THE UNSAPONIFIABLE FRACTION OF ALFALFA SEED OIL

Thesis for the Degree of M. S.
MICHIGAN STATE COLLEGE

Carroll King

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A Thesis

Submitted to the Faculty of

Michigan State College of Agriculture

and Applied Science in Partial

Fulfillment of the Requirements

for the Degree of Master of Science

by

Carroll King

June 1938

The writer wishes to thank Professor C. D. Ball for his cooperation, his consideration, and for his straightforward criticism.

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Introduction

In the literature it is customary to refer to that portion of a biological material which maybe extracted with petroleum ether, ethyl ether, or other similar solvents as a fat or as an oil. The term alfalfa seed oil as it is used in this thesis means that portion of alfalfa seed which can be extracted by ethyl ether.

The unsaponifiable fraction of a fat or oil may be defined as that portion which can be extracted from the saponified material by a suitable fat solvent. In the unsaponifiable fraction, one finds a complex mixture of chemical compounds, many of which have a physiological effect of some sort. The discovery of these biologically active compounds in the unsaponifiable fraction has had a stimulating effect on the chemical investigation of this fraction in various oils. As a result, new and interesting compounds are continually being discovered.

The work presented in this thesis deals with the unsaponifiable fraction of alfalfa seed oil (Medicago sativa, Hardigan variety). Apparently, there is no published account of any previous investigation of this fraction of the alfalfa seed.

Historical

The ether extract of the leaves and stems of the alfalfa plant, and particularly the unsaponifiable fraction of this extract, has been the subject of considerable interest to investigators in the past. At first, alfalfa was studied to provide some sort of an idea of its chemical composition as a feed. Later, the discovery of biologically active principals in the ether extract caused interest to be focused on this portion. Alfalfa has been much studied in connection with vitamin problems.

Étard and Moissan (1) in 1892 isolated from the carbon disulfide extract of dried alfalfa leaves an alcohol of the formula $C_{20}H_{41}OH$. They called this material "Medicagol". In 1911, Jacobson (2), of the Nevada Experiment Station, began an extensive study of the chemical composition of the alfalfa plant. From alfalfa meal (ground alfalfa plants) he isolated myristone, a ketone of formula $(C_{13}H_{27})_2CO$. In 1912 (3), while continuing the same investigation, he isolated a new ketone of formula $C_{21}H_{42}O$ to which he gave the name "Alfalfone". In 1913, Jacobson (4) discovered a saponin in the alcohol extract of alfalfa meal. He assigned to this material the empirical formula $C_{27}H_{37}O_{16}N$.

In 1919, Osborne and Mendel (5) noticed that alfalfa leaves in the diet would provide enough fat soluble vitamin to promote growth in young albino rats. In 1920, Steenbock and Gross (6) made a similar observation, using alfalfa meal as the dietary material. Later, in 1920, Steenbock and Boutwell (7) found that this growth promoting factor could be extracted from alfalfa meal with some of the common fat solvents. They also observed that the factor resisted saponification. In 1924, Steenbock, Nelson, and Black (8), by feeding alfalfa cured in the sunlight and in the dark, demonstrated the presence of two different vitamin-like activities; that is, a factor corresponding to Vitamin A and an antiricketic factor.

Almquist (9) in 1936 observed that alfalfa meal was a potent source of the fat soluble vitamin required by chicks to prevent hemorrhagic disease. He was able to concentrate this factor by chilling out fats and sterols. Later in the same year (10), he attained increased concentration by a vacuum distillation of the oily concentrate. In 1937 (11) he was able to separate a solid from the oily concentrate, which was even more potent as a source of the vitamin.

In 1916, Jacobson and Holmes (12) made and examination of the oil obtained from alfalfa seed (Medicago sativa L.) by a gasoline extraction. They determined all constants ordinarily used to characterize an oil and, in addition, identified the fatty acids which go to make up alfalfa seed oil. Some of the constants which they determined are reproduced in Table I.

In 1916, Gardner (13), while examining substances which might be used to furnish a drying oil for the manufacture of white paint, extracted alfalfa seed. He obtained 11.2% of a clear yellow oil having an iodine number of 165. Because of the low percentage of oil and of the expense of the seed, he did not push this investigation further.

Abbott (14) in 1929 published preliminary findings, indicating that germinated, ungerminated, and ten-day-etiolated alfalfa seeds were effective in protecting albino rats against xerophthalmia.

TABLE I

Constants for Alfalfa Seed Oil
as Given by
Jacobson and Holmes (12)

Index of Refraction	1.4783
Density	0.9117
Saponification Number	172.3
Iodine Number	154.2
Acid Number	2.85
Acetyl Value	19.8
Reichert-Meissl Number	0.40
Unsaponifiable Fraction	4.40%

Preparation of the Ether Extract

Certified alfalfa seed, Medicago sativa, Hardigan variety, was obtained from the Michigan State Farm Bureau. The seed used had the following analysis, according to the report of the "Michigan Crop Improvement Association":

Purity-------------------------99.7%

Inert Material-----------------0.18%

Foreign Seed-------------------0.012% including weed seed, non noxious.

The seed was ground twice, using a Hobart wheat-grinding mill set at the finest possible adjustment. This operation produced a fine, evenly ground mixture with no trace of any unground seeds.

The finely ground material, as it came from the Mill, was placed in a continuous extraction apparatus and was extracted with dry ethyl ether.

The extractor used was an adaptation of that described in Organic Synthesis by Gilman (15). It consisted of a five liter round bottom flask, a fourteen inch funnel, and a twelve liter round bottom flask. These were arranged in such a way that ether vapor produced by heating the solvent in the five liter flask passed up through the funnel and was condensed on the bottom of the twelve liter flask. A charge of alfalfa seed, usually about six hundred grams, was placed on a large filter paper fitted into the funnel. The ether, condensed on the large flask, extracted the seed as it returned to the heater flask. To prevent channeling, a perforated filter paper was placed on top of the charge of seed. This seemed to distribute the condensed ether evenly.

A sample of seed extracted for sixteen hours gave eleven and eight tenths percent oil. A second sample extracted for twenty hours gave a similar amount. To insure complete extraction of oil, all subsequent batches of seed were extracted for twenty hours.

After extraction was complete, the solution was removed from the flask and was placed in a distillation apparatus. The solvent was removed by distilling under an atmosphere of carbon dioxide. A water bath kept at a temperature of less than fifty degrees was used to heat the distillation flask.

Observation showed that the last traces of solvent could be removed only on prolonged heating. To avoid this heating, the material was placed in a vacuum oven maintained at room temperature and kept there for about eight hours. At the end of this period, the oil assumed a constant index of refraction which was taken to mean a constant composition. Data pertinent to this observation are shown in Table II.

TABLE II

Variation of Index of Refraction

with

Various Treatments of the Oil

Treatment	Index of Refraction 15°
1 hour heating at 50°	1.4768
2 hours in vacuum oven	1.4779
3 hours in vacuum oven	1.4779
8 hours in vacuum oven	1.4820
Over night in vacuum oven	1.4820

The oil obtained from alfalfa seed had a clear yellow color. After standing at a low temperature for about a week, a quantity of white, apparently amorphous substance separated. No attempt was made to characterize this material.

In order to get some idea of how the oil obtained from the Hardigan variety of alfalfa seed compared with that extracted by Jacobson from the seed of Medicago sative L., some of the ordinary fat constants were determined. In the determination of fat constants, the whole alfalfa seed oil was used, including that part which separated upon standing. Specific methods for the determinations were taken from the section on fat and oil analysis in the Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists (16). References for procedures were as follows:

1. Index of Refraction, by means of the Abbe Refractometer (Official) page 406.
2. Saponification number or Koettstorfer's number (Official). pages 412-413
3. Iodine number. Hanus method. (Official) pages 410-412.
4. Saturated and unsaturated acids by the Lead-Salt-Ether method (Official) pages 415-417. The separation of the saturated and unsaturated

acids by this method was somewhat incomplete. The values abtained were corrected according to the above method by utilizing the iodine number of the saturated acids obtained.

5. The unsaponifiable matter was determined by the F. A. C. method. (Official) page 420.

The constants obtained for alfalfa seed oil, using the methods outlined above, are given in Table III.

Preparation of the Unsaponifiable Material

The alfalfa seed oil was saponified by treating it in one hundred gram portion with six hundred milliliters of ninety-five percent ethyl alcohol and one hundred milliliters of fity percent aqueous potassium hydroxide. The resulting mixture was shaken vigorously and then was placed under a reflux condenser to boil for one hour.

The saponified material obtained as above was cooled, placed in a two liter separatory funnel, and extracted eight to ten times with petroleum ether. The resulting extracts were reduced to a volume of about two liters and then were washed with ten percent alcohol-water solution until the washings were free from potassium hydroxide; e.g., until the washings were neutral to phenolphthalein.

The solvent was then distilled from the solution until the volume was about fity milliliters. This

TABLE III

Constants of Alfalfa Seed Oil

	Sample I	Sample II	Sample III	Sample IV	Average
Index of Refraction	1.4820	1.4820	1.4820	1.4820	1.4820
Saponification Number	181.1	180.8			180.9
Iodine Number	156.2	156.1	156.4	155.7	156.1
Saturated Acids (percent)	11.0	10.1			10.55
Unsaturated Acids (percent)	89.0	89.0			89.45
Total Insoluble Acids (percent)	83.72	83.09			83.4
Unsaponifiable Fraction (percent)	4.07	3.95			4.01

concentrated petroleum ether solution of the unsaponifiable matter was transferred to a two hundred milliliter erlenmeyer flask and left to stand at room temperature.

Fractionation of the Unsaponifiable Material

After the petroleum ether solution of the unsaponifiable fraction had stood several hours, fine needle-like crystals began to appear. The amount of crystalline material increased each day, and in a week's time it seemed to occupy the entire solution. The material was then centrifuged and the crystalline portion separated. This material was recrystallized once from petroleum ether and twice from hot absolute alcohol. It melted sharply at 164.4°-164.8°, appeared to be homogeneous when examined under the microscope, and in general behaved like a pure organic compound. For reference purposes, these **crystals** will be designated as fraction I. A further characterization of this material will be given later.

A systematic fractionation of crystalline material which could be separated from the mother liquor of fraction I at various volumes and at various temperatures resulted in a large number of fractions of slightly different melting points. From the trend of these melting point data, it seemed that the fractions obtained were mixtures of a few compounds. Accordingly, a second fractionation was made; this time the procedure was adapted to bring out, if possible, more definite chemical compounds. The procedure used in this second fractionation is definitely imperfect; however, it does bring out a few fractions which seem to be definite compounds rather than mixtures.

In the second fractionation, the isolation of fraction I was carried out in the same manner as indicated above. For the remainder of the separation, one is referred to the flow sheet, figure I.

The material was separated from petroleum ether under four sets of conditions. Each of these was designated as a fraction; e.g., fractionI is the first material which separated, fraction II is the second, etc. In some cases, the main fractions were subdivided. When this occurred, respective divisions were indicated as fraction Ia or fraction Ib, etc. Each fraction obtained is listed on the flow sheet, permitting one to see at a glance the method used in its separation.

All fractions were purified by recrystallization from hot anhydrous ethyl alcohol until a constant melting point was obtained. In each case, after the material had been dissolved in the boiling solvent, it was set aside, and the crystalline material given time to grow. At least twenty-four hours elapsed between each recrystallization.

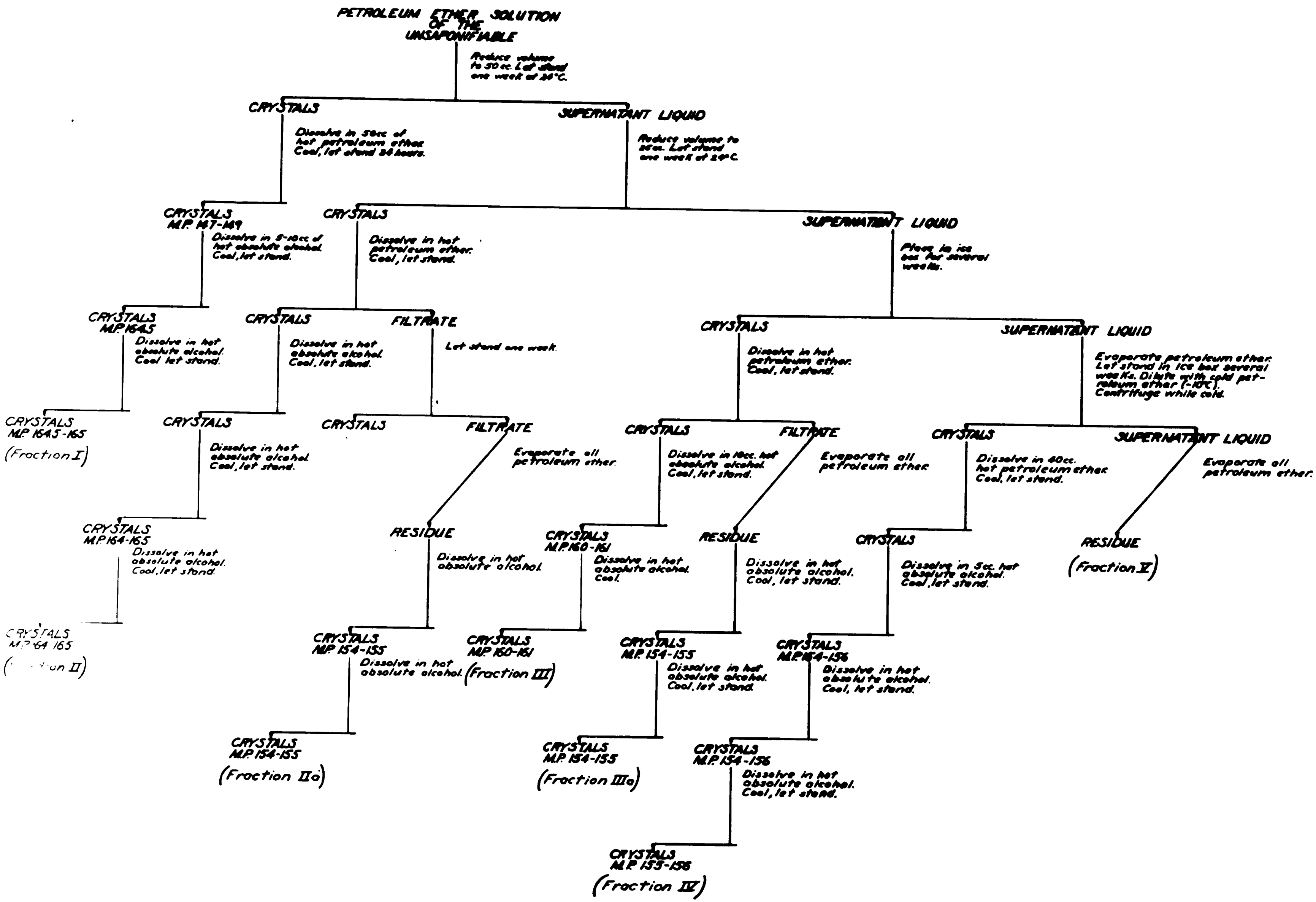


FIGURE I

Procedure Used in the Fractionation of the Unsaponifiable of Alfalfa Seed Oil

Examination of Fractions

Time did not permit detailed examination of all the fractions obtained. At present, only the first fraction has been characterized to any extent.

Fraction I was a homogeneous crystalline material. A microphotograph showing some of the characteristics of the crystals is shown in figure 2. A qualitative analysis following the procedure of Fisher (17), pages 48-52, showed carbon and hydrogen but no nitrogen, sulfur, or halogens.

The carbon and hydrogen content of fraction I was determined quantitatively according to the method of Pregl (18). The results are given in Table IV.

A constantly high value for water was observed in the Pregl apparatus. To correct for this, a blank was subtracted in each case. This blank was determined by making blank runs both before and after a series of determinations. It is believed that this extra water may be the result of an inferior grade of rubber tubing supplied for connections on this apparatus.

The data for fraction I given in Table IV show a carbon, hydrogen, oxygen ratio (oxygen by difference) of 20.5 : 34.42 : 1.

Calculated for $C_{20}H_{34}O$: Carbon 82.76% Hydrogen 11.72%

Calculated for $C_{41}H_{69}O$: Carbon 82.96% Hydrogen 11.46%

TABLE IV

Carbon and Hydrogen Content of Fraction I

Weight of Sample (mg)	Weight of CO_2 (mg)	Weight of H_2O (mg)	Water Blank (mg)	Weight H_2O minus Blank (mg)	Percent Carbon	Percent Hydrogen
2.053	6.233	2.238	0.086	2.152	82.75	11.64
2.128	6.485	2.317	0.086	2.231	83.09	11.65
2.272	6.864	2.472	0.086	2.386	82.39	11.62
3.110	9.450	3.363	0.086	3.277	83.09	11.70
3.436	10.487	3.674	0.086	3.588	83.23	11.60
1.617	4.921	1.821	0.086	1.735	82.99	11.91

Average Carbon----------82.92% Hydrogen--------11.69%

The molecular weight was next determined, using the method of Rast (19) as described by Niederl and Niederl (20).

In this method, the substance was placed in a weighed thin-walled tube; the tube and the substance were then weighed. Resublimed camphor was added, and the whole was weighed again. The tube was then sealed to a small glass rod and was pulled out to a suitable length for attachment to an Anchuetz thermometer. The thermometer and tube were then suspended in a sulfuric acid heating bath.

The melting temperature of the material dissolved in camphor was taken at the point where the last evidence of crystalline structure dissappeared. A strong light placed at ninety degrees to the line of vision assisted in the observation of this point. The mixture was melted and cooled repeatedly until readings within two tenths of a degree could be repeated consistently. The molecular weight was calculated from the melting point depression, taking the molecular freezing point constant of camphor as 40° C. The data obtained are given in Table V.

In the determination of molecular weight by this method, the assumption that no reaction takes place between the solvent and substance was made; actually there is no information relevant to the action of camphor on the compound in question.

TABLE V

Molecular Weight of Fraction I

	Weight of Sample (mg)	Weight of Camphor (mg)	M. P.	M. P.	Molecular Weight Calculated
1.	1.245	12.286	162.3	13.8	293.6
2.	1.011	17.211	168.1	8.0	293.7

Molecular Weight for $C_{41}H_{69}O_2$--------593

Molecular Weight for $C_{20}H_{34}O$ --------290

Since the compound was in the unsaponifiable fraction, the Liebermann-Burchard (21), Salkowski, and Whitly (22) sterol color reactions were tried. The Liebermann-Burchard and the Whitby B reactions were positive. The Salkowski and the Whitby A and C were negative.

Fraction I was soluble in cold concentrated sulfuric acid, ethyl ether, chloroform, and in hot absolute alcohol and hot acetic anhydride. It was insoluble in dilute hydrochloric acid, dilute potassium hydroxide, cold absolute alcohol, cold acetic anhydride, and in water. No absorption of bromine could be observed when a small amount of the material was dissolved in carbon tetrachloride and treated with a solution of five percent of bromine in carbon tetrachloride.

The following derivatives were prepared from Fraction I:

(a) The benzoyl derivative

About twenty milligrams of fraction I was dissolved in twelve milliliters of pyridine. To this was added about four milliliters of benzoyl chloride. The reaction mixture was heated over a low flame for a few minutes and then poured into forty milliliters of cold distilled water. A small amount of material which could not be separated by decantation was separated on a Hirsch funnel. The solid material was washed once by suspending in dilute sodium carbonate and once by suspending in water. Purification was accomplished by recrystallization, once from ninety-five percent alcohol and once from absolute alcohol.. The product was a white crystalline substance. It resembled its parent compound closely in appearance and had a melting point of 190-192° C., as determined on the Johns-Fisher melting point apparatus.

Note: At this time a Johns-Fisher melting point apparatus became available. This instrument gave melting point values which were within five tenths degrees of those recorded when using an Anchuetz thermometer in a sulfuric acid bath. This deviation was not considered a serious handicap. Accordingly, the Johns-Fisher outfit was used for all subsequent determinations except where information to the contrary is indicated.

An analysis of the benzoyl derivative for carbon and hydrogen gave the values summarized below in Table VI.

The molecular weight of the benzoyl derivative could

TABLE VI

Carbon and Hydrogen Content of a Benzoyl Derivative Prepared from Fraction I

weight of Sample (mg)	Weight of CO_2 (mg)	Weight of H_2O (mg)	Water Blank (mg)	Weight H_2O minus Blank (mg)	Percent Carbon	Percent Hydrogen
2.847	8.530	2.579	0.086	2.493	81.61	10.11
2.371	7.172	2.215	0.086	2.129	82.33	9.97
2.355	7.213	2.329	0.086	2.243	82.87	10.58
2.134	6.459	2.060	0.086	1.974	82.56	10.26

Average ------------------- Carbon--------82.34% Hydrogen-------10.23%

Required for $C_{27}H_{38}O_2$------Carbon--------82.23% Hydrogen--------9.64%

Required for $C_{48}H_{73}O_3$------ Carbon--------82.63% Hydrogen-------10.47%

not be obtained by the Rast method. When this material was melted with camphor, it began to darken; furthermore, the melting point of the mixture became lower with each successive melting, and a constant melting temperature could not be observed.

(b) The Acetyl Derivative

Twenty milligrams of fraction I was added to about three milliliters of acetic anhydride. The mixture was refluxed for about ten minutes. On cooling, a crystalline material separated. This was removed by filtration and recrystallized twice from absolute alcohol. The final product was a flaky, transparent, crystalline compound with a melting point of 178-179° C. An analysis of this material for carbon and hydrogen gave the values shown in Table VII.

The molecular weight determination for the acetyl derivative was not so satisfactory as that obtained for the original compound. The values obtained are given in Table VIII.

TABLE VII

Carbon and Hydrogen Content of an Acetyl Derivative Prepared from Fraction I

Weight of Sample (mg)	Weight of CO_2 (mg)	Weight of H_2O (mg)	Water Blank (mg)	Weight H_2O minus Blank (mg)	Percent Carbon	Percent Hydrogen
2.580	7.787	2.676	0.086	2.590	82.31	11.15
1.697	5.117	1.810	0.086	1.728	82.23	11.31
3.101	9.234	3.186	0.086	3.100	81.21	11.11

	Averages	Carbon------81.91%	Hydrogen---------11.19%
	Required for $C_{22}H_{36}O_2$	Carbon------79.51%	Hydrogen---------10.84%
	Required for $C_{43}H_{71}O_2$	Carbon------81.27%	Hydrogen---------11.18%

TAPLE VIII

Molecular Weight of the Acetyl Derivative

of Fraction I

	Weight of Sample (mg)	Weight of Camphor (mg)	M.P.	M.P.	Calculated Molecular Weight
Sample 1	0.612	5.770	159.3 (Anchuetz)	16.8	252
Sample 2	0.793	14.069	167.4 (Anchuetz)	8.7	259

Average-------------------256

Required for $C_{22}H_{36}O_2$-----332

Required for $C_{43}H_{71}O_3$-----635

The acetyl derivative was also prepared by using acetyl chloride as a reagent. A few milligrams of fraction I was dissolved in about five-tenths milliliters of acetyl chloride, and the mixture was heated gently. The solution was cooled and poured carefully into five milliliters of water. A crystalline compound separated. This was removed by filtration, using a Hirsch funnel. The material on the funnel was washed twice with distilled water and then was recrystallized from absolute ethyl alcohol. The product was a transparent, fragile, crystalline material having a melting point of 177-178° C. This corresponds to that given by the derivative prepared with acetic anhydride as a reagent.

The benzoyl derivative and the acetyl derivative of Fraction I give the following sterol color reactions: in each case, the Liebermann-Burchard and Whitby B were positive, and the Salkowski was negative.

A few milligrams of fraction I was boiled for twenty minutes in a solution of one part phenyl hydrazine, one part glacial acetic acid and two parts water. The crystalline material did not dissolve. The solution was filtered while hot. The crystals remaining on the filter were washed several times with water and then were washed twice with absolute alcohol. They had a melting point of 163-164° C., indicating no reaction had taken place with phenyl hydrazine. The filtrate was placed in an ice box, and no crystalline or oily material separated.

Fraction II was found to be identical with fraction I. The data which led to this conclusion are given in Table IX.

A microphotograph of fraction II is shown in figure 3.

Fractions IIa, III, IIIa, and IV could not be examined in detail as was fraction I. The data available for them are presented in Table X. Microphotographs of fractions IIa, III, and IV are shown in figures 4, 5, and 6 respectively.

In order to assist in the identification of the chemical individuals which are represented in fractions IIa, III, IIIa, and IV, acetyl derivatives were prepared from

TABLE IX

Summary of Properties of Fraction II

Melting point	164-165° C.
Molecular Weight	
0.921 mg. substance	
10.571 mg. camphor	
M. P.-----12.0° C.	
Calculated molecular weight	291.6
Liebermann-Burchard Reaction	positive
Salkowski Reaction	negative
Whitby B Reaction	positive
Melting point of acetyl derivative	177-178°
Mixed melting point of Fractions I and II	164-165°

TABLE X

Summary of Properties of Fractions IIa, III, IIIa, and IV *

Fraction	M. P.	Sterol Reactions: Liebermann Burchard	Sterol Reactions: Salkowski	Sterol Reactions: Whitby B.	Appearance of Crystals
I	164.4-164.8	Positive	Negative	Positive	White cholesterol like plates. Fig. 2
II	164.4-164.8	Positive	Negative	Positive	White cholesterol like plates. Fig. 3
IIa	154-155				Long, narrow hexagonal plates. Fig. 4
III	160-161	Positive	Negative	Positive	Twisted elongated hexagonal plates. Fig. 5
IIIa	154-155	Positive	Negative	Positive	Twisted elongated hexagonal plates.
IV	155-156	Positive	Negative	Positive	Twisted elongated hexagonal plates. Fig. 6

* Fractions I and II are included for comparison

each, using acetyl chloride as a reagent and proceeding in the same manner as in the preparation of the acetyl derivatives of fractions I and II. The acetates resulting were all fragile, plate-like, transparent crystals. Their respective melting points are given in Table XI

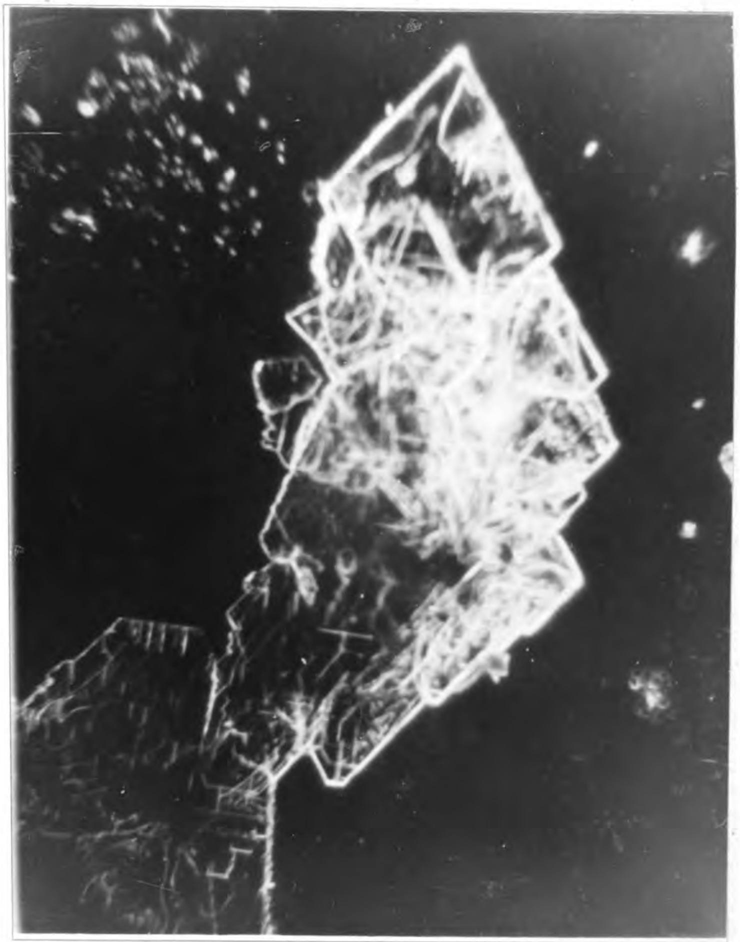
TABLE XI

Melting Points of Acetyl Derivatives of Fractions

Fraction No.	M. P.
I	178-179
II	177-178
IIa	164-166
III	174-176
IIIa	168-169
IV	168-170

After all possible crystalline material had been separated from the concentrated petroleum ether solution of the unsaponifiable fraction, the remaining solvent was removed by heating on the steam bath. The residue, a clear, orange-yellow liquid, was designated as fraction V. It weighed 2.59 grams and represented about 63% of the unsaponifiable fraction or about 2.5% of the whole oil. Fraction V gave the same sterol color reactions as the other compounds. The Liebermann-Burchard and Whitby B were positive and the Salkowski was negative. The material was not further characterized. It was placed under and atmosphere of carbon dioxide and stored in the ice box pending further investigation.

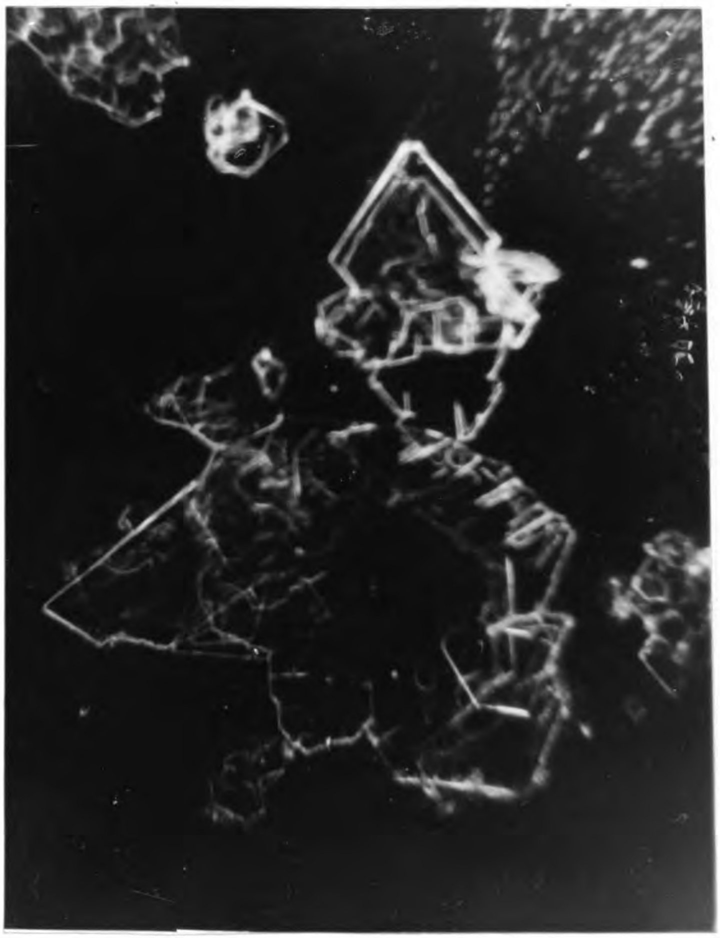
FIGURE 2



Fraction I

Magnification 120 times. Dark field illumination.

FIGURE 3



Fraction II

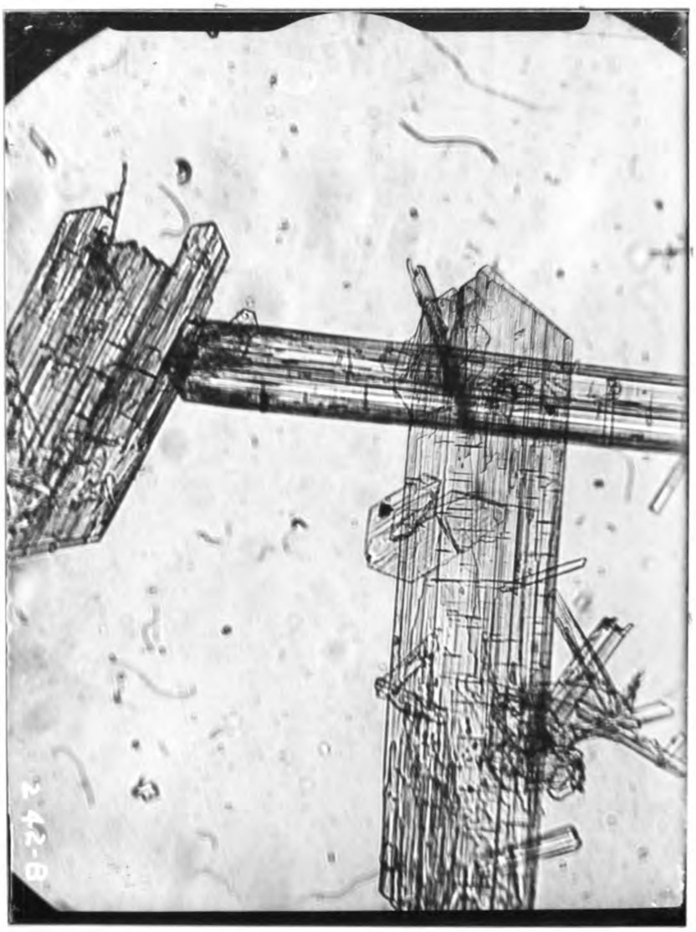
Magnification 120 diameters. Dark field illumination.

FIGURE 4

Fraction IIa

Magnification 120 diameters.

FIGURE 5



Fraction III

Magnification 120 diameters

FIGURE 6

Fraction IV

Magnification 120 diameters.

Discussion

The constants for the oil obtained from alfalfa seed, (Medicago sativa, Hardigan variety) grown in Michigan, show a reasonable agreement with those obtained by Jacobson from a western grown seed (Medicago sativa L.). An absolute comparison of the oils is impossible; the oil examined in this thesis was obtained by ether extraction; whereas, the oil examined by Jacobson was obtained by a gasoline extraction. The two oils show a fair agreement for several of the common constants; namely, the iodine number, the saponification number, the index of refraction, and the amount of unsaponifiable matter. This may indicate that the two are essentially similar. The differences observed are no more than might be expected in oils extracted with different solvents. Nothing can be said about the effect of growth conditions.

When alfalfa seed oil is allowed to stand for some time, a quantity of an amorphous substance separates. Gortner (23) reported the separation of a similar substance from wheat flour oil, and Ball (24) observed the separation of a substance from wheat embryo oil when it had stood for some time at a low temperature. Jacobson and Holmes (12) make no mention of any such observation for alfalfa seed oil.

The amount of unsaponifiable material in alfalfa seed is unusually high as compared to some of the more common oils. It consists of about 37% of perfectly white crystalline material which can be separated from concentrated petroleum ether solutions and of about 63% of a yellow-orange liquid.

The crystalline material in the unsaponifiable portion has been separated into four main fractions and two sub-fractions. A study of the fractions obtained indicates the probable presence of four chemical individuals.

Fractions I and II have been shown to be identical; e.g., they have the same melting point, 164-165° C., their crystalline structure is similar, they both form derivatives with acetyl chloride, and the derivatives formed have the same melting point 178-179° C. The existance of this chemical individual in two different fractions is caused by the incomplete separation procedure. It is hoped that in subsequent separations, this defect can be corrected.

Fraction IIa has a melting point of 154-155 degrees. Its crystalline structure distinguishes it sharply from Fractions II and I, but it does not distinguish it from fractions IIIa and IV. Fraction IIa, when treated with acetyl chloride, produced a derivative which melted at 164-165 degrees. Because of the small amount of material, the preparation of this derivative could not be repeated.

Accordingly, there is some question as to its existance as a distinct compound; that is, there is a possibility that it may be the same as fractions IIIa and IV.

Fraction III consists of thin elongated hexagonal crystals which have a curious twist along the long axis. This structural peculiarity is evident when the crystals are formed by cooling a hot absolute alcohol solution of the fraction and observing the crystals suspended in the alcohol. Fraction III melts at 160-161^{o} C. and forms a derivative with acetyl chloride which melts at 174-175^{o} C.

Fraction IIIa and fraction IV are probably the same. They melt at 154-155 degrees and 155-156 degrees respectively. Both fractions react with acetyl chloride to form derivatives which melt at 168-170 degrees. Their crystalline structure is indistinguishable under the microscope.

A summary of the data relevant to fraction I brings out the following facts:

1. It is soluble in cold concentrated sulfuric acid but not soluble in water, dilute hydrochloric acid, or in dilute sodium hydroxide.
2. It will form derivatives with acyl chlorides but not with phenyl hydrazine.
3. It does not add bromine.
4. It has a very low hydrogen content, much lower than any straight chain or branched chain aliphatic compound.
5. It gives some of the common sterol reactions.

From these data one may reasonably state that fraction I is a cyclic alcohol. Its refusal to add bromine suggests that it is saturated. The statement that it is a saturated compound may meet with some objection however, since Anderson and Nabenhaur (25) state that only unsaturated sterols give the Liebermann-Burchard reaction.

From the sterol color reactions, one may suggest that it belongs to the sterol family. This last point is, of course, subject to some question, since there are other substances which give these color reactions. Callow and Young (26) define sterols as substances which have a cyclopentanophenanthrene ring system as a nucleus. There is no evidence to indicate whether or not this ring structure is present in fraction I.

An attempt was made to assign fraction I a definite empirical formula. Unfortunately, the experimental data obtained by approaching the problem from different points of view gave conflicting impressions. Direct analysis for carbon and hydrogen indicated a probabe formula of $C_{41}H_{69}O_2$. A molecular weight determination by the method of Rast considered in connection with the percent of carbon and hydrogen suggested that $C_{20}H_{34}O$ was probably the correct formula. To clear up this difficulty, a benzoyl derivative and an acetyl derivative were prepared from the fraction.

The molecular weight of the benzoyl derivative could not be determined by the Rast method (15), but a carbon and hydrogen determination by the Pregl method (14) gave values which suggested that the formula of the original compound was probably $C_{41}H_{69}O_2$. It must be stated, however, that the agreement here left much to be desired.

The acetyl derivative gave an average carbon and hydrogen content which could not be well correlated with either of the possibilities suggested for the original compound. Although a single selected determination gave almost perfect agreement with $C_{41}H_{69}O_2$ as the formula for the original compound, a molecular weight determination of this material gave a value which was lower than that of the original compound. This rather surprising figure may possibly be explained by assuming the compound dissociated in the camphor solution causing an unusually low melting point for the mixture. Such an explanation is, however, purely speculative.

The exact molecular formula for fraction I cannot be given at this time. It is hoped that later work will either correlate the controversial facts known at present or bring to light possible errors which may have been made in the determinations.

Summary and Conclusions

1. Some chemical and physical constants for the oil extracted from alfalfa seed (Medicago sativa, Hardigan variety) have been determined.

2. The unsaponifiable fraction of this oil has been shown to consist of a clear yellow-orange liquid and of a quantity of a white crystalline solid.

3. The crystalline material in the unsaponifiable portion of this oil has been fractionated in such a way that several chemically homogeneous substances may be identified.

4. A derivative has been prepared from each of the solid fractions separated.

5. The first fraction obtained has been classified as a cyclic alcohol, probably belonging to the sterol family.

6. An attempt was made to determine the empirical formula of fraction I.

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