Robert Allen Carlton

Pharmaceutical Microscopy

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Preface

A simple glance at the table of contents demonstrates the breadth of microscopy techniques applied to pharmaceutical microscopy. We range from simple stereomicroscopy to polarized light microscopy, and from electron microscopy to IR and Raman spectroscopy. This can be an intimidating set of techniques and instruments to attempt to learn and to use in just a cursory fashion, much less to become expert in use and interpretation. I am fortunate to have been given the opportunity to learn to use all of these techniques over my career. I began microscopy by measuring glass fibers on a projection microscope in a closet and have moved from there to all of the techniques listed in this book. I did the majority of my education part time after I started in industry and have consciously directed my academic studies toward industrial microscopy. My doctorate is in chemistry (physical) and my dissertation covered quantitative analysis using energy dispersive X-ray spectrometry in the environmental scanning electron microscope. I am fortunate to have had supportive managers as well as supportive academic advisors.

I learned optical microscopy from McCrone Research Institute, from listening to talks by industrial microscopists, from reading microscopy works, and by long hours with eyes applied to eyepieces. Walter McCrone's courses and conferences are what helped me decide that my goal was to become a chemical microscopist. Skip Palenik and John Delly were the two microscopists I most admired, but I must say that there were so many fine industrial microscopists working in the 1980s. I use the past tense because industrial specialization in science is not as common today as it was then. I think we have lost something important but such opinions may be tilting at windmills. Still, I believe that the satisfaction of becoming a master craftsman (woman as well) is worth the effort and pays dividends to business. Oh, did I mention how much I learned from chats at the bar after conferences bending elbows and ears? I cannot forget that source of "learning."

I have worked in the pharmaceutical industry since 1992. In that time, I have worked on hundreds of compounds and had maybe a dozen approved. My work has extended from very early-stage discovery before first time in human studies to post launch. I have transferred microscope size methods to manufacturing facilities. Most pharmaceutical scientists work in a very narrow range of development – a development that can take a decade from start to finish. Solid-state scientists, on the other hand, often work from the very early stages looking at polymorphism and particle size all the way to marketing approval and manufacture. It is a fine way to learn the industry and the particular challenges to drug development.

I use personal pronouns such as ‘I’, ‘you’, and ‘we’ much more than is common in scientific books. I hope you do not find the practice too jarring or distracting. I chose to present the information in this fashion because so much of the contents involves personal preference and experience. Also, I am hoping to achieve the sens that you and I are sitting in front of a microscope and I am sharing with you what I have learned from more than 30 experience in industrial and pharmaceutical microscopy.

I am an enthusiastic proponent of microscopy in solid-state pharmaceutical development. I have gained a great deal of personal satisfaction from using microscopy to solve industrial problems and I hope this book helps you have the same experience.

April 2011

Robert A Carlton
Collegeville PA

Acknowledgments

I want to thank my wife, Brenda, for her help, support and understanding in this endeavor. I know, you are probably thinking this is the trivial, obligatory "thank the marriage mate" sentence. It is not. All it requires is one unruly sentence, a paragraph that wrests control and refuses to yield, one misbehaving figure to turn an otherwise chummy, congenial writer into a raving, mad lunatic. Brenda has been patient and understanding when I have needed it the most. I do appreciate the care and the sacrifices. Brenda also did a thorough edit of the book which was particularly helpful since she is an author in her own right (Drug Money, Accident on 13th and Jefferson). Thank you from the bottom of my heart.

My daughter Danielle Broadaway helped with a number of figures and with my Web site. Thank you. My parents, Joan and Bob Carlton are a continuing source of inspiration and encouragement.

I had the opportunity to work with two young interns from Singapore. Hwee-Jing Ong and Yuniatine who did much of the actual lab work related to caffeine and carbamazepine. It was a distinct pleasure to work with both of them. They are both bright, capable scientists and I expect good things from them in the future.

My editor at Springer, David Packer, has been a source of encouragement and good advice throughout the writing process.

Dr. Jeffrey Brum, Dr. Ronald Mueller, and Dr. Greg Webber of GlaxoSmithKline have all supported my work and graciously allowed me to use instrumentation in our labs. Jeff has been particularly understanding and supportive.

I have benefited from talks with Dr. Frederick Vogt and Dr. Mark Strohmeier of GSK about microspectroscopy. I have received help and advice on the same subject from Dr John Refner of the John Jay School of Criminal Justice and from Pauline Leary of Smiths Detection. Fran Adar of Horiba Jobin-Yvon has contributed good advice on Raman microspectroscopy.

I have had the benefit of many good microscopy teachers including Skip Palenik, John Delly, Walter McCrone, and Charles Lyman among many others. I also want to thank a generation of industrial microscopists who freely gave of their knowledge in technical meetings and in conversations afterwards. They are an inspiring example.

Finally, in order to finish this book in a reasonable time frame, I had to have help with our extensive gardens at home. Tina, Kalee, Sutton, Jasmine, George, Aubree, Gavin, and Dave provided that help willingly and with good humor. Without their help the task would have been much harder and taken much longer.

Contents

1 Introduction 1
1.1 Microscopy and Industrial Microscopy 1
1.2 Pharmaceutical Microscopy 1
1.3 Purpose of the Book 2
1.4 Plan of the Book 4
1.5 How Best to Use the Book 5
References 6

2 Polarized Light Microscopy 7
2.1 Introduction 7
2.2 Properties of Light 8
2.3 Basic Optics 11
2.4 Crystallography 15
2.5 Optical Crystallography 19
2.5.1 No Polars, Brightfield 22
2.5.2 One Polar 28
2.5.3 Crossed Polars 28
2.5.4 Summary of Optical Crystallography 37
2.6 Measurement of Refractive Indices 37
2.6.1 Introduction 37
2.6.2 Cubic Crystals and Amorphous Materials: Basic Immersion Technique 38
2.6.3 Uniaxial Crystals 41
2.6.4 Biaxial Crystal Search Method 42
2.6.5 Biaxial Crystals with Aid of Interference Figures 45
2.6.6 Summary of Refractive Index Measurements 46
2.7 Instruments and Köhler Illumination 47
2.7.1 Objectives 48
2.7.2 Oculars (Eyepiece) 49
2.7.3 Light Source 49
2.7.4 Substage Condenser 50
2.7.5 Microscope Stand and Stage 50
2.7.6 Polarizer and Analyzer 51

2.7.7 Compensators 51
2.7.8 Köhler Illumination 51
2.8 Stereomicroscopy 54
2.9 Photomicrography 55
2.10 Summary 57
2.11 Exercises 57
A. Sample Preparation and Preparation of Crystals 57
B. Microscope Setup: Köhler Illumination 58
C. Polarized Light 59
D. Interference colors 60
E. Refractive Index: Cubic, Uniaxial, Biaxial, High low 61
F. Optical Crystallography 62
References 63
Internet References 64

3 Thermal Microscopy 65
3.1 Introduction 65
3.2 Instruments and Methods 66
3.3 Calibration 69
3.4 Test Strategy 71
3.5 Observations 72
3.5.1 Melting Point 72
3.5.2 Solid–Solid Transformations 75
3.5.3 Solid–Liquid–Solid Transformations 75
3.5.4 Sublimation 76
3.5.5 Cooling 78
3.5.6 Dehydration and Desolvation 78
3.6 Fusion Techniques and Other Applications 79
3.7 Summary 81
3.8 Exercises 81
A. Sample Preparation and Experimental Setup 81
B. Calibration and Performance Checks 82
C. Effects of Heating Rate 82
D. Phase Transformations 83
E. Fusion 83
References 83
Internet References 84

4 Scanning Electron Microscopy and Energy-Dispersive X-Ray Spectrometry 85
4.1 Introduction 85
4.2 Scanning Electron Microscopy 86
4.2.1 Basic Operation of SEM 86
4.2.2 Electron Beam–Specimen Interactions 89
4.2.3 Backscatter Electron Emission and Detection 91

4.2.4 Secondary Electron Emission and Detection 94
4.2.5 SEM Instruments 98
4.3 Sample Preparation and Imaging Strategy for Pharmaceuticals 102
4.3.1 Drug Substance 102
4.3.2 Drug Product 107
4.4 Energy Dispersive X-Ray Spectrometry 108
4.4.1 Theory 108
4.4.2 Qualitative Analysis 114
4.4.3 Quantitative Analysis 117
4.4.4 Elemental Mapping 120
4.4.5 EDS with Low-Vacuum SEM 123
4.5 Summary 125
4.6 Exercises 126
A. Sample Preparation 126
B. Secondary and Backscattered Electron Imaging 127
C. Low-Vacuum SEM 127
D. EDS Qualitative Analysis 128
E. Elemental Mapping 128
References 129
Internet References 130

5 Infrared and Raman Microscopy 131
5.1 Introduction 131
5.2 Infrared Microscopy 132
5.2.1 Theory 132
5.2.2 Instruments 134
5.2.3 Sample Preparation and Testing 136
5.2.4 Calibration and Performance Checks 140
5.3 Raman Microscopy 141
5.3.1 Theory 141
5.3.2 Instruments 142
5.3.3 Sample Preparation and Testing 144
5.3.4 Calibration and Performance Checks 146
5.4 Applications 147
5.4.1 Solid-State Analysis 147
5.4.2 Contaminant Analysis 151
5.4.3 Chemical Imaging 152
5.5 Summary 153
5.6 Exercises 154
A. Basic Operation 154
B. Operating Parameters 154
C. Spectral Interpretation 155
D. Mapping 155
References 155
Internet Resources 156

6 Specialized Microscopy Techniques 157
6.1 Introduction 157
6.2 Confocal Microscopy 157
6.3 Scanning Probe Microscopy 159
6.4 Transmission Electron Microscopy 160
6.5 Differential Interference Contrast and Hoffmann Modulation Contrast 162
6.6 Phase Contrast 163
6.7 Dispersion Staining 165
6.8 Dark Field and Rheinberg Illumination 167
6.9 Flourescence Microscopy 168
6.10 Freeze-Dry Microscopy 170
6.11 Thermal Microscopy in the SEM 170
6.12 Summary 171
References 171
Internet Reference 172

7 Image Analysis 173
7.1 Introduction 173
7.2 General Principles 174
7.3 Sample Preparation 175
7.3.1 Introduction 175
7.3.2 Optical Microscopy Sample Preparation 176
7.3.3 SEM Sample Preparation 179
7.4 Image Production 181
7.4.1 General Considerations 181
7.4.2 Image Production with the Optical Microscope 182
7.4.3 Image Production with the SEM 182
7.4.4 Choice of Microscopy: Resolution and Magnification 183
7.5 Image Collection 183
7.6 Image Processing 185
7.7 Segmentation 187
7.8 Binary Operations 188
7.8.1 Feature Improvements 188
7.8.2 Removal of Unwanted Features and Noise 191
7.9 Measurements 193
7.9.1 General Considerations 193
7.9.2 Field Measurements 194
7.9.3 Object Measurements 194
7.10 Calibration, Performance Checks, and Size Standards 202
7.11 Data Presentation and Statistics 203
7.12 Exercises 207
A. Sample Preparation 208
B. Image Collection 208
C. Image Processing 208

D. Image Segmentation 209
E. Binary Operations 209
F. Measurements 209
G. Statistics and Data Presentation 210
References 210
Internet References 211

8 Polymorphism 213
8.1 Introduction 213
8.2 Theory 214
8.2.1 Solid-State Forms 214
8.2.2 Form Relationships 218
8.3 Solid-State Form Discovery and Selection 222
8.3.1 General Considerations 222
8.3.2 Form Discovery 223
8.3.3 Form Selection 226
8.4 Analytical Characterization of Solid-State Forms 227
8.5 Caffeine Polymorphism: A Practical Example 228
8.5.1 Introduction 228
8.5.2 Caffeine Properties 229
8.5.3 Caffeine Form Discovery – Initial Tests 230
8.5.4 Form Discovery – Polymorph Screening 234
8.5.5 Form Stability and Form Relationships 238
8.5.6 Thermodynamic Relationship of Anhydrous Polymorphs 240
8.5.7 Caffeine Hydrate 241
8.5.8 Caffeine Example Summary 242
8.6 Summary 243
References 244
Caffeine Polymorphism 245
Regulatory Guidance 246
Additional General References 246

9 Size and Shape Analysis 247
9.1 Introduction 247
9.2 Qualitative Shape Analysis 248
9.3 Image Analysis Examples 250
9.3.1 Image Analysis of Hydrocortisone Cream with Optical Microscopy 250
9.3.2 Image Analysis of Carbon Black with SEM 254
9.3.3 Image Analysis of Generic Naproxen Sodium Tablet: Backscatter and EDS 258
9.3.4 Summary of Exercises 264
9.4 Method Development and Error Analysis 264
9.4.1 General Considerations 264

9.4.2 Method Development 265
9.4.3 Error Analysis and Validation 266
9.5 Specifications 273
9.6 Automation 274
9.7 Summary 276
References 276
Internet Reference 276

10 Contaminant Analysis 277
10.1 Introduction 277
10.2 Robert's Rules for Contaminant Identification 279
10.2.1 Rule #1: Think Before You Act 279
10.2.2 Rule #2: Get All the Information Before You Start 279
10.2.3 Rule #3: Clearly Establish the Goal of the Investigation 280
10.2.4 Rule #4: Every Experiment Should Test a Hypothesis 281
10.2.5 Rule #5: Start Simple and Progress to Complex 281
10.2.6 Rule #6: Maintain Clear Distinction Between Fact and Hypothesis 283
10.2.7 Rule #7: Verify Conclusions 283
10.2.8 Rule #8: Document as You Work 284
10.2.9 Rule #9: Have a Clear Exit Strategy 285
10.3 Practical Examples 286
10.3.1 Introduction and Method of Analysis 286
10.3.2 Dust 286
10.3.3 Activated Charcoal in Powdered Sugar 289
10.3.4 Polymer Tubing in Corn Starch 294
10.3.5 Silicone Oil in Water 299
10.4 Laboratory Requirements for Contaminant Identification 301
10.5 Summary 302
Reference 303
General References 303
Internet References 303

11 Conclusion 305
11.1 General Summary 305
11.2 Training and Education 307
11.3 Experts vs. Generalists 308
11.4 Conclusion 309

Index 311

Chapter 1
Introduction

1.1 Microscopy and Industrial Microscopy

The art and science of modern microscopy began with the research and work of van Leeuwenhoek in the Netherlands and Hooke in England during the seventeenth century (Needham 1958). These initial developments were quickly followed by innovations in the design and manufacture of microscopes and in the range of applications. It was the nineteenth century, however, that saw the development of well-corrected optics for compound microscopes and the development of good microscope stands. These instrumental developments were part of the general advancement of science and technology in the industrial revolution, but were also a response to the interest in microscopy by amateurs. Evidently, it was common for Victorian age dinner parties to end with the gentlemen and ladies pulling out their microscopes and examining beautifully prepared slides of animals, plants, and minerals – in separate rooms, of course.

Most applications and uses of the microscope centered on academic and amateur interests until the mid-nineteenth century when a number of scientists and engineers began to apply microscopy to industrial problem-solving (Croft 2006). Microscopy was used to aid in food manufacture, in vaccine development, in metals, in chemistry, and so on. Industrial microscopy has surged in the twentieth century to embrace nearly every business enterprise, including paper, glass, polymers, and, yes, pharmaceuticals. Microscopy has been applied to drug development for some time both in biological and physical fields. Today, the schematic image of an optical microscope is a universal sign for science itself.

1.2 Pharmaceutical Microscopy

This is a textbook on the subject of pharmaceutical microscopy. Every drug that you take is delivered in a physical form: tablet, capsule, inhaler, topical, etc. The drug is composed of a number of different components which can be categorized as active drug substance (API – active pharmaceutical ingredient) or as an excipient.

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Excipients are used for a variety of purposes such as bulking agents, disintegrants, lubricants, etc. They can be both organic and inorganic in nature. Both the drug substance and the drug product (DP) are influenced by material properties that can be studied using different kinds of microscopy. Some of these material properties include particle size, polymorphism, particle morphology, crystallography, etc. Microscopy is heavily used in pharmaceutical research, development, and manufacturing. A wide variety of microscopy techniques are applied to this study and include polarized light microscopy, thermal microscopy, electron microscopy, microspectroscopy, digital image analysis, as well as other more specialized techniques. Microscopy is applied to both biological and physical sciences in drug development, but this book only discusses physical applications.

Pharmaceutical solid dosage forms impose a number of burdens on the drug substance (DS) besides being efficacious. For one thing, DS must be chemically and physically stable over the shelf life of the drug (often 2 years). It must be very pure. If direct compression is used to make tablets, the API (I use DS and API interchangeably in this book) must be stable to pressure. It needs to flow well. It must be well-behaved with regard to its physical form. In other words, the DS should not be a hydrated form that spontaneously converts to the anhydrate in the tablet, for example. The API needs to be somewhat soluble in the gastro-intestinal tract and the dissolved chemical should be permeable across the biological membranes of the tract. Ideally, the DS can directly crystallize to a specified particle size or, if not, it must be stable to comminution processes. With all of these requirements and burdens, it is amazing that any chemical can be made into a drug product.

Many of the properties of the API fall under the discipline of solid-state chemistry and the pharmaceutical industry employs quite a number of solid-state chemists. Virtually all prospective pharmaceutical molecules are studied using a wide variety of solid-state techniques such as X-ray diffraction (powder and single-crystal), thermal analysis (differential scanning calorimetry, thermal gravimetric analysis, etc.), gravimetric water vapor absorption, surface area analysis, particle size analysis (light diffraction, sieve analysis, etc.), solid-state nuclear magnetic resonance, among many others. Microscopy plays a prominent role in pharmaceutical development and this text presents the main uses of microscopy in physical pharmaceutical development.

Microscopy is principally used for three purposes: solid-state form analysis; size and shape analysis; and for the identification of contaminants. All three of these areas are covered in this book. The microscopy techniques to be discussed include optical and polarized light microscopy, scanning electron microscopy, thermal microscopy, microspectroscopy, image analysis, and sundry other techniques.

1.3 Purpose of the Book

The Shakers (a small nineteenth-century religious group) believed that a measure of one's faith is the devotion to excellence in tasks both large and small. Or as my father put it, "A job worth doing is a job worth doing well." That seems an antiquated idea these days and I do wonder whether, in the past, the ideal was honored more in the breach than the observance. In any case, I doubt I will hear that ideal espoused by managers and CEOs here at the beginning of the twenty-first century. The reason for this change probably has much to do with the need to maintain a lean workforce and workers who are flexible and can do many different things. Yet, paradoxically, it is technical excellence that is the engine of material progress and those enterprises that ignore technological advances quickly falter and fail. For us as individuals, though, managing a technical career today requires us to be nimble, at the forefront of advances, yet also an expert in the application of not only the new but the old.

That is all a long preamble to the statement of the purpose of this book and my quandary. The purpose of this book is to provide the general solid-state analyst the necessary tools to use microscopy to aid in the development of pharmaceuticals. Consequently, it is slim on theory and fat on practice. I have attempted to present sufficient theory for a basic understanding of practice. I have bursting bookshelves, chock full of excellent texts on nearly every topic in microscopy. Rather than attempt to better the best, I shall simply refer to many of these texts for an explanation of specific theoretical aspects of microscopy and highlight the ones that I find particularly helpful and useful in pharmaceutical research.

My quandary was whether I should write a text on pharmaceutical microscopy aimed at the expert working in the field or a text for the general solid-state analyst. Well, to be frank, there are only six of us left in the first category (OK, only a slight exaggeration) and the other five probably know more than I do, so there is not much sense in writing for them. There are, however, a great many fine pharmaceutical scientists who use or try to use microscopy in pharmaceutical research but for whom microscopy is one of a suite of useful tools. These folks need information and knowledge but have neither the time nor the inclination to become an expert microscopist. My goal is to provide the general analyst with the necessary information to do the minimum required with the microscope in pharmaceutical development, while pointing the way to developing expert skills.

The author has worked in the glass, polymer, metal, and pharmaceutical industries as a chemical microscopist for over 30 years. I have found much of interest and exciting problems to solve in all of these industries, but solid-state organic chemistry is unique in my opinion. There is so much that is undiscovered and unknown that it is much like a discoverer of a new forest. Of the hundreds of molecules that I have worked on, not one has failed to surprise me at some point. In pharmaceutics, we ask molecules not only to be safe, effective, and stable, but also to have physical properties that allow us to make tablets, capsules, ointments, and inhalers. It is these physical properties that often surprise us (i.e., bite us on the posterior) as a drug goes through development and manufacturing. This is an exciting field and the microscopist may have the best seat in the house to watch and participate in the show.

To aid in practice, I provide exercises at the end of most chapters and fully worked examples in other chapters. I have tried to use only materials in these exercises that are readily available to someone in the pharmaceutical industry or at a university.

I concentrate on substances such as caffeine, carbamazepine, naproxen sodium tablets, hydrocortisone creams, etc. In some places, however, I have not been able to find readily available materials that suit the purpose and have had to fall back on "Material A." I do encourage you to practice these exercises or make up your own. Microscopy is not a subject that you can learn just by reading a book. You have to do the task to truly learn it well.

1.4 Plan of the Book

I have divided the book into two general sections: Methods and Techniques; and Applications. I begin with a discussion of polarized light microscopy (PLM) and optical crystallography in Chap. 2. PLM is a superb tool for the analysis of solid-state forms but has fallen out of favor in recent years. I am not sure why this is so given the power of the technique, but I suspect it is due to the need to devote consider time and study to the discipline even to begin to use it. It may also have something to do with the observational nature of the resulting data. In any case, I believe that even a small amount of study of these techniques can yield good rewards and so I begin with this subject. I next move on to thermal microscopy in Chap. 3. This technique is used more often for form analysis than is PLM, but still is probably underutilized. The technique yields information that is beneficial in its own right, but also is supremely adapted as an aid in interpretation of data from other thermal methods.

Chapter 4 covers scanning electron microscopy (SEM) and energy dispersive X-ray spectrometry (EDS). The SEM is most often used for morphological studies of API and drug product. It can also be used in conjunction with EDS for mapping and for contamination analysis. Chapter 5 presents techniques with microspectroscopy, in particular IR and Raman microspectroscopy and their applications, which include chemical mapping and contamination examinations. Chapter 6 deals with a variety of other types of microscopy that have been applied to pharmaceutical development but are not in routine use at this time. It is useful to be aware of these techniques and how they have been applied. Chapter 7 covers the subject of size and shape analysis of drug substance particles using image analysis and the optical and scanning electron microscopes. Since this is a fairly involved topic, the chapter covers issues from instrumentation to statistics.

The next section of the book is devoted to applications of the previously discussed techniques. The first application is to polymorphism and solid-state form in Chap. 8. There is a discussion of how microscopy techniques can be applied to solid-state studies and an extensive discussion of caffeine solid properties. You will note that a number of other solid-state techniques are discussed as well. Solid-state analysis is so complex that we require many different probes to understand the behavior of solid organic molecules. There simply is no one technique that does it all and many are required. To paraphrase a common saying of Walter McCrone, there are very few problems for which microscopy will not make a major contribution and there are some that it can solve completely. Chapter 9 presents applications of size and shape analysis. The size and shape of API can have an influence on the bulk physical properties of the drug substance and an influence on drug product. Good correlations require that we have reliable quantitative values for the size and shape of the API particles. This chapter discusses how to obtain those values.

The applications part of the book ends with a discussion of contamination analysis in Chap. 10. Microscopy techniques are ideally suited for the analysis of contaminants, yet there is not much in the literature dealing with this subject. I suppose the dearth of literature references is related to the reticence of companies to publish details of defects. In that spirit, the actual applications in this chapter do not relate to any real cases. The examples are artificial in that I purposely contaminated materials. Rest assured, though, that the artificial examples have some basis in my own experience in this field.

Each chapter contains its own list of references. In some cases, I also include interesting articles or books that are not directly referred to in the text. I have also listed a few Web sites of interest but with some trepidation. It seems that sites are rather ephemeral and I cannot even be sure that all of them will be live at the time the book is published much less years later. I decided to include some Web sites though given our reliance on such sources for information.

This is a book about pharmaceutical microscopy, although many if not all of these techniques can be applied to other industries. Given the uncertain nature of the industrial enterprise, it behooves the industrial scientist to find ways to apply their craft across a wide variety of fields. The history of microscopy is replete with examples of new applications of old techniques and there is no reason to believe this trend will not continue well into the future.

1.5 How Best to Use the Book

I have designed this book with exercises in mind and exercises with commonly available materials. I hope you use this book next to the microscope and work exercises as you read. I have also tried to make exercises and sections independent of other sections. In other words, if you need to work on hot stage microscopy and have limited experience, I am hopeful you can read the chapter on that subject, work a few exercises and then go to your work. I think that is the best way to use the text.

In that vein, you will notice that not all figures and photographs are perfect – in fact some are far from perfect. Rather than present the ideal, I give you the real. I have not done any cleanup with an imaging program and you will see some inevitable dust ghosts. You will notice some issues with white balance. My intention in leaving these problems is to let you see the images as you might see them on your microscope. I once had a manager ask me why my images were not as fine as some he had seen in a magazine using the same microscope. Sure enough mine were not nearly so clear and perfect. I knew the author and learned that the images were heavily corrected in an imaging program. He showed me the originals and they were not very different from mine. So, in this text, I present the images to you as you will see them.

I have established a Web site at carltonmicroscopy.com. If you have comments, corrections or recommendations, please feel free to contact me at that site.

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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 ~5 μm 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

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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 pharmaceutics. 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 crystallo-graphy 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 micro-scopy. 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^2. 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:

$$f = \upsilon / \lambda, \tag{2.1}$$

where f is the frequency, υ is the velocity of the wave, and λ is the wavelength.

For the electromagnetic spectrum,

$$f = c / \lambda. \tag{2.2}$$

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.

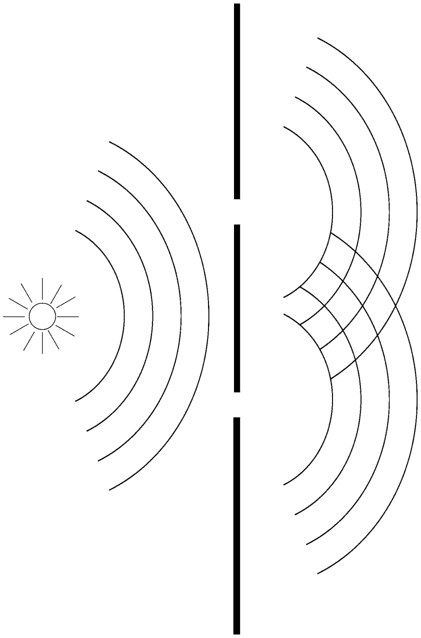

Fig. 2.1 Schematic illustration of light diffraction. Light exiting a slit behaves as a new light source in the sense that waves propagate from the slit as well as from the original source

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

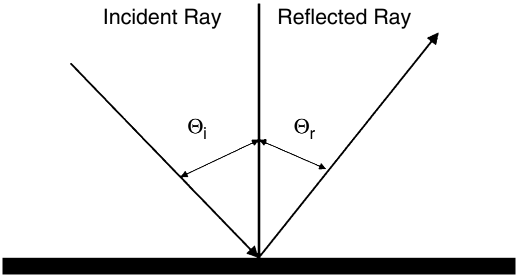

Fig. 2.2 Light reflection. Angle of reflection equals angle of incidence

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:

$$n = \upsilon_{\text{vacuum}} / \upsilon_{\text{medium}}, \tag{2.3}$$

where υ 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

$$\frac{n_\mathrm{r}}{n_\mathrm{i}} = \frac{\sin\theta_\mathrm{i}}{\sin\theta_\mathrm{r}}, \tag{2.4}$$

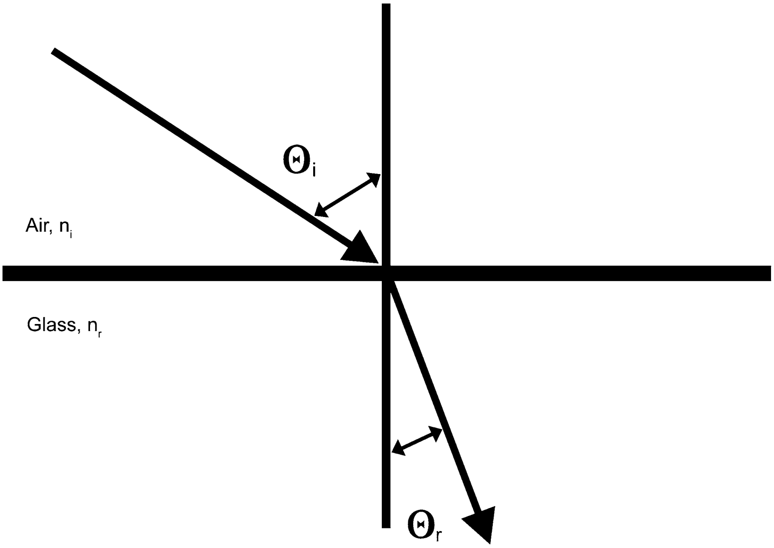

Fig. 2.3 Schematic illustration of Snell's law. Mathematical relationship governing ordinary refraction

where n_r is the refractive index of the refracting material, n_i is the refractive index of the incident material, and θ 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 pharmaceutics.

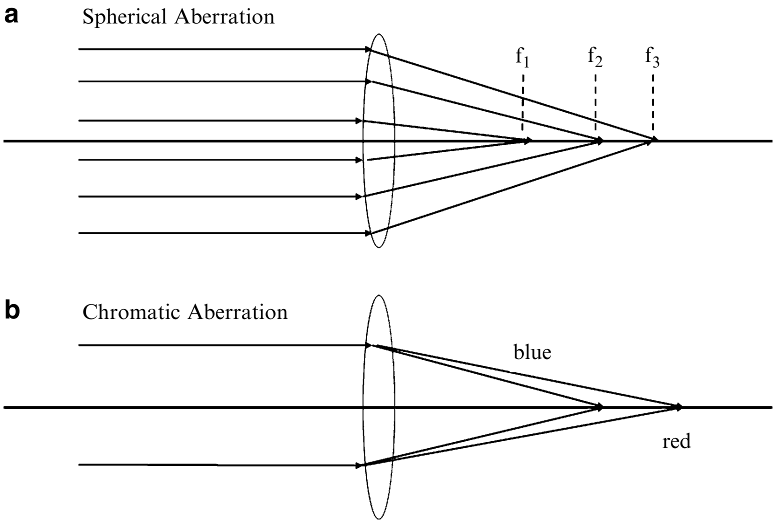

Fig. 2.4 Illustration of spherical and chromatic aberration. (**a**) Real lenses do not focus rays at exactly the same point. (**b**) Different wavelengths of white light focus at different points

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 μm 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 ½ the disc diameter. The key equation governing resolution in the microscope is as follows:

$$d_{\mathrm{r}} = \frac{0.61\lambda}{\mathrm{NA}}, \tag{2.5}$$

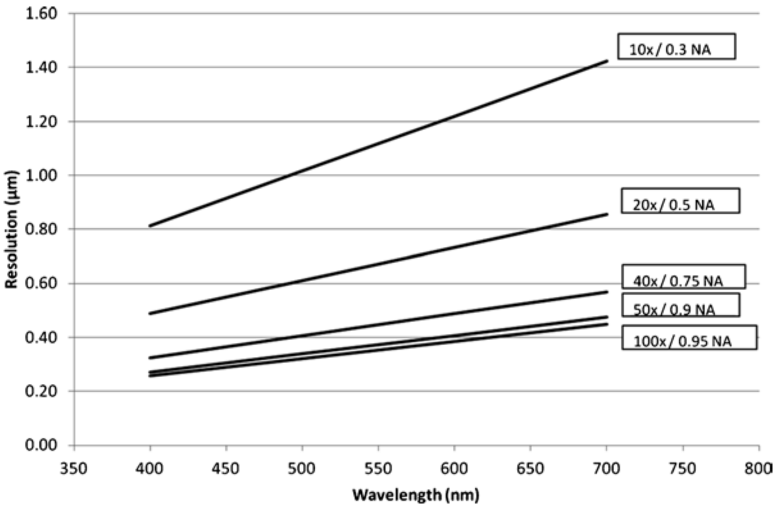

Fig. 2.5 Graph relating resolution to wavelength for some objectives. Resolving power is dependent on the numerical aperture of the objective and light wavelength. Higher NA and lower wavelength allows for highest resolution

where d_r is the resolution (diameter of smallest resolvable feature), λ 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
	Unequal spacing along that axis
Orthorhombic	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
	Unequal spacing along that axis
Triclinic	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

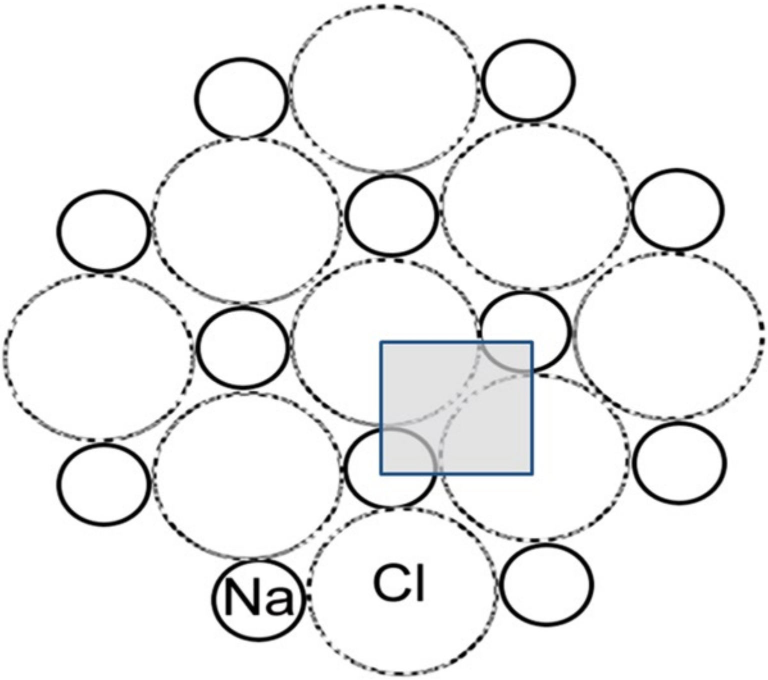

Fig. 2.6 Repeating pattern for sodium chloride crystal. The repeating pattern for sodium chloride crystals is made up of alternating sodium and chlorine atoms. This illustration shows the pattern in two dimensions. In a real crystal, the pattern extends in all three dimensions. The box illustrates the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

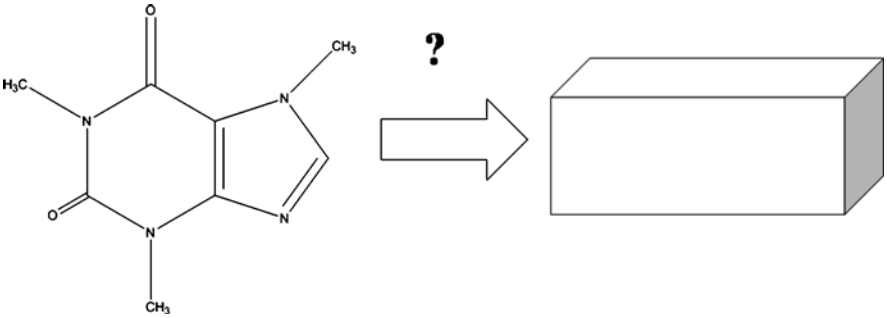

Fig. 2.7 Fit of caffeine molecule in tetragonal unit cell. It can be difficult to imagine how a caffeine (or other organic) molecule fits in the unit cell. The cell is not arranged so that each atom of the molecule sits at the junction of axes but one or more caffeine molecules sit within the unit cell. 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_α 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 Å; b – 16.751 Å, and c – 14.800 Å with a β angle of 95.8°. 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 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 $\varepsilon > \omega$ optic sign if positive, negative if opposite. For biaxial crystals, if value of β closer to that of γ, optic sign if negative, if closer to α then optic sign is positive
Optic axial angle	Angle between optic axes, evidenced as angle between dark regions in interference figure. Is 90° for uniaxial and orthorhombic crystals, and between 0 and 90° 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
No polars			
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
1 Polar			
Pleochroism		X	X
Refractive indices	X (polar not required)	X (ε, ω)	X (α, β, γ)
Birefringence (calculated)		X	X
R.I. Dispersion	X (polar not required)	X	X
Crossed polars			
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

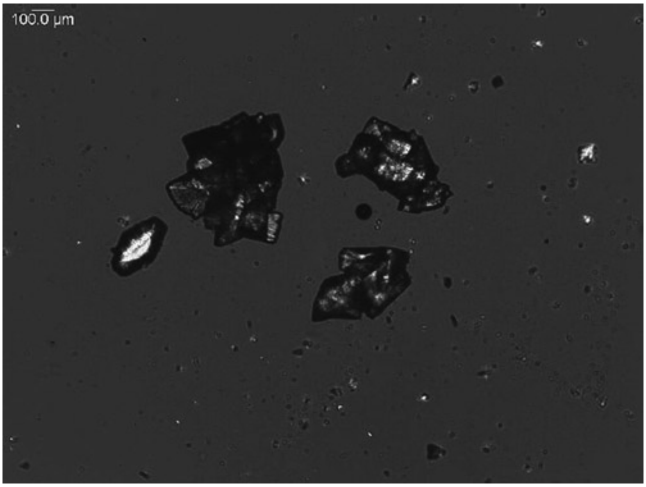

Fig. 2.8 Carbamazepine Form III. This image illustrates typical particle agglomeration as seen in the optical microscope. (Image size = 1,400 × 1040 μm)

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

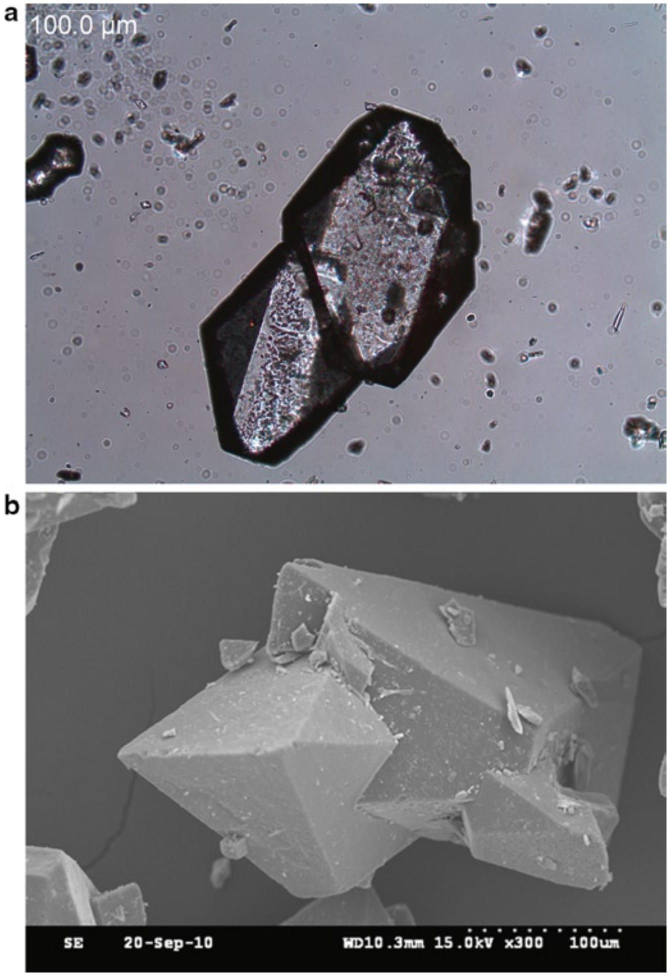

Fig. 2.9 (**a**) Optical photomicrograph and (**b**) SEM image of twinned carbamazepine. These images illustrate typical interpenetration twins. SEM is most useful for this type of twinning whereas PLM is better suited to twins joined along their length. (PLM image size = 2,800 × 2,080 µm; SEM image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

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. Twining 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.

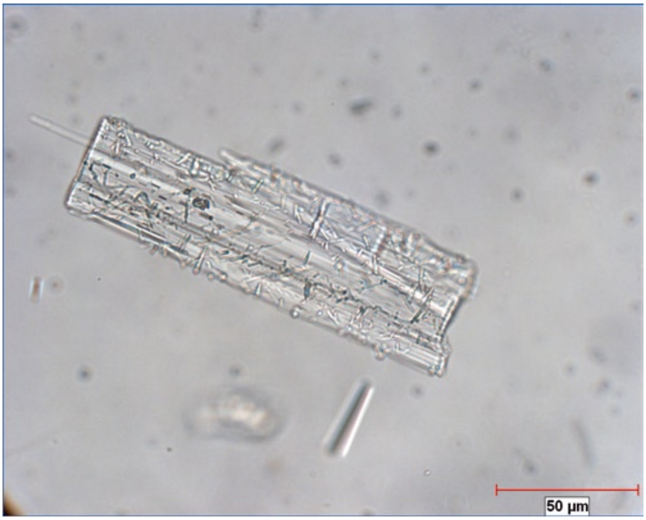

Fig. 2.10 Caffeine Form II growing on crystal of Form I. Appearance of crystals growing on the surface of the original crystals is one good application of assessment of surface texture during stability trials. (Image size = 210 × 170 µm)

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.

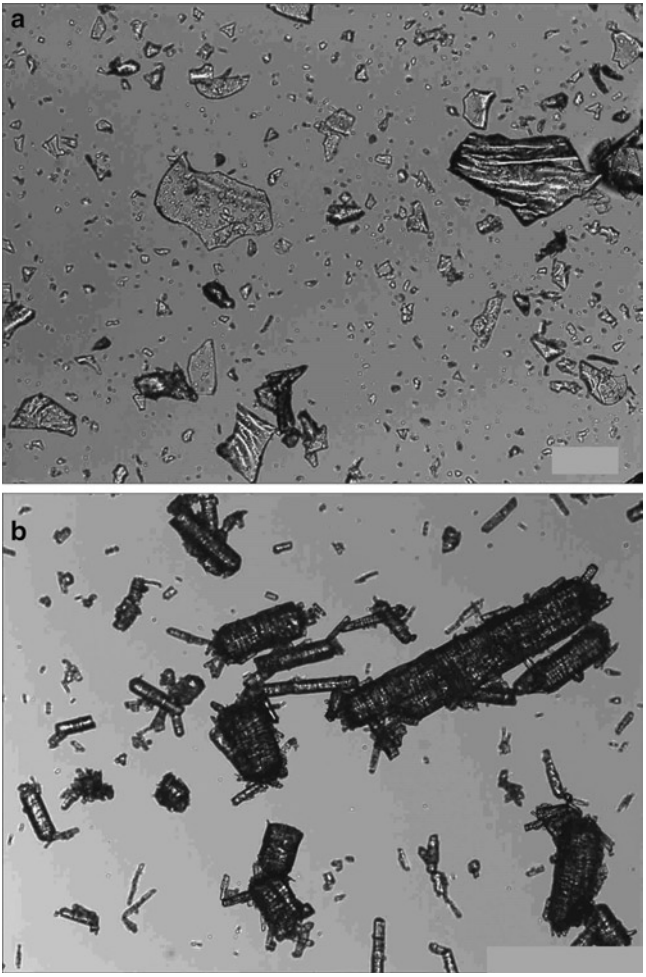

Fig. 2.11 Appearance of desolvated sample: (**a**) before desolvation and (**b**) after desolvation. Image (**a**) is the initial sample, whereas (**b**) is after storage at 30°C and 65% relative humidity for 3 months. The loss of transparency is one indication of a loss of solvent. (Image size = 1,400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

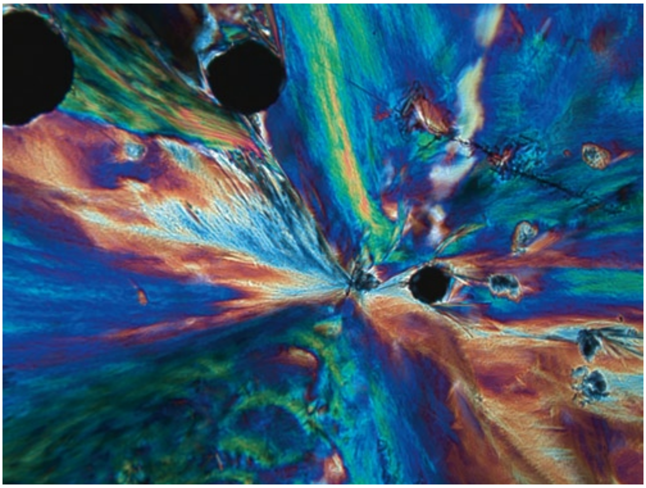

Fig. 2.12 Acetylsalicylic acid (aspirin) fusion preparation. Illustrates the beauty and utility of crystal interference colors. Note the color progression extending from the central region toward the *upper left* of the photomicrograph. The color progression proceeds from *black* to *white*, *yellow*,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

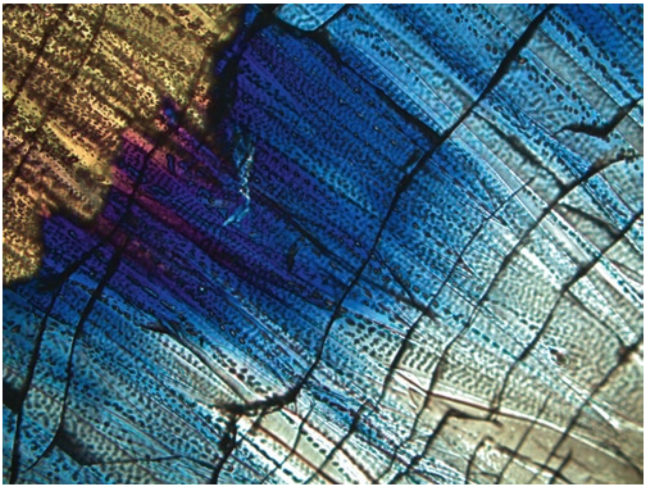

Fig. 2.13 Benzil fusion preparation. Anomalous polarization colors as displayed by a benzyl fusion preparation. Note the progression from *white* to *blue* and the difference in color progression as compared with acetylsalicylic acid in Fig. 2.12. (Image size = 2,800 × 2,080 µm)

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

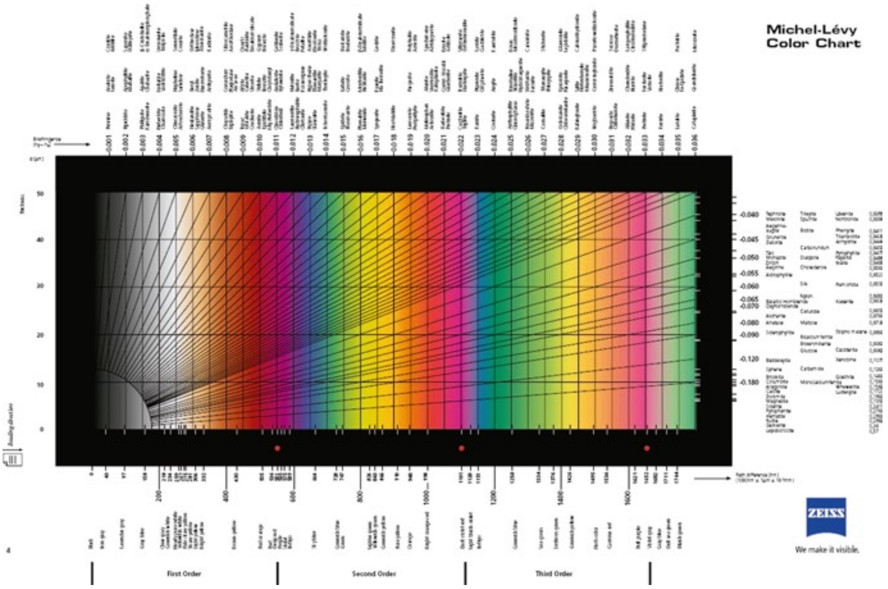

Fig. 2.14 Michel-Levy Chart (copied with permission by Carl Zeiss, Inc.). The Michel-Levy chart shows the progression of interference colors with thickness based on the birefringence of the material. The chart can be used to estimate birefringence as an aid in refractive index determinations

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-Levy chart (see Fig. 2.14 which is a copy of the Michel-Levy chart as produced by Zeiss) and the thickness of the specimen. Retardation is defined mathematically as follows:

$$r = 1{,}000\mathrm{t} \times B \tag{2.5}$$

where r is the retardation in nm, t is the thickness in μm, and B is the birefringence. We use the Michel-Levy 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 360º. 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 × 2,080 μm)

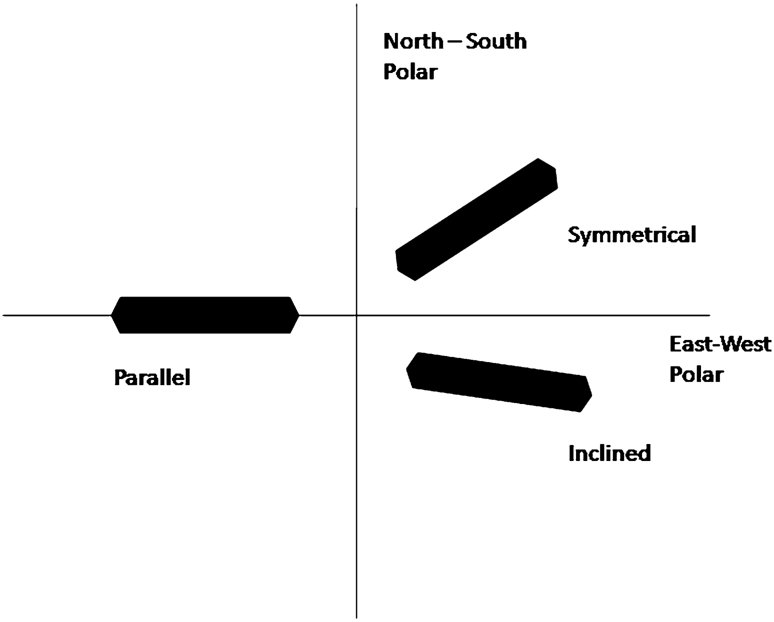

Fig. 2.16 Schematic drawing of extinction positions. Crystals can have parallel, symmetrical, or inclined extinction. Monoclinic and triclinic crystals are the only ones that display inclined extinction. The extinction angle is constant for crystals but can vary with wavelength

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

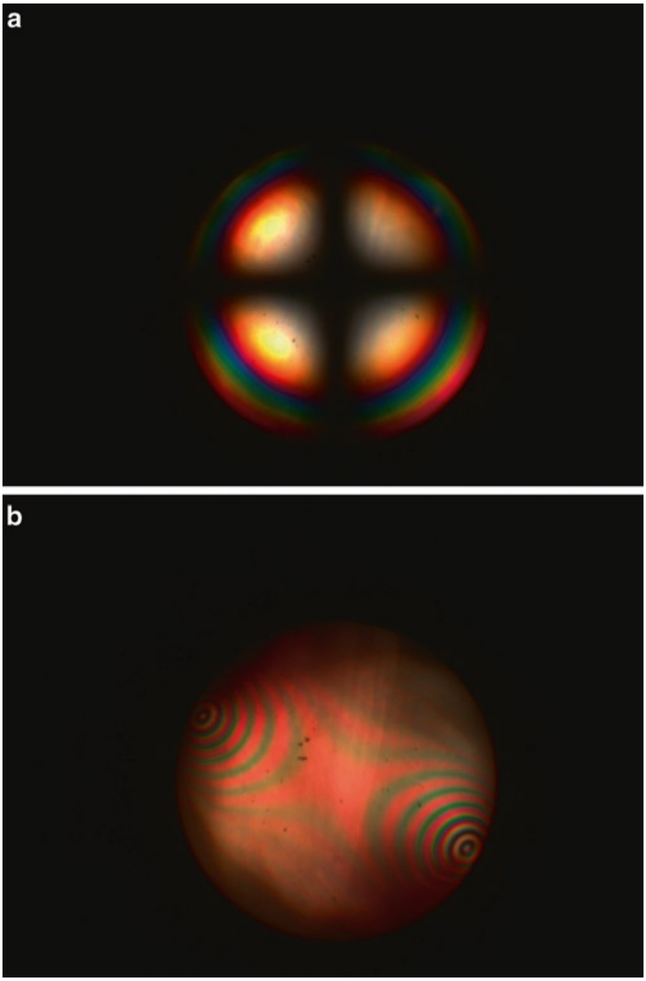

Fig. 2.17 (**a**) Sodium nitrate, uniaxial interference figure; (**b**) mylar, biaxial interference figure. A Sodium nitrate fusion preparation and Mylar show centered uniaxial and biaxial interference figures. Most crystals and crystal fragments show off-center figures

interference figures are called isogyres, whereas the focal point of each arm of the hyperbola in a biaxial interference figure is called a melatrope. The melatrope 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 β closer to that of γ, the optic sign is negative, if closer to α 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

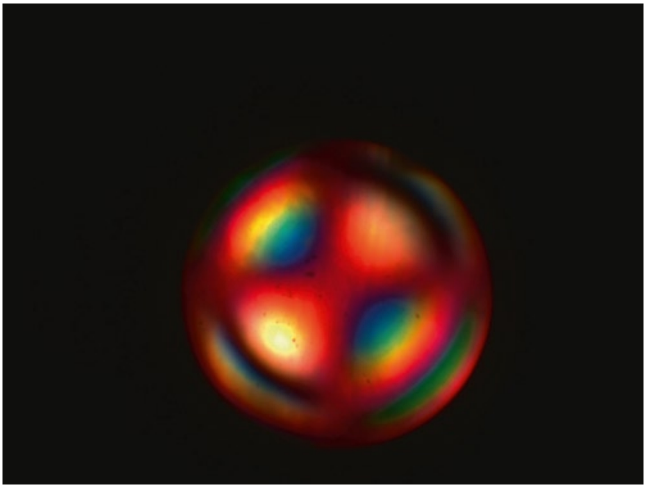

Fig. 2.18 Uniaxial interference figure with lambda compensator. The uniaxial interference figure of sodium nitrate (Fig. 2.17a) has *red color* in the second and fourth quadrants and *blue color* in the first and third quadrants. This is a negative optic sign

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

Determination of the exact angle (referred to as 2 V) 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° 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.

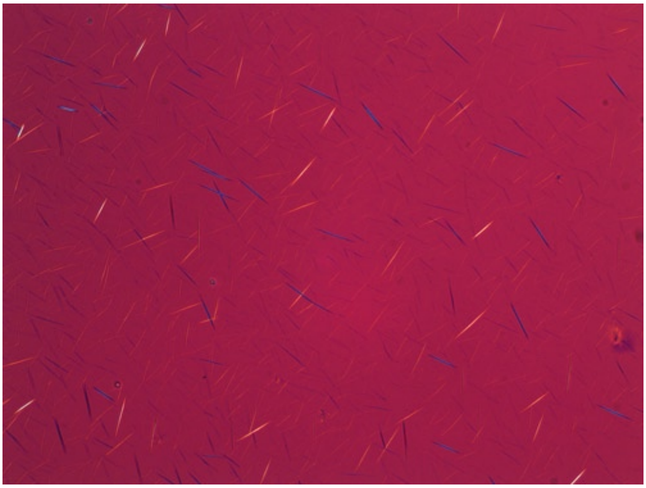

Fig. 2.19 Caffeine with negative sign of elongation. Sublimed caffeine with crossed polars and lambda waveplate. Crystals with the long direction in the second and fourth quadrants are *yellow*, whereas those with the long direction in the first and third quadrants are *blue*. This color indicates a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

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

→

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)

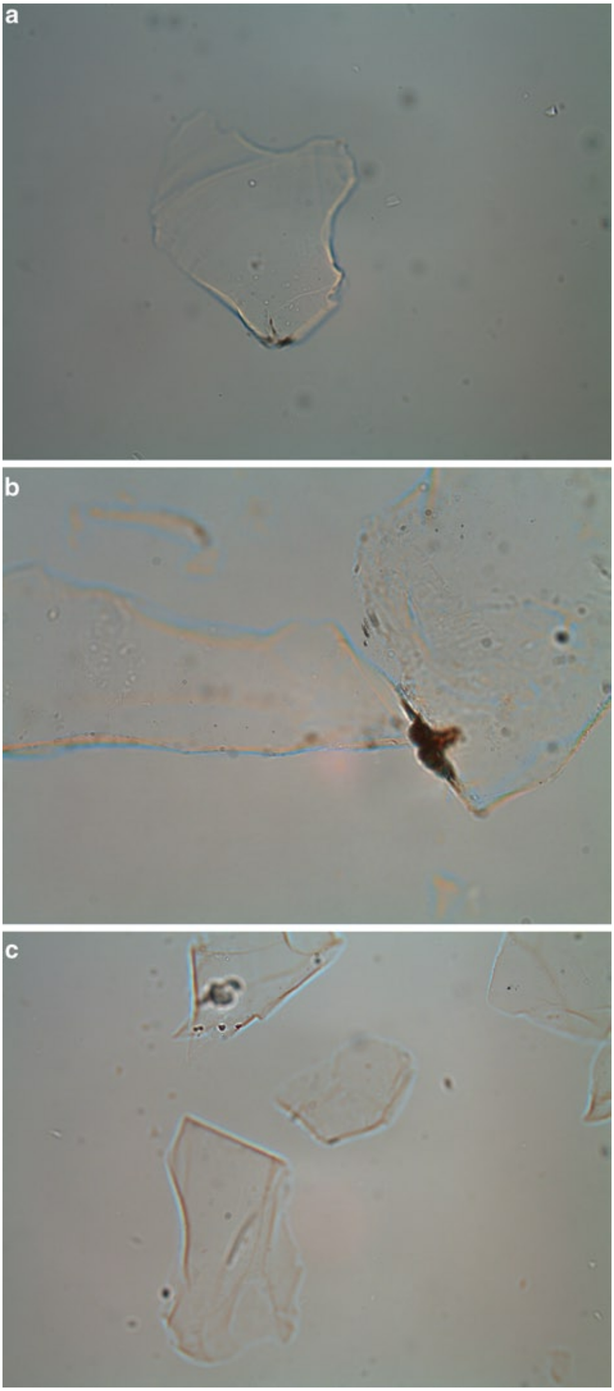

Fig. 2.20 Glass particles in RI liquids illustrating Becke line. Glass fragment with RI of 1.540 mounted in Cargille liquids with indices of (**a**) 1.536, (**b**) 1.540, and (**c**) 1.544. Note that the Becke

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× and 40×.
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

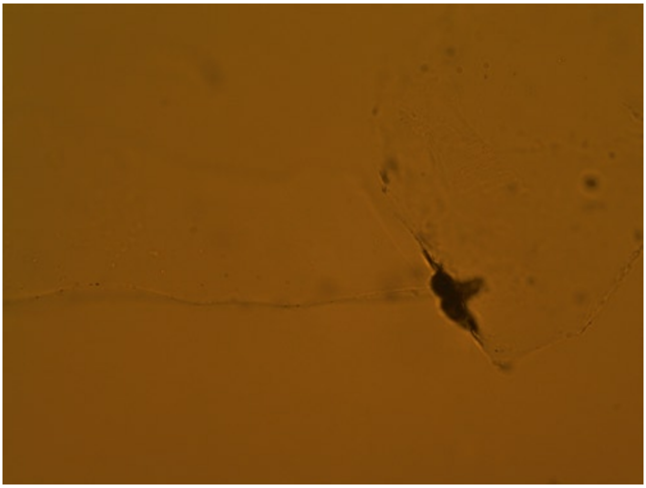

Fig. 2.21 Filter (589 nm) used for refractive index. A 589 nm interference filter has been inserted into the light path of the particles in Fig. 2.20b. No bright lines are observed. (Image size = 1,400 × 1,040 μm)

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 ε (extraordinary ray) and ω (ordinary ray), although there were other naming conventions used in the past and you will still occasionally see n_ε for the extraordinary index and n_ω 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 ω at one extinction position of the crystal and any index between ω and ε 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 ω 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 ω, 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 ω index. Figure 2.22a, b shows caffeine crystals at two extinction positions in a refractive index liquid close to the ω index of 1.702. Note the change in contrast of the fibers at each orientation. The ε index is measured, in the search method, by mounting crystals in successive liquids either higher or lower than ω until we reach the outer limit. That index is ε. If ε has a value greater than ω, the crystal is said to have a positive optic sign. If ε has a value lower than ω, 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 ε is that sections showing this index will have the highest interference colors when oriented 45° 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, α is the lowest index, γ is the highest index, and β is intermediate. Some authors use subscripts to designate the indices as n_α, n_β, and n_γ. 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 α, β, and γ (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 α to γ (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 β. 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).

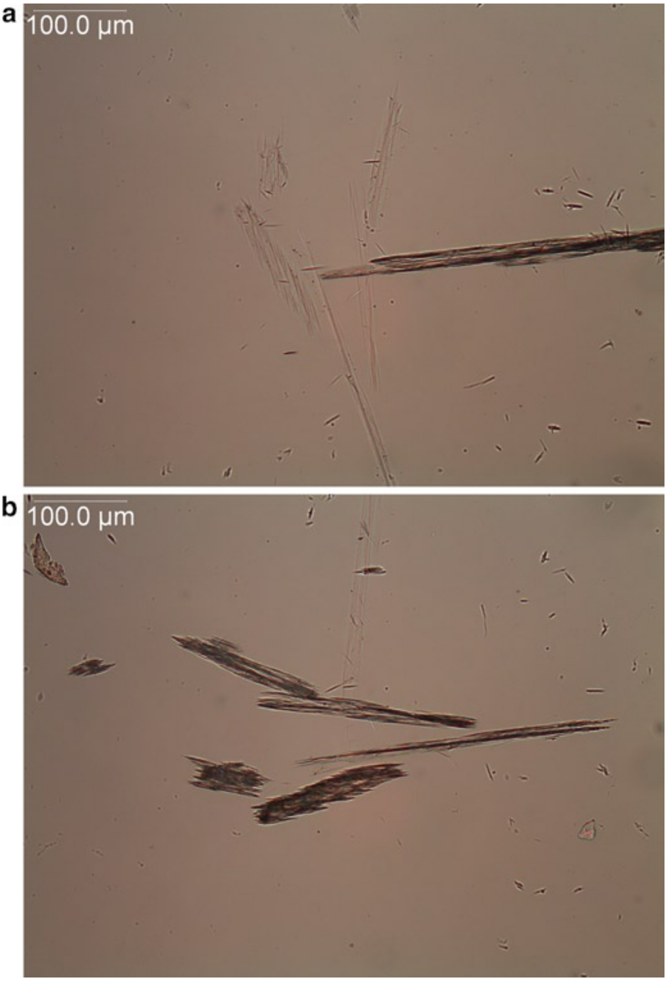

Fig. 2.22 Caffeine in 1.705 Cargille oil in two opposite orientations. Caffeine Form II is uniaxial with an ω index of 1.702. Note the difference in particle contrast at the two different orientations of the rods when in the extinction orientation. (Image size = 700 × 520 μm)

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° 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 α and γ are far removed from each other. If the interference color order is low, then we know they are close.

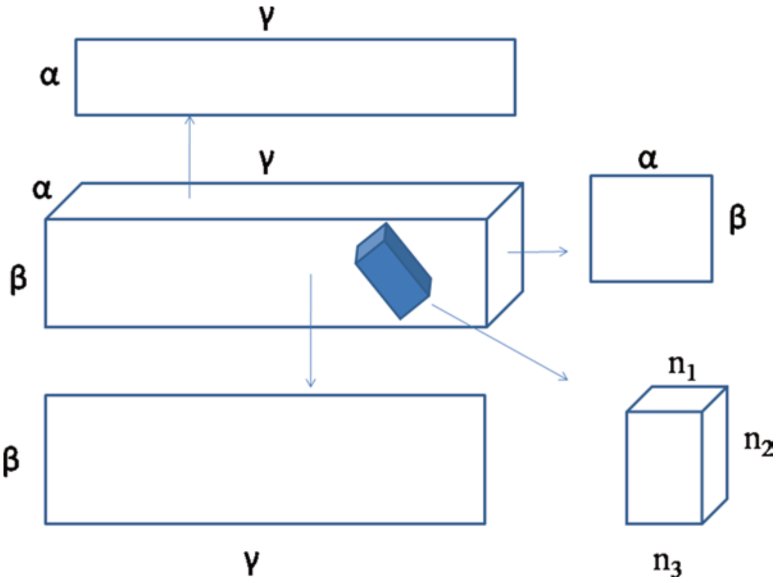

Fig. 2.23 Schematic drawing of refractive indices with biaxial crystal. With well-formed crystals we should be able to measure α, β, and γ by aligning the crystal with extinction positions. In practice, most analyses are made on crystal fragments that can display any indices from α to γ

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 γ 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 β requires recognition of the appropriate vibration direction and for that we need interference figures. We can approximate β, 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 β 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 β. If β is closer in value to α than γ, the crystal is said to have a positive optic sign. If β is closer in value to γ than to α, 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 α. 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
Uniaxial crystals		
Centered optic axis	+ or −	ω
Uncentered optic axis	+ or −	ω, ε'
Flash figure	+ or −	ω, ε
Biaxial crystals		
Acute bisectrix	+	α, β
Acute bisectrix	−	γ, β
Obtuse bisectrix	+	γ, β
Obtuse bisectrix	−	α, β
Optic normal	+ or −	α, γ
Single optic axis	+ or −	β

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 Betrand 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

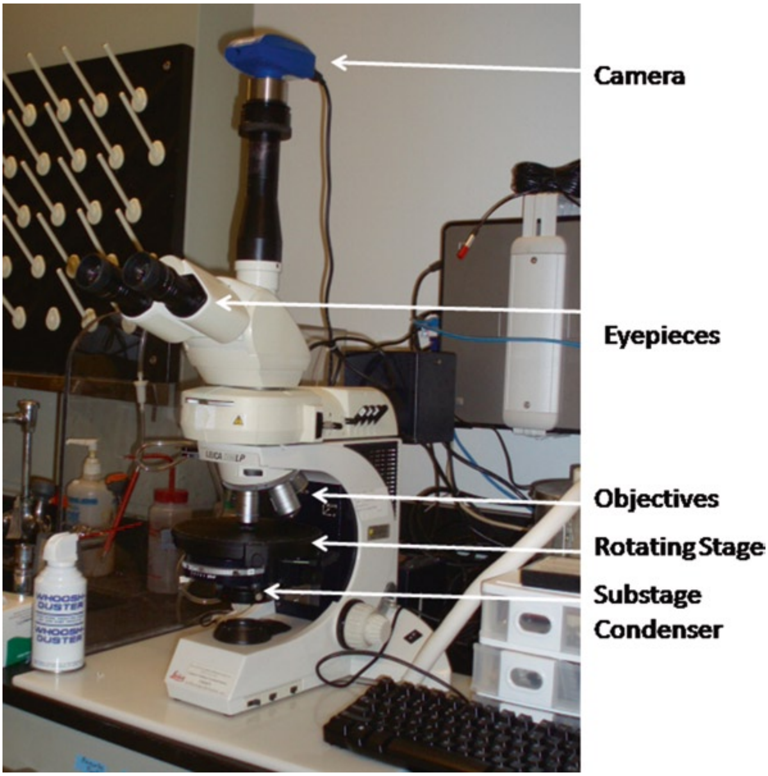

Fig. 2.24 Leitz DM/LM polarizing light microscope. This is one of the microscopes used to produce the images in this book. The other is the Olympus BH2 polarizing light microscope. The Leitz microscope uses infinity optics, whereas the Olympus BH2 has a fixed tube length

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 40×/0.85 NA 160 mm tube length fluorite objective; a Leitz reflected light 50×/0.55 NA fluorite infinity-corrected objective; a Leica 50×/0.50 NA achromat infinity-corrected objective, an Olympus 100×/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×. 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× ocular in one binocular slot and a 20× or 25× 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 λ waveplate that shifts the light spectrum one order. There are also ¼ λ 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 λ 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

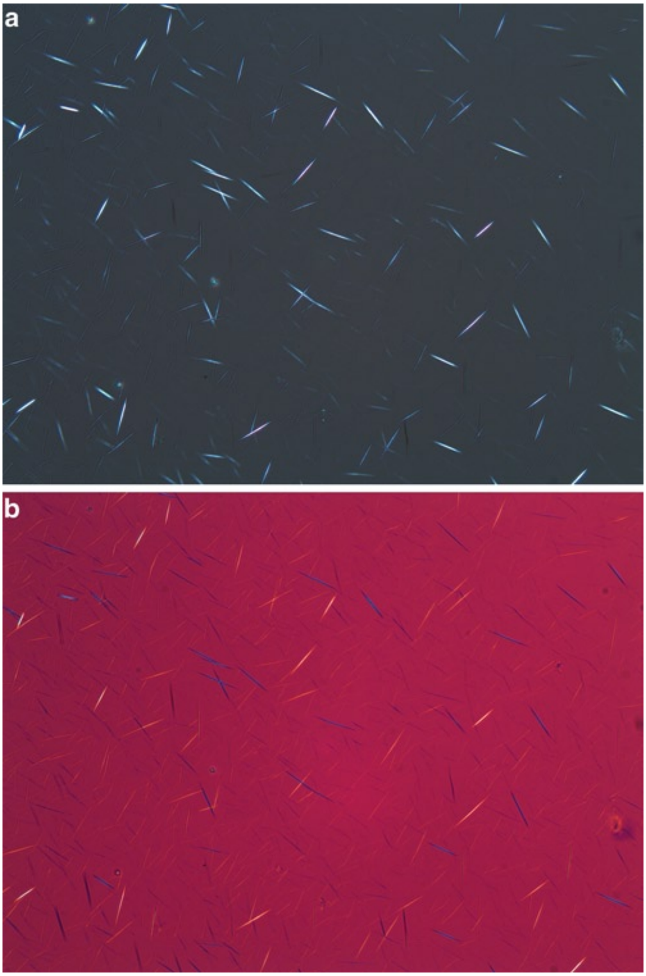

Fig. 2.26 Sublimed caffeine: (**a**) fully crossed polars and (**b**) lambda waveplate. Images (**a**) and (**b**) show sublimed caffeine in crossed polars and then with a full waveplate. The lambda waveplate image can be easier to use for demonstrations and in reports. (Image size = 700 × 520 µm)

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×, 40×).

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 λ 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 µm.

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×), 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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Leica. http://www.leica-microsystems.com/2.9 Photomicrography

Chapter 3
Thermal Microscopy

3.1 Introduction

Thermal microscopy simply requires the addition of a heating and/or cooling stage to an optical or scanning electron microscope. The range of applications of the optical microscope is greatly expanded when we observe the response of materials to heating and cooling. I have included the topic of thermal microscopy as a separate, albeit short, chapter because it has distinct advantages and a unique place in pharmaceutical development. The thermal behavior of pharmaceutical drug substance aids in our understanding of the nature of hydrates, polymorphs, and solvates. Thermal microscopy is not the only way to study the thermal behavior but it does have important advantages over other techniques, not least of which is that we directly observe phase transitions. Direct observations are a distinct aid in interpreting transitions detected and measured by other techniques such as differential scanning calorimetry, thermal gravimetric analysis, variable humidity, and temperature experiments with IR, Raman, and X-ray powder diffractometry. Thermal microscopy is rarely used in isolation and typically is one of a suite of instruments that may be used to understand the solid-state properties of a drug substance.

Kofler and Kofler (1954) and Kuhnert-Brandstatter (1971) in Austria and McCrone (1957) in the USA developed the modern practice of thermal microscopy. [Vitez et al. (1998), Vitez and Newman (2007) present succinct histories of thermal microscopy.] The early workers in the field invented and applied some quite unique approaches to solving solid-state problems. As applied in pharmaceutical development, we are most interested in melting behavior, in phase transitions (solid–solid, solid–liquid–solid, etc.), and in dehydration and desolvation. Nearly, all of this work is applied to the study and understanding of polymorphism and solid-state forms. The Koflers and Kuhnert-Brandstatter also developed fusion techniques for the identification of pharmaceuticals. This application has been supplanted to some extent by chromatographic techniques, but there are still some useful applications of the technique, besides the fact that fusion preparations can be quite beautiful and appealing in and of themselves. Another important application of the thermal stage is in the evaluation of freeze-drying parameters such as eutectic and collapse

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temperatures. Chapter 6 briefly describes about that technique. In this chapter, we cover the following topics: instruments and methods, calibration, basic observations, and fusion methods.

3.2 Instruments and Methods

There are any number of hot stages available both commercially and as described in the literature that individuals can build themselves (Vitez and Newman 2007; McCrone 1975; Wilson and McGee 1988). At the most basic level, one just needs a means of heating a slide preparation, measuring the temperature, and making observations. The simplest type is called the "poor man's" hot stage and consists of a slide with a transparent metallic coating with two leads soldered to the ends of the slides. The leads are connected to a variable voltage source that controls the voltage to the slide and hence the temperature. Koflers described a more sophisticated design and a variant of that design was sold by Thomas Scientific Supply. Both of these designs allowed for reasonably accurate measurement of temperature, a stable platform that served as a heat sink as well, and the ability to arrange heat baffles (glass plates or coverslips) arranged above the specimen so as to control heat loss. Both stages also allowed one to move the preparation on the stage so as to examine different areas. These stages have mostly been replaced by two other types in the pharmaceutical industry. The first is a series of stages built by Linkam Co. in the UK (see Fig. 3.1 and Internet References). These stages are mostly transmitted light instruments like the Kofler and Thomas ones and use a circular silver heating block for good heat conduction with a hole drilled through the block for observations. The second type in common use in pharmaceutics is made by the Mettler-Toledo

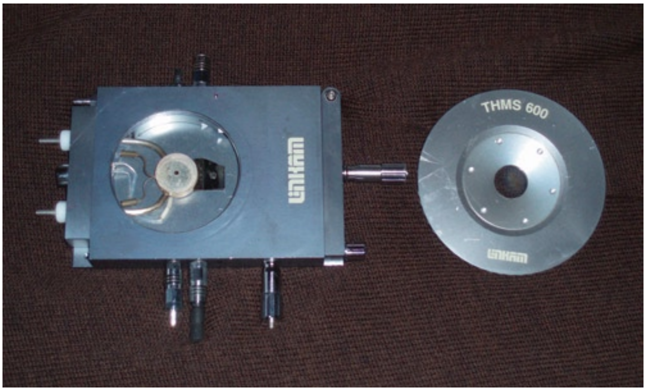

Fig. 3.1 Linkam THMS600 stage. In various configurations, this stage is capable to withstand temperatures between −196 and 600°C. It is probably the most common type of stage used in pharmaceutical development

company (see Internet References). An interesting accessory for this hot stage is a DSC module that allows one to simultaneously record the DSC thermogram and take photomicrographs of that sample. Vitez and Newman (2007) show an example of this application. Modern instruments have sophisticated control systems for heating and cooling cycles and often can be controlled by the same software that controls the digital or analog camera. The Linkam stages can also be fitted with a cooling device using liquid nitrogen or some other coolant that allows one to control the cooling rate as well as heating rate. It also allows one to cool the stage rapidly between tests to improve testing efficiency. In my experience, these control programs take some time to learn and are not particularly intuitive, but are well worth the trouble since they allow for quite a bit of operational flexibility.

Over my career, I have used nearly half a dozen different thermal stages for a variety of different purposes and I greatly prefer the modern stages, such as those made by Linkam and Mettler-Toledo, to the older stages. The modern stages are really excellent at giving the operator good control over heating and cooling rates and in maintaining a set temperature. With the addition of a digital or analog camera to the microscope and appropriate software to run the stage and the camera, one can concentrate on direct observations and less on ensuring proper stage control and documentation. With the older stages, we had to constantly be concerned about maintaining the proper heating rate. Since we often used a simple AC to DC electric transformer, we had to be concerned about the incoming voltage. On high-power usage days, the voltage delivered to the laboratory might be as many as 5–10 V less than on other days or even early in the morning. The incoming voltage irregularities could cause problems achieving reproducible heating rates. On the other hand, the older stages allowed much better access to the sample and enabled the microscopist to manipulate it during experiments if so desired. I think the best of both worlds is to have an older Kofler stage set up next to a newer one since each have particular advantages.

It is not necessary to use a highly sophisticated microscope with the thermal stage, but in my opinion polarized light with the ability to insert a full-wave plate is critical. Some of the solid-state transitions we observe can only be seen in polarized light and it is often easier to record these transitions with fully crossed polars and a full-wave plate than with direct transmitted light. The objectives can be of moderate quality, such as achromats, since we generally operate at low magnifications. Also, since we use relatively high temperatures (as high as 300–350°C) we do have to be careful not to damage the optics with heat, which is another good reason to use less-expensive objectives and substage condensers. For that reason, it is best to use long working-distance or even very long working-distance objectives that are available from most microscope manufacturers. We also have to careful with the substage condenser since it also can be close to the heating element. Personally, I find it useful to use either an intermediate magnifier (if available) or high-magnification eyepieces with the hot stage. Admittedly, I gain no improvement in resolution, but it does help me to see small particle behavior better – but then I have more aged eyes. In any case, if you have an intermediate magnifier or access to high-magnification eyepieces (15, 20×), I recommend you to give them a try.

Sample preparation is a key to obtaining good results with thermal microscopy. I will repeat this statement many times in this book because it is so important. It is common to prepare specimens in both the dry and wet states. With dry mounts, we are looking for not only solid transitions, but also for evidence of sublimation. With oil mounts, we are looking for evidence of solvent release as well as the typical transitions. In the case of dry mounts, we generally want to see a number of discrete particles in the field of view, but not too many since it is difficult to see subtle transitions or even the beginning of the melt in a large mass of particles. We may tend to have a heavier particle loading with oil mounts, since it is often easier to see bubble liberation from a mass of particles as opposed to just a few particles and bubbles. For dry mounts, I often place a small amount of the specimen onto one coverslip, add another coverslip on top, and then rotate one coverslip against the other in order to get an even distribution. The upper coverslip generally has a liberal amount of specimen on it after this operation and those particles impede observations of sublimation. So, I typically remove the upper coverslip and put a fresh one onto the preparation. I save the original upper coverslip for the next experiment. For oil mounts, I generally deposit a small amount of solid onto the coverslip and then add a very, very small amount of oil and mix. I favor little oil since the oil will flow at higher temperatures and too much makes a mess. Also the oil sometimes flows below the coverslip and can seriously degrade your observations.

It is typical to prepare quite a few dry and oil mounts and test them at different heating rates. It is useful to examine the difference in thermal behavior with wet and dry mounts and at different heating rates. When initially working with triamcinolone acetonide, I completely missed the dehydration and recrystallization near the melting point of the hemi-hydrate even at a heating rate of 4°C/min. Only when I dropped to 1°C/min did I observe this phenomenon. So, I like to use both very high and very slow heating rates.

I favor a baffling system, an illustration of which is presented in Fig. 3.2, for better control of the heat flow in the stage (most of the illustrations and examples

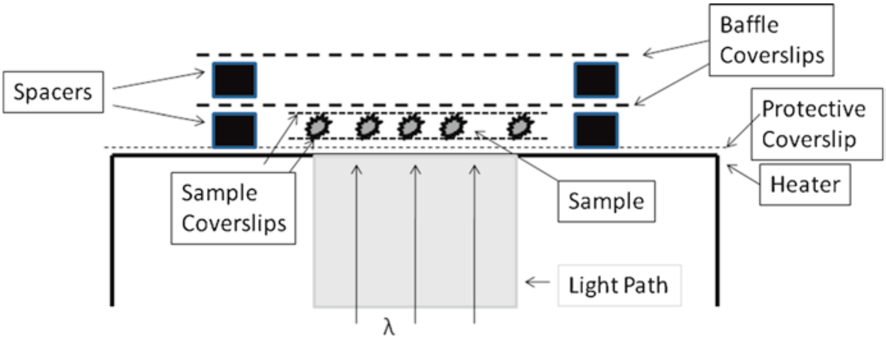

Fig. 3.2 Baffling system for HSM specimen. The specimen is sandwiched between two coverslips and then placed on the heater with a protective coverslip (particularly needed for oil mounts). Metal spacers are used for holding two glass coverslips as baffles to ensure even and reproducible heat flow

in this chapter are using the Linkam hot stage since that is what available to me). There is some divergence of opinion about the need for such extensive baffling. I favor it both by custom but also since it provides a more thermally consistent reservoir for the particles. In other words, the area of the upper coverslip is closer in temperature to the bottom coverslip containing the specimen with this type of baffle system. However, you chose to set up your test chamber, you do need to be sure to calibrate using that arrangement. You cannot be sure that the temperature readout will be correct if you test with a different arrangement from your calibration.

3.3 Calibration

It is good practice to verify calibration with performance checks on a frequent basis. Each stage is a bit different in the calibration details, but fundamentally the calibration operation requires measuring the melting temperature of a known material and adjusting the internal (or external if using a manual system) calibration curve to that known temperature. This requires a set of good melting point reference standards which is not as easy to obtain as you might think. We really need pure materials that do not undergo significant phase transitions at temperatures below the melting point and that have a sharp (1–2°C) melting point. Also, we should have the ability to test melting points along the range of 30–350°C. There are a variety of sets of standards available from a number of commercial suppliers. Most of these sets are specifically designed for the calibration of capillary melting point instruments and some of the standards may not be well suited to hot-stage work due to sublimation, solid-state transitions, and broad melting ranges. Table 3.1 lists the standards put together by Reichert specifically for hot stage and hot bench work and was developed based on the work of Koflers and Kuhnert-Brandstatter. I prefer this set of standards for performance checks. I have also used the sets by Thomas Scientific and by Sigma-Aldrich satisfactorily. One advantage of the Reichert set is that these chemicals are also used for the eutectic temperature

Table 3.1 Melting point standards (based on Reichert–Jung standard set)

Standard	Melting point (°C)	Observations
Azobenzol	68	Sharp melt
Benzil	95	Sublimation from ~85°C, sharp melt
Acetanilide	114.5	Sublimation from ~55°C, sharp melt
Phenacetin	134.5	Sublimation from ~90°C, sharp melt
Benzanilid	163	Sublimation from ~100°C, sharp melt
Salophen	191	Sublimation from ~160°C, sharp melt
Dicyandiamid	210	Sublimation from ~170°C, 2–3°C melt range
Saccharin	228	Sublimation from ~180°C, sharp melt
Phenolphthalein	263	Sublimation from ~230°C, ~4°C melt range

identification scheme published by Kuhnert-Brandstatter (1971) for pharmaceuticals. If you obtain the standards listed in Table 3.1 from a chemical supply house, be sure to order the highest purity samples. Residual solvents and impurities will lower the observed melting point and give false values for the standard melting point. As far as I know, none of the sets mentioned in this paragraph are available commercially. You will have to purchase the chemicals separately.

Systems, in my experience, seem to go out of calibration at random intervals and with little warning, hence the need for frequent verification of calibration. It is also good practice to record the values of these performance checks in order to monitor system performance. I recommend performance checks with melting point standards near the temperature of interest for your sample and with the same test heating rates. This practice is particularly important if the results of the work will be reported to regulatory agencies. In those cases, I do a performance check at temperatures just above and just below the temperature of interest. So, for example, for a melting temperature at 125°C, I might test phenacetin (m.p. 134.5°C) and benzanilid (m.p. 163°C). Performance checks above and below the compound melting point eliminate any question that the observed temperature is correct.

Many organic chemicals sublime before melting and this is true for many of the standards in the Reichert and in other standard sets. In solid-state analysis,

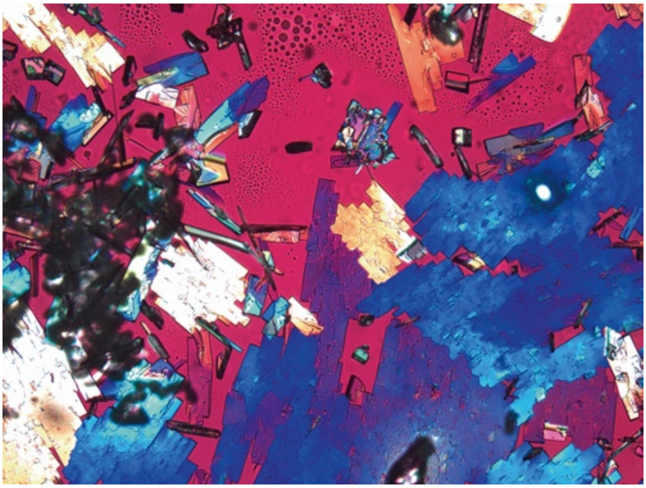

Fig. 3.3 Phenacetin sublimate. This sample was tested at a slow heating rate of 1°C/min allowing a thick crystalline sublimate to form above the standard specimen. The specimen is located on the left side of the image and is a dark mass that is slightly out of focus. This sample illustrates the difficulties that arise in testing standards and samples that sublime (image size = 1,400 × 1,040 μm)

sublimation can be an important means of searching for new polymorphs and forms. In melting point determinations, however, the presence of the sublimate can be distracting and can even lead to incorrect results. In general, the sublimate will appear to melt at a temperature a few degrees higher than the underlying standard specimen since the temperature on the underside of the coverslip is generally 1–2°C less than that of the coverslip in contact with the heating element. In some cases, it can be nearly impossible to see the underlying specimen below the sublimate and all of the standard specimen may sublime so that it is impossible to determine the melting temperature. If this occurs (both with standard and sample), add excess material to ensure that some is remaining at melting and use a high heating rate to just below the melting temperature so as to minimize the time the specimen sublimes. Also, an oil mount can frequently solve this problem. Figure 3.3 shows a crystalline sublimate of phenacetin nearly occluding the standard specimen on the slide (see left side of image for dark mass of particles that are out of focus). This sample was prepared with a slow heating rate of 1°C/min and so there was enough time for a thick crystalline sublimate to form well before the melting temperature.

3.4 Test Strategy

For a new, unknown sample with relatively little thermal information, I recommend the following strategy. Begin with a fast scan (~10–20°C/min) over a wide temperature range (ambient to ~300° or degradation) with a dry mount. Note the temperatures of interest and evidence of solvation, solid–solid transitions, melt, melt followed by recrystallization, etc. Retest at lower heating rates of 3–5°C/min. Use a higher rate (~20°C/min) up to the temperature of interest and then lower the heating rate at 5–10° below the minimum temperature of interest. For critical work, test at a low heating rate of 1°C/min. Repeat these tests with oil mounts. If the melting temperature is below the degradation temperature (pharmaceutical compounds often melt at ~150°C and degrade between 250 and 350°C), Do a dry mount test using a low heating rate (~5°C/min) from the melting temperature up to evidence of degradation. Figure 3.4a, b shows the typical appearance of carbamazepine melt undergoing degradation. Note the progression from clear droplets to orange. Degradation is best seen with brightfield illumination and no polars or compensation plates. There are times that polymorphs will crystallize from the melt at temperatures well above the melting point. It is sometimes difficult to observe these recrystallizations with high heating rates. Finally, prepare a specimen with a generous amount of sample in an oil mount and use a low heating rate from ambient up to ~120°C. With this test, look for bubble formation as an evidence for hydration or solvation.

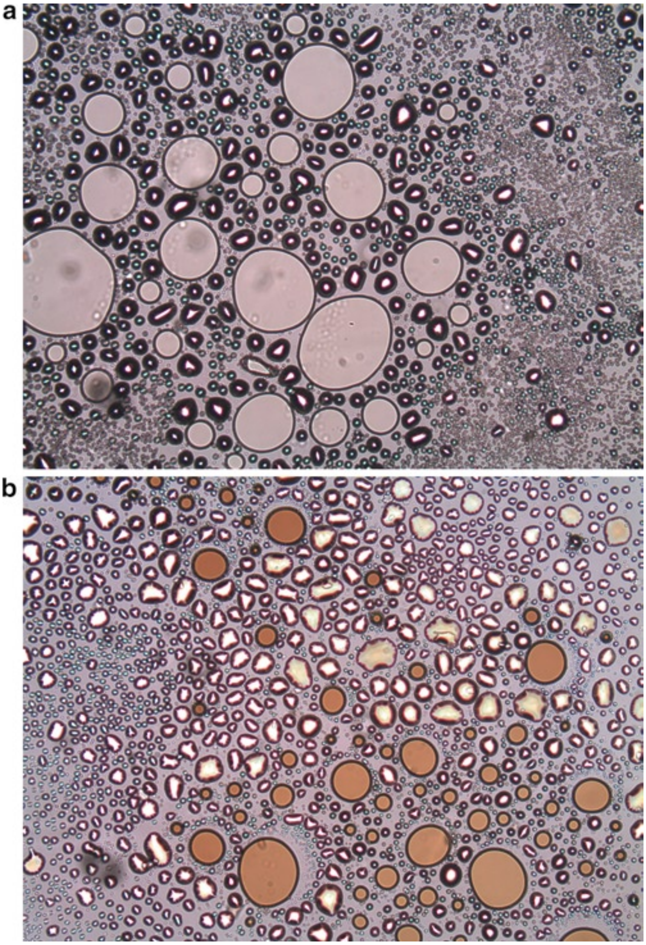

Fig. 3.4 Carbamazepine degradation. Photos (**a**) and (**b**) show the progression of color as the melt degrades. It is best to observe degradation with brightfield illumination and no polars or compensator plates (image size = 1,400 × 1,040 μm)

3.5 Observations

3.5.1 Melting Point

Melting point is the fundamental measurement of thermal microscopy. It is a basic thermodynamic property of the material, can be used for identification, and is always required for regulatory filings. There are, of course, other means of determining melting point such as DSC and the capillary melting point apparatus, but only thermal microscopy gives us a direct view of the particle melting behavior.

The thermal microscope can also be used to determine the equilibrium melting point, which is difficult to determine with other techniques.

There are three important melting temperatures that can be observed in a melting experiment on the thermal microscope: onset melting temperature, final melting temperature, and equilibrium melting temperature. Onset melting point can be defined as the first particle melt, whereas final melt is the last particle melt in the field of view. These melting points are observed as defined. Onset melt can be difficult to detect particularly if there are quite a few particles in the field of view. Onset melt is also not as diagnostic for the material unless it is exceptionally pure. Residual solvent reduces the melting point and, in my experience, may not be homogeneously distributed among individual particles. Final melt is the temperature at which the last solid particle disappears from view. Walter McCrone (1957) recommends using this value for the melting point since it is more reproducible than the onset. I use the final particle melt routinely, even though it has its limitations. If there is a mass of particles on the slide, then the last particle may not be representative of the whole. Final melt is considerably easier to obtain on a routine basis than the equilibrium melt. Certainly, it is a better value to use than the onset melting point. For critical work, make sure to have the appropriate field of view for making the correct observation. In other words, there are plenty of particles in the field of view (~20 or so) but they are not strongly agglomerated. Figure 3.5a–c shows the progression of melt of saccharin (m.p. ~229°C).

The equilibrium melting temperature lies exactly between the melting and recrystallization temperatures and that is how it is measured. Increase the temperature until you just begin to detect melting, and then cool looking for recrystallization. The equilibrium melting temperature lies just between melting and recrystallization. The equilibrium melting point is the true, thermodynamic value. As you might imagine, from the explanation, it is not easy to determine. It requires a deft finger on the heating and cooling buttons and a sharp eye. The recommendation is to actually melt the particle and then cool and note the recrystallization temperature. Then reheat the particle and try to time it so that you cool just as the particle melts. Most pharmaceuticals, though, do not readily recrystallize. Once you melt the particle, the recrystallization may not occur until the preparation reaches a low temperature and may not recrystallize for many hours or days even at ambient conditions. Also, remember that while the melting temperature is a thermodynamic value, recrystallization is not and has a significant kinetic component. For routine work, I favor the final melt and find it suffices for most pharmaceutical applications.

The melting point is significantly affected by the presence of residual solvents. That means that early development compounds, which often have high-residual solvent levels, may give anomalously low melting points. The solvent is likely to be inhomogeneously distributed throughout the sample and so we would not expect to see its loss like a true solvate. Instead, it will probably be liberated at the melt thereby depressing the melting point. So, do not be surprised if the measured melting points of batches made later in the development are higher than those of earlier batches. In fact, it is a good idea to repeat melting experiments with some of the

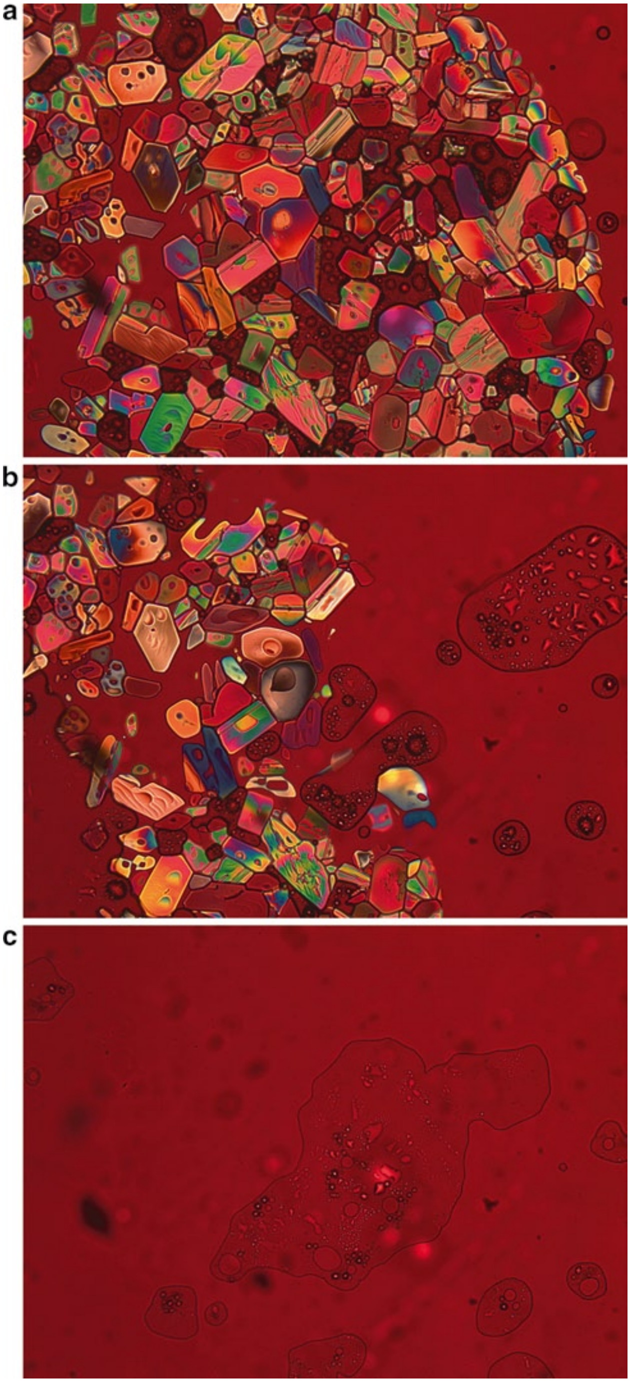

Fig. 3.5 Saccharin melting point. (**a**) 228.5°C, (**b**) 229.1°C, and (**c**) 229.5°C show the melting progression of saccharin. The heating rate for this experiment was 1°C/min (image size = 1,400 × 1,040 μm)

registration batches (those batches used for Phase III stability studies and for key clinical studies) and use those values in registration filings if it is needed.

3.5.2 Solid–Solid Transformations

The study of polymorphism is the main reason that thermal microscopy is well-supported and used in the pharmaceutical industry. By itself, the thermal microscope can be used to thoroughly study polymorphism, but its utility is enhanced when combined with other solid-state analysis instruments such as DSC, TGA, among others. While the melting point is an important component of these studies, it is the ability to directly observe phase transitions that makes it an invaluable aid in polymorphism studies.

One of the most common of these phase transitions is the solid–solid transformation. One polymorph will spontaneously convert to another without going through an intermediate liquid state. On the microscope, this type of transition can be seen as the progression of a crystal front through the particles, or as a change in particle characteristics such as interference color or crystal expansion and movement. These types of transitions can be quite subtle and difficult to detect and generally are best observed with very slow heating rates (1–3°C/min). A progressing crystal front or a change in birefringence is generally diagnostic for a polymorph transition or a desolvation event. Movement and possible expansion of the particles is not diagnostic since these observations can be due to simple thermal expansion effects. Without corroborating data, I would not call particle movement a solid–solid transformation. Figure 3.6a, b shows an example of a solid–solid transformation between carbamazepine Form III and Form I.

3.5.3 Solid–Liquid–Solid Transformations

Another common polymorph transition is the solid–liquid–solid transformation. The particles will melt then recrystallize to another polymorph. Usually, the new crystals will grow either simultaneously or just after the melt is complete. Some compounds, though, will not recrystallize until the temperature is much higher than the melting temperature. That is the reason to conduct a test or two where you use a slow heating rate from the melt to degradation looking for crystallization. This type of event is diagnostic for polymorphism or desolvation. We must be very careful if the melt is nearly simultaneous with degradation. The new polymorph may be a new polymorph or the result of desolvation, but it may also signify the loss of a functional group and subsequent crystallization of a degradant. I have seen this occur with carboxylic acids where CO_2 is liberated and the remaining chemical entity recrystallizes. Degradation is often seen as yellowing or browning of the crystals and/or the melt (see Fig. 3.4). Degradation most often occurs with pharmaceuticals in the temperature range of 250–350°C. Figure 3.7a–d shows the progression of a trehalose dehydrate particle as it melts and recrystallizes at ~100°C.

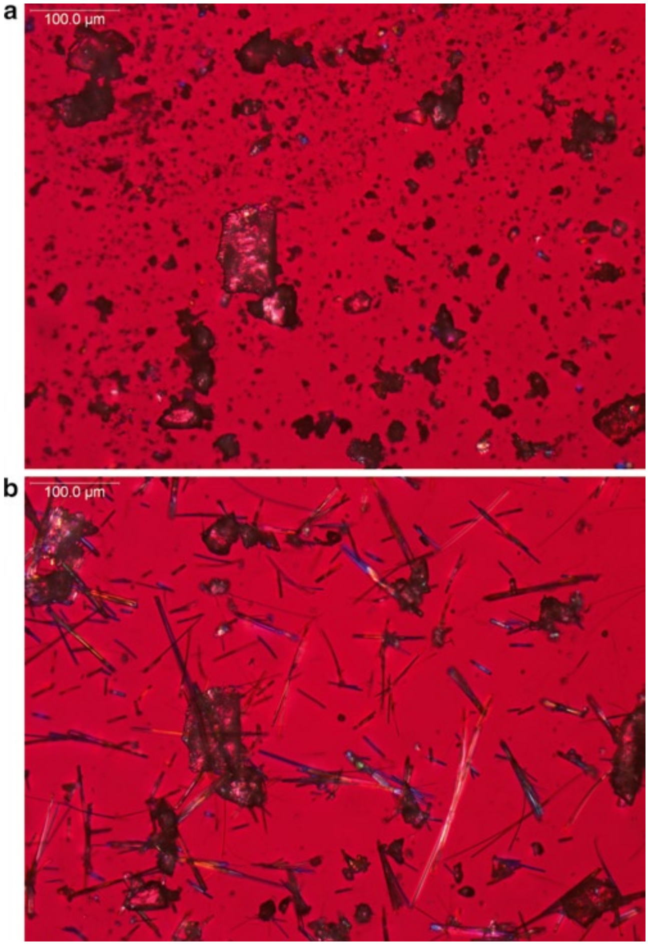

Fig. 3.6 Carbamazepine phase transition. At ~160°C, carbamazepine form III converts to form I in the form of needles growing at the surface of the particles. (**a**) Shows the initial sample of form III, and (**b**) shows the completed conversion at a temperature of ~160°C (image size = 1,400 × 1,040 µm)

At this temperature, the sample loses water and then recrystallizes to the anhydrate. Not all droplets display this behavior, and the nature of the recrystallization appears to be due to particle size (Taylor and York 1998).

3.5.4 *Sublimation*

With sublimation, the crystal passes directly from the solid to the gas phase without first going through the liquid phase. Sublimation is observed on the thermal

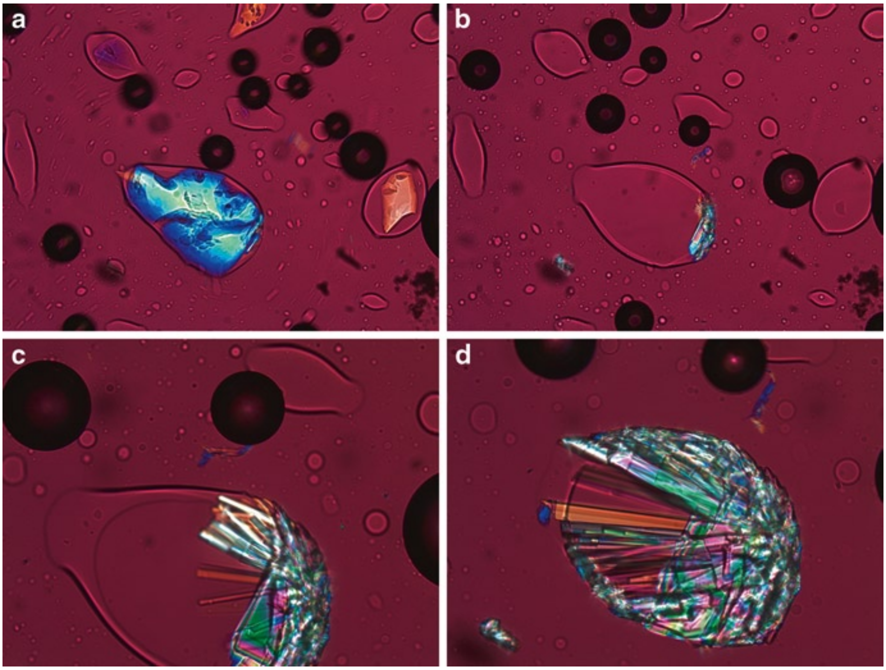

Fig. 3.7 (**a**–**d**) Trehalose dihydrate recrystallization. Trehalose dihydrate loses water at ~100°C, melts and then recrystallizes to the anhydrate. With large particles, the needle-like anhydrate particles will grow directly on the dihydrate particles in a solid–solid transition. With some small particles, the transition goes through the melt (image size = 1,400 × 1,040 μm)

microscope as crystals growing on the bottom side of the upper coverslip. We stand a better chance of observing crystalline sublimation if the upper coverslip is cooled. If we suspect sublimation but do not observe it or if we do have the formation of a liquid sublimate, then we may want to operate the stage without the upper cover while being careful not to contaminate the objective. I have blown air onto the coverglass surface to enhance sublimation or even put some dry ice on the coverslip for the same purpose. Figure 3.3 shows a crystalline sublimate of phenacetin.

Sublimation can be an evidence of polymorphism, but it is also possible for the sublimate to be the same form as that which melted. It is good practice to collect the sublimate and do further tests, (i.e., melting point, micro-IR, and Micro-Raman) to ensure that the sublimate is a different form. Sublimation can also be an excellent way to harvest good crystals for other polymorph studies such as establishing thermodynamic form stability relationships and for optical crystallography. For these purposes, it may be more convenient to use a hot plate, glass vial, and coverslip to make and easily harvest good crystals. Figure 3.8 shows caffeine Form I crystals sublimed by the latter technique.

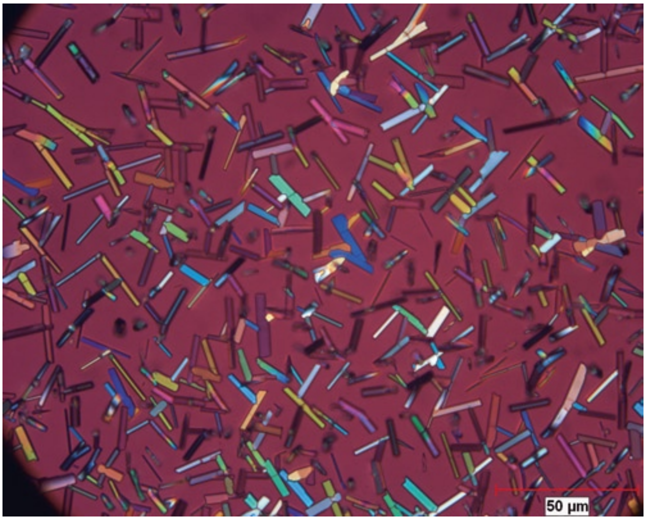

Fig. 3.8 Caffeine form I sublimed using hot plate. A sample of caffeine Form II was placed in crystallization dish with a glass cover and heated at ~150°C. This technique readily forms high-quality form I crystals for other experiments (image size = 700 × 520 μm)

3.5.5 Cooling

It is good practice to observe samples on cooling after a thermal experiment. Mostly, we are looking for recrystallization on cooling, the temperature of recrystallization, and the type of crystals formed. Frankly, it is rarely useful with pharmaceuticals since most drug molecules do not spontaneously recrystallize on cooling. If they do though, Ostwald rule states that the order of recrystallization is through metastable polymorphs. Cooling experiments are occasionally a good way to discover new polymorphs. Figure 3.9 shows the recrystallization of the saccharin sample melted in Fig. 3.5. It is good practice to test these crystals to ensure that they are the original solid-state form.

3.5.6 Dehydration and Desolvation

The evaluation of desolvation (to include dehydration henceforth) is probably the simplest and most common operation in pharmaceutical microscopy. It can be used as a confirmatory test that an endothermic event in the DSC and weight loss in TGA are related to the release of solvent or water from the crystal rather than a polymorphic transition. The experiment is conducted with an excess of crystals in oil and one looks for bubble formation. Bubbles are a sure sign of desolvation. An excess is used since it is possible for the gas to diffuse into the oil without forming bubbles.

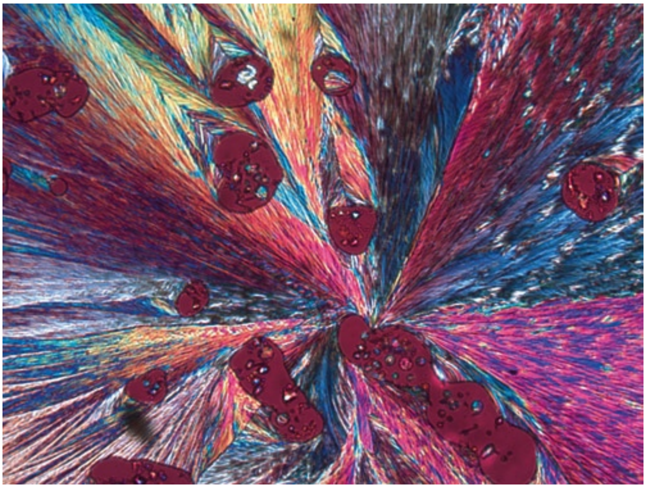

Fig. 3.9 Saccharin recrystallization. This photomicrograph shows how saccharin recrystallizes on cooling. This is the same sample as used for Fig. 3.5 (image size = 1,400 × 1,040 μm)

It is a good idea to use different heating rates with repeat experiments and slower rates are generally better than faster rates. The release of solvent can be, but is not always, accompanied by melting of the crystal. If the crystal melts with desolvation, then we can assume that the solvent is rather tightly bound in the crystal. No change in the crystal on desolvation is an indication, but certainly not absolute proof, that the solvent or water is labile and resides in lattice channels with relatively weak bonding to the molecule. If the crystals melt, one should be on the lookout for recrystallization since that can be indicative of a stable anhydrous or desolvated form. In practice, we need be careful not to confuse air bubbles formed in the process of making the sample with gas bubble generation. Figure 3.7 shows the appearance of the water vapor bubbles as trehalose dehydrates. Some bubbles were present in the initial preparation and these bubbles grew as a result of dehydration. Many new bubbles formed on desolvation. I favor a large sample size when testing for dehydration or desolvation.

3.6 Fusion Techniques and Other Applications

Many of the early developments in thermomicroscopy were directed toward chemical identification, estimation of purity, and determination of physico-chemical constants, along with the study of polymorphism and solvation. In the main, the hot stage is used for the last two subjects in pharmaceutical microscopy, but there are some interesting applications in the other areas. In particular, you can use fusion methods for the discovery of polymorphs and the observations mentioned previously can be used in the study of eutectics, solid solutions, and chiral compounds

(Teetsov and McCrone, 1965). The texts by McCrone (1957) and Kuhnert-Brandstatter (1971) present reasonably thorough descriptions of these techniques.

In brief, Koflers and Kuhnert-Brandstatter developed techniques for determining the identity of an unknown organic or pharmaceutical compound using the unknown's eutectic formation with certain compounds as well as the refractive index of the melt. This scheme has been replaced by others for identity but the fundamentals can still be used for polymorphism studies and for the study of chiral compounds. The microscopical technique can use the contact method as established by McCrone (1957) or one can use the observation of early eutectic melt as established by Kofler and Kofler (1954) and Kuhnert-Brandstatter (1971). In the fusion technique, one melts some of the higher melting compound under one side of the coverslip, allows it to recrystallize, then melts the other compound on the other side of the coverslip. The two compounds will meet and recrystallize near the middle and form a eutectic zone (see Fig. 3.10). The melting point of this eutectic can be diagnostic for that set of compounds. In the Koflers technique, the reference material and sample are mixed dry and then heated. The onset of melt is indicative of the eutectic and can be used for identification. If one uses two reference materials, we end up with three melting points (pure sample and two eutectics), a refractive index estimation of the melt (see Kuhnert-Brandstatter 1971 for details), and the thermal observations for the identification. We can use acetylsalicylic acid as an example. The eutectic with acetanilide melts at 81°C and the eutectic with phenacetin melts at 96°C. Acetylsalicylic acid melts at approximately 134°C and so we have three values for identification. If we observe lower melts or a higher initial melt, these observations may indicate that the solid-state form of the sample is not as expected and may be a new form.

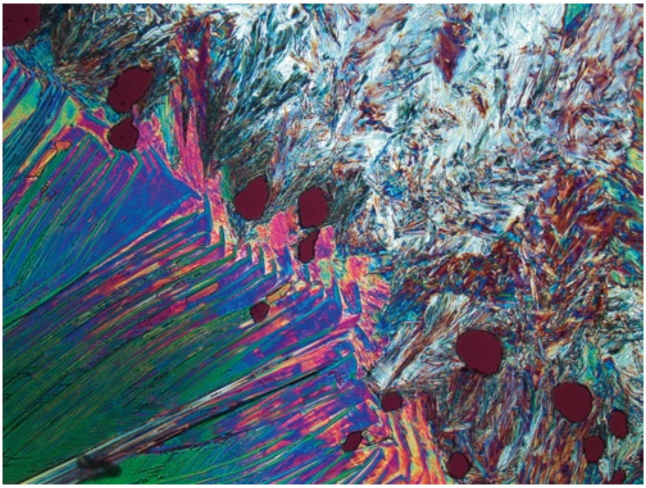

Fig. 3.10 Fusion of acetylsalicylic acid with phenacetin. Acetylsalicylic acid is on the right and phenacetin is on the left. The eutectic forms in the middle mixing zone (image size = 1,400 × 1,040 μm)

The experiment can also help us discover new polymorphs and, frankly, the experiments would be worthwhile for nothing more than their aesthetic value since incredibly beautiful images often result. New polymorphs can be discovered if more than one eutectic is observed. This scheme is not presently used often, but one worker in the industry swore by its effectiveness.

Clearly, the technique mentioned above is quite useful if you need to study a eutectic system and can be used with chiral compounds since enantiomers can form eutectics. One application of eutectic mixtures is the estimation of enantiotropic polymorph transition temperature (Teetsov and McCrone, 1965).

3.7 Summary

Hot-stage microscopy is a well-established technique in pharmaceutical development. Although at first glance, it may seem complicated and with experiments that are somewhat difficult to conduct, the technique is actually reasonably easy to learn with a moderate amount of practice. Modern hot-stage instrumentation is quite versatile and can make these measurements much easier to conduct and document than in the past. The ability to make movies from digital images means that one can readily display what one sees in the microscope. The results of thermomicroscopy can be used by themselves to understand thermal behavior and polymorphism, but are useful when combined with results from other techniques. The hot-stage microscope is particularly useful in interpreting and understanding results of techniques such as DSC.

3.8 Exercises

These exercises use some of the chemicals listed in Table 3.1 along with carbamazepine, caffeine, and acetylsalicylic acid.

A. Sample Preparation and Experimental Setup

A1. Sample Preparation

Practice preparing and manipulating small coverslips and small amounts of sample. Use caffeine as a test material. Place a small amount of caffeine on one coverslip, place another coverslip on top and then rotate each coverslip against the other. Place this preparation onto a glass microscope slide and examine under the microscope. Note that there are quite a few crystals adhering to the upper coverslip. Set this preparation aside and practice preparing a caffeine sample in high-viscosity oil.

Place a small amount of the caffeine onto one coverslip; add a small drop of oil and mix. Place a coverslip onto this preparation and again examine directly with the microscope. The oil preparation should be a small drop in the middle of the coverslip. If the drop extends across the coverslip, you probably have too much material. Set this preparation aside for later testing as well.

A2. Preparing the Hot Stage for Use

Prepare your hot stage for use according to the manufacturer's instructions. Place a dummy preparation onto the hot stage and heat to a high temperature, above 300°C if your stage allows. It is good practice to heat the stage initially to drive off any residual moisture that may be in the internal parts of the stage. This operation does not need to be repeated frequently, but you should do so if the stage has not been used for a week or two. Spend some time learning the operation of the stage controller and any associated software for collecting images or for controlling the stage. The controls and software often have a great deal of flexibility but may not be intuitive to use. Spending some time learning the instrument will pay dividends later.

B. Calibration and Performance Checks

Prepare specimens of acetanilide (114.5°C), benzanilid (163°C), and saccharin (228°C). These melting points of these chemicals span the melting temperature range for pharmaceutical compounds. Set a high heating rate (i.e., 20°C/min) from ambient to 5°C below the expected melting point and then a low rate (3–5°C/min) from 5°C below the melting point to 5°C above it for benzanilid. Test the sample and note the temperature of final melt. I typically allow a ±1.5°C melting point range for a passing performance check. If the value lies outside of this range, calibrate the system. Now test acetanilide and saccharin to ensure proper calibration. Be sure to note how each material recrystallizes as the specimens cool.

Practice with the other standards as well. I cycle through my set of standards as I run tests. At the beginning of the day (when I plan thermal tests), I test the next highest melting point standard compared with the day before. Over a period of 9 days, I will have tested all of the standards. I find these tests good practice and a good test of the instrument performance.

C. Effects of Heating Rate

Prepare a few specimens of phenacetin to study the effects of heating rate. Test first as described above in Exercise B with a 3–5°C heating rate through 134°C.

Test again but set the heating rate to 1°C/min from 110°C. Note the presence of the sublimate and how difficult it is to judge the melting point. Test again but with a 20°C/min heating rate through 134°C. Note that although there is less sublimate occluding the view, the observed melting point is much harder to accurately determine. Do similar tests with other standards.

D. Phase Transformations

In this exercise, test the caffeine and carbamazepine specimens prepared in Exercise A. Use the test strategy outlined in Sect. 3.4. Note all transformations and how dry samples differ from those in oil. Be sure to observe the samples on cooling. Also, test trehalose dihydrate with large and small particles both dry and in oil. Note how some melted drops recrystallize whereas others do not. Also, note that with the larger particles in a dry preparation, the anhydrate grows on the surface of the particles.

E. Fusion

Practice making pure fusion preparations of acetylsalicylic acid and saccharin. Note how each recrystallizes. Melt and recrystallize these same specimens a second time and not the similarities and differences to the first recrystallization.

Practice making mixed fusion preparations of acetylsalicylic acid with acetanilide and with phenacetin. Note the location of the eutectic zone. Conduct a melting experiment with each of these preparations and determine the melting temperatures of the eutectics. Now prepare specimens of acetylsalicylic acid with acetanilide by placing a small amount of each compound in a mortar and pestle and lightly grinding them together. Place a small amount of this mixture on a coverslip and conduct a melting test looking for evidence of the first melt. Do the same experiment acetylsalicylic acid and phenacetin. Again note the temperature of the first melt. The observed eutectic melting temperatures should be approximately 81 and 96°C, respectively.

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Chapter 4
Scanning Electron Microscopy and Energy-Dispersive X-Ray Spectrometry

4.1 Introduction

The scanning electron microscope (SEM) is used extensively in pharmaceutical development for both drug substance (DS) and drug product (DP). The vast bulk of the work is examination of size and shape of the individual particles making up the drug substance powder. Some of this work is quantitative in the sense that the size and shape of the particles are measured using image analysis (see Chaps. 7 and 9), but most of the work is more qualitative in nature. It is somewhat amazing that the SEM produces images that are readily interpreted by our visual system when you consider we are looking at the interaction of electrons with matter and that, in fact, there is no true optical system to the SEM. Even without numbers, one can get a sense of how the DS powder will behave based just on the appearance of the particles. If well-crystallized with sharp crystal edges, we may suppose the material will flow well. If it is highly agglomerated, with what appear to be particles fused together, we might suppose the powder will not flow as well. Even if those initial suppositions are somewhat naïve and general, just having an image of the particles can help workers understand the system they are dealing with. By the way, it is a rare technical presentation on drug substance that does not include at least one SEM image.

Energy-dispersive X-ray spectrometry (EDS) is not as extensively used in pharmaceutical development as is the SEM, but EDS is a valuable tool for elemental analysis. EDS is mostly used either for contaminant investigations or for tablet mapping. Active pharmaceutical ingredients are mostly composed of C, H, O, and N. Although C, O, and N can be detected by EDS, the instrument is not very sensitive to these elements. Drug substance can also contain Br, Cl, S, P along with a few other elements either in the main chemical structure or as salts. Drug product excipients frequently contain Ca, P, Ti, Mg, Si along with a few other elements. Low atomic number elements have a lower X-ray yield compared with high atomic number elements. The advent of silicon drift diode (SDD) detectors for EDS has improved the X-ray count rates and has made chemical imaging of tablet cross-sections practical. This application is likely to take on more prominence in the years to come since there is an interest in understanding the distribution of elements (and compounds) in tablets in order to improve product consistency and even dissolution properties.

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In this chapter, I discuss some of the theory and practice of SEM and EDS. As with optical microscopy, I am not going to delve deeply into the theory since there are quite a number of excellent texts on that subject (see Goldstein et al. 2003 and Reimer 1998). I present those theoretical ideas that I think are critical to a good understanding of the SEM and EDS for basic pharmaceutical applications. The interaction of an electron beam with materials is at the core of SEM/EDS and is complicated and interesting to say the least. I personally find the subject fascinating and recommend it to you both for profit and for intellectual satisfaction. This is an active area of research and so you can learn much from books, from technical meetings, and from schools. In this chapter, my intention is to introduce the subject in a manner sufficient to aid you in doing the majority of pharmaceutical tasks. You will find more detailed information of the applications of SEM/EDS in the section of this book on applications including Chaps. 8–10.

4.2 Scanning Electron Microscopy

4.2.1 Basic Operation of SEM

In an optical microscope, we image features either by passing light through them in transmission mode or by reflecting light from the surface in reflection mode. The SEM is a reflection instrument but with some significant differences from a light microscope. Electrons interact with the specimen with a variety of different mechanisms producing a complex set of signals. These signals include backscattered electrons, secondary electrons, Auger electrons, characteristic X-rays, Bremstrhalung X-rays, cathodoluminescince, and that is not all. There is a detector for every one of these signals and specific applications for which that detector and signal is ideally suited. If you add gas to the chamber, you can add a couple of other signals to the mix. In my opinion, it is the fecundity of signals and the cleverness of scientists and engineers exploiting those signals that has led to the popularity of the instrument.

The SEM forms an image by rastering an electron beam across the surface of the specimen and coupling that e-beam raster with the raster of a cathode ray tube, LCD, or plasma screen. Figure 4.1 shows a schematic drawing of the major elements of the image forming elements of the SEM. The signal from the detector is then used to adjust the intensity of the corresponding pixel of the screen. Naturally more signal leads to more intensity on the screen and the image is built up bit-by-bit in a back and forth, raster pattern. That is it in a nutshell.

In many respects, it is rather strange that an SEM image is as easily interpretable as it is when we consider the mechanism of image formation. In the SEM, a reasonably well-focused electron beam is produced and directed down the column to the specimen. In order to manipulate the electron beam and minimize interactions, the column must be at high vacuum. The beam is finely focused by an electromagnetic or electrostatic lens and a series of apertures. This beam is rastered across the surface

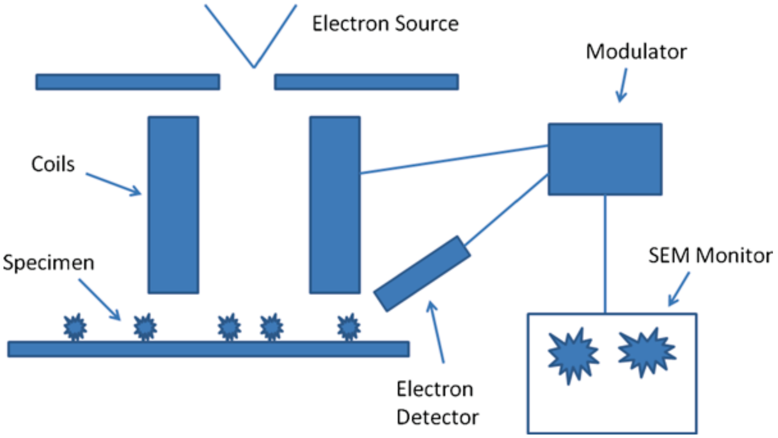

Fig. 4.1 Schematic drawing of SEM. An SEM image is formed by coupling the raster of the electron beam of the instrument with the raster of a cathode ray tube or a liquid crystal display. As the raster area of the sample is reduced, the specimen appears magnified since the raster size of the monitor is fixed

of the specimen using deflection scanning coils. The signal from specimen is collected and used to moderate the intensity of an accompanying monitor. The beam deflection coils are connected to and synchronized with the output monitor. It is all rather amazing that you end up with a photo of something recognizable such as the caffeine hydrate crystal shown in Fig. 4.2.

That explains the image but how does it magnify? It is rather simple and ingenious. One keeps the raster size of the monitor constant but reduces the raster size of the electron beam on the specimen. The smaller the size of the specimen raster region, the greater the magnification (M) of the image on the monitor. Assuming a square raster size, then

$$\mathrm{M} = \frac{\text{Width of monitor area}}{\text{Width of scanned area}}. \tag{4.1}$$

So, for instance, if we have a 10 cm × 10 cm image area on the monitor and our specimen image width is 10 cm then our magnification is 1. On the other hand, if the specimen area is 100 μm × 100 μm then the magnification is as follows:

$$\mathrm{M} = 10\,\mathrm{cm} / 0.01\,\mathrm{cm} = 10^3 = 1{,}000\times. \tag{4.2}$$

Similarly, if the specimen raster area is 10 μm × 10 μm then the magnification is 10,000× and so on. It is useful to keep these relationships in mind when using the SEM. For one thing, it helps understand why there is less damage to the specimen from the beam at low magnification compared with high magnification. If we keep all other SEM operating conditions the same, we are injecting the same current into a much smaller area as we increase magnification. Too much current in a beam-sensitive material can have deleterious effects – to say the least.

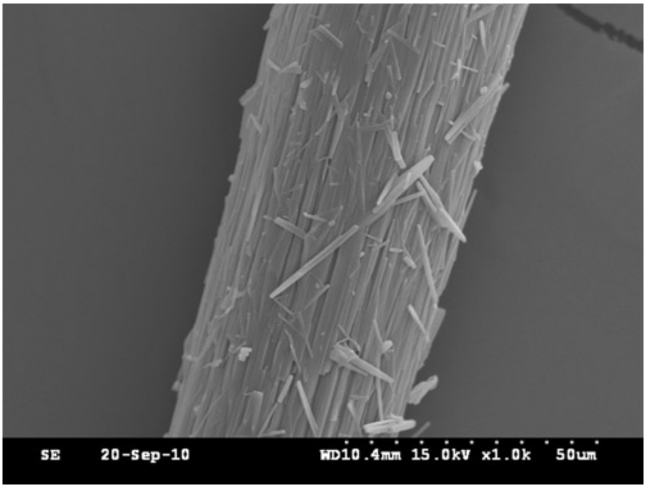

Fig. 4.2 Caffeine hydrate crystal. This photomicrograph illustrates the easy interpretation of SEM images. We see a near three-dimensional appearance of a bundle of crystals with other crystals lying on the surface. The appearance of the image seems to accord well with our sense of sight in the natural world. (Image size = 122 × 99 μm)

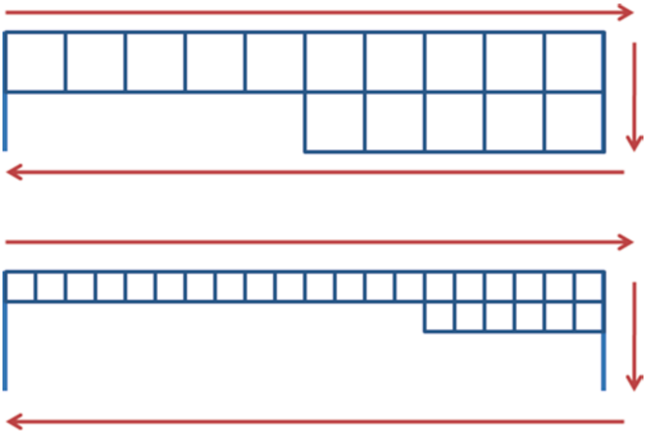

Fig. 4.3 Illustration of SEM raster pattern. This cartoon illustrates the nature of the raster pattern with a SEM. The upper raster contains 10 squares. If we suppose that the raster distance is 1 mm, then each square is 100 × 100 μm^2. In the lower raster, there are 20 squares and let us keep the raster distance the same. Now each square is 50 × 50 μm^2. An image using the lower raster has higher resolution compared with the upper one

Another important consideration in SEM imaging (actually all imaging) is the pixel size or picture resolution. The pixel size is controlled by the number of points sampled along the raster line in the SEM (see Fig. 4.3). The more raster points along the line, the higher the image resolution. Pixel size can be determined by length of raster line divided by number of points sampled along this line. In general, one has some control over the pixel size by specifying the image size (i.e., 640 × 480 pixels). It is important to consider this value. Ideally, one wants the smallest pixel size possible since this controls resolution. But we have a physical limit based on the interaction size and volume of the beam with the specimen. Little is gained by reducing the pixel size below the interaction area of the electron beam since at each raster point we will get signal contribution from adjacent areas. As noted below, interaction volume and the area from which signals are emitted depends on signal (SE, BSE, X-ray), instrumental variables (beam size, accelerating voltage, etc.), and sample type (average atomic number). In pharmaceutical development, we are mostly working with organics with relatively low average atomic number so the interaction volume is mostly dependent on signal and instrument variables.

As a practical matter, the use of digital electronics has complicated our view of magnification and pixel size in the SEM. The image on the monitor is variable as is the digital image. With varying resolutions selectable on the SEM, it can be quite confusing. For example, we may have a large liquid crystal display (LCD) SEM monitor and the SEM image is formed with ¼ the total area of the monitor allowing room on the monitor for other controls. Now if we zoom the monitor image to the full screen, we have changed the apparent magnification to our eyes but have not changed the original raster size. In other words, the resolution has not changed. In a sense, zooming the monitor image is similar to empty magnification in an optical microscope produced by using high magnification eyepieces. For this reason, we need to be careful in how we report magnification. It is becoming common practice to state the image size (i.e., 200 μm × 200 μm) of the photomicrograph and image rather than the magnification for publication.

4.2.2 Electron Beam–Specimen Interactions

It is necessary to understand the basics of electron–specimen interactions in order to intelligently use all of the features and imaging mechanisms of a SEM. I say the basics, because this subject quickly moves from a simplistic view of the atom to quantum physics. For most applications of the SEM in pharmaceutical development the basic view suffices and it is not necessary to delve deeply into the mysteries of quanta. On the other hand, it is a fascinating subject and if you have the interest, I recommend Reimer (1998). In this chapter, we are taking a cursory view with an eye toward practical applications.

The SEM electron beam is characterized by three parameters that are under the control of the operator: the beam diameter, the beam current, and the convergence angle of the beam. For high resolution, we want a small electron beam diameter with high current and low convergence angle – all of which is under the control of the operator. Once the beam hits the specimen, however, the operator has little control over the path of the electron beam and subsequent signal. So, the ultimate resolution of the SEM depends as much on electron–specimen interactions as on control of the instrument parameters. Hence, the need to be aware of the basics of how an electron beam interacts with a specimen.

Physicists have two categories for electron–specimen interactions: elastic and inelastic collisions. Elastic collisions are defined as those interactions in which the electron changes trajectory (direction) but loses no energy in the process. Inelastic collisions are defined as those with little to no electron trajectory change but the electron loses energy by transference to the specimen. Elastic collisions in the SEM are Coulombic in nature and can be thought of as the interaction of the negative charge of the electron with the positive charge of the nucleus (see Fig. 4.4). The beam electron can change direction through any angle from 0 to 180°, although most interactions result in a small angle change of 2–5°. This small angle change is particularly so with organic compounds made up of light elements. In some cases, though, the electron can be reflected back toward the pole piece of the SEM at an angle near 180°. These reflected electrons are referred to as backscattered electrons and are the source of the so-named signal. The probability of backscattering is greater with higher atomic number elements and with lower beam energy. We will discuss backscatter imaging in more detail in a later section.

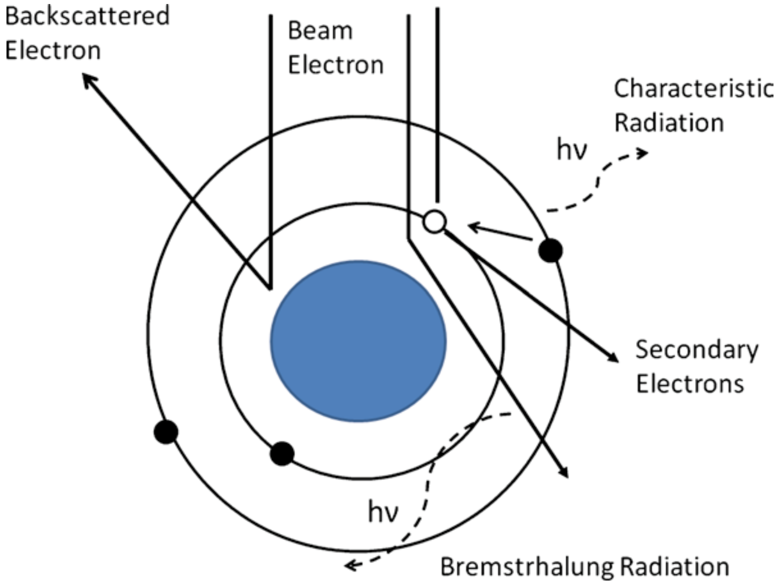

Fig. 4.4 Elastic and inelastic interactions of beam electrons with an atom. On the *left*, elastic interactions are illustrated resulting production of a backscattered electron. The beam electron is repelled by the positive charge of the nucleus with change in direction and but little to no change in energy. On the *right*, inelastic interactions are illustrated resulting in the production of a secondary electron (among other effects). An inner shell electron is expelled from the atom which is the primary mechanism for secondary electron production resulting in the high resolution signal for imaging. The excited atom relaxes by filling the inner shell with an electron from an outer shell. This process results in the production of characteristic X-rays. Bremstrahlung or braking radiation results from the slowing of a beam electron as it interacts with the nucleus of the atom

Inelastic scattering can be divided into the following broad categories:

Phonons – lattice oscillations (like ringing a bell).
Plasmons – excitation of a free-electron "gas" which is mainly observed in metals.
Secondary electron emission – a valence band electron is "knocked" out of the atom and moves through the sample, some of these escape the surface, secondary electrons are defined as having less than 50 eV energy.
Continuum (Bremstrhalung) X-rays – the braking radiation resulting from the deceleration of a beam electron. The electron can lose some small portion of its energy as an X-ray or all of its energy at once. Continuum X-rays therefore vary from 0 up to the corresponding accelerating voltage.
Inner shell ionization – inner shell electrons can be knocked out of the atom, leaving the atom in an excited state. The atom can relax by an outer shell electron moving to the inner shell, thereby releasing energy as an X-ray or by the emission of an Auger electron. See Fig. 4.4 for an illustration of the last three categories.

There are other processes at work as well including cathodoluminescence, fluorescence, and diffraction. I will not be discussing these latter subjects in much detail since they are not used often in pharmaceutical development. I will leave an in-depth discussion of X-ray generation to the section on energy-dispersive X-ray spectrometry.

It is useful to consider some of the factors affecting the backscatter and secondary electron emission since these are the fundamental imaging mechanisms in the SEM.

4.2.3 Backscatter Electron Emission and Detection

The backscatter electron yield increases with increasing atomic number and so these electrons can be used to produce images based on atomic number differences (see Fig. 4.5). Backscatter electrons are less sensitive to topology than are secondary electrons and so can be used with samples that are not flat where we want to highlight atomic number differences. Backscatter detection is also popular in low-vacuum SEMs that cannot use the patented detector technology of FEI (originally Electroscan). I have used BSE with samples that charge even with metal coating and with low vacuum (some aggregates are composed of plates with air gaps between – the classic definition of a capacitor). BSE is particularly useful when doing X-ray mapping or contaminant identification since metals and other high atomic number elements are easily detected.

Backscattered electrons can be likened to shooting a cannon ball at a piece of tissue paper and having it bounce back at you. Ernest Rutherford described its discovery as the single most incredible scientific observation of his life. It is, however, easy to imagine that the probability of backscattering is directly related to the mass of the atom being probed; denser atoms backscatter more electrons than less dense atoms. In other words, more beam electrons should be scattered backwards from gold than from carbon. The ratio of backscattered electrons to input

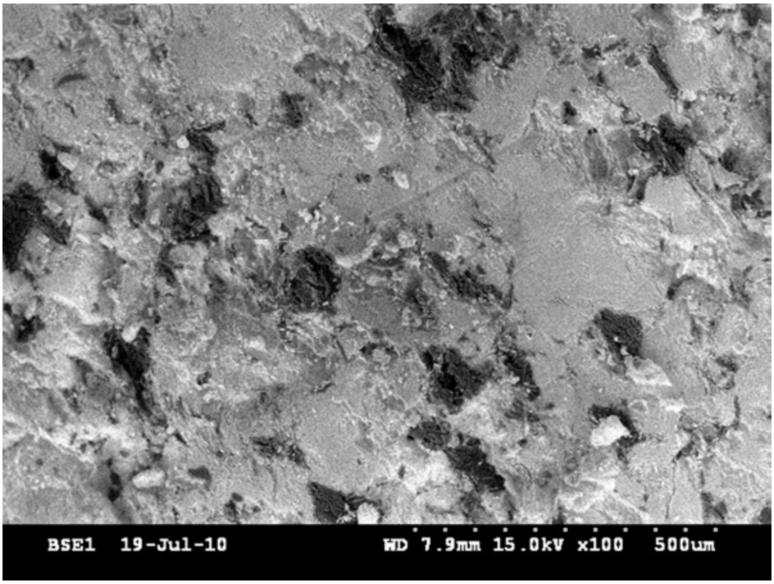

Fig. 4.5 BSE image of generic naproxen sodium tablet. The bright areas of the cross-section of the tablet are the drug substance, whereas the dark areas are cellulose used as a bulking agent. Naproxen sodium is bright due to the heavier Na atom in the DS, whereas the cellulose is composed of lighter elements. (Image size = 1,217 × 987 μm)

electrons is termed the backscattered electron yield and is commonly designated with the Greek letter η. The BSE yield does depend upon atomic number but the dependence is not completely linear (see Fig. 4.6). The relationship is close to linear for the light elements commonly found in pharmaceuticals.

The BSE yield of a molecule or complex compound is the weighted sum of each component's yield as in the following equation:

$$\eta = \sum\nolimits_{\mathrm{i}} C_{\mathrm{i}} \eta_{\mathrm{i}} \tag{4.3}$$

Where C is the weight percent of the element i. So BSE contrast will vary with the mean atomic number of each compound and higher atomic number compounds will, in general, be brighter than lower atomic number compounds – with some exceptions that mostly involve metals. For the light elements that make up pharmaceutical products, the relationship of brightness to higher atomic number holds. The backscatter yield is not particularly sensitive to beam energy in the 5–20 kV range, but the yield is highly sensitive to specimen tilt. When using the BSE detector it is best not to tilt the specimen.

The spatial resolution of the backscatter signal is not particularly high since backscattered electrons can be emitted some distance from the location that the beam electrons strike the specimen. When working in low vacuum this effect is exacerbated due to the spread of beam electrons by the chamber gas.

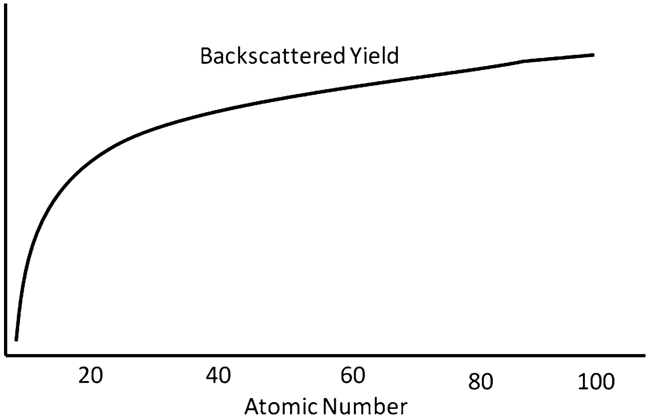

Fig. 4.6 Backscattered yield vs. atomic number. The backscattered electron yield directly depends upon atomic number but the relationship is not completely linear. For light elements, however, the yield is nearly linear and so we can assume that regions of the sample with lighter contrast have higher atomic number than regions with lower atomic number

Consequently, the BSE signal is not appropriate for high resolution work and the practical magnification limit is ~10,000× depending on the sample, the microscope and the detector. For most cases, this is not a significant limitation for pharmaceutical microscopy since our features of interest can be resolved at much lower magnifications.

The depth at which backscatter electrons are generated depends, of course, on the beam energy and the atomic number of the elements in the specimen. The generation depth can be on the order of more than 1 μm for light element materials as are in pharmaceuticals. Figure 4.7 shows two Monte Carlo simulations of the penetration depth of the electrons and the backscattered electrons expected with carbon and lead. Note the higher number of backscattered electrons with lead and the shallower depth of penetration compared with carbon. It is important to keep in mind that electrons, particularly backscattered electrons, can be emitted some distance from the location of the electron beam.

There are two general classes of backscattered electron detectors: light pipe and solid state. In the light pipe, or Robinson™ detector, a clear plastic light pipe is placed at the pole piece just where the electron beam exits the column. The plastic is coated with a scintillator material that is sensitive to electrons. The scintillator produces a flash of light when struck by an electron and this flash is detected by a photomultiplier just outside the sample chamber of the instrument. The charge from the photomultiplier is used to produce the SEM image on the monitor. A solid-state detector is placed in the same location as the Robinson™ detector, but instead of a light pipe, a silicon chip is used to detect electrons. The charge produced in the chip is used to form the SEM image on the monitor. Robinson™ detectors are often used with low-vacuum instruments since they operate in higher pressures whereas the solid-state detectors generally require a high vacuum for operation. The Robinson™

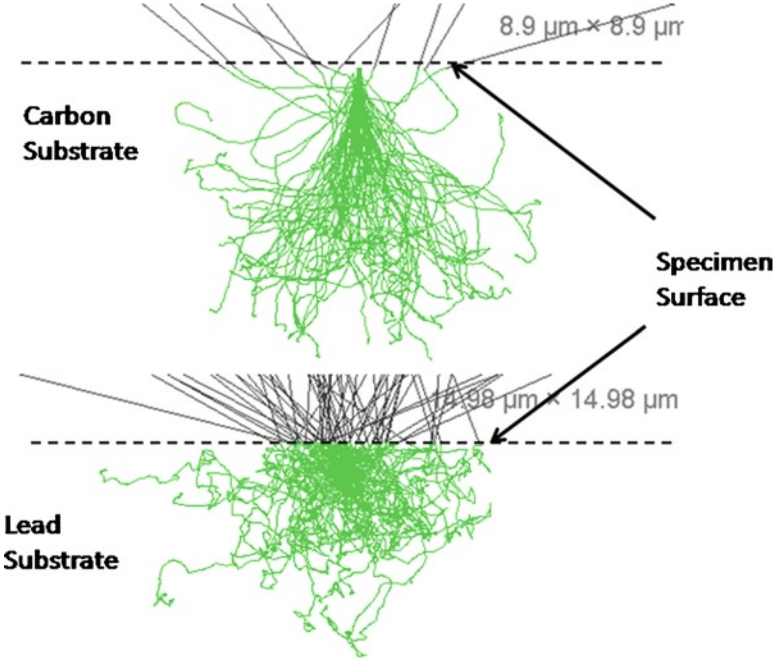

Fig. 4.7 Monte Carlo simulation of electron penetration depth. The upper Monte Carlo simulation shows the expected penetration depth and backscattered electron signal from carbon and the bottom is of lead. Note the difference in penetration depth between the two materials and the differences in backscattered signal. Carbon has fewer backscattered electrons and they are generated at a shallower depth than lead with more total backscattered electrons from deeper in the specimen

detectors are quite robust and work well but do not have the sensitivity of the solid-state detector. In my opinion, the solid-state detectors often produce a better image than the Robinson™ and are capable of higher resolution, although I have to admit I have not done a thorough comparison of the two types of detectors.

We will consider how BSE can best be used for pharmaceutical applications in a later section of this chapter.

4.2.4 Secondary Electron Emission and Detection

Secondary electrons originate from the interaction of the beam electrons with the valence shell electrons in the outer shell of the specimen atoms. By definition and design, secondary electrons have an energy value of less than 50 eV, although most secondary electrons (~90%) have an energy value of less than 10 eV. There are a number of factors that affect secondary electron yield (commonly designated as, δ) but it is somewhat difficult to formulate broad general rules that apply in every case.

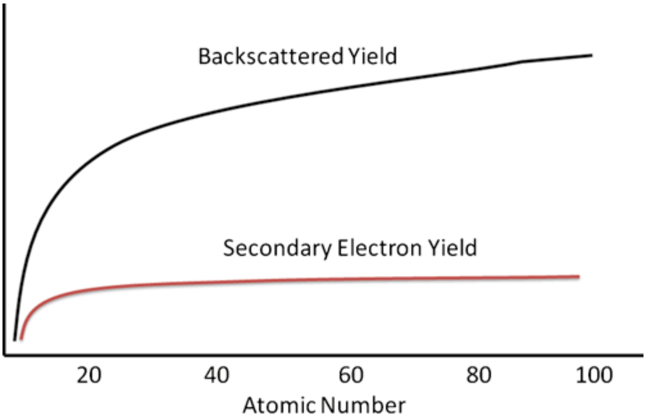

Fig. 4.8 Graph of secondary electron yield vs. atomic number. The *upper curve* is the backscatter yield from Fig. 4.6. The *lower curve* shows the relationship of secondary electron yield vs. atomic number. The secondary electron yield does not strongly depend upon atomic number although some of the lightest elements have low yield compared with heavier elements

The secondary electron yield is not particularly sensitive to specimen composition and atomic number, although there is some dependence of yield on atomic number. For instance, carbon has an anomalously low yield compared with other elements of similar atomic number (see Fig. 4.8 and Goldstein et al. 2003). This slight dependence is of little consequence for routine imaging of pharmaceutical products.

Of more consequence to pharmaceutics is the fact that the secondary electron yield increases as beam energy decreases. This is due to the increase in the probability of a beam electron interacting with a surface specimen electron at lower accelerating voltage. The greater this probability, the more secondary electrons that are both generated and emitted from the specimen surface. Increasing the secondary yield with lower accelerating voltage means that one can obtain better resolution surface images at lower voltages. Figure 4.9a, b shows the same carbamazepine specimen at 15 and 5 kV, notice the difference in surface detail between the two photomicrographs. This relationship of increasing secondary electron yield with decreasing beam voltage has lead to an interesting approach to imaging charging specimens without the need for metal coating. If we think of it, we are depositing electrons into the specimen and in turn emitting electrons. There is what is referred to as the crossover point (actually two) where the input electrons equal the emitted. At this crossover point, even materials with strong electrical resistance, such as most organic compounds, will not charge since there is an electrical charge balance. For carbon-based systems, this crossover point occurs at about 2 kV. Imaging at this accelerating voltage can be a useful strategy for some beam-sensitive samples. (See Goldstein et al. 2003, p. 90, for more information on this subject.)

Secondary electrons are generated all along the path of the input beam electron within the specimen, but those that are emitted originate in the upper 10–50 nm of the specimen surface. This fact means that the secondary electron signal should be

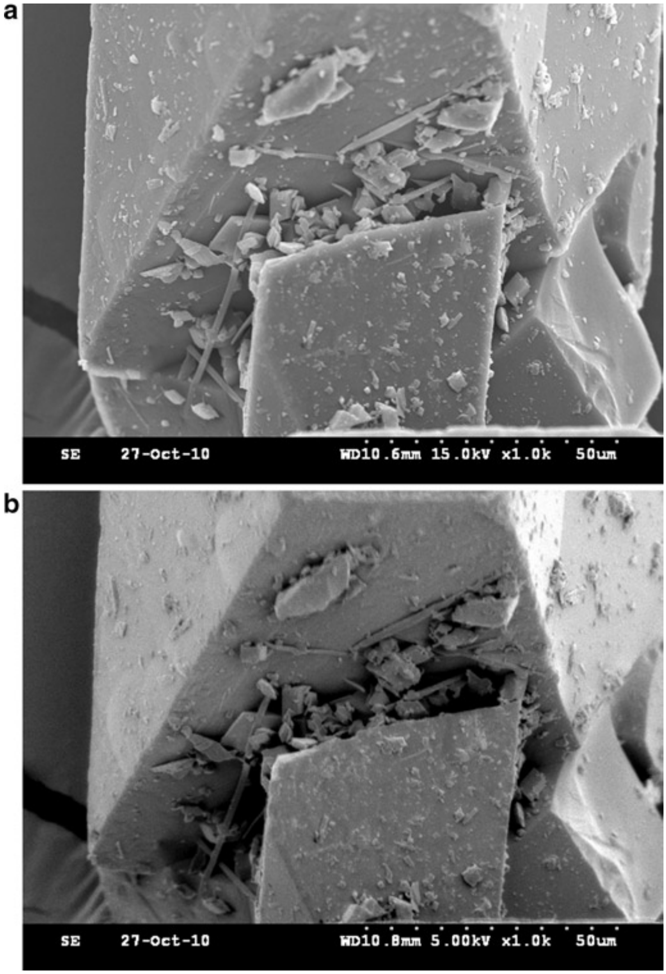

Fig. 4.9 Carbamazepine (**a**) 15 kV accelerating voltage (**b**) 5 kV accelerating voltage. These two photomicrographs illustrate the differences observed with different accelerating voltages. A lower voltage results in an image that provides more surface detail than does a higher voltage image. (Image size = 122 × 99 μm)

a very sensitive probe of the specimen surface and should produce high resolution images rather easily. While that statement is true, there are complications related to the generation of secondary electrons that we need to take into consideration when imaging. Most secondary electron detectors indiscriminately collect low-energy electrons and there are a number of different sources of secondary electrons in the SEM. For example, the backscattered electrons produce secondary electrons as they are emitted from the specimen surface. Secondary electrons from the primary electron beam are generally designated as $\mathrm{SE_{I}}$, whereas those from backscattered electrons are designated $\mathrm{SE_{II}}$. There are also $\mathrm{SE_{III}}$ and $\mathrm{SE_{IV}}$ related to electrons produced by the pole piece and other parts in the specimen chamber, but I am not going to discuss those in detail. The SE_{II} signal can be produced some distance from the location of the input beam since backscatter electrons can be emitted some distance from the probe electrons. These SE_{II} electrons then degrade resolution. Since the backscatter yield increases with atomic number, the contribution of SE_{II} electrons to the total secondary electron signal increases with increasing atomic number. Some have estimated that the SE_{II} electrons can represent as much as 20% of the total SE signal for light elements and can be the dominant signal for heavy elements (see Goldstein et al. 2003). Since in pharmaceuticals we generally deal with low Z materials at low resolution, the SE_{II} effect is not particularly significant. On the other hand, if you require maximum resolution for imaging the surface of wet-bead milled materials or inhaled products, you should spend some time studying the effects of the various SE signals and how to minimize the effects of such on your image.

The most common secondary electron detector is the Everhart–Thornley (E–T) secondary electron detector although there are quite a few unique detectors available to us. The last decade (2000–2010) has seen a revolution in SEM design occasioned mostly by the needs of the semiconductor industry. We, in other industries, are also the beneficiaries of this revolution. Still, the E–T secondary detector is probably the most common on routine SEMs and is the most commonly used for high-vacuum imaging of pharmaceutical products.

The E–T detector is similar to the Robinson detector in the sense that the electron is detected using a scintillator on a light pipe and the resulting flash of light is detected by a photomultiplier and used to form the image (Fig. 4.10). The differences are location of the detector, shape of the detector, and the presence of charged grids on the detector opening that attract low-energy electrons and repel high-energy electrons. Note that due to the charge on the detector cage, the E–T detector will draw electrons from any location on the specimen surface. In other words, the

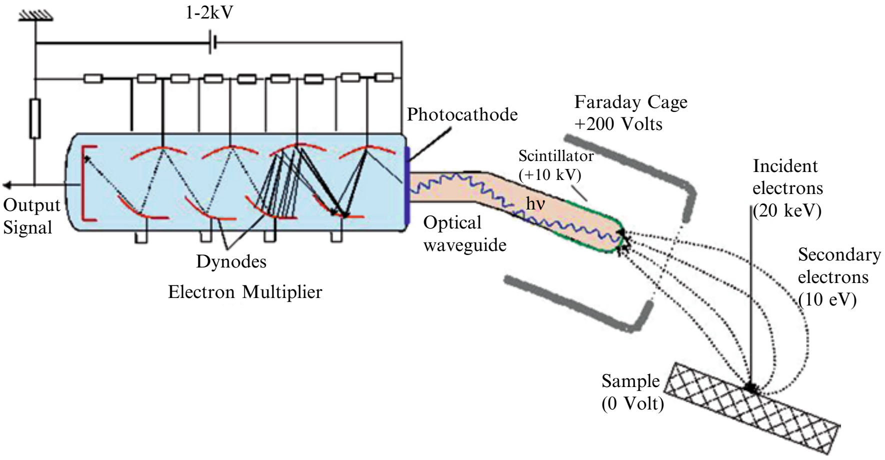

Fig. 4.10 Drawing of Everhart–Thornley secondary electron detector. Reproduced with permission of Emmanuel de Chambost from Wikipedia

E–T detector is not limited to line of sight and can collect electrons from areas that are shaded by protuberances. It is this property of the detector that allows us to obtain good topographical information on the specimen.

This concludes the bulk of my discussion about beam–specimen interactions as it impacts basic SEM imaging although I will return to the subject in the EDS section and the Low-vacuum section. This presentation is pretty simplistic and will not satisfy the purists in all regards, but I do hope you have a good appreciation for some of the complexities that we deal with behind the wonderful images. For the bulk of our work, we do not need to understand much about the physics of electron–atom collisions, but if you want to get the most from your instrument and be able to apply it creatively to new problems, you will be well rewarded for the time and effort spent studying this subject.

4.2.5 SEM Instruments

We can categorize SEM instruments into four divisions based on type of electron gun and on type of instrument (i.e., high and low vacuum). The categories are fuzzy and overlap to some degree, but I think this type of categorization is helpful to the pharmaceutical scientist who has no intention of becoming an expert but does want to understand the basics. In general, there are two types of electron guns: thermionic and field emission. There are also two types of instruments based on sample chamber vacuum: high vacuum and low vacuum. Finally, there is a new subcategory, the benchtop SEM which generally is a subcategory of the low vacuum instrument. If you understand the distinction among these categories, you will understand quite bit about SEM technology. So there are thermionic high-vacuum instruments, thermionic low-vacuum ones, field-emission high-vacuum instruments, and field-emission low-vacuum ones. Each category of instrument has a specific set of advantages and limitations.

4.2.5.1 Electron Guns

As mentioned, there are two categories of electron guns: thermionic and field emission. The thermionic gun has either a tungsten hairpin wire or a lanthanum hexaboride (LaB_6) crystal which are heated to high temperature. A potential field is applied to the tip region and electrons pulled from the tip. Thermionic guns produce good current but there are inherent limitations to the smallest tip size that can be manufactured for this purpose and tip size limits the ultimate resolution. In other words, there are physical limitations to thermionic guns that preclude their use for very high resolution. Since pharmaceutical applications rarely require the highest resolution, thermionic guns are generally quite suitable for our work – unless one does extensive work with inhaled products or with wet-bead milled products. Also, the tungsten thermionic gun requires frequent replacement of the tip compared with LaB_6 or field-emission tips.

With field-emission guns, a potential is applied to a very fine tungsten tip and electrons drawn from the tip by the electric field. The generation of the electron beam in this fashion requires a high vacuum at the tip. Again there are two types of field-emission guns categorized as cold and hot (or Schottky, wherein the emission is aided by heat) guns. Both are characterized by high brightness (good current, small initial beam size, and low convergence). They also have the advantage of long life. The cold emission guns may not generate quite the maximum current as do the thermionic guns, but they generally produce adequate current for most pharmaceutical work. The trade-off between thermionic and field emission, naturally enough, is cost. Field-emission instruments are much more expensive than comparable thermionic instruments.

For SEMs, sophistication equals cost. The more sophisticated the instrument the higher the cost. The field emission guns and instruments have distinct advantages over thermionic instruments but cost is not one of them. A very crude survey indicates that field emission instruments are roughly twice the cost of a thermionic SEM. If you need high resolution, then field emission is definitely the way to go. If you rarely exceed 10,000×, then you may be paying quite a bit of money for excess capability. Interestingly, in my experience, most modern field-emission instruments are better suited for a multi-user lab than are thermionic ones. If you plan to work with wet-bead milled (nano) suspensions or with inhaled dosage forms, then you do need a field-emission microscope. Even though it is possible to work with these dosage forms using a thermionic instrument, it is very difficult and you must ensure perfection in all the normal operating parameters to achieve barely adequate resolution. You will spend much more time fine-tuning the thermionic instrument than doing observations.

4.2.5.2 Low Vacuum and Benchtop Instruments

Low-vacuum SEMs are particularly useful in pharmaceutical analysis. That is because most of the items we examine are nonconductors which are also vacuum and beam sensitive. The low vacuum SEM helps ameliorate problems with vacuum sensitivity and with charging. In fact, pharmaceutical companies were among the first to purchase the initial instruments made by Electroscan (based on designs by Danilatos) in the late 1980s. Benchtop SEMs are relatively new and generally are low-vacuum instruments with extremely restricted operating conditions so that the accelerating voltage may be fixed as is the working distance, final aperture, and detector (generally backscatter detector). Even with these limitations, the benchtop instrument may be an appealing choice for some applications due to its low cost as well as its restrictive operating conditions. In particular, it may be an ideal instrument for a multi-user environment where many different analysts need to take reference photomicrographs. It may also have some advantages in particle size using image analysis.

Low vacuum instruments can be divided into two categories based on the type of gas in the specimen chamber and the type of detector. The first commercial instruments were offered by Electroscan and allowed for relatively high water (other gases available) vapor pressures in the chamber and used a patented secondary electron detector by Danilatos (1993) and were called the ESEM (Environmental Scanning Electron Microscope). The name ESEM was under trademark (lapsed in 1999) and so when other instruments were introduced they were referred to as low-vacuum SEMs. These latter instruments nearly always used air as the chamber vapor and a backscatter detector for image production. It is pretty onerous to have to say "ESEM and Low-Vacuum SEM" all the time, so I use low-vacuum SEM for the entire class of instruments. Confusingly, LV-SEM can also stand for Low-Voltage SEM so try not to be too confused by the acronyms. Really, the differences in these instruments have begun to blur since many modern instruments have both high-vacuum and low-vacuum modes, since secondary electron detectors have been developed for low-vacuum instruments and backscatter detectors for ESEMs. In the following paragraphs, I will discuss the critical operating characteristics of these instruments and how they impact pharmaceutical applications.

Danilatos developed the ESEM in an attempt to obtain good images of wool fibers (see Danilatos 1988, 1990a, b). First, he began by looking at using water vapor in the chamber in order to reduce charging effects and to keep the fibers hydrated. Even a small amount of water vapor (100–500 Pa) worked surprisingly well for these problems, but it was nearly impossible to obtain an image with conventional secondary electron detectors. Danilatos then experimented with using the water vapor as a means to enhance secondary electron detection. He did this by inventing a detector that is simply a charged plate with a variably applied potential (Danilatos 1990b). He found that if he negatively charged the plate and placed it some short distance above the sample, he induced a positive charge on the specimen surface and produced an electron cascade that could be detected and measured using the charged plate. Figure 4.11 shows a schematic diagram of the detector with cascade and Fig. 4.12 shows one instrumental configuration. The popularity of these instruments is a testament to their utility in many different industries, not least of which is the pharmaceutical industry.

There are quite a few advantages to low-vacuum instruments in pharmacy. One is the lack of any need to coat specimens. This advantage is not solely convenience. The coating process requires significant vacuum which can damage our pharmaceutical samples. We can place entire tablets in the SEM without having to do any advance preparation and we can obtain acceptable images. By manipulating temperature and water pressure, we can keep hydrated samples in close to ambient conditions. We can do dynamic experiments by adjusting the water vapor pressure and the temperature. We can use other gases in the chamber and experiment with solvates. These instruments are particularly useful for EDS since we do not need to coat the specimen with a metal that causes significant peak overlaps. In short, these instruments are quite useful in pharmaceutical development and are here to stay. They are not without their idiosyncrasies and I will have more to say about this subject in later sections.

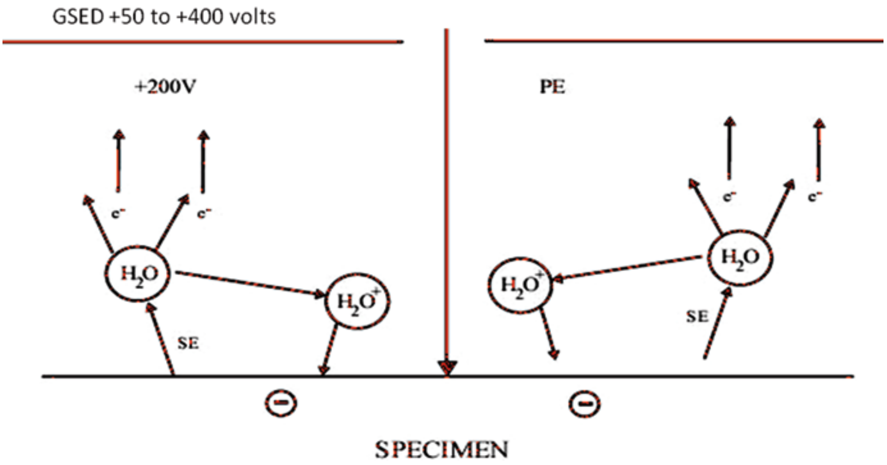

Fig. 4.11 Illustration of electron cascade in ESEM. As the beam electron strikes the specimen, secondary electrons are generated as in a high-vacuum SEM. These secondary electrons are drawn toward the detector and interact with water molecules. This interaction results in production of an additional electron and a positive ion. These electrons in turn interact with other water molecules resulting in an electron cascade and amplification of the secondary electron signal. The positive ions are drawn to the negative surface of the specimen and serve to neutralize the specimen charge and reduce specimen-charging effects

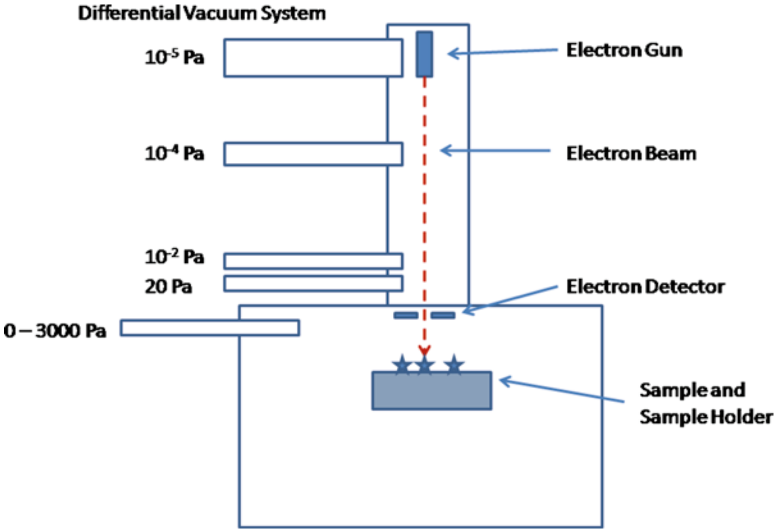

Fig. 4.12 Schematic Drawing of ESEM. The drawing is based on an early ESEM instrument from Electroscan and shows the differential pumping system

4.3 Sample Preparation and Imaging Strategy for Pharmaceuticals

It may seem a bit strange to connect these two subjects but in my mind they are intimately connected. It is hard to put too much emphasis on sample preparation. No matter how fine the microscope, the final analysis will be only as good as the sample. Sampling, from full batch to microscope specimen, could be the subject of its own text and so we do not have space to cover many of the details in this book. Also, how we approach imaging will very much depend on how we decide to sample and vice-versa. So, we will touch on the surface of the subject here and you will find practical sample preparation and imaging recommendations in the examples and practice exercises accompanying this and other chapters. Lyman et al. (1990) provide additional practical advice on sampling, imaging strategy, and instrument setup as does Goldstein et al. (2003).

Sample preparation and setup of the microscope depends upon whether we are looking at drug substance or drug product, and if drug product, they will depend on dosage form. Simply put, you will prepare and image particles from a wet-bead suspension differently than a tablet surface. Most importantly, sampling and imaging depend upon the objective of the analysis. It is always a good idea to question the requestor carefully about the objective of the analysis rather than assume that you know. It is quite common for folks to drop off samples and simply ask for pictures. Maybe they want photomicrographs just for reference and maybe they need more. A few good questions help sort the matter out. The following paragraphs will discuss sample preparation and imaging of drug substance and drug product. Table 4.1 lists a number of imaging considerations in instrument setup for the major objectives in SEM and EDS analysis.

4.3.1 Drug Substance

Let us start with the simplest case, crystallized drug substance. In my experience, the size of the primary particles generally ranges from 20 to 200 μm, although larger and smaller particles are not uncommon. The goal of this work is generally to aid the synthetic and process chemists in their efforts to consistently produce drug substance with the desired physical properties. The objective of the analysis is to produce photomicrographs of representative particles. The simplest sample preparation is to remove a small specimen from the vial with a spatula and sprinkle the particles onto double-sided carbon tape on an aluminum SEM stub. You can then either blow off the excess or knock the excess off by striking the edge of the stub onto the table top. You can then image directly in low vacuum or sputter coat with metal for high-vacuum work. Admittedly, this type of sample preparation is hardly precisely representative, but suits the need quite well. It is simple, fast, and yields the desired information. My rough guess is that maybe 70% of the routine

Table 4.1 SEM and EDS analysis strategy

Instrument parameter	High resolution	Depth of field	EDS	Vacuum sensitivity	Beam sensitivity	High image quality
Accelerating voltage	High	High	Sample dependent[a]	High	Low	High
Beam spot size	Low	Medium	High	Medium	Medium	Medium
Final aperture	Low	High	High	Medium	Low	High
Working distance	Low	High	Set[b]	Low	Low	Medium
Gas (for low vacuum mode)	High	NA	Low	Low	Low	High
Coating	Yes	NA	No	No	Yes	Yes
Detector	E–T	E–T	BSE	BSE	BSE	E–T

This table does not take into account some modern advances in SEM instruments. For instance, there are now many excellent secondary electron detectors for low-vacuum instruments and so it is not necessary to use a backscattered electron detector. Even though there are some obvious exceptions, this table can be a good starting tool for the SEM novice or occasional user

[a] The accelerating voltage (kV) must be 1.5–2 times greater than the energy (keV) of highest energy X-ray line expected

[b] The proper EDS working distance is set by the manufacturer for the proper detector geometry

Fig. 4.13 SEM Photomicrograph of Hydroxy Propyl Methyl Cellulose. This image illustrates a typical sample preparation and image of drug substance and excipient particles. (Image size = 488 × 396 μm)

work in our laboratories is done in this fashion. Figure 4.13 of hydroxyl propyl methyl cellulose (HPMC) is an example of this type of sample preparation.

There are a number of variants on this technique that produce more representative samples and are also simple and fast. One variant is to deposit your specimen onto a flat surface such as a glass microscope slide and spread the particles over the surface. Press the double-sided tape (on a SEM stub) onto the powder with a light pressure. The particles stick to the tape and you can then directly examine or sputter coat. Another variant is to use a jar with holes in the lid. Place the SEM stub with double-sided tape onto a raised platform on the bottom of the jar, deposit the sample onto the bottom of the jar, and close the lid, and then blow air into one of the holes in the lid. The particles will be suspended in the air blast and then fall onto the double-sided tape. After a few minutes (to allow the fine particles to fall), remove the stub and test.

If I am primarily interested in reference photomicrographs, I usually coat the specimen and image in high vacuum at relatively high kV (15) with a moderate spot size and final aperture. I then will take images at 100, 250, 500, and 1,000× at a minimum. I find standard-sized images useful for comparison and for presentations. While it should not be too hard for trained scientists to compare an image taken at 400× with one taken at 550×, I find it convenient to not require them to do it. If I have a set of common size photomicrographs, I am able to present a simple comparison of drug substance manufactured many years apart. It does not preclude taking additional photomicrographs at other magnifications, but at least I will have a similar set for each batch.

It is not uncommon in pharmaceutical labs for operators to get into something of a rut when it comes to setting up the SEM, including use of detectors and accelerating voltage. In some respects, this repetitiveness is both expected and useful. As mentioned, it can be awkward to compare images taken years apart with very different imaging conditions in a presentation to management. On the other hand, it is good to get out of the rut and experiment a bit. If you are accustomed to operating at 15 kV with a secondary electron detector in high vacuum and lots of specimen coating, try working at much lower kV with the same sample. Try using BSE on an uncoated sample in low vacuum. All of these images will have a somewhat different "feel" to them and it can be surprising what you can learn from this exercise about your sample.

The simple sample preparation techniques recommended for crystallized drug substance will not work as well for DS that is milled or micronized. These samples are much more challenging to prepare both because of the nature of the material but also because the objective of the analysis is often more demanding. Not only do we want representative reference photos, we may also want to have a reasonably accurate estimate of particle size and shape. The preparation and analysis are almost always complicated by the fact that milled and micronized drug substance is invariably agglomerated. These agglomerates can be both weak and strong referring to their resistance to breakage and separation. The requestor may be interested in the number, size, shape, and resilience of the agglomerates. Or they may be mostly interested in primary particle size and shape. The size of milled primary particles is typically less than 20 μm and a significant percentage of the particles can be less than 1 μm in diameter. We are generally on firmer ground with shape since the milling process nearly always produces an irregular shape. In fact, we think (only half jokingly) that there should be a shape category called "micronized," because micronized particles have a characteristic irregular shape. So, for all these reasons, we need to be careful how we prepare and image milled drug substance.

For an initial cursory examination, it may be okay to use one of the sample preparation techniques previously mentioned – particularly the powder in bottle technique. In fact, for some samples, the powder in bottle technique works well for obtaining representative specimens. Only some samples, though. In most cases, the cohesive nature of pharmaceutical drug substance works against that technique. There are some variants on the aerosolization of powder that have been developed by some commercial manufacturers such as Clemex (see Web reference). I have tested a couple of these devices and they work quite well with most samples. The only drawback is that they typically use quite a bit of material and so are best suited for work in late stage development.

The best way of getting a truly representative sample with micronized drug substance is to suspend it in a liquid and either filter it onto a Nucleopore™ filter or spray it onto a stub or other SEM sample holder (see Chap. 7 for more details on this technique). This type of sample preparation is more time consuming and makes more of a mess than the aforementioned techniques, but it produces representative specimens for every sample to which I have applied it. Once you get accustomed to the technique it is not that much more of a bother and the results are generally worth it. See Fig. 4.14 of caffeine hydrate for a representative image using this technique. For wet-bead milled DS, I think the only way of producing a representative image is with

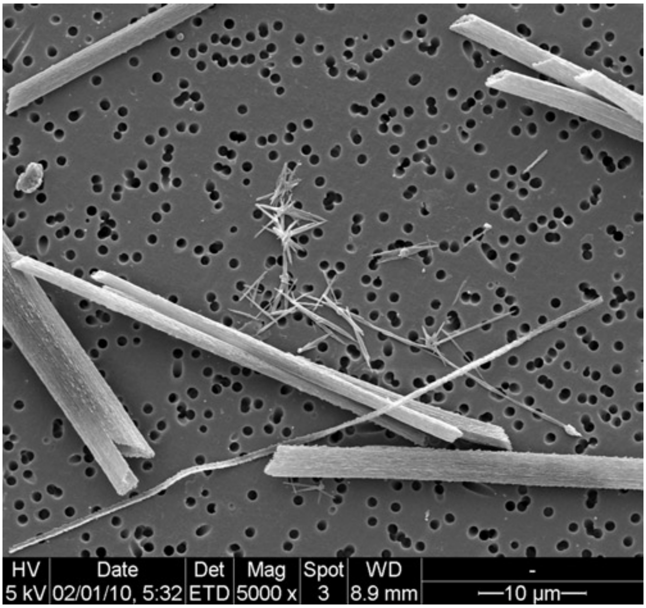

Fig. 4.14 Caffeine hydrate prepared with Nucleopore™ filter. Caffeine hydrate was suspended in cyclohexane and filtered through a 1 μm Nucleopore™ filter. Note the good separation of the particles. (Image size 55 × 55 μm)

the Nucleopore™ filter. You do have to dilute the suspension quite a bit and, in fact, I dilute until I can barely see the blue Tyndale effect of the submicron particles by holding the bottle of suspension up to light. That typically yields enough particles on the filter so that a number of particles are visible in each field of view but not so many particles that the particles are agglomerated due solely to sample preparation.

I generally coat these specimens even if I am using low vacuum. I think you get better particle definition with a coated particle. Milled particles can be imaged reasonably well in a thermionic high-vacuum instrument with secondary electron detection, but its best to operate at low kV, small spot size, and small aperture. It takes more trouble to set up good imaging conditions, but it is necessary if there are a significant number of particles less than 1 or 2 μm in diameter. It is quite challenging to obtain good images of wet-bead milled particles using a thermionic instrument since these particles are often less than 0.5 μm in diameter. It is better to use a field-emission type instrument at low kV. If that is not available, then plan to spend considerable time setting up the instrument since you will need to operate at ~50,000× to resolve the smallest particles. This is the upper practical magnification for thermionic SEMs with pharmaceutical materials. While it is true that the instruments are capable of better magnification with some samples, it is generally hard to go higher with pharmaceuticals.

I typically ensure that I have a small section of the aluminum specimen stub uncovered so I can use that area to set up the microscope. Most aluminum stubs have some quite small features in the metal that are suitable for high resolution imaging.

I will align and stigmate on the aluminum before I move to the particles. That is because the metal produces a better signal for setup. You may want to do some cursory examinations of wet-bead milled samples with a thermionic instrument, but to do critical work (quantitative size and shape), I recommend using a field-emission instrument. Thermionic instruments just do not have the capability for routine imaging of wet-bead samples in my opinion.

4.3.2 Drug Product

It is hard to generalize about drug product since there are so many different dosage forms on the market or in development. For instance, how do we generalize among a capsule, a subcutaneous injection of a suspension, and a skin patch? Sample preparation and imaging will be different for each dosage form and for the goals of the analysis. I will discuss some of the common dosage forms in this section and have some examples of other dosage forms in other chapters.

To start with the simplest case, we can treat capsule contents much like we treat drug substance. Capsule formulations are typically granulated in order to improve flow and so if you are interested in the drug substance in the capsule, you will need a method to break up and disperse the granules so as to liberate the primary drug substance particles. A mortar and pestle works reasonably well in most cases.

For the direct examination of tablet surfaces, you need no special preparation. You can attach the tablet to double-sided tape or glue it to a specimen holder and examine directly. It is generally best to use low vacuum since it is very hard to sputter coat an entire tablet. Since tablet surface features are relatively large, you should work with relatively high kV (~15), a medium spot size, and medium aperture. To examine the interior of the tablet, I recommend using a microtome. You can learn something by fracturing the tablet and it is possible to shave a cross-section with a razor and get reasonable images, but the most reproducible technique for producing representative cross-sections of tablets is with a microtome. An older, manual microtome is adequate and, of course, you will be looking at the surface remaining on the tablet, not the slices coming from the microtome blade as in typical microtomy. You may also need to fabricate a good holder for the microtome. As with tablet surfaces, you are not likely to be doing high-resolution work with tablet cross-sections so moderate kV (10–15), moderate beam size and moderate final aperture are best. Figure 4.15 shows a typical cross-section of a Naproxen tablet. I like to use backscatter detection since the components of the tablet generally have differing average atomic number.

For other dosage forms, I recommend spending considerable time and effort on developing the sample preparation recipe in an iterative fashion. Try a simple preparation and typical imaging conditions. Even if things look okay, attempt other imaging conditions on that sample and then try a radically different sampling technique. If all the images are similar, then you can have some confidence in your techniques. If they are very different, then you will need to expend more effort to

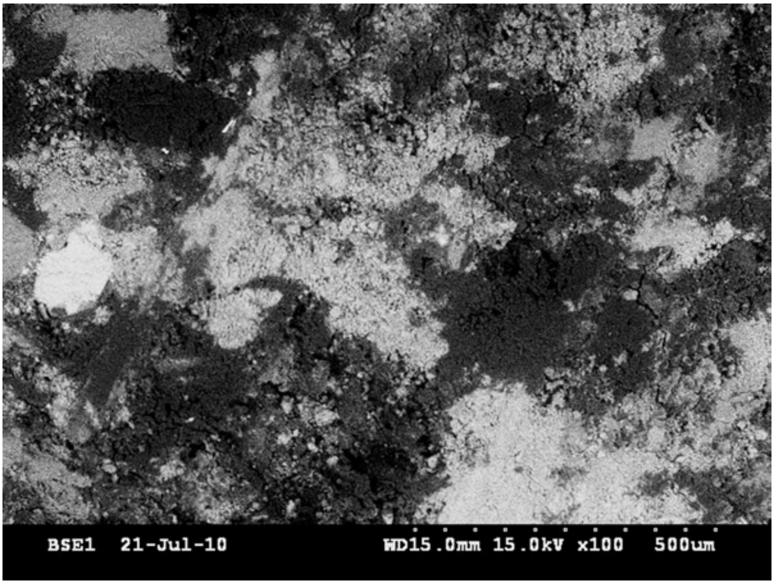

Fig. 4.15 Generic Naproxen sodium tablet cross-section. This sample was prepared using a microtome and is imaged using BSE. The bright areas are naproxen sodium and the dark areas are excipients, probably Avicel. (Image size 1,217×987 μm)

understand these differences and which technique is best for the analysis goal. Time spent getting the sample preparation and imaging correct is generally regained when you have to interpret and present the results.

4.4 Energy Dispersive X-Ray Spectrometry

4.4.1 Theory

The discussion of the SEM so far has concentrated on imaging and while that is appropriate for pharmaceutical applications, we inevitably desire some chemical information to go along with the images. Energy-dispersive X-ray spectrometry (EDS) can supply chemical information and has a number of important applications in pharmaceutics. The fundamentals of EDS can be difficult to understand without some grounding in atomic physics even though the actual process of collecting a spectrum and doing qualitative analysis is relatively straight-forward and not overly complex. Still the more that you know about the physics underlying the technique, the better able you are to correctly interpret spectra and to troubleshoot the system when problems in interpretation arise. We will cover some basics first, and then discuss qualitative and quantitative analysis, followed by elemental mapping.

As mentioned in the electron–specimen interaction section, two categories of X-rays are generated as a result of the electron interacting with a specimen atom.

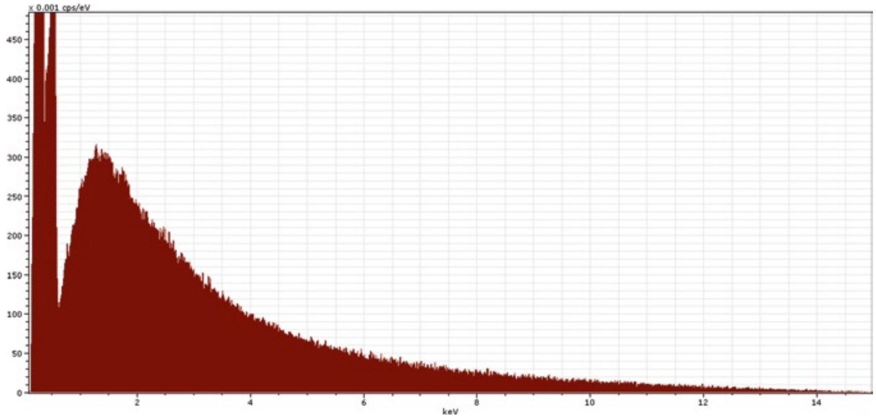

Fig. 4.16 EDS spectrum of carbon block. This spectrum illustrates the Bremstrahlung radiation that results from the Coulombic interaction of the beam electron with the atomic nucleus. X-rays are produced with energies extending from zero to the electron accelerating voltage. The apparent reduction in X-ray intensity in the low keV region is due to the detector. In fact, the curve extends upward and more X-rays are produced at low energies than are detected and counted

The first category is the braking or Bremstrhalung radiation which results from the interaction of the negatively charged electron with the positively charged atomic nucleus. Figure 4.16 shows an expanded spectrum of a carbon block with only the carbon peak but serves as an illustration of the braking radiation and X-ray background. As you can see, the energy extends from near zero to the corresponding accelerating voltage of 15 kV. In spectroscopy, we often deal with a background that is primarily noise, but with EDS the background is both noise and signal. This fact has some important consequences for EDS analysis and, in particular, quantitative analysis and elemental mapping. The presence of the background due to Bremstrahlung does not have only negative consequences. It is often used as part of peak-to-background quantitative analysis among other uses (Statham 1978; Griffin and Nockolds 1996).

The beam electrons can also interact with inner shell electrons in the atom and knock them out of their orbital shell. In the process of relaxing from the excited state, the atom can release characteristic X-rays, fluorescence, and Auger electrons. We are only here concerned with the characteristic X-ray emission. These characteristic X-rays are the basis of EDS and of X-ray wavelength spectrometry or the electron microprobe as it is called. In this latter instrument, crystals are arranged in specific locations around the sample so that X-rays of particular wavelengths are transmitted by diffraction. This instrument is quite good for quantitative analysis of inorganics, metals, and minerals but only has very few, if any, applications in pharmaceutical development. There are also instruments used to detect Auger electrons and are quite useful for surface analysis. Again, there are few pharmaceutical applications of Auger spectroscopy.

It is helpful to think of EDS with an atomic orbital model. That is that we have electrons circling the nucleus in specific orbits like the planets around the sun. Of course, this model is simplistic and leaves much to be desired but is helpful in our thinking of EDS. We are concerned with three orbits designated by the letters K, L, and M. When an inner shell electron is displaced, the atom stabilizes itself by replacing that electron with one from the K, L, or M orbits. The number of possible orbits depends upon the atomic number of the element. The light elements most commonly found in pharmaceuticals typically can only generate K orbit transitions. To complicate things a bit, each orbit can be divided into suborbits. There are two suborbits of interest in the K orbit, designated by the Greek letters α and β. So in a spectrum of a light element we would expect to see two peaks associated with K orbit transitions and these are designated as K_α and K_β. The K_α line is much more intense than the K_β one and the ratio of the two lines is constant. The l and m lines are more numerous and complex with a confusing and hard-to-remember set of subscript designations. As with the K lines, the L and M lines have a definite relationship to each and constant ratio to the most intense line. Figure 4.17 shows the ratio of line heights for each of the lines and the subscript labels. It is important to not only be aware of all of these lines but to know them reasonably well. Most software programs that control EDS detectors allow you to superimpose the various lines onto generated spectra and these lines can be quite helpful as a memory aid, but I would not trust them implicitly. It is helpful to have a table of lines available to consult either in paper format or in a computer table.

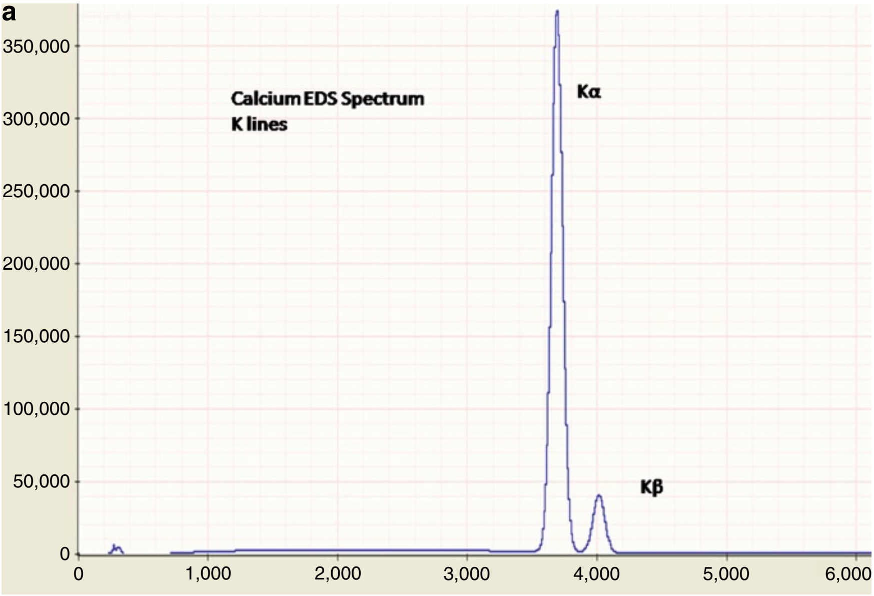

Fig. 4.17 EDS spectrum lines. (**a**) K lines for calcium, (**b**) L and M lines for barium, and (C) M lines for lead. These spectra illustrate the lines expected from an EDS spectrum of each of these elements. The ratio of line heights and presence of each line depends upon the accelerating voltage. These spectra are Monte Carlo simulations produced using the NIST program DTSA II (see Web site under Internet references for more details on this program)

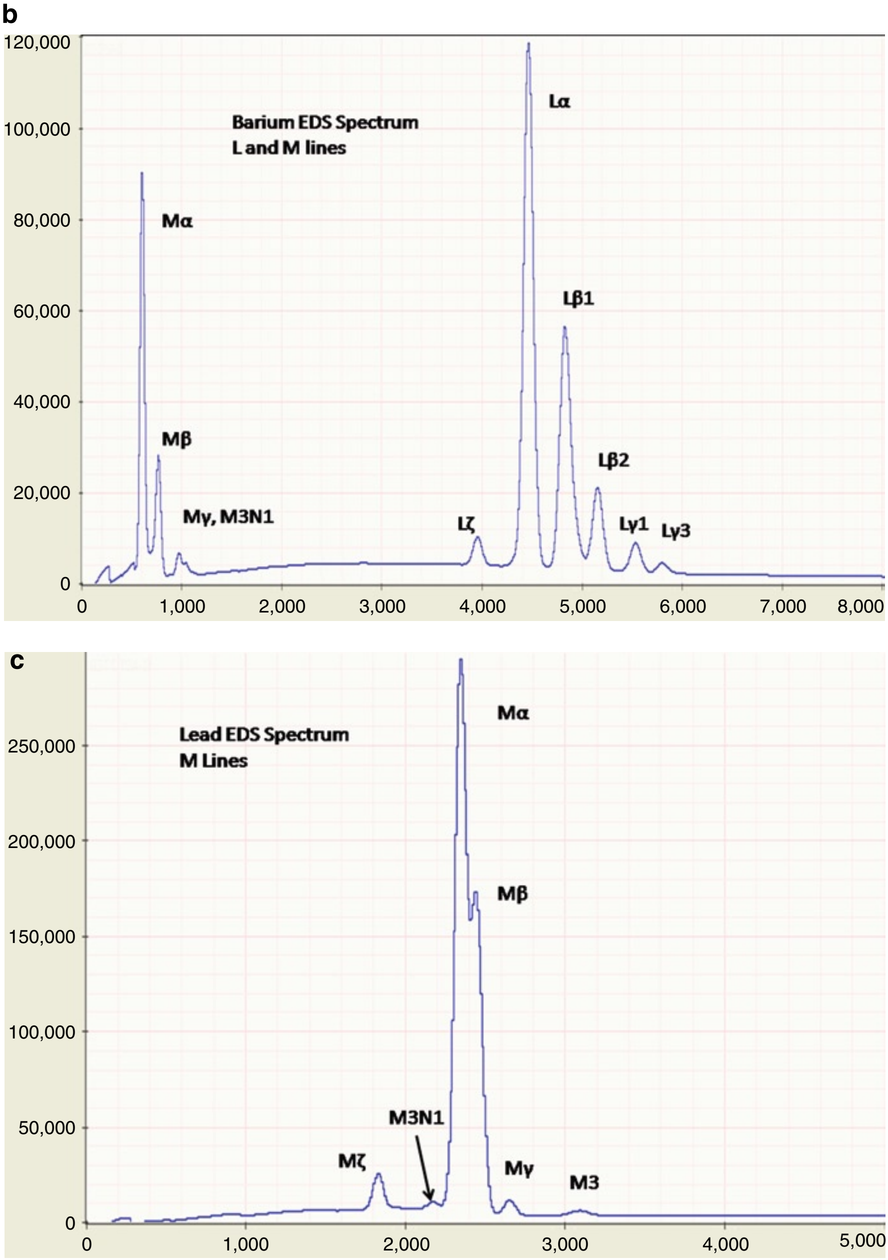

Fig. 4.17 (continued)

The EDS system measures the energy of the X-rays that are generated and produces a spectrum that relates intensity (number of X-ray counts) to energy. X-ray energy in microanalysis is generally measured in electron volts, eV, and should not be confused with the accelerating voltage measured in kV. The latter is the potential of the electric field, whereas the former is the electron energy. So, for instance, if the electric field in the SEM is 15 kV, an electron accelerated in this field has energy of 15 keV. Figure 4.18 shows a typical spectrum of a generic naproxen sodium tablet cross-section with lines for C, O, Na, Si, Al, Mg, Cl, and possibly some Ti.

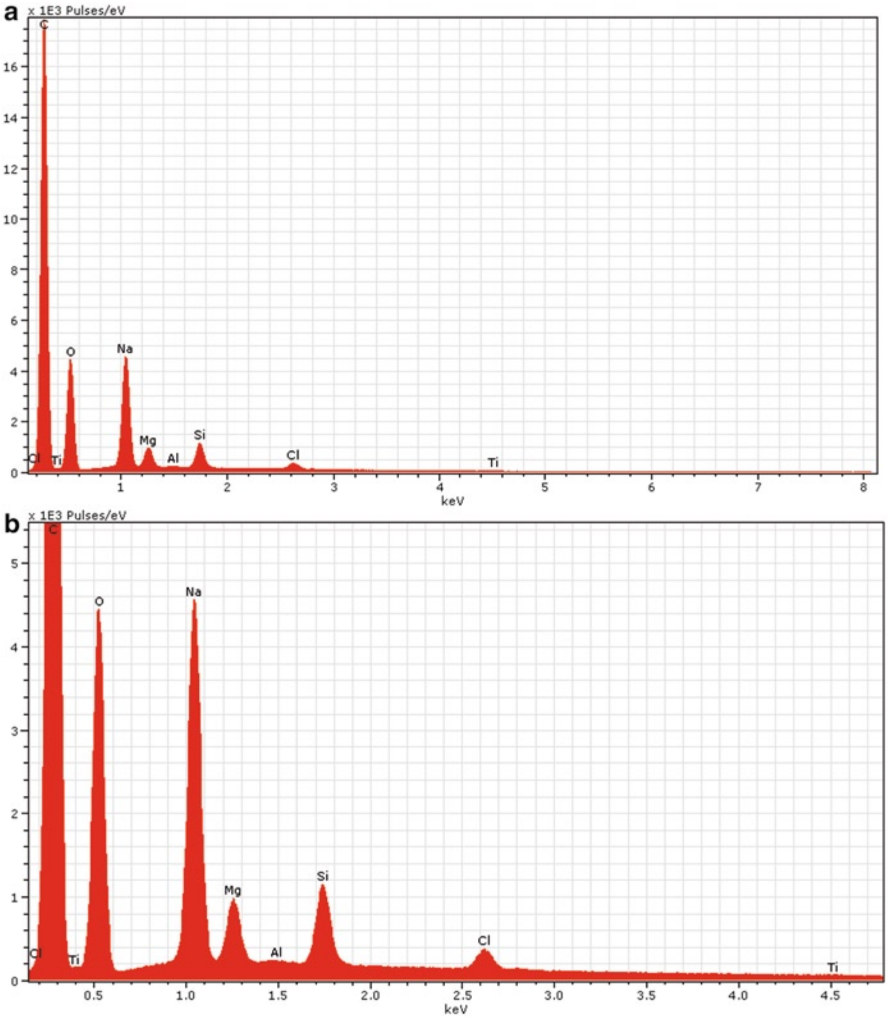

Fig. 4.18 EDS spectrum Naproxen sodium tablet cross-section: (**a**) full scale and (**b**) expanded scale using SDD detector. This spectrum is typical of those collected from pharmaceutical samples

There are two types of X-ray detectors in use today. The lithium-drifted silicon (SiLi) detector has been used for over 50 years and many systems are still in operation. A silicon chip is doped with lithium and when struck by an X-ray produces a charge that can be measured and amplified by the electronics. The strength of the charge is related to the energy of the input X-ray. The SiLi detector has to be maintained at liquid nitrogen temperatures both for the detector and for the field effect transistor that provides the initial signal amplification. Further, the silicon chip has to be protected from the chamber using a beryllium or polymer window. The polymer window is preferred since it allows for better light element detection than does the beryllium window.

Silicon drift diode (SDD) detectors are also made of a silicon chip but with a series of concentric wires so as to provide an induced electric field on the chip that sweeps electrons produced as result of X-ray collection to a central anode and field effect transistor. Dead time for signal shaping and measurement is much reduced compared with a SiLi detector. The improved efficiency of this detector is such that liquid nitrogen is not required and the chip and transistor can be cooled with a peltier type cooler.

The resolution of SiLi and SDD detectors is in the 125 to 140 eV range, whereas wavelength spectrometers have resolutions in the range of 10 eV. The inherent X-ray line widths are on the order of a few eV. The low resolution of EDS X-ray detectors has important implications for qualitative analysis as it is inevitable that peaks with certain elements will overlap. Overlapping peaks lead to uncertainty in elemental identification. Figure 4.18a, b shows a typical SDD EDS spectrum of a cross-section of naproxen sodium.

The concept of dead time is important in practical EDS operation. The detector requires a finite time to collect the X-ray, amplify and shape the voltage signal, and add the X-ray count to the spectrum. During this time, the detector is switched off and no X-rays are collected. We, the operator, have some control over this time both with the microscope as well as with the EDS instrument. Anything that increases beam current on the microscope, such as increasing spot size, increasing accelerating voltage, increase in final aperture size, will increase the X-ray signal. This increase in beam current and X-rays will quickly overwhelm the SiLi X-ray detector and the detector will spend more time switched off than on. Live time is the total time of analysis whereas real time is the time the detector is switched on. The difference is dead time and is typically presented as a relative percentage of real time to live time. In most cases, we want to keep the dead time to about 10% for the most efficient X-ray collection. One control that we have on the detector side is the shaping time. The longer we allow the instrument to accurately measure the voltage in the detector and to amplify and shape the charge, the higher the dead time. There are different terms for this variable but dwell time is somewhat common and easily understood. For that reason, it is common to decrease the dwell time for mapping and for quantitative analysis and to increase the dwell time for qualitative analysis.

The primary advantage of SDD detectors over SiLi detectors is the improved count rate. For pharmaceuticals, it is the reduced dead time possible with SDD detectors that leads to improved count rates. So, for example, in a direct comparison of X-ray counts between a SiLi and a SDD detector using a tablet cross-section and standardizing on the beam current, I found that the count rate increased from 2,500 to 17,000 cts/s (Carlton 2009). This represents an enormous improvement in count rate and allows for significant improvements in elemental mapping and in quantitative analysis. Nearly all new detectors today are SDD even though the SiLi detectors are still working well on many SEMs. Over time it is doubtless that all X-ray detectors will be SDDs.

We need to examine a final important aspect of EDS and that is the detector geometry. EDS detectors are line of sight. In other words, the detector "sees" a region of the sample and only X-rays from that region are collected. Consequently, X-ray detectors are installed in the SEM sample chamber in a particular orientation and the most common angle of the detector to the specimen is about 35°. In order to achieve that angle, the working distance of the specimen must be at a particular distance – often 10 or 15 mm. At any other working distance, you will not get the optimum efficiency as evidenced by reduced count rates. In practice, most of us alter the working distance slightly up and down from the recommended working distance in order to get the "sweet" spot of maximum count rate. For qualitative analysis and for X-ray mapping the correct detector geometry is not crucial to obtaining good results. For quantitative analysis, it is crucial to maintain and to know the exact detector geometry.

4.4.2 Qualitative Analysis

SEM/EDS is superbly suited for qualitative elemental analysis. It simultaneously and quickly detects nearly all of the elements of interest in pharmaceutical products with relatively simple sample preparation. Sometimes it is just that ease of operation that can get us into trouble. It is easy to collect a spectrum and modern software will automatically assign elements to the peaks. But, as with most scientific instruments, one has to be careful with the interpretation because there are a number of pitfalls and spectrum artifacts that can cause us problems. For that reason, it is best to always verify any identification.

First, it is always wise to check calibration before analysis. EDS systems will drift and some more than others. In other words, the computer assigns a numerical energy value to each incoming X-ray. The hardware and software, though, have to be calibrated first to ensure that the values are exact. You will see some drift over time so that the EDS markers for say, Cu, do not line up exactly with a known Cu peak. Once the markers are off by more than 10–30 eV, then it is time to recalibrate. Most modern systems have an automated calibration system. Also, it is good laboratory practice to record these performance checks and track the performance of the instrument over time. Generally we use the Cu L and K lines for calibration since they extend from low energy ($L\alpha 1$ – 0.93 keV) to middle energy ($K\alpha$ – 8.05 keV). If the work is not critical but solely informational, you may want to use the Al of the specimen stub as a standard. The $K\alpha$ line should be at 1.49 keV. It is not an ideal performance standard since it lies in the low-energy end of the spectrum and it is possible to be nearly on at low energy and off by quite a bit at high energy. If you decide to use Cu as a performance standard, it is useful to record intensities of the K and L lines. The ratio of these two lines should remain relatively constant over time. If the ratio changes by more than 10% or so, then it may be a good time to check with the service engineer since this can be a sign of problems with the detector. If you are recording the ratio of Cu K and L lines, then be sure and keep the accelerating voltage the same since the ratio will naturally be different at different beam energies.

The analysis strategy depends to some extent on the goal of the examination. In general, we use EDS in the qualitative mode to identify foreign material. We typically have some hypothesis about the general nature of the material such as that it may be inorganic or metallic. In this case, we should begin in a survey mode. The first consideration is sample preparation. In general, this should be quite simple. If it is a tablet with a spot, we may want to directly mount the tablet onto a stub and examine it directly. If we are dealing with a particle in a liquid matrix, we will want to filter it, mount the filter onto a SEM stub and examine it directly. Particles in powder or tablets can cause us some preparation issues since powder will generally adhere to the particle. Since, at this range of size, EDS is a surface technique, we may not be able to collect a good spectrum of the particle. We have to proceed carefully. Washing the particle with water or other solvent is an obvious way of removing the drug substance and/or drug product, but we certainly do not want to dissolve the particle. If we have an abundance of particles, a solubility test is in order. If we have only one or two particles, we have to try a different approach. One approach is to remove as much material as possible from the particle using a needle under a stereomicroscope. If we can reveal at least some small portion of the particle for analysis, then that may suffice for identity.

I generally begin EDS analysis in low vacuum mode with an accelerating voltage of 15 kV. High vacuum requires coating which should be avoided. An accelerating voltage of 15 kV is sufficiently high to excite most of the typical metallic contaminants and will also excite the lower atomic number elements as well. The rule of thumb is that the accelerating voltage in kV should be at least 1.5–2 times the X-ray energy in keV for good collection. Using the Cu Kα line at 8.05 keV as an example, in order to properly collect this line, the accelerating voltage needs to be 12 kV at a minimum and is better at >16 kV. I typically collect a spectrum for a short period of time in order to ensure that I have set up the SEM and EDS conditions correctly before collecting the final spectrum. You should pay particular attention to beam spot size and current in the SEM and dwell time in the EDS. Look for high X-ray count rates with low dead times. Once the conditions look right collect a longer spectrum and run a 300 s live-time spectrum. That amount of time is typically sufficient to ensure that minor and trace elements can be detected if present. In many cases, a 100 s live-time collection is sufficient.

The initial examination also calls for a consideration of SEM magnification. In general, for contaminant identification, we probably want to run in EDS spot mode which has the effect of keeping the electron beam in one spot without rastering. When using spot mode, monitor the specimen carefully because these conditions can frequently lead to beam damage. Before using spot mode, it is good to begin with a survey magnification at TV scan rates and set an area (either by altering the magnification or setting the area from the EDS software) that encompasses the item of interest and a bit more. Run this initial spectrum at 300 s and inspect the specimen for beam damage once the run is complete. Then you can select certain areas for spot analysis based on the backscatter image. You should compare the wide field examination with the spot mode.

At this point, we do an initial peak identification. It is okay to use the automatic peak identification software routine at this stage, since we are looking for possible peaks to verify by altering the kV. What I mean is that if we see a possible peak at the low-energy part of the spectrum that we suspect is an L or M line, we need to investigate the higher energy range of the spectrum for the K line. For some elements, the 15 kV accelerating voltage may be insufficient to excite the K line of the putative element. If we suspect that this is the case, then we need to increase the accelerating voltage to 20 or more kV, re-align the SEM, and re-collect the spectrum. Figure 4.19 overlays spectra of a multivitamin tablet collected at 10 and 20 kV. Note that Cu and Zn K line peaks are not present in the 10 kV spectrum but are clearly observed in the 20 kV spectrum.

At this point in the examination, it is appropriate to do a thorough qualitative analysis and I do this manually. First, identify the major elements as determined by peak height. This means accounting for all possible lines associated with that element. If a major peak is identified as a Kα line, then there should be a Kβ one at the appropriate location and of the appropriate height and shape. The same goes for any L lines and M lines. We have to account for sum peaks and escape peaks (more on these in the next paragraph). Then move on to the minor elements and proceed as above. We do not have to worry as much about sum or escape peaks but we now have to be concerned about not detecting all of the smaller peaks associated with

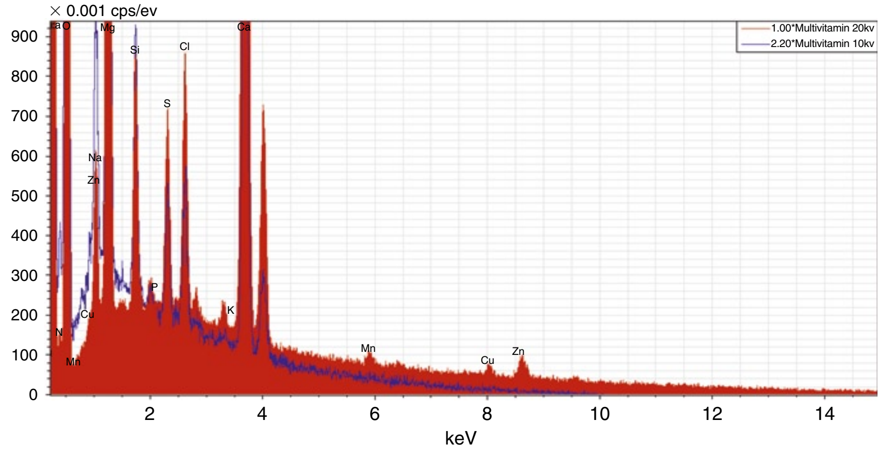

Fig. 4.19 EDS overlay of multivitamin collected at 10 and 20 kV. Note that the Cu and Zn K lines are not present in the 10 kV spectrum but are present in the 20 kV spectrum

the element. Once we have identified the minor peaks and marked the lines, we move to the trace elements. Trace element identification is tricky as you might expect and the identification can be somewhat uncertain – particularly if the small peak is in the low-energy region (0–5 kV). It can be quite difficult to be certain whether the line is associated with k, l, or m lines. That is one reason it is important to account for all the lines from the major and minor elements.

If you intend to do quite a bit of EDS or are doing critical work, I recommend that you read some texts on EDS artifacts (Goldstein et al. 2003; Lyman et al. 1990). It is well worth the time and trouble. It is even better to attempt to generate such artifacts yourself so that you know what they look like with your system. I am only going to discuss two of the important artifacts referred to as "Sum" and "Escape" peaks. Even though we work hard to ensure that we are counting and measuring one X-ray at a time, it is possible that we will detect and measure two of the same element X-rays simultaneously which results in 'Sum' peaks. Clearly, this phenomenon occurs more often with major elements with high X-ray intensities and less often with minor and trace elements. Let us take Ca as a practical example since calcium phosphate is a common excipient in tablets. The Kα line for Ca is at 3.69 keV. The Ca sum peak would be present at 7.38 keV (Ca + Ca). Escape peaks are due to the possibility that some of the detected X-rays will knock out a Si electron from the EDS detector material and will be counted. This sort of peak also is primarily a concern with major elements and is evident as a peak that has less energy than the primary peak by the energy of Si Kα which is 1.74 keV. So, for our Ca example, we would expect to see an escape peak at 1.95 eV for Ca. Some escape and sum peaks may overlap with other elemental peaks. Figure 4.20 shows a spectrum of calcium carbonate collected under conditions designed to generate sum and escape peaks.

The limit of detection for any particular element will depend upon a host of factors including overvoltage and possible elemental overlaps. If you need a minimum value in order to determine whether EDS is capable of detecting to the desired limit, then use 0.1 wt %. In other words, if you get a request to detect an element at the 0.01% level, then it is quite improbable that EDS will be capable of that level. It is possible to do a little bit better than 0.1% detection and quite possible to do worse. As a general rule, the detection limit is inversely related to the number of times the analysis is conducted, the line intensity, the analysis time, and the peak-to-background ratio (see Ziebold 1967 and Lifshin et al. 1999). In other words, the larger those values, the lower the detection limit.

4.4.3 Quantitative Analysis

Quantitative analysis with SEM/EDS is not for the faint of heart. Neither the SEM nor the EDS system is truly designed with quantitative analysis in mind. Since we rarely need to use the SEM/EDS for quantitative work in pharmaceutical development, the inherent difficulties in quantitative analysis are not of much concern to us.

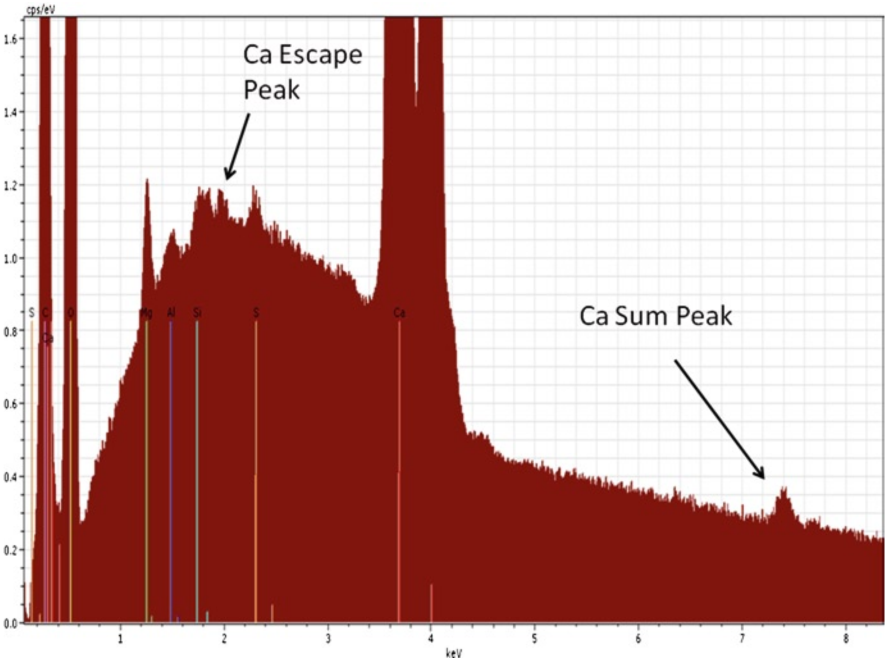

Fig. 4.20 Ca escape and sum peaks. An EDS spectrum collected under conditions (high dead time) designed to produce escape and sum peaks. Even under these conditions the peaks are small with a silicon drift detector. It is easy to see how these peaks can be mistaken for trace level elements and one should always check for the possibility of sum and escape peaks when doing qualitative analysis

Even so, I am going to present a small amount of information on the subject since you may be required to do some quantitative analysis and since knowledge of the subject is helpful for a good understanding of EDS as a whole.

The number of X-ray counts in an elemental peak is directly related to the concentration of that element in the specimen. Unfortunately, it is not possible to simply ratio peak intensities and get good quantitative values. For that reason, it is not a good idea to attempt to approximate the amount of each element present by visually comparing peak heights or even peak areas. There are a number of factors that impact the peak height and area including absorption, overvoltage, and X-ray yield.

The most common and basic quantitative correction technique is referred to a ZAF and is based on the following equation:

$$C_{\text{specimen}} = \frac{I_{\text{specimen}}}{I_{\text{standard}}} ZAF. \tag{4.4}$$

Where C is the concentration, I is the X-ray intensity, Z is a correction due to atomic number, A is an absorption correction, and F is a fluorescence correction. It is the atomic number and absorption corrections that make it impossible to approximate quantities using peak heights or areas. For quantitative analysis, we use a standard of the element of interest and compare its intensity with that of the specimen and then correct for the ZAF matrix effects. The standard can be a 100% (full) standard or can be part of a mixture so long as the amount is well known. Also, be aware that there are other correction techniques that might work better for your application than does the ZAF technique. This approach (measure an elemental standard using strictly controlled operating conditions, measure the specimen under exactly the same conditions, and modify the ratio using ZAF corrections) is referred to as the "Full" standards approach to quantitative analysis. There is also a one standard and a standardless approach. The one standard approach has you measure one material and develop some information about your system. You then, in essence, model the expected elemental peak intensities based on this one standard and use those modeled intensities to compare with the measured ones.

The standardless method of quantitative analysis is quite popular and it is hard to get a commercial instrument that does not have standardless analysis as a prominent feature of the software. Many of us who have experience with quantitative EDS are disturbed by this trend and I caution you to tread carefully when using this "oh so easy" set of quantitative buttons. The reason that we are cautious is the possibility for large error in the results. Errors as great as 100–200% can result when using the standardless routines (Newbury 1999). Standardless analysis is similar to the one-standard approach except that the intensity modeling is based solely on the input parameters for the SEM and for the EDS without any connection with actual performance. It is easy to see how large errors can result from so many assumptions. Standardless analysis was originally developed for quantitative analysis of steels and actually can work reasonably well in that application. It can also work reasonably well with other systems, but you must be diligent in testing the results. If standardless works well for a set of standards you have made then you may proceed but cautiously to test unknowns within that class of compounds and within the composition limits of your standards. I highly recommend against reporting standardless quantitative results for complete unknowns. For instance, when doing a contaminant analysis, I would be very nervous about reporting any kind of quantitative data from a standardless program.

It is not good analytical practice with quantitative EDS to report normalized results. One of the best checks of the correctness of the quantitative result is whether the total amount sums to near 100%. If not, then our ZAF corrections may be in error or we may have missed an element. That is another problem with one-standard and standardless analysis – they are forced to normalize.

There is another method of doing quantitative analysis that has some limited applications to our industry and that is called the peak-to-background method (Statham 1978; Griffin and Nockolds 1996). In this method, you measure the peak-to-background ratio for the elements of interest in a number of standards of varying percentages and generate a calibration curve. You can then collect your unknown spectrum under the same conditions (it is handy to count until you achieve the correct background) and use the measured P/B ratio and your calibration curve to determine amounts. This technique works best if the elements of interest are minor constituents and the matrix elements in the standards are the same as the unknown. For example, I was able to use this technique effectively on solid-phase synthesis beads in which I was measuring Cl, S, and Br in amounts of 2–10% in polystyrene beads. These beads were used for combinatorial chemistry in drug discovery. The technique worked well just because more than 90% of the material was the polymer matrix (Carlton 2000).

High quality quantitative analysis requires that the sample be perfectly flat, infinitely long, and infinitely thick (Infinity as viewed from an electron!). This sample requirement holds for all types of analysis whether it be full standards, one standard, or standardless. If you are evaluating any other type of sample, the measurement accuracy will be poor. You simply cannot accurately measure small particles standing proud on a substrate. The reason is that the equations that govern the quantitative analysis assume infinite thickness and flatness and extension. For instance, a key parameter in the correction procedures is the distance that the beam electron travels in the specimen before being fully absorbed. With particles, the electron may actually escape from the side of the particle before it is absorbed thereby reducing the expected X-ray yield. Be very, very cautious when reporting quantitative results from anything other than flat samples.

It is necessary to measure the beam current with a Faraday cage and a picoammeter when doing full standards analysis and a good idea with one standard and standardless techniques. The full standards comparison requires that the beam current be the same for the unknown as for the standards. Goldstein et al. (2003) and Lyman et al. (1990) present all the necessary details about full standards analysis.

Finally, it is a sign of good analytical practice to report analytical uncertainty (or the error) with quantitative results. The most common way of doing this is to measure the sample multiple (5–6) times and reporting the mean, standard deviation, and the 95% confidence limits.

I hope that I have succeeded in making you nervous about doing quantitative analysis with the SEM. I know I am. It can be done, but you really have to work hard at it.

4.4.4 Elemental Mapping

One of the more popular and powerful techniques in EDS is elemental mapping. In this technique, the location of each element in the sample is represented by a different color on the image. This elemental map is often superimposed onto the original secondary electron or backscatter electron image. Figure 4.21 shows an example of an X-ray map for a generic multivitamin cross-section with C, Mg, Si, Cl, and Ca represented by different colors overlaid on the backscattered image. In practice, you (or an automated function of the software) set an energy region-of-interest (ROI) that encompasses the energy of the element of interest. For example, for Ca the Kα line is at 3.7 keV and one could choose a ROI from 3.5 to 3.9 keV. Each time an X-ray is detected with that energy a pixel is turned on at the location of

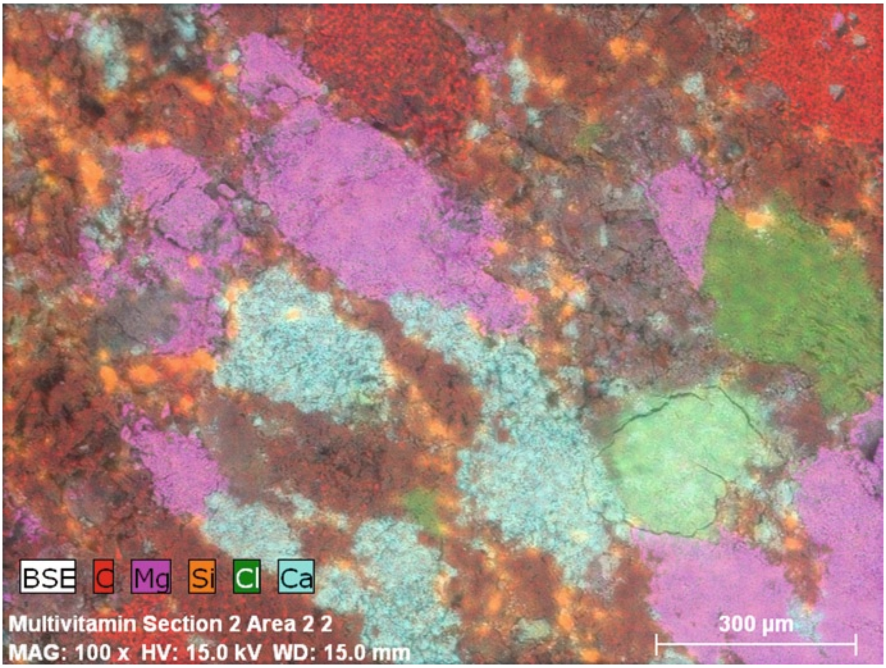

Fig. 4.21 EDS elemental map of a multivitamin cross-section. Each element is colored differently and then combined into a color coded map. For instance, the light blue regions have a heavy concentration of Ca whereas the magenta regions have a high concentration of Mg. These maps allow one to assess the distribution of different elements and compounds within the tablet. (Image size = 1,284 × 1,008)

the electron beam on the specimen as represented on the computer monitor. When another X-ray is detected with that energy at that location on the specimen, the intensity of the pixel is increased. Intensity is accumulated so that the brightness of the pixel is directly related to X-ray energy intensity. Therefore, a feature of the specimen that is rich in a particular element will have many pixels turned on in the feature area. If you collect enough X-rays then it is possible to begin to discern the size and shape of the features containing that particular element. The pixel can be white or any other color and is selectable by the operator.

Clearly, there are many possible applications using the elemental and molecular distribution within a specimen. In the pharmaceutical industry, the primary application is to tablet cross-sections and to granule cross-sections. One use of such a map is to assess the distribution of some excipients that are known to affect dissolution rate. Magnesium stearate is often added to tablets as a punch and die lubricant. Too much magnesium stearate can inhibit dissolution and whenever there is a dissolution problem, it is very common to be asked about the distribution of the magnesium stearate within the tablet. EDS elemental maps can be used to visualize the distribution based on a magnesium map. Another compound that is frequently implicated in tablet problems is sodium starch glycolate. Again we can visualize the distribution using a sodium map. Another use of maps that has not been widely applied is in validation of particle size measurements within the tablet itself. We mostly control particle size of the input drug substance since it is quite difficult to measure particle size of the drug substance particles within a tablet or capsule. We assume that the particle size is mostly retained through the tablet or capsule or manufacturing process and that is usually true most of the time and particularly for particles that are mostly less than approximately 100 μm in diameter. There are occasions though where it would be useful to be able to correlate the DS particle size in the tablet or capsule to that measured by other means. In one case, we were able to demonstrate with an EDS map that the rather large (100s of micrometers in diameter) agglomerates retained their size throughout the tableting process. The size of the DS agglomerates in the tablets was consistent with that measured by laser diffraction. Naturally, this would not be a routine test, but it was useful as part of the method development process.

Elemental maps are surrounded by quicksand. It is really quite easy to misinterpret a map due to a number of different artifacts and pitfalls. First, it is quite important to have a flat sample. Remember that the EDS detector is line of sight and if a feature protrudes above the surface, features on the far side of the protuberance will not be detected accurately. When we set up regions-of-interest, we assume that there will be a reasonably large elemental peak at that energy value. If there is no elemental peak, we may just detect the continuum radiation and X-rays will be detected but not necessarily from the element of interest. X-rays can also be emitted some distance from the location of the electron beam. Consequently, the spatial resolution is such that we may not be able to draw conclusions about very small areas. The intensity of the pixels is under our control and we can and should adjust it for ease of visualization. That adjustment can lead to artifacts. Finally, it is common to treat the images with an image analysis program to smooth some of the features, particularly if we are going to make feature measurements. Image processing can be useful but should be handled with care.

The requirement of flat samples can be difficult to satisfy. I generally use a microtome to produce a flat tablet cross-section. For pharmaceuticals and SiLi detectors, decent maps at a reasonable image resolution can take considerable time. For some applications, I have collected maps of one image for more than 4 h. I know some workers who collected maps overnight – for more than 12 h (you generally need a image drift correction for those long collection times). The SDD detectors have cut that time down considerably and what would take a couple of hours to collect with a SiLi now take 15–20 min with a SDD. The ability to collect maps in much shorter times allows us to begin to consider meaningful feature measurement of multiple EDS maps.

There are many other considerations to elemental maps that are covered well in other texts. If you are considering doing maps, I strongly urge you to practice with known samples before attempting unknown samples. I recommend beginning with a sample made by impressing small bits of different metals into holes you have made in an aluminum stub. For instance, make a small hole in the stub surface, place a small bit of copper into the hole and flatten the copper using a press. By putting a number of different metals next to each other, you can make a nice test sample for mapping. I would experiment with making maps of that sample and looking for artifacts and learning how to use the mapping software appropriately. Next, I recommend that you practice with a multivitamin tablet as outlined in the exercise below. Once you are comfortable with these samples, I think you are ready to progress to "real" samples.

4.4.5 EDS with Low-Vacuum SEM

Back in the early 1990s when I took ownership of my first ESEM, it was received wisdom that EDS in the ESEM was impractical. Not being overly fond of received wisdom, I did a Master's thesis on qualitative EDS analysis in the ESEM and a doctoral dissertation on quantitative analysis in the ESEM (Carlton 1999, 2000). The received wisdom was not correct, but the level of effort required to establish that fact was worthy of a couple of degrees. My point is that although EDS is possible in the ESEM, be acutely aware of the problems caused by the interaction of the beam electrons with the gas vapor producing what is termed as an electron skirt extending quite far from the location of the beam electrons. Of course, there were many other people who did as much and more than myself to establish EDS in the ESEM, most notably Eric Doehne (Doehne and Bower 1993; Doehne 1997) at the Getty Institute, Scott Wight at the NIST, Bilde-Soerenson and Appel (1997) in Sweden, Raynauld Gauvin (1999) in Canada and Brendon Griffin (with Nockolds 1996) in Australia among many others.

The primary complication of EDS in low vacuum SEM is the spread of the electron beam by gas vapor atoms in the specimen chamber. Remember that the working distance of the specimen for EDS is set at 10–15 mm so that the beam electrons have that distance to traverse before striking the specimen target. With a reasonable pressure in the chamber of 100–200 Pa, the electron skirt can then extend tens and hundreds of micrometers and even many millimeters away from the target spot (Carlton 1997). That means that X-rays are going to be produced quite some distance from the feature of interest unless you are looking at millimeter-size features. A number of correction techniques have been developed and all depend upon the fact that X-ray counts for elements in the feature being examined will increase with decreasing chamber pressure, whereas X-ray counts will decrease for elements in features outside the feature of interest (Carlton 2000). Figure 4.22 is a graph showing this relationship for glass beads (NIST SRM 2066) embedded in lead. Note the direction of the curve for lead versus the direction of the curves for the elements in the glass. It is well beyond the scope of this book to delve into all the mysteries of EDS in the ESEM (although a part of me is aching to do so). I do want you to be aware of some of the advantages and pitfalls since EDS in the low-vacuum SEM is commonly used in pharmaceutical development.

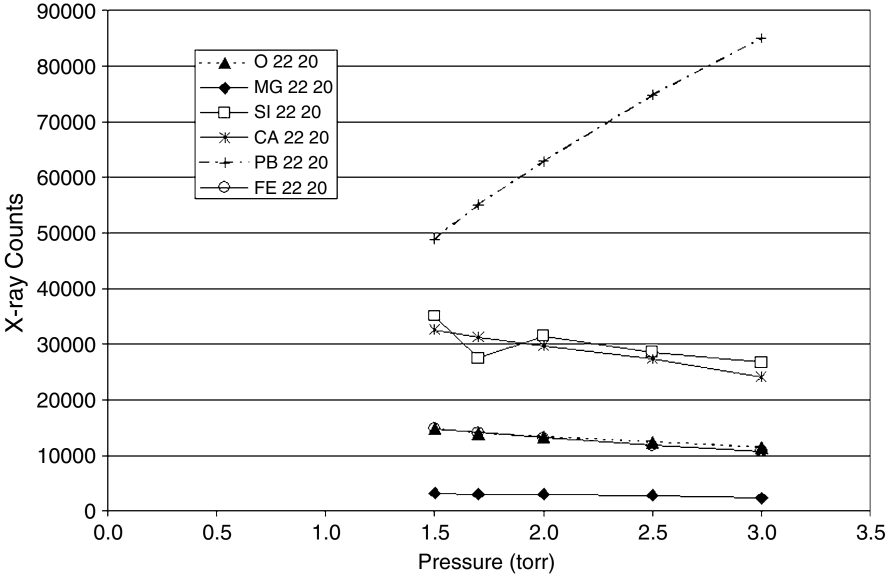

Fig. 4.22 X-ray counts vs. chamber pressure for glass beads embedded in lead. Glass beads of NIST SRM2066 are embedded in lead and tested at various chamber vapor pressures. Note that the lead X-ray counts increase with increasing chamber pressure whereas, in general, the glass element (Si, O, Mg, Ca, and Fe) counts decrease with increasing pressure. These relationships demonstrate the effect of the electron skirt on X-ray microanalysis (from Carlton, 2003)

First, the size of the electron skirt is directly related to gas path length, to chamber pressure and indirectly related to accelerating voltage. In other words, you can minimize the size of the skirt by reducing the gas path length, by reducing the chamber pressure and by increasing the accelerating voltage. The gas path length, of course, is related to the working distance and is defined as the distance from the bottom of the pole piece to the specimen. That working distance is set for EDS. One manufacturer that I know of, though, provides an adapter that reduces the effective gas path length. This adapter fits on the end of the pole piece and extends to within 2 mm of the specimen height (for the EDS working distance). This device has enormous effect on reducing skirt effects and I wish all the SEM manufacturers had such adapters available. The chamber pressure can be reduced quite a bit more than you might imagine if your primary purpose is to reduce charging and not to produce an image with the electron cascade. If you image with a backscatter electron detector it is possible to nearly eliminate charging effects with as little as 50–90 Pa even on tablets. You can also reduce the beam skirt by using an accelerating voltage as high as 20 kV, although that has some deleterious effects on light element EDS and so it is probably better to stick with 10 or 15 kV. As a side note, I used a metallic ring above the specimen grounded through a picoammeter to measure the charge on the specimen surface. I adjusted the voltage to the secondary electron detector so as to just balance the charges so that the specimen surface was neutral. I recommend this approach for critical work (Carlton et al. 2004).

So let us suppose you have minimized the beam spread as much as possible, how do you proceed with qualitative analysis? I recommend collecting spectra at two different pressures, say 50 and 100 Pa and compare the spectra. Peaks that are larger with lower pressure are usually in the feature of interest whereas those that are smaller with lower pressure are probably outside of the feature of interest. For important work, you may want to collect spectra at 4 or 5 pressures, determine the counts in each peak (minus background) and plot counts versus pressure. You can then extrapolate (either directly by drawing a line through the points on the plot or by using linear least squares regression) to zero pressure. Elements contributing X-rays from outside the field of interest should go to near zero counts at zero pressure. I do not recommend doing quantitative analysis in the low-vacuum SEM for pharmaceutical applications. If you need to do so, then consult the literature for application of the different correction techniques.

A problem obviously exists with the recommended approach. What if the same element is present both in the feature of interest and outside the feature? It is possible to get a false negative by collecting two spectra at different pressures and superimposing. It will look like the peak is decreasing with decreasing pressure and so you may conclude it is outside the feature of interest. If you suspect such a situation, then collecting spectra at a series of pressures and plotting the X-ray counts against pressure and then extrapolating to zero pressure is the best approach. If the plot of the suspect element does not go to zero, then it may be present in the feature.

On a practical note, since I mostly use the EDS for contaminant analysis and for mapping, the skirt poses few problems in day-to-day analysis. Typically, I am looking at a contaminant region for suspect elements. The elements contributed from outside the feature of interest are likely to be those of the tablet or expected components of the dosage form. Any unexpected elements are likely to be from the contaminant. The beam skirt has little influence on mapping. If you take a map of an element outside the field of view, it will be completely diffuse and will not be concentrated in any features of interest. In fact, I think this may be an alternative way of checking to see if the element is in the feature of interest. If you take a map and the elemental distribution is homogeneous then it is probably outside the field of view.

4.5 Summary

I hope you can see that SEM and EDS are important analytical tools in pharmaceutical development. It is true that you can do reasonable work with a cursory understanding of these techniques. I think, though, you will find a great deal of satisfaction and you can make important contributions to your company by studying and learning the subjects in more depth. The following exercises can help you develop some better understanding. Full competency, though, requires personal study, training from experts, and experience.

4.6 Exercises

Naturally, I assume that you have instrumentation available to use for these experiments. While I think it is possible to learn SEM imaging on your own by reading and experimentation, I do not think you can learn EDS qualitative analysis on your own. I am very skeptical that you can learn EDS quantitative analysis on your own. If you plan to do any EDS, get training from University and company courses.

A. Sample Preparation

A1. Powder on Stub

Take a small specimen of carbamazepine and deposit the powder directly onto double-sided carbon tape on an aluminum stub. Coat the specimen with metal such as gold-palladium. Prepare another specimen in the same manner but do not sputter coat this specimen. Examine both specimens using a stereomicroscope. Note the shape of the particles and the proximity of one particle to another. Keep the specimens for the imaging exercises.

A2. Powder in a Bottle

Obtain a bottle with lid that is approximately 4 in. tall and 3 in. in diameter (larger is ok but smaller may not work as well). Make a number of small holes in the lid. Place an aluminum stub (with double-sided carbon tape on the surface) in the bottom of the bottle. You may want to place the stub on a small platform and keep it in place with double-sided tape. Now place a small amount of caffeine or carbamazepine in the bottom of the bottle at the corners. Now close the lid. Use a gas bottle such as is used to clean computer keyboards and blow some gas into the bottle. The gas should aerosolize the powder which will then fall onto the double-sided carbon tape. Examine the stub with a stereomicroscope. If you did not achieve an even distribution and sufficient particle loading, then redo the sample preparation. Keep the specimen for examination on the SEM.

A3. Filtration

Prepare a suspension of caffeine in a suspension of cyclohexane (add a small amount of surfactant such as polyethylene glycol if needed). Sonicate the suspension for 30 s. Filter a small aliquot of the suspension through a 1 or 5 μm Nucleopore™ filter. Mount the filter onto double-sided carbon tape on an aluminum SEM stub. Examine the filter using a stereomicroscope. If the particle loading and distribution is good, then coat the specimen with a metal coating. This preparation technique requires some practice in order to produce good specimens. Also use a mortar and pestle and gently grind some of the caffeine and prepare a filter with that material. Mount on the SEM and compare particle appearance.

B. Secondary and Backscattered Electron Imaging

B1. SE and BSE Imaging Comparison

Put the coated sample prepared in Exercise A1 in the SEM. Examine the particles using the secondary and backscattered electron detectors. Alter the working distance (5–15 mm) and the accelerating voltage (5–20 kV) and notice the difference in appearance of the particles. Use a short working distance, 15 kV accelerating voltage and secondary electron detector to image particles at high resolution (~5,000–10,000×). Switch to backscattered electron detector and note the difficulty in achieving good focus at these magnifications.

B2. Elemental BSE Imaging

One of the simplest test samples for BSE imaging is an aluminum SEM stub with double-sided carbon tape and either a copper wire or better copper tape. If you make a cross-shape with the carbon tape and copper tape, the junction of the two strips gives you three materials with very different atomic numbers. Image this junction using BSE and note that the brightness is directly related to the atomic number. Another good test sample is double-sided carbon tape on an aluminum stub with glass particles embedded in the tape. Again you get three materials with very different atomic number contrast. Finally, prepare a cross-section of a multivitamin tablet either using a microtome or using a razor blade. Mount the cross-section onto double-sided carbon tape on an aluminum SEM stub and place in the SEM at low vacuum. Image in BSE mode and note the contrast differences in the various regions of the tablet. Keep this sample since we will be using it in later exercises.

C. Low-Vacuum SEM

Use the multivitamin sample prepared in B2. Begin by imaging with the BSE detector at moderate chamber vapor pressure. The exact pressures will depend upon the particular SEM but for most low-vacuum types, set the pressure at ~50 Pa. Obtain a good image. Now increase the pressure to ~90 Pa and note the differences in appearance. The image contrast will suffer and it will not be as easy to achieve good focus. Drop the pressure to 20 or 30 Pa and note the improvements in image quality. Repeat this exercise with a secondary electron, if available on your instrument. Note that image quality goes through a maximum at some pressure and that above and below that value, the quality suffers. Drop the pressure until you have either charging or a loss of image. This is the point at which the electron cascade fails.

D. EDS Qualitative Analysis

D1. Miscellaneous Specimens

There is really no substitute for collecting a large number of spectra from a variety of different materials. You can begin with calibration standards such as copper and aluminum and progress to steels and glass. Practice altering kV, beam current (spot size), and final aperture size. Note the changes in total count rates. Practice elemental identification on materials with some small amounts of a few elements. Minerals are often quite good for this type of practice. Talc is a good sample for practice. I also recommend collecting some dust from home and from the laboratory and attempt identification of the various particles. This exercise can be quite useful when it comes to identify unknowns.

D2. Multivitamin EDS

Mount the multivitamin cross-section prepared in B2 in the SEM. Use 15 kV accelerating voltage and ~50 Pa chamber vapor pressure with the BSE detector. Choose an area with a high degree of contrast with many light and dark areas. Collect spectra from each of these areas. Pay particular attention to areas with very bright, high contrast small regions. These may contain high atomic number elements such as Cu, Zn, and Fe. Attempt to connect the elemental assignments that you have made with the molecule listing on the label.

E. Elemental Mapping

Mount the multivitamin sample as described in D2. Collect elemental maps for all of the elements you identified in that exercise. Practice collecting maps with different resolutions and with different times. Note that the quality of the image improves with high resolution and long times, but also note that often there is little to no additional information contained in these maps compared with the shorter time and resolution maps. If the work is to be published internally or externally, it may be important to collect high quality maps, whereas if the information is to be used simply for analysis, it may be better to collect more maps with shorter times.

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Chapter 5
Infrared and Raman Microscopy

5.1 Introduction

Infrared (IR) and Raman spectroscopy are invaluable tools in pharmaceutical development since both yield information on the chemical structure of pharmaceutical molecules. IR and Raman are used for drug identification, for determination of molecular structure (along with nuclear magnetic resonance, mass spectrometry, single crystal X-diffraction, among other techniques), for solid-state analysis, for contaminant/particulate identification, and for chemical imaging of pharmaceutical dosage forms. IR and Raman microscopy are generally applied to the last three areas.

IR and Raman are often discussed together, as I am doing here, since the applications are similar and the resulting spectra are similar. It is good to keep in mind that they have very different physical origins. Infrared spectroscopy is based on the absorption of infrared radiation by molecules due to vibrational states of particular functional groups in the molecule. Raman spectroscopy is based on the inelastic scattering (energy transferred in scattering process) of light by the molecule again due to specific vibrational states of particular functional groups in the molecule. The different physical origins of the IR and Raman effects result in some bands (due to specific vibrational states) that are active in the IR but not the Raman and vice-versa. Generally, though, a pharmaceutical compound will have either a distinctive IR or Raman spectrum or both. Consequently, Infrared and Raman spectroscopy should be considered complementary techniques in pharmaceutical development.

Ask a spectroscopist, and he or she will say that a microscope is a useful accessory to the spectrometer. Microscopists know better since, clearly, the spectrometer is a most useful accessory to a microscope. In either perspective, there is a happy marriage between microscopy and spectroscopy and it is possible to do some very good work with IR and Raman microscopes (see Reffner and Martoglio 1995). In fact, I believe that most spectrometers sold to pharmaceutical R&D have a microscope attachment. The prime advantage of IR and Raman microscopy is the ability to obtain chemical information from a discrete and known location on a sample or of a small particle. Also, it is possible to get chemical information on individual small particles that can aid in polymorphism examinations.

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Clearly, to get the most from the instruments it is necessary to be skilled at both microscopy and spectroscopy. While you can get some valuable information from a cursory study of microspectroscopy, it requires quite a bit of study and even more practice to become proficient at the techniques. In this chapter, I am going to present an introduction: to the theory behind each type of spectroscopy; to the types of available instruments; to sample preparation; and to applications. I will end with a few exercises that can help you get started in this field. I find immense satisfaction in doing IR and Raman microscopy and recommend it to you both for its utility in our work and for its possibilities for personal satisfaction.

5.2 Infrared Microscopy

5.2.1 Theory

We can categorize the internal energy of a molecule into translational, rotational, vibrational, and electronic energies. When a molecule is irradiated with electromagnetic radiation, the energy can be absorbed by the molecule by exciting any one of these energy categories. IR and Raman spectroscopy are related to vibrational energy and, to be specific, infrared radiation of a specific wavelength and energy will be absorbed by specific types of vibrations by specific functional groups in the molecule. For example, the carbonyl functional group has a strong absorbance band at 1,550–1,900 cm^{-1} due to the stretching vibration of the C=O bond. Since molecular vibrational bands are directly dependent on molecular structure, we can compare the energy spectrums of the irradiating and emitted light to determine molecular structure from absorbance (or transmittance).

Only when the irradiating light energy is matched with the vibrational energy of the functional group will light be absorbed. By irradiating a sample with light having a wide range of infrared energies (or sequentially irradiating with monochromatic light), we can produce a spectrum relating absorption (or transmittance) to energy. Figure 5.1 shows a typical IR absorbance spectrum for carbamazepine Form III. The infrared portion of the electromagnetic spectrum extends from 10 to 14,000 cm^{-1} (various authors have various limits but these limits encompass all authors). Infrared radiation is generally divided into three categories: far IR, 10–400 cm^{-1}; mid IR, 400–4,000 cm^{-1}; and near IR, 4,000–14,000 cm^{-1}. IR microscopy utilizes the mid-IR range. Near-IR spectroscopy has become popular in the pharmaceutical industry in recent years because of its ability to collect spectra from whole tablets and other dosage forms. I have seen some articles on near-IR microscopy, but I do not believe the technique has routine uses in pharmaceutical microscopy as of yet and so I do not discuss the technique in this book.

Early IR spectrometers, and IR microscopes, were dispersive instruments. With these instruments, a grating monochromator is used to select individual wavelengths of light to irradiate the specimen in a sequential fashion. Later spectrometers, and nearly all IR microscopes today, use what is called Fourier Transform

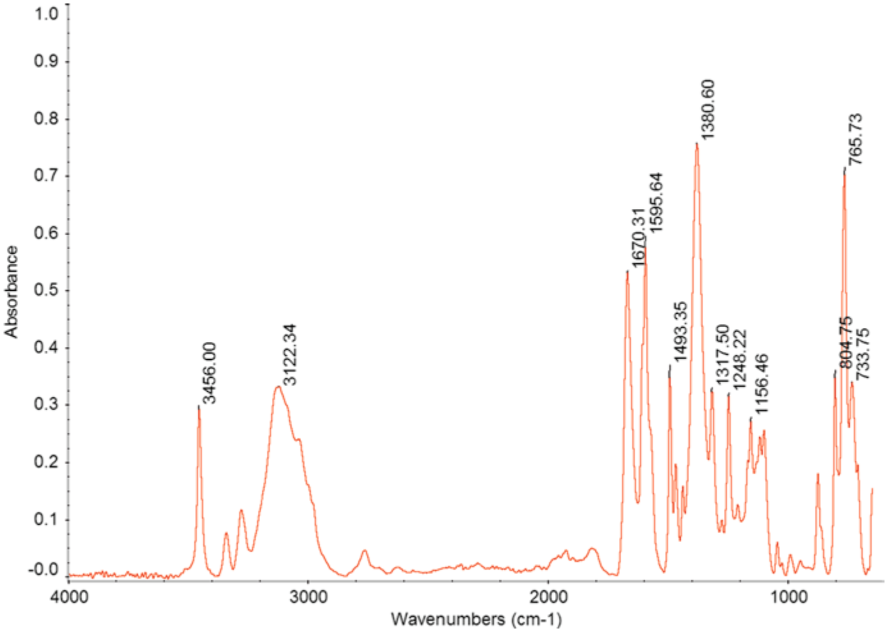

Fig. 5.1 IR spectrum of carbamazepine form III. This figure presents a typical IR spectrum of a pharmaceutical compound. There are two regions of interest: 3,600 to 3,000 cm^{-1}, and 1,600 to 500 cm^{-1}. The first region is primarily used to determine hydrogen bonding and the presence of water whereas the latter region is termed the fingerprint region since it often is distinctive for a particular compound or polymorph. All IR spectra presented in this chapter were collected by co-adding 128 scans at a resolution of 4 cm^{-1} using an IlluminatIR™ IR microscope with an ATR objective

IR (FTIR) spectroscopy (Griffiths and de Haseth 2007). In this instrument, the specimen is irradiated with broadband illumination through a Michelson interferometer. The resulting interferogram is operated on by a mathematical operation called a Fourier Transform that yields frequency information and the type of spectrum shown in Fig. 5.1. Figure 5.2 shows a schematic representation of an FTIR microscope attachment. Dispersive and FT instruments each have distinctive advantages and disadvantages, but two advantages of the FT technique for microscopy are speed (simultaneous irradiation) and superior low signal performance. The FT technique has better signal-to-noise performance and is better with low-level absorbance than is the dispersive instrument.

Microscopes and IR spectrometers were first connected in the late 1940s using dispersive spectrometers (Reffner and Martoglio 1995). The early IR microscopes were not very popular and their use was restricted to a limited number of applications and university laboratories. The Fourier Transform technique became popular in the 1970s for routine IR spectroscopy and microscopes were connected to these instruments in the 1980s. FTIR microscopy has enjoyed a great deal of success since that time and is a common instrument in modern industrial laboratories and, in particular, in pharmaceutical development labs. There have been a number of

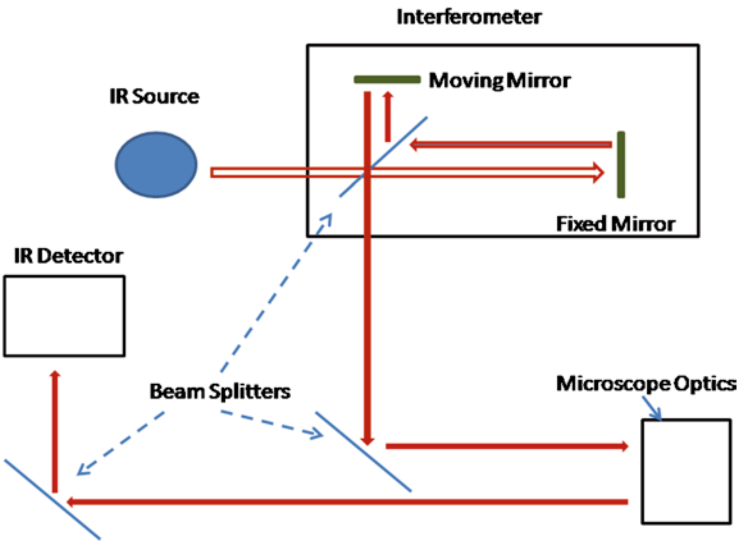

Fig. 5.2 Schematic representation of an FTIR microscope attachment (*view from top down*). This type of spectrometer is designed to fit onto the top of a regular microscope with infinity-corrected optics. This arrangement allows for both high-quality microscopy and spectroscopy. All IR spectrometers for microscopes are composed of similar elements: IR source, interferometer, optics to direct radiation to and from the sample, and a detector. The detector is coupled to a computer (not shown) for the Fourier Transform and for spectrum display

important advances in IR microscopy over the years, but one of the most notable is the IR attachment to a regular microscope, i.e., a small box fitted on the top of the microscope. The original IR microscopes were specially designed to fit a particular IR bench. The microscope was then just another sampling accessory such as diffuse reflectance or attenuated total reflectance attachments. With the advent of infinity-corrected optics for the optical microscope, it is now practical to use IR as an accessory to an optical microscope like reflectance or fluorescence attachments. This arrangement has, in my opinion, simplified IR microscopy and we get the best of both microscopy and IR spectroscopy.

5.2.2 Instruments

The FTIR microscope has the following components: IR source, interferometer, microscope, IR objectives, IR detector, and computer for the Fourier Transform operation, instrument control, and spectrum display (Fig. 5.2). Most systems also have a camera for photomicrography. The source provides broadband IR radiation that is directed to the interferometer. The interferometer splits the radiation sending part to the sample and part to a mirror of known distance. The beam going to the specimen is generally limited by an aperture so as to collect radiation from the specimen alone and to reduce stray radiation from outside the specimen. The beams are combined and interfere when recombined. The interferogram is focused onto the detector (e.g., a mercury telluride/cadmium telluride (MCT) detector cooled to liquid nitrogen temperatures). The resultant digital interferogram is operated on with a Fourier Transform producing a spectrum of absorbance vs. wavenumber. I am going to limit the rest of the discussion to IR optics for the microscope since there are a number of good sources for detailed information on the other components (Griffiths and de Haseth 2007; Colthup et al. 1990).

Objectives for an IR microscope are complicated by the fact that glass absorbs infrared radiation and so is a poor material for IR optics. A number of creative solutions have been applied to this problem and today there are good objectives that can produce both a reasonably good optical image as well as good IR spectra. Objectives for IR microscopy can work with either transmitted or reflected light. Transmitted light optics must have both a special objective and a special condenser. Reflected IR objectives serve both as condenser and as image former. Transmitted light objectives and condensers generally use metal reflective surfaces to focus and transmit the light much like a reflective telescope. These Schwarzschild objectives collect the light with one mirror, focuses it onto another mirror which collimates the light as it sends it to the sample or to the detector.

Schwarzschild objectives were the first type used in the original instruments of the late 1940s and early 1950s and are still in use. They can be used either in transmitted IR work or in reflected work. There are some limitations to magnification and the highest power is generally ~30× (which is true for ATR and other reflected light optics as well). With visible light the image is discernible but not of the same quality as glass optics, although modern Schwarzschild objectives come close to achieving glass objective images. If your material is naturally reflective, the sample substrate is not particularly important. Most pharmaceuticals, though, are not particularly reflective and to use this objective it is best to mount the specimen onto a reflective slide (aluminum, gold, low E glass, etc.) and ensure that the specimen is thin by crushing it or some other technique. This arrangement has the added advantage that absorption occurs both as the IR beam penetrates the sample but also as it reflected back from the substrate.

I think that the majority of the work done with pharmaceuticals today uses reflected light and if there is an IR attachment on top of a normal light microscope, then almost certainly it is operating in reflected light mode. In general, there are four types of reflective IR spectroscopy: reflection-absorption, diffuse reflection, specular reflection, and attenuated total reflectance (ATR). The Schwarzschild objective operates with reflection-absorption. It is also common to use objectives based on attenuated total reflectance. This objective is brought into direct contact with the sample. We get total reflection of light at the interface of the two materials of different (objective and sample surfaces) refractive index at what is called the critical angle. The angle depends upon the refractive index of the two materials and is defined as follows:

$$\theta_c = \sin^{-1}\left(\frac{n_2}{n_1}\right), \tag{5.1}$$

where, in our case, n_2 is the sample refractive index, and n_1 is the refractive index of the objective material generally diamond or zinc selenide (ZnSe). Diamond has a refractive index of 2.4 and most organic materials have an average refractive index of 1.6 so the ATR objective requires a critical angle of ~40°. The ATR objective works even though there is total reflectance of the light because some light protrudes below the surface due to the wave nature of light and this is called the evanescent wave. It is the evanescent wave that interacts with the specimen with resulting IR absorbance. The evanescent wave penetrates the specimen according to the following equation:

$$d_\mathrm{p} = \frac{\lambda}{2\pi n_1[\sin^2\theta - (n_2/n_1)^2]^{1/2}}, \tag{5.2}$$

where λ is the wavelength of light and n_1, n_2, and θ are as defined previously. Using this equation, the penetration depth is normally around 500 nm. This is a useful equation to have handy and programmed in your handheld calculator or in a spreadsheet since we are often asked about penetration depths. It is easy to see though, that there must be intimate contact of objective with slide in order to get a good signal since the evanescent wave only protrudes a half-micrometer or so below the objective. Figure 5.3 shows a schematic drawing of a typical ATR objective.

5.2.3 Sample Preparation and Testing

The quality of IR spectra is much influenced by sample preparation. In some cases, sample preparation is relatively simple such as for ATR spectra of polymers. Generally, these samples can be imaged directly by placing the polymer on a stiff

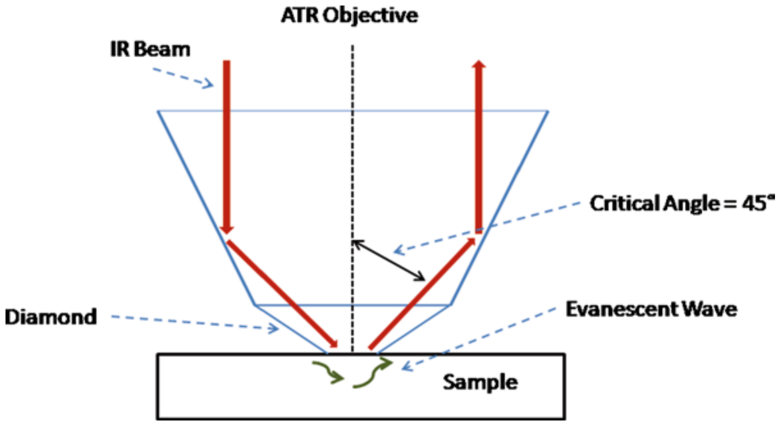

Fig. 5.3 ATR objective for IR and visible light. The ATR objective is used frequently for pharmaceutical applications

substrate such as a glass microscope slide. On the other hand, if we are dealing with very small particles in a parenteral, it may be quite difficult to isolate the particle and then mount it on an appropriate substrate. The details of the sample preparation depend upon the sample itself and the type of IR objective used in the analysis. For soft samples with an ATR objective, it is sufficient to mount the sample directly onto the glass microscope slide and then use the pressure of the objective to produce a flat sample that results in intimate contact of sample and objective across the whole face of the objective. Glass slides are good substrates and readily available in the microscope lab, but they break easily. I think everyone who has ever used an ATR objective has at some time has broken a glass slide. There are any number of other substrates that can be used because the nature of the substrate is not critical to collecting good spectra. As a practical matter, I look for absorbance in the range of 0.3–0.8 for the best spectra. Lower absorbance generally leads to unacceptably high noise levels and higher absorbance often leads to broad peaks. In talking with other spectroscopists, the target absorbance is a matter of preference. If there is a single particle to analyze, then clearly you take what you can get, but if there are a number of particles to chose from, then it may be best to target a specific absorbance range.

The nature of the substrate does make a difference in transmission and reflectance IR microscopy as does the thickness of the specimen. For transmission experiments, clearly the substrate must be as near transparent to IR radiation as possible and so glass is not suitable. In most cases, polished discs of KBr or NaCl are used. These discs are reusable, but are fragile and we can think of them as expendables. We wash with methanol and if there is a little water in the solvent, it removes surface layers of the salt in the cleaning process. This type of cleaning is generally an advantage since we often embed particles in the salt when we flatten the samples. In some rare cases, transmission samples will be sufficiently thin to pass the IR beam, but not so thin that little to no absorption takes place. In most cases, it is necessary to flatten the sample. It is common practice to use a metal roller to flatten particles. The roller works reasonably well if the material is not cohesive and sticky. It is disconcerting to roll your one suspect, contaminant particle and have it attach itself to the roller. For those samples, it may be better to compress the particles with a glass slide or to use a diamond compression cell that is designed for IR experiments. You can compress and then test directly since the diamond transmits IR radiation. I recommend spending some time practicing with sample preparation if you are doing transmission or reflectance IR microscopy since this type of sample preparation is a learned skill. Also, with some practice you begin to recognize particles on your salt cake that have the appropriate thickness. Particles of appropriate thinness have a peculiar translucent appearance that is distinctive.

Reflection IR microscopy has similar constraints on the thinness of the specimen but the substrate must be IR reflective. There are a number of different slides available for this purpose from microscopy equipment suppliers. We can use the so-called low E glass that is designed for transmitting visible light in buildings but restricting IR radiation. Most of these slides have a thin coating of tin oxide which reflects the IR. There are sample holders of aluminum, steel, and gold available as well.

Some of these holders have numbered regions so it is easy to keep track of sample location. The aluminum and gold have easily scratched surfaces and so we must be careful with them and again, to some extent, these slides should be considered expendable. It is useful to practice sample preparation and, as in the case with transmission particles, the appearance of sufficiently thin particles is distinctive.

It is important to be aware of the possibility of solid-state form changes induced by sample preparation. We think mainly of solvent and temperature effects on form change, but pressure can also induce a form change. The most obvious form change due to pressure is from crystalline to noncrystalline, from a highly ordered to a disordered state. There can be obvious changes to the infrared spectrum of such a change as with carbamazepine (see Fig. 5.4, for an example). It is also possible to convert to a metastable form on sample flattening or to make higher pressure forms that only slowly convert to the stable form at room conditions. If the sample is solvated or hydrated, flattening can liberate lightly bound solvent and water, thereby altering the spectrum and sufficiently high pressure may even remove tightly bound solvate and water molecules. The effects of the pressure changes may not be particularly obvious and we may need to be creative about how to determine the possible effects of sample preparation. One approach is to try different techniques and objectives and compare spectra. For instance, we can compare an ATR objective spectrum with a reflectance objective spectrum. Even with this comparison we need to be careful, since we are likely to observe some differences

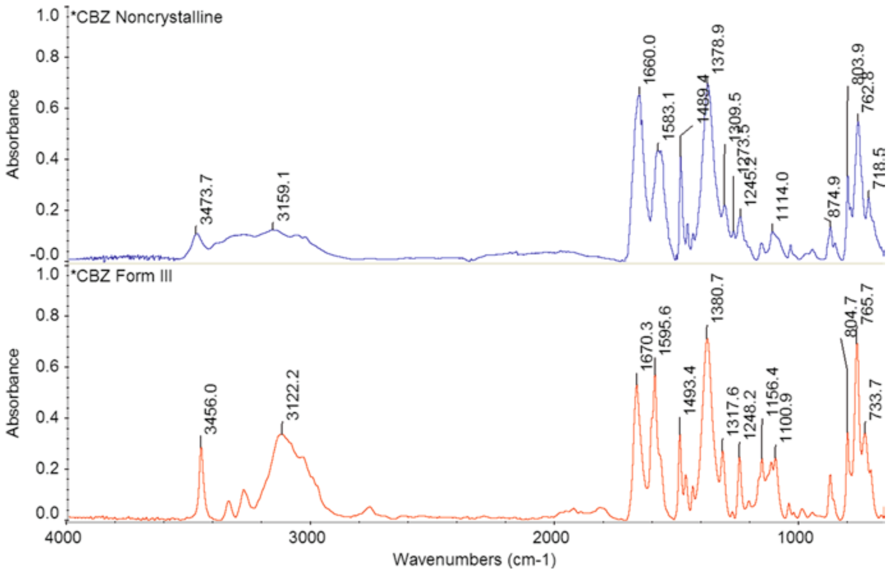

Fig. 5.4 IR spectra of crystalline and noncrystalline carbamazepine form III. The upper spectrum was collected from noncrystalline carbamazepine (CBZ) melt, whereas the bottom spectrum is from crystalline CBZ. Note the sharpness of the peaks of the crystalline CBZ compared with that of the noncrystalline one. Although the effects are subtle, one could draw incorrect conclusions from the spectra if a crystalline sample is rendered noncrystalline in the sample preparation process

due to the objective and the mechanism of radiation collection. It is quite beneficial, in my opinion, to experiment with different sample preparation techniques and to consult with other workers in the field. Most analysts have little tricks of the trade that rarely make their way into the literature.

IR microscopy requires collection of a background. It is good practice to collect the background in a region close to the specimen and under the exact conditions used for the specimen collection. I typically collect one background on a slide and use it so long as I am looking at specimens on that slide close to the background area. If I move far from the original background (>½cm or so), I collect a new background. I definitely recommend collecting a new background with a change of slides. The ATR objective collects a background in a different fashion. The stage is lowered so that the objective is not in contact with a specimen, clean the ATR surface well, and then collect a background. If I am doing critical work, I collect a new background after each specimen test and lens cleaning. For routine testing, I collect a new background for every new specimen (assuming I am doing 3 or 4 tests of each specimen). Usually the manufacturer of the system has recommendations for background collection.

It is important to set certain test settings before collecting the background and sample and these include aperture size, resolution, number of scans, and spectral range. Spectral range is the range of wavelengths displayed on the *x*-axis and generally extends from 4,000 to 400 cm^{-1}, although the default range is somewhat different for each instrument. The aperture size is selected based on the size of the feature to be examined. Some modern instruments have internal apertures that appear superimposed on the image either in the eyepiece or more typically on the computer monitor. Select the aperture so that the sampling spatial dimensions are just slightly less than the size of the feature. That maximizes the IR signal and reduces stray light. Some systems have manually adjustable apertures so that almost any sample size and shape can be accommodated. Again, the exact functioning and type of aperture is dependent on the manufacturer.

Resolution is an important consideration and is based on the type of analysis you are conducting. In general, solid samples have IR spectra with inherent bandwidths of around 10 cm^{-1} so we typically want our spectrum resolution to be less than this value although for some applications 16 cm^{-1} is adequate. The signal-to-noise ratio improves with better resolution (4 cm^{-1} resolution has higher signal-to-noise than does 8 cm^{-1} resolution) but the intensity of the signal is less. Consequently, we must optimize resolution against intensity. It is important to remember, though, that it is not possible to detect spectral peak shifts less than the resolution of the system, so if the resolution is set at 8 cm^{-1} and you need to detect a difference in peak location of 6 cm^{-1}, you are bound for disappointment. Most analysts that I know use a default resolution of 8 cm^{-1} for routine work.

Finally, there is the matter of number of scans. We scan multiple times in order to improve the signal-to-noise of the interferogram and resulting spectrum. The improvement is not linear and varies with the inverse of the square root of the number of scans. In other words, we quickly reach a point where we get only marginally better signal-to-noise with massive increases in number of scans and analysis time, of course.

Most systems scan with powers of 2 and a typical value for scans is 32 or 64. It is very rare for analysts to collect more than 256 scans, as there is only marginal improvement compared with the lower number of scans.

5.2.4 Calibration and Performance Checks

Calibration of an IR microscope is probably best done by a skilled technician. In one sense, we do a sort of calibration when we align our optics and interferometer at the beginning of a test session – which is a good practice, by the way. Most manufacturers recommend that the interferogram be examined and the signal maximized through alignment. Mostly we are aligning the mirrors and detectors. Although we do not routinely calibrate the IR microscope it is good practice to regularly conduct performance checks. One good check is a blank spectrum. Collect a background, and then collect it again as the sample. The spectrum should be flat with no peaks. Peaks associated with CO_2 peaks at around 2,400 cm^{-1} are commonly observed with microscopy tests. Some systems have methods of analyzing the blank spectrum for spectrometer and detector performance. I think it is also a good idea to monitor performance with some standard material. The traditional material of choice is polystyrene (Fig. 5.5). Collect the spectrum and then ensure that five or ten of the most intense peaks are in proper locations. We must account

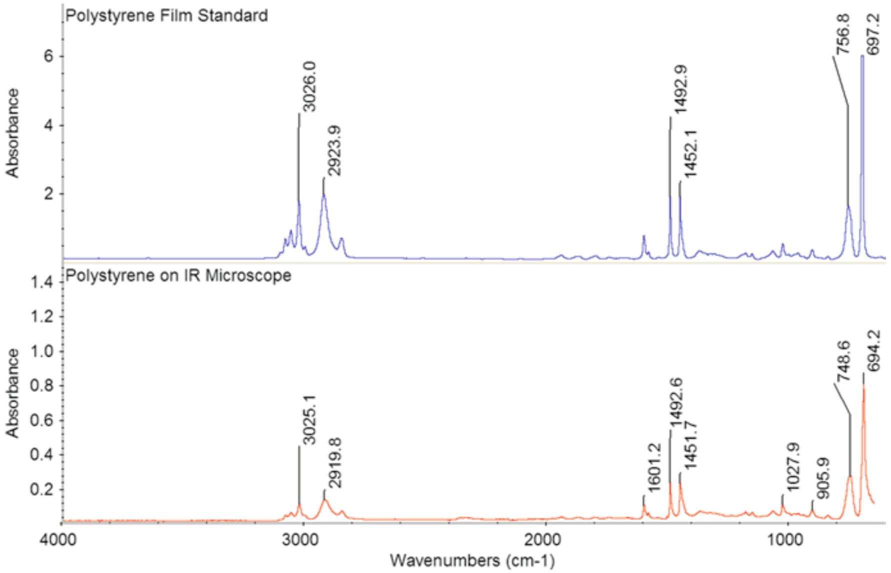

Fig. 5.5 IR spectrum of polystyrene. The upper spectrum is a polystyrene film reference, whereas the bottom spectrum was collected from a polystyrene film on a microscope IR. We can use the polystyrene as a performance check where we compare the expected high peak wavenumbers vs. the experimental ones. The peaks at 3,026; 2,924; 1,601; 1,493; and 1,452 cm^{-1} serve as good, routine performance checks

for the system resolution when making these comparisons. My rule of thumb is that the peaks must be divergent by more than 5 cm^{-1} for the performance check to fail and to cause an investigation. For example, the reference peak at 2,924 cm^{-1} is 2,920 cm^{-1} in the test sample. This difference constitutes borderline acceptance since the test spectrum was collected at 4 cm^{-1} resolution. If a peak fails, I first ensure that the peak location is correct. I generally use the automated peak determination programs but they are certainly not infallible. I do a manual peak location. If that value is still suspect, I will collect an additional spectrum. If the peak is still incorrect, then it is time for a service visit from the spectrometer vendor.

In general, IR microscopes are sturdy and require minimal maintenance. IR sources, like any microscope light source, have a limited lifetime and must be replaced regularly. It is obvious that a source is going bad when the recommended signal intensity cannot be attained on your interferogram – which is another good reason to monitor and keep a record of this value.

5.3 Raman Microscopy

5.3.1 Theory

Even though IR and Raman spectra look similar, the cause and nature of the spectra are fundamentally different. IR spectra are a result of the selective absorption of irradiating infrared radiation by a molecule's functional groups. Raman spectra are a result of light scattering and the selective absorption of a small amount of the irradiating light by the molecule's functional groups. IR bands are prominent for polar functional groups since a change in dipole moment is required for absorption in the infrared. Raman bands are more prominent for nonpolar groups since a change in polarization is required for absorption with Raman scattering. So, in this sense, the two techniques are complementary and are used as such in pharmaceutical development.

Raman is a light-scattering phenomenon. Most light irradiating a reflective, opaque object will be reflected with no change in energy and is referred to as Rayleigh scattering. Some light (1 in 10^4–10^5 photons) will undergo inelastic scattering and will either lose energy to the object or gain energy from the object. If the molecule is in the ground state (which is the most common case for our experimental setups in Raman microscopy), then the irradiating light will lose energy to the object (Stokes scattering). If the molecule is already in an excited state, then the light will gain energy from the object (anti-Stokes scattering). Most Raman experiments utilize Stokes scattering. Since the loss or gain of energy is relative to the energy of the irradiating light, then the x-axis of the Raman spectrum should properly be in Δcm^{-1}, although it is common to drop the delta sign and just label the x-axis as reciprocal centimeters. Figure 5.6 shows the Raman, Stokes scattering spectrum of carbamazepine Form III and can be compared with the IR spectrum of the same material shown in Fig. 5.1.

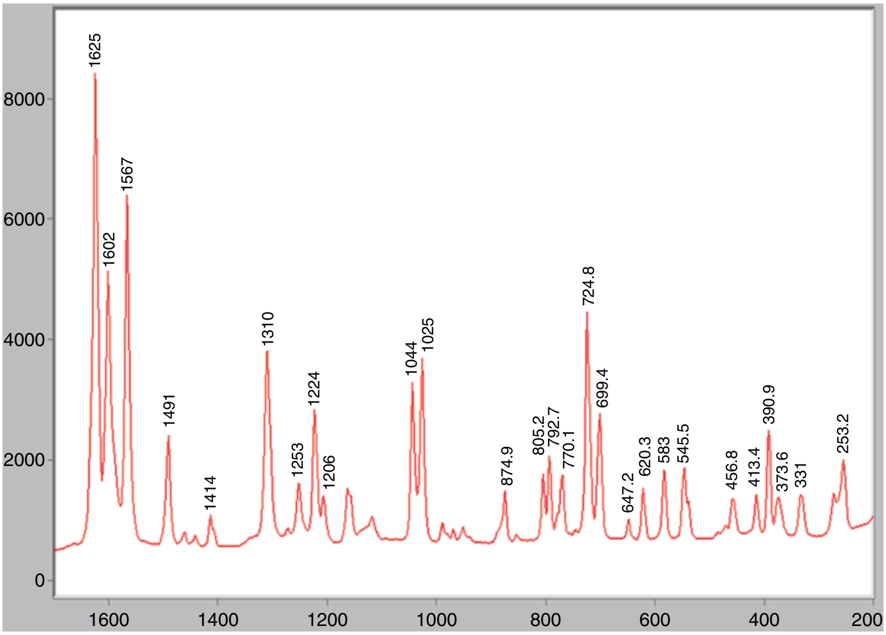

Fig. 5.6 Raman spectrum of carbamazepine form III. Compare this spectrum with the IR spectrum presented in Fig. 5.1 (only the region from 1,700 to 200 cm^{-1} is shown for the Raman spectrum). Note the differences in peak locations and in peak-to-noise between the two different spectra. Raman and IR spectra should be considered as complementary data. This Raman spectrum (and all those to follow) was collected using a 633 nm laser with a 5 s dwell time and 10 co-added scans using a ×50 long working distance objective

5.3.2 *Instruments*

Raman spectrometers and microscopes come in both dispersive and Fourier Transform flavors. Normally, a laser is used as the irradiating light source and lasers with different wavelengths are common and may exist on the same instrument. Some common lasers in use have wavelengths of 1,064, 785, and 633 nm. There are at least two significant hurdles to Raman spectroscopy and these practical difficulties are part of the reason Raman has lagged behind IR spectroscopy in instrument development and applications. First, the Raman signal is faint. As mentioned, only 1 in ~10^4 irradiating light photons will exhibit Raman scattering. Second, fluorescence is common with materials having a Raman signal and the fluorescence yield is much greater than the Raman one. Consequently, fluorescence can overwhelm the Raman signal. Many inventions and techniques have been applied to solving these practical difficulties so that neither low signal nor fluorescence pose too many limitations today. Still it is wise to pay attention to fluorescence in particular.

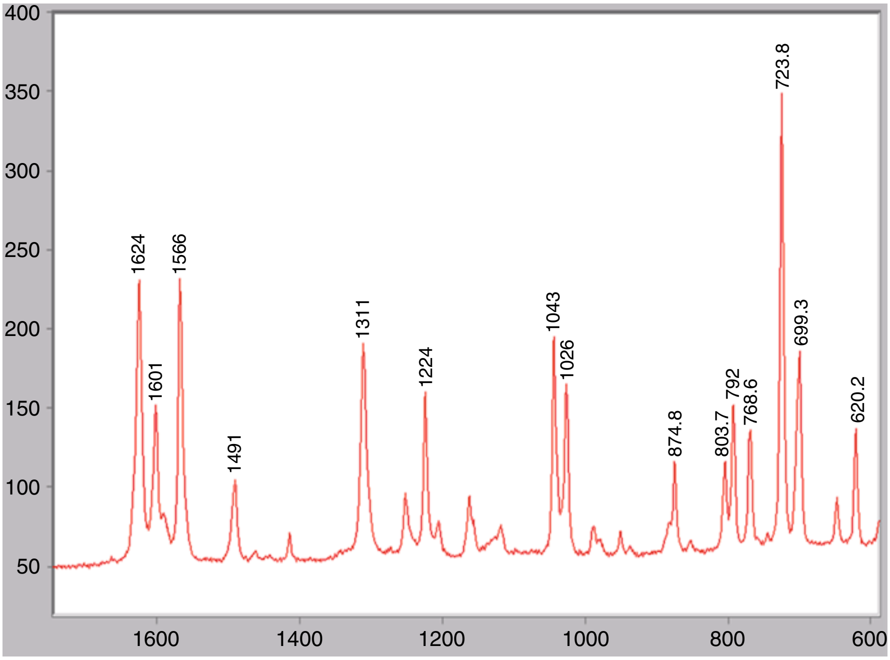

Fig. 5.7 Carbamazepine form III Raman spectrum using 785 nm laser. This carbamazepine spectrum was collected with a 785 nm laser and this figure should be compared with that presented in Fig. 5.6. Note the lower signal intensity (*y*-axis) from the 785 nm laser compared with that of the 633 nm laser (400 units full scale for the 785 nm laser vs. 9,000 units full scale with the 633 nm one)

One reason for multiple lasers on one instrument is to work around fluorescence problems. If we have fluorescence issues at 633 nm, then it may be more manageable with a 785 nm laser. Figure 5.7 presents a Raman spectrum (expanded scale) of carbamazepine collected with a 785 nm laser and this figure should be compared with that presented in Fig. 5.6. Note the lower signal intensity (*y*-axis) from the 785 nm laser compared with that of the 633 nm laser (400 units full scale for the 785 nm laser vs. 9,000 units full scale with the 633 nm one). Figure 5.8 shows a spectrum of carbamazepine Form III collected on a glass substrate and illustrates the appearance of a spectrum with significant fluorescence. The background is high and the signal curved. This spectrum was produced by using a medium magnification objective (20×) on a small particle that did not fill the entire field of view. Fluorescence due to the glass was collected along with the carbamazepine signal.

Since, in general, the irradiating illumination and the resulting Raman scattering are not much affected by glass elements in the microscope. It is possible to use a regular microscope with normal glass optics. Some Raman instruments simply combine the spectrometer with a slightly modified, off-the-shelf optical microscope, whereas other instruments have specially designed microscopes. There are also now some instruments that simply attach to the top of an infinity-corrected optics microscope similar to IR microscope attachments. These are generally FT

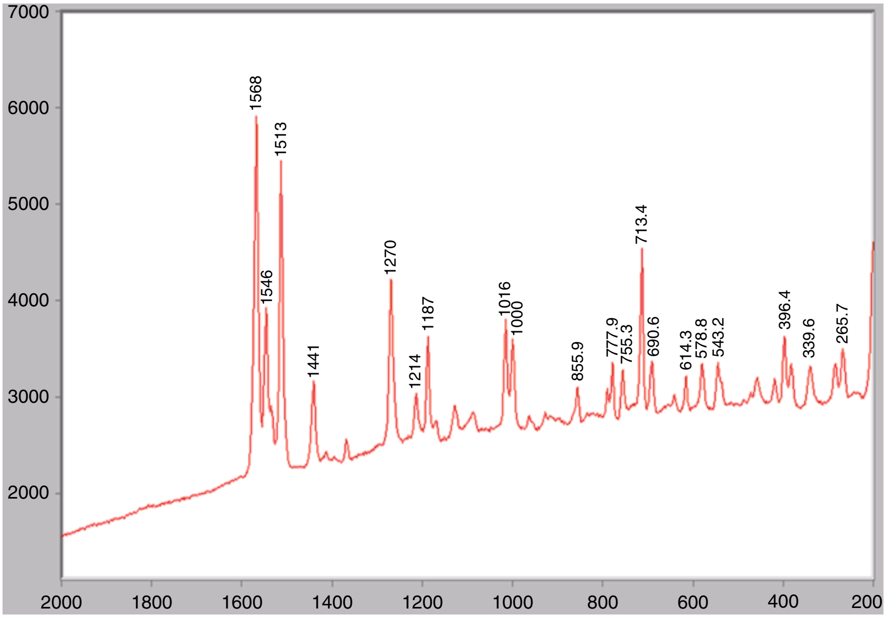

Fig. 5.8 Carbamazepine form III Raman spectrum illustrating effects of fluorescence. This spectrum of carbamazepine Form III collected on a glass substrate illustrates the appearance of a spectrum with significant fluorescence. The background is high and the signal curved. This spectrum was produced by using a medium magnification objective (×20) on a small particle that did not fill the entire field of view. Fluorescence due to the glass was collected along with the carbamazepine signal

type instruments. As a word of caution, be careful with specially designed microscopes or those in which the light source has been replaced. In my experience, the light intensity can be poor. If you are evaluating instruments, be sure and look at a typical sample and observe the specimen using the eyepieces. While the image may look fine on the computer screen, it may be quite poor through the eyepieces. This can be a disadvantage if you wish to do normal microscopy using the instrument and just use Raman as another accessory and if you wish to display chemical images where the location of certain molecules is superimposed onto the optical image. Particularly in the latter case is it important to have good microscope images. Raman microscopes generally use reflection optics and the resulting images are somewhat different that those collected with transmission optics. The appearance differences are particularly noticeable with polarized light images since root causes of polarization are different between transmission and reflected light.

5.3.3 Sample Preparation and Testing

Raman microscopy sample preparation is, in general, simple since it is a scattering technique. The specimen can be mounted on almost any substrate. A flat specimen is preferable and the techniques described in IR sample preparation are suitable for Raman as well. High power objectives can be used for Raman microscopy and it is possible to collect spectra from regions as small as 1 μm^2 which compares favorably with the 100 μm^2 size for IR. Sample stability can be an issue since the intense, focused light beam can easily damage most pharmaceutical materials. It is a good practice to examine your specimen after a test in order to assess damage. The damage usually can be seen as a discolored area. If the sample has been damaged, it is necessary to redo the test but with less light power or fewer scans. One interesting application of Raman is examining pharmaceuticals through glass, such as the sample container. It is not generally feasible for microscopy, but if there are some contaminants attached to the side-walls in glass parenteral vials then these particles can be tested directly by Raman microscopy.

The setup for an FT-Raman experiment is similar to that of a FTIR experiment in that the interferometer limits should be maximized by appropriate adjustments of the mirrors in the interferometer. Generally, FT-Raman detectors are cooled with liquid nitrogen. It is important to adjust the power setting and the number of scans. It is not uncommon to do some initial tests with relatively high power and few scans and then move to another, nearby region, reduce the power and increase the number of scans for the final tests. It is not necessary to establish a background with Raman microscopy. Do check with the manufacturer about the exact nature of the setup for the Raman system.

Dispersive Raman spectrometers have a few different operating parameters compared with FT-Raman systems as you might expect. The key variables are the following: choice of laser; grating size; size of the confocal aperture; spectrometer slit width; exposure time; number of scans; and a laser filter to cut intensity. I discuss all of these operating parameters save the last. It is ideal if your system has a variety of different lasers ranging from 600 to 1,000 nm in wavelength. As mentioned, fluorescence is probably the most important consideration in obtaining good Raman spectra. We generally get the most intense Raman signal from low wavelength light sources, i.e., 633 HeNe laser, but we are also more likely to encounter fluorescence interference with the lower wavelength source (Fig. 5.8). If we do have significant fluorescence then using a longer wavelength source may reduce the problem. Lower wavelength lasers generally have poorer spectral resolution but better spatial resolution than do higher wavelength ones. The spectrometer grating is used to produce monochromatic light of different wavelengths. A higher density of lines (number of grooves per cm) leads to higher spectral resolution, but lower signal and longer collection times. Clearly, we seek an optimum among all three variables and it is probably best to start at relatively low line density and increase to obtain the desired resolution.

The purpose of the confocal aperture is to reduce stray light and thereby improve the signal-to-noise ratio. Clearly, the smaller the aperture the better the signal-to-noise but the signal itself is reduced in intensity as well. It is best to start with small apertures and work your way up in size for small features of interest and vice-versa for large features of interest. The spectrometer slit size limits the size of the image entering the spectrometer with smaller slit sizes leading to better spectral resolution. In general, it is best to be as low as possible so as to maintain high spectral resolution. Finally, there is accumulation time and number of scans. Accumulation time is the residence time at any particular wavelength and number of scans is the number of co-added spectrums collected and averaged. As will all spectroscopy, we seek an optimum. Since signal-to-noise goes with the square root of accumulation time and number of scans, then we hit a point of diminishing returns where collecting twice as long with twice as many scans gives us much less than twice as much signal-to-noise. It is not possible to give hard and fast rules about collection times and scans since the optimum is sample dependent. It also depends on the purpose of the examination. If the spectrum is for corroboration and information only, we collect less than if the spectrum is for regulatory registration or publication.

5.3.4 *Calibration and Performance Checks*

As with any laboratory instrument, it is good practice to conduct routine performance checks and have the instrument calibrated regularly. There are a number of different compounds that can be used for performance checks including polystyrene and polyethylene (Fig. 5.9). Hutsebaut, Vandenabeele, and Moens (2005)

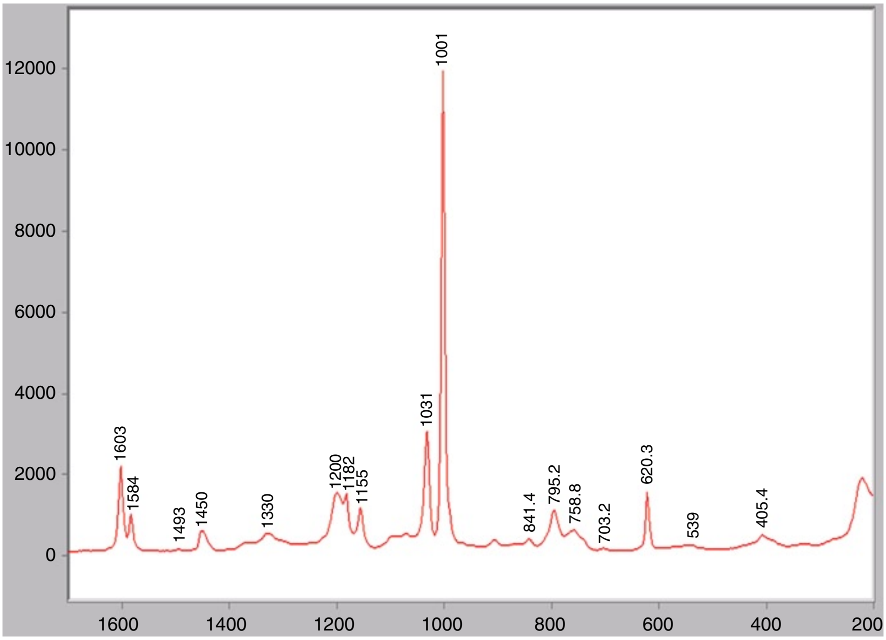

Fig. 5.9 Raman spectrum of polystyrene film. Polystyrene can also be used as a performance check for Raman microscopes. In this case, the peaks at 1,603; 1,031; 1,001; and 602 cm^{-1} can be used for performance checks. If the peak value varies by more than ~5 cm^{-1}, then the system should be re-calibrated using a standard such as a polished silicon chip

present a thorough discussion of calibration and performance checks with some alternative recommendations. For dispersive instruments, a silicon chip can be used to monitor the laser wavelength and then it is possible to use the position of the Stokes and anti-Stokes bands to assess calibration. Calibration is typically done by the instrument manufacturer. Raman shifts require determination of the irradiating radiation wavelength as well as the wavelength (wavenumber as displayed) associated with the excited vibrational energy state. It is easy to see how systematic and random errors in each measurement can lead to larger errors in the location of the subtracted wavenumber value, hence the importance of routine performance checks. It is, in my experience, quite easy to make mistakes in peak location due to poor calibration with a Raman microscope and the calibration shifts more than many other spectroscopic instruments. For routine work, the slight shifts in peak location may not be critical, but for published work or for patents, proper peak locations should be checked frequently as you test.

5.4 Applications

The major applications of IR and Raman microscopy in pharmaceutical development are to solid-state analysis, to contaminant identification, and to chemical imaging. We will briefly discuss each of these areas. There are quite a number of excellent references to IR and Raman spectroscopy and to microspectroscopy. The following articles are general discussions of the use of IR and Raman spectroscopy in pharmaceutical development: Aldrich and Smith 1995; Anderton 2003; Brittain 1991, 2011; Bugay and Williams 1995; Chalmers and Dent 2006; and Gendrin et al. 2008. The IR and Raman spectroscopy of carbamazepine and its different forms are discussed by Grzesiak et al. (2003), Rustichelli et al. (2000), and Tian et al. (2006). In the references section, I also list a number of useful Web sites dealing with the subject of vibrational spectroscopy.

5.4.1 Solid-State Analysis

In considering the application of any analytical technique it is important to keep in mind the key goals of the analysis. With regard to solid-state analysis, the chief goal of the analytical program is the identification and the progression of the optimal form (salt, polymorph, solvate, hydrate, etc.) for pharmaceutical development (see Chap. 8 for more details on this subject). The optimal form is defined in terms of the form's effects on efficacy, stability, and commercial viability. Solid-state form can affect efficacy through solubility and dissolution rate, i.e., the ability to deliver the proper drug into the biological system at the appropriate rate. Form can affect chemical and physical stability over time under different temperature and humidity conditions and has effects on final dosage form processing. Finally, solid-state forms can be patented, affecting the commercial viability of the molecule. It is crucial to identify the optimum form as early in development as possible since form changes during clinical trials inevitably lead to added time in the clinical program and to added cost due to required bio-equivalence clinical trials. IR and Raman microscopy directly affect the overall goal of solid-state analysis and the specific studies related to efficacy, stability, and commercial viability since solid-state forms often have distinctive IR and Raman spectra.

IR and Raman microscopy can be used both as a simple identification tool and as probes of molecular solid-state structure. IR and Raman spectra can function as "fingerprints" for identifying and distinguishing molecules and their polymorphs and solvates. For example, Fig. 5.10 presents the IR spectra of carbamazepine Form II and the dihydrate. Notice, particularly, the presence of the broad OH stretch at ~3,400 cm^{-1} with the hydrate and other more subtle differences in the spectra. On the other hand, the IR and Raman spectra of carbamazepine Forms I and II are not significantly different than the IR and Raman spectra of Form III (Figs. 5.11 and 5.12). This example illustrates the fact that polymorph differences are not assured with either the IR or Raman spectra of different forms. Observed differences can be useful if present, but we cannot always be sure that differences do exist. The closer the solid-state structure of the solid-state forms to each other, the fewer the spectral differences that can be observed with vibrational spectroscopy. Clearly, we expect greater differences among solvates, hydrates, and anhydrates than between

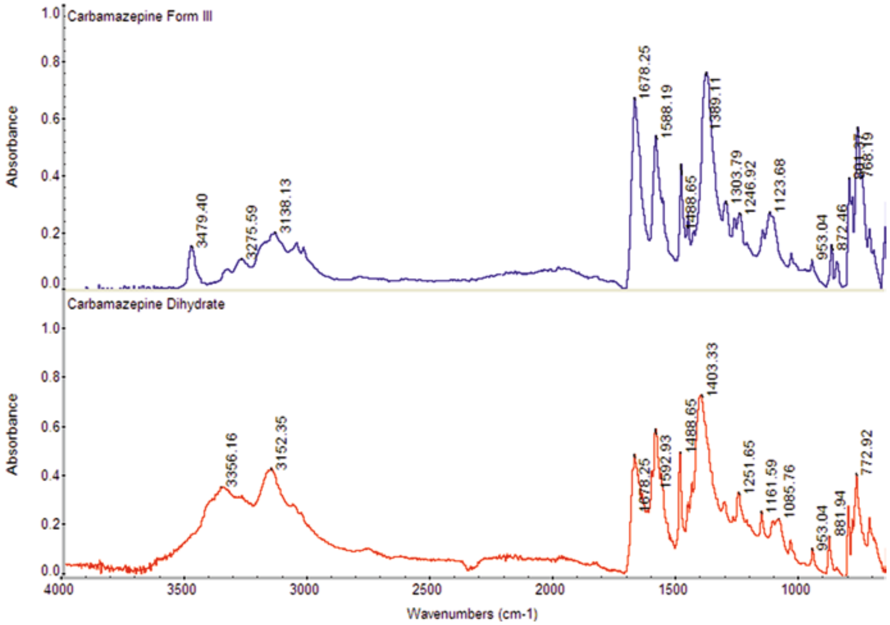

Fig. 5.10 IR spectra of carbamazepine form III and the dihydrate. Notice the spectral differences around 3,400 cm^{-1} where the dehydrate displays a broad peak due to OH stretching. There are also more subtle differences in the fingerprint region of the two spectra

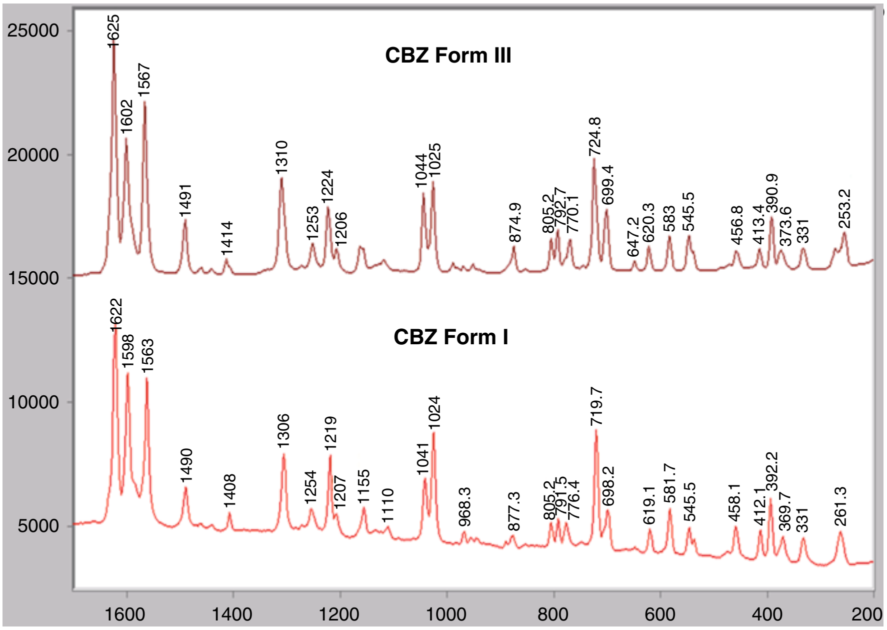

Fig. 5.11 Raman spectra of carbamazepine form III and form I. There are few if any differences in these two spectra indicating similar solid-state structures for the two polymorphs. The Raman spectrum of form II is also similar to that of forms III and I

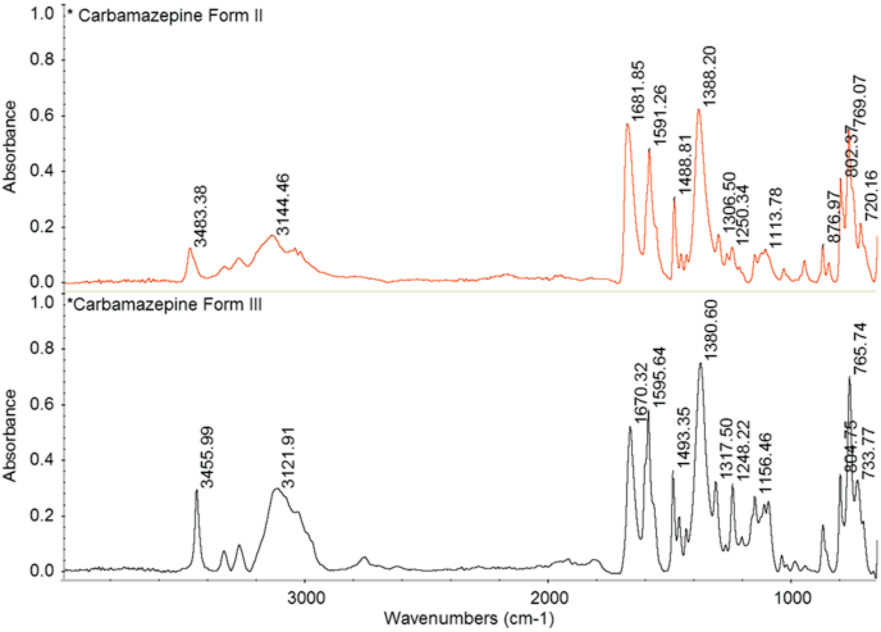

Fig. 5.12 IR spectra of carbamazepine form III and form II. The IR spectra of the different polymorphs of carbamazepine are similar to each other just as is the case with the Raman spectra

polymorphs since the former may contain different functional groups than does the latter. Both types of spectra can be used for identification, but are mostly used along with other techniques such as NMR and X-ray crystallography to aid in the understanding of chemical bonding. Edwards et al. (1997) present a well-constructed argument for the molecular basis of the solid-state differences between caffeine hydrate and the two anhydrous polymorphs.

Infrared and Raman microscopy are often used for the discovery of new forms. Automated form discovery platforms often use IR and Raman for analysis of precipitates from crystallization experiments. As mentioned, if the new forms are hydrates or solvates they may be easier to identify than if they are polymorphs due to the possible presence of new functional groups. Due to the complementary nature of IR and Raman, though, if little difference is observed with one technique then it is likely that more distinct differences will occur with the other one. That is one reason that these automated platforms will often use both techniques along with micro-X-ray diffraction. We need to be careful, though, about possible differences when we are interpreting spectra. If the quality of the crystals is not good or if our collection conditions are not the same, we may infer differences due to polymorphism when, in fact, the differences are sample related. This problem is particularly acute if we are interpreting small shoulders, small peaks, and intensity differences. We need to be especially careful with stating that intensity differences are due to polymorphism. Still, if we suspect polymorphism, we should proceed to the next stage (see Chap. 8) but with skepticism intact.

I think it is a good practice to do functional group vibration assignments with spectra both for general information as well as to aid in understanding structural differences between different forms. The presentation of Raman data and spectral assignments in Edwards et al. (1997) for caffeine and Grzesiak et al. (2003) for carbamazepine are good examples of this type of assignment. The authors list the peak assignments and the differences among the forms. I like the notation of type of functional group vibration in the first paper. This recommendation seems like it should be unnecessary to state since it is obviously a good practice, but given the time constraints in laboratories today, it is easy to make and present spectra without assignment. Actually, maybe I am reinforcing the recommendation for myself since I honor it more in the breach than in the observance.

Water and pharmaceuticals is a tricky subject. Water can be tightly bound in the lattice and fully stoichiometric or it can be loosely bound in channels – or both can exist in the same chemical. Water can also be associated with disordered states and can be adsorbed to the surface of the particles. IR microscopy can be particularly helpful in hydration studies since it is sensitive to the presence of water and the location of peaks associated with hydrogen bonding can be affected by the presence or absence of water – a subject that will be discussed in more detail in Chap. 8. There are some devices available for conducting IR experiments using thermal microscopy and using humidity stages. It is possible to directly monitor changes in IR (and Raman although not as common) spectra associated with the gain or loss of water due to either humidity changes or with temperature changes.

Another active area of research in the solid-state is the understanding of the thermodynamic relationship of polymorphs and the relative stability of solvates and hydrates. IR and Raman microscopy, coupled with the humidity and temperature stages mentioned above, can aid in these studies – providing, of course, that spectral differences are distinct enough to act as markers for form transitions. In particular, IR and Raman can be helpful with solution phase transformation experiments (see Chap. 8, for discussion). We can pull a small amount of the solid from the experimental setup and test it with the IR or Raman microscope to determine whether full polymorph transformation has occurred. Otherwise, we might stop the experiment, filter, and test with X-ray powder diffraction (XRPD) only to learn that the full transformation has not occurred.

I think it is also good practice to collect the IR and possibly the Raman spectrum of single crystals used in X-ray crystallography structural determinations. We test these crystals assuming they are the form of interest, yet we may need to use an exotic solvent in order to make good crystals. IR and Raman microscopy are well suited to this type of examination.

Infrared and Raman spectroscopy are often used for the quantitative analysis of forms – but not typically using microscopy since the specimen volume in a microscope examination is small and not ideal. Special care should be taken to ensure standardization of all operating parameters when developing IR or Raman microscopy quantitative analysis methods. Even small changes in these operating parameters can have a large impact on the accuracy and precision of the quantitative analysis and some of these operating parameters (such as the power output from the IR or Raman source) are difficult to control.

5.4.2 Contaminant Analysis

Infrared and Raman microscopy are quite well suited for particulate/contaminant examinations and are a common tool in forensic trace analysis. Naturally, the techniques are well suited to such examinations because we can obtain molecular data from 1 μm^2 particles in the case of Raman microscopy and from 10×10 μm particles in the case of IR microscopy. We can do the peak assignments directly or use libraries of spectra in order to identify the chemical (see Colthup et al. 1990). In general, IR libraries are more extensive and more useful than are Raman ones – but that will probably change with the more common use of Raman and the internet. I think, it is best to collect reference spectra of materials common to your operations using your IR and Raman microscope. It is a good idea to collect as many materials as possible from the contamination site when doing an investigation. Generally, those submitting the contamination samples are happy to supply miscellaneous materials that are implicated in the problem – at least until you identify the contaminant.

Sampling can be tough for contaminant examinations. Particle picking is best for solid dosage forms and a fine tip tungsten needle works well (Teetsov 1995).

For liquids, I think filtration is best and then particle picking, although there are samples that resist this technique (see Chap. 10 from more details on the subject). For filtration, and even for particle picking, we need to be aware of the possibility, if not the certainty, of introducing particles from our laboratory environment into our sample preparation. We should take care to minimize the problem but it is quite hard to do in a multiuse laboratory. Sampling in contaminant analysis is often given short shrift in comparison with the results from analytical techniques, but sampling is anything but trivial. It is a skill that must be learned through practice.

5.4.3 Chemical Imaging

It is a common trend today to look at the distribution of various parts of a tablet or granule using Raman, and less often IR, microscopy. These experiments produce maps relating to the distribution of the chemical to an image of the tablet or granule. The goal of the examination is to determine whether or not the excipients or drug substance is evenly distributed in the tablet or granule. It is thought that the uneven distribution of some excipients can lead to poor dissolution and other problems. Raman microscopy is well suited for this type of examination because it is a scattering technique and does not require contact of the objective with the object. For IR microscopy, it is difficult to use the Schwarzschild reflecting objective since there is no reflective substrate or the ATR objective since it must be in contact with the tablet surface. Mapping does require a reasonably flat surface. Raman scattering does not require perfect focus, but if the sample is far from flat, there must be an allowance for automatic refocusing if the map is to be collected over a long time without operator interference. It may be necessary to microtome or polish the tablet or granule. Coupling image analysis to chemical mapping allows for a more quantitative assessment of component distribution. Image analysis is more practical now with instruments designed particularly for mapping and with modern image analysis programs. Image analysis does, however, require that we collect quite a few images in order to generate good statistical comparisons. We will probably see many more pharmaceutical applications of chemical mapping as more groups explore the utility of the technique. Figure 5.13 shows a simple map of a cross-section of naproxen sodium as a illustration of the technique. These maps were collected at low image resolution and still required nearly an hour to collect. For high resolution maps, it is not uncommon to collect a set of maps overnight. This figure is black and white but most maps can also be produced in color.

In Chap. 4, I presented some uses of chemical maps with scanning electron microscopy and energy dispersive X-ray spectrometry (EDS). In our laboratories, Raman and EDS maps are considered complementary. Raman maps show the distribution of molecules whereas EDS maps show elemental distribution. The two types of maps present alternative views and both are useful depending upon the goal of the analysis.

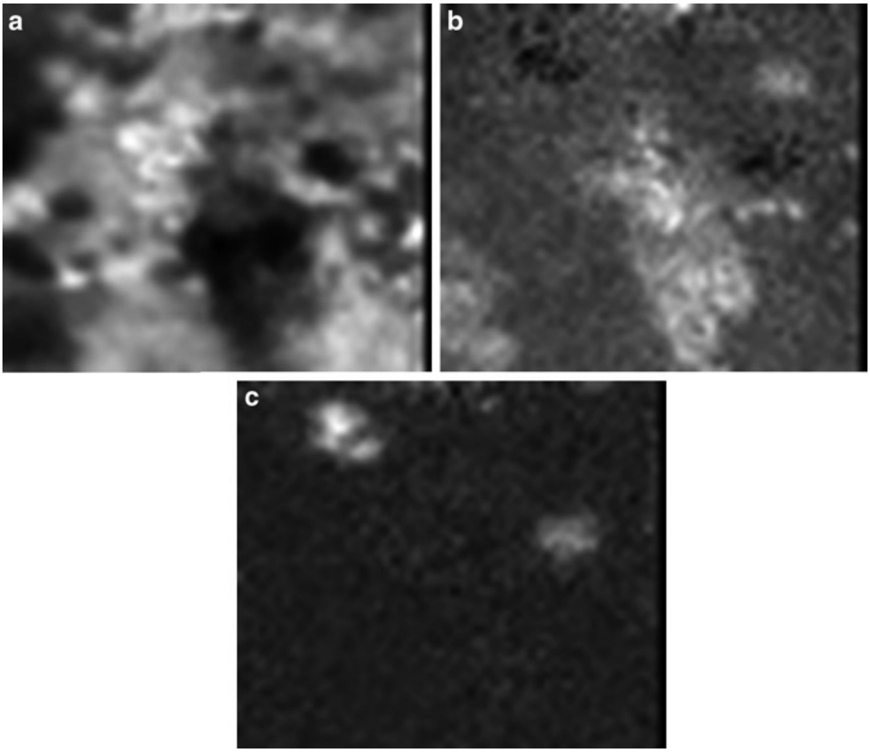

Fig. 5.13 Raman map of naproxen sodium tablet cross-section. (**a**) 1,300–1,500 cm^{-1} representing naproxen sodium, (**b**) 1,100–1,300 cm^{-1} representing cellulosic bulking agent, and (**c**) 600–700 cm^{-1} representing an unknown. These maps were collected with a ×20 objective and the area is 500×500 μm. This figure serves to illustrate the types of chemical maps that are possible with Raman mapping

5.5 Summary

Infrared and Raman microscopy are both important tools in pharmaceutical development. We have discussed a number of good applications to the field and the uses are certainly not limited to the few mentioned in this chapter. It is important, though, for us to understand the strengths and limitations of these instruments in order to get the best use of them. Like a hammer that is good for nailing wood but not so good for fixing the plumbing, spectroscopy can be just the right tool for the job and just an awful mess for another one. Study, in the sense of learning from books like this or from coursework, is necessary but to fully understand the technique you must experiment with it and apply it. The exercises in this chapter and book are a good start, but only that, a start. Your proficiency in the technique is directly related to the number of samples you have examined.

5.6 Exercises

I find that the software accompanying IR and Raman microscopes to require a steep learning curve. In other words, allot plenty of time to learn even the basics of the software since it is rarely intuitive in my experience. Also, it is fairly common to have connection issues between the software operating the camera and that operating the spectrometer. Again, you should allot sufficient time to learn these systems before beginning any exercises. Do keep in mind that you are working with three different components: microscope, spectrometer, and camera. Learning the idiosyncrasies of each component is important for good work. Also, if you find that you are only an occasional user, do plan to spend some time just refreshing your memory on the system operation before doing "real" tests.

A. Basic Operation

A1. Polystyrene Film

Collect good spectra on a film of polystyrene. Pay attention to how the system operates and displays data. Compare the peak positions that have been collected with the known peaks of polystyrene. There is probably a reference spectrum of polystyrene in your system and it is helpful to overlay this reference spectrum with the one you collect.

A2. Caffeine Form III and Hydrate

Prepare samples of caffeine hydrate. Collect IR and Raman spectra of anhydrous caffeine and then a hydrated sample. If there are different IR objectives on your system, such as a reflecting and an ATR objective, practice collecting spectra using each objective and with different sample preparation techniques. Note the subtle differences in peak positions and in peak intensities with each type of objective. For Raman, collect different spectra using low, medium, and high power objectives. In general, the higher power objectives yield better signal-to-noise but reduced intensities.

B. Operating Parameters

B1. IR Operating Parameters

Use caffeine Form II and practice adjusting the different operating parameters available to you with the IR microscope. In particular, change the resolution and the number of co-added scans and note the differences in the spectra. Change the size of the aperture and note the differences in the spectra.

B2. Raman Operating Parameters

Use caffeine Form II and practice adjusting operating parameters available to you with the Raman microscope. In particular, change the slit, the hole size, the grating size (if using a dispersive instrument), the dwell time, and the number of accumulations. In my work, I took one sample and changed each of these parameters in turn and made comments on the spectral effects or lack thereof. I refer to this reference on a regular basis. Also, evaluate the effect of using different objectives. Finally, take a known sample that fluoresces and investigate the effect of changing the laser wavelength (assuming, of course, there are multiple lasers on your system). A tablet with cellulosic excipients is one good example or small nonfluorescing particles on glass.

C. Spectral Interpretation

Identify the peak locations for your caffeine anhydrous and hydrous IR and Raman spectra. Make a table similar to that presented in Fig. 5.13 from Edwards et al. 1997. Compare your assignments with those recorded in the literature.

D. Mapping

D1. Map of Naproxen Sodium Tablet

Prepare a cross-section of a naproxen sodium tablet and collect low-resolution maps in order to learn the software and the manner in which maps are collected. There are a few new operating parameters for maps, the most important of which are the map resolution and the dwell time for each collection point. The purpose of collecting a low-resolution map with short dwell time is to learn the system. Once you have mastered that operation, collect a higher-resolution map with longer dwell times as well. Note the difference in the quality of the maps.

D2. Map of Multivitamin Tablet

Prepare a cross-section of a multivitamin tablet and collect maps as in Exercise D1. These maps will be considerably more complex and will require more care in the spectral assignments.

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Internet Resources

Spectroscopy: Solutions for Materials Analysis – an online magazine: http://spectroscopyonline.findanalytichem.com/spectroscopy

Spectroscopy Now: http://www.spectroscopynow.com/coi/cda/home.cda?chId=0

Spectroscopy Europe and Asia: http://www.spectroscopyeurope.com/

Internet Journal of Vibrational Spectroscopy. http://www.ijvs.com/archive.html

Chapter 6
Specialized Microscopy Techniques

6.1 Introduction

There are quite a few types of microscopy and microscopical techniques that are used occasionally in pharmaceutical microscopy but do not deserve chapter status. In some cases, these techniques are quite useful in biological applications but have only a few limited uses in pharmaceutics. In other cases, you simply need to be aware of the nature of the technique and the appropriate applications in case you get asked if that technique is applicable to the problem at hand. A pet peeve of mine is that it seems to be the considered the essence of good scientific management today to suggest alternative ways of looking at a problem – even if the manager has very little idea of the nature and limitations of the technique. I cannot count the number of times a manager, director, or vice-president has asked a question such as "Have you considered using cyclotron radiation for your IR experiment?" "Well, no I haven't because it's a really dumb idea and won't help solve the problem in any way, shape or form." Of course, I do not say those things, but I sure think them. Some of the techniques below have, as I mentioned, important applications in biological science or other fields but only limited applications in physical pharmacy. Some of these techniques, I think, are under-utilized in our industry and I think it may be worth our time to work them into our projects. A prime example of the latter is defect analysis by TEM. There are significant hurdles to this application but I think it is an under-studied area.

6.2 Confocal Microscopy

Confocal microscopy, and in particular laser scanning confocal microscopy, is a popular technique in biological work and in the semiconductor industry. There are only a few applications in physical pharmacy and these are well reviewed by Pygall et al. (2007). Confocal microscopy improves the depth of field of the microscope without degrading resolution. The technique is generally used on fluorescent materials or those materials that accept fluorophore staining, although it is not a necessity (see Murphy 2001 for biological applications).

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The confocal microscope was invented by Minsky in 1957 (Minsky 1988) and his initial work was on individual cells in microscope preparations of nervous system sections. Groups of neurons are quite difficult to image due to the density of the cells and their interconnectedness. Minsky's invention utilized pinholes in front of both the objective lens and the condenser. The pinholes served to exclude stray light and isolate only that light coming from the feature of interest. The pinholes were placed in a disk that spun. Minsky described both reflected and transmitted light applications. The main application of the instrument was imaging deeply embedded objects that were difficult to image due to diffuse light from the surrounding material. Figure 6.1 presents a confocal image of a biological system with multiple fluorophore stains.

Developments in electronics, computers, and lasers have improved confocal microscopy considerably, and in particular, have made possible the laser scanning confocal microscope. With this latter technique, a map is built of the object by scanning in the x and y directions at a particular z, then slightly moving z and building another map in x and y and so on. The computer holds all the information and puts together a three-dimensional image of the object by combining all of the x–y slices. This allows one to image whole objects within a thick prepared section of a biological object.

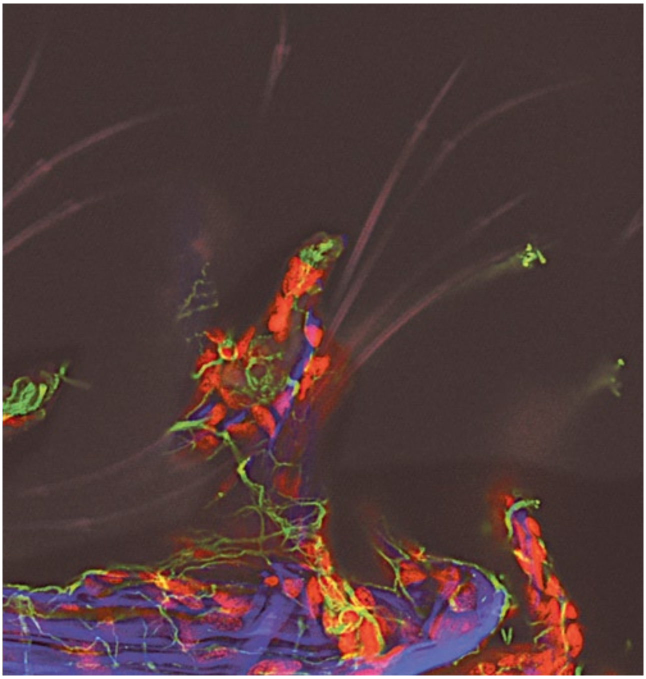

Fig. 6.1 Confocal image of Bristle Worm (*Platynereis dumerilii*). This sample was stained as follows: nervous system (Alexa 555), muscles (Alexa 488), and nuclei (Draq5TM). Reprinted with the kind permission of Carl Zeiss Inc.

There are a number of good reasons the technique is not routinely applied to pharmaceuticals. Drugs typically are not naturally fluorescent and, even if they are, the fluorescence is usually quite faint. It is not easy to stain drug compounds with fluorophores. I have not seen any references to drug staining in the literature, although my research in this area is not extensive. Another reason that confocal microscopy is not much used with drugs is that drug substance and drug product are well imaged by other techniques. One possible application is to drug substance within drug product. Tablets are opaque though and so not practical for confocal microscopy or its laser scanning cousin. Still, there are some possible applications to some drug product dosage forms, such as gels, that may help solve a very specific problem (see Ringqvist et al. 2003; Depypere et al. 2009 for some applications). Since the technique is often used in biological work, you may have access to such an instrument in the drug discovery division of your company or at a local university.

6.3 Scanning Probe Microscopy

Scanning probe microscopy encompasses a number of different techniques for achieving near atomic resolution by detecting the interaction of a fine metal probe with the surface of a material. In its most common guise, the position and deflection of the fine probe is measured using light (laser) reflected from the end of the cantilever where the tip is located. The technique can achieve atomic level resolution but only in a very small area, as we might expect (the higher the magnification, the smaller the area observed is a universal rule in microscopy). There are quite a few different techniques under the umbrella of scanning probe microscopy with different imaging modes such as contact, noncontact, tapping, etc. The bulk of the applications of the technique are probably to semiconductors although there are a few applications to pharmaceuticals.

The majority of the applications of scanning probe microscopy in pharmaceuticals utilize atomic force microscopy (AFM). AFM measures the force of the interaction between the metal probe tip and the surface of the specimen. The pharmaceutical applications of AFM have concentrated on drug–biomolecule interactions with only a few applications to physical pharmacy (see Turner et al. 2007 for a good review of pharmaceutical applications). There are some interesting studies on molecular size and on various excipients such as lactose. AFM has also been used to study disorder and changes in disorder over time. Disorder (related to degree of crystallinity) is important to us because we commonly mill materials, inducing crystal defects and affecting physical properties. There has also been some work reported looking at the adhesion of drug particles to lactose carrier beads in dry-powder inhalation devices.

At first blush, one could easily imagine that AFM would have important applications in pharmaceutical development. The fact that there is little reported in the literature on applications of AFM in pharmacy is not due to a lack of interest by companies and universities or to a lack of investment. Just the opposite is the case. Much effort has been expended on the technique by the industry but, frankly, there is little to show for that effort. I believe the lack of success is mainly due to the

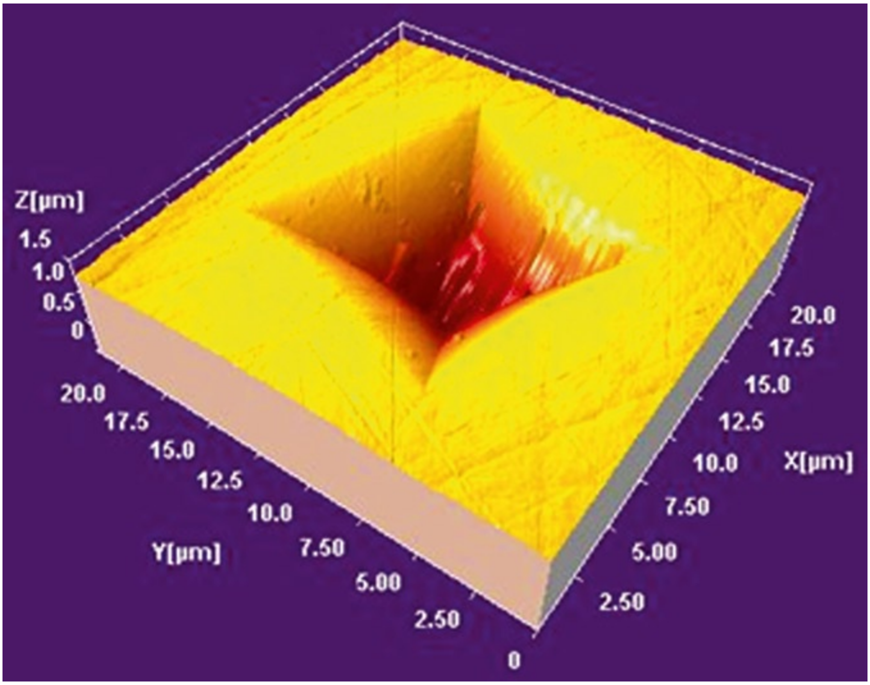

Fig. 6.2 AFM image of lactose indentation. This photomicrograph is an example of the kind of images the scanning probe microscopy techniques produce

working scale of the instrument. AFM is really good at looking at the atomic scale and nanometer-sized objects. It turns out that most problems in pharmaceutical development are in the micrometer to millimeter size range. We have problems extending the intense knowledge gained on very small sections of a very small particle to kilogram-sized batches of drug substance or even to 300-mg size tablets. Unless ways are found to extend the knowledge gained on the atomic scale to bulk properties of powders, then it is likely that AFM will have only a few limited applications in pharmaceutics. Like other techniques in this section, AFM may be able to solve a small number of niche problems, and you should be aware of those potential applications, but the technique will probably not become a routine tool in pharmaceutical microscopy. Figure 6.2 presents an AFM image of the indentation of lactose as an example of the kind of images the technique produces.

6.4 Transmission Electron Microscopy

The transmission electron microscope (TEM) was developed in the 1930s and has gone on to become a workhorse instrument in materials and biological science. It operates on the same principles as the light microscope but electrons are used to form the image instead of visible radiation. The TEM consists of an electron gun and focusing electromagnets (similar to those employed in SEM) and the image is directly detected on a fluorescent screen or indirectly using photographic film or a digital camera. The specimen must be thin (on the order of nanometers) and must be stable in an intense electron beam. These last two requirements, along with the small sample size, have limited the use of TEM in pharmaceutical development. Much like the AFM, we obtain very good information on a very small-sized specimen and must extend that to the bulk drug substance or drug product. Unlike the AFM, the TEM requires extensive sample preparation and modification and one must always question whether we are looking at "real" features or those induced by the sample preparation process.

In the biological field, the emphasis is on morphology and somewhat on chemistry (using primarily EDS). The TEM is used routinely for microbiology and the study of cells. In the materials field, morphology and chemistry are important applications as well, but it is also possible to study crystallography and defect structures among others. For physical pharmacy, the few applications have concentrated on morphology, such as for wet-bead milled particles, and on defects. There has been some recent work looking at crystallography as well (see Eddleston et al. 2010).

As mentioned, the primary impediments to the use of TEM in pharmaceutical development are sample preparation and sample stability in the electron beam. The particles can be no more than 500 nm in thickness and generally need to be on the order of 100–200 nm. Even our micronized drug substance only has a small proportion of particles in that size range. Further, the particle must be stable in the beam which also requires thin particles, particularly when doing diffraction studies.

I think there are three potential applications of TEM to pharmaceuticals. The first is to wet-bead milled (nanomilled) drug substance. The size of these particles ranges from 10s of nanometers up to 1 μm. It is not unusual to have 90% of the particles with a diameter less than 500 nm. The second potential application is to electron diffraction structure determination of small crystals that resist crystallization into sizes appropriate for X-ray diffraction. These are mostly particles with needle-like habit that tend to break along their length not width. Lastly, the study of defect structures in milled materials is particularly well suited to the TEM. We know that there is a high degree of disorder in our milled drug substance as well as in our freshly crystallized samples. We might profitably make use of the knowledge of these defect structures to better understand how best to mill, which drug substances are the best candidates for milling, and how these defects "anneal" over time – if we could study them directly. Although I believe these are profitable avenues for investigation, we should not minimize the hurdles to success. None of these applications are straight forward and all will require sustained diligent effort and not a little creativity.

If you wish to learn more about the use of transmission electron microscopy in materials science, I strongly recommend the text by Williams and Carter (2009). This book has chapters on imaging defect structures and on crystallography and is probably the best place to begin if you are considering TEM applications.

6.5 Differential Interference Contrast and Hoffmann Modulation Contrast

In pharmaceutical microscopy, we are accustomed to working with objects with a high degree of innate contrast. Drug particles generally have sharp edges, interior features, and we can improve edge contrast by choice of mounting media. Unstained and/or live biological samples do not have these properties and it can be quite difficult to image biological features due to the poor inherent contrast. Further, it is desirable to be able to generate a three-dimensional (or apparent 3-D) image of our objects on the optical microscope. Differential interference contrast (DIC), also called Nomarski interference contrast after its primary inventor, and Hoffman modulation contrast (HMC) microscopy were invented as means of furthering these ends. I have combined the two techniques together since the applications of the techniques are similar as are the images to some extent. The fundamental mechanisms of the two techniques are very different. See Murphy (2001) and McLaughlin (1977) for more details on the basic theory and applications of these two contrast methods.

In DIC, a Wollaston prism is used to change the phase of a small portion of the incoming beam. After the light travels through the specimen, the small phase-changed portion of the beam is recombined with the major unchanged portion of the beam. The recombined light beam is now is imaged normally but the edges and internal features of the object will have greater contrast due to the differences in phase between the unchanged and phase-altered light. I know, it sounds like I am speaking a foreign language. I am not going to expend much more effort explaining the technique since it is only rarely used in physical pharmacy, but it is used a great deal in biological work as well as in reflected light examinations of metals and semiconductors. You can learn much more about the technique from Murphy (2001) and Wayne (2009). Fig. 6.3 shows the appearance of a typical DIC image.

Hoffman modulation contrast does not affect the phase of the light but is a dark field technique in the sense that oblique light rays are used for imaging. The intensity of light traveling through the specimen is reduced (modulated) without altering the phase, then recombined at the back focal plane of the objective. Again, small contrast differences in the specimen are highlighted and the image can take on a three-dimensional appearance (Hoffman 1977). As in DIC, the technique can be used in both transmitted and in reflected light. Both DIC and Hoffman modulation contrast are quite useful in biological applications and in metals and semiconductors.

I have used both techniques for three main applications. The first is for enhancing contrast in low-contrast materials. Frankly, I do not do this often since the SEM has a similar effect (3-D) and I nearly always collect both optical and SEM images. Naturally, the SEM is a surface technique and DIC and HMC can be useful if you are trying to enhance the contrast of features within a particle. Second, the technique can be used for drug product such as topicals and patches. Again, I do not do this often since it is rarely needed. Lastly, there is the aesthetic affect (see Fig. 6.3). A pleasing image is an end in itself and can be a definite aid in, otherwise tedious, presentations or reports.

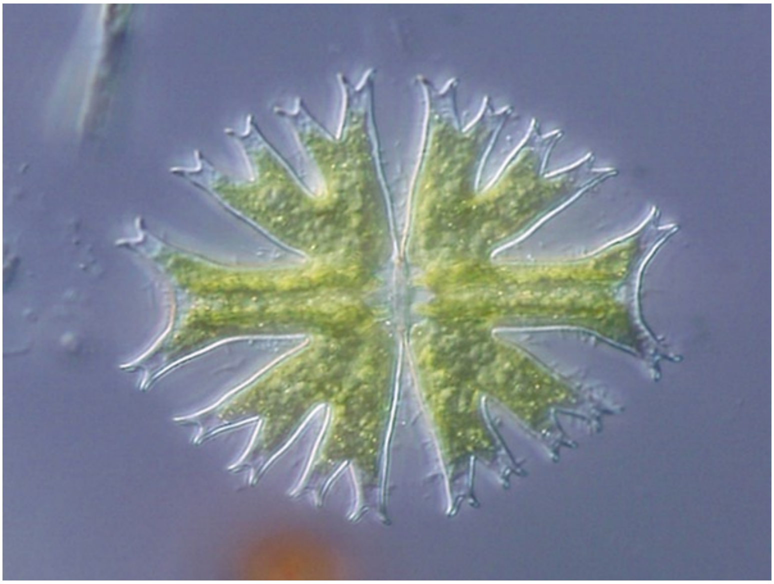

Fig. 6.3 DIC image of *Micrasterias radiata*. This image well illustrates the appearance of a DIC image and the improvement in contrast possible with this technique. DIC is also commonly used in reflected light images of metals and semiconductors. Used with permission from Wikipedia http://en.wikipedia.org/wiki/File:Micrasterias_radiata.jpg

6.6 Phase Contrast

Phase contrast microscopy is another technique used to improve low-contrast images and, again, is primarily used in biological microscopy (Murphy 2001; Needham 1958). Phase contrast is also used in methods used to count particles, such as asbestos, on air sampling filters. The technique is not used a great deal in physical pharmacy but does have one very important application, in my opinion, and that is to the evaluation of wet-bead-milled materials. Use of phase microscopy with wet-bead-milled materials allows us to qualitatively assess milling success in the sense that it allows us to determine whether most of the particles have been milled to the proper submicron size. It is particularly useful as a fast and reliable technique for evaluation during the milling process and as part of milling development. Although phase contrast microscopy does not allow us to circumvent the resolution limits of the microscope, we are able to detect submicron particles more easily than with normal brightfield illumination.

Frits Zernike developed phase contrast microscopy in the 1930s and received the Nobel Prize in physics in 1953 for this work (Zernike 1955). In brief, the phase microscopy attachments convert small phase differences into amplitude and contrast differences. This is accomplished by two sets of rings mounted in the substage condenser and in the back focal plane of the objective. The bottom ring is generally a black spacer with a narrow transparent ring. The upper ring is generally a transparent spacer with an opaque narrow ring. The sizes of the transparent and opaque rings are the same (as seen in the microscope) for selected substage condensers and objectives. In other words, the condenser attachment and objective are matched and both must be changed when changing objectives. The initial setup of the system requires that one look at the back focal plane with a telescope or Bertrand lens and aligns the two rings so that they are superimposed.

One would imagine that no light at all would reach the eyepiece from such an arrangement. And, in fact, the background of the phase contrast image is usually dark gray, but the features on the microscope slide will appear bright. While the rings are superimposed, it is possible for diffracted rays to enter the light path. Edges and features of the object on the slide will diffract light rays and hence, we can observe the objects with enhanced contrast. Figure 6.4 shows an image of a wet-bead-milled material. The background is made up of many very small particles that are exhibiting Brownian motion. The motion of the small particles is quite easy to detect on the microscope and quite difficult to image. The larger particle in the middle of the picture is an unmilled particle. Figure 6.5 shows a phase contrast image of a collection of diatoms and illustrates the improvement in contrast possible with this technique for biological specimens.

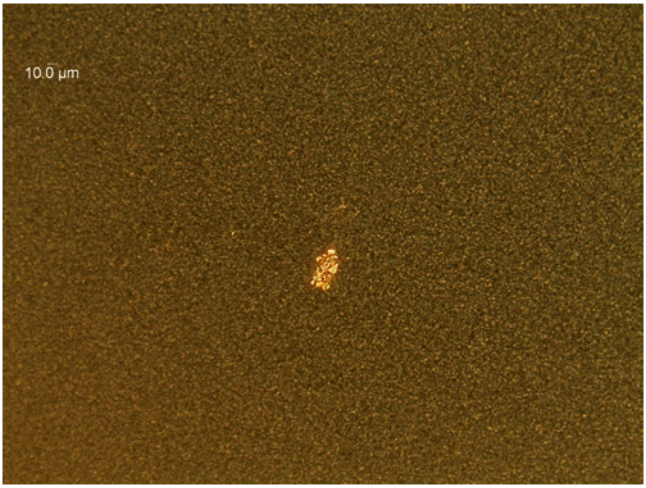

Fig. 6.4 Phase contrast image of wet-bead-milled drug substance. The background is made up of many very small particles that are exhibiting Brownian motion. The motion of the small particles is quite easy to detect on the microscope and quite difficult to image. The larger particle in the middle of the picture is an unmilled particle. (Image size = 350 × 260 µm)

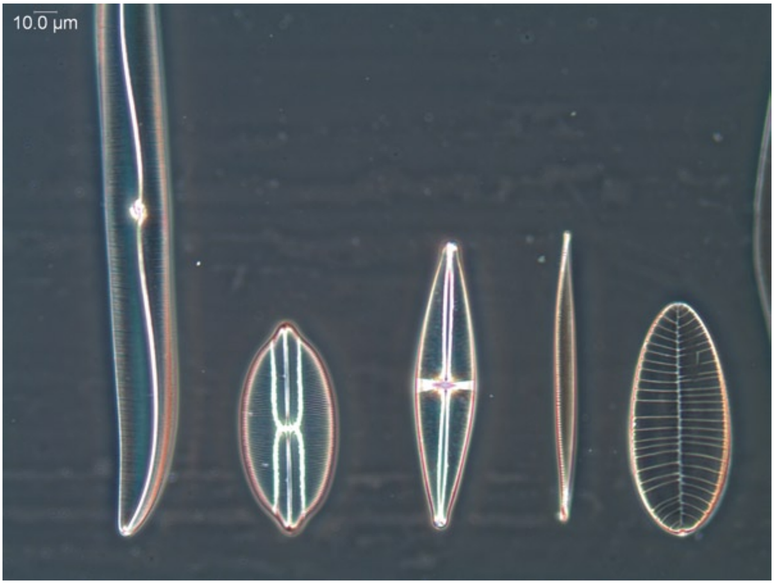

Fig. 6.5 Phase contrast image of diatoms. This image illustrates the improvement in contrast possible with this technique for biological specimens. (Image size = 700 × 520 µm)

6.7 Dispersion Staining

Dispersion staining is a technique popularized by Walter McCrone, among others, and has been used for the identification of a variety of different materials, such as asbestos, as well as an aid in refractive index measurements (McCrone et al. 1984). Its most common application is to the identification of asbestos. It is not often used in pharmaceutical development for identification but can have some applications in polymorph studies.

Dispersion staining is an optical staining technique (no physical staining of the material on the slide is done) utilizing the difference in refractive index dispersion between a liquid and the solid being examined. Remember that the refractive index of a material is dependent on the wavelength of light illuminating the object. That is the reason that near the refractive index match between a solid and a liquid, the boundaries of the particle will be blue and red if illuminated with broad spectrum white light. Dispersion is the name associated with the relationship of wavelength with refractive index and, in general, refractive index changes more with incremental wavelength changes in liquids than in solids (see Fig. 6.6). Dispersion Staining requires use of special high-dispersion liquids and a special objective. There are two variants to the technique referred to as annular (central opening) and central stop (opaque circle). One mounts the material in a high dispersion refractive index liquid near the refractive index of the solid. When using central stop, the stop is swiveled into place on the back focal plane of the objective and the substage condenser diaphragm reduced so that the central stop just encompasses the opening

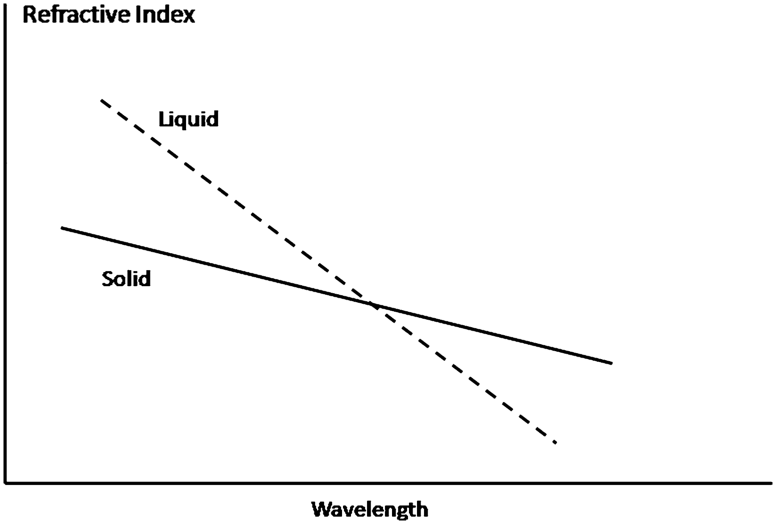

Fig. 6.6 Typical dispersion curves for solids and liquids. The difference in slope between liquid and solid refractive index vs. wavelength curves is the basis of the dispersion staining effect

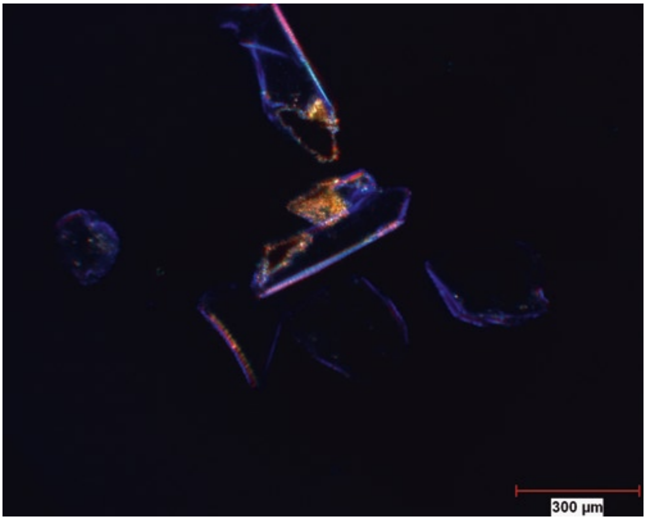

Fig. 6.7 Dispersion staining of glass flakes. This is a central stop image of glass fragments with a refractive index of 1.55 in Series E high dispersion Cargille refractive index liquid with an index of 1.5500 at 589 nm. (Image size = 1,400 × 1,040 µm)

(as seen using Bertrand lens or telescopic lens). Observing normally, the transparent particles will have colored fringes against a black background (see Fig. 6.7). Since the observed colors depend on the refractive index of the particle, in theory polymorphs and solvates should have different colors and allow one to quickly locate single particles of one polymorph in a preparation of the other polymorph. I say, in theory, because most pharmaceuticals have three refractive indices with views representing those indices plus all in-between the limits. In general, the refractive indices of polymorphs overlay to some degree, so only in a very narrow refractive index band will particles of one polymorph have a different color and only in certain particle orientations. That may be one reason the technique is not much used in pharmacy, but I think it is useful to know something about the technique for those rare situations that it is useful.

6.8 Dark Field and Rheinberg Illumination

Dark field microscopy is another optical staining technique with general applicability in biological studies and some materials studies. It is not often used in physical pharmacy but does have at least one important application and that is to the examination of parenterals when looking for foreign particles using a stereomicroscope. It is useful because dark field produces a dark background with bright objects. It is typically easier for us to observe particles in that type of lighting than with dark particles in a bright background.

Dark field microscopy is rather simple and requires only a specialized substage condenser that concentrates oblique light (refracted light, reflected light, etc.) onto the specimen and into the objective. A stereomicroscope must be specially fitted with a stop that covers most of the lower light source and generally must be purchased as such or retrofitted with the appropriate swing out stop. Figure 6.8 shows a typical

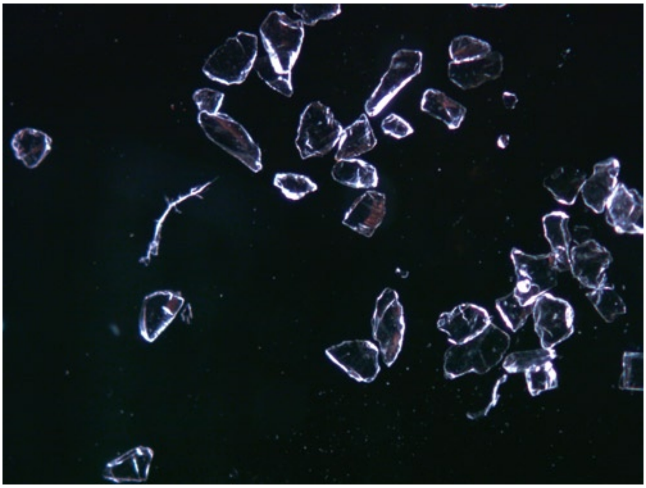

Fig. 6.8 Dark field image of glass particles using a stereomicroscope. This type of imaging is particularly useful for locating small, nearly transparent particles in a parenteral vial. (Image size = 3.4 × 2.5 mm)

dark field image of glass particles in a glass container using a stereomicroscope. This type of imaging is particularly useful for locating small, nearly transparent particles in a parenteral vial. Dark field can either be used in transmitted or in reflected light.

Rheinberg illumination is a variant of a dark field technique but produces images with the boundaries of particles in one color against a background of another color. Rheinberg illumination uses a central stop of one color and an annular stop of another that are sandwiched together. The filter arrangement is placed below the microscope condenser such that oblique light from the annular filter is collected by the objective. The boundaries of the object will have the color of the outside annular stop and the background the central stop color. In general, the central stop needs to be pretty dark and the technique works best with low power objectives. I do not know of any particular use of the technique other than aesthetics and I do not know of any suppliers of the filters. You must make your own. If you are interested in the technique, see the Olympus Microscopy Resource Web link for one of the best descriptions of the technique that I have seen.

6.9 Flourescence Microscopy

Fluorescence microscopy has become a nearly ubiquitous tool in biology and in the biomedical fields and there have been significant developments to the instrumentation and the fluorophores in recent years (Needham 1958; Murphy 2001). Fluorescence is the emission of light at one wavelength due to the excitation of a fluorophore by light of another wavelength. The exciting light is typically in the ultraviolet region, whereas emission must be visible to be of use in the microscope. In general, emitted light has a longer wavelength than the exciting light. In most cases, the object (structure in biological cell generally) is stained with a fluorophore since most objects do not naturally fluoresce or if they do, only faintly.

Fluorescence microscopy is nearly always done in reflectance mode and the critical accessories are the light source (xenon or mercury arc-discharge lamp) and the filter cube used for excitation and for emission. These cubes have excitation, emission, and dichroic filters that are matched and generally cannot be interchanged among different manufacturers. The filter cube generally has a narrow bandpass filter for the entering light from the source that excludes light outside the desired wavelengths. It also has a dichroic filter that passes all of the excitation light (by reflection) to the object and passes all of the light from the object back through the objective. There is also an emission filter that passes only the wavelengths from the emitted fluorescence. There are a large number of different cube sets and you must check with the microscope manufacturer to get the correct set for your application. Finally, if you wish to record your images, you have to have very good digital cameras that are specially designed for fluorescence. Fluorescence generally requires a cooled CCD-type camera. The emitted light is generally so low that it is barely visible through the eyepieces to say nothing of the digital camera. In fact, when doing fluorescence of drug substance, I generally darken the room entirely and allow my eyes to become accustomed to the low light for 15–20 min before using the microscope (otherwise known as taking a nap). Biological applications are not generally so problematic with low-level light since the features of interest are generally stained with a strong fluorophore. Staining is not generally practiced in physical pharmacy applications.

I have used fluorescence as a tool for assessing the distribution of the drug substance in the drug product (see Fig. 6.9a, b, for an example). I have used it for tablets but more commonly for topical drug products, since there are generally better techniques for imaging drug substance in tablets and capsules. Also the cellulose excipients generally fluoresce in the wavelengths of interest and interfere with the drug substance fluorescence. With suspensions and topical products though, the drug

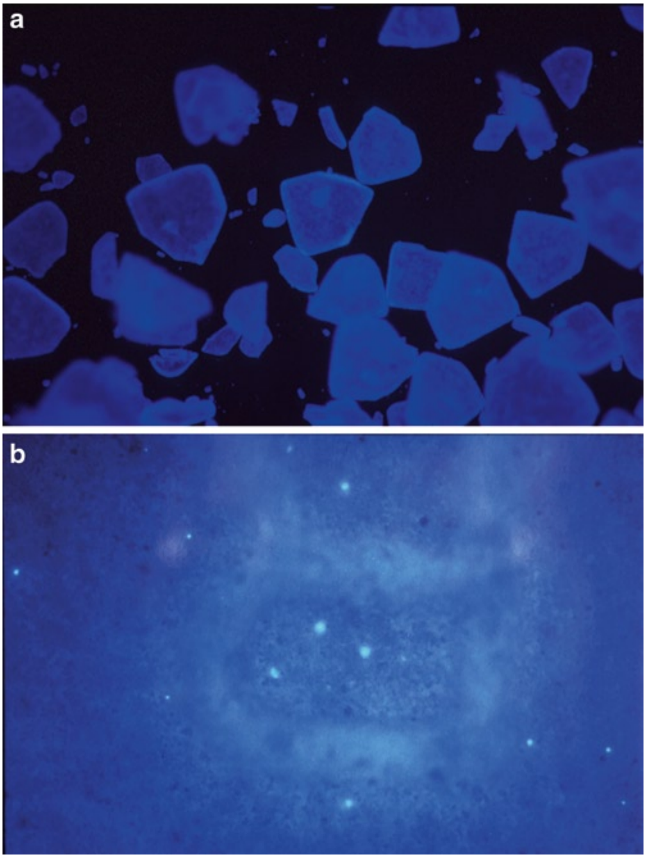

Fig. 6.9 Fluorescence images of (**a**) drug substance and (**b**) tablet. This drug substance had good natural fluorescence and so it was possible to detect small particles of the drug substance on the surface of the tablet. In this case, the fluorescence images helped understand accelerated dissolution with some tablets since significant amounts of DS were on the surface of the tablet. (Image size: (**a**) = 700 × 520 μm; (**b**) = 6.8 × 5.0 mm)

substance is generally the only fluorescing component and often will be quite visible. The primary application for topical products is assessing the association of the drug substance with a particular component of a two- or three-component suspension. Another interesting application that I have not personally tried is using fluorescence to determine whether observed particulates in parenterals (or other solution-based systems) are drug substance. Although I would be loathe to use fluorescence as a primary means of identification, it can be useful as a confirmatory tool.

6.10 Freeze-Dry Microscopy

Freeze drying (or lyophilization) is a manufacturing process used primarily with biopharmaceuticals to improve stability of the proteins and peptides. The method is also used to improve dissolution rate and to enhance bioavailability for some small molecule drug substance. Thermal microscopy is one of the many analytical techniques used to aid in the determination of the appropriate parameters in the freeze-drying cycle (Liu 2006). Lyophilization generally has three steps: solidification by freezing, ice sublimation, and moisture desorption. The most important parameters in setting the cycle are freezing temperature, pressure, and duration of each step. These will vary with each particular formulation and microscopy can aid in identifying the appropriate values for these important parameters. The primary uses of the microscope are to determine the cake collapse temperature and to ice morphology during the sublimation cycle.

The design of the cold stage is similar to that of the thermal stages described in Chap. 3 but with the difference that the stage is designed to work in a vacuum and, in better stages, the vacuum can be measured. There are a number of commercial stages available from thermal stage manufacturers and the setup of the instrument is relatively straight forward, although I allot quite a space of time to refreshing my memory on the technique if it has been some time since the last test. I do not recommend attempting freeze-drying experiments with regular thermal stages even if they can be operated at low temperatures. It is necessary to evacuate air from the stage before conducting any examinations, typically using a vacuum pump. Performance verification tests, of the system and the microscopist, using mannitol are useful in my opinion (see Kim et al. 1998).

6.11 Thermal Microscopy in the SEM

One of the most interesting applications of the Environmental Scanning Electron Microscope is to the study of “wet” samples. In fact, the earliest sales literature referred to the ESEM as the “Wet SEM.” It was recognized early in the development of the instrument that if one used a cooling stage and appropriate control of the water vapor, then it was possible to maintain 100% relative humidity in the chamber and keep specimens fully hydrated. This type of control has been applied to a number of biological samples, emulsions and other hydrated materials (Stokes 2001; McGregor and Donald 2010).

Although the ESEM is extensively used for the analysis of pharmaceuticals, there are only a few limited examples in the literature of the use of thermal stages with the ESEM. In fact, a literature search failed to find any recent (less than 5 years) articles combining the ESEM and a thermal stage with pharmaceutical analysis. Probably the most important application is to the study of labile hydrates. For most hydrates, we do not require 100% relative humidity in the sample chamber in order to study the sample since most hydrates are reasonably stable under the normal operating conditions of the low-vacuum SEM and ESEM. There are, though, some quite unstable hydrates that can best be studied using the cold stage and by manipulating the water vapor pressure to set an exact relative humidity. It also seems reasonable to evaluate the stability of some drug substance and even drug product in high relative humidity atmospheres and the ESEM cold stage is ideally suited to this type of investigation. In the main, though, the technique remains more of a curiosity than a practical tool in pharmaceutical analysis.

It is possible to achieve high relative humidities with simple Peltier stages that allow temperature control at approximately 3–5°C. Using a modest water vapor pressure of 4–5 Torr, one can achieve relative humidities in the 90–100% range.

6.12 Summary

In writing and researching this chapter, I was struck by two facts. First, there are some marvelously creative people who continue to revitalize and renew microscopy. There are any number of interesting microscopy techniques that I have not mentioned at all. Second, a good percentage of the techniques discussed in this chapter were invented in the last 100 years and many in the last 50. That is extraordinary in my opinion. Microscopy is about 350 years old and by all accounts a mature science. Yet, we continue to develop and invent new techniques. It is a testament to the vitality of the field, and I think, a good reason to be a professional microscopist.

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Olympus Microscopy Resources: http://www.olympusmicro.com/index.html

Chapter 7
Image Analysis

7.1 Introduction

Particles are ubiquitous in pharmaceutical drug substance and drug product. Our final crystallization steps are designed to produce particles of uniform dimensions and, failing that, we mill or micronize the powder to reduce the particle size and ensure uniformity. We routinely measure particle size and control it. It is common to set specifications for particle size distribution parameters such as the 10th, 50th, and 90th percentiles. It is a rare New Drug Application that does not have extensive presentations of particle size and its impact on drug product performance. For products designed to be inhaled (for the treatment of asthma, for instance), the limits on particle size distribution are tightly controlled. For these products, particle shape can also be an important performance attribute. It is difficult to over-emphasize the importance of particle size measurement and control to the pharmaceutical industry and to drug development. Image analysis (IA), by both optical and scanning electron microscopy, is a useful technique for determining the particle size and shape.

Microscopical image analysis (sometimes referred to as *digital* image analysis) can be defined as the use of a computer and stored image generated by a microscope to measure feature size and shape. While there are a variety of methods of determining particle size, the sole method of determining particle shape is image analysis. Shape analyses are not terribly common, at this time, but are required for the development of inhaled respiratory products. At its most basic, digital image analysis is simply the coupling of a computer to a microscope for image collection and for feature processing and analysis. There have been enormous improvements in both techniques and instrumentation over the last 50 or so years. The development of powerful, fast computers has had a profound effect on image analysis. Microscopes and cameras have improved as well, albeit at a slightly less frantic pace than computer improvements. Today, we have quite powerful computer-based systems for making routine particle size and shape measurements using microscopy.

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In this chapter, some of the basic principles and techniques of image analysis as applied to pharmaceutical development are presented. In Chap. 9, some applications of the technique to common pharmaceutical problems as well as a discussion of general shape analysis are discussed. In general, image analysis is used most often with the optical microscope, but there is no reason why one cannot also use the SEM. In some cases, the SEM has distinct advantages over optical microscopy and, increasingly, the SEM is being used for image analysis. I cover the use of both the optical microscope and the SEM with image analysis.

7.2 General Principles

Image analysis can be separated into the following discrete steps:

1. Sample preparation
2. Image production
3. Image collection
4. Image processing
5. Feature segmentation
6. Binary operations
7. Measurements
8. Data presentation and statistics

Each of these topics is discussed in turn.

As a preamble, there are a number of general principles that I find useful to bear in mind when embarking on the development of an IA (image analysis) method.

First, sample preparation is the key for good image analysis. I would like to put flashing, neon lights around this principle because I think good sample preparation is that important and too often given less attention than it deserves.

Second, produce the optimum image on the microscope then ensure that image is faithfully reproduced by camera and computer system. This means adherence to good methods of contrast such as Köhler illumination.

Third, minimize the number of necessary image processing steps by producing good particle separation in sample preparation and good particle contrast in the microscope.

Fourth, minimize the number and complexity of binary operations and test the ruggedness of those used (i.e., binary operations such as particle separation may work well with a few images but not so well with all).

Fifth, as with any analytical technique, good correlation of IA results with product properties or with results from another analytical technique lends credence to conclusions.

To be succinct, adequate prework and simple procedures lead naturally to robust and precise results.

7.3 Sample Preparation

7.3.1 Introduction

It is obvious that sampling techniques depend upon the type of sample being tested. Bulk powders are sampled differently than suspensions and creams and even more differently than powder in aerosol or dry-powder inhalers. Most of the discussion below is oriented toward bulk powders as that is the most common application of image analysis in pharmaceutical development. Many of the sample preparation techniques that I discuss in other chapters can readily be adapted to IA uses.

Sample preparation is the most important step in image analysis and the quality of the results will only be as good as the quality of the sample preparation. Well-prepared samples with the optimum number of nontouching particles in each field-of-view will inevitably lead to dependable image analysis results. Unfortunately, it is not really possible to prescribe one simple sample preparation technique that is guaranteed to work with each and every bulk powder sample. To some degree, each sample is unique and it is necessary to experiment with a variety of different techniques in order to discover the optimum procedure for that sample. Consequently, it is beneficial to become familiar with as many different sample preparation techniques as possible. In this section, a number of methods that I find useful are discussed, but the discussion certainly does not exhaust the subject.

We often cannot control the quality (i.e., sample representative of bulk) of the sampling from the bulk material, but we do have control over how we make specimens from our sample. We must always keep in mind that our samples are inherently nonhomogeneous. A powdered sample placed on a laboratory bench for any amount of time will begin to segregate with larger particles rising to the top. This phenomenon is known as the Brazil Nut Effect and particularly applies to noncohesive materials (Duran 2000). Some cohesive materials will segregate in this manner as well (Ellenberger et al. 2006). In any case, good mixing and sampling are required to get the best results. Allen (1997) is probably the best general source for information on the subject of powder sampling, in my opinion.

Clearly, the rigor with which we study and account for sample preparation effects depends upon the ultimate use of the data. If the results will only be used for an initial, cursory evaluation of particle size and shape, it may not be necessary to go to great lengths to assure representative sampling. Simply rotating the bottle and taking a small amount from the surface may suffice. If the results will be used as part of a material specification or as part of a stability assessment, then full rigor will be required to understand sampling effects.

Sample preparation is a skill and an art made better by practice. It is a wise idea for the novice to spend considerable time learning these techniques (and sharing any good new tricks with the general community) before attempting to undertake “real” analysis. This practice time will undoubtedly yield many benefits over time.

Also, if you have been away from this type of testing for some time, it is helpful to do some practice tests before attempting the real thing. It is my experience that the time spent attempting to do good analysis with a bad sample preparation is a false saving of time. No matter how harried and rushed you might be, making a new better preparation will inevitably be the most efficient approach.

7.3.2 Optical Microscopy Sample Preparation

7.3.2.1 OM-1 Powder in Oil, Direct Application

The most common technique is manual dispersion of a small specimen of the sample directly onto the microscope slide. This preparation can be examined dry or by using a liquid media such as dimethyl silicone fluid or mineral oil. For this wet dispersion technique, a small specimen of the sample is first placed onto the microscope slide. A drop or two of the liquid media is added to the specimen, and gently mixed using a small spatula. A coverslip is then placed over the preparation. The wet dispersion technique is preferred for the examination of small primary particles. For samples of larger size (i.e., large crystals, granules, etc.), the dry dispersion technique with no coverslip is preferred.

Some samples may contain large agglomerates that are outside the range of the lowest power microscope objective. For these samples, simply note the presence of large agglomerates in the experimental record and, if possible, provide an estimate of primary particle size and shape. A photographic image can suffice for this estimate.

You may wish to make permanent slides of samples using a variant of this technique with a permanent mounting medium such as Norland® optical adhesive. Prepare the slide as described above then put the specimen under a UV lamp. The adhesive hardens in approximately 5 min. These adhesives come with a few different refractive indices making it possible to select the type of mountant based on the desired contrast.

7.3.2.2 OM-2 Powder in Oil Dispersion with Vortexing or Sonication

In this method, we disperse a small amount of the DS powder in a small vial with silicone oil and then vortex or sonicate this preparation. A small drop of the suspension is then placed onto a microscope slide with coverslip. This method provides somewhat more representative specimens and can break up lose agglomerates.

7.3.2.3 OM-3 Gas Blown into Closed Container

This sample preparation technique either works spectacularly well or does not work at all. It is best used with noncohesive samples. The technique requires a shallow container with holes in the lid and compressed air (as in the gas containers used to blow dust off of computer keyboards). Place a glass slide with a small amount of silicon oil in the container. Place a small amount of the sample in the bottom of the container as well. Place the lid on the container and then blow the compressed gas into the container. The powder is blown into the air within the container and some particles settle onto the glass slide. It take some experimentation to determine the best container size, the location of the slide relative to the powder, the number and size of air holes in the lid, and the method of blowing gas into the container. The advantage of this method is that it nearly always gives you a good even distribution of particles on the slide. The disadvantage is that it does not work very well with particularly cohesive materials or those that are heavily agglomerated.

7.3.2.4 OM-4 Suspension Spray

The suspension spray technique is another good method for making IA samples (my thanks to Rudolph Ismay, GSK Harlow, UK for the initial demonstration of this technique). A suspension is made using powder, cyclohexane, and a surfactant such as lecithin or polyethylene glycol. The suspension is sprayed onto a glass slide using a TLC sprayer or fine paint sprayer. The spray apparatus I use was purchased at a hobby shop and is designed for painting of small objects such as model airplanes. It is necessary to experiment with the suspension concentration, air pressure, distance of spray nozzle to stub, and number of sprays; and you can expect some variations that are sample dependent. This technique works well, but you will note a small residue around each particle. The residue can easily be ignored in optical microscopy but is more difficult to ignore with SEM.

Figure 7.1 presents an OM image of a real sample preparation of caffeine and illustrates some of the challenges that we face doing image analysis on drug substance while Fig. 7.2 illustrates an ideal sample preparation but with nonpharmaceutical glass particles. First, note that in both figures there are a good number of individual particles that are well separated from each other. In Fig. 7.2, there are a number of touching particles, but it is easy to see that these could be separated from each other relatively easily and reproducibly. The situation is more complex with the particles in Fig. 7.1. Some of the particles are clearly just touching and should be separated. For other agglomerates, though, it is difficult to determine whether or not these agglomerates should be counted as one particle or if the agglomerates should be separated, ignored, or measured separately.

There is no simple, easy answer to the problem of agglomerates. If successive sample preparations lead to the same type images, even if the dispersing energy is different, then this state of agglomeration clearly represents the state of the particles. On the other hand, if these agglomerates can be relatively easily broken up, then we probably want to do that and make additional preparations. It is a very good idea to be in close contact with the person requesting the work about this subject. It may be that agglomerates are important and they want us to measure and

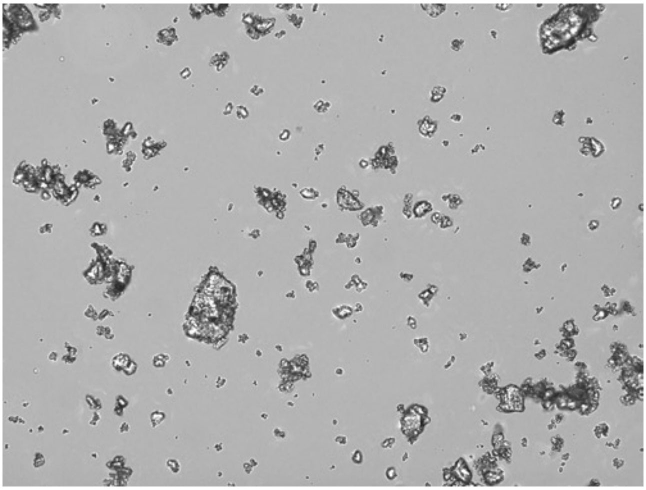

Fig. 7.1 Caffeine particles in silicone oil. This image is typical for pharmaceutical drug substance and illustrates some of the difficulties inherent in image analysis. The particles exhibit varying levels of agglomeration and many particles appear to be touching although not chemically bound (image size = 700 × 520 μm)

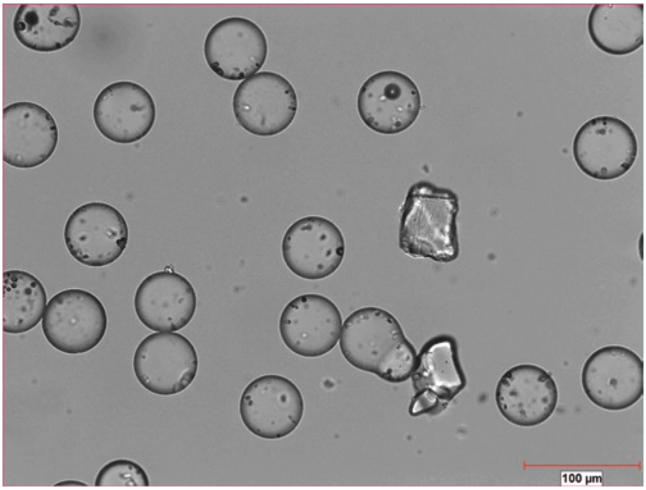

Fig. 7.2 Glass beads. The particles in this image are well separated and ideal for image analysis. This type of preparation is the goal for sample preparation (image size = 700 × 520 μm)

keep track of them. As an example, we may be testing a topical suspension to be used as an eye treatment. Large agglomerates are clearly undesirable since they can be irritating. On the other hand, the requestor may be more interested in primary particle size and less interested in agglomerates. Close contact and consultation saves time and provides better data.

7.3.3 SEM Sample Preparation

7.3.3.1 SEM-1 Direct Powder Application

The simplest method of making an SEM preparation is to sprinkle the powder onto double-sided tape on an SEM stub then sputter coat with metal to improve conductivity and image contrast (required for all the SEM sample preparation techniques). While this method is satisfactory for the assessment of primary particle morphology and size, the method does not generally work with pharmaceutical powders. This sample preparation method may work well with noncohesive powders, like glass beads, but it does not routinely produce good IA specimens with cohesive materials. It is certainly worth an initial experiment, but it is unlikely to be the preparation method of choice.

7.3.3.2 SEM-2 Press Tape into Powder

Another simple approach involves laying out a bed of powder on a flat surface and then pressing double-sided carbon tape (on a SEM stub) into the surface. This technique generally is better than SEM-1 but still does not routinely produce good images with cohesive materials.

7.3.3.3 SEM-3 Gas Blown into Closed Container

The technique outlined in OM-3 can also be used with SEM stubs. In this case, aluminum stubs with double-sided carbon tape are placed into the container instead of the glass slides. All of the practical notes mentioned in OM-3 apply to SEM-3.

7.3.3.4 SEM-4 Nucleopore™ Filtration

In my opinion, this is one of the two best techniques for preparing SEM samples for image analysis. In this technique, the powder is suspended in a liquid (such as acetonitrile or cyclohexane with a surfactant) and then filtered through a Nucleopore™ filter. The filter is then placed onto double-sided carbon tape on a SEM stub. The key to this technique is getting the suspension concentration correct and that will depend on the particle size. I generally use sequential dilutions for the initial tests. Naturally, if one is doing this work for routine release or for stability trials, the exact sample preparation recipe must be well established.

7.3.3.5 SEM-5 Suspension Spray

The suspension spray technique described in OM-4 is the other good method of making IA samples. The same suspension is made as in OM-4 and SEM-4 (powder in cyclohexane with surfactant), but in this case the sample is sprayed onto the SEM stub. In this case, it will be necessary to experiment with different types of substrate and with different imaging techniques since the residue around the particle can be problematic for IA. I have experimented with many different substrates and found glass slides and silicon chips to be acceptable for many applications. I may then work in low-voltage secondary electron detection in order to reduce the effects of the residue (most likely due to the surfactant).

Sample preparation for materials to be used in inhaled products is often driven by the need to replicate as closely as possible the action of the inhalation device. Consequently, the sample preparation is often simpler than that of micronized or milled DS. For inhaled DS, the challenge may be ensuring an adequate number of particles in each field of view for measurement efficiency.

Figure 7.3 presents a typical SEM image using the suspension filtration sample preparation with backscatter detection. Note that an image that is appropriate for IA may not make a particularly pleasing image. Figure 7.4 presents an inhaled sample on a glass coverslip with secondary detection and low kV.

As a final note on sample preparation, there are some commercial sample dispersers using more sophisticated approaches to this sample preparation technique. I have experimented with a couple of them and I think they hold good

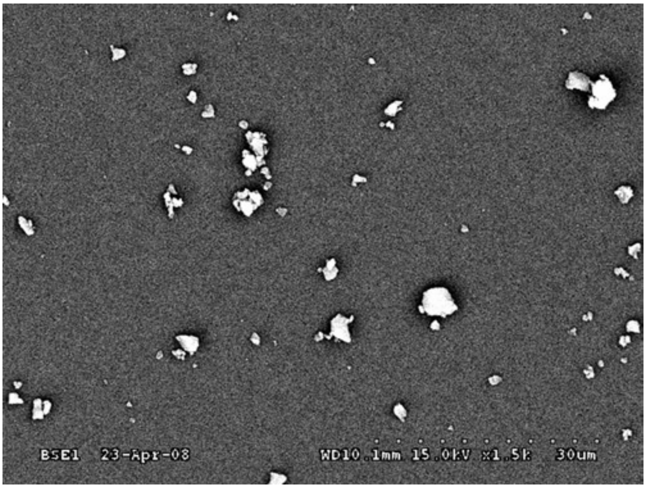

Fig. 7.3 SEM image using suspension filtration sample preparation. This caffeine image was made by suspending powder in cyclohexane with lecithin and filtering through a Nucleopore™ filter. The image is collected in backscatter mode. Although the image is good for IA, it is not ideal for simple visual examination (image size = 92 × 75 μm)

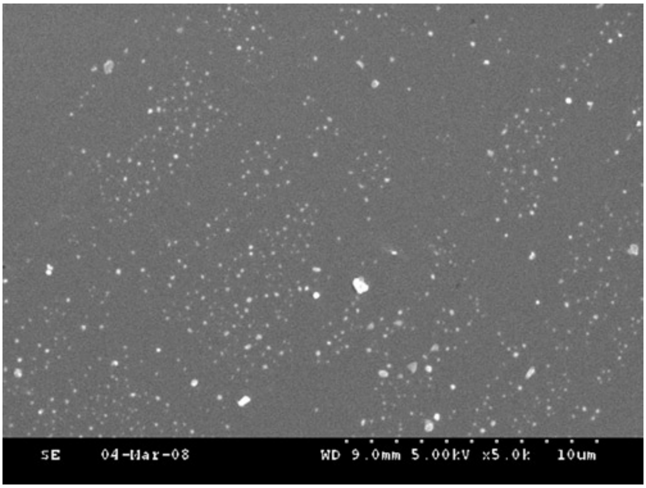

Fig. 7.4 SEM image inhaled spray onto glass coverslip. A commercial inhaled spray device was used to generate this sample on a glass coverslip. The low-voltage secondary electron signal is used to collect this image (image size = 24 × 20 μm)

promise for routine sample preparation. Their high cost, though, limits wide use at this time, in my opinion.

7.4 Image Production

7.4.1 General Considerations

The objective for image production is an image of particles or features with good contrast and well separated from each other. This type of image is often not ideal for regular visual examination. The basic principle underlying image production is that a good image on the microscope leads to good images for image analysis. Consequently, it is worthwhile taking adequate time to setup the microscope for good image quality. If 50% of your method development time will be devoted to sample preparation, then 25% of the time should be devoted to determining the appropriate imaging conditions on the microscope. The instrument, whether OM or SEM, should be in good operating condition and clean. All of the optical elements should be well aligned. It is good practice to examine a standard sample at the beginning of analysis in order to assess the operating condition of the microscope. For optical microscopy, I use a diatom test slide. The various diatoms have small features of different sizes that are detectable with various objectives. For SEM, I use an aluminum stub and examine imperfections on the aluminum surface.

7.4.2 Image Production with the Optical Microscope

Ensure that Köhler illumination is established (see Chap. 2, Sect. 2.4.8 for an explanation of Köhler illumination). Exact illumination conditions are sample dependent. The objective is to maximize the contrast between the particles and the surrounding field. Brightfield illumination with transmitted light is typically used for the analysis. If illumination conditions other than brightfield are employed (i.e., phase contrast, polarized light, fluorescence, etc.), then thoroughly document the selected technique.

It is best practice to maintain the bulb voltage at the recommended setting (typically 10 V for halogen bulbs) and to adjust the contrast and brightness using the substage aperture, field diaphragm, and neutral density filters. This procedure ensures the optimum image clarity and particle definition.

7.4.3 Image Production with the SEM

Accurate particle discrimination depends upon high contrast between particle and background. High-contrast images that are ideal for image analysis are typically poor for visual observation in the SEM. I recommend the use of backscatter electron detection or low-voltage secondary electron detection depending upon the sample. Backscatter detection is often ideal for image analysis. Backscatter images provide high-contrast particles compared with the background and the interior of the particles that have the same gray level throughout the particle interior. Also, in many low-vacuum microscopes, this is the only imaging mode available. Backscatter detection works well with the Nucleopore™ filters but not as well with the glass slides due to charging effects (see Chap. 4 for a general discussion of SEM imaging). In order to obtain a good backscatter electron yield, one must operate at low working distances (less than 10 mm) and high accelerating voltage (~15 kV). The metallic coating on the specimen will also enhance electron yield.

Low-voltage secondary electron detection works well with inhaled products prepared on glass slides. Ideally, one uses the upper cross-over accelerating voltage (the voltage at which the incoming electron current equals the outgoing current from secondary and backscatter electrons). This is an experimentally determined value, but for organic materials, it lies in the range of 1–3 kV. It is not necessary to use a metallic coating when using the cross-over voltage, although coating generally does not adversely affect imaging. In general, it is possible to adjust contrast and brightness so as to obtain images of particles that have an even gray level across the particle interior.

Figures 7.3 and 7.4 illustrate the different images one can obtain with backscatter detection and low-voltage secondary electron detection.

7.4.4 Choice of Microscopy: Resolution and Magnification

The primary factor affecting the choice of microscopy is the required resolution. The practical lower limit of quantization for the optical microscope is approximately 3 μm. While it is true that the limit of detection is much less than this for some objectives, the ability to accurately measure features less than 3 μm in size is much reduced due to diffraction effects. The apparent size of nearly all particles less than 3 μm is nearly the same due to the Becke lines surrounding the particles. Consequently, there is little point in trying to size particles less than 3 μm using the optical microscope and if the distribution of these particles is important then one should use an SEM. The practical lower size limit for the SEM is approximately 0.1 μm, although that value depends greatly on the microscope and can be much less.

In general, the magnification for the SEM should be set between 500 and 2,000 times. The exact magnification will depend upon two main factors: the size distribution of the particles and the chosen resolution. For previously untested samples, it is a good idea to examine one specimen on the SEM before analysis. This initial examination aids in estimating the size range of the particles and in choosing an appropriate magnification.

Resolution will also impact the choice of magnification. For the SEM, there are three components of total resolution, as follows: (1) the SEM resolution (influenced by the scan rate); (2) the SEM digital storage resolution (generally expressed as the pixel area, i.e., 640×480 pixels); (3) the image analysis resolution (also expressed as a pixel area). The ultimate resolution of the analysis is governed by the lowest of the three resolutions.

Whether using the SEM or the OM, it is important to set a lower size limit at the outset of the analysis. You, as the analyst, may not be in a position to know that lower limit. In that case, you should discuss it with the requestor and certainly, the lower limit should be clearly stated in the experimental results.

7.5 Image Collection

Once we have produced an image with the microscope, we have to collect and store it in some fashion. Modern image analyzers work with digital images. Images are stored as an array and can be imagined as a chart with x and y axes as the position and z as the gray level and/or color. The computer applies mathematical operations to each of the pixels (picture elements) in turn as part of the image analysis method. Consequently, all images must be in a digital format. Modern SEMs produce digital images and so image analyzers can work directly with SEM images. Optical microscopes, however, produce analog images that then must be converted into digital ones. Digital cameras do this analog-to-digital conversion internally, whereas analog cameras collect the analog image and one must have an analog-to-digital converter board in the computer. These boards are not generally standard on computers and must be added. Digital cameras are so common today that it is rare to find an analog camera even though they do have some distinct advantages in speed over the digital ones.

In most cases, the operator has some control over the size and resolution of the digital image and certainly has control over the manner in which the image is stored. In general, it is best to store images with formats that have a minimum or no image compression such as bitmap (.bmp) or TIFF (.tif). JPEG is a quite common storage format but does have varying levels of compression. The format removes "empty" pixels for storage then interpolates between informational pixels. This sort of operation works well for human vision since our eyes are quite good at smoothing out image irregularities. It does not work so well with image analyzers. Some image processing operations use pixels surrounding ones of interest as part of the mathematical operation. Interpolation or null pixels can produce ugly artifacts as a result of the operation.

Image sizes of 640×480 pixels produce reasonable images for human vision, but are at the lower limit for image analysis. With current computer power and storage capabilities, it is not unreasonable to store and operate on images in the 1,300×1,100 and 2,200×1,600 range. It is important to match the required minimum feature size with the image resolution. Let us say we wish to measure features down to 3 μm in an optical microscope. If we store our image from the optical microscope at 640×480 pixels and the magnification is such that the calibration works out to 0.5 μm per pixel, then the minimum size we can measure is 5 μm. This assumes that we must have at least 10 pixels to accurately measure a feature. If, on the other hand, we store the image at 1,000×800 then the calibration will be 0.19 μm per pixel and the minimum size is 1.9 μm. Alternatively, if we store at 2,000×1,600, then the minimum size is 0.5 μm and the added accuracy may not warrant the added time for analysis and the required storage space. In any case, it is clear that we must take such matters into account when developing a method for analysis.

Nearly every optical microscope will have some unevenness in the illumination of the field-of-view. This unevenness can be due to poor alignment of the microscope bulb and optics or by some dirt on the lens. The human eye is well able to accommodate quite a bit of image unevenness and artifacts, but computers are not so accommodating. Different image analysis software systems have different ways of correcting for background illumination irregularities. In general, though, one collects a reference image in an open area of the slide under the same conditions used for analysis. This reference image is then used to adjust the illumination of the final image. The need for shading correction can be reduced by ensuring good alignment and cleanliness.

Another pesky part of optical microscopy image collection is the white balance of the camera. If using black and white images, it is not so critical, but for recording and doing image analysis on color images, it is necessary to adjust the color balance. Again the precise manner of doing a white balance correction is different for different image analysis software and, frankly, the quality of the white balance is also dependent on the software manufacturer. Some are much better than others.

At the time of this writing, there are very few if any image analysis systems directly connected to SEMs. In other words, the image analysis program does not control the functions of the SEM such as the stage and/or the image collection. One collects and stores the appropriate images and then sets the image analysis program to work on them. On the other hand, there are a number of software packages that not only control the optical microscope, but also control a high-precision sample stage. Older systems had some problems with focus on the optical microscope, but newer systems have solved most of those issues. Still, if you plan to measure very small particles on an optical microscope with a high-power (100×) objective, be prepared for problems in the focus. The depth of focus is so small for the high-power objectives that it is quite difficult for any image analyzer to consistently determine focus from one field of view to another.

7.6 Image Processing

Image processing is common to most of us with the advent of digital cameras for personal use and the availability of image software to display those personal images. At a minimum, they allow us some control over brightness and contrast after the image has been collected and some programs also allow us to reduce noise or crop the picture. All image analysis software includes a broad range of different types of image processing steps to help us improve the image for analysis.

Image processing for personal images is designed to improve the overall visual impression of the image. Image processing for analysis is used to improve the features so that they can be separated easily from the background (segmentation) and all pixels in a feature are kept together (discrimination). These operations are a major source of potential error and should be treated with care. It is critically important to assess the effect of any image processing operations on the size and shape of the particles of interest. This requires some judgment. It is rare, indeed, that any imaging operation will exactly duplicate the size and shape of every particle of interest. There will undoubtedly be some error for some particles. We typically strive for an optimum so that most particles are accurately represented.

I typically only use two image processing operations. The first is boundary sharpening (delineation) and the second is a median filter to reduce noise. Delineation serves to accentuate the boundary of the particle. Figures 7.5a, b demonstrate the effect of the delineation step. This is a particularly difficult sample for image analysis. It is a topical antibiotic with drug particles suspended in the matrix of petrolatum. The petrolatum is semicrystalline with some acicular structure. The sample preparation is particularly challenging and specimen conditioning in a low-pressure environment is required to obtain reasonably good images. Still it is necessary to further highlight the boundaries of the particles using a delineation step. Notice in Fig. 7.5b that the general appearance of the particles has been altered in an unacceptable fashion for visual examination, yet the boundaries have been sharpened and made more distinct from the background. This is a

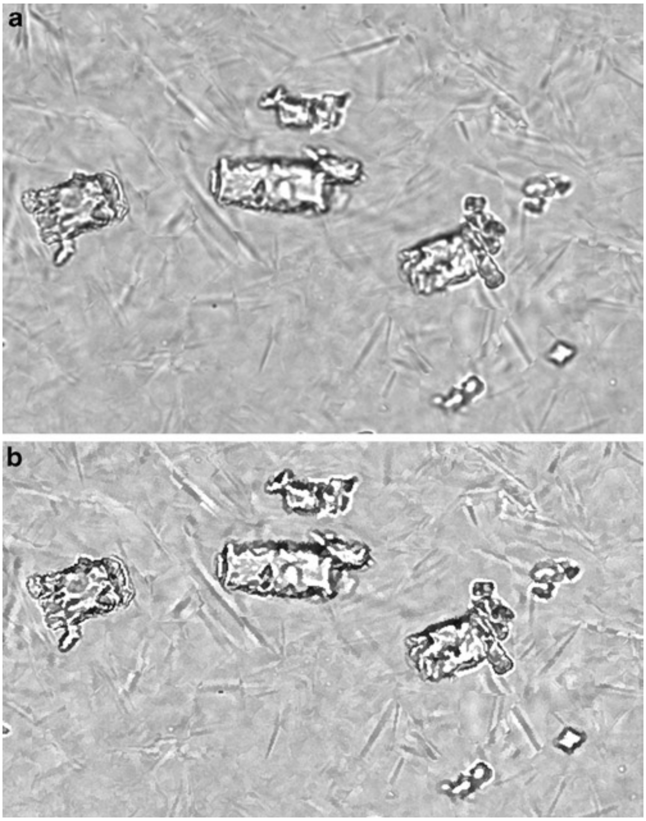

Fig. 7.5 Topical particles in petrolatum matrix (**a**) original image (**b**) boundary sharpened. The topical suspension was spread onto a glass slide and conditioned under vacuum. Note in (**a**) that the boundaries of the particles are quite difficult to detect for some particles. A delineation step was applied to the image in order to accentuate the particle boundaries and produce (**b**). Delineated boundaries aide the segmentation and discrimination operations (image size = 75 × 56 μm)

common feature of image processing. An operation which is beneficial for image analysis may result in poor images from a visual aspect. One should always test the effect of image processing on segmentation and on the final data.

Use of a median filter followed by a sharpen filter is a way to eliminate visual noise from an image. The median filter takes the median pixel value from a matrix (usually 3 × 3) of pixels and replaces the central pixel value with the median value. It is a way of smoothing an image and reducing noise that has been introduced in the image collection and digitization processes. The resulting image nearly always has softened edges and so a follow-up sharpen step is useful for regenerating good edges. These filters are not routinely used but can be quite helpful with noisy images.

There are a number of different gray-level image processing operations that may be helpful for specific situations. They can be just the ticket for some tricky images, but one needs to be very careful in using a processing operation on a large number

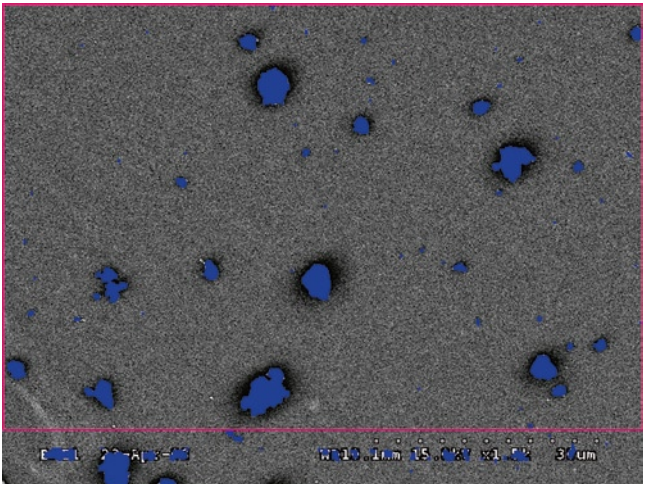

Fig. 7.6 SEM image after segmentation. The particles colored *blue* are those particles that have been segmented using a thresholding operation. The analyzer has selected those particles for further binary operations and for measurement. The *red box* outlines the field of view for measurement and eliminates the text at the bottom of the photomicrograph (image size = 92 × 75 µm)

of sequential images in an automated analysis. What works for the initial couple of images may not at all work for every image. The robustness of these operations must be evaluated and documented.

7.7 Segmentation

Segmentation can be defined as the process of dividing the image between regions that correspond to the object of interest and regions that correspond to the background or to objects that are not of interest (noise). Thresholding is one way of doing segmentation. The thresholding step defines the object of interest as having a set range of brightness values and defines all other brightness values as background. This operation results in a binary, black and white image (see Russ 1995, Chap. 6 for more details on this subject). Without question, thresholding is a key element in good image analysis. Rarely, though do we have images that allow for perfect segmentation for every object of interest. Segmentation, then, must be optimized for the particular sample being examined with the purpose of estimating the true size and shape of most of the particles. Modern image analyzers typically provide a number of different thresholding operations. Some of these combine a number of different steps and can be quite useful. An example is *Contrast Thresholding* from Clemex. Again, the cost of the power and flexibility of these operations is additional complexity and the necessity for a skilled operator to conduct the test.

When doing the gray-level threshold we are presented with a graph of gray levels (256) by intensity. Ideally, the items of interest will have high gray-level intensity whereas the background is low. Then we can select the high intensity region for further analysis. Normally, though, we have a broad distribution of gray-level intensity. Hence, the need to optimize the thresholding. Clearly, the more contrast we have in the image between the items of interest and the background, the more accurate the thresholding. There are situations, though, where we are limited with regard to our sample preparation and so there may be little difference between the gray levels of the objects of interest and other objects or the background (topical samples are an example). In those cases, we may need to be creative in our approach to thresholding.

It is always a good idea to collect an initial image at the start of analysis and set the gray threshold with that image. If you do that from within the routine and then save the step, the routine will automatically use the new thresholding conditions.

Figure 7.6 presents an SEM image where the blue colored particles have been segmented using a thresholding operation. These blue features have been selected for binary operations and for eventual measurement.

7.8 Binary Operations

The final size and shape measurements are made on the segmented, binary images. There are, however, a number of binary operations that can be used before measurement to improve the accuracy and precision of the analysis. As with gray-level image processing, there are any number of binary operations in most modern image analysis programs. Some of these operations are quite specialized and are not often used in routine analysis. The operations discussed below are the most common for pharmaceutical samples. I divide the following discussion into operations that improve the features prior to measurement and those operations that are intended to remove noise and unwanted features.

7.8.1 Feature Improvements

7.8.1.1 Closing

Closing connects the boundaries of objects. Optical microscopy images in particular may threshold such that the entire feature boundary is not segmented. The uneven illumination inherent in transparent particles with only edge contrast is the cause of this problem. Most modern programs allow for some flexibility in the manner of the closing such as the distance between pixels that will be connected as well as some geometrical options. For instance, diagonal closing along with straight lines can be available. As with nearly every image analysis operation, it is best to become familiar with the operation before applying it in "real" tests. Also, it is good to test a number of images in your sequence rather than just the first one. I have occasionally filled in every pixel in the image by being just a bit too heavy handed with this step. One has to be particularly careful using this operation with heavily populated fields of view. The Closing step may, in fact, join features together that should remain separate.

7.8.1.2 Fill

The Fill operation fills all of the open area within the boundaries of an object. This operation is particularly helpful with optical microscopy images. With a normal brightfield image, one nearly always gets a segmented boundary due to the Becke lines, but the transparent interior of the features is rarely completely segmented. The Fill step eliminates this problem.

7.8.1.3 Separate, Disconnect, Bridge Removal

One of the most important characteristics of good sample preparation is well-separated particles. It simply is not possible, though, to prepare a sample with no touching particles and so a particle separation step is often needed. We must be very careful with these operations. We will probably cut particles that should not be separated and the shape of those cuts will not be natural. So we should approach this subject with some trepidation. This operation must be tested with many real images to be analyzed. You will inevitably be forced to make some compromises between separating segments of a particle that ought not to be separate and not separating those that should be separated.

Figure 7.7 presents an example of this type of operation. Image A is a backscattered electron image with touching granules. Image B shows particle separation. This is a good example of the sorts of difficulties that we face with touching and agglomerated particles. Four of the granules are well separated whereas the central clump has what appears to be two or three touching granules. The program broke this central clump into two features. It is likely, though, that the lower pink feature is actually two granules in very close proximity to each other. It is simply not possible to judge with any degree of certainty the nature of the attachment of these granules to each other. Consequently, there is some judgment required by the operator. If you are using the method early in development and are only looking for approximate size and shape values, then you may wish to do no or only minimal separation. At the stage where you need to develop a project-specific method, then I recommend including an assessment of the feature separation operation as part of your method development.

There are a host of other binary operations that have quite specific uses, an example of which is the disconnection and reconnection of crossing elongated features. Since many drug substance particles are elongated and even fibrous, this

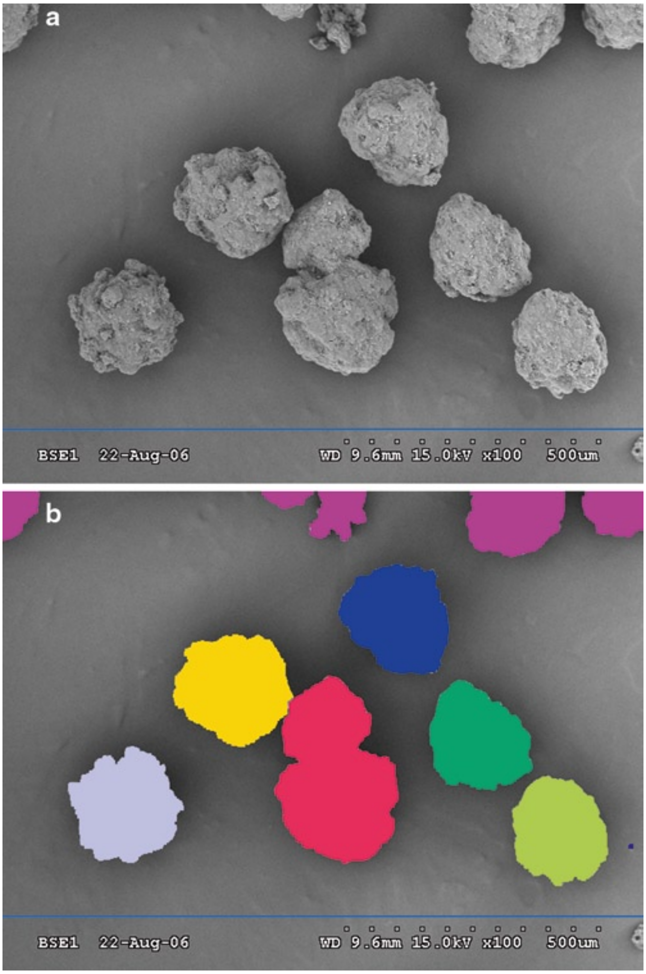

Fig. 7.7 Illustration of particle separation, (**a**) original image (**b**) segmented and separated particles. This figure illustrates the trade-offs that are required when doing a feature separation. The computer algorithm will separate the particles according to rigid rules that may not exactly correspond with our judgment of how the particle should be divided. Our judgment may also be unsure since many cohesive powders contain both resilient and easily broken agglomerates. Particle separation steps should be handled with great care (image size = 1220 × 990 µm)

operation can be quite helpful. There are also a number of operations that come from texture analysis such as erosion and dilation steps. Again these operations can be quite useful in many circumstances. In my programs, however, I tend to be sparing with both the image processing and binary operations. I depend more upon good sample preparation and good inherent feature contrast from the microscope. It is a matter of preference, but in my experience, the more processing steps there are in a program the more opportunity for the program to fail and yield unreliable results.

It is good, though, to be familiar with all of these processing operations since they do have specific and important applications.

7.8.2 Removal of Unwanted Features and Noise

7.8.2.1 Border Transfer

In general, every image will have features touching or cut-off by the borders of the image. Consequently, there is a need to deal with these partial features. There are a number of choices. One can minimize the problem by stitching together a series of images to make one large image and analyze it. It is true that there are still features touching borders, but they are fewer. The disadvantage to this choice is the stitching feature. It can cause feature artifacts wherein the superimposed features do not line up exactly. This produces a feature that appears to have a shadow and can lead to errors in the thresholding operation in particular. Another choice is to use the border transfer function to eliminate all features touching a border. One then just collects a number of different fields of view and counts all particles not touching borders. This leads to other statistical errors (get references). When using the border transfer function, the common approach is to ignore border particles on two sides of the image, say upper and right edges. This leads to the best statistical sampling. In Fig. 7.6, the red box is the measurement area and particles touching the red lines are subject to removal.

7.8.2.2 Object Transfer by Limits

This operation can be extremely useful. Figure 7.8 presents a segmented image of the glass beads in Fig. 7.2. We are interested in only the nearly spherical particles. We can use the "Object Transfer by Limits" (as it is referred to in the Clemex software) to highlight only the spherical particles. In Fig. 7.9, all objects with a roundness value less than 0.7 are transferred to the Purple color. You can simply eliminate these particles or measure them as a separate distribution. The red particles are those touching the borders and will not be counted. Note that touching particles have been separated as well. The next step is to eliminate noise with a Trap function.

Object transfer can be done on color, gray, and black and white images. There are a large number of descriptors that can be used for this purpose. In other words, in most modern programs, it is possible to use many different measurement parameters and other feature descriptors for object transfer.

7.8.2.3 Trap

The trap function is used to eliminate very small objects and noise. It is a necessary step for all routines. The size of the trap is given in pixels and so the appropriate

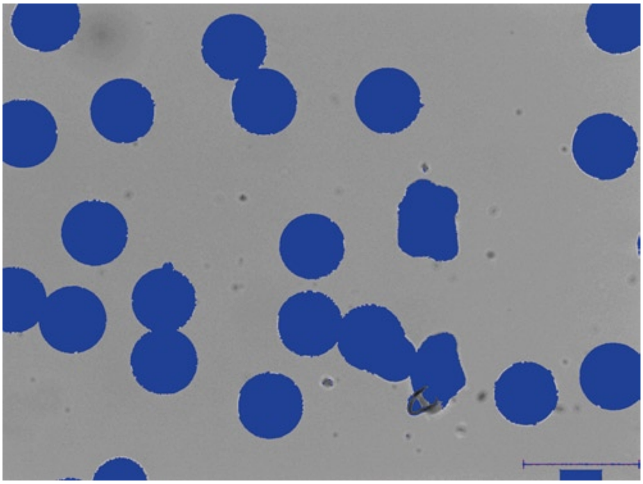

Fig. 7.8 Segmented glass beads (Fig. 7.2). In this image all of the *blue* particles are those that have been segmented. Partial particles are touching the boundaries, there are some features that are not spherical and there is noise. Binary operations can be used to eliminate all of the unwanted features (image size = 700 × 520 μm)

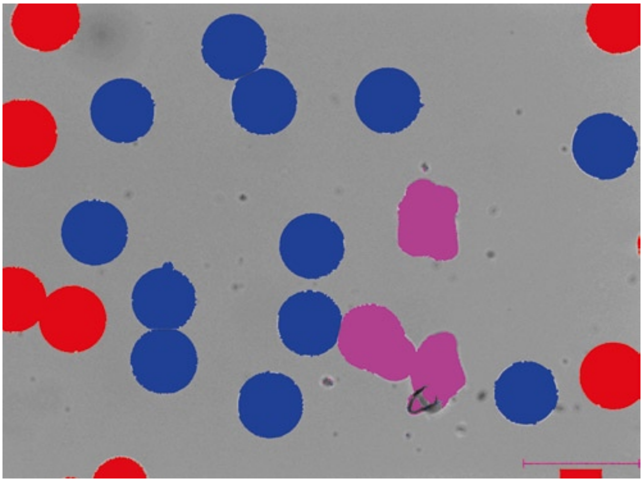

Fig. 7.9 Glass bead image that is segmented (*blue*), separated, border removed (*red*), and roundness deselected (*purple*). A number of binary operations have been applied to image 7–8 in order to select the desired features for measurement. Note that there are some very small *blue* features that still remain. The Trap function described below will remove the noise (image size = 700 × 520 μm)

size will depend upon the magnification and resolution of the image. It is good practice to determine beforehand the smallest feature of interest and set the trap to eliminate all particles smaller than that size. You can accomplish an equivalent operation by using the Object Transfer by Limits and using a circular diameter or

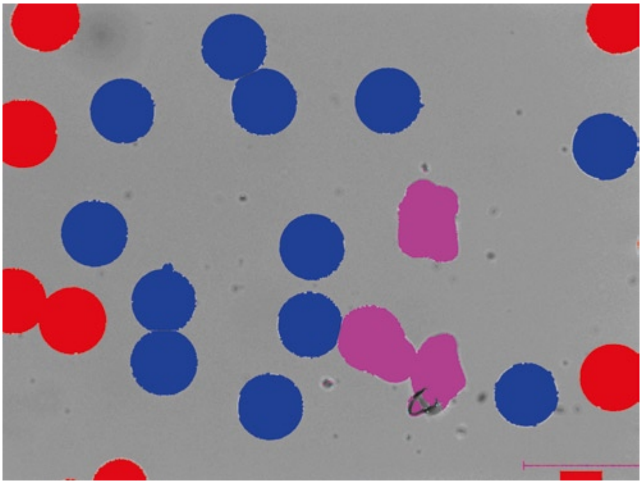

Fig. 7.10 Elimination of noise by trap function. The trap function has been applied to Fig. 7.9 and the small noise features have been eliminated. This image is now ready for measurement (image size = 700 × 520 μm)

area to eliminate small particles. The advantage to the latter is that the selection property is in micrometers and so is independent of magnification and resolution. Figure 7.10 illustrates the use of the trap function. The very small objects in the image have been moved to a different bitplane (blue-green) and will not be measured with the blue objects. One can, however, measure the size and shape of the green objects, if needed. It may be somewhat difficult to see these features in Fig. 7.10 on an initial glance. If you look at the oblong purple particle in the lower right, you can see a number of small green objects associated with this object. They are small noise features eliminated by the Trap function.

7.9 Measurements

7.9.1 General Considerations

There is a truly baffling variety of different size and shape parameters available with image analysis. Undoubtedly, each of these parameters has been found useful in some application or another, but only a few of them are useful for pharmaceutical analysis. In this section, some general principles around measurements are discussed. There are a number of excellent texts on this subject and those by John Russ (1988, 1995) are among the best. One book I have found to be particularly useful was produced by the Joyce-Loebl company of Great Britain in 1985 entitled *IMAGE ANALYSIS: Principles and Practice* (1985). Of course it is outdated and the book is unavailable except through used book stores, but I find it to be one of the finest and simplest books on basic topics and well worth the trouble in obtaining it.

7.9.2 Field Measurements

One important categorization of measurements is generally titled “Field” and “Object” measurements. Field measurements calculate values for the entire field-of-view without reference to individual features. For instance, the area fraction of API in a tablet cross-section could be calculated as a Field measurement. The utility of this type of measurement is based on the basic stereological law that states that the volume fraction of a phase is the same as the area fraction of that phase in a cross-section. The following authors present the principles and history of stereology: DeHoff and Rhines 1968; Underwood 1970; Weibel 1979; and Russ 1986. Before the advent of computer-aided analysis, Field measurements were much easier to make using manual methods than were object measurements and are still commonly used in mineralogy and metallurgy. Field measurements are rarely used in pharmaceutical analysis but you should be aware of them both because they can be useful and because most modern analyzers require that the operator chose between Field and Object measurements. Common Field measurements are area, count, perimeter, and chord size.

7.9.3 Object Measurements

The most common measurements in pharmaceutical development are Object measurements. In these, individual features are identified using some object recognition algorithm and then various measurements are made on these identified objects. The results of these measurements are then tabulated listing each feature and the corresponding values for each of the selected measurements.

The most common Object measurements in pharmaceutical analysis are the following: area, length, breadth, Feret diameters, perimeter, circular diameter, spherical diameter, aspect ratio, and roundness. Except for area, all of these measurements can be realized in a number of different ways (i.e., different algorithms for the calculation) or can be defined in different ways. Aspect ratio is most commonly defined as length divided by width, but some define it oppositely. Roundness and sphericity are defined in a host of different ways. A few mathematical formulas are presented below. It is crucially important to know the nature and definition of any geometric measurement that you use. It is, oh so easy, to just list any number of measurements in your program since computing power imposes few limitations on the number of and kind of measurements that can be collected. It is usually better to think carefully about what you want to measure before starting.

In the following sections, I make a distinction between direct and derived measurements. Direct measurements are those that use the number and arrangement of pixels to calculate geometric parameters. For example, area is simply the total number of pixels times the calibration factor. Derived measurements are those that use geometric parameters to calculate new geometric parameters. An example is aspect ratio which uses length and width to calculate a dimensionless value. I think this distinction is important when interpreting results and evaluating error.

I do list a number of miscellaneous parameters but the list is by no means complete and exhaustive.

7.9.3.1 Direct Measurements

Area

Area is the simplest of all measurements and the most straight-forward. Once the feature is segmented (i.e., recognized as a feature of interest), the area is defined as the sum of the pixels in the feature. In order to convert pixels to a recognizable size, one uses the calibration factor determined from measurements of known distances. For instance, if a feature has 215 pixels and the calibration factor is 1.0 μm^2/pixel, then the area of the pixel is 215 μm^2.

Perimeter

Perimeter is a useful concept that has some subtle measurement complications, particularly with image analysis on the microscope. The actual measurement itself is simplicity itself. One counts the number of pixels along the boundary of the feature and applies the calibration factor. It is the interpretation of the perimeter measurement that can be complicated. There are two complications in perimeter measurements that should be borne in mind by the analyst.

The first complication lies in the measurement itself. We are trying to measure a thin boundary line with square pixels. This is inevitably going to lead to some error just as a matter of pixelation. The second complication lies in the fact that perimeter is directly related to magnification. The greater the magnification, the larger the perimeter. It is this fundamental observation that lead to the development of Fractals (Mandelbrot 1983). For these reasons, we need be careful when using perimeter or when our derived measurements use this parameter.

Feret Diameter

The Feret dimension can be defined as the distance between two parallel tangents on the boundaries of the feature (Fig. 7.11). It is generally formed with reference to the horizontal and vertical axes of the screen. Many image analysis systems allow the user to specify the number of Feret's that are collected in measurement by number or by angle. For example, 8 Feret's would be developed at angles of 22.5° while 64 Feret's would be developed at 2.8°. Clearly, more Feret dimensions lead to greater accuracy in describing features but also require more computer memory.

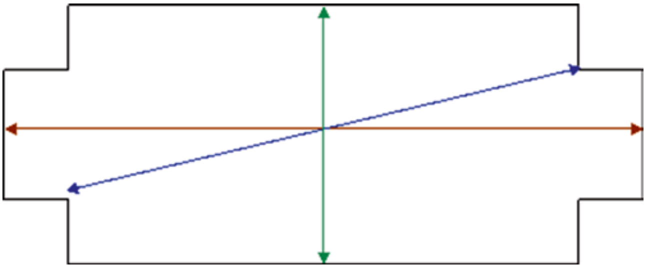

Fig. 7.11 Illustration of Feret measurements. Each of the *arrows* represents a different Feret diameter

Feret diameters were developed with manual microscope counting since they could be measured by centering the feature so that the eyepiece crosshairs are in the geometric center of the feature, then rotating the stage by different angles and measuring the edge to edge distance.

The Feret dimension is one of the most common measurements in image analysis and is used as the basis for quite a few other derived measurements such as length (largest Feret) and breadth (smallest Feret). Feret diameters can be quite useful for the description of irregularly shaped features that are approximately circular. Feret diameters can be quite misleading when dealing with acicular and with rectangular features and other unusual shapes.

You should spend some time experimenting with Feret functions and how they operate with different features since they are such a common measurement.

7.9.3.2 Derived Measurements

Equivalent Circular Diameter and Equivalent Spherical Diameter

Equivalent circular and spherical diameters are common measurement concepts in all particle size and shape science. The equivalent circular diameter can be defined as the diameter of a circle having the same projected area as that of the feature of interest. It can be calculated using the following formula:

$$D = 2\sqrt{\frac{\text{Area}}{\pi}}.$$

Circular diameter is a convenient comparative measurement. Numerical comparisons of area seem somewhat difficult to understand, whereas diameter (length) comparisons are fairly intuitive for most of us. As an illustration, consider two circles with diameters of 20 and 30 μm, respectively (Fig. 7.12). Intuitively, we think that the latter is a third larger than the former. The corresponding area values though are 314.2 and 706.9 μm^2. The larger circle has ~2.2 times the area of the smaller one.

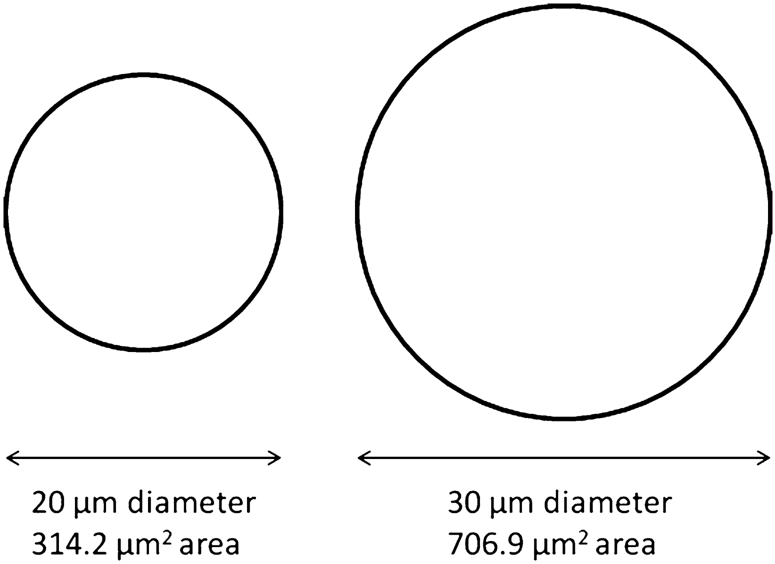

Fig. 7.12 Comparison of area and circular diameter of two circles. These *two circles* represent features with diameters of 20 and 30 μm each. The areas then are 314 and 707 μm^2. It is somewhat easier to understand the diameter difference than the area one

While this difference is quite meaningful and has important physical implications, the diameter comparison is more intuitive.

While the concept of equivalent diameters is useful, the application is host to a number of different errors of which I will only discuss a few here. In general, the equivalent diameters are susceptible to overestimations of true size. Both OM and SEM produce two-dimensional particle projections. Errors can be introduced by the projection of the edges and by the lack of measurement in the third dimension. Consider pyramidally shaped particles. Whether the point is up or down, the image analyzer will measure this particle as a square. Both size and shape measurements will be in error and, in general, the measured values will be greater than the real ones.

Another important three-dimensional effect is caused by the fact that most real particles will lie on the substrate (glass slide or SEM stub) with its shortest dimension parallel to the light or electron beam path. As an example, let us suppose that we have a cube of wood that is 100 cm in each dimension. No matter what edge we measure we come up with correct values for the size of the particle. Now consider the result of cutting a 10 cm slice from another cube and allowing that slice to fall onto a surface. The 100 × 100 × 10 cm slice will fall so that the 100 × 100 cm face lies flat (horizontally) on the surface and the 10 cm dimension is vertical. If we look down at the slice and look down at the original cube, we will make the same measurement for cube and slice – 100 × 100 cm and conclude that the two have the same dimensions – clearly an error. All microscope measurements suffer from this error of projection and should be considered as part of the error analysis in method development. This effect is particularly pronounced in measurement of plates and rods.

Consideration of the third dimension leads naturally to volume concepts in image analysis and in particular to equivalent spherical diameters. This parameter can be defined as the diameter of a sphere having the same volume as that of the object being measured. Since we compound assumptions (particle is a sphere, knowledge of third dimension) in this measurement, it is highly prone to large errors. Even so, it can be quite useful in pharmaceutics. The most common particle size instruments used for drug substance are those based on static light scattering. Light scattering instruments produce a volume weighted diffraction diameter, so comparisons of IA with light scattering should be on a volume basis, hence the need to convert two-dimensional IA measurements into volume values.

The measurement parameters that we have discussed so far have all been related to size. One important advantage to an image analysis system is its ability to provide information on particle shape. While there may be many different ways of approaching the measurement of shape, two of the most useful in pharmaceutical analysis are those related to elongation and circularity. Shape factors describing self-similarity (fractals) seem like they should be useful, but there is not much in the literature on applications of this parameter. In any case, the following discussions will revolve around ways of assessing these three measures of particle shape beginning with elongation.

Aspect Ratio

The most common shape factor used in pharmaceutics is aspect ratio. Despite being so common, there is no universally accepted definition of aspect ratio. I recommend you look at Table 1 of Almeida-Prieto et al. (2007) which lists many of the ways the aspect ratio has been defined in the pharmaceutical literature. Herein, I am using the definition of aspect ratio being the length divided by the width. One reason this geometric parameter is used so often is our intuitive understanding the concept. Using the above definition, if I say two particles have aspect ratios of 10 and 3, we can picture quite easily the idea that the first particle is longer and thinner than the second. While easy to understand, aspect ratio can be particularly tricky to measure in image analysis. The following paragraphs present a number of ways the aspect ratio can be determined. It is getting boring I know, but I will say again; do not use the default aspect ratio in your program without rigorously testing its applicability for your application. There is no substitute for a full error analysis.

The most common definition of aspect ratio is longest Feret (length) divided by shortest Feret (width) or by breadth. While common, this definition is susceptible to numerous errors. For instance, with a rectangle the longest Feret will be the diagonal with a consequent overestimation of the aspect ratio. It is useful for some applications and for some shapes, but is not recommended for routine use with pharmaceutical drug substance particles.

Another method of calculating aspect ratio that reduces some of the errors in the use of longest and shortest Feret diameters is the length squared divided by area. One way of determining this value is given as follows:

$$\frac{(\text{Feret Length})^2}{\text{Area}}. \tag{7.1}$$

This formula reduces some of the errors due to the measurement of width, but suffers from some inaccuracies for rod-like objects since the Feret length is likely to be the longest diagonal. A better variant on this calculation uses a different measure of the length – the Feret diameter at right angles is assumed to be the shortest Feret (called Main Length here and in the Clemex software) and is defined as follows:

$$\frac{(\text{Main Length})^2}{\text{Area}}. \tag{7.2}$$

I find that this parameter is one of the best for general image analysis and I typically try this aspect ratio before looking at others. It typically fails at very small aspect ratios (1–2) and very large aspect ratios (>15).

Another method of calculating aspect ratio uses the concepts of inner and outer circles and the diameter associated with each. Figure 7.13 illustrates these concepts and the aspect ratio based on them. Clearly, this aspect ratio is highly sensitive to projections and so should be used with care.

It may be frustrating, particularly to managers and supervisors, but there is no such thing as the correct definition of aspect ratio. All of the different definitions and formulas have been developed in order to circumvent problems with standard formulas. Each has its advantages and disadvantages and it behooves us as the analyst to choose the parameter that pragmatically fits the needs of the project. Inevitably, the final measure of the utility of a parameter is its ability to help explain and predict bulk product properties.

Roundness

Circularity measurements compare the shape of the particle to that of a circle. A value of 1 indicates that the shape of the object is close to that of a circle. The measurements nearly always involve measurements of area, perimeter, and length. As with aspect ratio, the terminology of circularity can be confusing. In every case, 1 is considered a circle but depending on the definition the values can go from 0 to 1 or 1 to infinity. It really does not matter which is used, providing one can develop a correlation between particle circularity and bulk product performance. Intuitively, it is difficult to say whether describing the circularity as 0.5 or 2 is more understandable.

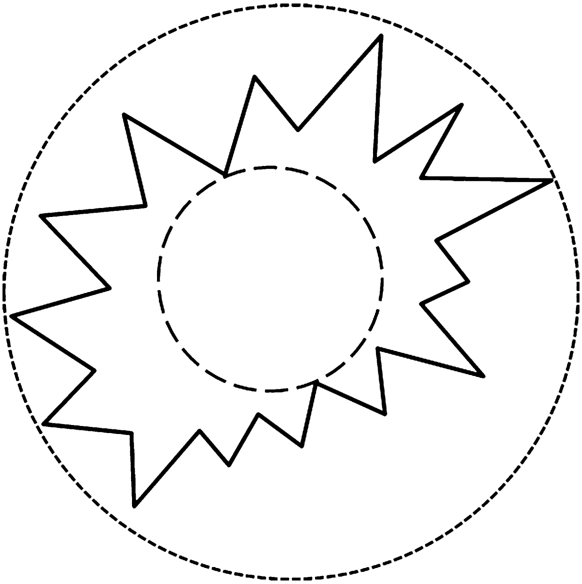

Fig. 7.13 Illustration of aspect ratio based on inner and outer circles. The central feature has a number of projections and the aspect ratio can be determined by the ratio of the outer diameter to the inner diameter. In this case, the aspect ratio is 2.6

Properly we are measuring circularity not sphericity. In every case, we are observing a two-dimensional projection of the object on the microscope. In the special case of a circle, however, the properties of the circle will be the same as the sphere – if it is truly spherical. That assumption must be tested for accurate projections to three dimensions.

Roundness and sphericity are generally measured using some variant of the following formulas:

$$\text{Roundness} = \frac{4 \times \text{Area}}{\pi \times \text{Length}^2}$$

$$\text{Sphericity} = 4 \times \pi \times \frac{\text{Area}}{(\text{Perimeter})^2}.$$

You should check your image analysis program for the exact equation. Most software allows you to develop your own parameters if you wish.

7.9.3.3 Miscellaneous Measurements

The software programs I routinely use (Clemex, ImagePro, Zeiss, etc.) list more than 60 different measurements. This number does not count the possible number of combinations of measurements. All of these measurements are (or have been) useful for one application or another and it is good to have such diversity available to us. On the other hand, the accidental selection of an inappropriate parameter is easy and can lead to unfortunate consequences, like the public, close questioning of the meaning of the parameter by a big boss. Not that I would know about that, of course. We need to be careful with the use of any geometric parameter and particularly careful with the use of derived ones. One should understand exactly what is being measured and the strengths and weaknesses of that measurement. Testing of the applicable limits of the measurement is crucial. Do be careful with terminology and mathematical formulas. Image analysis programs use different terms for the same formula and different formulas for the same terms. Be absolutely clear on the formula used for the measurement and include that formula with any report or experimental write-up. With those warnings, I will list a few measurements and a bit on their use.

Convex Perimeter. This parameter is the perimeter of a polygon whose sides just touch the protruding points of the feature. It is formed by joining the tangents of the Feret diameters. Imagine a star-shaped object and the convex perimeter is the box joining the star tips. The convex perimeter ought to correspond well with a sieve measurement.

Fractal Dimension. Fractals have been defined as “a rough or fragmented geometric shape that can be split into parts, each of which is (at least approximately) a reduced-size copy of the whole” (Mandelbrot 1983). It is particularly useful for irregular shapes that are difficult to compare with ideal geometric categories, such as circles or squares. Fractals are often said to have the property of “self-similarity” although that is not the only requirement for classification as a fractal. Fractals have a numeric value related to the self-similarity. This concept is appealing since it was formulated for shapes similar to those we see in pharmaceutical development. The irregular surface of a coastline is not that dissimilar to the shape of a micronized particle. The practical application of the fractal dimension to pharmaceuticals is not well-established and requires more study.

Inner and Outer Diameters. The outer diameter is the diameter of a circle that encompasses all the protuberances of the feature, whereas the inner diameter is the diameter of the largest circle that fits completely within the feature.

Martin’s Diameter. This value is the diameter cutting the feature into two parts having equal areas. It was used mostly with manual image analysis techniques and is less used today.

Roughness. This parameter provides a measure of the smoothness of the feature boundary. It is often calculated as the convex perimeter divided by the perimeter. Naturally, the measurement suffers from all of the problems inherent in perimeter measurements with the image analyzer. Also, one should be careful to make this measurement before using any smoothing or other image processing operations or, at a minimum, evaluate the effect of the image processing operation on the Roughness values.

String Length and Width. Long, thin, curved features are particularly difficult to measure and, yet, are quite common with pharmaceutical drug substances.

Measurements-based Feret diameters will be grossly incorrect. Consequently, there are a number of ways of determining string length and width. Most have formulas using the perimeter and the area, with all the caveats associated with perimeter measurements. Be particularly careful when dealing with fibrous materials. They pose a challenge not only to image analysis measurements, but also to light diffraction particle size analysis and even to product formulation. In my experience, once the aspect ratio goes beyond about 20:1, none of the image analysis parameters are particularly accurate and, in the end, just knowing that the particles are extremely elongated may be enough.

7.10 Calibration, Performance Checks, and Size Standards

Calibration is a crucial part of image analysis and must be done with care for both the optical and scanning electron microscopes.

While the general calibration operation is similar for all image analysis systems, the particular methodology can be quite different and it is important to spend some time to learning the vagaries of your system. In general, calibration operates as follows:

- Place a standard with known distance markings onto the microscope stage
- Set up the microscope with the objective to be used in the analysis
- Follow the calibration instructions in the image analysis program
- Test the calibration with a performance check

Calibration instructions vary from one image analysis system to another. Some systems allow you to calibrate using an electronic caliper in either the horizontal or vertical direction. In most cases, you place one end of the caliper over the left (or right) edge of the micrometer bar and the other end over the right (or left) edge of the micrometer bar a known distance from the first one. You then input the known distance into the program and it generates a value for the width (and height for square pixels) of each pixel, for example, 0.5 μm per pixel. Other systems use an electronic square so that you calibrate an area not a distance. Some require separate vertical and horizontal measurements that are made by rotating the stage with the micrometer on it. Most image analysis systems that I have used have adequate to good calibration instructions.

You can check the internal consistency of the calibration factors by noting the relationship of the objective magnifications with the calibration factor. For instance, the 40 times calibration factor should be half that of the 20 times one. The relationship may not be exact but should be quite close with modern high-quality objectives. If there are disparities, it is time to check the calibration. For optical microscopy, it is not necessary to calibrate the instrument frequently. Calibration is only necessary if the performance verification check indicates that the instrument is out of calibration.

Performance and calibration checks are relatively simple with the optical microscope. Most image analysis systems allow you to make interactive, real-time straight line measurements. It is then possible to place your standard onto the microscope stage, chose the appropriate objective and make a measurement. I generally expect the measurement to be within 5% of the known value. This may seem a large acceptable error, but there are inherent difficulties in using a cursor on a computer screen to make interactive measurements. Also, it is my experience that when calibration factors go wrong, they go very wrong and this type of check is sufficient to catch these large-scale errors. I recommend that performance checks be conducted daily before analysis using the objective and image setup to be used in the analysis. I do not think that it is necessary to do a performance check repetitively when using the same objective and image setup for many analyses in the same day. It is good practice, though, to do the performance check whenever changing objectives. Just because one objective is well-calibrated that does not mean that the others are. I generally use my calibration slide to do performance checks.

Calibration of an SEM is similar to that of an optical microscope in that one collects an image of the standard at the desired magnification and setup conditions and then uses the internal image analysis program and calipers to measure a known distance. I generally calibrate with each analysis. Modern SEMs are wonderfully stable compared with older ones, but I have trouble trusting that stability. Older SEMs suffered from lens hysteresis effects such that magnification and calibrations would change as much as 10% with just turning the instrument on and off. Further, the magnification (and calibration) was usually only accurate at one specified working distance and was only approximate at other distances. While modern instruments (post 1995–2000) are more stable, I still think it is good practice to re-calibrate with each analysis. It is not really practical, nor even necessary, to perform routine performance checks if one is calibrating with each analysis.

Accurate, traceable micrometer slides are available from a number of different suppliers. Most are traceable to the National Physical Laboratory (NPL) of Great Britain. The NPL will also recheck calibration standards and provide an accuracy statement. Generally, it is not necessary to re-calibrate a standard but some, overzealous in my opinion, quality assurance people require recalibration on a regular schedule. Size standards for the SEM are also available from a number of different suppliers. These standards can be problematic if one is operating at high magnifications since it is difficult to manufacture and test very small standards. Of course, standards traceable to accepted measurement laboratories such as the NPL and the National Institutes for Standards and Technology (NIST) in the USA are best.

7.11 Data Presentation and Statistics

Once a set of measurements has been collected, then it is necessary to present a summary of the data. As with other subjects, you are well-advised to spend some time studying statistics using standard texts in the field. On the other hand, I have found it pretty difficult to learn from either the texts or statisticians. So, even though it is necessary to study textbooks, there is no good substitute for practice and experience. The textbooks are often written for statisticians and mathematicians – even those textbooks purportedly for the scientist and engineer. Talking with statisticians has been, in general, an exercise in futility in my case – with the rare exception. So, I am going to present some very basic ideas in data presentation and statistics as they apply to image analysis and encourage you to explore the possibilities of your own image analysis software package and other software devoted to statistics.

The simplest and by no means least important means of data presentation is a representative photomicrograph. I believe it is important to always include such with a report or experimental write-up. It is much easier to illustrate the unusual shape of features with a photo than with either words or numbers. Be careful, though, to be sure you understand in a general sense the relationship of the features as seen in the photo and the statistical values. For instance, when looking at a photomicrograph of particles with a wide range of sizes, we will naturally weight the particles by number. In other words, the image may have one 20-μm diameter particle and twenty 1 μm particles and we will mentally say there are 20 times as many 1 μm particles as 20 μm ones which is correct. But the volume of the 20 μm diameter particle is approximately 4,200 μm^3, whereas the total volume of the twenty 1 μm particles is approximately 10 μm^3. Since mass is directly proportional to volume, it is clear that our sense of particles by number will not be much in agreement with distribution of mass. In any case, we should be prepared to discuss this subject and be sure that any intuitive sense of the particles as represented by the photomicrograph can be correlated with the numerical data.

The most common way of representing image analysis data is with histograms using frequency or cumulative frequency distributions of the data (see Fig. 7.14, for example). The values for each measurement parameter are segregated into size bins. For example, all particles with a circular diameter between 10 and 15 μm are counted and that number is used for the *y*-axis and is plotted against the mid-point of the size class (12.5 μm in this case). One can either use the absolute values for the count in each size class or use a percentage of the whole number. This is a normal frequency distribution. One can also plot the cumulative values against size classes. With cumulative distributions, the total number of counts less than the specified size class is plotted against the midpoint of that size class. It is possible and desirable to superimpose both the frequency and cumulative distributions on the same graph. It is also common to use a log-scale for the *x*-axis since it is common for particle size distributions to cover more than one order of magnitude. Although the log-scale is a popular representation of the distribution, I personally also like to look at the data with a linear *x*-axis. The linear representation is more intuitive in my opinion.

The graphical representation of data and distribution is as much an art as a science (Tufte 2001). There are any number of ways of presenting data, but remember we are attempting to illustrate some point with the graph. Possibly, we are demonstrating that the particle size distribution of two different batches of the

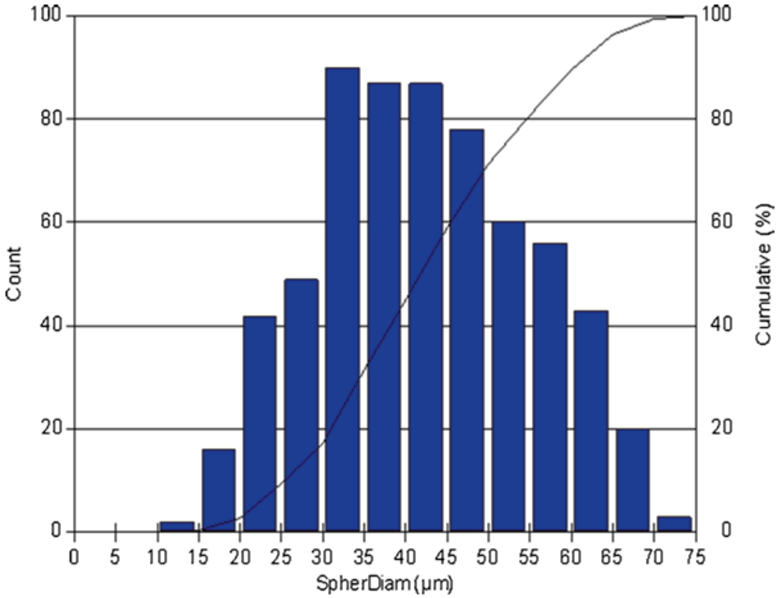

Fig. 7.14 Frequency and cumulative frequency histograms for glass beads. These graphs present the frequency and cumulative frequency histograms for the circular diameter of NIST SRM 1003b glass beads. The y-axis on the left is counts, whereas the one on the right is in cumulative percentage. The *box-shaped figures* are for frequency histogram and show the number of glass beads which have a diameter of each 5 µm size range. The *black curve* represents the cumulative frequency histogram and shows the percentage of beads with diameters more and less than the chosen value. For example, 60% of the particles have a diameter less than 45 µm, whereas 40% of the particles have a diameter greater than that value

same material is the same. Maybe we are demonstrating the agreement of image analysis and static light diffraction data. On the other hand, we may wish to explain why a static light diffraction measurement is not accurate due to the aspect ratio of the particles. In any case, our graph is attempting to make a point, to demonstrate some feature of the analysis results. An accurate graph that is pleasing to the eyes is the goal. So, we have to be careful of some representations that may look good but that are not reflective of the data. For instance, if we are presenting a bar graph of relative frequency versus circular diameter, we should not use three-dimensional bars (Fig. 7.15). Visually, a three-dimensional bar is seen as a volume, whereas a two-dimensional bar is an area. The three-dimensional representation may imply much greater differences than actually exist with the data. Another example is the limits of the y-axis scale. We can choose the scale such that we maximize or minimize the apparent variability in the data. We ought to strive to present the data honestly but artfully, so as to best illustrate the point of the graph.

We also need to provide numerical summary data along with the photomicrograph and the graphs. Every set of data can be characterized with a central tendency (average) and with a range. There are a variety of statistical parameters available to

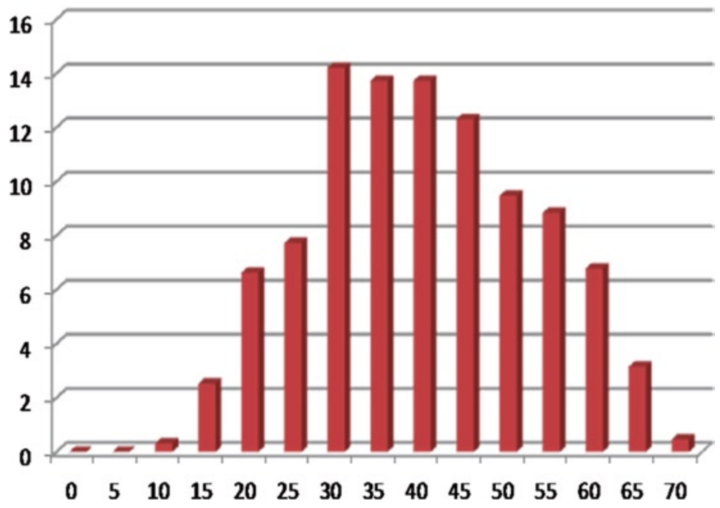

Fig. 7.15 Three-dimensional representation of chart in Fig. 7.14. The data from Fig. 7.14 are replotted as a three-dimensional frequency histogram. Since the depth values for each box are not real, one can chose any depth. Since we are comparing volumes and not areas, note that the difference between the 60 and 65 μm bins appears greater in Fig. 7.15 than in Fig. 7.14. The *y*-axis in this case is a percentage value not a count value as in Fig. 7.14

us and most modern image analyzers have quite a number of selectable parameters. I shall discuss only a few of the possible parameters and those that are in common use in image analysis.

Although population or count is not a calculated statistical parameter, it is clearly important. Population is just the total number of measured features. Since this value speaks directly to the adequacy of sampling, population should always be included in the table of statistics.

We are always interested in the central tendency of the distribution as an average. There are many different averages and all are useful for particular applications. The two most common ones in image analysis are the mean and the median. The median is the 50th percentile or that value for which half the data is smaller and half the data is larger. It can be determined directly from the cumulative distribution. The mean is defined as the total sum of the data divided the number of data points and can be calculated using the following equation:

$$\text{Mean} = \frac{\sum f_i x_i}{\sum f_i}, \tag{7.3}$$

where f_i is the frequency (number, mass, volume, etc.) at the *i*th size class and x_i is the mid-point of the size class. It is good practice to always provide these two values in a table of image analysis statistics. Two other estimates of central tendency that are used in image analysis are the mode (size class with greatest number of counts) and mid-range (maximum plus minimum size divided by 2). The mode can be useful when assessing secondary drug substance processing operations such as milling. We may be attempting to keep the mode the same but alter either the number of fines or larger particles by adjusting milling conditions. The mean and median will be much affected by size extremes, whereas the mode will not. The mid-range can also be useful if we are limited in data sampling but still wish to have some estimate of the central tendency for approximations. For instance, if we are looking at particles in a parenteral solution, we may only have 10s or 20s of particles. This population size is insufficient for good statistical analysis, but an average based on the mid-range may provide an adequate approximation.

Averages are quite useful but can be deceiving without an estimate of the dispersion of the data. For example, if we have 20 people with a height of 150 cm (5 ft) and 20 with a height of 180 cm (6 ft), the average (mean and median) is 165 cm (5.5 ft), yet no one individual is that height. The range (maximum minus minimum) is the simplest estimate of data dispersion. The standard deviation is also useful and common. It is calculated as follows:

$$s = \sqrt{\frac{\sum (x - \text{mean})^2}{n - 1}}, \tag{7.4}$$

where n is the population.

This calculation is the sample standard deviation and should be used when a small sample of the whole is measured. By itself, the standard deviation is not particularly useful in image analysis, but when divided by the mean to yield the coefficient of variation (CV) it can give a sense of the spread of the data in relation to the mean. The smaller the CV, the less spread to the data.

By far and away, the most popular representation for particle size data in the pharmaceutical industry is that based on the cumulative distribution with the median as the measure of central tendency and the 10th (×10, d10, etc.) and 90th (×90, d90, etc.) percentiles as the estimates of the dispersion of the population. In fact, if particle size specifications are required by regulatory authorities, such specifications are nearly always based on these three parameters. Consequently, it is good practice to include them in reports and experimental write-ups even if other statistical parameters are to be used.

7.12 Exercises

Any image analysis program can be used with these exercises. I worked them using Clemex and ImagePro depending on the application. These are both quite sophisticated programs and quite expensive. If such a program is not available to you, you can download ImageJ at no cost (http://rsb.info.nih.gov/ij/). It should be possible to do all of the following exercises with any modern image analysis program. I also assume that you have access to a high-quality optical microscope and SEM. I used either an Olympus BH-2 or a Leica DM/LM to do the OM experiments and either an Hitachi SN3500 or FEI Quanta 400F FEG for the SEM experiments.

For a beginner at image analysis or for someone who has not been active for awhile, I recommend spending some time with the exercises outlined below and with others that you develop. In a busy work schedule it may seem like wasted time, but it is not in my opinion. Remember one of the keys to good image analysis is producing good images with good feature contrast. The more you know how the microscope and image analysis work together, the more efficient you will be at doing your tests. So, take some time and experiment.

A. Sample Preparation

A1. Optical Microscopy

Prepare specimens of glass beads with the methods listed in Sect. 7.3.2. Examine these specimens using an optical microscope and note the differences in particle separation and loading. Now prepare specimens of caffeine form II (as received from the container) and note the differences among specimen preparation techniques and the differences with the glass specimen preparations. Clearly, noncohesive materials are much easier to prepare. If you have made a specimen of caffeine hydrate, duplicate the sample preparation with this sample. Note the fundamental difficulties in producing any specimen that is adequate for image analysis.

A2. SEM Sample Preparation

Now repeat the steps discussed in Exercise A1 using the sample preparation techniques outlined in Sect. 7.3.3. Note similar differences among different specimens and compare with specimens made with optical microscopy.

B. Image Collection

Use the specimens generated in Exercise A with both OM and SEM. Practice collecting images at different resolutions. Also use some of the different imaging techniques mentioned in Chaps. 2 and 4. Save some of these images for the next exercises.

C. Image Processing

Use both OM and SEM images and practice various image processing algorithms available in your image analysis program. In particular, try the delineate, sharpen, median, and smooth filters and note the effects on the image. Most software programs have various options available for each filter and it is useful to experiment with these options.

D. Image Segmentation

Use some of the images from both Exercises B and C for segmentation. You should have various methods available for the threshold step and practice using all of these methods. Note that some minor alterations in the threshold step can have profound effects on what is turned into a binary image and what is discarded. Most programs allow you to outline the features that are binary and overly with the original image. If so, try enlarging the image and examine different features to see how well the segmentation step worked. Also, note that what may work well for one feature in the image may not work so well with another one. We seek an optimum so that most particles are faithfully represented in the binary image.

E. Binary Operations

Use the segmented images from Exercise D and practice numerous binary operations. In particular, try the ones discussed in Sect. 7.8 such as Closing, Fill, Separate, etc. Again vary the options available for each operation and note the effect on the binary particles. As with segmentation, we seek an optimum whereby most particles are improved by the binary operation. Also, try noise removal and transfer by limits.

F. Measurements

All that work to get to this stage! Now that you have generated a number of binary images, practice making different types of measurements. I recommend starting with simple ones – area, length, breadth, circular diameter, and aspect ratio and then move on to more advanced measurements. Most image analyzers allow one to make manual measurements of length using a cursor. It is quite useful to make some comparisons of manual with automatic measurements. They usually will not be the same but should be relatively close (~10%). In particular, note how the aspect ratio can vary for acicular particles depending upon how it is measured.

Do also spend some time practicing making up derived measurements using the standard measurements provided. Some programs make this relatively easy, whereas some make it tough to find the subroutine and to work with it. Note that, in general, you need to name the new measurement and most programs have some limitations on the length of the name and in some cases, the particular letter or symbols are used.

G. Statistics and Data Presentation

Nearly all image analysis systems will give you the mean, median, standard deviation, minimum value, and maximum value for each of the measurements you have specified in the routine. Some programs will also provide some distribution properties such as the 10th percentile and the 90th percentile (50th percentile of course is the median). A few programs are quite flexible and allow you to specify a large variety of different distribution properties.

Collect a reasonable number of measurements (100–250) from some of your stored images and generate basic statistics using a couple of the selected measurements such as area and circular diameter. Look at the different options for generating statistics and, in particular, determine the ease of generating distribution statistics such as the 10th or 90th percentile.

Generate a regular frequency histogram. Adjust the bin sizes to study the effect of more or less bins on the appearance of the distribution. Generate a cumulative frequency distribution and note that it is possible to read the 10th and 90th percentiles directly from the graph.

Even if you do not work many of the exercises that I recommend, do spend some time learning the operation of your image analysis software program. This time is well-spent, in my opinion, since most of these programs are quite sophisticated and require a relatively steep learning curve. The more facility you have with the software, the more efficient you will be in collecting and reporting image analysis data.

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Image Pro (Media Cybernetics): http://www.mediacy.com/

Chapter 8
Polymorphism

8.1 Introduction

In 1996, a New Drug Application (NDA) for ritonavir from Abbott Laboratories was approved for the treatment of HIV. In 1998, many lots began to fail dissolution tests due to the appearance of a new, unknown polymorph. Within a short period of time, all lots of drug product failed dissolution testing. Samples of this new form were brought to the R&D laboratories for further testing, and within a few days, all lots of drug substance spontaneously converted to the new form and it was impossible to make the old polymorph by normal means (Bauer et al. 2001). In fact, when scientists from the R&D facilities visited a bulk manufacturing facility that was successfully using the old form, stocks of DS began to convert to the new form and that facility was unable to produce acceptable product. As Chemburkar et al. (2000) wrote, in a fine understatement, "This put Abbott into a market crisis." This common experience is a good reason to study polymorphism.

Polymorphism is of interest in pharmaceutical development because of the following three primary reasons. The first is that drug substance properties affect drug product performance. As in the case with ritonavir, different solid-state forms have different dissolution and solubility characteristics that can affect bioavailability. Different forms can also behave differently in manufacturing. These forms can also respond differently to accelerated stability trials. The second reason to study polymorphism is that regulatory agencies require companies to study polymorphism and report those studies in order to receive marketing approval. Regulatory agencies need to ensure that the product given to patients in clinical trials is the same product when marketed. Polymorph control is a means of ensuring a consistent product. Further, many regulatory agencies view good control of drug substance solid-state properties as evidence of good manufacturing. Third, different solid-state forms can be patented and marketed. Innovator companies can discover, develop, and conduct all the clinical trials on one solid-state form establishing the safety, efficacy, and stability of the medicine, while another company patents a different form of the molecule and can then piggyback on the work of the innovator. There is, then, a large financial incentive to study and understand the solid-state properties of pharmaceuticals.

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Solid-state studies of pharmaceuticals are empirical in nature. Solid-state chemistry and physics have not advanced to the stage that we can make accurate predictions about polymorphism, solvation, and hydration simply from the chemical formula. As Maddox put it in a 1988 Nature editorial (Maddox 1988),

> One of the continuing scandals in the physical sciences is that it remains in general impossible to predict the structure of even the simplest crystalline solids from a knowledge of their chemical composition.

Even though he was confident that the day was near, it has proven much more difficult to calculate crystal structure from chemical composition.

The study of the solid state in pharmaceutical science involves many different disciplines and analytical techniques with a heavy emphasis on microscopy including polarized light microscopy (PLM), hot-state microscopy, scanning electron microscopy (SEM), as well as IR and Raman microscopy. The objective of this chapter is to demonstrate how microscopy fits into these studies of pharmaceutical polymorphism.

We begin this chapter with a general discussion of solid-state theory with an emphasis on organic chemicals. We then discuss how new forms are discovered and the selection process for form development. The choice of form for development is an important element in pharmaceutical development. We then switch to a discussion of the analytical characterization of polymorphs, followed by an in-depth discussion of caffeine polymorphism as a model compound. Naturally, our orientation is how microscopy fits in all these elements of pharmaceutical development, not as an isolated technique but as part of the whole cloth. This entire book is reductionist in the sense that I isolate and separate microscopy and its contributions in order to aid you, the reader, in your understanding of the subject. In practice, of course, it does not really work like that. Pharmaceutical development is holistic, or should be, and microscopy is one piece of the whole just as one strand of thread is part of a whole carpet. The holistic nature of solid-state analysis is one reason I chose to concentrate on one model compound as an example.

8.2 Theory

8.2.1 Solid-State Forms

8.2.1.1 General Considerations and Nomenclature

Organic chemicals crystallize in a variety of ways. First, they can form salts or can crystallize as a free acid or free base. Each salt and the parent can crystallize in different geometric orientations and form polymorphs. Water or some other solvent can crystallize with the chemical in the unit cell to form hydrates and solvates. Finally, the chemical can solidify without crystallizing to form a noncrystalline or amorphous solid. A particular chemical may have a salt and free crystalline form.

It may have many polymorphs, hydrates, and solvates (Lu and Rohani 2009). Or the chemical may not make stable salts and exist in one form only with no hydrates or solvates. It is this rich diversity of solid-state possibilities that challenges (and excites, to be truthful) the solid-state scientist.

Unfortunately, we do need to discuss nomenclature before proceeding, although I will have more to say about the subject as we go along. To state that the nomenclature around the solid state is not standardized is to make an understatement – and, of course, everyone has strong opinions about correct nomenclature, including myself. I once asked Walter McCrone how he distinguishes among polymorphs, solvates, hydrates, etc. in his writings. He said he did not even bother. The name "polymorphism" was good enough in his opinion to refer to the whole field of solid-state analysis. To some extent, usage has followed his lead and I certainly do so (hence the title of this chapter). If you get a text on pharmaceutical polymorphism, there is some expectation that there will be a discussion of hydrates, solvates, and noncrystalline solids as well. To be succinct, salts and parent molecules from which they derive are completely different chemical entities in the solid state. Polymorphs are the same chemically, but have different three-dimensional order. Solvates and hydrates resemble salts in the sense that they are completely different chemically in the solid state from anhydrates. Solvates and hydrates can have polymorphs themselves and, in general, are crystallographically complicated since the water or solvent can reside in the crystal lattice in different ways. The terminology has branched out to include terms such as pseudopolymorphs, desolvated solvates, pseudomorphs, co-crystals, polyamorphous solids, etc. I personally am quite sparing with the last set of terms since I think salt, parent, hydrate, and solvate cover the field nicely. Most authors are considerate and define their terms as they go along, so just be sure that you know what they refer to when they use particular terms.

Different physical forms of the parent molecule can have different physical properties. The differences in physical properties can be quite small or quite large. In general, as noted, physical property differences must be determined experimentally and cannot be predicted from chemical structure. With ritonavir, the differences in solubility between Form 1 (the original form) and Form II were large enough that the drug product did not meet dissolution specifications for the marketed drug. With carbamazepine, polymorph properties are quite similar to each other but different than the dihydrate. Again the degree of difference and the significance of those differences in practical development must be determined experimentally.

8.2.1.2 Salts

A salt is an ionic compound and, in the case of pharmaceuticals, it is a molecular ionic compound. The charged portions of the compound are referred to as cations and anions and both acidic and basic pharmaceuticals can form salts. In a recent survey of pharmaceuticals, more than half are salts (Paulekuhn et al. 2007). Nearly 39% of the salts are formed from free base parent molecules, whereas nearly 13%

are formed from free acids. The primary driving force for the choice of a particular salt form is improved dissolution and bioavailability of the salt compared with the parent, although other physical properties can influence or drive the decision (Huang and Tong 2004). A number of different cations and anions have been used with pharmaceutical salts, but 53% of the free bases use chloride as the anion and 75% of the free acids use sodium as the cation.

For the purposes of the solid-state analysis, salts should be considered as completely different entities when compared with each other and with the parent. Salts can have polymorphs, solvates, and particularly hydrates. In fact, when I see a sodium salt, I immediately begin to look for complex hydration. Sodium salts do not universally have hydrates, but it occurs often enough that it is better to expect them than otherwise. The polymorphism of the parent has no influence on the polymorphism of the salts. In other words, just because the parent has numerous forms does not mean the salts will follow suit and vice versa. So, if a salt form is chosen, it must be studied as if it is a new entity with no preconceptions.

8.2.1.3 Polymorphs

Polymorphs are solid forms of a chemical that differ only in the geometric arrangement of the molecules in the solid. (It is common to refer to the polymorphs of elements as allotropes, but it is a distinction with no difference.) Some polymorphs can crystallize in very different crystal systems, whereas others have the same crystal system and same space group and only differ by the size of the unit cell. Polymorphs can be important in pharmaceutical development due to the potential differences in physical properties such as solubility and dissolution. Most pharmaceutical compounds exhibit polymorphism. The literature is replete with books and journal articles on the subject. Brittain (1999), Hilfiker (2006), Lu and Rohani (2009), Bettinetti (1988), and Haleblian and McCrone (1969) are all good places to learn more about this subject.

8.2.1.4 Hydrates

Hydrates are a special case of solvation wherein water is incorporated into the solid in a variety of ways. Hydrates can have an enormous impact on physical properties and must be carefully studied, if they are found to exist. The books by Brittain (1999) and Hilfiker (2006) and the article by Lu and Rohani (2009) have good discussions and plenty of references to hydration. The article by Newman et al. (2008) is also a good reference and I recommend that you spend some time studying the theory and practice of characterizing pharmaceutical hydrates.

Hydration of organic molecules is really a subject that deserves its own book, in my opinion. Hydrates can be the most complicated solid form for pharmaceutical development. In general, the hydrate is less soluble in the gastric system than anhydrates and yet, in some cases, the conversion of anhydrate to hydrate in the gastric system can be quite rapid. The anhydrate may absorb significant water at high relative humidities to form hydrates and this conversion can have bad effects on solid dosage drug product such as expansion of the tablet resulting in loss of integrity (tablet goes to fine powder). Hydration also can have an impact on dissolution and solubility testing of anhydrous forms if the hydrate readily forms in an aqueous environment.

Hydration is complicated by the fact that there are a number of different mechanisms for water inclusion in the solid and a particular hydrate may have multiple water mechanisms. Hydration mechanisms are listed below:

- Water of crystallization – water intimate part of lattice
- Channel water – water lightly bound in crystal channels
- Absorbed water in defect regions (associated with noncrystalline)
- Adsorbed water on the surface of the molecule

A particular lot of drug substance can have water of all four types. As an added gift, water absorption and content can be affected by impurities. A little bit of sodium chloride can make the sample look like an incredible water absorber. This subject requires a lot more attention than I can give it here and I urge you to always be wary of water and study it carefully.

8.2.1.5 Solvates

Solvates are solid forms of the compound but with solvent or some other molecule incorporated into the crystal lattice. Solvates are mostly a nuisance since they may limit the choice of solvent for final crystallization, although there are some solvated forms on the market and in development. If the incorporated molecule is solid at room temperature, the resulting solid is often referred to as a co-crystal for some inscrutable reason (Bond 2007). The physical state of the solvent at room temperature is the sole difference between a solvate and a co-crystal. There has been some interest of late in the latter since the use of solid solvents opens up a wide range of possible new compounds with superior properties over that of the parent molecule. Once again we have to treat solvates as we would an entirely new entity with its own solid-state properties including polymorphism.

You will likely run across a few really ugly words that substitute for solvates, such as pseudopolymorphs, pseudomorphs, and solvatomorphs. Many workers favor these terms, so there is no harm in using them (Brittain 1999), but there is also little to be gained from their use, in my opinion. I do not use them because they do not really aid in direct communication and, well, they are truly awful sounding words.

8.2.1.6 Noncrystalline State (Amorphous Solids)

There is continuing interest in the use of noncrystalline solids as pharmaceutical dosage forms (Yu 2001). The primary advantage of an amorphous solid is that its solubility and dissolution is often improved compared with crystalline forms. If we are dealing with a low solubility, low bioavailability drug, then any improvement is welcome. Amorphous solids come with a heavy price though since they can be chemically and physically unstable, they can spontaneously and unpredictably convert to a crystalline form and they are often hygroscopic. For some drugs, though, the advantages may be worth the disadvantages. In that case, we do want to be able to predict both the potential improvement in solubility (Murdande et al. 2010) and the physical stability of the amorphous form (Graeser et al. 2009). Microscopy can aid in the characterization of noncrystalline solids as a quick check of crystallinity by PLM and can be used as an adjunct technique for the understanding of thermal events in differential scanning calorimetry (DSC) thermograms. For instance, we can verify that a thermal feature on the thermogram is a glass transition temperature as opposed to a phase transition.

Terminology in the literature on disordered solids is not uniform. The term “amorphous” is most often applied to disordered pharmaceutical solids, but can be misleading. This is particularly so if we are referring to disordered regions in a mostly crystalline solid which may have resulted from milling or micronization. We need to keep it clear in our own minds that there are quite a few different causes of what appears to be amorphous content. For instance, we are likely to be introducing defects in the long-range crystal structure such as dislocations and strain. These defects may reduce the intensity and broaden our X-ray peaks, but then there is no real amorphous content. This confusion can be particularly problematic if we are preparing amorphous standards for the quantization of a small disordered phase in an otherwise crystalline solid. The nature of the long-range order may be very different in these two materials and any quantitative values we obtain with such standards are suspect.

8.2.2 Form Relationships

Discussion of form relationships and stability in the literature are often murky and confusing. There is often some mingling of physical and thermodynamic stability, a distinction that we need to keep close in mind when conducting our own studies. Any consideration of form stability has to include a kinetic component since in some cases it is appropriate to use a metastable form if the kinetics of transition to the thermodynamically stable form is slow. We have methods of determining the thermodynamic stability of a group of polymorphs but the situation is very different with salts, hydrates, and solvates. We can readily compare physical stability in various environments, but it is more difficult to speak of thermodynamic stability comparisons of one salt with another. I am not going to go into a great deal of discussion on this topic, but I do recommend you pay attention to distinctions between physical and thermodynamic stability.

A key goal of solid-state studies is the determination of the optimum form for development. We discuss more about that subject in the next section. Before we can rationally make that form progression decision, it is necessary to know the kinetic and thermodynamic relationship of the forms to one another. In essence, we are looking at stability relationships. In other words, we ask the following questions: "Under what conditions will one form convert into another?"; "Which form is the most physically and chemically stable under processing and storage conditions over time?" Normally, we want to choose the form that is both physically and thermodynamically stable, although that is not the only criterion that we apply to that choice. Polymorphs have a well-defined thermodynamic relationship that is discussed in more detail below. We have good predictability of the relative thermodynamic stability of the different forms at ambient conditions. Kinetic stability of different forms is less predictable and we rely on empirical evidence for that determination. Both thermodynamic and kinetic stability must be established empirically for solvates and hydrates. Microscopy can play an important role in all of these studies.

Each pair of polymorphs for a specific compound is divided into two classes based on the relationship of melting point to Gibbs free energy. Remember that free energy of a form is related to the following equation: $G=H-TS$, where G is the Gibbs free energy, H is the enthalpy, T is the absolute temperature, and S is the entropy. The driving force for change is related to the difference in free energy between two forms. The form with the lower Gibbs free energy is the more stable form at ambient conditions. Although we use relative terms such as "more stable" and "most stable," we should keep in mind that as a consequence of the phase rule only one solid-state form can be thermodynamically stable at a specific temperature and pressure. All other forms are metastable to that form at that specific temperature and pressure. We can represent the relationship of any pair of polymorphs with the following equation:

$$\Delta G = G_{\mathrm{II}} - G_{\mathrm{I}} = \Delta H - T\Delta S. \tag{8.1}$$

As a consequence then:

If $\Delta G > 0$, then Form I is stable at the specified T, P.
If $\Delta G < 0$, then Form II is stable at the specified T, P.

Each pair of polymorphs falls under two categories labeled as either monotropic or enantiotropic. For monotropic polymorphs, the higher melting form is thermodynamically stable at all temperatures below the melting point. For enantiotropic polymorphs, the higher melting form is not thermodynamically stable at all temperatures below the melting point and there is some transition temperature for which the stability reverses and some other polymorph is stable. Clearly, it is important to determine these relationships early in development since any metastable form has a driving force to spontaneously convert to the stable form.

Figures 8.1 and 8.2 present schematic representations of the relationship between energy and temperature for monotropic and enantiotropic polymorphs. For monotropic polymorphs, the following relationships hold:

Melting point of polymorph I > melting point of polymorph II
T_t (if it exists) > Melting point of polymorph I (T_t = transition temperature)

$\Delta G > 0$ since $G_{\mathrm{II}} > G_{\mathrm{I}}$

Polymorph I is stable at ambient conditions and all temperatures below the melting point of polymorph I

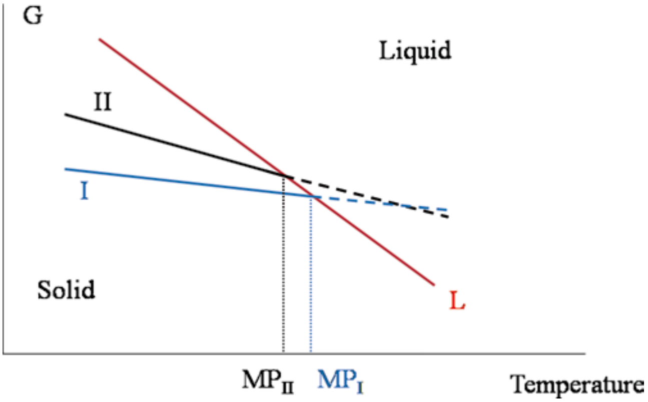

Fig. 8.1 Energy vs. temperature diagram for monotropic polymorphs. Note that higher melting form (I) has the lower free energy across all temperatures below the melt. The higher melting form (I) then is thermodynamically stable at all temperatures compared with Form II

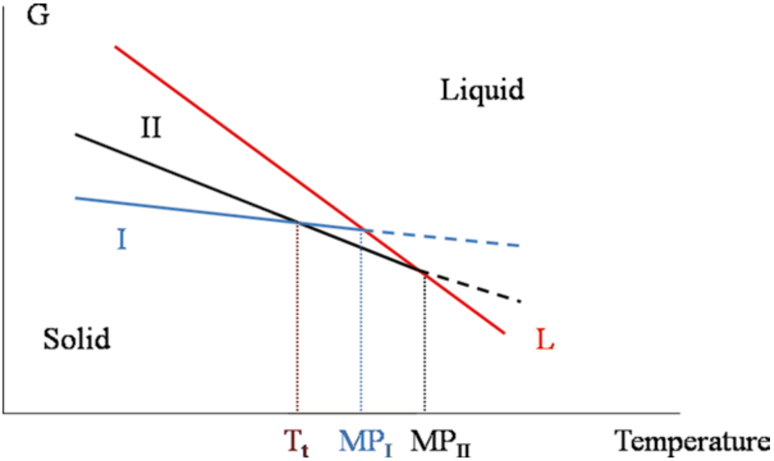

Fig. 8.2 Energy vs. temperature diagram for enantiotropic polymorphs. Note that in this case, the higher melting form (II) does not have the lower free energy across all temperatures below the melt. There is a transition temperature where the free energy relationship reverses. Below this temperature, Form I is stable and above this temperature, Form II is stable. Consequently, at the transition temperature one form will spontaneously convert into the other one. Of course, this diagram does not indicate the kinetics of the conversion process

For enantiotropic polymorphs, the following relationships hold:

The Gibbs free energy relationship reverses below the melting point of polymorph II
The melting point of polymorph I < melting point of polymorph II
Below T_t, $\Delta G > 0$, polymorph I is stable
Above T_t, $\Delta G < 0$, polymorph II is stable
At T_t, $\Delta G = 0$, both polymorphs are stable

Each set of polymorphs have an independent relationship with each other (see Fig. 8.3), and each salt, solvate, and hydrate has the possibility of forming polymorphs. As you can imagine, polymorphism can be quite complicated if there are many different forms and salts.

For any pair of polymorphs, a key study is the determination of whether the polymorphs have a monotropic or an enantiotropic relationship. If enantiotropic, we need to determine the value of the transition temperature. If that temperature is near to ambient conditions or near to synthetic processing temperatures, then the choice of polymorph for development can become complicated. There are a number of different analytical techniques for determining whether polymorphs are enantiotropic and the value of the transition temperature (see Burger and Ramsberger 1979a, b; Burger 1982; Brittain 1999; Hilfiker 2006; Carlton 2006). The determination of form relationships generally rely upon direct observation of transitions, upon thermal data for each polymorph, and upon solubility vs. temperature curves. There are some other techniques such as vapor pressure that are also used. Microscopy is useful for directly observing phase transitions and in two of the analytical techniques used to determine the enantiotropic transition temperature: eutectic temperatures and solvent-mediated phase transformation. I briefly discuss these two techniques.

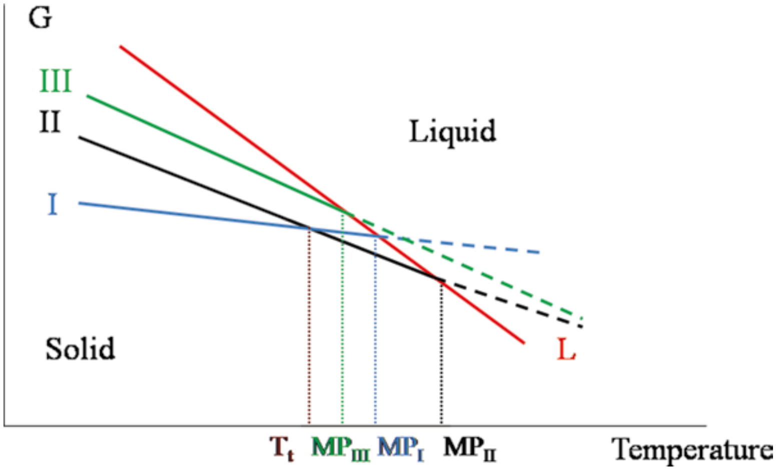

Fig. 8.3 Energy vs. temperature diagram for three polymorphs. Polymorph I and II are enantiotropically related to each other with a transition temperature, whereas Form I and Form III as well as Form II and Form III are monotropically related with no transition temperature below the melt. This diagram illustrates the fact that each pair of polymorphs have their own polymorph relationship

Solvent-mediated phase transformation (also known as solution phase transformation) makes use of the fact that different polymorphs have different solubilities in any particular solvent. If we saturate a solvent with the drug and then introduce crystals of each polymorph into the saturated solution, over time the metastable polymorph will convert into the stable one. In some cases, this conversion occurs quickly enough that the experiment can be conducted on a microscope slide. In other cases, it is better to conduct the experiment in a sealed system to allow for more time and to minimize evaporation. If the system evaporates any at all, we introduce kinetic factors into a thermodynamic experiment and we are likely to get incorrect results. In the microscope, we also depend upon some difference in particle characteristics such as shape or interference colors to make an accurate determination. If we conduct the experiment at various temperatures, we can also make an accurate estimation of the transition temperature (Carlton et al. 1996).

The eutectic method is based on the facts that the eutectics formed between each polymorph and a test compound have different melting points and that the eutectic formed with the more stable polymorph has a higher melting point than those formed with the less stable polymorph. Eutectics are best studied with the thermal microscope and we can make eutectics of each polymorph and a test compound. We then measure the melting point of each eutectic. If we use a number of test compounds with different melting points, we can develop a relationship between eutectic melting point and polymorph melting point that can help us establish enantiotropy and the transition temperature (Teetsov and McCrone 1965). This sort of test can also be conducted using DSC as described and established by Yu (1995). The eutectic melting point depression technique is not particularly common in physical pharmacy.

As a final note on form relationships and stability, much of the routine microscopic analysis involves the examination of stability samples at various time points, over time and often for many years. It is quite easy to get overwhelmed by the volume of such work and it is somewhat tedious. I think it is worthwhile to treat these samples carefully and with due attention. If a solid-state form transition is going to occur on stability, microscopy can often detect it quite early. Microscopy is easily the most sensitive technique for detecting different polymorphs since, in general, we only require one crystal for identification. In practice, our detection limits are influenced by differences in shape or other optical crystallographic properties, but still we can frequently see form transitions quite early in stability – providing we examine our samples carefully.

8.3 Solid-State Form Discovery and Selection

8.3.1 General Considerations

The solid-state form is an important consideration from the first time we crystallize the potential drug. The form may not be quite as important in the very initial stages of discovery compared with late-stage development since most early studies are aimed at determining whether or not the drug affects the biological target of interest. Solid-state form, though, will take on increasing importance as the potential drug moves to animal, toxicology studies, and then into man. Most regulatory authorities do not require much solid-state information for the first time in human studies, but still it behooves us to learn as much as possible, as early as possible. Even at early stages, the solid-state form can affect the delivered dose of drug (bioavailability) in safety studies and can affect the kinetics of absorption. By the time, the drug reaches Phase II clinical trials, solid-state form takes on added importance and certainly by Phase III, it is necessary to have chosen the optimal form for development (see Regulatory Guidance section of References for more information).

As a molecule progresses through development, there are two critical solid-state questions that must be addressed: (1) How many different solid-state forms are there?; (2) Which is the optimal form for development? Early in development we may just chose to monitor form by collecting a small set of analytical information on all early discovery batches. Microscopy (optical, hot-state, vibrational, and SEM) is particularly well suited to these early studies since it requires minimal sample when sample sizes are severely limited. Clearly, other techniques, such as X-ray powder diffraction (XRPD) and thermal analysis, need be applied as well when sample is available. Always, we need to use many different characterization tools. The choice of form for development is frequently a matter of balancing different properties such as bioavailability, stability, and ease of processing. There are few rules about this choice and it will depend heavily upon both the properties of the molecule and the intended dosage form.

8.3.2 Form Discovery

An important means of form discovery is simply monitoring form in early solid batches of the molecule. For these initial observational studies, the emphasis is on consistency of form in the sample and any evidence for multiple forms. A critical feature of early drug samples is that they often have a high level of residual solvents, water, and other impurities since the synthetic chemistry process has not been optimized. Also, the degree of crystallinity of these samples can be rather low. It is easy to be fooled and we ought to be careful in our interpretation of results. For instance, if we conduct a thermal test and see evidence of outgassing by the formation of bubbles and the crystal goes opaque, this could be the result of loss of solvent that is a part of the crystal or could be simply loss of residual solvent that is captured in noncrystalline regions in the crystal or in crystal defects. That being said, early studies are important for form discovery since it often is the case that metastable forms occur early in development.

Another caveat to early observational studies is that the crystals may exhibit different morphological characteristics due solely to processing conditions and not to form differences (see Fig. 8.4a, b). It is tempting to believe that a difference in habit is indicative of a difference in form. That is frequently the case, but also is frequently not the case. We require more proof than morphology and vibrational

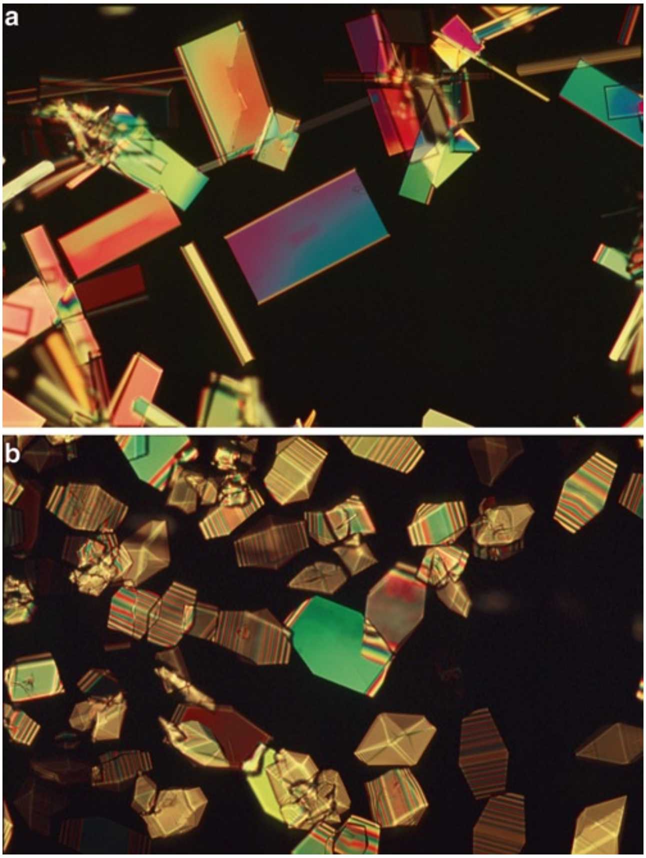

Fig. 8.4 (**a**, **b**) Estradiol hemi-hydrate crystallized from different solvents. Note the difference in habit between the two sets of crystals. This habit difference can be due to polymorphism but also can simply be due to the solvent. In this case, both crystals are the hemi-hydrate even though the habits are different (image size = 700 × 520 μm)

spectroscopy is one means of achieving that proof and powder X-ray diffraction is another. We should not forget melting point and refractive index, since these techniques are also distinctive for different forms.

At some point, though, we will need to conduct a thorough polymorph screen with the intent of discovering as many of the possible solid-state forms of the molecule as possible. These studies must be empirical. As McCrone (1957) put it:

> Those who study polymorphism are rapidly reaching the conclusion that all compounds, organic and inorganic, can crystallize in different crystal forms or polymorphs. In fact, the more diligently any system is studied the larger the number of polymorphs discovered.

Bettinetti (1988) put the problem similarly:

> There are no precise rules to predict the polymorphism of a drug, but it has been observed that the phenomenon comes up with a frequency proportional to the time used in its study, and therefore the number of experimental tests conducted.

The empirical nature of solid-state form discovery has given rise to systems of automated salt, polymorph, and solvate screening involving a large number of different solvents and crystallization conditions. Hundreds of small-scale experiments are conducted and the results examined in an automated fashion using techniques such as polarized microscopy, Raman spectroscopy, and XRPD. The goal of this work is to discover as many different forms as possible. Once discovered, they must be scaled up and characterized. If the aim of the study is the optimal form for development, the solvents and crystallization conditions will likely be related to potential, large-scale crystallization processing conditions. If the aim of the study is patent protection, then the solvents and conditions may be quite broad so as to include as many possibilities as possible (see Morissette et al. 2004).

Let us suppose, though, we have reason to do our polymorph discovery mostly with the microscope. How might we go about such studies? We might choose the microscope if sample is scarce since microscope studies, as outlined below, require a few hundreds of milligrams of material, whereas large-scale automated screens generally require many grams of sample. Also, if the molecule is quite potent or is dangerous to handle, we may want to conduct studies using smaller amounts. The first general technique is recrystallization from different solvents. We can either conduct the experiment by allowing a solution to evaporate or we can make a hot super-saturated solution and allow it to cool and crystallize. The latter is somewhat easier to conduct on the small scale using flat capillaries that are convenient for mounting and observing on the microscope. In both cases, we use a sparing amount of drug and solvent and make our observations directly on the microscope.

If we suspect different forms, we need to verify using refractive index, thermal microscopy, and vibrational microscopy. Raman microscopy is particularly useful with the glass flat capillaries since it is possible to collect a spectrum through the glass. On the microscope, solvent can be removed using a tissue filter to collect the liquid by capillary action and, if needed, we can wash the crystals in other solvents directly on the microscope slide. There is a variant of solvent techniques that involves what is called a hanging drop and gas diffusion. A solution of the molecule and solvent is prepared on a coverslip, then turned over and placed onto a well on a microscope slide so that the drop hangs suspended above the cavity. A small drop of another solvent is placed at the bottom of the cavity and the solvent's vapor diffuses through the hanging drop and may produce new solid-state forms on crystallization. Again, characterization can be accomplished by refractive index, thermal microscopy, and vibrational microscopy.

Thermal microscopy is also a powerful technique for polymorph discovery. As noted in Chap. 3, we can observe the progression of solid-state forms as we heat the solid, either dry or suspended in oil. We can observe solvation and hydration effects as gas bubbles and as changes in crystal transparency. We can observe solid-state form changes on temperature rise as solid-to-solid transformations and as solid-to-liquid transformations. Sublimation is a particularly useful technique for discovering new forms. We can also observe changes in solid-state form on cooling of the melt. Finally, there are fusion techniques that can also be employed.

8.3.3 Form Selection

In my experience, the choice of form for development ranges from the trivial to the "we have no good options, only less bad ones." In many cases, there is only one known form and no matter how we stress the compound, it only makes that one form. On the other hand, a compound I worked on had multiple polymorphs, multiple hydrates, and more than 15 known solvates. There was no good form choice for that compound, only less bad ones. Although there is no golden rule or rules for form choice, there are three (in my opinion) general principles influencing the decision.

First, we generally want to choose the thermodynamically stable polymorph at ambient conditions since all other polymorphs have a thermodynamic driving force to convert to that form. While it is desirable to choose the stable anhydrate, there are plenty of reasons to choose a different form. Hydrates generally have lower solubility and slower dissolution rates than the stable anhydrate, but if the anhydrate rapidly converts to the hydrate in GI tract conditions then we may be effectively dosing the hydrate and we may as well consider developing it. Also, if the anhydrate is particularly hygroscopic, the hydrate may be a better choice. Another compound I worked with had a enantiotropic polymorphs transition temperature near 36°C. The stable polymorph at 25°C converted to the metastable form with tablet processing. Since there was little evidence for solid-state conversion of the two forms on stability, we decided to progress the metastable form at 25°C (Katrincic et al. 2009).

Second, choose the solid-state form with the highest solubility and the fastest dissolution rate. Normally, we should progress the form that dissolves the fastest in the GI tract and that leads to the maximum bioavailability. In my experience, most polymorphs have similar solubility and similar dissolution rates in the drug product and so the form choice will not be overly dependent on solubility. There are enough exceptions though, like ritonavir, so we need to consider this criterion closely. If the drug is highly soluble and has good permeability across intestinal tissue (BCS Class 1 for aficionados of such classification schemes), then differences in form solubility are not likely to play a decisive role in form selection. The same is true at the opposite end of the spectrum where the drug has low solubility and low permeability (BCS Class 4). Differences in form solubility can have a significant impact on form selection if the compound has low solubility and high permeability (BCS Class 2) and can have an impact with high solubility and low permeability (BCS Class 3) compounds (see Amidon et al. 1995 and FDA Web site section in References). We do have to keep close in mind that our dissolution and solubility tests can induce form conversions with the result that we may not be measuring the dissolution rate or solubility of the desired form. This problem is particularly acute if the compound readily forms hydrates. For solubility experiments, in particular, it is good practice to test the form of the solid remaining at the conclusion of the test.

Third, choose the form with the best processing properties where best refers to physical and chemical stability and to ease of processing. By physical stability, I mean the form does not convert when processed. Clearly, the choice is affected by dosage form. If we are making a tablet and we need to wet granulate in order to improve flow properties before making the tablet, a more hygroscopic form is likely less desirable than another less hygroscopic one. One form may preferentially convert to another one under the pressure and temperature conditions in tablet processing (see Katrincic et al. 2009). In the case of paracetamol, the form that can be routinely crystallized cannot be compressed directly (Nichols and Frampton 1998). Since crystallization is important for the control of chemical impurities, we may have to accept some compromises (with additional unit cost of course) with regard to processing. It is not uncommon at all for a desirable form in all other respects to crystallize as needles. If those needles cannot be easily broken (i.e., milling results in smaller needles, but needles nonetheless), then it may be desirable to use a different nonneedle-like form since needles are notoriously hard to formulate.

As you can see, form selection can be a difficult choice and may involve a number of practical considerations (see Singhal and Curatolo 2004). As a side note, as an analytical microscopist, we are not likely to be called upon to make the final form decision. The more we know about the process and how the decision is made, though, the better able we are to supply appropriate data to aid that decision.

8.4 Analytical Characterization of Solid-State Forms

Virtually every property of a solid can and does vary for polymorphs, solvates, and hydrates. For pharmaceutical development, the following physical properties of each form have the greatest impact on the drug product (see a more complete list in Brittain 1999):

Density, hygroscopicity, melt temperature, enthalpy, entropy, free energy, solubility, dissolution rate, physical and chemical stability, surface energy, particle habit, hardness, ease of compaction, and flow.

We study these properties using the following analytical techniques, among others:

- Microscopy (polarized light, scanning electron, and hot-stage)
- XRPD (ambient, hi-humidity, and hi-temperature)
- DSC (regular and modulated)
- Thermal gravimetric analysis (TGA)
- Water vapor absorption (WVA)
- Vibrational spectroscopy (IR and Raman)
- Solid-state NMR
- Single-crystal X-ray diffraction structure determination
- Surface area analysis
- Solubility

- Intrinsic dissolution
- Bulk, tap density, and true density

As you can imagine (or know if you are in the business), the extent and range of different disciplines used in solid-state analysis requires both experts in the interpretation of the results from these techniques as well as a cadre of well-trained analysts to conduct the tests. Most analysts must be generalists to a great extent and be able to conduct quite a number of different tests using any number of different instruments (our laboratory has more than 30 different types of instruments for eight scientists). While microscopy is an integral aspect of pharmaceutical development, few of us have the time and opportunity to specialize completely. The best use of the techniques, however, does require expert use and interpretation. It is my hope that this book will help those scientists that need to use the microscope but do not have the time or inclination to become a dedicated microscopist.

The following texts discuss solid-state characterization in a comprehensive fashion and so I am not going to elaborate on these techniques: Lee and Webb (2003), Brittain (2011), Hilfiker (2006), and Yu et al. (1998). I will add a personal perspective, though. Since polymorphism does require such a wide variety of analytical techniques and scientific disciplines, it can be quite difficult to integrate all of the information into a coherent and consistent story. In other words, it is challenging to turn scientific data into scientific information leading to good project decisions. The microscopist has an important role in generating scientific data, but we should not get too immersed in our own perspective. I believe any solid-state scientist should learn as much as possible about the strengths and limitations of all applied analytical techniques. There are times when PLM is just the right tool for just the right application, but other times when microscopy is irrelevant. I believe a skilled craftsman knows the difference and is willing to say so. That, by the way, is true for all of the analytical techniques we use. The development of scientific information leading to good project decisions is the essence of industrial science (and industrial microscopy, of course) with great utility in commerce and great personal satisfaction.

8.5 Caffeine Polymorphism: A Practical Example

8.5.1 Introduction

In this section, we are going to study the polymorphism of caffeine as a model compound. My intention with this presentation is to show that different analytical techniques are used in concert with each other to develop understanding of the solid-state properties of a compound. I have, in previous chapters, shown how microscopy directly contributes to the understanding of particular solid-state properties. In this section, I strive to show how microscopy is a part of the whole study.

Caffeine, along with other model compounds such as carbamazepine (Grzesiak et al. 2003; Rustichelli et al. 2000), sulfathiazole (Hu et al. 2010), and mannitol (Braun et al. 2010) can serve as an excellent learning tool. I recommend that you obtain samples of these compounds and attempt to replicate the work described herein for caffeine and the published work for the other compounds. Although caffeine and the other compounds have been extensively examined, they are not as simple and straight forward as you might expect. Caffeine presents some difficulties in analysis and interpretation that are typical of pharmaceutical compounds. In other words, the analytical approach you use with caffeine and the other recommended compounds is exactly the same approach you will take with a new potential drug. I have a separate section in the references for caffeine. I am not going to refer to specific articles as we go through this section unless there is a particular point to make with that article. Clearly, I cannot list every article on caffeine solid-state properties, but the listing I give you is a good introduction to the subject.

8.5.2 Caffeine Properties

Caffeine is a xanthine alkaloid and works on the central nervous system of humans as a stimulant. Caffeine is the most widely used psychoactive substance in the world and, as you know, is one of the main attractions to our morning coffee and afternoon tea. Nature was not thinking of our morning needs when designing caffeine. It is a natural pesticide for some insects.

Figure 8.5 shows the chemical structure of caffeine. Caffeine has been intensively studied because of its economic importance and its solid-state properties continue to be studied. I did a simple literature search on caffeine and solid-state properties and received 3,956 journal article hits with the latest being in 2010 in the year of this writing. Caffeine is known to have three forms: two anhydrous polymorphs and a hydrate. While much is known about the nature of these forms, there are still some questions with regard to caffeine crystallography and hydration.

We are going to ignore the caffeine literature for the moment and treat caffeine as if it were a new compound delivered to us for solid-state analysis. We will follow

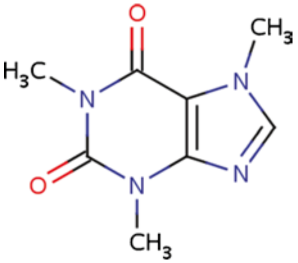

Fig. 8.5 Chemical structure of caffeine. Caffeine has a molecular weight of 194 and the following chemical formula: $C_8H_{10}N_4O_2$.

the progression of studies from form discovery to form characterization and form selection as if it were a new chemical entity. Once we are done we can compare our results with those found in the literature.

8.5.3 Caffeine Form Discovery – Initial Tests

My first step is always to simply look at the sample without any visual aids and then with the stereomicroscope. I look first at color. I expect to see pure colors and most of the time, the compound is white, although I may occasionally see yellow or orange. In all cases, I am looking for pure colors, not pastels or dingy shades. I expect all the particles to have the same shade of color and not have some very dark particles and some light. If I do see dingy colors or a grayish tint, then I am reasonably sure that I have not received a particularly pure compound. The sample is likely to contain residual solvents, synthesis residues, or some other type of contaminant. A lack of purity is no reason to reject the sample at this stage and, in fact, a certain lack of purity is expected. We do need to be aware though, that some, if not all, of our tests will need to be redone once we have received pure material. Many of our results will change from the impure to the pure material and a good example is melting point. Some impurities lower the melting point.

Next I look at the powder properties – again with the unaided eye and by stereomicroscopy. I am looking at the general appearance of the crystals. Are they large and well-formed or are they small and irregular? Large, well-formed crystals are easier to work with at early stages of development and are an indication of a good synthetic process. Well-formed crystals at the early stages of development, though, are the exception not the rule. I am also looking for particles with different habits. If I see mostly blocks, but also the occasional needle, then I have a good sign of polymorphism. As noted earlier, different particle habits can be a sign of different polymorphs but the observation does not constitute proof. The observation does call for further investigation. Figure 8.6 shows caffeine as received under the stereomicroscope. Note that the powder is pure white and that we do not see multiple particle habits. Also note that the particles are small and irregular.

The next step is to examine the sample using PLM and SEM. With PLM, we look for birefringence and the lack thereof. Birefringence is an indication of crystallinity and the lack of interference colors is an indication of an amorphous solid. Many of the measured physical properties are strongly influenced by the degree of crystallinity and the presence of amorphous particles is an important early observation. I also note the particle size and shape partly for the polymorphism study and partly to aid formulation. If we detect differences in particle habit, the differences may be due to polymorphism. The SEM examination is similar to that using PLM in the sense that we are looking for variations in particle habit and shape. Figures 8.7 and 8.8 show PLM and SEM images of caffeine as received. The particle habit is not variable and there is no evidence of amorphous particles.

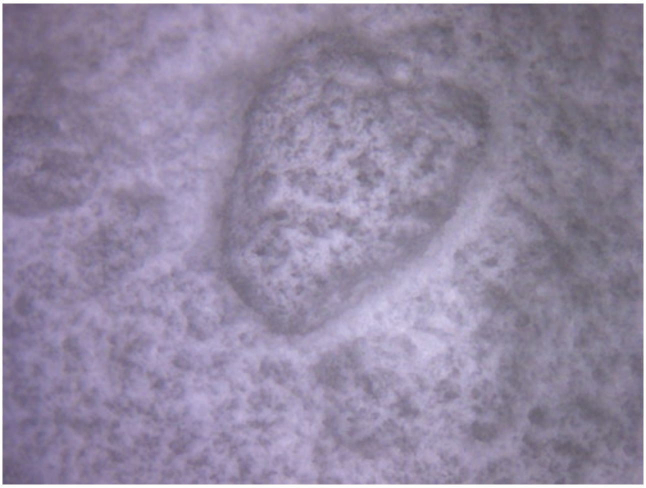

Fig. 8.6 Caffeine powder under the stereomicroscope. Note that the powder is pure white and that we do not see multiple particle habits. Also note that the particles are small and irregular (3.4 × 2.5 mm)

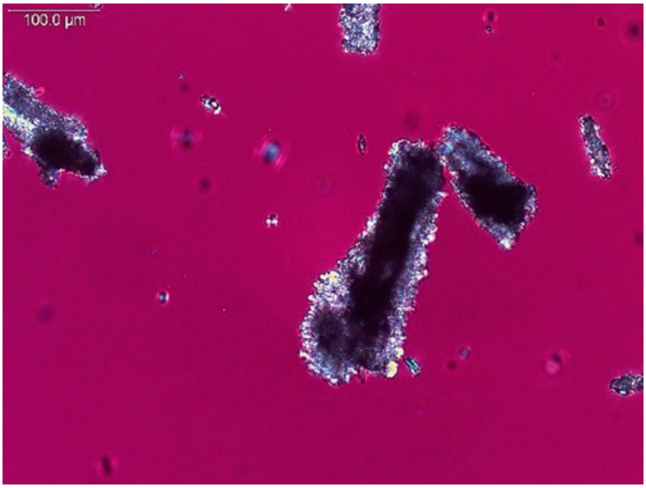

Fig. 8.7 Caffeine polarized light photomicrograph. This is a photomicrograph of caffeine as received. Note that the particle display interference colors indicating that they are crystalline. The larger particles appear to be aggregates of much smaller particles. Only one crystal habit is observed (image size = 350 × 260 µm)

At this point, I will collect some baseline characterization data including XRPD, DSC, TGA, IR, Raman, and water vapor absorption – providing I have enough material for all of these tests. XRPD yields a crystallographic fingerprint while IR and Raman provide molecular fingerprints. The sharpness of the XRPD peaks and the peak-to-background ratios can be indications of the degree of crystallinity

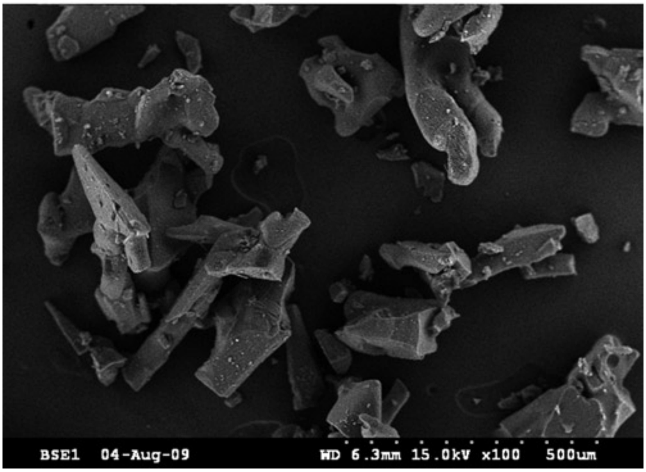

Fig. 8.8 Caffeine scanning electron microscopy photomicrograph. Note the irregular shape of all of the particles and that the larger particles appear to be aggregates of much smaller particles. Only one crystal habit is observed (image size = 1,217 × 987 μm)

(sharper peaks = better crystallinity). Infrared spectroscopy and Raman can be an early indication of possible hydration. At this stage, I am particularly interested in the DSC and TGA. A complicated DSC thermogram is often the first indication of polymorphism and solvation, whereas the TGA results tell us much about purity and solvation.

Figure 8.9 shows an overlay of the DSC and TGA of caffeine as received. All the tests conducted to this point (visual exam, PLM, SEM, XRPD, IR, Raman) do not show any indication of polymorphism or solvation. The DSC/TGA are otherwise. One of the most obvious features of the DSC is the small endotherm with an onset temperature at 146°C and an enthalpy value of 18 J/g. While there can be a number of different causes of such a peak, a polymorphic transformation is one of those causes. On the other hand, we do not observe an exotherm along with the endotherm so we have to be cautious in our interpretation of the event at this point. The final endotherm at 236°C is sharp with a large enthalpy value of 100 J/g, but the shape of the thermogram after the melt is somewhat unusual and does not appear to be degradation. All in all, the DSC presents a somewhat uncertain picture, but I definitely suspect polymorphism.

The TGA results provide additional clues and some confusion. First, there is little to no weight loss from the ambient temperature up to 146°C. The lack of weight loss argues against hydration and some solvates which will lose weight at a lower temperature. Interestingly, the sample displays a sharp weight loss at 160°C that continues until all of the sample is lost. This complete weight loss occurs at a temperature below that of the major endotherm. And yet, the DSC displays a sharp endotherm with a high enthalpy both of which are a sure indication of a melt process. So, if all of the sample is gone, what is melting?

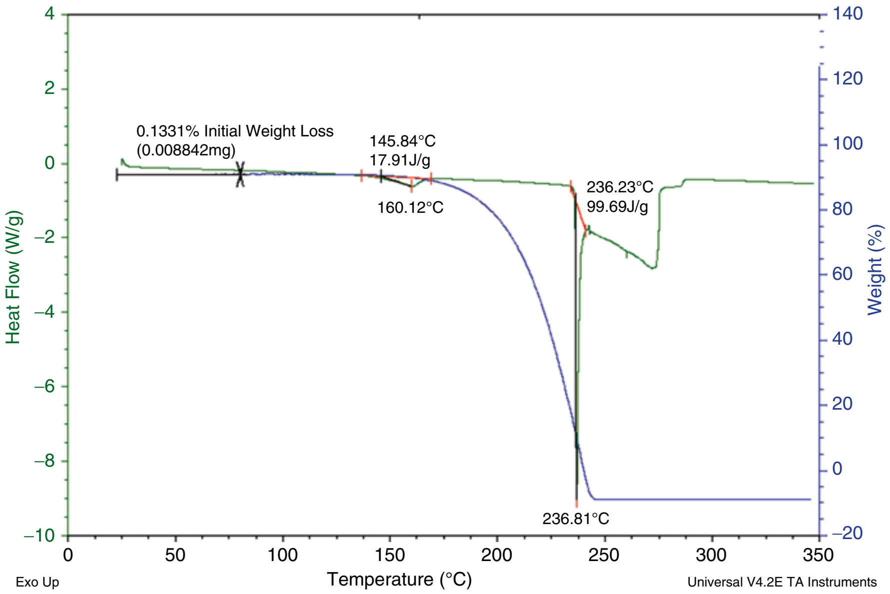

Fig. 8.9 Overlay of DSC and TGA for caffeine. The DSC thermogram has two endotherms at 146 and 236°C. The lower temperature endotherm may be an indication of a polymorphic transformation but is not followed by an exotherm which is expected for a transformation. The TGA is flat, with minimal weight loss up to 160°C, but then all of the weight is lost prior to the melt. The TGA does not lend support to a solid–solid transformation at ~150°C

The best way to answer these questions and to better understand the thermal analysis is to do some hot-stage microscopy examinations. I typically start by heating a dry sample at 10°C/min from ambient up to degradation in order to survey the heating behavior. I then conduct further tests with different heating rates and in oil in order to better understand any observed thermal features. With the initial test, we observe sublimation of the caffeine sample that begins below 150°C and the consequent melt of the sublimate at 236°C (see Fig. 8.10). The DSC/TGA results now make sense. The endotherm in the DSC at 146°C is due to sublimation. Naturally, there is no exotherm following the endotherm. The TGA trace begins to lose weight at that temperature, and since the test is conducted in an open pan, all of the sample is lost to sublimation during the course of heating and before the melt of the sublimate. In the DSC pan, however, the caffeine sublimes onto the upper lid (loose lid configuration) and it is that sublimed material that melts at 236°C.

Since caffeine does sublime, then there is an excellent chance that the sublimate is a different solid-state form. The best way of testing this hypothesis is to collect sufficient sublimed material for an XRPD test (~10 mg). I collected this material by placing a generous amount (20–25 mg) of caffeine into a shallow glass container and placed a glass plate onto the top of the container. I then heated this sample at the sublimation temperature of 150°C and collected the sublimate onto the upper glass plate. The XRPD of the sublimate is overlaid with that of the original material

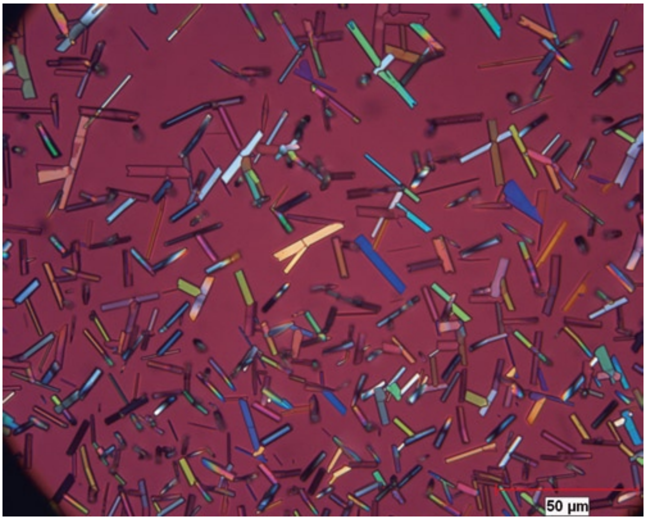

Fig. 8.10 Caffeine sublimate from hot-stage experiment. Caffeine sublimes onto the upper surface of the glass coverslip beginning at ~140°C. The observation of sublimation helps rationalize the unusual features in the DSC/TGA traces. The sublimate can be a different polymorphic form (image size = 700 × 520 µm)

in Fig. 8.11. The clear difference in XRPD patterns establishes that the sublimate is a new form of caffeine. We can now use the sublimed material to characterize the new form as we did with the original material using PLM, SEM, IR, Raman, DSC, and TGA. Beyond analytical characterization, we are also looking for ways of distinguishing one polymorph from another. XRPD is the best means of distinguishing the polymorphs. By convention, the as-received, stable polymorph of caffeine is named Form II, whereas the sublimed, metastable form is Form I.

We have learned quite a bit about our new compound with a very few, simple tests, in a short period of time (1–2 days) and with a small amount of material (~50 mg). At this point, it is appropriate to do an exhaustive search for polymorphs and solvates using 5–10 g of pure material.

8.5.4 Form Discovery – Polymorph Screening

Polymorph screening involves a series of tests using different solvents and crystallization techniques in order to stress the compound and to produce as many polymorphs and solvates as possible. Much of this work is now automated, but it can be done manually – it just takes longer. Most of the tests use recrystallization from solvents followed by a few simple analytical tests such as XRPD, IR, and Raman. If the goal of the work is the discovery of all possible polymorphs and solvates for patent purposes, then we will choose as many different solvents and solvent classes

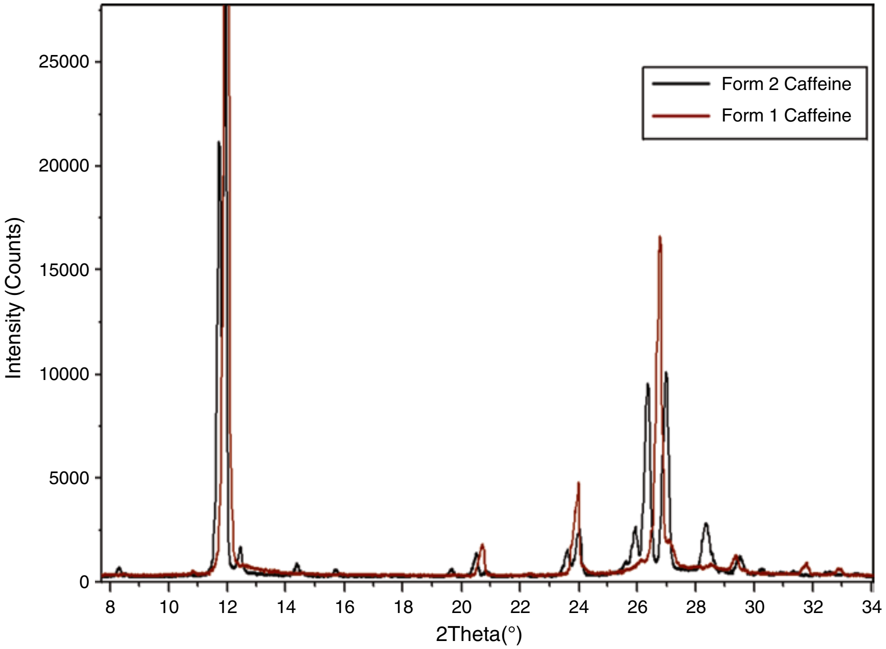

Fig. 8.11 XRPD overlay of Form II and Form I. There are clear differences in the XRPD patterns of the two forms indicating that they are different solid-state forms. Form II of caffeine is the stable form, whereas Form II is the sublimate and is metastable to Form I. The XRPD confirms that the sublimate observed by hot-stage microscopy is a new solid-state form

as possible. If the purpose of the examination is more to predict the possible polymorphism resulting from the use of the anticipated final crystallization solvents, then we may use fewer solvents but more recrystallization conditions. Of course, water is one of the primary solvents to be examined.

Although caffeine is not highly soluble in water, it is possible to dissolve a reasonable quantity of caffeine in hot water (50–60°C). If we then allow the solution to cool slowly over a few hours time, lo and behold, we observe particles crystallizing with a very different habit compared with either anhydrous form (see Fig. 8.12). The XRPD pattern of the hydrated material is different from that of the anhydrates (see Fig. 8.13) as are the DSC/TGA (Fig. 8.14) and the IR (Fig. 8.15). All of the data support the conclusion that this is a new form and that the form is hydrated. The hydrate may not be particularly stable since the TGA loss and the DSC endotherm begin near ambient conditions. The low temperature onset of water loss is indicative of a labile hydrate. The amount of water lost (3.5%) corresponds to about 0.4 mol of water per molecule of caffeine and if we redo this test multiple times with fresh samples we will likely get water loss corresponding to 0.5–0.8 mol of water per molecule of caffeine. The variable amount of water loss is another indication that the water is only lightly bound in the crystal lattice. Although we do

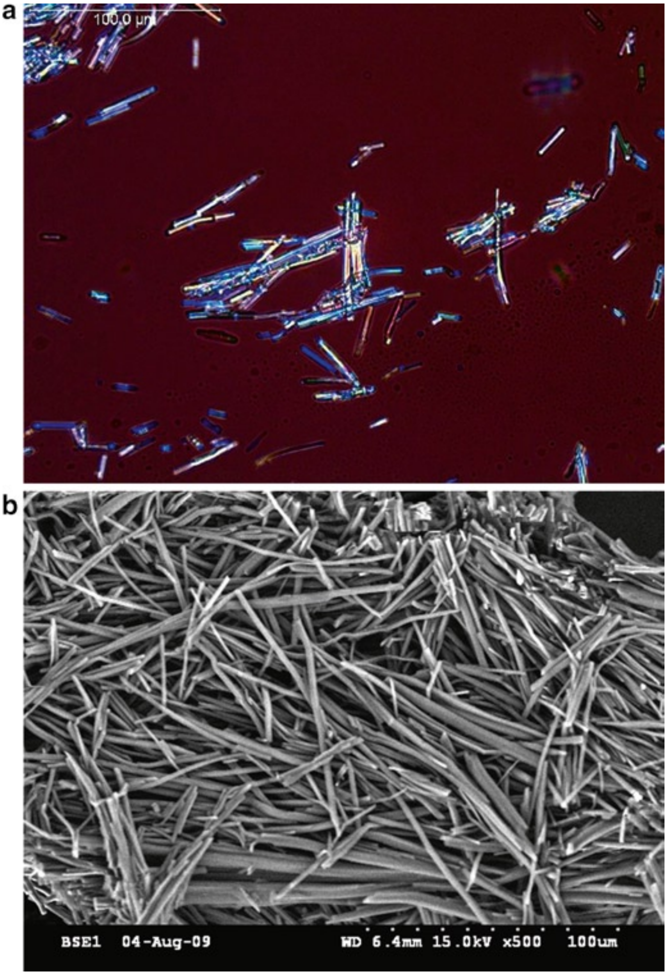

Fig. 8.12 PLM (**a**) and SEM (**b**) of caffeine hydrate. Caffeine hydrate has a very different habit and appearance compared with Form I and Form II

not generally like to use hydrates in pharmaceutical dosage forms due to reduced solubility and dissolution, the fact that this hydrate is variable and labile adds even more evidence against choosing it for development.

This work concludes this phase of form discovery, although we should bear in mind that it is still possible for new forms to be discovered later. It is important to monitor the form in new lots of drug substance and in stability trials through the development and even the manufacturing cycle. Also, as a side note, we should be on the lookout for large well-formed crystals when we conduct our polymorph screening. These crystals may be good for optical crystallography and for single-crystal X-ray crystallography. In particular, single-crystal X-ray crystallography can help us understand how caffeine molecules are connected in the lattice for each

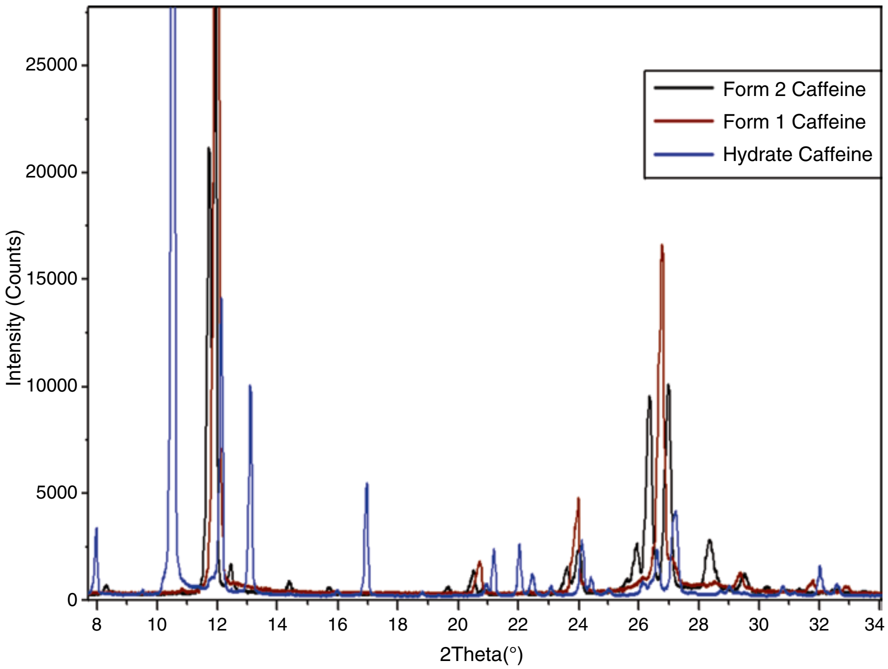

Fig. 8.13 XRD overlay of caffeine hydrate with the two anhydrous forms. The XRD pattern of the hydrate is clearly different than those of either anhydrous form. The XRD pattern is confirmation this form generated from water is different than the anhydrous ones

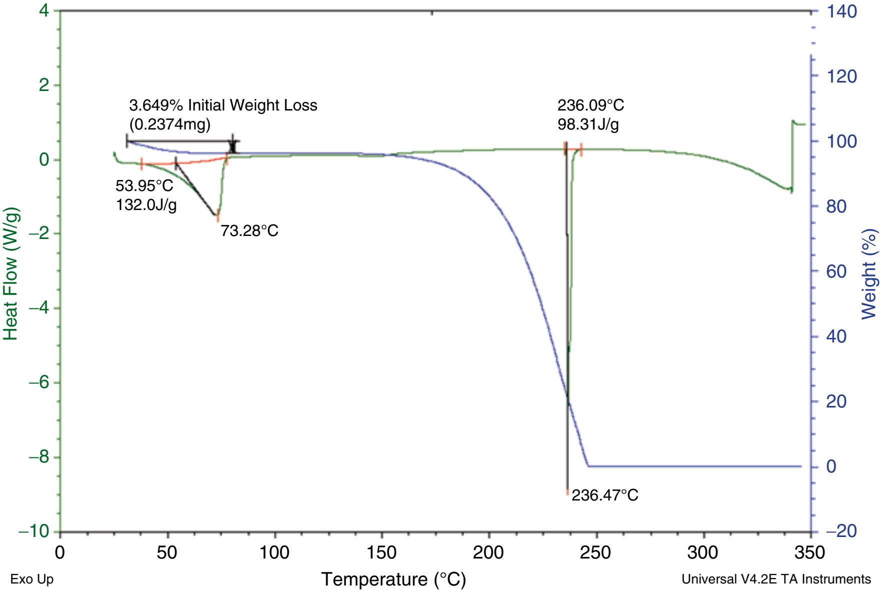

Fig. 8.14 DSC and TGA of caffeine hydrate. The DSC and TGA are different than those of the anhydrous forms. There is a broad endotherm from ~50 to ~75°C in the DSC and a 3.5% corresponding loss in the TGA. Not only does these data support the presence of a new form, but also evidence that the form is a hydrate

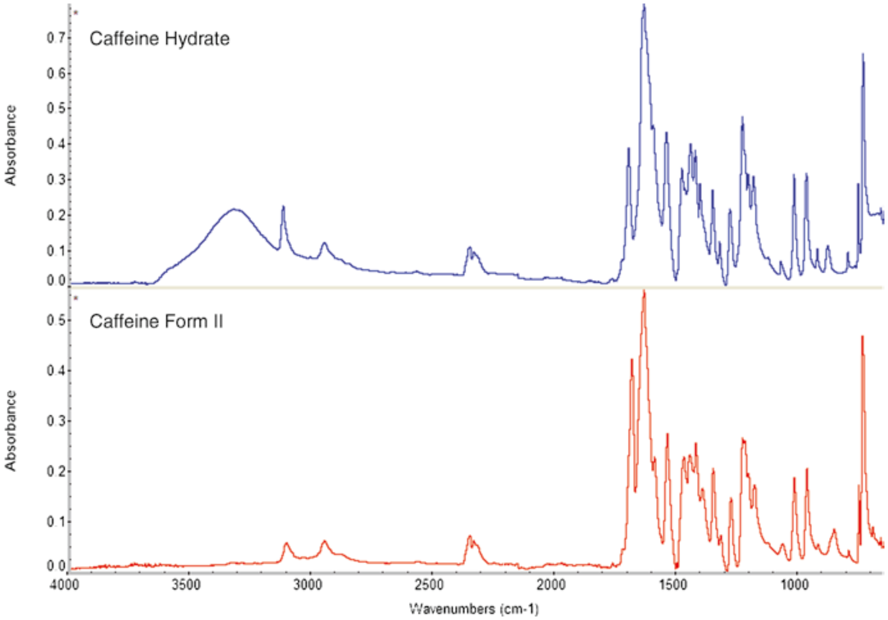

Fig. 8.15 IR of caffeine hydrate and caffeine Form II. The IR spectrum of the new form generated from water is further data supporting the generation of a new form and that the form is hydrated. Note the broad absorbance centered at ~3,500 cm^{-1}. This peak is generally associated with the O–H stretch and is indicative of water in the molecule

form. This information can be useful when we attempt to understand how the water is located and bound in the crystal and why it is easily removed. Although it is not a hard and fast requirement to have single-crystal X-ray diffraction data at the time of the NDA for marketing, the regulators do generally expect that we have attempted to obtain such information.

8.5.5 Form Stability and Form Relationships

Now that we have determined that our system has two anhydrous polymorphs and a hydrate, our next steps are to: (1) assess the form stability, in other words, determine which form is physically and thermodynamically stable, and (2) determine the thermodynamic relationship of the two anhydrous forms. These tests generally require substantial amounts of each from for analysis. A good target is 500 mg of each form. We may be able to get by with less but some of the tests, such as solubility, can consume lots of material.

The initial tests of stability can be purely physical. Small amounts of each form (~25 mg) can be placed in vials stored at different temperatures and relative humidities. Since the XRPD pattern of each form is distinctive, it is best to use a combination of PLM and XRPD for analysis. What we observe is fast conversion of Form I to Form II and even faster conversion of the hydrate to Form II. The conversion of Form I to Form II can initially be observed as crystals growing on the surface of Form I (see Fig. 8.16). The appearance of growing crystals is certainly a sure sign of Form conversion, but we cannot assume that the conversion is from I to II, it is necessary to confirm this result using another test such as XRPD. This test may be difficult since it can take many months for complete conversion to occur and because the XRD patterns do not allow for low limits of detection of Form II in Form I. Conversion of the hydrate

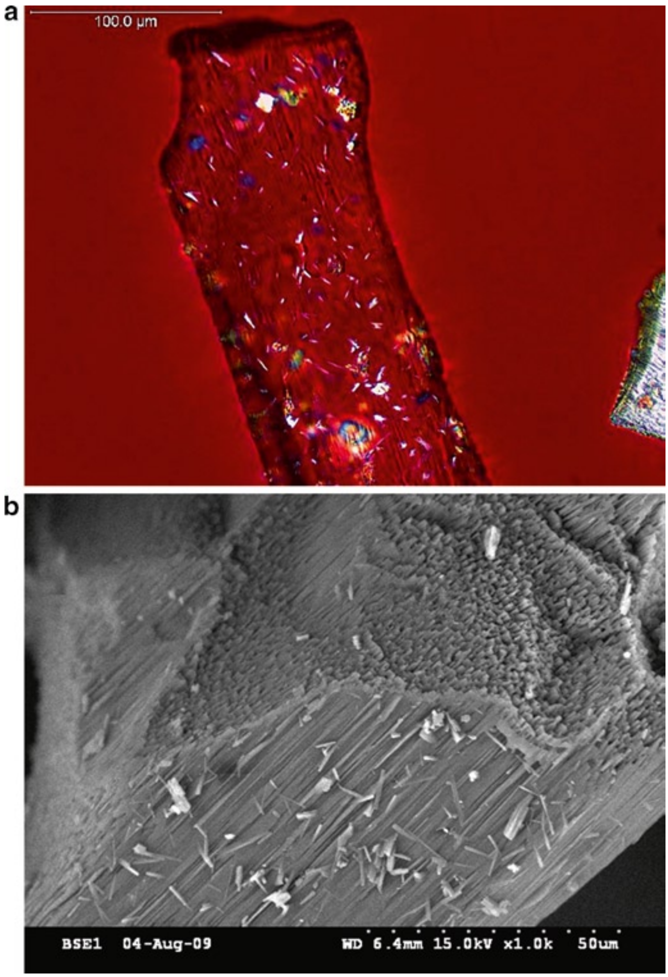

Fig. 8.16 Caffeine Form II crystals growing on the surface of Form I: (**a**) PLM (**b**) SEM. Within 24 h at ambient conditions, Form II crystals are observed to begin growing on Form I crystals. Although this process begins quickly, it can take a considerable amount of time for full conversion to occur (>1 year). The identity of the growing crystals must be confirmed by an analytical technique such as XRD (image **a** size = 350 × 260 μm; image **b** size = 122 × 99 μm)

to Form II occurs quite rapidly and can be easily monitored by XRPD and by IR. The hydrate conversion has also been monitored by microscopy and appears to begin at the ends of the hydrate needles (Suzuki et al. 1985).

Clear form conversions of this type are an indication of both physical and thermodynamic stability relationships. For caffeine, we can be confident at this point that Form II is the best candidate for development since both Form I and the hydrate spontaneously convert to that form in the solid state. For completeness, though, we do want to learn more about the thermodynamic stability relationship of the anhydrous polymorphs, the kinetics of transformation, and the nature of how water interacts with caffeine in the hydrated form. If we were developing caffeine, then these studies could be done later in development, but well before submission to the FDA for marketing approval. I believe that a thorough understanding of the solid state and a clear, lucid presentation of that understanding to regulators aids the submission and approval process.

8.5.6 Thermodynamic Relationship of Anhydrous Polymorphs

The caffeine polymorphs present an interesting case for determining their exact thermodynamic relationships due to the sublimation of Form II and the subsequent crystallization of Form I at approximately 150°C followed by the melt at 236°C. The fact that the higher melting form is not stable at ambient conditions implies that the polymorphs are enantiotropically related with a transition temperature probably near the sublimation temperature and the literature on the subject is in agreement on this position (see Griesser et al. 1999). Yet, it is somewhat difficult for me to wrap my mind around the complication of sublimation. How the sublimation of caffeine fits into the thermodynamic stability relationship of the two polymorphs is not directly addressed in the literature that I have been able to find. I think it could be a good subject for analysis and a journal article. This book is not really the place for such new information, so I am going to sidestep the issue a bit and discuss caffeine polymorphs as if they were somewhat normal enantiotropes.

The key practical issue that we face when dealing with enantiotropic polymorphs is the determination of the transition temperature. If this temperature lies near room temperature or near temperatures used in stability studies, we can face a challenge in choosing the form for development. Also, if the transition temperature is near that used in the final crystallization step of the synthesis, then the choice of form may be complicated. Finally, though we do not typically conduct experiments with pressure and polymorphs, we do need to keep in mind that pressure is another important variable, particularly when we mill or compress the drug substance. Katrincic et al. (2009) and Vogt et al. (2008) discuss a set of enantiotropically related polymorphs that had a transition temperature of ~36°C and clear conversions on milling. In this case, we chose to develop the stable form above 36°C since that form was physically stable at ambient temperature and because the other form converted to this higher temperature form on milling. The determination of the thermodynamic relationships in this case required extensive tests over a long time period. The program was discontinued for other reasons, so I cannot report on the long-term use of the higher temperature form and whether it converts to the lower temperature form on long-term storage.

In Sect. 8.2.2, I briefly presented some of the methods used for determination of the enantiotropic transition temperature (see Carlton 2006 for a full discussion of the subject). Most of these techniques would not work with caffeine since Form II sublimes and because both anhydrous forms spontaneously convert to the hydrate in solubility studies. The determination of the transition temperature is still an active area of research with workers reporting a range of temperatures from 130 to 162°C. Griesser et al. (1999) presents a good summary of the work done to that point and provide their own estimate of transition temperature based on vapor pressure measurements (see Caffeine Polymorphism section of References for more details).

In our laboratories, we attempted a variety of techniques for measuring the transition temperature and found the experiments quite difficult to conduct. In particular, we tried the eutectic technique recommended by Teetsov and McCrone (1965) and by Yu (1995) and were unsuccessful due to sublimation of the forms. We attempted solution phase transformations at different temperatures but ran into difficulties getting a good solvent for use at high temperatures. We only succeeded when we happened to note that we dissolved a small amount of the solid when using Cargille mounting liquids at high temperatures. We used this liquid and our estimate of the transition temperature is 141°C (to be published in a peer-reviewed journal at a later date). This value is well within the range of accepted values.

In a very real sense, the determination of the thermodynamic transition temperature is purely theoretical since caffeine begins to sublime close to 145°C. This temperature is truly the phase transition temperature of most practical significance to pharmaceutical development. It is instructive, though, to read the literature on caffeine thermodynamics and to attempt these experiments. It is the best way to learn the techniques. For an easier example, I recommend that you attempt to determine the transition temperature for carbamazepine Form III and Form I. Form III is stable at ambient conditions and Form I at higher temperatures. The determination of the transition temperature is relatively straight forward (see Grzesiak et al. 2003).

8.5.7 *Caffeine Hydrate*

Full characterization of the hydrate is the last remaining task in our study of the new–old compound. Remember that the IR and the DSC/TGA results indicated that caffeine can crystallize as a hydrate, but that the hydrate is not particularly stable at ambient conditions. This information is sufficient to eliminate this form for drug development, but we do need to know about the properties of this form. This is particularly so since it is evident (from our studies) that the hydrate forms readily in dissolution and in solubility studies. It is quite likely that the hydrate also forms in the gastrointestinal tract if we take a caffeine tablet.

The caffeine hydrate should have ~8.5% of water if it is a full monohydrate and ~4.4% if it is a hemi-hydrate. By TGA (and also by other techniques), we get water content values that range from 3 to 7%. We also can note that the more vigorously we dry the sample (particularly at temperatures above 50°C) the lower the water content. The variability in determined water content and the fact that the molar ratio of water is not stoichiometric are sure signs that caffeine is a channel or variable hydrate. Variable hydrates are difficult to use in drug manufacturing and so are rarely, if ever, chosen for development.

If you look again at the structure of caffeine in Fig. 8.5, you can note that there are no sites for strong hydrogen bonding and, as we know, water generally participates in hydrates through hydrogen bonds. There is, though, one nitrogen in the five-member imidazole ring that can have weak hydrogen bonding. Single-crystal X-ray studies of caffeine hydrate indicate that water is loosely associated with that nitrogen. We can also note from the structure that the purine is probably rigid and flat as, in fact, the single-crystal X-ray structures indicate. Stacking of the molecules in the unit cell leads to tunnels and channels along the *c*-axis of the crystal that allow for relatively easy movement of water molecules in the crystal lattice. And so, it appears that the imidazole nitrogen allows for just enough force from hydrogen bonding with water to allow formation of the hydrate but this weak bond can be relatively easily broken. The water molecules can then leave the molecule through the *c*-axis. Variable hydrates often have these crystallographic channels and, hence, the name channel hydrates.

The X-ray crystallography of the anhydrous polymorphs is also interesting but for a very different reason. It has been very difficult to obtain high-quality single-crystal X-ray data of either anhydrous form due to disorder in the crystals. The resulting patterns are also disordered and so structure solutions are poor in quality. Large Form I crystals can be made by sublimation but begin nearly immediately to convert to Form II. Form II nearly always crystallizes in polycrystalline masses that do not yield good X-ray diffraction patterns. One way of thinking of this situation is that the crystals of polymorph II contain so many defects that it is not generally possible to use techniques like single-crystal X-ray diffraction to probe its crystallography. There has been some recent success in obtaining single-crystal data for these forms (see Enright et al. 2007).

8.5.8 Caffeine Example Summary

I think that is probably enough for caffeine and hopefully not too much. I think you can see that some aspects of polymorphism studies can be quite simple in the sense that Form II caffeine has a number of desirable physical properties that can render it a good form for development. You can also see, though, that once we begin to study polymorphism in depth, we face difficult challenges in developing full understanding. The challenges faced with caffeine are not unusual, in my experience, even though the particulars are likely different from one compound to another. To borrow from Tolstoy, each compound has some dysfunctional properties but they are all uniquely dysfunctional. I hope that you also can see the need for a variety of analytical techniques, but even more, the need to stitch together all the analytical results into a whole, rational picture. Microscopy plays an important role in both the analytical characterization but also in the synthesis of the results.

There are a number of polymorphic drug compounds that have been thoroughly studied over the years and that are relatively easy to obtain. Carbamazepine has four polymorphs and a dihydrate (Grzesiak et al. 2003). Sulfathiazole has five polymorphs (Hu et al. 2010) and D-mannitol has three polymorphs, a hemi-hydrate and a stable noncrystalline form (Burger 1982; Braun et al. 2010). I think you will find it well worth your time to study all of these compounds, and in particular mannitol, since it is a common excipient in pharmacy.

8.6 Summary

Polymorphism is clearly an important consideration in the development of new pharmaceutical medicines. The study of the solid state is often quite complicated since some organic molecules have many polymorphs, solvates, and/or hydrates. If there are many solid-state forms from which to chose for clinical development, then the form decision will often be a compromise with no one solid-state form having all of the desired properties. The characterization and understanding of the solid state requires the application of a wide variety of different scientific disciplines and analytical techniques. Microscopy, in all its guises, plays an important role in the study of the solid state.

As a personal aside (soapbox really), I do encounter an attitude, now and again, that states categorically that other analytical techniques do everything microscopy can do, but only better. So, it is nice to have pictures but that is about as far as it goes. In response, first, it is simply not true. Sublimation by the hot-stage is unique and there are only poor substitutes for it. What other technique yields the refractive index of the solid? Second, the sensitivity of microscopy techniques is unmatched since we can obtain high-quality data on single, small crystals. Finally, even if we have some analytical duplication, why must we choose only one? Have we stopped using Raman and IR spectroscopy because solid-state NMR yields similar information? No, we use each to reinforce the other and supply data not available or not easily available from the other. The fact is that making an organic molecule into a safe, efficacious, stable medicine to fight disease is a Herculean task. We really need every tool at our disposal. That is about as much justification I wish to give for the use of microscopy in solid-state studies. Any manager willing to airily dismiss a major analytical tool like microscopy will undoubtedly get the end product they deserve!

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Chapter 9
Size and Shape Analysis

9.1 Introduction

Microscopy is an excellent tool for determining particle size and shape. Although there are much faster and more precise analytical techniques for measuring particle size, microscopy does have some particular advantages in size measurements. Microscopy is the only method that can yield qualitative and quantitative estimates of particle shape. This chapter is dedicated to the subject of size and shape measurements using the microscope.

In pharmaceutical microscopy, image analysis can be used as either a direct measure of particle size and shape or to provide complementary data in support of other techniques. Image analysis is used as a direct measure of particle size or shape with creams, suspensions and for inhaled products. It is often difficult to obtain good data using other techniques with these formulations. Image analysis is also used in a complementary fashion with laser diffraction particle size analysis and is often used as a check on method accuracy. In particular, it is useful to use image analysis in the development of particle size methods for nanosuspension products. It is also possible to use image analysis for the direct analysis of the distribution of drug substance and excipients in tablets and in capsules.

It is a fundamental tenet of microscopy that knowledge of the size and shape of the components of a system leads to understanding of the bulk system properties and allows us to predict the response of the system in different situations. While this tenet is probably true in theory, in practice system responses are generally a result of many different factors that not only directly affect bulk properties but that also interact with each other. For that reason, it is almost always necessary to study particle size and shape as one component of a multicomponent system. For example, a one-component regression model predicting tablet dissolution as a function of particle size is rarely, if ever, robust since tablet dissolution is also a function of other factors such as tablet hardness, coating thickness, dissolution testing methodology, and amount and kind of disintegrant to name just a few factors. Multifactor studies are required for good understanding and for accurate predictions. I am not going to discuss this subject in any great detail, but as a microscopist you should

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be aware that the best results from image analysis are part of a full system study and a multicomponent analysis.

In this chapter, we will discuss a number of practical examples of the use of image analysis in pharmaceutical development. I have tried to choose examples using readily available materials of pharmaceutical interest, although that is easier said than done. I have resorted to “compound A” in places to best make the point. We will also cover the subjects of image analysis method development, error analysis (validation), specifications for particle size and shape, and microscope automation. Before discussing quantitative image analysis, we will consider some aspects of qualitative shape analysis.

9.2 Qualitative Shape Analysis

As I have stated elsewhere, it is a rare technical project presentation in the pharmaceutical industry that does not include photomicrographs of particles of the compound being discussed. In many cases, the image stands alone and only qualitative descriptions of size and shape are presented. These types of photomicrographs can be very useful if, for instance, we are trying to demonstrate how our fibrous material maintains that shape even after aggressive milling operations. Naturally, we want to be able to describe the particle shape along with mentioning the size range. Although the terminology around particle shape is not completely standardized, there are some accepted terms and general definitions of these terms. A good source is the US Pharmacopeia section <776> (USP33 2011). This section applies specifically to optical microscopy determinations of shape, but is clearly applicable to the SEM as well. The USP divides the shape characterization into four parts: primary particle shape, associated (aggregated) particle shape, particle condition (edges, occlusions, etc.), and surface characteristics. We will discuss each of these parts of shape analysis listed in the USP. There is also an ISO document (13322-1, 2004) that deals with static image analysis. Frankly, I do not find the document particularly helpful and I will not discuss it further, but you should be aware of its existence.

The USP lists six descriptions of shape as follows: acicular, columnar, flake, plate, lath, and equant with general definitions for each category and a set of drawings illustrating each type of particle. Adrich and Smith (1995) add tablet, rod, ribbon, and fiber to those categories. They also provide relative axial lengths to the description to aid in the categorization and a set of drawings as well. In our laboratories, we have a similar categorization but we not only have drawings but representative photomicrographs of each type of particle as well. In my opinion, the number of categories and exact definitions of each category need not be standardized and can be particular to each company. It is useful to begin with the USP definitions but it is appropriate to add additional categories for your specific company. What is critical, in my opinion, is that the definitions be well explained and ideally they should be illustrated with real particle examples. I will give an example of an additional category, and that is one called “micronized.” Not every micronized particle looks the same, but the shape is common enough to deserve its own category, in my opinion.

The state of association is a critical shape description that is somewhat difficult to define. The USP uses terms such as lamellar, aggregate, agglomerate, conglomerate, spherulite, and drusy (small particles stuck to larger ones). There have been some fierce arguments over the exact definition of and distinction between an aggregate and agglomerate. The USP defines the first as a mass of adhered particles and the second as fused particles (a conglomerate is a mixture of different types of particles). I personally do not think it matters which word is used so long as your definition is clear. The basic problem with the distinction is that we have to make a judgment as to how easy it is to break the associated particles into primary particles. Although we can guess by looking at the morphology, it is still only a guess. It is my habit to look at particle association in a step-wise fashion beginning with visual observations and stereomicroscopy and then progressing to optical microscopy and SEM. Most pharmaceuticals are cohesive and left to stand alone in a jar will form loose compacts just by the action of normal vibrations. We get some idea of hardiness just by trying to remove a few of these for examination. Frequently, they break apart just as we remove them. In other cases, they are hard nuggets that cannot be broken at all. I believe these types of observations can be important to those doing the drug substance and drug product development and should be a part of our recorded observations.

Surface characteristics can also be important observations. Some categories of surface appearance are smooth, cracked, porous, rough, and pitted. We have successfully used some of these observations to aid in understanding unusual surface area measurements and unusual water vapor absorption values. After milling, material behavior can be opposite to expectations. For instance, the surface area measurements may increase in value rather than decrease as is most typical. An increase in surface area implies smaller particles and it is difficult to rationalize how surface area increases over time during storage. Some surface observations of cracking and pitting may help explain the unusual observations.

The USP also has a general category such as for edges, degree of transparency, defects, and color. Some of these observations may be useful on occasion but I do not find them helpful on a regular basis.

I would like to re-emphasis one point. It is quite important for you to have standard definitions of your qualitative shape categories within your company and group. Ideally, the categories and associated definitions should be written in a test method or in a protocol for everyone to use. Drawings are helpful, but I think a collection of pictures, both optical and SEM, is the best means of ensuring that your shape classifications are clear. These methods may take some time to put together, but I think it is time well-spent. On a similar note, if you are publishing shape information either to the wider scientific audience or to regulatory authorities, it is important for your terms to be well-defined.

9.3 Image Analysis Examples

In this section, I am going to go into some detail as to how we make measurements of size and shape using optical microscopy and the SEM. We have covered the details of image analysis in Chap. 7 and here we will apply what we learned there. The first example is a topical cream. I used a generic hydrocortisone cream as an example of a suspension. In many cases, image analysis is the only way we can measure the particle size of the suspension particles. In the second example, I examine the size and shape of carbon black particles on an SEM. I had hoped to simulate a nanosuspension with this sample, but it turned out to be quite difficult to separate primary particles from aggregates. Still, I think this example is instructive of how we work with wet-bead milled materials. We do not normally use image analysis as a primary technique for the size measurement of a nanosuspension. I use it as an accuracy check on both static and dynamic laser diffraction particle size measurements. If I achieve reasonably good agreement on the size of the nanoparticles with all three techniques, then I have high confidence in the faster, more precise laser diffraction measurements. Finally, we look at the components of a generic naproxen sodium tablet in cross-section. These tablets are excellent for learning and establishing good methodology since they are relatively simple tablets with only a few components. Once you have practiced with this sort of tablet (aspirin is also good for practice and has even fewer components than naproxen). To truly test your skills, take a look at a multivitamin. These tablets are interesting because of the large number of different components and elements.

9.3.1 Image Analysis of Hydrocortisone Cream with Optical Microscopy

I used generic 1% hydrocortisone cream for this example. I examined a number of different suspensions such as toothpaste and topical antibiotics. These suspensions are really interesting to examine but are a bit too complicated with too many different solid phases to serve well as a simple image analysis example. Hydrocortisone is better since it has only one solid phase. Hydrocortisone is a corticosteroid and is used for skin inflammation and irritation. It is readily available at the local pharmacy – or maybe in your home medicine cabinet.

Figure 9.1 shows an image of the hydrocortisone particles using a 20× objective. The sample was prepared by laying down two strips of tape on a microscope slide, depositing a small amount of the cream between the two strips, and smoothing with a razor blade. I then allowed the sample to “cure” for at least 2 h so that any air bubbles were removed. The hydrocortisone particles have good contrast with the background and are well separated from each other.

The image analysis routine was simple with steps for collecting the image, thresholding and segmenting the features. The binary features were filled, the border particles removed, and all features with an area less than 3 μm^2 were not measured.

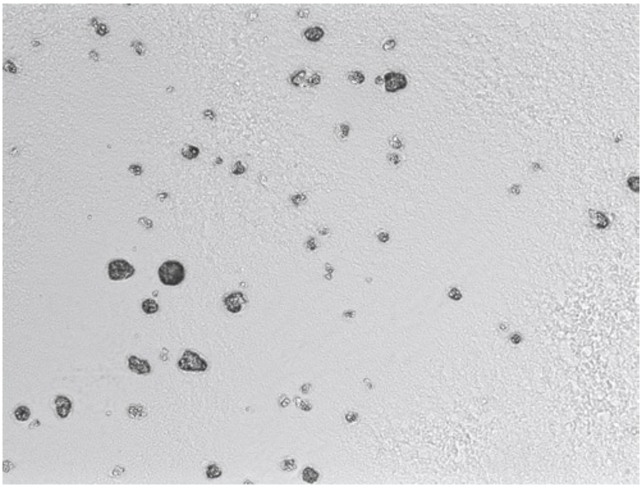


Fig. 9.1 Hydrocortisone suspension particles. The sample was prepared by laying down two strips of tape on a microscope slide, depositing a small amount of the cream between the two strips, and smoothing with a razor blade. The hydrocortisone particles have high contrast with the surroundings and so are good for image analysis (image size = 700 × 520 μm)

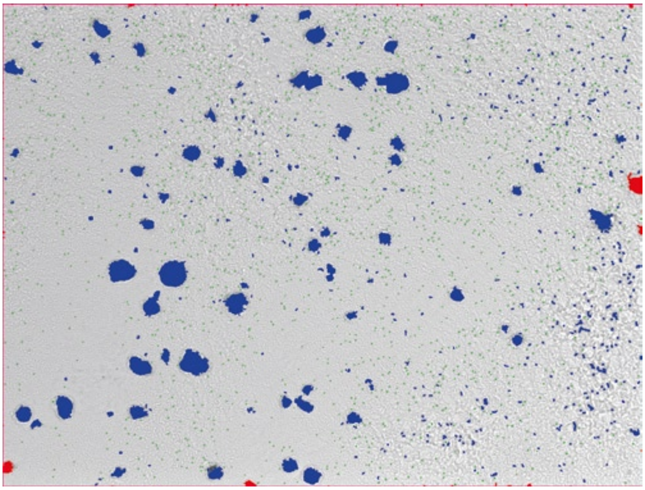


Fig. 9.2 Hydrocortisone image after image processing. The *blue particles* will be measured, the *red features* are particles touching the border and will not be counted and the *green features* are the small particles and noise that have been eliminated from the analysis (image size = 700 × 520 μm)

Figure 9.2 shows the resulting measurement image superimposed on the original image. The blue particles will be measured, the red features are particles touching the border and will not be counted and the green features are the small particles and noise that have been eliminated from the analysis.

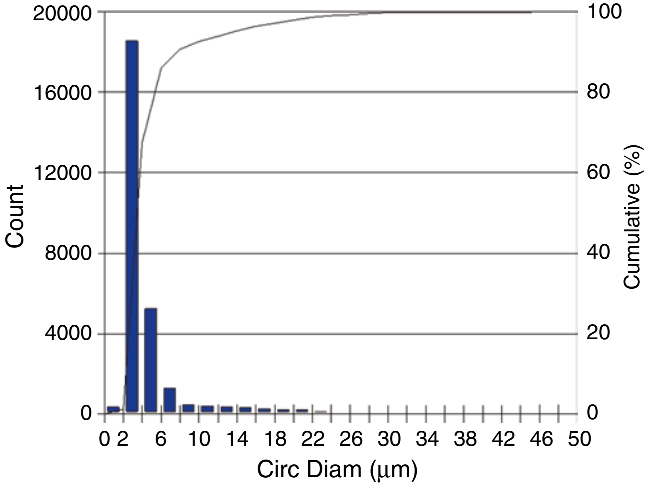


Fig. 9.3 Hydrocortisone size distribution: count vs. circular diameter. This graph shows the distribution of the circular diameter of the particles against the number of particles having that size. Count distributions give equal weight to each particle irrespective of size and are weighted toward the smaller particles

I chose a reasonably large number of measurement parameters including area, perimeter, sphericity, roundness, aspect ratio, fractal dimension, circular diameter, spherical volume, and aspect ratio (defined as the Feret diameter that is 90° to the minimum Feret as the length and the area in the formula $(\text{Length})^2/\text{Area}$. I refer to this measurement as the main length aspect ratio. I only intend on using the circular diameter and the main length aspect ratio in my analysis, but I think it is useful to have these other parameters available for future use if needed. I examined 20 fields from each of two slides. The analysis time was in the range of 5 min per slide not counting sample preparation. I measured approximately 28,000 particles. Figure 9.3 shows the distribution of sizes using the circular diameter by count. The mean circular diameter was 4.6 μm and the range was from 2 to 45 μm. For comparison, Fig. 9.4 shows the circular diameter weighted by the spherical volume and is a measure of the mass distribution. I expect these volume-weighted values to correlate better with the results from laser diffraction particle size. Here the mean diameter is 20.7 μm with the same range of course. Finally, Fig. 9.5 shows the distribution of the main length aspect ratio. A value of 1 is considered to be a perfect circle and larger aspect ratios indicate elongated particles. More than 75% of the particles have an aspect ratio less than 2 indicating that the bulk of the particles are nearly circular. This result implies that our use of the equivalent circular diameter is appropriate and that our weighting by volume is also appropriate. Table 9.1 presents a numerical comparison of the data generated in this test.

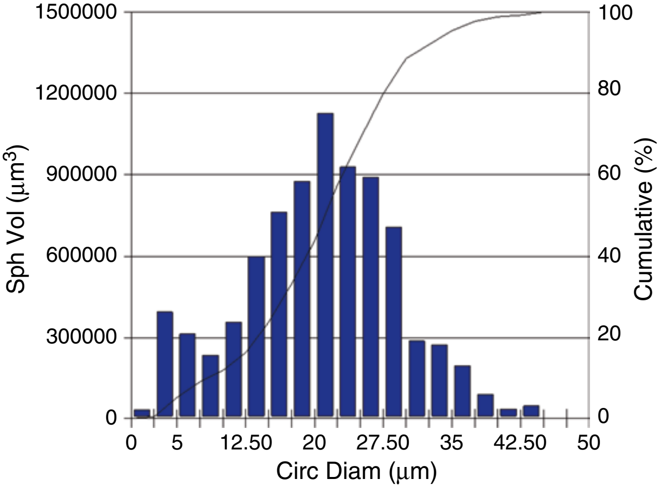


Fig. 9.4 Hydrocortisone size distribution: spherical volume vs. circular diameter. This graph weights the size by volume and is more nearly reflective of the mass distribution of the particles – providing the particles are approximately spherical

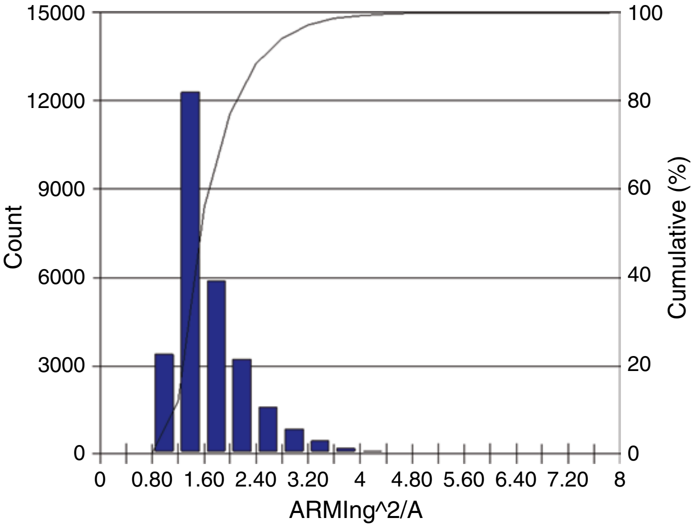


Fig. 9.5 Hydrocortisone distribution of particle aspect ratio. This graph shows the distribution of aspect ratio by count. The bulk of the particles have an aspect ratio less than 2 indicating that they are close to an ideal circle with aspect ratio of 1

Table 9.1 Hydrocortisone particle size and shape measurements, all values in μm except aspect ratio

Parameter	Mean	Std. dev.	×10	×50	×90
Circular diameter, count	4.6	4.1	2.3	3.3	7.4
Circular diameter, volume	20.7	8.4	8.3	21.1	30.4
Aspect ratio	1.7	0.6	1.2	1.5	2.5

Std. dev. = standard deviation, ×10 = 10th percentile, ×50 = 50th percentile, ×90 = 90th percentile

I chose to present this example first because it illustrates many of the points that I highlighted in Chap. 7. I devoted most of my development time to sample preparation and even though it is reasonably simple, it did take some time and experimentation to obtain good samples for analysis. Even though I state the sample preparation steps in a concise fashion, you will find that it does take some experience to get the particle distribution correct even with the aid of the transparent tape. The image analysis sequence is really very simple. I did not need to do any image processing nor did I need to do any particle separation. The binary steps were quite simple and few. I collected a number of different geometric parameters but only presented a few. I am confident that I could fully develop and validate this method and use it for both development and possibly even in manufacturing. I have no hesitation in presenting the data to regulatory authorities (after full development and error analysis of course). I do recommend that you practice on this product or a similar one in order to develop some facility with image analysis techniques.

9.3.2 Image Analysis of Carbon Black with SEM

For this example, we will switch from optical to scanning electron microscopy. The measurement of particle size and shape of small particles is a primary use of image analysis with the SEM. The analysis of nanoparticles by this technique is particularly useful since the effectiveness of the medicine directly depends upon the particle size. Another good use of the technique is with inhaled aerosol drug products where both size and shape are important to the effectiveness of the medicine. I decided to use carbon black for this example because of the small size of the primary particles and since it is, of course, readily available. It is a good sample for you to practice on but it does not quite mimic either nanoparticles or aerosolized particles. Carbon black particles are quite small and well within the nanoregion defined as less than 1 μm, but it is very difficult to break up carbon black aggregates. Notice in Fig. 9.6 that a number of the larger particles are really aggregates. Even with some of the drawbacks to carbon black, I still recommend it as a good practice compound.

I prepared the sample by mounting a small amount of the carbon black in water with a small amount of polyethylene glycol as a surfactant. I vortexed the sample for more than a minute and then sonicated it for approximately 20 min. I filtered a

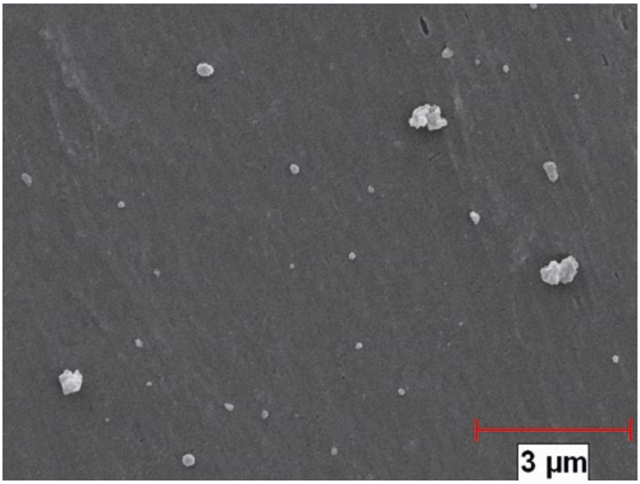


Fig. 9.6 Carbon black on Nucleopore™ filter. This is an example image of carbon black on a filter at 5 kV accelerating voltage and a magnification of 10,000 times. The image has been processed using a delineation step to improve edge definition of the particles and to improve contrast (image size = 12.2 × 9.9 µm)

small aliquot through a 0.01 µm Nucleopore™ filter, mounted the filter on double-sided carbon tape and sputter coated with gold-palladium. I examined the sample at 5 and 15 kV accelerating voltage in high vacuum mode. Although I collected quite a few images at each accelerating voltage, I used the 5 kV images for this analysis. The 5 kV images did not focus as well as the 15 kV images and the visual appearance was not as good. On the other hand, the 5 kV images had a more even illumination across each particle and were much easier to segment. Although the relatively poor focus was irritating to look at, the size and shape analysis was unaffected. In fact, I recommend experimentation with varying levels of focus on both an optical and a SEM image. It is surprising how poor the image can be for visual examination and still yield accurate size measurements.

For this demonstration, I only collected ten images from two specimens for a total of 20 fields. Although this number of images may be sufficient to yield a good estimate of the count statistics, it is insufficient for volume estimates. I did do some image processing as an edge delineation step. Thresholding and segmentation was straight forward although I did need to close and fill to ensure that the entire particle was measured. I eliminated border particles as in the hydrocortisone example. The elimination of noise and artifacts was somewhat more troublesome. The filter itself contributed some small feature noise just due to inhomogeneities in the height of the filter. I am not sure if these ridges and valleys are a property of the filter or of the way I laid the filter onto the double-sided tape. In either case, I had to remove these features. First, I used a size filter and removed anything less than 0.01 µm^2 in area. Second, I removed all elongated particles using a roundness filter. Figure 9.7 shows the resulting image ready for measurement. It is not a perfect representation

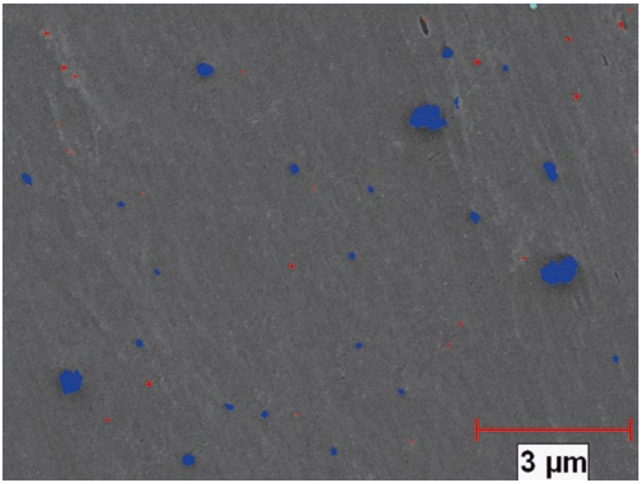


Fig. 9.7 Carbon black binary image. This is an example of a carbon black image after thresholding, segmentation (*blue*), noise removal (*red*), and border transfer (*green*). This image is ready for measurement (image size = 12.2 × 9.9 μm)

of what I consider to be the distinction between carbon black particles and artifacts, but it is acceptable in my opinion.

Figures 9.8 and 9.9 show the count and volume distributions for the circular diameter. The size analysis looks appropriate (see Table 9.2 for the statistical summary) and clearly most particles are less than 1 μm in diameter by both the count and the volume representations. Note, though, in Fig. 9.9 that many of the size bins are not filled and that there are no particles with a diameter near 1.5 μm. Only 176 particles were measured in this test and, clearly, more particles need to be measured for an accurate estimate of the volume distribution. Figure 9.10 presents the distribution of the aspect ratio of the particles. The mean aspect ratio is 2.5 suggesting that the particles are not as spherical as the hydrocortisone ones. It is possible that some of the particles with the larger aspect ratios are also related to the background features from the filter. I noted that many of these features were elongated. It might be a good idea to investigate this possibility further and setting an upper aspect ratio limit may be warranted.

Carbon black provides a good example of some of the challenges we face in size and shape analysis using the SEM. We nearly always have to face the issue of aggregated or touching particles and so sample preparation is a critical step. It is not, however, always a simple task to determine how much energy to put into the sample preparation process in order to break up the particle aggregates. We do want to have a sample preparation technique that mimics the way the dosage form will act in dissolution tests and in human use. That goal is nearly impossible to accurately determine and so judgment is called for on our part. I think the best strategy is to separate particles as well as we can with full documentation of the rationale for the separation procedure. Particle separation problems are acute with micronized and milled

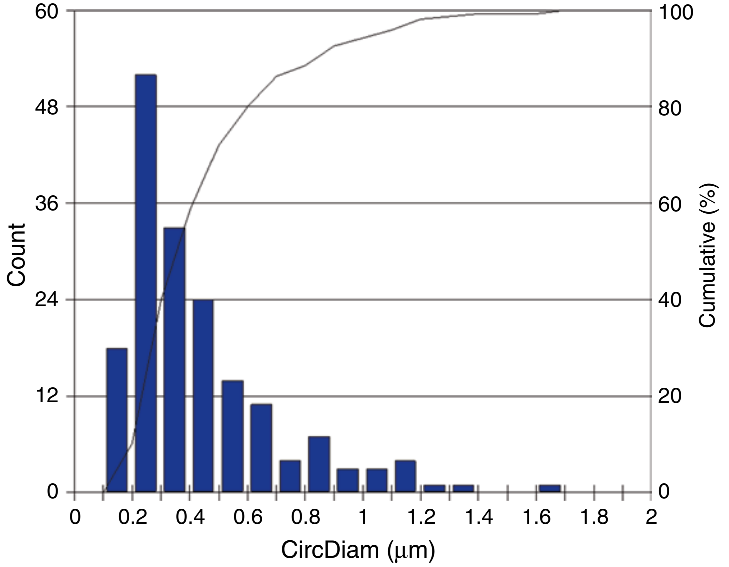


Fig. 9.8 Carbon black size distribution: count vs. circular diameter. Most of the particles are less than 1 μm in diameter. The distribution is in general agreement with the photomicrographs

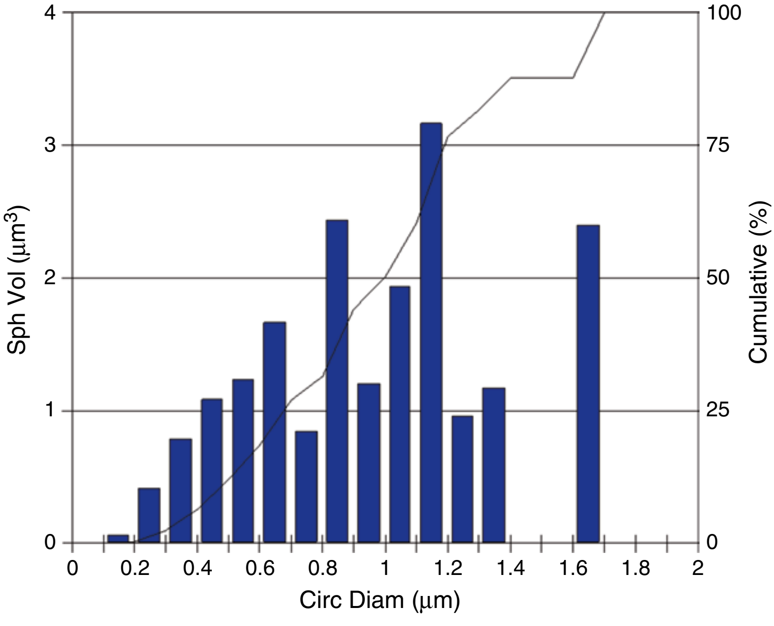


Fig. 9.9 Carbon black size distribution: spherical volume vs. circular diameter. This figure shows the distribution of particle circular diameter weighted by spherical volume. The volume distribution and statistical parameters correlate better with mass distributions than does the count distribution. This volume distribution presumes that the particles are spherical

Table 9.2 Carbon black particle size and shape measurements, all values in μm except aspect ratio

Parameter	Mean	Std. dev.	×10	×50	×90
Circular diameter, count	439	268	200	400	900
Circular diameter, volume	978	384	500	900	1,400
Aspect ratio	2.5	0.7	1.6	2.2	3.5

Std. dev. = standard deviation, ×10 = 10th percentile, ×50 = 50th percentile, ×90 = 90th percentile

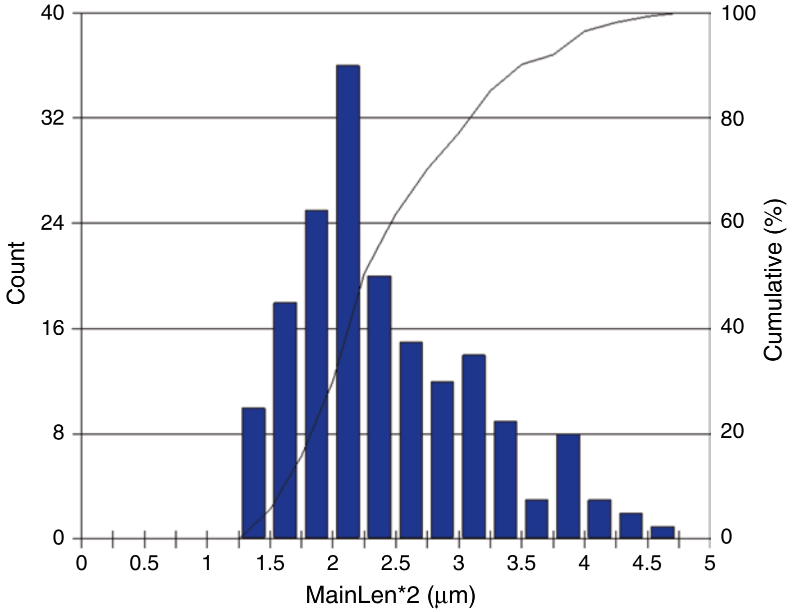


Fig. 9.10 Carbon black distribution by particle aspect ratio. This graph shows the distribution of aspect ratio by count. The bulk of the particles have an aspect ratio less than 2 indicating that they are close to an ideal circle with aspect ratio of 1

materials, but should be quite manageable with wet-bead milled suspensions. In the latter case, the challenge is to reproducibly prepare a dilute suspension for filtering and that is generally just a matter of experimentation.

9.3.3 Image Analysis of Generic Naproxen Sodium Tablet: Backscatter and EDS

I am going to switch gears in this example and look at features of a tablet cross-section. I chose naproxen sodium because it is easily obtained, has only a few components but also has some complications in the analysis. Naproxen sodium is a generic painkiller and readily available. Naturally, not every manufacturer uses

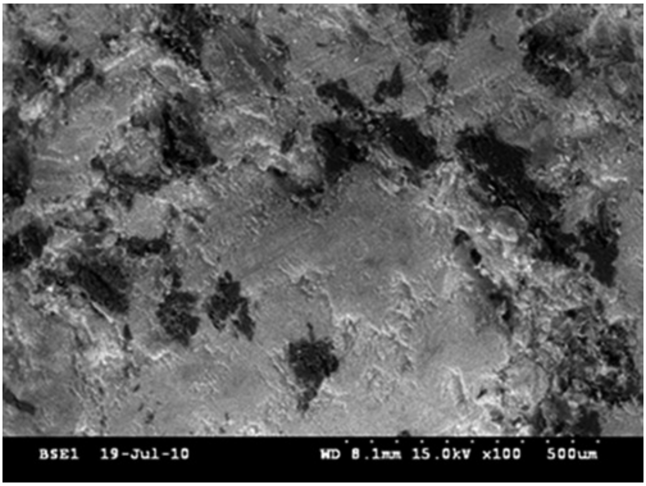


Fig. 9.11 Backscatter image of generic naproxen sodium tablet. This tablet cross-section shows at least three different contrast regions indicating at least three different components. The *darker region* is probably cellulose and the *background light region* is probably the drug substance

the same excipients so do not be surprised if you do not see the same elements and excipients in your tablet that I present in this section. Although my bottle does not list its ingredients, similar generic drugs contain cellulose (e.g., Avicel a microcrystalline cellulose) as a bulking agent, talc as a glidant and lubricant, silicon dioxide as a glidant, and magnesium stearate as a lubricant. The coating may include titanium dioxide. Figure 9.11 presents a backscattered image of the sectioned tablet and Fig. 9.12 shows the associated EDS spectrum. As expected, we detect carbon, oxygen, sodium, magnesium, and silicon. There is also a Cl peak for which I do not know the source and there are trace peaks of Al and Ti. The Al peak could be an artifact from the instrument but also might be in the talc. The Ti is probably from the coating and sample preparation process.

The most common use of this type of image is to assess the possibility that some of the excipients are not homogeneously distributed within the tablet. It is difficult to know how significant excipient inhomogeneities within a tablet might be to product performance. It is well-known that the level of magnesium stearate in a tablet can affect dissolution behavior with more magnesium stearate leading to reduced dissolution rate (Wang et al. 2010). I have not, however, seen any evidence suggesting that internal tablet excipient distribution differences contribute to this problem. There has been some work on magnesium stearate distribution using EDS, but most of the work is qualitative in nature (Neilly et al. 2009; Carlton 2010). On the other hand, we have not previously had practical techniques for evaluating this distribution within a tablet and so we probably do not know if an uneven distribution of excipients has any effects. The introduction of EDS silicon drift detectors and the consequent increase in map collection speeds provides a practical means for both

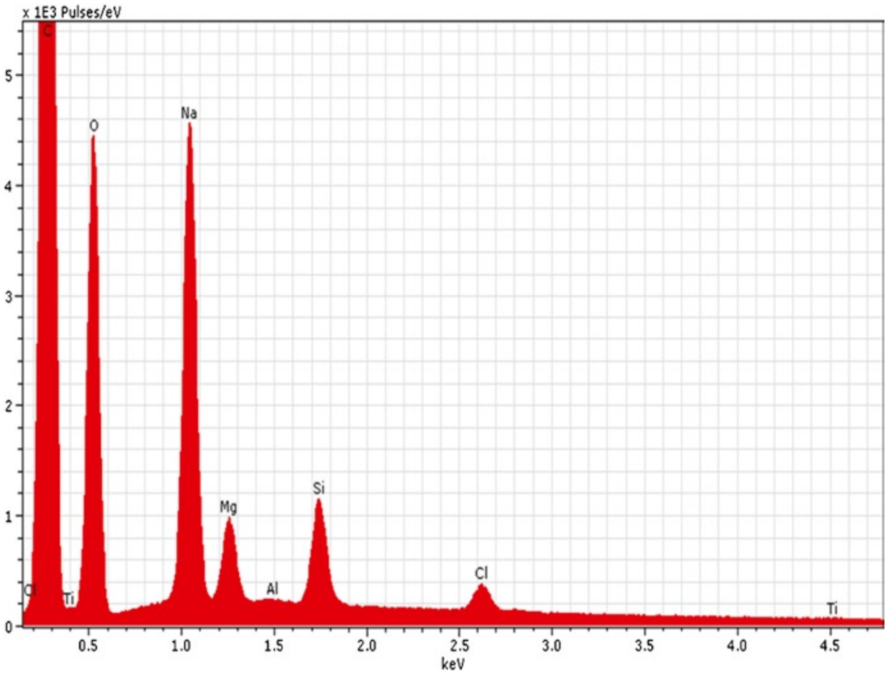
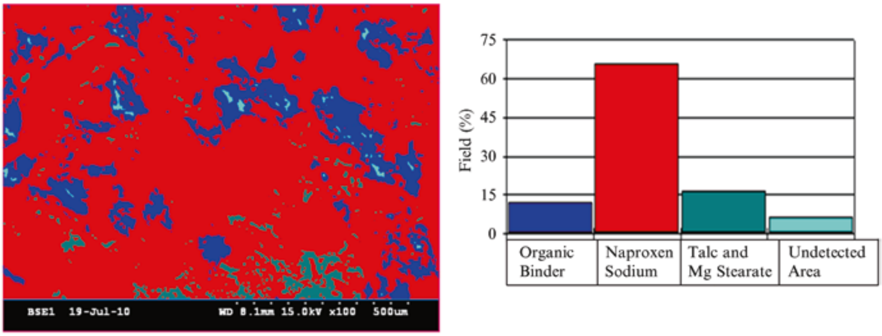


Fig. 9.12 EDS spectrum of naproxen sodium tablet. The EDS spectrum shows peaks for carbon, oxygen, sodium, magnesium, and silicon that are associated with the drug and suspected excipients. There is also a Cl peak of unknown source and there are trace peaks of Al and Ti. The Al peak could be an artifact from the instrument but also might be in the talc. The Ti is probably from the coating and sample preparation process

qualitative and quantitative analysis of excipients within tablets (see Chap. 4 for more information on silicon drift detectors and elemental maps).

Sample preparation can be particularly challenging with tablets. The ideal approach is to use a microtome to slice into the tablet and, of course, we are interested in the tablet face not the slices. This method can be approximated reasonably well if using a sharp blade and cutting the tablet by hand. These cutting techniques work well with tablets having good internal integrity but will not work so well with those tablets with poor integrity. I have had enormous problems making good sections of generic aspirin by any means. The generic aspirin that I have examined is nearly completely acetylsalicylic acid with a small amount of calcium phosphate used as a disintegrant. Although the tablet seems to hold together reasonably well with handling, sectioning it reduces the tablet to a heap of powder nine times out of ten. I have also tried mounting the tablet in a polymer and polishing the cut section as a metallographic mount. This method was no better than the microtome and required more time and trouble. I have had some success just polishing the tablet on fine sandpaper but I inevitably embed some of the polishing material into the surface. Sectioning problems, as with aspirin, are more the exception than the rule since most pharmaceutical tablets have organic binders to aid tablet integrity.



	Organic Excipient	Naproxen Sodium	Talc and Mg Stearate	Undetected Area
Area Percent (%)	11.7	66.0	16.1	6.1

Fig. 9.13 Naproxen sodium tablet field measurement using backscattered image. This image illustrates the use of a backscattered image with field measurements. In this case, the total area of each region that has the same gray level are ratioed to the total area. Features are not measured individually. Although field measurements are not commonly used, this image does illustrate the use of backscattered images for image analysis as well as the use of field measurements

Figure 9.13 presents a backscatter image of the naproxen sodium tablet section with superimposed field measurement results. Remember (see Chap. 7) that field measurements evaluate the entire field without distinguishing among features. In other words, it lumps all features with a certain gray level together. So, with area as an example, all the dark blue features are summed to yield a total area of dark blue – which corresponds to the organic cellulose binder. This value can then be ratioed to the entire area to yield an area percentage. We assume that the area percentage is equal to the volume and mass percentage of that phase reference. This type of analysis can be somewhat useful if we compare area percentages from a number of regions on one tablet with area percentages from a different tablet. For instance, we can compare excipient areas between good and poor dissolution tablets. In my opinion, though, field measurements are not particularly useful and we can obtain similar information for object-based measurements where every feature is individually measured and reported. Field measurements are simple to make and the only requirement is that the selected gray values correspond to each excipient and to the drug substance. In Fig. 9.13, the red corresponds with the drug substance, whereas the dark blue and green regions are excipients. The light blue regions had little backscatter signal and were not counted. This figure does illustrate the use of backscatter images for image analysis providing we have determined the elemental composition of each area by EDS.

Figure 9.14 shows the corresponding image analysis using EDS maps and object measurements instead of backscattered images and field measurements for oxygen, sodium, and magnesium. The oxygen (green) is associated with many of the excipients but can easily be used to measure the size distribution of the cellulose binder.

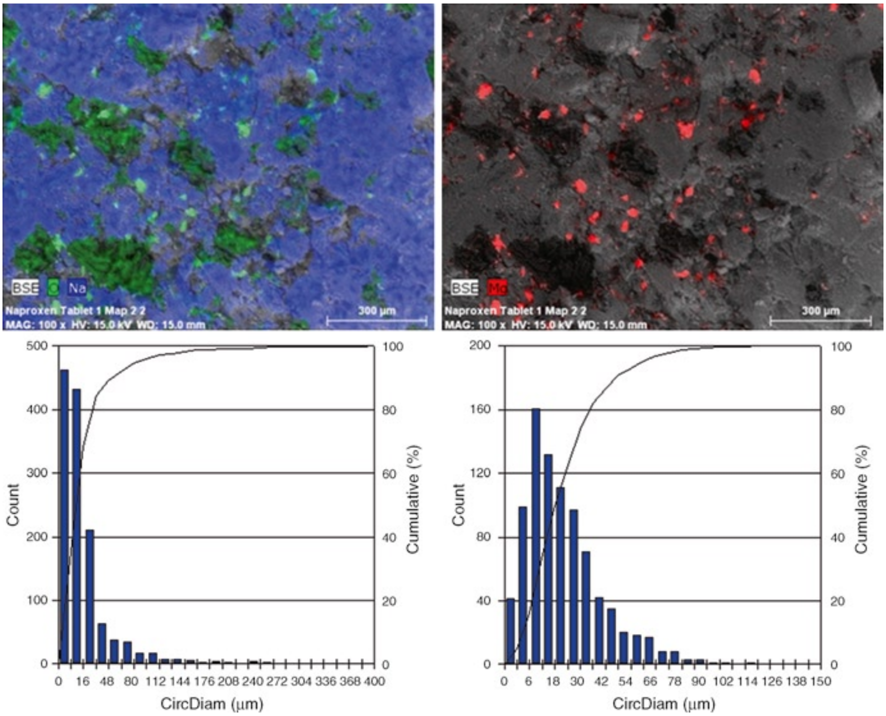


Fig. 9.14 Naproxen sodium object measurements using EDS maps of oxygen and magnesium. EDS maps were collected using a silicon drift detector. The resulting maps were evaluated using an image analyzer and multiple measurements were collected for each feature. Such data can be used to quantitatively compare the size and shape of the excipient particles within a tablet and among different tablets

The sodium (blue) is only associated with naproxen sodium and can be used for measurements of the drug substance. The magnesium (red) map is more complicated in the sense that it certainly represents the talc, but may also represent the magnesium stearate. A good way to distinguish the two sources is to subtract the silicon map from the magnesium map. That should just leave the magnesium stearate regions. In order to obtain suitable maps for this purpose it is necessary to collect the maps at higher image resolution and for longer than the 15 min collection time of these maps.

Also note the possibility of presenting size and shape data on the different features in the map. In other words, we should be able to compare size and shape data between different regions and different tablets. In this way, we can quantitatively determine whether excipient distributions are different among different tablets and tablet lots.

There are a number of different kinds and sizes of hurdles to jump in analyzing excipient distributions within tablets using EDS. We have already discussed the problems associated with good sample preparation which is a relatively high hurdle in some cases. We have not discussed magnification. Some of the features we want to measure can be quite large and a low magnification is required. Some of the excipients, such as silicon dioxide and magnesium stearate, are quite small requiring higher magnification. We can probably find an intermediate magnification that works for both sorts of particles in most cases, but we do need to explicitly study the matter. If we use low magnifications, we will be limited to only a few fields of view for measurement, depending upon the size of the tablet. In order to make good quantitative measurements, though, we probably need to assess many fields of view from many regions of many tablets. Estimating the required population before analysis is difficult and, since there is little in the literature on the subject, we cannot rely on previous published work to guide us. Even with silicon drift detectors, the analysis time is lengthy and if we require many tablets and sections, then the analysis may require many days or even weeks to complete. The time factor limits the use of the technique to nonroutine testing.

We also need to be concerned about the stability of our samples in the electron beam and the effects of beam spread on the results. EDS counting statistics improve with beam energy and current. The stability of the sample in the beam is opposite to that relationship and we risk destroying or burning our sample with a high beam current. Sputter coating with metal will not help the stability issue and we do want to work in low vacuum mode so we can minimize charging with such a large nonconducting sample. The chamber gas, though, will cause electron beam spread which will certainly degrade our spatial resolution when using EDS since X-rays will be generated some distance from the location of the electron beam. We do want to minimize the gas path length if our SEM has that option and we want to minimize the gas pressure as much as is required to inhibit charging.

Finally, there are a number of issues with segmenting EDS maps. Most EDS maps have quite a bit of granularity in the sense that a contiguous feature may not have an even color distribution. Most modern EDS systems have image processing options that smooth the colors and produce features with a good distribution of color. Those image processing steps may result in some inaccuracies in size and shape measurements. In other words, how confident are we that the software has filled in the gaps in a feature correctly? One solution to this problem is to collect longer maps, but that leads to other practical issues in that generally longer analysis time results in fewer collected images. We can also use the image analysis software to segment the features and decide upon the set of rules for segmentation after some experimentation. The electron beam spread can complicate the segmentation if many X-rays are collected far outside the target location of the primary electron beam. I think the best way of solving segmentation problems is to compare the EDS map with the corresponding backscatter image or to simply use the backscatter image if we can distinguish all of the compounds of interest using the gray levels of the backscattered image.

Even with all of the possible complications, I think we will see more work published looking at the distribution of drug and excipients in drug product. It will be interesting to see if it is possible to draw correlations between such distributions and drug product performance. Undoubtedly, quantitative image analysis will be required for such correlations.

9.3.4 *Summary of Exercises*

You may never be called on to assess excipient and drug inhomogeneities within tablets or to evaluate the size distribution of particles in a topical suspension. Still, I do think such work makes for worthy exercises and ones that will teach you quite a bit about practical applications of image analysis. At a minimum, I recommend that you use image analysis with the common dosage forms used at your development site and to add a few other examples for general information. There is no good substitute for practice and experience if you wish to be skilled at the image analysis of pharmaceutical drug substance and product.

9.4 Method Development and Error Analysis

9.4.1 *General Considerations*

There are a variety of reasons to develop and use test methods in pharmaceutical development and the particular reason governs the required rigor in method development and error analysis. For example, early in drug development, we may wish to minimize the amount of material used in determining particle size and we wish to get the maximum amount of information in a short period of time to aid in the establishment of the chemical synthesis. In this case, a very simple image analysis (IA) method may suffice to yield the required information. On the other hand, if we wish to use IA information as part of the registration stability package sent to regulatory agencies, then the method should be fully developed and validated. The requirements for the validation and error analysis of image analysis methods are not well established since such information is rarely used for drug substance or drug product specifications. The following advice comes from my own experience, from conversations with other microscopists, and from what is commonly required for particle size method development and validation (see also Zingerman et al. 1992; Larsen et al. 2003).

In general, we divide the making of a test method into two parts: method development and error analysis (accuracy and precision). I discuss both of these aspects in some detail in this section. As a side-note on terminology, it is common in pharmaceutics to refer to all method error analysis studies as "validation." I use the terms "error analysis" and "validation" interchangeably. In my opinion, while the term "validation" is appropriate for methods used to measure chemical impurities, it is less appropriate for particle size and shape methods. Chemical impurity methods are validated against independent requirements as established by regulatory authorities. We are validating that the method is fit for the purpose of determining specified amounts of chemical impurities. In particle size analysis, on the other hand, there are no independent requirements and any specifications generated must include the error due to the methodology. Whatever term is preferred, it clearly is important to know the sources of error in the measurements.

Table 9.3 Steps in method development and error analysis

Method development	Method error analysis
Sample preparation	Accuracy
Sampling statistics	Precision: repeatability
Choice of measurement parameters	Precision: reproducibility
Effect of image processing	Specificity
Effect of threshold	Range
Effect of binary processing	Detection limit
Range	Robustness
Detection limit	

In Table 9.3, I present the different aspects of method development and error analysis. Some aspects of method development and error analysis are shared and iterative. In method development, we chose magnification and transfer limits depending upon the purpose of the test and these set our detection limits, whereas in error analysis we need to ensure that, in fact, we can detect the size and/or shape we have chosen. In the next few sections, we will discuss each of these aspects of method development and error analysis that have not previously been presented in Chap. 7.

9.4.2 Method Development

The first step in method development is the choice of microscopy technique. This choice begins with OM vs. SEM and then the particular imaging conditions for each. The decision may seem trivial but can be complicated. For instance, I have developed IA methods for creams with suspended drug substance. The drug substance is micronized with an ×90 of less than 5 µm. Normally, we want to use SEM for the method given the size of the particles. I chose polarized light microscopy with a high NA objective and operated in fully crossed polars. The drug substance appeared as barely resolvable circles of white light on a dark background. Clearly, the accuracy of individual particle size measurements in this method is relatively poor given that OM does not size accurately below ~3 µm. On the other hand, the purpose of these methods was to detect particle growth on stability. The methods contained a qualitative aspect in that the particles were observed for morphology – sharp crystal faces indicate growth, but the quantitative aspect was suitable for the purpose. If crystals grow at all, the optical microscope/image analysis method will detect that growth. SEM was not chosen given the difficulties in sample preparation of a cream for SEM and the difficulties of separating the particles from the matrix. Much of the success of the IA method depends on a good choice of imaging technique and there are no hard and fast rules for that choice.

I have shared my obsession with sample preparation many times and will do so again here. The success of the image analysis method is directly related to sample preparation and, in my opinion, the vast bulk of your method development time should be spent on sample preparation.

The next steps in method development are interactive and iterative between routine development and sampling statistics. We typically start with a simple routine and establish the necessary image processing, segmentation and binary operations which requires testing a few fields of view. Then we choose the geometric measurement parameters (usually in consultation with the requestor, i.e., what are they looking for) and ensure that our overall routine accurately measures most particles in a field of view. Now we might begin to think about sampling statistics. As a general rule, we get better statistics if we measure few fields of view on many independent preparations. Clearly, we have to balance optimum sampling statistics against practical time considerations. Sample preparation is generally one of the most time-consuming aspects of image analysis. I generally begin with 25–30 fields of view from two independent sample preparations. I will adjust upward depending upon the intention of the method and the interim results.

Shape data is often more normally distributed than is size data. Consequently, it may not be necessary to measure as many particles for shape as for size. If the data from each sample preparation are compared and the 95% confidence limits for the mean and median overlap, then a couple of slides and a few thousand particles may be sufficient. If size is being estimated, on the other hand, and in particular estimations of mass-weighted size distributions, then care has to be taken with the number of measurements and sampling. For instance, the rule of thumb is that for mass distributions, every size bin in the distribution must contain at least 20 numbers (Allen 1997). If your distribution is skewed with a few large particles in each field of view, then more measurements of many sample preparations and fields of view will be required. I have developed methods requiring more than five sample preparations and more than 100,000 measured particles.

This is a good point at which to evaluate the ruggedness or robustness of the method. Slight changes to operating parameters such as focus, segmentation, image processing, and binary processing can be used to assess the effects on size results. The ruggedness studies help to determine the acceptable limits for each of the parts of the method. For example, for a number of methods that I have developed, I found that focus was not as important as I originally thought. Relatively wide variations in focus had little impact on the final values. Of course, this is not a general rule and must be tested in each case.

9.4.3 Error Analysis and Validation

Error analysis is made up of two components: accuracy and precision. Accuracy is the agreement of results with a well-characterized standard, whereas precision is agreement of repeat tests or experiments. Precision can be further divided into repeatability and reproducibility. Repeatability is agreement of results conducted by the same operator on the same sample using the same instrument on the same day.

Reproducibility is agreement of results where the operator, day, instrument, and sample are different. Precision studies are best conducted using a multifactorial design of experiments approach. This type of study allows us to estimate the error in each part of the analysis as well as the overall analysis.

Accuracy begins with the calibration of the system (optics, camera, computer) using a known size standard (see Chap. 7). The routine performance check is also a practical measure of accuracy. Calibration and performance checks, however, are probably not sufficient for determining size accuracy and certainly not sufficient for shape accuracy. Calibration and performance checks are generally two-point linear distance measurements which are no guarantee that the way in which the image analysis program converts these distances into areas, circular diameters, aspect ratios, etc., is accurate. Consequently, it is necessary to go beyond these measurements for a full error analysis and validation.

In most cases, we use a micrometer slide or micrometer stub for calibration and performance checks. It is preferable to use size standards that have been certified by a testing laboratory such as the National Physical Laboratory in Great Britain or the National Institutes for Science and Technology in the USA. I have, on occasion been asked by those in quality assurance as to the frequency of recalibration for these standards. In my opinion, they do not require recalibration, but my opinion has not always carried the day. If you are required to recalibrate then I think doing so 1 year after purchase and every third year after that is sufficient. I do recommend that you argue against the practice. It is hard to formulate a reasonable mechanism which results in a size change of the standard without ruining its use at the same time. Rarely, if ever, are microscope measurements used in specifications and when they are, the specifications rarely have an impact on safety, stability of efficacy of the drug product. Microscope specifications generally reflect good manufacturing practice rather than key use attributes of the drug product. I think these are persuasive arguments against recalibration but I have won the argument only about half of the time.

It is generally much easier to assess accuracy in size measurements than to assess accuracy in shape. That is due to the number of available size measurement standards and the lack of availability of shape standards. The NIST has a number of size standards, some of which have already been mentioned, as do some commercial companies. Most of these standards are not specifically designed for image analysis but can be adapted for that use. For instance, the NIST glass standards such as 1000 series (20–2,450 μm) are certified as particle size standards, but the certification is for the entire bottle of powder. If the bottle is subsampled, then the particle size distribution will only be accurate if you have taken the trouble to ensure good sampling, i.e., using a powder riffler. Still, we expect to get results that are reasonably close. It is tempting to use some of the polystyrene spheres from companies such as Duke Scientific Technologies. In my experience, these standards are somewhat difficult to use. In most cases, the particles are suspended in water which means that the specimen will begin to evaporate as soon as it is prepared. Evaporation will lead to segregation and movement of the particles. If polystyrene samples are used, be sure and pay extra attention to your sample preparation.

Good standards for shape are hard to come by. It is possible to obtain pretty good standards representing spheres. Glass and polystyrene standards are reasonably good for this purpose. Standards for other geometric shapes are more difficult to find. I personally have not been faced with the necessity of using a well-characterized and suitable shape standard. What I have done is use artificially generated computer shapes or well-behaved crystals (such as salt crystals or glass fibers) to demonstrate the capability of the instrument to distinguish among different shapes. A good set of shape standards would be useful to the discipline.

Let us consider three approaches to determining size accuracy: (1) Manual linear measurements of a size standard (Graticules LTD, 1 mm/0.01 divisions); (2) NIST Glass Microspheres SRM 1965; (3) Duke Scientific glass microspheres – No. 9020. In this case, a Leica DM6000 microscope and objectives were calibrated using a NIST traceable size standard from Clemex Technologies. For test 1, the Graticules micrometer slide was placed onto the scope and ten manual measurements were made using the following objectives: 5×/0.15 NA HC PL Fluotar, 10×/0.30 NA HC PL Fluotar, 20×/0.50 NA HC PL Fluotar, 40×/0.75 NA HC PL Fluotar, 50×/0.90 PL Apo, and 100×/0.95 PL Apo. The results are presented in Table 9.4 below.

These results constitute excellent accuracy in manual linear measurements. In a sense, it defines the capability of the system to make good measurements. But, since the measurements were made in a manual mode, the results do not prove good accuracy in an automated mode.

In the second test, 100 fixed, latex microspheres of NIST SRM1965 were measured at three magnifications using a simple routine involving collection of the image and thresholding followed by removal of noise, agglomerated particles, particles touching the boundary, and nonspherical particles. The results are presented in Table 9.5.

Table 9.4 Replicate measurements of micrometer slide, mean, 10 measurements each

Objective	Nominal size (μm)	Measured size (μm)	Standard deviation (μm)
5×	1,000	999.6	1.62
10×	500	499.9	0.46
20×	200	200.0	0.24
40×	50	50.0	0.13
50×	50	50.0	0.12
100×	20	20.0	0.12

Table 9.5 Replicate measurements of NIST SRM 1965, 100 measurements

Objective	Mean diameter (μm)	Standard deviation (μm)	Relative error (%)
10×	10.6	1.0	7.1
20×	10.1	0.7	2.0
40×	9.8	0.6	−1.0
Certified diameter	9.9	0.04	–

There is good agreement between the NIST certified diameter and the results using the 20× (2% relative error) and 40× objectives (1% relative error). While marginally acceptable, the 10× results (7% relative error) are clearly not as good as those with the 20× and 40×. This difference is reasonable as we expect that the higher magnification objectives are more accurate.

As a final accuracy check, we compare the stated values for the Duke 9020 glass beads with a measured distribution. In this case, the 9020 glass beads were suspended in silicone oil on four slides and 25 fields of view were measured on each slide. The results are presented below in Table 9.6.

The mean diameter for all of the slides was 21.0 μm vs. the certified value of 20.3 μm which represents a 3.4% relative error. As noted in Chap. 7, I believe better than 5% relative error represents good agreement for this type of testing. While spheres appear to be a good standard for testing, determinations of good focus and edges can be problematic. Figure 9.15 is a photomicrograph of the Duke 9020

Table 9.6 Replicate measurements for Duke Scientific 9020 glass beads

Slide	Mean diameter (μm)	Standard deviation (μm)	Population
1	21.3	1.71	814
2	21.0	1.71	1,233
3	20.7	1.71	1,237
4	21.2	1.72	530
All slides	21.0	1.71	3,814
Certified value	20.3	2.1	–

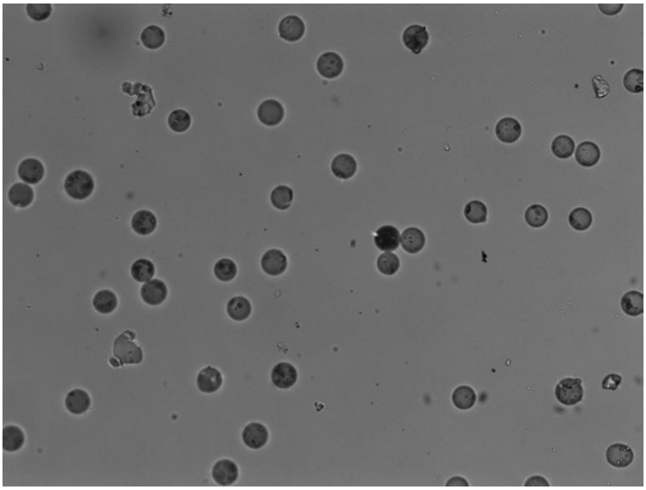


Fig. 9.15 Optical image of Duke Scientific No. 9020 glass beads. These glass beads are useful for error analysis since they contain a narrow distribution of bead diameters. Note the difficulties in focusing each particle in the field of view simultaneously. Also note that there are some glass fragments that are not spherical. These fragments and other noise need to be eliminated from measurements as part of the image analysis routine (image size = 700 × 520 μm)

spheres and you can see the difficulties in obtaining good focus across the entire field of view and in determination of the edges. Further, not all of the glass beads are perfectly spherical in shape and the distribution can be affected by how these artifacts are handled.

There are some aspects of the error analysis that will be the same for every method. I find it useful to deal with these aspects of accuracy and precision using standards and including this information in a separate report accompanying the validation documentation. For instance, the data presented in the accuracy section is the same for all analyses – providing the objective of interest has been evaluated with an appropriate sized standard. An omnibus accuracy report can be written that includes good tests of accuracy for all of the objectives normally used in your imaging applications. For precision studies, we should establish the error in certain operations but it is not necessary to use the material in question. For instance, we should establish the error due to repeat tests of the same image as well as the error if the same image is collected a number of different times. We anticipate that this error is quite small and establishing the values of the error once with standards should be sufficient.

As an example, I tested a collection of glass beads by determining the size by running the image analysis routine ten times on the same image. The mean value was 27.5 μm with a standard deviation of 0. This means, of course, that the same mean value was obtained each time the routine was run. Using the same field of view, I refocused and refit the threshold and ran the routine ten times. The mean value was 27.2 μm and with a 0.2 μm standard deviation. There is some variability but it is quite small (Carlton and Englehart 2005).

Because we are working with a distribution and because those distributions are not close to Gaussian, the precision of our measurements are strongly affected by the range of particle sizes. For example, let us suppose that we have a mean diameter of ~20 μm but we also have some agglomerates that are much larger – say 100 μm. The presence of these relatively rare large agglomerates will have an impact on the distribution values and hence the precision. While it is always the case that precision is made up of both the measurement error and the natural variability of the tested sample, particle size and shape analyses are particularly prone to larger error compared to some other analytical techniques such as chromatography. This fact complicates determinations of method error.

Precision studies are probably best illustrated by example. Let us take compound A which is representative of what we get with cohesive drug substance. Figure 9.16 is a light photomicrograph of a typical field of view. As you can see, it is a pretty tough sample to work with, but typical. Let us suppose we are interested in the size (diameter) of the particles and we have decided to treat the agglomerates as particles and are not interested only in primary particle size. Let us begin by testing five different locations on one slide and measure 25 fields of view at each location and then let us look at 25 fields of view from five slides. Tables 9.7 and 9.8 present the results of the tests.

First of all, note that there is quite a bit of variability within the slide itself. For instance, the mean circular diameter ranges from 9.6 to 14.1 μm. The ×50, ×90 and

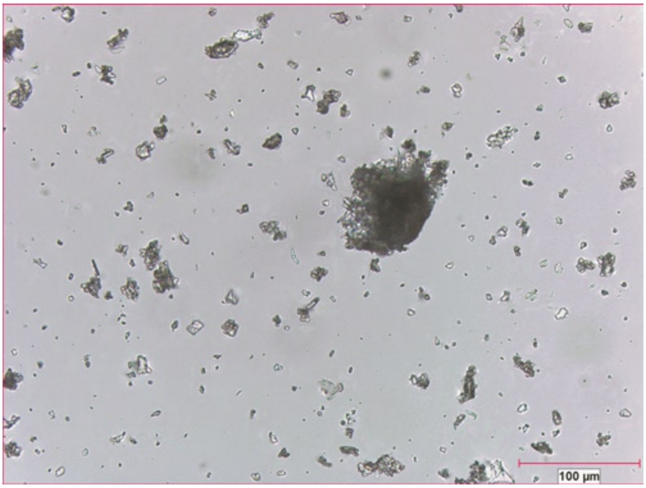


Fig. 9.16 Photomicrograph of representative field of view for typical drug substance. This image shows the typical appearance of cohesive drug substance mounted in silicon oil. The particles are reasonably well separated but there are definitely aggregates that have not been broken apart by the sample preparation technique (image size = 520 × 390 µm)

Table 9.7 Compound A: 5 slides, 25 fields of view, circular diameter (µm)

Slide	Count	Mean	×10	×50	×90	Maximum
1	3,621	12.4	5.5	12.4	23.4	130
2	5,572	12.8	5.8	10.1	23.0	138
3	3,444	10.2	5.4	8.2	16.9	66
4	4,448	12.4	5.5	9.2	21.7	111
5	3,345	10.1	5.5	8.7	16.8	179
Mean	4,086	11.6	5.5	9.7	20.3	125
Std. dev.	938	1.3	0.1	1.7	3.3	41
RSD (%)	23	11.2	2.5	17.1	16.0	33

Table 9.8 Compound A: 5 locations on 1 slide, 25 fields of view, circular diameter (µm)

Location	Count	Mean	×10	×50	×90	Maximum
1	3,345	10.1	5.5	8.7	16.8	179
2	3,076	11.9	5.6	9.6	20.2	142
3	1,595	14.1	5.8	10.6	27.1	92
4	2,212	9.6	5.6	7.8	14.7	89
5	5,572	12.8	5.8	10.1	23.0	137
Mean	3,160	11.7	5.6	9.4	20.6	128
Std. dev.	1,517	1.9	0.2	1.1	4.9	38
RSD (%)	48	16.0	3.9	11.9	24.2	30

maximum also have large ranges. The ×10 does not have a particularly large range and this is common in particle size analysis. Most distributions are skewed toward the larger particle sizes which primarily affects the other statistical parameters. The variability is particularly evident in the relative standard deviation (RSD) or coefficient of variation (defined as the standard deviation divided by the mean). For most analytical work, we like to target the RSD to less than 5% although we may relax that a bit to 10% for particle size analysis of small particles. For the within slide comparison, the RSD is 16% for the mean circular diameter and 12% for the ×50. Most of this variability is due to the wide distribution in size values and the difficulty in sampling. An obvious way of reducing the RSD is to count more areas on the slide and to ensure that sampling is representative. Practical constraints, however, are such that we may have to accept high RSD values. If that is the case, then any specifications applied to this sort of data must take the high RSD into account.

The data from multiple slides (see Table 9.7) displays similar variability to that of the within one slide data (Table 9.8). Interestingly, the mean values of the statistical parameters for the within slide and the between-slide comparison are nearly the same. For instance, the mean circular diameter is 11.7 μm for the within slide comparison, whereas it is 11.6 μm for the between-slide comparison. This result suggests that for this material it may not matter whether we sample many regions of one slide or sample many different slides.

There are a number of different ways of representing the precision. We can list the standard deviation, the RSD, the variance (square of the standard deviation) or confidence limits. I personally have a preference for 95% confidence limits since it is relatively easy to make comparisons of different samples with them. To refresh your memory, the 95% confidence interval is defined as

$$\mathrm{CI} = \overline{x} \pm ts / \sqrt{N}, \qquad (9.1)$$

where CI is the confidence interval, x is the mean value, t is the Student t value, s is the standard deviation, and N is the sample population. For five slides, we should use 2.571 as the t value for a two-sided 95% confidence interval, and the square root of 5 as 2.24. The confidence interval for the ×50 then is $9.7 \pm (2.571 \times 1.7)/2.24$ μm or 9.7 ± 1.9 μm (or 2.0 if you round up). I like this representation since the difference in distributions can easily be established if the 95% confidence intervals do not overlap. Clearly, there is much more that can be done around determining and improving precision and whether or not you do more will be governed by your companies' procedures and the eventual use of the method.

Specificity is not normally tested for image analysis methods since we normally deal with one compound. If multiple species (such as a two-component suspension) are present in the formulation then it may be necessary to establish specificity. A full characterization of each species according to the property being used for discrimination should be sufficient. If birefringent particles are being compared with nonbirefringent ones, it will need to establish that all birefringent particles belong to one species and all nonbirefringent to the other.

Range and detection limits are set by the choice of objective and the transfer settings in the image analysis routine. I recommend that you establish with the requestor the lower size or shape limit of interest and then chose the microscopy and routine according to that limit. The upper limit is a function of the magnification and the size of the field of view. We do have to be careful here since even if it is possible for a large particle to be encompassed within the field of view, there may be a very low probability that a whole complete particle will ever be centered in the field of view. You probably want the area of the field of view to be at least four times the area of the largest particle of interest which should give you a reasonable probability of detecting and measuring large particles.

When setting the lower detection limit, be mindful of the effective resolution limit of the objective when doing optical microscopy. For instance, a 40× objective with a numerical aperture of 0.75 has a lower resolution limit of ~0.5 μm with white light (remember that the resolution limit is defined as 0.61 times wavelength of light divided by the numerical aperture of the objective). It may actually be possible to measure smaller features with the image analyzer but these measurements cannot be correct.

We should check robustness of the sample preparation and the image collection and analysis steps. In particular, we should make small variations in the way we prepare samples and see if these variations affect the results (that is where the confidence interval comes in handy). We should also alter the substage condenser opening, the bulb voltage, the thresholding and any other variable that we suspect will affect the results. Again the most efficient approach to this subject uses design of experiments procedures.

As you can tell, a proper error analysis and validation is a time-consuming affair. I estimate that an average of 3 weeks is required for a full method development and error analysis. That may seem excessive and it probably is if we have a well-behaved sample and the validation gods are smiling on us. If the sample is contrary and we have angered the gods, then 4–6 weeks is not unreasonable.

9.5 Specifications

It is rare that we need to develop specifications for image analysis particle size results but it does occur. If needed, they generally include a limit for the central tendency (mean, median) and some limits on the spread of the distribution (10th and 90th percentiles). In some cases, there are in total six specifications – upper and lower limits on the ×10, ×50, and ×90. In general, specifications are based on results from tests of a number of DS or DP batches and, in particular, those clinical and stability batches. We may have to request provisional specifications if there are only batches used in development. Typically 50 to 100 batches are needed to set reasonable specifications. In most cases, we do not have that many batches before regulatory filing. We need then, to provide reasonable interim specifications based on both batch history and on method variability.

As an example, let us suppose we measure the ×50 of say ten batches and determine that the standard deviation of the samples is 0.6 μm with a mean value of 23.1 μm. Now suppose that the method standard deviation for the ×50 is 0.4 μm. An obvious approach is to simply use the sample standard deviation of 0.6 μm and multiply by 3 (95% confidence limits) and set the specification at 23.1 ± 1.8 μm. I have successfully argued that since we have so few batches, we ought to include the method variability in the specification as well. The limits then are $\sqrt{(0.6^2 + 0.4^2)} = 0.7\,\mu\text{m}$ with the resulting limits as 23.1 ± 2.1 μm. I am sure that some will blanch at the lack of statistical rigor at such an approach, but I think I have some justification for it. In general, the batch history will be collected on one or at most two instruments by one or at most two operators. Consequently, the batch tests will not include a component accounting for different instruments and operators. The final release of the DS or DP will generally be conducted by a different laboratory, instrument and operator(s). The method variability, if estimated correctly, will take into account the variability attributable to these additional factors. I think it is reasonable to combine the variabilities when designing provisional specifications using a limited number of batches.

Do not be surprised when your proposed specifications get kicked around and dissected by folks within your own organization and by regulators. Frankly, there does not seem to be an ideal method for setting particle size specifications and it seems that everyone has an opinion on the subject and is eager to share it with you. I have yet to go through a filing that did not require significant numbers of hours arguing over the specifications internally and then responding to requests and questions (actually demands) from regulators. We might be forgiven for just chucking out any old specifications, knowing it is all to do over and over again. I guess it is possible I am a bit cynical on the subject. To be fair though, the arguments make sense from each perspective. Regulators see no need to allow specifications beyond what has gone into the clinic. They know material with those properties works. From a manufacturing perspective, making the specifications too restrictive may result in too many batch failures with the consequent effects on the profitability of the drug. So there will naturally be this give and take between narrow and wide specifications.

9.6 Automation

I started using image analysis in the late 1970s using a projection microscope and a ruler. We used a carbon arc as the light source and projected the image of the particles on the wall. I used a ruler to measure each particle individually. I am not sure how that experience led to my passion for microscopy but it did. From that time, I have progressed to systems with greater and greater degrees of automation. With the projection scope, it generally took me 8 h to measure 500–1,000 particles. Now, I can measure 50,000 in 30 min on the optical microscope.

There are a number of confounding factors that you should be aware of with automation and the factors are somewhat different for optical and scanning electron microscopes. I have already mentioned the need to ensure that what is measured with the first field, generally the one used to test the routine, will remain the same to the last field. Mostly this means assessing the ruggedness of your thresholding step. Image collection is also an issue. Modern optical image analyzers have some very sophisticated ways of determining optimum focus. These techniques do typically take some time to master but it is well worth the effort. In many cases, I have chosen an imaging technique, such as fully crossed polars, that is not amenable to direct focus. Some image analysis systems allow us to set focus points across the measurement field and the instrument smoothly adjusts the focus from one point to another. This process provides a big advantage for some samples. Focus is a particular problem with high magnification and high numerical aperture objectives. The depth of field is so thin that the normal focusing operation will fail since the last focus point is so far from the new one. Some programs allow the user to specify that a lower magnification objective is used first to set the gross focus and the higher objective then swung into place for the fine focus.

It is a distinct advantage if most of your microscope parts are motorized and controllable by the imaging program. The need is clear with objectives but it is important that the field diaphragm, condenser aperture, and bulb voltage also be controllable. It is also useful to have the substage condenser controllable as well, although this is more difficult to achieve. We can then begin to make up programs that allow setting conditions in the original program development and then reproducibly go back to those exact conditions for future analyses. This degree of automation also means that relatively unskilled operators can conduct good analyses providing the program is followed exactly.

It is a more difficult task to automate the SEM. It is true that many energy dispersive X-ray systems have the ability to control the SEM stage and imaging conditions, but to date most of these systems have relatively meager image analysis systems (bet I will get a debate on that statement). I know of very few dedicated image analysis systems that control the SEM. Further, I do not have a great deal of confidence in automatic brightness and contrast or in auto focus functions on the SEM. If repetitive image analysis measurements on the SEM are required, then I recommend that looking into systems that allow for direct control or a system that allows the collection of many images into a repository that can be used by the image analysis program as well. In the late 1980s, I had a Zeiss SEM with Kontron image analyzer that worked quite well at automated image analysis. There have been quite a few changes since then and I am not aware of any such systems on the market today. Most modern SEMs, however, do have mechanisms for automated stages and can reasonably easily be set up to collect many images in an unattended state. It is relatively fast to then have the dedicated image analysis system measure all the images in the folder.

As a final caution, sometimes the effort to fully automate may not be worth the time. I have spent countless hours setting up automated image analysis, data storage, and report generation when I could have done much of the work manually in a much shorter time. If you are only doing a few tests of one or two samples, it may be better to manually store the data, image and make your report. If the time spent doing full automation can be recouped with future work, then surely it is worth the time, but beware that full automation can be a time sink hole.

9.7 Summary

Image analysis with the optical and scanning electron microscopes has a wide-ranging set of applications. We can look at drug substance particles and drug product features. We have some very powerful tools at our disposal for developing quantitative relationships between microscopic morphology and drug product performance. There are good reasons to do these studies including the interest of pharmaceutical companies and regulators in better product understanding. The major impediment to these types of in-depth studies is some lack of familiarity with the technique by technical managers and some lack of facility in conducting the study by pharmaceutical scientists. It really is necessary to know both microscopy and image analysis well if we wish to go beyond simple size and shape distributions and progress to studies that contribute to good product understanding. I hope this chapter has prepared you for learning how to use image analysis and whetted your appetite for exploring its possibilities.

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Chapter 10
Contaminant Analysis

10.1 Introduction

The identification of contaminants is the third area in which microscopy makes a major impact on pharmaceutical development. Interestingly, it may be the least published field in pharmaceutical microscopy and yet there are many high profile cases of particulate contamination and the resultant product recalls. The rapid identification of contaminants is critical to development and manufacturing success. The microscopist may not be able to solve the entire problem in the sense that he or she can point a finger at the exact source of failure and contamination, but the microscopist nearly always can point the industrial engineers in the right direction. In some cases, it may be as important to know what the contaminant is not as what it is.

The vast bulk of contamination samples in my experience have been detected internally in manufacture or during the quality control tests of the product. At times, though, the complaints may come from customers or from regulatory agencies. The analysis approach is the same in each case but the importance of the analysis may be different in that problems detected in manufacturing most likely have not involved the public or the customers. In other words, there are no safety risks to patients. If detected by the customer or by a regulatory agency, the importance of the analysis is much greater since patient safety may be involved. Again, this difference ought not to influence the diligence and thoroughness of the analysis, but the microscopist will be under much more pressure in the second case.

I will start with a few examples of contaminants from my own experience. I will begin with the very first contaminant test I conducted way back when I worked for the fiberglass insulation industry. We had filters on our incoming liquid polymer bins as a precaution against dirt. Since the liquid was contained from incoming railroad cars all the way to the application station (this was a heat setting polymer), these filters were changed every 3 months on preventative maintenance and typically were quite clean. They began failing every week in the sense that they were so loaded with particulates that they ceased passing liquid. I was sent the filters and asked to identify the particles. I was a newly minted microscopist fresh from Walter McCrone's course at his research institute and was anxious (both good and bad) to apply what I had learned. The filter had everything: quartz, fiberglass, carbon black,

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glass fragments, a variety of other minerals, many more things than my limited skills were up for. I provisionally identified the source as dust. I dutifully reported the results and was, in turn, laughed at. Simply not possible said the engineers. I heard nothing more about it for a few months when I was told through the grapevine that the cause had been found. Seems an engineer happened to be walking past the polymer storage drums on the third shift (~3 AM) when he observed the janitor dumping his sweepings into the holding tank! Dust it was. Why the janitor chose this spot to dump is anyone's guess. In any case, this first example taught me quite a bit about contamination and the strange sources of some contaminants.

At various times, I have identified rust on orthopedic implants, silicone oil in parenterals, steel in drug substance, carbon black in excipients, diatoms in patches, dried coating particles on tablets, mercury in drug substance (someone tried to stir the batch with a mercury thermometer), salt crystals, and the list goes on. Really, nearly anything we use can contribute particles to the drug product and drug substance. Some problems have been easy to solve. I was able to remove magnetic iron particles from powder using a magnet. That was pretty easy. I worked on a case where three or four of our tablets had different colored contaminants on the outside coating. Turns out, a customer had put many different pills (of different colors) in the same bottle for a trip. The pills rubbed against each other and the colored regions on our white tablets were from the other colored pills. The source of this problem was very hard to track down.

Contaminant identification is closely akin to forensic science and integrates a large number of skills. It is ideal to have a team of people (who work well together of course) working on the problem. Just a few of the instruments and techniques used in pharmaceutical contaminant identification are as follows: stereomicroscopy, photography, polarized light microscopy, IR and Raman microspectroscopy, scanning electron microscopy, energy dispersive X-ray spectrometry, solid-state NMR, chromatography of various sorts, classical chemical tests, spot tests, microchemical tests, etc. It is rare that one individual has sufficient skill in all of these instruments and techniques to not only conduct tests but also to interpret them. One can, though, become reasonably skilled at the various types of microscopy including PLM, SEM/EDS, and microspectroscopy.

There is no substitute for practice in pharmaceutical contaminant examinations. It is, of course, important to have training and McCrone Research Institute has led the way in this type of training for many years (see Internet References). Training will give you the basic skills but to become expert at contaminants, you must practice. It is ideal to work with someone experienced who is also willing to help explain the process as they involve you in the investigation. Personal practice is also good using test samples as suggested in the exercises in this chapter. Although there is a normal sense of urgency when contaminants arrive at the doorstep, it is worthwhile to pad your work with some practice. If you suspect silicone oil in the parenteral, obtain some placebo and add an appropriate amount of silicone oil to it as a practice in doing the "real" analysis. If you suspect a gasket (common problem) polymer, obtain a sample of the gasket and practice with it. Finally, learn from others. As much or more than other disciplines, microscopists learn from each other at technical meetings – both formally through talks and informally through conversations. If at all possible attend these meetings and look for the forensic talks. The process of forensic identification is nearly the same for all industries and you are sure to learn something good as others discuss how they solved their contaminant problems.

It is also useful to look at the literature in forensics and materials identification. Besides the cited articles, I have included useful sources of information under General References.

10.2 Robert's Rules for Contaminant Identification

Industrial contamination problems are so varied that it is neither possible nor desirable to adhere to a strict prescription or methodology for identification. The following "rules" really provide a general guideline for conducting a contaminant examination and present certain principles that should be borne in mind.

10.2.1 Rule #1: Think Before You Act

It has always seemed somewhat strange to me that many of us, who get paid to think, feel like we are wasting time when we do it. I can hardly think of an example of a contaminant problem that did not arrive at my desk wrapped with labels of "Urgent," "Fate of Mankind Hangs in Balance (or at least the company's fate)," etc. And the *coupe de grace* is invoking the holy name of the CEO or company president. "Cranston Snord is personally interested in this problem." So get it right and get it right, right now! Panic of that sort is contagious and so there is enormous pressure from without to get to work now and this pressure is sure to take up residence in your psyche. We need to resist that pressure. So, take a deep breath and think. Rule #2 can help.

In particular, after you do a test of a hypothesis, spend some time considering the results of the test. Let us say you have formed a hypothesis that the contaminant is a glass flake based on its appearance (conchoidal fracture), its transparency, and its lack of birefringence. Now you take it to the SEM for EDS and do not detect Si. Well so much for that hypothesis, but no reason to panic. Now it is time to think. Your first observations are correct – fracture, transparency, lack of birefringence, etc. What else is like that? So it is time to think and to research. (By the way, this is a real example and it turned out to be a specific polymer that breaks like glass. It is the only sample that I have seen like that in my career.)

10.2.2 Rule #2: Get All the Information Before You Start

Collect as much information as possible about the sample and the problem before conducting your first test. Frequently, the contaminant sample is delivered by someone only peripherally involved in the project. They may ask for identification and, in some cases, will ask just for a specific test – say elemental analysis by SEM/EDS. Providing just that information may suffice if you are only part of the identification process and you are providing the information to someone else who will be doing the interpretation. If, on the other hand, you will be expected to do the interpretation, then you need more information. Keep in mind that there is a Grand Canyon sized gulf between identification of the specific contaminant and identification of the source of the problem. Most requestors assume that knowledge of the source will simply appear from the ether once identification is done. Remember the janitor. Particulate identification did not aid in identifying the source.

Of course, it is not practical to obtain all the needed information in one bolus at the beginning of the analysis. We do need to establish communication with the individuals in charge of solving the problem and work back and forth with them, sharing information. Generally, a hypothesis has already been developed – let us use a polymeric gasket particle in the drug substance powder as an example. The particles may have been detected by the line operators – you should know that. They may suspect that one particular gasket is to blame and they looked at it and sure enough it came off in pieces. You should know that fact and request pieces of the suspect gasket and a similar new gasket for comparison purposes. Is this a common problem or rare? Exactly how were the particles were discovered. Ask lots of questions before or at least simultaneously with the beginning of your examination. Opening the lines of communication with the person in the know is one of the most important benefits of this quest for info.

10.2.3 Rule #3: Clearly Establish the Goal of the Investigation

As microscopists, we may be quite able to determine the identity of the contaminant but have only limited means of establishing the source and no ability to solve the problem. I have on occasion been asked whether a particular batch containing a contaminant should be released for clinical use. While I am flattered by the assumption that I know something, I am after all just a microscopist. The decision to release is not my job.

Even in the identification phase, we need to know the goal. For example, if we see a fiber in a parenteral, is it sufficient to say it is a fiber from an external source and not say drug substance or some component of the packaging? Or is it necessary to distinguish a natural from a synthetic one? If synthetic, do we need to know the chemical identity – nylon, polyester, etc? If nylon, do we need the mill where it was made? Clearly, when we say we want to identify something, there are varying levels of identification. It is good to establish this level at the beginning.

For myself, I begin asking whether the requestor is content with determining whether the contaminant is external or internal, and if internal whether it is drug substance or some other component. The requestor may just want to know whether their blending process is at fault or whether something contaminated the manufacturing process. Internal or external may be sufficient. If external, material class (animal, vegetable, mineral) is nearly always possible to determine just as part of the initial examination. Establishing the goal of the analysis is important since the time required for identification goes exponentially up with the specificity of that identification. You can imagine how long it might take to determine the manufacturer and mill of one nylon fiber. Whereas determining whether it is a fiber and nylon requires much less time.

10.2.4 Rule #4: Every Experiment Should Test a Hypothesis

Corollary: Do no experiment that does not test a hypothesis

There is a school of thought that says "let's just do the test and then let the data tell the story." I am not fond of this approach. Without a hypothesis to guide us, we will wander around and find ourselves travelling dead ends until we finally find our way. So, for example, let us suppose we have some hard nuggets of some white substance in our powder blend. We have a bright new shiny LC-MS (liquid chromatography–mass spectrometry) sitting on the bench, so we dissolve some stuff up, inject and lo and behold, we have a nice spectrum. Yes, it does have the drug substance fragments but then lots of other ones. What to make of that? We will probably begin to search for materials with those fragments and may end up with all sorts of possibilities, each one of which we will be obliged to track down. I think it is better to start by framing the hypothesis, then testing it, making new hypotheses, testing, and so on until we are confident in the identification.

Nearly always your first general hypothesis will be provided by the requestor. "We see some black particles on the tablet surface and we think it may be gasket material." So your first test may be the visual examination followed by the stereomicroscope where you test this hypothesis. On examination, you find the apparent solid black particle is actually a multiplicity of small purplish particles. When you touch them with a tungsten needle they are hard and brittle and not soft and ductile. The gasket hypothesis is unlikely, so you frame a new hypothesis that the particles are left over coating particles from a different product and test that hypothesis.

I do not always explicitly write down my hypotheses – particularly since I now have quite a bit of experience with contaminants. I may write them down, though, in particularly difficult cases. It is much like diagramming sentences – something I hated to do in grammar school seeing absolutely no practical application. Now though, when I have written a particularly intractable sentence, it helps to diagram. So it is with hypothesis testing. The very act of writing it down can help the identification process in knotty problems.

10.2.5 Rule #5: Start Simple and Progress to Complex

The very first thing to do with a contaminant sample is to look at directly, maybe with the benefit of a simple lens. This first step allows you to become acquainted with your sample. For instance, if it is powder and you see many, large black specs, then you can rule in or out a number of likely possibilities. If it is a parenteral and you see absolutely nothing in the liquid, that gives you a number of clues. For myself, I like to know whether this is going to be an easy or hard analysis. I can almost always guess this at the beginning. Further, as you look at the sample, a number of questions may come to mind.

I then progress to the stereomicroscope and, if at all possible, I like to examine the sample directly without any sampling. I take photomicrographs again without sampling even if the photograph quality is poor. These photomicrographs are partly for the final presentation and report and partly for the analysis. Let us say it is a parenteral and I later filter the solution onto a Nucleopore™ filter and examine it with the SEM. I will usually collect any number of particles on the filter and having the original photomicrographs can aid in locating the particle of interest.

The stereomicroscopy generally leads to a class hypothesis which I then use to direct the next studies. So, for instance, when looking at a black speck in a tablet coating, I may form the hypothesis that it is metallic (class). Clearly, a metal speck is not an internal contaminant and is likely to be from the manufacturing line. I may base this initial conclusion on the metallic luster of the particle and the fact that it resists penetration by a tungsten needle. I can then test this hypothesis using SEM/EDS looking for metal elements and, in particular, looking for Al, Fe, Cr, Ni, V, etc. I might also see Cu and Zn if it is brass and even Sn if it is related to a solder. In any case, I progress from the simple to the complex. Figure 10.1 presents a schematic diagram of the approach that I typically take with contaminants.

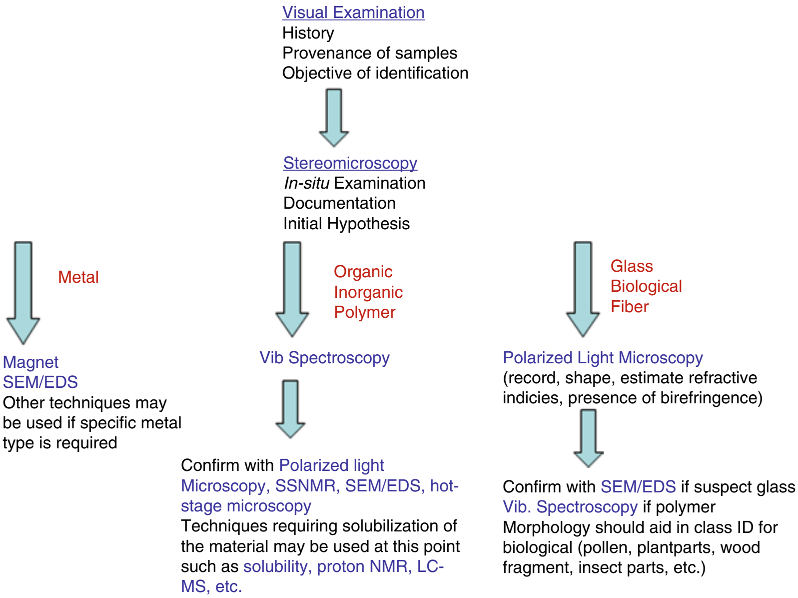

Fig. 10.1 Workflow for contaminant identification. This diagram presents one approach to identifying contaminant particles. It emphasizes the development of hypotheses early in the investigation and then testing these hypotheses with specifically designed analytical tests. Once the hypothesis is confirmed, it is necessary to verify the results by conducting additional analytical tests. The direction of the workflow is from less destructive to more destructive tests

I will give a brief example where I failed to do this initial examination. I was working on a metal piece that had an embedded particle of some sort. I rushed to the SEM thinking this is a easy problem and I was busy with other stuff. I did the analysis and it looked carbon rich, which it was – but I was not analyzing the embedded particle. Instead, I was looking at the residual glue from the tape used to mark the location of the particle. I had not taken the time to look at the piece with a stereomicroscope and noted that just below the particle was a collection of glue that had balled up when the tape was removed. Fortunately, I caught the mistake before embarrassing myself too much and reporting my results.

10.2.6 Rule #6: Maintain Clear Distinction Between Fact and Hypothesis

In other words, do not mix the map and the territory. If someone shows you a street map, you may say "Oh that's Philadelphia." To be anal and pedantic, no it is not. It is a map of Philadelphia streets. The real thing lives in Philadelphia. I cannot speak for anyone else, but I have problems mixing these up. To use a microscopy example, if you are looking at particles in crossed polars, you may say "these particles are crystalline because they are birefringent." That may be correct but it is not precise language. Crystallinity is an abstract concept as is birefringence. The fact you observe is color in particles between crossed polars on the polarizing light microscope. Those are the facts and interference, birefringence, and crystallinity are abstract concepts used to understand the facts – the map.

Why is this distinction important in contaminant analysis? We can be led down the wrong path when we are acting on what we think is a proven hypothesis, a fact, when it is not. Using the previous example, glass can exhibit strain birefringence. That is colors between crossed polars that is not due to long range crystallinity. If we have detected colors, assumed crystallinity and gone forward, we may miss the fact that the particle is, in fact, highly strained glass. I do not recommend you be that precise in all your relationships and activities. You may not often be invited to light-hearted gatherings for fun and cheer. Do trust me on this one. Such rigor in thinking is just the ticket, though, for contaminant analysis.

10.2.7 Rule #7: Verify Conclusions

This rule seems obvious but it can be devilishly difficult. Ideally, you would have two additional tests confirming the results of the first positive test of the hypothesis. The number and kind of additional confirmatory tests depend on the investigation. If the contaminant has important implications such as loss of clinical supplies, patient safety, product recalls, etc., then it is a very good idea to confirm with two tests. If you are doing development work and confirming a likely source, then it may not be necessary. So, for example, the mercury in drug substance identification was for confirmation. Although no one owned up to stirring powder with a mercury thermometer (if they were smart enough not to admit this, why were they not smart enough not to do it in the first place?), the mercury was obvious as was the broken thermometer. What we needed was analytical confirmation and I was able to supply that using SEM/EDS in the ESEM. One test was sufficient in this case. In most cases, though, I prefer two levels of verification.

For instance, if I hypothesize a nylon fiber that has been delustered based on polarized light microscopy, I might verify using IR microspectroscopy and EDS (for titanium commonly used in delustering process). It may prove quite difficult to verify for a number of different reasons. It may be that you are sample limited. There may be only one or two particles limiting you to one or two tests. The sample size may not be suited to some analytical techniques. In general, particles need to be at least 50 μm in diameter for IR microspectroscopy. The material may not have many unique identifying characteristics. Carbon black is a classic example. It is often identified more on what it is not rather than what it is. Verification is quite difficult.

Verification does not necessarily have to be as specific as the original identification. Solubility is often quite useful as a confirming test, but is certainly not very specific. For example, if you have determined that the particle in the parenteral is probably drug substance a simple solubility test or thermal test may be sufficient verification. Note that you should reserve destructive tests for the end of the examination and for when you are not sample limited.

Accept that in some cases it may not be possible to verify. In those cases, I am fairly circumspect about how I present the results. Of course, I nearly always use the forensic terminology "the result of this test is *consistent with the hypothesis* that the contaminating particle is a glass fragment." True proof is hard to come by. Nearly always we are working with some level of inference. So, I think it is appropriate in the final report to be clear about the level of confidence in the identification. If we have decided the contaminant is carbon black based on negative data (it is not this, it is not that, etc.) and morphology, then we should be clear about it.

10.2.8 Rule #8: Document as You Work

Probably, you wonder why I propose this rule since it is so self-evident. It is because I wantonly disregard it and dearly pay the price now and again. In those cases where you are taking photomicrographs or spectra, you will naturally store those and have them available. No, what I refer to here is the documentation of the results: all the observations that you make as you go along, many of which are highly observational in nature. I once had particles in a parenteral that produced a colored spectrum, a rainbow, as they rotated in the liquid under the stereomicroscope. While that piece of information did not turn out to be critical to the identification, I did need to note that observation. Those particles were from a glass cleaning process that left a thin residue on the inside of the glass that later broke away – I never did determine why they produced the spectrum. You cannot know beforehand which observation will be critical and they should not be left to fallible memory.

10.2.9 Rule #9: Have a Clear Exit Strategy

Yep, I stole that from Colin Powell but I think it applies to a lot more than just military intervention. What I mean by this rule is keep close in mind your own limitations and the limits of your work. You may be pressured to do more with your analytical techniques than is either warranted or feasible. It is up to you, to some extent, to set the limits and it most certainly is up to you to ensure that others know the analytical limits of your techniques.

The number of people involved in the problem is directly related to the potential business consequences. Yes, indeed, good ole' Cranston may have you on the phone if the problem is serious enough. And everyone who works for Mr. Snord will follow up frequently to be sure you truly appreciate the importance of the task. It does not happen often, but it sure is intimidating when it does. We do want to be cooperative and helpful, but we have to know our limits.

Most problems like this occur when it comes to decide what to do with the offending batch of material. These decisions can be quite difficult to make. Those responsible for making these decisions generally want quantitative data beyond the qualitative identification. How much of this contaminant is present and in how many products? Now if it took Herculean effort to identify the particulate, even Zeus himself will be unable to quantify it. Now is when an exit strategy comes in handy. For example, EDS is a wonderful qualitative tool for elemental analysis. Any attempt to do quantitative analysis can be quite challenging. While it may be possible to quantify the number of glass particles in the specimen on the microscope, extending that to your sample (~100 mg) and then to the batch (~10 kg) is a big stretch. Let us say though you succeed in making a good estimate, say ten glass particles per gram. Maybe this is determined to be acceptable, and then you may be in the position that future batches will be released based on your test. In my opinion, that is a step too far with a technique poorly suited to the task.

To be succinct, do your best to keep from getting involved in work that is beyond the capability of analytical expertise and instruments. Not everyone (and people I respect) that I have worked with agree with this rule. Their attitude is to do anything and everything in their power to solve the problem even if it means loosening a nut with a hammer and screwdriver. Solve the problem with the tools at hand. I can see the appeal of that approach. For myself, I prefer to know beforehand what I can do and do well and stick to that. One reason for that attitude is that contaminant analysis has been an afterthought, an add-on duty to those things I am expected to do. If solving manufacturing problems was my sole job, then, of course, getting the solution is paramount. In my case, and I bet in yours as well, you will get called on to do the identification while you are doing your "day" job. It is critical, in my opinion, to know when you have done enough and get on to your other work.

10.3 Practical Examples

10.3.1 Introduction and Method of Analysis

In this section, we will examine a number of artificial contaminant identification examples. As with the exercises and examples in other chapters, I recommend that you practice with similar samples. Even though you know the answer before you start, proceed as if the sample is an unknown. We will start with common household or laboratory dust; look at activated charcoal in sugar, polymer tubing particles in starch, and silicone oil in water. Each of these samples provides some unique challenges and range from quite easy to quite difficult. Most good forensic microscopists that I know have spent copious amounts of time collecting and examining known samples. Since contaminant identification is a kind of forensic microscopy, we also are well-advised to collect and study known samples that are specific to our environment and manufacturing processes.

10.3.2 Dust

There are some common elements in most dust samples. You will undoubtedly find cellulose and other polymeric fibers. They are ubiquitous. You should find quartz, calcite, and iron oxide. Glass fragments and glass fibers are commonly found in dust. You will observe some biological samples such as pollen, plant trichomes, and even bug parts. You will also have particles particular to your environment. I can hardly look at any sample from my home without finding a few dog and cat hairs. Our laboratories are the same and it is a good idea to become accustomed to the kind of airborne particles that are common in your lab. For most of us, contaminant identification is one of many duties that we have and our labs are designed for flexibility. That means that it will be quite difficult to completely eliminate laboratory dust particles from our contaminant samples. The more we know about the particles common to our laboratory, the better prepared we will be to distinguish between laboratory dust and the contaminant particles of interest. So studies of dust are useful both for learning and for actual identification of contaminants.

Figure 10.2 presents a stereomicroscope image of dust collected onto a white filter. I prepared the sample by using a small paint brush to push particles off of a rarely cleaned surface onto a filter. I collected the sample from my workbench in my garage. As you can see there are plenty of particles to keep us amused for some time. It is rare to get contaminant samples with this degree of complexity and if you do see something like this sample, then you can guess it has something to do with a cleaning system malfunction. Just by identifying a few particles from an environmental source that are not from the manufacturing line or from the drug product, we can point the requestor in the right direction.

I begin this analysis by categorizing the particles into broad classes based on morphology. Fiber particles would be one class. Metal flakes would be another

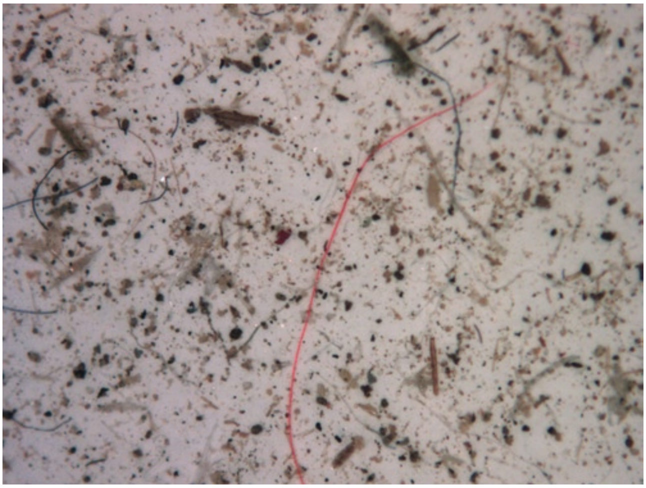

Fig. 10.2 Dust particles observed with a stereomicroscope. This sample was collected by brushing dust from a surface in a garage used for woodworking and gardening. It shows a wide variety of different particle types and is a useful sample for practicing techniques of particle identification (image size = 3.4 × 2.5 mm)

as would glass. Wood chunks are another feature of this sample and their presence is reasonable since some woodworking is done in the garage. There appear to be bits of carbon black and could be residue from lawn mowers or other gasoline engines. I think you get the point. Once I have these categories, I proceed with the analysis. Even though I have separated the particles into different classes, I am not rigid about this classification. It is an aid to structuring the analysis not a conclusion. In other words, I have to do the analytical tests before I can state definitively that metal flakes are indeed metal flakes and not something that just looks like metal flakes. Notice also that we are greatly aided by knowledge of the environment. Just given this sample and asked to identify the particles would be much more difficult if we do not know the origin of the sample and the kind of activities that take place at that location. Do be sure to obtain as much information and as many standard samples as possible from the person requesting the work.

Figures 10.3 and 10.4 present polarized light photomicrographs of some of the dust particles. In the first figure, there are quite a few different types of particles based on interference colors and morphology. If you see something like this in a real investigation, it is sure evidence of a malfunction in some cleaning procedure. For our purposes, though, we can see particles that are nearly completely transparent, some that are nearly opaque, some with high birefringence and some with low. Some particles we identify using other techniques such as IR, Raman, and EDS, whereas others are identified best by PLM. In particular, it is beneficial to look for biological materials such as pollen and plant trichomes. Microscopy is generally the only way to identify these particles.

Fig. 10.3 Dust particles by polarized light microscopy. This image shows a wide variety of different particles by PLM. Such a sample would be indicative of a cleaning process malfunction since there are so many environmental samples. Classifying the particles by morphology and by interference colors suffices for this type of sample (image size = 700 × 520 μm)

Fig. 10.4 Fibers from dust sample by polarized light microscopy. This image shows three types of fibers. The fiber on the *left*, with the particles on the surface, is delustered nylon whereas the one on the *right* in the twisted orientation is cellulose. These two fiber types are relatively easy to identify by morphology. The central fiber is less common and further tests are necessary to develop an initial hypothesis (image size = 700 × 520 μm)

Figures 10.5 and 10.6 present SEM/EDS results for two particles. Figure 10.5a is an SEM image of two particles from the dust sample. The one on the left is bright and probably metallic which is confirmed by EDS (Fig. 10.5b) as a particle of copper. The larger structure on the right has an interesting morphology but appears to be just carbon, oxygen with a small amount of Si and Al. EDS spectra was collected for both particles but only the spectrum of the larger particle is shown here. The copper, chrome, and tin peaks in the spectrum are probably due to electron beam spread by the chamber gas. This spectrum illustrates the care that must be taken when doing qualitative analysis in low vacuum mode in the SEM. Figure 10.6a shows a particle of unusual morphology with high contrast and smaller darker particles. Figure 10.6b shows the accompanying EDS spectrum and along with carbon and oxygen, there are peaks for iron, nickel, sulfur, and silicon along with small peaks for chrome and chlorine. The metal peaks are indicative of steel whereas the other elements are suggestive of a combustion particle. These results illustrate how most real particle samples are mixtures and it can be difficult to decide on identity because of material that is attached to the particle but is not part of it.

Figure 10.7 shows an overlay of an IR spectrum of a nylon fiber and of a cellulose fiber. There are clear differences between the two spectra and each particle spectrum can be compared against databases and against known samples. I personally prefer known samples for the identification that I have tested on the same instrument. Ideally, we are looking at samples from the manufacturing line or from the components of the drug product. The databases can be helpful, but there generally are some slight differences between the sample used for the database and the sample related to your investigation. It is a good idea to request reference materials, such as gaskets, once you have determined the contaminant class. For instance, if you have determined that the sample is a flexible polymer, then it is appropriate to ask for samples of gaskets used in the manufacturing line. I use that example because blown gaskets are a common source of contaminants.

This exercise is an excellent teaching tool and can aid you in developing good procedures for sample collection and analysis. Of course, it is not particularly realistic in the sense that you will rarely, if ever, get such a sample. On the other hand, as mentioned, the first contaminant sample of my career was just such a sample. By the way, if your significant other graciously does the bulk of the house cleaning, enthusiastically showing him or her of all the different kinds of particles you have discovered in the “clean” house may not be a good idea. The bad idea is probably made worse if you have discovered loads of bug parts.

10.3.3 Activated Charcoal in Powdered Sugar

Charcoal is often used in synthetic chemistry purification steps. If the filter downstream from the charcoal ruptures, you may have carbon black contamination. Carbon black is also a primary constituent of smoke and gas from combustion processes. Gasoline engines, gas, or oil fired heaters, smoke from cigarettes are all

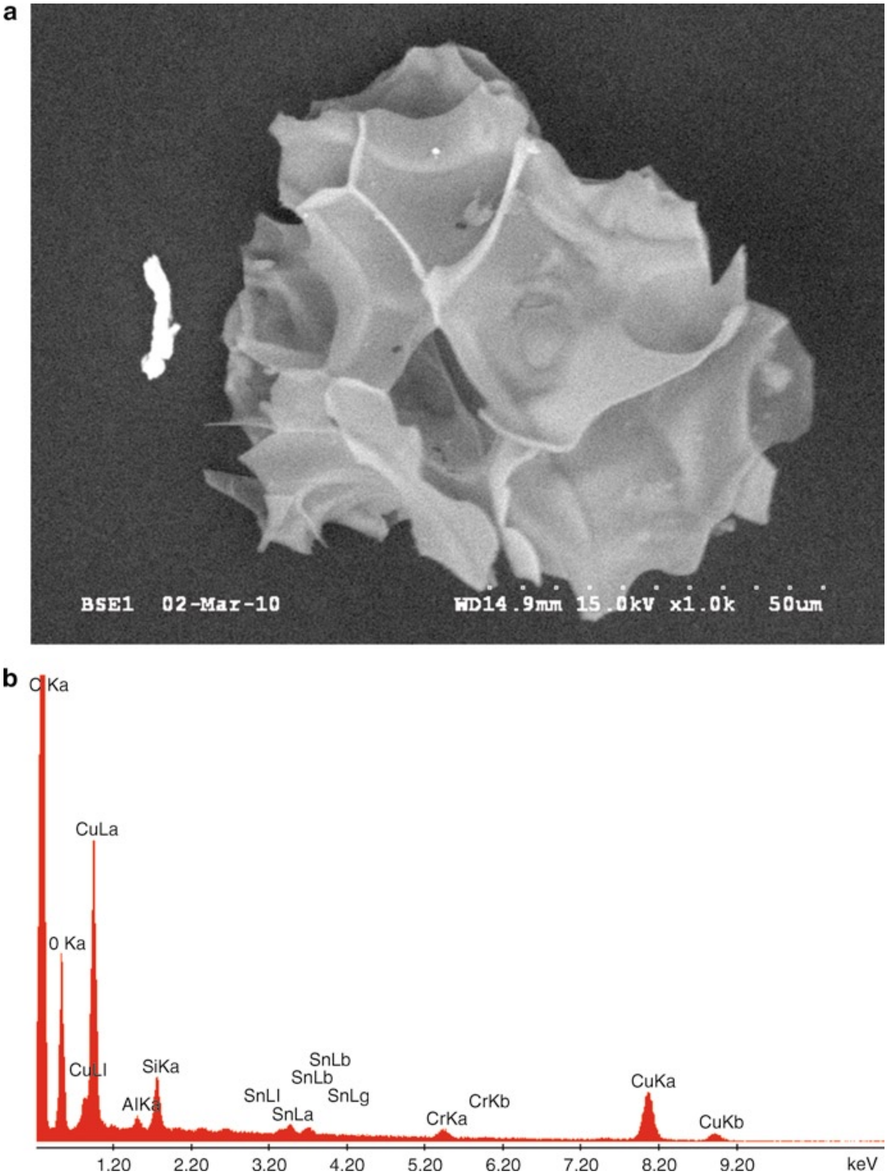

Fig. 10.5 SEM/EDS of dust particles. (**a**) This is an SEM image of two particles from the dust sample. The one on the *left* is bright and probably metallic which is confirmed by EDS (**b**) as a particle of copper. The larger structure on the *right* has an interesting morphology but appears to be just carbon, oxygen with a small amount of Si and Al. EDS spectra were collected for both particles but only the spectrum of the larger particle is shown here. The copper, chrome, and tin peaks in the spectrum are probably due to electron beam spread by the chamber gas. This spectrum illustrates the care that must be taken when doing qualitative analysis in low vacuum mode in the SEM (image size = 122 × 99 μm)

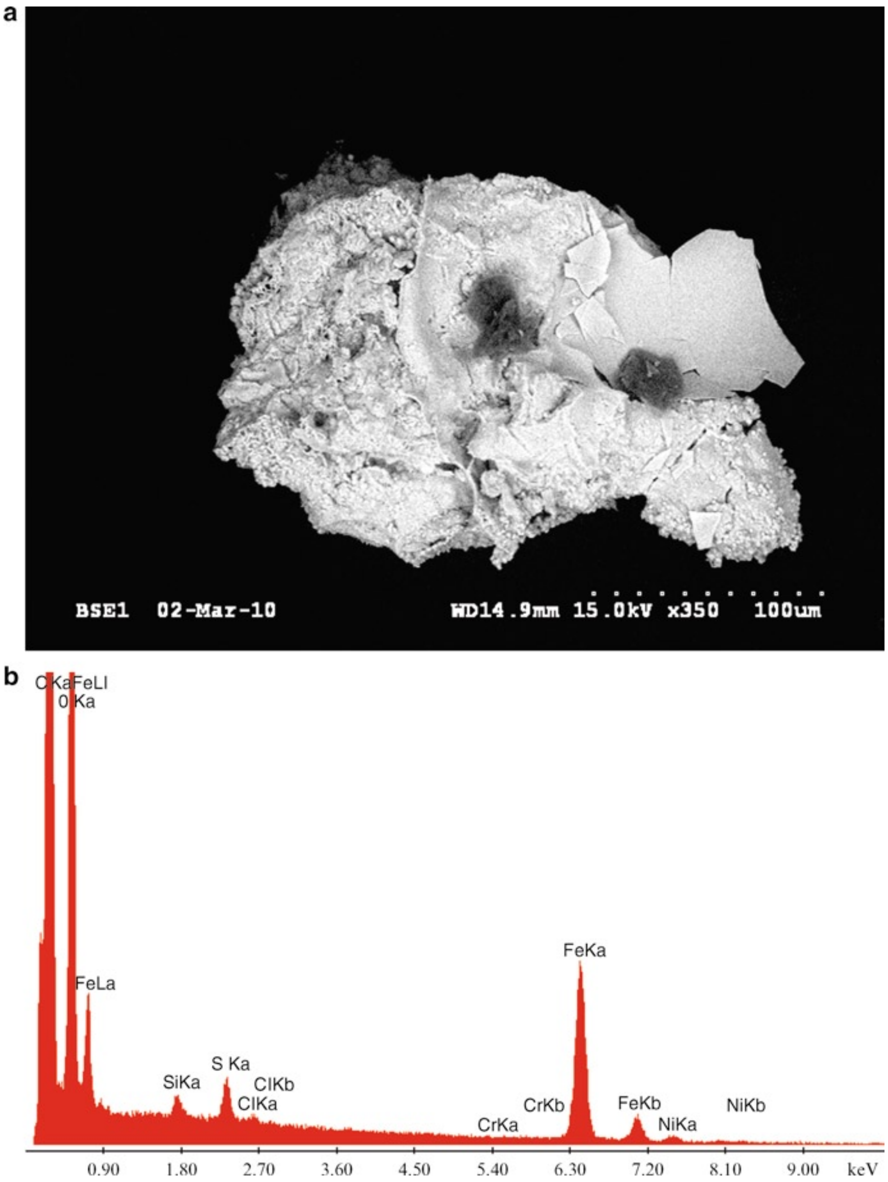

Fig. 10.6 SEM/EDS of dust particles. (**a**) This shows a particle of unusual morphology with high contrast and smaller darker particles. (**b**) This shows the accompanying EDS spectrum and along with carbon and oxygen, there are peaks for iron, nickel, sulfur, and silicon along with small peaks for chrome and chlorine. The metal peaks are indicative of steel, whereas the other elements are suggestive of a combustion particle. These results illustrate how most real particle samples are mixtures and it can be difficult to decide on identity because of material that is attached to the particle but is not part of it (image size = 366 × 275 μm)

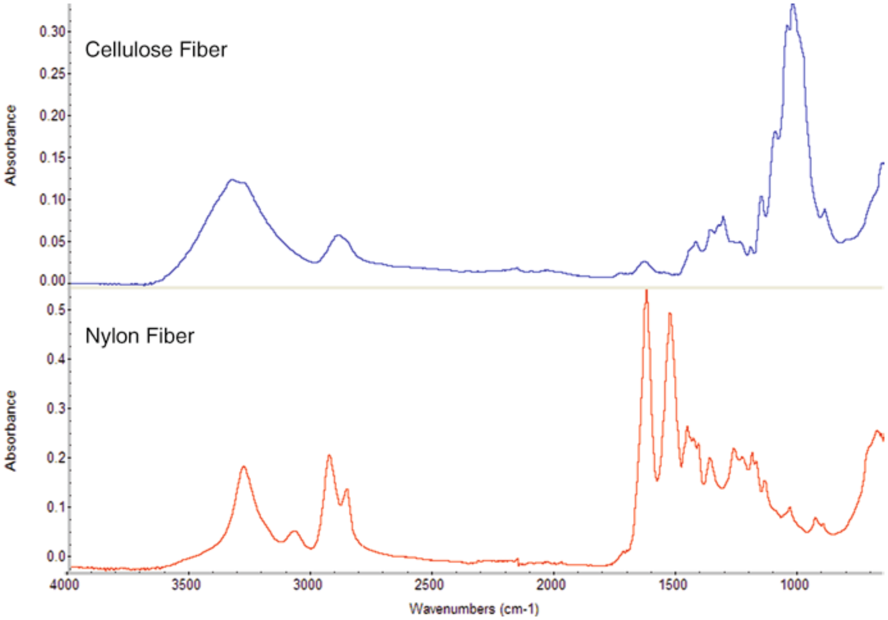

Fig. 10.7 Infrared spectra of nylon and cellulose fibers. These fibers can be positively identified using IR. We can compare with databases and with known samples from the manufacturing line or from the components of the drug product. I prefer the latter when available

potential sources of carbon black and those are only a few of the potential sources. Consequently, carbon black is a reasonably common contaminant particle as is activated charcoal. Both contaminants, though, are really quite tough to chemically identify. Very little in the way of chemical bonds and functional groups remain in the material and it is mostly composed of carbon, oxygen, and sulfur with a few other minor elements such as chlorine, sodium and phosphorous. On the other hand, carbon black and charcoal have a very distinctive appearance and are chemically inert. If you suspect charcoal, you will almost inevitably have to do some solubility tests for confirmation of the identification.

Charcoal is also a good example of relying on negative information. In this situation, we work through all the possibilities of what the contaminant is not, leaving us with only a few ideas of what it is. In the world of pharmaceutical contaminants, there are a few such problem materials. Carbon black is one and protein is another. We don't have many distinctive tests for either one. To be clear, in both cases, the material itself does not have distinctive elemental or molecular properties that we can use for positive identification. In order to study such a case, I recommend that you add a small amount of carbon black or activated charcoal to powdered sugar or some drug compound that you have thoroughly studied.

Figure 10.8 shows charcoal particles in powdered sugar under the stereomicroscope. The stereomicroscope is useful for documentation of the foreign object and for developing the initial class hypothesis. In this case, I am looking to establish

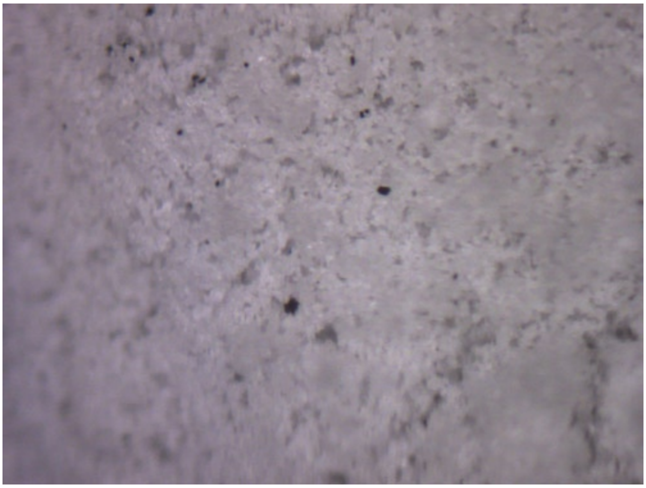

Fig. 10.8 Stereomicroscope image of charcoal particles in powdered sugar. This image illustrates how we can use the stereomicroscope to document the appearance of the foreign particles in the drug substance (image size = 3.4 × 2.5 mm)

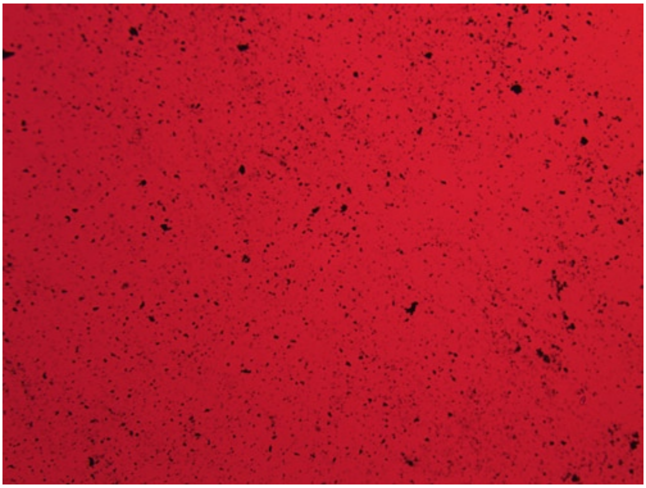

Fig. 10.9 Polarized light photomicrograph of charcoal particles. The particles are opaque and do not display interference colors. The larger particles appear to be aggregates of much smaller particles (image size = 350 × 260 μm)

whether the particle is most similar to metals, glass, polymers, drug substance, biological material, etc. In this case, I conclude that this object is not glass, metallic, polymeric (by pressing on the particle with a needle), and probably not biological. I suspect it is something related to drug substance such as charred drug substance or charcoal. I will mount some of the particles for examination by polarized light microscopy (Fig. 10.9) and find that it is opaque and does not display interference colors.

I will break apart a few particles to see if the black appearance is only on the surface. In this case, the particle breaks into many very much smaller particles. The PLM test has helped confirm that the particles are most likely to be charred drug substance or carbon black. Naturally, if I have not cleaned the particle of sugar particles, I am sure to detect them as well. That is a good reason to have collected good analytical data on the drug substance beforehand.

Figure 10.10a, b present an SEM image and EDS spectrum of an activated charcoal particle. The particle morphology is nondescript and irregular. The interior is porous with many cracks and there are numerous small particles adhering to the larger one. Although the morphology is not unique, we can say this morphology is consistent with that of reference charcoal particles. In other words, although the morphology does not add proof to the case for activated charcoal, it does not negate the hypothesis either. The EDS is similar in that we detect carbon, oxygen, silicon, and sulfur along with numerous other trace elements not shown in the spectrum. As with the SEM, the elemental analysis does not prove that the particle is charcoal, but the detected elements are consistent with what we expect with charcoal. The EDS is consistent with charcoal. As we collect data, we are building a case for charcoal or carbon black without any positive chemical identification.

The charcoal IR spectrum is interesting in that it is a nearly straight line but at high absorbance (Fig. 10.11). Given that the particle is black it is not surprising that it absorbs IR radiation. The IR spectrum does not provide a positive identification, but it does add more evidence to the hypothesis testing. The IR is consistent with the hypothesis. By the way, the program I use to collect the spectrum automatically compares the spectrum with a database and this spectrum had a good match with "activated charcoal." I guess if the computer says it is a match, it must be. At this point, some solubility tests are appropriate. If the particles do not dissolve in any of the normal solvents such as methanol, ethanol, acetone, etc., then I have high confidence in the identification. Even so, I do not think we have sufficient data to distinguish between charcoal and charred drug substance. Both possibilities should be suggested to the requestor for further investigation of possible sources in manufacturing.

10.3.4 Polymer Tubing in Corn Starch

In this example, we will be looking at a piece of rubber tubing in starch. I chose corn starch because it is a cohesive material much like most drug substance and because it is safe to use in relatively large quantities. Some of the most common pharmaceutical contaminants are polymeric materials used in the manufacturing line or in drug product packaging. In this case, I took a piece of orange vacuum tubing, cut it into small pieces and put it into the starch. Figure 10.12 shows the stereomicroscope image of this particle in the matrix. In general, these problems are straight forward and relatively easy to solve. Removal of the particle can easily be accomplished using a needle and the fact that the material is soft and resilient is a

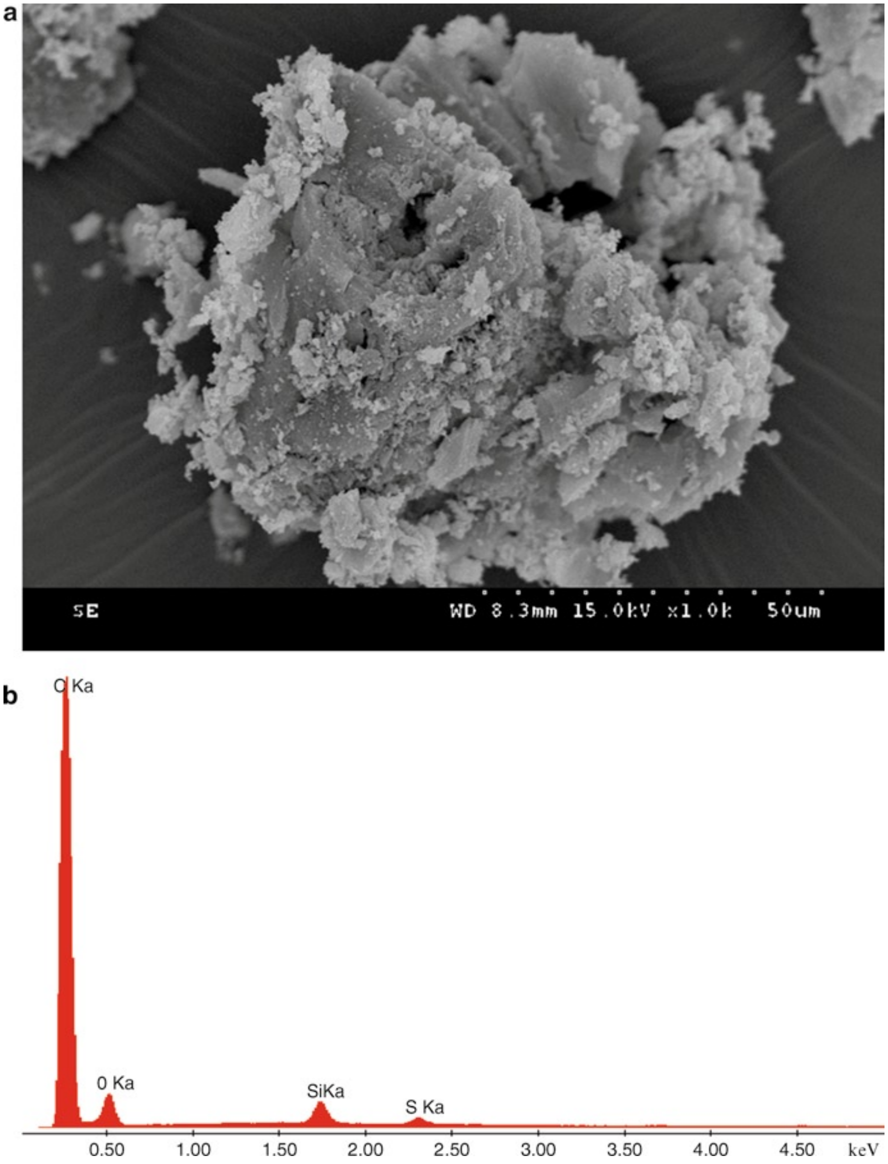

Fig. 10.10 Charcoal SEM/EDS. (**a**) The morphology of the particle is nondescript. The particle has a porous interior with many cracks. There are numerous small particles adhering to the larger one. (**b**) The EDS spectrum has carbon, oxygen, silicon, and sulfur. If one expands the scale many other elements can also be detected in trace amounts (image size = 122 × 99 μm)

clue that it is probably polymeric. If you have more than one particle, it is worthwhile to attempt to clean off the residual drug substance (starch in this case) using water or methanol. Generally, you know what dissolves the drug substance and so

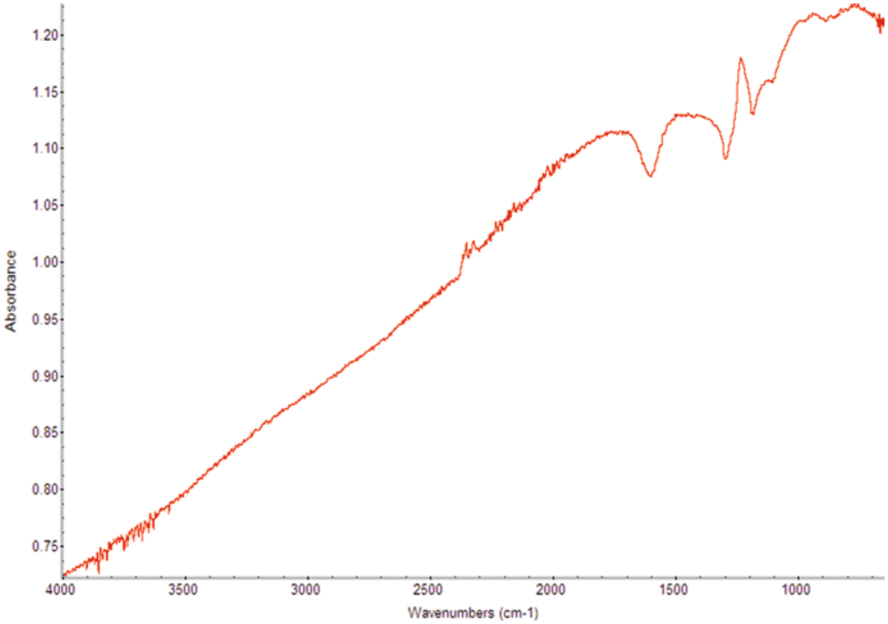

Fig. 10.11 Charcoal IR spectrum. The spectrum has few distinguishing features for identification. There is high absorbance, however, which is consistent with the behavior of charcoal

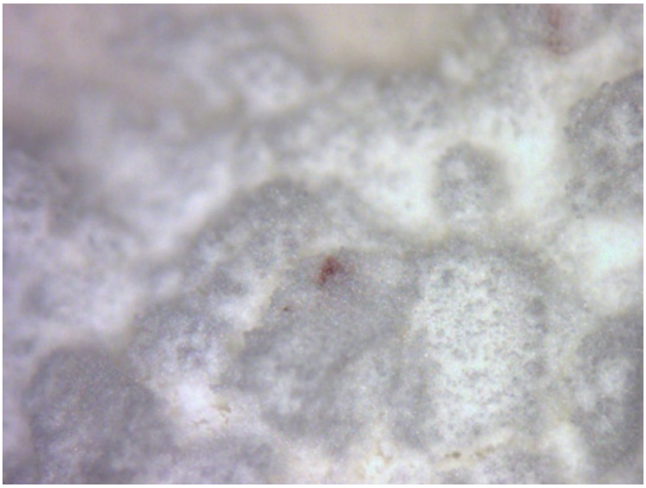

Fig. 10.12 Stereomicroscope image of polymer particle in corn starch. Polymer particles in drug substance and product are common contaminants. These particles are relatively easy to remove from the sample and are generally easy to identify (image size = 3.4 × 2.5 mm)

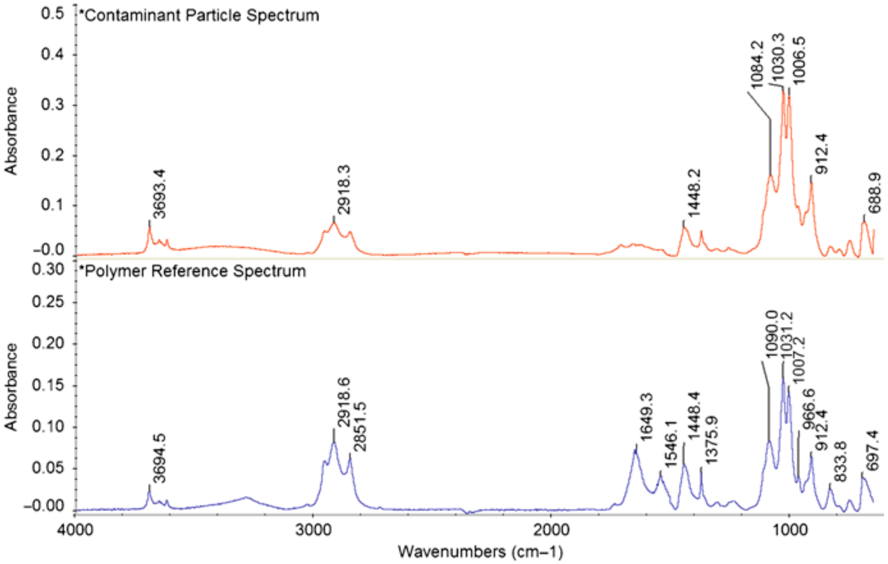

Fig. 10.13 IR spectra of the original tubing and the polymeric particle. As expected, the IR spectra of the reference and the particle the same. Polymer materials are generally easily distinguished using IR and Raman

can pick the appropriate solvent. The fact that the particle does not dissolve in that particular solvent is good information.

It is best to go directly to IR and Raman spectroscopy for putative polymeric particles. Figure 10.13 shows the IR overlay of the original tubing and the particle. The IR spectra show close resemblance to each other as expected. We can also use a database to attempt to identify the polymer. The IR was not difficult to collect and I used the ATR objective which is particularly well suited to polymeric materials. The SEM and EDS (Fig. 10.14) of this material is interesting. Most of these rubber type vacuum hoses have a filler material and in this case it appears to be mineral based with silicon and aluminum among other elements. The interesting thing about the fillers is that they may provide a distinctive fingerprint for the polymer comparison. So, it is a good idea to routinely collect EDS spectra for polymer contaminants even if the IR and Raman are persuasive. We can consider the EDS spectrum as verification of the identification.

The most common problems that I face with polymers is lack of a suitable reference standard and the small size of some particles. It is ideal if we can locate polymers used in the process or in the product, but this is not always possible. In that case, we will have to use a database of reference spectra. A more serious problem is the small size of some polymer fragments. If the particle is less than ~50 μm in diameter, it may be better to go directly to Raman spectroscopy. Unless you are quite experienced and skilled in IR microspectroscopy, it can be difficult to obtain good spectra of smaller particles.

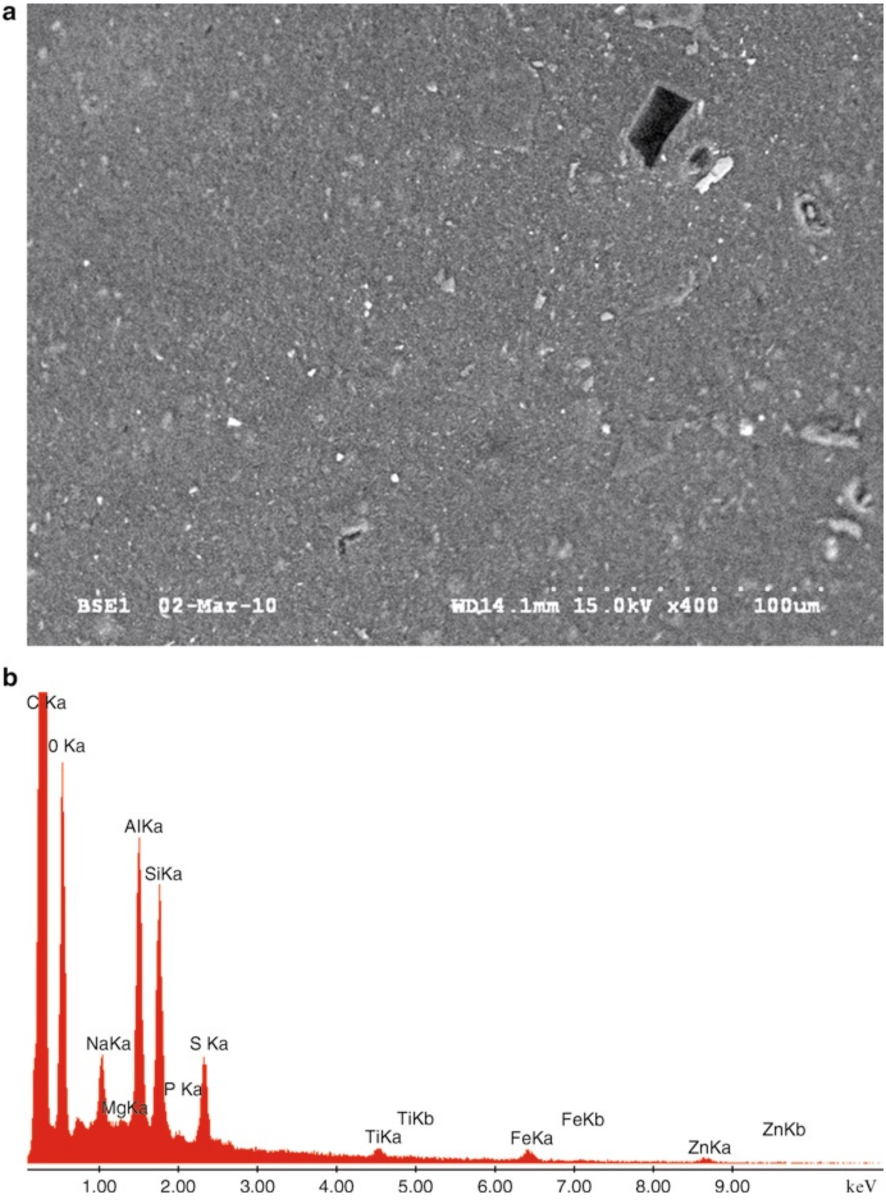

Fig. 10.14 SEM and EDS of polymeric particle. The backscattered electron image of the particle indicates that it has a higher atomic number filler associated with it as shown by the bright particle on the surface of the particle. The EDS spectrum has major peaks for Al, Si, Na, and S along with other minor peaks. Some of the minor peaks may be from the machine from which the tubing was taken. The major peaks may provide a fingerprint for the polymer since a variety of different fillers are used in rubber tubing (image size = 326 × 244 μm)

10.3.5 Silicone Oil in Water

I saved the best for last. Actually, it is the worst. It is one of the toughest contaminants to identify because of difficulties in getting a good sample for analysis. Silicone oil is used with parenteral formulations mainly for the lubrication of glass vials and stoppers. The amount of silicone oil used as lubricant is usually miniscule but errors occur and it is not uncommon to get silicone oil in parenteral products. The oil has a distinctive appearance but can be devilishly difficult to isolate for analysis. Most filtration operations will not filter out the oil and you cannot use a spatula or needle to manipulate it. IR is the best method for analysis but can be problematic if the oil drop is very small which is the case with most silicone drops. Figure 10.15 shows a silicone oil droplet in a vial of water and Fig. 10.16 shows a similar drop by polarized light microscopy. I prepared this sample by putting a small amount of silicone oil into water in a glass scintillation vial. The difference between a silicone oil droplet and an air bubble is difficult to describe but relatively easy to detect. I recommend that you make up a practice sample, shake the vial to make air bubbles and observe the differences between them and silicone. The silicone droplet is difficult to observe by PLM since it does not display interference colors. The fact that it is not miscible with water is an important observation, although there may be a few different oils that could be associated with the product that are not silicone. Residual solvent from the drug substance synthesis is another alternative.

The sample preparation method that I use with the greatest success involves drying a drop of the parenteral that contains a silicone oil droplet onto the IR substrate (Al or low-E glass slides). I use a micropipette and draw up a small amount of the

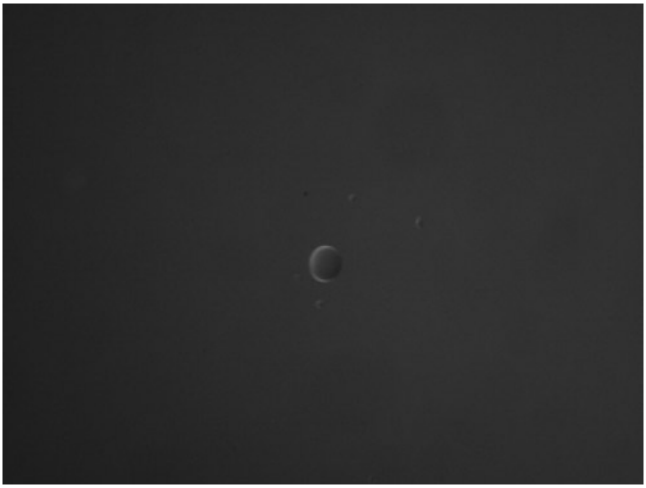

Fig. 10.15 Stereomicroscope image of silicone oil droplet in water. Silicone oil has a distinctive appearance that is quite different than the appearance of air bubbles. The silicone droplet is easier to see directly on the stereomicroscope than it is to photograph. The oil droplets are generally in motion and so difficult to keep in focus for the photomicrograph (image size = 3.4 × 2.5 mm)

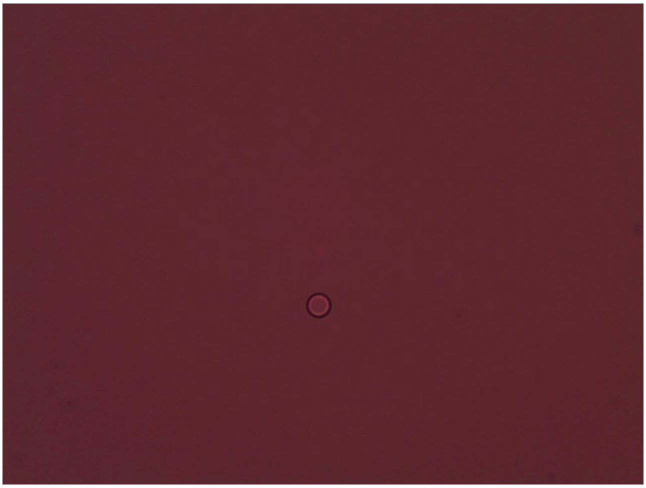

Fig. 10.16 Polarized light photomicrograph of silicone oil in water. The droplet is difficult to detect both in the photomicrograph and on the microscope itself. The silicone droplet is not miscible in water but does not display any interference colors. This observation is highly suggestive for silicone oil (image size = 350 × 260 µm)

parenteral liquid under the stereomicroscope. In that way, I am able to ensure that I also got the silicone drop into the pipette. Once I have the drop on the substrate, it is necessary to manipulate the silicone oil droplet so that it does not lie on top of dried drug substance and excipients. One way of aiding this process is to draw up most of the excess liquid with a filter paper and then to draw the silicone oil away from the drying droplet with a fine needle or glass rod. This process works most of the time, but the drug product liquid does sometimes dry under the silicone oil droplet. I try carefully to draw this oil away from the dried components with a fine needle. This process is laborious and time-consuming, not to mention frustrating since it may take numerous attempts to get an oil droplet suitable for analysis. If you only have a few droplets to work on, then you may not be able to get a suitable sample for IR.

Figure 10.17 shows an IR spectrum of a small silicone oil droplet compared with the reference standard. Note that the intensity of the droplet IR is quite low and the signal to noise is poor. Further note that the spectrum only has a few peaks so that even with a good spectrum it may be difficult to have great confidence in the identification. On the other hand, if the oil was residual solvent or some other type of oil, the spectrum would probably be much more complicated. As I said, silicone oil in a parenteral is a tough identification to make and it is rare that we have enough evidence for high confidence in our conclusion. At times, the only verification of a correct identification is a change in components that resolves the problem. If you do suspect silicone oil, I do recommend that you practice this analysis and ideally you should add a small amount of silicone oil to your parenteral liquid and practice on that sample before you attempt the real thing.

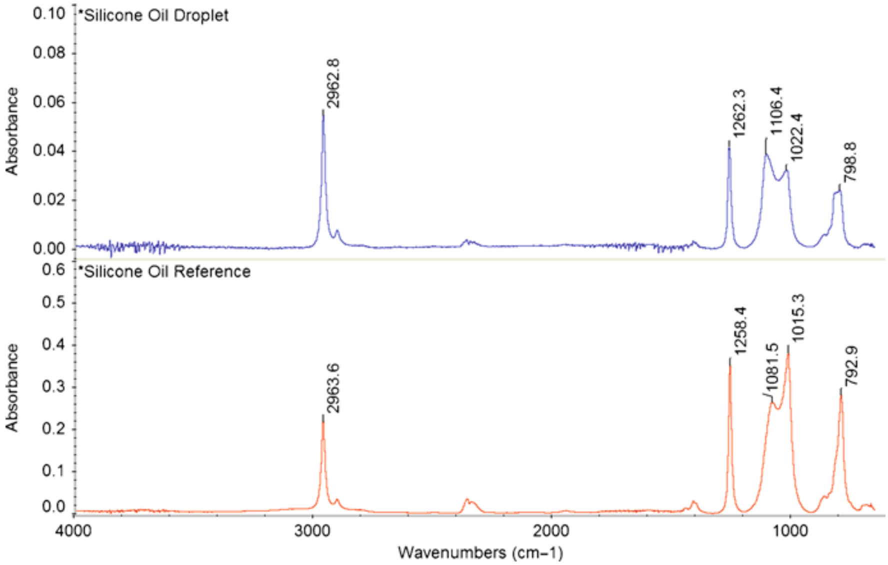

Fig. 10.17 IR spectra of silicone oil droplet and reference. The silicone oil droplet was collected from a drop that was drying on an E-glass slide. The silicone droplet was drawn away from the larger parenteral drop using a sharp needle. The spectrum was collected using a reflective objective. Note that the intensity is low and the signal to noise poor. Still it is possible to observe distinctive peaks associated with silicone oil

It is quite easy to think up many more practice samples. The most important aspect of this practice is sample manipulation, in my opinion. The most challenging samples are those where we only have a very few contaminant particles and they are small (less than 10 µm). Any practice where you manipulate and test particles which are that small and smaller is helpful.

10.4 Laboratory Requirements for Contaminant Identification

One confounding factor in contaminant identification is the possibility that the particle we analyze was introduced from our laboratory and is not the contaminant particle at all. This problem is particularly acute if we are dealing with one or two very small particles. If we have many particles, then those particles introduced in sample preparation will generally be much smaller in number that the contaminant particles. The opposite holds true if we only have a few contaminant particles. I emphasize examining and recording the size and shape of the contaminant particles in the original sample with the stereomicroscope because it allows us to compare the particles that we are analyzing with those we saw in the as-received condition. We also must do our best to control particulates in our sample preparation area.

For most of us, particulate identification is just one part of our job and we do not have dedicated clean rooms for this task. Most laboratory environments have heavy airborne particulate loads and so the possibility of contamination is high. I have two specific recommendations to mitigate this problem. The first is the use of controls. Make it a habit to prepare control samples with the same procedure you used for the contaminant. If you make the same particle identification on the blank as on the sample, then you probably have foreign particles introduced from your environment. The second recommendation is to obtain and use a positive pressure enclosure with a fine particle filter to prepare your samples. These enclosures take in laboratory air, filter it, and then blow it at you and your sample preparation region with laminar flow. You do have to be careful if you are working with dangerous materials and a glove box would be better for those types of samples. But for most common contaminants a laminar flow, positive pressure enclosure can be a good solution to environmental particulates.

10.5 Summary

Contaminant analysis can be exceptionally rewarding both for the analyst and for the company. There are few activities where the microscopist can point to such a direct contribution to the financial bottom line as in identifying contaminants. Intellectually, it is a fine challenge and engages all of your analytical capabilities. In my experience, the more you do this kind of work the more requests for work you will get and the more valued you will be within your company. It is also a portable skill and pharmaceutical companies are not the only companies with contaminants. As you may have guessed, I learned my initial skills in the glass and metal industries. While the nature of the problems are somewhat different, the approaches to solving them are similar.

I cannot emphasize enough that to develop contaminant analysis skills requires lots of practice. Get into the habit of making up practical examples and test cases for yourself. Do some reverse engineering and identify components of healthcare and pharmaceutical products. I doubt if there is any discipline with more "tricks of the trade" than microscopy and contaminant identification. Learn from the literature and from discussions with other analysts. After more than 30 years looking at contaminants, I still regularly learn new ideas from other people. When you are at meetings, ask about the details. How do you remove that particle adhering to the inside of the bottle due to surface tension? How do you keep extraneous particles from your filter? Pay particular attention to the forensic literature and to forensic speakers. Forensic microscopists are the best at the business of particle identification and we can learn a lot from them. Also, if possible, attend schools on the subject, such as the courses offered at McCrone Research Institute (see Chap. 12 for more information on training).

I will finish with a quotation from my first instructor in microscopy, Skip Palenik (Palenik, 1979):

> The skills possessed by the investigator are almost always the deciding factor in a successful analysis. Lack of advanced instrumentation is rarely a severe handicap. Consequently, an investigator with the most sophisticated instruments who lacks the basic skills and mental attitude stands almost no chance of success in this task.

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Internet References

McCrone Research Institute: http://www.mcri.org/home/
McCrone Associates: http://www.mccroneassociates.com/

Chapter 11
Conclusion

11.1 General Summary

I hope you have enjoyed this tour of pharmaceutical microscopy and have learned a thing or two. I know that I have. It is easy to think you know something until you actually have to explain it and write it down in a cogent and understandable fashion. That is when I found some things I did not know as well as I thought I did. Writing the book has afforded me the opportunity to find those weak spots and strengthen them.

We have covered numerous techniques related to microscopy and to the applications of microscopy in drug development. Optical microscopy is an excellent tool for looking at particle morphology and learning much about the crystal properties of a material. Optical crystallography is under utilized in drug development, but holds great promise for important contributions in my opinion. Thermal microscopy has long been a standard tool in drug development and continues to be so. It is quite useful as a stand-alone tool but is truly useful as a supplementary technique to other thermal analysis methods.

The scanning electron microscope is also a common tool in physical pharmacy but most work has been purely qualitative in nature. It is becoming more common to couple image analysis with SEM in order to provide quantitative data for size and shape analysis. I think we will see much more work with quantitative measurements since they hold the promise of correlating microscopic observations with bulk material properties. The use of energy dispersive X-ray spectrometry in drug development is limited to contamination analysis and some chemical mapping but I think the technique will be used more in the future. The use of silicon drift detectors in generating chemical maps and the consequent improvements in speed have opened up new ways of probing our solid dosage forms and will allow us to better understand and predict their properties.

Using an IR or Raman spectrometer with an optical microscope allows us to better understand the chemical nature of the particle we observe along with the morphological and crystallographic properties. Spectroscopy allows us to understand the chemical bonding of different forms of drug substance besides providing a fingerprint

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for identification. There are quite a number of other specialized techniques that are occasionally used in physical pharmacy. These techniques include transmission electron microscopy, fluorescence microscopy, phase contrast and interference techniques, among others. In fact, the list of microscopy techniques applied to pharmaceutical problems is lengthy and growing. Microscopy is alive and well in pharmaceutical development.

Microscopes are used to study drug substance polymorphism, particle size and shape and to identify contaminants. Microscopy has been, in many cases, the primary tool used for understanding but more often is one of a suite of techniques used to solve a problem. In Chap. 9 I provided a rather thorough explanation of the solid-state properties of caffeine. Microscopy certainly plays an important role in this explanation but is one of many different techniques used for the analysis. Even though this is a reductionist book in the sense that I have concentrated on the use and benefits of the microscope in drug development, I hope you understand that microscopy is practiced holistically in the industry. As much as I am dedicated to microscopy, I could not do my work without many other techniques and instruments such as X-ray powder diffraction, thermal analysis, and laser diffraction particle size analysis. It is a rare privilege to be given the opportunity to work on interesting problems with so many fine instruments.

Although I have not emphasized the matter in the book, microscopy is one of those disciplines that neatly match the aesthetic with the practical and scientific. Microscope images can be beautiful and useful at the same time. This combination of the beautiful with the utilitarian appeals to my sense of harmony in work and nature. I can still remember the first time that I saw interference colors with polarized light microscopy. I was amazed then and I am still amazed that something so pretty could also have much to say about the internal order of the observed crystals. One could do thermal microscopy just for the beautiful images.

In the introduction, I briefly touched on the history of microscopy but did not discuss developments of the twentieth century much. Astounding advances have been made in microscopy in that century and in the latter half in particular. Transmission electron microscopy, scanning electron microscopy, atomic force microscopy, confocal microscopy, and microspectroscopy were all invented and developed in that time. The optical microscope benefited from improved optics, convenient polarizing elements, the invention of phase contrast and interference contrast methods to mention just a few new techniques. I mention all of these inventions and developments to make the point that in our studies of nature, and in particular solid-state drug properties, we are not constrained by limitations in instrumentation. Maybe otherwise. It is possible that our ability to integrate our data into a cogent explanation that solves problems is made more difficult by the ease of collecting so much data from so many different instruments. In any case, we certainly have the tools to do nearly all the work necessary to develop, market, and manufacture new medications.

11.2 Training and Education

If I have met my goals in this book, you should now ask an obvious question: Where do I go to learn pharmaceutical microscopy? I do wish I had a simple answer. I know of no universities that offer programs in the subject either as a major field of study within pharmacy departments or even schools with a heavy practical emphasis on microscopy. The latter method of learning microscopy is quite common in materials science schools. Although these material science departments rarely have specific degree programs in microscopy, you can choose a dissertation that is heavily weighted toward that field. I used that approach in my dissertation from Lehigh University and it allowed me to learn electron microscopy from true experts in the subject. I know of no comparable programs in any pharmacy departments. I also do not know of any university that has concentrations in chemical microscopy either, although it is possible to learn optical crystallography in many geology departments.

There are a number of different institutes that offer short courses in pharmaceutical microscopy in the USA. I am not aware of any such institutes in the rest of the world, but that may just be due to my lack of awareness. McCrone Research Institute is where I learned most of my optical microscopy. I have taken four courses there and highly recommend them. To my knowledge they are the only school offering a course specifically on pharmaceutical microscopy. McCrone Associates supports a similar College of Microscopy with some instructional overlap with the McCrone Research Institute but with less emphasis on pharmaceutical microscopy (at the time of this writing). There are a number of different universities that offer short courses in infrared and Raman spectroscopy and Bowdoin College comes to mind. Lehigh University offers a number of short courses in the theory and practice of electron microscopy and associated techniques. I have taken a couple of these courses to great benefit. I am sure an internet search will provide you with a number of good sources of short courses on individual techniques in microscopy and a few on pharmaceutical microscopy.

It is also possible to learn much from meetings of microscopy societies. In the USA, the Microscopy Society of America (MSA) has summer meetings in conjunction with the Microbeam Analysis Society. There is a pharmaceutical focused interest group within MSA that sponsors speaking sessions, posters, and a social hour as part of the summer meeting. These sessions are quite useful since they combine both biological and physical pharmacy talks. We get some good cross-fertilization from the talks and from speaking with each other afterwards. There are also local microscopy chapters that can help new microscopists and for those located in areas with a number of pharmaceutical companies, the local chapter is sure to have pharmaceutical microscopists on hand. The McCrone Research Institute hosts the Inter-Micro conference every summer also. In years past, this meeting had at least half a dozen or so pharmaceutical microscopists in attendance. I have some hope that this practice can be revived in the future.

Finally, most microscopists I know learned much of their skills from books and journal articles. I have listed books and articles that I have found particularly helpful and those I recommend for learning in each chapter. Microscopy has an interesting tradition of producing books dedicated to specific industries such as ceramics, food, polymers, resins, metals, etc. I do hope this book follows well in that tradition and is worthy of it. I have a multitude of such microscopy books and have learned much from them. It is true that a book on ceramic and glass microscopy will probably not have much to say directly about pharmaceutical microscopy, but you might be surprised by how much we can learn from other industries. This cross-fertilization is another advantage of some of the large-scale microscopy meetings where scientists from academia, government and many different industries get together to discuss their craft.

11.3 Experts vs. Generalists

I have alluded to the trend to hire generalists as opposed to experts in industry and I have designed this book with the generalist in mind. I would like to present a brief argument for allowing the development of experts in microscopy as well as other disciplines. Over the years, I have met and listened to a large number of industrial microscopists. By the 1980s, these individuals had been working in the polymer, paint, glass, metal, food, etc., industries for decades. They were each craftsmen (really only a very few women) in microscopy but much more than that. I remember in particular a paper and wood microscopist who spoke regularly at InterMicro conferences in the 1980s. Yes he knew everything worth knowing about paper microscopy, but he also knew how to solve problems. He could section a piece of paper, put it under the microscope and tell you nearly instantaneously what had gone wrong and how to fix it. Yes, he was very, very particular about his craft but he could also see the big picture with regard to the business enterprise and the necessity of ensuring that his work contributed to the profitability of the company. He and others like him are my ideal for industrial microscopists. I am sorry we have lost much of that sense of craftsmanship and dedication to a discipline.

Let me give you an analogy. Virtually everyone reading this book knows how to drive an automobile and is a functional driver. Some more functional than others but still I wager all of us have reasonable driving skills. Now put us behind the wheel of a racing car and charge us with winning the race! Well, now that is different. Most of us might be lucky just to get around the track if we are the only ones on it. Even if we master the controls well enough to drive with the other racing cars, would anyone imagine that we could compete against the professional drivers, much less win the race? Not at all. We would not expect that level of expertise from someone who is a functional driver. So why do we expect a high level of technical expertise from a functional analyst? We should not. If industrial enterprises expect to compete and win in the competitive marketplace, should they not have experts who know their craft as well as a skilled professional driver knows his race car?

11.4 Conclusion

I recommend the art and science of pharmaceutical microscopy to you. The development and marketing of a novel medicine is a wonderful experience and yields profits for both user and manufacturer. Drug development is an amazingly complex business with a wide range of different challenges, not least of which is the subtlety of the solid-state properties of organic chemicals. Pharmaceutical microscopy plays an integral role in understanding these solid-state properties and helping to bring new drugs to patients.

Index

A
Abbe, E., 14
Abbott laboratories, 213
Absorbed water, 2, 16, 217, 227, 231, 249
Accelerating voltage, 89, 91, 95, 96, 99, 103–105, 109, 110, 112, 113, 115, 116, 124, 127, 182, 255
Accuracy, 19, 37, 38, 41, 42, 44–46, 56, 66, 83, 113, 120, 122, 151, 182–185, 188, 195–196, 200, 202, 203, 205, 214, 222, 247–248, 250. 255, 256, 263–270
Acetaminophen, 25, 62–63
Acetylsalicylic acid, 28, 29, 31, 60, 62, 80, 81, 83, 260
Achromatic objectives, 13, 48, 67
Acicular, 185, 196, 209, 248
Acute bisectrix, 21, 35–36, 46
Adrich, D.S., 248
AFM. *See* Atomic force microscopy
Agglomerates, 20, 22–23, 54–55, 57–58, 73, 85, 105, 106, 122, 176–178, 189, 190, 249, 268, 270
Agglomeration, 20, 22–23, 54–55, 57–58, 73, 85, 105, 106, 122, 176–178, 189, 190, 249, 268, 270
Aggregate, 23, 91, 231, 232, 248–250, 254, 256, 271, 293
Aggregation, 15, 20–23, 91, 231, 232, 248–250, 254, 256, 271, 293
Aldrich, D.S., 22, 147
Allen, T., 175
Almeida-Prieto, S., 198
Amorphous solid, 38–41, 214–218, 230
Analog image, 183–184
Analog to digital converter, 56, 183–184
Analyzer, 31, 40, 45, 47, 51, 59, 137, 140, 183–185, 187, 189, 194, 197, 201, 205–206, 209, 262, 273, 275
Anderton, C.L., 147
Anhydrate, 2, 8, 76, 77, 83, 148, 215, 216, 226, 235
Anion, 215–216
Annular stop, 165, 168
Anomalous interference colors, 20, 21, 28–30, 60
Antibiotic, 185, 250
Anti-Stokes scattering, 141
Aperture, 15, 33, 48, 54, 86–87, 99, 103, 104, 106, 134–135, 145, 182, 273, 275
  size, 107, 113, 128, 139, 154
Apochromatic objective, 13, 33, 48
Appel, C.C., 123
Area, 2, 8–9, 49, 50, 56, 66, 69, 79, 86, 87, 89, 91, 92, 97–98, 106–108, 116, 118–119, 121, 122, 128, 131, 139, 145, 147, 151–153, 157, 159, 183, 184, 189, 191–197, 199–202, 205–207, 209, 210, 228, 241, 249, 250, 252, 255, 261, 267, 272, 273, 277, 301, 307
Armstrong, J.T., 102, 120
Aspect ratio, 194, 195, 198–200, 202, 205, 209, 252–254, 256–258, 267
Atomic force microscopy (AFM), 159–161, 306
Atomic number, 85, 89–93, 95, 97, 107, 110, 115, 118–119, 127, 128, 298
ATR. *See* Attenuated total reflectance
Attenuated total reflectance (ATR), 133–139, 152, 154, 297

R.A. Carlton, *Pharmaceutical Microscopy*, DOI 10.1007/978-1-4419-8831-7,

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Auger electron, 86, 91, 109
Automation, 248, 274–275
Average, 37, 89, 107, 136, 146, 205–207, 273
Avicel, 108, 259

B
Back focal plane, 10, 20, 28, 32, 46, 47, 162–165
Backscattered electrons, 86, 90–94, 96–97, 103, 127, 298
Backscatter electron detection, 91–94, 103, 124, 127, 180, 182
Backscatter electron yield, 91–93
Backscatter image, 90, 100, 116, 120, 124, 127, 180, 182, 189, 258–263, 298
Bandli, B.R., 45
Bauer, J., 213
Bauer, P., 213
BCS class, 226
Beam current, 87, 89, 113–114, 120, 128, 263
Beam diameter, 89
Beam size, 89, 99, 103, 107, 115
Becke line, 38–41, 61, 183, 189
Benchtop SEM, 98, 99
Benzanilid, 69, 70, 82
Benzil, 29–30, 60, 69
Bertrand lens, 34, 54, 62, 164–166
Bettinetti, G., 216, 224
Biaxial crystal, 7, 20, 21, 33–38, 41–46
Biaxial interference figure, 20, 32–33, 45–46, 62
Bilde-Soerenson, J.B., 123
Binary, 250, 254–256, 265
  operation, 174, 186–193, 209, 266
Bioavailability, 170, 213, 216, 218, 223, 226
Birefringence, 20, 21, 28, 30–31, 34, 43, 44, 59–61, 75, 230, 272, 279, 282, 283, 287
Bitmap (Bmp), 183
Blanco-Mendez, J., 198
Bloss, F.D., 9, 18, 22, 35, 38, 45
Bmp. *See* Bitmap
Bogdonawich, S., 147
Border transfer, 191, 255, 256
Born, M., 8
Bowdoin college, 307
Bowen, A.M., 45
Brazil nut effect, 175
Breadth, 36, 194, 196, 198, 209
Bremstrhalung x-ray, 86, 91
Bridge removal, 189–191
Brittain, H.G., 147, 216, 228
Brownian motion, 164
Bugay, D.E., 147
Bulb voltage, 50–54, 56, 182, 273, 275

C
Caffeine, 3–4, 16–17, 25–26, 36, 42, 43, 51, 52, 57, 61, 77, 78, 81–83, 126, 127, 155, 177, 178, 180, 228–243, 306
  hydrate, 18, 58, 87, 88, 105, 106, 150, 154, 208, 236, 237, 240–242
  polymorphism, 214, 228–241
Calcium phosphate, 117, 260
Calibration, 44, 61, 66, 69–71, 82, 114, 119, 128, 140–141, 146–147, 184, 194, 195, 202–203, 267, 268
Carbamazepine (CBZ), 3–4, 23, 24, 57, 60–61, 71, 72, 75, 76, 81, 83, 95, 96, 126, 132, 133, 138, 141–144, 147–150, 215, 229, 241, 243
Carbon black, 250, 254–258, 277–278, 284, 287, 289–292, 294
Carbon tape, 102, 126, 127, 179, 254, 255
Cargille liquid, 38–40, 61, 166, 241
Cargille refractive index liquids, 38–40, 61, 166
Carlton, R.A., 240
Carter, C.B., 161
Cathode ray tude, 86, 87
Cathodoluminescince, 86, 91
Cation, 60, 106, 216
CBZ. *See* Carbamazepine
Cellulose, 59, 92, 104, 167, 259, 261, 286, 288–289, 292
Central stop, 20, 165–166, 168
Central tendency, 205–207, 273
Chalmers, J.M., 147
Chamot, E.M., 11
Channel water, 79, 150, 217, 242
Characteristic x-ray, 86, 90, 109
Charcoal, 286, 289–296
Chemburkar, S.R., 213
Chemical imaging, 85, 131, 144, 147, 152–153
Chord size, 194
Chromatic aberration, 12–14, 49
Chrome, 289–291
Cianderani, S., 241
Circular diameter, 192–194, 196, 197, 204, 205, 209, 210, 252–254, 256–258, 267, 270–272
Cleavage, 20, 21, 25, 38
Clemex, 105, 187, 191, 199, 200, 207, 268

Closing, 38, 40, 42, 43, 45, 62, 67, 92, 100, 104, 126, 135, 139, 176–180, 188–189, 199, 201, 202, 209, 218, 226, 241, 253, 255, 258, 267, 270, 285, 297
Coating, 66, 91, 93, 95, 100, 102–107, 115, 126–127, 137, 179, 182, 247, 255, 259, 260, 263, 278, 281, 282
Co-crystal, 215, 217
Coefficient of variation (CV), 207, 272
Cohesive, 22–23, 54–55, 105, 137, 175, 177, 179, 190, 249, 270, 271, 294
Cold emission, 99
Cold stage, 99, 170, 171
Collapse temperature, 65–66, 170
Color balance, 53, 56, 184
Columnar, 248
Compensator
  full wave-plate, 34
  quartz wedge, 44, 51
Compound microscope, 1, 47, 54
Conchoidal fracture, 279
Confidence interval, 272, 273
Confocal aperture, 145
Confocal microscopy, 157–159, 306
Conglomerate, 249
Contaminant identification, 54, 91, 115, 147, 277–286, 301–302
Contamination, 2, 4, 5, 54, 59, 77, 85, 91, 115, 119, 125, 131, 137, 145, 147, 151–152, 230, 277–302, 305, 306
Continuum x-ray, 91, 122
Contrast thresholding, 187, 188
Convergence angle, 89
Convex lens, 13
Convex perimeter, 201
Copper, 123, 127, 128, 289, 290
Corn starch, 294–298
Coulombic, 90, 109
Count, 109, 117–119, 123–125, 157, 163, 177, 191, 194–196, 200, 204–206, 235, 237, 251–258, 261, 263, 271, 272, 275
  rate, 85, 113–115, 128
Coverslip, 23, 28, 43, 55, 58–61, 66, 68, 69, 71, 77, 80–83, 176, 180, 181, 225, 233, 234
Cream, 4, 175, 247, 250–254, 265
Critical angle, 135, 136
Crossed polars, 9, 10, 19–21, 24, 28–36, 41–44, 46, 51, 52, 59, 60, 62, 67, 265, 275, 283
Crossover point, 95
Cross-over voltage, 182
Crystallinity, 9, 16, 28, 51, 159, 218, 223, 230–232, 283
Crystallography, 2, 4, 7–8, 10, 12, 15–37, 45–46, 50, 51, 57, 60, 62–63, 77, 150, 151, 161, 215, 222, 229, 231, 236–237, 242, 305, 307
Crystal system, 16, 20, 21, 32, 37, 41, 42, 46, 216
Cubic crystal, 10, 16, 22, 28, 31, 32, 37–41, 46
Cumulative frequency histogram, 204, 205
CV. *See* Coefficient of variation
Cyclohexane, 105, 106, 126, 177, 179–180

D
Danilatos, 99, 100
Danilatos, G.D., 100
Dark field illumination, 54, 167–168
Dead time, 113–115, 118
Defects, 5, 157, 159, 161, 217, 218, 223, 242, 249
Defect structures, 161
DeHoff, R.T., 194
Dehydration, 65, 68, 75–76, 78–79
Delineation, 185, 186, 208, 255
Delly, J.G., 8, 11, 22, 27, 34, 35, 37, 38, 51, 52, 55
Delustered nylon, 284, 288
de Matas, M., 150, 155
Deming, K., 213
Dent, G., 147
Depypere, F., 159
Derived measurements, 194–200, 209
Desolvated solvates, 215
Desolvation, 26, 27, 65, 75, 78–79, 215
Detection limit, 117, 183, 222, 238–239, 265, 272–273
Detector geometry, 103, 114
DeVincentis, J., 147
Dewettnick, K., 159
Diamond, 12, 18, 136, 137
Diatoms, 53, 164, 165, 181, 278
Diatom test slide, 181
DIC. *See* Differential interference contrast
Dichroic filter, 168
Diederich, A.M., 240
Differential interference contrast (DIC), 10, 48, 162–163
Differential scanning calorimetry (DSC), 2, 65–67, 72, 75, 78, 81, 218, 222, 227, 231–235, 237, 241
Diffraction, 2, 10, 11, 14, 18, 24–26, 28, 37, 54, 91, 109, 122, 131, 150, 161, 183, 198, 202, 205, 223, 224, 227, 238, 242, 247, 250, 252, 306

Digital camera, 52, 53, 55, 56, 67, 160–161, 168, 183–185
Digital image analysis, 2, 173
Digital images, 2, 13, 55, 56, 67, 81, 89, 173, 184
Direct measurements, 37, 194–196, 247
Disconnect, 189–191
Disordered solids, 218
Dispersion staining, 20, 21, 27, 165–167
Dispersive spectrometers, 133, 145
Dissolution, 85, 121, 147, 152, 169, 170, 213, 215–217, 226–228, 236, 241, 247, 256, 259, 261
Donaubauer, J., 213
Double refraction, 10, 19, 32, 46
DP. *See* Drug product
Drug product (DP), 2, 4, 5, 85, 102, 107–108, 115, 159, 161, 162, 169, 171, 173, 213, 215–217, 226, 227, 249, 263, 264, 267, 273, 274, 276, 278, 286, 289, 292, 294, 300
Drug substance (DS), 1–2, 4, 5, 7, 22–23, 37, 54–55, 65, 85, 92, 102–107, 115, 122, 152, 159, 161, 164, 168–171, 173, 178, 189–190, 198, 201–202, 206–207, 213, 240, 247, 259, 261, 265, 270, 271, 278, 280, 283–284, 293–296, 299, 300, 305–306
Drusy, 249
DS. *See* Drug substance
DSC. *See* Differential scanning calorimetry
Duke microspheres 9020, 268, 269
Duke Scientific Technologies, 267
Dziki, W., 213

E
Echlin, P., 102, 120
Edge angles, 20, 21, 26, 196
EDS. *See* Energy dispersive x-ray spectrometry
EDS artifact, 114, 117, 122
EDS map, 4, 85, 120–122, 152, 259–263
Edwards, H.G., 150, 155
Elastic collisions, 90
Electromagnetic radiation, 8, 9, 132
Electron beam, 89–93
  skirt, 123–125
  spread, 124–125, 263, 289, 290
Electron cascade, 100, 101, 124, 128
Electron diffraction, 109, 161
Electroscan, 91, 99–101
Elemental mapping, 108, 109, 114, 120–123, 125, 128, 152, 260
Elongation, 21, 22, 36, 58–60, 62, 189–190, 198, 202, 252, 255, 256
Enantiotropic, 81, 219–222, 226, 240, 241
Endotherm, 78, 232, 233, 235, 237
Energy dispersive X-ray spectrometry (EDS), 4, 19, 85–128, 152, 161, 258–263, 278–280, 282, 284, 285, 287, 289–291, 294, 295, 297, 298, 305
Energy *vs.* temperature diagram, 219–221
Enthalpy, 219, 227, 232
Entropy, 219, 227
Environmental Scanning Electron Microscope (ESEM), 100, 101, 123, 170, 171, 284
Equant, 248
Equilibrium melting temperature, 73
Error analysis, 197–198, 248, 254, 264–273
Escape peak, 116–118
ESEM. *See* Environmental Scanning Electron Microscope
E-T detector. *See* Everhart-Thornley (E-T) detector
Eutectic temperature, 65–66, 69–70, 80, 83, 221
Evanescent wave, 136
Evans, S.H., 45
Everhart-Thornley (E-T) detector, 97
Excipient, 1–2, 85, 104, 108, 117, 121, 152, 155, 159, 169, 243, 247, 258–264, 278, 300
Exotherm, 232, 233
Exposure time, 145
Extinction angle, 20, 21, 36
  dispersion, 31–32
Extinction position, 20, 21, 31–32, 41–44, 59
Eyepiece, 20, 31, 34, 44, 49, 50, 59, 61, 62, 67, 89, 139, 144, 164, 168–169, 196

F
Faraday cage, 97, 120
FDA, 226, 240
Feature segmentation, 174, 187–188
Feret diameter, 194–196, 199, 201–202, 252
Ferioli, V., 147
Feynman, R.P., 10
Fiber, 21, 25–26, 28, 36, 42, 45, 59, 100, 248, 268, 277–278, 280–282, 284, 286–289, 292
Fiberglass, 268, 277, 286
  insulation, 277
Ficarra, R., 147
Field diaphragm, 50–55, 182, 275
Field-emission, 98, 99, 107

Field measurements, 180, 187, 194, 261, 263, 266, 269, 274, 275
Fill, 54, 90, 143, 144, 189, 209, 250, 255, 256, 263, 297, 298
Final melting temperature, 71, 73, 75, 82
Fiori, C.E., 102, 120
Flake, 22, 166, 248, 279, 286–287
Flat capillary, 225
Fluorescence, 49, 91, 109, 118, 134, 142–145, 159, 182
  microscopy, 9, 50, 168–170, 306
Fluorite objective, 13, 48
Fluorophore, 157–159, 168, 169
Fluotar, 268
Forensic science, 278
Fourier Transform, 132–135, 142
Fourier Transform IR (FTIR), 132–134, 145
Fractal, 195, 198, 201, 252
  dimension, 201, 252
Frampton, C., 25, 63
Free acid, 214, 216
Free base, 214–216
Freeze dry microscopy, 170
Frequency histogram, 204–206, 210
Frits Zernike, 163
Full standards, 119, 120
Fusion, 20, 28–31, 33, 60, 62, 65, 66, 79–81, 83, 85, 225, 249

G
Gage, S.H., 8
Gamberini, G., 147
Gamberini, M.C., 147
Gasket, 278, 280, 281, 289
Gas path length, 124, 263
Gaussian distribution, 270
Gauvin, R., 123
Gibbs free energy, 219, 221
Glass, 1, 3, 11–13, 28, 38–41, 46, 58, 60, 61, 66, 68, 77, 78, 81, 104, 123, 124, 127, 128, 135, 137, 143–145, 155, 166–168, 177–182, 186, 191, 192, 197, 205, 208, 218, 225, 233, 234, 267–270, 278, 279, 282–287, 293, 299–302, 308
Goldstein, J.I., 102, 120
Gordon, K.C., 147
Grating, 132, 145, 155
Gray level, 182, 183, 186, 188, 261, 263
Griesser, U.J., 241
Griffin, B.J., 123
Grzesiak, A.L., 147, 150
Gunter, M.E., 45

H
Habit, 20–23, 25, 26, 31, 43, 62, 161, 223–224, 227, 230–232, 235–236, 249, 302
Haleblian, J., 216
Hartshorne, N.H., 21, 22, 26, 34, 35, 38, 45, 46, 51
Henry, R., 213
Hexagonal crystal, 10, 16, 18, 31–32, 35, 37, 41, 46
High vacuum, 86, 93–94, 97–102, 104–106, 115, 255
Hilfiker, R., 216, 228
Hoffmann modulation contrast, 10, 162–163
Hofmeier, U.C., 241
Hot stage, 5, 66, 67, 69, 79, 81, 82, 214, 223, 227, 233–235, 243, 282
Hot-stage microscopy, 81, 233, 235, 282
Hutsebaut, D., 146
Hydrate, 2, 8, 10, 18, 26, 58, 65, 68, 75, 87, 88, 100, 105, 106, 138, 147, 148, 150, 151, 154, 170–171, 208, 214–219, 221, 224, 226, 227, 229, 235–243
Hydrocortisone cream, 3–4, 250–254
Hydrogen bonding, 133, 150, 242
Hydroxy propyl methyl cellulose, 104

I
Image analysis, 2, 4, 53, 55, 85, 99, 122, 152, 173–210, 247–248, 250–267, 269, 270, 272–276, 305
Image collection, 55–56, 82, 173, 174, 180, 181, 183–186, 188, 203, 208, 210, 250, 255, 262, 263, 268, 270, 273, 275
ImagePro, 200, 207
Image processing, 56, 122, 174, 184–188, 190, 201, 208, 251, 254, 255, 263, 265, 266
Image production, 100, 174, 181–183
Industrial microscopy, 1
Inelastic collisions, 90
Inelastic scattering, 90–91, 131, 141
Infinity corrected objective, 48, 49, 134, 143
Infinity-corrected optics, 134, 143
Infrared (IR) microscopy, 19, 131–155, 278
Infrared (IR) microspectrscopy, 4, 147, 278, 284, 297
Infrared radiation (IR), 4, 8, 19, 65, 77, 131–155, 157, 214, 227, 231, 232, 234, 235, 238–241, 243, 278, 284, 287, 289, 292, 294, 296, 297, 299–301, 305
Infrared (IR) spectroscopy, 131–135, 142, 147, 148, 151, 231–232, 243, 297, 307
Inhaled drug product, 180, 254

Inhaler, 1, 3, 175
Inner diameter, 200, 201
Inner shell electron, 90, 91, 109, 110
Inner shell ionization, 91
Inoué, S., 55
Interference, 9, 28–36, 38, 40–46, 48, 51, 60–62, 75, 145, 152, 162, 227, 230, 231, 283, 287, 288, 293, 299, 300, 306
  colors, 10, 20, 21, 28–31, 36, 42–44, 51, 60–62, 75, 222, 230, 231, 287, 288, 293, 299, 300, 306
  figure, 10, 20, 21, 28, 32–36, 38, 44–46, 62–63
  filter, 40, 41
Interferogram, 10, 133, 135, 139–141
Interferometer, 133, 134, 140, 145
Inter-Micro conference, 307, 308
IR. *See* Infrared radiation
ISO, 248
Isogyre, 32–33, 35, 62,–63

J
Joyce Loebl, 193
Joy, D.C., 102, 120
Jpeg, 184

K
Katrincic, L.M., 240
Kim, K., 147, 150
Kinetic stability, 219
K-line, 110, 114, 116
Kofler, A., 65, 66, 69, 80
Kofler hot stage, 66, 67, 69
Kofler, L., 65, 66, 69, 80
Köhler illumination, 47–56, 58, 59, 174, 182
Kuhnert-Brandstatter, M., 65, 69, 70, 80

L
Lamellar, 249
Lang, M., 147, 150
Lanthanum hexaboride (LaB_6), 98
Laser diffraction particle size analysis, 37, 247, 250, 252, 306
Laser scanning confocal microscopy, 157–159, 306
Lath, 248
Lawson, E., 150, 155
LCD. *See* Liquid crystal display
Lee, D.C., 228
Lehigh University, 307
Leighton, R.B., 10
Length, 8, 9, 12–15, 18, 20, 21, 24–27, 30–32, 35, 36, 38, 40, 41, 45, 47–49, 56, 59, 61, 88, 109, 113, 124, 132, 136, 139, 142, 145–147, 155, 161, 165, 166, 168, 169, 175, 194–196, 198–202, 209, 248, 252, 263, 273, 306
Lens hysteresis, 203
Lewen, G., 147
Lifshin, E., 102, 120
Light
  amplitude, 9–10
  intensity, 9, 50, 53, 54, 144, 162
  interference, 9, 10, 20, 283
  phase, 9, 162
  reflection, 11–12, 22, 86, 135, 168
  scattering, 141, 142, 198
  wavelength, 8, 9, 12, 14, 15, 27, 40, 45, 56, 132, 136, 142, 145, 165, 168, 273
Limit of detection, 117, 183, 239, 265, 272, 273
Linkam, 66, 67, 69
Liquid crystal display (LCD), 86, 87, 89
Live time, 5, 113, 115
L-line, 110, 116, 117
Long, S.T., 240
Low E glass, 135, 137, 299
Low vacuum, 91–93, 98–103, 105–107, 115, 123–125, 127–128, 171, 182, 263, 289, 290
Low voltage, 95, 96, 100, 180–182
Lu, J., 216
Lyman, C.E., 102, 120
Lyophilization, 170

M
Magnesium stearate, 121–122, 259, 262, 263
Magnification, 13–15, 22, 48, 49, 53, 57–60, 67, 87, 89, 93, 104, 106, 115–116, 127, 135, 143, 144, 159, 183, 184, 191–193, 195, 202, 203, 255, 262–263, 265, 268, 269, 273, 275
Mannitol, 170, 229, 243
Martin, L.C., 8
Martin's diameter, 201
Mason, C.W., 11
Matsumoto, B., 52, 55
Matzger, A.J., 147, 150
McCrone Associates, 34, 307
McCrone, L.B., 8, 11, 22, 27, 34, 35, 37, 38, 51
McCrone Research Institute, 37, 278, 302, 307
McCrone, W.C., 4, 8, 11, 22, 27, 34, 35, 37, 38, 51, 65, 73, 80, 165, 215, 216, 224, 241, 277

McFarland, K., 213
McGoverin, C.M., 147
McKie, C., 18
McKie, D., 18
McLaughlin, R.B., 162
Mean, 18, 19, 22, 23, 26, 42, 47, 66, 70–73, 81, 92, 95, 96, 100, 115, 120, 122, 123, 162, 170, 174, 195, 197, 201, 203, 204, 206, 207, 210, 213, 216, 223, 224, 227, 234, 249, 252, 254, 256, 258–260, 266–273, 275, 280, 285, 286
Median, 15, 206–208, 210, 266, 273
  filter, 56, 185, 186
Melatrope, 32–33
Melia, C.D., 157
Melting point, 68–75, 77, 80, 82, 83, 219–222, 224, 230
Melting temperature, 69–71, 73, 75, 82, 83
Melt temperature, 69–71, 73, 75, 82, 83, 227
Mercury, 135, 168, 278, 284
Method development, 122, 181, 189, 197–198, 248, 264–273
Method validation, 248, 264
Mettler-Toledo, 66–67
Michael, J., 102, 120
Michel-Levy chart, 28, 30, 33, 34, 44, 60, 61
Microbeam Analysis Society, 307
Micrometer slide, 203, 267, 268
Micronized, 105, 161, 173, 180, 201, 248–249, 256–257, 265
Micronized drug substance, 105, 161, 265
Microscope objective, 13, 47, 48, 62, 67, 135, 152, 167, 168, 176, 185, 202–203, 214, 250, 265, 268, 273, 275
Microscopy Society of America, 307
Microspectroscopy, 2, 4, 132, 147, 278, 284, 297, 306
Microtome, 107–108, 122, 127, 152, 260
Milled drug substance, 105, 161, 164, 180
Mineral oil, 58, 176
Minsky, M., 158
M-line, 110, 116, 117
Mode, 86, 115, 116, 127, 135, 168, 180, 182, 206–207, 255, 263, 268, 289, 290
Moens, L., 146
Monoclinic crystal, 10, 16, 18, 20, 21, 32, 35–37, 42
Monotropic, 219–221
Monte Carlo, 93–94, 110
Morphology, 2, 4, 20, 161, 170, 179, 223–224, 249, 265, 276, 282, 283, 286–291, 294, 295, 305
Morris, S., 213
Morse, S.A., 22, 34, 35, 38, 45, 51
Mueller, R.L., 240
Multi-vitamin, 116, 120, 121, 123, 127, 128, 155, 250
Murphy, D.B., 55, 162

N

Nano-suspension, 99, 247, 250
Naproxen sodium, 3–4, 91–92, 108, 112, 113, 152, 153, 155, 250, 258–263
Naproxen sodium tablet, 3–4, 92, 108, 112, 153, 155, 250, 258–263
Narayanan, B.A., 213
National Institutes for Science and Technology, 267
National Physical Laboratory (NPL), 203, 267
NDA. *See* New drug application
Needham, G.H., 7–9, 11, 32, 37, 48, 51
Newbury, D.E., 102, 120
New drug application (NDA), 173, 213, 238
Newman, A.W., 65, 67, 147, 216
Nichols, G., 25, 57, 63
Nomarski interference contrast, 162
Non-crystalline solid, 138, 214, 215, 217–218
Norland optical adhesive, 58, 176
Normalized results, 119
NPL. *See* National Physical Laboratory
Nucleopore™, 105, 106, 126, 179, 180, 182, 254–255, 282
Numerical aperture, 15, 33, 48, 273, 275
Nylon, 280, 281, 284, 288, 289, 292

O

Object measurements, 194–203, 261, 262
Object transfer by limits, 191–193
Oblique light, 162, 167, 168
Obtuse bisectrix, 21, 35–36, 46
Ocular, 47, 49
Onset melting temperature, 73
Oostveldt, P.V., 159
Optical crystallography, 4, 7–8, 10, 12, 16, 18–37, 45, 50, 51, 57, 62–63, 77, 222, 236, 305, 307
Optical microscopy, 1, 2, 4, 7–9, 23, 24, 47, 55, 57, 65, 86, 134, 143, 162, 173, 174, 176–178, 181–184, 188, 189, 202, 203, 207, 208, 223, 248–254, 265, 273, 274, 305–307
Optical orientation, 21, 35–37, 45, 62
Optic axial angle, 20, 21, 62
  dispersion, 34–35
Optic sign, 20, 21, 34, 35, 42, 45
Orthopedic implants, 278

Orthorhombic crystal, 10, 16, 18, 20, 21, 31–32, 35, 37, 42
Ostwald rule, 78
Otero-Espinar, J., 198
Outer diameter, 200, 201
Outgassing, 223

P
Paint sprayer, 177
Palenik, S.J., 303
Paracetamol, 25, 57, 61–63, 227
Parenterals, 137, 145, 167, 168, 170, 207, 278, 280–282, 284, 299–301
Particle habit, 227, 230, 231
Particle shape, 105, 173, 175, 176, 185, 187, 189, 197–199, 222, 230, 247–249, 254, 258, 264, 266, 270, 306
Particle size, 2, 7, 37, 76, 99, 105, 122, 173, 175, 176, 178, 179, 183, 185, 187, 189, 196–199, 202, 204–205, 207, 222, 230, 247–250, 252, 254, 258, 264–267, 270, 272–274, 306
Patel, K., 213
Patent, 91, 100, 147, 148, 213, 225, 234
Peak-to-background, 109, 117, 119, 231–232
Performance check, 69, 70, 82, 114, 140–141, 146–147, 202–203, 267
Perimeter, 194, 195, 199–202, 252
Peters, K.R., 102, 120
Petrolatum, 185, 186
Pharmaceutical microscopy
  drug, 1
  excipients, 2
  solid dosage, 2
  use, 2
Phase contrast, 10, 48, 163–165, 182, 306
Phase I, (AU: Not found)
Phase II, 223
Phase III, 75, 223
Phase transition, 65, 69, 75, 76, 218, 221, 241
Phenacetin, 69–71, 77, 80, 82, 83
Phonon, 91
Photomicrograph, 22, 24, 29, 34, 47, 51–53, 55–57, 67, 79, 87, 89, 95, 96, 99, 102, 104, 134, 160
Photomicroscopy, (AU: Not found)
Photomultiplier, 93, 97
Picoammeter, 124
Pieters, J.G., 159
Pitt, C., 241
Pixel, 86, 88, 89, 120–122, 183–186, 188, 189, 191–192, 194, 195, 202
Plant trichomes, 286, 287
Plasmon, 91
Plate, 34, 36, 51, 52, 58–60, 62, 66, 67, 71, 72, 77, 78, 91, 100, 197–198, 233–234, 248
Pleochroism, 20, 21, 28, 59
PLM. *See* Polarized light microscopy
Polarization, 8, 9, 29–30, 141, 144
Polarized light, 2, 4, 7–63, 67, 144, 182, 214, 227, 231, 265, 278, 282, 284, 287, 288, 293, 299, 300, 306
Polarized light microscopy (PLM), 2, 4, 7–63, 67, 144, 182, 214, 225, 227, 231, 265, 278, 282, 284, 287, 288, 293, 299, 300, 306
Polarizer, 9, 31, 36, 40, 45, 47, 50, 51, 59
Polars, 9, 10, 19–36, 41–44, 46, 51, 52, 59–62, 67, 71, 72, 256, 275, 283
Pollen, 282, 286, 287
Polyester, 280
Polyethylene, 126, 146, 177, 254
  glycol, 126, 146–147, 177, 254
Polymer, 1, 3, 113, 120, 136, 260, 277–280, 282, 286, 289, 293–298, 307
Polymorphism, 2, 4, 8, 10, 18, 19, 25, 26, 37, 65, 71, 75, 77–81, 131, 133, 147–151, 165–167, 213–243, 306
Polymorphs, 8, 10, 19, 26, 65, 70–71, 75, 77–79, 81, 133, 147–151, 165–167, 167, 213–222, 224–227, 229, 230, 234–238, 240–243
Polymorph screen, 224, 234–238
Polystyrene, 120, 140, 146, 154, 267, 268
Porter, W., 213
Precision, 151, 185, 188, 264–267, 270, 272
Pseudomorph, 215, 217
Pseudo-polymorph, 215, 217
Pygall, S.R., 157

Q
Qualitative analysis, 108, 113–118, 123, 125, 126, 128, 248–249, 259–260, 285, 289, 290
Quantitative analysis, 108, 109, 113, 114, 117–120, 123, 125, 126, 151, 259–260, 285
Quarter-wave plate, 67
Quartz, 44, 51, 277, 286
Quick, J., 213

R
Rades, T., 147
Raman microscopy, 19, 131–155, 214, 225
Raman microspectroscopy, 4, 147, 278
Raman scattering, 131, 141–144, 152

Raman Spectroscopy, 131, 132, 142, 147, 151, 225, 227, 232, 243, 297, 305, 307
Range, 1, 7, 12, 15–16, 19, 37, 38, 44, 49, 65, 69, 71, 75–76, 82, 92, 102, 113, 115, 116, 132, 137, 139, 160, 161, 171, 176, 182–185, 187, 204–207, 217, 218, 226, 228, 241, 242, 248, 252, 265, 270–273, 283, 286
Raster, 86–89, 115–116
Rayleigh scattering, 141
Reay, A., 147
Reflected electron, 90
Refraction, 10, 12, 13, 19, 28, 34–46
α Refractive index, 42
β Refractive index, 42
ε Refractive index, 42
γ Refractive index, 42
ω Refractive index, 42
Refractive index (RI), 10, 12–13, 19–21, 27, 28, 30–31, 41–42, 61–63, 80, 135–136, 165–166, 176, 224, 225, 243, 282
  dispersion, 27, 28, 31, 45, 165
Region of interest (ROI), 120
Relative axial lengths, 248
Relative error, 268, 269
Relative standard deviation (RSD), 271–272
Repeatability, 265–267
Reproducibility, 56, 67, 68, 73, 97, 107, 174, 177, 258, 265–267, 275
Residual solvent, 70, 73, 223, 230, 299, 300
Resolution, 10, 13–15, 49, 55, 67, 88–90, 92–99, 103, 106, 107, 113, 122, 127, 128, 133, 139–141, 145–146, 152, 154, 155, 157, 159, 163, 183, 184, 192, 193, 208, 262, 263, 273
  limit, 10, 89, 98, 145–146, 163, 183, 273
Resolving power, 14, 15
Retardation, 28–31, 46
Reutzel-Edens, S.M., 216
Reutzel, S.M., 228
Rheinberg illumination, 167–168
Rhines, F.N., 194
RI. *See* Refractive index
Ribbon, 248
Riley, D., 213
Ritonavir, 213, 215, 226
Robinson™ detector, 93–94, 97
Robustness, 186–187, 265, 266, 273
Rod, 199, 248, 300
Rohani, S., 216
ROI. *See* Region of interest
Romig, A.D., 102, 120
Roughness, 201, 277
Roundness, 191, 192, 194, 199–200, 252, 255
RSD. *See* Relative standard deviation
Ruggedness, 174, 266, 274–275
Russ, J.C., 193, 194
Rust, 278
Rustichelli, C., 147

S
Saccharin, 69, 73, 74, 78, 79, 82, 83
Salt, 85, 137, 147, 214–216, 218, 221, 225, 268, 278
Sample preparation, 54, 57–58, 68, 81–82, 102–108, 114, 115, 126–127, 132, 136–140, 144–146, 152, 154, 161, 174–181, 185, 188–190, 208, 252, 254, 256, 259, 260, 262, 265–267, 271, 273, 299, 301, 302
Sandler, N., 147
Sands, M., 10
Saville, D.J., 147
Sawyer, L., 102, 120
Scanning electron microscopy (SEM), 2, 4, 19, 24, 54, 55, 85–128, 152, 160–162, 170–171, 173, 174, 177, 179–183, 185, 187, 188, 197, 203, 207, 208, 214, 223, 230, 232, 234, 236, 239, 248–250, 254–258, 263, 265, 275, 278–280, 282–284, 289–291, 294, 295, 297, 298, 305, 306
Scanning probe microscopy, 159–160
Scans, 2, 4, 65, 71, 85–128, 133, 139–142, 145, 146, 152, 154, 157–160, 170, 173, 183, 202, 214, 218, 227, 230, 232, 254, 274, 276, 278, 305, 306
Schottky, 99
Schwarzschild, 135, 152
Scintillator, 93, 97
Secondary electron, 86, 90, 91, 94–98, 100, 101, 103, 105, 106, 120, 124, 127–128, 180–182
  detection, 94–98, 100, 106, 180, 182
  yield, 94, 95
Segment, 174, 185–192, 195, 209, 250–251, 255, 256, 263, 266
Segmentation, 174, 185–188, 209, 255, 256, 263, 266
SEM. *See* Scanning electron microscopy
Separate, 1, 49, 62, 65, 174, 177, 178, 181, 185, 189–192, 202, 209, 214, 229, 250, 256, 270, 271, 287
Shape, 2, 4, 7, 11–13, 20–22, 24, 28, 85, 97, 105, 121, 126, 127, 157, 173, 174, 185, 187, 188, 193, 196–199, 201, 204, 205, 222, 232, 247–276, 282, 301, 305, 306
Sharpen filter, 186
Shields, L., 150, 155

Signal-to-noise, 133, 139, 145, 146, 154, 300, 301
Sign of elongation, 21, 36, 59, 60
Silicon dioxide, 259, 262–263
Silicon drift detector, 118, 259–260, 262, 263, 305
Silicone oil, 57–59, 176, 178, 269, 278, 286, 299–301
Silicon lithium (SiLi) detector, 113–114, 122
Single crystal x-ray crystallography, 236
Skip Palenik, 59, 303
Slayter, E.M., 8, 11
Slayter, H.S., 8, 11
Slit width, 145
Smith, M.A., 22, 147, 248
Soldani, M., 213
Solid-liquid-solid transformation, 65, 75–76
Solid-solid transformation, 75, 225, 233
Solid-state analysis, 3, 4, 7, 70, 75, 131, 147–151, 214–216, 228, 229
Solid-state detector, 93–94
Solid-state forms, 2, 4, 7–8, 65, 78, 80, 138, 147–148, 213–219, 222–227, 233, 235, 243
Solubility, 115, 147, 213, 215–218, 221, 222, 226–228, 236, 238, 241, 282, 284, 292, 294
Solution phase transformation, 151, 222, 241
Solvate, 10, 26, 27, 65, 73, 79, 100, 138, 147, 148, 150, 151, 166–167, 214–219, 221, 225–227, 232, 234, 243
Solvatomorph, 217
Solvent mediated phase transformation, 221, 222
Space groups, 16, 18, 216
Spanton, S., 213
Spatial resolution, 92, 122, 145, 263
Specification, 3, 9, 10, 12, 19, 22, 37, 45–46, 49, 55, 86, 98, 109–110, 131, 132, 137, 148, 159, 173, 175, 186–187, 189–191, 207, 215, 219, 229, 248–249, 264, 267, 272–274, 279–281, 284, 286, 302, 308
Specificity, 19. 265, 272, 280–281
Spectral range, 139
Spherical aberration, 12–14, 49
Spherical diameter, 194, 196, 198, 252
Spherical volume, 252, 253, 257
Sphericity, 194, 200, 252
Spherulite, 60, 249
Spiwek, H., 213
Spot size, 103, 104, 106, 107, 113, 115, 128
SRM 1965, 267, 268
Standard deviation, 120, 207, 210, 254, 258, 268–270, 272–274
Standardless, 119, 120
Steel, 119, 128, 137, 278, 289, 291
Stephenson, G.A., 228
Stereology, 194
Stereomicroscope, 22, 23, 54–55, 57–58, 115, 126, 167–168, 230, 231, 249, 278, 281–284, 286, 287, 292–294, 296, 299–301
Stereomicroscopy, 54–55, 230, 249, 278, 282
Stoiber, R.E., 22, 34, 35, 38, 45, 51
Stokes scattering, 133, 141
Strachan, C.J., 147
String length, 201–202
String width, 201–202
Stuart, A., 21, 22, 26, 34, 35, 38, 45, 46, 51
Student *t* value, 272
Sublimate, 70, 71, 77, 82–83, 233–235
Sublimation, 58, 61, 68–71, 76–78, 83, 170, 225, 233–235, 240–243
Sub-stage condenser, 50–52, 54, 55, 58, 67, 163–167, 273, 275
Sugar, 286, 289–294
Sulfathiazole, 229, 243
Sum peak, 116–118
Sun, Y.T., 240
Super-saturated solution, 225
Surface texture, 20, 21, 25–26
Su, S.C., 45
Suspension, 99, 102, 106, 107, 126, 169, 170, 175–180, 186, 247, 250, 251, 258, 264, 272
Szelagiewicz, M., 241

T
Tablet, 1–4, 85, 92, 100, 102, 107, 108, 112, 114–117, 121–125, 127, 132, 152, 153, 155, 159, 160, 169, 194, 216–217, 226–227, 241, 247, 250, 258–264, 278, 282
Teetsov, A.S., 241
TEM. *See* Transmission electron microscopy
Temperature, 12, 16, 18, 26, 38, 40, 58, 61, 65–71, 73, 75, 76, 78, 81–83, 98, 100, 113, 135, 138, 147, 150, 151, 170, 171, 217–222, 225–227, 232, 233, 235, 237, 238, 240–242
Tetragonal crystal, 10, 16, 18, 21, 31–32, 37, 41, 46
TGA. *See* Thermal gravimetric analysis
Thermal analysis, 2, 223, 233, 305, 306
Thermal gravimetric analysis (TGA), 2, 65, 75, 78, 227, 231–235, 237, 241, 242
Thermal microscopy, 2, 4, 19, 49, 65–83, 150, 170–171, 225, 305, 306

Thermal stage, 65–67, 170, 171
Thermionic, 98, 99, 106, 107
Thermodynamically stable form, 18, 218–220, 226, 238
Thermodynamic stability, 218, 219, 240
10th percentile, 210, 254, 256, 258
50th percentile, 206, 210, 254, 258
90th percentile, 173, 210, 254, 258, 273
Threshold, 187, 188, 191, 209, 250, 255, 256, 265, 268, 270, 273, 275
Tian, F., 147
Tiff, 184
Timmins, P., 157
Tin, 80, 137, 289, 290
Titanium, 259, 284
  dioxide, 259
TLC sprayer, 177
Tommasini, S., 147
Trace element, 115–118, 151, 294
Training, 49, 57, 62, 104, 125, 126, 137, 228, 278, 302, 307–308
Transition temperature, 81, 218–222, 226, 240, 241
Transmission electron microscopy (TEM), 157, 160–161, 306
Trap, 191–193
Trehalose dihyrate, 75–77, 79, 83
Triamcinolone acetonide, 68
Triclinic crystal, 10, 16, 18, 20, 21, 32, 35–37, 42
Tungsten, 49, 50, 98, 99, 151–152, 281, 282
Twinning, 20, 21, 23–24, 56–57, 60
Tyndale effect, 106

U
Underwood, E.E., 194
Undulating birefringence, 59
Uniaxial crystal, 20, 21, 32–34, 36–38, 41–42, 46, 62
Uniaxial interference figure, 20, 32–33, 35, 45, 62
Unit cell, 17, 18, 214, 216, 242
US Pharmacopeia, 248

V
Vandenabeele, P., 146
Variance, 272
Vibrational energy, 132, 147
Vibrational microscopy, 31, 36, 223, 225
Vibrational states, 131, 147
Vitez, I.M., 65, 67
Vogt, F.G., 240
Vortex, 176, 254–255

W
Wahlstrom, E.E., 22, 35
Water of crystallization, 217
Water vapor absorption, 2, 227, 231, 249
Wayne, R., 162
Weaver, R., 45
Webb, M., 228
Web site, 6
Weibel, E.R., 194
Wet-bead milled particles, 14, 106, 161
Whetstone, J., 157
White balance, 5, 53, 184
Williams, A.C., 147
Williams, D.B., 102, 120, 161
Wolf, E., 8
Wollaston prism, 162
Wood, E.A., 22
Working distance, 48–49, 67, 99, 103, 114, 123, 124, 127, 142, 182, 203

X
Xanthine alkaloid, 229
X-ray diffraction, 2, 18, 24, 25, 109, 150, 151, 161, 223–224, 227, 238, 242, 306
X-ray powder diffraction (XRPD), 2, 25, 151, 223–225, 227, 231–235, 306
XRPD. *See* X-ray powder diffraction

Y
York, P., 150, 155
Yu, L., 222, 228, 241

Z
ZAF, 118–119
Zernike, F., 163
Zinc selenide (ZnSe), 136
ZnSe. *See* Zinc selenide
Zografi, G., 216