A STUDY OF POLYMERIZED VEGETABLE OILS

PRITCHARD

OBSERVATIONS ON FRACTIONS PREPARED FROM THERMALLY POLYMERIZED VEGETABLE OILS AS RELATED TO THEIR EFFECTS ON THE NUTRITION OF THE RAT

A Thesis

by

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GENERAL INTRODUCTION

The shortage of edible oils and fats during the Second World War stimulated research in this laboratory on the utilization of linseed oil for edible purposes. This oil was available in good supply. Unfortunately hydrogenated linseed oil when incorporated into shortenings exhibited a marked tendency to produce off-flavours. This tendency to flavour reversion has been attributed to the isolinoleic acids produced by hydrogenation from linolenic acid present in large quantity in linseed oil.

A method of removing a portion of the linolenic acid was devised. The method consisted of thermally polymerizing the oil and solvent extracting the unpolymerized triglycerides. The hydrogenated extract displayed good stability as regards flavour recession and produced a shortening suitable in taste and baking properties. Unfortunately the shortening displayed markedly lowered nutritional value when incorporated in the diets of young rats.

Thermally polymerized peanut, corn, rapeseed, and soyabean oils all displayed lowered nutritive value, but a much longer heating time was required before results were obtained similar to those obtained with linseed oil.

Recent work in this laboratory has effected a separation of the monomeric portion of polymerized linseed oil ethyl esters into two fractions, viz., a very injurious fraction,

and a fraction comparable in nutritive value to the unheated esters. The injurious material constitutes about ten per cent of the whole oil. The present work has been aimed at identifying and characterizing the components of the injurious fraction as well as attempting to isolate nutritionally deleterious material from other thermally polymerized oils.

PART I

REVIEW OF THE LITERATURE

Thermal Polymerization

The heat-bodying of polyunsaturated oils has long been used by the paint and varnish industry to produce better drying oils. More recently polymerization has been applied to edible vegetable oils (Lips et al, 1953) to stabilize the oils against flavour reversion.

While the process has long been used, little was known of the reaction mechanisms or products formed until the last twenty-five years. Scheiber (1929) noticed that refractive indices increased with thermal polymerization times. He attributed this to thermal conjugation of double bonds within fatty acid residues to form conjugated dienes. Kappelmeier (1933) advanced the idea of a 1, 4-addition of a dienophile to a conjugated diene. These two proposals were combined into the Scheiber-Kappelmeier theory of thermal polymerization (Scheiber, 1936) which involves two steps: (1) thermal conjugation of unsaturated fatty acyl groups to conjugated dienes, and (2) the addition of an unsaturated fatty acid residue (dienophile) to the conjugated diene by a Diels-Alder type addition to produce a dimer containing a cyclohexene ring.

Empirical and theoretical evidence supports this theory as being one of the methods, if not the only method, of thermal polymerization. Bradley and Richardson (1940) showed

that conjugated diene absorption (Ultra violet) increases initially during polymerization and then decreases during the latter stages of the reaction, while Bradley and Johnston (1940) proved that conjugated oils polymerize faster than non-conjugated oils. Later Radlove and Falkenberg (1948) presented evidence that previously conjugated soyabean and linseed oils bodied to the same viscosity as the non-conjugated oils possessed superior drying properties. These facts tend to favour thermal conjugation as being the initial step in thermal polymerization.

Bradley and Johnston (1940) confirmed dimerization as being the principal reaction of methyl esters of olive, soya-bean, linseed, dehydrated castor, and tung oil during polymerization. They subsequently (1941) isolated a methyl linoleate dimer from polymerized methyl esters of dehydrated castor oil acids. Previously Brod, France, and Evans (1939) had shown that the polymerization products of 9,12- and 9,11-linoleates were identical. Hence it appears that dimers are part of the reaction products.

Farmer and Morrison-Jones (1940) found small quantities of crystalline cyclohexene derivatives in the total dimer of methyl sorbate, which indicated a Diels-Alder reaction had occurred.

Ahmed and Farmer (1940) polymerized 1,4-pentadiene, which has a bond group similar to linoleic acid. They found the thermal dimer to be a complex mixture containing cyclo-

hexenes from which o-phthalic acid was isolated in small quantities. The dimer was of such a structure as to indicate that 1,3-pentadiene had been added to a bond of unconjugated 1,4-pentadiene. Lawson and Spoerri (1947) have shown that co-polymerization does occur between unconjugated and conjugated fatty esters. Paschke and Wheeler (1949) have confirmed this observation by showing that n-methyl linoleate will polymerize by addition with conjugated linoleate isomers.

Oils containing trienoic acids (linolenic, elaeostearic) have been said to dimerize first to monocyclic dimers with subsequent reaction of the remaining ethenoid bonds with another conjugated diene resulting in formation of bicyclic trimers. In this connection Boelhouwer, Tien, and Waterman (1953) have applied their method of ring analysis to dimers of methyl linoleate and methyl linolenate. They found that linoleate dimers contained one ring per molecule while linolenate dimers contained two rings per molecule.

As well as conjugation, cis-trans isomerization has been shown to occur in fatty acyl radicals during heat bodying (Paschke, Jackson, and Wheeler, 1952). In studies with methyl linoleate these authors have shown that normal cis, cis-linoleate isomerizes to cis-trans and trans-trans forms. The trans-trans conjugated form has been shown to polymerize much more rapidly than either the cis-trans or cis-cis isomers. Molecular models (Fisher-Hirschfelder) suggest that neither cis-cis nor cis-trans conjugated linoleates can

swing into the proper planar semi-ring structure required for dienophile attack without severe molecular strain and the adjacent methylene groups present serious steric hindrance to dienophile approach. The trans-trans form, however, can rotate freely to the semi-ring structure and presents minimum interference to dienophile approach. From these considerations Wheeler (1951) proposes that the majority of the conjugated dienes isomerize to trans-trans forms before adding a dienophile. It is probable that other geometrical isomers could add dienophiles if sufficient activation energy was provided to distort the molecules to the correct planar position. Such an occurrence is quite possible, especially at the high temperatures of polymerization.

Although the evidence for the Diels-Alder type addition is quite convincing, classical organic structural proof of the nature of dimers or trimers has been lacking. Recently Clingman, Rivett, and Sutton (1953, 1954) have provided such a proof. They polymerized methyl-β-elaeostearate (trans,trans,trans-9,11,13-octadecatrienoate) for four and one half hours at 185° C. in a sealed bulb. The dimer, on substitutive bromination followed by debromination and then oxidation, gave prehnitic acid (benzene-1,2,3,4-tetracarboxylic acid). This acid has the required six-membered ring of carbon atoms substituted in four neighbouring positions that would result if a Diels-Alder type addition had occurred. The yield of prehnitic seemed to indicate that Diels-Alder addition

was the only operative reaction in this case.

These authors also isolated prehnitic acid from the dimers of methyl linoleate, methyl linolenate, and sunflower-seed oil. In these instances the yield of prehnitic acid seemed to indicate that another mechanism besides the 1,4-addition was operating. The polymerization temperatures may have destroyed some of the cyclic structures and thus decrease the yield of prehnitic acid.

The Scheiber-Kappelmeier theory has not been universally accepted, and in such a complex reaction process as thermal polymerization it is almost certain that other mechanisms are operative. The point of disagreement is whether these mechanisms are minor or major ones.

Brocklesby (1941) proposed a scheme in which two direct carbon-to-carbon bonds are formed between two ethenoid bonds in different residues forming a four carbon ring which then rearranges as shown:

$$\begin{matrix} R.CH{:}CH.R' \\ + \\ R.CH{:}CH.R'' \end{matrix} \rightarrow \begin{matrix} R.CH\text{-}CH.R' \\ | \quad | \\ R.CH\text{-}CH.R'' \end{matrix} \rightarrow \begin{matrix} R.CH_2.CH.R' \\ | \\ R.C{:}CH.R'' \end{matrix}$$

This author said that the reaction could only occur intermolecularly because of the shift in the fatty acyl chains which would rupture any intermolecular compound. This theory was advanced before the mobility of hydrocarbon chains was fully realized.

Bernstein (1946, 1949) does not believe that prior conjugation is necessary for polymerization. He proposed a

direct union between ethenoid groups subject merely to steric considerations.

Barker, Crawford, and Hilditch (1951 a) view polymerization as occurring between a reactive methylene group of one fatty acyl group and the conjugated diene system of another group by a modified 1,4-addition forming a dimer with a single cross link (cf. two cross links with Diels-Alder reaction). They based the empirical part of this theory on the fact that polymerized sunflowerseed oil exhibits more residual unsaturation (after the linoleic and linolenic acids have been accounted for) than would be expected from a Diels-Alder type addition. They also found that the mixed fatty acids of the polymerized oil became more soluble in acetone at -55° C. as the polymerization proceeded. Since linoleic and linolenic acids "were accounted for" by a spectrophotometric procedure, it is quite possible that with the short time alkali-isomerization procedure used by the authors (Hilditch, Morton, and Riley, 1945) they might have failed to isomerize all the difficultly-conjugable linoleate and linolenate compounds (Paschke, Tolberg, et al, 1952). If so, this would account for the high iodine value of the residual unsaturation (cf. Section VII, Experimental).

Paschke and Wheeler (1952) have demonstrated that methyl oleate possesses a slight but definite polymerization functionality. They isolated a small amount of dimer which contained one ethenoid group per molecule. No theory as to its formation was advanced by these authors.

Regardless of the method of formation, it has been established that dimerization (and trimerization) occur during thermal polymerization of polyene oils. There have been some considerations as to whether the dimerization occurs between acid groups in different molecules (intermolecular dimerization) or whether it occurs between acid groups in the same molecule (intramolecular dimerization).

Intermolecular dimerization increases the molecular weight by uniting two different gylcerides. Intramolecular dimerization, on the other hand, would not increase the molecular weight because reaction is between different parts of the same molecule.

Adams and Powers (1944, 1946) noticed thatm during the early stages of polymerization, the iodine value decreased rapidly with only slight increases in molecular weight and viscosity. They gave this as evidence of intramolecular dimerization.

Bradley and Pfann (1940) studied a sample of thoroughly heat-gelled drying oil. On conversion of the solid phase to methyl esters, analysis showed seventy-five per cent dimers and twenty-five per cent monomers. This, they said, is a higher proportion of dimers than would be expected by interpolymerization.

Barker, Crawford, and Hilditch (1951 a) contend that initial polymerization occurs by an intramolecular dimerization to produce cross-linked acyl groups. The sudden rise

in viscosity in later stages with little apparent change in linoleate and linolenate content suggested that there was a rearrangement from intra- to interdimerization by acyl interchange (interesterification). They demonstrated that ester interchange did occur at bodying temperatures, albeit slowly (Barker, Crawford, and Hilditch, 1951 b). These authors also converted some of the bodied oils to methyl esters and determined their molecular weights. From these weights they calculated the theoretical molecular weights of the triglycerides. In all cases the calculated molecular weights were greater than the observed values. This, they contend, is due to intrapolymer formation.

Joubert and Sutton (1952) separated a lightly-bodied pilchard oil into monomeric (sixty-five per cent) and polymeric (thirty-five per cent) fractions. The monomeric glycerides, after conversion to methyl esters, were distilled to afford only 1.5 per cent dimer esters. This indicated no appreciable intrapolymerization. Powers (1952) replied to Joubert and Sutton and said that the results confirmed rather than negated intrapolymerization. To this Sutton (1953) gave an equivocal answer. The polemic rests at present at that stage.

Polymerized linseed oil was investigated for intra- and intermolecular dimers by Paschke and Wheeler (1954). Linseed oil was bodied at 300° for 1.5, 3, and 6 hours. The glycerides were separated into a monomeric and a dimeric fraction by

molecular distillation, and the derived methyl esters were analyzed for monomer, dimer, and trimer. They found that the monomeric glycerides contained very little intradimers (three to four per cent of the acid groups in the whole oil)while the polymeric glycerides contained appreciable amounts (ten to twenty per cent). They contend that there is no evidence to support the contention that a shift from intra- to inter-dimerization is the major cause of the sudden increase in viscosity in the later stages of polymerization. It is probably due to the nature of the relationship of viscosity to molecular weight and of molecular weight to extent of reactions in the polyfunctional system present.

Numerous minor side reactions occur during thermal polymerization. The products include hydrocarbons, acrolein, aldehydes, free fatty acids, low molecular weight esters, etc. Some of these products originate from pyrolytic decomposition, others are formed as by-products of the main polymerization. In this latter connection, Paschke and Wheeler (1949) found monomeric iso-oleates which they could not hydrogenate to stearate, and in the present work the NAFD fractions isolated from sunflowerseed, soyabean, and linseed oils represent abnormal (theoretically) products.

Adduction Compounds of Urea and Thiourea

Bengen (1940) discovered the phenomenon of urea complex formation with aliphatic straight-chain compounds. He showed that normal straight-chain hydrocarbons form urea complexes by addition, but that cyclic or branched-chain molecules do not. Thus the petroleum and oil chemist were provided with an excellent analytical tool for a separation method based on molecular shape and chain length.

This rule is not rigid, for some cyclic and branched-chain structures will form adducts if the attached hydrocarbon chain is of sufficient length (Truter, 1951). An interesting pair in this connection is 3-ethyltetracosane and 1-cyclopentlheneicosane. Both have an entity of five carbon atoms attached to a straight-chain of twenty-one carbon atoms. While the rigid cyclic arrangement of five carbon atoms does not interfere with adduction, the freely rotating ethyl groups in 3-ethyltetracosane prevent urea adduction.

Detailed reports on this complex formation have been made (Bengen and Schlenk, 1949; Zimmerschied *et al*, 1950) since the original German patent came to light. Schlenk (1949) reported in detail on the formation of urea complexes in relation to the shape of the organic molecule, their composition, their crystal structure, and their energy of formation. These results have since been confirmed and extended by Redlich *et al* (1950 a) and Knight *et al* (1952).

In the adducts six urea molecules form a hexagonal lattice which has a hollow core. It is into this "tunnel" that the adductible materials go. All adducts exhibit the same crystalline form.

Adducts of unsaturated fatty acids have been shown to stabilize these acids towards oxidation (Schlenk and Holman, 1950 a, 1950 b).

Recently the difference in rate of adduct formation between saturated straight-chain acids and unsaturated straight-chain acids has been utilized for their separation. This fractional separation technique has been used by Abu-Nasr et al (1954) for the separation of highly unsaturated fatty acids, by Swern and Parker to separate oleic acid from inedible animal oils (1952 a), oleic and methyl oleate from olive oil (1952 b), and the concentration of natural linoleic and linolenic acids (1953).

The history and processes connected with urea adduct formation have been reviewed in a previous thesis (Wells, 1952). Since that time the range of application of the technique has widened, but the basic theory has not changed.

Thiourea forms a somewhat similar type of inclusion complex with certain types of organic molecules. This phenomenon was discovered by Angla (1947) and since has been described and studied by Schlenk (1951) and by Redlich et al (1950 b).

Thiourea forms adducts with more compact molecules than

those characteristic of urea adduction (i.e. long chain hydrocarbons). The interior channel in the thiourea spiral is larger than that of urea because of the larger radius of the sulfur atom. Schiessler and Flitter (1952) have said that the channel in these adducts has the dimensions of 5.0 by 6.8 Å. Their figures are based on many experiments with molecules of known dimensions.

Thiourea inclusion compounds are less stable than their urea analogues and hence possess a low heat of formation, lower free energy, and show a decrease in the molal ratio of thiourea to reactant compared with urea to reactant.

Thiourea and urea appear to offer possibilities of complementary applications, especially in petroleum technology where compact (aromatic) and long chain hydrocarbons are frequently encountered together.

Occurrence, Physiological Effect, and Nutrition Of Heated Oils and Fatty Acids

Heated Oils

Unsaturated vegetable oils develop undesirable odours and flavours when exposed to heat or light and thus their use as edible oils is restricted. Thermal polymerization renders such oils much more stable to flavour reversion (lips et al, 1953). This process has been used in the United States for herring oil (Lassen et al, 1949) and in Germany for fish oils (B.I.O.S. Report) to replace more expensive vegetable oils in canning and cooking.

The content of linolenic acid, considered the chief agent responsible for flavour reversion, was greatly reduced in linseed oil by polymerization process of Privett et al, (1945) in which the oil was heated at 275° for twelve hours under a stream of carbon dioxide. This heated oil was acetone segregated and the segregate was hydrogenated to produce a shortening of superior flavour stability. However, when this product was fed to rats, a high incidence of death occurred (Crampton and Miller, 1946). Since whole linseed oil is wholesome and nutritious (Molotkow, 1932), the polymerization process must have deleteriously effected the oil.

Gass and Mills (1947) prepared heated linseed oil and fed hydrogenated and non-hydrogenated fractions to rats. Both fractions were nutritionally inferior to the whole

unheated oil. This showed that the isomeric acids produced by hydrogenation were not responsible for the impaired quality of the oil. These workers further investigated the effect of lecithin and ergosterol on rat nutrition. These compounds, incorporated in commercial shortening and heated at 275° for fifteen hours, had no effect upon the value of the shortening. Furthermore, tests with crude and refined oils heated under carbon dioxide showed identical results. As a final test Gass fed the volatile condensate from thermally polymerized linseed (as shortening) to rats. They proved to be innocuous. Hence it seemed that the nutritional defect lay within the polymerized oil itself, and was not due to hydrogenation products, unsaponifiable matter, or the volatile pyrolysis products.

Previously Roffo (1944) had reported that continuous feeding of heated sunflowerseed and olive oils (350° for thirty minutes) produced carcinoma in rats. These results have never been confirmed, but Morris (1943) has reported that heated lard (300° for twenty minutes) causes reduced growth and weight losses when fed to rats as fifty per cent of the diet. He further noticed some incidence of paralysis and gastric ulcers. Recently Chalmers (1954) fed heated cottonseed oils to rats and found that some developed ulcers in their forestomachs. However, both Chalmers and the other two workers heated oils in the presence of air. This would cause excessive peroxidation. Privett heated oils under

carbon dioxide, a process which largely eliminates peroxide formation. Harris (1947), however, has shown that sardine oil heated under vacuum causes decreased growth.

Subsequent experiments with oils polymerized by the method of Privett showed that linseed, corn, rapeseed, peanut, soyabean, and herring oils all decreased in nutritional value on heating (Crampton, Farmer, and Berryhill, 1951). The effect was more apparent with increased heating time and with increased levels of oil in the diets. Linseed oil, the most unsaturated oil, produced the worst symptoms in the shortest time. It had been thought that heated oil interfered with proper absorption in the gut. When heated linseed oil was fed at a different time from the main diet no appreciable difference from simultaneous feedings was noted. Hence the deleterious effect is not merely due to interference with absorption of nutrients.

Kauritz (1953) demonstrated that oxidized hog fat caused a decrease in growth rates of rats. This decrease paralleled the intake of oxidized fat. Vitamin E is a natural growth factor (Fraenkel and Blewett, 1946) and anti-oxidant, and its absence from the diet can cause serious effects. (Mackenzie et al, 1940). Because heating oils destroys their natural anti-oxidants, NDGA (nordihydroguararetic acid, an anti-oxidant) and Vitamin E were fed along with the linseed oil diets in an experiment in this laboratory. No significant difference was noted between the diets with and without these

additions (Crampton, Common, et al, 1951 a). Therefore neither peroxidation nor lack of Vitamin E was responsible for the decreased nutritive value of the heated linseed oil. Farmer, Crampton, and Siddall (1951) have shown that pregnant rats fed heated linseed oil produced decreased numbers in their litters and that the offspring were underweight. This same effect was observed with pregnant rats fed Vitamin E as a supplement.

Wiseblatt (1950) formed the ethyl esters from the heated linseed oil by alcoholysis. These esters were solvent segregated then vacuum distilled. None of the fractions isolated by this method were equal in nutritional value to the esters of the unheated oil (Crampton, Common, et al, 1951 b). Six out of eight of the rats fed the polymeric residue died. Although these rats ate two grams per day, an amount that has proven to be sufficient for survival, the reduced digestibility of these esters might have lowered the caloric intake to a point where death by starvation would ensue. These rats all showed poor general health, matted coats, and stick feces. It therefore seems that part of the reduced value of heated oils is due to the presence of undigestible polymeric material. This material, however, does not seem to be toxic.

Destruction of essential factors in the oil during polymerization might also account for poor condition in the rats. Rokkanes (1953) has shown that linseed oil contains an

essential hair growth factor for rats. This factor seems to be associated with the linoleic and linolenic acids. Both these acids are also essential nutrients.

Wells (1952) urea segregated the distillable ethyl esters of heated linseed oil into two fractions: an adduct forming fraction, and a non-adduct forming fraction. The adduct forming fraction proved equal in nutritional value to the whole esters of the unheated oil (Crampton, Common, et al, 1953). which are but slightly inferior to the triglycerides. This was the first time that a nutritionally good fraction had been isolated from heated linseed oil.

The urea non-adducting fraction was found to be extremely injurious to growing rats. While, unlike the polymeric material, these fractions were well digested, they were not utilized for growth. All rats fed this material died. Thus it seems that the nutritionally deleterious material resides in the non-adducting portion of the distillable ethyl esters, and is composed of one or more of the following classes of compounds: cyclic fatty acids, branched-chain fatty acids, or highly unsaturated fatty acids. This follows from the fact that urea will not form adducts with such molecules.

Fatty Acid Isomers

As well as supplying a concentrated source of calories in the diet, fat provides a vehicle for essential nutrients as well as having a sparing action on other dietary

components. Three fatty acids are considered essential for the nutrition of the rat, of man, and possibly of many other higher vertebrates, viz.: linoleic (9,12-octadecadienoic) acid, linolenic (9,12,15-octadecatrienoic) acid, and arachidonic (5,8,11,14-octadecatetraenoic) acid. Some authorities refer to this group as "Vitamin F", however, few authors use the term. These acids are widely distributed throughout the plant and marine worlds. Lack of essential fatty acids causes hyperkeratosis in rats (Ramalingaswami and Sinclair, 1953), and leads to disturbed ovulation and numerous epidermal diseases in both humans and rats (Wooster and Blanck, 1950).

In thermal polymerization and hydrogenation a certain amount of change in oil structure occurs. Positional and geometric isomers of the fatty acids are formed, and as a result a portion of the essential fatty acid activity is destroyed.

Melnick and Deuel (1954) have made a study of the literature concerning the effects of hydrogenation of vegetable oils to determine whether the isomeric acids so formed differ from the naturally occurring fatty acids in nutritional value. It was found, using microbiological assay techniques, that iso-oleic acids are not anti-metabolites for natural oleic, but function as normal nutrients. The conjugated fatty acids are readily metabolized to carbon dioxide and water, and do not act as antagonists for the essential fatty acids.

Hydrogenated oils compare favourably with natural oils as sources of essential fatty acids. Although the per cent of these acids is reduced, it seems that the iso-acids exhibit essential fatty acid activity. These conclusions may be applied with reasonable validity to thermally polymerized oils as well as to hydrogenated oils for there is a certain similarity in the products of reaction.

Boughton (1953) has demonstrated that several mono-unsaturated fatty acids can replace oleic acid in the nutrition of corynebacterium "Q". These acids can have either the cis or trans configuration, and it is not essential that the chain be eighteen carbon atoms long.

Blaxter, Brown, and MacDonald (1953 a) have shown that calves fed the total unsaturated fatty acids of codliver oil supplemented with α-tocopherol developed severe muscular dystrophy. Calves fed the unsaponifiable matter or the saturated acids displayed no symptoms. A slight dystrophy occurred in calves fed the higher unsaturated fatty acids, but only a paleness in the muscles occurred when the lower unsaturates were fed. It was concluded that the toxicity of codliver oil is caused by its polyunsaturated fatty acids and not by hypervitaminosis A or D. This toxicity may be a general effect of polyunsaturation and hence not be due to any specific fatty acid. dl-α-tocopherol protected the animals against the effects of the oil only when administered orally (ibid, 1953 b).

It seems, therefore, that the body can handle most of the isomeric fatty acids, and that only polyunsaturates cause harm.

Branched-chain Fatty Acids

Chevreul in 1817 (cf. Hilditch, 1947) discovered isovaleric acid in the head oil of the dolphin. This was the first time that an odd-numbered fatty acid had been found in nature. It was not until much later that the branched-chain nature of the acid was definitely established. This remained the only naturally-occurring branched-chain fatty acid which could be isolated from triglyceride oils until 1950.

Anderson and his co-workers (1929) showed that the waxy envelope of certain acid-fast bacilli (leprosy and tuberculosis) contained appreciable amounts of branched-chain fatty acids. Anderson and Chargaff (1929) isolated tuberculostearic acid from tubercle wax, which was later proven to be 10-methylstearic acid by Spielman (1934). Phthioic acid was similarly isolated and investigated by Anderson and Spielman (1936, 1944, 1945). They considered it to be a C_{26} multi-branched chain acid. Polgar and Robinson (1943, 1945) have assigned it the structure of 3,13,19-trimethyltricosanoic acid. A C_{27}-phthioic acid, isolated from tubercle bacilli by Cason et al (1953), has been given the structure of a α-methyl-α,β-unsaturated acid. Velick and Anderson (1944) isolated a C_{20} acid from the acetone-soluble fats of Phylomonas tumefaciens which they called phytomonic acid. It is a 10 or 11-

monomethyl nonadecanoic acid. Very recent work by Polgar (1954 a, 1954 b) has elucidated the structure of two acids, mycolipenic and mycoceranic, from the lipids of tubercle bacilli, as tri-methyl branched fatty acid.

Up until 1950 the only other source of branched-chain fatty acids was wool wax. Weitkamp (1945) investigated the composition of Degras (wool fat). He isolated and identified several branched-chain fatty acids in the material which were mainly esters of sterols and triterpenoids. Recently Brouwer and Nijkamp (1953) have identified 3-methylbutanoic and 2-methylbutanoic acids in the hair grease of dogs. Branched-chain fatty acids have also been reported to occur as esters of octadecanol in the coccygeal glands of ducks (Weitzel, 1951).

Before 1950 no naturally-occurring branched-chain fatty acids, with the sole exception of isovaleric, had been reported as existing in natural triglycerides. However, in 1950 Hansen and Shorland published the first paper of a series in which they have reported the occurrence of small amounts of branched-chain fatty acids in triglycerides from many animal fats. The first paper reported the presence of a branched-chain acid in butterfat. In subsequent papers they reported the isolation of a C_{17}-methyl branched acid (1951 a), and a multi-branched C_{20} acid (1951 b) in butterfat triglycerides. In 1954 they identified two acids in the butterfat as (+)-12-methyltetradecanoic and 13-methyltetradecanoic acids.

The same authors (1952 a) examined the external tissue fat of oxen and found a C_{17}-dimethyl acid, then mutton fat yielded three fatty acids, two of them optically active: (+)-14-methylhexadecanoic acid (1952 b), and (+)-12-methyl tetradecanoic acid. Later (1953) the same fat yielded a 13-methyltetradecanoic acid. These latter two optically active fatty acids have also been isolated from wool wax. Shark liver oil has been shown to contain a C_{18} multi-branched fatty acid (Morice and Shorland, 1952). All the acids reported by Shorland and his co-workers are components of natural triglycerides. Hence the older notion that branched-chain fatty acids are rare and mainly confined to micro-organisms and marine mammals must be abandoned. It will also be seen that many of these acids contain odd numbers of carbon atoms, which is contrary to the older generalization that natural fatty acids are always even-numbered. Nothing is yet known about the metabolism of these acids, although it has been suggested that leucine, isoleucine, and valine play a role in their synthesis, especially those of low molecular weight. It is almost certain that the branched-chain acids follow different metabolic pathways from the normal, straight-chain fatty acids.

The physiological activity of branched-chain fatty acids has been investigated by several workers, especially since pathogenic bacteria have been shown to contain such acids.

Anderson (1929) has reported that injections of tuber-

culostearic and phthioic acids produce tubercle-like lesions at the injection site.

Robinson (1940) injected two synthetic branched-chain fatty acids into rabbits and obtained a reaction in both cases. Intraperitoneal injections of 2,2-dimethylundecanoic acid produced peritonitis and leukemia in rabbits, while 2,2-di-n-octylpropanoic acid produced a reaction similar to phthioic acid.

Buu-Hoi and Ratsimamanga (1943) obtained similar results when guinea pigs were injected with 2,2-dimethyloctadecanoic acid. The dose was fifty mg. of acid weekly for four weeks. A marked decrease in hemoglobin concentration - fifty per cent in three weeks - and an increase in the ratio of monocyte to lymphocyte (M/L) occurred. The M/L ratio rose to forty to ninety within twelve hours after injection. The control animals had M/L ratios of only ten to twenty.

Autopsy of the guinea pigs revealed extensive adhesions between organs of the peritoneal cavity. Lesions similar to those of tuberculosis were noted on the liver and the diaphragm.

The linear isomer, arachidic acid, when tested, proved innocuous.

Paraf et al (1945) gave guinea pigs four intraperitoneal injections over a two week period of an unsaturated branched-chain fatty acid (α , α-dimethyl-ω tridecylic acid). The result was cachexia ending in death. Subcutaneous injections

produced shock and loss of weight but not death. Massive, concurrent injections of nicotinamide countered the effect of the intraperitoneal injections, but they did not prevent the loss of weight.

Cagnant et al (1950) tested the esters of the same acid. Subcutaneous injections of 0.6 c.c. into mice or guinea pigs produced no toxic effects. The ester did appear to have a bacteriotropic effect when injected into guinea pigs infected with septic tuberculosis.

Polgar (1948) tested 3,13,19-trimethyltricosanoic acid and found effects comparable to those of natural phthioic acid.

Many fatty acids have been tested for bactericidal activity against B.leprae. Stanley, Jay, and Adams (1929) synthesized a series of hexadecanoic and octadecanoic acids in which the carboxyl group varied from terminal to central position on the carbon chain. The best effect was produced by acids with the carboxyl group near the centre of the chain.

These reports indicate that branched-chain fatty acids have definite physiological effects and that these effects are more pronounced in the higher molecular weight acids. The presence of a free carboxyl group has been shown necessary for physiological action in one instance and it may be that a free carboxyl group is prerequisite for biological activity in all cases.

Cyclic Fatty Acids

The tropical plants of the family Flacourticeae produce chaulmoogra, lukrabo, and gorli-seed oils which are rich in cyclic fatty acids (chaulmoogric, hydnocarpic, gorlic), all of which contain a terminal cyclopentenyl ring. These acids have been known for many years.

More recently Hofmann na d Lucas (1950) isolated a C_{19} cyclic acid from Lactobacillus arabinosis which they called lactobacillic acid. This acid contains a non-terminal cyclopropane ring at the 11-12 positions.

Hydrolyzates of the polymixins have yielded a 6-methyl octanoic acid (cf. Fracton and Simmonds, 1953).

Nunn (1952) isolated and characterized a cyclic fatty acid from the kernel oil of Sterculia foetida. This acid, called sterculic acid, has been identified as ω -(2 n-octyl-cyclo-prop-1-enyl)-octanoic acid. This acid has a non-terminal cyclopropene ring, and Nunn demonstrated that the acid only forms urea adducts with difficulty.

Chaulmoogra oil has long been used in the treatment of B.leprae, and investigators have thoroughly studied its component fatty acids (cf. Ralston, 1948). It has been established that cyclic fatty acids of the oil possess bactericidal activity. This activity is not greatly decreased by esterification. Many cyclic and branched-chain fatty acids have been tested in vitro for bactericidal action on B. leprae, and it has been established that a cyclopentenyl

ring is unnecessary. The principal requirement is high molecular weight (C_{16}-C_{18}) with the molecular configuration being of secondary importance. Many other cycli and branched-chain acids have been synthesized and tested for activity on B.leprae, and many of these have proven to be as effective as chaulmoogric acid in vitro.

Bernhard and Muller (1938) have shown that oral administration of chaulmoogric acid and its derivatives produce mild toxicity, hence, in prophylaxis, the dose must be strictly controlled. These authors failed to elucidate the metabolic role of the acid.

Buu-Hoi and Ratsimamanga (1941) investigated the toxicity of chaulmoogric and hydnocarpic acids. They ascribed the injurious effects to the cyclopentenyl ring.

Sartory et al (1950) studied natural and synthetic cyclopentenyl acids as well as some closely-related fatty acids. They injected rats and guinea pigs with 0.6 c.c. and 1.0 c.c. of acid respectively. The doses were administered both intraperitoneally and subcutaneously. The symptoms noted were: (1) Very severe convulsions, (2) respiratory spasms, (3) a decreased pulse rate, (4) elevated arterial pressure, (5) vaso-constrictions which arrested peristaltic movements of the digestive tract, (6) death (0.6 c.c. injected into rats caused death in three to six hours), and (7) in cases of delayed death, methemoglobin formation.

As a result of these studies the authors believe that

a five or six membered ring possessing an isolated ethenoid bond is responsible for toxicity. Removal of the double bond by hydrogenation, or addition of more double bonds to the ring, depressed the toxicity. This is in contrast to the bactericidal activity of the oil, where the double bond or even the ring had no special effect upon the activity.

PART II

METHODS

Alkali-refining and Bleaching

The oils were alkali-refined in batches of 1.5 kgm. in a 2000 ml. erlenmeyer flask with 20° Baume sodium hydroxide. The acid value of the oil was determined, and from tables (Bailey, 1945) the correct amount of alkali was calculated. This was added to the oil at room temperature while stirring vigorously with a mechanical stirrer. When the oil "broke" the stirring was discontinued and the flask was placed on a steam bath. The temperature was raised to 50° C. with occassional stirring, then removed and placed in a cold room (6° C) overnight. The clear oil was decanted and the gummy residue discarded. The refined oil was washed free of alkali with warm water and dried over sodium sulfate. The sodium sulfate was removed from the oil by filtration.

Batches of 500 gm. of refined oil were bleached with two per cent w/v activated bleaching clay (Super Filtrol). The batch of oil was heated to 50° under a stream of nitrogen then the bleaching clay was added with stirring. The flask was swirled for a few minutes to assure intimate mixing and then allowed to remain at 50° for fifteen minutes. The bleached oil was filtered free of clay.

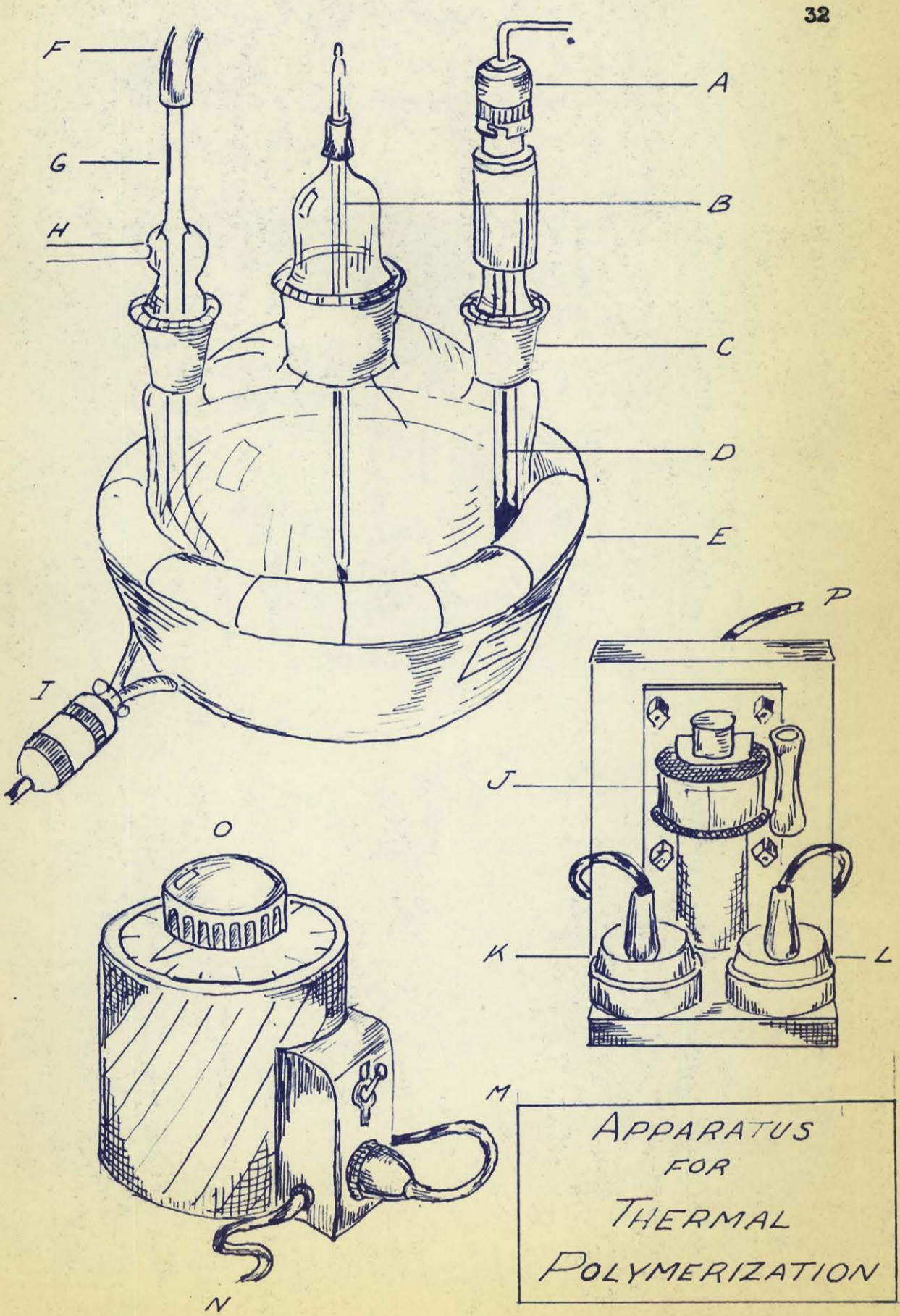
Thermal Polymerization

The polymerization apparatus is illustrated in Figure

1. It consists of a 2000 ml. three-necked flask having a 45/50 standard taper centre neck and 29/42 standard taper side necks. These necks accommodate a 360° thermometer, a thermoregulator, a gas inlet and outlet tube. They are described in the legend on page 33.

The polymerization flask is heated by a Glas-Col hemispherical mantle. The upper half of the flask is insulated with a layer of glass wool. Temperature control is effected by means of a thermoregulator (Precision Scientific Company) attached to a mercury relay. A Powerstat variable transformer (set at 85 volts) is inserted between the power supply (110 V.A.C.) and the relay. This provides a better temperature control and acts as a safeguard against relay failure. The temperature can be regulated to $\pm$ 2° C.

The polymerization flask holds 1000 to 1200 gms. of oil. Allowance must be made for the expansion of the oil on heating. When this amount of oil was used, little adjustment of the gas supply was necessary. A vigorous stream of carbon dioxide was bubbled through the oil during the heat-up and cool-down periods as well as during the actual polymerization. Timing was begun when the temperature reached 275°. Approximately two hours were required to reach this temperature. When the required heating time had elapsed, the power was shut off and the heating mantle and glass wool insulation removed from the polymerizer. The gas stream was increased to a violent bubble in order to facilitate removal of the



APPARATUS FOR THERMAL POLYMERIZATION

FIGURE 1.

KEY TO FIGURE 1

A. Metal canopy provided with bayonet lock, has prong terminals for contact with terminal fitting of the thermoregulator. A rubber-insulated lead connects this cap with L of the relay.

B. 360° thermometer held by a 45/50 inside joint drawn to fit snugly about the thermometer. A small piece of rubber tubing provides a seal.

C. A 29/42 inside taper joint which serves to hold the thermoregulator D in position. A gas tight seal is provided by a neoprene rubber tube.

D. Thermoregulator, merc-to-wire, adjustable with sealed contact (Precision Scientific Company).

E. Glas-Col 2000 ml. hemispherical heating mantle.

F. Rubber tube connection with carbon dioxide cylinder.

G. Gas inlet tube with 29/42 joint which reaches almost to the bottom of the polymerization flask.

H. Gas outlet tube. Provides for the escape of CO_2 and volatile products of pyrolysis.

I. Electrical connection of heating mantle. It is connected with terminal K of the relay.

J. Relay, merc-to-merc (Precision Scientific Company).

K. Terminal for mantle connection I.

L. Terminal for thermoregulator connection A.

M. Lead from relay (P).

N. Lead to 110 volt A.C. power supply.

O. Powerstat variable transformer.

P. Lead from relay, attaches to Powerstat at M.

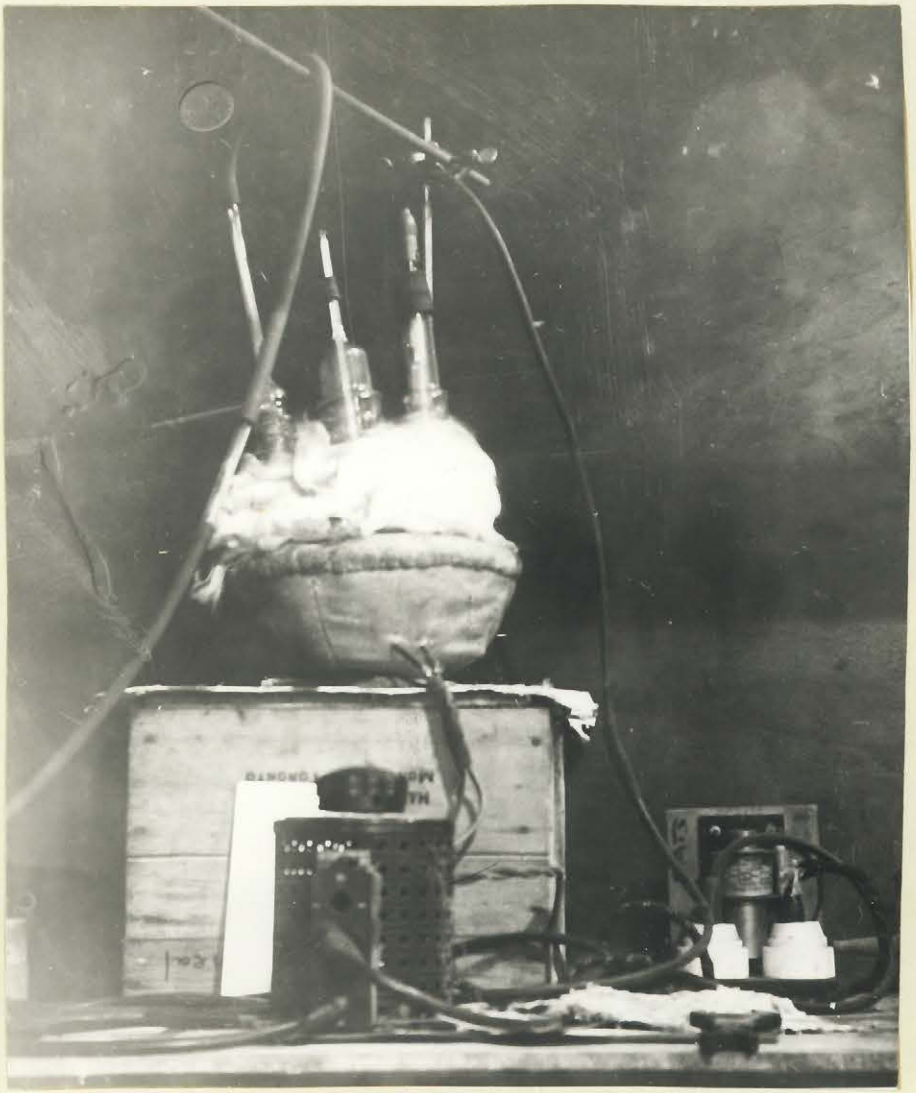


FIGURE 2. Apparatus for thermal polymerization

volatile products of pyrolysis. After the oil had cooled 30° to 40°, the stream of carbon dioxide was reduced and the oil was allowed to cool to room temperature. This required approximately one and one half hours.

Transesterification

Formerly an alcoholic 0.5 per cent solution of sodium hydroxide was employed as catalyst. This procedure had some defects, e.g. alcoholysis was often incomplete, much time was required for dissolving the alkali in ethanol or methanol, and the oil had to be pre-washed with alcohol. The present method uses 0.4 per cent sodium metal in anhydrous alcohol. The weighed sodium is dissolved in the correct volume of dry alcohol in a florence flask fitted with a reflux condenser. Thirty grams of ethanol or twenty grams of methanol were used for each 100 grams of oil. The oil was heated to 55° C in a round-bottomed flask and the sodium ethoxide (or methoxide) solution added quickly. The sodium solution does not normally require heating because of the exothermic nature of the reaction between sodium and alcohol. The mixture was shaken vigourously for about a minute and then replaced on the steam bath and held at 55° for three hours. The ester mixture was then allowed to cool to room temperature overnight. The dark red glycerol layer settled to the bottom.

The esters (upper layer) were decanted into a large separatory funnel (see page 36) and washed with large volumes

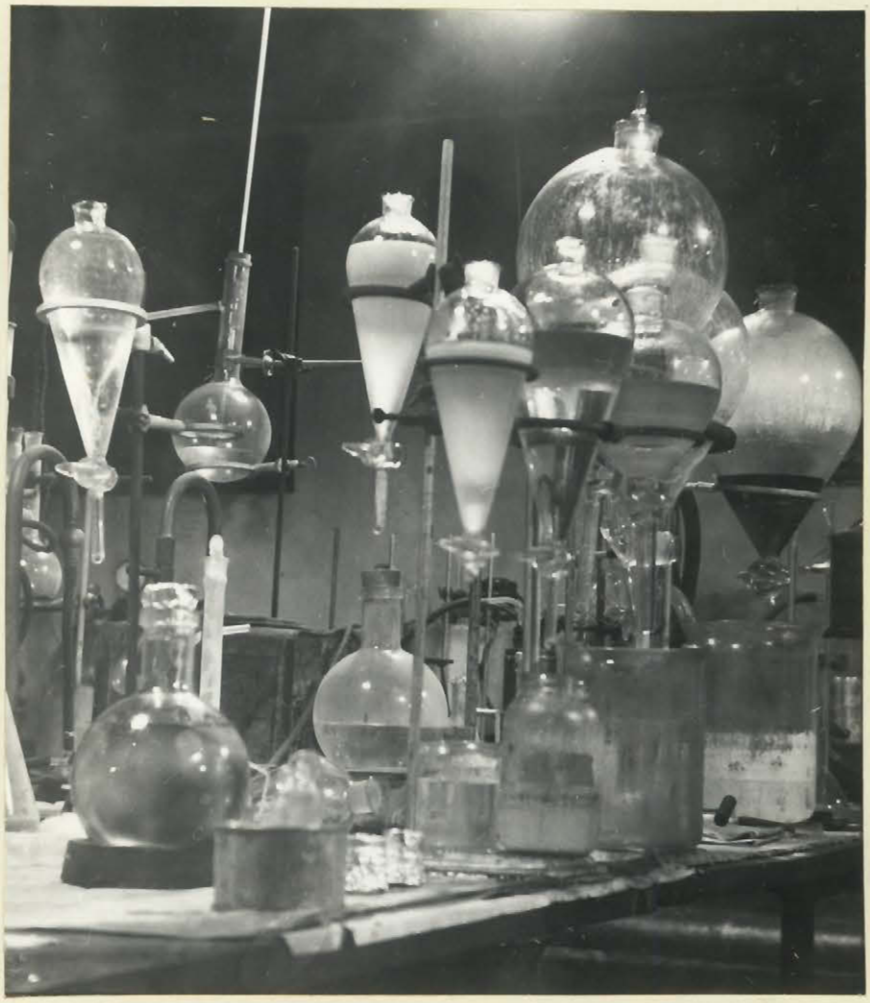


FIGURE 3. Separatory funnels used in oil research at Macdonald College.

of hot water until the washing were neutral to phenolphthalein. This served to removed the soaps and free alkali. The esters were then dried over sodium sulfate, filtered, and the last traces of moisture removed under reduced pressure.

If the transesterification does not go to completion (i.e. no glycerol layer appears), the reaction can be forced by the addition of more (five to ten ml.) of warm sodium-alcohol solution and the process repeated. In this case care must be exercised because more soaps are formed and the danger of emulsification during washing arises. Yields by this "forced" method are always lower than normal.

Vacuum Distillation

The esters were distilled in the apparatus illustrated in Figure 4. Claisen flasks of various sizes were used, depending upon the volume of oil to be distilled. The usual size for preparation of oils for nutritional feeding trials was 500 ml. Heat was supplied by a wax bath (Fisher Bath Wax). The distillation flask was filled with glass wool to minimize the bumping of the oils.

Distillation temperature limits were established for the esters because no manomater was available. The maximum allowable bath temperature was 240° although distillation of the monomeric material was usually complete at 210°. The estimated vacuum on the system was 1 to 3 mm. When distillation was complete, the bath was removed and the oil allowed to cool without breaking the vacuum. All fractions (before and

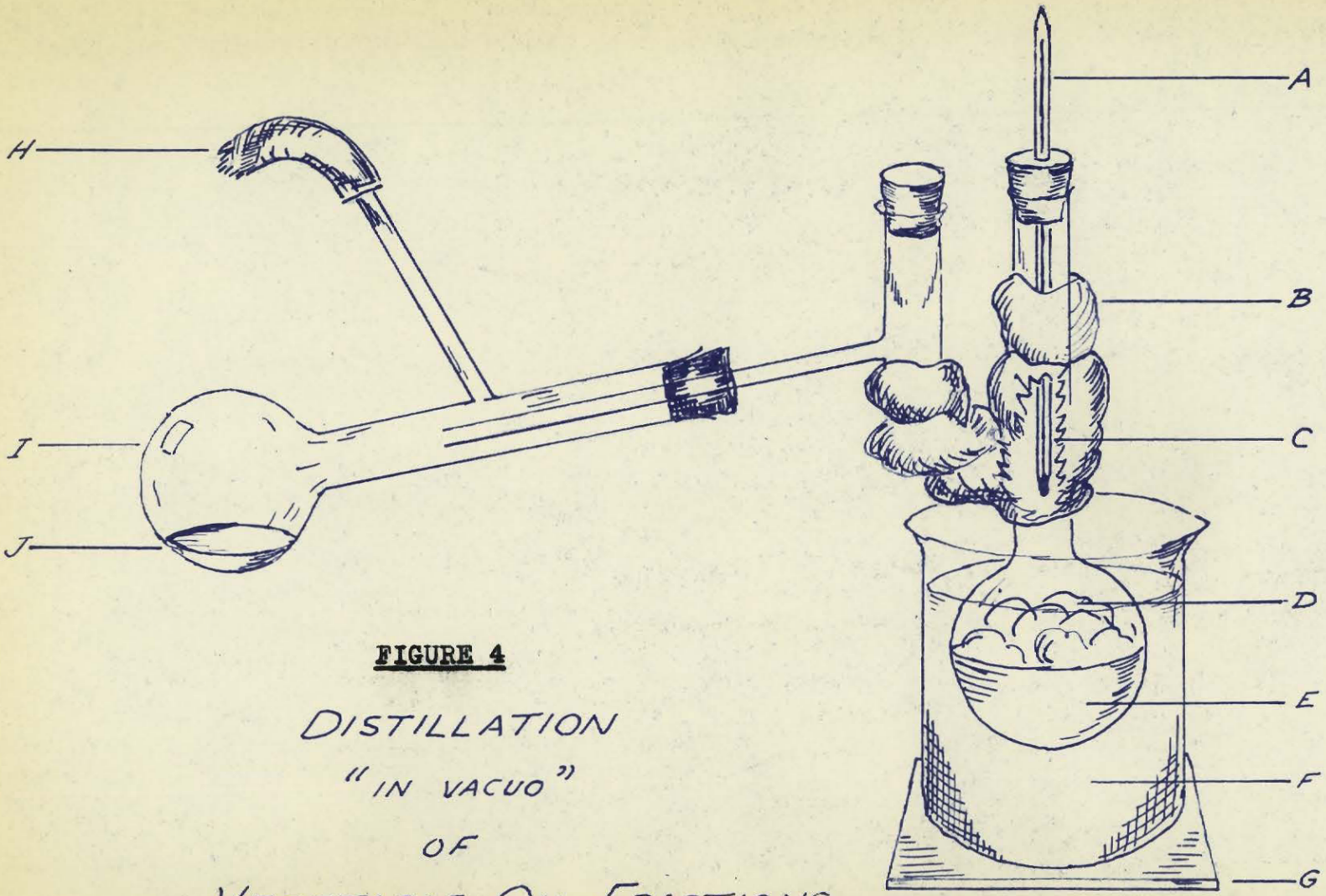


FIGURE 4

DISTILLATION
"IN VACUO"
OF
VEGETABLE OIL FRACTIONS

KEY TO FIGURE 4

A. 360° thermometer for determining the temperature of distilling esters.

B. Glass wool covering upper portion of Claisen flask to facilitate distillation by approximating adiabatic conditions.

C. Claisen flask

D. Glass wool in pot to prevent excessive bumping of the oils during distillation.

E. Oil or ester fraction.

F. Wax bath (Fisher Bath Wax).

G. Wire gauze to prevent excessive heat reaching eax bath.

H. Rubber connections to vacuum system.

I. Receiver for distillate.

J. Distillate.

KEY TO FIGURE 5

A. Distillation tube drawn to scale.

B. Diagram of distillation tube in position for distillation.

1. Thermometer, 300° C.

2. Wax bath (Fisher Bath Wax)

3. To the vacuum system.

4. Residue.

5. Distillate.

6. Point of severance.

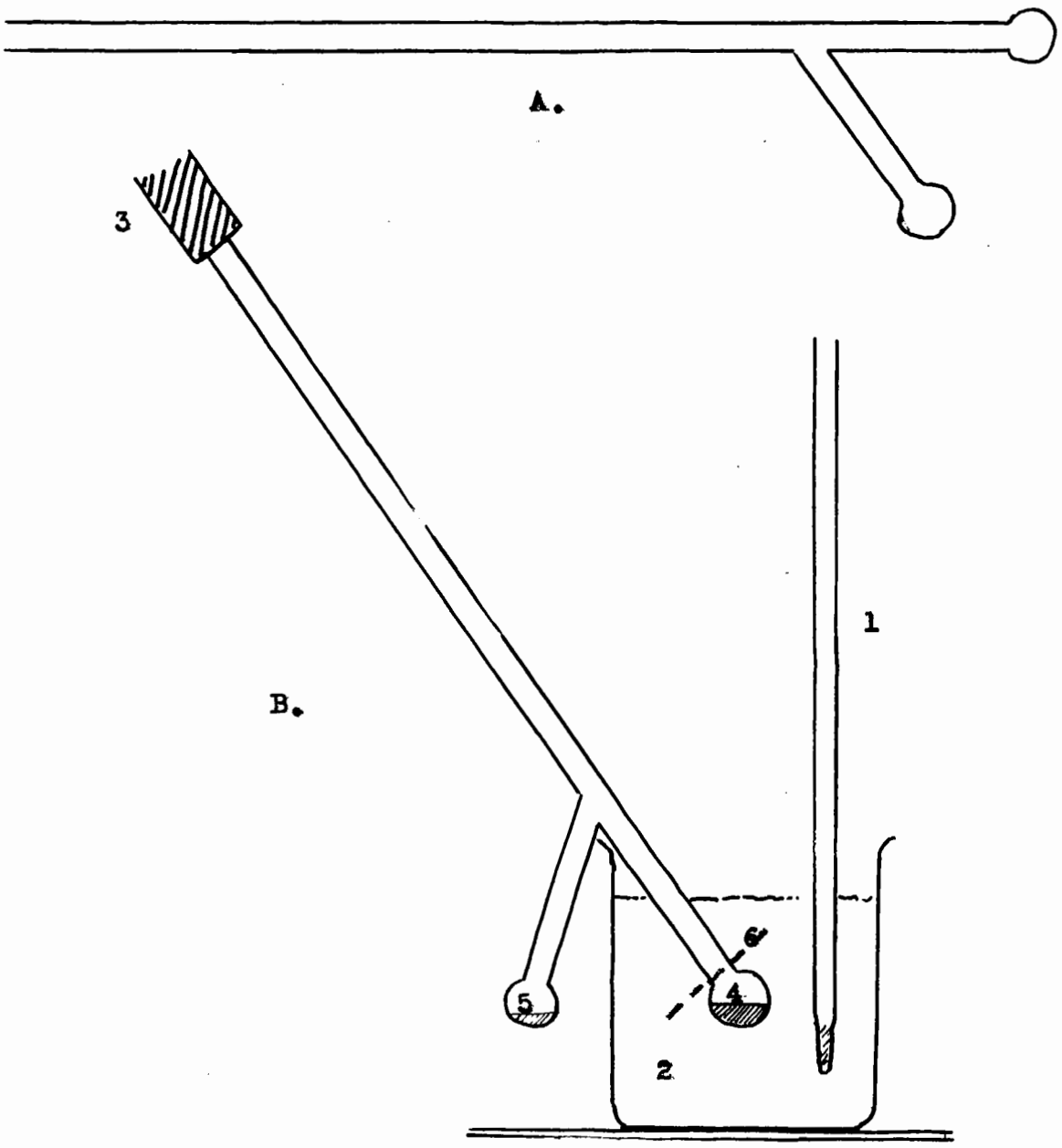


Figure 5. Apparatus for the semi-micro distillation <u>in vacuo</u> of vegetable oil fractions.

after distillation) were protected from oxidation with 0.05 per cent Tenox II (Tennessee Eastman Corporation) anti-oxidant.

A semi-micro distillation was used for the quantitative determination of the per cent distillable esters. The apparatus is shown in Figure 5. A small charge of oil (0.5 to 0.7 gm.) was placed in the bulb of the tared tube by means of a micro-pipette. The tube was re-weighed to determine the weight of the oil, and then the oil was distilled *in vacuo*. The distillate collected in the side arm of the tube. After distillation the tube was severed just above the bulb and both parts were weighed. Then each was washed free of oil and re-weighed. The weights of the distillate and residue were determined by difference.

Urea Segregation

Linear C_{18} fatty acid esters of ethanol or methanol combine with urea in the approximate ratio of one part ester to three parts urea. Therefore to insure complete segregation of all linear material in the esters, a weight of 4 gm. of urea to 1 gm. of esters was employed. The slurry technique, with 1 ml. of ethanol or methanol for every gm. of urea, was used because of the large quantities involved. A description of a typical batch preparation such as used in the preparation of oils for nutrition trials follows:

500 gm. of ester was dissolved in two liters of absolute

ethanol and the solution warmed to 50° C. Two kgm. of finely powdered urea was slowly added with stirring. Any lumps formed during the addition were broken up before more urea was added. The slurry was held at 50° for thirty minutes, and then allowed to cool gradually to room temperature overnight. The adducts were removed on a large Buechner funnel, washed with three portions of absolute ethanol saturated with urea, and the washings were added to the filtrate.

The adducts were decomposed with a large volume of hot water in which the urea dissolved and the oily esters rose to the surface. The aqueous portion was drawn off in a separatory funnel and the remaining esters were washed with warm water and dried over sodium sulfate. The last traces of moisture were removed under reduced pressure.

The combined filtrate and washings were diluted with warm water, poured into a separatory funnel, and the aqueous portion removed. The esters were washed and dried. Both fractions, adducts and non-adducts, were protected with 0.05 per cent Tenox II.

This procedure is a general one and can be used for any oils, heated or unheated. The procedure has an advantage over the solution method in that smaller volumes are handled, and the separation is almost as efficient. The processing temperatures are low (50° at the highest), hence there is little chance for the formation of peroxides.

In quantitative work the esters were recovered from the

aqueous solutions by extraction with ether. The extracts were dried over sodium sulfate and then the ether was removed under reduced pressure in a tared vessel.

Thiourea Segregation

The method closely followed that of Schiessler and Flitter (1952). The modification was in replacing the methanol with ethanol as solvent for the oil.

Mean Molecular Weights

Mean molecular weights were determined by cryoscopy in purified cyclohexane. The concentration of the solute was kept below five per cent for all determinations. The solutions were cooled in an ice-water bath and stirred with a stainless steel loop stirrer. A Beckmann thermometer enabled freezing points to be estimated within $\pm$ 0.002° C. Observed molecular weights were corrected for free fatty acid content by the method of Berstein (1948).

Cyclohexane was purified for cryoscopy as follows: A quantity of cyclohexane was mechanically shaken with about ten per cent of its volume of twenty per cent fuming sulfuric acid for twelve hours. The acid layer was drawn off and the cyclohexane was shaken for another twelve hours with a similar portion of fuming sulfuric acid. This again was drawn off and excess acid in the cyclohexane was removed by addition of granular barium hydroxide. The cyclohexane was then decanted and distilled through a Stedman column.

Iodine Value

The method of Benham and Klee (1950) was used exclusively throughout the present work. However, the reaction time has been increased from one minute to one hour. The method has been found satisfactory for comparative purposes.

Acid Values

A sample of about five grams of oil was dissolved in 100 ml. of a previously-neutralized mixture of toluene and ethanol (1:1 by volume). One ml. of the reagent of Kleinzeller and Trim (1944) was added, and the solution titrated with 0.1 N sodium hydroxide using vigorous stirring. The acid values were expressed as per cent oleic acid.

Peroxide Values

Peroxide values were determined by the method of Skellon and Wills (1948). They are reported as milligrams of peroxide oxygen per kilogram of oil (or ester).

Refractive Indices

Refractive indices were determined at 25° C. with a Zeiss dipping refractometer. A sodium vapour lamp was used for illumination.

Spectrophotometric Analysis

The absorption spectra and the fatty acid composition were determined using the method recommended by the Spectroscopy Committee, American Oil Chemists' Society (1949, 1951). Certain suggested modification advanced by Jackson, Paschke, et al (1952) were followed when analyzing heated oils.

The constant temperature bath used for alkali-isomerizations has been fully described previously (Wells, 1952). Only minor changes have been made by the author: these included insertion of two tubes filled with calcium chloride between the oxygen-absorption system and the gas manifold to remove moisture.

Viscosity

Viscosities were determined with the Gardner Bubble Viscometer at 25° C.

Lead Salt Separation

The method was that of Hilditch (1947)

Lithium Salt Separation

The method of Hilditch and Pedelty (1939) was used. This method incorporates the best features of many previous methods, and was considered suitable for the present work.

Low Temperature Crystallization

The procedure of Hiditch (1947) was employed in which acetone is the fatty acid solvent. In a subsequent crystallization attempt, Skellysolve B was used because the fatty acids are less soluble in it than in acetone (Foreman and Brown, 1943). No precipitable material could be obtained from the acetone solutions.

Multiple Fractional Extraction

The theory and methods of Bush and Densen (1948) were followed. The work of Rebello and Daubert (1951), who extracted dibasic acids from oxidized oil mixtures, was used as a guide. These procedures were found to give satisfactory results. In the ether-water solvent systems emulsification frequently occurred. These emulsions were temporary and separated on standing a sufficient length of time, but they served to prolong the extraction. A twenty-four extract (144 extractions) procedure required from five to seven days to complete because of the time consumed in allowing emulsions to settle out.

Determination of Saturates

The oxidation-adsorption method of Schuette and Dal Nogare (1951) was employed. The results of this method were found to agree well with the per cent saturates as obtained by using extraction procedures.

Oxidative Cleavage

Potassium permanganate in acetone solution will effect oxidative cleavage of unsaturated fatty acids at their double bonds. The products are monobasic and dibasic acids from the normal unsaturated fatty acids (i.e. oleic, linoleic, linolenic, etc.), whereas cyclic or branched-chain acids would give cyclic, branched-chain, or tribasic fatty acids from such a procedure.

(a) Permanganate oxidation

The procedure was that of Armstrong and Hilditch (1925) as modified by Nunn (1952). Some further minor manipulative changes were made by the author and these are noted as they arise.

(b) Nitric acid oxidation

A weighed sample of oil (ca. 5 gm.) was placed in a 250 ml. round-bottomed flask fitted with reflux condenser and 25 ml. of concentrated nitric acid was added through the condenser. A gas trap was fitted to the end of the condenser to collect escaping gas fumes and the mixture was gently warmed until the reaction started, then the heating was stopped. The reaction proceeded violently (exothermic) for several minutes before subsiding. After an hour, when the mixture had cooled, heating was resumed and the mixture was refluxed for two hours. The dark coloured product was treated similarly to the products from the permanganate oxidation (i.e. steam distilled).

After the author had completed the work on nitric acid oxidation (August, 1953) with the successful separation of monobasic and dibasic acid fractions, there appeared in the literature a patent which covered essentially the same procedure (U.S.Patent 2,662,908 Dec. 15th, 1953, issued to R.L.Logan). A summary of this method follows: 5 parts of fish liver fatty acids were boiled with 25 parts of 9 % nitric acid under a pressure of 15 lbs. of air per square inch for eight hours. The monobasic acids were steam distilled from the reaction mixture and the dibasic acids were recovered from the reaction mixture (undistilled).

Paper Chromatography

It is not necessary her to give a detailed description of chromatographic techniques. If a discussion is desired of the principles of chromatography, reference may be made to Strain et al (1952).

In the present wotk three distinct types of material were chromatographed: long-chain monobasic fatty acids of high molecular weight, aliphatic monobasic acids of low molecular weight (C_2 to C_{10}), aliphatic dibasic acids (C_2 to C_{10}).

In treating the high molecular weight fatty acids, reversed phase chromatography was sometimes used. This necessitated the preparation of specially treated papers. The methods of their preparation are as follows:

1. Silica-impreganated paper: strips of Whatman No. 1 paper (2.5 by 25 cm.) were immersed in a saturated solution of sodium silicate, drained, and transfered to a 6 N solution of hydrochloric acid. The strips were washed free of acid and

dried at 110° C. The method is that of Kirchner and Keller (1950).

2. Velan-waterproofed paper: similar strips of Whatman No. 1 paper were thoroughly dried at 110° for three hours in an air oven. They were immersed in a 0.5 per cent solution of 1-octadecyloxymethyl pyridinium chloride (Velan) in toluene kept at 90°. The papers were allowed to remain in this solution for three hours. The strips were removed, drained, and rinsed five times with absolute ethanol. They were then dried at 110°. The method is that of Baker (1953).

3. Mineral oil impreganated paper: 2.5 by 30 cm. strips of Whatman No. 1 paper were immersed in a bath of white mineral oil (White petrolatum), then hung for a day or two to remove the excess oil. They were used without further treatment.

When chromatographing the higher molecular weight fatty acids on Velan paper the method of Baker (1953) was used.

It should be noted that all chromatographic solvents will be referred to only by Roman numerals throughout this text. A list of the solvents, and their composition, will be found in the appendix.

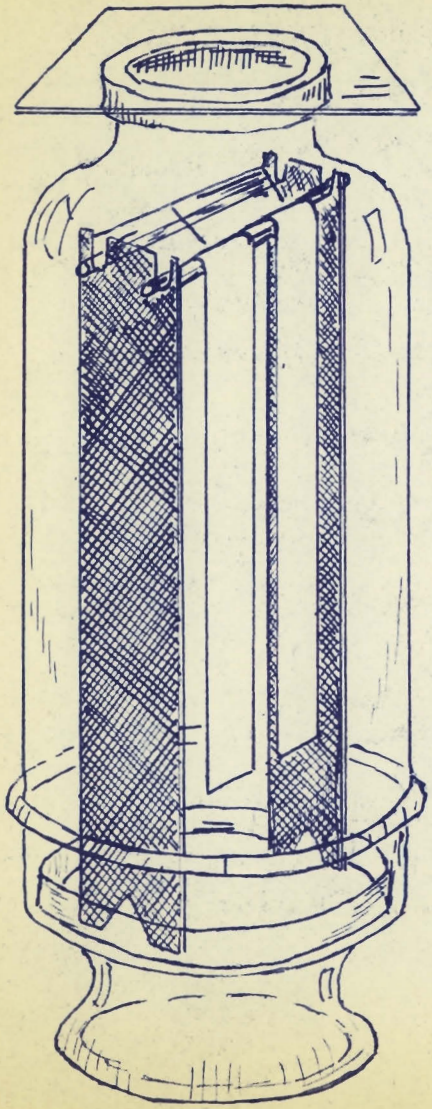
The higher fatty acids were run as the hydroxamic acids and as free acids on silica-impregnated and oil-impregnated papers. Two procedures were used to prepare the hydroxamic acids - that of Inouye and Noda (1951) and that of Fiegl (1937). The free acids were made by the process of Nunn (1952). Chromatography on the silica paper was exclusively done by

the ascending method. The strips were run in a large glass jar. It had all glass supports, and the papers were secured to the supports by means of cotton thread. Acids run on the oil-impregnated paper were chromatographed similarly (ascending).

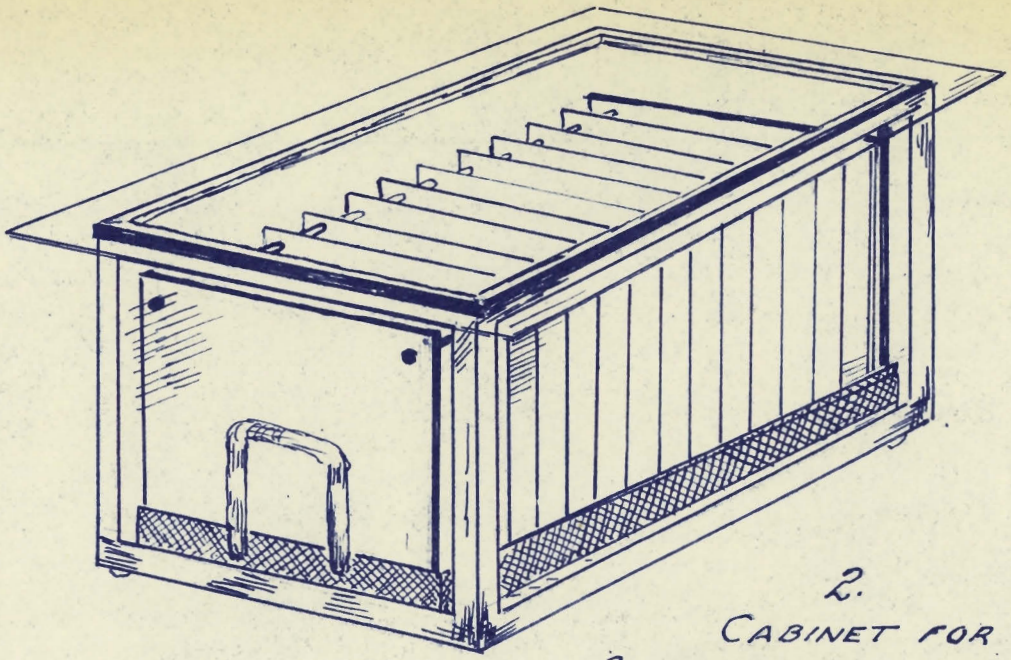
The lower monobasic acids were usually chromatographed as their ammonium salts. These were prepared by mixing an excess of concentrated ammonia with the acid in a test tube or, if insufficient acid was available (as with the acids from the fractional distillation), the acid was spotted on the paper and then concentrated ammonia was added to the spot dropwise. Each drop was allowed to dry before the next was added. Five drops were usually sufficient to form the salt.

Untreated Whatman No. 1 paper was used in chromatographing both the low molecular weight monobasic acids and the dibasic acids. These papers were run by ascending or descending methods as strips, cylinders (ascending), or sheets. Figure 6 shows the jar used for descending chromatography with paper strips. On the same figure is an illustration of a multi-sheet chromatography cabinet. With this cabinet eleven chromatograms (sheets 20 by 20 cm.) may be run simultaneously either one dimensionally or two dimensionally.

Two sprays were used for all acids except the hydroxamic acids for which an alcoholic solution of ferric chloride was used (Inouye and Noda, 1949). The sprays were bromocresol purple and bromocresol blue, and they were prepared as



1.
JAR WITH TROUGH
FOR DESCENDING
CHROMATOGRAPHY



2.
CABINET FOR
2-DIMENSIONAL
CHROMATOGRAPHY BY
ASCENDING METHOD

CHROMATOGRAPHY

Figure 6. Chromatographic apparatus used in analysis of heat polymerized vegetable oil fractions.

follows: 0.5 per cent w/v bromocresol purple in ethanol was diluted with formalin (1:5 formalin:ethanol) and then adjusted to pH 5 with 0.1 N sodium hydroxide (Reid and Lederer, 1952). The formalin provides better spot definition by forming urotropin (hexamethylene tetramine) with the ammonium salts present. The second indicator, 0.5 per cent bromocresol blue, was prepared in exactly the same manner. Exposing the chromatograms to ammonia vapour immediately after spraying usually gave more distinct spots.

PART III

EXPERIMENTAL

SECTION I

Preparation of Thermally Polymerized Sunflowerseed Oils and Characterization of the Various Fractions Derived from Each Oil

Introduction

Previously Wells (1952) had polymerized linseed oil for twelve hours at 275° C and obtained from the oil a urea non-adduct-forming fraction (1). This fraction proved to be injurious when fed to growing rats.

Linseed oil is classified as a linolenic acid group oil because of its high content of linolenic acid (9,12,15-octadecatrienoic acid). Because of this fact it had been suspected that linolenic acid might be partly or wholly responsible for the formation of the NAFD fraction. In order that this hypothesis be tested, an oleic-linoleic acid oil was examined. Sunflowerseed oil, which contains about ten per cent saturated acids, thirty per cent oleic acid, and sixty per cent linoleic acid, was chosen. It was treated in the same manner as that used by Wells (1952); batches of oils were polymerized for varying lengths of time, esterified to methyl esters, and then distilled *in vacuo*. In addition to these processes the distillable methyl esters were treated

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(1) The non-adduct-forming fraction of the distillable esters of an oil will henceforth be referred to as the NAFD.

with urea to see if any non-adduct-forming material was present. This latter procedure had not been tried with the soyabean oils.

Procedure and Results .

About three kilograms of raw sunflowerseed oil was alkali-refined with 3.5 per cent of 20° Baume sodium hydroxide, washed, and dried. The loss in refining was about fifteen per cent. The refined oil was bleached with two per cent activated bleaching clay (Super Filtrol, Johns-Manville), filtered, and then divided into batches of five hundred grams each.

Each batch was heated for a different length of time at 275° under a stream of carbon dioxide. The heating times chosen were 4, 9, 15, 22, and 30 hours. The refractive index, viscosity, mean molecular weight (cryoscopic), free fatty acids, and the iodine value were determined for each of these oils.

The methyl esters were prepared by transesterification. Methyl esters were prepared in preference to ethyl esters because of their greater ease of preparation and their lesser liability to emulsify. The average loss during esterification was ten per cent. The esters were washed, dried, and analyzed.

The methyl esters were distilled *in vacuo* and a monomeric fraction collected. The fraction came off in the temperature range 160 - 200° under a pressure which was estimated at two to three mm. of mercury. The upper limit for the monomeric

fraction was taken at 240° C. although distillation was usually complete at 210°. A semi-micro distillation was also carried out on each fraction in order to determine accurate monomer-polymer percentages.

One hundred grams of each distillate was treated with urea and a urea adduct and non-adduct were collected. The oil was recovered by dilution of each (adduct and non-adduct) with water followed by ether extraction. The ether extracts were dried, filtered, and evaporated to dryness on a steam bath in a tared vessel. The percentage of each component was calculated. No NAFD could be found in the oil that had been heated for 4 or 9 hours; a small amount appeared in the fraction that had been heated for 15 hours; the fractions heated for 22 and 30 hours contained appreciable amounts of NAFD.

A spectroscopic analysis of the refined and bleached sunflowerseed oil showed that linolenic acid was not present in detectable amounts.

Tables I, II, III, and IV, respectively, show the chemical and physical characteristics of the whole oils, the methyl esters, the distillable methyl esters, and the adduct-forming fraction of the distillable methyl esters. No results were obtained for the NAFD fractions.

Table V shows the relative proportions of distillable and polymeric material in the methyl esters as well as the percentages of AFD and NAFD. It should be noted that all

figures in this table are expressed as percentages of the total methyl esters.

Figures 7, 8, 9, and 10 are graphical representations of the data presented in Tables I, II, and III.

Tables VI and VII give the component fatty acids of the total methyl esters and the distillable methyl esters. The data in these tables were calculated from spectroscopic measurements. In calculating the amount of oleic acid, the percentage of saturated acids was considered to have remained constant throughout the heating times (i.e. the same as the unheated esters). This has been the view adopted by both Wells (1952) and Wiseblatt (1950) and stems from the fact that iodine values are too unreliable, when applied to heated oils, to afford a basis for calculation of the amount of oleic acid.

It should be noted that the "linoleic" and "linolenic" acids as given in Tables VI and VII also would include any isomeric acids which would give dienoic or trienoic conjugation on alkali-isomerization (1).

The data of Table VI is presented in Figure 14 to illustrate the relative changes in fatty acid composition with time. The abscissa has been taken as heating time rather than iodine value. Former workers have used iodine values and

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(1) For the purposes of the present thesis, therefore, "linoleic" denotes conjugable dienoic acid calculated as linoleic acid. "Linolenic" denotes conjugable trienoic acid calculated as linolenic acid.

have strongly criticized the use of heating time because of the variance of heat-up and cool-down times. However, in the present experiments these times were quite constant; two hours for heating up to 275°, and one and one half hours to cool to room temperature. The author considers that the error inherent in iodine values is as large, if not larger, than the error associated with bodying time.

In Figures 11 and 12 the decrease in absorption of the non-conjugated constituents of the methyl esters and the distillable methyl esters respectively has been shown. Essentially this provides confirmation of the iodine value determinations. Figure 13 shows the same data as Figure 11 arranged to show the rate of decrease in diene absorption with time.

Figure 15 shows the straight-line relationship of the refractive indices to per cent polymers. This shows that polymerized sunflowerseed oils of a desired polymer content can be obtained with reasonable accuracy by polymerizing to the appropriate refractive index.

Discussion

"Linolenic" acid was almost completely absent from the sunflowerseed oil fractions (1.1 per cent being the largest amount), yet a NAFD fraction was formed. This would seem to indicate that some agent other than linolenic acid is responsible for NAFD formation in sunflowerseed oil. However,

the possibility of trace amounts of linolenic acid in the oil being responsible, at least in part, cannot be excluded completely. Linolenic acid in small amounts could possibly undergo pyrolytic disintergration into two or more parts any of which, attaching onto another ester, could easily create an assymmetric compound incapable of urea adduction. The indisputable fact is that sunflowerseed oil will form a NAFD fraction when heated at 275° for fifteen hours or more.

Comparing the yields of linseed and sunflowerseed oil NAFD fractions, it is evident that linseed oil forms such a fraction much more rapidly and in larger quantity (12 hours for 12 per cent yield) than the sunflowerseed oil (30 hours for 10 per cent yield) does.

It is most probable that NAFD formation in linseed oil is related to the high content of linolenic acid. What causes formation of sunflowerseed NAFD is not known, but linoleic acid most certainly plays a role.

Wells (1952), in his study of heated soyabean oil, said that the polymerization process appeared to have three stages. In the first stage the "linolenic" and "linoleic" acids decline slowly in concentration as the oleic acid concentration and diene value increase slightly (A). Secondly, oleic acid concentration began to increase rapidly accompanied by a rapid decrease in "linolenic" and "linoleic" acid concentrations (B). In the final stage all reactions slowed up and levelled off

(C). Figure 14 shows the relation of the fatty acid concentration in heated sunflowerseed methyl esters to the heating time. On comparing the sunflowerseed polymerization to that of soyabean oil it will be noted that the induction stage (A) is missing. The first step in sunflowerseed polymerization seems to be a rapid decrease in "linoleic" acid with simultaneous increase in "oleic" acid concentration; the reactions then gradually slow up and level off, and at thirty hours the oleic acid begins to decrease slightly. These two steps approximate the B and C stages of soyabean polymerization.

Wiseblatt's results (1950) with polymerized linseed oil indicated a three stage polymerization procedure similar to the soyabean oil. Since both linseed and soyabean oils contain appreciable amounts of linolenic acid, it is possible that the linolenic acid is responsible for the characteristic induction period (A) of these two oils. This initial slow decline in the higher unsaturates' concentration could be explained by the prior conjugation of linolenic acid before it reacts in direct polymerization.

To summarize, it seems that in linseed and soyabean oils thermal polymerization has at least three stages before reaching the gel point. In sunflowerseed oil, the initial or induction stage is lacking possibly because of the absence of linolenic acid. Linolenic acid, however, is not essential for the formation of a NAFD fraction.

TABLE I

Characteristics of Thermally Polymerized Sunflowerseed Oils

Time of heating (hours)	Free fatty acid (per cent)	$n_D^{25^\circ}$	Mean molecular weight (cryoscopic)	Iodine value	Viscosity (poise) 25°
0	0.03	1.47145	723	133	---
4	0.94	1.47285	729	130	0.65
9	0.04	1.47343	817	128	0.80
15	1.03	1.47480	882	124	1.25
22	0.99	1.47644	1374	119	2.63
30	1.94	1.47869	1827	115	6.00

TABLE II

Characteristics of the Methyl Esters Derived from Thermally Polymerized Sunflowerseed Oil Batches

Time of heating (hours)	Free fatty acids (per cent)	$n_D^{25^\circ}$	Mean molecular weight (cryoscopic)	Iodine value
0	0.09	1.45576	299	132
4	0.09	1.45526	304	132
9	0.18	1.45661	296	125
15	0.24	1.45730	314	121
22	0.25	1.45891	317	116
30	0.24	1.46117	345	112

TABLE III

Characteristics of the Distillable Methyl Esters of Thermally Polymerized Sunflowerseed Oils

Heating time (hours)	Free fatty acids (per cent)	$n_D^{25^\circ}$	Iodine value	Saponification equivalent
4	---	1.45486	122	289
9	---	1.45459	120	294
15	0.84	1.45450	114	271
22	1.87	1.45400	119	263
30	4.92	1.45243	101	256

TABLE IV

Characteristics of the Adduct-forming Fraction (AFD) of the Distillable Methyl Esters of Thermally Polymerized Sunflowerseed Oil

Heating time (hours)	$n_D^{25^\circ}$	Iodine value
4	1.46155	120
9	1.46037	115
15	1.45603	113
22	1.45464	110
30	1.45711	103

TABLE V

Composition of the Methyl Esters of Thermally Polymerized Sunflowerseed Oil

Results Expressed as Percentage of Total Ester

Time of heating (hours)	Distillable Esters			Non-distillable esters (polymeric residue)
	Total	Adduct-forming (AFD)	Non-adduct-forming (NAFD)[1]	
4	91.3			8.7
9	89.5			10.5
15	82.2	77.0	5.2	17.8
22	78.6	70.7	7.9	21.4
30	65.5	56.4	10.0	34.4

1. These values were based on the weight of the AFD and hence are probably slightly high because of losses of AFD during processing.

TABLE VI

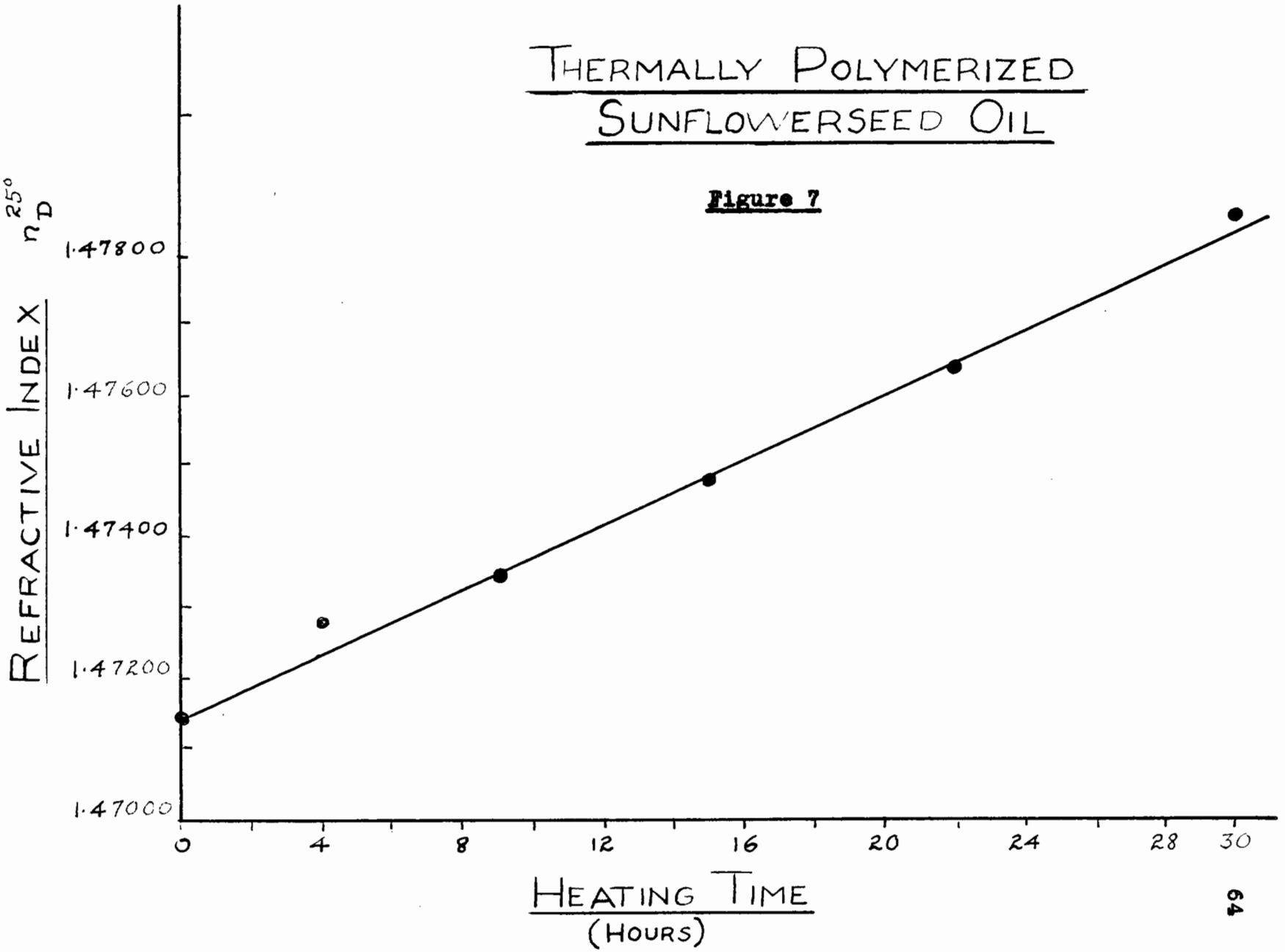
Fatty Acid Composition of the Total Mixed Methyl Esters of Thermally Polymerized Sunflowerseed Oil

Hours heated	0	4	9	15	22	30
% Conjugated diene	0	15.5	13.0	12.2	3.8	8.5
% "linoleic" acid	55.4	27.6	21.4	12.9	10.9	6.9
% "linolenic" acid	1.1	0.4	0.2	0.2	--	---
% "oleic" acid	30	43	52.2	61.2	71.7	71.1
% saturates	13.5	13.5	13.5	13.5	13.5	13.5

TABLE VII

Fatty Acid Composition of the Distillable Methyl Esters of Thermally Polymerized Sunflowerseed Oil

Hours heated	4	9	15	22	30
% Conjugated diene	13.56	15.7	14.0	10.1	2.4
% "linoleic" acid	30.1	27.5	17.1	9.6	8.8
% "linolenic" acid	0.05	---	---	---	---
% "oleic" acid	46.1	42.5	48.6	53.9	58.4
% Saturates	10.1	15	20	25	30


THERMALLY POLYMERIZED
SUNFLOWERSEED OIL
Figure 7
REFRACTIVE INDEX $n_D^{25°}$
1.47800
1.47600
1.47400
1.47200
1.47000
0
4
8
12
16
20
24
28
30
HEATING TIME
(HOURS)

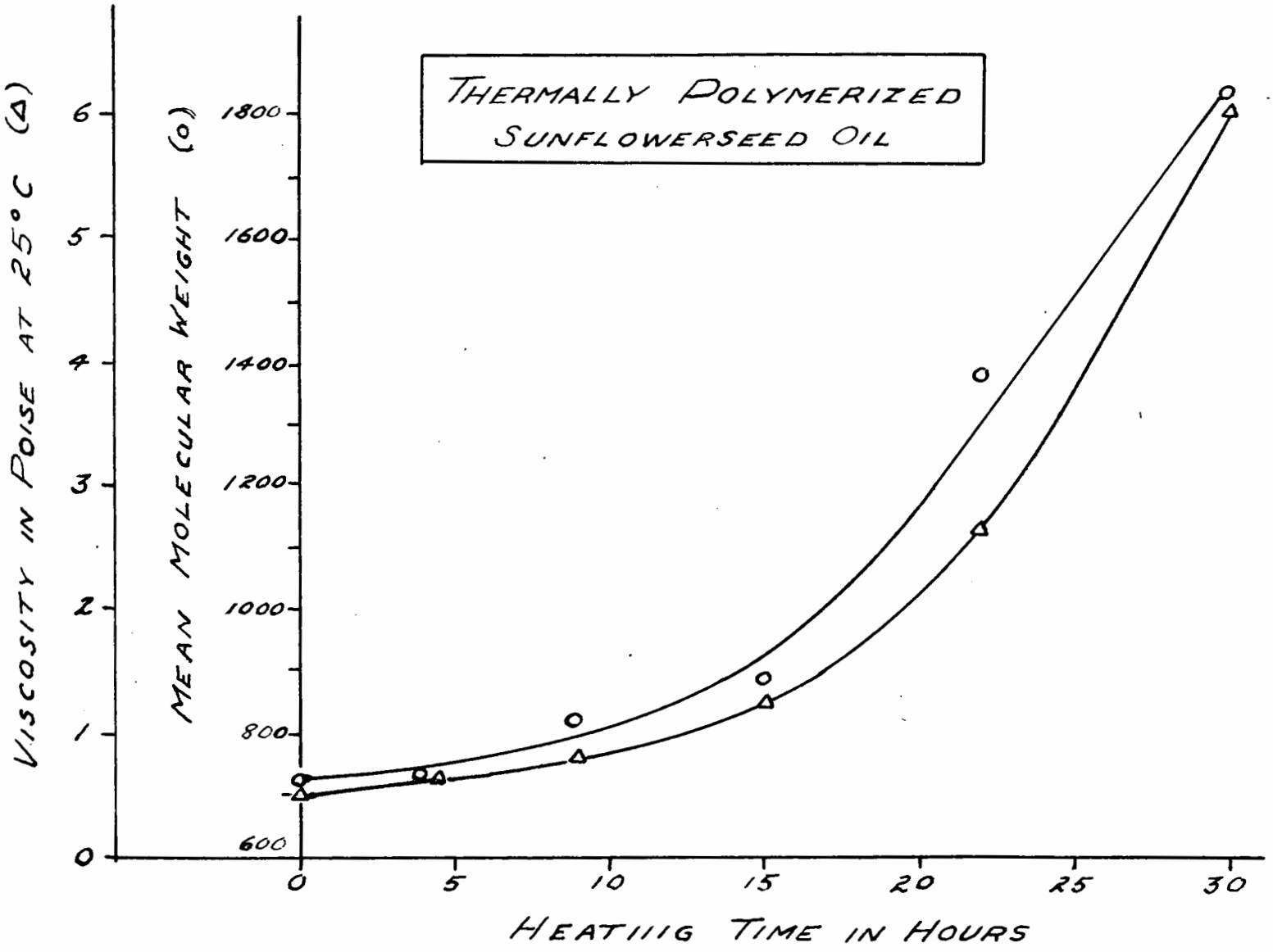


Figure 8. The relation of viscosity and mean molecular weight to the heating time.

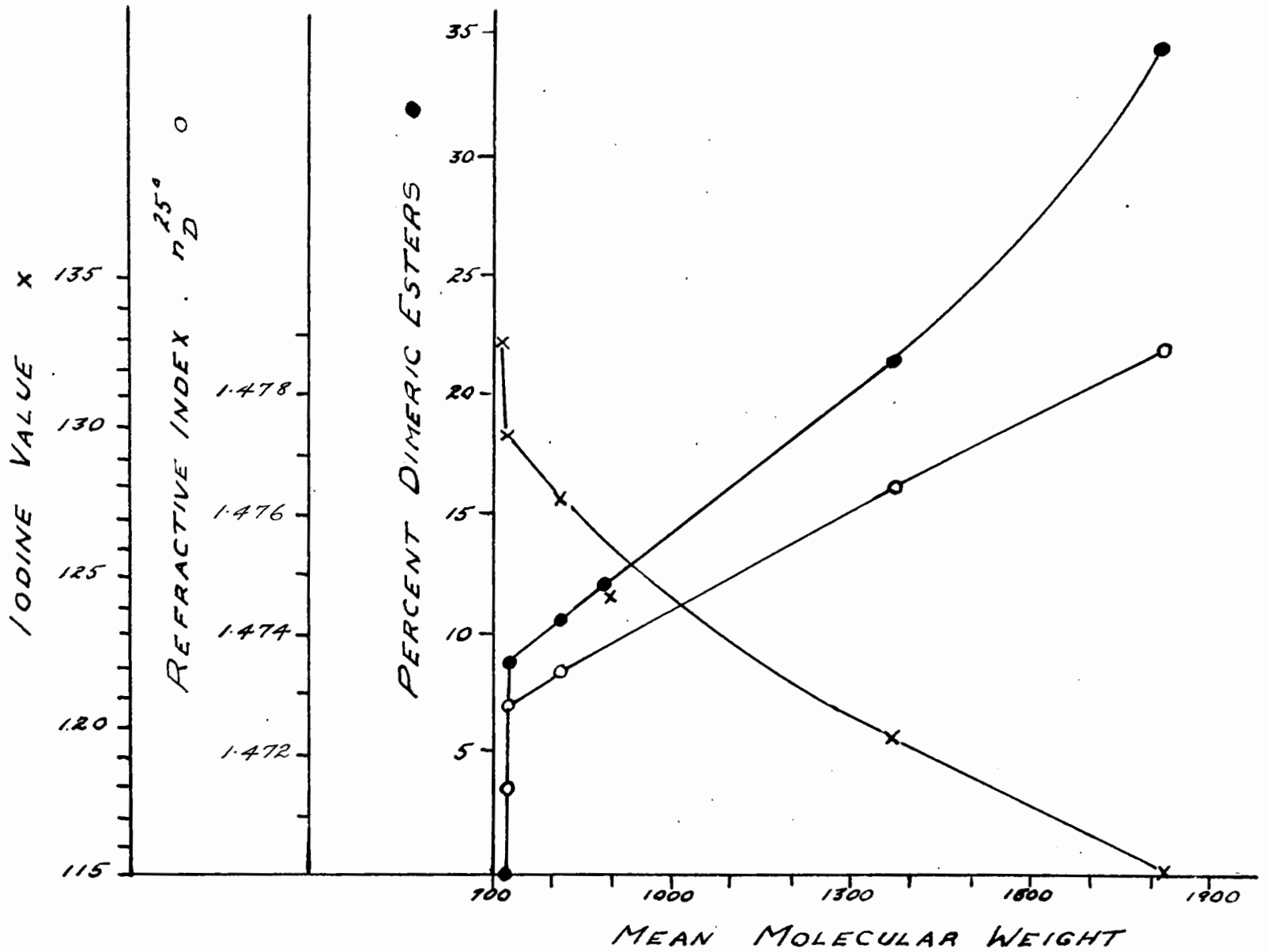


Figure 9. Thermally polymerized sunflowerseed oil: the relation of iodine values, refractive indices, and polymer content to the mean molecular weight (cryoscopic).

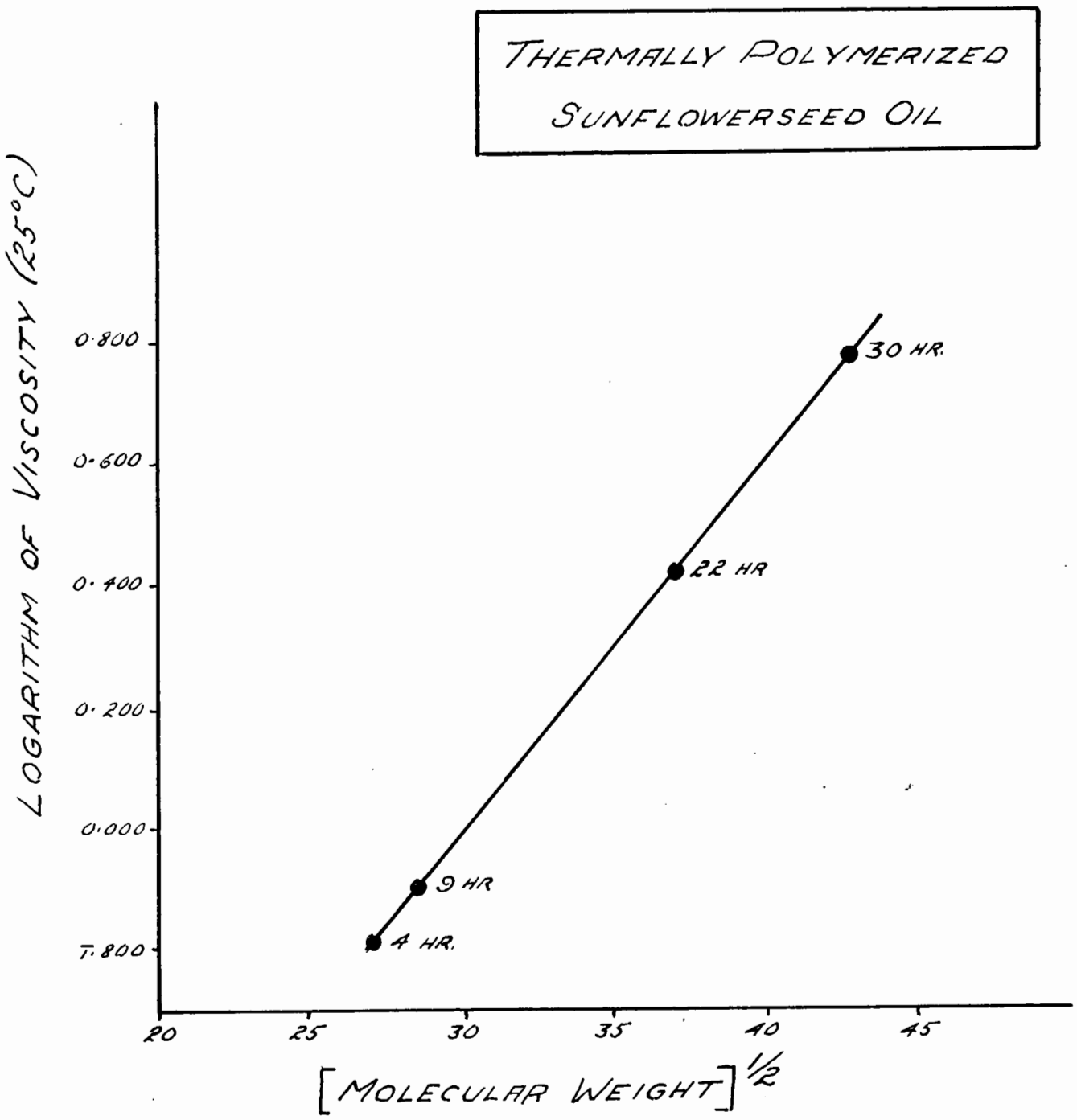


Figure 10. Relation of the logarithm of viscosity to the square root of the molecular weight in thermally polymerized sunflowerseed oils.

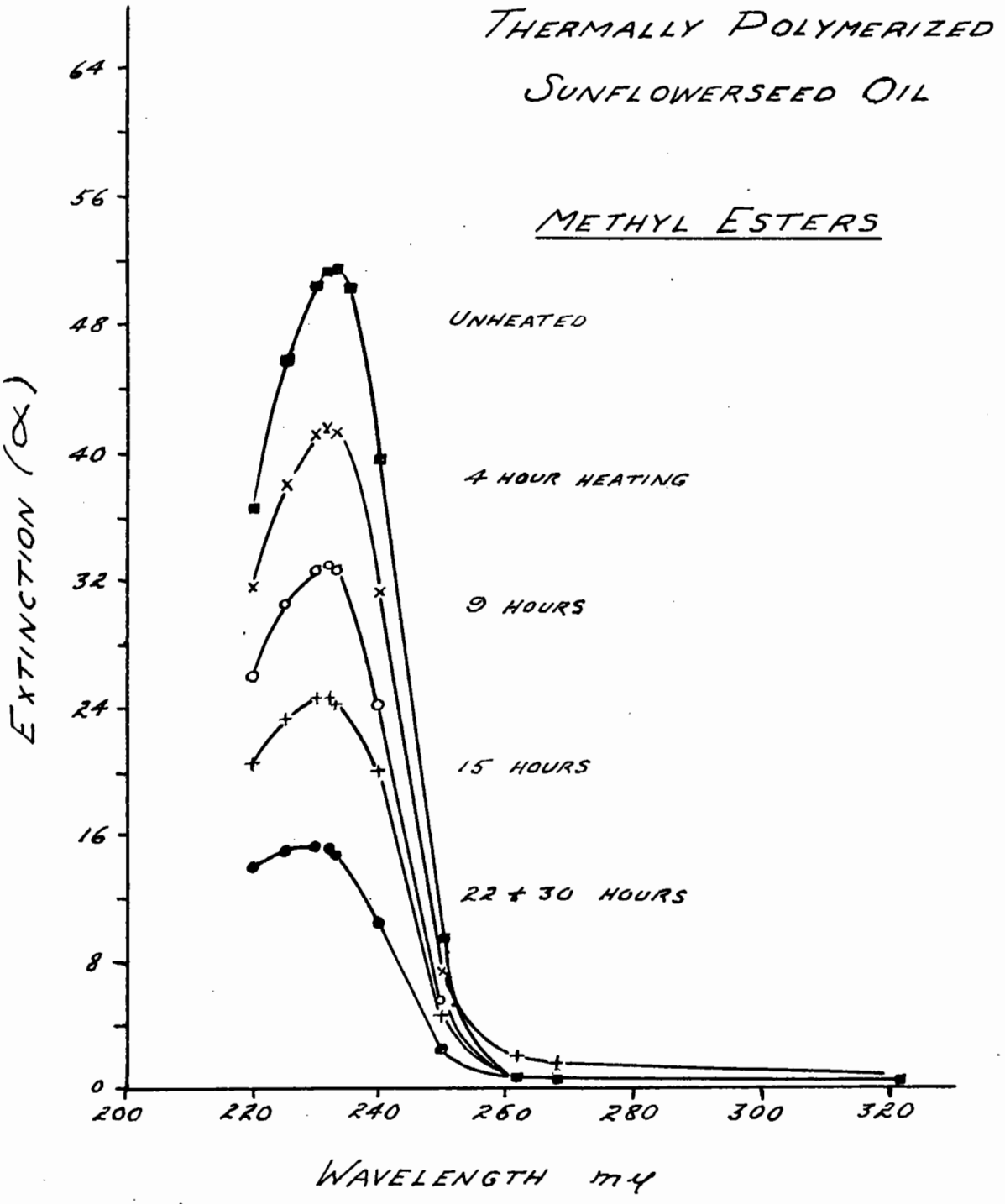


Figure 11. Comparison of the absorption spectra of the non-conjugated constituents of the total mixed methyl esters of thermally polymerized sunflowerseed oil at the various heating periods.

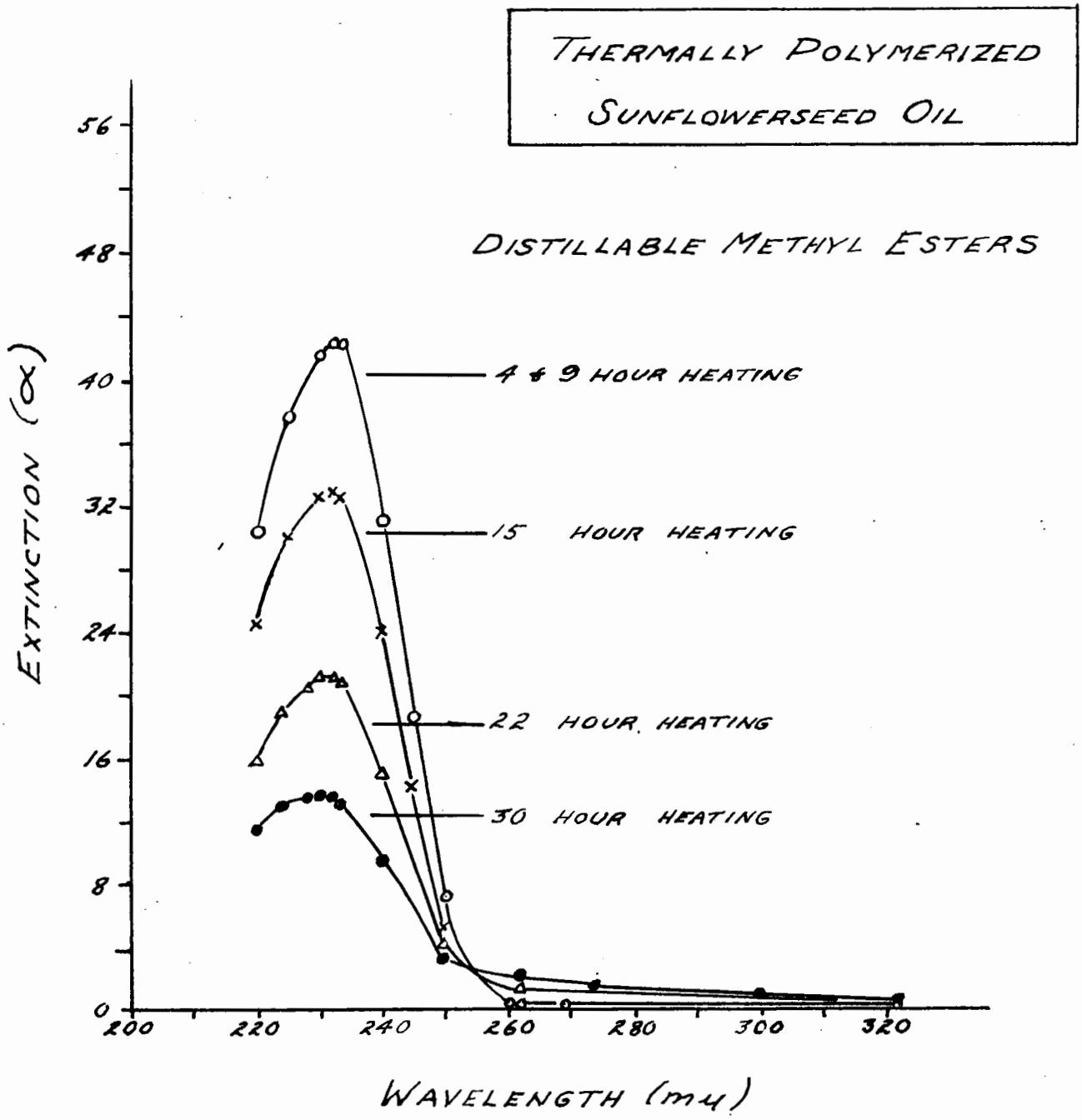


Figure 12. Comparison of the absorption spectra of the non-conjugated constituents of the distillable methyl esters of thermally polymerized sunflowerseed oil at the various heating periods.

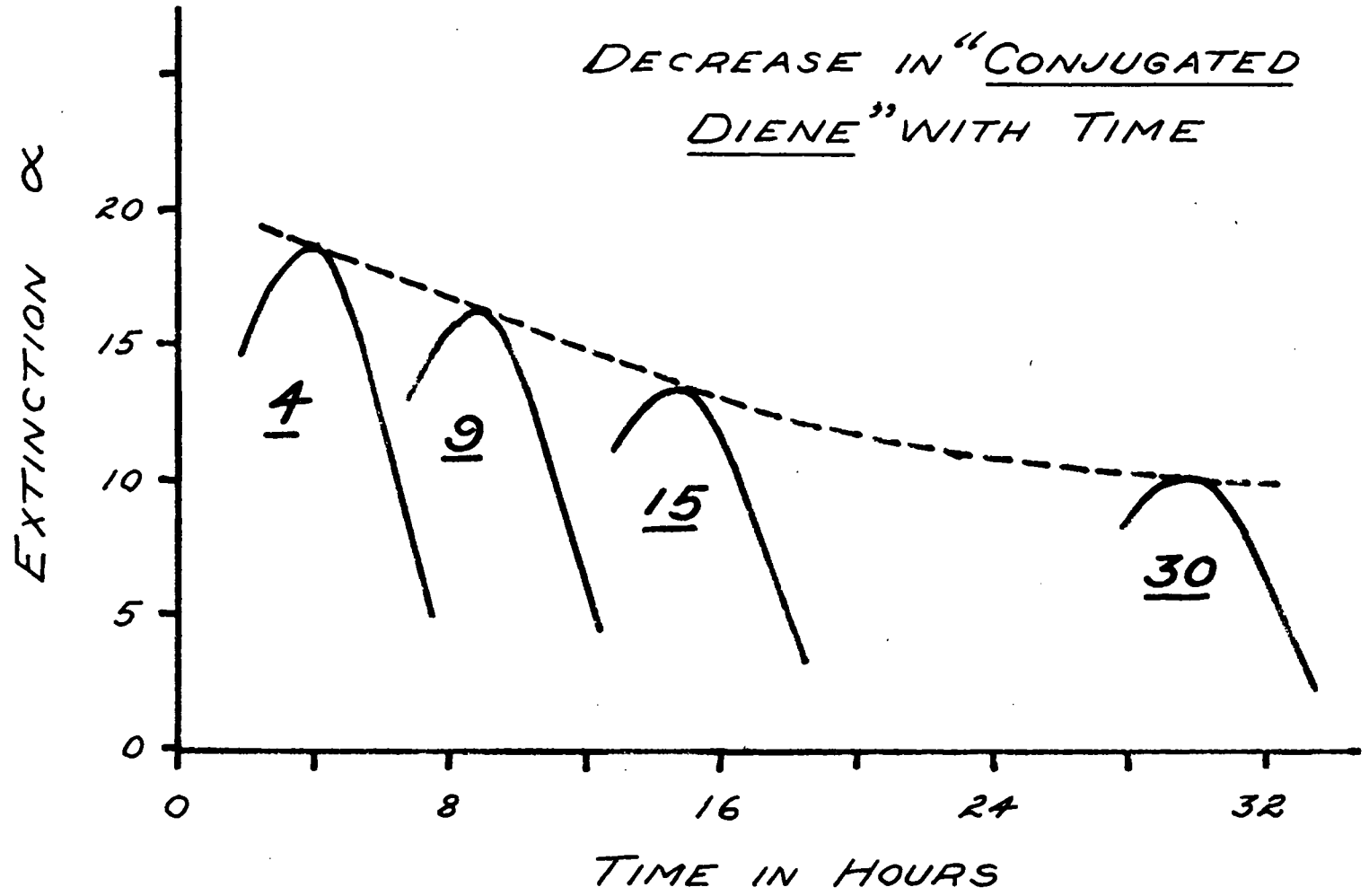


Figure 13. Decrease in "conjugated diene" with time in the mixed methyl esters of thermally polymerized sunflowerseed oil.

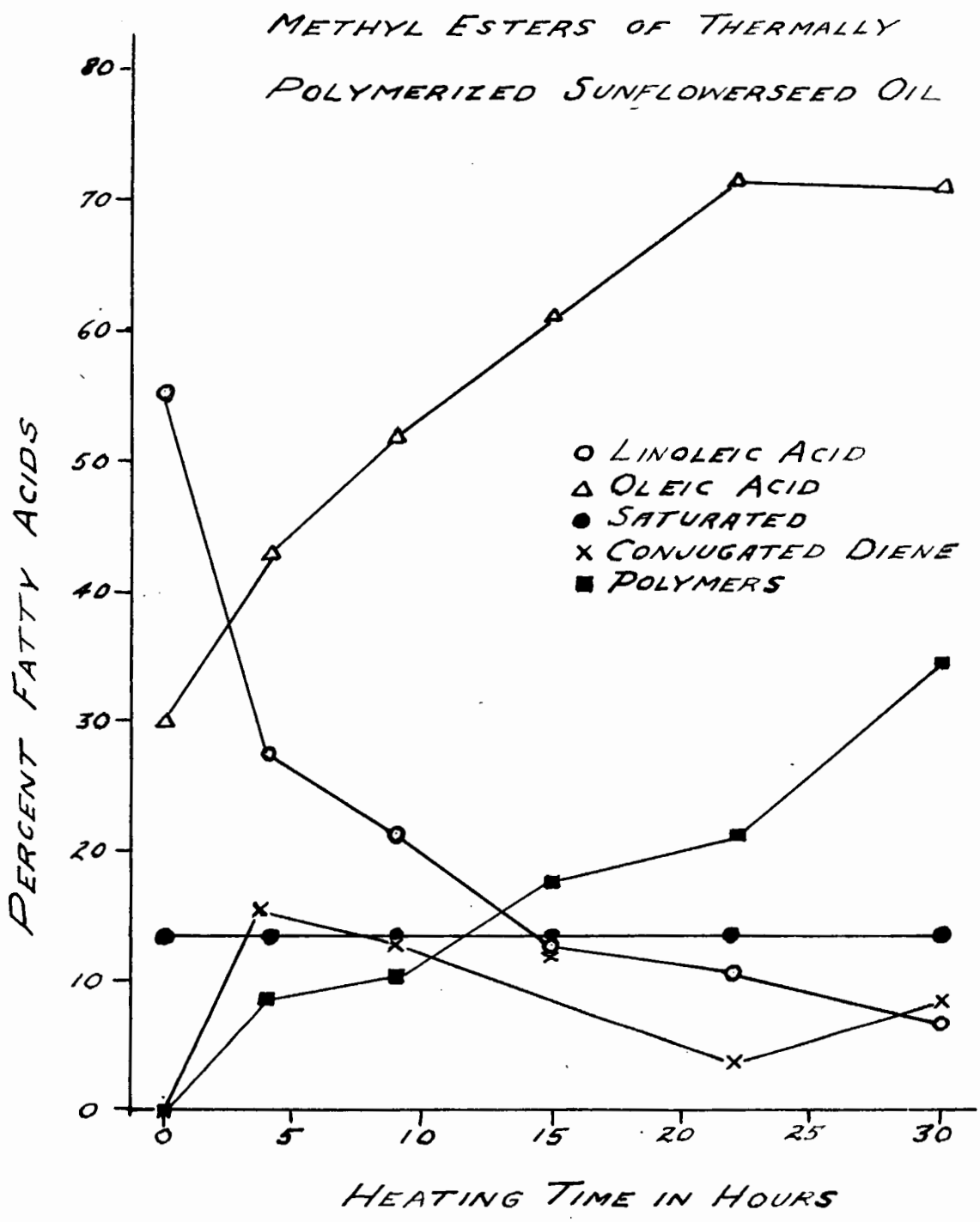


Figure 14. Relation of the heating time to the concentrations of the constituent fatty acids in the methyl esters of heated sunflowerseed oil.

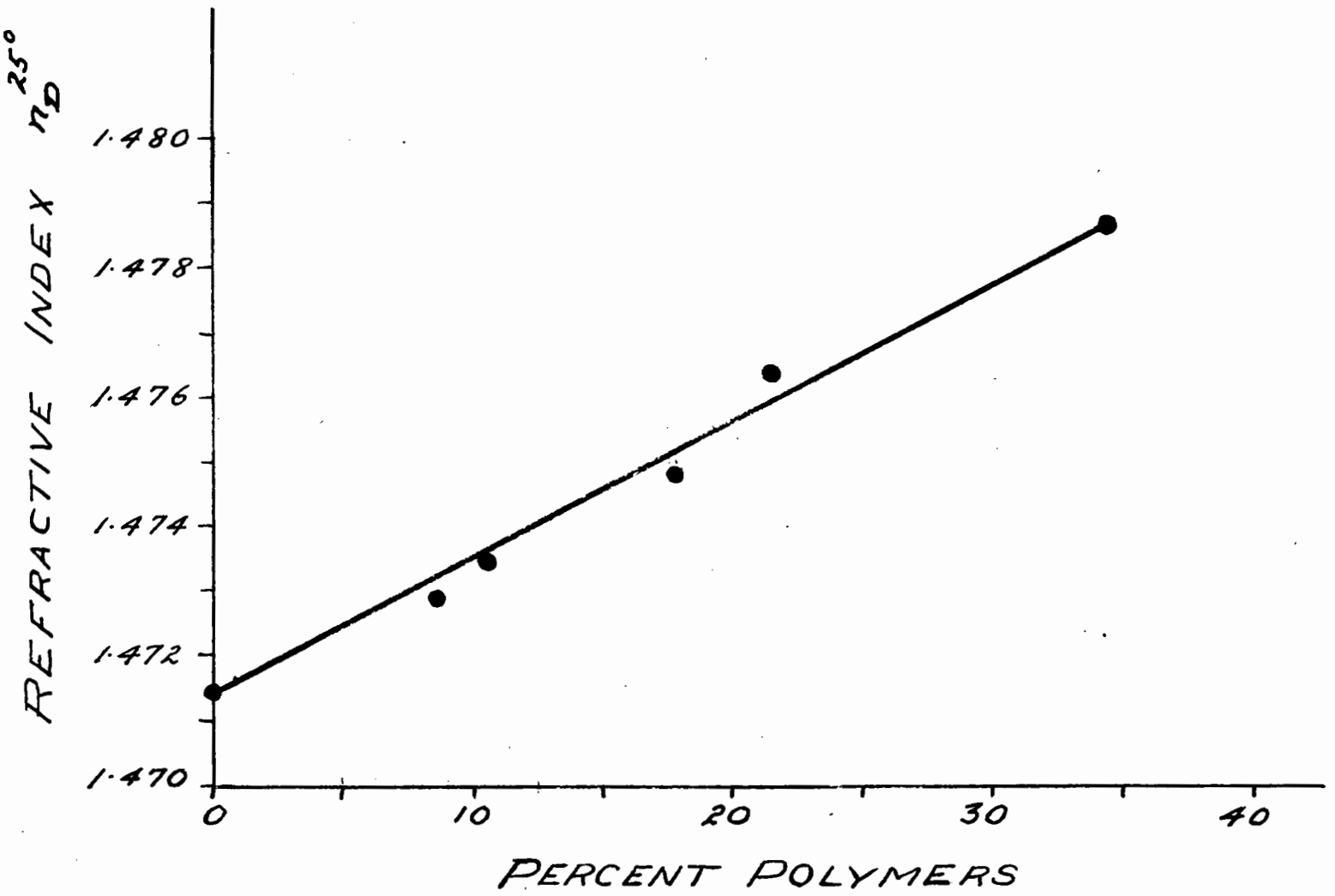


Figure 15. Relation of the polymer content to the refractive indices of thermally polymerized sunflowerseed oils

SECTION II

The Nutritional Value of the Fractions Obtained from Thermally Polymerized Sunflowerseed Oil

Introduction

The work of Wiseblatt (1950) had shown that the distillable ethyl esters of linseed oil were less nutritious than the unheated esters from the whole oil. Subsequently Wells (1952) isolated a urea non-adducting fraction (NAFD) from the distillable esters which proved to be extremely injurious when fed to growing rats. The adduct-forming part of the distillable esters (AFD) proved to be as nutritious as the unheated esters. This was the first time that a linseed oil fraction had been found that was equal in nutritional value to the esters from the unheated oil.

Since a NAFD fraction had been isolated from thermally polymerized sunflowerseed oil (Section I), it was thought desirable to assess the comparative nutritive value of this oil. Six fractions were prepared from the polymerized oil: urea adducts of the ethyl esters (AFE), urea non-adducts of the ethyl esters (NAFE), distillable esters, non-distillable esters (residue), urea adducts of the distillable esters (AFD), and urea non-adducts of the distillable esters (NAFD). In co-operation with the Department of Animal Nutrition at Macdonald College, a feeding trial was conducted in which growing rats were fed the six fractions in definite daily amounts.

Procedure and Results

Raw sunflowerseed oil was alkali-refined, bleached, and polymerized in the same manner as reported in Section I. A heating time of twenty-six hours at 275° C was chosen on the basis of previous experiments. In these experiments it had been found that there was not any appreciable amount of NAFD formation until the oil had been heated for twenty hours.

The ethyl esters were formed by transesterification, and a portion was fractionated with urea into adducts and non-adducts. The remaining esters were distilled *in vacuo* to yield a polymeric residue and a monomeric distillate. The distillable esters were segregated with urea into an adduct-forming portion (AFD), and a non-adduct forming fraction (NAFD). The scheme of separation is set out in Figure 16. The yields of the various fractions are reported in Table VIII.

The chemical characteristics were determined for four of the six fractions. The urea adducts and non-adducts of the whole esters were not reported because, due to limitations of available processing facilities and manpower, the feeding of these two fractions was discontinued after two weeks. Table VIII shows the characteristics of the fractions.

The absorption spectra of some of the fractions were determined for comparative purposes and are presented in Figure 17. A more comprehensive discussion of the NAFD will be found in Section VII.

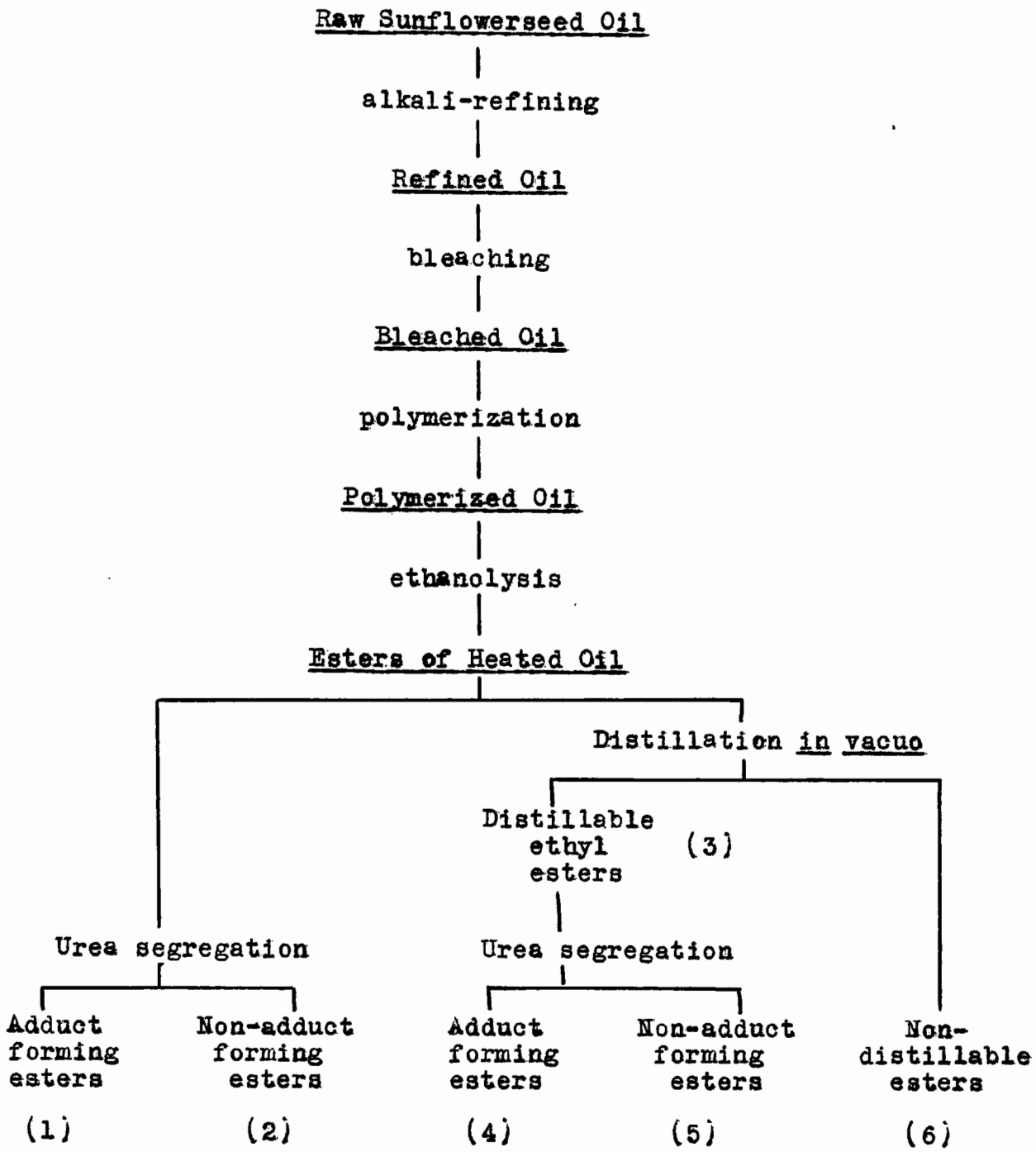


Figure 16. Flow sheet illustrating preparation of fractions of esters of heated sunflowerseed oil used in feeding trials.

Because some of the previous experiments had been partly vitiated by the presence of peroxides, a peroxide determination was conducted on each oil prior to its incorporation into the prepared rat diets. It was found that all values fell well within the safety limit. In additions all fractions were protected with 0.025 per cent Tenox II anti-oxidant during processing operations as well as before being incorporated into the diets.

The ester fractions were incorporated into standard diets at levels of ten and twenty per cent. The diet formulation is given in Table IX. It will be noted that whole wheat was substituted for white flour in the second replicate. The tests were conducted in duplicate with a group of ten rats for each ester fraction, thus making a total of 120 rats for the complete trial. Some of the rats had to be withdrawn from the trial for various reasons, and when the supply of two of the ester fractions was exhausted after only two weeks, the rats on these diets were also removed. Hence not all of the 120 rats finished the trial. These facts are recorded as footnotes to the tables.

The age of the animals varied from twenty to thirty days, and one rat in each group of ten was a female. The average diet allowed each rat per day was ten grams.

The results of the feeding trials are set out in Tables X, XI, XII, and XIII. A comparison between the mean weight gain of rats fed linseed oils and those fed sunflowerseed oils is

recorded in Table XIV. The data in these tables was compiled by Miss D.J.Crawford (1953) of the Nutrition Department who worked in conjunction with the author on the heated oil project.

Discussion

Even a cursory study of the preceding tables will show that interpretation of the data from the feeding trials presents considerable difficulty. While the author does not feel competent to attempt any comprehensive interpretation, nevertheless it is felt that three broad conclusions can be drawn without much risk of error:-

1. Linseed oil as a whole, and the NAFD fraction in particular, is much inferior to sunflowerseed oil nutritionally.
2. The nutritional study of sunflowerseed oil shows that all ester fractions, save the polymeric ones (NAFE and residue), are capable of supporting growth in young rats.
3. Increasing the amounts of ethyl esters from polymerized sunflowerseed oil in the diet from the ten to the twenty per cent level caused a significant decrease in the average adjusted gain. This has been attributed to a lowering of the feed efficiency (D.J.Crawford, 1953).

The chemical analysis of the NAFD fraction, which has proven to be nutritionally dissimilar to linseed, was also

chemically dissimilar. It may contain a small portion of the same type of material as the linseed NAFD, but the bulk of the non-adducting distillable esters must be of a different and less noxious character. A further discussion of the NAFD fraction will be found in Section VII.

TABLE VIII

Yields and Characteristics of Fractions of Ethyl Esters of Heated Sunflowerseed Oil Used in Feeding Trials

Oil fraction	Yield as per cent of total esters of heated oil	Iodine value	Mol. wt.	$n_D^{25^\circ}$
Whole esters	100	109	338	---
Adduct-forming of the whole esters	65	---	---	---
Non-adduct-forming of whole esters	35	---	---	---
Distillable ethyl esters	75	110	303	---
Adduct-forming of the distillable esters	65	107	295	1.45257
Non-adduct-forming of the distillable esters	10	130	296	1.45671
Non-distillable esters (polymeric residue)	25	106	613	1.47655

TABLE IX

Percentage Composition of Rat Diets Containing Ester Fractions of Polymerized Sunflowerseed Oil[1]

Ingredients	Percentage Composition	
	10 % fat diets	20 % fat diets
White flour or ground whole wheat	53.65	43.65
Skim milk powder	19.00	19.00
Casein	11.50	11.50
Ester fractions	10.00	20.00
Yeast	3.00	3.00
Bonemeal	2.00	2.00
Salt (NaCl)	0.50	0.50
Chromic sesquioxide (2)	0.25	0.25
Ferric citrate	0.10	0.10
	100.00	100.00

1. Supplement - each rat was given a weekly dose of one drop of corn oil containing 175 I.U. of Vitamin A and 35 I.U. of Vitamin D

2. For the determination of digestibility

TABLE X

The Effect of Feeding Fractions of Heat Polymerized Sunflowerseed Oil at the 10 per cent Level in the Diet

(10 rats per lot)

Heated oil fraction fed	Number surviving test period	Average 28 day gain (grams)	Average 28 day dry matter intake (grams)	Mean gains adjusted to intake of 1000 dig. cal. (grams)
Urea adducts of the whole ethyl esters	10	150	360	89
Urea non-adducts of the whole esters	5[1]	150	345	101
Distillable ethyl esters	10	162	366	90
Urea adduct-forming of the distillate	10	159	368	87
Urea non-adduct-forming of distillate	10	142	329	93
Non-distillable esters	10	143	384	84

1. Five animals developed severe diarrhea and were removed from the experiment

TABLE XI

The Effect of Feeding Fractions of Heat Polymerized Sunflowerseed Oil at the 20 per cent Level in the Diet

(10 rats per lot)

Heated oil fraction fed	Number surviving test period	Average 28 day gain (grams)	Average 28 day dry m matter intake (grams)	Mean gains adjusted to intake of 1000 dig. cal. (grams)
Urea adducts of the whole ethyl esters	10	143	323	82
Urea non-adducts of the whole esters	5[1]	107	327	69
Distillable ethyl esters	10	162	328	95
Urea adduct-forming of the distillate	10	151	314	92
Urea non-adduct forming of distillate	10	97	241	77
Non-distillable esters	10	90	351	65

1. Five animals developed severe diarrhea and were removed from the experiment

TABLE XII

Comparison of Data Accrued from Feeding Two Levels of Heat Polymerized Sunflowerseed Oil in the Diet (60 rats per level)

Level	Average 28 day gain (grams)	Average 28 day dry matter intake (grams)	Average 28 day gains adjusted to intake of 1000 dig. cal. (grams)
10 per cent	151	361	89
20 per cent	130	311	82

TABLE XIII

Comparison of Mean Weight Gains (Grams) Per 1000 Digestible Calories Ingested in Two Replicates Where Heated Sunflowerseed Fractions Were Fed (1)

Level of heated oil in diet	Replicate	Heated Fractions Fed					
		AFE	NAFE	Dist.	AFD	NAFD	Res.
10	1 (white flour)	87	--[2]	84	74	83	86
	2 (Ground wheat)	91	101	96	101	101	81
20	1 (white flour)	79	--[2]	81	78	74	46
	2 (Ground wheat)	84	69	108	103	79	84

1. Data rounded to nearest whole number
2. Five rats of replicate 1 developed severe diarrhea and were removed from the trial

TABLE XIV

Mean Weight Gains in Grams (Adjusted to Digestible Calorie Intake of 1000) of Rats Fed Sgregates of Heated Linseed and Sunflowerseed Oil (Average of 20 values)

Heated oil fraction fed	Fraction fed						
	AFE	NAFE	Dist.	AFD	NAFD	Res.	Mean
Linseed	61	--[1]	53	58	--[1]	58	58
Sunflowerseed	85	(90)	93	89	(85)	75	85

1. Rats died

Note: figures in brackets were not included in computing the mean

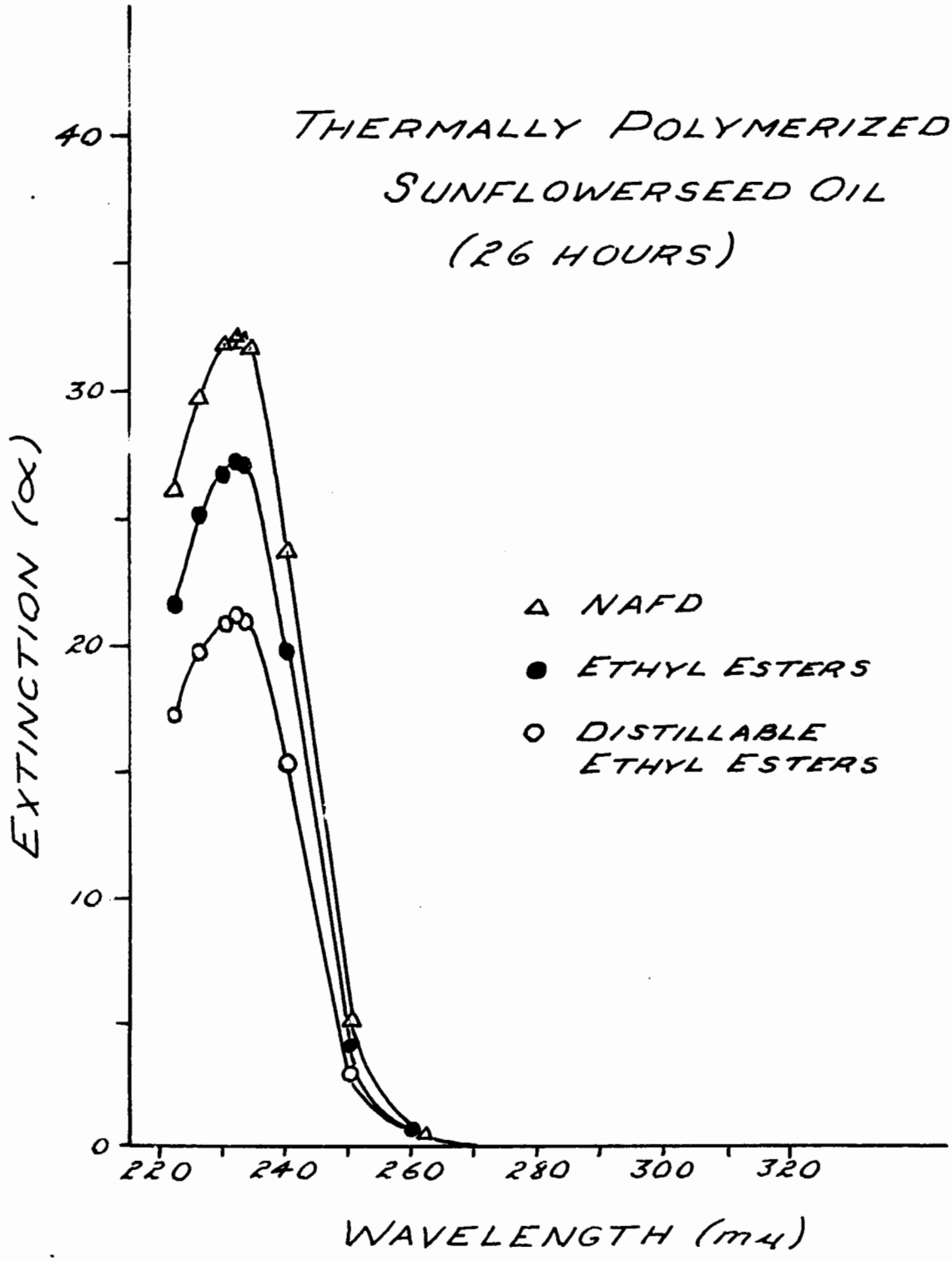


Figure 17. Absorption spectra of three ethyl ester fractions of twenty-six hour heated sunflowerseed oil.

SECTION III

Preparation of Thermally Polymerized Soyabean Oil and the Characterization of the Derived Fractions

Introduction

Previously urea non-adducting fractions (NAFD) from the distillable ethyl esters of polymerized linseed oil (Wells, 1952) and from polymerized sunflowerseed oil (Section I) had been isolated. The NAFD fractions from both oils were not nutritious, the linseed fraction being extremely injurious to growing rats. To get a logical sequence soyabean oil, an edible oil intermediate between linseed and sunflowerseed oils in properties, was chosen for study. The object was to isolate and characterize an NAFD from Soyabean oil prior to conducting a feeding trial with the NAFD on growing rats. After a survey of the properties of the methyl esters from heated soyabean oil (Wells, 1952) and a consideration of the relative yields of NAFD from linseed and sunflowerseed oils, an arbitrary time of twenty hours was chosen for polymerization of this oil.

Procedure and Results

Batches of raw soyabean oil were alkali-refined with 3.6 per cent 20° Baume sodium hydroxide, washed, and dried with sodium sulfate. The last traces of moisture were removed under reduced pressure. The losses during refining were about ten per cent of the whole oil. The oil was then bleached with two per cent bleaching clay (Super Filtrol), filtered, and polymerized at 275° for twenty hours under a stream of carbon

dioxide.

The ethyl esters were made by transesterification, washed, dried, and then distilled *in vacuo* to yield the distillable ethyl esters. These esters were subjected to urea fractionation.

The whole esters, before distillation, were also segregated with urea to yield two fractions - the non-adduct forming (NAFE), and the adduct-forming (AFE).

The chemical characteristics of the various isolated fractions were determined. The results are reported in Table XV.

The relative amounts of the fractions isolated from the mixed ethyl esters are given in Table XVI. The percentages in this table are based on the total methyl esters being 100.

Figure 18 shows the spectra of the non-conjugated constituents as determined by spectrophotometric analysis. The spectrum of the NAFD is given in Section VII. From this data the fatty acid composition of the mixed ethyl esters was calculated (Table XVII).

In washing the ethyl esters a distinct darkening of the oil was noted when distilled water from the building still was used. This darkening did not occur when water, purified by de-ionization (Illco De-ionizer, Illinois Water Treatment Company), was used. It was thought that the copper coils used in the commercial-type building still caused the distilled water to contain considerable copper. This dissolved copper

was suspected as a cause of the darkening of the esters. Ethyl esters washed with distilled water and ethyl esters washed with de-ionized water were analyzed for their copper content (Boulet and McFarlane, 1945). The results are reported in Table XVIII.

Discussion

As had been anticipated, an NAFD fraction was isolated from the twenty hour heated soyabean oil. This fraction constituted about ten per cent of the total ethyl esters (cf. linseed oil - 15 per cent at 12 hours, sunflowerseed oil - 10 per cent at 26 hours) which seems to indicate that a rate of polymerization intermediate between linseed and sunflowerseed oils occurred.

The amounts of the various fractions isolated indicated that production of enough material for a nutritional trial was feasible.

A more complete discussion of the chemical properties of the NAFD fraction will be found in Section VII.

Table XVIII showed that the darkened esters contained 100 per cent more copper than the light-coloured esters. This observation would seem to substantiate the view that the dissolved copper in the wash-water was responsible for the darkening of the ethyl esters.

The darkening of the ethyl esters had been noted previously by the author when processing sunflowerseed oil. At

that time little note was taken of the matter as it was thought to be a normal feature of the process. When de-ionized water became available and esters washed with it remained light coloured, it was thought advisable to investigate further.

The amount of copper present is well below the normal tolerance of animal bodies for copper. Hence the dissolved copper could not be responsible for the deleterious effects of the heated ester fractions.

TABLE XVIII

Amount of Dissolved Copper in the Ethyl Esters of Heated Soyabean Oil

Wash water used	γ / gram
Distilled water	8
De-ionized water	4

TABLE XV

Chemical Characteristics of the Fractions Obtained from Twenty Hour Heated Soyabean Oil

Fraction	Iodine value	$n_D^{25^\circ}$	Mol. wt.	Free fatty acids[1]
Ethyl esters	105	1.45612	327	0.9
Adduct-forming of the ethyl esters (AFE)	99	1.45302	295.5	0.7
Non-adduct-forming of the ethyl esters (NAFE)	125	1.46998	415	0.9
Distillable ethyl esters	106	-----	296	0.4
Residue from the distillation	114	-----	415	6--
Non-adduct-forming of the distillable (NAFD)	143	1.47001	294	0.1
Adduct-forming of the distillable (AFD)	92	1.44935	293.5	0.6

1. Calculated as per cent oleic acid

TABLE XVI

The Relative Proportions of the Fractions Isolated from Twenty Hour Heated Soyabean Oil (1)

Distillable Ethyl Esters			Polymers
Total	AFD	NAFD	
74.4	64	10.4	25.6
	AFE		NAFE
	63.3		36.7

1. Results expressed as per cent of total ethyl esters

TABLE XVII

Chemical Composition of the Mixed Ethyl Esters from Twenty Hour Heated Soyabean Oil

Constituent	Per cent
Conjugated diene	3.14
"linoleic" acid	16.45
"linolenic" acid	2.06
Oleic acid	45.4
Saturated material	7.3
Polymers	25.6

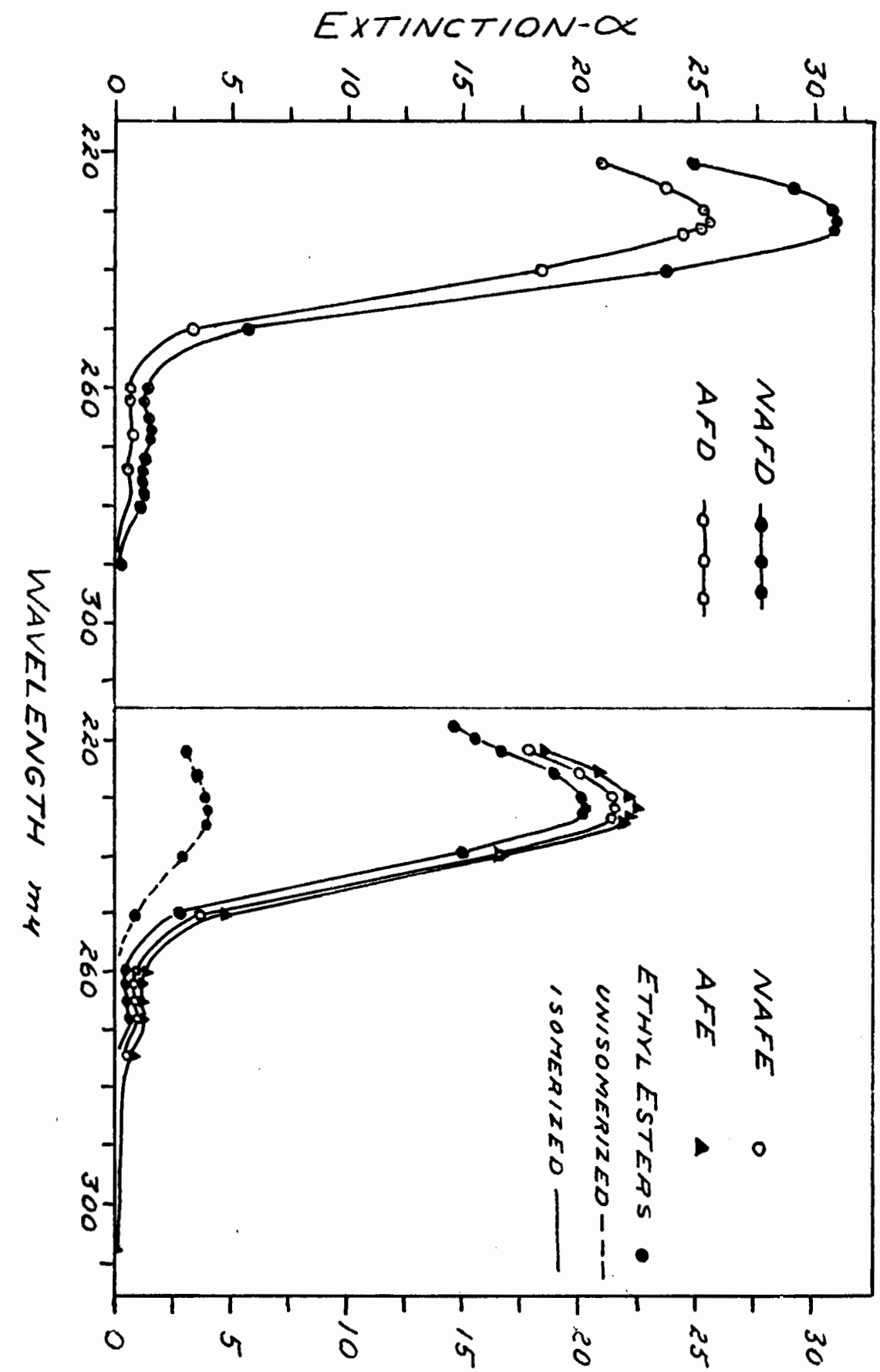

EXTINCTION-α
0
5
10
15
20
25
30
220
260
300
WAVELENGTH mμ
NAFD
AFD
NAFE
AFE
ETHYL ESTERS
UNISOMERIZED
ISOMERIZED

FIGURE 18.

SECTION IV

Nutritional Investigation of the Fractions From Thermally Polymerized Soyabean Oil

Introduction

The NAFD fraction from heated linseed oil, a linolenic acid group oil, has proven extremely toxic to growing rats. When fed at levels of ten per cent in their diets, all rats died within one to two weeks. On the other hand the feeding of sunflowerseed NAFD, an oleic-linoleic acid group oil, caused only a slight suppression of the growth rate. Soyabean oil, an oil intermediate between linseed and sunflowerseed oils in chemical composition, had been examined and a NAFD fraction had been isolated from the distillable ethyl esters. This fraction was fed to growing rats to determine its effect. If this proved to be intermediate between the other two oils, then it would be reasonable to assume that the injurious effects of heated vegetable oils are mainly due to the transformation products of linolenic acid.

Procedure and Results

Raw soyabean oil was alkali-refined, bleached, and polymerized in the manner outlined in Section III.

The polymerized oil was transformed to the ethyl esters by ethanolysis and the resultant esters were divided into two portions. The smaller portion was segregated with urea into adducts (AFE) and non-adducts (NAFE). The larger amount was distilled *in vacuo* to give a monomeric distillate and a

polymeric residue. The distillate was fractionated with urea into an adducting (AFD) fraction and a non-adducting (NAFD) fraction. The scheme of separation of these six fractions is illustrated in Figure 16.

These fractions possessed the same chemical characteristics as those described in Section III (Table XV). Their absorption spectra are given in Figure 16.

The six fractions were fed to young rats in definite daily amounts for a period of twenty-eight days. The diet (Table IX) was the standard Macdonald College diet previously used in heated oil trials. The procedure for feeding the rats has been outlined in Section II. One hundred and twenty rats, sixty on the ten per cent level, sixty on the twenty per cent fat level, were fed the six ester fractions. In each level ten rats were fed one ester fraction making a total of twenty rats on each ester fraction (i.e. ten rats on ten per cent fat level, ten rats on twenty per cent level). The appearance, weight gains, feed intake, and adjusted feed intake were tabulated (Table XIX).

Discussion

The rats on the diets of non-distillable esters at the twenty per cent levels all developed severe diarrhoea and produced extremely sticky feces by which many of the animals were fastened to their cages. A few died, and the remainder were removed from the trial. Similar results had been obtained with the polymeric material of heated linseed oil. In this

latter case it was thought that partial starvation and excess diarrhoea caused their death rather than any "toxic" material within the fraction itself. The same reasoning can be applied to the soyabean polymers. The fact that this fraction is not very digestible can be seen when it is realized that the polymeric material is essentially a "varnish". Both fractions 2 and 6 (NAFK and non-distillable esters, Table XIX) can be considered in the catagory of varnishes. The fact that soyabean polymers (less linolenic acid) produce similar effects to linseed polymers can be rationalized when it is considered that linseed oil contains much more polymeric matter at a given heating time than does soyabean oil (i.e. soyabean oil would have to be polymerized for twenty hours to obtain an amount of polymer equal to linseed oil polymerized for twelve hours).

The NAFD fraction from linseed oil had proven fatal to young rats while that from sunflowerseed oil was only slightly inferior nutritionally to the other monomeric fractions. In soyabean oil the NAFD seems to have an effect intermediate between these other two oils. The adjusted gains are much less than the other monomeric fractions (Table XIX) except the polymers, but they did not prove fatal. Hence it seems that some nutritionally deleterious material is present in the soyabean NAFD but only in sufficient quantity to cause suppressed growth, not sufficient to be fatal.

Therefore it appears that the supposition mentioned

previously, namely that the toxicity of heated vegetable oils is proportional to their linolenic acid content, is valid. It is thought by the author that the term linolenic acid should not be taken literally but rather should mean trienoic and other higher unsaturated acids (i.e. acids containing three or more ethenoid bonds).

TABLE XIX

Results of Feeding Six Ester Fractions from Thermally Polymerized Soyabean Oil to Growing Rats
(10 rats per lot)

Heated oil fraction fed	Per cent esters in diet	Average 28 day gain[1] (grams)	Average 28 day dry matter intake (grams)	Average digest. calories (grams)	Gains adjusted by regression to equal dig. calories (grams)
Urea adduct of whole ester (AFE)	10	99.0	246.0	994	80.3
	20	92.8	220.1	950	79.8
Urea non-adducts of the whole esters (NAFE)	10	62.9	213.7	809	68.2
	20	---[2]	---	---	---
Distillable ethyl esters	10	101.4	252.3	978	84.8
	20	83.4	217.6	932	72.7
Adduct forming esters of distillate (AFD)	10	98.9	247.2	948	86.2
	20	90.6	219.8	938	79.2
Urea non-adduct forming distillate (NAFD)	10	36.4	160.2	626	65.5
	20	15.9	114.8	472	65.0
Non-distillable esters (polymeric residue)	10	68.6	244.1	852	68.3
	20	---[2]	---	---	---
			Mean difference at P 0.05		9.6

1. Nine rats per lot used in the calculations. 2. Rats were removed from trial because of diarrhoea.

SECTION V

The Effect Upon Adult Rats of Subcutaneous Injections of Ethyl Esters of Thermally Polymerized Linseed Oil

Introduction

The NAFD fraction of heated linseed oil has proven fatal to young rats when incorporated into their diets at levels of ten per cent or more. It is not known how these esters act to produce death in such a short time (one to two weeks) for the diets are quite digestible and the feed intake is sufficient to maintain growth, yet the animals die.

The lack of essential fatty acids in the diet does not seem possible because analysis has shown that the oils contain sufficient amounts for normal metabolism. It is possible, however, that a substance is present in the NAFD which prevents their utilization. Isomeric acids (positional and geometric) have been shown to metabolize as well as the normal acids in animal bodies and not, as some authorities have thought, act as antagonists to the normal dietary fatty acids. Hence some injurious factor must be present.

It was suspected that esters of branched-chain and cyclic fatty acids formed part of the NAFD. They would be formed during the polymerization process from the normal acids. Previous workers have demonstrated the toxicity of such acids when injected into mice and guinea pigs. The chief response of the guinea pig is the production of tubercle-like lesions and ultimately death.

The NAFD esters with other innocuous (orally) esters as controls were injected subcutaneously into adult male rats. The rats were killed after completion of the test and were examined for abnormalities. Some workers have demonstrated that hemolysis occurs when branched-chain fatty acids are injected into animals, therefore the hemoglobin levels of the test rats were checked at the start and at the termination of the trials.

The results were expected to give some indication as to whether the toxicity of the linseed NAFD was a phenomenon associated with only oral administration or whether an injurious effect could also be produced on subcutaneous administration.

Procedure and Results

Six groups of four adult male rats were used in this study. Throughout the test they were fed the Macdonald College rat diet No. 6 which has the composition:

Wheat	38 %
Cereal grass	7
Fishmeal	15
Oat groats	10
Wheat germ	10
Corn	9.5
Soyabean oilmeal	5
Yeast	3
Bonemeal	2
Salt (iodized)	0.5

The rats were kept in individual wire-bottomed cages and given feed and water ad libitum. Injections of fractions of heated linseed oil esters were made as shown in Table XX.

TABLE XX

Schedule of Subcutaneous Injections of Ester Fractions of Heated Linseed Oil Into Adult Male Rats

Group	Fraction injected	Duration of test (days)	Injection schedule (days)	Original dose level (ml/dose)
1	Esters of whole unheated linseed oil	7	2nd,4th	0.5
2	Esters of whole unheated linseed oil	28	2nd,9th,16th 23rd	0.05[1]
3	Non-adduct forming fraction of distillate	7	2nd,4th	0.5
4	Non-adduct forming fraction of distillate	28	2nd,9th,16th 23rd	0.05[1]
5	Adduct forming fraction of distillate (AFD)	7	2nd,4th	0.5
6	Adduct forming fraction of distillate (AFD)	28	2nd,9th,16th 23rd	0.05[1]

1. When the animals survived from the first injection the dose was increased to 0.5 ml.

The site of injection of the esters was to the right of the spine, approximately half an inch anterior to the tail. The oil fractions were injected slowly using a hypodermic syringe into a shaved spot. On the first and the last days of each experiment samples of blood were taken for the estimation of hemoglobin level. The hemoglobin values were determined by the method of Gibson and Harrison (1945).

No signs of acute toxicity were evident either directly following the injections or throughout the experimental period. Some of the animals developed lumps at the site of the injection which, on autopsy, proved to be undispersed oil. These lumps, if present, usually became more pronounced after the second or third injections.

The hemoglobin levels are shown in Table XXI. In these tables the normal (average) rat hemoglobin level is taken as sixteen per cent. The animals on the seven day test had hemoglobin levels slightly below standard, the group average being about 1.1 per cent lower than sixteen per cent. Those on the twenty-eight day trial exhibited a group average either above or not more than 0.6 per cent below the standard.

The change in liveweight and feed consumptions were not abnormal. They were tabulated by Miss Barabara Clark of the Nutrition Department and are recorded in report on Project No. 263 (B.Clark, 1952). Because they were not significant they are not reported here.

At the end of the seven day test the rats were killed and given a macroscopic examination. Ten rats appeared normal. Two others exhibited enlarged blood vessels in the intestine but otherwise appeared normal.

The rats on the twenty-eight day test were transported alive by the author to the Animal Diseases Research Institute (Can. Dept. Agri.) at Hull, Quebec. There Dr. P.J.Plummer, a pathologist, examined the animals for possible presence of

TABLE XXI

Individual Initial and Final Hemoglobin Levels (1) of Rats Injected Subcutaneously With Ethyl Esters from Heated Linseed Oil

Group No.	Ethyl esters injected	Rat No.	1st day	Average	7th day	Average	28th day	Average	Difference (2)
L	Esters unheated linseed oil	4	16.4		16.5				
		9	16.0	16.3	14.6	14.9			1.4
		14	16.0		14.4				
		19	16.9		14.2				
2	Esters unheated linseed oil	5	17.1				16.4		
		10	16.1	16.2			14.7	15.4	0.8
		15	15.4				15.4		
		20	16.4				14.9		
3	Non-adduct forming fraction of the distillate (NAFD)	24	15.0		14.9				
		29	16.1	16.0	15.1	15.0			1.0
		34	17.3		16.2				
		39	15.4		14.0				

1. Standard rat blood assumed to be sixteen per cent hemoglobin
2. Difference between 1st and 7th days or 1st and 28th days

Continued

TABLE XXI (Continued)

Group No.	Ethyl esters injected	Rat No.	1st day	Average	7th day	Average	28th day	Average	Difference (2)
4	Non-adduct-forming fraction of the distillate (NAFD)	25	20.7				19.2		
		30	19.7	18.2			15.5	16.3	1.9
		35	15.9				15.5		
		40	17.1				15.0		
5	Adduct forming fraction of distillate (AFD)	44	15.4		15.5				
		49	16.1	16.3	15.1	15.8			0.5
		54	15.9		15.7				
		59	17.8		16.7				
6	Adduct forming fraction of distillate (AFD)	45	19.3				16.2		
		50	15.6	17.1			15.0	16.1	1.0
		55	16.1				15.8		
		60	17.3				17.4		

1. Standard rat blood assumed to be sixteen per cent hemoglobin
2. Difference between 1st and 7th days or 1st and 28th days

tubercle-like lesions or other abnormalities. His report verbatim:

"Each animal was subjected to thorough autopsy. All organs and tissues macroscopically were within normal limits. The subcutaneous tissue in the region of the right groin of rat No. 5 did appear more yellow than in other regions but there was no indication of additional change.
Histopathology - paraffin sections were prepared from the following organs; thyroid gland, cross section of the great vessels just anterior to the heart, heart, lung, stomach, three portions of the small intestine, two portions of the large intestine including the caecum, liver, spleen, kidneys, urinary bladder, adrenal gland, adrenals, also various lymph nodes. No significant changes were found in the above organs taken from any of the twelve rats."

Discussion

No significant differences were found in the hemoglobin levels, the liveweights, feed consumptions, or internal structures of any of the rats in the test after injections of heated oil fractions.

These results do not necessarily mean that the fatty <u>acids</u> of the NAFD are not injurious when injected subcutaneously, but they do seem to indicate that injections of NAFD <u>esters</u> cause no ill effects to the animals over a period of four weeks. The difference is important. Most of the toxic effects noted in the literature have been produced with

branched or cyclic fatty acids, not esters. It is quite possible that in the gut hydrolytic action frees the acids from the ester structure and the liberated fatty acids then are capable of exerting a physiological effect. Hydrolytic enzymes or secretions such as are found in the gut are absent from most other regions of the body, hence the injected esters would remain unchanged and innocuous. Much more work has to be done before we can say with any degree of certainty how the NAFD fraction exerts its fatal effect.

SECTION VI

An Investigation of the Chemical Nature of the Non-adduct Forming Fraction of the Distillable Ethyl Esters of Polymerized Linseed Oil

Introduction

The NAFD fraction from polymerized linseed oil has proven extremely injurious to growing rats, much more so than NAFD from either soyabean or sunflowerseed oil. Because of this fact, it was adjudged the best fraction on which to conduct a chemical investigation. Prior to the present work the characteristics shown in Table XXII had been established for this fraction.

TABLE XXII

The Chemical Characteristics of the NAFD Fraction of Heated Linseed Oil

Terminal methyl number	1.70
Iodine value (average)	170
Mean molecular weight (cryoscopic)	300
Per cent hydroxy groups	0.34
Saponification equivalent	330
Refractive index (25° C.)	1.46986
% conjugated diene	2.7
% conjugated triene	0.0
% "linoleic" acid	3.1
% "linolenic" acid	2.5
Unidentified	91.7

The work presented in this section comprised two distinct lines of attack on the problem of the composition and constitu-

tion of the NAFD fraction. Firstly, a general study of the characteristics of the fraction as a whole; and, secondly, a study of the products of disruptive oxidation of the NAFD. The general experimental scheme was as follows:-

INVESTIGATION OF THE FRACTION AS A WHOLE

1.1 Preparation of the free acids

1.2 Determination of the saturated material

1.3 Lead salt separation

1.4 Lithium salt separation

1.5 Low temperature crystallization

1.6 Chromatography of the hydroxamic acids

1.7 Thiourea segregation

DISRUPTIVE OXIDATION

2.0 Permanganate oxidation

- 2.1 Oxidation
- 2.2 Removal of the neutral matter
- 2.3 Isolation of the steam volatile and non-volatile matter
- 2.4 Treatment of the isolated fractions

Monobasic acids

- 2.4.1 Duclaux constants
- 2.4.2 Chromatography
- 2.4.3 Fractional distillation
- 2.4.4 Chromatography of fractions from fractional distillation

Dibasic acids

2.4.5 Neutralization equivalent

2.4.6 Preliminary solvent separation

2.4.7 Chromatography of the dibasic acids

2.4.8 Multiple fractional extraction

2.4.9 Chromatography of the solvent-separated fractions

3.0 Nitric acid oxidation

3.1 Oxidation

3.2 Steam distillation

3.3 Residue from steam distillation

3.4 Distillate

3.5 Fractional distillation

3.6 Neutralization equivalent

3.7 Chromatography

3.8 Derivatives

INVESTIGATION OF THE FRACTION AS A WHOLE

1.1 Preparation of the Free Acids from the NAFD Esters

Procedure and results: saponification of the oil was effected by mixing the NAFD with a cold conc. alcoholic solution of sodium hydroxide and allowing the mixture to stand overnight. The solution was acidified with sulfuric acid and extracted with ether. The ether extracts were combined and washed with aqueous sodium hydroxide. The alkaline washings were acidified and then extracted with ether,

the extracts then being dried, filtered, and taken to dryness on a steam bath in a tared vessel. The product was a dark red viscous oil. The recovered acids constituted eighty per cent of the NAFD sample. The product was stored in a vacuum desiccator at 6° C.

Discussion The abrupt darkening of the oil when alkali is added seems to be a characteristic of alkali-oil reactions. It is almost certain that the saponification process causes some changes in the chemical constitution of the fatty acids, but it is most likely to be of a minor nature (i.e. migration of double bonds) rather than to involve any structural changes in the carbon skeleton.

1.2 Determination of the Saturated Material

Procedure and results: 0.5083 grams of the NAFD esters was refluxed with potassium permanganate in acetone for two hours. The oxidized esters were then transferred to a beaker, the oxides of manganese decomposed with sodium sulfite, and the solution acidified with sulfuric acid. The acidified solution was transferred to a separatory funnel and extracted with chloroform. The combined extracts were washed with water, dried, and allowed to percolate through an alumina column tinted with bromo phenol blue. The percolate was collected until the yellow band of acid material approached the lower end of the column. The eluted fraction was then taken to dryness. Found 0.0087 grams of

saturated esters were present. This is equivalent to 1.7 per cent of the total esters.

Discussion The result of 1.7 per cent confirms the supposition that this fraction is almost completely composed of unsaturated material. This value also agrees well with the 2.2 per cent obtained by ether extraction of the sodium soaps of the oxidized NAFD (Section VI, 2.2).

Both these results (1.7 and 2.2) tend to invalidate the contention that the discrepany between molecular weights calculated from saponification equivalent (330) and from cryoscopy (300) is caused by neutral hydrocarbons.

1.3 Lead Salt Separation

Procedure and results: 2.6 grams of the free acids, prepared from the NAFD esters, was dissolved in anhydrous ethanol, heated to boiling, and a boiling five per cent solution of lead acetate in ethanol was added (100 ml.) with stirring. The mixture was stirred for several minutes with heating and then allowed to stand overnight at room temperature. No precipitate formed. The solution was kept at 6° C. for three weeks. No precipitate settled out during this time.

Discussion The lead salt separation will precipitate saturated fatty acids from alcoholic solution as lead soaps. However, some oleic, iso-oleic acids, and certain conjugated acids (elaeostearic) will also precipitate. The

absence of any precipitate indicates that little or no acids of these types are present. The fraction is most likely composed of unsaturated acids of such a structure that they will not form urea adducts. No recorded data are available as to how branched-chain or cyclic fatty acids react with lead salts. The result is in agreement with the determination of saturated material.

1.4 Lithium Salt Separation

Procedure and results: 0.7875 g. of the free fatty acids of the NAFD fraction was dissolved in 15 ml. of anhydrous acetone. The solution was warmed and titrated with a saturated aqueous solution of lithium hydroxide to pH 9. pHydrion paper (Micro Essential Laboratory) was used as indicator because the solution was too dark to use an internal indicator. The solution became progressively darker as the end point was approached. Water was added to make a ninety-five per cent acetone solution, the mixture was swirled several times and then allowed to stand at room temperature overnight.

A dark red precipitate settled out. This was collected on a Hirsch funnel, washed with ninety-five per cent acetone and the washings were added to the filtrate. The filtrate (soluble soaps) was acidified with hydrochloric acid, the acetone was removed on the steam bath, and the remaining solution was extracted with ether. The ether extracts were dried, filtered, and evaporated to dryness. The product was a red, mobile oil. Yield 473.5 mg.

The insoluble soaps (residue) were acidified with dilute hydrochloric acid, transferred to a Buechner funnel and washed with a little chloroform. The filtrate was then extracted with ether and the extracts were dried over sodium sulfate, filtered, and the filtrate taken to dryness in a tared vessel. Found 260.2 mg. of a dark red, viscous oil.

The weight of the acids treated was 787.5 mg., while the recovered acids weighed 733.7 mg. This represents a loss of six per cent.

Table XXIII shows the relative proportion of the soluble and insoluble fatty acids of the linseed NAFD when separated as the lithium soaps. Table XXIV gives the iodine values of the two fractions.

TABLE XXIII

The Resolution of the Fatty Acids from Linseed Oil NAFD as the Lithium Salts

	Soluble fatty acids	Insoluble fatty acids
Expressed as per cent of total esters	61	33
Expressed as per cent of recovered acids	64.5	35.5

TABLE XXIV

The Iodine Values of the Fatty Acid Fractions Separated by the Lithium Salt Method

Fraction	Iodine value
Soluble fatty acids	230
Insoluble fatty acids	120

Discussion Crystallization of the lithium salts from ninety-five per cent acetone solution resolves the fatty acids into two fractions: a highly unsaturated soluble fraction, and a more saturated insoluble fraction. The iodine values as shown in Table XXIV confirm this fact.

From Table XXIII it seems that sixty-six per cent of the fraction is composed of polyethenoid acids. No references are available on cyclic and branched-chain fatty acid reaction with lithium hydroxide in acetone.

It should be noted that the original lithium salt separation (Tsujimoto, 1920) was used to effect a separation of the polyethenoid fatty acids of the C_{20} and C_{22} series found in whale and fish oils. This raises an interesting point concerning the soluble fraction. It is possible that this fraction might be composed of monomers of higher molecular weight than the mean molecular weight (300) would indicate (cf. S.E. 330). It could reasonably be assumed that, if the first supposition is true. extra carbons are attached at

non-terminal positions so as to form branches. The insoluble fraction could possibly be composed of such acids which have lost part of their carbons through breakage of the chain (i.e. at the double bonds).

1.5 Low Temperature Crystallization

Procedure and results: 12.9 grams of the free acids of the NAFD were dissolved in sixty-five ml. of acetone (anhydrous) in a large test tube. The tube was placed in a Dewar flask fitted with thermometer and stirrer and the whole cooled to -70° C. gradually by means of dry ice and ethanol. The solution was kept at -70° for thirty hours with occasional stirring. No insoluble material separated out during this period.

The free acids (8.5 g.) were dissolved in ninety ml. of Skellysolve B in a test tube. The tube was cooled to -70° and maintained at that temperature for thirty hours. A small precipitate had formed by this time. The upper liquid was decanted and the remainder removed with a sintered glass filter stick using suction. The precipitate was isolated and weighed 0.45 grams (five per cent of the total weight). It was dark red and viscous. The refractive index and iodine value were determined:

$n_D^{25^\circ}$ 1.48399

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Discussion The only normal, natural fatty acid, saturated or unsaturated, which is appreciably soluble in Skellysolve B at -70° is linolenic acid (4.27 g. in1000 g. solvent). All others are practically insoluble, i.e. their solubilities are less than 0.5 gram per 1000 grams of solvent. In acetone solution solubilities increase somewhat (e.g. linolenic - 4.23 g. to 17.6 g. per 1000 g. solvent) but, besides linolenic, only linoleic is appreciably soluble (4 g. per 1000 g.). The negligible amount of precipitated matter (less than five per cent) indicates that linolenic acid is the only normal acid that could possibly be present. Spectroscopic data have shown that about six per cent linolenic (or isomers) is present. This would account for the precipitated matter. Therefore over ninety per cent of the fraction is composed of soluble fatty acids other than linolenic or linoleic acids.

Little information is available on the solubility of cyclic fatty acids in Skellysolve B or acetone solutions. However, information on the lower molecular weight cyclohexyl acids (cyclohexylacetic to cyclohexylcaproic) indicate only moderate solubility (Dow, 1950) in acetone. This, of course, does not necessarily mean that the higher homologues behave in a similar manner. Nothing is known of the solubilities of cyclic polyethenoid fatty acids.

It is known that the branched-chain fatty acids are extremely soluble in acetone and petroleum ether. They will

not crystallize out at low temperature or else they will come out in the last fraction at the lowest temperature. It has been observed (Morice and Shorland, 1952) that two or more methyl groups, or alkyl groups other than methyl, increase the solubility of a given fatty acid. These considerations tend to support the contention that the NAFD fraction contains branched-chain fatty acids.

1.6 Chromatography of the Hydroxamic Acids

Procedure and results Whatman No. 1 paper and three specially-treated papers were used in chromatographing the hydroxamic acids. The three special papers were Velan-waterproofed, silica-impreganated, and oil-impregnated. The methods of preparation of these papers and of the hydroxamic acids are listed under methods. The acids were spotted by means of a small glass rod in spots not more than one cm. in diameter. In this preliminary study concentrations were not exactly determined - just sufficient was added to get a discernable spot. The spots were placed two cm. above the solvent surface in the case of ascending chromatograms, and four cm. from the reservoir in the case of descending ones. The paper strips were 2.5 cm. wide, while the length varied with the cabinet used - twenty to forty cm.

Ferric chloride (FC), bromocresol blue (BCB), and bromocresol purple (BCP) were the sprays employed.

Eight different solvent systems were tried in conjunction with different papers. The various solvents are listed in the

appendix and will be referred to throughout this section by Roman numerals only.

Table XXV shows the result of the chromatography of the hydroxamic acids.

TABLE XXV

Paper Chromatography of the Hydroxamic Acids Derived from the NAFD Fraction of Linseed Oil

Solvent	Paper	Method	R_f x 100[1]	Spray[2]
XIV	Silica-impregnated strips	Ascending	0	FC
X	Silica-impregnated strips	Ascending	0	FC
XIV	Whatman No. 1	Ascending	100	BCG_2
VIa	Whatman No. 1	Descending	100	BCG
VIb	Whatman No. 1	Descending	0.5	BCP
V	Whatman No. 1	Descending	16.5	BCP
V	Silica-impregnated strips	Descending	16.5	BCP
XI	Paraffin oil impregnated	Ascending	60	BCG
II	Whatman No. 1	Ascending	24.2	BCG
II	Whatman No. 1	Ascending	32	BCP
XVIII	Velan paper	Descending	--	--
XII	Whatman No. 1	Ascending	20.2	BCP

1. R_f is equal to $\frac{\text{distance moved by acid}}{\text{distance moved by front}}$

2. Sprays: FC, ferric chloride; BCG, bromocresol green; BCP, bromocresol purple.

Discussion The fact that the NAFD fraction is not homogeneous was demonstrated by the lithium salt separation. Therefore at least two spots should appear on a satisfactory chromatogram. No satisfactory resolution has been accomplished.

1.7 Thiourea Segregation

Procedure and results: 1.2 grams of linseed NAFD was dissolved in twenty-five ml. of anhydrous ethanol and the solution saturated with thiourea at room temperature. The mixture was then shaken vigorously and allowed to stand at 6° for two weeks. The crystals were separated on a Buechner funnel and the filtrate collected, decomposed with warm water, extracted with ether, and the ether extracts dried over sodium sulfate. On evaporating the extract to dryness, the recovered non-adduct forming material weighed 1.2 grams.

Discussion Only compact, short-chain molecules conforming to an outside diameter of 5.8 to 6.8 Å. form thiourea adducts (Schiessler and Flitter, 1952). Hence branched-chain and olefinic C_{18} molecules do not form adducts. Because of these facts the polyethenoid acids of the NAFD were not expected to form adducts. They did not.

DISRUPTIVE OXIDATION PROCEDURES

2. Permanganate Oxidation

2.1 Oxidation: 6.8528 grams of NAFD were placed in a 500 ml. round-bottomed, ground glass stoppered flask fitted with reflux condenser and Glas-Col heating mantle. Seventy-five ml. of anhydrous acetone was added and the whole gently refluxed. Forty-two grams of finely-powdered potassium permanganate was added gradually over a period of four hours. The permanganate was washed down the condenser with small portions of dry acetone. The mixture was allowed to reflux for twenty-four hours. The acetone was then distilled off and water added. Large lumps were broken up and sufficient sulfurous acid added to decolourize the solution, followed by sulfuric acid until the solution was acid. The mixture was then filtered. The filtrate contained the oxidized esters and the neutral or unoxidized esters.

2.2 Removal of the Neutral Matter A dark red oil was noted floating on top of the aqueous filtrate, this was decanted (Fraction 1). The remaining solution was extracted with ether and the extracts were combined and washed with ten per cent w/v aqueous potassium hydroxide. The colour passed from the ethereal layer to the aqueous during these washings. The ether solution was washed, dried, and filtered. On evaporation it yielded 152.2 mg. of a clear yellow oil (Fraction 2). This composed 2.2 per cent

of the total weight of the oxidized NAFD and is the neutral meterial, most probably comprising the saturated esters (cf. Section VI, 1.2).

2.3 Isolation of the steam volatile and non-volatile acids:

The alkaline washings from 2.2 were acidified with sulfuric acid and steam distilled. A few ml. of concentrated ammonia was placed in the receiver to trap the more volatile acids as their ammonium salts. About a liter and a half of distillate was collected. The distillate was made alkaline with sodium hydroxide and concentrated to 100 ml. on the steam bath. The concentrate was acidified with sulfuric acid and extracted with ether. The colour passed to the ether layer. The ether extracts were combined, dried, filtered, and evaporated. The product was a viscous, yellow oil which had a pungent smell (Fraction 3). Weight 18.8 mg., equivalent to 0.27 per cent of the total weight. This fraction is composed of the monobasic acids.

An alternative procedure was sometimes used to free the fraction from sodium salts which interfere in chromatography and other determinations. After concentrating to a small volume on the steam bath, the solution was passed through a cation exchange column (Dowex 50) which had been conditioned with dilute hydrochloric acid. The sodium ions were removed and the free organic acids were collected as percolate. This can be extracted with ether or used directly inc chromatography.

The residual liqour in the distilling flask was extracted with ether. The extracts were combined and dried. The dried extracts were concentrated to 100 ml . on the steam bath. The resultant solution was then washed with twenty per cent aqueous sodium carbonate. The colour passed to the aqueous layer. The remaining ether layer was dried and yielded, on evaporation, 23.5 mg. of a dark-coloured oil (Fraction 5). This is equivalent to 0.34 per cent of the total weight.

The carbonate washings were acidified with sulfuric acid and extracted with ether. The yield from the extracts was 1.297 grams of a white, waxy solid (Fraction 4). This constituted nineteen per cent of the total weight and was composed of dibasic or polybasic acids.

Discussion As described above, five fractions have been isolated from the oxidized NAFD esters, to wit:-

1. A neutral oil, immiscible in water, of unknown characteristics (about fifteen per cent).
2. A neutral fraction (2.2 per cent) composed of saturated esters.
3. Steam distillable fraction (0.27 per cent) composed of volatile monobasic acids.
4. A non-steam volatile fraction (nineteen per cent) consisting of the polybasic acids - mainly dibasic.
5. Oil of unknown composition (0.34 per cent).

Fraction 1 may yet prove to be the most important one for it

could possibly contain saturated, branched-chain fatty acids. The amount of this particular fraction seemed to remain fairly constant whether the oil was oxidized for eight, twenty-four, or forty-eight hours. This indicated a material quite resistant to oxidation and not, as first suspected, merely unreacted NAFD.

The procedure outlined above is the quantitative one, usually the process is carried out qualitatively to get sufficient material for the analytical work.

The five fractions listed above account for thirty-seven per cent of the NAFD. Sixty to sixty-five per cent has not been accounted for, or has been lost. Because the thirty-seven per cent amount has remained constant during many oxidations, it is thought that the remainder of the fraction must have been completely oxidized, under the conditions employed, to unrecoverable fragments.

2.4 Treatment of the Isolated Fractions

STEAM DISTILLABLE FRACTION

2.4.1 Duclaux constants: the aqueous steam distillable fraction (no ammonia added to the receiver) was used. The distillate was arbitrarily divided into two portions: the first 150 ml. of distillate, called distillate No. 1. and the remainder of the distillate (ca. 800 ml.) called distillate No. 2. The Duclaux constants were determined on both. The results are given in Table XXVI.

TABLE XXVI

Duclaux Constants of the Steam Distillable Fractions Obtained by Permanganate Oxidation of the NAFD from Heated Linseed Oil

Fraction	Constants
Distillate No. 1	28.7, 6.2, 3.9
Distillate No. 2	13.1, 7.9, 7.7

2.4.2 Chromatography of the monobasic acids: the aqueous distillates obtained as described in 2.4.1 were chromatographed as their ammonium salts with solvent XIII by the ascending method. The distillates and known monobasic acids (standards) were spotted on a cyclinder of Whatman No. 1 paper (untreated) and run in a Bell jar. The results are shown in Table XXVII.

Table XXVIII shows the results of chromatography of the whole monobasic acid fraction (2.3) from an alcoholic solution. The ascending method was used with solvent II.

2.4.3 Fractional distillation A small sample of the monobasic acids was placed in an Emich tube and covered with a wad of asbestos. The closed end of the tube was placed in a warm wax bath (Fisher Bath Wax) and the temperature gradually raised by means of a micro-burner. Fractions were removed at intervals with a micro-

TABLE XXVII

Results of the Chromatography of the Monobasic Acid Fraction Obtained by Permanganate Oxidation of the NAFD from Heated Linseed Oil[1]

Acids chromatographed	R_f x 100
No. 1 distillate	35.6 59.7
No. 2 distillate	34.3 77.1
Acetic acid	59.7
Propanoic acid	67.5
Butanoic acid	73.6
Levulinic acid	66.4
Hydracrylic acid	57.8
Lactic acid	62.7
Glycolic acid	54.5

TABLE XXVIII

Results of the Chromatography of the Monobasic Acid Fraction Obtained by Permanganate Oxidation of the NAFD from Heated Linseed Oil[2]

Acids chromatographed	R_f x 100
Monobasic fraction	100
Hydracrylic acid	54
Levulinic acid	88

1. Ascending method with Solvent XIII.
2. Ascending method with solvent II.

pipette. The list of fractions given in Table XXIX is for two typical distillations.

The p-toluidide of fraction 8 (I) was prepared (Schriner and Fuson, 1948). It was found to melt at 68-70°; mixed melting point with p-toluidide of butanoic acid was 68 - 69°.

Fraction 7 (I) was treated in a similar manner. The p-toluidide melted at 60° C.

2.4.4 Chromatography of the fractions from fractional distillation: the fractions were chromatographed by the ascending method on sheets (20 by 20 cm.) of Whatman No. 1 filter paper in a multi-paper cabinet (Figure 6). The solvents are listed under Table XXX.

Discussion The monobasic acid fraction from the permanganate oxidation has been shown to contain acetic and butamoic acids. Chromatographic study of the mixed monobasic acids and fractions 1 and 2 from the fractional distillation showed the presence of acetic acid. Butanoic acid was detected by chromatography, and its presence was confirmed by a mixed melting point on the p-toluidide of fraction 4 with p-toluidide of butanoic acid.

The R_f values for fractions 6 to 11 (Table XXX) show only one spot which appeared in all chromatograms in the same general region. The R_f values of these spots are almost identical with that of hexanoic (caproic) acid, but it will be

noted that these fractions, when run alongside hexanoic acid, their R_f values were slightly lower than that of hexanoic. This must signify that hexanoic or a very closely related acid is present.

Perhaps it should be mentioned here that in collecting these fractions (6 to 11), a very definite odour was detected. It was almost identical with that of hexanoic acid.

It has been the experience of the author and that of others (Reid and Lederer, 1952) that the R_f of a normal monobasic acid is somewhat larger than that of the corresponding isomer (i.e. butyric, isobutyric; valeric, isovaleric; caproic, isocaproic). Hence the fact that fractions 6 to 11 have R_f's slightly lower than that of hexanoic acid could mean the presence of an isomer - likely 4-methylpentanoic acid (iso caproic).

The p-toluidide of fraction 7 was made. It melted at 60° (without recrystallization). Isocapro-p-toluidide melts at 61 - 62.5° (Underwood and Gale, 1934). It seems reasonable to assume that isocaproic acid is present.

Table XXIX presents the relative amounts of the various cuts from the fractional distillation. It will be seen that butyric and isocaproic acids together constitute the major portion of the monobasic acids. Acetic acid formed only a relatively small percentage. The absence of acetic acid in large amount may be significant, or it may be that it was lost during the steam distillation and/or during fractionation

TABLE XXIX

Results of the Micro Fractional Distillation of the Monobasic Acid Fraction Obtained by Permanganate Oxidation of the NAFD from Heated Linseed Oil

First fractionation (I)			Second fractionation (2)		
Fraction No.	Temperature range[1]	Amount (rel.)	Fraction No.	Temperature range[1]	Amount (rel.)
1	-100	small	1	-100	small
2	100-110	medium	2	100-110	medium
3	110-130	large	3	110-130	
4	120-130	large	4	120-130	
5	130-140	v.large	5	130-140	
6	140-145	large	6	140-145	
7	145-150	large	7	145-150	v.large
8	150	large	8	150	
9	150-155	small	9	150-155	
10	155-172	minute	10	155-172	small
11	172-216	minute	11	172-200	small

1. Represents the bath temperature. The temperature inside the Emich tube was probably higher because of the temperature differential within the heating bath.

because of its volatility.

In the light of the foregoing evidence it can be said that acetic, butyric, and isocaproic acids have been established as the major components of the isolated monobasic acid fraction.

TABLE XXX

Results of the Chromatography of Acids from the Monobasic Acid Fraction Separated by Fractional Distillation[1]

Standard acids and fractions	R_f x 100 of Monobasic acids[2]					
	1	2	3	4	5	6
Propanoic acid			71.7			
Butyric acid				80.0		
Isovaleric acid			76.5			
Fraction No. 5		67.7			85	
10					84	
11					83	
Propanoic acid			66.6			
Butyric acid				72		
Fraction No. 11						81
Butyric acid				77.5		
Caproic acid						93.6
Fraction No. 2			70.3			
9						92
Propanoic acid			76			
Caproic acid						92.8
						88.5
Fraction No. 9						88.5
Acetic acid		65.2				
Butyric acid				80		
Caproic acid						84.5
Fraction No. 4				77.5		
Butyric acid				81.1		
Caproic acid						87
Fraction No. 4				80.5		

Continued next page

TABLE XXX (Continued)

Standard acids and fractions	R_f x 100 of Monobasic acids[2]					
	1	2	3	4	5	6
butyric acid				79.3		
						89.1
Fraction No. 6						89.0
Acetic acid		63				
Propanoic acid			67			
Butyric acid				79.3		
Caproic acid						85.3
Capric acid						(95)
Fraction No. 4			70			
3		61				

1. Solvent XX was used in these chromatograms, and the spray was bromocresol purple.
2. The numbers 1, 2, 3, ... etc. refer to the number of carbon atoms in the acid molecule.

NON-STEAM DISTILLABLE FRACTION

2.4.5 Neutralization equivalent: weighed samples of the mixed acids were dissolved in ethanol and titrated with sodium hydroxide to phenolphthalein end point. A blank was run on the ethanol. Results are presented below.

Weight of mixed acids, grams		0.13438	0.07687
ml. of sodium hydroxide	0.152 N	8.65	
	0.0305 N		25.14
Correction for blank, ml.		0.045	0.225
Calculated mean molecular weight assuming dibasic acids present		204.9	202.2

2.4.6 Preliminary solvent separation The dibasic acid fraction was extracted with cold water, filtered, and the filtrate taken to dryness. The dry solid was dissolved in benzene, refluxed for thirty minutes, and then filtered. The filtrate was taken to dryness. Three fraction were isolated by this method:

1. Water soluble fraction - a waxy solid of distinctive odour and having a granular texture. M.Pt. 78-84°.
2. Water soluble, benzene soluble fraction - a waxy brown solid. M.Pt. 64-67°.
3. Water soluble, benzene insoluble fraction - a small amount of a greasy solid.

In a separate determination it was found that about ninety-five per cent of the dibasic acid fraction was water soluble, five per cent insoluble. The former fraction, i.e. the water soluble part, was not composed of completely water

soluble acids, for it was noted that the soluble fraction contained suspended matter. This material could not be removed by filtration and remained in suspension even after standing several days. Therefore when the term soluble dibasic acids is used this consideration must be borne in mind.

2.4.7 Chromatography of the dibasic acids The dibasic acid fraction as a whole and the fractions obtained by preliminary solvent separation were chromatographed. The results of these investigations are reported in Tables XXXI, XXXII, and XXXIII. The solvents used are cited in footnotes to the tables. Whatman No. 1 paper was used in all the chromatograms.

2.4.8 Multiple fractional extraction About one gram of the water soluble portion of the dibasic acid mixture was added to 100 ml. of ether-saturated water. This was transferred to a separatory funnel and fourty ml. of water-saturated ether added. Twenty-five extractions were performed according to the procedure outlined in methods. Ten extracts were isolated; five ether, and five water. They were taken to dryness on the steam bath and their appearance and melting points noted where possible. The results are reported in Table XXXIV.

A sample of the water soluble fraction (0.661 g.) of the dibasic acid mixture was dissolved in sixty ml. of ether-saturated water and poured into a separatory funnel. Twenty

ml. of water-saturated ether was added. One hundred extractions were carried out in the usual manner, and twenty extracts isolated. The fractions were evaporated to dryness and their characteristics noted (Table XXXV).

The water insoluble portion of the dibasic acid mixture was given a simple six stage extraction and three fractions were isolated. The melting point determinations showed:

1. m.p. 105-107° C.
2. m.p. 114
3. m.p. 126

2.4.9 Chromatography of the solvent separated fractions

Fractions from both the ten and twenty multiple fractional extractions were chromatographed. The results are presented in Tables XXXVI and XXXVII.

Discussion The neutralization equivalent on the mixed acids was 200 (calculated as a dibasic acid), which suggests that the higher dibasic acids predominated in the mixture, i.e. pimelic, suberic, azelaic, and sebacic. This view was further supported when the meting point of the fraction (65°) was considered. Hence the presence of appreciable amounts of the lower dibasic acids with high melting points (oxalic, malonic, adipic) is excluded. The acids present in appreciable amounts were most likely glutaric, pimelic, suberic, azelaic, and sebacic.

The chromatograms of the mixed acids showed four spots. From Table XXXI it appears that suberic and sebacic acids

were present. However the R_f values of the mixed acids are different from that of an acid run singly on a chromatogram. Other factors (time, temperature, etc.) being constant, the relative proportion and the number of acids in a mixture have an influence on the R_f value of any single acid of the mixture. The situation could be compared with melting point mixtures. From Tables XXXVI and XXXVII, it seems that, with the solvents studied, mixtures of four very closely related fatty acids of low molecular weight have larger R_f values than mixtures of similar acids of higher molecular weight. For example, pimelic acid, when incorporated into a mixture of dibasic acids of higher molecular weight than itself, has an R_f four to ten units lower than if it were mixed with dibasic acids of lower molecular weight. These facts must be kept inmmind when reading the tables of R_f values.

The probability that hydroxy acids form part of the dibasic acid mixture because of the oxidative procedures was disproven. It was shown that hydroxy acids have uniformly low R_f values, much lower than the majority of the dibasic acids of the non-volatile fraction when they were chromatographed together in the same solvent.

Tricarballylic acid, a tribasic acid, had an R_f of zero when chromatographed in the ammoniacal solvent usually used in dibasic acid chromatography. Therefore the tribasic acids form no appreciable proportion of the residue.

Two dimensional chromatography indicated the presence of

suberic acid (Table XXXII). It is of interest to note that only one spot from the dibasic acid fraction appeared in the second dimension - all others disappeared in the second solvent (phenol).

In the preliminary solvent separation, the water soluble portion contained too many acids to give any definite knowledge of the composition. Chromatography, in most cases, will show trace amounts of acids, hence give an unsatisfactory picture of the mixture as a whole. However, the water insoluble portion, on chromatographing, seemed to contain suberic and sebacic acids. The relatively high melting point of this portion (2.4.8) supported the chromatographic evidence (m.p. suberic 140°, sebacic 134°).

The two multiple fractional extractions did not clarify the picture greatly because it seems that the extractions were not numerous enough to give complete separation. Hence the chromatograms showed spots for acids which were really present in only very minute amounts. It was not possible to arrive at any conclusion as to which of the spots was the most significant. These extractions, however, verified the previous supposition that the dibasic acid mixture is composed mainly of high molecular weight dibasic acids -pimelic to suberic.

No dibasic acids have been definitely identified. The possibility exists that branched-chain acids such as methyl succinic or ethyl malonic are present. Samples of such acids were not available for examination.

TABLE XXXI

Results of the Descending Chromatography of the Dibasic Acid Fraction Obtained by Permanganate Oxidation of the NAFD from Heated Linseed Oil[5]

Fraction or standard acid	R_f x 100					
Dibasic acid fraction	2.5			15.0	26.9	35.8
Tartaric acid		4.0				
Citric acid	3.0					
Malic acid		5.8				
Maleic acid			8.3			
Fumaric acid			7.3			
Azelaic acid					29.3	(1)
Dibasic acid fraction (2)	2.64		8.5	17.6	22.6	
	2.12		7.9	18.5	23.6	
Malonic acid		3.63				(2)
Dibasic acid fraction (3)		5.9	14.7	19.8	25.2	
		5.7	14.1	18.5	23.2	
		5.1	10.6	18.5	22.0	
Oxalic acid	1.2					
Adipic acid		5.5				(3)
Tricarballylic	0.0					(4)

1. Solvent XVIII
2. Solvent XII
3. Solvent XII
4. Solvent XII
5. The lines between the groups of figures means that each section was run on the same paper, i.e. identical conditions.

TABLE XXXII

Results of
Two Dimensional Chromatography on the
Dibasic Acid Fraction Obtained
By Permanganate Oxidation
of NAFD from Heated
Linseed Oil[1,2]

Fraction or standard acid	R_f x 100	
	First dimension	Second dimension
Dibasic acid fraction (4)	20.3	91.3
	18.1	90.6
	20.7	92.0
	21.0	90.0
Malonic acid	3.6	68.7
Succinic acid	3.8	78.6
Adipic acid	8.0	85.5
Azelaic acid	19.5	92.6
Dibasic acid fraction (2)	15.4	87.4
	12.9	86.6
Tricarballylic acid	0.0	73.6
Malonic acid	0.7	69.3
Succinic acid	1.5	72.8
Azelaic acid	12.4	87.0

1. Ascending method using multi-paper apparatus illustrated in Figure 6.
2. Solvent XII in the first dimension, Solvent II in the second dimension.

Explanation of Chromatography Tables XXXIII, XXXVI, XXXVII

The horizontal lines separating the various sections of these tables means that the substances within the lines were chromatographed at the same time in the same solvent. A comparison between substances within these enclosures is more valid than a comparison between enclosures.

Solvents and Methods

Table XXXIII:
1. Descending strips with Solvent XXI
2. Descending strips with Solvent XXII
3. Ascending sheet with Solvent XX
4. Ascending sheet with Solvent XX
5. Descending strips with Solvent XIX
6. Descending strips with Solvent XIX

Table XXXVI:
1. Descending strips with Solvent XXI
2. Descending strips with Solvent XXII
3. Descending strips with Solvent XXI

Numbers 4 to 9 inclusive were chromatographed by the ascending method on sheets using Solvent XX.

Table XXXVII: All parts of this table used the same solvent and method - descending strips with Solvent XII.

TABLE XXXIII

Results of Preliminary Chromatographic Experiments with Solvent Extraction on the Dibasic Acid Fraction

Fractions Chromatographed	R_f x 100								
	Oxalic	Malonic	Succinic	Glutaric	Adipic	Pimelic	Suberic	Azelaic	Sebacic
Standard acids						54	61.3	67.6	74.6
Standard acids			34.9	41.8	50.3	57			
Standard acids				49			64.8		75.8
Benzene soluble							62.2	69	75.6
Benzene insoluble							63.5	71	
Water soluble, ether soluble							61.7	68.3	
Standard acids [1]						51.3	58.2	64.5	71.5
Standard acids						75.8	80	86.7	92.5
Standard acids			69.3	74.3	79.0	82.0			
Benzene soluble								84.2	
Benzene insoluble [2]							78.9	85.4	
Standard acids						42.0	47.3	53.3	58.6
Benzene soluble								53.3	60
Water soluble, ether soluble [3]							45.2		
Standard acids						44.5	51.6	58.1	64.6
Water insoluble [4]***							54.6		61.3

Continued

TABLE XXXIII (Continued)

Results of Preliminary Chromatographic Experiments with Solvent Extraction on the Dibasic Acid Fraction

Fractions Chromatographed	R_f x 100								
	Oxalic	Malonic	Succinic	Glutaric	Adipic	Pimelic	Suberic	Azelaic	Sebacic
Standard acids		52.2			63.4			83.7	
Benzene soluble						73.6	84	90.2	97.1
Benzene insoluble		48.5				74.2			98.5
Water soluble, ether soluble						76.1	83.1		96.4
Water soluble, ether insoluble						76.2			97.3
Water insoluble							83		96.7
Dibasic acids [5]		54.4				76.8		89.5	96.6
Adipic acid					65.5				
Azelaic acid								82.5	
Succinic acid			60.7						
Malonic acid [6]		50.8							

TABLE XXXIV

Characteristics of Ten Extracts of Multiple Fractional Solvent Extraction

Aqueous Extracts			Ether Extracts		
No.	M.Pt.	Amount	No.	M.Pt.	Amount
1.		large	6.	70-85°	large
2.	70-74°	medium	7.	80-85°	large
3.	ca. 65°	medium	8.	71-77°	large
4.	ca. 65°	small	9.	64-67°	small
5.	70-90°	medium	10.		large

TABLE XXXV

Characteristics of Twenty Extracts of Multiple Fractional Solvent Separation

Ether Extracts			Aqueous Extracts		
No.	M.Pt.	Amount	No.	M.Pt.	Amount
1.	102-104°	medium	11.	65-84°	medium
2.	66-75°	large	12.	72°	medium
3.	94-95°	medium	13.		large
4.	87-90°	large	14.		---
5.	83°	large	15.		medium
6.		medium	16.		medium
7.	95°	small	17.		medium
8.	124°	small	18.		large
9.		minute	19.		large
10.		minute	20.		minute

TABLE XXXVI

Results of the Chromatography of the Products from Ten Counter Current Extraction of the Water-soluble Portion of the Dibasic Acids

Fractions Chromatographed	R_f x 100								
	Oxalic	Malonic	Succinic	Glutaric	Adipic	Pimelic	Suberic	Azelaic	Sebacic
Standard acids						54	61.3	67.6	74.6
Standard acids			34.9	41.8	50.3	57			
Standard acids				47			64.8		75.8
Fraction No. 5							64	69.8	
Fraction No. 7							62		
Fraction No. 8							63.1	70.2	
Fraction No. 9							65.2		
[1]									
Standard acids						40	49	61	78.5
Standard acids				20.6			55.3		81.6
Fraction No. 8							52.4	71.6	
Fraction No. 9							49.7		
[2]									
Standard acids						34.3	39.4	46.5	56
Standard acids						29.8	37.1	44	53.5
Fraction No. 5						32.2			
Fraction No. 7						30.1			
Fraction No. 8						28.8	38.2		
Fraction No. 9						30.9			
[3]									
Standard acids						44.5	51.6	58.1	64.6
Fraction No. 3							52.2		
Fraction No. 8						44.8	51.3		
[4]									

Continued

TABLE XXXVI (Continued)

Fractions Chromatographed	R_f x 100								
	Oxalic	Malonic	Succinic	Glutaric	Adipic	Pimelic	Suberic	Azelaic	Sebacic
Standard acids						43.6	51.3	57.3	64.6
Standard acids				26.7			40		53.3
Fraction No. 8 [5]						41.3	48.6		
Standard acids						42	47.3	53.3	58.6
Fraction No. 7 [6]						45.6			
Standard acids						46.8	52.5	59	64.8
Fraction No. 5						42.8			
Fraction No. 9 [7]						45.5			
Standard acids						46.6	55	62.6	68
Fraction No. 5						51.2	58.5		
Fraction No. 7 [8]						52.7			
Standard acids						43.6	51.1	57.8	64.6
Fraction No. 9 [9]							48.8		

TABLE XXXVII

Results of the Twenty Fraction Counter Current Extraction
of the Water Soluble Portion of the Dibasic Acids

Fractions Chromatographed	R_f x 100								
	Oxalic	Malonic	Succinic	Glutaric	Adipic	Pimelic	Suberic	Azelaic	Sebacic
Standard acids									
Standard acids		14	24.4		32.9			46.7	
Fraction No. 1		14.9					40.4		52.8
Fraction No. 2		15.6		30				45.5	56.2
Fraction No. 3		13.6		29.6				49.3	
Fraction No. 4		12.8		29.5					50
Standard acids	8.8	15.8		20.2				41.0	
Fraction No. 5		14.9				32.2	36.1		
Fraction No. 6*		13.3 (sl.)				29.8			
Fraction No. 7		13.1 (sl.)				31.2			
Fraction No. 8									
Standard acids					16.4			41.6	
Fraction No. 11					18.3	31.3			
Fraction No. 12					18.0	30.9			
Fraction No. 13					16.7				
Fraction No. 15					17.2				
Fraction No. 16**					14.0**	26.0**			
Fraction No. 17					17.9				
Standard acids**					13.6			33.6	
Fraction No. 18					15.6		29.2		
Fraction No. 19					14.8			32.9	
Fraction No. 20							24.1		

* No. 6 should read No. 7, etc. ** This belongs with standards below (indicated by **)

NITRIC ACID OXIDATION

3.1 Oxidation: twenty-five ml. of concentrated nitric acid was added to four grams of NAFD in a round-bottomed flask fitted with a reflux condenser. The condenser was fitted with a gas trap to catch gases liberated during the reaction. The mixture was gently warmed until a reaction started - as evidenced by production of much brown gas - then the heat was withdrawn until the first, violent phase of the reaction subsided. During this period a violent effervescence liberated brown fumes of nitrogen oxides which indicated the reduction of the nitric acid. When the effervescence stopped, another twenty-five ml. of nitric acid was added, and the heating was continued. The last twenty-five ml. of acid dissolved the dark-coloured material that had formed during the initial phase of the oxidation. Heating under reflux was continued for two hours. The product was a dark-coloured solution with a black oily layer floating on top. This oil resembled fraction 1 (2.2) which was isolated from the potassium permanganate oxidation.

3.2 Steam distillation: the acid mixture was steam distilled and a volatile and non-volatile fraction collected.

3.3 Residue from steam distillation The non-volatile acids (and neutral matter) were extracted with ether, and the extracted washed

with ten per cent w/v aqueous sodium carbonate. At this point a dark oil separated at the interface between the water-ether layers (cf. 2.2). This was removed. The carbonate extracts were acidified with sulfuric acid and extracted with ether. The ether extracts, on evaporation, yielded a dark-red viscous oil. This should contain the dibasic acids.

3.4 Distillate The aqueous distillate was a clear solution with a very sharp, pungent odour, not unlike that of formic or acetic acids. The Duclaux constants were determined on the first 100 ml. of distillate and were found to be: 12.4, 9.9, and 9.7. The material used in the Duclaux determination was added to the main body of distillate and the whole neutralized with potassium hydroxide and concentrated to small volume on the steam bath. The concentrate was acidified with sulfuric acid and extracted with ether. The ether extracts, on evaporation to dryness, yielded 158 mg. of a brown, waxy solid. This constituted approximately 4.5 per cent of the total NAFD oxidized. It should contain the monobasic acids.

3.5 Fractional distillation A portion of the monobasic mixture (3.4) was fractionally distilled by the Emich tube technique. The fractions collected are shown in Table XXXVIII. The amounts and boiling points are also noted.

3.6 Neutralization equivalent: a weighed sample of monobasic acids when titrated with sodium hydroxide gave a neutralization equivalent of 135. It is thought that this figure is somewhat high because of the difficulty encountered in the weighing of the sample.

3.7 Chromatography: the monobasic acid fraction as a whole and the fractions from the fractional distillation were chromatographed. The results are reported in Tables XXXIX and XL.

3.8 Derivatives: attempts to make derivatives from the cuts obtained from the fractional distillation failed. The derivatives were invariably lost during the processing. This may be understood when it is considered that only one or two milligrams of each fraction was available. However the anilide on the forerun of the steam distillation was made and was found to melt at 114-116° C. (anilide of acetic acid, m.p. 114° C.).

Discussion The nitric acid oxidation procedure was quite violent, but this does not necessarily mean the oxidation was more complete than the permanganate oxidation. In point of fact, more monobasic acids were recovered from the nitric acid oxidation - four per cent (cf. 0.3 per cent from permanganate). The dibasic acids, however, were dark-coloured, viscous semi-solids as compared to the white wax obtained from the permanganate process. These dark-coloured

acids are probably highly oxidized. No satisfactory method has yet been devised to crystallize this viscous fraction from solution.

The monobasic acid fraction seems to have been composed of acetic, propanoic, and some acids of the C_4 to C_6 range ("smelly acids"). The odour from the higher fractions of the fractional distillation (fractions 4 to 6) was characteristic of these type acids.

The presence of a low molecular weight acid had been inferred from the value of the Duclaux constants on the forerun of the steam distillation. Chromatography showed the probable presence of acetic acid. This was confirmed by making the derivative (anilide). Acetic acid could be expected to occur as a fragment from the complete oxidation of some of the oil constituents.

The presence of butyric (or isobutyric) seems possible from chromatographic results, and an acid (or acids) of higher molecular weight than butyric is present.

To summarize, it seems that acetic acid, butanoic acid, and at least one acid of molecular weight greater than butanoic are present in the monobasic acid fraction from the nitric acid oxidation of the NAFD. No resolution of the dibasic acid portion has been accomplished.

TABLE XXXVIII

Results of the Fractional Distillation of the Monobasic Acid Fraction Obtained by Nitric Acid Oxidation of the NAFD from Heated Linseed Oil

Fraction number	Boiling point °C	Amount	Odour
L	85-90°	small	sharp
2	118-121	large	
3	119-121	large	
4	135.5	medium	butyric
5	143-153	large	
6	152-153.5	medium	
7	190	minute	caprylic

TABLE XXXIX

Results of the Chromatography of the Monobasic Acids Obtained by Nitric Acid Oxidation of the NAFD from Heated Linseed Oil[1]

Fraction	R_f x 100		
Monobasic acids		76.6	88.5 86.3
Formic acid	54.6		
Butyric acid		76	
Formic, propanoic, butanoic mixture	54	65.2	75.5

1. Ascending method, solvent XX.

TABLE XL

Results of the Chromatography of the Fractions Obtained by Fractional Distillation of the Monobasic Acid Fraction from Nitric Acid Oxidation of the NAFD from Heated Linseed Oil[1]

Fraction or standard acid	R_f x 100		
Acetic acid	25.9		
Isobutyric acid		52.6	
Acetic, isobutyric acid mixture	23.7	68.3	
Fraction No. 3	24.3		
4	23.1		
6	16.7		76.1

1. Ascending method with Solvent XII

SECTION VII

A Comparison of the NAFD Fractions from Thermally Polymerized Linseed, Soyabean, and Sunflowerseed Oils

Introduction

Very little new experimental work will be introduced in this section, which is a compilation and correlation of the experimental work described in other sections - principally Sections I, II, III, IV, and VI. In the following discussion the work of other authors will be compared to the present work, and conclusions drawn when possible. The experimental procedures are mentioned in the discussion as they occur.

Discussion

The three NAFD fractions have one property in common, to wit, they will not form urea inclusion compounds. This fact means that their structure must be different from the normal, straight-chain, saturated ethyl esters which will readily form such molecular compounds.

There are three possible structural types which would not form urea adducts: branched-chain esters, cyclic esters, and highly unsaturated esters. If the alkyl chain is sufficiently long, terminal branching or cyclization will not prevent adduction; however, non-terminal groups will inhibit urea inclusion.

The tendency for fatty acids to form inclusion compounds with urea decreases with increasing unsaturation. Linoleic

and linolenic acids form urea adducts least readily of the normal vegetable oil acids. In fractional segregation of the vegetable oil acids (Swern and Parker, 1952 a, 1952 b, 1953), linoleic comes out with the last segregate, and linolenic acid usually remains in the liquid filtrate unless low temperatures are employed (6° C.). In complexing the ethyl esters of the heated oils, room temperature (23° to 27°) was the lowest processing temperature reached. Hence some adductible esters might have remained in the NAFD.

The effect of cis-trans isomers and conjugation on urea inclusion has not been extensively studied because most of the compounds have not been isolated yet in pure form. However, in a preliminary study (Schlenk and Holman, 1950) conjugated isomers were found to form urea adducts more readily than the normal acid, and elaidic acid (trans) yielded slightly more adduct than the normal acid, oleic (cis). Therefore, thermal polymerization would tend to favour the production of more adducting esters in so far as it causes conjugation and elaidinization.

Branched-chain acids do not form adducts and, if present in the distillable portion, would appear in the NAFD after urea adduction. The terminally-branched acids only form adducts with difficulty, and would most likely also augment the NAFD.

Cyclic fatty acids of chaulmoogra oil do not form urea adducts. This was demonstrated by Wells (1952), who made the

ethyl esters and subjected them to urea segregation. Ninety per cent could not be made to form adducts.

TABLE XLI

Typical Analysis of Chaulmoogra Oil[1]

Constituent	Per cent
Palmitic acid	4.0
Oleic acid	14.8
Lower cyclic homologues	0.4
Hydnocarpic acid	35.3
Chaulmoogric acid	22.7
Gorlic acid	22.8

1. This table was taken from Hilditch (1947).

Table XLI shows that about eighty per cent of the oil is composed of C_{16} to C_{18} fatty acids possessing a terminal cyclopentenyl ring. In general, it is probable that cyclohexyl, cyclohexenyl, and cyclopentyl rings will also prevent adduct formation. Nunn (1952) has shown that cyclopropyl rings form adducts with difficulty. Terminal phenyl rings only form inclusion compounds with difficulty, and only if the chain is of sufficient length.

From the preceding discussion, it is evident that the possible number of compounds that could compose the NAFD fraction is quite large. Eliminated are the straight-chain, saturated and monounsaturated esters. In the doubtful class fall the terminal-branched or terminal cyclic esters. With such compounds it is uncertain whether they would adduct under

the experimental conditions. Most probably they would form part of the NAFD.

Nutritionally, the oils are linseed, soyabean, and sunflowerseed in decreasing order of injuriousness. The same sequence holds for some of the chemical properties. The iodine values, refractive indices, and the polymerization time required to form an NAFD, all comply with this observation. The data are recorded in Table XLII.

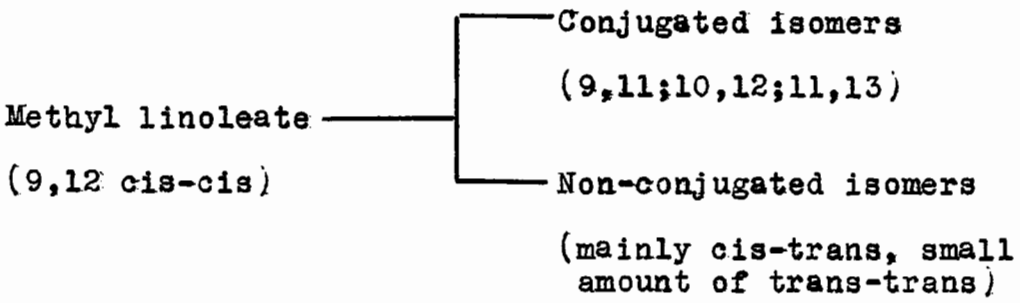
Figure 19 shows a plot of the per cent polymers vs. iodine value for linseed, soyabean, and sunflowerseed methyl esters (from which the NAFD can be obtained). The slopes (Δ I.V./Δ % polymer) of all three are similar, except that sunflowerseed oil lacks the initial steep decrease which both other oils exhibit.

The esters differ most markedly in respect to the results of spectral examination. Figures 20, 21, and 22 show the absorption spectra of sunflowerseed, soyabean, and linseed NAFD before alkali-isomerization, after a twenty-five minute isomerization, and after a six hour isomerization. Figure 23 shows the spectra of Figures 20 to 22 on the same paper for easier comparison.

Linseed NAFD is the most unusual of the three oils. It has a very low absorption at all wavelengths when compared to the other two oils. The latter show much absorption at 233 mu. and this increases greatly after a six hour isomerization. The sunflowerseed oil NAFD has the greatest absorption at both

times.

The remarkable increase in absorption with soyabean and sunflowerseed oils can be explained on the basis of cis-trans isomerization. Paschke, Jackson, and Wheeler (1952) have shown that methyl linoleate on thermal polymerization reacted as follows:-



Of course, a portion must have also interacted to form dimeric and other polymeric material. The cis-trans, non-conjugated isomer comprised a large portion of the monomeric fraction. This compound requires a five to six hour isomerization to reach maximum absorption (i.e. completely conjugated), while the normal cic-cis ester required but twenty to thirty minutes. Therefore a twenty-five isomerization time (Official A.O.C.S.) gives a false result when applied to heated oils. The six hour period is more nearly correct. In Table XLIII the spectral analysis are reported from data obtained by both twenty-five minute and six hour isomerization times. It will be seen that a seriously false impression of composition would be entertained if only the twenty-five minute time had been used. This table shows that both

sunflowerseed and soyabean NAFD contain twenty-five per cent more "linoleate" than the official analysis procedure would have shown. Linseed NAFD would only be three per cent in error.

Weighed samples of sunflowerseed NAFD were alkali-isomerized for varying lengths of time. The absorption at 233 mu., the maximum absorption peak, were noted. From these results the K'_{233} was calculated, and then plotted against time. The graph is shown in Figure 24. This curve is almost identical with that obtained by Jackson, Paschke, Tolberg, et al (1952) when they isomerized cis-trans, non-conjugated linoleate. This further substantiates the claim that much of the NAFD fractions of soyabean and sunflowerseed oils are composed of cis-trans isomers.

No results are available on the transformation of linolenate (cis,cis,cis-9,12,15-octadecatrienoate) on thermal polymerization, but it is probable that a similar cis-trans change would occur and that the non-conjugated, cis-trans isomers formed would require long isomerization times (i.e. six hours). It is strongly suspected, therefore, that a considerable portion of the NAFD fractions of soyabean and sunflowerseed oil is composed of cis-trans compounds. Linseed NAFD contains a much lesser proportion of this type of ester. Of course, there must be other modifications of structure in these esters to prevent adduction, for all positional isomers complex equally well with urea. The point is that somewhere in the alkyl chain of these non-

adducting esters, a cis-trans configuration must exist.

With sunflowerseed and soyabean NAFD it could be that the majority of the unsaturation is contained in these cis-trans isomers. Highly unsaturated compounds do not form urea inclusion compounds. The other esters comprising the NAFD (ca. fifty per cent) must possess some structural modification that prevents adduction. This reasoning cannot apply to the NAFD of linseed oil because only a small percentage is composed of cis-trans isomers (six per cent) and the iodine value is quite high (178). The six per cent could not possibly contain all the unsaturation, or even a major portion of it, and still retain its stability towards peroxidation. The linseed NAFD is comparatively stable towards autoxidation (Wells, 1952). Hence all the linseed esters must contain appreciable unsaturation. The possible structures of the fractions have been tabulated in Table XLIV.

Table XLIII shows that ninety per cent of the linseed NAFD, fifty per cent of the soyabean NAFD, and fifty per cent of the sunflowerseed NAFD is composed of non-conjugable esters. These portions are most probably composed of non-terminal cyclic or branched-chain fatty esters. In the case of linseed oil the ring or branch probably is situated between the double bonds. This would account for the difficulty in conjugating the esters by alkali-isomerization.

Figure 25 shows the absorption spectrum of the ethyl esters of chaulmoogra oil after a twenty-five minute isomerization. This diagram is inserted for comparative purposes.

TABLE XLII

A Comparison of the Chemical Characteristics of the NAFD Fractions from Heated Linseed, Soyabean, and Sunflowerseed Oils

Characteristic	Linseed	Soyabean	Sunflowerseed
Iodine value	178	143	130
Molecular weight	300	294	296
Saponification Eq.	330		
Refractive index at 25°	1.46986	1.47001	1.45671
Solid matter on standing at 6° C. (saturates)	none	none	none
Required time of polymerization for ten per cent yield of NAFD (hours)	12	20	26

TABLE XLIV

Possible Structure of Compounds in the NAFD Fractions of Polymerized Oils

Linseed NAFD	Soyabean NAFD	Sunflowerseed NAFD
Cyclic and/or branched-chain	Cyclic and/or branched-chain	Cyclic and/or branched-chain
Highly unsaturated	Less	Less
Low per cent of cis-trans isomers	25 per cent of cis-trans isomers	25 per cent plus cis-trans isomers
90 per cent non-conjugable	50 per cent non-conjugable	50 per cent non-conjugable

TABLE XLIII

Per cent Constituents in the NAFD Fractions
As Calculated from Spectroscopic Data

Constituent	Linseed	Soyabean	Sunflowerseed
A. 25 minute isomerization			
Conjugated diene	2.7	2.54	3.25
Conjugated triene	---	---	---
"linoleic" acid	2.5	28.8	30.6
"linolenic" acid	3.1	2.8	---
Saturates	2	little	little
B. Six hour isomerization			
Conjugated diene	2.7	2.54	3.25
Conjugated triene	---	---	---
"linoleic" acid	6.0	53.6	59.0
"linolenic" acid	2.5	---	---

Summary

It seems that, in the case of the three vegetable oils studied, a high degree of unsaturation is associated with a high degree of injuriousness. Linseed is the most harmful and sunflowerseed the least harmful. The amount of non-conjugable material in the NAFD fraction increases with the "toxicity" of the fraction. This material, which varies in degree of

unsaturation and highest in linseed and lowest in sunflowerseed, is probably composed of non-terminal, branched-chain or /and cyclic groups attached to the main fatty acyl chain. All three NAFD fractions display a degree of cis-trans isomerization. However, the most injurious one, linseed, has the least amount of such isomeric material, and hence it is reasonable to assume that such compounds have little effect upon the nutritional value of the oils.

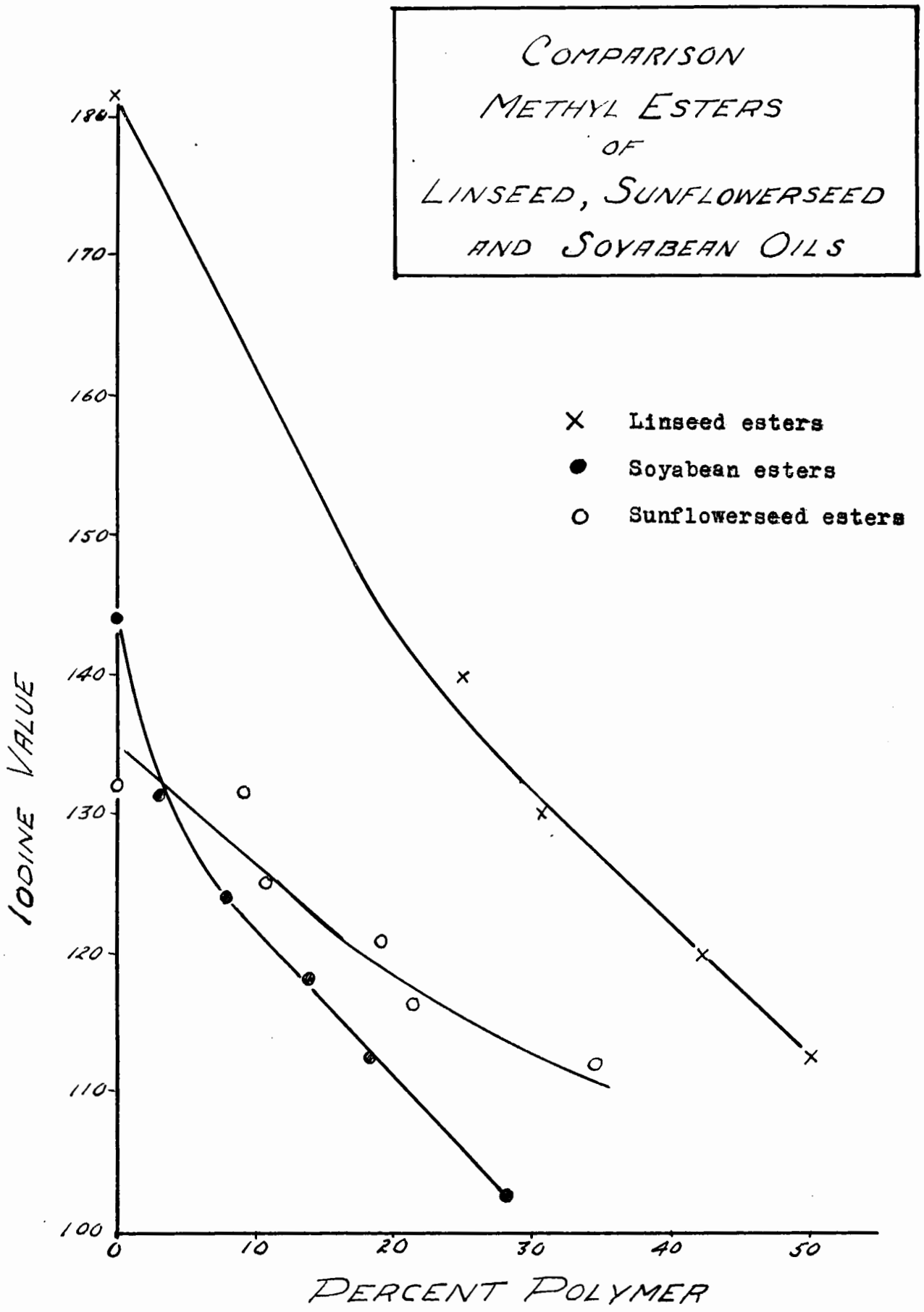

COMPARISON
METHYL ESTERS
OF
LINSEED, SUNFLOWERSEED
AND SOYABEAN OILS
Linseed esters
Soyabean esters
Sunflowerseed esters
IODINE VALUE
180
170
160
150
140
130
120
110
100
0
10
20
30
40
50
PERCENT POLYMER

Figure 19.

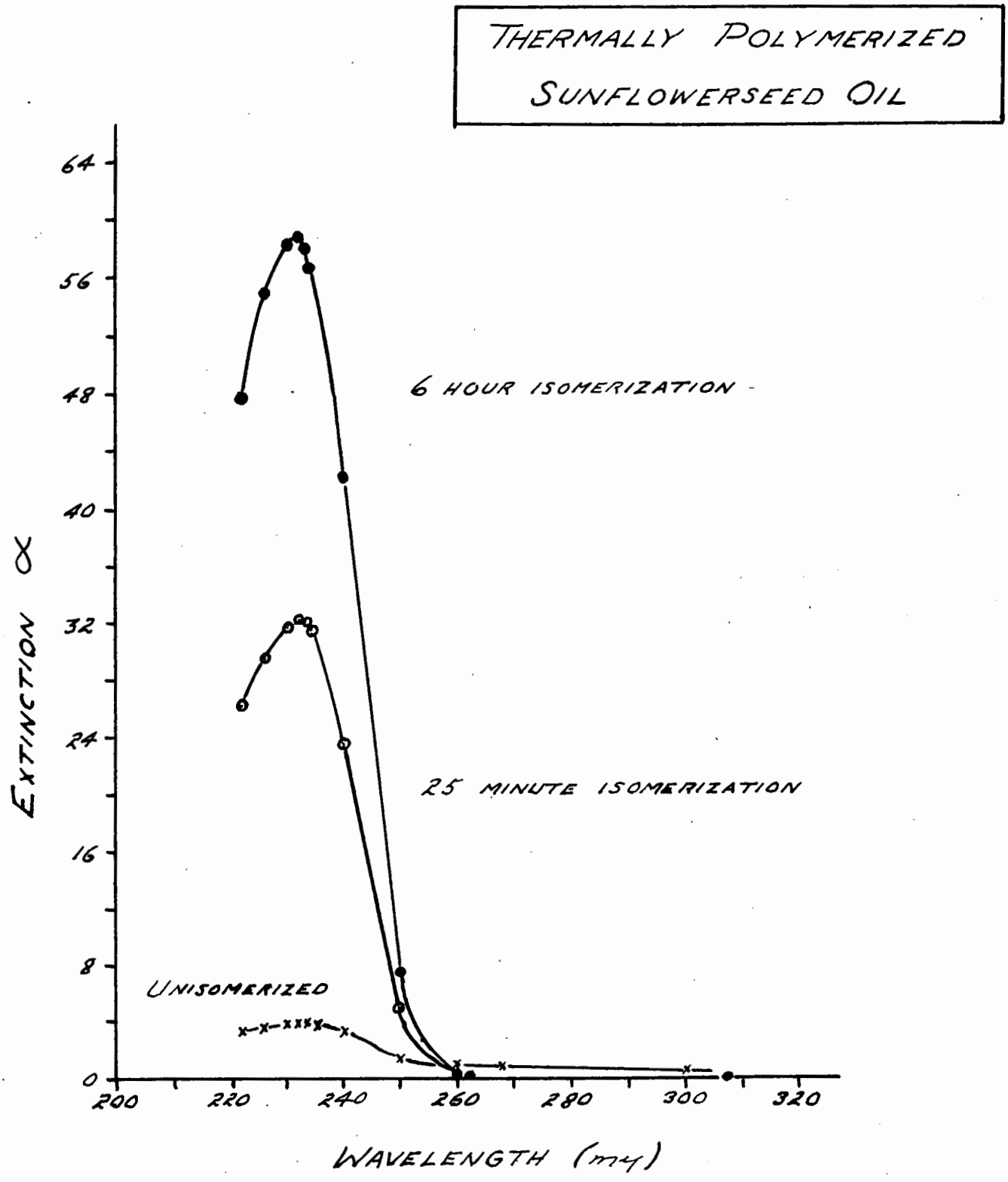


Figure 20. The absorption spectra of the NAFD fraction from thermally polymerized sunflowerseed oil showing the absorption before isomerization, after a 25 minute isomerization, and after a six hour isomerization.

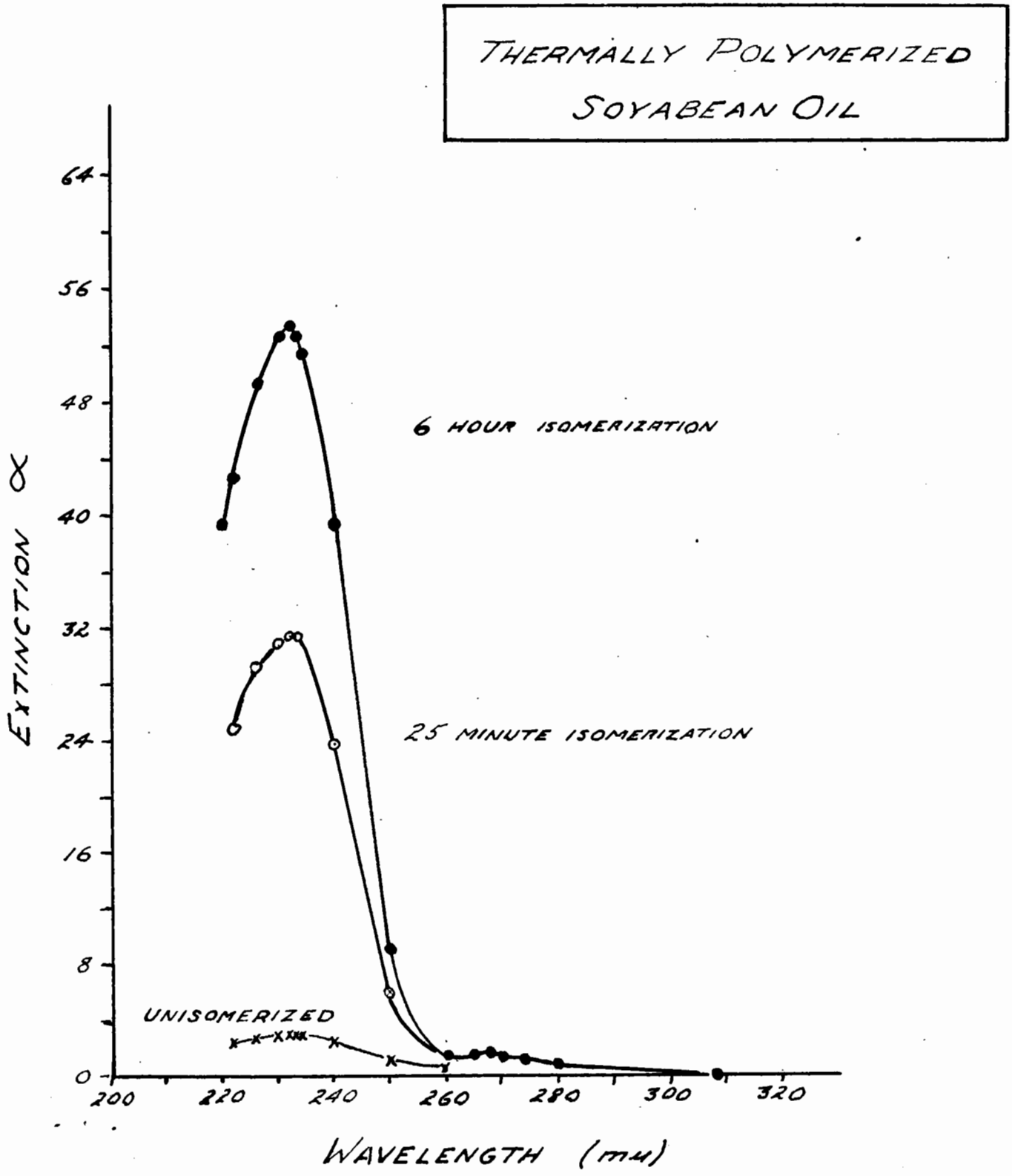


Figure 21. The absorption spectra of the NAFD fraction from thermally polymerized soyabean oil showing the absorption before isomerization, after a 25 minute isomerization, and after a six hour isomerization.

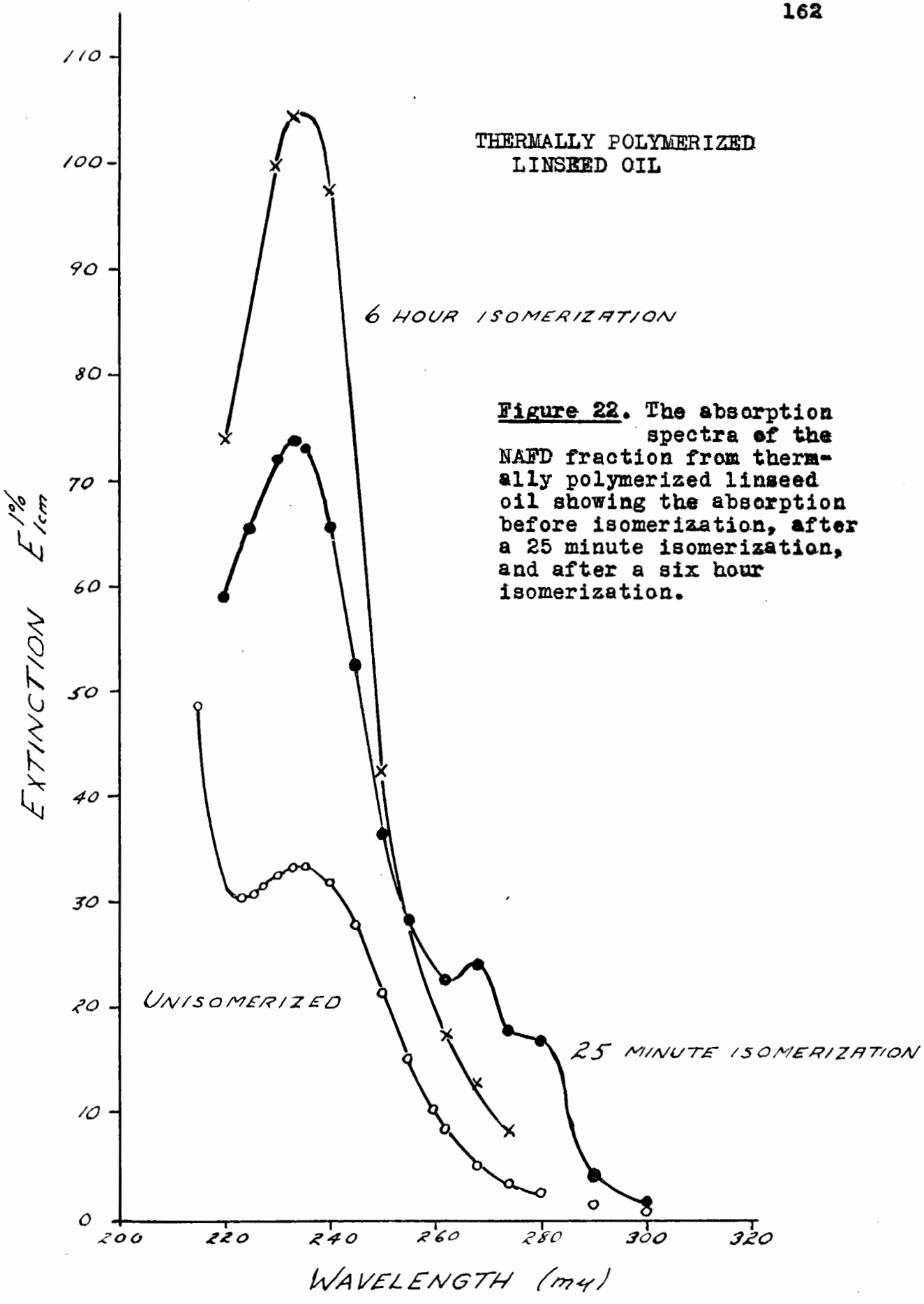


Figure 22. The absorption spectra of the NAFD fraction from thermally polymerized linseed oil showing the absorption before isomerization, after a 25 minute isomerization, and after a six hour isomerization.

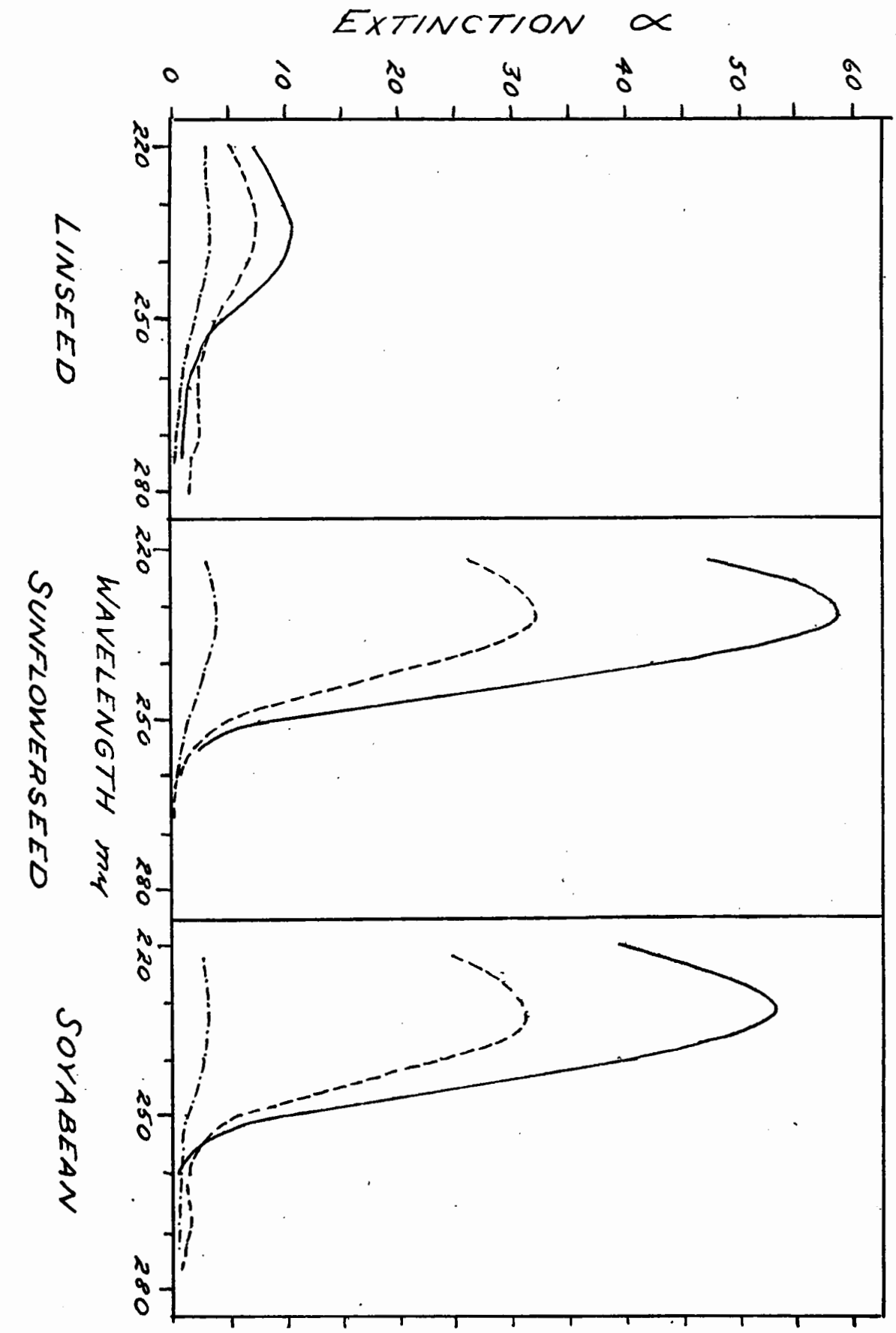

EXTINCTION α
0
10
20
30
40
50
60
220
250
280
WAVELENGTH mμ
LINSEED
SUNFLOWERSEED
SOYABEAN

FIGURE 23

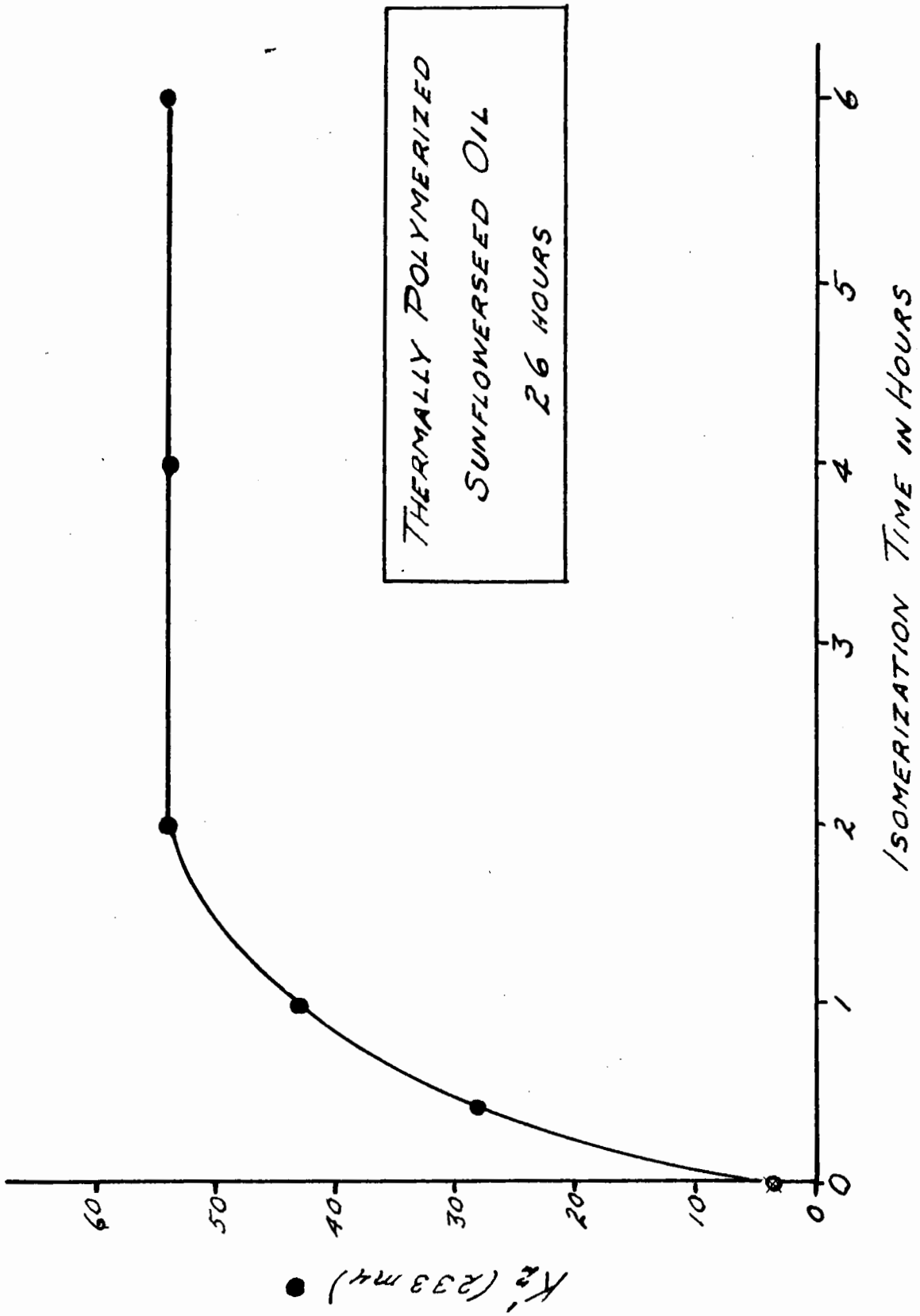

THERMALLY POLYMERIZED
SUNFLOWERSEED OIL
26 HOURS
K_2' (233 mμ)
60
50
40
30
20
10
0
0
1
2
3
4
5
6
ISOMERIZATION TIME IN HOURS

FIGURE 24

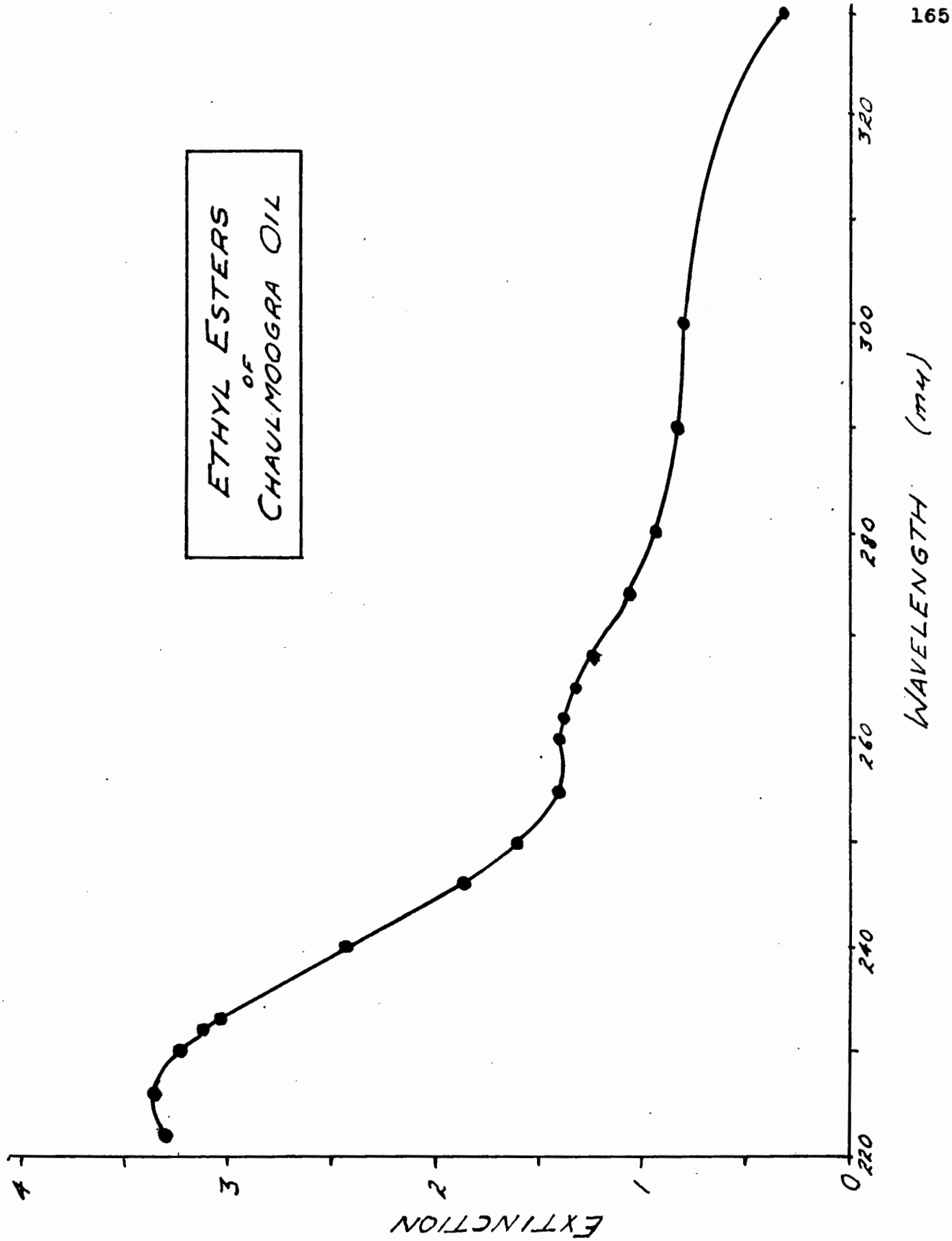

ETHYL ESTERS
OF
CHAULMOOGRA OIL
EXTINCTION
0
1
2
3
4
WAVELENGTH (mμ)
220
240
260
280
300
320

FIGURE 25

25

SECTION VIII

Some Observations on Two Heat-treated Oils of Industry

Introduction

It has been known for some time (B.I.O.S., 1947) that commercial use has been made of thermal polymerization for stabilizing polyunsaturated vegetable oils against flavour reversion. When this laboratory was fortunate enough to receive two samples of industrial oils - a heated herring oil and a heated soyabean oil - it was decided to examine them for the possible presence of NAFD fractions. In the light of the nutritionally deleterious effects of heated soyabean and linseed oil NAFD fractions, two oils that have been studied in this laboratory, it would be of interest to see if these commercial oils contained any material (NAFD) which might possibly be harmful. At this time, the separated fractions have not yet been examined nutritionally.

Both herring and soyabean oils contain appreciable unsaturation, soyabean oil being the most unsaturated (total) although herring oil contains a portion of more highly unsaturated fatty acids (e.g. clupanodonic acid, a pentaenoic acid). This small amount of highly unsaturated matter in the herring oil would, on the basis of previous results, be ideal for the production of a NAFD.

The ethyl esters were made from oils by transesterification and the esters were segregated with urea. The non-

adducting fraction was then distilled *in vacuo* to give the NAFD.

This procedure varied slightly from that usually used in this laboratory in that the ethyl esters were segregated before distillation rather than afterwards. The small amount of material available favoured the adoption of this modification.

Procedure and Results

A sample of 212 gm. of heated herring oil was warmed on the steam bath to 50° C. and the calculated amount of sodium in warm (50°) ethanol was added with stirring. The solution immediately turned an intense ruby red colour from the previous light yellow. The dark red glycerol layer was allowed to settle and then removed in a separatory funnel. The esters were washed free of alkali and dried.

Herring oil esters tended to solidify when chilled. The whole oil exhibited the same tendency and, therefore, had to be warmed before it could be handled for weighing. This is a characteristic property of fish oils because of their large content of totally saturated acids.

The ethyl esters were segregated with urea in the usual manner and gave a non-adduct forming (NAFE) fraction and a adduct forming fraction (AFE). The NAFE was distilled *in vacuo*. The monomeric distillate collected was the NAFD.

Soyabean oil was treated in the same manner.

The percentages of each fraction, based on the ethyl

esters as 100, has been reported in Tables XLVI and XLVII. The chemical characteristics were determined when feasible (i.e. enough material) and have been listed in the same tables.

A portion of the whole heated oil and of the NAFD from herring oil was alkali-isomerized (Official Method, A.O.C.S.) for twenty-five minutes and for six hours. The spectrum of the whole oil increased slightly (not shown) indicating that conjugation was not at a maximum after twenty-five minutes of isomerization. The NAFD, however, had a lower absorption at six hours than at twenty-five minutes (Figure 26). Herb and Riemenschneider (1953) have pointed out that polyunsaturated fatty acids should be alkali-isomerized for fifteen minutes (ten minutes less than usual) with twenty-one per cent potassium hydroxide in glycol (cf. six per cent in Official Method) for best results. Hammond and Lundberg (1953) decreased this time to five minutes. These authors showed that, while dienoic and trienoic acids will show some increase in absorption with a longer heating period, the polyunsaturated acids undergo destruction more rapidly than they conjugate after four or five minutes and the total absorption will decrease. This is applicable to herring NAFD which contains the majority of the unsaturates. Figure 26, therefore, does not show the maximum absorption of the NAFD fraction. The graph is suitable, nevertheless, for comparing the spectra to those of other vegetable oil NAFD fractions.

Discussion

Table XLVII showed that soyabean oil contained only five per cent NAFE. On distillation this yielded negligible distillate (NAFD), hence the amount of NAFD was below one per cent. Unless the effect of NAFD is accummulative, which is doubtful (although the problem has never been examined), the oil is harmless.

The heated herring oil, however, contained approximately twelve per cent NAFD. The iodine value was 220, the highest yet obtained with a NAFD fraction. In Table XLV it is seen that the majority of the unsaturated fatty acids contain carbon chains of twenty or twenty-two atoms, this would mean that the average number of double bonds per molecule would be about two (cf. linseed NAFD, two bonds per molecule). The fact that the fraction is highly unsaturated and does not form urea adducts tends to support the previous contention (Section VII), to wit, that polyunsaturated fatty acids probably form a large part of all NAFD fractions. What other structural modifications are present in the herring NAFD are unknown, although with tetraenoic and pentaenoic fatty acids, a great variety of products could be formed.

Because of the large percentage (12 per cent) of NAFD in heated herring oil, it is thought by the author that this oil should be given a thorough nutritional examination before being allowed on commercial markets in any form.

TABLE XLV

Typical Analysis of Herring Oil[1]

Saturated Fatty Acids		Unsaturated Fatty Acids	
No. carbons	Per cent	No. carbons	Per cent
C_{14}	8	C_{14}	----
C_{16}	15.7	C_{16}	4.6
		C_{18}	22.2
C_{18}	0.2	C_{20}	22
		C_{22}	27.3
		C_{24}	----

1. Hilditch (1947)

TABLE XLVII

Chemical Characteristics of Some Fractions From Heated Soyabean Oil

Fraction	Per cent	Iodine value	$n_D^{25°}$
Whole heated oil	100	124.2 121.0	1.47214
Ethyl esters (AFE)	100	124.3	1.5606
Adduct forming of esters (AFE)	95	----	1.45410
Non-adduct forming of esters (NAFE)	5	----	-----

TABLE XLVI

Chemical Characteristics of Some Fractions From Heated Herring Oil

Oil Fraction	Per cent	Iodine value	$n_D^{25^\circ}$
Heated oil	100	105.5	1.47431
Ethyl esters	100	98.5	1.45732
Adduct forming of esters (AFE)	71	62	1.44847
Non-adduct forming of esters (NAFE)	29	191	-------
Non-adduct forming of distillate (NAFD)	12	220	-------
Residue	17.4	--	-------

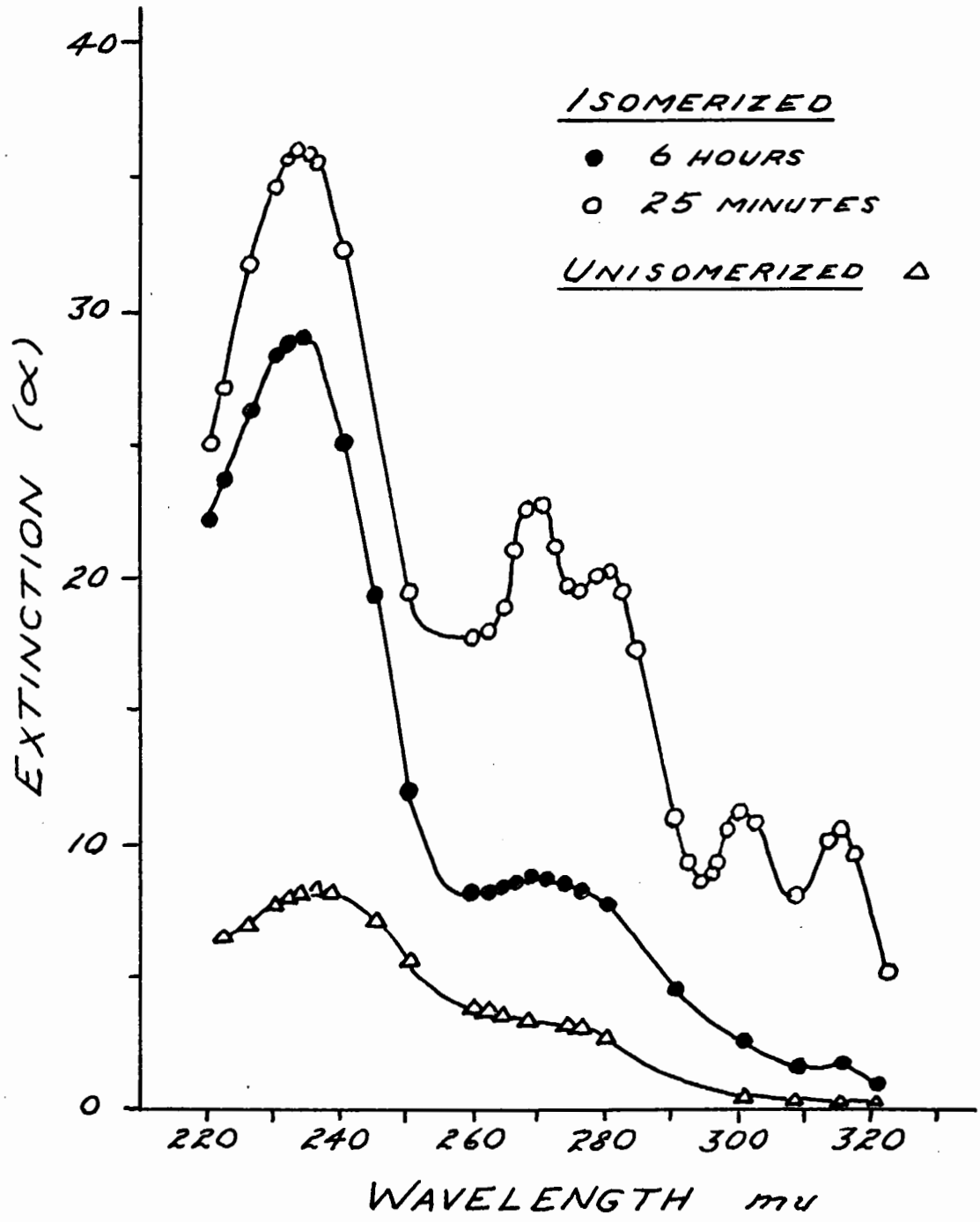


Figure 26. The absorption spectrum of the NAFD fraction from thermally polymerized herring oil before alkali-isomerization, after a 25 minute alkali-isomerization, and after a six hour isomerization.

SECTION IX

Preliminary Studies of Heated Ethyl Esters of Linseed Oil

Introduction

The monomeric portion of linseed oil that has been polymerized for twelve hours yields a non-adduct forming fraction (NAFD). Studies on the structure of this fraction have been reported in Section VI. Only a preliminary investigation of the possible mechanism of formation has so far been reported (Wells, 1952). As a preliminary study on mechanism of NAFD formation, the ethyl esters of linseed oil were polymerized in exactly the same manner as the whole oil. It was of interest to find if the polymerized esters would yield a NAFD. If so, then it can be inferred that the triglyceride structure is not necessarily a prerequisite for NAFD formation. It has previously been shown (Wells, 1952; Section I, Section III) that fatty acid composition of the oils is important to the quantity and quality of the NAFD produced on thermal polymerization. The importance of the triglyceride structure can be gauged by the ethyl ester polymerization.

Procedure and Results

A small batch of alkali-refined linseed oil was transesterified with ethanol to form the ethyl esters. These mixed esters were polymerized at 275° C. for twelve hours under a constant stream of carbon dioxide. The heated esters were

then distilled *in vacuo* to yield a monomeric distillate. The distillate was segregated with urea, and a urea non-adducting and a urea adducting fraction were collected.

Table XLVIII shows the yields of the fractions as per cent of the heated ethyl esters.

The chemical characteristics of the oils are presented in Table XLIX.

In Figure 27 the absorption spectra of the alkali-isomerized and unisomerized NAFD of the heated esters has been contrasted to the NAFD isolated from heated linseed oil. Figure 28 shows the absorption curves of the various ester fractions from the polymerized ethyl esters.

Discussion

The results indicate that the triglyceride structure is not essential for the formation of a NAFD.

In recent years much study has been given to the polymerization of simple (monohydroxy alcohols) esters of fatty acids because experiments with the whole oils yield a very complex mixture of products, and authorities preferred to analyze the relatively simple products of ester polymerization before tackling the more complex triglyceride structure.

Polymerization involves two main steps: (1) thermal conjugation to conjugated diene or triene acids - a slow reaction, and (2) addition of dienophile to the conjugated diene to form a polymeric compound - a fast reaction. Most evidence points to this latter reaction as occurring via a

Diels-Alder type addition, although other mechanisms undoubtedly contribute to polymer formation (Barker, Crawford, and Hilditch, 1951 a). In this present study we are mainly concerned with the monomeric portion of the heated esters and reaction (2) is not too important in monomer formation and transformation, unless, of course, dimers are pyrolyzed to simple monomeric products. An outline of some possible reactions of simple unsaturated fatty acid esters is shown below:-

$N_1 \longrightarrow C_2$

C and/or N $\longrightarrow$ iso-acids with same number of double bonds per molecule

C and/or N $\longrightarrow$ iso-acids with fewer double bonds per molecule

C and/or N $\longrightarrow$ cyclization

C and/or N $\longrightarrow$ pyrolysis to simpler products, with C, perhaps branched

C and/or N $\longrightarrow$ addition of pyrolysis products to molecule to form branches

C + N $\longrightarrow D_3$

N + N $\longrightarrow$ D

C + C $\longrightarrow$ D

D, N, C + D $\longrightarrow$ Polymers

D $\longrightarrow$ Decarboxylation

D $\longrightarrow$ Pyrolysis

---

1. N represents the normal unsaturated fatty acid group
2. C represents the conjugated fatty acid group
3. D represents a dimeric molecule

On polymerization of normal methyl linoleate at 290° to 300° for six to ninety-six hours, Paschke and Wheeler (1949) found that approximately twenty per cent of the monomeric fraction contained non-conjugable material based on a twenty-five minute isomerization with alkaline glycol. An examination of this twenty per cent portion showed that about forty per cent could be hydrogenated to stearate, the remaining sixty per cent could not be identified. The authors thought that it was probably composed of cyclic monomers.

In subsequent work (Paschke, Jackson, and Wheeler, 1952) the non-conjugable portion was isomerized for six hours with alkaline glycol, and forty per cent was found to be composed of cis-trans or trans-trans isomers of linoleate. These isomers require a six hour isomerization to reach maximum absorption, hence would remain undetected in a short twenty-five minute isomerization. There was still ten to fifteen per cent of the <u>total</u> monomer fraction composed of unknown esters - called the "X" fraction by the authors - which were non-conjugable, yet exhibited appreciable iodine values. It is thought by the present author that this material would behave most probably as a NAFD.

The NAFD fraction from polymerized whole ethyl esters of linseed oil constituted about fifteen per cent of the monomeric material (Table XLVIII). This compares favourably with the ten per cent of the "X" component isolated by Paschke <u>et al</u> from polymerized methyl linoleate. Spectral analysis of

the NAFD yielded data from which the amount of conjugable material in the fraction was calculated. It was found to be composed of five per cent thermally conjugated matter, three per cent conjugable matter, and over ninety per cent of non-conjugable esters. The results are based on a twenty-five minute isomerization time. It is almost certain that, in view of the previous results of Paschke, Jackson and Wheeler (1952), a six hour isomerization would show more conjugable material - but at least seventy per cent of the NAFD is probably composed of non-conjugable esters. The structure of this portion could be comparable to the "X" fraction of the methyl linoleate. The NAFD fractions of all the oils studied seem to be characteristically composed of large proportions of non-conjugable material (Section VII).

Figure 28 shows the absorption curves of the various ester fractions from the polymerized ethyl esters of linseed. It will be seen that the spectrum of the AFD postion of the monomer closely resembles that of the unheated esters. This strongly suggests that the AFD contains a majority of unreacted ethyl esters plus some straight-chain isomeric esters of low idone value (i.e. more saturated), while the NAFD, whose absorption spectrum bears no resemblance to that of the unheated esters, contains the thermally transformed esters which would be of an entirely different constitution.

A comparison of the NAFD from the whole heated linseed oil and the NAFD from the polymerized ethyl esters is shown

in Figure 27. The comparison is remarkable. It will be seen that neither fraction shows a great increase in diene conjugation when alkali-isomerized. The increase in both cases can be traces directly to a small amount of "linolenate" which further isomerizes to increase the diene absorption. If the ester NAFD had been isomerized for six hours, it is most probable that the diene absorption would have increased to a limited extent. However, the linseed NAFD (polymerized oil), on six hour isomerization, only increased from three to six per cent, a very small amount. This still left ninety per cent of non-conjugable matter. It is assumed that the ester NAFD would act in a similar manner.

The small amount of difficultly-conjugable matter can be explained on the basis of cis-trans isomerization. The normal cis-cis esters isomerize to cis-trans non-conjugated esters on heating. These cis-trans esters require a four to five hour alkali-isomerization period to reach maximum absorption. It is most proabable that such esters are also non-adducting with urea, hence would form part of the NAFD. It has been shown that esters of this type form a very large portion of the sunflowerseed and soyabean NAFD fractions.

The chemical characteristics of the linseed oil NAFD and the linseed ester NAFD are set out in Table L. Both results are quite similar, it will be noted.

It should be mentioned that Boelhouwer *et al* (1953) in studies on polymerized linseed oil, methyl linoleate, and

methyl linolenate found that the monomeric fractions contained small amounts of cyclic material. These compounds were determined by a method of ring analysis based on the conversions of the esters to hydrocarbons (Vlugter, Waterman, and van Westen, 1935 a, 1935 b). These analytical methods are not absolutely infallible because a mathematical treatment of the physical constants is used rather than classical organic analytical techniques.

Summary

1. The linear ethyl esters of linseed oil will produce a NAFD on thermal polymerization.
2. The percentage of NAFD formed from the ethyl ester polymerization and the whole oil (triglyceride) polymerization is almost the same (ten to twelve per cent).
3. Both NAFD fractions (ester and oil) contain similar amounts of non-conjugable material of high iodine value.
4. Both NAFD fractions contain a small amount of difficultly-conjugable esters. These are most probably geometrical isomers of linoleate and linolenate.
5. The results with the heated ethyl esters of the whole linseed oil agree well with previous results of other authors on simpler esters.

TABLE XLVIII

Composition of the Heated Ethyl Esters of Linseed Oil[1]

Distillable esters			Polymeric residue
Total	AFD	NAFD	
95	83	12	5

1. Results expressed as a percentage of heated esters

TABLE XLIX

Characteristics of the Heated Ethyl Esters of Linseed Oil

Fraction	$n_D^{25°}$	Mean molecular weight (cryoscopic)	Iodine value
Whole oil	--	--	184
Unheated ethyl esters	1.46124	339	195
Heated ethyl esters	1.46790	379	141
Distillable esters	1.45837	300	139
Adduct forming esters of distillate (AFD)	1.45508	313	116
Non-adduct forming esters of distillate (NAFD)	1.46975	346	154

TABLE L

Comparison of the Characteristics of the NAFD Fraction of Polymerized Linseed Ethyl Esters to the NAFD from Polymerized Linseed Oil

Characteristic	Linseed ethyl esters	Whole linseed oil
Non-conjugable material	ca. 80 %	ca. 80 %
Small amount of difficultly-isomerizable material	yes	yes
Thermally conjugated esters (before isomerization)	large	large
Spectrum	Both fractions exhibit similar spectra before and after the isomerization	
Iodine value	154	170
Molecular weight	346	300
$n_D^{25^o}$	1.46975	1.46986
Per cent of the total esters	12	12
Number of double bonds per molecule, calculated from I.V. and Mol. Wt.	-2	2
Conjugated diene %	4.37	2.7 (2.7)[1]
"linoleic" acid %	---	2.5 (6.0)
"linolenic" acid %	3.47	3.1 (2.5)

1. ( ) indicates a six hour isomerization time.

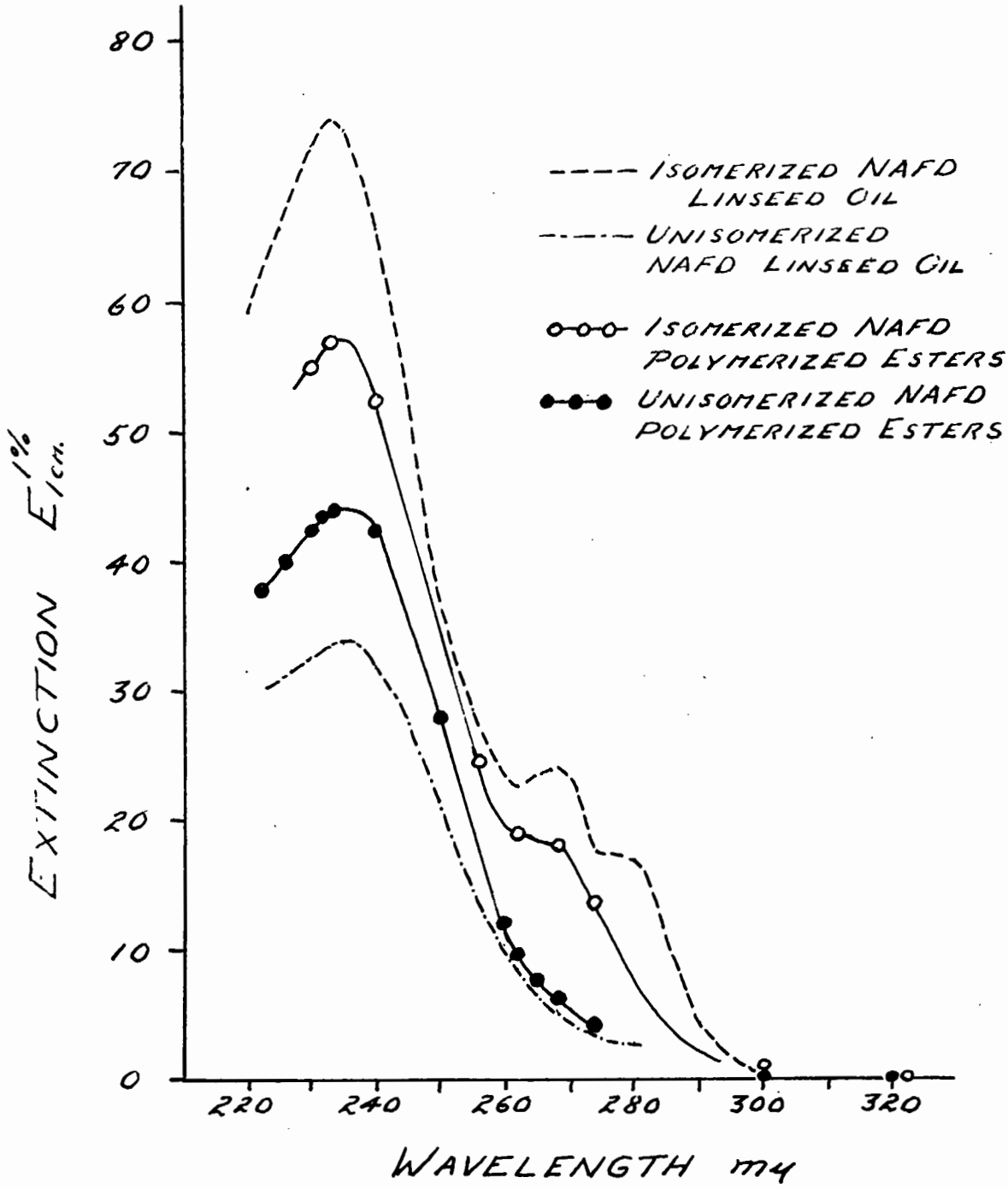


Figure 27. Contrast of the absorption spectra of the NAFD fractions from polymerized linseed oil and polymerized linseed oil ethyl esters.

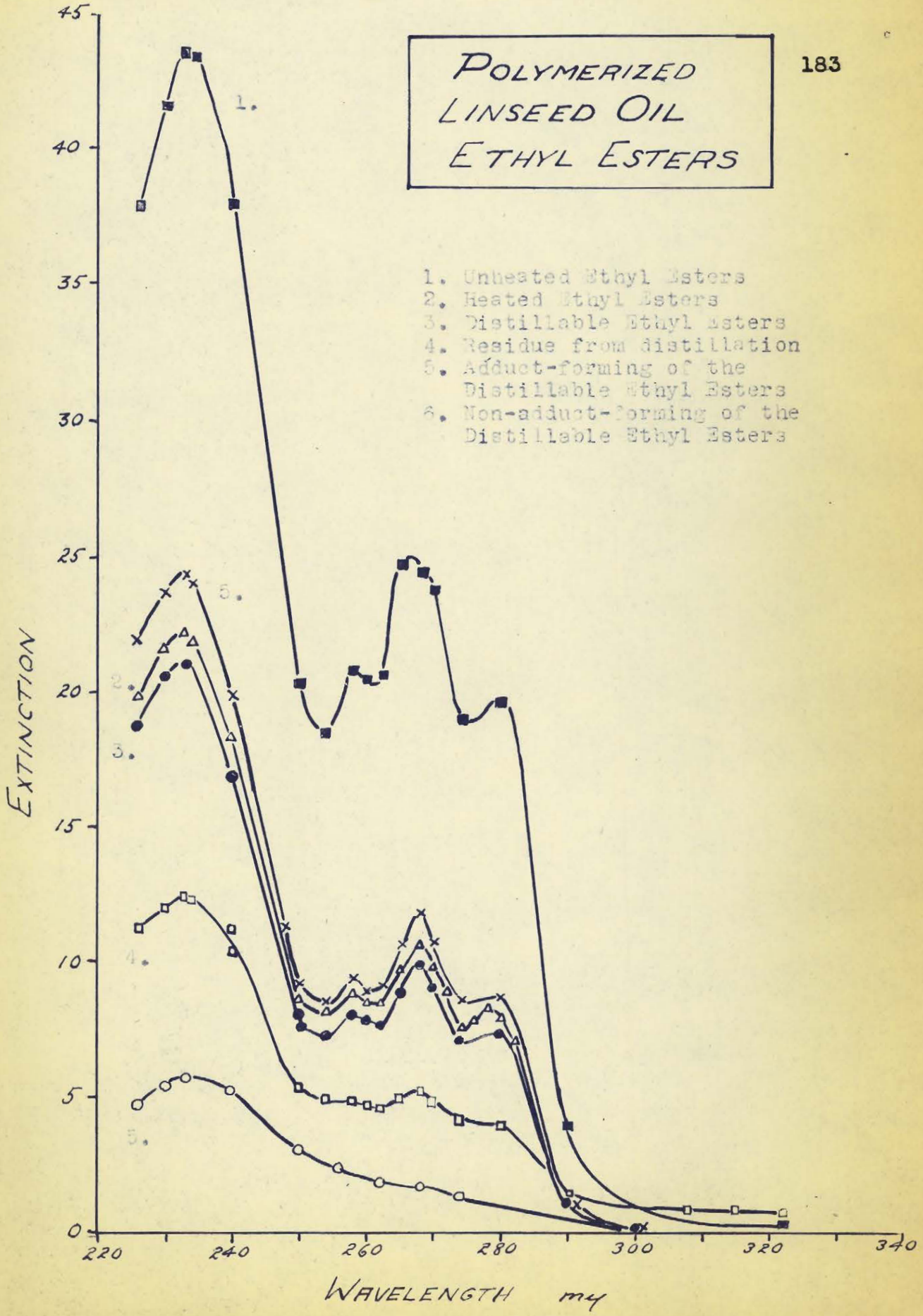


Figure 28. Absorption spectra of the various fractions from thermally polymerized ethyl esters of linseed oil.

GENERAL DISCUSSION

The purposes of these researches have been twofold: firstly to investigate the character and composition of the non-adduct forming (NAFD) fraction of the distillate from heated linseed oil, and, secondly, to examine other heated vegetable oils for the presence of injurious material similar to that found in heated linseed.

The examination of the linseed NAFD has shown that:-

(a) Branched-chain acids were present in the NAFD because isocaproic acid had been isolated from oxidatively cleaved NAFD.

(b) The six hour alkali-isomerization illustrated that the fraction contained about three per cent of difficultly-conjugable material, probably cis-trans isomers. Ninety per cent was composed of non-conjugable esters.

(c) The fraction contained the majority of the unsaturated material as shown by the high iodine value (170-180).

(d) The percentage of saturates was negligible (less than two per cent) as evidenced by the failure to form insoluble lead salts and by two different direct determinations of unsaturated material.

(e) The non-homogeneity of the fraction has been proven by lithium salt separation which divided the NAFD into thirty-three per cent insolubles and sixty-six per cent solubles.

(f) The fact that the linseed oil ethyl esters, on polymerization, formed a NAFD fraction showed that triglyceride structure was not essential for the formation of a NAFD in linseed oil.

(g) Comparative studies with soyabean and sunflowerseed oils indicated that the presence of polyunsaturated fatty acids favoured formation of a NAFD. The greater the concentration of such acids, the shorter was the heating time required to produce a NAFD. Studies with a marine oil (herring), containing tetraenoic and pentaenoic acids, showed that the acids are concentrated in the NAFD. Nutritional studies have shown that the injuriousness of a heated vegetable oil parallels the linolenic acid concentration.

(h) The fraction was composed of very soluble fatty acyl groups as evidenced by its refusal to crystallize from solution at low temperature.

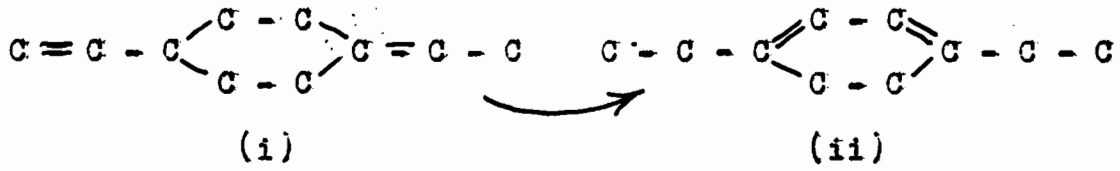
These are the facts. Recent review articles have pointed out the non-injurious nature of the geometrical and positional isomers of normal unsaturated fatty acids. These acids, whose presence in the NAFD is certain, cannot account for the deleterious effect of the material. Therefore, only three types of fatty acids can be considered: cyclic, branched-chain, and polyunsaturated. All these three types have been proven toxic to experimental animals.

Polyunsaturated fatty acids have proven toxic to rats and calves and they also only form urea adducts with difficulty. The iodine value of the NAFD is quite high, indicating dienoic esters (average). But two factors mitigate against polyunsaturates as being the major toxic factor. The iodine value is not extremely high, although a portion of the NAFD may be highly unsaturated, this would mean that another portion would have to have little unsaturation. Secondly, if straight-chain polyunsaturates were present, then a much higher absorption spectrum would be expected both before and after alkali-isomerization (cf. herring oil). In point of fact, the linseed NAFD spectra are less than that of either the sunflowerseed or soyabean oil NAFD fractions, and very much less than that of herring oil. Hence polyunsaturation has not been seriously considered as the major lethal factor.

Cyclic fatty acids are known to be harmful, both when administered orally and when injected subcutaneously or intraperitoneally. Most will not form urea adducts. If cyclic acids are present they must, of necessity, contain some unsaturation. The location of the double bonds must be such that they cannot conjugate, for the majority of the NAFD is composed of non-conjugable material. This would mean that the double bonds would have to be on opposite sides of a non-terminal ring. This structure would give a dibasic acid with a ring in the alkyl chain on oxidation. If a bond were

in the ring, oxidative cleavage would have given a tribasic or tetrabasic acid, and none were found among the oxidized fragments.

It is not known with certainty how well a ring would prevent conjugation occurring on alkali-isomerization. Laughton (1954) considers that the presence of a non-terminal cyclohexane ring would not prevent conjugation, albeit it might be slow. He thinks that, if the bonds were on opposite sides of the ring, conjugation would finalize in the ring thusly -



(i) (ii)

Such a structure (ii) would lead to greater substitution about the pi-bonds and stabilizes the configuration. Such a structure might exhibit a different absorption than that of normal conjugated fatty acids.

A branched-chain acid, isocaproic, has been found among the oxidative products of the NAFD fraction. It is not likely that this results from rupture of a cyclic structure during permanganate oxidation as the conditions of oxidation were quite mild. Hence it is fairly certain that some of the fatty acids, perhaps the majority, contain alkyl side chains. Laughton (1954) has stated that alkyl branched unsaturated compounds will not isomerize as readily as normal straight-chain compounds. It is thought that double bonds on opposite sides of an alkyl branch would not conjugate. If this is so,

then the ninety per cent of the esters of the NAFD, which will not conjugate, could be rationalized. Laughton, however, was merely applying theoretical knowledge to the problem, no experimental evidence esists, as far as is known, concerning alkali-isomerization reactions with branched-chain (or cyclic) fatty acids. The position of the carboxyl group would also influence bond shifts in the alkyl chain.

It is known that branched-chain saturated fatty acids melt at a much lower temperature than the normal acids of corresponding carbon content. It is therefore almost certain that the solubilities of such branched-chain acids in most solvents will be much greater than that of corresponding normal acids, and similarly one would expect the lithium and lead salts to be relatively soluble in acetone, alcohol, etc. (Hilditch, 1954). The cyclic compounds are not nearly so soluble (cf. chaulmoogric acid) hence, the experimental evidence suggests branched-chain fatty acids as being the major constituents of the NAFD fraction.

The presence of deleterious material has been discovered in soyabean oil. This was to be expected because of the appreciable concentration of trienoic acid in the oil. Sunflowerseed oil, predominately dienoic in character, produced a NAFD after a long thermal polymerization period. However, the NAFD so-produced had only slightly impaired nutritional value. The presence of polyunsaturated fatty acids, therefore, must be essential for production of harmful material in the

heated oils studied. Herring oil, which contains a fair amount of unsaturated fatty acids, has been shown to contain a NAFD fraction. Whether or not this fraction is toxic has not yet been established.

In view of the foregoing facts it is rather doubtful if thermal polymerization is a satisfactory means of reducing flavour reversion unless a more selective polymerization process can be developed or urea segregation employed to separate the nutritious straight-chain monomers (AFD) from the toxic non-adducting monomers (NAFD). This latter process, however, might prove too expensive for widespread commercial adoption.

SUMMARY

1. The toxic fraction (NAFD) from the distillable ethyl esters of heated linseed oil has been investigated. It was found to contain little saturated material, some cis-trans isomers (about three per cent), and a main portion composed of non-conjugable material. This latter portion has been shown to contain unsaturated branched-chain fatty acids.
2. Subcutaneous injections of the NAFD esters from heated linseed oil produced no detectable physiological effects.
3. A urea non-adducting fraction (NAFD) from the distillable ethyl esters of heated sunflowerseed oil has been isolated. Nutritional tests have shown that the heating only slightly impaired its nutritive value.
4. A urea non-adducting fraction (NAFD) from the distillable ethyl esters of heated soyabean oil has been isolated. Nutritional tests have shown that this material is intermediate between sunflowerseed and linseed oil NAFD fractions in nutritive value.
5. A comparative study has been made of the NAFD fractions from heated sunflowerseed, soyabean, and linseed oils.
6. Two commercially polymerized oils, a soyabean oil and a herring oil, have been examined for NAFD content. A NAFD fraction was obtained from the heated herring oil. This fraction has not yet been examined nutritionally.

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APPENDIX

Chromatographic Solvents

Solvent I

Composition: Ether ..... 13 parts (vol)
Acetic acid ... 3 parts
Water ..... 1 part

Application: Forms a homogeneous mixture which is generally used in ascending chromatography.

Reference: Denison and Phares (1952)

Solvent II

Composition: Phenol ..... 75 % by volume
Water ..... 25 % by volume
Formic acid .. 1 % of total

Application: Forms a homogeneous mixture which has been exclusively employed in ascending chromatography. In two dimensional work this forms the second solvent- to difficult to remove to be used for first dimension.

Solvent III

Composition: Benzyl alcohol ..... 5 ml.
n-butanol ..... 5 ml.
water ..... 1 ml.
Formic acid ..... 1 % total

Application: Homogeneous mixture that is used in ascending chromatography.

Reference: Denison and Phares (1952)

Solvent IV

Composition: n-butanol ..... 100 volumes
water ..... 15 volumes
diethylamine .... 1 volume

Application: Homogeneous solvent that has been used in ascending and in descending chromatography.

Reference: Jones, Dowling, & Skraba (1953).

Solvent V

Composition: Isobutanol saturated with 1.5 N ammonia.

Application: The upper organic phase is used to run the chromatograms in, the aqueous lower layer is used to place in cabinet to saturate the atmosphere with ammonia.

Reference: Reid and Lederer (1951).

Solvent VI

Composition: n-butanol .... saturated with water

Application: The butanol saturated with water is called VIa, the water saturated with butanol is called VIb. Two phase system.

Reference: Inouye and Noda (1951 c).

Solvent VII

Composition: n-pentanol is saturated with a 50 % v/v solution of ammonia-water.

Application: Use organic phase.

Reference: Inouye and Noda, ibid.

Solvent VIII

Composition: Ethanol ..... 1 part by volume
Water ..... ½ part by volume

Application: homogeneous solvent.

Reference: Inouye and Noda, ibid.

Solvent IX

Composition: Benzene ..... 1 part by volume
Butanol (n) .. 1 part by volume
Saturate the mixture with water

Application: Use the organic phase.

Reference: Inouye and Noda (1952).

Solvent X

Composition: Chlororform ..... 1 part
Ether ..... 1 part

Application: Homogeneous solvent

Solvent XI

Composition: n-propanol ..... 9 parts
water ..... 2 parts

Application: Homogenous solvent

Solvent XII

Composition: Isobutanol ..... 80 parts
Ammonia ..... 15 parts
Glycol ..... 5 parts

Application: Homogeneous solvent used in both ascending and descending work, especially with dibasic acids.

Reference: Zahn and Wolf (1951)

Solvent XIII

Composition: n-propanol ..... 50 volumes
Water ..... 12 volumes
Ammonia ..... 12 volumes

Application: Homogeneous solvent

Reference: Isherwood and Hanes (1953)

Solvent XIV
Composition: Ethanol ..... 25 parts
Ammonia ..... 2 parts
Application: Homogeneous solvent
Reference: Kennedy and Barker (1951)

Solvent XV
Composition: Isobutanol ..... 80 volumes
Ammonia ..... 10 volumes
Glycerine ..... 10 volumes
Application: Homogeneous solvent

Solvent XVI
Composition: Isobutanol ..... 80 volumes
Monoethanolamine 10 volumes
Ammonia ..... 10 volumes
Application : homogeneous solvent

Solvent XVII
Composition: Isobutanol ..... 20 volumes
Ammonia ..... 4 volumes
Glycol ..... 10 volumes
Application: Homogeneous solvent

Solvent XVIII
Composition: 70/30 methanol:water mixture saturated with decalin.
Application: the lower aqueous methanol portion is used.

Solvent XIX
Composition: Isobutanol ..... 24 volumes
Ammonia ..... 6 volumes
Glycol ..... 3 volumes
Application: Homogeneous solvent.

Solvent XX
Composition: Propanol ..... 60 volumes
Ammonia ..... 40 volumes
Application: Homogeneous solvent
Reference: Isherwood and Hanes (1953)

Solvent XXI
Composition: Isobutanol ..... 80 volumes
Ammonia ..... 10 volumes
Glycol ..... 10 volumes
Application: Homogeneous solvent

Solvent XXII
Composition: Isobutanol ..... 70 volumes
Ammonia ..... 20 volumes
Glycol ..... 10 volumes
Application: Homogeneous solvent