GC-MS Determination of an Unknown Pesticide. II. An Experiment Investigating Quality of Analysis Using Random Sampling and Estimation of Variance

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Abstract: Newly devised components are described which significantly enhance both the scientific and pedagogical components of a previously published, qualitatively oriented experiment for students in an undergraduate instrumental analysis course. To provide a laboratory exercise containing as much real-world experience as possible, the earlier experiment was modified in such a way as to allow its use as a quantitative experiment illustrating such principles as sampling, experimental design, analysis of variance, and the use of internal standards.

Introduction

Students in an undergraduate instrumental analysis course are commonly asked to perform laboratory experiments to familiarize themselves with instrumental techniques and chemical manipulations. Frequently, however, students see only the essentially trouble-free, final result of the design, testing, and redefinition of those experiments. Consequently students sometimes develop little appreciation for the thought, creativity, and planning that precedes the performance of a reliable experiment. There is a likelihood that students encountering only the final, fully tested procedures will develop the false impression that analysis, particularly quantitative analysis, is a simple and, usually, correctly working pursuit, with few pitfalls or uncertainties.

In the current analytical environment of falling limits of detection and increasing regulatory, legal, and journalistic implications, it is more important than ever that the chemistry curriculum lead the student to appreciate the factors affecting the quality and reliability of chemical measurement. In recognition of the budding chemist's need for such an appreciation, we have significantly modified a previously published, qualitative experiment [1] by incorporating quantitative determination and, perhaps more importantly, experimental design and estimation of variance to examine the factors influencing variability in the determinations.

Virtually every instructor in any chemical laboratory course has encountered student "reasons" for inaccuracy or imprecision in analyses such as, "the instrument reading fluctuated," or "we must have measured incorrectly." Tacit or explicit acceptance by instructors of such reasons, perhaps frequently appropriate in lower-level chemistry courses, may lead the more advanced student to the incorrect conclusion that these reasons are the principal ones for variations in correctly performed experiments. Our modifications to the earlier experiment are designed to illuminate and quantitatively estimate the variational influences of factors related to sampling, work up, and instrumentation on series of multiplicate measurements.

In the current incarnation of this experiment, we have retained the most important pedagogical aspects of the previous version [1] while adding significant enhancements. Specifically, the modified experiment asks the students to perform the following activities:

- implement a random sampling protocol, representative of a limited population of possibly contaminated grapes,
- prepare representative samples of contaminated and uncontaminated grapes,
- identify by gas chromatography–mass spectrometry (GC-MS) a target component in a standard mixture of chlorinated pesticides and to calculate its response factor relative to an included standard (hexachlorobenzene),
- determine the concentration of an unknown pesticide in each sample prepared,
- use a simple estimation of variance to estimate the relative contributions of sampling, work up and instrumentation to the overall analytical variations.

Experimental

Experimental Model. Excessive variance can arise from many sources. The most obvious is sampling; even preparing an instrument-sized sample from a homogeneous gross sample requires careful attention to statistics to ensure all components of the gross sample have an equal chance to be present in the analysis step. Gas, liquid, and solid homogeneous samples require strict protocols to ensure analytical results have some relevant meaning. For heterogeneous samples, the importance of these considerations are greatly compounded. To identity, quantify, and then delineate relative sizes of variances encountered is a difficult prospect even in advanced laboratories; for most, this will be the only encounter with such a procedure during their undergraduate education.

Two samples are prepared in parallel. The first is composed of ordinary white seedless grapes. The second consists of approximately 100 commercial seedless grapes which have been sprayed with a dilute (3%) methanol solution of commercial chlordane (from our archives—this product is no longer available over the counter in the United States) and allowed to dry. Chlordane was chosen as a particularly good subject for GC with electron-capture detection; it is not central to the experiment outlined. The students are told only that some of the grapes have been contaminated. Questions they must answer using gas chromatography with mass spectrometric detection involve the identity and level of the contamination and quantitative estimation of the sources of variance in the analysis. Grapes were

extracted with hexane to remove fat-soluble compounds, including the active ingredient of the unknown (chlordane). Qualitative analysis was made through comparison of gas chromatographic retention times with the components of a certified standard (EPA 508/508.1). Mass spectra for peaks matching retention times were compared; molecular formulas were deduced from isotopic ratios of the molecular ion peaks. Once the formula was derived, mass spectral data were compared to those available for examination on the NIST website [2] to provide confirmation of qualitative identification. The qualitative program has been described previously [1]. Details regarding quantification and variance are now of concern.

Quantification. Determination of chlordane levels was achieved by calculating a response factor from differences in chromatographic integrator response for samples and standards of known concentration. Our standard in this case was purchased from Supelco as the Certified EPA 508/508.1 Chlorinated Pesticides Mix, which contains chlordane, hexachlorobenzene (HCB), and other compounds at the 1000 µg/mL level [3]. We chose HCB as an internal standard to be added to all samples for quantification. This allowed for calculation of unknown levels according to the following relationship:

$$\mu g_{\text{Chlordane}} = R \times \left(\frac{\text{area}_{\text{Chlordane}}}{\text{area}_{\text{HCB}}} \right) \times \mu g_{\text{HCB}} \qquad (1)$$

where $\text{area}_{\text{chlordane}}$ is the integrated area of the chromatographic peak attributable to the γ- or α-chlordane isomers, and area_{HCB} is the integrated area of the chromatographic peak for hexachlorobenzene.

Estimating Variance. What are possible sources of variance? Sampling must be considered, particularly in a heterogeneous sample of this sort. Since the pesticide must be extracted and concentrated before analysis, work up variance is a contributing factor. Variance between replicate runs of the same sample (instrumental variance) should be very small for an instrument in good repair, but not necessarily zero. Since sample injection by inexperienced analysts can result in significant variation in injection volume (and hence detector signal), this source of error (operator variance) must be considered. Expressed mathematically:

In order to keep the number of terms from becoming too unwieldy, operator variance was minimized by expressing analysis results as a ratio of chlordane peak areas (t_R = 10.11 min, 10.40 min. for γ- and α-chlordane, respectively) to areas for HCB internal standard added to all samples (t_R = 7.30 min). This minimizes the effect of $\sigma^2_{\text{operator}}$ and allows calculated variances to be unitless and comparable.

$$\sigma^2_{\text{Total}} = \sum_{i=1}^{n} \sigma_i^2 = \sigma^2_{\text{Sampling}} + \sigma^2_{\text{Workup}} + \sigma^2_{\text{Instrumental}} \qquad (2)$$

(n = number of contributors to variance)

Now that the three-component model is agreed upon, it is necessary to design a sampling protocol to support it [4]. In order to prepare a representative sample, students set up their grapes in a two-dimensional (x, y) grid. Students were divided into three groups, each to work up their samples independently, one group each to prepare the samples used to assess instrumental, work up, and sampling variance.

Not having any population statistics from previous runs, proper sample size is unknown. Each group used a spreadsheet to generate five random (x, y) pairs and selected those from the two-dimensional grid for analysis.

Preparation of the Total Variance Sample. Five grapes were selected at random, each grape was ground to a semisolid pulp and shaken vigorously with 50 mL of hexanes (Malinckrodt AR grade) to extract fat-soluble compounds. The liquid was decanted from the solid material and the shaking was repeated with 50 mL of hexanes. The solvent was stripped away by rotary evaporation, and the pale, thin liquid remaining was redissolved in 1.00 mL of hexanes spiked with a 400 µg/mL hexachlorobenzene standard solution. This gave five presumably identical samples for gas chromatography. Reagent grade hexachlorobenzene used to make the internal standard was purchased from Aldrich and used as received.

Preparation of the Composite Sample. Five grapes were selected at random, *combined*, and were ground into a semisolid pulp. The resultant mass was shaken thoroughly with 50 mL of hexanes, and the solvent was decanted from the solid. This was repeated with an additional 50 mL of hexanes. The volume of this solution was measured and divided into five portions of equal volume with a pipette. Hexanes were removed from each individual portion by rotary evaporation and the remaining liquid was spiked with 1.00 mL of the HCB standard as described above.

Preparation of the Instrumental Variance Sample. Five grapes were selected at random worked up in parallel fashion as described above. One of the five spiked samples so prepared was removed for analysis and was injected into the gas chromatograph five times.

The rationale for this experimental design is to mathematically or experimentally isolate the contributions to the overall experimental variance, and to demonstrate to relative importance of various components to the reliability of the overall analysis.

Instrumentation Particulars. Gas chromatography was performed on both contaminated and uncontaminated samples. The certified chlorinated pesticide standard against which both were compared, EPA 508/508.1, was purchased from Supelco, Inc. Chromatographic equipment consisted of an HP 5890 GC coupled to a 5970 Series Mass Selective Detector, which was held at approximately 2×10^{-5} torr with a Varian turbo pump. Mobile phase was a 5% methane/95% argon mix for the ECD detector. The mobile phase for the HP 5890 was helium. A Supelco SPB-5 column of diameter 0.25 mm and film thickness 0.25 µm was used; either 15 m (HP 5880A) or 30 m (HP 5890). Injection port temperature was 300 °C in each case. Temperature program for both standard and samples: initial column temperature was 120 °C, increasing at a program rate of 30 °C/min to 180 °C, then increasing at a rate of 10 °C/min to 280 °C, and the column was held at that temperature for 15 minutes. This program was sufficient to elute all major components from all samples. The ^{63}Ni detector was held at 350 °C; the GC-MS detector was set to 280 °C. Injection volumes were 0.5 µL for the ECD, and 1 µL for the GC-MS.

Results

Qualitative determination of the unknown pesticide was performed as previously described [1]. Quantitative results are obtained using equation (1) and are reported either as µg chlordane/sample or µg chlordane/grape.

Following injection of the five total variance samples, five composite samples, and five injections of one replicate sample, data for peak areas obtained from the total ion chromatogram should appear similar to that displayed in Table 1.

Using our experimental model, variances were calculated from these data according to:

a) σ^2_{total}: 5 grapes individually worked up and injected; $\sigma^2_{\text{instrumental}} + \sigma^2_{\text{work up}}$: 5 equal portions of a 5-grape composite sample, each worked up and injected;
b) $\sigma^2_{\text{instrumental}}$: 5 replicate injections of a single portion of a 5-grape composite sample;
c) $\sigma^2_{\text{work up}}$: calculated as (b) – (c)
d) $\sigma^2_{\text{sampling}}$: calculated as (a) – (b)

$$CL = \bar{x} + \frac{ts}{\sqrt{N}} \qquad (3)$$

Table 1. Typical Data Obtained From the Total Ion Chromatogram

Samples	Integrated Area: Pesticide	Integrated Area: Hexachlorobenzene	Ratio (Pesticide/HCB)
	Composite Sample		
1	20011323	170877502	0.1171092
2	27244668	161394374	0.1688080
3	4199748	37409983	0.1122628
4	18818055	149389259	0.1259666
5	20529871	166556544	0.1232607
	Replicate Sample		
1	16814622	161311627	0.1042369
2	21589404	167624576	0.1287962
3	21835189	157941586	0.1382485
4	20207318	171704036	0.1176869
5	19870586	169620436	0.1171474
	Total Variance Sample		
1	17681575	166386392	0.1062682
2	53081976	180449891	0.2941646
3	65545071	173836972	0.3770491
4	114133521	157999773	0.7223651
5	44135204	159907149	0.2760052

Table 2. Typical Student Results

	Composite	Replicate	Total
Mean unknown mass (μg)	46.510	43.543	127.577
Standard deviation	8.126	4.631	81.763
Variance	66.034	21.446	6685.147
CL (95%)	7.123	4.059	71.667
V(ratio)	0.000409	0.000133	0.003280

Students also calculate confidence intervals according to where the symbols have their usual meanings in a determination where the population standard deviation is unknown.

Following calculations (equations 1, 2, and 3) done with a spreadsheet program, typical student results as they might appear in a submitted report are shown Table 2.

Thus, the total variance sample indicates levels of ~128 ± 72 μg chlordane/grape, the composite sample indicates 46 ± 7 μg chlordane/grape, and the replicate sample indicates 44 ± 4 μg chlordane/grape.

Once the variances are calculated (this can be done from Table 1 using any spreadsheet program), their relative magnitudes can be compared as shown in Figure 1.

This is quite an eye-opening experience for undergraduates. Most of them are trained to think in terms of minimization of error in laboratory procedures only; even those who recognize variances due to work up are rarely exposed to situations where 99% of the error is engendered before the sample even enters the laboratory. Education at the undergraduate level rarely conveys the complete context of an analysis, i.e., analyst involvement from gross sample to data interpretation. We all tell our students, beginning in sophomore organic laboratory, that precise measurements on impure samples are a waste of time. However, this statement, while apropos to synthesis, is not applicable to environmental analysis. We implemented this two-week experiment in order to demonstrate to students how absolute numbers often are misleading or do not tell the entire

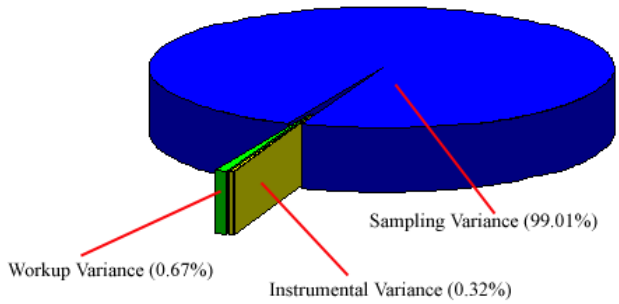

Figure 1. Relative contributions to experimental variance determined according to experimental model.

story. Sampling is a critical component of analysis, one with which the responsible analyst must be integrally involved.

One final calculation is useful in demonstrating the importance of sampling. We ask students, once data is in hand, to calculate how many grapes would need to be sampled in order that the total variance measurements would exhibit the same degree of precision as the composite measurements. Using the expression

$$N = \left(\frac{ts}{E} \right)^2 \qquad (4)$$

where t = 2.57 (95%), s = 81.76 (from experimental data), and E = 7 (maximum allowable variance), then one obtains the result that more than 900 grapes would have to be sampled in order to obtain a degree of precision similar to that observed for the composite sample! For the same exhibited degree of precision as the instrumental variance sample, more than 2700 grapes would need to be analyzed. These numbers help convey the actual *meaning* of analysis, beyond the much simpler concepts of limits of detection or micrograms recovered.

Conclusions

We find this exercise to be a highly successful one in that it accomplishes important pedagogical goals while stimulating much student interest. While the levels of chlordane doped on the grapes do not represent realistic levels, this exercise still simulates many components of a real-world analysis. Again, due to the tight scheduling of undergraduate laboratory education, it is very difficult to work in sampling exercises to student experiments.

This laboratory can be performed at any point in the semester. We have tried it just before fall break, after four other chromatographic exercises, as representing a denouement in that field. It might be best offered, however, as an initial exercise, as the concepts demonstrated serve throughout the semester. The only difficulty in an early assignment is with mass spectrometry, which is only necessary to determine a molecular formula. This procedure can be covered sufficiently in one hour of lecture time. We find it also alerts students that "this course is going to be different" with subsequent higher levels of effort and excitement demonstrated.

The experiment can be improved in several ways, which we are now investigating. One is to lower the amount of contamination. The levels of chlordane used are unrealistically

high, as this is a *simulation* of a real-world experiment. We felt it was more important to stress the concepts described here than make the laboratory an exercise in signal-to-noise levels. An intriguing variation would be to increase the size of the gross sample to incorporate different levels of contamination. This would allow students to observe the increase in the relative contribution of work up variance as analyte level decreases. Other, more complex sample matrices could be examined. Grapes, while heterogeneous, do not offer nearly the sampling challenge that melons or unshelled nuts would. Depending on the size of the class, a worthwhile variation is to have a identical set of groups perform parallel analyses to those described above with non-representative samples (grapes selected from the center of the gross sample, for example) for comparison. We used chlordane as our contaminant because of its low limits of detection with GC-MS and, especially, GC-ECD. Many other satisfying possibilities exist, particularly using GC-MS. We are currently considering ways to incorporate a chemical surrogate into the grapes prior to analysis in order to have students investigate analyte recovery, in addition to the factors mentioned here.

In conclusion, this experiment has been one of the most successful in our program. It generates surprising (and gratifying) excitement among the students who feel that they are learning something relevant and applicable.

References and Notes

1. Holder, G. N.; Breiner, S. J.; Farrar, D. G.; Gooden, D. M.; McLure, L. M. *Chem. Educator* [Online] **1999**, *4*(6): S1430-4171(99)06341-1.
2. National Institute of Standards and Technology. http://webbook.nist.gov/chemistry/ (accessed April 2000).
3. SUPELCO, Inc., 1999 Chromatography Products catalog, p. 387.
4. In this experiment, we ask students to model the various contributions to total variance as “instrumental”, “workup” and “sampling.” From the instructor’s point of view, we recognize that “sampling” variance actually contains variational components related both to sampling and to application of the target pesticide. From the standpoint of the student, however, without knowledge of the application methods or origin of the population, the third mentioned component is reasonably treated as a sampling-related one. Such an approach realistically simulates the situation which might occur in a farmer's field or garden.