

## Chapter 2

# Polarized Light Microscopy

### 2.1 Introduction

The optical microscope is used extensively in pharmaceutical development with the primary application being solid-state analysis. The applications range from simple images of drug substance to illustrate particle size and shape to full optical crystallography. The range of utility of the microscope is considerably extended by the use of polarized light which allows us to obtain crystallographic data on small individual crystals. I will use the term Polarized Light Microscopy (PLM) for all light microscopy discussions in this chapter.

Polarized light microscopy provides us a unique window into the internal structure of crystals and at the same time is aesthetically pleasing due to the colors and shapes of the crystals. The use of PLM as a tool for crystallography extends back at least 200 years. For many of those years, it was the prime tool for examining the crystal properties of minerals and inorganic chemicals, as well as organics. Pasteur's seminal studies in the handedness of organic chemicals were initially conducted on an optical microscope. Needham (1958) provides some information on the development of PLM but frankly I am not aware of any good articles or books that detail the history of both the development of the polarizing light microscope and its applications.

The use of polarized light on the optical microscope allows us to determine the optical crystallographic properties of the crystal. Optical crystallography is related to but different from X-ray crystallography. Each technique provides unique information about the crystal structure and the combination of the two is powerful indeed. For example, optical crystallography uniquely yields the sign and magnitude of the angle between optic axes in a biaxial crystal. This value, among others, can be used for the definitive identification of the solid-state form. This determination can be made on crystals as small as  $\sim 5\ \mu\text{m}$  and can be done in a mixture of particles of different forms. PLM was originally developed for studies in mineralogy and petrography. It has since expanded so that virtually every field that deals with crystalline materials uses the techniques of optical crystallography.

Optical crystallographic measurements can yield as many as 20 different characteristics, many of which are numerical. This set of values is unique for each

solid-state form. A hydrate will have different optical crystallographic values than its anhydrate and different polymorphs of the anhydrate (or hydrate for that matter) will have different optical properties. Optical crystallography, then, is a superb tool for the *in situ* identification of different solid-state forms. There are two caveats: First, we must have good reference data for each of the forms; second, the microscopist must be skilled in the art and science of optical crystallography. It seems to me that the decline in the use of this science is related to both of these requirements. Optical crystallographic references are scattered and incomplete and there are relatively few scientists that are skilled in the necessary techniques.

I believe that there are a number of good reasons to attempt a revival of optical crystallography in pharmaceuticals. First, it does provide a set of unique values for the identification of solid-state forms. Second, optical crystallography can be quite useful in the study of thermodynamic form relationships (see Chap. 8). Third, if the optical and X-ray crystallographic characteristics have been related, then it is possible to determine which faces and thereby which functional groups of the chemical are exposed by comminution processes (Nichols, 1998). Fourth, a thorough understanding of optical crystallography is an excellent tool for the understanding of crystallography overall. It is a wonderful teaching aid.

For all of these reasons, I begin this book with a discussion of polarized light microscopy and optical crystallography. I also use this opportunity to discuss optics and the basic use of the optical microscope. Optical crystallography and PLM require that we set the microscope up well and in the process of learning these subjects one must also learn proper microscopy technique.

## 2.2 Properties of Light

There are a number of excellent texts on light and optics in relation to optical microscopy. Much of the discussion below follows Slayter and Slayter (1992), McCrone et al. (1984), and Needham (1958). Gage (1943) and Martin (1966) provide a more detailed and extensive discussion of light and optics as they relate to microscopy. Born and Wolf (1980) is the standard for optics.

Visible light is a narrow part of the electromagnetic (EM) spectrum usually considered to extend from 400 to 700 nm ( $10^{-9}$  m) in wavelength. Wavelengths of the entire electromagnetic spectrum extend from  $10^{-16}$  m for gamma rays to  $10^{10}$  m for long radio waves. The spectrum is divided into the following categories based on short to long wavelength (high to low frequency): gamma rays, X-rays, ultraviolet radiation, visible radiation, infrared radiation, microwave radiation, short radio waves, and long radio waves. These categories are somewhat arbitrary in nature and all electromagnetic radiation share common features. The key properties of electromagnetic radiation are intensity, frequency (wavelength), polarization, phase and angular orbital momentum. The first four properties are directly used in PLM.

Intensity is defined as power over area, for example, watts/m<sup>2</sup>. We have an intuitive sense of intensity in normal life as we may turn on more lights in an area

if we feel it is dimly lit. While intensity is of great interest in microscopy, we do have some control over the property since most microscopes use artificial illumination with variable power bulbs. Historically, intensity was a major issue in microscopy since most microscopes used the sun or candles as the light source. Even today, we are interested in increased intensity for low light level applications like fluorescence microscopy. For that reason, some specialized techniques also have specialized high-intensity light sources.

Electromagnetic radiation can be considered as a wave phenomenon. As such, the waves have a frequency (number of waves in a unit measure) and the inverse – a wavelength. Wavelength is frequently used in optical microscopy and is defined as the wave distance from peak-to-peak or from trough-to-trough. These relationships can be mathematically represented as follows:

$$f = v / \lambda, \quad (2.1)$$

where  $f$  is the frequency,  $v$  is the velocity of the wave, and  $\lambda$  is the wavelength.

For the electromagnetic spectrum,

$$f = c / \lambda. \quad (2.2)$$

Since the velocity of the wave is the speed of light. We often use the term and concept of wavelength in optical microscopy and commonly use nanometers as the unit of measure (spectroscopy more commonly uses wavenumber, see Chap. 5). Various filters of specific wavelengths of light are used with the microscope and the most common of these are 589 nm (D line), 486 nm (F line), and 656 nm (C line). The D line (589 nm) is yellow light and is situated in the middle of the visible spectrum. Some microscopists also use monochromators with their microscope to produce narrow wavelengths of light, although the practice is not as common today as it once was.

Polarization refers to limitations on the direction of wave oscillation. A polarizer acts like a filter to allow only light oscillating in one orientation to pass. We use polarized light for many applications in optical microscopy. For example, we can determine crystallinity of a sample using a few crystals and crossed polars. The test is sensitive, fast, and prone to only a few errors. Polarization is one of the more difficult concepts to describe since there really are not many large scale analogous phenomena to act as an example. As Needham (1958, pg 164) states, “It is much easier to demonstrate polarized light than to clearly describe what it is.” I think I could title this entire chapter with that statement. Bloss (1961) presents a cogent and well-illustrated introduction to polarized light.

Phase refers the time progression of the wave. In other words, at  $t_0$  the wave may be at a peak,  $t_1$  halfway between peak and trough,  $t_2$  at the bottom of the trough, etc. In general, we do not use phase information directly in microscopy but only as the phase relates to other waves through interference. Light waves can either constructively or destructively interfere. The waves may add in amplitude, subtract in amplitude, or cancel each other. If the phase of the combining light waves is the same, the waves will constructively interfere and add amplitudes. If the waves are out of phase,

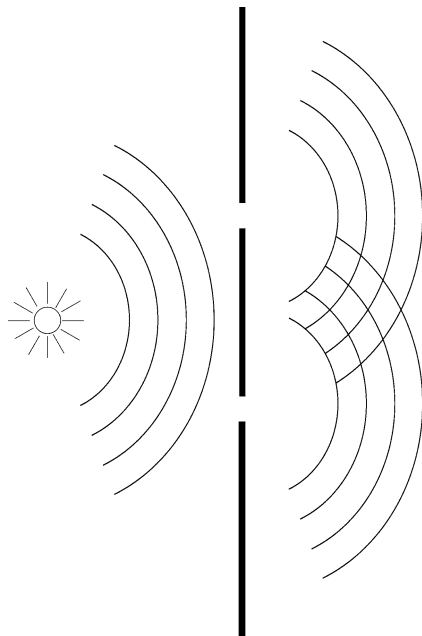
then amplitudes will be diminished or even canceled. Phase differences are used in a variety of microscopy techniques primarily to improve contrast. Phase contrast, differential interference contrast, and Hoffman modulation contrast are just a few of the methods utilizing phase differences to enhance contrast. These latter techniques are more commonly applied to biological than to physical pharmacy.

Some crystals have the property of double refraction and incoming light is split into different optical axes (directions where speed of light is different). When this light is recombined, such as in a polarizing light microscope, we get interference and with PLM a visual interferogram is formed at the back focal plane of the objective. The properties of this interference figure are characteristic of the material and can be used in understanding the crystallography and molecular properties of that material. The underlying physical principle is alteration of light speed by interactions of light with different functional groups in the molecule. The properties of the interference figure can also be used to identify the crystal and even to distinguish among polymorphs, hydrates, and solvates.

Double refraction is the phenomenon underlying all of optical crystallography and it behooves us to understand it well. Simple refraction occurs when a light beam traverses a refractive index interface. A pencil placed in a half-full cup of water appears bent where the air meets the liquid. Some crystalline materials not only have this ordinary refraction but split the light into two or three beams, each of which now travels at a different speed. These different refraction properties are based entirely on the crystallography of the particles being examined. Cubic crystals, like sodium chloride, do not display double refraction and hence do not produce interference colors in crossed polars. Hexagonal and tetragonal crystals split the incoming beam into an ordinary ray that follows typical refraction properties and an extraordinary ray which does not. Crystals in these systems display interference colors in crossed polars. Orthorhombic, monoclinic and triclinic crystals split the light into three rays and also display interference colors. It is by observing and measuring the specific refraction properties of specific materials that we are able to collect a number of observations and values that aid in understanding the underlying solid symmetry properties of the molecule and allow us to rapidly identify unknown particles.

Another important property of light waves is diffraction as the light interacts with a slit or small opening. In a sense, light bends around the opening so that it appears that the small slit or hole is the source of new waves (see Fig. 2.1). Diffraction is a key concept in theories about the resolution limits of microscopes. This brings us to the particle-wave duality concept of light. Einstein showed, in a brilliant piece of work, that light has particle as well as wave properties (Weaver 1987). Consideration of the implications of this fact led to the development of wave (or quantum) mechanics. Quantum mechanics has some direct applicability in electron microscopy and in vibrational spectroscopy and is a fascinating and worthy field of study. It has little place in a book on practical applications of pharmaceutical microscopy and will not be discussed further. Feynman et al. (1965) has a reasonably cogent and understandable discussion of quantum theory requiring only a moderate understanding of physics.

**Fig. 2.1** Schematic illustration of light diffraction. Light exiting a slit behaves as a new light source in the sense that waves propagate from the slit as well as from the original source

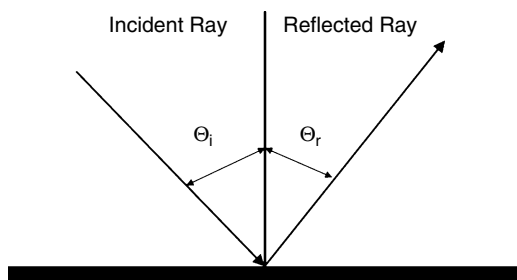


## 2.3 Basic Optics

It is not necessary to be an expert at optics to intelligently use the microscope, but it is necessary to know and understand a few basic concepts. There are many excellent texts on optics in the microscope and I recommend Slayter and Slayter (1992), McCrone et al. (1984), and Chamot and Mason (1958) along with Needham (1958) for further study. I will discuss a few basic ideas in this section. At the heart of optics is the interaction of light with materials and, of course, for microscopy the material of most interest is glass in different shapes.

When visible light irradiates an object, the light can be reflected, transmitted, or both. Reflection and transmission can occur such that the light retains all of its energy or some energy can be absorbed in the process. The rich complexity of the interaction of light with objects, in particular glass, allows for the versatility and power of modern microscopes.

The law of reflection states that the angle of reflection is equal to the angle of incidence (see Fig. 2.2) for any particular light ray irradiating a smooth, reflective surface. If the surface is irregular and a broad beam of light is used, then we get diffuse reflection. If the surface is polished and we use a narrow beam of light, then we get specular reflection and this reflected light can be polarized. Fishermen are familiar with this phenomenon and use polarizing sunglasses to reduce light reflection so as to better see the fish below the surface of the water. It is easy to see



**Fig. 2.2** Light reflection. Angle of reflection equals angle of incidence

that if we make mirrors of different shapes, particularly concave mirrors, we can use the curved surface to focus light rays to a point and produce an image. Reflected light telescopes, such as the Hubble telescope, use this principle. The image is generally not perfect since the rays may not focus to exactly the same point (spherical aberration) and light of different wavelengths will not focus precisely (chromatic aberration). These natural limitations can be corrected to some extent and are more easily accomplished with transmitted light optics than with reflected light.

The special theory of relativity posits that the speed of light is the same for all observers, which is the speed of light in a vacuum. The speed of light is certainly not a constant in all mediums. The ratio of the speed of light in the vacuum to the speed of light in the medium is called the refractive index. Refractive index is designated by lower case letter,  $n$  and is defined as follows:

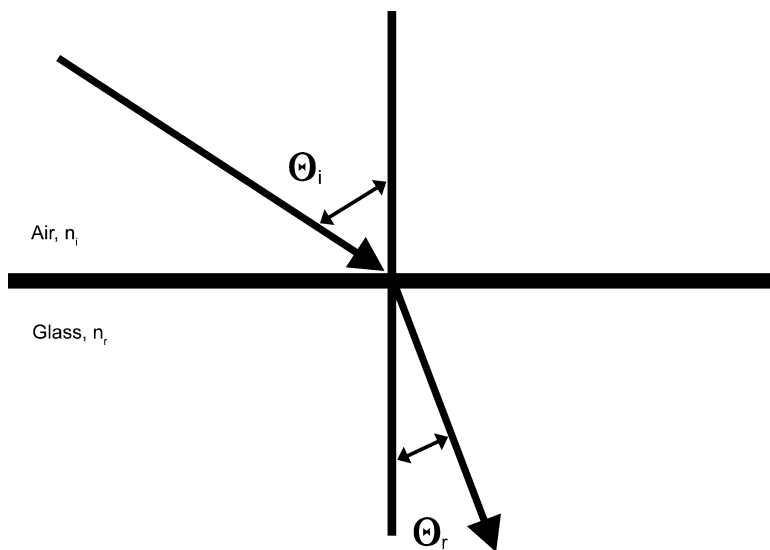
$$n = v_{\text{vacuum}} / v_{\text{medium}}, \quad (2.3)$$

where  $v$  is the speed of light. Air has an index of approximately 1.0029 and is most often directly substituted for the vacuum value. Water has a refractive index of 1.33, whereas indices of pharmaceutical compounds are closer to 1.60. Diamond has a high refractive index of 2.42 and glass has a broad range of values from near 1.3 up to 1.9 depending upon the glass composition.

Refractive index is dependent on both temperature and upon wavelength. This dependence of optical properties on wavelength (called dispersion) is a prominent factor in optical crystallography and many properties exhibit dispersion. Dispersion of some optical properties can be hard to measure but are generally specific to a material and good for identification. In microscopy, we are most interested in the temperature range from 20 to 30°C and light wavelengths from 400 to 700 nm.

Light incident on a transparent material changes not only its speed but also its direction. This phenomenon is called refraction and is commonly observed by placing a knife or spoon in a glass of water and noticing that the image of the knife or fork is bent. Mathematically, refraction obeys Snell's law

$$\frac{n_r}{n_i} = \frac{\sin \theta_i}{\sin \theta_r}, \quad (2.4)$$

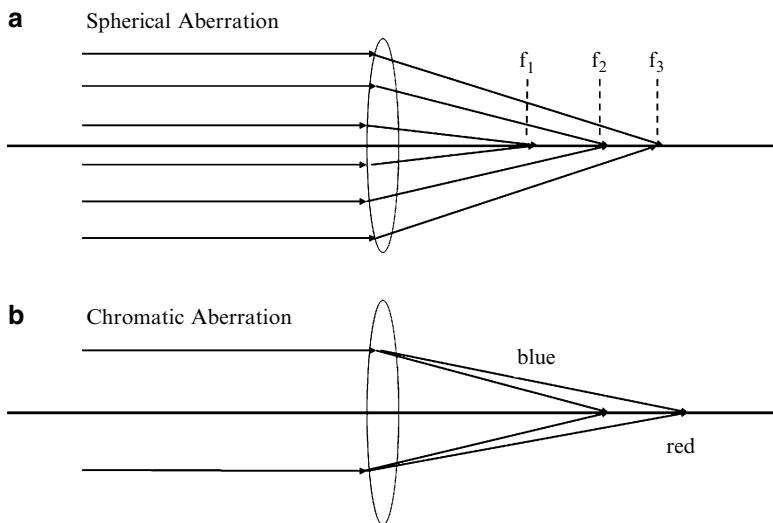


**Fig. 2.3** Schematic illustration of Snell's law. Mathematical relationship governing ordinary refraction

where  $n_r$  is the refractive index of the refracting material,  $n_i$  is the refractive index of the incident material, and  $\theta$  is the angle of the light ray with the perpendicular direction to the interface (see Fig. 2.3). This basic law provides the fundamental relationship for the manipulation of light by glass lens and in microscopes.

Let us consider a convex lens and see how this equation can be applied to image formation as well as its inherent aberrations (see Fig. 2.4). Light from an object is refracted through the lens to a focus point and forms an image. The size (magnification) of this image in relationship to the original object depends upon the shape and size of the lens, the distance from the object to the lens, and the distance of the image from the lens, not to mention the size of the object itself. Microscope objectives have multiple optical elements (in the teens for apochromats) to produce the image and to correct for spherical and chromatic aberrations. Spherical aberration refers to the fact that not all light rays from the object will have exactly the same focus point (see Fig. 2.4a). Chromatic aberration refers to the fact that different wavelengths of light will be refracted by the lens to different degrees and hence different wavelengths have different focus points (see Fig. 2.4b). Objectives are rated according to the level of spherical and chromatic corrections and are priced accordingly. Achromats have the least corrections and apochromats the most. Fluorites have intermediate corrections.

The topics of refractive index and aberrations in objectives are the most important aspects of optics used for practical work in pharmaceutical microscopy. Clearly, the more we know about the fundamentals of optics, the better our work, but one can do good basic work with just the parts of optics we have covered along with a firm understanding of magnification and resolution. Concepts around resolution are probably the most important for correct application of microscopy in pharmaceuticals.



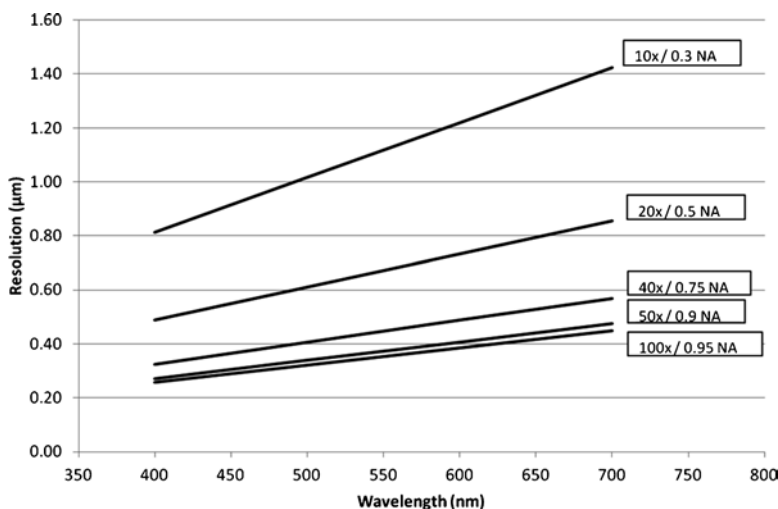
**Fig. 2.4** Illustration of spherical and chromatic aberration. (a) Real lenses do not focus rays at exactly the same point. (b) Different wavelengths of white light focus at different points

It is crucial to always keep in mind the distinction between magnification and resolution. Resolution is loosely defined as the smallest-sized features that can be distinguished from each other. So, for example, if we are examining wet-bead milled particles that are less than  $1\text{ }\mu\text{m}$  in diameter, what is the smallest-sized particle we can detect and resolve when they lay next to each other? Magnification is related to resolution but we can greatly increase our magnification without significantly affecting our resolving power. We have probably all had the experience of magnifying an image to the point that we begin to see the individual elements making up the image and have lost all sense of the object itself. Magnification is simply increasing the apparent size of an object. In microscopy, it is much more important to have good resolution than high magnification.

Resolution is affected by a number of factors, not least of which is the performance of the human eye. Diffraction theory, as applied to microscopy by Ernst Abbe in 1873, is the most common approach to resolution used by practicing microscopists (Needham 1958; McCrone et al. 1984). If we consider a small object in the microscope, in reality we are viewing a central object surrounded by alternating bright and dark diffraction rings. If the object is large with respect to the rings, then the rings will not affect resolution. If, on the other hand, the rings and object are near the same size, then our ability to resolve the two objects depends as much on the size of the rings as on the size of the objects themselves. Abbe based his theory on the principle that for two features to be distinguished their diffraction discs should not overlap more than  $\frac{1}{2}$  the disc diameter. The key equation governing resolution in the microscope is as follows:

$$d_r = \frac{0.61\lambda}{\text{NA}}, \quad (2.5)$$





**Fig. 2.5** Graph relating resolution to wavelength for some objectives. Resolving power is dependent on the numerical aperture of the objective and light wavelength. Higher NA and lower wavelength allows for highest resolution

where  $d_r$  is the resolution (diameter of smallest resolvable feature),  $\lambda$  is the wavelength of light, and NA is the numerical aperture of the objective (listed on objective and is a measure of light capturing ability). Figure 2.5 presents a graph based on this equation for a standard set of Leica objectives. For example, let us compare resolution of a 50× dry objective with NA of 0.90 with a 100× dry objective with and a NA of 0.95. Using a value of 0.589 μm as the median wavelength of white light, we find that  $d_r$  is 0.399 μm for the 50×/0.90 NA objective and 0.378 μm for the 100×/0.95 NA objective. We doubled the magnification but only marginally improved the resolving power.

How do we choose an objective for a particular task? In practice, the choice is empirical. It is always a good idea to work from lowest to highest magnification when examining a new specimen or sample. Low power examination allows us to survey a large part of the slide and look for obvious regions of interest. We can then move to higher power objectives within those regions in order to better resolve fine features. For ultimate resolution, we need to use oil immersion objectives.

## 2.4 Crystallography

According to Webster's Ninth New Collegiate Dictionary (Merriam Webster 1983), crystallography is defined as the "Science dealing with the system of forms among crystals, their structure, and their forms of aggregation." The atoms or molecules of a crystalline solid usually are oriented in space with a repeating pattern in three dimensions. Think of bricks in a wall or even better, of patterned ceramic tiles. This is the crystalline state. In some solids, there is no long-range repeating pattern, though there

may be short range order and this condition is referred to as the amorphous or glassy state. There are also imperfections in most real crystals and this condition is referred to as disorder. Crystallinity and disorder are important topics in physical pharmacy since the degree of crystallinity can affect some important bulk properties of a solid such as the amount of water it can absorb as a function of temperature and relative humidity.

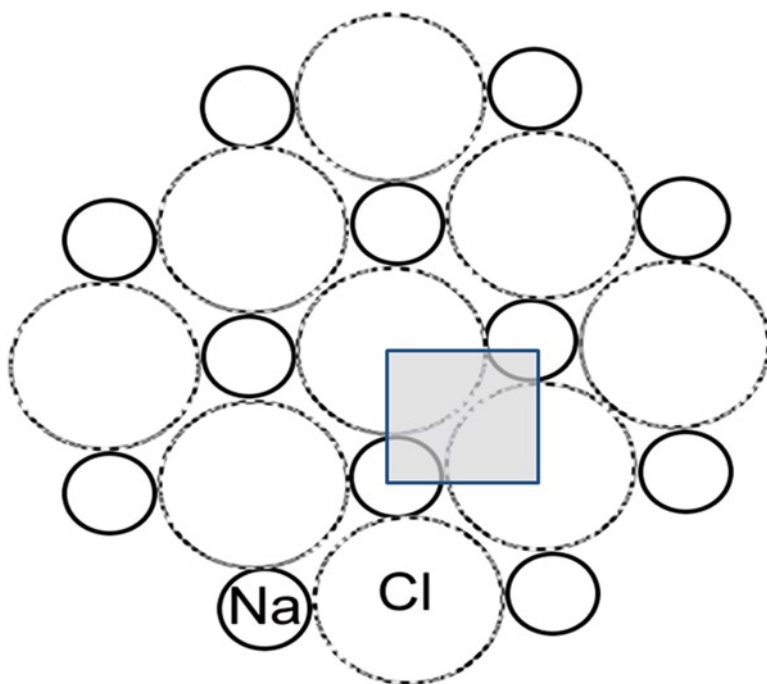
Surprisingly, there are only a few repeating patterns. The patterns can be categorized as follows into six crystal systems with decreasing degrees of symmetry: cubic, tetragonal, hexagonal, orthorhombic, monoclinic, and triclinic. The trigonal system, which is referenced in older literature, is now considered part of the hexagonal system. Within the six systems there are 32 classes and 230 space groups (See McCrone et al. 1984, Table XVII, p. 110 for a full list of classes and space groups). Classes and space groups are particular to the six systems. Although classes and space groups are quite important, we only have access to direct knowledge about crystal system with optical crystallography. Although this limits the utility of optical crystallography for structure determination, we are still able to use PLM for identification and for some corroborative crystallography. Table 2.1 presents the axis orientation and spacing of the six crystal systems.

Repeating patterns in crystallography are fairly simple to imagine in a cubic system like sodium chloride (see Fig. 2.6). Imagine a box with alternating sodium and chloride atoms at each of the corners of the box. These boxes then are stacked in each dimension so far as the eye can see. Although it is slightly more difficult, it is still relatively easy to imagine the repeating patterns of atoms for materials crystallizing in the other crystal systems. It is much more difficult to imagine the crystal structure for a simple organic molecule like caffeine (see Fig. 2.7). We clearly cannot

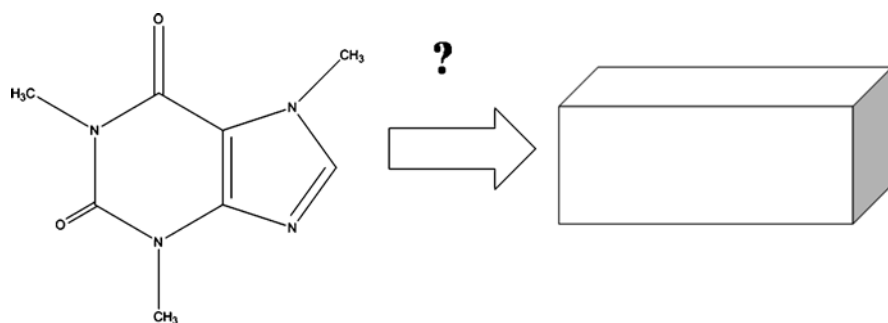
**Table 2.1** Crystal systems and orientation

Crystal system	Axis orientation and spacing
Cubic	Three axes at 90° to each other Equal spacing along each axis
Tetragonal	Three axes at 90° to each other Equal spacing along two axes, unequal spacing along one axis
Hexagonal	Three axes at 120° to each other in same plane Equal spacing along those axes One axis at 90° to plane of other axes
Orthorhombic	Unequal spacing along that axis Three axes at 90° to each other Unequal spacing along each axis
Monoclinic	Two axes oblique to each other in one plane Unequal spacing along each axis One axis normal to plane of two axes
Triclinic	Unequal spacing along that axis Three oblique axes Unequal spacing along each axis

Crystal systems can be categorized by the angle of the crystal axes the spacing of elements along those axes



**Fig. 2.6** Repeating pattern for sodium chloride crystal. The repeating pattern for sodium chloride crystals is made up of alternating sodium and chlorine atoms. This illustration shows the pattern in two dimensions. In a real crystal, the pattern extends in all three dimensions. The box illustrates the two-dimensional face of the unit cell. It also extends in three dimensions



**Fig. 2.7** Fit of caffeine molecule in tetragonal unit cell. It can be difficult to imagine how a caffeine (or other organic) molecule fits in the unit cell. The cell is not arranged so that each atom of the molecule sits at the junction of axes but one or more caffeine molecules sit within the unit cell. Many caffeine molecules are arranged for optimum fit within the unit cell. The unit cell then becomes an abstract boundary for the molecules

arrange the repeating structure based on individual atoms sitting at the junction of axes or even at spaces along the axes. It is pretty hard to imagine even the molecules themselves sitting at the corners like with sodium chloride. It can aid visualization to think of the crystal as a box with one, two, or more molecules sitting within the box. The molecule (or multiple molecules) sits in a particular orientation within the box and the size of the box is dependent on the orientation of the molecules and on the size of the molecules. These boxes are then stacked in three dimensions to form the crystal. Clearly, this is an abstract concept using imaginary boxes, but it is surprising how successful this artifice has been in understanding molecular structure in organic crystals. Rarely, do organic molecules fit into cubic boxes or into the other high symmetry systems such as the tetragonal and hexagonal. Most organic molecules crystallize into the orthorhombic, monoclinic, or triclinic systems.

One of the most surprising phenomena in nature, to my mind, is chemical polymorphism. Polymorphism is the ability of chemicals to arrange themselves into different geometric patterns that are physically stable in the solid state. One of the best examples of polymorphism is that of the element carbon (to a purist pure-element crystal forms are called allotropes). In one physically stable orientation, carbon forms graphite which is soft and used as a lubricant and as pencil “lead.” Carbon atoms in graphite are arranged in sheets with strong bonds within a sheet and relatively weak bonds between sheets. In the other orientation, carbon forms diamond which is the hardest natural mineral with very different properties than graphite. Carbon atoms in diamond are arranged in a tetrahedron repeating pattern. Interestingly, at ambient conditions of temperature and pressure, graphite is the thermodynamically stable form, whereas diamond is metastable – which means that diamond should spontaneously convert to graphite. The time period for this conversion is estimated at longer than the estimated age of the Universe. So, no need to worry about our diamond rings. This subject of polymorphism is critical to pharmaceutical development and we will discuss it in much more detail in Chap. 8. It is crucial to understand basic concepts of crystallography in order to understand chemical polymorphism.

As a final note on this section, the most common and accepted technique for probing crystallography is single crystal X-ray diffraction structure analysis. A single crystal is placed into a goniometer (device for orienting crystals) and irradiated with X-ray radiation of a single wavelength (usually Cu  $k_{\alpha}$  line at 8.05 keV). The resulting diffraction pattern (pattern of bright dots with varying intensities) is collected and then the crystal moved and another pattern collected. Thousands of patterns may be collected and the spot positions, angles and intensities are measured. From these measurements, it is possible to make a map of the crystallographic structure of the crystal and determine key parameters such as unit cell dimensions and angles as well as space group. For instance, single crystal X-ray experiments have determined that caffeine hydrate is monoclinic with space group  $P2_1/C$ . The unit cell dimensions are as follows:  $a - 3.974 \text{ \AA}$ ;  $b - 16.751 \text{ \AA}$ , and  $c - 14.800 \text{ \AA}$  with a  $\beta$  angle of  $95.8^\circ$ . There are four molecules in the unit cell (Edwards et al. 1997). Although X-ray diffraction is the standard for crystallography, optical methods have some applications in physical pharmacy and will be discussed next. The following texts are two among many that are helpful for learning the principles of crystallography: Bloss 1971; McKie and McKie 1986.

## 2.5 Optical Crystallography

I have 10–15 books dealing in whole or in part with the methods of optical crystallography and I do not have anywhere near an exhaustive library on the subject. I think the number of texts is a testament both to the usefulness and the difficulty of the subject. Optical crystallography is most commonly applied in mineralogy but is also directly applicable to organic chemicals. Optical crystallography is based on the fact that light travels with different speeds in different directions in the crystal. The methods of optical crystallography take advantage of this property. Given the range and specificity of optical crystallographic properties of pharmaceutical compounds and a good reference database, it is possible to identify single small crystals even to the point of distinguishing between similar polymorphs. Add in thermal microscopy, IR and Raman microscopy, and SEM/EDS and we have a powerful identification scheme, indeed.

To be frank, I struggle with the best way to approach the subject of optical crystallography in the context of pharmaceutical microscopy. The subject could easily take over and use up all the available space. Then we would have a text on pharmaceutical optical crystallography and not pharmaceutical microscopy. This section may seem complex and, in part, that is due to the complexity of the subject matter. In this section, I hope to introduce the topic and give you a few exercises to get started. I have also concentrated on those topics, such as refractive index measurements, that are immediately useful in our field and are not very hard to learn and perfect. I am also going to concentrate more on the nature of the observations and less on the underlying crystallography. I am not going to introduce the optical indicatrix even though it is the central means of structuring optical crystallographic properties. While useful, it requires too much space to adequately explain. In some cases, I will only list the property and a reference to more information. I do hope you develop an interest in the subject. It is gratifying on a personal level and I think has great utility in pharmaceutical development. I believe that if more of us develop and use the technique, we will continue to find more and more applications for it.

Tables 2.2 and 2.3 present a list of observations that one can make with the polarizing light microscope and the observations that are particular to each crystallographic system. I list the properties according to how one sets up the microscope: no polars, one polar, crossed polars (although there is plenty of crossover). As mentioned, my emphasis is on the observations and not as much with the theory. We will briefly discuss each of these properties in the next section. I have chosen this format since it corresponds with the way that we set up polarized light on the microscope. I intend the two tables as a quick reference for the types of observations and measurements that can be made using polarized light. I discuss refractive index in the next section (2.6) since it is the most common microscope measurement in pharmaceutical development and deserves to be emphasized. On the other hand, in order to make accurate measurements of refractive index, one must be familiar with optical crystallography.

**Table 2.2** Description of optical properties of crystals

Optical property	Description
Color	Color of thick sections in brightfield
Habit, shape	Habit for well-shaped crystals, morphology of particles
Aggregation, agglomeration	Crystals or particles mechanically or chemically fused together Appearance and resistance to breakage
Twining	Individual crystals sharing face(s)
Cleavage	Description of manner in which crystals break
Surface texture	Appearance of crystal, particle surface
Transparency	How easily does light pass through particle
Edge angles	Angles between crystal faces
Dispersion staining colors	Central stop colors in high dispersion R.I. liquids near refractive index of particles
Pleochroism	Color change on stage rotation
Refractive indices	Formally, speed of light in vacuum divided by speed of light in particle. Measured by comparing particle relief in known R.I. liquids. Number of principal indices dependant on crystal system
R.I. Dispersion	Variation of refractive index with wavelength
Interference colors	Color of crystal between crossed polars, depends on birefringence, crystal thickness, and crystal orientation. Saturation of color dependant on order
Anomalous interference colors	Different sequence of colors related to crystal thickness
Birefringence	Difference between high and low refractive indices
Extinction position	All crystals and crystal fragments will go dark four times on rotation of the stage (unless the crystal is oriented so that the optic axis is in parallel to the light path). Refractive index measurements are generally made at extinction positions for anisotropic crystals
Extinction angle	The angle of a crystal edges with the positions of the crossed polars (generally aligned with crosshairs of the eyepiece) when the crystal is dark. Only pertinent to crystals in the monoclinic and triclinic crystal systems
Dispersion of extinction angles	Variation of extinction angle with wavelength
Interference figure	An image of light interference at the back focal plane of an objective for anisotropic crystals. Uniaxial interference figures have Maltese cross appearance or part of the cross depending on crystal orientation. Biaxial interference figures have appearance of hyperbola or parts of the hyperbola depending on crystal orientation and optic axial angle
Optic sign	Sign of birefringence, for uniaxial crystals if $\epsilon > \omega$ optic sign if positive, negative if opposite. For biaxial crystals, if value of $\beta$ closer to that of $\gamma$ , optic sign if negative, if closer to $\alpha$ then optic sign is positive
Optic axial angle	Angle between optic axes, evidenced as angle between dark regions in interference figure. Is $90^\circ$ for uniaxial and orthorhombic crystals, and between $0$ and $90^\circ$ for biaxial crystals (monoclinic and triclinic)
Dispersion of optic axes	Variation of angle of optic axes with wavelength

(continued)

**Table 2.2** (continued)

Optical property	Description
Acute bisectrix	Vibration direction bisecting acute angle between optic axes
Obtuse bisectrix	Vibration direction bisecting obtuse angle between optic axes
Optical orientation	Relationship between crystallographic directions and optical directions. Fixed in uniaxial crystals, but varied in biaxial
Sign of elongation	For elongated uniaxial crystals (and fibers that act like uniaxial crystals), if the high index is associated with the length then the sign is positive, negative otherwise. Not related to crystal symmetry

**Table 2.3** Relationship of optical properties to crystal system

	Isotropic	Anisotropic, uniaxial	Anisotropic, biaxial
Optical property	Cubic, disordered	Tetragonal, hexagonal	Orthorhombic, monoclinic, triclinic
<i>No polars</i>			
Color	X	X	X
Habit, shape	X	X	X
Aggregation	X	X	X
Twining	X	X	X
Cleavage	X	X	X
Surface texture	X	X	X
Transparency	X	X	X
Crystal edge angles	X	X	X
Dispersion staining colors	X	X	X
<i>1 Polar</i>			
Pleochroism		X	X
Refractive indices	X (polar not required)	X ( $\epsilon$ , $\omega$ )	X ( $\alpha$ , $\beta$ , $\gamma$ )
Birefringence (calculated)		X	X
R.I. Dispersion	X (polar not required)	X	X
<i>Crossed polars</i>			
Interference colors		X	X
Anomalous interference colors		X	X
Birefringence		X	X
Extinction position		X	X
Extinction angle			X (Monoclinic and triclinic Only)
Dispersion of extinction angles			X
Interference figure		X	X
Optic sign		X	X
Optic axial angle (measured)			X
Optic axial angle (calculated)			X
Dispersion of optic axes			X
Acute bisectrix			X
Optical orientation			X
Sign of elongation		X	

Adapted from Table 3 (Relationship of Interference Figure to Optical Orientation) of Hartshorne and Stuart (1970, p. 360)

The following texts are in my opinion the best to consult to learn more about optical crystallography. McCrone et al. (1984) and Stoiber and Morse (1994) are currently available for purchase and are the best place to start. Hartshorne and Stuart (1970) is not currently available but is well worth the trouble to obtain on the used book market. Bloss (1961) is considered the standard by many people and is available but in a newer version than the one I list. Wahlstrom (1979) is also considered a standard text, although I have not used the text extensively myself. For the best short introduction to the entire subject of crystals and optical crystallography, see Wood (1977). There are also a number of excellent Web sites with descriptions of these properties and applications (see Web site section in References below).

### ***2.5.1 No Polars, Brightfield***

#### **2.5.1.1 Color**

Some drug substance is naturally colored due to absorption and can be seen in reflected light as well as transmitted. The initial observation of color is best made visually without magnification since the colors are generally less intense in the PLM. I also use the stereomicroscope and, if necessary, record an image to document the color. We need to be careful about stating color since the specific color depends on the light used and whether we are looking in transmission or reflection.

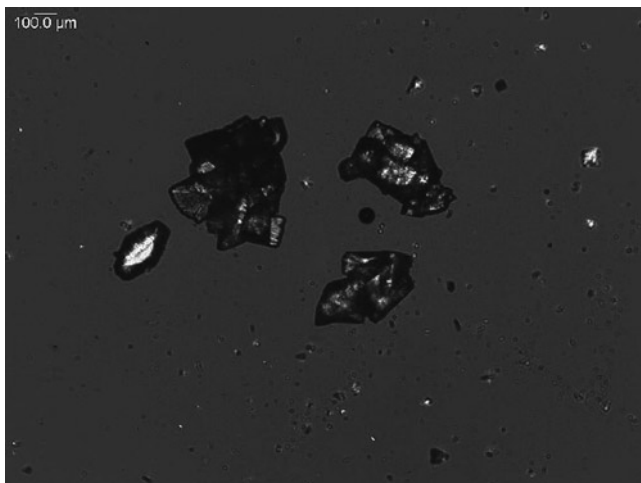
#### **2.5.1.2 Habit, Shape**

Habit and shape are related concepts but with some important distinctions. Habit refers to an ideal crystal shape. For instance, cubic crystals can grow as cubes or as rhombohedra. Rarely, do we see ideal crystals on the microscope and the actual crystal shape may be distorted compared with the ideal. Shape refers to actual geometrical features such as elongation of one or another crystal face forming rods, flakes, needles, etc. Descriptions of habit are standardized whereas those of shape are not. Aldrich and Smith (1995) have a good description of shape that I use and we will consider the subject in more detail in Chap. 9. In any case, be consistent. Actual photomicrographs of drug substance that illustrate the meaning of shape terms are useful for reference.

#### **2.5.1.3 Aggregation, Agglomeration**

These terms refer to the tendency for particles to stick to each other. Again the usage of the terms is not standardized and it is a good idea to define your terminology in journal articles or presentations. In general, the distinction between the two terms refers to the resilience of the particle clumps and the ease of breaking them apart. When you open a bottle of cohesive drug substance, you generally will see round





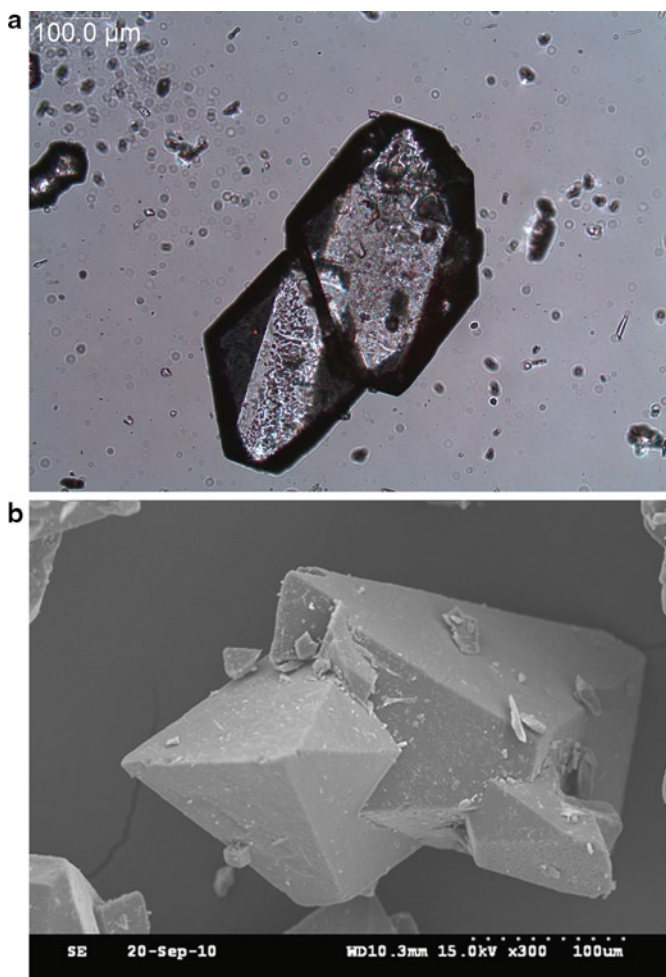
**Fig. 2.8** Carbamazepine Form III. This image illustrates typical particle agglomeration as seen in the optical microscope. (Image size =  $1,400 \times 1040 \mu\text{m}$ )

clumps of particles. In many cases, they are so loosely stuck together that the clump cannot be removed without breaking it whereas, in other cases, you cannot break the clump without exerting strong forces. There is no standardization with regard to whether aggregates or agglomerates are the resilient clumps and vice versa. Out of habit, I use the term agglomerates for all types of clumping with an adjective describing degree of resilience.

Those agglomerates that cannot be easily broken generally have developed chemical bonds between particles. I generally note the degree of agglomeration with reference to primary particles. In other words, if I see large agglomerates visually, are these agglomerates of agglomerates or agglomerates of primary particles? Figure 2.8 is carbamazepine directly from the bottle and illustrates what I mean by a highly agglomerated system. Note that 10 s of primary particles are joined together in large agglomerates. In this case, the agglomerates are resilient and do not break apart easily. I first judge the presence of agglomerates visually on the stereomicroscope as I am making slides (i.e., ease of breaking clumps under coverslip) and then using the PLM. These observations can be of practical interest to the process engineer as he or she develops the final crystallization process.

#### 2.5.1.4 Twinning

Twins are crystals that share a crystallographic plane or face. It should not be confused with crystals that may happen to be joined together by electrostatic forces or even are connected by a bridge of material. In other words, there is a significant difference between twinning and agglomeration, although some samples may display both phenomena. Twinning is not uncommon in pharmaceuticals but only



**Fig. 2.9** (a) Optical photomicrograph and (b) SEM image of twinned carbamazepine. These images illustrate typical interpenetration twins. SEM is most useful for this type of twinning whereas PLM is better suited to twins joined along their length. (PLM image size =  $2,800 \times 2,080 \mu\text{m}$ ; SEM image size =  $407 \times 330 \mu\text{m}$ )

rarely has any direct effects on bulk powder processing or use. Twinning can be a particular problem when attempting to grow good crystals for single crystal X-ray diffraction studies. In my experience, the most common twins are joined along their length and form a chevron shape. Figure 2.9a, b illustrate interpenetration twins with carbamazepine. Twinning of this type can be somewhat difficult to detect using optical microscopy but is relatively easy to detect with the SEM. Twins joined along their length may be easier to detect with PLM since, in general, each member of the twin will go to extinction in crossed polars at different stage orientations.

### 2.5.1.5 Cleavage

Cleavage in science refers to the manner in which minerals and other crystals break. Two good mineral examples are mica which breaks into large, thin, flat sheets and asbestos that breaks along its length into very fine fibrils. Pharmaceutical compounds do not usually break along distinctive cleavage planes and so most often are cited as having irregular cleavage. Preferential breakage can be highly important in bulk processing and so it is good to get in the habit of looking for distinctive cleavage. A good example of this impact is highlighted by Nichols and Frampton (1998) in their study of paracetamol (acetaminophen). Form I cannot be directly compressed in part due to how it cleaves apart, whereas Form II is suitable for direct compression.

### 2.5.1.6 Surface Texture

Surface texture is not properly a crystallographic property but is most often related to crystallization conditions, solvent loss or to polymorphism. Solvent loss may show up as pockmarks on the surface whereas polymorphism often results in the presence of small crystals on the surface. The latter can be particularly useful in stability trials since the presence of these growing crystals may be detected with the microscope before being detected by X-ray powder diffraction or calorimetry. Figure 2.10 illustrates this last point with caffeine Form I converting to Form II.



**Fig. 2.10** Caffeine Form II growing on crystal of Form I. Appearance of crystals growing on the surface of the original crystals is one good application of assessment of surface texture during stability trials. (Image size=210×170 μm)

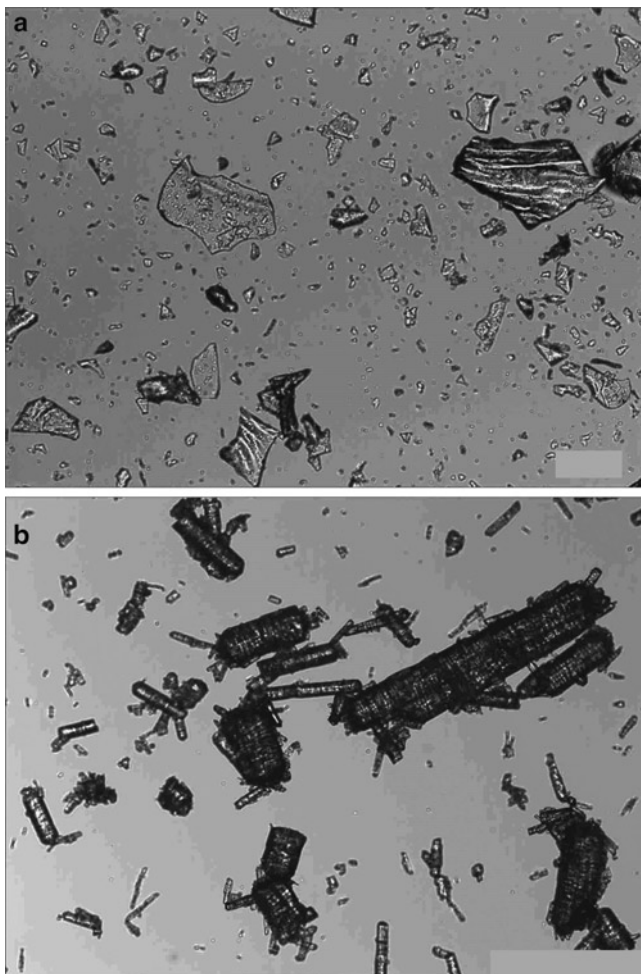
You can see a number of fibers of Form II on the larger particle of Form I. Note that caffeine Form II is the stable form at ambient conditions, not Form I, and so there is a thermodynamic driving force for the solid-state conversion from I to II. Also, be careful with these observations. Just because we see growing crystals does not necessarily mean a form change, although that is the most probable cause. Such observations call for more study and one may need to apply significant effort in order to characterize the growing form.

#### 2.5.1.7 Transparency

Most pharmaceutical compounds are transparent in transmitted light. There are a number of reasons why the particles may not be transparent and the observation can be helpful in development. The lack of transparency could be related to inclusions of gas or liquid as a result of crystallization conditions such as solvent system, mixer stir speed, presence or lack of crystallization seeds, among others. One of the most important causes of a lack of transparency is due to loss of solvent in a solvated system. Figure 2.11 shows a sample that loses solvent on stability. The first photo (a) is the initial sample, whereas (b) is after 3 months stability at 30°C and 65% relative humidity. The change in transparency is an indication of loss of solvent and that the system is solvated. Typically we think of desolvation occurring as the result of high temperature or even low humidity if the system is hydrated. Some-times, though, the freshly crystallized material will show signs of desolvation. This observation is generally due to desolvation as part of the final crystallization step.

#### 2.5.1.8 Edge Angles

There are two laws of crystallography that affect crystal habit and shape. The first is the law of constancy of interfacial angles which states that angles among faces of crystals of the same compound are constant. In other words, no matter how distorted the shape, the face angles are all the same for the same compound, providing, of course, each crystal is the same polymorph. While this law may seem self-evident today, it was important in the development of crystallography. The law of rational indices states that the ratio of lengths of crystal face intercepts can be reduced to small integers. This is the genesis of the Miller indices as a method of describing crystal faces. The application of these laws to practical crystal measurement has a long history but is not particularly useful in pharmaceutical microscopy. Hartshorne and Stuart (1970, pgs 255 – 257) have an excellent discussion of the practical means of making these measurements on the microscope. I urge caution in these measurements, since it is easy to make large errors.



**Fig. 2.11** Appearance of desolvated sample: (a) before desolvation and (b) after desolvation. Image (a) is the initial sample, whereas (b) is after storage at 30°C and 65% relative humidity for 3 months. The loss of transparency is one indication of a loss of solvent. (Image size = 1,400 × 1,040 μm)

### 2.5.1.9 Dispersion Staining Colors

Dispersion staining is an optical staining technique that takes advantage of the difference in relationship between light wavelength and refractive index between solids and liquids. The refractive index of liquids changes much more with wavelength compared with solids. The technique requires the use of high dispersion refractive index liquids and a special objective. I discuss this technique in more detail in Chap. 6 and McCrone et al. (1984) has an extensive discussion of the method and its applications.

## **2.5.2 One Polar**

### **2.5.2.1 Pleochroism**

Pleochroism is observed as different crystal colors depending upon orientation of the crystal to the vibration direction of the one polar. Pleochroism is due to selective absorption of the refracted ray (Nichols 2006). It is a common test in mineralogy and is seen by inserting one polar and rotating the stage. It is not particularly common for pharmaceuticals but can be diagnostic as an identification tool if the colors can easily be detected in small particles.

### **2.5.2.2 Refractive Indices (Dispersion of)**

Discussed below in [Sect. 2.6](#)

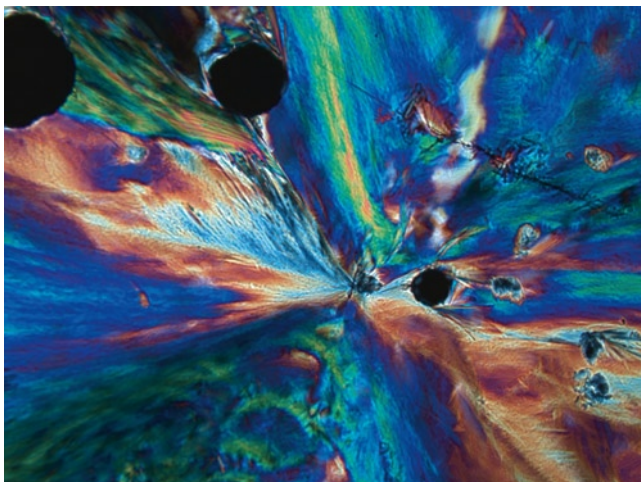
## **2.5.3 Crossed Polars**

### **2.5.3.1 Interference Colors (Anomalous)**

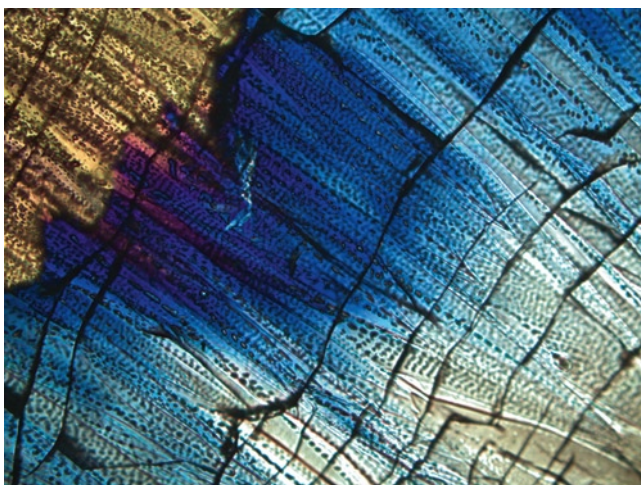
Interference colors produce beautiful images of crystals in crossed polars such as acetylsalicylic acid (aspirin) which has been melted and allowed to recrystallize in a thin film under a coverslip (see Fig. 2.12). Recrystallization from the melt is often referred to as a fusion preparation. Besides the aesthetic pleasure, interference colors are useful in determining optical crystallographic properties. Interference colors result from recombination of doubly refracted rays. If we look at the back focal plane of the objective, we see an interference figure which is the diffraction pattern of the same recombination. The particular color observed depends upon the birefringence (difference between high and low refractive index which is indicative of the retardation of light speed experienced by the refracted ray also referred to as retardation) and the thickness of the particle. The color sequence with thickness goes as Newton's series and can be divided into orders which can be seen in Fig. 2.12 in the fan-shaped crystal wedge extending from the center of the photo toward the upper left (see also Fig. 2.14 of the Michel-Levy chart). It is useful to note the interference color and order of most particles in a field of view.

Since cubic crystals and disordered materials have only one vibration direction and one refractive index, they do not display interference colors. This fact is used as a sensitive test for crystallinity. Interference colors (and the lack thereof) can also result from other factors so we should be careful in using this test for crystallinity. For instance, strained glass will show faint interference colors at the edges of the particles. Organic fibers display interference colors as do liquid crystals, even though they are not crystals in the classic sense. These exceptions are easily detected with experience.



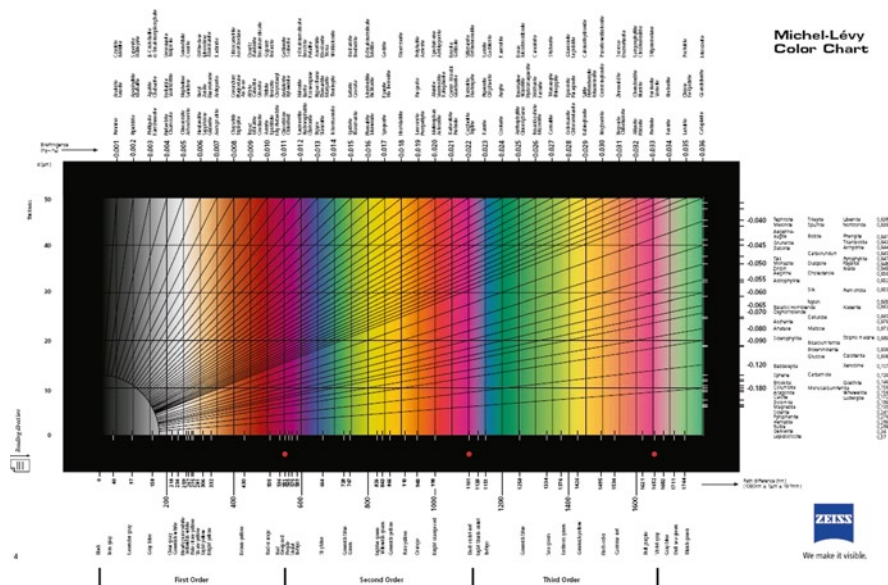


**Fig. 2.12** Acetylsalicylic acid (aspirin) fusion preparation. Illustrates the beauty and utility of crystal interference colors. Note the color progression extending from the central region toward the *upper left* of the photomicrograph. The color progression proceeds from *black* to *white*, *yellow*, *orange blue*, and *red*. The colors are related to retardation and crystal thickness. (Image size=2,800×2,080  $\mu\text{m}$ )



**Fig. 2.13** Benzil fusion preparation. Anomalous polarization colors as displayed by a benzil fusion preparation. Note the progression from *white* to *blue* and the difference in color progression as compared with acetylsalicylic acid in Fig. 2.12. (Image size=2,800×2,080  $\mu\text{m}$ )

Some materials will display anomalous polarization colors that do not follow Newton's series (black, gray, white, yellow, orange, red, blue, and green). This phenomenon can be useful for identification since materials displaying anomalous polarization colors are relatively rare. Figure 2.13 shows an example of anomalous



**Fig. 2.14** Michel-Lévy Chart (copied with permission by Carl Zeiss, Inc.). The Michel-Lévy chart shows the progression of interference colors with thickness based on the birefringence of the material. The chart can be used to estimate birefringence as an aid in refractive index determinations

colors with a fusion preparation of benzil. Note that the progression (lower right to upper left) proceeds from white to blue. This phenomenon is due to selective absorption of certain wavelengths by the material.

### 2.5.3.2 Birefringence

Birefringence is the difference between the highest and lowest refractive indices for anisotropic crystals and is represented by upper case  $B$ . It is a fundamental optical crystallographic property and should be quoted with refractive index values. Birefringence can also be estimated using the Michel-Lévy chart (see Fig. 2.14 which is a copy of the Michel-Lévy chart as produced by Zeiss) and the thickness of the specimen. Retardation is defined mathematically as follows:

$$r = 1,000t \times B \quad (2.5)$$

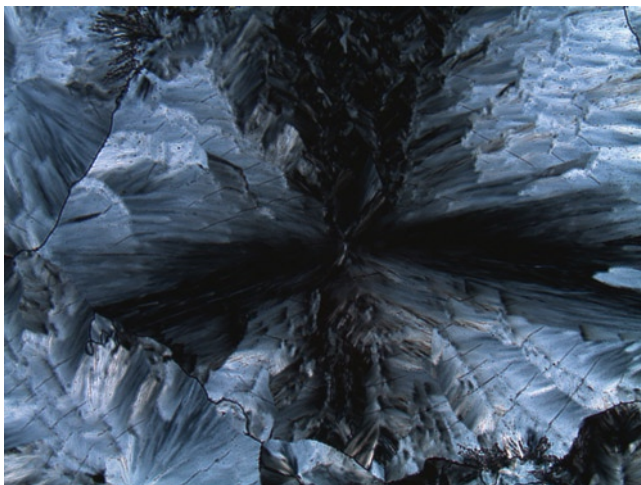
where  $r$  is the retardation in nm,  $t$  is the thickness in  $\mu\text{m}$ , and  $B$  is the birefringence. We use the Michel-Lévy chart in multiple ways, but one good use is to estimate birefringence when doing refractive index measurements. We can estimate the retardation based on color and then measure the crystal thickness. Using the equation above or the graphical solution with the chart, we can then estimate birefringence.



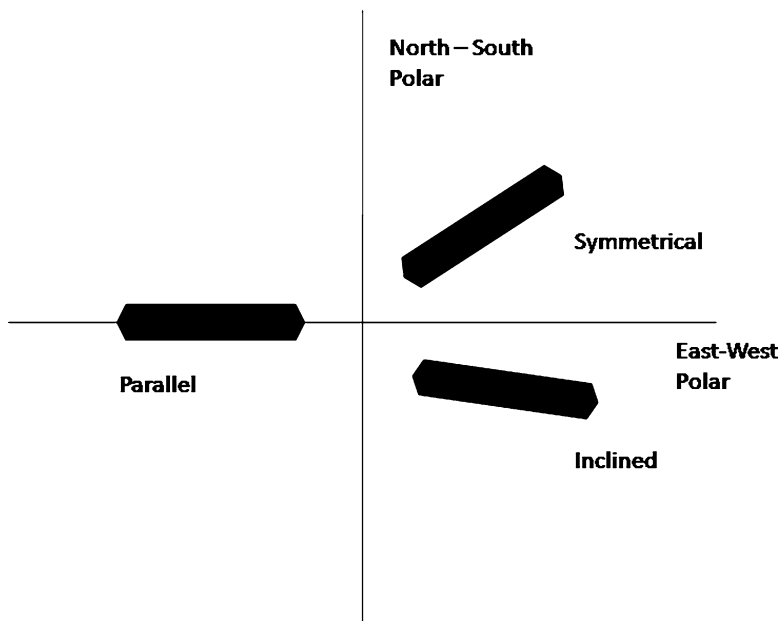
Knowing birefringence and one measured index, we have a good idea what the other index will be. Birefringence can depend upon the variation of refractive index with wavelength.

### 2.5.3.3 Extinction Position and Angle (Dispersion of)

On rotation of the microscope stage, the vibration direction of the crystal will be parallel to the vibration direction of the crossed polars four times on a complete rotation of  $360^\circ$ . When the vibration direction of the crystal and the directions of the polars are aligned there will be no interference and, hence no color. The crystal will be black against a black background (see Fig. 2.15 showing an acetylsalicylic acid fusion preparation with crystals at different orientations). Cubic and disordered materials, of course, do not go to extinction since they do not have interference colors. Anisotropic materials can display different extinction properties. There are three types of extinction related to crystal habit called parallel, symmetrical, and inclined or oblique. If we orient the vibration direction of our polars with the eyepiece crosshairs, we can use some distinctive feature of the crystal habit to determine the type of extinction and the angle if appropriate. Figure 2.16 is a drawing with the representation of the different types of extinction and angles. As a cautionary note, some modern microscopes allow you to rotate both analyzer and polarizer independently. Be careful that the polars are completely crossed and aligned with the eyepiece crosshairs when measuring extinction angle. Materials crystallizing in the tetragonal, hexagonal, and orthorhombic systems will have either parallel or



**Fig. 2.15** Extinction positions of acetylsalicylic acid. This fusion preparation of acetylsalicylic acid shows the different extinction positions of the crystals oriented in a radial fashion. The dark crystals are oriented with the polars and show parallel extinction. (Image size =  $2,800 \times 2,080 \mu\text{m}$ )

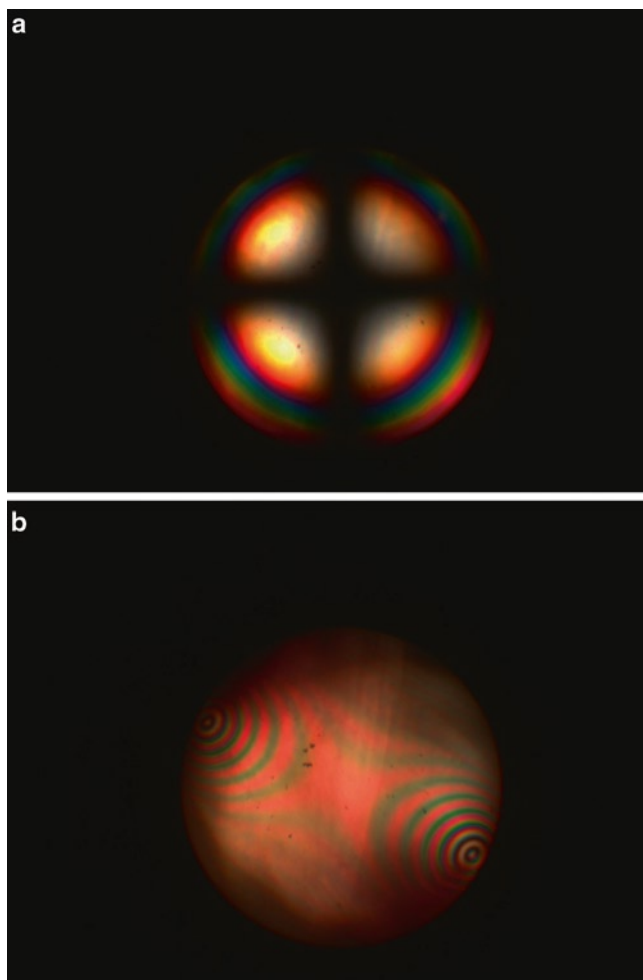


**Fig. 2.16** Schematic drawing of extinction positions. Crystals can have parallel, symmetrical, or inclined extinction. Monoclinic and triclinic crystals are the only ones that display inclined extinction. The extinction angle is constant for crystals but can vary with wavelength

symmetrical extinction and hence no extinction angles. Monoclinic and triclinic crystals do have extinction angles. The extinction angle can depend upon wavelength and should be checked with the appropriate filters.

#### 2.5.3.4 Interference Figure

Materials that exhibit the phenomenon of double refraction are said to be anisotropic, whereas cubic crystals and disordered materials are said to be isotropic. As mentioned, interference figures are formed at the back focal plane of the objective in crossed polars when the refracted rays of anisotropic materials recombine and interfere. Materials in crystal systems with two vibration directions (tetragonal and hexagonal) are said to be uniaxial and form a distinctive Maltese cross interference figure. Materials in crystal systems with three vibration directions (orthorhombic, monoclinic, and triclinic) are said to be biaxial and form a distinctive hyperbola type interference figure. At this point, I will invoke Needham again and demonstrate rather than describe. Figure 2.17 presents uniaxial and biaxial interference figures for sodium nitrate and Mylar, respectively. These materials are good for demonstration purposes since they generally show centered interference figures, whereas most crystals and crystal fragments show off-center interference figures. The black brushes of the uniaxial and biaxial



**Fig. 2.17** (a) Sodium nitrate, uniaxial interference figure; (b) mylar, biaxial interference figure. A Sodium nitrate fusion preparation and Mylar show centered uniaxial and biaxial interference figures. Most crystals and crystal fragments show off-center figures

interference figures are called isogyres, whereas the focal point of each arm of the hyperbola in a biaxial interference figure is called a melatropes. The melatropes corresponds with a particular vibration direction in the crystal. The Zeiss brochure on the Michel-Levy Chart and Polarized light (see Web site reference) has some instructive images of conoscopic figures.

How do we set up the microscope so as to observe interference figures? First, we want to use objectives with a high numerical aperture. Use of apochromatic objectives is ideal. We need to perfectly center the objective to the stage as evidenced by the crystal remaining centered on full rotation of the stage (see microscope

manufacturer on how to center the objective – some are easy and some are quite awkward). The polars need to be completely crossed and aligned with the eyepiece crosshairs. In order to see the figure, we can use an intermediate lens called a Bertrand lens and ideally it is focusable. Bertrand lenses are available with most modern petrographic microscopes. Alternatively, we can remove the eyepiece and replace it with a pinhole cap. The cap is, as the name implies, a very small hole in the eyepiece cap. You can see the interference figure directly and then observe its motion upon rotation of the stage.

When the interference figure is centered, it is easy to recognize its character and to make measurements on it. In general, though, we see off-center interference figures that are much more difficult to characterize and to measure. This is particularly the case when examining crystal fragments and small particles. There is an art to the recognition and use of such off-center figures and Stoiber and Morse (1994) have an extensive discussion of how to recognize these figures and to make the most of them. I recommend that you consult that text to pursue the topic in more detail. Although not particularly difficult to master, it does require a great deal of practice to become skilled at the recognition and use of off-center interference figures.

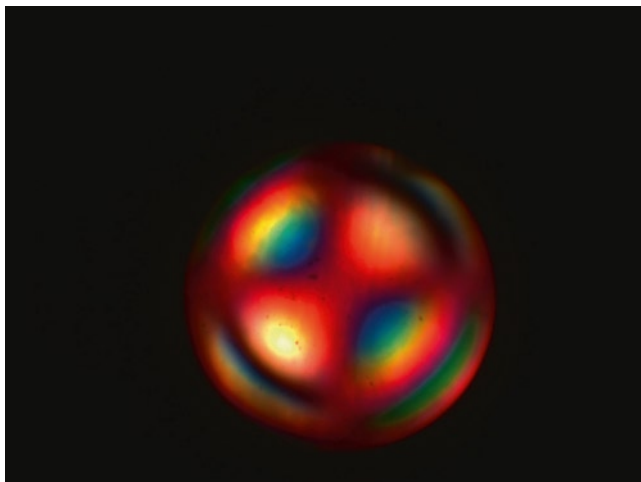
You might notice that the photomicrograph of the Mylar interference figure in Fig. 2.17b is poor. In fact, that image is representative of centered interference figures and a Bertrand lens. The image clarity is somewhat better with a pinhole eyepiece but do not expect high clarity with interference figures. They can be quite difficult to see well.

### 2.5.3.5 Optic Sign

The optic sign is also referred to as the sign of birefringence. For uniaxial crystals, if  $\varepsilon > \omega$  the optic sign is positive, and is negative if opposite. For biaxial crystals, if the value of  $\beta$  closer to that of  $\gamma$ , the optic sign is negative, if closer to  $\alpha$  then the optic sign is positive. Alternatively, we can directly determine the optic sign using a full waveplate compensator. The color in different quadrants of the interference figure after insertion of the compensator indicates the optic sign. This property is another one of those better demonstrated than described tests (see Fig. 2.18 for the appearance of the color seen with a lambda compensator indicating the optic sign). Stoiber and Morse (1994), Hartshorne and Stuart (1970), and McCrone et al. (1984) all discuss the use of compensators. Delly's articles (Delly 2003a, b) on the Michel-Levy chart and Senarmont compensation also have discussion of compensators (see internet references, McCrone Associates Modern Microscopy Web page).

### 2.5.3.6 Optic Axial Angle (Dispersion of)

The refracted rays of light travel in different vibration directions in the crystal. The angle between these vibration directions is called the optic axial angle. It can be seen as the angle between dark regions in an interference figure. Since all



**Fig. 2.18** Uniaxial interference figure with lambda compensator. The uniaxial interference figure of sodium nitrate (Fig. 2.17a) has *red color* in the second and fourth quadrants and *blue color* in the first and third quadrants. This is a negative optic sign

crystallographic angles are  $90^\circ$  for uniaxial (not true for hexagonal crystals but is true of hexagonal interference figure) and orthorhombic crystals, the optic angle will be  $90^\circ$ . The vibration directions for biaxial crystals will lay between 0 and  $90^\circ$  for biaxial crystals (monoclinic and triclinic) as will the optic axial angles.

Determination of the exact angle (referred to as  $2V$ ) for monoclinic and triclinic crystals is complicated by the fact that the angle we infer from the microscope is larger than the angle in the crystal since the light we observed is refracted by the oil in the mount and the air between the coverglass and objective. McCrone et al. (1984, p. 155), has a good discussion and illustration of this effect. See Bloss (1961, 1981) for more detailed approaches to calculating and measuring the optic axial angle. It is also possible to make approximate estimates using the appearance of the isogyres. McCrone et al. (1984, p. 159) discusses this approach and provides a nice schematic illustration of this approximation.

As with many other optical crystallographic properties, the optic axial angle can have dispersion due to wavelength. Dispersion of the optic axes can be an important identifying characteristic.

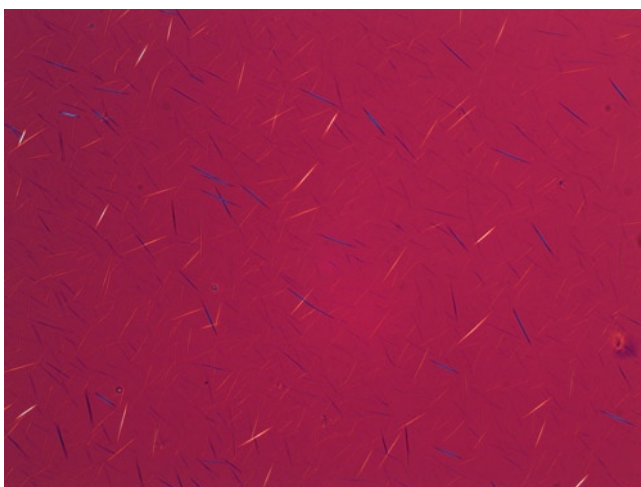
### 2.5.3.7 Acute Bisectrix, Obtuse Bisectrix, Optical Orientation

I am simply going to mention these crystallographic properties and recommend the following references for more information (Bloss (1961), Stoiber and Morse (1994), Hartshorne and Stuart (1970), and Wahlstrom (1979)). These properties are important in the complete description of the optical crystallography of a

biaxial crystal, but are a bit beyond this text. The key point to remember though is that the high, low, and intermediate refractive indices correspond with particular crystal faces and edges and there is no *a priori* way of determining this orientation. The actual orientation of the optic axes will be skewed with regard to crystal faces and edges for monoclinic and triclinic crystals and must be determined. Again we use interference figures and extinction angles to make these determinations.

### 2.5.3.8 Sign of Elongation

For elongated uniaxial crystals (and fibers that act like uniaxial crystals), the color of the crystal with crossed polars and inserted full waveplate will depend upon crystal orientation. The color depends on whether the high refractive index is associated with the length or the breadth of the elongated particle. If the high index is associated with the length then the sign is positive, negative otherwise. One determines the sign of elongation by orienting the long axis of the crystal at  $45^\circ$  to the microscope polarizer vibration directions and then inserting the full waveplate. If the color increases the sign is positive, if the color decreases (by comparison with Newton's series), then the sign is negative. Figure 2.19 shows sublimed caffeine with a negative sign of elongation. This property is used with fibers that have internal strain that results in interference colors and resultant sign of elongation. Since most pharmaceutical compounds crystallize in low symmetry systems, this property is not often used in pharmaceutical development.



**Fig. 2.19** Caffeine with negative sign of elongation. Sublimed caffeine with crossed polars and lambda waveplate. Crystals with the long direction in the second and fourth quadrants are yellow, whereas those with the long direction in the first and third quadrants are blue. This color indicates a negative sign of elongation. (Image size =  $700 \times 520 \mu\text{m}$ )

### 2.5.4 Summary of Optical Crystallography

If you are new to the subject, I am quite sure that this section is somewhat overwhelming. The subject is complex but actually is not quite as difficult as it may seem since, to invoke Needham for a final time, the properties are relatively easy to demonstrate but quite difficult to describe with words. The best way to learn these techniques is with a talented and knowledgeable instructor. There are a few schools that are excellent at teaching polarized light microscopy and the McCrone Research Institute and Hooke College of Microscopy come to mind. The second best way to learn is to duplicate the work of others. Take some known materials and practice determining each of the properties. Finally, there are books such as this one and the examples from the literature. I hope I have shed some light on the techniques and we will revisit the subject in Chap. 8 when we look at specific applications of optical crystallography in the study of polymorphism.

## 2.6 Measurement of Refractive Indices

### 2.6.1 Introduction

The measurement of refractive indices is the most common operation in pharmaceutical optical crystallography and we will spend some space on its discussion. Refractive index (RI) is used for identification of compounds and in the study of polymorphism. It is also a key component of static laser diffraction particle size analysis using Mie theory. That theory requires knowledge of the average refractive index for accurate size measurements.

Refractive index measurements for cubic and disordered materials are relatively straight forward, but the measurements are complicated in anisotropic materials due to the fact that materials crystallizing in these crystal systems will have more than one principle refractive index. The observation of these indices in any specific crystal depends upon the optical orientation and the faces presented to the observer. The key points to remember about refractive index measurements are as follows: *cubic* crystals and disordered/amorphous materials have one refractive index; *uniaxial* crystals in the tetragonal and hexagonal systems have two principle refractive indices; and *biaxial* crystals in the orthorhombic, monoclinic, and triclinic systems have three principle indices. A fragment of a uniaxial or biaxial crystal, though, will show a range of indices from the high to the low values. So we must be able to recognize vibration directions in the crystal for the most accurate measurement of refractive index. Recognition of vibration directions is relatively easy in uniaxial crystals and anything but easy in biaxial crystals. Since most pharmaceutical drug substance is biaxial, highly accurate measurement of refractive index with pharmaceuticals requires a great deal of skill and experience.

On the other hand, there are simpler, less accurate techniques that generally serve the purpose for our work and these techniques are relatively easy to learn and apply. I am going to break the discussion of refractive index into four parts: (1) measurement of materials with one refractive index (cubic and disordered); (2) uniaxial crystals with two indices; (3) biaxial crystals with three indices by the crushed particle search method; (4) biaxial crystals using interference figures. I am not going to go into a great deal of detail with the fourth method since it is quite involved. For more information on interference figures with biaxial crystals see Stoiber and Morse (1994), McCrone et al. (1984), Hartshorne and Stuart (1970), and Bloss (1961)

### 2.6.2 *Cubic Crystals and Amorphous Materials: Basic Immersion Technique*

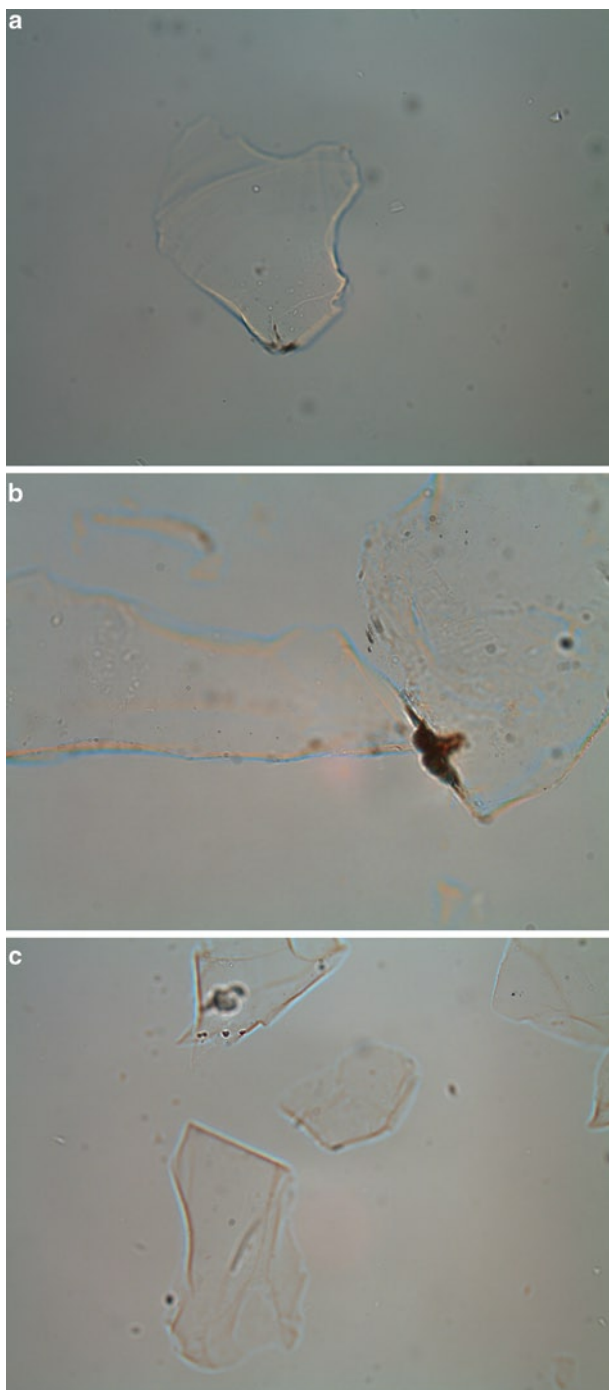
All refractive index measurements in solids compare the index of the sample with that of a known liquid and these measurements are generally referred to as immersion techniques. The R. P. Cargille, Inc. (Cedar Grove NJ USA) company manufactures a range of liquids with known refractive index and most of us use these liquids as our reference materials (if in doubt we can determine the index of the liquid using a refractometer). For samples with one index, the measurement is straight forward and simple. Mount particles of the sample in the reference liquid and compare the index of the solid with that of the liquid. If the solid index is higher, mount in a liquid of higher index, if the solid is lower vice versa. Continue in this fashion until you get a match. Once the refractive index is determined then correct the value for temperature and wavelength and report as such. The question then is how do we compare indices?

I favor the Becke line test and will describe it herein, although I know of many proponents of the oblique illumination method (see Allen 1962). The Becke line test uses the contrast of the particle in the liquid and the presence of a halo around the particle for the index comparison. When particle and liquid are exactly matched, it is not possible to detect the particle (although there may be inclusions and cleavage marks that are visible). This impossibility is due to the fact that there is no refraction of light at the interface between the liquid and solid, and hence, no contrast. When the liquid and solid refractive indices are close but not exactly the same, then you will see some particle contrast as well as a bright or dark halo around the particle. This bright halo is called the Becke line and the direction it moves when raising the focus of the microscope (generally by lowering the stage) is the mechanism for determining RI. The Becke line travels toward the material with the higher index. Figure 2.20 shows the appearance of the Becke line with glass fragments with an index of 1.540. Figure 2.20a shows a glass fragment in Cargille index liquid

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**Fig. 2.20** (continued) line (*bright line*) moves toward the material with the higher index as the focus is raised (generally as stage is lowered). Note the *blue* and *yellow colors* in (b). These colors are indicative of an RI match and are caused by the dependence of liquid and solid RI on wavelength. (Image size = 1,400 × 1,040 μm)





**Fig. 2.20** Glass particles in RI liquids illustrating Becke line. Glass fragment with RI of 1.540 mounted in Cargille liquids with indices of (a) 1.536, (b) 1.540, and (c) 1.544. Note that the Becke

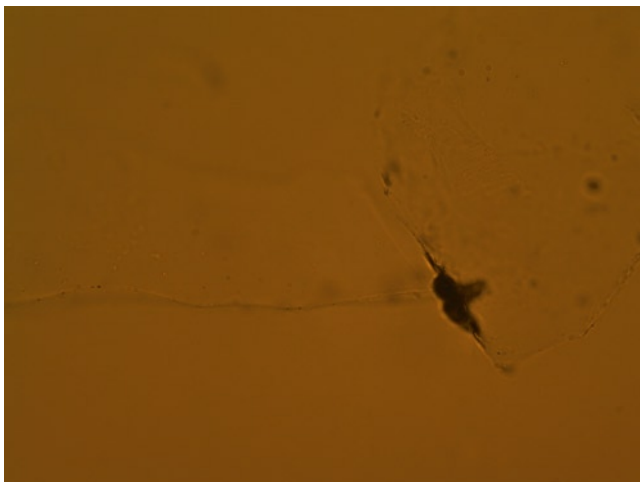
1.536 with the Becke line within the particle as the focus has been raised indicating that the particle has a higher refractive index than the liquid. Figure 2.20c shows a different glass fragment in Cargille oil 1.544 with the Becke line outside the particle as the focus is raised indicating that the index of the liquid is greater than that of the particle. Figure 2.20b shows a glass fragment in Cargill oil 1.540 with an index match. Note the blue and yellow colors of the particle in Figure 2.20b. These colors are due to RI dispersion and the fact that the index of the liquid and particle depends upon light wavelength.

I generally follow the steps listed below for RI measurements.

1. Place a small amount of the specimen onto a glass slide and cover with a small coverglass.
2. Place a small amount of the reference liquid next to the coverglass and allow the liquid to flow into the sample using capillary action.
3. Put the sample onto the microscope stage and observe with medium power lenses, for example 20 $\times$  and 40 $\times$ .
4. Close down the substage iris (for cubic crystals and disordered materials it is not necessary to insert either the polarizer or analyzer).
5. Note the contrast of the particles. With very high contrast the Becke line test may be ambiguous and it may be better to mount in two liquids much higher and much lower than the current one.
6. Raise the focus of the microscope (lower stage generally) while observing a particle of interest.
7. If the bright halo (Becke line) moves toward the particle, its index is greater than that of the liquid and the next liquid used should have a higher index.
8. If the bright halo (Becke line) moves toward the liquid, the particle index is lower than that of the liquid and the next liquid used should have a lower index.
9. Chose the next liquid based on the degree of contrast. If the contrast is low, chose a liquid close in value with the current one and vice versa.
10. Continue sequentially until you achieve a match (see description of the match in the next paragraph).
11. Verify by testing the next highest and next lowest index values.
12. Use interference filters (589, 486, and 656 nm) and verify the index. The indices are likely to be different at 486 and 656 compared to 589. Note these indices.
13. Measure the temperature on the stage and correct for temperature using the equation on the refractive index liquid bottle.
14. Report the index as temperature corrected and list the values for all three wavelengths.

It may seem like quite a few steps, but one can determine the index of a cubic or disordered material in 15 min or so with practice. I do recommend practice with known glass standards. Some sets of the Cargille liquids come with glass standards and they are excellent for practice. In fact, if I am measuring refractive index after an extended break, I will measure a few known materials just for the practice.

The exact index match can be determined by alternating with white light and monochromatic light (using a filter). At the match, you will see blue and yellow bands



**Fig. 2.21** Filter (589 nm) used for refractive index. A 589 nm interference filter has been inserted into the light path of the particles in Fig. 2.20b. No bright lines are observed. (Image size =  $1,400 \times 1,040 \mu\text{m}$ )

around the particle due to dispersion of index with wavelength (see Fig. 2.20b). These bands act like the Becke line as you raise focus. If you then insert your filter, you should see nothing at all. Figure 2.21 shows the effect of the 589 nm interference filter with the glass particles in Fig. 2.20b; note the lack of any bright lines. The particle should be invisible and there should be no Becke line. I have often thought I have a match and then used the 589 nm filter and determined I was slightly off. That's another reason I like to test one index liquid higher and lower, just to be absolutely sure of the correct value.

This exact same operation is used to measure the indices in uniaxial and biaxial crystals – once you have identified the principle vibration directions.

### 2.6.3 Uniaxial Crystals

Uniaxial crystals have two vibration directions and two principle refractive indices. Materials crystallizing in the tetragonal and hexagonal crystal systems are uniaxial and it is rare for pharmaceutical compounds to crystallize in these systems. The principle refractive indices are labeled using the Greek letters  $\epsilon$  (extraordinary ray) and  $\omega$  (ordinary ray), although there were other naming conventions used in the past and you will still occasionally see  $n_e$  for the extraordinary index and  $n_o$  for the ordinary index. In order to accurately measure uniaxial indices, it is necessary to recognize these two principle vibration directions, which turns out to be relatively easy to do. Although we measure the index with 1 polar inserted, we use both polars in the crossed position to determine vibration directions (extinction). Hence, in practice we

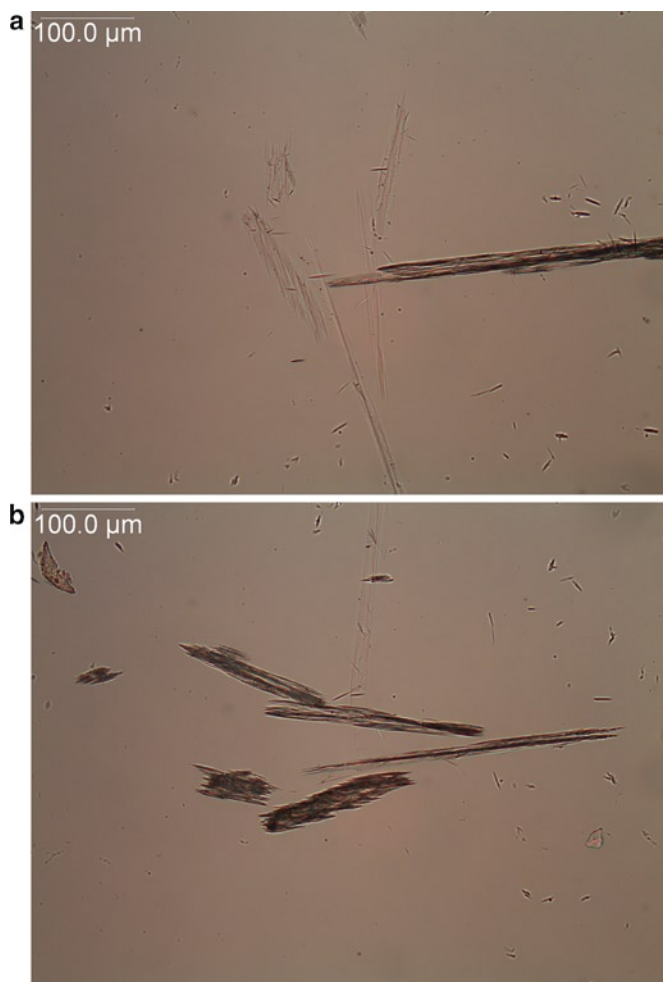
go back and forth between 1 and 2 polars. The reason for this practice is that you **MUST** measure indices when the crystal is in the extinction position (see below). That means that you measure indices at two rotation positions on the stage.

The key to recognizing uniaxial vibration directions is that all fragments of uniaxial crystals show  $\omega$  at one extinction position of the crystal and any index between  $\omega$  and  $\varepsilon$  at the other extinction position. It is this fact that allows us to determine vibration directions and accurately measure refractive indices. Since every particle shows  $\omega$  then all crystals in the field of view will have the same relief (contrast) at two positions on rotation of the stage – with one polar inserted. When we are close to a match with  $\omega$ , all crystals will go invisible at two rotation positions and as noted when making the measurement we want to ensure that we are at extinction with the  $\omega$  index. Figure 2.22a, b shows caffeine crystals at two extinction positions in a refractive index liquid close to the  $\omega$  index of 1.702. Note the change in contrast of the fibers at each orientation. The  $\varepsilon$  index is measured, in the search method, by mounting crystals in successive liquids either higher or lower than  $\omega$  until we reach the outer limit. That index is  $\varepsilon$ . If  $\varepsilon$  has a value greater than  $\omega$ , the crystal is said to have a positive optic sign. If  $\varepsilon$  has a value lower than  $\omega$ , the crystal is said to have a negative optic sign. The actual measurements of refractive index follow the recipe in the cubic section with the following additions: (1) the particle indices must be measured at extinction; (2) the index should be measured with 1 polar inserted (it is not necessary but is good practice). One helpful hint in recognizing  $\varepsilon$  is that sections showing this index will have the highest interference colors when oriented  $45^\circ$  to the extinction position (allowing for thickness of course).

## 2.6.4 Biaxial Crystal Search Method

This procedure is designed for biaxial crystals with three principle refractive indices. Materials that crystallize in the orthorhombic, monoclinic, and triclinic crystal systems are biaxial. By convention,  $\alpha$  is the lowest index,  $\gamma$  is the highest index, and  $\beta$  is intermediate. Some authors use subscripts to designate the indices as  $n_\alpha$ ,  $n_\beta$ , and  $n_\gamma$ . The technique described herein is useful for relatively rapid refractive index determinations and is the most common technique used in physical pharmacy.

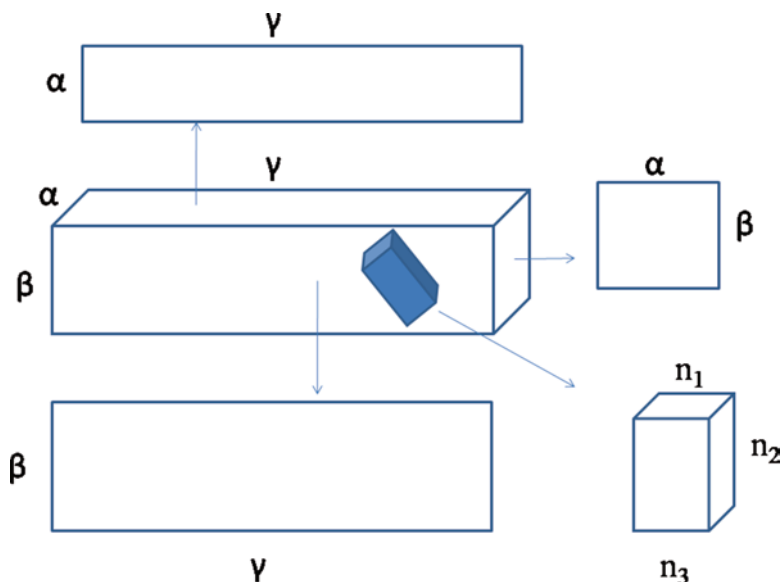
It is helpful to begin by referring to a schematic of a biaxial crystal (orthorhombic) with edges marked as  $\alpha$ ,  $\beta$ , and  $\gamma$  (see Fig. 2.23). The shaded box is meant to represent a fragment of the larger crystal. Note particularly that only in a well-formed crystal will the faces show the principle indices and that crystal fragments have values ranging from  $\alpha$  to  $\gamma$  (represented by  $n_1$ ,  $n_2$ ,  $n_3$ ). Compared with uniaxial crystals, refractive index measurements of biaxial crystals are more difficult since we do not have the aid of having one index always represented with each crystal fragment. In the simple search method, we use the recipe described in part 1 and apply it to the highest and lowest indices and then examine the preparation for  $\beta$ . The latter index can be recognized in crystals with a uniform gray color that does not change on rotation (do not be confused by crystals with high order interference colors that do not change on stage rotation).



**Fig. 2.22** Caffeine in 1.705 Cargille oil in two opposite orientations. Caffeine Form II is uniaxial with an  $\omega$  index of 1.702. Note the difference in particle contrast at the two different orientations of the rods when in the extinction orientation. (Image size =  $700 \times 520 \mu\text{m}$ )

I use the following procedure for measuring the indices of a biaxial material.

1. Begin by mounting some crystals in a viscous liquid that allows us to roll the crystal under the coverslip. Observe the habit and appearance of the crystal with one polar inserted, and then do the same with crossed polars. I am particularly interested in the interference colors (see description in next section) displayed by crystals oriented  $45^\circ$  to the extinction positions since this yields maximum colors. Visually make an estimate of the order of birefringence (1st, 2nd, 3rd, etc.) and if high, we know that  $\alpha$  and  $\gamma$  are far removed from each other. If the interference color order is low, then we know they are close.



**Fig. 2.23** Schematic drawing of refractive indices with biaxial crystal. With well-formed crystals we should be able to measure  $\alpha$ ,  $\beta$ , and  $\gamma$  by aligning the crystal with extinction positions. In practice, most analyses are made on crystal fragments that can display any indices from  $\alpha$  to  $\gamma$

2. Next determine the approximate thickness of the crystal using the eyepiece micrometer (calibrated first, of course) and then make a more accurate estimate of the interference colors using crossed polars and a quartz wedge compensator. Once thickness and interference color are known, use the Michel-Levy chart to determine birefringence (see next section). Since birefringence is defined as the difference between the highest and lowest indices, once one index is determined, we have a good idea what the next one should be.
3. Now mount crystals in refractive index liquids of 1.55, 1.60, and 1.65. In my experience, most pharmaceuticals have indices in this range. Suppose that we determine that the lowest index is between 1.55 and 1.60. We can now determine the exact index by sequentially mounting crystals in liquids between those values. Clearly, the best strategy is to mount in a liquid halfway between 1.55 and 1.60, say 1.575, and continue by halves until we get a match. Knowing the birefringence, we can approximate then the  $\gamma$  index, in this case, and mount in liquids around that estimated value. As with any index measurement, it is good practice to confirm the value by mounting in a liquid just a bit higher than your presumed match. Remember that you must make your measurements with crystals that are at extinction.

An accurate determination of  $\beta$  requires recognition of the appropriate vibration direction and for that we need interference figures. We can approximate  $\beta$ , however, by searching for particles, in crossed polars, with uniform illumination that does not change on rotation of the stage with only 1 polar inserted. Also, particles showing just  $\beta$  should have the same relief on rotation of the stage. Depending upon

how the crystal breaks, you may or may not be able to make a good estimate of  $\beta$ . If  $\beta$  is closer in value to  $\alpha$  than  $\gamma$ , the crystal is said to have a positive optic sign. If  $\beta$  is closer in value to  $\gamma$  than to  $\alpha$ , then the optic sign is negative.

While this technique is simple and relatively fast (normally it takes 30 to 45 min), it rests on the presumption that crushed particles will show all optic directions. Reconsider Fig. 2.23, if we lengthen the long direction until we have a fiber, then it may be difficult to crush particles that will show  $\alpha$ . Some materials maintain the fiber-like character even after harsh milling. It is particularly difficult to get accurate indices of these types of materials with any technique and it is good practice to asterisk your values with an explanation of the difficulties in the analysis. For truly accurate work, we must use the interference figures in order to locate vibration directions. Stoiber and Morse (1994) present an extensive discussion of refractive index determinations by search techniques.

### 2.6.5 *Biaxial Crystals with Aid of Interference Figures*

The biaxial crystal search method is quite useful for an initial estimate of the refractive indices and in many cases will suffice for project needs. If, on the other hand, we require accurate refractive index values, we must relate refractive index to optic orientation and, hence, to interference figures. It is possible to use interference figures with crystal fragments and crushed particles directly or one can use well-formed crystals and rotational devices, such as a spindle stage or goniometer, to measure all the indices on one crystal. Bloss (1981) and Gunter et al. (2004) discuss this latter technique in detail. Bowen (2009) published a specific example of the use of the spindle stage in optical crystallography with silver sulfadiazine.

Table 2.4 presents the relationship of various uniaxial and biaxial interference figures with observed refractive indices. Stoiber and Morse (1994) and Hartshorne and Stuart (1970) have clear presentations of the appearance of these interference figures and how to distinguish them. The particular interference figure that you observe with a crystal fragment depends on the orientation of that particle to the vibration directions of the microscope polarizer and analyzer. Unless you have aligned the optic orientation of the particle with the vibration direction of the microscope polarizers, you will see a partial interference figure. There are clever things that can be done with these partial figures, but it does require experience and skill to interpret them. Most particles in a field of view will display partial interference figures and only a few of the ideal figures are listed in Table 2.4. In order to determine accurate indices, we must orient the crystal so as to present the appropriate crystal face for measurement of the indices of that face. We orient the crystal using the interference figure.

As noted previously, refractive index depends upon light wavelength. This dependence is termed dispersion. In general, dispersion is low (a few units in third decimal place) for solids, but can be quite high. Dispersion is related to preferential absorption of light along specific crystallographic directions. Although not often used in pharmaceutical crystallography, I think it has the potential to aid in

**Table 2.4** Relationship of observed interference figure with principle refractive index and optical sign (adapted from Hartshorne and Stuart 1970, p. 360)

Interference figure	Optical sign	Indices
<i>Uniaxial crystals</i>		
Centered optic axis	+ or –	$\omega$
Uncentered optic axis	+ or –	$\omega, \varepsilon'$
Flash figure	+ or –	$\omega, \varepsilon$
<i>Biaxial crystals</i>		
Acute bisectrix	+	$\alpha, \beta$
Acute bisectrix	–	$\gamma, \beta$
Obtuse bisectrix	+	$\gamma, \beta$
Obtuse bisectrix	–	$\alpha, \beta$
Optic normal	+ or –	$\alpha, \gamma$
Single optic axis	+ or –	$\beta$

See Sect. 2.5.3.1 for more information

understanding how the orientation of certain functional groups in the crystal can affect bulk properties. Further study of this phenomenon is warranted, I believe.

## 2.6.6 Summary of Refractive Index Measurements

To summarize refractive index measurements, the number of principle indices depends upon the crystal system. Cubic crystals and disordered materials do not split light into multiple rays with different speeds and so have one refractive index in all orientations and do show an interference figure in crossed polars.

Uniaxial crystals (tetragonal and hexagonal systems) split light into two rays that travel through the crystal at different speeds. One ray proceeds as if in glass, whereas the other ray proceeds at a speed either greater or less than the normal ray. This phenomenon is termed double refraction and the difference in speeds termed retardation. Uniaxial crystals have two principle refractive indices. When the two refracted light rays recombine at the back focal plane of the objective, they interfere and produce a characteristic figure that is similar in appearance to a Maltese cross.

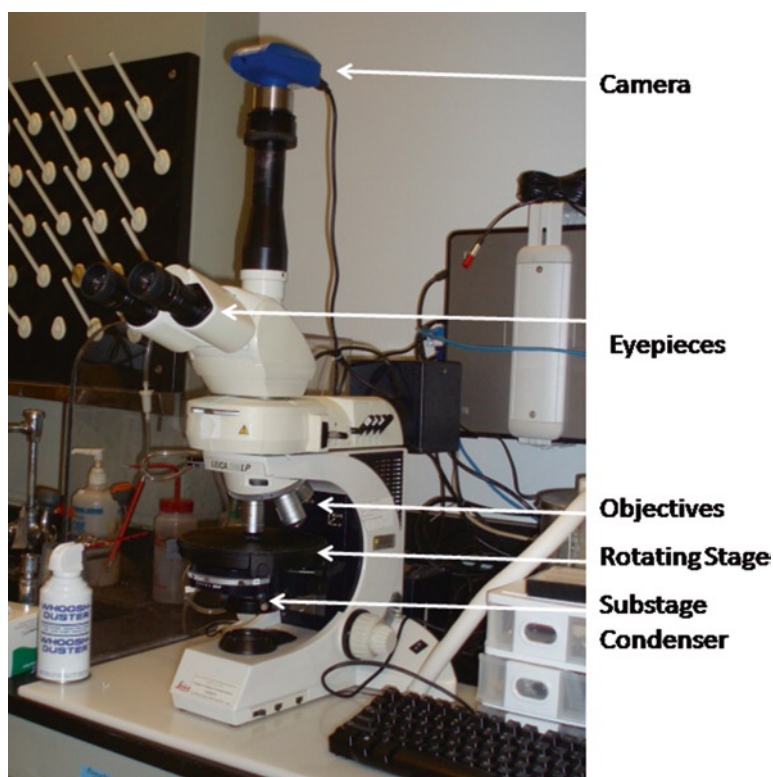
Biaxial crystals split light into three rays, one of which acts like it would in glass. The other two rays proceed with either an increased or decreased speed. This form of double refraction leads to three principle refractive indices. The recombination of the three rays results in interference in the back focal plane of the objective and can be detected as a hyperbola figure between crossed polars.

Refractive index measurements are simple with isotropic materials, relatively easy with uniaxial crystals and difficult with biaxial crystals. For the most accurate work, it is necessary to relate the vibration directions of the crystal to refractive index with the aid of the interference figure.



## 2.7 Instruments and Köhler Illumination

At its simplest, a compound microscope has objectives to form the image, oculars to magnify that image and present it in a convenient fashion to the observer, a light source to illuminate the sample, a light condenser to concentrate the light in a small region of the specimen, a stage (preferably rotating) to hold the specimen, a stand to hold all the components and allow for fine and coarse focusing, a polarizer below the specimen, a polarizing analyzer above the specimen, a slot for insertion of various compensators, a Bertrand lens for observing the back focal plane of the objective, and some means for taking photomicrographs. Figure 2.24 is a photo of a Leica DM/LM polarizing light microscope showing the location of all of the components. There are any number of books describing in detail the optical microscope. In fact, almost any book that discusses applications of the microscope will have a section on the microscope itself and accessories. Needham (1958) is an



**Fig. 2.24** Leitz DM/LM polarizing light microscope. This is one of the microscopes used to produce the images in this book. The other is the Olympus BH2 polarizing light microscope. The Leitz microscope uses infinity optics, whereas the Olympus BH2 has a fixed tube length

excellent place to start in my opinion, even if some of the information is dated. There are also many internet Web sites that not only describe the microscope but also have tutorials on many applications. I have listed a few of these Web sites in the References section. In my opinion, Molecular Expressions is a very good place to start and I routinely use it. I have also listed Web locations for microscope manufacturers and they also have quite a bit of basic information available for free download. I will present some basic information on microscopes in this section, but for really detailed information, I recommend you go to the literature and to the Web sites.

### 2.7.1 Objectives

There are three types of objectives depending upon the degree of correction: achromats, fluorites, and apochromats. Achromats have the least correction and apochromats the most. Naturally, cost follows along with the degree of correction. Objectives today are listed with the magnification, whether it is corrected for a planar field, the tube length for fixed tube length objectives or an infinity sign for infinity-corrected objectives, the numerical aperture, and may have the working distance. Also reflecting light objectives are usually specially designated as such. If the objective is designed for oil immersion, phase contrast, or differential interference contrast, there will be a special designation for such. Figure 2.25 shows four objectives for different applications including a reflected light infinity-corrected objective and an oil



**Fig. 2.25** Microscope objectives. From left to right, Nikon 40x/0.85 NA 160 mm tube length fluorite objective; a Leitz reflected light 50x/0.55 NA fluorite infinity-corrected objective; a Leica 50x/0.50 NA achromat infinity-corrected objective, an Olympus 100x/1.35 NA apochromat oil immersion 160 mm tube length objective

immersion fixed tube length objective. There are also long working distance objectives for use in thermal microscopy. Some designations are common among microscope manufacturers and some are specific. Most manufacturers have a table available that lists all of the objectives and their designations. Nearly all objectives and microscopes today are made with infinity objectives so that the tube length is not critical and it is possible to place a number of accessories, such as fluorescence and reflected light modules onto the microscope with minimal disruption. Objectives are clearly the most critical part of the microscope and should be treated with care. They should be regularly cleaned and stored away from dust when not in use.

### **2.7.2 *Oculars (Eyepiece)***

There are two main types of oculars but many subtypes. The two main types are Ramsden (positive) and Huyghenian (negative) and these can be compensating, wide field, high eyepoint, etc. The microscopy references have extensive discussions of the different types so we will emphasize only three important aspects of the ocular. First, it is important to determine whether a particular objective requires a specific compensating eyepiece for full correction. Modern microscope manufacturers do most spherical and chromatic aberration corrections in the objective but in some cases corrections occur in specific eyepieces. It is worth your while to find out from the manufacturer what oculars are recommended with each objective and to follow those recommendations. The second aspect of ocular use is that the useful range of magnification is 10–20 $\times$ . The ocular is a simple magnifier and does not usually contribute to resolution, but added magnification at the eyepiece can be helpful for the microscopist. I find high magnification oculars quite helpful in thermal microscopy. I often will have a 10 $\times$  ocular in one binocular slot and a 20 $\times$  or 25 $\times$  in the other slot. This arrangement allows me to observe a wide field and simultaneously to observe small features and transitions. The third aspect is that most eyepiece combinations and microscopes allow for separate focusing to accommodate poor vision. In most cases, you focus the image with a stationary eyepiece that is matched with your camera. Then you focus the other eyepiece to bring both images into correct focus for each eye. That being the case, with a multiuser instrument you will probably need to adjust the focus of the eyepieces for your eyes at each use. As mentioned, there are a number of specialized oculars available and you should check with the manufacturer if you have interest in these.

### **2.7.3 *Light Source***

Most microscope light sources today use tungsten-halogen bulbs of high brightness. It usually is necessary to adjust the position of the bulb with respect to the light train to obtain even illumination of the entire field of view. Some sources are automatically

centered, but I prefer to have a centerable bulb to ensure evenness of illumination. Some manufacturers place a diffusing filter in front of the bulb for even illumination but this has the disadvantage of reducing light intensity. Microscopes with built-in illuminators also have a field diaphragm just below the substage condenser to reduce stray light. The diaphragm is adjusted for each objective and must be centered (see Köhler illumination section for details). There are other illumination systems for special techniques such as fluorescence microscopy.

### ***2.7.4 Substage Condenser***

The purpose of the substage condenser is to concentrate the light into a small area so that just the field of view is illuminated. Most condensers have a swing-out lens that is used for 10× and higher power objectives. A significant part of the setup for Köhler illumination is the adjustment of the height of the condenser and the width of the diaphragm in the condenser. The condenser must also be centered. Most substage condensers used in optical crystallography either have a slot for the polarizer or have a built-in rotatable polarizer. Adjustments to the substage condenser are the key to critical imaging. If you are unfamiliar with this condenser, I recommend that you spend some time working with it. The two most common adjustments made by good microscopists are the fine focus and the substage condenser. Since the depth of focus is thin for light microscopy, the microscopist must continually raise and lower focus in small increments in order to image the entire feature from top to bottom. Similarly, the substage condenser controls the contrast and brightness of the image and generally must be frequently adjusted.

### ***2.7.5 Microscope Stand and Stage***

It is necessary to keep the microscope as steady as possible when in use. Consequently, microscope stands are usually heavy and rigid. Most modern microscope stands have a built in voltage transformer since tungsten-bulbs require DC current instead of the AC current common to laboratories. Modern microscopes have a number of conveniences available including tilting eyepiece modules for viewing convenience. Stands also generally have slots for filters between the light source and the field diaphragm. If fitted with neutral density filters, it is possible to cut the intensity of the light without limiting specimen contrast. Manufacturers spend a great deal of time and effort considering stand design. There is, of course, the artistic design component of the microscope, but there are also considerations of use. Many of us spend long hours looking through the microscope and if it is poorly designed then we can strain our necks, hands, arms and backs. In truth, the ergonomic designs of most modern microscopes are such that it is usually the placement of the microscope on the laboratory bench and the adjustments to the laboratory chair that result in bodily strains, not the microscope design. I find location on the laboratory bench a

significant problem as it is the custom in modern laboratories to move locations frequently. It is rare that one has the opportunity to design a microscope bench for convenience and for proper ergonomics.

### **2.7.6 Polarizer and Analyzer**

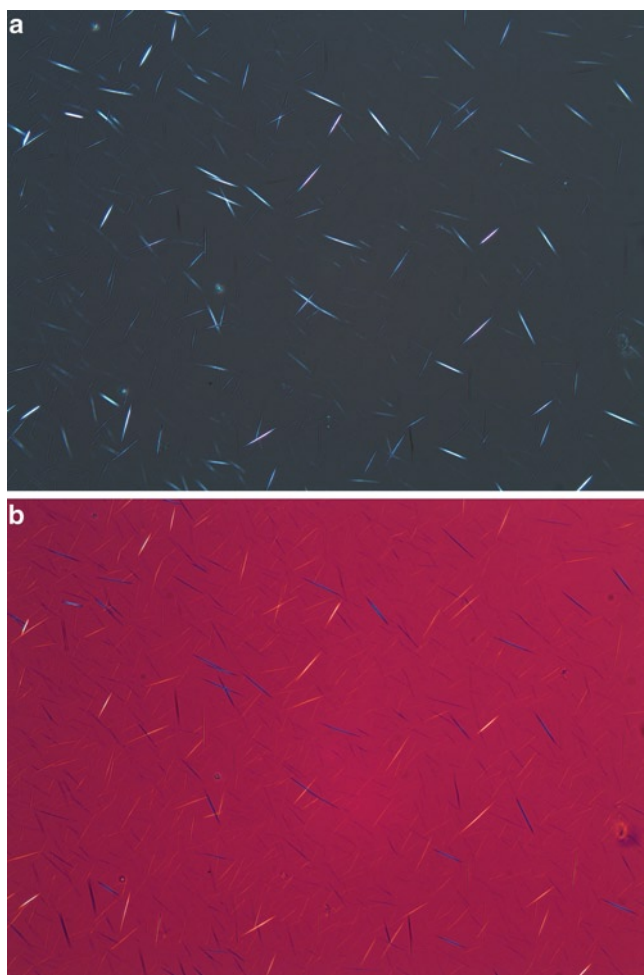
For polarized light microscopy we need a polarizer below the substage condenser and one just above the objective. By convention the lower polarizer is called the polarizer, while the upper polarizer is called the analyzer. Ideally, we want the analyzer to rotate and have markings indicating the angle of rotation. It is common for both analyzer and polarizer to rotate in modern microscopes. As previously noted, we need to be sure that the orientation of each is correct when doing some optical crystallography operations.

### **2.7.7 Compensators**

There are quite a few different compensators available for polarized light examinations. The most common is the full  $\lambda$  waveplate that shifts the light spectrum one order. There are also  $\frac{1}{4}$   $\lambda$  waveplates, quartz wedges, Berek compensators, and Senarmont compensators to mention a few. McCrone et al. (1984) and Stoiber and Morse (1994) both give simple, brief descriptions of the uses of many different compensators while Hartshorne and Stuart (1970) give a rather full treatment. For pharmaceutical microscopy, the compensator of greatest utility is the full  $\lambda$  waveplate. This waveplate is most often used to demonstrate crystallinity and to aid in photomicrography since the particles are colored on a red background in crossed polars. It can be challenging to produce good images of interference colors that are intense against a black background. Figure 2.26a, b show the same image of sublimed caffeine in crossed polars and then with a full waveplate. The second image can often be easier to present in slides and reports than the first image.

### **2.7.8 Köhler Illumination**

There are six basic adjustments to the microscope that must be made before one even begins to examine a specimen. There are a variety of different ways of making these adjustments (see McCrone et al. (1984) and Needham (1958) for a discussion of the different illumination methods). The most commonly used procedure today is referred to as Köhler Illumination. While it is possible to obtain an image without establishing Köhler illumination, this procedure ensures that you have gotten the optimum image. Each of the tasks listed in the remainder of this book presumes you have set up Köhler Illumination. The six basic adjustments are as follows: bulb



**Fig. 2.26** Sublimed caffeine: (a) fully crossed polars and (b) lambda waveplate. Images (a) and (b) show sublimed caffeine in crossed polars and then with a full waveplate. The lambda waveplate image can be easier to use for demonstrations and in reports. (Image size =  $700 \times 520 \mu\text{m}$ )

voltage; filament centering and location; specimen focus; field diaphragm opening size; substage diaphragm opening size; substage condenser location.

Köhler illumination provides a procedure for making each of these adjustments in turn. In my opinion, the very best discussion of this technique is in Delly's *Photography Through the Microscope* which is Kodak Publication P-2 published in 1988 (Delly 1988). As of this writing, both new and used copies of this book are available. I highly recommend that you obtain a copy of it, not only for illumination but also for other presentations of basic microscopy and photography. The book discusses the use of various films and not analog or digital cameras, but most of the information still applies to making good photomicrographs. Matsumoto (2010) also

has well-illustrated and easy-to-follow instructions for setting up Köhler illumination. His book focuses on digital photomicrography.

### 2.7.8.1 Köhler Illumination Setup Steps

1. *Set bulb voltage to manufacturers recommended level (for 12 V halogen bulbs that is generally 10 V).*

There are a couple of reasons for this step. The color spectrum and intensity of the light output depends on this voltage. When using color film, it is/was important to have the correct color for proper color balance in the film. While a bit less important for analog or digital cameras, the white balance of the camera and subsequent colors in the image will be affected by the voltage. The more important reason is image sharpness and contrast. Both are simply better at the higher voltage. But do not trust me, give it a try. Set your scope up with a specimen with fine interior detail (diatoms work wonderfully for this), get Köhler Illumination then raise and lower the voltage. You decide.

2. *Center the filament and adjust its location relative to the microscope stand according to the manufacturer's instructions.*

This adjustment primarily affects the light intensity at the specimen. Almost always more is better and this requires a well-positioned filament. It is much better to reduce illumination using gray filters than to realize you just do not have quite enough light intensity. Also, a poorly positioned filament directly affects the evenness of light in the background. This unevenness is deadly for image analysis and quite irritating for all work. It is best to do the initial filament centering with the lamp housing removed from the microscope. You can project the bulb image onto the wall or place a piece of paper or translucent plastic over the opening in order to test for centration and focus.

Alas, you may not be able to make these adjustments with your modern microscope. Manufacturers have moved to nonadjustable or barely adjustable light sources. It is easy to beat up the manufacturers over this change but really they are simply reacting to customer demands. In truth, the more knobs and dials to adjust, the more likely folks are to mess it up. It is not much of a problem with skilled users, but these users do not drive the market. Occasional users buy the most microscopes. So, if your microscope has one of these nonadjustable light sources, you simply have to make the best of it. In my experience, the filament centering and position, while not ideal from my perspective, is quite acceptable for most work. If you truly need something better, buy a different microscope.

3. *Swing a relatively high magnification objective into place (20× or 40×) and focus on an object with reasonably sized features and contrast. Ensure that the appropriate condenser is in place. Focus the features of the image.*

It is necessary to have the features focused in order to proceed to the next steps.

4. *Center and Focus the Field diaphragm.*

This step is probably the most critical step in the procedure. You perform this operation by first reducing the field diaphragm to its smallest size and then

centering it in the field of view. Next, focus the edges of the diaphragm by raising or lowering the substage condenser. You should see a red and blue colored fringe at the edge of the leaves due to diffraction effects. Focus is determined by hitting that point just between the two colors. Now open the field diaphragm until the edges are just outside the field of view.

5. *Ensure the lamp filament is focused and fills the field of view.*

In order to image the lamp filament, it is necessary to either swing in the Bertrand lens or to pull out an objective and view the filament directly. You should not need to use the centering apparatus since that was the first step. There generally is a focusing knob on the lamp housing to adjust the filament and in some cases there is also a parabolic mirror which ensures maximum illumination. The mirror yields two filament images and these should be superimposed (see manufacturer's instructions for how this step is accomplished).

6. *Adjust the substage aperture for optimum specimen contrast.*

Probably the second most common error made in critical microscopy is to use the substage aperture to control light intensity while the most common error is to use bulb voltage for the same purpose. You set the substage aperture to optimize feature contrast. If that results in light intensity that is too high, which is often the case, then the proper way of reducing light intensity is to use neutral density gray filters. Using the substage aperture for light intensity adjustments leads to either washed out images or too much contrast.

At this point, you should have an image with even illumination and good feature contrast. It is necessary to check steps 4 and 6 every time you change objectives. In practice, it is usually only necessary to make small adjustments. I find that setting up Köhler illumination requires no more than 5–10 min once you have developed some facility and practice in the procedure. In daily practice, I skip step 2 unless I know someone else has used the microscope before me or if I am changing filaments.

## 2.8 Stereomicroscopy

Oftentimes, stereomicroscopes seem like the ugly stepchild to compound microscopes, yet I find that I use the stereomicroscope on a regular and routine basis. It is good for sample preparation, for examining bulk samples, and for contaminant identification. There are a number of different illumination techniques for stereomicroscopy. The simplest illumination method is reflected light that ideally can be directed from multiple directions and heights. It can be quite useful to illuminate the object with light from the side so as to assess surface or to use a type of dark field illumination.

I routinely use the stereomicroscope when preparing samples for light or for SEM analysis. First, I like to examine the contents of the sample bottle directly. Most cohesive drug substance will form spherical agglomerates on standing. I think it is useful to note the size and shape of the agglomerates as part of a routine microscopic



examination. Also, attempting to crush these agglomerates can give some qualitative sense of their resilience. I recommend the practice. I always keep a good stereomicroscope near my optical microscope and SEM. It is also useful to have it connected to a camera system so as to document observations with photomicrographs.

## 2.9 Photomicrography

The rapid pace of changes in digital camera technology makes it nearly impossible to discuss the current state of the art in photomicrography in a book to be published some months hence. The Web sites associated with Microscopy-UK, Molecular Expressions, Nikon, and Olympus (see internet references) all have current information on digital imaging and digital cameras. I have mentioned Delly's (1988) photomicrography book as an excellent general reference and Inoué (1986) is a treasure trove of good information on digital imaging, although dated. Matsumoto (2010) and Murphy (2001) are good sources for modern digital imaging and photomicrography. I also have a specific section in Chap. 7 that discusses digital imaging related to image analysis. In this section, I present a few basic principles of photomicrography.

The first basic principle of photomicrography is that the quality of the photo cannot be better than the quality of the image in the microscope. In other words, it is necessary to spend some time and trouble ensuring that the microscope is set up correctly. One common problem that we encounter is an unevenness of illumination across the image that is difficult to see in the microscope but that is readily apparent in the photomicrograph. This problem is nearly always due to poor set up of the microscope illuminator or in poor centration of the field diaphragm. The reason that you may not detect the unevenness in the microscope is that the eye is really quite good at smoothing unevenness where as film or digital images do not smooth at all. Still, it is good policy to begin by optimizing the image on the microscope. Another annoying problem that crops up frequently is out-of-focus spots due to dust and dirt on the microscope or on the slide. In particular, dust on the coverslip and on the substage condenser is readily visible in the image even if it is out of focus. Ensuring good cleanliness is necessary to solve that problem.

A digital imaging system consists of the microscope to produce the image, camera attachments to the microscope, optics for transmitting the image from the microscope to the digital camera, the camera itself, and then a computer and software for displaying and storing the images. There are quite a number of different ways of combining different elements to achieve the purpose. First, it is important to consult with the microscope and camera manufacturer's literature on the best way of setting up a digital photomicrography system. Second, although it is possible to mix and match components, in practice you need to have extensive knowledge of optics, cameras, and computers for this approach to work well. A number of different companies, such as Nikon, Olympus, Media Cybernetics, and Pax-it, to name a few, have complete systems from microscope coupling devices to software. To give an example of the problems with mix and match systems, many software programs collect images from only

a few select cameras. If you purchase that software and try to connect it to your camera, it will not work. So, unless you are expert at this area, I advise getting a complete setup from a company specializing in digital photomicrography.

It is important to keep in mind that the image as constructed in the microscope is analog and must be converted to a digital format to be stored in the computer. That conversion process always has limitations and it is important to match the resolution of the image with the resolution of the camera and computer. I address this issue in more detail below in Chap. 7.

One of the most vexing problems in color digital photomicrography is color balance, in other words, ensuring that the colors seen in the microscope image are accurately reproduced in the digital image. The fundamental problem in color balance is that the detecting elements in digital cameras have an uneven response to light of different wavelengths and must be adjusted accordingly. Most digital cameras for hobby use do these adjustments automatically or semiautomatically for the two main illumination conditions of daylight or indoor incandescent light bulbs. The microscope though can have a number of different illuminators and the color of the light is directly affected by the voltage applied to the bulb. The voltage settings on most illuminators are not reproducible enough to ensure the same color balance on resetting the values. Consequently, most imaging software programs have either an automated or manual color balance program. The operation of these varies from one program to another and I strongly recommend that you carefully read the software operation manual for this program. In general, the program requires that you set up the microscope as you intend to take the photo and then move to a blank field of view. You can then call up the color balance program which automatically sets the color. Alternatively, some programs allow you to select a small area of the image you are examining that is white and using that limited space for the color adjustment. Even so, be prepared for frustration with some programs and cameras. Proper color balance does require patience and persistence.

While it is not possible to rectify fundamental problems in an image due to poor microscope or camera setup, it is possible to remove many imperfections using programs such as Adobe Photoshop®. One example of the type of adjustment is the color balance can be altered using that program. Such programs can also be used to adjust contrast and brightness. In my opinion, simple adjustments of that type need not be documented. If, however, you decide to perform a median filter to remove noise or some other type of image processing, then I believe it is important to document those steps. The documentation can be simple and maintained for future questions if the images are strictly internal, but I think a description of the steps should accompany the image if used for publication. To be honest, the need for such documentation is not universally accepted and there are good arguments on both sides of the issue.

Finally, we need to be aware of the aesthetic and artistic component of our images. A photomicrograph is a representative illustration of what we, as the microscopist, see in the microscope. We may have looked at tens or possibly even hundreds of fields of view before selecting a few to record. Naturally, the recorded images should accurately represent what we observe and what points we wish to discuss and illustrate. So, for instance, Fig. 2.9a, b do illustrate the nature of twinning in caffeine, but these are two of probably 20 photomicrographs that I took

from approximately 50 fields of view. I selected these two photos for their aesthetic and artistic value as well as for their scientific value. I am not an artist and have no artistic training at all, but I do think that with many years of experience and practice, I can see an image that is aesthetically pleasing even though I cannot articulate what makes it so. I do recommend that you consider the artistic qualities of any image that you collect. One of the most important advantages of microscopy is its ability to transmit information in a nonverbal fashion, as the “picture is worth a thousand words” saying goes. An aesthetically pleasing picture certainly aids the nonverbal information transmission.

## 2.10 Summary

I have devoted quite a bit of space to a discussion of the polarized light microscopy and optical crystallography. In my opinion, these topics are one of the foundations of pharmaceutical microscopy. Skill at these subjects can have a direct impact on the drug development process, aiding us in our understanding of drug substance solid-state and material properties. I get somewhat frustrated with the common “It’s just a microscope” attitude I often encounter, but it is easy to see how pervasive that attitude is given the fact that the microscope is one of the first scientific instruments we use in our childhood. I think the microscope does have an important role to play in drug development, but that the key to its use is the skill of the microscopist. I hope that this chapter has whetted your appetite for learning more and working more with the optical microscope.

As a final note, there is no general agreement upon how polarized light microscopy data should be presented although there is a recommended practice document for ACS publications (Mason 1945) that is fairly old and not particularly prescriptive. Personally, I favor the manner in which Nichols (1998) presented his optical properties of paracetamol. I think the presentation is in general accord with X-ray crystallographic data presentation and is clear and easy to understand. I recommend his paper as a model.

## 2.11 Exercises

### *A. Sample Preparation and Preparation of Crystals*

#### **A1. Simple Sample Preparation with Silicone Oil**

Mount a small specimen of carbamazepine or caffeine in silicone oil. Take some time to examine the bottle contents directly without magnification and then with a stereomicroscope. Take a small needle or spatula and manipulate the powder directly in the bottle and then take a spatula and remove a small amount and

examine it under the stereomicroscope at varying magnifications. Note large spherical agglomerates and whether they break easily or are resilient. Put a small amount of powder onto a glass slide, then put a small drop of silicone or mineral oil on the powder and gently disperse with a needle. Now place a coverslip onto the oil preparation and observe the preparation at low magnification on a polarizing light microscope. Do not be surprised if you have too much material on the slide with your first preparation. Make new slides with less material and continue until you get an even distribution of particles across the coverslip and only a few particles in the field of view when using a high power objective (20 $\times$ , 40 $\times$ ).

## **A2. Sublimation**

Place 5–10 mg of caffeine in a small flat container and cover with a large coverslip or glass slide. Place the container onto hot plate at 180–200°C. You should begin to see crystals sublime onto the cover and allow the cover to collect a large number of caffeine crystals. Once completed, remove a small portion of the crystals and mount in silicone or mineral oil. You should observe well-formed, elongated rectangles. Observe these crystals with various objectives.

## **A3. Permanent Mount Using Norland Optical Adhesive**

Take a small amount of the sublimed caffeine crystals and mount in Norland optical adhesive on a microscope slide and cover with a coverslip. Put this preparation under a UV light and allow the specimen to harden. Once complete, observe the crystals using the PLM.

## **A4. Preparation of Caffeine Hydrate**

Put approximately 10 mL of water into an appropriate scintillation vial and heat to approximately 50°C. Slowly add and dissolve caffeine to the heated water. Once you have dissolved a reasonable amount of caffeine, place a lid on the preparation and reduce the temperature slowly over a period of 4–5 h. You should see the formation of relatively large caffeine hydrate crystals. Remove a few of these crystals and mount in silicone oil as in A1. Note the differences in appearance between anhydrous and hydrated caffeine.

## ***B. Microscope Setup: Köhler Illumination***

Use the slide prepared in experiment in A2 or A3 to set up the microscope with Köhler illumination according to the instructions in 2.4.8. Experiment with different settings of the substage condenser and observe the differences in feature contrast.

## ***C. Polarized Light***

### **C1. Basic Observations**

Use the slide prepared in experiment A2 or A3 and ensure the microscope is set up with Köhler illumination. Cross the polars and observe the preparation at 10× or 20× magnification. Note the colors of the particles, they should vary from uniformly white to blues, reds, and greens. The difference in color is due to differences in thickness. Note that particles with the length oriented north–south or east–west have little to no color while those particles with the length oriented at other angles are colored. The most intense colors occur with those particles at 45° to the eyepiece crosshairs (or at 45° to the north–south and east–west orientations). Insert a full  $\lambda$  waveplate. Note the red, magenta background and the yellow and blue colors of those particles that were white in crossed polars. Rotate the stage and note the change in colors. Note that the particles oriented with the length at 45° to the crosshairs and that are located in the 2nd and 4th quadrants are yellow, whereas those particles 90° to that are blue. These color orientations are indicative of a negative sign of elongation.

### **C2. Orientation of Polars**

Ensure that your polarizer and analyzer are oriented correctly and correspond with the eyepiece crosshairs (if you do not have crosshairs in your eyepiece you should obtain a proper reticule for this purpose). At one time, Olympus supplied a slide with their polarizing light microscopes that could be used to check orientation. Also Skip Palenik of Microtrace provides a pleochroic fiber that can be used for the same purpose (see internet references for Microtrace). If you do not have access to these aids, you can check the microscope polar using sublimed caffeine rods. They have parallel extinction and the crystals should go to extinction when the particle length is parallel with the crosshairs.

### **C3. Undulating Birefringence with Cellulose Fibers**

Mount a few cellulose fibers, from tissues or Kimwipes, on a microscope slide with silicone oil and a coverslip. Observe the fibers with fully crossed polars. Note that the fibers exhibit undulating extinction. In other words, the entire fiber does not go to extinction at any one position. Rather, regions of the fiber go to extinction more or less independently of other regions. This type of extinction is due to the helical nature of the cellulose molecules and is reasonably distinctive for cellulose. Since cellulose fibers are among the most common of particulate contaminants in slide preparations, it behooves you to learn to recognize cellulose fibers by sight.

## ***D. Interference colors***

### **D1. Examination of Interference Colors with Fusion Preparations**

Prepare fusion slides of sodium nitrate, benzil, and acetylsalicylic acid. Place a small amount (~1 mg or less) of each compound onto a clean slide and cover with a coverglass. Heat each preparation on a hot plate just until the compound melts. Allow the preparation to cool. If practical, observe the melted compound as it cools. The manner of recrystallization is interesting and often beautiful to watch. You should have a reasonably flat, thin film of recrystallized material. Examine acetylsalicylic acid first using a low to medium magnification objective (5–20×). It generally recrystallizes into radial spherulites that have first-order white to green interference colors depending upon thickness. Insert the full lambda waveplate and observe the differences in colors between it and the fully crossed polars. There may be some individual needles of the compound at the edges of the preparation and note that they have positive sign of elongation.

Next look at the sodium nitrate fusion preparation with low to medium magnification (5–20×) and with fully crossed polars. Sodium nitrate generally crystallizes into thin flat films that are randomly oriented with regard to crystallography. Note that in some sections the color is a uniform dark gray that does not change much on stage rotation, whereas other sections have nearly saturated white colors indicating high order interference colors, there also should be some regions with first-order greens, reds and blues. Save this slide for a later experiment (G).

Finally, examine the benzil fusion preparation. Note the difference in the progression of interference colors with thickness compared with sodium nitrate and acetylsalicylic acid. Benzil displays anomalous interference colors.

If your preparations are too flat and you do not have sufficient differences in thickness to see the progression of interference colors, then you can prepare a wedge by using a bit more compound and placing a few glass fragments under one edge of the coverslip, which should provide the necessary thickness progression.

### **D2. Michel-Levy Chart**

Examine the Michel-Levy chart and note the progression of colors. Compare this color progression with what you see with the fusion compounds examined in D1. You do not always or even usually get the progression in real preparations that you expect from the Michel-Levy chart. The reason for this disparity is generally due to discontinuous changes in thickness within the film and particles.

### **D3. Carbamazepine Birefringence**

Mount some carbamazepine (CBZ) in a viscous mounting media such as Cargille immersion oil type VH (this immersion oil is used for some of the thermal experiments as well). Most bottles of CBZ I have examined have rather large, twinned crystals.

Try rolling crystals by gently moving the coverslip in one direction or another. Note the changing appearance of the crystals on rotation. You usually will not see much difference in interference colors since the crystals are so thick that you generally have very high order interference colors. You are likely to have some crushed fragments. If not, put some pressure on the coverslip and break a few large crystals. You should see some crystal fragments with lower order interference colors such as greens and blues. Roll these crystals and note the change in color as the fragments rotate. Try determining the birefringence of a crystal fragment by measuring its thickness (using a calibrated eyepiece micrometer) and working with the Michel-Levy chart. You locate the thickness and color on the chart and then follow the line to the appropriate birefringence. Also, do the calculation,  $B = r/1,000t$  where  $B$  is the birefringence,  $r$  is the wavelength (color) of the crystal in nm, and  $t$  is the crystal thickness in  $\mu\text{m}$ .

### ***E. Refractive Index: Cubic, Uniaxial, Biaxial, High low***

#### **E1. Glass Refractive Index**

Mount some glass fragments of known refractive index in liquids with the same index. Sets of Cargille refractive index standards often contain a few vials of known glass particles. Also mount some of the glass in index liquid slightly above and below the RI match of the glass. Observe the different contrast and the Becke lines as you adjust the focus up and down. For the match sample, place a 589 nm filter in the light path and observe glass fragments. You should not see any Becke lines at all. In fact, the particles should be quite difficult to detect. Now measure the temperature on the stage and adjust the RI value using the equation on the bottle. Observe the Becke lines when inserting a 486 and a 656 nm filter in the light path. Note the presence of the Becke lines with those filters. Note on the side of the refractive index liquid standard the refractive index of the liquid at those wavelengths and the dispersion.

#### **E2. Uniaxial Refractive Index**

Mount some caffeine crystals prepared by sublimation in 1.705 and 1.436 refractive index liquids. Observe the 1.705 preparation first using one polar. Note that on rotation of the stage, every crystal becomes invisible at certain orientations but that the crystals have relatively high contrast 90° to that rotation. Observe the 1.436 RI preparation next and note that while some crystals go to extinction on rotation, not all do. Prepare some additional mounts in refractive index liquids just higher and just lower than 1.705 and 1.436 and note the contrast.

#### **E3. Biaxial Refractive Index**

Measure the refractive indices of paracetamol Form 1 (1.580, 1.704, and 1.643). Note the inclined extinction at 36°.

## ***F. Optical Crystallography***

### **F1. Uniaxial and Biaxial Interference Figures**

Examine the fusion preparation of sodium nitrate and look for dark-gray regions that only change contrast slightly on stage rotation. Use a high power objective (40 $\times$ ), focus on the surface and insert the Bertrand lens (if you do not have Bertrand lens on your microscope, pull an eyepiece from the tube and look down the tube. Alternatively use a pinhole eyepiece). You should see a Maltese cross near the center of the field of view. Rotate the stage and observe the appearance of the cross. Insert the lambda waveplate and note the locations of the yellow and blue colorations near the center of the Maltese cross.

Next, mount a small fragment of Mylar onto a microscope slide and examine it as described for sodium nitrate. Note the appearance of the two isogyres and the concentric circles around each. This is an ideal biaxial interference figure and, in general, you will observe a Maltese cross like the uniaxial interference figure and as you rotate the stage, the isogyres will separate from the Maltese cross and move toward the edges of the field of view. A preparation with mica crystals is useful for training since you generally see many different optical orientations depending upon the crystal fragment. I recommend that you practice with mica for some time before working with “real” samples.

### **F2. Acetylsalicylic Acid Uniaxial Interference Figure**

Examine the fusion preparation of acetylsalicylic acid as in exercise G1 and compare the appearance of the uniaxial interference figures in this preparation with those observed with sodium nitrate. In general, you will see more partial figures with acetylsalicylic acid than with sodium nitrate. Insert the lambda waveplate and observe the blue and yellow colors as the figure is rotated.

### **F3. Paracetamol (Acetaminophen) Interference Figure**

Mount a small specimen of acetaminophen in the high viscosity VH Cargille immersion oil. Observe the crystal habit (elongated prisms and plates) with direct transmitted light and then with fully crossed polars. Note the interference colors and that in thick sections the particles display high order interference colors, although it is generally possible to find thin fragments with low order interference colors. Center some thin crystals in the field of view and examine using the Bertrand lens (or pinhole eyepiece). Note that, in general, you get off-center interference figures that appear close to uniaxial. The optic axial angle for form I acetaminophen is reported to be 86°. Consequently, with stage rotation, the isogyres of the interference figure will pull apart only slightly. This a good sample with which



to experiment since the optical properties have been described well by Nichols (1998) and Nichols and Frampton (1998). I recommend recrystallizing this material from different solvents and practicing optical crystallographic measurements including refractive index.

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