in the laboratory, with a resultant pickup of moisture by the glycerol which had the effect of inactivating a portion of the catalyst. In addition, the pilot-plant batches required a much longer heat-up time than the laboratory preparations and agitation of the pilot-plant batches was not as efficient.

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A Method for the Determination of the Water-Insoluble Combined Lactic Acid Content of Shortenings Containing Lactylated Emulsifiers

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Shortenings containing glycerol lactopalmitates and glycerol lactostearates are analyzed for water-insoluble combined lactic acid content using a procedure adapted from that of Barker and Summerson (1). Water-soluble constituents are extracted from a chloroform solution of the shortening. The water-washed shortening is saponified and then acidified to release the lactic acid, which is degraded to acetaldehyde by heating with concentrated sulfuric acid. The acetaldehyde is reacted with p-phenyl phenol in concentrated acid solution to produce a purple colored reaction product. The intensity of the color is proportional to the concentration of the acetaldehyde. Absorption is read at 570 mμ using a lithium lactate solution as a standard.

The method has been applied to the analysis of shortenings containing from 0.70 to 1.10% water-insoluble combined lactic acid.

UNTIL RECENTLY, monoglycerides of long chain fatty acids were the chief emulsifiers in shortenings. Within the past few years they have been supplemented with, and partially replaced by, glycerol lactopalmitates (GLP) and glycerol lactostearates (GLS), which are mixed glycerol esters containing varying amounts of lactic and fatty acids. The effectiveness of these emulsifiers in a shortening depends on the concentration of GLP or GLS in the shortening and on the chemical composition of the emulsifier itself. Those esters which contain 1 molecule of fatty acid and 1 or more molecules of lactic acid per molecule of glycerol are the most effective emulsifiers.

Current shortening analysis methods, including monoglyceride determinations and saponification values, are not adequate to evaluate a lactylated shortening. Although the saponification value is influenced by the lactic acid content, the small amount of lactic acid esters present in a shortening, compared to the fatty acid esters, is insufficient to raise the overall saponification value of the shortening by more than 2 or 3 units. Since esterified lactic acid is the critical component in these shortenings, a determination of combined lactic acid content would serve as a basis for shortening evaluation.

In the manufacture of GLP and GLS, varying amounts of mono-, di-, and trilactin, as well as small amounts of free lactic acid, are present in the final product. These do not contribute to the emulsification properties of the GLP or GLS and must therefore be removed before determining combined lactic acid on the shortening. Upon removal of these products by washing, the lactic acid can be released from its esters by saponification and measured in its free state.

The literature contains various methods for lactic acid determination in biological fluids, dried milk, dried eggs, and wine. In the Hillig (5) method, lactic acid is extracted from aqueous solution with ethyl ether by a continuous liquid-liquid extraction. The extract is taken up in water solution and lactic acid determined colorimetrically with ferric chloride. There are several methods of lactic acid determination based on its degradation to acetaldehyde, which may then be determined colorimetrically with veratrol (6), p-phenyl phenol (1), or hydroquinone (2), or may alternatively be absorbed in bisulfite solution and the addition product determined titrimetrically (3). Lactic acid may also be determined colorimetrically after oxidation to pyruvic acid (4).

An adaptation of the Barker and Summerson procedure for determining lactic acid in biological materials (1), in which the lactic acid is degraded to

acetaldehyde and determined by its color reaction with p-phenyl phenol in concentrated sulfuric acid solution, was chosen for determining combined lactic acid in shortenings. Because percentages of lactic acid involved are low, a colorimetric procedure is desirable. The p-phenyl phenol is more sensitive to small amounts of lactic acid than other colorimetric reagents.

Analysis Procedure

Due to the very small amounts of lactic acid used in the color development, there is a serious risk of contamination, and all glassware should be scrupulously clean. Contamination from perspiration should be especially avoided.

The procedure consists of five basic steps:

1. Removal of water-insoluble glycerol esters from the shortening.
2. Saponification of the water-insoluble glycerol esters, followed by acidification of the potassium salts to release the lactic and fatty acids.
3. Degradation of the lactic acid to acetaldehyde by heating with concentrated sulfuric acid. (Heating time is important in this step since underheating will give incomplete conversion, whereas overheating may cause loss of the volatile acetaldehyde.)
4. Reaction of acetaldehyde in concentrated acid solution with p-phenyl phenol to give di-p-hydroxydiphenylethane, which is gradually oxidized to a purple colored compound of unknown composition. (The color reaction is catalyzed by copper sulfate. After oxidation has been allowed to progress at a low temperature for a time, it can be accelerated to completion by holding the reaction mixture at an elevated temperature for a short time. If the solution is then cooled to 20°C. immediately the color is stable for several hours.)
5. Measurement of light absorption of the colored complex at 570 mμ.

The above analysis is carried out as follows:

Apparatus:

Extraction cylinders—25 ml., with siphons to fit
Separatory funnels—60 ml.
Volumetric flasks—100 ml.
Test tubes—19 × 150 mm. Pyrex, and rack for tubes
Pipets—1 ml. measuring, calibrated in 0.01 ml.
1 ml. volumetric
Buret—50 ml.
Microburet—calibrated in 0.02 ml.
Water baths—80°C., 30°C., and ice water
Stop watch
Spectrophotometer with 1 cm. cells

Reagents:

Chloroform, U.S.P.

Alcoholic potassium hydroxide—Add 5 to 10 grams of potassium hydroxide to 1–1.5 liters of 95% alcohol in a 2-liter flask and reflux for 1 hour. Distill the alcohol and dissolve 40 grams of reagent grade KOH in 1 liter of the distilled alcohol, keeping the temperature below 15.5°C. The solution should be clear

Sulfuric acid—concentrated

Sulfuric acid—dilute 1 volume of acid with 1 volume of distilled water

Petroleum ether, low boiling

Copper sulfate solution—4% $CuSO_4 \cdot 4\ H_2O$ in distilled water

p-Phenyl phenol solution—Dissolve 1.5% p-phenyl phenol (Eastman) in 2% sodium hydroxide solution. Filter. Prepare fresh daily

Lithium lactate standard solution—Dissolve 1.067 grams lithium lactate (Harleco) in distilled water and dilute to 1 liter. This solution contains the equivalent of 1 mg. lactic acid per ml. It will keep several months stored in the refrigerator

Removal of Water-Soluble Glycerides. Weigh accurately a 0.5- to 0.6-gram sample of melted shortening into an extraction cylinder. Dissolve the sample in 10 ml. of chloroform. Add 10 ml. of distilled water to the cylinder and extract by inverting the cylinder 25–30 times. Siphon off the separated water layer and repeat the extraction two more times, each time with 10 ml. of water. Set up a blank determination on the reagents, using 10 ml. of chloroform instead of the sample solution.

Transfer the washed chloroform solution to a 50-ml. beaker and rinse the cylinder with two 3-ml. portions of chloroform, followed by a final rinse with 3 ml. of acetone, adding the rinsings to the 50 ml. beaker. Evaporate the solvent under a stream of clean air on a steam bath until no odor of chloroform remains.

Saponification and Acidification to Release Lactic Acid. Add 5 ml. of alcoholic potassium hydroxide to the beaker containing the water-washed sample. Heat the beaker on the steam bath to saponify the shortening. When almost all of the alcohol has evaporated, add 2–3 ml. of water and again evaporate until no odor of alcohol remains. Add 5–10 ml. of distilled water and continue heating on the steam bath to dissolve the soap.

Acidify the sample by the addition of 3 ml. of 1:1 sulfuric acid solution. Keep the sample on the steam bath a short time until the fatty acids have completely melted, then cool to 60°C. and add 20–25 ml. of petroleum ether.

Transfer the beaker contents quantitatively to a separatory funnel, rinsing the beaker with about 5 ml. of 60°C. distilled water. Draw the water layer through a fluted filter paper (Whatman No. 12) into a 100-ml. volumetric flask. (A fluted paper is used to avoid any perspiration contamination from the hands which might occur in folding a filter paper.) Rinse the beaker again with about 10 ml. of 60°C. water, pour into the separatory funnel, and after cooling to room temperature, swirl the funnel contents to extract the petroleum ether layer. Draw off the water layer through the filter as previously, and repeat the washing once more.

Dilute the contents of the flask to 100 ml. Prepare a dilution from the solution such that the final concentration is equal to 2–8 micrograms of lactic acid per ml. of solution.

Degradation to Acetaldehyde. Pipet 1 ml. of the diluted sample solution into a Pyrex test tube. Add exactly 0.10 ml. of 4% $CuSO_4 \cdot 5\ H_2O$ solution to the tube, followed by 6 ml. of concentrated sulfuric acid, added from a buret.

Shake the sample solution for 10 seconds to disperse the lactic acid in the sulfuric acid, stopper it loosely with a glass stopper, and place it in an 80°C. water bath for exactly 5 minutes. Immediately cool the sample solution to below 20°C. by placing it in an ice water bath for 5 minutes.

Color Reaction and Measurement. Add 0.10 ml. of p-phenyl phenol solution to the cooled acetaldehyde

solution, taking care not to get any reagent on the sides of the tube. Disperse the reagent immediately by shaking the tube. Place the tube in a 30°C. water bath for 30 minutes. Shake it once during this interval to dissolve any remaining reagent. Place the tube in an 80°C. water bath for exactly 90 seconds. Remove, cool for 5 minutes in an ice water bath, and read the optical density of the solution at 570 mμ against the blank solution which has been treated the same as the sample throughout.

Calculation of Lactic Acid Content. Determine the concentration of lactic acid by referring to a standard curve which is set up as follows: Prepare a tenfold dilution of the lithium lactate standard solution. Dilute it further to give solutions containing 1, 2, 4, 6, and 8 micrograms of lactic acid per ml. Pipet 1 ml. of each of these solutions into a Pyrex test tube, convert the lactic acid to acetaldehyde, and develop the color as shown above for a sample solution. Use 1 ml. of distilled water as a blank. Plot the absorption at 570 mμ against the lactic acid concentration in micrograms. The curve obtained obeys Beer's Law in this range.

$$\% \text{ lactic acid} = 100 \frac{(\text{micrograms lactic acid} \times \text{dilution factor})}{\text{Sample weight in grams} \times 10^6}$$

Results on Commercial Shortening Analyses

The method has been used for over a year in analyzing commercial shortenings for combined water-insoluble lactic acid content. Shortenings analyzed ranged from about 0.70 to 1.10% water-insoluble lactic acid. As a check on the precision of the method in this range, a sample of shortening which had analyzed 0.90% water-insoluble lactic acid was blended with an amount of shortening containing no emulsifier to give a product with 0.70% water-insoluble lactic acid. Another portion of the lactylated shortening was blended with sufficient GLP, having a determined water-insoluble lactic acid content of 11.4% to give a mixture containing 1.10% water-insoluble lactic acid. Results of analyses on these 3 shortenings are shown in Table I.

TABLE I

Water-Insoluble Combined Lactic Acid Content of Shortenings

Sample No.	Calculated % water-insoluble lactic acid	Determined % water-insoluble lactic acid	Average
1	.....	0.90, 0.88, 0.87, 0.90, 0.91	0.89
2	0.70	0.72, 0.68, 0.68, 0.69	0.69
3	1.10	1.09, 1.10, 1.08, 1.07	1.08

During the year's time, a control shortening sample was analyzed 41 times for combined water-insoluble lactic acid content. Three different analyzers participated in these analyses. The range of results on the analyses was 0.88 to 0.99% water-insoluble lactic acid with a sample mean of 0.92%. The standard deviation was .028%.

The method has also been applied to the analysis of GLP and GLS emulsifiers for water-insoluble combined lactic acid content. Sample size is reduced to 0.1 gram for these analyses where water-insoluble lactic acid content is of the order of 10 to 13%.

Discussion

In the development of the lactic acid analysis procedure, several modifications of the acetaldehyde-p-phenyl phenol procedure were tried. In the literature there is some controversy concerning the use of the copper catalyst in color development. It was used by Barker and Summerson (1); however, J. Velasco and C.R. Noll (7) did not use it in determining lactic acid in dried milk products. Both procedures were tried in the above work. It was found that, without the use of the copper catalyst, complete color development is not attained and the extent of color development is highly dependent on the temperature. At any given temperature, maximum color development is achieved after 3 hours and no further change is noted after an additional 2 hours. In developing colors of standard solutions containing 1, 3, 5, 7.5, and 10 micrograms of lactic acid at 8°, 20°, 30°, and 45°C., it was found that greatest color development is attained at the lowest temperature with lesser color development as the temperature increases. There is a considerable decrease in extent of color development between 30° and 45°C. When a copper catalyst is used, color development is complete and reaches completion in ½ hour when the method is followed as given above.

In the course of this investigation it was noted that color development is very sensitive to small changes in quantities of reagent or in procedure. To be sure that optimum time and temperatures were used, as well as optimum amounts of reagents throughout, the following variables were studied:

p-Phenyl Phenol Concentration. To standards containing from 1–10 micrograms of lactic acid, p-phenyl phenol was added in these amounts: (a) 0.05 ml. of 1.0% solution; (b) 0.05 ml. of 1.5% solution; (c) 0.10 ml. of 1.5% solution; (d) 0.15 ml. of 1.5% solution. Both (c) and (d) gave full color development though (d) was difficult to dissolve in the acid solution. Incomplete color development was given by (a) when more than 5 micrograms of lactic acid were present, and (b) was inadequate for more than 7.5 micrograms. Based on these results, 0.10 ml. of 1.5% p-phenyl phenol solution was chosen.

Copper Catalyst Concentration. Using the same standards as above, it was found that 0.02 ml. of 4% solution of $CuSO_4 \cdot 5\,H_2O$ is insufficient for complete color development, and 0.05 ml. is somewhat low for 10 micrograms of lactic acid. Either 0.10 or 0.15 ml. is satisfactory. To keep the sulfuric acid as concentrated as possible during color development, 0.10 ml. was chosen.

Bath Temperature. The effect of bath temperature on conversion of lactic acid to acetaldehyde was checked at both 80° and 100°C. There was no difference. The same was true for bath temperature for final color development. Either 80° or 100°C. is satisfactory.

Time for Color Development. In developing the violet color, there is a noticeable change from blue-

violet to red-violet in the final 90 seconds when color development is completed in the 80°C. bath. Standard lactic acid solutions were checked at a final color development time of 60, 90, and 180 seconds. Identical results were obtained after 60 and 90 seconds at 80°C., but there was a marked decrease in color intensity when the solutions were held in the bath for 180 seconds. Ninety seconds was chosen for the time, which must be accurately measured.

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The Nutritive Value of the Brazil Nut Oil

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The chemical composition of the decorticated Brazil nut in the natural and fat-free state was determined, and the results confirm that this seed contains significant amounts of oil and protein. In three growth experiments carried out in rats, the Brazil nut oil had a nutritive value comparable to that of butterfat and the common table oils, olive oil, cottonseed oil, and corn oil. The use of levels of the Brazil nut oil up to 20% of the diet did not reduce growth or food consumption of rats. Heat treatment of the oil up to 120 min. at 140°C. did not reduce its nutritive value. The coefficient of digestibility of the Brazil nut oil was similar to that of the other oils, fresh and heated. The digestibility of the Brazil nut oil averaged 98% and was not affected by the heat treatment. The data indicate that Brazil nut oil is comparable to other animal and vegetable fats and oils in common use for human consumption.

ONE OF THE GREAT number of seeds in the Latin American countries that offers unexploited possibilities for development and use (8) is the fruit of the tree *Bertholletia excelsa,* known as the Brazil nut. Available chemical analyses of this nut (9) indicate that it contains oil and protein in amounts that have practical significance for both animal and human nutrition.

Pechnik *et al.* (10) classified the oil as a semi-drying type from studies for its density, refractive index, saponification, and iodine number, and similar findings were reported by Noriega (9). The digestibility of the oil was found to be 97.7% (10). Hilditch (6) and Schuette *et al.* (11) investigated the fatty acid composition of Brazil nut oil and showed that the concentration of oleic acid varied between 48 and 58% while the linoleic acid content varied from 23 to 30%. Chaves and Pechnik (3) showed that the carotene in Brazil nut oil is relatively stable. Costa and Mota (4) indicated that, for the extraction of the oil, screw-pressing is very efficient and more practical than solvent extraction because with the latter the oil retains traces of the solvent and the fat-soluble vitamins are completely removed from the meal.

The present study was undertaken to determine whether the oil is of a sufficiently good nutritive value to recommend its industrial production on a large scale as a table oil. This, in turn, might make the press cake available for use in animal and human nutrition.

[1] International Cooperation Administration fellow and Research Assistant. INCAP Publication I-150.

Materials and Methods

The nuts used for the extraction of the oil were obtained from Belem, Pará, Brazil.[2] The decorticated nut was analyzed for its proximate composition by A.O.A.C. methods (2), and the oil was obtained from it by solvent extraction with petroleum ether in a 3-liter Soxhlet extractor and by pressure. The solvent-extracted oil was purified by filtration with Celite [3] after removal of the solvent by vacuum. The pressure-extracted oil was obtained with a Carver laboratory press, using 10,000 p.s.i. pressure, and was also purified with Celite. Approximately 85% yields were obtained by using pressure and 90–95% by using the solvent method. The purified oil had a clear yellowish color. The pressure and solvent-extracted oils were analyzed for their iodine value, unsaponifiable matter, and color by the American Oil Chemists' Society Methods and Analysis (1) while the fatty acids were calculated from the areas under their gas-liquid chromatographic curves, using a Barber-Colman Model 20, tritium detector.

Nutritive value was assessed by rat-growth trials and digestibility studies, using albino rats of the Wistar strain. In all experiments the animals were distributed among the experimental diets by weight and sex and were placed in individual all-wire cages with raised screen bottoms. Food and water were provided *ad libitum,* and growth and food consumption were noted every 7 days for a total of 28 days.

The first of the three growth trials with rats was designed to test the nutritive value of the solvent-extracted oil. Brazil nut oil was tested at 5, 10, and 20% levels in a basal ration composed of vitamin-free casein [4] 25%, alphacel 1.80%, mineral mixture [5] 4.00%, L-cystine 0.20%, cod liver oil [6] 2.00%, and cornstarch to 100%. Five ml. of a vitamin solution (7) per 100 g. were also added. Each ration was fed to groups of six female rats. An additional group of rats was fed the basal diet with 20% olive oil.

The second growth trial was similar to the first except that the pressure-extracted oil was used at

[2] Obtained through the courtesy of Frank Lowenstein.

[3] Johns-Manville.

[4] Nutritional Biochemicals Corporation, Cleveland, O.

[5] Hegsted Mineral Mixture (5), Nutritional Biochemicals Corporation, Cleveland, O.

[6] 1,800 U.S.P. units of vitamin A and 175 U.S.P. units of vitamin D per gram from Mead-Johnson, Evansville, Ind.