

NOTE

Use of Phenol-Sulfuric Acid Assay to Determine Composition of Starch-Oil Composites

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Recent studies at this center have reported the preparation of jet-cooked starch-oil composites (Eskins and Fanta 1994, Fanta and Eskins 1995, Knutson et al 1996), that demonstrate unusual stability, both in dry form and in solution, and are useful as fat replacers-reducers in food products. Composites containing up to 500 mg of oil per gram of starch can be dried to a powder that forms a stable suspension when redissolved in water. Oil does not separate from suspensions spontaneously, and hexane extraction is difficult and inefficient. From the dried product, oil cannot be completely recovered by extraction with hexane; as much as 25% of the original weight of oil is retained in the starch matrix. This retained oil is designated as tightly bound oil (Knutson et al 1996), the extractable portion is designated as loosely bound, and any oil that separates spontaneously is designated as unbound oil.

Characterization of these composites requires a method to determine carbohydrate content of fractions containing high concentrations of oil. The phenol-sulfuric acid assay (Dubois et al 1956), a widely used method for measurement of carbohydrates, is rapid, reproducible, and accurate, but its efficacy for analyzing samples with high lipid content and the possibility of interference by lipids has not been established. This study was undertaken to determine whether the phenol-sulfuric acid assay could be used in the presence of excess lipid. In the course of the investigation, it was found that reaction of mixtures containing carbohydrate and unsaturated lipids formed a second chromophore, which could be used to estimate the oil content of the samples.

MATERIALS AND METHODS

Reagent-grade phenol and sulfuric acid were used. Glucose, corn starch, oleic, linoleic and linolenic acid methyl esters, and lauric and stearic free fatty acids were obtained from Sigma Chemical Co. (St. Louis, MO). Corn, vegetable (soybean), and olive oils were obtained from a local supermarket. Lipid samples were dissolved in absolute ethanol. Carbohydrate and composite samples were prepared by suspending in water.

The phenol-sulfuric acid procedure was conducted as described by Dubois et al (1956), with the modification that the solvent for samples at final dilution (at concentrations low enough for absorbance measurement) was a 1:1 mixture of ethanol and water. Although lipids were insoluble in this mixture, they remained suspended in the solvent as emulsions or finely divided droplets. This solvent was suitable for mixtures of oils or fatty acid methyl esters with either glucose or starch, and for suspended starch-oil composites. Samples containing 0–100 μg of carbohydrate and 0–1,000 μg of lipid in 1 mL of solvent were mixed with 1 mL of 5% phenol in a

test tube cuvette with a 19-mm path length; 5 mL of concentrated sulfuric acid was added rapidly to generate heat to drive the reaction. The reaction mixture was allowed to cool to room temperature, and absorbance was measured at 490 nm in a spectrophotometer (Sequoia-Turner, model 690) against a water blank. Absorbance spectra were measured at 5-nm intervals for 380–600 nm.

RESULTS

In initial experiments, samples containing carbohydrate and soybean oil developed a red-orange color rather than the amber color typical of the phenol-sulfuric acid assay. Intensity of the red color increased with increasing lipid concentration, but absorbance at 490 nm (the wavelength of maximum absorbance for glucose and starch) did not vary significantly. These results indicated a second chromophore with an absorbance maximum at a higher wavelength that was independent of the carbohydrate chromophore. The presence of this chromophore was verified by measuring absorbance spectra of mixtures containing varying concentrations of carbohydrate and lipid. Spectra are shown in Fig. 1 for samples containing: 1) 1,000 μg of oleic and linoleic acid methyl esters; 2) 50 μg of glucose; 3) mixtures of 50 μg of glucose with either 1,000 μg of oleic or linoleic acid methyl ester. All mixtures containing carbohydrate and unsaturated lipid exhibited a shoulder on the carbohydrate absorbance curve at 530–540 nm, the size of which varied with the concentration of carbohydrate or lipid. Spectra of glucose and starch, both with and without lipid, were equivalent. The spectra for mixtures of linolenic acid with carbohydrate (data not shown) were similar to those of linoleic acid. Samples of unsaturated lipid in the absence of carbohydrate were yellow, as shown by the absorbance <460 nm. Absorbance of these samples was elevated throughout the spectrum but became constant >580 nm. To eliminate baseline interference from lipids, absorbance at 580 nm was routinely used as the zero value for all

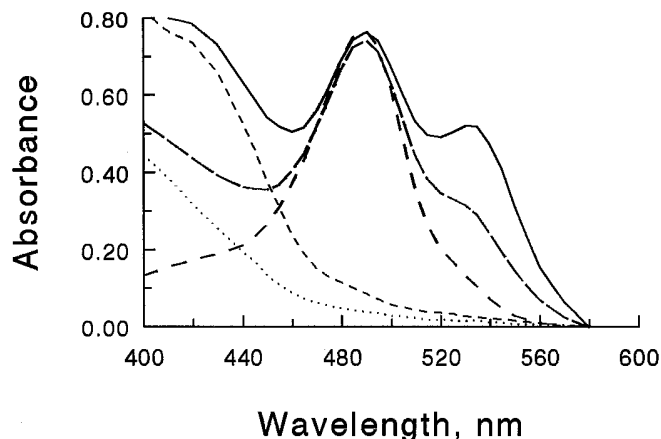


Fig. 1. Phenol-sulfuric acid absorbance spectra of glucose, unsaturated fatty acid methyl esters, and mixtures of glucose with unsaturated fatty acid methyl esters: oleic acid ($\cdot \cdot \cdot$); linoleic acid ($- \cdot -$); glucose ($- - -$); glucose + oleic acid ($- - -$); glucose + linoleic acid ($-$).

¹Biomaterials Processing Research Unit, National Center for Agricultural Utilization Research, ARS, USDA, 1815 N. University, Peoria, IL 61604. Mention of a product is for informational purposes only and is not meant to imply recommendation by the U. S. Department of Agriculture over others that may be suitable.

spectra. Saturated lipids developed no color in the absence of carbohydrate, and in the presence of carbohydrate, only developed color typical of pure carbohydrate, with no shoulder at 530–540 nm.

To isolate the spectrum of the chromophore at 530–540 nm, absorbance spectra of carbohydrate alone and lipid alone were subtracted from spectra of mixtures of carbohydrate and lipid at equivalent concentrations, leaving a difference spectrum consisting of a single symmetrical peak with absorbance maximum at 535 nm, absorbance of which varied with concentration of both carbohydrate and lipid.

To further evaluate the formation of this chromophore, reactions were run in the absence of phenol and by reacting lipid with phenol and sulfuric acid, allowing the reaction mixture to cool to room temperature, and adding glucose to the mixture. Spectra of these mixtures were measured periodically for up to 500 hr. These experiments showed that the 535-nm chromophore formed in the absence of phenol and in the reaction mixture did not immediately form upon addition of glucose to a cooled lipid-sulfuric acid reaction mixture. As absorbance at 490 nm gradually increased after a period of many hours, formation of the 535-nm chromophore also occurred. Control samples of glucose added to a cooled reaction mixture containing no lipid developed absorbance at 490 nm at the same rate but with no maximum at 535 nm. These experiments demonstrated that formation of the 535-nm chromophore required the presence of the chromophore responsible for carbohydrate absorbance at 490 nm.

Absorbance at 490 and 535 nm was measured in mixtures containing 0–100 μg of glucose or starch and 0–1,000 μg of oleic, linoleic or linolenic acid methyl esters, or corn, soybean, or olive oil. Net absorbance of the 535-nm chromophore was calculated by correcting for the proportion of absorbance at that wavelength caused by the carbohydrate chromophore. The ratio of absorbance at 535 nm to that at 490 nm, measured on glucose samples at concentrations of 10–100 μg , was calculated to be 0.1496, with a standard deviation of 0.0035. Net absorbance of carbohydrate-lipid mixtures at 535 nm, after correction, varied linearly with concentration of both lipid and carbohydrate.

Data for mixtures of glucose with each lipid were evaluated using the equation for a three-dimensional linear plot: $z = k \times x \times y$, where x is μg of carbohydrate, y is μg of lipid, z is absorbance at 535 nm, and k is the constant of proportionality. A k value was calculated for each lipid studied. For fatty acid methyl esters, values of ($k \times 10^6$) were: oleic acid, 4.39; linoleic acid, 8.69; linolenic acid, 6.92. For triglyceride oils values of ($k \times 10^6$) were: olive, 4.51; soybean, 5.64; and corn, 6.02. Linear correlation coefficients were >0.96 . A representative plot using mixtures of glucose and linoleic acid is shown in Fig. 2. Rearranging the equation to: $y = z/(k \times x)$ made it possible to determine lipid content of carbohydrate-lipid mixtures by determining carbohydrate content of the sample and calculating the appropriate k value. Results from calculation of composition of starch-oil composite fractions were equivalent to those obtained by enzyme hydrolysis of the starch followed by extraction of oil and determination of carbohydrate content of the residual sample (C. A. Knutson, unpublished data).

DISCUSSION

The initial goal of this study was to determine whether the phenol-sulfuric acid assay could be used to determine carbohydrate content of samples with high oil content. This was satisfied by ascertaining that neither saturated or unsaturated lipids interfered with the absorbance maximum at 490 nm.

Discovery of the formation of the 535-nm chromophore enhanced the utility of the assay for our use by making it possible to determine both carbohydrate and oil content of high-oil samples in a single procedure. Because of the low absorptivity of the 535-nm

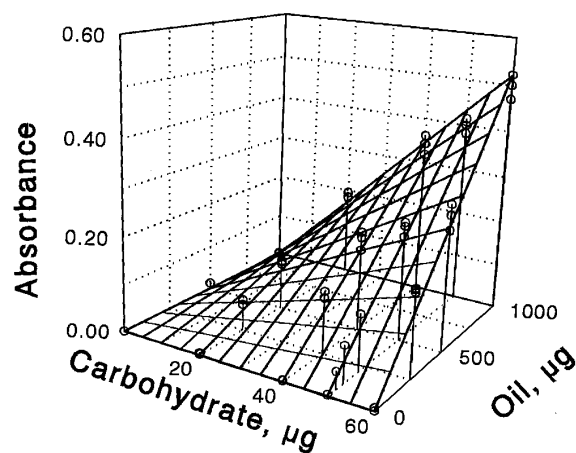


Fig. 2. Absorbance of 535-nm chromophore formed from glucose + linoleic acid methyl ester.

chromophore, sensitivity is not great enough to accurately assay samples at low oil concentrations. Because k values are specific for each lipid, the identity of a lipid must be known for quantitative assay, but the relatively narrow range of k values for triglyceride oils allows the method to be used for preliminary estimates of oil content samples of unknown composition.

Formation of the 535-nm chromophore appears to be due to the interaction of two products formed in concentrated sulfuric acid: (5-hydroxymethyl)-2-furaldehyde derived from carbohydrate (Wolfrom et al 1948, 1949) and sulfated oils formed by the action of sulfuric acid on double bonds of unsaturated oils. The reaction to form sulfated oils has been long known and was one of the earliest methods for producing synthetic surfactants (Jungermann 1979).

Significantly, although development of the chromophore at 535 nm was linear with respect to lipid concentration, it was not proportional to the degree of unsaturation of the lipid. The k value for linoleic acid (two double bonds) was approximately double that of oleic acid (one double bond) but was also higher than the value for linolenic acid (three double bonds), indicating steric interference either in sulfation of multiple double bonds or in development of the chromophore. The k values of the three oils in the study were in the expected range given their approximate fatty acid compositions (e.g., olive oil contains twice as much oleic acid as corn oil and about one-fifth as much linoleic acid as soybean oil) and the values for pure fatty acids.

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