defatted almond meal, it is advantageous to employ quarter-saturated ammonium sulfate.

Another method which seems to preserve the activity of the enzymes very well was tried by Krishnamurti and Subrahmanyan (35). They froze the extract in ammonium sulfate solution and evaporated in vacuo.

In addition, Sumner and Somers (60, pp.43-44) showed that any dilute salt solution can be used for the extraction of enzymes. In 1939, Ball and Lineweaver (6) crystallized papain from an extract of papaya latex in dilute solutions of ammonium sulfate or sodium chloride.

D. Methods for Determining Proteolysis

Such factors as accuracy, facility, cost of equipment and chemicals were taken into consideration in the selection of a method for the determination of proteolysis. It was on this basis that several methods were investigated.

1. Colorimetric methods

The first colorimetric method considered for the determination of the products of proteolysis was that of

Folin and Ciocalteu (20). They described the preparation of a phenol reagent which gives colors with certain amino acids. This method has its limitations in that it determines only those amino acids which have a phenolic ring and gives different colors with different amino-acids of this nature. The phenol reagent is difficult to prepare and is not very stable over long periods.

The ninhydrin test, originally designed for qualitative determination of protein and amino-acids (47, p.108), was modified by Merton (45) for quantitative determinations using an interferometer. Shaw and McFarlane (55) used the photoelectric colorimeter in their modification. They made a calibration curve using 15 different concentrations of tryptophane solutions between the range of 0.007 to 0.141 mg. per ml. Other modifications were those of Virtanen and Laine (68, 69, 70). The latter were designed with the purpose of determining individual amino acids. One of the limitations of the ninhydrin test is that it gives the color reaction at the very low pH of 2.5, where some proteins could not be kept in solution. The other limitation was that it could be used successfully only for quantitative determinations of amino acids in very low dilutions. This characteristic could, however, be of great advantage in certain cases.

In 1929, Waser (73) developed a color test for amino-acids based on the formation of oxazolones. The test shows the presence of the $CH(NH_2)COOH$ group. To a few ml. of amino-acid solution are added 3 to 4 ml. of 10% sodium carbonate solution and the mixture is boiled. A pinch of p-nitrobenzyl chloride is then added. If alpha-amino acids are present, a dark wine-red to violet-blue color develops. Kerrer and Christoffel (29) who studied this test found that the color of alpha-amino acids in pyridine or dilute alkali was due to the formation of 2-(p-nitro-phenyl)-4-alkyl-5-oxazolones. They observed that 1 mg. of oxazolone in 5 ml. of water, ethyl alcohol, acetone, pyridine, ether or dioxane, with dropwise addition of 0.1 N NaOH to the appearance of color, gave red-violet, red-violet, red-violet, blue, blue, blue, and red-violet shades respectively, which have no apparent relation to the corresponding dielectric constants of the solvents. Kerrer and Keller (30) reported in 1943 that the Waser Color was due to the formation of a mesomeric system containing many conjugated double bonds. Most of this work has been confirmed by Kurtz (36), and Edlbacher and Litvan (19).

There are other qualitative color tests for amino acids such as the one developed by Vangas (64, 65) based

on color formation by reaction between aromatic amino acids and bindone in acetic acid solution. Some histochemical tests were developed by Sera (54) and Gomori (23). These were based on staining reactions. A more comprehensive histochemical test was described by Tauber (62, 63).

Charney and Tomerelli worked out a very good colorimetric test for the determination of the proteolytic activity of duodenal juice (11). It is based upon the loss of the color of Azo-protein, due to hydrolysis by an enzyme.

2. Nephelometric methods

Leubner (37, 38) published his nephelometric method for determining protein hydrolysis in 1938. In this method, 1 ml. of 1:20,000 aqueous dilution of duodenal content is incubated in a water bath at 38° C. with 10 ml. of a mixture consisting of 5 ml. of neutralized 1% casein solution, 10 ml. of phosphate buffer (85 vol. M/15 Na_2HPO_4 plus 15 vol. M/15 NaH_2PO_4) and 35 ml. of quinidine solution (3 gms. quinidine-HCL in 100 ml. of warm distilled water); diluted with water to a volume of 45 ml. The turbidity is measured by means of Pulfrich step photometer. From the turbidity of the mixture, as compared with that of casein buffer mixture, incubated without enzyme, the quantity can be calculated by means of tables. This method was used later for determining the activity of

trypsin (38). Lipase activity was determined using triolein turbidity as the index of hydrolysis.

Svershkov (61) used the same principle to determine the extent of enzyme hydrolysis of proteins in fruit and berry juices.

3. Viscosity and solubility methods

Balls used Northrup and Hussey's method (49) which depends on the decrease in viscosity (4; 5) of the substrate (gelatin) as an index of proteolysis. Using basically the same principle, Moore, Stern and Bergman (46) separated the products of hydrolysis on the basis of the solubility in various solvents and used them as the index of proteolysis.

4. Titrimetric methods

Leubner (37) also described a titrimetric method in which 1 ml. of 1:50 dilution of the duodenal content or enzyme (33) was incubated at 38° C. with 2 ml. of neutralized 2% casein solution, 0.5 ml. of buffer solution (22.6 gm. KH_2PO_4, 35.7 gm. citric acid, distilled water to vol. 500 ml., 2.5 N.NaOH to pH 8.00) and 2 ml. of distilled water. The blank mixture without enzyme was also incubated. The solution was titrated according to Sorensen's formol titration method, using phenolphthalein as the indicator.

Melnick, Oser and Weiss (42) and Melnick and Oser (43) made a thorough study of the formol titration and modified it to give results which correlate very closely with that of nitrogen determined by the classical Kjeldahl method. In carrying out the formol titration, the test solution containing approximately 30 mg. of nitrogen was first adjusted to pH 7.00 in a total volume of 20 ml. Two ml. of a 37% solution of formaldehyde, adjusted to pH 7, was added. This caused a decrease in the pH of the solution, in most cases to pH 5 or 6. The solution was titrated to pH 9.5 with 0.1 N NaOH. This pH was chosen as the end point because at this point the formol titratable nitrogen equalled the Kjeldahl values for ammonium chloride, valine (a typical monoamino-monocarboxylic acid), and for glutamic acid (a typical monoamino-dicarboxylic acid). Further details of the titration are discussed by them.

5. Loehlein-Volhard method

It was considered desirable to investigate in detail the method reported by Niedercorn, Thayer and Evans (48), because they followed a very well-organized procedure which can be successfully applied to the determination of proteolysis brought about by enzymes. The procedure followed by them was as follows:

a. Elution

The sample of pancreatin was weighed into 40 ml. beakers and covered with 200 ml. of 0.75% NaCl solution. $(NH_4)_2SO_4$ can be substituted for NaCl.

After standing at 25° C. for half an hour with occasional stirring, the solutions were filtered through 18.5 cm. Whatman No. 1. filter paper, and 50 ml. aliquots added to the bottles containing the substrate (also at 25° C.). High dilutions and low temperatures were preferred for the elution, since they favored complete extraction and lessened inactivation.

b. Digestion

Each digestion bottle containing 50 ml. of 5% protein solution or 2.5 gm. protein and 50 ml. of buffer, received 50 ml. of the elute and was placed in a bath maintained at 40° C. The blanks were the same except that 0.75% NaCl or $(NH_4)_2SO_4$ solution was added instead of the elute.

c. Precipitation

The precipitating solution consists of 0.06 N H_2SO_4. The digests were allowed to stand for 15 minutes after the addition of 100 ml. of this solution and then filtered through Whatman No. 1. filter paper.

d. Soluble nitrogen was determined by titration with 0.1 N NaOH using Brom Cresol purple as the indicator or by the Kjeldahl method.

E. Addition of Proteolytic Enzymes to Cheese

For many years, investigators have been interested in the relation between proteolysis and cheese ripening. Allen, Trillat and Sauton (1) realized the importance of proteolysis in cheese ripening and studied its relation with flavor. They found that some relationship did exist between ripening and proteolysis, since more breakdown products were found in aged cheese than in green or less ripened samples. Harper and Swanson have successfully used certain amino-acids as

Allen (1) appears to be the first to have studied the biochemistry of cheese in detail. According to him, the extent of nitrogen degredation can best be measured by the formaldehyde titration of an extract of the cheese in 80% alcohol. He agreed with earlier workers that cheese made from raw milk undergoes a quicker and more extensive ripening than that made from pasteurized milk.

Hawesson (25) reported in 1930 that the addition of supplementary quantities of rennin in the production of cheese causes a considerable increase in the rate of

ripening. Eagles and Sadler (17, 18) showed that the ripening of cheese is due to rennin and peptic-like enzymes liberated by bacteria. The action of rennin in cheese ripening was confirmed by later work of Sherwood (56). He pointed out that ripening was due to the presence of an enzyme other than the rennet itself. Kelly (32) found that the ripening of cheese was brought about by galactase (indigenous to the milk), pepsin in the rennet extract and enzymes produced by bacteria. Doan and Freeman (15), and Kay, Hiscox and Davies (31) agreed with their predecessors in most of their work. They also demonstrated that commercial rennet contained enzymes capable of four different functions: (i) clotting the milk at pH 6.5, (ii) breaking down the protein at pH 2 (pepsin), (iii) proteolysis at pH 4.6 (papainase), and (iv) proteolysis at pH 4.6 (peptidase). They showed that although cheese can be made from pepsin, bitterness is likely to develop.

Freeman and Dahle (21) added pepsin and trypsin to the curd in order to study the effect of these enzymes on the ripening of cheese. They found that pepsin increased the rate of proteolysis during ripening, particularly at the beginning of the ripening period at lower ripening temperatures. Added pepsin did not accelerate the development of flavor, but produced an aged cheese with an

appreciably higher score. Trypsin increased markedly the rate of proteolysis during the early part of the ripening periods, after which its effect was reduced greatly. The enzyme also increased slightly the rate of flavor development, but reduced the maximum flavor score attained. In a later publication, Doan and Freeman (16) reported that cheddar cheese was ripened more rapidly by producing a cheese of relatively low acidity, adding the two enzymes and ripening at a higher temperature.

CHAPTER III

EXPERIMENTAL PROCEDURES

A. Preparation of Filbert Extracts

Sodium chloride was chosen as the extractant because it has been used with success for the extraction of proteolytic enzymes and is available readily in pure form. In addition, it is accepted as a common food ingredient by the Food and Drug Administration of the United States Department of Agriculture and does not have to be declared on the label when used in food products. This would facilitate the use of filbert extracts in foods.

Of several concentrations of sodium chloride tried in the preparation of the extracts, it was found that concentrations between five and ten per cent produced very good results. The method for the preparation of the extracts was as follows:

1. Filbert kernels were chopped in an ordinary kitchen nut-chopper to a fine size and the material was sieved through a No. 20 mesh screen. If the nuts were not dry, it was necessary to dry them before chopping, otherwise it was very difficult to get the fine particle size.

2. Twenty-five grams of the powdered nuts were soaked in 100 ml. of five per cent sodium chloride for a

period of two and a half hours. During this period, the mixture was stirred several times to obtain proper soaking of the powder.

3. At the end of two hours, the suspension was shaken well and filtered through a single layer of cheese cloth at first and then through four layers of the cloth. In order to get proper extraction, it became necessary to squeeze the material during the first filtration and recover as much of the filtrate as possible.

4. The extract was left at 2° C for about three hours. This resulted in the separation of the extract into two layers. The top layer contained the fat while the bottom layer was almost fat free.

5. The bottom layer was siphoned out by means of a pipette and this fraction was used for the study of proteolytic activity. The pH of this extract was 6.30-6.40.

It was essential to take all possible aseptic precautions during the preparation of the extracts in order to keep microbial contamination as low as possible. The filbert nuts were cleaned by wiping them with a clean dry cloth, dried and stored in a clean dry place at 2° C. All the equipment used in the extraction was sterilized and handled with aseptic precautions. If this was not done, the microbial contamination interfered with the

hydrolytic activity of the extracts and results showed wide variations.

B. Preparation of Substrates

The first factor taken into consideration in the preparation of the substrates was the use of native proteins, because if the filbert extracts are used in foods such as cheese, it would be concerned with the hydrolysis of these proteins.

1. Egg albumin

Egg albumin substrate was tried first. It is of interest to note that most of the literature on the use of this substrate reports the use of denatured egg albumin.

In order to have the same concentration of the substrate throughout the work, it was decided to prepare the substrate in a large volume.

Albumin was separated from two dozen fresh eggs and dissolved (peptized) in three per cent sodium chloride solution. Total solids were determined, and the solids content adjusted to two per cent with more sodium chloride solution of the same strength. Fifty ml. of toluene were poured on the surface of this substrate in a gallon jar and stored at 2° C. Aseptic conditions were maintained during its preparation.

2. Casein

Casein is another substrate which can be obtained with comparative ease in un-denatured form. It has been used quite extensively for the study of proteolysis, though in denatured form.

The casein substrate was prepared as follows:

a) 1500 ml. of skim milk was saturated with 600 grams of sodium chloride. The viscous solution, on standing overnight at 35° C, settled down a little.

b) Next morning, the mixture was centrifuged for 15 minutes. The supernatent liquid was discarded, the settled casein was suspended in 40 per cent sodium chloride solution, and centrifuged again. The casein was washed four times in this manner until the supernatent liquid was clear and did not give any precipitate with hydrochloric acid after filtration. This indicated that other proteins were removed by the washing. The colorless liquid indicated, also, the absence of pigments.

c) The final sediment was suspended in 500 ml. of distilled water.

d) The sodium chloride content of the suspension was determined by titration against silver nitrate using dichlorofluorescein as the indicator. It was found to contain 1.3% sodium chloride.

e) The total solids content of the mixture,

determined by the association of official agricultural chemists' method, was 13.13 per cent.

f) A suspension containing 3 per cent casein was prepared.

3. Non-fat milk solids*

Preliminary experiments showed that the filbert extracts were very active when non-fat milk solids were used as the substrate. Besides, the use of filbert extracts in cheese ripening was considered an important aspect of the application of this research and a substrate which resembled cheese to a large extent was needed. Other important requirements of the substrate were that it be comparatively free from fat so that the hydrolysis of fat by the presence of lipase in the extract would not interfere with the results of the proteolysis, and the substrate be available readily in uniform composition.

Low pre-heat dried non-fat milk solids (skim milk powder) appeared to satisfy the above requirements in spite of its limitations with regard to the presence of impurities, most of which are present also in cheese. The non-fat milk solids used here were prepared by preheating the skim milk between 115° and 117° F, by

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* Non-fat milk solids is the commercial term used for dry skim milk. Its fat content is about 1½ per cent.

condensing it to a ratio of 4:1 and spray-drying in a stainless steel Roger drier, using a 58-2 Monarch nozzle. The particle size of the powder was about five microns. Freshly prepared powder was obtained and stored at 2° C. The reason for the choice of this powder was that it had been pre-heated to a lower temperature than the conventional type of powder and, hence, the protein in it was denatured to a much lesser degree.

The substrate was prepared by stirring nine grams of the powder into 91 ml. of distilled water by means of a Hamilton Beach milk shake machine for one and one-half minutes. Fifteen minutes were allowed for the foam to subside and then the mixture was poured into a two-liter Erlenmeyer flask. Aseptic conditions were maintained as far as possible in order not to contaminate the substrate beyond the level of contamination present in the original powder.

C. Determination of Proteolysis

1. Preliminary Investigations

Several colorimetric methods were investigated at first since these are the most modern and time-saving ones. These seem to have certain limitations for the kind of determinations desired here. The first requirement is to have a method which is based upon the

determination of as many products of proteolysis as possible, not individually but in mass. The second qualification desired is that it should be efficient for the determinations in a comparatively large quantity of the digest. The colorimetric methods did not seem to meet these requirements since most of them make use of an index based upon the determinations of a few components. The method reported by Charney and Tomarelli (11), while expensive, meets the requirements more closely than other colorimetric methods.

The nephelometric method of Loubner (37, 38), which measures the decrease of turbidity in protein solutions as an index of hydrolysis, was not found to be of any value because of the kind of substrate used in these investigations. He has used, also, Sorenson's formol titration with phenolphthalein as the indicator. The limitation of this method, however, is the indicator, the color of which turns pink around pH 8.3, while a number of amino acids are not titrated below pH 9.5 according to the very comprehensive work of Molnick, Oser and Weiss (42), and Molnick and Oser (43). The titration of the hydrolysate, using Brom Cresol purple, has been reported by Niedercorn, Thayer and Evans (44), which also has the same limitation of being dependent upon a color indicator.

The classical kjeldahl method was not tried because of its time-consuming character. Besides, Melnick and Oser (43) have shown that their modification of the formol titration is almost as efficient as the kjeldahl method.

On the basis of the foregoing experience and the literature survey, the following method was developed. It depends, to a large extent, upon the Melnick and Oser modification of Sorensen's formol titration.

2. Preparation of the Samples

a) Thirty ml. of the extract were added to 300 ml. of the substrate and mixed thoroughly by stirring. At the same time, a control sample (blank) was prepared by adding 30 ml. of five per cent sodium chloride solution, instead of the extract, to another sample of 300 ml. of the substrate. At first, some experiments were run in which the control had 30 ml. of inactivated (by boiling for five minutes) filbert extract as shown in the case of the hydrolysis of casein in Table II. Little or no difference was found between the control containing the inactivated extract and the one containing the five per cent sodium chloride solution.

In most of the experiments, the digests were prepared as outlined already but, in some places, it was

necessary to make slight changes. These changes are indicated wherever made.

The pH of the digest was 6.55-6.60.

b) Seven 40 ml. aliquots were poured into seven 125 ml. erlenmeyer flasks, followed by 2 ml. of toluene in order to 'prevent' microbial fermentation. This was done in addition to all the aseptic precautions observed during the preparation of the samples.

c) The samples were incubated at 30° C for multiples of 24 or 48 hour periods.

3. The Modified Formol Titration

a) At the end of the regular intervals of 24 or 48 hour periods, the samples were removed from the incubator and the coagulum was broken by stirring with an air driven glass stirrer. This facilitated the precipitation of unhydrolysed protein.

b) Ten ml. of approximately one normal trichloroacetic acid were added to each of the 40 ml. samples in the erlenmeyer flasks and allowed to stand for five minutes. Acid of the same normality was used throughout the experiments.

c) The acidified digest was filtered through a 15 cm. Whatman No. 5 filter paper.

d) After the precipitation was complete, a 25 ml. aliquot of the filtrate was pipetted into a 250 ml.

beaker and an equal volume of water was added to cover the electrodes. The pH of this hydrolysate was adjusted to 7.0 with 0.1 normal sodium hydroxide, using a Beckman H-2 AC operated pH meter with the type E glass electrode. This electrode is resistant to alkali and requires very little sodium ion correction. In some of the preliminary experiments, the Model G pH meter was used, but the Model H-2 was found just as efficient for this work. In addition, it had the advantage of having a line operated circuit. (A picture of the titration equipment is shown on page 30.)

e) Two ml. of neutralized (pH 7) formaldehyde were added to the hydrolysate. This lowered the pH of the solution to between 6.8 and 6.5, depending upon the degree of hydrolysis.

f) The solution was titrated then to pH 9.5 with 0.1 N. sodium hydroxide solution.

g) The sodium ion correction was applied and all the results were expressed as ml. of 0.1 N. sodium hydroxide required to titrate 100 ml. of the hydrolysate to pH 9.5 after the addition of formaldehyde. This was done by subtracting the titration results of the control (blank) from that of the experimental sample and multiplying the difference by five.

Precautions

In order to get correct results which could be duplicated with a small standard error, it was necessary to take the following precautions:

1. Trichloroacetic acid used as the precipitant should be about one normal. If it falls below 0.5 normal, the precipitation time has to be increased in order to get correct results.
2. Any fine filter paper may be used provided that it is used throughout the work; otherwise, the results vary.
3. Filtration should be complete even though an aliquot is taken.
4. Normality of the sodium hydroxide used should be constant and exact.
5. The same electrodes should be used throughout the experiments. It is necessary to use Beckman type E glass electrode because the titrations are usually run between pH 7.0 and 9.5. This electrode is alkali resistant and has a very low sodium ion correction.
6. In order to keep the pH meter in proper adjustment, it was advisable to check it against buffers of pH 7 and/or 9.5 after at least four

titrations.

7. Excessive speed of the stirrer caused vibrations which interfered with the pH readings. It should be controlled.
8. A uniform substrate should be used.

Taking all these precautions into consideration, the standard error of the means of six samples (three containing Barcelona and three containing Du Chilly filbert extracts) was calculated to be 1.42 as shown on pages 113, 114 and 117 of the appendix. The F value was insignificant for the columns, indicating that the results were duplicated with negligible error, as shown on pages 115 and 116 of the appendix for both the extracts used. The determination of F value included another variable, the method of extraction. This indicated that the variations in the method of extraction of the filberts were also negligible.

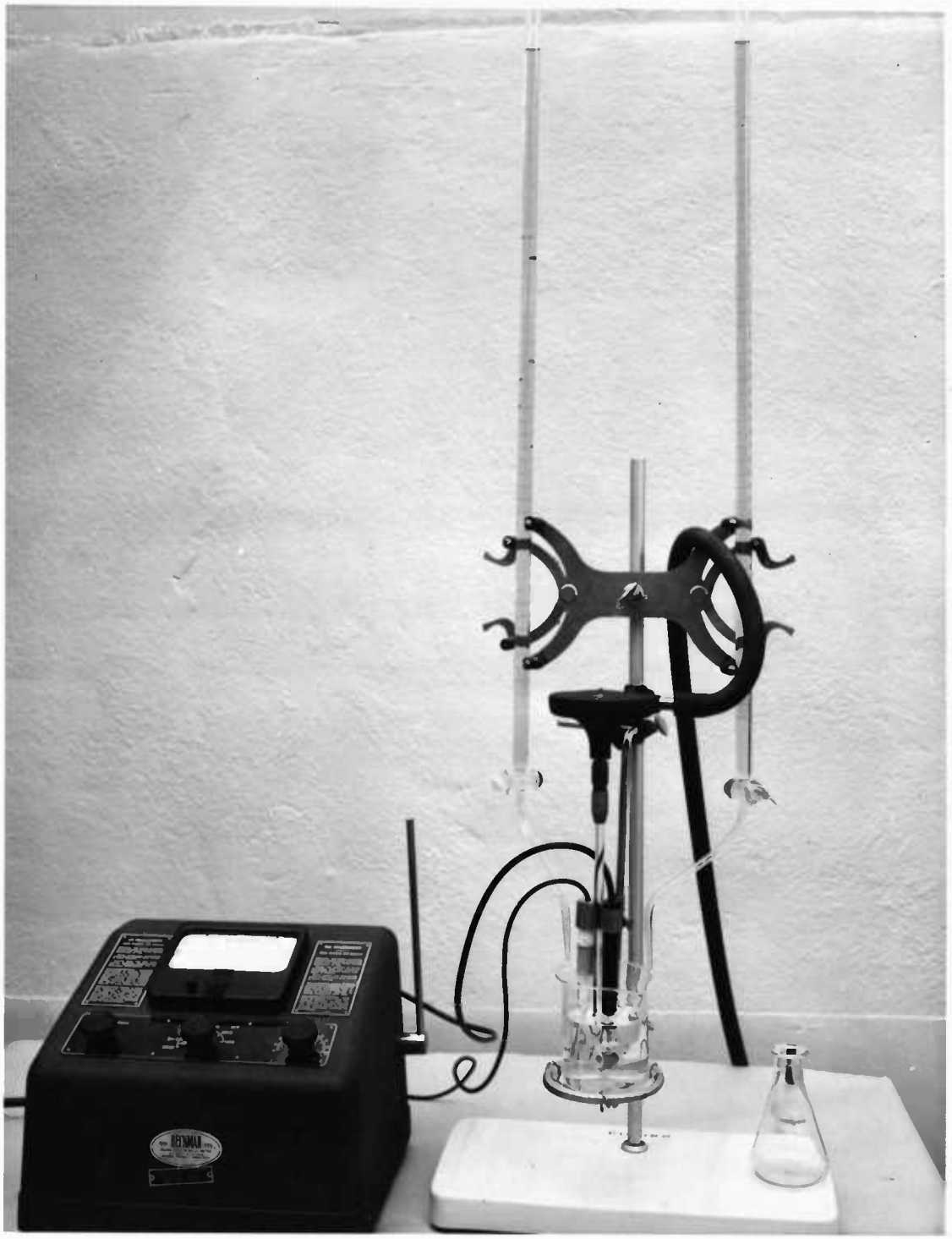


PLATE I - Titration Equipment.

CHAPTER IV

RESULTS AND DISCUSSION

A. Comparison of the Substrates

The hydrolysis of 2 per cent egg albumin, 3 per cent casein and 9 per cent non-fat milk solids (having about 1½ per cent fat and about 3.8 per cent proteins) substrates by Barcelona and Du Chilly extracts was compared. The non-fat milk solids substrate showed the maximum rate as well as the extent of hydrolysis and was finally chosen as the substrate for all further investigations.

Egg albumin showed the least degree of hydrolysis, casein was the next and non-fat milk solids surpassed both in the rate as well as the extent of its hydrolysis; the data for this are summarized in Tables I, II, III (first column) and IV (first column). The results are graphically presented in Figures I and II. The extent of hydrolysis for casein is about four times that for egg albumin, and that for non-fat milk solids substrate is about twice that for casein or eight that for egg albumin when the first 48 hour period is taken into consideration. This was found to be the period of maximum activity for all substrates. The activity of the extracts continues at a fast rate on casein and non-fat milk solids

for the first 72 hours, then starts to slow down and comes almost to an end after 180 hours.

There could be several possible explanations for the greater degree of hydrolysis shown for the non-fat milk solids. The latter has received heat treatment during its preparation and, hence, has undergone a certain amount of denaturation, perhaps resulting in a modification of protein linkages. Besides, the non-fat milk solids are a complex substrate and have many substances present in the powder, besides the proteins, which might have acted as activators for the proteolytic action. Presence of a few sporeforming thermophilic bacteria might have had a little to do with the higher results for the non-fat milk solids; however, this is very doubtful because all the experiments were run at 30° C and 2 ml. of toluene was poured on the surface of each 40 ml. of the digest. This also prevented any further contamination of the digest.

The hydrolysis of the casein substrate was slow perhaps because it was in an undenatured form and most of the other substances present with it had been washed out during its preparation.

The hydrolysis of the egg albumin substrate came to an end after the first 48 hours. A possible explanation for this could be the presence of an enzyme inhibitor or that the enzyme or enzymes present in the extracts did

not attack all the linkages of this protein.

To try and explain the difference in activity of the extracts for the substrates without any further work would be speculation.

B. Effect of Storage on Filbert Extracts

In order to study the effect of storage, Barcelona and Du Chilly extracts were stored at 2° C in a refrigerator. The extracts were aged for one, two and four weeks, and after each storage period, their activity was determined using the non-fat milk solids as the substrate. The results are compared with those of the fresh extracts in Tables III and IV and illustrated by Figures III and IV. For all these samples, the incubation temperature used was 30° C.

This study shows that the activity of both, Barcelona and Du Chilly, extracts drop to a little more than half on storage for a week, and a little less than half in two weeks, when the first 24 hour period of incubation is considered. The extracts stored for one week lost about 33 per cent of their activity when the extent of hydrolysis for 48 and 72 hours at 30° C is compared with that of the fresh extracts. For the same periods of hydrolysis, the two weeks old extracts lost about 60 per cent activity. When 96 and 180 hour periods are examined,

TABLE I

HYDROLYSIS OF EGG ALBUMIN SUBSTRATE BY FILBERT EXTRACTS
(Incubation temperature 30° C)

Time in hours	Ml. 0.1 N. NaOH			B-C On the basis of 20 ml. substrate	B-C On the basis of 100 ml. substrate	D-C On the basis of 20 ml. substrate	D-C On the basis of 100 ml. substrate
	B	D	C				
0	0.60	0.60	0.60	0.00	0.00	0.00	0.00
24	0.90	0.80	0.60	0.30	1.50	0.20	1.00
48	1.00	0.85	0.50	0.50	2.50	0.35	1.65
72	1.10	0.95	0.55	0.55	2.75	0.40	2.00
96	1.15	1.00	0.60	0.55	2.75	0.40	2.00
120	1.20	1.00	0.60	0.60	3.00	0.40	2.00
144	1.20	1.00	0.60	0.60	3.00	0.40	2.00

B - 10 ml. of Barcelona extract per 100 ml. of substrate.

D - 10 ml. of Du Chilly extract per 100 ml. of substrate.

D - 5% NaCl solution substituted for the extract.

pH of the digest was 6.7-6.8.

All the above results were obtained according to the method outlined on pages 26 and 27.

FIGURE I

COMPARISON OF THE PROTEOLYTIC ACTIVITY OF BARCELONA AND DU CHILLY EXTRACTS

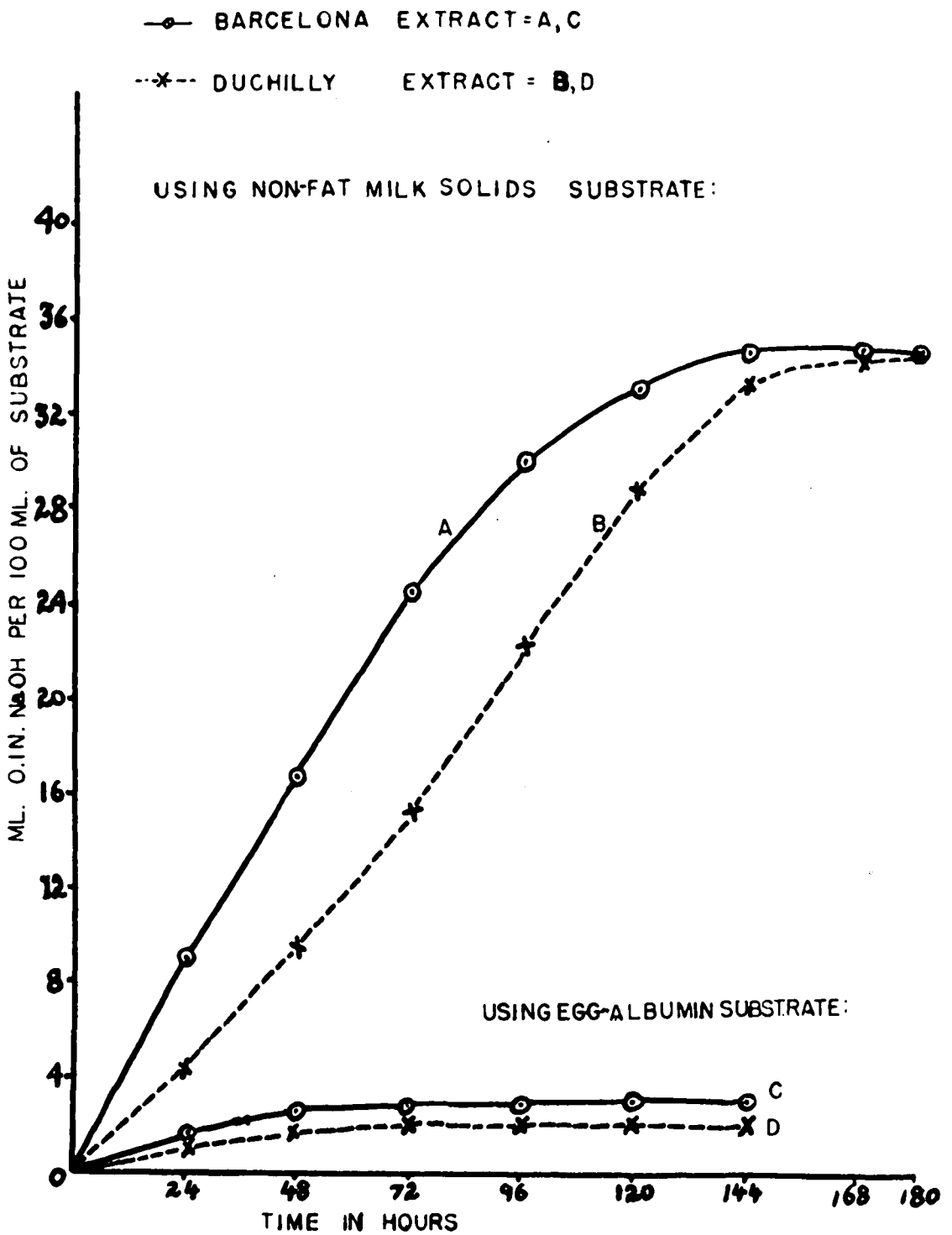


TABLE II

HYDROLYSIS OF CASEIN BY FILBERT EXTRACTS
(Incubation temperature 30°C)

Time in hours	Ml. 0.1 N. NaOH				B-BC On the basis of 20 ml. substrate	B-BC On the basis of 100 ml. substrate	D-DC On the basis of 20 ml. substrate	D-DC On the basis of 100 ml. substrate	C
	B	BC	D	DC					
0	1.20	1.20	1.20	1.20	0.00	0.00	0.00	0.00	1.20
24	2.20	1.10	2.00	1.10	1.10	5.50	0.90	4.50	1.10
48	3.25	1.25	2.55	1.25	2.00	10.00	1.30	6.50	1.25
72	4.00	1.40	3.00	1.40	2.60	13.00	1.60	8.00	1.40
96	4.25	1.45	3.45	1.45	2.80	14.00	2.00	10.00	1.45
120	4.40	1.50	3.70	1.50	2.90	14.50	2.20	11.00	1.45
144	4.40	1.50	4.00	1.50	2.90	14.50	2.50	12.50	1.50
168	4.40	1.50	4.20	1.50	2.90	14.50	2.70	13.50	1.50
192	4.50	1.60	4.50	1.60	2.90	14.50	2.90	14.50	1.55
216	4.50	1.60	4.50	1.60	2.90	14.50	2.90	14.50	1.60

B - 10 ml. of Barcelona extract per 100 ml. of substrate.
BC - Control containing Barcelona extract inactivated by boiling for 5 minutes.
D - 10 ml. of Du Chilly extract per 100 ml. of substrate.
DC - Control containing Du Chilly extract inactivated by boiling for 5 minutes.
C - Control without any inactivated extract, containing 5% sodium chloride instead. pH of the digest was 6.80.

All the above results were obtained according to the method outlined on pages 26 and 27.

FIGURE II

HYDROLYSIS OF CASEIN BY FILBERT EXTRACTS

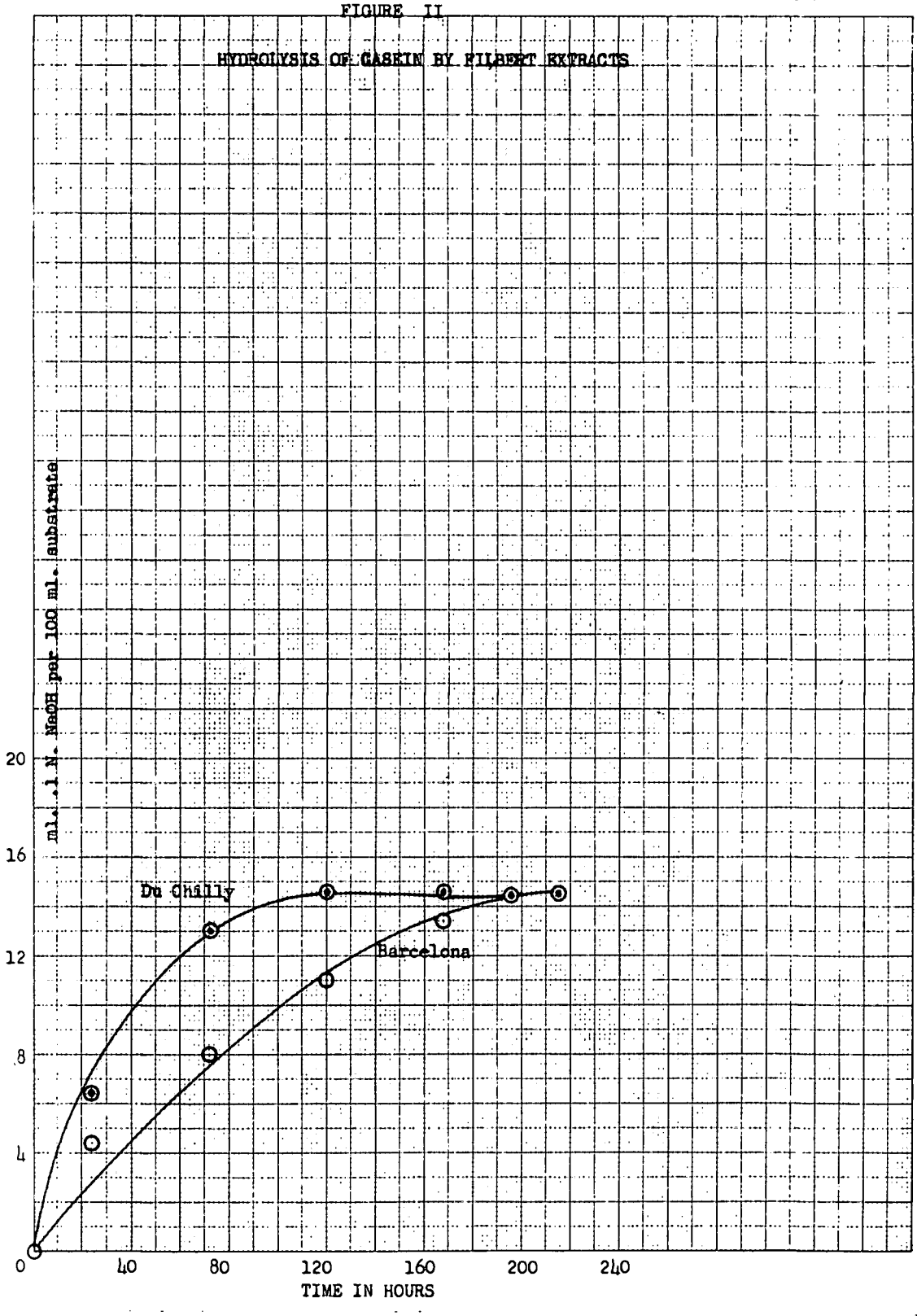


TABLE III*

EFFECT OF STORAGE AT 2° C ON THE ACTIVITY OF BARCELONA EXTRACT (Incubation temperature 30°C)

	Fresh B-C	Stored One week B-C	Stored two weeks B-C
Time in hours		Ml. 0.1 N. NaOH	
0	0.00	0.00	0.00
24	9.00	5.25	4.00
48	16.75	11.25	10.00
72	24.50	17.25	14.50
96	30.00	22.00	15.75
120	33.00	23.25	16.25
144	34.75	23.50	16.25
168	34.75	23.50	17.00
180	34.75	23.50	17.25

B - 10 ml. of Barcelona extract per 100 ml. of substrate.

C - Control - 5% NaCl substituted for the extract.

pH of the digests was 6.55.

All the above results were obtained according to the method outlined on pages 26 and 27.

* For details of the results presented in this table, refer to Tables 1, 2, and 3 in the appendix.

FIGURE III

EFFECT OF STORAGE ON BARCELONA EXTRACT

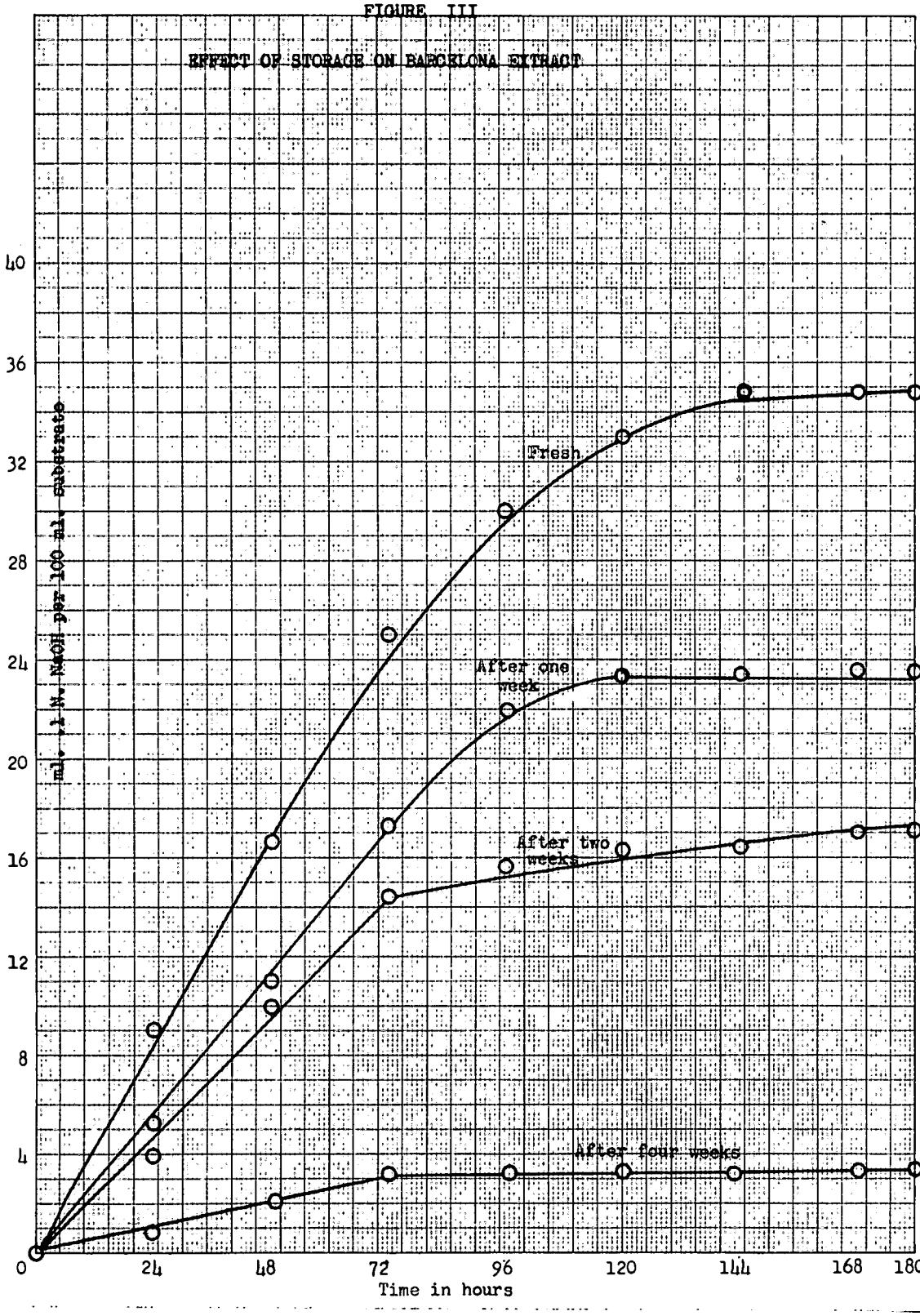


TABLE IV*

EFFECT OF ATORAGE AT 2° C ON THE ACTIVITY OF DU CHILLY EXTRACT (Incubation temperature 30°C)

	Fresh D-C	Stored One week D-C	Stored two weeks D-C
Time in hours	Ml. 0.1 N. NaOH		
0	0.00	0.00	0.00
24	4.25	2.75	1.50
48	9.50	6.75	5.75
72	15.25	11.25	9.00
96	22.25	15.00	11.50
120	28.75	18.25	13.00
144	33.50	23.00	15.25
168	34.25	23.50	16.25
180	34.75	23.50	16.25

D - 10 ml. of Du Chilly extract per 100 ml. of substrate.

C - 5% NaCl solution substituted for the extract.

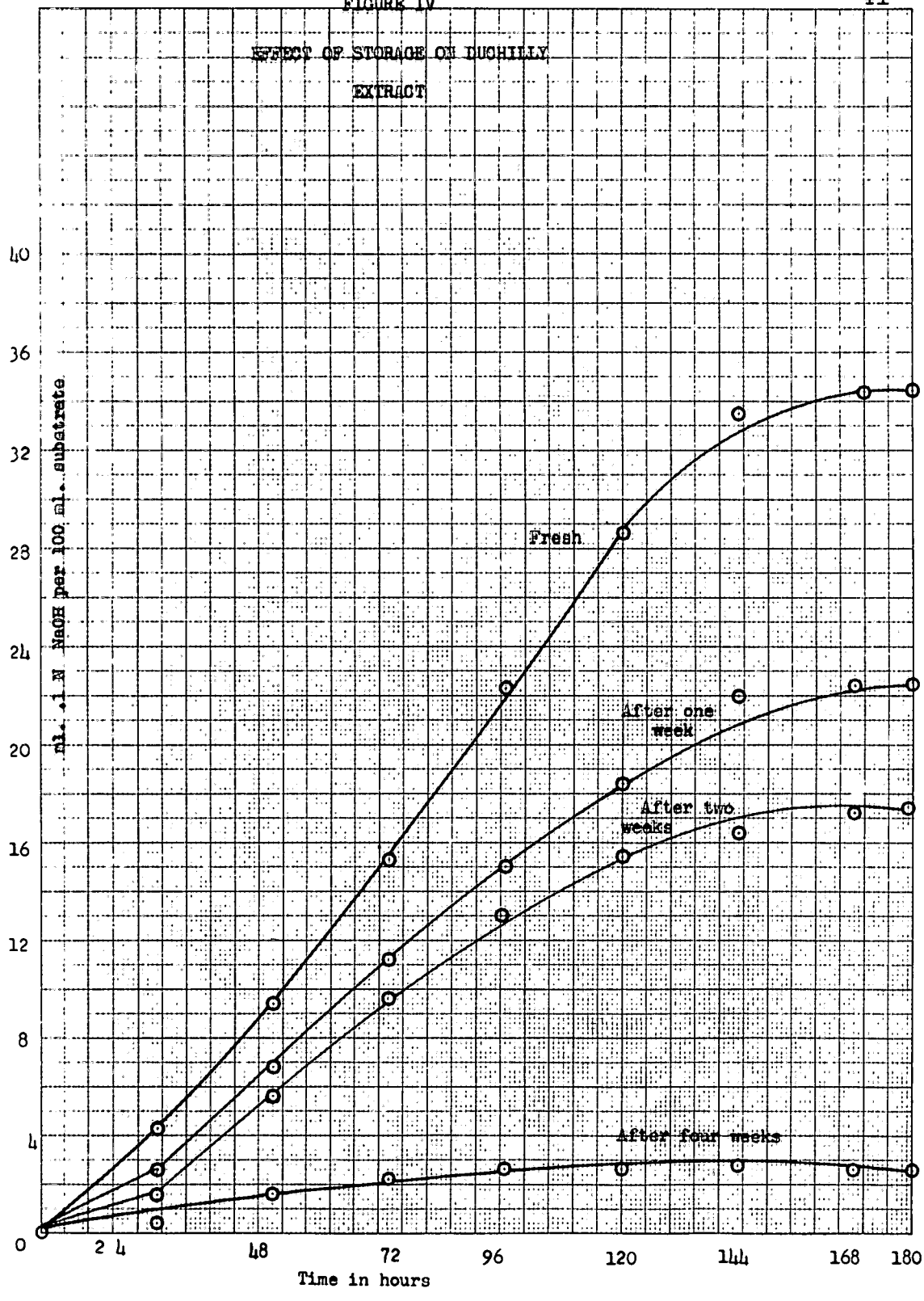
pH of the digests was 6.55.

All the above results were obtained according to the method outlined on pages 26 and 27 and expressed on the basis of 100 ml. of the substrate.

* For details of the results presented in this table, refer to Tables 1, 2, and 3 in the appendix.

FIGURE IV

EFFECT OF STORAGE ON DUCHILLY EXTRACT



it is found that the activity of the extracts stored for one week remains at the same level, while that of the one stored for two weeks falls to half that of the fresh extracts (or stops increasing at an earlier period). The rise in the activity after 24 hours appears to be due, perhaps, to their activation by the incubation temperature of 30° C which is much higher than the storage temperature of 2° C. Extracts stored for four weeks lost most of their activity.

The effect of storage on Barcelona and Du Chilly extracts appears to be very similar if not the same. The results indicate that the extracts do not keep well in storage at 2° C. It is advisable to make fresh extracts whenever required. At the same time, this work suggests that methods should be devised for concentrating, drying, or isolating the enzyme or enzymes from the extracts so that the material can be stored without loss of activity.

C. Effect of Different Incubation Temperatures on the Activity of Filbert Extracts

Proteolytic activity of Barcelona and Du Chilly extracts was studied at incubation temperatures of 10°, 20°, 30°, 37°, and 50° C. The data obtained from the results of this study are summarized in Tables V and VI and illustrated in Figures V and VI.

Of the temperatures studied, the optimum was at 37° C. The activity at this temperature was considered 100 per cent. The activity at 30° C was about 65 per cent as compared to that at the optimum for most intervals of the time. At 20° C, it was about 20 per cent and at 10° C, it was about 13 per cent for the intervals of 48 and 96 hours and falls to a lower percentage with the increase of time. Heat inactivates these extracts, for the degree of proteolysis at 50° C is less than that at 20° C for the first 48 hours of incubation and later becomes almost the same as that at 10° C. It was found later that if the extracts were heated to 60° C, they were completely inactivated; the difference between the control and the sample containing the inactivated extract was zero.

It would be ordinarily expected that although the hydrolysis at the lower temperatures proceeds at a slower rate, the total extent of hydrolysis for all the temperatures would be the same if permitted to continue for a long enough time. Contrary to this, the hydrolysis of all the samples almost ceases by the end of 240 hours, but the extent of hydrolysis for all the samples does not become equal. At this point, the hydrolysis at 10° C is 9.9 per cent, at 20° C is 20.3 per cent, at 30° C is 95

per cent and at 50° C, it is 8.1 per cent of the hydrolysis at 37° C for the Barcelona extracts. The results for Du Chilly are similar to those for Barcelona extracts.

A possible explanation for the foregoing results may be that either the proteolytic enzyme (or enzymes) in the extracts get slowly inactivated or there may be some unknown causes for this phenomenon.

At this stage, an attempt was made to find a rate law for the activity of Barcelona extract at 30° and 37° C.

The units used in the calculations are defined as follows:

E - Concentration of the extract. This was considered equal to one, where 10 ml. of the extract (representing 2.5 gms. of filbert nuts) were added per 100 ml. of 9% non-fat milk solids substrate.

S - Initial active substrate. This was assumed to be equal to 46.5 ml. of NaOH because that was the highest value of proteolysis obtained at 30° C. The maximum value at 37° C was very close to that at 30° C. It was assumed that if a large number of experiments were run, the values would have been about the same.

N - ml. of 0.1 N. NaOH used. This is proportional to the amount of substrate hydrolysed.

S - N = Amount of active substrate left at time t for the value of N.

t - Time of the reaction in hours.

Having defined the above units, the following equation was found to apply at pH 6.55:

$\frac{dN}{dt} = k(E)(S-N)$ where k is the rate constant-------I.

The integrated form of this equation is as follows:

$2.3 \log_{10} \frac{(S\ \ )}{(S-N)} = k(E)t$----------II.

The power of (E) was determined to be one according to the initial rate method (62, p.9). This proved that the reaction is of the first order with respect to (E) and (S-N).

The value of k was calculated as 8.43×10^{-3} with a standard error of 1.87×10^{-3} (or an approximate 20 per cent maximum deviation of t for a given value of N) at 30° C. The dimensions of k being $\frac{1}{E \times t}$, that is, k is the reciprocal of the relative extract concentration (E) units times the number of hours (t). At 37° C, the same equation can be applied when the value of k is 15.14×10^{-3} (or an approximate 15 per cent maximum deviation of t for a given value of N).

It is not possible to calculate the value of k at 20° C using this equation because the value of k

decreases as the hydrolysis proceeds at this temperature. This happens because some new variable comes into play at lower temperatures as a result of the complex nature of the extract and/or the substrate.

The values of k at 10° C and 50° C were not determined because the amount of hydrolysis at these temperatures is too low to be of any importance.

D. Effect of Different Concentrations of Filbert Extracts on Non-fat Milk Solids Substrate

Three different concentrations of the Barcelona and Du Chilly extracts were compared for their activities, using non-fat milk solids as the substrate.

The following digests were prepared for these studies:

First Series

B1 (or D1) - 250 ml. 9 per cent non-fat milk solids, 12.5 ml. Barcelona (or Du Chilly) extract, 37.5 ml. distilled water; making the total volume 300 ml.

C1 - Same as B1 (or D1), except that 12.5 ml. of 5 per cent sodium chloride solution was substituted for the extract.

Second Series

B2 (or D2) - 250 ml. 9 per cent non-fat milk solids,

TABLE V*

EFFECT OF INCUBATION TEMPERATURES ON THE HYDROLYSIS OF NON-FAT MILK SOLIDS BY BARCELONA EXTRACTS

Time in hours	B-C at 10° C	B-C at 20° C	B-C at 30° C	B-C at 37° C	B-C at 50° C
	Ml. 0.1 N. NaOH				
0	0.00	0.00	0.00	0.00	0.00
48	1.25	4.50	18.00	27.00	3.00
96	2.50	7.25	28.75	34.00	2.25
144	3.00	7.50	35.50	41.00	5.25
192	4.00	8.00	40.00	43.00 (168 hrs.)	3.50
240	4.25	8.75	41.00	43.00	3.50

B - 20 ml. of Barcelona extract per 100 ml. of substrate.

C - Control - 5% NaOH substituted for the extract.

pH of the digests was 6.55.

All the above results were obtained according to the method outlined on pages 26 and 27 and expressed on the basis of 100 ml. of substrate.

* For details of the results presented in this table, refer to Tables 10, 11, 12, 13 and 14 in the appendix.

FIGURE V

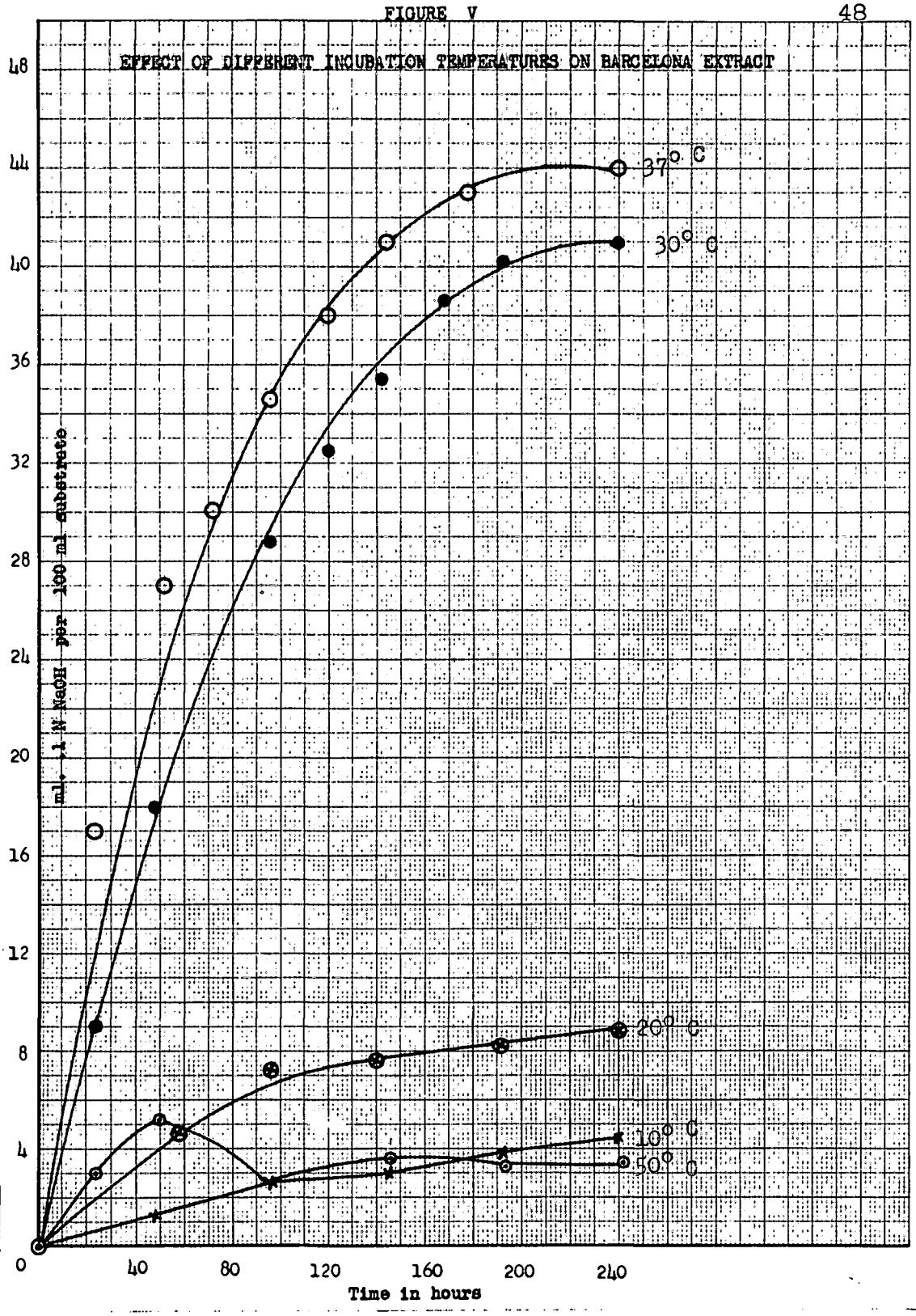

EFFECT OF DIFFERENT INCUBATION TEMPERATURES ON BARCELONA EXTRACT
37° C
30° C
20° C
10° C
50° C
c.c. 1 N NaOH per 100 gms. substance
0
4
8
12
16
20
24
28
32
36
40
44
48
40
80
120
160
200
240
Time in hours

TABLE VI*

EFFECT OF INCUBATION TEMPERATURES ON THE HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY DU CHILLY EXTRACT

Time in hours	D-C at 10° C	D-C at 20° C	D-C at 30° C	D-C at 37° C	D-C at 50° C
	Ml. 0.1 N. NaOH				
0	0.00	0.00	0.00	0.00	0.00
48	2.00	4.50	10.00	14.00	3.00
96	3.00	7.00	20.00	25.00	1.75
144	3.50	9.00	-	-	2.50
168	-	-	32.00	36.50	-
192	4.25	9.50	-	-	3.25
240	4.25	9.50	38.00	40.00	3.25

D - 10 ml. of Du Chilly extract per 100 ml. of substrate.

C - Control - 5% NaCl substituted for the extract.

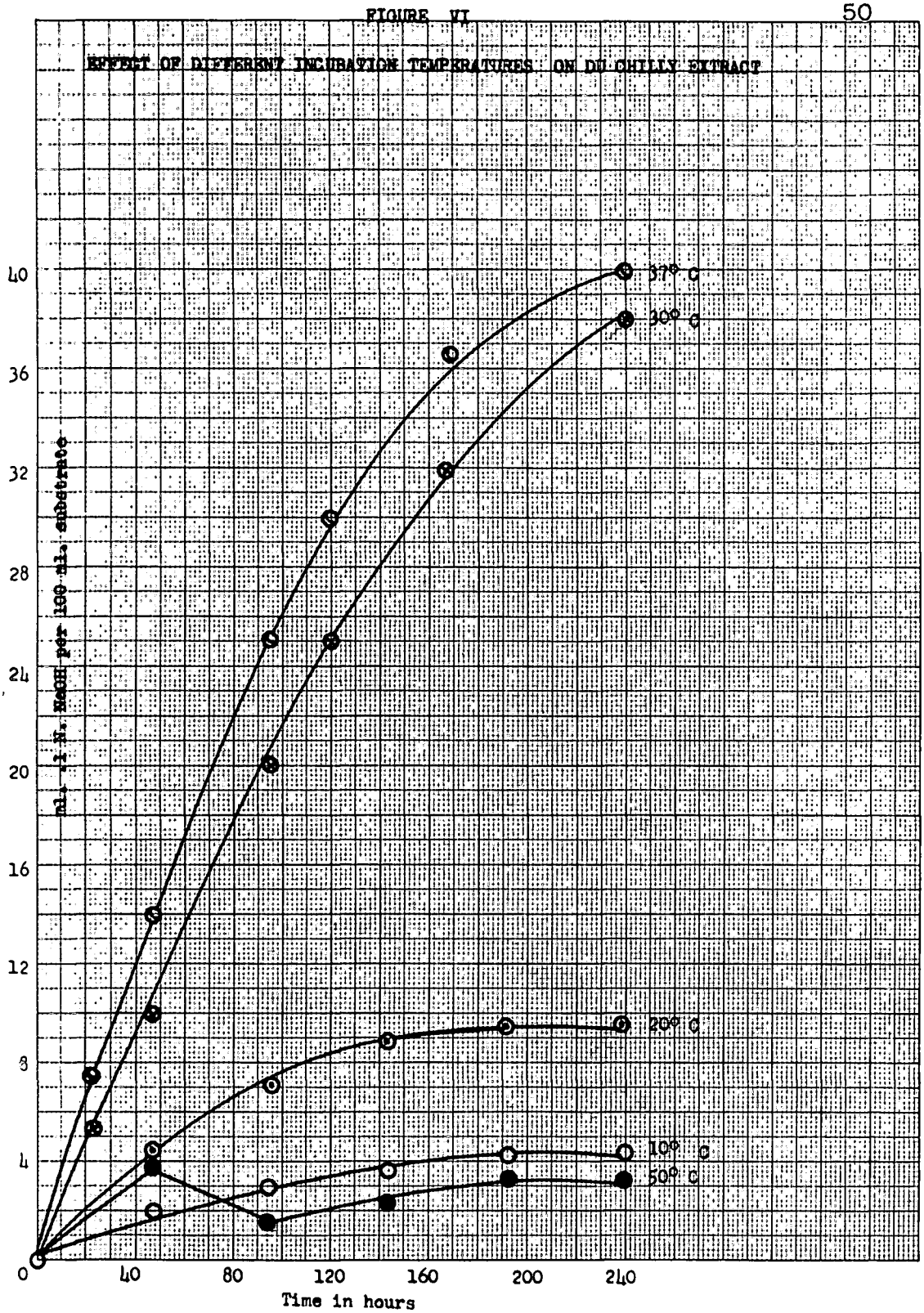
pH of the digests was 6.55.

All the above results were obtained according to the method outlined on pages 26 and 27 and expressed on the basis of 100 ml. of substrate.

* For details of the results presented in this table, refer to Tables 15, 16, 17, 18, and 19 in the appendix.

FIGURE VI

EFFECT OF DIFFERENT INCUBATION TEMPERATURES ON DU CHILLY EXTRACT



25.0 ml. Barcelona (or Du Chilly) extract, 25.0 ml. distilled water; making the total volume 300 ml.

C2 - Same as B2 (or D2), except that 25.0 ml. of 5% sodium chloride solution was substituted for the extract.

Third Series

B3 (or D3) - 250 ml. 9 per cent non-fat milk solids, 50 ml. Barcelona (or Du Chilly) extract; making the total volume 300 ml.

C3 - Same as B3 (or D3), except that 50 ml. sodium chloride was substituted for the extract.

B - Barcelona

D - Du Chilly

C - Control

The ratio of filbert extract to the substrate in the second series is 1:10 (or E = 1) which is standard for all the studies in the entire investigation. The first series has half the standard amount of the extract (or E = ½) and the third series has twice the standard amount of the extract (or E = 2).

All the samples were incubated at 30° C and the hydrolysis determined at regular intervals of 48 hours

until they reached a stage at the end of 240 hours beyond which the hydrolysis would not proceed. The results are summarized in Tables VII and VIII and illustrated by Figures VII and VIII.

The results indicate that the hydrolysis of non-fat milk solids substrate varies with the extract substrate ratio. The larger the amount of extract per substrate, the faster the rate as well as greater the total hydrolysis.

Application of the equation on Page 45 in Section C to these results indicates that the value of k for E=1 and E=2 is just about the same as indicated farther down on Page 45. The equation cannot be applied to the results when E=$\frac{1}{2}$ because the value of k reduces as the hydrolysis proceeds further after the first 48 hour period. Perhaps a new variable comes into existence because of the complex nature of the substrate and/or the extract.

E. Effect of pH on Filbert Extracts

This investigation was carried on with a view to find the optimum pH for the proteolysis of non-fat milk solids by the filbert extracts.

TABLE VII*

EFFECT OF DIFFERENT CONCENTRATIONS OF BARCELONA EXTRACT ON NON-FAT MILK SOLIDS SUBSTRATE
(Incubation temperature 30° C)

	B1-C1	B2-C2	B3-C3
Time in hours	Ml. 0.1 N. NaOH		
0	0.00	0.00	0.00
48	6.50	9.75	22.50
96	7.50	20.50	33.75
144	9.75	29.50	44.00
192	10.25	31.00	46.50
240	10.50	35.50	46.50

B - 10 ml. of Barcelona extract per 100 ml. of substrate.

C - 5% NaCl substituted for the extract.

pH of the digests was 6.60.

All the above results were obtained according to the method outlined on pages 26 and 27, and expressed on the basis of 100 ml. of substrate.

* For details of the results presented in this table, refer to Tables 4, 5, and 6 in the appendix.

FIGURE VII

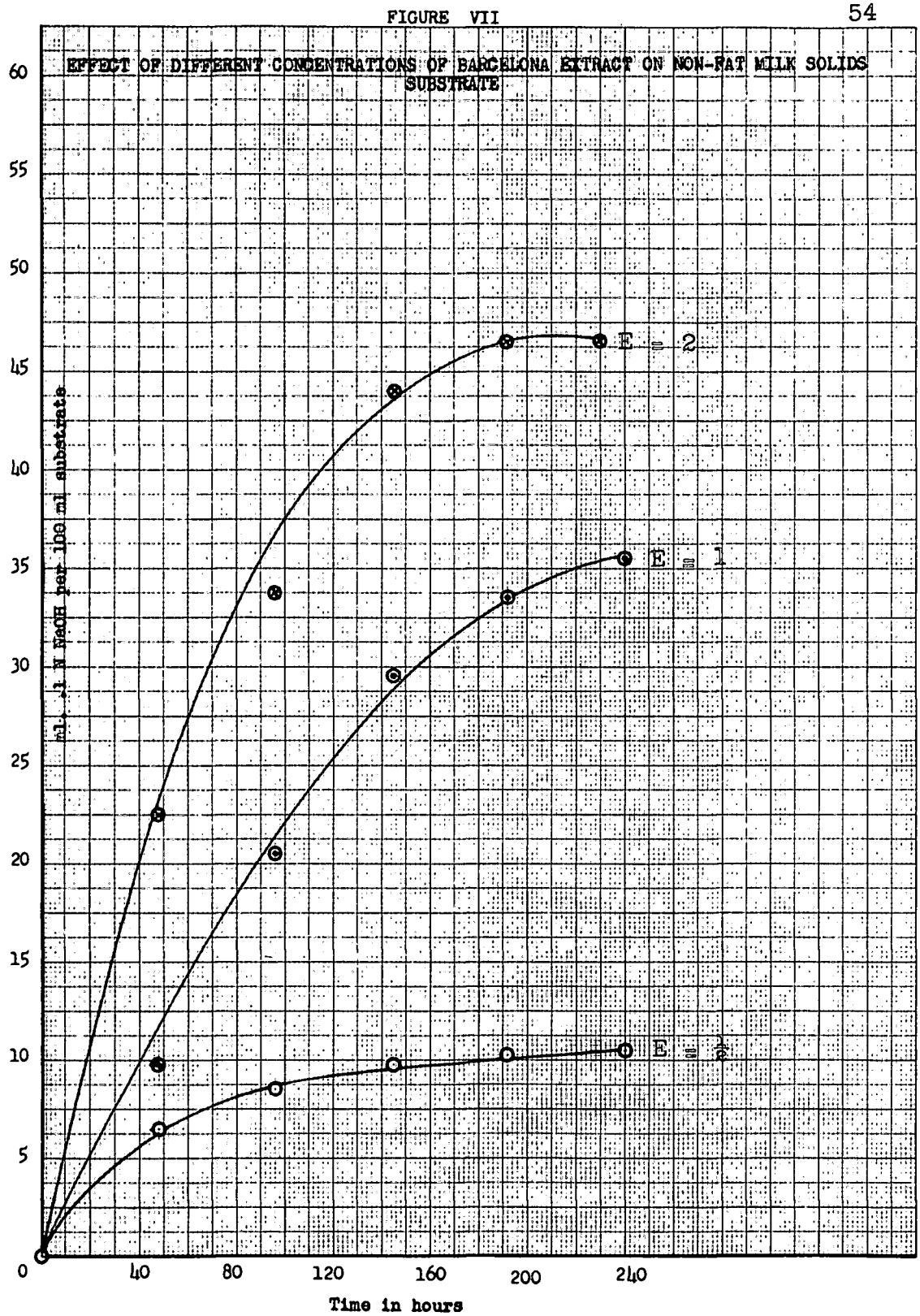

EFFECT OF DIFFERENT CONCENTRATIONS OF BARCELONA EXTRACT ON NON-FAT MILK SOLIDS SUBSTRATE
ml. .1 N NaOH per 100 ml substrate
Time in hours
E = 2
E = 1
E = ½
0
5
10
15
20
25
30
35
40
45
50
55
60
40
80
120
160
200
240

TABLE VIII*

EFFECT OF DIFFERENT CONCENTRATIONS OF DU CHILLY EXTRACT ON NON-FAT MILK SOLIDS SUBSTRATE
(Incubation temperature 30° C)

	D1-C1	D2-C2	D3-C3
Time in hours	Ml. 0.1 N. NaOH		
0	0.00	0.00	0.00
48	5.75	16.25	25.00
96	10.00	25.50	42.00
144	13.00	32.75	49.75
192	15.00	38.75	58.25
240	15.00	42.25	60.75

D - 10 ml. of Du Chilly extract per 100 ml. of substrate.

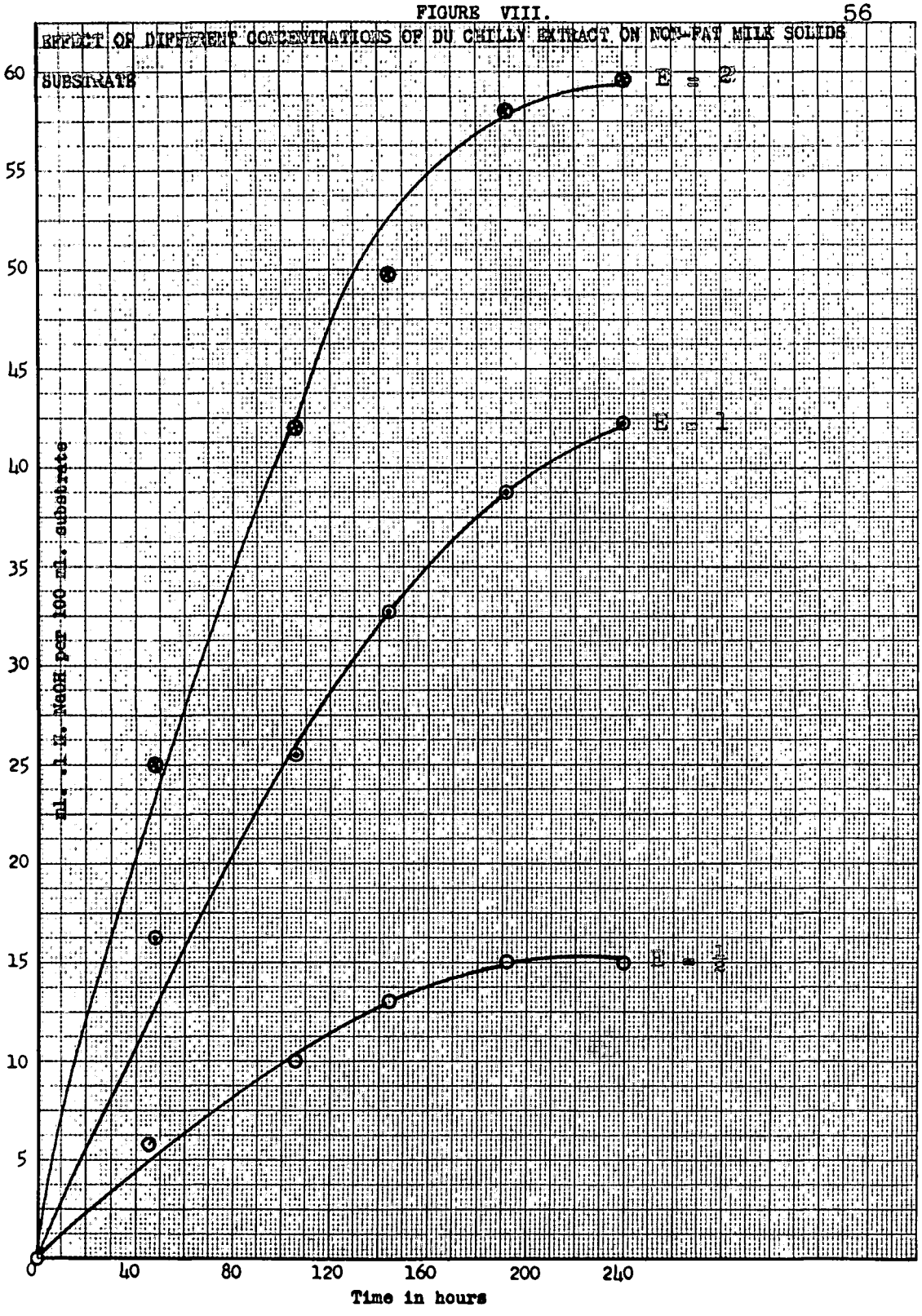
C - Control - 5% NaCl substituted for the extract.

pH of the digests was 6.60.

All the above results were obtained according to the method outlined on pages 26 and 27, and expressed on the basis of 100 ml. of substrate.

* For details of the results presented in this table, refer to Tables 7, 8, and 9 in the appendix.

FIGURE VIII.


EFFECT OF DIFFERENT CONCENTRATIONS OF DU CHILLY EXTRACT ON NON-FAT MILK SOLIDS SUBSTRATE
E = 2
E = 1
E = ½
ml. of N. NaOH per 100 ml. substrate
Time in hours
0
40
80
120
160
200
240
5
10
15
20
25
30
35
40
45
50
55
60

In order to adjust the non-fat milk solids substrate to different pH values, it became necessary to determine its buffer capacity as shown in Table IX and in Figure IX. Using this information, the following substrates were prepared with the desired pH values.

1. pH 5.00 - 400 ml. of 10 per cent non-fat milk solids, 13.50 ml. 1 N. HCl, 6.5 ml. distilled water.
2. pH 5.50 - 400 ml. of 10 per cent non-fat milk solids, 8.5 ml. 1 N. HCl, 11.50 ml. distilled water.
3. pH 6.55 - 400 ml. of 10 per cent non-fat milk solids, (natural) 15.00 ml. distilled water.
4. pH 7.50 - 400 ml. of 10 per cent non-fat milk solids, 5.00 ml. 1 N. NaOH, 15.0 ml. distilled water.
5. pH 8.5 - 400 ml. of 10 per cent non-fat milk solids, 8.00 ml. 1 N. NaOH, 12.00 ml. distilled water.

All the above substrates were made up to the same volume and contained the same percentage of solids. Two 200 ml. volumes from each of the substrates were poured into 500 ml. erlenmeyer flasks. To one of them was added 20.0 ml. of the extract and to the other 20.0 ml. of 5 per cent sodium chloride solution for the control. These were divided into 40 ml. portions in 125 ml. flasks, 2 ml. of toluene poured on the surface and each sample

incubated at 30° C. The hydrolysis of these samples, determined at regular intervals, is shown in Tables X and XI and Figures X and XI.

The optimum for both the extracts was found to be at pH 5.50. When the pH was decreased or increased from this point, there was a decrease in the activity of the extracts. Comparison of the effect of pH on the two extracts, Barcelona and Du Chilly, is discussed in the next section.

The optimum pH for the proteolytic activity of the extracts indicates that their activity is neither peptic nor tryptic in nature, because pepsin and trypsin have their optima around pH 1.5-2 and 7.8 (60,pp.165,175) respectively. The optimum for the activity of the filbert extracts is very close to pH 5.35, the optimum for the proteolytic activity of animal rennet (60, p.172). Sherwood (56) found strong evidence that rennet is the only important proteolytic agent attacking cheese protein during ripening. Both rennet and the filbert extracts have their optimum activity around 37° C.

The close similarity between the filbert extracts and rennet suggests that the former may be of value in cheese ripening.

TABLE IX

BUFFER CAPACITY OF THE 10% NON-FAT MILK SOLIDS SUBSTRATE

pH	Ml. of 1 N. HCl per 50 ml. substrate
6.55	0.00
6.20	0.25
5.90	0.50
5.70	0.75
5.45	1.00
5.30	1.25
5.20	1.50
5.05	1.75
4.95	2.00
4.85	2.25
4.70	2.50

FIGURE IX

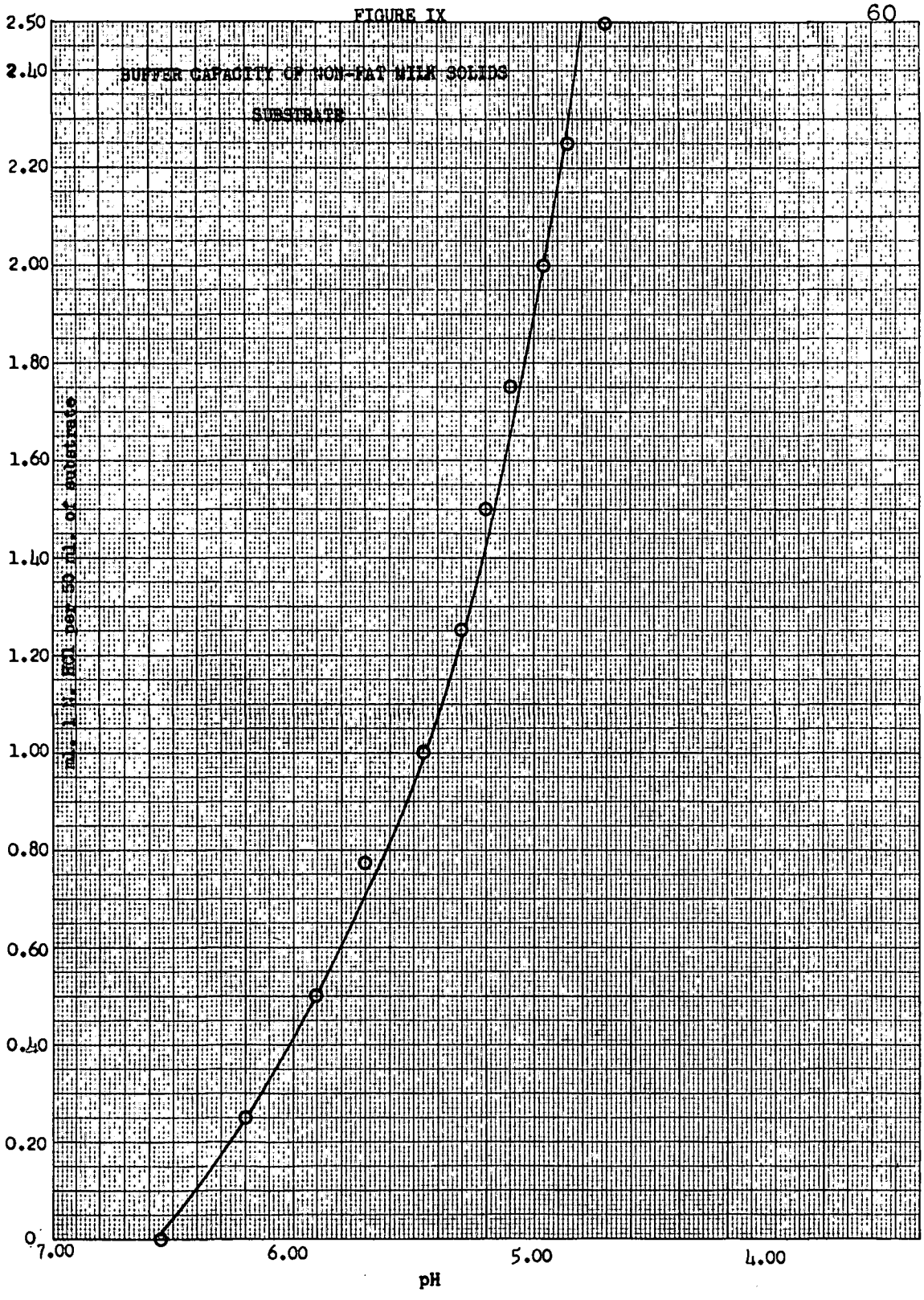

BUFFER CAPACITY OF NON-FAT MILK SOLIDS
SUBSTRATE
m.l. 1/. HCl per 50 ml. of substrate
2.50
2.40
2.20
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0
7.00
6.00
5.00
4.00
pH

TABLE X*

EFFECT OF pH ON THE HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY BARCELONA EXTRACT
(Incubation temperature 30° C)

Time in hours	B-C at pH 5	B-C at pH 5.5	B-C at pH 6.6	B-C at pH 7.5	B-C at pH 8.5
	Ml. 0.1 N. NaOH				
0	0.00	0.00	0.00	0.00	0.00
48	21.25	25.00	15.75	19.00	13.75
96	27.75	34.00	24.00	22.75	20.00
144	32.25	37.00	31.00	27.75	26.00
192	35.25	38.00	34.75	32.00	30.00
240	36.75	40.00	34.75	33.50	31.00

B - 10 ml. of Barcelona extract per 100 ml. of substrate.

C - 5% NaCl substituted for the extract.

All the above results were obtained according to the method outlined on pages 26 and 27, and expressed on the basis of 100 ml. of the substrate.

* For details of the results presented in this table refer to Tables 20, 21, 22, 23, and 24 in the appendix.

FIGURE X

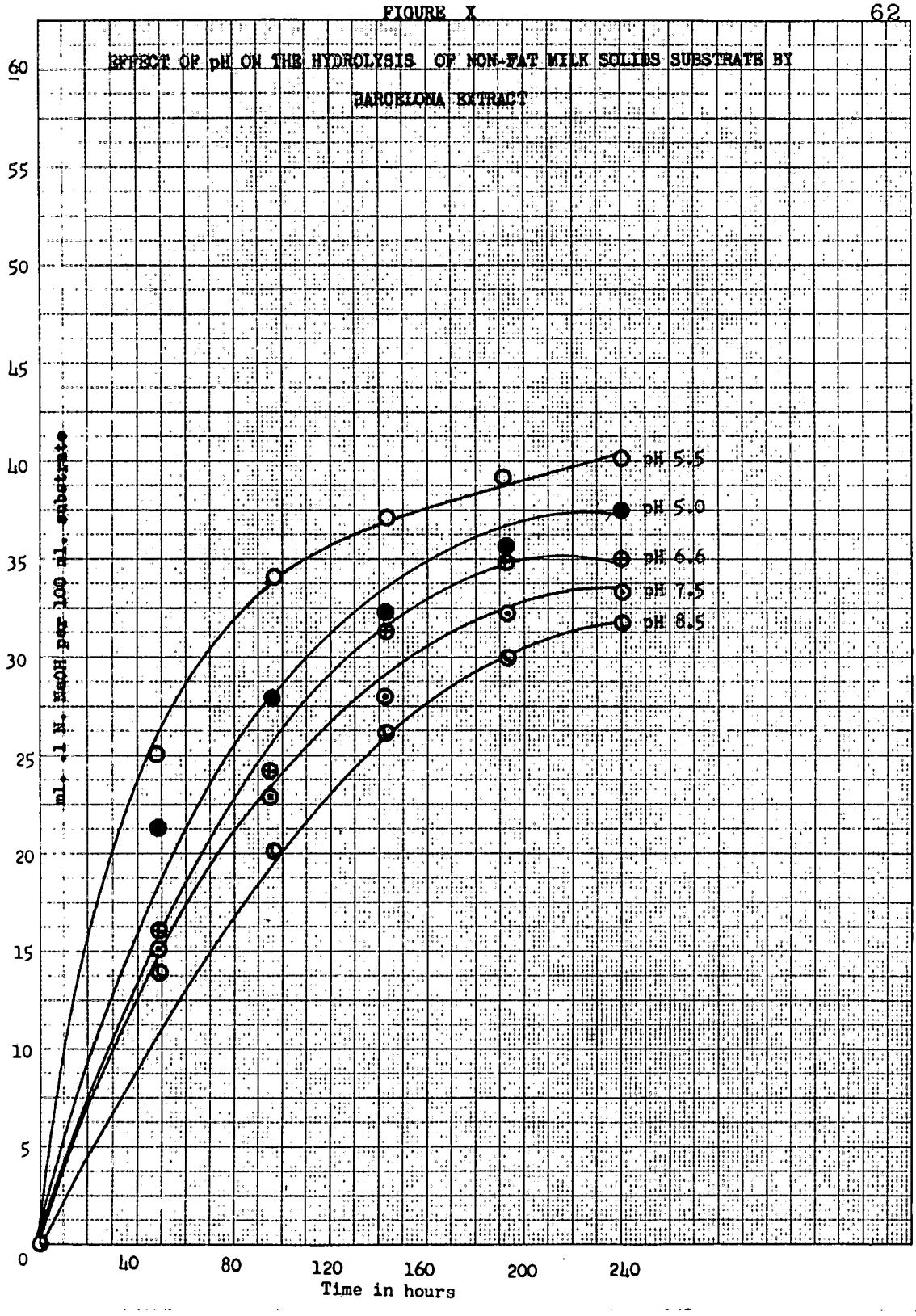

EFFECT OF pH ON THE HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY BARCELONA EXTRACT
ml. 1 N. NaOH per 100 ml. substrate
Time in hours
pH 5.5
pH 5.0
pH 6.6
pH 7.5
pH 8.5
0
5
10
15
20
25
30
35
40
45
50
55
60
40
80
120
160
200
240

TABLE XI*

EFFECT OF pH ON THE HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY DU CHILLY EXTRACT
(Incubation temperature 30° C)

Time in hours	D-C at pH 5	D-C at pH 5.5	D-C at pH 6.6	D-C at pH 7.5	D-C at pH 8.5
	Ml. 0.1 N. NaOH				
0	0.00	0.00	0.00	0.00	0.00
48	25.00	28.75	19.25	17.50	16.00
96	36.50	41.00	31.50	25.00	22.00
144	47.25	52.50	36.50	34.00	29.50
192	53.00	57.50	42.75	37.50	33.00
240	61.50	62.25	45.50	40.00	35.75

D - 10 ml. of Du Chilly extract per 100 ml. of substrate.

C - Control - 5% NaCl substituted for the extract.

All the above results were obtained according to the method outlined on pages 26 and 27, and results expressed on the basis of 100 ml. of substrate.

* For details of the results presented in this table, refer to Tables 25, 26, 27, 28 and 29 in the appendix.

FIGURE XI

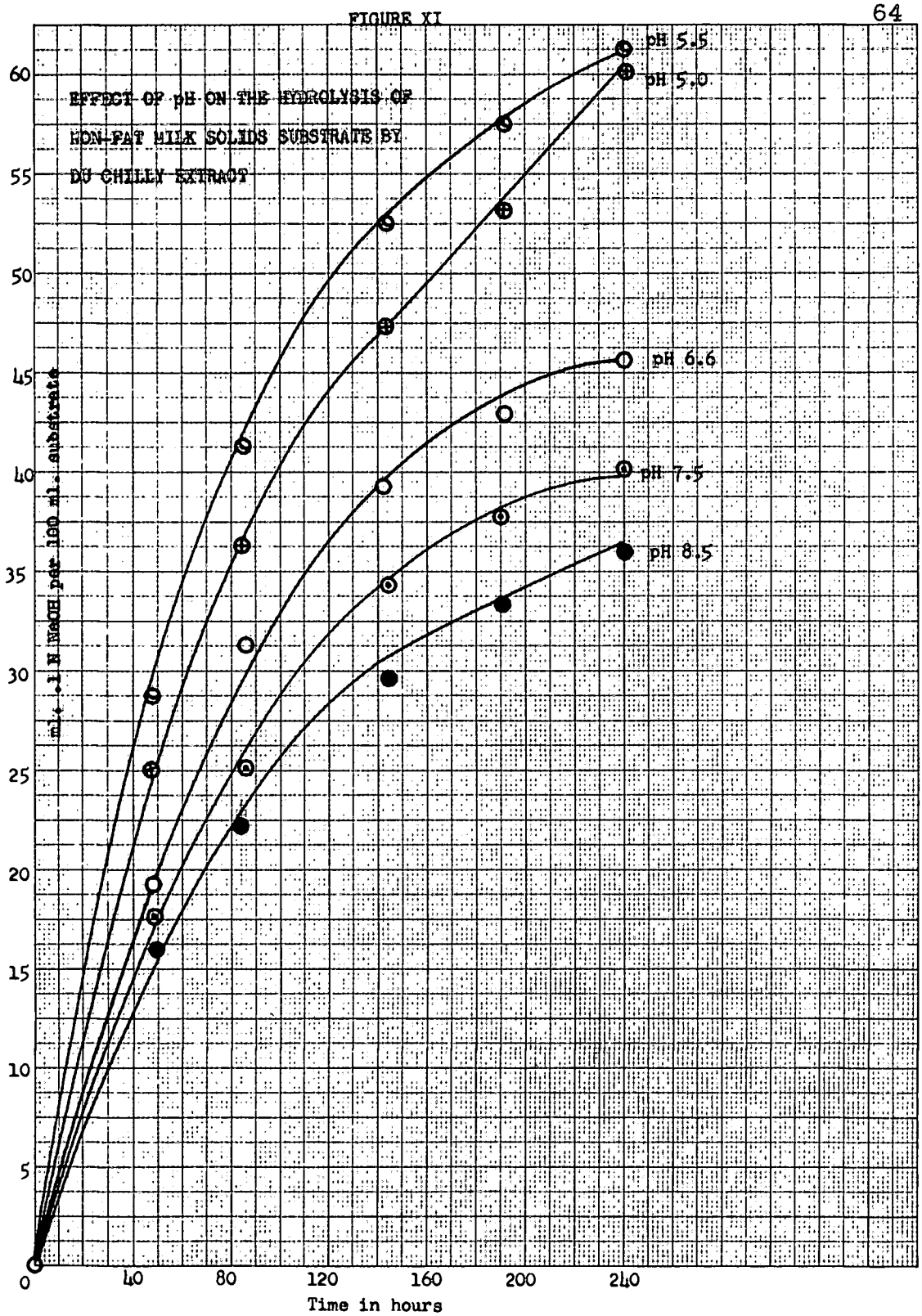
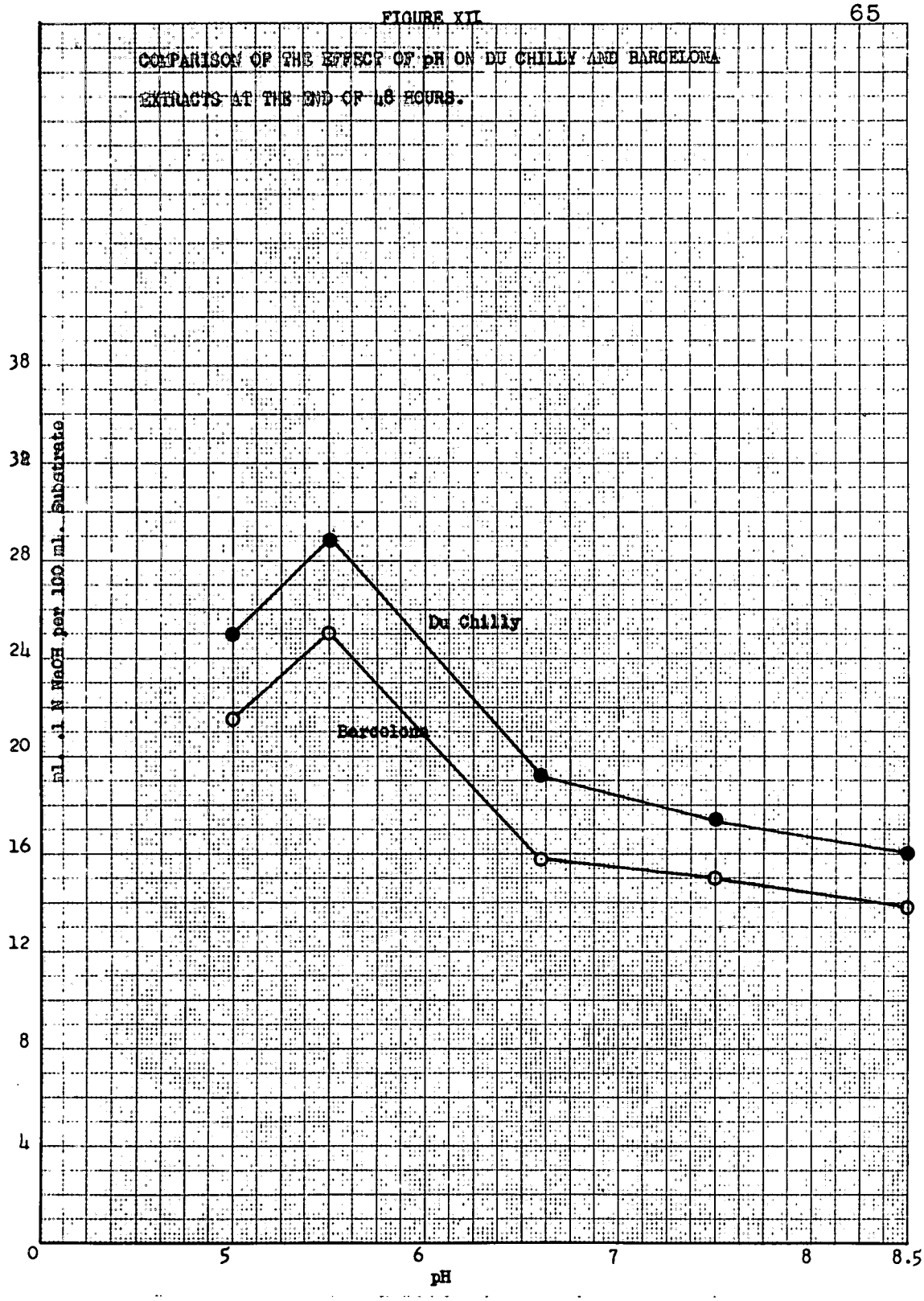

EFFECT OF pH ON THE HYDROLYSIS OF
NON-FAT MILK SOLIDS SUBSTRATE BY
DU CHILLY EXTRACT
pH 5.5
pH 5.0
pH 6.6
pH 7.5
pH 8.5
ml. .1 N NaOH per 100 ml. substrate
Time in hours
0
5
10
15
20
25
30
35
40
45
50
55
60
40
80
120
160
200
240

FIGURE XII

COMPARISON OF THE EFFECT OF pH ON DU CHILLY AND BARCELONA EXTRACTS AT THE END OF 48 HOURS.



F. Comparison of the Two Extracts

The two extracts obtained from the Barcelona and Du Chilly filberts appear to be the same in their proteolytic activity.

Barcelona extract has shown a little more activity in the earlier stages of hydrolysis as illustrated by Figure I, but the Du Chilly extract brings about the same extent of hydrolysis at a slower rate until both become equal at the end of 180 hours. Similar results were obtained when casein was used as the substrate (Figure II). However, on egg albumin, the Barcelona extract has shown slightly more activity, but it is too low to be of any probable significance. Since Du Chilly extract showed slower activity in some of the earlier work (Figure I), it would have been possible to assume the presence of some kind of retarder or inhibitor; but at a later date, some extract was prepared from another stock of this variety of the nuts which showed more activity than the corresponding Barcelona extract. This was demonstrated by the extracts used for the study of different concentrations of the extracts and also during the study of the effect of pH on their activity, where the Du Chilly extracts have shown more activity than the Barcelona extracts (Figures VII, VIII, X, XI and XII). There are

other evidences to prove that the two extracts contain the same enzyme or enzymes responsible for the proteolytic action. Both have the same optimum temperature and pH. Both the extracts are affected in the same manner if their ratios are varied with respect to the substrate (Figures VII and VIII). Both are inactivated by heating at 60° for one minute. Figure XII illustrates that the curves of pH versus hydrolysis (ml. 0.1 N. NaOH) for both the extracts run parallel as if they were curves for the same enzyme or enzymes in different concentrations.

It can be concluded on the basis of the above discussion that the concentrations of the proteolytic enzyme (or enzymes) vary in each of the two extracts depending upon the stock of filberts from which they were made. The concentration of the enzyme or enzymes in the nuts varies with the condition of growth, variety and methods of handling as discussed in the chapter on the review of literature.

It might be of interest to mention, at this point, that as a result of the complexity of the extract and the substrate, most of the results obtained might be due to the complementary action of more than one enzyme as well as the presence of other substances.

CHAPTER V

USE OF FILBERT EXTRACTS IN CHEESE RIPENING

Several investigators have used proteolytic enzymes such as pepsin, trypsin and rennin and shortened the ripening period of cheese with some success. It was on this basis that the effect of filbert extracts was investigated in cheese ripening.

Five hundred and fifty grams of filberts were ground in an Enterprize meat grinder, using the smallest disc. Five hundred grams of this finely ground material was soaked for two and one-half hours in one liter of 10 per cent sodium chloride solution. The mixture was then run through a stainless steel centrifugal puree machine and filtered through a double layer of cheese cloth. The extract was prepared usually late in the evening and stored overnight at 2° C. Next morning, the bottom layer of the extract was separated from the top fatty layer by siphoning the former with a pipette. The extract was introduced in the cheese by the following two methods:

A. Five six-pound samples of the cheese curd were taken out of a commercial vat after milling. Each sample was put in a short five-gallon milk can and the cans were placed in a water bath at 96° F ± 2° F. The curd was

stirred for 15 minutes and then the different extracts were added to different samples, sodium chloride solution being added to the control sample. Stirring was continued for five minutes and then 74 grams of salt was added to each of the samples. After stirring for 10 more minutes with wooden spoons, each of the samples was put into a five-pound cheese hoop. These were pressed for an hour, removed from the hoops, bandaged, and pressed overnight. After this, each sample was marked according to the treatment it had received, wrapped in Marathon Cheese Wrapping Paper (Parakote) and stored at 50° F. The following samples were prepared:

Group 1

a. Using Du Chilly Extract

i) 100 ml. of defatted extract in 10 per cent NaCl was added.

ii) 100 ml. of total (not defatted) extract in 10 per cent NaCl was added.

b. Using Barcelona Extract

i) 100 ml. of defatted extract in 10 per cent NaCl was added.

ii) 100 ml. of total (not defatted) extract in 10 per cent NaCl was added.

c. Control Sample

i) 100 ml. of 10 per cent NaCl solution added.

B. Five six-pound samples of cheese curd were taken out from a commercial vat after salting, put in five-pound hoops and pressed for an hour. Then 50 ml. of the filbert extract were sprayed or spread on all sides of the samples. They were bandaged and pressed overnight. Next morning, they were taken out of the hoops, unwrapped from the bandage, rewrapped in Marathon Cheese Wrapping Paper (Parakote) and stored at 50° F. The following samples were prepared by this method:

Group 2

a. Using Du Chilly Extract

i) 50 ml. of defatted extract was sprayed on the surface of the cheese.

ii) 50 ml. of total (not defatted) extract was sprayed on the surface of the cheese.

b. Using Barcelona Extract

i) 50 ml. of defatted extract sprayed on the surface of the cheese.

ii) 50 ml. of total (not defatted) extract sprayed on the surface of the cheese.

c. Control Sample - no treatment

Results of Flavor Tests

A. At the end of one month

The samples to which filbert extract had been

added after milling (Group 1) showed slight rancidity as compared to the control sample. Samples to which total extracts had been added were a little more rancid than the ones to which defatted extracts had been added. Texture of all these samples had become firm and brittle, with the exception of the controls. Very little difference was observed between the effects of Barcelona and Du Chilly extracts at this stage.

Samples of Group 2 showed no marked difference from the control.

B. At the end of two months

A marked difference was observed between the control and the experimental samples of Group 1. Rancidity had disappeared in samples to which the fat-free extracts were added, and these samples had stronger flavor than controls. Samples with total extracts were slightly rancid. There was a little after taste in all the samples except the controls. Group 2 samples showed similar changes, but to a much lesser degree.

At the end of four and one-half months, opinions of three expert cheese tasters were obtained on the cheese samples of Group 1. They were presented with all the five samples and requested to record their opinions on a

sheet of paper. The identity of the samples was not disclosed to them until they were through tasting the cheese. Their remarks are tabulated in Table XII.

The bitterness found in some of the samples disappeared after one more month of storage. Cheese containing defatted extracts was preferred over the samples containing total extracts. All the results of this taste test tend to indicate that there is improvement in flavor or at least the ripening time is shortened by adding the filbert extracts to the cheese curd. However, all these results should be considered preliminary. Results of many more experiments will be required before any positive conclusion can be reached. The shortening of the ripening period may not be due to the action of one enzyme alone but a combination of several enzymes present in the extracts, in the cheese and the bacteria.

It would be safe to conclude that the results of this work appear promising enough to warrant further investigations of the effect of filbert extracts in the cheese ripening.

TABLE XII

OPINIONS OF THREE EXPERT CHEESE TASTERS
ON SAMPLES OF GROUP 1 AFTER
FOUR AND A HALF MONTHS' STORAGE

Extract added	1	2	3
Defatted Barcelona	Less bitter than total Barcelona. Weak body.	Much better odor than control. Slightly bitter.	Good odor. Slightly bitter.
Total Barcelona	Slightly bitter aftertaste but good aged flavor. Weak body.	More pronounced flavor than above sample.	Considerably bitter, undesirable flavor. Weak body.
Defatted Du Chilly	Much better flavor than all the other samples. Aged.	Very good flavor. Much better than control. More aged.	Outstanding, aged flavor. Better than all other samples.
Total Du Chilly	Good, aged flavor.	Very good flavor. Stronger than above.	As good as above, except more aged.
Control	Mild, clean flavor.	Mild, clean flavor.	Mild flavor.

CHAPTER VI

SUMMARY AND CONCLUSIONS

1. The literature survey showed that no work has been reported on the proteolytic activity of nuts. Information available on the use of proteolytic enzymes in cheese ripening was surveyed also.

2. Five per cent sodium chloride solution was shown to be a good extractant for filbert nuts.

3. The modified formal titration was found to be an efficient method for the determination of proteolysis.

4. The filbert extracts showed maximum proteolysis of non-fat milk solids substrate as compared to casein and egg albumin substrates. Of the incubation temperatures (10°, 20°, 30°, 37°, and 50° C) studied, 37° was the optimum; and of the pH values (pH 5.00, 5.50, 6.55, 7.50 and 8.50) investigated, pH 5.5 was the optimum for the hydrolysis of non-fat milk solids substrate. An attempt was made to calculate the rate constant for the reaction involved.

5. Storage of the filbert extracts at 2° C results in a progressive decrease in their activity. Most of the activity was lost after four weeks.

6. The rate as well as the extent of proteolysis was decreased or increased by decreasing or increasing

the extract concentrations with respect to the substrate.

7. Considerable evidence has been obtained to show that the enzyme or enzymes responsible for the proteolysis are the same in both the varieties of the nuts used.

8. Preliminary determinations of the use of filbert extracts in the cheese ripening are promising enough to warrant further investigations.

9. Additional studies should be conducted in isolating, concentrating and identifying the enzymes present in the filberts and determining their kinetic behavior.

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APPENDIX

TABLE 1

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY FILBERT EXTRACTS

(Freshly made) (Incubation temperature 30° C)

Time in hours	Ml. 0. 1 N. NaOH			B-C On the basis of 20 ml. substrate	B-C On the basis of 100 ml. substrate	D-C On the basis of 20 ml. substrate	D-C On the basis of 100 ml. substrate
	B	D	C				
0	5.70	5.70	5.70	0.00	0.00	0.00	0.00
24	7.95	7.00	6.15	1.80	9.00	0.85	4.25
48	9.60	8.15	6.25	3.35	16.75	1.90	9.50
72	11.80	9.80	6.80	4.90	24.50	3.05	15.25
96	13.00	11.45	7.00	6.00	30.00	4.45	22.25
120	13.80	12.95	7.20	6.60	33.00	5.75	28.75
144	14.35	14.10	7.40	6.95	34.75	6.70	33.50
168	15.15	15.00	8.15	6.95	34.75	6.85	34.25
180	15.15	15.15	8.20	6.95	34.75	6.95	34.75

B - 10 ml. of Barcelona Extract per 100 ml. of substrate.

D - 10 ml. of Du Chilly Extract per 100 ml. of substrate.

C - Control - 5% NaCl solution substituted for the extract.

pH of the digests was 6.55.

The above figures represent Ml. of 0. 1 N. NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 2

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY FILBERT EXTRACTS AFTER ONE WEEK'S STORAGE AT 2° C
(Incubation temperature 30° C)

Time in hours	Ml. 0. 1 N. NaOH			B-C On the basis of 20 ml. substrate	B-C On the basis of 100 ml. substrate	D-C On the basis of 20 ml. substrate	D-C On the basis of 100 ml. substrate
	B	D	C				
0	6.20	6.20	6.20	0.00	0.00	0.00	0.00
24	7.20	6.65	6.15	1.05	5.25	0.55	2.75
48	8.75	7.65	6.25	2.25	11.25	1.35	6.75
72	10.40	9.00	6.75	3.45	17.25	2.25	11.25
96	11.30	8.10	6.90	4.40	22.00	3.00	15.00
120	11.65	10.65	7.00	4.65	23.25	3.65	18.25
144	12.55	12.45	7.85	4.70	23.50	4.60	23.00
168	12.20	12.20	7.50	4.70	23.50	4.70	23.50
180	12.35	12.35	7.65	4.70	23.50	4.70	23.50

B - 10 ml. of Barcelona Extract per 100 ml. of substrate.

D - 10 ml. of Du Chilly Extract per 100 ml. of substrate.

C - Control - 5% NaCl substituted for the extract.

pH of the digests was 6.55.

The above figures represent Ml. of 0. 1 N. NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 3

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY FILBERT EXTRACTS AFTER TWO WEEKS' STORAGE AT 2° C
(Incubation temperature 30° C)

Time in hours	Ml. 0. 1 N. NaOH			B-C On the basis of 20 ml. substrate	B-C On the basis of 100 ml. substrate	D-C On the basis of 20 ml. substrate	D-C On the basis of 100 ml. substrate
	B	D	C				
0	6.00	6.00	6.00	0.00	0.00	0.00	0.00
24	6.70	6.20	5.90	0.80	4.00	0.30	1.50
48	8.00	7.15	6.00	2.00	10.00	1.15	5.75
72	9.75	8.65	6.90	2.90	14.50	1.80	9.00
96	10.60	9.75	7.45	3.15	15.75	2.30	11.50
120	10.80	10.05	7.45	3.25	16.25	2.60	13.00
144	10.70	10.50	7.50	3.25	16.25	3.05	15.25
168	11.00	10.85	7.60	3.40	17.00	3.25	16.25
180	11.10	10.85	7.65	3.45	17.25	3.25	16.25

B - 10 ml. of Barcelona Extract per 100 ml. of substrate.

D - 10 ml. of Du Chilly Extract per 100 ml. of substrate.

C - Control - 5% NaCl substituted for the extract.

pH of the digests was 6.55.

The above figures represent Ml. of 0. 1 N. NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 4

EFFECT OF DIFFERENT CONCENTRATIONS OF BARCELONA EXTRACT ON NON-FAT MILK SOLIDS SUBSTRATE
(Incubation Temperature 30° C)

Time in hours	Ml.0.1N.NaOH		B-C On the basis of 20 ml. substrate	B-C On the basis of 100 ml. substrate
	B	C		
0	4.90	4.90	0.00	0.00
48	6.40	5.10	1.30	6.50
96	7.00	5.50	1.50	7.50
144	8.05	6.10	1.95	9.75
192	9.15	7.10	2.05	10.25
240	9.70	7.60	2.10	10.50

The above results are based upon the following ratio of substrate to extract:

B - 250 ml. of non-fat milk solids (9%), 12.5 ml. of Barcelona extract, 37.5 ml. of distilled water; making the total volume 300 ml.

C - 250 ml. of non-fat milk solids (9%), 12.5 ml. of 5% NaCl, 37.5 ml. of distilled water; making the total volume 300 ml.

pH of the digests was 6.60

The above figures represent ml. of 0.1N.NaOH required to titrate 25 ml. of the neutralized (pH7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH9.5 after the addition of 2 ml. of formaldehyde (pH7.0).

TABLE 5

EFFECT OF DIFFERENT CONCENTRATIONS OF BARCELONA EXTRACT ON NON-FAT MILK SOLIDS SUBSTRATE

(Incubation Temperature 30° C)

Time in hours	Ml.0.1N.NaOH		B-C On the basis of 20 ml. substrate	B-C On the basis of 100 ml. substrate
	B	C		
0	4.90	4.90	0.00	0.00
48	7.05	5.10	1.95	9.75
96	9.60	5.50	4.10	20.50
144	12.00	6.10	5.90	29.50
192	13.80	7.10	6.70	31.00
240	14.70	7.60	7.10	35.50

The above results are based upon the following ratio of substrate to extract:

B - Barcelona - 250 ml. of non-fat milk solids (9%), 25 ml. of extract, 25 ml. of distilled water; making the total volume 300 ml.

C - Control - 250 ml. of non-fat milk solids (9%), 25 ml. of 5% NaCl, 25 ml. of distilled water; making the total volume 300 ml.

pH of the digests was 6.60.

The above figures represent ml. of 0.1N.NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 6

EFFECT OF DIFFERENT CONCENTRATIONS OF BARCELONA EXTRACT ON NON-FAT MILK SOLIDS SUBSTRATE

(Incubation Temperature 30° C)

Time in hours	Ml.0.1N.NaOH		B-C On the basis of 20 ml. substrate	B-C On the basis of 100 ml. substrate
	B	C		
0	4.90	4.90	0.00	0.00
48	9.40	5.10	4.30	22.50
96	12.25	5.50	6.75	33.75
144	14.90	6.10	8.80	44.00
192	16.40	7.10	9.30	46.50
240	16.90	7.60	9.30	46.50

The above results are based upon the following ratio of substrate to extract:

B - Barcelona - 250 ml. of non-fat milk solids (9%), 50 ml. of extract; making the total volume 300 ml.

C - Control - 250 ml. of non-fat milk solids (9%), 50 ml. of 5% NaCl; making the total volume 300 ml.

pH of the digests was 6.60.

The above figures represent ml. of 0.1N.NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 7

EFFECT OF DIFFERENT CONCENTRATIONS OF DU CHILLY EXTRACT ON NON-FAT MILK SOLIDS SUBSTRATE

(Incubation Temperature 30° C)

Time in hours	Ml.0.1N.NaOH		D-C On the basis of 20 ml. substrate	D-C On the basis of 100 ml. substrate
	D	C		
0	4.80	4.80	0.00	0.00
48	6.00	4.85	1.15	5.75
96	6.60	4.60	2.00	10.00
144	7.45	4.85	2.60	13.00
192	8.50	5.50	3.00	15.00
240	8.55	5.55	3.00	15.00

The above results are based upon the following ratio of substrate to extract:

D - Du Chilly - 250 ml. of non-fat milk solids (9%), 12.5 ml. of extract, 37.5 ml. of distilled water; making the total volume 300 ml.

C - Control - 250 ml. of non-fat milk solids (9%), 12.5 ml. of 5% NaCl, 37.5 ml. of distilled water.

pH of the digests was 6.60

The above figures represent ml. of 0.1N.NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 8

EFFECT OF DIFFERENT CONCENTRATIONS OF DU CHILLY EXTRACT ON NON-FAT MILK SOLIDS SUBSTRATE

(Incubation Temperature 30° C)

Time in hours	Ml.0.1N.NaOH		D-C On the basis of 20 ml. substrate	D-C On the basis of 100 ml. substrate
	D	C		
0	4.80	4.80	0.00	0.00
48	8.10	4.85	3.25	16.25
96	9.70	4.60	5.10	25.50
144	11.40	4.85	6.55	32.75
192	13.25	5.50	7.75	37.75
240	14.00	5.55	8.45	42.25

The above results are based upon the following ratio of substrate to extract:

D - Du Chilly - 250 ml. of non-fat milk solids (9%), 25 ml. of extract, 25 ml. of distilled water; making the total volume 300 mj.

C - Control - 250 ml. of non-fat milk solids substrate, 25 ml. of 5% NaCl, 25 ml. of distilled water; making the total volume 300 ml.

pH of the digests was 6.60.

The above figures represent ml. of 0.1N.NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 9

EFFECT OF DIFFERENT CONCENTRATIONS OF DU CHILLY EXTRACT ON NON-FAT MILK SOLIDS SUBSTRATE

(Incubation Temperature 30° C)

Time in hours	Ml.0.1N.NaOH		D-C On the basis of 20 ml. substrate	D-C On the basis of 100 ml. substrate
	D	C		
0	4.80	4.80	0.00	0.00
48	9.85	4.85	5.00	25.00
96	13.00	4.60	8.40	42.00
144	14.80	4.85	9.95	49.75
192	17.15	5.50	11.65	58.25
240	17.70	5.55	12.15	60.75

The above results are based upon the following ratio of substrate to extract:

D - Du Chilly - 250 ml. of non-fat milk solids (9%), 50 ml. of extract; making the total volume 300 ml.

C - Control - 250 ml. of non-fat milk solids substrate, 50 ml. of 5% NaCl; making the total volume 300 ml.

pH of the extracts was 6.60.

The above figures represent ml. of 0.1 N. NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 10

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY BARCELONA EXTRACTS AT 10° C

Time in hours	Ml. 0.1N.NaOH		B-C On the basis of 20 ml. substrate	B-C On the basis of 100 ml. substrate
	D	C		
0	5.10	5.10	0.00	0.00
48	5.25	5.00	0.25	1.25
96	5.45	4.95	0.50	2.50
144	5.50	4.90	0.60	3.00
192	5.95	5.15	0.80	4.00
240	6.00	5.15	0.85	4.25
288	6.10	5.15	0.95	4.75

The above results are based upon the following ratio of substrate to extract:

B - 10 ml. of Barcelona extract per 100 ml. of substrate.

C - Control - 5% NaCl substituted for the extract

pH of the digests was 6.55.

The above figures represent ml. of 0.1N.NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 11

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY BARCELONA EXTRACTS AT 20° C

Time in hours	Ml.0.1N.NaOH		B-C On the basis of 20 ml. substrate	B-C On the basis of 100 ml. substrate
	D	C		
0	5.10	5.10	0.00	0.00
48	6.00	5.10	0.90	4.50
96	6.25	4.80	1.45	7.25
144	6.35	4.85	1.50	7.50
192	6.45	4.85	1.60	8.00
240	6.60	4.85	1.75	8.75
288	6.80	4.90	1.80	9.00

The above results are based upon the following ratio of substrate to extract:

B - 10 ml. of Barcelona extract per 100 ml. of substrate.

C - Control - 5% NaCl substituted for the extract

pH of the digests was 6.55.

The above figures represent ml. of 0.1N.NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 12

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY BARCELONA EXTRACTS AT 30° C

Time in hours	Ml.0.1N.NaOH D	Ml.0.1N.NaOH C	B-C On the basis of 20 ml. substrate	B-C On the basis of 100 ml. substrate
0	5.10	5.10	0.00	0.00
24	6.80	5.00	1.80	9.00
48	9.20	5.60	3.60	18.00
72	10.00	5.75	4.25	22.25
96	11.60	5.85	5.75	28.75
120	13.50	7.00	6.50	32.50
144	14.60	7.50	7.10	35.50
168	14.35	6.60	7.75	38.75
192	14.65	6.65	8.00	40.00
240	14.60	6.40	8.20	41.00

The above results are based upon the following ratio of substrate to extract:

B - 10 ml. of Barcelona extract per 100 ml. substrate.

C - Control - 5% of NaCl solution substituted for the extract

pH of the digests was 6.55.

The above figures represent ml. of 0.1N.NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 13

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY BARCELONA EXTRACTS AT 37° C

Time in hours	Ml.0.1N.NaOH		B-C On the basis of 20 ml. substrate	B-C On the basis of 100 ml. substrate
	B	C		
0	5.10	5.10	0.00	0.00
24	7.45	4.90	3.40	17.00
48	9.25	4.85	4.40	27.00
72	11.10	4.90	6.20	31.00
96	11.60	5.00	6.60	34.00
120	12.80	5.20	7.60	38.00
144	14.20	6.00	8.20	41.00
168	14.70	6.00	8.70	43.00
240	15.10	6.30	8.88	44.00

The above results are based upon the following ratio of substrate to extract:

B - 10 ml. of Barcelona extract per 100 ml. of substrate.

C - Control - 5% NaCl substituted for the extract.

pH of the digests was 6.55

The above figures represent Ml. of 0. 1 N. NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 14

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY BARCELONA EXTRACTS AT 50° C

Time in hours	Ml. 0.1N. NaOH B	Ml. 0.1N. NaOH C	B-C On the basis of 20 ml. substrate	B-C On the basis of 100 ml. substrate
0	5.10	5.10	0.00	0.00
24	5.60	5.00	0.60	3.00
48	6.00	4.95	1.05	5.25
72	5.65	5.05	0.65	3.00
96	5.40	4.90	0.05	2.25
144	5.80	4.50	0.70	3.50
192	6.40	5.70	0.70	3.50
240	6.60	5.90	0.70	3.50

The above results are based upon the following ratio of substrate to extract:

B - 10 ml. Barcelona extract per 100 ml. of substrate.

C - Control - 5% NaCl substituted for the extract.

pH of the digests was 6.55.

The above figures represent Ml. of 0. 1 N. NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 15

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY DU CHILLY EXTRACT AT 10° C

Time in hours	Ml.0.1N.NaOH D	C	D-C On the basis of 20 ml. substrate	D-C On the basis of 100 ml. substrate
0	5.10	5.10	0.00	0.00
48	5.10	4.70	0.40	2.00
96	5.40	4.80	0.60	3.00
144	5.60	4.90	0.70	3.50
192	5.70	4.85	0.85	4.25
240	5.80	4.95	0.85	4.25

The above results are based upon the following ratio of substrate to extract:

D - 10 ml. of Du Chilly extract per 100 ml. of substrate.

C - Control - 5% NaCl substituted for the extract.

pH of the digests was 6.55.

The above figures represent Ml. of 0. 1 N. NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 16

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY DU CHILLY EXTRACT AT 20° C

Time in hours	Ml.0.1N.NaOH		D-C On the basis of 20 ml. substrate	D-C On the basis of 100 ml. substrate
	D	C		
0	5.10	5.10	0.00	0.00
48	5.60	4.70	0.90	4.50
96	6.20	4.80	1.40	7.00
144	6.60	4.80	1.80	9.00
192	6.00	4.10	1.90	9.50
240	6.60	4.70	1.90	9.50

The above results are based upon the following ratio of substrate to extract:

D - 10 ml. of Du Chilly extract per 100 ml. of substrate.

C - Control - 5% NaCl substituted for the extract.

pH of the digests was 6.55.

The above figures represent Ml. of 0. 1 N. NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 17

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY DU CHILLY EXTRACT AT 30° C

Time in hours	Ml.0.1N.NaOH D	C	D-C On the basis of 20 ml. substrate	D-C On the basis of 100 ml. substrate
0	5.10	5.10	0.00	0.00
24	6.05	5.00	1.05	5.25
48	6.90	4.90	2.00	10.00
96	9.10	5.10	4.00	20.00
120	10.50	5.50	5.00	25.00
168	12.20	5.35	6.85	32.00
240	13.70	6.15	7.60	38.00

The above results are based upon the following ratio of substrate to extract:

D - 10 ml. of Du Chilly extract per 100 ml. of substrate.

C - Control - 5% NaCl substituted for the extract.

pH of the digests was 6.55.

The above figures represent Ml. of 0. 1 N. NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 18

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY DU CHILLY EXTRACT AT 37° C

Time in hours	Ml.0.1N.NaOH		D-C On the basis of 20 ml. substrate	D-C On the basis of 100 ml. substrate
	D	C		
0	5.10	5.10	0.00	0.00
24	6.50	5.00	1.50	7.50
48	7.50	4.70	2.80	14.00
96	10.00	5.00	5.00	25.00
120	11.20	5.20	6.00	30.00
168	12.70	5.50	7.20	36.50
240	14.10	6.10	8.00	40.00

The above results are based upon the following ratio of substrate to extract:

D - 10 ml. of Du Chilly extract per 100 ml. of substrate.

C - Control - 5% NaCl substituted for the extract.

pH of the digests was 6.55.

The above figures represent Ml. of 0. 1 N. NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 19

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY DU CHILLY EXTRACT AT 50° C

Time in hours	Ml. 0.1N. NaOH		D-C On the basis of 20 ml. substrate	D-C On the basis of 100 ml. substrate
	D	C		
0	5.10	5.10	0.00	0.00
48	5.20	4.85	0.60	3.00
96	5.75	5.20	0.35	1.75
144	5.90	5.40	0.50	2.50
192	6.75	6.05	0.65	3.25
240	6.80	6.20	0.65	3.25

The above results are based upon the following ratio of substrate to extract:

D - 10 ml. of Du Chilly extract per 100 ml. of substrate.

C - Control - 5% NaCl substituted for the extract.

pH of the digests was 6.55.

The above figures represent Ml. of 0. 1 N. NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 20

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY BARCELONA EXTRACT AT pH 5.00

(Incubation temperature 30° C)

Time in hours	Ml.0.1N.NaOH		B-C On the basis of 20 ml. substrate	B-C On the basis of 100 ml. substrate
	B	C		
0	0.00	0.00	0.00	0.00
48	8.75	4.50	4.25	21.50
96	10.25	4.80	5.55	27.75
144	12.50	5.05	6.45	32.25
192	12.30	5.25	7.05	35.25
240	12.90	5.55	7.35	36.75

The above results are based upon the following ratio of substrate to extract:

B - 10 ml. of Barcelona extract per 100 ml. of substrate.

C - Control - 5% NaCl solution substituted for the extract.

The above figures represent Ml. of 0. 1 N. NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 21

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY BARCELONA EXTRACT AT pH 5.50

(Incubation temperature 30° C)

Time in hours	Ml.0.1N.NaOH B	C	B-C On the basis of 20 ml. substrate	B-C On the basis of 100 ml. substrate
0	0.00	0.00	0.00	0.00
48	9.65	4.65	5.00	25.00
96	11.20	4.40	6.80	34.00
144	12.20	4.80	7.40	37.00
192	11.90	4.30	7.60	38.00
240	13.00	5.00	8.00	40.00

The above results are based upon the following ratio of substrate to extract:

B - 10 ml. of Barcelona extract per 100 ml. of substrate.

C - Control - 5% NaCl substituted for the extract.

The above figures represent Ml. of 0. 1 N. NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 22

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY BARCELONA EXTRACT AT pH 6.55

(Incubation temperature 30° C)

Time in hours	Ml.0.1N.NaOH		B-C On the basis of 20 ml. substrate	B-C On the basis of 100 ml. substrate
	B	C		
0	0.00	0.00	0.00	0.00
48	8.10	4.95	3.15	15.75
96	10.30	4.50	5.80	24.00
144	12.00	5.80	6.20	31.00
192	11.95	5.00	6.95	34.75
240	12.20	5.25	6.95	34.75

The above results are based upon the following ratio of substrate to extract:

B - 10 ml. of Barcelona extract per 100 ml. of substrate.

C - Control - 5% NaCl solution substituted for the extract.

The above figures represent Ml. of 0. 1 N. NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 23

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY BARCELONA EXTRACT AT pH 7.5

(Incubation temperature 30° C)

Time in hours	Ml.0.1N.NaOH		B-C On the basis of 20 ml. substrate	B-C On the basis of 100 ml. substrate
	B	C		
0	0.00	0.00	0.00	0.00
48	7.55	4.55	3.00	15.00
96	9.85	5.30	4.55	22.75
144	11.85	6.30	5.55	27.75
192	12.50	6.10	6.40	32.00
240	13.20	6.50	6.70	33.50

The above results are based upon the following ratio of substrate to extract:

B - 10 ml. of Barcelona extract per 100 ml. of substrate.

C - Control - 5% NaCl substituted for the extract.

The above figures represent Ml. of 0. 1 N. NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 24

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY BARCELONA EXTRACT AT pH 6.5

(Incubation temperature 30°C)

Time in hours	Ml.0.1N.NaOH		B-C On the basis of 20 ml. substrate	B-C On the basis of 100 ml. substrate
	B	C		
0	0.00	0.00	0.00	0.00
48	8.30	5.55	2.75	13.75
96	9.80	5.80	4.00	20.00
144	12.20	7.00	5.20	26.00
192	12.50	7.50	6.00	30.00
240	12.90	6.70	6.20	31.00

The above results are based upon the following ratio of substrate to extract:

B - Barcelona extract per 100 ml. of substrate.

C - Control - 5% NaCl substituted for the extract.

The above figures represent Ml. of 0. 1 N. NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 25

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY DU CHILLY EXTRACT AT pH 5.00

(Incubation temperature 30° C)

Time in hours	Ml.0.1N.NaOH		D-C On the basis of 20 ml. substrate	D-C On the basis of 100 ml. substrate
	D	C		
0	0.00	0.00	0.00	0.00
48	9.60	4.60	5.00	25.00
96	12.80	4.50	7.30	36.00
144	14.15	4.70	9.45	47.25
192	15.90	5.30	10.60	53.00
240	17.25	4.80	12.45	62.25

The above results are based upon the following ratio of substrate to extract:

D - 10 ml. of Du Chilly extract per 100 ml. of substrate.

C - Control - 5% NaCl substituted for the extract.

The above figures represent Ml. of 0. 1 N. NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 26

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE
BY DU CHILLY EXTRACT AT pH 5.50

(Incubation temperature 30° C)

Time in hours	Ml.0.1N.NaOH		D-C On the basis of 20 ml. substrate	D-C On the basis of 100 ml. substrate
	D	C		
0	0.00	0.00	0.00	0.00
48	10.60	4.85	5.75	28.75
96	12.90	4.70	8.20	41.00
144	13.30	4.80	10.50	52.45
192	16.70	5.20	11.50	57.50
240	17.00	4.70	12.30	61.50

The above results are based upon the following ratio of substrate to extract:

D - 10 ml. of Du Chilly extract per 100 ml. substrate.

C - Control - 5% NaCl substituted for the extract.

The above figures represent Ml. of 0. 1 N. NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 27

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY DU CHILLY EXTRACT AT pH 6.55

(Incubation temperature 30° C)

Time in hours	Ml.0.1N.NaOH		D-C On the basis of 20 ml. substrate	D-C On the basis of 100 ml. substrate
	D	C		
0	0.00	0.00	0.00	0.00
48	8.85	5.00	3.85	19.25
96	11.60	5.30	6.30	31.50
144	14.00	6.70	7.30	36.50
192	14.70	6.15	8.55	42.75
240	15.20	6.10	9.10	45.50

The above results are based upon the following ratio of substrate to extract:

D - 10 ml. of Du Chilly extract per 100 ml. of substrate.

C - Control - 5% NaCl substituted for the extract.

The above figures represent Ml. of 0. 1 N. NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 28

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY DU CHILLY EXTRACT AT pH 7.50

(Incubation temperature 30° C)

Time in hours	Ml.0.1N.NaOH		D-C On the basis of 20 ml. substrate	D-C On the basis of 100 ml. substrate
	D	C		
0	0.00	0.00	0.00	0.00
48	8.50	5.00	3.50	17.50
96	11.30	6.30	5.00	25.00
144	13.60	6.80	6.80	34.00
192	15.10	7.60	7.50	37.50
240	15.80	7.80	8.00	40.00

The above results are based upon the following ratio of substrate to extract:

D - 10 ml. of Du Chilly extract per 100 ml. of substrate.

C - Control - 5% NaCl substituted for the extract.

The above figures represent Ml. of 0. 1 N. NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

TABLE 29

HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY DU CHILLY EXTRACT AT pH 8.50

(Incubation temperature 30° C)

Time in hours	Ml.0.1N.NaOH D	C	D-C On the basis of 20 ml. substrate	D-C On the basis of 100 ml. substrate
0	0.00	0.00	0.00	0.00
48	8.00	4.80	3.20	16.00
96	11.20	6.80	4.40	22.00
144	13.30	7.40	5.90	29.50
192	14.50	7.90	6.60	33.00
240	16.15	9.00	7.15	35.75

The above results are based upon the following ratio of substrate to extract:

D - 10 ml. of Du Chilly extract per 100 ml. of substrate.

C - Control - 5% NaCl substituted for the extract.

The above figures represent Ml. of 0. 1 N. NaOH required to titrate 25 ml. of the neutralized (pH 7) filtrate (equivalent to 20 ml. of the hydrolysate) to pH 9.5 after the addition of 2 ml. of formaldehyde (pH 7.0).

FOLLOWING ARE THE THREE OBSERVATIONS OF THE HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY BARCELONA EXTRACTS DETERMINED BY THE MODIFIED FORMOL TITRATION METHOD:

Time in hours	Ml. of 0.1 N. NaOH per 100 ml. of substrate 1	2	3	Means of 1, 2, 3
0	0.00	0.00	0.00	0.00
48	16.75	18.00	17.50	17.42
96	30.00	28.75	29.50	29.42
144	34.75	34.50	35.00	34.75
192	35.00	38.00	36.00	36.33
240	35.00	38.00	34.75	36.33

Each of the above observations was made at a different time. Extracts were made from the same stock of filberts but at three different occasions. The calculations of the F value and the standard error are shown on following pages.

FOLLOWING ARE THE THREE OBSERVATIONS OF THE HYDROLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BY DU CHILLY EXTRACTS DETERMINED BY THE MODIFIED FORMOL TITRATION METHOD:

Time in hours	Ml. of 0.1 N. NaOH per 100 ml. of substrate 1	2	3	Means of 1, 2, 3
0	0.00	0.00	0.00	0.00
48	9.50	10.00	10.00	9.83
96	22.00	20.00	20.50	20.83
144	33.50	29.50	33.00	32.00
192	34.75	35.00	35.00	34.92
240	35.00	38.00	36.00	36.33

Each of the above observations was made at a different time. Extracts were made from the same stock of filberts but at three different occasions. The calculations of the F value and the standard error are shown on following pages.

ANALYSIS OF VARIANCE CALCULATIONS

Experiment: DETERMINATION OF THE PROTEOLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BROUGHT ABOUT BY BARCELONA EXTRACTS USING THE MODIFIED FORMOL TITRATION METHOD

Preliminary Calculations

(1)	(2)	(3)	(4)	(5)	(6)
Source of Variation	Total of Squares	No. of Items Squared	Observations per Squared Item	Total of Squares per Observation (2)÷(4)	Sum of Squares (5)-correction
Correction	214,137.56	1	15	14,275.8375	0
Column	71,395.8125	3	5	14,279.1625	3.3250
Row	45,148.188	5	3	15,049.3958	773.5583
Ind. obs.	15,060.438	15	1	15,060.4375	784.6000

Analysis of Variance

Variation Due to:	Sum of Squares	Degrees of Freedom	Mean Square	F	Remarks
Method	3.3250	2	1.6625	1.72	Not significant
Time	773.5583	4	193.3896	200.48	Significant
Error	7.7167	8	0.9646		
Total	784.6000	14			

ANALYSIS OF VARIANCE CALCULATIONS

Experiment: DETERMINATION OF THE PROTEOLYSIS OF NON-FAT MILK SOLIDS SUBSTRATE BROUGHT ABOUT BY DU CHILLY EXTRACTS USING THE MODIFIED FORMOL TITRATION METHOD

Preliminary Calculations

(1)	(2)	(3)	(4)	(5)	(6)
Source of Variation	Total of Squares	No. of items Squared	Observations per Squared Item	Total of Squares per Observation (2)÷(4)	Sum of Squares (5)-correction
Correction	161403.0625	1	15	10760.20516	0
Time	36846.0625	5	3	12282.02083	1,521.-81667
Method	53801.0625	3	5	10760.81250	0.60834
Ind. Obs.	12298.5625	15	1	12298.56250	1,538.-35834

Analysis of Variance

Variation Due to:	Sum of Squares	Degrees of Freedom	Mean Square	F	Remarks
Method	0.60834	2	0.30417	0.15	not significant
Time	1,521.81667	4	380.45417	191.02	significant
Error	15.93333	8	1.99167		
Total	1,538.35834	14			

Pooled ss = 27.5834

s^2 = 1.37917

$\sqrt{s^2}$ = 1.1744

$2.09\sqrt{s^2}$ = 2.4545

$\frac{2.09\sqrt{s^2}}{\sqrt{3}}$ = 1.4171

If one determination is made, the true value could be anywhere between

$$x \pm 2.45$$ (on 95 per cent probability basis)

If three determination is made, the true value could be anywhere between

$$\bar{X} \pm 1.42$$ (on 95 per cent probability basis)

where $\bar{X}$ is the average of the three determinations.