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How to grow crystals for X-ray crystallography

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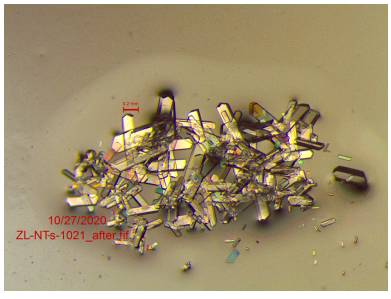


Growing high-quality crystals remains a necessary part of crystallography and many other techniques. This article tabulates and describes several techniques and variations that will help individuals grow high-quality crystals in preparation for crystallographic techniques and other endeavors, such as form screening. The discussion is organized to focus on low-tech approaches available in any laboratory.

1. Introduction

Sample preparation is a key aspect of any analytical technique. In X-ray crystallography, this means growing crystals of sufficient size and quality to fit the instrument and the technique. A modern instrument can harvest diffraction data from needles as thin as 20 microns. Instruments of an earlier vintage may need crystals with dimensions closer to ten times that. Neutron diffraction experiments require crystals still larger. In all cases, the quality of the crystals grown is a deciding factor for success. This article presents techniques for growing high-quality crystals for single-crystal X-ray diffraction (SCXRD). The focus is on techniques available in nearly any chemistry laboratory doing organic/organometallic chemistry. Many of these will be well-known conventional approaches. There is also a mention of more systematic approaches that offer high-throughput screening for industrial applications. Comments about refinement to these techniques are born of experience chasing crystals of uncooperative samples that only eventually yield diffraction-quality crystals. There are myriad variations on the themes below. Consider this an outline, with tips and tricks and resources that might further your efforts in pursuing great crystals. Also available are numerous books (Holden & Morrison, 1982), papers (Müller, 2009; Etter *et al.*, 1986), and websites with contributions from respected crystallographers that offer advice for crystal growth (Boyle, 2023; Blake, 2024; Mueller, 2024; Dinger & Klosin, 2015).

The time, effort, material, and resources needed to grow crystals ranges widely depending on the compound, from 'minimal' to 'impractically high' on all four fronts. So why do this? Why advocate for growing crystals on fast-moving projects where material is precious and resources may be limited? The answer is the same as always: unambiguous structural characterization moves projects along, keeps them on track (or gets them back on one), and provides insight to the chemical process. X-ray analysis of diffraction-quality crystals can unlock a project's potential from almost any point in its cycle: from early confirmation of regiochemistry or stereochemistry in starting materials, to the 'cherry-on-top' structure at the end of a multi-step synthesis. In the middle, crystal structures of isolated intermediates support and direct

synthetic routes and mechanistic studies, while also giving process-relevant details for later scale-up. Crystallization is still favored today by industry as a purification technique because it is scalable. It also offers myriad choices for selecting the form that is isolated – a key factor in the pharmaceutical field. Either from a user or facility manager point of view, getting the best data possible is most important. Users depend on the facility to advance their projects, careers, hopes, and dreams. The crystallographer needs to minimize the required instrument time and refinement effort to make the structure publishable. This frees up resources for more samples and experiments, and deepens the relationship between the users and the facility. The crystal-growing effort is also an opportunity to collaborate openly on the project. The most value from the crystal-growing effort may be seen when the chemical drawing of the proposed structure *might not be* the structure of the actual material. And all parties must keep an open mind about what is actually in that vial. This fosters a culture that benefits everyone involved. The resources needed to start growing crystals are as little as a few milligrams of material and a weekend of quiet evaporation. So why not do this?

2. Options to consider

2.1. Chemical assessment

A crystallization begins with an assessment of the compound's solubility and chemical stability. Is the material air/water sensitive? Is it thermally stable? Does the reactivity of the compound preclude the use of certain solvents? Many of these answers are usually known by the time a crystallization is undertaken. If not, they are readily addressed with a few qualitative tests.

2.2. Solubility profile

The most common medium for growing crystals is liquid solution. It is also the most convenient approach, requiring the least equipment and infrastructure. A first step here is to collect information about the solubility of the material in question. It is also critical at this point to have a chemical context for the compound of interest. Look at the reaction steps leading up to it, and keep in mind that impurities such as solvents or by-products from previous chemical steps can be carried through the process despite careful work and good separation technology. A simple chemical drawing can be highly useful in making solvent choices. Based on the functional groups present, some chemical reasoning can be used to make rational choices about where to start. A 'like dissolves like' approach is reasonable. Nonpolar compounds, or those with significant nonpolar regions, may be highly soluble in ethers or aromatic solvents. Polar protic compounds will likely need polar protic solvents to get them into solution. Other factors to be considered are molecular shape and the presence of hydrogen bonding. Large flat molecules, and systems with extensive intermolecular hydrogen bonding, often have lower solubility in common solvents. Fig. 1 shows a typical solubility profile at the bench. *N*-(*o*-Tolyl)acetamide demonstrates a range of solubilities as the polarity/polarizability of the solvent increases.

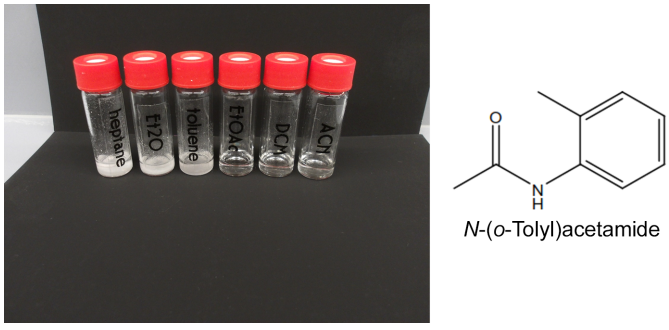


Figure 1
The 'line-of-vials' approach to solubility testing for 20 mg samples of analyte in 0.5 ml of solvent. From left to right: heptane, diethyl ether (Et_2O), toluene, ethyl acetate (EtOAc), dichloromethane (DCM), and acetonitrile (ACN).

At the bench, a good tactic is to assemble a line or grid of vials containing a small amount of material. This is as simple as scooping some material into a vial, adding a few drops of solvent, swirling the vial, and observing. Starting with solvents that evaporate quickly allows easy recovery for further tests before progressing to polar solvents until significant solubility is observed. The range of solvents used in this process will inform decisions about crystallization protocols. The choice for this range will vary by analyte. Solvents like dimethylformamide (DMF), dimethyl sulfoxide (DMSO), and water may be reserved for cases when nothing else works because they are difficult to remove.

3. Techniques

This section organizes crystallization techniques with an eye toward the economical use and re-use of material. Observations from the solubility tests can guide decisions about what solvents and techniques to try. Comments for each technique include a description of the procedure, discussion of limitations/risks, and how to avoid common mistakes.

3.1. Slow evaporation

Sometimes, making a solution and leaving the cap off produces a viable batch of crystals that diffract sufficiently for SCXRD. This technique can directly follow the solubility screening by simply walking away. The real advantage here is that slow evaporation requires minimal effort. Other examples of this technique include:

– NMR tubes left in the back of the hood over a holiday.

– A vial with a loose cap placed on a quiet shelf. Or a sealed septum vial with a needle inserted.

– Schlenk line apparatus with a slow purge of inert gas.

– High-throughput nanodrop evaporation under oil (Tyler *et al.*, 2020).

There are some drawbacks to the slow evaporation approach. If experiments go to dryness before the crystals are discovered, harvesting the crystals by scraping the vial may

degrade or destroy them. Solvated crystals may lose their interstitial solvent, resulting in poor diffraction. The compound of interest may decompose over time due to oxygen or water vapor in the laboratory, or other chemical pathways. In addition, loose solvent vapor from slow evaporation is unwelcome in the environment. Good technique, knowledge of reactivity, and monitoring the progress can decrease the risks associated with slow evaporation. Evaporation experiments can also be set up in sealed chambers by using the vapor-diffusion concepts discussed later.

3.2. Slow cooling

Slow cooling exploits the change in solubility as a function of temperature. In the case of the example compound in Fig. 1, toluene would be a good choice. A large ΔT between ambient and boiling temperature typically translates into a greater recovery of solid when the mixture returns to room temperature. Heat a slurry until a clear solution is obtained. It is useful to have a reserve of heated solvent available to add if needed. Don't skimp on the size of the vessel, as it may take more solvent than anticipated. Remove the heat source and allow the solution to cool slowly in an insulated container. If low-boiling solvents are used, cool a saturated solution in the freezer. Some examples of this technique:

– Beaker of solution on a hotplate. Turn off the hotplate and let stand to cool.

– Place a vial of hot solution inside a vacuum jacketed container. Pack with glass wool. Refrigerate.

– Run a Soxhlet extractor to make a concentrated solution. Remove the solvent vessel, seal it, and place it in a vacuum flask, cover with glass wool and allow to cool.

– A thermostat-controlled oven with software to design temperature ramping and cycling strategies.

Some drawbacks to the slow-cooling technique are obvious. Heating flammable solvents is inherently dangerous and should be done with proper safety measures in place. Cooling flammable solvents requires a refrigeration unit that is suitably safe for that purpose (*i.e.* 'explosion proof') and vials should be well sealed. Thermally-sensitive compounds may have a limited temperature range available. It sometimes requires very slow cooling to produce X-ray-quality crystals of sufficient size. Dissolution of some materials might be slow, and an impatient person can end up with too much solvent. In this case, the experiment becomes slow evaporation. Or the sample is returned to the rotovap for reduction and restart.

Slow cooling is a simple, accessible, and effective way to grow X-ray-quality crystals for many compounds. There are easy ways to use basic equipment to make effective setups. There are also advanced systems (Technobis, 2023) to control temperature and other conditions so that complex crystallization protocols can be developed. A variation of slow cooling that deserves mention is thermal cycling. This moves the system back and forth across the boundary of supersaturation. As a result, crystals are ripened to larger size and sometimes better quality (Mullin, 2001).

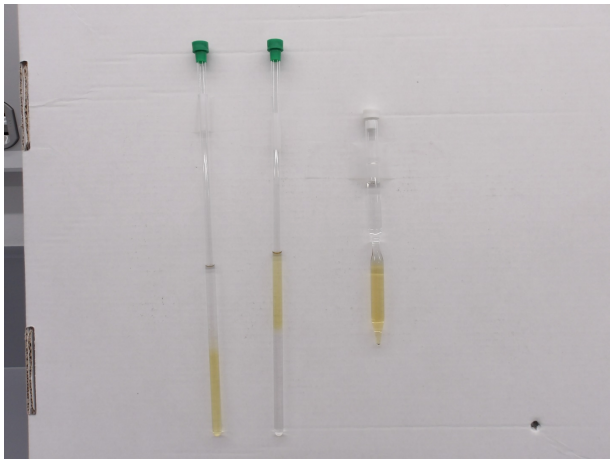


Figure 2
NMR tube prepared for crystallization by (left) layering heptane onto a yellow THF solution, (middle) layering a yellow THF solution onto dichloromethane counter-solvent and (right) layering solution/counter-solvent in a lab-built constriction tube made from a Pasteur pipet.

3.3. Layering

Solvent layering relies on the solubility difference of the analyte in two solvents. A typical setup starts with a solution of the material in a polar solvent. A (usually less polar) counter-solvent is gently layered on top of the solution. The vessel is capped, and diffusion slowly moves one layer into another. This lowers the polarity of the system to the point that precipitation begins, hopefully producing crystals. This is best done in a narrow vessel like an NMR tube or capillary. The choice of solvent/counter-solvent pairing is critical. Planning is needed to ensure the denser liquid is the bottom layer. The typical arrangement is solution on the bottom and counter-solvent on top. Miscible liquids from opposite ends of the 'line-of-vials' are good choices for this technique. This method is gentle, relatively quick (1–5 days), simple, and inexpensive. Example setups are shown in Fig. 2.

There are several pitfalls related to the layering approach. Mixing outside of diffusion is the enemy. Even holding the tube in hand cause enough temperature fluctuation to produce unwanted stirring. Sometimes mixing goes too quickly and only tiny crystals or amorphous precipitate are recovered. Layering on a counter-solvent requires some skill. These challenges can be addressed with some simple strategies: a layer of 'blank' solvent can be added to the tube before the counter-solvent is layered. This helps prevent disturbing the layer of solution in the bottom. Another approach is to flash freeze the lower solution in liquid nitrogen, then add the counter-solvent layer on top of the solid. It is also possible to make tubes with a tapered middle, which slows mixing and prevents disturbing the lower layer during setup.

3.4. Mixed evaporation

Evaporation of a mixed-solvent solution offers the simplicity of slow evaporation with some insurance against the sample drying out. A low-boiling solvent is paired with a higher-boiling counter-solvent, and slow evaporation is

Table 1
Examples of solvent pairs useful for mixed evaporation crystallizations.

Solvent (b.p. in °C)	Counter-solvent choices (b.p. in °C)
Dichloromethane (35)	Heptane (98), toluene(110), *n*-butyl acetate (126)
Tetrahydrofuran (66)	Water (100), toluene (110)
Acetone (56)	Water (100), toluene (110)

allowed to progress. As the volume of solvent decreases, the solubility of the analyte in the vial drops. The high-boiling counter-solvent ensures that the mixture does not go to dryness. Mixed evaporation does not require the dexterity needed for layering. A good starting ratio is 2:1 solvent–counter-solvent. If needed, more solvent can be added, knowing that it will evaporate later. Table 1 lists a few handy solvent pairs for mixed evaporation with boiling points in °C.

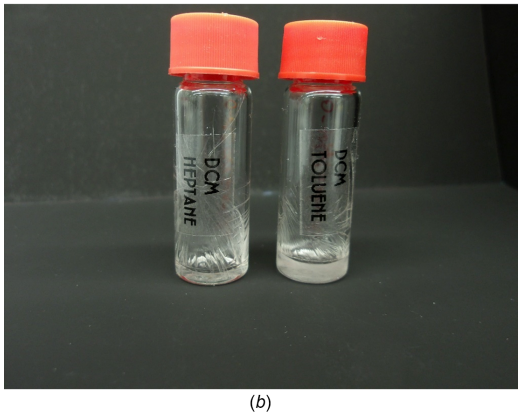
This approach is well suited for an intentional 'first try'. The DCM/heptane and DCM/toluene pairings, shown in Fig. 3, typically finish in 24 to 48 h. As little as 10 mg can permit three or four tests in the first set of vials. An additional advantage is that the material and vials themselves are generally easy to reuse for a next set of solvents.

Mixed evaporation works best with a large difference in boiling point between solvent and counter-solvent. This approach limits the choice of high-boiling liquids to those that are also poor solvents for the material. Materials that require polar high-boiling solvents like DMF or DMSO are less well suited to this approach. Whenever other crystallization attempts are combined in a larger vial to recover material, a mixed evaporation experiment is inadvertanlty started. Documentation and reproducibility become difficult when this mixture turns out to be where the crystals grow.

3.5. Vapor diffusion

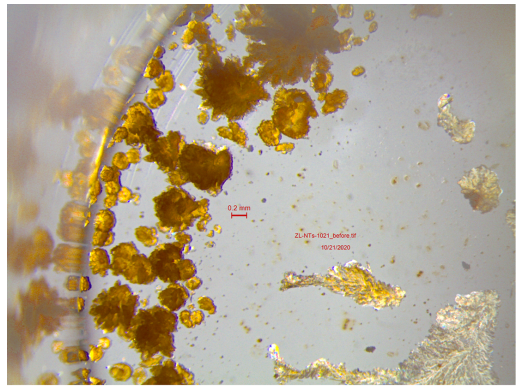
Vapor diffusion is perhaps the gentlest and most versatile approach to crystal growth that is readily available in a standard chemistry laboratory. The analyte is dissolved in the less-volatile solvent and paired with a volatile counter-solvent. The pair should be miscible, with the analyte having a much lower

(*a*)



(*b*)

Figure 3
(*a*) Mixed evaporation crystallizations using 20 mg of *N*-(*o*-tolyl)acetamide in 0.5 ml DCM, with 0.2 ml of counter-solvent added. The caps are loosened 1/4 turn. (*b*) t = 24 h.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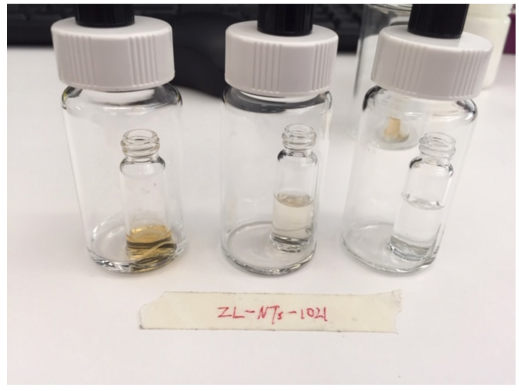

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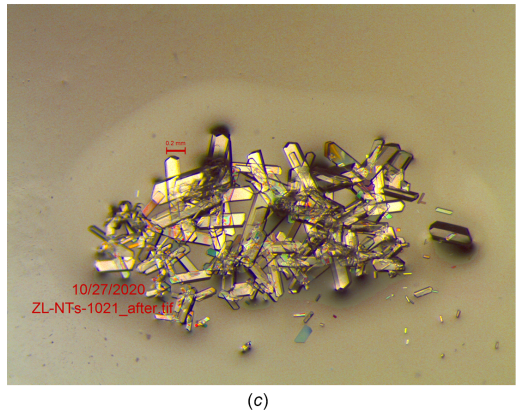

(*c*)

Figure 4
(*a*) Before, (*b*) during, and (*c*) after pictures of successful vapor-diffusion crystal growth. The solvent/antisolvent pair was ethyl acetate/pentane.

Table 2
Some common solvent/counter-solvent pairs useful for vapor-diffusion crystallzations.

Solvent (b.p. in °C)	Counter-solvent choices (b.p. in °C)
Ethyl acetate (77)	Pentane (36)
Acetonitrile (82)	Diethyl ether (35), pentane (36)
Methanol (65)	Dichloromethane (35)
Water (100)	Ethanol (78)
DMF (153)	Diethyl ether (35)
DMSO (189)	Water (100)

solubility in the counter-solvent (from opposite ends of the line-of-vials in Fig. 1). The experiment is arranged as nested vials. The inner vial contains the analyte solution and the outer holds a reservoir (the 'pool') of the counter-solvent. There should be headspace available for vapor exchange to happen. As the counter-solvent vapor dissolves into the solution, the solubility of the analyte decreases. This continues until the solvent is saturated with counter-solvent, or the pool is completely transferred to the inner vial. The timeframe depends widely on solvent choice and conditions. Diethyl ether and pentane will transfer overnight, while higher-boiling counter-solvents take more time. Fig. 4 shows microscope images of before and after, as well as the setup at various stages of vapor transfer.

Vapor diffusion tends to work best starting with a saturated solution of material. A dilute starting solution may mean that nothing precipitates. It is also important not to overfill the inner vial. Otherwise, it may overflow when counter-solvent transfers in. Reproducibility can be a challenge without careful portioning of the material and solvents. Some recommendations are listed in Table 2.

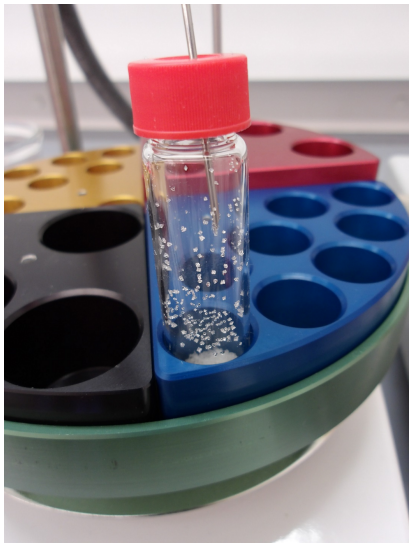

Figure 5
Crystallization of adamantane by sublimation in a septum-top vial. The temperature was maintained at 65 °C using a hotplate and the vacuum held at ∼200 mbar (1 bar = 10^5 Pa) using house vacuum and a syringe needle pushed through the septum. Sublimed crystals are seen on the vial walls.

There are many more options for vapor-diffusion conditions (Spingler *et al.*, 2012). For example: (i) if a crystallization using MeOH/DCM crashes out, giving microcrystals, then charge the pool with 1:1 MeOH–DCM instead of neat DCM. This will decrease the driving force for transfer and provide a gentler approach to a supersturation in the inner vial. (ii) If slow evaporation of DCM leaves only desolvated crystals trapped in a crust, slow evaporation can be equilibrated before dryness by setting the DCM solution in a vapor chamber with *just the right amount* of methanol (or a DCM/MeOH mix as above) in the 'pool'. (iii) If crystallization of a particular form requires just the right relative humidity, the vapor chamber can be charged with salt solutions to regulate that variable. Biochemists have numerous techniques to explore a vast landscape of conditions for vapor-diffusion crystallizations (McPherson & Gavira, 2014). There are also many commercial products to control vapor-diffusion conditions.

3.6. Sublimation

Sublimation can be a good option for some compounds as a purification and crystal-growth method. It also offers a good way to grow crystals that are unlikely to be solvated forms. The variable of solvent choice is replaced by the combination of pressure and temperature. Thermal stability of the analyte is an important consideration. Some materials like iodine, phenol, and ferrocene can grow dramatically large and beautiful crystals. Many other materials grow preferentially into very thin needles and plates. The setup can be as simple as a flask or test tube on a hot plate or as elaborate as a specialty apparatus built for vacuum sublimation. Fig. 5 shows a simple arrangement used to crystallize adamantane by sublimation.

3.7. Dealing with oils

It is common in organic chemistry that new molecules are first isolated as oils even after careful purification. Residual solvent, stopcock grease, or other contaminants may be the issue. If drying under vacuum doesn't help, stirring the oil with a small amount of hexanes or ether may extract these adulterants, and yield something that can be recrystallized. This doesn't always work. But it is worth trying something like this to see if the material can be isolated as a solid.

3.8. Salts and cocrystals

When sufficiently good specimens are not available from the above techniques, a salt or cocrystal of the desired material may afford better crystals. This topic is larger than the scope of this article (Quere & Wouters, 2011; Wouters & Quere, 2011). Suffice it to say that these represent their own chemical endeavor that deploys an understanding of molecular structure and intermolecular interactions that may result in achieving large high-quality crystals suitable for SCXRD. There are also techniques deploying chaperone molecules (Virovets *et al.*, 2021), complexing agents (Etter & Baures, 1988), and molecular networks (Fujita, 2021; Carroll & Coles, 2023) that may also yield SCXRD-quality crystals containing stubborn molecules.

4. Conclusion

Beyond the structural insights gained from crystallography, other benefits come from the work put into crystallization. Notably, documenting the chemical behavior in multiple systems (solubility, stability, *etc*.) is of use for advancing the project or program. For uncooperative samples that 'never' crystallize, the question is 'When do I give up and move on?'. There is always another thing to try, right? This can only be answered by, and with support from, the team. Regardless of the outcome of crystallization attempts, the discussion, collaboration, and hands-on experience built around the effort unlocks another benefit – comaradery with others in the field which gives strategies, options, and encouragement for making a regular repeating habit of growing great crystals. Good hunting.

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