after storage, as was concluded also from a study of the spectra of treated and control crude oils.

Bleaching removes all traces of carotenoids, and there is no significant difference in the spectra of the treated and the control screw-pressed cottonseed oils after alkali-refining and bleaching (Fig. 4).

In this study the carotenoids of the cottonseed oils have not been identified, but the *p*-aminobenzoic acid removes interfering pigments to such an extent that the treated oils should serve as an excellent source of material for further studies on these yellow pigments normally found in crude cottonseed oils. Preliminary experiments with the objective of separating and identifying the carotenoids of such a treated cottonseed oil have been already initiated in this laboratory.

Discussion

The use of *p*-aminobenzoic acid to precipitate gossypol from freshly prepared crude cottonseed oils prevents color reversion on storage of these oils under conditions which promote color reversion. The fact that gossypol was precipitated by the reagent was proved by the recovery of a gossypol derivative, di-*p*-carboxyanilinogossypol, from the preciptate and by spectrophotometric data. Other materials of unknown nature are co-precipitated by this treatment, but their role in color reversion has not been established. These findings establish the principle of the cause and prevention of color reversion in crude cottonseed oil.

This work suggests an effective approach to the inhibition of color reversion during the storage of crude cottonseed oil. In order to be practical however the reagents used to precipitate the gossypol must be economical or the gossypol derivative must have sufficient value of its own to pay for the extra cost of treatment.

Summary

Treatment of freshly prepared crude cottonseed oils with *p*-aminobenzoic acid and subsequent removal of the di-*p*-carboxyanilinogossypol formed makes it possible to store the oils at a relatively high temperature (37-38°C.) and for an extended period of time (30 days) without incurring any adverse changes in the refining and bleaching properties of the oils. In addition, a considerable decrease in the refining loss of the crude oil is obtained, and the stability of the bleached oil is not affected by the treatment.

Spectrophotometric studies made during all phases of the chemical treatment and during the refining and bleaching procedures show that the *p*-aminobenzoic acid removes almost completely the gossypol-like pigments which are present in the crude oils and yields oils having the characteristic carotenoid spectrum.

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Analysis of Butylated Hydroxyanisole in Paper and Paperboard

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THE use of butylated hydroxyanisole (BHA) in paper and paperboard used for packaging fats and oils, and in foods containing fats and oils has made a method of analysis necessary. This discussion includes qualitative and quantitative methods for BHA in paper and paperboard. A new method for the rapid quantitative determination of BHA in paper is given, which makes an analysis possible in 25 minutes.

During the past few years a number of antioxidants have been proposed for use in paper for food packaging. Among these are gum guaiac, resorcinol, lecithin, soya bean flour, and oat flour. Bentz (1) has now reported the use of butylated hydroxyanisole in paper. Before any chemical can be accepted for use in paper in contact with foods however, accurate methods of analysis must be available.

The use of 2,2′-bipyridine and ferric chloride has been widely accepted (5) as entirely satisfactory for the analysis of butylated hydroxyanisole in fats and oils when other phenolic antioxidants are not present. Since this method was already available (2), the only problem involved in the analysis of BHA in paper and paperboard was to remove the BHA from these materials and get it into an alcoholic solution.

Quantitative Analysis of BHA in Paper

Long Method

When BHA is applied to paper and paperboard by the usual techniques, it is dispersed in extremely fine particles over a vast surface area, which means that any method of removing BHA from these materials must involve intimate contact of a suitable solvent for BHA with all the fibers of the paper and paperboard. It appeared that a soxhlet extraction would

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be most suitable for this purpose. It had been reported (5) that 72% alcohol was entirely satisfactory for removing BHA from fats and oils. In our laboratories we have generally favored the use of 80% alcohol due to its slightly greater solubility for BHA, and our standard curves were based on this percentage.

In order to determine whether BHA could be removed by this technique, 15 g. of untreated parchment paper were cut into strips approximately 1/4 x 2 in. long and packed in a 33 x 94-mm. fiber thimble. Into this thimble was placed an accurately weighed quantity of BHA. Soxhlet extractions were run for varying lengths of time, and it was found that the BHA could be recovered quantitatively.

Our next experiment was to take a paper which had been treated with BHA in the laboratory and extract it for 10 hours. Duplicate analyses of 0.0138 and 0.0140% BHA were obtained. Six samples of this same paper were extracted for varying lengths of time with 150 cc. portions of 80% ethyl alsohol. On cooling, the alcoholic extracts were filtered and diluted to 200 cc. The results are shown in Table I.

TABLE I
Effect of Extraction Time on BHA Analysis

Hours Extracted	Percentage BHA
2	0.0107
4	0.0119
6	0.0120
8	0.0135
10	0.0150
12	0.0142

From these data it is seen that an extraction time of 10 hours is satisfactory.

Before the analysis itself can be carried out, it is necessary to prepare the following reagents:

Ethyl alcohol	100% absolute ethyl alcohol and 80% ethyl alcohol diluted with distilled water
2,2′-Bipyridine	0.1% in absolute ethyl alcohol
Ferric Chloride (hexahydrate)	0.0832% solution in absolute ethyl alcohol

It is necessary next to prepare a standard curve. We used the same curve for BHA in paper and paperboard as we used for BHA in fats and oils. This curve is set up so that the concentration of BHA extracted from 7.5 g. of sample and diluted to 100 cc. can be read directly as percentage BHA from the standard curve. In this curve percentage transmittance *versus* percentage BHA is plotted on semi-log paper and a straight line is obtained. (It should be noted also that optical density can be plotted *versus* concentration on regular graph paper to give a straight line.) Most common spectrophotometers require 10 to 20 cc. of solution for an analysis plus a suitable blank. Over the range of 10 to 50 micrograms of BHA in 10 cc. of solution, the color formed between BHA and the color-developing reagents obeys Beer's Law. It is therefore a simple matter to make up suitable solutions of BHA in 80% ethyl alcohol.

The mixing should be carried out in very dim light, preferably less than 1 foot candle. For the blanks use 4 cc. of 80% ethyl alcohol plus 8 cc. of the 2,2′-bipyridine reagent and 8 cc. of ferric chloride reagent. For the different concentrations of BHA use 4 cc. of each sample and 8 cc. of each of the color developing reagents. The samples should be mixed in either glass-stoppered flasks or small glass-stoppered bottles. Once they are mixed, they should be placed immediately in the dark. All mixing and aging should be done in an air-conditioned laboratory at 72°F.

Several researchers have reported an aging time of 30 minutes to be adequate. Our tests showed that for the blanks the color was slightly more stable between 40-50 minutes after mixing. For samples containing BHA anywhere between 20 minutes and 2 hours is satisfactory. For simplicity in running the samples an aging time of approximately 40 minutes is used for all samples. At the end of 40 minutes aging in the dark, the samples are run on a spectrophotometer at a wavelength of 522 millimicrons, and either the per cent transmittance or the optical density is recorded, depending on which standard curve is being used. Then the concentration of BHA in the solutions, and hence in the original samples, is calculated.

Due to the influence of unknown materials or dyes present in practically all papers and paperboards, it is necessary that papers not treated with BHA be extracted and analyzed along with the treated ones. The values of apparent BHA should be subtracted from the values obtained for the treated papers. Only in this way can an accurate analysis of the actual BHA added be obtained. We have found a number of papers and paperboards which give quite high apparent BHA values even though they have not been treated. In particular, many paperboards made with reclaimed stock contain impurities which make the analyses for per cent BHA quite difficult. When such difficulties are encountered, it is preferable to dilute the alcoholic extracts to obtain a readable range. Vegetable parchment paper is perhaps easiest to analyze since it is made entirely from virgin pulp. Glassine paper generally gives a slight amount of trouble due to the dyes present in the paper.

Since 2,6-dichloroquinonechloroimide is quite specific for BHA, it may be used in an alternate method to minimize interference. Spectrophotometer readings are made at 610 millimicrons. However we have found some untreated papers and paperboards whose extracts react with this reagent.

Since we have not been able to treat papers with an accurately known quantity of BHA under conditions resembling commercial operations, the accuracy of the method is not known. However the precision of this method is quite high (Table II). From Table II it is seen that the results could be reported as $0.0555\% \pm 0.0002\% \left(\frac{0.0007}{\sqrt{8}}\right)$.

TABLE II
Results of Analyses on One Sample of BHA-Treated Vegetable Parchment Paper

Analysis No.	Percentage BHA	Deviation from the Mean
1[a]	0.0560	0.0005
2	0.0570	0.0015
3	0.0554	0.0001
4	0.0552	0.0003
5	0.0542	0.0013
6	0.0538	0.0017
7	0.0554	0.0001
8	0.0552	0.0003
Mean	0.0555	Average Mean Deviation 0.0007

[a] Analysis No. 1 was run 6 weeks before the other samples. All other analyses were run at the same time.

Quantitative Analysis of BHA in Paper

Rapid Test Method

It has long been known (3) that 2,6-dichloroquinonechloroimide (2,6-D) reacts with phenols. The

color reaction is sensitive to pH, giving maximum color at pH 9.4, and the color formed is stable for at least five hours. Maximum color intensity is formed within 15 minutes after adding the reagents. This would obviously be a great improvement over the previous method if a way could be found to remove BHA quickly from paper and paperboard. To avoid confusion it will be stated now that no success was obtained with paperboard. Hence the following discussion is for papers such as vegetable parchment and glassine only. Since this method is new and different from the previous one, it is reported in some detail.

Reagents

2,6-Dichloroquinonechloroimide [2,6-D] — 0.01% solution in absolute ethyl alcohol. This reagent must be freshly prepared for optimum results.

Borax buffer—2.0% aqueous solution of $Na_2B_4O_7 \cdot 10H_2O$.

Ethyl alcohol—100% absolute and 80% by volume with distilled water.

Equipment

Eighteen 50-cc. color comparison tubes with a suitable rack The rack should be equipped with a fluorescent light and the tubes viewed against a white background.

Six 250-cc. Erlenmeyer flasks, preferably wide-mouth, and equipped with ground glass stoppers.

Balance sensitive to 0.10 g. or less.

Procedures

Make up a standard solution of BHA in 80% alcohol at a concentration of 10 micrograms per cc. Next prepare a set of 10 solutions as shown in Table III. The 50-cc. color comparison tubes should be filled with the solutions and viewed horizontally since the colors are too intense for vertical viewing. Since these blue indophenol solutions are not stable beyond five hours, it is necessary to prepare permanent standards

TABLE III
Preparation of Standard Solutions

Number	BHA Standard Solution, cc.	80% Alcohol[a] cc.	Concentration of BHA, micrograms per cc.
1	2.5	57.5	0.3125
2	5.0	55.0	0.6250
3	7.5	52.5	0.9375
4	10.0	50.0	1.2500
5	12.5	47.5	1.5625
6	15.0	45.0	1.8750
7	17.5	42.5	2.1875
8	20.0	40.0	2.5000
9	22.5	37.5	2.8125
10	25.0	35.0	3.1250

[a] Next add 10 cc. of 2,6-D reagent and 10 cc. of borax solution to each sample.

from suitable dyes. In order to duplicate the actual colors obtained, it is necessary to use either a blue and a purple or a blue and a red. The particular dyes used are not important so long as excellent color comparisons can be obtained.

Many experiments were tried to develop a rapid method for the removal of BHA from paper. Analyses in all cases were compared with those obtained, using the long method previously given. The following method was found adequate for our needs:

Weigh 5 g. of BHA treated paper to the nearest 0.1 g. Cut the paper into small pieces (¼ x ½ in.) and put it into a glass-stoppered 250-cc. wide-mouth Erlenmeyer flask. Add 50 cc. of absolute ethyl alcohol and shake well for at least five minutes. Decant the alcohol into a graduated cylinder or volumetric flask. Extract the paper a second time with 50 cc. of 100% absolute ethyl alcohol and combine the alcoholic extracts. Determine the volume of the extracts or dilute to a known volume. Take an aliquot of the extracts and dilute it to 48 cc. with 80% ethyl alcohol. Add 8 cc. of the 2,6-D reagent and 8 cc. of the borax reagent. Let stand 10 to 15 minutes and compare it with the color standards. It may be necessary to dilute two or more aliquots to obtain a solution within the range of the standards. A typical comparison of the analytical results obtained using the two methods is as follows:

Comparison of Rapid Test Method with Long Test Method

Number	Percentage BHA Rapid Test Method	Percentage BHA Long Test Method
1	0.050	0.053
2	0.090	0.099

The calculations of BHA present in paper are as follows:

$$\frac{\text{conc. from std. scale}^{2} \times \text{cc. prepared solution} \times \dfrac{\text{cc. of extract}}{\text{cc. of aliquot}} \times 100}{\text{weight of paper}} = \%\ \text{BHA}$$

For example:

Five g. of paper are extracted and the extracts diluted to 100 cc. Ten cc. are diluted to 48 cc. with 80% alcohol, and 8 cc. of each reagent are added. After 15 minutes a color rating of 6 is obtained. The calculations are as follows:

$$\frac{1.8750 \times 10^{-6} \times 64 \times 100/10 \times 100}{5} = 0.024\%\ \text{BHA}$$

From these data it is apparent that an experienced operator can run an accurate BHA analysis on paper in approximately 25 minutes.

Qualitative Test for BHA in Paper

A rapid qualitative test for BHA in paper is frequently very useful to paper manufacturers and converters. The test can be run as follows:

Weigh approximately 5 g. of paper and cut it into small pieces (¼ x ½ in.). Put the paper in a 250-cc. wide-mouth, glass-stoppered, Erlenmeyer flask. Add 50 cc. of 100% ethyl alcohol and shake for 5 minutes. Decant 5 cc. of the alcoholic extract into a test tube. Add 1 cc. of a 2% solution of borax [$Na_2B_4O_7 \cdot 10H_2O$] in distilled water. The pH of the resulting solution should be very near 9.4. Next add 1 cc. of a 0.01% solution of 2,6-dichloroquinonechloroimide in 100% absolute ethyl alcohol. The formation of a blue color within 15 minutes indicates the presence of BHA. A pink to light brown color indicates a negative test. Untreated papers should be run along with treated ones.

Due to the presence of impurities in reclaimed paper this test is generally not satisfactory for paperboard. It has been used with good results on glassine, vegetable parchment, sulfite, sulfate, and a few laminates such as foil-sulfite and foil-parchment. The test will generally not work on waxed papers since alcohol does not dissolve the wax.

Test to Determine Which Side of the Paper Is Treated with BHA

For food packaging often only the side of the paper next to the food is treated with BHA. Hence a problem sometimes arises to detect the treated side at the point of converting the paper into finished wrappers. A simple technique has been worked out which gives very reliable results. The operator should run tests on papers of known origin and treatment to familiarize himself with the colors produced. The test may be run on most papers as follows:

Prepare two cylinders of the paper to be tested with opposite sides inward. The cylinders should be about one inch in diameter and two to three inches long. The paper should overlap only enough to fasten it together. Either straight pins

[2] Convert to grams by multiplying by 10^{-6}.

or paper clips are satisfactory. Next pour about 175 cc. of 80% alcohol into a 250-cc. beaker and add 10 cc. of the 2,6-D reagent solution. Stir well. Then add 10 cc. of the borax solution and mix well. Allow the solution to become quiet and stand the cylinder upright in the center of the beaker. Note that the cylinder should be placed in the solution within one minute or less after the borax has been added. Let the paper stand without mixing and examine it by viewing both vertically and horizontally against white backgrounds. The presence of a blue ring near the paper shows the BHA is present. A more positive test is generally obtained if the treated side is inside the cylinder, hence the preparation of two cylinders. It is again most desirable to run untreated paper along with the paper in question for a comparison of the results. The cylinder should be viewed at least at 5-, 10-, and 15-minute intervals and more often if desired. Once the blue color has formed, it diffuses slowly throughout the solution. It is necessary therefore to follow the formation of the blue rings. If both sides of the paper are treated, the test will indicate by the rates of formation and intensities which side contains the higher concentration of BHA. Again if the paper does not contain BHA, a light pink color will form within the 15-minute test period. This test has been tried on vegetable parchment, sulfite, sulfate, and glassine with excellent results. It will not work however on waxed paper or paperboard. Simple as this solution is, it solves a problem that is frequently very baffling.

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Molar Refraction, Molar Volume, and Refractive Index of Fatty Acid Esters and Related Compounds in the Liquid State[1]

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THE use of refractive index and molar refraction as aids in establishing the identity, purity, or structure of organic compounds is well known. Measured values of these constants do not appear to have been fully exploited for such purposes however in the chemistry of pure fatty acids and derivatives even though refractive index has been widely used in the characterization of natural fats and oils.

Recently in this laboratory a supposedly pure methyl docosahexaenoate, prepared from hog brain (1), was found to have a molar refraction higher than the value calculated from atomic constants. The refractive index and molar refraction were also higher than empirical values reported by Farmer and Vandenheuvel for a methyl docosahexaenoate prepared from cod liver oil (2). Moreover these authors also described a simple straight line relationship between refractive index and unsaturation for methyl esters of long chain fatty acids (3) that was consistent with, and in part dependent on their observed refractive index for the docosahexaenoate.

A study was therefore undertaken of molar refraction, molar volume, and refractive index of methyl esters of fatty acids as functions of two parameters, carbon chain length and unsaturation, with a view to developing equations that would be useful in determining identity, purity, and structure. The development of such equations for the methyl esters of common types of fatty acids (excluding those with conjugated double bond systems and those with double bonds in the *trans* configuration) is reported. Similar principles and relationships appear to hold for the fatty acids themselves and other derivatives.

General Relationships

Molar refraction, R_m, refractive index, n, and molar volume, V_m, are related by the well known equation of Lorenz (4) and Lorentz (5):

$$R_m = \frac{n^2 - 1}{n^2 + 2} V_m \qquad \text{I}$$

It is also well known that R_m may be calculated to a first approximation for any compound by the summation of certain atomic "constants." The "constant" for any particular atomic component is subject to second order variations, depending on the nature of other components and molecular structural relationships. It is reasonable to expect however that for a family of compounds which differ only in carbon chain length and unsaturation, for example, methyl esters of normal fatty acids, a single set of constants could be found which would yield calculated molar refractions in very close agreement with observed values for all members of the family of compounds. The equation could take the form:

$$R_m = k_1C + k_2D + k_3 \qquad \text{II}$$

where C is the number of carbon atoms in the fatty acid chain (not including the carbon of the alcoholic methyl group), D is the number of double bonds, and k_1, k_2, and k_3 are constants. The relationship to which II reduces in the absence of double bonds has been found to hold for saturated fatty acids at 80°C. by Dorinson *et al.* (6).

It has also been found previously that V_m is a linear function of the carbon chain length for saturated fatty acids and esters; further, the introduction of an isolated double bond in members of such a series of compounds has a uniform effect on molar volume (6, 7). It is to be anticipated therefore that a general equation for molar volume and density such as follows might be applicable:

$$V_m = \frac{M}{d} = k_4C + k_5D + k_6 \qquad \text{III}$$

where M is the molecular weight, d is the density, and k_4, k_5, and k_6 are constants.

R_m, which is considered to be a function of the volume actually occupied by the molecules of a mol of substance, is nearly independent of temperature. This is not true of V_m, and the constants k_4, k_5, and k_6 will

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