A New Colorimetric Assay of Tabletop Sweeteners Using a Modified Biuret Reagent

An Analytical Chemistry Experiment for the Undergraduate Curriculum

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Several spectrophotometric procedures for determination of aspartame concentration in solutions have been reported in the chemical literature. These protocols include reagents such as ninhydrin *(1),* chloranilic acid *(2),* diethyldithiocarbamate *(3),* benzoquinone *(4),* and *p*-dimethylaminobenzaldehyde *(5).* Unfortunately, these methods involve hazardous reagents, tend to be time consuming, and are not conducive to teaching applications. A rapid, colorimetric method for assay of aspartame in tabletop sweeteners would be of considerable benefit in undergraduate teaching laboratories since most students are familiar with this artificial sweetener and find the, somewhat controversial, history of this substance quite interesting *(6).*

Described herein is a fast, convenient qualitative and quantitative analysis experiment for use in introductory to advanced-level laboratories that takes advantage of the unique color changes observed when copper(II) ions react with proteinaceous molecules. These experiments demonstrate the usefulness of a modified biuret reagent for differentiation and identification of solutions containing various tabletop sweeteners. Most notable is the quantitative, colorimetric analysis of tabletop sweeteners containing aspartame (i.e., NutraSweet).

The diagnostic reddish-purple color resulting when proteins react with alkaline solutions containing copper(II) ions has long been recognized as an effective means by which to identify aqueous protein solutions *(7).* As a result, colorimetric assay of total protein content in solutions has been an area of intense study. A literature report of the biuret method in 1949 definitively established the effectiveness of using copper(II) solutions in quantitative analysis of protein solutions *(8).* The name of this method is derived from the organic compound, biuret, which forms a purple–violet-colored copper(II) complex when reacted with an alkaline copper(II) sulfate solution *(9).* This copper(II)–biuret complex is similar in color to that observed when proteins react with alkaline copper(II) solutions. Accordingly, the biuret reagent, as it is now described, consists of a solution of copper(II) sulfate, potassium sodium tartrate, and sodium hydroxide. The biuret method is considered only useful for the assay of proteins and peptides with three or more amino acid residues present since simpler peptides and amino acids yield a blue-colored complex when reacted with the biuret reagent (*7* and references therein).

Subsequent to the publication of the biuret method, numerous other methods for determination of total protein concentration appeared in the chemical literature. These include the Lowry *(10),* Coomassie blue G-250 dye binding (Bradford) *(11),* bicinchoninic acid *(12),* and the pyrogallol red-molybdate *(13, 14)* assays. In recent years each of these methods has undergone scrutiny with respect to potential assay interference by various proteinaceous and non-proteinaceous materials. Cross reactivity with amino acids, carbohydrates, peptides, antibiotics, analgesics, sodium dodecyl sulfate, and even vitamins have been reported within the various colorimetric assay's *(8, 15–17).* Indeed, the classical biuret reagent is remarkably similar to Fehling's reagent for qualitative detection of reducing sugars, indicating the inherent difficulty associated with using this copper-based reagent for detection of amino acids and proteins in solutions that contain dextrose.

Amino acid and dipeptide interference within the biuret method is of particular interest. Amino acids and dipeptides react with the biuret reagent to provide distinctly dark blue complex ion solutions. The visual appearance of these solutions is quite different from the corresponding, purple, protein complexes providing for easy differentiation between these types of compounds. The indicative blue color observed when amino acids and small peptides react with aqueous copper(II) solutions has largely been disregarded as a quantitative tool even though this diagnostic color change has been known for over 75 years *(18).* Indeed, amino acid cross-reactivity is typically considered an undesired problem in applications of the biuret assay *(7)*! Conversely, reaction of 1 M copper(II) sulfate solution with amino acid and amine solutions has been shown to be an effective qualitative assay for use in instructional settings *(19).*

The distinctive blue complex that results when aspartame reacts with copper(II) ions allows for qualitative and quantitative analyses of solutions containing this important artificial sweetener. This blue complex was shown to be the result of coordination of the carboxylate and β-amino groups of aspartame to copper(II) ion forming a unique trinuclear copper(II) complex *(20).*

Care must be taken when attempting to assay tabletop sweeteners with copper(II)-based reagents since a large component of these products is, in fact, dextrose. Sugars such as dextrose are well known to readily react with caustic copper(II) sulfate solutions (Fehling's reagent) *(21).* Significantly, we found if the solution pH is kept below 11, dextrose will not interfere with the analysis of aspartame in tabletop sweeteners. This is an important component of the new method described herein.

Experimental Procedure

Students are provided a standard sample of aspartame and several individual serving packages of different tabletop sweet-

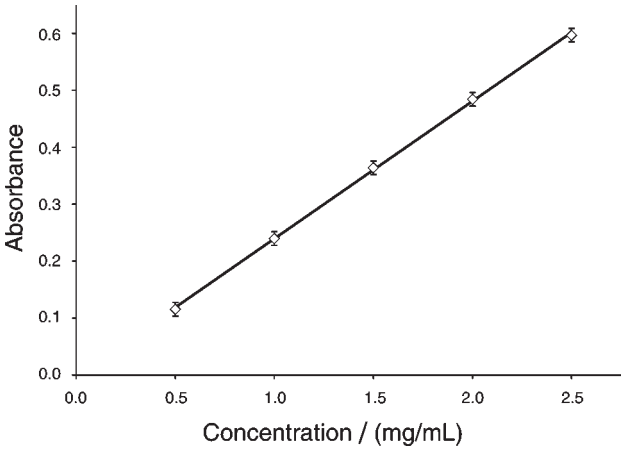

Figure 1. Plot of absorbance versus concentration/(mg/mL) for aspartame standards.

eners. Typically these sweeteners include those that contain sugar, aspartame, acesulfame potassium, saccharine, and sucralose. An aspartame stock solution is prepared by dissolving 250 mg aspartame into 50.00 mL water (5.00 mg/mL). This reference standard is compared to solutions of each of the sweetener samples. Sweetener solutions to be tested are made by dissolving approximately 1 g of each sweetener into 15 mL water.

Each of the sweetener samples is then reacted with the modified biuret reagent. This reagent may be prepared up to two weeks in advance and consists of 60 mmol copper(II) sulfate pentahydrate, 210 mmol potassium sodium tartrate tetrahydrate, and 500 mmol sodium carbonate monohydrate dissolved in 1 L of water.

Samples are compared most effectively using a 24-well plate. The first two wells of the plate are labeled "water" and "aspartame", respectively. The water serves as a blank. Subsequent wells are labeled to identify each sweetener being tested. A disposable pipet is used to deliver about 1 mL of each sample to a labeled well in the 24-well plate. Then, five drops of biuret reagent are added to the water, aspartame standard, and each subsequent sample. Observations are made and recorded.

Once the sweetener containing aspartame is identified, the quantity of aspartame in a single serving packet may be determined by colorimetric analysis at a wavelength of 629 nm and applying the Beer–Lambert law. Reference standards are prepared with concentrations of 0.50 mg/mL, 1.00 mg/mL, 1.50 mg/mL, 2.00 mg/mL, and 2.50 mg/mL. These may be readily obtained by dilution of the stock aspartame solution (5.00 mg/mL). In practice, the 0.50 mg/mL reference standard is made by mixing 1.00 mL of stock aspartame solution with 2.00 mL biuret reagent and dilution to 10.00 mL total volume with water. The other standards are prepared in an analogous manner using 2.00 mL, 3.00 mL, 4.00 mL, and 5.00 mL of stock aspartame solution, respectively. A reagent blank is prepared by adding 2.00 mL biuret reagent to a 10.00 mL volumetric flask and dilution to volume with water. The spectrophotometer is calibrated with the reagent blank and absorbance readings of the standards are made after allowing at least 15 minutes for the samples to react.

Sweetener samples are prepared in a manner similar to the reference standards. Students deliver sweetener samples with masses ranging from 0.2000 g to 0.5000 g directly to a 10.00 mL volumetric flask. Water (5.0 mL) is added to the flask to dissolve the sample and biuret reagent (2.00 mL) is added to the solution. Finally, the sample is diluted to volume with water. Absorbance readings are, once again, made after allowing at least 15 minutes for the samples to react.

Equipment and Chemicals

All spectroscopic data were collected with a Thermo Electron Corporation Spectronic 20D+ spectrophotometer. Chemicals are reagent grade, with the exception of commercial sweeteners, which were purchased at a local retail outlet.

Hazards

Copper(II) sulfate pentahydrate is toxic and dangerous for the environment. It is harmful if swallowed and irritating to the eyes and skin. This compound is known to be very toxic to aquatic organisms and is a possible sensitizer. Target organs of copper(II) sulfate include the liver and kidneys. Although aspartame is commonly ingested as an artificial sweetener, caution should be exercised to avoid direct contact and inhalation. Sodium carbonate monohydrate and potassium sodium tartrate tetrahydrate are irritating to eyes and the respiratory tract. Avoid contact and inhalation.

Results and Discussion

Qualitative analysis of tabletop sweeteners containing aspartame using the modified biuret reagent is straightforward. A typical package of artificial sweetener contains around 1 g of material. Dissolving the entire package into 15 mL water provides a solution of appropriate concentration to give an easily recognized comparative analysis. Addition of biuret reagent reveals the sweetener that contains aspartame as a result of the diagnostic blue color. Other sweeteners do not react (see the Supplemental Material[W]).

Quantitative analysis of sweetener packages using this modified biuret reagent provides excellent results (Figure 1). Comparison of standard plots over a series of 12 student trials gave consistently linear relationships (r^2 = 0.9996). Absorbance measurements on the standard graph had a deviation of 0.025 or less. Student analyses of 44 different samples of Equal gave, on average, a total aspartame content of 37.6 mg/g sweetener as compared to a reported value of 38 mg/g sweetener *(22)*. Variance of this measurement was as low as ±0.60 mg/g sweetener depending upon the student group conducting the assay. Overall, students in introductory laboratory sections were able to obtain results within ±2 mg of the reported aspartame content value. More advanced students were able to get values of 38.2 mg/g ± 0.85 mg/g sweetener on a consistent basis.

Of particular note in this procedure is the ability to obtain consistently reliable results with a minimum of manual manipulations. Overall sample preparation consists of three steps: (i) addition of the analyte to a 10.00 mL volumetric flask, (ii) addition of biuret reagent, and (iii) dilution with water. Spectroscopic analysis of the samples completes the process.

Students can work effectively in small groups (three or fewer) and use a minimum of glassware. Each group requires

six volumetric flasks (10 mL), a 5 mL graduated volumetric pipet, a 2 mL volumetric pipet, and two cuvettes. Introductory students may be supplied with a stock aspartame solution (5.00 mg/mL) prepared by the instructor in advance of the laboratory session. Students then use a graduated 5 mL volumetric pipet to prepare the aspartame standards. Addition of the biuret reagent to the standard solutions using a 2 mL volumetric pipet and dilution to volume with water completes the process of preparing the reference standards. A reagent blank is prepared similarly.

Direct addition of the sweetener sample to the volumetric flask simplifies the procedure and minimizes error. Dissolution of the sweetener into water may be facilitated by immersing the flask into an ultrasonic cleaner for about one minute. Upon addition of biuret reagent and dilution to volume with water, each sample must be allowed to react for a minimum of 15 minutes. Absorbance readings are variable prior to this time and have not reached a maximum value. Also note that dextrose and maltodextrose do not interfere with this assay. However, use of the classical, sodium hydroxide-based, biuret reagent results in rapid oxidation of these carbohydrates and causes the assay to fail.

The optimum wavelength for data collection may be determined by analyzing any one of the aspartame–copper (II) standard solutions using a scanning UV–vis spectrophotometer. A representative spectrum collected using a Beckman model DU 520 UV–vis spectrophotometer, scanning the region between 400 nm and 850 nm, revealed that the maximum absorbance for the aspartame–copper(II) complex is found at 629 nm. Each sweetener sample is subsequently evaluated using visible light spectroscopy at this wavelength. A Spectronic 20 spectrophotometer and glass or plastic cuvettes may be employed for this assay since the analysis is conducted in the visible region of the light spectrum. This eliminates the need for expensive quartz or special UV-transparent plastic cuvettes and more expensive, UV-capable spectrophotometers.

The entire experiment may be readily completed in a three-hour laboratory period. Introductory students may simply follow a predefined procedure to obtain results while advanced students may be challenged with an inquiry-based approach. Advanced students are given the appropriate theoretical background with respect to colorimetric analysis and are challenged to develop a protocol to determine aspartame content in Equal samples based upon the expected aspartame content of 38 mg/g sweetener. They can then prepare their own stock solutions and conduct the computations necessary to determine appropriate standard solution concentrations.

Conclusions

A new colorimetric analysis of aspartame in the tabletop sweetener Equal is presented. Students obtain results that are reliable and accurate in comparison to the reported accepted values. Student analyses of 44 different samples of Equal gave a total aspartame content of 37.6 mg/g ± 2 mg/g sweetener as compared to the reported value of 38 mg/g sweetener. The procedure is a simple four-step process involving addition of the analyte to a 10.00 mL volumetric flask, addition of modified biuret reagent, dilution with water, and spectroscopic analysis. Introductory to advanced-level undergraduate students may complete the analysis in a typical three-hour laboratory period. Inquiry-based learning methods may be readily incorporated into this experiment owing to its relative ease and straightforward nature.

[w]Supplemental Material

Instructions for the students including detailed background material and postlab questions are available in this issue of *JCE Online*.

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