Quantitative HPLC Analysis of Rosmarinic Acid in Extracts of *Melissa officinalis* and Spectrophotometric Measurement of Their Antioxidant Activities

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Melissa officinalis (common name: lemon balm), a *Lamiaceae*, is an herb native to South Europe. The leaves emit a distinct fragrant lemon odor when bruised and are used to make teas and herbal infusions. Lemon balm leaves are traditionally used for their sedative and antispasmodic effects, as well as for treating gastrointestinal disorders *(1)*. The hot water extracts also have antimicrobial and antiviral activities (against *Herpes simplex* virus) and inhibit division of tumor cells *(2, 3)*.

Studies correlating lemon balm's medicinal properties with its antioxidant activity and phenolic profile have been published *(4–6)*. There is consensus that the significant antioxidant properties and other medicinal properties exhibited by lemon balm are mainly due to the large quantities of rosmarinic acid (Figure 1), an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, which can also be found in several other medicinal plants, herbs, and spices *(5, 7)*.

Antioxidants are substances that neutralize harmful free radicals in our bodies. A free radical is any species capable of independent existence containing one or more unpaired electrons. Free radicals are produced by normal aerobic metabolism; however, excess quantities of free radicals are harmful because they damage DNA, proteins, and lipids *(8, 9)*. Free radical formation is controlled naturally by antioxidants and the study of antioxidants present in food is a growing area of research. Methods to determine antioxidant activity are generally based on the inhibition of certain reactions by the presence of antioxidants. The most widely used methods involve the generation of radical compounds, which will be quenched by the antioxidant compounds present in the plant extracts *(10)*.

Several articles have appeared in this *Journal* concerning the application of HPLC techniques to consumer products such as foodstuffs *(11–15)* and drugs *(16–19)*, but none have focused on herbal teas. The medicinal properties associated with herbal teas and other foodstuffs are in part related with their protective properties against free radical damage.

We describe an HPLC experiment for the quantitative assay of rosmarinic acid in teas made from different samples of *Melissa officinalis*. A spectrophotometric method employing DPPH• (2,2-diphenyl-1-picrylhydrazyl) is used to determine the antioxidant activities of the different teas. Finally an attempt is made to correlate the antioxidant properties of the different teas with their quantities of rosmarinic acid.

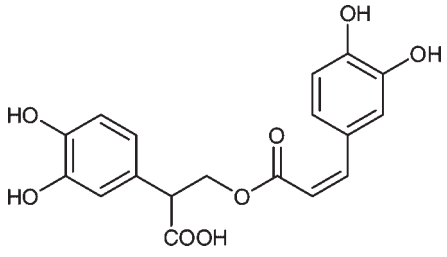

Figure 1. Rosmarinic acid chemical structure.

Overview

This laboratory experiment was devised for a third-year course on analytical chemistry with emphasis on chromatographic techniques, but was also used for a second-year course on natural product chemistry. All the students had taken general chemistry and organic chemistry courses. Students perform this experiment in groups of three and the experiment requires two class periods (six hours total). The HPLC analysis of the tea samples and the calibration curve are completed in the first class period, while the antioxidant activities are measured during the second class period.

Laboratory Summary

Commercial sources of *Melissa officinalis* tea can be found in supermarkets and health food stores. To prepare the extract, a weighed sample (2.00 g for fresh samples, 0.60 g for dry tea) is soaked in 20.0 mL of near-boiling water for 5 minutes, and the solid is removed by filtration.

The instructor provides a standard solution of rosmarinic acid (0.200 mg/mL in water), and the students prepare additional standards of 0.100, 0.050, and 0.025 mg/mL by dilution. The HPLC peak areas (average of three measurements) for the four standards and the tea extracts are obtained using a 250 mm × 4.6 mm column packed with 5 mm C-18 stationary phase particles and a diode array detector (DAD) scanned from 200 to 550 nm (chromatographic profile recorded at 254 nm). The isochratic mobile phase, which is composed of 30% acetonitrile and 70% aqueous solution of acetonitrile (2.5% v/v) and formic acid (0.5% v/v), is pumped at 1.0 mL/min.

The tea extracts are stored in the refrigerator until the following laboratory period for the antioxidant study. The instructor provides 100 mM DPPH• in methanol. Students place 2.975 mL of DPPH• into two test tubes and add 25 μL aliquots of either 1 mM ascorbic acid or the tea extract. After waiting 30 minutes for color development (samples kept in the dark), the absorbances are read at 515 nm versus the untreated DPPH• as the blank.

Hazards

Rosmarinic acid, DPPH•, formic acid, and acetonitrile may cause skin, eye, and respiratory irritation. Acetonitrile is also highly inflammable. Appropriate caution should be exercised in handling these compounds, and students should wear chemical safety goggles and compatible chemical-resistant gloves.

Results and Discussion

Measuring Quantity

We have chosen to provide students with the stock solutions but they could prepare all the solutions and the mobile phase if an additional 2 hours of laboratory time are available. Owing to limitations of the chromatographic equipment, each student group was assigned a standard concentration and a single calibration curve was done per class, but this is also a matter for the instructor to decide.

Typical chromatograms for rosmarinic acid and *Melissa officinalis* tea sample are shown in Figures 2 and 3, respectively. Rosmarinic acid shows a retention time, t_R, of 7.04 min. Despite the fact that several peaks appear in the tea chromatogram, the rosmarinic acid peak is always the major peak and appears with t_R = 7.04 min. The equipment had a DAD that generated the UV spectra of the rosmarinic acid standard (Figure 2) online and then was also used to evaluate purity of the peak with t_R = 7.04 min in the tea sample (data not shown). The students typically have trouble understanding the differences and advantages of a DAD versus a variable wavelength UV detector and this work helped to elucidate these differences.

A typical plot of peak area versus concentration of rosmarinic acid is shown in Figure 4. The calibration curve shows the linear relationship over the concentration 0.025 to 0.200 mg/mL with a correlation coefficient of 0.996. Students usually get correlation coefficients greater than 0.980.

The percentage of rosmarinic acid in the tea samples is highly variable. For example the results obtained by students in the fall semester of 2005 ranged from 0.47 mg/mL to 0.78 mg/mL for dry plant commercial and noncommercial sources and 0.14 mg/mL to 0.40 mg/mL for fresh sources (see the Supplemental Material[W]). Differences were observed when the same quantity of plant was analyzed fresh or dried, when the same plant was collected in different times of the year, or when different samples of a commercial tea were analyzed. Variation is also expected to occur when the same plant is dried under different conditions or when changes are made to the tea preparation.

As it is well known, chemical and biological diversity of aromatic and medicinal plants depends on such factors as cultivation area, climatic conditions, vegetation phase, and genetic modifications *(20)*. As students bring in samples from different origins, the gathered data usually generates a stimulating discussion on the causes for the differences on the rosmarinic acid content measured.

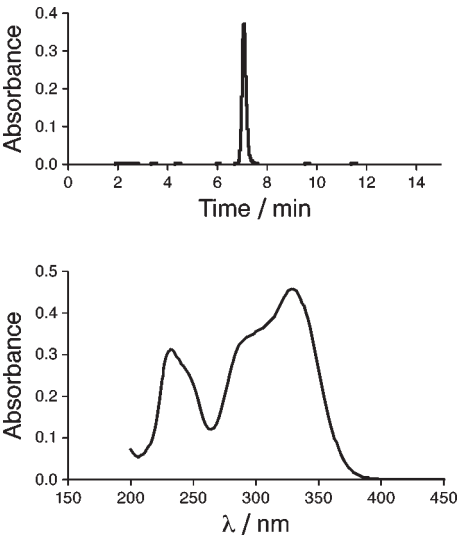

Figure 2. (top) Chromatogram and (bottom) UV spectrum obtained from rosmarinic acid standard solution.

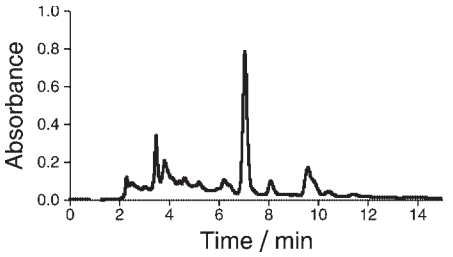

Figure 3. Chromatogram obtained from *Melissa officinalis* infusion.

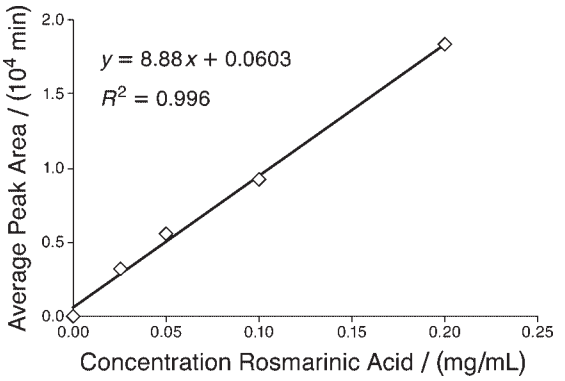

Figure 4. Rosmarinic acid calibration curve.

Measuring Activity

The antioxidant properties of foodstuffs is often reported in the news, and students are very interested in the subject, which has also been reviewed in this *Journal (21)*. There are several methods to measure the antioxidant properties of a plant extract *(10)*, and we chose the DPPH• method for its simplicity. This method measures the relative antioxidant activity of plant extracts to scavenge the radical DPPH• as compared to a standard quantity of L-ascorbic acid (AA), 1 mM. When DPPH• reacts with an antioxidant compound that can donate hydrogen it is reduced. The change in color (deep-violet to yellow) is measured at 515 nm on a UV–vis spectrophotometer. This change in absorbance is then used to generate the value of AEAC (L-ascorbic acid equivalent antioxidant activity) *(22)*,

$$\mathrm{AEAC} = \left(\frac{\Delta A}{\Delta A_{\mathrm{AA}}}\right) C_{\mathrm{AA}}\, V \left(\frac{100}{W}\right)$$

where ΔA is the change of absorbance after addition of plant extract, C_{AA} is the concentration of AA standard solution, ΔA_{AA} is the change of absorbance when the same volume of AA standard solution as that of plant extract was added, V is the volume of filtrate, and W is the weight of plant used for extraction.

The AEAC value is a measure for the antioxidant activity of the tea sample and is expressed as mg of L-ascorbic acid (AA) per 100 g of plant. The values obtained were variable and ranged from 0.81 mg AA/100 g plant to 10.93 mg AA/100 g plant (see the Supplemental Material[W]). As stated previously, there are reports in the literature that correlate the antioxidant properties of the *Mellisa officinallis* tea with their content in rosmarinic acid *(23),* and, for the dried tea samples studied, a good correlation (0.956) was observed (Figure 5). The data for the fresh plants were not plotted because only two samples were studied.

An exciting outcome of this laboratory experiment was the stimulating classroom discussions after the results from the different classes were compiled. Students became aware that special care should be taken in the preparation and commercialization of medicinal plants to maximize their medicinal properties and also to question the validity of the health benefit claims associated with some herbal supplements.

Modifications

Several modifications can be made to the experiment. The experiment can be reduced to a single period if only rosmarinic acid quantification is performed. For a more biochemistry- or food chemistry-oriented class other methods for antioxidant measurement can also be performed, namely, the ABTS, 2,2′azinobis(3-ethylbenzthiazoline-6-sulfonic acid), radical cation assay *(24),* and the generation of the EC_{50} (quantity of antioxidant necessary to decrease the initial $DPPH^{\bullet}$ or $ABTS^{\bullet+}$ concentration by 50%) for each tea sample *(25).* When enough replicas are studied, the teas made from different plants can also be compared by statistical analysis (e.g., ANOVA).

[W]Supplemental Material

Instructions for the students and notes for the instructors, including more information concerning radicals, measurement of the antioxidant properties by the DPPH• method, the derivation of the AEAC formula, and examples of expected results and required calculations, are available in this issue of *JCE Online*.

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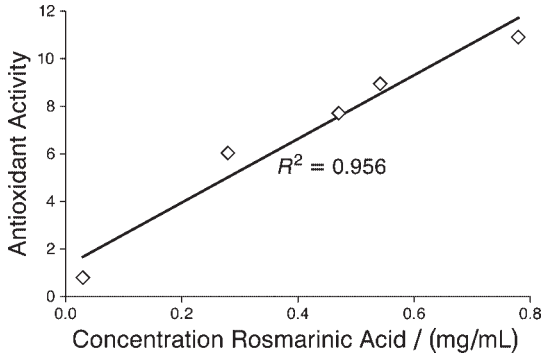

Figure 5. Correlation between AEAC and concentration of rosmarinic acid.

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The structures of rosmarinic acid and DPPH are available in fully manipulable Jmol and Chime format as *JCE* Featured Molecules in *JCE Online* (see page 1496).

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