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Two Methods of Determining Total Phenolic Content of Foods and Juices in a General, Organic, and Biological (GOB) Chemistry Lab

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The introduction of students to spectroscopic techniques of identifying and quantifying chemicals is fundamental and consistently covered in introductory laboratory manuals. Instrumental techniques of analysis are widespread in the contemporary medical environment, so students in health-related disciplines would benefit from experiences in spectroscopic chemical analysis. Unfortunately, general, organic, and biological (GOB) chemistry laboratory manuals offer few, if any, engaging exercises involving application of scientific instrumentation. Another challenge associated with the GOB chemistry laboratory, which is usually required in some form by schools of nursing, is showing how the chemistry being taught is relevant *(1)*. In an effort to introduce instrumental techniques of analysis and more health-related course content into the GOB course, we developed and implemented laboratory exercises involving the determination of phenolic concentration in various foods and beverages by visible absorption spectroscopy.

Phenolics have reached the popular vernacular owing to their attributed support of the immune system, success against maladies from cancer to cardiovascular disease, and their common presence in fruits, vegetables, whole grains, legumes, nuts, chocolate, tea, and wine *(2, 3)*. Health benefits related to phenolics are primarily attributed to their strong antioxidant capacity *(4)*. In addition, phenolics are important in fruit quality, such as taste and color *(2, 5, 6)*. Their natural ubiquity, widely recognized benefits, and prominence in the popular culture make phenolics an interesting and relevant class of chemicals to analyze.

There are various methods of quantifying total phenolic content in food and drink. The most generally accepted method appears to be that of Singleton and Rossi *(7)*, which assays antioxidant capacity using the Folin−Ciocalteu (FC) reagent. Modifications of this method along with sample preparation *(8)* were introduced to the students enrolled in GOB chemistry laboratories in fall, 2007 *(9)*. Students were able to complete the analysis in a three-hour laboratory with results comparable to values found in the literature *(8)*. Simplifications in the analytical procedure evolved over the years and allowed students more time to understand the importance of a calibration plot, discuss their experimental results, and appreciate the significance of their findings *(10)*. Our original technique, involving the use of analytical glassware, is more suitable to general introductory chemistry laboratories *(11)*.

In summer, 2009, the laboratory assay was limited to total phenolic content of white grape juices using the ferrous ammonium sulfate (FAS) reagent *(6, 12)*. Using juices eliminated extraction techniques but provided time to compare different samples and discuss the results. The use of the FAS reagent generated more consistent results than the FC reagent. Additionally, FAS is not as susceptible to contamination, is less toxic, and less harmful to the environment *(13)*.

Experiment

There are a variety of different phenolic compounds in foods and juices, each with their unique absorptivity in the ultraviolet to visible region. As a result, there is a distinction between specific and total phenolic assay. To identify and quantify specific phenolics, high-performance liquid chromatography (HPLC) can be used *(8)*, but this method is beyond the scope of a typical GOB laboratory course. To determine total phenolic content (concentration), three assumptions are made: (i) reagents such as FC and FAS react similarly with all phenolics *(14)*; (ii) only phenolics react with the FC and FAS reagents *(13)*; and (iii) the relationship between absorption of reacted FC or FAS reagents as a function of phenolic concentration is linear, following the Beer−Lambert law. Typically, gallic acid is used as the standard reference phenolic compound. Total phenolic content is then expressed as the gallic acid equivalent (GAE) *(15)*.

Folin−Ciocalteu (FC) Reagent

The FC reagent was used to determine the GAE of solid foods, primarily apples. A small quantity, ∼10 g, of finely cut solid food sample was ground by mortar and pestle in 10 mL of 70% (v/v) ethanol for 10 min. The solid was washed and the filtrate collected using vacuum filtration through 1 cm of Celite. The filtrate was then diluted to 50 mL with 70% ethanol. Separately, five gallic acid solutions between 0.003% (m/v) and 0.015% (m/v) were prepared from a stock solution of 0.15% (m/v) gallic acid in 70% (v/v) ethanol. Small volumes, 0.6 mL of each solution of gallic acid, the sample filtrate, and a 70% (v/v) ethanol blank were transferred to 13 × 100 mm test tubes. To each of the seven test tubes, 2.0 mL of the FC reagent and 1.5 mL of 7.5% (m/v) sodium carbonate were added and mixed. After the addition of these solutions, the concentrations of the five gallic acid standards ranged from 4 to 22 μg/mL. The test tubes were placed in a 60 ± 5 °C water bath for 10 min and then quenched in an ice bath. The solutions were transferred to cuvettes and absorptions collected at 765 nm. The GAE content of the food sample from the absorbance value was multiplied by 34 mL/g (the dilution factor) to obtain units of (μg GAE)/(g food sample).

Ferrous Ammonium Sulfate (FAS) Reagent

The FAS reagent was used to determine the GAE of white grape juice. A small volume, 1 mL, of white grape juice was placed

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Table 1. GOB Chemistry Student Results from Fall 2007 to Spring 2010

Technique	Food Type	Gallic Acid Equivalent Average/[(μg GAE)/(g sample)]	Standard Deviation/(μg/g)	Number of Groups
F−C Reagent	Apple	290	150	16
F−C Reagent	Pear	290	190	5
F−C Reagent	Banana	290	330	4
F−C Reagent	Applesauce	420	370	3
F−C Reagent	Orange	550	190	3
F−C Reagent	Kiwi	410	-	2
F−C Reagent	Strawberry	1000	-	2
F−C Reagent	Fruit Juice	580	-	2
F−C Reagent	Pomegranate	3900	-	1
F−C Reagent	Cherry	200	-	1
F−C Reagent	Melon	170	-	1
F−C Reagent	Peach	280	-	1
F−C Reagent	Plum	330	-	1
F−C Reagent	Onion	360	-	1

Technique	Food Type	Gallic Acid Equivalent Average/[(μg GAE)/(mL sample)]	Standard Deviation/(μg/mL)	Number of Groups
FAS Reagent	White Grape Juice: National Brand	2900	620	33
FAS Reagent	White Grape Juice: Store Brand	1100	220	13

in a 13 × 100 mm test tube, neutralized by sodium bicarbonate, and diluted to 4 mL with water. Separately, a 4 mL water blank and four, 4 mL gallic acid standard solutions between 0.03 and 0.15% (m/v) were prepared in 13 × 100 mm test tubes from a stock solution of 0.15% (m/v) gallic acid in 70% (v/v) ethanol. Two drops of 1% (m/v) aqueous ferrous ammonium sulfate (FAS) were added to each of the six test tubes. The solutions were shaken and placed in a 40−50 °C water bath for 15 min. The solutions were cooled in a cold water bath, transferred to cuvettes, and absorbance was measured at 575 nm.

Hazards

The FC reagent is a commercially available mixture of sodium tungstate, sodium molybdate, phosphoric acid, hydrochloric acid, lithium sulfate, and water. It is corrosive and dangerous if inhaled or if it comes in contact with any part of the body. All wastes generated from the FC test should be disposed of in accordance with local, state and federal regulations. Sodium carbonate is an irritant. Aqueous ethanol is flammable. Gallic acid and ferrous ammonium sulfate may cause irritation to skin, eyes, and respiratory tract and may be harmful if swallowed or inhaled.

Results

Folin−Ciocalteu (FC) Reagent

The value of 290 ± 150 (μg GAE)/(g sample) for apples (Table 1) compares to the values found in the literature of 430 ± 160 μg/g *(8)* with a range of 50−600 μg/g *(2)*. The values are dependent on the type of apple *(7, 8, 15)*, and, in the case of this exercise, the extraction technique. With a mortar and pestle, a 70% ethanol solution was more efficient in extraction than the acetone or methanol solutions mentioned in the literature.

Variations in water bath temperatures and length of reaction between FC reagent and phenolics affected the magnitude of the slope of the line modeling the relationship between absorption and gallic acid concentration. When 765 nm absorption was plotted as a function of gallic acid concentration by 43 groups or teams of GOB students, the slope was 0.065 ± 0.016 mL/μg, with an intercept of −0.02 ± 0.06, giving a squared correlation coefficient (R^2) of 0.7 ± 0.2.

Ferrous Ammonium Sulfate (FAS) Reagent

The total phenolic content of a national brand of grape juice, determined in the literature using the FC reagent, was 2.6 ± 0.4 mg/mL *(16)*. The experimentally determined total phenolic content of the same national brand of grape juice by 33 groups or teams of GOB students using the FAS reagent was 2.9 ± 0.6 mg/mL (Table 1). The slope of the line representing absorption as a function of gallic acid concentration for these same groups was 0.97 ± 0.03 mL/mg, with an intercept of −0.05 ± 0.03 and an R^2 = 0.89 ± 0.02.

Discussion

Determination of the total phenolic content of solid foods introduces students to the following laboratory techniques: extraction, vacuum filtration, volumetric analysis, and (Spec 20) spectrophotometer instrument use and calibration. In addition, the Beer−Lambert law is introduced and applied. The analyses of juices and other solutions such as wine and teas do not expose students to extraction and vacuum filtration techniques.

Absorption at 765 nm is used to determine the GAE of foods when the FC reagent is employed. As a result, the total phenolic content of a wide variety of juices, wines, chocolate, teas and solid foods can be determined with the FC reagent *(3, 15, 16)*.

Conversely, the GAE is determined by absorption at 575 nm using the FAS reagent. Most colored food and drink appreciably absorb and interfere at this wavelength, limiting this application to low-colored foods (low absorptivity at 575 nm) such as white wine and white juice.

As mentioned in the introduction and experimental section, the FC and FAS reagent assay the antioxidant capacity of the sample by comparing to known quantities of gallic acid. The measured GAE is assumed to represent "total phenolic content" of a sample. It is important to note that, although the preponderance of antioxidants are due to phenolics in plant-derived foods, other antioxidants may be either inherent or added to food and drink, such as ascorbic acid, sulfites, and sugars *(4, 7, 14, 15, 17)*.

Using a multitude of experimental techniques in an exercise opens opportunities for greater error. Perhaps the greatest source of error in the analysis of solid food phenolic content was the extraction technique. The literature describes preparing homogenized samples under reduced temperature using a Polytron blender. This is contrasted to the room temperature use of the mortar and pestle in introductory laboratories. Of course, student ability and enthusiasm to crush food with a mortar and pestle over 10 min varies considerably. This is reflected in a relatively low average value for apple phenolic content (Table 1). In addition, total phenolic content of pears, bananas, and oranges is generally lower than that of apples *(2, 15)*; however, student results for these softer fruits show comparable phenolic content with apples. Commercial applesauce, which is expected to have less GAE phenolics than fresh fruits owing to oxidation, is soft and often has added ascorbic acid, which may contribute to the GAE *(14)*.

The literature suggests mixing the FC reagent, buffer, and phenolics and letting this solution stand at room temperature for 1−2 h *(7, 15)*. To accommodate a 3-h laboratory period, the reaction time was limited to 10 min, which required heating the solution to obtain measurable absorptions. Temperatures of the water bath generated by hot plates varied, and although slopes of 765 nm absorption versus gallic acid concentration varied as a function of time and temperature, there was no correlation between the degree of the slopes and the determined quantity of GAE for similar sample food sources. The purpose of the ice bath is to slow down the reaction, allowing both gallic acid standards and sample phenolic solution to react with the FC reagent for the same time and temperature.

The change in slope of absorption versus gallic acid concentration using the FAS reagent was not as sensitive to changes in temperature and time. In addition, student determination of the GAE of grape juice using FAS is close to the accuracy and precision of values found in the literature that used the FC reagent.

After students generate the standard gallic acid solutions, measuring additional juice samples requires little time and reinforces the relevance of the technique. For one semester, students compared the GAE of the national brand white grape juice to the store brand. As the data (Table 1) indicate, the national brand contained twice the GAE of the store brand. As very little grape juice is needed for the lab, a natural extension is to test the GAE of semester-old grape juice, varying storage methods, to new grape juice. Comparisons between other light-colored fruit juices are also possible and easy. As with applesauce, commercial juices often have added ascorbic acid; however, we found that ascorbic acid does not contribute to the GAE using the FAS reagent.

Conclusion

Since fall 2007, students in GOB chemistry lab have been successfully introduced to absorption spectroscopy, using a Spec 20, to determine the total phenolic content (GAE) in food or juice. They generated calibration graphs and applied the Beer−Lambert law. The analysis of solid food samples involved extraction, which was the dominant source of error. In one 3-h lab period, student teams did not have time to analyze and compare different solid food samples. In contrast, the preparation of juice samples is simple and allows comparison of different beverages.

The laboratory exercises apply unit and volumetric analysis; they introduce students to instrument calibration, preparation of standards, graph interpretation, and ultimately exposes students to determining analyte concentrations spectroscopically. Students responded well to what they learned. Of the 20 laboratory exercises required of the GOB lab students, this lab was among the most popular along with "Saponification and Soaps" and the "Preparation of Aspirin". Student comments indicated that they enjoyed comparing phenolic concentrations between samples. As a result, students preferred the FAS technique to the FC technique. Determination of the total phenolic content for a wide range of food and juice is possible using the FC reagent. Conversely, the FAS reagent is limited to low-colored samples but appears to be a more consistent indicator of GAE. The FAS reagent is also less hazardous than the FC reagent. We anticipate expanding the FAS analysis to other low-colored beverages and allowing for more comparisons between samples.

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Supporting Information Available

Two laboratory exercises are enclosed. Notes to the instructor for each lab include, hazards, required preparation, needed chemicals and equipment, and suggested topics for prelaboratory discussion. Each lab, as presented to our students includes sections on Principle, Introduction, Experimental Procedure, Prelab exercises, Report sheet, and Postlab questions. This material is available via the Internet at http://pubs.acs.org.

