EFFECT OF MOISTURE LEVELS ON THE OXIDATION OF UNSATURATED FATS

by

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T H E S I S

submitted to the

UNIVERSITY OF GLASGOW

in accordance with the regulations

governing the award of

the DEGREE of

MASTER of SCIENCE

in the Faculty of Science

Biochemistry Laboratory,

Department of Applied Microbiology and Biology,

University of Strathclyde

(formerly The Royal College of Science & Technology)

GLASGOW

JUNE 1965

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Ann Arbor, MI 48106 – 1346

C O N T E N T S

ACKNOWLEDGMENTS

The author wishes to take this opportunity to express her sincere thanks to Dr.J.A.Blain,B.Sc.,Ph.D.,A.R.I.C. for his valuable advice,suggestions and help during the course of these studies.

Thanks are also due to Dr.J.Patterson,B.Sc.,Ph.D. for his co-operation.

To them and to others who have helped in the preparation of this thesis the author extends her heartiest thanks.

ABSTRACT

This thesis contains an account of a general study carried out partly in collaboration with Dr. J. A. Blain at the University of Strathclyde during the years 1963 to 1965. The subject of investigation is the effect of moisture levels on the catalysed oxidation of unsaturated fats in meals and in model systems using a starch matrix.

Initially the effect of moisture levels and other factors on lipoxidase catalysed oxidation of linoleate and soya bean oil is described. In most of these studies soya beans were used as a source for lipoxidase, although other sources were examined.

The effect of moisture levels on haematin compounds catalysed fat oxidation was investigated. In most studies haemoglobin was used as a catalyst although other haematin compounds have been examined. In addition the effect of moisture level on the action of the enzyme lipoxidase has been compared with results for the haemoglobin.

In this study, model systems were used, since they provided for closer control of the variables than would be possible with normal foodstuffs.

Fresh foods are known to contain natural substances

which protect them from rapid deterioration, the antioxidants. There are also substances likely to promote oxidative deterioration, among the haematin compounds and lipoxidase, the importance of which, in influencing stability of food fats has been the subject of much speculation.

The results of these studies indicate the possible implication of haematins in the oxidation deterioration of lipids in foodstuffs even when these are very dry.

CHAPTER I.

INTRODUCTION AND LITERATURE SURVEY

THE STATE OF WATER IN BIOLOGICAL MATERIALS

During the last fifty years the attentions of the biologist, physiologist, and biochemist have been directed towards the solutions of problems which involve a knowledge of the state of water in living structures, and it has become evident that the water in living tissues and inanimate colloidal systems does not exist wholly as free water, but that it is in part intimately bound to the organic structures.[1] In studying the important role of water in different natural materials and its influence on their mechanical, physical, chemical, and enzymic behaviour, it has been found necessary to distinguish between the different forms of water-binding to hydrophilic macromolecules.

Many phenomena observed in living systems have indicated that not all the water present can function in the capacities which would be expected of ordinary water. For example, a part of the water present appears to be unable to act as a solvent or medium in which reactions can take place. Seeds containing as much as 50 % of their weight as removable water will remain dormant, but if the water content is increased by just half again as much, the seeds start to germinate and respiration will increase many times. Certain fungus spores will remain dormant when placed on substrate containing water sufficient to give relative vapour pressure of 75 % but will grow if the relative

vapour pressure is raised to 80 %.[2] If added water contains electrolyte or sugar in sufficient concentration to retain the relative vapour pressure at 75 % , this addition of water in amount still will not serve to allow the mold to start growing.

Such observations have led to the idea that water in an organism (or any colloid system) may be partially or entirely bound water, with the obvious connotation that it is not free to act as water is required to act in order to promote those processes which are dependent on the presence of free water.

The concept of bound and free water has been applied in attempts to supply a basis of explanation for such physiological processes as swelling of animal tissues as in edema, the drought- and frost-hardiness in plants, and for the imbibition process in colloidal systems generally.[3,4,5,6,7,8]

Water binding in foodstuffs

The conception of bound water is of special importance in food processing where water is removed by evaporation, dehydration or other methods. Irreversible changes may occur in the products when bound water is partially removed.

Food -especially those having structure- are not at all uniform in their microscopic dimensions and there is therefore no regular distribution of water particles in them and different types of water binding will always be found in one and the same

product. The different groups of substances could be distributed in the water and more or less bound to it in the following ways:

(a) The water serves as media for molecular dispersion of soluble substances like sodium chloride or sugar. Such dispersion normally gives the most uniform distribution of the solute, provided its diffusion is not influenced by a semipermeable membrane or other means and provided the solution is not saturated.

(b) The water forms a colloidal solution by diluting hydrophilic macromolecules such as proteins.

Depending on the water content, two different forms of structure are possible, sol and gel, which may be changed from one to the other reversibly. The solubility of colloids and therefore their water binding capacity depends on pH and is minimum at the isoelectric point.

(c) The water forms an emulsion with substances of low solubility, thus giving a coarse dispersion.

<u>The nature of bound water and methods whereby it can be estimated</u>

The concept of bound water as a physiological factor apparently arose independently with three groups of investigators. Balcar, Sansum, and Woodyatt,[9] in 1919, were the first persons to suggest a bound water $\rightleftharpoons$ free water equilibrium, but they were unable to devise a technique which would differentiate these

forms of water. Unaware of their suggestion Newton and Gortner[10] in 1922, proposed that the bound water be determined by cryoscopic technique, in the presence of a (theoretical) molar solution of sucrose. They were interested in the relation between the hardiness of winter wheat and the moisture content, and they suggested that in the hardening-off process a portion of the water became intimately associated with the hydrophilic colloids so that it was essentially removed from the liquid state and became to all intents and purposes a part of the solid phase. They devised a technique whereby they could test the bound water theory as a determining factor in the phenomena of winter hardiness. Rubner and co-workers[11] arrived at essentially the same view point with respect to the ability of protoplasmic colloids to bind water and remove it from the liquid state.

Bound water has been estimated by a variety of methods for over forty years without universal acceptance of a precise definition. Probably the most acceptable is that of Kuprianoff[8] who considers bound water as that which remains unfrozen even if the sample is submitted to very low temperature down to -50 or -70°C. and which can only be expelled by heating normally to about 105°C.[8]

Many methods have been used for its estimation, among

which are:

(1) Freezing methods

The amount of 'bound water' is considered to be equal to unfrozen water; the free water or frozen out water is determined calorimetrically from the difference of enthalpy[11,12,13] or dilatometrically from the change in volume of sample taking place due to freezing out water.[14]

(2) Solvent methods

For estimating the 'bound water' in liquid the depression of its freezing point or the depression of its vapour pressure after adding a solution of known solute concentration can be used. The depression of the freezing point is greater if more of the water in the tested liquid is bound. 'Free water' in a food will be determined as such, which gives normal vapour pressure depression when a known substance is added to this food.[8]

(3) Chemical methods

Cobalt chloride hexahydrate has a pink colour which turns blue when it loses water and becomes pure $Co\ Cl_2$. By adding the hexahydrate to a sample of food and drying it at 25^0 to 30^0C. it is possible to check when the colour turns blue showing that free water is no further present. The amount of bound water could then be determined by weighing.[8]

Copper sulphate and other substances could be used in a similar way.

(4) Many other different methods have been proposed for measurement of 'bound water' , such as the specific heat method,[15] direct pressure method,[16] drying method,[17,18] the vapour pressure method,[19,20,21] refractometric method,[22,23] polarimetric method,[24] dielectric constant method[25,26] ..... etc. but they have not often been applied.

THE EFFECT OF VARIATION IN MOISTURE CONTENT ON THE STORAGE DETERIORATION RATE IN FOODSTUFFS

In food processing and storage many substances undergo changes; for example, meat goes 'bad', milk turns sour, .. etc. and many oils such as linseed oil thicken and ultimately solidify; changes that occur can be chemical, microbiological, or biochemical. The storage life is limited by these changes.

It has been long known that dried foods can be kept at ordinary temperature without risk of bacterial deterioration. According to Scott[27] such spoilage is delayed and fewer types of organisms develope in at a relative humidity of 75 % . At 70 % spoilage is greatly delayed and did not always result even during prolonged storage. At relative humidity of 65 % very few organisms are known to grow and spoilage would be most unlikely to occur in less than one to two years.

Even in foodstuffs strongly protected by low moisture content against the development of organisms, experience shows that enzymic reactions can occur. Among the other possible effects are those that modify smell, taste, and texture.

Generally it is not simple to decide which kind of reaction leads to spoilage in such dry foods. It can be often shown that enzymes are still present in the dry product and one might expect participation of enzymes in the reactions, but the

multiplicity of the possible and overlapping reactions make many investigations difficult.

It has been shown that increasing surface, and hence better access to oxygen, may play a role too. For example, it has been found that grinding tissues is often an important factor in such enzymatic reactions, because it brings enzyme and substrate into a close contact. Thus a cereal that can be stored for years at a suitable moisture content, will, in a rough-ground state, spoil in few weeks at the same moisture content.

Sorption isotherms

To predict the storage life of low-moisture foods it is necessary to run extended storage experiments unless sufficient experience is already available. Methods that permit the advance determination of the most favourable moisture range for storage are valuable.

One could, for certain enzymes, say that it is best to have moisture as low as possible, but the moisture content of many foods cannot always be lowered without damage, since oxidation of lipids is to be feared at low moisture contents. It should be however be possible to find the moisture level at which these spoilage reactions are minimal.

Empirical studies have been carried out on different food items to determine the optimum moisture contents for long-term

stability. These studies led to an investigation of the moisture-vapour pressure relationships in many different materials. The functional connection between relative humidities and moisture content is expressed as the sorption isotherm.

Sorption isotherms serve a number of useful functions. They may be employed: to define limits for the dehydration of fruits, vegetables, nuts, ... etc.,[28] to estimate moisture content changes under any given temperature,[29] to evaluate processing variables and to distinguish differences between cereal grades,[30] and to define moisture or humidity conditions under which product deterioration[31,32,33] and microbial growth[34,35,36] can be inhibited.

Hygroscopic equilibrium data are difficult to obtain experimentally at extreme humidities because of the limitations of available hygrometers or because of the rapid deterioration and development of molds at high humidities. Several equations have been derived on empirical or theoretical bases for the description of sorption phenomena and tested against observed data.[37-45]

The sorption isotherm can be easily obtained by allowing a food in a suitable container to establish equilibrium with saturated solutions of salts which give different relative humidities.[27] When equilibrium has been established the moisture

content is measured and plotted against the relative humidities. Figure 1 shows an idealized isotherm. The slopes and points of inflection in this figure are indicative of the water-binding capacity or of the relative amounts of free and bound water. According to Rockland[46] and Mekower,[28] a low moisture food would be protected against fat oxidation if its moisture content, or the equilibrium relative humidities, lies below the inflection point of the sorption isotherm. The inflection point below which a low-moisture food could be protected, is indicated in the figure by the symbol (a_i).

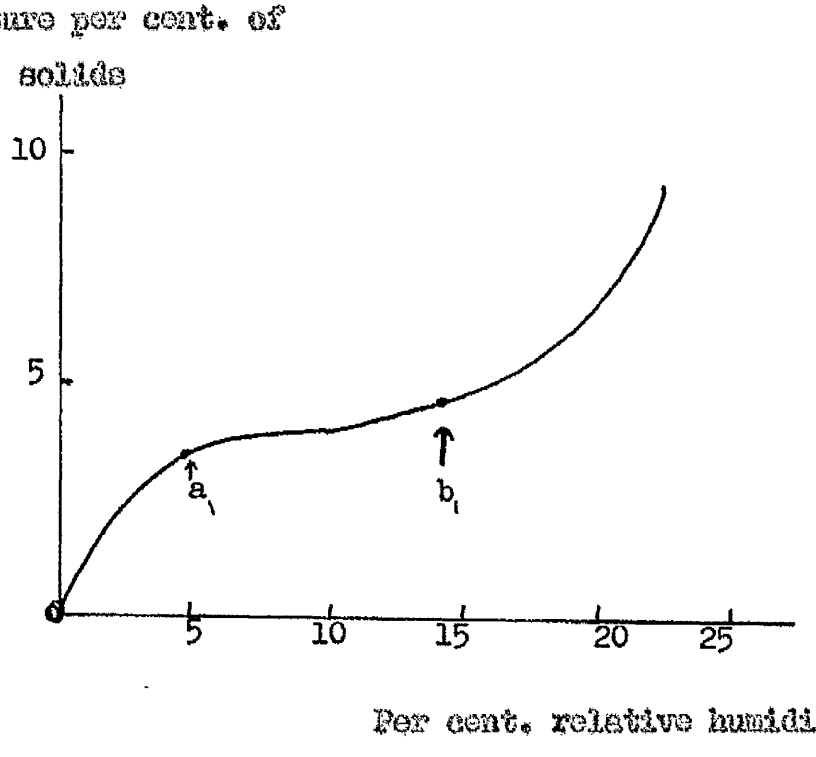


Figure 1. Idealized moisture sorption isotherm.

The monolayer value (explained in the following section) is indicated by the symbol a_1. Water content below this value is called bound water. Between a_1 and b_1 is designated dissolved water and above b_1, free water.

The adsorption theory of Brunauer, Emmett and Teller (B.E.T.)

The B.E.T. equation has frequently been applied to food materials. A few examples are its application to proteins by Shaw[47], Bull,[40] Dunford and Morrison,[48] and Mellon and co-workers;[49] to dehydrated eggs by Makower;[50] to rice by Hogan and Koran;[51] and to wheat by Becker and Sallans.[52] None of these applications, however, was for establishing moisture levels for optimum stability.

The B.E.T. equation for moisture sorption isotherms is:

$$\frac{p}{a\,(p_o - p)} = \frac{1}{a_1 c} + \frac{c-1}{a_1 c} \cdot \frac{p}{p_o}$$

in which

a = g of water per 100 g dry solids at moisture-vapour pressure p

p_o = vapour pressure of pure water at the same temperature

c = a constant related to the heat of adsorption

a_1 = g of water equivalent to a monomolecular layer adsorbed on 100 g of dry solids

A plot of $\frac{p}{a}(p_o - p)$ against $\frac{p}{p_o}$ is a straight line with y-axis intercept equal to $\frac{1}{a_1 c}$ and slope equal to $\frac{(c-1)}{a_1 c}$

The amount of water which represents a monomolecular layer according to the B.E.T. theory may be regarded as a protective film which protect the particles of food from attack by oxygen. There is evidence in the literature that oxidation and rancidification are aggravated by drying to very low moisture levels. This may lead to pigment instability and loss of vitamins and sometimes initiates nonenzymatic browning reactions.[53] The statistical monomolecular layer may not in fact represent a continuous film but rather corresponds to the number of available reactive adsorption sites in the protein, carbohydrate and fat components of the foods. Water attached to these sites, probably by hydrogen bonding, should protect them from reaction with oxygen. In other terms, the relative humidity or moisture vapour pressure at this point represents a partial pressure of water vapour which is competitive with the oxygen partial pressure to the extent of being protective. The adsorbed water might also inhibit interactions between adjacent polar groups, thereby preserving their hydrophilic properties, and facilitating rehydration. On the other hand, moisture in excess of the monolayer value represents free water, which may promote hydrolysis, caking, and other defects.[54]

Oxygen adsorption at low moisture levels

It has been indicated that the monolayer amount of water does, in fact, protect against adsorption of atmospheric oxygen. Molecular oxygen adsorbed by potato starch and by dehydrated beef was measured with the polarograph. At moisture content above the monolayer value, the amount of oxygen adsorbed was very low and was not affected by changes in moisture content. However, as the moisture was reduced below the calculated monolayer value, the amount of adsorbed oxygen increased sharply. The adsorbed oxygen could not be displaced by allowing the food to readsorb moisture from an atmosphere of higher humidity. In other words, moisture prevented the adsorption of oxygen, but oxygen already adsorbed was not displaced by moisture.[55] Thus it appears that water at least in part exerts its influence by preventing the adsorption of oxygen.

Recently, Salwin[54] undertook a series of investigations which indicated that the moisture content which corresponds to a theoretical monomolecular layer of adsorbed water, according to the B.E.T. equation, should represent an optimal amount of water for preservation of dried foodstuffs.

Reactions in food of low moisture levels

Enzymes are considered to be inactive in the "dry" state

and under conditions of low water contents their speed of action is reduced. According to Nelson et al.,[56] the velocity of hydrolysis of sucrose by invertase decreased when the substrate concentration was greater than 10 %. This decreasing in velocity was shown to be due to the resulting decrease of water content below 90 % of the system. Their results have been verified by Harold et al.[57] However, recently published results have shown that for other enzymes reactions can occur in solid materials with very low moisture contents.[58]

(a) Foods of animal origin

(1) Dried egg

Due to the high lipid content of the egg, the changes that occur during storage of dried egg were first thought to be an enzyme-hydrolysis of these lipid substances. Brooks[59] ascribed the increase in acidity of the egg powder to the formation of free fatty acids by lipases, and concluded that this increase showed a definite dependence on moisture content. Later it was reported[60] that the higher acidity that Brooks[59] noted can probably be attributed to the effect of bacterial enzymes formed during development of bacteria in the liquid egg before drying and which can still be active even when the microorganisms themselves have died during processing or storage.

(2) Dairy products

Lipid oxidation in milk products has been a serious and intensively investigated problem. The problem is mainly one of flavour.

The mechanism of lipid oxidation in milk follows two different paths, depending on the presence or relative absence of water as a solvent for the reactions.

In the aqueous phase of milk, the triglycerides are relatively stable and the phospholipids are preferentially oxidized. When water is absent, as in dried milk products, the triglycerides are relatively susceptible to autoxidation whereas the phospholipids are more stable and when present in the triglycerides, they serve as an antioxidant.[61-63]

(3) Meat products

In dried meat, it has been found that the acidity of the fat content of air-dried raw beef increased during storage at a rate dependent on the moisture content and that the rate was much higher than that in precooked dehydrated meat. That increase in acidity has been due to the lipase action in dried raw meat.[64] Tappel[65] showed that freeze-drying has led to much better products in meat particularly. Matheson[66] has found that enzymes in meat survive accelerated freeze-drying and storage, that they appear to react in the dry state provided the relative humidity is high

enough and that the rate of glycogen disappearance in meat is also somewhat moisture dependent, which suggests that enzymes might be responsible.

Sharp[67] has shown that in freeze-dried raw meat it is possible that enzymes such as ⍺- amylase and maltase may be active during storage and may produce from available glycogen or intermediary dextrins a supply of reactant sugars for non-enzymic browning reactions.

It has been claimed that sodium chloride catalyses fat oxidation in meat and that the effect of salt in lipid oxidation depends upon free moisture in the system. As moisture is removed by dehydration or by freezing the salt progressively increases the rate of oxidation.[68]

(b) Foods of plant origin

Grains and flours

The high proportion of cereal products in human nutrition makes careful protection from spoilage an important task for scientists. Furthermore, enzymes can seldom be inactivated by a heat treatment without harming properties important to further processing. High moisture content permits the growth of molds. It has been reported that hydrolytic rancidity may become of of consequence in stored whole grains.[69,70] Kizel[71] found that

the lipases of wheat require a moisture content in the grains of at least 15 % for activity.

It has been said that the hydrolytic endogenous enzymes of grains should play no great role in changes on storage when the moisture content is at equilibrium with relative humidities of less than 75 %.[72-77] There is strong evidence that the changes which do occur during such storage can be traced back to mold enzymes that were able to form before the cereal reached a low moisture content.

When grains are milled to produce meals and flours, the equilibrium characterizing living seed is destroyed. The fat, which in the seed was localized, is now distributed widely. Some enzymes are inactivated, and others are released and distributed widely. Hydrolytic, enzymatic and autoxidative rancidities may now develop.

Reports dealing with changes during storage of flours frequently omit data on moisture content. The development of fat acidity by hydrolysis is accelerated by moisture, whereas oxidative rancidity appears to be enhanced when the moisture content of flour is reduced to a low level. Guendet et al.[32] have pointed out that fat acidity increases sharply at higher moisture contents, at 14 % moisture 50 % of the lipids were split. He concluded that the level of fat acidity that was reached after

52 weeks was dependent not only on the lipase but also on the amount of substrate present.

Thomas and Rothe[78], from their work on oat products, assumed that the bitterness which sometimes developes occurs in two steps: (1) formation of peroxide, and (2) destruction of the peroxide to form the bitter substance, and concluded that the reaction is enzymic and the enzyme involved could be lipoxidase. Rothe[79] concluded that the bitter material could be ascribed to the oxidation of unsaturated fatty components that leads to the formation of peroxides.

Moisture content and enzyme activity

It can be seen from the observations made above that while enzymes are likely to be involved in the reactions which take place in 'dry' foodstuffs, only fragmentary and empirical observations have been made in this field. In general, studies on this topic can be divided into these carried out on liquid systems in which part of the water is replaced by another solute and those on meals or other solid materials.

It is also obvious from the above that the enzyme reactions can occur in the solid materials. If one accept the idea that enzyme reaction goes via the intermediate enzyme-substrate complex - and there is no reason to disagree in this particular

case--then the formation and decomposition of this complex (and hence the reaction velocity)will depend on the rate at which the substrate reaches the enzyme and the rate at which the products diffuse. It is very likely that diffusion rates determine the over-all rate for reactions in the solid state. The limiting effect of the moisture would reflect,not its property as a reaction partner,but rather its ability to facilitate the diffusion of these partners.[80] In this case,therefore,the amount of moisture available would not be as important as the form of moisture in which it is available.

Kiermeier and Coduro,[81] showed that it is not possible to set a limiting relative humidity for such enzymatic reactions as can be done to a certain extent for microbiological changes. No visible hydrolysis occurs in a mixture of starch and diastase in equilibrium with relative humidity of 70 %. When cellulose or some other substances with a capillary structure, is substituted for part of the starch however, starch hydrolysis begins at a relative humidity of 46 %. Capillarity, therefore, is credited with having a major effect upon such enzymatic reactions. Ackor and Luck[82], from their work on lecithinase, concluded that the enzyme reactions occur in the freely mobile capillary-condensed water, for their own investigations showed that the enzyme activity increased strongly in the region of the capillary condensation.

They believed that enzyme catalyzed reactions in solid substances can occur only in the liquid phase, i.e. in the water filled capillaries. According to their concept, the moisture adsorbed in the region of the first part of the sorption isotherm can make no contribution to hydrolysis, or it can have only a very small effect in view of the difficulties imposed on diffusion of the reaction partners and reaction products in this region. The conditions here are apparently similar to the reactions in frozen foods, where it is assumed that the changes occur in the liquid phase. The concept developed by Acker and Luck[82] would also explain why the degree of splitting reaches a different final value for each relative humidity. In samples that differ only in moisture content - not in enzyme and substrate concentrations - it would be expected that the splitting would occur at different rates but approach the same final value in every case.

This could be explained as follows: in the region of capillary condensation the moisture is condensed at first (at medium relative humidity) in the small capillaries. In these moisture filled pores only the substrate lying in the immediate vicinity of the enzyme can be reached. The expected equilibrium will be established locally, but the splitting of the substrate, calculated on total substrate in the sample, will stop at a lower level. As the relative humidity is increased, increasingly larger

pores are filled so that an increasing amount of substrate can be dissolved and brought into reaction with the enzyme. The extent of splitting will therefore increase, and the reaction rate will increase because of better diffusion possibilities. On this basis, an enzymatic reaction could occur to a great extent only when absorption of moisture on the surface is complete and liquid water is beginning to form in the pores.

It can be assumed that, with the beginning of capillary condensation, the soluble constituents dissolve in the condensed water so that saturated solutions are formed at first, and in such case, most enzymes are weakly active or have no activity. In any case, Acker and Luck[82] believe that enzymatic reactions do not proceed below the inflection point of the sorption isotherm, or only at a greatly reduced rate.

It is true that the halt in enzymatic reactions after some time at different degrees of splitting could also be explained by the enzymes becoming inactive. It has been shown[83] that the enzyme activity of phospholipases, and phosphatase[84] on lecithin and phenylphosphate respectively, in samples equilibrated at a lower relative humidity increases immediatly if the samples are brought into a higher relative humidity and therefore to a higher moisture content. Thus it is not sufficient to dry foods to a low moisture content; care must be taken to assure that they

remain at this moisture content. Acker and Luck[84] showed that phenolphthalein phosphate is split much more rapidly by barely-malt extracts than is phenylphosphate, but the situation is reversed in the solid state. In dry material the hydrolysis of phenolphthalein phosphate is observed only above 80 % relative humidity, whereas phenylphosphate is hydrolyzed at 25 % relative humidity, although very slowly. Perhaps this is because the larger molecule does not fit into the narrow capillaries, and is therefore split only when water condensed in the large capillaries.

CATALYSED OXIDATION OF UNSATURATED FATS

General introduction

Many materials containing even small percentages of fat undergo, during storage, certain changes which give rise to odours described as "rancid".

Rancidity in food products may be the result of either oxidation or hydrolysis of the lipid components. The development of rancidity is potentially one of the major types of chemical change in fatty foods which can lead to economic losses and has long been a problem in the food industry. Rancid foods are characterized not only by disagreeable flavours and odours, but also by lower nutritional value because of vitamin destruction[85] and by poor colour due to pigment co-oxidation.

Hydrolytic rancidity in fats and oils results in the formation of free fatty acids, di and monoglycerides and glycerol, and is sometimes caused by enzymes. Control of hydrolytic rancidity may be accomplished by inactivation of enzymes, and by storage at low moisture levels and low temperature.

Oxidative rancidity is caused by chemical changes in the fat due to the action of oxygen. The molecules susceptible to autoxidation are chiefly those with unsaturated linkages at which the oxygen becomes attached during autoxidation. The resulting compounds which contain unstable peroxide linkages

then break up into aldehydes and other decomposition products which cause the odour of rancid fat.

This rancid odour is only apparent when oxidation is well advanced and many tests for incipient rancidity have been devised.

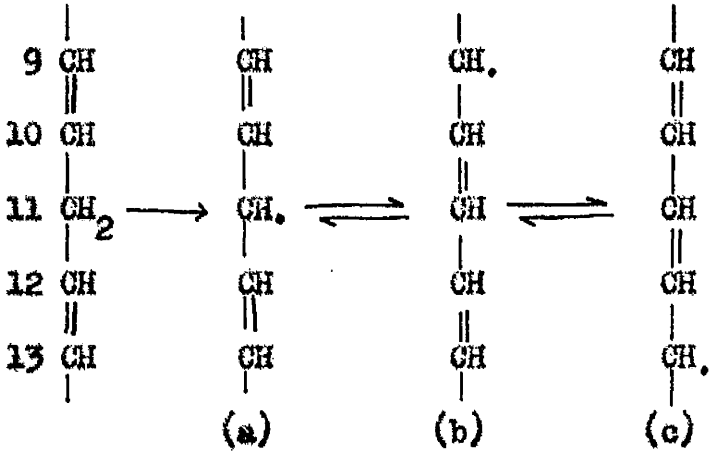
Light, heat, concentration of oxygen, moisture, and the presence of catalysts or inhibitors affect the rate of oxidative rancidity, often with seemingly very different results. At a certain stage the rate of oxygen uptake increases and this marks the end of what has become known as the induction period. At the end of this period, when the deterioration of flavour of the fat becomes markedly apparent, the oxidative rancidity becomes organoleptically perceptible. The induction period is therefore a measure of stability of the oil or fat in respect of this type of oxidation, and since it must be subject to many external factors, the conditions under which any determination of the length of this period is made must be strictly defined.

It has been generally accepted that the induction period, measured under conditions resulting in a very considerable acceleration of the normal processes of oxidation is of practical significance in predictions of stability.

The mechanisms involved in autoxidation have been adequately reviewed and need not be elaborated here.[86-88] Suffice to say

that it has been widely accepted that at ordinary temperature, the autoxidation of unsaturated fats proceeds by a free-radical chain-reaction which results in the formation of conjugated diene peroxides, this, as shown by Farmer[89]; involves:

(1) Initiation: the first step in the oxidative decomposition of unsaturated fats is believed to be a dehydrogenation from the activated methylene group to yield a free radical (a) which may form the conjugated radicals (b) and (c).



(2) Propagation: there then follows the addition of oxygen to the free radical.

$$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$$

This radical in turn brings about a dehydrogenation from an activated methylene group of another fat molecule, forming the hydroperoxide product and a fresh free-radical molecule, and the cycle continues.

$$ROO^{\bullet} + RH_2 \longrightarrow ROOH$$

Chains may be terminated by interaction of free radicals.

Decomposition of hydroperoxides may produce additional free radicals, initiating more chains. The major primary product is thus conjugated hydroperoxide.

Measurement of oxidation

Many tests have been devised for following the course of oxidation.

(1) Of these, many make use of the peroxide formation which occurs at the double bonds.[90-94] Iodometric and thiocyanate assays have been most used.

(2) A number of tests used industrially, such as the Kreis test[95-97] measure secondary oxidation products. Such tests, at best, are normally only semi-quantitative.

(3) A frequent method of examination is to measure the actual uptake of oxygen during the period of storage. This is followed by standard Warburg manometric techniques.[98-99]

(4) Spectrophotometric techniques have been used for measuring oxidation.[99] These are based on the production of conjugated diene which absorbs strongly at 232 millimicrons.

Many other methods have been devised in this field, and the review given by Link et al.[100] is useful in this respect.

Haematin compounds and fat oxidation

(a) Importance of haematin compounds

Haematin (ferriprotoporphyrin) and haem (ferroprotoporphyrin) are the prosthetic groups of many enzymes, co-enzymes, and "half-enzymes". These include the enzymes, catalase and peroxidase, the cytochromes, haemoglobin and myoglobin. All these compounds differ in the nature of the protein attached, the mode of attachment, and the number of iron protoporphyrin groups per molecule.

The haematin compounds, haemoglobin, myoglobin, catalase, peroxidase, and cytochromes are of wide-spread occurrence in living organisms, as are the readily oxidised unsaturated fatty acids linoleic and linolenic. There is thus a potential instability towards oxidation in all organisms.

There has been speculation on haematin catalysis of unsaturated fatty acid oxidations as the primary reaction in certain types of non-metabolic unsaturated lipid oxidations in living animal tissue. It appears also to be involved in spoilage of frozen meat products by oxidative rancidity.[101]

It has been found that lipid peroxides occur in measurable amount in body lipids of vitamin E - defficient animals.[102] Since lipids peroxides are also destroyed by haematin compounds they could subsequently be broken down by haematins present in

the tissue. It has been suggested[102] that peroxide breakdown products react further to oxidise sulfhydryl groups of enzymes, thereby inactivating them and disturbing normal metabolism. Furthermore, it has been found that a catalysis of unsaturated fatty acids oxidation by haematin compounds is responsible for the yellow fat disease of animals which could be prevented by adding large amounts of vitamin E or antioxidants to diets which contain large amounts of unsaturated fats.[103]

(b) Catalytic activity on unsaturated fatty acids

(1) In animal material

That iron plays a part as a catalyst in living cell was first shown by Warburg.[104]

Robinson[105] was the first to observe the catalysis of unsaturated lipids oxidation by haematin compounds. She indicated that haemoglobin, methemoglobin and hacmin catalyze the oxidation of linseed oil-water emulsions, while haematoporphyrin was ineffective and concluded that the catalytic activity should be attributed to the complexed iron and not to the oxygen released by oxyhaemoglobin. Barron and Lyman[106] suggested that the reaction proceeded by a chain reaction mechanism. Haurowitz and Schwerin[107] claimed that the catalytic oxidation of linoleic acid by haemin occurs only in emulsion systems.

Haurowitz et al.[108] found that the catalysis of linoleic

and linolenic oxidation by haemin or haemoglobin is coupled with a destruction of the pigments. They concluded that inorganic iron was released during the reaction, and suggested that the pigments were destroyed by the intermediate formation of fatty acid peroxides.

Banks[109] postulated that performed linoleate peroxide was necessary for haematin catalysis, and showed that haematin had no immediate effect on peroxide-free linoleic acid.

Watt and Peng[110] suggested that unsaturated-fat oxidative activity which they found in meat was due to its haemoglobin and myoglobin content rather than to the presence of lipoxidase.

Tappel[111] proved that the haematin compounds are responsible for the catalytic oxidation of meat lipids. Rat stomach, beef muscle and heart, liver, kidney; pork loin, muscle and adipose tissue; chicken and turkey muscle and fat; and fish muscle were all tested and gave positive results for haematin catalysis and negative results for lipoxidase activity.

Boyd and Adams[112] also used a differential technique to substantiate the results of Watts and Peng[110] and of Tappel.[111] They showed that linoleate oxidation was catalyzed by the haematin pigments - in the extracts of beef and pork adipose tissue, Kidney, heart, brain and lungs - and not by lipoxidase.

Tappel[113] showed that haematin compounds are non-specific

catalysts for the decomposition of a wide variety of unsaturated compounds which form peroxides during oxidation. These included linseed alcohol, conjugated linoleic acid, squalene. The mechanism of action of haematin compounds catalyzed unsaturated fatty acid oxidation proposed by him involves an initiation reaction in which the haematin compound reacts with a lipid hydroperoxide to form an intermediate complex, followed by the decomposition of this intermediate complex into two free radicals:

$$ROOH + FeOH \longrightarrow ROOFe + H_2O$$

$$ROOFe \longrightarrow RO^{\bullet} + FeO^{\bullet}$$

where ROOH represents the hydroperoxide, and Fe represents the haematin compounds.

The catalyst is regenerated by abstracting a hydrogen atom from another fatty acid (RH) molecule:

$$FeO^{\bullet} + RH \longrightarrow FeOH + R^{\bullet}$$

The fatty acid free radicals produced can undergo free radical chain propagation and chain termination by the generally accepted mechanisms of autoxidation. His suggestion was that carbonyl compounds could be produced when -C-O- bond acission is accompanied by -C-C- bond rupture.

The destruction of the haematin catalyst which occurs during the reaction was thought to be caused by a random reaction of the haematin compounds with free radicals.

A good deal of work has been done on the specific reactions of iron in the haemoproteins[114,115] and the decomposition of organic hydroperoxides by iron or metal coordination compounds has been extensively studied by Baxendale,[116] Waters,[117] Weiss,[118] and others.

Maier[119] studied in detail the catalysis of unsaturated fatty acid oxidation by haematin compounds. He concluded that the initiation reaction between haemoglobin and linoleate hydroperoxide is independent of total linoleate concentration. He suggested that the haemoprotein is first oxidized to methaemoglobin (ferric form) either as a direct result of the catalytic decomposition of hydroperoxides or as a secondary reaction between the hydroperoxide decomposition product and the haemoprotein and that this is followed by a degradation of the methaemoglobin to the point where the porphyrin is ultimately destroyed.

(2) In plant material

In the year 1892, Petit[120] originated the research on the organic iron derivatives in plants. He discovered in barley an organic substance containing both iron and nitrogen. Similar substances were discovered by Stoklesa[121] in onion.

The presence of haematoid iron in plants was first laid by Gola[122] who indicated that a great part of the plant iron occurs in an organic substance "very similar although not identical"

with the haematin of the blood. However his theory was based on rather unconvincing experiments. Keilin[123] confirmed and extended these findings and showed that the pigments are widely distributed not only in the tissues of higher animals and plants but also in yeast and bacteria. Keilin[123] named these pigments the "cytochromes" meaning cellular pigments. Schum[124] confirmed Keilin's data and also added some particulars. He succeeded[125] in establishing the identity of the porphyrin derived from the plant haematin with that prepared the same way from blood haematin. Cytochrome was also found in algae[126] and mushrooms.[127] Subsequent investigations have shown that there is a good deal of haematins in the roots of cabbage, radish, cauliflower and turnib, and that the secondary roots are specially rich in haematins.[128] All forms of haematin compounds previously found in animals have since been found in plants including haemoglobin itself in leguminosae.[129,130]

It was noted by Hopson et al.[131] that extracts of peas contained two factors which involved in the linoleate-coupled oxidation of glutathione and they had evidence which suggested that one factor behaved like a haematin compound. Blain et al.[132] recently postulated the existence of two factors in soya, one having a true lipoxidase action by acting on unoxidised linoleate and causing the concurrent bleaching of carotene, while the other, shows lipoperoxidase action, like cytochrome c, in utilising

preformed peroxide to bleach carotene.

Carotenoid-bleaching enzymes

Carotene loss in damaged green leaves has long been associated with enzymic attack.[133] The catalytic factor involved could be partially inhibited by cyanide and was most active at pH 4.[134,135] Similar observation have been made by Friend et al.[136] who found that homogenates and chloroplasts prepared from sugar beet leaves destroy carotene.

Recently Booth[137] reported an enzymic destruction of endogenous - B - carotene by plant tissues containing chlorophyll after maceration. Friend et al.[138] ascribed carotene-bleaching to an enzyme which inhibition studies indicated as likely to contain iron.

More recent studies indicated that the destruction of crocin by the particulate preparations from sugar beet leaves was inhibited completely by boiling, and that mitochondrial preparation from sugar beet leaves homogenate also catalysed the destruction of crocin and have two optimum pH, one near pH 4 and the other at pH 7.5.[139]

The idea of a direct carotenase action by these enzymes still a matter of doubt since it is well-known that the lipoxidases and haematin compounds occur in association with lipids[131] and all of the observations on carotene destruction which have appeared would be attributed to coupled fat oxidations catalysed by

haematins or lipoxidase.

LIPOXIDASE CATALYZED FAT OXIDATION

(a) Discovery of lipoxidase

In the year 1932 Andre and Hou[141] discovered the presence of an enzyme in soya beans which they named lipoxidase. Hass and Bohn[142] patented the use of soya bean extracts for bleaching the yellow pigments of wheat flour. Later, Frey et al. [143] showed that these extracts not only destroyed carotene but also vitamin A activity as well. Sumner et al.[144] studied an enzyme system present in soya bean which caused the bleaching of carotene; this "carotene oxidase" was also found to cause the oxidation of unsaturated fats to peroxides. It was shown by Sumner[145] and independently by Tauber[146] that the destruction of carotene is brought about by lipoxidase and these authors put the view that lipoxidase catalyses the oxidation of unsaturated fatty acids and that it was during this process that carotene was co-oxidised.

(b) Distribution in nature

So far, the evidence indicates that lipoxidases are found only in higher plants. Reis and Fraps[147] presented quantitative data on lipoxidase activities of numerous varieties of peas and beans. Strain[148] detected the enzyme in several legume seeds. Sumber[149] investigated the activities in a variety of soya beans

products. Kirsanova[150] reported the presence of the enzyme in potato juice and in radishes. Its presence in wheat flour and potato was demonstrated earlier by Sumner.[151] Mitchell[152] found that lucerne contains lipoxidase activity which was inactivated by the dehydration process and added that carotene destruction during storage in stored dehydrated lucerne is probably not enzymic in nature. Irvine et al.[153] undertook a detailed study of wheat lipoxidase.

The above report shows that lipoxidase has a wide distribution in the plant kingdom. In fact, plants appear to be the only source for this enzyme.

(c) Substrate specificity

Lipoxidase is a non-metallic enzyme which catalyzes the peroxidation of certain fatty acids with molecular oxygen.[157] The enzyme has been found to act only on linoleic, linolenic and arachidonic acids and their esters, fatty acids which contain methylene interrupted double bonds, $-CH=CH-CH_2-CH=CH-$, with both bonds cis; trans isomers are not attached.[158] In general, the primary oxidation products formed by the action of lipoxidase are the same as those formed in the ordinary autoxidation of these acids,[159] these are conjugated dienes later followed by peroxide formation.

(d) pH activity

Using neutral fat or linoleate esters as substrate, in a

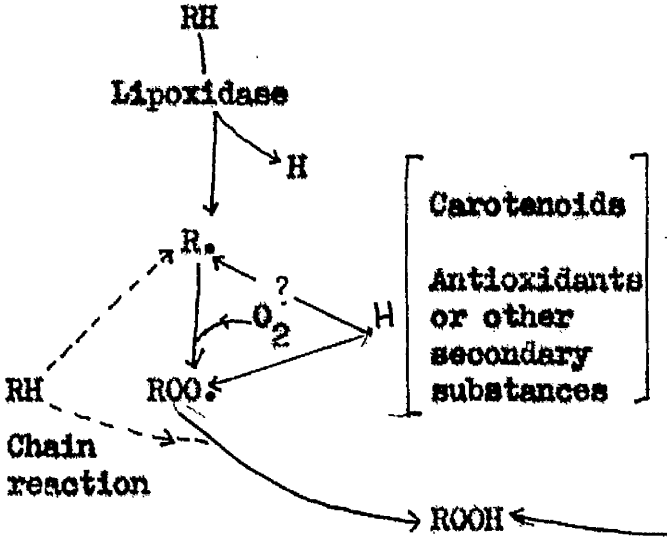
coupled carotene bleach system, lipoxidase was found to have optimum activity at pH 6.5.[132,154,155]

Holman[155] using diene conjugation and thiocyanate for peroxide estimation showed that lipoxidase has optimum activity on sodium linoleate at pH 9.3. Other workers[156] have reported that the pH for optimum activity varies with the various sources of lipoxidase and also with varying the substrate. For example, Navy beans extracts showed optimum at pH 7.5, whereas peanuts lipoxidase on the same substrate (linoleic acid) gave an optimum at 8.1. Lima beans gave optimum of 7.5 on linoleic acid and 5.5 and 7.0 on trilinolein.

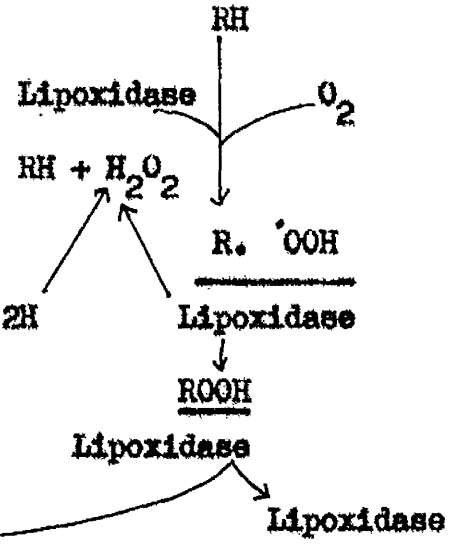
(e) Mechanism of action

The mechanism of lipoxidase action is controversial and two hypotheses are indicated below.

Bergstron and Holman



Tappel, Boyer, and Lundberg



Conjugated linoleate hydroperoxides

Bergstrom and Holman[159] postulated that lipoxidase initiates a free radical chain similar to that involved in linoleate autoxidation since oxidation products were similar and since coupled oxidation of carotenoids and other substrates might be explained on this basis.

Tappel and his co-workers[160] believed a chain reaction of this type to be unlikely partly because the kinetics of the system were not typical of chain reactions, because they considered the action of antioxidants was not typically chain breaking and because they found no induction period.

More recently Tappel[161] has conceded that lipoxidase catalysis of unsaturated fatty acids could be regarded as a modulated free radical chain reaction.

Blain[162] subsequently suggested that neither the system proposed by Bergstrom and Holman nor that proposed by Tappel et al. seems to be fully adequate to account for observations made on the coupled oxidation of carotenoids and vitamin A.

Lipoperoxidase

Blain and Styles[134] indicated the existence of an enzyme system in crude soya extracts which decomposed conjugated linoleate peroxide with concurrent bleaching of carotene. This action is similar to that known for haematins.

Subsequently, it has been found that linoleic acid hydroperoxides could be shown to be decomposed by an enzymic factor in soya

extracts,[163-165] in the absence of carotene.

This type of activity was found to occur at pH 4 - 6, and completely inactivated by heating at 75°C. It has also been found that this enzymic factor could be inhibited with cyanide.[164]

Comparison between haematin and lipoxidase activity

Tappel[166] undertook a series of investigations on the possible role of haematin catalysts in fat oxidation. He made the observation that haemoglobin catalysis linoleate oxidation only in emulsion systems whereas soya bean lipoxidase catalyses oxidation both in emulsified and homogenous substrates. This difference between haematin catalysts and lipoxidase gives a method by which the two catalysts could be differentiated.

The haematin compounds have been shown to be more active at pH's around 4,[134,135] whereas lipoxidases are more active at higher pH's which inhibit haematin catalysis of linoleic acid.[161]

Cyanide and ascorbic acid were found to be specific for haematin inhibition.[112]

EFFECT OF MOISTURE LEVELS ON FAT OXIDATION

There have been a number of conflicting views as to the exact role that water plays when present.

Fisher[167] observed that the rate at which flour absorbed oxygen at 60°C. increased as the moisture content was decreased

at low levels.

Smith[168] ascribed the best keeping quality of fat to a low moisture content. Ryan et al.[169] maintained that water levels have little to do with the production of rancidity. Winkel[170] in earlier publications considered moisture as a catalyst for the production of rancidity, but in later work discounted it as a factor.

Experiments of Fine and Olsen[171] indicated that fat rancidity (in patent flour, wheat germ oil and other products) developed rapidly in samples of moisture content of 2 % or less, but moisture content of 3 % or over were definitely protective. Glycerol was also found to have a protective action with grain products.

In connection with their studies on the keeping quality of milk powders, Holm and Greenbank[172,173] observed that the presence of water retards the development of tallowy odours. In discussing autoxidation and the influence of moisture in this reaction, Greenbank and Holm[174] stated the explanation for greater tallowiness at lower humidities, or lower moisture contents, lies undoubtedly in the fact that in the absence of moisture, the autoxidation proceeds to the aldehyde stage, while when moisture is present it proceeds directly to the acid stage, thus giving little of the tallowy odour produced by the aldehydes and the other by-products. Their results have been confirmed by Anderegg and Nelson.[175]

Guedet et al.[176] indicated that the development of fat acidity is accelerated by moisture, whereas oxidative rancidity is enhanced

when the moisture content of flour is reduced to low levels. Their results were in agreement with that obtained by Andrews[177] who found that the extent of rancidity was in inverse relation to the moisture content. Samples of flour containing 1.5, 4.0, and 8.0 % moisture were rancid after 6 month's storage at 40°C., whereas these stored at 11.0 and 13.5 % were entirely free from rancidity.

The general conclusion from these observations would be that either too low or too high a moisture level may promote oxidative rancidity.

CHAPTER II.

EFFECT OF VARIATION IN MOISTURE CONTENT ON LIPOXIDASE CATALYZED FAT OXIDATION

The effect of moisture level on fat oxidation in general has been the subject of only a few studies and no work appears to have been done previously on the effects of moisture on fat-oxidising enzymes.

Blain et al.,[178] using water/glycerol mixtures, indicated that the activities of peroxidase and lipoxidase are modified by the availability of water much as those of the hydrolytic enzymes.

The activity of peroxidase appeared to be much more sensitive to change in water levels between 50 and 100 per cent. than did that of haemoglobin.

Lipoxidase (crude soya extract) appeared to have little activity below 20 per cent. water content and above that the reaction rate was most susceptible to change in water levels up to about 70 %.

It has been well known that soya bean meal does not readily show oxidative rancidity despite its high content of lipoxidase. This might be due to its relatively low moisture content, to the natural antioxidants of soya bean oil, to the lack of contact between enzyme and substrate or a combination of these factors.

Little is known on these points and the experiments described here were undertaken to attempt some clarification.

While it is known that lipoxidase is the major fat oxidising catalyst in soya bean meal, it is recognized that other factors may be important at low moisture levels.

This section is divided into four parts:

Part (1) deals with experiments in which fresh soya flour was conditioned to different moisture levels, either by humidification as described in appendix (I) or simply by adding water in small amounts with mixing and weighing to reach the desired levels, at a temperature of 20°C. and the oxidation was followed from time to time both by means of diene conjugation measurements and by the thiocyanate technique.

Part (2) deals with the experiment in which soya flour was freed from fat with ether, and the dried residue being divided into two parts, one part was mixed with 10 % of soya bean oil, and the second was mixed with 10 % of methyl linoleate. The same techniques for humidification and measuring the oxidation were carried out.

Part (3) deals with experiment in which defatted soya bean flour was first moistened in order to modify any natural state of distribution of lipoxidase, then freeze-dried. Oxidation was followed at regular intervals in the usual way.

Part (4) deals with experiments in which soya lipoxidase was

extracted with water then freeze-dried and the dried material was mixed with potato starch.

In addition, lentils and potato were examined, since they have been reported to contain lipoxidase.[121,122]

A note on the composition of soya bean meal will be found in appendix VII.

PART (1)

Initially, experiments were carried out to find how soya bean meal lipids were affected by oxidation in the meal at different moisture levels.

Soya bean seeds were ground to pass a 60 mesh sieve (the yield was 91.6 %) of the total weight of beans), being well mixed and then kept in tightly stoppered jar. The flour had initially 20 % oil and 7.5 % moisture.

To determine the effect of varying moisture content upon the rate of oxidation, samples were stored over saturated solutions of chemical materials at a temperature of 20°C. (see appendix I). The relative humidities used were 20, 33, 52, 58, 72.6, and 81.7 % , which correspond to moisture contents of 6.4, 7.1, 10.1, 10.7, 15.2, and 21.2 % respectively at equilibrium. An absorption isotherm was plotted and is shown in Figure 2.

Oxidation was followed by making ethanol extracts and measuring the diene conjugation at 232.5 millimicrons on one sample and by determination of hydroperoxide in another (see appendix II.)

A second experiment was run at the same time and in the same humidification vessels to find whether the actual distribution of lipids within the structured elements of the meal afford protection from oxidation. To do this a 50 gm. sample of the

meal was moistened with 50 mls. ether which is an effecient fat-extractor solvent. The ether was then evaporated off from the meal under vacuum. It was assumed that this process would cause redistribution of the soya oil within the meal. This flour was then treated exactly as was the sample which had not been exposed to ether.

Tables 1 and 2 show the results obtained. From the tables it is readily apparent that the rate of soya bean oil autoxidation seem to be very slow within this range of moisture levels (1.3 to 21 %).

At all moisture levels examined here only negligible peroxide values were obtained, whereas the diene conjugation values did increase to some slight extent. This disparity may be due to hydroperoxide decomposition, which might be catalyzed by the haematin compounds present, naturally, in soya beans or by the enzymic factor which acts on preformed linoleic hydroperoxides, to which Blain et al.[132,134] have given the name lipoperoxidase. This factor destroyed hydroperoxide without a corresponding destruction of diene conjugation.

The increase in the diene conjugation values noticed after 45 days would not necessarily be due to enzyme action.

It will be seen that the ether-treated, but not extracted, sample was not more prone to oxidation than was the untreated sample.

TABLE 1. Spectral absorption of the products of soya bean oil autoxidation at 20°C. (diene contents at 232.5 m μ)

R.H. %	Mois. cont. %	Sample	Optical density at 232.5 millimicrons 4	10	14	21	28	35	49	68	83 days
P_2O_5	1.3	A	.002	.042	.043	.032	.038	.063	.060	.140	.089
		B	.061	-	.067	.033	.019	.064	.034	-	-
20	6.4	A	-	-	-	-	-	.103	.105	.159	.204
		B	-	-	-	-	-	.075	.098	.180	.193
33	7.1	A	.026	.002	.064	.030	.042	.083	.045	.160	.143
		B	.067	-	.054	.038	.037	.095	.103	.165	.125
52	10.1	A	.112	.026	.092	.106	.067	.120	.134	.158	.206
		B	.091	.039	.101	.085	.101	.151	.127	.082	.182
58	10.7	A	.065	.020	.095	.070	.119	.095	.130	.054	.150
		B	.051	.030	.089	.072	-	.150	.100	.082	.202
72.6	15.2	A	.093	.031	.096	.070	.080	.137	.130	.118	.240
		B	.067	.041	.111	.059	.058	.111	.080	.179	.196
81.7	21.2	A	.132	.046	.150	.111	.155	.153	-	-	-
		B	.072	.031	.150	.120	.122	.126	-	-	-

A = untreated B = ether-treated

TABLE 2. Spectral absorption of the products of soya bean oil autoxidation at 20°C. (peroxide contents at 470 m u).

R.H. %	Moist. cont. %	Sample	Optical density at 470 millimicrons 4	10	14	21	28	35	49	68	83 days
			← no increase →								
P_2O_5	1.3	A	-	-	-	-	-	-	-	.016	.010
		B	-	-	-	-	-	-	-	.013	.005
20	6.4	A	-	-	-	-	-	-	-	.013	.012
		B	-	-	-	-	-	-	-	.013	.007
33	7.1	A	-	-	-	-	-	-	-	.015	.005
		B	-	-	-	-	-	-	-	.017	.002
52	10.1	A	-	-	-	-	-	-	-	.013	-
		B	-	-	-	-	-	-	-	.026	.005
58	10.7	A	-	-	-	-	-	-	-	-	.007
		B	-	-	-	-	-	-	-	.006	.010
72.6	15.2	A	-	-	-	-	-	-	-	.006	.008
		B	-	-	-	-	-	-	-	.006	.007
81.7	21.2	A	-	-	-	-	-	-	-	-	-
		B	-	-	-	-	-	-	-	-	-

In the second experiment the autoxidation of soya bean oil at higher moisture contents (from 1 to 47 %) was studied. A new sample of soya bean was used. Preparation of the samples was the same as in the first experiment. A few drops of chloroform were added as a precaution against possible microbial growth which might take place at the higher relative humidities. Tables 3 and 4 and figures 2 - 7 show the data obtained.

TABLE 3. Lipoxidase catalysis on soya bean oil at high moisture levels (diene contents at 232.5 millimicrons).

R.H. %	Moist. cont. %	Optical density at 232.5 millimicrons 2	5	9	13	16	20	26 days
P_2O_5	1.0	.036	.046	.045	.015	.028	.063	.054
15	5.3	.030	.043	.052	.025	.024	.047	.040
81.7	19.7	-	.191	.168	.130	.137	.193	.168
86	24.8	.132	.252	.260	.240	.287	.317	.257
93.1	41.0	.224	.513	.715	.610	.640	.705	-
97	47.3	.054	-	.440	.660	1.130	-	-

TABLE 4. Lipoxidase catalysis on soya bean oil at high moisture levels (peroxide contents at 470 millimicrons).

R.H. %	Moist. cont. %	Optical density at 470 millimicrons 2	5	9	13	16	20	26 days
P_2O_5	1	0.005	0.015	0.020	0.010	0.018	0.018	0.013
15	5.3	0.015	0.025	0.010	0.010	0.010	0.010	0.010
81.7	19.7	-	0.025	0.010	0.010	0.015	0.030	0.020
88	24.8	0.050	0.055	0.035	0.035	0.030	0.045	0.025
93.1	41.0	0.080	0.135	0.265	0.140	0.155	0.155	-
97	47.3	-	0.030	0.155	0.370	0.800	-	-

From the results obtained, shown in tables 3, 4 and figures 2 - 7, it will be observed that the rate of oxidation developed corresponds very closely with the amount of moisture present in each of the samples. In the range of moisture levels examined here, it is readily apparent that the rate of oxidation increases as the moisture level is raised, and that the reaction increases notably at higher moisture levels (at 25 % and over), figure 7.

It has also been shown that the oxidation started early

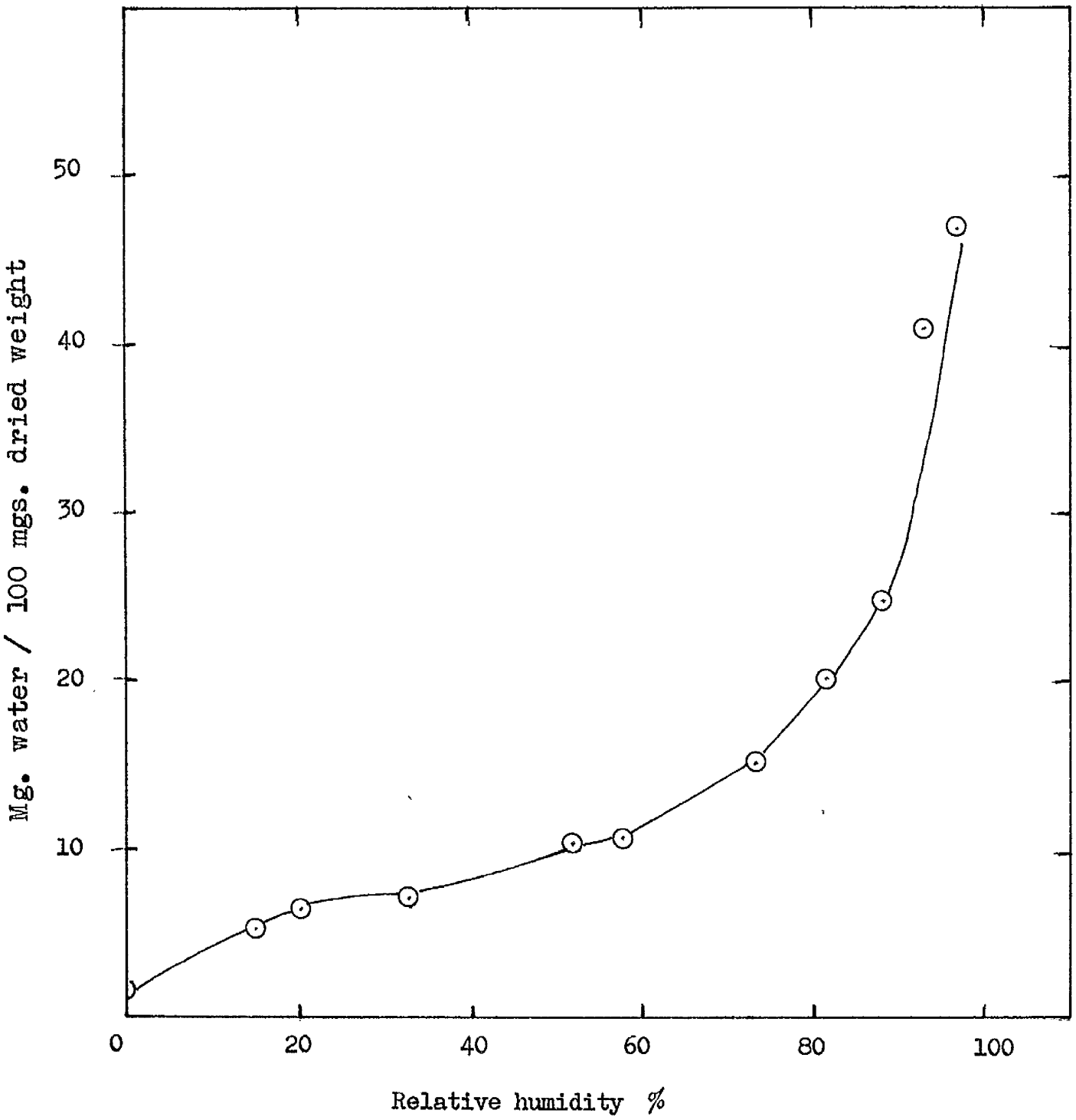


Fig.2 Variation of moisture content of soya bean flour with change in relative humidity at 20°C.

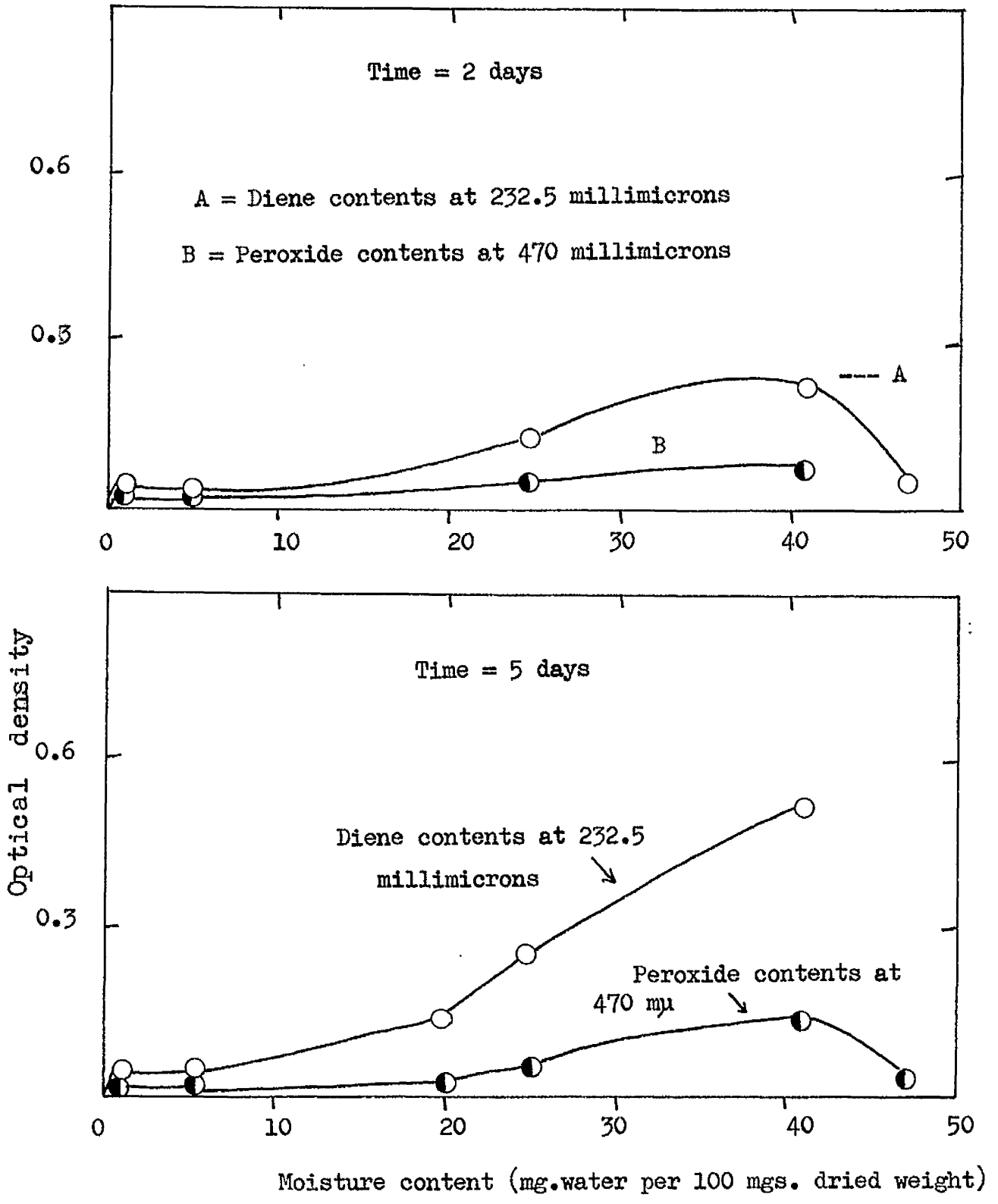


Fig. 3 Variation of oxidation levels of soya bean oil with change in moisture content of meal.

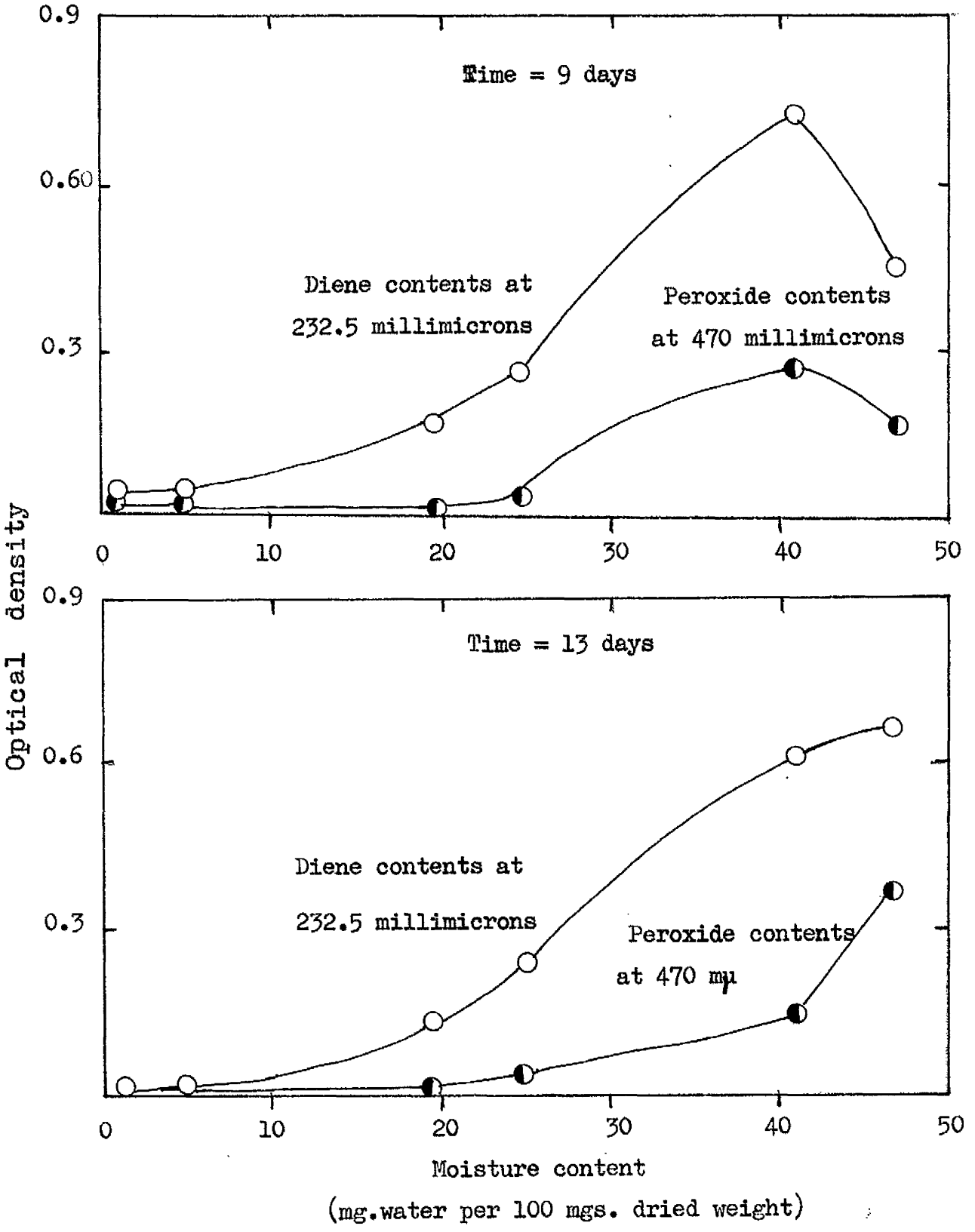


Fig. 4 Variation of oxidation levels of soya bean oil with change in moisture content of meal.

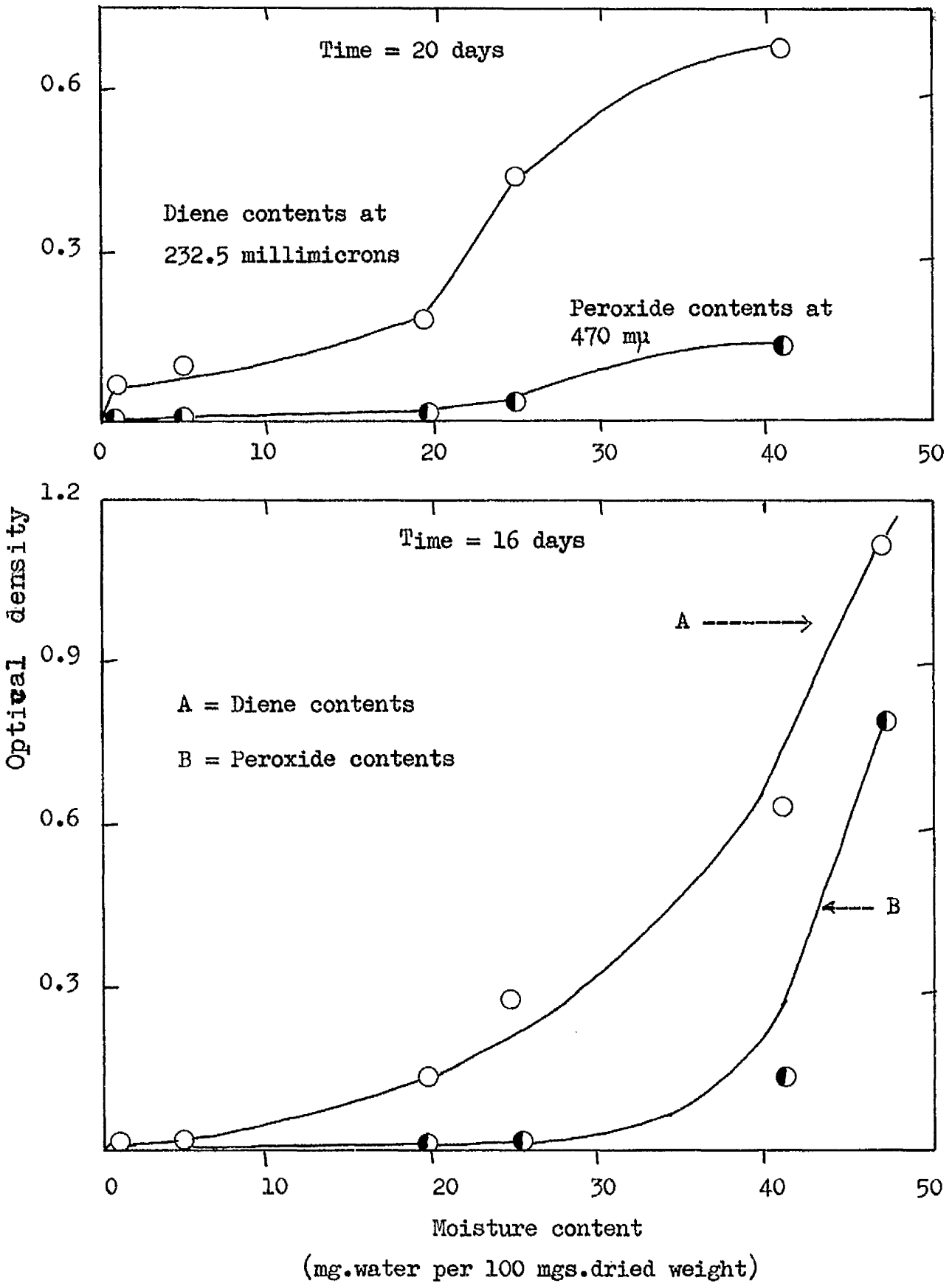


Fig. 5 Variation of oxidation levels of soya bean oil with change in moisture content of meal.

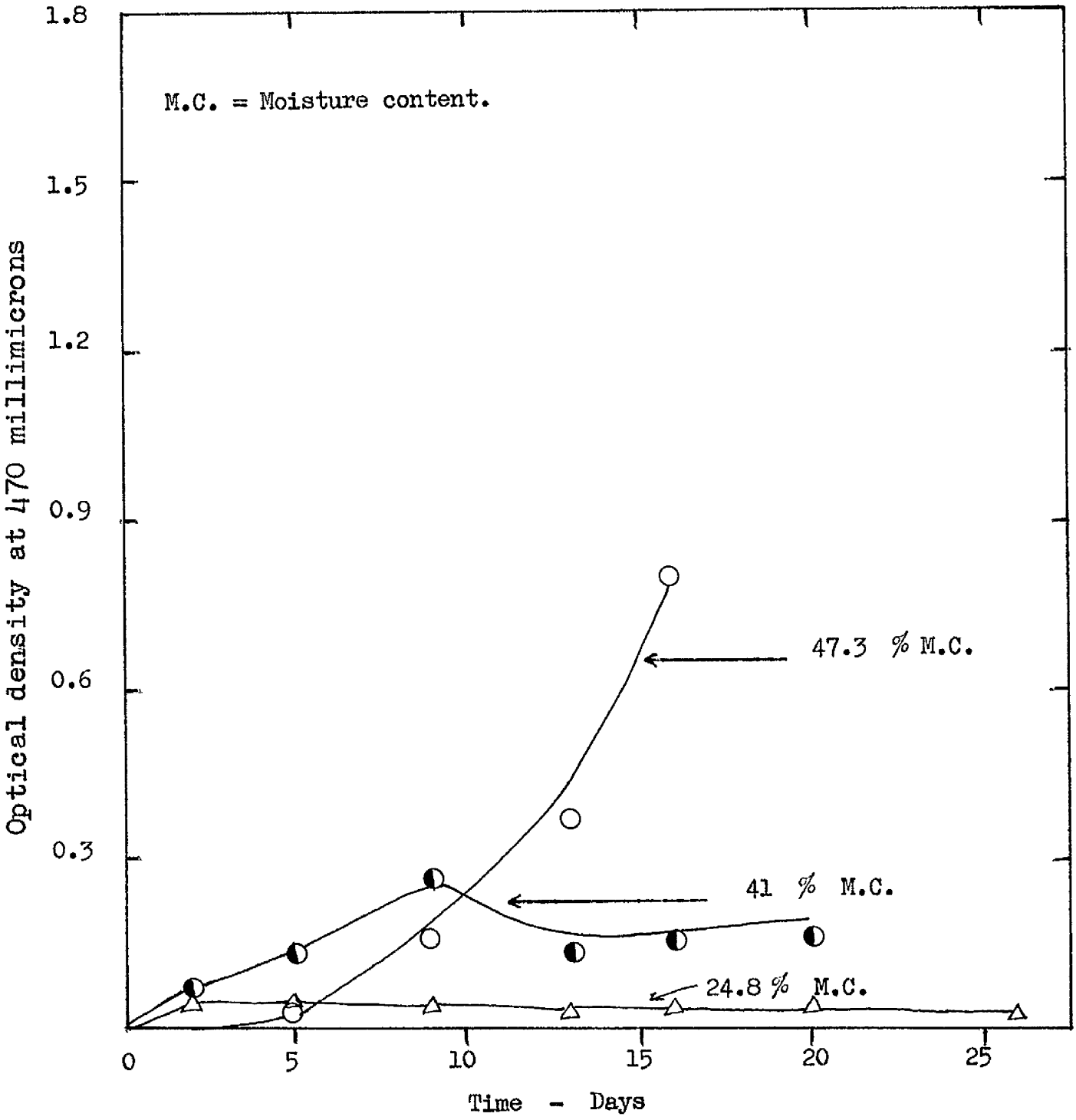


Fig. 6 Rate of change of oxodation levels of soya beam oil .

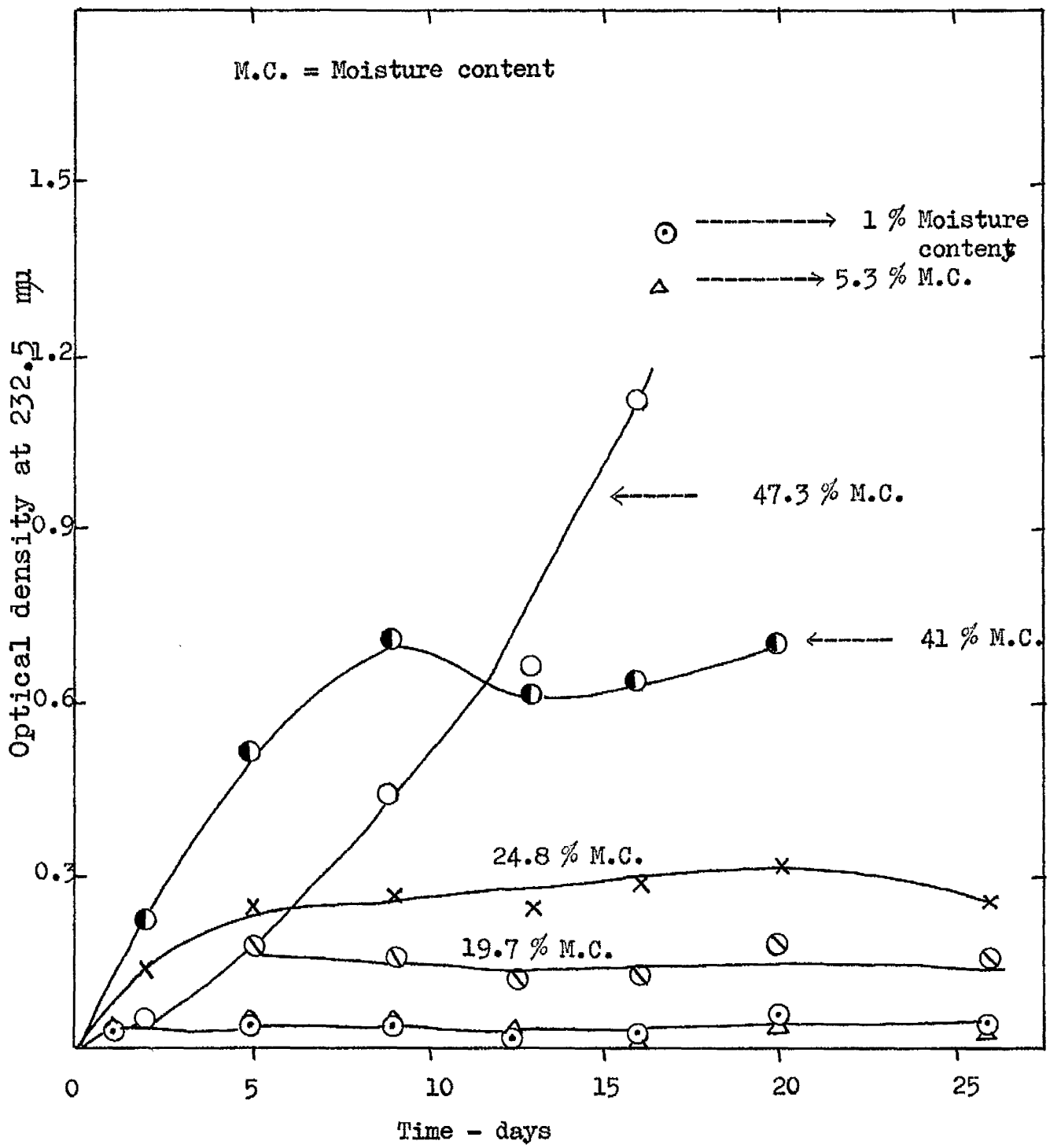


Fig. 7 Rate of change of oxidation levels of soya bean oil

at the very high moisture levels (40 %) and that it was followed by the decomposition of both the peroxides and the dienes produced during the oxidation. At moisture content of 47 % the rate of soya bean oil oxidation seemed to be faster than that found at 40 % moisture content but a longer induction period was noticed. The reason for that is unknown, but possibly a high value had been reached before 2 days and decreased again as is sometimes seen.

With the object of comparing both of the two methods for adjusting the moisture in the samples (humidification and direct addition of water) a third experiment in which water was added by mixing was carried out. A freshly milled sample of soya bean flour was so adjusted to moisture contents of 20, 30, 40, and 50 % . Oxidation was followed from time to time both by measuring conjugated diene and hydroperoxide. Results obtained are shown in tables 5, 6.

TABLE 5. Lipoxidase catalysis on soya bean oil at 30 % moisture content (direct addition of water).

Time	Peroxide value (at 470 m μ)	Conjugated diene (at 232.5 m μ)
1 hour	0.157	0.225
3 "	0.170	0.324
5 days	0.186	0.554

TABLE 6. Lipoxidase catalysis on soya bean oil at high moisture levels (40 and 50 %).

Moisture content %	Peroxide value		Conjugated diene	
	Zero time	4 days	Zero time	4 days
40	0.510	1.191	0.738	0.795
50	0.600		0.940	1.774

It was found that at 20 % water content no oxidation was observed even after 4 days.

At all the other levels oxidation was very rapid and in fact even when samples were extracted with ethanol just after mixing in water, oxidation was already well-advanced.

Probably it is not possible to avoid local high concentrations of water for a short time after addition and it would seem that this produces too rapid catalysis for the method to be as reliable as the humidification technique.

However it would seem that at levels below 20 % the binding of water is sufficiently rapid for no reaction to take place.

Although the fact that an increase in both absorption at 232.5 millimicrons and thiocyanate values made it appear

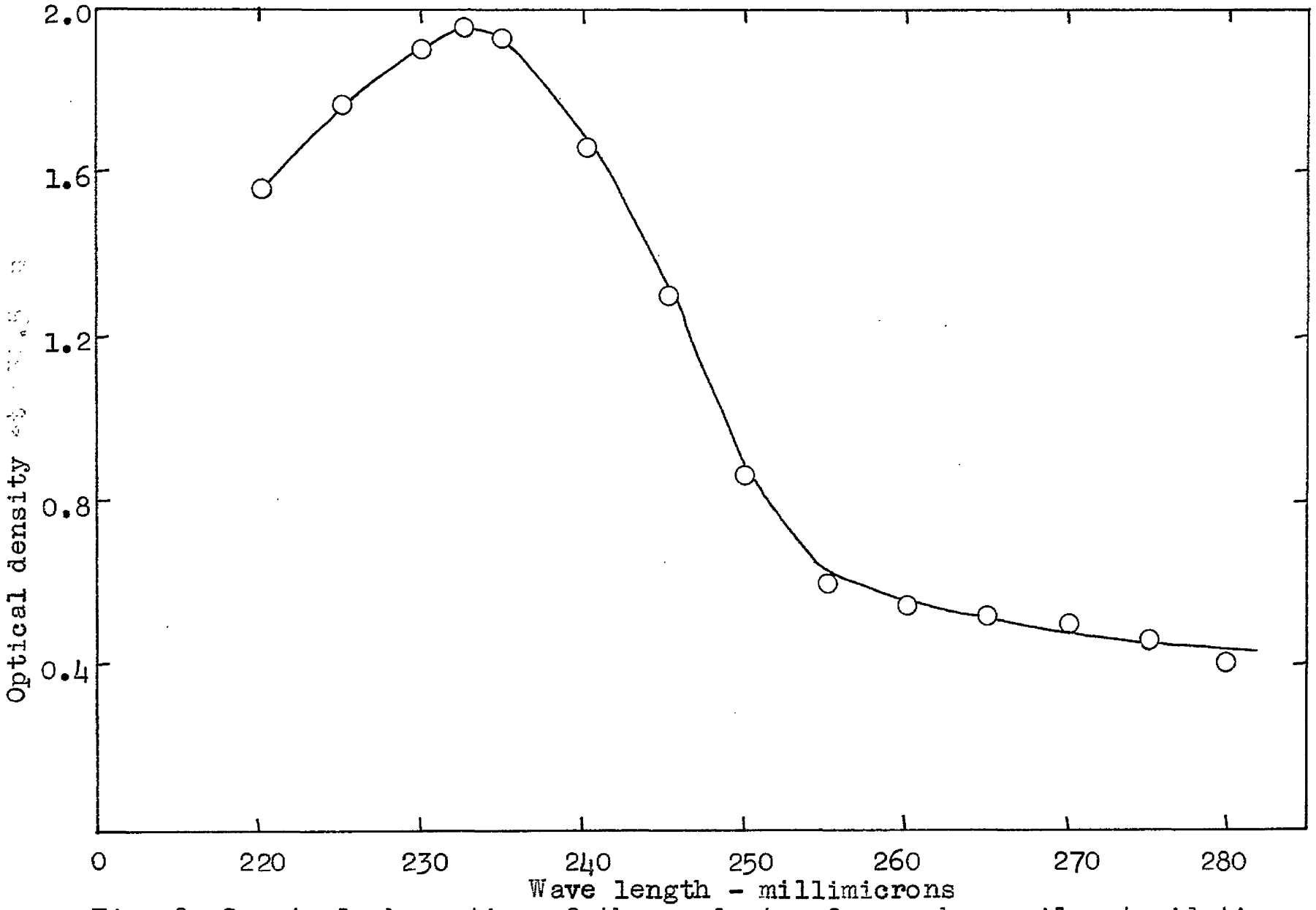


Fig. 8 Spectral absorption of the products of soya bean oil autoxidation with variation in wave length

very probably that the effects measured were in fact due to fat oxidation. It was thought advisable to confirm this by plotting an absorption curve on the ethanol extract in the U.V. range.

TABLE 7. Spectral absorption of the products of soya bean oil autoxidation with variation in wave length.

Moisture content %	Wave length (millimic.)	Optical density
50	220	1.570
	225	1.770
	230	1.900
	232.5	1.950
	235	1.930
	240	1.660
	245	1.300
	250	0.850
	255	0.600
	260	0.548
	265	0.534
	270	0.512
	275	0.456
	280	0.408

It can be seen from table 7 and figure 8 that the data

obtained indicates an absorption peak at 232.5 millimicrons which is consistent with formation of conjugated diene.

Discussion

The results of this series of experiments indicate that lipoxidase can catalyze the oxidation of soya bean oil under conditions of restricted moisture. The rate at which the changes occur increases as the moisture content was raised. At all the moisture contents examined soya bean oil shows an induction period. It has also been shown that both the peroxides and the dienes values may decreased markedly at moisture level of 40 % as the oxidation progressed. This decrease in optical density noticed at the high moisture levels was suggested to be due to the destruction of both the dienes and peroxides.[132] However, it has also been shown that ether extractable lipids of flour becomes "bound" when the flours are wetted or draughed.[179] Phospholipids were found to be bound preferentially in comparison with other flour lipids, and most of the lipids were found associated with that fraction of gluten proteins usually referred to as glutenin.[180] This suggestion has been also made by McCaig[181] who claimed that a protein-lipid complex is formed when wheat flour is made into dough.

Taking this into consideration it is possible that the

decrease in both diene and peroxide values noticed at 40 % moisture content was due to lipid binding in soya flour, since soya bean contains about 41.3 % proteins, although not glutenins.

The dependence of the reaction upon the proportion of water present gave rise to the thought that the enzyme lipoxidase, might react only in the liquid phase, since it has been indicated that the dry flour in dough binds 27.3 % of its weight of water.[182] If a similar situation exists in soya meal as might be expected from its sorption isotherm it should explain why the oxidation did not proceed markedly at moisture contents below 25 % and that the rate of oxidation was considerable at moisture contents of 40 % and over.

The results of these series of experiments may be compared with the previous work on wheat products of Irvine[183] who indicated that the rate of destruction of xanthophyll pigments during the mixing stage of macaroni processing increases with increasing absorption of water to a maximum at about 33 % . He believed that the destruction of pigment occurs through a coupled reaction involving the peroxidation of unsaturated fat by the enzyme lipoxidase.

Adding the water directly to the samples by spraying did show the same general pattern of results although the reaction was much faster than the humidified.

It has also been demonstrated that the ether-treated, but not extracted, sample of soya meal showed that the ether treatment did not affect the rate of oxidation. From observation by Howard[184] it would seem that an analogous situation exists for solvent treatment of wheat flour.

PART (2)

INFLUENCE OF ANTIOXIDANTS ON THE RATE OF AUTOXIDATION OF SOYA BEAN OIL

The experiments described in part (1) suggested that the amount of moisture available would not be as important as the form of moisture in which it is available, since much smaller peroxide and diene values have been obtained at moisture contents below 25 % , where the water molecules are believed to be bound by functional groups of proteins and carbohydrates. On the other hand there is an assumption that the natural antioxidants present in soya bean oil, are of great importance.

To check this point the following experiment was carried out, in which soya bean oil was compared with methyl linoleate.

A freshly milled sample of soya bean flour was ether-defatted, then divided into two lots. One was mixed with 10 % of its weight of soya bean oil, and the other with 10 % of its weight of fresh unoxidized methyl linoleate which is known to be free from antioxidants. Methyl linoleate used was of high purity (Hormel Inst.) which had been repacked in vacuum in 100 mg. quantities. Soya bean oil was prepared as in appendix III.

Small quantities (0.2 gm.) of each sample were brought into equilibrium with relative humidities of the air, in small

desiccators as described previously. Relative humidities used were: 0, 52, 58, 72.6, and 81.7% which are corresponding to moisture contents of 1.3, 10.1, 10.7, 15.2, and 21.2 % respectively. Measurement of oxidation was carried out as described before.

TABLE 6. Lipoxidase catalysis on methyl linoleate at low moisture levels (conjugated diene values at 232.5 m u).

R.H. %	Moist. cont. %	Optical density at 232.5 millimic. 8 days S.B.O.	8 days M.L.	19 days S.B.O.	19 days M.L.	26 days S.B.O.	26 days M.L.
P_2O_5	1.3	.010	.027	.071	.097	.021	.010
52	10.1	.006	.013	.067	.078	.021	.034
58	10.7	.010	.025	.070	.066	.021	.016
72.6	15.2	.025	.100	.060	.138	.036	.117
81.7	21.2	.041	.310	.083	.366	.074	.361

S.B.O. = Soya bean oil

M.L. = Methyl linoleate

TABLE 9. Lipoxidase catalysis on methyl linoleate at low moisture levels (peroxide values at 232.5 m u).

R.H. %	Moist. cont. %	Optical density at 470 millimicrons 8 days S.B.O.	8 days M.L.	19 days S.B.O.	19 days M.L.	26 days S.B.O.	26 days M.L.
P_2O_5	1.3	-	-	.070	.009	-	-
52	10.1	-	-	.010	.005	-	-
58	10.7	-	-	.004	-	-	-
72.6	15.2	-	-	-	-	-	-
81.7	21.2	.026	.035	-	.020	-	.026

S.B.O. = Soya bean oil M.L. = Methyl linoleate

It can be seen from tables 8, 9 and figure 9 that at lower moisture levels (below about 15 %) the methyl linoleate shows little more susceptibility to oxidation than the soya bean oil and only at higher moisture levels is there much difference. At 21 % moisture content it is clear that the soya bean oil is much more resistant to oxidation. At this level of moisture methyl linoleate was oxidized within eight days in contrast to the soya bean oil which showed an induction period of 26 days. This was presumably due to the effect of the natural antioxidants in soya bean oil, which without them would be expected to be

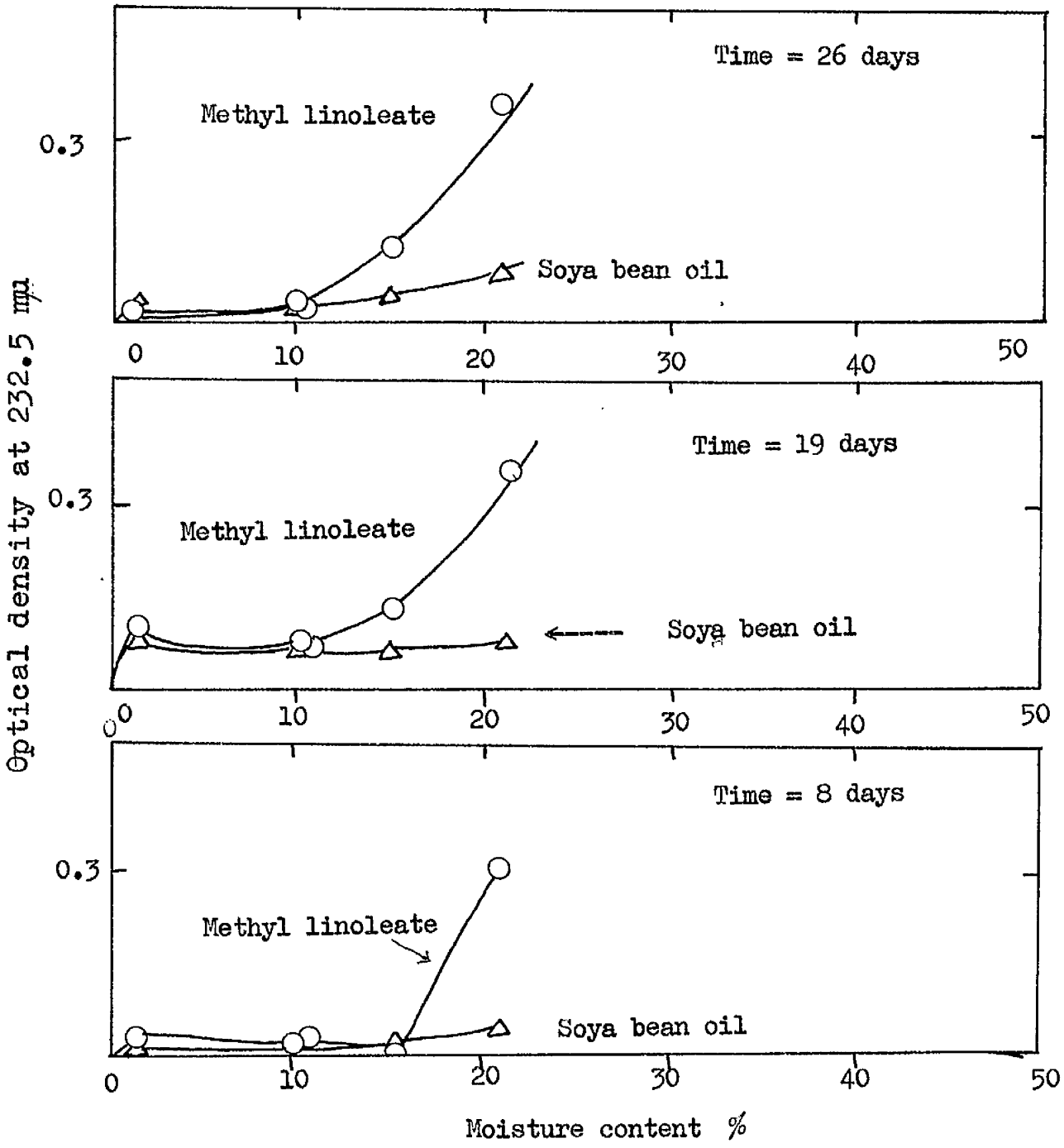


Fig. 9 Variation of oxidation levels of methyl linoleate with change in moisture content of defatted soya bean meal

more readily oxidised than linoleate because of its content of linolenate.

Again there is a marked peroxide decomposition at all the moisture levels examined here.

Effect of the antioxidants on lipoxidase activity (using liquid system)

In the previous experiment, it was demonstrated that the natural lipid soluble antioxidants in soya bean meal are of more importance at low moisture levels than the moisture content in maintaining stability, since there was a considerable difference between the behaviour of soya bean oil and methyl linoleate in the dried system.

In the experiment described here confirmation of this was sought by examining the relation between the natural antioxidants and lipoxidase activity in the liquid system.

Materials and methods

The system used here is given in appendix VIII. The results obtained are shown in table 9A and figure 9A.

Results

It can be seen from figure 9A that the natural antioxidants, present in soya bean oil, affected the reaction to a marked extent. Since soya bean oil has a very high linolenic acid

TABLE (9A. Effect of the antioxidants on lipoxidase activity (using liquid system).

Time	Optical density at 470 millimicrons	
	Methyl linoleate	Soya bean oil
15 seconds	0.060	0.042
1 minute	0.140	0.110
2 "	0.220	0.078
3 "	0.268	0.080
4 "	0.345	0.100
5 "	0.340	0.112
6 "	0.380	0.112
7 "	0.385	0.110
8 "	0.405	0.135
9 "	0.412	0.148
10 "	0.450	0.142
1 hour	0.520	0.200
2 "	-	0.208
3 "	-	0.180
4 "	-	0.188

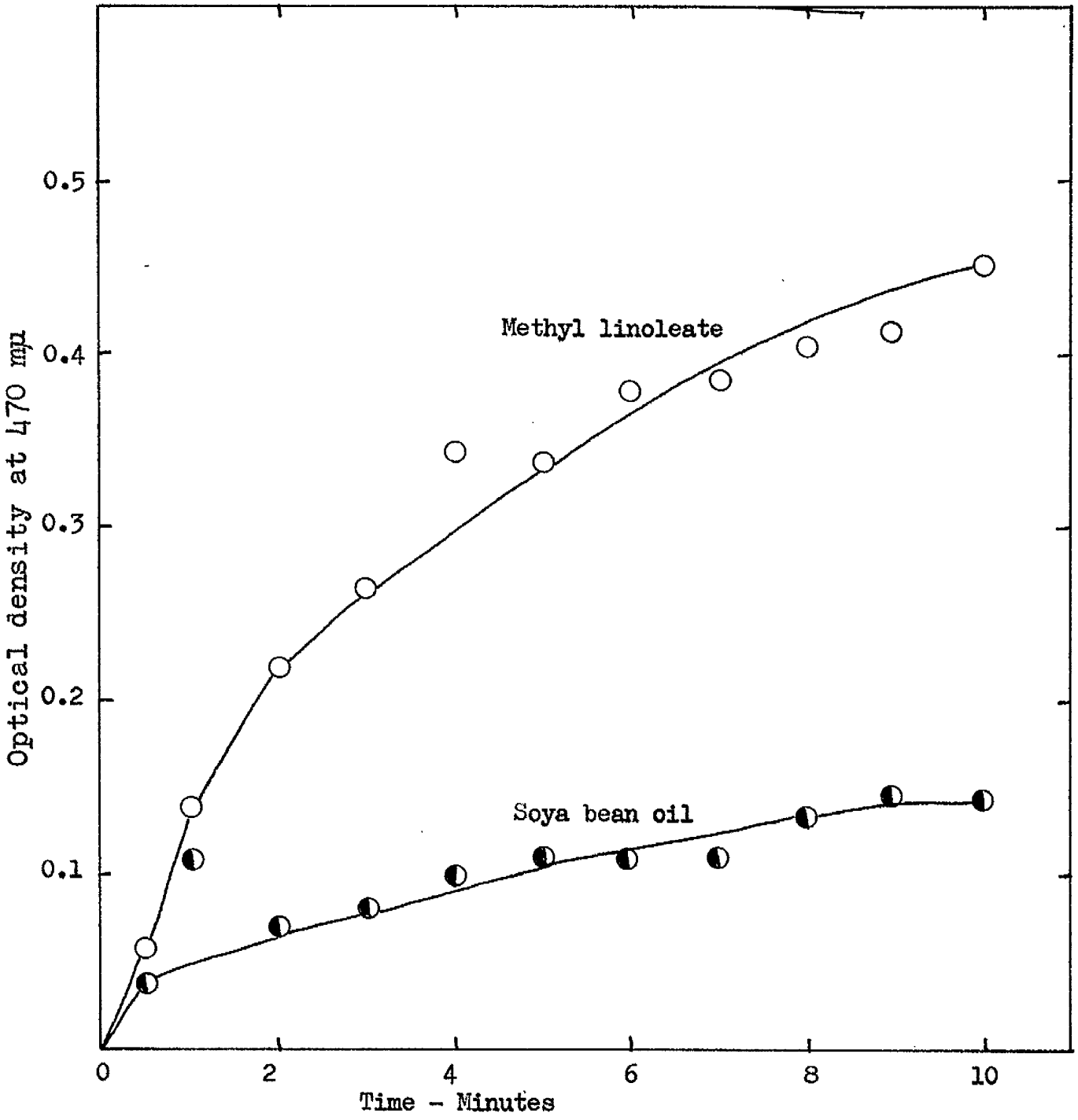


Fig. 9 a Effect of the antioxidants on lipoxidase activity (using liquid system)

content and the other fatty acids present would manifest competitive inhibition to an insignificant extent at the levels present,[185] it seems reasonable to attribute the diminished reaction rate to antioxidant present. Soya bean oil is known to contain tocopherols.

PART (3)

The experiments described suggested that only when free water was available could the enzyme have access to substrate. This raised the point as to whether, if the enzyme were redistributed by wetting and then drying, differences might result.

In the experiment described here, defatted soya flour was first moistened and vacuum dried in the frozen state (see appendix IV.) being well mixed and kept over silica gel until constant weights were achieved. The dried material was then mixed with 10 % of its weight of freshly prepared soya bean oil.

The procedures for humidification and measuring the oxidation were the same as used previously.

Tables 10, 11 and figures 10 to 15 show the data obtained

TABLE 10. Effect of wetting and freeze-drying on lipoxidase catalysis on soya bean oil (diene contents at 232.5 m μ).

R.H. %	Moist. cont. %	Optical density at 232.5 millimicrons 3	4	5	7	10	12	14	22	24 days
P_2O_5	1.3	.020	.054	.027	.015	.018	.009	.022	.053	-
20	7.9	.015	.055	.040	.007	.017	.005	.010	-	-
58	11.0	.037	.085	.063	.042	.060	.058	.060	.104	.069
65	16.6	.175	.195	.195	.133	.150	.143	.130	.233	.185

TABLE 10. (continued)

R.H. %	Moist. cont. %	Optical density at 232.5 millimicrons 3	4	5	7	10	12	14	22	24 days
72.6	15	.107	.145	.110	.110	.118	.150	.110	.430	.281
81.7	21	.165	.245	.192	.171	.208	.189	-	.335	.239
88	25.5	.264	.297	.297	.273	.325	.360	-	-	-
93	40	1.255	1.440	1.400	1.400	1.550	1.450	1.202	1.500	-

TABLE 11. Effect of wetting and freeze-drying on lipoxidase catalysis on soya bean oil (peroxide values at 470 m u).

R.H. %	Moist. cont. %	Optical density at 470 millimicrons 3	4	5	7	10	12	14	22	24 days
P_2O_5	1.3	.040	.040	.040	.045	.012	.030	.020	.040	-
20	7.4	.040	.040	.035	.035	.022	.045	.020	-	-
58	11.0	.040	.045	.035	-	.020	.005	.030	.030	.015
65	16.6	.090	.090	.075	.065	.060	.070	.050	.102	.105
72.6	15.0	.070	.070	.080	.065	.050	.075	.035	.310	.225
81.7	21.0	.070	.120	.060	.065	.040	.058	.050	.070	.065
88	25.5	.162	.170	.170	.225	.200	.200	-	-	-
93	40.0	1.600	1.620	1.520	1.520	1.570	1.755	1.600	1.670	-

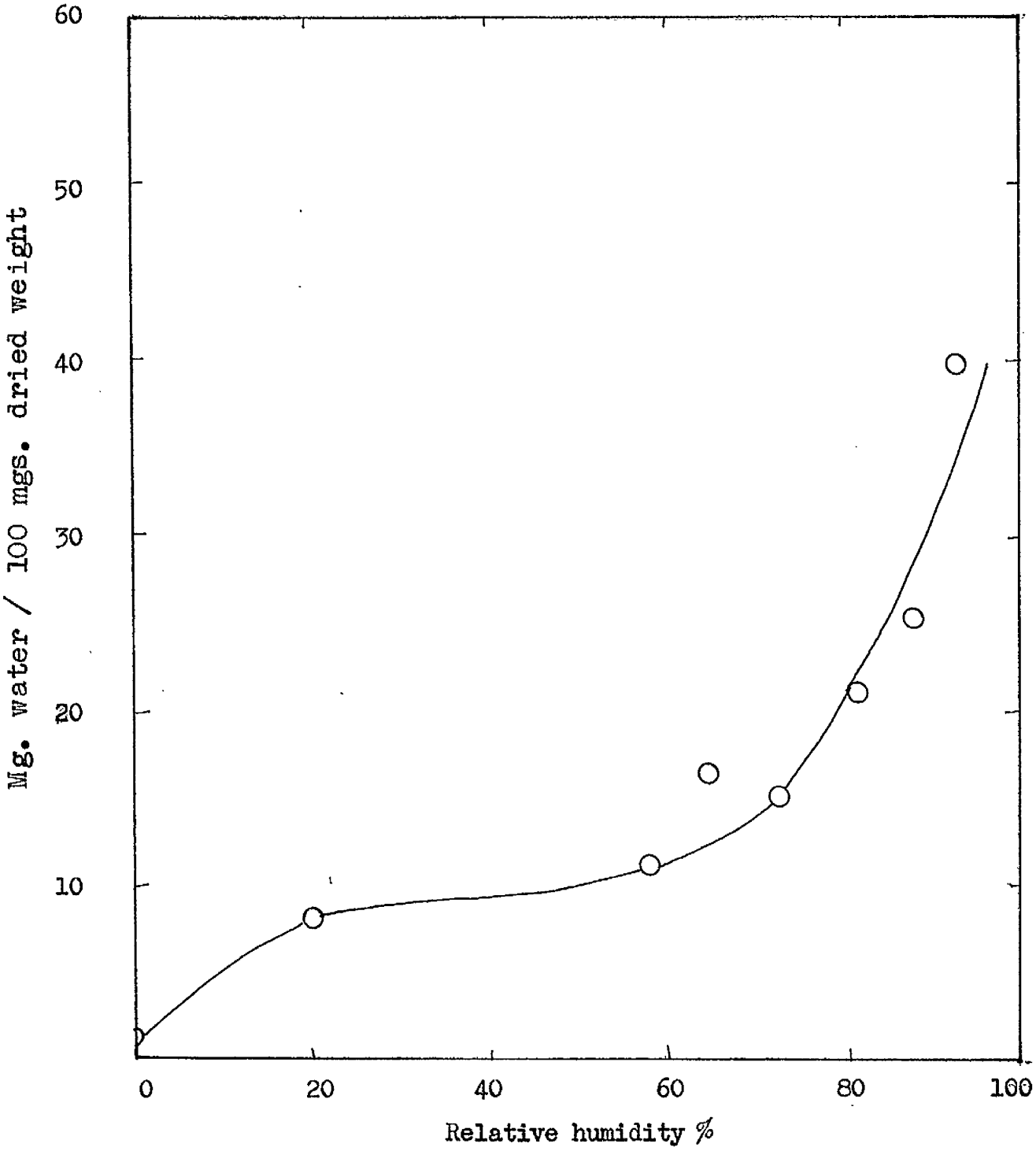


Fig. 10 Effect of freeze-drying on the sorption isotherm of soya bean flour

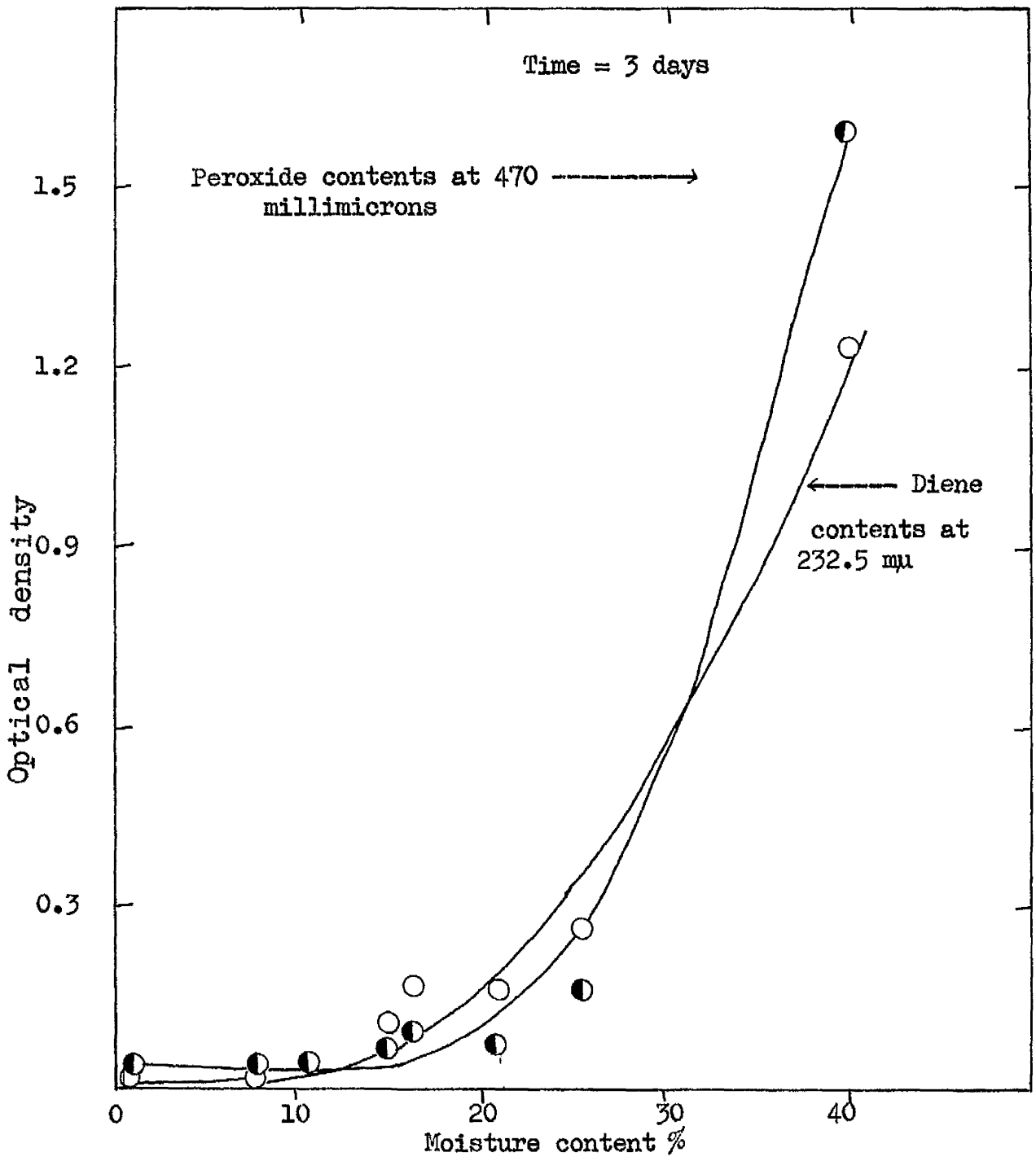


Fig. 11 Variation of oxidation levels of soya bean oil with change in moisture content of soya meal after pretreatment by wetting and freeze-drying

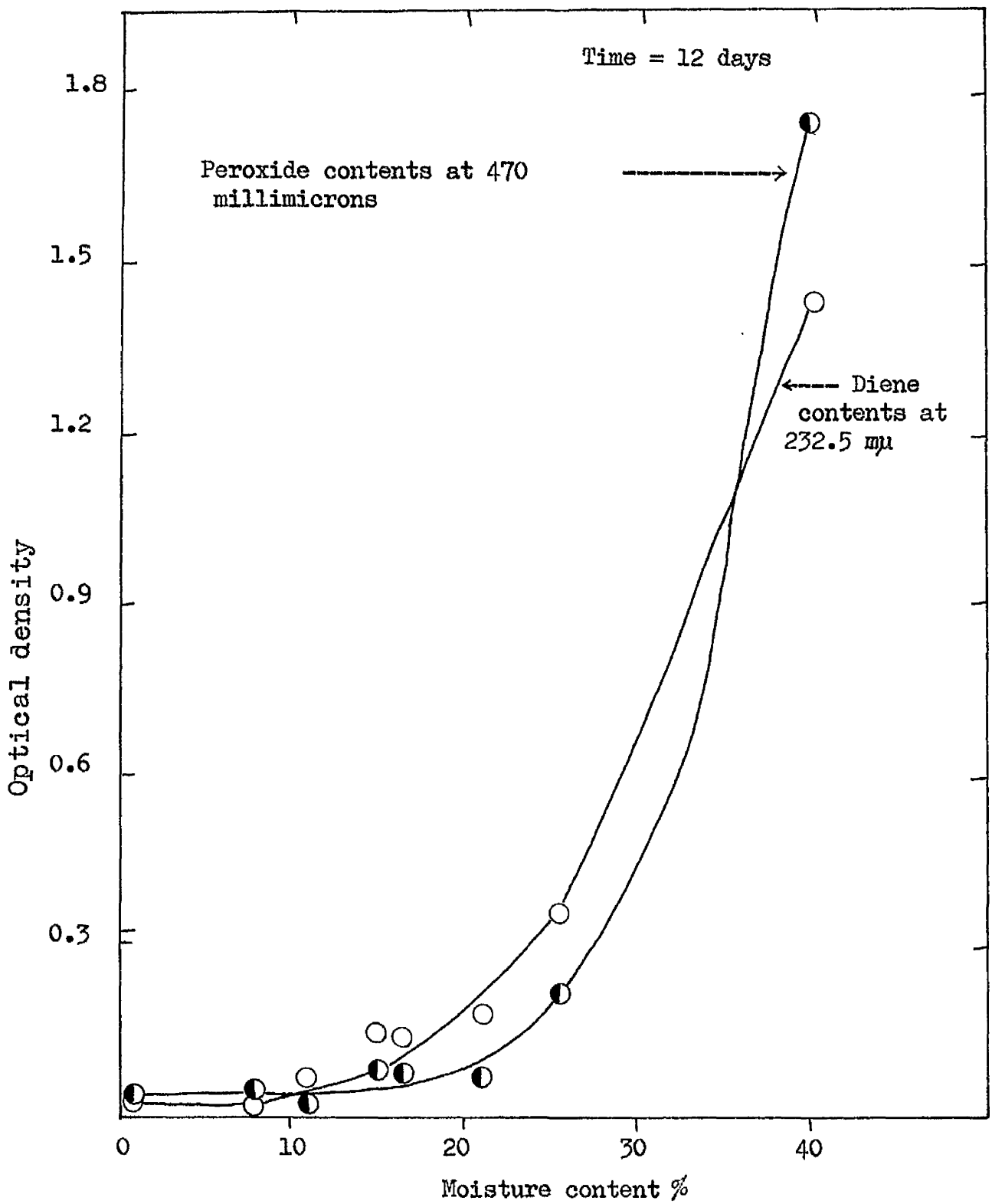


Fig.12 Variation of oxidation levels of soya bean oil with change in moisture content of soya meal after pretreatment by wetting and freeze-drying

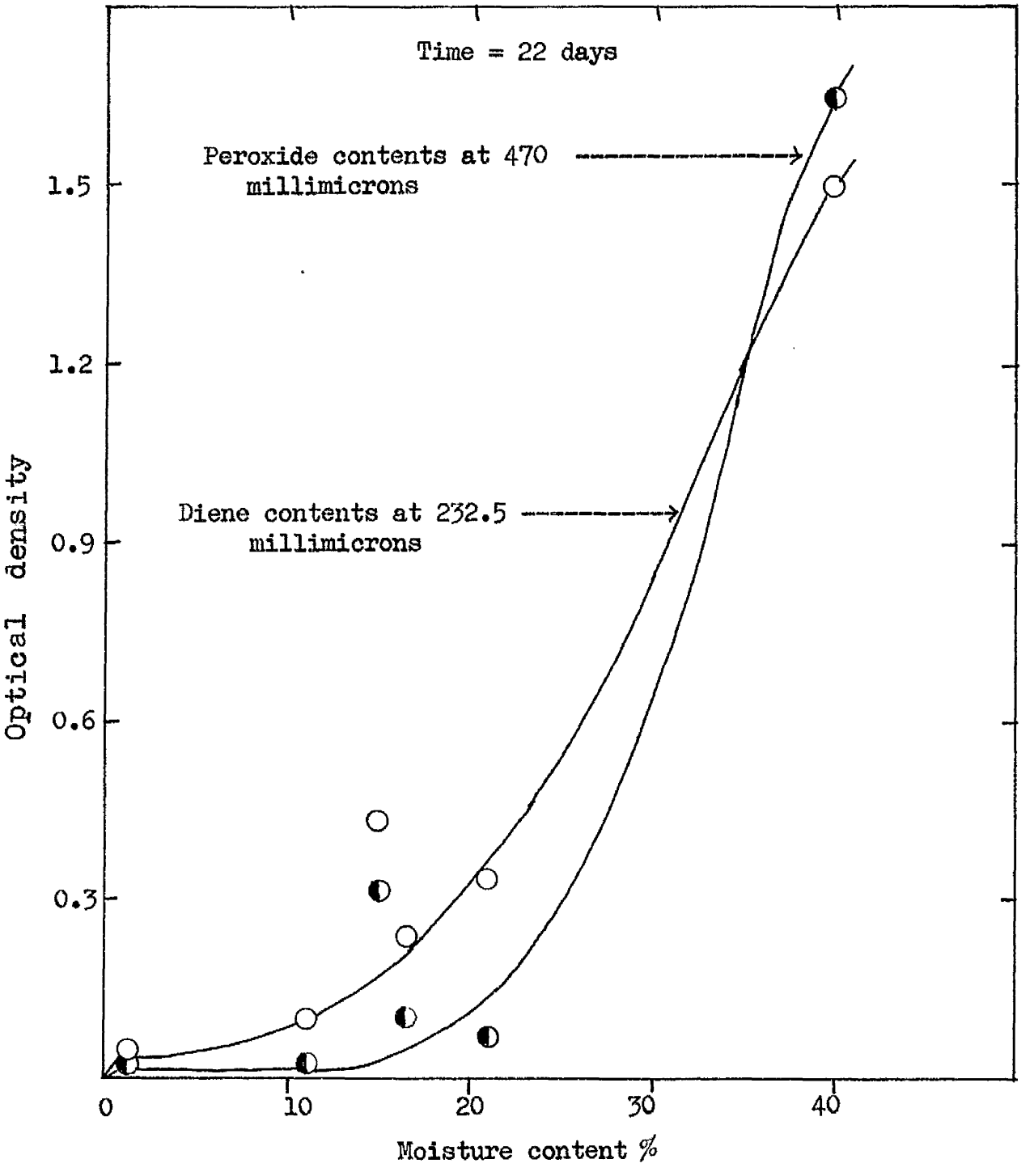


Fig. 13 Variation of oxidation levels of soya bean oil with change in moisture content of soya meal after pretreatment by wetting and freeze-drying

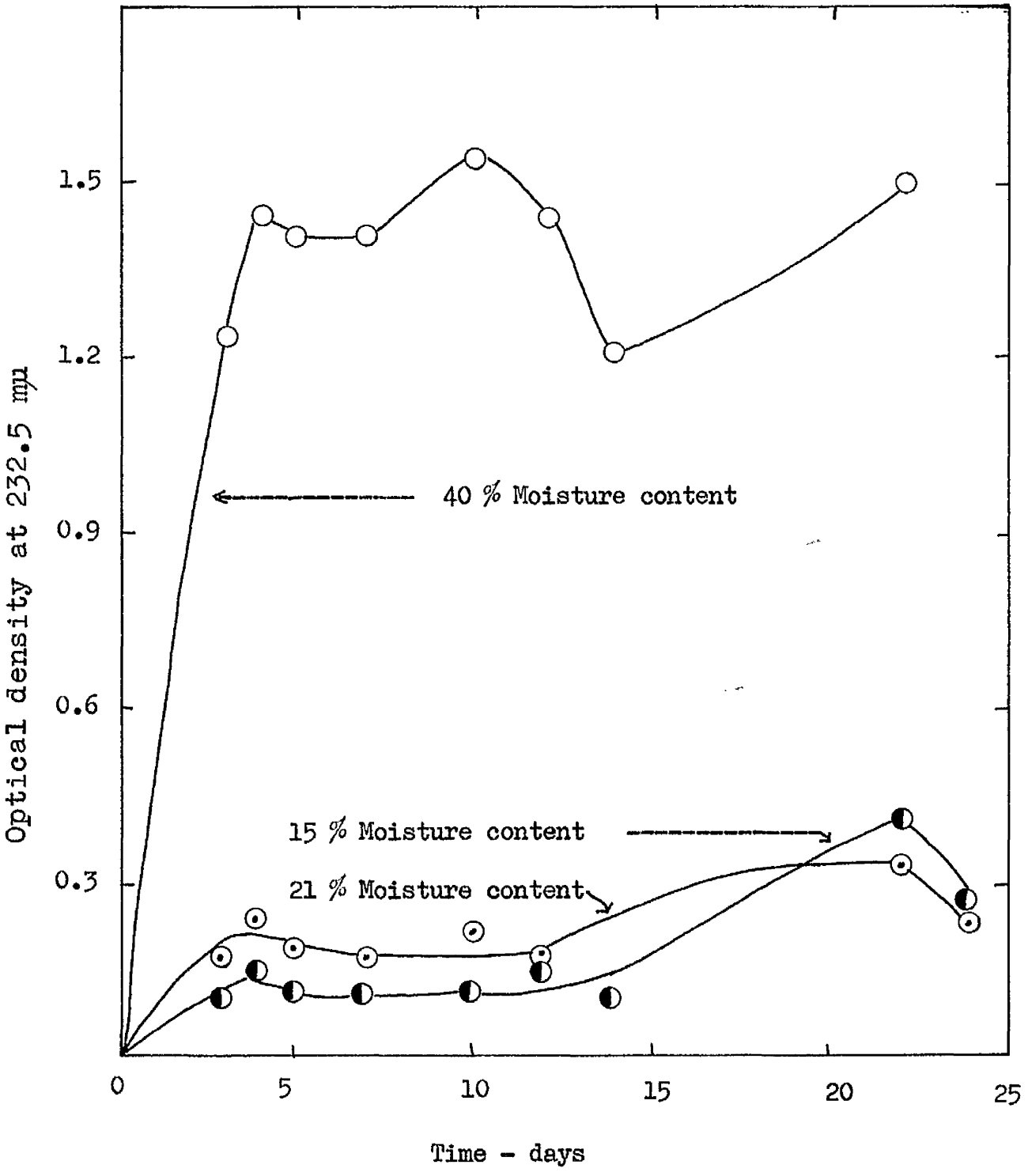


Fig. 14 Rate of change of oxidation levels of soya bean oil

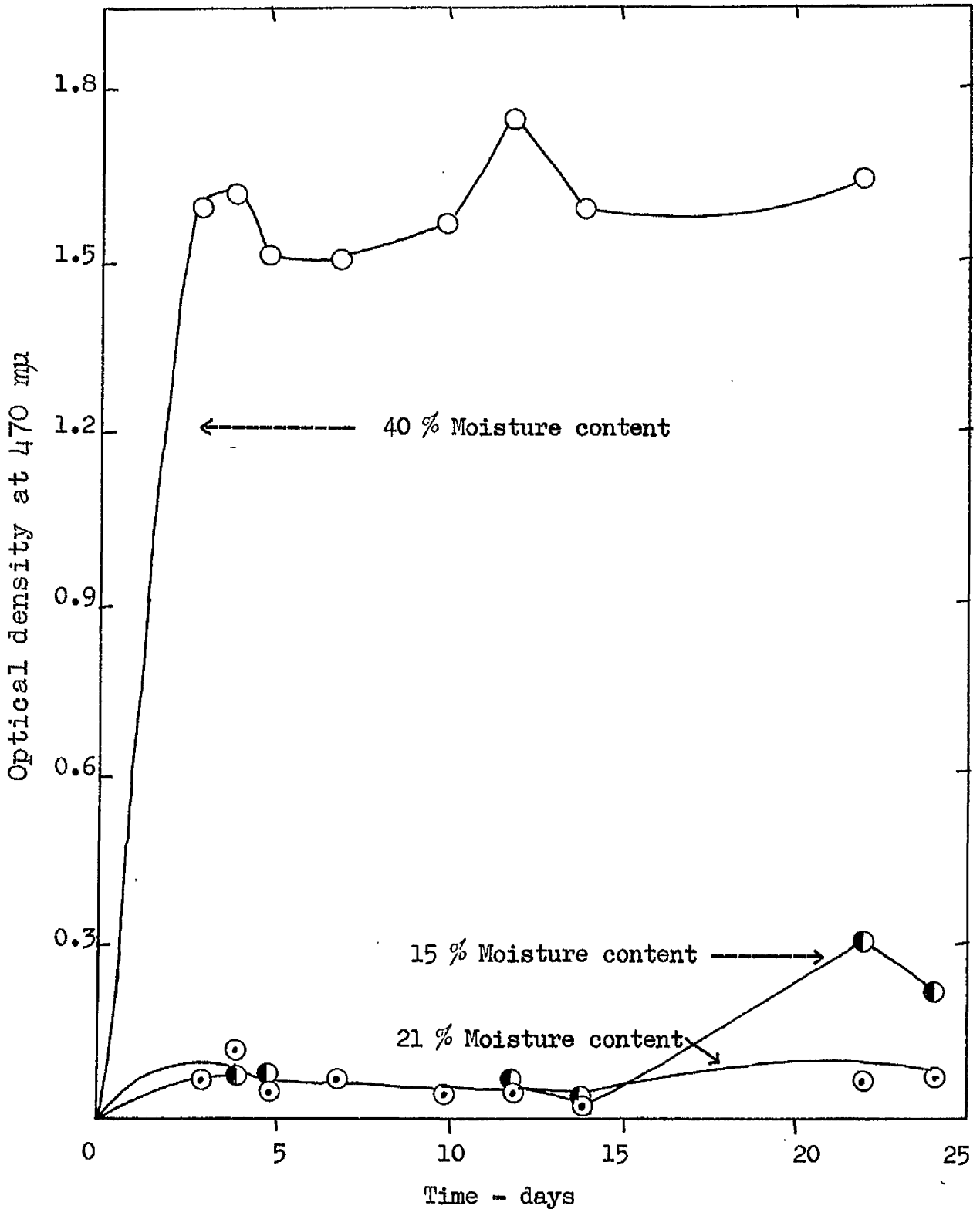


Fig. 15 Rate of change of oxidation levels of soya bean oil

The data obtained, shown in tables 10, 11 and figures 10 to 15 , indicated that altering the ordered state in soya bean flour through moistening and freeze-drying has affected the rate of autoxidation of soya bean oil to some extent. At all the moisture levels examined here, the peroxide values were higher than that obtained in part (1) and part (2) and in general rates of oxidation were very much increased.

The pattern of oxidation being greater at higher moisture levels than at the lower ones persisted. At moisture levels as low as 15 % , oxidation is now observed.

From figures 14 and 15 it can be seen that at 40 % moisture content, oxidation is very rapid and thereafter the level of oxidised product fluctuates. This phenomenon is not uncommon in fat oxidation. The rate of decomposition of linoleate hydroperoxide and conjugated diene tends to become greater as its concentration increases so that an unsteady equilibrium is reached.

PART (4)

Influence of the matrix on lipoxidase activity

It has already been shown that wetting and drying gives high oxidation rate in soya bean meal presumably by making enzyme and substrate more available to each other. In the experiment described here the lipoxidase (and other water-soluble substances) were removed and mixed with another matrix to see whether the matrix was of major importance in oxidation rates.

Crude lipoxidase was first extracted from defatted soya flour, with water as described in appendix V., being freeze-dried and kept in a desiccator over silica gel to remove as much water as possible. The dried material was then mixed with potato starch (2 parts of freeze-dried crude lipoxidase : 3 parts of potato starch) so that the lipoxidase extracted would be present in the same weight of mixture as in the original soya.

The mixture was then mixed with 10 % of its weight of freshly prepared sample of soya bean oil and humidified as described in appendix I.

Oxidation was followed from time to time by measuring both conjugated diene and peroxides.

TABLE 12. Influence of the matrix on lipoxidase catalysis of soya bean oil (diene contents at 232.5 m u).

R.H. %	Moist. cont. %	Optical density at 232.5 millimicrons 2	5	7	9	12	16	23	27 days
P_2O_5	1.3	.030	.031	.005	.034	.009	.040	.103	.073
20	7.1	.025	.030	.006	.035	.006	.040	.108	.070
33	8.3	.025	.041	.009	.035	.009	.045	.130	.075
58	14.6	.040	.055	.023	.045	.012	.067	.118	.095
66	15.7	.045	.060	.058	.128	.282	.129	.256	.266
72.6	18.5	.050	.071	.040	.072	.050	.098	.171	.143
81	20.5	.068	.070	.046	.080	.085	.095	.175	.136
88	24.0	.100	.100	.096	.120	.138	.141	.238	.220
93	36.4	.117	.122	.113	.175	.180	.281	-	-

TABLE 13. Influence of the matrix on lipoxidase catalysis on soya bean oil (peroxide contents at 470 m u).

R.H. %	Moist. cont. %	Optical density at 470 millimicrons 2	5	7	9	12	16	23	27 days
P_2O_5	1.3	.020	.025	.045	.040	.075	.065	.100	.100
20	7.1	.020	.025	.035	.026	.050	.035	.120	.100
33	8.3	.015	.030	.050	.050	.058	.050	.130	.120
58	14.6	.050	.050	.085	.060	.065	.050	.130	.130
66	15.7	.050	.095	.200	.415	.960	.350	.600	.865
72.6	18.5	.065	.090	.100	.125	.152	.150	.230	.275
81.7	20.5	.092	.100	.090	.130	.140	.155	.220	.215
88	24.0	.212	.215	.245	.280	.315	.300	.400	.485
93	36.4	.225	.290	.310	.420	.550	.745	-	-

TABLE 14. Influence of the matrix on lipoxidase catalysis on soya bean oil (peroxide contents at 470 mμ).

R.H. %	Moist. cont. %	Optical density at 470 millimicrons 2	3	4	5	6	9	10	11 days
P_2O_5	2	.040	.065	.070	.090	.120	.120	.140	.110
20	8.9	.010	.045	.030	.045	.032	.065	.053	.045
33	10.0	.010	.040	.050	.060	.050	.055	.062	.055
58	14.3	.020	.065	.040	.070	.060	.060	.085	.060
65	19.7	.100	.100	.160	.330	.375	.320	.315	.310
72.6	17.2	.080	.115	.120	.140	.180	.165	.170	.135
81.7	20.4	.070	.125	.140	.175	.190	.210	.245	.225
88	24.8	.125	.315	.345	.555	.810	1.030	.880	1.060
93	31.0	.930	1.610	1.610	1.520	1.520	1.900	1.635	1.620

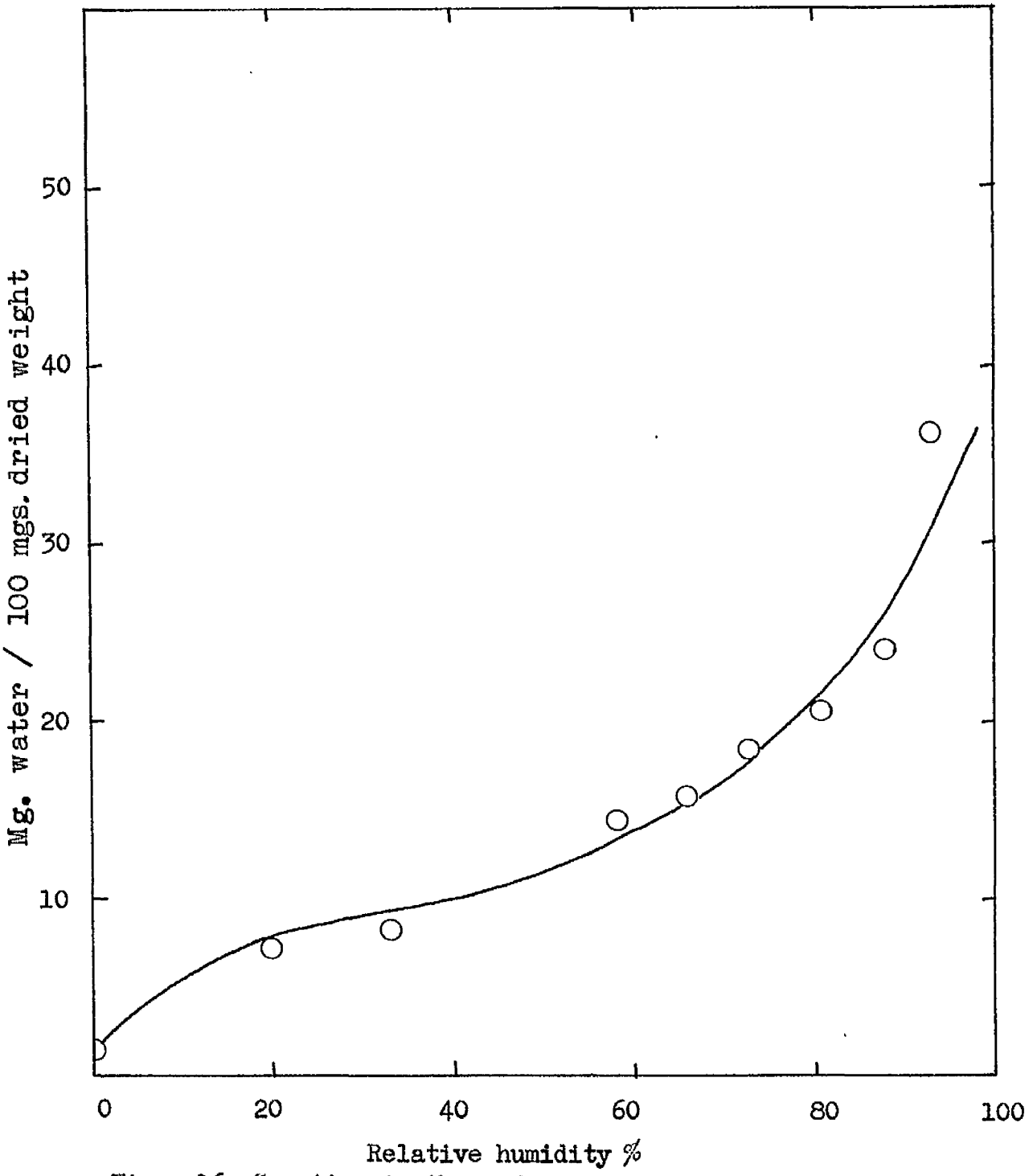


Fig. 16 Sorption isotherm for potato starch +freeze-dried soya extract at 20°C.

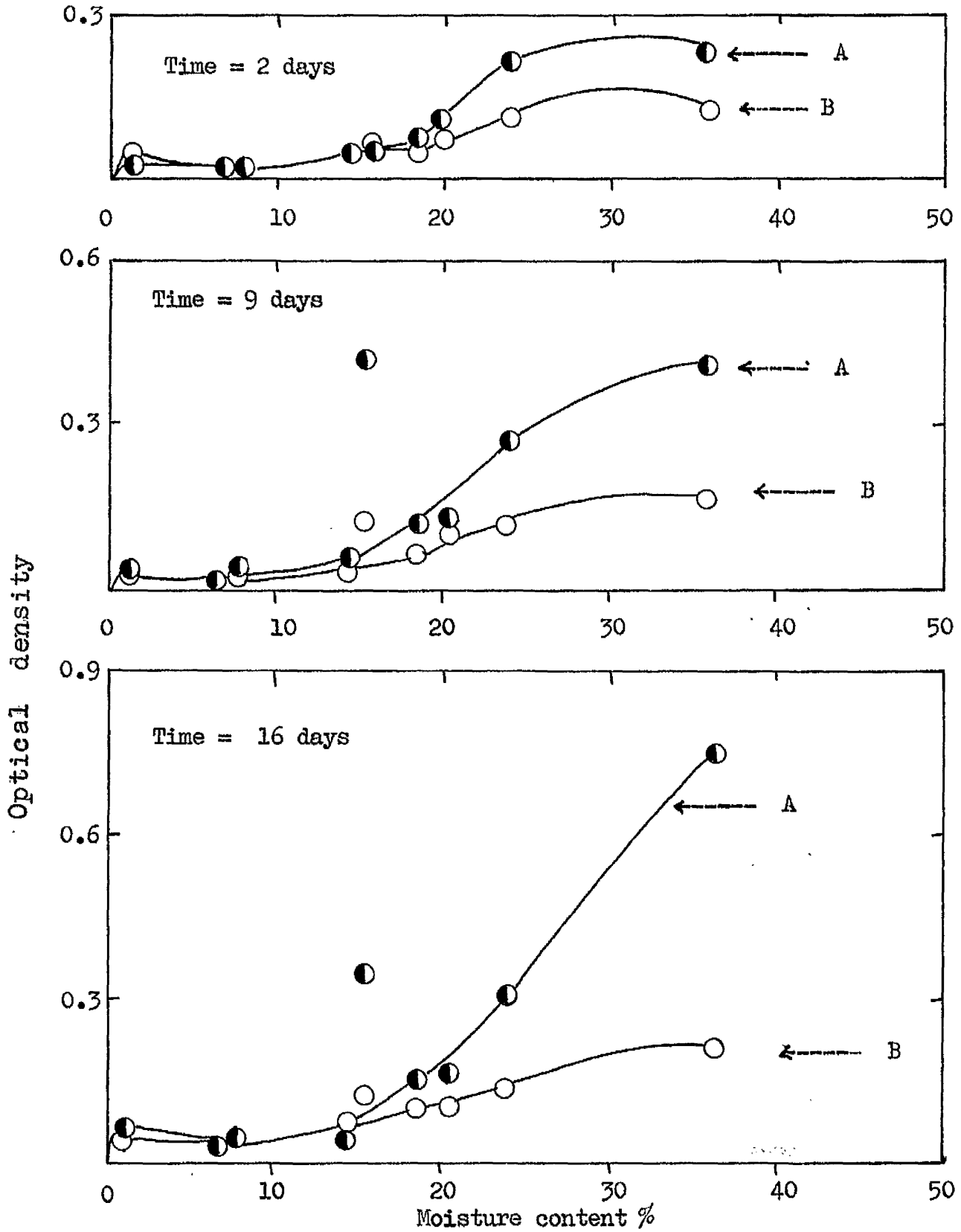


Fig. 17 Influence of moisture levels on oxidation of soya bean oil using matrix of starch with freeze-dried crude lipoxidase

A = Peroxide contents at 470 millimicrons

B = Diene contents at 232.5 millimicrons

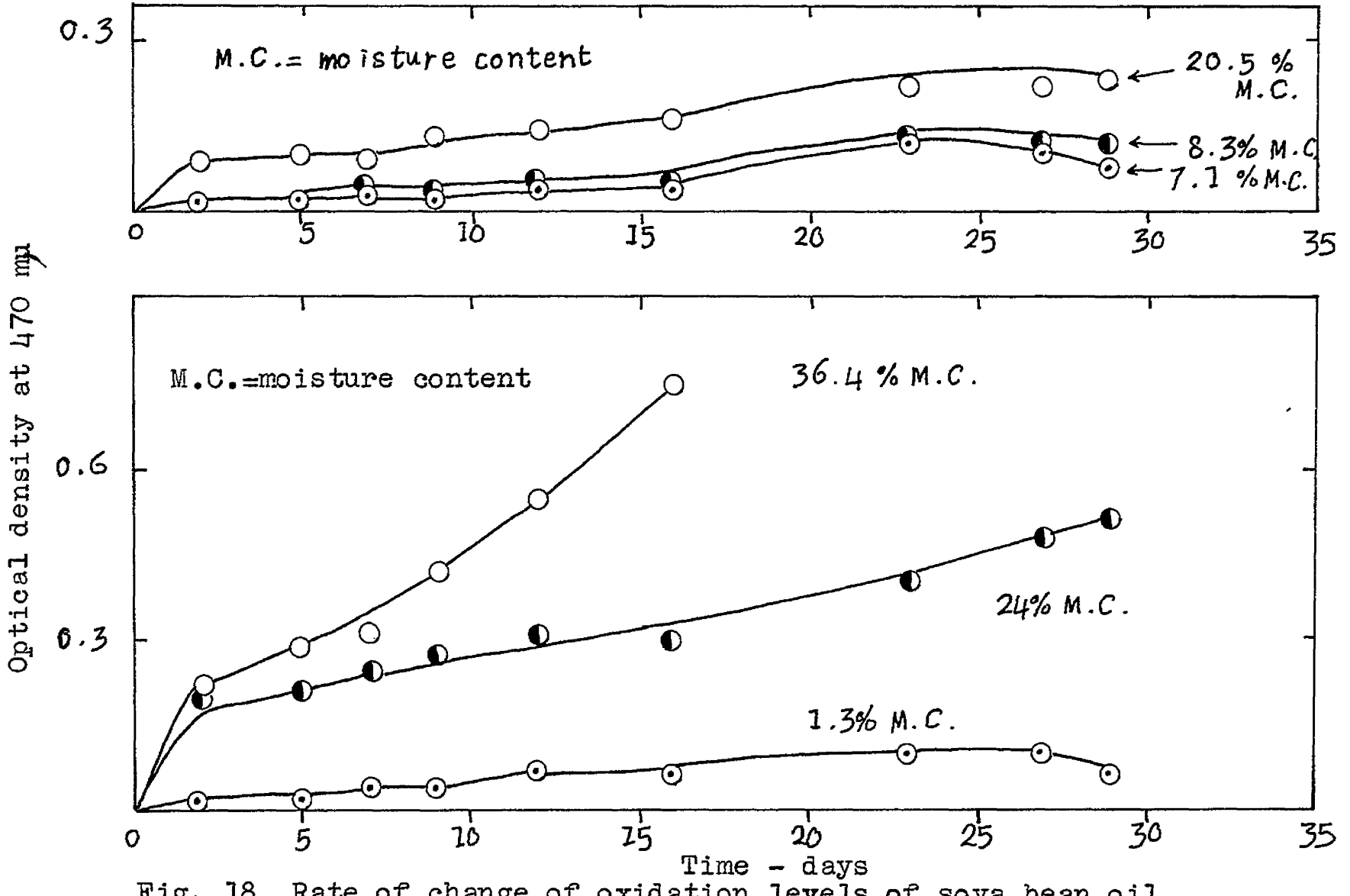


Fig. 18 Rate of change of oxidation levels of soya bean oil using matrix of potato starch + freeze-dried crude lipoxidase

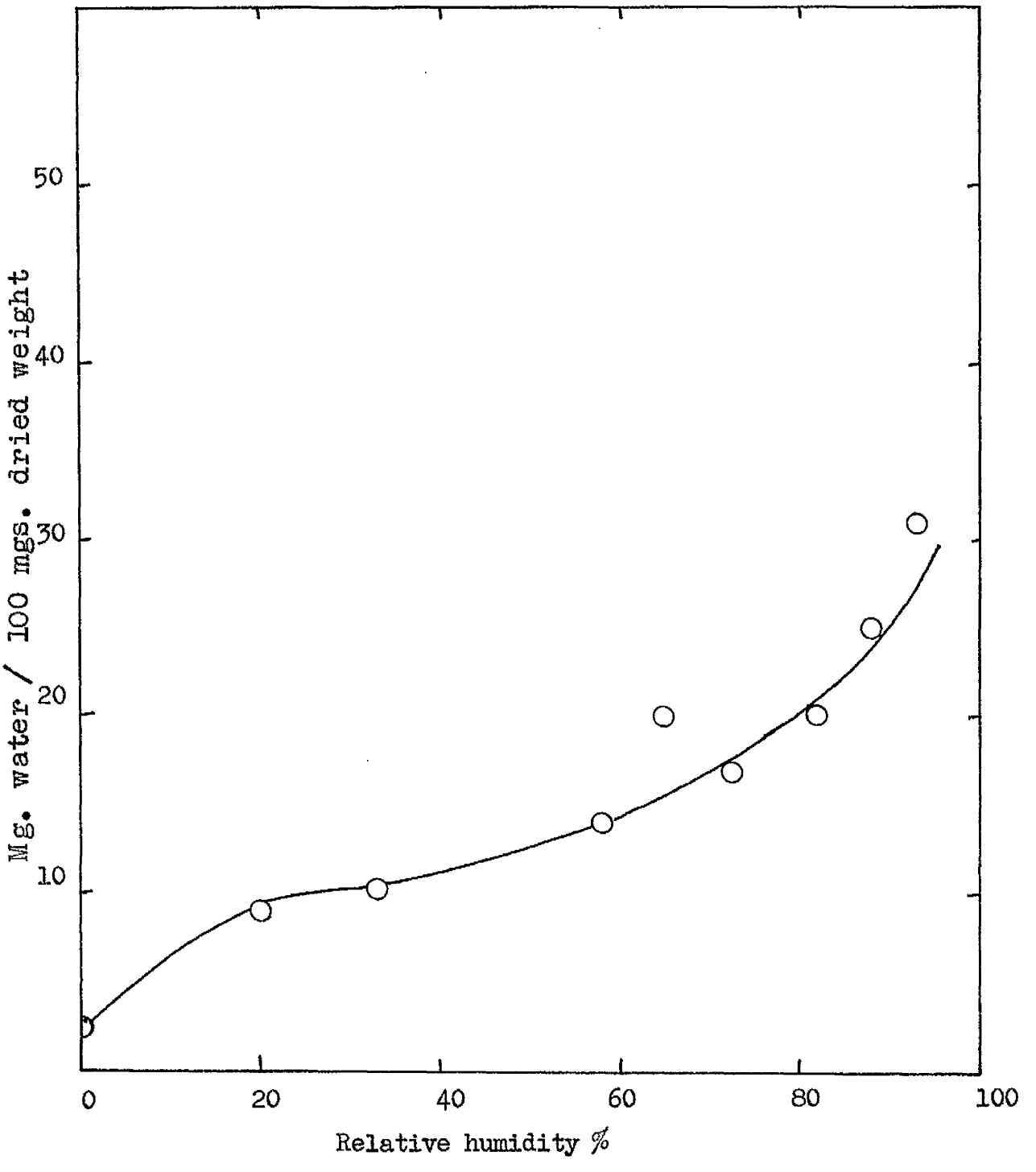


Fig. 19 Variation of moisture content of potato starch + freeze-dried soya extract with change in relative humidity at 20°C.

Results

The results obtained, shown in tables 12, 13 and figures 16 - 18, indicated that the matrix has a considerable effect on lipoxidase activity since oxidation rates were greater and in general, the peroxide values obtained were higher relative to the conjugated diene values than in previous findings. This point will be discussed subsequently.

It would appear that at a moisture content of 14 % to 15 % there is a peak in lipoxidase activity. However the peroxide value seemed anomalously high in relation to diene.

To check this point another experiment was commenced. In the second experiment magnesium acetate was used for humidity control instead of sodium nitrite. The results of this experiment are shown in table 14.

Although magnesium acetate is said to give the same percentage of relative humidity at 20°C. (R.H. 65 %), unfortunately it gave a high moisture content than predicted by the tables as can be seen in figure 19.

It was assumed that the apparent high oxidation using sodium nitrite was due to decomposition and liberation of oxides of nitrogen.

Lentils and potato as sources for lipoxidase

The purpose of this experiment was to compare the effect of moisture levels on lipoxidase activity in both of lentils and potato, which have been shown to be sources of lipoxidase action with a pattern similar to that of soya, lentil being a source of comparable activity to soya,[186] and dried potato being considerably less active on a weight basis.[187] The potato provides a matrix low in proteins.

Lentils was first milled to pass a 60 mesh sieve, defatted in a soxhlet apparatus for about 18 hours and the ether was evaporated under an infrared lamp.

Potato was first washed and the skin removed, then homogenised and freeze-dried. The freeze-dried material was ether-defatted in a soxhlet apparatus for about 18 hours and the ether was evaporated under an infrared lamp, to avoid moistening.

Both defatted lentils and defatted potato were stored in a desiccator over P_2O_5 for about one week in order to remove as much water as possible, then each mixed with 1 % of its dried weight of fresh methyl linoleate. Both lentil flour and freeze-dried potato powder were conditioned to a different relative humidities as described in appendix I.

The results obtained are shown in tables 14 - 17 and figures 20 - 23.

TABLE 14. Influence of moisture levels on oxidation of methyl linoleate with defatted lentil flours (diene contents at 232.5 m u).

R.H. %	Moist. cont. %	Optical density at 232.5 millimicrons 3	5	6	8	11	19	21 days
P_2O_5	1.7	.000	.009	.019	.033	.030	.052	.035
15	9.2	.007	.000	.009	.020	.020	.010	.005
32	10.5	.009	.010	.009	.018	.023	.017	.005
58	15.0	.027	.010	.020	.027	.035	.038	.018
88	24.6	.029	.027	.039	.010	.048	.055	.050
93	37.3	.031	.087	.134	.139	.130	.091	.075
97	40.6	.123	.124	.118	.114	.112	.092	.075

TABLE 15. Influence of moisture levels on oxidation of methyl linoleate with defatted lentil flours (peroxide contents at 470 m u).

R.H. %	Moist. cont. %	Optical density at 470 millimicrons 3	5	6	8	11	21 days
P_2O_5	1.7	.014	.015	.015	.015	.020	.040
15	9.2	.000	.010	.010	.010	.010	.010
32	10.5	.005	.010	.005	.010	.010	.010
58	15.0	.005	.010	.005	.010	.010	.025
88	24.6	.005	.010	.010	.035	.010	.040
93	37.3	.010	.075	.135	.240	.165	.080
97	40.6	.072	.190	.140	.115	.080	.070

TABLE 16. Influence of moisture levels on oxidation of methyl linoleate with freeze-dried potato (diene contents at 232.5 m u).

R.H. %	Moist. cont. %	Optical density at 232.5 millimicrons 2	4	7	15	17 days
P_2O_5	1.0	.023	.158	.365	.137	.100
15	6.3	.073	.212	.343	.104	.085
33	12.4	.002	.024	.000	.025	.023
64	18.5	.031	.045	.003	.030	.032
86	25.2	.085	.095	.008	.061	.058
93	40.3	.098	.092	.013	-	-
97	44.4	.093	.085	.000	-	-

TABLE 17. Influence of moisture levels on oxidation of methyl linoleate with freeze-dried potato (peroxide contents at 470 m u).

R.H. %	Moist. cont. %	Optical density at 470 millimicrons 2	4	7	15	17 days
P_2O_5	1.0	.090	.440	.940	.710	.425
15	6.3	.210	.600	.920	.245	.235
33	12.4	.020	.018	.015	.030	.030
65	18.5	.032	.010	.015	.020	.025
88	25.2	.062	.030	.015	.028	.030
93	40.3	.080	.030	.020	-	-
97	44.4	.080	.030	.015	-	-

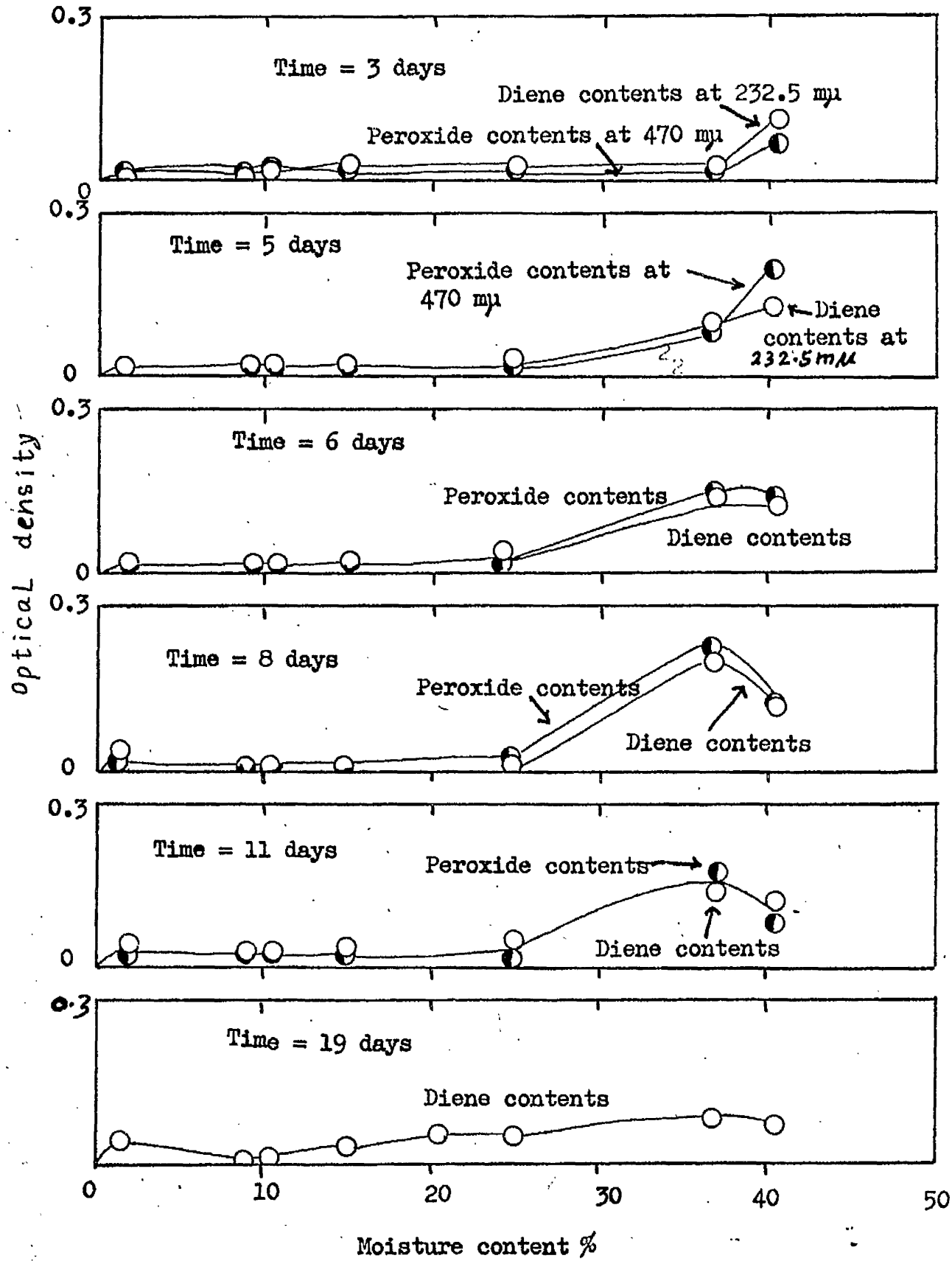


Fig. 20 Influence of moisture levels on oxidation of methyl linoleate with defatted lentil meal.

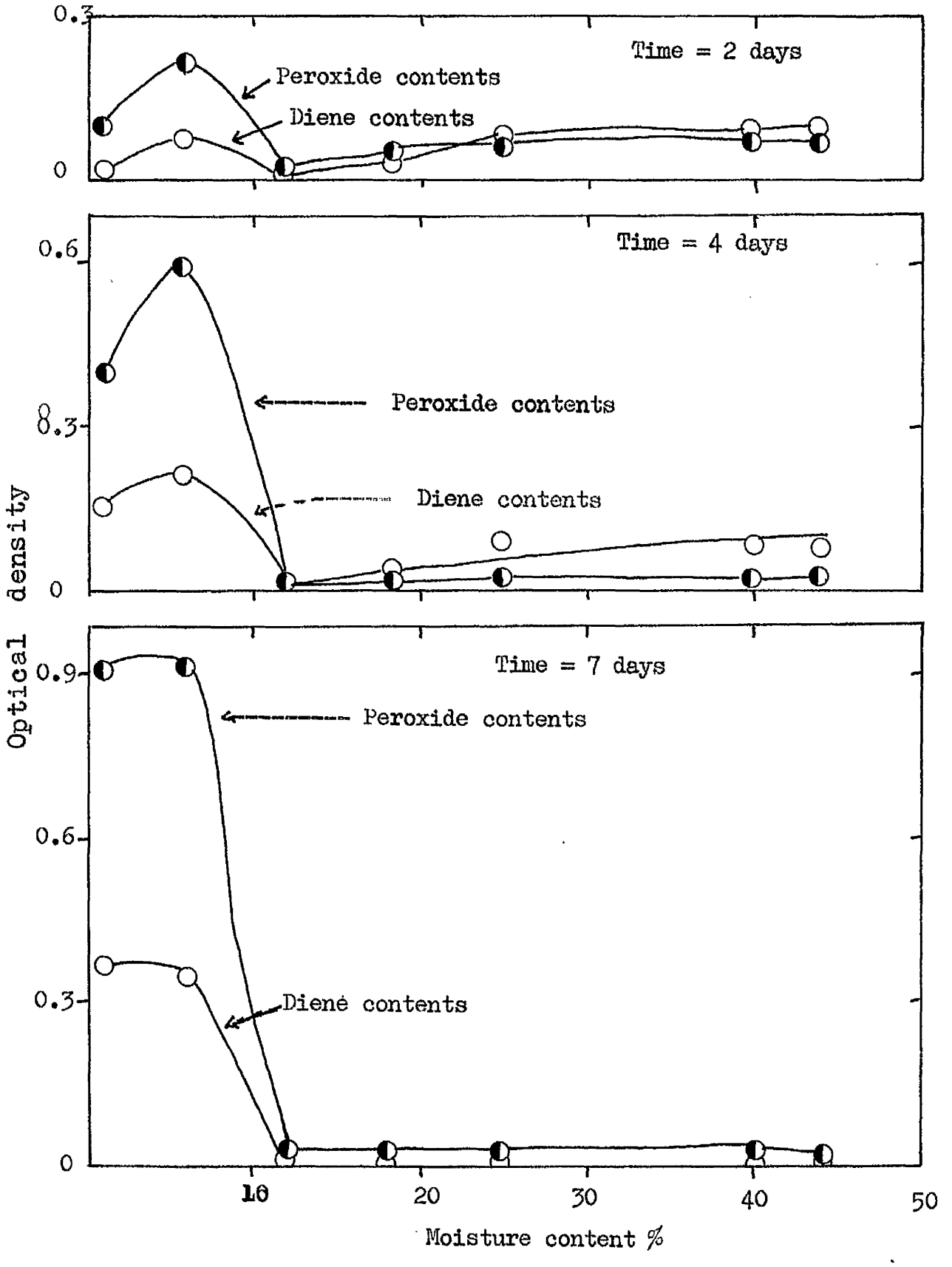


Fig.21 Influence of moisture levels on oxidation of methyl linoleate with freeze-dried potato

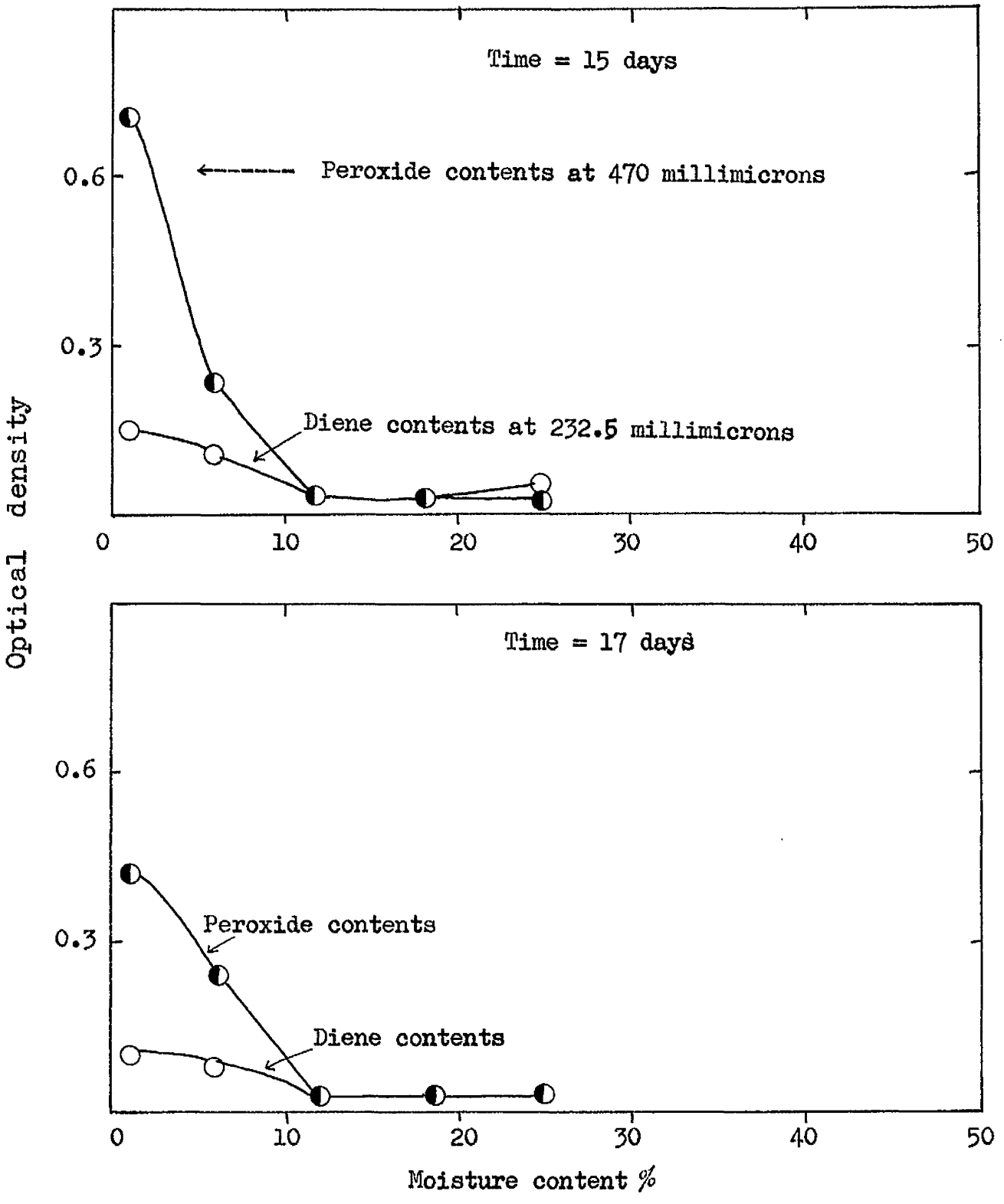


Fig. 22 Influence of moisture levels on oxidation of methyl linoleate with freeze-dried potato

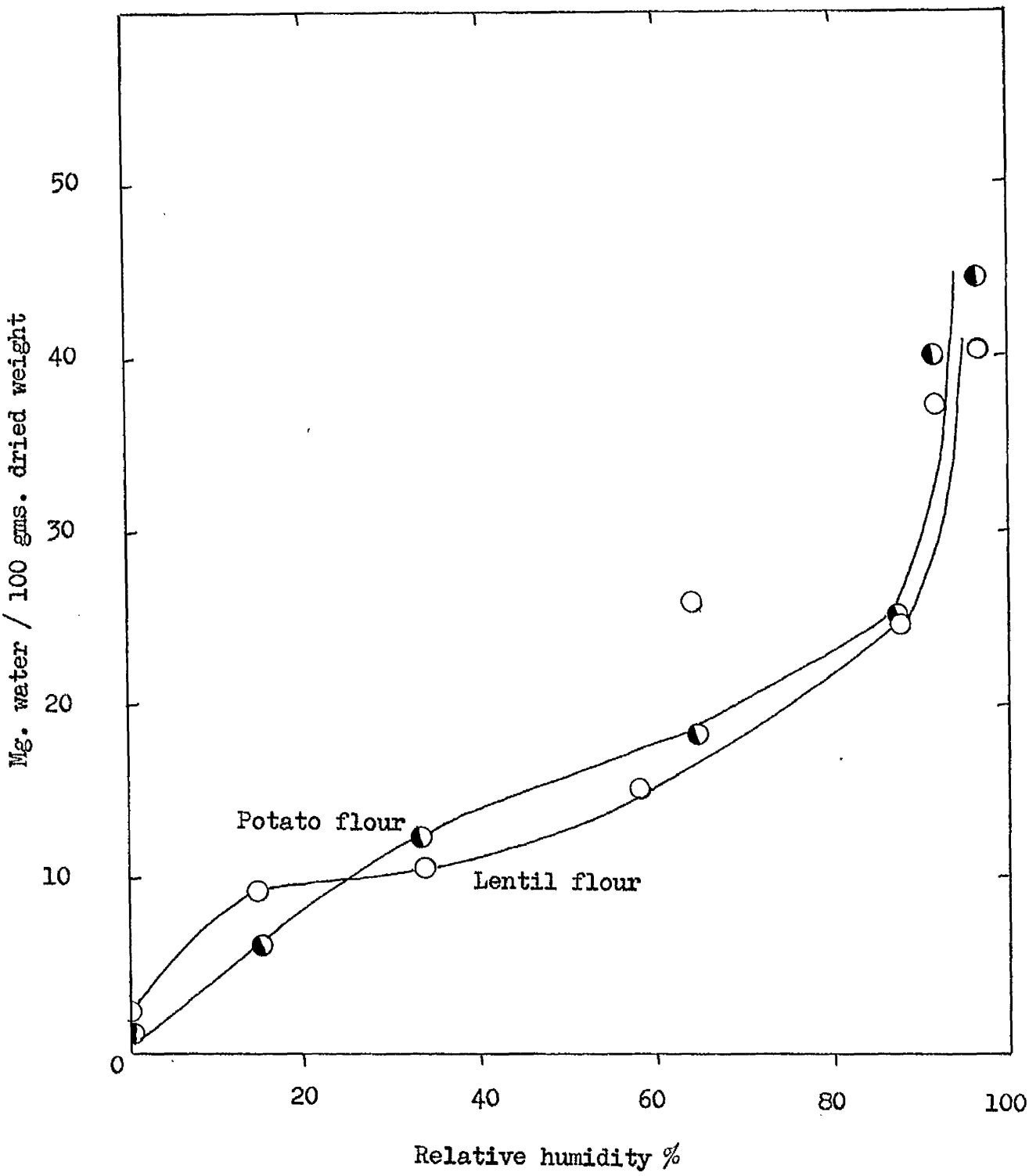


Fig. 23 Sorption isotherms for lentil and potato flours

The results for lentils are not strictly comparable with these for soya since the concentration of linoleate was smaller ($\frac{1}{10}$), in conformity with natural levels of fat.

The lentil flour behaved in a fashion rather similar to the soya flour which had not been wetted in that oxidation at the lower moisture levels (under 25 %) was negligible even after three weeks. Again the resistance of well dispersed methyl linoleate to oxidation is surprising since one assumes that the natural antioxidants of the seed material are removed by ether-defatting.

The potato gives a very different picture from previous results in that oxidation at moisture levels below 10 % occurs more rapidly than at higher levels. This is more in keeping with the general observations by many workers that dried foods oxidise readily at these low moisture levels, where it will be noticed that the ratio of peroxide to conjugated diene is high.

General discussion and conclusions

The results obtained in this section indicated that the effects of moisture levels on the oxidation of unsaturated fats in ground seed material rich in lipoxidase are complex and involve a number of factors but certain points can be made.

In the absence of previous work on the effect of water content on oxidative enzymes it might have been anticipated lipoxidase would have resembled lipase in seeds since both act on fatty substrates. However while lipase is active at moisture levels down to 6 - 7 % it seems that lipoxidase would require levels of between 20 and 30 % unless disrupted from its natural state by wetting and drying when a level of 14 - 15 % appears to initiate action.

It is also clear that the stability of soya meal towards oxidation is not caused only by oil soluble antioxidants in the oil since at low moisture levels methyl linoleate is more stable with soya bean meal than might be expected, as will be seen from subsequont observations with methyl linoleate on starch.

Since the use of freeze-dried crude lipoxidase with starch matrix leads to more rapid oxidation, the non-fat portion of soya, whether due to protein, or some other factor insoluble in ether, appears to be important in stabilizing the fat against oxidation.

A complicating factor is the lack of constant ratio between conjugated diene and thiocyanate peroxide values which, it is suggested, may result from different rates of destruction of the hydroperoxide and conjugated diene parts of the molecule since there appears to be destruction of both at higher oxidation levels.

At the level of accuracy which the method permits one cannot pinpoint any definite moisture level as permitting enzyme action but it is clear that this is likely to be significant at moisture levels over 15 % . This data confirm the work of Hutchins,[188] who found that the soya bean oil quality depends on the moisture content of the soya bean meals at the time of milling. His results indicated that the oil obtained from soya beans of 8 - 12 % moisture content was good, and that between 12 and 13 % moisture content the quality began to deteriorate and became rapidly worse above 13 %.

The oxidation was found to increase more rapidly as higher levels reached about 25 %.

It seems that points of inflection on the absorption isotherm might coincide with these values as can be seen in figure 2 suggesting changes in modes of water binding.

Whether because of difference in catalytic factors or low protein matrix or some other unidentified factors the behaviour

of freeze-dried potato is very different from that of the seed-meals.

The amount of haematin compounds present in vegetable material may also affect both formation of oxidised fat and its destruction but this is very difficult to measure. It was decided to examine haemoglobin (as a typical haematin readily obtained in the pure state) as a catalyst. This seems valid because the work of Tappel suggested that the iron-porphyrins such as haemoglobin, cytochrome c, catalase, and peroxidase are similar in their action in oxidising fats.

Humidification methods are used in subsequent experiments since it was shown in this section that mixing in water is likely to give results which are not consistent with equilibrium moisture content effects.

The humidification methods have the advantage of permitting simultaneous measurements to be made on many samples at different humidities with very simple equipment. Moreover, the level of moisture in all the samples remains practically the same throughout the time of storage.

While it is conceivable that drying at an elevated temperature might give a slightly high values for moisture content, this method has been used because it is relatively rapid and practicable.

CHAPTER III.

EFFECT OF VARIATION IN MOISTURE CONTENT ON HAEMATIN CATALYSED FAT OXIDATION

It was suggested in the previous chapter that haematin compounds in soya bean meal might complicate the oxidation of lipids by lipoxidase and it seemed useful to devise model systems to examine the effects of moisture level on haematin-catalysed oxidation of unsaturated fats. This is also of intrinsic interest since the importance of haematins in oxidation rancidity in meat products is known and there has been speculation on their role in producing fat oxidation in plant products. In view of their high activity towards unsaturated fats and their occurrence in most natural products this seems likely.

Haemoglobin was used in most of these studies since it may be obtained as a relatively pure and defined material. Tappel has shown that the other iron-porphyrin proteins act in a very similar fashion to haemoglobin in promoting oxidation of linoleate.

Potato starch (B.D.H. Chem. Division) was used as a matrix except where otherwise stated. Soya bean oil has been used as a substrate mostly.

No previous reports have appeared on the effect of moisture levels on haematin catalysed fat oxidation although the experiments of Koch[201] have indicated that haemoglobin has greater activity in freeze-dried emulsions of unsaturated oils than in the untreated emulsions.

Before examining the action of haemoglobin on lipids in dry systems it seemed advisable to confirm that haemoglobin would oxidise not only methyl linoleate but also soya bean oil in liquid emulsion systems to obtain some impression of the antioxidants of soya bean oil with haemoglobin since it has been shown that lipoxidase catalysis and haemoglobin catalysis show different sensitivity to different antioxidants.[189]

The procedures used here were the same as those given in appendix VIII. The results obtained are shown in table 18.

TABLE 18. Haemoglobin catalyzed fat oxidation (using liquid system).

Time	Optical density at 470 millimicrons			
	A Methyl linoleate		Soya bean oil	
	A	B	A	B
Blank	.014	.029	.030	.050
10 minutes	.084	.089	.037	.020
30 "	.107	.094	.014	.011
1 hour	.114	.114	.014	.024
2 "	.129	.131	.024	.024
3 "	.169	.149	—	.015
4 "	.164	.154	—	.015
24 "	.255	.235	.135	.205
26 "	.245	.225	.195	.185
45 "	.170	.200	.065	.085
48 "	.180	.215	.095	.085

From table 18, it can be seen that the soya bean oil show a markedly greater induction period.

It can also be seen that peroxide values reach a maximum value then tend to decrease, a feature which is marked in the "dry" systems.

Comparison between lipoxidase and haemoglobin catalysis of soya bean oil oxidation at different moisture levels

The object of this experiment was to compare the effect of moisture levels on lipoxidase and haemoglobin activity.

In experiment described here, purified haemoglobin (Sigma chemical Co.) was used.

Lipoxidase was prepared from defatted soya flour as described in appendix V, then freeze-dried. The freeze-dried material was stored in a desiccator over silica gel for seven days in order to remove as much water as possible.

Starch was dried over P_2O_5 for one month to achieve an initially low moisture level. This was 3.7 % since natural granular starch dries very slowly. Water was then mixed in simply by adding in small amounts with mixing and weighing to reach the desired levels. The crude lipoxidase or haemoglobin, dispersed in dried starch, was then mixed in.

Measurement of oxidation was carried out by extracting samples with ethanol and using aliquots for hydroperoxide and conjugated diene estimation (see appendix II).

The results obtained are shown in tables 19 and 20. Figures 24 - 30 show the difference between the two catalysts.

TABLE 19. Comparison between lipoxidase and haemoglobin catalysis of soya bean oil oxidation at different moisture levels.

Moist. cont. %		Optical density at 232.5 millimicrons (diene contents) 3	4	6	10	11	12	13	17	20 days
3.7	L.	.010	-	-	.000	.010	.015	.062	.011	.025
	Hb.	.012	-	-	.023	.012	.029	.072	.036	.113
8.7	L.	.012	-	-	.018	.010	.016	.061	.034	.025
	Hb.	.013	-	-	.166	.178	.194	.261	.083	.046
13.7	L.	.014	-	-	.020	.011	.017	.067	.046	.023
	Hb.	.014	-	-	.172	.180	.194	.270	.099	.051
23.7	L.	.018	-	-	.025	.012	.018	.077	.032	.024
	Hb.	.015	-	-	.056	.033	.052	.110	.181	.130
33.7	L.	.021	-	-	.040	.014	.038	.104	.050	.134
	Hb.	.021	-	-	.075	.067	.110	.184	.171	.184
43.7	L.	.041	-	-	.066	.046	.076	.171	.169	.201
	Hb.	.041	-	-	.245	.268	.283	.353	.181	.075

L. = Lipoxidase Hb. = Haemoglobin

TABLE 20. Comparison between lipoxidase and haemoglobin catalysis of soya bean oil oxidation at different moisture levels.

Moist. cont. %		Optical density at 470 millimicrons (peroxide contents) 3	4	6	10	11	12	13	17	20 days
3.7	L.	-	-	-	-	-	.020	-	-	.015
	Hb.	-	-	-	.030	.030	.045	.040	.075	.290
8.7	L.	-	-	-	-	-	.015	-	.018	.023
	Hb.	-	-	-	.422	.515	.635	.715	.236	.179
13.7	L.	-	-	-	-	-	.033	-	-	.018
	Hb.	-	-	.083	.427	.511	.611	.683	.337	.188
23.7	L.	-	-	-	-	-	.035	-	-	.062
	Hb.	-	-	.025	.062	.081	.087	.100	.181	.362
33.7	L.	-	-	-	.030	.035	.057	.057	.075	.242
	Hb.	-	-	.071	.121	.164	.242	.285	.435	.500
43.7	L.	-	.055	.083	.116	.150	.183	.183	.466	.608
	Hb.	-	.081	.358	.616	.783	.650	.850	.455	.233

L. = Lipoxidase Hb. = Haemoglobin

The data obtained from these studies indicates that haemoglobin acts as a more powerful oxidative catalyst than does lipoxidase at the concentration used. It was found to catalyze the oxidation of soya bean oil at low and high moisture levels, whereas the activity of lipoxidase appears to be negligible below 30 % moisture and the reaction rate is most susceptible to

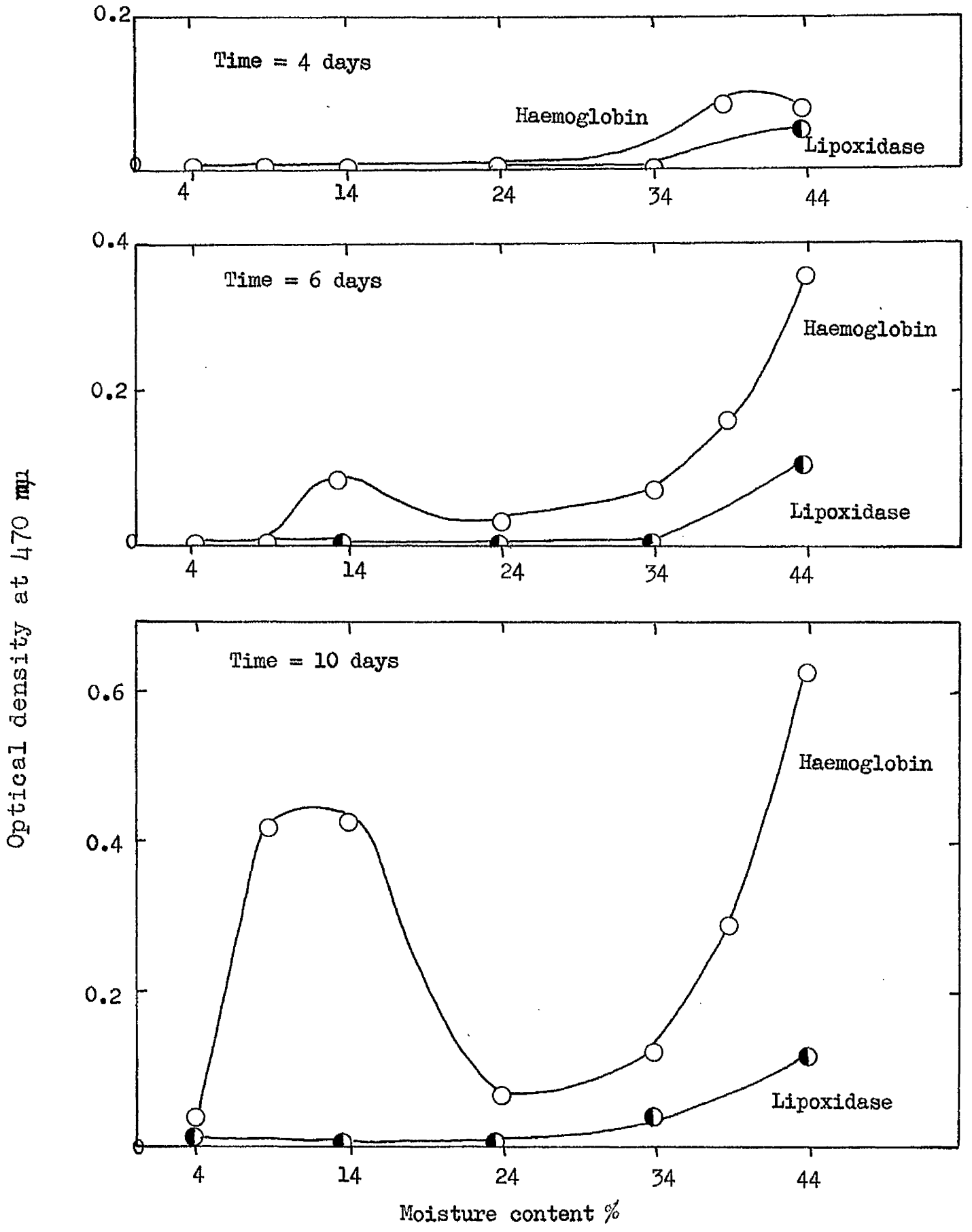


Fig. 24 Comparison between haemoglobin and lipoxidase activity at the different moisture levels

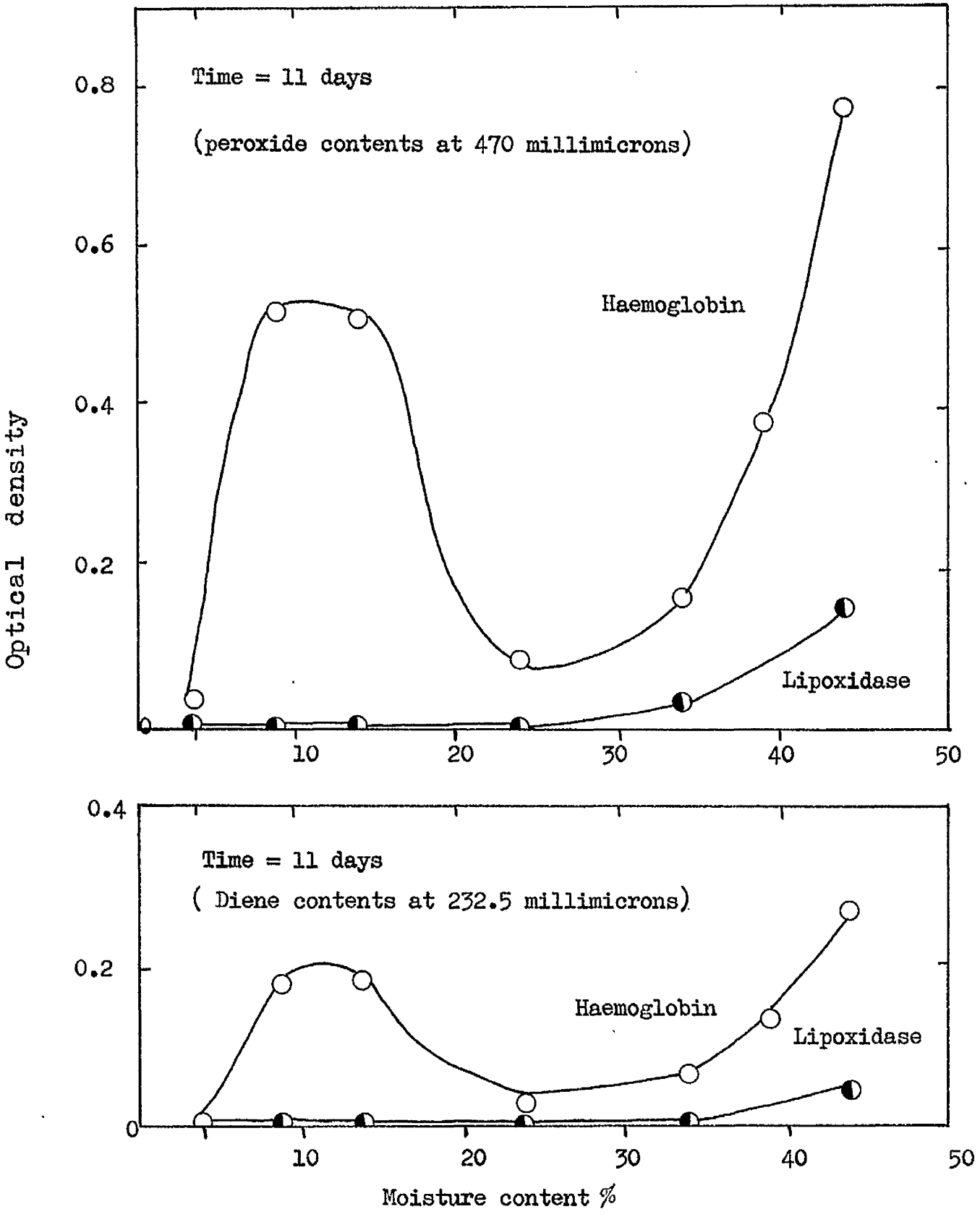


Fig. 25 Comparison between haemoglobin and lipoxidase activity at the different moisture levels

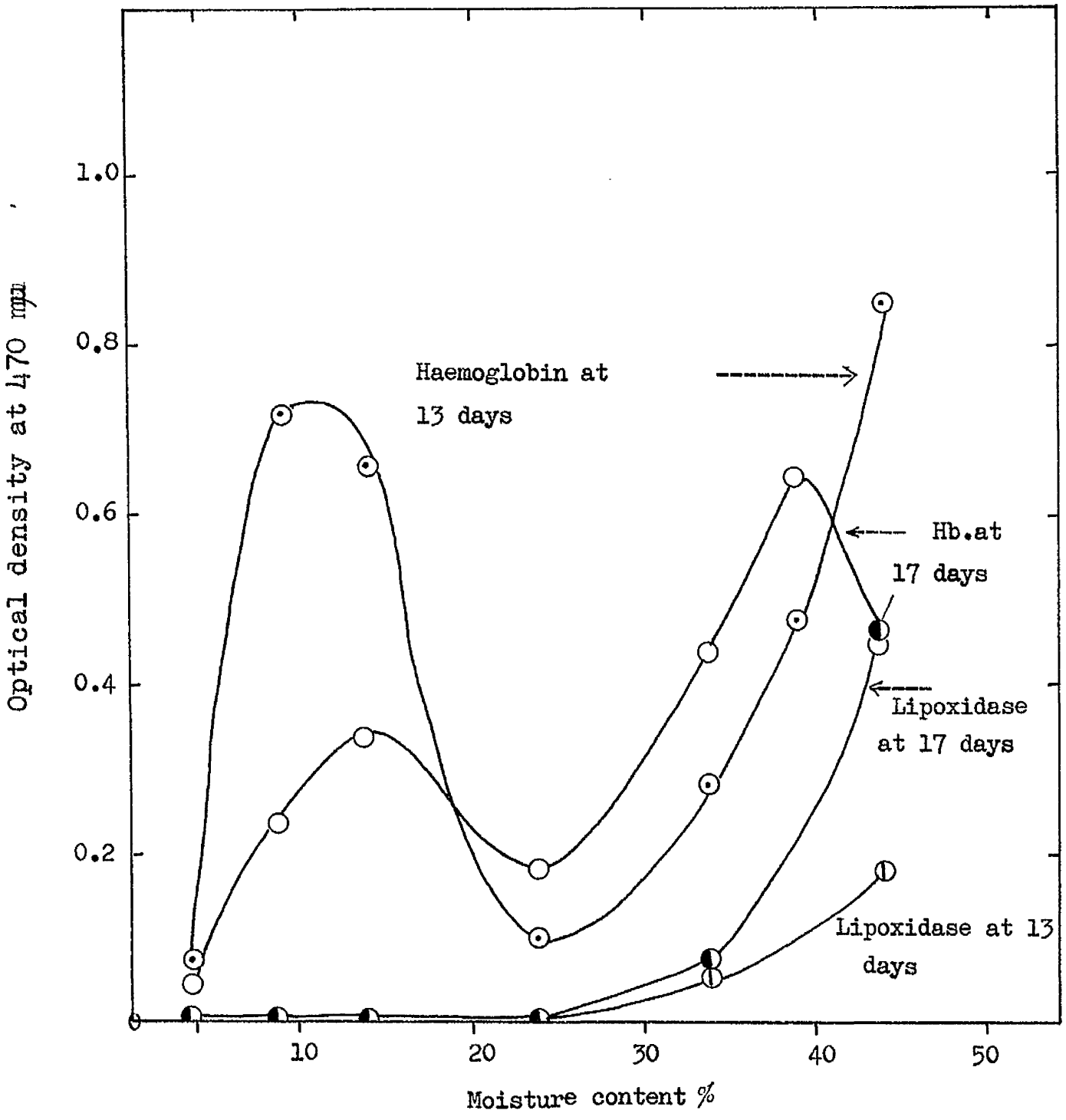


Fig. 26 Comparison between haemoglobin and lipoxidase activity at the different moisture levels

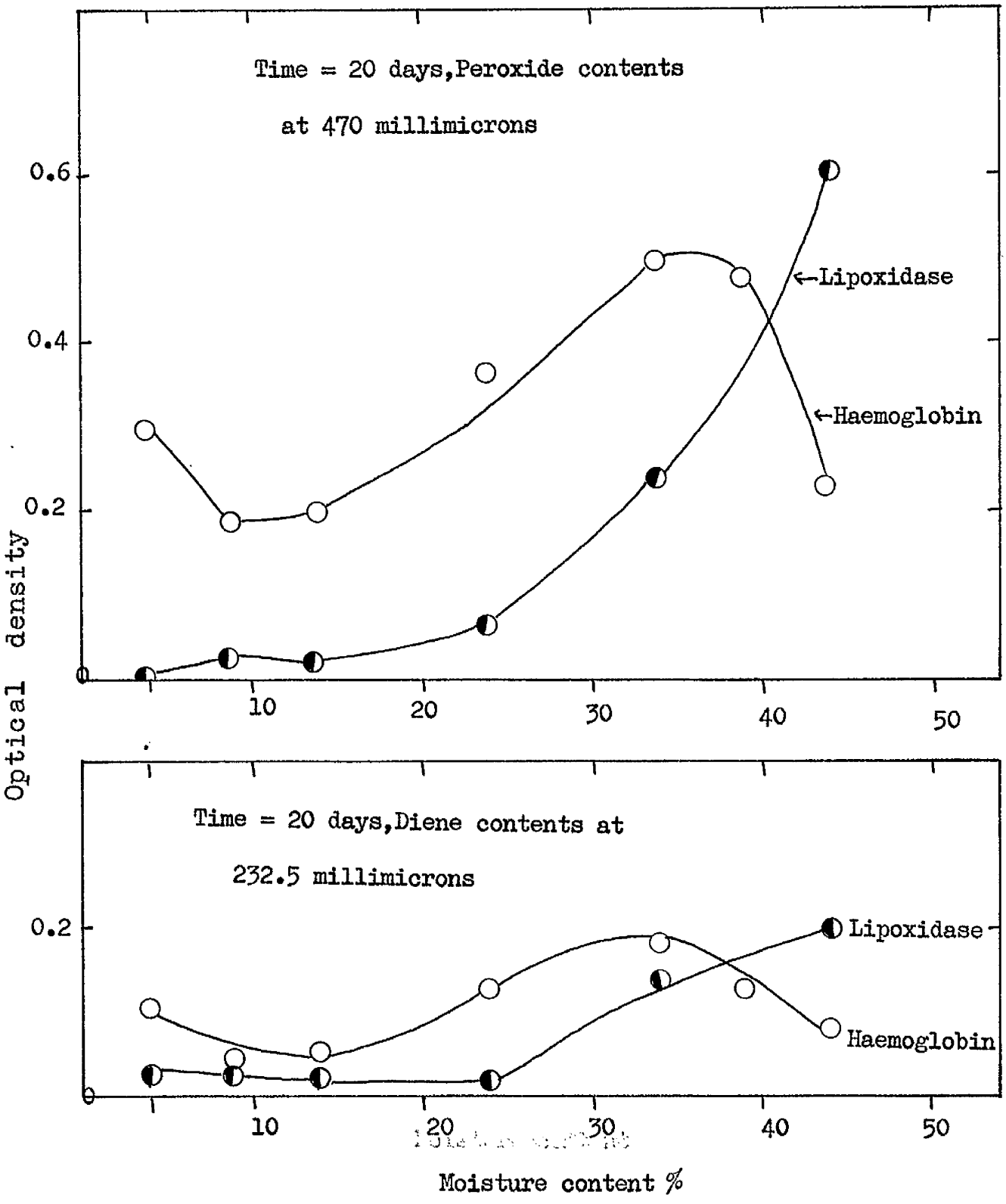


Fig. 27 Comparison between haemoglobin and lipoxidase activity at the different moisture levels

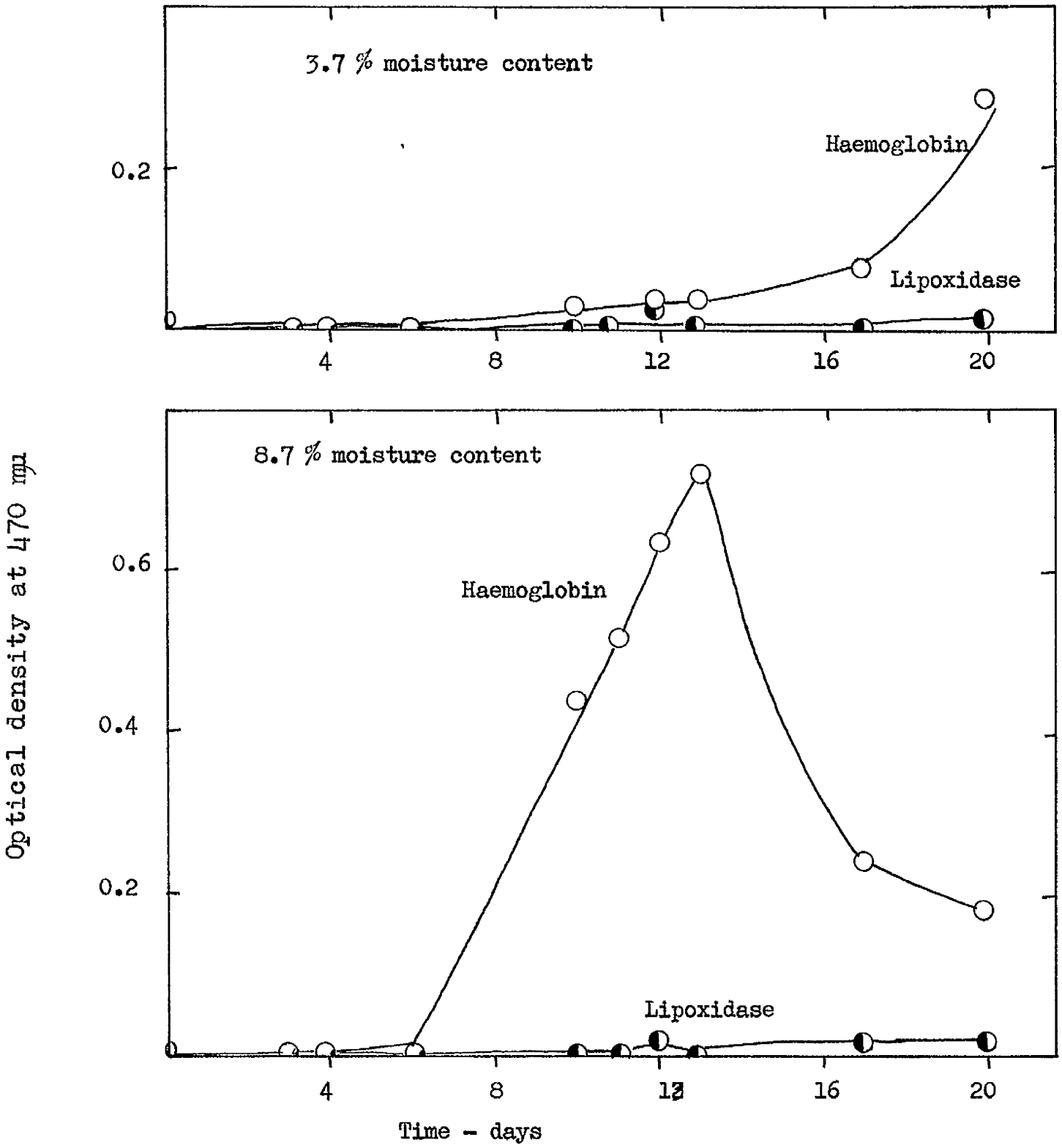


Fig. 28 Rate of change of oxidation levels of soya bean oil

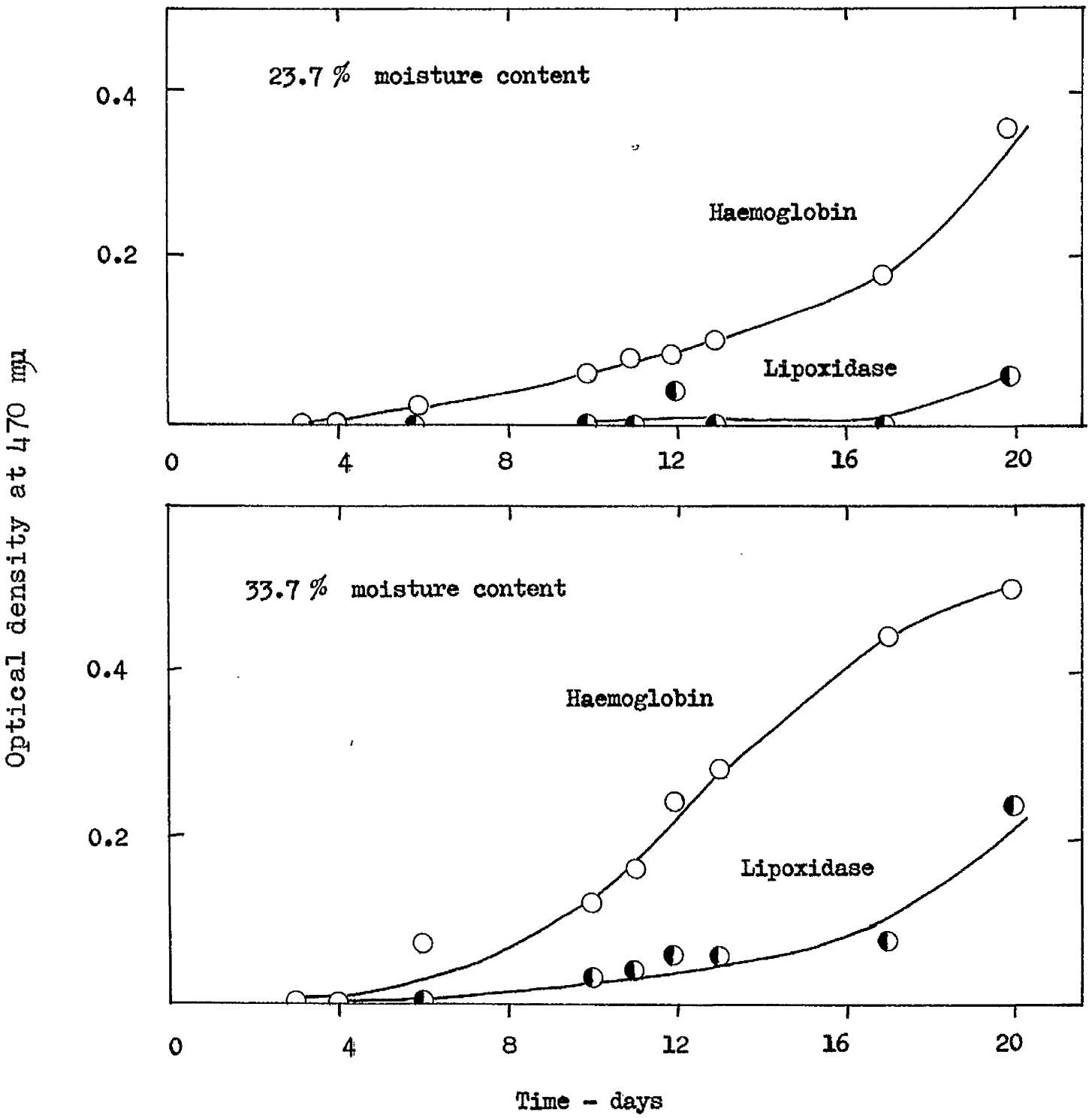


Fig. 29 Rate of change of oxidation levels of soya bean oil

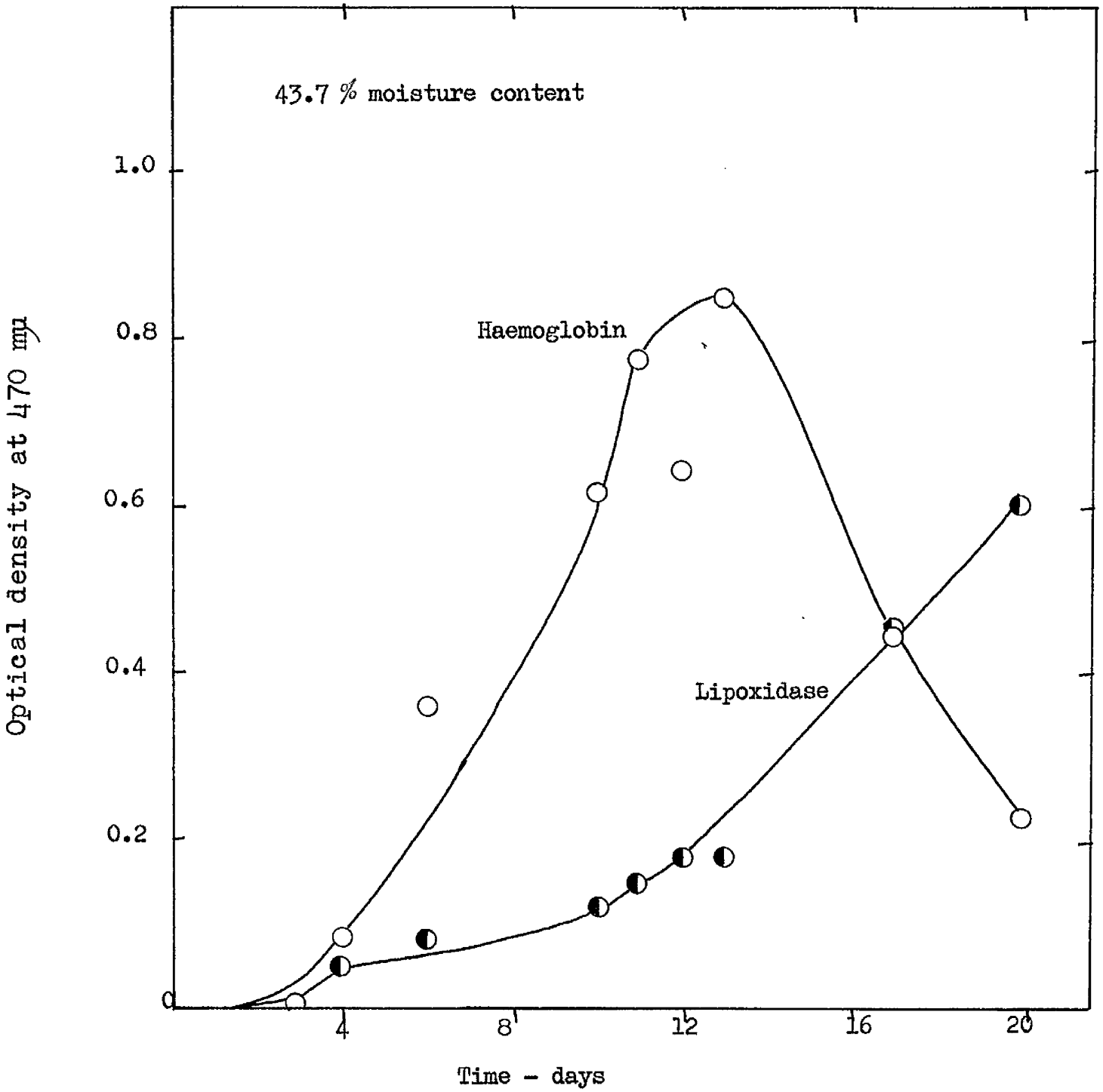


Fig.30 Rate of change of oxidation levels of soya bean oil

change in water levels up to 40 % .

According to Mann[128] concentration of the order of 5 mgs. haemin per 100 mgs. dry weight may be found in seed tissue. The amount added here is roughly equivalent.

The sequence of formation and breakdown of peroxides and conjugated diene in the system is shown in figures 28 and 30 at moisture levels of 8.7 % and 43.7 % .

Here the oxidation appears to take place most readily at values around 9 - 14 % moisture level, but further drying decreases the tendency to oxidation.

To check this point another experiment was carried out. The procedures used here in the present experiment were the same as those employed previously except that chloroform was added in a few drops to stop the bacterial action and mould's growth. The results are given in tables 21 and 22.

The results of this experiment, given in tables 21,22, confirmed those previously obtained.

Adding chloroform did not appear to modify the rate of oxidation to a great extent.

TABLE 21. Effect of moisture levels on haemoglobin activity (direct addition of water)

Moisture content %	Optical density at 232.5 millimicrons 4	8	15	17 days
3.7	.074	.031	.050	.063
8.7	.100	.200	.057	.043
23.7	.075	.038	.027	.043
23.7(no chlorof.)	.080	.031	.031	.030
33.7	.035	.037	.040	.034
33.7(no chlorof.)	.060	.037	.071	.028
38.7	.043	.061	.250	.090
43.7	.150	.180	.160	.200
Blank (3.7)	.057	.025	.016	.015

TABLE 22. Effect of moisture levels on haemoglobin activity (direct addition of water)

Moisture content %	Optical density at 470 millimicrons 4	8	10	15	17 days
3.7	.065	.050	.035	.090	.155
8.7	.180	.660	.705	.213	.223
23.7	.015	.052	.044	.072	.077
23.7(no chlorof.)	-	.065	.044	.037	.087
33.7	.045	.078	.056	.080	.130
33.7(no chlorof.)		.086	.056	.080	.130
38.7		.134	.107	.173	.283
43.7	.215	.540	.610	.950	.870
Blank	.055	.020	.018	.020	.015

Effect of variations of the type of starch on haemoglobin catalyzed soya bean oil oxidation

This experiment was conducted to determine whether changing the type of starch would alter rate of soya bean oil oxidation in the presence of haemoglobin, and hence to gain some information of whether the observed effects of moisture level on haemoglobin activity were general or peculiar to the system.

Materials and methods

Haemoglobin and soya bean oil and potato starch were as described previously; soluble starch (M & B Chem. Co.), was tested also.

In the previous exploratory experiment water had been mixed in, but in view of previous findings which indicated that this method was less reliable, the humidification technique was used here.

Starch was first dried by storing in a desiccator for two months over silica gel to 4 % moisture content, being mixed with 1 % of its weight of soya bean oil, then haemoglobin-starch mixture similarly dried was mixed in. Samples of the mixture (starch + oil + haemoglobin) were equilibriated with atmospheres ranging from 0 to 97 % relative humidity.

TABLE 23. Effect of moisture levels on the rate of oxidation of soya bean oil using a matrix of potato starch + haemoglobin.

R.H. %	Moist. cont.%	Optical density at 470 millimicrons 3	5	6	10	13	14	17	18	21	25 days
P_2O_5	2.4	.005	.025	.035	.050	.060	.075	.115	.142	.245	.520
15	9.0	.007	.020	.010	.045	.215	.215	.525	.610	.660	.240
33	16.0	.025	.020	.015	.025	.015	.015	.020	.020	.020	.040
58	19.0	.015	.025	.025	.032	.025	.050	.055	.040	.020	.220
65	22.5	.015	.020	.020	.020	.025	.025	.020	.020	.020	.040
81.7	25.5	.010	.020	.015	.020	.010	.020	.020	.020	.020	.030
88	28	.015	.010	.015	.018	.015	.013	.010	.010	.020	.028
93	35.5	.025	.025	.030	.045	.050	.065	.035	.060	.090	.095
97	39.0	.010	.045	.065	.085	.080	.150	.105	.240	.275	

TABLE 24. Effect of moisture levels on the rate of oxidation of soya bean oil using a matrix of soluble starch + haemoglobin.

R.H. %	Moist. cont.%	Optical density at 470 millimicrons 3	6	7	10	11	14	18	21	25 days
P_2O_5	2.5	.015	.025	.040	.030	.035	.045	.055	.115	.133
15	9.1	.012	.025	.020	.030	.020	.090	.420	.650	.590
33	16.5	.014	.010	.020	.025	.020	.020	.045	.040	.045
58	19.5	.010	.010	.030	.020	.020	.020	.050	.035	
65	24.7	.015	.010	.015	.020	.020	.020	.050	.025	.030
81.7	27.0	.015	.010	.020	.020	.020	.020	.050	.035	.043
88.0	29.0	.014	.015	.020	.030	.020	.020	.040	.030	.035
93	36.5	.015	.025	.035	.035	.050	.085	.140	.170	.230
97	40.7	.018	.050	.100	.200	.200	.235			

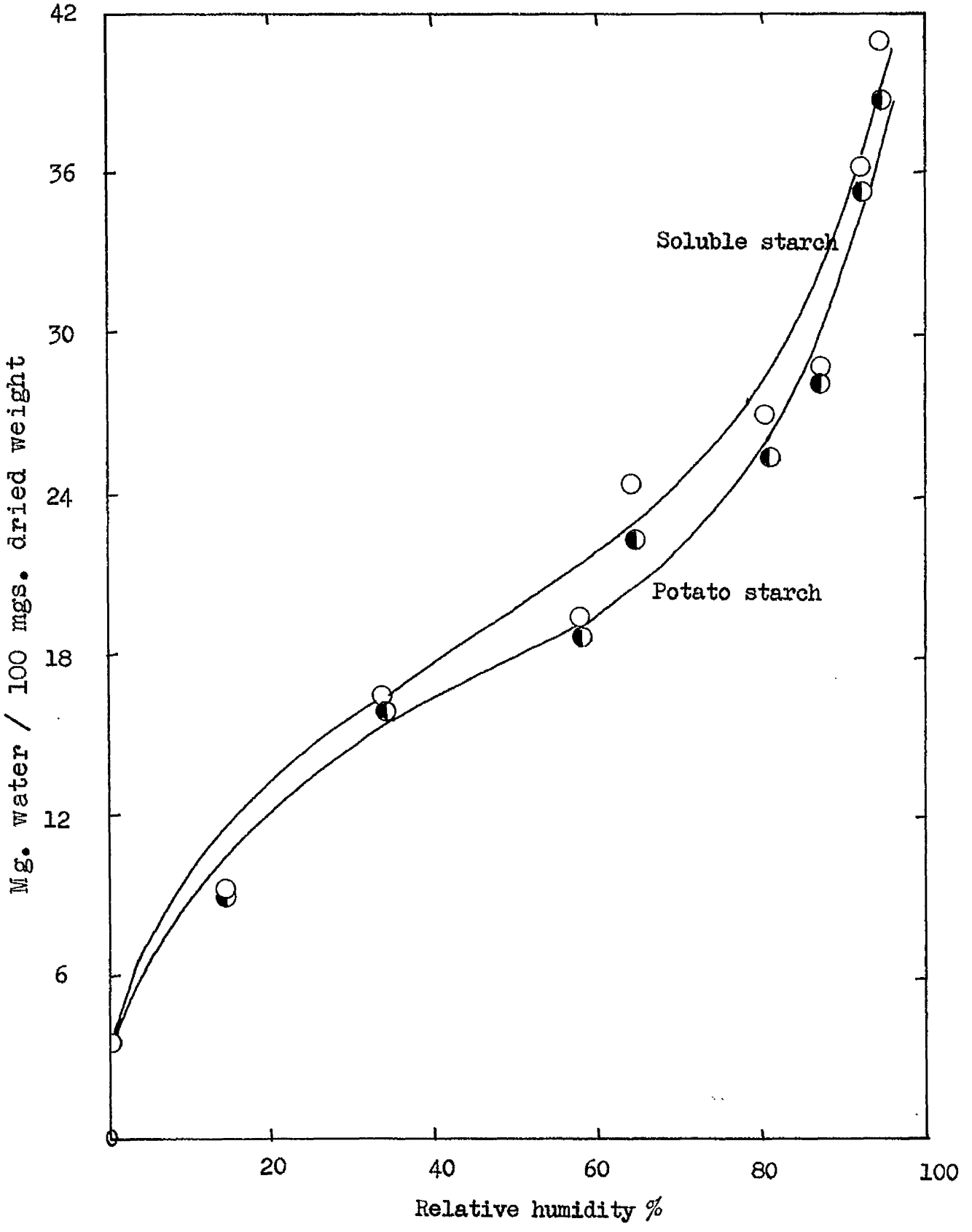


Fig. 31 Variation of moisture levels of starch with change in relative humidity at 20°C.

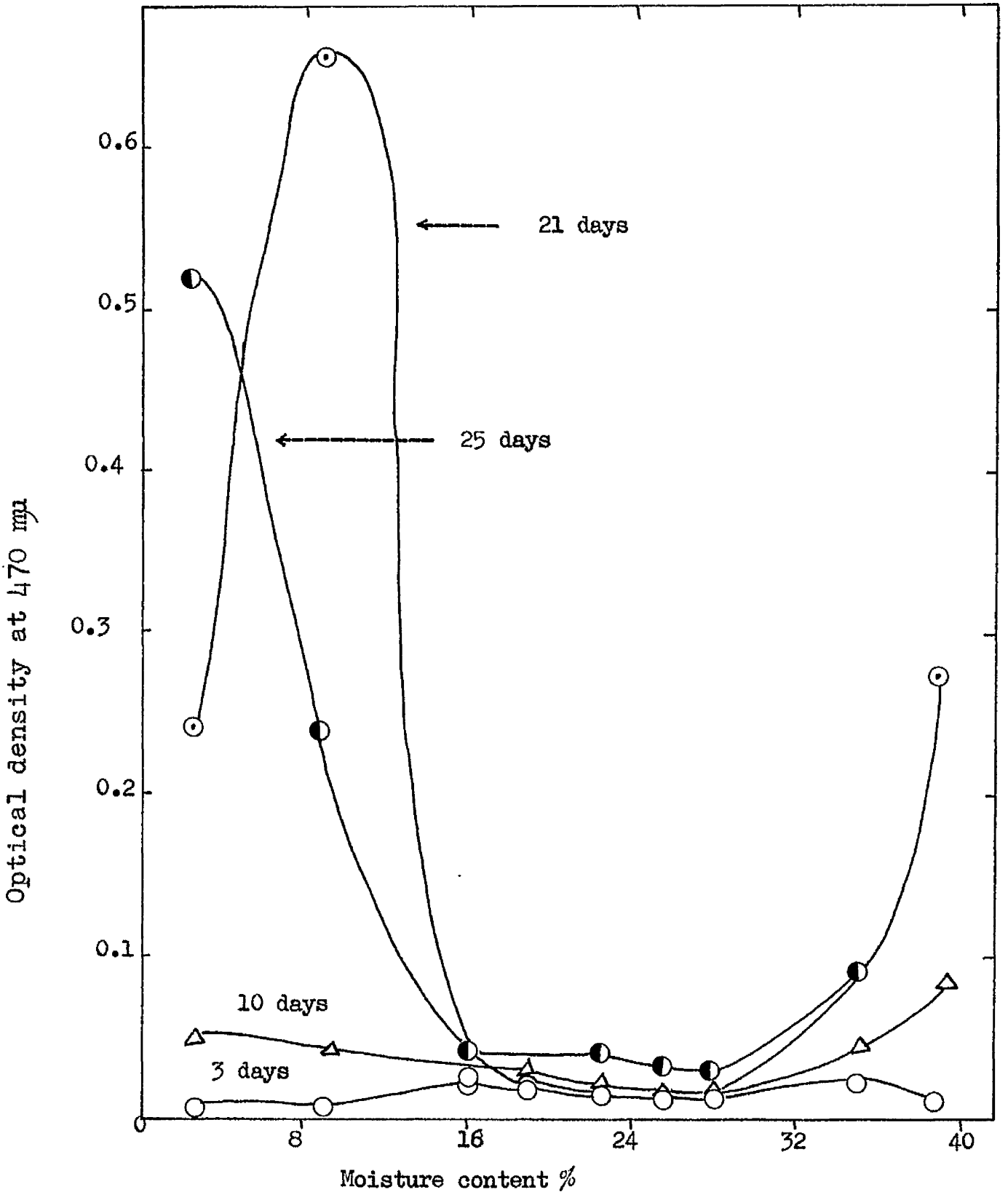


Fig. 32 Effect of moisture levels on haemoglobin activity using matrix of potato starch

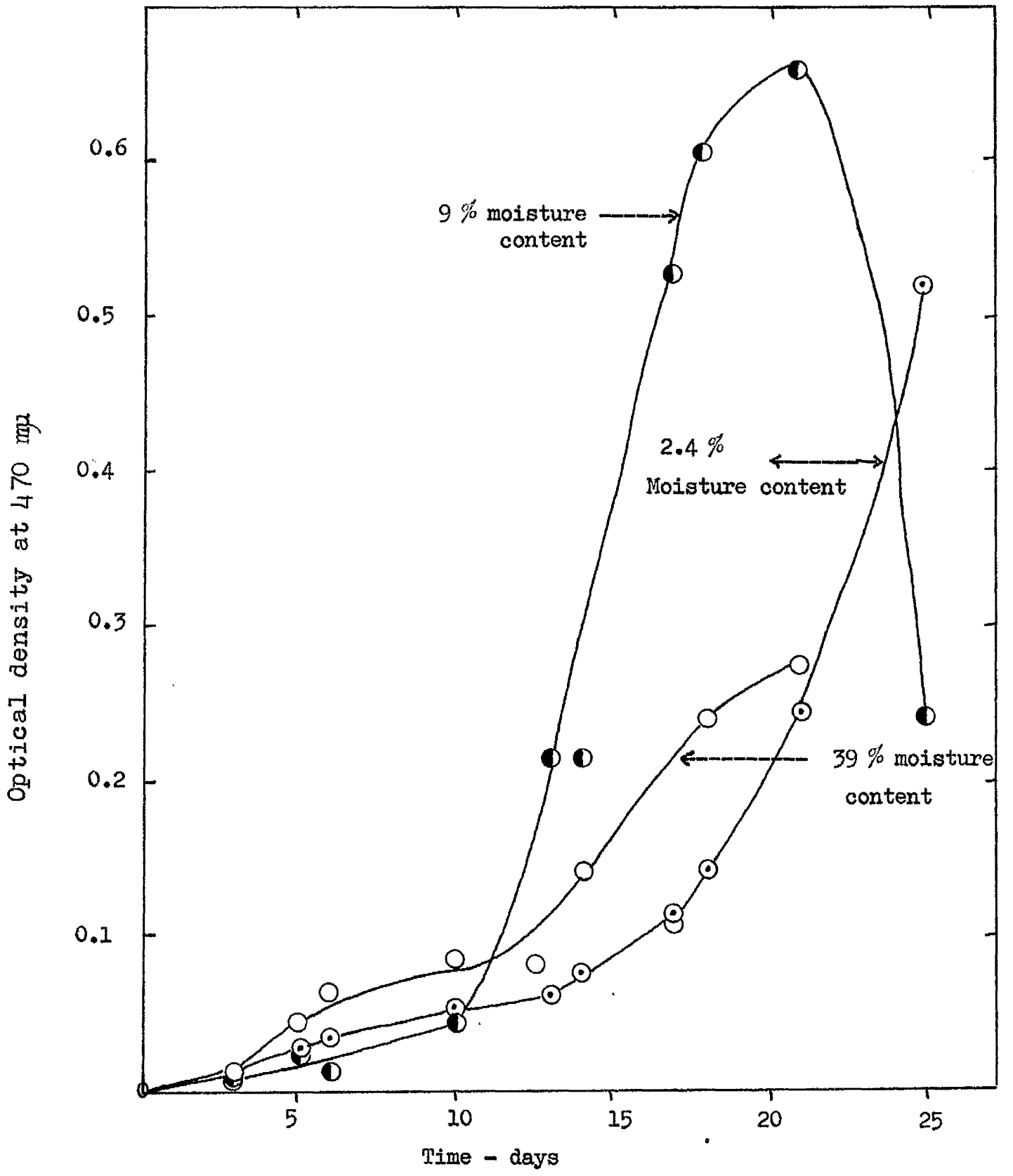


Fig. 33 Rate of change of oxidation levels of soya bean oil using matrix of potato starch and haemoglobin

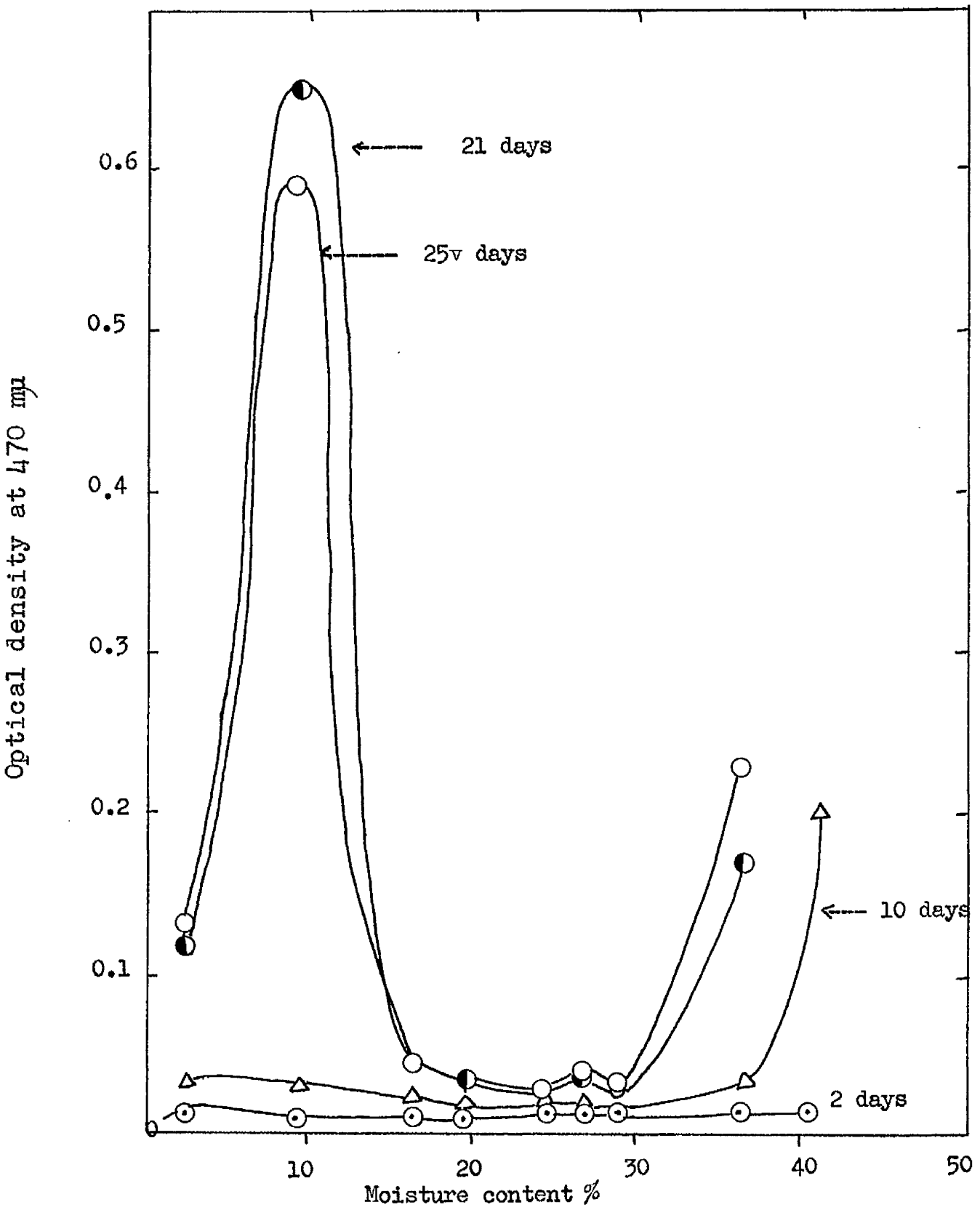


Fig. 34 Effect of moisture levels on haemoglobin activity using matrix of soluble starch

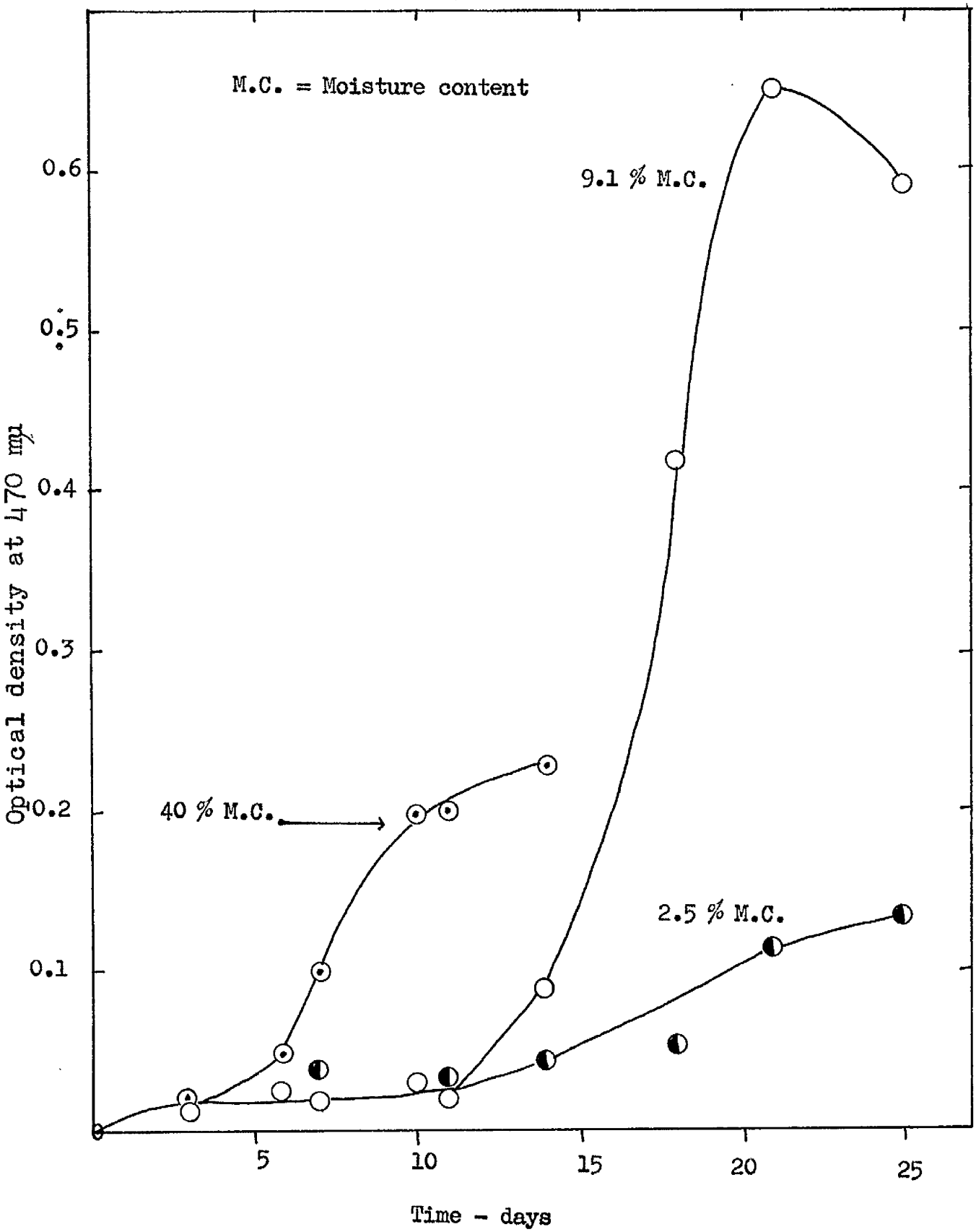


Fig. 35 Rate of change of oxidation levels of soya bean oil using matrix of soluble starch + haemoglobin

The data obtained from this experiment, shown in tables 23,24 and figures 31 - 35, in general confirms the previous findings that haemoglobin is much more active at lower moisture levels (of the order of 9 - 14 %) than in the range from 16 to 29 % ,

It has also been found that, at the very high moisture levels, the reaction started early and that was followed by a decomposition in the peroxide value, figure 35.

In general while the soluble starch mixture, at low moisture levels, is more resistant to oxidation than that of the potato starch, the pattern is very similar.

Discussion

The experiments described in this part show conclusively that haemoglobin acts in low concentration as an efficient catalyst in the oxidation of soya bean oil at both low and high moisture levels.

In studying the relationship between soya bean oil oxidation and moisture levels ranging from (2 % - 43 % - dry basis), in the presence of haemoglobin, it was observed that there was an apparent range of moisture levels (from 16 to 29 %), above and below which the development of fat oxidation occured more rapidly during storage. The induction period was shorter at the very high moisture levels (40 % and over).

Generally the peroxide and diene conjugation concentrations reached a maximum values and then decreased as shown in figures 28, 30, 34, and 35. This appears to be due to the decomposition of both the peroxides and the diene formed during the oxidation. This correlates with similar results on liquid systems where it has been shown that while soya extracts destroy hydroperoxide without destruction of diene[190] haemoglobin destroys both conjugated diene and hydroperoxides.[191]

The major point brought out by these experiments is that, unlike lipoxidase, haemoglobin is an active catalyst at low moisture levels. In general it has been accepted that haematin oxidation of unsaturated fatty acids is similar to autoxidation in pattern. Here, haemoglobin catalysis, unlike that of lipoxidase, appears to lean closer to the pattern which many workers have observed for fat oxidation in that drying increases the tendency to oxidise.

It seems clear that if the pattern of fat oxidation in foods is similar to that in this model system, then the haematins are more likely to be involved in most materials than are lipoxidases since moisture levels in meals and flours are most frequently of the order of 8 - 15 % .

It must be remembered that the tests used here for measuring fat oxidation are crude compared to organoleptic effects and that

rancidity will probably occur before the end of the induction period.

It has been found that changing the type of starch did not greatly alter the main pattern. With both potato and soluble starch the rate of soya bean oil oxidation, in the presence of haemoglobin, was much greater at moisture level around 9 % and 40 % , but while haemoglobin was active in potato starch at 2 % moisture content, it has been noticed that soluble starch mixture was resistant to oxidation at this level of moisture, although the sorption isotherm for both the two starches was similar, figure 31.

It can be taken that the oxidation in all cases is due to haemoglobin catalysis since only negligible peroxide and conjugated diene values have been obtained when this is omitted, tables 21 and 22.

Effect of moisture levels on cytochrome catalysis on soya bean oil oxidation

The experiment described here is concerned with the possible effects of variation in moisture content on cytochrome c activity.

It was found in the previous experiments that haemoglobin, unlike lipoxidase, is an active catalyst at low moisture levels and it seemed of interest to see whether cytochrome c would behave like haemoglobin.

The system previously derived to study haemoglobin activity at the different moisture levels was used here in an examination of cytochrome c.

Materials and methods

Pure cytochrome c (Horse heart, "100 % purity," Seravac Laboratories) was used as a catalyst. The weight used was the same as for haemoglobin, so that on a molar equivalent basis the haematin concentration is somewhat higher. Soya bean oil and potato starch were the same as described previously.

The procedures used here for preparation, humidification, and measuring oxidation were as those employed in the previous experiment.

The relative humidities used here were: 0, 15, 20, 33, 58, 65, 81, 88, 93, and 97 % , which correspond to moisture

contents of 2.3, 11.2, 11.7, 12.6, 17, 23, 23.5, 27, 35, and 36.5 % respectively.

The sensitivity of cytochrome c catalysis to the different moisture levels is shown in tables 25 and 26, and figures 36 - 38.

TABLE 25. Effect of moisture levels on cytochrome c catalysis on soya bean oil oxidation.

R.H. %	Moist. cont. %	Optical density at 470 millimicrons 3	5	7	11	13	17	20 days
P_2O_5	2.3	.010	.015	.010	.010	.025	.065	.120
15	11.2	.015	.015	.010	.020	.025	.065	.070
20	11.7	.020	.015	.020	.020	.020	.065	.065
33	12.6	.020	.020	.052	.175	.250	.620	.780
58	17.0	.010	.010	.040	.080	.110	.200	.235
66	23.0	.005	.010	.020	.030	.015	.045	.040
81.7	23.5	.005	.010	.010	.010	.010	.035	.040
88	27.0	.005	.010	.020	.020	.010	.030	.025
93	35.0	.005	.010	.020	.025	.030	.065	.066
97	36.5	.005	.010	.010	.035	.040	.100	.100
15*	11.2	zero	zero	—	—	—	.010	.010
97*	36.5	zero	zero	—	—	—	.010	.010

* = Control

TABLE 26. Effect of moisture levels on cytochrome c catalysis on soya bean oil oxidation.

R.H. %	Moisture cont. %	Optical density at 232.5 m μ 3	17	20 days
P_2O_5	2.3	.053	.056	.054
15	11.2	.045	.053	.042
20	11.7	.052	.050	.038
32	12.6	.023	.243	.200
58	17.0	.043	.103	.110
65	23.0	.030	.049	.033
81.7	23.5	.042	.055	.030
86	27.0	.027	.044	.025
93	35.0	.045	.054	.038
97	36.5	.067	.064	.055
15 (cont.)	11.2	.043	.030	.016
97 (cont.)	36.5	.045	.029	.018
Blank	—	.042	—	—

From the results of this experiment, shown in tables 25,26 and figures 36 - 38, it is clear that cytochrome c could catalyze the oxidation of soya bean oil at the low moisture levels and that the highest oxidation rate was at moisture level of 12.6 % .

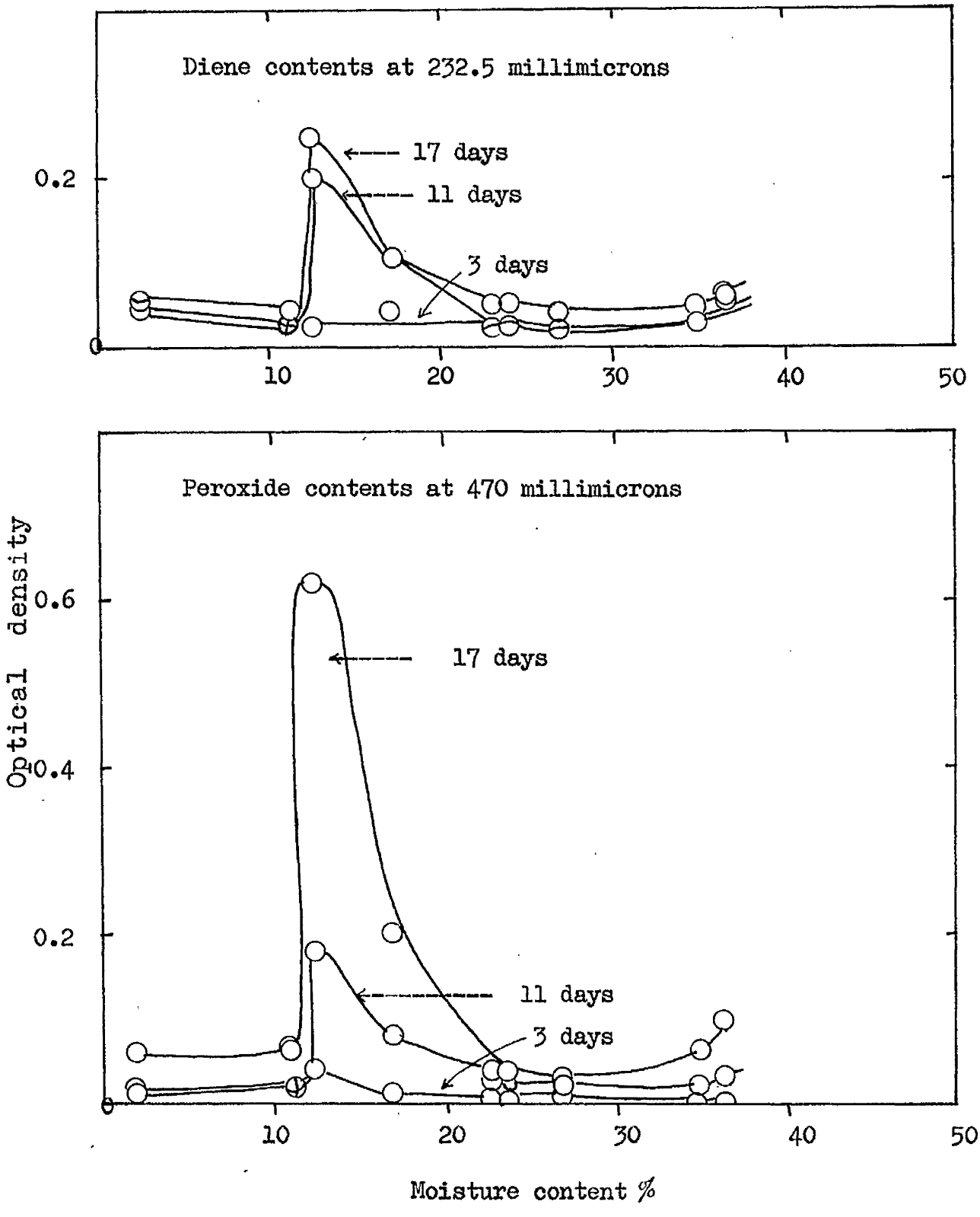


Fig. 36 Variation of oxidation levels of soya bean oil with change in moisture content (using matrix of potato starch + cytochrome c).

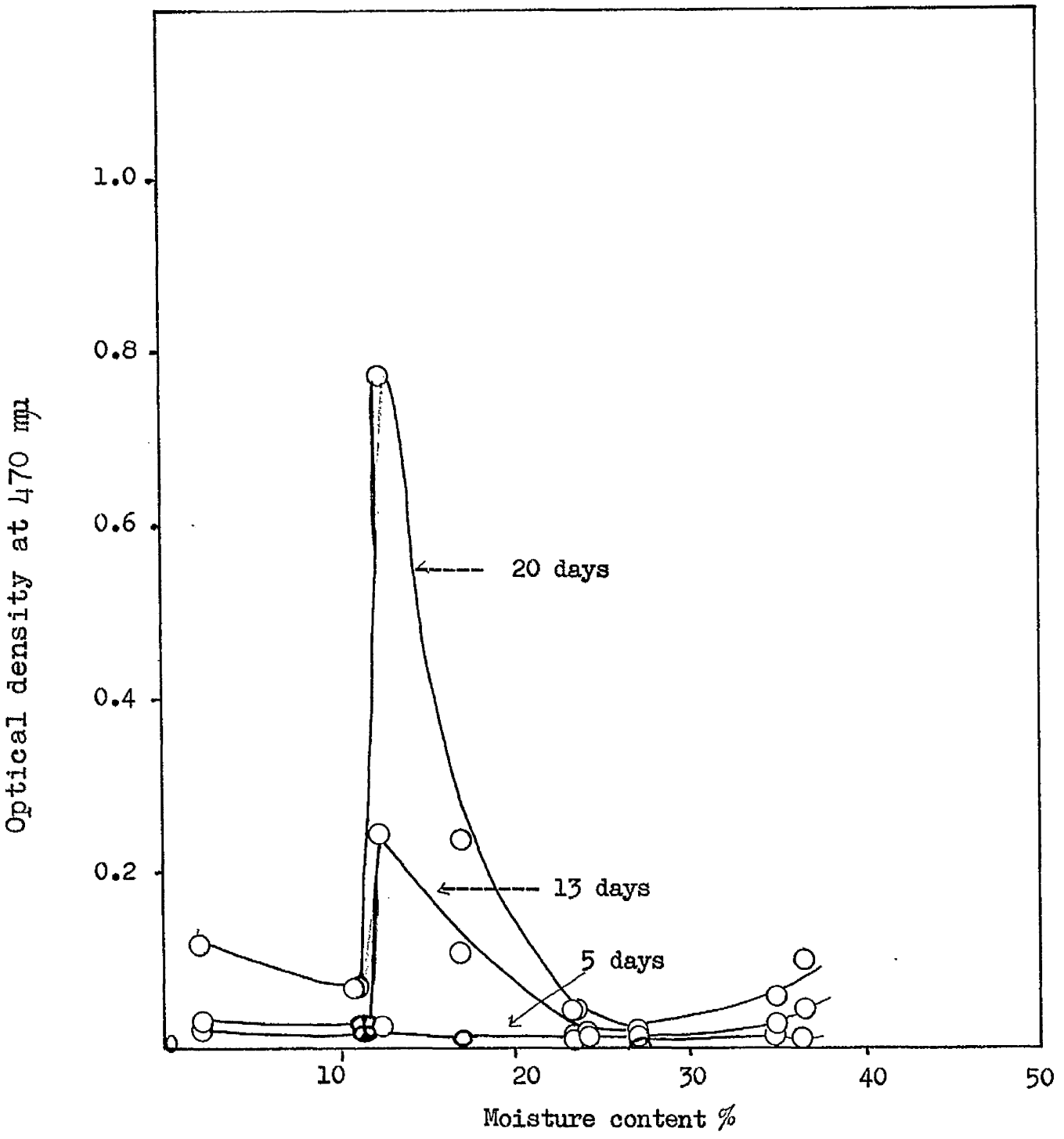


Fig. 37 Variation of oxidation levels of soya bean oil with change in moisture content (using matrix of potato starch + cytochrom c)

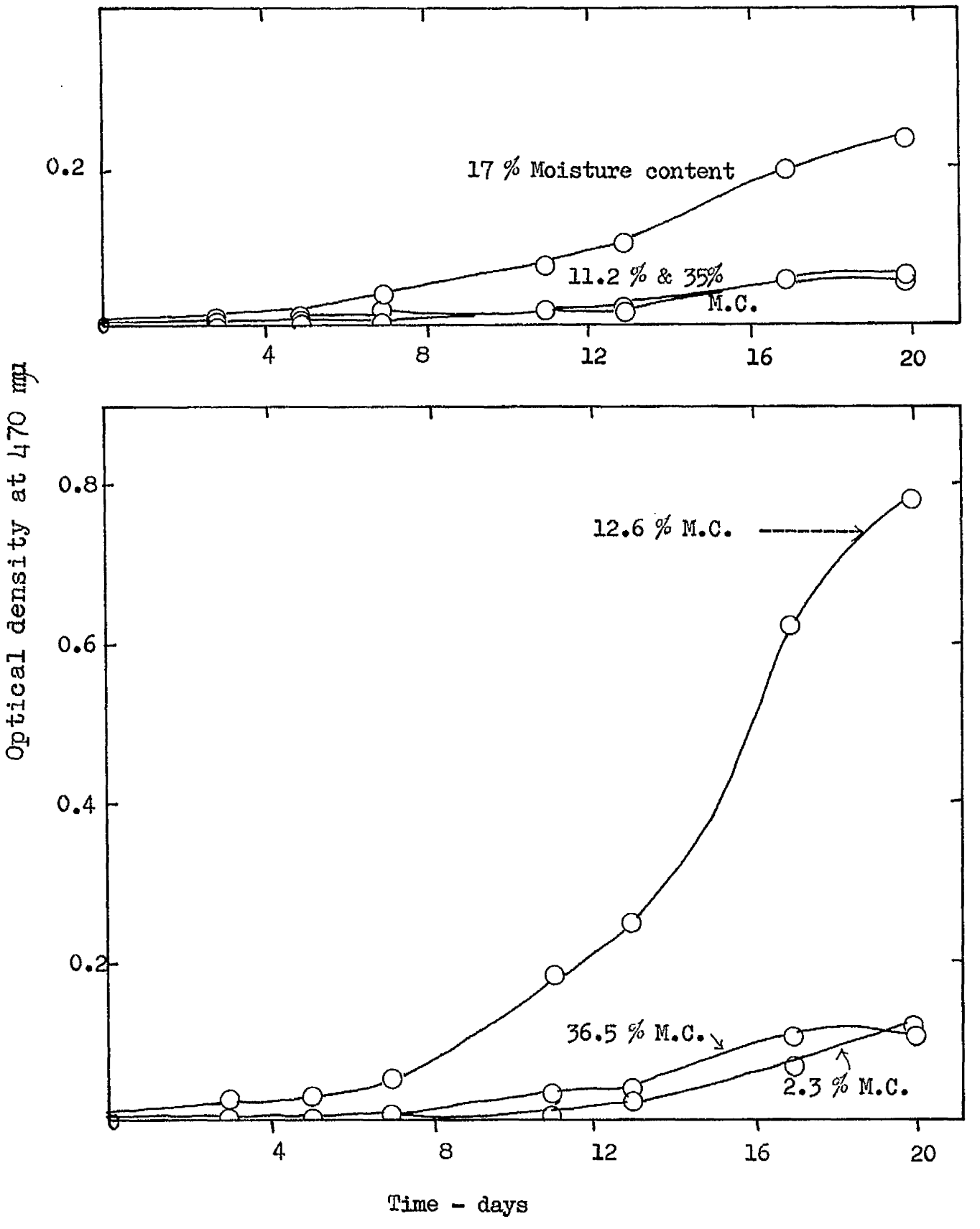


Fig. 38 Rate of change of oxidation levels of soya bean oil using matrix of potato starch + cytochrome c

At moisture levels of 17 % the rate of oxidation was higher than that at 23 % moisture content and over.

To check this point a second experiment was commenced, in which cytochrome c activity was examined only at the low moisture levels.

Materials and methods were the same as those employed previously. The results obtained are shown in table 27.

TABLE 27. Cytochrome c catalyzed soya bean oil oxidation at low moisture levels.

R.H. %	Moist. cont. %	Optical density at 470 millimicrons 5	9	11	15	23 days
P_2O_5	2.0	.010	.020	.025	.035	.070
15	99.1	.010	.020	.045	.060	.140
20	11.0	.010	.040	.040	.060	.115
33	12.0	.010	.210	.300	.600	.283
58	17.0	.010	.120	.175	.270	.575
66	22.0	.010	.090		.220	.370
33(cont.)	12.0	—	—	.010	.010	.010
58(cont.)	17.0	—	—	.010	.010	.010

cont. = no cytochrome c

It can be seen from the data obtained, table 27, that the cytochrome c is similar to haemoglobin in showing low activity at both high and very low moisture levels but appears to differ somewhat in the actual range found.

It would appear that cytochrome c is most active under the conditions examined between 12 % and 22 % although over longer periods it is active at moisture level as low as 9 % . Haemoglobin has a corresponding high activity range at lower levels and it is close to its maximal at 9 % moisture content while cytochrome c is very much more active at 12 % moisture content than that 9 %. Again cytochrome c is active at 17 % moisture content whereas haemoglobin is relatively inactive at this level.

Effect of moisture levels on peroxidase activity

In this experiment an attempt was made to discover the effect of variation in moisture content on peroxidase activity. It was hoped to make some comparison between the activity of haematins in forming and destroying lipid peroxide with their action on hydrogen peroxide, using peroxidase as the haematin compound.

Since hydrogen peroxide is unstable in the presence of organic matter the more stable compound, urea peroxide, was used with o-dianisidine which gives a reddish colour on oxidation.

Materials and methods

Potato starch, described before, was used as a matrix. Urea hydrogen peroxide was used as a substrate. Purified horse radish peroxidase (Sigma Chemical Co.) was used as a catalyst.

The starch was initially 20 % moisture content, it was mixed with 0.1 % of its weight of urea hydrogen peroxide and 0.1 % of o-dianisidine. Samples of that mixture were adjusted to moisture levels of 40, 50, 60, 70, and 80 % . The solid peroxidase was then mixed in, in concentration equal to that used for haemoglobin preparation.

Results

It was noticed that the colour in the mixture, after the addition of peroxidase, developed immediately and no further changes in the colour were noticed when the samples were stored for longer time. Although many solvents were used it was not found possible to extract the colour which developed.

It was measured in the starch using an E E L reflactance spectrophotometer at 601 millimicrons. All the readings were taken against a blank of starch and urea hydrogen peroxide mixture with the same moisture contents.

It was also found difficult to measure the samples all at once without changing in the moisture content, since a great deal of water has been noticed to evaporate when preparing the sample for measurements. The difficulties of technique were too great to obtain useful results and the experiments were not continued. However the results obtained by reflectance measurements are included for interest (figure 39). Because of the unsatisfactory nature of the technique lower moisture levels were not examined.

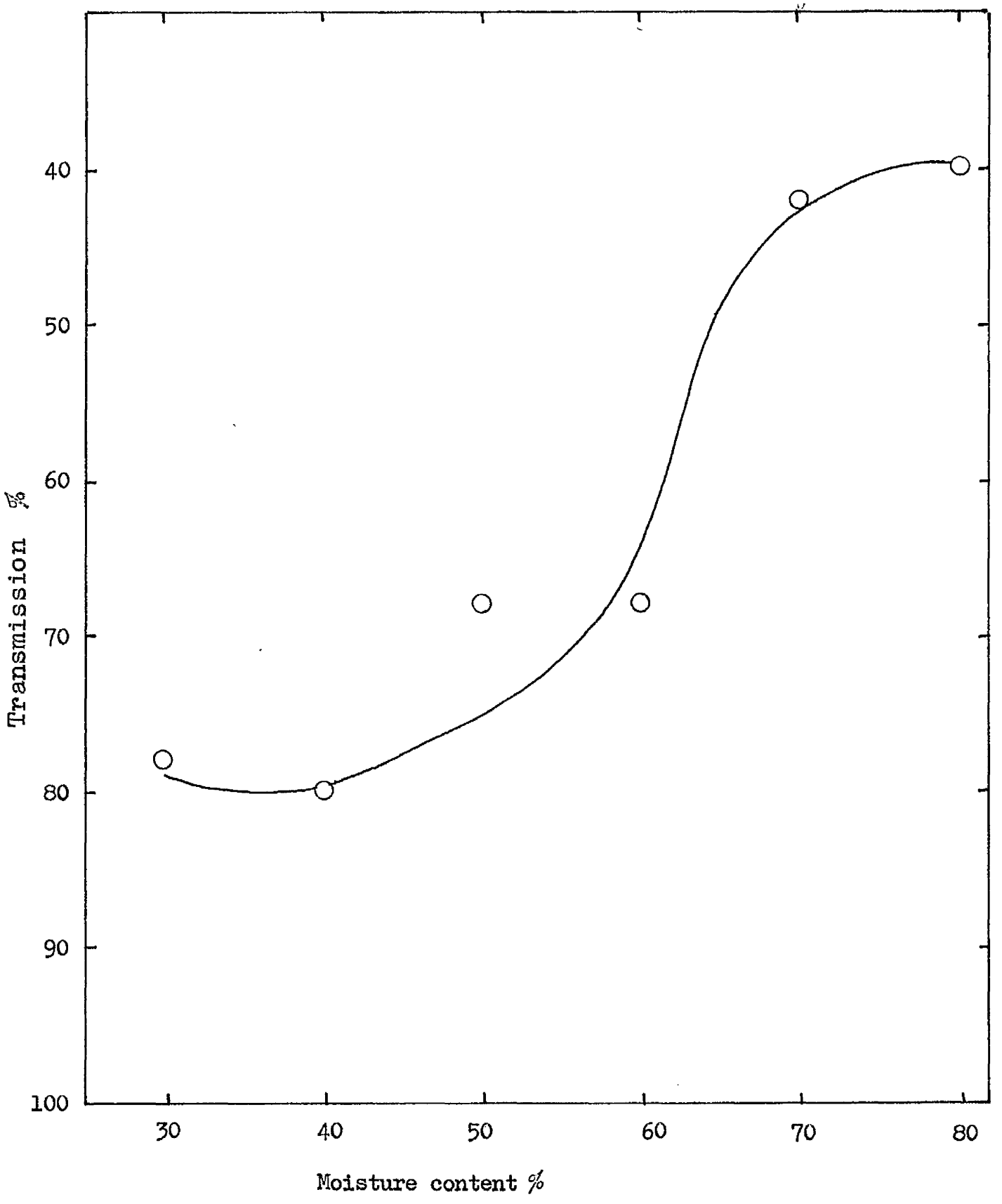


Fig. 39 Effect of moisture levels on peroxidase activity.

SUMMARY AND CONCLUSIONS

The results obtained in this work are, on the whole, empirical. There is no comprehensive theory available to which observation may be referred and the results obtained here can only add to the empirical data which exists. It is probably the case that a great deal more empirical data will have to become available before any major generalization can be made.

Since haematin compounds are found to be more active at the low moisture levels, probably that oxidative rancidity in stored food materials may be promoted by haematins present. On the other hand lipoxidase is unlikely to act in stored meals in spite of its high activity, since it requires relatively high moisture levels which would permit its action. This would apply to situations such as those which occur in doughs.

Results with cytochrome c and haemoglobin show the same general pattern and this is not significantly altered by altering the type of starch used so that it would seem valid to suggest that the pattern of fat oxidation by haematin compounds, naturally present in foods, might under some circumstances simulate that found here. This would not be expected to confirm to observations made on food materials which have undergone blanching or heat treatment before dehydration. While observations on dehydrated potato would confirm to this, these on soya bean might be expected

to show indications of the haematin compounds known to be present by a peak in activity at moisture levels of 9 - 12 %. Possibly difference of these and lentil to potato may be due to high protein content, as amino acids are known to inhibit fat oxidation under some conditions. Unfortunately it was not possible to study protein effects in the time available. At moisture levels above 25 % it seems that haemoglobin and lipoxidase respond to increase in moisture levels in a very similar fashion suggesting that below 25 % moisture in starch is not free. This is consistent with what is known of water-binding by starch.

The peak in haemoglobin activity at moisture levels around 9 % to 13 % is difficult to account for on the basis of known theories. Some qualitative experiments which have not been reported in the preceding section showed that haemin added to soya bean oil in acetone (which was then removed by distillation in vacuo) caused rapid oxidation in the absence of moisture. This was shown manometrically. Here the haemin was actually in solution in its substrate. Thus if the haematin and substrate are effectively brought into contact water is not essential. It may be that below a certain level of hydration the lipophilic properties of the haematin protein are more pronounced.

However, on the model systems the number of variables which may operate are considerable. Swelling of the matrix on

hydration, the degree of hydration of the protein catalyst, the ratio of chain reactions in this physically complex system, and diffusion of the catalyst at levels of hydration above the monolayer value may all be involved and there is insufficient knowledge to justify speculation.

It is also obvious that the rate of linoleate hydroperoxide formation and breakdown may both be altered by moisture levels and since the ratio of diene conjugation to hydroperoxide varies according to the catalyst these results confirm earlier observations (in liquid systems) that destruction of conjugated diene need not follow that of hydroperoxide.

Most studies on fat oxidation do not seem to have taken this factor into account in following the course of oxidative rancidity in stored materials by a single technique.

In general the results stress that for lipoxidase to act in foodstuffs moisture levels must be much higher than for haematins. Since the two types of catalyst respond in a different fashion to different antioxidants these results might be of value in indicating a natural basis for selection of antioxidants according to the level of moisture in foodstuffs where both lipoxidase and haematins are present as in animal food mixes. At present antioxidants are used very much on an empirical basis.

APPENDICES

APPENDIX I. HUMIDIFICATION TECHNIQUE

Samples of soya bean flour of 0.2 grams were placed in aluminium foil containers in small desiccators each of which served as a constant-humidity chamber. The vapour pressure of water in a desiccator was controlled by saturated solutions of chemical salts of known relative humidity, and the desiccators were kept at constant temperature, so that the relative humidity within the desiccator was fixed. The water content of the samples placed in the desiccators changed with the time until equilibrium was reached. To determine the water contents corresponding to the relative humidity used, two samples from each desiccator, were removed at various intervals and weighed. When no further change in weight occured, as indicated by two successive weighings, the sample was considered to have reached equilibrium. In all cases equilibration was virtually complete in 24 hours at 20°C. The moisture content of the sample was obtained from the difference between the weight at equilibrium and the weight of the sample after complete removal of water by drying at 110°C. for 24 hours.

The desiccators were all glass, about 6 inches in diameter.

The samples were thin and have large surface to facilitate the exchange of water vapour between the interior of the sample and the surrounding atmosphere. Each desiccator was capable of holding about ten samples.

In the case of comparing two enzymic catalysts, larger desiccators, 12 inches in diameter and capable of holding 40 samples each, were used.

Ten desiccators were used, each one containing a different saturated solution to cover a range of relative humiditied from about 0 to 97 per cent.

The humidity in the desiccators was obtained from tables given by The British electrical and allied industrial research association[192] for the percentages relative humidities over saturated solutions and related information.

Salt	Percentage relative humidity at 20°C.
Phosphorus pentoxide (P_2O_5)	0
Lithium chloride ($LiCl\,H_2O$)	15
Potassium acetate (CH_3COOK)	20
Magnesium chloride ($MgCl.6H_2O$)	33
Sodium hydrogen sulphate($NaHSO_4.H_2O$)	52
Sodium bromide ($NaBr.2H_2O$)	58
Magnesium acetate $[(CH_3COO)_2 Mg.\ 4H_2O]$	65
Sodium nitrite ($NaNO_2$)	66
Ammonium chloride+Potassium nitrate mixture	72.6
Ammonium sulphate $[(NH_4)_2 SO_4]$	81.7
Potassium chromate (K_2CrO_4)	88
Ammonium dihydrogen phosphate($NH_4 H_2PO_4$)	93.1
Potassium dihydrogen phosphate ($KH_2 PO_4$)	97
Potassium sulphate ($K_2 SO_4$)	99.4

APPENDIX 11. DETERMINATION OF HYDROPEROXIDE AND CONJUGATED DIENE

(A) For the determination of conjugated diene 10 mls.of redistilled ethanol were added to 0.2 gm.of the reaction system,from each desiccator,in a centrifuge tube,to extract the products resulting from the oxidation and the mixture was centrifuged.Then 1 ml.of the extract was pipetted into 12.5 mls.of 100 % redistilled ethanol to dilute for measuring the diene concentration. The absorbency was read, against a blank of 100 % ethanol, with the use of an Optica 04 Spectrophotometer and 1 cm. cuvette at 232.5 mμ.

(B) Determination of hydroperoxide

The thiocyanate method of Koch[193] was chosen to measure the hydroperoxides produced during the oxidation. The detailed procedure used in this work follows:

To 0.2 gm. of the reaction mixture from each desiccator, 10 mls. of redistilled ethanol were added to extract the hydroperoxides, and the mixture was centrifuged. Then 1 ml. of the supernatent liquid was pipetted into 12.5 mls. of redistilled ethanol and this dilution was used for peroxide estimation.

To carry out the colour reaction, 0.1 ml. conc. hydrochloric acid and 0.025 ml. of 2.5 % ferrous ammonium sulphate solution in 3 % hydrochloric acid were added. Precisely 30 seconds after the Fe^{++} addition, 0.5 ml. of 20 % ammonium thiocyanate was added

Exactly 3 minutes later the absorbance of the coloured solution was measured using an E E L Spectra Spectrophotometer with 1 cm. cells, at 470 millimicrons.

APPENDIX III. SOYA BEAN OIL PREPARATION

20 grams of soya beans were first milled to pass a 20 mesh sieve and extracted with 500 mls. pure ether. The extract was filtered and the ether was evaporated under vacuum as a precaution against any oxidation. The oil was packed in small ampoules in 300 mgs. quantities in vacuo and stored at $-22^{\circ}C$.

APPENDIX IV. FREEZE-DRYING TECHNIQUE

For freeze-drying material was rapidly frozen in a shallow layer in a round bottom flask (1 liter size) by immersion in solid CO_2- ethanol. The flasks were then connected to a vacuum apparatus and the material dried at about 0.1 mm Hg. The dried material was removed and stored over silica gel, in order to remove as much water as possible.

APPENDIX V. SOYA BEAN EXTRACT FOR FREEZE-DRYING

Soya bean flour was extracted with water (10 gm.flour/100 mls. of water) and the mixture was centrifuged; the clear supernatant solution was freeze-dried as in appendix IV.

APPENDIX VI. DIRECT ADDITION OF WATER TO MATRICES BY MIXING

A simple technique was used for studying the effect of moisture levels on fat oxidation; it consisted in bringing the enzyme and oil into close contact. The starch was first mixed with 1 % of its weight of unsaturated oil. Samples of that mixture (1 - 2 gm.) were adjusted to the desired moisture contents by manually mixing in the amounts of water which had been calculated to give the desired moisture content. The water was added in increments of 0.01 ml. from a micro-burette over a period of about ten minutes. Actual final content was obtained by dried weight. The samples were then mixed with 10 % of their weight of haemoglobin and starch mixture (14 mgs. haemoglobin for 1 gm. dried starch) and kept at fixed temperature (20°C.).

APPENDIX VII. THE CHEMICAL COMPOSITION OF SOYA BEAN

Soya beans consist principally of lipids, proteins, carbohydrates, and mineral constituents. Tables 1 and 2 show the chemical composition of the component parts of soya beans.

TABLE 1.

Part	Portion of the seed %	Moisture	Proteins	Sugars	Fat	Ash
Cotyledons	90-92	10.57	41.33	14.60	20.73	4.38
Embryo	1.5-2.0	12.01	36.93	17.32	10.45	4.08
Seed coat	6 - 8	12.53	7.00	21.02	00.60	3.83

L.H. Bailey et al., Cereal Chem., 12 , 441, (1935).

TABLE 2.

Comparison of iodine value and distribution of fatty acids derived from various soya beans oil.

Seed variety	Saturated fatty acids %	Unsaturated fatty acids			
		Total	Oleic	Linoleic	Linolenic
Dunfield	12.0	88.0	60.0	25.1	2.9
Peking	12.4	87.6	24.2	56.2	7.2
Seneca	11.9	88.1	24.5	55.6	8.0
Wild beans	13.5	86.5	11.3	63.1	12.1

F.G. Dollear et al. , Oil & Soap , 17 , 120 - 121 , (1940).

APPENDIX VIII. LIPOXIDASE ASSAY IN LIQUID SYSTEM

Materials: Methyl linoleate and soya bean oil were used as a substrate in concentration of 1 mg./ml. ethanol.

(2) Phosphate/citrate buffer pH 6.5. This was made up as follows:

14.2 mls. of 0.2 M disodium hydrogen phosphate + 5.8 mls of 0.1 M citric acid. The mixture was diluted to 100 mls, with water.

(3) Enzymes:

a. lipoxidase preparation: to 0.5 gms. of defatted soya flour 40 mls. of distilled water were added. After thoroughly mixing for 10 minutes the aqueous extract was centrifuged at 4000 rpm for 15 minutes in an M S E Major centrifuge. The clear supernatant was filtered and the filtrate was used for experiment.

b. Haemoglobin was prepared in a concentration of 9 mgs. of pure haemoglobin/10 mls. distilled water.

Reaction mixture was prepared as follows:

10 mls. phosphate/citrate buffer pH. 6.5, 1 ml. substrate and 1 ml. of the enzyme solution.

On the addition of enzyme solution to the reaction mixture a stop-clock was started and 1 ml. was taken into 10 mls. of 100 % ethanol at various time intervals.

The thiocyanate assay was used for measuring the oxidation as described in appendix II., except that here 10 ml. of the ethanolic solution of oxidised fat were used instead of the 12.5 mls. used previously. The quantities of reagents were correspondingly reduced.

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