Kinetics of the Kreis Test Through Response Surface Methodology

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The Kreis test is one of the procedures for early detection of oxidative products of fats and oils. The reaction is complicated, sensitive and continuous. The kinetics of the Kreis test have been studied as a function of reagent, phloroglucinol, oil quantity and incubation period for three oils with different fatty acid profiles, storage life and presence of compounds other than triglycerides. A central composite rotatable design with each factor at five levels required 20 experiments for each oil. The results of experiments carried out in duplicate have been examined by analysis of variance, and polynomials of appropriate degree were fitted to the data. The polyhedron search method was used to compute optimum conditions for carrying out the test for each oil. Three-dimensional graphs were generated to bring out the differences in the oils and the kinetics for each oil. Though "compromised" optimized conditions have been reported, the results indicate the necessity of more detailed investigation into the reaction.

KEY WORDS: Kinetics, Kreis test, optimization, rancidity.

Edible oils are subjected to various tests routinely, such as peroxide value, free fatty acids (FFA), thiobarbituric acid and sensory analysis, for detecting the onset of rancidity. In addition to these analyses, the Kreis test is also used occasionally. The method, originally recommended by Kreis (1), involves the production of a red color when phloroglucinol (PG) reacts with oxidized fat in acid solution. The reaction itself, as well as reagent and oil levels, has been studied on and off in this test because of its promising role in early detection of rancidity, particularly the by-products of the oxidation, such as aldehydes. Following the work of Powick (2), Patton *et al.* (3) made a thorough study of the Kreis test. They confirmed the observation of Lea (4) and Hilditch (5) that basically, when fats are oxidized, certain unsaturated fatty acids undergo rearrangement of malonic dialdehyde to form epihydrin aldehyde, which in turn gives a red color in the Kreis test. Being isomers, both malonic dialdehyde and epihydrin aldehyde have the characteristic structure to give a Kreis positive test, along with a large number of other compounds. Their study was with particular reference to oxidized milk fat, and they stated that the Kreis test could be safely used to identify early stages of oxidation of fats. The test has, however, come under criticism by Mehlenbacher (6) and by Gray (7) because it does not parallel development of rancidity, which is not well defined. However, the test continues to evince interest among researchers and is quoted in several research papers (8–10); it is also adopted in national standards (11). It was considered worthwhile to study the kinetics of the Kreis test because PG is a sensitive reagent, and the color development is almost instantaneous. Identification of conditions with respect to quantities of oil, PG and incubation time from the study of reaction kinetics is required to indicate how convenient and satisfactory or relatively stable measurements can be made. It was decided to study the above three factors with a suitable experimental design that covered a wide range in each factor.

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The kinetics thus studied on palmolein oil (PO), rice bran oil (RBO) and double-filtered groundnut oil (GNO) have been reported in this paper.

MATERIALS AND METHODS

Materials. PO was procured from the public distribution system, RBO from a solvent extraction plant and GNO from the local market.

Oils were analyzed for PV, FFA and sensory quality immediately after procurement, and these values were referred to as "initial value" and were found to be within permissible limits.

Kreis test. Oil was dissolved in 5 mL chloroform, which was made acidic with 10 mL of trichloroacetic acid (30%) in glacial acetic acid and mixed well. PG (1% solution in glacial acetic acid) was added, mixed well and incubated for predetermined durations at 55°C as per the design. The mixture was then cooled to room temperature and quenched with 4 mL ethanol. Absorbance was read at 547 nm against a blank, run simultaneously. The absorbance maximum of 547 nm was selected as reported in earlier studies and also confirmed from the absorption spectrum recorded on a double-beam spectrophotometer between 330–770 nm.

Design of the experiments. The factors considered important in the Kreis test were PG (1–5 mL of 1% in acetic acid); oil (1–5 mL); and incubation period (10–90 min). A central composite rotatable design (CCRD) for the three factors, each at five levels, was selected (12), which required 20 experiments to be conducted. Table 1 gives the

TABLE 1

Design of Experiments for the Kreis Test[a]

Experiment number	PG (mL)	Oil (mL)	Incubation period (min)
1	1.8	1.8	15.6
2	4.2	1.8	15.6
3	1.8	4.2	15.6
4	4.2	4.2	15.6
5	1.8	1.8	57.6
6	4.2	1.8	57.6
7	1.8	4.2	57.6
8	4.2	4.2	57.6
9	1.0	3.0	30.0
10	5.0	3.0	30.0
11	3.0	1.0	30.0
12	3.0	5.0	30.0
13	3.0	3.0	10.0
14	3.0	3.0	90.0
15	3.0	3.0	30.0
16	3.0	3.0	30.0
17	3.0	3.0	30.0
18	3.0	3.0	30.0
19	3.0	3.0	30.0
20	3.0	3.0	30.0

[a]The coded values were calculated as follows: for phloroglucinol (PG): X_1 (PG level − 3)/1.19; for oil: X_2 (oil level − 3)/1.19; for incubation period: X_3 [Ln (incubation period) − 3.4012]/0.6532.

details of the experimental points as per the design. The experiments were run in duplicates.

Statistical analysis and optimization. The data on absorbance in the experiments with the three variables were subjected to analysis of variance (ANOVA), appropriate to the design. Appropriate multinomial models were fitted, and minimum levels of the three factors were worked out to maximize the absorbance by using a transcripted version (in Turbo Pascal) of the polyhedron search method as described by Nelder and Mead (13). The general multinomial fitted follows:

$$Y = b_0 + \Sigma\, b_i X_i + \Sigma\Sigma_{i \leqslant j}\, b_{ij} X_i X_j + \varepsilon \qquad [1]$$

where Y is the predicted absorbance, b_0 the constant intercept, b_i the linear regression coefficients, b_{ij} the first-order interaction coefficient when i is not equal to j and quadratic coefficients otherwise, X_i and X_j are levels of different factors and ε is the random error.

The analysis was carried out on an Intel 80386-based IBM compatible PC-AT with software developed in this laboratory (14). Three-dimensional (3-D) graphs of the fitted response surfaces were generated by using a graphics package developed in this laboratory (15).

RESULTS AND DISCUSSION

The responses of the three oils were quite different from each other, and the three factors chosen showed different types of interactions. For this reason, the results for each oil are reported separately.

PO. The ANOVA indicated that variation in PG level did not show any effect on the absorbance ($P > 0.05$) nor did it show any interaction with oil or incubation period ($P > 0.05$). Therefore, PG was not considered in the model. The level of oil and incubation duration showed highly significant effects ($P \leqslant 0.05$) on the absorbance (Table 2). The model, fitted with multiple correlation of 0.92 follows:

$$\text{absorbance} = \exp\{-1.029 + 0.307X_2 + 0.0798X_3 - 0.047(X_2)^2\} \qquad [2]$$

where X_2 and X_3 are explained as in Table 1.

The experimental and the model-predicted absorbance values are given in Table 2. The closeness of the two reflects the adequacy of the fitted model, as well as the transformation used while planning the experiments as per CCRD. Although PG did not appear in the model, it is essential for the reaction to take place. The results presented here clearly indicate that even the lowest level of PG used in the study (1 mL) was sufficient to react even with the highest level of oil used (5 mL).

RBO. It is apparent from Table 2 that all three factors, namely PG, oil and incubation duration, showed their influence on the final absorbance ($P \leqslant 0.05$). PG showed significant interaction with incubation time ($P \leqslant 0.05$), while only oil indicated existence of quadratic effect ($P \leqslant 0.05$). The fitted model, with a multiple correlation of 0.88, follows:

$$\text{absorbance} = \exp\{-0.122 + 0.127X_1 + 0.153X_2 + 0.0957X_3 + 0.0769X_1X_3 + 0.0694(X_2)^2\} \qquad [3]$$

where X_1, X_2 and X_3 are as in Table 1.

Because all coefficients are positive, the absorbance increased with increasing levels of all the factors. The observed and model-predicted absorbances are again given in Table 2. For some experiments, the deviation between the two absorbances was sometimes 0.2 or greater at higher levels of absorbance.

TABLE 2

Observed and Model-Predicted Absorbances for the Three Oils[a]

Experiment number	PG (mL)	Oil (mL)	Time (min)	PO		RBO		GNO	
				Observed	Predicted	Observed	Predicted	Observed	Predicted
1	1.8	1.8	15.6	0.237	0.232	0.706	0.704	0.220	0.219
2	4.2	1.8	15.6	0.223	0.232	0.696	0.777	0.244	0.181
3	1.8	4.2	15.6	0.431	0.428	0.978	0.956	0.310	0.213
4	4.2	4.2	15.6	0.350	0.428	1.070	1.056	0.338	0.311
5	1.8	1.8	57.6	0.318	0.272	0.830	0.731	0.316	0.272
6	4.2	1.8	57.6	0.314	0.272	1.154	1.098	0.336	0.388
7	1.8	4.2	57.6	0.546	0.502	1.132	0.992	0.482	0.517
8	4.2	4.2	57.6	0.532	0.502	1.625	1.491	1.625	1.296
9	1.0	3.0	30.0	0.340	0.357	0.614	0.715	0.192	0.225
10	5.0	3.0	30.0	0.374	0.357	1.091	1.095	0.354	0.417
11	3.0	1.0	30.0	0.162	0.187	0.978[b]	0.885	0.161	0.173
12	3.0	5.0	30.0	0.566	0.524	1.245	1.393	0.362	0.466
13	3.0	3.0	10.0	0.356	0.312	0.778	0.753	0.142	0.200
14	3.0	3.0	90.0	0.316	0.409	0.814	1.039	0.814	0.799
15	3.0	3.0	30.0	0.319	0.357	0.848	0.885	0.177	0.153
16	3.0	3.0	30.0	0.358	0.357	0.820	0.885	0.143	0.153
17	3.0	3.0	30.0	0.342	0.357	0.899	0.885	0.151	0.153
18	3.0	3.0	30.0	0.380	0.357	0.868	0.885	0.160	0.153
19	3.0	3.0	30.0	0.380	0.357	0.934	0.885	0.155	0.153
20	3.0	3.0	30.0	0.364	0.357	1.021	0.885	0.151	0.153

[a]PO, palm olein; RBO, rice bran oil; GNO, groundnut oil; see Table 1 for other abbreviation.
[b]Single value, rest in duplicates.

GNO. The absorbance showed dependence on all three factors as well as the interactions between them ($P \leq 0.05$). The incubation time also showed its quadratic influence (Table 2). The fitted model (multiple R = 0.95) follows:

$$\text{absorbance} = \exp\{-1.877 + 0.183X_1 + 0.295X_2 + 0.411X_3 + 0.246(X_1)^2 + 0.142X_1X_2 + 0.136X_1X_3 + 0.218(X_2)^2 + 0.167X_2X_3 + 0.340(X_3)^2\} \quad [4]$$

where X_1, X_2 and X_3 are as in Table 1.

Again, as in the case of RBO, all terms in the model have shown positive effects and add to the absorbance. For some experiments, deviations between observed and predicted values at higher levels of absorbance were up to 0.3.

Optimization. The minimum levels of PG, oil and incubation periods were computed so that absorbance comparable to 0.5 could be attained for convenient measurement. The oil was fixed at 1 mL, and incubation periods of $\leq$ 25 min were sought. The absorbance responses were searched by using the Nelder and Mead algorithm as detailed earlier. The optimum conditions thus obtained are presented in Table 3. The expected absorbances are also given under those conditions.

Response surfaces (3-D graphs). PO: At a fixed time of 25 min, the dependence of absorbance on oil quantity and PG is shown in Figure 1. It is clear that the minimum quantity of PG (1 mL) was capable of reacting with increasing levels of oil and producing linearly increasing absorbances. This indicates that the proportion of oil to reagent is important, and the smaller quantities of oil will be preferable to conclude reaction within 25 min. Figure 2 shows absorbance as a function of PG and incubation duration. The response remained unaffected by PG for the reasons given earlier for Figure 1, while with incubation time the absorbance increased but at a much lower rate, especially beyond 25 min of incubation. The absorbance as a function of incubation duration and oil is shown in Figure 3. At the lowest level of oil (1 mL), there was hardly any gain in absorbance with incubation. On the other hand, the absorbance increased with oil at all levels of incubation. At higher levels of oil, the absorbance increased slowly with incubation.

RBO: Figure 4 gives the 3-D plot relating absorbance to oil quantity and PG at incubation fixed at 15 min. Up to 2.5 mL of oil, there was hardly any increase in absorbance irrespective of the PG level. And beyond 2.5 mL oil, the absorbance increased sharply, depending on the level of PG. The absorbance increased marginally with PG at all levels of oil. The dependence of absorbance on the incubation period and PG is shown in Figure 5 at 1 mL oil. The incubation hardly showed any effect on absorbance

TABLE 3

Optimum Conditions for the Kreis Test for Different Oils[a]

Factor	PO	RBO	GNO
PG (1% in acetic acid) (mL)	1.0	1.0	1.0
Oil (mL)	1.0	1.0	1.0
Incubation time (min)	25	15	18
Expected absorbance	0.18	0.71	0.50

[a]See Tables 1 and 2 for abbreviations.

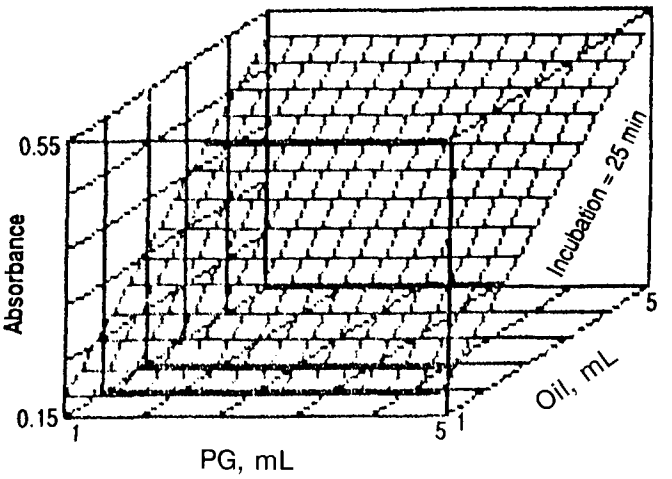

FIG. 1. Absorbance at 547 nm as a function of phloroglucinol (PG) and oil quantities for palm oil at 25 min of incubation.

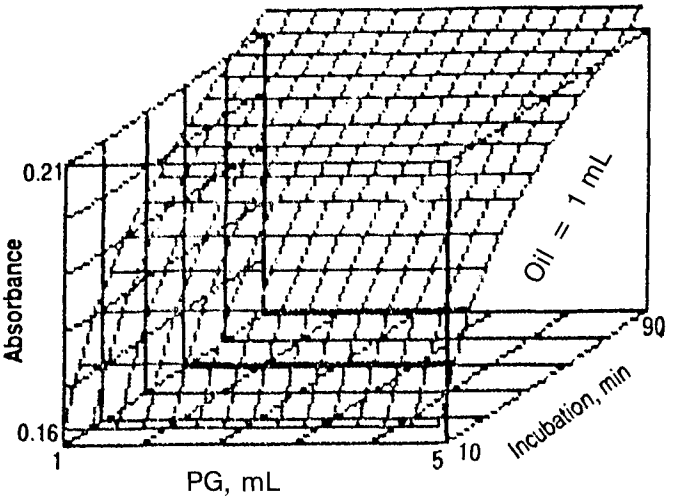

FIG. 2. Absorbance at 547 nm as a function of PG quantity and incubation period for palm oil with 1 mL oil. See Figure 1 for abbreviation.

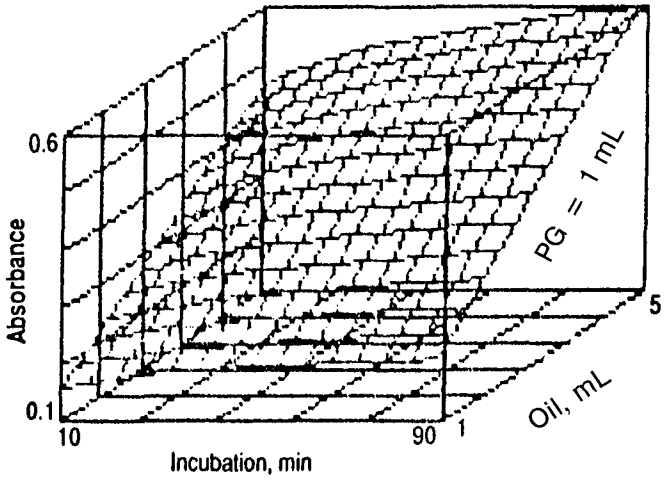

FIG. 3. Absorbance at 547 nm as a function of incubation period and oil quantity for palm oil with 1 mL PG. See Figure 1 for abbreviation.

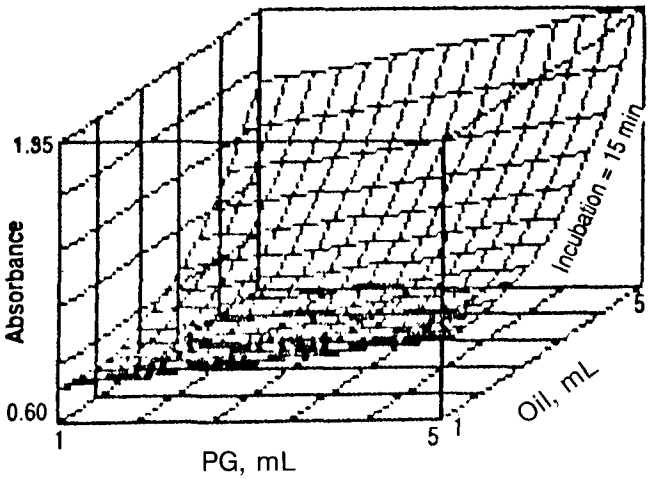

FIG. 4. Absorbance at 547 nm as a function of PG and oil quantities for rice bran oil at 15 min of incubation. See Figure 1 for abbreviation.

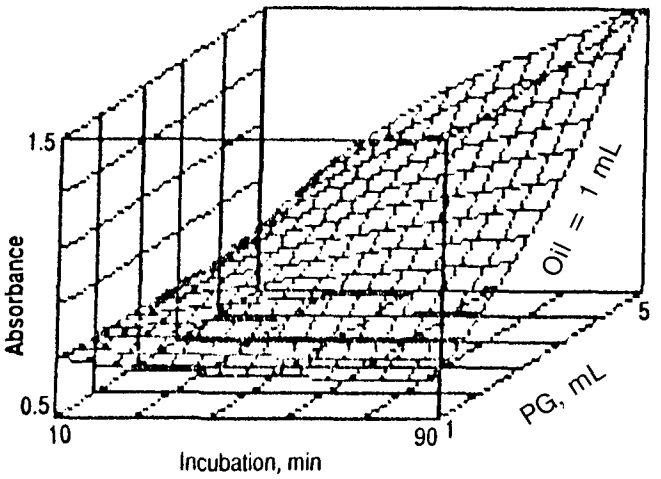

FIG. 5. Absorbance at 547 nm as a function of incubation period and PG quantity for rice bran oil with 1 mL oil. See Figure 1 for abbreviation.

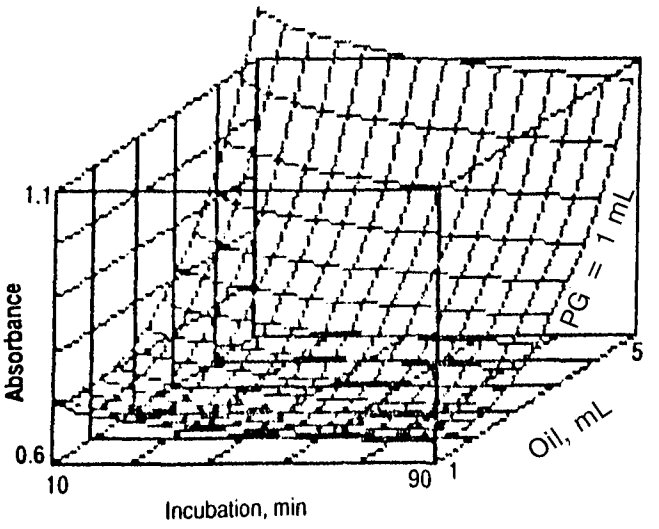

FIG. 6. Absorbance at 547 nm as a function of incubation period and oil quantity for rice bran oil with 1 mL PG. See Figure 1 for abbreviation.

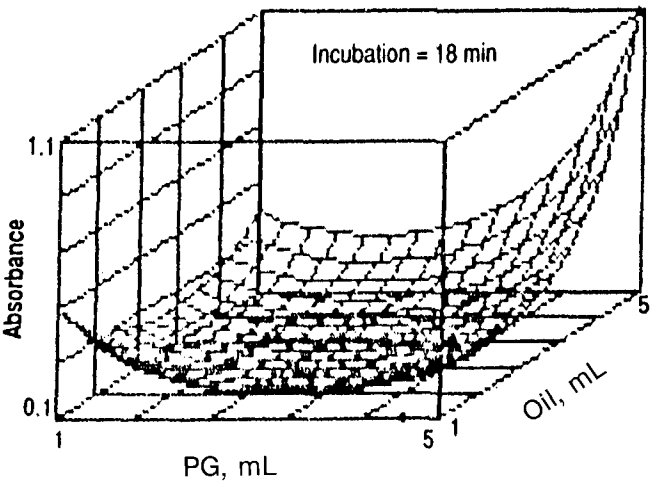

FIG. 7. Absorbance at 547 nm as a function of PG and oil quantities for groundnut oil at 18 min of incubation. See Figure 1 for abbreviation.

at 1 mL PG, while at 5 mL PG it increased with incubation. At shorter incubation (10 min), the absorbance did not increase with PG, while at longer incubations it increased with PG, particularly beyond 40 min of incubation. The effect of incubation and oil on absorbance at 1 mL PG is shown in Figure 6. The absorbance decreased slightly with increasing incubation periods at all levels of oil. However, the differences were neglible after 15 min of incubation. The absorbance increased sharply with oil as described earlier. The pattern was parabolic in nature, and below 1.8 mL oil there was hardly any change in the absorbance.

GNO: The absorbance as a function of PG and oil quantity at 18 min of incubation is shown in Figure 7. Parabolic response with both PG and oil was observed, with the minimum occurring at approximately 3 mL PG and 3 mL oil. The absorbance showed increasing trends on either sides of the minimum point (at 3 mL each of oil and PG). Higher predicted absorbance corresponded to the highest levels of PG and oil. The lowest levels of PG and oil (1 mL each) resulted in high absorbance of 0.5, justifying the chosen levels of PG and oil (Table 3). Figure 8 depicts the effect of PG and incubation on the absorbance at 1 mL of oil. At 1 mL PG, there was a sharp decrease in absorbance, up to 20 min of incubation, and then it increased slowly. At higher levels of PG (5 mL), the absorbance increased sharply with incubation of more than 25 min. The absorbance decreased sharply with increasing PG up to 2.5 mL at 10 min of incubation, while at 90 min of incubation the trend was different and the highest absorbance corresponded to 5 mL PG. Figure 9 shows the effect of oil and incubation on absorbance at 1 mL PG. The absorbance decreased with increasing oil at 10 min of incubation, and the trend was similar to that described for PG in Figure 8. Similarly, the pattern of absorbance with oil

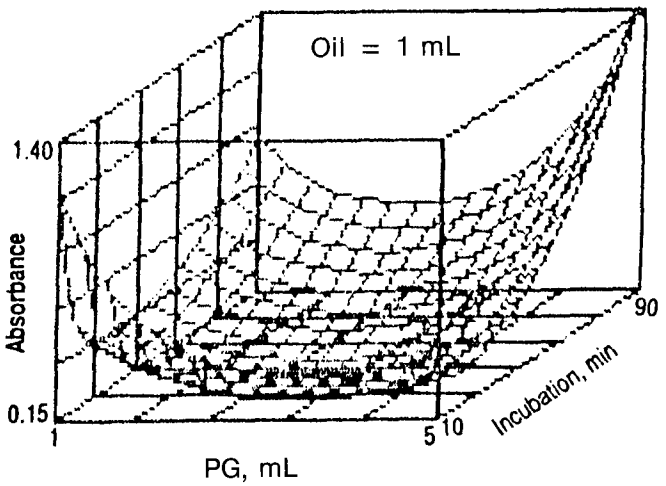

FIG. 8. Absorbance at 547 nm as a function of PG and oil quantities for groundnut oil at 18 min of incubation. See Figure 1 for abbreviation.

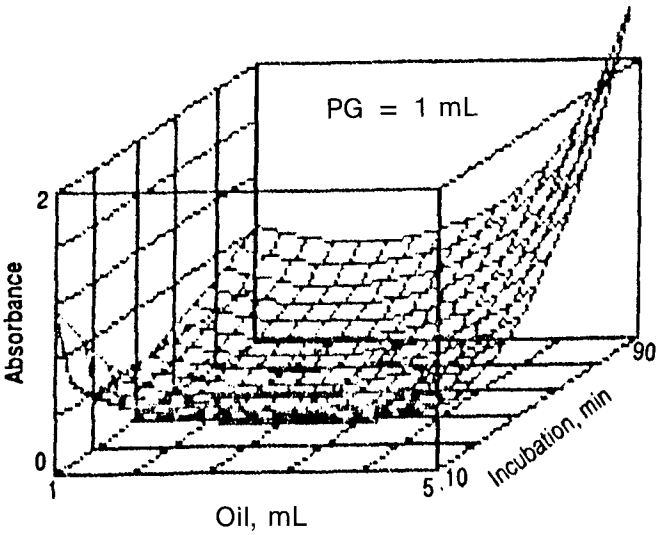

FIG. 9. Absorbance at 547 nm as a function of oil quantity and incubation period for groundnut oil with 1 mL PG. See Figure 1 for abbreviation.

at 90 min of incubation was similar to that described earlier for PG. The effects of incubation at 1 or 5 mL oil were similar to that described for PG in Figure 8.

It is apparent from the results that the Kreis test reaction is complex, but fast, and that it reflects continuous changes in color complex with incubation. The three oils have shown different behavior, e.g., even the smallest quantity of PG (1 mL of 1% solution) was capable of reacting with PO at all levels (1 to 5 mL), and the reagent did not show any difference ($P > 0.05$) from 1 to 5 mL. For RBO and GNO, the reagent showed its effect, and for the latter, all three factors (PG, oil and incubation) showed significant effects. However, for none of the oils did the reaction show a stationary phase to achieve realistic optimization. The results necessitate further investigation into the nature of the colored complex, its stoichiometry and standardization of reaction conditions.

The optimum levels reported in this paper for the three oils represent the best conditions reported so far. Varying quantities of oil (or fat), the reagent (PG), and incubation time and temperature have been reported by Kreis (1) and Patton *et al.* (3).

In summary, the Kreis test is a complicated and sensitive reaction with immediate appearance of color, which intensifies continuously. The colored complex showed a maximum absorbance at 547 nm at which the color monitoring was carried out with minimum time lag. The development of color as a function of PG, oil and incubation time was different for all three oils studied. Increasing levels of PG did not show any effect on the color production of PO, while all three factors (PG, oil and incubation time) showed their effects on the color development for GNO. Polynomials of suitable degree were fitted to the Kreis test data for each oil. The optimum levels of PG, oil and incubation periods for the three oils were worked out for a fixed minimum amount of oil of 1 mL with incubation within 25 min by means of fitted polynomials, which resulted in a practical set of conditions in each oil.

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