Research Instrumentation Used in Education: Liquid Chromatography–Mass Spectrometry Experiences in Upper-Class Laboratory Settings

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The purpose of this workshop was to explore the ramifications of using complex, research-grade instrumentation in undergraduate science education. Our thesis is that the use of complicated instruments—meaning complex in theory, data interpretation, operation, and/or sample preparation—in undergraduate laboratory settings introduces unique pedagogical elements. We identify and discuss those elements based on our experience in using such instrumentation, particularly liquid chromatography with mass spectral detection, in an integrated laboratory.

The use of modern instrumentation in chemistry laboratories is of course quotidian. Excepting the occasional discussions regarding the use of more complicated instruments in introductory labs (1), there is no doubt that modern chemical education demands the exposure of students to relevant instrumentation. However, the most complicated instruments bring a set of pedagogical issues into the laboratory. Our definition of a complex instrument is one that 1) requires extra care, uncommon techniques, or multiple steps in sample preparation; 2) has an abstruse or complicated theory of operation, resulting in difficult operation or sophisticated data interpretation; and/or 3) has complicated hardware. By this definition, examples include Fourier-transform nuclear magnetic resonance (FT-NMR), liquid chromatography coupled with mass spectrometry (LC-MS), time-resolved spectroscopy, and multidimensional mass spectrometry. Some, such as FT-NMR, can definitely be streamlined to the point where users do not "see" the complexity. The list of "simple" instruments includes pH meters, absorption spectrophotometers (including flame atomic absorption), most Fourier-transform infrared spectrometers, and even gas chromatography with flame ionization detection. There are clearly "in-betweeners," such as near-infrared absorption spectrophotometry, where the instrumentation is pretty simple, but data analysis can be complex, or liquid chromatography, which is pretty reliable but potentially has complicated method development.

The distinction between simple and complex instruments is not really important of and by itself; it does, however, serve to highlight the issues that arise when using certain instruments. With a simple instrument, a student can put in a dilute sample, get a response, and essentially be within a few knob turns or another dilution of having optimal, linear response. Data interpretation consists of simple readouts or, at worst, a peak measurement of some sort. A subtler addendum is that a simple instrument will be adjustable toward optimal, linear response by easily interpretable signs. That is, troubleshooting can be easily mastered by beginning students. For example, in flame atomic absorption (AA) spectrometry, there are few choices for the instrument itself. Well-known methods provided by numerous sources give recipes for sample types and metals, and the manufacturer gives dynamic range and instrument settings. Even if, upon aspirating the first sample, the sample is out of calibration range, this problem will be obvious from the readout, and the remedy dilution will also be obvious. Of course, there are plenty of things that can go wrong, and many situations require moderately complex sample digestions. But 95% of the time, AA is simple. More importantly, 95% of the time, this is how the AA is used in teaching laboratories.

By contrast, an LC-MS requires choice of a method for the separation and for the electrospray, both of which can be quite complicated, even with published methods as a guide. For liquid chromatography (LC), column type and packing,

flow rate, mobile phase, gradient, and injection volume are all variable (though there are rules of thumb for certain types of samples, such as water-soluble proteins); for electrospray, cone voltage, postcolumn buffer additives, mode, and needle voltage are variable. Furthermore, decisions of mobile phase, column size, and flow rate depend on the electrospray mode, which in turn depends to a certain extent on what type of information is desired. Experienced users get a good feel for default settings, but experience is the key word. Because the sample goes into an LC, sample preparation is often somewhat more complicated, requiring at least filtering and dilution, but often multiple extractions as well. Finally, interpretation of the mass spectrum can be complicated, especially if there is fragmentation or if the sample has multiple charges.

In addition, the theoretical underpinnings of the instrument tend to be subtler. Troubleshooting a method or an instrumental problem, which always occurs to some extent when using complex instrumentation and/or complex experimental protocols, absolutely requires students to understand how the instrument works, not just in terms of knobs and software, but also in terms of chemistry and physics. It also requires knowledge of how the sample will interact with the instrument. For example, in one relatively simple experiment, students were separating nucleotides, using a method that was adapted from a fairly old literature protocol. They had to adapt the method to a modern packing material (higher carbon load, highly pure silica, smaller particles), column size, gradient method, and a buffer compatible with mass spectrometry (MS) detection (though we used ultraviolet [UV] detection during development). That required detailed understanding of LC theory and practice. They then had problems with background: rising during the run and variance from day to day and run to run. Questions arose: Was it an instrumental problem (artifact)? A column problem? A sample or mobile phase contaminant? Learning to sort through these questions required that the students devise their own experiments and test them. It also required that they were familiar with standard test methods (i.e., the column test). In this case, they narrowed it down to contaminated trifluoroacetic acid (TFA) and loss of TFA because of sparging. To formulate the questions in the first place, then devise experiments for their testing, run the experiments, and correctly interpret the results, students had to know how LC works and what questions to ask TAs and faculty. The use of complex instrumentation, in contrast to simple instruments, automatically begets practical reinforcement of theory and higher-order learning. These observations also belie the idea that instruments are black boxes whose use robs students of the opportunity to develop chemical intuition.

Exposing the students to the kind of instruments that chemists use, and that they themselves are likely to use in their careers, is a clear and easily defensible argument for including complex instruments in the curriculum. LC-MS has become a ubiquitous method in nearly every kind of laboratory that makes (bio)chemical measurements, and this is especially true in biotechnology and pharmaceutics. Because of its versatility, LC-MS (and many other techniques) is used in a wide variety of measurements (that is, it can be applied to a wide variety of samples). This means, in turn, that it can be used across a wide variety of projects in a wide variety of disciplines. Our hypothesis is that such use will forcibly demonstrate that chemistry and chemical research is interdisciplinary by its nature (2,3).

To reinforce the interdisciplinary concept, we at Duquesne University stopped teaching analytical, physical, inorganic, and biochemistry laboratories several years ago and instead began teaching a two-semester course for majors that we call Integrated Laboratory (IL). Many other departments have similar curricula (e.g., Maruca [4]). By the time students begin IL, they have had all of their lecture courses, except second-semester physical chemistry, second-semester biochemistry (which they take concurrently), and any advanced courses they might take. Most have completed their core requirements in biology, mathematics, and physics. In addition, we have sophomore students take a course called Research Laboratory Techniques, which is probably best described in the vernacular as "Quant." The latter was developed because we needed the IL students to have basic laboratory skills before entering IL. What is not clear to us is whether the students need such extensive classroom preparation before entering IL or using complex instruments. Certainly, the reinforcement of classroom learning is an important part of IL.

The first semester of IL is designed to get students familiar with instrumentation, computational methods, common data manipulation and analysis tools, good notebook protocols, working in teams, and communication methods. We also wean them off of the "cookbook" laboratory experience.

The students are formally expected to be in the laboratory two afternoons per week, although they usually need extra time, and we have recitation and discussion once a week for 1 or 2 hours. Recitation is student-driven, with students giving the prelabs and showing and interpreting data. The student groups work several projects, beginning with a protein unfolding exercise (5). This is followed by an experiment on protein isolation and characterization (including LC-MS) and one of several metal compound syntheses. Exact details are beyond the scope of the present chapter. These experiments all have known outcomes, or, at least, we are fairly confident that we know what is supposed to happen. This sort of experiment is fairly common in the integrated laboratory format. Examples from the literature include (6–15). Note that all of these experiments vary in complexity and in ease for the student. In our own case, we start with experiments that are conceptually simple and work our way up to more involved projects. The final project in the semester is typically a small-scale research project, culled from faculty research interests, normally with fairly rigid expectations for what will work, either because of experience with similar experiments or because of well-documented literature. Groups may cooperate on complicated projects or work on different projects.

In the second semester, students work only on research-based problems. Faculty assigned to teach the course and highly qualified TAs mentor them. We have a number of goals for the projects, beyond gathering useful data and beyond the intrinsic experience of reinforcing what they learned in their classes. Students have to learn how to plan experiments (question to sample to answer), how to prepare in advance of running instruments, how to adjust their plans when initial plans go awry or when an instrument is not available, how to work with samples from a variety of sources, how to persist in getting quality data, how to put up with the tedium of running many samples for good statistics, and how to communicate their results to their peers. This can be called peer-led learning, cooperative learning, problem-based instruction, or what have you, but the bottom line is that they learn to do laboratory work like all chemists do.

Three examples of the use of LC-MS will help to illustrate the value of using complex instrumentation with multidisciplinary projects: extraction and identification of ellagitannins from oak, caffeine in wastewater runoff, and purification and characterization of alcohol dehydrogenase.

Ellagitannins are polyphenols on sugar backbones. They are extracted from oak by alcoholic solutions and are therefore present in wine and whiskey aged in oak. It turns out that the tannin profile of oak may be helpful in matching oak staves for barrel production because tannin production is strongly correlated with genome. The overall project is to prove this hypothesis and will eventually involve genetic typing and measurement of the ellagitannin profile. For this example, the focus is on the LC-MS method development. General LC methods were suggested by developing the chromatography of gallic acid (the polyphenol "monomer") and catechin (a simple ellagitannin that is commercially available). The method was then translated for LC-MS of the oak extract and validated against a standard spectrophotometric method based on gallic acid. Figure 1 shows the mass spectra at two of the LC-UV absorption peaks that correspond to two known ellagitannins, apparently as their ethanol-bound derivatives. The project was only moderately successful, because the signal was not strong enough to identify most tannins nor could it quantitate them for profiling. But from a standing start with no commercially available standards, it is impressive that any tannins were positively identified. The students found that, for this kind of sample, a much more involved sample preparation scheme was needed for success. This is a common observation and

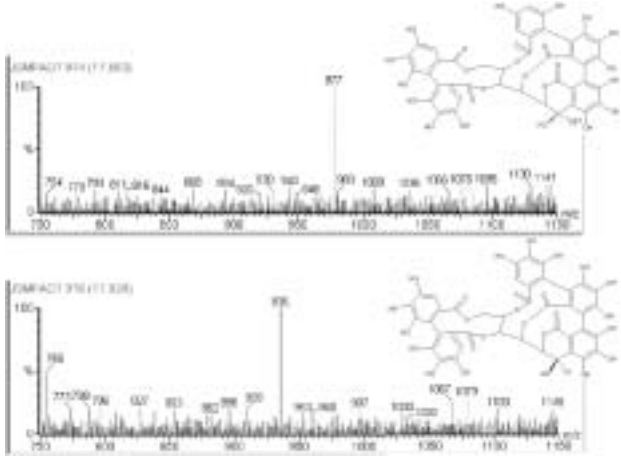

Figure 1. Mass spectra of time slices from the maxima of two UV-detected peaks in a liquid chromatogram of a 24-hour ethanol extract of American White Oak. The spectra and their corresponding chromatogram peaks were assigned to the ethanol adducts of castalagin and vescalagin (top and bottom). Structures of these two compounds are inset.

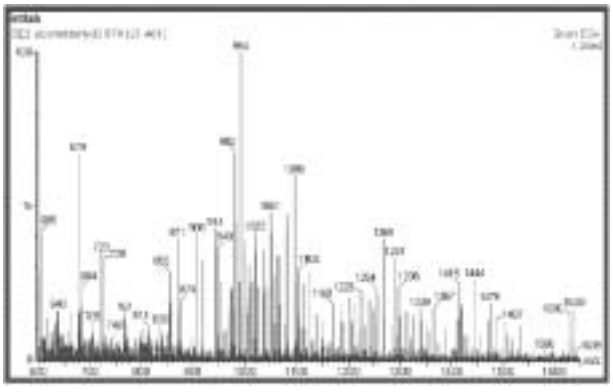

Figure 2. Mass spectrum from a time slice of a chromatogram from the purification of bovine liver alcohol dehydrogenase. The UV-detected peak corresponded in retention time to purchased alcohol dehydrogenase. The mass spectrum shows the multiple-charge envelope. The MS also corresponded to that from a known, pure sample.

an issue with which practicing chemists deal, but it is not learned with clean, or preprepared, samples. In addition, a tremendous amount of trial and error with instrument parameters was required just to get a decent signal from any tannins. In the process, the students discovered that an in-depth knowledge of the instrument and considerable thought applied beforehand is a preferable substitute for brute force trial and error. There are simply too many different combinations and interrelations in the settings to map the parameter space.

Caffeine is anthropogenic, except in geographical regions where caffeine is synthesized in plants. As a consequence, its use as a marker for waste in watershed downstream from waste treatment plants has been proposed. In the experiment here, students collected samples from a local creek upstream and downstream from an entering tributary and from the tributary. The samples were extracted by solid phase extraction and stored. A second group developed an LC–electrospray ionization single-ion MS method for caffeine determination, primarily because we anticipated that the sensitivity of LC-UV would not be sufficient. The biggest problem the students encountered was varying calibration sensitivity on a day-to-day basis, which entailed learning the (somewhat complex) set of conditions that set sensitivity for the LC-MS. As Table 1 shows, once students mastered the instrument, they obtained excellent precision, and the accuracy of the match between caffeine mass flow entering and exiting the confluence of the streams was high.

Alcohol dehydrogenase was isolated from bovine liver as part of a long, complicated, multi-team effort. The focus here is on LC-MS of the purified enzyme. Figure 2 shows the mass spectrum. The important point here is that these kind of data are quite complicated and require comparison with standards, data manipulation and analysis, and high-quality technique for an optimal, reproducible signal. Even with that kind of effort, students find out that sometimes your best guess is the best you can do. In fact, for this particular spectrum, the quality of the enzyme was pretty poor because of some corners that were cut during purification. The students found out that familiarity with the instrument was the only way to get quality spectra. They also found out that one try is never enough when working with real samples. Finally, students also learned that sometimes there is no answer to the question, "Why isn't it working?" except, "Devise experiments to find out."

On the whole, the experience for the students seems to be quite positive. Feedback in the form of student and TA questionnaires has established, with moderate certainty, that the students definitely feel a sense of project ownership when they are forced to put so much time into learning and using advanced instrumentation. For many students, this is reinforced if they perceive the instrument to be modern and rel-

Table 1. Concentrations and Mass Flow Rates from Caffeine Extracted from Lick Run and from Peter's Creek Above and Below the Confluence of Peter's Creek and Lick Run

Sample	Concentration of sample (μM)	Mass flow rate (g/min)
1: Peter's Creek below Lick Run	9.567	0.1105
2: Peter's Creek above Lick Run	3.461	0.0304
3: Lick Run tributary	18.520	0.0810

Mass flow rates were calculated from average flow velocity data.

evant. It is not at all clear that they perceive the issue of interdisciplinary research, but means either that they do not "get it" or we have done the job so well that disciplinary boundaries were never there in the first place.

Teaching the students to master the instruments, in terms of basic operation, is quite easily done. Most students master their instruments pretty quickly. Interestingly, they seem to be the ones most nervous about breaking the instruments, though in fact they almost never do anything that causes serious harm (it is usually graduate students who do that). By the end of the first semester, typically, students have learned enough that they are comfortable with getting on a new instrument or going and using one they have used before without significant supervision. By the end of the year, they have learned, for the most part, properties of most of the instruments to the point where they can, when faced with a new problem, decide which instrument to use—or at least what questions to ask. It is important that the faculty and TAs be quite familiar with the instruments they are using. Students do not usually respond well to tasks that are beyond the capabilities of their instructors, and, what is more important, it is impossible for instructors to guide students to a solution, rather than telling them what to do, if they do not have a fairly profound familiarity with the instrument.

Teaching the students how to prepare for and design experiments when faced with a question originating from biology, chemistry, or in between is clearly the hardest task. They come from a culture of cookbook experiments, and many of them take time to get over the frustration of the experiment not "working" or "going right" on the first try. This situation is exacerbated by the need to sort out instrumental and procedural problems. Teaching students how to deal with daily frustrations and adjustments requires patience and good TAs. Most students actually seem to prefer the known outcome, even if the experiment itself is complicated. However, teaching them to work through problems will ultimately give them very valuable tools. Complex instrumentation usage teaches this, as do problem-based laboratories.

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