Abstract

INFRARED MICROSCOPIC TECHNIQUES FOR THE QUALITATIVE ANALYSIS OF CROSS-SECTIONED RENAL CALCULI AND EMBEDDED MINERALIZED DEPOSITS

by Jennifer C. Anderson

This thesis presents investigations into the qualitative analysis of renal stones and embedded deposits using infrared (IR) microspectroscopy. The incorporation of a microscope into infrared analysis improves spatial resolution, allowing the interrogation of small areas of interest, and the identification of microscopic particles. The use of IR reflection/absorption (R/A) techniques as a means to identify mineral deposits embedded in tissue is discussed, as is the use of IR reflectance off the surface of cross-sectioned calculi. These techniques have been found to be more accurate and less time-consuming than the traditional practices of contrast staining, especially for samples containing multiple components. In an attempt to quantify a particular component, attenuated total internal reflection (ATR) IR microspectroscopy calibration curves were created, against which unknown samples were measured. However, more work is needed for this method to be considered truly quantitative. Finally, a spectral library of common renal stone components is presented.
INFRARED MICROSCOPIC TECHNIQUES FOR THE QUALITATIVE
ANALYSIS OF CROSS-SECTIONED RENAL CALCULI AND EMBEDDED
MINERALIZED DEPOSITS

A Thesis

Submitted to the
Faculty of Miami University
in partial fulfillment of
the requirements for the degree of
Masters of Science
Department of Chemistry and Biochemistry

by
Jennifer C. Anderson
Miami University
Oxford, Ohio
2004

Advisor____________________________
André Sommer

Reader____________________________
Gary Lorigan

Reader____________________________
Neil Danielson

Reader____________________________
Susan Forest
# Table of Contents

<table>
<thead>
<tr>
<th>Chapter 1</th>
<th>Introduction</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Introduction</td>
<td>2</td>
</tr>
<tr>
<td>1.1.1</td>
<td>Purpose for Renal Calculi Analysis</td>
<td>2</td>
</tr>
<tr>
<td>1.1.2</td>
<td>Methods of Analysis</td>
<td>6</td>
</tr>
<tr>
<td>1.1.3</td>
<td>Treatments</td>
<td>7</td>
</tr>
<tr>
<td>1.2</td>
<td>Infrared Analysis Techniques</td>
<td>8</td>
</tr>
<tr>
<td>1.2.1</td>
<td>Introduction</td>
<td>8</td>
</tr>
<tr>
<td>1.2.2</td>
<td>Transmission</td>
<td>9</td>
</tr>
<tr>
<td>1.2.3</td>
<td>Reflection</td>
<td>10</td>
</tr>
<tr>
<td>1.2.4</td>
<td>ATR</td>
<td>14</td>
</tr>
<tr>
<td>1.2.5</td>
<td>Molecular Microspectroscopy and the Infrared Microscope</td>
<td>16</td>
</tr>
<tr>
<td>1.2.6</td>
<td>Infrared Imaging</td>
<td>19</td>
</tr>
<tr>
<td>1.3</td>
<td>Histology</td>
<td>20</td>
</tr>
<tr>
<td>1.3.1</td>
<td>Introduction</td>
<td>20</td>
</tr>
<tr>
<td>1.3.2</td>
<td>Tissue Staining Procedure Background</td>
<td>20</td>
</tr>
<tr>
<td>1.3.3</td>
<td>Stone Preparation History</td>
<td>23</td>
</tr>
<tr>
<td>1.4</td>
<td>Pathology</td>
<td>23</td>
</tr>
<tr>
<td>1.5</td>
<td>Goals</td>
<td>24</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Chapter 2</th>
<th>A Protocol for the Analysis of Renal Mineral Deposits in Biopsied Tissue Using Infrared Microanalysis</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Introduction</td>
<td>26</td>
</tr>
<tr>
<td>2.1.1</td>
<td>Tissue Analysis</td>
<td>26</td>
</tr>
<tr>
<td>2.1.2</td>
<td>Visual Identification</td>
<td>27</td>
</tr>
<tr>
<td>2.1.3</td>
<td>Histological Preferences</td>
<td>28</td>
</tr>
<tr>
<td>2.2</td>
<td>Materials and Instrumentation</td>
<td>29</td>
</tr>
<tr>
<td>2.3</td>
<td>Results and Discussion</td>
<td>30</td>
</tr>
<tr>
<td>2.3.1</td>
<td>Initial Attempts via Transmission Analysis</td>
<td>30</td>
</tr>
<tr>
<td>2.3.2</td>
<td>Photometric Correctness</td>
<td>32</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>-------</td>
<td>------</td>
</tr>
<tr>
<td>2.3.3</td>
<td>Low-E Slide Homogeneity</td>
<td>34</td>
</tr>
<tr>
<td>2.3.4</td>
<td>Reflection/Absorption Analysis</td>
<td>34</td>
</tr>
<tr>
<td>2.4</td>
<td>Conclusions</td>
<td>39</td>
</tr>
<tr>
<td>3.</td>
<td>Reflection Analysis of Isolated Renal Stones Using Infrared Microspectroscopy</td>
<td>40</td>
</tr>
<tr>
<td>3.1</td>
<td>Introduction</td>
<td>41</td>
</tr>
<tr>
<td>3.1.1</td>
<td>Transmission</td>
<td>41</td>
</tr>
<tr>
<td>3.1.2</td>
<td>Specular and Diffuse Reflectance Techniques</td>
<td>42</td>
</tr>
<tr>
<td>3.1.3</td>
<td>ATR Techniques</td>
<td>42</td>
</tr>
<tr>
<td>3.2</td>
<td>Sample Histology</td>
<td>43</td>
</tr>
<tr>
<td>3.3</td>
<td>Instrumentation</td>
<td>43</td>
</tr>
<tr>
<td>3.4</td>
<td>Results and Discussion</td>
<td>44</td>
</tr>
<tr>
<td>3.4.1</td>
<td>A Comparison of Transmission and ATR Spectra</td>
<td>44</td>
</tr>
<tr>
<td>3.4.2</td>
<td>A Comparison of Specular and Diffuse Reflectance</td>
<td>47</td>
</tr>
<tr>
<td>3.4.3</td>
<td>Reflection Imaging of Kidney Stones</td>
<td>52</td>
</tr>
<tr>
<td>3.5</td>
<td>Conclusions</td>
<td>77</td>
</tr>
<tr>
<td>4.</td>
<td>A Quantitative Study of Renal Stone Components</td>
<td>78</td>
</tr>
<tr>
<td>4.1</td>
<td>Introduction</td>
<td>79</td>
</tr>
<tr>
<td>4.1.1</td>
<td>Quantitative Analysis</td>
<td>79</td>
</tr>
<tr>
<td>4.1.2</td>
<td>Relation to Renal Crystal Analysis</td>
<td>80</td>
</tr>
<tr>
<td>4.2</td>
<td>Experimental</td>
<td>82</td>
</tr>
<tr>
<td>4.2.1</td>
<td>Instrumentation</td>
<td>82</td>
</tr>
<tr>
<td>4.2.2</td>
<td>Sample Preparation</td>
<td>82</td>
</tr>
<tr>
<td>4.2.3</td>
<td>Particle Size Analysis</td>
<td>83</td>
</tr>
<tr>
<td>4.2.4</td>
<td>Reproducibility of the Method</td>
<td>83</td>
</tr>
<tr>
<td>4.2.5</td>
<td>Peak Areas</td>
<td>83</td>
</tr>
<tr>
<td>4.3</td>
<td>Results and Discussion</td>
<td>84</td>
</tr>
<tr>
<td>4.3.1</td>
<td>ATR Calibration Curves</td>
<td>84</td>
</tr>
</tbody>
</table>
4.3.2 Sensitivity of the Method 92
4.3.3 ATR 3% Reproducibility 92
4.3.4 Raman Calibration Curve 95
4.3.5 Unknown Analysis 97
4.3.6 Particle Size 97
4.4 Conclusions 98

A. Reference Library 99
A.1 Introduction 100
A.2 Experimental 100
A.2.1 Instrumentation 100
A.2.2 Methods 100
A.2.3 Calcium Urate 101
A.2.4 Materials 101
A.3 Results and Discussion 101
A.3.1 Reference Library 101
A.3.2 Other Libraries 110
A.4 Conclusions 110
<table>
<thead>
<tr>
<th>List of Tables</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.1</td>
<td>4</td>
</tr>
<tr>
<td>Common Components of Renal Calculi</td>
<td></td>
</tr>
<tr>
<td>Table 4.1</td>
<td>87</td>
</tr>
<tr>
<td>ATR Calibration Data 770-790 cm(^{-1})</td>
<td></td>
</tr>
<tr>
<td>Table 4.2</td>
<td>88</td>
</tr>
<tr>
<td>ATR Calibration Data 1310-1330 cm(^{-1})</td>
<td></td>
</tr>
<tr>
<td>Table 4.3</td>
<td>94</td>
</tr>
<tr>
<td>ATR Reproducibility</td>
<td></td>
</tr>
<tr>
<td>List of Figures</td>
<td>Page</td>
</tr>
<tr>
<td>----------------</td>
<td>------</td>
</tr>
<tr>
<td>Figure 1.1: Diffuse Reflectance</td>
<td>11</td>
</tr>
<tr>
<td>Figure 1.2: The Reflection/Absorption Process</td>
<td>13</td>
</tr>
<tr>
<td>Figure 1.3: IRE/Sample Interaction</td>
<td>15</td>
</tr>
<tr>
<td>Figure 1.4: Infrared Microscope Diagram</td>
<td>18</td>
</tr>
<tr>
<td>Figure 1.5: Stained Tissue Sample</td>
<td>22</td>
</tr>
<tr>
<td>Figure 2.1: Visible and False-Color Images of Tissue Section</td>
<td>31</td>
</tr>
<tr>
<td>Figure 2.2: Transmission Spectra of Tissue Section</td>
<td>33</td>
</tr>
<tr>
<td>Figure 2.3: Stained Tissue Sample on Low-E Slide</td>
<td>36</td>
</tr>
<tr>
<td>Figure 2.4: Human Biopsy Spectra</td>
<td>37</td>
</tr>
<tr>
<td>Figure 3.1: ATR and Transmission Spectra Comparison</td>
<td>46</td>
</tr>
<tr>
<td>Figure 3.2: DRIFTS and Specular Reflectance Spectra Comparison</td>
<td>48</td>
</tr>
<tr>
<td>Figure 3.3: DRIFTS and Reflectance Spectra Comparison for Oxalate Monohydrate</td>
<td>50</td>
</tr>
<tr>
<td>Figure 3.4: DRIFTS and Reflectance Spectra Comparison for Hydroxyapatite</td>
<td>51</td>
</tr>
<tr>
<td>Figure 3.5: Visible Image of Brushite Stone</td>
<td>54</td>
</tr>
<tr>
<td>Figure 3.6: Total Absorbance Map of Brushite Stone</td>
<td>55</td>
</tr>
<tr>
<td>Figure 3.7: False-Color Image Brushite Stone at 1050 cm(^{-1})</td>
<td>57</td>
</tr>
<tr>
<td>Figure 3.8: Brushite Stone and Unidentified Component</td>
<td>58</td>
</tr>
<tr>
<td>Figure 3.9: Visible Image of Cystine Stone</td>
<td>60</td>
</tr>
<tr>
<td>Figure 3.10 Total Absorbance Map of Cystine Stone</td>
<td>61</td>
</tr>
<tr>
<td>Figure 3.11 False-Color Image of Cystine Stone from 1300-1600 cm(^{-1})</td>
<td>62</td>
</tr>
<tr>
<td>Figure 3.12 False-Color of Cystine Stone at 1050 cm(^{-1})</td>
<td>63</td>
</tr>
<tr>
<td>Figure 3.13 Visible Image of Two-Component Stone</td>
<td>65</td>
</tr>
<tr>
<td>Figure 3.14 Total Absorbance Map of Two-Component Stone</td>
<td>66</td>
</tr>
<tr>
<td>Figure 3.15 False-Color Image of Two-Component Stone at 1050 cm(^{-1})</td>
<td>67</td>
</tr>
<tr>
<td>Figure 3.16 False-Color Image of Two-Component Stone at 1600 cm(^{-1})</td>
<td>68</td>
</tr>
<tr>
<td>Figure 3.17 Cluster Image of Two-Component Stone</td>
<td>70</td>
</tr>
<tr>
<td>Figure 3.18 Spectra From Face of Two-Component Stone</td>
<td>71</td>
</tr>
<tr>
<td>Figure 3.19 Visible Image of Oxalate Stone</td>
<td>73</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------------</td>
</tr>
<tr>
<td>3.20</td>
<td>False-Color Image of Oxalate Dihydrate Components</td>
</tr>
<tr>
<td>3.21</td>
<td>False-Color Image of Oxalate Monohydrate Components</td>
</tr>
<tr>
<td>3.22</td>
<td>False-Color Image of Hydroxyapatite Components</td>
</tr>
<tr>
<td>4.1</td>
<td>Spectrum of Human Renal Biopsy</td>
</tr>
<tr>
<td>4.2</td>
<td>ATR Spectra of Hydroxyapatite and Oxalate</td>
</tr>
<tr>
<td>4.3</td>
<td>ATR Calibration Curves</td>
</tr>
<tr>
<td>4.3</td>
<td>Ratio Calibration Curves</td>
</tr>
<tr>
<td>4.4</td>
<td>ATR Spectra of 1 % and 3 % Calcium Oxalate</td>
</tr>
<tr>
<td>4.5</td>
<td>Raman Calibration Curve</td>
</tr>
<tr>
<td>A.1</td>
<td>List of Library Samples and Spectra</td>
</tr>
</tbody>
</table>
Dedication:

To my amazing husband, Nathan, who will always inspire me to be my best, no matter what circumstances I may face.
Acknowledgements

I would like to thank my God, for giving me the opportunity to study at Miami University over the last three years, and my beloved for supporting me even when times were hard. I thank my committee, Drs. Rakovan, Lorigan, Danielson and Forest for their comments and suggestions, and especially thank Andy for his guidance in learning how to write in an intelligible manner and pointing out all of my non-words. I thank Kathy for being the glue of the lab, Luis for checking my grammar, Meghan for keeping me company on some long evenings, and Brian for his abounding wisdom and immeasurable help in learning what the MML is all about.
CHAPTER 1
INTRODUCTION
1.1 Introduction

Although they are the focus of countless studies in many nations, renal stones, their evolution, and diagnosis continue to be a source of angst for many pathologists and patients. Renal stones affect large portions of the population, most significantly among industrialized nations, and cost billions of dollars a year in diagnosis and treatment. [1] In addition, this debilitating condition contributes to loss of profit and productivity in industry, reduced income for families, and places the afflicted individual in extreme physical pain. A patient’s lifestyle, family history, and ethnicity all play a role in the formation of renal stones, though the chemistry of formation has yet to be definitively determined. The health and ages of patients range from the very young to the elderly, with the majority of calculi appearing in Caucasian males. [2-5]

This thesis will encompass work that is aimed at making the analysis and diagnosis of renal stones faster, easier, and more accurate. Methods will be presented that require less training than is currently given for the present-day pathologist, sample preparation methods that require less work on the part of the histologist, and analysis procedures that will greatly reduce the amount of misdiagnoses currently occurring in the field of pathology.

1.1.1 Purpose for Renal Calculi Analysis

The analysis of renal calculi is important for several reasons. First, the chemistry behind stone formation is not well-understood, due in part to the amount of time it takes for stones to develop, which is typically years. Unfortunately, by the time the stones are removed or passed, biochemical studies of the blood and urine will not elucidate the exact causes of stone nucleation. [3] Estepa and Daudon have compiled a listing of possible causes correlated to the nucleus material in a variety of calculi. Though this list is not all-inclusive, many plausible generalizations are presented that give insight and clarification to the possible origins of several categories of renal stones.

Recurring stone formers often repeatedly produce stones containing the same components, allowing the identification of an early stone to dictate the treatment thereafter. The vast majority of stones have a calcium oxalate, uric acid, or hydroxyapatite center with few exceptions. Most of these exceptions are chemically
induced and are not naturally occurring. [3, 6] The majority of renal stone components are formed naturally in the body or are ingested during the course of normal dietary habits. Table 1.1 illustrates the common formulas and names of minerals strongly associated with renal calculi.
Table 1.1  Common components found in renal calculi.

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Formula</th>
<th>Common Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium oxalate monohydrate</td>
<td>CaC₂O₄ ⋅ H₂O</td>
<td>Whewellite</td>
</tr>
<tr>
<td>Calcium oxalate dihydrate</td>
<td>CaC₂O₄ ⋅ 2H₂O</td>
<td>Weddellite</td>
</tr>
<tr>
<td>Calcium phosphate</td>
<td>Ca₃(PO₄)₂</td>
<td>Whitlockite</td>
</tr>
<tr>
<td>Calcium phosphate (mixed anions)</td>
<td>Ca₅(PO₄)₃(OH,F,Cl)</td>
<td>Apatite</td>
</tr>
<tr>
<td>Basic Calcium phosphate</td>
<td>Ca₁₀(PO₄)₆(OH)₂</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>Basic Calcium phosphate carbonate</td>
<td>Ca₅(PO₄,CO₃)₃(OH)</td>
<td>Dahllite</td>
</tr>
<tr>
<td>Calcium hydrogen phosphate</td>
<td>CaHPO₄</td>
<td>Monetite</td>
</tr>
<tr>
<td>Calcium hydrogen phosphate dihydrate</td>
<td>CaHPO₄ ⋅ 2H₂O</td>
<td>Brushite</td>
</tr>
<tr>
<td>Magnesium ammonium phosphate hexahydrate</td>
<td>MgNH₄PO₄ ⋅ 6H₂O</td>
<td>Struvite</td>
</tr>
<tr>
<td>Uric acid</td>
<td>C₅H₄N₄O₃</td>
<td>N/A</td>
</tr>
<tr>
<td>Sodium urate monohydrate</td>
<td>NaC₂H₃N₄O₃ ⋅ H₂O</td>
<td>N/A</td>
</tr>
<tr>
<td>Cystine</td>
<td>C₆H₁₂N₂O₄S₂</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Secondly, over 64% of the American population is considered overweight, 30% of that number being termed obese [7], having a body mass index (BMI) of over 30. [8] In addition, 1 in 50 Americans is termed morbidly obese, and 1 in 400 is super obese, having a BMI of over 40 and 50, respectively. [7, 9] Renal stone growths have recently been studied in conjunction with obesity and its treatment. [9-12] One popular medical treatment for obese patients is a procedure known as gastric bypass surgery. In this process, the stomach is reduced to a much smaller size, resulting in a drastic loss of excess weight; unfortunately, some of the patients that have had this procedure become stone formers. [13]

A third reason the analysis of renal calculi is important is because it is expected that space travel will increase over the next several decades. The formation mechanisms of stones in space travel and microgravitational atmospheres are better understood [14-18], in contrast to other situations of renal stone development, where the chemistry behind the formation remains obscure. The effects of microgravity on the human body have been found to increase desorption of calcium salts from the bones, creating a urinary state of saturation [19]; urinary volume, citrate intake, and pH are decreased during space flight, sharply increasing the potential to form renal stones. As longer space missions become more routine, understanding the chemistry and conditions for renal stone development in microgravity situations will become critical.

Lastly, the current analyses of renal calculi are often incorrect, with estimations of misdiagnoses being as high as 40%. [20, 21] Subjectivity on the part of both the histologist and pathologist contribute to this large number, as well as non-selective staining procedures and methods of renal stone component identification that have changed little over the past several decades.

These statistics are important in that they emphasize the necessity of this research. Through the research conducted here, the potential exists to have a more accurate and faster method of analysis for both tissue-embedded crystal deposits and isolated renal calculi that is both highly reproducible and easy to perform. Not only will this procedure save money, it will also save time and training, and will increase the accuracy of diagnosis in the field of pathology.
1.1.2 Methods of Analysis

Many methods of renal stone analysis involve the destruction of the stone or tissue sample due to histological preferences or instrumental limitations. One of the most common methods of calculi analysis is X-ray diffraction (XRD). XRD involves the diffraction of penetrating X-rays due to unique crystal structure of the sample, and has the potential to detail information such as phase identity, crystal size, texture, strain and defects, as well as other structural parameters. Though XRD is a non-destructive technique, calculi samples are often ground prior to analysis so as to ensure a reading that represents the entire calculus and not only the top 0.01-0.1mm. [22]

The benefits of XRD include spectra that are relatively simple and are independent of excitation conditions and chemical state. XRD does have some drawbacks, however, including the shallowness of the X-ray penetration, and spectra that can suffer from interelement effects that may be substantial and require computer correction. [22]

Infrared analysis is also very common in the analysis of calculi and embedded mineral deposits. Preparation of the sample often includes the grinding of the stone with a mortar and pestle and the subsequent mixing with KBr to form a pellet. Because KBr is transparent in the mid-infrared region (MIR), only the stone component is responsible for the absorption bands observed in the spectrum. The obvious drawback to this technique is the destruction of the sample.

A previously conducted study compared XRD and infrared (IR) data against wet chemical methods to determine the composition and quantity of components in renal stones. [23] This study found that XRD worked well for differentiating small differences in the forms of urate, but was less than adequate for determining minor constituents. Infrared analysis was found to be superior for physically small samples and easily identified minor components in calculi. Both IR and XRD were found to be more accurate and time-favorable than wet chemical methods.

Carmona, et al. [24] published a review of both infrared and Raman spectroscopy of urinary calculi that goes into great detail regarding the absorption bands seen in individual calculi spectra. Likewise, many other authors have also published critical data in the area of stone analysis. [23, 25-27] As important as these reports are, the old
tradition of pulverizing the stone and mixing it with KBr or a similar salt is continued. The majority of the above research is time-consuming and is performed in a macroscopic state. In contrast, our analysis relies on in-situ reflectance microspectroscopic infrared techniques, which will be shown not only to be less time consuming and more accurate, but can also provide information as to the beginning stages of nucleation as well.

Renal stone components are often embedded in a tissue matrix, and are referred to as crystal or mineral deposits. A thin section of the tissue sample is often sandwiched between two alkali halide or barium fluoride windows for analysis. This mounting method allows the sample to remain flat over an area of approximately 1cm².

Diem et al. have written a series of articles on the infrared spectroscopy of human cells and tissues, and of the most interest to this research are issues VII and VIII on the infrared microanalysis and IR-mapping of liver tissue. [28, 29] In another procedure, Ouyang et al. used infrared microspectroscopic imaging to determine fracture healing in rat femurs. [30] This work is similar to ours in that the authors imaged a mixed sample containing both soft tissue and hardened mineralized portions, producing very high quality infrared images. Though these three reports contain information and techniques similar to our own, there are several differences regarding procedures between the above articles and the research presented here. The first of these differences is that the tissue samples of two of these reports [28, 29] do not contain mineralized deposits embedded in the tissue. Secondly, the tissue samples in these three particular publications have been stained. [28-30] A third and more significant difference is the substrate used with these methods of analysis. For the imaging of tissue sections, the authors mounted the tissue section between two infrared-transparent windows, while in the present study, infrared-reflective slides are employed.

1.1.3 Treatments

It is estimated that the total costs of evaluation, treatment, and outpatient care due to renal stones in the United States are approximately $1.83 billion annually [1], with the treatment of stones absorbing the majority of costs. The treatment and removal of renal calculi is often dependant upon the composition and hardness of the stone, as well as stone size and placement in the urinary tract. Though the main components of renal
stones tend to be calcium oxalate, calcium phosphate, and uric acid (see Table 1.1),
calculi with up to 85 components have been previously analyzed. [3]

Renal stones are treated through several different techniques, two of the more
well-known methods being extracorporeal shockwave lithotripsy (ESWL) and surgical
removal. Unfortunately, ESWL causes renal tissue trauma and hemorrhaging with
virtually every treatment [24], while surgical methods are invasive and require a longer
recovery time. Other popular methods of treatment include percutaneous
nephrolithotripsy, nephrolithotomy, ureteroscopy, and natural passing.

ESWL subjects the patient to a series of intense ultra-sonic pulses directed at the
stone position in the urinary tract. The depth of the pulse that passes through the body is
determined by the scope of the pulse: broad pulses have a larger field of effectiveness but
are not as powerful, while a more focused pulse is more powerful but also more difficult
to direct to the exact location of the renal stone. In addition, the more narrow pulses tend
to injure internal tissue faster and more dramatically than the broader pulses. [20]

Percutaneous nephrolithotripsy (the shattering of stones) and percutaneous
nephrolithotomy (the removal of stones) involves the insertion of a narrow endoscope
through the back of the patient into the kidney in order to break up or remove the stone,
respectively. This procedure is often used when ESWL has failed and is usually
attempted prior to surgery, which is considered a last resort due to extended recovery
time. Ureteroscopy is similar to percutaneous nephrolithotomy, but in this case, the
endoscope is inserted through the urethra instead of through the back of the patient.

1.2 Infrared Analysis Techniques
1.2.1 Introduction

The source of energy for an infrared system is a broadband source, meaning that a
large range of wavelengths are incident upon the sample during analysis. Infrared
wavelengths, from 2.5 to 25 µm, may be absorbed by molecules in the sample. As the
incident electromagnetic radiation impinges upon the sample, certain wavelengths are
absorbed, corresponding to specific harmonic vibrations of covalent bonds within the
molecule. The absorption of specific wavelengths produces a unique fingerprint for each
molecule based on such nuclear displacements. This absorption is detected by the
detector and is proportional to the Beer-Lambert equation (equation 1.1), which states that the absorbance is a function of the extinction coefficient $\varepsilon$ (Å cm$^2$g$^{-1}$), the path length of radiation $b$ (cm), and the density of the sample, $\rho$ (g/cm$^3$). [31] The absorption, $A$ in equation 1.1, is also defined as the log of the power of the incident radiation divided by the power of the transmitted radiation.

$$A=\varepsilon b \rho$$  \hspace{1cm} (1.1)

1.2.2 Transmission

When a beam of radiation is transmitted through a medium, the energy of the beam is attenuated as the direct result of several factors. Energy is lost both as it enters and exits the sample in a reflection off a phase boundary as the result of differences in the refractive indices of the medium and its surroundings. Energy is also lost due to the scattering and absorption that takes place by the sample itself. All of these losses of radiation combine to yield a transmitted beam of energy less intense than the incident beam. However, absorption by the sample should be the only mechanism responsible for loss of radiant intensity. Great care must be taken to ensure that the other components (reflection, scattering) are in most cases negligible, should one want to attempt a quantitative analysis. [22]

In transmission mode, the sample must be freestanding or placed on a non infrared-absorbing substrate, such as a KBr, KCl, or BaF$_2$ window. The path length of the radiation is considered to be the sample thickness since the angle at which radiation enters the sample is at or near normal incidence.

Transmittance, related to absorbance through equation 1.2, is the usual manner in which infrared spectra are displayed for qualitative comparisons. In any infrared analysis, it is desirable to have the transmitted bands registering between 80 and 20 %T. Bands producing an absorbance lower than 20 %T indicate that the sample is too thick, and bands less than 80 %T indicate that the sample is too thin. Reproducibility in the thickness of hand-pressed samples is difficult due to the micrometer-level thicknesses required for photometrically accurate transmission analysis.

$$A=-\log T$$  \hspace{1cm} (1.2)

Here, $T$ is equal to the ratio of the power of the transmitted energy to the incident energy.
1.2.3 Reflection

Four types of reflection techniques are referred to in this thesis: specular, diffuse, reflection/absorption (R/A), and attenuated total internal reflection (ATR). Both specular and diffuse reflectance can be employed for the analysis of renal stones, however, each method requires that the sample have specific properties. For specular reflectance, the surface of the stone must be highly polished and optically flat. [32] In specular reflectance, the angle of the incident radiation \( (\theta_i) \) equals that of the reflected radiation \( (\theta_R) \) according to Snell’s Law.

\[
\sin \theta_i = \sin \theta_R
\]

For most materials, only 5-10% of the energy is reflected from the surface. Specular reflectance spectra exhibit absorptions resembling the first-derivative of a normal absorption band, though computer corrections can be applied to the spectrum to make it appear as a normal infrared transmission spectrum.

Diffuse reflectance requires that the sample be a highly scattering powder with individual crystal sizes that approach that of the wavelength of radiation being employed in the analysis. Radiation enters the sample and undergoes a complex path through the sample, which can involve reflection, refraction, scattering, and absorption, as is shown in Figure 1.1 [33]; there is no angular relationship between the incident and diffusely scattered electromagnetic radiation. As the crystal size increases, the surface of the sample appears more flat and smooth, increasing the amount of specular reflection.
Figure 1.1  Diffuse Reflectance
Diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) is a technique often used in pharmaceutical analysis and industry to determine enantiomeric purity and homogeneity of active drugs dispersed in benign excipients. [34-46] The field is well established, and several quantitative methods using this technique have been approved by the Food and Drug Administration (FDA). The DRIFTS process involves reflected radiation that is independent of the incident angle, meaning that the intensity of the energy reflected from the sample is identical no matter what angle the sample is viewed from. [33] This lack of dependence on Snell’s Law arises from the processes occurring in Figure 1.1.

DRIFTS has been used as a tool for the analysis of urinary calculi with some success. [47] Though the method produced quantitative results, the preparation of the calculi samples was time consuming. In the above report [47], the calculus was dissected layer by layer; this process often leaves small amounts of stone for analysis, and in the event of error, may not provide enough material for an accurate study.

Several problems potentially exist when a sample is ground and ratioed with KBr for DRIFTS analysis. Some of these complications can include bromine ion exchange with sample components, or water absorption by the KBr, which is hygroscopic to a certain degree. [47] However, when the sample is correctly prepared and analyzed under proper conditions, these interferences are minimized.

For the tissue research presented here, a technique termed reflection/absorption (R/A) analysis was used. This method is also referred to as reflection-transmission or trans reflectance. In this method, a reflective substrate or low-energy (low-E) glass slide is utilized. When a sample is mounted on the reflective substrate, the path of the incident radiation proceeds through the tissue twice and reflects off the surface of the substrate once. This process allows the path length of electromagnetic radiation to be approximately doubled, which can enhance the absorbance for thin films. A diagram of the reflection/absorption process is given in Figure 1.2. A doubling of the path length results in a larger absorbance, theoretically increasing the signal while maintaining the same level of noise. However, for highly absorbing materials, a path length that is too long can create absorption bands that are photometrically inaccurate, as was discussed earlier.
Figure 1.2  Infrared electromagnetic radiation as it enters a sample is refracted and reflected and exits the sample in a reflection/absorption (R/A) process.
1.2.4 ATR

The final reflectance method discussed in this section, attenuated total internal reflection (ATR) analysis, involves the use of an internal reflection element (IRE), and an evanescent wave penetrating the sample to a depth that is related both to the wavelength of the radiation and the angle at which it is incident upon the sample. This direct-contact method of analysis allows for photometrically accurate spectra to be collected with minimal sample preparation. However, it also carries with it the slight potential to damage the sample.

Attenuated total internal reflection (ATR) spectroscopy is a technique in which a single crystal internal reflection element (IRE) of a high refractive index material in crystalline form is placed into intimate contact with a sample (see figure 1.3). IR radiation is passed through the crystal and is nearly totally internally reflected. The radiation internally reflects inside the crystal as long as it is at an angle greater than the critical angle of the IRE, \( \theta_c \), which is given by equation 1.4.

\[
\theta_c = \arcsin \left( \frac{n_s}{n_c} \right)
\]  

(1.4)

In the above equation, \( n_c \) is the refractive index of the crystal and \( n_s \) is the refractive index of the sample.
Figure 1.3  The path of infrared electromagnetic radiation as it interacts with the sample and is internally reflected inside the ATR germanium (Ge) IRE.
The small fraction of energy that is not internally reflected, termed the evanescent wave, penetrates into the sample to a depth $d_p$, which is similar to the path length discussed in the previous section. This depth of penetration is determined by equation 1.5.

$$
d_p = \frac{\lambda}{2\pi \sin^2 \phi - \left(\frac{n_s}{n_c}\right)^2}^{1/2}
$$

(1.5)

Here it can be seen that the depth of the evanescent wave is increased by the wavelength of the radiation, but is reduced by increasing the angle of the incident beam, $\phi$. In addition, the depth is increased as the ratio of the refractive index of the sample ($n_s$) to the crystal ($n_c$) decreases. [48] A more extensive discussion about ATR analysis, also involving ATR point-by-point imaging, takes place in section 1.2.6.

The ATR technique is very popular because the sample can vary in thickness and does not need to be scattering or optically smooth in order for a spectrum to be collected. An even larger advantage of this technique is the lack of sample preparation needed to obtain quantitative and qualitative information. However, because the depth of the evanescent wave varies with wavelength, the absorption will also vary. Thus for comparison of an ATR spectrum with that of a reflection or transmission spectrum, it is necessary to perform an ATR correction, which corrects the band intensities and compensates for a greater penetration depth (path length) at larger wavelengths. [48]

1.2.5 Molecular Microspectroscopy and the Infrared Microscope

Molecular spectroscopy differs from atomic spectroscopy in many respects. In atomic spectroscopy, a determination of the atoms present is the goal; information on how the atoms are oriented or linked to each other can be difficult to determine. Molecular spectroscopy, or the determination of molecular groups, yields data concerning molecular structure. Molecular groupings characteristically absorb energy at certain wavelengths in the infrared region, allowing a rapid determination of common functional groups, such as carbonyls, phosphates, and amines. Molecular microspectroscopy is the coupling of molecular spectroscopy with microscopy, creating a powerful analytical tool useful in countless areas of industry and research.
In the past, standard microscopy techniques have been used to prepare the sample, define the area of interest, and determine what parameters will be included in the analysis. Spectroscopy was then typically used to determine the chemical composition and form of the analyte. In microspectroscopy, however, these two methods are indistinguishable: spectroscopic imaging can be used to isolate an area of the analyte in question using an adjustable aperture, and areas of interest can be determined by obtaining a spectral map of the specimen.

J.A. Reffner writes that microscopy is the science of creating, recording and interpreting magnified images, while analytical spectroscopy is the science of emission, reflection, and absorption of radiant energy to determine the structure and composition of matter. [49] Microspectroscopy, a complementary combination of microscopy and spectroscopy, has the advantages of both techniques with few limitations. A diagram of a standard infrared microscope is presented in Figure 1.4.
Figure 1.4  A schematic of an infrared microscope.
The infrared microscope can be used in several different sampling modes: transmission, diffuse reflection, specular reflection, and ATR. The instrument specifications and sampling modes will be discussed further in each section of this thesis relative to the analysis of different sample types.

As with all microscopes, the infrared microscope is limited by diffraction through the wavelength of energy that is employed for the analysis. The smallest sample size that can be analyzed without artifacts is given by equation 1.6 below.

\[ d = \frac{1.22\lambda}{n \sin \theta} \]  

(1.6)

In equation 1.6, the sample size or beam waist is represented by \( d \), \( n \) is the refractive index of the medium in which the measurement is conducted, and \( \lambda \) is the wavelength. The value of \( \theta \), the angle of the incident radiation from the normal, is set by the manufacturer; in most cases, \( \sin \theta = 0.6 \). For transmission and reflectance measurements, the beam waist approximates \( 2\lambda \) and \( 4\lambda \), respectively.

1.2.6 Infrared Imaging

Infrared imaging refers to the generation of images that are for specific molecular absorptions. This process is often referred to as mapping or quilting, and can be performed in all three instrumental modes: transmission, reflection, and ATR.

Two modes of imaging on the infrared microscope are serial (point-by-point) and parallel imaging. The serial mode involves a single-point detector that collects an infrared spectrum at every point over the entire designated area of the sample. This process yields a high signal to noise, but is significantly slower than the parallel imaging mode. In parallel imaging, a linear or 2 dimensional detector collects radiant information (i.e. spectra) from multiple points on the sample at the same time. For linear arrays, 16 spectra can be simultaneously collected, whereas with a focal plane array detector, \( 256^2 \) spectra can be collected at any given time. Both the wavelength range and the signal to noise are smaller with the array, but the sampling time is greatly reduced. Examples of transmission imaging are many, and yield excellent results both qualitatively and quantitatively. [21, 29, 30, 50-67]
Infrared mapping in reflection mode has been performed with excellent results, and has specific applicability in the biological and pathological sciences. [68] This method of analysis is not as common as transmission mapping, and is the focus of Chapter 2.

ATR, the last mode of imaging in infrared discussed here, also produces a map by a point-by-point process. [69] The drop-down Ge IRE comes into intimate contact with the sample, as was described in section 1.2.4. This method of imaging produces possibly the best and most photometrically correct set of spectra, with the least amount of sample preparation of any other method. However, the technique is burdensome in the fact that the IRE must be physically lifted after each scan and placed at the next sampling point by the user. In addition, there remains the possibility that particles from the sample may adhere to the IRE, contaminating other sections of sample and yielding false absorbance bands. ATR point-by-point imaging has been performed previously on soft samples. [69]

An alternative ATR imaging mode has been suggested that incorporates a macro hemisphere into an infrared imaging microscope. [70] In this method, the IRE is securely in contact with the sample, with the infrared electromagnetic radiation scanning off-axis to produce an infrared spectral image map. Due to the refractive index of the crystal, the sample experiences a greater magnification, however, the area that can be imaged is limited to the size of the crystal.

1.3 **Histology**

1.3.1 **Introduction**

Histology is the anatomical study of microscopic structures of tissue, and a general knowledge and understanding of the histological process is pertinent to this research. Histology in the context of this thesis deals with the preparation of the tissue and stone samples, and does not apply to the interpretation of results or the application of treatments to patients.

1.3.2 **Tissue Staining Procedure Background**

A common staining method for tissue sections is the H&E (Haematoxylin and Eosin) procedure. This method has approximately ten steps, depending on the level of
staining needed, many of which include intricate timing in order to achieve a properly prepared sample slide. To maintain the integrity of the sample, the slide must be passed thorough a series of liquids during the course of dehydrating, embedding, mounting, and staining, all of which require attention to detail and a high level of involvement by the histologist.

Dehydration of the tissue section entails passing the tissue through a series of alcohol/water gradients taking a minimum of 3.5 hours, all the while increasing the alcohol to water ratio. [71] The sample is then drained and cleared. Clearing agents are non-polar liquids that are miscible in alcohols but are immiscible in water. Clearing, in this case the removal of the alcohol product from the slides, involves passing the slide through at least two changes of clearing agent, usually xylene or toluene. Afterwards, the sample can be infiltrated with wax through three 30-minute sessions of wax immersion, and may remain in this state until slicing and staining. The sample can then be de-waxed and hydrated via steps outlined elsewhere. [71]

At the stage of preparation where the staining procedure takes place, personal preferences and judgments are often used by the histologist as to the amount of contrast needed for each sample type. The staining process produces a contrast between tissue areas with different properties, or between proteinaceous tissue and mineralized deposits. Quite often, the diagnosis of a patients’ renal stone relies solely on the staining outcome of the tissue section, as well as the interpretation and experience of the pathologist.

Figure 1.5 displays a section of tissue that has been stained with Yasue stain [72], a stain similar to H&E that is preferential for calcium phosphate. The dark areas toward the center are the crystal deposits that are embedded in the tissue. This sample was one of the controls for the research presented in this thesis.
Figure 1.5  Section of stained tissue mounted on glass substrate. The black areas towards the center and the bottom right of the tissue are crystal deposits.
1.3.3 Stone Preparation History

Many studies have been performed on renal stones and large embedded mineral deposits by infrared analysis, the vast majority of which use the KBr pellet technique. [11, 23, 25-27, 73, 74] Though this process has been a standard method for renal calculi component determination in the past, it involves time-consuming and sometimes difficult sample preparation. In addition, the sample is often completely destroyed via grinding during the sample preparation process, therefore limiting the amount of study that can be performed on a particular sample.

Regardless of whether or not the isolated stone is cross-sectioned or ground prior to analysis, the same steps of dehydrating, clearing and embedding the sample must be performed on the stone. The stone must be dehydrated in the same fashion as tissue samples: any residual water will negatively influence the amount of infiltration by the polymer, weakening the stone and making it more susceptible to breakage or crumbling during the course of slicing or analysis. The process of embedding the stone in resin is relatively straightforward, and involves a polymerizing agent to create a resin hard enough to keep the stone intact during the act of slicing. [71]

The analysis of cross-sections of renal stones is the focus of Chapter 3, and alternative methods of analysis and preparation will be discussed further in that chapter.

1.4 Pathology

While histology is the science of organizing and preparing tissue or renal stone sections, pathology is the science of understanding and analyzing the sample. The precise pathology of tissues is just as much of an art as it is a science, and the visual identification of renal stones or renal crystal deposits has led to many misdiagnoses in the past. [20, 21]

In many hospitals and clinics, visual diagnosis is the preferred, and often only, method of determining the composition of renal calculi or crystal inclusions. A tissue sample that has been stained or dyed produces a contrast of shade or color between hardened mineral deposits and the surrounding tissue. These differences in color can vary from subtle to drastic, and it is the job of the pathologist to correctly interpret and diagnose what these contrasts in color indicate. Using a different percentage of stain or
altering the time a sample remains in the alcohol for a dehydrating stage affects the
differentiation ability of the stain and can produce either stronger or weaker counter
stains, depending on the alterations made. Several other examples exist where procedural
differences in the process produce slight variations in the result. [71]

1.5 Goals

The goal of this thesis is to present methods of analysis that are highly qualitative
in nature. It is hoped that the procedures outlined in the following chapters successfully
reduce the possibility of calculi misdiagnosis, while simultaneously easing the burden of
sample preparation by the histologist.
CHAPTER 2
A PROTOCOL FOR THE ANALYSIS OF RENAL MINERAL DEPOSITS IN BIOPSIED TISSUE USING INFRARED MICROANALYSIS
2.1  Introduction

For well over 160 years, disease detection in biopsied tissue has relied on the painstaking preparation of thin sections followed by contrast staining to visibly signal the presence or absence of disease. Subsequent to this preparation is the interpretation of the visible and chemical data by a trained pathologist. For most tissue biopsies, thin sectioning and contrast staining procedures are considered routine. The study of mineralized crystals in renal biopsies presents a challenge in that two distinct phases are present in the same thin section. The first is a relatively soft gelatinous material that comprises the tissue itself; the second is a hard mineral phase, namely the renal crystal deposits. As such, great care must be taken in preparing the thin section so that the hard mineralized portion remains intact during the sectioning and multi-step preparation of the sample. In addition to these challenges, the subsequent diagnosis can be highly subjective.

This chapter will present a protocol of analysis for mineralized materials embedded in tissue sections that uses readily or easily obtainable materials and instrumentation. The selectivity of infrared microspectroscopy allows tissue sections to remain unstained, alleviating the tedious and time-consuming constraints of earlier methods of visual analysis. The method presented in this chapter will save time and training, while simultaneously offering an unbiased analysis of mineralized components that is more accurate and conducive to patient treatments than previous methods.

2.1.1  Tissue Analysis

In recent years, several studies have shown that infrared microanalysis is useful in the detection of disease states in thin sections of biopsied tissue. [56, 75-78] Infrared markers have been identified for several diseases, including cirrhosis of the liver, breast cancer and lung cancer. [28, 79, 80] Infrared absorption spectroscopy provides a unique molecular fingerprint of the material being studied. Due to the inherent selectivity of the method, the potential exists for the multi-step staining procedure of tissues to be eliminated altogether. Most of the aforementioned studies have employed transmission infrared microanalysis, though here as well the study of renal crystals presents a challenge. While the tissue can be optically thin and infrared transmitting, the
mineralized deposits are optically dense, non-transmitting, and scattering. These characteristics have led us to develop a protocol for their analysis, which is the subject of this investigation.

2.1.2 Visual Identification

The analysis of renal crystal deposits is important for several reasons, including the growing rate of obesity, space travel, and the rate of misdiagnosis as discussed in section 1.1. Though there are many reasons for the analysis of renal stones, the methods used to determine constituents are often tedious or inadequate. These shortcomings continue to be a source of uncertainty in component identification for many pathologists. The visual identification of renal crystal deposits using stained tissue samples is one method of crystal identification commonly used today. Haematoxylin and Eosine (H&E) stains remain the most popular contrast stain, though more accurate information is usually accessible through other staining combinations. [71] Staining provides contrast between hardened areas of, for example, calcium phosphate, and the softer areas of tissue. Though some stains have the ability to specifically detect certain elements in the crystal deposit, renal crystals often have several main components that are similar in elemental composition, making differentiation based on elemental composition alone difficult.

Several problems exist in the determination of mineral deposit components using a visual identification method, the foremost of these being the subjectivity of both the histologist and the pathologist. The process of staining tissue samples is long and tedious, yielding ample opportunity for slight variations in the staining time, and thus, the staining outcome. Histology is often touted as being just as much an art form as a scientific process. Though the staining process is much more streamlined than 160 years ago, the many steps of dehydrating, clearing, fixing, staining and interpreting the sample add together to create a long process in which personal subjectivity influences the final product.

Infrared microspectroscopic imaging on tissue sections has been previously performed with very positive results. [28, 29, 53, 58, 61, 81-83] Some of this work is similar to ours and deals with embedded calcified tissue [30], but there remain many
differences between the work presented in this thesis and the previously published reports. For instance, the substrates used varies widely, but as of yet does not include the use of low-energy slides for mineral inclusion analysis.

2.1.3 **Histological Preferences**

One of the difficulties with traditional staining procedures is that histologists prefer to mount stained tissue sections on glass microscope slides, which absorb strongly in the mid-infrared region (MIR), and thus, are not conducive to infrared analysis. In contrast, infrared microspectroscopists prefer alkali halide windows that are transparent in the MIR region. These differential preferences require the histologist to prepare two thin sections; the first section is mounted on glass for pathological analysis, while the other is mounted on an infrared transparent window for infrared analysis. This parallel procedure is time consuming and carries with it the potential to damage the tissue and render it useless. In addition, infrared windows are costly. If a new method is to be accepted by practicing histologists and pathologists, it must employ technology that is traditionally accepted.

The research presented here incorporates a Low-Energy (low-E) reflective slide substrate that has similar characteristics to a common microscope slide. The histologist prepares the section in the traditional manner, but mounts the tissue upon the reflective slide. This same reflective slide can be employed for visible as well as infrared analysis on a single tissue section. When combined with infrared analysis, this practice allows fast and easy examination of mixed samples, omitting the process of staining tissue samples while allowing the analysis and identification of both tissue and embedded crystal deposits. Both reflection/absorption (R/A) as well as attenuated total internal reflection (ATR) analysis of the tissue and embedded material can be performed on the low-E substrate, yielding qualitative, unbiased, and accurate information about the mineralized stone components and the embedding tissue media.
2.2 Materials and Instrumentation

Tissue sections, both stained and unstained, were analyzed using a Perkin-Elmer Spectrum Spotlight 300 infrared imaging microscope. This microscope has a 16 X 1 array detector for the rapid acquisition of molecular images, and a 100 X 100 μm single point detector for the acquisition of high signal to noise spectra with a lower wavenumber cutoff of 580 cm$^{-1}$. Both detectors are based on the well-established mercury cadmium telluride (MCT) technology. The minimum sample size that can be analyzed using either detector is approximately two wavelengths (ca. 6 micrometers). [68] The majority of spectra presented in this report were collected using the single point detector and represent the average of 64 individual scans collected at a spectral resolution of 4 cm$^{-1}$. The microscope can be operated in transmission, reflection or attenuated total internal reflection (ATR) modes. In this latter mode, a drop-down Ge (n=4) internal reflection element (IRE) was employed. A 50 X 50 micrometer confocal aperture was employed to isolate the sample region of interest for the transmission and reflection modes (see section 1.2.5 on confocal microspectroscopy). The same aperture was employed for the ATR mode, however, the Ge IRE provides an additional 4X magnification due to the refractive index of the crystal [69, 70, 84], resulting in a sampling area of ~ 13 X 13 micrometers.

The substrate used in this investigation was a low-E glass slide (Kevley Technologies, Chesterland, Ohio). [85] The slides have the same dimensions as those of standard glass microscope slides, making the mounting of tissue samples routine for histologists.

Tissue, mineralized deposits, and stone samples were obtained from the Indiana University Medical School with the informed consent of the patients and were prepared by a certified histologist. The patients varied in age, symptoms and backgrounds. The unstained tissue sections on the low-E substrates were imaged using the visible CCD camera and frame grabber on the Spectrum Spotlight. A serial section stained with Yasue silver replacement stain was employed as a control to confirm the location of the calcium. [72] This serial section was reviewed using a standard visible microscope in an attempt to visually determine the general area of the mineralized crystals of interest.
2.3 Results and Discussion

2.3.1 Initial Attempts via Transmission Analysis

Initial attempts to study the tissue sections involved the use of transmission infrared microspectroscopy because this method typically yields the best results when the long-term goal includes quantitative analysis. However, this mode requires extensive sample preparation to ensure that the sample thickness is on the order of two to six micrometers. This thickness yields infrared spectra of proteinaceous materials in which the minimum transmittance is no less than 20%, a requirement for quantitative analysis and photometrically accurate band intensities. Further, the sample must be mounted flat on an infrared transparent window in order to avoid sloping baselines or artifacts in the spectrum. Most transmission analysis of tissue samples involves mounting a wet or dry section between two barium fluoride (or similar) windows [28-30, 53, 58, 61, 81-83, 86], which are transparent from 50,000 down to 750 cm\(^{-1}\). [87] Barium fluoride is hard and non-hygroscopic, unlike alkali halide windows such as NaCl or KBr. Mounting the tissue between two windows in a low compression cell ensures that the sample remains relatively flat over an area of approximately 1 cm\(^2\).

Figure 2.1 displays both a visible image and a false-color infrared image of a 4µm thick tissue section that has been embedded in paraffin and compressed between two BaF\(_2\) windows. The patient had been previously diagnosed as a calcium oxalate stone former, though currently, infrared analysis classifies the mineralized deposits to be calcium phosphate.
Figure 2.1  Visible (top) and false-color image (bottom) of a section of tissue mounted between two BaF₂ windows. The blue areas indicate a non-absorbing species (the tissue), while the green, yellow and red areas indicate a highly absorbing species (the embedded mineral deposit.)
Transmission spectra were collected from the boxed area on the visible image in Figure 2.1 to produce the false-color infrared image on the right. The false-color image is made up of approximately 4000 spectra, which were collected in parallel during a 15-minute experiment. This image is based on the peak height of the asymmetric stretching vibration (1050 cm\(^{-1}\)) of the ortho-phosphate group associated with hydroxyapatite. The image exemplifies the benefit of infrared imaging for the study of tissue. In addition to eliminating the staining procedure, interpretation of the results is much less subjective than current methods of crystal deposit identification.

The major drawback to the transmission mode of analysis is the extensive sample preparation and the introduction of substrate materials that are considered foreign to the histologist. In order for a method to be quickly accepted by the medical community, it should employ methods that are commonly practiced in that community. In addition, barium fluoride windows are expensive, somewhat brittle, and are commonly furnished as round disks with a diameter of 13mm. and a thickness of 2mm. Although micro-arrays of tissue sections deposited on barium fluoride have been developed [88-93], they can be very costly and time consuming to prepare. Finally, barium fluoride is only transparent down to 750 cm\(^{-1}\). The region below this is sometimes useful for further differentiation of mineralized deposit components.

2.3.2 Photometric Correctness

In the context of the present study, mineral deposits present a problem in that they absorb strongly and scatter electromagnetic radiation, which ultimately affects the results and potential quantitative capacity of the method. Figure 2.2 illustrates the spectra of a crystal (top) and tissue (bottom) extracted from the infrared image. The spectrum of the tissue is photometrically accurate, whereas that of the crystal is not. The feature (1050 cm\(^{-1}\)) associated with the hydroxyapatite is totally absorbing, exhibiting percent transmission values that approach zero. In addition, the entire spectrum has a positive sloping baseline going toward lower wavenumbers. These artifacts arise from the strong absorbance of the deposit and the propensity for scattering, respectively. It should be noted that as the size of the crystal decreases, scattering becomes more problematic, which could make identification difficult.
Figure 2.2  Spectra of the crystal deposit (top) and tissue (bottom) taken from the transmission analysis of Figure 2.1.
Strong negative transmission bands can be noticed in the spectra of both the crystal and tissue near 2900 and 1450 cm\(^{-1}\). These are due to uncompensated matrix material. The embedding material is a hydrocarbon, which absorbs near 2900, 1450 and 720 cm\(^{-1}\).

2.3.3 Low-E Slide Homogeneity

Following the analysis of tissue sections using a transmission process, reflection/absorption measurements were conducted using the low-E substrate. [85] These slides have similar physical characteristics as conventional glass microscope slides with the exception of a thin three-layer reflective coating on the surface of one side. Tissue analysis using low-E slides has been performed previously with much success. [94]

The homogeneity of the low-E substrate was tested by spin coating a thin film (approximately 0.6 micrometers) of poly(methylmethacrylate) on the surface of the substrate. Six slides taken from two different batches produced by the manufacturer were spin-coated, with the resultant films exhibiting a faint purple hue. The peak area of the carbonyl (C=O) absorption from 125 infrared spectra in each map collected over a \(~4.0 \times 4.0\) mm\(^2\) area for each slide was determined. The average relative standard deviation of the peak area was less than 10.7% over the mapped area. These results demonstrate that the reflection properties of the low-E slide are homogeneous over an area of 4.0 \times 4.0\) mm\(^2\), which should be sufficient for most histological analyses.

2.3.4 Reflection/Absorption Analysis

Figure 2.3 illustrates the visible image of a stained tissue sample mounted on the low-E glass slide, while Figure 2.4 illustrates infrared spectra obtained on a stained mineral deposit using R/A and ATR sampling modes. Based on infrared features observed in the R/A spectrum, it appears as though the deposit is comprised mostly of tissue. Again, the sloping baseline indicates the presence of scattering. The most prominent features in the infrared spectrum are the N-H asymmetric stretch and the amide I and amide II bands located at 3250, 1650 and 1550 cm\(^{-1}\), respectively. All of these features are characteristic of protein. Upon closer inspection, the spectrum
reveals an inverted absorption located near 780 cm$^{-1}$. This feature is present in all R/A spectra of the deposits collected from this particular series of samples, and is absent in spectra of the tissue. The patient had previously been diagnosed with a calcium oxalate stone; since calcium oxalate has a feature near the absorption in question, the presence of the inverted band was employed as evidence for the presence of calcium oxalate. The negative absorption arises from anomalous dispersion, which is caused by increased Fresnel reflection near strong absorption bands. The spectrum demonstrates that R/A is a viable method in these particular cases.
Figure 2.3 Visible image of a stained tissue section mounted on a low-E slide. The black areas are the embedded crystal deposits.
Figure 2.4  Spectra comparing results from R/A and ATR analysis of a stained human biopsy.
Although the R/A spectrum is useful from a qualitative perspective, quantitative considerations exemplify two shortcomings. First, in the reflectance mode, the optical path length is approximately double when compared to the transmission mode (see Figure 1.2). It is approximately double since the electromagnetic radiation must pass through the sample, reflect from the substrate, and pass back through the sample again before reaching the detector. As such, the thickness of the section should be one-half that required for transmission should any quantitative analysis be attempted. Second, scattering and reflection artifacts are still present, which could significantly affect the interpretation of results. Finally, the identification of calcium oxalate should be based on more than one feature, even if it is a well-behaved and reproducible one as in the present case presented here.

In contrast to the R/A spectrum, Figure 2.4 also presents a spectrum obtained on the same mineralized deposit using the ATR method. In this method, a shaped germanium IRE is placed in contact with the sample (see Figure 1.3). The tip of the IRE is ~100 µm, however, the sampled area is dependent upon the refractive index of the IRE and the size of the confocal aperture. [84] For the same aperture size as used in transmission or reflection/absorption analysis, the sampled area is four times smaller in the ATR analysis due to the 4X magnification associated with the refractive index of the germanium IRE. [84] The spectrum obtained on the deposit clearly shows the asymmetric and symmetric stretching modes of the oxalate anion located at 1620 and 1318 cm\(^{-1}\), respectively. A reference ATR spectrum of calcium oxalate is provided for comparison. An added benefit of the ATR method is that the penetration of the infrared energy into the sample is less than 1 µm (see equation 1.5). Thus, for samples thicker than this penetration depth, the optical path through the sample is independent of sample thickness. As a result, photometrically accurate spectra can be obtained on all the materials associated with renal stones without having to worry about the thickness of the section. The reduced path length also reduces scattering that is not only particle size dependent, but path length dependent as well. The only drawbacks to the ATR method are that the IRE contacts the sample, potentially damaging it, and that spectra often need an ATR correction to correct for larger absorbences at longer wavelengths. However, where spectral resolution and photometric accuracy are the main focus, the quality of the
spectrum using ATR spectroscopy is, by far, the best of all the methods presented when taking all things into consideration.

2.4 **Conclusions**

The results demonstrate that mixed sample types containing tissue and mineralized deposits are easily analyzed while mounted on a low-E slide using the ATR method. R/A absorption analysis allows one to quickly survey a tissue section and provides qualitative information about its components. Once interesting sites have been identified by R/A analysis, ATR analysis can then be used to collect the best data possible. ATR analysis provides spectra free from many of the artifacts associated with transmission and R/A analysis, and completes the full picture of the components contained in the crystal deposits and tissue. ATR analysis of tissue samples has been presented in the past [79, 95], but we are unaware of embedded mineralized deposits analyzed using this method.

The procedural novelty of this approach to tissue and mineral deposit analysis is the ability to qualitatively, and soon we hope quantitatively, determine the components of crystal deposits without extensive histological preparations or the possible subjectivity of a pathologist. In using a low-E slide, the histologist is presented with a familiar substrate as well as decreased responsibility in that the tissue need not be stained. In addition, the pathologist is relieved of the task of transferring the tissue sample to a substrate complementary to infrared analysis, while simultaneously having the ability to determine mineralized material as well as tissue components.
CHAPTER 3
REFLECTION ANALYSIS OF ISOLATED RENAL STONES USING INFRARED MICROSPECTROSCOPY
3.1 Introduction

The analysis of isolated renal stones is complex for many reasons. Not only is the stone initially embedded in a gelatinous tissue material, but when removed, the stone can become brittle and difficult to handle. Some methods of stone analysis require the destruction of the stone via grinding, while others use thin sections, which are difficult to produce and if produced, are often easily damaged. Few methods maintain the structural integrity of the stone, which is important when considering the chemistry of formation and the evolution of the stone in the renal system.

While chapter 2 focused on the ability of ATR to work in a complementary fashion with the R/A analysis of embedded mineralized deposits, this chapter will show that ATR is not the optimal method for the analysis of cross-sectioned renal calculi samples. Instead, this chapter will present an easy manner in which to analyze cross-sectioned renal stones using the reflectance mode of the infrared microscope. A cross-section of stone, regardless of thickness, will yield spectra similar to diffuse reflectance. Though some evidence of specular reflection may be present in the spectra, the interference is slight and does not interfere with qualitative analysis of the sample.

3.1.1 Transmission

Recent studies have focused on the infrared imaging of hardened areas of calcified tissue and bone sections, but these studies have employed transmission methods. [50, 59, 60, 62, 67, 96] Thin sections from 0.5 µm to 5 µm thick are mounted on or between BaF₂ windows for the analysis. [59, 97] Most of these reports utilize thin sections of bone, which are structurally more sound than thin sections of renal calculi. This difference in structural stability is the result of the makeup of the sample: bone contains not only the mineral portion, hydroxyapatite, but also has an organic component, collagen, which is intertwined into a triple helical structure that cross-links with other components in the bone, reinforcing the triple helix and thus strengthening the bone. [60] Renal stones, however, are composed of particles that are more circular or flat in nature, and are less structurally stable. Thus reproducibility in thickness, though difficult to attain, is easier with bone samples than with renal stone samples.
3.1.2 Specular and Diffuse Reflectance Techniques

Specular reflectance is a Fresnel, or front surface reflection that occurs on optically flat and reflective surfaces, and has been previously discussed (see section 1.2.3). The reflected electromagnetic radiation obeys Snell’s Law of reflection in that the incident and reflected angles are identical. Specular reflectance is characterized by derivative-shaped absorbance bands, and is present in most samples where the particle size of the sample exceeds that of $2\lambda$.

Diffuse reflectance arises from highly scattering powders whose particle size approximate the size of the wavelength of radiation. The method is commonly employed by the pharmaceutical industry to analyze drug formulations in the powdered or tablet form. [34-46, 98, 99]

3.1.3 ATR Techniques

A third reflectance method, ATR microspectroscopy, has been successfully applied to the analysis of crystalline deposits in tissue [68], as was previously described in Chapter 2. Although this method is useful for the analysis of single points on a sample, a mapping type experiment presents several problems. [69] Successful mapping using a point-by-point process depends on the stability of the renal stone: the sample must be stable so as not to deposit particles onto the IRE, contaminating the remaining sample and yielding non-representative absorption bands. If material adheres to the IRE, that component will register on all subsequent scans, leading to erroneous spectral data.

Macro ATR imaging using a focal plane array detector has been previously presented for the analysis of polymer and biological samples. [70] However, macro ATR imaging presents a different challenge in that it is not adhesion to the IRE that is of concern, but is instead the ability of the IRE to maintain intimate contact over a large area. [70] The pressure of the ATR IRE needed to ensure good contact may be large enough to crush the sample. Macro ATR imaging functions on similar principles as micro ATR, where an evanescent wave penetrates a small distance into the sample, usually around 1 $\mu$m. ATR in general allows for much less sample preparation than other methods, but suffers from the drawbacks stated above.
3.2 Sample Histology

Human renal stones were obtained during percutaneous nephrolithotomy. Intact stones were infiltrated with methyl methacrylate, after which, polymerization was completed by the addition of Perkadox 16. Stone cross-sections between 1-2 mm thick were cut using a diamond wire saw (Well Saw, Delaware Diamond Knives, DE) and mounted onto a plastic slide and subsequently covered with a cover slip.

The diamond-saw finish held by the stones makes the stones dull reflectors necessary for diffuse reflectance spectroscopy. The specular reflectance produced arises from the particle sizes present in the sample. As the particle size approaches $2\lambda$, spectral reflectance increases. However, reflectance mapping using the above process produces more than adequate images for qualitative determination of stone components, regardless of possible spectral interferences.

3.3 Instrumentation

The DRIFTS spectra of reference materials presented in this report were collected using the single point mercury cadmium telluride (MCT) detector on the Perkin-Elmer Spectrum Spotlight 300 infrared imaging microscope, and represent the average of 32 individual scans collected at a spectral resolution of 4 cm$^{-1}$. A 50 X 50 $\mu$m confocal aperture was employed to isolate the sample region of interest for both the DRIFTS and reflectance imaging modes. Background spectra were collected using KBr that was prepared in an identical manner to the sample.

Images of sectioned stones were collected on the Perkin-Elmer Spectrum Spotlight 300 infrared imaging microscope using the 16 X 1 linear array MCT detector. Spectra collected and extracted from false-color images using this detector represent the average of 8 scans at a spectral resolution of 8 cm$^{-1}$. Though the wavenumber range and signal to noise are both lower for the linear array than for the single point detector, the speed of acquisition is greatly increased. Background spectra were collected using a blank section of the reflective surface of a low-E slide.

ATR analysis of reference samples was performed on a Harrick Split-pea ATR microscope. This system employs a single-bounce silicon IRE (n=3.5) with a standard
deuterium triglycine sulfate (DTGS) detector on the Perkin-Elmer Spectrum 2000 macro bench. Spectra collected using this device represent the average of 32 individual scans possessing a spectral resolution of 4 cm$^{-1}$. The samples were brought into intimate contact with the IRE using a loading pressure mass of 0.5 kg.

HyperView software (Perkin-Elmer Corporation, CT) was employed to conduct principle component analysis (PCA) on reflectance maps of stone cross-sections. The program was run on a Dell Optiplex GX150 computer with a Pentium IV chip.

3.4 Results and Discussion

Alternative microspectroscopic attempts to study individual renal stones have included transmission analysis, but thin sections of stones can be burdensome to prepare and are prone to damage. [3, 25, 27, 100-110] Thin sections of renal stone become fragile when isolated from the matrix of the tissue sample, and though they are embedded in a hard resin can be damaged through mishandling or general instability. Transmission analysis of samples typically produces excellent results when quantitative analysis is the long-term goal [68], but in this particular case, the amount of sample preparation involved and the lack of reproducibility in the section thickness makes transmission analysis cumbersome and next to impossible.

3.4.1 A Comparison of Transmission and ATR Spectra

Figure 3.1 illustrates a comparison between transmission and ATR spectra of neat (pure) calcium hydroxyapatite (HAP) and calcium oxalate monohydrate (CaOx). Transmission spectra were obtained by flattening a small amount of sample using the tip of a metal probe and placing the sample onto a KCl substrate. The transmission spectrum is inadequate for quantitative analysis since the bands are not photometrically accurate. Photometric accuracy is defined as having the percent transmission lie between 80 %T and 20 %T, implying that the sample is neither too thin nor too thick. Both transmission spectra are, in reality, nearly totally absorbing, having a %T approaching zero. For hydroxyapatite, this is demonstrated in the band near 1050 cm$^{-1}$, and for oxalate, it is present in the band near 1600 cm$^{-1}$. 
The ATR spectra, however, are photometrically correct, and have the ability to be used for both quantitative and qualitative analysis. ATR data were collected by pressing a small pellet of the neat material and placing the pellet into direct contact with the IRE, as was mentioned in section 3.3. The fundamentals of ATR analysis have been previously discussed in sections 1.2.4 and 2.1.3. ATR involves the nearly total reflection of energy through an IRE crystal, and the penetration of the sample by the remaining energy to a distance $d_p$ that interacts with the sample.
Figure 3.1  A comparison of ATR (top) and transmission (2\textsuperscript{nd}) spectra of calcium hydroxyapatite and ATR (3\textsuperscript{rd}) and transmission (bottom) spectra of oxalate monohydrate.
3.4.2 A Comparison of Specular and Diffuse Reflectance Spectra

Figure 3.2 compares reflectance spectra of HAP and CaOx obtained using two different methods. The first method involved pressing a pellet of a neat sample and obtaining a reflectance spectrum. Because the pellets were pressed, the surface became very flat and reflective, leading to the domination of specular reflectance over diffuse reflectance. Specular reflectance manifests itself in the form of derivative-shaped artifacts adjacent to high intensity absorption bands.

The other method utilized diffuse reflectance to collect the spectrum. The electromagnetic radiation, instead of reflecting at the same angle as the incident, undergoes the process outlined in Figure 1.1. The samples are not optically flat or shiny in diffuse reflectance, limiting the amount of specular reflectance seen in the spectra to the point where it can be considered negligible.

The only difference between the specular and diffuse reflectance studies was the form of the sample. For the specular reflectance trial, neat pellets were manually pressed using a pellet press (Parr Instruments, Moline, IL), creating a compact sample that was both optically flat and highly reflective. These pellets were subsequently placed on a reflective slide and analyzed using the reflectance mode of the infrared imaging microscope. For the diffuse reflectance studies, an approximate 1:10 ratio of sample to KBr was ground in an agate mortar and pestle and subsequently placed in a diffuse reflectance cup, where the reflectance mode of the infrared imaging microscope was once again used.
Figure 3.2  DRIFTS hydroxyapatite (top), specular reflectance hydroxyapatite (2\textsuperscript{nd}), DRIFTS calcium oxalate (3\textsuperscript{rd}), and specular reflectance calcium oxalate (bottom).
Figures 3.3 and 3.4 illustrate a comparison of DRIFTS spectra of reference materials to reflectance spectra obtained from images of cross-sectioned kidney stones. The two examples shown, calcium oxalate monohydrate and calcium hydroxyapatite, are both primary components in many renal calculi (see Table 1.1). The reflectance spectra of the stones approximate DRIFTS spectra because of the particle sizes in the stone; since the particles are smaller than approximately $2\lambda$, the radiation takes a more random and complicated path, resulting in mostly diffuse reflectance with very few specular components (see Figure 1.1). Specular reflectance is present due to the existence of a slight derivative shape on the leading edge of the absorption bands. Had the particles been larger than $2\lambda$, they would have appeared as a flat, smooth surface, yielding more specular interference.
Figure 3.3  Comparison of a reflectance spectrum (top) to a DRIFTS spectrum (bottom) for oxalate monohydrate.
Figure 3.4  Comparison of a reflectance spectrum (top) to a DRIFTS spectrum (bottom) for calcium hydroxyapatite.
As has been discussed previously, slight specular reflectance remains present in the reflectance spectra taken from the surface of the renal calculi. In Figure 3.3, this feature can be seen at 1730, 1340, and 800 cm\(^{-1}\) for calcium oxalate monohydrate, and in Figure 3.4, at 1150 cm\(^{-1}\) for hydroxyapatite.

### 3.4.3 Reflection Imaging of Kidney Stones

The formation of kidney stones and their chemical etiology is not known. By studying the radial projection of a cross-sectioned kidney stone, it may be possible to determine the chemistry from nucleation to some distant point in time. The process is similar to geochemistry, but with the time and distance scales being highly compressed. Infrared imaging of cross-sectioned stones allows one to precisely obtain molecular information and thus eventually determine the chemistry of stone formation.

The process of analysis and imaging for a renal stone section is similar to that described in section 2.1.2 for tissue sections. Though the calculus cross-section can undergo absorption much like the mineralized deposits, the spectra are far more diffuse-like due to the small particle size. Crystal sizes discussed in Chapter 2 were larger than 2\(\lambda\), which yielded data more influenced by specular reflectance and other effects, creating absorption bands that were not photometrically accurate. The difference in spectra of the stone cross-sections from the embedded mineralized portions arises from the diffuse reflectance taking place as outlined in Figure 1.1.

In infrared imaging, spectra are used to generate molecular images of renal stone cross-sections. The following figures will illustrate the dramatic information-gathering ability of the infrared imaging process; as many as 128,000 spectra (Figure 3.13) are contained in some images. Each point on the stone cross-section contains a spectrum that is the average of eight individual scans ranging from 4000 to 720 cm\(^{-1}\), allowing the image to be displayed with respect to the average absorbance at any wavenumber within this range.

Figure 3.5, containing approximately 94,000 individual spectra, displays a photomicrograph of the cross-section of a brushite stone. This stone was thought to be 100 % brushite, but was determined to have an unknown component by the imaging
process reported in this chapter. Following the visible image of the stone is the total absorbance infrared image map (Figure 3.6) of the boxed section in Figure 3.5.
Figure 3.5  Photomicrograph of a brushite stone cross-section (top) and the spectrum of brushite (bottom) obtained from the surface of the stone.
Figure 3.6  Total absorbance image map of a selected area of the brushite stone. The dark blue areas representing low absorbance are associated with the embedding media.
As stated above, the absorption intensity map can be displayed with respect to any wavenumber within the range over which the sample was scanned. Figure 3.7 illustrates this advantage by displaying an image map colored with respect to the absorbance intensity of the 1050 cm$^{-1}$ band. This stone was found to contain not only brushite, but an unidentified component as well, which is imaged in Figure 3.8.
Figure 3.7  An image of a brushite stone (top) based on the absorbance band near 1050 cm$^{-1}$ (bottom).
Figure 3.8  Image of brushite stone (top) with respect to the absorbance band of the unidentified compound (bottom). The unidentified compound is highlighted in red and yellow.
Another example of infrared imaging on renal stone cross-sections is displayed in Figure 3.9, containing over 65,000 spectra. This example of a cystine stone was previously thought to be 100% pure. However, upon infrared analysis using the described method, it was determined that a large amount of the stone corresponded to hydroxyapatite. Following the photomicrograph is the stone’s total absorbance map, Figure 3.10, and an absorbance map (Figure 3.11) taken with respect to the absorbance bands in the 1300 to 1600 cm\(^{-1}\) range (also Figure 3.11), which is a characteristic absorbance region for cystine. The red and light green areas in Figure 3.11 correspond to the cystine, however, the faint blue areas represent hydroxyapatite, which is profiled in greater detail in Figure 3.12.
Figure 3.9  Photomicrograph of a cystine stone cross-section, and the reflectance spectrum off the face of the cystine stone.
Figure 3.10  Total absorbance image map of cystine.
Figure 3.11  Image based on cystine absorption bands from approximately 1300-1600 cm\(^{-1}\). The red and yellow areas correspond to cystine.
Figure 3.12  An image of the cystine stone (top) taken with respect to the hydroxyapatite absorption band at 1050 cm$^{-1}$ (bottom). The small flecks of red and yellow correspond to hydroxyapatite, while the blue and green areas are cystine.
Figure 3.13 displays the visible image of a renal stone that contains two distinct components: calcium oxalate and calcium hydroxyapatite. There is a visible layer structure to the stone, and one might assume each color to be indicative of a different component. However, this layer structure can be misleading, since colors can vary due to the amount of compression in a sample as well as due to the components. Calcium oxalate (see Table 1.1) has a set of several characteristic IR absorbance bands, while hydroxyapatite has only one significant absorption band. This makes differentiation using IR analysis much more accurate than visual analysis, which relies on a difference in color between the stone layers. Figure 3.14 contains the false-color reflectance image of a section of the stone based upon average absorbance, followed by Figures 3.15 and 3.16, demonstrating the ability of the program to differentiate between hydroxyapatite and calcium oxalate based on specific absorption bands.
Figure 3.13  Visible image of hydroxyapatite/oxalate stone cross-section. Selected area of interest is boxed in red. Below, the spectra from the surface: oxalate (top) and hydroxyapatite (bottom).
Figure 3.14  False-color image of the total absorbance for a hydroxyapatite/oxalate stone cross-section.
Figure 3.15  Image of hydroxyapatite/oxalate stone section based on hydroxyapatite band centered near 1050 cm$^{-1}$.

**spectrum 11 at -3469.30 (stone04)**
Figure 3.16  Image of hydroxyapatite/oxalate stone section based on oxalate band centered near 1600 cm\(^{-1}\).
PCA (principle component analysis) provides data for clustering processes, and serves a two-fold purpose: not only does this technique distinguish different components and species, but it also serves to confirm homogeneity within a sample. PCA categorizes the different types of spectra present in an image, serving the purpose of differentiation. Clustering uses the data from the PCA process and combines under one color areas with nearly identical spectra.

Figure 3.17 displays an image map of the same area as outlined in Figure 3.13. However, in Figure 3.17, PCA and clustering analysis have been performed and compiled using HyperView software, revealing the placement of the two components and embedding material. The distinctive layers now visible in the PCA image map represent the two different components, and at any point on the map, the spectrum of the component making up that area can be obtained. The reflectance spectra obtained from the two different areas of composition (oxalate and hydroxyapatite) are presented and compared to ATR reference spectra in Figure 3.18. As was discussed earlier, a small amount of specular reflectance is present in the spectra from the stone. This can be seen in the leading edge of large absorbance bands.
Figure 3.17  False-color image of hydroxyapatite/oxalate stone after PCA and cluster analysis. The blue areas indicate calcium hydroxyapatite, while the red areas are calcium oxalate.
Figure 3.18  Calcium oxalate (top) and calcium hydroxyapatite (bottom) spectra originating from the reflectance off of the stone face (tops) and from ATR reference analysis (bottoms).
Not only is infrared imaging useful as a tool to distinguish between different renal stone components, but it is also used to distinguish between different forms of the same component. Calcium oxalate is produced in two forms: oxalate monohydrate and oxalate dihydrate. Both oxalates are present in the stone photographed in Figure 3.19, as is a small amount of hydroxyapatite, but it is impossible to visually determine the areas where each oxalate type or apatite resides. Figures 3.20 and 3.21 feature two sets of images: the first set imaged with respect to the absorbance band characteristic of oxalate dihydrate, and the second set imaged with respect to the absorbance band characteristic of oxalate monohydrate. Figure 3.22 verifies the placement of hydroxyapatite in the stone.
Figure 3.19  Visible image (top) and total absorbance image (bottom) of an oxalate stone containing two types of oxalate: monohydrate and dihydrate.
Figure 3.20  Image (top) of oxalate stone with respect to oxalate dihydrate band (bottom).
Figure 3.21  Image (top) of oxalate stone with respect to oxalate monohydrate band (bottom).
Figure 3.22  Image of stone (top) with respect to hydroxyapatite band (bottom).
3.5 Conclusions

Presented in this chapter is a new and easy method of intact renal stone analysis using instrumentation and software that are both easy to use and are qualitative in nature. This method is less susceptible to error or misdiagnosis than alternative visible-based methods of stone analysis, and bypasses the sectioning and grinding steps that have often accompanied renal stone analysis in the past.

A total of twelve stones from different sources have been analyzed to date in this manner, each yielding spectra similar to the diffuse spectra seen here. It is a fortunate coincidence that the particle sizes of renal stones are conducive to this type of infrared analysis, allowing us to differentiate between components that are often very similar in makeup and appearance. It is hoped that as this technique becomes more prevalent, the rate of misdiagnosis will decrease, increasing patient treatment, comfort, and satisfaction.
CHAPTER 4

A QUANTITATIVE STUDY OF RENAL STONE COMPONENTS
4.1 Introduction

Perhaps the most dramatic finding of this research has been the fact that infrared analysis has proven the pathologist diagnosis incorrect in approximately 40% of the cases examined. [20] For example, Chapter 3 introduced several cases where renal calculi that had originally been classified as being a pure substance were in fact found to be mixtures of different components. Detractors of our findings argue that infrared analysis is more sensitive for apatite than oxalate, making IR unsuitable for this type of analysis. However, we will show that this is an untrue statement. Chapter 4 offers the calibration and sensitivity data that supports the work of the previous three chapters, and alludes to a potentially quantitative method that will be the focus of future research in this area.

The focus of chapter 2 was the analysis of embedded mineral deposits using the complementary techniques of ATR and R/A analysis, while chapter 3 examined the use of reflectance measurements as a means to qualitatively determine the components in cross-sections of renal calculi. The intended goal of chapter 4 is to prove that there exists a linear relationship between the absorbance of calcium oxalate and the percentage of oxalate in a matrix of calcium hydroxyapatite using ATR and Raman spectroscopy (a complementary technique to infrared spectroscopy). An additional goal of this chapter is to determine the approximate detection limits for mixtures where calcium oxalate is either the major or minor component.

4.1.1 Quantitative Analysis

Calcium oxalate and calcium hydroxyapatite are among the most common components in human renal stones (see Table 1.1). Though the analysis of both of these components with respect to their formation, structure, and role in renal stone development and elsewhere is a common practice [2-6, 11, 25-27, 47, 73, 74, 99-133], quantitative methods often continue to employ the destruction of the sample.

For any quantitative research to be achieved in the area of infrared renal stone analysis, a general statement or set of calibration curves must be made, associating the percentage of renal stone components to an absorbance value. While many calibration curves for renal stone components currently exist [107, 115, 118, 132, 134, 135], they are
primarily for the use of the laboratory that has created them, and are seldom transferable
due to procedural and instrumental differences.

4.1.2 Relation to Renal Crystal Analysis

As an example of an application of the calibration curves to be presented in this
chapter, Figure 4.1 displays the spectrum of an embedded mineralized deposit in a human
renal biopsy that was previously diagnosed via visual identification methods to contain
only calcium oxalate. Upon the application of the calibration equation formed in this
chapter to the experimental area over a designated range in the sample, it was determined
that the percentage of calcium oxalate in the sample was only approximately 2.16 %.
The other constituent known to be present, calcium hydroxyapatite, is known to not have
any interfering absorption bands in that general area, and was determined to be the major
component in the sample.
Figure 4.1 Human renal biopsy thought to contain a small percentage of calcium oxalate monohydrate in the tissue-embedded mineralized deposit. Arrows denote bands common to calcium oxalate.
In Figure 4.1 above, a combination of bands occurring from hydroxyapatite, calcium oxalate, and tissue are visible. The band near 1620 cm\(^{-1}\) is broad, and occurs from the overlap of the amide I band (~1650 cm\(^{-1}\)) from the tissue and a nearby oxalate band at 1619 cm\(^{-1}\). The major absorption band at 1020 cm\(^{-1}\) is indicative of hydroxyapatite, as is the band at 600 cm\(^{-1}\). However, the bands at 1317 and 780 cm\(^{-1}\) are indicative of calcium oxalate, and are highlighted by arrows.

4.2 Experimental

4.2.1 Instrumentation

ATR analysis was performed on a Harrick Split-pea ATR microscope. This system employs a single-bounce silicon IRE (n=3.5) with a standard deuterium triglycine sulfate (DTGS) detector on the Perkin-Elmer Spectrum 2000 macro bench. Spectra collected using this device represent the average of 32 individual scans possessing a spectral resolution of 4 cm\(^{-1}\). The samples were brought into intimate contact with the IRE using a loading of 0.5 kg.

Raman spectra were collected using a Perkin-Elmer Spectrum 2000 FT-Raman spectrometer. Samples were excited with a Nd/YAG (1064 nm) laser. The 180° back scattering geometry was employed in the conventional macro sampling accessory. Power at the sample did not exceed 200 milliwatts. Spectra were collected at 4 cm\(^{-1}\) resolution and represent the average of 64 individual scans.

4.2.2 Sample Preparation

Mixtures of calcium oxalate (CaOx) (Alfa Chemical) and calcium hydroxyapatite (HAP) (Spectrum Chemical) were created possessing 1 to 99 % oxalate. Subsequent to the addition of oxalate to the apatite, the mixture was ground for five minutes using an agate mortar and pestle. The mixtures were then stored in air-tight glass vials until analysis. Certain oxalate samples were ground in a Wig-L-Bug (Rinn Corporation, Elgin, IL) for one minute prior to the combining of the two species. These same samples were once again Wig-L-Bugged one minute to grind the sample prior to analysis.

Samples were pressed using a manual pellet press (Parr Instruments, Moline, IL) prior to analysis. Great care was taken to ensure all pellets were manually pressed in the
same manner, including the time of pressing and the force applied. For ATR analysis, no fewer than six pellets of each sample ratio were used in order to determine the average, standard deviation, and percent relative standard deviation.

4.2.3 Particle Size Analysis

Individual samples of CaOx and HAP were prepared as described above and analyzed via a particle size analyzer (Precision Detectors, Bellingham, MA) at all stages of grinding. In addition, CaOx and HAP directly from the reagent bottle were analyzed. It was found that the sizes of the particles were not altered drastically by grinding using a Wig-L-Bug method, and were appreciably similar in original size so as to create a homogeneous mixture. Therefore, in all pellets equal to or greater than 5 % CaOx, a mortar and pestle was the sole method of grinding the components.

4.2.4 Reproducibility of the Method

The reproducibility of both the ATR and Raman methods were tested by creating three batches of approximately 3 % (3.04 %, 3.06 %, and 3.01 %) CaOx in a HAP matrix. No fewer than six pressed pellets from each batch were analyzed, creating an average, standard deviation, and relative standard deviation percentage for each sample batch. For each analysis, every attempt was made to ensure that the pellets were equal in size and compactness.

A fourth batch of 3 % CaOx in HAP was created and served two purposes: the first as another sample for a reproducibility measurement on the ATR, and the other as a test of general homogeneity of the two samples. This batch was ground via mortar and pestle, but was not Wig-L-Bugged. It was instead ground and mixed into a slurry with MeOH, which was allowed to evaporate off over several days in a protected vessel.

4.2.5 Peak Areas

Spectra were displayed in absorbance mode and analyzed using the peak height/area function in the Spectrum v.5.0.1 program (Perkin-Elmer, Shelton, CT). Calcium oxalate monohydrate peak areas from both 770-790 cm⁻¹ and 1310-1330 cm⁻¹ were collected, as well as calcium hydroxyapatite peak areas from 970-1120 cm⁻¹. For
peak area ratios, the 770-790 cm\(^{-1}\) range of oxalate monohydrate was divided by the 970-1120 cm\(^{-1}\) peak area of hydroxyapatite.

4.3 **Results and Discussion**

4.3.1 **ATR Calibration Curve**

Samples from 0 to 100 % CaOx in HAP were pressed and analyzed using an ATR method. ATR spectra of neat CaOx and HAP, as well as a 20 % CaOx sample, are presented in Figure 4.2. The spectrum of the 20 % CaOx in HAP sample is a linear combination of the two main component spectra, and the major absorption bands of each component type are clearly visible.
**Figure 4.2** ATR spectra of neat CaOx and HAP, as well as a 20 % spectrum of CaOx in HAP.
The corrected peak areas (Tables 4.1 and 4.2) for the ranges of 1310-1330 cm\(^{-1}\) and 770-790 cm\(^{-1}\) were tabulated, averaged, and graphed to produce Figure 4.3. Two separate linear calibration curves exist: the first from 0 to 20 %, and the other from 20 to 100 %, both existing with acceptable linearity. The data from 0 to 20 % CaOx appeared to be unreasonable with respect to the rest of the data, and was run a second time after the data for all other percentages had been collected. The orange markers represent this additional trial, and were found to be reproducible with the originally collected data.
Table 4.1  ATR calibration curve data from 1310-1330 cm\(^{-1}\).

<table>
<thead>
<tr>
<th>% Oxal.</th>
<th>Peak</th>
<th>Area:</th>
<th>Avg:</th>
<th>SD:</th>
<th>RSD:</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.024</td>
<td>0.076</td>
<td>0.074</td>
<td>0.058</td>
<td>0.008</td>
</tr>
<tr>
<td>20</td>
<td>0.027</td>
<td>0.150</td>
<td>0.150</td>
<td>0.140</td>
<td>0.104</td>
</tr>
<tr>
<td>30</td>
<td>0.036</td>
<td>0.305</td>
<td>0.310</td>
<td>0.140</td>
<td>0.008</td>
</tr>
<tr>
<td>40</td>
<td>0.039</td>
<td>0.362</td>
<td>0.362</td>
<td>0.362</td>
<td>0.008</td>
</tr>
<tr>
<td>50</td>
<td>0.042</td>
<td>0.467</td>
<td>0.467</td>
<td>0.467</td>
<td>0.008</td>
</tr>
<tr>
<td>60</td>
<td>0.046</td>
<td>0.603</td>
<td>0.603</td>
<td>0.603</td>
<td>0.008</td>
</tr>
<tr>
<td>70</td>
<td>0.051</td>
<td>0.661</td>
<td>0.661</td>
<td>0.661</td>
<td>0.008</td>
</tr>
<tr>
<td>80</td>
<td>0.056</td>
<td>0.735</td>
<td>0.735</td>
<td>0.735</td>
<td>0.008</td>
</tr>
<tr>
<td>90</td>
<td>0.065</td>
<td>0.771</td>
<td>0.771</td>
<td>0.771</td>
<td>0.008</td>
</tr>
<tr>
<td>95</td>
<td>0.081</td>
<td>0.835</td>
<td>0.835</td>
<td>0.835</td>
<td>0.008</td>
</tr>
<tr>
<td>97</td>
<td>0.084</td>
<td>0.866</td>
<td>0.866</td>
<td>0.866</td>
<td>0.008</td>
</tr>
<tr>
<td>99</td>
<td>0.086</td>
<td>0.879</td>
<td>0.879</td>
<td>0.879</td>
<td>0.008</td>
</tr>
</tbody>
</table>
Table 4.2  ATR calibration curve data from 770-790 cm⁻¹.

<table>
<thead>
<tr>
<th>% Oxal.</th>
<th>Peak Area:</th>
<th>Avg:</th>
<th>SD:</th>
<th>RSD:</th>
</tr>
</thead>
<tbody>
<tr>
<td>770-790</td>
<td>0.028</td>
<td>0.079</td>
<td>0.013</td>
<td>18.9</td>
</tr>
<tr>
<td>80</td>
<td>0.020</td>
<td>0.102</td>
<td>0.016</td>
<td>24.9</td>
</tr>
<tr>
<td>90</td>
<td>0.022</td>
<td>0.111</td>
<td>0.016</td>
<td>14.5</td>
</tr>
<tr>
<td>100</td>
<td>0.025</td>
<td>0.120</td>
<td>0.018</td>
<td>15.0</td>
</tr>
<tr>
<td>110</td>
<td>0.028</td>
<td>0.129</td>
<td>0.020</td>
<td>15.9</td>
</tr>
<tr>
<td>120</td>
<td>0.031</td>
<td>0.138</td>
<td>0.013</td>
<td>18.3</td>
</tr>
<tr>
<td>130</td>
<td>0.034</td>
<td>0.147</td>
<td>0.017</td>
<td>15.9</td>
</tr>
<tr>
<td>140</td>
<td>0.037</td>
<td>0.156</td>
<td>0.021</td>
<td>13.7</td>
</tr>
<tr>
<td>150</td>
<td>0.040</td>
<td>0.165</td>
<td>0.025</td>
<td>15.8</td>
</tr>
<tr>
<td>160</td>
<td>0.043</td>
<td>0.174</td>
<td>0.029</td>
<td>16.3</td>
</tr>
<tr>
<td>170</td>
<td>0.045</td>
<td>0.183</td>
<td>0.031</td>
<td>17.0</td>
</tr>
<tr>
<td>180</td>
<td>0.048</td>
<td>0.192</td>
<td>0.033</td>
<td>17.6</td>
</tr>
<tr>
<td>190</td>
<td>0.051</td>
<td>0.201</td>
<td>0.035</td>
<td>18.2</td>
</tr>
<tr>
<td>200</td>
<td>0.054</td>
<td>0.210</td>
<td>0.037</td>
<td>18.9</td>
</tr>
</tbody>
</table>

770-790 cm⁻¹ Oxal. Peak Area: Avg: SD: RSD:
Figure 4.3  Calibration plots for 770-790 cm\(^{-1}\) (top) and 1310-1330 cm\(^{-1}\) (bottom) of CaOx percentages.

**770-790 cm\(^{-1}\) range**

- From 100% to 0%: 
  \[ y = 0.009x + 0.267 \]
  \[ R^2 = 0.996 \]

- From 0% to 20%: 
  \[ y = 0.024x + 0.003 \]
  \[ R^2 = 0.998 \]

**1310-1330 cm\(^{-1}\) range**

- From 100% to 0%: 
  \[ y = 0.005x + 0.278 \]
  \[ R^2 = 0.988 \]

- From 0% to 20%: 
  \[ y = 0.021x + 0.007 \]
  \[ R^2 = 0.995 \]
After the trial of isolated peak areas of calcium oxalate monohydrate were found not to create a single linear calibration line, the corrected peak areas of the 770-790 cm\(^{-1}\) CaOx and 970-1120 cm\(^{-1}\) HAP were ratioed in an attempt to determine if any obvious errors had occurred (Figure 4.4). Peak areas for CaOx over the above range were divided by the HAP peak area for the range 970-1120 cm\(^{-1}\) and were subsequently averaged. The result is an exponential curve growing towards infinity near 95 % CaOx, which is expected since the area under the calcium oxalate curve is growing larger with concentration while the area under the HAP curve is approaching zero.

Also in Figure 4.4 is the maximized area from 1-60 % CaOx of the same exponential graph. This lower range appears linear, though the points above this range rapidly digress to produce the exponential graph.
Figure 4.4  Ratioed areas of 770-790 cm\(^{-1}\) of CaOx to 970-1120 cm\(^{-1}\) of HAP producing an exponential graph, and the same graph maximized from 1-60 % CaOx to produce a linear graph.

\[ y = 0.003x - 0.004 \]
\[ R^2 = 0.994 \]
4.3.2 Sensitivity of the Method

As was discussed in the introduction to this chapter, some opponents of the results of this research comment that infrared analysis is more sensitive to hydroxyapatite than to oxalate. However, this argument is unfounded and incorrect, since previous research performed in this area determined that infrared analysis of oxalate (1632 cm\(^{-1}\) band) is actually twice as sensitive as that of hydroxyapatite (1029 cm\(^{-1}\) band). [13] Sensitivity was measured by individually comparing the absorbance of both materials under identical circumstances. The molar absorptivity of the oxalate band at 1632 cm\(^{-1}\) is much larger than that of the oxalate 780 cm\(^{-1}\) band. This difference in molar absorptivity means that the ability of the instrument to detect calcium oxalate at 1 % using this weaker band dramatically disavows prior comments from detractors as to the sensitivity of the method being preferential to hydroxyapatite. Though the 1632 cm\(^{-1}\) band of oxalate was not used in this particular quantitation method, similar calibration curves using this band could easily be created.

4.3.3 ATR 3% Reproducibility

It was determined that calcium oxalate monohydrate could be observed at 1%, as can be seen in Figure 4.5. The value of 3 % CaOx (also shown in Figure 4.5) was chosen for the reproducibility study because it was felt that this percentage was more visible and reproducible than 1 % CaOx. The results of the reproducibility analysis are displayed in Table 4.3, with batch D being the 3 % slurry in MeOH (as mentioned in 4.2.3). The corrected areas under two calcium oxalate monohydrate peaks were taken and tabulated. As can be seen, the relative standard deviations ranged from 9.54-16.28 % for the area 770-790 cm\(^{-1}\), and from 7.28-13.99 % for the area 1310-1330 cm\(^{-1}\). These results, however, do not include the 3 % MeOH slurry, which experienced high percent relative standard deviations for both peak areas measured. When the slurry sample, made to check homogeneity as explained in section 4.2.4, is taken into account, the total RSD %’s climb to over 25 and 31 for the 770-790 cm\(^{-1}\) and 1310-1330 cm\(^{-1}\) ranges, respectively.
Figure 4.5  Neat (bottom), 3% (middle) and 1% (top) CaOx. Arrow denotes absorbance band at \(~780\, \text{cm}^{-1}\).
Table 4.3  
ATR reproducibility of 3 % CaOx in HAP using the corrected peak area.

<table>
<thead>
<tr>
<th>cm⁻¹ range:</th>
<th>3a</th>
<th></th>
<th>3b</th>
<th></th>
<th>3c</th>
<th></th>
<th>3d (slurry)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>770-790</td>
<td>1310-1330</td>
<td>770-790</td>
<td>1310-1330</td>
<td>770-790</td>
<td>1310-1330</td>
<td>770-790</td>
</tr>
<tr>
<td>corr. peak area :</td>
<td>0.0940</td>
<td>0.0866</td>
<td>0.0712</td>
<td>0.0452</td>
<td>0.0878</td>
<td>0.0597</td>
<td>0.0364</td>
</tr>
<tr>
<td></td>
<td>0.0807</td>
<td>0.0763</td>
<td>0.0698</td>
<td>0.0496</td>
<td>0.0797</td>
<td>0.0522</td>
<td>0.0409</td>
</tr>
<tr>
<td></td>
<td>0.0838</td>
<td>0.0741</td>
<td>0.0585</td>
<td>0.0440</td>
<td>0.0909</td>
<td>0.0634</td>
<td>0.0561</td>
</tr>
<tr>
<td></td>
<td>0.0699</td>
<td>0.0584</td>
<td>0.0550</td>
<td>0.0396</td>
<td>0.0742</td>
<td>0.0557</td>
<td>0.0486</td>
</tr>
<tr>
<td></td>
<td>0.0873</td>
<td>0.0813</td>
<td>0.0626</td>
<td>0.0458</td>
<td>0.0717</td>
<td>0.0414</td>
<td>0.0397</td>
</tr>
<tr>
<td></td>
<td>0.0584</td>
<td>0.0656</td>
<td>0.0568</td>
<td>0.0415</td>
<td>0.0768</td>
<td>0.0527</td>
<td>0.0434</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.0714</td>
<td>0.0433</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Average: | 0.0790 | 0.0737 | 0.0636 | 0.0441 | 0.0802 | 0.0542 | 0.0477 | 0.0378 |
| SD: | 0.013 | 0.010 | 0.007 | 0.003 | 0.008 | 0.008 | 0.010 | 0.011 |
| %RSD: | 16.3 | 14.00 | 11.2 | 7.3 | 9.54 | 14.00 | 21.3 | 28.7 |

<table>
<thead>
<tr>
<th>All</th>
<th>770-790</th>
<th>1310-1330</th>
<th>No 3d</th>
<th>770-790</th>
<th>1310-1330</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average:</td>
<td>0.0654</td>
<td>0.0506</td>
<td></td>
<td>0.0737</td>
<td>0.0567</td>
</tr>
<tr>
<td>SD:</td>
<td>0.017</td>
<td>0.016</td>
<td></td>
<td>0.012</td>
<td>0.015</td>
</tr>
<tr>
<td>RSD:</td>
<td>25.5%</td>
<td>31.6%</td>
<td></td>
<td>16.2%</td>
<td>25.5%</td>
</tr>
</tbody>
</table>
4.3.4 Raman Calibration Curve

A reproducibility study using 3% ratios was also performed on the Raman system. As with ATR, six pellets from each of three batches were used, with data being collected over the area 1450-1500 cm\(^{-1}\). The percent relative standard deviation for all trials combined was 7.56%, leading to the conclusion that achieving a reproducible result would be much easier and possibly more accurate using the Raman system.

A calibration curve was also attempted on the FT-Raman instrument, producing a linear range from 1 to 90% with an \(R^2\) value of 0.9902 over a wavenumber range of 1450-1500 cm\(^{-1}\). This calibration curve is displayed in Figure 4.6. Raman spectroscopy was attempted as a parallel study to ATR not only because of the complementary information it provides, but also because of the ability to analyze smaller particles, which results from the wavelength of radiation utilized. As was described in Chapter 1, the size of the particle that can be analyzed is related to the wavelength of radiation used. Raman spectroscopy uses a shorter wavelength of radiation, 1064 nm (approximately 10,000 cm\(^{-1}\)), when compared to infrared analysis, which uses wavelengths on the order of 10,000 nm (1000 cm\(^{-1}\)).
Figure 4.6  Raman calibration curve using 1-90 % CaOx in HAP.

Raman 1450-1500 cm$^{-1}$

\[ y = 21.9x + 43.1 \]
\[ R^2 = 0.990 \]
4.3.5 Unknown Analysis

Three pellets each of two unknown samples of ratios of calcium oxalate to hydroxyapatite were run on the ATR, averaged, and applied against the calibration curve. Sample A yielded an experimental percentage of 15.84 % CaOx using the area under 770-790 cm\(^{-1}\). The actual value was calculated to be 16.99 %, a difference of 1.15%. With an experimental standard deviation of 0.39% (an RSD of 2.47%), the actual value was not technically within experimental limits, however, this value may be near enough to the true value to be acceptable to the medical community. The percentage of CaOx using the 1310-1330 cm\(^{-1}\) range equaled 12.99 %, which is a large deviation from the true value of 16.99 % and is well out of experimental error range.

The second unknown sample, sample B, yielded an experimental percentage of 62.93 % with a standard deviation of 4.9 % in the 770-790 cm\(^{-1}\) range. The actual value of the unknown was 59.17 %, and is well within experimental error. For the 1310-1330 cm\(^{-1}\) range, the experimental value nearly matched the calculated value at 59.79 % and 59.17 %, respectively.

4.3.6 Particle Size

An analysis of the particle sizes of both calcium oxalate and calcium hydroxyapatite using a particle-sizing instrument (Precision Detectors, Bellingham, MA) was performed. For this process, neat and mixed samples were suspended in water, and were examined at all stages of sample preparation, including before and after Wig-L-Bugging for 1, 3, 5 and 10 minutes, grinding with a mortar and pestle, and unaltered.

The data from this analysis is inconclusive. The measurements were taken in triplicate, though there was little reproducibility. For several samples, particle size often varied from approximately 0.3 \(\mu\text{m}\) to 10 \(\mu\text{m}\) or more between samplings within the same run. Since this was the case with both hydroxyapatite as well as calcium oxalate, it was determined that there were enough similarities in size that a homogeneous mixture would result after grinding with a mortar and pestle rather than Wig-L-Bugging the samples. This was confirmed with the calibration curve.
4.4 Conclusions

The ATR method appears to have the potential to give a quantitative approximation of the percentage of calcium oxalate in a sample, even though there exist two separate and distinct regions of the calibration curves for the association of peak area with the percentage of calcium oxalate.

The Raman technique appears to have better reproducibility and a single calibration curve, both of which imply Raman is a technique worth pursuing for the quantitative analysis of renal stones. Both of these methods will require more work in order to achieve a suitable calibration curve for the quantitative analysis of calcium oxalate.

The sensitivity of the method for oxalate is proven in the detection of calcium oxalate at the 1 % level. The detection of hydroxyapatite as minor component in a calcium oxalate matrix could not be achieved until 5 % hydroxyapatite, indicating that detractors of our research were incorrect in their initial assessment of the method being more sensitive for hydroxyapatite over calcium oxalate.
A.1 Introduction

Many reference libraries of kidney stone components have been created; virtually every researcher in the field of renal calculi must run a set of reference materials in order to have access to the information needed to correctly categorize renal stones.

Comprehensive databases are available from several commercial sources and research groups, however, the method used for the database and the ongoing research presented in this thesis may differ enough that intensities or wavenumbers may be slightly shifted. In addition, running reference spectra on the current instrument of analysis is always preferred to ensure proper identification of absorbance bands.

Found here are the reference spectra obtained for the purposes of this research. Major absorbance bands have been listed, along with the spectrum and chemical formula. Though reflection and transmission spectra were also collected, only the ATR data is presented here because of its high quality and clarity.

A.2 Experimental

A.2.1 Instrumentation

All ATR spectra were taken on the Perkin-Elmer Split Pea ATR system, which uses a Harrick ATR and has been previously described in this thesis. The samples were scanned at 4 cm\(^{-1}\) resolution and are the average of 32 individual scans.

A.2.2 Methods

All reference specimens were ground manually into a fine powder using an agate mortar and pestle if not initially in powder form. A neat sample pellet was then created using a manual pellet press (Parr Instruments, Moline, IL), and was subsequently transferred to the ATR crystal or to a low-E slide (Kevley Technologies, Chesterland, OH) for ATR or reflectance analysis, respectively.

For several of the samples, small amounts of the compound had to be chipped away from larger sections of rock or crystal. This was done using a hammer on the mineral while it was wrapped in a paper towel. Every attempt was made to keep samples as pure as possible.
A.2.3 Calcium Urate

Calcium urate was prepared from uric acid and calcium hydroxide by a method published by P. Carmona.[129] While stirring, 800 mL of 0.00125 M uric acid was brought to 100°C, to which 100 mL of 0.0005 M calcium hydroxide was added drop-wise. This solution was boiled down to approximately 170 mL, at which point it was allowed to cool to room temperature. A precipitate formed, which was subsequently vacuum filtered and washed with a small amount of cold doubly-distilled deionized water. Ample amount was recovered for analysis with ATR, and was kept in a desiccator until needed. A natural sample of the mineral was unable to be obtained through other methods.

A.2.4 Materials

The following chemicals were obtained in their natural mineral form from Excalibur Minerals, Peekskill, NY, and were ground as controls: brushite (Pigs Hole, VA), struvite (Skipton Caves, Australia), aragonite (Serfou, Morocco), carbonate hydroxyapatite (Casa Collina Quarry, Pitigliano, Italy), calcite (Chihuahua, Mexico), whitlockite (Custer, SD), weddellite (Biggs, OR) and monetite (Mono Lake, CA). In addition, hydroxyapatite (Holly Springs, GA) and fluoroapatite (Cerro Mercado, Durango, Mexico) were obtained in their natural crystalline form from a private donor. Calcium phosphate monobasic, dibasic and tribasic (Mallinckrodt, NY), uric acid (99+% pure; Aldrich, Milwaukee, WI), L-cystine (99% pure; Aldrich, Milwaukee, WI), cholesterol (95% pure; Aldrich, Milwaukee, WI), calcium hydroxyapatite (from bone meal; Spectrum Chemical, Gardena, CA), uric acid sodium salt (Aldrich, Milwaukee, WI), calcium oxalate monohydrate (99% pure; Alfa Chemical, Danvers, MA), and calcium sulfate (reagent grade; Aldrich, Milwaukee, WI) were also used.

A.3 Results and Discussion

A.3.1 Reference Library

The compounds of interest are presented in Figure A.1, along with individual spectra, the chemical formula and wavenumber assignments. The structures of complex
compounds are also given. In all, twenty compounds were created or obtained for spectral analysis.
Figure A.1  List of control specimens, complete with spectra and absorbance bands. There are many spectral similarities in several of the phosphate-containing groups, though each has distinctive identification bands as well.

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Brushite (Australian)</strong> Calcium phosphate dihydrate ( \text{Ca(HPO}_4\text{)}\cdot2\text{H}_2\text{O} )</td>
<td>3158 (w) 1646 (w) 1413 (w,br,d) 1345 (vs, shoulder) 1056 (m) 869 (m) 796 (m, d?) 669 (w) 562 (m, shoulder) 523 (vs)</td>
</tr>
<tr>
<td><strong>Aragonite</strong> ( \text{Ca(CO}_3\text{)} )</td>
<td>1782 (w) 1449 (vs,d,shoulder) 1083 (m) 855 (vs) 713 (m) 700 (m)</td>
</tr>
<tr>
<td><strong>Calcium Hydroxyapatite</strong> ( \text{Ca}_5(\text{PO}_3\text{)}_3(\text{OH}) )</td>
<td>3571 (w) 1989 (w) 1087 (m) 1024 (vs) 962 (s) 628 (m) 598 (s) 562 (s)</td>
</tr>
<tr>
<td>Compound</td>
<td>Wavenumber (cm(^{-1}))</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>Calcium Phosphate Dibasic</td>
<td></td>
</tr>
<tr>
<td>(\text{Ca(H}_3\text{PO}_4)(\cdot\text{2 Cl})</td>
<td>1396 (w, br)</td>
</tr>
<tr>
<td></td>
<td>1342 (w, br)</td>
</tr>
<tr>
<td></td>
<td>1124 (m)</td>
</tr>
<tr>
<td></td>
<td>1057 (s)</td>
</tr>
<tr>
<td></td>
<td>989 (s)</td>
</tr>
<tr>
<td></td>
<td>884 (s, br, d?)</td>
</tr>
<tr>
<td></td>
<td>535 (s, d)</td>
</tr>
<tr>
<td>Calcium Phosphate Monobasic</td>
<td></td>
</tr>
<tr>
<td>(\text{Ca(H}_3\text{PO}_4)(\cdot\text{2 Cl})</td>
<td>2924 (w)</td>
</tr>
<tr>
<td></td>
<td>1648 (w)</td>
</tr>
<tr>
<td></td>
<td>1217 (m, d)</td>
</tr>
<tr>
<td></td>
<td>1157 (w)</td>
</tr>
<tr>
<td></td>
<td>1080 (s)</td>
</tr>
<tr>
<td></td>
<td>955 (s)</td>
</tr>
<tr>
<td></td>
<td>856 (m)</td>
</tr>
<tr>
<td></td>
<td>668 (m)</td>
</tr>
<tr>
<td></td>
<td>568 (m)</td>
</tr>
<tr>
<td></td>
<td>548 (m)</td>
</tr>
<tr>
<td></td>
<td>481 (vs)</td>
</tr>
<tr>
<td>Calcium Phosphate Tribasic</td>
<td></td>
</tr>
<tr>
<td>(\text{Ca}_3\text{(H}_3\text{PO}_4)(\cdot\text{6 Cl})</td>
<td>3571 (w)</td>
</tr>
<tr>
<td></td>
<td>1089 (m)</td>
</tr>
<tr>
<td></td>
<td>1024 (vs)</td>
</tr>
<tr>
<td></td>
<td>966 (m)</td>
</tr>
<tr>
<td></td>
<td>630 (m)</td>
</tr>
<tr>
<td></td>
<td>600 (m)</td>
</tr>
<tr>
<td></td>
<td>560 (s)</td>
</tr>
<tr>
<td></td>
<td>472 (w)</td>
</tr>
<tr>
<td>Calcium Hydroxide</td>
<td></td>
</tr>
<tr>
<td>(\text{Ca(OH)}_2)</td>
<td>3640 (s)</td>
</tr>
<tr>
<td></td>
<td>1415 (w, br)</td>
</tr>
<tr>
<td></td>
<td>873 (w)</td>
</tr>
</tbody>
</table>
**Calcite**
\[ \text{CaCO}_3 \]

2511 (w)
1794 (w)
1399 (vs)
872 (s)
712 (m)

**Cholesterol**
\[ \text{C}_{27}\text{H}_{46}\text{O} \]

3389 (w, br)
2930 (s)
2866 (m)
1464 (m)
1435 (m, d)
1365 (m, d)
1055 (s)
1022 (m)
953 (m)
840 (m)
799 (m)
593 (m)
501 (m)

**Monetite**
\[ \text{CaHPO}_4; \text{Calcium hydrogen phosphate} \]

3200 (w, br)
1603 (w, br)
1360 (w, br, d)
1024 (vs, br)
555 (s)
<table>
<thead>
<tr>
<th>Compound</th>
<th>cm&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>%T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorapatite Ca&lt;sub&gt;5&lt;/sub&gt;(PO&lt;sub&gt;4&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;F</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3538 (w)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1995 (w, br)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1090 (m)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1025 (vs)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>964 (s)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>599 (s)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>566 (s)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>473 (w)</td>
<td></td>
</tr>
<tr>
<td>Struvite MgNH&lt;sub&gt;4&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;•6 H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3265 (w)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1649 (w)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1234 (w)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1162 (m)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1056 (s)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1019 (s)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>884 (m)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>649 (m, br)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>505 (s)</td>
<td></td>
</tr>
<tr>
<td>Weddellite CaC&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;4&lt;/sub&gt;•2 H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1161 (m)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1063 (s)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>792 (m, d)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>695 (w)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>451 (s)</td>
<td></td>
</tr>
<tr>
<td>Whitlockite Ca&lt;sub&gt;9&lt;/sub&gt;(Mg, Fe)H(PO&lt;sub&gt;4&lt;/sub&gt;)&lt;sub&gt;7&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1161 (m)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1056 (s)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>922 (m, shoulder)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>795 (m)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>777 (m)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>694 (w)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>604 (w)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>559 (w)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>450 (vs)</td>
<td></td>
</tr>
<tr>
<td>Compound</td>
<td>Molecular Formula</td>
<td>Vibrational Frequencies (cm(^{-1}))</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>Uric Acid</td>
<td>C(_5)H(_4)N(_4)O(_3)</td>
<td>2993 (s, br) 2822 (s,d) 2025 (w) 1659 (vs,d) 1587 (s) 1485 (m) 1435 (m) 1400 (m) 1349 (m) 1302 (m) 1121 (s) 1026 (m) 991 (s) 875 (m) 778 (s) 742 (vs) 705 (s) 618 (s) 572 (s) 520 (s) 470 (vs)</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>C(_6)H(_12)N(_2)O(_4)S(_2)</td>
<td>3008 (br) 2093 (w) 1656 (w) 1620 (m) 1579 (s) 1482 (s) 1403 (vs) 1381 (s) 1337 (s) 1296 (s) 1193 (m) 1124 (m) 1089 (m) 1040 (m) 962 (m) 872 (m) 845 (s) 775 (s) 674 (s) 615 (m) 537 (vs) 451 (vs)</td>
</tr>
</tbody>
</table>
Calcium Urate

$\text{Ca}\left(\text{H}_{2}\text{NCONH}_2\right)\cdot \frac{1}{2}\text{Ca}$

- $3434 \text{ (m, shoulder)}$
- $2897 \text{ (m, br)}$
- $2769 \text{ (m, br)}$
- $2140 \text{ (w)}$
- $2033 \text{ (w)}$
- $1635 \text{ (vs, shoulder)}$
- $1586 \text{ (s)}$
- $1488 \text{ (w)}$
- $1423 \text{ (w)}$
- $1402 \text{ (m)}$
- $1326 \text{ (m)}$
- $1283 \text{ (w)}$
- $1216 \text{ (w)}$
- $1117 \text{ (m)}$
- $1036 \text{ (w)}$
- $993 \text{ (m)}$
- $873 \text{ (w)}$
- $777 \text{ (s)}$
- $745 \text{ (s)}$
- $705 \text{ (s)}$
- $659 \text{ (w)}$
- $621 \text{ (m)}$
- $573 \text{ (m)}$
- $515 \text{ (m)}$
- $467 \text{ (s)}$

Carbonate Hydroxyapatite

$\text{CaCO}_3(\text{OH})(\text{PO}_4)$

- $3569 \text{ (w,br)}$
- $1469 \text{ (w)}$
- $953 \text{ (m, shoulder)}$
- $902 \text{ (m, shoulder)}$
- $846 \text{ (m,br)}$
- $624 \text{ (w)}$
Uric Acid Sodium Salt

3598 (w, sh)
3033 (m, br, d)
2924 (m, shoulder)
2672 (w, shoulder)
2056 (w)
1738 (m)
1654 (s)
1610 (s)
1530 (m)
1430 (m)
1383 (s)
1349 (m)
1258 (m)
1200 (w)
1137 (w)
1046 (w)
1003 (m)
885 (m)
831 (m)
798 (s)
764 (s)
740 (s)
721 (s)
657 (m)
626 (m)
597 (m)
548 (m, br)
529 (s)
491 (s)

Whewellite
CaC₂O₄•H₂O
Calcium oxalate monohydrate

3421 (w)
3329 (w)
3048 (w)
1611 (s)
1379 (w)
1314 (s)
777 (m)
659 (m)
590 (m)
511 (m)
A.3.2  Other Libraries

Numerous other libraries are currently available, though most research groups prefer to generate their own; libraries are often developed for use in conjunction with computer programs to quantitatively evaluate components present. [26, 136] The difference between these libraries and the library presented here is that we have created a reference library with very little sample preparation. While other libraries have used a KBr pellet technique requiring a homogeneous mixture of sample and KBr, we have used the neat (pure) sample, obtained directly from the mineral source in most cases.

A.4  Conclusions

While several commercial libraries of renal stone spectra exist, it is preferable to create a new library for use with our instruments and samples. The next step in broadening this library is to make quantitative ratios of some of the more common species for analysis, similar to what was done in Chapter 4.
References


