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Laboratory Experiment

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UV−Vis Spectrophotometric Analysis and Quantification of Glyphosate for an Interdisciplinary Undergraduate Laboratory

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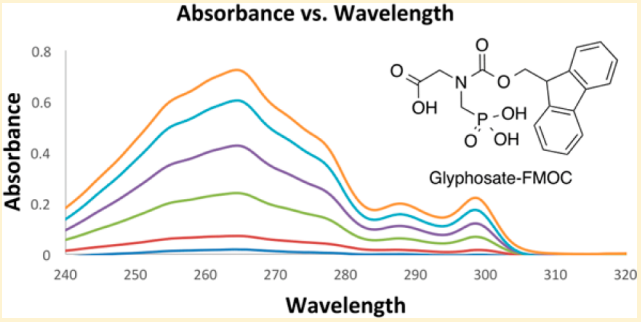
Supporting Information

ABSTRACT: Glyphosate (*N*-(phosphonomethyl)glycine) is the most widely used herbicide on earth. A simple assay to quantify glyphosate concentrations in environmental samples was developed as part of an interdisciplinary effort linking introductory laboratory courses in chemistry, biology, and microbiology. In this 3 h laboratory experiment, students used UV−vis spectroscopy to quantify glyphosate in prepared unknowns and supernatants from glyphosate-treated soil samples. Regression analysis indicated that the assay is linear up to 20.0 ppm, making it particularly useful for detection of low levels of glyphosate in environmental samples. The assay can be used to quantify the activity of glyphosate-degrading soil microorganisms by comparing glyphosate levels between autoclaved and nonautoclaved soil slurries.



KEYWORDS: *UV−Vis Spectroscopy, First-Year Undergraduate/General, Interdisciplinary/Multidisciplinary, Inquiry-Based/Discovery Learning, Laboratory Instruction*

BACKGROUND

Glyphosate (*N*-(phosphonomethyl)glycine), **1**, the active ingredient in Roundup (Monsanto Corporation), has become the most widely used broad-spectrum herbicide in the world. Over the past four decades (1975−2015) Roundup use in the United States has increased more than any other herbicide[1] and was estimated at 280−290 million pounds in 2015 (USGS, National Water Quality Assessment Program, NAWQAP).[2] Glyphosate kills the entire plant, as the herbicide is absorbed by the leaves and then transported to the roots.

Glyphosate inhibits the shikimate pathway by binding the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase), which is produced by plants, algae, fungi, and bacteria. When the EPSP synthase is inhibited, the plant is unable to synthesize aromatic amino acids[3] ultimately preventing *de novo* protein synthesis. Most animals do not synthesize aromatic amino acids and must acquire these amino acids from their diet. Hence, animals lack the target enzyme EPSP synthase, and animal cells should be unaffected by glyphosate. However, glyphosate may affect the many microbes that are part of the animal microbiome.[4]

The discovery of glyphosate as a broad-spectrum herbicide was followed by the construction of glyphosate-resistant, genetically modified soybean plants. The first glyphosate-resistant soybean (*Glycine max*), introduced in 1994, was engineered to express a mutant form of EPSP synthase gene, *aroA*, from the soil bacterium, *Agrobacterium tumefaciens* str. CP4. Since then, many other crop plants, including corn, sugar beets, alfalfa, and cotton, have been engineered to be glyphosate-resistant (GR),[1b] and many courses and course materials now contain components relating to the genetic modification to organisms.[5]

Yield of GR crops is often increased since repeated herbicide treatment during the growing season decreases competition for soil nutrients between weed and crop plants and significantly reduces topsoil depletion by facilitating no till operations eliminating competition from weeds.[3,6,7] Hence, the benefits to farming and the availability of GR crops ultimately are responsible for the rapid increase of glyphosate use in agriculture. However, increased glyphosate use has raised issues with respect to the effects that residual glyphosate may have on the health of the ecosystem.[8]

The use, fate, and persistence of herbicides in agricultural fields and in urban areas are extremely important to farming and environmental health. Manufacturers report that the half-life of glyphosate in the soil is a few days, but this depends on the type of soil, the microbial community of the soil, and the temperature.[9] Proper quantification of glyphosate persistence, accumulation, and potential adaptions of the soil microbiome is increasingly important.[10] Recent reports highlight the changing

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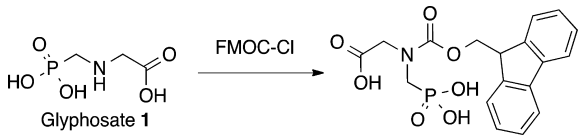
soil biota in areas of glyphosate use as well as the discovery of many glyphosate-degrading bacteria.[11] Glyphosate-degrading bacteria have evolved with metabolic pathways to utilize glyphosate either as a carbon source, a nitrogen source, or both,[12] as well as a phosphorus source,[13] and may present a biological method to prevent excessive glyphosate accumulation in the environment. These factors make this molecule an intriguing compound to discuss and quantify in freshman undergraduate courses.

Two weeks were devoted to the glyphosate analysis laboratory exercise. The lab was designed for analysis to be completed in a 3 h lab period aimed at developing a standard linear regression for glyphosate in the sub-20 ppm range and quantification of a prepared unknown (week 1). The assay utilizes the functionalization of glyphosate **1** to a FMOC−glyphosate adduct, **2**, that will absorb light in the UV range (λ_{max} 264 nm) as shown in Scheme 1 and described in previous reports.[14]

Scheme 1. Reaction of Glyphosate 1 with FMOC-Cl To Produce the UV-Active Adduct 2



The assay was then used to quantify unknowns from aqueous biological and agricultural samples (week 2). The glyphosate samples assayed by the chemistry students originated from experiments conducted by students in biology and microbiology laboratories. The curricula of both of these laboratories are currently undergoing a redesign process to become a CURE (course-based undergraduate research experiences); as providing open-ended projects with more devoted time has been shown to be positive by others.[15] Students were expected to evaluate their results against known standards and the environmental samples obtained from an interdisciplinary link to the biology and microbiology courses.

As a land grant institution in a mostly rural and agricultural state, many of our students have experiences in agriculture, farming, and crop and pest management. Selecting glyphosate as a target for analysis in a multidisciplinary laboratory project provides an authentic lab experience with a real world application in addition to emphasizes the interdisciplinary nature of the study of chemistry as it applies to biological systems, and agricultural and environmental health.

HAZARDS

In addition to goggles, the use of nitrile gloves in this laboratory is recommended, although glyphosate, a known herbicide, is classified with low toxicity to humans.[16] Fluorenyl orthochloroformate (FMOC-Cl) is an irritant. Since acetonitrile and dichloromethane are volatile solvents, we recommend a well-ventilated lab space.

EXPERIMENT

The experiment was designed to occur over two sequential laboratory periods. In the first lab period the students prepared the samples for the standard curve and tested a prepared unknown sample. In session 2, the students analyzed the samples supplied by the biology and microbiology laboratory. Students are expected to analyze multiple sample dilutions on the basis of the sample information provided. This sequence imitates an authentic analytical laboratory testing an unknown for a client. Students worked individually or in pairs.

To create a successful experiment, it was necessary to overcome several obstacles. First, glyphosate does not absorb well in the UV−vis range. However, the fluorenyl orthoformate derivative, commonly known as the FMOC functionality, absorbs at 264 nm (λ_{max}).[18] The reaction time length reported by Waiman et al. was too long for a typical lab period, but Fidencio et al. found that FMOC incorporation occurred immediately with no significant reduction in sensitivity.[14b] On the basis of this report, we modified the assay by reducing the FMOC-Cl incubation time from 2 h to 30 min to fit within the lab period. Another issue was the limited stability of the FMOC-Cl solution. It was necessary to make fresh stock each day.

Sample Preparation

FMOC-Cl was added to the samples at a ratio 50 times larger than the highest expected glyphosate concentration. Following incubation, a 6 mL aliquot was removed, treated with 8 mL dichloromethane, and centrifuged. The aqueous layer (3 mL) was transferred to a quartz cuvette and scanned across the entire UV−vis region. Absorbance at 264 nm was used to quantify the FMOC−glyphosate complex. All measurements were taken using the UV−vis spectrophotometers used in the teaching laboratories (scan rate at 3000 nm/min; photometric range −0.3−3 A; 0−200% T; silicon photodiode detector). The assay was performed using incubation times varying from 15 min to 2 h and found that the longer incubation periods yielded more reproducible results. Since 30 min seemed the longest incubation time that would allow students to finish within one lab period, a second dichloromethane extraction was added which greatly improved reproducibility of the assay. FMOC-Cl at amounts of 50 times in excess to glyphosate may have accounted for variability in the assay. Addition of the second extraction after the initial 30 min incubation yielded more consistent linear regressions (>0.90 with many above 0.95).

Student Laboratory Work

During the first lab session, chemistry undergraduate students were provided with a stock solution of glyphosate (180 ppm) in 0.1 M aqueous KCl solution. The students used a micropipette and volumetric flasks to prepare dilutions of the glyphosate stock solution at concentrations of 1, 5, 10, 15, and 18 ppm for a standard curve. These samples underwent the FMOC-Cl assay along with an "unknown" sample for testing. The students consistently measured the concentration of the unknown within 1−2% of the actual concentration. During the second lab session, the students repeated the construction of the calibration curve as well as the samples provided by the students from the biology or microbiology laboratories. The chemistry students did receive sample information including treatment time and concentration of glyphosate exposure as might be expected in an analytic laboratory setting.

The microbiology faculty designed a separate procedure provided in the Supporting Information (SI) collecting local soil samples to assess whether resident soil bacteria were able to degrade glyphosate, i.e., utilize glyphosate as a nutrient (source of carbon or nitrogen).

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Example of Soil Sample Preparation from Microbiology Students

A stock solution of 1% glyphosate was diluted to a concentration of 1000 ppm (0.1%). Enrichments were set up using sterilized 25 mM MOPS medium, pH 6.8, inoculated with 0.25 g of sterilized or nonsterilized soil (control). The medium was supplemented with ammonia (enriches for use of glyphosate as carbon source), acetate (enriches for use of glyphosate as nitrogen source), both (enriches for use of glyphosate as carbon and nitrogen source), or nothing (control). After incubation, the samples were centrifuged, and the supernatant was removed to assay. For the FMOC-Cl assay, the samples were diluted and subjected to analysis.

The results were then shared with the microbiology students to augment the individual projects. These types of analysis and data sharing provided an important aspect of the learning experience to incoming students illustrating the interdisciplinary nature of science. Although sampling was completed on time, compiling and comparing data sets across the multiple lab periods occurred after the course finished. Sharing software preferences vary; however, an example utilizing Google Sheets is provided (SI).

PEDOGOGICAL GOALS

The goals of developing a CURE for students include that they understand the

- Interdisciplinary nature of scientific knowledge
- Importance of applying skills to novel scenarios
- Use of linear regression analysis applied to determine the concentration of a solute (here, glyphosate) in a variety of different unknown and biological samples
- Importance of precision in laboratory analysis
- Applicability of commonly used and known chemicals and their molecular relevance

MATERIALS

Glyphosate (*N*-(phosphonomethyl)glycine) was purchased from Bio Basic Canada Inc. in Ontario, Canada, and used as received. FMOC-Cl was purchased from AK Scientific and used as received. Acetonitrile and dichloromethane were purchased from Fisher Scientific at the spectrophotometric grade. KCl was purchased from Fisher Scientific and used as received. For preparation of 180 ppm glyphosate solution, 0.360 g of glyphosate was weighed using an analytical balance, transferred into a 2 L volumetric flask, and dissolved in 0.1 M KCl. The borate buffer was prepared by adding 15.255 g (0.04 mol) of $Na_2B_4O_7{\cdot}10H_2O$ to 1 L of deionized (DI) water. For the preparation of 1% (w/v) glyphosate stock solution, 100 mg of glyphosate was dissolved to 100 mL with 20 mM MOPS buffer, pH 6.8, with trace minerals. For each sample, a 15 mL centrifuge tube was filled with 6 mL of aqueous glyphosate solution (varied concentrations), 1 mL of borate buffer, and 1 mL of (1 g/L) FMOC-Cl in acetonitrile.

RESULTS

The modified Waiman assay incorporating the fluorenyl group onto the glyphosate molecule and resultant UV−vis spectrophotometric analysis provided accurate quantification of glyphosate in the 1−18 ppm range. An example of the data obtained by students is shown in Figure 1. Although calibration curves were calculated at 264 nm, the curves also provide consistent linearity (R^2) with equally high confidence at many

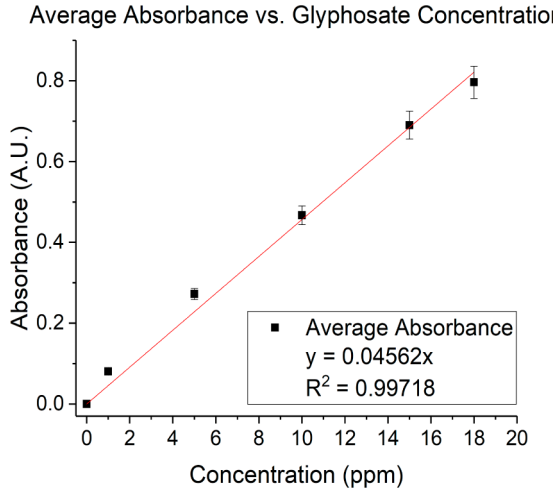

Figure 1. Normalized average absorbance at 264 nm vs concentration for three trials of glyphosate standards. Error bars indicate standard deviation.

points along the graph (shown in gray in Figure 2) as previously noted by Fidencio and co-workers.[14b] Decreasing

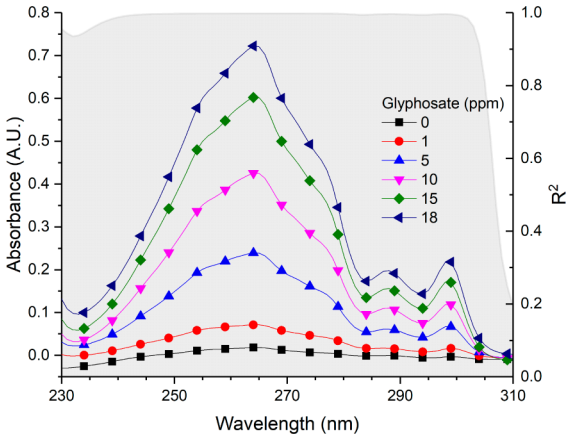

Figure 2. Absorbance curves for the linear regression of glyphosate. Curve fitting was performed at 264 nm, yet the linearity of the curves could be performed with high confidence (in gray) at many wavelengths.

the incubation time of aqueous glyphosate solutions with FMOC-Cl acetonitrile from 2 h to 30 min gave optimal results by adding a second dichloromethane extraction. Using this assay a majority of student groups succeeded in obtaining linear regression correlations above 0.95. Further, the students' results improved when repeating the assay in week 2 of the experiment. This positive result is from the repetitive nature of performing the lab in two sequential laboratory periods. These results provide consistent data across the >250 students performing the laboratory.

Analysis of the results obtained from the biological samples supported the hypothesis that soil bacteria were able to consume glyphosate either as a carbon source, as a nitrogen source, or as both. The data below show results for multiple tests on five different soil samples provided by the microbiology students. In every sample, over 50% of the glyphosate was consumed as shown in Figure 3. Results varied on the basis of soil sample source. Glyphosate consumption seemed lower in enrichment where glyphosate was the sole carbon *and* nitrogen

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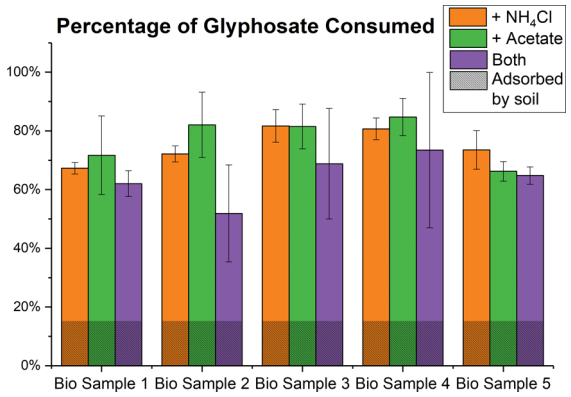

Figure 3. Data from soil samples incubated with glyphosate plus a nitrogen source (NH_4Cl) or a carbon source (acetate) or with no addition. Samples show the amount of glyphosate consumption when given either a C source or a N source, yet overall consumption was deterred when samples needed glyphosate as both a C and N source.

source. The microbiology students performed Gram stains and identified bacteria using 16S rDNA sequence analysis. The bacteria identified belonged to the genera *Arthrobacter*, *Microbacterium*, and *Rhodococcus* (Gram positive), and *Brevundimonas*, *Phyllobacterium*, and *Agrobacterium* (Gram negative).

In addition to biodegradation of glyphosate in soil, glyphosate can be inactivated through interaction with metals and/or minerals.[17] Students in the biology lab addressed the inactivation of glyphosate in different types of soil. To distinguish between biological degradation and sequestration by soil particles of glyphosate, biology students were interested in determining the glyphosate concentrations that remained after incubation with regular and sterilized soil. The chemistry students were able to help them by analyzing the concentration of glyphosate in regular and sterilized (autoclaved) soil. Shown in Figure 3, the results provided by the chemistry students also showed that glyphosate adsorption to the soil accounted for roughly 15% of total glyphosate consumption in the experiment. This value is consistent with previous reports of glyphosate interactions in soil.[18] In our experiments the high percentage of glyphosate added to the soil samples (at neutral pH) ensured glyphosate was not limiting during the incubation period.

CONCLUSIONS

Here we describe a laboratory exercise of a commonly used herbicide, glyphosate. The procedure was modified to ensure completion in a single 3 h laboratory period. Students obtained consistent and repeatable results with >0.90 regressions and accurately measured unknowns and biological samples. The integrated nature of this laboratory provides a connection between biological sciences and chemistry that has made the lab experience more authentic for students. The realization that accuracy and repeatability of experimental results are critical to a third party is an important aspect of this exercise.

This project is part of a larger effort to update and modernize the freshman laboratory curricula by designing authentic, research-based experiments that allow students to formulate their own research question, develop a hypothesis, and conduct experiments that attempt to answer these questions. Further, we strive to demonstrate the interdisciplinary nature of science by providing research topics that illustrate that knowledge and skills gained in one field of science are valid and adaptable to other scientific fields. Collaboration with others not only is essential in science but also is a valued asset in every facet of life. When students see relevance in their laboratory and lecture courses and connect personal experiences to compounds in their daily life, a notable increase in participation and interest can be observed. Moreover, working on a topic of current importance stimulated questions and led to an exchange of viewpoints. Observing students during class revealed many incidences of students sharing their thoughts on the use of herbicides, pesticides, and GMOs.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.7b00440.

Laboratory exercises, pre- and postlab questions, and sample graphs and data sets (PDF, DOCX)

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Notes

The authors declare no competing financial interest.

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