

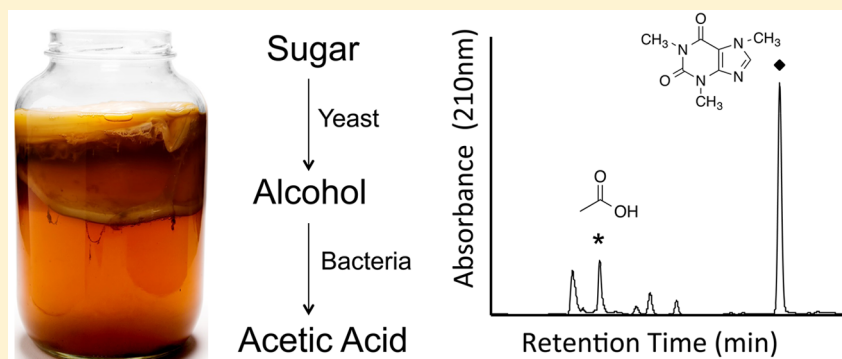
Titration and HPLC Characterization of Kombucha Fermentation: A Laboratory Experiment in Food Analysis

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S Supporting Information



ABSTRACT: Quantification of the many constituents that make up our food, whether they are desirable (vitamins, antioxidants, nutrients) or undesirable (pesticides, toxins), is one of the most practical applications of chemistry. In this study, kombucha, a popular fermented tea beverage, was analyzed using acid–base titration and high-performance liquid chromatography (HPLC). Kombucha is made via the fermentation of sweetened black tea with a symbiotic culture of bacteria and yeast (SCOBY), which produces acetic acid in addition to a variety of other organic acids and vitamins. The aim of this study was to analyze the acid content of kombucha over a 21-day fermentation period in order to characterize the fermentation kinetics. Titrimetric analysis revealed that the total acidity increased linearly with fermentation time at a rate of 1.5 mM/day. The acetic acid content was also quantified by HPLC at 7-day intervals by standard addition and compared to the total acidity determined by titration. A paired Student's *t* test was used to validate the methods. In an extension of the lab, HPLC was also used to identify and quantify the caffeine content of kombucha. The experiments utilized in this study provide a means to characterize beverages and fermentation as well as teach important quantitative and statistical skills. This study was implemented as a three-week teaching lab in a quantitative analysis course for undergraduate chemistry majors.

KEYWORDS: Analytical Chemistry, Laboratory Instruction, Titration/Volumetric Analysis, Food Science, Quantitative Analysis, Applications of Chemistry, Chromatography, First-Year Undergraduate/General, Upper-Division Undergraduate, Collaborative

National Chemistry Week 2014 brought attention to the chemistry of food with the theme “The Sweet Side of Chemistry—Candy”. Although geared toward younger children, this theme highlighted the pedagogical value of using food as a teaching tool at all educational stages in chemistry.¹ Food is a fascinating subject with broad appeal and can effectively engage students in college-level chemistry courses. In addition, food science is an important field of chemistry, though it is often avoided in the undergraduate laboratory due to the complexity of the sample matrix. An introduction to food analysis at the college level can both teach essential analytical skills and inspire interest in the practical application of chemical analysis. Recent papers in this journal^{2,3} suggest that curricula are beginning to reflect an increased interest in food chemistry among students.

In this laboratory, students analyzed the acid content of kombucha, a fermented tea beverage. This ancient beverage is

prepared by allowing a symbiotic culture of bacteria and yeast (SCOBY) to grow in a preparation of sweetened black tea. The yeast in the SCOBY consumes sugar to produce ethanol and carbon dioxide, while the bacteria (primarily *Acetobacter* and *Gluconabacter* genera) metabolize the ethanol produced by the yeast to form acetic acid.⁴ Together, the bacteria and yeast also produce a number of other metabolites, including vitamins C, B1, B2, B3, B6, and B12; folic acid; organic acids, including gluconic, L-lactic, and glucuronic; and various enzymes.^{5,6} In addition, the black tea used to prepare kombucha is a source of caffeine. All of these components are excellent candidates for analysis in a chemistry lab.

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The aim of this experiment was to investigate the acidity of kombucha as a means of understanding its fermentation kinetics. As fermentation proceeds, the bacteria in the SCOBY produce increasing amounts of acetic acid as well as trace amounts of other organic acids. Therefore, the kinetics of the fermentation process can be monitored by measuring changes in acid content over time. The kombucha was allowed to ferment in the lab over the course of 21 days, and the instructor and a student aide collected samples every 2–4 days throughout the fermentation period. The samples were stored at $-20\text{ }^{\circ}\text{C}$ to halt the fermentation process, and then later thawed and distributed among students for titrimetric analysis in the first week of the experiment. The kombucha samples were subjected to a potentiometric titration using sodium hydroxide (NaOH) as the strong base and Gran Plot analysis for end point determination. Each student then calculated the titratable acid (TA) concentration in their sample and compiled the class data to produce a plot of acidity as a function of fermentation time.

In the second week of the experiment, high-performance liquid chromatography (HPLC) was used to quantify the amount of acetic acid in kombucha at 7-day intervals during fermentation using the method of standard additions. Because titrimetric analysis is unable to discriminate between the dominant acid (acetic acid) and other trace organic acids, a chromatographic method such as HPLC must be used to separate the various sugars and acids for selective quantification. Students compared the acetic acid content determined by HPLC to the total acid content determined by titration and used a *t* test to validate the two methods of analysis.

Instructors may also implement the experiment as an independent project or a three-week experiment. In the third week, HPLC can be used to identify caffeine or other organic constituents of kombucha, such as gallic acid.⁷ In this study, students used standard additions to quantify the amount of caffeine present in the same samples studied for acetic acid content.

These experiments were implemented over 3 weeks in a quantitative analysis course for college chemistry majors ($n = 44$ students). The teaching objectives of week 1 (titrimetry) included the use of a buret and pH meter, standardization of a titrant, proper titration technique, and identification and meaning of titration end point. The teaching objectives of week two (chromatography) encompassed the fundamentals of HPLC, the use of the standard addition method for analyte quantification, and statistical analysis. Depending on the aims of the course, this experiment could also be used to discuss the chemistry of fermentation. In addition, the titrimetric portion of the experiment could be implemented independently for a general or nonmajor chemistry course.

METHODS

Kombucha Preparation

Kombucha was prepared by first making a 10 L batch of sweetened black tea. Looseleaf Lipton black tea and white sugar were purchased from a local supermarket. The tea was prepared by steeping 1.5 g/L (total: 15 g) of loose tea for 5 min in 8.75 L of boiling distilled water. The tea leaves were removed by filtration through a standard coffee filter, and white granulated sugar was then added at 65 g/L (total: 650 g). Afterward, the sweetened tea mixture was allowed to cool to $25\text{--}30\text{ }^{\circ}\text{C}$. Next, 12.5% v/v of a liquid kombucha mother culture (total: 1.25 L)

was added, bringing the total brew volume to 10 L. The kombucha mother was obtained from a local kombucha homebrew, but any plain-flavored kombucha beverage from a commercial health food retailer can also be used. The tea preparation was allowed to ferment in a 2.8-gallon glass beverage dispenser in a dark corner at room temperature for 21 days. The opening of the dispenser was covered with a paper towel secured by a rubber band to allow gas exchange and prevent contamination. Samples were collected at 0, 2, 4, 7, 10, 14, 17, and 21 days. To ensure homogeneity of the kombucha during sample collection, the entire kombucha brew was first stirred for 1–2 min using a stir plate and stir bar. The desired sample volume was then dispensed from the spigot on the bottom of the beverage container. Each sample was labeled and stored in a sealed plastic container at $-20\text{ }^{\circ}\text{C}$ until the time of analysis. Freezing the samples was found to be a necessary step to halt the fermentation progress. Prior to analysis, samples were removed from the freezer and thawed in a lukewarm water bath for 1–2 h.

Characterization of Fermentation Kinetics by Titration

The kombucha samples were subjected to acid–base titration using a nominal 0.1 M sodium hydroxide (NaOH) solution standardized with dried potassium hydrogen phthalate (KHP). The titration progress was monitored using a pH meter and electrode (Vernier) connected to a computer interface running LoggerPro software. The resulting titration curves were analyzed using a Gran Plot to determine the titration end point. Each student then calculated the acid concentration in their kombucha sample, and the class data were recorded in a shared Google Docs spreadsheet. The combined data set was used to produce a plot of the total acid content as a function of fermentation time.

HPLC Analysis of Acetic Acid and Caffeine

HPLC analysis was performed on samples fermented for 0, 7, 14, or 21 days using a Shimadzu DGU-28 series liquid chromatograph system equipped with a SPD-20A photodiode array detector set to record at 210 nm (acids) and 273 nm (caffeine). Samples were separated with a Phenomenex C18 reverse-phase column ($5\text{ }\mu\text{m}$, $150\text{ mm} \times 4.6\text{ mm}$) using a low-pressure gradient mobile phase of 0.1% H_3PO_4 in H_2O with a 20–40–20 MeOH gradient (20%–40% MeOH from $t = 0\text{--}6$ min, 40%–20% from $t = 6\text{--}9$ min, hold at 20% MeOH for 1 min). The flow rate was 0.75 mL/min, and the total run time was 10 min. The column was operated at room temperature, and the sample injection volume was $20\text{ }\mu\text{L}$. Standard additions were prepared for acetic acid (Sigma-Aldrich) and caffeine (J.T. Baker). All samples were filtered prior to HPLC analysis using a $0.45\text{ }\mu\text{m}$ filter. A full student procedure and instructor notes are available in the [Supporting Information](#).

HAZARDS

Sodium hydroxide is a strong base and should be handled with care. Gloves and protective eyewear should be used at all times. The kombucha prepared in this lab will come into contact with laboratory glassware and should NOT be consumed.

RESULTS AND DISCUSSION

Students were first asked to standardize the communal sodium hydroxide solution used for titrimetric analysis. Using dried potassium hydrogen phthalate as a standard, each student titrated four samples of NaOH, and individual data was shared in a class spreadsheet. Using the combined class results, the

NaOH solution was found to have a concentration of 0.0987 ± 0.0008 M. Students were then given a 100 mL kombucha sample collected on a single fermentation day for titration analysis. Working in pairs, the students titrated each sample three times using the standardized 0.0987 M NaOH (Figure 1A). The titration progress was monitored using a standard pH

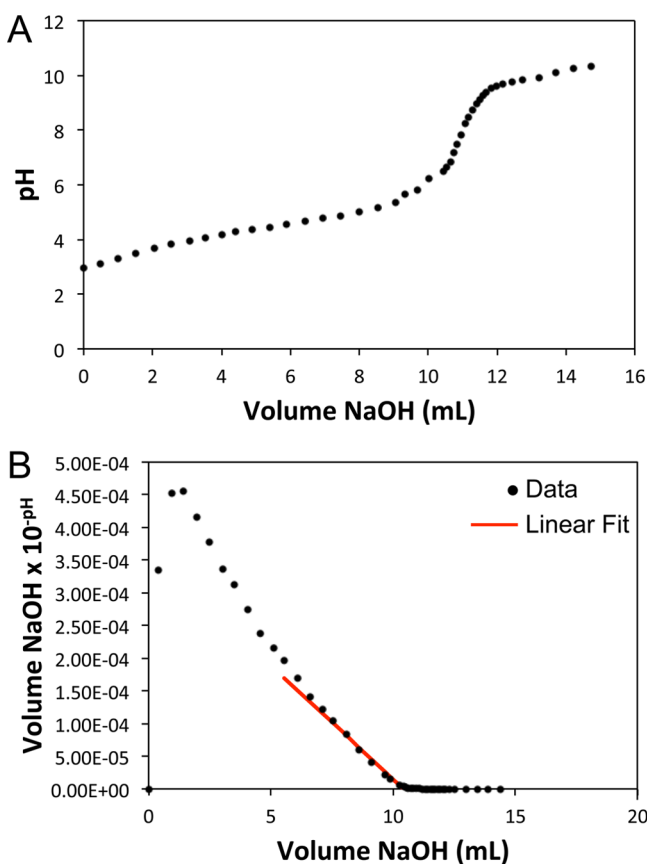


Figure 1. Titration results. (A) The pH of a kombucha sample fermented for 14 days was monitored as a function of the volume of titrant added. (B) The titration data was plotted using the Gran plot equation. The solid red line is a fit to the linear region of the Gran plot, where the x -intercept is equal to the equivalence volume of the titration. Here, the best-fit line gave a slope of $-(3.5 \pm 0.1) \times 10^{-5}$ and a y -intercept of $(3.6 \pm 0.1) \times 10^{-4}$. The equivalence volume calculated from this equation was 10.4 mL.

meter connected to a computer interface. A visual acid–base indicator was not used because the color of the kombucha obscured visual end point observation. Instead, students determined the approximate titration end point by observing the shape of the titration curve.

The titration end point was then determined quantitatively for each data set using a Gran plot (Figure 1B and Figure S1) (for an excellent tutorial, see the experimental supplement to Harris' *Quantitative Chemical Analysis*).⁸ The titration data were plotted according to the Gran Plot equation:

$$V_b \times 10^{-pH} = K_A(V_e - V_b)$$

where V_b is the volume of base (NaOH) added and pH is the pH value at the added volume of base V_b . K_A is the acid dissociation constant (1.75×10^{-5} for acetic acid) and is equivalent to the slope of the linear region of the Gran plot; V_e is the equivalence volume and is determined by finding the x -

intercept for the linear region of the Gran plot nearest the titration end point. At the equivalence volume, the number of moles of base added is equal to the number of moles of acid present in the sample. The students used the equivalence volumes calculated from their Gran plots to determine the total acid concentration in each of their three kombucha samples in units of moles per liter and then recorded these values in a class spreadsheet. Each time point was analyzed by at least three student groups, giving a total of at least nine independent concentration measurements for each day. The nine (or more) acid concentrations were used to find the average and standard deviation of the acid concentration for a given fermentation time. The average acid concentration was then plotted as a function of fermentation time in days (Figure 2). The acid

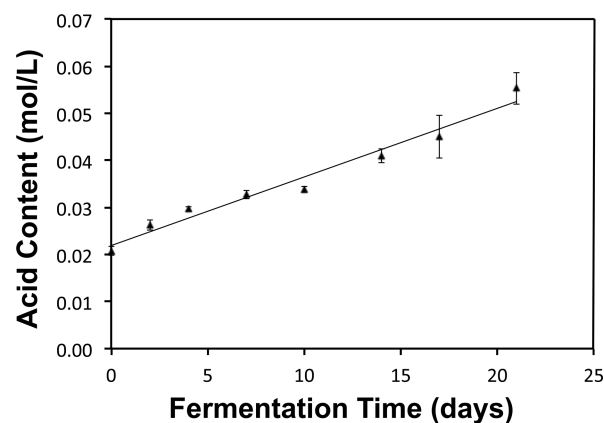


Figure 2. Fermentation kinetics. The total acid content of the kombucha brew was monitored as a function of fermentation time to understand the fermentation kinetics. Data points and error bars represent the average and 95% confidence intervals, respectively, of at least nine individual titrations. The acid concentration was found to increase linearly with time at a rate of 1.5 ± 0.1 mM/day. A linear fit to the data revealed an equation of $y = 0.0015(\pm 0.0001)x + 0.022(\pm 0.001)$.

concentration was shown to increase linearly as a function of fermentation time, reaching a maximum value of 0.055 ± 0.004 M on day 21 of the 21-day fermentation period. To illustrate the precision of the titration data, students used 95% confidence intervals to add error bars to each of the concentration measurements on the kinetic plot.

Interestingly, kombucha fermentation has previously been shown to follow complex kinetics influenced in part by bacteria and yeast growth. One study showed that the counts of acetic acid-producing bacteria in kombucha increased up to 4 days of fermentation and then declined thereafter.⁹ Another study showed an initial lag in metabolism, followed by a steady increase in acid content after 8 h of fermentation;¹⁰ however, the kinetics were not monitored past the first 12 h of fermentation. Our data revealed more straightforward fermentation kinetics, with the acid concentration increasing linearly with time at a rate of 1.5 ± 0.1 mM/day. Later time points exhibited greater variability in concentration and required longer equilibration times to produce stable pH readings (as exhibited by larger error bars in Figure 2). Since the samples were not filtered before titration, these anomalies may be due to the buildup of additional acids and other interfering metabolites from an increasingly complicated sample matrix.

Following complete titration analysis of the kombucha samples, students were asked to quantify the acetic acid

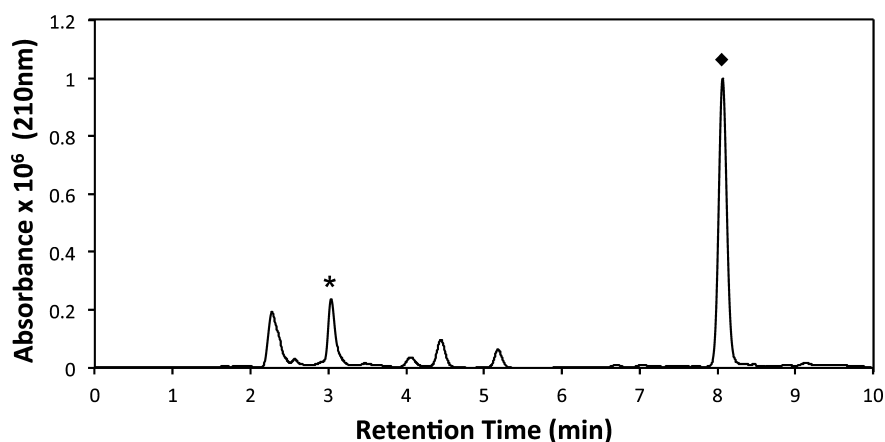


Figure 3. HPLC analysis of a kombucha sample fermented for 14 days. Acetic acid eluted at 3.05 ± 0.01 min (peak indicated by the asterisk), and caffeine eluted at 8.084 ± 0.008 min (peak indicated by the diamond). Values represent the averages and standard deviations of three runs for each analyte. The chromatogram was normalized to the caffeine peak height because the caffeine concentration should remain constant regardless of fermentation time.

concentration at select time points using HPLC. Prior to classroom implementation of the HPLC analysis, we experimented with various mobile phases, including mixtures of methanol/water and acetonitrile/water, following the work of Wang et. al.⁷ Although these authors used isocratic elution, we found that the best separation of the acidic components of the kombucha was achieved using a gradient of 20–40–20 methanol as the organic component and 0.1% orthophosphoric acid in water as the aqueous component of the mobile phase. Acetic acid and caffeine were monitored at 210 and 273 nm, respectively. Under these conditions, acetic acid eluted at 3.05 ± 0.01 min and caffeine eluted at 8.084 ± 0.008 min (Figure 3). The method of standard addition was used both to identify the acetic acid and caffeine peaks and to quantify the amounts of acetic acid and caffeine in kombucha samples fermented for 14 days (Figures 4 and 5). For quantification, three separate samples (kombucha and two standard additions) were analyzed twice each. The peak heights were plotted as a function of the concentration of the added standard, and the plots were then analyzed by linear regression. For acetic acid, the standard additions yielded a linear fit of $y = 5.7 (\pm 0.4)x + 0.22 (\pm 0.02)$. For caffeine, the standard additions yielded a linear fit of $y = 0.025 (\pm 0.003)x + 0.98 (\pm 0.03)$.

Data analysis is a central component of this lab, and students should be asked to compare the acid content determined by HPLC analysis to that determined by titrimetry (Figure 6). Here, for example, titration analysis yielded a day-14 acid content of 41 ± 3 mM, while HPLC yielded a day-14 acetic acid concentration of 39 ± 1.5 mM. Importantly, titration measures all acids in the kombucha sample, while HPLC is able to selectively quantify acetic acid. It is therefore reasonable to expect that the HPLC acetic acid concentration should be lower than the total acid content determined by titration. A paired Student's *t* test was used to compare the two methods, with the null hypothesis (H_0) stating the two methods would yield significantly different results. A paired *t* test was used to evaluate the average difference between [acetic acid] results for each day of fermentation, where $t_{\text{calc}} = 0.692$. Since $t_{\text{calc}} < t_{\text{critical}}$ (≈ 3.18 for $DF = 3$), H_0 was rejected and no significant difference was found between the methods at the 95% significance level.

In addition to the acid analysis, students can compare the measured caffeine concentration to the expected caffeine

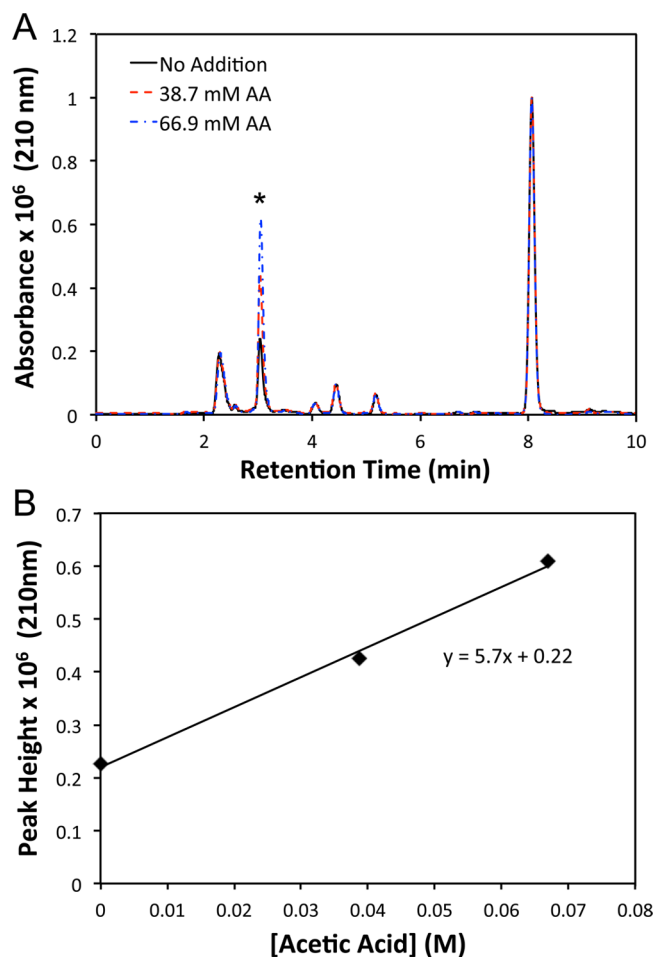


Figure 4. Acetic acid HPLC results. (A) Identification of acetic acid using standard additions. Chromatograms were collected from three 14-day samples: kombucha (no acetic acid addition), kombucha + 38.7 mM acetic acid, and kombucha + 66.9 mM acetic acid. (B) Quantification of acetic acid using standard additions. The height of the acetic acid peak was plotted as a function of the added concentration, yielding a linear regression fit of $y = 5.7 (\pm 0.4)x + 0.22 (\pm 0.02)$ and $R^2 = 0.99559$.

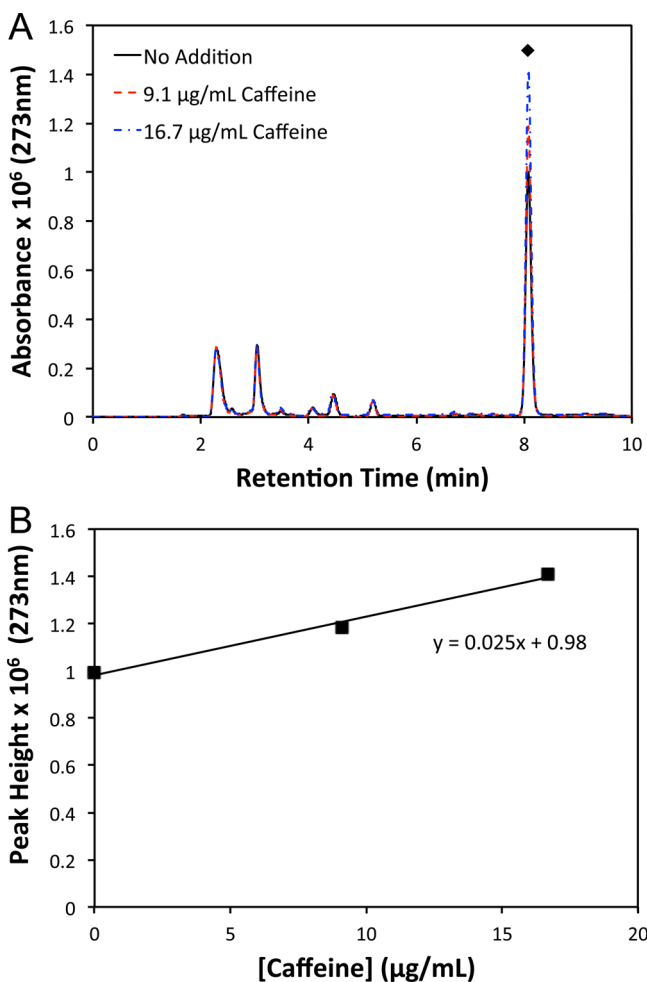


Figure 5. Caffeine HPLC results. (A) Identification of caffeine using standard additions. Chromatograms were collected from three 14-day samples: kombucha (no caffeine addition), kombucha +9.1 µg/mL caffeine, and kombucha +16.7 µg caffeine. (B) Quantification of caffeine using standard additions. The height of the caffeine was plotted as a function of the added concentration, yielding a linear regression fit of $y = 0.025(\pm 0.003)x + 0.98 (\pm 0.03)$ and $R^2 = 0.98899$.

content for black tea. According to the nutritional information from the tea manufacturer, each 2 g bag of tea contains 55 mg of caffeine. Because the kombucha brew was prepared using 1.5 g of tea per liter, the total initial caffeine concentration according to the manufacturer would be 41.3 µg/mL (this is a calculation the students can perform themselves). The caffeine concentration determined by HPLC in this study was 39 ± 5 µg/mL, with a relative error of 4.1%. Students were graded on the accuracy of their sample preparation in relation to the magnitude of their relative error for caffeine analysis.

CONCLUSION

Overall, students appreciated the practical aspect of this teaching lab and were excited to learn about and discuss food chemistry and fermentation chemistry. The lab uses cheap and nonhazardous reagents, can be completed in two 3-h laboratory sessions, and is an excellent platform for teaching acid–base titration and chromatography. The data present numerous opportunities to practice statistical analysis, such as the determination of standard deviation and propagation of error. For a general chemistry course, it is suggested to perform only

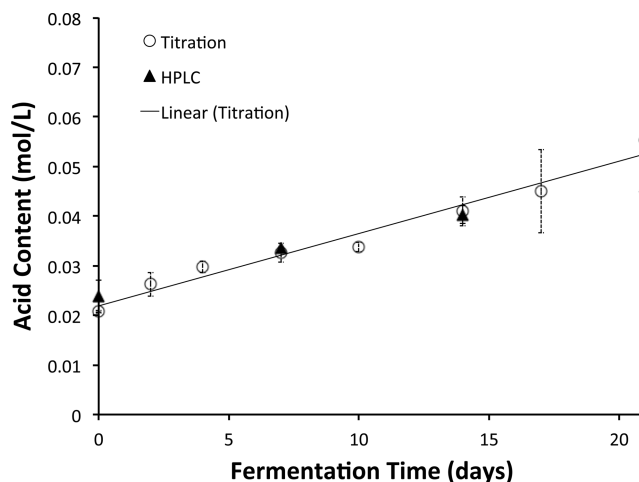


Figure 6. Comparison of titration and HPLC quantification of acid content in kombucha. Data between the two methods were compared using a paired Student's *t* test. The null hypothesis stated there would be a statistically significant difference between the two methods, and H_0 was rejected at a 95% significance, where $t_{\text{calc}} = 0.69 < t_{\text{crit}} = 3.18$. The slopes of the two lines are also in good agreement, where $m_{\text{titration}} = 1.5$ mM/day and $m_{\text{HPLC}} = 1.1$ mM/day.

Week 1 of the lab (acid–base titration). The entire three-week lab, including HPLC analysis, is suitable for an upper level quantitative analysis course or fermentation science course. In addition, further aspects of the fermentation can be readily explored: for example, the effect of temperature, sugar concentration, or SCOBY mass on the fermentation rate.¹¹ As a more advanced project, students could optimize the HPLC conditions to give full separation of other analytes in kombucha, such as catechins.^{12–14}

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.6b00329.

Gran plot analysis, HPLC chromatograms, and peak heights from standard addition chromatograms (PDF, DOCX)

Student lab manual (PDF, DOCX)

Instructor guide (PDF, DOCX)

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Notes

The authors declare no competing financial interest.

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