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# A Modified Monomolecular Film Test for Micro-quantities of Lipids in Foods

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## SUMMARY

Several sources of experimental error in the monomolecular film test for micro-quantities of lipids in food products were enumerated and evaluated. The test results are affected by factors causing differences in the oxidized oil film pressure surrounding the area of lipid spread on the surface of the acetic acid solution. Factors which increase the film pressure decrease the lipid spread area.

Time lapse after application of the lipid, temperature of solution, oil characteristics, and readjustment of pressure after application of lipid were studied. An alternate method to measure the area of the lipid spread was introduced. The method consisted of a tray equipped with a movable bar which approximates the area of the lipid spread and corrects for film pressure variation neglected by previous published methods.

LABORATORY STATE  
METHODS  
PROCEDURE

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This bulletin is a report on Department  
of Poultry Husbandry research  
project 17, Egg Utilization



# A Modified Monomolecular Film Test for Micro-quantities of Lipids in Foods

JAY T. COLBURN,\* O. J. COTTERILL AND E. M. FUNK

## INTRODUCTION

The quality of many food products is greatly affected by the fat, or lipid, content of their milk and egg constituents. This study had to do with evaluating and developing methods of determining micro-quantities of lipids. A new indirect method of measuring the lipid content was devised.

The quality of nonfat dried milk solids is affected by the presence of too much fat in the powder. This fat increases off-flavors. In the egg products industry the albumen that is contaminated with lipid material has limited commercial use. Albumen with too great a lipid content cannot be used in angel cakes as the functional properties of the albumen are adversely affected by the lipid. Normally, the fresh egg has a minute amount of lipid material present in the albumen portion. It has been postulated by Smith (1959) that there is a diffusion of glycerides from the yolk to the albumen. There is also an opportunity for yolk materials to contaminate the albumen during the breaking and separation of the shell egg.

A method for determining very small amounts of lipid that has been used in the egg products and dairy industries is based on the ability of the lipids to form a monomolecular layer on an aqueous solution. The lipids are extracted from the material being analyzed and then redissolved in an aliquot of solvent. A film of oxidized oil is spread on an aqueous solution until a given color is reflected on the surface. This color is assumed to have a constant surface pressure. A given amount of the redissolved lipid is then applied to the oxidized oil film, which is displaced by the lipid extract. The outline of the lipid spread is then traced on glass, transferred to paper, and measured as an indication of the lipid content.

This study is limited mainly to factors that may cause variation after the extract has been applied to form the oil film. The extraction procedure and factors that vary after the application of the lipids may be assumed to be of general nature and apply to various materials being analyzed.

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Some of the factors that are included in this study are: variations in the oxidized oil; effect of surface pressure on the lipid spread areas; effect of time on the spread area; and temperature effects on the film. An indirect method of measuring the lipid content has been devised. This method and the instrument used are described. The indirect method eliminates the necessity of tracing and measuring the lipid area, thus reducing the time required for analysis with about the same degree of accuracy. This method also corrects for some error that may be encountered due to variations in surface pressure of the oil film after application of the lipid extract.

## REVIEW OF LITERATURE

The study of the spread of oil films on aqueous solutions has received considerable attention for many years.

Pockels (1891) and Rayleigh (1899) were among the first to investigate the effect of surface films on the surface tension of aqueous solutions in a scientific manner. They found that they could manipulate surface films by the use of surface barriers. By this means they discovered that the pressure exerted by the films was a function of amount of compression.

Langmuir (1917) contributed much to the knowledge of surface films. He suggested that the materials making up the film were oriented in a particular manner on the surface. The length of the carbon chains of the material did not affect the cross sectional area of the molecule.

Gorter and Grendel (1925) were able to utilize this information as a means of estimating the lipid contents of blood.

Blodgett (1934) showed that monolayers could be deposited on a solid surface to a sufficient depth to produce interference colors due to structural interference of the light waves.

Jones (1950) utilized these findings to develop a general method for the determination of micro-amounts of lipid material spread on an aqueous solution. He used a third order green interference color of an oxidized oil film to obtain a surface pressure of 20 (+ 1) dynes per centimeter. He prepared the oxidized oil by oxidation at 300° F. for a period of eight hours.

Heinemann and Rohr (1950) modified the extraction procedure for use in the dairy industry.

Another modification of the general method was made by Bergquist and Wells (1956) for application in the egg products industry. They used the method to determine small amounts of lipid material in egg albumen after it was separated from the yolk.

Smith (1959) states that there are very minute amounts of lipid material in the albumen of the egg. He also states that this lipid material may diffuse to the albumen from the yolk upon aging of the egg.

Romanoff and Romanoff (1949) state that the lipids constitute about 33 percent of the yolk on a wet weight basis. The lipid materials consist of: glycerides (62.3%), phospholipids (32.8%), sterols (4.9%), and cerebrosides (in trace amounts). They also state that the lipid content of the albumen is only at a trace level.

Harkins (1952) stated that oils, when spread on an aqueous solution exhibit, upon compression, a display of colors. These colors change from a dark yellow at low compression to gold, red, purple, blue, and green and then repeat the series of colors. At higher compressions the red and green colors predominate. Each of these repetitions of a color comprise an order of color. The film, during this process, is thickening from a few hundred angstroms to several thousand angstroms.

Sachanen (1945) stated that considerable variation may exist in oils taken from various oil fields. Some of the variations that may be found in paraffin-base and asphaltic-base oils are shown in Table 1. He

TABLE 1 - A COMPARISON OF THE PROPERTIES OF PARAFFIN-BASE AND ASPHALTIC-BASE LUBRICATING OILS

Molecular Weight	Paraffin Base 350-650	Asphaltic- Base 260-450
Percentage of paraffinic side chains	75	45
Percentage of naphthenic aromatic rings	25	55
Average number of rings	1.7	3.5
Specific gravity (60°)	0.86-0.89	0.91-0.97
Viscosity at 100° F. (S.U.)	100-2350	100-7000
Viscosity at 210° F. (S.U.)	39-150	37-150
Flash (°F)	390-550	335-465

stated that mixtures of the two different base oils have properties which are intermediate between the two extremes but more closely resemble the paraffin-base oils. This author also stated that the products formed on oxidation of petroleum oils are: peroxides, acids, alcohols, aldehydes, ketones, and resinous and asphaltic compounds.

## PROCEDURES AND RESULTS

### Lipid Extraction

Extraction Procedure: The extraction of the lipids from the contaminated egg albumen was carried out according to the procedure of Bergquist and Wells (1956). The lipids were added to fresh egg albumen in the form of yolk to approximate the commercial situation.

Briefly, the extraction involved an ethanol precipitation of the protein in the presence of 0.2 percent ammonium hydroxide followed by an ethyl ether ("purified for fat extraction") and petroleum ether (B.P. 30° - 60° C.) extraction of the lipid materials. Twenty-five ml. of the ether layer were pipetted to an aluminum drying dish and the ether solvent was evaporated on a warm "hot plate." After evaporation of the ether the residue was cooled and redissolved in petroleum ether. In the actual determination the weight of the petroleum ether was constant at 2.5 grams. A small amount of this redissolved lipid material was delivered to the oxidized oil surface by gently expelling the micropipette contents just below the surface.

Stock Solution Preparation: To eliminate the variation that might occur in the extraction procedure, a "stock" lipid-petroleum ether solution was prepared. The yolk concentration in the original yolk-albumen mixture was increased to provide a greater amount of lipids than would usually be measured by the monomolecular film method. The lipid material was extracted in the manner already outlined and then was diluted to a volume of about 50 ml. with petroleum ether. Different concentrations of the lipid-petroleum ether solution were used to study the factors occurring in the film test and their effect on different area spreads. Preparation of a standard curve series will be discussed as a separate point.

### Micropipettes

For delivery of the lipid-petroleum ether solution to the surface, a modified Breed and Brew pipette was used. Originally, the pipette was calibrated to contain 0.01 ml. at 20° C. The tip of the pipette was drawn out to form a smaller orifice. The pipette was calibrated with mercury and found to contain 0.0088 ml. at 20° C.

### Acid Solution

A 0.2 percent acetic acid solution, as used by Bergquist and Wells (1956), was employed as a surface on which to spread the oxidized oil and lipid films.

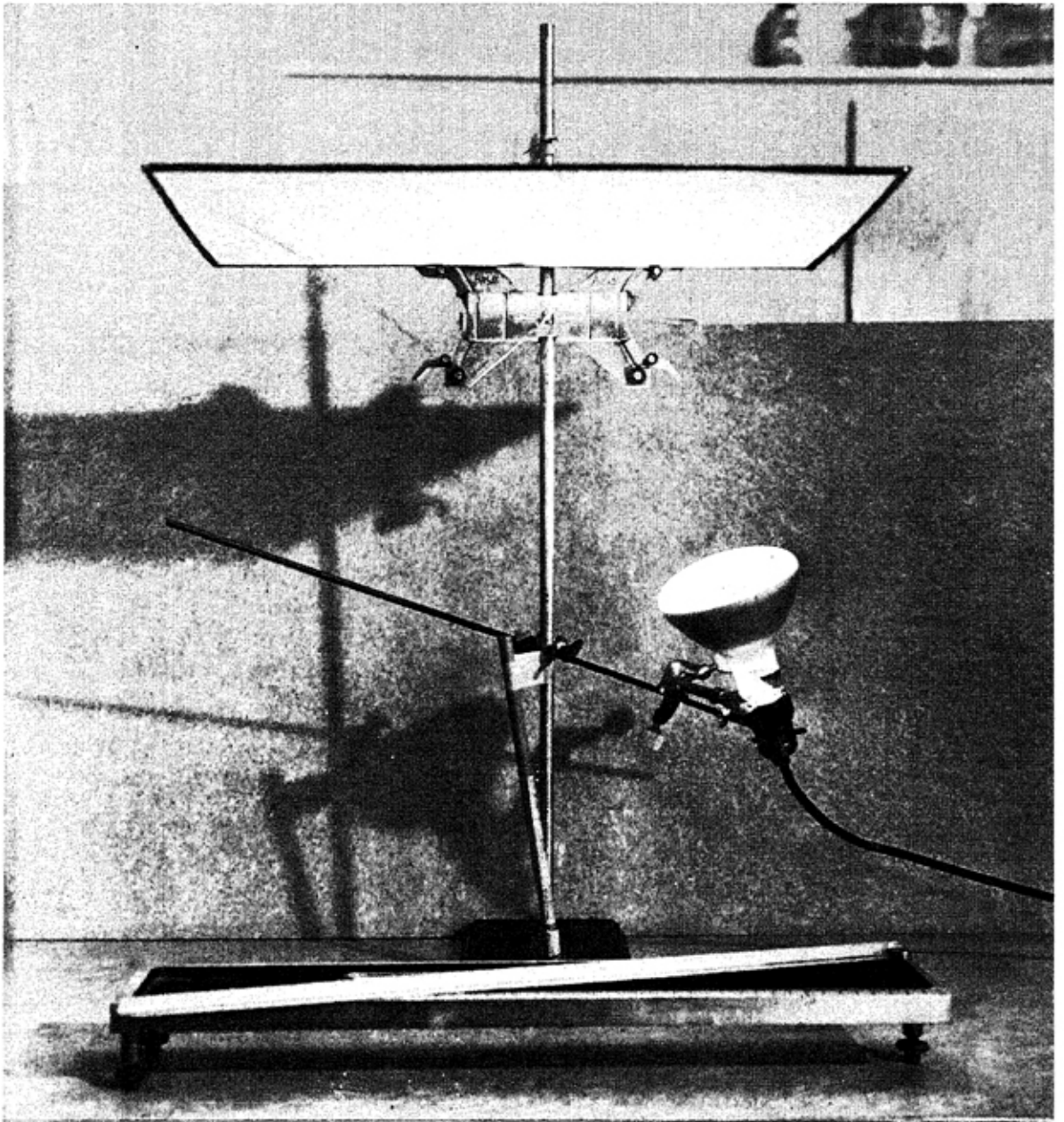


Figure 1. Lighting Apparatus for reflected light and the measuring tray.

### Light Source

The apparatus for providing reflected light was similar to that used by Bergquist and Wells (1956). This arrangement is shown in Figure 1. A 150 watt reflector floodlight was trained to reflect the light from a 14 x 21 inch fiber board panel painted with white enamel. The panel was placed 24 inches above and parallel to the surface of the solution.

A black background was provided for each container used for the acetic acid solution. The glass pie dish container was painted on the outside and coated on the inside with paraffin. The metal tray, to be discussed later, was painted on the inside and then coated with paraffin, which was nonspreading.



## Oxidized Oil

Purpose: Oxidized oil was used in this test as a boundary for the lipid spread on the acetic acid solution. The oil film interference colors also provide a means of attaining a measure of the surface pressure without resorting to more elaborate and expensive equipment in quality control analysis. Interference color is a measure of the film thickness. Oils were oxidized for study of variations that might occur within an oil and between different oil samples. Oils that did not contain detergent were purchased in quart cans at service stations. In general, it might be said that the oils were the least expensive oils available. Most of the oils were 20 SAE viscosity rating but several 30 SAE viscosity oils were included.

Oxidation Procedure: The samples were oxidized by bubbling compressed air through the oil at 300° F. in a ventilated oven. The only means of regulating the air flow was to feed the air through glass pipettes of the same size in an attempt to obtain an equal air flow to each sample.

Refractive Index and Spreading Properties: Samples were withdrawn at intervals to check spreading properties and the refractive indices at different stages of the oxidation process. Refractive indices of the oils were determined with an Abbe' refractometer with a circulating water bath temperature control at 21.5° C. Results are shown in Tables 2 and 3. There was no great change in the refractive index during the

TABLE 2 - CHANGE IN REFRACTIVE INDEX OF OIL DURING  
OXIDATION PROCESS (21.1° C.)

Oxidation Time (Hrs.)	A 20	F 30	D 20	E 20	G 20
0	1.5135	1.5113	1.4865	1.4892	1.4859
2	1.5140	1.5090	1.4861	1.4889	1.4860
4	1.5144	1.5103	1.4863	1.4887	1.4871
6	1.5146	1.5107	1.4866	1.4892	1.4861
7	1.5148	1.5107	1.4868	1.4892	1.4871
8	1.5148	1.5107	1.4868	1.4891	1.4867
9	1.5137	1.5080	1.4863	1.4891	1.4865

TABLE 3 - CHANGE IN REFRACTIVE INDICES OF OXIDIZED  
OIL SAMPLE AT THREE TEMPERATURES

Temp. (°C)	Oil		
	A	B	C
16.1	1.5170	1.4892	1.4895
20.0	1.5153	1.4879	1.4888
21.1	1.5145	1.4873	1.4870
30.5	1.5110	1.4833	1.4832

oxidation process. However, there was a decided difference in the oils in regard to darkening of color during oxidation. Some of the oils became dark and opaque and others retained their initial translucence with little darkening.

All of the oils tested showed a change in refractive index with temperature change. The refractive index of the oil decreased as the temperature increased. It should be noted that the refractive indices of the different oils varied at any given temperature. Of many more oils compared, there was a grouping at refractive indices of 1.48 and 1.51. Variation within these groups is of a lesser nature.

Although no objective measurements were taken, the variations in the oxidized oils were observed in respect to interference colors of the film. Some of the oils were found to be unsatisfactory for use in this test. The factors considered as a means of evaluating the oil for test use were ability to form a uniform interference color on the surface, general spreading ability, and absence of spots in the film which apparently were formed by a residue of an unknown nature. Oils A, B, and C were considered generally good in respect to these criteria.

The ability of the oil sample to spread as a film with a homogeneous interference color was the criterion at this time. Freedom from residue spots was also considered.

The study of the oil properties in other respects was confined mainly to oils that will be known as Oil A (20 SAE), Oil B (30 SAE) and Oil C (20 SAE). These three oils were studied in regard to surface pressure variations which will be discussed in detail.

### Lipid Spread Measurement

The area of the lipid spread was used as an indication of the lipid content of the material being analyzed. This area was measured in different ways.

Pie Dish Method: The first method was the pie dish method of Bergquist and Wells (1956). The procedure involves the use of a glass pie dish with the underside painted black. The dish was filled with the acetic acid solution. At the proper time the oxidized oil was delivered to the surface of the acetic acid solution with a number 27 needle on a 1 ml. tuberculin syringe until a third order green color was observed. The lipid-petroleum ether solution was then applied to the oxidized oil film with the micropipette. The outline of the spread was traced with a sharpened wax pencil on a pane of glass which was supported 0.25 inch above the surface. Triplicate tracings were made of each spread when possible.

Modification of the Pie Dish Method. A modification of the pie dish method is shown in Figure 2. A glass barrier was used as a means of regulating the surface pressure of the film. A drop of oil from the needle was delivered to the surface and then the glass barrier was

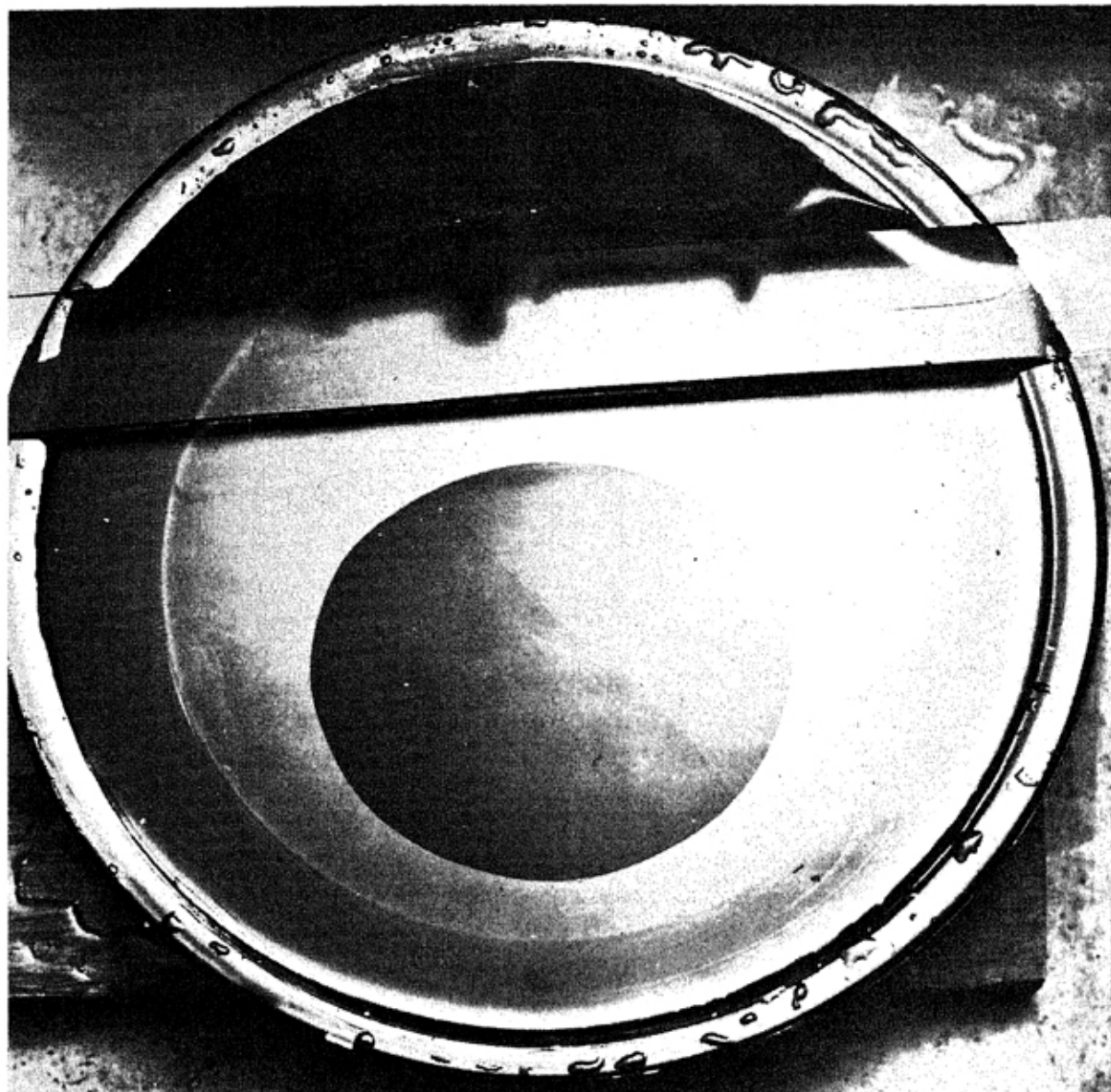


Figure 2. Pie dish apparatus with sliding bar barrier modification.

used to adjust the film pressure to the third order green. Care must be taken that there is no leakage past the barrier at the ends. This modification allows readjustment of the surface pressure. In place of a glass barrier or a knife edge, a waxed thread may be used to adjust the oil film pressure.

Tracing and Measurement. The outline traced on the glass was traced on paper. The lipid spread area was then measured with a compensating polar planimeter (K. & E. #4236) graduated to 0.1 square centimeter (0.0115 square inch). This arrangement is shown in Figure 3. Lipid spreads that moved on the surface were discarded as the area traced would not be accurate. A uniform procedure of tracing was used as much as possible. The inside of the wax pencil line coincided with the outside of the lipid spread. The tracing on the paper followed the inside of the wax pencil line. The planimeter pointer traced the inside of the pencil line.

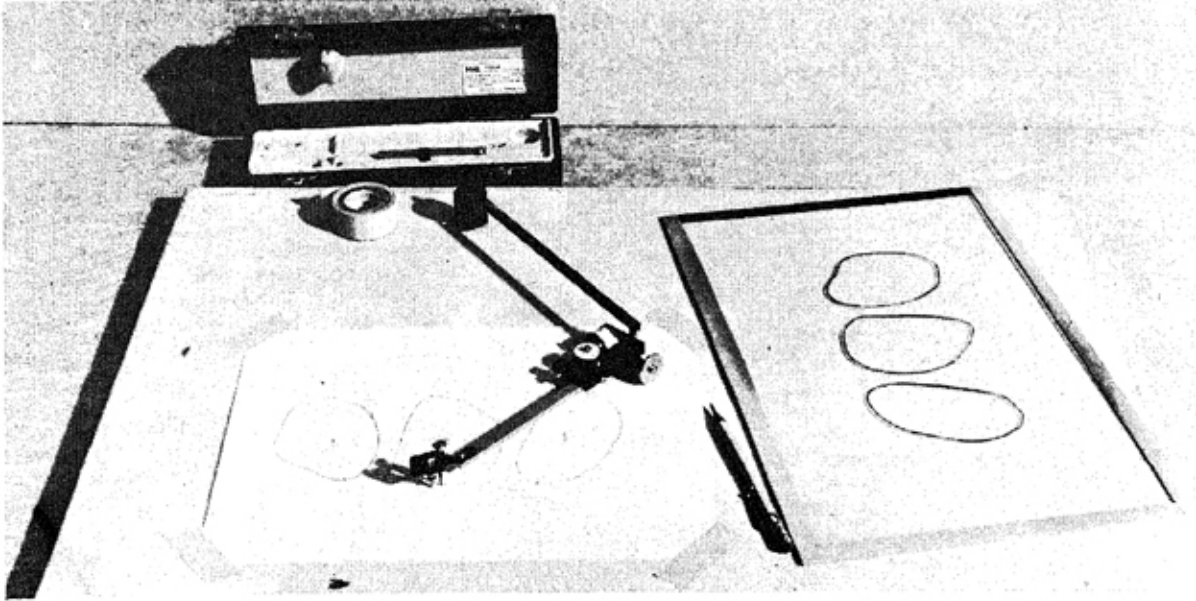


Figure 3. Polar planimeter and tracings of one lipid spread.

Measuring Tray Method. An indirect method of measurement of the lipid spread was devised. This method utilized an instrument designed by the senior author and constructed in the science instrument shop of the University of Missouri. The instrument will be referred to as the "measuring tray." The construction details are shown in Figures 4 and 5. The tray was machined from brass stock but a more acid resistant metal would be superior. The interior of the tray was painted black and coated with paraffin to prevent reaction of the acid solution with the metal tray. The paraffin was nonspreading.

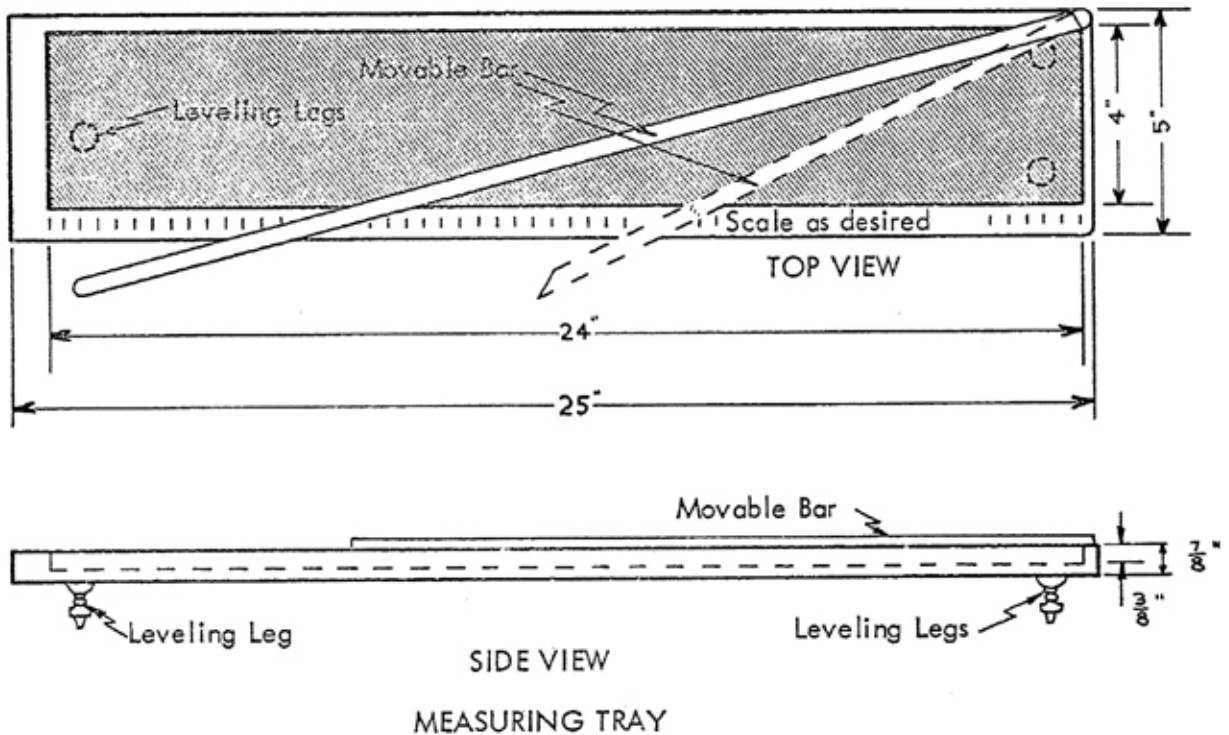


Figure 4. Measuring tray design.

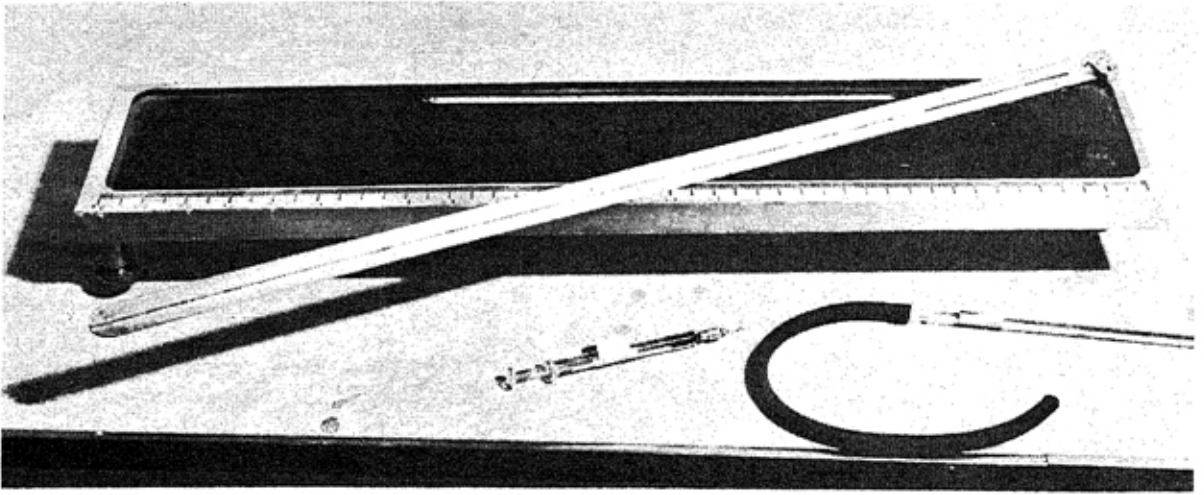
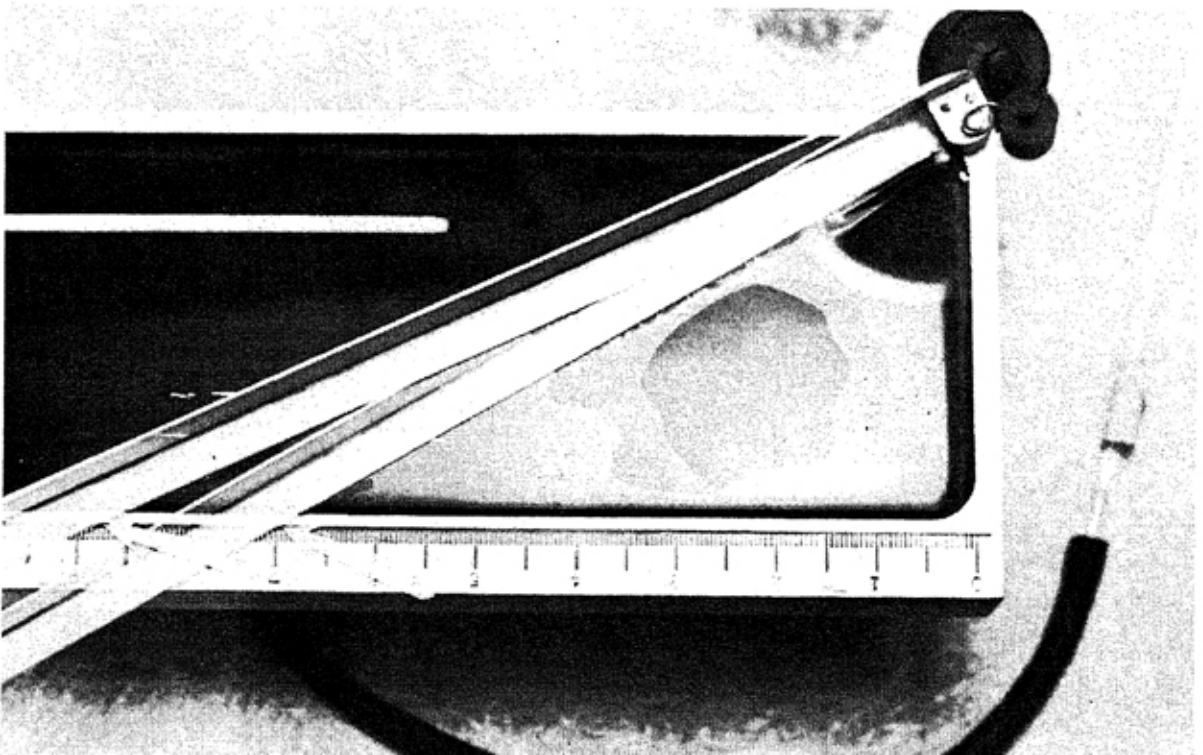


Figure 5. The measuring tray.

Inside measurements of the tray were 4 x 24 inches. A movable hypotenuse bar is pivoted at the base of the triangle so that the bar may be made to coincide with the base or the side of the tray opposite the scale side. In this way a variable area triangle is formed by moving the hypotenuse (bar). The scale in this particular tray is calibrated in inches graduated to 0.05. The point of intersection is taken at the scale edge. This enables further estimation of the height of the triangle to 0.01 inch.

Use of the tray is quite simple. The acetic acid solution is added to the tray until it is slightly higher than the top edge of the tray. A random amount of oxidized oil is delivered to the triangle formed by the scale side, the movable bar, and the end of the tray at the lesser end of the scale. The bar is moved until the oil surface film is a third order green (point A in Figure 6). The lipid-petroleum ether extract solution

Figure 6. Double exposure of measuring tray shown two positions of the sliding bar. The area of triangle between bar position A & B approximates the area of the lipid spread monolayer.



is then delivered to the oil-covered surface. The addition of the lipids increases the surface pressure of the oil film as indicated by the change of interference colors of the film. The intersection point of the movable bar on the scaled side is recorded.

The volume of oil delivered to the surface determines the area of the initial triangle covered with oil. This may be controlled to a certain extent by watching the size of the droplet that forms on the needle tip, and touching the acid solution surface when the desired amount of oil is exposed.

The color of the film is readjusted to the third order green by increasing the area of the oil film by moving the bar along the scale. The new point of intersection (point B in Figure 6) is then recorded.

Calculation of the area is based on the formula for the area of a triangle,  $A = 1/2 bh$ . The scale readings serve as the height (h) of the triangle and the end of the tray serves as the base (b) of the triangle. The difference in the area of the two triangles is taken as the area value for that lipid content.

To test the accuracy of the indirect method, a series of dilutions of egg yolk lipids were measured. These dilutions of lipids were prepared as outlined under "Stock Solution Preparation." The process of measuring the resultant areas was repeated as a check of accuracy. The coefficients of variation were calculated and are shown in Table 4. Generally, the coefficient of variation appears to increase as the area of the spread decreases. A method for correcting this change will be discussed later.

TABLE 4 - LIPID SPREAD AREAS DETERMINED  
BY MEASURING TRAY METHOD

Lipid Solution	Number of Determinations	Mean Area (in. <sup>2</sup> )	Standard Deviation (in. <sup>2</sup> )	Coefficient of Variation (%)
1	6	7.04	.189	2.69
2	6	5.01	.22	2.38
3	4	3.68	.12	3.26
4	6	2.82	.10	3.62
5	5	1.06	.05	4.71

### Surface Pressure

The oxidized oil film is assumed to have a constant surface pressure at a third order green. This color has been stated by Jones (1950) to have a surface pressure of 20 (+ 1) dynes per centimeter. Several aspects of surface pressure and its effect on this test have been studied.

Hydrophile Balance Measurements: The surface pressures of oils A, B, and C were measured at the first four orders of green at different temperatures by using the hydrophile balance shown in Figure 7. The

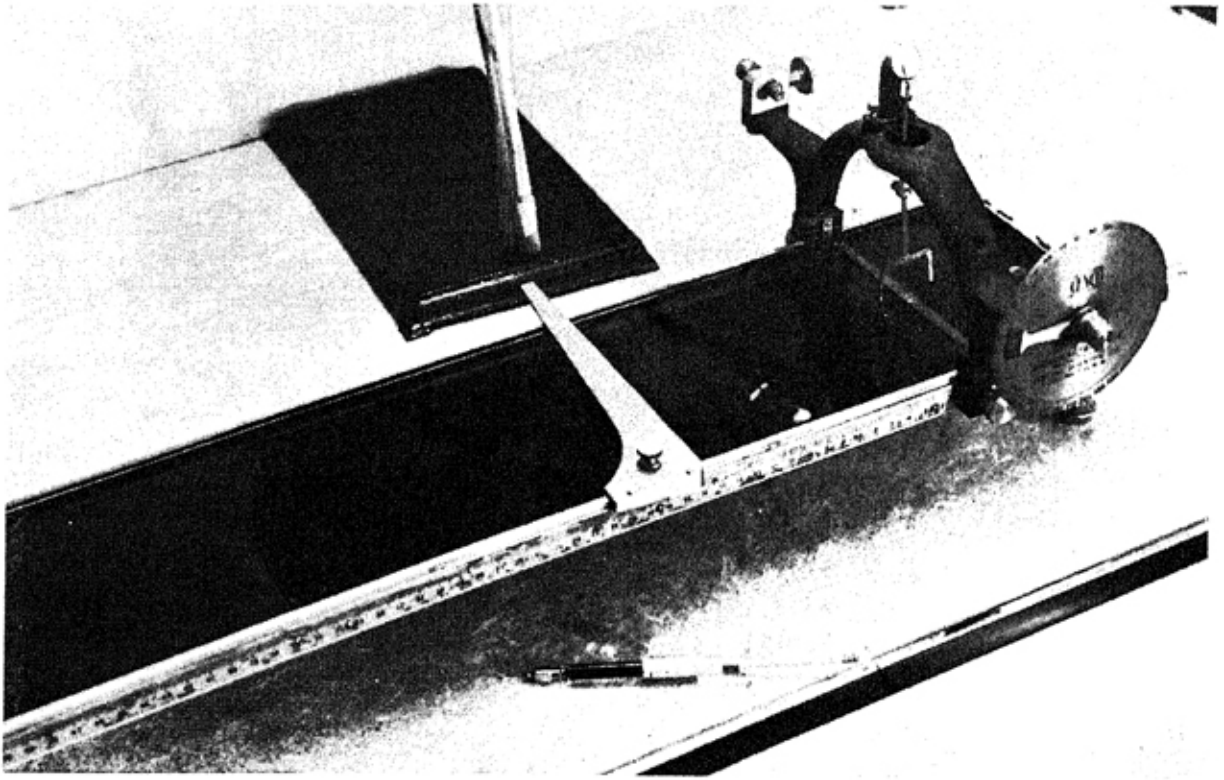


Figure 7. Hydrophile balance used to measure surface pressure.

balance measures the difference in surface tension between the oil film covered portion and the clear surface of the solution. The 0.2 percent acetic acid solution was used to spread the oil film.

The result of temperature variations on the surface pressure of the film was studied. A constant room temperature of  $15^{\circ}$  C. was used for one series of observations and room temperatures of  $20^{\circ}$  and  $250^{\circ}$  C. were used for the higher temperature observations. All equipment and materials were allowed to equilibrate to each of these temperatures before measurements were made. Temperature of the solution was measured by placing a thermometer in the bottom of the hydrophile balance tray.

The lighting apparatus described under "Light Source" and shown in Figure 1 was used for this experiment. It was necessary to cut a piece of glass to fit the bottom of the tray. This glass was painted black and coated with paraffin.

The hydrophile balance was calibrated before each series of observations according to the method of Adam (1941). Calibration of the instrument involved the determination of the value of a scale division by placing known weights on the arm extending from the torsion wire at a right angle to the float. The procedure after calibration of the instrument was as follows: The oil was delivered to the area of the solution to the left of the torsion head. The barrier with hair attachments at each end was used to contain the oil film. The movable barrier was used to compress the oil film to each of the orders of green. A reading was taken at each of these colors.

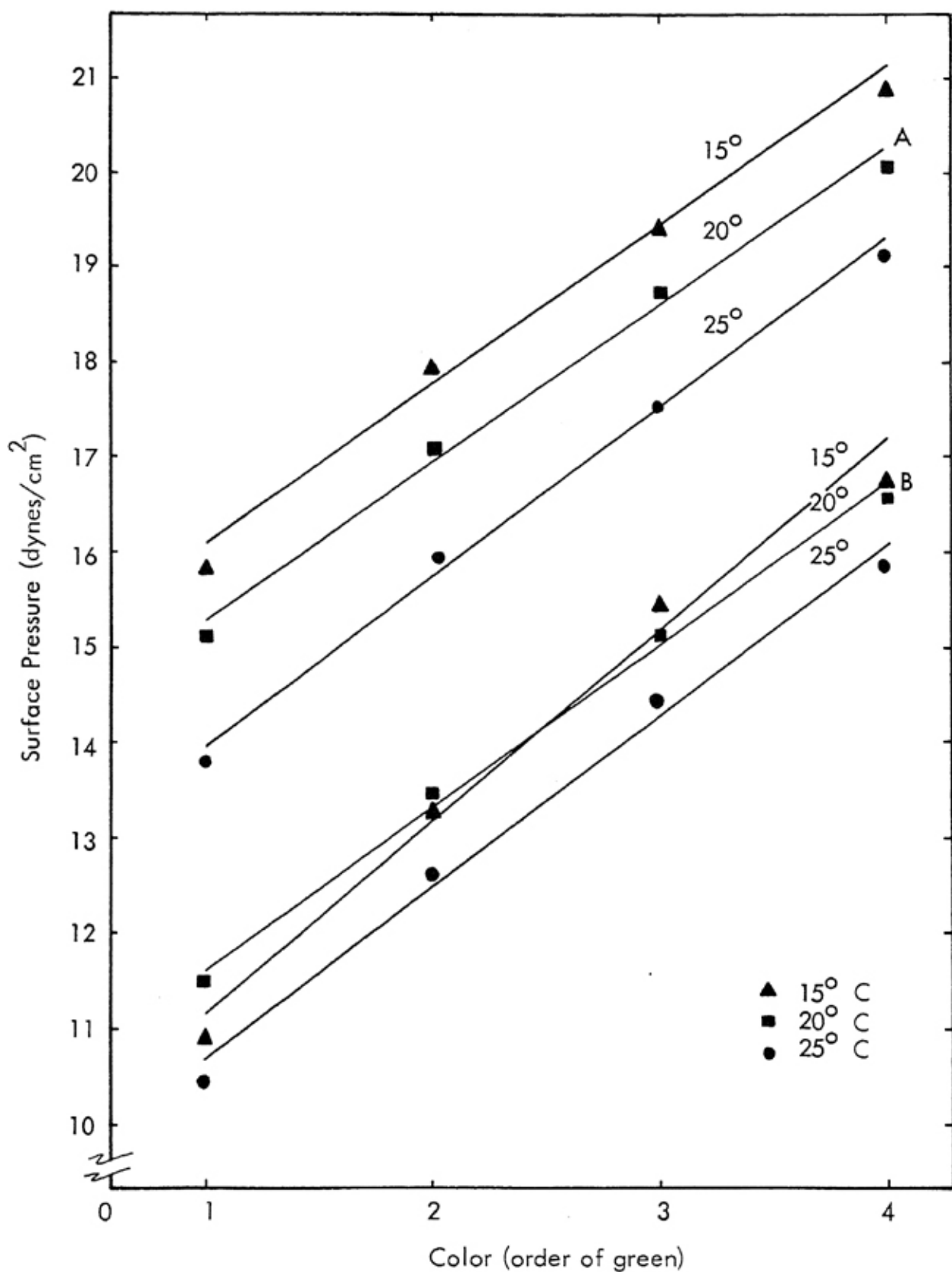


Figure 8. Surface pressure of two different oil films at three temperatures.

Three separate observations were made of each condition; the average was used for the values expressed in Figure 8. It was necessary to exercise extreme care that the area of the tray, not intentionally covered with oil, be kept free of accidental surface films. The varia-



tions found in the surface pressures are subject to error due to lack of sensitivity of the hydrophile balance at the pressures measured. The hydrophile balance was calibrated and found to have a sensitivity of 3.4 (+ 0.1) dynes per centimeter per scale division. However, generally good agreement was obtained for the values averaged to determine the points shown on the graph. The range for the value averaged to determine the points shown on the graph was 0.2 (+ 0.1) dynes per centimeter.

The interference colors of the oxidized oil film are used as an indication of the surface pressure and are supposedly constant. Actually, this is a measure of film thickness. The results of surface pressure measurements of two oils oxidized under similar conditions are shown in Figure 8.

There was a difference in the pressures of the two oils at any order of green. There was also a variation within the same oil at different temperatures. Oil A was a 20 SAE viscosity oil and oil B was a 30 SAE viscosity oil. A third oil was also used in this study, but the results for it are not given. The third oil, C, had a 20 SAE viscosity rating. Oil C differed from oil A in rate of change in surface pressure per degree of green. At the first order green the surface pressure averaged 1.4 (+ 0.1) dynes per centimeter lower than that of oil A at all temperatures. Between the second and third order green, the surface pressure exceeded that measured for oil A, until finally at the fourth order green, the surface pressure averaged 0.45 dynes per centimeter greater than that of oil A at 20° and 25° measurements.

There was an increase in surface pressure as the temperature decreased. Difficulty was encountered working with the higher viscosity oil films at the lower temperatures.

Area Change: Another aspect of the surface pressure effect on the test result was that of the change in the area at different pressures of the film. This experiment was carried out at 24° (+ 0.5) C. using the measuring tray and a photographic process of recording the area of the spread. The film of oil was applied and adjusted to a color less than that of the first order green. The lipid material was applied and the color of the film was adjusted to each of the different orders of green. A 45-second interval was maintained after the regulation of the color of the film before the picture was taken. The picture was then enlarged and the area was measured from the enlargement as a means of determining the scale of enlargement. The results of these measurements are shown in Figure 9. It was noted that the area of the spread diminished as the order of green was increased.

Pressure Readjustment. In this test the surface pressure of the oxidized oil film is regulated to a color characteristic of the third order green interference. The addition of the lipid-petroleum ether solution to the surface changes the interference color by displacement of the oxidized oil. This is an indication of a change in the surface

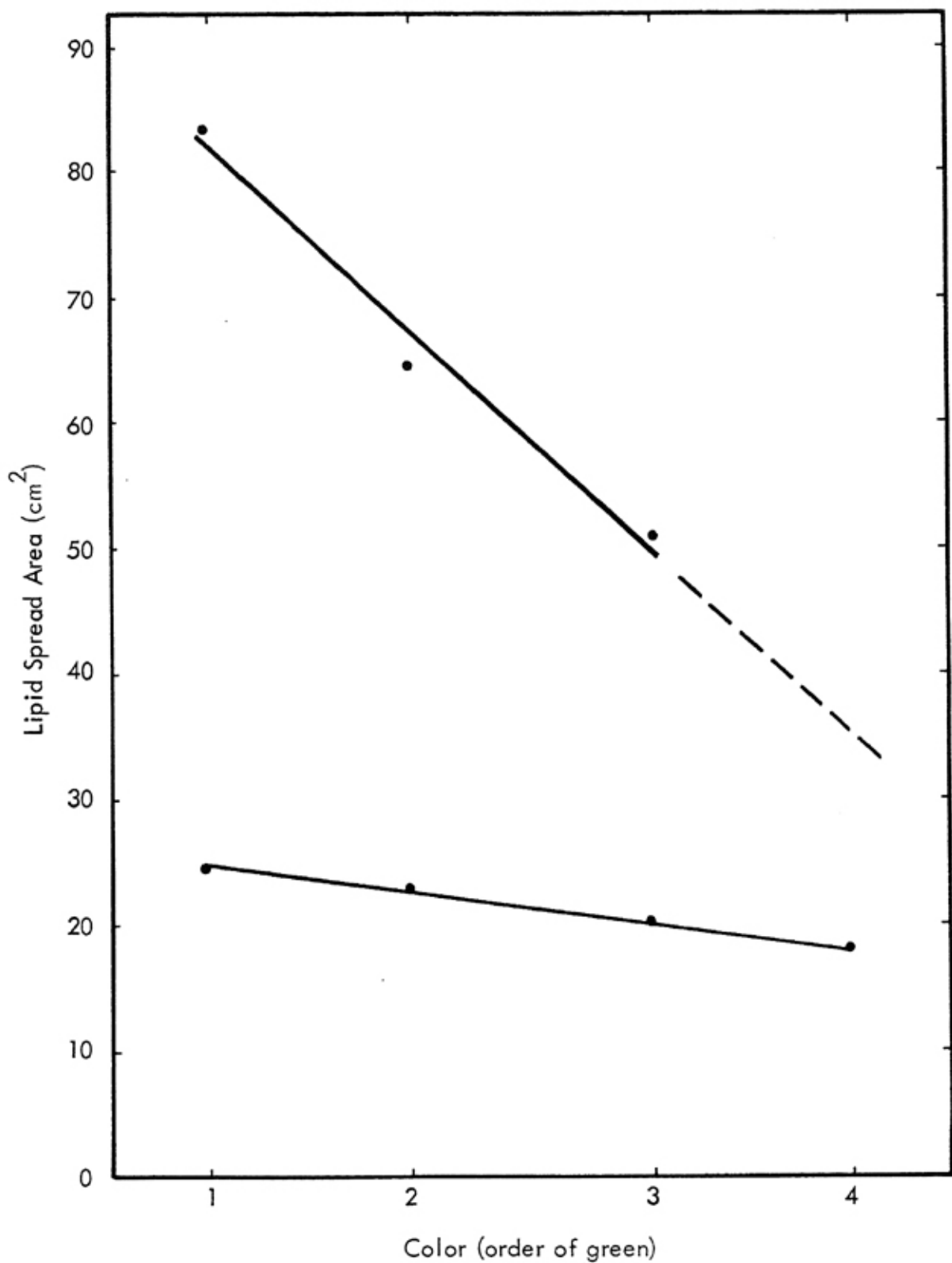


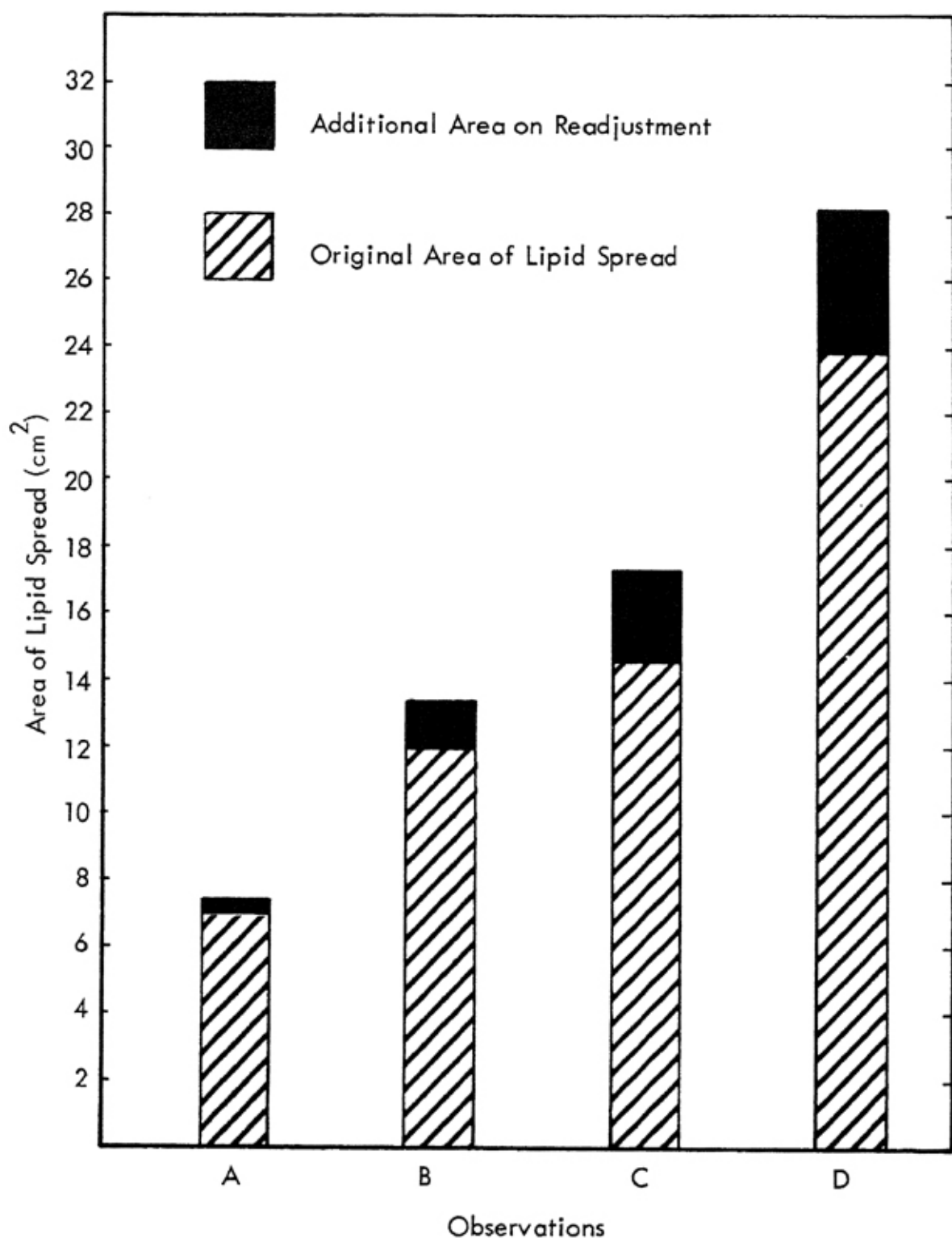
Figure 9. Effect of increasing surface pressure on area of lipid spread.

pressure of the oil film. The effect of this change in surface pressure on the area of the lipid spread was studied in the following way.

The oxidized oil was applied to the surface of the acetic acid solution and the interference color was adjusted to the third order green by moving the barrier. The lipid-petroleum ether solution was added to the surface by micropipette. After a 45 second interval, a picture of the spread was taken with a camera placed above the spread. The interference color of the film was then readjusted to the third order green by moving the barrier to expand the area covered by the oxidized oil film. After another 45-second interval a second picture was taken. Enlargements of these pictures were made. A portion of the scale was included in the enlarged picture as a means of determining the scale of enlargement. The enlarged picture of the lipid spread was measured with the polar planimeter. The necessary corrections for enlargement were made to obtain the original area of the spread. The change in the area of the lipid spread after readjustment of the interference color to the third order green is shown in Figure 10.

When the lipid is applied to the oxidized oil spread, the surface pressure of the oil film is increased as evidenced by the change in interference color. When the pressure is again regulated to the third order green after the application of the lipid, the area of the spread is increased. The increase in the pressure was dependent on the area of the spread and the total area of the oxidized oil film. This is shown by the graph bars C and D for larger area spreads. The larger the spread, the greater the tendency to minimize the area of the lipid spread, and thus the lipid content of the material being analyzed when no readjustment of pressure is utilized.

Figure 6, which is a double exposure of a particular lipid spread shows that an increase in the area of the spread over the original area occurred with readjustment of pressure as used in the measuring tray. The spread lying mostly to the right was the original area.



**Figure 10.** The Effect of surface pressure readjustment on the area of lipid spread for four different observations.

#### Effect of Temperature on Area

Preliminary studies indicated that there was a change in the area of the lipid spread with temperature.

Tracing and pie dish methods were used to measure the temperature effect on several sizes of spreads. The original pressure of the film was regulated using the glass barrier. There was no readjust-

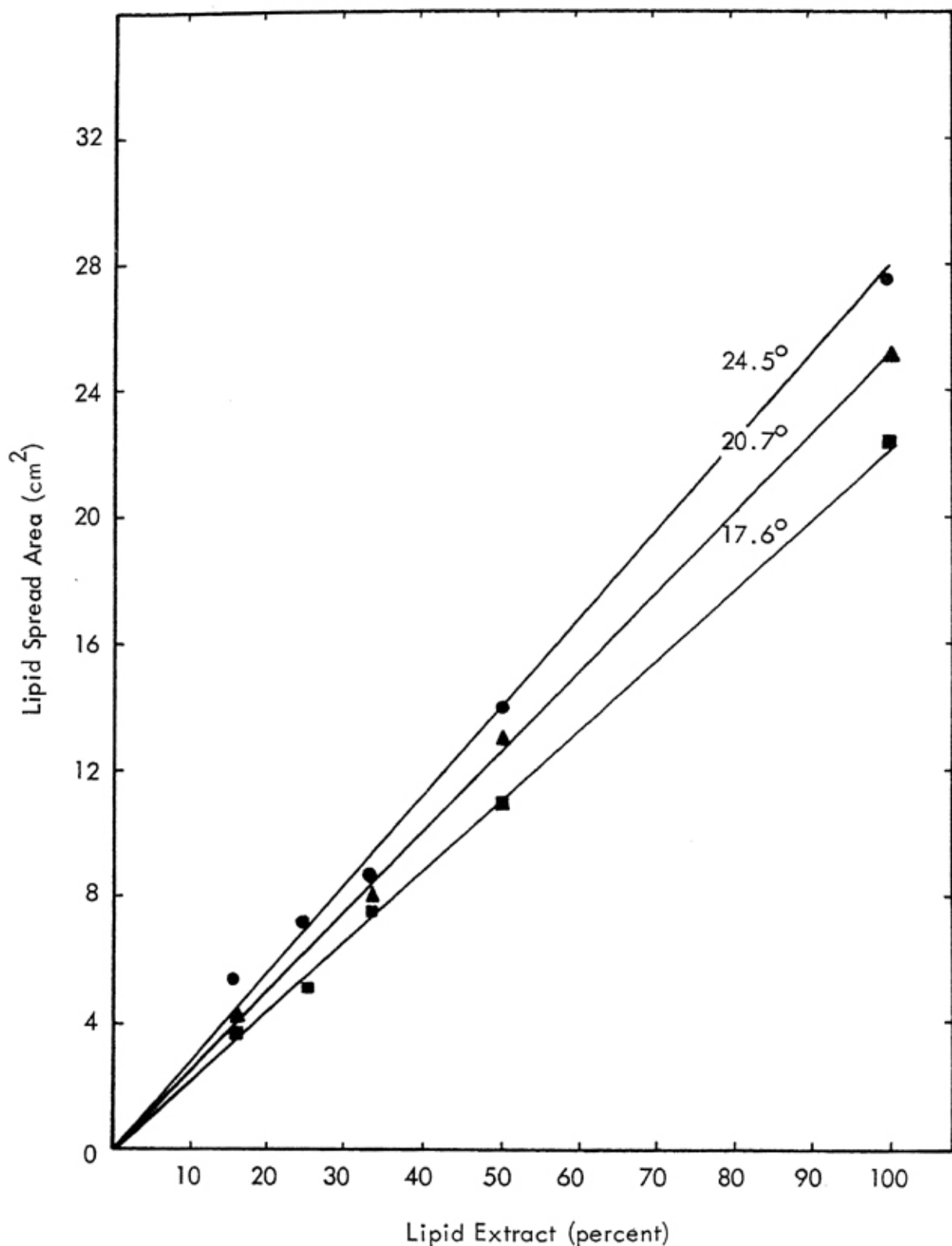


Figure 11. Effect of temperature on the lipid spread area.

ment of the surface pressure after the lipid-petroleum ether solution had been applied.

Different dilutions of a stock solution as described under "Stock Solution Preparation" were used. Oil A was used for this study. The temperature was regulated by a water bath surrounding the pie dish. Each spread was allowed to age for 4 minutes; then triplicate tracings were made. The results of this study are shown in Figure 11.

There was a variation in the area of the lipid spread with temperature change. The experiment was carried out using the tracing method. The data are not shown in table form, but a series of spreads were measured at different temperatures as low as 7° C. and a definite difference in the area of the spread was found. As would be expected, the area of the spread diminished as the temperature decreased.

### Effect of Time on Spread Area

The pie dish and tracing method used was similar to that described for the temperature effect on area. A stop watch was started as soon as possible after application of the lipid-petroleum ether solution. Tracings were made of the spread as often as possible. The time at the start of tracing each spread was recorded within the outline. Area measurement was made by the method described previously under "Tracing and Measurement."

The results are shown in Figure 12. These results were confirmed by the photographic method, but the measurements are not shown due to the greater time lapse used for the photographic recording.

The spreads increased in size and apparently approached an equilibrium size after aging. The larger the area of the spread, the more rapid the increase in the area initially.

Other oils than the one used for this study (oil A) were tried and found to vary in this respect. One of the other oils studied apparently came to an equilibrium pressure quite rapidly as no change in the area was observed by the tracing method. This oil was not satisfactory in other respects and was not utilized other than in this study.

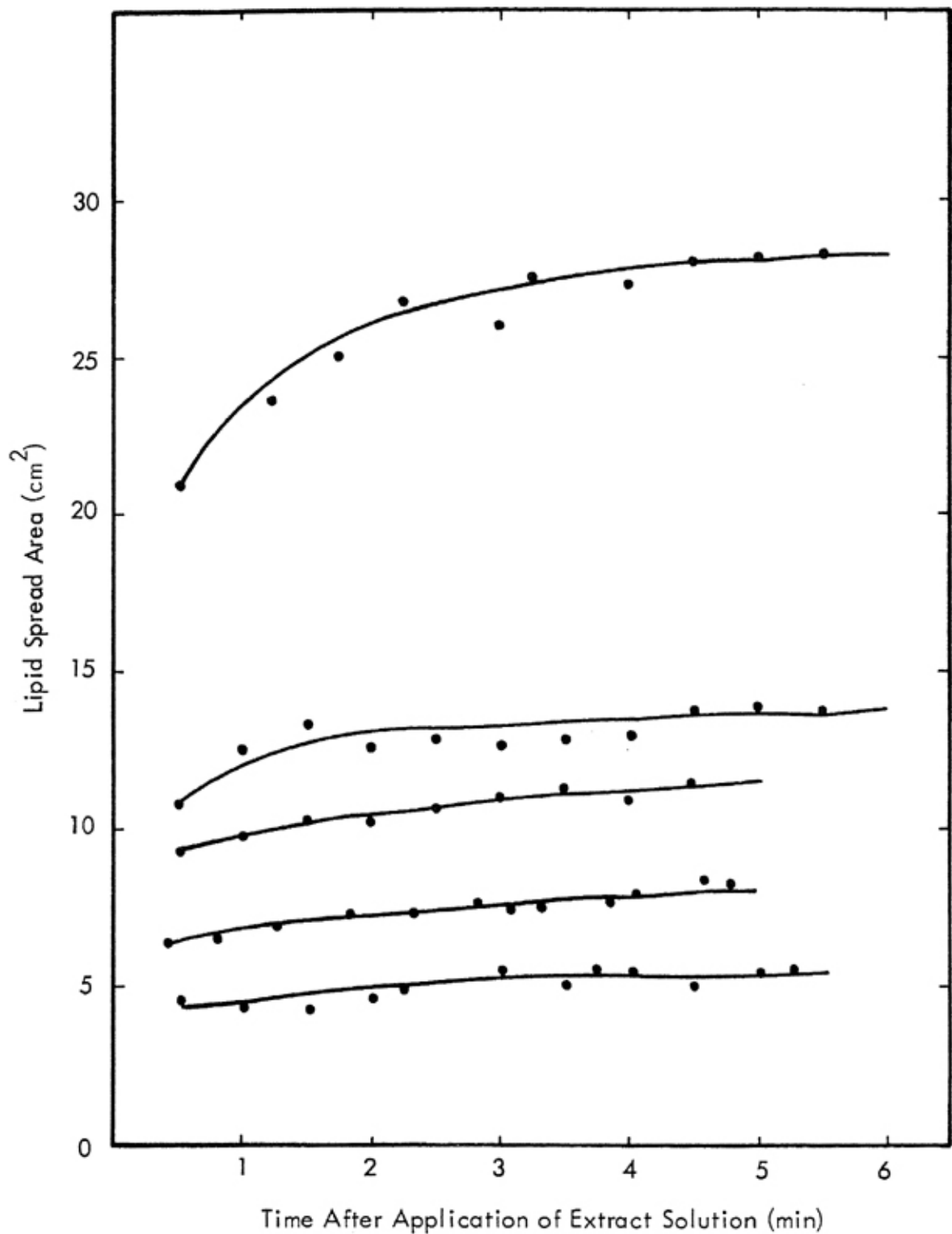


Figure 12. Effect of time after application of lipid extract on the area of lipid spreads.

## Standard Curve

A standard curve was constructed for lipid concentration versus area of the spread utilizing the measuring tray method. The lipids were extracted according to the method given under "Lipid Extraction." After evaporation of the ether solvent, the lipid material was weighed into a 50 ml. volumetric flask and then filled to the mark with petroleum ether at room temperature. Two different dilutions of this solution were made by careful pipetting. The original concentration of the solution was 1.02 mg. of lipid per ml. of petroleum ether. Dilutions of 0.19 mg. of lipid per ml. and 0.51 mg. per ml. were prepared for use in the standard curve.

The micropipette (0.0088 ml.) was used to deliver the lipid-petroleum ether solution to the surface. The acid solution temperature was 24° C. A time interval of 15 seconds was maintained before readjustment of the surface pressure. Six spreads of each concentration were applied and measured to determine the points.

The standard curve prepared for lipid concentration versus area of the spread is shown in Figure 13. The values used for this curve are given in Table 5. The coefficient of variation for the areas determined by this means increase as the area of the spread decreases. The "Y" intercept for the line determined is greater than zero because of a small spread that results from the petroleum ether only.

TABLE 5 - STANDARD CURVE DATA USING THE MEASURING TRAY AS A MEANS OF DETERMINING LIPID CONTENT OF EGG ALBUMEN

Concentration of Egg Lipids in Petroleum Ether (mg./ml.)	No. of Determinations	Equivalent Yolk in Egg Albumen (%)	Average Area (in. <sup>2</sup> ) Using .0088 ml.	Coefficient of Variation (%)
1.02	6	.30	.94	8.86
.51	6	.15	.49	10.80
.20	6	.06	.24	12.50



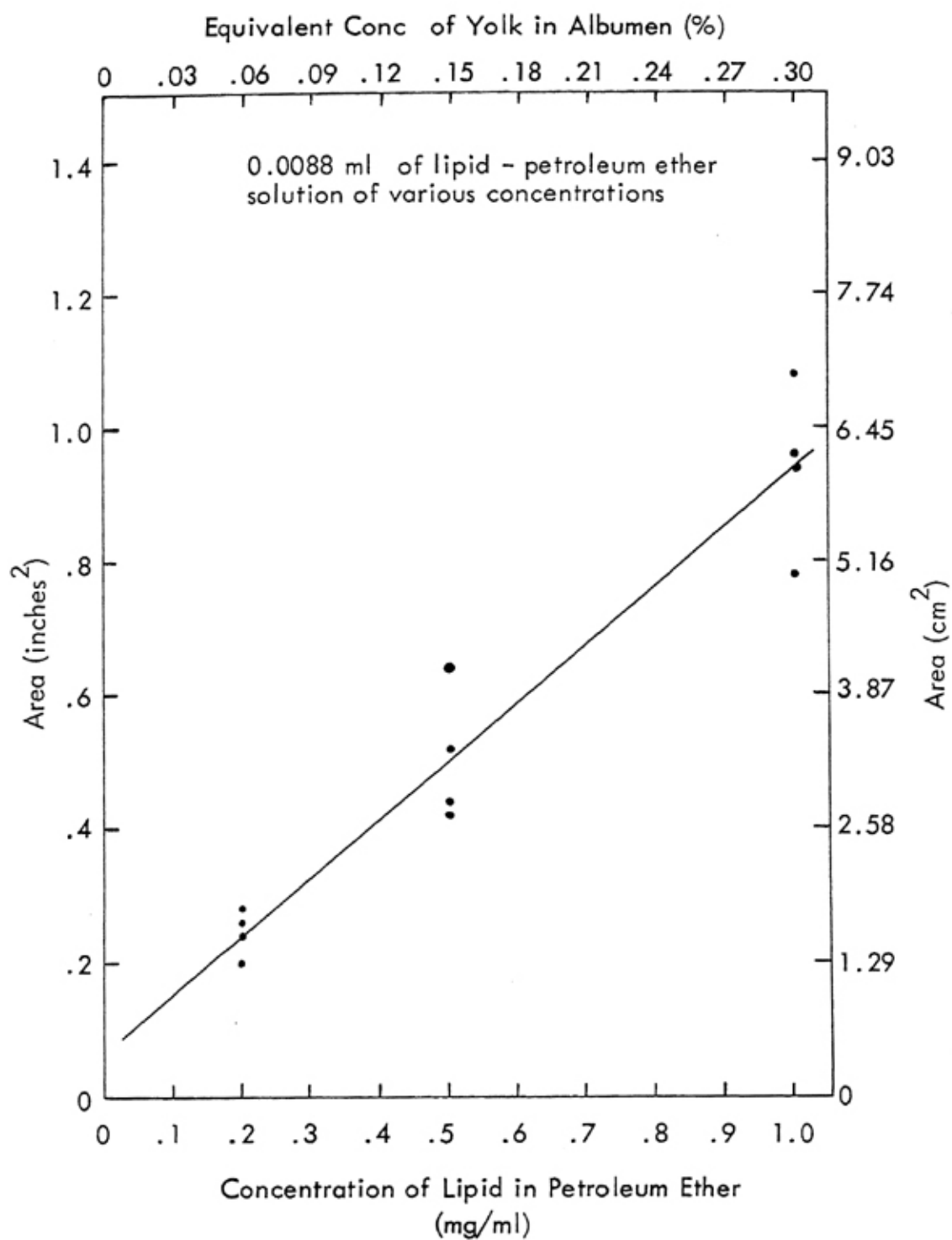


Figure 13. Standard curve for lipid determination using the measuring tray.

## DISCUSSION

The monomolecular surface film method has been discussed by Heinemann and Rohr (1950) and Bergquist and Wells (1956) for their particular applications. These workers enumerated a number of sources of experimental error considered to be important.

Heinemann and Rohr (1950) listed sources of error that they encountered working with milk lipids. They found that the use of a second order green resulted in a greater coefficient of variation than when the third order green was used. They pointed out that a temperature variation between 20° and 30° C. had little effect on the area of the spread. Variations in pipetting, tracing from the glass to paper, and planimeter measurement, were also discussed. Extraction error was thought to be small. The tracing error was considered to be a major problem.

Bergquist and Wells (1955) listed possible errors that they considered to be important. The listed sampling, extraction, solvent measurement, weighing, and contamination of equipment and solvents. Temperature variation was not considered to be important in the 20° to 30° C. range. Tracing error was also mentioned.

This study was oriented to evaluate the other possible sources of variation in the determination. The major factor that has not been covered by these other workers is the effect of surface pressure of the oil film and its effect on the area of the lipid spread.

Factors which affect the surface pressure of the film, and therefore the area of the lipid spread, are: temperature, oil, and time.

It has been assumed in previous papers that the surface pressure of an oxidized oil film, when adjusted to the thickness of the third order green interference color, is constant. The study reported in this bulletin has indicated that this assumption is not valid.

Variations in oil samples used for this test contribute to the differences that may be found. Properties that may be concerned with the variation in behavior of the oils, as used in this test, include refractive index and compositional difference. The composition of the oil may vary depending on the geographical source of the crude oil, variations in the refining process, and the inclusion of additives. Composition of the oil affects the refractive index. Also, refractive index of the oil is determined by density, which varies with temperature.

The interference color of an oil film is a function of the film thickness. However, a given interference color is not an absolute measure of the thickness of the film since it is also affected by the refractive index of the material.

Composition of the oil sample also has a bearing on the reaction of the oil during oxidation. It was not determined just what groups were affected but it seems reasonable to assume that reaction of groups in the oil samples might vary. Variation in this respect would have a bearing on the affinity that might be developed at the oil interface when the oil is spread as a film. This would affect the surface tension or, more correctly, the interfacial tension. The surface pressure of the

film was determined by the use of the hydrophile balance which actually measures differences in surface tension between a film-free surface and a film-covered surface.

There are reasons for possible variation in the surface pressure of oil films at a particular interference color. The effect that these variations have on the accuracy of a lipid determination by this method might be considered in terms of the spreading coefficient. The spreading coefficient, as used in this application, is an expression of units of lipid per unit of lipid spread area.

Variations in the surface pressure used for determination of a spreading coefficient would affect the area of the spread and, therefore, the spreading coefficient. Use of a spreading coefficient calculated by one person is not necessarily valid for use by another individual unless the oils used have the same surface pressure at the designated interference color. The net result is an error in the lipid content of the material being analyzed.

In addition to variation between surface pressures of different oil samples, variation in pressure during one determination is thought to be important. The variation in surface pressure that occurs at this time is caused by displacement of the oxidized oil film by the lipid materials being analyzed. The variation in surface pressure is dependent on the size of the lipid spread and the total area covered by the oxidized oil film. The change in surface pressure is indicated by interference colors. An increase in surface pressure minimized the area of the lipid spread.

Reduction of the lipid spread area would accentuate the importance of tracing error. Each unit of area represents a greater concentration of lipid as the area is diminished by an increase in the surface pressure. This reduction in lipid spread area would affect the spreading coefficient calculated for a particular lipid material and would appear to give a variation in the lipid spreading coefficient depending on the concentration.

The effect of pressure variation can be reduced by spreading the oxidized oil film over a large area. The variation in the surface pressure may be eliminated by readjustment of the film thickness to the original thickness. Readjustment of the thickness may be accomplished by increasing the total area covered by the oxidized oil film and the lipid spread.

The measuring tray method is believed to have certain advantages over the tracing method. The most obvious advantage is the elimination of the tracing and planimeter measurement procedure. Tracing has been cited previously as a source of considerable error. A less obvious advantage is the improvement in representing the actual lipid content as a lipid spread area, by the use of constant pressures. The effect of not readjusting the surface pressure after application of the lipid material to the surface has been pointed out.

The results obtained by this method are not an actual measure of the lipid spread area. The values represented are differences in the areas

that the oxidized oil is spread over to maintain a uniform thickness. The lipid materials added displace the oxidized oil and then occupy a given area per molecule on the surface of the film at the surface pressures used. The oil that is confined to a given area is then forced to increase in thickness. This changes the interference color of the film. When the area of the oil film is then readjusted to the original thickness, as judged by the interference color, a measure of the lipid content is obtained by observing the change in the area.

It has been stated that the actual area of the lipid spread is not measured. However, a straight line relationship exists with the oil used for this test. A standard curve may be prepared for this test. The accuracy of this method can be increased over what has been presented. A certain amount of judgement must be exercised to accomplish this. No modification of the actual procedure is required. All that is required is an adjustment of the initial amount of oil that is applied to form the film. A smaller initial area increased the accuracy of the test when an extremely small amount of lipid was anticipated. If a large area, relative to the size of the spread, is used the sensitivity is decreased. The result may be no apparent change in the interference color. Coefficients of variation shown in Tables 4 and 5 vary considerably by areas of the spreads that were measured. A random amount of oil was delivered to the surface to form the film. The "random" amount of oil was used in the sense that no regulation was exercised by the operator. However, the amount of oil present in the form of a drop would be influenced by temperature of oil and generally does not vary greatly during a series of measurements.

The coefficient of variation was less for the larger area spreads. This was due to increased sensitivity for the oxidized oil film areas that resulted from the amount of oil delivered to the surface. As the size of the spread was diminished sensitivity to color change was also diminished and the coefficient of variation increased. The results shown for the smaller area spreads are not considered valid.

In use, as a quality control method, the amount of lipid may be approximated by an estimation of the relative amount of lipid residue in the aluminum drying dish prior to redissolving the lipid in the petroleum ether.

The operator's color perception is a factor which must be considered. This test does not require a greater degree of color perception than the other method of measuring the lipid spread but care is required that the same color is repeated. It is not necessary that the third order green be used but the color sequence in that thickness of film is favorable. At greater thickness and lesser thickness than the third order green, two shades of pink occur. This provides a good contrasting color and a small change in the area results in a noticeable color change.

Although the viscosity of the oil was not studied as a factor in this test it is suspected that a variation in the viscosity of the oil would have a bearing on the results obtained.

There are several other factors that have not been discussed which may have a bearing on the results of a lipid determination by either of the methods described. The importance of the variation caused by temperature may not be great, but, since it affects the area of the spread, it should be considered. The most reliable results for this test would be obtained by using a constant temperature for the analysis. Temperature would be expected to have an effect on the results of an analysis by the measuring tray method.

Temperature variations could be expected to affect surface pressure, density, and refractive index in this test. Temperature increases would lower surface pressure and decrease the density. A decrease in density would result in a lower refractive index. These variations would occur in the oxidized oil film.

The effect of time on the lipid spread may also vary depending on the oil. The oil used in this experiment allowed an increase in the area of the lipid spread, while some oils did not seem to allow this change.

Jones (1950) has suggested that the lipid spread be allowed to age for three minutes. This procedure would not eliminate the change in area, particularly in large spreads, but would minimize the error. A constant aging time might be utilized to an advantage when the measuring tray method is used.

Many of the changes that occur in the oil film might be minimized or measured with a standard oil modified for use in this test. Factors which might be considered in the selection of a standard oil are:

1. Uniformity of oil film interference color.
2. Refractive index of the oil.
3. Stability of the film pressure exerted by the film.
4. Stability of the oil during storage.
5. Uniform viscosity.

An oil designed or treated to have known properties in regard to these factors would allow further specifications for its use.

## BIBLIOGRAPHY

- Adam, N.K., 1941. *The Physics and Chemistry of Surfaces*. Oxford University Press, London.
- Bikerman, J. J., 1948. *Surface Chemistry for Industrial Research*. Academic Press, Inc., N. Y.
- Bergquist, Dwight H., and Frank Wells, 1956. The Monomolecular Surface Film Method for Determining Small Quantities of Yolk or Fat in Egg Albumen. *Food Technol.*, 10: 48-50.
- Blodgett, K. B., 1934. Monomolecular Films of Fatty Acids on Glass. *J. Amer. Chem. Soc.*, 56: 495.
- Brode, W. R., 1947. *Chemical Spectroscopy*. John Wiley & Sons, Inc., N. Y.
- Gorter, E., and F. Grendel, 1925. On Bimolecular Layers of Lipids on the Chromocytes of the Blood. *J. Exp. Med.*, 41: 439-443.
- Harkins, William D., 1952. *The Physical Chemistry of Surface Films*. Reinhold Publishing Corp., N. Y.
- Heinemann, Burdet, and M. Robert Rohr, 1950. A Micro Method for Routine Determination of Fat in Skim Milk and Nonfat Dry Milk Solids. *J. Dairy Sci.*, 33: 703-709.
- Jones, K. K., 1950. A Micro Method for Fat Analysis Based on Formation of Monolayer Films. *Science*, 111: 9-10.
- Langmuir, Irving, 1917. The Constitution and Fundamental Properties of Solids and Liquids. II. Liquids. *Amer. Chem. Soc. Jour.* 39: 1848-1906.
- Pockels, Agnes, 1891. Published Correspondence to the Editor. *Nature*, 43: 437-439.
- Rayleigh, Lord, 1899. XXXVI. Investigations in Capillarity: The Size of Drops, the Liberation of Gas from Super-saturated Solutions, Colliding Jets, The Tension of Contaminated Water-Surfaces. *Phil. Mag.* 48: 321-337.
- Rideal, E. K., 1930. *An Introduction to Surface Chemistry*. Cambridge University Press, Longon.
- Romanoff, A. L., and A. J. Romanoff, 1949. *The Avian Egg*. John Wiley & Sons, Inc., New York.
- Sachanen, A. N., 1945. *The Chemical Constituents of Petroleum*. Reinhold Publishing Corp., N. Y.
- Sears, Francis Weston, and Mark W. Zemansky, 1950. *University Physics*. Addison-Wesley Press, Inc., Cambridge, Mass.
- Smith, C. F., 1959. Shell Egg Deterioration: Diffusion of Yolk Lipids into Albumen as the Natural Cause of Failures in Performance. *Poultry Sci.*, 38: 181-192.
- Sobotka, Harry, 1954. *Monomolecular Layers*. Amer. Assoc. Adv. Sci., Washington, D. C.