TABLE II
Statistical Analysis of Data on 24 Shipments

Test	Avg. diff. % cont. flow minus off. probe	Standard deviation of diff.	Calculated value of "t"	0.05 Significance level for "t" @ 24 degrees of freedom
Protein, moisture-free	+0.067	±0.460	+0.714	+1.71[a]
Crude fiber, moisture-free	+0.080	±0.217	−1.819	+1.71[a]
Moisture	+0.138	±0.299	±2.26	±2.06

[a] Single-tailed test.

found, a possibility which might occur once in 20 attempts. The test which was made is commonly called a one-sided test, meaning that the continuous flow method of sampling would be rejected only if it had a higher protein content of a definite magnitude and the continuous method would be accepted even if it differed markedly on the low side.

For crude fiber, since the calculated value of "t" is less than the limiting value, there is insufficient evidence that the continuous flow sample has a lower crude fiber than the official sample. Therefore the alternate hypothesis, that the crude fiber in the continuous flow sample is not lower than the official method, is accepted. Here again a single-sided test was used, requiring rejection of the continuous method only if the method obtained low values for crude fiber. Since the sampling method leads to obtaining slightly higher values, the continuous flow sample is satisfactory.

The moisture content was run on the samples as a necessary part of reducing the analysis for crude fiber and protein to a dry basis. It was desirable to know whether the moisture content on the continuously drawn sample differed from that of the official sample. Note that the question was "is the moisture different?" The calculated value of "t" is higher than the limiting value from the tables, and the hypothesis that the two methods differ is accepted. The moisture content is higher on the continuous sample, as would be expected, since this sample is protected from loss by evaporation whereas the probe sample is not as well protected. In this respect this study therefore indicates that the continuous flow sample is a better sample, hence a safeguard against shipment of high-moisture meal.

Summary

In an effort to avoid the operational errors introduced by the human factor in taking official probe samples of meal, a continuous flow sampler was designed and tested in comparison with the official probe sample on 50% Protein Soybean Oil Meal. The protein content was found not to be higher on the continuously taken sample. The crude fiber content was found not be lower on the continuously taken sample. The moisture content on the continuously taken sample differed from the official probe sample. The difference was on the high side as would be expected from consideration of the degree of exposure to air.

The continuous flow method described does not conform to the tentative A.O.C.S. Committee recommendation on automatic meal sampling, hence has no official standing. Any such installation as that described should be evaluated to reveal whether its operation is satisfactory. An experiment similar to the one described here should be used in such an evaluation.

These conclusions were reached as the result of objective statistical tests of data provided by a soundly designed experiment. The value of statistical planning for an experiment is demonstrated.

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Comparison of Lipoxidase Oxidation and Autoxidation of Cottonseed Oil by Rat Bio-Assay[1]

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THE FRACTIONATION of lipoxidase enzymes from legumes and studies of the properties of the different fractions (12) led to the work reported in this presentation. Studies on purified fractions showed (12) a rapid loss of enzyme activity with time. As an explanation for this loss of activity, it was of interest to determine whether the products of the enzyme reaction were inhibitory or whether the enzyme was particularly sensitive to the experimental conditions.

[1] This paper reports research undertaken by the Quartermaster Food and Container Institute for the Armed Forces, QM Research and Engineering Command, U. S. Army, and has been assigned No. 962 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

It was also noted that the highly oxidized oil had an odor quite different from both unoxidized and autoxidized oils. At no time was the odor of the enzyme-oxidized oil as offensive as that in autoxidized oils, even those of very high peroxide values. This extreme difference in the odors typical of the two treatments is a strong indication that the oxidation products must differ. The usual analytical procedures for oxidation products are not adequate to account for these observed differences.

A number of workers (3, 8, 9, 10, 11) have reported that thermally oxidized oils have less nutritive value than the comparative fresh oils using the rat bio-assay. It was decided to use a bio-assay to determine whether the differences in odor between the enzyme-

oxidized and the autoxidized oils could be related to nutritional adequacy, which in turn would indicate that enzyme oxidation produced products differing structurally from autoxidized oil.

Other workers have investigated structural differences between enzyme-oxidized linoleic acid and autoxidized acid. Privett *et al.* (14) found that the principal products of lipoxidized Na-linoleate were monomeric monohydroperoxides. Some optically active polymers were found, but in small amounts. Friend (5) recently reported that coupled oxidation of β-carotene with a linoleate-lipoxidase system produced oxidized β-carotene products that differed from products obtained by β-carotene oxidation with autoxidizing linoleate. Holman (7) studied the effect of enzyme-oxidized ethyl linoleate on fat deficient rats and concluded that, in the absence of ethyl linoleate, the linoleate peroxide exhibited a toxic effect. However it is believed that the present research is the first reporting comparative studies between enzyme-oxidized and autoxidized triglycerides.

Experimental

The navy beans were ground in a Wiley mill to pass through a No. 20 sieve. The freshly ground material was slurried with iced, distilled water (10 g. per 100 ml. w/v) by gentle stirring for 10 min. This entire slurry was added to cottonseed oil 1.25:1 v/v, and the mixture was stirred in a high-speed blender for 10 min. The initial temperature of the blender contents was 17°C., and the final temperature was kept below 22°C. by a cooling coil. The resulting emulsion was separated in a continuous supercentrifuge at 40,000 r.p.m., and the spent aqueous phase was discarded. Oil recovery was about 80% of the original charge.

The enzyme-oxidized oil was then substituted for the original refined oil, and the same oxidation procedure was followed as many times as desired or until insufficient oil was recovered to go farther.

A sample of the same refined oil was used for peroxide production by autoxidation. Air was bubbled through the oil while heating at an average of 85°C. until it had reached the desired peroxide value.

The method of Wheeler (16) was used for peroxide determinations. Carbonyl content was measured by the method of Henick (6) and percentage diene conjugation by the method of Mitchell (13), using the constants of Brice *et al.* (1).

In the bio-assay procedure three samples of oil, control (C), autoxidized (A), and enzyme-oxidized (E), were incorporated into purified-type diets at 10% and 20% by weight for rat assay. The diets were made by addition of oil and sucrose to 80 parts of casein diet base, as described by Calloway *et al.* (2) except that all vitamins were fed by dropper daily. The odor of the oil was masked by spraying the diets with anise.

Each of these diets was fed to a group of ten weanling, male, albino rats of the Holtzman Sprague-Dawley strain. Half of each weight-matched group was fed *ad libitum*. The remaining animals were trio-fed within each fat level by limiting food consumption to the intake of the poorest eater. Food intake was recorded daily and body weight three times weekly for four weeks. The diets were stored in a freezer, and portions were removed for feeding as required.

Results and Discussion

Two kg. of refined cottonseed oil were treated in the manner described above for enzyme oxidation, and the recovered oil was returned to the cycle with fresh increments of navy bean extract (holding v/v ratio constant) for a total of 10 such treatments. After each recovery of oil it was assayed for peroxide value, carbonyl value, and diene conjugation. Results of these determinations are presented in Figure 1. It will be noted that straight lines best fit the experimental points when total change is plotted against the number of times the oil was enzyme-oxidized. Analysis of the oil after the tenth lipoxidase treatment showed the following composition: P.V. = 1139 meq./kg., carbonyl = 305 μM/g., and deine conjugation 11.7%.

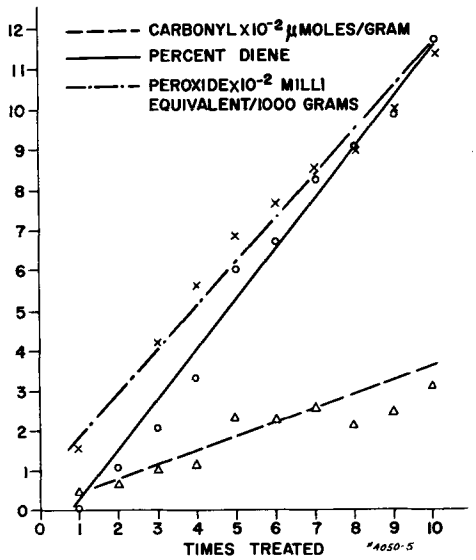

FIG. 1. Lipoxidase oxidation of cottonseed oil.

The straight-line relationship for increase in oxidation products with increase in number of treatments indicates that the products of enzyme action are definitely not inhibitory to enzyme. The observed rapid decrease in rate of enzyme activity within a short period of time (enzyme action practically ceases after 20 min.) must be related to the instability of the enzyme when removed from nature's protective mechanism in the raw navy bean. In dry navy beans the fat-oxidizing enzymes are stable for extended periods at temperatures in excess of 70°C. However, after water extraction of the enzyme, it loses activity fairly rapidly when stored at 0°C.

Another observation made from the plots in Figure 1 is the indication that carbonyls are probably not formed by autoxidative breakdown but by enzyme action. If the carbonyls arose from autoxidative breakdown, there should be a greater production of carbonyls as the peroxide content becomes larger.

The mild off-odor (not at all similar to that of, and much less pronounced than, autoxidized oils), produced in the enzyme-oxidized oil despite the rather high carbonyl value, led to speculation on the differences which exist in the nature of the final products. Since the rat bio-assay has been used to study toxicity of autoxidized oils, this method was selected to see if differences between the oils could be detected in this manner.

Samples of cottonseed oil were freshly prepared for rat bio-assay. Oxidation by navy bean lipoxidase was accomplished in three steps and by autoxidation in 39 hrs. Analyses of these oils are given in Table I. The higher carbonyl content and lower percentage of diene conjugation in the autoxidized oil indicated greater breakdown of peroxide than occurred in the enzyme-oxidized oil of nearly the same final peroxide value. These differences may be partially responsible for the differences observed in the bio-assay.

TABLE I

Comparison of the Peroxide, Carbonyl, and Diene Conjugation of Enzyme *vs.* Autoxidized Cottonseed Oil

Oil	Treatment	P.V. meq./kg.	Carbonyl μM/g.	Diene conjugation %
E	Enzyme-oxidized	690	202	8.1
A	Autoxidized	640	293	6.8
C	Control	0	4	0.3

Figure 2 shows the results of *ad libitum* feeding studies. The control oil diet showed the best response, followed by the enzyme-oxidized oil diet, with the autoxidized oil diet running a poor third. Growth supported by both of the oxidized oils was significantly less than that of the control sample at both the 10% and 20% levels, as judged by t-test (4). At the 20% level, superiority of oil E over A was evident. In terms of caloric efficiency, the grams of body weight gained per 100 calories consumed, the

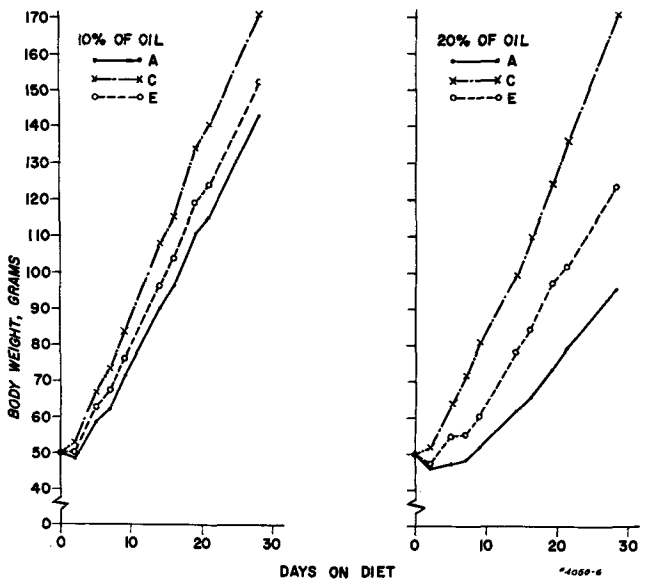

FIG. 2. Body weight of rats fed, *ad libitum*, diets containing oxidized oils: A—autoxidized; C—control; E—enzyme-oxidized.

same relationships existed except that at the 10% level oil E could not be differentiated from C and was superior to A (Table II).

Because of the marked variation in *ad libitum* caloric intake, shown in Table II, the results obtained with trio-feeding are perhaps of more pertinence. As is apparent from Figure 3 and Table II, all differences among oils fed at the 10% level were eliminated by equating food intake. However, at the 20% level, oil A was found to be distinctly inferior to oils C and E, which did not differ from each other

TABLE II

Caloric Intake and Efficiency of Rats Fed Diets Containing Oxidized Oils

Diet	Fed *ad libitum* Intake cal./4 wks.	Fed *ad libitum* Efficiency G. gain/ 100 cal.	Trio fed Intake cal./4 wks.	Trio fed Efficiency G. gain/ 100 cal.
10% of Oil[a]				
C	1,275	9.48 ± 0.45	905	10.09 ± 0.25
A	1,097	8.45 ± 0.63	904	9.50 ± 0.41
E	1,090	9.35 ± 0.44	899	9.60 ± 0.35
20% of oil[a]				
C	1,259	9.54 ± 0.40	686	9.10 ± 0.61
A	720	6.38 ± 0.68	677	6.73 ± 0.54
E	915	7.95 ± 1.34	682	8.53 ± 0.80

[a] C—control oil; A—autoxidized oil; E—enzyme-oxidized oil.

by analysis of paired comparisons (15). Thus it appears that the differences between C and E in the *ad libitum* test may have been caused by preference rather than toxicity but that sample A shows a definite impairment to nutritive quality.

The rat bio-assay appears to be useful to show qualitative differences in enzyme *vs.* autoxidized oil.

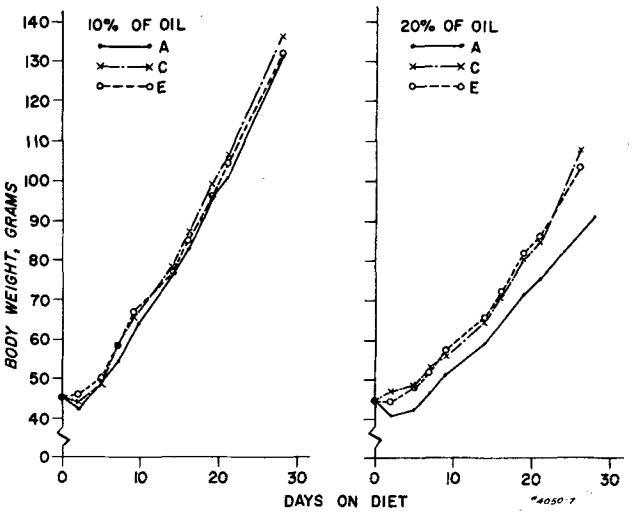

FIG. 3. Body weight of rats trio-fed diets containing oxidized oils: A—autoxidized; C—control; E—enzyme-oxidized.

However the results must be verified because of the differences in carbonyl and diene conjugation between the oils.

Summary

A stepwise oxidation of cottonseed oil with navy bean lipoxidase shows a straight-line relationship for increases in peroxide value, carbonyl value, and diene conjugation.

The linear increase in peroxide value for the stepwise enzyme oxidation shows that the substrate is not a limiting factor in enzyme action and that the peroxide content of the oil does not exert an inhibitory effect on the enzyme, at least, within the range obtained in this work.

A rat bio-assay showed that cottonseed oil enzyme oxidized to a P.V. of 690, while not as acceptable, was as adequate as a control oil under conditions of paired feeding. Autoxidation of cottonseed oil to a P.V. of 640 showed definite loss in caloric efficiency compared to the control oil and enzyme oxidized oil.

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Sulfation with Sulfur Trioxide: Ethenoxylated Long-Chain Alcohols[1]

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SULFATED ETHENOXYLATED long-chain alcohols are of increasing interest as detergents because of favorable detergency, solubility, and cost (2). The preparative procedure involves reaction of a long-chain alcohol derived from animal fats, coconut oil, or petroleum with any desired ratio of ethylene oxide to form a mixture of polyether alcohols, which is then sulfated and neutralized for use.

Various reagents have been used experimentally for the sulfation step (Table I). Chlorosulfonic acid without a solvent has been most generally employed commercially. The purpose of the present study is to evaluate it in comparison with a newer reagent, sulfur trioxide vapor, which is commercially available from stabilized liquid sulfur trioxide marketed under the trade name "Sulfan" (5). Only one patent reference was noted (1) on sulfation of an ethenoxylated long-chain alcohol with sulfur trioxide in vapor form although this reagent has shown promise with long-chain alcohols and with ethenoxylated alkylphenols (3). Sulfur trioxide with liquid SO_2 solvent is used to only a minor degree industrially because of the difficulty in recovering and recycling the solvent.

Raw Materials

Samples used in the present study, as obtained from five different commercial sources, are listed in Table II; A–D were liquids, E and F solids, melting below 35°C.

Experimental Procedure

Sulfation. The sample (200–400 g.) was sulfated with sulfur trioxide vapor diluted with dry air by the method previously used by the authors for sulfating lauryl alcohol (6). Heat evolution was steady during sulfation (15 to 30 min.); external cooling was used as necessary to maintain a reaction temperature of 25–35°C. for liquid samples A–D, or 50–55°C. for solid samples E and F, which were sulfated as melts. These two samples were premelted before sulfation; below 45°C. foaming was excessive with these materials. Weight loss during sulfation was less than 2%, based on total weight of both reagents.

Chlorosulfonic acid was added dropwise with stirring and cooling to 25–35°C. for liquid samples A–D, or 40–50°C. for solid samples E and F, which were sulfated as melts. No difficulty was experienced with stirring even though no solvent was used. During the addition of the first half of the acid there was no gas evolution, but heat evolution was substantial, corresponding to the sum of the heat of sulfation and the heat of solution of hydrogen chloride. Toward the end of the acid addition, endothermic evolution of hydrogen chloride occurred, and heating was required to maintain the desired temperature range until the evolution ceased. The reaction time (20–45 min.) was somewhat longer with this reagent because of substantial foaming during the final stage of reaction.

Neutralization. To avoid gelation the acid sulfates prepared with both reagents were neutralized dif-

TABLE II
Data on Raw Materials Studied

Sample	Alcohol	Mol. wt.[a]	Approx. moles oxide
A	Lauryl	334	3
B	Lauryl	338	3
C	Tridecyl	325	3
D	Tridecyl	332	3
E	Tallow[b]	440	4
F	Tallow	425	4

[a] As reported by manufacturer.
[b] Stated to comprise 65% stearyl and 35% cetyl alcohols.

[1] For previous papers in this series see (3) and other references cited therein.

TABLE I
Sulfation of Ethenoxylated Long-Chain Alcohols-Literature Summary

Alcohol	Moles ethylene oxide	Reagents used	Reference
Trimethylnonanol	3, 5	100% H_2SO_4 with dioxane	8
Butyloctanol	3–5	100% H_2SO_4; SO_3 with liquid SO_2	10, 11
Tridecyl	1–10	100% H_2SO_4; SO_3 with liquid SO_2; SO_3 vapor; $ClSO_3H$ with ethyl ether; NH_2SO_3H	1, 7
Ethylmethylundecanol	5	100% H_2SO_4 with CCl_4; SO_3 in liquid SO_2	9
Hexa-, octadecanol	2, 10	$ClSO_3H$ with tetrachloroethylene	2
Lauryl, oleyl, etc	1–3	$ClSO_3H$ (no solvent); $ClSO_3H$ with urea	13