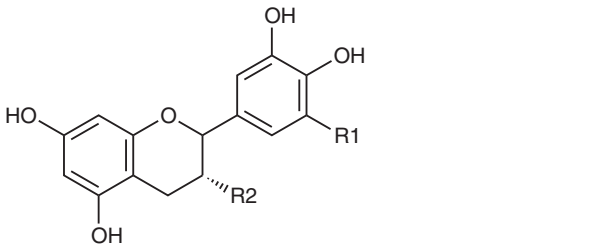
Quantification of Tea Flavonoids by High Performance Liquid Chromatography

Jessica D. Freeman and Emily D. Niemeyer*

Department of Chemistry and Biochemistry, Southwestern University, Georgetown, TX 78626; *niemeyee@southwestern.edu

Tea remains one of the most popular beverages worldwide because of historical and cultural traditions as well as increasing interest in its reported health benefits *(1).* Produced from the *Camellia sinensis* bush, tea is manufactured by harvesting the plant's leaves and leaf buds, then drying and processing them. Drinking green tea is correlated with inhibition of certain types of cancer (primarily of the digestive system) *(2, 3),* while black and green tea consumption is associated with the prevention of heart disease *(4, 5).* Although brewed tea is known to be a complex chemical mixture containing caffeine, lignin, chlorophyll, and various amino and organic acids, most researchers agree that the flavonoids found in tea are likely the origin of its protective health properties *(5, 6).* Flavonoids are natural antioxidants produced as secondary plant metabolites, and are therefore widely distributed in fruits, vegetables, and many plant-based products.

The majority of the flavonoids present in tea belong to a classification of polyphenolic compounds known as catechins *(7).* Catechins share a general flavan-3-ol structure (see Figure 1) and are known to have potent, radical-scavenging abilities *(8, 9).* Research has shown that epigallocatechin gallate (EGCG), one of the most well-studied catechin isomers, may inhibit carcinogenesis, mutagenesis, and tumorigenesis in vitro *(10, 11).* Catechins are found naturally in a number of foods and beverages, such as juice, beer, wine, and chocolate *(12),* although they occur in particularly high concentration in tea, especially the unfermented green and white varieties.



flavonoid	R1	R2
epicatechin	H	OH
gallocatechin	OH	OH
epigallocatechin gallate	OH	galloyl

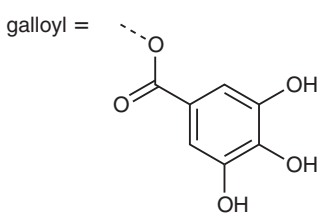


Figure 1. Chemical structures of catechin isomers analyzed in this laboratory experiment.

Despite increasing public interest in polyphenolic compounds and their role in human disease prevention *(13),* few experiments published in this *Journal* have introduced students to dietary flavonoids or aimed to quantify flavonoid levels in plant-based products. Anthocyanins in berries have been determined by spectrophotometry *(14)* and thin-layer chromatography *(15),* while wine phenolics have been analyzed using solid-phase extraction techniques *(16)* and high performance liquid chromatography (HPLC) *(17).* Although it is not a flavonoid, the plant metabolite rosmarinic acid has been quantified in lemon balm infusions by HPLC *(18).*

In this paper we describe a simple and optimized HPLC protocol to quantify catechin levels in a variety of commercial teas.[1] HPLC is the most prevalent technique to determine catechin concentrations in plant-derived products *(19)* and this laboratory exercise is ideal for introducing students to chromatographic separations using an analysis technique important in agricultural chemistry and nutrition. The experiment can be completed within a typical four-hour laboratory period by upper-level undergraduates in courses such as quantitative analysis or instrumental methods of analysis.

Experimental Procedure

The most common flavonoids in tea exist as four pairs of stereoisomers: catechin and epicatechin; epigallocatechin and gallocatechin; catechin gallate and epicatechin gallate; and epigallocatechin gallate and gallocatechin gallate. Due to time constraints in a typical laboratory period, we analyze three of the catechin isomers: epicatechin, gallocatechin, and epigallocatechin gallate.[2] Prior to the laboratory session, concentrated standards of epicatechin, gallocatechin, and epigallocatechin gallate are prepared for the students in methanol:citric acid solution (800 mg/L) and stored in amber vials at 4 °C.[3]

Students prepare their samples by brewing a tea bag in boiling water for 5 min, cooling the solution to room temperature, adjusting the pH, adding citric acid to stabilize the catechins, and finally diluting the sample with methanol *(20).* All sample solutions are then passed through a 0.45 µm Whatman filter to remove particulates prior to injection in the HPLC. During a given laboratory period, students analyze three tea samples with varying degrees of fermentation (black, oolong, green, white) and caffeine levels (regular, decaffeinated).

We typically have students work in pairs to make the tea samples, dilute solutions of the individual catechins, and mixed calibration standards. After completing the solution preparation, student groups inject individual standards of gallocatechin, epicatechin, and epigallocatechin gallate to determine the retention time for each compound and the elution order of the analytes in the mixed standard. Students then analyze their four mixed calibration standards (containing all three analytes) prepared over a wide concentration range, usually between 5–75 mg/L for each catechin.

Two phosphate buffer mobile phases (0.025 M, pH = 2.0) are prepared for the students prior to the experiment: A with 5% acetonitrile, and B containing 25% acetonitrile. Separation of the catechins is achieved by HPLC using a typical C-18 column with a linear gradient elution of 85% A to 20% A over 10 minutes at a flow rate of 1 mL/min. All catechins are conveniently monitored using absorbance detection at 278 nm.

Hazards

Hazards associated with this experiment are minimal. However, safety precautions should be exercised when handling the acetonitrile:phosphate buffer mobile phases to minimize any inhalation and skin contact. Acetonitrile may cause eye irritation and can cause damage to the respiratory system, nervous system, and kidneys.

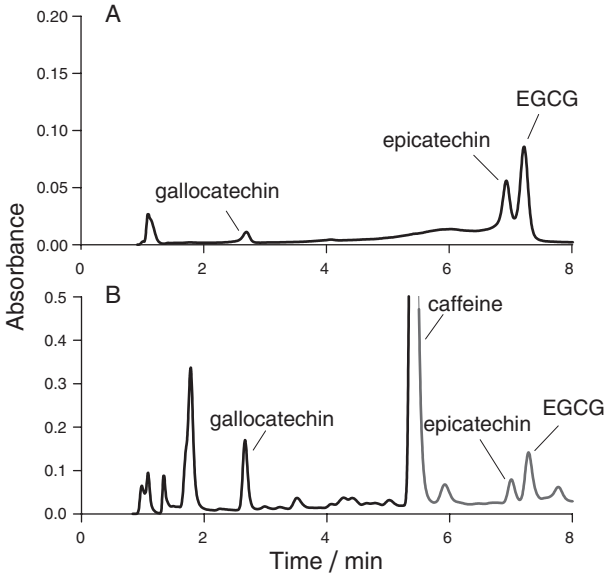


Figure 2. Student-generated chromatographic data of (A) a 50 mg/L mixed standard of gallocatechin, epicatechin, and epigallocatechin gallate (EGCG) in methanol:citric acid; and (B) an organic Earl Grey tea sample.

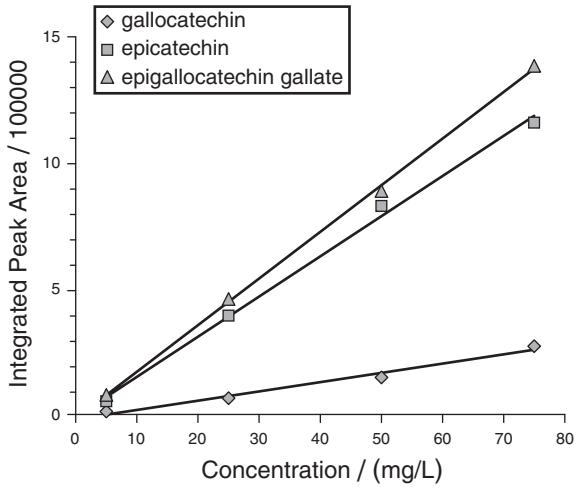


Figure 3. Representative student calibration data for the HPLC analysis of gallocatechin (R^2 = 0.981), epicatechin (R^2 = 0.996), and epigallocatechin gallate (R^2 = 0.999) mixed standards.

Results

Figure 2A shows a typical student's HPLC separation of a 50 mg/L mixed gallocatechin, epicatechin, and epigallocatechin gallate standard solution. Although complete baseline separation of the epicatechin and epigallocatechin gallate is not achieved using our rapid analysis conditions, the compounds are sufficiently separated to yield linear calibration data for this experiment. The chromatogram shows that the analytes elute quickly: average retention times were 2.7 min for gallocatechin, 6.9 min for epicatechin, and 7.2 min for epigallocatechin gallate.

Using the four chromatograms obtained from their mixed catechin standard solutions, students integrate the area under each chromatographic peak using software provided with the HPLC, and generate calibration plots for gallocatechin, epicatechin, and epigallocatechin gallate (Figure 3). Students use the linear least-squares method within a standard spreadsheet program to determine regression lines, correlation coefficients, and the uncertainties associated with each of their calibration curves.

Figure 2B presents a student-generated chromatogram of an organic Earl Grey tea sample. Although the chromatogram is dominated by the large caffeine peak at 5.4 min, gallocatechin, epicatechin, and epigallocatechin gallate are easily identifiable within the tea sample based on their average retention times. Students compare the integrated peak area of each catechin in their sample chromatogram to their calibration curves and, after correcting for dilutions, calculate the individual catechin concentrations in their tea samples. Table 1 presents the gallocatechin, epicatechin, and epigallocatechin gallate concentrations (in mg/L), determined for organic Earl Grey tea, decaffeinated green tea with peach, and English Breakfast tea. Students are required to calculate the uncertainties associated with their catechin concentrations by propagating the error determined for each of their calibration curves *(21)*. In addition, students are asked to determine the total catechin concentrations with associated uncertainties for all of their tea samples.[4]

Of the teas listed in Table 1, the black English Breakfast tea contained the highest total catechin concentration (464 mg/L), while the decaffeinated green tea with peach contained the lowest (64 mg/L). It has been previously shown that, in general, decaffeinated teas contain significantly lower flavonoid concentrations than their caffeinated counterparts *(22)* and fruit teas are also known to have low catechin levels *(23)*. Gallocatechin was found to be the most abundant catechin in both of the black teas studied (Earl Grey and English Breakfast), while epigallocatechin gallate, the primary flavonoid found in green tea, was confirmed as the most abundant catechin in the decaffeinated green tea sample.

Our students have observed large variations in individual catechin concentrations and overall catechin levels in the teas they have analyzed. Even within a single variety of tea, differences in catechin concentrations may be observed due to the geographic origin of the tea blend, how the tea leaf was processed, or the age of the tea bag *(23)*. In addition, beverage preparation conditions such as the temperature of the water,

Table 1. Comparison of Catechin Concentrations[a] Determined for Some Common Brewed Teas

Type of Tea Analyzed	Gallocatechin/ (mg/L)	Epicatechin/ (mg/L)	EGCG/ (mg/L)	Total[b] Catechins/ (mg/L)
Organic Earl Grey Tea	323.9 ± 28.3	35.2 ± 3.3	72.0 ± 1.6	431.1 ± 28.5
Decaffeinated Green Tea	14.6 ± 8.0	19.4 ± 3.5	30.0 ± 1.6	64.0 ± 8.9
English Breakfast Tea	364.6 ± 32.2	53.9 ± 3.2	45.3 ± 1.5	463.8 ± 32.4

[a]The catechins analyzed were gallocatechin, epicatechin, and epigallocatechin gallate; uncertainty values are provided.
[b]Total catechin concentrations are determined by summing the individual concentrations of gallocatechin, epicatechin, and epigallocatechin.

brewing time, and use of agitation or stirring have also been shown to significantly alter catechin levels in tea brews *(23).*

Conclusions

This laboratory experiment introduces students to basic principles of chromatography and quantitative analysis using a simple and efficient HPLC protocol to quantify catechin levels in commercial teas. Students also have the opportunity to learn more about dietary flavonoids and their relation to human disease prevention, while discovering the importance of instrumental analysis within agricultural chemistry and nutrition.

Acknowledgements

Financial support for this work was generously provided by the Robert A. Welch Foundation (AF-0005). The authors wish to thank Kristen Carranco, Coty Maypole, and Lynn Tarkington for initially developing this laboratory experiment as part of their independent project, Instrumental Methods of Analysis.

Notes

1. This same procedure may also be used to analyze catechins in other beverages such as juice, wine, and beer.
2. Any of the catechin isomers may be chosen for this analysis, however, we typically choose the least expensive catechin standards available commercially.
3. Instructors may also provide a caffeine standard solution since caffeine is present in large quantities in many tea samples.
4. Total catechin concentrations are determined by summing the individual concentrations of gallocatechin, epicatechin, and epigallocatechin for each tea sample. Although there are likely to be other catechin isomers present in the tea samples and chromatograms, we ask students to neglect other catechins' contributions to the overall analysis.

Literature Cited

1. Demeule, M.; Michaud-Levesque, J.; Annabi, B.; Gingras, D.; Boivin, D.; Jodoin, J.; Lamy, S.; Bertrand, Y.; Béliveau, R. *Curr. Med. Chem. Anti-Cancer Agents* **2002,** *2,* 441–463.
2. Higdon, J. V.; Frei, B. *Crit. Rev. Food Sci. Nutr.* **2003,** *43,* 89–143.
3. Neuhouser, M. L. *Nutr. Cancer* **2004,** *50,* 1–7.
4. Peters, U.; Poole, C.; Arab, L. *Am. J. Epidemiol.* **2001,** *154,* 495–503.
5. Gardener, E. J.; Ruxton, C. H. S.; Leeds, A. R. *Eur. J. Clin. Nutr.* **2007,** *61,* 3–18.
6. Arts, I. C. W; Hollman, P. C. H. *Am. J. Clin. Nutr.* **2005,** *81 (suppl),* 317S–25S.
7. Beecher, G. R. *J. Nutr.* **2003,** *133,* 3248S–3254S.
8. Kondo, K.; Kurihara, M.; Fukuhara, K. Mechanism of Antioxidant Effect of Catechins. In *Methods in Enzymology, Flavonoids, and Other Polyphenols,* Vol. 335; Academic Press: San Diego, CA, 2001; pp 203–217.
9. Zhao, B.; Guo, Q.; Xin, W. Free Radical Scavenging by Green Tea Polyphenols. In *Methods in Enzymology, Flavonoids, and Other Polyphenols,* Vol. 335; Academic Press: San Diego, CA, 2001; pp 217–222.
10. Lambert, J. D.; Hong, J.; Yang, G.; Liao, J.; Yang, C. S. *Am. J. Clin. Nutr.* **2005,** *81,* 284S–91S.
11. Surh, Y.-H. *Nature Rev. Cancer* **2003,** *3,* 768–780.
12. Arts, I. C. W.; van de Putte, B.; Hollman, P. C. *J. Agric. Food Chem.* **2000,** *48,* 1752–1757.
13. Several "popular press" magazines have published recent articles on the health benefits of flavonoids, including: (a) Horowitz, J. *Time* **2002,** *159,* 76–81; (b) *Consumer's Research Magazine* **2001,** *84,* 28–31; (c) Raloff, J. *Science News* **2005,** *167,* 27–29.
14. Curtright, R. D.; Rynearson, J. A.; Markwell, J. *J. Chem. Educ.* **1994,** *71,* 682–684.
15. Curtright, R.; Rynearson, J. A.; Markwell, J. *J. Chem. Educ.* **1996,** *73,* 306–309.
16. Brenneman, C. A.; Ebeler, S. E. *J. Chem. Educ.* **1999,** *76,* 1710–1711.
17. da Queija, C.; Queirós, M. A.; Rodrigues, L. M. *J. Chem. Educ.* **2001,** *78,* 236–237.
18. Canelas, V.; Teixeira da Costa, C. *J. Chem. Educ.* **2007,** *84,* 1502–1504.
19. Dalluge, J. J.; Nelson, B. C. *J. Chrom. A* **2000,** *881,* 411–424.
20. Arts, I. C. W.; van de Putte, B.; Hollman, P. C. H. *J. Agric. Food Chem.* **2000,** *48,* 1752–1757.
21. For a straightforward discussion of propagation of uncertainty with a calibration curve, see: Harris, D. C. *Quantitative Chemical Analysis,* 7th ed.; W. H. Freeman and Company: New York, 2007; pp 69–72.
22. Henning, S. M.; Fajardo-Lira, C.; Lee H. W.; Youssefian, A. A.; Go, V. L. W.; Heber, D. *Nutr. Cancer* **2003,** *45,* 226–235.
23. Khokhar, S.; Magnusdottir, S. G. M. *J. Agric. Food Chem.* **2002,** *50,* 565–570.

Supporting *JCE Online* Material

http://www.jce.divched.org/Journal/Issues/2008/Jul/abs951.html

Abstract and keywords

Full text (PDF)
Links to cited *JCE* articles

Supplement
Student instructions and handouts
Detailed experimental procedures and instructor notes