A MODIFIED METHOD FOR THE DETERMINATION OF AMYLASE ACTIVITY IN BARLEY AND MALT AND ITS APPLICATION IN PLANT BREEDING

by

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ABSTRACT

A colorimetric procedure for the determination of amylase activity in barley and malt was developed. Measurement of the end-products of diastasis is based on the reaction between reducing sugars, produced by the action of amylases on a starch substrate, and 3,5-dinitrosalicylic acid. The total amylase activity is measured and, after differential inactivation of beta-amylase by a phenyl mercuric salt before diastasis, alpha-amylase activity is determined. Beta-amylase activity is obtained by difference.

Sources of error and reproducibility were determined. A variation of 1° C. from the 20° C. diastasis temperature causes an error of 5 per cent in total amylase activity and 4.5 per cent in alpha-amylase activity. Errors of 4 per cent and 2.5 per cent arise from a 15 second deviation from the 5 minute diastasis time. Differences between days and between duplicate determinations were not significant. The correlations between values found by the modified methods and results of the official methods of the American Society of Brewing Chemists were 0.99 for each pair of measurements. These correlations are ideal for the substitution of one method for another. Standard errors of estimate in predicting total amylase and alpha-amylase activities in conventional units from results obtained by the modified are equivalent to 4 per cent of the mean values. Considering the curvilinearity of the Lintner scale the error in total amylase activity is satisfactory, particularly as the error is lowest in the central part of the range. The alpha-amylase activity error is also considered satisfactory, when the sources of error in the official method, arising principally from visual judgement of the end-point, are taken into account.

The total and alpha-amylase determinations are made using the

same malt extract and alpha-, beta- and total amylase activities are expressed in the same units. The methods are time - saving and convenient.

The method described above, in conjunction with a new process for malting small samples (20 gm.) of barley was applied to some aspects of barley breeding. Correlations between barley amylase and the alpha- and beta-amylase of corresponding malts showed that while malt beta-activity can be predicted from a barley amylase determination, prediction of malt alpha-amylase is less reliable. This shows the necessity of malting to measure certain barley quality criteria. A study of the distribution of amylase activities among lines in a hybrid population indicated that alpha depends upon the quantitative action of several genes and that beta is due to non-dominant single gene action. Amylase activity does not appear to be associated with earliness of maturity in barley. A study of the daily variation of nitrogen and amylase activity prior to harvest indicated that harvest date may have a critical effect on the biochemical properties of barley.

INTRODUCTION

Analytical tests for quality factors that are most useful in a barley breeding program are not necessarily those that find the most favor in the malting barley industries. The industrial chemist is concerned with the technical efficiency of a process and the uniformity of a product. He is dealing with two or three barley varieties and he is very familiar with their general behavior. His efforts are therefore geared to the detection of small differences between batches of the same barley variety and to the estimation of their effect on an industrial process. The plant breeders' chemist, on the other hand, deals with large numbers of lines with unknown characteristics from hybrid populations. He must select from this material those lines that have a high probability of becoming acceptable malting varieties. Further, if breeding programs are to proceed with maximum efficiency, the selection must be accomplished between harvest and the following seeding time. Finally, he must use only a small sample of grain. Thus, while they have a common concern with the complexities of malting quality, the techniques that appeal to the industrial chemist are not always appropriate for the chemist concerned with plant breeding and its associated research.

The screening of hybrid barley populations, that is the selection of lines with promising malting quality, is essentially a process of rejection. The chemist compares the behavior of unknown barley lines with that of already acceptable standard varieties, grown and tested under the same environmental conditions. Those lines that show marked inferiority or departure from the mean performance of standard varieties

are discarded from the breeding program. Those that are not rejected are subjected to more rigorous tests the following year. This process is continued until the most promising lines have been selected and can be transferred from the plant breeding station to a laboratory where their actual industrial worth can be assessed.

At the earliest stage selection for malting quality can be based to some degree on barley nitrogen content. A generation later there are tests for the prediction of total extractable matter and of amylase activity that do not involve malting and which have been used successfully for a long time. However, progress in breeding for malting quality has resulted in narrower ranges in quality variation in hybrid material and it has become necessary to look for more discriminatory tests on which to base selection. The relations between various barley and malt properties have been investigated and have been useful in the development of prediction tests for malting quality. There are, however, certain quality criteria that can not be predicted with any reliability unless the barley is first malted. Among them is alpha-amylase activity.

It has proved possible to scale down laboratory malting to the point where as little as 20gm. of barley can be made into malt for reliable analytical determinations. This enables assays of alpha-amylase activity to be made at an early generation in a breeding program. The saccharifying property of the amylases is the basis for several methods for determination of their joint activity, including the standard procedures of professional chemists' associations in America and Europe. For the determination of alpha-amylase acting alone, however, its dextrinizing property is used in official methods. These methods are, of course, satisfactory for use in the

examination of plant breeding material, but they have the drawback of being time-consuming, which limits the number of samples that can be analyzed.

Both alpha- and beta-amylases function to liberate reducing sugars by their action on starch, and within a certain range of conditions their activities in this respect are additive. There seemed to be no reason why this property should not be used, along with a suitable colorimetric method for the determination of reducing sugars, to measure the activities of both enzymes, provided that one enzyme could be inactivated in the presence of the other. A new procedure that is precise and economical in time and materials was developed. Used in combination with a small-scale malting technique, the new method enables determinations of amylase activities to be made on large numbers of small-size barley samples. It should prove useful in testing hybrid populations from breeding programs, as well as in genetic studies and other experiments that are necessary adjuncts to plant breeding.

REVIEW OF LITERATURE

1. General Properties of Amylases

By definition, the amylases are enzymes hydrolyzing glucosidic linkages and acting upon glycogen, starches and dextrins. They are outstanding in that they are involved in a wide variety of industrial processes. Amylases may be divided into two main types, the alpha- and beta-amylases, which differ in their action on starch. However, the designation does not refer to the configuration of the glucosidic bond that is hydrolyzed; both enzymes hydrolyze alpha-1,4 linkages.

There appears to be only one kind of beta-amylase (27, 46). It is not found in animals, bacteria or fungi but is widespread in green plants, being found predominantly in ungerminated cereals and in sweet potato and soybean. In barley, beta-amylase is present in two forms:

a. A "free" form, readily extractable in water, is a fraction varying from 50 per cent to 80 per cent of the total. Sandegren and Klang (49) found 55 per cent of the total beta-amylase readily soluble from a two-row barley and 72 per cent from a six-row barley. Among Canadian six-row barleys, the writer found the readily soluble fraction to be about 74 per cent, with exceptions in the case of certain varieties in which the readily soluble portion was of the order of 50 per cent. Thus the amount of water soluble enzyme varies with barley type, though not apparently with seasonal conditions.

b. A "latent" form that is extractable only by some special treatment that releases, produces or maintains the sulphydryl (SH) groups on which the enzyme activity depends (52). Digestion of barley in papain or in reducing agents such as cysteine or glycollic acid results in higher beta-amylase activity than treatment with water alone (11, 43, 49, 56). It has been demonstrated (43) that free and latent beta-

amylases are substantially identical in their ultracentrifugal and electrophoretic properties.

In malting, the increase in beta-amylase activity is considered to be due to the release of additional SH groups from malt protein during germination (22,43,48). This suggestion is supported by the fact that papain, a proteolytic enzyme, increases amylolytic activity in barley by "liberating" the latent beta-amylase. Further evidence for this assumption is provided by the fact that conditions that favor the removal of SH groups result in the inactivation of the enzyme. Phenyl mercuric salts cause loss of beta- activity while having little effect on alpha-amylase (52); oxidizing agents such as iodine (28) have a similar effect, as does a mixture of ascorbic acid and cupric ions (47).

During the germination of barley, a second form of amylase is produced which is able to dextrinize starch and destroy the iodine-color property of starch. Apparently all germinating cereals produce this enzyme, known as alpha-amylase (32), and in contrast to beta-amylase, there are many types of alpha-amylase. They are widely distributed in nature and are derived from both plant and animal sources. The enzyme is secreted by animals in saliva and pancreatic juice and is produced by many bacteria and certain fungi, especially molds of the genera Mucor, Aspergillus and Penicillium.

The activity of pancreatic amylase has been shown to depend on free primary amino groups and to be independent of SH groups (17). The dependence of malt amylase on free amino groups has also been suggested (44). That SH groups are unimportant to alpha-amylase activity is indicated by the fact that treatments that inactivate the SH grouping in beta-amylase have little effect on alpha-amylase. Calcium ions

have a special role in the functioning of this enzyme, acting as a stability factor and not as an activator. Possibly calcium maintains the enzyme in a configuration unfavorable for proteolysis. Calcium salts increase the resistance of alpha-amylase to thermal inactivation but have the opposite effect on beta-amylase (26,33). A preparation of alpha-amylase free from active beta-amylase can be obtained by heating malt extract at 70° C. for 15 minutes in the presence of 0.1 per cent calcium acetate (33). On the other hand, all alpha-activity can be destroyed by a sodium triphosphate treatment due to fixation of calcium. All alpha-amylases are now regarded as metallo-proteins containing calcium, an element essential for their activity.

2. Amylolysis of Starch

Cereal starches consist of two components, both polymers of glucose. A linear polymer, amylose, consists of long unbranched chains of glucose units joined by alpha- 1,4 glucosidic linkages. The chain length varies with the source of the amylose, 200 to 300 glucose units being typical. The other component is amylopectin, a branched polymer with a ramified structure. Each branch consists of 20 to 30 glucose units joined by alpha- 1,4 linkages and a molecule of amylopectin contains hundreds of branches. The branch points are connected by alpha- 1,6 linkages. The detailed structure of amylopectin, that is, whether there is any order in the arrangement of the branches, is not yet known. In fact, the presence of a significant proportion of alpha- 1,2 or alpha- 1,3 linkages has been postulated (1,8,57). Amylopectin is not homogeneous and could consist of branched polymers having different molecular weights. In barley, the linear fraction, amylose, is 20 to 25 per cent of the whole starch. Malted barley starch has a higher proportion

of amylose, due to limited degradation of amylopectin by enzymes during germination.

Acting on whole starch, beta-amylase rapidly produces maltose to the extent of about 60 per cent of the theoretical yield (27). There is no rapid change in the viscosity of the starch paste and the residual 40 per cent is virtually non-reducing and gives a blue color with iodine. Hydrolysis begins at the non-reducing end of each chain and splits off maltose units. There is presently a controversy (23 and references cited) as to whether beta-amylase attacks amylose in a single chain pattern (attacking one chain completely before commencing on another), in a multi-chain pattern (removing one maltose only from a chain, then attacking other chains in the same way) or a multiple attack (removing several but not all maltoses, then attacking another chain). Theoretically, the amylose component is hydrolyzed completely, as it contains no branches, whereas the amylopectin fraction is hydrolyzed to about 50 per cent. There is no dextrinization in the true sense and beta-amylase attacks only the alpha- 1,4 linkages in starch. In the branched component beta-amylolysis results in the removal of outer chains down to two or three glucose units from the 1,6 branching point. The limit of this action is used in studies on the structure of amylopectin (1). The residue is known as beta-limit dextrin, though it is not an absolute limit dextrin, as its end groups are never shorter than two or three glucose units.

Pure crystalline beta-amylase does not achieve complete conversion of amylose to maltose. Investigation has shown that the reaction ceases when 70 to 80 per cent of the theoretical yield of maltose is attained, depending on the source of the substrate (10,40,41,42). It appears that the molecular structure of amylose is not simple, that it

is heterogeneous and contains anomalous branch points which cannot be degraded by beta-amylase. The branching differs from that of amylopectin in that the number and frequency of amylose branches are very much lower and that chain lengths in amylose are considerably longer than those of amylopectin. An enzyme factor, Z, has been postulated as necessary to enable beta-amylase to complete its hydrolysis (41). The Z-enzyme has been reported to be a beta-glucosidase which attacks beta-glucosidic linkages at the presumed branch points in amylose. Though the Z-enzyme is able to attack these anomalous linkages, it has been shown to have no action on alpha- 1,3, alpha- 1,4 or alpha- 1,6 glucosidic linkages, nor on beta-linked disaccharides (18). The Z-enzyme is not inhibited by mercuric chloride, which distinguishes it from alpha- and beta-amylases. However the identity of the Z-enzyme has not yet been decided, nor has the nature of the branchings that it attacks; its function is to enhance the beta-amylolysis limit of amylose. A linear starch molecule is completely degraded to maltose if it contains an even number of glucose units; otherwise the last three glucose units at the reducing end of the chain remain as maltotriose. Residual maltotriose units may be converted to maltose and glucose by beta-amylase, but the action proceeds very slowly.

The outstanding characteristic of alpha-amylase is its ability to dextrinize starch and the enzyme is often referred to as a dextrinogen (29). The iodine color property of starch is destroyed and the viscosity of starch paste sharply decreased. In contrast to beta-amylase, alpha-amylase hydrolyzes glucosidic bonds of either amylose or amylopectin in somewhat random fashion to produce initially a variety of dextrins and finally a mixture of maltose, small amounts of maltotriose and glucose, and limit dextrins (46). The starch-liquefying

property, aiding the solubilization of starch, is due to the reduction in size of starch molecules and to the disruption of intermicellar network within the starch molecules and belongs to alpha-amylase alone. Liquefaction proceeds at temperatures as high as 78° C., where normal dextrinizing, saccharifying and beta-amylase activities are inhibited. It has not been possible, however, to separate the liquefying factor from preparations of alpha-amylase (45).

Initially, alpha-amylase ruptures the more centrally located alpha- 1,4 glucosidic linkages in the chains of both components of starch, producing both linear and branched low molecular weight dextrins (27). The linear dextrins from amylose are further slowly cleaved to reducing sugars, mainly maltose. Hydrolysis proceeds with attacks on 1,4 linkages either side of amylopectin branching points, and alpha-amylase is able to break the branch at the last 1,4 linkage, whereas beta-amylase cannot shorten the side chains in amylopectin within two or three glucose units of the branch point. In this respect alpha-amylase may well be an essential precursor to the action of the enzyme limit-dextrinase, which is also present in some malts and which further degrades dextrins. Like beta-amylase, however, alpha-amylase cannot hydrolyze alpha- 1,6 bonds, and bonds between glucose residues immediately adjacent to the 1,6 bonds are similarly generally immune from attack (45). The saccharifying action of alpha-amylase, that is the production of beta-maltose, along with small amounts of glucose and maltotriose, from linear and branched dextrins, proceeds much more slowly than the dextrinizing action. Nevertheless, alpha-amylase can reach a hydrolysis limit of 80 to 90 per cent of the theoretical value, provided that the action is prolonged over a period of two to four

weeks (46). The residual limit dextrins, whose yield depends on the length of the reaction time, are derived from portions of the branched fraction of starch that contains the alpha- 1,6 bonds. These limit dextrins, which are unfermentable, usually contain five to seven glucose units, though panose, composed of three glucose units, has been obtained from malt reactions (53).

Joint Action of the Amylases

Acting together, alpha- and beta-amylase cause more rapid and extensive hydrolysis of starch than equivalent concentrations of either acting alone (27,45). The main product of this action is maltose, but the presence of glucose and iso-maltose has also been reported, as well as several tri- and tetra-saccharides along with residual limit dextrins. Alpha-amylase is the fundamental enzyme with its high affinity for starch and its power of rapid dextrinization. Beta-amylase, acting in conjunction with alpha-amylase, fulfils the role of an accessory which accelerates and furthers the saccharification process. It is probable that the alpha-enzyme, acting alone, could achieve as complete a conversion of starch as the combined amylase action, but this would be a prolonged process. Beta-amylase, acting alone, does not hydrolyze starch to products that give no color with iodine. It has less affinity for starch and a greater affinity for dextrins than alpha-amylase.

Factors Affecting Amyloysis of Malt

In reactions that are catalyzed by enzymes, it is necessary to consider several factors that influence the rate of change. To determine the extent of these influences it is usual to measure one factor while keeping all others constant. This procedure has the limitation that the optimum effect of the factor may shift with a change in another variable

factor and any information obtained could be of doubtful value. It is necessary, therefore, to consider the contribution of several factors simultaneously, or to recognize that the effect of a factor determined separately gives optimum activity under set specified conditions. This is especially true when considering the enzymatic hydrolysis of malt during the industrial mashing process (45).

Amylolysis of starch is a major part, but only a part, of mashing. Even if proteolysis and other enzyme action could be excluded from consideration of the activity taking place during the process, it is virtually impossible to decide on external optima for amylolytic action. The usual controlling factors apply in amylolysis: relative concentration of the two amylases, hydrogen ion concentration, temperature, duration of the reaction, inactivation of the enzymes, quality and concentration of the substrate and the influence of other ions. Numerous studies have been made on the effect of these factors, singly or in combination, on the mashing process as a whole and on starch amylolysis as a separate entity. In many cases the optima for various factors have been given numerical values. For example, the optimum pH for beta-amylase action is said to be 4.6, while that for alpha-amylase is reported at 5.7 (29). Also, beta-amylase is said to operate most efficiently at 55° C., whereas alpha-amylase has an optimum closer to 70° C.(27,45). However while certain generalizations are possible, the extent of interaction between all factors and the fact that each amylase appears to have different optima lessens the practical value of these data. It is, nevertheless, always possible that optimum conditions for amylolysis may vary among malts made from different barley varieties. Such varietal differences may be useful criteria in selecting in hybrid barley populations.

While optimum conditions for amylase activity, both in industry and in the laboratory, are at present indefinable, the results of investigations have been valuable in setting up working limits for amylolysis. Mashing processes in industry may not include all, if any, absolute optima, but they do yield a product that is acceptable, in quality and quantity. Laboratory assays of amylase activity, run ordinarily at pH 4.7 and 20° C., and employing a starch substrate that is as uniform as possible, do provide useful data for industry (29) and for barley breeder (37).

3. Amylases in Malting

One of the main reasons for converting barley into malt is the desired production of alpha-amylase (45). It has been mentioned that this enzyme is fundamental for starch hydrolysis, and that it is not present in ungerminated barley. At the same time, the malting process prepares the starch substrate for final amylolytic action by decreasing the resistance of barley starch to enzymic degradation. In fact during germination a certain amount of beta-amylolysis of amylopectin takes place but amylose undergoes no change.

The malting process is divided into three stages. Barley is steeped in cold water until its moisture content reaches a level that is considered optimum, often about 40 per cent. Steeping is accompanied by some form of aeration and it is during this stage that alpha-amylase production is initiated. The second stage is a controlled germination period of several days in a cold, moist atmosphere. During germination alpha-amylase development increases, slowly at first and rapidly after two to four days. Both forms of beta-amylase present in barley are very greatly increased during the growth period. The last stage of the conversion from barley to malt is a drying process using air-flow and

heat. The purpose of kilning is the production of a stable, friable substance and the introduction of color and flavor. These effects are accompanied by some destruction of the amylases, especially beta.

There are many systems of making malt, and while each includes the principles of steeping, germination and kilning, operational details vary considerably. The three stages may be carried out as distinct, separate processes, or steeping and germination may be combined, or all three may become a continuous process. Designs for malting equipment are numerous and new apparatus is being developed continually. Malting is, essentially, an air and water treatment of barley under certain time and temperature conditions. The treatments and conditions are governed by the type of malt it is desired to produce and by the quality of the barley as determined by varietal and environmental characteristics. There are, therefore, many components of malting technology; amylase activity, along with other malt properties, is affected by their variation.

Some Effects of Malting Conditions on Amylases

In the evaluation of new strains of barley from plant breeding programs, a laboratory scale malting plant is used. Malting conditions are precisely controlled so that among groups of strains grown in the same environment variations in the malts are due to varietal characteristics only. However, the apparatus does lend itself to a limited study of malting variables, and some of the results are described below.

The data in Table 1 show the variation in saccharifying activity, that is, the combined action of both amylases, and in alpha-amylase activity with germination time. The results (unpublished) were obtained by the writer on Canadian grown barley varieties malted in the laboratory equipment. Before germination the samples were steeped in

Table 1

Effect of Germination Time on Amylase Activity

Barley Variety	Germination Time - days	Saccharifying Activity - degrees Lintner	Alpha-amylase Activity - dextrinizing units
Montcalm (six-row)	6	174	38
	8	190	60
	10	194	70
Swan (six-row)	6	201	44
	8	220	65
	10	225	72
Kenia (two-row)	6	135	60
	8	143	90
	10	155	100

water at 11° C. until the moisture content reached 44 per cent. Germination temperature was constant at 11° C., and the malts were all kilned under constant conditions.

The data show the increase of amylase activity with germination time, though in these examples which are typical of Canadian grown barleys, there is a levelling off after 8 days. The difference in relative amylase production between two-row and six-row barleys is also shown.

Amylase development in germinating barleys is also influenced by the moisture content of the grain during growth. Table 2 gives the results (unpublished) of an experiment on this factor. Samples of a six-row barley, grown in Canada, were steeped in water at 11° C. for various moisture contents and then germinated at 11° C. for 5 and 6 days. Moisture content was maintained during growth, and the malt was kilned by the standard process.

Table 2

Effect of Moisture Content during Germination on Amylase Activity

Germination Moisture %	Germination Time - days 5		6	
	Sacch. Activity	Alpha-amylase Activity	Sacch. Activity	Alpha-amylase Activity
38	140	34	161	44
42	163	35	182	46
44	183	34	191	45
46	185	35	193	46

In this experiment, alpha-amylase production was not affected, but higher moisture content resulted in higher saccharifying activity and, by inference, more beta-amylase production.

Higher germination temperatures would be expected, like higher moisture content, to stimulate metabolic activity in the growing barley. However, there appears to be a maximum temperature, above which amylase production begins to decline. Results of the writer's own experiments in a germination temperature range between 10° and 15° C. showed higher malt saccharifying activity at the higher temperature. On the other hand, data quoted by Hopkins and Krause (29) indicate that amylase activity of a malt produced at 24° is lower than that of a malt grown at 14° C. According to Dickson, Olson and Shands (19) duration of growth is the most important factor for alpha-amylase production, regardless of temperature or of barley variety. They add that at 16° C., although the enzyme is produced more rapidly than at lower temperatures, the total yield is scarcely affected.

The kilning treatment has a marked effect on the amylases. Their relative activities, especially that of the decidedly less heat-

stable beta-amylase, are altered considerably. The kilning process may occupy one to three days with gradual temperature increases, the final temperature depending upon the enzymatic and other properties desired in the finished malt. The effects of kilning on the amylases produced during germination vary greatly with temperature conditions and with the rate of drying, and they are so complex that, unless one particular kilning program is considered, only very general statements are possible. Though in most systems, alpha-amylase is only slightly affected, beta-amylase is inactivated to an extent dependent on heat application. High temperatures early in the kilning process, when the malt moisture content is high, cause extensive uncontrolled destruction of beta-amylase. Low temperatures at first, followed by higher temperatures when the malt is almost dry, will result in less enzyme inactivation, in accordance with the principle that enzyme stability increases in drier situations. Low temperatures throughout the process result in less destruction of beta-amylase and the finished malt is of the high saccharifying type used in the distilling industry. The amylases are active during the kilning process while moisture is still present in sufficient amount. Starch degradation initiated during germination continues, accompanied by the production of reducing sugars.

4. Measurement of Amylase Activity

In a review of the subject, Wildner cites some 150 methods for detrmining amylase activity (55). The majority are based upon one of the manifestations of amylase action:

a) Measurement of decrease in viscosity of fluids during enzymatic hydrolysis of starch (liquefaction).

b) Observation of the change in the starch-iodine color reaction (dextrinization).

c) Measurement of increase in reducing sugars (saccharification).

Enzyme assay methods have been classified into five types by Landis (34) and nearly all of the amylase activity methods fall into four of these, in fact the majority into two of them. Type I employs the principle that when two enzyme solutions are diluted to the point where they produce identical conversions in equal times, the strengths of the enzymes are proportional to their concentrations. In type II assays time is the variable and is inversely proportional, within limits, to enzyme activity required to produce a constant conversion. Time is constant in type III assays and enzyme strength is related to a variable degree of conversion. Type IV assays vary both time and conversion and have been used very rarely in studies of amylases. Finally, type V assays are based upon reaction kinetics. The majority of amylase determinations fall in types II and III.

While beta-amylase has some slight effect on starch-liquefying activity, for all practical purposes the reduction in viscosity of starch pastes can be considered as a measure of alpha-amylase activity. The best known methods using the liquefying property are those described by Jozsa and Johnston (30), Landis and Redfern (35) and Blom and Bak (I5). The viscosity method measures the initial steps of starch breakdown and can be the basis of a very sensitive method for measuring alpha-amylase activity. However there are disadvantages in that a relatively large amount of specially prepared substrate is required for each determination and the method tends to be tedious and time consuming.

At the present time, the most widely used procedures for the determination of alpha-amylase depend on the dextrinizing property of the enzyme as indicated by the disappearance of the blue iodine color of starch. One of the earliest techniques developed was that of Wohlgemuth (56), which is a type I assay. In the earlier procedures it was assumed

that a dextrinizing method measured alpha-amylase exclusive of beta-amylase activity. Later investigation showed that the combined effects of both enzymes were measured. A type II, modified procedure was developed by Sandstedt, Kneen and Blish (50) in which the rate of dextrinization is measured in the presence of an excess of beta-amylase. Under this condition changes in the rate are attributable to variations in alpha-amylase activity alone. Alpha-amylase activity is expressed in terms of the digestion time required for the enzyme to convert starch substrate to products which give a red-brown color with iodine. Various modifications of the original method have been made, among them a change in dextrinizing temperature (39) and the use of a colorimeter and a standard graph to determine the end-point (16). The Sandstedt, Kneen and Blish method is the basis of the Official Method for the Determination of Alpha-Amylase of the American Society of Brewing Chemists (5). The method was adopted as official by the Society after several years of careful study by committees which investigated reaction temperature, substrates, preparation of malt infusion, standard end-point color and methods of expressing results. The method is reliable but is not the ultimate in convenience if a large number of determinations have to be carried out in a day.

Saccharifying methods, in which the production of reducing sugars by amylases acting on a starch substrate at 20° to 40° C. is measured, are in general use. Applied to malt extracts this principle, a type II assay, measures the combined saccharifying effects of the alpha-and beta-amylases, present in the malt. This estimation is related to the so-called diastatic power of cereals and is the basis for the Official Method of the American Society of Brewing Chemists, (5) the American Association of Cereal Chemists (3) and the Association of Official

Agricultural Chemists (9) for the measurement of this property. The Official Methods specify the use of Fehling's solution or the ferricyanide reagent (6) for the estimation of reducing sugars after hydrolysis of starch. Many other reagents have been suggested for the estimation of reducing sugars, among them 3,5- dinitrosalicylic acid (31,51 and references cited).

Diastatic power represents the saccharifying effect of both amylases. Graesser and Dax (24) described a method of using the saccharifying property to estimate the activity of alpha-amylase alone. The beta-amylase was differentially inactivated by heating malt extract at 70° C. in the presence of calcium ions. This approach has considerable merit in that both diastatic power and alpha-amylase can be determined quickly and simply using the same techniques and apparatus. Within a certain range of conditions, the saccharifying activities of the amylases are additive, and the activities of both enzymes can be expressed in the same units.

An essential step in malt measurement procedure is the extraction of the enzymes from the grain. Maximum extraction is not obtained by the use of distilled water but by employing dilute salt solutions. Wiener and Hopkins (54) reported that proportion of malt alpha-amylase extractable by water at 21° C. varied from one-fifth (green malt) to four-fifths (kilned malt) of that extractable by 0.2 percent calcium acetate solution. They also noted that water soluble fractions do not run parallel to calcium acetate soluble proportions. Lowry and Olson (36) stated that the amount of alpha-amylase extracted from malt by 0.5 N sodium chloride at 20° C. is 1.46 times that extracted by water. The American Society of Brewing Chemists Alpha-amylase Committee reported that 0.4 percent sodium bicarbonate is the most efficient extractant but the extra over

2.5 per cent sodium chloride is not statistically significant (4). Thus various extractants have been tried. Erlich and Burkert (20) carried out an extensive study of the effects on amylase activities of extraction procedures at 20°C. and 30°C. using a wide variety of electrolytes in various concentrations. Sodium chloride solution is used in the official method of the American Society of Brewing Chemists (5) but calcium acetate solution finds favor with other workers.

The amylases are proteins and have been classified as water-soluble albumins. However Wiener and Hopkins (54) suggest that, as the amylases are more soluble in salt solution than they are in water, they resemble globulins. The possibility exists that the enzymes are protected from the extracting water by globulins in which they are embedded and thus are only brought into solution when the globulin is dispersed. Another factor that may influence solubility is that aqueous extraction of barley and malt takes place approximately at the iso-electric point of malt amylases. It is also possible that much of the amylase that is insoluble in water is adsorbed on the starch granules of malt (54).

A MODIFIED METHOD FOR THE DETERMINATION OF AMYLASE ACTIVITY IN BARLEY AND MALT

1. Introduction

A simplified, rapid method for the determination of the combined and individual activities of alpha- and beta-amylase is useful in plant science projects where large numbers of barley samples have to be examined. Amylase activities of barley and malted barley are reflected in the amount of reducing sugars formed by the action of grain infusions on starch. Within a certain range of conditions the activities of alpha- and beta-amylases are additive.

The use of 3,5- dinitrosalicylic acid (DNS) for the colorimetric estimation of reducing sugars has been described on several occasions (31,38,51 and references cited) and its reliability has been demonstrated. In order to determine alpha-activity independently of beta-amylase, the beta-enzyme may be differentially inactivated. This procedure was suggested by Graesser and Dax (24) using heat in the presence of calcium ions. Their technique is, however, more tedious than the use of organic mercury salts at room temperature. Weill and Caldwell (52) have reported that phenyl mercuric salts cause loss of beta-amylase activity while having little influence on alpha-amylase action. Accordingly, the colorimetric method using DNS and the differential inactivation procedure using phenyl mercuric salts were combined into a technique that determines total and alpha-amylase activities, while beta-activity is determined by difference.

The method was found to be rapid and reliable. The results may be expressed in terms of the amount of maltose formed by a unit weight of grain in unit time, or they may be expressed by conversion into the units used by official methods (3,5,9) for amylase activity.

2. Study of Reagents

(a) 3,5- dinitrosalicylic acid

Preparation. 1.0 gm. of purified reagent is moistened with a little water. While stirring, 20 ml. 2N sodium hydroxide are added. The mixture is diluted with 50 ml. water, and 30 gm. sodium potassium tartrate are added and dissolved. The solution is made up to 100 ml. with water and filtered through Whatman #1 paper.

Optical Absorption. Maximum absorption was found to be 505 mμ, using a Coleman 14 spectrophotometer. There was sufficient absorption at other wavelengths in the region 500 to 540 mμ to enable the use of colorimeters. The Coleman 8 colorimeter filter #209 has a peak at 525 mμ and this instrument was found to be suitable for use in routine amylase determinations. For the purposes of this investigation, the spectrophotometer was used and the wavelength setting was 505 mμ.

Formation of Maltose-DNS Reaction Product. A mixture of 2 ml. maltose solution of known concentration (0.2 mgm. to 2.4 mgm. maltose per 2 ml. solution) was heated in boiling water with 2 ml. DNS reagent, then cooled quickly in ice water. The mixture was diluted with 20 ml. water and the optical density read. Table 3 shows the effect of boiling time on optical density. It was decided that the optimum practical time for heating is 5 minutes. Due to variation in optical density with time it is necessary to ensure that the water bath is maintained in a vigorously boiling state and that the time is controlled precisely, otherwise errors arise. This source of error was investigated and is discussed in section 4 b.

Stability of Maltose-DNS Reaction Product. The optical densities of several series of samples were read at intervals, from 10 minutes after boiling to 18 hours after boiling. There was no change in the

Table 3

Effect of Heating Time on Optical Density of Maltose Reaction Product

Maltose Sample	Optical Density after boiling for 5 mins.	7.5 mins.	10 mins.
1	.235	.280	.302
2	.090	.120	.155
3	.305	.340	.375
4	.395	.430	.465
5	.435	.485	.515
6	.50	.54	.58

optical densities, which covered a range from .08 to .72, during this time. This indicates that, having completed other analytical operations the final reading is not affected by storage of at least 18 hours.

Relation between Optical Density and Maltose. A series of solutions was prepared so that 2 ml. contained 0.2 to 2.4 mgm. anhydrous maltose. So that optical densities could be used for the determination of maltose produced by the action of a malt infusion on starch, 1 ml. starch solution was added to each maltose sample. A blank was prepared by mixing 2 ml. water and 1 ml. starch. The samples were heated in a boiling water bath with 2 ml. DNS for 5 minutes, cooled quickly and diluted with 20 ml. water, then read in the spectrophotometer. Triplicate determinations were made for each maltose concentration, and the whole experiment was repeated one month later. Figure 1 is a graph showing the relation between optical density of maltose-DNS reaction products and maltose. Mean density values were used. In this range the relation is linear. For experiments on starch substrate volume, additional graphs were prepared. These are not given, as they have no value other than in the starch experiments, which are described later.

(b) Starch Substrate

Preparation. The starch substrate used in this method is a 1 per cent solution of starch manufactured specifically for diastatic power determination by Merck and Co. Inc. The solution is buffered to pH 4.7. In practice, 5 gm. starch, dry basis, made into a paste with a little water, are poured into 400 ml. boiling water. The mixture is boiled for 2 minutes, cooled, 10 ml. buffer solution added and the whole made up to 500 ml. For some experiments the amount of buffer was 40 ml. The buffer used for starch is a solution of 41 gm. anhydrous sodium acetate and 30 gm. glacial acetic acid in 1 litre of water.

Volume and Buffer Concentration. Extracts of three samples of malt, with saccharifying activities of 120, 150 and 160 degrees Lintner, were diluted so as to produce a range of 0.4 to 2.0 mgm. malt equivalent in 2 ml. of the infusions actually used in the tests. Each infusion sample hydrolyzed each of four starch substrates: 1 ml. and 2 ml. of starch buffered with 20 ml. acetate buffer per litre and with 80 ml. buffer per litre. Hydrolysis temperature was 20° C. After 5 minutes hydrolysis the reaction was stopped by the addition of 2 ml. DNS and the mixture was heated in boiling water for 5 minutes. After cooling and dilution the optical density was read and the readings converted to mgm. maltose by reference to Figure 1. The results of the experiment on the 150 degrees malt, which are typical, are given in Table 4. They show that, under the conditions of this experiment, the volume of substrate has no effect on the amount of maltose produced, but that an increase in buffer concentration results in a lower maltose production. It was decided that optimum conditions in the proposed method of amylase assay should include 1 ml. starch substrate, buffered with 20 ml. per litre of acetate buffer solution, as described above.

FIGURE 1 MALTOSE CALIBRATION

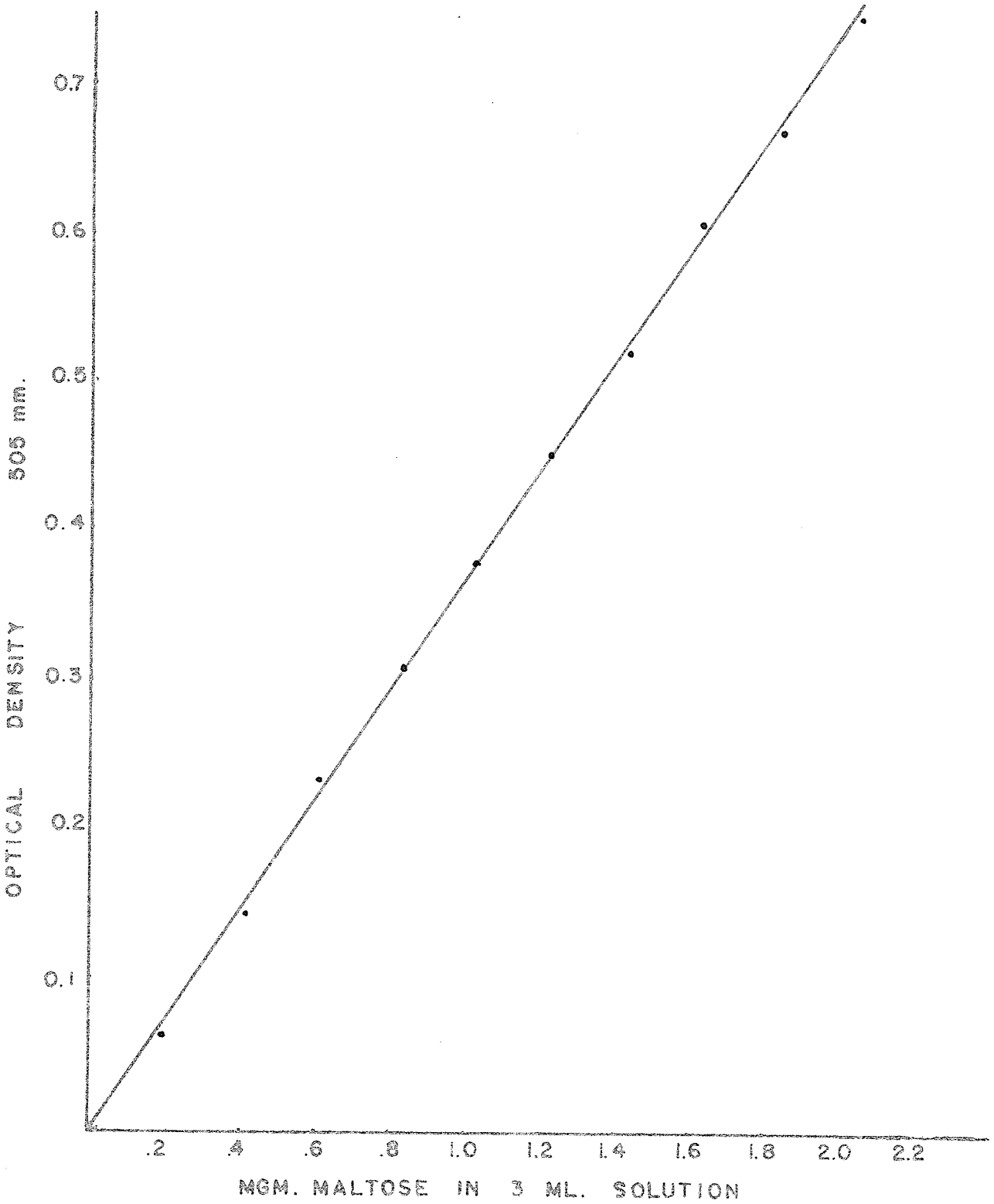


Table 4

Effect of Starch Volume and Buffer Concentration in Diastasis

	Starch Volume			
	1 ml.	2 ml.	1 ml.	2 ml.
Buffer, ml/litre	20	20	80	80
Mgm.malt per 2 ml.infusion	Mgm. maltose found			
0.4	.47	.48	.45	.44
0.8	.90	.90	.88	.92
1.0	1.13	1.13	1.10	1.09
1.2	1.30	1.28	1.26	1.25
1.5	1.57	1.57	1.50	1.49
2.0	1.98	2.00	1.90	1.90

The use of 1 ml. starch has a practical advantage in that pipette transfer and mixing of the starch with malt infusion is accomplished in less time than is the case with 2 ml. starch. The lower buffer concentration results in a steeper graph slope.

3. Development of Method

(a) Preparation of Barley and Malt Infusions

Extracts of barley and malt were prepared according to the official methods of the American Society of Brewing Chemists (5). In these procedures, 2.5 gm. ground barley are extracted overnight at 20° C. with 50 ml. of 1 per cent papain solution and 12.5 gm. milled malt are extracted for 2.5 hours at 20° with 250 ml. 0.5 per cent sodium chloride solution. A series of experiments was made to determine the amount of dilution of these extracts required so that the yield of maltose during amylolysis would be in the range 0.4 to 2.0 mgm. This is the range of the proposed colorimetric procedure (see Figure 1). It was found that the optimum dilution for total amylase (saccharifying) activity is 2 ml. extract made up to 200 ml. with water. The 2 ml. test aliquot of this diluted infusion contain the equivalent of 1 mgm. barley or malt. In the case of alpha-amylase determination, where beta-amylase is first inactivated, 5 ml. malt extract are diluted to 200 ml.

2 ml. of this solution contain the equivalent of 2.5 mgm. malt.

(b) Inactivation of Beta-amylase

In the first experiments on alpha-amylase determination, beta-amylase was inactivated by heating the malt extract at 70° C. for 15 minutes in the presence of calcium acetate. A more convenient procedure was sought, that would not require a controlled hot water bath. Various organic mercury salts have been reported to inactivate beta-amylase while having little or no effect on alpha-amylase. Two of these were investigated. The sample of 5 ml. malt extract, in a 200 ml. volumetric flask, was treated with 10 ml. saturated aqueous solution of phenyl mercuric chloride (PMC), or with 5 ml. 0.001 M chloromercuribenzoate (CMB) , for 30 minutes at room temperature and then diluted to 200 ml. with water. A series of 18 malt samples, covering a range of low to high alpha-amylase activity as determined by the method of the American Society of Brewing Chemists (5), was used in a comparison of the three beta-amylase inactivation methods. Diluted infusions of beta-inactivated malt extract were allowed to hydrolyze 1 per cent starch for 5 minutes at 20°after which DNS was added. The mixture was heated in boiling water for 5 minutes, then cooled and diluted. The optical density was read and converted to maltose equivalent (Figure 1).

The results on representative malt samples are given in Table 5. They show that while the mercury salts probably inactivate alpha-amylase to a slight extent, they are useful,as well as more convenient than the heat-calcium treatment, in an alpha-amylase activity determination. It was decided that either of the mercury salts is suitable, though PMC is preferable because it is cheaper and the preparation of a saturated solution is a very simple process.

Table 5

Effect of Beta-amylase Inactivating Agents

Malt Sample	Alpha-activity ASBC	Total sacch. activity ASBC	Mgm. maltose produced Calcium	PMC	CMB
D6	29	85	.72	.72	.72
D9	36	75	.85	.84	.84
D3	40	119	.95	.94	.94
C2	46	168	1.04	1.00	1.00
C7	50	136	1.14	1.10	1.08
D22	54	107	1.15	1.11	1.10
C18	64	140	1.37	1.33	1.33
C11	70	195	1.47	1.42	1.43

(c) Diastasis

Effect of Reaction Time

A malt sample having medium total amylase activity, about 120 degrees Lintner, and medium alpha-amylase activity, about 40 dextrinizing units, was used to study the increase in maltose production due to lengthening the time of starch hydrolysis. An extract of malt was prepared and diluted as described above. Aliquots of 2 ml. of untreated and beta-amylase inactivated infusions, attemperated to 20° C., hydrolyzed 1 ml. starch substrate for 3, 4, 5, 6, and 7 minutes. Hydrolysis was stopped by the addition of 2 ml. DNS and the mixture was treated as in previous experiments. Determinations of maltose produced for each hydrolysis time were made in triplicate for total and alpha-amylase activity, and the entire experiment was repeated on a second day.

The results are given in Table 6 and the increase in maltose production with increasing diastasis time is shown graphically in Figure 2. The increase in maltose production from 3 to 7 minutes (8 minutes in the case of alpha-amylase) was linear. There seemed to be

no reason to change the previously selected reaction time of 5 minutes. An increase in time would render the assay of high amylase activity malts difficult, if other analytical conditions were unchanged, due to high production of maltose and consequently too high optical density. A reduction in diastasis time would have the opposite effect with low activity malts. The 5 minute period, therefore, seemed to be the most convenient for routine work.

Table 6

Effect of Diastasis Time on Maltose Production

a. Total saccharifying activity

Time (minutes)	Mgm. maltose produced by 1 mgm. malt						
	Day 1			Day 2			
	1	2	3	1	2	3	Mean
3	.68	.70	.68	.70	.70	.67	.69
4	.82	.78	.82	.80	.80	.84	.81
5	.97	.97	.98	.96	.96	.95	.97
6	1.13	1.15	1.14	1.12	1.12	1.14	1.13
7	1.28	1.30	1.30	1.28	1.28	1.29	1.29

b. Alpha-amylase activity

Time (minutes)	Mgm. maltose produced by 2.5 mgm. malt						
	Day 1			Day 2			
	1	2	3	1	2	3	Mean
3	.68	.68	.72	.72	.72	.72	.71
4	.82	.84	.84	.79	.80	.82	.82
5	.90	.91	.91	.94	.93	.93	.92
6	1.04	1.02	1.01	1.02	1.03	1.03	1.03
7	1.12	1.12	1.12	1.10	1.10	1.12	1.11
8	1.22	1.23	1.23	1.24	1.23	1.22	1.23

Effect of Reaction Temperature

A malt sample having similar amylase properties to that used in the time experiment was used to study the rate of activity at various temperatures. Diluted malt infusions were prepared and maltose determinations were made as in the time experiment. Hydrolysis time was

constant at 5 minutes and hydrolysis temperatures were 10°, 20° and 30° C. for total amylase with an additional temperature of 40° for alpha-amylase. As in the time experiment, tests were made in triplicate on each of two days.

Results are given in Table 7 and are shown graphically in Figure 3. Increase in maltose production was linear over the range of temperature studied. Temperature coefficients (Q_{10}) for total amylase activity were 1.88 for 10°/20° and 1.48 for 20°/30°. For alpha-amylase activity, Q_{10} values were 1.80 for 10°/20° and 1.50 for 20°/30°. No modification of the standard 20° used in most amylase assay methods was considered. The main reason for this experiment was to determine the effect of small variations from the 20° C. temperature. The data were used in assessing this possible source of error, which is discussed later.

Table 7

Effect of Diastasis Temperature on Maltose Production

a. Total saccharifying activity

Temp. °C.	Mgm. maltose produced by 1 mgm. malt						
	Day 1			Day 2			
	1	2	3	1	2	3	Mean
10	.56	.57	.58	.59	.61	.58	.58
20	1.06	1.09	1.07	1.10	1.10	1.10	1.09
30	1.60	1.59	1.61	1.62	1.62	1.64	1.61

b. Alpha-amylase activity

Temp. °C.	Mgm. maltose produced by 2.5 mgm. malt						
	Day 1			Day 2			
	1	2	3	1	2	3	Mean
10	.52	.53	.54	.55	.56	.56	.54
20	.96	1.00	.97	.96	.94	.96	.97
30	1.42	1.44	1.45	1.46	1.47	1.47	1.45
40	1.86	1.85	1.88	1.89	1.87	1.86	1.87

FIGURE 2 EFFECT OF DIASTASIS TIME

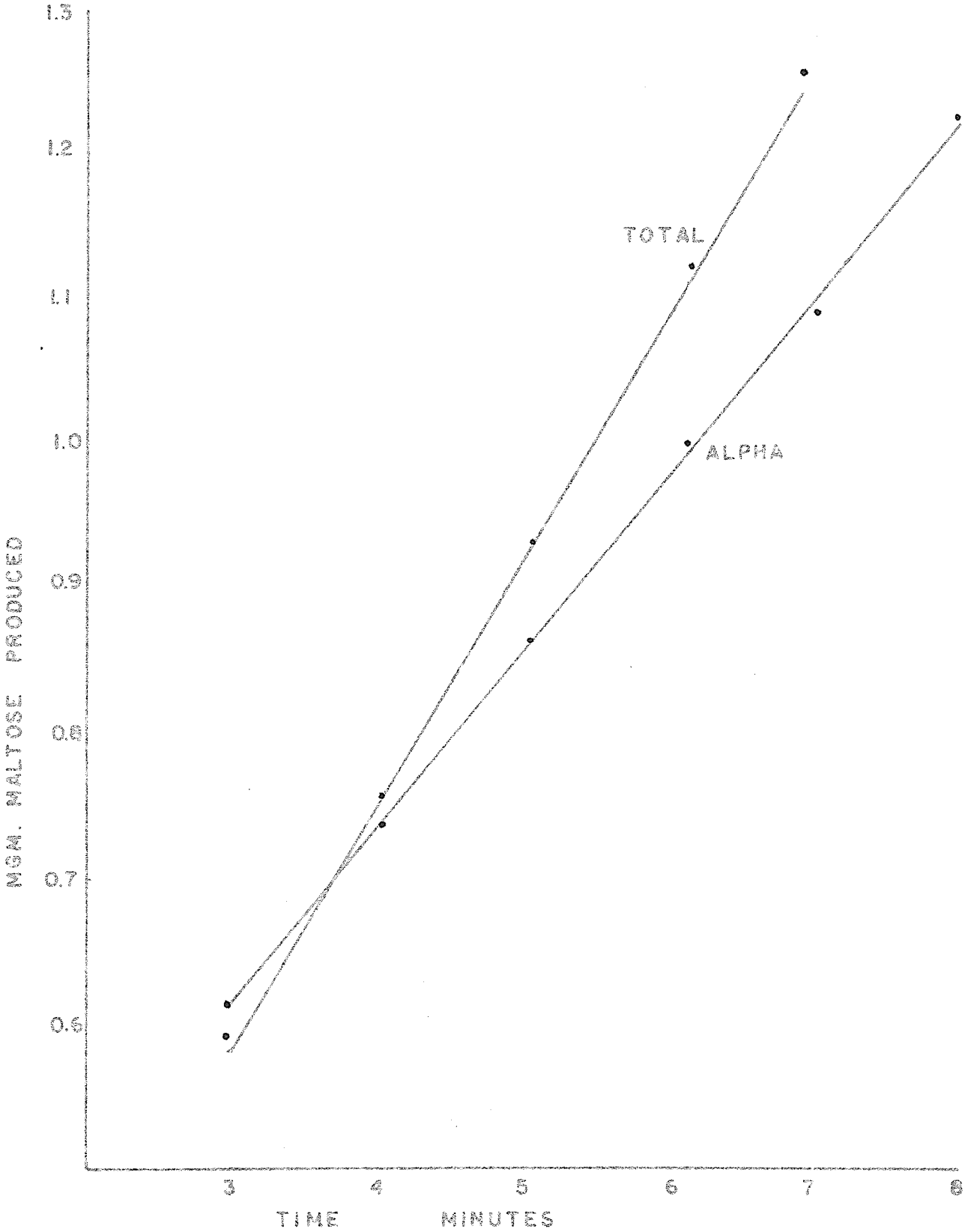
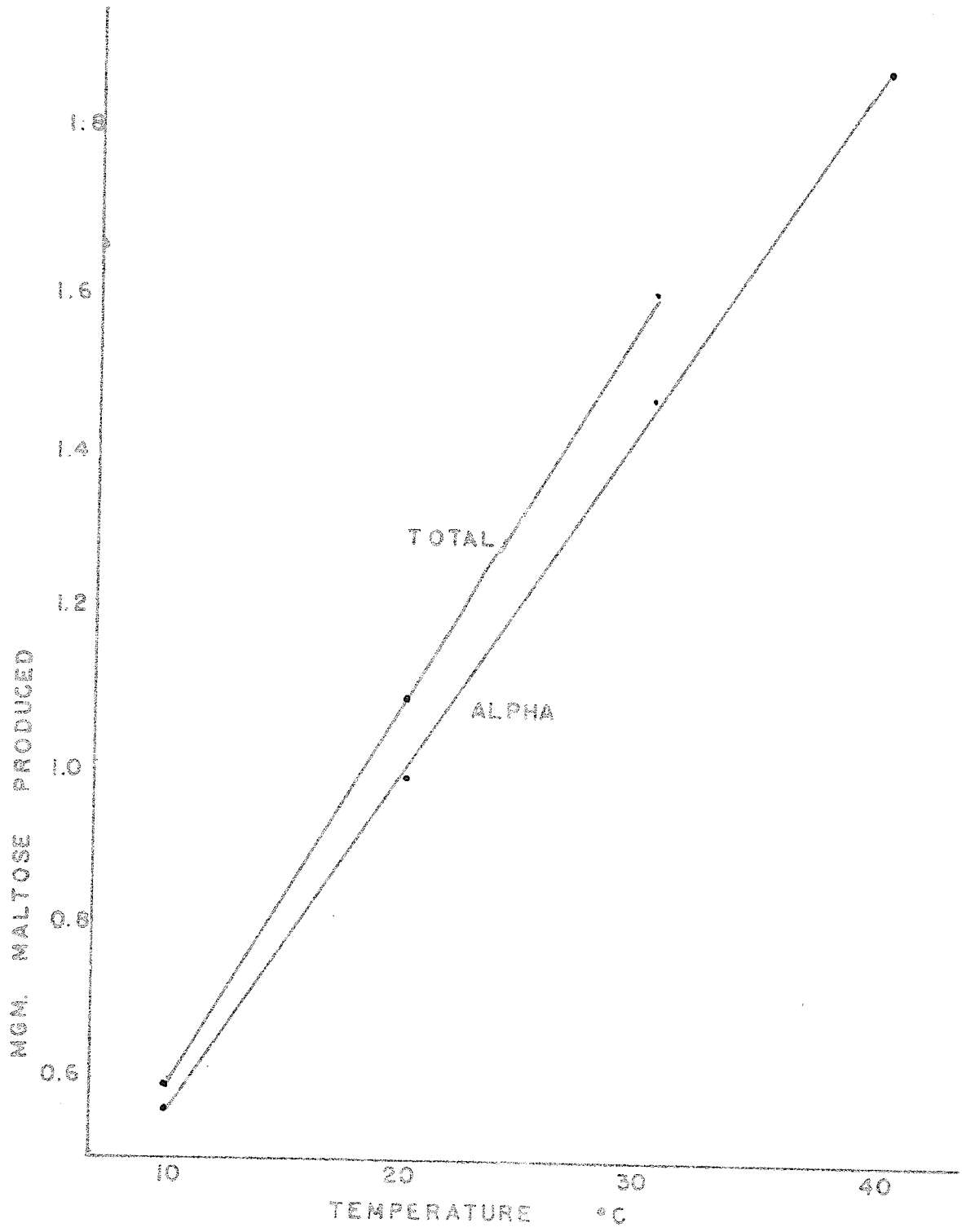


FIGURE 3 · EFFECT OF DIASTASIS TEMPERATURE



Substrates for Alpha-amylase Assay

The official method for alpha-amylase activity determination of the American Society of Brewing Chemists (5) specifies a beta-limit dextrin substrate, that is, a starch solution treated for several hours with an excess of beta-amylase. The behavior of this substrate in the modified method was investigated.

In a preliminary experiment raw starch and beta-amylase treated starch were compared in the official method. Results on a series of malt samples showed that the raw starch test took three times as long as the beta-dextrinized starch test to reach the standard iodine-dextrin color used as an end-point. This was expected, as the official method measures alpha-dextrinization, and the rate of alpha-amylase dextrinizing activity increases in the presence of additional beta-amylase. Thus when only the beta-amylase of the malt itself is present the dextrinizing rate is slower than is the case when the substrate has been treated with beta-amylase.

An attempt to use the beta-limit dextrin substrate in the modified method was unsuccessful due to the excessive amount of maltose present after hydrolysis and the consequent dark color of the DNS reaction product. The beta-amylase treated substrate contains maltose and an increase in maltose during hydrolysis with malt infusion might reflect the amount of alpha-amylase present. Further dilution of the malt infusion to the point where it ceases to be practical was equally unsuccessful. Although dilution of the DNS-maltose reaction product will produce a solution of optical density within the colorimeter range, the process, consisting of several dilutions, is cumbersome and inconvenient.

Even though these results were not unexpected, it seemed

reasonable to investigate the utility of the beta-limit dextrin substrate in the modified method. The results show that in a method that measures saccharification, raw starch is the better substrate. Ithas been shown (38) that the relation between the saccharifying and dextrinizing activities is linear and therefore the relation between the results of determinations made by the official method and the modified method should also be linear.

4. Precision of Method

(a) Reproducibility

In order to test the reproducibility of the method for the determination of maltose after diastasis, six malt samples were analyzed on each of four days. The malts were chosen to cover a range of total amylase activity and on each day extractions were made from each malt in duplicate. On each extract the diastasis reaction was also carried out in duplicate. Thus, a total of 16 maltose determinations was made on each sample of malt.

The results were tabulated and an analysis of variance carried out. The data are shown in Table 8. Statistical analysis illustrates the ability of the analytical technique to differentiate between malt samples. The reproducibility of the method between days is also shown. Using duplicate extractions as the error term, the variance ratio of days and that of the interaction of days and samples are not significant, while the sample variance ratio is highly significant. The very small variance due to duplicate determinations indicates the precision of the method. Some variation due to withdrawing whole malt samples from bulk lots for analysis is to be expected and this is shown by the significant variance ratio of duplicate extractions compared with duplicate determinations. However, there is no doubt that the method

is a reliable means of determining the amylase activity of barley and malt samples.

Table 8

Reproducibility of Analytical Method

a. Differences in mgm. maltose formed per mgm. malt

Malt sample	Total maltose (mean)	Day 1		Day 2		Day 3		Day 4	
		D	E	D	E	D	E	D	E
A	2.93	.03	.03	.03	.05	.03	.05	.02	.02
B	3.44	.02	.02	.01	.03	.04	.08	.02	.06
C	3.75	.03	.03	.03	.01	.01	.05	.03	.01
D	4.41	.03	.05	0	.04	.02	.06	.02	.07
E	4.70	.04	.04	.01	.05	.02	.06	.03	0
F	5.30	.03	.05	.03	.07	.02	.02	.01	.07

D - differences in duplicate determinations
E - differences in duplicate extractions

b. Analysis of variance

Source	DF	Sum of squares	Mean square	Stand.error
Days	3	.0032	.00107	
Malt samples	5	3.8105	.76210**	
Days x samples	15	.0072	.00048	.022
Duplicate extractions	24	.01355	.000565**	.024
Duplicate determinations	48	.000101	.000002	.0014

** Significant at 1% level

(b) Sources of Error

Extraction of Malt

It did not seem necessary to carry out a detailed investigation of the effect of extraction time on the amylase activities of malt samples, as there is already a good deal of information on this factor. The official method of the American Society of Brewing Chemists (5) requires a 2.5 hour extraction at 20° C. followed by 30 minutes filtration time. In the writer's laboratory, reduction of the extraction time to 2 hours has been found to have no significant effect on the results of amylase activity measurements. If the time is reduced to

30 minutes, saccharifying activity values of malt samples have been found to be about 4 per cent less than those of the 2.5 hour extraction.

Various extraction temperatures have been suggested, ranging from 20° to 35° C. (20,21,35). It is now generally accepted that the most satisfactory and convenient temperature for extraction of barley and malt is 20° C. The extractant solution has also been the subject of investigation (20,36,54). Distilled water alone is not as efficient an extractant of amylases from malt as are solutions of various salts. The use of 0.5 per cent sodium chloride solution has wide acceptance (5.9) and there seemed to be no reason to attempt any modification of this procedure.

In practice malt extractions were made using sodium chloride solution at 20° C. The time can be a matter of choice. If, for example, a comparison of the amylase activities of lines from a barley hybrid population is to be made, reduction of the extraction period to 1 hour, or less, will not affect any selection or screening made on the basis of the results. For inter-laboratory comparisons of analyses, the longer time of 2.5 hours is advisable.

Diastasis

The effect of variations in the time and temperature of diastasis (starch hydrolysis) was investigated in the development of the method and results of these experiments have been reported in section 3. Data taken from these results are shown in Tables 9 and 10. The effects of a deviation of 5 per cent from the specified time of 5 minutes and the temperature of 20° C. have been calculated and are included in the tables.

Formation and Stability of DNS-maltose Reaction Products

Extracts of four malt samples were prepared and diluted as described in previous experiments. A set of 12 2ml. aliquots of each

Table 9

Effect of Errors in Diastasis Time

Diastasis time (minutes)	Saccharifying activity mgm.maltose produced	Saccharifying activity equivalent deg.Lintner	Alpha-amylase activity mgm.maltose produced	Alpha-amylase activity equivalent dextrin.units
4.0	.82	95	.81	35
4.75	.93	113	.89	39
5.0	.97	120	.92	41
5.25	1.01	127	.94	42
6.0	1.12	146	1.02	47

Effect of 5 percent (15 seconds) error in time

Determination	Per cent error maltose	degrees Lintner	dextrinizing units
Sacch.activity	4.0	5.8	
Alpha-amylase	2.5		1.5

Table 10

Effect of Errors in Diastasis Temperature

Diastasis temp. °C.	Saccharifying activity mgm.maltose produced	Saccharifying activity equivalent deg.Lintner	Alpha-amylase activity mgm.maltose produced	Alpha-amylase activity equivalent dextrin.units
18	1.00	125	.90	40
19	1.05	134	.94	42
20	1.10	143	.99	45
21	1.15	151	1.03	47
22	1.20	160	1.08	50

Effect of 5 per cent (1 degree C.) in temperature

Determination	Per cent error maltose	degrees Lintner	dextrinizing units
Sacch.activity	5.0	6.3	
Alpha-amylase	4.5		2.5

diluted infusion provided, after diastasis at 20° for 5 minutes and the addition of 2 ml. DNS, triplicate samples for each of four boiling times. All determinations were repeated on a second day. After boiling for 3, 5, 7 and 9 minutes the samples were cooled quickly, diluted and the optical densities read in the spectrophotometer. The variation in optical density with boiling time is shown in Figure 4, which is a graph of the data in Table 11.

It had been necessary in the preliminary studies on the DNS-maltose reaction to examine the effect of variation in boiling time and exploratory data are reported in Table 1. At that time it was decided that a reasonable boiling time was 5 minutes and this has been used in the development of the modified method. Data from Figure 4 are tabulated in Table 12 and they indicate that a deviation of 5 per cent (15 seconds) in boiling time results in an error of 1 per cent in saccharifying and alpha-amylase activity values.

In practice the optical density readings are taken a few minutes after the boiled maltose mixture has been cooled and diluted. In section 2 it was noted that optical densities did not change on standing for several hours. This result was re-checked in the experiment on boiling time and the same conclusion was reached. While it is generally convenient to read the optical density very soon after other operations have been completed, no error would appear to be introduced if the samples are stored for some time before reading in the spectrophotometer.

FIGURE 4

EFFECT OF BOILING TIME ON OPTICAL DENSITY OF MALTOSE-DNS REACTION PRODUCT

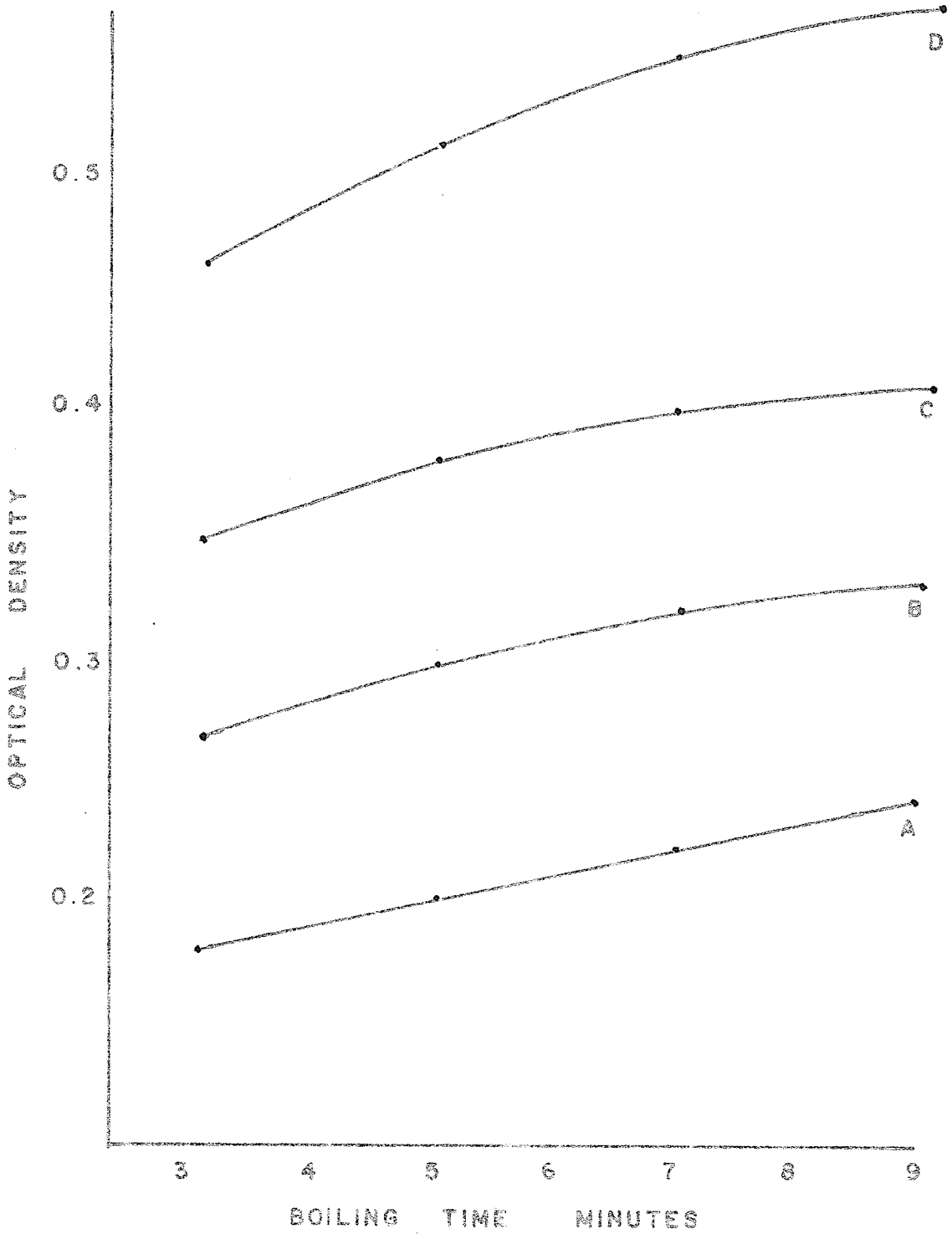


Table 11

Effect of Boiling Time on Optical Density

Boiling time (minutes)	Optical Density - Means Malt A	Malt B	Malt C	Malt D
3	.181	.261	.339	.462
5	.202	.294	.378	.509
7	.221	.313	.400	.543
9	.242	.325	.409	.558

Table 12

Effect of Errors in Boiling Time

Boiling time (minutes)		Results calculated on 5 minute boiling time mgm.maltose produced	sacch.activity	alpha-amylase activity
A	4.0	.75	83	31
	4.75	.78	88	32
	5.0	.79	89	33
	5.25	.80	91	34
	6.0	.82	95	35
B	4.0	.96	116	43
	4.75	.99	123	44
	5.0	1.00	125	45
	5.25	1.02	128	47
	6.0	1.04	132	48
C	4.0	1.30	178	63
	4.75	1.35	186	65
	5.0	1.36	188	66
	5.25	1.38	190	67
	6.0	1.41	196	69

(c) Relation between Modified Method and Official Methods

Barley and malt samples were selected from material that had analyzed by the official methods of the American Society of Brewing Chemists (5). For total amylase activity (saccharifying activity) 30 barley samples of various varieties and activities evenly distributed over a range of 110 to 290 degrees Lintner were selected. These were augmented by 10 malt samples whose activities ranged from 70 to 120

degrees Lintner. To compare alpha-amylase measurements 36 malt samples distributed over a range of 25 to 74 dextrinizing units were selected from the barley varieties grown in the 1962 Western Co-operative Barley Test at various locations.

Triplicate determinations of total and alpha-amylase activities, as appropriate, were made on the test samples by the modified method. The results were expressed in terms of mgm. maltose produced in 5 minutes per mgm. malt (total amylase) or per 2.5 mgm. malt (alpha-amylase). A mean value for each sample was calculated from the triplicate results. The correlation coefficient between the official and modified methods and the regression equation for predicting saccharifying activity in degrees Lintner from the maltose units of the modified method were calculated. The statistical data are given in Table 13a and the regression line is shown in Figure 5. The data for alpha-amylase activity were treated similarly and the results are shown in Table 13b and Figure 6.

The results show that data obtained by the modified method are significantly correlated to those obtained by the official method. Determinations of maltose produced during diastasis by total amylase activity and by alpha-amylase activity, in samples where beta-amylase has been inactivated, can be reliably converted into the conventional units used to express these activities.

Table 13

Relation between Modified and Official Methods - Statistical Data

a. Total saccharifying activity

X = mgm. maltose formed per 1 mgm. malt
Y = degrees Lintner, ASBC method

Regression equation $Y = 169.26 X - 43.06$

Standard error of estimate 6.82 [a]

Confidence intervals for estimate of Y

95%	X = 1.0	Y = 126.20 ± 2.73
	X = 1.5	Y = 210.83 ± 2.69
99%	X = 1.0	Y = 126.20 ± 3.65
	X = 1.5	Y = 210.83 ± 3.61

Correlation coefficient $r_{xy} = .99^{**}$

b. Alpha-amylase activity

X = mgm. maltose formed per 2.5 mgm. malt
Y = 20° dextrinizing units, ASBC method

Regression equation $Y = 56.11 X - 9.94$

Standard error of estimate 2.24 [b]

Confidence intervals for estimate of Y

95%	X = 1.0	Y = 46.17 ± 0.92
	X = 1.5	Y = 74.22 ± 1.62
99%	X = 1.0	Y = 46.17 ± 1.23
	X = 1.5	Y = 74.22 ± 2.19

Correlation coefficient $r_{xy} = .99^{**}$

[a] Allowing for the curvilinearity of the Lintner scale, this error is considered satisfactory, particularly as the errors are lowest in the central part of the range.

[b] This error is considered satisfactory when the sources of error in the ASBC method, arising principally from visual judgment of the end-point, are taken into account.

Note : Experimental results are given in Appendix Table 1, page 71.

FIGURE 5 TOTAL SACCHARIFYING ACTIVITY

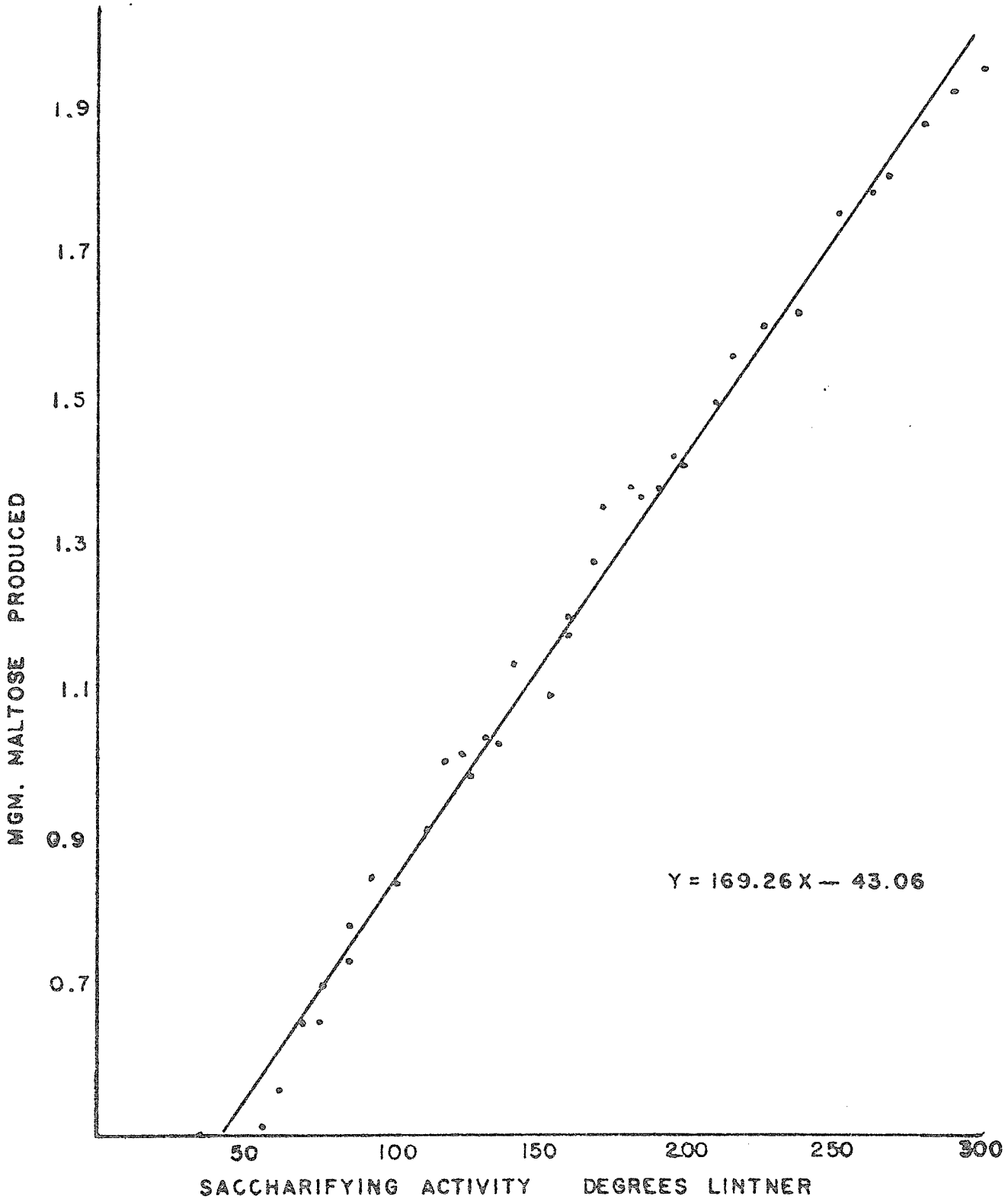
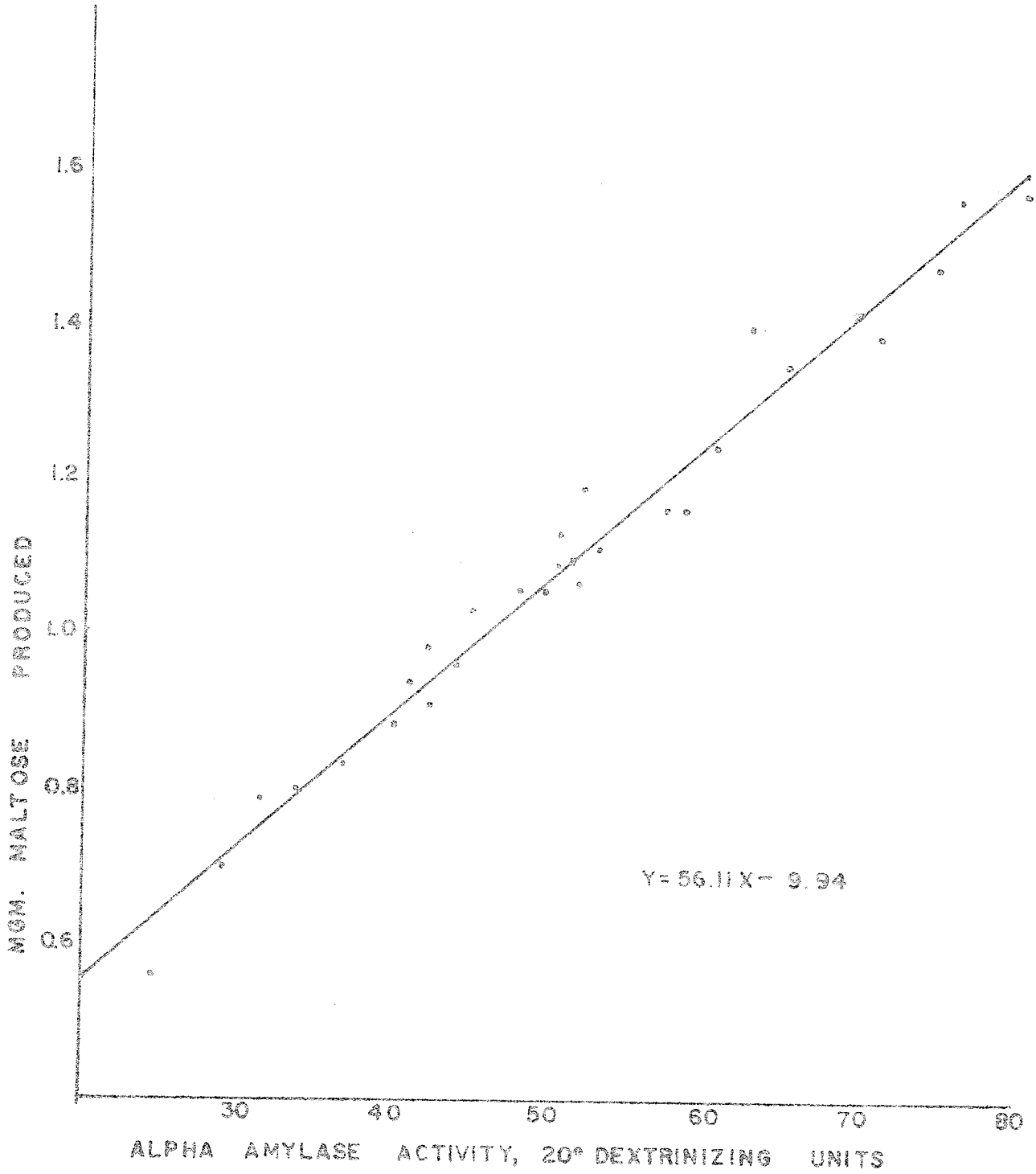


FIGURE 6 ALPHA AMYLASE ACTIVITY



5. Detailed method for routine determination of barley and malt amylase activities.

The amylase activities are determined by colormetric measurement of the amount of maltose produced by the action of extracts of the grains on a starch substrate. Saccharifying activity, that is, beta-amylase activity of barley or combined alpha- and beta-amylase activity, in the case of malt, can be conveniently and quickly measured. In the alpha-amylase determination, the beta-enzyme of malt is differentially inactivated. Maltose measurement depends on the color produced by its reaction with 3.5- dinitrosalicylic acid. Results may be expressed either in terms of the amount of maltose produced by a unit weight of malt, or they can be related to the conventional units for the expression of saccharifying and alpha-amylase activities.

The analytical procedure is in four stages:

a. Extraction of barley or malt and preparation of a suitably diluted infusion.

b. Diastasis or starch hydrolysis.

c. Maltose determination.

d. Calculation of results.

a. Extract preparation

Reagents:

i. Papain solution, 1 per cent. 10 gm. papain of high proteolytic activity are made into a paste with a little water, then washed into a 1 litre volumetric flask. The mixture is made up to the mark with water, allowed to stand for 2 hours, then filtered. The weight of papain and volume of solution should be scaled to the number of samples that are to be tested.

ii. Sodium chloride solution, 0.5 per cent.

iii Phenyl mercuric chloride (PMC), saturated aqueous solution.

Barley is ground in a Wiley mill to pass a 0.5 mm. sieve. A 2.5 gm. sample, in a 125 ml. Erlenmeyer flask, is extracted for 21 hours at 20° C with 50 ml. papain solution. The mixture, after extraction, is filtered through Whatman no. 4 filter paper, and 2 ml. of the filtrate are diluted to 200 ml. with water, in a volmetric flask.

Malt is weighed and then milled in either a Miag or a Brabender mill that has been adjusted to give a suitable grind. Reference should be made to the Methods of the American Society of Brewing Chemists regarding standardization of malt mills. Ordinarily 12.5 gm. malt are milled and extracted for 2.5 hours with 250 ml. sodium chloride solution, the mixture being shaken every 20 minutes. Where only small malt samples are available 5.0 gm. malt and 100 ml. salt solution can be used with equal accuracy. Extraction time may be shortened to 1 hour, provided that all samples in the series being compared are treated in the same way. Two dilutions of the extract are made. For total saccharifying activity, 2 ml. extract are diluted with water to 280 ml. For alpha-amylase activity, 5 ml. extract are pipetted into a 200 ml. volumetric flask and 10 ml. PMC solution added. This mixture is allowed to stand, at room temperature, for 30 minutes, then made up to 200 ml. with water.

b. Diastasis

Reagents

i Starch substrate: 5 gm. starch, dry basis, are made into a paste with a little cold water and then poured into 400 ml. boiling water. The mixture is boiled for 2 minutes and then cooled. 10 ml. buffer solution are added and the mixture made up to 500 ml.

ii Buffer solution: 41 gm. anhydrous sodium acetate and 30 ml. glacial acetic acid in 1 litre water.

iii DNS reagent: 1.0 gm purified 3,5-dinitrosalicylic acid is moistened with a little water. While stirring 20 ml. sodium hydroxide are added. The mixture is diluted with 50 ml. water and 30 gm. sodium potassium tartrate are added and dissolved. The solution is made up to 100 ml. with water and filtered through Whatman #. 1 paper. It is stored in a stoppered bottle.

In all amylase activity determinations 2 ml. of diluted infusion are used. This quantity is pipetted into a 20 x 150 mm. test tube. A series of tubes can be held in a rack in a water bath thermostatically controlled at 20° C. Diastasis is started by blowing 1 ml. of starch solution, attemperated to 20° C., into the malt infusion, mixing by shaking the tube for 5 seconds. As the starch is blown into the tube a stop watch is started. Diastasis is stopped after exactly 5 minutes by the addition of 2 ml. DNS reagent, which is also blown in and mixed thoroughly. Practice will ensure that there is no error incurred by blowing these reagents from pipettes. It is convenient to process the samples in batches of 10, adding reagents at intervals of 0.5 minutes. The first addition of starch is at time zero and the tenth at 4.5 minutes. At 5.0 minutes DNS is added to the first sample, and so on until the tenth sample receives DNS at 9.5 minutes. The pipette used for DNS is wiped with absorbent paper before adjusting and transferring its contents. Failure to observe this precaution leads to variable errors in colorimetry. Pipettes, volumetric flasks and test tubes should be cleaned with chromic acid after each day's operations. Plugging of pipette mouthpieces with cotton wool is advisable to remove the risk of contamination with salivary amylase.

c. Maltose determination

The test tubes from the diastasis reaction are transferred to a rack in a boiling water bath for exactly 5 minutes. The rack is withdrawn and placed quickly in a bath of ice water. After cooling for about 3 minutes, 20 ml. water are added to the contents of each tube, and the whole is thoroughly mixed. A suitable amount of each sample is poured into a clean, dry colorimeter cuvette for optical density determination. With each day's samples a blank is prepared in the same manner as the test samples except that 2 ml. water are substituted for the malt or barley infusion.

The colorimeter or spectrophotometer method to be used for optical density measurement must be standardized so that the optimum wavelength is known and so that optical densities can be related to the amounts of maltose present. A series of maltose solutions is prepared so that 2 ml. contain 0.4 to 2.0 mgm. anhydrous maltose. A mixture of 2 ml. maltose, 1 ml. starch and 2 ml. DNS is heated, cooled and diluted as above, and a blank is prepared. Optical densities of the samples are read at the pre-determined wavelength. Several sets of maltose samples should be used so that an accurate calibration curve can be prepared.

d. Calculation of results

The sample of diluted malt infusion for the total saccharifying activity determination contains the equivalent of 1 mgm. malt. The amount of maltose produced in diastasis by this sample ranges between 0.4 mgm. and 2.0 mgm. depending upon the barley or malt being tested. Saccharifying activity can be expressed as maltose produced per unit weight of malt.

The alpha-amylase test sample contains the equivalent of 2.5 mgm.

malt. Maltose produced is multiplied by 0.4 and then alpha-amylase activity can be expressed on the same basis as saccharifying activity. The saccharification effect due to beta-amylase is obtained by subtracting that due to alpha-amylase from the total saccharifying activity.

By using a standard curve or a regression equation, maltose produced can be converted to degrees Lintner or 20° dextrinizing units. These units are used for reporting barley and malt amylase activities when they are measured by official methods. In all cases, results are corrected for the moisture content of the barley or malt sample and are reported as dry basis.

Utility of the Method

The methods for the determination of total and alpha-amylase activity are reliable and reproducible. When applied to alpha-amylase determination, the modified method has an operational advantage by removal of the appreciable error that can arise in the standard method in the visual judgement of end-point of iodine color. The need for consecutive visual readings on the starch digest is eliminated by substitution of a single quantitive measurement. Applied to total amylase activity determination, the method eliminates lengthy starch digests and titrations. It therefore offers decided advantages in saving of time and simplicity of operation. Compared with the standard methods, the modified methods make possible at least twice as many determinations of amylase activities per man per week. In malt analysis, total and alpha-amylase activities can be made using the same extract of malt and total, alpha- and beta-amylase activities can be expressed in the same units of maltose produced per unit weight of malt.

APPLICATION OF THE MODIFIED METHOD IN PLANT BREEDING

1. Introduction

Malting quality tests are used in plant breeding primarily to select the most promising lines from hybrid populations. There are, however, studies that might be called adjuncts to plant breeding in which quality tests that can be applied to large numbers of samples are useful. Examples of such studies are: quality characteristics of parental varieties, the inheritance of quality factors in barley, relation or independence of various quality criteria, variation in field plots and tests of precision with which high quality lines are selected, the effect of cultural conditions on malting quality and the development of quality factors in the barley plant. Experiments on some of these aspects with regard to the amylase activity factor were carried out using the modified method for the determination of the activity of the two amylases.

While analysis of barley itself can provide some reliable information on probable malting behavior (14), certain properties, including alpha-amylase, can only be measured on malt. It was necessary, therefore, to consider methods of preparing malt samples. The standard laboratory malting procedure used in Canada (7) for quality evaluation of lines that are grown in more advanced field tests requires a minimum of 250 gm. of dry, graded barley. At earlier generations in plant breeding the samples available for testing are necessarily much smaller. In many of the auxiliary researches mentioned above, studies proceed more quickly and economically if quality analyses are made on small samples.

Experiments in malting 25 gm. of barley were carried out a few years ago (12). The malt was not kilned, because it was thought that kilning would introduce unknown variables in small samples. The analytical data obtained on the un-kilned or green micro-malts compared closely with those obtained on kilned malts made by the standard macro-procedure (12, 13). Green malt, however, must be analyzed immediately the germination period is over. This is frequently inconvenient. It proved possible to extend micro-malting to include kilning. With a reliable micro-malting procedure available, studies on barley and malt amylases could proceed.

2. Materials and Methods

Micro-malting

To check the efficiency of a micro-malting process that included kilning, barley samples from the 1962 Western Co-operative Barley Test, that were being given the standard malting test, were used. The micro-test consisted of steeping 20 gm. (dry basis) of barley, contained in an 8-ounce perforated polyethylene bottle, in cold water (11°C) until the moisture content reached 44 per cent. The samples were transferred to the laboratory malting germinator (7), wedged in among the routine standard samples, and germinated for 6 days at 11°C. The sprouted barleys were then placed in cylindrical containers of wire mesh, 1½ inches in diameter and 6 inches long, for kiln-drying. Kilning followed the standard schedule used for larger malt samples.

Total amylase (saccharifying) activity and alpha-amylase activity determinations were made on 36 small malt samples. The analyses were compared with those made on standard malts of the same varieties by official methods.

Plant Breeding

Experiments were carried out on three topics related to plant breeding:

(a) The distribution of alpha- and beta-amylase activities among lines in a hybrid population.

(b) The relation between barley amylase activity and malt alpha- and beta-amylase activities.

(c) Variation in amylase activity and other properties with date of harvesting.

The same material was used for experiments (a) and (b). A cross of Parkland X Olli, designated 5591, was made at the Experimental Farm, Beaverlodge, Alberta in 1959. The F_2 was space planted in the field in 1960 and 1,436 early maturing plants were retained. These were planted in the field in 1961 and 147 F_3 lines were selected. Seed from these F_3 lines were planted in Winnipeg in 1962. The plots were arranged in 9 ranges and each range included two checks of each parental variety. The progeny of these bulk F_3 lines, each of which traces back to an F_2 plant and in turn to an F_1 plant were used in the experiments.

The harvested material was dried and sufficient heads removed to provide about 75 gm. of a representative and uniform grain sample. Considerable care was taken during this operation. After threshing, the samples were stored until November 1962. For various reasons, 12 samples were discarded.

In experiment (a), micro-malts were made from 135 samples of hybrid lines and from a number of Olli and Parkland samples. The micro-malts were made from 20 gm. barley (dry basis) by the process described above. Total and alpha-amylase activities of the malt samples were

determined by the modified method and beta-amylase activity was calculated by subtraction. The results were expressed in terms of mgm. maltose produced in 5 minutes per mgm. malt.

In experiment (b), 40 of the 135 samples were selected on the basis of their total amylase activity. They were evenly distributed at various levels between maximum and minimum activity. Malt analysis data were already available. Samples of the original barley were ground and total barley amylase activity was determined on each by the modified method. Barley results were expressed in the same units as for malt.

For the third experiment, (c), plots of the barley variety Parkland were seeded on 25th May 1962. Each plot consisted of 10 rows, 12 feet long, and spaced 1 foot apart. Although 8 plots were seeded, only the 4 showing the most uniformity were selected for sampling. At each sampling, a handful of culms was harvested from each of the 4 plots, avoiding border rows and ends adjacent to pathways. The 4 samples were combined on each sampling day.

On 10th August, when sampling began, the earliest heads were in the late milk stage. There was little change in characteristics until 15th August when green color had virtually disappeared. Samples taken between 16th August and 18th August did not show kernel shrinkage on drying. By 20th August when the barley was recorded as mature, kernel hardness ranged from a stiff to a hard dough stage. The last sample was taken on 21st August.

All samples were allowed to dry and in October heads that gave a uniform representation of the sample in kernel color and size were selected. Sufficient heads were selected to yield 80 to 100 gm. of threshed grain.

The moisture content, weight per thousand kernels and total nitrogen content were determined on each day's barley sample. Duplicate micro-malts were made from 20 gm. samples of barley as described above, and these were analyzed for total and alpha-amylase activity by the modified method.

3. Results and Discussion

Micro-malting

Analytical data for amylase activities obtained on kilned micro-malts showed very close agreement with data on corresponding standard malting test samples. (Appendix Table, p. 72) It was decided that the micro technique was satisfactory for use in experiments that necessitate the production of malt from small samples of barley.

(a) Distribution of alpha- and beta-amylase among lines in a hybrid population.

The alpha-amylase activity of the 135 lines ranged from 0.33 units to 0.59 units, while beta-amylase activity ranged from 0.39 units to 0.80 units. The ranges of values were divided into classes of 0.04 units each and the distribution of the 135 lines among these classes is shown, for alpha- and beta-amylase activities, in Table 14. Data on the parental varieties are also given. The percentage distribution was calculated and is shown in Figure 7.

The symmetrical shape of the alpha-amylase distribution curve indicates that the enzyme activity might be inherited quantitatively and without dominance. This hypothesis was tested, assuming full heritability, no linkage effects and that all gene pairs are equally effective. The procedure was based upon Allard's discussion of quantitative inheritance (2,ch.8). The results were as follows:

Number of gene pairs	Chi-square	Probability
3	7.799	0.3 to 0.2
4	3.744	0.9 to 0.8
5	5.840	0.9 to 0.8
6	10.926	0.7 to 0.5

Within the range of the number of gene pairs tested, all results afforded a good fit to the hypothesis. However, four and five gene pairs have a higher probability than the others.

The shape of the beta-amylase distribution indicates single gene inheritance with no dominance, that is, a 1:2:1 ratio. This hypothesis was tested, using classes 2 to 5 (32 lines), 6 to 8 (62 lines) and 9 to 12 (41 lines), and again assuming full heritability. The chi-square value was 2.111, corresponding to a probability of 0.5 to 0.3, indicating no significant deviation from a 1:2:1 ratio.

An attempt was made to estimate the heritability by comparing the variance within families with the total variance of the population (2, ch. 9). This worked out to be 76 per cent for alpha-amylase and 83 per cent for beta-amylase. The shape of the beta-amylase distribution curve indicates that a high heritability value might be expected. However, the calculated heritability values cannot be rated as very reliable, as the families were very small.

(b) Relation between beta-amylase activity of barley and the alpha- and beta-amylases of malt.

The amylase activities of malt samples and their relation to barley amylase (saccharifying) activity are shown in Figure 8. Correlation coefficients between barley and malt and within malt properties were calculated and are given in Table 15. The data show that, within this particular cross, barley amylase activity is closely related to the total amylase activity of the malted sample. This is in agreement with results reported on other material (37,48) and is, in fact, the basis of a method for predicting malt saccharifying activity without actually

Table 14

Distribution of Alpha- and Beta-Amylase Activities in a Hybrid Barley Population from the cross Olli x Parkland

Amylase activities expressed in mgm. maltose produced per mgm. malt.

a. Parents

	Alpha	Beta
Parkland (mean of 3 samples)	.33	.47
Olli (mean of 15 samples)	.57	.70

b. Population

Class		Class centre	Distribution Alpha-amylase No.	Alpha-amylase per cent	Beta-amylase No.	Beta-amylase per cent
1	.33-.36	.345	4	2.96		
2	.37-.40	.385	19	14.07	2	1.48
3	.41-.44	.425	29	21.48	6	4.44
4	.45-.48	.465	35	25.92	14	10.37
5	.49-.52	.505	29	21.48	10	7.41
6	.53-.56	.545	16	11.85	16	11.85
7	.57-.60	.585	3	2.22	30	22.22
8	.61-.64	.625			16	11.85
9	.65-.68	.665			12	8.89
10	.69-.72	.705			16	11.85
11	.73-.76	.745			8	5.93
12	.77-.80	.785			5	3.70

malting the barley sample (14,37). The high correlation between barley amylase and malt beta-amylase may be expected, as barley amylase activity is in fact a beta-amylase activity. The relation between barley amylase and malt alpha-amylase, while significant, is less close. Among the malt properties, beta-amylase is more closely related to total(saccharifying) activity than is alpha-amylase.

FIGURE 7 DISTRIBUTION OF OLLI X PARKLAND LINES

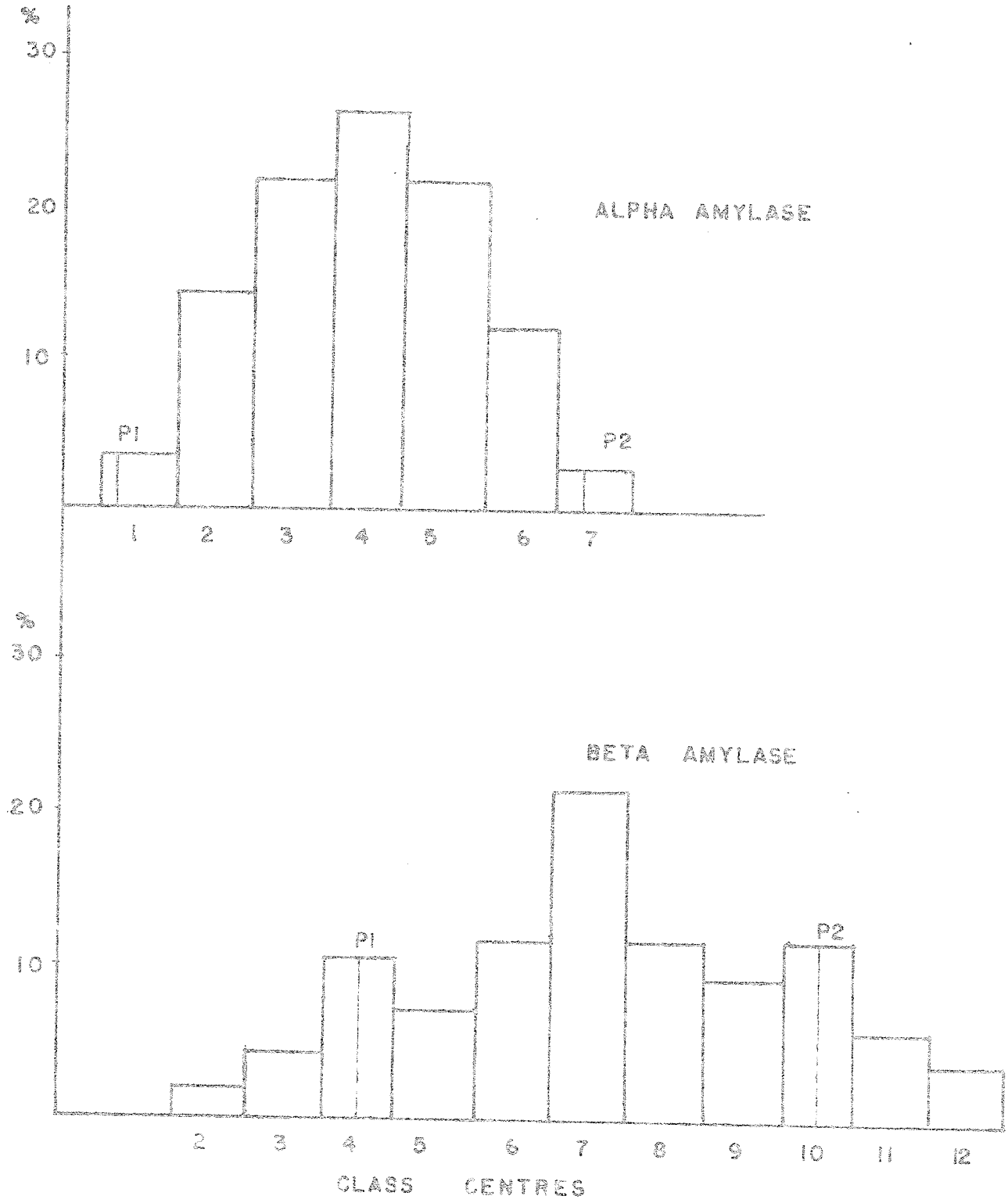


Table 15

Relations between Amylase Activities of Barley and Malt

a. Correlation

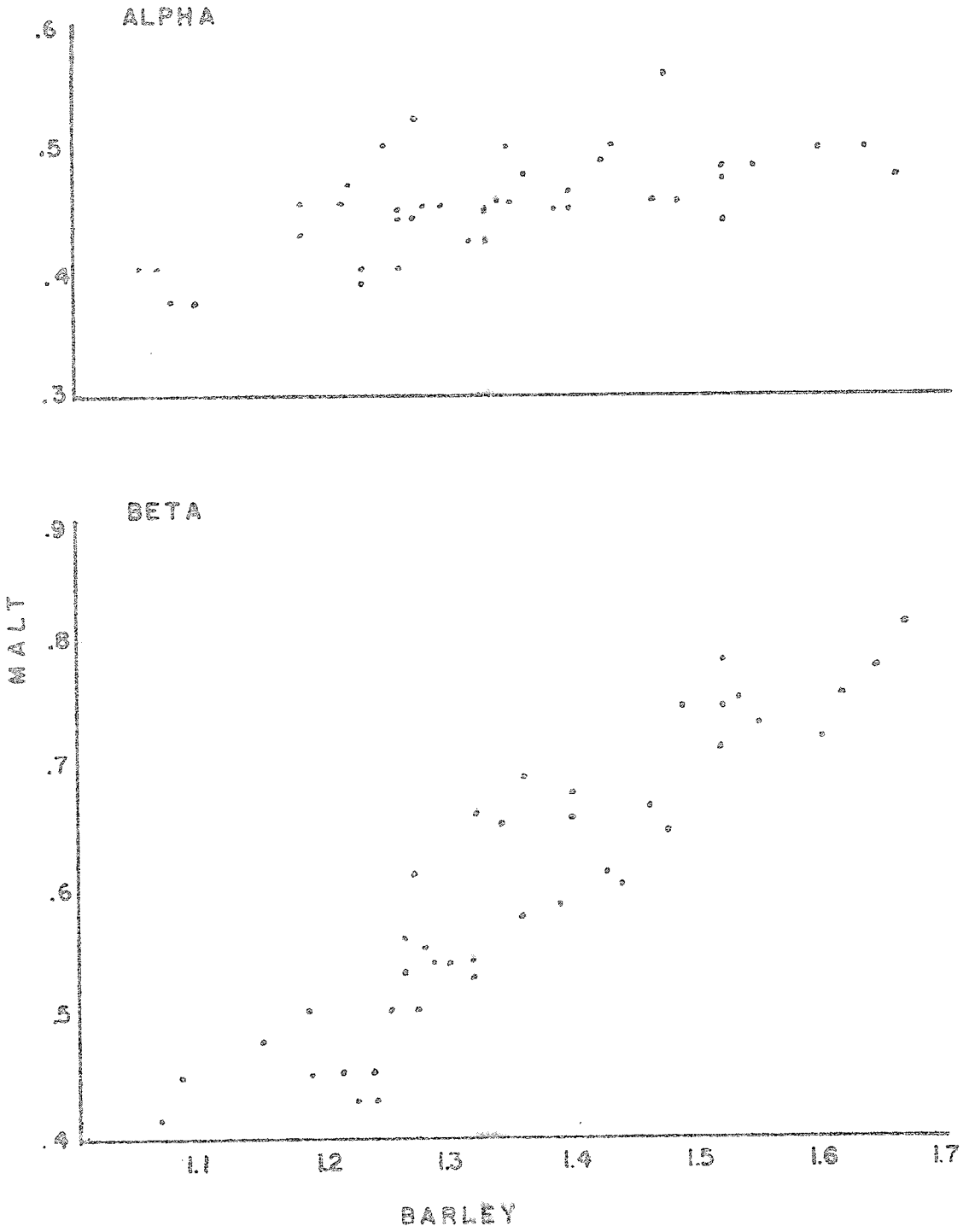
Properties	r	r^2
Barley and total malt	.949	.900
Barley and malt beta	.942	.887
Barley and malt alpha	.689	.475
Total malt and malt beta	.976	.953
Total malt and malt alpha	.772	.596

b. Differences between correlation coefficients

Properties	t
Barley and malt beta vs. barley and malt alpha	4.065
Total malt and malt alpha vs. total malt and malt beta	5.278

The correlation coefficients and differences are significant at the 1% point.

FIGURE 8 RELATION BETWEEN BARLEY AMYLASE AND MALT AMYLASES



(c) Variation in amylase activity and other properties with date of harvesting.

The results of barley and malt analyses made on the samples taken between 10th August and 21st August are given in Table 16 and the variation in properties is shown in Figures 9 and 10. The weight per thousand kernels (dry basis) increased during the first days in which the samples were taken, levelling off by 16th August. Nitrogen content decreased to a minimum around 17th August, after which it increased, slowly at first, then rapidly, showing a tendency to become constant when the last samples were taken.

Total malt amylase (saccharifying) activity followed a pattern closely similar to that of the barley nitrogen. This is not unexpected in view of the well-known relation between these two properties. The beta- and alpha-amylase components also reached minimum values by 18th August and then increased. The change, however, was much less marked in alpha-amylase than in beta-amylase in the last five samples. The ratio of beta- to alpha-amylase activity in the malt samples is interesting in that it shows a steadily increasing trend, independent of other properties measured. This may indicate that the precursors of alpha-amylase are laid down by the plant before the development of beta-amylase activity is completed.

Table 16

Effect of Harvest Date on Barley and Malt Properties

Date Aug. 1962	Sample	Barley 1000 K.Wt. gm.	Barley Nitrogen %	Malt Sacch. activity	Malt Alpha-activity	Malt Beta-activity	Malt Beta/Alpha ratio
10	1	26.8	2.13	1.02	.47	.55	1.17
13	2	30.2	2.09	.97	.42	.55	1.30
14	3	30.0	2.01	.93	.39	.54	1.38
15	4	33.2	1.98	.88	.36	.52	1.44
16	5	34.8	1.92	.82	.34	.48	1.41
17	6	35.0	1.88	.77	.32	.45	1.41
18	7	35.0	1.89	.72	.30	.42	1.40
19	8	35.4	1.92	.74	.30	.44	1.47
20	9	33.4	2.07	.81	.31	.50	1.60
21	10	35.8	2.09	.83	.32	.51	1.59

Amylase activities expressed in mgm. maltose produced per mgm. malt

FIGURE 9 HARVEST DATE AND AMYLASE ACTIVITIES

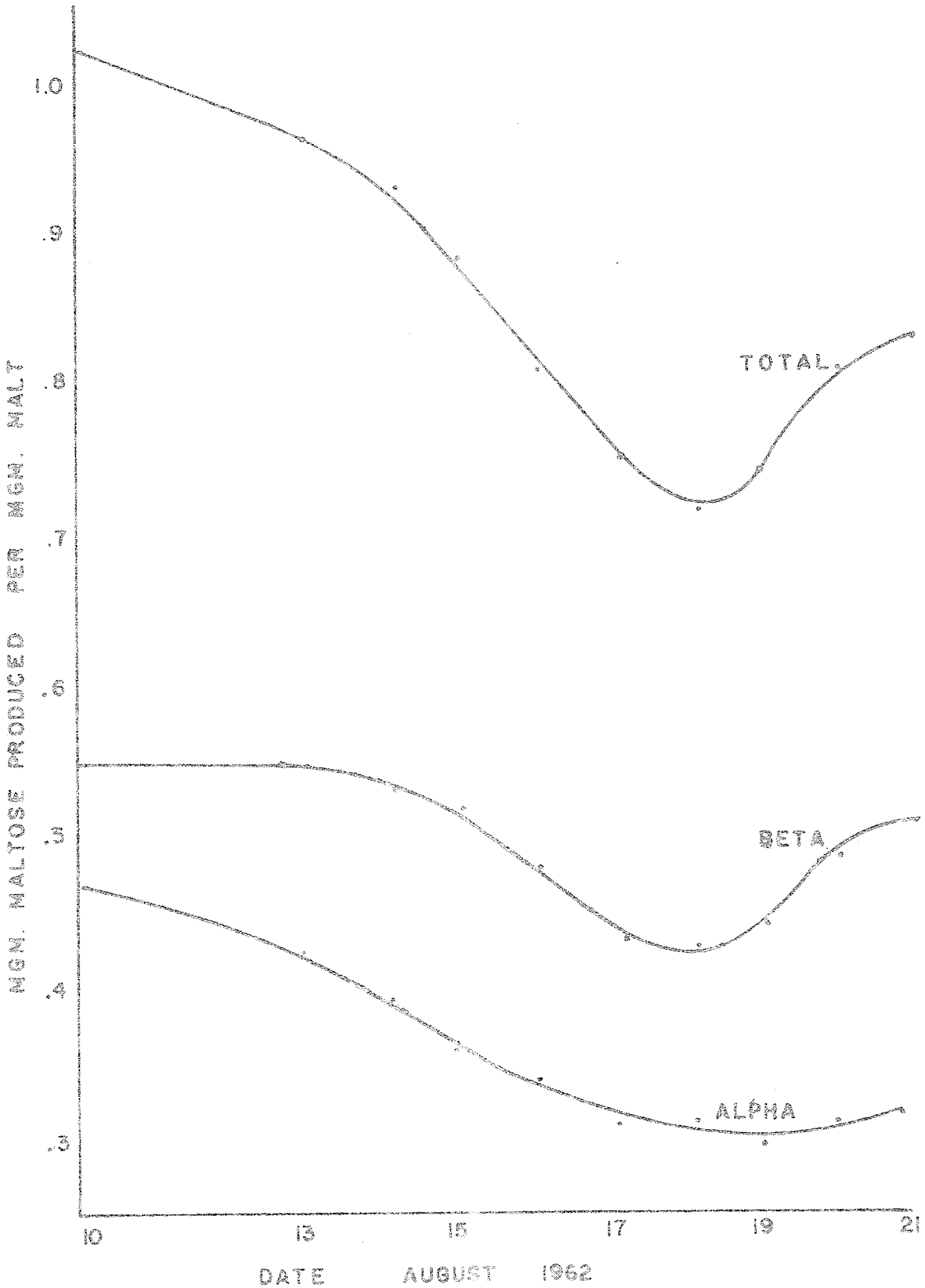
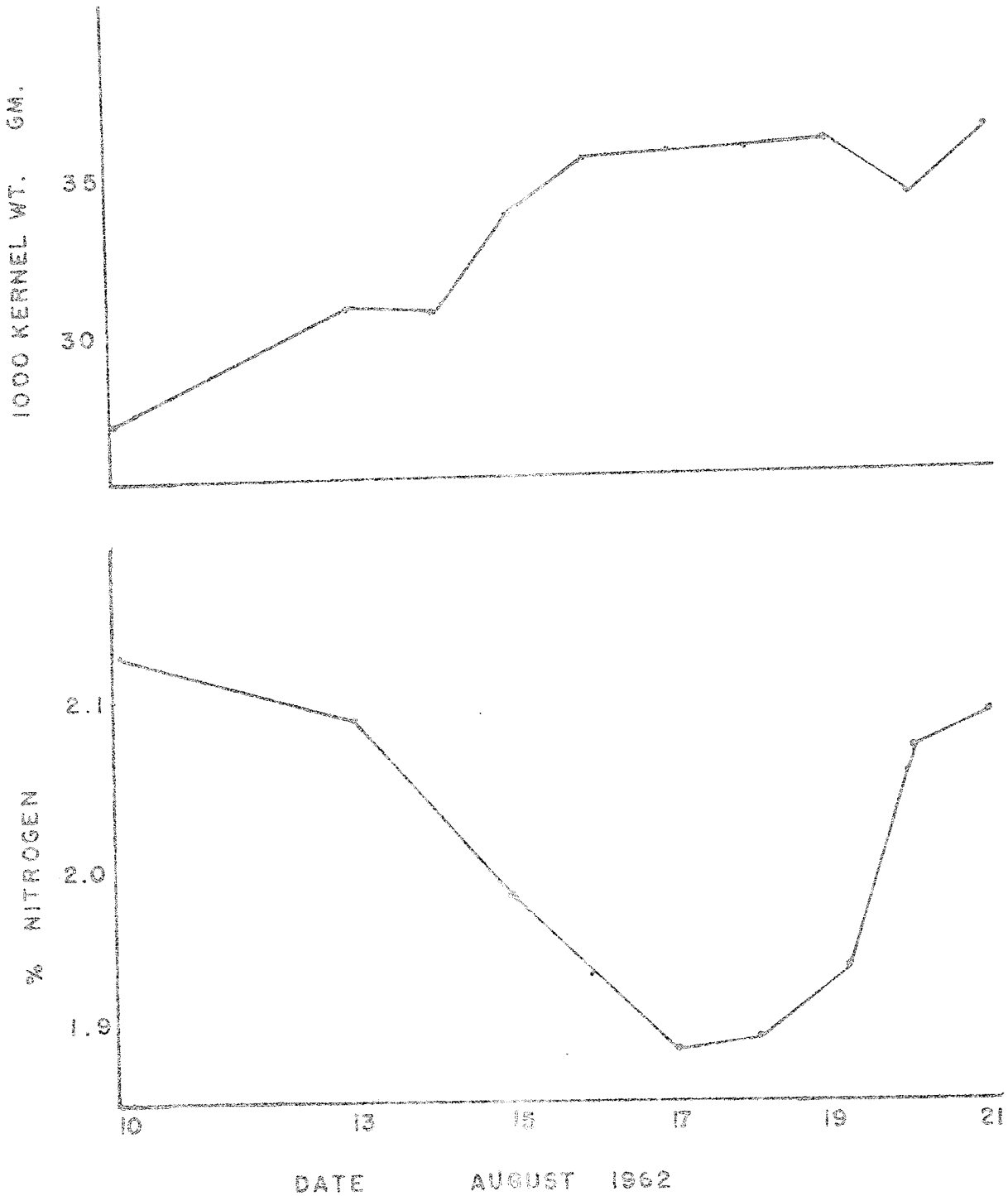


FIGURE 10 HARVEST DATE AND BARLEY PROPERTIES



4. General Discussion

The experiment on the distribution of amylase activities in a hybrid population was exploratory in nature. It dealt with a relatively small population from one cross only. Certain tentative conclusions are, however, possible. As has been suspected from other studies on the two enzymes, it would appear that the inheritance of alpha-amylase is independent of that of beta-amylase. Alpha-amylase activity seems to be the result of the quantitative action of several gene pairs, possibly four or five, and beta-amylase appears to depend on single gene action, without dominance. The heritability of both enzymes could be quite high. This study was made on lines selected for earliness of maturity resembling that of the Olli parent. The distribution of amylase activities between the values for the two parents indicates lack of association between amylase activity and the factors that contribute to earliness in barley.

The results of the study of the relation between barley and malt amylases indicate some degree of independence between barley amylase and the alpha-activity of malt. It would appear that malt alpha-amylase activity cannot be reliably predicted by the barley amylase measurement, and that alpha-amylase activity, while essential for malt saccharifying activity, is not necessarily reflected by the total amylase determination in malt. The results of this experiment support the suggestion that barley analyses alone are not completely adequate for the prediction of malting quality in a hybrid population. Malting, even on a small scale, is necessary to provide reliable information on quality criteria such as alpha-amylase activity. Further, in the analysis of malt itself, the separate determination of alpha-amylase is a useful additional test of the suitability of the barley for malting purposes, especially when the

malt is to be used in conjunction with unmalted grains. The experiments also suggest independence in the inheritance of the two malt amylases.

The data in this study are representative of one cross only. Similar correlations may not exist when other parental varieties are used. Some anomalies in the relation between barley and malt saccharifying activities have been reported (14,37) and others have been found by the writer in the examination of numerous parental varieties used by barley breeders as sources of factors such as disease resistance. This strengthens the case for using malt rather than barley analyses in the screening of hybrid populations, not only for the assay of alpha-amylase, but for total amylase activity as well. However, where anomalous relations are believed to exist, the method used in this experiment could be applied to parental varieties, singly and in crosses. The barley saccharifying activity test has been useful in screening hybrid material (14), but it requires cautious application, and a thorough understanding of the amylase characteristics of the barley varieties being used as parents by the plant breeder.

The results of the harvest date experiment, while not conclusive, indicate that harvest date can have a critical effect on the amylase activity of barley that is intended for malting. At a time when kernel weight, appearance and other readily observable criteria appear to have reached stability, significant changes may still be taking place in enzyme activity. This experiment was terminated before enzyme properties could be said to be stable, though the last sample indicated that they were reaching constancy. It is estimated that though the barley was visually judged to be mature on 20th August, biochemical maturity as measured by amylase activity may have required an additional two days of growth.

These three experiments are examples of the value of a new method for amylase determination that is less time-consuming. The necessary analytical work was accomplished in a relatively short time. Although malt is a prerequisite to alpha-amylase measurement, the malting of small barley samples did not present any difficulty. In this connection, it should be mentioned that while existing laboratory malting equipment was used in this study, an investigation, not yet reported, showed that barley can be satisfactorily made into green malt in simple apparatus housed in an ordinary domestic refrigerator. It should not be difficult to adapt apparatus found in any laboratory to the production of small malt samples. Malt analysis provides more information on quality factors than can be obtained from tests on unmalted barley, at least at the present time.

In early generations of a breeding program, the size of barley samples is small of necessity. In other studies sample size is not necessarily limited, but if the required data can be obtained on small samples by reasonably rapid techniques, the result is a saving in labor as well as space. The saving may be treated simply as an economy; alternatively it may be used to extend projects or to initiate additional investigations.

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Appendix Table 1

Relations between Modified and Official Methods - Analytical Data

Saccharifying Activity		Alpha-amylase Activity	
mgm.maltose per mgm.malt	degrees Lintner	mgm.maltose per 2.5mgm.malt	20° D.U.
.50	54	.54	23.4
.65	75	.79	32.0
.85	91	.85	35.6
.78	85	.66	28.2
.74	85	.80	33.6
.56	62	.93	40.0
.70	75	.91	42.7
.84	100	.98	42.7
.91	110	1.02	44.7
1.01	121	.93	41.7
1.01	119	1.05	49.2
.99	126	1.05	48.0
1.04	132	.94	43.6
1.03	136	1.12	50.5
1.14	138	1.18	53.5
1.10	153	1.05	51.9
1.18	159	1.10	54.8
1.28	168	1.09	51.9
1.36	172	1.08	50.5
1.38	181	1.40	64.0
1.37	185	1.22	60.0
1.38	190	1.33	66.2
1.42	194	1.57	76.2
1.41	200	1.39	71.1
1.46	206	1.58	80.0
1.52	210	1.48	74.0
1.56	215	1.16	57.2
1.60	228	1.16	58.1
1.62	235		
1.70	245		
1.76	253		
1.78	261		
1.81	267		
1.88	279		
1.92	289		
1.95	300		

Appendix Table 2

Relations between Micro-malts and Standard Laboratory Malts

Sample no.	Soluble nitrogen %		Saccharifying activity °L.		Alpha-amylase activity 20°D.U.	
	micro	standard	micro	standard	micro	standard
1	.86	.72	154	148	34.3	33.8
2	.96	.80	150	157	52.7	52.2
3	.87	.73	132	133	68.6	67.6
4	.80	.64	153	156	50.5	51.0
5	.85	.66	140	145	47.0	45.1
6	1.04	.83	196	204	47.0	48.5
7	.64	.50	97	99	24.0	24.4
8	.73	.56	140	146	27.6	27.6
9	.89	.72	159	170	42.9	43.2
10	.78	.64	127	132	32.7	32.7
11	.87	.71	161	165	41.2	40.5
12	.99	.81	178	184	42.9	43.0
13	.93	.75	172	175	75.2	73.5
14	.90	.70	136	137	33.8	34.9
15	1.12	.89	208	217	52.6	53.8
16	.76	.65	177	178	40.2	38.9
17	1.03	.80	169	173	48.5	48.5
18	.84	.67	172	179	59.0	58.6
19	.87	.68	185	188	44.2	44.2
20	.80	.65	120	125	52.6	53.6

Note : soluble nitrogen determined on cold extract of micro-malts and Congress extract of standard malts.