1944-1945—W. W. Wynn, Jr., Barrow-Agee Lab's., Cairo, Ill.
1945-1946—L. B. Forbes, L. B. Forbes Lab's., Little Rock, Ark.
Russell Haire, Planters Mfg. Co., Clarksdale, Miss.

R. R. HAIRE
F. F. HASBROUCK
L. H. HODGES
T. C. LAW
R. C. POPE
J. J. VOLLERTSEN, chairman.

CORRECTION

The words linoleic and linolenic were interchanged in the equations and sample calculations shown on page 142 of the May, 1946 issue of Oil & Soap in the article on "Applied Ultraviolet Spectrophotometry of Fats and Oils," according to the author, B. W. Beadle, American Meat Institute, Chicago.

Comparison of a Simplified, Quantitative Kreis Test With Peroxide Values of Oxidizing Fats*

BETTY M. WATTS and RUTH MAJOR
Home Economics Division, Agricultural Experiment Station,
State College of Washington, Pullman, Wash.

THE two chemical tests most widely employed in recent years to indicate rancidity in fats are (1) estimation of the peroxides by titration of the iodine liberated and (2) estimation of the color produced by reaction between oxidizing fats and phloroglucinol in acid solution (Kreis test).

Earlier work on the Kreis test has been well summarized by Lea (1). One of the chief objections to the test in its original form is the fact that the color development took place in a two-phase system and that the color was often distributed between the acid and the ether phases, in an irregular manner. Attempts to make the test quantitative in this form were not highly satisfactory (2). Walters, Muers, and Anderson (3) succeeded in developing the color in a single phase by dissolving the phloroglucinol in amyl acetate and substituting trichloracetic for hydrochloric acid. They found the test to be highly sensitive as compared to the older Kreis method and subject to quantitative measurement in a Zeiss-Pulfrich photometer.

White (4) compared the Walters, Muers, and Anderson method with peroxide oxygen values and with other methods of estimating rancidity on bacon fat. He found that the modified Kreis test was the most sensitive, gave excellent precision, and best correlation with the peroxide value. However, he concluded that the peroxide test was somewhat easier to apply.

Pool and Prater (5) developed a simplified modification of the Walters, Muers, and Anderson procedure, using glacial acetic acid in place of amyl acetate. The essential features of their method were obtained by private communication some months before its publication. In the present paper the simplified procedure has been applied to a number of oxidizing fats, and the results have been compared with peroxide values and iodine numbers on the same fats.

Analytical Methods

Peroxide Value. The Wheeler method (6) was used with minor modifications. The results were found to be more reproducible if the period of standing was increased to 10 minutes before titrating with thiosulfate. Stansby (7) has emphasized the importance of using the same weight of fat in this test; 0.5 gm. were used throughout. The peroxide numbers were expressed as millimols of peroxide per 1,000 gm. oil, i.e., peroxide number =

$$\frac{0.5\ (\text{ml. thio. used in titration})\ (\text{normality of thio.}) \times 1{,}000}{\text{wt. of fat}}$$

Iodine Numbers. The Hanus method as outlined by Woodman (8) was used.

Modified Kreis Test. Since this test as carried out differed in a number of minor details from that later published by Pool and Prater (5), the exact procedure used is described here.

Reagents. (A) Thirty gm. trichloracetic acid plus 100 ml. glacial acetic acid. (B) One gm. phloroglucinol plus 100 ml. glacial acetic acid (see note 6 on the method).

Procedure. Aliquots of chloroform solutions containing 0.2 gm. of fat (see Note 3) were transferred to colorimeter tubes and made up to 3 ml. with chloroform. Six ml. of reagent A and 1 ml. reagent B were added, the tube shaken (note 5), allowed to stand exactly 15 minutes at 37° C. (note 1), and cooled 3 minutes. A blank containing the fat but omitting the phloroglucinol and using 7 instead of 6 ml. of reagent A was prepared at the same time and treated in the same way (note 2).

The color was read in an Evelyn photoelectric colorimeter, using a 540 filter and setting the instrument at 100% transmission with the blank. The Kreis value was expressed as the optical density (i.e., 2-log galvanometer reading) divided by .02 (the concentration of fat in gm. per ml. of solution in the colorimeter tube).

NOTES ON THE KREIS METHOD

1. *Time and Temperature of Color Development.* The conditions for color development specified above were chosen arbitrarily. As pointed out by Walters, *et al.* (3) the red color does not reach a maximum at any time or temperature but changes gradually to a yellow color. Hence, the absolute values recorded for the Kreis test depend upon the time and temperature chosen for color development. The temperature effect

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was very marked; the color developed in 15 minutes at 37° C. was approximately equal to that obtained after 1 hour at 25° C.

The rate of color development in the animal fats studied fell off rapidly after the first 10 to 15 minutes at 37° C. The Kreis value after 30 minutes at 37° C. was approximately 10 to 15% higher than after 15 minutes at the same temperature. On the other hand, with vegetable oils high in linoleic acid (cottonseed and corn), color development continued at a rapid rate, giving values approximately 50% higher at 30 minutes than those obtained at 15 minutes. Pool and Prater (5) suggest the addition of alcohol to stop color development during the reading of the tubes.

2. *Blank Setting.* Since the phloroglucinol was omitted from the blank, it was essential to check the transmission of a phloroglucinol blank, from which the fat was omitted. With good samples of phloroglucinol, the galvanometer reading with phloroglucinol did not differ appreciably from that obtained with chloroform and reagent A alone. Impure samples of phloroglucinol on the other hand, may show considerable color and should not be used. With colorless fats, such as lard, fresh refined vegetable oils, etc., the fat blank may be omitted and the instrument set with phloroglucinol blank, but with chicken fat, butter, etc., the fat should be included in the blank.

3. *Effect of Varying Fat Concentration.* As pointed out by White (4) the Kreis value is not constant when the concentration of fat is varied. However, the change in Kreis value with fat concentration was reasonably constant for the various fats tested. Correction values are shown in the accompanying curve (Fig. 1).

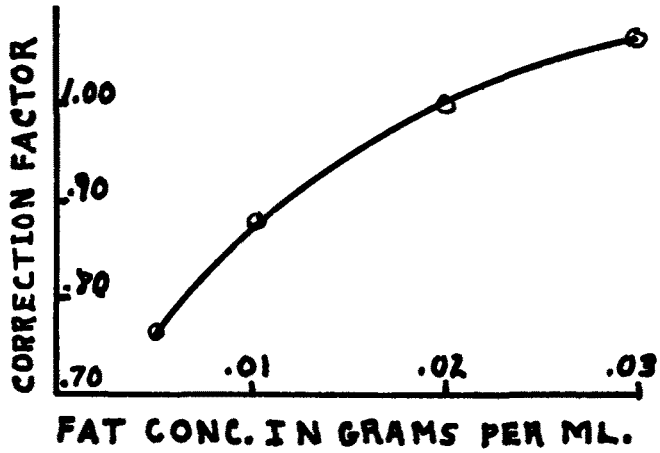

FIG. 1. Correction factors for converting Kreis values obtained with various concentrations of fat to values obtained with .02 gm./ml.

4. *Change in Kreis Value on Standing.* Kreis values on several samples of pork fat increased by an average of 9% on standing in chloroform solution for 24 hours. The determinations should, therefore, be completed on the same day that the fat is extracted. Under the same conditions no change in the peroxide value was noted.

5. *Shaking.* Walters, *et al.* (3) were unable to obtain reproducible results when the method of shaking was changed even slightly and recommend bubbling air through the tubes during the time of color development. With the modification of the method described above this seems not to be an important factor. No difference in reading was obtained between tubes shaken with a rotary motion just long enough to mix (about 10 seconds) as compared to tubes given an additional vigorous aeration for a period of 1 minute.

6. *Stability of Solutions.* Both reagents kept for a period of at least 7 months in dark bottles without showing any visible depreciation or any change in the ratio of the Kreis to peroxide values obtained on individual fats. Most of the results reported in this paper were obtained with a single lot of reagents. However, toward the end of the work reported here, new reagents were prepared, using a different lot of trichloracetic acid. The values obtained with the new trichloracetic reagent did not check with values on the same fat using the old reagent. Differences as great as 30% were obtained. These discrepancies are believed to be due to differences in the amount of water present in the trichloracetic acid. It was observed that the second lot of trichloracetic used was practically completely liquefied, whereas the first was solid. Addition of 5% water to Kreis reagent A caused a 34% reduction in the Kreis number of a sample of pork fat. Evidently careful drying of the trichloracetic is essential for reproducible results. Kreis numbers here reported were made with the same reagents and are comparable among themselves.

Experimental

Accelerated Tests. The fats used in this study had all passed their induction periods and were oxidizing rapidly at the time these comparisons were made. In all cases 2 gm. samples of the respective fats were weighed out into 100 ml. beakers and placed in an electrically heated, thermostatically controlled air oven at 105° C. At intervals the beakers were withdrawn from the ovens, the fats made up to 20 ml. with chloroform, and aliquots of the chloroform solution used for peroxide number, Kreis value and iodine number respectively.

Results for several representative fats are shown in Fig. 2. Several points of interest are brought out by these curves:

(1) Peak values for the Kreis test varied greatly with the fats tested whereas the peak attained by the peroxide number was fairly constant. In the case of refined cottonseed oil the curves were not plotted since at peroxide values greater than 150 color development in the Kreis test was so intense that even when the fat concentration in the colorimeter tube was reduced to .005 g./ml. the galvanometer readings were within 1 or 2 spaces of zero transmission and hence the Kreis values could not be accurately determined. Corn oil showed the same intense color development. The absolute values but not the shape of the curves were changed by varying the time for color development in the Kreis test, i.e., peak values were higher if a longer time was allowed for color development, but the time at which peak values were obtained was not shifted.

The intensity of the Kreis reaction seemed to parallel roughly the linoleic acid content of the fats studied, so far as this could be determined by values for linoleic acid given in the literature (Table 1). Such a correlation might be expected, since as pointed out by Lea (1) epihydrin aldehyde could easily be formed from the $=CH.CH_2CH=$ grouping of lino-

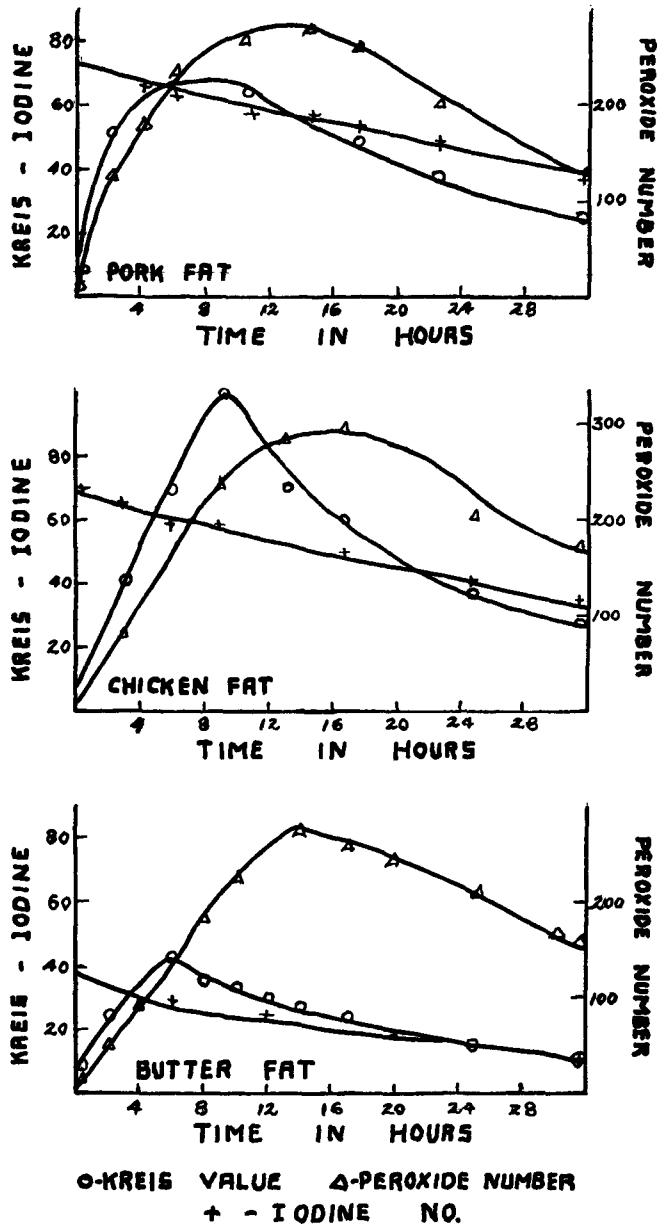

FIG. 2. Comparison of Kreis values, peroxide numbers and iodine numbers of several animal fats undergoing accelerated oxidation at 105° C.

leic (and linolenic) acids by peroxide formation and splitting at the double bonds, but a preliminary desaturation would be necessary to produce it from oleic. For vegetable oils containing considerable amounts of linoleic or linolenic acids a modification of the Kreis test employing greater dilution or lesser concentration of acid would need to be devised.

(2) As has been pointed out previously, both the peroxide number (11) and the Kreis test (3) go through a maximum during prolonged oxidation of a fat, but the relative positions of the maxima were not determined in earlier studies. Although peroxide formation is believed to be a preliminary step in the formation of epihydrin aldehyde, the peak of the Kreis test was in all cases attained long before the peroxide number reached its peak.

It was considered possible that the relatively early attainment of peak values in the Kreis test was due to the fact that the measurements were being made on

TABLE 1

Correlation Between Linoleic Acid Content and Kreis Values

Fat or Oil	Linoleic Acid*	Maximum Kreis Value Obtained at 105° C.
	%	
Butterfat	2- 4	40
Lard	6	68
Chicken fat	18-29	98
Corn oil	34-42	>300
Cottonseed oil	48-51	>300

* Figures for chicken fat taken from Nutter, *et al.* (9), others from Bailey (10).

mixed glycerides, the fatty acid components of which differed in the ease with which their peroxides were converted to epihydrin aldehyde. For example, peroxidation of linoleic acid precedes that of oleic acid (12) and the linoleic peroxide might be expected to decompose more rapidly to form epihydrin aldehyde. The comparisons were, therefore, repeated on pure ethyl oleate. The conditions of the experiment were the same except that an oven regulated at 93° C. had to be used for carrying out the accelerated tests. Because of the lower temperature, the absolute values for the ethyl oleate, shown in Fig. 3 are not compar-

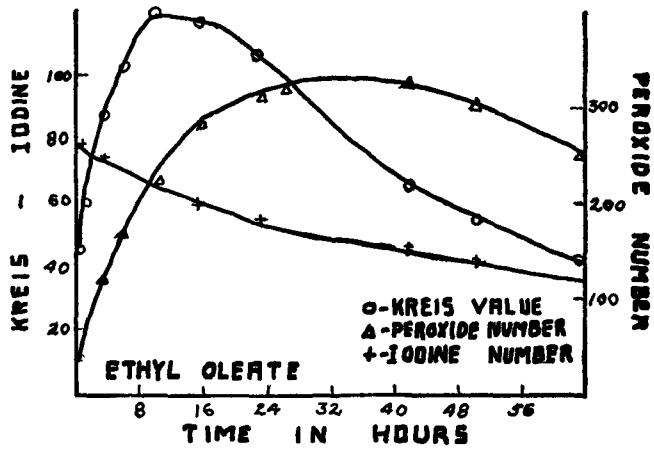

FIG. 3. Comparison of Kreis values, peroxide numbers and iodine numbers of pure ethyl oleate undergoing accelerated oxidation at 93° C.

able with those for the triglycerides (Fig. 2). However, with the pure oleate, as with the mixed triglycerides, the peak of the Kreis test was definitely earlier than the peroxide peak. It is obvious that, while good correlation between Kreis and peroxide values may be expected during early stages of rancidification, no such correlation exists at advanced stages of rancidification, where peroxide numbers continue to increase while Kreis values fall off rapidly.

3. Corroborating earlier work (9) a high degree of rancidity, as indicated by both Kreis and peroxide tests, is attained at a time when a relatively small proportion of the double bonds have been oxidized. At a stage of rancidity sufficiently high to be detected organoleptically the reduction in the iodine number was scarcely as great as the experimental error of the method.

Effect of Temperature of Storage on Kreis and Peroxide Values

A number of investigators have called attention to the falling off of peak peroxide values with increasing temperature. This has been ascribed to a relatively

greater increase in rate of peroxide breakdown than in rate of peroxide formation as temperature is increased (7). Since epihydrin aldehyde is presumably a secondary product, formed from the decomposition of peroxides, it was hoped that the Kreis test might prove more constant than the peroxide number with changing temperatures. The opposite proved to be the case. Not only peak values for the Kreis test but also ratio of Kreis to peroxide decreased with increasing temperature. At a temperature of 105° C. peak value for the Kreis test on pork fat was approximately 68 and the peroxide peak 250, a ratio of Kreis to peroxide of .27; at 38° C. peak value for the Kreis was 144 as compared to a peroxide of 327 (ratio = .44).

Aliquots of the same sample of pork fat used for the accelerated test (Fig. 2) were prepared in the same manner as for the accelerated tests (2 g. samples placed in 100 ml. beakers) but stored in a refrigerator at 5 to 8° C. Whereas at the higher temperature the Kreis value had reached its peak in 8 hours, in the refrigerated samples Kreis as well as peroxide values were still going up steadily at the end of 237 days of storage. At this time the Kreis value was 99 and the peroxide 159.

Refrigerated butter samples showed even greater stability. The sample of butter which reached its peak Kreis value at the end of 7 hours at 105° C. gave only faint peroxide or Kreis tests (values less than 5) after 300 days of refrigerated storage, although most of the color had bleached out before 200 days in storage.

Correlation Between Kreis Test and Peroxide Values in Non-rancid Pork Fats and Corn Oil. Fig. 4 shows the Kreis and peroxide values obtained during early stages of rancidification. A peroxide value of 20 was accepted as the point at which rancidity was definite. Stored frozen sausages, after thawing, were in the large majority of cases pronounced rancid when the peroxide value was over 20. "Slight rancidity" was very often noted in samples having peroxide values between 10 and 20.

The accelerated tests plotted in Fig. 4 were all done on fat from a small group of hogs on the same diet. On the other hand, the frozen sausages were taken from different hogs on a variety of diets, which may account for greater spread of values in the latter group. The relatively greater Kreis to peroxide ratio at the lower storage temperature is again apparent.

Several points on the curve for corn oil (a single sample of oil held at 93° C.) were plotted for comparison. Not only were the Kreis values at any given peroxide number much higher than for pork fats, but also color development was increasing rapidly at the arbitrary time limit of 15 minutes at which all readings were made.

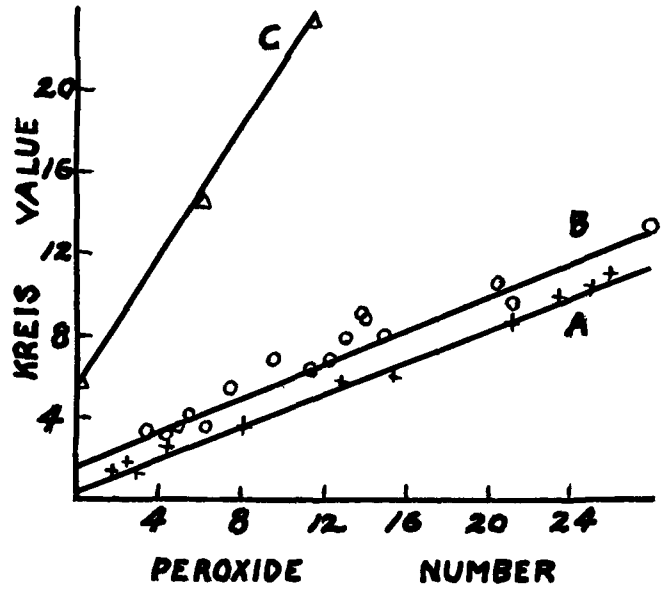

FIG. 4. Correlation between Kreis values and peroxide numbers of pork fat and corn oil during early stages of rancidification. A—pork fat at 93° C., B—fat extracted from frozen pork sausages, C—corn oil at 93° C.

Summary

1. A simplified quantitative Kreis test has been compared with peroxide numbers and iodine values during prolonged rancidification of several animal and vegetable fats.

2. The ratio of Kreis to peroxide values varied with the kind of fat and seemed to parallel its linoleic acid content. With vegetable oils containing a large amount of linoleic acid (corn and cottonseed) the intensity of color at the time of peak color development was too great to be read on the colorimeter.

3. With all fats for which accurate determinations were possible (butter, chicken, pork, and ethyl oleate), the Kreis values reached maximum and began falling off while peroxide values were still increasing.

4. Temperature of fat storage had a greater effect on the Kreis test than on the peroxide number. Increasing the temperature caused a decrease not only in the peak values obtained for the Kreis test, but also in the ratio of Kreis to peroxide values.

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