The Application of Near Infrared Spectroscopy to the Characterisation and Quality Control of Pharmaceutical Materials of Natural Origin

by

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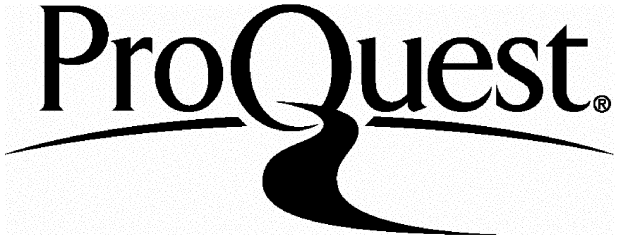

ABSTRACT

Natural products are of increasing interest as sources of novel pharmaceuticals and there are frequent questions as to their authenticity and purity. The use of Near Infrared (NIR) spectroscopy provides a method for rapid analysis of such materials and requires no sample preparation. Although there are many references detailing the use of NIR spectroscopy in the pharmaceutical industry, the majority of these are concerned with the analysis of pharmaceutically active drugs or pharmaceutical excipients.

The aims of this PhD were to demonstrate the potential of NIR spectroscopy as a tool in the quality control of a variety of natural materials. The emphasis, therefore, was on studying a range of different products in both a qualitative and quantitative manner. Qualitative methods included the identification and qualification of natural products by the use of constructed spectral libraries. Statistical discrimination methods such as Correlation in Wavelength Space (CWS) and Maximum Distance in Wavelength Space (MDWS) were employed to create Library methods that allowed an unknown spectrum to be identified with, or distinguished from, spectra used to construct the Library. Quantitative methods included the quantification of certain analytes at different concentrations in certain materials by developing calibration equations using the Multiple Linear Regression (MLR) method. Methods of sampling, preparation and measurement were developed to carry out these investigations in the most appropriate manner and to overcome problems encountered with data acquisition and analysis. British and European Pharmacopoeia assays for the quantification of certain chemical constituents in essential oils such as Eucalyptus and Lemon oils have been used as standard reference methods and compared with NIR methods developed as part of this research and it has been shown that the NIR method is similar in precision and accuracy to the conventional assay methods. In addition, it has been demonstrated that NIR spectroscopy can be used to identify and distinguish between different essential oils and that the water content of Agar samples can be determined. Initial studies have also been carried out into the detection of adulteration of Rosemary oil with Eucalyptus oil and the contamination of Fennel with Hemlock.

To summarise, the potential of NIR spectroscopy for the quality control of natural pharmaceuticals has been demonstrated and it is possible that this technique could replace more complicated and expensive traditional methods of analysis in the future.

ACKNOWLEDGEMENTS

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On a more personal note, I would like to thank my mum and dad for their financial support and patience and especially to Gareth for just about everything and without whom life would be a lot less fun.

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ABBREVIATIONS

BP	**British Pharmacopoeia**
CWS	**Correlation in Wavelength Space**
EP	**European Pharmacopoeia**
KF	**Karl Fischer**
MCA	**Medicines Control Agency**
MDWS	**Maximum Distance in Wavelength Space**
MLR	**Multiple Linear Regression**
NIR	**Near Infrared**
PC	**Principal Component**
PLS	**Partial Least Squares**
PLSR	**Partial Least Squares Regression**
SEC	**Standard Error of Calibration**
SEP	**Standard Error of Prediction**
SNV	**Standard Normal Variate (corrected)**
2ndD	**Second derivative**

LIST OF SYMBOLS

nm	**nanometres**
λ	**wavelength**

CHAPTER ONE: INTRODUCTION

1.1 Background and History

Natural products are of increasing interest as sources of novel pharmaceuticals and there are frequent questions as to their authenticity and purity. There is also increasing use of natural products as alternative and complementary medicines. It is reported that retail sales of herbal products have increased by 43% in the period from 1994 to 1998 and that retail sales of licensed herbal medicinal products were in the region of £50million in 1998 (Barnes *et al*, 2002a).

The need for a rapid, yet accurate alternative to the traditional techniques used in natural product analysis has never been greater due to the proposed introduction of new legislation requiring any herbal product sold in the UK to have been tested and justified in terms of their safety and quality (MCA website 2002a). Herbal remedies on the UK market with a marketing authorisation or Product Licence (PL) have to meet systematic quality and manufacturing requirements, designed to give public assurance that the product is made from correct ingredients to appropriate quality standards. Other herbal remedies are exempt at present from licencing requirements if they meet conditions set out in Section 12 (2) of the Medicines Act 1968. However, some manufacturers of unlicenced products voluntarily meet these same standards. The proposed European (EU) Directive on Traditional Herbal Medicinal Products would put in place consumer protection by requiring herbal remedies with a traditional use registration to be subject to the same systematic quality controls as licenced herbal remedies. The objective is that the public should have access to a wide range of safe, high quality herbal remedies with appropriate information about the use of the product. There needs to be a suitable balance between consumer choice and public safety.

The proposed standards in the Directive include:

- reliable identification of materials
- use of good quality herbal ingredients
- systematic checks to ensure there are not unacceptable pesticide residues, heavy metals or other forms of contamination
- adequate premises and equipment

- proper record keeping
- a suitably qualified person to take responsibility for the release of batches onto the market

The consultation exercise by the Medicines Control Agency (MCA), an executive agency of the Department of Health on this proposed EU Directive on Traditional Herbal Medicinal Products was extended to end on July 31st 2002. The Health Minister Lord Hunt expressed the opinion that this extended consultation exercise will allow increased communication between the MCA and the herbal sector due to concerns raised that the Directive could have a detrimental effect on the availability of products. At a conference of the Heath Food Manufacturer's Association, Lord Hunt said:

"The clear message we are receiving from many respondents, including representatives of small UK manufacturers of traditional remedies and herbal practitioners, is that the Directive is needed to ensure adequate safety and quality standards and to give consumers systematic information about the safe use of the product."

The MCA has also set up an online information service to give up-to-date advice on herbal medicines safety known as the "Herbal Safety News" (MCA website 2002b). The service will also provide details of concerns of specific herbal ingredients and any advice issued by the MCA or the Committee on Safety of Medicines (CSM), along with details of potential interactions with conventional or other herbal medicines and contamination of herbal remedies with heavy metals or the illegal inclusion of prescription-only medicines. One aim of the site is to promote to the general public the concept that herbal remedies are genuine medicines that have an effect on the body and so should be manufactured to high standards and used with care.

The European Agency for the Evaluation of Medicinal Products has set up a Herbal Medicinal Products Working Party (HMPWP) at the request of the European Parliament and the European Commission. Their mandate is to facilitate mutual recognition of marketing authorisations in the field of herbal medicinal products and one of their aims is to produce guidelines on how to adequately prove quality, safety and efficacy of herbal

medicinal products with particular reference to new scientific data and the well established use of herbal medicinal products.

Factors determining the safety and quality of a herbal product will include species used, geographical origin, time of harvesting and storage conditions. NIR spectroscopy may prove to be an ideal tool to determine or confirm identity of plant material and geographical origin and to quantify chemical constituents and physical parameters such as water content or particle size. The speed of data acquisition and interpretation together with the lack of or minimal sample preparation required mean that NIR may prove to be invaluable to manufacturers of herbal remedies allowing them to adhere to the Directive in terms of the reliable identification of materials and the use of good quality herbal ingredients.

The origins of the technique of NIR spectroscopy date back to the beginning of the 19th Century when Sir William Herschel carried out an experiment to confirm his curiosity as to which colour in the spectrum of visible light was responsible for the heat in sunlight. He used a glass prism to separate the visible light by refraction into the separate colours and then moved a thermometer from one region of colour to the other. On positioning it below the red end of the spectrum , the temperature began to rise and he named this region of the spectrum (past the visible range) the Infrared, using the Latin prefix meaning below (Burns and Margoshes, 1992). Coblentz carried out further work into this region when he invented an IR spectrometer using a rock salt prism and a thermopile as the detector which was attached to a mirror galvanometer. He charted the IR region by painstakingly moving the prism through a tiny angle, leaving the room and then taking the galvanometer reading by viewing from outside using a telescope. Two readings, one with and one without the sample to be measured was taken at each angle of the prism. In this manner, Coblentz measured several hundred compounds across the mid-IR region, between 1000 and 15000nm and found that no two compounds had the same spectrum. He identified the region between 2000 and 15000nm as giving each compound a unique 'fingerprint'. Coblentz also noticed that similar chemical groups had absorption bands in similar regions of the spectrum. When commercial IR instrumentation became available in the 1940s, scientist could obtain an IR spectrum of a compound in about 15 minutes, which could then be used to gain

information about the structure of that material. At this time, the Near Infrared region was avoided by most scientists, as there were distinct disadvantages to the use of the spectra of compounds in this region in terms of a 10 to 100 times decrease in sensitivity, loss of baseline resolution and the poorly defined absorption bands that could not be used for structure elucidation due to the overtones and combination bands present (Burns and Margoshes, 1992).

The number of papers published on the field of NIR spectroscopy were small in number, one of the most important being that of Kubelka and Munk (1931) which dealt with the principles of diffuse reflection and discussed the benefits of little or no sample preparation being required (Shenk *et al* 1992). However, transmission measurements were mostly used at this time, although work was carried out by Ellis and Bath (1938) who determined the water content of gelatin, as it was known that water had a high absorption in the NIR region. Barr and Harp (1943) obtained the NIR spectra of vegetable oils.

Although the principles and possibility of using the NIR region to quantify certain constituents in certain materials gradually became known during this period, one piece of work to launch NIR spectroscopy as a technique that could be used commercially was that of Ben-Gera and Norris (1968a) which discussed their work in quantifying fat and moisture in meat, moisture in soybeans and fat and moisture in milk. The agricultural industry was quick to realise the potential of this technique and embraced it whole-heartedly, especially with the advent of computers that had a high enough computing power to perform quick calculations as more sophisticated chemometrics came into being. The first commercial use of NIR spectroscopy occurred in the agricultural industry in the late 1960s and the technique has been successful in the quality control of natural materials for many years. The early developed NIR techniques were used to study the nutrient composition including protein content and the moisture content in grains and soybeans. The NIR spectrophotometers used were filter instruments, capable of detection of diffuse reflectance absorbance at a fixed number of wavelengths at which specific absorption bands relevant to agricultural materials were exhibited. In the early 1980s, commercial companies began to supply NIR instrumentation and software for forage and feed analysis. These instruments covered a greater number of wavelengths or were scanning instruments,

capable of covering the majority of the NIR wavelength range.

NIR spectroscopy was slow to be accepted within the pharmaceutical industry for several reasons. Firstly, the conventional techniques used for pharmaceutical analysis are separation methods such as High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) and the concept of a method that could be used to quantify one or more constituents without the need for prior separation was an alien one. Secondly, NIR spectroscopy was considered inferior to conventional IR spectroscopy due to the lack of primary absorption bands and so was less useful in structure elucidation and material identification. However, NIR spectroscopy eventually found its niche in the pharmaceutical industry for material identification and qualification and rapid quantification of certain chemical and physical parameters. The rate-limiting step for the progress of NIR spectroscopy as a technique was the computing power required to carry out the calculations, and the introduction of NIR spectroscopy to the pharmaceutical industry was initially greeted with caution as it was viewed as a 'black box' method by some sceptics and much work had to be done in identifying the benefits the technique had to offer (Shenk *et al* 1992).

There are many references detailing the use of NIR spectroscopy in the pharmaceutical industry, the majority of these are concerned with the analysis of pharmaceutically active drugs or pharmaceutical excipients. The work that has been done to date with natural products has demonstrated precedent for the capability of NIR spectroscopy for identifying and qualifying herbal materials or essential oils and for the determination of chemical constituents in natural products. Corti *et al* (1990) found it possible to distinguish between Ginseng, Silybum and Grapeseed powders without prior sample preparation using NIR reflectance spectroscopy and to further distinguish between two species of the Silybum. This indicated the possibility of not only being able to identify and distinguish between different plant materials, but also to discriminate in terms of species or geographical origin. In addition, it was found to be possible to distinguish between samples of Ginseng and those that had been adulterated with starch, down to 5% of starch in the sample. Furthermore, they were also able to show that NIR spectroscopy could determine the

amount of Ginsengosides present in Ginseng samples using HPLC as the reference method. However, due to the small number of samples included in the study, it was not possible to construct a robust full quantitative method, but it was shown that it was possible to distinguish between samples with varying amounts of Ginsengosides in a semi-quantitative manner.

Cho *et al* (2000) noted that geographical origin and cultivation period have always been considered very important in quality grading of Ginseng. They were able to show that very high levels of discrimination could be achieved for these two parameters and also that quantification of the medicinal components of ginseng could be achieved. Woo *et al* (2000) were also able to demonstrate the application of NIR spectroscopy for the classification of cultivation period of ginseng using pattern recognition techniques that converted the spectral data to information that allowed the visual separation of different types of sample. Kwon and Cho (2000) developed a method to identify the geographical origin of Sesame seeds by NIR spectroscopy. In addition they showed that the adulteration of Korean and Chinese Sesame seeds could be determined to an approximate 10% error level.

Fehrman *et al* (1996) showed that NIR spectroscopy could be used to determine in a fully quantitative manner the amount of several different chemical constituents present in Caraway and Fennel seeds. Previously, conventional steam distillation of the sample followed by GC was used to detect and quantify the essential oils present in these seeds, but this is labour intensive and time-consuming. Caraway is a well used spice and is also used in the pharmaceutical industry and for flavouring of food products. Fennel is used in Aromatherapy and is highly appreciated as a natural remedy for disorders of the digestive system. The work conducted in this study indicated that it may be possible to use NIR spectroscopy to select those seeds that contained the highest quantity of those oils which are extracted from the seeds for such uses, thereby maximizing production and minimizing cost.

A further example of quantification of chemical constituents of natural products is the quantification of Sennoside content of Senna plants using NIR in the diffuse reflectance mode by Molt *et al* (1997). A calibration was set up using reference data obtained from HPLC measurements of the Sennoside content. The accuracy of the NIR method was

shown to be comparable with that of the reference method. Woo *et al* (1998, 1999) were able to discriminate between herbal medicines of the same type but from different geographical regions for purposes of quality control, as it is known that the quality of such herbal treatments varies according to their source.

In the case of essential oils, it has previously been shown that Fourier Transform NIR spectroscopy can be used to distinguish between different essential oils using a reflectance probe inserted into the oil sample (Schultz and Lösing, 1995). To date, most of the work concerning the identification of essential oils and quantification of their chemical constituents has been by traditional techniques such as Thin Layer Chromatography (TLC), GC and HPLC. Steuer *et al* (2000) used a HPLC method to determine the quantity of nine different components in dried Rosemary leaves, including the total essential oil content, Cineole and Borneol. NIR calibrations developed were generally of comparable accuracy to the reference method, although three of the components could be less accurately identified. This work has paved the way and encouraged further investigation into the limits and possibilities of NIR spectroscopy for natural product analysis. Work to date at the Centre of Pharmaceutical Analysis, London School of Pharmacy, include the identification of *Digitalis purpurea* and the discrimination of Fennel and Hemlock (Kudo *et al* 1998, 2000). A review paper on the use of NIR spectroscopy at the Centre for the characterisation of herbal natural products has also been published (Watt 2001).

The European Pharmacopoeia was created in 1964 and since then the number of monographs for herbal drugs used either in their natural state after drying or extraction or for the isolation of natural active ingredients has increased to over one hundred and twenty. Many of these are reproduced in the British Pharmacopoeia. Along with specific monographs, general tests such as for foreign matter, ash value and microbial contamination are included (Barnes *et al* 2002b).

1.2 Purpose and Aims of the Research Project

It has been shown that the need for rapid, accurate methods of quality control or natural materials and natural products are required, especially with the imminent introduction of

legislation requiring herbal remedy manufacturers to demonstrate the quality and safety of their products. Furthermore, the use of NIR spectroscopy in both the agricultural and pharmaceutical industries suggests that it may be an ideal tool for the quality control of natural materials.

Several different natural materials either used in complementary medicine or the pharmaceutical field were investigated and methods of sampling, preparation and measurement were developed for the quality control of these materials. Solid materials were studied and although it was originally intended that only solid herbal materials and mixtures with possible adulterants be investigated, this has been extended to include work on essential oils for which NIR spectroscopy has proved to be a useful tool in their identification. It also seems likely that qualification of samples and determination of certain chemical constituents in these oils is possible and may provide as accurate and precise a technique as others in existence such as TLC and GC.

The questions listed below summarise the intended areas(s) of investigation and possible problems which may arise. It is hoped that methods of preparation, sampling, data acquisition and data analysis will be developed to overcome or minimise these problems.

- Is it possible to distinguish between different herbal materials and if so, how important is sample preparation in terms of sample size, particle size of the sample and species difference?

- Is it possible to quantify mixtures of two or more herbal materials? If so, how accurate is the quantification, what is the limit of detection and how important is sample presentation in terms of homogeneity of the mixture and sample mixture size?

- Is it possible to determine one or more specific constituents of a herbal material (including essential oils) using NIR spectroscopy correlated with a suitable reference method? What is the minimum number of samples required in terms of

calibration and validation sets?

- What chemometric (statistical) methods are best suited for data analysis?

1.3 Advantages and Disadvantages of NIR Spectroscopy

Advantages

Reviews by Blanco *et al* (1998), Shenk *et al* (1992) and work published by Brunner *et al* (2000) illustrate the use of NIR spectroscopy in the agricultural and pharmaceutical industry. The advantages of the technique are shown below.

- The low molar absorptivity of NIR bands (absorbance is 10 to 100 times smaller than in the conventional mid IR region) means there is no need to 'dilute' the samples prior to analysis. As such, samples can be scanned directly with little or no sample preparation.

- Quick analysis time. It takes an average of 40 seconds to obtain a single spectrum from a sample.

- Samples in many different forms can be studied – for example powders, liquids and oils and solids such as tablets.

- Analysis of 'unknown' samples is rapid once a library containing spectra of similar samples has been constructed.

- Samples can be subjected to both qualitative (for example identification or process conformity) and/or quantitative analysis (for example quantification of an excipient or constituent).

- There is no need for expensive or dangerous chemicals as are often required by

other methods such as HPLC or Karl Fischer titration.

- The ease of data acquisition and the simplicity of the NIR spectrophotometers allows quality control to be carried out by relatively unskilled technicians.

- The spectral data obtained are dependent not only on chemical information which allows the identification, qualification and quantification of materials in terms of their chemical constituents but also physical information, which allows for the measurement of particle size (O'Neil *et al* 1998).

Disadvantages

Although there are many advantages to NIR spectroscopy and the rapidity of the technique, and minimal sample preparation requirements are ideal for appropriate use in quality control, it will never remove the need for conventional techniques such as HPLC. It may be appropriate to replace traditional assays with NIR methods for the quality control of certain constituents in a number of materials or particle size analysis, but this is limited by the quantification limit of the technique and the accuracy of quantification, which in many cases will be poorer than that for HPLC and GC. In addition, NIR spectroscopy cannot be used to identify a substance unequivocally unlike techniques such as IR spectroscopy or Nuclear Magnetic Resonance (NMR) spectroscopy, although a substance can be identified to an acceptable degree of accuracy through the use of databases. However, quantitative calibrations are usually based on reference methods. A large number of samples sufficiently representative must be collected and scanned which may take a considerable amount of time. Data analysis and interpretation requires skilled personnel to create validated methods and the sample sets will need to be periodically updated to ensure the reliability of calibrations.

1.4 Theory and Principles of Near Infrared Spectroscopy

NIR spectroscopy in the pharmaceutical industry has been reviewed by Blanco *et al* (1998), which includes principles of the technique along with data analysis methods (see Chapter 2). The application of NIR spectroscopy to agricultural products has been reviewed by

Shenk *et al* (1992).

1.4.1 Introduction

The basic purpose of the technique of NIR spectroscopy is to obtain optical data from a set of samples and then to set up libraries of calibration sets containing the samples and 'teach' the system to link properties of these samples with their NIR spectra. For this reason NIR spectroscopy is entirely dependent on the information introduced to the system - before any meaningful data can be obtained, relevant information regarding the samples must be put in. Figure 1.4.1 gives a brief description of the steps involved in setting up a qualitative or quantitative NIR method. These steps generally include the acquisition of the NIR data of the samples to be used and secondly to analyse this data and turn them into either a meaningful qualitative method for identification and quality control purposes, and/or a quantitative calibration model which is then used to predict the property as defined by the method for incoming samples not used in the calibration.

1.4.2 Theory of NIR Spectroscopy

Spectroscopy can be defined as the study of the interaction between electromagnetic waves and matter. Electromagnetic radiation, which is in the form of energy travelling as a simple harmonic wave with a certain wavelength, interacts with the molecules or the atomic particles within molecules of the sample being studied. In the Ultraviolet and Visible regions this involves the transitions of electrons to higher excited energy states. In the mid IR and NIR regions vibrations of the molecules themselves occur.

The electromagnetic spectrum can be described in terms of frequency or wavelength, where wavelength is defined as the distance travelled in a complete cycle measured in nanometres (nm). The term wavenumber is often used, which is the reciprocal of the wavelength expressed as cm^{-1}. Although either term may be used when discussing NIR spectroscopy, the wavelength term is more commonly used.

Near Infrared is that part of the electromagnetic spectrum from 780nm to 2500nm and is located between the Visible (380nm to 780nm) and Infrared (2500nm to 25000nm) regions (Figure 1.4.2). Both NIR and IR spectroscopy arise from vibrational interactions between the electromagnetic radiation and the sample.

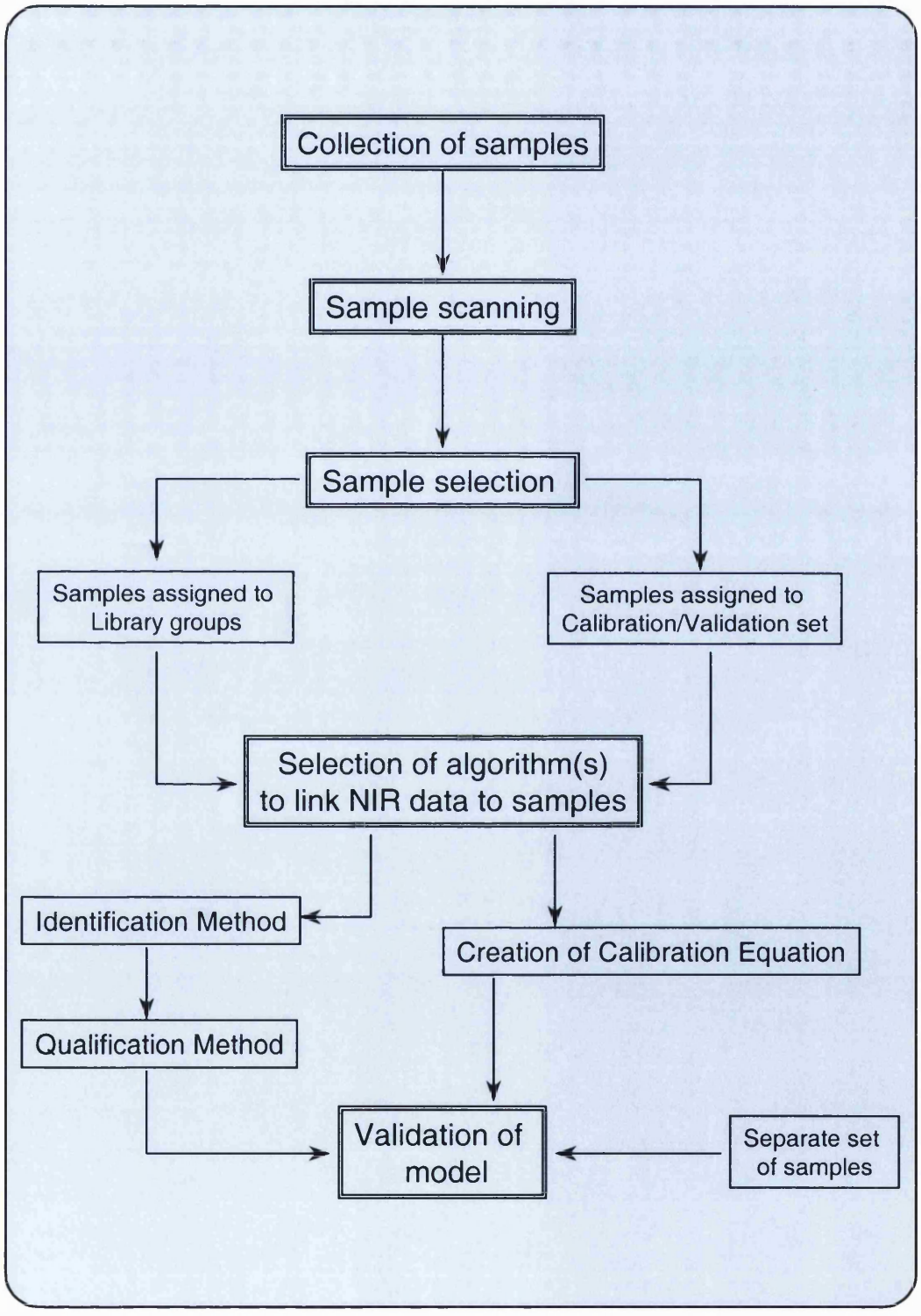


Figure 1.4.1 Creating a Qualitative / Quantitative Model using NIR Spectroscopic Data

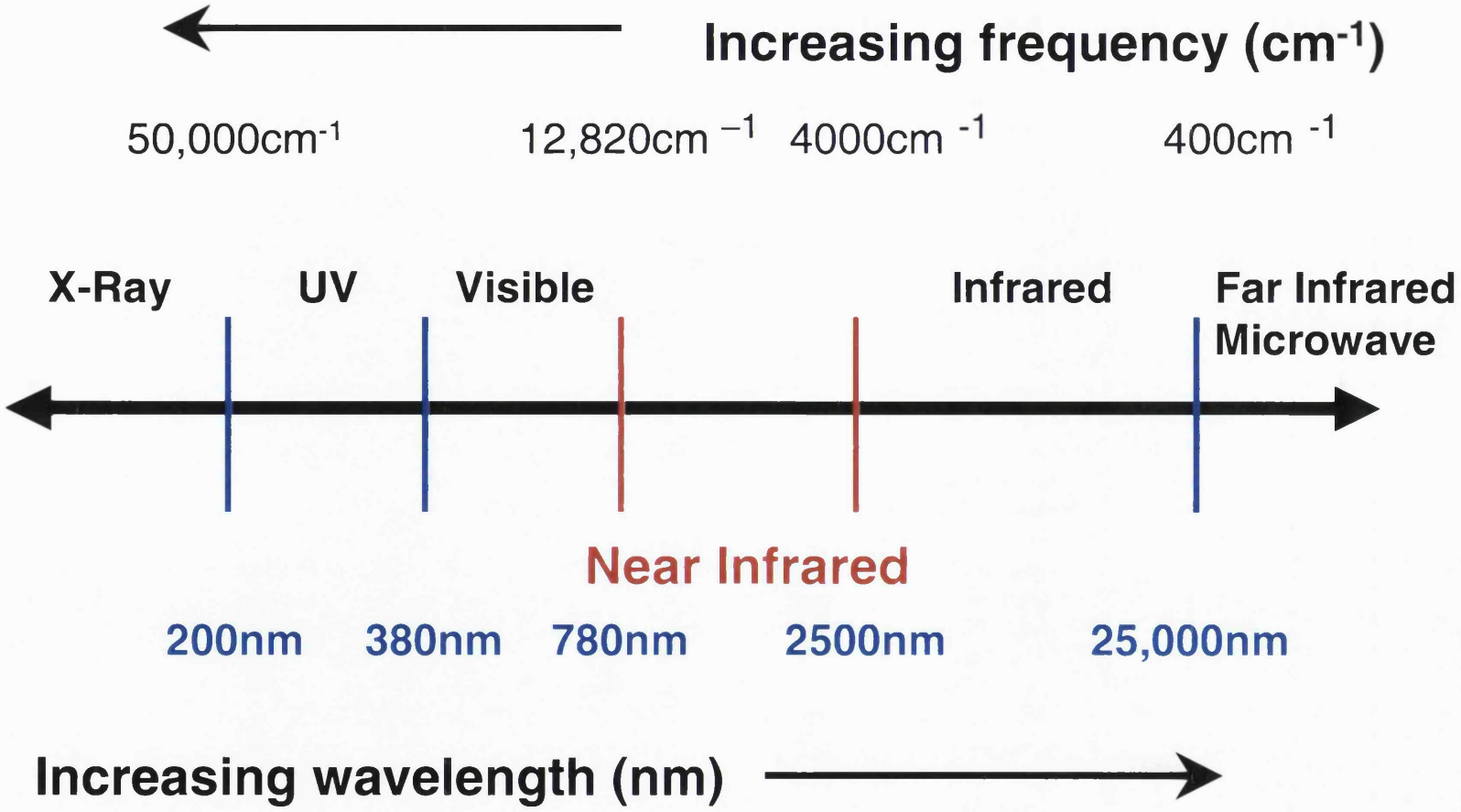


Figure 1.4.2 The position of the Near Infrared region in relation to other parts of the electromagnetic spectrum

Quantum Theory suggests that the energy of a molecule can only exist at certain values, or energy levels. Absorption of photons of the correct energy can cause the molecule to change between these vibrational energy levels. At room temperature, only the ground state has a significant proportion of this energy and so transitions due to absorption at these temperatures occur from the ground state (lowest energy level).

The absorption of IR radiation arises from the quantisation of vibrational energy within molecules, thus only certain frequencies (energies) of IR radiation will be absorbed by a given molecule. Such absorptions correspond to the stretching and bending frequencies of the bonds in covalent molecules. In the absorption process those frequencies which correspond to the fundamental bond vibrational frequencies, together with harmonics and overtones, are absorbed from the IR radiation.

Not all bonds within a molecule absorb in the IR region, even if the frequency of the radiation exactly matches that of the bond vibration. Symmetric bonds such as those in Cl_2 or H_2 do not show IR (or NIR) absorption. For absorptions to occur in both the IR and the NIR region, the bond must contain a changing dipole in order for energy to be absorbed and produce vibrational changes. Harmonic motion describes the fundamental bands that occur in the mid IR region.

Molecular vibrations can be described as harmonic or anharmonic. Harmonic oscillations obey Hooke's Law which describes the lateral movement of the molecule about its equilibrium, often described as the 'ball and spring' model. Many of the fundamental frequencies can be calculated roughly by assuming that band energies arise from the vibration of an ideal diatomic harmonic oscillator, essentially obeying Hooke's Law, which describes the stretching of a spring. The restoring force (F) exerted by the spring is proportional to the distance (x) travelled from the equilibrium and is given equation 1.4.1.

$F = - k x$ where k is the force constant (1.4.1)

Most molecules contain covalent bonds that share electrons between atoms. Although these

bonds are elastic, they do not obey Hooke's Law exactly. This gives rise to anharmonic vibrations. As two atoms approach, the repulsion between the nuclei causes the potential energy to rise more quickly than the harmonic approximation. The curve is steeper at the lower displacement (bond length) end of the curve. When the interatomic distance approaches the dissociation point, the potential energy levels off (Figure 1.4.3).

Absorption bands are produced when NIR radiation at specific frequencies (wavelengths) resonates as the same frequency as a molecular bond in a sample. The vibrational movement of molecules affects the degree of anharmonicity of the molecular vibrations. The greater the anharmonicity the greater the intensity of absorption. Vibrations within a molecule are known as either stretching or bending. Vibration in which there is a continuous change in the interatomic distance along the axis of the bond is known as stretching and may be symmetrical or asymmetrical. Bending vibrations involve a change in bond angle which are either in plane or out of plane. Bending vibrations may be further classified into scissoring, rocking, wagging and twisting. For any given molecule, stretching occurs at the highest frequency (lowest wavelength), followed by scissoring, wagging, twisting and rocking (Figure 1.4.4). Stretching vibrations have a larger amplitude of movement than bending and so give rise to the strongest intensity absorptions. R-H groups have the strongest overtones because the dipole moment is high. O-H, N-H, C-H and S-H bonds are also strong NIR absorbers. R-H stretch or R-H stretch/bend vibrations form most NIR bands. Hydrogen being the lightest atom and having the greatest degree of bond stretching and therefore anharmonicity gives strong absorption spectra.

The harmonic motion describes the fundamental bands that occur in the IR region and in this region the overtones are forbidden as Hooke's Law is obeyed. In the NIR region the energy transitions to an excited state higher than the first excited state are known as overtones (1st, 2nd, 3rd etc.) and transitions between multiple states gives rise to what is known as a combination band. The overtone bands are observed in approximately the 700nm to 1800nm region and the combination bands are observed in the 1800nm to 2500nm region.

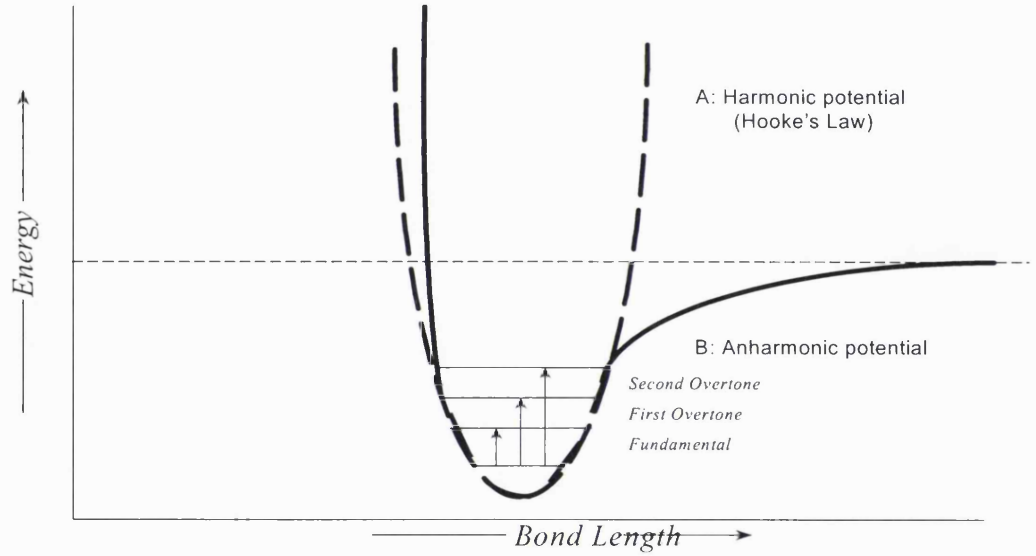


Figure 1.4.3 Energy Diagram

(Adapted from Burns and Ciurczak (eds.) Handbook of Near Infrared Analysis

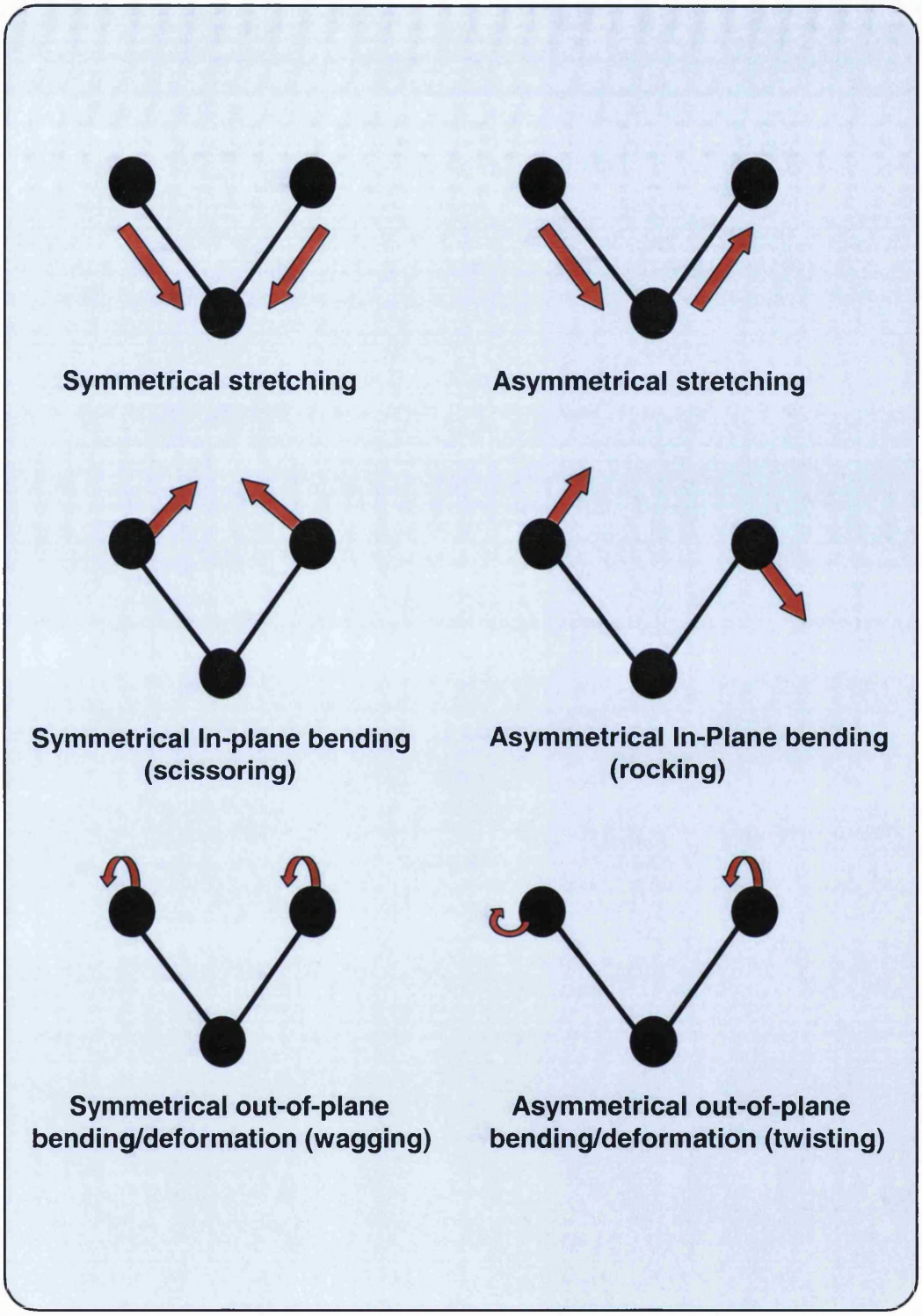


Figure 1.4.4 Modes of Vibration of a Molecule of the Type AX_2

For each fundamental absorption band there are a series of overtones with decreasing intensity. These weaker bands that occur in the NIR region are due to anharmonicity and are 10 to 100 times weaker than in the IR region and they occur at roughly ½ and ⅓ the fundamental absorption wavelength (resulting in the need for no or minimal sample preparation). The combination bands arising from a single fundamental absorption have increasing intensity with increasing wavelength (decreasing frequency). When using IR spectroscopy the sample has to be 'diluted', either by mixing with a liquid hydrocarbon (e.g. 'Nujol') or by pressing into a disc under a vacuum after mixing with powdered potassium chloride or bromide.

The relationship between NIR absorbance spectra and concentration in terms of quantitative NIR calibration is discussed in Section 2.2.

1.4.3 Band Assignments and Interpretation of NIR Spectra

In the mid IR region the fundamental vibrations occurring in specific regions of the spectrum can be used to identify unknown molecules. In simple cases it is possible to identify precisely each absorption. However, more complex IR spectra still provide valuable information, since each different type of bond has a different natural frequency and since the same type of bond in two different compounds is in a slightly different chemical environment, no two molecules will have exactly the same pattern of IR absorption. Although some of the frequencies absorbed might be the same, in no case will the IR spectra of two different molecules be identical. However, although this happens in the NIR region, and the vibrational overtones can be identified, these are more overlapping and cannot usually be placed in relation to the rest of the molecule. A particular band on a NIR spectrum consists of many bands containing information on more than one type of molecular vibration. There are, however, known regions of the NIR spectrum in which different bond vibrations occur and so some information can be gained regarding the structure of a substance (Ciurczak 1992, Halsey 1996, Osborne *et al* 1993a). Knowledge of the positions of the fundamental bands in the IR region allow the prediction of their corresponding overtone bands in the NIR region. The literature indicates the wavelengths of bond absorptions occurring for generic functional groups as well as those functionalities

most important to the NIR analyst, for example band characterisation wavelengths for moisture, carbohydrate and protein in the agricultural industry. The O-H vibrations of water exhibit a large absorption in the NIR region with two broad bands at approximately 1450nm and 1940nm. These bands contain information on hydrogen-bonded subspecies. Variations in hydrogen-bonded molecular subspecies (intermolecular and intramolecular) can cause band broadening and peak position shifts. Drying can be monitored through the use of NIR spectroscopy, although drying methods which result in the loss of volatile materials will give rise to different band positions and intensities as the ratio of pure water to bound or trapped water will vary.

The benefits of NIR spectroscopy in terms of its requiring no sample preparation , speed of spectra acquisition and the fact that both physical and chemical information are yielded by the spectra are exploited instead. As such NIR spectroscopy is not used to identify true unknowns. The advent of rapid-processing computers and sophisticated software programs able to manipulate data (spectral information) has opened up the great potential of NIR spectroscopy. It can be used as an identification and qualification tool through the comparison of NIR spectra with others compiled in a database. In addition, the correlation between analyte concentration or particle size and the intensity of an absorption in the NIR region allows accurate quantification using NIR calibrations.

1.5 INSTRUMENTATION

The main features of NIR instrumentation are given below:

- Light source: a tungsten-halogen filament lamp providing electromagnetic radiation in the wavelength region of 380nm to 3000nm

- Monochromator (if a pre-dispersive instrument). NIR Fourier Transform instruments also exist.

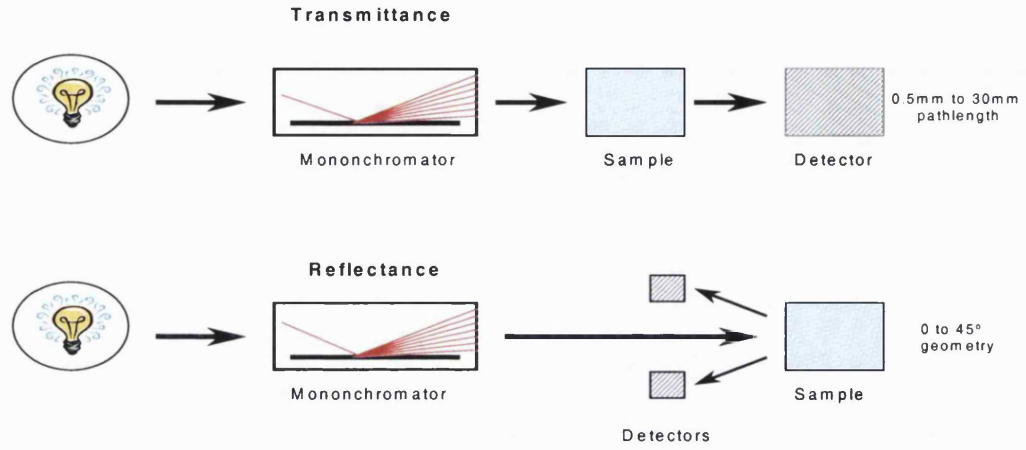
- Detector: This may be a reflectance or transmission (transmittance) detector. They

are made from lead sulphide for the 1100nm to 2500nm region and silicon for the 400nm to 1100nm region.

The monochromator is usually an oscillating concave holographic grating. The grating consists of a reflecting surface with fine laser-indented line. This converts white light into discrete wavelengths across the Visible/NIR region as the monochromator moves across in front of the light source. Figure 1.5.1 shows the basic set-up of NIR instrumentation.

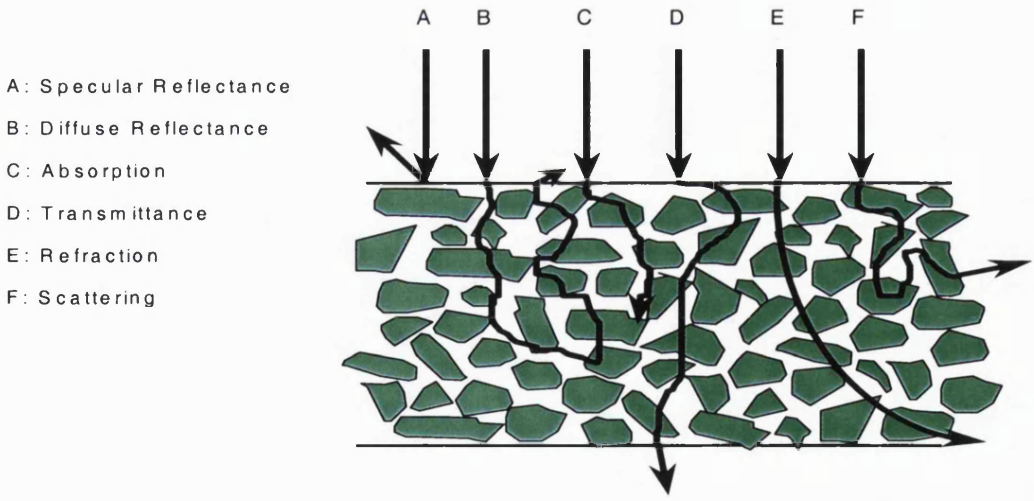
Instruments may make reflectance or transmittance (transmission) measurements depending on the sample module attached to the instrument. Throughout this work either reflectance or transflectance measurements were made and this will be discussed in more detail. Figure 1.5.2 shows the different ways NIR radiation may interact with the solid particles in a sample. With reflectance measurements, the detectors are positioned at 45° to the surface of the sample to achieve the maximum spectral information (from the diffuse reflectance) with the minimum of interference (from the specular reflectance). Figure 1.5.3 shows the FOSS NIRSystems Rapid Content Sampler module used to take diffuse reflectance measurements. The six detectors can be seen below the sample tablet. Specular reflectance occurs when the light directed at the sample is reflected directly back from the lower surface of the sample or the sample holder (for example a glass vial). This therefore gives no useful information about the sample itself and thus should be minimized. As such the detectors are placed at 45° to the bottom of the sample platform. Diffuse reflectance occurs when the light penetrates into the sample and interacts with it causing molecular vibrations. Much of this light is absorbed and scattered in all directions but some will be directed back from the sample towards the light source or the detectors.

There are several different measurement modules for NIR spectrophotometers. 'Static' transmittance or reflectance sampling modules are available which can be used to measure samples ranging from solid tablets, to powders and liquids. Fibre optic probes are also available which take reflectance measurements and can be used in an 'on-line' fashion in industrial situations, where the probe is 'dipped' into the incoming powder or liquid in the warehouse. The spectra obtained are then compared against a database and the samples tested for quality or conformity.



(Adapted from: FOSS NIRSystems Reference Manual)

Figure 1.5.1 Instrument Configurations



Adapted from: Burns and Ciurczak (eds.) Handbook of Near-Infrared Analysis

Figure 1.5.2 Interaction of NIR Radiation with Solid Particles in a Sample

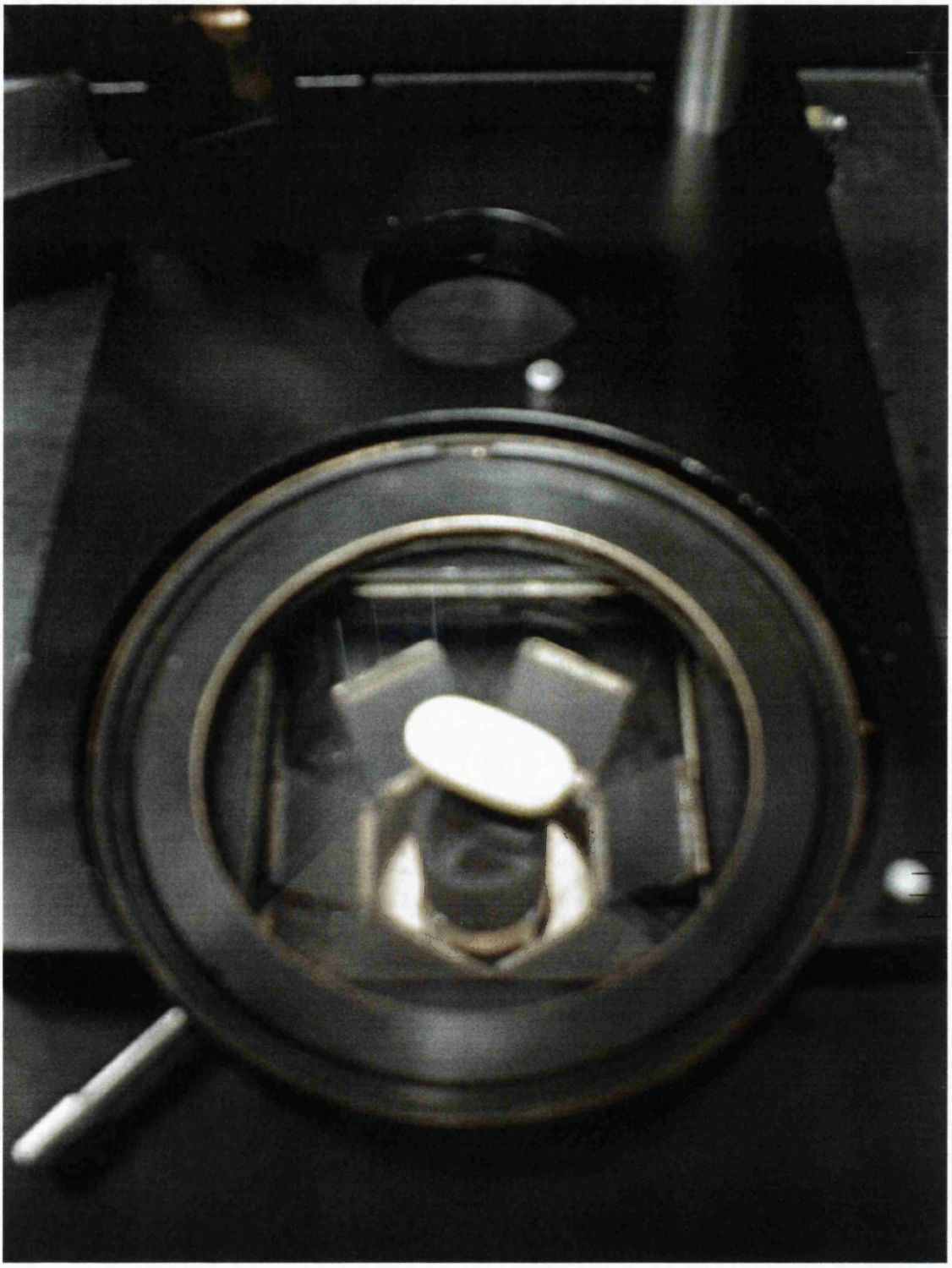


Figure 1.5.3 Position of Six Detectors on the FOSS NIRSystems Rapid Content Sampler so as to Minimise Specular Reflectance Interference

1.6 INSTRUMENT DIAGNOSTICS

Instrument manufacturers will include instrument diagnostics with the software that accompanies the equipment. Although the criteria may vary slightly, diagnostics are based on several tests, which are listed in the Pharmaceutical Analytical Science Group (PASG) Guidelines on the Development and Validation of Near Infrared (NIR) Spectroscopic Methods (PASG NIR Subgroup 2001). The criteria are described as follows:

Wavelength Accuracy: verified using a suitable standard at around 1200nm, 1600nm and 2000nm. The results should be within ± 1nm at 1200nm and 1600nm and ± 1.5nm at 2000nm.

Wavelength repeatability: verified using a suitable standard such as polystyrene or rare-earth oxides. The standard deviation of the wavelengths is consistent with the manufacturer's specification.

Response repeatability: verified using a suitable standard such as reflective thermoplastic resins doped with carbon black. The standard deviation of the maxima response is consistent with the manufacturer's specification.

Photometric linearity: verified by using a set of transmittance or reflectance standards (eg. Spectralon™ or carbon black). The observed response is plotted against the expected response. The slope of the line should be 1 ± 0.05 and the intercept 0.0 ± 0.05.

Photometric noise: this is determined using a suitable reflective standard, such as white reflective ceramic tiles or reflective thermoplastic resins. The reflectance standard is scanned in accordance with the manufacturer's recommendation and the photometric noise is calculated either peak-to-peak or, for a given wavelength, the standard deviation of the responses. The photometric noise should be consistent with the manufacturer's specification.

Vision® software provides a complete set of diagnostics functions to test whether the FOSS NIRSystems instrument performs according to the manufacturer's instructions. Instrument performance tests verify the reliability of NIRSystems spectrophotometers, which assures linearity, limits of detection and precision of NIR methods. It also performs a 'Wavelength Linearization' test, which checks whether the internal polystyrene reference peak positions correspond to the nominal positions and calculates the set of linearization constants that minimizes deviations from these nominals. This set of constants can be downloaded to the instrument and used in spectral acquisition.

CHAPTER TWO: DATA ANALYSIS AND INTERPRETATION

2.1 INTRODUCTION TO QUALITATIVE SPECTRAL DATA ANALYSIS

Unless otherwise stated, information is taken from Halsey (1996, 1998) and the PASG Guidelines on the Development and Validation of NIR Spectroscopic Methods (PASG NIR Subgroup 2001). NIR spectroscopy calibration basics have also been reviewed by Workman *et al* (1992).

Qualitative analysis can fall into the categories of either identification, qualification (quality control), or both. For identification work, a spectral Library may be set up to identify an unknown sample. A number of batches of the same material are scanned which are sufficiently representative of that material. When an unknown sample spectrum comes in, it is compared with the spectra in the database using one or a combination of different chemometric methods. In this way an unknown sample may be identified from the database if its statistical similarity to a spectrum in the database exceeds a pre-set threshold and there are no other matching spectra. This method is useful for confirming the identity of an incoming sample to a warehouse, the advantage of this technique being that the NIR method will be far quicker than conventional identification testing techniques such as HPLC.

Alternatively, or in combination, a spectral Library may be set up to monitor the quality of an incoming sample. For instance, several different batches of Aspirin may be set up in a database which specify the statistical criteria for the quality of further batches of the material. If an incoming sample spectrum is compared with the spectral Library, NIR spectroscopy and the associated chemometric methods will be able to either 'pass' or 'fail' the sample - a process known as qualification. Such a technique is often used 'on-line' in the pharmaceutical industry in the warehouse as pharmaceutical preparations and excipients are received.

The main software program used for data analysis in this work is that provided by the manufacturers of the NIR instrument, FOSS NIRSystems. This section will briefly cover some of the chemometric methods used in this Vision® software for the spectral data analysis in this research, but it must be borne in mind that there are several other equally

valid spectral data analysis programs, each with slightly different methods of data analysis.

2.2 CONSTRUCTION OF QUALITATIVE LIBRARIES

A Library is composed of Products, each Product containing sets of spectra representing the same data set. For example, each Product may contain three or more representative spectra, each of different species of the same plant material, or different species of the same genus. The Library allows identification and qualification of incoming materials. It is suggested that for a spectral Library containing chemically different samples, then 3-5 batches for each material is sufficiently representative, whereas for a Library containing several very similar materials, 10-30 batches are required.

Sample selection identifies statistically outlying spectra using one of several methods.

Identify method development is a global identification method set up for all spectra in the database and incoming 'unknown' samples.

Library clustering is another method of identifying spectra with different 'local' identification methods.

Qualify method development helps to confirm the identity of the unknown using more (or extra methods) and establishes the quality of the sample using pre-defined statistical thresholds.

Library Validation (not used) evaluates the suitability of the Library for use in identifying/qualifying incoming samples.

The statistical methods that may be used to create the qualitative Library are described in Section 2. 3. It is important when setting up a spectral Library that samples be included that contain the variability that would expect to be encountered in future incoming samples in routine laboratory use, but which would still be intended to fall within the specified

statistical thresholds. On the other hand, if using a Library for qualification purposes, it is equally important that the Library parameters be specified such that samples which one would not consider to be off sufficiently high quality would fall outside the pre-defined thresholds. Examples of sample variability are shown below:

- moisture
- particle size
- degradation products (for example with 'old' samples)
- similar species of plant material
- presentation (for example non-homogeneity of samples and probe insertion)

These factors and the extent to which they are considered depend on the intended scope of the spectral Library and the required discriminatory powers of the method.

A full or reduced wavelength range can be used. Sections of wavelengths can be removed if it is known that these contribute to irrelevant data (for example moisture content). Alternatively, specific wavelength regions can be used to enhance small but unwanted differences (Gerhäusser and Kovar, 1997).

2.3 QUALITATIVE IDENTIFICATION/QUALIFICATION METHODS AND THRESHOLDS

Only two of the four available identification methods available in the Vision® software will be discussed here, which have been used for all work to date. These two methods were employed by Blanco *et al* (2000) for identification and quantification assays for intact tablets and by Gerhäusser and Kovar (1997)to confirm the identity of 117 different drugs by using libraries based on full-range 2nd derivative spectra.

2.3.1 Correlation in Wavelength Space (CWS)

The identity of a sample is determined by comparing the sample spectrum with the average spectrum of each Product in the Library by means of the correlation coefficient. The

sample is identified as the Product representing the highest correlation coefficient whenever it exceeds its preset value.

In Vision®software, Libraries are constructed which contain the spectra of the samples of interest. Each spectrum is part of a Library Product, each Library Product containing a number of spectra of the same sample, or spectra of very similar samples. The CWS algorithm compares each individual spectrum in the Library, or the unknown sample spectrum being studied, to the mean spectrum of each Library Product. A diagrammatical representation of the CWS method is shown in Figures 2.3.1 and 2.3.2. Two spectra are marked 'R' and 'S' and the absorbance values (with or without spectral pre-treatments) for these two spectra are plotted. The Match Value is calculated by equation 2.3.1. The highest correlation is noted and if the correlation Match Value of a spectrum is lower than a specified threshold value (the default is 0.85 in Vision®) then a failed identification (ID) is given. If the Match Value is higher than the threshold value, a positive ID is given. The Match Values are in the range of 0 to 1, with two identical spectra having a Match Value of 1. A sample would be expected to be identified as part of the Library Product it is in, and not any other. If more than one Product has a correlation above the threshold value, the Products are identified as ambiguous. The CWS method is very sensitive to wavelength shifts, but not to absorbance shifts. It is therefore useful for chemically dissimilar materials (Vision® software manual 1999, Halsey 1996).

$$r = \frac{\sum RS}{\sqrt{\sum R^2 \sum S^2}} \qquad (2.3.1)$$

2.3.2 Maximum Distance in Wavelength Space (MDWS)

The MDWS algorithm checks the individual spectra in a Library Product against the mean spectrum for each Library Product and calculates the standard deviation at each wavelength. The one highest standard deviation is recorded as the distance value and not the average of all of them. If there is one peak or more where the standard deviation envelopes do not overlap (Figure 2.3.3), then the spectrum being studied will be distinguished from that Library Product.

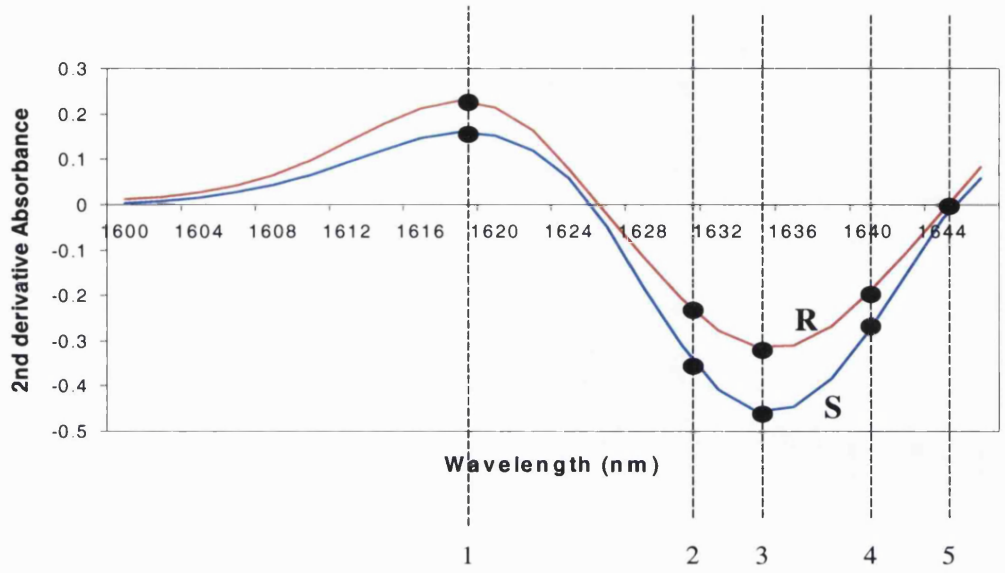


Figure 2.3.1 Diagrammatical Representation of Correlation in Wavelength Space (A)

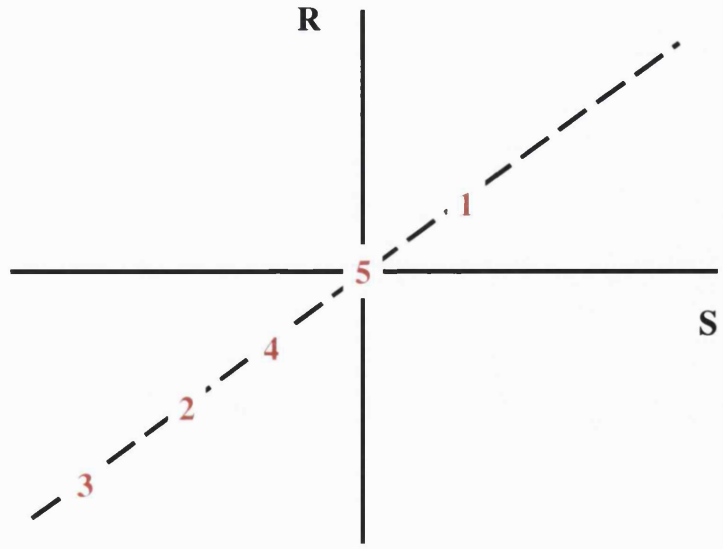
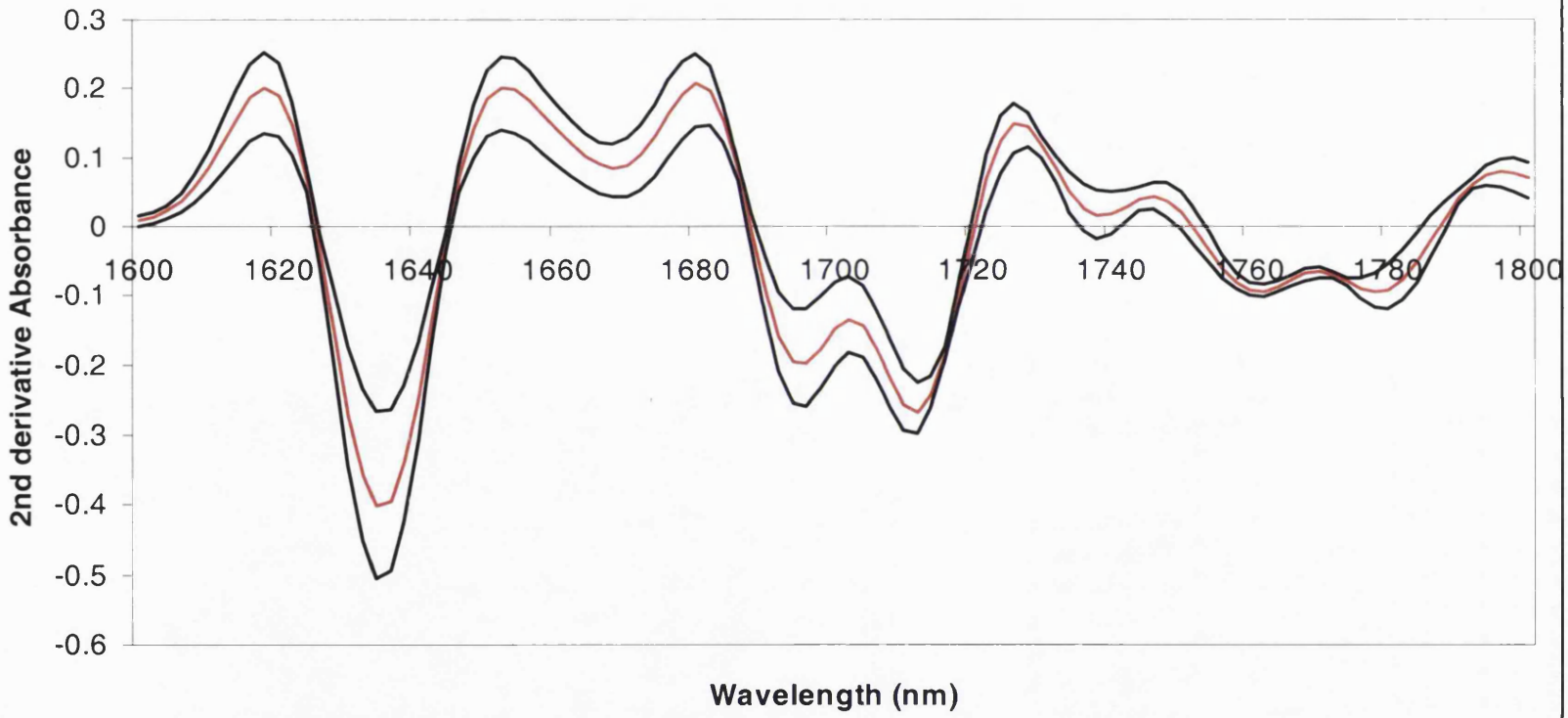


Figure 2.3.2 Diagrammatical representation of Correlation in Wavelength Space (B)

Figure 2.3.3 Plot of a mean 2nd derivative spectrum and its Maximum Distance in Wavelength Space envelope (plus and minus five standard deviations)



The threshold value is expressed in terms of standard deviations e.g. ∓6 s. A single value outside these control limits at any wavelength will cause the sample to be rejected. In contradiction with the CWS method, this algorithm is sensitive to absorbance changes and is therefore used for the identification and/or qualification of closely related materials. The lower the threshold, the more specific the identification method. See equation 2.3.2 below:

$$dxy = \max_{i=1}^{p} \left[\left| \frac{xi - \overline{yi}}{siy} \right| \right] \qquad (2.3.2)$$

This parameter describes the maximum distance d_{xy} between an unknown spectrum x and the average of each Product in the Library $\bar{y}$ in units of standard deviations s for the Product's training set. With the use of p wavelengths the distance can be calculated by the above equation.

The average spectrum at each wavelength point $\bar{y}_i$ and the standard deviation s_{iy} of a representative training set are calculated according to equations 2.3.3 and 2.3.4 below, where n is the number of spectra in the training set.

$$\bar{y}_i = \frac{1}{n} \sum_{i=1}^{n} y_i \qquad (2.3.3)$$

$$s_{iy} = \left[1 + \frac{1}{2(n-1)} \right] \sqrt{\frac{\sum_{i=1}^{n} (y_i - \bar{y}_i)^2}{n-1}} \qquad (2.3.4)$$

Gerhäusser and Kovar (1997) suggest that the training sets required for calibration of a Library by distance tend to be larger than when correlation is used. The distance threshold has to be determined individually for each drug substance, depending on the expected range

of variation of such physicochemical parameters such as the water content (Vision® software manual 1999, Halsey 1996).

2.4 Polar Qualification Analysis (Centre of Gravity Plots)

It is also possible to convert spectra from the Vision® software to Excel® format. This allows other methods of data analysis to be performed. Plotting of absorbance against reference values at selected wavelengths is a very simple way of visualising the spectral data and may be useful in identifying particular wavelength points along the spectrum that could be used for identifying and separating similar samples. The disadvantage of this technique is that as only a single wavelength is used for plotting absorbance at each axis, it is very sensitive to small changes in the NIR spectra of samples and a small change at that wavelength will have a marked effect on the plot. Another similar way of visualising data is to use the Polar Qualification System (PQS), which allows wavelength ranges to be used to construct the plot. This is far less sensitive to small changes in the NIR spectrum, but has the disadvantage that spreadsheet equations are required to transform the NIR data into the visual PQS system. The PQS system had successfully been used in the analysis of food products and was then applied to pharmaceutical substances from different sources (Van der Vlies *et al* 1995). They demonstrated that the method is able to detect smaller differences in chemical and/or physical properties than conventional algorithms which makes it an ideal tool for qualifying pharmaceutical substance by fingerprinting. A NIR spectrum is transformed to a single point on a plane. First the spectra are converted to Polar Co-ordinates and the centre of gravity of the resulting polar plot is calculated. Thus, these plots are also known as Centre of Gravity plots. The spectra are represented in a Polar Co-ordinate system in which the absorbance values are the radii and the angle α is a function of the wavelength. Often the spectra have some kind of mathematical pre-treatment, such as 2^{nd} derivative transformed spectra. The reason for this transformation is the fact that a polar spectrum has a well-defined centre of gravity that may represent a specific quality parameter. In such a polar spectrum, noise is randomly distributed around the circle of the polar co-ordinated system. Therefore, noise has very little effect on the centre of gravity

itself and noticeable differences between the spectra must be due to effects other than noise. By converting the NIR data into Centre of Gravity plots, the wealth of information present in each spectrum is reduced to one or more points, each lying in a two-dimensional plane and defined by two co-ordinates and a wavelength range. The PQS system was utilised in work carried out by Plugge and Van der Vlies (1996), which describes the use of NIR spectroscopy as a tool to improve quality by using the PQS method of data analysis to distinguish between a large number of samples supplied by various companies using a FOSS NIRSystems 5000 spectrophotometer. Use of the PQS system allowed differentiation between different grades of lactose (in terms of particle size) and also between crystalline, anhydrous and 'powder' forms of lactose. Further work by Kaffka *et al* (1998, 2000) on several food and industrial products, demonstrated that PQS is a drastic, but meaningful data-reduction method. They then further developed the system and three interpretations were given for the 'centre' spectrum, resulting in different formulae for determining the x and y co-ordinates of the quality point. The aim of the work was to introduce new spectral manipulation methods that allowed wavelength optimisation using PQS. Yoon *et al* (2000) used a PQS very effectively enabling the identification and qualification of raw pharmaceutical excipients.

Vision® software allows the export of the NIR data (as raw or pre-treated data) to Microsoft Excel® format. An in-house Excel® spreadsheet was used to construct the Centre of Gravity plots presented in this thesis.

2.5 Principal Component Analysis (PC Analysis)

2.5.1 Reasons for using PC Analysis

PC Analysis is a data analysis method whose development has relied on the introduction of computers with increased processing speeds sufficient to handle the many calculations required. It is a very sophisticated but powerful technique that allows spectral data to be visualised which can either be used for qualitative identification or qualification purposes, or which can then be used to create quantitative NIR calibrations (in combination with Multiple Linear Regression or MLR).

Much of the qualitative analysis in this thesis was carried out using the CWS and MDWS method. PC Analysis was used in addition to these methods for one or more of the following reasons:

- to detect possible outliers (see Section 4.3.5.2). An outlier is a spectrum that is different from the other samples in a data set that is either not well described by the qualitative calibration model or influences the model too much (Workman 1992).

- to identify samples which may be clustered with other samples. Samples lying in clusters are similar in nature.

- to identify the factor responsible for the majority of the variability within the samples. A high correlation between Principal Component (PC) 1 values (see below) and the reference data suggests that the majority of the sample variation is due to that variable, for example water content.

2.5.2 Definition of Principal Component Analysis

The definition of the American Society for Testing and Materials is as follows:

"Principal component analysis - a mathematical procedure for resolving sets of data into orthogonal components whose linear combinations approximate the original data to any desired degree of accuracy. As successive components are calculated, each component accounts for the maximum possible amount of residual variance in the set of data. In spectroscopy, the data are usually spectra, and the number of components is smaller than or equal to the number of variables or the number of spectra, whichever is less." (Mark 1992).

2.5.3 Principles of Principal Component Analysis

PC Analysis uses a mathematical algorithm that reorders the information in the spectral data matrix of the calibration set by defining a set of factor spectra, known as Principal

Components, which best describe the variance in the calibration set. The largest variance is described by the first factor (PC1) and with each following factor the amount of variance decreases until only the noise remains.

The PC Analysis method is a very useful tool of reducing the number of variables in a data seta and for obtaining useful two-dimensional views of a multi-dimensional data set. A data set consisting of 15 samples, for example, exists in 15-dimensional space , as this is the number of dimensions required to simultaneously plot all of the variables against one another. This cannot be represented by visual means but is the same concept as one-, two- or three-dimensional space. When all the data points are plotted they will form a cloud of points which may have an oval to circular cross-section in any particular direction (Figure 2.5.1). There will be the same number of axes as variables. The longest axis is defined as the first Principal Component (PC1), the next is PC 2 etc. The first Principal Component is that line which explains the greatest amount of variability in the data set. After this first factor (Principal Component) has been extracted from the data, another line representing the second Principal Component is drawn through the cloud of data points that represents the second largest variability in the data. Because each consecutive factor is defined to maximise the variability that is not captured by the preceding factor, consecutive factors are independent of each other. This means that they are uncorrelated or orthogonal to each other.

The total number of Principal Components will be equal to the number of variables in the data table. In this case, the variables are the number of samples in the data set. The maximum number of Principal Components that can be used to describe the data is the number of samples in the data set minus two (degree of freedom). The Principal Components form a new set of co-ordinate axes (Figure 2.5.2), replacing the original set of axes (the original variables). The Principal Components are orthogonal (uncorrelated) to each other. With a single variable, the points lie along a line, with two variables , they define a point in space and are described by a plane. With three variables, a three-dimensional plot is required, with a plane fitting through the data.

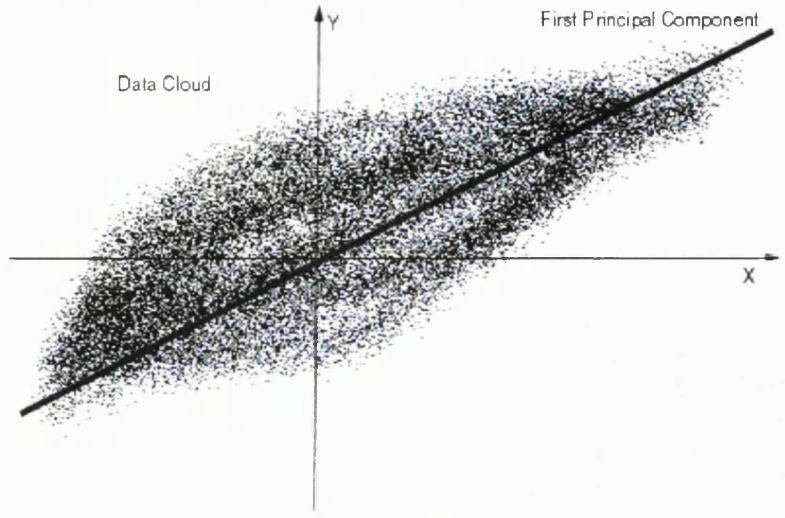


Figure 2.5.1 The First Principal Component in a Two-Dimensional Cloud of Data-Points. The first Principal Component is in the direction of maximum variance.

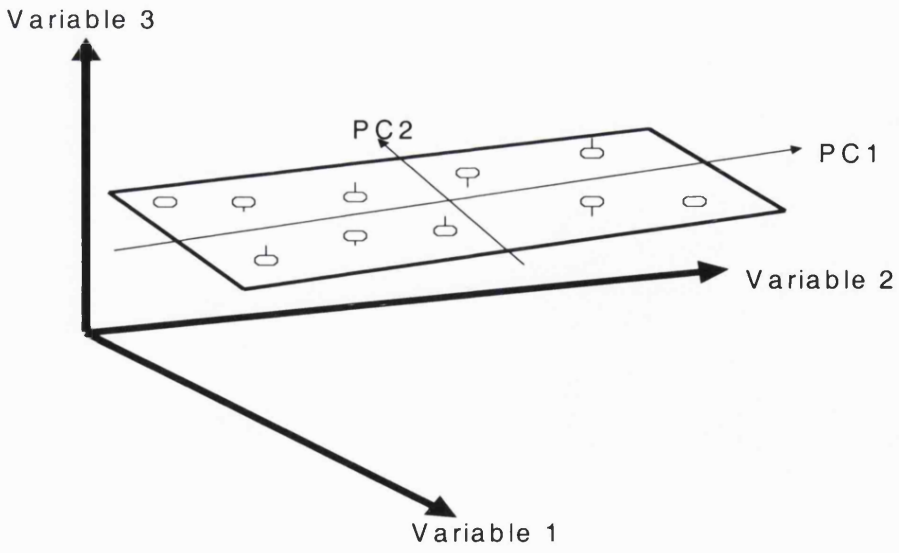


Figure 2.5.2 Principles of Projection for Principal Component Analysis (Adapted from The Unscrambler® Help System, version 7.6, CAMO Inc.)

With more than three variables, it is not possible to draw or create the plot, but the process remains the same where the axes are rotated so as to maximise the variance of the new factor. Usually, only the first Principal Components contain genuine information, while the later Principal Components are more likely to describe noise (The Unscrambler® 2000, Klaviter 2000, Statsoft 2002).

2.5.4 Information Provided by Principal Component Analysis

Each component of a PC Analysis model is characterised by three complementary sets of attributes, variances, loadings and scores.

Variances

Variances shows the proportion of the total variability between the data set samples described by each Principal Component. The higher the explained variance, the greater the proportion of variability within the data set is explained by that Principal Component.

Scores

Scores are calculated where information carried by several variables is concentrated onto a few underlying variables. Each sample in the data set has a score along each model Principal Component. The scores show the locations of the samples along each model component and can be used to detect sample patterns, groupings, similarities or differences (as described in Section 2.1.5.1). A two-dimensional scores plot can be constructed by plotting the value for a particular Principal Component against the score for another for each sample. Generally the most useful information is described using PCs 1 and 2.

Loadings

Loadings describe the data structure in terms of variable (wavelength) correlations. Each variable has a loading on a Principal Component. Variables with high loadings (close to +1 or -1) contribute greatly to that particular Principal Component. In The Unscrambler® software, a plot of the X-loadings against the X-variables (wavelengths) is given, which looks similar to a NIR spectrum. The wavelengths that contribute the most to a Principal

Component will have a high negative or positive loading value, which appears as a peak maxima or minima on this 'spectrum'. In this way it may be possible to determine what factor is contributing to the Principal Component. If the plot looks like a water spectrum with peaks at ~1430nm and ~1950nm that it is likely that water content is causing the variability described by that Principal Component. As another example, a loading plot may look similar to the NIR spectrum of a particular excipient in a tablet formulation, meaning that the excipient is likely to be the cause of the variability described by that Principal Component (The Unscrambler® 2000).

2.6 Principles of Quantitative NIR Analysis

2.6.1 The Relationship Between Absorbance and Concentration in Quantitative NIR Analysis

The ultimate goal of calibration is to calculate a mathematical model of the calibration data which is most sensitive to changes in concentration of the sample and least sensitive to non-concentration related factors, such as physical, chemical and instrumental variables. The best mathematical model is one which when applied to an NIR spectrum obtained from a sample of unknown concentration will provide a reliable, accurate and reproducible prediction of concentration for the analyte of interest.

In transmittance spectroscopy, the attenuation of the radiation transmitted by an absorbing sample is described by the Beer-Lambert Law, which states that absorbance is proportional to the concentration (C) of an analyte (equation 2.6.1):

$$A = \varepsilon C l \qquad (2.6.1)$$

where A is the absorbance of the sample at a particular wavelength, ε is the molar extinction coefficient of the sample at this wavelength and l is the path length (of the sample cell). The absorbance (A) is defined in equation 2.6.2:

$$A = \log\frac{1}{T} = \log\frac{I_0}{I} \qquad (2.6.2)$$

where T is the transmittance, I_0 is the measured radiant energy through the system at an appropriate wavelength in the absence of a sample and I is the measured radiant energy through the system at the same wavelength with the sample present.

Once the extinction coefficient is measured the equation can be used to find the concentration of other samples of the same analyte with an unknown concentration.
Where the Beer-Lambert Law rigorously holds, a plot of absorbance against concentration will result in a straight line through the origin and so in this situation only one sample with a known concentration would be required to produce this calibration line. However, in practice several samples need to be measured for the analyte concentration and a least-squares method is used to produce a line of best-fit (see Section 2.6.2 below).
Absorbances deviate from Beer Lambert Law at higher concentrations due most often to non-linearity of detection systems, scattering effects which are wavelength dependent, reflection, stray light and chemical interactions between components. Even when measurements are restricted to lower concentrations where the relationship between change in absorbance and concentration is linear, the calibration seldom passes through the origin and so a more realistic representation of the relationship between absorption and concentration is given in equation 2.6.3:

$$A = \varepsilon C l + A_0 \qquad (2.6.3)$$

where A_0 is the intercept.

The Beer Lambert law has been shown to be valid for the analysis of mixtures of hydrocarbons and amides and for water bound to protein. For a mixture of methanol and aniline on the other hand, deviations from additivity were observed and this effect was thought to be due to hydrogen bonding.

An approach to the problems of overlapping peaks (for example where a contaminant has

a peak which overlaps with the peak of the analyte of interest) and baseline correction involves the use of derivatives (see Section 2.10.2). The Beer Lambert Law will still apply to derivative spectra, i.e. the derivatised absorbance is still proportional to the concentration. The use of derivative data pre-treatment is often used to reduce non-zero bias, but derivatives do not remove multiplicative error and non-linearities.
Factors other than overlapping absorption bands that affect the Beer Lambert Law include particle size, interactions between the analyte and the solvent or other constituents, temperature changes and instrumental variations such as non-linearity and drift. The principal of the Beer Lambert Law still holds in simple cases and can be applied in such situations. Simple cases arise when the samples to be measured are clear solutions, or the solvent or matrix has no absorbance at the wavelength of analytical importance, if the analyte of interest has absorbance bands or if those absorbance bands are no interfered with by bands of the solvent or other components in the sample.

Diffuse reflectance NIR spectroscopy measurements are popular and this technique was used for all measurements of solid samples carried out as part of this research. These measurements are affected by the physical nature of the sample and the radiation is lost through forms of interaction with the sample (other than absorbance) as shown in Figure 1.5.2. The Beer Lambert Law is a rigorously derived model applying only to transmittance spectroscopy only.

Scattering is a major factor in diffuse reflectance NIR spectroscopy. For a sample that is opaque and non-absorbing, the incident radiation will be reflected in a manner which is described by the laws of mirrors. If the radiation that is transmitted through the first interface undergoes absorption then the transmitted radiation will be attenuated according to the Beer Lambert Law. The radiation emerging from this interface becomes diffused by random reflections, refractions and scatter at further interfaces inside the sample. When the radiation encounters discrete particles within the sample which are much larger than the wavelength, destructive interference becomes incomplete and the radiation propagates in all directions, a phenomenon known as scattering. The exact path of the propagating

radiation is extremely difficult to describe mathematically for particulate samples particularly if they also contain a heterogeneous distribution of absorbers. As such a definite reflectance theory does not exist, as the convolution of an infinite number of integrals would be required to describe all the combined light interaction effects at all surfaces under varying conditions. Consequently the Beer Lambert Law is often shown to illustrate the properties of NIR spectroscopy for lack of an ideal model. The absorption data is converted into apparent absorbance values (A), see equation 2.6.4.

$$A = \log R'/R = \log 1/R + \log R' \qquad (2.6.4)$$

where R' is the reflectance of a non-absorbing standard (e.g. ceramic reference standard) and R is the reflectance of the sample (R'>R). For monochromatic radiation log R' is constant and may be ignored.

The assumption is that the diffuse reflectance of an incident beam of radiation is directly proportional to the quantity of absorbing species interacting with the incident beam and so R depends on analyte concentration. Therefore this pre-treatment can be used to linearise the data. All references to absorbance values of NIR spectra in this thesis refer to the log 1/R corrected form. The use of this form means that NIR spectroscopic theory does not have to assume a linear relationship between the optical data and the constituent concentration, as data transformations or pre-treatments are used to linearise the reflectance data. The Kubelka-Munk function, which can also be used to linearise the data and includes normalised scattering and absorption terms is not generally used in NIR spectroscopy except for particle size measurements of for highly scattering solid samples. If the matrix absorbs at the same wavelength as the analyte, log 1/R will prove to be the most useful to relate reflectance to concentration.

Scattering is not a constant and depends on a number of properties of the sample, the most important of which are particle size and moisture content. Since scattering is inversely proportional to mean particle size, the radiation penetrates deeper into samples of larger

particle size and therefore log 1/R increases. The effect is to displace the diffuse reflectance spectrum along the y axis as a function of particle size. Scattering from particles is wavelength dependent with the high frequency/high energy (low wavelength) light subject to the most scattering and the higher the frequency the greater the sample penetration. In highly scattering samples, NIR energy may penetrate to only one or two particle thicknesses in depth. With highly absorbing samples, penetration may be up to several millimetres. The presence of water in a sample gives rise to characteristic absorption bands but also affects the overall spectrum since scattering depends on the ratio of the refractive index of the particles n, to that of the surrounding medium, n_o. As the moisture content increases so does the partial pressure of water vapour around the particles. Since the refractive index of water is greater than that of air this leads to a decrease in n/n_o, and hence scattering and therefore log 1/R increases. The factors affecting the degree and extent of scattering mean that scattering becomes a new unknown for each different sample and therefore the analytical utilization of diffuse reflectance spectra must be carried out on an empirical basis unless steps are taken to negate this effect.

Although the Beer Lambert Law is still the basis of the method applied in NIR spectroscopic analysis today, a simple measurement of absorbance is not sufficient to accommodate the variety of conditions encountered in many situations of analysis. Linear calibrations, also referred to as univariate calibrations, are most commonly used for simple calibrations of a single component with the relations for concentration versus optical data assumed to follow the Beer Lambert Law. Multi-component methods, also termed multivariate techniques, are used when a single wavelength will not predict concentration due to band overlap or when more than one component is to be measured simultaneously (Workman 1992, Osborne *et al* 1993b, Mark 1992).

2.6.2 Regression Analysis Using the Least Squares Method

It is not possible for a calibration equation to be constructed from only two data points in NIR spectroscopy due to the existence of errors and Regression Analysis needs to be carried out, where a best-fit line is generated through a number of points by 'Least Squares

Regression'. Regression Analysis is the use of the observations of the variables being considered to calculate a line of best-fit so that predictions can be made about the behaviour of the variables. The calculated line of best-fit is known as the regression line. A line is created that minimizes the residual distance between the data points and that line. Least squares regression can be carried out as y on x or x on y (Caswell 1999). The least squares regression method can be employed in three-dimensional space; for example a calibration plane where two wavelengths are selected in a MLR method.

2.6.3 The use of Multiple Linear Regression (MLR) in Spectral Data Analysis

Vision® software allows regression by MLR or Partial Least Squares (PLS). Most of the data analysis included in this thesis used MLR to construct the calibration equations. Data analysis should be kept as simple as possible, whilst remaining scientifically sound and valid. MLR is a simple but effective technique that can be easily explained and validated and if sufficient for the purpose should be used where possible. MLR is particularly suited to simple matrices where absorbances at specific wavelengths can be used to describe the spectral data and related to the reference data.

When applying Regression Analysis to NIR data, the absorbance of the sample at one or a combination of two or more wavelengths is correlated to the reference value. If a single wavelength is chosen this reduces to simple Linear Regression and is effectively the same as plotting the absorbance of the samples at that wavelength against the reference data. Where the data points represent absorbances of the data samples at a single wavelength, a two-dimensional best-fit line can be drawn. In the situation where more than one-wavelength has to be taken into account, for example a two-wavelength MLR method (as described previously), a three-dimensional best-fit plane rather than a line has to be created.

MLR selects that wavelength which has the highest correlation with the reference data. With a single wavelength a correlation coefficient is calculated, with MLR a multiple correlation coefficient (R^2) is given, where a value of 1 signifies a perfect fit and a value of 0 denotes a complete lack of relationship between the NIR spectra and the reference

values. Inclusion of more wavelengths in MLR increases the multiple correlation coefficient up to a point but there is danger of 'over-fitting' the spectral data which will make the calibration equation unfit for use in quantifying incoming unknown samples. 'Over-fitting' of a calibration means that predicted values in very close agreement with the samples in the calibration set, but will results in very poor predictions for incoming samples. The problem of co-linearity should be avoided. This occurs when two or more wavelengths selected in MLR describe aspects of the data set that are related. While such a model may describe the calibration set very well, it may be very sensitive to noise or systematic errors in the calibration data which may not be representative of the samples in general. Consequently, MLR models with co-linearity among analysis wavelengths may not provide reliable analysis of real samples. As a very general rule of thumb, one wavelength per ten sample spectra in the calibration set can be used.

2.6.4 Equation Describing the MLR Calibration

The Vision® software describes calibration equations developed in terms of the intercept of the line K(0) and the slope of the line K(1) at the wavelength chosen, as shown in equation 2.6.5:

$$Y = K(0) + K(1)\lambda_1 + K(2)\lambda_2 \ldots\ldots\ldots + K(n)A\lambda_n \qquad (2.6.5)$$

where Y is the predicted analyte concentration, $A\lambda_1$ and $A\lambda_2$ represent the absorbance values at the wavelengths specified.

2.7 Feasibility Study and Visual Interpretation of NIR Spectra

By visually inspecting the NIR spectrum of each individual component in the matrix, it may be possible to identify a unique region of absorbance for the constituent of interest. It may also be advisable to determine if the absorbance is linear with increasing concentrations of the constituent of interest. This can be done by producing a series of mixtures of the constituent to be quantified in a simple diluent. However, as it is known that the sample

matrix can have a profound effect on the NIR spectrum of a product, spiking the samples to be analysed with increasing amounts of the constituent may be a more valid method. If the absorption band for the constituent of interest is in a simple matrix and is not masked by interfering absorbance bands from other substances present in the matrix, it is likely that a simple MLR method could be used. If the sample matrix is more complex, a PLS method may be more suitable.

Although it is possible to carry out a feasibility study in this manner for a pharmaceutical material, where the separate constituents are readily available and samples can be spiked easily, the situation for the quantification of constituents in natural products is much more difficult. For example, with an essential oil, it would be impossible from a practical point-of-view to scan in the individual components (either after separation by HPLC, GC or from laboratory standards) of the oil and then to visually to compare the spectra. In this case, simply comparing the NIR spectrum of the oil with the NIR spectrum of a pure sample of the constituent to be quantified would be more sensible and time-efficient.

As part of the feasibility study it is also necessary to decide on the number of scans per spectrum to be used in the data collection. The most commonly used setting (and default when using the FOSS NIRSystems 6500 spectrophotometer) is 32, which means that the sample is scanned 32 times and each spectrum obtained is an average. By increasing the number of scans per sample, the reproducibility of the sample spectra is improved, although this is offset by the extra time taken to obtain a spectrum (it takes about 40 seconds to perform 32 scans). The number of spectra to be obtained from each sample should also be ascertained. For samples with larger particles size and/or a greater degree of non-homogeneity, it is necessary to take an average of a greater number of samples, in order that the spectrum representing the sample is reproducible. This will influence greatly the precision of an NIR method and should be considered carefully. However, the amount of time it would take for a reproducible average spectrum to be obtained by increasing the number of spectra used is outweighed by the need for the NIR method to remain a rapid technique and a balance should be achieved between the two (Halsey 1998).

2.8 SAMPLE COLLECTION

Often a great deal of time, even more than that of obtaining the NIR data from the samples to be used in a calibration, is spent on the collection of the samples. It is important that several criteria are satisfied by the sample set for fully validated methods. These are outlined below.

- The sample set must be representative in terms of physical and chemical properties of any samples that may be encountered in the future, for which the calibration is called upon to accurately predict the parameter of interest

- The calibration set samples should contain a linear range of values for the analyte of interest that extend beyond the lower and upper limits of samples to be tested

- A sufficient number of samples should be included in the calibration set

The number of samples required to construct a calibration depends on the regression method used and the complexity of the sample matrix. A PLS regression method will require more samples than an MLR method, and more samples will be required with an increasing number of wavelengths used in an MLR equation. As a general rule, 10 samples should be included for every wavelength factor employed although this will also depend on the complexity of the matrix; complex substances naturally requiring more samples to be included, as the sample set needs to take into account the varying amount of constituents other than that to be quantified present in the samples.

As mentioned previously, the work carried out outlined in this thesis is intended as a demonstration of the potential capability of NIR spectroscopy for quality control in this area. For this reason, sample collection was limited to obtaining samples of as many different brands and batch numbers as was possible in terms of time and the number of sources as possible.

2.9 REFERENCE ANALYSIS

Choice of reference method is extremely important. Reference methods include HPLC and GLC for separation and quantification of chemical constituents, Karl Fischer titration analysis for moisture or specified British Pharmacopoeia (BP) and European Pharmacopoeia (EP) assays in monographs for specific materials.

Several points must be noted here. Firstly, although a NIR calibration method may be more precise than the reference method, it can never be proved to be more accurate than the reference method, as the calibration is constructed from the reference data. The only way an NIR method could be described as more accurate than the reference method is if both methods are compared against a third method. Even then, however, this conclusion cannot necessarily be drawn. In the instance of moisture content determination, for example, the Karl Fischer titration method will produce different water content determinations from the oven loss-on-drying method because the water of crystallisation etc. is also taken into account and mass lost from the oven method may also include volatile materials. In this case, an NIR method calibrated using Karl Fischer data will compare very poorly with loss-on-drying data for the same samples.

Following on from this point, when carrying out data analysis on calibration results, the reference method is assumed to be accurate, whether it is or not! Quite clearly, the reference method should be as accurate as possible because errors will be transferred into the calibration equation and the equation may be totally useless in terms of predictive ability, even if the calibration statistics are acceptable.

The reference method should be carried out more than once on at least one of the samples in order to determine the precision of this method which can be later compared to the NIR method reproducibility. In addition, if any outliers are detected in the validation set, the possibility of an error in the reference method result can be discounted. Ideally the reference analysis should be carried out on samples as soon as they have been scanned to minimize the possibility of sample changes over time. Samples should be analysed in a random order if it is known what the nominal analyte content of the sample is, as this will minimize systematic errors.

2.10 MATHEMATICAL PRE-TREATMENT OF SPECTRA

Often many different combinations of mathematical pre-treatments will be tried before the most suitable one is found, although a knowledge of the type of pre-treatments available, how they work and their effect upon the spectra is useful. Often, raw spectra are mathematically treated in one or a combination of ways in order to maximize spectral information. This section covers those mathematical treatments that have been used in this research.

2.10.1 Standard Normal Variate (SNV)

This is a scatter correction method and normalises spectra when the effective path length varies between samples in the same data set. This is particularly useful when scanning samples such as natural products which have been crudely ground to a course powder using a pestle and mortar. Pathlength variation arises when particle size varies between samples and/or when sample presentation in the cell is not reproducible. The spectrum is mean centered and then divided by its standard deviation (equation 2.10.1.):

$$SI^{SNV} = \frac{(si - \bar{s})}{S.D.} \qquad (2.10.1)$$

where the standard deviation, S.D. is calculated by the equation 2.10.2

$$S.D. = \sqrt{\frac{\sum_{i=1}^{n} (s_i - \bar{s})^2}{n-1}} \qquad (2.10.2)$$

where $\bar{s}$ is the mean absorbance across the whole wavelength range, n is the number of wavelengths and s_i is the absorbance value (units will vary according to the method of pre-treatment of the spectrum, if any) at each wavelength i (Barnes *et al*, 1989, Vision® software manual 1999).

As an increase in particle size results in increased absorption, NIR spectroscopy can be used for particle size determination. Clearly, in this case the use of the SNV correction would be inappropriate. The underlying upwards curvature of spectra in the NIR region is due to the multiplicative scatter effect. The spectra are closer together at 1100nm than at 2500nm. There is a high degree of co-linearity (correlation) between data points in the log 1/R spectra which is a function of scatter and variable path length. A comparison between the raw (un-treated) NIR spectra and the same spectra with the SNV correction is shown in Figure 2.10.1

2.10.2 Derivatives

Derivatives are a widely used mathematical pre-treatment method, but it has really only been possible with the onset of computers easily capable of such data manipulation and is used to enhance spectral data. The use of increasing derivatives (that is 1st, then 2nd, then 3rd etc) gives increasing resolution but is offset by a decrease in signal-to-noise (S/N) ratio, but because this is so high in the NIR region this is still useful. Generally, only the 1st or 2nd derivative is applied to the spectra.

Derivatives are employed to improve peak shape and resolution. They are calculated as the difference between absorbances at adjacent wavelengths (i.e. absorbance is differentiated with respect to wavelength). Figure 2.10.2 shows spectral derivatives and Figure 2.10.3 compares the raw (un-treated) spectrum of a powdered Agar sample and its 1^{st} and 2^{nd} derivative counterparts.

The segment and gap sizes (measured in nm) used to calculate derivatives are very important. NIRSystems instruments record data every 2nm and so a segment of 20nm corresponds to 10 data points. However, an odd number of data points is always used and so an extra one is added to allow for a central data point.

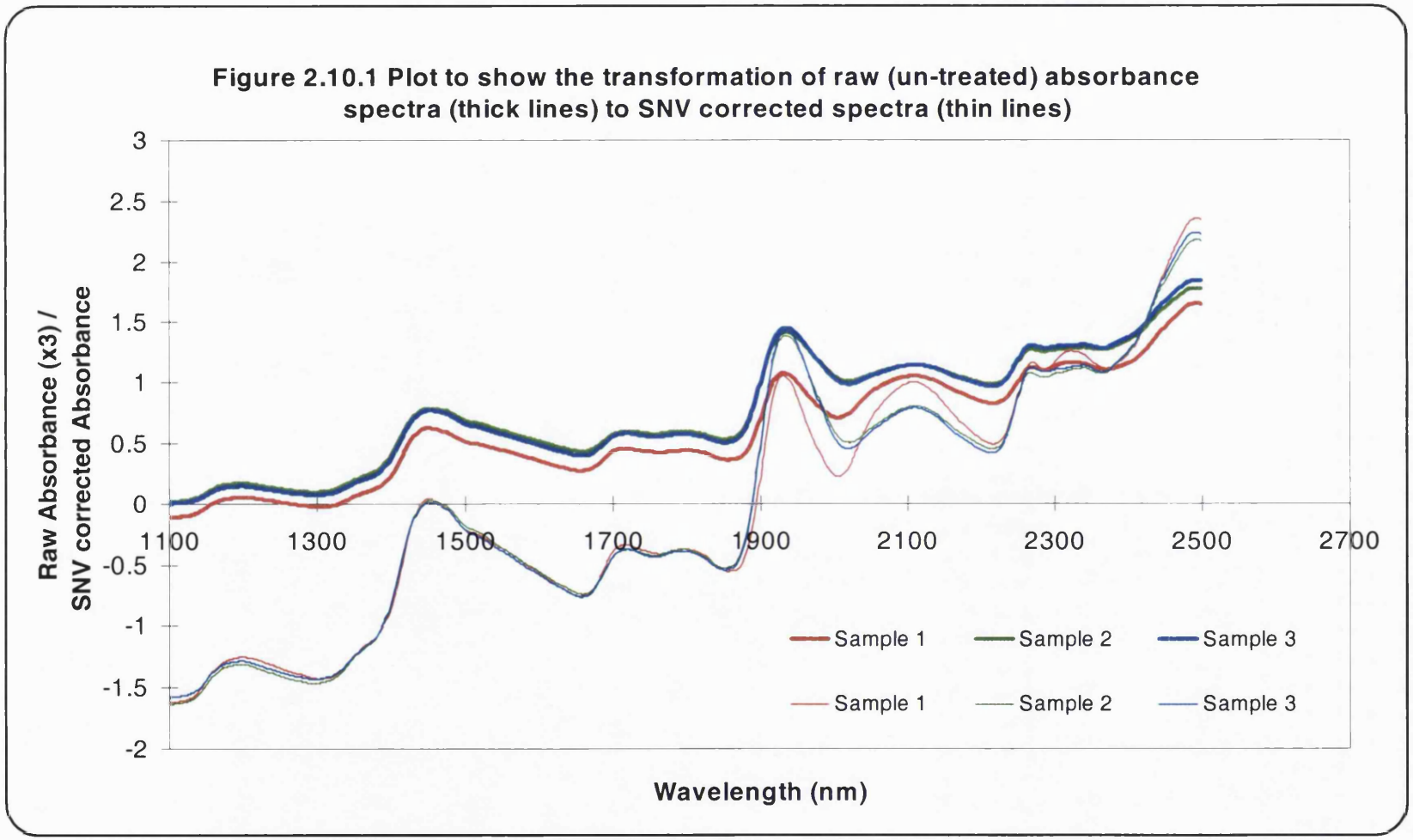


Figure 2.10.1 Plot to show the transformation of raw (un-treated) absorbance spectra (thick lines) to SNV corrected spectra (thin lines)
Raw Absorbance (x3) / SNV corrected Absorbance
3
2.5
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
1100
1300
1500
1700
1900
2100
2300
2500
2700
Sample 1
Sample 2
Sample 3
Sample 1
Sample 2
Sample 3
Wavelength (nm)

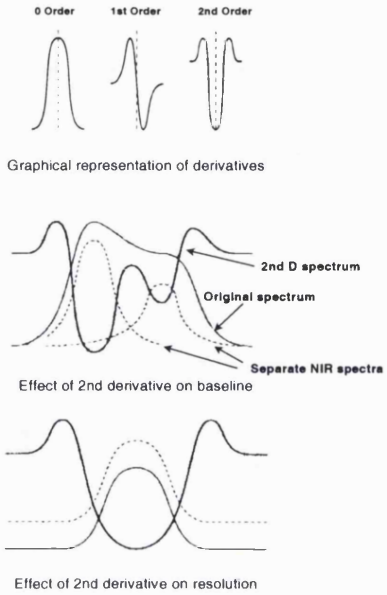


Figure 2.10.2 Spectral Derivatives and their Effects

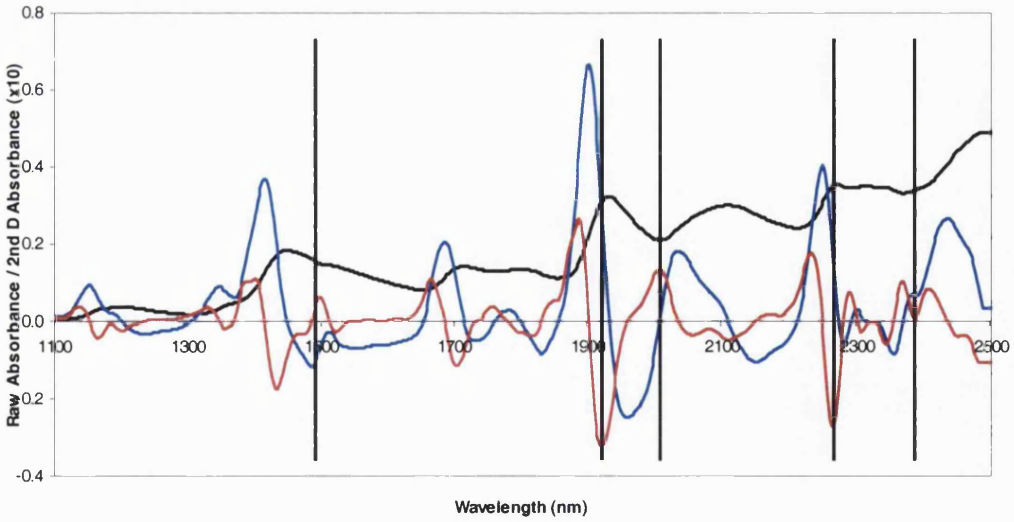


Figure 2.10.3 Comparison of a Raw (un-treated) NIR Spectrum and its 1st Derivative and 2nd Derivative Counterparts

With increasing gap size, there is more smoothing (because of fewer data points) and spectral information may be lost. However, a segment size of 20 data points may lead to a more robust calibration for quantitative analysis than a segment size of 10 data points. These and many other parameters may have to be tried in data analysis to find the most suitable combination. In NIR analysis , it is generally this that takes up the time. Gap size will not be discussed here and in all data analysis using derivatives a gap size of 10nm was employed. The 1st derivative is used most commonly to eliminate baseline offset variation within a set of spectra. The 2nd derivative is used to minimise both offset and slope (Halsey 1996).

2.11 Selection of Samples for the Calibration Set

The type of sample selection method available are specific to the software package used for data analysis. Vision® software allows sample selection by a three different methods. Samples may be split randomly (in a proportion defined by the user) between the calibration and internal validation sets. Alternatively each sample can be manually designated as a calibration, validation or outlier set sample. This can be useful where it is important to ensure that an even distribution of samples in terms of analyte concentration are included in the calibration set. Another method of sample selection is by statistical analysis of the spectral data, where outliers may be detected and removed. This method is useful for a large number of samples, but is less valid when only a relatively small number of samples is used.

2.12 Wavelength Selection in Multiple Linear Regression

Wavelength selection in MLR can be done manually, after visual inspection of the spectra, or selection can be made by the software. Vision® software uses a forward search method whereby the first wavelength selected for use in the calibration is that found to have absorbance values for the samples with the highest correlation to the reference data. If a second wavelength is required, then this one is chosen because it provides the highest

correlation to the reference data in combination with the first selected wavelength. More wavelengths can be selected by the software in the same way. Wavelengths can be added by linear summation, where the second wavelength absorbance is added to that of the first wavelength, or linear division, where the second term is divided into the first wavelength. Several problems may be encountered with either method of wavelength selection and it is vital that the wavelengths selected can be justified scientifically. With manual selection, it is found that the wavelength which looks as if it should have the highest correlation with the reference data is often very poor. Alternatively, the wavelength selected by the software may seem quite obscure.

If selecting a wavelength manually, it may seem sensible to select the wavelength at the peak of greatest absorbance band of the analyte of interest (in the appropriate direction if using derivatives). However, the signal-to-noise ratio is constant across the absorbance band and so there is no reason to prefer one wavelength over another within this spectral range. However, greater sensitivity is achieved if the largest peak is chosen to reduce possible interference from peak due to other compounds present.

With each addition of a wavelength to a MLR calibration, the calibration accuracy improves. However, there is the likelihood of 'over-fitting' the data (as mentioned previously in Section 2.6.3) Auxiliary statistics provided by Vision® when performing regression analysis help to decide the most appropriate number of wavelengths to be used in generating the equation (see Section 2.14.3 for further details).

2.13 Interpretation of the Calibration Equation Results

The following questions should be answered in order to justify the developed calibration equation. Justification of the wavelengths selected will improve confidence in the calibration in terms of predictive ability of future sample analyte determination.

Does the correlation peak increase in the same direction of the analyte peak?

It is vital that the first wavelength corresponds to the Absorbance of the analyte, in other words, that wavelength corresponds to an increase in the analyte concentration, and not the reduction of an interfering Absorbance from another constituent present in the sample. If using second derivative NIR spectra in the calibration, increasing absorbance from an increase in analyte concentration will occur in a negative direction, and so the wavelength selected must occur somewhere along a negative peak.

Is the equation robust, yet sensitive?

The correlation peak displayed by Vision® software can also be used to determine the stability of an equation. It should be as broad and flat if possible, which is an indication of a more robust wavelength, whereby small changes in absorbance do not adversely affect the calibration. Conversely, the calibration should also be sensitive. Vision® software plots the slope coefficient K(1) of the calibration equation against wavelength. The smaller the coefficient of the slope, the more sensitive the calibration is to small changes in the spectra. The sensitivity of the calibration should tend towards zero.

2.14 AUXILIARY REGRESSION STATISTICS

The Vision® software provides several different statistical values that are useful in interpreting an NIR calibration and other calculations can be made to assess its quality. The factors used in the work covered in this thesis are shown below.

- Multiple Correlation Coefficient
- Standard Errors
- F value
- % mean accuracy and bias and % relative error
- Comparison of the reference and NIR predicted results: residuals and confidence intervals for testing of statistical difference between the two

2.14.1 Multiple Correlation Coefficient

The correlation is a measure of the degree of linear relationship between two variables. A regression line calculated by Least Squares Analysis when considering NIR calibrations, may either be the absorbance (NIR data) values *vs.* the Concentration (reference) values or the NIR predicted *vs.* the Actual (reference) values.

The correlation, r, may be positive or negative and the close the value to 1, the higher the degree of linear relationship between the two variables. It is obviously easier for 10 points to lie close to a straight line in a scatter diagram than for 100 points to do so. Therefore a lower value for a correlation coefficient would be expected for a regression line based on a large number of observations (and thus being more representative of the 'true' relationship between the two variables), even though the actual degree of relationship between the two variables was the same. One disadvantage of the correlation coefficient is that it does not take into account the degrees of freedom (number of variables or wavelengths) used in the calibration. For this reason the F value (see below) is also given by Vision® software. The correlation coefficient also gives no indication of the predictive ability of the equation, and therefore over-emphasis of this value should be avoided. In NIR calibrations, linear regression only is acceptable, but in other techniques when one variable is linked to another, a low correlation coefficient does not mean there is no relationship, just that the relationship is not linear. However, it should be borne in mind that when plotting predicted values against actual values using a non-linear NIR equation, a line of $y = x$ would still be expected.

2.14.2 Standard Errors

The Standard Error of Calibration (SEC) is a statistical parameter that indicates the upper limit of accuracy in future predictions. When the calibration equation is applied to the calibration set of samples themselves, the SEC is calculated from the residual, f the difference between the predicted and the actual values at data point i equation 2.14.1.

$$SEC = \sqrt{\frac{\sum fi^2}{n-K-1}} \qquad (2.14.1)$$

where n is the number of samples and K is the number of wavelengths selected.

This equation takes into account the fact that the more factors used in the construction of the NIR calibration, the fit to the calibration data can appear to be better than it really is. In the absence of instrumental error, the Standard Error is a measure of the error of the reference method. It is also an indication of whether the predictions provided by the calibration equation will be sufficiently accurate for the purposes for which they are being generated.

The Standard Error of Prediction (SEP) is the same as the SEC but this time the calibration equation is applied to the validation set (i.e. those samples not included in the calibration set). One disadvantage of using the Standard Error is that it is an absolute value and therefore does not take into account the analyte concentration values within the range of the calibration and validation sets. Even with a supposedly small SEC it is clear to see that if the relative error of the predictions are high (for instance over 5%) then the accuracy of the calibration would not be acceptable. For this reason, the % mean accuracy (Section 2.14.5) is also calculated.

2.14.3 The F Value

The F value (equation 2.14.2) is a useful estimate of goodness of fit of spectral and reference data. It can also be used as a tool for how many wavelengths should be used in an equation and for determining which samples to eliminate as outliers from the calibration set.

$$F = \frac{R^2(N-K-1)}{K(1-R^2)} \qquad (2.14.2)$$

where N is the number of samples, K is the number of wavelengths used in the equation and R^2 is the Multiple Correlation Coefficient.

If the F value increases, then the addition of another wavelength to the calibration may be appropriate, if it decreases, then 'over-fitting' may occur and the SEP may increase in value to demonstrate this. However, these are not hard and fast rules and occasions may arise whereby an decrease in the F value (linked to the SEC) may be accompanied by a decrease in the SEP, in which case addition of another wavelength may be appropriate. The F value may also indicate some samples to be outliers if it increases on deletion of the sample from the calibration set.

2.14.4 Residuals

Vision® software displays plots of the differences between the NIR predicted and the reference values, against the NIR or reference values. In general, the residuals should be evenly distributed about the zero line, effectively showing there is no bias in the calibration equation. Some calibrations are non-linear over a large range and this can be seen by the residual points fall into a curve with increasing constituent value. If this occurs, then it may be appropriate to split the analyte range into two or more groups for separate calibrations.

2.14.5 % Mean Bias and Accuracy and % Relative Error

The SEC can often be deceptive, as it does not take into account the size of the analyte concentration being predicted. For this reason the % mean bias and accuracy (equations 2.14.3 and 2.14.4) are calculated. These are relative values and take into account the size of the reference value, by comparing the differences between the NIR predicted and reference values for each sample used in the calibration/validation set.

$$\%\ \text{Mean Bias} = \frac{\sum_{i=1}^{n} \frac{(\text{Predicted}_{\text{value}} - \text{Reference}_{\text{value}})}{\text{Reference}_{\text{value}}}}{n} \text{x}100 \qquad (2.14.3)$$

$$\% \text{ Mean Accuracy} = \frac{\sum_{i=1}^{n} \frac{|(\text{Predicted}_{value} - \text{Reference}_{value})|}{\text{Reference}_{value}}}{n} \text{x100} \qquad (2.14.4)$$

The % mean accuracy is essential an average of the relative error and this may be adequate in determining if a particular calibration is performing well enough or not. However, if it is imperative that no sample is predicted outside a certain accuracy, then the % relative error should also be calculated. This is calculated from equation 2.14.5.

$$\% \text{ Relative Error} = \frac{\text{Predicted}_{value} - \text{Reference}_{value}}{\text{Reference}_{value}} \text{ x 100} \qquad (2.14.5)$$

2.14.6 Confidence Intervals for the Detection of Statistical Difference Between the NIR Method and the Reference Method

The mean and confidence interval of a number of repeat measurements are found for both the NIR method and the Reference method. If the Confidence Intervals overlap, then there is no statistical evidence for difference in values obtained by the two methods and have equal precision. The Confidence Interval is calculated by equation 2.14.6.

$$\bar{x} \pm \left(t \times \frac{\sigma_{n-1}}{\sqrt{n}} \right) \qquad (2.14.6)$$

where $\bar{x}$ is the mean reading, t is the Students t distribution with (n-1) degrees of freedom, where n is the number of readings and σ_{n-1} is the standard deviation calculated by equation 2.14.7.

$$\sigma_{n-1} = \sqrt{\frac{n\sum x^2 - (\sum x)^2}{n(n-1)}} \qquad (2.14.7)$$

2.15 Other Methods of Quantitative Analysis

Although the majority of NIR calibration models in this work were constructed using MLR on occasion calibrations were created using the Principal Component Regression (PCR) method and the Partial Least Squares Regression (PLSR) method. These two methods are briefly described in this section. The main difference between MLR and the PCR and PLSR methods is that the former uses one or more discrete wavelengths to construct the NIR calibration model, whereas the latter two uses the whole spectrum or specified portions of it.

2.15.1 Principal Component Regression

Principal Component Regression is a two-step procedure. Firstly, the spectral data is analysed by PC Analysis (as described in Section 2.5) and then MLR is used to fit a calibration equation to the plane described by the Principal Components used in the PC Analysis. In this way the Principal Components instead of the raw data are used as predictors. Like with conventional MLR, PCR models only one Y-variable at a time (The Unscrambler® 2000).

2.15.2 Partial Least Squares Regression

PLSR combines the use of Principal Components with regression analysis. It uses Principal Components to explain the variation in the spectra, whilst as the same time giving the best fit to the reference data. In other words, PLSR is an improvement on the PCR method as it creates functions that can better relate to the reference data (for example constituent content assay values) rather than the Principal Components being simply those that are best related to the NIR spectra. PLSR often required fewer Principal Components to reach the optimal solution because the focus is on the dependent variables. PLSR may also give better results if there are large spectral variations which are not related to the properties of interest. A PRESS value statistic (Prediction Sum of Squares) is the calculation that determines the optimum number of factors (or PCs) to be used in the calibration. The PRESS value is calculated as follow. First, a single sample is removed from the calibration set and the remaining samples are used to create the calibration. This calibration is then

used to predict this sample and the difference is noted between the predicted and the actual value and used to calculate the sum of squares. This procedure is repeated for the other samples in the calibration set.

A second term, the Standard Error of Cross Validation (SECV) is used, which is similar to the PRESS value except that it is calculated from the square root of the mean squares for residuals. Each factor used in the creation of the NIR calibration (determined by the PRESS value) has an associated SECV (Halsey 1996,1998, Workmann 1992, The Unscrambler® 2000).

2.16 Full Method Validation: Fulfilling the ICH and PASG Guidelines

The International Conference on Harmonisation (ICH) is responsible for setting down requirements for the registration of pharmaceuticals for human use. A ICH Harmonised Tripartite Guideline was developed for the validation of analytical procedures included as part of registration applications submitted within the European Community (EC), Japan and the USA (ICH Tripartite Guidelines 1995). The text (known as the ICH Parent Guideline) states that it is not intended to provide direction on how to accomplish validation but serves as a collection of terms and their definitions. A tabular summation of the characteristics applicable to identification, control of impurities and assay procedures is included in the document. A second companion text: 'Validation of Analytical Procedures: Methodology' goes into further detail about the required validation characteristics and suggests how these may be fulfilled (ICH Tripartite Guidelines1997).

According to the ICH:

'The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose'.

Although the natural products industry at present (apart from those herbal medicines that hold a marketing authorisation/Product Licence) are not subject to these guidelines. Most

of the companies working within this industry will have their own 'in-house' validation guidelines to a greater or lesser extent.

The purpose of this research was not only to assess the suitability of NIR spectroscopy for the quality control of natural materials, but as these are intended for pharmaceutical (therapeutic use) in some form or other, to incorporate similar validation tests that are applied to pharmaceutical preparations and materials. As such, when constructing a NIR calibration, the ICH guidelines for validation may be applied to the calibration. It must be noted again at this point that fulfilment of the these ICH criteria by the calibration developed does not provide conclusive proof that the technique could be used in industry, as it has already been stressed that many more samples would need to be included in a commercially developed calibration. However, fulfilment of these criteria do serve to more fully validate the calibration and suggest strongly that such a technique would be suitable.

The typical validation characteristics that the ICH guidelines propose to be considered are shown below:

- accuracy
- precision: repeatability (short-term) and intermediate
- specificity
- detection limit
- quantitation limit
- linearity
- range
- robustness

Moffat *et al* (2000) published work which successfully applied these ICH Guidelines to a NIR reflectance assay of paracetamol in intact tablets. All of the validation criteria were considered in turn and provided an interpretation of the way in which the ICH Guidelines could be applied to an NIR calibration. The paper emphasised that in fact these Guidelines

were written with separative techniques such as HPLC and GC in mind and not for 'stand-alone' spectroscopic methods. It was also pointed out that the criteria specified were more suited to univariate mathematical method than for multivariate calibrations as are commonly used in NIR spectroscopy. A European Pharmacopoeia (EP) monograph and a British Pharmacopoeia (BP) monograph exist for the general use of NIR spectroscopy for pharmaceutical identity testing but does not include guidance on the criteria to be fulfilled as part of the validation of a method (EP2002a, BP 2001a). As such, the Pharmaceutical Analytical Sciences Group (PASG) set up a sub-group on NIR spectroscopy in collaboration with other experts in the field to provide guidance on the qualitative and quantitative analysis of pharmaceuticals by NIR spectroscopy (PASG NIR Subgroup 2001). The work by Moffat *et al* (2000) which interpreted the Guidelines for application to an NIR calibration was carried through in the Guidelines later published by the PASG, entitled 'Guidelines for the Development and Validation of Near Infrared (NIR) Spectroscopic Methods'. Earlier work had also been carried out by Plugge and Van Der Vlies (1993). The PASG acknowledge the NIR Technical Note supplied by Sheelagh Halsey for FOSS NIRSystems customers (Halsey 1998) and similar comprehensive notes regarding the development of an NIR calibration are reproduced in the Guidelines.

The validation guidelines set out by the PASG cover NIR Identification, Qualification and Quantification tests. The possibility of re-validation should be borne in mind, especially in certain circumstances such as changes in the synthesis of a drug substance, changes in the composition of the finished product, changes in the finished product manufacturing process or sources/grades of materials and changes in the analytical procedure of the NIR instrumentation. The document also contains guidelines for NIR instrumentation specifications, equipment validation and other qualification criteria and advice on the setting up of Identification and Qualification libraries and Quantitative calibration is also included.

2.17 SYSTEM SUITABILITY TESTING

This is discussed in the PASG Guidelines and is described as qualification tests appropriate to the intended use of the NIR spectrophotometer. The tests is proposes as a guide are outlined in the section on instrumentation. Moffat *et al* (2000) demonstrated system suitability by using those tests incorporated into the software of the manufacturer, and included, wavelength accuracy (linearisation), wavelength repeatability and bandwidth accuracy.

2.18 ROUTINE NIR ANALYSIS

The use of Routine NIR Analysis for use in an industrial/commercial environment is beyond the scope of the work covered in this thesis. When setting up a calibration for use in an industrial/commercial environment it is vital that the calibration model be fully validated by a second, separate set of samples covering the same analyte range. A more comprehensive validation would include samples that had been collected over a period of months, to ensure that adequate variation has been accounted for by the calibration.

The Full Method Validation, also beyond the scope of this work is covered by the ICH Guidelines criteria as described in Section 2.16. However, these criteria were adapted and used to assess the initial calibrations developed during the work, as the set-up of a full commercially acceptable calibration model was not the purpose of this research.

Routine NIR Analysis covers the set-up of a program that performs the quantitative calibration model on samples immediately after their NIR data have been obtained. The program may also combine a qualitative method that identifies and performs quality control on all incoming samples. If several calibrations are included in the program, then the results of the qualitative analysis determines which calibration model to be applied to the sample.

CHAPTER THREE:

MATERIALS AND METHODS

This chapter will cover the different instrumentation and sample presentation accessories used and also the reference methods applied for each piece of work. For the purposes of clarity, each piece of work will be considered separately in the Results Chapters and each set of results will also include a brief section covering the specific materials and methods used, along with an introduction to the intended purpose of the work.

3.1 INSTRUMENTATION, SOFTWARE AND SAMPLE ACCESSORIES

Instrumentation

FOSS NIRSystems 6500 spectrophotometer (NIRSystems Inc., Silver Spring, MD, USA)

FOSS NIRSystems Rapid Content Sampler module with HPLC glass vial holding plant material on the sample stage (Figure 3.1.1).

Software

NSAS™ software for NIR measurements and averaging of sample spectra (FOSS NIRSystems, Silver Spring, USA)

Vision® software version 2.50 for NIR measurements, spectral averaging and data analysis (FOSS NIRSystems, Silver Spring, MD, USA)

Sesame software version 3.1 for data analysis (Bran+Luebbe GmbH, Norderstedt, Germany)

The Unscrambler® software version 7.6 for data analysis (CAMO Inc., Corvallis, Oregon, USA)

The different types of data analysis used by the Vision®, Sesame and The Unscrambler® software are given in Table 3.1.1.

Sample presentation accessories

Waters® HPLC 4mm quartz glass vials (disposable)

Agricultural liquid sample cell (FOSS NIRSystems, Silver Spring, USA)

Reflectance vessel (FOSS NIRSystems, Silver Spring, USA)

Stainless steel reflectance disc (manufactured at the School of Pharmacy, London, UK)

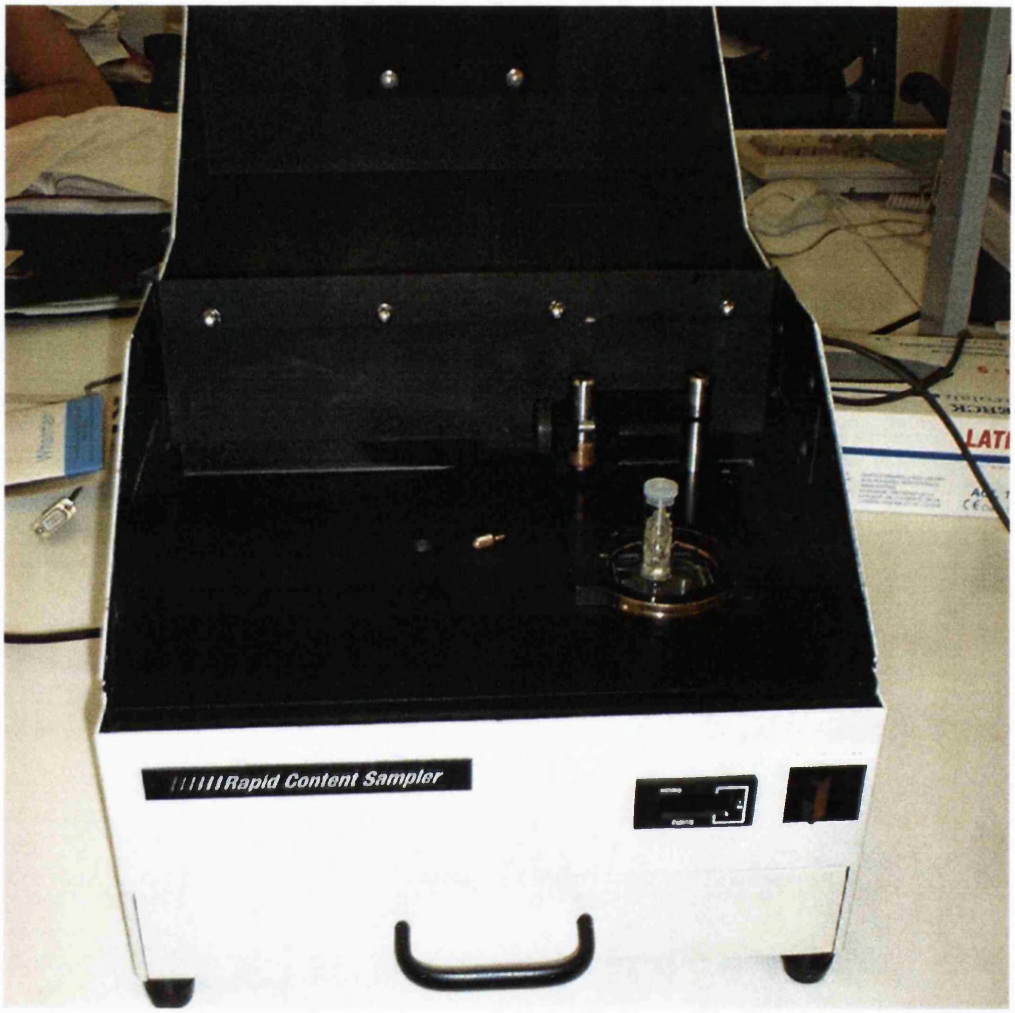


Figure 3.1.1 Rapid Content Sampler Module used for Diffuse Reflectance Measurements

	Software		
	Vision® 2.50	**Sesame 3.1**	**Unscrambler® 7.6**
Company	FOSS NIRSystems, Inc.	Bran+Luebbe GmbH	CAMO Inc.
Address	Silver Spring, MD, USA	Norderstedt, Germany	Corvallis, Oregon, USA
Qualitative techniques:			
Library method	Correlation in Wavelength Space ✔ Maximum Distance in Wavelength Space ✔	Principal Component Analysis	
Cluster method	Principal Component Analysis (✔)	(✔)	✔
Quantitative techniques:			
Multiple Linear Regression (MLR)	✔	✔	(✔)
Partial Least Squares Regression (PLSR)	✔	✔	(✔)
Principal Component Regression (PCR)	✘	(✔)	✔
Data pre-treatment methods:			
SNV	✔	✘	✘
2nd derivative	✔	✔	✔

Table 3.1.1 Data analysis methods for the Vision®, Sesame and Unscrambler® software

✔ = option used, ✘ = not available, (✔) = option available but not used

3.2 NIR Sample Presentation and Scanning Methods

It is possible to measure liquids and solutions by transmittance using either fibre-optic probes or quartz glass vials or tubes that fit into a specially designed holder. The path length (and therefore the width for glass vials) is typically between 0.5mm and 4mm but this depends on the transparency of the sample. The presence of air bubbles should be eliminated and the sample temperature kept constant. Air is taken as the reference. Transmittance measurements are popular for solids and have both advantages and disadvantages over the use of reflectance measurements. Specially designed tablet and capsule holders are available commercially, along with those available for the use of powders in glass vials.

Transmittance is generally more suitable for those tablets which are coated and capsules because reflectance measurements where the NIR radiation only passes into a small area of the sample will give information about the coating or capsule only and a relatively small amount of information about the tablet itself or the capsule content.

Sample accessories for the measurement of the diffuse reflectance of samples include, vials holders, sample cups and reflectance probes either for direct scanning by insertion into the sample or indirect scanning through a packaging material. An appropriate reflectance standard, one that is completely reflecting (non-absorbing) such as ceramic or Spectralon™ should be used.

The PASG Guidelines (PASG 1997) note the effective sample size that provides NIR measurements. In comparison to conventional techniques, even though the amount of sample included in the presentation accessory may be comparable, the area illuminated by the NIR beam is significantly smaller. This means that the technique is capable of detecting non-homogeneity of samples and although this may be used in specific applications for powder blending for example, in most other applications a method of averaging the NIR measurements of sample to minimise this must be found. This may be accomplished by the use of sample cups that spin the sample through the NIR beam, or taking the average of a number of samples prior to data analysis and calibration or Library development.

For liquids, a 'transflectance' method of sample presentation was employed. Measurement by transflectance is suitable for liquid samples and solid suspensions. An inert diffuse reflector such as dispersed titanium dioxide or a reflecting metal surface is used. Again the presence of air bubbles should be eliminated and the sample temperature kept constant. All transflectance measurements for the work covered in this thesis were taken using a reflectance vessel, described below. Aqueous liquids cannot be scanned in glass vials because the path length is far too long and most of the NIR light is lost as transmitted radiation from the other end of the vial. However, an alternative to the use of the reflectance vessel is through the use of barium sulphate powder at the bottom of the vial, as this reduces the path length sufficiently and effectively changes the sample presentation from reflectance to transflectance.

An appropriate reference dependent on the presentation method should be used. Some types of sample may be non-homogenous in terms of particle size and/or the mixing of constituents. Often samples are ground to an appropriate particle size range before sample scanning which will be of considerable help in the development or an NIR calibration, because even with the mathematical pre-treatment of data (see Section 2.10), effects due to particle size are not always totally removed. The benefits of minimising particle size variation has to be balanced with the extra time and money this will entail and of course, all incoming samples to be tested will have to be ground in the same way. The problems of sampling of materials in various forms is something that would have to be taken into consideration in the commercial environment; especially so when sampling raw natural materials from a larger batch sample.

3.3 PRESENTATION METHODS

The following accessories were used for sample presentation to the Rapid Content Sampler® module of the NIR Spectrophotometer.

3.3.1 Vials for Solid Samples

4ml disposable quartz glass Waters™ HPLC vials were used for the solid samples, which

were all in powdered form of varying particle sizes. These vials are ideal for use in NIR analysis because the bottoms are spectroscopically clear and provide minimal interference to the sample spectra (Yoon *et al* 1998) and also allow for an appropriate depth of sample to be used. For fine powders a depth of approximately 0.5cm is adequate, with samples with larger particle sizes of mixtures of samples with significantly different particle sizes, both or which have larger gaps between the particles will require a depth of approximately 1cm.

3.3.2 Agricultural Liquid Sample Cell

This cell is suitable for moderately viscous liquid samples and as such was used for the characterisation and identification of essential oils (see Section 4.1). The liquid sample cell comprises two circular ceramic discs; one with a reflective anodised stainless steel surface and the other with a circular quartz glass window. A few drops of the oil are placed on the reflective surface and the two discs screwed together. Light from the instrument is passed through the sample stage window, then through the quartz glass window, and then the sample, where it then reflects off the reflective surface and passes back through the cell. The measurements, although carried out by a diffuse reflectance measurement module are essentially 'transflectance' measurements. The total path length is 2 x 1mm and it is this small path length that allows oils to be studied by NIR. Five spectra were taken, the cell being rotated about the centre between each reading. Each reading was the average of 32 scans.

To minimise differences in spectra due to the presence of air bubbles, the sample cell was required to be cleaned and application of the sample to be repeated twice more, resulting in a total of 15 spectra obtained for each oil. These spectra were then averaged using NSAS® software before this single spectra for each sample was transferred to Vision® software for data analysis. Although only a few drops of oil are required, the oil is not recoverable for practical purposes and the necessity for repeat measurements increases the amount of oil required.

3.3.3 Reflectance Vessel for Liquid Samples

The reflectance vessel is also suitable for measurement of liquid samples in the 'transflectance' mode. This method of sample presentation replaced the agricultural sample cell for later work because it was significantly easier to eliminate air bubbles. Although this method uses a greater amount of oil (approximately 2g), the oil is recoverable with care. The reflectance vessel is an optically clear circular quartz glass open-topped container. A circular stainless steel disc is also required which has dimensions of 3.7cm in diameter and 9.0mm thickness, with a small ridge 1.0mm in depth around the rim of the disc. This rim allows a 1mm layer of oil to be sandwiched between the disc and the reflectance vessel. The path length for measurements is therefore 2 x 1mm. The reflectance disc also has three small grooves arranged symmetrically around the rim which allow for easy removal or air bubbles. Sufficient oil to cover the bottom of the vessel is added and the reflectance disc slowly lowered into the liquid. Three to five spectra were obtained for each sample, the vessel being rotated about the centre between spectra. Each spectrum was the mean of 32 scans. The vessel was cleaned and refilled with the sample the measurements repeated. The spectra were then averaged on the NSAS™ software to obtain a single mean spectrum for each sample and the data transferred to Vision® software for data analysis. Figures 3.3.1 and 3.3.2 shows the three sample presentation accessories used for the NIR measurements whilst Figure 3.3.3 is a diagrammatical representation of the reflectance vessel and the transflectance disc.

3.4 REFERENCE METHODS

It should be noted that the natural material samples used are detailed with each relevant piece of work in the Results section.

3.4.1 The Determination of the Water Content of Agar Samples using the Karl Fischer Titrimetric Method

3.4.1.1 Introduction

This is a sensitive technique that can be used to measure both residual water in samples and water of hydration in crystals.

Figure 3.3.1 Sample Presentation Accessories Viewed from Above (HPLC glass vial, agricultural cell and reflectance vessel with stainless steel disc)

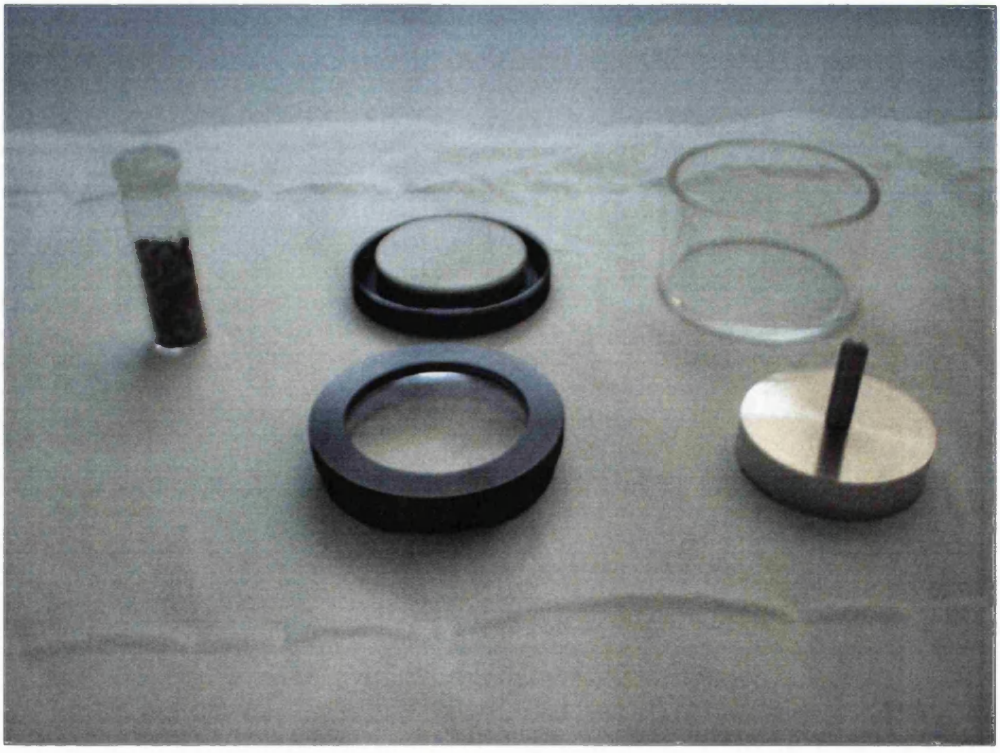


Figure 3.3.2 Sample Presentation Accessories Viewed from the Side (HPLC glass vial, agricultural cell and reflectance vessel with stainless steel disc)

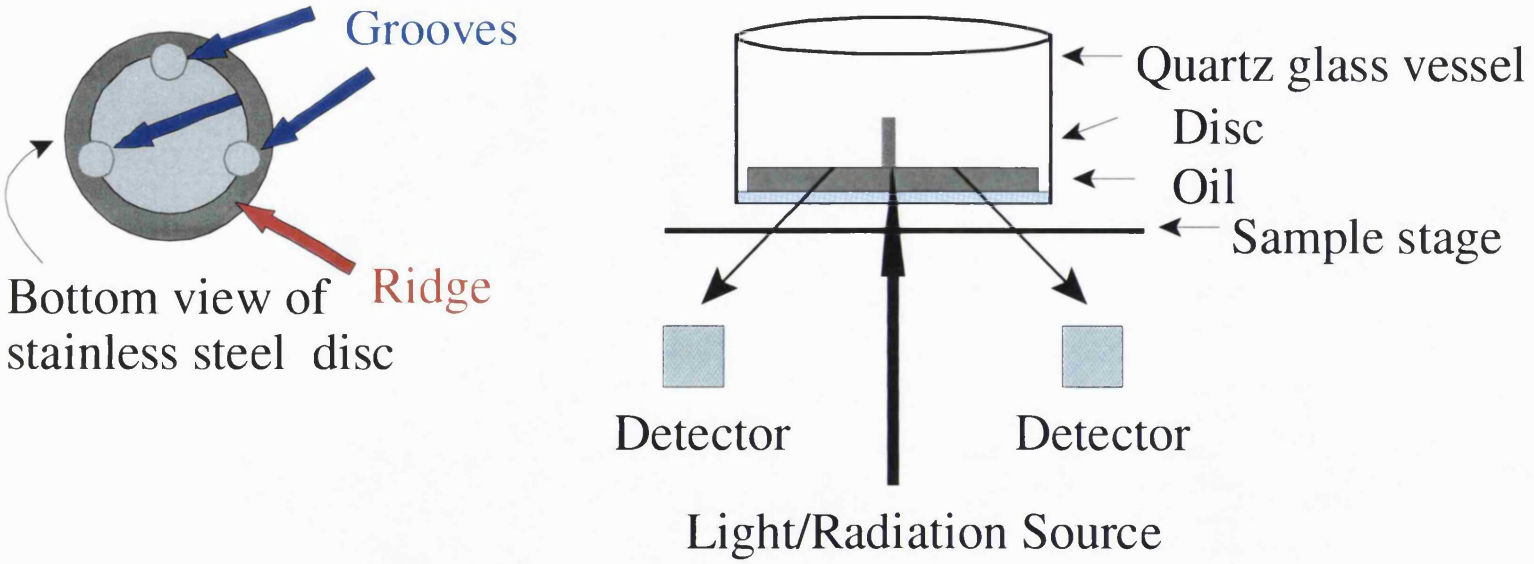


Figure 3.3.3 Reflectance vessel and stainless steel disc used for transflectance measurements of liquids

In 1935 Karl Fischer proposed the determination of small amounts of water using a reagent prepared by the action of sulphur dioxide upon a solution of iodine in a mixture of anhydrous pyridine and anhydrous methanol. The end-point of the reaction was determined by a dead-stop end-point procedure.

The Karl Fischer (KF) reaction is shown by equation 3.4.1.

$$I_2 + 2H_2O + SO_2 \rightarrow 2HI + H_2SO_4 \qquad (3.4.1)$$

The reaction takes place in the presence of a base, for example imidazole or primary amines and a solvent, such as methanol.

In order to achieve an equilibrium shift to the right, it is necessary to neutralise the acids that are formed during the process (HI and H_2SO_4) and pyridine was used for this purpose. The following two step reaction occurs (Figure 3.4.1):

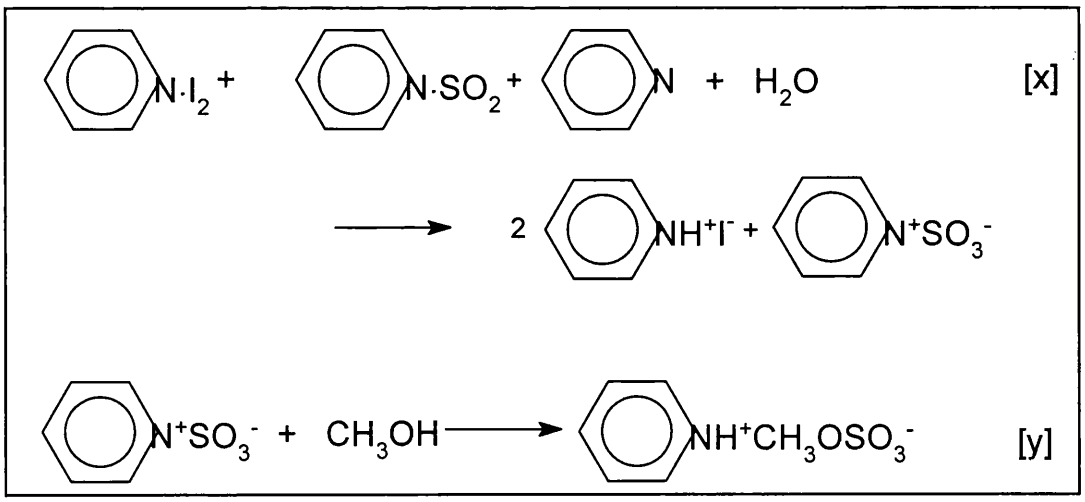


Figure 3.4.1 Karl Fischer reaction

According to these equations, methanol not only acts as a solvent but also participates directly in the reaction itself. In an alcoholic solution, the reaction between iodine and water takes place in the stoichiometric ratio of 1:1. A nominal concentration of iodine in

the KF reagent is given as ≈5mg/ml and so the exact concentration of iodine in the KF reagent needs to be found before the water content of the sample can be determined. This titration is carried out using di-sodium tartrate, which has an accurately known water content. The formula of di-sodium tartrate is $Na_2C_4H_4O_6.2H_2O$ with a M_R of 230.08. The water content is therefore $\frac{36.03}{230.08} \times 100 = 15.66\%$ m/m. Pyridine is not directly involved in the reaction and only acts as a buffering agent and can therefore be replaced by other bases. The rate of the KF reaction depends on the pH value of the medium and a buffer is required in the medium because titration is preferentially carried out within a pH range of 4-7. If the pH drops too low, endpoint attainment becomes sluggish or an endpoint will not be reached at all (Mettler Toledo website 2002, Harris 1991).

3.4.1.2 Reagents

The KF reagent consists of iodine, sulphur dioxide and pyridine. Methanol was used as the solvent.

3.4.1.3 Detection of End-point - the Principle of Bivoltametric Indication

A small constant current, the polarisation current "Ipol" is applied to a double platinum pin electrode. As long as the added iodine reacts with the water, there is no free iodine in the titrating solution. A high voltage is necessary to maintain the specified polarization current at the electrode. As soon as all the water has reacted with the iodine there will be free iodine in the titration solution. This free iodine causes ionic conduction and the voltage needs to be reduced to keep the polarization current constant. When the voltage drops below a defined value, the titration is terminated.

The ionic conduction occurs as follows:

an iodine molecule is attracted to the negatively charged platinum pin. It then acquires two electrons and turns to iodide ($2I^-$). The two negatively charged iodide ions are immediately attracted to the positively charged platinum pin, where they donate the electrons and form an iodine molecule again. The titration solution must be mixed thoroughly to ensure constant ionic conduction (Mettler Toledo website 2002 and Metrohm 2000).

3.4.1.4 Titer Determination of KF reagent

The determination of the exact iodine content of the Karl Fischer reagent is determined through the use of di-sodium tartrate. Under normal conditions di-sodium tartrate has a water content of 15.66% and is stable and non-hygroscopic. It should be reduced to a fine powder before using as it only dissolves slowly in methanol. For this reason a delay of 2 minutes was allowed before titration initiated. A sample of approximately 150mg was used (calculated by back-weighing), which allowed a titration of approximately 5ml with the 10ml burette. No more than three determinations were performed in the methanol in the vessel (~40ml). The methanol was then removed and replaced with fresh methanol and conditioned prior to further determinations. A relative standard deviation of less than 1% for three determinations was considered acceptable. An average of these determinations was used for sample analysis. The titer determination was repeated each day and whenever new reagent was added to the reagent vessel feeding into the burette.

3.4.1.5 Equations for calculation

The titer for the amount of iodine present in the KF reagent which must be calculated prior to sample analysis is given by the following equation 3.4.2:

$$\text{Titer (mg/ml)} = \frac{S \times F \times D}{V} \qquad (3.4.2)$$

where S is the sample size in g and V is the volume of KF reagent dispensed in ml. The Factor, F of 0.1566 (15.66%) is used to calculate the amount of water (mg) in the di-sodium tartrate and the Divisor, D of 1000 is used to convert the sample size to mg.

The formula used to calculate the water content of a sample is given in equation 3.4.3.

$$\text{water content (\% m/m)} = \frac{(V - B) \times T \times F}{S \times D} \qquad (3.4.3)$$

where V = dispensed KF reagent volume, B = blank volume, T = titer, F = factor (0.1), S = sample size and D = divisor

For example, if the titer (T, amount of iodine in the KF reagent) is 4.4519mg/ml and the sample size (S) is 0.1714g and the dispensed KF reagent volume for titration of the sample (V) is 5.536ml, then the water content of the sample in equation 3.4.4 is:

$$\text{water content (\% m/m)} = \frac{(5.536-0)\times 4.4519\times 0.1}{0.1714\times 1} = 14.38 \text{ (2dp)} \qquad (3.4.4)$$

where D=1 and:

$$\text{Factor, F} = \frac{1}{1000} \text{ [to convert S from g to mg] x 100 [to convert to a \%]} = \frac{1}{10} = 0.1$$

3.4.1.6 Materials Suitable for Water Content Determination by the KF method

The method is clearly confined to those cases where the rest of the material being analysed does not react with either of the components of the reagent nor with the hydrogen iodide which is formed during the reaction with water. Such substances include oxidising agents, reducing agents, basic oxides and salts of weak oxy-acids. Aldehydes and ketones react with methanol to form an acetal or ketal and water. This results in a higher than the 'true' water content of the sample. Strong acids also react with methanol to form an ester and water, also resulting in a higher water reading.

3.4.1.7 Factors to be Considered when using the KF method

The drift is the total ingress of water into the titration stand during a defined period of time and is specified in μg H_2O/min. The titration can be terminated using a pre-determined drift-stop such that when the drift falls below this value, the titration finishes. The titration is terminated when the actual drift is less than the sum of the initial drift and the relative drift. The value used was 10μg/min. This is also the value that determined the end of the pre-conditioning period which is required before sample analysis can be carried out.

It is known that the extraction time may cause significant differences in moisture content for some materials. For this reason an initial study was carried out in order to determine the most appropriate extraction time. Very long extraction times may be considered to be too time consuming, but too short an extraction time will results in poor precision in repeat

moisture content determinations of the same sample. The extraction time may have a significant effect on the water content reading of the Karl Fischer method, although it depends on the nature of the sample. In general, the greater the extraction time the greater the determined water content, until it reaches a maximum. A sample that consists entirely of 'free' water (i.e. that water that is not bound covalently as part of the sample structure) will yield its water more readily and therefore more quickly than a sample that consists of bound water as 'water of crystallisation', which will take a far greater extraction time before this is released, if at all. It is possible to leave the reaction mixture in the vessel for a certain amount of time before the titration is started or it is possible to set a length of time the titration should take place for, allowing for small increases in drift caused by a slow release of water from the sample to be taken into account. It must be noted here that the purpose of this work is to ascertain how closely the NIR spectra of a sample can be related by construction of a calibration to the water content as determined by the reference method. As such the determined water content need not be the 'true' content as long as all the samples are determined under the same reaction conditions. However, a long extraction time was considered appropriate because this allowed for more reproducible results in terms of repeat determinations of the same sample.

3.4.1.8 Instrumentation

The instrumentation is shown in Figure 3.4.2 and consists of the following units:

701 KF Titrino: consisting of the titrator, responsible for calculating and detecting the end-point of each titration and the exchange unit which houses the highly precise 10ml burette (10,000 increments), automatic burette filler and reagent vessel

703 Titration Stand: which holds the titration vessel, stirs the contents, aspirates spent solution and adds solvent

701 KF Titrino keyboard: used to program in the amount of sample in the reaction vessel and to change parameters of the titration.

Figure 3.4.2 Metrohm 701 Titrino Karl Fischer Titrator with 701 Keyboard and 703 Titration Stand

3.4.1.9 Materials

Agar samples were courtesy of Mark Bellchambers, also of the Centre for Pharmaceutical Analysis

Disodium tartrate: BDH (AnalaR®) Laboratory Supplies, Poole, Dorset, UK, product number 103334X and lot number A886601 732

Methanol, dried: BDH (AnalaR®) Laboratory Supplies, Poole, Dorset, UK, product number 104736J and lot numbers 1017585 138, 993685 119, 963085 041

Karl Fischer reagent: BDH (AnalaR®) Laboratory Supplies, Poole, Dorset, UK, product number 193205F and lot number 45524086

3.4.1.10 Procedure

The exact iodine concentration of the reagent was ascertained (titer) prior to sample analysis. Conditioning of the fresh dried methanol in the reaction vessel (removal of any moisture by addition of the KF reagent) is essential prior to sample analysis. Water content of the Agar samples was expected to range from approximately 5% to 15%m/m water. For a high level of accuracy, the amount of sample in the reaction vessel should be between 30 and 70% of the burette volume and so a sample mass of approximately 300mg accurately calculated by back-weighing was used. A relative standard deviation of less than 1% was considered acceptable for repeat determinations. No more than three determinations were carried out before the reaction vessel and emptied and fresh methanol added and pre-conditioned with the KF reagent. The stirring speed in the reaction vessel was adjusted such that a small vortex appears, but not be so high that bubbles form in the solution, as they falsify the measured values.

Samples were measured in a random order but care was taken such that the KF method was carried out as soon as practically possible after the sample spectra were obtained by NIR spectrophotometry. In addition, repeat determinations of each sample were carried out in a random order.

3.4.1.11 Titration Parameters and Configuration

Stop criteria: drift	Stop drift: 10µl/min	Stop volume: 40ml
Start volume: 0ml	Polarizer: I(pol)	I (pol): 50µA

EP: 250mV

Extraction time: Disodium tartrate titer (-)120s and sample analysis (-)1800s

Calculation parameters: D = 1 and F = 0.1 for sample analysis and F = 156.6 for KF reagent titer using di-sodium tartrate (Mettler Toledo website 2002).

3.4.2 BP Assay for the Determination of the Cineole Content of Eucalyptus Oil

3.4.2.1 Introduction

The assay (British Pharmacopoeia 2001b and European Pharmacopoeia 2002b) method for the determination of Cineole content in Eucalyptus oil was adopted in 1934 from a freezing-point method (Tusting Cocking, 1920). Melted o-cresol is added to the Eucalyptus oil and the o-cresol forms an addition compound with the Cineole present in the oil (referred to as 'cresineol'). The freezing point is noted and the Cineole content of the oil is obtained by reference to a table showing the freezing points of complexes of known Cineole content. The accuracy of the procedure is reported to be approximately ± 3% w/w (Tusting Cocking, 1920).

3.2.4.2 Materials

Thirty Eucalyptus oils of different brands and batch numbers were obtained from pharmacies. Twenty-one of these oils were of BP standard and nine were pure essential oils intended for use in Aromatherapy. O-cresol was obtained from Lancaster (Morecambe, Lancs, UK) and was stated to be 98+ %w/w pure. Cineole of 99% w/w purity was obtained from Avocado Research Chemicals Ltd., Heysham, Lancs, UK, product number 12269 and lot number F5731A.

3.2.4.3 Procedure

The apparatus consisted of a test-tube approximately 25mm in diameter and 150mm long, placed inside a test-tube approximately 40mm in diameter and 160mm long. The inner tube

was closed by a stopper which carried a thermometer about 175mm long and graduated in 0.2°C intervals, fixed so that the bulb was about 15mm above the bottom of the tube. The stopper had a hole allowing the passage of the stem of a stirrer made from a glass rod formed at one end into a loop of about 18mm overall diameter at right angles to the rod. The inner tube with its jacket was supported centrally in a 1 litre beaker containing a suitable supercooling liquid (water) to within 20mm of the top. A thermometer was supported in the cooling bath.

A 3.00g sample of the oil, recently dried with anhydrous sodium sulphate, was weighed into a dry test tube and 2.10g of melted o-cresol added. (These quantities correspond with the molecular masses of Cineole and o-cresol respectively). The masses weighed out were within ± 0.02g of the stated amounts. On cooling the mixture, at the onset of crystallisation there was a small rise in temperature. The highest temperature reached during solidification (t_1 °C) was noted. The mixture was re-melted on a water-bath at a temperature that did not exceed t_1 by more than 5°C and the tube placed in the apparatus, with the supercooling liquid in the beaker maintained at a temperature 5°C below t_1. At the onset of crystallisation, or when the temperature had fallen to 3°C below t_1, the mixture was stirred continuously. The highest temperature at which the mixture crystallised (t_2 °C) was noted. The operation was repeated until the two highest values obtained for t_2 did not differ by more than 0.2°C. If supercooling occurred, crystallisation was induced by adding a small crystal of the complex consisting of 3.00g of Cineole and 2.10g of melted o-cresol. The Cineole content of the Eucalyptus oil sample was determined by referral to a table containing values of t_2 and their respective apparent % w/w Cineole, if necessary by interpolation.

3.4.3 BP Assay for the Determination of the Citral Content of Lemon and Lemongrass Oil

3.4.3.1 Introduction

The BP (2001c) and European Pharmacopoeia (2002c) monographs for Lemon oil state that the oil must be obtained from the fresh peel of *Citrus limon* (L.) and contain not less than

2.2 % m/m and not more than 4.5% m/m of carbonyl compounds, calculated as Citral. Hydrochloric acid is liberated upon the reaction of hydroxylamine hydrochloride with the aldehyde and then titrated against a standardised solution of ethanolic potassium hydroxide.

The following reaction occurs as given in equation 3.4.5:

$$R \cdot CHO + NH_2OH \cdot HCl \rightarrow RCH = NOH + H_2O + HCl \qquad (3.4.5)$$

It must be noted that other aldehydes (including citronellal) are known to be present in Lemongrass oil. Citronellal and other aldehydes are also present in Lemon oil in tiny amounts. It is a suggestion, therefore that the BP assay for Citral in Lemon oil is more accurately an assay for total aldehyde content.

3.4.3.2 Materials

25 samples of pure Lemon essential oil and 18 samples of pure Lemongrass essential oil were obtained from retail outlets such as pharmacies, health food stores.

Citral (a mixture of *cis* and *trans*): Avocado Research Chemicals Ltd. Heysham, Lancs, UK (stated to be 95+% pure), product number 16169 and lot number G9601A

Hydroxylammonium chloride (hydroxylamine hydrochloride): BDH (AnalaR®) Laboratory Supplies, Poole, Dorset, UK, product number 101293X and lot number A998703 805.

Bromophenol blue sodium salt: Avocado Research Chemicals Ltd., Heysham, Lancs, product number 16899 and lot number F7654A

Potassium hydroxide pellets: Fisher Chemicals UK Ltd., Loughborough, Leicesteshire, UK, product number P15640153, batch number 0060757 070.

Sodium hydroxide pellets: BDH (AnalaR®) Laboratory Supplies, Poole,Dorset, UK, product number 301675N, lot number B281548 835

Hydrochloric acid 0.1M: Prolabo/Merck eurolab, Z.I de Vangereau 5250 Briare le Canal, product number 31 954.290, lot number 00070

Silver nitrite: Sigma-Aldrich Co. Ltd., product number 23,271-3, lot number L105628K1

Details for the preparation of solutions required for the assay are given in the Britsih and European Pharmacopoeia (BP2001d and EP2002d).

3.4.3.3 Procedure

Approximately 9g of the Lemon oil was accurately weighed (3sf) and mixed with 20ml absolute ethanol. 10.0ml of hydroxylamine hydrochloride solution and 0.4ml bromophenol blue solution were added. The mixture was titrated slowly with 0.5M ethanolic alcoholic potassium hydroxide until the colour changed from yellow to olive-green. The titrated mixture was then allowed to stand for 5 minutes and titrated again, if necessary, until changed from yellow to olive-green.

Each ml of 0.5M alcoholic potassium hydroxide is equivalent to 76.1mg of carbonyl compounds (molecular mass of Citral is 152.2).

Lemongrass oils were assayed for Citral content in the same way, but because the Citral content is much greater, the mass of oil used and the volume of reagents used in the assay was modified accordingly.

3.5 Introduction to Results Section

The results section will be split into two chapters for the purposes of clarity- one concerning the study of solid plant materials and the second concentrating on liquid plant products, namely essential oils. It is the intention to demonstrate the relative advantages of the use of NIR spectroscopy for the quality control of plant materials in these two forms and the different types of problem that arise through the data acquisition and material sampling in the solid and liquid forms, along with its uses and limitations. Each chapter will also deal with several different types of investigation, that of the identification and discrimination of pure plant materials, the study of mixtures of similar materials including inferior and adulterated samples and the quantification of certain constituents present in a particular sample. The data analysis techniques described in Chapter Two are used for all investigations. It is the hope that this will show that a few simple data analysis methods can be used in a wide variety of different studies and ultimately to demonstrate the potential of NIR spectroscopy for the quality control of pharmaceutical materials of natural origin.

CHAPTER FOUR: RESULTS

SOLID NATURAL PRODUCTS

4.1 Study of Plant Material in a Similar Pharmaceutical Medium

4.1.1 Introduction

Although NIR spectroscopy has been used extensively in the agricultural and the food industries to develop calibrations for the quantitative determination of certain chemical constituents present in plant materials, for example water in rice and compost, protein in cattle feed and starch in corn (Choi *et al* 2000, Suehara *et al* 2000 and Shenk *et al* 1992), there has been little published work concerning the quality control of plants where there is a possibility of adulteration or contamination with other plant materials. Traditionally, the best way of determining the authenticity or purity of a sample would be through macroscopic and microscopic examination, which is time-consuming and requires a knowledge of the plant morphology and features specific to that plant. Conventional techniques such as TLC, GC and HPLC whose aim is to separate out the different chemical constituents present are subject to a variety of problems. Firstly, a knowledge of all constituents present in the plant are required along with a rough estimate as to their quantity. Only then can peaks be identified as belonging to the sample and unexpected or peaks isolated and studied further. An additional problem is that without a knowledge of possible contaminating plant material and its constituents, the particular conditions used for chromatographic separation may result in adulterating peaks having similar retention times to constituents present in the authentic product, thus resulting in the analyte peak being obscured or hidden. It is therefore appropriate to investigate as to whether NIR spectroscopy could be used to determine the authenticity and purity of plant material. The benefits of the technique are well known including the fact that little or no sample preparation is required and data acquisition is rapid. Ideally, an NIR method would allow an adulterated or contaminated plant material sample to be distinguished from a pure plant material sample at as low a level of contamination as possible. (If this could be achieved, the next step would be to determine if NIR spectroscopy would be used to identify the contaminant present and to quantify the level of contamination). For the purposes of this investigation, two different plant materials were chosen and mixed with a pharmaceutical product of natural origin itself to from a series of binary mixtures. Valerian roots and

rhizomes and Cinchona bark were chosen. Valerian is an erect perennial growing up to 1.2m in height. It is native to Europe and northern Asia and grows wild in damp conditions. The roots and rhizomes are used therapeutically for conditions such as insomnia, nervous exhaustion, anxiety and high blood pressure. Constituents include volatile oil (up to 1.4% containing bornyl acetate among others), iridoids (also known as valepotriates which are though to be responsible for the sedative and depressant activities) and alkaloids. Cinchona is an evergreen tree reaching about 25m in height. It is native to mountainous tropical regions of South America (it is also known as Peruvian Bark) and is cultivated on tree farms as a source of quinine, widely known for its antimalarial and antibacterial properties. Cinchona bark is also used for fevers and digestive problems. The bark is stripped from the trunk, branches and root of 6-8 year old trees and then dried in the sun. The annual production of Cinchona bark is estimated to be roughly 8,000 tonnes a year. The main constituents are alkaloids, including quinine at levels of approximately 15%, bitter triterpenic glycosides, tannins and quinic acid (Evans 1996a,b and Chevallier, 2000a,b).

The British and European Pharmacopoeias contain monographs for both Valerian and Cinchona bark (BP2001e,f and EP 2002e,f). Valerian BP is defined as Valerian root, consisting of the whole underground parts of *Valeriana officinalis*, including the rhizome surrounded by the roots and stolons, or by fragments of these parts. The monograph describes minimum levels of essential oil per kilogram and includes identification tests and tests for foreign matter (inspection by eye or with a 6x lens), extractable matter and loss on drying. Cinchona bark BP is described as consisting of the dried bark of *Cinchona pubescens* Vahl *(Cinchona succirubra* Pavon) or of its varieties or hybrids. The monograph also describes tests for identification, foreign matter, total ash and an assay for the alkaloid content. Fennel herb was also used later on in the investigation. Microcrystalline cellulose (Avicel®) was employed as the pharmaceutical medium, commonly employed as an adsorbent, suspending agent, a tablet and capsule diluent and a tablet disintegrant. Microcrystalline cellulose is also used in the cosmetics and food industries. It is a purified, partially depolymerised cellulose that occurs as a white, odourless, tasteless, crystalline

powder composed of porous particles. It is commercially available in different particle sizes and moisture grades which have different properties and applications. Its moisture content if stored correctly is typically less than 5% w/v (Wade 2002). It was used in this study as its structure is comparable to the cellulose present in the cell walls of plant materials. Lactose was used later on in the study which is less similar in structure to the cellulose present in plants, to ascertain if it was easier to distinguish between these mixtures than those containing the microcrystalline cellulose.

Apart from serving the purpose as to investigate the possibility and scope of NIR spectroscopy to successfully determine between different mixtures, the use of several different data analysis techniques will be employed which will also serve to highlight the relative benefits and disadvantages of the different methods, which would then be used for data analysis in other investigations. It is acknowledged that the contamination of plant material with Avicel® or other similar pharmaceutical excipients and *vice versa* is far from likely, but it is emphasised that the purpose of this study was to ascertain if NIR spectroscopy could be used to detect differences in binary mixtures where the two materials were similar in structure. For this reason there was no benefit either way of studying plant material in Avicel® or lactose or *vice versa* (Avicel ® or lactose in plant material). All terms used here refer to the amount of plant material present in binary mixtures with Avicel® or lactose.

4.1.2 Investigation Aims

The aims of the investigation were ascertain if NIR spectroscopy could be used for:

- The identification / discrimination of pure plant materials in raw, ground and powdered forms and to determine if particle size affects the level of discrimination

- The distinction between the pure plant material for each of the Valerian and Cinchona species and the samples adulterated with Avicel®, and if so, to determine the level of discrimination obtained

- The accurate quantification of mixtures of plant material and adulterant and to determine the limits of quantification

4.1.3 Materials and Methods

- One sample each of dried *Cinchona officinalis*, *Cinchona lancifolia* and *Cinchona succirubra* barks (School of Pharmacy Pharmacognosy stores)
- One sample each of English Valerian (*Valeriana officinalis*), Indian Valerian (*Valeriana wallichii)* and a further sample of Valerian, species unknown (School of Pharmacy Pharmacognosy stores). The dried roots and rhizomes of the plants were used.
- A single sample of Fennel herb
- Microcrystalline cellulose (Avicel®) and lactose
- Waters 4ml clear glass vials with caps

The plant samples were used in forms known as 'raw' 'ground' and 'powdered' in this investigation. The raw samples were broken into small pieces and the ground samples were broken down to a rough particle size of from the small pieces using a coffee grinder. The powdered samples were prepared from the ground samples and further broken down to a smaller particle size using a pestle and mortar. All samples were ground in the coffee grinder for the same amount of time and although no attempt was made to control the particle size of the three forms, on visual inspection the particle size was similar for all samples for each form. NIR spectra were collected for the raw, ground and powered forms of Valerian, as it was considered necessary to study the effect of particle size on the ability to discriminate between different plant samples.

Several mixtures were prepared and their exact content (in terms of % m/m) noted. These were approximately 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 75, 85 and 95% m/m plant material in Avicel®. The use of a smaller particle size is likely to result in more homogeneous mixing. In addition, spectra obtained are likely to be more reproducible the smaller the particle size.

Although not part of this investigation, the method of sampling used for any type of quality control of solid plant material is likely to significantly affect the results. For example, with the Valerian consisting of roots and rhizomes, different samples containing different proportions of these plant structures may be significantly different in terms of the NIR spectra obtained. Furthermore, when sampling from a larger sample of material that may be contaminated, the degree (% m/m) of contamination is likely to differ in different areas of the sample. The size of the sampled material in comparison to the degree of contamination is therefore important. In a commercial situation where a method of quality control of plant material is required then a Library containing many different samples and batches of the plant material of interest is necessary to build up sufficient variability to be representative of the differences likely to be encountered in practice.

4.1.4 Data Analysis Techniques

- Library construction and study of CWS and MDWS Identification methods
- Polar Co-ordinate plots over selected wavelength ranges
- PC Analysis over selected wavelength ranges
- Construction of MLR calibrations for the quantitative determination of plant material in the binary mixtures

4.1.5 Results

4.1.5.1 Visual Inspection of the NIR Spectra

Figure 4.1.1 shows the SNV corrected 2nd derivative spectra of the three Cinchona bark samples, two of the Valerian samples and the Avicel®. A clear difference can be seen between the Cinchona bark samples and the Valerian samples. Although structurally similar to plant material, clear differences can also be seen between the Avicel® and the plant samples. Different species of the same plant have very similar spectra (except at certain peak maxima and minima). The main difference seen between them is at the water absorption bands of ~1450nm and ~1930nm.

Figure 4.1.2 shows the SNV corrected 2nd derivative spectra of the powdered *Cinchona succirubra* sample and its mixtures with Avicel®. Clear differences can be seen between the different mixtures at several points along the NIR spectrum, for instance at ~1660nm,~2100nm and the region 2200nm to 2500nm. The samples are also in the 'correct order' at these wavelength regions.

4.1.5.2 Library Construction for Valerian in its Raw, Ground and Powdered forms

A Library was constructed from the raw, ground and powdered forms of the three different Valerian samples. Each Library Product consisted of three spectra (three averages of four spectra for each sample) for each sample in a single form. In addition, a second sample of raw English Valerian (a second sample taken from the same English Valerian material) and a second sample of powdered English Valerian (the second sample powdered from the second raw sample) was included in the Library, giving rise to 11 Library Products in total. CWS and MDWS methods were applied separately to the Library. The threshold for Match Value was lowered for the CWS method and raised for the MDWS method in order to see the nearest neighbours to the spectrum in the Library Product being studied. The Match Values for all samples when compared with a powdered English Valerian spectrum were obtained. As there were three spectra in each Library Product, three Match Values for each sample were obtained and so these three values were averaged. The procedure was repeated for all samples (Library Products) when compared with a raw Indian Valerian spectrum.

Ideally, the three particle size samples for each species should be co-identified. However, if particle size took total precedence over the species, then the raw samples would be grouped together, then all the ground samples, then the powdered samples. To minimise the possibility of this happening SNV correction of all NIR spectra was used throughout this investigation, which helped to reduce particle size differences in the spectra.

An extract of the Library results is given in Table 4.1.1 (for the CWS method) and Table 4.1.2 (for the MDWS method).

Figure 4.1.1 SNV corrected 2nd derivative spectra of pure Avicel, three Cinchona bark species and two Valerian species

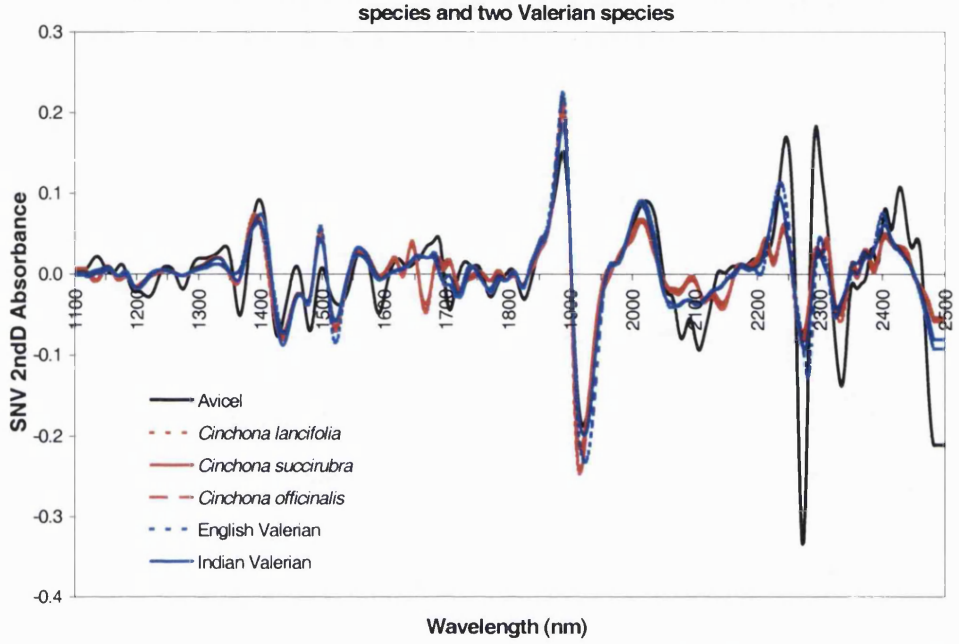
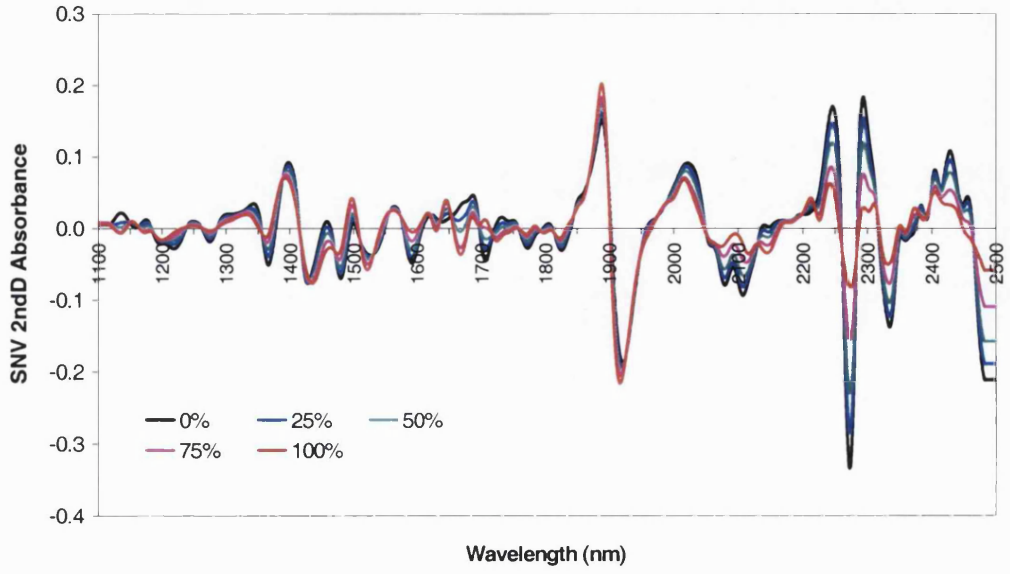


Figure 4.1.2 SNV corrected 2nd derivative spectra of mixtures of powdered *Cinchona succirubra* in Avicel



Spectrum name	Product Name	Match Value
English Valerian powder 1a	English Valerian powder 1	1.0
	English Valerian powder 2	0.9983
	English Valerian ground	0.9862
	Unknown Valerian powder	0.9846
	Unknown Valerian ground	0.9834
	Indian Valerian powder	0.9826
	Indian Valerian ground	0.9635
	Indian Valerian raw	0.9609
	English Valerian raw B	0.9560
	English Valerian raw A	0.9513
	Unknown Valerian raw	0.9486
Indian Valerian raw 1a	Indian Valerian raw	0.9999
	English Valerian raw B	0.9922
	Indian Valerian ground	0.9847
	English Valerian raw A	0.9800
	Unknown Valerian raw	0.9786
	English Valerian ground	0.9725
	Indian Valerian powder	0.9713
	Unknown Valerian ground	0.9708
	English Valerian powder 2	0.9695
	Unknown Valerian powder	0.9693
	English Valerian powder	0.9600

Table 4.1.1 Extract from the results of the Library containing Valerian in different forms using the Correlation in Wavelength Space Identification Method for SNV corrected 2nd derivative spectra over the whole wavelength range 1100nm to 2400nm

Spectrum name	Product name	Match Value
English Valerian powder 1a	English Valerian powder 1	0.7698
	English Valerian powder 2	50
	Indian Valerian raw	90
	Indian Valerian ground	126
	English Valerian raw A	170
	English Valerian raw B	268
	Unknown Valerian ground	285
	English Valerian ground	354
	Indian Valerian powder	527
	Unknown Valerian powder	1361
	Unknown Valerian raw	2553
Indian Valerian raw 1a	Indian Valerian raw	0.7698
	Indian Valerian ground	64
	English Valerian raw A	103
	English Valerian raw B	115
	English Valerian powder 2	207
	Indian Valerian powder	267
	English Valerian powder	815
	English Valerian ground	1137
	Unknown Valerian ground	1320
	Unknown Valerian powder	1963
	Unknown Valerian raw	3038

Table 4.1.2 Extract from results of the Library containing Valerian in different forms using the Maximum Distance in Wavelength Space Identification Method for SNV corrected 2nd derivative spectra over the whole wavelength range 1100nm to 2400nm

Only a general observation could be made with the CWS method, which was that in general the raw samples were grouped together but there was less distinction between the ground and powdered samples. For each sample, the powdered and ground samples were nearest neighbours. The two powdered English Valerian samples were nearest neighbours, as were the two raw English Valerian samples. There was no apparent trend noted with the MDWS method.

To summarise, powdered plant material samples 'perform better' than those with a larger particle size and SNV correction does not fully abolish the effects of particle size on the results.

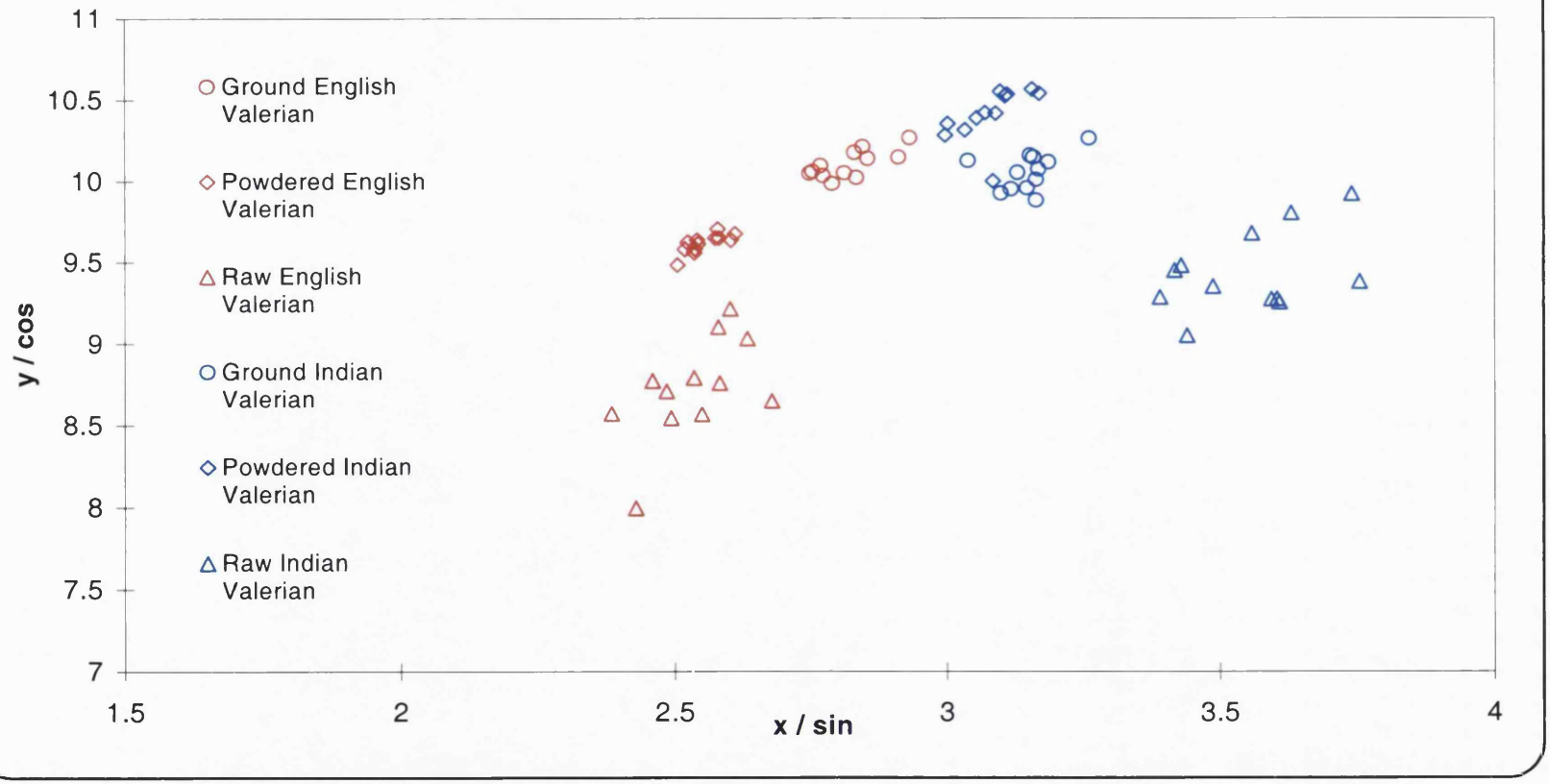
4.1.5.3 Polar Co-ordinate Plot for Pure Plant Material

A Polar Co-ordinate plot was also constructed to demonstrate the effect of particle size on the NIR spectra obtained. Figure 4.1.3 shows each of the twelve spectra obtained for the three Valerian samples in their different forms for SNV corrected 2nd derivative spectra over the wavelength range 2200nm to 2400nm. This plot seems to support the data from the CWS Library method, that is the powdered and ground samples are closer together than the raw samples and that different ground samples are just as likely to be co-identified as either the ground and or the powdered form of the same sample.

4.1.5.4 Library Construction of Binary Mixtures

Separate libraries were constructed for each set of binary mixtures of the six plant samples. CWS and MDWS methods over the whole wavelength range (1100nm to 2500nm) were applied to the libraries. Bearing in mind the default Match Value thresholds given in the Vision® software (0.85 for CWS and 4 for MDWS), the results were studied to see how high the plant material level in the mixtures had to be before it could be distinguished from the pure Avicel® sample. In addition, it was be reasonable to assume that if the method was successfully picking out the plant material as the differences between the mixtures, the Match Values assigned for the mixture would correlate with the amount of plant material present. The construction of the Library was such that all binary mixtures were compared against the pure Avicel®.

Figure 4.1.3 Polar Co-ordinate plot of SNV corrected 2nd derivative spectra of English and Indian Valerian in raw, ground and powdered forms (wavelength range 2200nm-2400nm)



A second sample of Avicel® (different batch number) was introduced into the Library containing the *Cinchona succirubra* samples, in order to ascertain how closely this was matched to the original sample of Avicel® in comparison with the adulterated sample with a low plant material content.

Due to the size and number of the libraries constructed and analysed for this investigation, not all the data are shown. However, extracts from the Libraries that typify the results are shown in Tables 4.1.3, 4.1.4 and 4.1.5. The results are summarised as follows:

- It is only the CWS method where the Match Values correlate with the amount of plant material present, that is, they are identified in the 'correct order' of nearest neighbours. The MDWS Match Values do not have correlating values with the plant material content, which suggests that this technique is not suitable for this particular purpose (see Tables 4.1.3, 4.1.4 and 4.1.5).

- From a study of Tables 4.1.3 and 4.1.5 it can be seen that all mixes have very similar CWS Match Values and many mixtures are above the threshold value for the CWS method, meaning there is little detected difference between the mixtures. The Match Value only drops below the threshold for mixtures of 85% and over for the *Cinchona succirubra* sample, 85% and over for the Indian Valerian sample and 75% and over for the Fennel sample.

- In Table 4.1.3 the Match Value for the Library Product containing the second batch of Avicel® when compared against the first sample is the same or less than (0.9996) that for the samples of 5% and lower. This means that this method shows that there is less difference between these mixes and Avicel® than a second sample of the same material, suggesting that samples below about 10% cannot be accurately be identified as being 'adulterated'. The MDWS method (Table 4.1.4) shows a greater distinction between the mixtures, with the second batch of Avicel® being the most closely matched to the first.

Spectrum name	Product name	Match Value
Avicel 1a	Avicel 1	1.0
	1%	0.9998
	2.5%	0.9998
	Avicel 2	0.9996
	5%	0.9996
	10%	0.9991
	25%	0.9944
	50%	0.9726
	75%	0.9018
	85%	0.8573
	95%	0.7981
	100%	0.7684

Table 4.1.3 Extract from the results of the Library containing *Cinchona succirubra* mixtures using the Correlation in Wavelength Space Identification Method for SNV corrected 2nd derivative spectra over the whole wavelength range of 1100nm to 2500nm

Spectrum name	Product name	Match Value
Avicel 1a	Avicel 1	0.7698
	Avicel 2	48
	0.5%	66
	0.25%	98
	2.5%	138
	10%	174
	1%	211
	25%	340
	5%	453
	85%	710
	95%	1128
	50%	1330
	100%	1379
	75%	2477
	100%	3610

Table 4.1.4 Extract from the results of the Library containing *Cinchona succirubra* mixtures using the Maximum Distance in Wavelength Space Identification Method for SNV corrected 2nd derivative spectra over the whole wavelength range 1100nm to 2500nm

Spectrum name	Product name	Match Value
Avicel 1	Avicel	1.00
	0.25%	0.9997
	0.5%	0.9997
	1%	0.9996
	2.4%	0.9995
	5%	0.9993
	10%	0.9992
	25%	0.9986
	50%	0.9948
	75%	0.9769
	85%	0.8718
	95%	0.8103
	100% Indian Valerian	0.7776
Avicel 1	Avicel	1.0
	5%	0.9993
	10%	0.9986
	25%	0.9920
	50%	0.9471
	75%	0.7789
	85%	0.6647
	95%	0.5307
	100% Fennel	0.4512

Table 4.1.5 Extract from the results of the Library containing Indian Valerian and Fennel mixtures using the Correlation in Wavelength Space Identification Method for SNV corrected 2nd derivative spectra over the whole wavelength range 1100nm to 2500nm

- It was found that if the wavelength range was reduced to 1600nm to 1800nm using the CWS method, this resulted in slightly greater discrimination between the mixtures.

These results will not be repeated in further tables but plots of the CWS Match Values against the plant material content for all 7 sets of sample mixtures for both the full wavelength range and the reduced wavelength range are given in Figures 4.1.4 and 4.1.5. Using the full wavelength range, it can be seen that the Match Values for the three forms of the same type of plant for both the Cinchona bark and Valerian samples are close together. In addition, the figure highlights the fact that the Match Values are over the default threshold value for all but the highest % of plant material in the mixtures. The Fennel mixtures show a marked difference in Match Value (over the default threshold value) from the Avicel® for mixtures above 70% over the full wavelength range. The high Match Values mean that it would not be possible to use this method for identification of the adulterated samples.

Using the reduced wavelength range, there is a marked difference in Match Value for samples of 20% and above for the Fennel samples. The Match Values for the Valerian sample mixtures are close together with greater separation between the species at higher plant material contents. For the Cinchona bark mixtures, the Match Value for the three species at each mixture are close together, with a marked drop in Match Value below the threshold for samples over 20%. This suggests that the use of the CWS method over this wavelength range would be more useful for the identification of the adulterated samples.

4.1.5.5 Polar Co-ordinate Plot of Binary Materials

Polar Co-ordinate plots were constructed over the 1600nm to 1800nm wavelength region on the SNV corrected 2nd derivative spectra of all 7 sets of sample mixes to ascertain if these could be used in conjunction with the Library identification/discrimination methods to determine the purity of material. The results are shown in Figure 4.1.6.

Figure 4.1.4 Plot of Correlation in Wavelength Space Match values against plant material content for SNV corrected 2nd derivative spectra over the wavelength range 1100nm to 2500nm

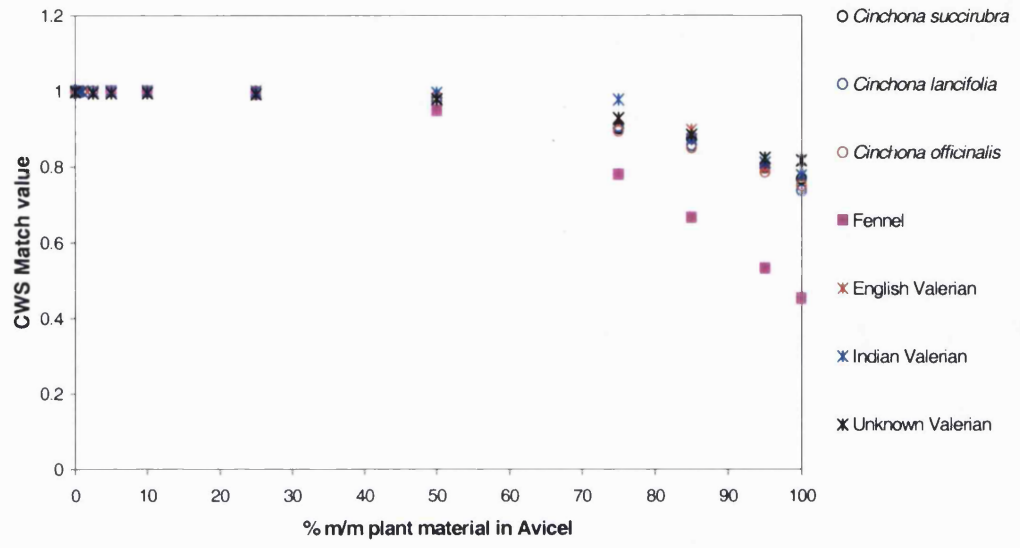


Figure 4.1.5 Plot of Correlation in Wavelength Space Match values against plant material content for SNV corrected 2nd derivative spectra over the wavelength range 1600nm to 1800nm

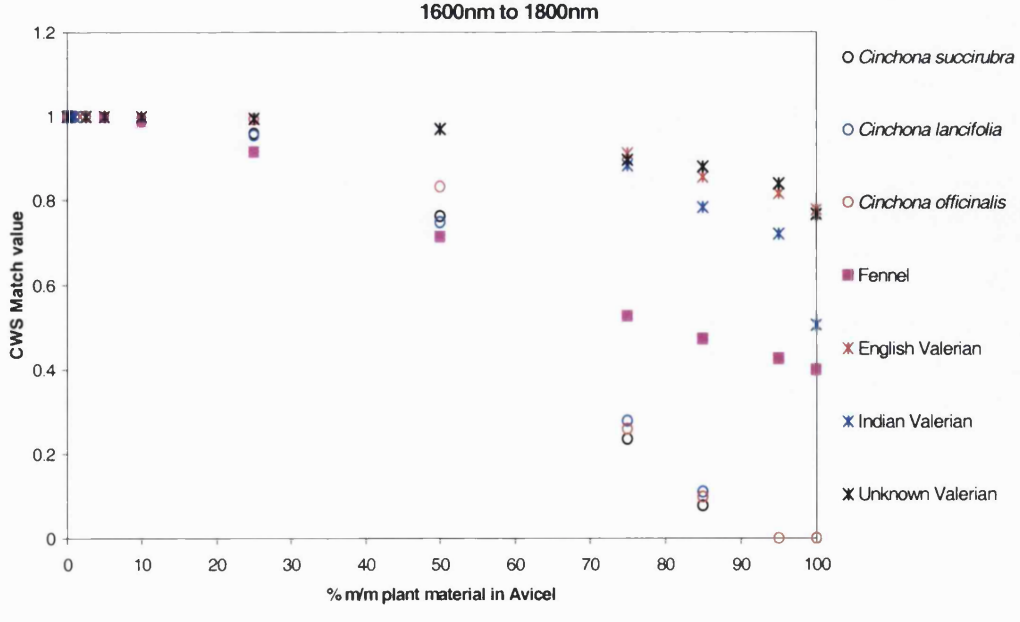
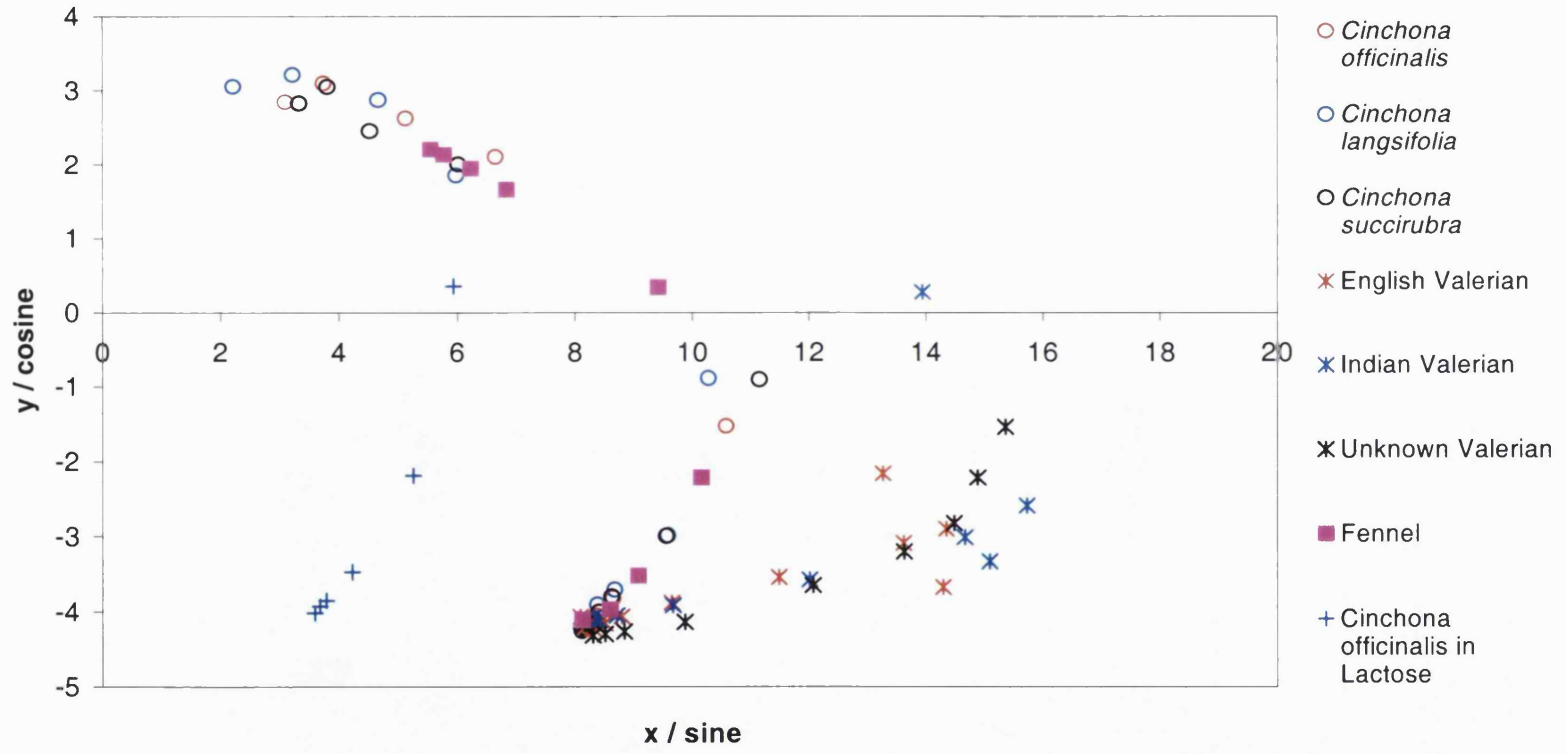


Figure 4.1.6 Polar Co-ordinate plot of SNV corrected 2nd derivative spectra of ground plant material in Avicel for Cinchona bark, Valerian and Fennel samples over the wavelength range 1600nm to 1800nm



In addition a set of mixture of *Cinchona officinalis* in lactose are also included for comparison. The plot carries a lot of information but several conclusions can be drawn. It can be seen that there appears to be a greater separation between the different Cinchona bark and Fennel mixes than for the Valerian mixtures. The 'change of direction' for the points in the plot occur for the Cinchona and Fennel mixtures at about the 50% level. This is illustrated by Figure 4.1.2 showing the NIR spectra for the mixtures of *Cinchona succirubra*. In the wavelength region 1600nm to 1800nm (used for the Polar Co-ordinate plot) it can be seen that there are changes in the peak directions of the NIR spectra - some of the mixtures have NIR spectra that are in a positive direction and the other in a negative direction. This wavelength region was refined further and it was found that the region 1660nm to 1710nm proved useful for the visual distinction of the binary mixtures. For the purposes of clarity, the sine values and cosine values were considered separately against the plant material content. Figures 4.1.7 and 4.1.8 show the results for the Indian Valerian and the *Cinchona succirubra* mixtures. There is a good trend for both plant mixtures, and although the points for the Indian Valerian mixtures appear fairly flat and therefore less discriminating, this is partly due to the expanded *y*-axis used to show both the sine and the cosine values.

4.1.5.6 Principal Component Analysis of Binary Materials

PC Analysis (using The Unscrambler® software) was carried out on the mixtures. To minimise the number of calculations and therefore the length of time required by the computer to carry out the data manipulation, an average of the twelve (untreated) NIR spectra obtained for each mix and plant material sample was used. It must be noted here that whilst Polar Co-ordinate plot values for each NIR spectrum are completely independent of any other data considered at the same time, PC Analysis takes into account all the NIR data for all samples included in the analysis set. In other words, if only a single set of mixtures for a single plant sample is included in the PC Analysis, the Principal Component values obtained for each mixture will be different if additional sets of mixtures are included. To keep matters simple, all sets of mixtures were analysed at the same time. The Unscrambler® software does not have an equivalent to the SNV correction method and so

a 2nd derivative transformation was carried out prior to PC analysis. This would mean that any differences in particle size of the mixture sets may be emphasized to a greater extent than analysis carried out using the Vision® software. Figures 4.1.9 and 4.1.10 show plots of the first two Principal Components for all mixtures over the full wavelength range (1100nm to 2500nm) and the 1600nm to 1800nm wavelength range respectively. To help with the interpretation of these plots, the first Principal Component (PC1) values are also plotted against the plant material content for all mixture sets, as shown in Figures 4.1.11 and 4.1.12.

The number of Principal Components used for the analysis over the full wavelength range and the 1600nm to 1800nm region was 8 and 5 respectively. The variability between the spectra described by PCs 1 and 2 for both wavelength ranges is also fairly small, suggesting that there are a number of other factors responsible for the variation in the data. From a study of Figure 4.1.11, it can be seen that there is correlation between PC 1 and the plant material or Avicel® content. Similar Principal Component values were obtained for the majority of the Cinchona bark and Valerian mixtures, but a difference was noted between these and the Fennel mixtures.

From a study of Figure 4.1.12, it can be seen that there is clearly a large difference between the Fennel mixes (the points for mixtures above 50% are off the scale of the plot) and the Cinchona bark and Valerian mixtures, whilst there are similarities between the Cinchona bark and Valerian mixes. For both wavelength ranges used, there is a correlation between PC1 and the plant material content but the exact PC value is specific to the type of plant material in the mixtures. Thus it is unlikely that PC1 is describing the Avicel® content, rather that it is describing the plant material present. However, it is also possible that the variation described by PC1 is attributable to water (moisture) content, as this could be proportional to the plant material or Avicel® content in the mixtures. The use of PC analysis may therefore, on selection of the most appropriate wavelength region, be useful in the identification of adulterated samples.

Figure 4.1.7 Plot of Polar Co-ordinate SNV corrected 2nd derivative spectra sine and cosine values against plant material content for mixtures of Indian Valerian in Avicel over the wavelength range 1660nm to 1710nm

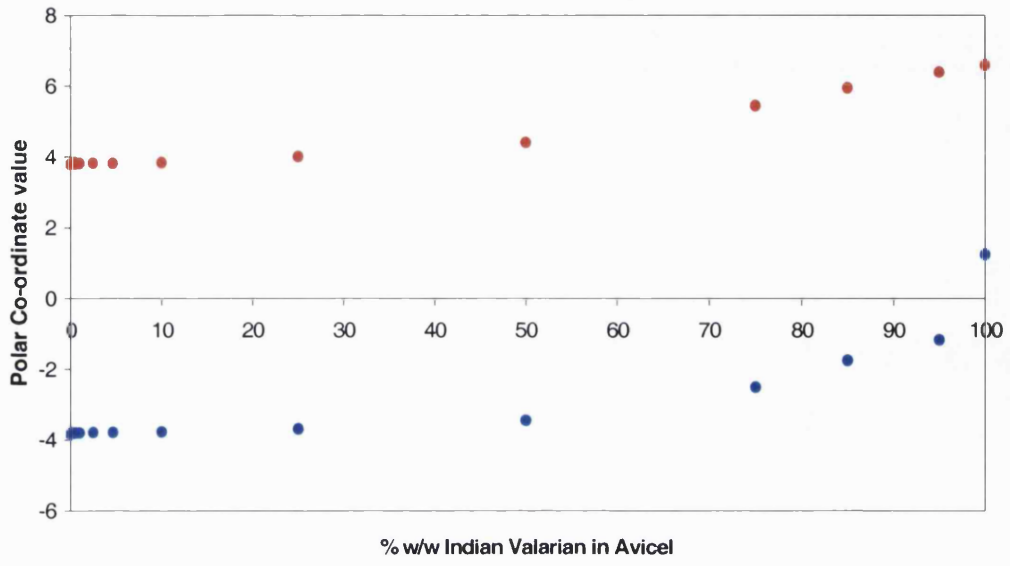
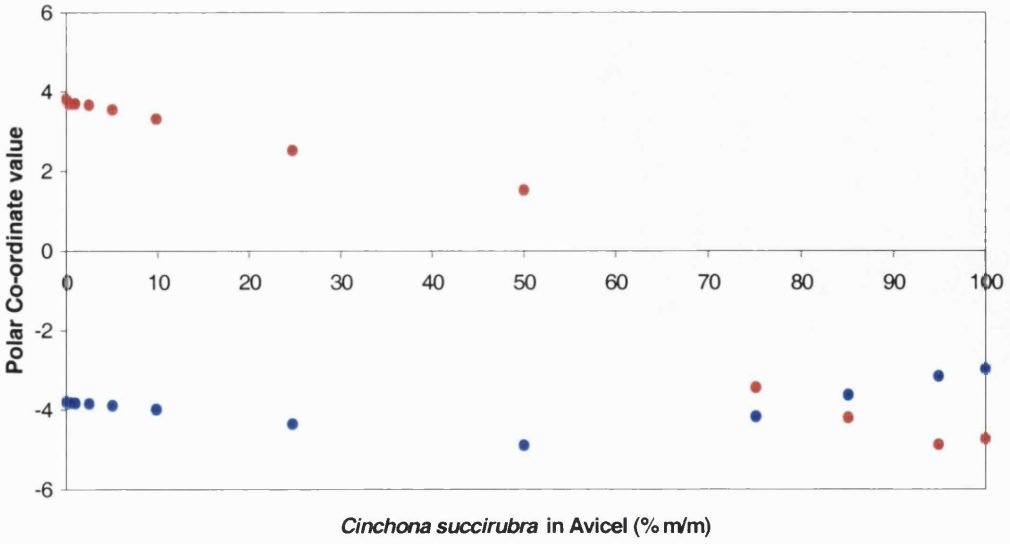


Figure 4.1.8 Plot of Polar Co-ordinate SNV corrected 2nd derivative spectra sine and cosine values against plant material content for mixtures of *Cinchona succirubra* in Avicel over the wavelength range 1660nm to 1710nm



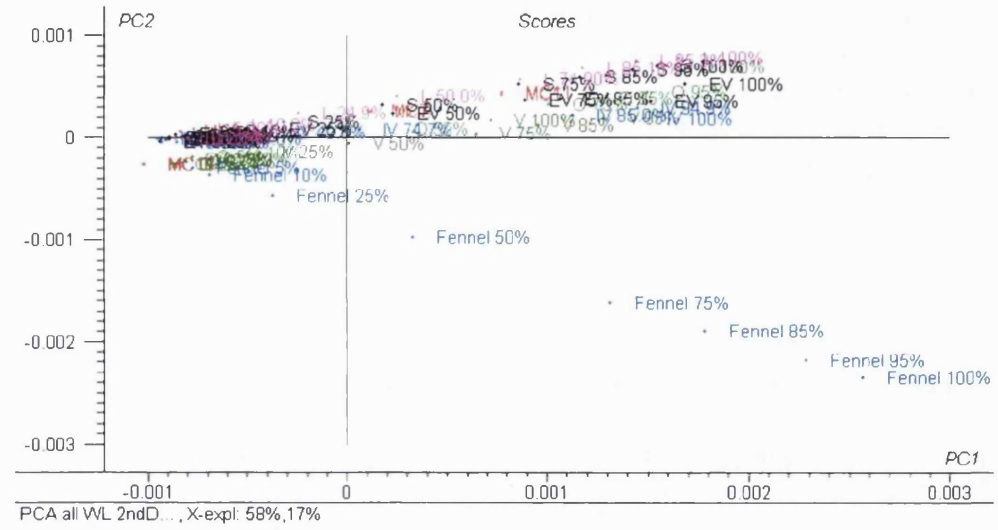


Figure 4.1.9 Principal Component plot of SNV corrected 2nd derivative spectra of mixtures of Cinchona bark, Valerian and Fennel in Avicel over the wavelength range 1100nm to 2500nm

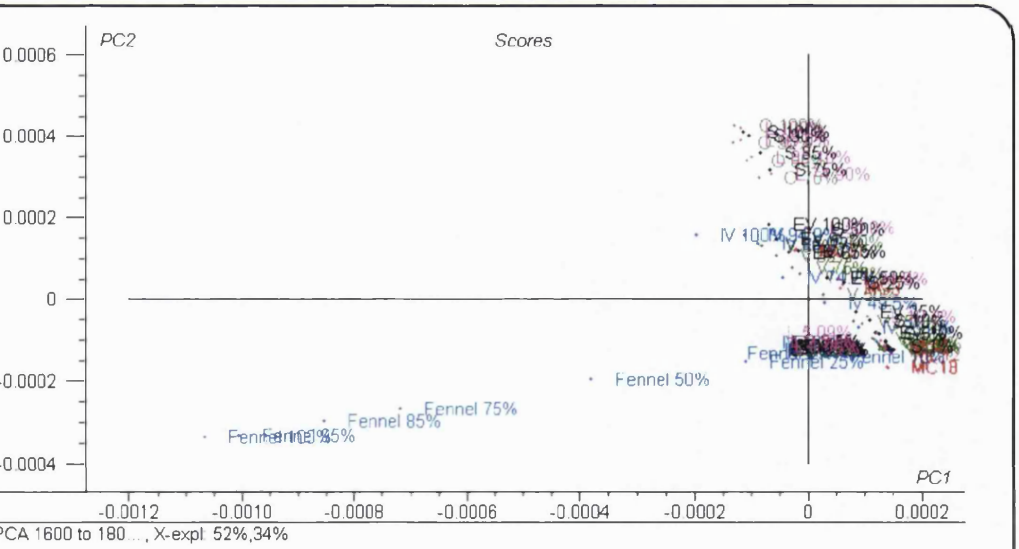


Figure 4.1.10 Principal Component plot of SNV corrected 2nd derivative spectra of mixtures of Cinchona bark, Valerian and Fennel in Avicel over the wavelength range 1600nm to 1800nm

Figure 4.1.11 Plot of PC1 values against plant material content for Cinchona bark Valerian and Fennel in Avicel mixtures over the wavlength range 1100nm to 2500nm

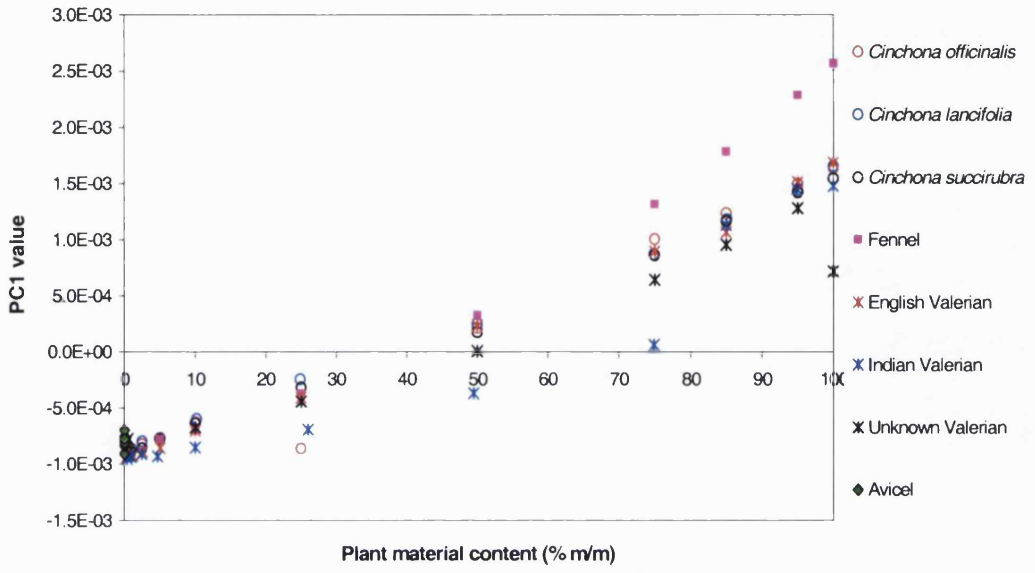
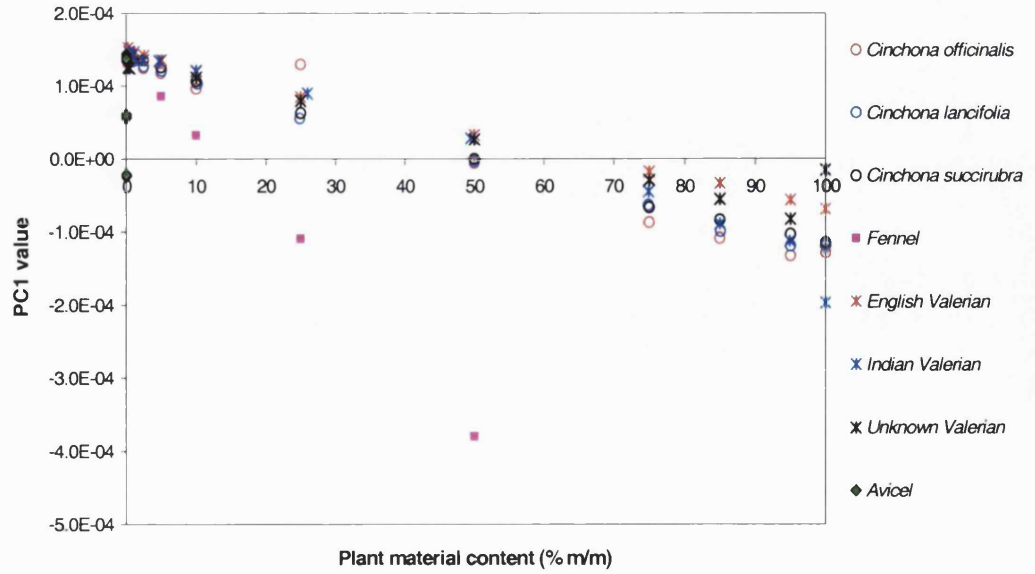


Figure 4.1.12 Plot of PC1 values against plant material content for Cinchona bark Valerian and Fennel in Avicel mixtures over the wavlength range 1600nm to 1800nm



4.1.5.7 Creation of Multiple Linear Regression Calibrations

A single NIR calibration was constructed with a sample set containing both the single and random mixture sets for all three Cinchona bark species (known as Sample Set 1). Due to the small number of samples in the individual mixtures sets, the construction of separate calibrations would not be appropriate. Similarly, a NIR calibration was constructed whose sample set combined the mixtures for the three different Valerian samples (English, Indian and 'unknown'). This was known as Sample Set 2.

The combination of the different species of Cinchona and Valerian in the two sample sets would allow for a more robust calibration. A forward search MLR method using Vision® software were employed. The wavelengths selected by the software would either be specific to the Avicel® present in the mixtures or to the plant material present. If it is taking into account the plant material content then it means that there is a negligible difference between the NIR spectra for the different species at the wavelength(s) chosen. Poor calibration results may suggest that the wavelengths selected do not correspond to the Avicel® and the difference between the NIR spectra of the different species would necessitate the creation of separate NIR calibrations for the separate species or the use of an alternative method to create the calibration model, such as PLSR or PCR. However, these procedures could not be considered in this study due to the low number of samples in the calibration sets.

The calibration model was set up in two different ways for both Sample Sets 1 and 2. The first calibration model involved assigning of all the samples to the calibration set only (no validation set). The second calibration model resulted from the mixtures being assigned in a 70%/30% ratio to the calibration and validation sets respectively. The advantage of the former method is that there is a larger number of samples in the calibration set, so allowing for a more accurate MLR calibration to be constructed. The drawback is that without a validation set, the accuracy of the calibration cannot be tested and the calibration equation may be too specific to the samples included in the set. The advantage of inclusion of a validation set is that the calibration equation can be tested, but a smaller number of samples were used to create the calibration model. The results for both are shown in Table 4.1.6.

	Cinchona bark (Sample Set 1)			Valerian (Sample Set 2)		
	Calibration only	Calibration set	Validation set	Calibration only	Calibration set	Validation set
Number of samples in set	37	27	10	35	25	10
Wavelength(s)	1710nm	1710nm		1172nm	1232nm	
R^2	0.998	0.999		0.990	0.992	
F-value	>10,000	17539		3270	2704	
Standard Error	1.785	1.59	2.21	3.92	3.67	6.15
% mean accuracy	49.82	16.4	126.5	56.4	45.12	51.25
Estimated limit of quantification	> 10%	>10%		~75%	~75%	

Table 4.1.6 Summary of the MLR calibration model results for Sample Set 1 (containing the three sets of Cinchona bark mixtures in Avicel) and Sample Set 2 (containing the three sets of Valerian mixtures in Avicel)

The addition of a second wavelength was considered for the calibration models with the validation set, but for both calibration models the F value decreased and the Standard Error of Prediction increased. The relative merits of the types of statistic used to assess MLR equation results are illustrated here. The Standard Error (of calibration or validation) is an absolute value. On first inspection the results seem low and therefore favourable. However, when the relative size of the plant material content is taken into account, by calculating the % mean accuracy, it can be seen that these errors are very large, ranging from 16% to as much as 127%. Mixtures with a low plant material content will significantly influence the size of this error and at first it may seem that the calibration equation is unsuitable. However, if the relative error for each mixture is calculated separately and plotted against plant material content (Figure 4.1.13 for the Cinchona bark calibration and Figure 4.1.14 for the Valerian calibration) it can be seen that the relative errors decrease with increasing plant material content. Note that the *x*-axis is not a continuous variable but represents different categories of plant material content. The errors decrease to a reasonable level (below approximately 5%) for a plant material content for the Cinchona bark species at 10% and above, but only at levels of 75% and above for the Valerian sample set. This suggests that the NIR calibration constructed for all three Cinchona bark species may be suitable for the determination of plant material content but that created for the Valerian samples is not. Figures 4.1.15 and 4.1.16 show the plot of predicted against actual plant material for the Sample Sets 1 and 2 respectively. It is noted that despite the extremely poor prediction accuracies for the Valerian sample calibration (Figure 4.1.14), the correlation is still very high. This demonstrates the need to consider the correlation along with other statistical information.

One of the reasons that the calibration equation is not suitable for the Valerian sample set is that there are significant differences between the different Valerian samples suggesting that they cannot be grouped together for a successful calibration, but they may be suitable on their own. For this reason, a separate calibration model was created for each of the three Valerian mixture sets. Due to the small number of samples in each set it was appropriate only to have a calibration set with no validation set.

Figure 4.1.13 Relative Error (%) for individual samples in Sample Set 1(Cinchona bark in Avicel mixtures)

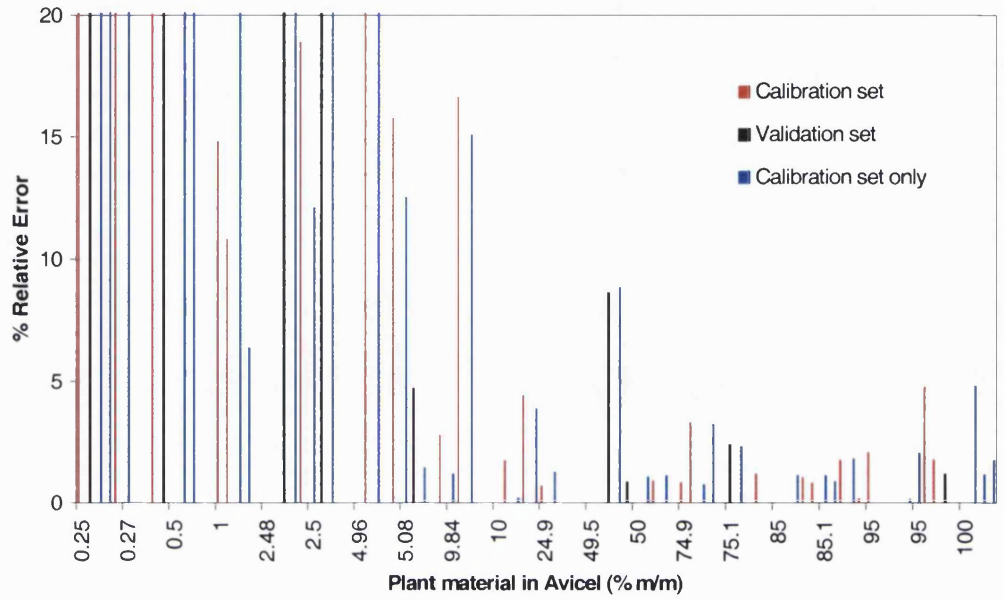


Figure 4.1.14 Relative Error (%) for individual samples in Sample Set 2 (Valerian in Avicel mixtures)

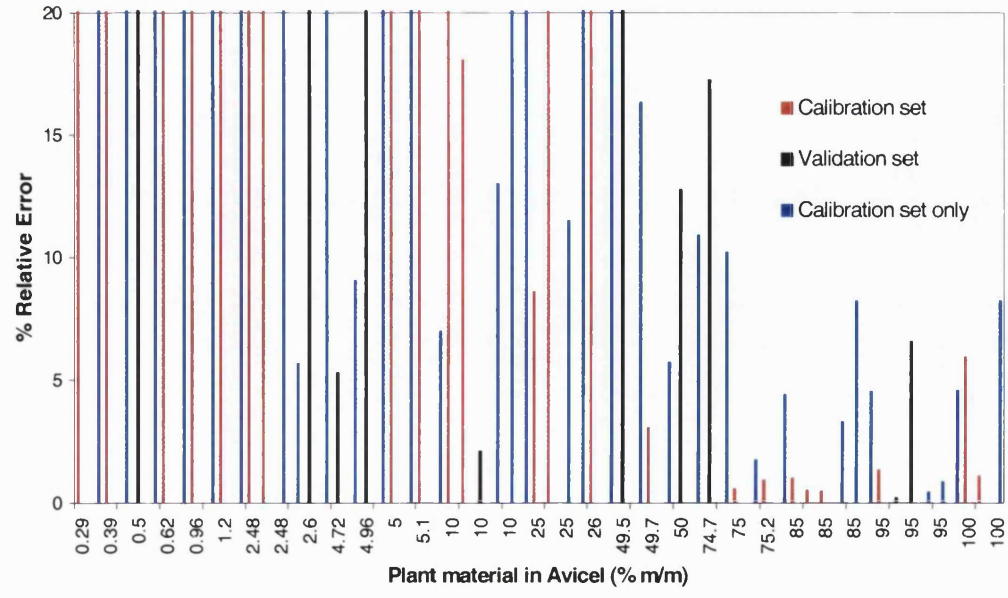


Figure 4.1.15 Plot of predicted against actual plant material content for Sample Set 1 (Cinchona bark in Avicel mixtures)

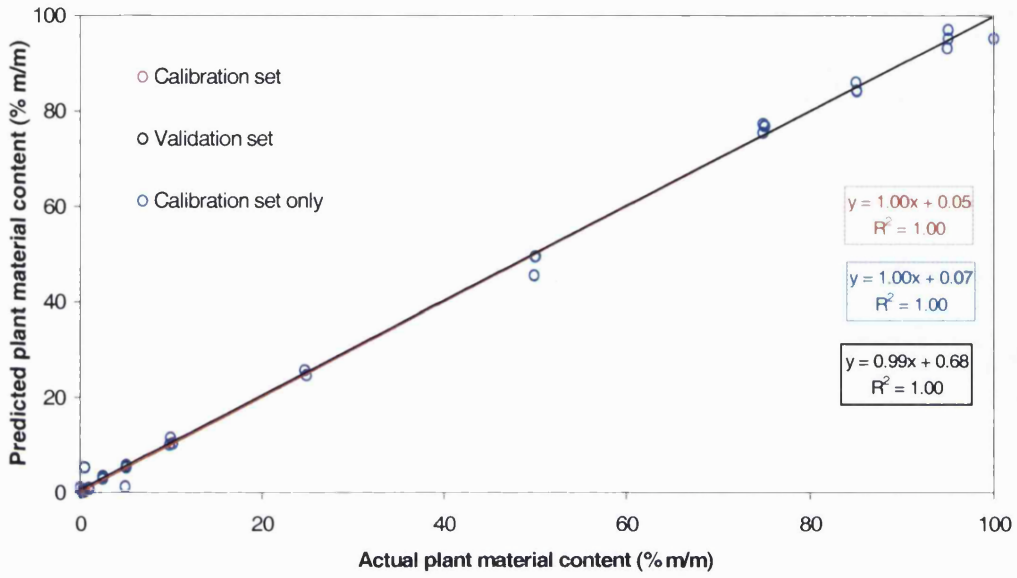
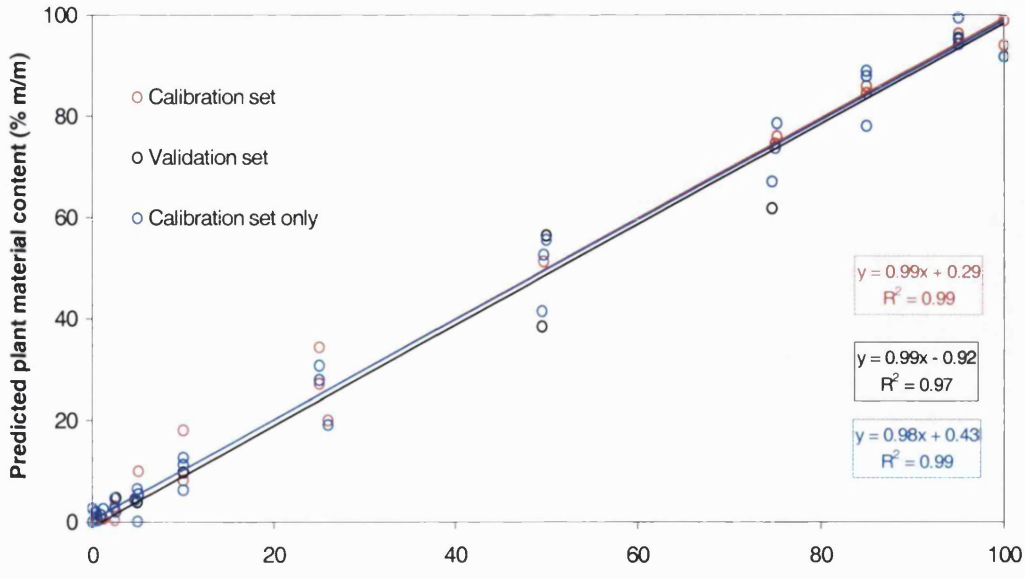


Figure 4.1.16 Plot of predicted against actual plant material content for Sample Set 2 (Valerian in Avicel mixtures)



The relative errors for each mixture for each calibration is shown in Figure 4.1.17. It can now be seen that the relative error is much less than that for the combined calibration set, with 'reasonable' errors for plant material mixtures of 25% or more. Figure 4.1.18 shows the plant material content predicted for the Indian and unknown Valerian mixtures using the calibration equation developed for the English Valerian mixture set. Ideally, the equation of the lines should be $y = x$. The equations of the regression lines show that there is a difference in the calibration model with both a bias (the line does not cross through 0) and skew (the slope of the line is not 1). It can be concluded that for MLR calibrations separate calibration models are required.

4.1.5.8 Prediction of Plant Material Content in an Alternative Adulterant

It is not known if the wavelength of 1710nm selected in the calibration for the Cinchona bark samples (Sample Set 1) was used as a measure of the plant material content or the Avicel® content, although inspection of Figure 4.1.19 suggests that it is taking the Avicel® content into account as the peak with the (negative) maxima at this point is that for the pure Avicel® and not the pure plant material. In order to test this theory, the calibration model for Sample Set 1 (calibration set only) was used to predict the plant material content of the *Cinchona officinalis* sample in a series of mixtures with lactose as the adulterant. The result is shown in Figure 4.1.20. Interestingly, although the predictions are extremely poor, there is an absolute correlation between the predicted and the actual plant material content values. The negative slope of the regression line is explained by the fact that at this wavelength (1710nm), the Avicel® is being taken into account and not the plant material. Although on initial inspection is appeared that the lactose and the Avicel® spectra crossed at this point and therefore had the same absorbance values, in fact the difference between them is very large (~ -0.09 absorbance units), which explains why the more lactose present in the sample the poorer the prediction.

The NIR spectra were studied from Figure 4.1.19. The intention was to show how selection of different wavelengths to create the NIR calibrations changes the ability of that calibration to accurately predict the plant material (Cinchona bark content) in an alternative adulterant.

Figure 4.1.17 Relative Error (%) for individual samples in MLR calibrations for mixtures of English Valerian in Avicel, Indian Valerian in Avicel and the unknown Valerian sample in Avicel

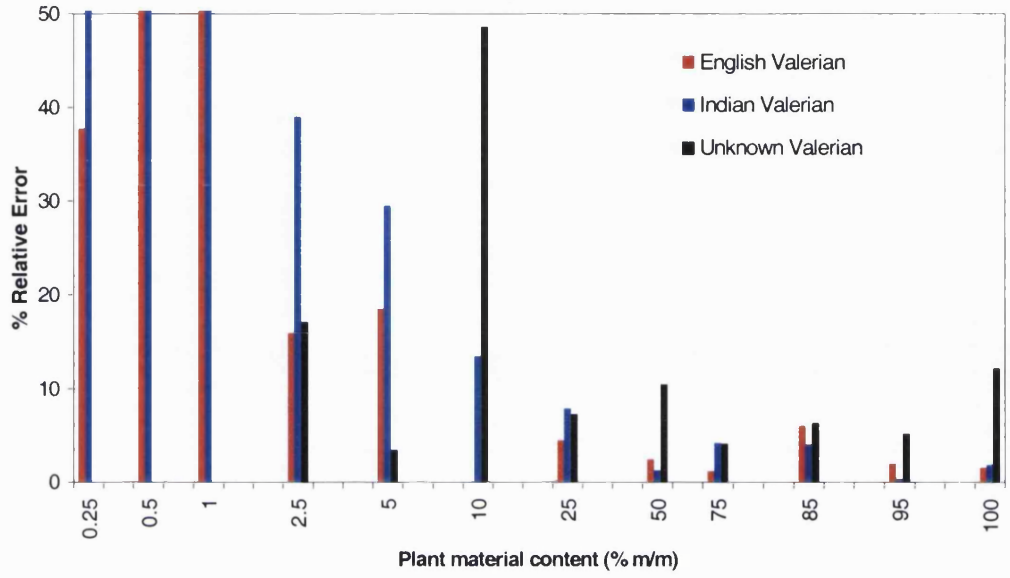


Figure 4.1.18 Plot of predicted against actual plant material content for samples of Indian Valerian in Avicel and the unknown Valerian sample in Avicel using the MLR equation model developed for English Valerian in Avicel

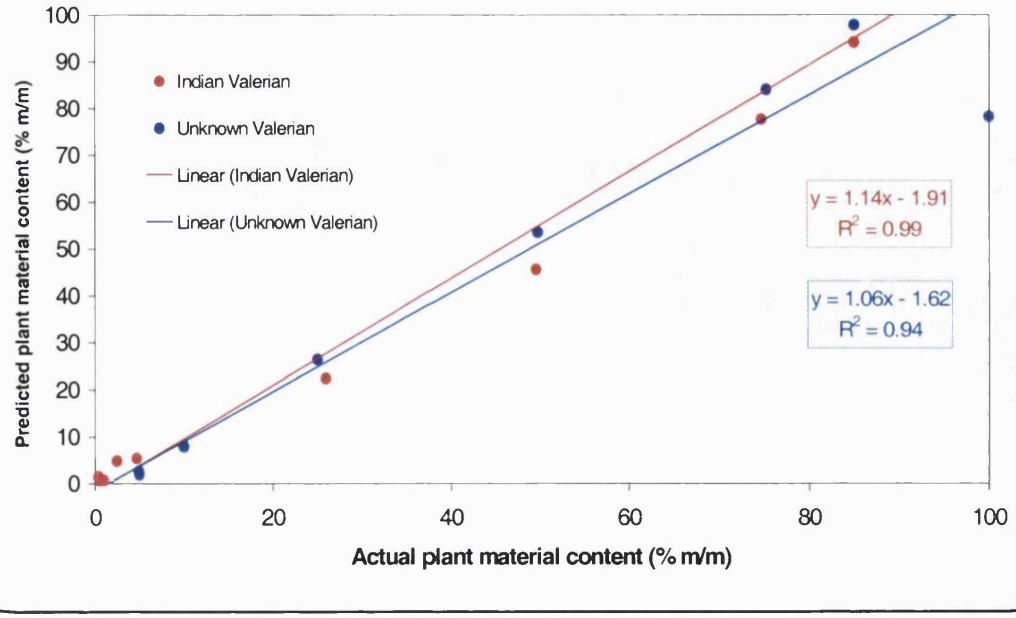


Figure 4.1.19 SNV corrected 2nd derivative spectra of Avicel, lactose and powdered *Cinchona lancifolia*, *Cinchona succirubra* and *Cinchona officinalis* over the wavelength range 1600nm to 1800nm

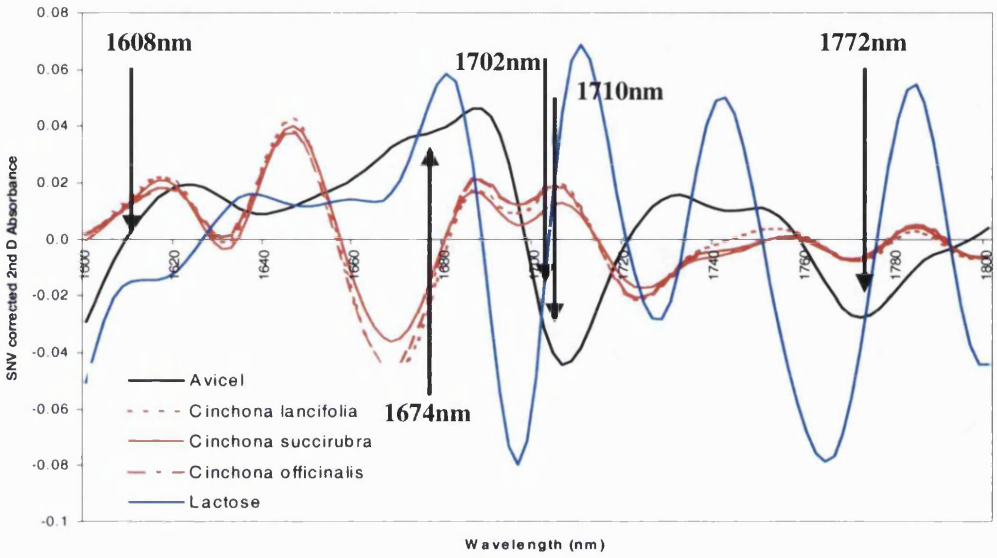
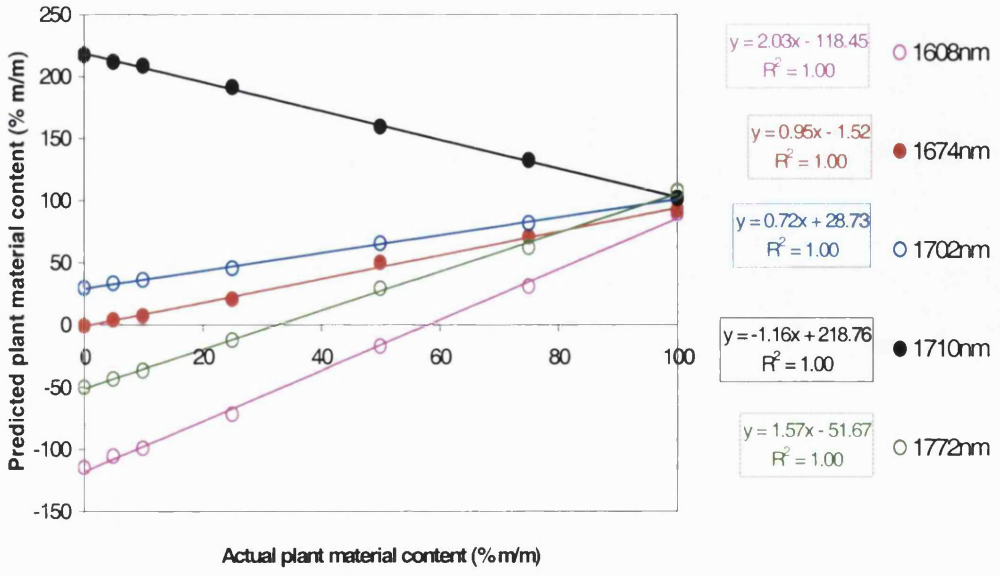


Figure 4.1.20 Plot of predicted against actual plant material content for mixtures of *Cinchona officinalis* in lactose using MLR calibration models developed for Sample Set 1 (Cinchona bark mixtures in Avicel) using different single wavelengths



The differences in these predictions are linked to the absorbance values of the Cinchona bark, Avicel® and lactose at that particular wavelength. The chosen fixed wavelength would therefore replace the 1710nm wavelength chosen by the forward search method. The wavelength at 1608nm was chosen first, at which point the Avicel® has an absorbance of zero, the Cinchona barks have a positive absorbance value and the lactose a negative absorbance value. As with the regression line obtained using the 1710nm wavelength, there is a large difference in the absorbances of the lactose and Avicel®, but the value is less and the sign reversed (0.015 absorbance units), resulting in a lower predicted lactose content and hence a change in the slope of the line. The calibration model using the 1674nm wavelength provided very accurate predictions, explained by the fact that there was minimal difference between the lactose and the Avicel® (0.0003 absorbance units). For the two calibration models created using the remaining fixed wavelengths, the difference in value between the predicted and the actual content is again due to the difference in the absorbance of the lactose and the Avicel® (0.0136 and 0.0104 absorbance units for the 1702nm and 1772nm calibration models respectively). As even a small difference in absorbance value between the lactose and the Avicel® resulted in a very large difference between the predicted and the actual lactose content. Therefore it is necessary to check from the numerical data the exact difference between the two spectra. For all calibrations, the accuracy of prediction for the 100% *Cinchona officinalis* sample is dependent on the closeness of the spectra for the three species of Cinchona bark. The different Standard Errors of Calibration (there was no validation set), the correlation and the F values of the MLR calibrations for the different wavelengths used are shown in Table 4.1.7.

	1710nm forward search	1608nm	1674nm fixed	1702nm	1772nm
Correlation (R^2)	1.00	0.99	0.99	1.00	0.99
F value	>10,000	4847	4823	8436	5666
SEC (% m/m)	1.79	3.43	3.44	2.61	3.18

Table 4.1.7 Summary of MLR calibration model statistics for Sample Set 1 (Cinchona bark samples in Avicel®) with different single wavelength selections

It can be concluded that in this study the wavelength that allows for the most accurate prediction of the Cinchona bark content in Avicel® is not able to provide an adequately accurate MLR calibration for the Cinchona bark in lactose. Either a balance would have to be achieved or separate NIR calibration models with different wavelengths used. However, this study has shown that by careful selection of wavelengths by study the individual NIR spectra and their relationships with others, it may be possible to create MLR calibrations that can be used to predict the amount of adulterant in a material regardless of what the adulterant is, although the greater the similarity in character to the material being studied and its adulterant the more difficult it will be to develop a MLR calibration that can achieve this. This illustrates another benefit of the use or MLR for the construction of NIR calibration models. Over the whole wavelength range there are clear differences between the lactose and the Avicel® and so it would not be possible to use NIR calibrations created using the PLSR and PCR methods, as a range of wavelengths and not single wavelengths are used in these methods.

4.1.6 Summary of Results and Conclusions

Although it can in no way be said that a procedure has been set up that allows the use of NIR spectroscopy for the determination of plant material in mixtures of physically similar materials, the results demonstrate the problems that arise with this technique, which can then be taken into consideration with further work employed. This was one of the main reasons for undertaking this work and the use of different methods of data analysis can be used to extract different kinds of information from the same pieces of data, namely the NIR spectra. The benefits of manual wavelength selection has also been demonstrated. A few conclusions can be drawn from the work which are summarised below.

- Sampled materials for NIR analysis should be similar in particle size, as the use of SNV corrected NIR spectra only minimises the differences, not removes them.

- The use of CWS as a Library identification method is more suitable than the use of the MDWS method.

- It is not simply enough to set a threshold value for the Library method in order to distinguish between pure samples and adulterated or contaminated samples - it is necessary to study a range of mixtures in order to determine the limits of detection/quantification of the method.

- The limits of detection and quantification are summarised in Table 4.1.8 as determined for the several data analysis methods used in this investigation. These are only very rough estimates made from the Figures presented in this section of work and only serve as a guide to the relative usefulness of the techniques.

- Use of a single technique alone does not extract sufficient information from the NIR data and a combination of these methods must be used for any useful procedure to be developed for this or a similar type of work.

Method	Limit
CWS Library method	>75%
Polar Co-ordinate plot	>50%
Principal Component Analysis	>50%
MLR calibration	>25%

Table 4.1.8 Estimates of the limit of quantification for plant material content in Avicel for the methods employed in the investigation

Although not successful in its aims, it must be remembered that at present it is unlikely that any non-NIR technique could be used to solve the same problem. This work has shown that with care, NIR spectroscopy could be used in similar work providing its limitations are known and several methods of data analysis are applied to the problem.

4.2 THE IDENTIFICATION AND DISTINCTION OF UMBELLIFERAE SEEDS AND THE DETECTION OF CONTAMINATION OF FENNEL WITH HEMLOCK

4.2.1 Introduction

Fennel and Hemlock belong to the Umbelliferae family, of which there are approximately 300 genera and 3000 species in total. The name derives from the Latin word *umbellula* which means "a little shade" - the individual flower stalks arise form the same point, resulting in clusters of flowers closely arranged to produce the decorative, lacy 'parasol' effect.

The Umbelliferae family is very versatile and members are used as food crops (e.g. Wild Carrots and Parsnips), herbs and spices (e.g. Caraway, Coriander and Cumin), Aromatherapy oils (Fennel), perfumes and flavourings (Fennel, Aniseed and Dill). Dill (*Anethum graveolens*) and Anise (*Pimpinella anisum*) are still commercially grown to produce crops for medicinal use The most well-documented poisonous species, Hemlock was probably responsible for the death of Socrates and contains the alkaloid coniine (Grieve 1992a,b). A NIR method has been developed for the essential oil content and composition of Fennel, Caraway, Dill and Coriander by Schulz *et al* (1998).

4.2.2 Aims of the Work

Fennel (*Foeniculum vulgare*) and Hemlock (*Conium maculatum*) are very similar in appearance and grow in the same climates and conditions, leading to a potential for accidental contamination when harvesting or even mis-identification. Previous work carried out at the Centre for Pharmaceutical Analysis (Kudo *et al*, 1998) showed that it was possible to distinguish the difference between these two species using NIR spectroscopy. Consequently, this opened up the possibility that this technique could be employed to identify contaminated Fennel samples. However, only 'pure' samples of Fennel and Hemlock were studied, and so the aim of this work was to ascertain if it was possible for to detect the difference between 'pure' Fennel samples and prepared mixtures (adulterated samples) of Hemlock in Fennel and if so, to what level of contamination.

The investigation was designed so as to answer the following questions.

Qualitative Analysis

- Is it possible to distinguish between members of the Umbelliferae family?
- Can a Library and identification method be set up such that Fennel samples adulterated with Hemlock can be distinguished from the 'pure' samples? If so, at what levels of contamination?

Quantitative Analysis

- Can a calibration be set up to accurately quantify the level of adulteration (contamination) and if so to what levels of contamination?

In general:

- How sensitive or robust are these qualitative or quantitative methods?

4.2.3 Materials and Methods

4.2.3.1 'Pure samples'

Twelve samples of Fennel seeds and at least three seed samples for each of eleven other members of the Umbelliferae family were purchased from health food stores. The five samples of Hemlock seeds were obtained from the Pharmacognosy stores at the School of Pharmacy. These seeds were used whole and placed in Waters® 5mm HPLC vials to a depth of approximately two centimetres.

4.2.3.2 Preparation of adulterated (contaminated) samples

One sample of Fennel seed and one sample of Hemlock seed were used to prepare a series of mixtures (known subsequently as the adulterated samples) from 0 to 100% (a total of 16 including the Fennel sample at 0% and the Hemlock sample at 100%). These were known collectively as the single mixture set. The seeds were ground in a coffee grinder to produce a coarse powder prior to mixing. The particle size when grinding the samples was not controlled, although all Fennel and Hemlock samples were ground for the same amount

of time and the particle size was similar on visual inspection. Ideally, any method developed that could detect the adulteration/ contamination of Fennel samples should not require for samples to be of identical (controlled) particle size, but it was borne in mind that this may have an adverse affect on the method development.

In order to test for the possibility of creating a more robust qualitative or quantitative calibration (that is, not specific to a single sample each of Hemlock and Fennel), a second set of mixtures (15 adulterated samples in total) ranging from 0 to 100% was prepared. Each mix consisted of a different randomly chosen Fennel and Hemlock seed sample and were known collectively as the random mixture set. Table 4.2.1 shows the exact ratio of Fennel to Hemlock for both sets of samples.

4.2.3.3 Instrumentation and Equipment

All measurements were carried out on the FOSS NIRSystems 6500 spectrophotometer with Rapid Content Sampler module. The data acquisition software was NSAS™, which was also used for the averaging of the spectra. Waters® 4mm clear glass vials were used as the sample presentation method. The vials were filled to a depth of approximately two centimetres. Spectral data analysis was performed using Vision® and The Unscrambler® software.

4.2.3.4 Near Infrared measurements

Twelve spectra were obtained for each sample, the vial being shaken in between each spectral measurement. An average of the twelve spectra was used for the data analysis. The whole seeds were scanned for the comparison of the Umbelliferae seeds, whilst the two mixture sets consisted of the coarse powder samples.

4.2.4 Results

4.2.4.1 Identification of and Differentiation of Umbelliferae seeds.

A Library (*Library 1*) was constructed such that all twelve types of Umbelliferae seeds were present as a separate Product.

Single set of mixtures	Random set of mixtures
Hemlock content in Fennel (% m/m)	
1.49	0.94
3.64	1.13
4.58	1.73
6.16	2.5
8.21	3.38
10.35	4.10
21.17	4.79
32.2	5.21
39.2	7.81
51.1	10.15
61.6	10.74
71.6	15.23
78.5	24.52
90.1	51.77
	67.54
$n = 14$	$n = 15$

Table 4.2.1 Exact content of Hemlock in Fennel (% m/m) for the single mixture set and the random mixture set of adulterated samples

The Umbelliferae seeds used in the Library were Angelica, Anise, Caraway, Celery, Chervil, Coriander, Cumin, Dill, Fennel, Hemlock, Lovage, and Parsley . A MDWS method with a threshold Match Value of 4.9 on SNV corrected 2nd derivative spectra allowed for complete distinction between the twelve different Umbelliferae seeds, the greatest similarity (lowest Match Value) being between the Fennel and the Hemlock seeds (4.98). Table 4.2.2 below shows the relative number of failures as the threshold Match Value using this method is increased. The total number of spectra in the Library was 57.

Match Value	% of failures	Number of samples
4.9	0	0
5	1.8	1
6	12.3	7
10	68.4	39
15	89.5	51
20	98.2	56

Table 4.2.2 Relative number of failures with increasing Match Value for *Library 1* containing SNV corrected 2nd derivative spectra of 12 different Umbelliferae seeds

PC Analysis was carried out on 2nd derivative spectra of the Umbelliferae samples. Several different wavelength ranges were studied. The aim was to achieve separation between all the different seeds, but especially the Fennel, Hemlock and Anise seeds, which are the most similar. No wavelength range was found which allowed complete visual separation of all the Umbelliferae species, but use of the full wavelength range (1100nm to 2500nm) allowed for the greatest separation of the Fennel, Hemlock and Anise seeds (see Figures 4.2.1 and 4.2.2).

Polar Co-ordinate plots were then constructed to ascertain if these allowed for visual separation of the Umbelliferae seeds. As for the PC Analysis, several different wavelength ranges were tried.

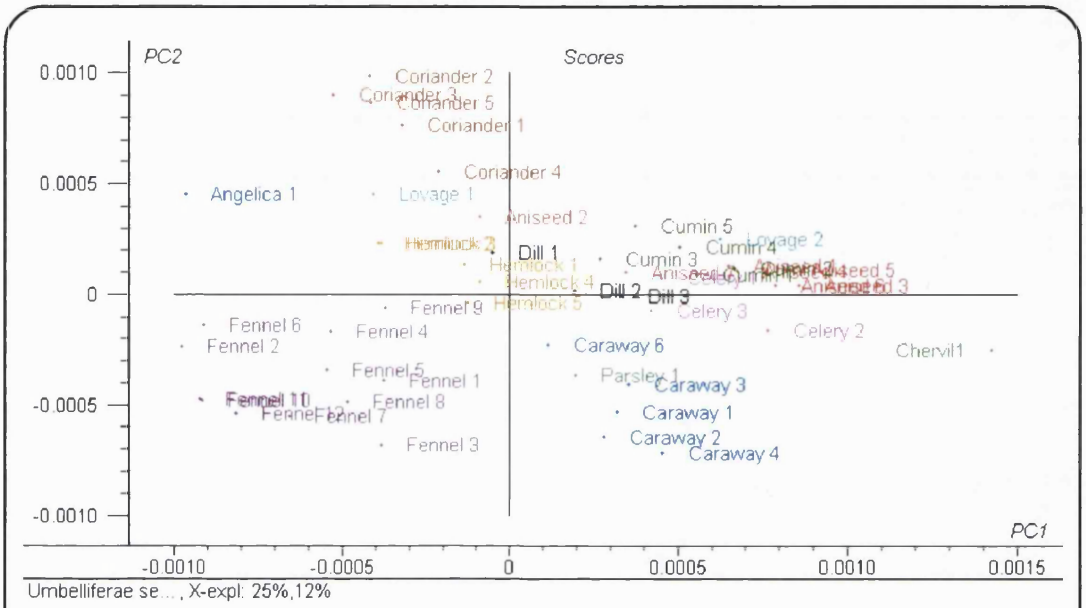


Figure 4.2.1 Principal Component plot of 2nd derivative spectra of Umbelliferae seeds over the full wavelength range 1100nm to 2500nm

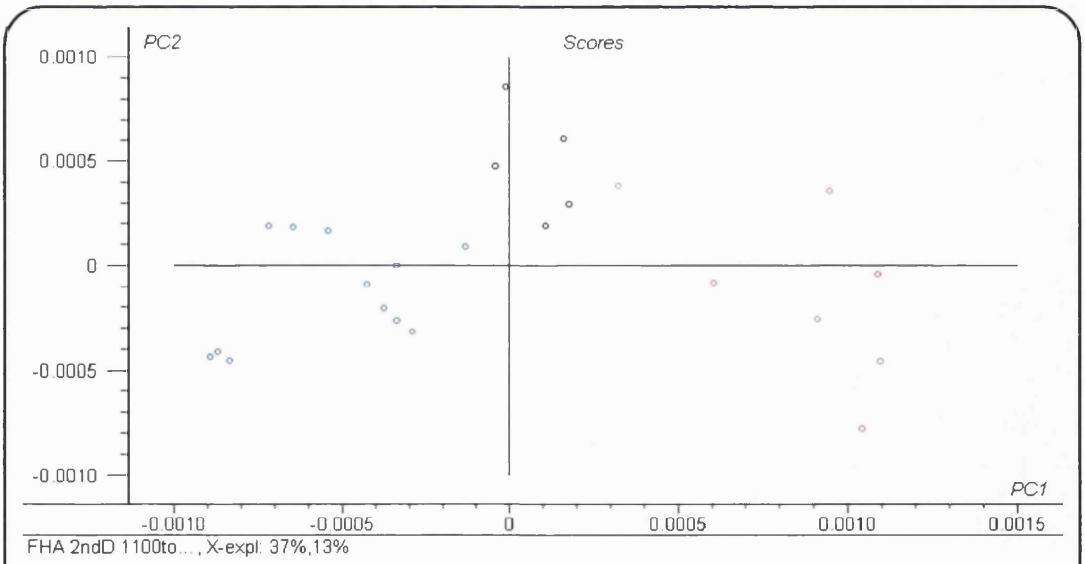


Figure 4.2.2 Principal Component plot of 2nd derivative spectra of Fennel (blue), Anise (red) and Hemlock (black) seeds over the wavelength range 2200nm to 2400nm

The greatest visual separation for the Fennel, Hemlock and Anise seeds was achieved by using the full wavelength range (Figure 4.2.3), whereas the greatest separation for all the Umbelliferae seeds was achieved using the 2200nm to 2400nm wavelength region (Figure 4.2.4).

4.2.4.2 Comparison of pure Fennel and Hemlock samples with adulterated samples

Figures 4.2.5 and 4.2.6 show the raw and SNV corrected 2nd derivative spectra of the 12 samples of Fennel seeds and 5 samples of Hemlock seeds. Upon visual inspection it can be seen that there are differences between the Fennel and the Hemlock samples, seen in the wavelength region 2200nm to 2400nm (shown in Figure 4.2.6) and other regions of the NIR spectrum. Most of these differences are peak height differences as opposed to wavelength shifts in the peaks. Differences are also seen in the water content seen in the wavelength region approximately 1900nm to 1950nm. There are distinct differences amongst the Fennel samples at the water peak and other in other regions of the spectrum, whereas the Hemlock spectra are more closely grouped. Differences noted are likely caused by factors such as ageing, storage conditions and geographical origin.

Library methods

Libraries were constructed such each Library Product represented a single mix. Each adulterated sample was present as a single Library Product. *Library 1* consisted of the single mixture set, whilst *Library 2* consisted of the random mixture set. The CWS method on SNV corrected 2nd derivative spectra was applied to *Library 1*. The Match Value of the Fennel Product against the Library Product of each mix was noted. As there were three averaged NIR spectra present in each Product, the average Match Value was calculated. It was found that samples containing at least 21% Hemlock had increasing Match Values corresponding with an increase in Hemlock content. For samples containing less than 21% Hemlock, there was no correlation between the Match Value and the amount of Hemlock present in the sample (all the samples had Match Value of 0.9997 or above).

Figure 4.2.3 Polar Co-ordinate plot of SNV corrected 2nd deriviatve spectra of Umbelliferae seeds over the wavelength range 1100nm to 2500nm

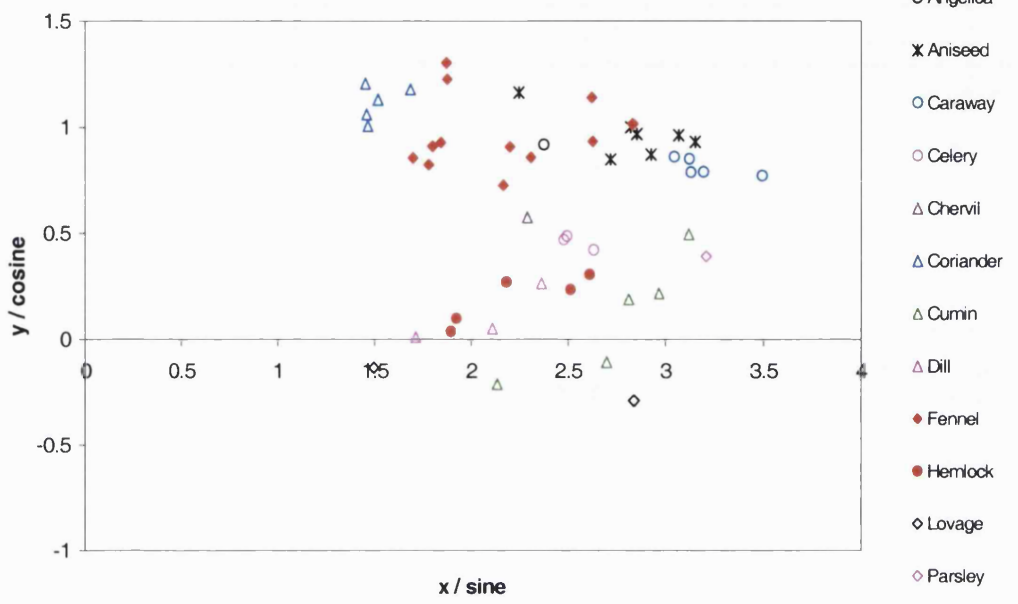


Figure 4.2.4 Polar Co-ordinate plot of SNV corrected 2nd derivative spectra of Umbelliferae seeds using over the wavelength range 2200nm to 2400nm

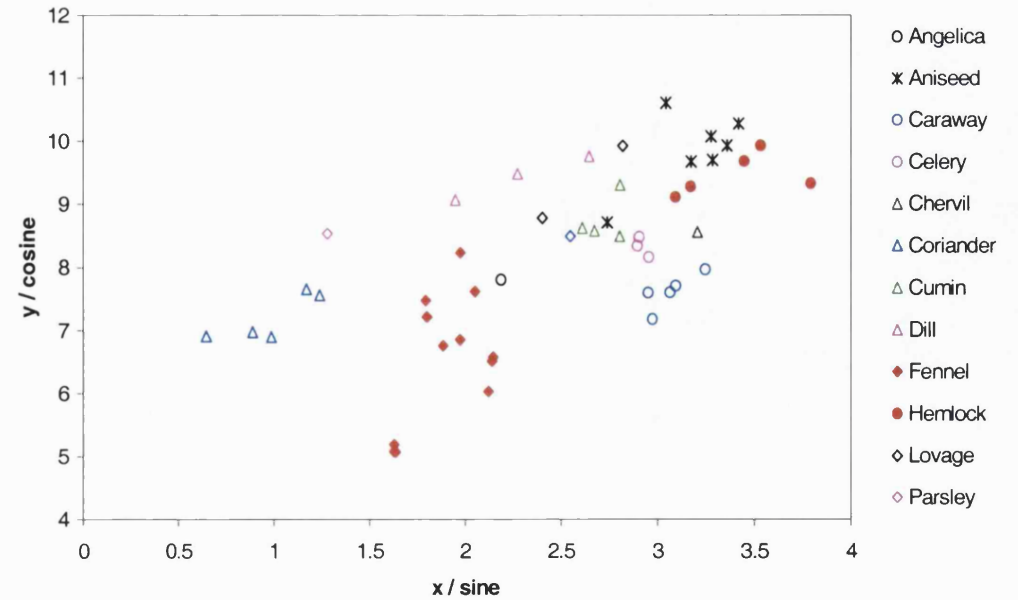


Figure 4.2.5 Raw (untreated) Absorbance spectra of Fennel seeds (blue) and Hemlock seeds (red) over the wavelength range 1100nm to 2500nm

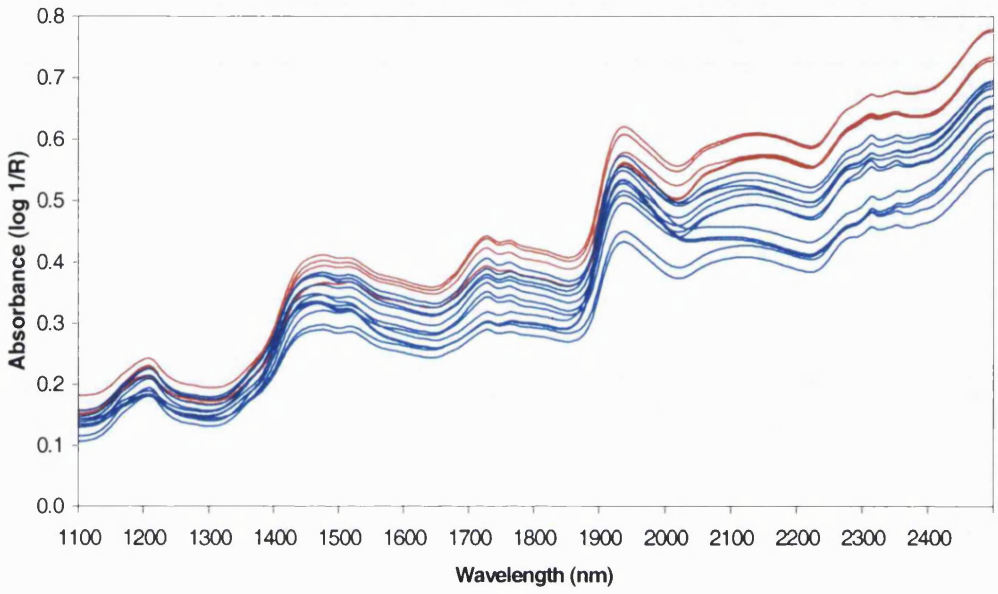
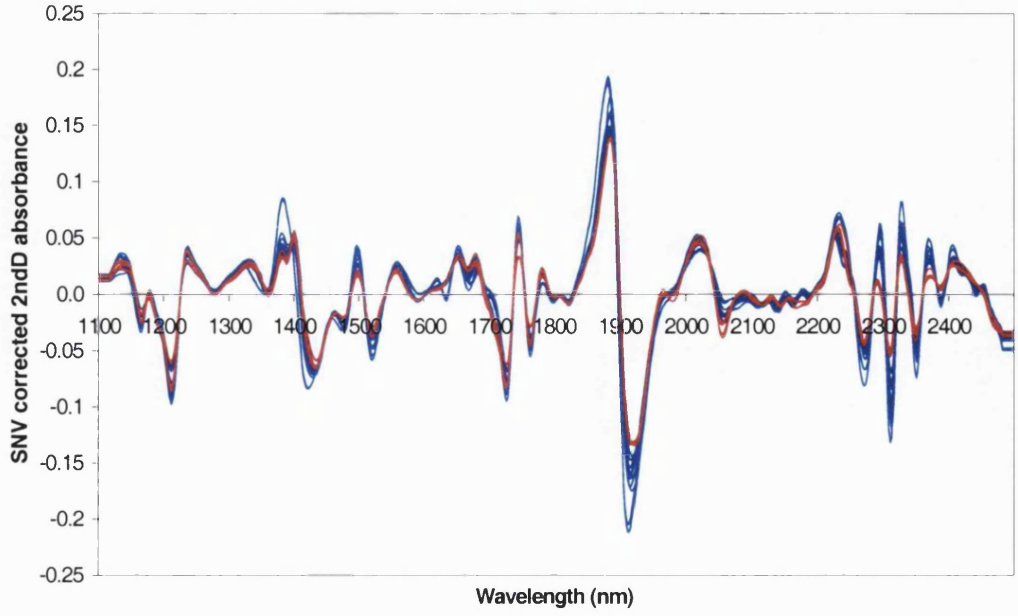


Figure 4.2.6: SNV corrected 2nd derivative spectra for Fennel seeds (blue) and Hemlock seeds (red) over the wavelength range 1100nm to 2500nm



However, despite the correlation between Match Value and higher Hemlock content samples, the Match Values were still 0.98 and above, and so it is unlikely that this method could be used in practice for the detection of adulterated samples of Fennel (data not shown).

The MDWS method was then applied to *Library 1*. A plot of the Match Value against the Hemlock content of the samples is shown in Figure 4.2. 7. The red line is the line of best-fit for all the mixtures plus the corresponding ground Fennel and Hemlock samples, whilst the blue dotted line covers all samples with a Hemlock content of less than 15%. It can be seen that there is a reasonable correlation between the MDWS Match Value and the Hemlock, content for the higher Hemlock content mixtures, but the equation of the line and the correlation is significantly altered for the lower Hemlock content samples. It may therefore be possible to use this method for the detection of adulterated with a Hemlock content of over 15%, but the method is not suitable for lower Hemlock content samples.

When the CWS method was applied to the *Library 2* containing the random mixture set, no correlation was found between the Match Values and the Hemlock content for even the high Hemlock content mixtures. However, a correlation was seen between the MDWS Match Value and the Hemlock content, as seen in Figure 4.2.8, which can be compared directly with Figure 4.2.7. It can be seen that not only is there a lower correlation between the Match Value and Hemlock content for this sample set, the Match Values themselves are greatly lowered. A further three samples of Fennel were added to the Library as a separate Product and the highest Match Value for the Product containing the whole set of Fennel samples was found to be 2.2. From Figure 4.2.8 it can be seen that the Match Values for adulterated samples of 15% or over have Match Values of 5 or more. This suggests that although the Match Values for the random sample mix are lower than for the single sample mix, they appear larger than the likely Match Values for additional samples of Fennel. It is therefore possible that this method could be used as an indication of the adulteration of Fennel samples.

Figure 4.2.7 Plot of Maximum Distance in Wavelength Space Match Value against Hemlock content in ground adulterated samples of Fennel (single mixture set)

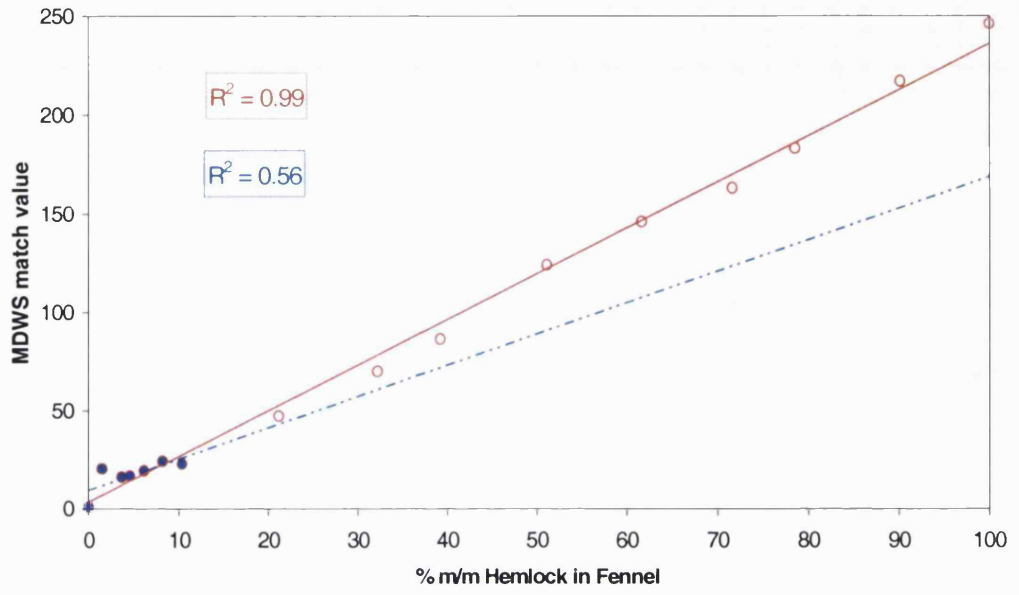
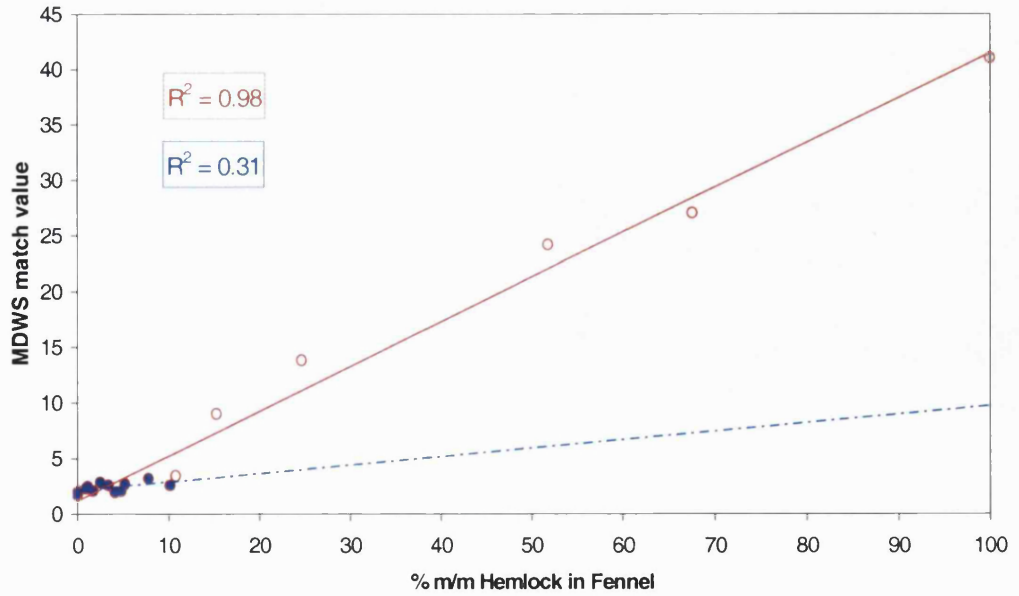


Figure 4.2.8 Plot of Maximum Distance in Wavelength Space Match Value against Hemlock content in ground adulterated samples of Fennel (random mixture set)



Polar Co-ordinate Plots

Several wavelength ranges were studied to find the Polar Co-ordinate plot that allowed for the greatest visual separation of the adulterated Fennel samples. The 2200nm to 2400nm wavelength range performed the best out of those tried (Figure 4.2.9). For ease of interpretation of the plot, only some of the adulterated samples are labelled individually, the others are marked with a black cross. All the adulterated samples were in the 'correct order' in terms of Hemlock content, connecting the pure Fennel and Hemlock samples. Figure 4.2.10 shows the same Polar Co-ordinate plot but with the sine and the cosine values each plotted against the Hemlock content. It can be seen that there is a good correlation between these parameters. The apparent flatness of the lines is due to the expanded y-axis scale to accommodate both the sine and cosine values. From inspection of Figures 4.2.9 and 4.2.10 it is suggested that this plot would able to show adulterated samples of Fennel for Hemlock contents of above approximately 20%.

The same wavelength ranges were tried for the second set of mixtures using the random samples and again the 2200nm to 2400nm wavelength region provided the best results. However, it can be seen from Figures 4.2.11 and 4.2.12 that there is little correlation between the sine and cosine values and the Hemlock content and therefore it is concluded that the use of Polar Co-ordinate plots are unlikely to be of use for the detection of adulterated Fennel samples.

Principal Component Analysis

PC Analysis was attempted over a number of different wavelength ranges to ascertain if there was a correlation between the Hemlock content of the samples. For a successful PC Analysis method, the major difference between a mixture and the others would have to be due to the Hemlock (or Fennel) content and not any other factor such as water content. Therefore, the Principal Component values for Principal Components 1 and 2 were plotted against the Hemlock content for the set of single mixtures (Figure 4.2.13) and the random set of mixtures (Figure 4.2.14). The number of Principal Components recommended to describe of all the variability of the data was 5 for the single sample mixtures and 8 for the random sample mixtures.

Figure 4.2.9 Polar Co-ordinate plot of the single mixture set of adulterated samples of Fennel over the wavelength range 2200nm to 2400nm (SNV 2ndD spectra)

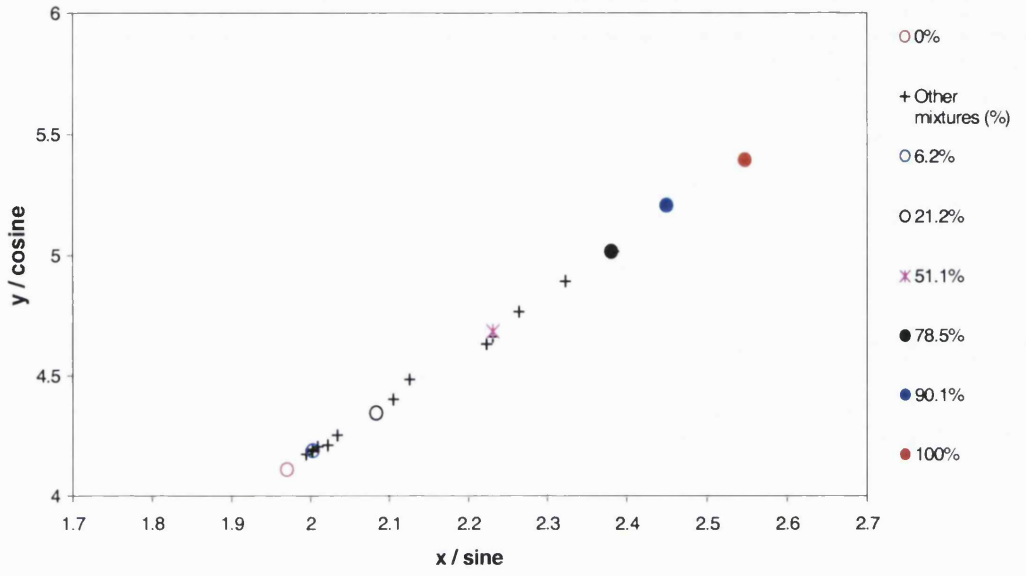


Figure 4.2.10 Polar Co-ordinate sine (red) and cosine (blue) values for adulterated samples of Fennel over the wavelength range 2200nm to 2400nm (single mixture set)

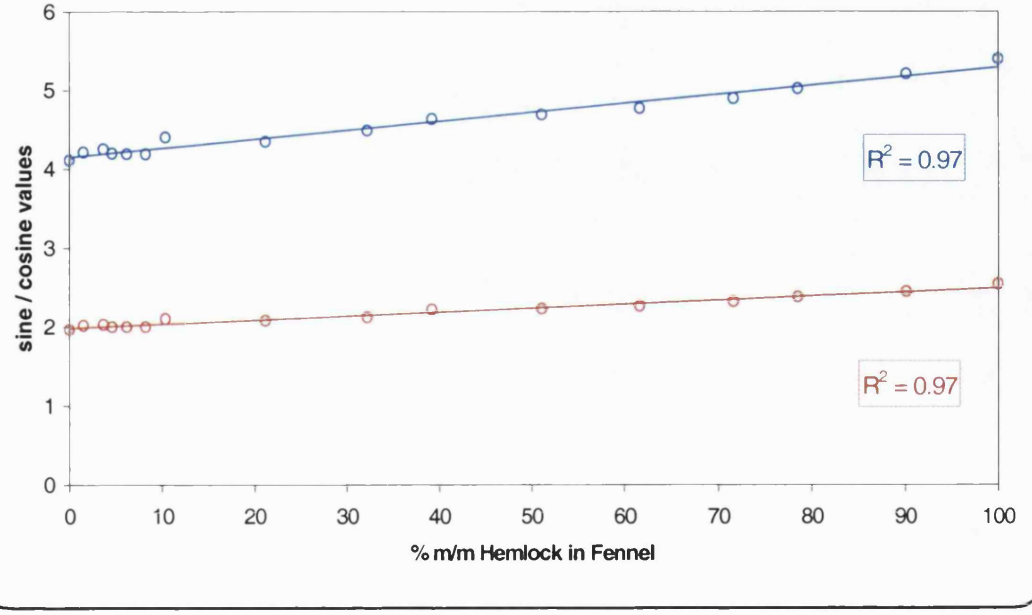


Figure 4.2.11Polar Co-ordinate plot of the random mixture set of adulterated samples of Fennel over the wavelength range 2200nm to 2400nm (SNV 2ndD spectra)

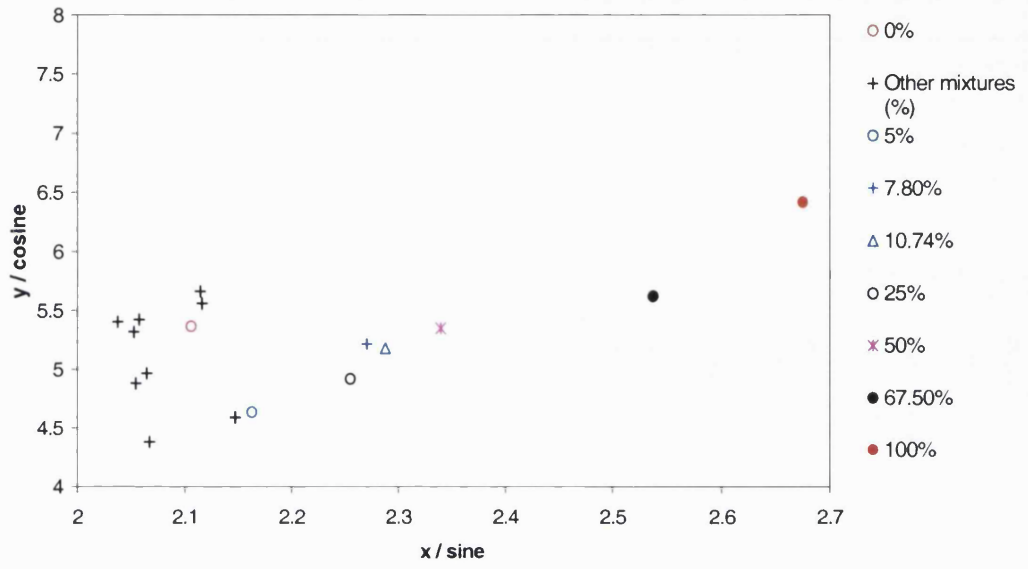


Figure 4.2.12 Polar Co-ordinate sine (red) and cosine (blue) values for adulterated samples of Fennel over the wavelength range 2200nm to 2400nm (random mixture set)

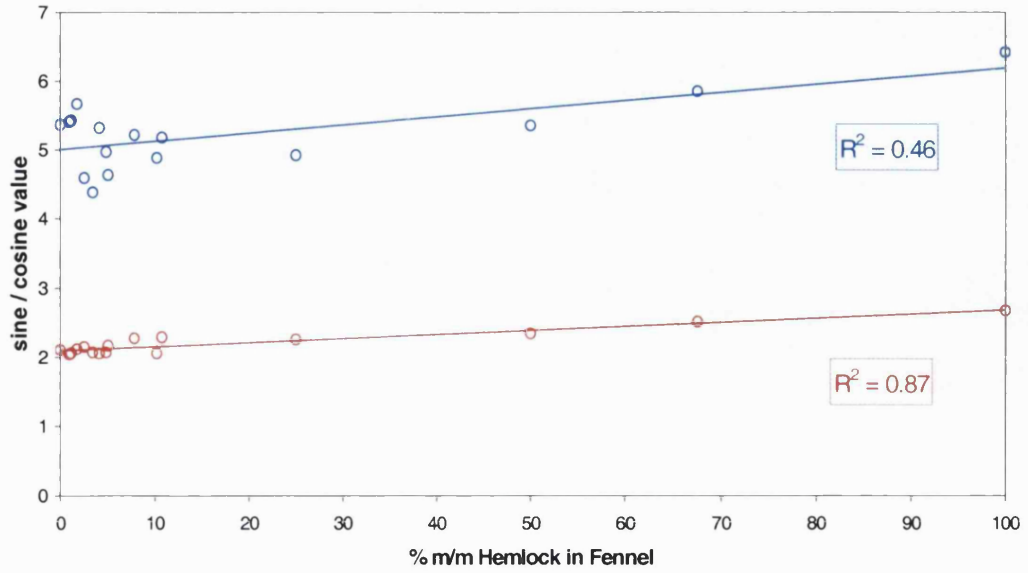


Figure 4.2.13 Plot of PC1 (red) and PC2 (blue) values against Hemlock content for single mixtures of adulterated samples of Fennel (2nd derivative spectra, 1100nm to 2500nm)

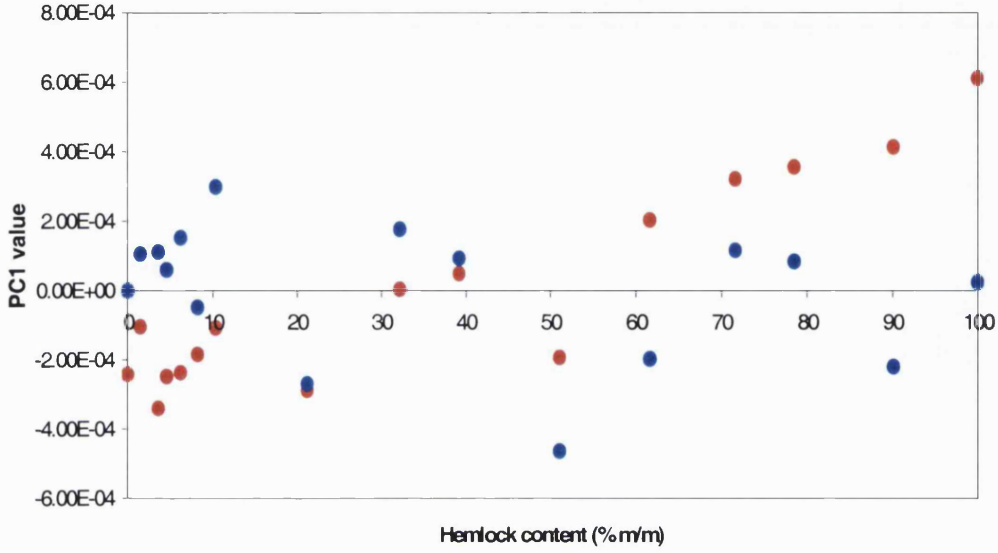
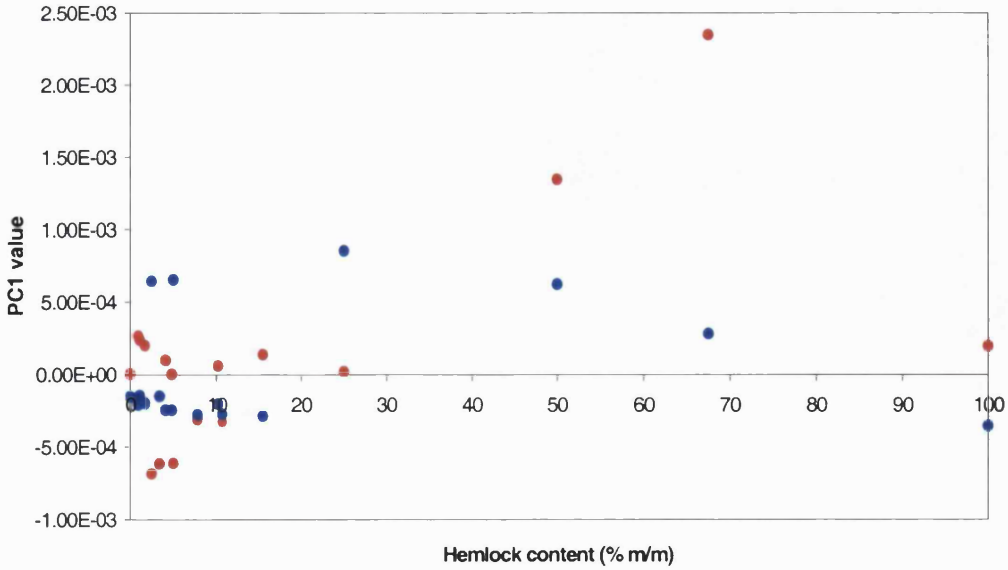


Figure 4.2.14 Plot of PC1 (red) and PC2 (blue) values against Hemlock content for random mixtures of adulterated samples of Fennel (2nd derivative spectra, 1100nm to 2500nm)



For the single mixtures of adulterated samples, if the 50% sample is disregarded, there may be a correlation between the PC values and Hemlock content for samples 30% and above. No correlation was found between the Hemlock content and PCs 1 and 2 for the random mixture set of adulterated samples, suggesting that factors other than Hemlock content were responsible for the majority of the data variability. One possibility was that water could be responsible and so PC Analysis was carried out after the removal of the wavelength region responsible for water. To be certain of removal of the complete water absorbance band, the wavelength region 1850nm to 1950nm was removed. This did not significantly alter the plot. It was also possible that the particle size of the mixtures was responsible for a large proportion of the variability of the data. For this reason the twelve whole Fennel seed samples (F1 to F12) and the five whole Hemlock seed samples (H1 to H5) were added to both the single mixture set and the random mixture set and PC Analysis was carried out, again discounting the water peak region. The results for the single mixtures set are shown in Figure 4.2.15. It can be seen that the whole seed samples are distanced from the remainder of the ground mixtures, suggesting that the first Principal Component variability is describing the particle size. It is possible that this is due to the fact that only 2nd derivative spectra were used here as SNV correction is not an option with The Unscrambler® software program. However, the plot shows the adulterated samples extending from the Fennel sample used in the mixtures to the Hemlock samples (the sample marked as 'Hemlock ground' was used in the mixtures). The adulterated samples were in roughly the correct order of Hemlock content, especially for the higher Hemlock content samples. The same analysis was also carried out on the random mixture set. The adulterated samples in this plot do not extend from the Fennel samples to the Hemlock samples. Again, it can be seen that particle size is responsible for a large proportion of the variability of the data (Figure 4.2.16).

Quantitative NIR Calibration construction

Due to the small number of samples in each mixture set, the creation of a quantitative calibration was limited to the use of the MLR method.

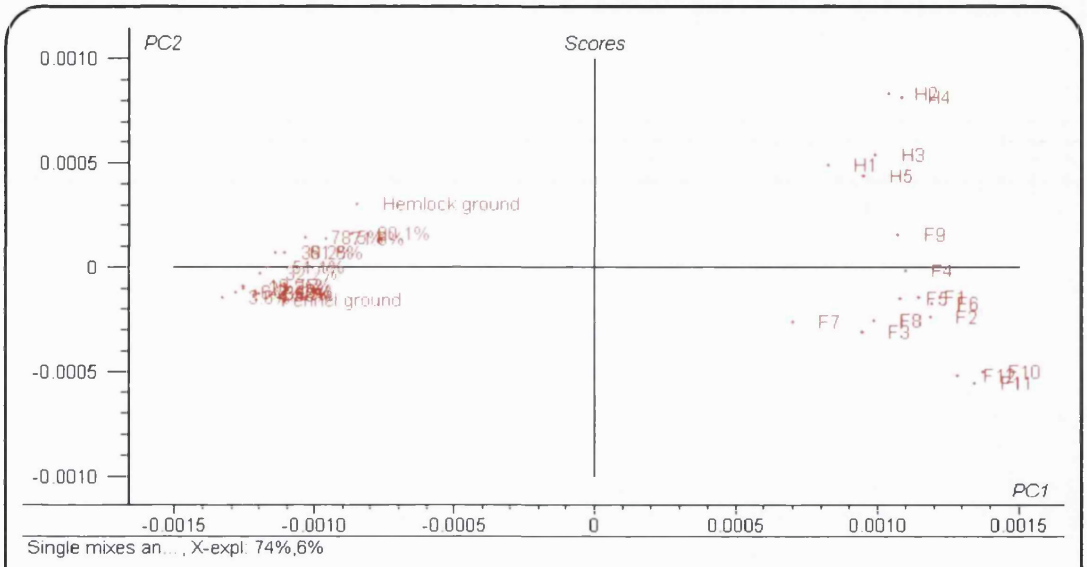


Figure 4.2.15: Principal Component plot for single mixture samples of adulterated Fennel, ground Fennel and Hemlock seeds and whole Fennel and Hemlock seeds (2nd derivative spectra minus the water peak wavelength range)

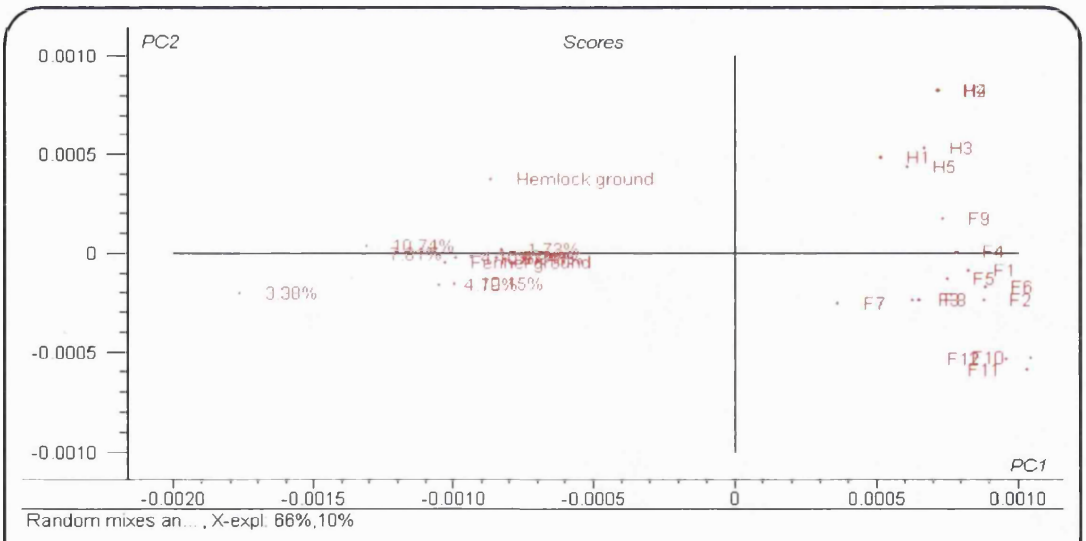


Figure 4.2.16: Principal Component plot of random mixture samples of adulterated Fennel, ground Fennel and Hemlock seeds and whole Fennel and Hemlock seeds (2nd derivative spectra minus the water peak wavelength range)

It was decided that the most appropriate use of the data would be to confine the single mixture set to the calibration set and the random mixture set to the validation set to create a NIR calibration (*Calibration 1*). A second NIR calibration (*Calibration 2*) with the reverse situation was created so that the random mixture set was in the calibration set and the single mixture set was in the validation set. This way it was possible to compare and relate the NIR data obtained for the two sets of mixtures. A single averaged spectrum was obtained from the twelve Fennel samples and a single averaged spectrum was obtained from the five Hemlock samples and these were included in the NIR calibrations.

It is likely that the NIR calibration created from the single mixture set samples (*Calibration 1*) would be too specific and therefore would not be able to accurately predict the Hemlock content of the random mixture validation set. Ideally, however, *Calibration 2* should be able to predict accurately the Hemlock content in the single sample validation set. The results are summarised in Table 4.2.3. Wavelengths were added to the calibration equation until either the F value decreased or the Standard Error for the validation set increased.

Statistic	*Calibration 1*	*Calibration 2*
Calibration set samples	Single mixture set	Random mixture set
Wavelength (s) (nm)	1630 + 2086 + 2298	2338 + 2282 + 1224
Correlation (R^2)	1.00	0.98
SEC (% m/m)	0.81	4.22
F value	9157	184
SEP (% m/m)	23.22	4.55
Validation set Correlation (R^2)	0.53	1.00

Table 4.2.3 Summary of results for *Calibration 1* created using the random mixture set as the calibration set and *Calibration 2* created using the single mixture set as the calibration set

The % Relative Errors were also calculated for both the calibration and validation samples. The % Relative Errors for the single mixtures in the validation set of *Calibration 1* were

high (poor) at 20% or less for samples with a Hemlock content of 20% and above. For samples with a Hemlock content of 50% and above the % Relative Error decreased to 5% or less.

The main problem is that a MLR quantitative method for adulterated samples is unlikely to work because there is no wavelength where there is sufficient minimal difference in (SNV 2ndD) absorbance between the Fennel samples, which show distinct differences in their spectra, yet which show a distinct difference between the Fennel spectra and the Hemlock spectra (even using ground samples which tend to give more reproducible spectra and through the use of the SNV correction which minimises effects due to particle size). To demonstrate this a NIR calibration was constructed that contained only the pure ground Fennel and Hemlock samples in the calibration set (no validation set). MLR, PLS and PC Analysis methods were applied to ascertain if any of these methods could be used to accurately predict the Hemlock content of the Fennel samples as 0% and the Hemlock samples as 100%. MLR1 (Table 4.2.4) shows the results obtained for a single wavelength only (selected by the forward search method in Vision® software as having the highest correlation with the reference data). MLR2 shows additional summation wavelengths added to improve the calibration. These were added to demonstrate that even with three wavelengths, the results obtained are still poor. The use of additional divisor wavelengths did not produce improved calibrations. A number of different mathematical pre-treatment methods were also tried but none appeared to be significantly better than the SNV corrected 2nd derivative treatment used for all previous data analysis.

The main factor that unduly influences the construction of a NIR calibration is the large variation in the NIR spectra of the pure Fennel and Hemlock samples compared to the relative differences between the two species. Unless these factors are removed or minimised the NIR technique described here is not suitable for the detection of Fennel contaminated with Hemlock.

	MLR1	MLR2	PLS	PCR	
Pre-treatment method	SNV 2nd D		SNV 2nd D	Un-treated	2nd D
Range of predicted Fennel contents (% m/m)	-12 to 20	-4 to 4	-2 to 2	-15 to 21	-13 to 18
Range of predicted Hemlock contents (% m/m)	88 to 109	98 to 102	98 to 102	92 to 107	66 to 101
Wavelength(s) (nm)	2292	2292 2184 1644	N/A	N/A	N/A
Number of factors PCs recommended	N/A	N/A	6	11	7

Table 4.2.4 Prediction accuracy (Hemlock content) for pure Fennel and Hemlock samples using three different methods of calibration construction.

4.2.5 Conclusions

Whilst this investigation has not provided a global NIR method for the detection of contaminated/adulterated Fennel, it has highlighted the type of problems that have to be overcome with NIR analysis. The conclusions are summarised below.

- Umbelliferae seeds can be successfully identified and distinguished using a Library with the MDWS method.

- Although it had been shown previously that Fennel could be distinguished from Hemlock, further investigation has shown that it is likely to be very to be difficult to develop a single method that can be successfully used for the detection of contamination. The results show that detection of Hemlock contamination below 20% is unlikely, but greater care with sample selection and the inclusion of greater sample and mixture numbers is likely to improve the results. It cannot be concluded from this work that mixtures of other members of the Umbelliferae

family (or indeed any other family) cannot be distinguished from the pure material and accurately quantified. This may be successful for other pairings of seeds which show a greater difference than that between Fennel and Hemlock (Figure 4.3.2).

- Particle size may have to be better controlled in future to minimise unwanted influences on a NIR method/calibration. It is also desirable (although not always practical) to minimise differences in moisture content, or to take steps to eliminate its effect on any NIR method developed.

- A combination of two or more data analysis methods, such as a Library Identification and a quantitative NIR calibration may have to be used before any sound judgements can be made regarding the results obtained.

- There appears to be a great deal of difference between the Fennel samples which may be due to age in addition to geographical origin and other factors. The detection of contaminated samples would be greatly eased if the difference between the Fennel samples was minimised. This may be achieved in practice if a company obtained samples from the same supplier and it may be possible therefore to create a Library method robust yet sensitive enough to detect levels of contamination down to levels of 10% or even less. However, the Hemlock as contaminant cannot be controlled and this is also likely to limit the possibility of creating an accurate calibration.

4.3 THE USE OF NIR MICROSCOPY FOR THE DETERMINATION OF THE MOISTURE CONTENT OF AGAR

4.3.1 Introduction

NIR spectroscopy has been used in the food and agricultural industries for many years and has been used for the determination of water (moisture) content in a wide range of materials, such as rice (Choi *et al* 2000), ethanol (Pasquini *et al* 2000),compost (Suehara *et al* 2000) and soybeans (Ben-Gera and Norris, 1968b). There is also published work demonstrating the ability of NIR spectroscopy to determine the moisture content of pharmaceutical materials such as lactose (Hammond *et al* 1998 and Hogan and Buckton 2001) and ampicillin-trihydrate (Plugge and van der Vlies, 1993). The purpose of this particular study is to ascertain if NIR spectroscopy can be used to determine the water content of Agar, a natural raw material used in the pharmaceutical industry as a suspending or thickening agent and as a component in solid microbiological culture media (Martindale 1999).

Traditional methods used in the determination of water content include the measurement of loss of mass on drying in an oven, the Karl Fischer titration method and gas chromatography. Such techniques are either expensive, time consuming or destructive in nature. NIR spectroscopy could provide a rapid and non-destructive alternative to these methods. The purpose of this work was to ascertain the potential of NIR spectroscopy as an alternative method of water determination in Agar, using the Karl Fischer titration method as the reference method. NIR calibrations created using MLR, PLSR and Principal Component Regression (PCR) will be compared in terms of their prediction accuracy.

Whether or not a NIR calibration is deemed successful depends on the criteria set for it, which are in turn determined by the setting and the purpose for which the quality control is intended. A 'rough' determination of moisture content may be adequate for incoming samples, or more accurate predictions (for example less than 5% relative error for a sample) may be necessary in the pharmaceutical industry.

4.3.2 Origin and Uses of Agar

Agar is the hydrophilic colloidal substance extracted from the cell walls of certain red algae. The name Agar is derived from a Malaysian word "agar-agar" which literally means "seaweed". The best Agar is extracted from species of the red algal genera Pterocladia and Gelidium, which are harvested by hand from populations in Spain, Portugal, Morocco, the Azores, California, Mexico, New Zealand, South Africa, India, Chile and Japan. Agars of lesser quality are extracted from Gracilaria and Hypnea species. Agar quality is seasonal in Pteroclaid species, being low in the colder months and high in the warmer seasons. Commercial Agar has been shown to contain, in addition to insoluble debris, proteinaceous material and soluble and insoluble salts, and two major separable polysaccharides known as agarose and agaropectin. Agarose is a strongly gelling non-ionic polysaccharide which is regarded as consisting of 1,3- linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose units (Figure 4.3.1). Agaropectin is a less clearly defined more complex polysaccharide having sulphate groups attached to it.

Modern Agar is a purified form consisting mainly of the neutral fraction known as agarose; the non-ionic form of the latter makes it more suitable for a range of laboratory applications. Agar in a crude or purified form also finds wide usage in the food industry where it is used in various kinds of ices, canned foods and bakery products and in pharmaceutical manufacturing as a suspending or thickening agent (Martindale 1999). Approximately 10,000 tonnes of Agar are produced world-wide at present. There is currently a shortage of exploitable populations of Agar-producing seaweeds and Agar is consequently an expensive product. At present, more than 50% of all Agar produced is food-grade Agar being produced from the genus Gracilaria (B&V Company website 2002, Guiry 2002).

A monograph for Agar is detailed in the European Pharmacopoeia (2002g) and British Pharmacopoeia (BP2001g). The BP defines Agar as the polysaccharides from various species of Rhodophyceae belonging to the genus Gellidium. It is prepared by treating the algae with boiling water, the extract is filtered whilst hot, concentrated and dried.

4.3.3 Water and Hydrogen Bonding

Two main absorption bands on 2nd derivative spectra at approximately 1450nm and 1930nm and are due to water or hydrogen bonding. The band at 1450nnm is a OH overtone band whilst the band seen at 1930nm is a OH combination band. This OH combination band consists of multiple overlapping bands and the apparent location of the band moves due to the changes in the relative proportions of the individual bands making up the composite bands. Peak shifts also depend on whether the water present in a sample is bonded intra- or inter-molecularly. It is known that hydrogen bond formation results in shifts to longer wavelengths whereas hydrogen bond breakage results in band shifts to shorter wavelengths. Shifts as large as 20nm to 50nm may be observed. Such peak shifts occur when changes in hydrogen bonding occur. This may happen when chemical changes happen in mixtures. It is possible that some Agar samples have water present that is not just 'free' water, but which is bound to some component in the sample. If these proportions are different between samples then this may lead to interference in a NIR calibration. This would be especially so for the loss-on-drying method of water content determination, as this measures only the 'free' water. The Karl Fischer technique being a chemical method can also be measure the bound water present in a sample.

The water absorption bands are also highly prone to temperature changes within the samples being measured and so care should be taken to minimise these differences. These differences can be manifest as peak shifts and a change in the peak maxima (or minima) value (Osborne *et al* 1993a, Shenk *et al* 1992).

4.3.4 Materials and Methods

4.3.4.1 Instrumentation and Software

The Karl Fischer determination equipment consisted of a Metrohm 701 KF Titrino, 703 Titration Stand, 701 KF Titrino keyboard and Sartorius printer. A FOSS NIRSystems 6500 spectrophotometer with Rapid Content Sampler module was used for acquisition of spectra. Data acquisition and averaging of spectra was carried out using Vision® version 2.21 software.

4.3.4.2 Materials

43 different samples of Agar were obtained courtesy of Mark Bellchambers, also of the Centre for Pharmaceutical Analysis. The samples were from various pharmaceutical excipient suppliers, several of these samples were different batches from the same supplier. Although no details were supplied along with the batches, it is likely that these Agar samples were not of BP standard and were in fact of varying specifications tailored for different commercial uses. B & V of Gattatico RE, Via Don Minzoni, Italy supplied many different grades of Agar including for uses in the pharmaceutical, confectionary and canned meat and fish industries. The Agars supplied from this company are all from the same class (Rhodophya) but different families (Philophoraceae, Gelidiaceae and Gracilariaceae) and within these families 8 different genus and approximately 30 different species. It is likely therefore that the 43 samples obtained from this and several other companies encompass a fairly wide range of Agar grades and types (B&V website, 2002). Disodium tartrate, dried methanol and Karl Fischer reagent were obtained from BDH (AnalaR®) Laboratory Supplies, Poole, Dorset, UK.

4.3.4.3 Karl Fischer measurements

Details concerning the Karl Fischer titration method can be found in the Materials and Methods Chapter, Section 3.4.1. A test for linearity of the reaction was first carried out. The Karl Fischer reagent must be titrated against a material with a known water content in order to known exactly how much iodine is present in the KF reagent. Five separate titrations of the KF reagent were carried out with disodium tartrate (five different masses were used), which has a known water content of 15.66%.

Moisture determinations for each sample were carried out within an hour of taking the NIR measurements in order to mimise the possibility of sample moisture intake / loss in between taking measurements. After introduction of the sample to the titration vessel, a time lapse (extraction time) can be set before the titration is carried out. For many different types of sample, the longer the extraction time the higher the determined water content until a plateau is reached. The benefit in time saved for shorter extraction times is offset by the possibility of less repeatable results. For this reason a single sample of Agar was assayed

using different extraction times, to find the shortest extraction time allowing the threshold water content to be reached. After study of the results (Figure 4.3.2) an extraction time of thirty minutes was chosen.

This study is concerned with the comparison of the developed NIR method with the Karl Fischer moisture content determinations as the reference data. The Karl Fischer method used may therefore not be the optimal method for the moisture content of Agar, but care has been taken to optimise the method to obtain the most reliable results. The apparent moisture content of a single sample will vary according to the developed method in terms of extraction time etc.

4.3.4.4 NIR measurements

Each sample of Agar was placed in a 4mm quartz glass disposable Waters® HPLC vial to a depth of at least 1cm and sealed in order the keep the samples as dry as possible in between taking the NIR measurements and the Karl Fischer water determinations. Each spectrum obtained was the average of 32 scans and an average of 5 spectra for each sample was taken for use in the development of the NIR method. The glass vial was shaken and tapped in between NIR spectra measurements.

4.3.4.5 NIR method development and calibration

MLR (Vision®), PLSR (Vision®) and PCR (The Unscrambler®) methods were used to create several calibrations and these were directly compared. Several different mathematical pre-treatment methods were also tried. Samples were selected manually and assigned to the calibration sets in an approximate 65%/45% split. The samples in the calibration set were chosen to have water contents across the full range of water contents for all samples tested. The samples were split between the calibration and validation sets in the same manner a second time with different samples in the calibration set but still across the full water content range. These two sample sets used to create separate NIR calibrations were known as Sample Set 1 and Sample Set 2. The purpose of using two different sample sets was to ascertain if similar results could be obtained from different NIR calibrations provided that samples were chosen equally from the lower, middle and upper range of water contents.

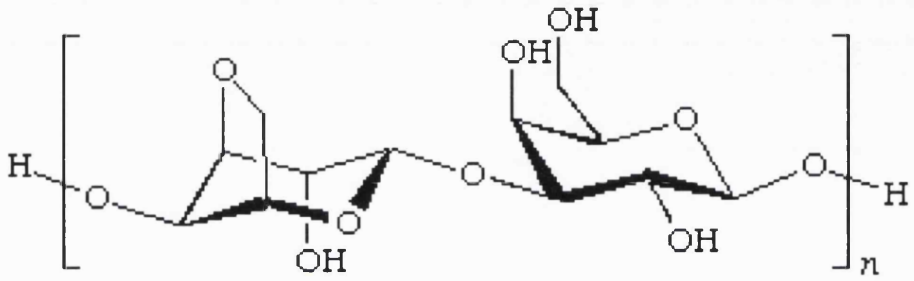
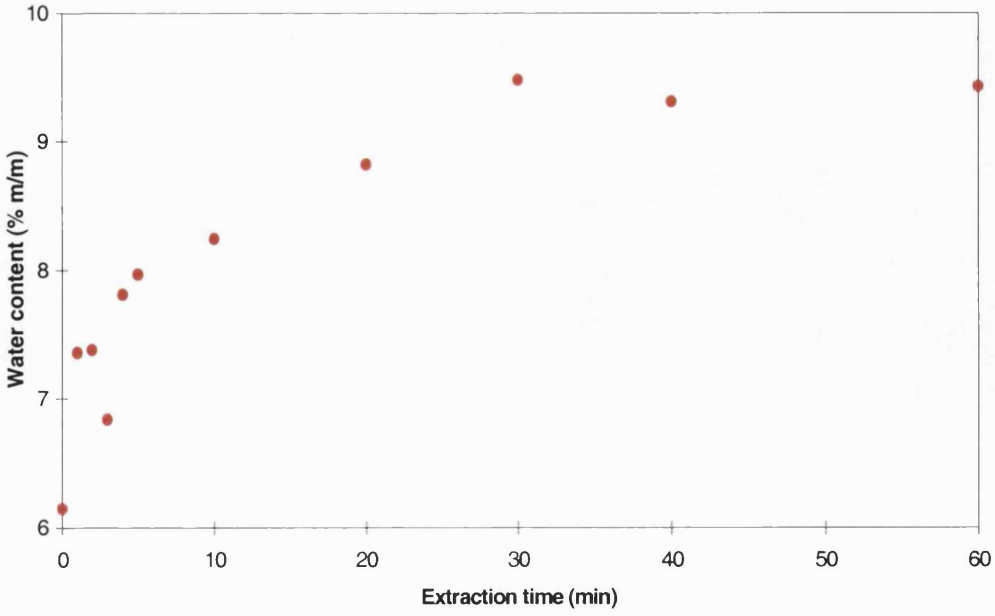


Figure 4.3.1 Agarose polysaccharide unit

Figure 4.3.2 Plot of water content of an agar sample against extraction time as determined by the Karl Fischer method



When using Vision® software to create a MLR calibration equation and two or more wavelengths are required, the additional wavelengths selected are those that provide the highest correlation to the reference data in combination with the previously chosen wavelength(s). This is known as a step-up method of wavelength selection. Sesame is a Bran & Luebbe NIR spectral analysis software program that offers a wavelength selection method known as combination, in which all possible combinations of wavelengths are compared to find the wavelength combination that has the highest correlation with the reference data. This often provides better wavelength selections than the step-up method but the disadvantage is that for a three wavelength combination the process takes about 5 hours. This wavelength method was used to compare with the MLR calibration created by the Vision® software as only one or two wavelengths were used to create the calibrations (which took a couple of minutes) as the use of more wavelengths was highly likely to result in over-fitting of the calibration to the calibration set samples. Vision® software has the option of using a second wavelength as a divisor term (that is as a ratio with the first wavelength, $\lambda 1/ \lambda 2$). This option was also studied in addition to the use of the 2nd wavelength being used in summation with the first wavelength.

Two methods of validation were used for both the Vision® PLSR and The Unscrambler® PCR methods. The first was Full Cross Validation and the second was with an internal validation set (as used for the MLR method). Full Cross Validation® is different for the Vision® and The Unscrambler® programs. In The Unscrambler® program, the same samples are used both for creation of the NIR calibration model and the validation set. Samples are left out from the calibration set and the model is calibrated on the remaining data points. Then the values for the left-out samples are predicted and the prediction residuals are computed. With Full Cross Validation only one sample is left out at a time and the process is repeated until all prediction residuals are combined. Cross Validation is most commonly used when employing PCR as it is recommended that test (validation) sets only be used with sample sets of 50 samples or more. However, the test (validation) set method was included to allow direct comparison with the other NIR calibrations created. In Full Cross Validation in Vision®, again a single sample is left out from the calibration set and the

model is calibrated on the remaining data points. However, this model is then applied to the validation set to give a SEP. Although both the PLSR and the PCR methods are forms of PC Analysis, PLSR takes into account the reference values of the calibration set samples (see Section 2.1.6).

4.3.4.6 Precision of the BP and NIR methods

A single Agar sample was assayed for water content six times using the Karl Fischer method. Six spectra for a single sample of Agar were obtained on a single day for determination of repeatability (short term precision) and six spectra were obtained on six consecutive days for determination of intermediate precision. These spectra were used to obtain NIR predicted water contents using the MLR calibrations created and the results used to compare the precision of the reference (Karl Fischer) and the NIR methods using the statistical equations described in Section 2.4.2.5.

4.3.5 Results and Discussion

4.3.5.1 NIR spectral interpretation

Figure 4.3.3 shows the results for the linearity of the KF method. A R^2 correlation of 0.9998 was obtained. The distribution of water contents as determined by the Karl Fischer reference method for the 43 samples used in the calibration are shown in Figure 4.3.4. Water contents ranged from 5.89 to 19.70 % m/m. The raw (un-treated) and SNV corrected 2^{nd} derivative NIR spectra for 15 Agar samples are shown in Figures 4.3.5, 4.3.6 and 4.3.7. It can be seen that the spectra for all samples are very similar, with differences between the spectra at the water peaks at ~1430nm and ~ 1920nm . At these wavelengths peak shifts are seen in addition to changes in peak maxima (negative) values. These wavelength shifts are likely to be due to temperature differences between the samples or differences in the type and extent of hydrogen bonding in the samples. It is possible that difference species are associated with varying degrees of 'bound' water (either intra- or inter-molecular). There are also spectral differences and peak shifts noted in the ~1380nm and ~2320nm regions.

Figure 4.3.3 Test for linearity of the Karl Fischer method using the standard di-sodium tartrate

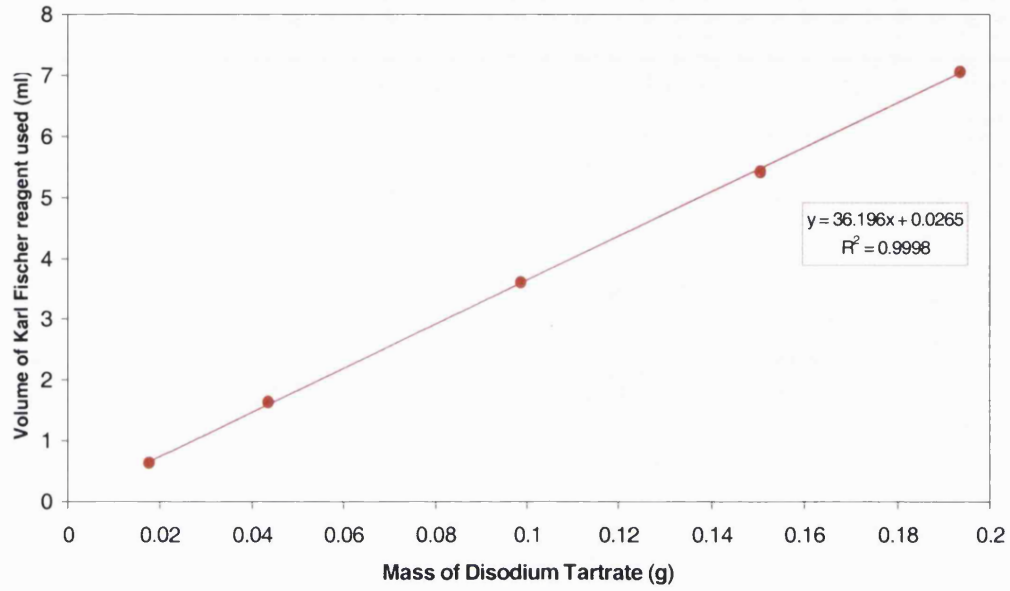


Figure 4.3.4 Frequency distribution of the water content of 43 agar samples

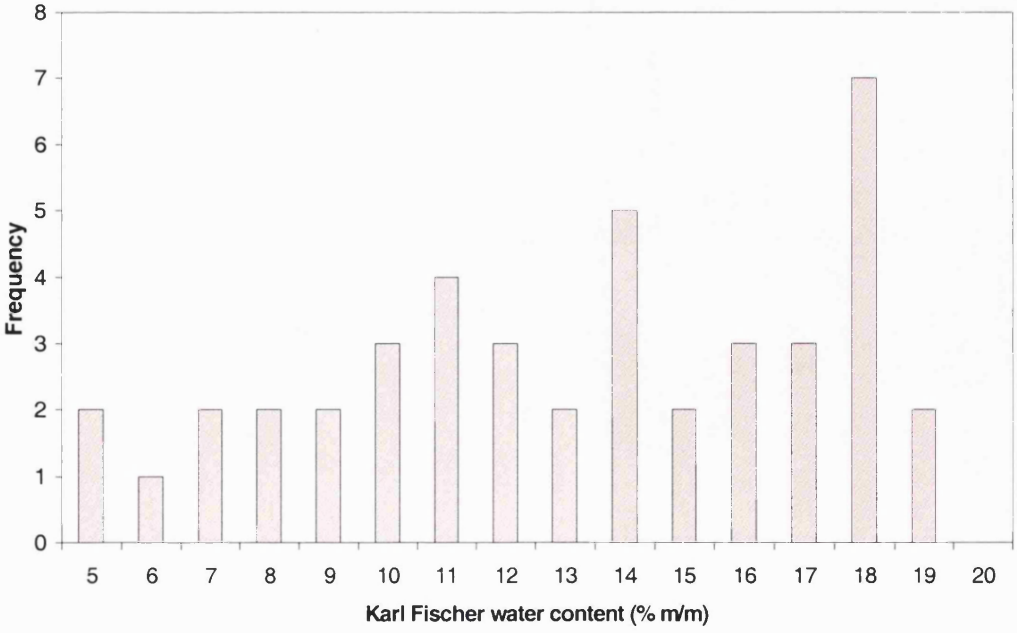


Figure 4.3.5 Raw (un-treated) absorbance spectra of 14 agar samples over the full wavelength range of 1100nm to 2500nm

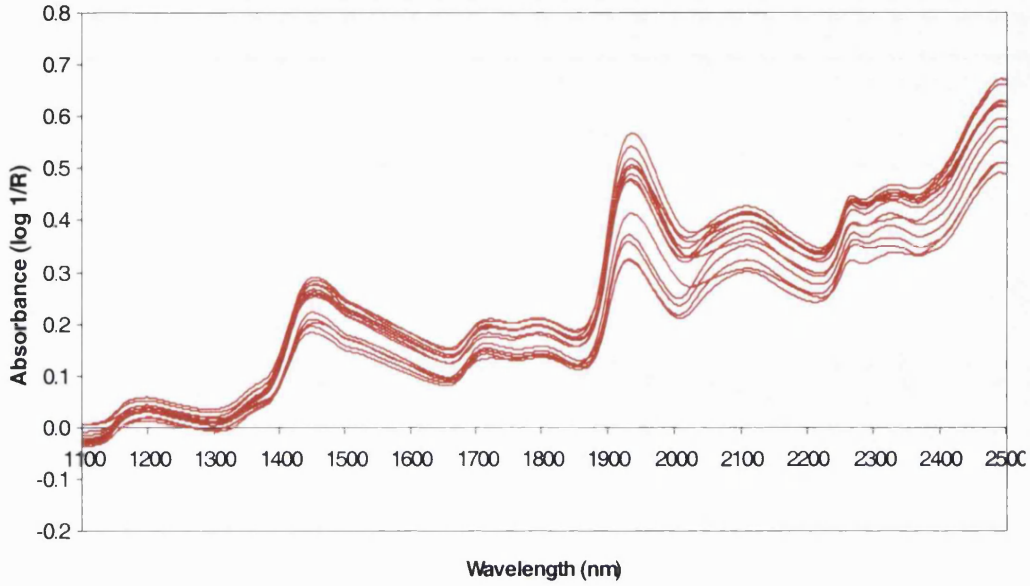


Figure 4.3.6 SNV corrected 2nd derivative spectra of 14 agar samples over the wavelength range 1100nm to 1700nm

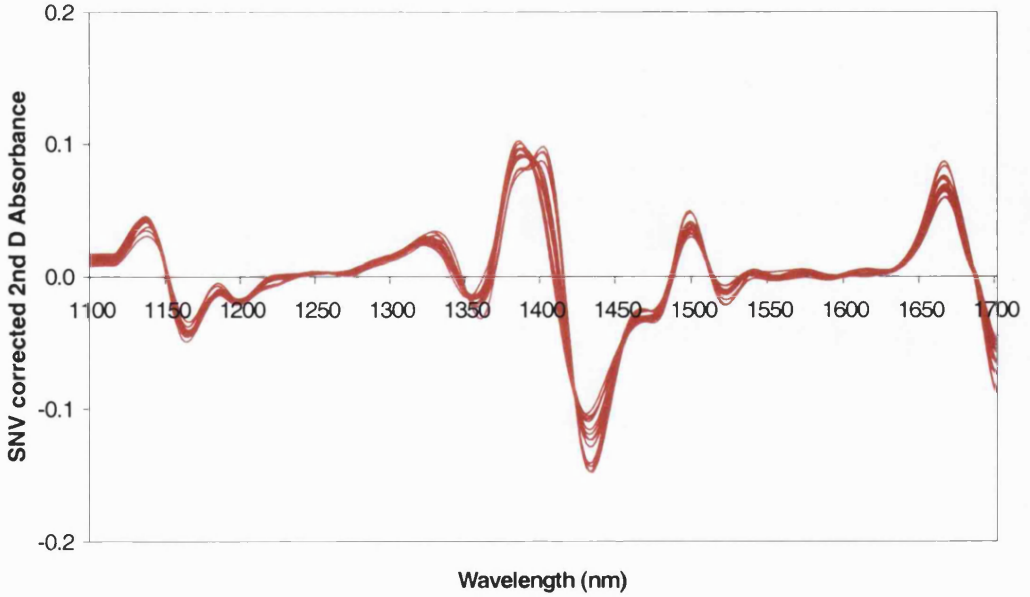
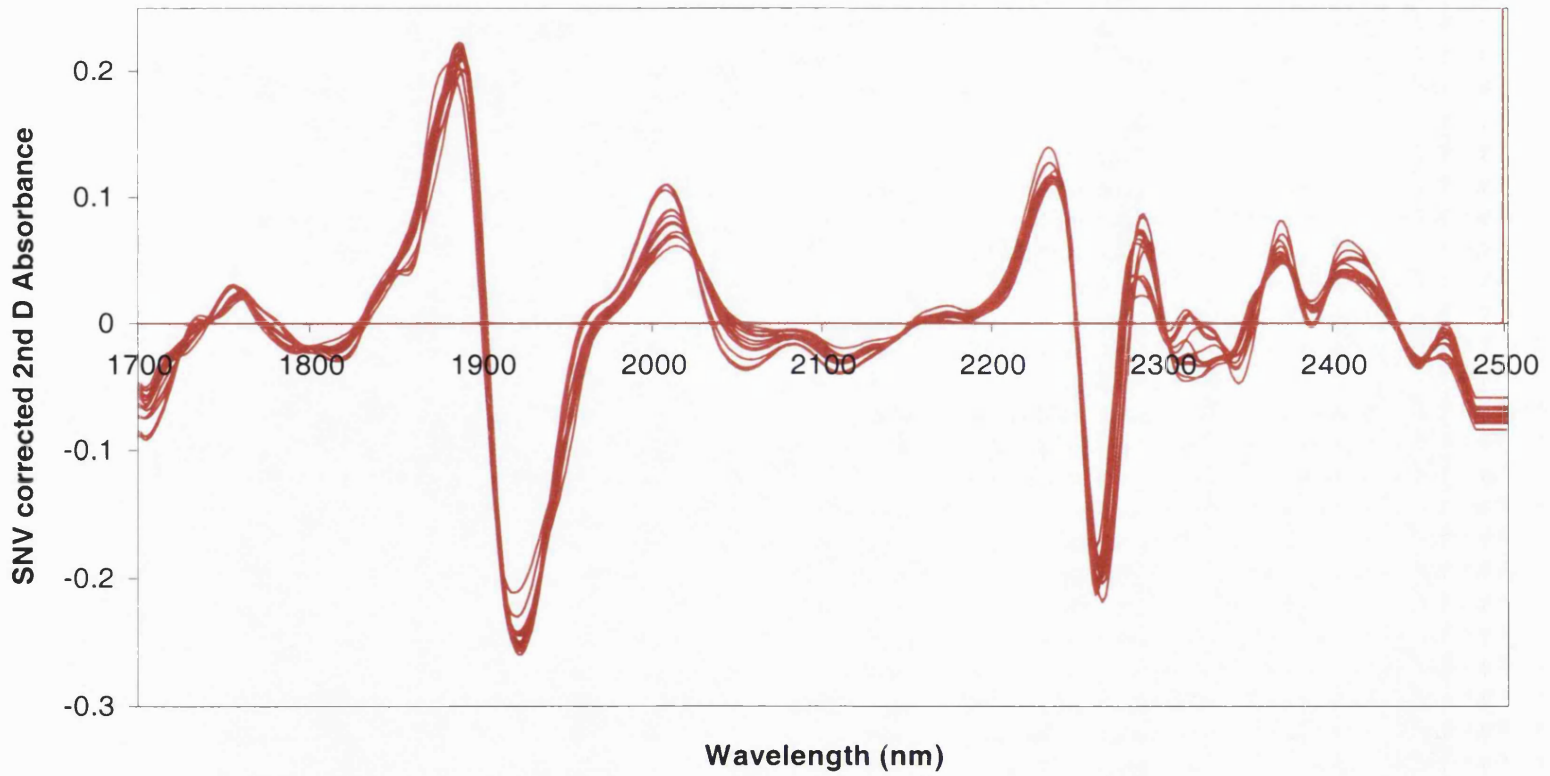


Figure 4.3.7 SNV corrected 2nd derivative spectra of 14 agar samples over the wavelength range 1700nm to 2500nm



4.3.5.2 Detection of 'outliers'

Outliers are those samples which poorly fit a qualitative or quantitative calibration. Samples can be outliers for the following reasons:

- the sample is a spectral outlier, that is the NIR spectral measurement has been improperly taken. Re-scanning the sample will therefore allow the sample to be re-introduced into the calibration model

- the sample is a reference measurement outlier, that is the reference value is incorrect (relative to the other samples). Re-measurement of the sample will therefore allow the sample to be re-introduced into the calibration model

- the sample is a genuine outlier, having different physical and/or chemical characteristics to the other samples in the calibration and should therefore not be included, as the calibration model does is not taking into account that particular variation. However, if additional samples similar in physical and chemical character to the outlier sample are also used in the calibration, it may be possible to retain the sample in the data set, otherwise a separate NIR calibration would have to be created for this and other similar samples.

The 43 sample spectra were used in a single calibration set (no validation set) to create a MLR calibration in Vision® and Sesame. A single wavelength forward search method was used. An inspection of the predicted versus the actual values from the calibration created using the Vision® software showed that one sample was very poorly predicted. This was removed from the sample set leaving a total number of Agar samples of 42. PC Analysis was carried out on the samples using The Unscrambler® software The PC Analysis did not show any obvious outliers on visual inspection of the results (Figure 4.3.8). However, it must be borne in mind that 'outliers' present in a MLR calibration will not necessarily correspond with results from PC Analysis, as the latter is taking into account the whole NIR spectral region, where as an MLR calibration uses only specific wavelengths. Most of the samples were in two distinct groups, with a third group containing samples less

distinct groups, with a third group containing samples less closely grouped together. The recommended number of Principal Components was 13, suggesting there were a number of differences between the samples other than water content. As mentioned previously there is likely to be wide range of Agars in terms of species and this may account for at least some of the differences in the NIR spectra. Particle size may also contribute to these differences, although all samples were in powder form and appeared on visual inspection to be of similar particle size. The Principal Component 1 and 2 (PC1 and PC2) values were plotted against the water contents of the samples (Figure 4.3.9). A fairly high R^2 correlation of 0.9185 was obtained for Principal Component 1, suggesting that the variation between the samples described by this the first component was in fact water. A plot of the X-loadings against wavelength is shown in Figure 4.3.10 for Principal Components 1 and 2. This is similar in appearance to a water spectrum and has negative peak maxima at ~ 1410nm and 1950nm. This is also suggestive that the PC1 is describing the difference in water content between the samples. It should be noted however that the first and second Principal Components described only 30% and 22% of the variability in data respectively. This also indicates that there are differences between the spectra caused by factors other than water.

4.3.5.3 Construction of quantitative NIR calibration models

After some investigation, it was found that the use of 2nd derivative spectra was the most appropriate for construction of the NIR calibrations for all software programs, giving improved (lower) Standard Errors. The use of the SNV correction in Vision® did not confer any additional benefit. Sample Sets 1 and 1 contained 27 samples in the calibration set and 15 samples in the validation set.

Tables 4.3.1 and 4.3.2 show a summary of the results from the creation of the different calibrations for Sample Sets 1 and 2. It can be seen that the best calibration in terms of % mean accuracy for the calibration and validation sets for Sample Set 1 is that given by the Sesame MLR two wavelength equation, showing the benefit of using the combination wavelength search as opposed to the step-up method. The Vision® MLR method shows two results, the first for a single wavelength only and the second with an additional wavelength in the calibration model.

Figure 4.3.8 Principal Component plot of 2nd derivative spectra of 43 agar samples over the wavelength range 1100nm to 2500nm

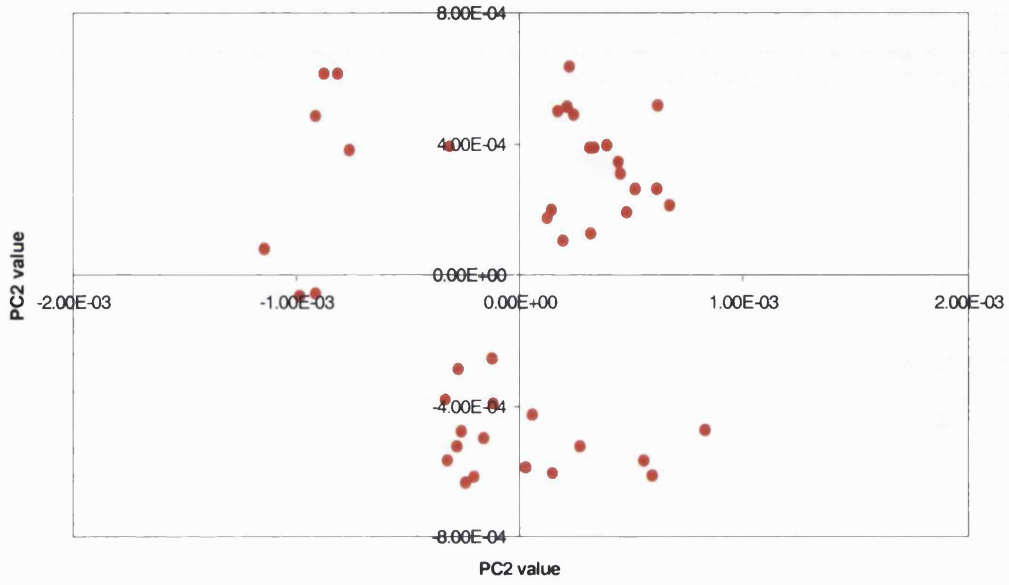
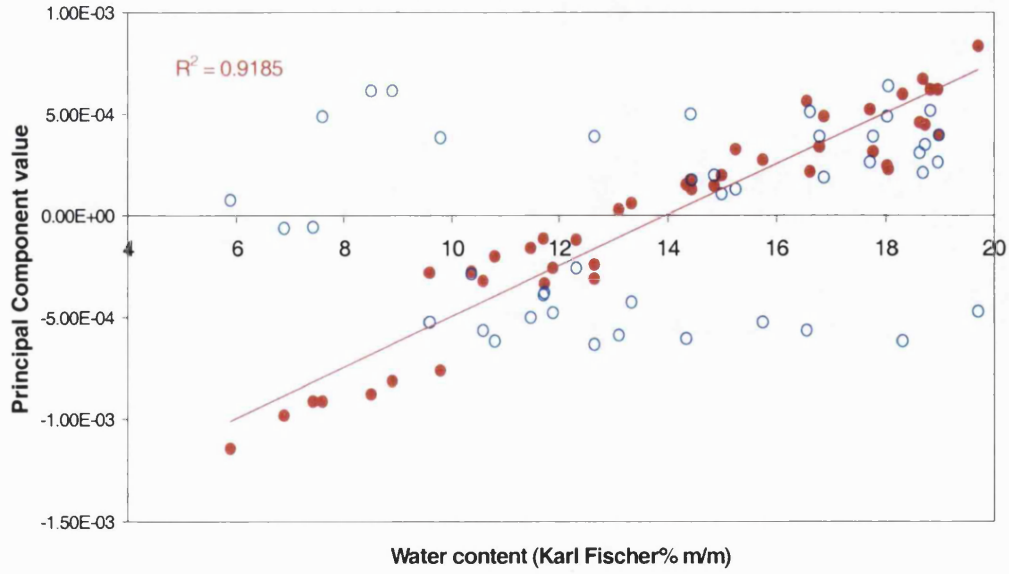


Figure 4.3.9 Plot of Principal Components 1 (red) and 2 (blue) values against water content for 2nd derivative spectra of 43 agar samples from a PC analysis over the full wavelength range 1100nm to 2500nm



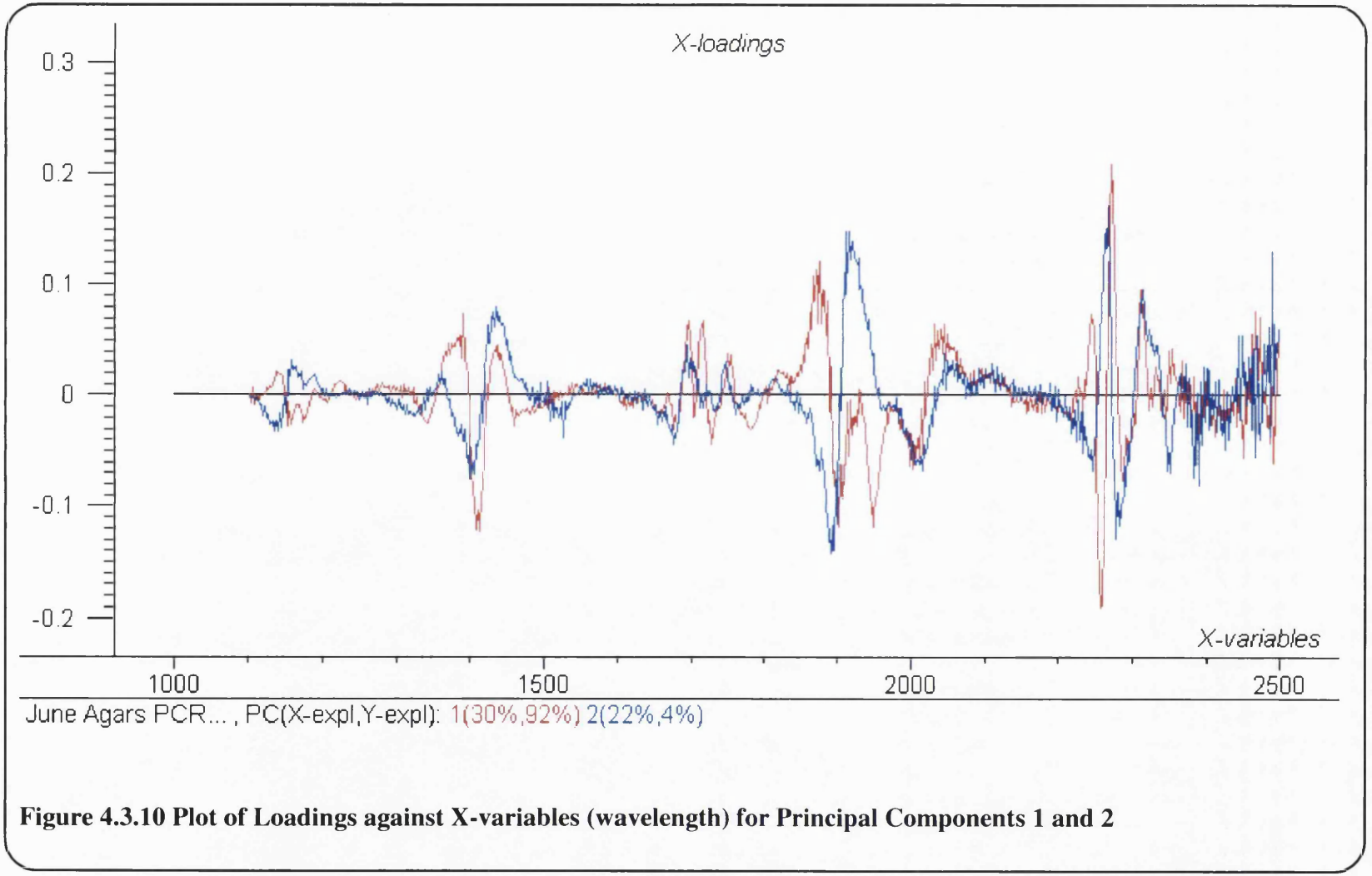


Figure 4.3.10 Plot of Loadings against X-variables (wavelength) for Principal Components 1 and 2

	Vision® MLR		Sesame MLR 2λ combination	Vision® PLSR		Unscrambler® PCR	
Calibration set:				**Full cross validation**	**Validation set**	**Full cross validation**	**Validation set**
Spectral pre-treatment	2nd D	2nd D	2nd D	2nd D	2nd D	2nd D	2nd D
Wavelength(s) / Number of Factors or PCs	1824	1824 1978	1816nm 2302nm	3	4	2	2
Correlation R^2	0.9706	0.9758	0.990	0.971	0.979	0.979	0.981
F value	825	484	568	260	258	N/A	N/A
SEC (% m/m)	0.6997	0.6481	0.599	0.825*	0.629	0.796	0.783
% mean accuracy	3.88	3.65	2.89	3.97	3.10	4.96	5.27
Validation set:							
Correlation R^2	0.9583	0.9707	Not given	0.971	0.981	0.975	0.974
SEP (% m/m)	0.8108	0.6719	Not given	0.686	0.590	0.881	0.912
% mean accuracy	4.56	3.82	3.32	4.32	3.75	5.61	6.10

Table 4.3.1 Summary of NIR calibrations created using MLR, PLSR and PCR techniques (Sample Set 1)

*** = Standard Error of Cross Validation**

	Vision® MLR		Sesame MLR 2λ combination	Vision® PLSR		Unscrambler® PCR	
Calibration set:				**Full cross validation**	**Validation set**	**Full cross validation**	**Validation set**
Spectral pre-treatment	2^{nd} D	2^{nd} D	2^{nd} D	2^{nd} D	2^{nd} D	2^{nd} D	2^{nd} D
Wavelength(s) / Number of Factors or PCs	1828	1828/2408	1492nm 1818nm	3	6	As before	2
Correlation R^2	0.9701	0.9793	0.993	0.970	0.984		0.977
F value	810	1185	802	245	207		N/A
SEC (% m/m)	0.6678	0.5551	0.479	0.785*	0.544		0.805
% mean accuracy	4.07	2.91	2.70	4.05	2.73		4.80
Validation set:							
Correlation R^2	0.9644	0.9587	Not given	0.979	0.979	As before	0.983
SEP (% m/m)	0.9573	0.9630	Not given	0.815	0.707		0.813
% mean accuracy	6.79	6.00	5.14	4.89	4.84		5.55

Table 4.3.2 Summary of NIR calibrations created using MLR, PLSR and PCR techniques (Sample Set 2)

*** = Standard Error of Cross Validation ***

The two wavelength Vision® MLR calibration provides the next best results, although it can be seen that the F value decreased on addition of the second wavelength. A decrease in F value usually indicates over-fitting of the calibration equation to the calibration set data, although in this case the SEP was lowered. Generally the SEP would be expected to be roughly the same or larger than the SEC. The reason for the reduced SEP in this case may be that the validation set is less comprehensive than the calibration set and needs to be increased in size.

For Sample Set 2 the best calibration in terms of % mean accuracy of the calibration set is achieved using the Sesame MLR method, whilst the Vision® PLSR method (no Full Cross Validation) provides the best results in terms of validation set. It should be noted that the best wavelength combination selected by the Sesame software is that which provides the lowest SEC and it does not take into account the SEP value. Differences can be seen in the mean accuracies for calibration models created using the Full Cross Validation and validation set methods for both the PLSR and PCR methods. It is worth using both methods of validation when creating calibration models to see if there are large differences between the results. This will depend on the number of samples present in the calibration.

The PLSR method performed better than the PCR method for both Sample Sets 1 and 2. This may be due to the fact that the reference values (water content) of the calibration samples are taken into account. To summarise, these results show the benefit of trying several different calibration methods with different parameters. It is likely that in this case the use of a MLR calibration model would be more suitable, as the PLSR and PCR methods are more suited to larger sample sets.

The calibration model containing all 43 samples in the calibration set (no validation set) was studied again. The five most poorly predicted samples (absolute difference between the predicted and the actual water content) were removed and these were subsequently known as the 'outliers', although it must be reiterated that it was not known if these samples were indeed genuine outliers or just required re-scanning (NIR spectrum) and/or assaying for water content. The results for the MLR calibration with the remaining 38

samples in the calibration set (no validation set) is compared with the calibration containing the 43 samples in Table 4.3.3.

	n = 43	**n = 38**
Wavelength (nm)	1826	1826
Correlation (R^2)	0.965	0.979
F value	1121	1682
SEC (% m/m)	0.744	0.575
% mean accuracy	4.27	3.7

Table 4.3.3 Summary of NIR calibration results for Agar sample sets with and without the five outliers

As can be seen the SEC and the % mean accuracy were improved on removal of all the 'outliers'. In addition, the F value increased each time one of the samples was removed, suggesting that they were indeed outliers. These samples were re-scanned and re-assayed. There was a <5% relative difference between the original and the new determined water contents and there was little difference in the NIR calibration results with the updated spectra. This suggests that these samples should either be removed or additional samples similar in physical and chemical character need to be added to the sample set to ascertain if improved predictions for these samples can be obtained. If the samples are genuine outliers and similar samples are added, then the wavelength selected may be altered, or an additional wavelength would be required to improve the calibration. Alternatively samples similar to these outliers may have to be excluded from the calibration and a separate NIR calibration set up for them.

In practice it is not sensible to remove more than 10% of all samples analysed, but a NIR calibration equation without these outlier samples was created to determine if the prediction accuracy was improved. It should also be noted that the wavelength remained the same (1826nm) on removal of the 'outliers'. This is very similar to the first wavelength selected

using the Vision® MLR calibration and one of the Sesame wavelengths for both Sample Sets 1 and 2.

A third sample set known as Sample Set 3 was then created with the remaining 38 Agar samples. 25 samples were assigned to the calibration set and 13 samples to the validation set. Two methods were used for the creation of this Vision® MLR method. The first allowed the (first) wavelength to be selected by the Vision® software and the second was to apply a fixed wavelength of 1826nm (the wavelength selected for the MLR model with the calibration set only as shown in Table 4.3.3). The results are shown in Table 4.3.4.

	Wavelength selected by Vision®		**Fixed wavelength of 1826nm**	
	Calibration set	Validation set	Calibration set	Validation set
Wavelength (nm)	1824		1826	
Correlation (R^2)	0.984	0.9769	0.98	0.985
F value	1432		1140	
SEC / SEP	0.531	0.737	0.594	0.587
% mean accuracy	3.38	3.79	3.9	3.7

Table 4.3.4 Summary of the two MLR calibrations for Sample Set 3, one using the forward search method and the other a fixed wavelength of 1826nm

As can be seen, the wavelength selected by Vision® is virtually the same as the fixed wavelength. On inspection of the SEC and SEP values given by Vision® software when creating the MLR calibration, it appears that an improved result is obtained using the fixed wavelength of 1826nm, as the SEP drops from 0.737 to 0.587. However, on calculation of the % mean accuracy (which has to be done manually and is not a statistic given by Vision® software), it can be seen that the results are very similar for the validation set. The wavelength of 1824nm was selected by Vision® software as having the highest correlation with the reference data and so it would be expected that the SEC is better for this than for

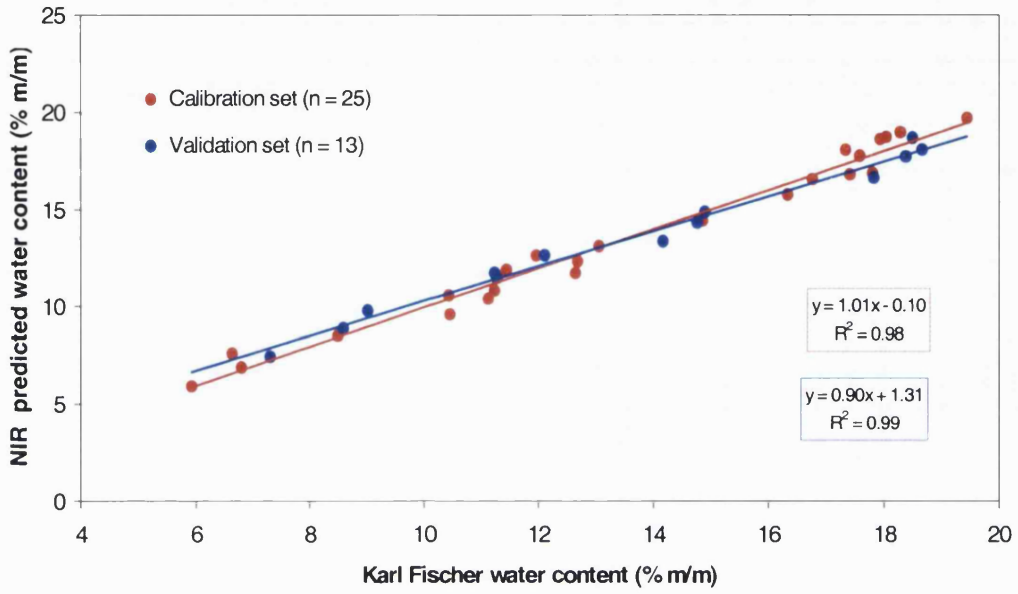
the calibration using the fixed wavelength of 1826nm. A plot of predicted against actual water content using the two different wavelengths is given in Figure 4.3.11.
The limits set down to assess the quality of a NIR calibration depends of course on the purpose for which it is intended. The % mean accuracies for each sample using this NIR calibration model are shown in Figure 4.3.12. All but one sample has a % relative error of less than 10%,whilst 28 out of the 38 samples are have prediction errors of less than 5%, which is often considered an appropriate limit of error.

4.3.5.4 Correlation of Library Identification Match Values with Water Content in Agar Samples

A Library was created using a selection (21) of the 43 Agar samples, including those samples thought to be genuine outliers. Each Library Product represented one sample, with each Product containing five spectra obtained for each sample. First a MDWS method and then a CWS method was applied to the Library. The lowest reference water content was 5.89 and an average of the (five) Match Values obtained for all other samples when compared against this sample were noted. No correlation was found between the MDWS value and the water content ($R^2 = 0.46$), but a correlation (R^2) of 0.92 was found between the CWS Match Values and the water content (Figure 4.3.13). Removal of the five 'outliers' resulted in an improved R^2 of 0.97 (n = 16), although interestingly the equation of the line remained virtually the same. This may mean that these samples are not outliers in relation to the CWS Match Values, as it can be seen from the Figure 4.3.13 that there is no obvious difference between these samples and the others in terms of residual difference, apart from the sample containing 18.82% water which has been over-predicted.

In practice, it is not possible to say that the use of the CWS Identification method is better or more accurate than the Karl Fischer reference method because the regression line was created using the same reference method. However, it has been shown that the CWS Identification method has a high correlation with this particular reference method for water content. Although it is likely, it cannot be assumed that the CWS method has a high correlation with the water content of samples measured by alternative methods such as loss-on-drying.

Figure 4.3.11 Plot of NIR predicted against Karl Fischer reference water content for the calibration model using Sample Set 3 at a fixed wavlength of 1826nm



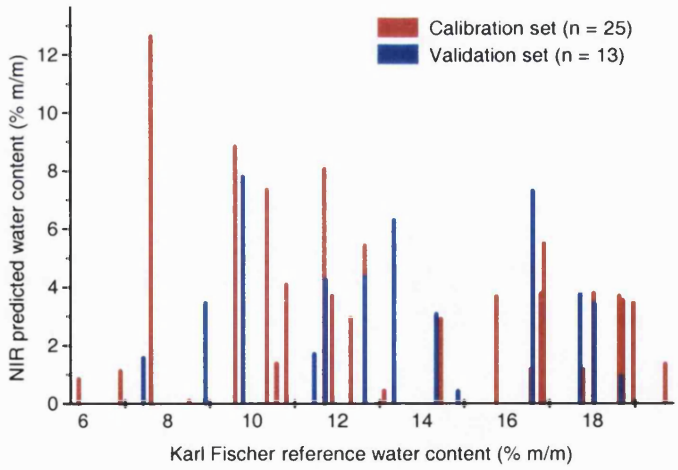
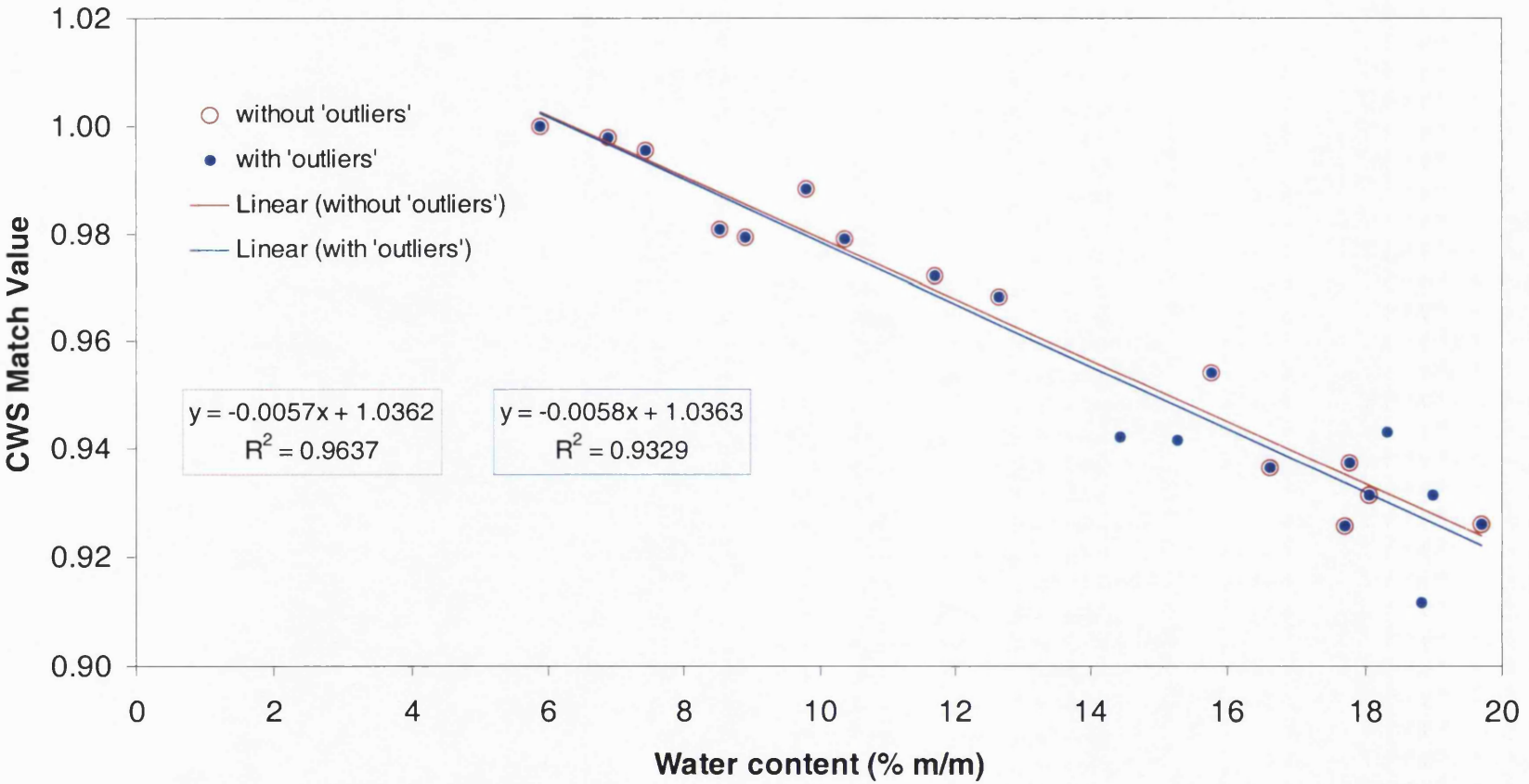


Figure 4.3.12 Plot of % Relative Error against Karl Fischer reference water content for individual agar samples in Sample Set 3 using the MLR calibration created using a fixed wavelength of 1826nm

Figure 4.3.13 Plot of Correlation in Wavelength Space Match value against reference water content for a selection of agar samples, including those identified as outliers



However, the use of this method and the high correlation between the two methods used here does show that the CWS Identification method may be used to help identify possible outliers. These samples can then be re-scanned and re-assayed to see if they can be re-introduced into the calibration, or if they are genuine outliers which should be removed or where similar samples should be added to the calibration set.

4.3.5.5 NIR Repeatability and Intermediate Precision

The fixed wavelength NIR calibrations for sample Sets 1 and 2 were used to predict the water content for a single sample of Agar six times in one day for repeatability (short-term precision) and intermediate precision. Table 4.3.5 shows the results obtained along with the results for the Karl Fischer determinations. All three calibration results have confidence intervals (at the 95% level)with values common to the Karl Fischer confidence interval for water content. This suggests that there is no significant random or systematic error for the NIR calibrations.

4.3.6 Conclusions

The results obtained in this study lead to the following general conclusions:

- The of Multiple Linear Regression appears to be suitable for the creation of an NIR calibration for the quantification of water content using the Karl Fischer technique as reference method. However, with additional samples in the calibration set, the use of the PLSR and PCR methods may be more suitable.

- Some samples were indicated as 'outliers', in which case more samples similar in physical and chemical characteristics need to be added before any NIR calibration is created. Alternatively these samples may need to be removed and a separate NIR calibration model employed.

- The CWS Identification method has a strong correlation with the reference water content. This could be used as an additional check on the water content determined

by a NIR calibration method for incoming samples, identifying samples that are genuine outliers or that may need re-scanning or re-assaying.

- It is likely that the Agar samples were from different sources in terms of species and even genus, thus resulting in a wide range of physical characteristics. It is possible, therefore, that a calibration consisting of entirely Agar samples falling within BP standards or those Agars of a specification required for use in the pharmaceutical industry (a more specific NIR calibration) would result in improved prediction accuracy. Figure 4.3.9 showed that there was at least two distinct groups of Agar sample. Use of separate NIR calibration models based on these groupings may result in improved prediction accuracies, although the calibration will be less robust and more specific.

- The benefits of using different sample assignments to the calibration and validation sets in addition to more careful study of wavelength selection for MLR calibrations has also been demonstrated. Widely differing results for calibration and validation set using different sample assignments suggest that the sample sets are not comprehensive enough and do not take into account the variation encountered within all the samples. Addition of more samples is likely to improve the prediction accuracy and lead to more consistent results between different sample sets.

	Mean water content (% m/m)	Standard Deviation (% m/m)	Coefficient of variation (%)	Confidence Interval (% m/m)	Common Confidence Interval ?
Karl Fischer	**11.50**	**0.22**	**0.98**	**11.37 to 11.62**	
Repeatability:					
Sample Set 1	11.53	0.18	1.59	11.33 to 11.74	✔
Sample Set 2	11.21	0.19	1.65	11.00 to 11.42	✔
Sample Set3	11.44	0.19	1.64	11.22 to 11.65	✔
Intermediate Precision:					
Sample Set 1	11.55	0.27	2.32	11.24 to 11.85	✔
Sample Set 2	11.29	0.21	1.86	11.05 to 11.53	✔
Sample Set 3	11.52	0.21	1.86	11.27 to 11.76	✔

Table 4.3.5 Summary of results for determination of short-term precision (repeatability) and intermediate precision for the NIR assay of one agar sample using a MLR method with a single fixed wavelength of 1826nm

CHAPTER FIVE: ESSENTIAL OILS

5.1 The Rapid Identification of Pure Essential Oils by Near Infrared Spectroscopy

5.1.1 Introduction

The use of essential oils in Aromatherapy and alternative medicine is becoming increasingly popular and there is demand for oils of high quality and purity. Several organisations exist to promote and maintain high standards of oils and Aromatherapy use as a complementary medicine such as the Aromatherapy Organisations Council (governing body for the profession in the UK), the Aromatherapy Trade Council and the International Society of Professional Aromatherapists, but of course membership of such organisations is voluntary. The use of volatile oils dates as far back as the ancient civilisations of India, China, Egypt, Babylon and Greece and their use by mainstream practitioners survived until the advent of scientific chemistry in the 17th century. However, since the 1920s their use has increased significantly in popularity. René Maurice Gattefossé (1881-1950) was the father of the modern thinking behind Aromatherapy. An accident in a laboratory lead him to the realisation that Lavender oil healed his burns rapidly and gave rise to less scarring than expected. The use of essential oils is often viewed as a holistic, complementary medicine. The philosophy of the healing effects of essential oils is that illness occurs when there is an imbalance between the mental, emotional and physical processes of the body and that Aromatherapy serves to restore the body's natural balance. Essential oils are described as the volatile, organic constituents of fragrant plant matter and contribute to both flavour and fragrance, but are not present in all plants. An essential oil should not have any of its normal components removed following extraction, nor have any other substance added. Essential oils can be obtained from flowers (such as Rose oil), leaves (such as Peppermint oil), fruit (such as Lemon and other citrus oils), grasses (such as Lemongrass oil), woods (such as Cedar oil), barks (such as Cinnamon oil), bulbs (such as Garlic oil) and dried flower buds (such as Clove oil). The essential oils are prepared through several methods such as cold-pressing and steam distillation - often the method by which the oils is obtained determines the quality of the oil. In steam distillation, the method used to produce the majority of essential oils, the essential oil present in the sample is evaporated by steam and

then condensed by cooling. Fresh plant material typically yields between 1% and 2% by weight of essential oil on distillation. The smaller the amount of oil produced per kilogram of plant material, the more expensive the oil becomes. Monographs of plant materials in the British and European Pharmacopoeias often specify the amount of oil that should be produced per kilogram. Other factors that determine the quality of an oil include geographical origin, species, time of harvesting and weather conditions during the growing season. The oils are thought to act systemically after absorption through the skin during massage or inhalation (after dilution with a suitable vegetable carrier oil). It is accepted by the scientific establishment that many of the oils contain chemical constituents that have significant pharmacological activity, although the dose received during Aromatherapy treatment being sufficient to cause these effects is debatable (Tisserand and Balacs, 1998a).

No medicinal claims must be made for Aromatherapy oils unless the manufacturer/ supplier has been granted a Product Licence and if so the safety and quality of the oils is governed by regulations under the Medicines Act. If no Product Licence is held, regulations under the cosmetics safety regulations must be followed. There are several monographs for essential oils in the British and European Pharmacopoeias, including Clove, Eucalyptus and Lemon oils. These monographs will typically contain a definition regarding the genus or species to be used, the method of extraction of the oils from the plant and a description of the colour and odour of the oil. Some of these monographs (for example for Eucalyptus and Lemon oil) will also specify that for the oil to be of BP quality, it has to contain a certain amount of one or more of its chemical constituents (see Section 5.2 and 5.3).
Sadly, there is a tendency amongst the general public to assume that anything natural is synonymous with being safe, whilst this is known as being far from the truth by the medical and scientific establishments. Tisserand and Balacs (1998a) compiled a book about essential oil safety, intended for health-care professionals, which details the main constituents of many oils, along with their toxicity and pharmacological actions. In addition, the pharmacokinetics and pharmacodynamics of these constituents are discussed. Several essential oils contain constituents that contain toxic components, where toxicity may occur upon contact with skin, inhalation, ingestion or a combination of these. For example, Pennyroyal oil contains Pulegone, a terpenoid ketone, present at levels of ~ 55 to

80%, which is toxic to the liver upon metabolism to epoxides. Thujone, another terpenoid ketone present in Tansy (66-81%) and Thuja (39-80%) can cause convulsions and fatty degeneration of the liver on chronic administration. Both the UK and EC 'standard permitted proportion' of Thujone in food flavourings is 0.0005 g/kg. It can be argued that the levels of certain identified toxic components are not present at levels that are dangerous when used as Aromatherapy and massage oils, and this may be true, but it must be borne in mind. For instance, Cinnamon bark is no longer recommended as it contains Cinnamaldehyde at levels of ~ 55-75% (and Eugenol at levels of ~5-18%). Cinnamon bark oil is a known strong dermal sensitiser, due to the presence of the Cinnamaldehyde. Cinnamon leaf oil, however, which does not contain Cinnamaldehyde (but contains Eugenol at levels of 70-90%) is not a dermal sensitiser. Certain oils are contra-indicated in people with certain medical conditions, such as Garlic oil which should not be used by those with thyroid disease, as many of its components can inhibit iodine metabolism at sufficient levels. Essential oils containing a high level of Citral such as Lemongrass (~ 75%) are not recommended to be taken orally by those with glaucoma as Citral can cause a rise in ocular tension. This illustrates the importance of correct identification of essential oils and even distinction between oils produced from different parts of the same plant. The increasing use of essential oils by the general public and by health care professionals in hospices and hospitals further increases the demand for essential oils of known quality and composition (Tisserand and Balacs 1998a).

Traditionally, separation techniques such as TLC, HPLC and GC or GC-MS have been employed for identification and quality control of these oils. However, these methods can be lengthy and require sample preparation before analysis. The aim of this investigation was to ascertain if NIR spectroscopy could be used to distinguish between different pure essential oils and indicate the level of discrimination attained. Application of this technique would have several distinct advantages over the current methods employed in that it is rapid, simple to use and require no sample pre-treatment. Schulz et Lösing (1995) also looked at the possibility of using NIR spectroscopy for the quality control of essential oils. They used a Bühler Fourier Transform NIR-VIS spectrophotometer with fibre optic probe (transflectance measurements) and the manufacturer's software for data analysis. A smaller

number of different essential oils was studied but similar results were obtained in terms of the ability to distinguish between essential oils types. One of the advantages of the NIR method employed in the present investigation over the previous technique is that a much smaller volume of oil is required for sampling. In addition, this investigation included the use of Polar Co-ordinate plots and PC Analysis as additional methods of identification / discrimination.

5.1.2 Materials and Methods

Twenty three different pure essential oils (more than one brand for several oils) were scanned on the FOSS NIRSystems 6500 spectrophotometer using the Rapid Content Sampler module in an agricultural liquid sample cell on NSAS software. A single spectra obtained was the average of 32 scans. 5 separate spectra were obtained for each oil, the cell being rotated about the centre before recording each spectrum. To minimise differences in the spectra due to air bubbles in the cell, the cell was cleaned and the whole procedure repeated twice more. An average of the 15 spectra obtained for each oil with SNV corrected 2^{nd} derivative pre-treatment was used for all data analyses. It was assumed that all oils used were 'pure' and neither adulterated or contaminated.

5.1.3 Results

5.1.3.1 Visual interpretation of spectra

Figure 5.1.1 shows the raw (un-treated) spectra for Orange, Tea tree, Lemon, Geranium and Lavender essential oils. SNV corrected 2^{nd} derivative spectra over the 2200nm to 2400nm wavelength range of the same oils are shown in Figure 5.1.2. There are obvious differences between these five oils, although the difference being between the Orange and the Lemon oils is much less. To illustrate the similarity between the citrus oils, SNV corrected 2^{nd} derivative spectra for six Citrus oils over the same wavelength range are shown in Figure 5.1.3. Six samples of the same type of oil, namely Lemon oil, are included to show that there is very little differences between the oils meaning that the variability between samples of the same type of oils are unlikely to interfere with the discrimination between different types of oil. A sample of Eucalyptus oil (not of the same family as the citrus oils) shows a much more marked difference between oils of the same plant family.

Figure 5.1.1 Raw (un-treated) NIR spectra of five different pure essential oils

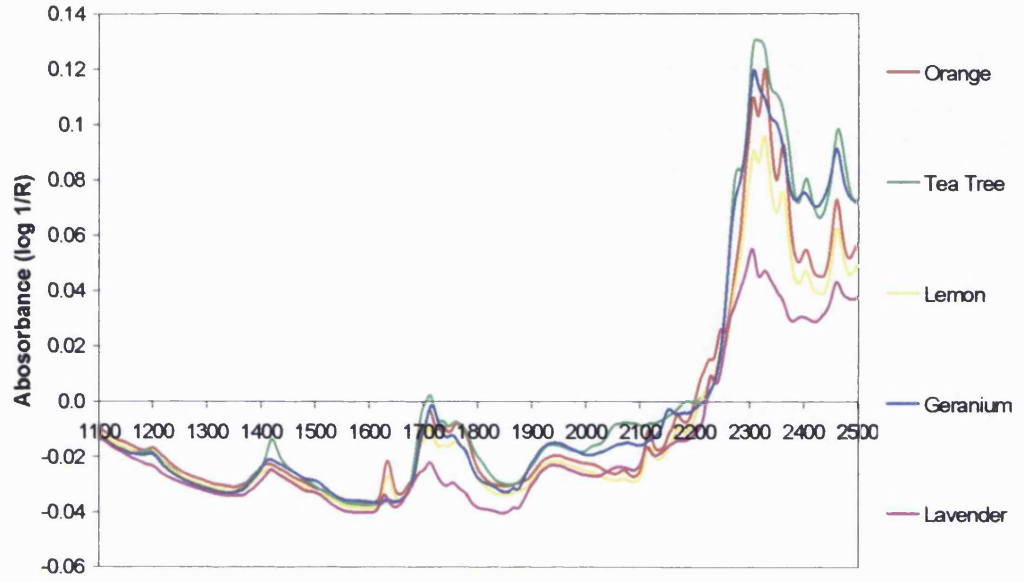
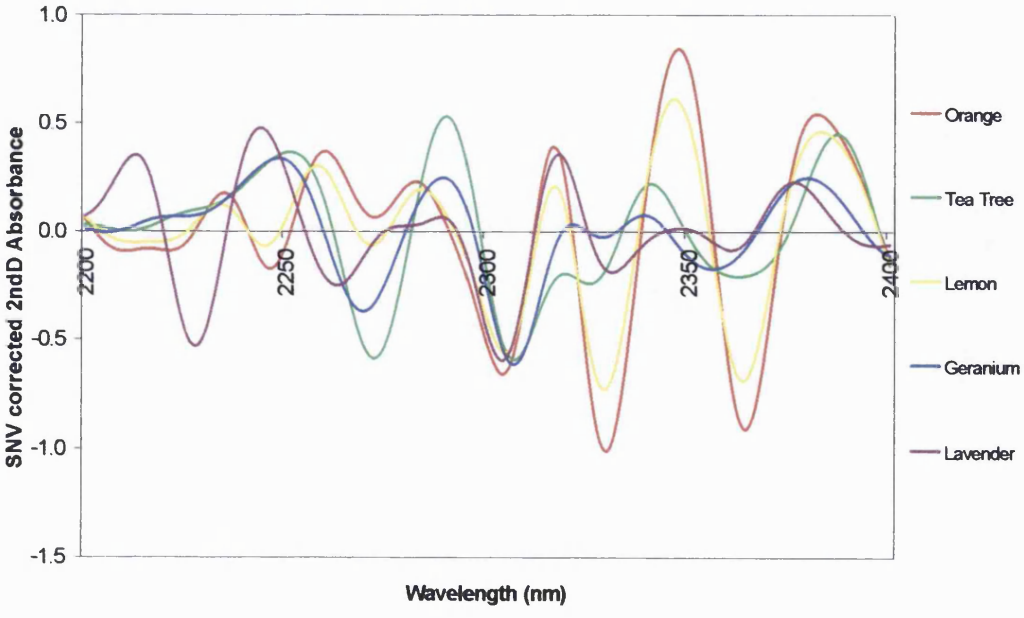


Figure 5.1.2 SNV corrected 2nd derivative spectra of five different pure essential oils



5.1.3.2 Library Evaluation

A Library was constructed, such that a separate Library Product was used for each type of essential oil (i.e. there were 25 different Library Products). Both the CWS and MDWS were investigated as possible Identification methods. SNV corrected 2nd derivative spectra were used after looking at several different mathematical pre-treatments, as these type of spectra allowed greater discrimination and therefore easier identification than other mathematical pre-treatments used. Each Library Product must contain at least three spectra and an averaged spectrum for each oil sample was used. The threshold value was lowered for the CWS method and the threshold raised for the MDWS method in order to see the Match Values for all oils in comparison to each Library Product. Table 5.1.1 shows data taken from the Library Identification results using both the CWS and MDWS methods (employed separately). As can be seen, using CWS the Match Values for the nearest neighbours of each Product are very similar. As the recommended minimum threshold value for use with a CWS method (the default value in the Vision® software) is 0.85, it can be concluded that this method is not suitable for the identification and discrimination of the different essential oils. However, the nearest neighbours using the CWS method are often similar in chemical character, as can be seen for the Rosemary oil which has nearest neighbours White Camphor, Niaouli and Eucalyptus. These oils oil contain Cineole in differing amounts and it is this constituent that is likely to be the cause of the similarity between the spectra using this method. This can be seen more clearly from Table 5.1.2 for a sample of Lemon oil. The nearest neighbours are those oils that are also part of the citrus family.

The results using the MDWS method were considered next. There was a much greater difference between the oils using this method and a threshold value of below 13.83 allowed for complete discrimination of all the essential oils. It is not clear why essential oils are classed as nearest neighbours using the MDWS method.

The use of both the CWS and the MDWS method allow for distinction of essential oils produced from different parts of the same plant. As described earlier, it is important that Cinnamon leaf oil and Cinnamon bark oil are not confused.

Spectrum name	Product name	Match Value
Correlation in Wavelength Space		
Eucalyptus 1	Eucalyptus	1.00
	Niaouli	0.97
	Camphor	0.96
	Rosemary	0.94
Rosemary 1	Rosemary	1.0
	Camphor (White)	0.98
	Niaouli	0.96
	Eucalyptus	0.94
Lavender 1	Lavender	1.00
	Bergamot	0.90
	Citronella	0.66
	Nerol puriss	0.63
Geranium 1	Geranium	1.00
	Citronella	0.88
Maximum Distance in Wavelength Space		
Eucalyptus 1	Eucalyptus	1.5
	Cedarwood Atlas	46
	Lavender	75
	Niaouli	76
Rosemary 1	Rosemary	0.77
	Cedarwood Atlas	33
	Cinnamon bark	65
	Eucalyptus	44
Lavender1	Lavender	1.5
	Cinnamon bark	31
	Clove	86
	Geranium	110
Geranium 1	Geranium	1.0
	Cedarwood Atlas	35

Table 5.1.1 Extract from the essential oil Library using the Correlation in Wavelength Space and Maximum Distance in Wavelength Space methods on SNV corrected 2nd derivative spectra over the wavelength range 1100nm to 2500nm

Spectrum name	Product name	Match Value
Lemon 1	Lemon	1.00
	Mandarin	0.99
	Grapefruit	0.98
	Orange	0.98
	Lime	0.96
	Bergamot	0.92
	Spearmint	0.80
	Citronella	0.78
	Cedarwood Atlas	0.74
Spectrum name	**Product name**	**Match Value**
Lemon 1	Lemon	1
	Eucalyptus	85
	Lavender	95
	Tea Tree	100
	Cedarwood Atlas	107
	Orange	118
	Clove	150
	Mandarin	204
	Bergamot	249
	Spearmint	268

Table 5.1.2 Match Values for the essential oils compared to a sample of Lemon oil using the Correlation in Wavelength Space and Maximum Distance in Wavelength Space methods on SNV corrected 2nd derivative spectra over the wavelength range 1100nm to 2500nm

Table 5.1.3 shows that Cinnamon Leaf and Cinnamon Bark can be distinguished easily using both the CWS and the MDWS methods, as there is a large difference in Match Value.

	Correlation in Wavelength Space	
Sample name	**Product name**	**Match Value**
Cinnamon leaf (1-6)	Cinnamon Leaf	>0.98
	Cinnamon Bark	<0.31
Cinnamon bark (1-6)	Cinnamon Bark	>0.99
	Cinnamon Leaf	<0.25
	Maximum Distance in Wavelength Space	
Sample name	**Product name**	**Match Value**
Cinnamon leaf (1-6)	Cinnamon Leaf	<1.6
	Cinnamon Bark	>141
Cinnamon bark (1-6)	Cinnamon Bark	<1.50
	Cinnamon Leaf	>40

Table 5.1.3 Difference in Match Values for Cinnamon leaf and Cinnamon bark oils using the CWS and MDWS Identification methods using SNV corrected 2nd derivative spectra over the wavelength range 1100nm to 2500nm

Figure 5.1.4 shows the SNV corrected 2nd derivative spectra of six Cinnamon leaf oils and Cinnamon bark oils. After study of these twelve oils, it was noted that the corresponding spectrum of one oil labelled as Cinnamon leaf oil appeared to be very similar to the bark oils with distinct differences between it and the 'other' Cinnamon leaf oils. Study of the IR spectra of these oils showed that this oil was indeed Cinnamon bark oil and the supplier of this sample was notified of the error. The analyst was grateful for the mistake being brought to his attention, and this illustrates the lack of and the need for stricter controls on the safety and quality of essential oils. A simple method such as NIR spectroscopy would be ideal for this purpose.

Figure 5.1.3 SNV corrected 2nd derivative spectra of six samples of Lemon oil, five other citrus oils and a sample of Eucalyptus oil

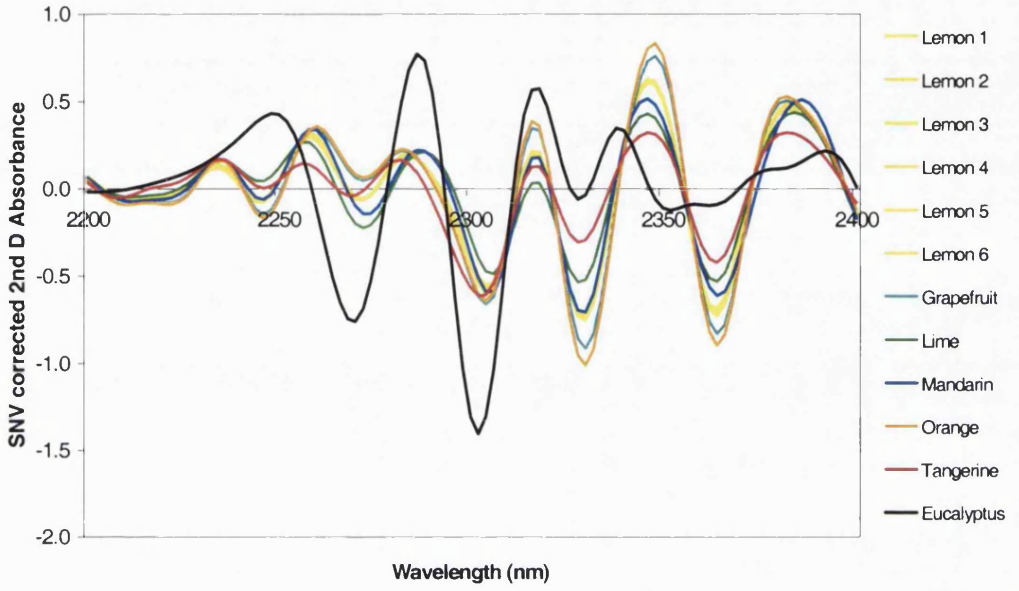
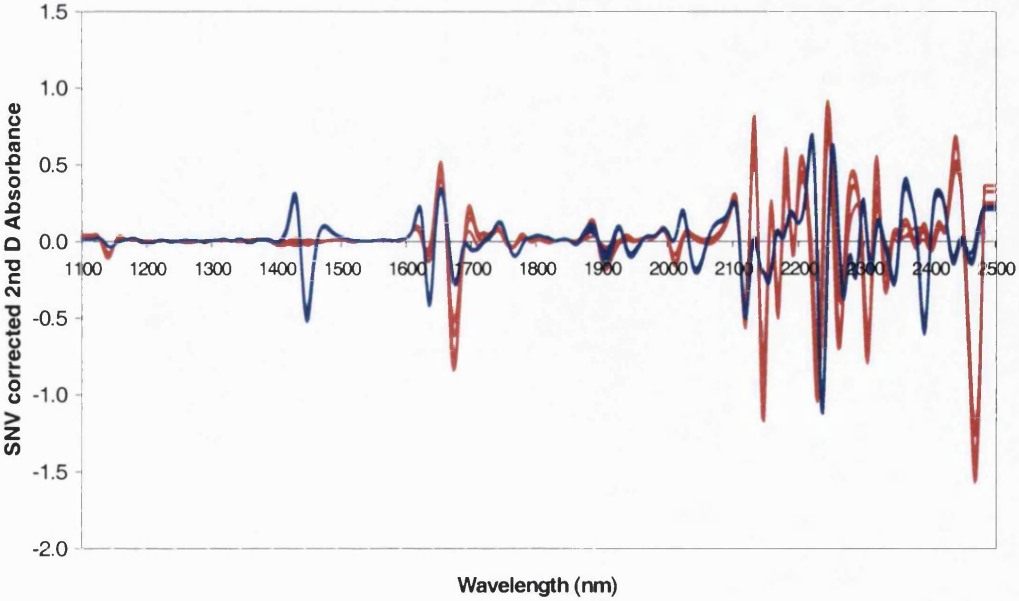


Figure 5.1.4 SNV corrected 2nd derivative spectra of six Cinammon leaf oils (blue) and Cinammon bark oils (red)



It is suggested that if an unknown essential oil was added to the Library in order to identify it using a certain Library method, then a CWS Identification method followed by a Qualification method (effectively a second Identification method) of MDWS would provide the best means for identifying a sample. The use of a CWS would allow for identification of nearest neighbours which would provide further evidence as to the identity of the unknown oil.

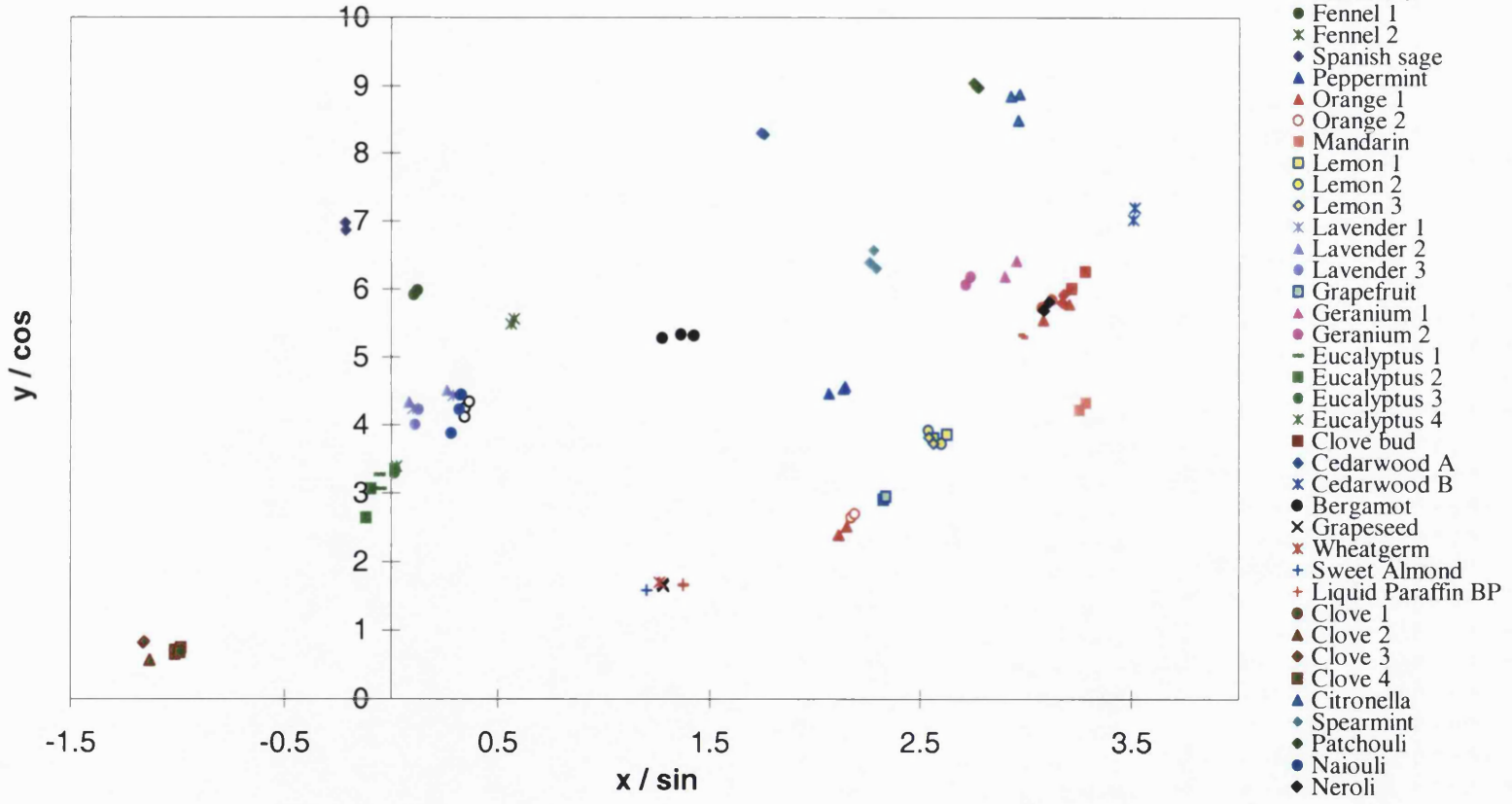
5.1.3.3 Polar Co-ordinate Plots

A Polar Co-ordinate plot was constructed for all the essential oils, allowing for a simple visual way of distinguishing between essential oils. Several different wavelength ranges were tried and the one that provided the greatest level of discrimination between the essential oils was the 2200nm to 2400nm region on SNV corrected 2nd derivative spectra (Figure 5.1.5). 39 samples of the 22 different essential oils are shown on the plot, along with four different oils commonly used to dilute essential oils to produce massage oils. Although some of the oils overlap with others of a different type (such as Lavender, Niaouli and Camphor) it may be possible to obtain complete distinction between all the essential oils by employing alongside for visual interpretation a second Polar Co-ordinate plot using a different or a larger wavelength range. Use of the CWS and more ideally the MDWS method would, however, allow for a complete distinction of the Lavender, Niaouli and White Camphor oils, as shown in Table 5.1.4. An average of the three Match Values is shown for each oil type. It should be noted that Camphor is the name of a raw material, the name of its essential oil and the name of a chemical constituent found in Camphor oils. Camphor oil is separated into four distinct essential oils by fractional distillation and are known as white, brown, yellow and blue camphor. None of these, therefore, can be classed as true essential oils.

5.1.3.4 Principal Component Analysis

PC Analysis was employed on 21 different essential oils on 2nd derivative spectra over the whole wavelength range and the 2200nm to 2400nm region in order to determine if the Principal Component plot could distinguish between the oils to a greater extent than the Polar Co-ordinate plot. The results are shown in Figures 5.1.6 and 5.1.7.

Figure 5.1.5 Polar Co-ordinate plot of SNV corrected 2nd derivative spectra over the wavelength range 2200nm to 2400nm of 22 different pure essential oils and four commonly used diluents



Sample name	Product name	Match Value
Lavender 1	Lavender	1.00
	Niaouli	0.58
	White Camphor	0.55
Niaouli 1	Niaouli	1.00
	Lavender	0.59
	White Camphor	0.98
White Camphor 1	White Camphor	1.00
	Niaouli	0.98
	Lavender	0.55
Sample name	**Product name**	**Match Value**
Lavender 1+2+3	Lavender	1
	Niaouli	182
	White Camphor	367
Niaouli 1	Niaouli	1
	Lavender	65
	White Camphor	116
White Camphor 1	White Camphor	1
	Niaouli	88
	Lavender	89

Table 5.1.4 Match Value for the Lavender, Niaouli and White Camphor oils when compared to each other using the Correlation in Wavelength Space and Maximum Distance in Wavelength Space methods on SNV corrected 2nd derivative spectra over the wavelength range 1100nm to 2500nm

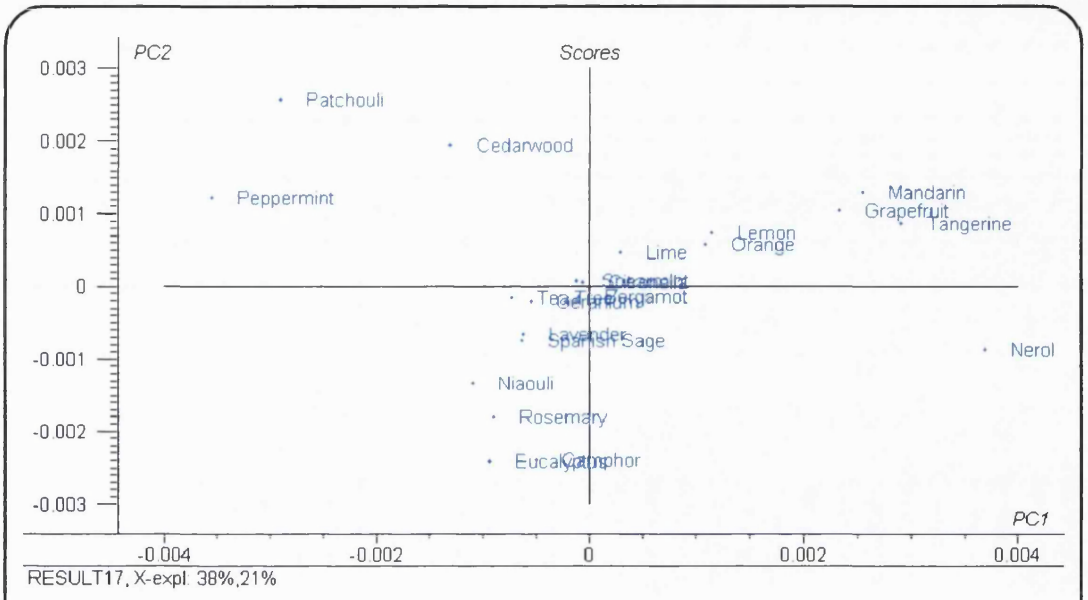


Figure 5.1.6 Principal Component plot of 2nd derivative spectra of pure essential oils over the wavelength range 1100nm to 2500nm

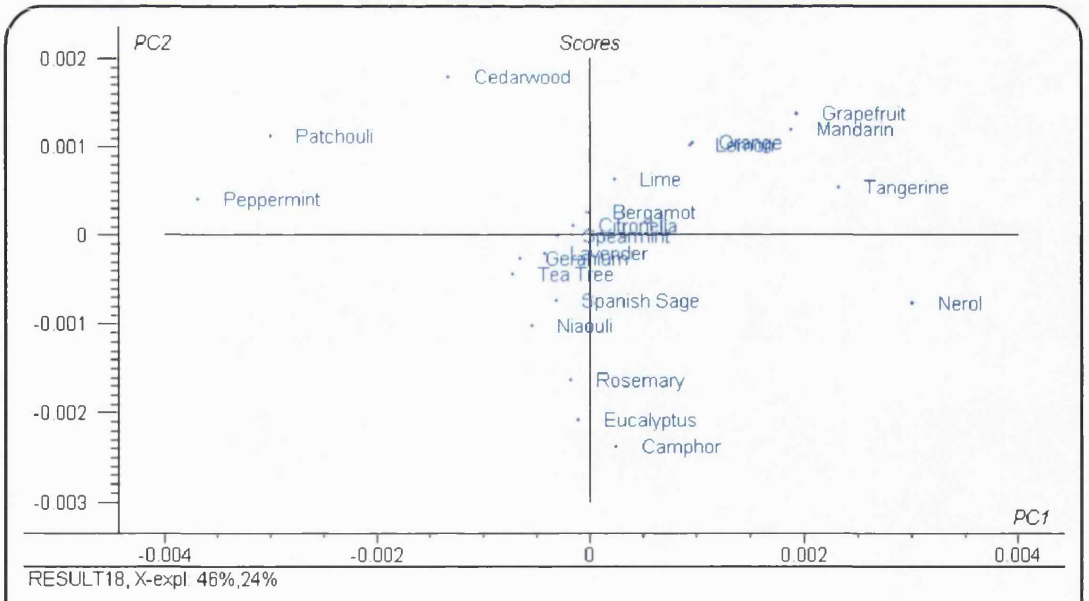


Figure 5.1.7 Principal Component plot of 2nd derivative spectra of pure essential oils over the wavelength range 2200nm to 2400nm

The first two Principal Components do not describe most of the information and describe roughly only 40% and 20% of the variability in the data. Both plots are very similar and the citrus oil groupings are still seen, along with the Eucalyptus, Niaouli, Camphor and Rosemary groupings as seen using the CWS Library Identification method. The PC Analysis method may also prove useful in providing supporting evidence as to the identity of an unknown essential oil sample.

5.1.4 Conclusions

NIR spectroscopy can be used to identify and different essential oils and also to discriminate between oils of a similar nature, for example different oils from the Citrus family and those oils produced from different parts of the same plant such as Cinnamon bark and leaf oils. A combination of Library Identification methods and Polar Co-ordinate and / or Principal Component plots may provide supporting evidence as to the identity of an oil and allow for discrimination between those oils whose identity cannot be confirmed by a single method alone.

5.2 A NEAR-INFRARED METHOD FOR THE ASSAY OF CINEOLE IN EUCALYPTUS OIL AS AN ALTERNATIVE TO THE OFFICIAL BP METHOD

5.2.1 Introduction

Eucalyptus trees come from the family *Myrtaceae* and are native to Australia. In addition they are grown for commercial purposes in those areas of the world with a subtropical or Mediterranean climate such as Portugal, Spain, China, Brazil and India. The stated content of volatile oil in the Eucalyptus genus varies, but is approximately 0.5 to 3.5% m/m, with the main constituent of the oil being Cineole in quantities of approximately 55 to 95% m/m (Newall *et al* 1996, Bisset & Wichtl 1994). Although there are many species of Eucalyptus, only a small number are suitable for medicinal use. The chief requirement is a high Cineole content and the absence of appreciable quantities of aldehydes and phellandrene, tests for which are described in monographs for Eucalyptus oil in the British and European Pharmacopoeias (BP 2001h and EP 2002h). Cineole is classified as a monoterpene, the terpenoids being widely distributed in nature and found in abundance in higher plants. Terpenoids (old name terpenes) are defined as natural products whose structures may be divided into isoprene units which are five carbon units containing two unsaturated bonds. Monoterpenoids are composed of two units and have the molecular formula $C_{10}H_{16}$. There are more than 1000 naturally occurring monoterpenoids and their characteristic features of volatility and a pungent odour are the most common compounds responsible for plants' smell and behaviour. The other constituents of Eucalyptus include moderate amounts of other terpenes, flavonoids, aldehydes and ketones and tannins. The structure for Cineole is shown in Figure 5.2.1 below.

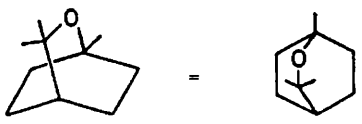


Figure 5.2.1 Structure of Cineole (Eucalyptol)

Eucalyptus oil of BP standard is obtained by steam distillation and rectification from the fresh leaves and terminal branchlets of various species of Eucalyptus oil rich in 1,8-Cineole. It contains not less than 70.0% m/m 1,8-Cineole (Eucalyptol). Eucalyptus oil is a colourless or pale yellow liquid with an aromatic and camphoraceous odour and a pungent and camphoraceous taste, followed by a sensation of cold. The assay (BP 2001b and EP 2002b) for the determination of Cineole content in Eucalyptus oil was adopted in 1934 from a freezing-point method (Tusting Cocking 1920). Melted o-cresol is added to the Eucalyptus oil and the o-cresol forms an addition compound with the Cineole present in the oil (referred to as 'cresineol'). The freezing point is noted and the Cineole content of the oil is obtained by reference to a table showing the freezing points of complexes of known Cineole content. The accuracy of the procedure is reported to be approximately ± 3% m/m (Tusting Cocking 1920) but there are several disadvantages associated with this method; the experimental procedure can be time consuming and has no internal standards. In addition, because o-cresol is hygroscopic, the presence of water may lower the apparent Cineole content by as much as 5% m/m. The method is also suitable for the determination of Cineole content of oils such as Camphor, wild Marjoram and other Cineole-containing oils. Cajuput and Niaouli oils were also used in this investigation to extend the range of Cineole contents used in the NIR calibrations. Cajuput (or Cajeput, *Melaleuca cajeputi*) and Niaouli (*Melaleuca viridiflora*) are from the same family as Tea Tree (Myrtaceae). Cajuput grows wild in Malaysia, Australia and south-eastern Asia whilst Niaouli is native to Australia , New Caledonia and the French Pacific islands. Both oils are obtained by steam distillation of the fresh (needle-like) leaves and twigs of the trees. The Cineole content is ~14 to 65% and ~ 38 to 58% for the Cajuput and the Niaouli oils respectively (Lawless 1999a,b). They have common indications, such as for cystitis, rheumatism, spots, muscular aches and pains and colds and flu.

Other methods such as GC are suitable for the determination of Cineole in certain essential oils (Watson 1994). Such a method can quantify such substances to a high degree of accuracy, but is time consuming and cumbersome. Although there is a general monograph for the use of NIR spectroscopy in both the British Pharmacopoeia (BP 2001a) and the European Pharmacopoeia (EP 2002a), as yet there is no monograph for the use of NIR spectroscopy for the quantitative determination of a constituent in a pharmaceutical

material. It is proposed that NIR spectroscopy is a suitable alternative to this BP method, in that it is simple to use, requires no sample preparation, is non-destructive, rapid and is comparable in accuracy to the traditional method.

5.2.2 Materials and Methods

5.2.2.1 Materials and Sample Preparation

Thirty Eucalyptus oils of different brands and batch numbers were obtained from pharmacies. Twenty-one of these oils were of BP standard and nine were pure essential oils intended for use in Aromatherapy. O-cresol was obtained from Lancaster (Morecambe, Lancs, UK) and was stated to be 98+% w/w pure. Cineole of 99% m/m purity was obtained from Avocado Research Chemicals Ltd., Heysham, Lancs, UK.

In order to extend the range of the NIR assay and to establish linearity at Cineole concentrations in Eucalyptus oil at those higher than those occurring naturally, five oils were 'spiked' with the pure Cineole and assayed to determine their exact Cineole content. This allowed for extension of the Cineole calibration with higher values of Cineole content. As it was not practicable to obtain Eucalyptus oil samples with Cineole contents of lower than 70% m/m, to extend the calibration, three samples of pure Niaouli oil and two samples of Cajuput oil, (both known to have a lower Cineole content than Eucalyptus oils) were assayed using the BP method and incorporated into the sample sets for creation of additional NIR calibration models.

5.2.2.2 BP Method

See Materials and Methods Chapter Section 3.4.2. All Eucalyptus oil samples were assayed, along with the additional samples that had been 'spiked' with the Cajuput or the Niaouli oils.

5.2.2.3 Instrumentation and Equipment

A FOSS NIRSystems 6500 spectrophotometer with Rapid Content Sampler module was used. The data acquisition and spectral averaging software was NSAS™. A reflectance

vessel with stainless steel disc allowing for transflectance measurements was employed for presentation of the sample. Spectral data analysis was performed on Vision® software.

5.2.2.4 Near-infrared Measurements

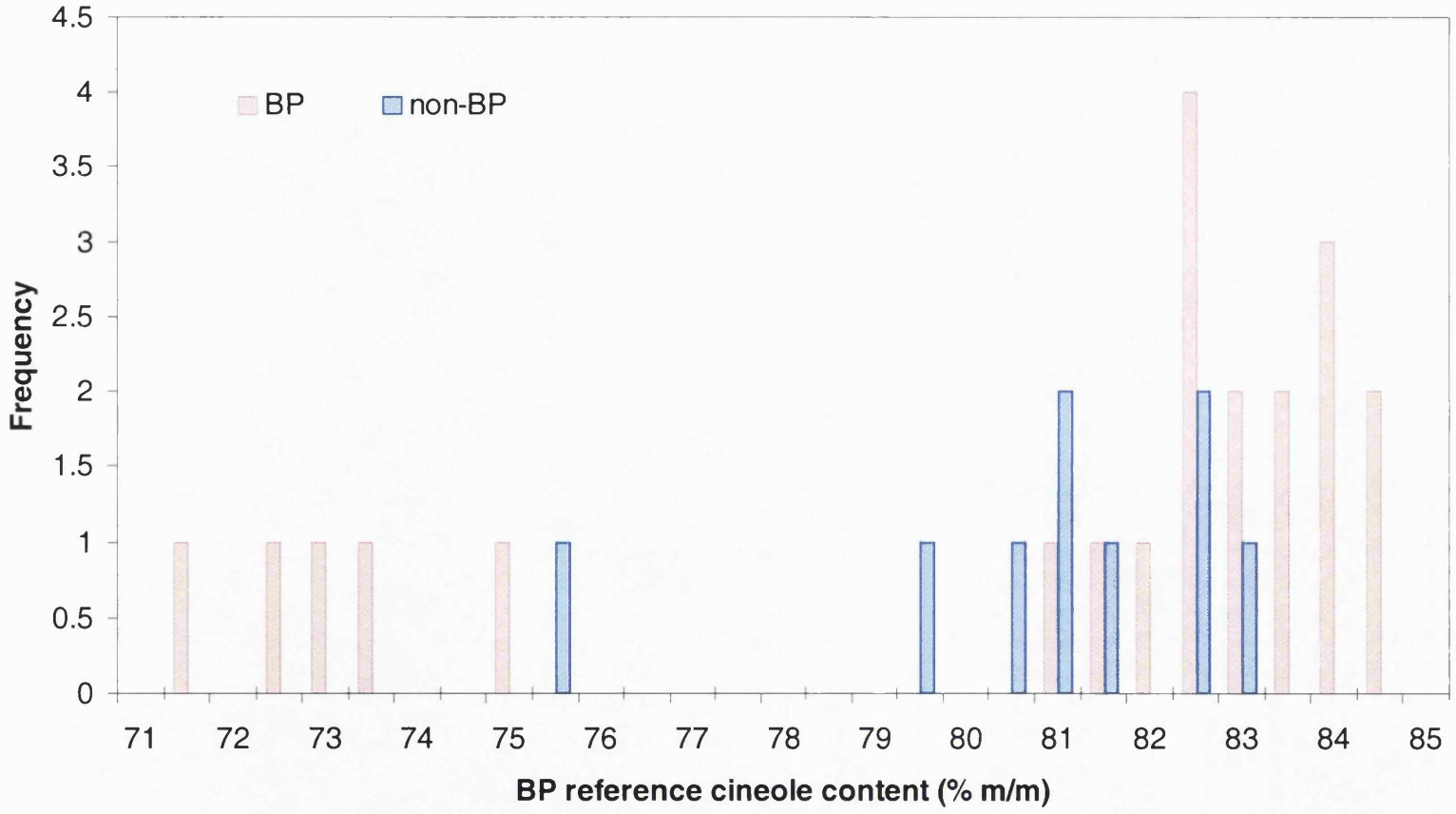
The Eucalyptus oils were scanned over the wavelength range of 1100nm to 2500nm. The Cineole content of all thirty of the Eucalyptus oil samples was determined using the BP assay as the reference method. Five spectra were obtained for each sample, the vessel being rotated about the centre between spectra. Each spectrum was the mean of 32 scans. The vessel was cleaned and refilled with the sample and a further five spectra obtained. These ten spectra were then averaged on the NSAS™ software to obtain a single mean spectrum for each sample and the data transferred to Vision®software.

5.2.2.5 Data Analysis and Construction of NIR Calibrations

A manual sample selection method was applied which involved assigning samples to the calibration set to obtain a representative set of Cineole contents for both the calibration and internal validation sets. Samples from the lower, middle and end of the Cineole content distribution (see Figure 5.2.2) were assigned, with 20 samples in the calibration set and 10 samples in the internal validation set. This procedure was repeated, assigning different representative samples between the calibration and validation set to obtain a second set of calibration and validation samples (known as Sample Sets 1 and 2). These two sets of calibration and validation samples were each used to obtain a NIR calibration equation; Calibration 1 from Sample Set 1 and Calibration 2 from Sample Set 2. The rationale behind the construction of two calibration equations was to verify that providing samples were chosen equally from the lower, middle and upper range of Cineole concentrations, the samples within these could be randomly chosen.

A MLR method in forward search mode (no pre-selected wavelengths) across the full wavelength range of 1100nm to 2500nm was employed for construction of the two calibration equations.

Figure 5.2.2 Frequency distribution of Cineole contents (% m/m) of Eucalyptus oil samples (21 BP samples and 9 non-BP samples)



There were several available mathematical pre-treatments of spectra, each of which could either be used alone or in combination. A number of mathematical pre-treatments of the spectral data set was investigated using the MLR method in order to select the most appropriate one(s) for use in the calibrations. The use of additional wavelengths was considered appropriate or not after consideration of either F value and the Standard Errors of Prediction; if the F value was lowered or the SEP increased than a second wavelength was considered unnecessary as it may have lead to 'over-fitting' of the NIR calibration to the reference set.

5.2.3 Results and Discussion

5.2.3.1 BP method

Figure 5.2.2 shows the frequency distribution of Cineole content of the 30 Eucalyptus oil samples, as determined by the BP method. These Cineole content values were then used as the reference values to develop the NIR method.

Use of the traditional BP and EP freezing point method for the determination of Cineole in the commercial Eucalyptus oil samples yielded values ranging from 71.5 % m/m to 85% m/m, with the values clustered into two distinct groups. This may be due to species difference, difference in geographical origin or time of harvesting. It was also noted that the non-BP samples used in this investigation were above the 70 % m/m minimum Cineole content as specified in the monographs. The range and distribution of Cineole contents used in the calibrations is therefore limited by the availability of commercial samples of Eucalyptus oils.

5.2.3.2 NIR method development and calibration

It was found that the use of SNV corrected 2nd derivative pre-treatment of the spectra was the most suitable for use in the development of the NIR calibrations. Figures 5.2.3 and 5.2.4 show the mean un-treated and SNV corrected 2nd derivative spectra obtained for Cineole and a single sample of Eucalyptus oil. These two spectra are very similar because of the high Cineole content of Eucalyptus oil.

Figure 5.2.3 Raw (un-treated) NIR spectra of Cineole (red) and a sample of Eucalyptus oil BP (blue) over the wavelength range 1100nm to 2500nm

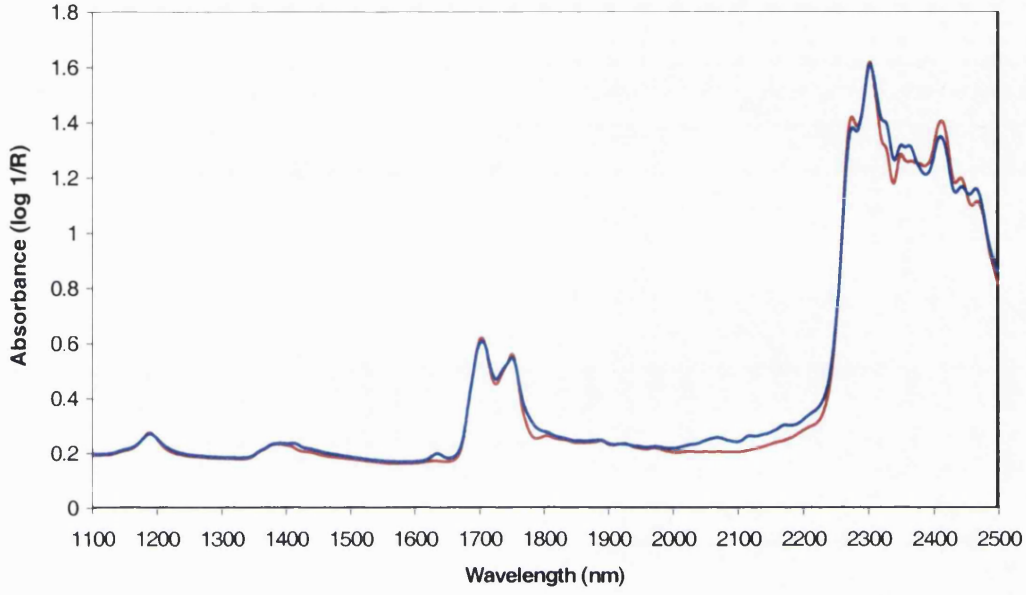
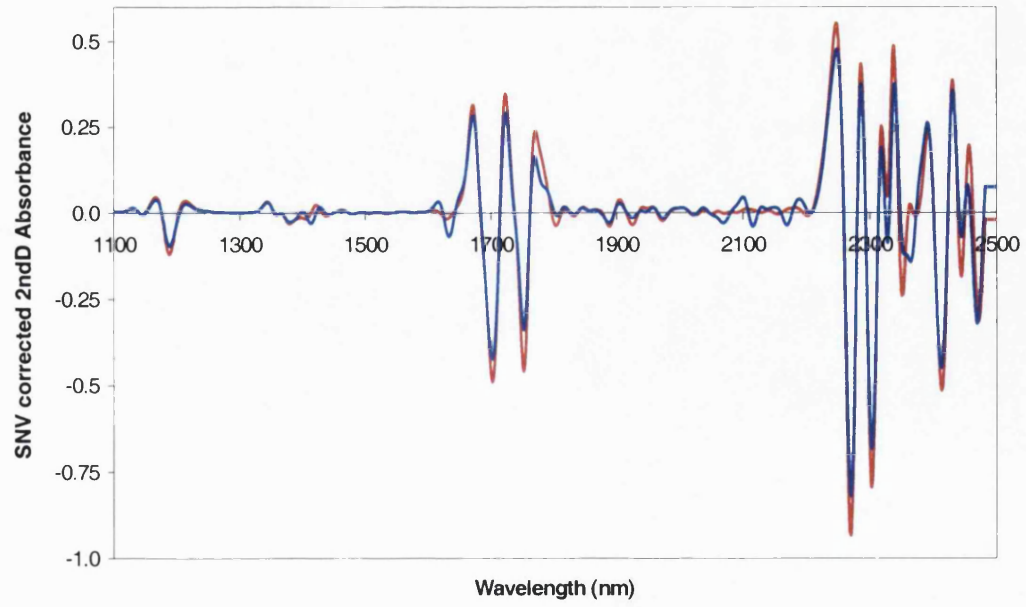


Figure 5.2.4 SNV corrected 2nd derivative mean spectra of Cineole (red) and a sample of Eucalyptus oil BP (blue) over the wavelength range 1100nm to 2500nm



The accuracy of a calibration was taken as how close the NIR values were to the BP reference values. The % mean bias and % mean accuracy were calculated for the two NIR calibrations and the results are summarised in Table 5.2.1. Correlation of the NIR spectra with the reference data (BP method) shows that these two methods are comparable for the estimation of the Cineole content, with a mean accuracy difference of 1.01% or less for both calibration equations.

The NIR method showed little evidence of bias, being ± 0.33% or less. Thus, providing samples are chosen equally from the lower, middle and upper range of Cineole concentrations, the samples within these can be randomly chosen.

	Calibration 1		**Calibration 2**	
	Calibration set	**Validation set**	**Calibration set**	**Validation set**
Correlation (R^2)	0.96	0.9	0.94	0.96
Wavelength(s) (nm)	1758		1756	
F value	382		262	
Mean bias (%)	0.01	-0.33	0.01	0.27
Mean accuracy (%)	0.85	1.01	0.88	0.86

Table 5.2.1 Summary of results for two calibration equations developed for the determination of Cineole content in Eucalyptus oils

The absorbance value at only a single wavelength was employed for construction of both calibration equations after consideration of the F values. The calibration equations take the form of that shown in equation 5.2.1.

$$C = K_0 + K_1 A_\lambda \qquad (5.2.1)$$

where K_0 is the intercept, K_1 is the slope for a plot of absorbance (A) at wavelength λ nm against reference values (concentration, C) for a single wavelength calibration.

The formulae for Calibrations 1 and 2 are given (equations 5.2.2 and 5.2.3) respectively.

$$C = 8.427 - 333.7A_{1758} \quad (5.2.2)$$

$$C = 4.053 - 252.8A_{1756} \quad (5.2.3)$$

5.2.3.3 Precision of the BP and NIR methods

A single sample of Eucalyptus oil was assayed for Cineole content using the BP method six times. Six spectra for a single sample of Eucalyptus oil were obtained on a single day for determination of repeatability (short term precision) and six spectra were obtained on six consecutive days for determination of intermediate precision. These were used to obtain NIR predicted Cineole contents using the two calibration equations constructed previously. The results are summarised in Table 5.2.2, where the standard deviation and the Coefficient of Variation are given, along with the mean (± 95% confidence limit) BP reference method and NIR predicted values.

	BP assay	NIR method			
		Repeatability		Intermediate Precision	
		C1	C2	C1	C2
Mean (% m/m)	75.3	75.4	74.8	74.5	74.8
Standard deviation (% m/m)	0.13	0.49	0.5	0.83	0.8
Coefficient of variation (%)	0.17	0.65	0.67	1.11	1.07
Confidence Interval (% m/m)	75.2 to 75.4	74.8 to 76.0	74.3 to 75.3	73.6 to 75.4	74.0 to 75.6

Table 5.2.2 Summary of results for determination of short-term precision (repeatability) and intermediate precision for Calibrations 1 and 2 (C1 and C2)

Precision of the NIR method was good, both on a short-term and intermediate time scale. The confidence interval for Sample Sets 1 and 2 for determination of both short-term and intermediate repeatability overlapped with that for the BP method, suggesting that there was no evidence for a difference in values obtained by the BP and NIR methods. The reason

for the high precision of the BP method is due to the measurement being repeated until the two highest values obtained differ by no more than 0.2°C.

5.2.3.4 Linearity / Range

The 'spiked' samples were found to be of Cineole content 89.8, 93.7, 95.6, 96.6 and 99.0% m/m and the Cineole content in the Niaouli and Cajuput oils was found to be between 50% m/m and 60 % m/m.

Three different Cineole content ranges were therefore used to create separate NIR calibrations for both Sample Sets 1 and 2 (with or without the additional samples in the calibration sets). The original range of 71.5 to 85.0% m/m [low] was increased to 71.5 to 99.0% [medium] on inclusion of the Cineole-spiked samples and the range was increased to 52.5 to 99.0% [high] on inclusion of both the Niaouli and Cajuput oils and the Cineole spiked samples. The results are shown in Table 5.2.3. MLR in forward search mode was again employed, with a single wavelength employed for each set of samples after consideration of the F values. Figures 5.2.5 and 5.2.6 show highlighted ranges of the NIR spectrum for pure Cineole and the Eucalyptus, Cajuput and Niaouli oils. 1758nm and 1756nm were the single wavelengths selected for Calibrations 1 and 2 for the low Cineole range samples. It can be seen from Figures 5.2.5 and 5.2.6 that across much of the wavelength range, including at these two wavelengths, the peak maxima and minima of the essential oils are due to their Cineole content. The wavelengths selected using the medium range Cineole content samples were 1774nm and 2462nm for Calibrations 1 and 2 respectively. It is less clear why these two wavelengths were selected. A similar wavelength of 1772nm was selected for Calibrations 1 and 2 using the high Cineole content range samples. In this region the peak is inverted (positive value in the 2nd derivative), suggesting that at this wavelength the presence of something other than Cineole (another constituent present in the essential oils at correlating levels to the Cineole) is being taken into account. At 2462nm the peak is positive but there is a peak shift between the pure Cineole and the essential oils. The % mean accuracy obtained using the low and medium Cineole content range samples are very similar. When using the high Cineole content range samples, the % mean accuracy for the calibration set was increased.

Figure 5.2.5 SNV corrected 2nd derivative spectra of Cineole, Eucalyptus oil, Cajuput oil and Niaouli oil over the wavelength range 1600nm to 1800nm

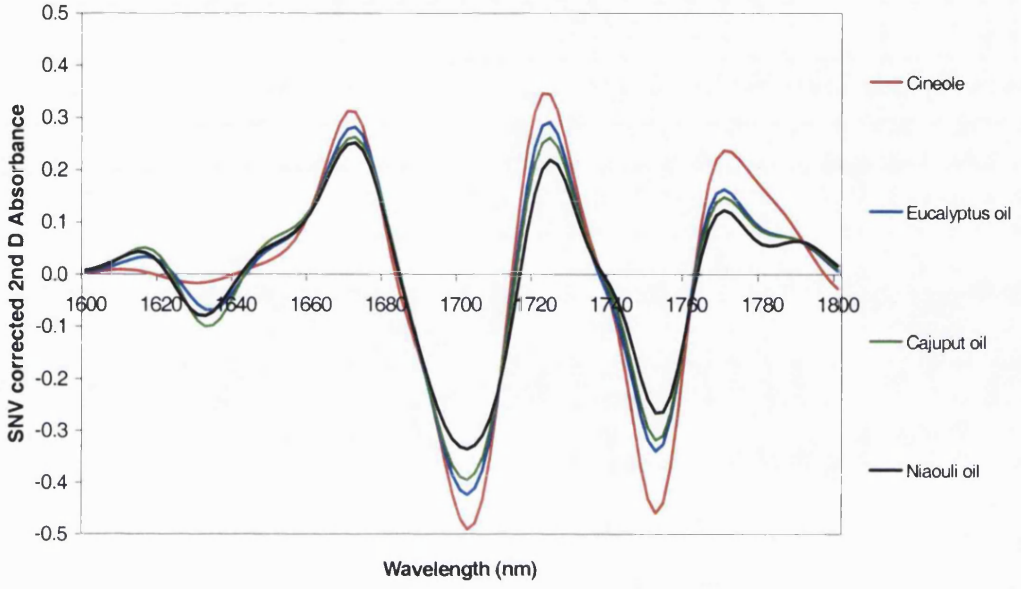
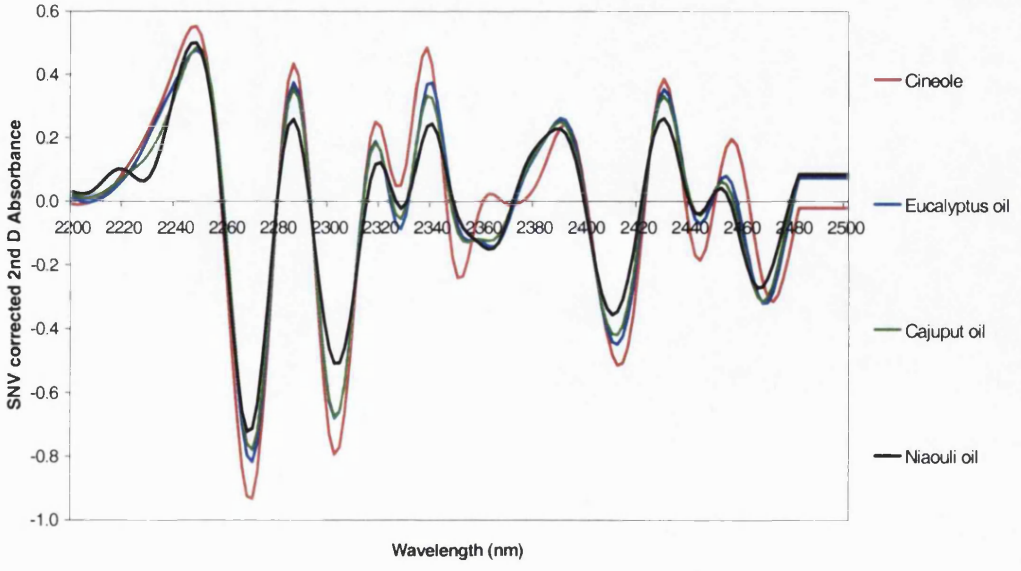


Figure 5.2.6 SNV corrected 2nd derivative spectra of Cineole, Eucalyptus oil, Cajuput oil and Niaouli oil over the wavelength range 2200nm to 2500nm



	Calibration 1		Calibration 2	
	Calibration set	**Validation set**	**Calibration set**	**Validation set**
Wavelength (nm)				
Low	1758		1756	
Medium	1774		2462	
High	1772		1772	
F value				
Low	382		262	
Medium	1147		1086	
High	2145		2138	
% mean bias				
Low	0.01	-0.33	0.01	0.27
Medium	0.01	-0.28	0.02	-0.22
High	0.49	-0.50	0.03	0.22
% mean accuracy				
Low	0.85	1.01	0.88	0.86
Medium	0.90	0.77	0.89	0.99
High	1.26	0.96	1.26	0.93

Table 5.2.3 Summary of results for the determination of linearity for the extension of the range of Cineole content values (% m/m)

This is due to either the fact that the matrix (chemical constituents) of the lower Cineole content Niaouli and Cajuput oils is different from that of Eucalyptus oil and / or the fact that the same absolute error in samples with a lower analyte concentration will result in a higher % mean accuracy. However, the % mean accuracy for the validation set is similar to those obtained for the other Cineole content ranges. Inclusion in the data set of Eucalyptus oils of lower Cineole content would provide a more accurate calibration equation than that obtained with the use of two different pure essential oils.

It would have been possible to construct a calibration equation for the determination of Cineole content in Eucalyptus oil using a single sample of Eucalyptus oil with a known Cineole content and spiking with different amounts of pure Cineole to obtain an appropriate range of Cineole contents. However, because the matrix of Eucalyptus oil will vary between samples, although a more accurate calibration is likely to have been achieved, it would be sensitive to small changes in the matrix of other samples and would therefore lead to poor determination of Cineole content in new samples. This is why the alternative method of spiking a random selection of Eucalyptus oils was used, with subsequent determination of their Cineole content using the BP assay method.

To assess if extension of the calibration range with the samples with or without the oils of lower Cineole content was appropriate, the 95% confidence intervals for the slope and intercept of the predicted versus reference values plot, were calculated (data not shown). Confidence intervals for the slope of the calibration and validation sets for Calibrations 1 and 2 include 1 and the confidence intervals for the intercept include 0. Thus there was no evidence for a relative systematic error in either calibration equation. In addition the F values increased which suggested that these new calibrations incorporating the two extended ranges (medium and high) fitted the reference data better.

Plots of NIR predicted values against BP method reference values for both Calibrations 1 and 2 for the low, medium and high Cineole content ranges, along with their R^2 values and equations of the lines of best-fit are given in Table 5.2.4 and Figures 5.2.7, 5.2.8, 5.2.9, and 5.2.10.

Although there are no limits of accuracy stated in the BP for this assay, experimental work detailed in the original literature stated a ± 3% m/m error (Tusting Cocking 1920). The results obtained for accuracy, linearity, precision and repeatability of the NIR method

compare favourably with that of the BP method. The NIR method also has advantages over the BP method in that once the calibration equation has been developed and validated, it is simpler to carry out, no sample preparation is required and it is more rapid. In addition, no other chemicals are required for the NIR method and the amount of sample used is considerably less.

5.2.4 Conclusions

In conclusion, the results show that the use of NIR spectroscopy with a reflectance vessel as the sample presentation method allows the prediction of Cineole content in a series of Eucalyptus oil samples to within accuracies that compare highly favourably with the official BP method, and that NIR spectroscopy could be used as an alternative method for the determination of Cineole content in Eucalyptus oil.

	Line equation		R^2	
	Calibration set	**Validation set**	**Calibration set**	**Validation set**
Calibration 1				
Low	$y = 0.95x + 3.7$	$y = 0.93x + 5.5$	0.96	0.90
Medium	$y = 0.98x + 1.7$	$y = 1.0x - 0.52$	0.98	0.95
High	$y = 0.99x + 1.1$	$y = 1.0x - 0.33$	0.99	0.95
Calibration 2				
Low	$y = 0.94x + 5.1$	$y = 0.97x + 2.8$	0.94	0.96
Medium	$y = 0.98x + 1.7$	$y = 0.83x + 13$	0.98	0.98
High	$y = 0.99x + 0.99$	$y = 0.95x + 4.4$	0.99	0.96

Table 5.2.4 Equation of the best-fit lines for the plots of predicted NIR values (y) against BP method reference values (x) for the three ranges of Cineole content values (% m/m)

Figure 5.2.7 Plot of NIR predicted against BP reference Cineole content for the Eucalytus oils of Sample Set 1 used in the calibration set

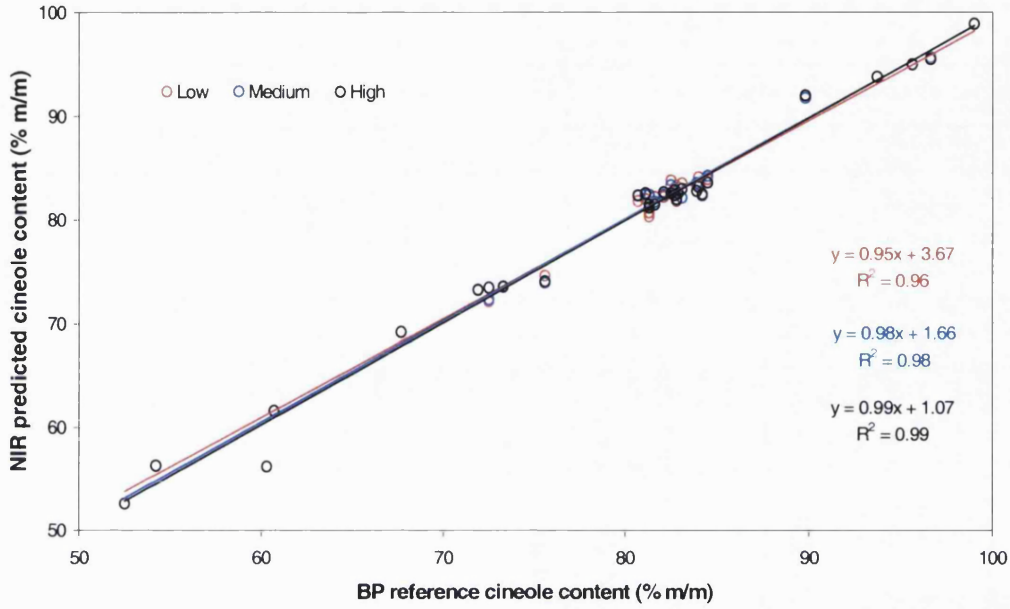


Figure 5.2.8 Plot of NIR predicted against BP reference Cineole content for the Eucalyptus oils of Sample Set 1 used in the validation set

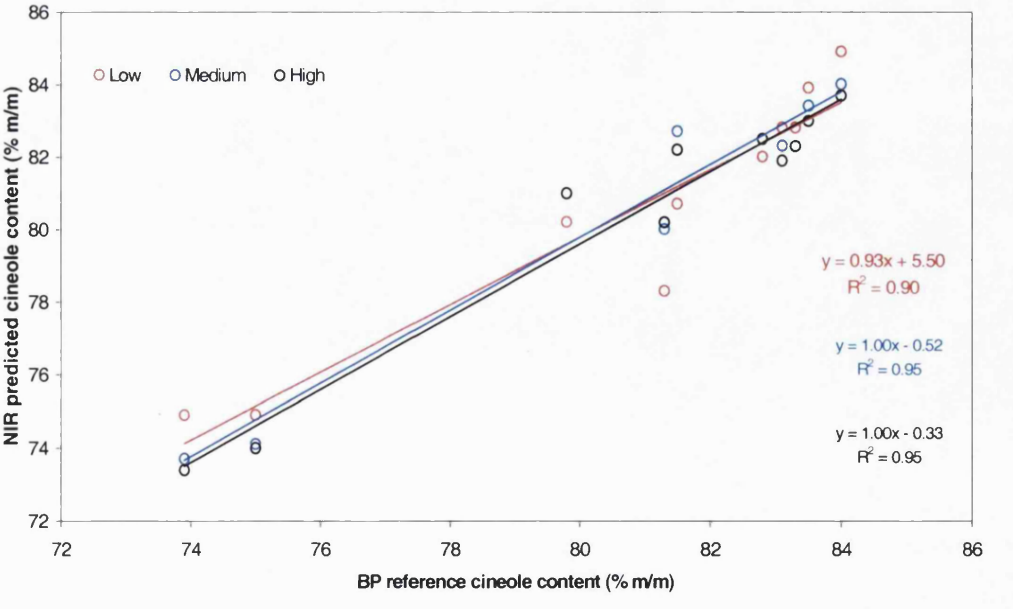


Figure 5.2.9 Plot of NIR predicted against BP reference Cineole content for the Eucalyptus oils of Sample Set 2 used in the calibration set

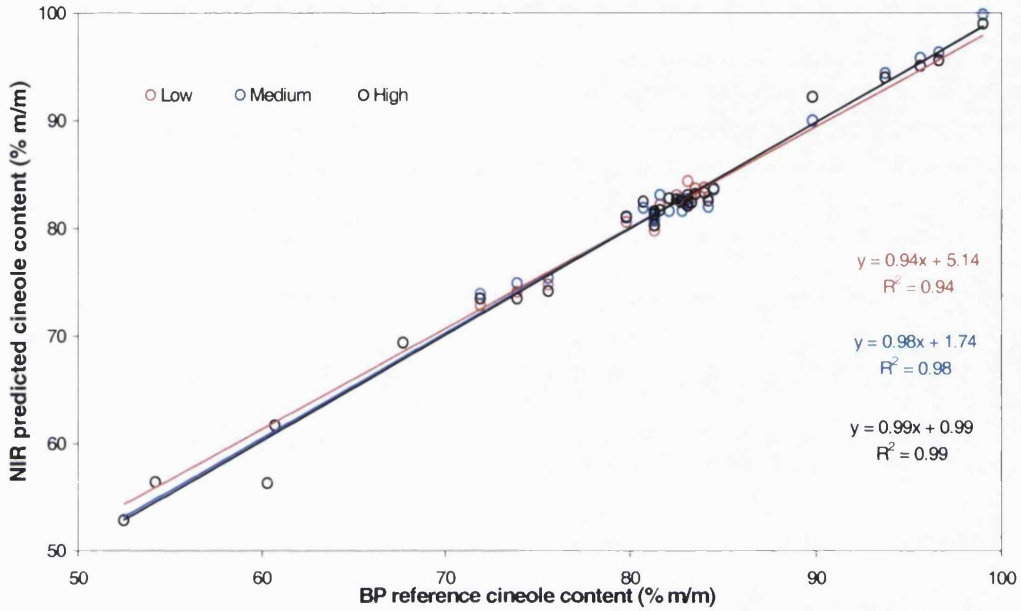
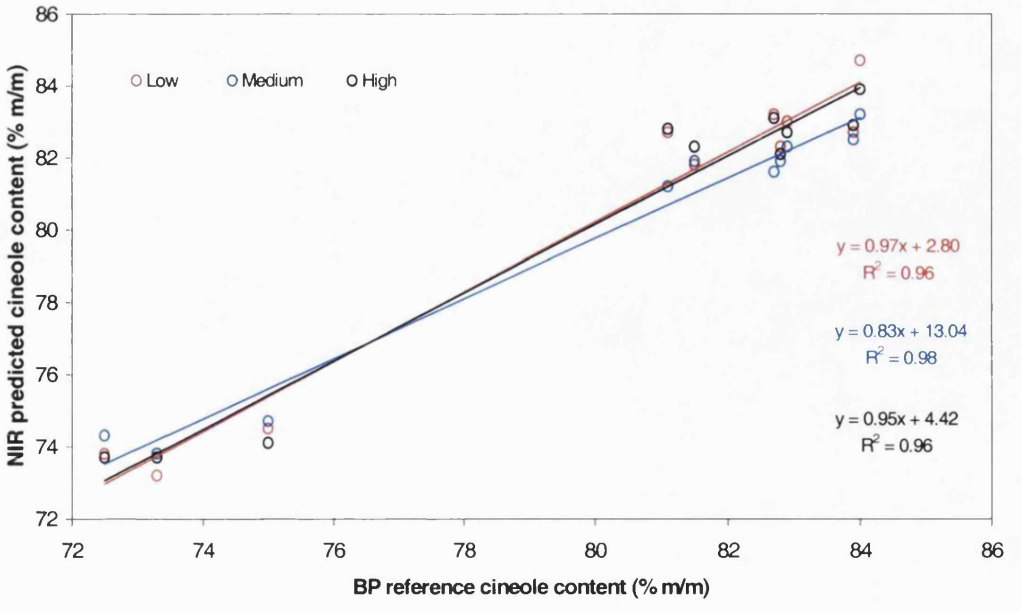


Figure 5.2.10 Plot of NIR predicted against BP reference Cineole content for the Eucalytus oils of Sample Set 2 used in the validation set



5.3 The Quantification of Citral in Lemongrass and Lemon oils by Near Infrared Spectroscopy

5.3.1 Introduction

NIR spectroscopy has been used to develop a robust yet sensitive calibration for the determination of Cineole (Eucalyptol) content in Eucalyptus oils which is present at levels in excess of 70.0% m/m. The aim of this present study was to determine if NIR spectroscopy was capable of quantifying chemical constituents other than Cineole in essential oils at similar concentrations. A second aim of this study was to ascertain if NIR spectroscopy could accurately quantify compounds present at much lower levels in essential oils using a simple means of data analysis. There are only a few reports concerning quantification of low concentration analytes in the literature. The quantification of secondary metabolites in tea, drugs and spice plants has been carried out (Schulz *et al* 1999a), as has the quantification of constituents present in the leaves and oil of peppermint (Schulz *et al* 1999b). Citrus oils have been studied both qualitatively and quantitatively (Steuer *et al* 2001, Schulz *et al* 2002), including the total aldehyde content of a number of citrus oils. Although the Standard Errors of Calibration appear low, the small amount of constituent present also has to be taken into account for these results to be meaningful. In all these cases more complicated techniques such as PLS and/or PC Analysis was carried out. The purpose of the present work was to show that the use of more simple techniques such as Multiple Linear Regression (MLR) for the construction of NIR calibrations can be also be useful. Moreover, this serves to dispel the 'black box' image often viewed by those not thoroughly acquainted with NIR spectroscopy.

Lemongrass oils and Lemon oils were studied, both of which contain the carbonyl compound Citral. Lemongrass oil contains Citral at levels of approximately 65 to 85% m/m (Lawless 1999c) and Lemon oil contains Citral at a concentration of approximately 2 to 5% m/m (Tyler *et al* 1988). By using these two oils it can be ascertained if a single calibration could be constructed to accurately quantify Citral at both high and low levels. Lemongrass (*Cymbopogon citratus*) oil is obtained by steam distillation from finely chopped fresh and partially dried leaves. There are two main types of Lemongrass oil -

West Indian and East Indian, each giving different characteristics to the oil (Lawless 1999c). It is thought to have antiseptic, deodorant and insecticidal properties and is also used in the cosmetic industry (Evans 1996c).

Lemon oil is produced around the Mediterranean, California and North America, Australia and parts of Africa. It is known to have antiseptic properties and is claimed to have medicinal benefits in the treatment of high blood pressure, dyspepsia and arthritis, although its main use is in the cosmetic and flavouring industries (Evans 1996c). The quality of Lemon oil in terms of Citral content varies greatly depending on country or area of origin, time of harvesting and other factors. It is also known that the Citral content of Lemon oil depends on the method used for extraction (Guenther 1952a). Methods of production of Lemon oil such as steam distillation will cause hydrolysis and oxidation of Citral and thus considerably lower the quality of the oil (Samuelsson 1999). The main constituent of Lemon oil is Limonene, a monoterpene hydrocarbon (at levels of approximately 90% m/m). Although some oils have a Citral content as high as 13% m/m, a high quality oil has an optimum range of 2 to 4% m/m Citral. Citral content is an important factor in determining the purity of Lemon oil and commercial purchasers of Lemon oil will carry out a determination of Citral content, along with a physical and chemical examination of the oil, as it is common to adulterate Lemon oil with Citral obtained from cheaper sources such as Lemongrass oil (Tyler *et al* 1988).

Citral (3,7-dimethyl-2,6-octadienal) from natural sources is a mixture of two geometric isomers geranial and neral, otherwise known as Citral 'a' and 'b', respectively (Merck Index 1996). It is the Citral 'a' form which largely predominates in nature and is responsible for the odour and flavour of Lemon and Lemongrass oils (Guenther 1957). The structure of the two forms is shown in Figure 5.3.1.

The British and European Pharmacopoeia monographs for Lemon oil (BP 2001c and EP 2002c) state that the oil must be obtained by suitable mechanical means, without the aid of heat, from the fresh peel of *Citrus limon* (L.) Burman fil. and contain not less than 2.2% m/m and not more than 4.5% m/m of carbonyl compounds calculated as Citral. The assay described in this monograph for the determination of Citral content was used as the

reference method in this study and NIR calibrations constructed for the determination of Citral in Lemongrass and Lemon oils.

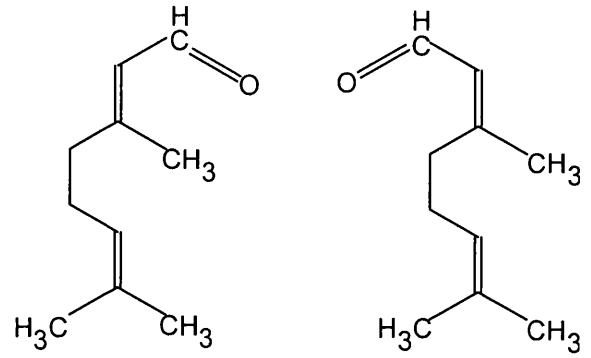


Figure 5.3.1 Chemical structure of Citral (*cis*- and *trans*-)

The BP assay is a titration method whereupon hydrochloric acid is liberated upon the reaction of hydroxylamine hydrochloride with the aldehyde (Citral) in the oil and then titrated against a standardised solution of ethanolic potassium hydroxide.

It must be noted that other aldehydes (such as Citronellal) are known to be present in Lemongrass oil and to a lesser extent in Lemon oil. Thus, the BP assay for Citral in Lemon oil (and the reference method for both types of oil used in this study) is more correctly an assay for total aldehyde content rather than for Citral content alone.

Development of an NIR calibration for the accurate determination content in Lemongrass and Lemon oils would result in a rapid, simple and non-destructive procedure that could be used to replace conventional methods.

5.3.2 Materials and Methods

5.3.2.1 Materials

Citral (a mixture of geranial and neral) was obtained from Avocado Research Chemicals Ltd. Heysham, Lancs, UK and was stated to contain more than 95% of the pure substance. Samples (25) of pure Lemon oil and pure Lemongrass oil (14) were obtained from retail outlets such as pharmacies and health food stores. To expand the range of concentration of initial Citral content in the samples obtained, after consideration of the Citral content,

samples of Lemon oil (10) and Lemongrass oil (11) were 'spiked' by adding a few drops of Citral.

5.3.2.2 BP method

See Materials and Methods Chapter, Section 3.4.3.

5.3.2.3 Instrumentation and Equipment

A FOSS NIRSystems 6500 spectrophotometer with a Rapid Content Sampler module was used. Data acquisition and spectral data analysis were performed using FOSS NIRSystems Vision® software. A reflectance vessel, in conjunction with a stainless steel cylindrical disc was employed for presentation of the sample

.

5.3.2.4 Near-infrared Measurements

The Lemon and Lemongrass oils were scanned over the wavelength range of 1100nm to 2500nm. Three spectra were obtained for each sample (each spectrum was the average of 32 scans) the vessel being rotated about the centre. The spectra were averaged prior to data analysis.

5.3.2.5 Data Analysis and Construction of NIR Calibrations

The Lemongrass oil sample set contained 26 samples, consisting of the 14 'pure' Lemongrass oil samples, the 11 'spiked' samples and the Citral sample. The Lemon oil sample set contained 35 samples, consisting of the 25 Lemon oil samples and the 10 'spiked' samples.

Samples from the lower, middle and end of the Citral content distribution were assigned to either the calibration or validation set. Of the Lemongrass oils samples, 18 were assigned to the calibration set and 8 samples were assigned to the validation set. For the Lemon oil samples, 25 were assigned to the calibration set and 10 to the validation set.

A MLR method initially in forward search mode (no pre-selected wavelengths) was employed across the full wavelength range of 1100nm to 2500nm. The use of spectral pre-treatments was investigated using the MLR method in order to select the most appropriate one(s) for use in the calibrations. The use of additional wavelengths was considered

appropriate or not after consideration of the F value and the SEP; if this value increased or the F value lowered then a second wavelength was considered unnecessary as it may have lead to 'over-fitting' of the NIR calibration to the reference set. A number of mathematical pre-treatments of the spectral data set was investigated using the MLR method in order to select the most appropriate one(s) for use in the calibrations. The addition of a second wavelength in the form of a denominator to that selected initially by the Vision® software (that is, a ratio of the two wavelengths) was considered as an alternative to summation of the two wavelengths for the construction of the MLR calibration equations. NIR calibrations using the PLS method were also developed for direct comparison with the MLR method NIR calibrations.

5.3.2.6 Precision of the BP and NIR Methods

Single samples of Lemon oil and Lemongrass oil were assayed for Citral content six times using the BP method. Six spectra for a single sample of the oils were obtained on a single day for determination of repeatability (short term precision) and six spectra were obtained on six consecutive days for determination of intermediate precision. As for all NIR readings obtained in this study, each spectrum was the average of three spectral readings taken.

5.3.3 Results and Discussion

5.3.3.1 BP method

Figures 5.3.2 and 5.3.3 show the frequency distribution of Citral content for the 26 Lemongrass oil samples and the 35 Lemon oil samples. For the Lemongrass oil samples, the range of Citral contents was 69.89% to 76.95% m/m.

The addition of the spiked Lemongrass oil samples increased the upper limit to 94.6% m/m. In addition to this a 'pure' sample of Citral was added to the calibration (a twelfth 'spiked' sample), which was found to contain 99% m/m Citral by the BP method.

Although there is a BP monograph for Lemon oil, no samples of Lemon oils of 'BP' standard were obtained. It was found that the Lemon oil samples yielded values ranging from 2.24 to 3.70% m/m. The ten spiked Lemon oil samples increased the Citral content range to 15.75% m/m.

Figure 5.3.2 Frequency distribution of total aldehyde (Citral) content in Lemongrass oil samples (14 'pure' samples, 11 'spiked' samples and one pure Citral sample)

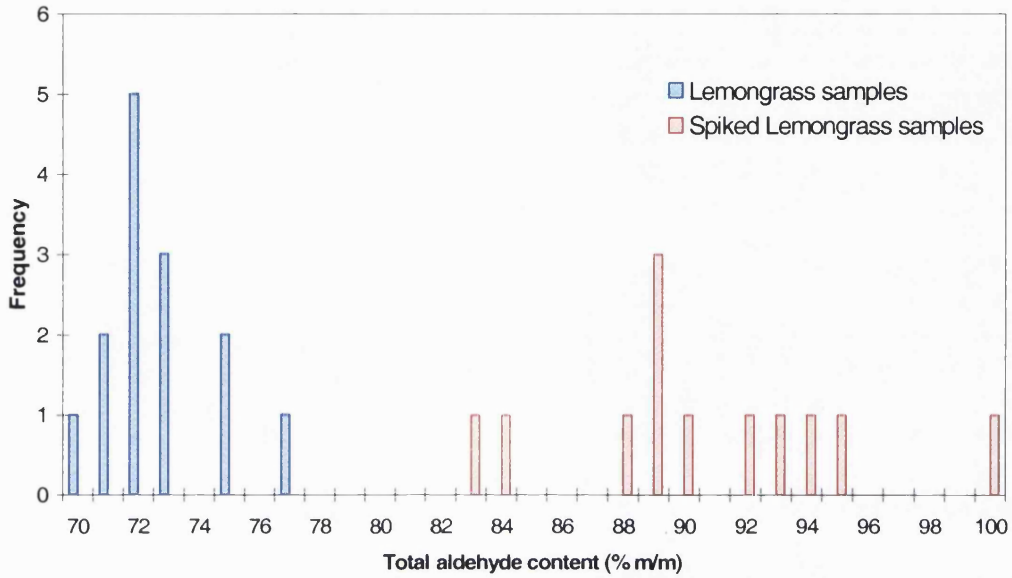
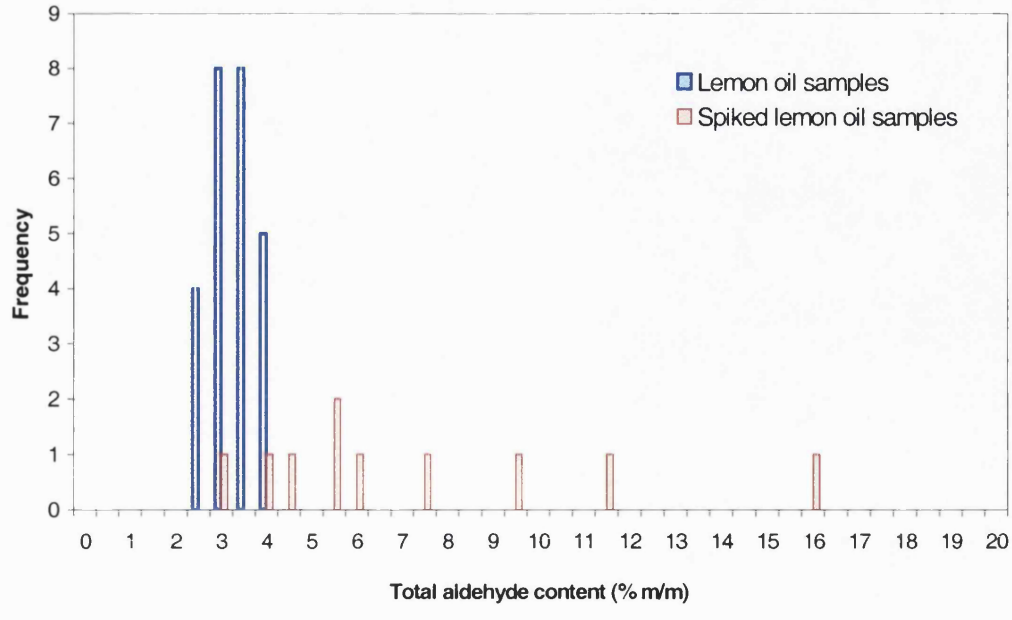


Figure 5.3.3 Frequency distribution of total aldehyde (Citral) content in Lemon oil samples (25 'pure' samples and 10 'spiked' samples)



5.3.3.2 NIR Method Development and Calibration

Figure 5.3.4 shows the mean spectrum obtained for Citral and a single sample of each of Lemongrass oil and Lemon oil. The most suitable mathematical pre-treatment of the spectra was found to be SNV corrected, 2nd derivative spectra.

The Lemongrass oil sample set was considered first, as it had a high Citral content and a good calibration in terms of accuracy and precision was expected. A wavelength of 2212nm (selected by the Vision® software) was found to have absorbance readings that had the highest correlation with the reference data. This region of the spectrum in its SNV corrected 2nd derivative form is shown in Figure 5.3.5. From a visual inspection of this region of the spectrum it can be seen that 2212nm is an acceptable wavelength to be incorporated into the equation, as it shows the increase in absorbance with an increase in Citral content (the peak is negative because the absorbance peaks are reversed in the 2nd derivative). The selection of this particular wavelength is supported by the fact that the aldehydic C-H group (contained in Citral) exhibits two characteristic combination bands near 2210nm and 2250nm (Whetsel 1968).

A second wavelength (selected by Vision® software as providing the highest correlation to the reference data in combination with the first wavelength) was added to the equation, but after consideration of the SEP and the F value it was not considered beneficial to the calibration. The results for this calibration are summarised in Table 5.3.1. The relative % error (similar to the % mean accuracy, but is calculated for the single sample alone) was less than 2.61 for all samples in the calibration and validation sets. A plot of the NIR predicted versus reference values for the calibration and validation sets is shown in Figure 5.3.6.

Figure 5.3.4 Raw (un-treated) NIR spectra of Lemongrass oil, Lemon oil and Citral

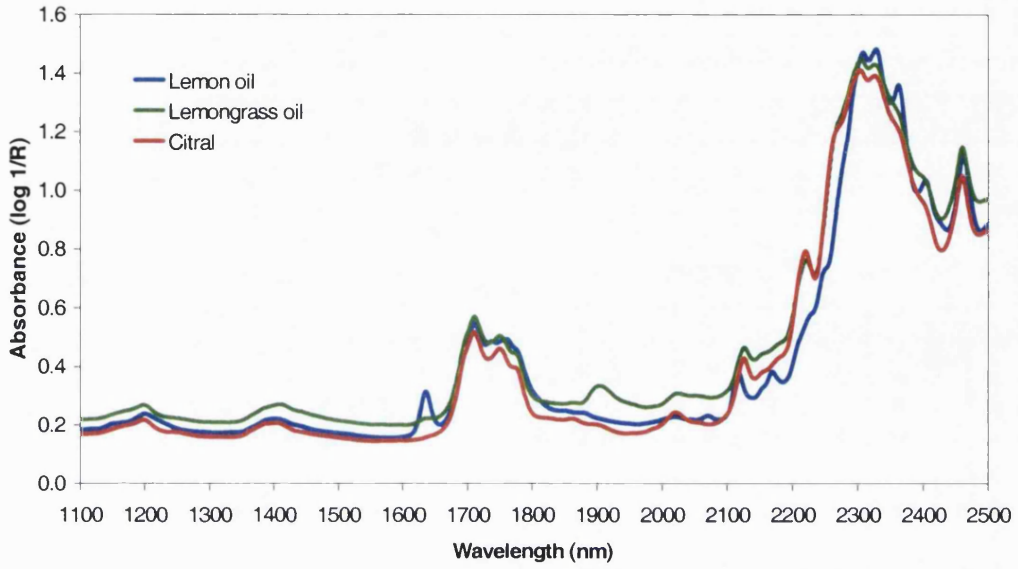
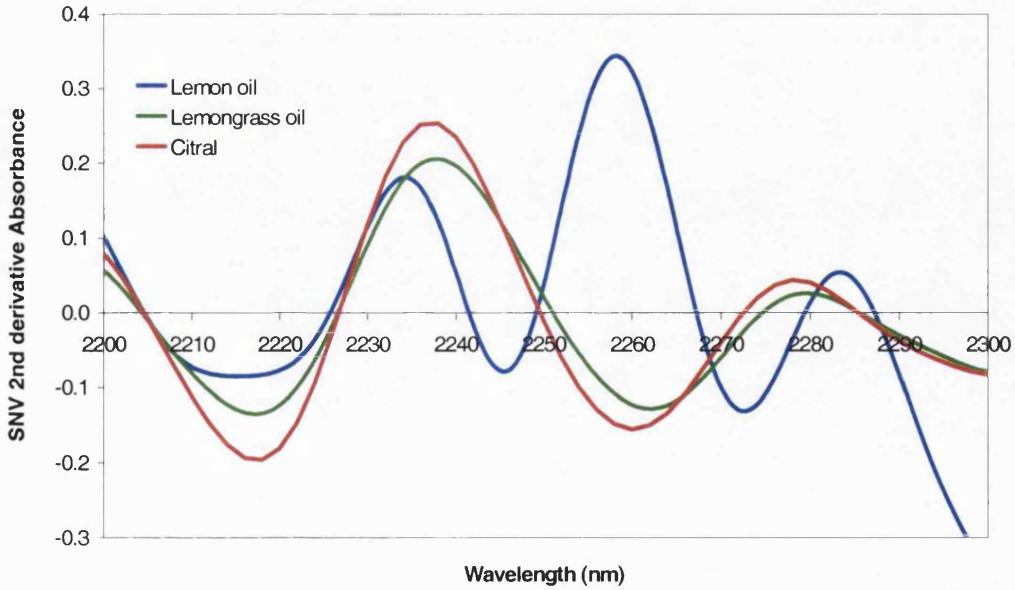


Figure 5.3.5 SNV corrected 2nd derivative spectra of Lemongrass oil, Lemon oil and Citral over the wavelength range 2200nm to 2300nm



	Lemongrass oils		Lemon oils	
	Calibration set	**Validation set**	**Calibration set**	**Validation set**
Correlation Coefficient R^2	0.988	0.997	0.996	0.994
Wavelength(s) (nm)	2212		2212	
F-value	1260		2872	
SEC / SEC (% m/m)	1.16	0.48	0.20	0.12
% mean bias	0.02	0.09	0.20	-0.71
% mean accuracy	1.00	0.52	4.28	2.86

Table 5.3.1 Summary of results for Lemongrass oils and Lemon oils for the determination of Citral (total aldehyde) content

The equation of the line for the calibration set samples was $y = 0.99x + 1.02$ and $y = 0.98x + 1.59$ for the validation set, which are in close agreement to $y = x$. The % mean accuracy for the calibration set was 1.00% and 0.52% for the validation set. The errors for the validation set in a calibration produced from a much larger group of samples such as that would be constructed for a commercial purpose would be expected to be roughly the same or larger than for the calibration set. The relatively small number of samples employed in the validation set for this calibration may account for the improved accuracy relative to the calibration set. The use of a PLS method to construct a MLR calibration equation means that the bias is as close to zero as possible for the reference (calibration set), but it is useful to compare this with the bias of the validation set. The mean bias was 0.02% for the calibration set and 0.09% for the validation set.

The Lemon oil sample set was then considered. Use of the same MLR program allowing the Vision® software to select the wavelength with the highest correlation to the reference data resulted in the selection of the absorbance data for the calibration at 2256nm, with a % mean accuracy of 9.65 and 9.01 for the calibration set and validation set respectively (data not shown). Although it is known there is a characteristic aldehyde band in this region of the NIR spectrum, inspection of the SNV corrected 2nd derivative spectra at this point (Figure 5.3.5) shows that the Lemon oil has a positive peak at this value whereas Citral has a negative peak. This is due to the fact that the aldehyde absorption band at this point is overlapped with a positive 2nd derivative peak due to a component other than Citral. This is likely to be Limonene, which is present at levels of roughly 90% m/m in Lemon oils. Addition of a second wavelength of 2230nm (selected by Vision® software) improved the calibration to give a % mean accuracy of 6.48 and 5.57 for the calibration and validation set respectively (data not shown). The selection of this wavelength may be explained by the fact that the absorbance value for the pure Citral is the same as for the Lemon oil.

A second approach to the construction of the calibration was thus considered, in order to ascertain if it was possible to further improve the calibration by the use of manually selected wavelengths. If the Lemon oil calibration was linear over a much greater range, extending to that of the Citral content for the Lemongrass oils, the wavelengths selected for both sample sets would be expected to be the same. For this reason the wavelength selected for the Lemongrass oils calibration (2212nm) was used in the construction of the Lemon

oil calibration. A second wavelength (selected by the Vision® software) was added to the calibration after consideration of the F values and the Standard Errors. This wavelength was found to be 2258nm, similar to the 2256nm wavelength selected originally. The use of the 2258nm wavelength enabled the presence of Limonene to be taken into account in the construction of the calibration equation, thus resulting in an improved calibration with greater quantification accuracy. The SNV 2nd derivative NIR spectra for Limonene, Lemon oil and Citral over the wavelength range 2200nm to 2300nm are shown in Figure 5.3.7. The mean bias and accuracy for the Lemon oil for the calibration set were 0.20% and 4.28% respectively and for the validation set were (-) 0.71% and 2.86% respectively. The summary of this calibration for the Lemon oil samples is shown in Table 5.3.1. The relative % error ranges from 0.36 to 10% or less for all but two of the calibration set samples, the remaining two being 13.8 and 15.8%. Table 5.3.2 shows the reference and NIR predicted Citral content for the individual samples of the Lemongrass and Lemon oil sets, along with these % relative error values. Re-titration of the oils, visual inspection of the NIR spectra and a brief look at Principal Components data for the set of Lemon oils gave no sign that these two samples were 'outliers' which could legitimately be removed from the calibration. Only one of the ten samples in the validation set (8.56 % relative error) was predicted to an accuracy poorer than 4.17%. Figure 5.3.8 shows the NIR calibration results for the Lemon oils in terms of NIR predicted versus reference values for the calibration and validation sets. The equation of the line was $y = 1.00x + 0.02$ and $y = 1.03x - 0.13$ for the calibration and validation sets respectively.

Comparison with the calibration for the Lemongrass oils also given in Table 5.3.1 shows that by taking the relative value of the Citral content of the oils into account (given by the % mean accuracy) the prediction error for the Lemon oil equation (4.28%) is considerably poorer than that developed for the Lemongrass oils (1.00%). The results for the Lemon oil samples may be considered to be unacceptably high. The accuracy of prediction of this method may also be improved by increasing the number of Lemon oils in the sample set to take into account other physical and chemical differences (apart from Citral content) between the oils, resulting in a more robust calibration.

Figure 5.3.6 Plot of NIR predicted values against reference Citral content of Lemongrass oils

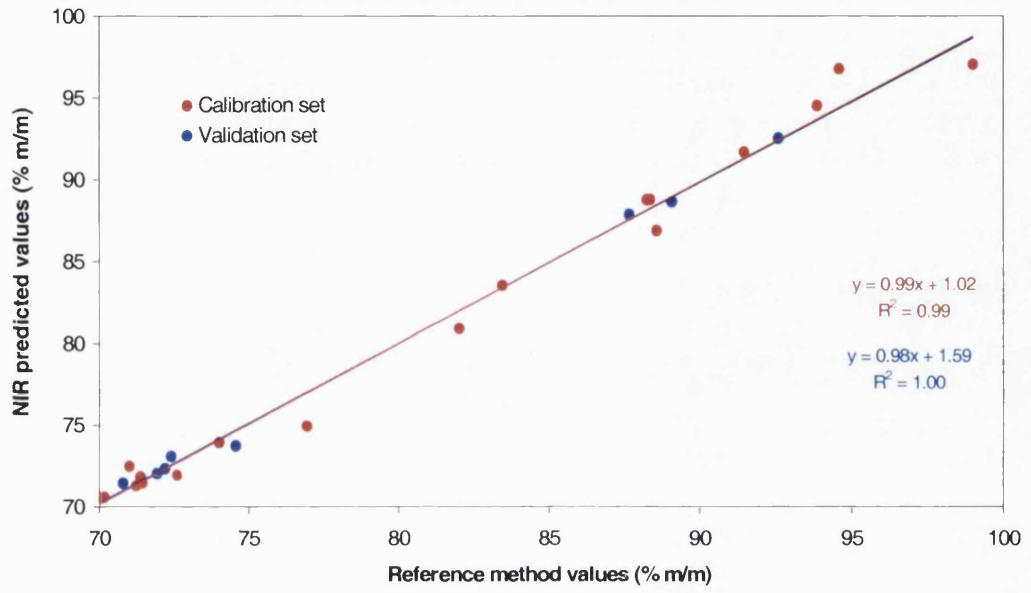
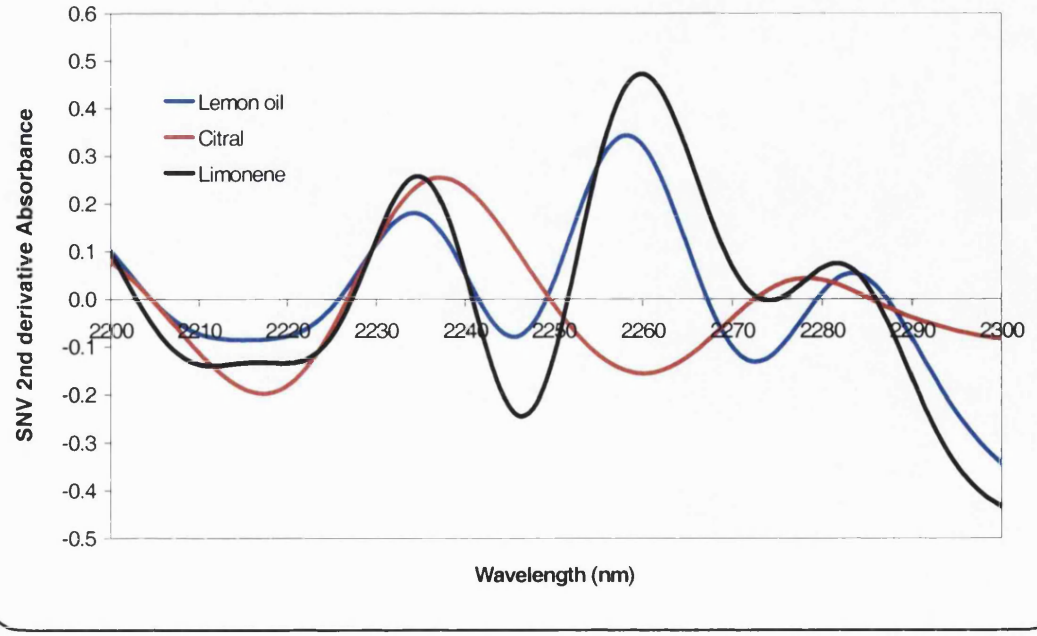


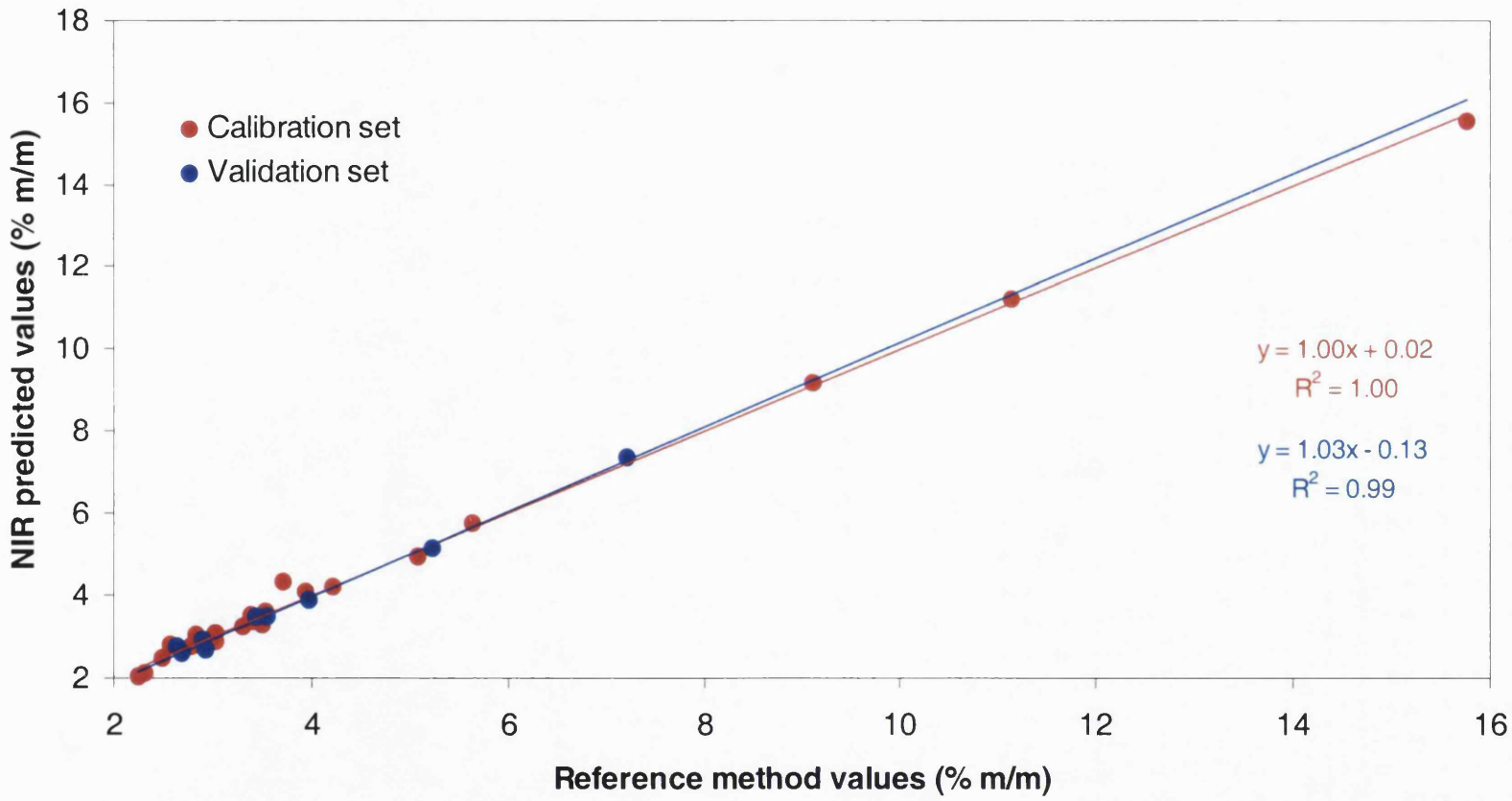
Figure 5.3.7 SNV corrected 2nd derivative spectra of Limonene, Citral and Lemon oil over the wavelength range 2200nm to 2300nm



Sample	Lemongrass oils			Lemon oils		
	Reference	NIR	Error %	Reference	NIR	Error %
1	70.18	70.6	0.6	2.24	2.05	8.48
2	69.89	70.81	1.32	2.24	1.93	13.84
3	71.24	71.29	0.07	2.31	2.14	7.36
4	71.45	71.45	0	2.48	2.49	0.4
5	71.38	71.85	0.66	2.57	2.82	9.73
6	72.6	71.94	-0.91	2.78	2.77	0.36
7	71.01	72.49	2.08	2.83	3.05	7.77
8	74.03	73.95	-0.11	2.9	2.76	4.83
9	76.95	74.95	-2.6	2.91	2.81	3.44
10	82.02	80.91	-1.35	3.02	3.07	1.66
11	83.45	83.55	0.12	3.02	2.87	4.97
12	88.59	86.9	-1.91	3.3	3.23	2.12
13	88.26	88.78	0.59	3.39	3.55	4.72
14	88.37	88.81	0.5	3.4	3.36	1.18
15	91.47	91.71	0.26	3.49	3.27	6.3
16	93.87	94.53	0.7	3.53	3.61	2.27
17	94.6	96.77	2.29	3.55	3.53	0.56
18	99	97.05	-1.97	3.7	4.35	17.57
19	70.8	71.44	0.9	3.94	4.08	3.55
20	71.93	72.04	0.15	4.21	4.22	0.24
21	72.19	72.33	0.19	5.08	4.95	2.56
22	72.4	73.08	0.94	5.63	5.73	1.78
23	74.58	73.72	-1.15	9.1	9.14	0.44
24	87.68	87.86	0.21	11.14	11.19	0.45
25	89.09	88.65	-0.49	15.75	15.56	1.21
26	92.6	92.53	-0.08	2.64	2.75	4.17
27				2.69	2.59	3.72
28				2.89	2.91	0.69
29				2.92	2.67	8.56
30				3.43	3.5	2.04
31				3.43	3.49	1.75
32				3.55	3.47	2.25
33				3.96	3.88	2.02
34				5.22	5.14	1.53
35				7.21	7.36	2.08

Table 5.3.2 NIR predicted and reference Citral (total aldehyde) content for Lemongrass and Lemon oils (calibration set = red, validation set = blue)

Figure 5.3.8 Plot of NIR predicted against reference Citral content of Lemon oils



The use of a second wavelength in the from of a denominator in combination with the first wavelength for the MLR calibration was discounted after investigation. The use of PLS did not result in a NIR calibration that performed better than the MLR equations in for both sets of oils (Table 5.3.3).

	Lemongrass oils				Lemon oils			
	Calibration set		Validation set		Calibration set		Validation set	
	MLR	PLS	MLR	PLS	MLR	PLS	MLR	PLS
% mean accuracy	1.00	1.05	0.52	0.53	4.28	5.35	2.86	3.5
% mean bias	0.02	0.02	0.09	-0.08	0.2	-0.1	-0.71	-0.37

Table 5.3.3 Comparison of MLR and PLS NIR calibration results

5.3.3.3 Precision of the BP and NIR Methods

The NIR spectra obtained for the precision experiment were used to obtain NIR predicted Citral contents using the two calibration equations constructed previously. The results are summarised in Table 5.3.4 for both sets of oils, where the standard deviation, s and the coefficient of variation, CV are given, along with the mean (± 95% confidence limit) BP reference method and NIR predicted values.

Precision of the NIR method was good, both on a short-term and intermediate time scale. The confidence interval for determination of both short-term and intermediate repeatability for both calibrations overlapped with that for the BP method, suggesting that there was no evidence for a difference in values obtained by the BP and NIR methods.

5.3.4 Conclusions

In conclusion, it is proposed that the use of NIR spectroscopy with a reflectance vessel as the sample presentation method allows the prediction of Citral content in a series of Lemongrass oil samples with a % mean accuracy (relative difference) of 1% or less, and that this NIR method is comparable in accuracy to the reference method. It would therefore be a suitable method for the determination of Citral content in such oils. The NIR method also has advantages over the BP method in that once the calibration equation has been developed and validated, it is simpler to carry out, no sample preparation is required and it is more rapid. In addition, no other chemicals are required for the NIR method and the amount of sample used is considerably less.

There are few reports on the study of NIR spectroscopy as a suitable method for the determination of constituents in sample matrices at levels as low as the Citral content of Lemon oil. The NIR method is less accurate than the BP titration method for the assay of Lemon oil, although being a simple and rapid technique it could be used to give the approximate Citral content. The % mean accuracy is decreased (and therefore improved) with increase in concentration of the constituent of interest. It is suggested that the limit of quantification (% mean accuracy) for complex essential oils using this NIR method is approximately 10% m/m or above but further work would have to be carried out to support this conclusion.

	Reference method		NIR method			
	Lemongrass oils	**Lemon oils**	**Lemongrass oils**	**Lemon oils**	**Lemongrass oils**	**Lemon oils**
Mean (% m/m)	75.6	4.29	75.8	4.25	75.1	7.32
Standard Deviation (% m/m)	0.27	0.03	0.50	0.10	0.17	0.16
Coefficient of Variation (%)	0.36	0.75	0.23	2.44	0.66	3.66
Confidence Interval (% m/m)	75.3 to 75.8	4.26 to 4.33	74.9 to 75.30	4.14 to 4.36	75.2 to 76.3	4.15 to 4.48
Common values in Confidence Interval?	N/A	N/A	✔	✔	✔	✔

Table 5.3.4 Summary of results for the determination of short-term precision and intermediate precision of the NIR method for a single sample of Lemongrass and Lemon oils

5.4 INVESTIGATION INTO THE POTENTIAL USE OF NIR SPECTROSCOPY FOR THE DETECTION OF ADULTERATION AND CONTAMINATION IN NATURAL PRODUCTS

5.4.1 Introduction

Many natural materials may become contaminated accidentally due to poor storage, labelling of the material and poor control over picking and harvesting conditions and possibly other factors. Deliberate contamination of natural medicinal products may also occur, generally for financial gain, where a lesser quality product is mixed with a small amount of a higher quality product in order to pass certain tests, such as those in the BP monographs or other quality control assays by HPLC, TLC etc. Conversely, a high quality material may be diluted with a lower quality material, such that the material being sold will still pass any quality assays, but financial gain can be made by supplying a cheaper and often inferior product. This concerns the deliberate adulteration of pure Rosemary oil with cheaper Eucalyptus oil for financial gain.

5.4.2 The Detection of Adulteration of Rosemary Oil

It has been suggested that Rosemary oil is often deliberately diluted by wholesale suppliers for financial gain. Rosemary oil contains Cineole at levels of about 20 to 50% and is similar in character to Eucalyptus oil (~ 70% Cineole). Other oils containing Cineole at lower levels such as Spike Lavender and Camphor or a combination of these oils may also be used as diluents, as these are also lower in cost than Rosemary oil. Pure Cineole may also be used to dilute Rosemary oils. Rosemary oil diluted with any of these components will often still pass the traditionally used quality tests and as such it is difficult to determine if a Rosemary oil sample has been contaminated or adulterated. A study of the price lists of eleven different suppliers of essential oils (Chemist and Druggist May 2002) revealed that on average Rosemary oil was 13.5% higher in cost than Eucalyptus oil. Rosemary oil and Eucalyptus oil are especially similar in appearance and smell. A paper entitled "Rosemary oil mistaken for Eucalyptus oil?" (in German, Ihrig *et al* 1990) addresses the

issue from a reverse viewpoint. Samples of an oil accepted as being Eucalyptus oil were submitted to the Central Laboratory of the German pharmaceutical industry on the suspicion that they were in fact Rosemary oils. A TLC technique was developed to detect and identify Borneol , a chemical constituent present in Rosemary oil but not Eucalyptus oil. However, for obvious reasons this method could not be used for the quality control of Rosemary oil, but serves to illustrate the point that these oils are indeed very similar in character and as such pose a problem in terms of their distinction. Techniques such as TLC or GC can be used to determine if a constituent is present that should not be there if different constituents are present in the diluting oils, but often the diluent contains the same constituents (albeit in different amounts) as Rosemary oil. This is true for Eucalyptus oil, which contains many of the same chemical constituents as Rosemary oil. For this reason determination of the Cineole content is often the only way to determine if a sample may have been adulterated by Eucalyptus oil. If the Cineole content is suspiciously high, it suggests that Cineole, Eucalyptus oil or another oil with Cineole present has been used as a diluent/adulterant.

A monograph (no. 1846) has been submitted for adoption to the European Pharmacopoeia Commission for inclusion in the European Pharmacopoeia for both Spanish type Rosemary oil and Moroccan and Tunisian type Rosemary oil (Pharmeuropa 1999, European Pharmacopoeia website 2002). The definition of Rosemary oil is that the oil is obtained by steam distillation of the flowering aerial parts of *Rosmarinus officinalis* L. It is described as a clear, colourless pale yellow liquid with a characteristic, fresh, rustic odour of camphor and Cineole. The identification tests include TLC with reference solutions of Borneol, Bornyl acetate and Cineole. The GC identification test uses several reference solutions which are shown in Table 5.4.1, along with the amount present in the two types of Rosemary oil. Figure 5.4.1 shows the structures of several chemical constituents present in Rosemary and/or Eucalyptus oil.

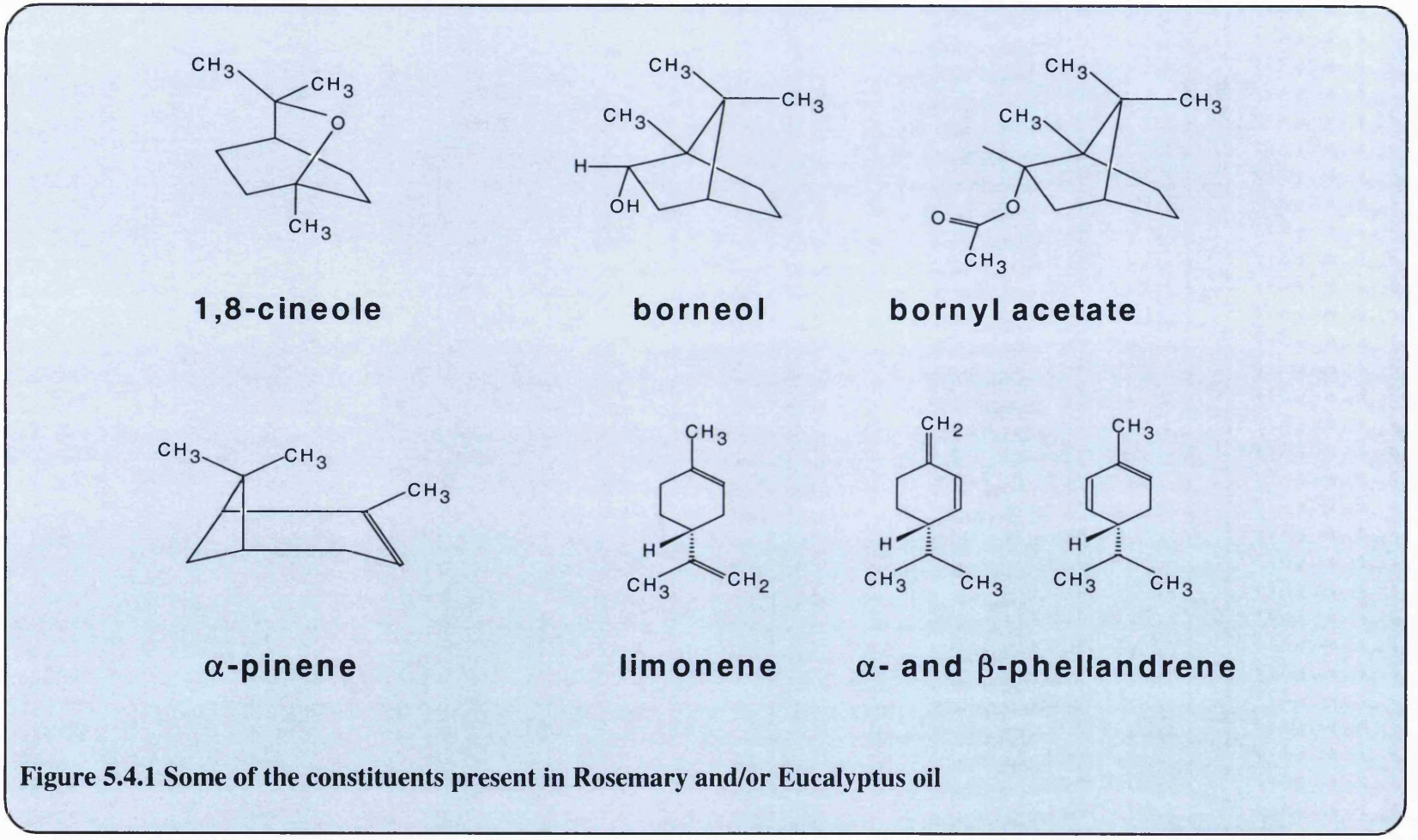


Figure 5.4.1 Some of the constituents present in Rosemary and/or Eucalyptus oil

Constituent	Amount present (% m/m)			
	Spanish type		Moroccan and Tunisian type	
	Minimum	Maximum	Minimum	Maximum
α-pinene	18	26	9	14
camphene	8	12	2.5	6
β-pinene	2	6	4	9
β-myrcene	1.5	5	1	2
limonene	2.5	5	1.5	4
cineole	**16**	**25**	**38**	**55**
p-cymene	1	2.2	0.8	2.5
camphor	13	21	5	15
bornyl acetate	0.5	2.5	0.1	1.5

Table 5.4.1 Percentage composition of chemical constituents in Rosemary oil

The BP and EP monographs for Eucalyptus oil (BP 2001h and EP 2002h) give details of the limits of certain constituents present as determined by Gas Chromatography. These are shown in Table 5.4.2.

Constituent	Specified range (% m/m)
α-pinene	2 to 8
β-pinene	less than 0.5
α-phellandrene	less than 1.5
limonene	4 to 12
1,8-Cineole	not less than 70
camphor	less than 0.1

Table 5.4.2 Limits of chemical constituents for Eucalyptus oil BP/EP

The main common constituent of Rosemary and Eucalyptus oil is Cineole and the two types (the Spanish type and the combined Moroccan and Tunisian type) have distinctly differing ranges of Cineole contents. The range of Cineole content for Rosemary oils of non-BP or EP standard is known to be 40 to 44% for Tunisian type and 17 to 25% for Spanish type Rosemary oil (Tisserand and Balacs 1998b). As will be discussed in more detail, the work being considered here involves the adulteration of Rosemary oil with Eucalyptus oil. The most important question that will have to be answered is whether NIR can be used to identify the presence of Eucalyptus oil (as a whole) in Rosemary oil, or whether it is capable only of identifying the presence of a particular constituent, such as Cineole.

Rosemary is described as being indicated for general debility, hypotension, impotence, asthma and other respiratory disorders, scabies and lice infestations, migraine, vertigo and fainting, among many others. An early reference to Rosemary was the 'elixir of youth' documented in 1310 which was apparently obtained by distilling cedar, Rosemary and turpentine. It used to have the name "Water of the Queen of Hungary" and supposedly to have transformed a paralytic, gout-ridden septuagenarian princess into a seductive girl whose hand was sought in marriage by a king of Poland (Valnet 1986).

5.4.3 Aims of the Work

As this is one of the most common methods of adulteration, the addition of Eucalyptus oil only was studied. Data analysis was carried out in stages, from both a qualitative and quantitative point of view. The purpose of the investigation was to answer the questions listed below.

Qualitative analysis

- Can a method be set up within a Library that will allow the complete distinction between the pure Rosemary and Eucalyptus oils?
- Can this same method be then used to identify the difference between the pure Rosemary oils and the adulterated Rosemary oils and if so, down to what levels of adulteration?
- Which property is best described by the NIR data: the Eucalyptus content of the adulterated Rosemary oil, or is it more specifically the Cineole content of the oil?

- What data analysis methods are most suitable for this purpose?

Quantitative analysis

- Can a calibration be constructed to accurately predict the Eucalyptus oil content of the adulterated Rosemary oils?
- If not, could an alternative calibration be set up to accurately predict the Cineole content of the adulterated samples?
- Can a universal calibration be constructed to predict the Cineole content of both Eucalyptus and Rosemary oil, and others?

Section 5.2 of this thesis describes the creation of a calibration to determine the Cineole content of Eucalyptus oil. It was postulated that it may be possible to use or adapt this calibration to determine the Cineole content of Rosemary oil samples for quality control purposes and detection of possible contaminants. It is important to note however that the investigation did not include the determination of Cineole content of the Rosemary oils used. The use of Polar Co-ordinate plots and two-wavelength plots for visual separation and identification of mixtures and the two oils was also investigated.

5.4.4 Materials and Methods

5.4.4.1 Pure Samples

Ten samples of Rosemary oil and ten samples of Eucalyptus oil were obtained from various health food stores, pharmacies and web-based Aromatherapy oil suppliers. In all cases the oil was marketed as 'pure' and as such, prior to data analysis, each oil was considered to be a pure essential oil.

5.4.4.2 Preparation of Adulterated Rosemary Oil Samples

Mixtures of Rosemary oil and Eucalyptus oil were prepared and subsequently known as the 'adulterated samples'. A single sample of Eucalyptus oil and a single sample of Rosemary oil were randomly chosen for each mix. The reason for this was to include enough variability into an NIR calibration such that it is not too sensitive to different samples of Rosemary oil applied to the calibration. In this way an NIR calibration should

be robust enough to cope with the naturally encountered variations in the oils. Figure 5.4.2 shows the distribution of mixes (0 to 100% Eucalyptus oil in Rosemary oil) prepared for the quantitative work.

5.4.4.3 Instrumentation and Equipment

All measurements were carried out on the FOSS NIRSystems 6500 spectrophotometer with Rapid Content Sampler module. The data acquisition software was NSAS, which was also used to average the spectra. The reflectance vessel, previously used for the Lemon and Lemongrass oil work was employed for presentation of the sample. Spectral data analysis was performed using Vision® and The Unscrambler® software.

5.4.4.4 Near Infrared Measurements

Approximately 2g of each oil was required to produce sufficient depth for the spectral measurements in the reflectance vessel. Each spectrum obtained was the average of 32 scans. Five spectra were obtained, the vessel being rotated about the centre before each spectral measurement. The reflectance vessel was then washed, dried and refilled with the same sample and the procedure repeated until three sets of five scans were obtained. An average of the fifteen spectra obtained for each oil was then used for data analysis. Unless stated elsewhere, all data analysis was carried out using SNV corrected 2nd derivative spectra for all Vision® software analysis. Analysis using The Unscrambler® software was carried out on 2nd derivative spectra.

5.4.5 Results

5.4.5.1 Qualitative Data Analysis

Figures 5.4.3 and 5.4.4 show the raw (untreated) and SNV corrected 2nd derivative absorbance spectra of a pure Rosemary oil sample, a pure Eucalyptus oil sample and three adulterated samples of 25%, 50% and 75% m/m Eucalyptus oil in Rosemary oil. Visual inspection alone shows that the mixtures and the pure oils can be distinguished from each other, with the peaks increasing in size in the 'correct' order in terms of increasing or decreasing Eucalyptus oil content. However, it should also be noted that there are obvious similarities between the pure Rosemary and Eucalyptus oils.

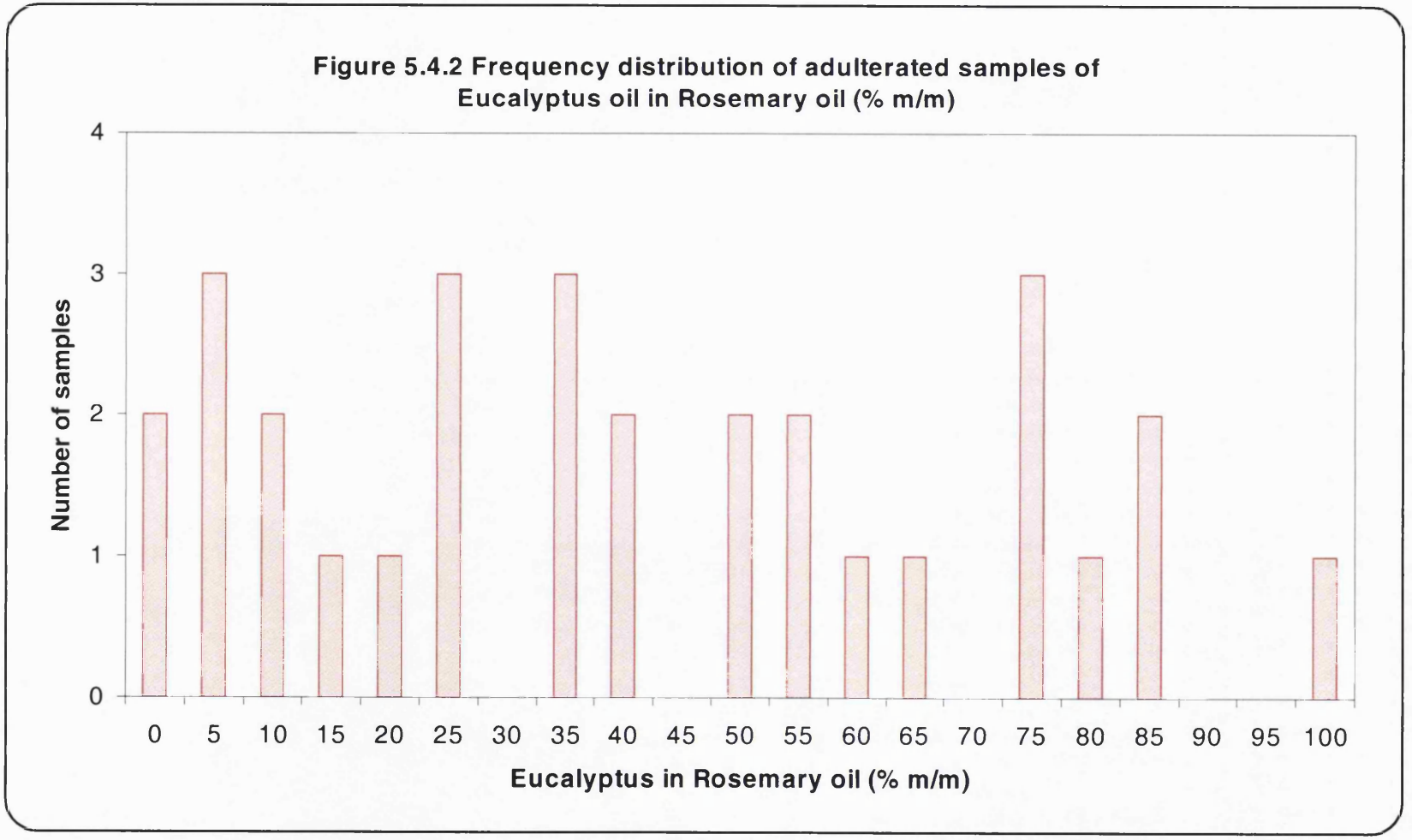


Figure 5.4.2 Frequency distribution of adulterated samples of Eucalyptus oil in Rosemary oil (% m/m)
Number of samples
0
1
2
3
4
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100
Eucalyptus in Rosemary oil (% m/m)

Figure 5.4.3 Raw (untreated) absorbance spectra of pure Rosemary and Eucalyptus oils and mixtures of 25%, 50% and 75% m/m Eucalyptus oil in Rosemary oil

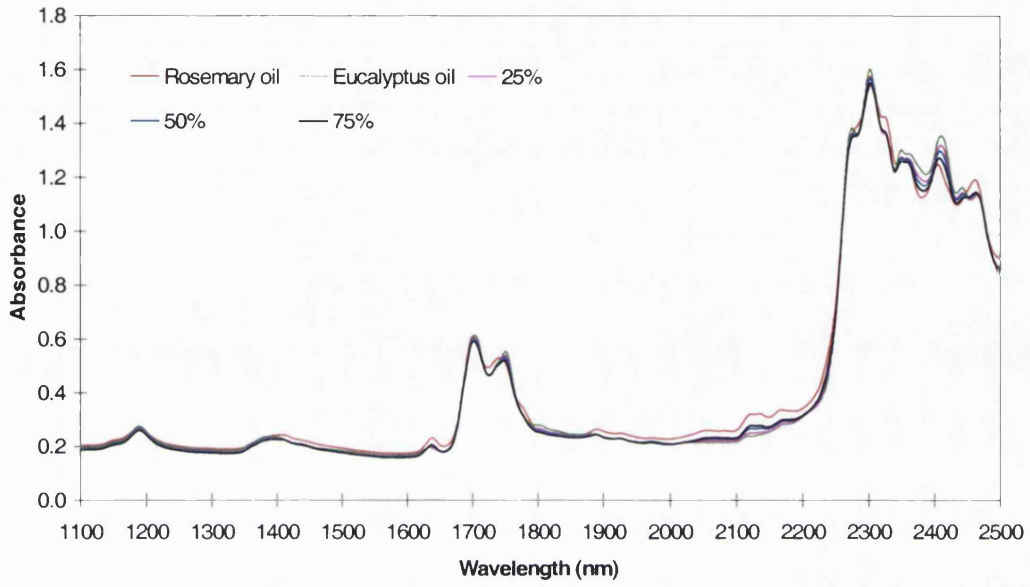
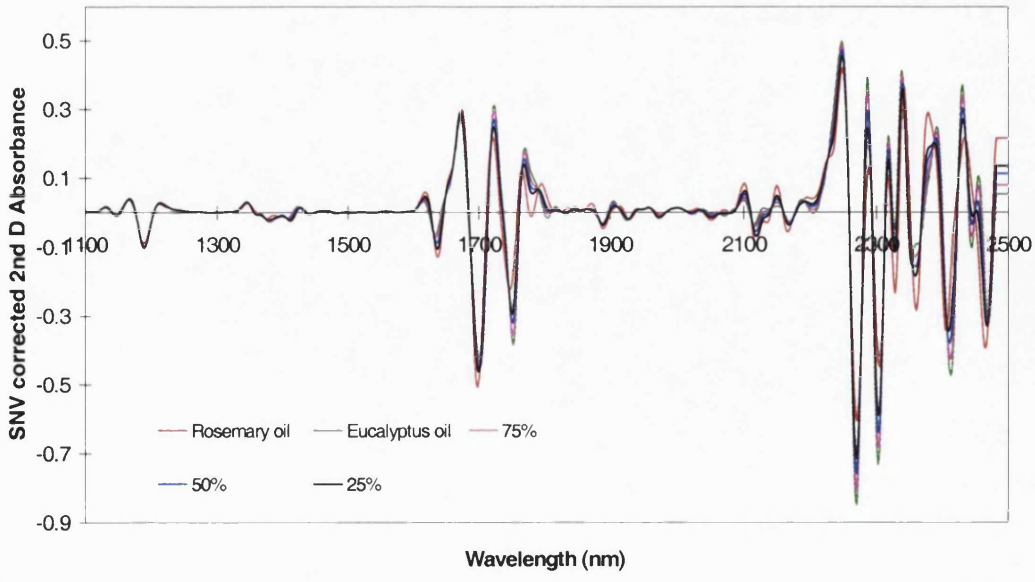


Figure 5.4.4 SNV corrected 2nd derivative spectra of a pure Rosemary and Eucalyptus oils and mixtures of 25%,50% and 75% m/m Eucalyptus oil in Rosemary oil



It is necessary to show that the pure Rosemary oils can be distinguished easily from the pure Eucalyptus oils used in this experiment. If this cannot be done then it is not unreasonable to assume that adulterated samples will not be distinguished from the pure Rosemary oils, or if they can be, that the method used could not be appropriately validated in the future. The Library can be could be constructed in two ways. The first (*Library 1*)is where all the Rosemary oils are in one group (Product) and the Eucalyptus oils are in another. *Library 2* consisted of separate Library Products for each of the ten pure Rosemary oils and the ten pure Eucalyptus oils, giving a total of 20 Products. This second Library would allow any similarities between a single oil and any others to be identified. Two libraries were therefore created.

A CWS Match Value threshold of 0.97 and a MDWS Match Value threshold of 12 either alone or in combination would allow for complete distinction between the two types of oil for *Library 1*.

For both the CWS and MDWS methods used on *Library 2*, the Eucalyptus oils were all closely grouped together and there was a distinct difference between the Match Values for the neighbouring Eucalyptus oils and the Rosemary oils. However, certain of the Rosemary oils were more closely identified to some of the Eucalyptus oils than some of the other Rosemary oils, suggesting that there were distinct differences within the Rosemary oils. An extract of the Library Identification results using the CWS method are shown in Tables 5.4.3 and 5.4.4. The ten Rosemary oils have been retrospectively labelled R1 to R10 and the Eucalyptus oils E1 to E10. This is for the purposes of clarity as these results will be referred to again after the findings from further investigations are shown. Rosemary oil R3 is grouped together with the other nine Rosemary oils and there is a distinct drop in Match Value from 0.9485 for the furthest away Rosemary oil to 0.8686 for the nearest Eucalyptus oil. The terms 'nearest' and 'furthest' refer to how close the Match Values of the oil samples are to the sample being studied. However, with Rosemary sample R10 , all the Eucalyptus oil samples are closer than the Rosemary oil samples R1 to R5.

Product Name	Match Value
R3	1
R4	1
R5	0.992
R2	0.9981
R1	0.9941
R6	0.9759
R7	0.9633
R8	0.9626
R9	0.9527
R10	0.9485
E10	0.8686
E9	0.8677
E8	0.844
E2	0.844
E3	0.8426
E1	0.8412
E7	0.8411
E5	0.839
E4	0.839
E6	0.838

Table 5.4.3 Extract from Library Identification CWS method results for pure Rosemary and Eucalyptus oils as individual Library Products compared against spectra R3a (*Library 2*).

Product Name	Match Value
R10	1
R9	0.9997
R8	0.9976
R7	0.9968
R6	0.9944
E10	0.9741
R9	0.9715
E8	0.9649
E2	0.9649
E3	0.9647
E1	0.9639
E7	0.9638
E5	0.9628
E4	0.9628
E6	0.9623
R5	0.9575
R4	0.9502
R3	0.9489
R2	0.9362
R1	0.9216

Table 5.4.4 Extract from Library Identification CWS method results for pure Rosemary and Eucalyptus oils as individual Library Products compared against spectra R10c (*Library 2*).

This suggests that there may be two distinct groupings of Rosemary oil, although it is not possible from this evidence alone to determine the cause of these differences.

The results for the Library Identification using the MDWS method (data not shown) were compared with the results in Table 5.4.3 obtained using the CWS method. As obtained previously, for Rosemary sample R3 the rest of the Rosemary oils were nearest neighbours followed by the Eucalyptus oil samples. However, for Rosemary oil sample R10 instead of the Eucalyptus oils being sandwiched between groups R1 to R5 and R6 to R10 as obtained previously, all the Rosemary oil samples were nearest neighbours with the Eucalyptus oil samples further away.

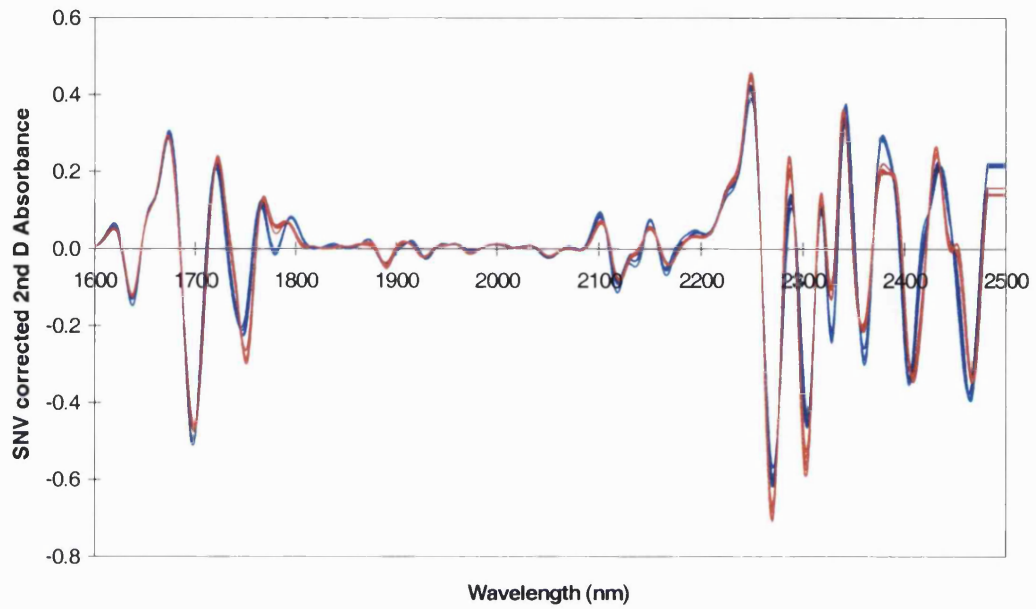
Two distinct groupings of the oil can also be seen by studying the SNV corrected 2nd derivative spectra of the ten Rosemary oil samples (Figure 5.4.5). To investigate this further, PC Analysis on The Unscrambler® software was carried out on the samples.

PC Analysis Results for the Pure Oils

Figure 5.4.6 shows the two distinct groups of Rosemary oils if PC Analysis (The Unscrambler®) is applied to 2nd derivative spectra of the samples. 93% of the difference is explained by the first Principal Component (PC1), whilst the second (PC2) describes 2% of the variability.

PC analysis was also carried out using Vision® software in the form of cluster analysis (see Section 2.1.6). Library clustering allows discrimination between very similar Products. The results are shown in Figure 5.4.7. Each axis represents the first three Principal Components. Again distinct groups of oils can be seen and the six clusters obtained have been labelled C1 to C6. Table 5.4.5 shows the cluster to which the Rosemary and Eucalyptus oils have been assigned.

Figure 5.4.5 SNV corrected 2nd derivative Absorbance spectra showing two groups with distinct differences between the spectra



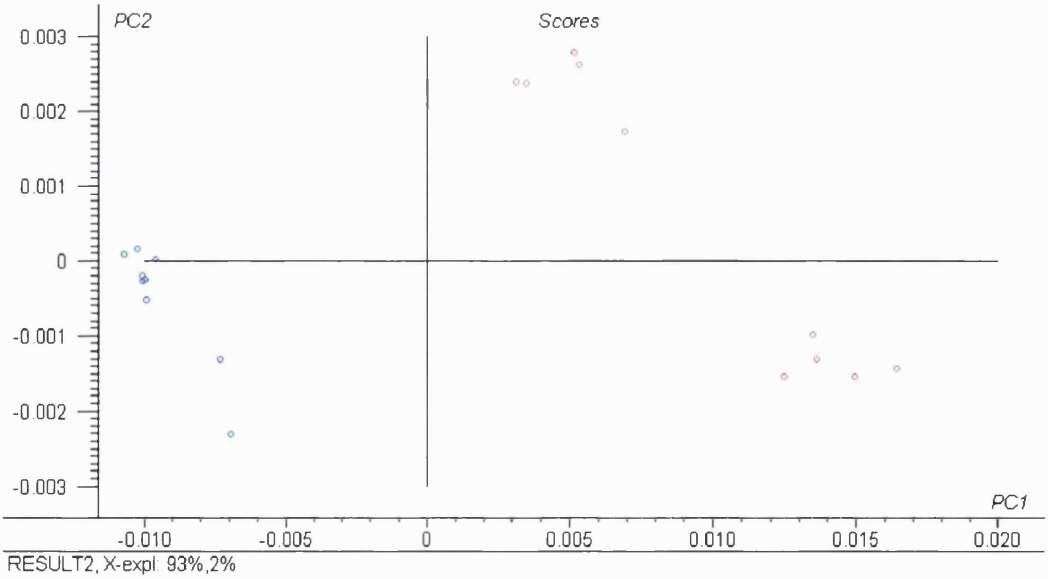


Figure 5.4.6 Principal Component plot (The Unscrambler®) for pure Rosemary and Eucalyptus oils

Cluster	Assigned oils
C1	R1, R2, R3, R4, R5
C2	R6, R7, R8
C3	R9, R10
C4	E9
C5	E10
C6	E1, E2, E3, E4, E5, E6, E7, E8

Table 5.4.5 Assignments of the pure oils to Principal Component cluster analysis results obtained using Vision® software

The nearest clusters containing Rosemary oils to those containing the Eucalyptus oils are those containing samples R6 to R10 and the remaining (furthest away) cluster contains samples R1 to R5. This information corresponds with the Principal Component results obtained using The Unscrambler® software, showing two distinct groups of Rosemary oils; one containing samples R1 to R5 and the other containing samples R6 to R10, with the majority of the variability being described by the first Principal Component.

Prediction of Cineole Content using the Eucalyptus Oil Calibration

It seemed likely that the difference between these two groups of Rosemary oils was due to Cineole content, but further evidence needed to be found to support this conclusion. The next step would be to use the NIR calibration developed for Eucalyptus oils (Section 5.2) to determine the Cineole content (hereafter known as the 'Cineole calibration') to give a predicted Cineole content. The 'Cineole calibration' was created using the BP assay for Cineole (a freezing-point method) as the reference method. It is important to note here that as no reference analysis was carried out to determine the actual Cineole content of these Rosemary oils, then this can be considered only as an unsubstantiated Cineole content. However, the calibration will be able to show the relative difference in Cineole contents of the Rosemary oils and could thus confirm the potential of the hypothesis. The predicted Cineole contents for both the Rosemary and Eucalyptus oils are shown in Table 5.4.6.

Rosemary oils		Eucalyptus oils	
Sample name	**Cineole content**	**Sample name**	**Cineole content**
R1	37.9	E1	74.27
R2	34.53	E2	73.28
R3	34.34	E3	74.84
R4	34.29	E4	73.99
R5	34.59	E5	74.31
R6	48.54	E6	76.05
R7	52.59	E7	75.08
R8	53.33	E8	73.28
R9	53.73	E9	65.53
R10	53	E10	64.09

Table 5.4.6 Predicted Cineole content (% m/m) of the pure Rosemary and Eucalyptus oils using the 'Cineole calibration' developed for the determination of Cineole content of Eucalyptus oils

The Eucalyptus oils were labelled E1 to E10 and the Rosemary oils were labelled R1 to R10 retrospectively. As can be seen, Rosemary oils R6 to R10 have a higher Cineole content than oils R1 to R5. These Cineole content values correlate with the results obtained from the Library Identification methods and the Principal Component analyses. Referring back to Table 5.4.3 showing the CWS Library Identification results for sample R3 it can be seen that Eucalyptus oil sample E10 which has the lowest Cineole content is the nearest Eucalyptus oil sample to Rosemary oil sample R3 whilst sample E6 which has the highest Cineole content is the furthest away. Similarly, when comparing to Rosemary oil sample R10 (Table 5.4.4) samples R6 to R9 which are the nearest neighbours are those samples that are in the group with the higher Cineole content, explaining why the Eucalyptus oil samples are the next nearest neighbours followed by Rosemary oil samples R1 to R5 which form the low Cineole content group.

The two distinct groups of Rosemary oils from PC analysis as seen in Figure 5.4.6 correspond to groups R1 to R5 and R6 to R10. As postulated, the PC1 describing 93% of the variation is highly likely to be due to the Cineole content of the oils.
On re-inspection of the Rosemary oil samples, their packaging and price it was noted that of the R1 to R5 oils with the lower Cineole content, three were marketed as organic oils and all five were higher in cost than the second group of oils. It is suggested, therefore, that the value of Rosemary oil (in terms of retail price) inversely correlated to the Cineole content and may well be correlated with a constituent present in Rosemary oil such as Borneol. These difference may be due to the species difference, as opposed to being organic with the Spanish oils having the lower Cineole content and therefore being the higher priced (and better quality) oils.

Testing the Hypotheses that CWS Match Values are Correlated to Cineole content

From the observations made concerning the correlation between the CWS Match Values and the predicted Cineole content using the 'Cineole calibration' from Tables 5.4.3 and 5.4.4, the next step would be to test this theory. Figure 5.4.8 shows a plot of CWS Match Value against predicted Cineole content for the Eucalyptus oils and the Rosemary oils, along with the related equations for the lines of best-fit. The results suggest that either the

CWS method is correlated with the Cineole content (as the lines are almost parallel) but that there is a bias or that a bias is present in the 'Cineole calibration' for the prediction of Cineole content in Rosemary oil. Bearing in mind the Cineole contents for the Rosemary oils are merely predicted and not necessarily accurate, but that the Eucalyptus oil contents are almost certainly accurate (the 'Cineole calibration' has been validated for Eucalyptus oils) it is a possibility that the actual Cineole contents of the Rosemary oils are lower than those predicted but in direct proportion with them. This is borne out by the fact that if the Cineole content of the Rosemary oils is calculated from the line equation for the Eucalyptus oils, a plot of these two sets of Cineole contents gives a correlation R^2 of 0.922. However, it should also be borne in that as a single wavelength MLR calibration was used (1756nm) it is possible that the presence of constituents other than Cineole (Figure 5.4.1) may interfere at this wavelength . If this occurs then it is unlikely that this calibration model could be corrected for bias and/or skew for use with Rosemary oils, rather than an alternative wavelength(s) should be used or an alternative method such as PLSR. Although it is not possible to definitively say if either CWS or the 'Cineole calibration' can be used to accurately predict Cineole content of Rosemary oils it is evident that Cineole content contributes significantly to the differences shown by several techniques (PC cluster method and PC analysis which correlate with the CWS Identification method and the 'Cineole calibration') between Rosemary and Eucalyptus oils. The MDWS Match Values were also plotted against Cineole content (data not shown), but no correlation was found between the two factors (R^2 is 0.50).

Use of CWS and MDWS Methods for the Qualitative Analysis of the Adulterated Rosemary oils

Having characterised the pure Rosemary and Eucalyptus oils used in this study, the adulterated Rosemary oil samples were then considered. A Library was constructed in the way that it would be constructed if in commercial use; that is, an 'unknown' incoming sample would be compared against a Library Product containing pure Rosemary oils.

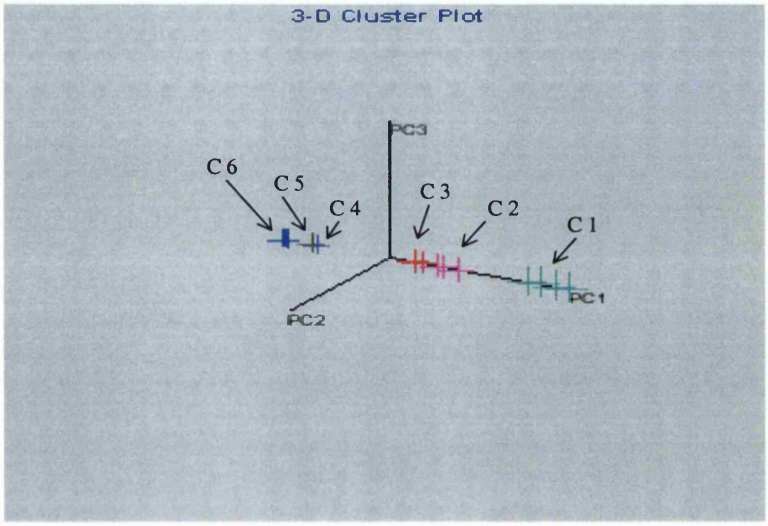
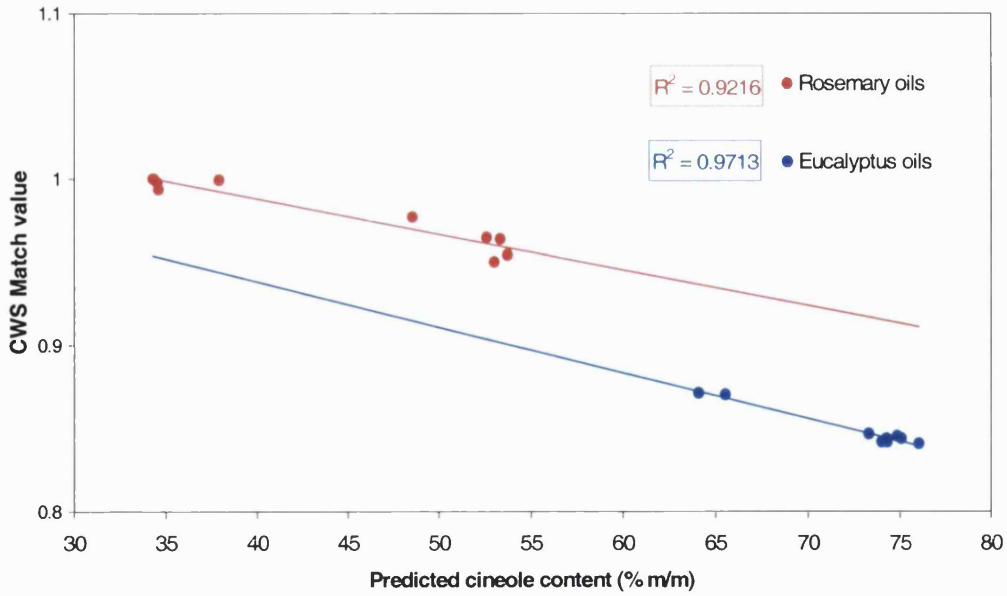


Figure 5.4.7 Principal Component cluster plot (Vision®) for pure Rosemary and Eucalyptus oils

Figure 5.4.8 Plot of Correlation in Wavelength Space Match Value against predicted Cineole content for pure Rosemary and Eucalyptus oils



As mentioned in the Introduction to Data Analysis chapter, a minimum of three spectra need to be present in a Library Product before spectral comparisons can be made with other samples. Thus, an average of each of the three sets of five spectra obtained for each oil was used to create its corresponding Library Product. The adulterated samples were compared to each other and a single Library Product consisting of all ten of the pure Rosemary oil spectra. Thus, when comparing the 'unknown' sample Product (consisting of three spectra) to the Rosemary oils group there will be three Match Values generated. An average of these three results for each adulterated sample was calculated and plotted against its Eucalyptus oil content.

Figures 5.4.9 and 5.4.10 show the results using the CWS and MDWS methods on SNV corrected 2nd derivative spectra respectively. It must be remembered that the purpose of creating such a Library is to be able to determine a threshold value that could be set such that any Rosemary oil would be above the threshold for a MDWS method or below for a CWS method, in other words, the 'unknown' adulterated Product would not be co-identified with the Rosemary oil Product. The default threshold probability value for the CWS method as suggested by the Vision® software is 0.85; the higher the threshold value that has to be used to give complete discrimination the more similar the samples are. As can be seen from Figure 5.4.9, even the mixtures with the highest Eucalyptus oil content has a probability value of at least 0.92. It has been established in the previous section that CWS is likely to correlated directly with the Cineole content in a sample (Figure 5.4.8) and this is why the information shown in Figure 5.4.9 shows a poor correlation between Match Value and Eucalyptus oil content. However, the high correlation (R^2=0.96 for samples over 18% m/m) between MDWS and Eucalyptus oil content (Figure 5.4.10) suggests that this method could be used to detect Eucalyptus oil content. However, the default threshold Match Value for the MDWS method in the Vision® software is 4 and only samples of above approximately 30% m/m Eucalyptus oil in Rosemary oil gives Match Values above 4. This therefore limits the usefulness of this method for the detection of adulteration of Rosemary oil with Eucalyptus oil. As it has been suggested earlier, it is possible that Cineole content would be a better marker for adulteration.

Figure 5.4.9 Plot of Correlation in Wavelength Space Match Value against Eucalyptus oil content for the adulterated mixtures

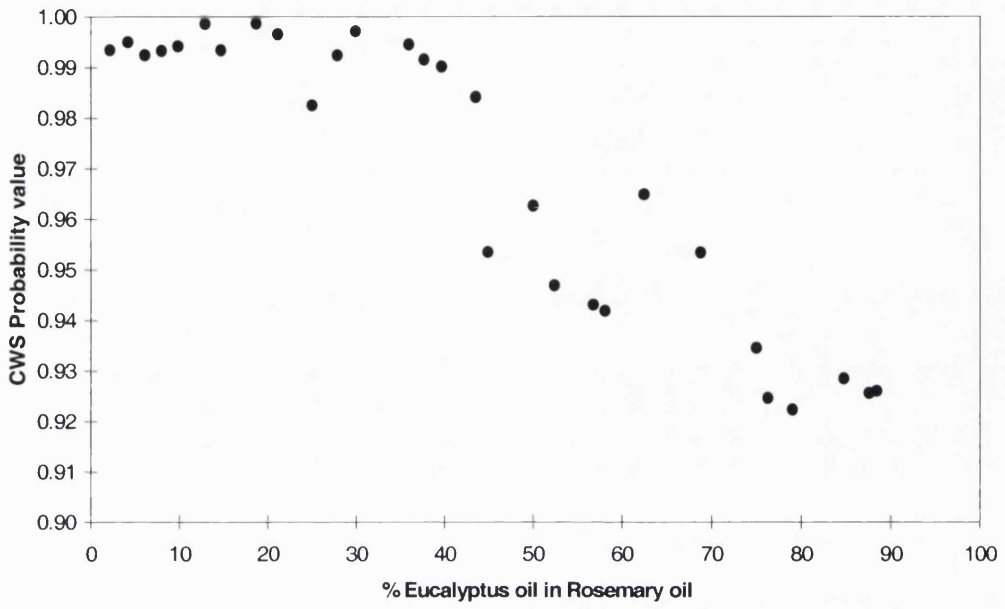
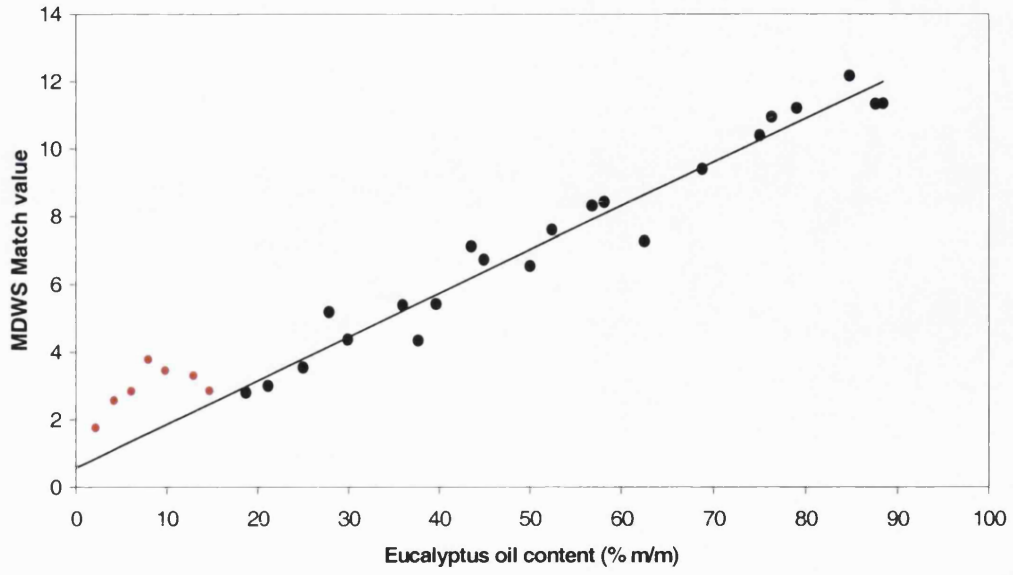


Figure 5.4.10 Plot of Maximum Distance in Wavelength Space Match Value against Eucalyptus oil content for the adulterated mixtures (the samples not included in the linear regression are shown in red)



5.4.5.2 Quantitative analysis of the adulterated Rosemary oil samples

It is not possible to construct a quantitative MLR calibration for the adulterated Rosemary oil samples in terms of Cineole content as these are only predicted values and therefore the results obtained would be meaningless.

Two MLR equations were set up using the Eucalyptus oil content as the reference value. The first one contained samples over 10%, with a total of 26 samples, of which 18 were in the calibration set and 8 were in the validation set. The second MLR equation contained samples over 30%, with a total of 20 samples, of which 12 were in the calibration set and 8 were in the validation set. It is acknowledged that only a limited amount of information can be gathered from these MLR calibrations due to the low number of samples present. However, they are still useful as a feasability study and can indicate if it would be possible in the future to create an accurate NIR calibration for the detection of Eucalyptus oil in Rosemary oil. Samples containing less than 10% Eucalyptus oil were not included in the calibrations as the existing evidence suggested that these would be predicted very poorly. Table 5.4.7 summarises the results for the two equations. A second divisor wavelength was added, as opposed to a second wavelength in summation as this provided the lowest Standard Error for the validation set, with a corresponding increase in F value. It can be seen that for both NIR calibrations the same two wavelengths were chosen (by the software). In addition, the removal of the samples containing 10 to 30% Eucalyptus oil lowered the SEC and the SEP. It is not immediately clear from inspection of the spectra why the these two wavelengths have been selected. However, at 2236nm (which may correspond to a C-H stretch/C=O stretch combination absorption band) the three spectra of Rosemary oil, Eucalyptus oil and Cineole are positive with Cineole having the highest value followed by Eucalyptus oil then Rosemary oil. This suggests that something other than Cineole present in either oil produced an interfering band, which may be Borneol as it has a CHO structure (see Figure 5.4.1). The 1126nm wavelength is at the very lower limit of the NIR detection region and it is not clear why this particular wavelength was chosen. A second approach was taken whereby a wavelength was chosen manually from visual inspection of the NIR (SNV 2ndD) spectra (Figure 5.4.11). At 2360nm there is no absorbance due to Cineole (it is zero) and the other two oils are approaching the negative peak with Rosemary oil having the greatest (negative) value. As the F value increased for

both calibrations, a second wavelength was added to the calibration as a summation term. For both calibrations this term was 2236nm, which is the same wavelength selected originally for both the NIR calibration equations. A summary of these results are shown in Table 5.4.8 which can be compared directly with Table 5.4.7. The SEC and the SEP were improved for both NIR calibrations in both the calibration and validation sets. It is likely that the improvement of the NIR calibrations was due to the fact that the wavelengths selected do not have Cineole absorbing at this point and the calibration is modelling the presence of something else, possibly Borneol. A plot of predicted against actual Eucalyptus oil content for both the >10% and the >30% NIR calibrations is shown in Figure 5.4.12. Figures 5.4.13 and 5.4.14 show the % Relative Errors for the individual samples. All % Relative Error values are less than 8% for the samples >30% Eucalyptus oil content.

Principal Component Analysis of the Adulterated Rosemary Oils

Figure 5.4.15 shows the same plot as given in Figure 5.4.6 with the addition of the adulterated Rosemary samples. It can be seen that PC1 describes well the increase in Eucalyptus oil content moving from right to left, however it is not known the exact property that is responsible for the information described by PC1. It is noted that the R1 to R5 adulterated samples extend towards the pure R1 to R5 Rosemary oils and similarly the R6 to R10 adulterated samples extend towards the pure R6 to R10 Rosemary oils. The fact that the Eucalyptus oils are a negative number suggest that PC1 may be describing the presence of something in the Rosemary oil (possibly Borneol) as opposed to the Eucalyptus oil or Cineole content of the samples. However, plots of the first Principal Component value against Eucalyptus oil content and Cineole content are shown in Figures 5.4.16 and 5.4.17 and the high correlation obtained for Figure 5.4.17 (R^2=0.973) indicates that it is likely PC1 is modelling the Cineole content in the samples, and it is this which is responsible for the majority of the variation in the samples. It is possible that the amount of Cineole present in the samples is inversely proportional to another constituent present, explaining the negative Principal Component value seen in Figure 5.4.15, yet the high correlation with the Cineole content (Figure 5.4.17).

Figure 5.4.11 SNV corrected 2nd derivative spectra of pure Rosemary and Eucalyptus oil and Cineole over the wavelength region 2200nm to 2400nm

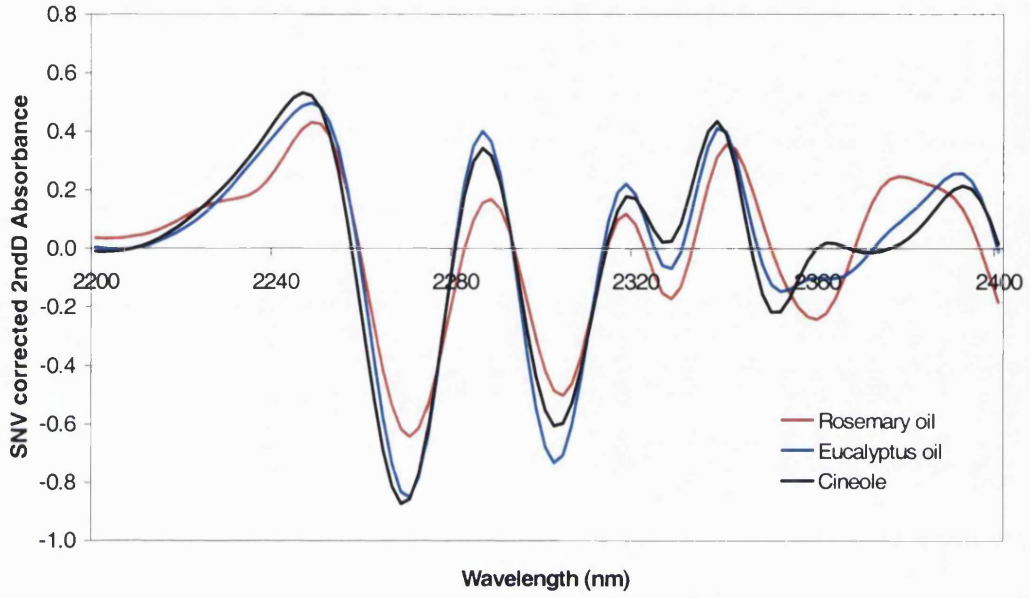
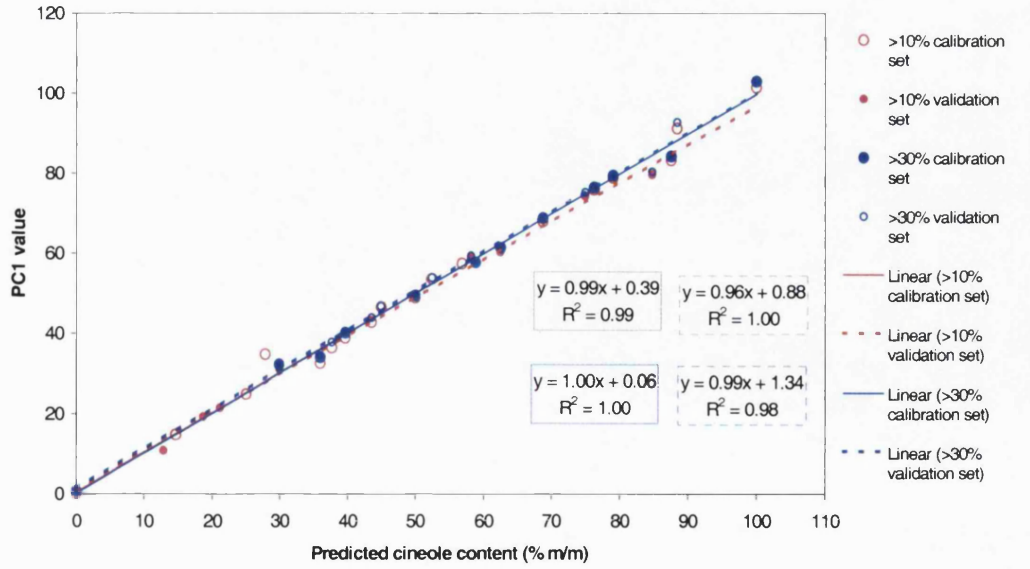


Figure 5.4.12 Plot of predicted against actual Eucalyptus oil content for the adulterated mixtures for both MLR calibrations (>10% and >30%) including a fixed wavelength of 2360nm



	Eucalyptus oil content in Rosemary oil (% m/m)			
	10 to 100%		30 to 100%	
	Calibration set	Validation set	Calibration set	Validation set
Correlation (R^2)	0.9887	0.9948	0.9895	0.9927
Wavelength(s) (nm)	2236 / 1126		2236 / 1126	
SEC / SEC (% m/m)	2.99	2.62	3.01	1.66
F value	1400		939	
% mean accuracy	4.61	8.8	3.47	2.5

Table 5.4.7 Calibration statistics for the MLR equations constructed for the Eucalyptus oil content of the adulterated Rosemary oil samples

	Eucalyptus oil content in Rosemary oil (% m/m)			
	10 to 100%		30 to 100%	
	Calibration set	Validation set	Calibration set	Validation set
Correlation (R^2)	0.9923	0.9955	0.9963	0.9847
Wavelength(s) (nm)	2360 + 2236		2360 + 2236	
SEC / SEC (% m/m)	2.55	2.21	1.89	2.35
F value	968		1199	
% mean accuracy	3.79	4.6	2.35	2.44

Table 5.4.8 Calibration statistics for the MLR equations constructed for the Eucalyptus oil content of the adulterated Rosemary oil samples using a first fixed wavelength of 2360nm

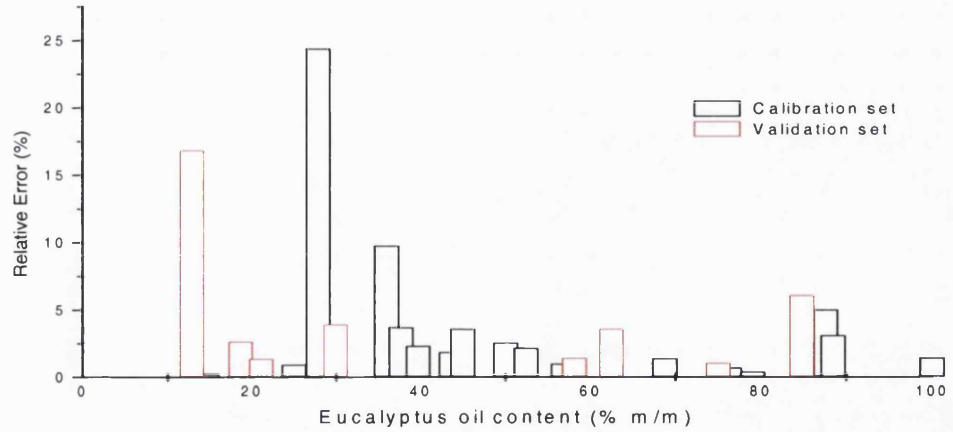


Figure 5.4.13 Plot of % Relative Error against Eucalyptus oil content for the individual adulterated samples in the >10% MLR calibration including a fixed wavelength of 2360nm

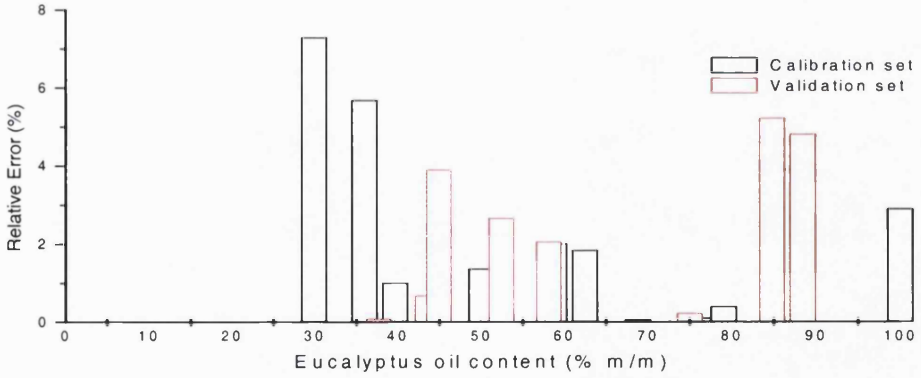


Figure 5.4.14 Plot of % Relative Error against Eucalyptus oil content for the individual adulterated samples in the >30% MLR calibration including a fixed wavelength of 2360nm

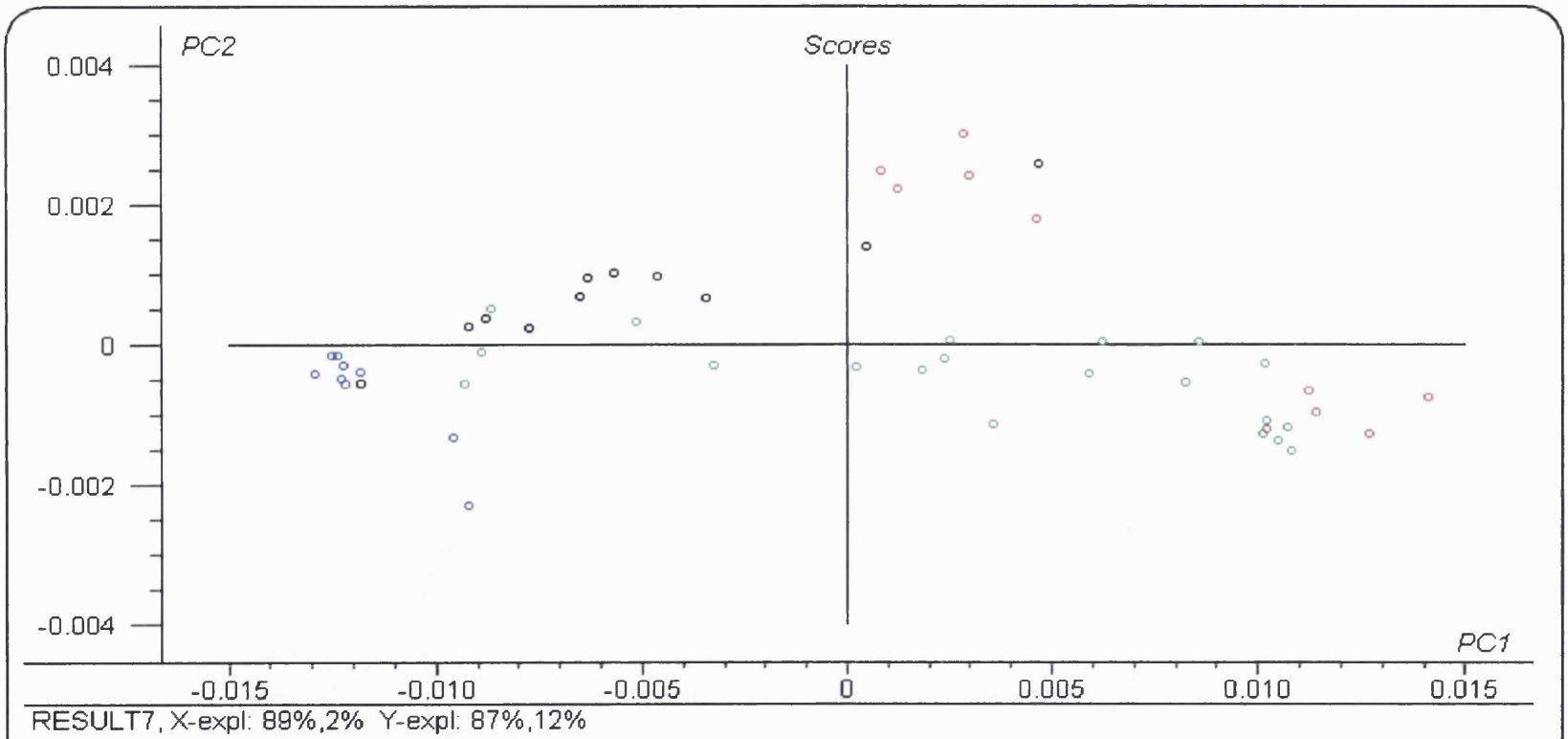


Figure 5.4.15 Principal Component plot for 2nd derivative spectra of the pure Rosemary (red), Eucalyptus (blue) oils and the adulterated samples comprising Rosemary oils R1 to R5 (green) and R6 to R10 (black)

Figure 5.4.16 Plot of Principal Component 1 values against Eucalyptus oil content for pure Eucalyptus and Rosemary oils and the adulterated samples

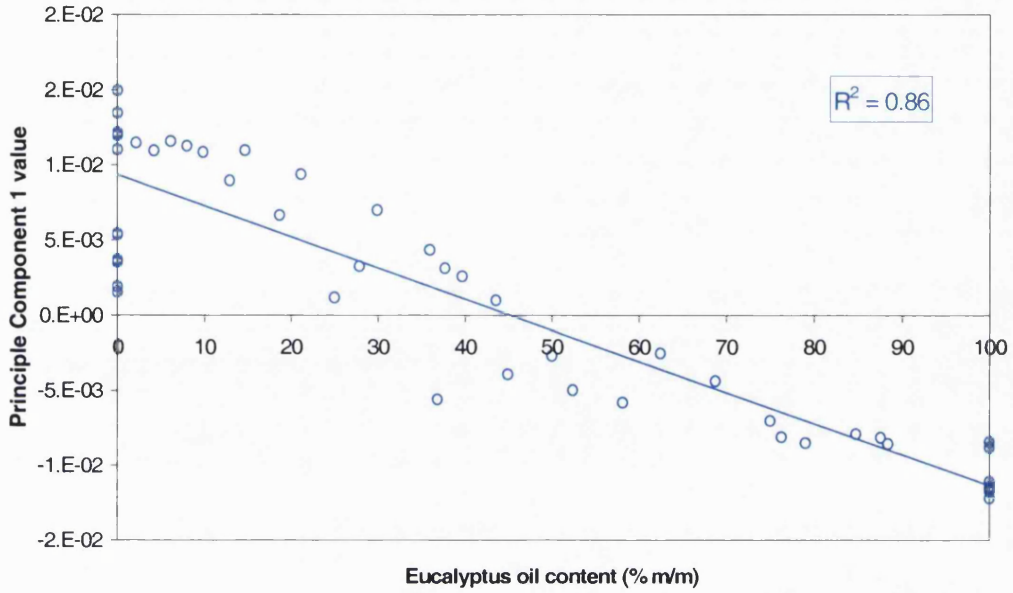
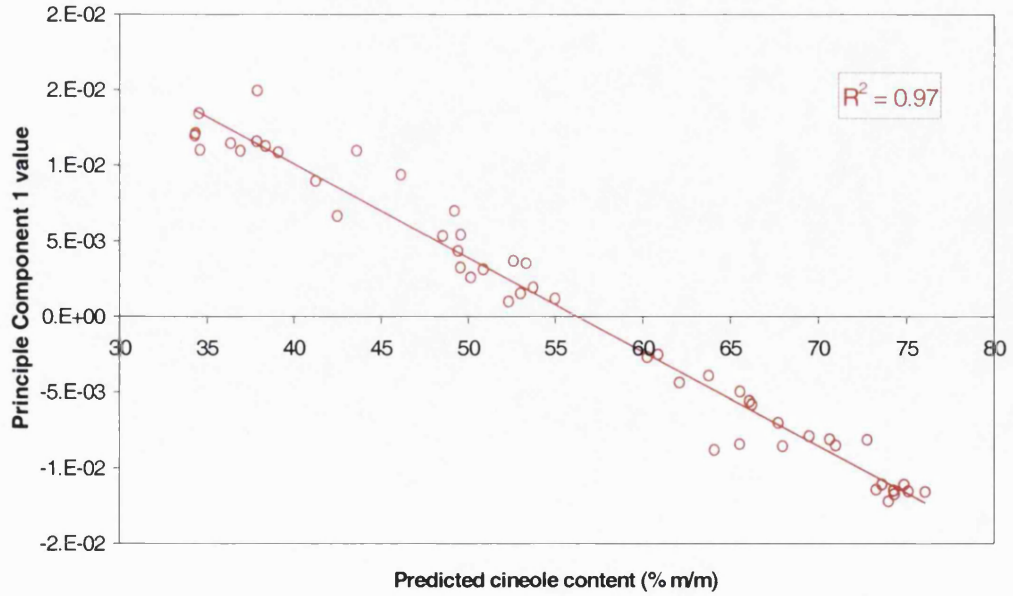


Figure 5.4.17 Plot of Principal Component 1 values against predicted Cineole content for pure Rosemary and Eucalyptus oils and the adulterated samples



Polar Co-ordinate Plots

As used before for the identification of different types of essential oil, Polar Co-ordinate plots were created to look at the data for the pure Rosemary and Eucalyptus oils visually. Previous experience has shown that the 2200nm to 2400nm wavelength region often provides the most useful visual interpretation of the spectra and this is shown in Figure 5.4.18. Little information can be gathered from this plot and there is no distinct differentiation between the two types of oil along the x-direction and a small but distinct split along y-axis. A second wavelength range of 1600nm to 1800nm was tried and the results are shown in Figure 5.4.19. Interestingly, although there is no differentiation between the two types of oil the R6 to R10 Rosemary oils are grouped alongside the Eucalyptus oils. This supports the previous data that these five oils have a considerably higher Cineole content than the oils R1 to R5.

These plots are much simpler than the Principal Component plots but can supply useful information as seen for the pure essential oils work (Figure 5.1.5). In this case, unless a more appropriate wavelength range can be found, it seems that PC Analysis is more useful.

Two-wavelength plots

From visual inspection of the NIR spectra, a two-wavelength plot of SNV corrected 2nd D absorbance at 2214nm over that at 1644nm was chosen. At these two wavelengths there is separation between the two types of oil and all oils of the same type are grouped closely together. The results are shown in Figure 5.4.20. Upon initial inspection it can be seen that not only are the Rosemary oils clearly separated from the Eucalyptus oils, but the adulterated samples form points linking the two sets of oils. At levels of 10% Eucalyptus oil content and below, however, the position of the mixture on the plot heavily depends on the specific Rosemary oil sample used to prepare the mixture. This can also be seen by the Rosemary oil sample, R2, which is separated from the rest of the cluster. C9.81, C7.96 and C6.10 were prepared using this sample, explaining their position away from the rest of the cluster. This two-wavelength plot illustrates nicely the advantages and disadvantages of using this type of plot in qualitative data analysis.

Figure 5.4.18 Polar Co-ordinate plot over the wavelength range 2200nm-2400nm on SNV corrected 2ndD spectra of pure Rosemary and Eucalyptus oils

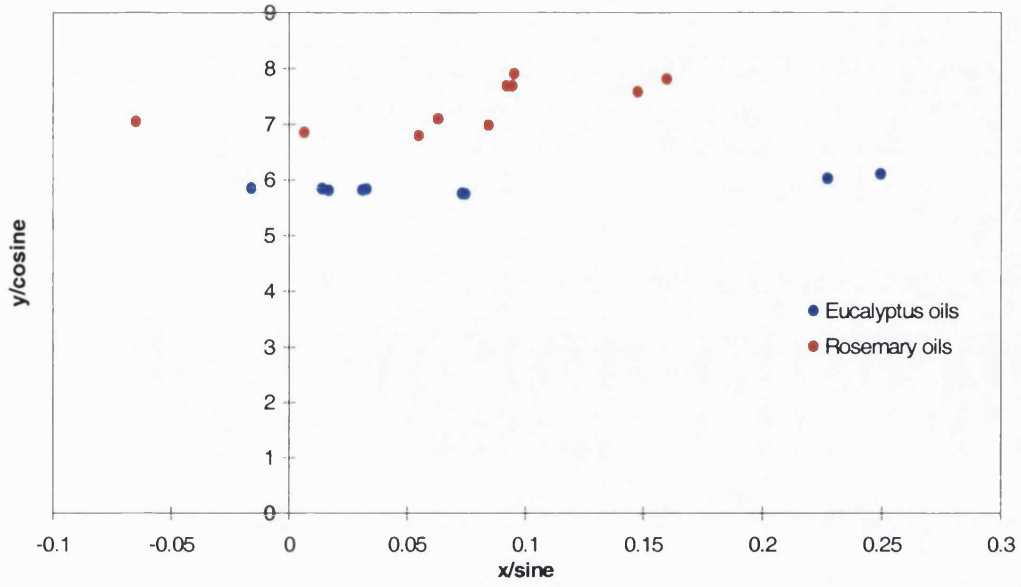
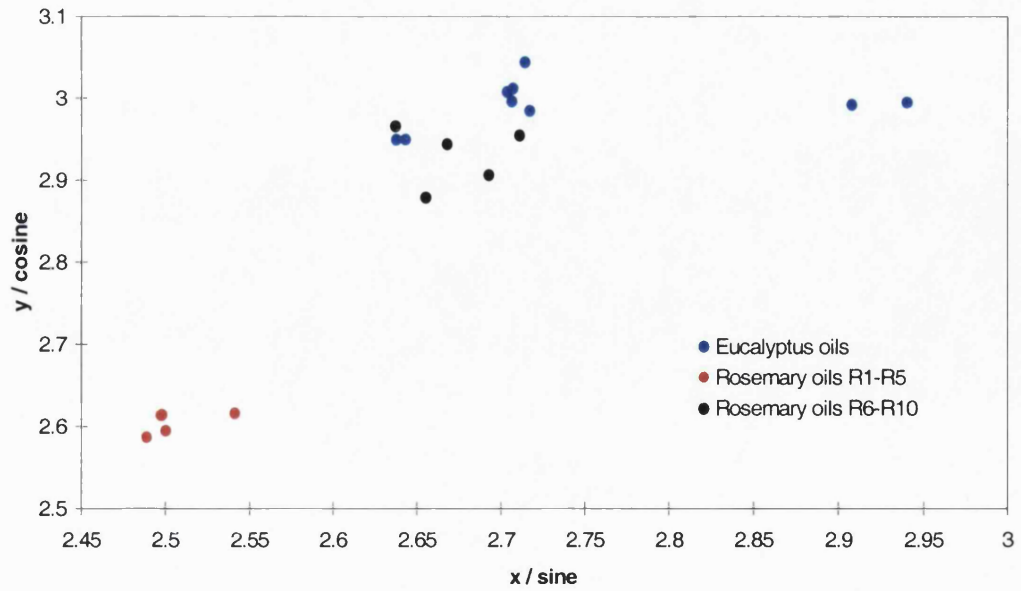


Figure 5.4.19 Polar Co-ordinate plot over the wavelength range 1600nm to 1800nm on SNV corrected 2nd D spectra or pure Rosemary and Eucalyptus oils



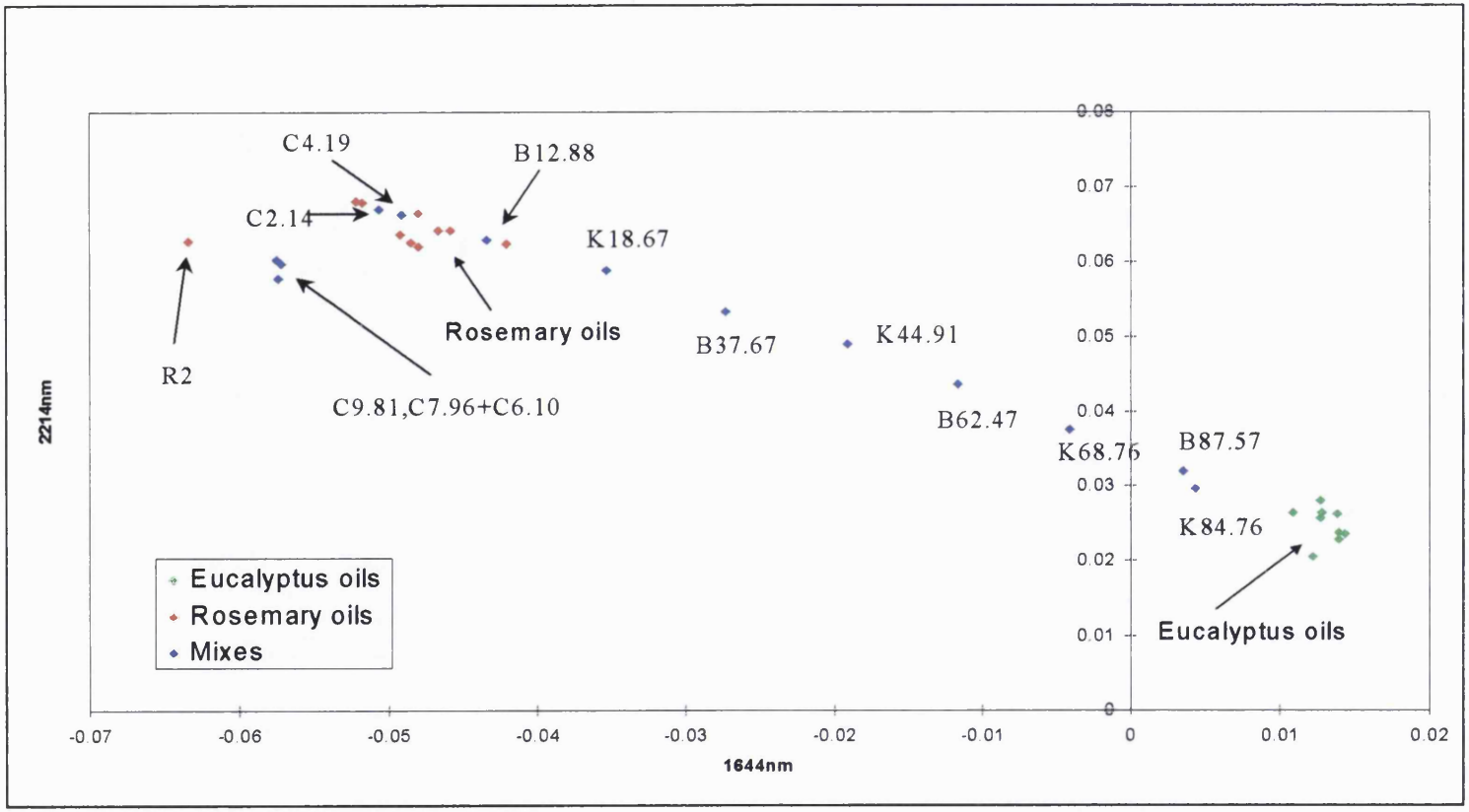


Figure 5.4.20 Two wavelength plot at 2214nm and 1644nm for the pure Rosemary and Eucalyptus oils and a selection of the adulterated samples

Although trends can be determined easily and could be used as part of the quality control of such materials, a small change in the NIR spectrum of a sample will result in a visual difference in the resulting point on the plot and this must be taken into account. Conversely, whilst a Polar Co-ordinate plot will be far less susceptible to changes in the NIR spectrum, trends between types of oil and between samples and mixtures are less likely to be detected and the plot could be considered to be too robust.

5.4.6 Conclusions

Several methods of data analysis have been employed to determine how appropriate NIR spectroscopy is as a technique for the detection of adulteration of Rosemary oil. The following main conclusions can be drawn.

- The results suggest that there are two distinct groups of Rosemary oil which differ in Cineole content, with the lower Cineole content oils likely to be Spanish Rosemary oil and the higher Cineole content samples Moroccan or Tunisian type oils. The species type may be responsible for the differences in value of the oils and their corresponding quality.

- The majority of the variation between Rosemary oil and Eucalyptus oil and the sample mixtures is most likely due to the change in Cineole content, as a high correlation is achieved between the first Principal Component and the Cineole content. However, the negative value for the Eucalyptus oils may mean that it is accounting for a constituent other than Cineole which is present in proportional amount to Cineole.

- The Library Identification method CWS correlates highly with Cineole content, whilst the MDWS method appears to model more closely the Eucalyptus oil content.

- Use of the CWS method in combination with the Cineole contents predicted by the 'Cineole calibration' suggest that the calibration could be used to accurately predict the Cineole content of Rosemary oils, but that there is a bias in the equation which would need to be corrected for. Use of Rosemary oils with known Cineole contents would have to be applied to the 'Cineole calibration' in order to test this hypothesis.

- The detection and quantification of Eucalyptus oil as a whole present in Rosemary oil is more difficult that the quantification of Cineole content alone. The NIR calibrations constructed suggest that it may be possible to predict to within an acceptable level adulterated Rosemary oil samples with a Eucalyptus content greater than 30%. Addition of more adulterated samples to the calibration set should improve the NIR calibration. The MDWS Identification method could also be used in combination with a NIR calibration, as Match Values also give a reasonable degree of prediction for levels above 30% Eucalyptus content.

- If the difference in the two groups of Rosemary oil is due to species type then it would be possible to create separate NIR calibrations for the two species of oil, as either a Principal Component method or a Library identification method could be used to determine the Rosemary oil type before quantitative analysis was carried out. This would result in more accurate (yet specific) calibrations for Eucalyptus oil content.

CONCLUSIONS

CONCLUSIONS

The work carried out in this thesis has shown the potential of NIR spectroscopy for the characterisation and quality control of pharmaceutical materials of natural origin. It has highlighted factors that need to be overcome or minimised associated with the use of this method for herbal materials which, being subject to far fewer controls than those materials used in the pharmaceutical industry, exhibit a great variation even between batches of the same plant material. The factors causing variation include differences in variety, geographical origin and growing, harvesting and storage conditions. In addition, sampling problems, homogeneity and repeatability of spectral data pose the main problems for solid samples. The work on essential oils demonstrates that NIR spectroscopy is well suited for the analysis of natural remedies in liquid form.

Detection and quantification limits of mixtures of similar materials are indicated, such as seen with the Fennel adulterated with Hemlock and the mixtures of Valerian and Cinchona bark with microcrystalline cellulose. It has also been shown that water content can be accurately quantified in Agar samples of several different species and origins using a single NIR calibration. NIR spectroscopy has been shown to be an ideal tool for quality control of essential oils as it has been demonstrated that identification, characterisation and quality control in terms of quantification of certain excipients. It appears from the work carried out here that essential oil constituents present at levels in excess of 10% m/m can be accurately quantified and constituents present at levels of 5% or over can be quantified less accurately but nevertheless at a level useful for the quality control of such materials, although this will depend on the number and presence of other constituents.

The use of the simple method of MLR for the construction of quantitative NIR calibrations proved to be as useful in many cases as other more complicated methods of data analysis such as PLSR and Principal Component Regression. It has been shown that simple two-wavelength plots can provide information complementary to that provided by PC Analysis. The use of the CWS method in qualitative analysis, whilst less effective at distinguishing between materials similar in character is useful for determining possible correlations between constituents, whilst the MDWS method is effective at distinguishing between very similar materials, such as members of the same plant family. The use of several data

analysis methods has demonstrated the need for these techniques to be used in combination with others to give a fuller picture and to allow optimisation of the NIR method. While lessons can be learned from the work here and it has done much to promote the use of NIR spectroscopy and demonstrate its potential, assessment of the suitability of application of NIR spectroscopy to a particular problem is entirely empirical, in that although previous experience with similar materials may be useful in determining the probability that a successful qualitative model or quantitative model can be made, it is not until an initial feasibility study on the exact materials of interest has been carried out that a proper assessment can be made.

Further work that could be carried out that is directly related to the work carried out in the thesis include the creation of a calibration for Cineole content in essential oils other than Eucalyptus oil and further investigation into the limits of detection and quantification of both solid and liquid natural pharmaceutical samples when present as mixtures.
Other areas which are likely to benefit from the use of NIR spectroscopy as a quality control tool include the setting up of a database for Chinese and Ayervedic plant medicinal materials which would allow for rapid identification of a sample, as mis-identification or substitution of materials has been known to occur. In Belgium and France, the substitution of *Aristolochia fangchi* root in formulations as a substitute for *Stephania tetrandra* resulted in over 100 cases irreversible nephropathy. In Chinese herbal medicine, substitution of one plant for another is commonplace. In addition, the adulteration of herbal remedies with synthetic drugs such as antidiabetics and steroids or the contamination with heavy metals is a great cause for concern (Barnes *et al* 2002c and MCA website 2002b). The likely introduction of tighter regulations concerning natural herbal remedies reinforces the need for a rapid and easy-to-use technique. NIR spectroscopy fills a gap in the analytical lab as techniques such as HPLC and GC are separative methods and do not concern a sample as a whole. In addition, a single spectrum could be used to identify and qualify a material and quantify one or more of its constituents. It is also possible that the use of NIR spectroscopy may prove useful in the quality control of cannabis plant material and even possible quantification of constituents such as Tetrahydrocannabinol (THC) and Cannabidiol (CBD).

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