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Simple and efficient modifications of well known techniques for reliable growth of high-quality crystals of small bioorganic molecules

Denis A. Rychkov,[a,b]* Sergey G. Arkhipov[b,a]* and Elena V. Boldyreva[b,a]*

[a]REC-008, Novosibirsk State University, Pirogova 2, Novosibirsk 630090, Russian Federation, and [b]Institute of Solid State Chemistry, Siberian Branch of the Russian Academy of Sciences, Kutateladze 18, Novosibirsk 630128, Russian Federation. Correspondence e-mail: rychkov.dennis@gmail.com, arksergey@gmail.com, eboldyreva@yahoo.com

A number of modifications to traditional techniques are suggested in order to overcome problems that frequently arise when growing crystals from solution. These improvements, and their combination, help to avoid problems such as poor nucleation, the spontaneous precipitation of many poor-quality small powder-like crystals, crystals adhering to the crystallization vessel or to each other, and chemical degradation of the solution. The proposed techniques can be used to crystallize desirable metastable polymorphs reliably. None of the suggested methods demands the usage of any special or expensive equipment, or specific skills, and they can be implemented in the chemistry curriculum even at secondary school level. Examples are given for the crystallization of small organic molecules such as carboxylic acids, amino acids, pharmaceuticals *etc.*, but the same techniques are applicable to other classes of compound.

1. Introduction

Since the introduction of X-ray diffraction for structure determination, there has been a pursuit, by both academia and industry, to improve the quality of the collected data. This has led to innovations in engineering, experimental techniques, data-processing software and the methods of crystal growth. The achievements in the development of hardware and software are impressive (Moseley & De Broglie, 1962; Sheldrick, 2008; Dolomanov *et al.*, 2009), but obtaining single crystals of suitable quality remains a serious problem. Obtaining a single crystal by crystallization from solution is apparently simple, and even children are encouraged to try this technique, *e.g.* at crystal growth competitions (IUCr, 2014). However, whilst well established procedures work well for some compounds, they fail in the case of many others. Even for compounds that are readily soluble in water or another solvent, numerous problems can arise upon crystallization from solution. The most common problems include a too low or too high nucleation rate, the spontaneous precipitation of many poor-quality small powder-like crystals, crystals adhering to the crystallization vessel or to each other, and chemical degradation of the solution. The existence of these problems only becomes clear for the selected compound during the crystallization procedure itself and is often frustrating, especially for young researchers, who may give up and stop experiments at this stage. The usual advice given in such problematic cases is to change the solvent or even the crystallization method (*e.g.* to use sublimation or gel crystallization), but the compound may be poorly soluble in other solvents or oxidize easily at high temperatures. The problems of crystallization may force researchers to look for very complicated crystallization techniques (Dhanaraj *et al.*, 2010) or even to change the substances selected for a research project.

Another issue is that there are a number of articles and books that provide very detailed information about the crystallization of certain substances, but it is not always straightforward to transfer the same technique to a new compound. The conditions may be different and the crystallization process can be influenced by a wide range of factors, ranging from a different climate (*e.g.* different humidity) to different reagents and equipment used. Some books give a broad variety of explanations (sometimes even contradictory) for a number of processes but suggest no practical tips for laboratory work. In general, information is scattered between many sources and is not immediately accessible in problematic crystallization cases. The aim of this laboratory note is to describe simple and efficient improvements to well known crystal growth techniques to help overcome some common and difficult problems that may arise during crystallization from solution. The application of these improvements is illustrated using amino and carboxylic acids as examples, together with two pharmaceutical and biologically active compounds. As a 'side effect' of our investigation, we have developed a technique that allows metastable polymorphs to be found reliably and reproducibly. None of the methods suggested demands the usage of any special or expensive equipment, or specific skills, and they can be implemented in the chemistry curriculum even at secondary school level.

2. Experimental procedures

The following reagents were used: serotonin creatinine sulfate monohydrate (Sigma–Aldrich, purity $\geq$98%), serotonin

adipate (Pana-Life Bio-chemical, China, purity ≥98% according to high-performance liquid chromatography data), L-serine (Sigma–Aldrich, purity ≥99%), DL-serine (ICN Biomedicals), L-alanine (Sigma–Aldrich, purity ≥99.5%), L-valine (Fluka, purity >99%), oxalic acid dehydrate (Reahim, purity ≥98%), maleic acid (Himreaktiv, purity ≥98%), malonic acid (Fluka, purity 99%), paracetamol (Merck, purity ≥99%), metacetine [synthesized at Novosibirsk Institute of Chemistry, Siberian Branch of the Russian Academy of Sciences (SB RAS)], piroxicam (synthesized at Irkutsk Institute of Chemistry, SB RAS), *p*-acetotoluidine (synthesized at Novosibirsk Institute of Chemistry, SB RAS) and ascorbic acid (a pharmaceutical sample; Lumi, Russia). Standard glassware (glass plates) was used. The microscopes used were LOMO MSP-1 and LOMO Polam 213-M.

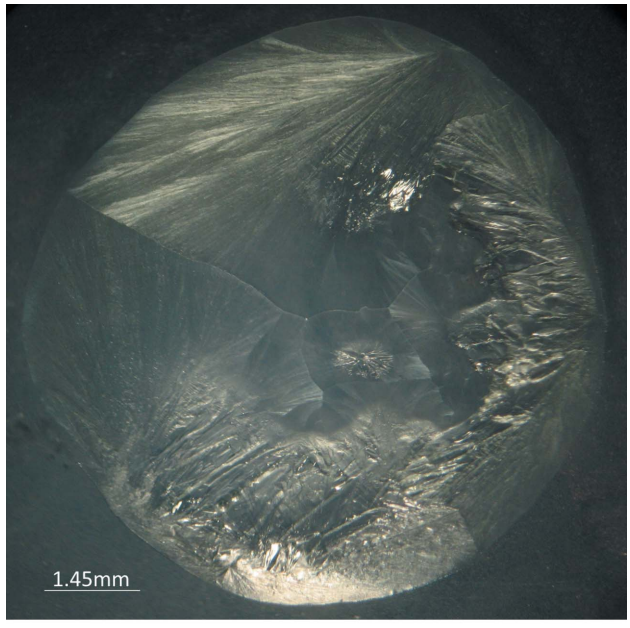
All crystallization procedures were performed in sitting drops (Bergfors, 2009) (slow evaporation method) with diameters not larger than 10 mm, to decrease the time of crystal growth and the consumption of a substance. A large number of drops helps to obtain statistics on the influence of different conditions on crystallization and to be sure that the results are not accidental. Crystallization procedures can be very sensitive to temperature, humidity *etc*., which were therefore controlled for each selected compound.

2.1. Avoiding multiple nucleation and dendritic or spherulitic growth

Multiple nucleation and dendritic or spherulitic growth (Fig. 1) present themselves as some of the most common problems preventing a yield of high-quality single crystals. They occur when the supersaturation is too high, which can be overcome using a wide range of seeding procedures.

Of course, seeding itself is not a novelty. It is widely used for the crystallization of proteins, even in automatic procedures (D'Arcy *et al*., 2007; Obmolova *et al.*, 2010; Shaw Stewart *et al.*, 2011). Another field where seeding is used as a common application is the pharmaceutical industry, where it is used, in particular, to ensure robust crystallization (Rohani, 2010; Tung, 2013), or in industrial crystallization when large single crystals are required. The only initial problem with these applications is that the seeds themselves must somehow be obtained. What is regarded as a 'seed' for the growth of a large industrial crystal would suffice as the 'goal crystal' for many other applications, *e.g.* for single-crystal X-ray diffraction analysis, but securing this very first small but perfect crystal may present a real problem. The technique that we propose is aimed at initially obtaining seed crystals in one solution and then transferring these seeds into another solution for subsequent growth.

A drop of approximately 90% saturated solution (drop No. 1) with a volume of 50–200 µl is placed on a glass surface and its slow evaporation is observed through a microscope until the first tiny crystals appear. Any of the newly produced crystals is then touched with a needle or, preferably, a hair or cat whisker. The same needle (hair, whisker) is then used to 'scratch' the surface of a freshly prepared drop of the same solution (drop No. 2) from one border to another (in Fig. 2*a* one can see small crystals growing along the 'streak'). The second drop has a lower supersaturation than drop No. 1 at the moment of seed extraction. This procedure is termed 'streak seeding' and is a variant of microseeding, which can be widely applied to protein crystallization (Bergfors, 2003). If this procedure is successful, small crystals that are well separated from each other form at the surface (Fig. 2*a*), which then grow over time and either fall to the bottom or stay in the bulk of the solution (Fig. 2*b*). If no crystals are observed after streak seeding, this usually means that drop No. 2 was not saturated enough, and the procedure should be repeated after approximately 10 min. Usually, the crystals that form using streak seeding come off the glass more easily than in the original technique of slow evaporation.



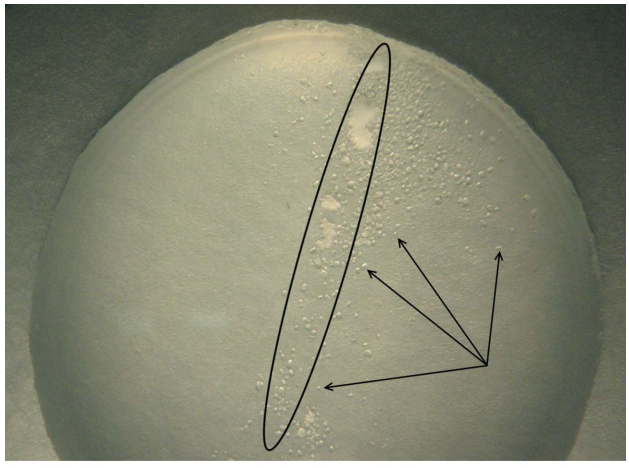
(*a*)

(*b*)

Figure 1
Microphotographs of (*a*) spherulitic crystallization of co-crystals of L-ascorbic acid and L-serine and (*b*) multiple nucleation on crystallization of serotonin creatinine sulfate monohydrate.

This simple technique has been successfully used to grow single crystals of a series of compounds that would otherwise not crystallize and instead give sticky syrup-like phases [L-serine–L-ascorbic acid co-crystal, L-serinium semi-maleate, DL-serinium semi-maleate, bis(L-serinium) oxalate; Arkhipov & Boldyreva, 2014; Arkhipov *et al.*, 2013]. For other systems, our technique showed better results when compared with 'ordinary' slow evaporation, in terms of the shape and size of the crystals.

Another interesting application of the technique is in obtaining crystals of metastable polymorphs (the crystal structures of which were then checked using X-ray diffraction). According to Ostwald's 'stage rule' (Ostwald, 1897), the first phases to crystallize are usually metastable. If left in the same solution where they initially form, metastable phases normally convert into other, more stable, polymorphs. When a seed of such a metastable polymorph is extracted from drop No. 1 and transferred to drop No. 2, it continues to grow, which results in a reasonably large single crystal of the metastable polymorph being obtained successfully. As an example, we can refer to growing crystals of a metastable polymorph of *p*-acetotoluidine from 80% alcohol solution, which succeeded using this technique.



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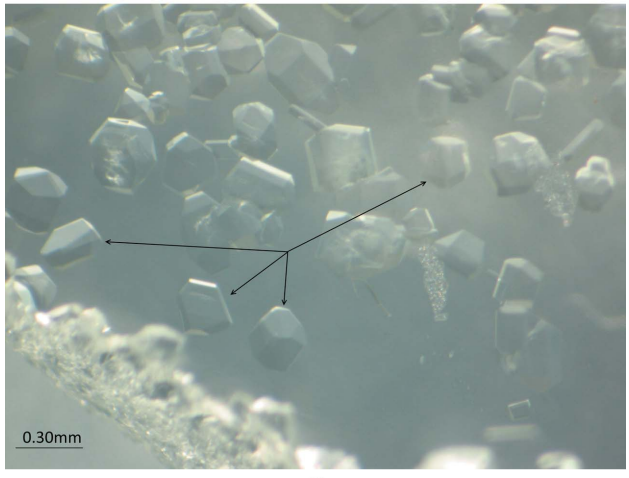


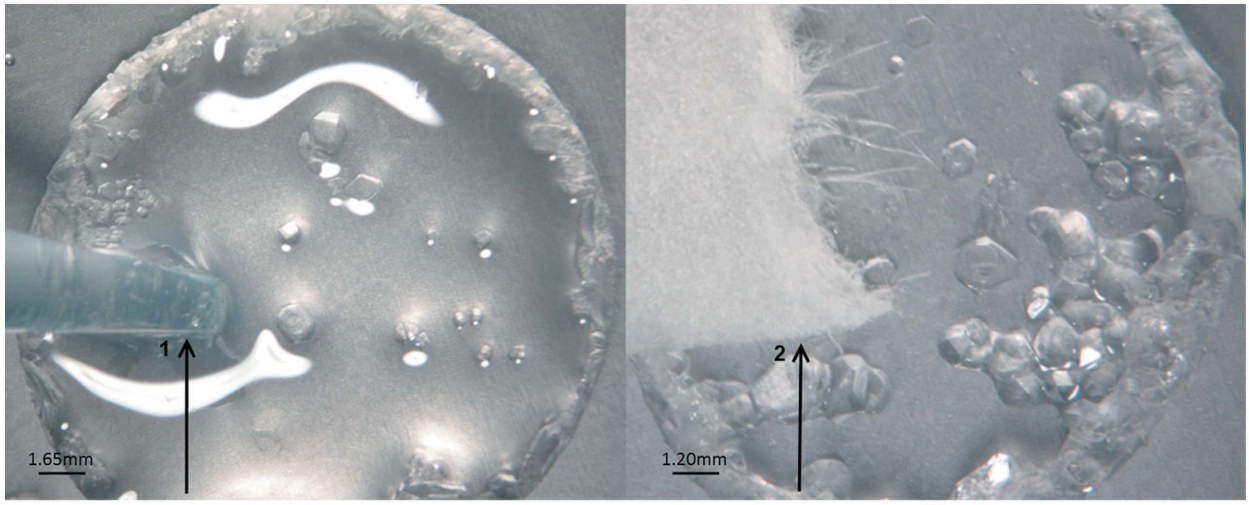
(*b*)

Figure 2
Microphotographs of (*a*) initially small and (*b*) already grown in size well separated co-crystals of L-ascorbic acid with L-serine obtained using the streak seeding procedure. Several crystals are indicated with arrows. The streak line is encircled.

If the size of a crystal in drop No. 2 is still too small for the desirable application, or the number of crystals growing simultaneously is too large so that the crystals start giving intergrowths, it is possible to extract a crystal from drop No. 2 and place it onto a new, freshly prepared, drop No. 3 of the saturated solution. In this case, the saturation of the solution should be controlled with high precision (carefully watching its evaporation under the microscope), in order to prevent the crystal accidentally dissolving. It is recommended to transfer a crystal onto a new drop, together with a small amount of solution, in order to prevent any crystallization at the surface of the crystal and to smooth the difference in the levels of supersaturation during this procedure. If multiple precipitation does accidently occur at the surface of the transferred crystal, despite precautionary measures being taken, it is recommended to dilute the drop carefully with a small amount of distilled water (5–7% of drop volume, aspirated and dispensed *via* pipette) to dissolve the smaller crystals at the surface, after which the 'main' crystal will grow with time up to a desirable size (the target size depends on the final purpose of the crystal). This procedure can be repeated as many times as necessary. The target crystal can then be extracted from the drop as soon as it has reached a desirable size. The procedure usually gives crystals with well defined crystal faces, which is important in the case of polarized single-crystal Raman spectroscopy, for example. The technique makes it possible to produce relatively large crystals (up to 4–5 mm), which can be used to study the surface properties and surface interactions of different organic crystalline materials. In this case, crystals can be prepared in the drop after adding a drop of new solution of the desired substance. Most of the crystals are dissolved at this moment, with the exception of the largest one. Depending on the substance, solvent and drop size, in 20–50 min the remaining large crystal grows larger. In the case of malonic acid, for example, extra mother liquor must be added and mixed using an automatic pipette (aspirating and dispensing). Mother liquor is added every time small crystals appear, and this must be done five or six times before 4–5 mm crystals are obtained, which usually takes several hours.

2.2. Avoiding crystal adhesion

Streak seeding also helps to solve the problem of crystal adhesion, since fewer crystals grow and they grow mainly in the bulk of the solution or at its surface, not adhering to the substrate. However, crystals often stick to each other when they grow or when one is attempting to extract them from solution, especially if they grow as plates with a thickness of approximately 0.05 mm. A simple and elegant way of avoiding crystal adhesion is to stop crystallization at the moment the target crystals are considered large enough, and when they are still separated from each other, by removing the solvent cautiously with an automatic pipette or a small piece of filter paper (Fig. 3). Though this trick might seem obvious, most



(*a*) (*b*)

Figure 3
Microphotographs demonstrating the removal of the crystallization solution, (*a*) by aspiration with an automatic pipette and (*b*) by soaking up with a small piece of filter paper, when the target crystals are considered to be ready for extraction. (1) the tip of the automatic pipette; (2) a piece of filter paper.

manuals and textbooks on crystallization recommend a reverse sequence of actions: 'simply' extract a crystal and then dry it using filter paper. The problem with this standard recommendation is that it is very difficult to avoid the spontaneous crystallization of multiple tiny crystals at the surface of the main crystal, as the residual solution evaporates faster than it can be soaked up. In addition, it is quite difficult 'to soak up the solution with filter paper' from the surface of a crystal with linear dimensions of less than 0.3 mm immediately after its extraction.

The simple soaking up of solution from the vessel prior to crystal extraction enables the extraction of high-quality small single crystals efficiently and without damage, even without using special tools like MicroGrippers, MicroMounts (MiTeGen; http://www.mitegen.com/products/micromounts/micromounts.shtml) and CryoLoops (Bergfors, 2009).

For many compounds that can withstand contact with both water and oil, oil can also be used to facilitate crystal extraction from solution, as long as the oil does not interfere with the planned experiments with the target crystal, *e.g.* X-ray diffraction. Either the crystal and a small residual amount of mother liquor can be transferred into oil or, *vice versa*, oil can be added to the crystal in the mother liquor. Oil replaces the mother liquor at the crystal surface and protects it from desolvation (in the case of solvates).

Another technique that can be used to avoid crystals adhering to each other is to spread the same volume of solution over a larger surface area once the seeds have formed, so that the final crystals form further apart from each other. The proposed surface should be completely clean (washed with soap and rinsed with distilled water), and spreading should be performed only after some small crystals have already appeared. In this way it is possible physically to separate small crystals from each other by spreading the liquid in which they float. This procedure can be combined effectively with streak seeding, when small crystals have already formed and need to be separated. The technique gives positive results in all cases, unless the solvent is so viscous that the solution evaporates in hours or days, instead of minutes.

The adhesion of crystals to the surface of the crystallization vessel can be prevented by using different modifiers of the surface, which can decrease the wettability of the surface and result in a more spherical shape of the crystallization drop (Fujii & Hirayama, 1999). Parafilm and paraffin modifiers were shown to work efficiently enough in almost all the problematic cases, especially when growing crystals from water or alcohol solutions (Fig. 4). The technique involves covering the surface with a layer of paraffin, or stretching Parafilm over the glass, and placing a drop of solution directly onto this layer. A more spherical shape of the crystallization drop usually results in larger crystals, especially if streak seeding is used, and the crystals sink in the solution with time. This approach was efficiently used to crystallize L-serinium

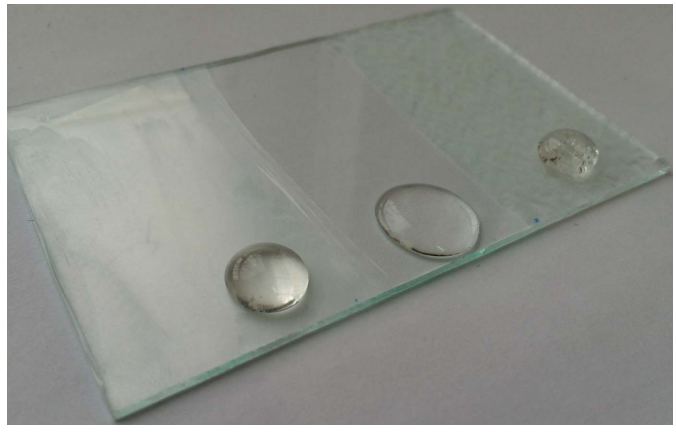
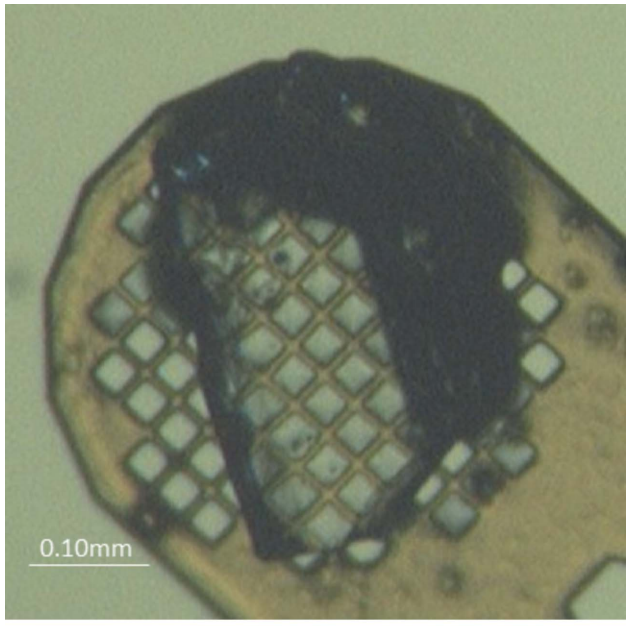


Figure 4
Microphotographs of 200 µl drops on the surface of a microscope slide, modified with Parafilm (left), the original glass surface (middle) and modified with paraffin (right). One can clearly see the change in the shape of the drops, depending on the surface wettability.

semi-maleate and DL-serinium semi-maleate. The approach is much simpler than that described in the literature (Fermani *et al.*, 2001; Falini *et al.*, 2002) but no less effective.

An interesting application of this approach is in growing crystals of compounds that can degrade easily. For example, we readily obtained high-quality well faceted co-crystals of L-ascorbic acid with L-serine of controlled size using this method (Fig. 5). This mixture is unstable in solution because L-ascorbic acid is slowly oxidized by atmospheric oxygen, so special procedures for obtaining crystals are normally required using an inert atmosphere (Sudhakar *et al.*, 1980). For comparison, we could also reproduce the co-crystallization of L-ascorbic acid with L-serine under an inert atmosphere (glassware containing the solution was blown through with nitrogen, no air contact with the solution) but were unable in that case to obtain well faceted crystals. Problems with substances that degrade in solution are discussed in §2.3, and here we should confirm that a simple procedure with surface modification allowed us to grow perfectly faceted crystals of monoclinic paracetamol (from alcohol solution), metacetin (from water–alcohol 50:50 *v*/*v* solution), piroxicam (from acetonitrile), malonic and maleic acids (from water solution), L-valine and L-alanine (from water solution), and L-ascorbic acid and some amino-acid-containing co-crystals [L-serinium maleate, DL-serinium maleate, bis(L-serinium) oxalate dehydrate] (from water solution) (Arkhipov & Boldyreva, 2014; Arkhipov *et al.*, 2013).



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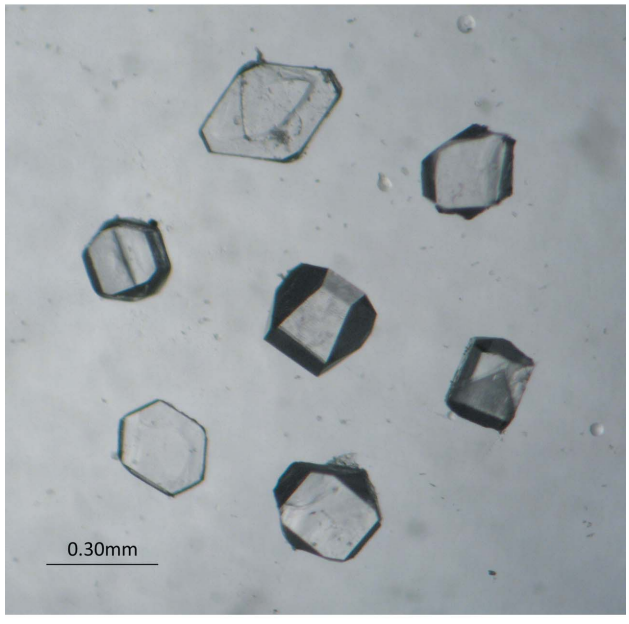


(*b*)

Figure 5
Microphotographs of crystals of L-ascorbic acid. (*a*) Obtained using the traditional technique of slow evaporation (10 d) under an inert atmosphere used in an X-ray experiment, mounted on a MiTeGen MicroMount, and (*b*) obtained by fast crystallization from a spherical crystallization drop without any special precautions. Crystals obtained by the technique proposed here (*b*) are well faceted, while the crystal obtained by the traditional technique (*a*) has crooked facets (especially the upper and right facets, which are dark on the microphotograph).

The processes underlying the crystallization of well faceted crystals when using this procedure deserve a special study which would be beyond the scope of this note. We can note that the technique gives several advantages when compared with traditional slow evaporation from larger volumes, without transferring seeds from one solution into another. First of all, air contact is minimal because the evaporation of a drop does not usually take more than 10–20 min, depending on the drop size. Another advantage is that the surface of a spherical drop is smaller than that of a flat drop, which provokes fewer crystals to grow on the surface. We did not find any evidence that the process of crystal growth depends on the volume of a drop but not on its shape – despite numerous attempts, almost all the substances we tried did not crystallize if the plain glass substrate was used, irrespective of the drop volume.

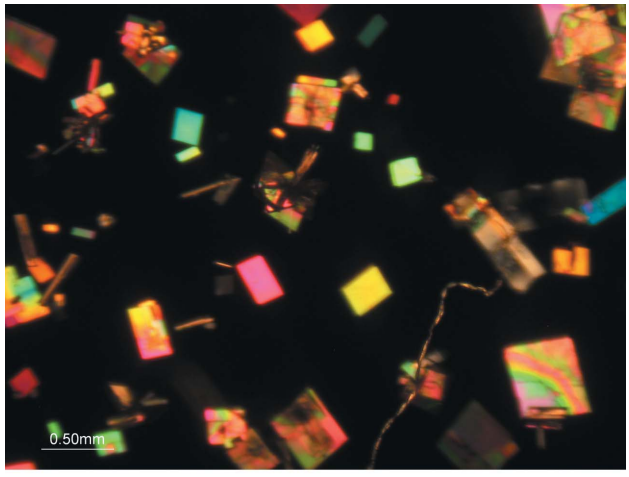
2.3. Avoiding substance degradation in solution

The problem of chemical degradation often arises when crystallizing biologically active compounds. Instead of attempting to change the crystallization method completely, one can still use crystallization from aqueous solution by evaporation, but modifying the traditional technique. This would shorten the time that the unstable substance stays in the liquid phase, especially at high temperatures, usually above 313–333 K, depending on the substance. The crystallization time can be reduced significantly by using 'crystallization drops', which have been described above. In addition, it is advisable to lower the temperature as far as possible (even down to below 273 K by putting the drops in a freezer) and not heat the sample at all, and, if heating is absolutely unavoidable, to decrease the heating time and temperature. Obviously, one should also pay close attention to the pH of the solution and change it accordingly, if the substance is especially unstable in a certain pH range. The whole crystallization

procedure can be more complicated than with chemically stable compounds and usually demands more effort.

An example of the crystallization of a very unstable compound is that of serotonin (Hoyer *et al.*, 2002). The traditional techniques of antisolvent crystallization (Rapport *et al.*, 1948), slow evaporation (Aniy *et al.*, 1978) or cooling of a saturated solution (Karle *et al.*, 1965) do not give positive results. This shows that traditional techniques do not allow one to obtain crystals of high quality without serotonin degradation, which can be clearly seen through a colour change from transparent to pink or even dark brown, induced by chemical degradation of the serotonin. Basic problems related to the crystallization of serotonin and its salts include the degradation of this compound in solution within several hours, its high sensitivity to temperature, crystal fragility and the spontaneous crystallization of many tiny intergrown crystals (powder-like precipitation).

To avoid each of these obstacles, we have developed a crystallization procedure comprising several simple steps. A saturated solution was prepared under ambient conditions and then briefly heated to a temperature not exceeding 313 K, to saturate the solution further at this higher temperature by dissolving an additional amount of a substance. At the same time, carefully cleaned microscope slides (washed with soap and rinsed with distilled water) were frozen in a freezer (*ca* 263 K). Drops with a total volume of less than 100 µl were placed on the pre-frozen microscope slides outside of the freezer and then returned to the freezer. Upon removal from the freezer, the supercooled solution was found to convert to ice following a light knock of the microscope slide. The drops were subsequently melted. When the first crystals were observed under the microscope, the drops were spread over the surface of the microscope slide by tilting the glass, doubling the contact area of the original drop with the surface. The solution was allowed to evaporate without disturbance. This resulted in large amounts of powder and a substantial number of single crystals, which formed separately from each other. In this case, we also separated the nucleation stage (resulting from consecutive cooling for 10 min at 277 K and



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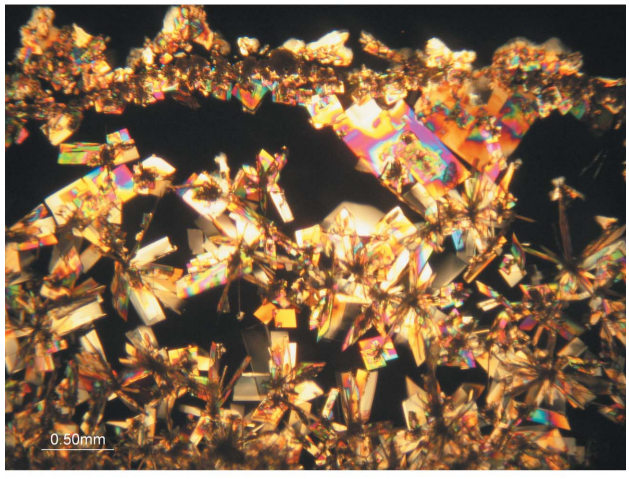

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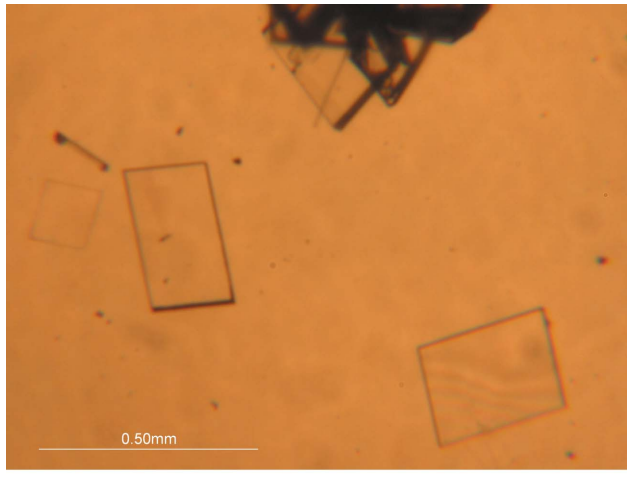
Figure 6
A comparison of the results of crystallizing serotonin creatinine sulfate monohydrate using two different experimental procedures (microphotographs obtained using polarized light): (*a*) crystals grown using solution freezing, and (*b*) crystals grown using the traditional slow evaporation technique.



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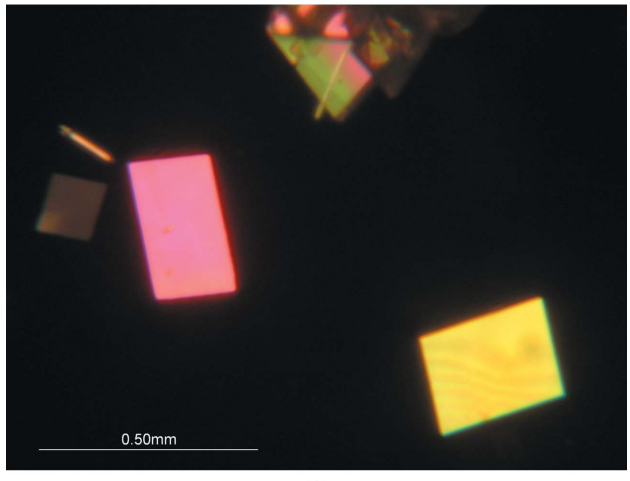

(*b*)

Figure 7
Photographs demonstrating the quality of the crystals of serotonin adipate obtained using the technique described in §2.3. The crystals are shown as seen in (*a*) unpolarized and (*b*) polarized light.

freezing for 15 min at 263 K) and the crystal growth stage (occurring during evaporation of the cooled solution). The results of the crystallization of serotonin using different techniques are compared in Fig. 6.

This procedure may be performed without the freezing stage (with only cooling down to 277 K) but usually displays worse results. We suppose that freezing the solution influences nucleation, though the mechanism of this effect remains unclear. One of the possibilities is that the ice forms cages around the nuclei to separate them from each other or prevent some of them from future growth after melting, if a nucleus is not thermodynamically stable or if concentration gradients exist (as melting is non-equilibrious).

The same procedure was applied to obtain high-quality single crystals of serotonin creatinine sulfate monohydrate and serotonin adipate (Fig. 7) with dimensions of $0.15 \times 0.12 \times 0.05$ mm. The perfection of the crystals was clear even after visual inspection and was additionally proved by single-crystal X-ray diffraction (Rychkov *et al.*, 2013). We also used this technique to grow crystals of ascorbic acid, which normally degrades in solution over several hours.

3. Conclusions

In this paper we have described simple but effective modifications of well known techniques of crystal growth from solution by methods of slow evaporation or by cooling of saturated solutions. These modifications help to overcome several of the most common problems that can arise during crystal growth attempts. Time spent obtaining perfect crystals is never in vain, and one is rewarded at the later stages of research by a significant facilitation in attaining and interpreting high-quality diffraction or spectroscopic data. All implemented techniques were tested for amino and carboxylic acids and their salts, crystallized from water–alcohol mixtures, or from water or alcohol solutions, but they can also be applied for crystallization of other classes of organic molecules from other solvents.

In conclusion, even simple modifications of well known procedures can improve the crystallization results significantly. If traditional techniques do not succeed when attempting to crystallize a compound, this does not mean that the same method will not work after the introduction of some modifications. Usually, it will take a day or two (maybe a week) to identify the necessary modification and there is no need to change the whole crystallization method completely. The streak seeding method described here usually helps to avoid multiple and spherulitic nucleation. Modifying the surface and changing the method of extracting the crystals helps to prevent crystal adhesion. Reducing the heating time and freezing the solutions assists in fighting against chemical degradation.

A new improvement to well known techniques is the implementation of the streak seeding procedure for the crystallization of small organic molecules without using complicated and expensive equipment. Another new suggestion is to use simple and readily available surface modifiers such as Parafilm and paraffin for the sitting drop to obtain well faceted crystals, while only substances and materials designed specifically for the purpose were used before. Consecutive freezing and evaporation in drops was implemented to grow crystals of biologically active unstable compounds. All these procedures were implemented successfully to obtain high-quality crystals of more than 15 compounds that would not crystallize with standard techniques, which shows significant progress in this field.

It is highlighted that some of the suggested procedures, in particular those using the sitting-drop technique, were partly borrowed from protein crystallization procedures (Bergfors, 2009) and implemented to improve the crystallization of small molecules, which is also often a challenge. Nevertheless, we must point out the considerable differences and the simplicity of our technique: in contrast with protein crystallization procedures, the supersaturation in our method is achieved by solvent evaporation, instead of antisolvent vapour diffusion. Another distinction is that none of the described techniques requires any special equipment (like that used for protein crystallization), and the techniques can be used in any chemical laboratory, even by beginners. These proposed techniques can be useful not only for research but for educational purposes as well. This paper provides examples for courses in crystal growth, showing that a proper analysis of the crystallization difficulties and an intelligent combination and modification of well known crystallization techniques allow one to grow crystals of high quality, overcoming major problems.

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