NEAR-INFRARED MICROSCOPY AND IMAGE ANALYSIS
FOR PHARMACEUTICAL PROCESS CONTROL

A thesis submitted in partial fulfilment of the requirements of The University of London for the degree of Doctor of Philosophy in the Faculty of Medicine by

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This thesis describes research conducted in the School of Pharmacy, University of London between August 1999 and July 2006 under the supervision of Professor Anthony Moffat and Doctor Roger Jee. I certify that the research described is original and that parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.
ABSTRACT

Near-infrared microscopy (NIRM) provides low-level matrix information on the composition and distribution of ingredients within an intact pharmaceutical blend or tablet. The technique couples microscopy with spectroscopy, allowing spectral information to be obtained from small regions (~625 μm²), and with sample movement provides information on a specified area. The combination of spatial and spectral information gives rise to a hyper-spectral data cube, whereby following a defined pixel though the data cube gives rise to a NIR spectrum. These cubes contain over 25,000 spectra and therefore data manipulation and image analysis are critical to producing an informative chemical image which provides a physical fingerprint of pharmaceutical solid dosage forms.

To address the applicability of NIRM to pharmaceutical samples, it was necessary to evaluate the optimum data acquisition conditions, considering where spectral data actually came from. The sampling volume was explored by examination of the lateral resolution and depth of penetration of the NIR radiation. It was found for NIRM that 95% of the signal came from a minimum depth of 109 μm. Lateral resolution was found to be dependent on magnification and aperture size.

Using NIRM, a wide range of pharmaceutical samples have been examined, ranging from pre-tabletting blends through to finished dosage forms. By use of multivariate and image analysis methods it has been possible to extract useful chemical information and create ‘visual’ images of the sample. These images provide information on the size, shape and distribution of each component within the formulation. This information has been utilized in studies of blend homogeneity, dissolution and manufacturing production issues. In each example, NIRM provides novel insight into the matrix changes induced upon processing, improving knowledge and understanding of pharmaceutical processes and potentially showing the applicability of this technology for pharmaceutical process control.
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I hope what follows will not disappoint, Happy Reading!
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<tr>
<td>2D</td>
<td>two dimensional</td>
</tr>
<tr>
<td>AOTF</td>
<td>acoustic optical tuneable filter</td>
</tr>
<tr>
<td>ASTM</td>
<td>American Society for Testing and Materials</td>
</tr>
<tr>
<td>API</td>
<td>active pharmaceutical ingredient</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
</tr>
<tr>
<td>cm⁻¹</td>
<td>wavenumber (reciprocal centimetres)</td>
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<tr>
<td>CDER</td>
<td>Centre for Drug Evaluation and Research</td>
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<tr>
<td>CGMPs</td>
<td>Current Good Manufacturing Practices</td>
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<td>DCP</td>
<td>dibasic calcium phosphate</td>
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<td>FPA</td>
<td>focal plane array</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>K-M</td>
<td>Kubelka-Munk</td>
</tr>
<tr>
<td>L1</td>
<td>location 1 for manufacture of product Y</td>
</tr>
<tr>
<td>L2</td>
<td>location 2 for manufacture of product Y</td>
</tr>
<tr>
<td>LCTF</td>
<td>liquid crystal tuneable filter</td>
</tr>
<tr>
<td>LDA</td>
<td>linear discriminant analysis</td>
</tr>
<tr>
<td>LVF</td>
<td>linear variable filter</td>
</tr>
<tr>
<td>mg</td>
<td>milligrams</td>
</tr>
<tr>
<td>mm</td>
<td>millimetres</td>
</tr>
<tr>
<td>mins</td>
<td>minutes</td>
</tr>
<tr>
<td>MCC</td>
<td>microcrystalline cellulose</td>
</tr>
<tr>
<td>MCR</td>
<td>multivariate curve resolution</td>
</tr>
<tr>
<td>MCT</td>
<td>mercury cadmium telluride</td>
</tr>
<tr>
<td>MgS</td>
<td>magnesium stearate</td>
</tr>
<tr>
<td>MSC</td>
<td>multiplicative scatter correction</td>
</tr>
<tr>
<td>MSD</td>
<td>mean of PLS score distribution</td>
</tr>
<tr>
<td>MIR</td>
<td>mid-infrared</td>
</tr>
<tr>
<td>nm</td>
<td>nanometre</td>
</tr>
<tr>
<td>NA</td>
<td>numerical aperture</td>
</tr>
<tr>
<td>NIR</td>
<td>near-infrared</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NIRM</td>
<td>near-infrared microscopy</td>
</tr>
<tr>
<td>NIRS</td>
<td>near-infrared spectroscopy</td>
</tr>
<tr>
<td>NIR-GI</td>
<td>near-infrared global imaging</td>
</tr>
<tr>
<td>NIR-PM</td>
<td>near-infrared point mapping</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NN</td>
<td>nearest neighbour</td>
</tr>
<tr>
<td>OTC</td>
<td>oxytetracycline hydrochloride</td>
</tr>
<tr>
<td>PCA</td>
<td>principal component analysis</td>
</tr>
<tr>
<td>PC</td>
<td>principal component</td>
</tr>
<tr>
<td>PLS</td>
<td>partial least squares regression</td>
</tr>
<tr>
<td>PP</td>
<td>pyrantel palmoate</td>
</tr>
<tr>
<td>Pra</td>
<td>prazosin hydrochloride</td>
</tr>
<tr>
<td>p.s.i</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>PRESS</td>
<td>predicted residual error sum of squares</td>
</tr>
<tr>
<td>PTFE</td>
<td>polytetrafluoroethylene</td>
</tr>
<tr>
<td>QAL</td>
<td>quality alert limit</td>
</tr>
<tr>
<td>RGB</td>
<td>red green blue</td>
</tr>
<tr>
<td>RSTD</td>
<td>relative standard deviation</td>
</tr>
<tr>
<td>S1</td>
<td>stage one of dissolution testing using US pharmacopeia methodology</td>
</tr>
<tr>
<td>S2</td>
<td>stage two of dissolution testing using US pharmacopeia methodology</td>
</tr>
<tr>
<td>S3</td>
<td>stage three of dissolution testing using US pharmacopeia methodology</td>
</tr>
<tr>
<td>SIMS</td>
<td>secondary ion mass spectrometry</td>
</tr>
<tr>
<td>s/n</td>
<td>signal to noise ratio</td>
</tr>
<tr>
<td>SNV</td>
<td>standard normal variant</td>
</tr>
<tr>
<td>SOP</td>
<td>standard operating procedure</td>
</tr>
<tr>
<td>SSG</td>
<td>sodium starch glycolate</td>
</tr>
<tr>
<td>SVD</td>
<td>singular value decomposition</td>
</tr>
<tr>
<td>STD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>Tio</td>
<td>ticonazole</td>
</tr>
<tr>
<td>TOF-SIMS</td>
<td>time-of-flight secondary ion mass spectrometry</td>
</tr>
<tr>
<td>μm</td>
<td>micrometres</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>w/w</td>
<td>weight of individual component divided by weight of total formulation</td>
</tr>
</tbody>
</table>
**List of Symbols**

- $\alpha$: extent of the Airy ring, minimum resolvable spatial resolution
- $a$: absorptivity
- $a'_{i}$: image contrast value
- $a'$: proportionality constant
- $a_{n}$: intercept of the least squares regression line
- $A$: apparent absorbance
- $A_{i}$: the surface sampling area in NIR microscopy experiment
- $A_{2}$: the inner area
- $A_{x}$: absorbance at position $x$ in the line-scan
- $A_{p_{1}}$: absorbance values of the pure component 1
- $A_{p_{2}}$: absorbance values of the pure component 2
- $b$: intercept on the y-axis for exponential function
- $b_{1}$: linear co-efficient of score vectors $t$ and $u$ in PLS
- $b_{n}$: slope of the least squares regression line
- $c$: concentration of an absorbing analyte
- $c_{s}$: $\log_{10}$ of the number of calcium silicate domains
- $C$: concentration matrix
- $\delta$: number of times a spectral ordinate is differentiated to give derivative spectrum
- $d$: offset of data from zero for exponential function
- $d_{v}$: dissolution value at 45 minutes
- $D$: diameter of objective
- $DP_{50}$: depth radiation penetrates into a sample to give signal corresponding 50% intensity of pure substrate
- $D$: data matrix – two dimensional matrix of spectral data
- $\eta$: refractive index
- $\epsilon$: molar absorptivity
- $e_{m}$: the residual spectrum after MSC fitting
- $e$: residual score vector from PLS analysis
$E$ total energy of particle

$E_b$ energy required to compress or extend a bond

$E_v$ Vibrational energy levels of diatomic molecules

$E$ residual / noise matrix of data matrix $D$

$f$ focal length

$F$ F-Number of Objective (related to the objective diameter and the focal length)

$F$ residual noise matrix of the concentration matrix $C$

$G$ residual noise matrix of concentration matrix $C$ after PLS decomposition

$h$ Planck's Constant ($6.626 \times 10^{-34}$ J s)

$h$ Planck's Constant ($1.054 \times 10^{-34}$ Joule seconds)

$i$ the angle that light disperses away from the 'apertured' area

$I$ number of spectral classes in PLS library

$ID$ information depth

$j$ individual wavelength of set $J$

$J$ wavelength range

$\kappa$ absorption coefficient

$k$ bonding force constant

$\lambda$ wavelength of light

$\lambda_2$ unique component 2 absorption

$l$ latent variable (integer 1, 2, ..., $n$)

$L$ total number of latent variables, $l$, in a model.

$\mu$ reduced mass

$m$ mass of single particle

$\nu$ vibrational quantum number

$\Delta \nu$ change in vibrational state

$n$ an integer

$N$ number of nuclei in a polyatomic molecule

$NA$ numerical aperture

$p$ number of principal components in PCA decomposition

$P_i$ eigenvector of the 1st principal component with direction through $D$ that explains the most variability in the data
eigenvector of the 2nd principal component with direction through $\mathbf{D}$ that explains the most of the remaining variability in the data and is orthogonal to $p_1$

loading vector from PCA analysis of $\mathbf{D}$ for the $p^{th}$ principal component

$P$ Loadings Matrix after decomposition of $\mathbf{D}$

$Q$ quantity of dissolved active ingredient specified in product monograph as a percentage of the labelled content

$Q$ loadings matrix after decomposition of $\mathbf{C}$

$r$ internuclear distance

$r_{eq}$ bond length of system when the energy is at a minimum

$r_{unk}$ vector of responses from unknown sample

$\text{rows}$ represents the number of rows in data matrix $\mathbf{D}$.

$R^2$ coefficient of determination for linear regression and is the proportion of the variance explained by the model (if perfect predictability $R^2 = 1$)

$R$ relative reflectance, the ratio of the intensity of light reflected by a sample to that of a standard

$R_\infty$ is the absolute reflectance of an infinitely thick opaque sample

$\sigma$ standard deviation of spectrum over all $J$ wavelengths

$s$ scattering coefficient

$sr$ spatial resolution

$\sin \theta$ sin of the angle of incident light divided by the refractive index of the reflecting surface.

$\tau$ rate constant

$t$ thickness penetrated to reach the substrate

$t_1$ score vector of the 1st principal component determined by projection of $\mathbf{D}$ onto $p_1$

$t_2$ score vector of the 2nd principal component determined by projection of $\mathbf{D}$ onto $p_2$

$t_p$ score vector for $p^{th}$ principal component from decomposition of $\mathbf{D}$

$t$ is the score vector (for any latent variable $l$ ) from decomposition of variation in $\mathbf{D}$ in PLS

$t_{unk}$ score vector from unknown sample response in PLS

$\mathbf{T}$ scores Matrix after decomposition of $\mathbf{D}$

$u$ un-processed signal

$\bar{u}$ mean signal of unprocessed spectrum over all $J$ wavelengths
- \( \mathbf{u} \): score vector for latent variable \( l \) which describes the variation in the data set from decomposition of variation in \( \mathbf{C} \) in PLS
- \( \mathbf{u}_{\text{unk}} \): estimate of score vector of \( \mathbf{C} \) for an unknown sample response (\( \mathbf{r}_{\text{unk}} \))
- \( \mathbf{U} \): scores Matrix after decomposition of \( \mathbf{C} \)
- \( V(x) \): potential energy function of single particle of mass \( m \) moving in the \( x \) direction
- \( w \): number of wavelength channels in 3 dimensional image cube (z-axis)
- \( x \): direction
- \( x \): \( x \)-axis dimension in 3D image cube
- \( y \): \( y \)-axis dimension in 3D image cube
- \( y_{50} \): absorbance value that corresponds to half the pure drug response
- \( y_m \): mean spectrum for set of \( z \) spectra
- \( y_n \): raw spectrum from a set of \( z \) spectra (\( i=1,2...z \))
- \( y_{\text{MSC}} \): MSC transform of raw spectra
- \( y_{ij} \): SNV transformed signal at wavelength \( j \)
- \( \zeta \): fraction of light incident on a surface that is reflected rather than absorbed
- \( z \): number of spectra in a set
- \( z \): \( z \)-axis dimension in 3D image cube, contains spectral information
- \( \phi \): angle of incidence of light
- \( \chi_e \): anharmonicity constant
- \( \psi(x) \): wavefunction of the \( x \) co-ordinate
- \( \omega \): vibrational frequency of an elastic bond
- \( \omega_e \): oscillation frequency with a corresponding anharmonicity constant of \( \chi_e \)
1. INTRODUCTION

1.1 QUALITY CONTROL OF PHARMACEUTICAL SOLID DOSAGE FORMS

One of the most common pharmaceutical dosage forms is a tablet. A tablet is a solid object that has been formed by firstly mixing a number of components together and then placing 'units' of the mixture into cavities and applying a compression force that binds the materials together. This mixture can be named the pre-compression blend and consists of the active pharmaceutical ingredient (API) and a number of excipients. Each excipient is present in the formulation to serve a specific role, e.g. magnesium stearate is typically used as a lubricant, which aids ejection of the tablet from the punch after compression (Carstensen, 1993). Despite the presence of at least two other components, the main quality control on the tablet during manufacture relates to the mixing of the API component in the pre-compression blend and on the even distribution of API between dosage units in the final dosage form.

Typically a tablet is released from production to sales based on bulk measurements (chemical and physical properties) of a small sample of tablets from each batch. These measurements include potency, where the average amount of API in 20 tablets is measured, and content uniformity where the potency of 10 individual tablets taken across a batch are compared (European Pharmacopoeia, 2006a). Physical parameters are also measured such as hardness and mass, along with chemical stability. Tablets are also tested for dissolution properties, which is a measure of bioavailability but could also expose differences in excipient concentration in each tablet. The main focus of all these various tests is the amount of API present, and the amount released from the tablet. No method is currently used which allows the performance of the individual ingredients to be compared, relative to each other, in one measurement.
One method, which has the potential to provide information on all components in one analysis, is near-infrared microscopy (NIRM). Near-infrared spectroscopy (NIRS) (Section 1.2) is now a widely accepted technique for the analysis of pharmaceutical products with applications ranging from raw material assessment through to final product testing. At this ‘macro-scale’, NIRS provides chemical information from a bulk sample. Chemical interactions may also be determined due to peak shape or wavelength shifts but information on the matrix composition, i.e. individual components distribution cannot be truly determined.

NIRM (Section 1.5) relates to the coupling of NIRS with a microscope. It offers the opportunity to explore what chemical species are present at a micro-scale level (by NIRS) but also provides spatial information on their distribution within a sample, either by sample movement or multi-element detectors, hence exposing spatial (physical) information on the sample. With spatial and chemical information it is possible to generate chemical images of each ingredient within the formulation. NIRM may therefore be applicable to pharmaceutical samples both in process testing and quality control, as it should provide a means to visualise the pharmaceutical sample and describe the relative distribution and cluster sizes of the ingredients within either the pre-compression blend or in the finished tablet. This would provide a method that would allow all components to be measured in one analysis and the differences in the microscopic structural information obtained can be correlated to the macroscale properties of the tablet.

1.2 FUNDAMENTALS OF NEAR-INFRARED SPECTROSCOPY

William Herschel first identified the NIR region of the electromagnetic spectrum in 1800. Herschel had been attempting to find which colour in the visible spectrum could be attributed to the heat from the sun. To answer this question, Herschel used a glass prism to disperse white light into its individual coloured components and passed a thermometer through each region. Herschel noticed a slight difference on passing
through the visible spectrum, but nothing that could be equated to the heat from the sun. However, on passing the thermometer beyond the red region of the spectrum, the thermometer temperature changed dramatically and Herschel assumed that an invisible band existed and named it the region infra red. This was the first discovery of what is now referred to as NIR radiation as glass will typically absorb mid-range infrared radiation.

The NIR region is defined by the European Pharmacopoeia (2006b) as having a wavelength range from approximately 780 nm to 2500 nm (wavenumber range 12800 to 4000 cm\(^{-1}\)). The absorptions in this region represent the overtones and combinations from the fundamental molecular vibrations found in the mid-infrared (MIR) region. These overtones and combinations are mainly associated with X-H stretching modes (e.g. C-H, O-H, N-H and S-H). Theoretically, the overtone and combination absorptions in the NIR region are forbidden as the Schrödinger equation approximates most bonds to a harmonic oscillator, where selection rules state that the vibrational change (\(\Delta \nu\)) can only be \(\Delta \nu = \pm 1\) (Alberty and Sibley, 1992). However, overtones occur when \(\Delta \nu = \pm 2, \pm 3\ldots\) and combination absorptions cover transitions from the ground state to an excited state occurring concurrently in different vibrational states. In practice, overtones and combination absorptions are observed because of anharmonicity.

Anharmonicity is best explained by first looking at the simple harmonic oscillator model for a bond. Without the influence of external energy, two atoms will form a stable covalent molecule due only to electronic rearrangement, which balances the forces. The atoms settle at a mean distance where the forces are balanced and the energy of the system is at a minimum. If an attempt is made to pull the atoms apart, there is a resistance from the attractive forces and if pushed together there is opposition from the repulsive forces. In either case an attempt to distort the bond requires an input of energy. The compression and extension of a bond may be considered similar to the
behaviour of a spring and hence obeys Hooke's Law. The energy required to compress or extend the bond \( E_b \) is described by:

\[
E_b = \frac{1}{2} k (r - r_{eq})^2
\]  

(1.1)

Where \( k \) is the bonding force constant (a measure of the strength or rigidity of a chemical bond in its equilibrium position), \( r \) the internuclear distance and \( r_{eq} \) is the bond length when the energy of the system is at a minimum. Hence, as energy into the system increases, the degree of compression or extension will be greater but the vibrational frequency will not change. This is because, like a spring, an elastic bond has a certain vibrational frequency \( \omega \) that is dependent on the reduced mass \( \mu \) of the system and the bonding force constant \( k \) and for a simple harmonic oscillator is described by:

\[
\omega = \frac{1}{2 \pi} \sqrt{\frac{k}{\mu}}
\]  

(1.2)

The fundamental vibrational frequency of simple diatomic molecules and the average values of two-atom stretches within polyatomic molecules do not deviate greatly from this calculation but the solution is only an average or centre frequency of the diatomic bond (Ciurczak, 2001a). If the reduced mass of bonds are similar, e.g. C-H \( (\mu = 0.85) \), N-H \( (0.87) \) and O-H \( (0.89) \), Equation 1.2 implies that the frequencies of these pairs would be similar. This does not occur in practice as in real molecules the electron donating or withdrawing properties of neighbouring atoms/groups influence the bond strength \( k \) and length \( r_{eq} \), which in turn affect the frequency of the X-H bond.

Unlike a classical spring model, molecular vibrations do not have an infinite number of energy levels. Instead of a continuum of energies, vibrational energies are quantised forming discrete energy levels described by quantum theory. Allowed vibrational energies may be calculated from the time-independent Schrödinger equation for a
single particle of mass \( m \) moving in the \( x \) direction with a potential energy function \( V(x) \):

\[
\frac{-\hbar^2}{2m} \frac{d^2 \psi(x)}{dx^2} + V(x) \psi(x) = E \psi(x)
\]  (1.3)

where the wavefunction \( \psi(x) \) is a function of the \( x \) co-ordinate, \( \hbar \) is \( h/2\pi \) where \( h \) is Planck's constant, and \( E \) is the total energy of the particle (Alberty and Sibley, 1992). This equation is solved using the vibrational Hamiltonian for a diatomic molecule. The solution of this equation leads to somewhat complicated values for each discrete energy level, but a simplified version of these levels may be written for the energy levels of diatomic molecules given by

\[
E_v = (v + \frac{1}{2}) \omega \quad v = 0, 1, 2
\]  (1.4)

where \( v \) is the vibrational quantum number and \( \omega \) the vibrational frequency (1.2). Hence, the energy levels are equally spaced with a separation of \( \omega \), Figure 1.1a. This figure also shows that even with lowest vibrational energy (\( v = 0 \)) diatomic bonds do not have zero vibrational energy and hence they are never completely at rest relative to one another.

In real molecules, the simple harmonic oscillator model is an over simplification. Although real bonds are elastic, they are not so homogenous as to obey Hooke's Law. If the bond between two atoms has the same force applied for extension and compression, the same level of distortion will not always occur. There also comes a point during stretching of the bond that the molecule dissociates into atoms. As such, more complicated behaviour is observed than represented by a simple harmonic oscillator, instead a more typical shape for the energy curve is shown in Figure 1.1b, an anharmonic oscillator.
The Morse function is an empirical expression that is a good approximation to this curve. When this equation is used in solving the Schrödinger equation, the resultant pattern of allowed vibrational energy levels is found to be

\[ E_v = (\nu + \frac{1}{2})\omega_e^2 - (\nu + \frac{1}{2})^2 \omega_e^2 \chi_e \]  

where \( \omega_e \) is the oscillation frequency with a corresponding anharmonicity constant of \( \chi_e \). In Figure 1.1b it can also be seen that as \( \nu \) increases the vibrational levels crowd closer together, losing the equal spacing observed in the harmonic oscillator. Rearranging Equation 1.5 and comparing with the harmonic oscillator (Equation 1.4) gives rise to a vibrational frequency described by Equation 1.6.

\[ \omega = \omega_e (1 - \chi_e (\nu + \frac{1}{2})) \]  

\( E_v \) is the energy level, \( \nu \) is the vibrational quantum number, \( \omega_e \) is the oscillation frequency, and \( \chi_e \) is the anharmonicity constant.
This implies that the anharmonic oscillator behaves like the harmonic oscillator but with a vibrational frequency that decreases steadily with \( \nu \). The selection rules for the anharmonic oscillator are found to be \( \Delta \nu = \pm 1, \pm 2, \pm 3, \ldots \pm n \), where \( n \) is an integer, which unlike the harmonic oscillator allows the possibility of larger jumps. These are predicted by theory and in practice only the lines of \( \Delta \nu = \pm 1, \pm 2, \pm 3 \), at the most, have observable intensity. The transition with \( \Delta \nu = \pm 1 \), is called the fundamental frequency and has considerable intensity. It is these fundamental frequencies that give rise to the bands in the MIR region. The wavenumber position of these bands is given by

\[
E_{\nu=1} - E_{\nu=0} = \omega_\nu (1 - 2 \chi_\nu) \tag{1.7}
\]

As the anharmonicity value is usually seen to be between 1 \(- 5\%\), the right hand side of Equation 1.7 is usually approximated to \( \omega_\nu \). Higher frequency bands are also observed as selection rules state that \( \Delta \nu = \pm 2 \) or \( \pm 3 \), these are called overtones and form the basis of NIRS. These bands appear at frequencies two or three times higher than the fundamental frequency, i.e. \( \Delta \nu = \pm 2 \) means that \( \Delta E = 2 \omega_\nu (1 - 3 \chi_\nu) \approx 2 \omega_\nu \) and similarly for \( \Delta \nu = \pm 3 \) it follows that \( \Delta E \approx 3 \omega_\nu \).

Another prominent feature of the NIR spectrum is the large number of combination bands. In addition to bands being produced at twice or three times the frequency of the fundamental, there is a tendency for two or more vibrations to combine (through addition or subtraction of the energies) to give a single band. To explain combination bands, it is good to examine polyatomic molecules. In non-linear polyatomic molecules there are \( 3N - 6 \) energy levels, where \( N \) is the number of nuclei. Hence, in a system such as water, there are three fundamental vibrations (two symmetric and one asymmetric stretch). These fundamental frequencies may exhibit simultaneous changes in the energies of two or more of the vibrational modes, the frequency of the
observed bands will be either, the sum of \( (\omega_1 + \omega_2, 2\omega_1 + \omega_2, \text{etc.}) \) or the difference between \( (\omega_1 - \omega_2, 2\omega_1 - \omega_2) \) the individual fundamental frequencies. The result is very weak bands named combination and subtraction bands, although the later is rarely observed at room temperature in the NIR region, (Blanco et al, 1998).

As stated previously, the major contributions to the NIR region are those frequencies associated with overtone and combination bands, in particular those associated with X-H bonds. The reason for their presence in the NIR region is that the small mass and large force constant for hydrogen results in a high fundamental frequency for this atom (Blanco, 1998). A high fundamental frequency (e.g. asymmetric stretch in water at 3756 cm\(^{-1}\)) results in a first and second overtone in the NIR region (7512 cm\(^{-1}\) and 11268 cm\(^{-1}\)). However, groups such as C-Cl have a fundamental vibration at low frequencies in the MIR region (~750 cm\(^{-1}\)) and therefore the first few overtones also exist in MIR region (1500 cm\(^{-1}\), 2250 cm\(^{-1}\) and 3000 cm\(^{-1}\)).

1.3 Near-infrared Diffuse Reflectance Spectroscopy

There are three different means by which NIR spectra can be obtained: transmission, reflection or transflection measurements. Transmittance is described by the American Society for Testing and Materials (ASTM, 2000) as the ratio of radiant power transmitted by the sample to the radiant power incident on the sample, shown in Figure 1.2a. Reflectance is described as the ratio of the radiant power reflected by the sample to the radiant power incident on the sample, shown in Figure 1.2b. Finally, transflectance is described as an experimental method whereby radiant energy that is transmitted through the specimen is returned through the specimen by the means of an external reflector, shown in Figure 1.2c. In this research only reflectance measurements have been used and hence will be discussed further.
Reflection spectroscopy, Figure 1.2b, measures the amount of light reflected from a sample 'surface'. The resultant signal contains a specular component and a diffuse component. The specular component is described by Fresnel's Law (Blanco, 1998). It describes the fraction of light ($\zeta$) incident on a surface that is reflected rather than absorbed (Equation 1.8) and is a function of the angle of incidence of light ($\phi$) and the refractive index ($\eta$) of the surface. Hence, the specular component contains little composition information and therefore detectors in NIR spectrometers are positioned to minimise the specular contributions, e.g. as shown in Figure 1.2b.

$$
\zeta = \frac{\sin^2(\phi - \theta) + \tan^2(\phi - \theta)}{\sin^2(\phi + \theta) + \tan^2(\phi + \theta)} \quad \text{where} \quad \sin \theta = \frac{\sin \phi}{\eta}
$$

Therefore it is the diffuse component of reflected radiation that contains the information required for NIRS. There are a number of different theories on diffuse reflectance, but the most widely accepted is that of Kubelka and Munk from 1931. (N.B. Many people have interpreted this work, (Blanco, 1998; Olinger et al, 2001) and it is these examples which have been interpreted for this research.) The Kubelka-Munk (K-M) function is described in Equation 1.9, where $R_\infty$ is the absolute reflectance of an infinitely thick opaque sample with an absorption coefficient $\kappa$ and a scattering coefficient $s$. 

Figure 1.2 - Schematic of a) transmission, b) reflection and c) transflection methods for acquiring NIR spectra
In practice it is more common to use relative reflectance \((R)\), which is the ratio of the intensity of light reflected by a sample to that of a standard. NIR reflectance standards can be made from a selection of different materials, including magnesium oxide, barium sulphate, ceramics, Spectralon and Teflon (polytetrafluoroethylene). The K-M function can be used for quantitative analysis e.g. to determine the concentration \((c)\) of an absorbing analyte,

\[
f(R) = \frac{(1 - R^2)}{2R} = \frac{\kappa}{s} = \frac{\log \epsilon c}{s} = \frac{c}{a}
\]

where \(\epsilon\) is the molar absorptivity and \(a = s/2.303 \epsilon\). This relationship implies that a plot of \(f(R)\) versus concentration \((c)\) would be linear with a slope of \(1/a\). This statement is only true when there are weak absorption bands and when the product of concentration and absorptivity is low. With the absorptions of organic materials being related to vibrational overtone and combination bands, and not the fundamental vibration, the bands have a low absorptivity. As such most organic analytes are weakly absorbing in the NIR region, without the need for dilution and therefore follow the K-M equation. However, deviations from this equation can arise because the analyte is not isolated from other components. The surrounding matrix often absorbs the incident radiation at least as strongly as the analyte and at the same wavelength as the analyte causing deviations from the K-M equation.

A practical alternative to the K-M equation is a relationship between concentration \((c)\) and relative reflectance \((R)\). This relationship is similar to Beer’s Law and is given by Equation 1.11, where \(A\) is the apparent absorbance, \(R\) is the relative reflectance, \(c\) the concentration and \(a'\) a proportionality constant.
\[ A = \log \frac{1}{R} = a'c \] (1.11)

Hence, although there have been many detailed investigations into the theory of diffuse reflection spectroscopy, there are few results which are useful for both quantitative and qualitative analysis. In fact, simply converting reflectance values to the log \(1/R\) is effective for many powdered samples in NIR reflectance spectroscopy (Olinger et al., 2001).

1.4 Near-infrared Spectroscopy in the Pharmaceutical Industry.

The application of NIRS in the pharmaceutical industry has taken a while to come to fruition. Although the NIR region was first discovered in the early 19th century, the technique was not widely used because the bands in the NIR region are not distinct for discrete bonds. In addition, they have a tendency to overlay and are difficult to resolve, and even if resolved the bands are difficult to interpret and assign. As a result of this, up until the 1980's, NIRS was not a widely used technique, in fact Wetzel (1983) described NIR as "a sleeper among spectroscopic techniques". Around this time only 40 references existed for analytical applications of NIRS, with MIR being the method of choice because of the strong, distinct bands. Over the next ten years, the applications of NIRS dramatically increased, mainly due to computer technology which allowed the data to be treated with complex mathematics and hence provided the opportunity to resolve the NIR absorption bands and associate them with the sample being studied. In 1994 an article was entitled “Near-infrared spectroscopy. The giant is running strong” (McClure, 1994) and the literature is now widely filled with applications of NIRS in the chemical, agricultural, textile and pharmaceutical industries.

The use of NIRS in pharmaceutical analysis is varied and ranges from testing of the input raw materials through all stages of pharmaceutical production (Plugge and van der Vlies, 1992; Hammond et al, 1998a) and to correlate directly with end product tests,
e.g. dissolution and hardness (Ghaly, 2004; Hoag et al, 2005). Utilisation of NIRS through the different pharmaceutical manufacturing stages means it serves in both a qualitative and quantitative role (Ciurczak and Drennen 2001b, Blanco 1998). NIRS has been used not only to look at raw excipient materials, but also for the examination of different polymorphs of the API (Norris et al, 1997; Gimet and Luong, 1987). The technique has also been used for the identification of the API during blending with excipients (Aldridge et al, 1996) as well as within dosage forms (Trafford et al, 1999 and Corti et al, 1999). With NIRS being sensitive to the presence of water, the technique has been utilised for moisture determination and monitoring drying of the API (Derksen et al 1998; Maggard et al 1999). NIR spectra also contain a high content of physical information and for this reason the technique has been exploited in particle size determinations (Ciurczak et al, 1986, O’Neil et al, 1998, 1999, and Higgins et al, 2003).

1.5 Principles of Near-infrared Microscopy
The technique of NIRM relates to the coupling of a microscope with a NIR spectrometer. The idea of the marriage between microscopy and spectroscopy is not new, with the first reported article on the coupling of the two appearing in Nature in 1949 (Barer, 1949). At the same time, a second article was published in Science by Gore (1949) which alongside the Nature paper established the possibility of combining light microscopy with MIR spectroscopy. Over the past 50 years the instrumentation has been improved but also extended into different fields of spectroscopy, including Raman (Delhaye and Dhamelincourt, 1975), fluorescence (Hanley et al, 1999), nuclear magnetic resonance (Nebgen et al, 1995) and NIR (Hammond, 1998b) spectroscopy.

NIRM is used here as a general term to cover the collection of NIR spectra through microscope optics. The instrumentation for the coupling of the microscope with a spectrometer is relatively simple: a source of radiant energy, a means to separate this radiation into discrete energies, an interface allowing this energy to be transmitted or
reflected by the sample, a detector, and a data recording and analysis system. The means by which these data can be obtained can be separated into three different categories, which are shown in Figure 1.3. At this time, all three different methods can be found in commercially available instrumentation.

![Diagram of the modes of data collection for NIRM](image)

Figure 1.3 - Diagram of the modes of data collection for NIRM, where a) is point mapping, b) line mapping and c) global imaging

Although there are a variety of methods to obtain NIR spectra at a micro-level, the results from all experiments are the same: that is a spatial and spectral (chemical) set of information, which can be described as a three dimensional data cube, shown in Figure 1.4. Figure 1.4a shows a hypothetical situation for three components (different shapes) with different spectral signatures. If these three components are mixed together the output data from the microscopy experiment is a cube where the x and y dimensions of the image contains spatial information, and the z axis represents the spectral axis, Figure 1.4b. If a xy plane is examined at a specific wavelength e.g. 1675nm only the component with a response at this wavelength gives a chemical image (component 2 in Figure 1.4b) but if a slice is taken at specific x and y coordinates then through the z-plane it is possible to obtain a spectrum at that point, shown in Figure 1.4b for a component 1 region. Using this set of information together produces chemical images of a sample through identification using the spectral information and determination of size and distribution of a component from the spatial information.
In point mapping experiments (NIR-PM), each acquisition obtains a full spectral component from a fixed point (e.g. an area 20 μm by 20 μm), and then sample movement is required to obtain the spatial information. The light from the spectrometer is focused onto the sample surface (using a Cassegrain lens) and then after interaction with the sample, is focused through a set of apertures onto the detector; as shown in Figure 1.5.

**Figure 1.4 - Schematic representation of a three component model system with different shapes and a) spectral features with b) as the output from a NIRM experiment**

**Figure 1.5 - Optical configuration and light path within near-infrared microscope**
Line mapping experiments (NIR-LM) are very similar to point mapping, in that sample movement is required to build the spatial set of information. However, time to build the spatial component is greatly reduced because of the multi-element detector (16 elements in commercially available systems). The instrumentation utilised in point mapping (a) and line mapping (b) experiments are shown in Figure 1.6.

a)  

b)  

*Figure 1.6 - Photographs of a) Autoimage point mapping and b) Spotlight line mapping instruments*

NIR global imaging (NIR-GI) systems have only really been developed over the past eight years after the de-classification of focal plane array (FPA) technology from the US military. These arrays function as cameras for the NIR region, with each pixel in the array allowing spatial information to be obtained. The spectral component of the data set is obtained by movement through the wavelengths of interest using a wavelength filter, including liquid crystal (LCTF) or acoustic optical (AOTF) tuneable filters (Lewis et al, 1992, 1994a) and more recently linear variable filters (LVF) (Lewis et al, 2002a). This has meant that spectra are no longer collected in a step-wise fashion. Instead, spatial information is obtained from the two-dimensional detector and ‘spectra’ are obtained by building up the wavelength images sequentially changing the tuneable filter, schematically shown in Figure 1.7. The actual instrumentation used for NIR-GI experiments are shown in Figure 1.8.
1.6 APPLICATIONS OF NEAR-INFRARED MICROSCOPY

Over the past ten years, applications of NIRM have been explored but previously, microspectroscopy techniques were focused in other spectral regions, mainly Raman and MIR. The applications have ranged from chemical based, such as determination of component distributions within emulsion systems (Andrew et al, 1998) through to many applications in biological systems. Examples of biological applications have included investigations into the spatial distribution of two different zinc phthalocyanines in cell lines (Freeman et al, 1998), images of human breast cells (Small et al, 2003)
and utilisation of chemical images in tissue biopsies (Centeno and Johnson, 1993; Haka et al, 2001). Infrared imaging systems have also been shown to be applicable to satellite imaging of space (Lewis et al, 1998). Applications in pharmaceuticals have not been widely reported but of those, examples include the use of $^1$H-NMR microscopy for understanding porosity in solid dosage forms (Nebgen et al, 1995) and characterisation of controlled release beads using time-of-flight secondary ion mass spectrometry (TOF-SIMS) to examine the different mass ions (Luk et al, 2003). MIR images have been most widely applied, with applications including the assessment of blend homogeneity of a two component system consisting of ethyl cellulose and captopril (Lin and Lee, 2004) and in the understanding of dissolution performance of tablets prepared using different granulation parameters (Yoggo et al, 2005) also MIR imaging has shown success in the monitoring of the swelling occurring in solid dosage forms during dissolution (Kazarian et al, 2004a, b).

Although other microspectroscopy techniques are available and used to obtain chemical images, the main focus of this research is involved in the NIR region and its application to pharmaceutical samples. The application of NIRM in the pharmaceutical industry is a recent event, with instrumentation only becoming commercially available around eight years ago, ~ 1998. Up until this point, NIRM results were obtained by pushing MIR mercury cadmium telluride (MCT) detectors to their limits (Smith et al, 1989). The benefits offered by NIRM are very similar to macroscale NIRS (widely exploited in pharmaceutical analysis) but the use of NIRM has not been widely reported. In 1995 Lachenal challenged the NIR community — "If NIR was a sleeping giant among spectroscopy techniques $\mu$NIR is perhaps a sleeping dwarf. Try to wake up the dwarf and good luck!"

There are a number of areas that have explored the potential of NIRM, including the biological, pharmaceutical, chemical and food industries. The first biological role for NIRM was proposed in 1995. A NIR confocal microscope was developed to examine
layers of human breast tissue non-destructively, without the need for individual 20 μm thick sections (Murphy et al, 1995). Here the short NIR wavelengths (750 – 1000 nm) were used to penetrate the layers, and the difference in intensity of transmitted signal allowed the normal and diseased tissue states to be distinguished. In work by Lodder et al (1998), the difference in the intensity of lipid signals from rabbits with and without cholesterol feeding was observed as a distribution within the aorta. Lipid signals were also measured in rats, to evaluate NIR imaging as method to monitor lipid metabolism (Buice et al, 1998). More recently, the hydration state of human skin has also been evaluated (Attas et al, 2002a, b), with the strong response from water in the NIR region allowing changes in the epidermal layer to be evaluated after depositing skin treatments and the water response has also been useful in the assessment of burn depths in human skin (Cross et al, 2005). One of the most recent biological applications is in mapping tissue oxygenation in the beating heart, where oxy and deoxy-hemoglobin was resolved using peak area ratios (Shaw et al, 2003). In all biological examples, typically only one species is evaluated in a complex matrix, meaning that the majority of spectral/spatial information obtained is not utilised.

NIRM has also been evaluated as a method for document authentication (Tran et al, 1998; Roux et al, 2005), allowing comparison of ink characteristics at both a spatial and spectral level. There have been proposals that NIRM can be used to determine the sugar content in melon flesh (Sugiyma, 1999) and soluble solids in kiwi-fruits (Martinsen et al, 1998), utilising the NIR spectra of the component of interest to locate its spatial location within the fruit. NIRM has also been shown to be able to detect bruising on pickling cucumbers, although prediction is affected by the age of bruising (Ariana et al, 2006). Faecal contamination in poultry carcasses has also been explored as an application area for NIR imaging (Lawerence et al, 2001) using principal component analysis to resolve breast skin from three different faecal contaminants. The non-destructive nature of NIRM makes it an ideal method for examining works of art (Attas et al, 2002c), allowing different pigments to be examined, classified into
carbon/elemental, iron oxide and organic based compounds and associated to a location within the artwork.

A different approach to utilising NIRM has been undertaken to evaluate synthesis of peptides at a microscale level (400 – 500 µg) (Tran et al, 1999a and 2001). By utilising a camera array it is possible to monitor simultaneously the conversion of amino acids into peptides within multiple wells, hence only the spectral component is used. The same research group has shown that with NIR imaging it is also possible to determine the reaction kinetics of epoxy curing (Tran et al, 1999b, 2000 and 2002) and sol-gel formations (Tran et al, 2002) by monitoring images at known wavelengths.

The reported applications of NIRM to date have tended to either use the chemical information to identify one component within a complicated matrix or use the multi-element detection system to allow multiple features to be monitored simultaneously. It is only when pharmaceutical applications are explored that there seems to be full utilisation of the three-dimensional data obtained from NIRM experiments. This is particularly the case in the first reported article on the potential of NIRM to control the quality of pharmaceuticals (Hammond, 1998b). In this example, identification of three different components of a tablet formulation was possible, and provided information on the spatial distribution of components relative to one another. In fact, it is commented that the active had formed clusters, information unobtainable by any other technique.

NIR-GI has also been utilised to identify the different layers present in time release granules (Lewis et al, 2001). Food and Drugs Administration (FDA) investigations into the potential of NIRM for the analysis of powder blend homogeneity have also proved successful (Lyons et al, 2002). NIR imaging provided a means to visualise the distribution of the API in the formulation, with well blended tablets said to have a general contribution from the API across the entire area analysed but poorly blended tablets having localised API regions. Work by Josefson et al (2006) has also explored blend homogeneity, but with an emphasis on trying to numerically describe different
spatial distributions by use of wavelets. The blend homogeneity of an API was also examined within a V-blender unit by Drennan et al (2001). In this example, the imaging system was not used for ‘micro-scale’ analysis but instead the multi-element FPA was used as a means to monitor variance across the top of a pre-compression blend over a 30 minute blending period, with a two minute data collection interval. It was shown that blending was complete, when the standard deviation between the pixels was minimised. It has also been suggested (Lewis et al; Veronin and Youan, 2004) that NIR chemical images can be used in the assessment of counterfeits, as the API distribution can be compared between manufacturers.

With reported applications of NIRM, there is still a trend to measure either one single component’s distribution or utilise the multiple spectral information without the chemical images. The question arises, why collect all this data and only utilise a small fraction?

In terms of pharmaceutical applications, NIRM has been useful for understanding the distribution of an API within an excipient matrix but with the full compliment of NIR spectral data it should have been possible to also determine the chemical images for each excipient.

With knowledge on all components within a pharmaceutical formulation, NIRM could serve to determine the blend homogeneity of all components, not only the API. Exposing the micro-scale interactions of a sample may also be a useful method to understand process variation, and how it affected product performance. NIRM can then become a viable method for pharmaceutical process control, which in the last year has also been suggested by other researchers (Reich, 2005).
1.7 NEAR-INFRARED MICROSCOPY AND IMAGE ANALYSIS FOR PHARMACEUTICAL PROCESS CONTROL

The applications of NIRM reported in the literature have been limited, but in theory NIRM should be able to fill a knowledge gap within the pharmaceutical industry. Instead of formulators theoretically determining the impact of process or ingredient changes, NIRM provides the opportunity to visualise the reality.

Therefore the aim of this research is to understand areas where NIRM can add value in the pharmaceutical industry and understand the limitations for the technology. The ultimate goal is to be able to utilise the information obtained in process control, rather than in a laboratory environment. However, for this to be attainable it is necessary to understand how best to utilise and interpret the output from a NIRM experiment.

To reach this objective, experiments will be performed to determine the optimum method for working with high volumes of spectral information and how best to utilise the output to produce chemical images. Currently a knowledge gap exists in how accurate chemical images are at describing a pharmaceutical solid dosage form and this research will examine the lateral and depth resolution in a NIRM experiment, and analyse how this contributes to the potential applications for this technology. Real world samples will be measured during this research, including products with production issues (dissolution and processing (tablet sticking)), to provide specific examples of what knowledge can be obtained from NIRM experiments, and any potential for this methodology to be used for pharmaceutical process control. Overall this research will provide increased understanding of NIRM and its role in the pharmaceutical industry.
2. DEVELOPMENT OF DATA PROCESSING METHODS

2.1 DATA PROCESSING METHODS AVAILABLE FOR EVALUATING THREE DIMENSIONAL SPECTROSCOPIC DATA CUBES

In any NIRM experiment there are two components of the resultant data; that is spectral and spatial information. The spatial information represents two dimensions of the resultant data cube (x- and y-axis), and the spectral information is described in the 3rd dimension (z-axis), as previously shown in Figure 1.4. In a typical NIR-PM experiment, around 10,000 spectra may be obtained, but this number can dramatically increase to over 82,000 spectra in a NIR-GI experiment. Therefore data analysis approaches are complex due to the large number of spectra and also the inclusion of spatial information. However, it is also known that NIR spectra obtained from reflectance measurements will contain both chemical (due to absorption of radiation) and physical (due to scatter effects) information. In a microscopy experiment the physical information is also added to by the effects of non-optically flat surfaces, which cause offsets in spectral intensity due to focusing variations. Therefore data processing regimes are essential to evaluate the complex set of data obtained in a NIRM experiment.

2.1.1 Pre-processing Approaches

The physical components in NIR spectra can be addressed by the use of pre-processing methods. These techniques have been widely used in macro-scale NIR. The most common approaches are normalisation (used to remove the spectral offset induced by scatter differences) or derivatives that also remove baseline scatter but also help in removing the slope variation in the NIR spectra caused by particle size variations and enhance the resolution of overlapping information (Bokobka, 1998). The two most commonly used methods for normalisation are multiplicative scatter correction (MSC) (Ilari et al, 1988) and standard normal variate (SNV) (Barnes et al, 1989).
**Standard Normal Variate**

The standard normal variate (SNV) transform mean centres the spectra, taking the mean value of each spectrum (over all wavelengths) from the response at each individual wavelength, and then scales them using the standard deviation of each spectrum (over all wavelengths). In this transformation each individual spectrum is treated in isolation. Equation 2.1 describes the transformation, where $y_{sj}$ is the SNV transformed signal at wavelength $j$ with the original signal at wavelength $j$ of $u$. The mean signal of the original spectrum is $\bar{u}$ and the standard deviation of the spectrum is $\sigma$, both calculated over all $J$ wavelengths. Spectra treated in this manner have a zero mean and variance equal to one. SNV effectively removes variation in spectra caused by particle size variations but also reduces scattering effects.

$$y_{sj} = \frac{(u_{sj} - \bar{u})}{\sigma}$$  \hspace{1cm} (2.1)

**Multiplicative Scatter Correction**

Multiplicative scatter correction (MSC) produces similar results to SNV pre-treatment as both are linearly related (Dhanoa et al, 1994). However, MSC transform utilises the mean of a spectral set, rather than working on isolated spectra. Each spectrum in the set is then regressed against the set mean spectrum to obtain the multiplicative and additive correction factors, for which the linear regression co-efficients (intercept and slope) provide estimates. Therefore for a set of $z$ raw spectra, $(n = 1,2 \ldots z)$, with a set mean of $y_m$, the ordinary least squares regression line for the $n^{th}$ spectrum is given in Equation 2.2, where $a_n$ is the intercept of the least squares regression line, $b_n$ is the slope of the least squares regression line, and $e_n$ is the residual spectrum after MSC fitting (usually attributable to chemical information).

$$y_n = a_n + b_n y_m + e_n$$  \hspace{1cm} (2.2)
The MSC transformed spectrum \( y_{n_{\text{MSC}}} \) is obtained by subtraction of the additive factor, \( a_n \), from the raw spectrum \( y_n \) followed by division of the multiplicative factor, \( b_n \) as described in Equation 2.3 for the \( n \)th spectrum in the set.

\[
y_{n_{\text{MSC}}} = \frac{(y_n - a_n)}{b_n}
\]  

(2.3)

Spectra treated in this manner have a non-zero mean and variance related to the set-mean spectrum variance. Therefore, in terms of spectral imaging experiments, SNV approaches have been applied throughout this work, due to functioning on an individual spectrum basis with no dependence on the set of data per image. This is believed to have allowed for correction of not only scatter effects but also variations caused by small focusing variations on analysis surface, by the fact that all spectra have a common zero mean and have been scaled to unit variance.

**Derivative Pre-Treatment**

Derivative spectroscopy is a technique in which the absorbance (or other spectral ordinate) is differentiated \( \delta \) times with respect to wavenumber / wavelength to give the \( \delta \)th derivative spectrum (Bertie, 2002). The derivative treatment is used to transform changes of slope in the original spectrum into more prominent features in the derivative spectra. Because of the resolution enhancement caused by derivative treatments, the method is widely applied in NIRS due to poorly resolved component information in complex spectra (O’Haver and Begley, 1981). The approach is certainly not new with discussions around the applicability of the method for quantitative analysis extending back into the mid-1970’s (O’Haver and Green, 1976). Along with improving spectral features, derivative treatments also degrade the signal to noise ratio (SNR) and it is often necessary to apply a smoothing procedure before derivation (Bokobka, 1998; O’Haver and Begley, 1981). Typical smoothing approaches utilised are based on Gaussian and Savitsky-Golay smoothing filters (O’Haver and Begley, 1981; Bromba
and Ziegler, 1983). In NIRS, first and second order derivatives are most commonly utilised. First order derivatives of absorbance data give rise to a curve with peaks and valleys that relate to the point of inflection on either side of absorbance peak whereas second order derivatives result in the absorbance peaks pointing down (Shenk et al., 1992). Second derivatives are often easier for spectral interpretation due to the fact that band intensity and peak location are maintained with respect to the original absorbance peak.

2.1.2 Extracting Chemical Information

As in typical spectroscopic approaches, after pre-processing methods it is possible to extract chemical information from an image cube by only univariate approaches. There are advantages and disadvantages to univariate methods. Primarily univariate methods can be considered simple methods, as they only require examination of either a unique wavelength or integration of a unique wavelength region. However, because unique wavelengths need to be identified, it requires prior knowledge of the sample and often the approach is slow and very user dependent.

With NIR data, second derivative spectra give the operator a greater chance of specifically identifying the material at any pixel. Materials with very similar structures give differences in their second derivative spectra, which may not have been obvious in the raw spectra, e.g. discrimination of free water and water of crystallization, similar carbohydrates or even polymorphic forms. Images of each ingredient can easily be generated by monitoring of single wavelength absorption or by integration of wavelength regions, typically unique to a component, to give a single value for each spectrum. The method relies on the operator to determine when a pixel is one ingredient or another.
A less user dependent approach to generating a single value for each spectrum is to correlate the map spectra to the respective pure component spectrum. A correlation value is obtained by calculating the vector dot product between the reference and sample spectra. Correlation approaches are advantageous as a larger number of wavelengths can be utilised to identify each component.

Even with the advantages of correlation approaches, univariate methods are very reliant upon the analyst, allowing for variation in one sample analysis to the next. Often exclusion of large volumes of data occurs and prior knowledge of the sample system is almost essential. As such there is a desire to examine the data in a more automated manner with an ability to have no prior knowledge of the sample and also to use all of the spectral information collected. Multivariate approaches have been widely used and satisfy all of the desired criteria for image data processing. Such an approach would allow data processing to become more robust and less user dependent. However, with the number of approaches available a question arises about which multivariate approach to use. Many approaches have been taken and there seems to be no right or wrong answer to this question. The most suitable technique depends on the purpose of the analysis and the actual data set.

**Principal Component Analysis**

One of the most frequently used multivariate tools is principal component analysis (PCA). In fact, it is typically thought of as the workhorse of multivariate image analysis (Geladi and Grahn, 1996, Baronti et al, 1996). The key to the use of any multivariate approach for imaging data is the re-organisation of the 3 dimensional image cube into a 2 dimensional matrix (De Juan et al, 2003 and 2004). Once in the matrix form traditional PCA approaches can be taken to describe the important spectral information in a smaller number of components (Davies, 1992; Davies and Fearn, 2004). These principal components describe the variation in the spectra. For each component, a vector or “loading” is produced along with a score. Each spectrum can therefore be
described as a combination of principal components, with the contribution of each principal component to the individual spectrum being known as the score. The loading represents the spectral dependence of the principal component, that is to say the loading is the contribution of each wavelength to the principal component and as such it would be expected that loadings would look similar to spectra.

If an image is considered to have \( x \) by \( y \) pixels with \( w \) wavelength channels, then it can be turned into a 2 dimensional matrix by reshaping to a two-way array (Data matrix - \( D \)) that is \((x \times y)\) by \( w \), such that each row in the matrix is a spectrum related to a different pixel (matrix unfolding), Figure 2.1. The resultant data matrix, \( D \), is identical to normal spectroscopic methods and therefore PCA can then be performed on the matrix using traditional approaches. PCA decomposes the data matrix \( D \), into scores \( T \) and loadings \( P \) matrices as described in Equation 2.4, where \( T \) and \( P \) each consist of \( p \) orthogonal column vectors associated with \( p \) principal components and \( E \) is the residual/noise matrix.

\[
D = TP^T + E
\]  

(2.4)

The decomposition of \( D \) is based on eigenvector decomposition of the covariance matrix, which can be calculated using Equation 2.5, due to mean centring and unit variance scaling of the data prior to PCA, where \( \text{rows} \) represents the number of rows in data matrix \( D \).

\[
\text{cov}(D) = \frac{D^T D}{(\text{rows})^{-1}}
\]  

(2.5)

The 1st principal component (PC) is calculated by finding the direction through the data \((w \) dimensional data) that explains the most variability in the data. This gives rise to an eigenvector \( p_i \) and the matching score vector \( t_i \) which can be determined by
projection of $D$ onto $p_1$. The second eigenvector ($p_2$) must be orthogonal to $p_1$ and explain the maximum amount of the remaining variability in the data set. This continues until all the variance has been accounted, such that Equation 2.4 actually can be written as Equation 2.6 ($E$ has been removed as presumed to contain random noise fluctuations, hence $T$ and $P$ describe all the structure in the data).

$$D = t_1 p_1^T + t_2 p_2^T + t_3 p_3^T + \ldots + t_p p_p^T = t_1$$

(2.6)

After PCA modelling, the scores matrix can be folded back up to reform images and the loadings interpreted in the usual way. This allows the maximum variation in the data to be represented by chemical images and the loadings can be used to assign chemical species — although often a PC cannot be unambiguously assigned to one component due to the presence of positive and negative peaks in a loading vector.

**Figure 2.1 - Schematic description of reduction of image cube to two-way array for PCA analysis and subsequent matrix re-folding**

PCA is an unsupervised method and hence information on the composition of the formulation is not needed to be able to use the algorithm. Therefore it is an ideal
method for rapid screening of large image sets. PCA has been utilised to differentiate between API and excipients in an over the counter formulation (Lewis et al., 2002b). Unsupervised PCA allowed the API regions to be easily visualised and the raw spectra from these regions matched back to pure component spectra. Further multivariate approaches were required to extract concentration information, by utilisation of alternating least squares but this simply allowed a percentage of the total area of image that was API pixels to be determined. The ability of PCA to study samples with little prior knowledge has also been shown in the examination of biological samples (Feld et al., 2002). In this work, PCA was successfully used to examine breast tissue and identify the ductal units (cells) from the collagen matrix although assignments of the loadings back to the known spectral features were difficult to achieve. However, it was commented that PCA modelling could be utilised to build more sophisticated models for further tissue studies. Even away from the field of vibrational spectroscopy, PCA has been used successfully in the analysis of x-ray photoelectron spectroscopy (XPS) images, where it has been used to examine the degradation of polymer blends and successfully identify critical reference data (Fulghum and Artyushkova, 2001).

As applications of imaging develop, and with the introduction of larger FPAs, the amount of data generated per image is dramatically increasing. Research continues into making data processing regimes faster, in particular PCA methods (Vogt and Tacke, 2001) using wavelet transforms. Singular value decomposition (SVD) was then performed in the wavelet domain involving only relevant wavelet co-efficients (any small wavelet coefficients were set to zero). Hence, acceleration was due to the smaller matrix in the SVD stage. The results of the accelerated PCA had good agreement with traditional methods, with also a noise reduction in the finished spectra being observed. This approach could readily be applied to imaging data sets after matrix unfolding but as with all PCA methods, image contrast would be observed in the score images but association to unique chemical components would still be challenging.
Partial Least Squares Regression

On the other hand a technique such as partial least squares (PLS) regression, which is based upon PCA, requires information on the spectral composition of the sample (Van der Broek et al, 1996). PLS (method 1) was first applied to NIR data in 1983 (Martens, 1983), and allows a relationship between a single variable (e.g. concentration) and a set of variables (e.g. absorbance at many $\lambda$'s) to be developed. The technique is ideal for NIR spectroscopy as it allows for extraction of data from complex spectra with overlapping peaks. PLS fundamentally works by exploiting the correlation that already exists between the spectral data and the constituent concentrations although pure component information must be supplied before the analysis is performed, often in the form of a training set (library). The library can be built using individual spectra or regions from different experiments. However it is collated, the library should include all spectral variations. In the pharmaceutical industry, PLS approaches are applied to the use of NIR to predict API concentration in bulk tablets (Corti et al, 1999) by reference to a traditional slower analytical method that provided the original potency data to the calibration.

PLS is derived from PCA methodology, but deviates with an additional step during the decomposition of the data matrix $D$ into the scores ($T$) and loadings ($P$) matrices. This step involves a concentration matrix ($C$) also being reduced into scores ($U$) and loading ($Q$) matrices resulting in not only Equation 2.4 (as described for PCA) but also Equation 2.7, where $F$ is the residual noise matrix of the concentration matrix $C$.

$$C = UQ + F \quad (2.7)$$

Therefore PLS modelling estimates the underlying factors in both the data matrix $D$ and the concentration matrix $C$, defining the subspace in $D$ which best models $C$ (Bebbe and Kowalski, 1987; Geladi and Kowalski, 1986). This is achieved using the columns of $C$ to estimate the factors for $D$, and at the same time the columns of $D$
are used to estimate the factors for \( \mathbf{C} \). Unlike PCA, in PLS the \( \mathbf{T} \) factors estimating the columns of \( \mathbf{D} \) are not optimal as they are rotated to simultaneously describe \( \mathbf{C} \). In an ideal situation the variations in \( \mathbf{D} \) would be exactly the same sources of variation in \( \mathbf{C} \) and factors would be identical, but in real data \( \mathbf{D} \) and \( \mathbf{C} \) vary in a non-correlated manor. Their relationship is typically linear which allows for the use of a linear predictor. This is described in Equation 2.8 where \( \mathbf{u} \) is the score vector (for any latent variable, \( l \); where \( l = 1 \) is the variable which describes the largest variation in the data set) from decomposition of variation in \( \mathbf{C} \), \( \mathbf{t} \) is the score vector (for any latent variable \( l \)) from decomposition of variation in \( \mathbf{D} \), \( \mathbf{e} \) is the residual. In this equation \( b_l \) is the inner relationship between \( \mathbf{t} \) and \( \mathbf{u} \) that can be used to calculate subsequent factors. If a good relationship exists between \( \mathbf{D} \) and \( \mathbf{C} \) then the vectors \( \mathbf{u} \) and \( \mathbf{t} \) will be similar in the model space and \( \mathbf{e} \) will have a small value, if \( \mathbf{t} \) is compared to that from PCA decomposition of \( \mathbf{D} \) it would differ slightly due to rotation towards \( \mathbf{u} \).

\[
\mathbf{u} = b_l \mathbf{t} + \mathbf{e} \tag{2.8}
\]

Equation 2.8 can be used to describe the model for \( \mathbf{C} \) (if \( \mathbf{D} \) has more than one dimension) such that Equation 2.9 represents the original concentration matrix (\( \mathbf{C} \)). This equation means that the score values of \( \mathbf{C} \) can be estimated from score values of \( \mathbf{D} \). The final model consists of scores matrices \( \mathbf{T} \) and \( \mathbf{U} \), for \( \mathbf{D} \) and \( \mathbf{C} \) respectively, which are linearly related with a coefficient of \( b_l \) that describes the relationship for \( L \) latent variables. \( \mathbf{G} \) is the residual noise matrix.

\[
\mathbf{C} = b_l \mathbf{T} \mathbf{Q} + \mathbf{G} \tag{2.9}
\]

Having established a model that describes the variation in both the data and concentration matrices, it becomes possible to predict the concentration of an unknown
sample. The vector of responses \( r_{unk} \) of the unknown sample is taken through the calibration model and a score vector \( t_{unk} \) is calculated. Using the \( b_j \) factors and Equation 2.7, gives rise to an estimate of score vector \( u_{unk} \), which can be transformed into concentration estimates using the calibration model for \( C \).

With chemical images this method of quantification by PLS is not strictly valid as, although a representative area of a single tablet is taken, the measurement consists of many thousands of spectra, each which have a different response. That is to say that some pixels will have a higher concentration from others, perhaps a defined region of API. Therefore the appropriate reference method will give a bulk concentration measurement, which is harder to correlate to individual pixel responses. NIR microscopy data allows for identification of all components in a tablet matrix – not just focused on the API component – hence reference methods for each individual component are difficult to obtain. PLS is used in a classification rather than a quantification method (Ståhle and Wold, 1987) as an approach for chemical imaging data throughout this research with PLS method 2 being used to evaluate the data matrix and all library classes in one single analysis.

In this manner the calibration set consists of the spectra of the pure components of the tablet matrix of interest, with each component being assumed to have a concentration set to 1. The PLS model is a result of decomposition of the pure component spectral set, examining the variation and presuming equivalent concentration. This gives rise to \( I \) image planes (where \( I \) is the number of pure components (spectral classes)). The library data set is evaluated by assuming that the number of principal components \( (I) \) is equal to the number of spectral classes in the library \( (I) \) where spectra in each class contain a concentration matrix with ones and zeros (one for concentration of its class and zero for all other classes). When the data matrix is subsequently examined, the factors are computed by utilising the concentration matrix to rotate factors from the
spectral matrix such that they describe the maximum co-variance between the spectral and concentration matrix. The resultant scores data cube has $I$ image planes which are scaled from 0 to 1 for each component, showing whether a material is present or not i.e. a class membership image.

In the literature there are limited examples of the use of PLS with chemical images, successful implementations of PLS and image analysis have employed the use of Kernel-PLS (Lindgren et al, 1993, 1994) which has been termed Image PLS (IPLS). This approach has been termed IPLS as the number of pixel elements in an image (which are typically far greater than the number of spectral channels) can be reduced by using blocks of data which are related by their latent variables and hence allows for optimisation of data analysis. When the data have been mean-centred and scaled to unit variance, all variables have equal variance weights and Kernel-PLS decompositions pertain to correlations (Lied and Esbensen, 2001). In this work a PLS approach was shown to be successfully utilised in the assessment of Swedish crispbread - in the identification of faults (broken, perforated, burnt) from acceptable surfaces. The model is spatially complex (many pixels) but has only 3 spectral variables - red, green and blue channels of visible camera (but potentially could be applied to higher channel systems). IPLS has also been used to study the aging of a banana. Ageing was determined by colour (green to yellow to brown). Simply counting the number of brown pixels in an image did not give a quantitative estimate of age as no account is made of the degree of brownness. Therefore what results is a spatially and spectroscopically related development process, where the calibration parameter is storage time. PLS approaches were also utilised to relate imaging data of cheese (electron micrographs) to rheological results e.g. fracture stress and strain (Huang et al, 2003) in such a way that rheological properties could be predicted from cheese images.
Using this supervised approach allows rapid analysis of images and one with little operator input. It allows the identification of known spectral properties to be screened from an image data set. However, using PLS analysis or any other supervised algorithm requires care to ensure that any material not included in the library is not simply overlooked.

**Other Multivariate Approaches**

The multivariate approaches above have been utilised throughout this research. The following approaches have been reported in the literature as other methods for analysing spectroscopic chemical images.

Analyses of spectroscopic image data sets have included the use of Euclidean and Mahalanobis distances, as means of classification. One such method was shown in the use of Kohonen networks (Hutter et al, 1997) in secondary ion mass spectrometry (SIMS) images as a means to interpret chemical phases, which were typically impossible to visualise due to mass interferences. By utilising scatter plots and Euclidean distance rules it was possible to identify elemental groupings which could be assigned to regions on the original image along with interactions between different chemicals. Mansfield et al suggested that the use of Fuzzy C-Means Clustering (FMC) dramatically increased the information obtained from NIR spectroscopic images (Mansfield et al, 1997). The algorithm they suggested detected novel and unanticipated spectral features from sub-regions of the image, without any prior knowledge of the chemical composition, and at the same time improved the signal-to-noise ratio of the spectra. The FMC algorithm was also used alongside NIR imaging for art conservation applications (Mansfield et al, 1999). At the same time as the unsupervised method of FMC was applied, supervised linear discriminant analysis (LDA) was used. The LDA was found to give better results, but required prior knowledge of the sample.
Single value decomposition (SVD) has been used to optimise the sample to background signal ratio of NIR spectroscopic images and the data classified with multivariate image rank analysis (Van den Broek et al, 1995 and Wienke et al, 1995). Identification of the three main components in wheat, using NIR spectral imaging was possible using stepwise discriminant analysis and canonical discriminant analysis (Robert et al, 1992). Another approach taken to improve signal to noise in imaging data was the use of wavelet transformations (Nikolov et al, 1996) where the de-noising of SIMS images were achieved. Wavelets have previously been proposed as a means to smooth, de-noise and compress spectroscopic data (Barclay and Bonner, 1997), including specific examples using Raman (Ehrentreich and Sümmchen, 2001), NIR (Walczak et al, 1996), MIR (Alsberg et al, 1997) and nuclear magnetic resonance (NMR) / ultra violet (UV) spectra (Leung et al, 1998). Advantages of reduced noise, and smaller data sets have not been picked up and exploited in spectroscopic imaging despite the proven applicability to spectroscopic data, in particular interest to this field should be the data compression offered by wavelet approaches.

With the large volume of spatially resolved data obtained from imaging experiments, it makes an ideal data set from which to try and extract pure component spectral information. There are many different techniques that can be utilised for resolution of pure spectral components, but probably the most widely accepted is multivariate curve resolution, MCR. This technique has been used to resolve successfully the different components present within an emulsion system examined using Raman chemical images (Hancewicz and Andrew, 1998). Not only were the spatial distributions of each component observed, but also by utilisation of MCR it was possible to extract a spectrum from each region, which was related to the known pure components. MCR has also been used in Fourier Transform Infrared (FT-IR) microscopic analysis of corn kernels to determine the chemical composition, and understand what caused variation in crops (Budevska, 2003). In this study MCR was used to determine the pure component spectra which were present in the corn kernels. By matching to a reference
spectral library they identified and presented the different distributions of two different proteins (which were not resolved by univariate methods) and showed a clear relationship to the age of the kernels. MCR has also been successfully implemented to separate the spectral composition of mixed biopolymer systems, despite the highly overlapping Raman spectra (Pudney et al, 2003) and the different components absolute concentration determined.

Currently, there is no standard approach to data manipulation and no one algorithm or mathematical treatment can deal with all data sets or problems. The following sections in this chapter present the different approaches and methods utilised throughout this research work.

2.2 EVALUATION OF SAMPLES FOR UNDERSTANDING OF DATA PROCESSING TECHNIQUES

This section will evaluate the different approaches, which can be taken to look at the chemical information extracted from spectral data cubes and with spatial information used to produce chemical images. For each processing method, a six-component system will be evaluated which consists of images of six pure component materials that have been analysed individually and joined together after analysis. Hence each area should contain only spectral information on one component. An example of a true application of each processing regime will also be presented on a tablet sample made using a mixture of the six pure components.

The six pure components were selected to represent the materials used in a tablet dosage formulation. Samples of each of the following components, voriconazole (Pfizer Inc.), povidone, pregelatinised starch, lactose monohydrate, croscarmellose sodium and magnesium stearate (300 mg of each) were individually compacted into discs using 100 p.s.i. pressure, each disc was ~3 mm thick. An area on the surface of each disc (5 by 5 mm in size) was analysed using a line imaging system (Figure 1.6b),
whereby 16 spectra were collected in one acquisition from a line of detectors. Each spectrum came from a square pixel with dimensions of 25 x 25 μm. Ratio spectra were collected using a gold mirror as the reflectance standard. Each spectrum was the average of 4 scans across the wavelengths 1300 – 2600 nm, with ~2 nm data intervals. In total over 30,000 spectra were obtained for each sample, taking ~30 minutes to collect. After data collection, the resultant unprocessed data cubes were translated into a format (.spf) readable by image analysis software (ISYS, Spectral Dimensions, MD) and then concatenated (image sets joined together by y-axis) to give rise to a data cube with spatial arrangement shown in Figure 2.2. The colours in this figure represent the colour that will be utilised in display of the spectrum of each component.

![Diagram of six components](image)

**Figure 2.2 - Spatial position and colour referencing for six component (spatially resolved) system**

Using the same experimental parameters, a data cube was obtained from the core of a tablet made using these six ingredients, the tablet core was exposed by milling the tablet surface down to the widest point of the tablet. A wet granulation process was used to produce the tablets and the weight/weight relationship of the ingredients is listed in Table 2.1. The tablets were sourced from Pfizer Inc.
Table 2.1 - Chemical composition of a six component, non-spatially resolved system

<table>
<thead>
<tr>
<th>Component</th>
<th>Weight in 600 mg Tablet (mg)</th>
<th>% Weight/weight (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voriconazole</td>
<td>200</td>
<td>33.3</td>
</tr>
<tr>
<td>Lactose Monohydrate</td>
<td>250</td>
<td>41.7</td>
</tr>
<tr>
<td>Pregelatinised Starch</td>
<td>84</td>
<td>14</td>
</tr>
<tr>
<td>Croscarmellose Sodium</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>Povidone</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>Mg Stearate</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

2.3 UNDERSTANDING THE IMAGE CUBE

To enable the processing of spectral data cubes into ‘useful’ information, it is firstly required to understand how spectral and spatial information are correlated. Figure 2.3 shows the SNV corrected NIR spectra for each of the six pure components shown in Figure 2.2. (N.B. SNV pre-treatment has been used to remove the baseline offset in the spectra; insert in Figure 2.3 shows the raw absorbance data). From this figure it can be seen that there are wavelengths where only one component has a unique response e.g. 6072 cm\(^{-1}\) for voriconazole component. In the figure the three unique wavelengths of the major components have been identified and marked.

![Figure 2.3 - SNV corrected NIR spectra for each of the six pure components with unique wavelength for each of the major components identified. The insert shows the raw absorbance spectra for each pure component.](image)
In terms of spectral processing, the concept of normalisation is accepted but when the spatial component is taken into consideration, normalisation also works to enhance the image features. Analysis of the raw absorbance data cube for the tablet produced from the six pure components gives rise to three primary information sets, shown in Figure 2.4. The first set is the spectral information where each spectrum is associated with one pixel in the image. Secondly the image, where the image contrast is a function of the absorbance at 6072 cm\(^{-1}\) and finally the third set of information is a histogram distribution of the number of pixels that have value \(a\)' at a specific image contrast feature, in this example absorbance at 6072 cm\(^{-1}\). From this figure it can be seen that the white pixels have NIR spectra which are different, depending on which pixel is selected (red spectra in Figure 2.4) and it can be concluded that despite a unique wavelength response for the voriconazole, the chemical image does not only show the spatial arrangement of the component of interest, but includes spatial features from spectrally different materials.

![Figure 2.4](image)

**Figure 2.4 - Evaluation of the 3 sets of information obtained from a raw absorbance data cube; where a) represents spectra from individual pixels (red = white pixels and black = black pixels), b) absorbance response for each pixel at one wavelength (image plane at 6072 cm\(^{-1}\)), c) histogram of number of pixels relative to absorbance value at 6072 cm\(^{-1}\)**

However, if the spectra are normalised, the image contrast changes (shown in Figure 2.5) and the NIR spectra from white pixels are all similar and match that of pure voriconazole material. Information can also be obtained on the spatial arrangement of the voriconazole component because of the change in the distribution of pixel intensity (Figure 2.5c), with the long tail on the right hand side of the distribution being...
associated with very intense regions of voriconazole, whereas the centre of the distribution is around a negative value, which would be where most of the other components would absorb.

![Diagram](image)

**Figure 2.5 - Evaluation of the 3 sets of information obtained from a normalised absorbance data cube; where a) represents spectra from individual pixels (red = white pixels and black = black pixels), b) absorbance response for each pixel at one wavelength (image plane at 6072 cm⁻¹), c) histogram of number of pixels relative to absorbance value at 6072 cm⁻¹.**

Therefore the analysis of spectral data cubes gives rise to three sets of information, spectral, image and distribution of pixel intensity. Although the latter two parameters have been presented above in terms of single wavelength intensity, both could be generated using other values such as score values from PCA or PLS analysis (presented in Section 2.5 and 2.6 respectively). It should also be noted that although the spectral information is important for identification of chemical species, it plays a more significant role in the middle of data processing rather than the end result of a NIRM experiment.

### 2.4 Normalisation and Univariate Data Processing

With the wide range of tools available to analyse spectral data, it is often a challenge to determine where to begin with data processing. However, it is often best to look first for the simple information that can be obtained by simply observing single wavelength data. Using the six pure component data set, Figure 2.6 shows the effect of monitoring single wavelengths. From the images presented it can be seen that each of the three unique component wavelengths, previously identified for the major components (Figure
2.3), correctly identifies only the component of interest (shown as bright white pixels). However, in terms of the other components only 4 other peaks are identified in the histogram plot, showing that each component is not resolved. This is not surprising as at the wavelengths chosen, the other components have similar absorbance response.

To allow understanding of the relative distributions of each component it is common to overlay chemical images of each unique component to form a combined image. Similar to televisions there are only three colour channels available for image overlap, red, green and blue giving rise to a RGB image, shown in Figure 2.6. In this case, the three components identified by single wavelengths are visible uniquely as the RGB channels, and the three components not identified are shown as black/navy in colour. The colour scaling for each image is based simply on the intensity range at each wavelength.

![RGB Image](image1)

*Figure 2.6 - Image contrast observed at unique wavelengths (for major components) in spatially resolved system along with histogram showing the number of pixels relative to normalised absorbance at each wavelength. Each of the images produced for unique wavelengths are summed to produce an RGB image, respective channel indicated by arrows.*

When this approach is used for the tablet matrix, where the six components are now spatially close to one another the same defined information is not obtained, Figure 2.7. The first difference is in the distribution of pixel intensity. For the pure component data
at least 5 peaks were identified in the histogram. However, for the tablet data, only one peak is found for each component when within the tablet matrix. The reason for this becomes more apparent when the spectral data of the identified pixels for each component are compared to the pure component spectral data, shown in Figure 2.7. From the spectra it can be seen that although each spectrum associated with white pixels has features of the pure component, there are also other component spectral features present. (N.B. Reasons for this will be discussed in Chapter 3 when lateral and depth resolutions are examined.)

![Figure 2.7 - Image contrast observed at unique wavelengths (for major components) in non-spatially resolved system along with mono-modal histograms showing the number of pixels relative to normalised absorbance at each wavelength. The spectra under each image represent a comparison between the known pure component spectra (shown in black) and the white pixels from each image (component specific colouring).](image-url)

When the component images are combined to form an RGB image, overlap of the components are observed making the RGB image less defined in terms of spatial distribution of the components, Figure 2.8a. Overlap of components can be reduced by only including pixels with certain absorbance values at the given wavelengths; Figure
2.8b shows the situation when each component’s ‘unique’ pixels are selected. ‘Unique’ is used here to describe an absorbance value that allows only pixels with absorbance values similar to that of the pure component to be included, i.e. the separation point between the histogram of the pure component of interest and the other excipient responses in Figure 2.6. That is at 6072 cm⁻¹ pixels with absorbance >0 are used in the red channel of the image, for 6272 cm⁻¹ absorbance >-0.2 allows inclusion in green channel and at 6888 cm⁻¹ absorbance >-0.3 is included in blue channel. However, this method can result in significantly different interpretations of the chemical images, as the scaling intensity of each channel is dependent on the range selected.

Figure 2.8 - Overlaid images of voriconazole (red), lactose monohydrate (blue) and pregelatinised starch (green) obtained at unique wavelengths and scaled a) 3 standard deviations around mean absorbance from all pixels and b) only pixels with absorbance similar to pure component material (determined from Figure 2.6)

The evaluation of single wavelength data may provide some information on 3 of the 6 components in the tablet formulation, but as not all components have a unique wavelength it is not possible to identify every component within the tablet matrix using a univariate approach and hence if information on all components is required it is essential to move towards multi-variate methods of analysis.

2.5 Principal Component Analysis
PCA can be useful for processing spectral cubes, as the method is unsupervised and therefore no prior knowledge of the components in the system are required. The six
pure material data cube was processed using PCA and 10 factors were included in the model. Figure 2.9 shows the resultant score images for each principal component (PC), with the x-axis representing the number of PCs and the y-axis represent the score value at that PC. The line is an example of the typical trend observed at each pixel. It can be seen that up to and including PC six, the score images contain spatial information on the different materials, greater than component 7 the images appear to simply show noise in the system. The values shown in brackets represent the percentage variance described by that PC.

If the loadings are examined for each of the first seven PC’s, Figure 2.10, it can be seen that the 1st loading looks like an average of all pure component spectra i.e. sloping baseline. This is similar to previous investigations of PCA in image analysis (Baronti et al, 1997) where the first principal component was thought to simply be linked to the average reflected light. This loading also describes 90.49% of the variance in the data cube. The 2nd and 3rd loadings describe 5.27% and 3.04%,
respectively of the variance in the data cube although from the loadings it is not apparent which spectral features of the pure materials are described between these components. The same can be said of the 4th thru 6th loadings, which all describe 0.2 - 0.7% of the variance. When the lower loadings are examined, they account for less than 0.01% of the variance, and spectroscopically start to describe the noise in the system. If the resultant RGB image from combination of score 1 to 3 images is examined, each component can be observed as a different colour, Figure 2.9b, each channel being colour-scaled based on individual score value range. As PC 1 score values were far more intense than any other PC, the RGB image was also prepared using score images 2 - 4, also shown in Figure 2.9b, where all components again were identified by a different colour. Therefore, although it may not be possible to identify the spectroscopic features associated with each score, PCA has the potential to resolve all six components.

![Figure 2.10 - Loadings obtained from each principal component of spatially resolved data cube](image)

Therefore, it can be concluded that when the components are spatially resolved the spectroscopy allows for identification of each pure material using PCA. It would therefore follow that if the same analysis were performed on materials that are combined and not spatially resolved, PCA should still be able to resolve the six
different components. When PCA was performed on such a system, this was not found to be the case. Figure 2.11 shows the score images from each PC, with the percentage variance described by each PC shown in brackets.

![Plot of score values observed relative to the number of principal components used in the decomposition of non-spatially resolved data cube, with insert of image obtained for each PC and the percentage variance described by each PC](image)

**Figure 2.11** - Plot of score values observed relative to the number of principal components used in the decomposition of non-spatially resolved data cube, with insert of image obtained for each PC and the percentage variance described by each PC.

Similar to the pure component system, up to and including PC six, the score images contain spatial information on the different materials and greater than component 7 the images start to show noise in the system. If the loadings associated with each score image are evaluated (Figure 2.12), it can be seen that again the first PC describes almost an average spectral response, with sloping baseline. This PC describes 99.82% of the variance in the system. The second loading shows a strong match to the pure voriconazole spectrum and the spatial arrangement of white pixels is similar to the single wavelength image shown in Figure 2.7. However, this PC only represents 0.08% of the variance in the system. For PC 3 loading, there is some similarity to the povidone component and it could be thought that the image describes mainly this component. However, this component only describes 0.04% of the variance. For PC's
4 – 6, between 0.003 and 0.005 % of the variance is described and for higher PC’s less than 0.002% variance is described.

Figure 2.12 - Loadings obtained from each principal component of non-spatially resolved data cube

When the first three score images are overlaid, Figure 2.13a, there appears to be overlap of the different components (shown by cyan and yellow colours) and also the distinct colours observed for the pure component system are not observed. As the first PC is thought to be an average spectral response, the 2nd – 4th PC score images were overlaid. The resultant image is shown in Figure 2.13b, and overlap of components is still observed. This is further clarified if the spectra from each coloured region are investigated, Figure 2.13c. It can be seen that the green/cyan regions have strong bands that can be associated with voriconazole. The blue and green have similar shapes in the region associated with the lactose component and the red and black spectra have an increased peak at the region identified as pregelatinised starch. Therefore the three main components have been identified in the chemical image but the regions identified are strongly overlapped (hence presence of cyan/magenta and yellow in the image). Hence, it can be concluded that PCA has the potential to resolve the major components in a formulation, but struggles to pull out the low level
components. Even when the major components are spatially resolved, it is difficult to spectroscopically resolve the components.

![Image of score images](image)

**Figure 2.13 - a) Overlay of score images obtained for PC 1,2, and 3 and b) score images obtained for PC 4,2, and 3 of decomposition of non-spatlally resolved data cube in channel order red, green and blue, c) spectra from corresponding colours in image (b) showing overlapped spectral features**

### 2.6 Principal Component Analysis with Data Reconstruction

When PCA was used on the image data cube, whether on pure component or tablet matrix, the first PC described the major variation in the spectral set, >90.5% and >99.8% of the variance in the spectral set respectively. For both sample sets, the loading obtained for the 1st PC appeared to be an average response from all pixels. Therefore it was thought that it might be possible to improve the resolving ability of the different pure component’s spectra from the image set by removal of this ‘average’ spectral component. In an attempt to extract pure substance spectra from the data cube, the contribution from the first PC was deleted from the scores and loading matrices. The new spectral data set was constructed by multiplication of the score matrix with the loading matrix for the remaining 9 PC's.

Figure 2.14 shows the effect that this has on the spatial and spectral response from each of the pure materials. Having removed the spectral contribution from the 1st PC, it is possible to identify wavelengths where each component has an identifiable response; although peak position is not unique the component has the strongest feature in the wavelength region. At these wavelengths, the correct spatial
arrangement of each component is observed. However, spatially there are small responses (grey regions) from other components, due to the non-unique absorption band, where there are only small intensity variations between components of interest and other components. This is particularly true for the croscarmellose sodium component. The resultant individual component images can be overlaid to give a unique colour region (RGB) for each component, shown in Figure 2.14. The top RGB image is a combination of voriconazole, povidone and pregelatinised starch wavelengths images and the bottom RGB image is a combination of lactose monohydrate, croscarmellose sodium and magnesium stearate wavelength images.

![Figure 2.14](image)

**Figure 2.14** - Spectral response observed from each spatially defined region after PCA reconstruction of original spatially resolved data cube – and unique wavelengths that can be associated with each pure component. The bottom left hand side of this figure shows the RGB images generated by overlay of i) voriconazole, povidone and pregelatinised starch and ii) lactose monohydrate, croscarmellose sodium and magnesium stearate wavelength images in the order red, green and blue.

If the same analysis is performed on the tablet system and the unique wavelengths identified in Figure 2.14 are utilised, the same spatial contrast is observed for the three main components, shown in Figure 2.15. In this figure it can be seen that the defined substance colours, have spectra that are similar to the pure substance spectra shown in Figure 2.14. Hence PCA reconstruction has allowed for identification of material
distribution and also relationship of spectral information back to the pure substance for the three main components.

Figure 2.15 - Image contrast and associated spectral response of white pixels observed for each of the major pure components in the non-spatially resolved system after PCA reconstruction, along with overlay of each image to produce an RGB image of voriconazole, pregelatinised starch and lactose monohydrate respectively.

When the three lower level concentration components are examined, Figure 2.16, it is possible to see that although the three components have been identified and a distribution obtained the spectral and spatial information for the povidone component appears to be dominated by API information (Figure 2.15). However, the spatial information for the povidone component can be observed to have many more pixels than that of only the API, shown in Figure 2.17 by green pixels. Using this new spatial configuration for povidone and re-examining the spectral information for this component, shows two distinct spectral groups (Figure 2.16), where dark green spectra are representative of the API regions and light green spectra are the actual povidone regions.
Figure 2.16 - Image contrast and associated spectral response of white pixels observed for each of the minor pure components in the non-spatially resolved system after PCA reconstruction, along with overlay of each image to produce an RGB image of povidone, croscarmellose sodium and magnesium stearate respectively.

It can therefore be concluded that the wavelength chosen for the povidone component, although unique in a matched concentration configuration, is dominated by the API spectrum in a mixed concentration configuration.

Figure 2.17 - Image contrast at wavelengths associated with API (voriconazole) and povidone after PCA reconstruction of non-spatially resolved system, which show similar spatial arrangements but when overlaid in the order red and green, shows yellow pixels which are common to both images and the green pixels which can be described as povidone.

This example has shown that PCA reconstruction can be utilised to determine the spatial distribution of multiple, spatially unresolved materials. Along with obtaining
spatial information on the materials, the spectral information can be obtained and related back to pure material spectra. Therefore PCA reconstruction appears to be more applicable for the analysis of NIR chemical images than just PCA. However, the applicability of PCA reconstruction is limited as it still has the disadvantages of univariate methods whereby there is user interpretation and single wavelengths required to make the analysis a success, hence having limited applicability. However it is a good routine for obtaining ‘clean’ spectral information from a mixed component matrix.

2.7 **Partial Least Squares Regression**

A partial least squares (PLS) classification model was established which allowed for identification of each ingredient within the tablet matrix. The model was established by building a library with six classes – one for each ingredient in the matrix. PLS can be used as a classification tool and hence the classification image for each component consists of the degree of class membership (scaled from 0 to 1). The PLS model used seven components, were the minimum error (determined by predicted residual error sum of squares (PRESS) plot) was observed which included a ‘noise’ class.

The six pure material data cube was regressed against the PLS model and the resultant score images and score pixel distributions are represented in Figure 2.18, where it can be seen that for all six substances the spatial location of each has been identified correctly, with no interaction from any other material regions. The reason for this is obvious from the pixel distributions, where only two classes are observed for each material *i.e.* the pixels that match the library data are centred close to a score value of 1 and those that don’t match centre around a 0 score value. This approach requires no user input, and is therefore a rapid approach for analysing the NIR spectral data cubes and from examination of the pure, spatially resolved data set would appear to be the best approach for analysing spectral data sets.
Figure 2.18 - Score images and histograms of score value versus number of pixels for each of the six materials in the spatially resolved data cube, showing the pure component of interest to have a score value around 1 and all other regions in the image to have a score value of ~0.

However, when the same materials in a spatially complex system are examined (Figure 2.19), the pixel distributions for each substance are not as defined as observed in Figure 2.18. Instead of two discrete distributions, one for materials that are and a second distribution for those that are not the material of interest, only one distribution is observed, shown in Figure 2.19. In this figure the image shown next to each histogram has been grey scaled across the entire distribution (indicated by blue dotted lines); hence it is very difficult to see individual material distribution within the matrix as all pixels have a contributing intensity.
Figure 2.19 - Score images and histograms of score value versus number of pixels for each of the six materials in the non-spatially resolved data cube, showing only mono-modal distributions for each component.

If for example the three main substances are overlaid to form a RGB images, shown in Figure 2.20, it is very difficult to determine the spatial location of each material, with the edges of each being fuzzy. Also in the image the lactose material, known to be present at 41.7% of the formulation, is the dominant material with >50% of the pixels having a lactose response, resulting in a very strong blue image.

Figure 2.20 - Summation of score images for lactose monohydrate (blue), pregelatinised starch (green) and API (voriconazole, red) into a combined RGB image
Therefore the question is posed: how can each of these different single distributions represent all materials within the tablet matrix. Mono-modal pixel distributions imply that every pixel has a contribution from every material in the formulation. This may not be surprising as PCA showed that the most significant variation between all pixels was an average spectral response, with sloping baseline. Therefore it is known that each pixel has a very similar spectral response. Another thought for observing a contribution for every material at each pixel could also be due to the contributing volume of the spectral response, discussed later in Chapter 3 – whereby a single component does not occupy the true volume of material sampled at each pixel. However, what is noted from the different material histograms is that the centre of the distribution varies depending upon material concentration within the tablet matrix. Each histogram has been overlaid in Figure 2.21. It can be seen that each material’s distribution is centred on a different PLS score value, which appears to be proportional to concentration. A comparison between component concentration and histogram features are presented in Table 2.2.

![Histogram Overlay](image)

*Figure 2.21 - Overlay of histograms (score value versus number of pixels) for each pure component in PLS classification model showing a decrease in mean of distribution with respect to % w/w in formulation*
Table 2.2 - Comparison of pure component % w/w in non-spatially resolved system relative to parameters associated with distribution of pixel score value

<table>
<thead>
<tr>
<th>Component</th>
<th>% w/w</th>
<th>$\text{MSD} \pm \text{STD}$</th>
<th>$(\text{MSD} \pm \text{STD}) \times 100$</th>
<th>Skew</th>
<th>Kurtosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voriconazole</td>
<td>33.3</td>
<td>0.2961 ± 0.0603</td>
<td>29.6 ± 6.0</td>
<td>2.7</td>
<td>11.7</td>
</tr>
<tr>
<td>Lactose Monohydrate</td>
<td>41.7</td>
<td>0.4747 ± 0.0615</td>
<td>47.5 ± 6.1</td>
<td>0.88</td>
<td>1.6</td>
</tr>
<tr>
<td>Pregelatinised Starch</td>
<td>14</td>
<td>0.2284 ± 0.0751</td>
<td>22.8 ± 7.5</td>
<td>0.97</td>
<td>2.0</td>
</tr>
<tr>
<td>Croscarmellose Sodium</td>
<td>5</td>
<td>0.0735 ± 0.0635</td>
<td>7.3 ± 6.3</td>
<td>-0.42</td>
<td>1.3</td>
</tr>
<tr>
<td>Povidone</td>
<td>5</td>
<td>0.0409 ± 0.0178</td>
<td>4.1 ± 1.8</td>
<td>-0.58</td>
<td>1.2</td>
</tr>
<tr>
<td>Magnesium Stearate</td>
<td>1</td>
<td>0.01385 ± 0.0163</td>
<td>1.4 ± 1.6</td>
<td>-0.11</td>
<td>0.17</td>
</tr>
</tbody>
</table>

The mean of the score distribution (MSD) is presented with the standard deviation (STD) around this mean. If this parameter is scaled by a factor of 100, the value obtained is similar to the % w/w concentration. When the STD range is taken into consideration, 5 of the 6 components show score values that match the true concentration, shown in Figure 2.22.

![Figure 2.22](image)

**Figure 2.22** - Plot of true pure component concentration (% w/w) in non-spatially resolved system versus the mean score value ($\times 100$) from distribution of score values for each components relative to number of pixels

The pregelatinised starch component's score value is slightly higher than the true concentration and is over predicted by the score value approach. A potential
explanation for this could be due to the strong water band observed in pregelatinised starch (~5250 cm\(^{-1}\) (~1910 nm)). The tablets are produced using a granulation process and water is used with povidone to make the granulating agent. From the three major materials in the formulation, pregelatinised starch is the only one with a strong response around 5250 cm\(^{-1}\) and water spectral contributions could be mixed in with this component, which is not modelled in the library data set.

Another interesting comparison in the MSD values is between povidone and croscarmellose sodium, as both materials are present in the formulation at 5% w/w but the MSD for povidone is slightly lower, ~4% whereas the croscarmellose sodium prediction is slightly higher ~7%. Including the STD, both components fall with the actual % w/w but this variation could be a result of material properties (such as density, particle size or molar absorptivity) or simply due to spatial distribution of each component in the tablet matrix (povidone is larger with very defined domains whereas croscarmellose sodium is closer to the individual pixel level – making spatial detection of this component more challenging). The relationship between MSD from PLS classification of image data is explored further in Chapter 4 but further work is still required to explore the effect of material properties on concentration prediction.

2.8 CONCLUSIONS

In terms of data processing methods, chemical imaging data can be analysed in a very similar fashion to any other spectroscopic method. Obviously spatial information adds an extra factor but in terms of processing regimes the algorithms are completely ignorant of any spatial information. This is due to the fact that spatial information is removed in the first step of any analysis by translation into a two-dimensional (2D) matrix. The spatial information is only used after processing when the 2D matrix is re-folded to give rise to the processed image cube. In this research, there is typically a three-stage method to the evaluation of an image data cube. The first stage is to normalise the data and evaluate the changes in the images at each wavelength. This
step allows an understanding of the significant spectral changes in the sample, which can be referenced to the pure component for further clarification. Similarly a decision around the need for derivatives can also be obtained. The second step is to perform PCA to determine the major spectral variations in the data set and the effect this has on spatial distribution. Both of these steps serve to increase understanding of the first sample, and optimise knowledge around ingredient distribution and its relationship to pure spectral information. With this understanding, a movement to the third stage is possible; the use of PLS approaches. By performing stages one and two, it ensures that the unsupervised nature of PLS does not run the risk of mis-identifying a component of interest. Throughout this research this three stage approach has been utilised and determination of optimised processing methods has been possible.
3. EFFECTIVE VOLUME SAMPLED IN NIR MICROSCOPY

3.1 INTRODUCTION

3.1.1 Lateral Spatial Resolution

NIR microscopy provides the means to look at a sample’s microscopic matrix and gain an understanding of the position and particle size of chemical components within that matrix. How representative this chemical information is depends from where the detected NIR signal is actually obtained. There are two key attributes that define from where the interacted NIR radiation is detected, namely the lateral spatial resolution and the depth of penetration.

Lateral spatial resolution in a NIR-PM experiment is defined as the ability to measure the spectrum from an object defined by the apertures without impurity radiation from neighbouring objects (Messerschmidt, 1988). In NIR-GI the spatial resolution is a function of magnification and array size. Very little work has been reported on how effective these methods are for defining the true area of contribution to each spectrum. The factors affecting spatial resolution are discussed in more detail in Section 3.2. The second area that is key to obtaining a representative chemical image of the sample of interest is how far the NIR radiation penetrates into the sample and more importantly where a signal is actually detected. This is vital for accurate understanding of chemical images, as it is necessary to know whether the image obtained simply models only the top layer of particles, or whether the image is an average of multiple layers. This chapter addresses lateral spatial resolution and the contributing depth of penetration in an attempt to understand the limitations of NIR microscopy.

3.1.2 Factors Affecting Lateral Spatial Resolution

With NIR microscopy being the coupling of microscopy and spectroscopy, there are a number of optical principles that must be recognised as well as the need for an understanding of NIRS. Snell’s Law of refraction for mirrored surfaces states that when
a ray of light encounters a reflective surface, the angle of incidence of that ray with respect to the surface will equal the angle of reflection. However optical aberrations do occur within the optical path, such as spherical or chromatic aberrations, which affect the light path of the ray. However, these aberrations are usually corrected within the actual instrument design. The one area that can not be corrected for is diffraction. In microscopy, diffraction causes the image of the sample to blur, i.e. the returned signal to the detector contains spectral information from a larger physical area than is expected. Diffraction can occur at a number of points within the microscope optics, but the small apertures employed to spatially isolate the sample of interest are responsible for most of the diffraction that occurs, (Sommer and Katon, 1991).

When a wave passes through an aperture, it undergoes diffraction and so the image of a point source is not a point but a diffraction pattern. Specific geometric shapes have pre-determined solutions to their diffraction patterns. For a point source this diffraction pattern is known as Airy Rings, shown in Figure 3.1.

![Figure 3.1 - The diffraction pattern of a point source (Airy Rings)](image)

The distance from the centre of the inner bright ring to the centre of the first dark ring is defined, as the extent of the Airy ring (α), Equation 3.1, where f is the focal length of the objective, λ the wavelength of light and D the diameter of the objective.

\[
\alpha = \frac{1.22 f \lambda}{D} \quad (3.1)
\]
The Rayleigh Criterion is that 'two images can just be resolved when the central maximum of one pattern coincides with the first minimum of the other', (Benson, 1991), which is another method for describing the extent of the Airy ring. Hence, $\alpha$ is actually the minimum resolvable spatial resolution. The value of the objective typically used in microscopy is the numerical aperture ($NA$) which is related to the objective diameter and the focal length by the $F$-number. The $F$-number of the objective is given by Equation 3.2.

$$F = \frac{1}{2NA} = \frac{f}{D}$$

(3.2)

which means that the minimum spatial resolution ($sr$) can be described as Equation 3.3.

$$sr = \frac{1.22\lambda}{2NA}$$

(3.3)

The NIR-PM instrument used a wavelength range from 1111 nm through to 2500 nm with a $NA = 0.6$. This implies that the theoretical minimum resolvable spatial resolution is in the order of $1 - 2.5 \mu m$.

To date this limit has not been reached in NIR-PM. This is due to energy limitations, which are caused by the aperturing method, and secondly to detector size which becomes shoot noise limited at smaller than $10 \mu m$. The most realistic prospect of achieving these sorts of spatial resolution lie with NIR-GI, because these simply require increased magnification with an appropriate amount of radiation hence the true limitations are diffraction (Lewis et al, 1995).

No figures exist in the literature for NIR-PM experiments, but some studies have been performed to examine the spatial resolution in a MIR point mapping experiment.
(Sommer and Katon, 1991). In this study the effect of stray light on spatial resolution was evaluated using cellulose acetate films. The authors reported that even when 8 μm apertures were employed; the spatial resolution at best was 56 μm – a factor of three times greater than that predicted using the Airy Ring model. The spatial resolution in Raman line imaging has been examined (Hayden and Morris, 1996) looking at the effects of slit width and magnification using polystyrene spheres. The work showed that spectral features of 10 μm polystyrene spheres could be identified within an epoxy matrix, but the chemical images lacked the resolution to define the spherical shape of the polystyrene. Spheres seem like an ideal sample choice due to their defined edges but, because of their volume, they show variations with depth of field and positioning. Using this method for spatial resolution studies requires careful sample preparation, to ensure similar depth of field for each sphere but is ideal because of spectral definition.

Polystyrene micro-spheres were also used by Treado et al., 1999, to evaluate the spatial resolution in Raman imaging. This investigation showed the resolving power of the instrument at 992 cm⁻¹, to be able to identify and resolve two micro-spheres (2 μm in diameter) separated by a spacing of only 200 nm. Obviously these sorts of limits are not achievable in the NIR region but the spheres, in this instance with correct positioning, were ideal for obtaining an estimate of spatial resolution. However, no spectral information was presented for an understanding of the spectral response of these small samples. Similarly in a study by Lewis et al. (1995) a resolution target was used to evaluate spatial resolution, with objects 17.5 μm in size and separation, being resolved at a wavelength of 3 μm but without the presence of supporting spectral information. These two spatial resolution figures relate to the smallest resolvable object and not necessarily to the actual area of spectral contributions. One method currently expanding in use is infrared microscopy using synchrotron radiation as a source. The synchrotron radiation serves as a diffraction-limited point source and the spatial resolution of the wavelength of light used have been reported (Carr, 2001).
The system can allow imaging at the diffraction limit because good S/N is observed in the spectra even when the apertures are set at below diffraction levels.

The literature examples which mention spatial resolution seem to be mainly discussions on how to improve instrumentation to minimise the effects of stray light induced by diffraction and not necessarily useful for an analyst to use in understanding spatial resolution. Various methods of evaluating spatial resolution have been discussed and each will be exploited to understand the spatial resolution limits in both NIR-PM and NIR-GI.

3.1.3 Depth of Penetration of NIR radiation

In the literature there are no discussions on the depth that NIR radiation penetrates into a sample in NIR microscopy. There are examples of measurements of the penetration of NIR radiation in macro-scale experiments and these are discussed in this section. It should be noted that the depth of penetration is an important consideration for microscopy experiments, as the depth will affect whether the resultant chemical images simply are a ‘surface’ description of the sample or are actually an average of a number of layers of material.

In macro-scale NIRS, various studies have been performed to estimate the sample size examined using diffuse reflectance NIRS. These evaluations have included studies of the ‘information depth’, which is the depth of sample contributing to the measured reflected radiation, which is different from the maximum physical penetration depth of the radiation (Berntsson et al, 1998). Various figures exist in the literature for the information depth, ranging from as small as 100 μm (Andersson et al, 1999), through to 5000 μm (MacDonald and Prebble, 1993). Olinger and Griffiths (1992) reported a means to estimate the information depth in diffuse reflection NIRS using mixtures of carbazole, graphite and sodium chloride. Using the measured absorbance values,
known particle size and absorptivity for the mixtures, it was possible to calculate the number of particles that the NIR radiation reaching the detector had passed through. For small (33 μm in diameter) and large (100 μm in diameter) particles, at 1672 nm, the information depth was in the order of 1 mm, but because of scattering effects the true information depth was believed to be much smaller, around one-third of this value (~300 μm). Haanstra et al (1998) investigated the information depth for polyethylene films and their experiments suggested a value around 600 μm in the wavelength range 1800 - 2200 nm.

When the information depth was investigated for pharmaceutical materials, different values were again observed for the information depth. Hammond, et al (1997) performed a study on pharmaceutical powders compacted to a density of 0.5 gcm$^{-3}$, and found that the information depth was in the order of 500 μm. Studies by Hammond et al (1999) based on lactose monohydrate (bulk density of 0.575 gcm$^{-3}$) showed that, using a NIR reflectance probe, the information depth was no greater than 500 μm and that 4 mg of sample was analysed. Berntsson et al (1999) also discussed both penetration issues within pharmaceutical materials and the effective sample size in reflectance NIRS. Their work was based around film-coated pellets with different powder thickness and also samples of microcrystalline cellulose. Using microcrystalline cellulose the sample size was 20 mgcm$^{-2}$ and an information depth of 670 μm was determined at 1500 nm. For the film-coated pellets, the values above 1500 nm were around 70 mgcm$^{-2}$ for the sample size and 1 mm for the information depth. Film coated tablets were also investigated by Andersson et al (1999) but in their investigations the information depth was found to be in the order of 100 – 200 μm. This was predicted from an extension of a PCA score plot after NIR analysis of film-coated (10 – 50 μm) bi-layer tablets. Hence, the studies to date have shown the variability in the actual value of the information depth and also shown dependence of this value upon the wavelength of light along with the actual density, particle size and absorptivity of the material studied, this is summarised in Table 3.1.
Table 3.1 - Literature values for depth of penetration of NIRS radiation into a variety of different materials and at a number of different wavelengths.

<table>
<thead>
<tr>
<th>Depth of Penetration (µm)</th>
<th>Wavelength(s) of interest</th>
<th>Material and Physical Information</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 - 200 µm</td>
<td>1450 - 1650 nm &amp; 2050 - 2200 nm</td>
<td>Film-coated bi-layer tablet</td>
<td>Andersson et al, 1999</td>
</tr>
<tr>
<td>&lt;500 µm</td>
<td>Above 1800 nm</td>
<td>Pharmaceutical blend (loose powder)</td>
<td>Hammond et al, 1997</td>
</tr>
<tr>
<td>500 µm</td>
<td>1000 - 1721 nm</td>
<td>Lactose monohydrate (loose powder)</td>
<td>Hammond et al, 1999</td>
</tr>
<tr>
<td>~500 µm</td>
<td>1400 - 1800 nm</td>
<td>Pharmaceutical blend (loose powder)</td>
<td>Hammond et al, 1997</td>
</tr>
<tr>
<td>600 µm</td>
<td>1800 - 2200 nm</td>
<td>Polyethylene film</td>
<td>Haanstra et al, 1998</td>
</tr>
<tr>
<td>670 µm</td>
<td>1600 nm</td>
<td>Microcrystalline cellulose (loose powder)</td>
<td>Berntsson et al, 1999</td>
</tr>
<tr>
<td>&lt;750 µm</td>
<td>&gt;1600 nm</td>
<td>Pharmaceutical powders (loose powders)</td>
<td>Berntsson et al, 1998</td>
</tr>
<tr>
<td>900 µm</td>
<td>1600 nm</td>
<td>Film coated pellets (loose powder)</td>
<td>Berntsson et al, 1999</td>
</tr>
<tr>
<td>~1 mm</td>
<td>1672 nm</td>
<td>Powder mixture of carbazole, graphite and sodium chloride</td>
<td>Olinger and Griffiths, 1992</td>
</tr>
<tr>
<td>&gt;1 mm</td>
<td>&lt;1400 nm</td>
<td>Pharmaceutical blend (loose powder)</td>
<td>Hammond et al, 1997</td>
</tr>
<tr>
<td>2.33 mm</td>
<td>900 nm</td>
<td>Microcrystalline cellulose (loose powder)</td>
<td>Berntsson et al, 1999</td>
</tr>
<tr>
<td>2.5 mm</td>
<td>400 - 900 nm</td>
<td>Polyethylene film</td>
<td>Haanstra et al, 1998</td>
</tr>
<tr>
<td>4.6 mm</td>
<td>900 nm</td>
<td>Film coated pellets (loose powder)</td>
<td>Berntsson et al, 1999</td>
</tr>
<tr>
<td>Up to 5 mm</td>
<td>1100 - 2500 nm</td>
<td>Pharmaceutical samples</td>
<td>MacDonald and Prebble, 1993</td>
</tr>
</tbody>
</table>

All factors affecting the information depth in macro-scale NIR spectroscopy will be applicable to NIR microscopy. However, the actual reported information depths have referred to purely macro-scale NIR spectroscopy and these values may differ for micro-scale measurements. This difference will arise because of variation in the intensity of the radiation reaching the sample and also because of the variation in focusing of the radiation. It could also be caused by the fact that some of the reported values have
been estimated from extrapolation of experimental results and others have been limited by sample preparation restrictions.

Previous work in the current studies on combining Raman and NIR point mapping data suggested that both techniques have a common sampling area, (Chapter 7). Typically Raman spectroscopy is thought of as a surface technique and therefore the information depth in NIR microscopy was not thought to be as large as some of the previously reported penetration values, e.g. <500 μm.

From the reported literature data, there is no agreed value for the information depth in NIR spectroscopy. This is not critical for macro-scale NIRS but with NIRM sampling a surface area of ~20 μm × 20 μm for each spectrum acquired, it is important to know whether the light penetrates just the immediate particle (say 20 μm) or several layers below (say 200 μm). The results from the experiments presented here should lead to an accurate description of the actual depth sampled and used for spectral information in reflectance NIR microscopy.

3.1.4 Determination of the volume sampled in NIR microscopy

This investigation will examine the lateral resolution of NIRM instrumentation, which is a result a two different factors, the actual area of sample analysed and also the area from which a spectral contribution is obtained. Ideally these two factors would be equivalent, but to date have not been determined. The spectral contribution will also be affected by the depth to which the NIR radiation penetrates and therefore this value will be measured. With these values an approximation of the volume of material sampled during a NIRM experiment can be determined, and therefore determination of the mass and percentage of a unit dose measured.
3.2 EXPERIMENTAL

3.2.1 Area of Sample Contribution in NIR microscopy

To investigate the area of contribution in a NIRM experiment it was necessary to explore samples with known sizes to allow the smallest resolvable object to be determined.

Samples and Preparation

Two different objects with known sizes were selected. Firstly, polymer micro-spheres (20 μm) were obtained from the National Institute of Standards and Testing (NIST, Gaithersburg, MD, USA) as dispersion in an aqueous solution and were prepared for analysis by dispersion onto a clean microscope slide and being left to dry (at room temperature). Secondly a United States Air Force (USAF) 1951 reflective resolution target (glass with blue chrome target bars) was obtained from Sine Patterns LLC, NY, USA. The target bars are placed into groups with each group having six elements and each element has six lines with specified line-widths. In this work group two to five elements were examined, covering a line width from 8.77 μm - 114.5 μm (actual line widths for each group and element are presented in Appendix 1).

Instrumentation

The Autoimage NIR point mapping system (shown in Figure 1.6a) was used to analyse both the polymer micro-spheres and the USAF target. The spectrometer collected spectral data over the range 4000 – 7500 cm⁻¹ (1333 nm - 2500 nm) with a 16 cm⁻¹ data interval and an average of 25 scans. The area of analysis was varied dependent upon the sample but the aperture width was fixed at 20 μm. The MatrixNIR™ global imaging system (shown in Figure 1.8) was used to analyse the USAF target. An image was generated at 1570 nm using 16 co-added frames with the ×10 objective in front of the target.
Data Collection
Two different areas on the slide with the micro-sphere dispersion were analysed using NIR-PM. Both were 500 by 500 μm in size (676 spectra) but one contained a cluster of micro-spheres approximately 300 μm in diameter and the other contained 16 micro-spheres with different inter-sphere distances.

For NIR-PM experiments on the USAF target, maps were set-up around group two (2.18 mm by 3.54 mm), three (1.16 mm by 2.22 mm) and four (0.58 mm by 1.08 mm) elements. The line width of the smallest element in group four was 17.54 μm and it was reasoned that as this size was smaller than the aperture size that the bars would not be resolvable and as such group five elements were not examined. For NIR-GI experiments on the USAF target, it was possible to examine group four and five elements in a single measurement. Group 3 elements and above were not examined as these had the smallest line width of 35 μm, which is three times greater than the instrument manufacturer’s suggestion of a lateral resolution of 10 μm.

Software and Data Processing
The raw NIR data collected from NIR-PM experiments were converted into ASCII files and translated into a format (.spf) readable by image analysis software (ISYS, Spectral Dimensions, MD). The data from the NIR-GI experiments were directly opened in the image analysis software.

Chemical images of the micro-spheres were generated at a single wavelength, with contrast being based on the actual absorbance response. Comparison between visible and chemical images was performed using Paint Shop Pro 5, Jasc Software. For the USAF targets by NIR-PM the raw absorbance data was used but processed using PCA (Section 2.5) with five factors before generation of the chemical images. In NIR-GI only one wavelength image was obtained and as such the chemical image was obtained by the variation in absorbance at 1570 nm. From the chemical images of the USAF target
generated, line projections through the target elements were saved as ASCII files and opened in Excel (Microsoft Corp., Seattle, USA). The limit of identification for size was determined when three lines were not observed for an element set.

3.2.2 Area of Spectral Contributions in NIR microscopy

Samples and Preparation
In NIR-PM it was thought the simplest method to measure the spectral contribution would be to limit the amount of sample exposed to the NIR radiation, by employing the use of a second aperture. Optical apertures were sourced from Lenox Lasers Inc, (MD, USA). The optical apertures were made from 300-series stainless steel discs (shiny or blackened surface), thickness \( \geq 15.5 \, \mu \text{m} \), with circular apertures of varying size at the centre of the discs generated using laser drilling. The blackened discs did not work, due to light absorption, and as such the shiny discs were employed. Five different aperture sizes were selected (400 \( \mu \text{m} \), 200 \( \mu \text{m} \), 100 \( \mu \text{m} \), 50 \( \mu \text{m} \) and 25 \( \mu \text{m} \)) and using a small clamp were secured onto the measurement surface. The clamp ensured that the aperture was in close contact with the sample surface, such that both were in focus under the microscope. Spectralon standard (Labsphere Inc, North Sutton, NH, USA) was used to generate the background spectrum. A 300 mg sample of oxytetracyline hydrochloride (Pfizer Inc.) was compressed into a wafer (1 cm diameter, 3 mm thick) and used for the sample spectrum, due to its strong known absorptions.

Due to the poor results obtained from the aperture experiments, a second method was employed to evaluate spectral contributions. Interfaces were made between five sets of two spectrally different materials. The interface was generated by firstly compressing one material into a barrel, using hand pressure, and then adding the second material on top and compressing the two materials together using 4000 p.s.i. The disc was then removed and was cut in half using a scalpel (across the perpendicular axis to the interface) to expose the interface for analysis.
Five different API's from Pfizer Inc. were used in this investigation, oxytetracycline hydrochloride (OTC), prazosin hydrochloride (Pra), fluconazole (Flu), pyrantel palmoate (PP) and ticonazole (Tio). In general, API's were selected because they had very distinct bands at specific wavelengths (normalised SNV spectra shown in Appendix 2) but also oxytetracyline hydrochloride and pyrantel palmoate were selected as they were coloured and allowed for easy interface identification. Table 3.2 provides the four combinations of APIs used, along with information on the spectral and colour variations of the different interface sets.

Table 3.2 - Combination of materials and variations present in the sample interface

<table>
<thead>
<tr>
<th>Interface</th>
<th>Materials 1</th>
<th>Materials 2</th>
<th>Significant Colour Variation</th>
<th>Wavenumber Responses Measured (cm⁻¹) Material 1</th>
<th>Wavenumber Responses Measured (cm⁻¹) Material 2</th>
<th>Absorbance values similar at strongest absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>OTC</td>
<td>Flu</td>
<td>Yes</td>
<td>4472, 5207, 5992</td>
<td>4408, 4712, 6112</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>OTC</td>
<td>Tio</td>
<td>Yes</td>
<td>5207</td>
<td>4344, 4648, 5808, 6120</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>OTC</td>
<td>PP</td>
<td>No</td>
<td>4480, 5207</td>
<td>4338, 6104</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>Flu</td>
<td>Pra</td>
<td>No</td>
<td>4680, 5968, 6120</td>
<td>4960</td>
<td>No</td>
</tr>
</tbody>
</table>

Instrumentation and Data Collection

The Autoimage NIR point mapping system (shown in Figure 1.6a) was used to analyse both the apertured and interface samples. The spectrometer collected spectral data over the range 4000 - 9000 cm⁻¹ (1111 nm - 2500nm) with a 16 cm⁻¹ data interval and an average of 25 scans using an aperture width of 20 μm. For the secondary aperture experiment, a spectrum was collected through the aperture and the absorbance spectrum generated by referencing the sample to the Spectralon data file, as previously described in Equation 1.11.

When the interface samples were examined using NIR-PM a line-scan (spectral collection with movement in only one axis) was performed across each interface. Each line scan covered a distance of 440 μm (23 spectra) with a spectrum being collected every 20 μm (equivalent to aperture size). In all line-scans the interface was aligned.
with point 12 ± 1 point in the line-scan which meant that each component was analysed from 0 μm through to at least 200 μm from the interface, as shown in Figure 3.2.

Figure 3.2 - Schematic of line scan experiment across sample interface

The MatrixNIR™ global imaging system (shown in Figure 1.8) was also used to analyse the sample interfaces. A data cube was collected from 1100 - 1700 nm using a 5 nm data interval with each wavelength being the average of 16 co-added frames and 2 repeat scans. A reference data cube was collected under the same conditions using a Spectralon standard. A ×10 objective was utilised such that the pixel size was ~ 9 μm.

Software and Data Processing
In the case of the apertures and the line-scan data there was no need to translate the data into the image analysis software. The spectra obtained through the apertures were examined as percentage reflectance signals within the data acquisition package (AutoImage). The line-scan data were converted in ASCII files and analysed within Delight (DSquared Development, La Grande, OR, USA), which is a spectral processing package. The spectral sets for each interface were truncated to cover only the spectral range with useful information (4200 - 7500 cm⁻¹). The spectra were then treated with an SNV transform to remove baseline offsets, and then smoothed (3 point) and second derivatives (9 point) calculated.
The unique absorptions for each component were identified and the second derivative absorbance value at each wavenumber was determined across the entire line-scan. The difference in absorption between the two components was determined at each wavenumber and used to calculate the percentage contribution of each component, Equation 3.4. Equation 3.4 represents the percentage of component 1 present at a unique component 2 absorption ($\lambda_x$), where $A_x$ is the absorbance at position $x$ in the line-scan, and $A_{P2}$ and $A_{P1}$ are the absorbance values of the pure components at this wavenumber.

$$\%\text{Comp}_{12} = \left( \frac{A_x - A_{P2}}{A_{P1} - A_{P2}} \right)$$

To determine the percentage contribution of each component throughout the line-scan, the average of the percentage of each component at each unique wavelength was calculated. This value was then used to determine the percentage of each component that contributed to the spectrum at each position.

For the NIR-GI interface data, the absorption spectra were calculated by dividing the sample data cube by the standard data cube and then the inverse logarithm calculated. The spectra were then mean centered and scaled to unit variance, and a three point smooth performed. Due to the large number of spectra in the data set, the spectra were processed using PLS (Section 2.7). A library of all six components (five API's and off-sample spectra) were generated and used to allow PLS 2 to be performed on the data cubes. Using the score images, it was possible to identify the amount of each component of interest that was present at each pixel. Three different percentage regions were selected, 90 – 100%, 75 – 90% and 50 – 75%. These were represented in a colour image, showing where the component of interest was greater than half the contribution to the overall spectrum. The two interface score images for >50% were
combined, to identify any regions of spectral overlap and hence determine the position of spectral contributions.

3.2.3 Depth of Penetration in NIR microscopy

The depth of penetration of NIR radiation into a sample was investigated measuring the absorption profile changes of a substrate when the interacting radiation had to penetrate increasing amounts of material to reach the substrate. Layers of material were stacked on top of the substrate, as seen in Figure 3.3. It was not possible to make compressed pharmaceutical excipients to make these layers and so it was necessary to mimic the typical nature of pharmaceutical materials. Cellulose and its derivatives are commonly used as one of the main ingredients in pharmaceutical formulations and they are also widely available in day to day life with a large range of properties. It was therefore decided to use cellulose (paper) as the main basis for this investigation. To ensure that the paper modelled the physical nature of pharmaceutical blends/tablets, a second form of cellulose was also used. In this case the cellulose was prepared as slurry and applied thinly onto the surface of the substrate.

![Figure 3.3 - Schematic of the experimental model established, with the NIR light path shown being only one example of the possible interaction of the radiation](image)

**Samples and Preparation**

Nine types of paper were obtained from various sources (Table 3.3). The thickness of each paper was measured using a micrometer and the values ranged from 20 - 114 μm, with each measurement having an associated reading error of ± 6 μm, Table
3.3. All nine paper types were analysed using NIR-PM but only the thinnest paper was used for NIR-GI experiments.

Table 3.3 - Thickness and make of each paper used for depth of penetration investigation

<table>
<thead>
<tr>
<th>Class</th>
<th>Type</th>
<th>Make</th>
<th>Thickness (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A4</td>
<td>Bright White</td>
<td>Hewlett Packard</td>
<td>114</td>
</tr>
<tr>
<td>A4</td>
<td>Photocopy</td>
<td>Ikon</td>
<td>89</td>
</tr>
<tr>
<td>A4</td>
<td>Laboratory Notebook</td>
<td>Dudley – Code 4555</td>
<td>79</td>
</tr>
<tr>
<td>Tissue</td>
<td>Medicated</td>
<td>Izal</td>
<td>41</td>
</tr>
<tr>
<td>Cigarette</td>
<td>Corner Cut</td>
<td>Swan</td>
<td>28</td>
</tr>
<tr>
<td>Cigarette</td>
<td>Medium Weight</td>
<td>Swan</td>
<td>26</td>
</tr>
<tr>
<td>Cigarette</td>
<td>Medium Weight</td>
<td>Rizla</td>
<td>25</td>
</tr>
<tr>
<td>Cigarette</td>
<td>Light Weight</td>
<td>Rizla</td>
<td>20</td>
</tr>
<tr>
<td>Cigarette</td>
<td>Kingsize</td>
<td>Rizla</td>
<td>20</td>
</tr>
</tbody>
</table>

Meclizine hydrochloride (300 mg) was compressed into a disc (3 mm in depth) using 4000 p.s.i pressure and utilised as the substrate (due to strong absorptions in different regions from cellulose). The sample was prepared for analysis by mounting the API disc on to a microscope slide, using cyanoacrylate adhesive to prevent movement during analysis. Each layer of paper was secured across the drug surface using transparent adhesive tape at each edge. After each paper addition, the spectral data were collected from the sample surface, and then layers were subsequently added until at least 150 μm of cellulose was present on the drug surface. After the analysis of each set of paper layers, a pure sample of each paper (folded to give thickness greater than 150 μm) was analysed with no substrate present.

To enable the wavelength dependency of the NIR radiation to be monitored, two other materials were used as the substrate. Magnesium stearate and confectioner’s sugar (milled sucrose with 3% corn starch) were selected because they were spectrally different from meclizine hydrochloride and cellulose. Each material (300 mg) was separately compressed, as above using 4000 p.s.i. pressure. The light weight paper
was selected for this investigation, and for both magnesium stearate and sugar the same procedure was followed as for the API.

Two types of cellulose (Type 50 and Type 20 Sigmacell® with 50 μm and 20 μm mean particle sizes respectively) were obtained from Sigma Aldrich for preparation of the cellulose slurries. The substrate for this investigation could not be a pharmaceutical powder because of the ‘wet’ nature of the cellulose, which may have been absorbed by or penetrated into the pharmaceutical powder affecting its spectral characteristics. Therefore, an impervious substrate was used - the base of a plastic weighing boat. As a change in the substrate’s physical characteristics was made, it was necessary to repeat the light weight paper layer experiment using the plastic substrate, to ensure that similar results were obtained. The manufacturer’s recommended preparation of the cellulose was as 15 - 20% aqueous slurries, but these were found to be difficult to apply as a layer on the plastic, producing droplets instead of spreading. To minimise this effect, the slurries were prepared in methanol, which also assisted rapid drying. The slurries were then applied as a layer onto the plastic surface. The thickness of each layer was measured using the microscope focusing point because the use of micrometers was not feasible as the nature of the sample caused it to simply crumble under the force of the micrometers. It should be noted that the layers of cellulose were not of a constant thickness due to practical and sample limitations, and would have contained voids and the thickness not increased uniformly with each layer addition. The thickness of each layer was therefore an average of the thickness values measured at each data collection point, giving rise to Type 20 layers in the range 3 - 56 μm and for Type 50 layers in the range 18 - 88 μm.

Instrumentation and Data Collection
The Autoimage NIR point mapping system (shown in Figure 1.6a) was used for NIR-PM experiments and the MatrixNIR™ system (shown in Figure 1.8) was used for NIR-GI experiments. In the NIR-PM experiments the remote apertures were set with a
width of 20 μm, and the spectral data was collected over the range 7500 - 4200 cm⁻¹ with the spectrometer resolution set at 16 cm⁻¹ and each spectrum was the average of 25 co-added scans. An area of 3 × 4 mm was analysed for each sample, with spectra collected at 1 mm intervals. Therefore, for each sample, 20 spectra were obtained and the average of these was taken to be representative of the sample. In the NIR-GI experiments a ×10 objective was used to allow the depth of penetration in NIR global imaging to be compared with that observed in NIR point mapping (×15 objective). NIR images were collected at 5 nm wavelength intervals over the wavelength range 1100 – 1700 nm. Each image was collected as 16 co-additions and as an average of 4 scans.

Software and Data Processing
In NIR-PM experiments the data were collected through the instrument Autoimage software but the spectral data files were converted into ASCII format to allow for analysis in Delight software. The spectra were then converted in absorbance units, as previously described in Equation 1.11. To allow the twenty spectra from each thickness value to be averaged it was necessary to use SNV transform to remove physical offsets in the data. The second derivative spectra were then calculated using a 4 point smooth and 8 point derivative to improve spectral definition.

The reflection spectra obtained from NIR-GI experiments were firstly transformed into absorption spectra using the inverse logarithm relationship (Equation 1.11). The spectra were then mean centered and scaled to unit variance by sample, and then the average of the 76,800 spectra was calculated. As for the NIR-PM spectra, derivatives were calculated and, to allow for comparison to the NIR-PM data, the spectra were treated with a 2 point smooth and 3 point second derivative.

To enable a description of the line of best fit through the thickness versus absorption profiles, a non-linear regression analysis program, NLREG (Phillip H. Sherrod) Version 5.2, was used.
3.3 RESULTS AND DISCUSSIONS ON AREA OF SAMPLE CONTRIBUTION IN NEAR-INFRARED MICROSCOPY

3.3.1 Cluster of Polymer Micro-Spheres

The diameter of the polymer micro-spheres (20 µm) was equivalent to the aperture width in the NIR-PM instrument. However, they are not matched in shape so some of the apertured area will be outside the micro-sphere. When the spectral data were collected it was observed that the polymer micro-spheres did not give true absorption spectra, with the only variation between the glass and the micro-spheres being a change in % reflectance, glass 100% but micro-spheres ~10% reflectance across the entire wavelength range, shown in Figure 3.4b. The % reflectance response was used to generate the chemical image, Figure 3.4a. In this image, all pixels giving a NIR reflectance signal greater than 85% were coloured white and any pixel with less than 15% reflectance were coloured black. Grey-scaling was used in between these values.

![Figure 3.4 - a) NIR image of cluster of polymer micro-spheres with b) associated NIR spectra](image)

Having generated the NIR chemical image of the micro-sphere cluster it was possible to compare with the visible image features, Figure 3.5. This figure shows good correspondence between the two, and as such the visible image (Figure 3.5a) was subtracted from the NIR image (Figure 3.5b) giving the resultant image in Figure 3.5c. The resultant image shows that the NIR identified cluster is in fact slightly smaller than the visible image. This implies that the NIR spectral response comes from within the apertured area. The difference is thought to be caused by the curvature of the spheres that cause the apertured area to not be filled completely by a micro-sphere and hence
causing a lower % reflectance response at the edge of the cluster. From this it can be concluded that the aperture size is effectively defining the sample area and that an object much greater than the aperture size can be resolved without enlargement. It is also interesting to observe that the shaded pixels away from the main cluster in the NIR image (Figure 3.5b) can be associated with dried water droplets present on the glass slide.

![Image](image.png)

**Figure 3.5 - Comparison of visible (a) and NIR (b) images of the polymer micro-spheres, with image (c) representing the difference between the NIR and visible images (b - a). All images have a x- and y-axis dimension of 500 μm**

### 3.3.2 Dispersion of Polymer Micro-Spheres

From evaluation of the micro-sphere cluster it was observed that the threshold for % reflectance of a polymer micro-sphere is < 60%. If the % reflectance is greater than this threshold then the contributing factor is the glass slide. Using this threshold it was possible to generate the NIR chemical image for the dispersed micro-spheres, the resultant NIR image for the dispersed polymer micro-spheres, using the % reflectance limit set above, is shown in Figure 3.6b. The visible image of the spheres is also shown for comparative purposes in Figure 3.6a, but it should be noted that the NIR image is the mirror image of the visible image, through the x-axis.
Figure 3.6 - a) Visible and b) NIR image of the polymer micro-spheres dispersed on microscope slide with apertured areas shown and c) NIR image with the mapping grid removed (interpolated pixels)

On first examining the NIR image (Figure 3.6b), it would appear that it is not possible to spatially resolve the 20 μm diameter micro-spheres as the images show, on average, more than one pixel per micro-sphere. Individual spheres are more easily identified when the map grid is removed and spatial interpolation used to give Figure 3.6c. In this image individual spheres can be identified, although spheres in close proximity appear to merge into one area. An explanation for this may be found in the visible image with grids, which shows that during mapping the apertured areas were not exactly matched to a micro-sphere. The only possible spheres that fill an entire apertured area are number 3 and 11, marked in Figure 3.6b. In the NIR image, these two spheres each have one clearly defined dark grey box with very little shading surrounding it which suggests that there is very little contributions from outside the actual apertured area.

Two other methods for presenting the data of the individual micro-spheres were evaluated: a 3-dimensional plot and a contour plot. These two examples were generated at one wavelength rather than as an average of all wavelengths. With a flat NIR absorption profile it did not matter at which wavenumber the images were generated, but 7000 cm⁻¹ was used. Figure 3.7a shows the 3-D projection of the micro-spheres and Figure 3.7b the contour plot. In the 3-D plot all sixteen micro-
spheres can be identified but in the contour plot only fourteen peaks are present, with spheres 7 and 8 as well as spheres 15 and 16 being overlapped. In the sets where the spheres were not resolvable, the visible image (Figure 3.6), showed a spacing between these micro-spheres less than 20 \( \mu m \).

![Figure 3.7 - NIR image of polymer micro-spheres as a) 3D plot and b) contour plot](image)

This work has shown that although the 20 \( \mu m \) diameter micro-spheres can be identified, there could be some justification for believing that some contributions arise from out with the apertured area. However, at the same time the experiment could be considered non-ideal as the spheres do not always lie within the apertured area. What can be concluded from this work is that objects that are the same size as the aperture do give rise to an identifiable NIR signal, and that objects must be separated by the aperture size to be physically resolved. One point of interest is that when the micro-spheres were present as a cluster, they gave a NIR reflectance signal between 0 - 60 \% reflectance but when individually examined this value was always in the order of 60\% and lower percentages were not observed. This would suggest that the reflectance response identified from the sample does not solely arise from the apertured area and that although it is possible to resolve objects in the size order of the aperture it is not a pure component signal that allows this identification.
3.3.3 USAF 1951 Reflective Resolution Target by Near-infrared Point Mapping

For NIR-PM experiments group two, three and four elements on the resolution target were examined, covering a wide range of object sizes (17.54 - 114.5 μm). The NIR response from the target elements could be described as a linearly increasing reflectance response with respect to decreasing wavenumber but the glass slide gave the same % reflectance whatever the wavenumber. Therefore PCA was employed to separate the two components, allowing the entire wavelength range to be used for identification of the target elements. Group 2 elements (70.2 - 111.4 μm) were firstly examined, to ensure that the method defined to identify the target elements worked as the minimum line width was greater than three times the aperture size (> 70 μm / 3 pixels). Figure 3.8a shows the score image obtained for the group 2 elements and shows the elements to be clearly resolved. This figure also shows the average number of pixels associated with each element set, which can be multiplied by the aperture size, to give an estimate of the experimental element size. The calibrated element widths associated with the resolution target are provided in Appendix 1 for comparative purposes. It can be seen that the smallest element should be 70.2 μm in width and the NIR image has 3 pixels for this element, suggesting a width of 60 μm, showing that the NIR image is produced only from signal from within the apertured area. This can be further confirmed by analysis of the score value through all elements, Figure 3.8d, which shows three bars per element set in group two and also shows all bars have similar intensity (24,500 counts which is independent of line width). It can be concluded that the resolution target should be a good method for determining the minimal resolvable object in a NIRM experiment.

In the same manner, group 3 elements were examined (35 - 56 μm) with the resultant score image shown in Figure 3.8b. This figure shows that the elements are resolvable under the experimental conditions and implies that the minimum resolvable object is smaller than 35 μm. When the score responses relative to position through the elements were examined, Figure 3.8d, each set of elements was split into 3 bars all
with similar intensities (~24,500 counts). This implies that the spectral contributions come from only within the defined sample area and therefore NIRM can resolve objects 35 µm in size separated by a gap of 35 µm.

![Image](image_url)

**Figure 3.8 - PCA score two images for a) group 2, b) group 3 and c) group 4 resolution target elements along with d) score values as a function of distance through elements in each group**

Group 4 elements were challenging as they covered the range 17.54 µm - 31.25 µm. The resultant score image shown in Figure 3.8c and it can be seen that the separation of the different element bars is not well defined and the instrumentation is starting to
struggle to resolve the individual bars. If the score values through all elements of this group are monitored, Figure 3.8d, it can be seen that the number of counts ranges from 12,000 to 18,500, which is 50 - 75% of the number of counts observed for groups 2 and 3. This implies that the area sampled includes something other than the target elements. For elements 2 - 5 it is possible to observe three defined peaks, but the intensity varies between and within an element set.

If the number of pixels per element is examined (shown in Appendix 1) it can be seen that for element 2 (theoretically 27.84 μm) that in the score image it has 2 pixels per bar (40 μm) so in size terms is over predicted although the 3 bars are resolvable. Similarly for element 3 (theoretically 24.8 μm) and element 4 (theoretically 22.1 μm) both are observed on average at ~30 μm (between 1 and 2 pixels) so are again over predicted in size but can be resolved from each other. When the target size drops below the aperture size, element 5 (theoretically 19.69 μm), only 1 pixel per bar is observed and the bars can be resolved, although the intensity of the score value is greatly reduced (relative to other sizes). By element 6, which is only 17.54 μm, the system is no longer able to resolve the different bars, although score intensity is similar as for element 5. This implies that the smallest resolvable object is equivalent in size to the apertured area, but the objects must also be separated by a gap that is also equivalent to the apertured area. When the object of interest becomes close to the size of the apertured area, although it can be spatially resolved there is obviously some contribution from the surrounding area, which would suggest that spectral contributions are not solely from the apertured area but include some affects from the surroundings.

3.3.4 USAF 1951 Reflective Resolution Target by NIR Global Imaging

The field of view using the ×10 objective was found to be 2.8 × 2.1 mm, and as such each pixel was 8.8 μm by 8.8 μm. Hence it was thought that objects in the order of 10 μm should be resolvable under the instrument configuration used. Figure 3.9 shows
the resolution target at 1570 nm with group 4 and 5 elements being the main focus of the investigation (8.77 - 31.25 μm). A visual inspection of the target suggests that the limit of resolution is in the order of element 5 or 6 in Group 5.

Figure 3.9 - Chemical image of USAF 1951 Resolution Target at 1570 nm using NIR-GI

The NIR reflectance counts for group 4 and 5 elements were examined, both the vertical and horizontal elements, presented in Figure 3.10 as a function of arbitrary distance. This figure shows that all elements in group 4, whether 31.25 μm apart (elements 2) or 17.54 μm (elements 6), can be resolved in both the x and y dimension. When the elements are clearly separated the centre of the elements give an average NIR reflectance count of ~1650 whereas the glass gives a reflectance count of 2600, a difference of 950 counts. The difference is reduced to ~800 counts in group 4 elements 6, which implies that the elements are no longer contributing to the entire pixel response. This is also observed when the Group 5 elements are examined in Figure 3.10. This figure shows that for this set of elements the reflectance count difference ranges from 800 to 450 counts, as the elements reduce in size, confirming that the elements no longer cover an entire pixel. Also when moving from elements 1 in group 5 through to elements 6 the three elements become less clearly resolved, in particular with element 6. This would suggest that the smallest identifiable (resolvable) object in NIR global imaging using a ×10 objective is in the order of 10 μm (element 5)
and that objects which are 9 μm (element 6), although giving contributions to the NIR reflectance will not be identified as individual components.

![Graph showing NIR reflectance counts for group 4 and 5 target elements with respect to distance across the resolution target when analysed with NIR-GI Instrumentation](image)

**Figure 3.10 - NIR reflectance counts for group 4 and 5 target elements with respect to distance across the resolution target when analysed with NIR-GI Instrumentation**

### 3.4 Results and Discussions on Area of Spectral Contribution in Near-Infrared Microscopy

#### 3.4.1 Optical Apertures

It was thought that by limiting the amount of sample available for interaction with the NIR radiation that a better understanding of the area of spectral contributions could be determined. A choice of blackened or shiny surfaced stainless steel was available, with the first being thought to be better as it would reduce any specular reflectance effects. However, when a 100 μm blackened aperture was placed on the sample surface a ~75% decrease in the energy response was observed, which resulted in an API spectrum which was dominated by noise. The signal to noise (s/n) was dramatically reduced making the distinction of absorption bands unachievable. Therefore it was concluded that the blackened apertures were absorbing too much...
radiation, and as such the experiment with different aperture sizes was performed using the shiny stainless steel apertures.

Figure 3.11a shows the background energy response for each aperture size, relative to the 99% reflectance standard with no aperture in place. This figure shows that the response of the Spectralon is dependent on the size of aperture, but the change is minimal after 100 μm. Using the highest energy value (~4750 cm⁻¹) it can be determined that the 400 μm aperture reduces the signal of the reflectance standard to ~70%, the 200 μm aperture to ~50% and the 100 μm (and smaller) to ~35%. When the actual NIR spectra of the API are measured through the various apertures (Figure 3.11b), it can be seen that the 400 μm apertured sample has a spectrum similar to that from using no aperture, although s/n is slightly degraded, this is also the case for the 200 μm apertured sample but s/n is certainly poorer. When the 100 μm and smaller apertured samples are examined, a variation now exists between all three smaller aperture sizes, with the 25 μm apertured sample giving a straight line which implies no diffuse reflectance from the API.

Therefore it can be concluded that the blackened surface was not responsible for the reduced energy or the poor spectral quality, but instead the actual aperture size was affecting performance (blackened and shiny stainless steel apertures give very similar responses). This investigation has therefore shown that to obtain a good NIR spectrum the NIR radiation needs to interact with a sample that is greater than the size of the remote apertures. This is known as over-aperturing because the remote apertures only come into play after the sample interaction point that allows more energy to interact with the sample. Conversely it implies that perhaps the spectral signal comes from an area much larger than just that defined by the aperture – which would follow what was observed with the individual micro-spheres.
3.4.2 Sample Interfaces using Near-infrared Point Mapping

Oxytetracycline hydrochloride and fluconazole differ in colour (showing a visible interface) and have very different NIR spectra, shown in Figure 3.12b. In this example there are three fluconazole and three oxytetracycline hydrochloride peaks that can be monitored, indicated on Figure 3.12b. Figure 3.12c shows the actual spectra obtained when the line scan of data was collected through the interface and shows approximately three spectra that have different intensities from the pure component, implying signal may be detected up to 60 μm from the interface.
Figure 3.12 - Examination of the spectral contributions at the interface between oxytetracycline hydrochloride and fluconazole, where a) are pure component spectra, b) chemical image at 6112 cm$^{-1}$ and c) changes in NIR response with position relative to interface.

As explained in Equation 3.4, the percentage of oxytetracycline hydrochloride at unique fluconazole peaks was determined and vice versa giving rise to values which can be used to determine the overall component contribution at each pixel. The average of the three different component wavelengths was calculated and is shown in Figure 3.13. From this figure it can be seen that at the interface there is a 55% signal from fluconazole and 45% signal from oxytetracycline hydrochloride.

When the oxytetracycline hydrochloride side of the interface was examined, it could be observed that at 20 µm from the interface there is a 30% contribution from fluconazole (cf. 75% for oxytetracycline hydrochloride) but at 40 µm there is only a 10% contribution from fluconazole (cf. 100% for oxytetracycline hydrochloride). As movement is made further into the oxytetracycline hydrochloride region, the average
oxytetracycline hydrochloride contribution is >95% and the average fluconazole contribution is <2.5%. Therefore it can be concluded that the fluconazole contribution at >60 µm from the interface is negligible.

If the same information is examined on the fluconazole side of the interface at 20 µm from the interface there is a 30% contribution from oxytetracycline hydrochloride (cf. 70% for fluconazole) but at 40 µm there is still a 20% contribution from oxytetracycline hydrochloride (cf. 85% for fluconazole). At 60 µm there is a 12% contribution from oxytetracycline hydrochloride (cf. 88% for fluconazole) and a similar situation is observed from 80 – 120 µm. Only after 140 µm from the interface does the average fluconazole contribution become greater than 95% and the average fluconazole contribution less than 3%.

![Graph](image)

*Figure 3.13 - Average fluconazole contribution (blue) and average oxytetracycline hydrochloride contribution (red) at each point in the line scan of the interface between the two materials*

Therefore there is a variation in the spectral contribution from adjoining material, with oxytetracycline hydrochloride contributing in a fluconazole environment for what appears greater than 140 µm whereas the fluconazole contributions are only observed 60 µm into the oxytetracycline hydrochloride environment. The interface between the two materials is obviously not 20 µm exactly, which may explain some of the
It was thought that the oxytetracycline hydrochloride might be observed more in the fluconazole environment, as oxytetracycline hydrochloride has stronger NIR absorptions. Therefore an evaluation was made of oxytetracycline hydrochloride and ticonazole, because ticonazole also has strong NIR absorptions. Figure 3.14 contains the % contribution response for this interface set. It can be observed the ticonazole response in the oxytetracycline region is minimal 40 μm from the interface but the oxytetracycline response in the ticonazole region becomes less than 5% after 140 μm from the interface, which is similar to the observations from oxytetracycline in fluconazole. Therefore it can be concluded that the magnitude of the NIR absorptions do not impact the spectral contributions across the interface.

Figure 3.14 - Average % contribution for each component at every point in the line scan of the interface sample sets, where the left hand side of the interface is the pure component and the right hand side is the component contribution on the opposite side of the interface.

The question therefore arises to what other characteristic of oxytetracycline makes it different from both ticonazole and fluconazole. Oxytetracycline is a brown coloured material whereas both fluconazole and ticonazole are white, so it could be colour...
affecting the API response and perhaps the white material caused different scattering effects.

Pyrantel palmoate is a yellow coloured API and it was prepared with oxytetracyline to determine the effects of colour on interface response. Figure 3.14 contains the % contribution response for this interface set. It can be observed the oxytetracyline contribution in pyrantel palmoate is less than 10% at only 60 µm from the interface. The opposite is also true for the pyrantel palmoate contributions within the oxytetracyline side of the interface, therefore implying that colour contrast at the sample interface does impact the spectral contributions. To further confirm, the fourth interface set (fluconazole and prazosin) was examined as both are white materials. Figure 3.14 contains the % contribution response for this interface set and it can be observed that both materials show less than 10% contribution at 80 µm from the interface. Therefore, as observed for two coloured materials, two white materials forming an interface show similar absorptions into the other material's environment.

The interface experiments are not perfect as the interface is not sharp; therefore there is some variability in the expected region that constitutes the interface. However, the interface experiments have shown that when materials have very different spectral responses that this does not affect the region from which spectral signal is detected from. If dark materials are present within a white matrix, then the spectral contributions from the darker material will have stronger contributions in the region of the white spectral information, due to changes in absorptivity. Figure 3.14 shows that at the interface of all sample sets each component has a 40 - 60% spectral contribution, and at any other point on the line scan a 50% spectral contribution is obtained from the component of interest i.e. with the apertured area (20 x 20 µm). Movement into the identified component side of the interface sees a return to > 75% spectral contribution after 40 µm. Movement into the opposite side of the interface shows an interfering spectral response of > 25% after 60 µm and > 10% after 140 µm. Therefore in NIR-PM
spectral contributions do not solely arise from the apertured area of interest, but from up to 60 µm away (obtaining ~75% spectral purity).

3.4.3 Sample Interfaces using NIR Global Imaging

Using NIR-GI it was not possible to map a line across the interface samples, but instead an image of the interface was obtained at each NIR wavelength. Figure 3.15 (column one) shows each interface set at 1635 nm (6116 cm⁻¹), with absorbance colour-scaling on all images being equivalent, showing slightly different absorptions for each component at this wavelength. This wavelength was selected as the strongest absorptions were observed (Appendix 2) but all API's had a response in this region. Therefore, a PLS model was established to allow the discrimination of the five components (based on spectral shape).

Figure 3.15 - Column one presents the NIR chemical images at 1635 nm, column two and three the PLS score image for component 1 and 2 respectively where red represents score values >0.95, green 0.75 - 0.95 and blue 0.5 - 0.75. Column four describes the combined PLS score images for component 1 and 2 in the interface sets a) OTC/Flu, b) Tio/OTC, c) PP/OTC and d) Flu/Pra where red represents component 1 and blue component 2 pixels with score values >0.75.
Figure 3.16 shows the resultant PLS score histograms obtained for interface set four (fluconazole and prazosin hydrochloride) and shows that both components give rise to a bi-modal score distribution (scores >0.5 and <0.5). As described for the NIR-PM experiments the percentage contribution of each component over the entire image provides an understanding of the spectral contributions observed on the opposite side of the interface. Therefore in the score images obtained for each component a pixel with a score of >0.9 (>90% contribution) was coloured red, pixels in the range 0.75 - 0.9 coloured green, blue pixels represented score values in the range 0.5 - 0.75 and any pixel falling out of this classification appeared black in the score image, presented in Figure 3.15 columns two and three for all interface sets.

![Figure 3.16 - Histogram plots of the score values versus number of pixels for the interface set four with a) representing fluconazole and b) prazosin hydrochloride](image)

From Figure 3.15 it can be observed that the score images for each component contain very few blue pixels at the interface, with the greatest width of pixels being observed for pyrantel palmoate. However it was also observed that the outer edge of the samples had lower score values and was thought to be due the sample surface not being optically-flat. To evaluate the interface, the two component's score images were combined into one image, using a threshold of 75% spectral purity, shown in column four of Figure 3.15. From these combined images the largest interface (black pixels) is observed in the fluconazole/oxytetracycline interface set, with a width of ~14 pixels (~125 μm) and the smallest interface for the ticonazole/oxytetracycline interface set (~8 pixels / ~70 μm). When a 50% spectral contribution was defined for each pixel, a black interface region was still observed between the two components, although this was typically no greater than 2 - 3 pixels (17.6 - 26.4 μm).
The values obtained for NIR-GI are in a similar magnitude to the observed values for NIR-PM, despite NIR-GI having a supposedly smaller area for spectral collection. Therefore it can be concluded that the spectral contributions in a NIR-M experiment come from out with the defined sample area. For >50% spectral purity the area sampled is 20 x 20 μm but for greater >75% spectral purity the contributing regions can be from 80 μm away from the data collection point, implying a sampling area of 100 x 100 μm.

3.5 RESULTS AND DISCUSSIONS ON DEPTH OF PENETRATION STUDIES IN NEAR-INFRARED MICROSCOPY EXPERIMENTS

It should be noted that in this section that the wavenumber responses have been converted into wavelengths to allow for comparison with previous reported literature values. Therefore the wavenumber region examined was 4200 - 9000 cm⁻¹ which equates to the wavelength region 1111 - 2381 nm.

3.5.1 Utilising Varying Thicknesses of Paper to determine Depth of Penetration

To allow the effects of depth of penetration to be evaluated on the NIR-PM instrumentation, the SNV absorbance spectra of the light weight paper on top of the API disc were evaluated for changes in spectral response with respect to increased depth of penetration, Figure 3.17. The NIR spectra changed as more paper layers were added and this variation could be monitored at one of three positions (i.e. where one substance has little or no absorption and the other has a strong absorbance) either at the unique drug absorption at 1675 nm or the unique cellulose absorptions at 2100 nm and 1480 nm.
Figure 3.17 - NIR absorbance spectra (after SNV pre-treatment) collected from the NIR-PM instrument, showing the change in absorbance as the drug substrate (peak at 1675 nm) was covered with increasing thickness of paper (20 to 200 μm, which is equivalent to 1 to 10 layers).

Second derivative spectra of the absorbance data were calculated. As this involves the rate of change of slope and the fact that the drug peak is asymmetrical, the drug peak minimum in the derivatised data does not occur at exactly the same point as the drug peak maximum in the raw absorbance data. The peak minimum in the derivatised data shifts by one data point (8 cm⁻¹) to 1671 nm. Due to initial studies comparing the raw and derivatised spectra, the relationship between thickness and absorbance, was monitored at 1675 nm for each paper type. Thickness and absorbance appeared to be related exponentially, Figure 3.18, confirming the relationship previously reported (Berntsson et al, 1998). To enable determination of the information depth, it was necessary to determine the point where the exponential no longer changed. However, this is often difficult and preferentially in exponential analysis the end point is not used, but instead the earlier, linear section of the exponential plot is used.
Figure 3.18 - The change of second derivative response at 1675 nm with increasing thickness of paper where the relationship between absorbance and thickness can be seen to be exponential with $R^2 = 0.986$

The linear section contains the absorbance value that corresponds to half the pure drug response ($y_{50}$) and hence, the corresponding thickness value will be approximately half of the true information depth. This is identical to the calculation of radioactive half-life. In this work, the term has been named $DP_{50}$, and represents the depth to which the radiation penetrates into the sample giving a signal corresponding to an intensity of 50% of the pure substrate. If the equation for the exponential is determined, by calculating the exponential fit to the data, then it would be possible to use the relationship to determine the $DP_{50}$ value for each paper type.

The exponential function can be described by Equation 3.5 where $A$ is the apparent absorbance value, $t$ the thickness penetrated to reach the substrate and $b$, $\tau$, $d$ constants. The constants all have significance; $b$ is the intercept on the y-axis, $\tau$ is the rate constant and $d$ is the offset of the data from zero.

$$A = be^{-\tau t} + d$$  \hspace{0.5cm} (3.5)
One method for the determination of $DP_{50}$ would be to calculate all the constants and the value for the 50% drug response, but it is possible to calculate the $DP_{50}$ value simply by using the rate constant $\tau$. This is due to the fact that the $DP_{50}$ value can be described by Equation 3.6. Hence the $DP_{50}$ values were determined by calculation of the rate constant (rate of decay of spectral intensity) using the best-fit exponential line.

$$DP_{50} = \ln \frac{2}{\sqrt{\tau}}$$ (3.6)

When the exponential was calculated for all the different paper responses, the fit of the exponential gave $R^2 > 0.99$, determined using the non-linear regression analysis programme. This was thought to be a good fit to the data and the exponential fits and rate constants were then calculated for all paper types, Table 3.3. The A4 papers were excluded from this part of the investigation due to the lack of data at low thickness values. The $DP_{50}$ value for each paper type was calculated using the rate constant and is also given in Table 3.4. The $DP_{50}$ values ranged from 39 - 57 $\mu$m. As all papers gave a $DP_{50}$ value within a similar range, the data sets were combined to find a general $DP_{50}$ value for cellulose materials and in this model the A4 data were included. The rate constant was found to be $0.017 \pm 0.001$ and hence the mean $DP_{50}$ value was within the range 39 - 43 $\mu$m.

Table 3.4 - Rate Constant and DP50 value calculated for each paper type

<table>
<thead>
<tr>
<th>Paper Type</th>
<th>$R^2$</th>
<th>$\tau$</th>
<th>$DP_{50}$ (um)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light (Rizla)</td>
<td>0.986</td>
<td>0.014 ± 0.002</td>
<td>50 ± 7</td>
</tr>
<tr>
<td>Kingsize (Rizla)</td>
<td>0.990</td>
<td>0.013 ± 0.001</td>
<td>53 ± 4</td>
</tr>
<tr>
<td>Medium (Rizla)</td>
<td>0.994</td>
<td>0.014 ± 0.002</td>
<td>50 ± 7</td>
</tr>
<tr>
<td>Medium (Swan)</td>
<td>0.987</td>
<td>0.014 ± 0.002</td>
<td>50 ± 7</td>
</tr>
<tr>
<td>Corner Cut (Swan)</td>
<td>0.996</td>
<td>0.017 ± 0.001</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>Medicated (izal)</td>
<td>0.989</td>
<td>0.015 ± 0.001</td>
<td>46 ± 3</td>
</tr>
</tbody>
</table>
3.5.2 Utilising Cellulose Slurries to Determine Depth of Penetration

As a direct result of changing the substrate to a non-pharmaceutical material, it was necessary to compare the results from the light weight paper layers on top of the pharmaceutical material with the results for the plastic material. When the second derivative absorbance values were compared it was found that the drug gives a minimum at 1671 nm but the plastic at 1678 nm. Calculating an exponential fit at this wavenumber provided a rate constant of $r = 0.013 \pm 0.002$, which corresponds to a $DP_{50}$ value of $53 \pm 8 \, \mu m$, (cf. $50 \pm 7 \, \mu m$ when using the drug as the substrate). Hence it was concluded, that within experimental error using the plastic material as the lower substrate was comparable to the paper/drug model previously established.

Figure 3.19 shows a comparison of the second derivative absorbance profile at 1678 nm for the two types of powdered cellulose with respect to the light weight paper data. This plot shows that the paper and powdered cellulose have a similar response to the NIR radiation. Using the data from the powdered cellulose layers, gives a rate constant, $r = 0.013 \pm 0.002$, and hence a $DP_{50}$ value of $53 \pm 8 \, \mu m$. This value is identical to that determined using the light weight paper layers on top of the plastic. It was therefore concluded that the paper layer model must be a close approximation to real pharmaceutical materials in terms of the physical as well as chemical information.

![Figure 3.19 - The change of second derivative response at 1678 nm with increasing thickness of powdered cellulose layers](image-url)
3.5.3 Effects of Depth of Penetration in NIR Global Imaging Experiments

Figure 3.20 shows the absorbance spectra for the light weight paper on top of the drug disc which was collected on the global imaging instrument. The figure shows that the NIR spectra obtained on the imaging instrument are equivalent, and have improved signal to noise, when compared to the FT-NIR spectra, Figure 3.17. (It should be noted that the FT-NIR InGaAs detector has poor response below 1333 nm for typical pharmaceutical mapping experiments). The comparison of the two spectral data sets show that the NIR-GI spectra change in a similar fashion to those obtained on the NIR-PM instrument.

![Figure 3.20 - NIR absorbance spectra collected from the NIR global imaging instrument, showing the change in absorbance as the drug substrate is covered with increasing thicknesses of paper (from 20 µm to 240 µm which is equivalent to 1 to 12 layers)](image)

The unique drug absorption occurred at 1675 nm and the change in second derivative response, at this wavelength, in relationship to thickness is shown in Figure 3.21. In this figure the results from the mapping instrument are presented for comparison and show the two techniques to have similar second derivative absorbance values for similar samples, but with a slight variation in the degree of change in absorbance with respect to layer thickness. The global imaging data had an exponential fit of $R^2 = 0.999$ and hence, the rate constant, $\tau$, was determined as $0.016 \pm 0.0005$ (cf. point
mapping with \( r = 0.014 \pm 0.002 \). \( DP_{50} \) for the global imaging data was calculated to be \( 43 \pm 2 \mu m \) which is in a similar order of magnitude to the value of \( DP_{50} \) for the light weight paper in the point mapping experiment (\( DP_{50} = 50 \pm 7 \mu m \)). The variation in \( DP_{50} \) is most likely to be caused by experimental errors associated with the preparation of the cellulose layers. If the \( DP_{50} \) value from the NIR-PM data on the combined cellulose data set (\( DP_{50} = 41 \pm 2 \mu m \)), which will be an average of layer preparations, is compared to the global imaging data (\( DP_{50} = 43 \pm 2 \mu m \)) it can be seen that there is good agreement between these two data sets.

![Figure 3.21](image)

**Figure 3.21** - The change of second derivative response at 1675 nm with increasing thickness of paper where the relationship between absorbance and thickness can be seen to be exponential with \( R^2 = 0.9992 \)

### 3.5.4 Wavelength Dependency of Depth of Penetration

To investigate the wavelength dependency of the depth of penetration the lower substrate was changed from the drug material to magnesium stearate, then confectioner’s sugar and scanned on the NIR-PM system. The second derivative spectra of all three substrates and cellulose were compared and seven different wavelengths were identified where the information depth could be investigated, listed in
Table 3.5. For each of the seven wavelengths, the fit of an exponential was calculated and used to provide the rate constant and the $D_{P_{50}}$ value, listed in Table 3.5.

Table 3.5 - Rate Constant and $D_{P_{50}}$ value calculated for each wavelength region

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Wavelength (nm)</th>
<th>$R^2$</th>
<th>$\tau$</th>
<th>$D_{P_{50}}$ (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confectioner Sugar</td>
<td>1437</td>
<td>0.986</td>
<td>0.010 ± 0.001</td>
<td>69 ± 7</td>
</tr>
<tr>
<td>Meclizine HCl</td>
<td>1674</td>
<td>0.986</td>
<td>0.014 ± 0.002</td>
<td>50 ± 7</td>
</tr>
<tr>
<td>Confectioner Sugar</td>
<td>1690</td>
<td>0.989</td>
<td>0.013 ± 0.001</td>
<td>53 ± 3</td>
</tr>
<tr>
<td>Mg Stearate</td>
<td>1726</td>
<td>0.993</td>
<td>0.016 ± 0.002</td>
<td>43 ± 6</td>
</tr>
<tr>
<td>Mg Stearate</td>
<td>1768</td>
<td>0.992</td>
<td>0.016 ± 0.002</td>
<td>43 ± 6</td>
</tr>
<tr>
<td>Meclizine HCl</td>
<td>2151</td>
<td>0.982</td>
<td>0.021 ± 0.002</td>
<td>33 ± 3</td>
</tr>
<tr>
<td>Mg Stearate</td>
<td>2305</td>
<td>0.992</td>
<td>0.026 ± 0.002</td>
<td>27 ± 2</td>
</tr>
</tbody>
</table>

From Figure 3.20 it can be seen that for the global imaging data there are two wavelengths where the information depth can be monitored, 1675 nm and 1145 nm. The rate constant for the lower wavelength peak was calculated, $\tau = 0.0038$, and hence a $D_{P_{50}}$ value of 180 μm was determined at this wavelength.

Both data sets show that the $D_{P_{50}}$ value is dependent upon the wavelength of light. This relationship is not unexpected, as the depth of penetration will be related to the scatter of the radiation, which is known to be wavelength dependent. The relationship between $D_{P_{50}}$ and wavelength was found to be exponential with a fit of $R^2 = 0.993$ and the line of best fit described by Equation 3.7. This equation could be utilised to predict the $D_{P_{50}}$ values for other wavelengths in the NIR region examined.

$$D_{P_{50}} = 30.5 + 18457e^{-0.0042t}$$

(3.7)

3.5.5 Determination of Information Depth

In all the experiments performed using the cellulose layers, at 1675 nm the $D_{P_{50}}$ value has been found to be within the range of 39 - 57 μm. However, the key aim of this
experiment was to determine the information depth. If an estimate of the information depth was to be quickly ascertained, it would be possible to inspect the exponential plots shown in Figure 3.18, Figure 3.19 and Figure 3.21 to see where the graphs begin to tail off. This tailing would be the point where the absorption changes are minimal and hence is a rough estimate of the information depth. Visual examination of these plots shows that minimal substrate contributions can be seen above 120 μm, and hence suggests that the information depth is in the order of 120 μm. However, with calculation of the $DP_{50}$ value (which is known to be a more accurate description of the exponential) the question which actually needs to be addressed is how does the $DP_{50}$ value relate to the actual information depth?

Knowing that the $DP_{50}$ value represents 50% of the information depth and that the relationship between the $DP_{50}$ value and the information depth is exponential it follows that $2 \times DP_{50}$ will be 75% of the information depth and similarly $3 \times DP_{50}$ represents 87.5% of the information depth. Following this decay series, eventually 100% of the information depth will be obtained after 25 iterations, but how significant is this value? Typically it is accepted that a 95% confidence limit is an acceptable result, and so if we consider this to be appropriate then $4.32 \times DP_{50}$ represents 95% of the information depth. This implies that with at least 95% confidence the information depth is within the range 168 – 246 μm. The $DP_{50}$ value has been found to vary exponentially with wavelength, and so the information depth for NIR microscopy ranges from 109 μm at 2380 nm to as much as 777 μm at shorter wavelengths (1100 nm). This figure has been determined for cellulose materials and when different particle sized materials are involved the value for the information depth may vary because of scattering differences, although previous work shows there to be minimal variation between particles less than 100 μm (Olinger and Griffiths, 1992), which is an average size for pharmaceuticals excipients.
Interpreting this value in terms of implications for NIRM suggests that for pure particle identification, the material being studied must have a depth which is greater than the information depth (>109 μm for longer wavelengths and >777 μm for shorter wavelengths). This implies that if the particle size was small, then it would be better to use the longer wavelength regions for identification of these components. However, it should be noted that half of the contribution to any spectrum obtained will come from the top ~100 μm of material (i.e. $DP_{50}$ range of 25 – 180 μm, for the spectral range 1100 – 2500 nm) and that contributions change exponentially below this depth. Hence, any particle that is smaller than the information depth but larger than the $DP_{50}$ value, at the surface of the sample, will have a greater contribution to the overall spectrum than from anything further into the sample. With the availability of multivariate analysis techniques e.g. PCA and MCR it is not always necessary to obtain pure component spectra, as these methods allow the user to extract the pure component spectral information from a ‘mixed’ spectrum. Hence, even if particles exist in their input size range, (typically 1 – 100 μm for pharmaceutical excipients), it is possible that small particles which are <109 μm from the surface of a tablet or compacted powder can still be distinguished from other materials in the matrix.

3.6 DETERMINATION OF THE ACTUAL SAMPLE SIZE IN NEAR-INFRARED MICROSCOPY EXPERIMENTS

Berntsson et al (1998) suggested a means to determine the sample size contributing to each spectrum to be obtainable by multiplying the density of the powder by the information depth and the sample surface area. The density of paper has been reported to be in the range 0.7 - 1.15 gcm$^{-3}$ (Handbook of Chemistry and Physics, 53rd Edition). In terms of the sample surface area it has been shown in this investigation that the spectral contribution from an area of 20 x 20 μm is responsible for over 50% of the spectral response and was therefore used to evaluate sample size. It follows that the mass of sample analysed in NIRM ranges from 0.03 - 0.05 μg at long wavelengths (2380 nm) to 0.22 - 0.36 μg at short wavelengths (1100 nm).
This is a simple model and does not take into account any interactions the NIR radiation will have with the sample. The Cassegrain lens focuses light on to the sample with a maximum angle of 37 degrees. If it is presumed that the light continues into the sample at this angle, then a square-pyramidal frustrum would be a good representation of the maximum volume that would contribute to each spectrum, this is shown in Figure 3.22 along with the first cuboid shape described. However, it is likely that the light will not follow the same path when it reaches the sample but the angle $i$ will decrease due to the change in refractive index caused by interaction with the sample. The angle $i$ will also depend on the actual wavelength of light, but using $i = 37$ degrees provides a good estimate of the maximum amount of sample that can contribute to one spectrum.

![Figure 3.22 - Schematic of the estimated regions of contribution to any one spectrum in NIR microscopy. The shaded region represents the area of greatest contribution, $i$ is the angle that the light disperses away from the 'apertured' area and $ID$ is the information depth.](image)

Obviously further interactions will take place in the sample, and so the actual light path of the NIR radiation will be dependent upon particle reflectivity and size but this model should provide a good estimate of the possible range of sample size. The volume of the model can be calculated using Equation 3.8, where $ID$ is the information depth, $A_1$ the surface sampling area and $A_2$ the inner area.

$$Volume = \frac{1}{3} ID(A_1 + A_2 + \sqrt{A_1 \times A_2})$$ (3.8)
Using this volume calculation for NIRM data (1100 – 2500 nm) gives rise to a sample size range of 0.22 - 1.59 µg, for materials with a density of 0.7 g cm\(^{-3}\) and increases to 0.37 - 2.62 µg, for materials with a density of 1.15 g cm\(^{-3}\).

### 3.7 Conclusions

In NIRM experiments it has been found that areas cut with the desired measurement region (the aperture size in NIR-PM (20 x 20 µm) and pixel size in NIR-GI (8.8 x 8.8 µm)) contribute to the detected signal. However >50% of the actual spectral information is obtained from a 20 x 20 µm area for both NIRM instruments. However, investigations have shown that components can contribute to the defined area despite being located up to 80 µm away from the defined area.

This work has found that by considering the typical nature of a pharmaceutical formulation to be modelled by use of cellulose material, then in reflectance NIR microscopy the depth from which spectral information is obtained is typically from the surface (focus point) to at least 200 µm below, observed at 1675 nm. The information depth has been found to be dependent upon the wavelength of light, where longer wavelengths cause a decrease in the information depth. This means that within the spectral range used in NIRM (1100 – 2500 nm) the information depth varies from 109 – 777 µm. These information depths suggest that when a NIR spectrum is collected in a typical contributing sample size will be somewhere between 0.22 µg and 2.62 µg of sample.

The information depth determined is smaller than the figures reported for other NIR instrumental methods. In the literature the values for the information depth are 500 µm or more for greater than 1400 nm. In NIR microscopy the information depth at 1400 nm is ~ 350 µm and at longer wavelengths (2380 nm) is as small as 109 µm.
The information depth determined for NIR microscopy is small enough to allow accurate mapping of the sample matrix. Used alongside chemometric methods it is possible to resolve the main ingredient contribution to a spectrum, from the weaker contribution from the particles directly below the surface. If small particles are present with a sample this work has shown that longer wavelengths would be more appropriate for these studies, with over half the spectral contributions coming from the top 50 μm of sample. However, if the sample contains larger particles then the actual wavelength region used is not as critical, with half the spectral contributions coming from the top ~200 μm of sample. With appropriate selection of the wavelength regions used, it is possible to see why NIR microscopy has been able to produce the same images as Raman microscopy, and hence why NIR microscopy can be considered a useful technique for pharmaceutical analysis.
4. NIR MICROSCOPY AS A QUANTIFICATION TOOL

4.1 INTRODUCTION
Quantifying the amount of API in a solid dosage form is critical to the eventual final product release to market. For content uniformity and potency, the assays described by the European Pharmacopoeia are based on destructive chromatography measurements. Not only are these time consuming measurements, but are single component driven with a focus on the chemistry of the API. A more proactive approach to content uniformity would be to look at not only the API, but also other components in the solid dosage matrix. Although the excipient concentrations are not critical to product release, a measurement of their concentrations in the formulation would help in process understanding, due to their impact upon ability to manufacture and solid dose physical properties e.g. disintegration.

Measurements such as potency are chemical based. However, solid dosage manufacture is a physical process. Although some tests are used to measure physical properties e.g. hardness, weight and friability, these measurements do not directly provide a physical fingerprint of the process. Therefore control of the physical parameters is still a challenge during pharmaceutical manufacture and the real emphasis resides in measurement of the chemical properties.

With this thought, an ideal measurement for product release would be one where API concentration could be determined using a method which gives a physical fingerprint of the process and measures the excipients concentrations simultaneously. An analytical tool which has potential to perform such a measurement is NIRM. The spatial information obtained from the chemical images can be used to understand the effects of the physical manufacturing process, such as distribution and size of all components in the final solid dose matrix. The determination of component concentration was first explored in Chapter 2 where the possibility of determining concentrations using PLS
approaches was shown. This chapter will examine further the potential of NIRM at predicting concentrations of components in solid dosage matrices by use of laboratory scale blends.

4.2 Method
Solid dosage form matrices were simulated by the use of four different excipients (corn starch (CS), lactose monohydrate (LM), microcrystalline cellulose (MCC) and hydroxypropyl cellulose (HPC)) and two different API's (ziprasidone hydrochloride (API-A) and doxycycline carrageenate (API-B)). The respective suppliers are listed in Table 4.1 with the bulk densities and the mean diameter of all materials by both number and volume measurements. By number distribution both API's have similar fine material, but API-B has a much larger mean particle size by volume distribution. For the excipient materials, corn starch has the smallest mean particle size and hydroxypropyl cellulose the largest, both microcrystalline cellulose and lactose monohydrate have very similar particle sizes. However, in terms of bulk density lactose monohydrate has almost double the value of microcrystalline cellulose.

<table>
<thead>
<tr>
<th>Component</th>
<th>Abbreviation</th>
<th>Supplier</th>
<th>Mean Particle Size / (μm)</th>
<th>Bulk Density / (g cm⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microcrystalline Cellulose</td>
<td>MCC</td>
<td>Emcocel 50M, Penwest</td>
<td>22.07</td>
<td>85.01</td>
</tr>
<tr>
<td>Lactose Monohydrate</td>
<td>LM</td>
<td>FastFlo, Foremost Farms</td>
<td>20.38</td>
<td>107.17</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>CS</td>
<td></td>
<td>10.5</td>
<td>18.18</td>
</tr>
<tr>
<td>Hydroxypropyl Cellulose</td>
<td>HPC</td>
<td>Klucel, Aqualon</td>
<td>21.52</td>
<td>254.35</td>
</tr>
<tr>
<td>Ziprasidone Hydrochloride</td>
<td>API-A</td>
<td>Pfizer Ltd.</td>
<td>5.0</td>
<td>18.5</td>
</tr>
<tr>
<td>Doxycycline Carrageenate</td>
<td>API-B</td>
<td>Pfizer Ltd.</td>
<td>6.2</td>
<td>48.46</td>
</tr>
</tbody>
</table>

In total twelve different blends were prepared, with the lowest concentration of any component being 5% w/w. The blends produced were selected to cover a broad range of both concentrations and combinations of ingredients. Blends were also produced...
from two, three and four components to allow transferability to real pharmaceutical formulations. The actual % w/w used in each blend is described in Table 4.2. It can be observed that all blends contained MCC and eleven of the twelve blends contained LM; this was performed to allow a wide but populated range of concentrations to be monitored along with ‘reference’ materials to allow the effects of different matrices on concentration prediction to be determined.

Table 4.2 - % w/w of components used in production of the twelve simulated blends

<table>
<thead>
<tr>
<th>Blend</th>
<th>LM</th>
<th>MCC</th>
<th>CS</th>
<th>HPC</th>
<th>API-A</th>
<th>API-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>80</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>50</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>3</td>
<td>25</td>
<td>75</td>
<td></td>
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</tr>
<tr>
<td>4</td>
<td>10</td>
<td>90</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>5</td>
<td>75</td>
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<tr>
<td>6</td>
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<tr>
<td>7</td>
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<td>60</td>
<td>15</td>
<td>20</td>
<td>5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each blend (10 g total) was prepared in a centrifuge tube and blended in a turbular blender for five minutes. From this blend, five sub-samples weighing 400 mg each were taken and pressed using a single punch hydraulic press (using 200 bar pressure) into tablet wafers ~4 mm thick and 1 cm in diameter. The dies used in the press gave rise to surfaces which were optically flat and hence required no further sample preparation for analysis on the NIRM instrumentation. Sub-samples of each of the pure raw materials were prepared in the same way, to allow a spectral library to be built for data processing.
The Spotlight NIR Line Imaging system (shown in Figure 1.6b) was utilised throughout this investigation to analyse both the raw materials and the final blends. The spectrometer was set to collect spectral data over the range 3800 – 7600 cm\(^{-1}\) (1316 nm – 2632 nm) with an 8 cm\(^{-1}\) data interval and an average of 4 scans. A sample area of 25 mm\(^2\) was utilised, which was a result of collection of 200 spectra in both the x- and y-axis, with each spectrum being from a sample area of 25 x 25 \(\mu m\). Each sample took \(\sim\)35 minutes to obtain, and the spectra were output as relative reflectance \((R)\), based on a gold mirror reference.

A sixth wafer was made of the blend 9 to allow a study of what sampling was required of a blend wafer to give representative results. The entire surface of this wafer was analysed which resulted in a square of 387 x 391 data points (7.175 mm x 9.775 mm) and then six different depths into this wafer were cut using a Leica EM Trim at \(\sim\)0.5 mm intervals. Prior to sectioning of the sample, the wafer was stuck to the microscope slide using cyanoacrylate adhesive. At each depth, the entire surface was analysed using the conditions described previously. The image obtained from the surface of the wafer was truncated to allow only features from the sample surface to be present in the chemical image, making a rectangular rather than circular area, which resulted in a data set which was 275 x 295 data points (6.875 mm x 7.375 mm). To understand the impact of reductions in sampling area, this region was also divided into quadrants which were compared, but also reduced sequentially by 10\% on each axis. The data from each layer studied was then truncated to the pre-determined optimised sampling area. Both sampling area and layer images were compared to determine for NIRM experiments what is a representative sample of a wafer.

4.3 DATA PROCESSING AND CHEMICAL IMAGE GENERATION

After data collection, the resultant unprocessed data cubes were translated into a format (.spf) readable by image analysis software (ISYS, Spectral Dimensions, MD).
The spectra were firstly transformed into absorbance units, according to Equation 1.11 and then normalised using SNV to remove any effects from scatter. It was not necessary to perform any further pre-processing as the spectra all had unique signatures, shown in Figure 4.1, although it should be noted that MCC and CS have the most similar spectral features. Individual libraries were then prepared for each blend, with only the raw materials used in the blend being incorporated into the library data set. Each blend data set was then correlated to the respective library using PLS 2 methodology, as previously described in Section 2.7. Two outputs from each experiment were obtained: the distribution of score values based on the number of pixels and the chemical image showing the distribution of components based on the score values. The latter is useful for evaluating the relative distributions of components, but the pixel histogram shows the potential relationship between the chemical image and component concentration. Both data outputs will be presented in this chapter.

![Figure 4.1 - SNV normalised FT-NIR spectra for raw materials a) excipients and b) API used in simulated blends](image)

The chemical images of each component were all prepared by a standard approach. This approach was to use the component % w/w in the formulation as the percentage of pixels to include from the score distribution. This is described in Figure 4.2 using Blend 1 as the example and was verified by examination of the spectral features of included and excluded pixels.
4.4 Quantification of Component Concentration in Two Component Systems

The ability to predict concentrations of only a two component system is not a realistic model. However, such systems can be used to understand the ability to predict component concentrations using NIRM at the simplest level. From Table 4.2 it can be observed that 5 different blends (1 – 5) were prepared using only two components. With the exception of blend 5, which was prepared from MCC and API-A, the other four blends were prepared using LM and MCC at varying % w/w concentrations. Figure 4.3 shows chemical images that were prepared for each blend, and the increase in MCC and decrease in LM concentrations from blends 1 to 4 can easily be visualised. In each image the area occupied by each component is similar to the % w/w. This is not unexpected as the appropriate percentage of pixels was selected to represent each component. From the images it can be seen that each component is well resolved, as
few overlapping regions can be observed and therefore it can be concluded that the percentage pixels can be used to allow visualisation of chemical distributions. These images show that with two components, visualisation of different concentrations is possible, but this is obviously a subjective approach.

![Images of chemical distributions](image)

**Figure 4.3 - NIRM chemical images of the distribution of components in blends 1 – 5 with green representing LM, blue as MCC and red as API-A**

As described in Section 4.3, chemical images are only one output of NIRM and the second output is a histogram of score values. These are presented for each two component blend in Figure 4.4. The first observation from the pixel score distributions is that for each two component set, the histograms are mirror images of each other around 0.5. This is not unexpected, as in the PLS algorithm each spectrum is required to be either one component or the other, such that each pixel would give rise to a total value of 1. Therefore, when a low score value is observed for one component, it follows that the other component must have a high score value, but it would not be expected that the mean of these individual score distributions (MSD) would centre on a value close to the % w/w. It would be thought that each component would have pixels with a bimodal distribution, at low and high score values, but this is not the case. For blends 1 – 4 it can be observed that the distributions of each component are quite broad, especially when compared to the distributions observed for blend 5. The reason for this variation could be due to the larger spectral or particle size differences between API-A and MCC (18.5 μm and 85.0 μm respectively, Table 4.1) which makes resolution of these two components simpler than that of MCC and LM (85.0 μm and 107.2 μm)
respectively, Table 4.1) which have many coinciding spectral features and similar particle sizes.

- **a) Blend 1**
  - $MSD_{MCC} = 0.179 \pm 0.055$
  - $MSD_{LM} = 0.825 \pm 0.055$

- **b) Blend 2**
  - $MSD_{MCC} = 0.498 \pm 0.079$
  - $MSD_{LM} = 0.509 \pm 0.079$

- **c) Blend 3**
  - $MSD_{MCC} = 0.767 \pm 0.073$
  - $MSD_{LM} = 0.238 \pm 0.074$

- **d) Blend 4**
  - $MSD_{MCC} = 0.875 \pm 0.047$
  - $MSD_{LM} = 0.129 \pm 0.047$

- **e) Blend 5**
  - $MSD_{MCC} = 0.782 \pm 0.016$
  - $MSD_{API-A} = 0.239 \pm 0.017$

*Figure 4.4 - Histograms of score values for each two component blend using green for LM pixels, blue for MCC pixels and red for API-A pixels, the MSD for each distribution is also included*
The broadness of the distributions are reflected in the standard deviation around the MSD value, with blend 5 standard deviation (0.017) being ~25% of the value observed for blend 3 (0.074) (where both blends differ only in the components used and not the % w/w values). This standard deviation reflects the spread of the pixel score values within an image, so could potentially be used to understand how well the two components have interacted. As five replicates of each blend were analysed it is also possible to determine the standard deviation between images.

Table 4.3 presents the MSD values with the standard deviations around these means for each component in each blend and also presents the average value of these five replicates and the deviation between the replicates. The deviation in the MSD values would be a good indicator of the variation in the measurement and the standard deviation of individual MSD values would be an indicator of pixel mixing (or potentially homogeneity). From Table 4.3 it can be observed that the variation between the MSD values is less than the variation in the spread of the pixel score values.

Table 4.3 - Replicate MSD values, means and standard deviations for each component identified in blends 1 - 5

<table>
<thead>
<tr>
<th>Component</th>
<th>Replicate</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>LM₅₀</td>
<td>0.825 ± 0.055</td>
<td>0.752 ± 0.083*</td>
</tr>
<tr>
<td>MCC₅₀</td>
<td>0.179 ± 0.055</td>
<td>0.252 ± 0.085*</td>
</tr>
<tr>
<td>Sum</td>
<td>1.004 ± 0.055</td>
<td>1.004 ± 0.084</td>
</tr>
<tr>
<td>LM₂₀</td>
<td>0.509 ± 0.079</td>
<td>0.490 ± 0.075</td>
</tr>
<tr>
<td>MCC₅₀</td>
<td>0.498 ± 0.079</td>
<td>0.517 ± 0.075</td>
</tr>
<tr>
<td>Sum</td>
<td>1.007 ± 0.079</td>
<td>1.007 ± 0.075</td>
</tr>
<tr>
<td>LM₂₅</td>
<td>0.238 ± 0.074</td>
<td>0.249 ± 0.068</td>
</tr>
<tr>
<td>MCC₇₅</td>
<td>0.767 ± 0.073</td>
<td>0.757 ± 0.067</td>
</tr>
<tr>
<td>Sum</td>
<td>1.005 ± 0.074</td>
<td>1.006 ± 0.068</td>
</tr>
<tr>
<td>LM₁₀</td>
<td>0.129 ± 0.047</td>
<td>0.124 ± 0.050</td>
</tr>
<tr>
<td>MCC₇₅</td>
<td>0.875 ± 0.047</td>
<td>0.879 ± 0.049</td>
</tr>
<tr>
<td>Sum</td>
<td>1.004 ± 0.047</td>
<td>1.003 ± 0.050</td>
</tr>
<tr>
<td>MCC₇₅</td>
<td>0.782 ± 0.016</td>
<td>0.781 ± 0.014</td>
</tr>
<tr>
<td>APL₁₂₅</td>
<td>0.239 ± 0.017</td>
<td>0.240 ± 0.015</td>
</tr>
<tr>
<td>Sum</td>
<td>1.021 ± 0.017</td>
<td>1.021 ± 0.015</td>
</tr>
</tbody>
</table>

* indicates a high standard deviation within pixel distribution
In Table 4.3 two results (*) have been indicated as having unusually high standard deviations around the MSD, Blend 1 wafer 2 and Blend 5 wafer 4. If the distributions and chemical images for these two samples are examined, Figure 4.5, it can be observed that the mixing of these two materials is not homogenous. Figure 4.5a shows the MCC to be distributed mainly at the bottom of the image and when the pixel distribution is compared to Figure 4.4c it can be seen that both distributions are much broader. What is most significant is the maximum of each distribution which is \(\sim 1500\) pixels in Figure 4.4c but only \(\sim 750\) in Figure 4.5a. In Figure 4.5b API-A can be seen as a large clump on the left hand side of the image, this poor distribution is observed in the pixel distribution by the extended tail at the high end of the API distribution and at the lower end of the MCC distribution. Therefore the standard deviation of the MSD is a good indicator of blend homogeneity.

![Figure 4.5](image)

**Figure 4.5** - Chemical images representing the distribution of LM (green), MCC (blue) and API-A (red) and associated pixel score distributions for a) blend 1 replicate 2 and b) blend 5 replicate 4

From Table 4.3 it can also be observed that for each blend sample examined, the sum of the MSD values is always equal to 1. This implies that the mixture is well described using the PLS model. Interestingly for blend 5 (contains API), the sum is slightly larger (1.02 cf. 1.005). To understand how good the MSD is at predicting concentration in
the binary mixtures, the % w/w was plotted relative to the MSD x 100 value and is shown in Figure 4.6. If all the individual values are used, a linear correlation of $R^2 = 0.9925$ is observed, which is improved to 0.9955 when the average of five samples is used. From Figure 4.6b it can be seen that the best fit line through the average data is similar to the actual concentration line.

\begin{align*}
\text{Blend 6} & : 33\% \text{ LM} + 33\% \text{ MCC} + 33\% \text{ CS} \\
\text{Blend 7} & : 25\% \text{ LM} + 60\% \text{ MCC} + 15\% \text{ CS} \\
\text{Blend 8} & : 20\% \text{ LM} + 50\% \text{ MCC} + 30\% \text{ CS} \\
\text{Blend 9} & : 45\% \text{ LM} + 45\% \text{ MCC} + 10\% \text{ API-A}
\end{align*}

\begin{figure}
\centering
\begin{subfigure}{0.4\textwidth}
\centering
\includegraphics[width=\textwidth]{figure46a.png}
\caption{Plot of MSD x 100 value for a) each ingredient in two component matrices and b) average value of each ingredient in blend versus % w/w of component}
\end{subfigure}
\begin{subfigure}{0.4\textwidth}
\centering
\includegraphics[width=\textwidth]{figure46b.png}
\caption{Figure 4.6 - NIR chemical images of the distribution of components in blends 6 – 9 with green representing LM, blue for MCC and red for CS/API-A}
\end{subfigure}
\end{figure}

\section*{4.5 Quantification of Component Concentration in Three Component Systems}
In Table 4.2 it can be observed that four different blends were prepared utilising three different components (blends 6 - 9). All four blends utilised LM and MCC but varied in the third component which was either CS or API-A. Figure 4.7 shows a chemical image that was prepared for each blend. From these variations, the different concentrations of components can be observed.
The histograms of pixel score values are presented in Figure 4.8 and once again show the variation in component concentrations in each blend. Blends 7 and 9 show good correlation between the MSD and the actual component concentrations. In both blends the LM and MCC are the major components representing over 85% w/w the total blend. However, in blends 6 and 8 the MSD does not trend the % w/w of each component, and in these blends LM and MCC are not the major components. It can be seen in Figure 4.8 that the CS distributions for blends 6 and 8 are located closest to 1, despite not being the largest component of the blend, and the MCC distributions and MSD values are lower than expected. This will be discussed further in Section 4.6.

**Blend 6**
- \( \text{MSD}_{\text{MCC}} = 0.182 \pm 0.060 \)
- \( \text{MSD}_{\text{LM}} = 0.364 \pm 0.077 \)
- \( \text{MSD}_{\text{CS}} = 0.463 \pm 0.060 \)

**Blend 7**
- \( \text{MSD}_{\text{MCC}} = 0.609 \pm 0.079 \)
- \( \text{MSD}_{\text{LM}} = 0.239 \pm 0.076 \)
- \( \text{MSD}_{\text{CS}} = 0.158 \pm 0.043 \)

**Blend 8**
- \( \text{MSD}_{\text{MCC}} = 0.405 \pm 0.077 \)
- \( \text{MSD}_{\text{LM}} = 0.135 \pm 0.075 \)
- \( \text{MSD}_{\text{CS}} = 0.465 \pm 0.060 \)

**Blend 9**
- \( \text{MSD}_{\text{MCC}} = 0.463 \pm 0.075 \)
- \( \text{MSD}_{\text{LM}} = 0.456 \pm 0.086 \)
- \( \text{MSD}_{\text{API-A}} = 0.096 \pm 0.020 \)

*Figure 4.8 - Histograms of score values for each three component blend using green to indicate LM pixels, blue for MCC pixels, and red for CS/API-A pixels, the MSD for each distribution is also included*
The actual MSD values for each component in individual wafers are presented in Table 4.4. As observed for the two component blends, the sum of the individual MSD values is equal to ~1 for each blend, implying that the PLS model describes each mixture well. It is interesting to observe that, as in the two component system, it is blend 9 which includes the API which has the highest summated value. It can also be observed that the variation between the MSD is again smaller than the individual standard deviations (STD) for each histogram. When the MSD values are compared to the actual % w/w concentration it can be seen (as observed from histogram location) that the ability to predict concentration from the MSD is poor for blends 6 and 8. To further understand why this occurs, the actual % w/w was plotted relative to the MSD $ \times 100$ value for a) the individual values and b) the average of the five replicates, Figure 4.9.

Table 4.4 - Replicate MSD values, means and standard deviations for each component identified in blends 6 - 9

<table>
<thead>
<tr>
<th>Component</th>
<th>Replicate 1</th>
<th>Replicate 2</th>
<th>Replicate 3</th>
<th>Replicate 4</th>
<th>Replicate 5</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>LM</td>
<td>0.305 ± 0.091</td>
<td>0.342 ± 0.068</td>
<td>0.364 ± 0.077</td>
<td>0.323 ± 0.080</td>
<td>0.372 ± 0.082</td>
<td>0.342 ± 0.027</td>
</tr>
<tr>
<td>MCC</td>
<td>0.144 ± 0.066</td>
<td>0.207 ± 0.063</td>
<td>0.182 ± 0.060</td>
<td>0.185 ± 0.065</td>
<td>0.189 ± 0.064</td>
<td>0.181 ± 0.023</td>
</tr>
<tr>
<td>CS</td>
<td>0.558 ± 0.098</td>
<td>0.461 ± 0.060</td>
<td>0.463 ± 0.060</td>
<td>0.5022 ± 0.067</td>
<td>0.450 ± 0.060</td>
<td>0.487 ± 0.044</td>
</tr>
<tr>
<td>Sum</td>
<td>1.01 ± 0.085</td>
<td>1.01 ± 0.064</td>
<td>1.01 ± 0.066</td>
<td>1.01 ± 0.071</td>
<td>1.01 ± 0.069</td>
<td>1.01 ± 0.0003</td>
</tr>
<tr>
<td>LM</td>
<td>0.259 ± 0.077</td>
<td>0.249 ± 0.078</td>
<td>0.261 ± 0.075</td>
<td>0.259 ± 0.074</td>
<td>0.257 ± 0.070</td>
<td>0.256 ± 0.005</td>
</tr>
<tr>
<td>MCC</td>
<td>0.600 ± 0.080</td>
<td>0.592 ± 0.080</td>
<td>0.564 ± 0.078</td>
<td>0.572 ± 0.077</td>
<td>0.570 ± 0.075</td>
<td>0.580 ± 0.015</td>
</tr>
<tr>
<td>CS</td>
<td>0.148 ± 0.043</td>
<td>0.168 ± 0.042</td>
<td>0.182 ± 0.044</td>
<td>0.179 ± 0.043</td>
<td>0.180 ± 0.043</td>
<td>0.171 ± 0.014</td>
</tr>
<tr>
<td>Sum</td>
<td>1.007 ± 0.066</td>
<td>1.007 ± 0.066</td>
<td>1.007 ± 0.065</td>
<td>1.007 ± 0.065</td>
<td>1.007 ± 0.062</td>
<td>1.007 ± 0.000</td>
</tr>
<tr>
<td>LM</td>
<td>0.144 ± 0.074</td>
<td>0.127 ± 0.075</td>
<td>0.135 ± 0.075</td>
<td>0.201 ± 0.074</td>
<td>0.132 ± 0.074</td>
<td>0.149 ± 0.035</td>
</tr>
<tr>
<td>MCC</td>
<td>0.463 ± 0.078</td>
<td>0.447 ± 0.074</td>
<td>0.405 ± 0.077</td>
<td>0.420 ± 0.075</td>
<td>0.483 ± 0.081</td>
<td>0.439 ± 0.034</td>
</tr>
<tr>
<td>CS</td>
<td>0.398 ± 0.058</td>
<td>0.432 ± 0.055</td>
<td>0.465 ± 0.060</td>
<td>0.385 ± 0.052</td>
<td>0.390 ± 0.056</td>
<td>0.418 ± 0.038</td>
</tr>
<tr>
<td>Sum</td>
<td>1.005 ± 0.070</td>
<td>1.006 ± 0.068</td>
<td>1.005 ± 0.071</td>
<td>1.006 ± 0.067</td>
<td>1.006 ± 0.070</td>
<td>1.006 ± 0.0005</td>
</tr>
<tr>
<td>LM</td>
<td>0.456 ± 0.086</td>
<td>0.4717 ± 0.084</td>
<td>0.504 ± 0.089</td>
<td>0.458 ± 0.082</td>
<td>0.473 ± 0.083</td>
<td>0.473 ± 0.019</td>
</tr>
<tr>
<td>MCC</td>
<td>0.463 ± 0.075</td>
<td>0.457 ± 0.072</td>
<td>0.426 ± 0.078</td>
<td>0.469 ± 0.071</td>
<td>0.459 ± 0.074</td>
<td>0.455 ± 0.016</td>
</tr>
<tr>
<td>APU</td>
<td>0.096 ± 0.020</td>
<td>0.087 ± 0.026</td>
<td>0.082 ± 0.020</td>
<td>0.088 ± 0.020</td>
<td>0.083 ± 0.020</td>
<td>0.085 ± 0.006</td>
</tr>
<tr>
<td>Sum</td>
<td>1.016 ± 0.061</td>
<td>1.015 ± 0.061</td>
<td>1.015 ± 0.062</td>
<td>1.015 ± 0.067</td>
<td>1.014 ± 0.058</td>
<td>1.013 ± 0.0005</td>
</tr>
</tbody>
</table>

Figure 4.9 shows that the prediction of concentrations from the MSDs is poor for the three component blends. The individual values have a linear correlation where $R^2 = 0.7644$, and even when the average values are utilised, the $R^2$ value is still only 0.915.
The lower value is observed for the individual replicates due to the spread in MSD across the five replicates for blends 6 and 8. A poor correlation is also observed for the averaged data as Figure 4.9b shows that the CS (at >20% w/w) is always over predicted and that the MCC and LM are under predicted when included in a blend with a high percentage of CS. If only blends 7 and 9 are used a linear correlation with $R^2 = 0.9987$ is observed, showing that the move to three components has not reduced the prediction ability. Prediction of % w/w from MSD does not appear to be possible when CS is present in blends at >20% w/w.

![Graphs showing correlation](image)

**Figure 4.9 - Plot of MSD x 100 values for a) each ingredient in 3 component matrix and b) average value of each ingredient in blend versus % w/w of component in blend**

### 4.6 Understanding Why The Inclusion of Corn Starch in Blends Impacts Concentration Prediction

CS is a regularly used excipient and the poor prediction of this component needs to be understood. In terms of physical properties, from Table 4.1, it can be seen that CS is the excipient with the smallest particle size (18.2 μm), but this is in a similar range to API-A (18.5 μm). When CS is present in a blend at low concentrations (similar to API blends) the prediction of concentration is accurate, but when the concentration is increased prediction performance decreases. As no blends have been produced with high API concentrations the physical parameter may play a role in poor prediction but this cannot be confirmed at this point. However, it is known that in NIRM the depth of penetration is greater than the particle size of these fine materials and therefore the spectrum obtained within a solid dose matrix will be diluted due to over-sampling.
Spectroscopically, CS has similar absorptivity to both LM and MCC, therefore this should not be a factor to consider. When the SNV normalised pure component spectra are compared (Figure 4.1a) it was observed that MCC and CS had very similar spectra. Therefore the CS spectral resolution (definition from other components) and spectral purity (from depth of penetration) may impact the ability to predict accurate concentrations, but will not for MCC or LM due to their large particle size or API due to its sharp unique bands.

![Figure 4.10 - Comparison of NIR spectra from blends 7 and 8 where a) represents the mean spectrum of the image, b) the pure raw materials, c) LM domains cf. MCC, d) MCC domains cf. MCC and e) CS domains cf. MCC](image)

Figure 4.10a shows the average spectra from each blend, and it can be seen by comparison with the pure component spectra (Figure 4.10b) that the mean spectra of both blends are most similar to that for MCC, which is not unexpected due to MCC being >50% w/w of the blend. There are, however, some deviations from the MCC spectrum for both blends, occurring at ~4000 cm\(^{-1}\) and in the region 5300 - 6500 cm\(^{-1}\) for blend 7, and can be associated with the increased LM concentration (20% cf. 25% w/w). Two regions of variation are also identified for blend 8, at 5200 cm\(^{-1}\) (strong CS response) and at 7200 cm\(^{-1}\) (reduced MCC response).
Therefore from the mean spectra it can be seen that blend 7 has a higher LM concentration and lower MCC concentration than blend 8 which has a higher CS concentration. A set of individual domain spectra, from each component, in the different blends were compared, Figure 4.10c – e. From this figure it can be seen that there are small spectral variations between the same components in different blends but in all cases the domain spectra are dominated by MCC contributions. This is due to the over sampling effects described in Chapter 3 which means overall matrix composition impacts spectral features, such that the depth of penetration results in all spectra being based on the major component – in this case MCC.

Exploring the domain spectra provides an opportunity to understand why the low and high concentrations of CS in a MCC matrix results in different prediction abilities. From Figure 4.10c it can be seen that the LM domains give spectra which differ to that for MCC in the region 5300 – 7000 cm$^{-1}$ and 5000 – 5200 cm$^{-1}$, which therefore allows for discrimination of these domains as LM. There is only one region of spectral difference between blends 7 and 8 LM domain spectra, occurring at 5168 cm$^{-1}$ and is attributable to the increased LM concentration – implying that the domains of LM are purer in blend 7, despite only a 5% increase over blend 8. In Figure 4.10d it can be observed that the MCC domains from blend 7 have identical spectra to pure MCC but the domains from blend 8 differ in intensity at 5200 cm$^{-1}$ (region where CS is a strong absorber). Therefore in blend 8 the MCC domains do not appear to be pure, with some contribution from the CS. When the CS domains are examined, Figure 4.10e the spectra show a number of deviations from the pure MCC spectrum, 5200 cm$^{-1}$ and 5500 - 6000 cm$^{-1}$, which should allow discrimination of the CS component. Blend 8 which has a higher CS concentration has the higher response at 5200 cm$^{-1}$ but no variation between the two blends is observed in the 5500 – 6000 cm$^{-1}$ region. Therefore discrimination in CS concentration would appear to be based on the response at 5200 cm$^{-1}$, which is the region where variations in the spectra from MCC.
domains from pure MCC spectrum were also observed for blend 8. This therefore explains why CS is over-predicted and MCC under-predicted in the PLS score distributions, as the two components cannot be easily spectrally resolved using only a normalised spectrum.

Derivatives are effective methods to improve spectral definition and examine changes in peak shapes. Therefore first and second derivative pre-treatments were explored to understand the impact on concentration prediction. Figure 4.11 shows the normalised absorbance, first derivative (Savitsky-Golay filter, 13 point interval, 2nd order polynomial) and second derivative (Savitsky-Golay filter, 13 point interval, 3rd order polynomial) NIR spectra for LM, MCC and CS. The first derivative provides better definition of the spectral variations around $\sim$4300 cm$^{-1}$ between MCC and CS which is further improved in the second derivative data. Moving from first to second derivative data also improves the resolution of the three components at 5200 cm$^{-1}$. To understand the impact on concentration prediction, blend 8, sample 3 was processed using different pre-processing in the PLS model.

Figure 4.11 - NIR spectra of LM (green), MCC (blue) and CS (red) using different pre-treatments with a) normalised absorbance, b) first derivative and c) second derivative
From Figure 4.12 it can be observed that the three different pre-processing methods give rise to very different histograms but do not change the actual chemical image (same percentage of pixels utilised). The most significant change was in the CS distribution which shifts from the highest distribution in Figure 4.12a to between LM and MCC distributions in Figure 4.12c. This shift obviously impacts the MSD which reduces from 0.465 (a) to 0.356 (c) and brings the value closer to the actual 30% w/w. The impact of the CS changes are also observed in the MCC histogram which gradually shifts to higher score values from Figure 4.12a to Figure 4.12c and the MSD shifts from 0.405 (a) to 0.511 (c) which again brings the value closer to the actual 50% w/w. LM was not significantly impacted by the pre-processing methods with a similar shape observed in all histograms and MSD values ranging from 0.124 - 0.139.

\[
\begin{align*}
MSD_{MCC} &= 0.405 \pm 0.077 \\
MSD_{LM} &= 0.135 \pm 0.075 \\
MSD_{CS} &= 0.465 \pm 0.060 \\
\text{Sum} &= 1.005 \\
MSD_{MCC} &= 0.429 \pm 0.094 \\
MSD_{LM} &= 0.124 \pm 0.076 \\
MSD_{CS} &= 0.458 \pm 0.062 \\
\text{Sum} &= 1.011 \\
MSD_{MCC} &= 0.511 \pm 0.102 \\
MSD_{LM} &= 0.139 \pm 0.085 \\
MSD_{CS} &= 0.356 \pm 0.065 \\
\text{Sum} &= 1.006
\end{align*}
\]

Figure 4.12 - Histograms of score values and RGB images for components in blend 8 sample 3 using three different pre-processing methods a) normalisation, b) first derivative and c) second derivative where green represents LM, blue MCC and red CS

As changes were observed in the output from the PLS model using second derivative data, it would suggest that this pre-processing approach is better for resolving CS from MCC. As minimal changes were also observed in the LM distributions it would imply
that the spectral resolution in normalised spectra was an issue for discrimination of MCC from CS, but the differences in normalised LM spectra were significant enough to appropriately assign LM pixels, with the derivative pre-treatment maintaining this discrimination.

Table 4.5 presents a comparison between the MSD values and STD obtained using the original normalisation method and second derivative pre-processing for blends 6 and 8. It can be seen that using a second derivative decreases the MSD value for the CS component and increases that of the MCC component but no obvious trends can be observed for the LM component as in blend 6 the values decrease after derivatisation but they increase in blend 8.

Table 4.5 - Replicate MSD values, means and standard deviations for each component identified in blends 6 and 8 using a) normalisation and b) second derivative pre-processing

<table>
<thead>
<tr>
<th></th>
<th>Replicate 1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a-LM_{33}</td>
<td>0.308 ± 0.091</td>
<td>0.342 ± 0.068</td>
<td>0.364 ± 0.077</td>
<td>0.323 ± 0.080</td>
<td>0.372 ± 0.082</td>
<td>0.342 ± 0.027</td>
</tr>
<tr>
<td>b-LM_{33}</td>
<td>0.241 ± 0.100</td>
<td>0.266 ± 0.093</td>
<td>0.285 ± 0.096</td>
<td>0.246 ± 0.093</td>
<td>0.287 ± 0.098</td>
<td>0.265 ± 0.021</td>
</tr>
<tr>
<td>a-MCC_{33}</td>
<td>0.144 ± 0.066</td>
<td>0.207 ± 0.063</td>
<td>0.182 ± 0.060</td>
<td>0.185 ± 0.065</td>
<td>0.189 ± 0.064</td>
<td>0.181 ± 0.023</td>
</tr>
<tr>
<td>b-MCC_{33}</td>
<td>0.358 ± 0.095</td>
<td>0.445 ± 0.092</td>
<td>0.412 ± 0.080</td>
<td>0.411 ± 0.091</td>
<td>0.410 ± 0.091</td>
<td>0.407 ± 0.031</td>
</tr>
<tr>
<td>a-CS_{33}</td>
<td>0.558 ± 0.098</td>
<td>0.461 ± 0.060</td>
<td>0.463 ± 0.060</td>
<td>0.502 ± 0.067</td>
<td>0.450 ± 0.060</td>
<td>0.487 ± 0.044</td>
</tr>
<tr>
<td>b-CS_{33}</td>
<td>0.426 ± 0.098</td>
<td>0.322 ± 0.059</td>
<td>0.336 ± 0.059</td>
<td>0.373 ± 0.070</td>
<td>0.332 ± 0.060</td>
<td>0.358 ± 0.043</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a-LM_{20}</td>
<td>0.144 ± 0.074</td>
<td>0.127 ± 0.075</td>
<td>0.135 ± 0.075</td>
<td>0.201 ± 0.074</td>
<td>0.132 ± 0.074</td>
<td>0.149 ± 0.035</td>
</tr>
<tr>
<td>b-LM_{20}</td>
<td>0.142 ± 0.085</td>
<td>0.133 ± 0.085</td>
<td>0.139 ± 0.085</td>
<td>0.149 ± 0.085</td>
<td>0.135 ± 0.084</td>
<td>0.140 ± 0.006</td>
</tr>
<tr>
<td>a-MCC_{50}</td>
<td>0.463 ± 0.078</td>
<td>0.447 ± 0.074</td>
<td>0.405 ± 0.077</td>
<td>0.420 ± 0.075</td>
<td>0.483 ± 0.081</td>
<td>0.439 ± 0.034</td>
</tr>
<tr>
<td>b-MCC_{50}</td>
<td>0.526 ± 0.105</td>
<td>0.520 ± 0.103</td>
<td>0.511 ± 0.102</td>
<td>0.499 ± 0.101</td>
<td>0.539 ± 0.105</td>
<td>0.519 ± 0.015</td>
</tr>
<tr>
<td>a-CS_{30}</td>
<td>0.398 ± 0.058</td>
<td>0.432 ± 0.055</td>
<td>0.465 ± 0.060</td>
<td>0.385 ± 0.052</td>
<td>0.390 ± 0.056</td>
<td>0.418 ± 0.038</td>
</tr>
<tr>
<td>b-CS_{30}</td>
<td>0.342 ± 0.065</td>
<td>0.362 ± 0.064</td>
<td>0.356 ± 0.065</td>
<td>0.349 ± 0.062</td>
<td>0.343 ± 0.020</td>
<td>0.350 ± 0.008</td>
</tr>
</tbody>
</table>

Table 4.5 also shows that standard deviations between replicates of blend 8 also decreases with the use of a second derivative but this does not occur with blend 6. In blend 6 the relative standard deviation (RSTD) for LM and MCC is ~8% and for CS is
12%. However, in blend 8, which has the same components, but at different concentrations, these values are halved as LM has a RSTD of 4.6%, MCC of 2.9% and CS of 2.4% suggesting the components in blend 6 have non-homogeneous distributions, in particular with CS. Figure 4.13a shows an example chemical image from blend 8 (sample 1) and in this image a cluster of CS is visible in the bottom right hand corner. This cluster alters the score distribution resulting in a tail at the higher score values. This impacts the MSD value (increase observed) and also causes a greater spread in the distribution which results in a much larger STD. Therefore, the distribution of the CS is critical to the resultant MSD value obtained with higher than expected MSD values being observed due to a heterogeneous distribution. This implies that blend uniformity can be assessed as a function of the pixel score distribution, which was also previously shown in Section 4.4.

![Figure 4.13 - a) Chemical image representing the distribution of LM (green), MCC (blue) and CS (red) and b) associated CS pixel score distribution for blend 6 replicate 1](image)

If the second derivative MSD values are used in the prediction of concentration for three component blends with CS, and the normalised data are used for the other two blends then the prediction model changes to $R^2 = 0.978$, although if blend 6 was excluded, due to high RSTD between replicates, then the MSD values would show a linear correlation with $R^2 = 0.990$. With this correlation it can be concluded that MSD values are a good predictor of the % w/w in three component systems. The reasons for previous over-prediction of CS was due to heterogeneous distribution of this component when present at >30% w/w, along with poor spectral discrimination.
between MCC and CS which was also impacted by the smaller particle size of CS which resulted in over-sampling of the CS domains.

4.7 QUANTIFICATION OF COMPONENT CONCENTRATION IN FOUR COMPONENT SYSTEMS

In Table 4.2 it can be observed that three different blends were prepared utilising four different components (blends 10 - 12). All three blends utilised LM and MCC, but varied in the third and fourth components which were either CS, HPC, API-A or API-B. Blends 10 and 11 had the same components but different concentrations. As blend 12 contained CS in a similar concentration to MCC it was processed using normalisation followed by second derivative pre-processing, as described in Section 4.6. Figure 4.14 shows an example chemical image that was prepared for each blend, and shows variations in component size and concentration of components.

![Blend 10 - 45% LM + 35% MCC + 10% HPC + 10% API-B](image1)

![Blend 11 - 40% LM + 35% MCC + 15% HPC + 10% API-B](image2)

![Blend 12 - 60% LM + 15% MCC + 20% CS + 5% API-A](image3)

Figure 4.14 - NIR chemical images of the distribution of components in blends 10 – 12 with green representing LM, blue for MCC, black for HPC/CS and red for API-A / API-B

The histograms of pixel score values are presented in Figure 4.15 and once again show the variation in component concentration in each blend. The histograms for blend 10 and 11 are interesting to compare as they both have the same components present but at differing concentrations. Both the MCC and API-B are present at the same % w/w in both formulations and on examination of the histograms appear to be very similar despite the changes in HPC and LM concentrations. Another interesting observation from the histograms is that the MCC and API-B distributions are normal whereas the LM and HPC distributions are positively skewed. If the chemical images from Figure 4.14 are examined, the skewing may be explained by the slightly larger
less well distributed domains of both of these components. If the shapes of the histograms for blend 12 are also examined, all distributions with the exception of LM give rise to normal distributions. The LM distribution is again slightly skewed to the right hand side and, as observed previously, can be related to the larger domains.

\[
\begin{align*}
\text{Blend 10} \\
MSD_{\text{MCC}} &= 0.332 \pm 0.064 \\
MSD_{\text{LM}} &= 0.450 \pm 0.077 \\
MSD_{\text{HPC}} &= 0.094 \pm 0.078 \\
MSD_{\text{APIB}} &= 0.140 \pm 0.054
\end{align*}
\]

\[
\begin{align*}
\text{Blend 11} \\
MSD_{\text{MCC}} &= 0.352 \pm 0.069 \\
MSD_{\text{LM}} &= 0.381 \pm 0.088 \\
MSD_{\text{HPC}} &= 0.156 \pm 0.097 \\
MSD_{\text{APIB}} &= 0.129 \pm 0.052
\end{align*}
\]

\[
\begin{align*}
\text{Blend 12} \\
MSD_{\text{MCC}} &= 0.166 \pm 0.084 \\
MSD_{\text{LM}} &= 0.557 \pm 0.094 \\
MSD_{\text{CS}} &= 0.279 \pm 0.068 \\
MSD_{\text{APIA}} &= 0.066 \pm 0.018
\end{align*}
\]

Figure 4.15 - Histograms of score values for each four component blends using green to indicate LM pixels, blue for MCC pixels, black for HPC/CS and red for API-A/API-B pixels, the MSD for each distribution is also included.

The actual MSD values for each component in individual wafers are presented in Table 4.6. As observed for the two and three component blends the individual MSD values summate to \(~1\) for each blend and all show values \(>1.01\) which is indicative of the API being present. It can also be observed that the variation between MSDs is smaller.
than the individual STD's for each MSD. Therefore, although the spread of pixel values changes, this does not impact the actual MSD which is a precise measurement of the chemical image. In each blend, the STD is also similar between the different replicates, implying good homogeneity. When the MSD values are compared to the actual % w/w values it can be seen (as observed from histogram locations) that the ability to predict concentrations from the MSDs are close to the actual % w/w values. When the actual % w/w values are plotted against the MSD × 100 values the individual values show a linear correlation with $R^2 = 0.9544$ which improves to $R^2 = 0.9888$ when the average values for each component in each blend are used.

Table 4.6 - Replicate MSDs, means and standard deviations for each component identified in blends 10 - 12

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>LM45</td>
<td>0.450 ± 0.077</td>
<td>0.445 ± 0.075</td>
<td>0.418 ± 0.075</td>
<td>0.441 ± 0.077</td>
<td>0.441 ± 0.080</td>
</tr>
<tr>
<td>MCC35</td>
<td>0.352 ± 0.064</td>
<td>0.341 ± 0.058</td>
<td>0.348 ± 0.063</td>
<td>0.350 ± 0.064</td>
<td>0.354 ± 0.063</td>
</tr>
<tr>
<td>HPC10</td>
<td>0.094 ± 0.078</td>
<td>0.080 ± 0.070</td>
<td>0.105 ± 0.086</td>
<td>0.097 ± 0.077</td>
<td>0.075 ± 0.070</td>
</tr>
<tr>
<td>APIb10</td>
<td>0.140 ± 0.054</td>
<td>0.150 ± 0.055</td>
<td>0.145 ± 0.050</td>
<td>0.129 ± 0.055</td>
<td>0.146 ± 0.053</td>
</tr>
<tr>
<td>Sum</td>
<td>1.016 ± 0.073</td>
<td>1.016 ± 0.067</td>
<td>1.017 ± 0.075</td>
<td>1.016 ± 0.073</td>
<td>1.016 ± 0.071</td>
</tr>
<tr>
<td></td>
<td>LM40</td>
<td>MCC35</td>
<td>HPC15</td>
<td>APIb10</td>
<td>Sum</td>
</tr>
<tr>
<td></td>
<td>0.381 ± 0.088</td>
<td>0.378 ± 0.088</td>
<td>0.387 ± 0.081</td>
<td>0.380 ± 0.081</td>
<td>0.368 ± 0.082</td>
</tr>
<tr>
<td></td>
<td>0.332 ± 0.069</td>
<td>0.347 ± 0.070</td>
<td>0.357 ± 0.064</td>
<td>0.358 ± 0.064</td>
<td>0.390 ± 0.066</td>
</tr>
<tr>
<td></td>
<td>0.156 ± 0.097</td>
<td>0.173 ± 0.101</td>
<td>0.161 ± 0.084</td>
<td>0.142 ± 0.090</td>
<td>0.129 ± 0.078</td>
</tr>
<tr>
<td></td>
<td>0.129 ± 0.052</td>
<td>0.121 ± 0.058</td>
<td>0.113 ± 0.045</td>
<td>0.137 ± 0.054</td>
<td>0.129 ± 0.057</td>
</tr>
<tr>
<td></td>
<td>1.018 ± 0.085</td>
<td>1.018 ± 0.086</td>
<td>1.018 ± 0.076</td>
<td>1.018 ± 0.076</td>
<td>1.016 ± 0.071</td>
</tr>
<tr>
<td></td>
<td>LM50</td>
<td>MCC15</td>
<td>CS20</td>
<td>APIb5</td>
<td>Sum</td>
</tr>
<tr>
<td></td>
<td>0.544 ± 0.087</td>
<td>0.548 ± 0.085</td>
<td>0.557 ± 0.094</td>
<td>0.544 ± 0.089</td>
<td>0.552 ± 0.090</td>
</tr>
<tr>
<td></td>
<td>0.161 ± 0.086</td>
<td>0.167 ± 0.085</td>
<td>0.166 ± 0.084</td>
<td>0.167 ± 0.089</td>
<td>0.167 ± 0.090</td>
</tr>
<tr>
<td></td>
<td>0.285 ± 0.057</td>
<td>0.268 ± 0.056</td>
<td>0.279 ± 0.058</td>
<td>0.270 ± 0.055</td>
<td>0.259 ± 0.056</td>
</tr>
<tr>
<td></td>
<td>0.064 ± 0.018</td>
<td>0.061 ± 0.018</td>
<td>0.066 ± 0.018</td>
<td>0.062 ± 0.018</td>
<td>0.062 ± 0.018</td>
</tr>
<tr>
<td></td>
<td>1.054 ± 0.074</td>
<td>1.044 ± 0.072</td>
<td>1.068 ± 0.076</td>
<td>1.043 ± 0.074</td>
<td>1.040 ± 0.074</td>
</tr>
</tbody>
</table>
4.8 Determination of How Accurately the Mean Value of Image Pixel Score Distributions Predicts the Actual Concentrations of Components in a Solid Dosage Matrix

In the previous three sections the correlation between the MSD value and the % w/w concentrations of two, three and four component blends has been evaluated. In all cases linear correlations were observed with $R^2$ values between 0.9784 and 0.9955, using the average of the five replicates of each concentration evaluated. This section will evaluate the data set as a whole, independent of how many components are in the solid dosage matrix.

Figure 4.16 presents the relationship between the MSD x 100 and the actual % w/w for each component in all blends examined. From this figure it can be seen that a linear correlation with $R^2 = 0.9869$ is obtained, with a scattering of all data points around the actual concentration line. Examination of these data shows that CS is a component which is typically over-predicted using the MSD and LM is often under predicted (but only when in a matrix with CS).

Figure 4.16 - Plot of average MSD x 100 value for each ingredient in each of the 12 blends prepared
As observed in Section 4.6, CS is a much finer particle size material, which appears to agglomerate when in the formulation at >20% w/w which results in heterogeneous distributions, which cause a skew in the pixel score distributions and hence over prediction of this component. As the sum of pixel scores must add up to one, this over-prediction of CS results in the reduced MSD value for LM. From Figure 4.16 it can be observed that this mis-prediction is around 5% w/w.

Using all data collected, the linear correlation shows that to predict the actual % w/w from the MSD values it is necessary to divide the MSD by 1.0057, which implies that there is on average a 0.57% over prediction by using MSD values. However, if the actual average differences between true and predicted concentrations are calculated, an error of $0.36 \pm 3.47\%$ w/w is observed and if calculated as a relative variation the error increases to $3.86 \pm 14.61\%$, implying that a relative difference of 18.5% could be observed. At high concentrations (90% w/w) this would result in a mis-prediction of ~16% w/w, but at low concentrations (5% w/w) would only result in ~1% w/w mis-prediction. Therefore currently using MSD as a means to predict concentration would not be appropriate for release testing (such as assay or content uniformity) but provides instead an estimate of concentration, which on average is within 0.36\% the actual concentration but the difference can be as large as 3.83\%. Further work is required to understand the impact of particle size and spectral definition on the prediction of concentration from PLS scores distributions.

4.9 A REPRESENTATIVE SAMPLE SIZE FOR NEAR-INFRARED MICROSCOPY

Having established a relationship between the MSD and the % w/w concentration of a sample, it becomes easier to understand what makes a representative sample area for NIRM experiments. It is often asked whether a 25 mm² area (30 minute analysis time) from a cross-sectional surface of a wafer satisfactorily represents the entire sample volume.
Firstly, the sample area was explored by analysis of the entire surface of blend 9 wafer 6. The image was reduced to the largest rectangle which would sit on the wafer surface representing $276 \times 295$ points ($\sim 50 \text{ mm}^2$), shown in Figure 4.17a. This data file was too large for the memory of the data processing software, and the file was cut in two, SNV normalised and then analysed using PLS before the resultant score images were combined to provide the score distributions for the whole area. The chemical image of the distribution of components is shown in Figure 4.17 along with the score distributions and MSD values for each component.

![Figure 4.17 - Visible and chemical image (a) for largest area analysed on wafer 6, blend 9 along with MSD values (b) and pixel score distributions (c) for API-A (red), MCC (blue) and LM (green) ](image)

This image was then divided into four to understand the variability in score values in a reduced image. A quarter of this image gave rise to an area which was 139 by 148 pixels ($3.45 \times 3.7 \text{ mm}$) and it can be seen from Table 4.7 that if the MSD values were used to determine the % w/w concentrations then LM would have a STD of 1.3%, MCC a STD of 1.3% and API-A only 0.3%. As RSTD these values increase to 2.55%, 3.17% and 3.41% respectively. Therefore it would appear that an area which is less than 13 mm$^2$ can be utilised to understand concentration effects, as all quadrants give very similar results.

Although concentration measurements may be possible using an area less than 13 mm$^2$, in any NIRM experiment it is also essential to be able to understand the size
of the different component domains. Therefore the sampling area also needs to provide constant image statistics. Figure 4.18 presents the MSD statistics and the chemical image of LM (green) in the matrix (blue) of blend 9 when each axis was sequentially reduced by 10%. The chemical images presented are not to scale, but instead are kept as a constant size to show changes in domain clarity as image size was reduced.

Table 4.7 - MSD and STD values for each quadrant of the largest area measured on wafer 6, blend 9

<table>
<thead>
<tr>
<th>Area</th>
<th>LM (MSD ± STD)</th>
<th>MCC (MSD ± STD)</th>
<th>API-A (MSD ± STD)</th>
<th>Sum of MSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top LHS (25 –TL)</td>
<td>0.519 ± 0.084</td>
<td>0.403 ± 0.073</td>
<td>0.093 ± 0.019</td>
<td>1.015</td>
</tr>
<tr>
<td>Bottom LHS (25 – BL)</td>
<td>0.506 ± 0.079</td>
<td>0.408 ± 0.068</td>
<td>0.101 ± 0.019</td>
<td>1.015</td>
</tr>
<tr>
<td>Top RHS (25 – TR)</td>
<td>0.493 ± 0.084</td>
<td>0.427 ± 0.073</td>
<td>0.096 ± 0.021</td>
<td>1.015</td>
</tr>
<tr>
<td>Bottom RHS (25 – BR)</td>
<td>0.521 ± 0.090</td>
<td>0.397 ± 0.078</td>
<td>0.097 ± 0.020</td>
<td>1.016</td>
</tr>
<tr>
<td>Average of 4 Sections</td>
<td>0.510 ± 0.084</td>
<td>0.409 ± 0.073</td>
<td>0.097 ± 0.020</td>
<td>1.015</td>
</tr>
<tr>
<td>STD (RSTD) of MSD</td>
<td>0.013 (2.55%)</td>
<td>0.013 (3.17%)</td>
<td>0.003 (3.41%)</td>
<td>0.0005 (0.05%)</td>
</tr>
</tbody>
</table>

From these images it can be seen that an image which is 0.3% of the largest area (0.12 mm²) has MSD values which are very different to all other values observed and also the area is so small that the chemical image shows very poor image contrast. In fact if the RSTD is calculated for all areas a value of 3.5% is observed. However, if the 0.3% area is excluded, a value of only 0.4% RSTD is observed. If the 25% area (bottom quadrant, LHS) is also included the RSTD increases to 1% which is still good agreement between all areas greater than 0.12 mm². Although MSD values imply similar results for a sample area of only 1% (~ 0.5 mm²) of the entire sample surface, when the images are visually examined the LM distribution suggests that at least 16% of the large area is necessary to perform image statistics (>8 mm²). This larger area for image statistics may be due to the input particle size of the LM (107 µm) which is ~21% of the linear dimension of the 1% sample area, compared with 1.3% of the linear dimension for the 16% sample area. It is also known that with the MSD being related to concentration effects that the values will be based on an over-sampled analysis.
area, due to the depth of penetration, whereas image statistics are based only on the number of pixels. It should also be noted that these values would only be true for a homogenous blend.

<table>
<thead>
<tr>
<th>Area</th>
<th>MSD_{MCC} = 0.410 ± 0.074</th>
<th>Area</th>
<th>MSD_{MCC} = 0.411 ± 0.073</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>MS{L_M} = 0.510 ± 0.085</td>
<td>81%</td>
<td>MS{L_M} = 0.506 ± 0.084</td>
</tr>
<tr>
<td></td>
<td>MSD_{APIa} = 0.097 ± 0.021</td>
<td></td>
<td>MSD_{APIa} = 0.098 ± 0.020</td>
</tr>
<tr>
<td>64%</td>
<td>MSD_{MCC} = 0.411 ± 0.073</td>
<td>49%</td>
<td>MSD_{MCC} = 0.412 ± 0.072</td>
</tr>
<tr>
<td></td>
<td>MS{L_M} = 0.506 ± 0.084</td>
<td></td>
<td>MS{L_M} = 0.501 ± 0.084</td>
</tr>
<tr>
<td></td>
<td>MSD_{APIa} = 0.099 ± 0.020</td>
<td></td>
<td>MSD_{APIa} = 0.099 ± 0.020</td>
</tr>
<tr>
<td>36%</td>
<td>MSD_{MCC} = 0.411 ± 0.072</td>
<td>25%</td>
<td>MSD_{MCC} = 0.410 ± 0.069</td>
</tr>
<tr>
<td></td>
<td>MS{L_M} = 0.506 ± 0.084</td>
<td></td>
<td>MS{L_M} = 0.509 ± 0.081</td>
</tr>
<tr>
<td></td>
<td>MSD_{APIa} = 0.098 ± 0.020</td>
<td></td>
<td>MSD_{APIa} = 0.098 ± 0.020</td>
</tr>
<tr>
<td>25%</td>
<td>MSD_{MCC} = 0.408 ± 0.068</td>
<td>16%</td>
<td>MSD_{MCC} = 0.410 ± 0.072</td>
</tr>
<tr>
<td>Area</td>
<td>MS{L_M} = 0.506 ± 0.079</td>
<td></td>
<td>MS{L_M} = 0.508 ± 0.084</td>
</tr>
<tr>
<td>(Bot)</td>
<td>MSD_{APIa} = 0.101 ± 0.019</td>
<td></td>
<td>MSD_{APIa} = 0.098 ± 0.020</td>
</tr>
<tr>
<td>LHS)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9%</td>
<td>MSD_{MCC} = 0.411 ± 0.071</td>
<td>4%</td>
<td>MSD_{MCC} = 0.406 ± 0.073</td>
</tr>
<tr>
<td>Area</td>
<td>MS{L_M} = 0.508 ± 0.084</td>
<td></td>
<td>MS{L_M} = 0.513 ± 0.085</td>
</tr>
<tr>
<td></td>
<td>MSD_{APIa} = 0.097 ± 0.020</td>
<td></td>
<td>MSD_{APIa} = 0.097 ± 0.019</td>
</tr>
<tr>
<td>1%</td>
<td>MSD_{MCC} = 0.409 ± 0.070</td>
<td>0.3%</td>
<td>MSD_{MCC} = 0.455 ± 0.052</td>
</tr>
<tr>
<td>Area</td>
<td>MS{L_M} = 0.507 ± 0.082</td>
<td></td>
<td>MS{L_M} = 0.450 ± 0.043</td>
</tr>
<tr>
<td></td>
<td>MSD_{APIa} = 0.010 ± 0.019</td>
<td></td>
<td>MSD_{APIa} = 0.113 ± 0.017</td>
</tr>
</tbody>
</table>

Figure 4.18 - Comparing the MSD statistics and chemical images of LM (green) distribution in the matrix (blue) of blend 9 when image size is reduced. (The images presented are not to scale, but are the same size to show variation in image quality).

To allow comparison of the image features numerically, contours were drawn around the LM particles using the mean value of 2 STD around the pixel score distribution to determine where to draw the contour. The values obtained are presented in Table 4.8.
From this table it can be seen that poor agreement is observed between the four different quadrants of the large area, suggesting that an area greater than 13 mm$^2$ is required for good image statistics. In fact at ~50% of the original image size, half of the number of domains are still observed, and the values for this size of area (25 mm$^2$) are similar to that observed from the original large image. Therefore the data suggest that an area of at least 25 mm$^2$ is necessary for good image detail but also for concentration determination. It is possible that a smaller area could still give precise results but as the analysis time for an area of 25 mm$^2$ is in the order of 30 minutes this is an optimum size for both data collection time and precise and accurate representation of the sample. Also at this size data manipulation occurs on the whole image at once, as current computer specifications can handle an image no larger than 205 by 205 pixels (26 mm$^2$ with 25 μm pixels) analysed in a single measurement. Therefore when sample volume is explored (evaluating different sample layers), surface areas of ~25 mm$^2$ will be processed.

**Table 4.8 - Variation in image statistics when the area of analysis is reduced**

<table>
<thead>
<tr>
<th>% Area</th>
<th>Area (mm$^2$)</th>
<th>Number of domains (% of total)</th>
<th>Area of Image Contoured (%)</th>
<th>Mean Diameter ± STD (pixels)</th>
<th>Nearest Neighbour (pixels)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>50.9</td>
<td>195 (100%)</td>
<td>50.1</td>
<td>16.4 ± 38.7</td>
<td>15.7</td>
</tr>
<tr>
<td>81</td>
<td>41.1</td>
<td>167 (86%)</td>
<td>49.4</td>
<td>15.7 ± 38.4</td>
<td>14.3</td>
</tr>
<tr>
<td>64</td>
<td>32.5</td>
<td>123 (63%)</td>
<td>49.9</td>
<td>16.4 ± 44.1</td>
<td>14.6</td>
</tr>
<tr>
<td>49</td>
<td>25.0</td>
<td>100 (51%)</td>
<td>49.4</td>
<td>15.9 ± 42.1</td>
<td>14.6</td>
</tr>
<tr>
<td>36</td>
<td>18.5</td>
<td>63 (32%)</td>
<td>49.6</td>
<td>17.2 ± 42.1</td>
<td>16.5</td>
</tr>
<tr>
<td>25</td>
<td>12.8</td>
<td>54 (28%)</td>
<td>49.6</td>
<td>15.5 ± 36.8</td>
<td>15.2</td>
</tr>
<tr>
<td>16</td>
<td>8.3</td>
<td>39 (20%)</td>
<td>49.8</td>
<td>14.7 ± 29.3</td>
<td>15.1</td>
</tr>
<tr>
<td>9</td>
<td>4.7</td>
<td>22 (8%)</td>
<td>49.7</td>
<td>14.7 ± 27.7</td>
<td>15.0</td>
</tr>
<tr>
<td>4.1</td>
<td>2.1</td>
<td>8 (3%)</td>
<td>49.6</td>
<td>16.3 ± 16.3</td>
<td>18.3</td>
</tr>
<tr>
<td>1</td>
<td>0.51</td>
<td>3 (1%)</td>
<td>50.6</td>
<td>13.2 ± 7.5</td>
<td>17.3</td>
</tr>
<tr>
<td>0.3</td>
<td>0.12</td>
<td>4 (1%)</td>
<td>54.1</td>
<td>5.8 ± 6.8</td>
<td>6.2</td>
</tr>
<tr>
<td>25-TL</td>
<td>12.8</td>
<td>42 (22%)</td>
<td>50.3</td>
<td>17.6 ± 33.6</td>
<td>17.4</td>
</tr>
<tr>
<td>25-BL</td>
<td>13.0</td>
<td>42 (21%)</td>
<td>49.6</td>
<td>17.3 ± 34.2</td>
<td>18.3</td>
</tr>
<tr>
<td>25-TR</td>
<td>12.5</td>
<td>57 (29%)</td>
<td>49.3</td>
<td>15.2 ± 28.3</td>
<td>14.5</td>
</tr>
<tr>
<td>25-BL</td>
<td>12.8</td>
<td>57 (29%)</td>
<td>50.1</td>
<td>16.4 ± 38.7</td>
<td>15.7</td>
</tr>
</tbody>
</table>
To determine if the analysis of a 25 mm$^2$ area from the sample surface represented the entire volume, different depths (7 layers) in the sample were examined. The surface of the wafer was named layer 0, with 6 sub-sections ~0.5 mm apart forming the further six layers. The height of the wafer at each layer is shown in Table 4.9 along with the MSD values and the spread of the histogram distributions, along with the average and STD of MSD for each component. From this table the RSTD for each component can be determined. For LM the RSTD is 2.81%, for MCC is 2.99% and for API-A is 6.45%. The RSTD for API-A is double that of LM or MCC but, as it was present at a lower concentration, this is not unexpected. If MSD $\times$ 100 values were used for concentration prediction then the average for API-A would be 8.9 ± 0.6%. In Table 4.4 the MSD and STD values for the 5 different wafers of blend 9 are presented; using this data it can be seen that the RSTD for LM was 4.07%, MCC was 3.49% and API-A was 6.63%. Therefore the variation observed in the different layers of one wafer is less than the variation observed between five different wafers.

Table 4.9 - MSD and STD values of each component at different depths into tablet wafer 6 (blend 9)

<table>
<thead>
<tr>
<th>Layer</th>
<th>Height (mm)</th>
<th>LM</th>
<th>MCC</th>
<th>API-A</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (surface)</td>
<td>4.04</td>
<td>0.501 ± 0.084</td>
<td>0.412 ± 0.072</td>
<td>0.099 ± 0.020</td>
<td>1.012</td>
</tr>
<tr>
<td>1</td>
<td>3.51</td>
<td>0.485 ± 0.064</td>
<td>0.436 ± 0.057</td>
<td>0.090 ± 0.016</td>
<td>1.011</td>
</tr>
<tr>
<td>2</td>
<td>2.79</td>
<td>0.506 ± 0.062</td>
<td>0.414 ± 0.055</td>
<td>0.090 ± 0.018</td>
<td>1.010</td>
</tr>
<tr>
<td>3</td>
<td>2.31</td>
<td>0.500 ± 0.058</td>
<td>0.427 ± 0.084</td>
<td>0.084 ± 0.017</td>
<td>1.011</td>
</tr>
<tr>
<td>4</td>
<td>1.71</td>
<td>0.483 ± 0.065</td>
<td>0.436 ± 0.058</td>
<td>0.092 ± 0.017</td>
<td>1.011</td>
</tr>
<tr>
<td>5</td>
<td>1.16</td>
<td>0.508 ± 0.062</td>
<td>0.412 ± 0.056</td>
<td>0.090 ± 0.016</td>
<td>1.010</td>
</tr>
<tr>
<td>6</td>
<td>0.55</td>
<td>0.524 ± 0.061</td>
<td>0.405 ± 0.084</td>
<td>0.081 ± 0.017</td>
<td>1.010</td>
</tr>
<tr>
<td>Average ± STD</td>
<td>-</td>
<td>0.501± 0.014</td>
<td>0.420 ± 0.013</td>
<td>0.089 ± 0.006</td>
<td>1.011 ± 0.0007</td>
</tr>
</tbody>
</table>

It can also be concluded that for concentration prediction, that the surface of the wafer gives rise to values which are representative of the entire wafer using an area of ~25 mm$^2$. Although the measurements observed for different layers of one wafer were precise, the actual values obtained for wafer 6 were within the variation observed for
the 5 replicate wafers but in fact are not 100% accurate. It can be seen from Table 4.9 that if the concentration of each component was predicted using the MSD values then LM would be over predicted (50.1 ± 1.4%), and both MCC and API-A under predicted (42.0 ± 1.3% and 8.9 ± 0.6%). However, as these values are similar between layers this must be representative of the composition of wafer 6. Therefore it may be concluded that there is more benefit in scanning multiple samples from one blend than scanning multiple layers from a single sample to obtain an accurate representation of the concentration of components using NIRM.

As discussed in understanding the affect of sampling area, the results of analysing different depths into the sample should also be examined in terms of image statistics. Table 4.10 presents the image statistics for LM at different depths in wafer 6 and also for the five replicate wafers of blend 9. From this table it can be seen that the surface of the wafers have fewer but larger domains than any of the layers from within the wafer. Therefore the surface layer of wafer 6 has not been used in the average of layers but instead utilised as wafer 6 in the average of surface layers.

Table 4.10 - Variation in image statistics between internal layers from wafer 6 and surface layers of wafers 1 - 6 from blend 9

<table>
<thead>
<tr>
<th>Layer</th>
<th>Number of domains</th>
<th>% Area</th>
<th>Mean Diameter ± STD (pixels)</th>
<th>NN* (pixels)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>138</td>
<td>50.2</td>
<td>13.6 ± 31.4</td>
<td>12.8</td>
</tr>
<tr>
<td>2</td>
<td>132</td>
<td>49.6</td>
<td>13.9 ± 29.6</td>
<td>14.4</td>
</tr>
<tr>
<td>3</td>
<td>140</td>
<td>50.3</td>
<td>13.4 ± 30.2</td>
<td>12.7</td>
</tr>
<tr>
<td>4</td>
<td>121</td>
<td>50.3</td>
<td>14.5 ± 35.0</td>
<td>13.4</td>
</tr>
<tr>
<td>5</td>
<td>127</td>
<td>49.5</td>
<td>14.1 ± 31.6</td>
<td>11.8</td>
</tr>
<tr>
<td>6</td>
<td>119</td>
<td>50.2</td>
<td>14.6 ± 41.2</td>
<td>13.4</td>
</tr>
<tr>
<td>Average Internal Layers</td>
<td>125</td>
<td>49.9</td>
<td>14.3 ± 35.1</td>
<td>13.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Number of domains</th>
<th>% Area</th>
<th>Mean Diameter ± STD (pixels)</th>
<th>NN* (pixels)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>80</td>
<td>49.5</td>
<td>15.6 ± 37.0</td>
<td>16.9</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>47.2</td>
<td>15.3 ± 37.0</td>
<td>15.3</td>
</tr>
<tr>
<td>3</td>
<td>71</td>
<td>50.3</td>
<td>16.9 ± 45.4</td>
<td>13.6</td>
</tr>
<tr>
<td>4</td>
<td>76</td>
<td>50.5</td>
<td>16.2 ± 39.3</td>
<td>15.7</td>
</tr>
<tr>
<td>5</td>
<td>81</td>
<td>49.7</td>
<td>15.6 ± 39.8</td>
<td>16.2</td>
</tr>
<tr>
<td>Average Surface Layers</td>
<td>81.3</td>
<td>49.4</td>
<td>15.9 ± 40.1</td>
<td>15.4</td>
</tr>
</tbody>
</table>

*NN = nearest neighbour
The average of internal versus surface layers show that a similar percentage of image area is used in the determination of LM statistics, but that in the internal layers the LM domains are 10% smaller but there are \(-\frac{1}{3}\) more domains which results in a lower number for the nearest neighbour. This variation implies that the surface is obviously impacted by compression more than the internal structure, i.e. the surface particles are more compressed, having larger surface area. However, if comparisons are made between like surfaces (for image statistics) then it does not matter whether an internal or surface layer is used as long as it is held constant within an experimental study, as RSTD for number of domains is 10.9% between layers and is 12.1% between different surface layers and is 5.8% and 3.6% respectively for mean diameter. This is obviously different from what was observed from the MSD values, where the surface had values within the spread of the internal layers.

4.10 CONCLUSIONS
This chapter has shown that there is a strong relationship between the MSD value and the % w/w of a component in a blend formulation. The ability to predict concentration is however impacted by the individual component and the matrix environment in which it resides, particularly with relation to NIR spectral resolution. Concentration predictions of materials with similar NIR spectra can be improved by the use of second derivative pre-treatments, but the best prediction is achieved if materials have different SNV normalised NIR spectra which can be distinguished using PLS.

If MSD is to be used to predict concentration it would be necessary to evaluate model systems to understand the true relationship between MSD and % w/w. However, if this work was not performed, the MSD value could still be utilised to understand changes in concentration from blend to blend as this chapter has shown that the MSD value is a precise measure of concentration. The STD between replicates is less than 2% w/w for excipients (RSTD <5%) but does increase with larger particle sized materials. When API is present in the matrix, the STD between replicates is less than 1% w/w.
(RSTD of 3.5%) and this improvement is thought to be attributable to the improved spectral definition of the matrix components from the API. If spectral features are similar, then mis-classification during PLS analysis can occur, as observed for blends with LM, MCC and CS. Therefore using MSD to predict concentration is not always accurate and for the samples evaluated in this work on average the predicted concentration was within 3.86% relative standard deviations of the actual concentration but could be as large as 18.5%.

Having a simple numerical value to compare components allowed an understanding of representative sampling of a dosage form to be made. Using an area of ~0.5 mm² allowed enough blend to be sampled and utilised for precise concentration prediction, but at this level there is no value in the imaging data obtained. It was found that the optimum area for NIRM analysis was ~25 mm² when using spatial resolution of 25 × 25 μm, as this gave rise to MSD values which could be used for prediction and also an image size which allowed for good image statistics whilst only requiring 30 minutes to acquire the spectra. Having evaluated different depths in one wafer, it is possible to conclude that concentration prediction is not affected by the position in the depth axis but this does hold true for image statistics. It was found that the size and number of domains of a component differs between the surface of a sample and subsequent layers within the wafer. The surface of the wafer had larger domain sizes (fewer domains) than internal depths which imply that the surface is impacted more by compression than the internal matrix.

The investigations have also shown that NIRM can be used to assess blend homogeneity, with the spread of the pixels around the MSD value being an indicator of uniformity. If the width of the distribution increases, i.e. the STD around the mean score value increases, it follows that the component is not homogenous. Non-homogenous materials give rise to regions of the image which have very intense (high score value) pixels, i.e. where a cluster of a component forms. If a typical blend score
distribution was known it would be possible from the score distribution to determine any
changes in concentration or component distribution, making NIRM a viable process
understanding technique.

It is believed that the relationship between the mean value of the pixel score
distribution and the component concentration exists because in any NIRM experiment
the NIR radiation interacts with not only the exposed surface but also penetrates some
way into the sample. As such, the spectrum from each pixel is most similar to an
average response from all components, where the spectral features are weighted
based on bulk concentration. The spectrum from a pixel only deviates slightly from this
mean response and this can be attributed to the component closest to the analysis
'surface', as it has greatest contribution to the overall spectrum (as discussed in
Chapter 3). It is easiest to see this affect in the two component blends where it was
expected that from the image of each component that a bi-modal distribution of score
values would be observed, centred on 0 and 1. When the two component blends were
analysed a mono-modal distribution was actually observed which centred on the
average concentration. The average response would be the mean spectrum (so actual
% w/w concentration) and the spread of score values would be a result of small
deviations in the spectral features relative to the mean spectrum. Therefore when the
spectrum from each pixel is compared to the library, where the score value for each
pure component is 1 and equally weighted to all other components, it follows that the
mean spectrum would give rise to score values similar to concentration. As such,
NIRM could be developed as a process control tool to understand firstly the variability
in concentration and distribution of all raw materials in the final dosage form – which
would occur by monitoring batch to batch variability with no prior work required.
However, by preparing some model samples, NIRM could be utilised to predict the
actual concentration of all raw materials in the final dosage form.
5. CHARACTERISATION OF TABLET MATRICES AND THE RELATIONSHIP TO PRODUCTION ISSUES

5.1 TABLET STICKING, A REAL WORLD PRODUCTION EXAMPLE

Within pharmaceutical production there are many different issues which can arise during manufacture which impact the movement of the pre-compression blend through the process (e.g. flow) or the appearance of the final solid dosage form (e.g. picking/sticking/capping). These manufacturing related issues will often impact the end product test (e.g. dissolution or simply appearance) and therefore require in-process testing to minimise the occurrence. Testing within the manufacturing location means it is often possible to identify issues during manufacture and make changes in equipment/process to rectify the situation, such as a reduced speed on a tablet press if compression issues are experienced. Although problems are often overcome, it is good practice to investigate why changes in material properties have occurred to minimise re-occurrence. This chapter will show that NIRM is a good tool for providing insight into variations in solid dosage forms which occur during manufacture which are currently not measured (or possible) using other analytical methodologies.

The focus of this chapter will be the evaluation of the solid dosage form, Norvasc® (Pfizer Inc) which is used in the treatment of hypertension (high blood pressure). At the time of this investigation Norvasc® was manufactured at 15 different Pfizer locations and sporadically experienced tablet sticking during manufacture at some of the sites. Sticking relates to the pre-tabletting blend adhering to the tablet punch face during production and Figure 5.1 shows an example of tablet dies with and without blend sticking and also the effects on the finished dosage form if sticking occurs. It was noted that all manufacturing locations use the same formulation, but equipment and raw materials varied dependent upon site.
Norvasc® is a five component formulation made by direct blending of the raw materials followed by compression to create the solid dosage form. The tablets varied in shape and size dependent on manufacturing location and dose. The main components of the formulation, microcrystalline cellulose (MCC) and dibasic calcium phosphate (DCP), account for 93.5% of the total weight of the formulation (62% w/w and 31.5% w/w respectively) and act as both bulking and binding agents. The active pharmaceutical ingredient (amlodipine besylate) is present at 3.5% w/w. The disintegrant is sodium starch glycolate (SSG) which is present at 2% w/w and the lubricant is magnesium stearate (MgS) which is only 1% w/w of the formulation. For this investigation a batch of typical (no production issues identified) Norvasc®, pre-compression blend and tablets, were received from 15 different drug product sites. If any production problems had been observed then a sample of the blend and tablets from these lots were also received (5 batches), giving rise to 20 different batches for analysis by NIRM.

5.2 LIMITATIONS IN NEAR-INFRARED MICROSCOPY EXPERIMENTS IN 1999
At this point it should be noted that this investigation was the first performed using NIRM as a means to understand production issues. This project set the framework for all future investigations, but had to utilise older technology and limited computer processing capabilities. All data were collected in 1999 prior to the development of an instrument capable of line mapping. Instead data collection was performed using point mapping (Perkin Elmer Autoimage microscope coupled with a FT-NIR Spectrum Identichcheck spectrometer shown previously in Figure 1.6a). Originally, an investigation was performed to establish the conditions for optimising signal to noise ratio as a
function of the spectral resolution, aperture size and number of scans utilised. Although this work was performed, the steps in determining optimum conditions will not be presented here, only the conclusions. It was found that the smallest aperture size which could be used was $20 \times 20 \mu \text{m}$ and to optimise signal to noise a spectral resolution of $16 \text{ cm}^{-1}$ was required with 25 scans per point. Using these conditions, it took over 12.5 hours to scan the surface analysis area of $1 \text{ mm}^2$ (2500 spectra) which is very slow in comparison to the system (Figure 1.6b) used in 2006 which takes $\sim 30$ minutes to acquire over 40,000 spectra.

A small study was performed to understand if a $1 \text{ mm}^2$ area could be representative of an entire dosage form. Figure 5.2 presents the chemical images obtained from four different analysis areas on a compressed blend wafer's surface (see Section 5.3), with blue representing MCC, black as DCP, red as API and green as SSG. These images were prepared using univariate processing in the data acquisition software. The smallest area was $0.25 \text{ mm}^2$ ($0.5 \text{ mm} \times 0.5 \text{ mm}$) and took $\sim 4$ hours to collect and the largest area was $4 \text{ mm}^2$ ($2 \text{ mm} \times 2 \text{ mm}$) which took over 50 hours to collect. The mid-size areas ($1 \text{ mm}^2$ & $2.25 \text{ mm}^2$) took 12.5 hours and 25 hours respectively. It can be seen from Figure 5.2 that except for the smallest map, the information obtained from the various images is very similar and includes defined regions from each of the four components. In each image the size of the domains also look similar. As the experiments were time consuming it was decided that the information from the $1 \text{ mm}^2$ area would provide a compromise between analysis time and amount of data obtained.

![Figure 5.2 - Different sizes a) 0.25 mm², b) 1 mm², c) 2.25 mm² and d) 4 mm², of chemical images of MCC (blue), DCP (black), SSG (green) and API (red) distributions in Norvasc® blend samples](image-url)
Not only was the instrumentation slower in 1999, but also computer processing was memory limited. Any data processing was also time consuming, with processing of one data cube taking ~12 hours. At this point, the image analysis software (ISYS, Spectral Dimensions, MD) was in its infancy (v1.5) as the benefits of NIRM had not yet been realised. There was a good collaboration with the vendor, which allowed extra processing tools to be incorporated. These changes are now present in the current version of the software, but unfortunately were not available through the lifespan of this investigation. In this work, image statistics were determined by a simple pixel counting approach shown in Figure 5.3 where the blend particle size is calculated using the individual chemical images. When an ingredient is present, it is shown in white and light grey within the image. The blend particle size is measured by counting the number of white/light grey pixels in each dimension. The number of pixels is multiplied by the aperture size and the average of the two axes is taken to be the domain size. To determine blend particle size for each lot the average from all particles in the three replicate blend images will be calculated. Therefore blend particle size is a crude approximation of the numerical variation in the chemical images.

Figure 5.3 - Visualisation of method used to determine blend particle size

The main benefit of using the image analysis package was that it provided an opportunity to use PCA and PLS routines which were considered more robust methods for interpreting the hundreds of spectra from a NIRM experiment. Prior to the
availability of this software, routine data processing had been performed in a univariate manner, but this approach utilised less than 20% of the data collected and was a highly user dependent, slow and variable method for data processing.

Despite the limitations in equipment, software and computer technology this investigation was significant to the advancement of NIRM in pharmaceutical analysis at the time. However, it should be recognised that the quality and understanding of the resultant data was without the knowledge which has been presented in Chapters 2 – 4.

5.3 Method

For each of the 20 batches investigated, samples of pre-compression blend and tablets were received. The lot number, tablet dose size and location of manufacture are provided in Table 5.1. The focus of the investigation was the pre-compression blend, due to variability in tablet size and shape with respect to location. The use of the pre-compression blend removed any variability induced by compression equipment at the individual site. Any differences in the process can be observed at this stage and prediction of sticking may be possible. As a loose powder the pre-compression blends had to be compacted to provide a flat surface for the data collection. Three subsamples of each blend, weighing 300 mg each, were taken and pressed using a single punch hydraulic press (using 200 bar pressure) into tablet wafers ~4 mm thick. Subsamples of each raw material (representative lots) were also prepared in the same way, to allow a spectral library to be built for data processing.

The Autoimage NIR Point Mapping system (shown in Figure 1.6a) was utilised throughout this investigation to analyse both the raw materials and the compressed blend wafers. The spectrometer was set to collect spectral data over the range 4000 – 7000 cm⁻¹ with 8 cm⁻¹ data intervals and an average of 25 scans per pixel. A sample area of 1 mm² was utilised for two of the blend wafers in each lot, but the third wafer was scanned over a much larger area (4 – 9 mm²). This was performed to ensure that
all minor components were observed if above the spatial limit of detection. The spectra were output as relative reflectance (\( R \)), based on the reference of a gold mirror.

Table 5.1 - Lot number, location and manufacturing experiences of pre-compression blends and tablets received

<table>
<thead>
<tr>
<th>Location</th>
<th>Pre-Compression Blend Lot No.</th>
<th>Tablet Dose Size (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Typical Performance</td>
<td>Sticking Observed</td>
</tr>
<tr>
<td>Australia</td>
<td>914-05916</td>
<td>914-05925</td>
</tr>
<tr>
<td>Belgium</td>
<td>901-05962</td>
<td>901-05759</td>
</tr>
<tr>
<td>Brazil</td>
<td>904-05032</td>
<td>904-05030</td>
</tr>
<tr>
<td>Canada</td>
<td>990153</td>
<td>n/a</td>
</tr>
<tr>
<td>China</td>
<td>958-05026</td>
<td>n/a</td>
</tr>
<tr>
<td>Germany</td>
<td>227510</td>
<td>n/a</td>
</tr>
<tr>
<td>Indonesia</td>
<td>929-05008</td>
<td>n/a</td>
</tr>
<tr>
<td>Italy</td>
<td>907-05111</td>
<td>n/a</td>
</tr>
<tr>
<td>Japan</td>
<td>n/a</td>
<td>905-01005</td>
</tr>
<tr>
<td>Korea</td>
<td>3390-9016</td>
<td>3390-9079</td>
</tr>
<tr>
<td>Mexico</td>
<td>918-05011</td>
<td>n/a</td>
</tr>
<tr>
<td>Morocco</td>
<td>131299</td>
<td>n/a</td>
</tr>
<tr>
<td>Puerto Rico</td>
<td>T9QP256-05770</td>
<td>n/a</td>
</tr>
<tr>
<td>South Africa</td>
<td>935-00501</td>
<td>n/a</td>
</tr>
<tr>
<td>Turkey</td>
<td>917-05121</td>
<td>n/a</td>
</tr>
<tr>
<td>UK</td>
<td>990-05118</td>
<td>n/a</td>
</tr>
</tbody>
</table>

5.4 DATA PROCESSING AND CHEMICAL IMAGE GENERATION

After data collection, the resultant data files were translated into a format (.spf) readable by the image analysis software (by conversion of image into individual spectra with co-ordinates). The spectra were firstly transformed into absorbance units, as previously described in Equation 1.11 and then normalised using SNV to remove any effects from scatter. The NIR spectra for MCC and SSG were found to be very similar, and derivatives were required to improve the spectral definition between these components. To evaluate the different derivatives and the ability of PLS to resolve all components, the image sets of the pure components were combined together and analysed as a single data set using PLS regression. Figure 5.4 shows the score values obtained for each class when pixels were selected from the image area of the
component in that class. From this figure it can be seen that both the second derivative treatments give rise to highly variable score values for each component and that the values obtained from MCC are weighted between class 2 (MCC) and class 4 (SSG). The variation was thought to be due to the increased noise in the spectra caused by the second derivative. With the amount of variability observed, second derivatives will not be utilised. Figure 5.4 also shows that the first derivative pre-treatment gives rise to precise and accurate classifications, with each class showing a value very close to 1 for the correct components. It was also observed that using a higher gap size (13, 2) gave better accuracy as the score values are slightly closer to 1. Therefore, a first derivative calculated with a 13 point interval and fitted with a 2nd order polynomial, will be used as a pre-treatment to PLS for the Norvasc® blends.

**Derivative Order (Gap Size, Polynomial Order)**

<table>
<thead>
<tr>
<th>Class</th>
<th>1&lt;sup&gt;st&lt;/sup&gt; Derivative (9,2)</th>
<th>1&lt;sup&gt;st&lt;/sup&gt; Derivative (13,2)</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; Derivative (13,4)</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; Derivative (25,4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class 1</td>
<td>(API)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class 2</td>
<td>(MCC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class 3</td>
<td>(DCP)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class 4</td>
<td>(SSG)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class 5</td>
<td>(MgS)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Figure 5.4 - Comparison of score values obtained for the five components in Norvasc® using PLS with different derivative pre-treatments, where the x-axis in each chart represents the class number in library and the y-axis the PLS score values*
5.5 Matrix Evaluation of Typical and Sticking Pre-compression Blends

To establish the matrix distribution of components within the blend samples, each image set was processed using PLS to generate chemical images based on score intensities for each component. Figure 5.5 presents the images of each of the five components in the large analysis area from both the typical and sticking Australian blend. It can be seen from this figure that the distribution of the API is significantly different between the two blends (many small clusters in the typical blend, but one large region in the sticking blend). There are also some variations in the distributions of the SSG and MgS. From the images, it would also appear that the DCP distribution is more homogenous in the typical blends. In the sticking blend, the MCC appears to occupy the bottom left hand side of the image and the DCP weighted more to the top right hand side. Therefore by eye, there are a number of differences between these two blends.

![Images of blend matrix comparison](image)

*Figure 5.5 – Comparison of the blend matrix of Australian typical and sticking Blends where a) represents MCC, b) DCP, c) API, d) SSG and e) MgS chemical distributions*

To understand the significance of this variation the two blends from Brazil were compared, Figure 5.6. In this figure it can be seen that there are more MgS clusters in the sticking blend, but the most significant variation appeared to be in the distribution of the DCP. In the typical blend, DCP appears as even clusters but is much finer and more evenly dispersed in the sticking blend. Therefore the only factor which is common between the blend performance and the two different manufacturing locations
is the relative distributions of the DCP and MCC (if DCP changes it will impact the MCC distribution as they account for 93.5% of the matrix).

![Figure 5.6 - Comparison of the blend matrix of Brazilian typical and sticking blends where a) represents MCC, b) DCP, c) API, d) SSG and e) MgS chemical distributions](image)

To evaluate these variations further, the blends from Belgium and Korea where also evaluated – as both sites had submitted typical and sticking blends for analysis. From Figure 5.7 it can be seen that the sticking Belgian blend has a cluster of API, which is not visible in the typical blend. In the typical blend, the MCC and DCP clusters also appear larger which is also observed for the Korean blend. In the Korean blends the API and MgS have larger clumps in the blend with typical performance.

Having evaluated blend samples with both typical and sticking performance from four different locations it appeared that the distribution of MCC and DCP was critical to tablet sticking issues. Although other variations were observed, such as changes in the API or MgS distributions, these where site specific and therefore not necessarily directly related to tablet sticking. However, the images presented represent only 8 of the 20 lots examined and the image data of the other lots will not been presented as it requires many images to represent these 13 lots, and visually the images are similar to those already presented. For this reason, and also if NIRM has any role in process control, it is necessary to consider the results in a more numerical format, as previously described in Section 5.2.
The blend particle size of each component was determined for every lot investigated and the values are presented in Figure 5.8. From this figure it is possible to see that the blend particle size of all the components varied with location. It is not unexpected that MCC (Figure 5.8a) has the largest blend particle size, with an average of ~280 μm, as it is the major component and forms the backbone of the tablet matrix. There is no obvious trend in the size of the MCC domains in sticking lots. When the DCP component was examined in the chemical images it was observed to form clumps in the formulation, and from Figure 5.7b it can be seen that the average blend particle size is ~250 μm. It can also be observed that the blends which resulted in tablet sticking had similar blend particle sizes, ~200 μm which were at the lower blend particle size for this component. Although no size trend had been observed in the MCC component when the blend particle size plots of MCC and DCP are compared (Figure 5.8a vs. Figure 5.8b) it can be seen that the size of these two components follows a similar trend, with the exception of the sticking lots and this relationship may be an indicator for tablet sticking.
In terms of the smaller components in the formulation, the API (Figure 5.8c) was found to have an average blend particle size of 72 μm but showed variability between the different sites. Almost half the sites had clusters >100 μm, and the other half had a blend particle size of ~20 μm. At this low level the size of the domains are equivalent to one apertured area, which is therefore the level of detection. When components are
at this size, it implies that the component is finely distributed within the tablet matrix. The variation in sites was due to different sources of the API, and implementation of a milling step of the API prior to production at some sites. When the SSG blend particle size is examined (Figure 5.8d) a variation between lots is observed, average of 60 μm, but there is no obvious relationship to tablet sticking. The average MgS blend particle size (Figure 5.8e) is 79 μm, but only 5 locations have clusters of MgS which are greater than the level of detection. Although the size of the MgS domains do not appear to be directly related to tablet sticking, four of the five locations experiencing sticking showed clusters of MgS. The appearance of clusters of this component is interesting as it is a very fine material which is in the blend to lubricate and would not be expected to agglomerate. The site variation is thought to be due to different suppliers of this raw material, which give different physical properties which are out with the scope of this research.

The blend particle size statistics have shown that the ratio of MCC and DCP may be directly related to tablet sticking, as the size of these two components follow similar trends except in sticking lots. Figure 5.9 presents a ratio of the blend particle size for these two components, and it is possible to observe an explanation for tablet sticking. In a lot which gives rise to good tableting performance, the blend particle size of the MCC and DCP are similar resulting in a ratio of blend particle size of 1. If the size of the DCP becomes reduced relative to that of MCC, then tablet sticking is probable, resulting in ratio values in the order of 0.6 – 0.7.
5.6 UNDERSTANDING MATERIAL PROPERTIES AND THEIR IMPACT ON TABLET STICKING

Both MCC and DCP are present in the formulation as bulking and binding agents but when compressed, the two materials undergo different physical processes. MCC crystals undergo plastic deformation but DCP crystals undergo brittle fracture (Rowe et al, 2003a, b). During the tabletting process, the blend is subjected to the compression force from the punches and dies, and initially all the particles will elastically deform. As the force continues, the elastic limit for the components will be reached. Beyond this limit there is either breakage or flow of the particles, otherwise known as brittle fracture or plastic deformation (Cartensen, 1993). Therefore DCP can be considered like the bricks in a wall, and MCC the cement. In the situation of building a good wall, there must be enough cement around the surface of bricks to hold the wall together. Therefore, when the DCP size is reduced relative to the MCC, (as observed in the sticking matrix), the surface area of the DCP increases but the MCC remains constant, and the MCC cannot cover all surfaces of the DCP which results in a weaker tablet matrix. Therefore, the DCP domains will easily separate when the die is removed after
compression, resulting in adhesion of the DCP to the faces of the die rather than within the tablet matrix.

Investigations using NIRM have therefore found a difference between sticking and typical batches of Norvasc® blends, relating to the size and distribution of the two main components. This investigation has shown that NIRM can be used to confirm theories which could be determined by formulation science, but provide scientific justification and visual representations of solid dosage forms. At the same time, by comparing tablets with typical performance and those experiencing manufacturing issues (tablet sticking), it is possible to gain insight into production issues building process knowledge and understanding. In this investigation the data has been converted into a numerical value (ratio of DCP and MCC) that may be used to control the process, a means by which to predict if tablet sticking was determined. If NIRM was capable of real time measurements of pre-compression blends, at-line to the process, it would be possible from the chemical images to predict the tablettng performance of the blend, in this case by looking at the blend particle size ratio of DCP to MCC. However, current NIRM instruments are laboratory focused and are too lengthy to be used as realistic process control tools but NIRM does have the potential with instrumentation improvements to be a useful process control tool.
6. **Utilising Chemical Images and Relevant Image Statistics to Understand Dissolution Performance**

### 6.1 Tablet Dissolution, A Critical Quality Attribute of Pharmaceutical Solid Dosage Forms

Until recently the pharmaceutical industry was considered risk adverse, with pharmaceutical manufacture being considered more of an art than a science (Dept. Health and Human Services, FDA, 2004). Over the past 2 – 3 years this mindset has been steadily changing with the introduction of the FDA’s Pharmaceutical Current Good Manufacturing Practises (CGMPs) for the 21st Century. This initiative aimed to enhance and modernise the regulation of pharmaceutical manufacturing and product quality. One of the key accomplishments of this initiative was the novel science-based regulation of product quality, such that a scientific framework was seen as a means to mitigate risk and continuously improve product quality. Such an approach opens the door to new technologies (such as Process Analytical Technologies (PAT)) to allow improvements in the efficiency and effectiveness of pharmaceutical manufacturing. As a direct result of the CGMPs initiative a separate guidance document for PAT was produced and issued in September 2004 (Centre for Drug Evaluation and Research (CDER), FDA, 2004). This guidance characterises the desired state of pharmaceutical manufacturing and regulation as a situation where ‘product quality and performance are ensured through the design of effective and efficient manufacturing processes’ and ‘product and process specifications are based on a mechanistic understanding of how formulation and process factors affect product performance’. With such approaches, the quality of a pharmaceutical product is not tested in but instead is either built-in or is by design. These requirements are now being unified across European, Japanese and American regulatory authorities under the International Conference on Harmonisation (ICH) 8 guidelines.
With these changes in place, a number of well established analytical technologies are being challenged around the value of the results obtained and the significance to the critical quality attributes of the finished pharmaceutical product. Currently, dissolution of a solid dosage form is one of the primary tests performed on the end product which holds any significant clinical relevance (CDER, FDA, 1997). Formulators rely on the dissolution characteristics of dosage forms to gain information on the performance of the formulation. Not only is dissolution testing a key performance indicator, but it is also the only finished product test which has any clinical relevance. When a patient takes a solid oral dosage form, the drug absorption is dependent upon a number of different factors including the dissolution or solubilisation of the drug under physiological conditions and the permeability across the gastrointestinal tract. Bioavailability studies are performed to correlate the in-vitro results with what is obtained in the laboratory using dissolution equipment, and these correlations are used in regulatory submissions and control. The equipment and methodology required for dissolution testing is described by the US Pharmacopeia General Chapter <711>. The important attribute from a dissolution test is the quantity, \( Q \), of dissolved active ingredient specified in the product monograph as a percentage of the labelled content. The acceptance limits on the first stage of testing (S1) is that each of the six units tested must not be less than \( Q \pm 5\% \). If any unit falls out with this acceptance criterion then a further six units can be tested (stage two (S2)), with the average of the 12 units being equal to or greater than \( Q \) and each unit no less than \( Q - 15\% \). If the lot still does not meet the acceptance criteria then stage three (S3) testing is performed where a further 12 units are tested, where the average of the 24 units must be equal or greater than \( Q \), with no unit less than \( Q - 25\% \) or not more than 2 units less than \( Q - 15\% \). Therefore dissolution failure would imply a significant change in performance.
Although a correlation exists between the dissolution test and bio-availability the reasons behind a dissolution profile are not generally well understood, with their being no true scientific understanding behind changes in dissolution profile. In fact the dissolution test does not truly allow for understanding of the critical quality attributes of the finished dosage form, i.e. what is the parameter which impacts how the product will perform downstream. If operating under a scientific framework and mechanistic understanding is to be obtained on how a formulation or process factors influence product performance then a conventional dissolution test is perhaps not the most appropriate test. Methodology is required which can be used to show bio-availability, but at the same time allow for true scientific understanding of why a formulation or process change has impacted product performance. One technique which offers this potential is NIR microscopy, where the chemical images produced would allow for understanding of the impact of a process variation or a change in formulation (such as input raw material particle size change) as it allows for visualisation within the solid dosage matrix. If visualisation of these changes can be observed, then it would follow that correlation to finished product performance (such as dissolution) would be possible. This chapter will examine two products (Product X and Product Y, Pfizer Inc.) exhibiting variable dissolution performance and how the matrix revealed by NIR chemical images can be potentially correlated to dissolution and provide an opportunity for performance prediction.

6.2 METHOD
Products
Product X is a five component tablet formulation made by wet granulation. The API, crospovidone and calcium silicate are the three major ingredients and magnesium stearate and silicon dioxide minor components at <1% w/w in the formulation. All ingredients have a unique NIR response and therefore it should be possible to visualise all within the tablet matrix. This product was under evaluation as the dissolution performance was variable, and resulted in lots not reaching the quality alert limit (QAL
The process was altered using a design of experiments to push the process to extremes, although only seven different lots were received for NIR microscopy investigations, Table 6.1. Each lot was tested by the quality operations laboratory for dissolution using the standard operating procedure (SOP) for this product, and the values obtained are shown in Table 6.1.

### Table 6.1 - Lot number and dissolution values for each lot of Product X analysed

<table>
<thead>
<tr>
<th>Lot Number</th>
<th>% dissolution at 45 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>3002102</td>
<td>87</td>
</tr>
<tr>
<td>3015200</td>
<td>86</td>
</tr>
<tr>
<td>03T2000V</td>
<td>82</td>
</tr>
<tr>
<td>3016200</td>
<td>79</td>
</tr>
<tr>
<td>3000200</td>
<td>76</td>
</tr>
<tr>
<td>03T2020</td>
<td>64</td>
</tr>
<tr>
<td>3017200</td>
<td>50</td>
</tr>
</tbody>
</table>

Product Y is a five component formulation produced by wet granulation. The API and lactose monohydrate represent ~75% of the total mass of the formulation. The formulation also contains croscarmellose sodium (5% w/w), pregelatinised starch (14% w/w), povidone (5% w/w) and magnesium stearate (1% w/w). The QAL for dissolution of this product was $Q >85\%$ at 30 minutes. All components have a unique NIR spectral response, and it should be possible to identify each component within the dosage form. This product was manufactured at two different Pfizer locations, but the API was sourced from the same vendor. During production at location one (L1) a tablet lot actually failed S3 dissolution testing and the root cause of this issue was thought to be the increased particle size of the API utilised. However, the API lot in question was also used at the second manufacturing location (L2) with no production issues, and therefore the root cause was questioned. Table 6.2 provides the lot numbers for each of the ten tablet lots received along with the dissolution performance (as tested by the quality operations laboratory using the SOP for this product) and the associated API lot numbers. From this table it can be seen that lot 0386K03 manufactured at location L1
was the problem lot, failing S3 dissolution testing but this lot utilised the same API lots as lot 310268704 from location L2.

Table 6.2 - Lot number and dissolution values for each lot of Product Y analysed

<table>
<thead>
<tr>
<th>Lot Number</th>
<th>Location of Manufacture</th>
<th>Dissolution Performance (average % at 30 minutes)</th>
<th>API Lot Number(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1159K01</td>
<td>L1</td>
<td>Pass S1 (101%)</td>
<td>13680417</td>
</tr>
<tr>
<td>1160K01</td>
<td>L1</td>
<td>Pass S1 (93%)</td>
<td>13680417</td>
</tr>
<tr>
<td>0765K02</td>
<td>L1</td>
<td>Pass S1 (91%)</td>
<td>23680420</td>
</tr>
<tr>
<td>0154K03</td>
<td>L1</td>
<td>Pass S2 (89%)</td>
<td>23680530 + 23680491</td>
</tr>
<tr>
<td>166K03</td>
<td>L1</td>
<td>Just Failed S1 / Pass S2 (97%)</td>
<td>33680143 + 23680530</td>
</tr>
<tr>
<td>167K03</td>
<td>L1</td>
<td>Pass S1 (96%)</td>
<td>33680143</td>
</tr>
<tr>
<td>0386K03</td>
<td>L1</td>
<td>Failed S3 (82%)</td>
<td>33680215 + 33680143</td>
</tr>
<tr>
<td>210315602</td>
<td>L2</td>
<td>Pass S1</td>
<td>23680420</td>
</tr>
<tr>
<td>310268704</td>
<td>L2</td>
<td>Pass S1</td>
<td>33680215 + 33680143</td>
</tr>
<tr>
<td>310315302</td>
<td>L2</td>
<td>Pass S1</td>
<td>33680250 + 33680215</td>
</tr>
</tbody>
</table>

Sample Preparation

For both products the tablets were prepared for analysis, by milling of the tablet using a Leica EM Trim. Milling allows a flat surface to be obtained and also exposes the core of the film coated tablets. For each lot of tablets the cross-section of five different tablets were examined, which allowed assessment of the significance of the sample variations. A wafer of each ingredient was also prepared using 300 mg of powder (which was compacted at 100 p.s.i. pressure into a 1 cm disc, which was ~3 mm thick) to allow for a spectral reference library to be established.

Instrumentation

The Spotlight FT-NIR microscope (Figure 1.6b) was utilised throughout this investigation to analyse both the raw materials and the cross-sectioned tablets. The spectrometer was set to collect spectral data over the range 3800 – 7600 cm⁻¹ with 8 cm⁻¹ data interval and an average of 4 scans per pixel. The spectra were output as relative reflectance (R), based on the reference of a gold mirror. To enable image data to be obtained, the sample was moved with steps of 25 µm in the x and 400 µm in the y direction, with a line of 16 pixels being acquired in each step (line imaging) i.e.
each spectrum was obtained from a 25 x 25 μm area. For each tablet sample, an area of 3.2 mm² was examined, although the x and y dimensions varied. For each pure ingredient wafer, an area of only 1 mm² was examined, allowing for 1600 spectra of each material to be included in the library.

**Software and Data Processing**

After data collection, the resultant unprocessed data files were translated into a format (.spf) readable by image analysis software (ISYS, Spectral Dimensions, MD). The spectra were firstly transformed into absorbance units, as previously described in Equation 1.11 and then normalised using SNV to remove any effects from scatter. For Product Y it was not necessary to perform any further pre-processing as the spectra all had unique signatures (previously presented in Figure 2.3). However a first derivative had to be employed for Product X due to the presence of an inorganic material in a high percentage. The calcium silicate has no strong absorptions and the slope in the spectrum (which is a function of the increasing scatter with longer wavelengths), is the discriminating spectral feature in any processing regime. However, as this slope was also present in all materials it makes classification of this material challenging using only normalised spectra. From Figure 6.1 it can be seen that the first derivative pre-treatment (Savitsky-Golay filter using 13 point gap, with 2nd order polynomial) used for the three major ingredients in Product X removes the slope and increases the resolution between the three components, allowing for identification in the tablet matrix.

![Figure 6.1](image.png)

*Figure 6.1– a) Raw absorbance and b) first derivative spectra of major components from Product X*
Identification of each raw material and API in the tablet samples was possible by correlating the tablet spectral data to a spectra library for the pure ingredients, using PLS methodology. Individual libraries were prepared for each tablet type, with only the raw materials used in the blend being incorporated into the library data set. Each tablet data set was then correlated to the respective library using PLS 2 methodology, as previously described in Section 2.7. For each tablet examined a chemical image of each component was generated, using the same scaling for each component independent of lot number. Images produced by this method can be translated into numerical information which allows for a fair comparison between images.

Numerical information can be generated from a chemical image by use of a contour tool. A contour level is set for each component based on a unique score value for each component e.g. 0.225 as shown in Figure 6.2a. Any pixel with a value less than this defined number will be given a zero value (black pixel), and any pixel with a value greater or equal to this number will be given a value of one (white pixel), giving rise to a binary image (shown in Figure 6.2b). Using this binary image, pixels with a value of one which are located next to each other will be captured by a single contour, such that it is possible to measure the size of these pixel domains. For each component it is possible to obtain statistics on an individual domain (Figure 6.2c represents statistics from the domain enclosed by the red box in Figure 6.2b) or a domain size distribution can be obtained, Figure 6.2d (based on the entire image in Figure 6.2b). As information is generated on a domain by domain basis, it is also possible to simply look at the total number of domains for each component, which can be used along with domain size to describe the distribution of components. Obviously the number of domains will be dependent upon image size, but as all images in this chapter are from identical size areas only the number of domains will be presented.
By generating numerical information on the chemical images, as well as pictorially describing the distribution of ingredients within the final blend, it is possible to discriminate samples on a statistical basis, although to the naked eye these differences are not always obvious. The statistics calculated by the software are able to consistently discriminate the samples in terms of product matrix.

6.3 MATRIX EVALUATION OF PRODUCT X FAILING QUALITY ALERT LIMIT FOR DISSOLUTION

To establish the matrix distribution of components within the blend samples, each image set was processed using PLS to generate chemical images based on score intensities for each component. Evaluations of magnesium stearate and silicon dioxide were not performed as their low concentrations coupled with the fact that both had particle sizes that were smaller than the spatial resolution, meant that it was not possible to identify them within the tablet matrix and as such have been excluded from further investigations. From the pixel score distributions, it was decided to use a value of 0.6 to contour the API domains, 0.23 to contour the crospovidone domains and 0.44
to contour the calcium silicate component. These values were based on both the concentration of each component in the formulation and the spectral response, an example is shown in Figure 6.3. From Figure 6.3a it can be seen that the limit for both crospovidone and calcium silicate is located at a similar position on the tail of the distribution, and is a result of these components being present at almost equal levels, 16% and 15% w/w respectively. The API score limit is located to the left hand side of the peak maximum due to the higher concentration of this component, ~60%. The exact positioning of these limits is made by assessing the spectral features at each score value, and it can be seen from Figure 6.3b, c and d that the limits selected result in pixels above the score limits having different spectral features to those with lower values. Figure 6.3e shows an example of how the individual images overlay each other, almost like completing a jigsaw puzzle to generate a description of the distribution of components in the tablet matrix – it should be noted that this image was generated using only binary images based on score limits and describes each components distribution in a defined manner.

Figure 6.4 presents an example image of the three major components within four different tablet lots – selected to span the entire dissolution range observed. In Figure 6.4 the chemical images have been presented with decreasing dissolution (with (d) having the lowest dissolution performance). From these images it can be observed that the number of calcium silicate domains appears to decrease with the same trend as dissolution, as many more calcium silicate are visible in Figure 6.4a and b. However there does not appear to an obvious variation in the crospovidone or API distributions. To allow the variations in the chemical images to be further explored, and understand if variation is significant, the statistics of each component were calculated, as described by Figure 6.2 using the values determined from the PLS histograms.
Figure 6.3 - Evaluation of tablet matrix from lot 3015200 of Product X where a) represents PLS score distributions and image limits for crospovidone, API and calcium silicate, b - d) representing the respective image and spectral features related to above and below this limits and e) combined RGB of binary images.
It can be seen from Table 6.3 that lot 3017200 has a significantly larger API domain diameter than any of the other five lots, as observed by the more solid API domains in Figure 6.4. The average value for the other six lots is $314 \pm 64 \mu m$ compared with $648 \pm 150 \mu m$ for lot 3017200; therefore the API has more than doubled in size in this very slow dissolving lot. With this increase in size it is not surprising that the number of API domains is significantly less in lot 3017200, $41 \pm 14$ domains compared with an average of $123 \pm 27$ domains for the other six lots. Therefore, it can be concluded that the API size and distribution is a factor in the dissolution of this product, with increased domain size (less domains) resulting in slow dissolution. However, it is not the sole factor as no trend in either the size or number of API domains is observed in the other six lots examined.
Table 6.3 - Size and image statistics of API component for six lots of Product X

<table>
<thead>
<tr>
<th>Lot No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Average ± STD</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Average ± STD</th>
</tr>
</thead>
<tbody>
<tr>
<td>3002102</td>
<td>452</td>
<td>450</td>
<td>415</td>
<td>297</td>
<td>260</td>
<td>375 ± 90</td>
<td>109</td>
<td>105</td>
<td>107</td>
<td>94</td>
<td>130</td>
<td>109 ± 13</td>
</tr>
<tr>
<td>3015200</td>
<td>307</td>
<td>317</td>
<td>325</td>
<td>280</td>
<td>380</td>
<td>322 ± 37</td>
<td>126</td>
<td>119</td>
<td>107</td>
<td>128</td>
<td>88</td>
<td>114 ± 16</td>
</tr>
<tr>
<td>03T2000</td>
<td>322</td>
<td>290</td>
<td>387</td>
<td>272</td>
<td>260</td>
<td>306 ± 51</td>
<td>123</td>
<td>141</td>
<td>85</td>
<td>149</td>
<td>166</td>
<td>133 ± 31</td>
</tr>
<tr>
<td>3016200</td>
<td>412</td>
<td>320</td>
<td>248</td>
<td>402</td>
<td>275</td>
<td>331 ± 73</td>
<td>72</td>
<td>120</td>
<td>153</td>
<td>79</td>
<td>129</td>
<td>111 ± 34</td>
</tr>
<tr>
<td>3000200</td>
<td>312</td>
<td>337</td>
<td>322</td>
<td>212</td>
<td>235</td>
<td>284 ± 56</td>
<td>115</td>
<td>108</td>
<td>113</td>
<td>165</td>
<td>190</td>
<td>138 ± 37</td>
</tr>
<tr>
<td>03T2020</td>
<td>248</td>
<td>255</td>
<td>280</td>
<td>305</td>
<td>258</td>
<td>269 ± 23</td>
<td>148</td>
<td>144</td>
<td>120</td>
<td>116</td>
<td>144</td>
<td>134 ± 15</td>
</tr>
<tr>
<td>3017200</td>
<td>632</td>
<td>508</td>
<td>532</td>
<td>885</td>
<td>680</td>
<td>648 ± 150</td>
<td>41</td>
<td>52</td>
<td>56</td>
<td>21</td>
<td>34</td>
<td>41 ± 14</td>
</tr>
</tbody>
</table>

When the statistics for the crospovidone component were examined, Table 6.4, it can be observed that this component shows a similar size and number of domains in all tablet lots. The average size of this component in all lots was 227 ± 28 µm and all lots were within 10% of this. For the number of domains, the average for all lots was 125 ± 20 domains with all lots showing no more than 16% deviation from this mean. In both cases there is some variation but there is no trend with regard to dissolution and it can be concluded that this component does not impact on the dissolution performance of this product.

Table 6.4 - Size and image statistics of crospovidone component for six lots of Product X

<table>
<thead>
<tr>
<th>Lot No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Average ± STD</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Average ± STD</th>
</tr>
</thead>
<tbody>
<tr>
<td>3002102</td>
<td>285</td>
<td>243</td>
<td>252</td>
<td>245</td>
<td>225</td>
<td>250 ± 22</td>
<td>85</td>
<td>92</td>
<td>95</td>
<td>125</td>
<td>123</td>
<td>104 ± 19</td>
</tr>
<tr>
<td>3015200</td>
<td>215</td>
<td>190</td>
<td>218</td>
<td>218</td>
<td>183</td>
<td>205 ± 17</td>
<td>140</td>
<td>145</td>
<td>137</td>
<td>132</td>
<td>146</td>
<td>140 ± 6</td>
</tr>
<tr>
<td>03T2000</td>
<td>212</td>
<td>220</td>
<td>218</td>
<td>247</td>
<td>230</td>
<td>225 ± 14</td>
<td>133</td>
<td>153</td>
<td>121</td>
<td>116</td>
<td>153</td>
<td>135 ± 17</td>
</tr>
<tr>
<td>3016200</td>
<td>190</td>
<td>228</td>
<td>228</td>
<td>183</td>
<td>202</td>
<td>206 ± 21</td>
<td>125</td>
<td>134</td>
<td>134</td>
<td>142</td>
<td>146</td>
<td>136 ± 8</td>
</tr>
<tr>
<td>3000200</td>
<td>265</td>
<td>250</td>
<td>218</td>
<td>282</td>
<td>212</td>
<td>245 ± 30</td>
<td>103</td>
<td>114</td>
<td>129</td>
<td>95</td>
<td>148</td>
<td>118 ± 21</td>
</tr>
<tr>
<td>03T2020</td>
<td>278</td>
<td>258</td>
<td>260</td>
<td>200</td>
<td>208</td>
<td>241 ± 35</td>
<td>99</td>
<td>98</td>
<td>105</td>
<td>154</td>
<td>157</td>
<td>123 ± 30</td>
</tr>
<tr>
<td>3017200</td>
<td>222</td>
<td>247</td>
<td>222</td>
<td>190</td>
<td>190</td>
<td>214 ± 24</td>
<td>113</td>
<td>103</td>
<td>129</td>
<td>118</td>
<td>131</td>
<td>119 ± 12</td>
</tr>
</tbody>
</table>

It was observed from the actual chemical images that there could be a potential correlation between the number of calcium silicate domains and the dissolution performance. Table 6.5 presents the image statistics for this component within each tablet lot. From this table it can be seen that size of this component is variable at the
extremes of dissolution performance, with the lot with 87% dissolution having a mean
domain diameter of 105 µm and the lot with 50% dissolution showing a mean domain
diameter of 90 µm compared to an average of 55 µm for the other five lots. The reason
for this size increase at the extremes of dissolution is unknown, but as no trend exists
between sizes and decreasing dissolution it is not thought that the size of this
component is critical to the dissolution performance of this product. However, the
same cannot be said for the number of calcium silicate domains where a steady
decrease in the number of domains is observed as the dissolution performance
decreases. It is thought that the reason that size and distribution of this component are
not directly related is due to the granulation/binding of the calcium silicate where
improved granulation masks the number of calcium silicate domains. From the
statistics, it would appear that granulation does not impact the size of this component.
However it does change the number of domains, with this component being
incorporated into granules which results in the variation in number of domains.

Table 6.5 - Size and image statistics of calcium silicate component for six lots of
Product X

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Average ± STD</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Average ± STD</th>
</tr>
</thead>
<tbody>
<tr>
<td>3002102</td>
<td>85</td>
<td>132</td>
<td>100</td>
<td>93</td>
<td>117</td>
<td>106 ± 19</td>
<td>572</td>
<td>666</td>
<td>464</td>
<td>528</td>
<td>692</td>
</tr>
<tr>
<td>3015200</td>
<td>52</td>
<td>55</td>
<td>55</td>
<td>45</td>
<td>47</td>
<td>50 ± 5</td>
<td>605</td>
<td>579</td>
<td>395</td>
<td>559</td>
<td>518</td>
</tr>
<tr>
<td>03T2000</td>
<td>60</td>
<td>62</td>
<td>57</td>
<td>57</td>
<td>57</td>
<td>59 ± 2</td>
<td>375</td>
<td>383</td>
<td>317</td>
<td>512</td>
<td>425</td>
</tr>
<tr>
<td>3016200</td>
<td>50</td>
<td>60</td>
<td>72</td>
<td>67</td>
<td>52</td>
<td>60 ± 9</td>
<td>285</td>
<td>432</td>
<td>242</td>
<td>269</td>
<td>350</td>
</tr>
<tr>
<td>3000200</td>
<td>47</td>
<td>52</td>
<td>50</td>
<td>55</td>
<td>45</td>
<td>50 ± 4</td>
<td>376</td>
<td>214</td>
<td>227</td>
<td>223</td>
<td>292</td>
</tr>
<tr>
<td>03T2020</td>
<td>53</td>
<td>55</td>
<td>58</td>
<td>63</td>
<td>53</td>
<td>56 ± 4</td>
<td>108</td>
<td>135</td>
<td>79</td>
<td>114</td>
<td>118</td>
</tr>
<tr>
<td>3017200</td>
<td>72</td>
<td>100</td>
<td>70</td>
<td>112</td>
<td>95</td>
<td>90 ± 18</td>
<td>59</td>
<td>14</td>
<td>43</td>
<td>19</td>
<td>17</td>
</tr>
</tbody>
</table>

The average number of calcium silicate domains for each lot can be plotted against the
dissolution value at 45 minutes, presented in Figure 6.5, and it can be observed that an
exponential relationship exists. Obviously the exponential relationship can be
transformed into a linear relationship by calculating the logarithm of the number of
calcium silicate domains, which gives rise to a linear relationship with regression coefficient of 0.9959.

\[ y = 0.6425e^{0.0786x} \]

\[ dv = 29.2cs - 5.91 \] (6.1)

To understand if this model could be used for prediction of dissolution a further three lots were requested with the dissolution properties unknown. The three lots received were 03T2034, 03T2018 and 03T2080. The data files were processed using the same treatments as the previous lots examined. It was found that the image statistics for the API component for each lot fell within the criteria of the previously established model.
The API domain size was 315 ± 45 µm for lot 03T2034, 344 ± 32 µm for lot 03T2018 and 329 ± 68 µm for lot 03T2080 and the number of domains were 130 ± 18 domains, 113 ± 30 domains and 125 ± 25 domains respectively. It was thought that dissolution of these lots could be predicted using the number of calcium silicate domains, presented in Table 6.6.

**Table 6.6 - Size and image statistics of calcium silicate component within three supplementary lots of Product X**

<table>
<thead>
<tr>
<th></th>
<th>Mean Domain Diameter (µm)</th>
<th>Number of Domains</th>
<th>Average ± STD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>03T2080</td>
<td>50</td>
<td>55</td>
<td>48</td>
</tr>
<tr>
<td>03T2018</td>
<td>60</td>
<td>55</td>
<td>53</td>
</tr>
<tr>
<td>03T2034</td>
<td>60</td>
<td>67</td>
<td>67</td>
</tr>
</tbody>
</table>

Using Equation 6.1 and the number of calcium silicate domains from Table 6.6, the average predicted dissolution values for the three lots with unknown dissolution performance were calculated. Table 6.7 presents the average predicted dissolution values (determined from the average number of calcium silicate domains for the five tablets per lot examined) along with the predicted range of dissolution values (based on the standard deviation of the average number of calcium silicate domains from the five tablets).

After the prediction was performed the manufacturing location was contacted to obtain the true dissolution results, which are presented in Table 6.7. The predicted values were all within 1% of the actual dissolution values for each lot. It can be concluded that using the number of calcium silicate domains is a good method for the prediction of dissolution performance for Product X. The benefit of such a method would be that it allows for prediction of blend performance rather than finished goods testing. At the blend stage in a process it is possible to make process changes, which could result in a batch going forward and passing the dissolution specification or stopping a blend that would not make satisfactory tablets for further processing.
Table 6.7 - Predicted and actual dissolution values for the three supplementary lots of Product X

<table>
<thead>
<tr>
<th></th>
<th>Average Predicted Dissolution (%)</th>
<th>Predicted Dissolution Range (%)</th>
<th>Actual Dissolution Measured (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>03T2080</td>
<td>81.3</td>
<td>80.0 – 82.5</td>
<td>80</td>
</tr>
<tr>
<td>03T2018</td>
<td>77.2</td>
<td>75.9 – 78.4</td>
<td>77</td>
</tr>
<tr>
<td>03T2034</td>
<td>88.1</td>
<td>87.4 – 88.7</td>
<td>89</td>
</tr>
</tbody>
</table>

This work has not only shown a means in which to predict dissolution but has also provided mechanistic understanding of the dissolution of this product. The number of calcium silicate domains is critical to the dissolution performance of this product, with more domains resulting in improved dissolution. The function of calcium silicate in this formulation is to preferentially absorb the water over the API. Therefore with less domains of calcium silicate less water can be absorbed by the excipients and instead goes to the API, which results in reduced dissolution performance, due to gelling of the API.

6.4 Matrix Evaluation of Product Y Failing Stage Three Dissolution Specifications

With Product Y being manufactured at two separate locations and a potential assignable root cause of the input API particle size it was decided to firstly examine only the API component. Utilising the unique API response at 6072 cm⁻¹ (determined from Figure 2.3) it was possible to visualise the API distribution within the tablet matrix. Figure 6.6 shows an example image from both locations with S1 performance and also an example of an S2 passing and S3 failing lot from location, L1. It can be seen from this figure that the API domain size, in lots from L1, increase in going from S1 to S2 performance (Figure 6.6a to Figure 6.6b), and also a further increase is observed in the S3 failing lot (Figure 6.6c). It could therefore be thought that the API domain size is critical to satisfactory dissolution of this product. However, Figure 6.6d presents the
distribution of the API in a S1 passing lot from L2, and it can be seen that the API
domain size in this lot is similar to the S3 failing lot from L1.

The size of these domains can be measured using image contours. A normalised
absorbance value of -0.5 was used for contouring, with pixels greater than this value
being counted in the image statistics. The resultant average domain statistics for each
lot showed lot 0765K02 to have the smallest domains (105 µm), then lot 0154K03 with
a domain size of 130 µm. Lot 0386K03 showed domains in the order of 175 µm which
was comparable to the size observed for lot 310315302 (180 µm). Therefore it can be
concluded that the API domain size is not the sole contributing factor to variable
dissolution of this product, and as such PLS methodology was utilised to further
examine the excipients distributions within these tablet matrices.

Figure 6.6 – Distribution of API component within Product Y tablet matrix where a) represents lot
0765K02 (passed S1 from L1), b) lot 0154K03 (passed S2 from L1), c) lot 0386K03 (failed S3 at L1)
and d) 310315302 (passed S1 from L2).

Figure 6.7 shows example chemical images for each component within the Product Y
tablet matrix from each of the four different types of lots, three from L1 with different
dissolution performance (passing S1 & S2 and failing S3) and one from L2 with good
dissolution (passing S1). The rows in Figure 6.7 represent different components within
the formulation, with i) representing the distribution of the API (red), lactose (green) and
croscarmellose sodium (blue) and ii) representing pregelatinised starch (blue),
povidone (green) and magnesium stearate (red). It can be seen from Figure 6.7 that
with the exception of the povidone component, the tablet matrix fits together like a jigsaw puzzle. The povidone component on the other hand appears to be mixed in with different components, which is not unexpected as this component is the binder added during the granulation process. Therefore the chemical images confirm that the binder is mixed in with other components, but it is interesting to observe larger solid domains of this component which may be a result of the depth as well as surface response from this component.

![Image of chemical images](image)

**Figure 6.7** – Chemical images of the component distributions in Product Y tablet matrix where row i) represents the API (red), lactose (green) and croscarmellose sodium (blue) and row ii) pregelatinised starch (blue), povidone (green) and magnesium stearate (red) and the lots are presented in the columns labelled a) 0765K02, b) 0154K03, c) 0386K03 and d) 310315302

Examination of the chemical images show no obvious variation between the different lots which indicates a reason for the change in dissolution performance. As such it was decided to explore the image statistics using the contour tool. To determine the contour level, the score distributions from each component were examined, Figure 6.8.

It can be seen from this figure that there is a trend in the position of the score distributions which is linked to concentration of each component within the tablet matrix and only the pregelatinised starch is over predicted (which is behaviour observed in Chapter 4 with corn starch). From these score plots it was decided to use a value of
0.42 for lactose domains, 0.33 for API domains and 0.36 for pregelatinised starch based on concentration and spectral features. For the croscarmellose sodium a value of 0.16 was utilised, 0.08 for povidone and a value of 0.05 for the magnesium stearate component. In the case of both the croscarmellose sodium and the magnesium stearate components their distributions showed the material to be sized in the order of 1 pixel (25 μm), therefore detection is more on a number of domains basis than an accurate size assessment.

To evaluate the variation in the size of the component domains in the tablet matrix the average mean domain diameter, from all five tablets per lot, were plotted relative to lot number, Figure 6.9. From this figure it can be seen that there is no significant change in the pregelatinised starch, croscarmellose sodium or magnesium stearate across all lots examined. It should be noted that the variation in these lots would be expected to be lower than the other components due to the size being close to the resolution of the system, with each component occupying on average only 1 – 3 pixels (25 – 75 μm). When the sizes of the API and povidone components are examined, it can be observed that these two materials are similar in size with the exception of lot 0386K03, which failed S3 dissolution testing. Unlike the other lots the API domains are almost double the size of the povidone domains in lot 0386K03, suggesting that povidone and API distributions are critical to the dissolution properties of these tablets. When the lactose component was examined it was observed that there were smaller domains in lot
0386K03, but that lot 0154K03 also had a similar domain size. It should be noted that
this lot (0154K03) passed S2 dissolution testing but lot 0166K03 which also passed S2
dissolution testing had a lactose domain size more similar to the S1 passing lots. This
suggests that the variability in lactose is not directly correlated to dissolution properties,
but this component may cause some of the variability.

![Graph showing mean domain diameter for each component in 10 lots of Product Y tablet matrix with variable dissolution.](image)

Figure 6.9 - Mean domain diameter for each component in 10 lots of Product Y tablet matrix with variable dissolution

To further assess the impact of component distributions on dissolution profiles the
number of component domains was also examined, with the average value for each lot
and the standard deviation of all five tablets examined plotted with respect to lot
number, shown in Figure 6.10. As all components had a significant difference in the
number of domains it was decided to plot each component individually to allow
potential correlation to dissolution performance to be examined. From Figure 6.10c, e
and f it can be observed that there is no significant variation in either the number of
pregelatinised starch, croscarmellose sodium or magnesium stearate domains. Using
this information along with the domain size information previously observed, it can be
concluded that the distribution of any of these three components is not critical to the
dissolution performance of this product. The same cannot be said for the lactose, API and povidone domains which show variation which could be related to dissolution performance.

![Figure 6.10](image)

Figure 6.10 - Number of domains for a) lactose, b) API, c) pregelatinised starch, d) povidone, e) croscarmellose sodium and f) magnesium stearate in 10 lots of Product Y tablet matrix with variable dissolution

From Figure 6.10a it can be observed that three lots have a higher number of lactose domains than the other seven lots. These lots have been identified as 0154K03, 0167K03 and 0386K03, as these three lots underwent different levels of dissolution
testing (S2, S1 and S3 respectively) it can be concluded that the variation is not directly linked to dissolution performance. When the API component is examined, Figure 6.10b, it can be seen that lot 0167K03 and 0386K03 show variation from the other five L1 samples. It is interesting that lot 0167K03 which passed S1 dissolution testing has a greater number of domains than any other lot examined. Lot 0386K03, although it has fewer domains than all other L1 lots, has a number of domains equivalent to that observed from samples from L2. Therefore, as observed for the lactose component, a direct correlation to dissolution is not observed. A slightly different situation is observed when the povidone component is examined (Figure 6.10d) as only the lots going beyond stage one testing show a variation from the other seven lots examined. However, the lots passing S2 testing have less povidone domains but the lot failing S3 testing has the highest number of domains compared to the average for the S1 lots. Therefore, it again has not a direct correlation with dissolution performance.

From the number of povidone, API and lactose domains it can be concluded that there is variation in these components which could relate to dissolution performance but this variation is dependent upon all three components. As such the number of component domains for each of these three materials was plotted to assess whether any dissolution performance groupings could be observed. Figure 6.11 presents this three dimensional plot and it can be observed that in this three dimensional space it is possible to group the lots based on dissolution performance. Lots going to S2 dissolution testing have significantly less povidone domains than S1 passing lots, and for the lot which failed S3 dissolution testing it can be observed that this lot has more povidone domains and different lactose to API domain ratio and hence why it forms a very separate group in the 3D plot.
In an attempt to provide a means to predict dissolution performance, principal components analysis (PCA) was employed to reduce the data into a two dimensional system. The number of domains for lactose, povidone and API were analysed using PCA with five factors. The resultant scores obtained for the first principal component allowed for lot 0386K03 to be resolved, but plotting the second and third components together showed a grouping of lots based on dissolution performance, shown in Figure 6.12.

It can be observed from Figure 6.12 that the grouping of lots is based only on dissolution performance, and is independent upon manufacturing location. Obviously this plot is based on the variation within this 10 lot data set, but it could be possible to analyse further lots of this product and find similar groupings due to the changes in povidone, lactose and API distributions. Unfortunately further lots were not available to test this method for dissolution performance prediction.
Figure 6.12 - Plot of score 2 versus score 3 for principal component analysis of the number of povidone, lactose and API domains in Product Y tablets with variable dissolution

It can be concluded from this investigation that the input particle size of the API is important for successful dissolution but it is the size and distribution of this component relative to the lactose and povidone domains which is actually critical to the dissolution performance. At location L2 the size and distribution of these three components was consistent and resulted in consistent dissolution, which always passes stage one dissolution testing. However, from the chemical images it would appear at location L1 that the size and distribution of the three components varies from lot to lot, which is a direct result of the granulation performance. This results in variable dissolution and the need for more than stage one dissolution testing, with the granulation quality impacting how the dissolution of this product occurs, due to water interaction with the matrix.

6.5 USING NEAR-INFRARED CHEMICAL IMAGES FOR THE PREDICTION OF DISSOLUTION PERFORMANCE

The two investigations presented within this chapter have shown the possibility of utilising NIR chemical images as a means to build mechanistic understanding of the dissolution performance of solid dosage forms. In both cases it has only been possible to ascertain this knowledge by examination of a sample set with a range of dissolution attributes, but with this data set it has been possible to establish a model which could
be utilised for further lots of these products to understand the dissolution performance. For Product X it was apparent from the NIR chemical images that the number of calcium silicate domains is critical to the performance of this product, but is subject to the API also having a particular size and distribution. The model established was able to predict the dissolution performance of three subsequent lots, and it would appear to be an accurate method for the prediction of dissolution performance. For Product Y the NIR chemical images showed that the API particle size input played a role in dissolution of this product, but also the distribution and size of the main ingredient and the granulating agent were critical to the dissolution performance of this product. For this product a clear predictive relationship between the number of domains of each of these three components and the dissolution performance was not possible, but utilisation of principal component analysis did allow the tablet lots to be classified based on the stage of dissolution testing required. Unfortunately no supplementary lots of this product were available to test the model established, but the investigation did show that the equipment used at two different manufacturing locations gives rise to a difference in the variation of granulation performance and that more benefit would be gained by a change in granulation equipment rather than tighter control of the input API particle size.

For the models established to be improved it would be necessary to perform dissolution on a dosage form firstly analysed by NIR microscopy, which would allow direct correlation to be determined. This may be more beneficial that using the average response from an entire lot of tablets, and could be an area for further work. However, this initial investigation into correlating chemical images to dissolution performance has shown potential for this methodology. Not only can chemical images show a direct relationship to dissolution performance, but also provide the opportunity to understand the reason for performance variation and allow understanding of the mechanism for dissolution.
7. **Chemical Image Fusion**

7.1 **Introduction**

Chemical Image Fusion (CIF) is the synergy of FT-NIR and Raman mapping microscopy methods as a means to enable a more complete visualisation of pharmaceutical formulations. In Chapters 4-6 the application of NIR microscopy to generate chemical images of pharmaceutical dosage forms has been shown. The technique provides the opportunity to visualise the majority of ingredients within solid dosage forms, over a 25 mm\(^2\) area, with a typical spectrum being collected from a 625 \(\mu\)m\(^2\) area. Spectroscopically, NIR can resolve the majority of API and excipients commonly used in pharmaceuticals. However, not all materials give a strong NIR response, with the lack of H-bonding in the molecules, e.g. dibasic calcium phosphate. If such components are present in the dosage form, it is often possible to identify regions where the NIR response is low and associate this with the NIR inactive material. However, this approach only works if there are high concentrations and only one NIR inactive component present. Therefore, for some dosage forms, NIR microscopy may not allow for complete matrix visualisation, which could be an issue when troubleshooting. Like NIR microscopy, Raman microscopy is also utilised in the pharmaceutical industry for the analysis of solid dosage forms (Brody, 2003 and Šašić, 2006). The most widely explored applications have been in the identification of polymorph conversion and impurities within solid dosage forms. Raman spectroscopy is typically ideal for drug molecules and inorganic salts, due to the strong spectra obtained from the symmetrical bonds; however it would struggle to resolve similar cellulose derived materials. Therefore Raman microscopy alone does not provide a complete solution for understanding solid dosage forms.

There would be a business benefit in being able to identify and visualise every ingredient within a pharmaceutical formulation to build complete product and process knowledge, especially when investigating the effects of changes in the defined
formulation matrix, manufacturing process and / or sources of raw materials. A single spectroscopic technique would not be capable of providing such information for all dosage forms, due to the wide range of formulation materials used (cellulose derived to complex drug molecules). Therefore limitations in individual spectroscopies have driven a desire to combine two complementary spectroscopies, such as Raman and Mid-Infrared (MIR) or Raman and NIR to provide a method for visualizing the composition of an entire pharmaceutical formulation and the distribution of each component.

MIR and Raman spectroscopy are complementary techniques with the functional groups that give strong MIR bands typically being weak in the Raman spectrum and vice versa (Katon et al 1986; Lewis et al, 1994b). This is because not all molecular vibrations give rise to absorption bands in the infrared (either MIR or NIR) spectrum, as this requires a change in a dipole moment. In diatomic molecules, e.g. O₂ and molecules with a centre of symmetry, the symmetric vibrations result in no vibrating electric dipole. This means that electromagnetic radiation cannot be absorbed, and hence the molecules are infrared in-active. To detect symmetrical vibrations, and other vibrations which do not generate a vibrating electric dipole, it is necessary to employ the inelastic scattering of light (Raman scattering). With Raman excitation, the wavelength of excitation is scattered into components at altered wavelengths with the energy related to the vibrational or rotational energy levels of the molecule. The scattering of light is associated with the induced electric dipole, which polarizes the molecule producing electromagnetic radiation. (Keresztury, 2002).

Raman and MIR are also complementary in terms of mapping, with MIR allowing higher sensitivity than Raman methods particularly for pharmaceutical excipients. MIR mapping is limited in lateral spatial resolution to ca. 10 μm, whereas Raman can achieve lateral spatial resolution in the order of 1 – 2 μm. In 1998, Sostek et al filed for a patent to cover their work on combining MIR and Raman microscopy. In this work
they discussed the approach of analysing a sample on one instrument and then moving it to the second instrument with the corresponding spectral information from the two instruments being analysed. Discussions then moved on to cover an instrument that performed both spectroscopic measurements without the need to move the sample, an instrument that was commercially produced. This form of instrumentation is beneficial as it provides a microscopic method for collecting data from two complementary techniques, but it was primarily designed for single point measurements. This technology has not been embraced by the scientific community for problem solving so, where complementary spectroscopic data are required, the practical alternative is still to use two different instruments.

Despite the complementary nature of IR and Raman spectroscopes, combination of the two is not thought of as an ideal approach for analysis of pharmaceuticals. This is due to the practical considerations when trying to bring the two techniques together. The majority of pharmaceutical samples studied are tablets, capsules or compressed powder blend and the poor reflectance performance of MIR microscopes makes measurements from these samples difficult. Therefore, MIR and Raman CIF is not an option.

As NIR spectroscopy involves the overtone and combination absorptions of the fundamental MIR absorptions, it follows that Raman and NIR spectroscopy must also be complementary. Minimal sample preparation is required by either technique, with most analyses possible directly on the compressed powder blend, or on cross-sectioned tablets (cut to provide a flat analysis area). There is a benefit of distinguishing inorganics and organics, with the inorganic species having strong Raman signals but in the NIR spectrum they are weak, or more typically have no NIR absorptions. This is true of dibasic calcium phosphate, an inorganic species typically used as a binding material in tablet formulations. In contrast, Raman spectroscopy struggles with separation of similar carbohydrate species used in pharmaceutical
formulations but there are clear NIR spectral differences. A specific example of this is in differentiating the diluent, such as microcrystalline cellulose, from the ingredient used for disintegration, commonly sodium starch glycolate. NIR spectroscopy also provides a means for identification of water, which in the MIR saturates the spectra, whereas in Raman spectroscopy water is typically a very weak scatterer and is difficult to detect. NIR spectroscopy is therefore useful for the detection of moisture or for identifying varying hydration states. Raman spectroscopy is spectrally richer than NIR spectroscopy for APIs, hence Raman spectroscopy is often more useful for the identification of different polymorphic forms. Hence, if the two techniques could be combined then almost all pharmaceutical ingredients would be detected.

Unlike the work performed by Sostek et al. 1998, no instrumentation is currently available which allows for concurrent analysis of samples by both Raman and NIR microscopy. This chapter will look at a “virtual instrument” which has been developed, where exactly the same area is analysed by the two individual micro-spectrometers. This should provide complementary data about the chemical composition of samples, which will lead to better understanding of the material under investigation and provide increased confidence in the results generated by either individual technique.

7.2 Method
The overall objective of this work was to establish a method that allowed for the complete visualization of solid dosage pharmaceutical formulations. The method selected for doing this was to combine Raman and FT-NIR map data, to create a visual representation of the samples chemical composition through a process called ‘Chemical Image Fusion’ (CIF). The approach taken was to map the sample on separate FT-NIR and Raman microscope systems, ensuring that the same sample area was analysed on both instruments. To enable this, it was necessary to determine the exact location of where the sample would be mapped. This was achieved by placing reference marks on the microscope slide, prior to sample analysis, Figure 7.1a.
The x and y positions of these marks were determined on each separate instrument. Figure 7.1b and c show the co-ordinate measurements from the two different instruments, and demonstrating that the positional errors of the xy stages on the two instruments are negligible. The data showed a maximum spatial error of 6 µm (0.024%) in the x direction and 7 µm (0.07%) in the y direction. In each experiment, a 20 µm pixel size was generated and the reproducibility can be said to be better than ±1 pixel in all generated chemical images. This is equivalent to the xy stage error specified for both the FT-NIR and Raman instruments. Therefore it was concluded that it would be possible to merge Raman and NIR chemical images with complete confidence with respect to the spatial positioning of samples.

Figure 7.1 - Schematic of microscope slide with reference marks (a) along with co-ordinate positions obtained on b) Raman and c) NIR microscopy instruments with the distances between marks as calculated using the co-ordinates

Samples and Preparation

Two products were used for the evaluation of Chemical Image Fusion. Cardura XL® API blend (Product A) was used to allow a four component formulation to be examined, with 96.1% composed of the two major ingredients (polyethylene oxide and hypromellose). The other 3.9% of the formulation was the API material and magnesium stearate. The two major ingredients had strong NIR and Raman spectra and hence it would be possible to identify both using either mapping technique. Only one pre-tabletting blend batch of this product was analysed and used to confirm that CIF was working correctly.

Norvasc® (Product B) was a five component formulation. The composition of the mixture was such that some ingredients would be identified by Raman microscopy (API
and dibasic calcium phosphate) and others by FT-NIR microscopy (microcrystalline cellulose and sodium starch glycolate, two similar cellulose derivatives). This formulation had experienced tabletting issues during production, where the tablets had been found to stick to the tablet tooling. One problem batch and one good pre-tabletting blend were received and examined using the CIF approach. CIF was applied to allow for identification of all components within the formulation and provide a means for differences in the matrix of good and sticking blends to be determined, hence providing a possible explanation for the tablets sticking to the tooling.

The analysis of blend samples required a 300 mg sub-sample to be pressed into a wafer. This wafer had an optically flat surface and was ideal for microscopic examination. The wafer was mounted (using cyanoacrylate adhesive to prevent movement during analysis) to a microscope slide with reference marks.

**Instrumentation and Data Collection**

All NIR data were collected using a Perkin Elmer Autolimage microscope with a ×15 Cassegrain objective coupled with a FT-NIR Spectrum Identicheck spectrometer, as shown in Figure 1.6a. Samples were mounted on a xyz motorized stage. The sampling aperture was set at 20 × 20 μm, and reflectance measurements were collected over the range 7000 – 4000 cm⁻¹. The resolution of the spectrometer was set at 16 cm⁻¹ and each spectrum collected was an average of 25 scans.

All Raman data were acquired on a Renishaw System 1000 Ramascope with a ×20 glass objective, 1200 per cm grove grating, equipped with both 633 nm HeNe and 782 nm diode laser excitation sources. Samples were mounted on an encoded xyz motorized stage and spectra acquired in static mode with a 30 second acquisition time. The grating was centred to give a spectral range that provided the best differentiation between the components present, for product A the grating was centered at 1100 cm⁻¹.
with data collected over the range 637 – 1536 cm\(^{-1}\). The grating was centred at 1200 cm\(^{-1}\) for Product B with the spectral range set at 744 – 1630 cm\(^{-1}\).

To allow for overlay of the data from both instruments, the step size for the stage was selected as 20 \(\mu\)m in both the x and y directions. Although the step size was identical for both instruments, the area from which data were collected had variations. This difference arose because of the fundamental difference in the focusing of the beam used for data collection. In Raman mapping the beam was focussed tightly at the centre of the area defined by the step size (Figure 7.2a) whereas the NIR beam was dispersed over the whole apertured area, Figure 7.2b. Therefore Raman spectra are collected from a much smaller area (ca. 5 \(\mu\)m spot) than the NIR spectra (20 \(\times\) 20 \(\mu\)m). This is a factor that must be considered during data interpretation.

Prior to the first analysis, the samples were examined using light microscopy to identify a suitable area for analysis. The slide reference marks were checked to verify good agreement between the xy stage controls on both instruments. For Product A the area analysed was 1.2 mm \(\times\) 1.2 mm, collecting 3721 spectra. The area for Product B varied, 1.18 mm \(\times\) 1.18 mm for the good blend (3600 spectra) and 1.38 mm \(\times\) 1.38 mm for the sticking blend (4900 spectra).
Data Processing
Chemical images were prepared using Renishaw Wire software for the Raman data and Perkin Elmer AutoImage software for the NIR data. The chemical images were overlaid, using Paint Shop Pro 5, Jasc Software, to produce the complete image of the blend/tablet composition.

Both NIR and Raman data sets used peak area measurements of baseline corrected single spectroscopic bands, which were unique to each component, to generate chemical images. Raman spectra were examined at frequencies unique to each ingredient. These images were three dimensional, where x and y directions gave the spatial information while the third (z) dimension represented colour intensity, where bright pixels represented positive identification. To allow identification of unique NIR peaks, it was necessary to convert the raw data into second derivatives. This process was performed to improve spectral definition and at the same time removed any baseline variations in the spectra (previously discussed in Chapter 2, Section 2.1.1).

Figure 7.3a presents the Raman spectra and Figure 7.3b the NIR spectra of the components in Product A. In each figure the peaks available for identification of each of the four components are shown, where the area of the peak would be used to generate intensity values in the individual chemical images. It can be seen from Figure 7.3b that the strongest response from the polyethylene oxide overlaps with the magnesium stearate component, but due to the large concentration of this component it was decided to utilise this peak and explore the chemical images for any magnesium stearate contribution.
The spectra obtained of the excipients used in Product B, measured by the different techniques are presented in Figure 7.4. The Raman spectra are shown in Figure 7.4a, where it can be observed that the sodium starch glycolate component has no sharp bands, and identification of this component using a peak area is not possible. The second derivative FT-NIR spectra are shown in Figure 7.4b and it can be seen that the dibasic calcium phosphate has no sharp bands and is challenging to identify in the NIR region. It was also not possible to identify the sodium starch glycolate using a single peak, but as sharp distinct bands were present in this spectrum, it was decided to identify this component using alternative methodology.
The identification of the sodium starch glycolate in the NIR images was made by correlating the spectra from the images to that of the pure component, by calculating the vector dot product between the reference and sample spectra (Hoult, 1991). In the example shown in Figure 7.4b the identification of the sodium starch glycolate was possible by correlation to the region 4600 – 4200 cm\(^{-1}\), shown by the black box. In this region there are peak shifts which allowed the sodium starch glycolate to be differentiated from the microcrystalline cellulose. Before any correlation could be performed, it was necessary to map a small sample of the pure components of interest. The average spectra obtained from the map data were then correlated to all the pure ingredient spectra contained in the formulation and allowed threshold limits to be established, with a correlation value of >0.85 representing sodium starch glycolate pixels.

### 7.3 Evaluation of Chemical Image Fusion
Product A was selected to determine if the combination of images from the two different spectroscopic techniques would be feasible. Although this was a four component system, it was decided to concentrate on only the polyethylene oxide and the hypromellose, as they represented 96.1% of the formulation. It was observed in Figure 7.3b that the polyethylene oxide had no unique absorptions in the NIR region, but the strongest peak, (4375 – 4300 cm\(^{-1}\)) was used as it overlapped only with the magnesium stearate (low level component). During processing, the images obtained for this ingredient were examined and no spectral contributions were observed. As such, it was concluded that the polyethylene oxide had been mapped successfully, without interference from the magnesium stearate. Using the peak areas identified in Figure 7.3 it was possible to generate chemical images of both the polyethylene oxide and the hypromellose from FT-NIR and Raman experiments. These are presented in Figure 7.5 along with the overlaid images from each individual technique. (This figure is provided to show the individual technique results and set the base line for CIF comparisons.)
From Figure 7.5 it is possible to see that both approaches give very similar chemical images for the two constituents in terms of spatial distribution. The images of the polyethylene oxide are comparable, with only some variation in intensity due primarily to focusing. This is a result of an uneven sample surface, which the FT-NIR eliminates using automatic focusing. In addition crystal orientation effects may cause intensity variation, especially in the Raman data but is not considered a significant issue. The images for the hypromellose show some variation, where the NIR images suggest large clumps of particles and the Raman images show smaller lumps of particles, which are found close together. This difference is thought to be caused by the manner in which data are collected by the two techniques, as presented in Figure 7.2, where in spite of an identical step sizes, $(20 \times 20 \, \mu m)$, the FT-NIR collects data from the entire $20 \times 20 \, \mu m$ area and the Raman from only $5 \times 5 \, \mu m$ due to beam focusing variations. When the individual component chemical images are overlaid, the resultant images give a very similar picture for the distribution and size of the two main ingredients, indicating the possibility for success with CIF. This can be further explored by placing a grid over each of the chemical images, as shown in Figure 7.6 where cross-referencing of each component's domains is possible, e.g. in the bottom two rows, pixels 3, 4 and 5 from the left in both images contain hypromellose contributions. It
should be noted that the ‘black holes’ in the overlaid image are areas where no one component dominates the spectrum.

As the two techniques give comparable images the chemical image of polyethylene oxide (by NIR) was overlaid with the chemical image of hypromellose (by Raman), shown in Figure 7.7a. The Raman image of polyethylene oxide was overlaid with the NIR image of hypromellose and is shown in Figure 7.7b. From Figure 7.7 it can be observed that in both cases the images fit together like a jigsaw puzzle with hypromellose filling the major black areas in the image of the polyethylene oxide. This experiment showed that the results obtainable from fusion of the images gives rise to an image, which provides an accurate description of what was present in the sample, in terms of distribution, size and identity of each ingredient, and implies CIF of solid dosage forms is possible.
Figure 7.7 - Chemical images of the distribution of polyethylene oxide (green) and hypromellose (blue) within Product A matrix using a) NIR and Raman and b) Raman and NIR images respectively

7.4 APPLICATION OF CHEMICAL IMAGE FUSION

It was observed in Figure 7.4 that the five components of Product B did not all have sharp spectral features in either the NIR or Raman spectra. Therefore GIF provides an opportunity to fully characterise all the components within the blend matrix and assist in determining the cause of product sticking, which was hypothesised as being due to a poor lubricant distribution, as this material has a function to reduce tablet sticking to the tooling (Cartensen, 1993).

When only the Raman mapping data were utilised, it was possible to map four of the five components. The sodium starch glycolate was not identifiable because the spectrum was unsuitable for image construction due to the lack of sharp peaks and coincidence with bands from other components. The resultant chemical images based on Raman data for both the good and bad blend samples are shown in Figure 7.8. From these images it can be seen that there is a variation in the distribution of the dibasic calcium phosphate between problem and good batches, but also of interest is the distribution of the magnesium stearate which appears to be present in clusters within the matrix.
When only the NIR mapping data were utilised, it was again possible to spectroscopically map four of the five components. The lack of a NIR spectrum for dibasic calcium phosphate prevented positive identification of this ingredient, but it was thought that any major black regions observed could be associated with the presence of the inorganic material. The chemical images obtained by NIR for the good and bad samples can be seen in Figure 7.9 and it is also interesting to see clusters of magnesium stearate in both good and sticking batches.
From Figure 7.8 and Figure 7.9 it can be observed that both instruments have mapped the same sample area, as the chemical images of the same components are similar in size and shape. Therefore to enable CIF, the most appropriate NIR and Raman chemical images were selected. Using the NIR region it had been possible to map the active ingredient but the spectra obtained for the regions with API were dominated by the microcrystalline cellulose spectra. Hence, the pixels identified as API by NIR were not considered as reliable as the Raman data. When considering the images for the microcrystalline cellulose, one of the main considerations is being able to distinguish the different types of carbohydrates. With the Raman spectra the microcrystalline cellulose and sodium starch glycolate spectra strongly overlap, but in the NIR, although spectrally similar, there are regions where peak shifts occur and hence the two materials can be distinguished. As a direct result of this, the NIR images of the microcrystalline cellulose and sodium starch glycolate were preferentially used for CIF. The magnesium stearate image created using the NIR data was used for CIF, because in regions identified as magnesium stearate the spectra obtained matched that of the pure component. This is unlike the Raman where spectra in the magnesium stearate regions had only a small magnesium stearate contribution and were instead dominated by the microcrystalline cellulose spectra. The dibasic calcium phosphate could be positively identified in the Raman but had no spectral features in the NIR and hence the Raman image was used for CIF. Therefore API and dibasic calcium phosphate chemical images determined using the Raman data and the sodium starch glycolate, microcrystalline cellulose and magnesium stearate chemical images determined using the NIR data were combined to provide a complete visualization of the pre-tabletting blends.

Figure 7.10 shows the images obtained for the good blend, for all the ingredients, using the most appropriate spectroscopy. The centre picture shows the CIF on the complete data set. This fused image shows the size and distribution of the materials within the blend. Figure 7.11 shows the identical set of images for the sticking blend.
Figure 7.10 - Chemical Image Fusion of two Raman chemical images with three NIR chemical images to give complete visualisation of the matrix of a good blend

Figure 7.11 - Chemical Image Fusion of two Raman chemical images with three NIR chemical images to give complete visualization of the matrix of a sticking blend
Although the presence of the API is clearly visible in its own chemical image, it is more difficult to see in the CIF data. This is because the drug is present at low concentration and forms an intimate mixture with the microcrystalline cellulose, resulting in a composite spectrum with a low API contribution. The low intensity of the API in the CIF images makes its visualisation challenging, despite the strong spectral features observed. In both Figure 7.10 and Figure 7.11 it can be seen that the CIF image provides visualisation of all components in the blend matrix. It should be noted that in both fusion images the presence of black areas is due to no dominant spectral features of any one ingredient in that region.

Comparisons of the two fused images show that differences exist between the two blends, Figure 7.12. These differences do not relate to the distribution of the magnesium stearate as anticipated, but actually relate to how the microcrystalline cellulose and calcium phosphate distribute themselves within the formulation. The magnesium stearate distribution does not match the theoretical distribution, where it has always been considered to finely distribute itself between the surfaces of the other ingredients. These images actually suggest that the magnesium stearate clumps together and has a bimodal domain size distribution centred on 20 and 100 µm. These sizes were typical in both the good and sticking blends. The images suggest that the sticking issue is related to the distribution of the dibasic calcium phosphate within the microcrystalline cellulose. In the good batch the dibasic calcium phosphate has a much larger domain size (40 – 150 µm) within the blend. This also seems to be true of the microcrystalline cellulose. On the other hand, in the sticking formulation the dibasic calcium phosphate seems to have a smaller domain size (20 – 60 µm) and a more intimate mixture with the microcrystalline cellulose (cyan coloured regions, which are a result of the mixing of blue (microcrystalline cellulose) and green (dibasic calcium phosphate pixels).
This is in sharp contrast to the typical particle size of the dibasic calcium phosphate before it is added to the process. At this stage in the production, the dibasic calcium phosphate has a median particle size in the order of 2 μm, see Figure 7.13. [These data were collected using a particle size vision system from Pharmavision Systems AB., Sweden]

It would appear that in order to make a good blend, an even distribution of small particle size excipients is not required, but instead a blend with clumps of particles seems to give good processability. This work has suggested that good excipient homogeneity gives rise to tablets that process poorly, a premise that is not normally considered in the pharmaceutical industry. Therefore it can be concluded that small particles with an even distribution are not always the best way to produce a
pharmaceutical blend from which a tablet with optimum properties can be manufactured.

The CIF results caused a change in the problem solving approach moving from one purely focussing on the magnesium stearate, to investigations into other excipients (e.g. microcrystalline cellulose and dibasic calcium phosphate). The current theory about the cause of differences between sticking and good batches is thought to be due to a variation in particle size between the raw ingredients. Figure 7.13 shows the particle size volume distributions for two different batches of the inorganic binder. The batch shown in Figure 7.13a had been used in production and known to be associated with sticking batches and Figure 7.13b had been used in production of good tablets. These figures show that in the material used for the production of good tablets, the mean particle size was larger than that of the material used in the sticking batches. It was calculated that 50% of the particles in the ‘good’ material have a mean particle diameter of less than 26 μm but in a ‘sticking’ material the diameter is dramatically decreased to 5 μm. This outcome from this investigation resulted in the particle size of the dibasic calcium phosphate being more tightly controlled with an on-going move to tighten all the raw material specifications.

7.5 CONCLUSIONS
Chemical image fusion improves the information available from individual NIR and Raman mapping experiments. The combination of the two techniques removes ambiguity from the data collected from the two separate techniques. This is due to the complementary nature of the two spectroscopies, which allows for the production of a complete visual representation of the chemical composition of pharmaceutical blends or tablets.
The synergy of the two mapping experiments has not only improved the chemical information obtainable, but has also demonstrated the good accuracy and precision between the XY stages on the two different (FT-NIR and Raman) microscopy systems.

The practical aspects of CIF have been demonstrated, along with other applications to which the method can be applied. CIF provides a visual image from which a greater understanding of the matrix properties of formulated materials can be obtained. This information can subsequently be used to improve product quality, through better formulation design and understanding of manufacturing processes. However, if CIF was to become a routinely used method for pharmaceutical analysis it would be necessary to combine the instrumentation. Although the virtual instrument provides results which can be combined together, it is a timely process which could be improved.

**7.6 ACKNOWLEDGEMENTS**

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8. **Conclusions**

The results of this research clearly demonstrate that NIRM has a role to play within the pharmaceutical industry, although there are still some obstacles which would prevent full utilisation of this methodology in pharmaceutical process control.

With the large volumes of data generated by such an approach, there will always be a time constraint to any experimentation performed. However, this work has shown that there is an optimum method for the analysis of pharmaceutical dosage forms. Experiments have shown that a sample area of >8 mm$^2$ is required to obtain good image information, but this is dependent upon the input particle size of the raw materials, and has been determined for a dosage form where the largest mean particle size was ~110 μm. It has also been found for homogenous systems, that there is more information to be obtained from analysing a number of samples per lot than from many layers within a single sample. However, it was observed that the information obtained on images collected from the surface of a sample are different to those from within, due to an increased sample surface area caused by higher compression forces at the sample surface. It has been concluded that an area of 25 mm$^2$, using a spatial resolution of 25 x 25 μm is optimum for NIRM experiments as it takes ~30 minutes to complete the experiment but the information obtained is representative of the entire sample.

This optimum sample size results in over 25,000 spectra which must be processed to generate chemical images. Firstly, it is necessary to normalise (SNV) the spectral data obtained, which not only removes spectral scatter but also enhances the features of the chemical images. Dependent upon the variation in spectral features, in the dosage form of interest, it may be necessary to include a derivative in the pre-treatment of the data. This was found to be necessary during this research when materials such as calcium silicate were included in the dosage form (Chapter 6) and also when similar concentrations of microcrystalline cellulose and corn starch (Chapter 4) were utilised.
Chemical images could be generated based on a change in wavelength absorbance values, but applicability is generally limited by the spectral resolution between materials. Therefore univariate approaches are not considered as a practical approach for preparing chemical images but instead, PLS algorithms were considered to be the best method for analysis of dosage forms with known compositions. If such an approach is utilised with spatially resolved materials, classification of spectra is simple. Image pixels have either a zero (no classification match) or one (classification match) score value, but this is not the case in most dosage forms where the materials are inter-dispersed. In such systems, PLS classification values are weighted by the relative concentrations of components and was found to be a direct result of the over-sampling that occurs during spectral measurement (function of lateral resolution and depth of penetration).

Although PLS methods were found to be ideal for this research, it is acknowledged that the exact composition of solid dosage forms is not always known and hence, in these situations, PCA reconstruction is probably a better approach to generating a chemical image. Due to the unsupervised nature of PCA methodology it was found to be an ideal method for understanding how many components are causing a change in spatial features in the image, although it was shown that low level components are not always identified in PCA approaches (Chapter 2). Therefore PCA reconstruction is superior, as the average spectral response (due to over-sampling with each spectrum measured) is removed, allowing the lower level components to be revealed. If PCA reconstruction is to be used for more than simple understanding of matrix composition, and actually for chemical image generation, it is limited by the same constraints as any univariate approach – the spectral resolution between components of interest.

In all experiments performed, the over-sampling of the dosage form at each spectral measurement was found to reduce the expected response from a material. Every spectrum obtained was dominated by the spectral features of the highest concentration
components. Although data processing methods were employed to overcome this, experiments were performed to understand the true volume of sample contributing to a single spectral measurement. The volume sampled was a function of both the lateral resolution and depth of penetration of the NIR radiation. Although objects equivalent in size to the defined measurement area could be resolved, it was found that there was a spectral contribution from out with this desired region. For a material of interest a 50% pure spectral response was all that could be guaranteed from a defined measurement area (20 x 20 μm). To obtain spectral purity of >75% the area of contribution was thought to be 100 x 100 μm, twenty-five times the desired measurement area. This obviously causes no issues if materials with particle sizes in this size magnitude are present in the dosage form, but obviously the spectral response from materials smaller than 100 μm in diameter will be diluted by the surrounding matrix. Over-sampling was also observed in the depth axis, as 50% of the spectral response was found to come from the top 39 – 57 μm of the sample, dependent upon the NIR wavelength used. It was found that the depth of penetration had an exponential relationship with NIR wavelength, with shortest wavelengths penetrating deepest into the sample. The information depth (depth to which spectral response is measured) was found to be 109 μm at 2380 nm and 777 μm at 1100 nm. Knowing both the lateral and depth resolution in NIRM allowed the actual mass of material being sampled during a spectral measurement to be determined, ranging from 0.22 - 1.59 μg for materials with a density of 0.7 gcm$^{-3}$ and increasing to 0.37 - 2.62 μg for materials with a density of 1.15 gcm$^{-3}$.

Although the over-sampling of the measurement area occurs, it is not an issue for component identification as multivariate methods (such as PLS) are able to resolve the individual component spectral features from a spectral response of the average of the entire dosage form. In fact, it has been found that this over-sampling actually makes NIRM a very good method for examining component concentrations within a solid dosage form, as the PLS score distributions for all pixels shows a direct relationship with component concentration (Chapter 4). If no over-sampling occurred then PLS
analysis of a solid dosage form would result in bi-modal score distributions, but in reality a mono-modal distribution is observed, with the mean of the distribution centered on a value close to the actual component concentration. In the blends prepared for this research a linear relationship between the mean of the score distribution and the actual % w/w concentration was observed with an average $R^2$ value of 0.9869. The relationship identified could be used to predict concentrations, but an error of 0.36 - 3.83% was observed, which is not significant for high concentration components but could impact the accurate determination of low level concentrations, with relative standard deviations ranging from 3.86 – 18.50%. Therefore it was concluded that although a correlation exists, without further understanding of factors impacting the relationship, the mean value of the score distributions would only be useful for comparing concentrations from lot to lot, rather than for accurate concentration values.

This research went beyond building the fundamental knowledge around NIRM experiments and has shown that NIRM can in fact be used to increase understanding of solid dosage forms, by application to real world samples. The chemical images obtained from NIRM have been shown to successfully visualise components within a solid dosage form, and that the distribution and size of these components can be correlated with product performance (dissolution and tablet sticking).

In terms of tablet sticking, NIR chemical images showed a direct correlation with performance, with the size of the two major components (dibasic calcium phosphate and microcrystalline cellulose) being shown to impact tablet strength (Chapter 5). With this relationship identified it was possible to bring understanding to the tablet sticking investigation, the two components had to be similar in size for a strong matrix, and when the dibasic calcium phosphate was smaller than the microcrystalline cellulose the matrix was weakened. Due to the different deformation processes which the two materials experience under compression, it is possible to equate the backbone of this
dosage form to a wall; with dibasic calcium phosphate acting as the bricks and microcrystalline cellulose as the cement. Therefore when the dibasic calcium phosphate is smaller than the microcrystalline cellulose, there is not enough ‘cement’ to hold the ‘bricks’ together and hence the tablet matrix is subject to sticking to the dies during compression. As well building understanding, by converting the NIRM result to a numerical value (ratio of DCP and MCC) it would be possible to control the process, and predict at the blending stage if tablet sticking would occur. Currently this is not a realistic situation as NIRM instruments are laboratory focused and are too lengthy to be used as realistic process control tools but if technology could be accelerated NIRM does have the potential to be a useful process control tool.

The samples from the tablet sticking investigation were also evaluated by ‘Chemical Image Fusion’, a virtual method to combine Raman and NIR microscopy. Although the samples had successfully been imaged by solely NIRM the samples were ideal for a CIF approach, as dibasic calcium phosphate does not give strong NIR absorptions and as was only identified in NIRM by a lack of NIR response. Raman and NIR microscopy are complimentary techniques, as NIR is able to distinguish very similar carbohydrate species (which Raman cannot) and Raman has strong responses from inorganic materials (which NIR does not) therefore combined would give full pharmaceutical excipient identification. The virtual instrument used showed that data from two separate instruments could be combined, and the spatial information obtained was complementary, and for the tablet sticking investigation showed that the size of the dibasic calcium phosphate was critical to performance (as was observed for the sole NIRM experiments in Chapter 5). Measurement of the input particle size of the dibasic calcium phosphate showed a significant difference between lots used in good and sticking tablet batches. As a direct result of the experiments performed the raw material specifications were tightened. Although the virtual instrument was successful for routine application of this combined approach it would be necessary to have a real combined instrument manufacture to reduce data collection times.
With the investigation of tablet sticking, the chemical images showed direct correlation with product performance which was also the situation when the tablets with dissolution issues where examined. For one product the chemical images showed that the number of calcium silicate domains in the dosage form was critical for successful dissolution. When the number of domains in each lot investigated was compared to dissolution performance an exponential relationship was observed. By taking the logarithm of the number of calcium silicate domains it was possible to use linear regression to obtain an equation for the prediction of dissolution performance. When a second product was examined the relationship between chemical images and dissolution performance was not as obvious, as the variation in the chemical images was based on three different materials. The number of API, lactose and povidone (binder) domains was found to vary with dissolution performance, such that it could be concluded that differences in dissolution were a direct result of variations in granulation performance. For both products the differences observed in the chemical images could be used to build mechanistic understanding of dissolution, as the changes observed in the tablet matrix could be correlated with the means in which water (dissolution media) could interact with the individual components during dissolution.

The correlations with product performance could be improved by directly performing NIRM experiments on the sample prior to the product performance test. This would improve the models established in this research, and provide further understanding of the variation within a single lot. Further work is also required in improving the correlations between concentration and the mean of the PLS score distributions. Although this work has shown that a correlation does exist, further work is required to understand what parameters of raw material impact this relationship, e.g. input particle size. In this work, issues where observed in predicting the concentration of corn starch and the exact reasons for this are still not understood. Therefore further evaluations of more excipients, in differing concentrations would aid to improve the error of prediction observed in this research.
Throughout this research, the application of NIRM has been in a root cause analysis mode and further work is required before the technology can be used as a process control tool. Although the information obtained can be used to predict performance, with the speed of acquisition it would not be realistic to expect measurements to be taken at-line of the process, and the process held until the results obtained. Therefore one of the key areas for the successful implementation of NIRM in process control would be to develop the instrumentation to acquire images faster. However, this research, despite the inability to measure samples in real time, has shown that NIRM has real potential as an analytical method for pharmaceutical analysis. In this research, information has been obtained on a solid dosage form which has only previously been theoretically determined. Therefore the value of this methodology has already been shown, but this only touches the tip of the iceberg for the potential applications for this technology within the pharmaceutical industry, with the future being an exciting place for near-infrared microscopy.
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APPENDIXES

Appendix 1 – Line Widths for USAF Resolution Target

<table>
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Appendix 2 - SNV pre-treated absorbance spectra of the five API’s used to generate interfaces collected by a) NIR-PM and b) NIR-GI instruments

a)

![Absorbance Spectra](image1)

b)

![Absorbance Spectra](image2)
Appendix 3 – List of Publications

1. ‘Chemical Image Fusion. The Synergy of FT-NIR and Raman Mapping Microscopy To Enable a More Complete Visualisation of Pharmaceutical Formulations’
   Clarke, F.C., Jamieson, M.J., Clark, D.A., Hammond, S.V., Jee, R.D., and Moffat, A.C.

2. ‘A near infrared view of pharmaceutical formulations’
   Clarke, F., Lewis, E.N. and Carroll, J.

3. ‘The development of NIR Microscopy for Process Control in Pharmaceutical Manufacturing’
   Clarke, F., Hammond, S.V., and Mattisson, C.A.

   Clarke, F.C., Hammond, S.V., Jee, R.D., and Moffat, A.C.

5. ‘Near-infrared Microspectroscopy’
   Clarke, F.C., and Hammond, S.V.
   Handbook of Vibrational Spectroscopy, Chalmers, J.M., and Griffiths, P.R., Wiley and Sons Ltd, Chichester

6. ‘NIR microscopy of pharmaceutical dosage forms’
   Clarke, F.C., and Hammond, S.V.,

7. ‘Extracting process-related information from pharmaceutical dosage forms using near infrared microscopy’
   Clarke, F.