ARTICLE
The Influence of Assay Design, Blinding, and *Gymnema sylvestre* on Sucrose Detection by Humans

Max G. Aleman*, Lauren J. Marconi*, Nam H. Nguyen*, Jae M. Park*, Maria M. Patino*, Yuchi Wang*, Celeste S. Watkins, and Chris Shelley

*Department of Biology, Franklin and Marshall College, Lancaster, PA 17604. *These authors contributed equally to this work.*

The detection and grading of tastes corresponding to different taste modalities can be tested in engaging laboratory sessions using students themselves as test subjects. This article describes a series of experiments in which data pertaining to the detection of salty and sweet tastes are obtained, and the ability of the herb *Gymnema sylvestre* to disrupt the detection of sucrose is quantified. The effects of blinding and different assay designs on EC50 estimation are also investigated. The data obtained allow for substantial data analysis, including non-linear regression using fixed and free parameters to quantify dose-response relationships, and the use of often under-utilized permutation tests to determine significant differences when the underlying data display heteroscedasticity.

Key words: sensory transduction; taste; Gymnema sylvestre*; dose-response; permutation test*

Sensory systems allow organisms to detect and respond appropriately to their environment. The detection of exogenous chemicals is essential and in its most basic form exists in all cellular life, as receptor-mediated signal transduction. In animals, taste and olfaction have evolved as specialized systems to detect external chemicals, with the primary role of the taste system being to detect nutrients for ingestion, and to avoid ingestion of potentially toxic chemicals. In mammals, five major taste modalities of salty, sour (acid), bitter, sweet, and umami (glutamate and other related compounds) have been definitively identified, and there is growing evidence for a sixth taste specific to fats (Besnard et al., 2015). Taste compounds (tastants) are detected at the apical ends of taste buds that are comprised of several different types of taste receptor cells, found on the tongue and soft palate.

The molecular mechanisms and types of cells underlying tastant detection vary considerably, and therefore the recent review by Chaudhari and Roper, (2010) is recommended as appropriate background reading for both instructors (and students) as required. The initial step in the detection of bitter, sweet, and umami tastes is the activation of G protein-coupled receptors (GPCR). Umami tastants bind to heteromeric receptors comprised of T1R1 and T1R3 subunits (Li et al., 2002; Nelson et al., 2002), whereas sweet tastants bind to heteromeric receptors comprised of T1R2 and T1R3 subunits (Nelson et al., 2001). Bitter tastants bind to members of the T2R family of GPCRs, subunits of which are encoded by at least 28 different genes in humans (Bachmanov et al., 2014). In contrast, salt (Na^+) detection is primarily mediated by the direct influx of Na^+ into taste cells via amiloride-sensitive epithelial Na^+ channels (ENaC) (Heck et al., 1984; Doolin and Gilbertson, 1996). The molecular basis for the detection of sour tastants is less well understood, but is known to involve the activity of the ion channel polycystic kidney disease 1-like channel 3 (PKD2L1) (Huang et al., 2006). In addition a Zn^{2+}-sensitive proton channel has been found sufficient to depolarize sour-detecting taste cells via proton blockage of the inward rectifier K^+ channel Kir2.1 (Chang et al., 2010; Ye et al., 2016). Successful detection of any of the major tastants leads to activation of either the chorda tympani or glossopharyngeal afferent nerves. This activation is believed to be via ATP release and subsequent activation of neuronal purine receptors in the case of bitter, sweet, and umami tastants (Finger et al., 2005), and via serotonergic synapses between sour taste receptor cells and afferent nerves (Huang et al., 2005). Little is known regarding how taste cell ENaC conductances ultimately depolarize the afferent gustatory nerves.

It is well know that the tastes of food can be modified by different tastant combinations and there are substances that modify taste perception. One of the best characterized are extracts from *Gymnema sylvestre*. *G. sylvestre*, also known as gurmar, is a member of the dicotyledonous class of plants in the family Apocynaceae, subfamily Asclepiadoideae. It has been known for many years that extracts of *G. sylvestre* can reduce the sensations of sweetness associated with sweet tastants and inhibit activation of the chorda tympani nerve in response to these tastants (Shore, 1892; Diamant et al., 1965; Warren et al., 1969), and many sweet-suppressing compounds have been isolated and identified from extracts of *G. sylvestre*. Many of these are triterpenoids termed gymnemic acids that have been shown to elicit biological activity in mammals (Sinsheimer et al., 1970; Di Fabio et al., 2014). An additional compound, the 35 amino acid polypeptide gurmarin isolated from *G. sylvestre* extracts, has also been shown to reduce responses to sweet compounds, including sucrose, in the rat (Imoto et al., 1991) and in mice (Ninomiya and Imoto, 1995). Other biological activities attributed to *G. sylvestre* compounds are inhibition of the Na^+-K^+ ATPase (Koch et al., 1973), and gymnemic acids

isolated from *G. sylvestre* have been shown to inhibit the glucose transporter SGLT1 (Wang et al., 2014).

The experiments described here formed two laboratory sessions (each of four hours) of a Pharmacology course attended by undergraduate upper level Biology/Biological Foundations of Behavior students. They are partly based on the experiments of Schroeder and Flannery-Schroeder, (2005) but were expanded to incorporate the quantification of dose-response relationships to determine the effects of assay design on EC50 estimates, the effects of blinding on sucrose detection, and the effects of *G. sylvestre* extracts on sucrose detection. The materials can be readily obtained and for minimal cost, and the data obtained are very robust and therefore suitable for rigorous analysis. In addition, as Schroeder and Flannery-Schroeder (2005) notes, student engagement is increased when they are the test subjects. Prior to the experimental work, a brief lecture was given outlining the aims of the laboratory sessions, the methodology to be used, and the different molecular mechanisms that underlie the detection of the five major taste groups. The major aims of the sessions included learning the different molecular mechanisms of the major tastant classes, to demonstrate how simple experiments can yield robust and reproducible data, to be able to analyze and compare dose-response data and draw appropriate conclusions from such data, and to identify potential sources of bias in experimental procedures. Following data collection and preliminary analysis, a tutorial session was hosted in the style of a research laboratory meeting where it was discussed how to present the data for publication, and pertinent points that should be included in the introduction, methods, results, and discussion sections.

MATERIALS AND METHODS

Each individual taste test was performed by soaking a sterile cotton-tipped applicator (Puritan) in the test solution. The cotton tip of the applicator was then placed on the tip of the tongue of a student for five seconds. Responses were recorded as either graded values (0 to 10, with 10 being the strongest and 0 being no detection) or as all-or-none data, in which a positive or negative response to the question, “Can you detect the tastant?” was recorded. The concentrations ranged from 1 mM to 5 M (NaCl) and 1 mM to 2 M (sucrose). This range was chosen as it was expected that no subjects would be able to detect tastants at 1 mM, and all subjects would be able to detect that tastants at the highest concentrations. Each subject conducted a single test per compound concentration for the graded data. For the sucrose all-or-none experiments each subject conducted taste tests on five replicates of eight different concentrations of sucrose in labelled tubes (unblinded tests) or forty coded tubes containing sucrose solutions (blinded). For the blinded experiments, five replicates of each of eight different concentrations of sucrose were labelled with a sequentially assigned random number between 1 and 40, generated using the RANDBETWEEN function in Microsoft Excel. After every individual taste test, subjects rinsed their mouths with distilled water.

To determine the effect of *G. sylvestre* on the subjects’ perception of sucrose taste, an extract was made from cut and sifted *G. sylvestre* leaves (Starwest Botanticals) by adding 9 g (approximately one half measuring cup) *G. sylvestre* to 1000 ml boiling water. The solution was allowed to steep for ten minutes and was then filtered to remove solids. The extract was allowed to cool to room temperature before use. To apply the extract, subjects were instructed to rinse their mouth completely with 25 ml *G. sylvestre* extract for 30 seconds, and to then discard the solution in waste receptacles provided. Taste tests were performed three minutes after the rinsing with the extract using the solutions from forty coded tubes that comprised five replicates of eight different blinded concentrations of sucrose.

Curve fitting was performed by the students in SPSS Statistics version 22 (IBM) with the Regression Module using the Levenberg-Marquardt method with the following form of the Hill equation. For some fits R_{max} was fixed to 100%.

$$R = R_{max}\left(\frac{A^{nH}}{EC50^{nH} + A^{nH}}\right)$$

If SPSS Statistics is not available, it should be possible to perform the curve fitting using the open source software R (R, version 3.1.2; The R Foundation for Statistical Computing; http//www.R-project.org). The Levenberg-Marquardt method can be implemented in R with the package minpack.lm (available at http/www/cran.r-project.org).

To highlight potential heteroscedasticity (unequal variance across sucrose concentrations,) in all-or-none data, the means and standard errors of the mean for the unblinded, blinded, and post *G. sylvestre* data were fitted with a parabola of the following form,

$$y = a(x - h)^2 + k$$

Significant differences between sucrose detection before and after *G. sylvestre* at all concentrations tested were determined using permutation tests (Drummond and Vowler, 2012). Briefly, for each sucrose concentration the data in the presence and absence of *G. sylvestre* were pooled, and all possible data set combinations were computed (two groups of six samples drawn from twelve data points). A distribution of differences between means was determined, from which the *p* value of the experimental data could be directly determined. Permutations test were computed using code written in R. *p* values less than 0.05 were deemed significant. Error bars denote standard errors of the means.

Approval for these experiments with human subjects was obtained from the Franklin and Marshall College Committee on Grants, which functions as an Institutional Review Board for research and laboratory sessions at Franklin and Marshall College that involve human subjects.

RESULTS

To determine the ability of subjects to detect sucrose and NaCl, two approaches were utilized. In the first, responses were graded from 0 to 10, with 0 representing no detection of the test substance, and 10 representing the detection of the strongest concentration of the test substance. In the latter tests, detection was quantified as an all-or-none response, and represented as the mean percentage of positive responses for each tested concentration. Fig. 1 shows the graded dose-response curves for NaCl (Fig. 1A) and sucrose (Fig. 1B). The EC50 values for NaCl and sucrose were similar, 243 mM and 276 mM, respectively, as were the Hill slopes of the dose-response curves, 1.05 and 1.07 for NaCl and sucrose, respectively.

Fig. 2A shows the detection of different concentrations of sucrose for both blinded and unblinded conditions, assayed using the all-or-none procedure. Both curves were left-shifted compared to the graded response sucrose assay. Interestingly, although the EC50 values under both blinded and unblinded conditions were similar, there was a notable difference in the slopes of the Hill plots, with the unblinded experiments having a much steeper slope than that of the blinded experiments (Table 1). It is possible that the much steeper slope of the unblinded Hill Plot arose entirely due to the variability and submaximal response observed with 300 mM sucrose (the origin of which is unknown). To investigate the effect of this variability on the Hill slope, all data were refitted with the maximal response (R_{max}) fixed to 100% (Fig. 2B). This reduced the Hill slope estimate of the unblinded data to 4.0, similar to the 3.5 obtained with the blinded data. The EC50 values were very similar, 44.7 mM for the unblinded experiments and 47.3 mM for the blinded conditions (Table 2).

To determine the effect of the *G. sylvestre* on the detection of sucrose, blinded tasting experiments were performed following the rinsing of the subject's mouths with *G. sylvestre* extract. There was a notable rightward shift in the sucrose detection dose-response curve, with a nearly 3-fold increase in the EC50 following extract application (Fig. 2C, 2D). A rightward shift of the dose-response curve was observed both when R_{max} was a free parameter, and when R_{max} was fixed 100% (Tables 1 and 2).

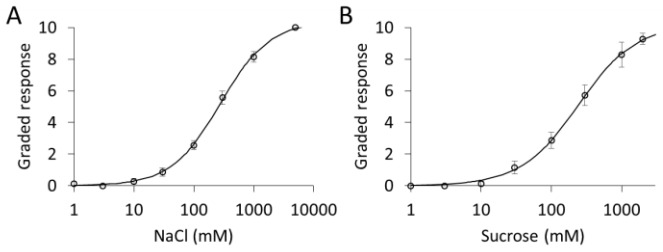

Figure 1. Graded responses to the detection of NaCl and sucrose. Responses ranged from 0 (no detection) to 10 (strongest possible detection). Each data point represents n = 7 subjects. Curves are fits to the data of the Hill equation. (*A*) Responses to NaCl. (EC50 = 276 mM, Hill slope = 1.07, maximal response = 10.4). (*B*) Responses to sucrose (EC50 = 243 mM, Hill slope = 1.06, maximal response = 10.2). Note that the fitted maximal response can be greater than 10 if this provides a better fit of the data than restricting the maximal response to values of 10 or less.

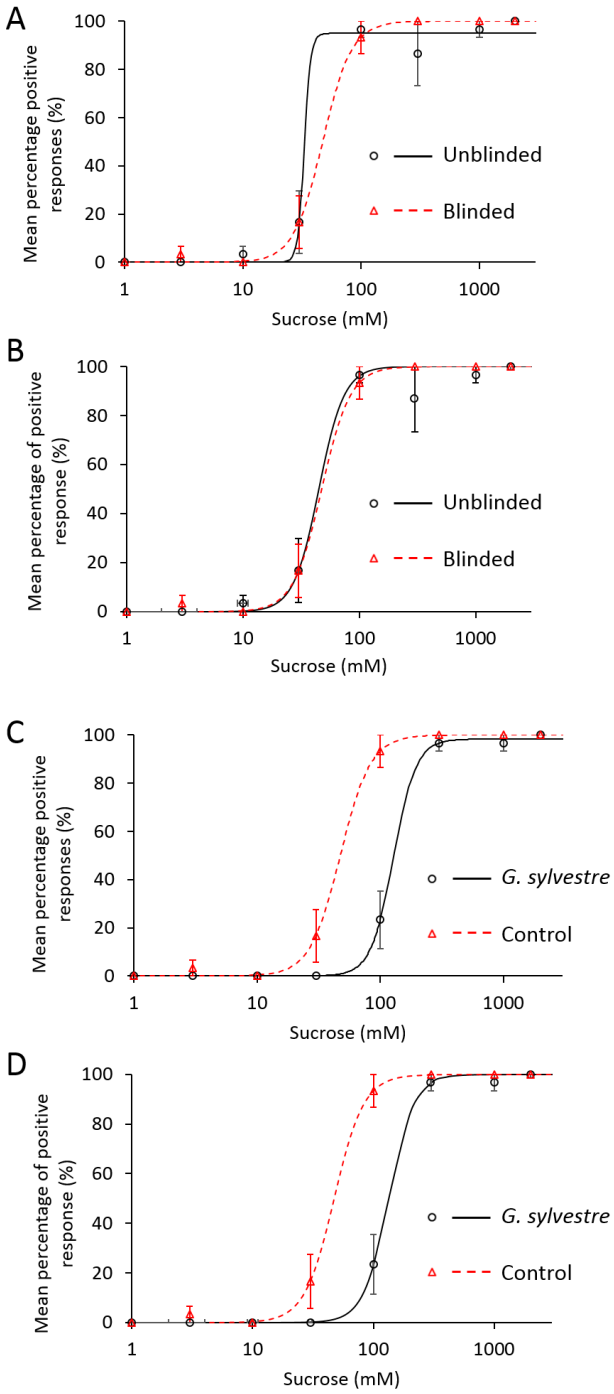

Figure 2. The effect of blinding and *G. sylvestre* on the detection of sucrose. (*A*) All-or-none dose-response curves in response to unblinded sucrose and blinded sucrose solutions fitted with Hill equations with all parameters free. (*B*) All-or-none dose-response curves in response to unblinded sucrose and blinded sucrose solutions fitted with Hill equations with the maximal response fixed to 100%. (*C*) All-or-none dose-response curves in response to blinded sucrose solution before (control) and after the administration of *G. sylvestre* extract fitted with Hill equations with all parameters free. (*D*) All-or-none dose-response curves in response to blinded sucrose solution before (control) and after the administration of *G. sylvestre* extract with the maximal response fixed to 100%. In all graphs each data point represents n = 6. Fit parameters are listed in Tables 1 and 2.

In order to determine whether *G. sylvestre* significantly influenced the detection of sucrose, a permutation test was performed for each concentration of sucrose tested. Permutation tests were used due to the failure of assumptions regarding the distribution of the data, and expected heteroscedasticity of the data. This can be seen clearly in Fig. 3A, in which a parabolic relationship between percentage of positive responses and the standard error of the mean of responses is observed. Fig. 3B shows a histogram of the differences between sample means for all possible permutations of the 100 mM sucrose data before and after *G. sylvestre* administration, clearly showing the effect of *G. sylvestre*. Administration of *G. sylvestre* significantly decreased the mean percentage of positive responses with 100 mM sucrose (p = 0.006). No significant differences in sucrose detection were found at the other sucrose concentrations.

	Unblinded	Blinded	Blinded following *G. sylvestre*
EC50 (mM)	33.1	47.4	127.8
95% C.I. (mM)	*	43.0 – 51.8	115.1 – 140.6
Hill slope	15.8	3.5	4.8
95% C.I.	*	2.9 – 4.1	2.9 – 6.6
R_{max} (%)	95.0	100.0	98.3
95% C.I. (%)	88.1 – 101.9	97.8 – 102.3	96.4 – 100.3

Table 1. Parameters from Hill fits of the unblinded, blinded, and blinded following *G. sylvestre* experiments. * The 95% C.I. for these estimates were essentially undefined.

	Unblinded	Blinded	Blinded following *G. sylvestre*
EC50 (mM)	44.7	47.3	133.3
95% C.I. (mM)	23.8 – 65.7	42.9 – 51.7	119.8 – 146.8
Hill slope	4.0	3.5	4.1
95% C.I.	-0.2 – 8.2	2.9 – 4.2	2.8 – 5.4
R_{max} (%)	100.0	100.0	100.0
95% C.I. (%)	n.a.	n.a.	n.a.

Table 2. Parameters from Hill fits of the unblinded, blinded, and blinded following *G. sylvestre* experiments with the maximal response (R_{max}) fixed to 100%. n.a. = not applicable.

DISCUSSION

The experiments described here were designed to enable undergraduates to obtain and analyze pharmacological response data pertaining to salt and sweet tastants, and to determine the effects of sample blinding, and pre-administration of an extract of *G. sylvestre* on responses. The graded response sucrose EC50 obtained was approximately 5-fold greater than the all-or-none sucrose EC50. This is expected, as the threshold for detection of

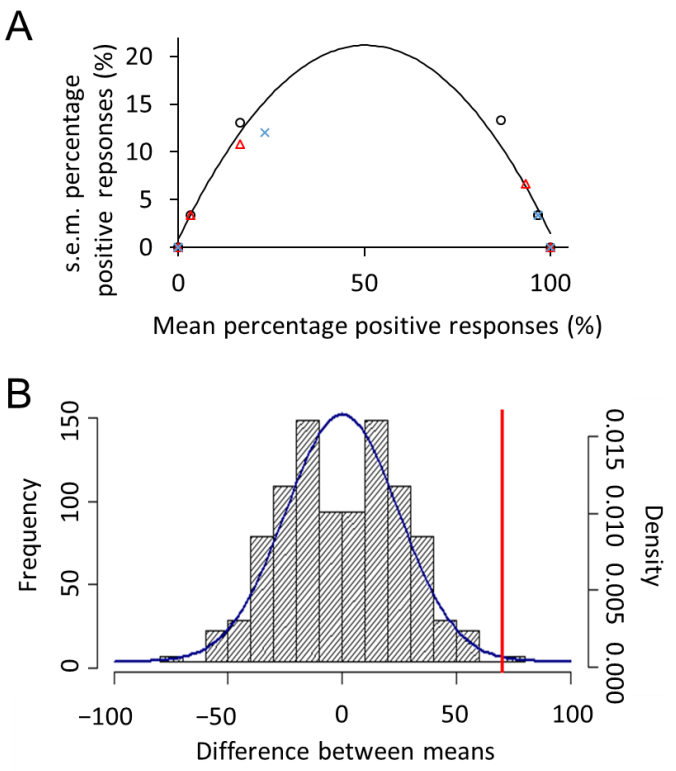

Figure 3. (A) The relationship between means and standard errors of the mean for all of the sucrose all-or-none data, unblinded (black circles), blinded control (red triangles), *G. sylvestre* treatment (blue crosses). The combined data were fitted with a parabola yielding a = -0.008, h = 50.4, k = 21.2. (*B*) The distribution of differences between means using experimental data from blinded 100 mM sucrose solutions with and without *G. sylvestre*. The permutations test yields 924 data sets. The blue line is the normal distribution probability density function (right y axis scale). The red line indicates the experimentally obtained difference between the means (mean = 70, n = 6 per condition, p = 0.006).

sucrose must, by definition, be at the lower end of a graded sucrose detection scale. By determining EC50 values on a graded scale and an all-or-nothing scale it is demonstrated to the students that the EC50 value of a receptor-agonist complex is dependent not just on the physicochemical properties of the molecular interaction but also on the type of response measured. This helps to reinforce the principle of the non-equivalence of an EC50 and binding affinity, despite the similarity in form of dose-response curves and dose-occupancy curves.

Preliminary data from previous labs had determined that the all or none assays produced data that was more readily reproducible than the graded response assays, and so the former was used to determine the effects of blinding and *G. sylvestre*. The graded response sucrose EC50 of 243 mM is greater than has been determined previously. For example, the midpoints in an intensity scale for sucrose taste have been found to span 100 mM to 180 mM sucrose (Lawless and Skinner, 1979). The EC50 for sucrose detection from the all or none assays of 47 mM is very similar to the 44 mM threshold value obtained by Heath et

al. (2006), but higher than the 12 mM value that has been reported in several other studies (Richter and Campbell, 1940; Pepino and Mennella, 2007; Joseph et al., 2016). However, there are differences in the assays across studies, and it should be noted that a large range of threshold values have been reported. To give one example, the range of sucrose thresholds obtained by Joseph et al. (2016) were 0.2 mM to 154 mM.

Blinding subjects to the sucrose solutions did not affect the dose-response relationship of sucrose. This could indicate either that blinding was not necessary to obtain unbiased results in this experimental paradigm, or that biases were consistent across the two sets of experiments and that the blinding conducted was not sufficient to overcome these biases. It should be noted that the blinded data experiments were singly-blinded not double-blinded. That is, the subjects were unaware of the true sucrose concentrations, but the instructor was. It is believed that this would be a source of minimal bias given that all six subjects were performing the taste tests concurrently, and there were forty differently coded tubes, making the monitoring of individual taste tests by the instructor unfeasible. However, because of the concurrent testing procedure possibly the most substantial source of bias may have been due to the subjects being aware of nearby subjects' responses to specific coded solutions. To minimize this, the protocol emphasized that subjects should come to their own conclusions regardless of the responses of other participants. Another potential source of bias is that the viscosity of sucrose solutions was visibly different at the highest concentrations of sucrose, and some subjects reported being able to distinguish the highly viscous solutions from the less viscous solutions during the taste test. However this is expected to have minimal influence on the data as it would only apply to highly concentrated sucrose solutions, concentrations at which there is little ambiguity as to whether sucrose can be detected.

We found that rinsing the tongue with *G. sylvestre* led to a reduction in the ability to detect sucrose, as reported previously (Meiselman and Halpern, 1970; Brala and Hagen, 1983; Schroeder and Schroeder-Flannery, 2005). We were able to quantify the effects over a range of sucrose concentrations and determine the effect on EC50 for sucrose detection. It has been shown previously that *G. sylvestre* extract reduces the amplitude of sucrose-evoked gustatory potentials from the chorda tympani nerve, but has no effect on the amplitudes of potentials evoked by bitter, sour, or salty tastants (Diamant et al., 1965; Min and Sakamoto, 1998). In addition, heteromeric T1R2 + T1R3 GPCR complexes responsible for the initial binding of sweet tastants in humans have been shown to be directly inhibited by both the *G. sylvestre* peptide gurmarin (Margolskee et al., 2007), and by gymnemic acids (Sanematsu et al., 2014). However it should be noted that the inhibition of sweet tastant detection by *G. sylvestre* lasts for several hours and persists after multiple washings of the mouth (Ninomiya and Imoto, 1995; Gent et al., 1999; Schroeder and Flannery-Schroeder, 2005). This suggests that the effects of *G. sylvestre* go beyond simple pharmacological inhibition of the cell surface sweet receptor, and may potentially involve receptor or pathway desensitization or receptor internalization.

A student experience questionnaire (n=7) was administered after the conclusion of the course. Students gave a mean rating of 4.1 ± 0.3 (standard error of the mean) for the relevance of the exercises as a learning tool in understanding taste pharmacology (1 representing not relevant, 5 representing very relevant). It was also of interest to determine student perceptions of their improvements in six specific areas following the exercises. The results were quantified on a five point scale, 1 indicating no perceived improvement and 5 indicating a lot of perceived improvement. Perceived improvements were generally ranked high and were as follows, "understanding of the interpretation of EC50 values" (mean score = 4.6 ± 0.2), "the importance of considering potential bias in experimental results" (mean = 4.4 ± 0.2), "understanding the planning of a research article" (mean = 4.3 ± 0.2), "understanding analysis of dose-response data" (mean = 4.3 ± 0.3), "understanding practical and software issues of curve fitting" (mean = 4.3 ± 0.4), and "understanding of taste receptor biology" (mean = 4.0 ± 0.4). Students reported that the most challenging aspects of the exercise were analyzing the data and preparing the figures. This is understandable given the considerable data analysis employed in these experiments, even though 86% of students had already completed a statistics course that is mandatory for their majors prior to these exercises.

The experiments detailed here are simple to perform and use readily available, inexpensive equipment and reagents so they are suitable for undergraduates. Despite the simplicity of the experimental setup, the data obtained by the students are comparable to those in the published literature, and enabled substantial data analysis, including the plotting of dose-response relationships, the use of non-linear regression, and statistical tests and the exercises improved students perception of their understanding of key pharmacological concepts. The experiments could be readily expanded in future sessions to determine, for example, the IC50 of *G. sylvestre* extract on sucrose detection, or the effects of *G. sylvestre* on the detection of other sugars and artificial sweeteners.

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Received June 01, 2016; revised August 04, 2016; accepted August 08, 2016.

This work was supported by Franklin and Marshall Biology Department teaching funds. All authors except Dr. Shelley were undergraduate students who participated in the experiments, analyzed data and helped develop the manuscript.

Address correspondence to: Dr. Chris Shelley, Biology Department, Franklin and Marshall College, PO Box 3003, Lancaster, PA, 17604. Email: cshelley@fandm.edu