The Analysis of Orange Oil and the Aqueous Solubility of *d*-Limonene[1]

Two Complementary Gas Chromatography Experiments

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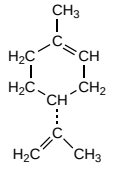
The importance of chromatographic methods in all areas of chemistry and related disciplines is indisputable, and laboratory exposure to chromatographic instrumentation is recognized as a necessary component of all chemistry degree programs. However, proper use of these techniques, especially quantitation methods, should also be taught in courses directed to majors in related sciences. For students to appreciate practical applications, it is also desirable to have a variety of experiments with real-world samples.

At the University of Florida, quantitative GC is now a component of the introductory analytical chemistry laboratory, which is heavily enrolled by majors in microbiology, engineering, food science, etc., as well as chemistry. This paper describes a new experiment for this student audience on the analysis of *d*-limonene in orange oil. Another experiment for the physical chemistry laboratory on the solubility of *d*-limonene in water is also reported. Although these are instructionally complementary, each is a stand-alone experiment that can be incorporated into other laboratory courses (e.g., instrumental analysis, organic chemistry, or as applications of food chemistry in a survey course for nonmajors.)

The Central Compound

The monoterpene *d*-limonene, (4*R*)-(+)-limonene (**1**), is the major component of all citrus essential oils. In commercial applications, limonene serves as a precursor for the manufacture of flavorings such as carvone and L-menthol, as a solvent (especially as a substitute for CFCs), as a penetrating oil, and as a carbon source for various syntheses of terpene resins (*1*). It is also used as an inhibitor of tetrafluoroethylene polymerization (*2*). This compound is ideal for use in a teaching scenario because it provides a "Florida flavor" and, although a possible irritant to skin, eyes, and mucous membranes, it has low oral toxicity. (The Low Toxic Dose to produce questionable carcinogenic effects in mice is 67 g/kg body weight administered orally over 39 weeks [*2*].)



d-limonene (**1**)

Analysis of Orange Oil

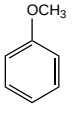
An experiment for the introductory analytical laboratory must work efficiently with relatively large student groups (18 students per section, 8 sections per week at UF). Separations must be complete within a few minutes, so that students can make multiple injections in a reasonable time. The GC columns and other necessary materials should also be inexpensive and present minimal safety concerns. At the same time, the experiment must demonstrate proper GC quantitation using the internal standardization method. For a "real" mixture, this requires finding a compound that (i) is not present in the sample, (ii) does not react with the sample, and (iii) does not co-elute with a component in the sample. For grading purposes it is also highly desirable to have a selection of similar, but quantitatively different, unknowns.

Background

These stringent demands are all met in the determination of diluted cold-pressed orange oil. In the cold-pressing process the oil from the peel is extracted with flowing water while the juice is being squeezed from the orange. After extraction the oil/water emulsion is allowed to sit for several hours, and the denser aqueous layer is drained from the bottom. The oil fraction is subsequently centrifuged to remove residual water.

Shaw and Coleman (*3*) analyzed cold-pressed oils from seven orange cultivars (one early-season, two midseason, four late-season). Their results showed that the variation in *d*-limonene content is small, with a mean and standard deviation of $(95.0_3 \pm 0.2_5)$% limonene by weight. Thus, a range of "real" unknowns can be prepared by diluting varying weights of orange oil with *n*-hexane (ca. 2% oil by weight). Students determine the weight percent of *d*-limonene in their respective unknowns and divide by 0.950_3 to obtain the wt % orange oil.[2]

The components of citrus oils include a wide variety of hydrocarbons, aldehydes, alcohols, esters, ketones, and some miscellaneous compounds (*4*). However, there are very few ethers, and in particular, anisole (**2**) has not been detected. This compound was chosen as the internal standard for the student experiment because it can be separated from both *d*-limonene and *n*-hexane on a 5-m megabore (0.53 mm) open-tubular column with bonded silicone (5% phenyl/95% methyl) stationary phase. These columns are provided free with the HP5890A GCs used for the analysis. Although some co-elution undoubtedly occurs, the minor components are buried in the baseline with the integrator setting that keeps the limonene and anisole on scale. Anisole also has the advantage of limited toxicity (LD_{50} = 2800 mg/kg in mice).



anisole (**2**)

Experimental Conditions

To perform the analysis, a set of five standards with limonene/anisole (L/A) weight ratios varying from about 0.5 to 2.0 must be prepared, as well as a solution containing

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known weights of the unknown and anisole. Each student is provided with a set of empty 1-dram vials, plus three vials separately containing the unknown and standard solutions (each ca. 0.02 g of compound per gram of solution) of limonene and anisole in hexane. (In small classes it may be feasible to have students prepare the standards themselves.)

Preparation of the mixed standards and unknown requires especial care to prevent solvent loss during the procedure. (Postpreparation loss should not be a concern because the L/A ratios do not change if only the solvent volatilizes.) To avoid problems with pipetting volatile materials, all mixtures are prepared by weighing the appropriate solutions. Students are instructed to line the vial caps with foil. At the analytical balance they tare six capped vials and use a Pasteur pipet to add approximately 0.1 g of the anisole standard to each, taking care to keep vials capped as much as possible. To five of the vials they then add weights of limonene standard needed to span the target range of weight ratios. A known weight (ca. 0.1 g) of the orange oil unknown is added to the sixth vial.

The laboratory is equipped with three HP5890A GCs, each with dual thermal conductivity detectors and integrators.[3] The columns described above are operated isothermally at 60 °C, so that students working on the same GC do not have to coordinate injections with temperature programs. The injectors and detectors are held at 250° and 300°, respectively. A typical chromatogram is shown in Figure 1. Although retention times vary depending on the student's actual column, the anisole elutes immediately after the hexane tail (t_R ~ 1.5 min), and the limonene peak is well separated with a t_R of about 3 min. Students inject 1-μL samples of each pure standard (to assign retention times), each mixed standard, and the mixed unknown, for a total instrument time of ca. one-half hour. If time allows, students should be instructed to inject the unknown several times and to calculate the average result and standard deviation. Results are best if all solutions are prepared and chromatographed during the same laboratory period. A full section of 18 students can finish the experiment in one 3-hour laboratory period, but it is less stressful if two weeks are allotted, with half the students working each week.

The section on chemical separations in the introductory analytical lecture course is often not encountered until the end of the semester. To provide background information, the laboratory manual includes chapters on chromatographic principles, GC instrumentation, and the particulars of the orange oil analysis. This material is also presented with suitable demonstrations in a videotape produced by the instructor and shown before the experiment.

Data Analysis and Results

Many texts present the internal standard method, but usually as a single-point calibration. In this experiment students are required to construct an internal standard calibration plot, and the instructions in the laboratory manual include a rather detailed explanation of the data analysis. Plots (e.g., Fig. 2) are routinely very linear, and there is little scatter in the points. In their reports, students are required to discuss the advantages and limitations of the internal standard method. Having experienced the usual difficulties with 1-μL injection volumes, they readily appreciate the importance of the ratioing procedure.

Students use the least-squares equation from the internal standard plot to calculate the limonene/anisole weight ratio, R, in the mixed unknown. The weight percent orange oil in the original unknown is subsequently calculated from the weights of the anisole standard and the orange oil sample (w_A and w_{sam}) in the mixed unknown, the concentration of the anisole standard, C_A, in grams anisole/gram solution, and the known *d*-limonene content of orange oil, according to the relationship

$$\text{wt \% oil} = \frac{w_{oil}}{w_{sam}} \cdot 100 = \frac{R \cdot w_A \cdot C_A \cdot 100}{0.950_3 \cdot w_{sam}} \quad (1)$$

For a typical class, the median result has a relative error of about 3%. When the experiment was first developed the results were much less accurate. The cause of the problem was improper storage of the standards and samples before they were given to the students. Now these solutions are prepared within a few days of the scheduled laboratory sessions, and all materials are kept in the refrigerator except when being dispensed.

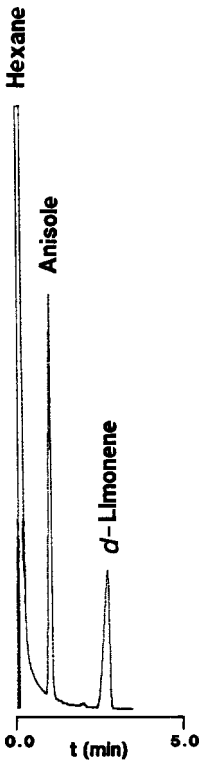

Figure 1. Typical gas chromatogram of ca. 1% orange oil in *n*-hexane with anisole added as the internal standard. A Hewlett-Packard 5 m × 0.53 mm open-tubular column of bonded silicone (5% phenyl/95% methyl) was operated isothermally at 60 °C. The injector and detector temperatures were 250 and 300 °C, respectively.

Solubility of *d*-Limonene

Because of its varied uses, knowledge of the physical properties of *d*-limonene is important both commercially and in fundamental studies. The GC procedure already described has been expanded for use in a physical chemistry experiment on the aqueous solubility of this compound. The method, based on the work of Massaldi and King (*6*), involves analyzing the headspace vapor in equilibrium limonene/water mixtures. Although the experiment is designed as a fundamental study of equilibrium properties, it also gives students experience in headspace sampling, a technique in many environmental monitoring procedures.

Background

If Henry's law is obeyed, the partial pressure of limonene in the headspace gas, P_L, is equal to $K_{L(aq)} \cdot X_{L(aq)}$ below the solubility limit, where $X_{L(aq)}$ is the mole fraction of limonene in water, and $K_{L(aq)}$ is the Henry's law constant. At saturation, a limonene-rich phase separates from solution, and, since the solubility of water in limonene is very small, P_L becomes equal to P_L^0, the vapor pressure of pure limonene at the system temperature. Thus, a plot of limonene vapor pressure (or proportional quantity) versus $X_{L(aq)}$ should have two linear regions (positive slope before saturation; near-zero slope after saturation), which intersect at the saturation limit. In this experiment, the concentration of limonene in the aqueous phase is so small that linear plots are also achieved using the total amount of added limonene, expressed conveniently using the added volume, V_{add}, as the abscissa.

The total number of moles of limonene in the system, n_{LT}, is distributed between the aqueous and vapor phases. Using the ideal gas result that the mole fraction of limonene in the vapor, X_{LV}, is the fraction of the total pressure due to

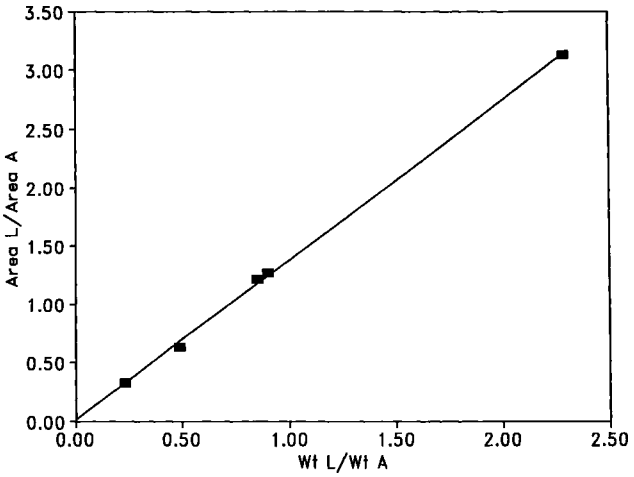

Figure 2. Typical internal standard plot. In this example, the least-squares slope and intercept are 1.37 and 0.013, respectively.

limonene, P_L/P_T, the following relationship holds:

$$n_{LT} = n_V \frac{P_L}{P_T} + n_{aq} \cdot X_{L(aq)} = \frac{P_L V_V}{RT} + n_{aq} \cdot X_{L(aq)} \qquad (2)$$

where n_V and n_{aq} represent the total number of moles in the vapor and aqueous phases, and V_V is the total volume of the headspace vapor. Thus, when the saturation limit (designated by *) is reached,

$$X^*_{L(aq)} = \frac{n^*_{L(aq)}}{n_{aq}} = \frac{n^*_{LT} - \frac{P^0_L V_V}{RT}}{n_{aq}} \qquad (3)$$

Because n_{aq} is essentially equal to the number of moles of water, there is a direct proportionality between n_{aq} and the volume of water, V_{aq}. Thus, the molar solubility of limonene can be written as

$$[L]^* = \frac{n^*_{L(aq)}}{V_{aq}} = \frac{n^*_{LT} - \frac{P^0_L V_V}{RT}}{V_{aq}} = \frac{n^*_{LT} - n^*_{LV}}{V_{aq}} \qquad (4)$$

where n^*_{LV} is the number of moles of limonene in the saturated vapor.

Experimental Conditions

Students are given a set of 12 matched 250-mL Erlenmeyer flasks (same lot) with average total volume (determined by the instructor by filling each flask to the lip with water and weighing) equal to 274 mL and 95% confidence limits of ± 4 mL. After adding 150 mL of water[4] to each flask, students add the following volumes (μL) of limonene to successive flasks: 0.3, 0.5, 0.7, 1.0, 1.5, 2.0 with a 1-μL syringe; 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 with a 10-μL syringe. A 10-μL aliquot of anisole (above the saturation limit for this compound) is also added to each flask. The internal standard helps to eliminate some of the chromatographic uncertainties, but, as discussed below, unnormalized limonene peak areas must be used as part of the data analysis.

The flasks are sealed with aluminum foil and Parafilm. To help prevent the foil from tearing during later headspace sampling, a piece of tape is placed across the top of the foil. The sealed flasks are then equilibrated for 2 h in a 25 °C shaker bath borrowed from the biochemistry laboratory. During the waiting period, the GC is calibrated for measurement of the vapor pressure of pure limonene. Students prepare a standard limonene solution (0.25 g accurately weighed and diluted with *n*-hexane in a 10-mL volumetric flask), and make at least four 1-μL injections to achieve an average calibration value.

At the end of the equilibration period, a gas-tight syringe is used to withdraw and inject a 2.00-mL aliquot of the headspace vapor from each Erlenmeyer flask. The needle hole is immediately covered with a second piece of tape, in case a second injection is needed. Peak area data are obtained for both limonene and anisole. After satisfactory GC data have been acquired, the flasks are cleaned with sequential acetone/hexane/acetone/water rinses and dried in the oven.

Data Analysis and Results

As described above, a plot of limonene vapor pressure versus $X_{L(aq)}$ (or X_{LT}) should have two linear segments that intersect at the saturation limit, but the same information is obtained by plotting GC peak area directly versus V_{add}, the volume of limonene added to each flask. To help control uncertainties due to injection volume, each limonene peak area is divided by the corresponding anisole area, and these area ratios are plotted, as shown in Figure 3. Students use least squares analysis to evaluate the two straight-line equations, and then equate them to calculate the saturation volume of added limonene (and its propagated uncertainty). The known density (0.84 g/mL) and formula weight (136 g/mol) of limonene are used to calculate the number of moles at saturation, n^*_{LT}, and the vapor pressure of pure limonene. The average peak area per mole of limonene, obtained from the data for the standard limonene solution, is used with the average limonene peak area (*not* the limonene/anisole area ratio) in the saturation region to calculate the number of moles of limonene vapor at saturation, n^*_{LV}. Equation 4 is then used to calculate the molar solubility in water, which is compared to the value in ref *6*. For the data shown in Figure 3 the solubility is $(6.3 \pm 0.6) \times 10^{-5}$ M, compared to the literature value of $(9.9 \pm 0.2) \times 10^{-5}$ M. Systematically low results are not uncommon. Presumably, this is due to poor equilibration of the liquid and vapor phases. The Erlenmeyer flasks must be shaken consistently during the experiment to avoid this problem.

The Henry's law constant, $K_{L(aq)}$, for limonene in water can be obtained from values of the vapor pressure and the mole fraction, $X_{L(aq)}$, of limonene in the aqueous phase before reaching the saturation limit. These two parameters are given by

$$P_L = \frac{(A_L/A_A)}{(A_L/A_A)^*} \cdot P^0_L \qquad (5)$$

and

$$X_{L(aq)} = \frac{n_{LT} - \frac{(A_L/A_A)}{(A_L/A_A)^*} \cdot n^*_{LV}}{n_{aq}} \qquad (6)$$

where (A_L/A_A) is the least-squares value of the limonene/anisole area ratio for each sample in the presaturation region,

$(A_L/A_A)^*$ is the average area ratio after saturation, and n^*_{LV} is the average number of moles of limonene in the saturated vapor. The Henry's law constant could be evaluated from the slope of a P_L versus $X_{L(aq)}$ plot. However, (A_L/A_A) and n_{LT} are both related to the volume of added limonene, V_{add}, via the least-squares equation obtained previously, and the density, ρ, and formula weight, M, of limonene. Using appropriate substitutions, it can be shown that the value of $K_{L(aq)}$ is given in simple form by

$$K_{L(aq)} = \frac{P_L^0 \cdot m \cdot n_{aq}/\left(A_L/A_A\right)^*}{\rho/M - n^*_{LV} \cdot m/\left(A_L/A_A\right)^*} \qquad (7)$$

where m is the least-squares slope of the (A_L/A_A) versus V_{add} plot. The P_L^0 result is usually low, but for internal consistency the experimental value should be used to calculate $K_{L(aq)}$. A value of $(5.5_5 \pm 1.5_2) \times 10^5$ torr was obtained using the data shown in Figure 3.

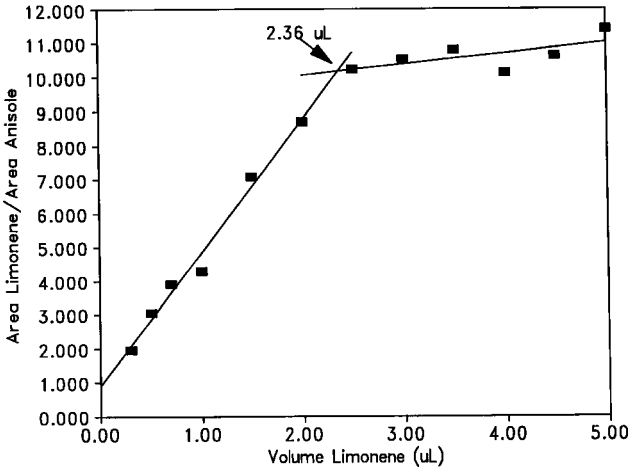

Figure 3. Plot of normalized limonene peak area (Area Limonene/Area Anisole) versus volume of limonene added. The straight lines intersect at the saturation limit.

Conclusion

The analysis of orange oil and the determination of the solubility of *d*-limonene are complementary, but stand-alone, experiments. Both are of interest to a variety of student audiences, and the instructor can alter the complexity of the data analysis (e.g., error analysis requirements) to suit the desired level of difficulty.

Acknowledgment

We thank Robert J. Braddock, Citrus Research and Education Center, Lake Alfred, FL, for his helpful suggestions and information about the cold-pressing process.

Notes

1. Presented at the Annual Meeting of the Florida Sections of the American Chemical Society, Orlando, FL, May 1994 and the Annual Meeting of the Southeast Association of Analytical Chemists, Tallahassee, FL, September 1994.

2. Although it would be desirable to have students obtain orange oil directly from the fruit, the cold-pressing procedure requires special equipment, and the maximum yield is less than 1 g per orange. Valencia oil with the composition given by ref *3*, as well as distilled *d*-limonene, may be purchased from *Tastemaker*, 4705 US Highway 92 East, Lakeland, FL 33801 (941/665-1040).

3. University of Florida students who continue to Instrumental Analysis use FID, ECD, and hyphenated MS detectors, as well as temperature and pressure programming, in that course.

4. The 150 mL of water may be added by pipet (e.g., 3×50 mL), but a graduated cylinder is adequate and requires much less time. Detailed error analysis shows that use of a graduated cylinder is not the major source of uncertainty in this experiment. If a pipet is used, an aspirator should be provided to aid in drawing the liquid above the line.

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