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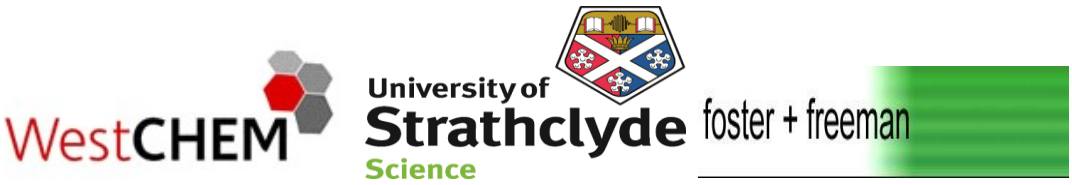

Multivariate Profiling of Gel Inks:

A Novel Tool for the Discrimination of Within and Between Brand Variation

By

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This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

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Abstract

The gel ink pen is the fastest growing pen class available on the modern market. Consequently, its prevalence in forensic casework is expected to increase. This poses a challenge to forensic scientists, since the chemistry of gel inks differ to other commonly encountered inks, thus discrimination by traditional methods like Thin Layer Chromatography (TLC) is limited and a new analytical methodology is required for distinguishing different formulations effectively. A study to evaluate discriminating potential of modern spectroscopic techniques alongside traditional methods of ink examination for within and between brand variations of two commonly encountered colours was undertaken. Within brand variation was detected between multiple samples of a limited number of inks using filtered light, FTIR-ATR and specifically Raman Spectroscopy. For 31 blue gel inks, a discriminating power of at least 0.93 was achieved using a combined analytical sequence of TLC, filtered light (IR absorption and IR fluorescence), Vis-MSP (transmittance), FTIR-ATR and Raman Spectroscopy (514.5 nm, 685 nm, 785 nm and 830 nm combined). A discriminating power of 0.98 was achieved for 25 red gel inks using a combination of TLC, filtered light (IR fluorescence only), FTIR-ATR and Raman Spectroscopy (785 nm only). Hybrid gel inks were readily identified using solubility testing, TLC and Raman Spectroscopy combined, with 92% of red inks tested found to contain both dyes and pigments. A library of Raman spectra from 200 known pigments was compiled and CI Pigment Blue 15:1 and 15:3, and Pigment Red 112 and 254 identified as likely colour components of several blue and red gel inks respectively. An objective multivariate statistical methodology incorporating peak selection, data binning and normalisation was developed and successfully applied to both IR and Raman Spectroscopic data. Hierarchical Cluster Analysis (HCA) and Self-Organising Feature Maps (SOFM) were more effective than Principal Component Analysis (PCA) for successful clustering.

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Presentations and Publications

Poster Presentations

1. **Reed, G**; Nic Daeid, N; Savage, K and Edwards, D. A preliminary investigation of the hyperspectral imaging capabilities of the Foster and Freeman VSC 6000/HS for the analysis of gel inks. 6th European Academy of Forensic Sciences (EAFS) Meeting, 20 – 24 August 2012, The Hague, Netherlands

2. **Reed, G**; Nic Daeid, N and Savage, K. The discrimination of black gel inks by FTIR-ATR and the application of a statistical methodology to achieve objective classification. 6th European Academy of Forensic Sciences (EAFS) Meeting, 20 – 24 August 2012, The Hague, Netherlands

3. **Reed, G**; Nic Daeid, N; Savage, K; Faulds, K and Ismail, Dzulkiflee. Comparison of two Raman Spectrometer systems for the analysis of blue and red gel pen inks using 785 nm excitation wavelength. 6th European Academy of Forensic Sciences (EAFS) Meeting, 20 - 24 August 2012, The Hague, Netherlands

4. **Reed, G**; Nic Daeid, N; Savage, K; Faulds, K and Ismail, D. The analysis of blue gel pen inks by Raman Spectroscopy using four excitation wavelengths in combination with a multivariate statistical methodology for discrimination. 6th European Academy of Forensic Sciences (EAFS) Meeting, 20 – 24 August 2012, The Hague, Netherlands

5. **Reed, G**; Nic Daeid, N; Savage, K and Faulds, K. A comparison of two Raman Spectrometer systems for the analysis of gel pen ink writings on white office paper using 785 nm excitation wavelength. Postgraduate Research Day, 9 June 2010, University of Strathclyde, Glasgow, UK

6. **Reed, G**; Nic Daeid, N; Savage, K, Faulds, K and Keeley, C. The analysis of blue, black and red gel pen inks by Raman Spectroscopy – preliminary findings. 5th European Academy of Forensic Sciences (EAFS) Triennial Meeting, 8 – 11 September 2009, Glasgow, UK

Oral Presentation

1. **Reed, G**; Nic Daeid, N; Savage, K; Faulds, K and Ismail, D. The application of Raman Spectroscopy to the analysis of blue, red and black gel pen writing inks. American Academy of Forensic Sciences (AAFS) 63rd Annual Scientific Meeting, 21 – 26 February 2011, Chicago, Illinois, USA

Peer Reviewed Journal Publication

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Webcast Video Presentation

1. **Reed, G**. Ink analysis for forensic investigators. Webcast available online "Strathclyde Uni" YouTube channel at http://www.youtube.com/watch?v=I34tz5nIPCs, uploaded 7 March 2011, Centre for Forensic Science, Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow, UK

Dedicated to my Dad, you are sorely missed by both Mum and I, forever in our hearts and minds

Reginald Henry Reed

July 15 1927 – October 15 2004

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Abbreviations

A	Absorbance
Abs/nm	Absorbance/Wavelength
AE	Auto Exposure
Al	Aluminium
ANN	Artificial Neural Network
AR	Annular
AU	Arbitrary Units
AUS	Australia
BMU	Best Matching Unit
Ca	Calcium
CA	Cluster Analysis
CCD	Charged Coupled Device
CI	Colour Index
cm	Centimetres
cm^{-1}	Wavenumber (see also $\tilde{\nu}$)
CO_2	Carbon Dioxide
Cu	Copper
DA	Discriminant Analysis
DART	Direct Analysis in Real Time
DMF	Dimethylformamide
DP	Discriminating Power
DRIFTS	Diffuse Reflectance Infrared Micro-Spectrometry
E	Energy
EDS	Energy Dispersive X-Ray Spectrometry
ESI-MS	Electron Spray Ionisation Mass Spectrometry
EtAc	Ethyl Acetate
EtOH	Ethanol
FD	First Derivative
FDMS	Field Desorption Mass Spectrometry
Fe	Iron
FLE	Filtered Light Examination
FTIR-ATR	Fourier Transform Infrared – Attenuated Total Reflectance
GC	Gas Chromatography
GCMS	Gas Chromatography Mass Spectrometry
gsm	Grams per Square Metre

HCA	Hierarchical Cluster Analysis
HCl	Hydrochloric Acid
HK	Hong Kong
H_2O	Water
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
HSI	Hyperspectral Imaging
ICP-AES	Inductively Coupled – Atomic Emission Spectroscopy
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
INTL	International
IR	Infrared
IRL	Infrared Fluorescence/Luminescence
IRR	Infrared Absorption/Reflectance
JAP	Japan
J/s	Joules per Second
KBr	Potassium Bromide
LA-ICP-MS	Laser Ablation - Inductively Coupled Plasma - Mass Spectrometry
LDA	Linear Discriminant Analysis
LCTF	Liquid Crystal Tuneable Filter
LDMS	Laser Desorption Mass Spectrometry
LED	Light Emitting Diode
LIBS	Laser Induced Breakdown Spectroscopy
m	Ground State
M	Measurement
Λ	Wavelength
Λ_{max}	Lambda (Wavelength) Maximum
Λ_{min}	Lambda (Wavelength) Minimum
MAL	Malaysia
MALDI-MS	Matrix Assisted Laser Desorption Ionisation Mass Spectrometry
MeOH	Methanol
mg	Milligram
Mg	Magnesium
Mn	Manganese
m/s	Metres per Second
MSP	Microspectrophotometry
mW	Milliwatts
Na	Sodium

N.A.	Numerical Aperture
NaCl	Sodium Chloride
Ni	Nickel
NIR	Near Infrared
n	Number or Lowest Ground State
nm	Nanometres
Pb	Lead
PC	Principal Component
PCA	Principal Component Analysis
PLM	Polarising Light Microscopy
R	Resolution
R_f	Retention Factor
RI	Refractive Index
RPI	Relative Peak Intensity
% RSD	% Relative Standard Deviation
s	Second
SA	South Africa
Si	Silicon
SIMCA	Self-Independent Modelling of Class Analogies
S:N	Signal to Noise Ratio
SEM	Scanning Electron Microscopy
SERRS	Surface Enhanced Resonance Raman Spectroscopy
SOFM	Self-Organising Feature Map
SOP	Standard Operating Procedure
Sr	Strontium
T	Transmittance
TLC	Thin Layer Chromatography
ToF-SIMS	Time of Flight Secondary Ion Mass Spectrometry
µ	Micron
UK	United Kingdom
µm	Micrometer
US	United States
UV	Ultra-Violet
v	Frequency
$\tilde{\nu}$	wavenumber (see also cm^{-1})
Vis	Visible
VSC	Video Spectral Comparator

XRF	X-Ray Fluorescence
~	Approximately
<	Less Than
>	More Than
#	Number

Brand/Model Combinations of Gel Pen Abbreviations

BIC	BIC
COF	Corner Office
FBC	Faber Castell
GRE	Great Expressions
GSF	G'Soft
ICM	Inoxchrom
MG	M & G
PKR	Parker
PLT	Pilot
PPM	Papermate
PTL	Pentel
PTN	Partner
STB	Stabilo
STD	Staedtler
STP	Staples
UNI	Uniball
WHS	WH Smiths
WKE	Works Essentials
ZBR	Zebra

Chapter One

Introduction

1.1 Introduction

Traditional methods for analysing writing inks can provide a certain level of discrimination between two or more ink entries by means of their morphological characteristics, their behaviour under different lighting conditions and their dye and chemical composition. Furthermore, where a reference database is available, a possible identification of the brand and/or model of ink may be afforded. Should the microscopy, filtered light and thin layer chromatographic (TLC) data for two or more ink entries "match" it can be inferred that they could have originated from the same source, i.e. the same pen. However, this does not serve as conclusive evidence that two or more ink entries have a common origin as it is conceivable that the inks are from different sources containing the same ink formulation. Moreover, any suggested identification of formulation is limited by the completeness of any database used. Taking this into consideration along with the mass production of pens and knowledge that ink manufacturers supply a number of different pen producers, this can significantly influence evidential value. Therefore it would be desirable to develop an analytical technique and/or analytical protocol capable of improving the discriminating potential of ink analysis. This thesis is concerned with the examination of gel pen writing inks in this context. With this in mind, the overall aims of this research were two-fold. Firstly to investigate the potential of spectroscopic methods for the analysis and discrimination of gel inks both on their own and in combination with traditional methods i.e. filtered light examination and TLC. Secondly, to develop and evaluate a multivariate profiling methodology based on Chemometric analysis of the spectroscopic data.

The specific objectives of this research were to:

1. Investigate the ability of traditional and conventional techniques (filtered light examination (by use of the VSC 6000/HS) and TLC) to characterise gel inks including an assessment of within and between brand variation
2. Investigate the ability of spectroscopic methods (Vis-MSP, FTIR-ATR and Raman Spectroscopy) to characterise gel inks, including an assessment of within and

between brand variation, and the creation of a pigment spectral library for potential colorant identification by Raman Spectroscopy

3. Calculate the Discriminating Power (DP) of each analytical technique both alone and in combination
4. Develop and evaluate a multivariate profiling methodology of the spectroscopic data using Chemometric methods
5. Develop an analytical scheme, together with validated Standard Operating Procedures (SOP's) for the examination of gel inks for use in routine casework.

Chapter One provides a brief introduction into the different types of writing instrument available on the modern day market with particular in depth focus on the gel pen and its ink composition. Attention is also given to the chemistry of colour, the traditional analytical approach to forensic examination, the analytical instrumentation involved and the associated difficulties of applying these approaches to gel inks specifically. Data analysis and interpretation in terms of both discriminating power and multivariate statistical techniques are also discussed.

Chapters Two through **Six** each deal with a specific analytical technique and its applications to the investigation of within and between brand/model discrimination of two common colours of gel ink, (blue and red). Each Chapter incorporates a summary of the relevant literature before describing the experimental procedures used, followed by a discussion of the results for each colour group and overall conclusions reached.

Chapter Seven deals with data analysis and interpretation, initially focussing on the discriminating power of each analytical technique by colour group, both alone and in combination, before moving into a discussion around the development and evaluation of a multivariate profiling methodology based primarily on Raman and IR Spectroscopic data.

Chapter Eight, the final Chapter, discusses the overall conclusions drawn from all studies. This Chapter also provides a final evaluation of the analytical methodology employed for the within and between brand/model discrimination of the two colour groups of gel ink pen examined. Any further work to be conducted is identified together with a reference to emerging techniques considered of future potential value highlighted.

1.2 Types of Writing Ink and Pen Class

The history and development of writing inks and writing instruments can be viewed in combination and broadly split into pre and post-World War II (WWII) eras. Modern day writing instruments have predominantly been developed since the end of WWII. The history of pens (i.e. Reed and Quill pens) and inks (i.e. Carbon and Iron-Gallotannate ink) prior to this period will not be discussed in depth, but the reader is referred to several texts which provide a valuable insight [1-10]. For the purposes of this thesis, modern day writing instruments can be categorised into five different pen classes: the fountain pen, ballpoint pen, porous tip pen, rollerball pen and gel ink pen.

1.2.1 Fountain Pen

The invention of steel and then later metal alloy pen nibs, rubber inkwells and mechanical machinery to enable mass production led to the development of the first practical fountain pen by American Businessman, Lewis Edson Waterman, in 1884. The pen consisted of a barrel into which the ink could be deposited by dropper and subsequently fed to the metal alloy nib [3]. An iron-gallotannate blue-black ink was used in these new pens to deposit an insoluble stable writing line onto paper. By optimising the quantity of iron salts in the composition, a permanent fade resistant and smudge proof ink was created, suitable for maintaining important documentary records, and became known as record ink [9]. However, the acidic nature of this ink damaged the metal nibs of the fountain pen [9] and was not suitable for routine use. As a consequence, a second class of fountain pen ink was developed for everyday writing. These inks, in contrast to the blue-black inks, contained minimal amounts of iron compounds and an increased proportion of synthetic dyestuffs in a water based solution. The acidity of these fountain pen inks was greatly reduced minimising corrosion damage to the metal pen nibs [8, 9] and the use of synthetic dyes provided a range of bright colours making them popular with the general public [3]. However, the lack of iron components and increased use of synthetic dyestuffs meant these inks were not resistant to water or light fast, and were therefore not recommended for record keeping [8-10]. Fountain pens rapidly became the writing method of choice and remained so until the development of the ballpoint pen in the mid-20th Century.

1.2.2 Ballpoint Pen

A number of attempts at creating the first ballpoint pen were made in the late 19th and early 20th Century. All unsuccessful, until Ladislao Biro and his brother George patented their invention in the late 1930s. Their design incorporated a small metal ball bearing (~1 mm diameter) housed in a socket at the end of the pen barrel. The barrel contained a paste like oil based dyestuff ink in sufficient quantity to write for several months, and was deposited onto paper via the rolling ball mechanism and capillary action [3].

Milton Reynolds, an American Entrepreneur, developed this concept further and devised a way to feed the ink to the rolling ball by gravity action. In 1945, Reynolds introduced his version of the ballpoint pen to the US market at Gimbells Department Store in New York City, and it became an instant hit with sales reaching several million within only a few years. However, these pens had imperfections, mainly arising from the nature of the ink being used. The ink contained an oil based solvent such as castor oil [10] or linseed oil [3], combined with a basic dye such as Methyl Violet. The ink had a tendency to adhere to and accumulate to the metal ball and its housing (gooping of point assembly), which clogged the writing mechanism causing problems with direction and stopping of the ink line, as well as acidic corrosion of the metal ball bearing. The ink also had poor light fastness, was slow to dry and did not absorb well into the paper fibres so lacked a degree of permanency. Reynolds pens were also prone to leaking of the ink from the reservoir [3, 10].

The solution to these problems was provided in the early 1950s by Frans Seec, a Hungarian Chemist who developed a more suitable ink for use with these pens which was soon adopted for mass production. Seec's ink used a glycol based solvent such as ethylene glycol, phenoxyethylene glycol or glycerine [3]. In conjunction with chelated metalised dyes soluble in such solvents, e.g. copper phthalocyanine [3], and other acid or basic dyes [10]. This new formulation offered improved drying time and better light fastness, as well as the opportunity to utilise a greater range of colour. Over the years, additional ingredients have been added to improve the properties of ballpoint inks. Modern day formulations can be either water or solvent based, and may contain non-ionic surfactants to prevent clogging of the pen mechanism, xanthan gums to improve ink drying time, and resins to assist with binding the ink to the paper fibres [10], amongst others, in addition to water soluble dyestuffs.

1.2.3 Porous Tip or Fibre Tip Pens

In the early 1960s, Pentel launched a new writing instrument in the form of the porous tip pen [3]. This pen is more commonly known as the fibre tip or felt tip pen, so called because unlike the metal nib of the fountain and rolling metal ball of the ballpoint pens, its writing tip is composed of densely packed fibres. Fluid ink is capillary fed through the spacing's between the fibres onto the surface of the paper. The width of the writing line can be either very fine or very thick dependent on how densely packed the fibres are within the stylus tip [11]. These pens can therefore be used for both handwriting or for artistic purposes, i.e. colour drawing. The composition of the ink can be water or solvent based, i.e. xylene, and contains dyestuffs, typically metalised chelated dyes to provide bright fade resistant colours, as well as additives to increase properties such as ink drying time [3].

1.2.4 Rollerball Pens

The porous tip pen was quickly followed by the rollerball pen in 1968 [3], so called because it uses the same rolling ball mechanism of ink deposition as the ballpoint pen [11] whilst providing a generally more comfortable writing experience. Unlike the ballpoint, it uses a more fountain pen like fluid ink to provide a smooth fine ink line for handwriting. The ink is aqueous based and contains a reduced concentration of water soluble dyestuffs to provide colour, e.g. metalised chelated dyes or acidic dye salts, as well as solvents (e.g. formamide), to reduce the risk of the ink drying out at the ball tip [3].

The invention of the ballpoint pen in the mid-20th Century caused a significant shift away from the use of the fountain pen for everyday handwriting, and rapidly became the global writing method of choice to the present day. In the 1980's it was estimated that of the handwritten documents submitted for examination in forensic casework, 80% were written with ballpoint pens [1]. However, a new type of writing instrument, the gel ink pen, containing a revolutionary ink emerged in the early 1980s and over the past 30 years has become the fastest growing group of pens available on the market today [12].

1.3 Gel Ink Pens

Gel ink pens can be defined as pens which "utilize a rolling ball mechanism to transfer…a water based gel…(whose) colorants maybe dyes or pigments, but are typically pigments" to

the surface of paper [13]. It is the uniqueness of the pigment based gel that distinguishes the gel pen from other classes of writing instrument previously described, and which poses a problem for forensic scientists, thus forming the basis of this research.

1.3.1 History and Development of the Gel Ink Pen

Detailed information about gel ink, specifically its composition and manufacture, is scarce in the available literature, primarily because of confidentiality issues surrounding specific ink brand formulations. As with previous researchers [12, 13], attempts were made to contact nine major pen and ink manufacturers who include gel ink products in their product portfolio (BIC; Uniball; Pentel; Parker; Pilot; Staedtler; Zebra; Dockumental and National Ink) to seek out further information, but these attempts were met with limited success. Despite this, previous researchers [12, 13] have managed to successfully acquire some information concerning gel inks .

The gel ink pen was created by the Sakura Color Product Corporation of Japan, specialists in manufacturing stationary for the artistic market [14]. Sakura were keen to enter the writing instrument market by developing a rollerball pen to rival those of their competitors; a pen that retained the smooth writing ability of the rollerball whilst eliminating the problem of reduced ink flow resulting in poor writing line quality that dogged the rollerball through continued use over the lifetime of the pen [13]. Sakura discovered the solution was to create a gel based ink that worked on the principle of thixotropy and possessed the advantages of both water based fluid ink and solvent based ballpoint ink [14]. The principle of thixotropy had been used previously in the Fisher Space pen created in 1969 and subsequently used by Astronauts on NASA space missions because of its unique ability to write under zero gravity conditions [13]. Thixotropy is a change in chemical state, and in the case of gels means that when a gel is static it is in a solid state, but upon some form of disturbance, such as the friction of a rolling metal ball, the gel turns into a liquid, thus providing a constant and consistent ink flow throughout the duration of the pens life [14].

On October 20th 1982, Sakura patented the first gel ink containing xanthane gum as the key thixotropic ingredient to provide the necessary gel like qualities [14]. Further work was conducted over the subsequent two years to correct imperfections, design a completely new pen assembly and develop a suitable manufacturing process to incorporate the gel ink

into the pens [14]. In 1984, the first ever gel pen containing a dye based gel ink was made available in Japan and was called the Ballsign 280. This was quickly superseded by the Ballsign 150 in 1985, which contained a superior pigment based gel ink formulation; and the Ballsign 80 in 1987 with improved writing performance and availability in three colours; blue, red and black, becoming the most popular of the three available gel pens in Japan at that time. In 1989, the Ballsign 80 was rebranded for the North American market, and was henceforth known as the Gelly Roll® [14]. Some authors claim gel pens also began to emerge on other international markets, i.e. Europe, in the early 1990s [15]. However there seems to be some uncertainty when gel pens were available within the United Kingdom (UK), but were known to be available to a limited extent at the latest by 1997 [16]. By the mid-1990s, other pen and ink manufacturers not wanting to miss out on a sales opportunity had started developing and marketing their own versions of the gel ink pen with at least three major pen manufacturers, i.e. Pentel, Zebra and Mitsubishi (Uniball) offering gel ink pens amongst their product range in the United States (US) [17]. However, despite some popularity, it wasn't until 1997 that serious interest in the gel pen by the general public began to emerge when Sakura of America, an affiliate of Sakura Product Corporation of Japan, began marketing a new metallic version of the gel ink pen [14]. These metallic gel ink pens, available in nine colours, contained fine powdered metal, i.e. aluminium [18], to impart a sparkling effect to the writing line. These were followed by the introduction of Gelly Roll Lightening® pens in 1999, which provided a silver outline to the writing line, and Gelly Roll Stardust™ in 2000 [14], available in 12 colours, which provided a glitter-like appearance to the writing line from the inclusion of finely ground cosmetic grade glass particles [18]. In 2001, a further 12 dark colours were added to the Gelly Roll Stardust™ range and Gelly Roll Moonlight™, which provides a glow-in-the-dark writing line was marketed in the US [14]. To date, the Gelly Roll® brand consists of 65 different colours, with more likely to be added as the gel ink pen increases in popularity [14]. In 2009, the majority of major pen manufacturers appeared to sell at least one model of gel pen in their global product range, with others such as Uniball (Mitsubishi Pencil Co.), Pilot and Pentel marketing several models with their own unique selling points. Even discounted brands of gel pens are available in stores such as Poundland, The Works, and Poundmart in the UK. The gel ink pen continues to grow in popularity and is favoured by children and artists because of the wide range of colours and textures of ink available [13, 19].

1.3.2 Physical Characteristics and Mechanism

Gel ink pens are sometimes marketed as rollerball gel pens as they use the same rolling ball mechanism as both the rollerball and ballpoint pen, but differ in that the metal ball is polished to a greater degree in order to assist with an unhindered flow of ink from the fill tube to paper [13]. The plastic fill tube which contains the gel ink is blocked at the distal end with a ¼ inch clear silicon grease plug which acts to prevent the ink from both leaking and leaving a residue as the ink supply diminishes through repeated use of the pen [17]. The metal ball, housed in a nib holder, is attached at the proximal end of the fill tube, which itself is typically stored in an airtight clear or semi-transparent barrel through which the writer can see both the colour and volume of ink remaining [17].

1.3.3 Manufacture

The commercial and, therefore, confidential nature of the processes involved with the manufacture and composition of writing inks means that such information is not readily available in the public domain. A few references [20, 21] describe the manufacture and composition of inks, but these do not provide information relevant to modern day writing inks. A search of the available literature has revealed little concerning the manufacture of gel ink. Brunelle and Reed [22] describe the basic processes involved in the manufacture of ballpoint and fluid writing ink, the steam jacket kettle and flow reactor methods respectively, but it is not clear if either process is relevant to gel inks. Sakura of America [14] and Florence *et al* [13] highlight the need for centrifugation to remove air bubbles when depositing the gel ink into the fill tube of the pen assembly, suggesting that a new method of manufacture had to be developed, but this seems more relevant to the manufacture of the pen itself rather than the ink. Despite this lack of information, some knowledge as to what chemicals go into a gel ink formulation is available.

1.3.4 Writing Ink Formulations and Batches

Any writing ink is made according to a formula, which can be described as “a set of instructions for making ink from a list of specified materials” [22]. These ink formulations may remain unchanged for long periods of time, or be altered relatively frequently in response to factors such as the following outlined in Brunelle and Reed [22]:

- The need to adjust flow or drying properties
- To compensate for the regional climate of the particular end user consumer market
- To make improvements to the product or writing performance
- Fluctuations in the availability of ink ingredients and pen components
- Changes in customer specification

Ink formulations are prepared in batches, the definition of which is "dependant on both the capabilities of the manufacturer to produce and that of the needs of the customer" [22]. Many pen manufacturers do not possess the capabilities to manufacture their own ink, and therefore outsource their requirements to ink manufacturers such as Dockumental (Germany) and National Ink (US) [23]. The pen and ink manufacturer work closely together to ensure that a suitable ink is chosen for the particular class of writing instrument being produced, and it is unlikely that the pen manufacturer will be aware of the exact formulation to be used [24]. Batch-to-batch, or within brand variation, may result from a number of possibilities as outlined in Brunelle and Crawford [25]:

- During manufacture of the ink, emphasis is placed on achieving a "consistent colour and viscosity" rather than ensuring "precisely the same proportion of ingredients" are added
- Leftover batches arising from discontinued orders or incorrect ink formulations are generally stored and maybe added to batches of ink with the same or similar formulation to reduce losses from manufacturing costs
- The quality of the raw materials used in ink formulations, i.e. dyestuffs, may vary over time between or within suppliers
- The same reaction vessels will be used continually to produce batches of ink with different formulations, and therefore may result in contamination of a batch if not cleaned correctly between use
- Mistakes made in the measurement of ingredients to be used in an ink formulation

These batch variations, although apparently unusual [25], are of great interest to the forensic scientist who may be able to detect such subtle variations thus enhancing the evidential value of the analytical results.

1.3.5 Chemical Composition

The specific composition of gel ink formulations are closely guarded commercial secrets, but some general information about the types of chemicals that may be found within the inks is available in the literature [12, 13, 15, 17-19, 26-28]. Gel inks are aqueous based, some containing as much as 80% water [18]. The colour is predominantly provided by microscopic sized (<0.5 μm) insoluble organic and/or inorganic pigment particles which provide gel inks with a wide array of bright colours, as well as the traditional blues and blacks [12, 17]. However, modern gel ink formulations are known to contain dyes instead of, or as well as, pigments, for example in hybrid gel ink pens [13]. In fact, the first gel ink pen produced by Sakura used a dye based gel ink, but was rapidly superseded by a superior pigment based gel ink [14].

It is believed that commercial competition has led to some manufacturers (who lack the technical expertise and may be prohibited by patent issue rights) to use dyes in their own gel ink formulations rather than pigments [15, 27]. Whilst pigments provide a range of added benefits, soluble dyes can be used in varied amounts to fine tune the hue and brightness of ink colour [15, 27]. Other gel ink ingredients may include solvents, resins, lubricants, biocides, surfactants, corrosion inhibitors, sequestrants, sheer thinning agents, emulsifying agents, pH buffers and adjusters, polymerisation agents and pseudoplasticizers [12]. Table 1.1 provides details of the functions of each ingredient together with examples based on information acquired from Brunelle and Crawford [12].

INGREDIENT	FUNCTION	EXAMPLES
Dyes	Colouring Agent	Food Dyes: CI Food Yellow 3 Basic Dyes: CI Basic Yellow 1; CI Basic Yellow 2; CI Basic Violet 1; CI Basic Green 4
Pigments	Colouring Agent	Organic: Azo Lakes; Insoluble Azo Pigments; Chelate Azo Pigments; Phthalocyanine Pigments; Perylene Pigments; Perinone Pigments; Anthraquinone Pigments; Quinacridone Pigments; Dye Lakes; Nitroso Pigments; Condensed Polyazo Pigments; Dioxazine Pigment; Indigoid Pigments; Thioindigoid Pigments Inorganic: Titanium Oxide; Carbon Black; Metal Powder; Iron Oxide; Melamine Pigment
Solvents and Vehicles	Carrier for the Colouring Agent	Alkylene Glycols; Polyalkylene Glycols; Triols; Glycerols; Thiodiethanol; N-Methyl-2-Pyrrolidone; 1,3-Dimethyl-2-Imidazolidinone Organic Solvents: Ethylene Glycol; Diethylene Glycol; Propylene Glycol; Glycerine; Polyhydric Alcohols; Propylene Glycol Monomethyl Ether; Glycolethers; Propylene Glycol Monomethyl Ether Acetate; Glycol Ether Esters
Resins	Adjust Viscosity; Increase Film Strength; Enhance Lubrication of Rolling Metal Ball; Bind Ink to Paper	Carboxymethyl Cellulose; Xanthan Gum; Water-Soluble Acrylic Resin; Synthetic Resins; Water-Soluble Maleic Acid Resin; Water Soluble Styrene Resin; Water-Soluble Styrene Acrylic Resin; Water-Soluble Styrene-Maleic Acid Resin; Polyvinyl Pyrrolidone; Polyvinyl Alcohol; Water-Soluble Urethane Resin
Lubricants	Lubricate Rolling Metal Ball in Socket	Alkali Metal Salts or Alkanol Amine Salts of Fatty Acids; Phosphorus Surfactants
Biocides	Prevent Microbial Growth in Ink	Methyl p-Hydroxybenzoate; Propyl p-Hydroxybenzoate; 1-(3-Chloroallyl_-3,5-7-Triazo-1-Azoniaadamantane
Surfactants	Adjust Surface Tension of Ink; Sufficient Wetting of Rolling Ball to Provide Suitable Rate of Ink Delivery	Non-Ionic: Polyoxyalkylene Higher Fatty Acid Esters; Higher Fatty Acid Partial Esters of Polyhydric Alcohols; Higher Fatty Acid Esters of Saccharide Anionic: Alkylated Sulfonates of Higher Fatty Acid Amides and Alkylallysulfonates
Corrosion Inhibitors	Preserve the Rolling Metal Ball and Socket	Benzotriazole; Tolytriazole; Dicyclohexyl Ammonium Nitrate
Sequestrants	Maintains Ingredients in Solution	Tetrasodium EDTA or Versene 100 RTM of Dow Chemical; Trisodium Phosphate; Sodium Hexametaphosphate; Sodium Glucoheptanate
Shear Thinning Agents	Promote Free Flow of Ink	Polymeric Material e.g. Alpha-Methyl-Styrene-Styrene-Acrylic Acid Terpolymer
pH Controllers(buffers)	Maintain pH of Ink	Triethanolamine; Morpholine; Diethanolamine; Ethylamine; Monoethylamine
pH Adjusters	Adjust pH of Ink to Suitable Level	Sodium Hydroxide; Sodium Carbonate; Alkanol Amine; Ammonia
Polymerization Agents		Xanthan; Carboxymethyl Cellulose
Rust Preventatives	Prevent Rust Forming on Metal Ball	Benzotriazole and Derivatives; Dicyclohexylammonium Nitrate
Antiseptics		Potassium Sorbate; Sodium Benzoate; Sodium Pentacholorphenolate; Sodium Dihydroacetate; 1,2-Benzisothiazoline-3-on
Pseudoplasticizers	Influences Viscosity of Gel Ink in Fill Tube Necessary to Maintain as a Static Solid	Xanthan Gum; Tamarind Gum; Carrageenan Gum; Tragacanth Gum; Locust Bean Gum; Gum Arabic; Guar Gum; Curdlan; Pectin; Agar; Gelatine; Mannans; Cellulose; Urethane Synthetic Polymers; Smectite; Montmorillonite; Methyl Cellulose; Ethyl Cellulose; Carboxymethyl Cellulose Natural Polysaccharides e.g. Welan Gum; Xanthan Gum; Cyamoposis Gum; Locust Bean Gum; Ramsan Gum with Polymeric Formula Composed of Monosaccharide's e.g. Glucose, GA lactose, Rhamnose; Mannose; Glucuronate Salt; Semi-Synthetic Cellulose Polymers

Table 1.1: Types of chemicals found in gel ink formulations, their functions and some examples (information acquired from Brunelle and Crawford [12])

1.3.6 Advantages and Disadvantages of Gel Pens

The advantages of gel ink pens have been widely highlighted in the available literature and appear to far outweigh the potential disadvantages. The vast selection of brilliant colours available and creative textures appeals to the artistic imagination of the general public [13, 15, 28]. Insoluble pigment composition provides a permanent writing medium suitable for maintaining important documentary records; resistant to light, water and chemical degradation as well as acid free preventing damage to the paper substrate [13]. The high water based content of gel inks as opposed to the solvent based nature of ballpoint pen inks, coupled with the use of primarily organic pigments and other non-toxic ingredients, i.e. resins and additives, makes gel inks appeal to the environmentally friendly consumer [13, 15, 17, 18]. Whilst the smooth, fast and consistent ink flow makes gel ink a more attractive choice of writing instrument than the standard ballpoint pen [14, 18].

Despite these advantages, the gel ink pen is not perfect, and may possess a number of disadvantages that could impede its popularity. It is claimed that gel ink pens do not last as long as their ballpoint counterparts, with suggestion that eight gel ink pens are required to fill the same number of written pages as a single ballpoint pen [18]. If accurate, the comparatively short writing life combined with the increased demand for disposable gel ink pens would counterbalance the ecological benefits of their ink formulations. However, others argue that gel ink pens possess a long writing life [15] and with availability of gel ink refills [13], this apparent disadvantage may not be as detrimental as suggested. Another downside lies in the manufacture and specialised pen design required to support the use of the gel ink adding to its cost of production and ultimately its retail price [13]. In 2012, a standard ballpoint pen typically retailed below 60p in the UK, compared to between £1 - £7 for a gel ink pen dependent upon brand and model. This additional expense combined with shorter writing life may deter some from choosing the gel ink pen over other types of writing instrument, although for others the additional benefits they offer maybe considered worth the extra expense. Perhaps the most significant drawback lies in the tendency of gel ink to dry out quickly on the metal ball nib, which can affect the quality of the writing line, producing problems with ink starting, hesitation and skipping [13]. However, manufacturers have taken steps to reduce this problem by producing capped models and retractable models with removable protective seals for the ball nib [13].

1.4 Colour

Dyes and pigments appear coloured as a result of the interaction of electromagnetic radiation with their chemical structure. Many types of electromagnetic radiation exist, which together form the electromagnetic spectrum shown in Figure 1.1. Regions of electromagnetic radiation that are of importance to the analysis of ink include: UV (~200 – 400 nm), Visible (~400 – 800 nm) and Infrared (~800 – 1000 nm). UV and Infrared (IR) will be discussed later, but visible radiation is essential in providing an explanation for the appearance of colour.

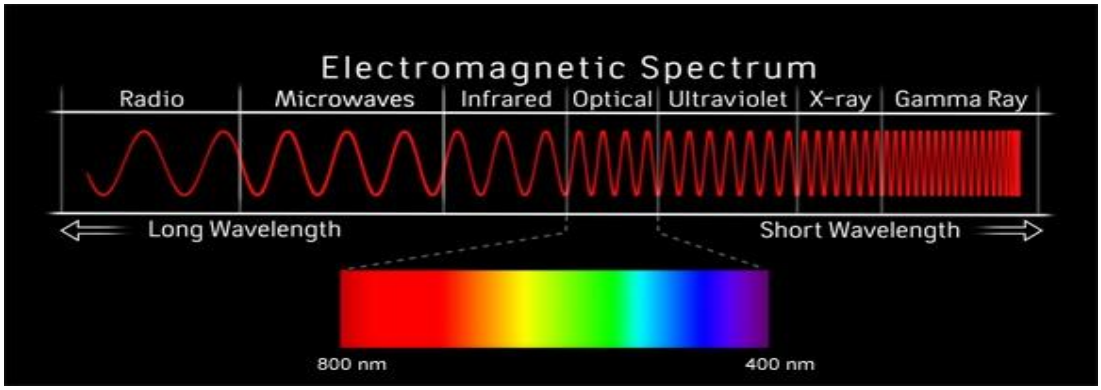


Figure 1.1: The electromagnetic spectrum (reproduced from [29])

Visible radiation occupies the region of the electromagnetic spectrum between approximately 380 – 780 nm, and is so named because it is the only type of electromagnetic radiation that can be detected unaided by the human eye [30]. Natural daylight or white light occurs as a result of the entire visible wavelength range striking the eye. However, the visible region can be broken down into several component wavelength regions that correspond to a specific colour [30]. These component wavelength regions can be represented as the visible spectrum of colour, ranging from violet at the lowest wavelength range (~400 – 435 nm) to red at the highest (~605 – 750 nm) as shown in Table 1.2. If light of these specific wavelengths is registered by the human eye then that colour is perceived. For example, if light of wavelength 650 nm is viewed, then the colour red will be perceived. The three primary colours of light, red (R), green (G) and blue (B), can be combined in a process called additive colour mixing, to produce other colours of light, including, cyan (blue and green), magenta (red and blue), and yellow (red and green) [31]. Another way to produce colour is by a process called subtractive colour mixing, and this is the process by which we perceive ink and other matter as coloured [30].

In subtractive colour mixing, when white light interacts with a coloured material such as an ink, certain wavelength ranges will be absorbed by the dyes or pigments whilst others will be reflected. It is these combined wavelengths of reflected light that the human eye detects and perceives as the colour of the ink, and are known as the complimentary colours [30]. Table 1.2 lists the complimentary colours associated with the absorption of specific wavelength ranges of visible light. This process is called subtractive colour mixing because absorption of a particular wavelength range from the full range of wavelengths that make up white light results in the remaining reflected wavelengths combining to form a particular colour perceived by the eye [30]. For example, a blue ink appears as blue because it absorbs yellow (580 – 595 nm) light and reflects a combination of all other wavelengths to give the complimentary colour blue; whilst a red ink appears red because it absorbs bluish-green (490 – 500 nm) light and reflects all other wavelengths combined to give the complimentary colour red. If all wavelengths of visible light are reflected by the ink, it will appear white. If all wavelengths are absorbed, the ink will appear black [30].

Wavelength Range	Colour	Complimentary Colour
400 – 435	Violet	Greenish – Yellow
435 – 480	Blue	Yellow
480 – 490	Greenish – Blue	Orange
490 – 500	Bluish- Green	Red
500 – 560	Green	Purple
560 – 580	Yellowish – Green	Violet
580 – 595	Yellow	Blue
595 – 605	Orange	Greenish – Blue
605 – 750	Red	Bluish - Green

Table 1.2: Complimentary Colour Relationships [30]

1.4.1 Colorimetry [32]

Colorimetry is the term used to define the “science of measurement of the observed colour and its translation into a mathematical form to determine and specify colours” [33] within the visible spectral range. An objective means to define colour by numbers was devised by the Commission de L’Eclairage in 1931, and is known as the CIE System. It is based on the two fundamental principles that (a) the human eye responds only to three primary colours, and (b) that all colours perceived by the human eye are a combination of two or more of these primary colours. A person’s perception of colour is influenced by the lighting and viewing conditions under which that colour is observed, and therefore the CIE System defines standard lighting and viewing conditions for the analysis of colour. The system

numerically defines the human eye response to these primary colours in the form of tristimulus values, denoted X, Y and Z for red, blue and green respectively. Tristimulus values for the contribution of each primary colour to a specific colour can be measured by Microspectrophotometry (MSP), and in turn be used to calculate chromaticity co-ordinates (x, y and z) using Equations 1.1 – 1.3.

$$X = X/X+Y+Z \qquad y = Y/X+Y+Z \qquad z = Z/X+Y+Z \text{ [33]} \qquad \text{Equations 1.1 – 1.3}$$

Normally the chromaticity co-ordinates x and y only are calculated, since the sum of all three is always 1. A chromaticity diagram like the one in Figure 1.2 consists of the chromaticity co-ordinates for all spectral colours within the visible range. By plotting the x and y co-ordinates for a colour under study on the chromaticity diagram, it is possible to determine an accurate visual definition of that colour.

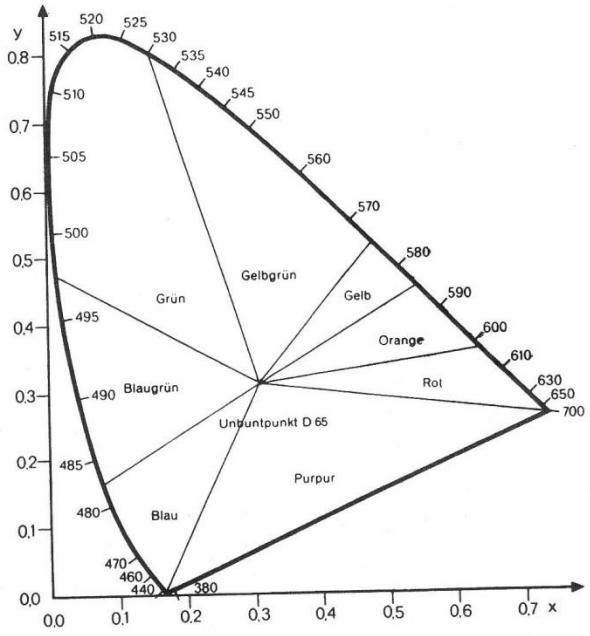


Figure 1.2: CIE Chromaticity diagram (reproduced from [33])

It is important to note that chromaticity co-ordinates are based on % transmission spectra. The concentration of a colorant in a sample can influence the % transmittance, therefore, if the concentration of a colorant varies within the sample, the % transmittance will be affected, which in turn will vary the tristimulus and chromaticity co-ordinate values. To overcome this, complimentary chromaticity co-ordinates, denoted x', y' and z', can be

calculated from the measurement of absorbance spectra instead. These values can also be used to numerically define the colour of a sample using the same chromaticity diagram.

1.4.2 Dyes and Pigments

The colour of a gel ink is typically provided through the incorporation of coloured pigments into its formulation. However, some manufacturers use gel inks that contain dyes rather pigments to impart colour to the ink for reasons of technicality and commercial competitiveness [15, 27]. Absorption of visible radiation by dyes and pigments occurs as a result of two key structural features common to most organic colorants, chromophores and auxochromes. Chromophores are "unsaturated organic functional groups" normally attached to aromatic rings that provide the colour e.g. the azo group (-N=N-) [34]. Auxochromes are "basic salt forming groups" that enhance the depth of colour, e.g. the hydroxyl (-OH) or amino group (-NH) [33]. These two structural groups are attached by a conjugated system of double and triple chemical bonds, the length of which can influence the wavelength range at which an organic colorant absorbs visible radiation and thus influence its colour [30]. Two types of valence electrons associated with these chemical structures, π (pie) and n electrons, absorb the visible radiation causing excitation and promotion of π–π* and n-π* electronic transitions, thus imparting colour to the dye or pigment molecule [30, 33]. It should be noted that this process relates to organic dyes and pigments only, since inorganic pigments are coloured as a result of a different type of electronic transition, i.e. charge transfer [35, 36]. The colour of an ink may therefore be as a result of a single colorant or a combination of colorants, reflecting light of a particular colour [11, 37]. This is why two or more inks may appear to be indistinguishable in colour to the naked eye, but may be different chemically and therefore distinguishable from one another only by a thorough scientific examination [11].

Although the primary function of dyes and pigments is to provide colour and they are similar in terms of their chemistry, they are very different types of compounds and each possess their own specific properties [30]. The key distinction between these two compounds is that, whilst in their raw form they exist as coloured powders, dyes are soluble in solution, whilst pigments remain insoluble [30]. It is this key difference that is at the heart of the challenge facing forensic scientists wishing to discriminate between the different gel inks.

1.4.3 Classification of Dyes and Pigments

The Society of Dyers and Colourists are responsible for publishing and updating a reference list identifying all known dyes and pigments used for commercial applications referred to as the Colour Index (CI) [30]. When a colorant is classified within the Colour Index it is given a CI Generic Name, which is defined as "a classification name and serial number which when allocated to a commercial product allows that product to be classified within any Colour Index Application Class" [38]. It consists of the method of application, hue and a number, which is a reference to its place in the chronological order of introduction of different commercial colorants [30]. An example of a CI Generic Name is CI Pigment Blue 15 for Copper Phthalocyanine.

An additional five or six digit CI Constitution Number (i.e. CI 74160) may also be listed alongside some colorants where their chemical constitution has been disclosed by the manufacturer [38]. Sometimes, the CI Generic Name and/or its CI Constitution Number will be sub-divided by a colon (i.e. CI Pigment Red 48:1 (CI 15865:1). This is to highlight some slight difference in its properties or chemical structure to that of the related parent coloured compound classified with the same CI Generic Name and/or CI Constitution Number [38]. Additional information provided by the Colour Index include the end use application of the colorant, its fastness properties, commercial trade name(s) and the contact details of those manufacturers that produce it [30, 38]. When a colorant with identical chemical structure is produced by different manufacturers, it will be given the same CI Constitution Number, but its commercial name may differ by manufacturer since it is they who provide the trade names for their products [39].

Essentially, dyes and pigments are classified in two different ways: by chemical structure and by method of application [30]. Classification of colorants by their chemical structure is of greater relevance to ink manufacture than the method of application, which is of greater relevance to the textile dyeing industry [30]. Dyes and pigments are classified into groups based on the presence of a particular feature in their chemical structure. Three chemical classes of colorant dominate the commercial market: azo, phthalocyanine and carbonyl, whilst many other classes are of less or no commercial importance [30]. In terms of colorants specifically relevant to writing ink manufacture, Brunelle and Crawford [12]

suggest that four chemical classes are particularly relevant: azo, phthalocyanine, triarylmethane and azine.

1.4.3.1 Azo Colorants

Azo compounds, an example of which is presented in Figure 1.3, can be used as both dyes and pigments and are synthesised in a two-step reaction process called diazotisation and azo coupling [40]. This involves the reaction of an organic compound containing a diazo group with an organic compound possessing a coupling component, to produce a compound containing an azo group (-N=N-). This provides a link between two carbon atoms each of which usually form part of an aromatic ring structure, i.e. phenols and primary aromatic amines [40]. Colorants of this chemical class may contain one azo group (monoazo) or more than one azo group (i.e. diazo, triazo, etc.) [40]. With respect to colour, azo compounds are normally used to impart yellow, orange and red colours, particularly important in pigmented form, but are available in almost any intense bright colour [35, 40]. They tend to be of poorer quality with regards to their technical properties compared to the other chemical classes, (such as phthalocyanines), but do provide satisfactory light fastness as well as heat, water and solvent resistance, which in combination with their low cost and ease of manufacture, make azo compounds the predominant chemical class of organic colorant [40]. The number of azo colorants range in their thousands [41] and they are used for most applications where colour is required [40]. Recent developments have led to the creation of high performance azo pigments which possess improved technical properties whilst retaining the bright intense colours characteristic of azo compounds [35]. Azo pigments are typically used in printing inks and toners associated with inkjet printers, colour laser printers and photocopiers. CI Pigment Yellow 12 and 13, and CI Pigment Red 57:1 are examples of azo pigments frequently used in the colouration of yellow and magenta toners respectively [35].

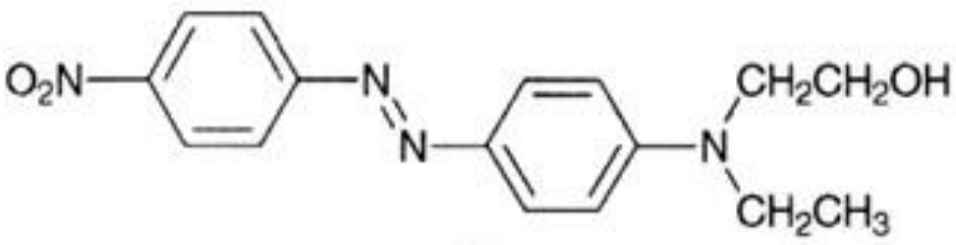


Figure 1.3: CI Disperse Red 1, an example of an azo dye compound (reproduced from [40])

1.4.3.2 Phthalocyanine Colorants

Metal phthalocyanines are formed from high temperature reactions involving a phthalic acid derivative, a nitrogen containing compound and a metal derivative, typically a first transition series metal such as copper [42]. The resulting aromatic molecule is a centrosymmetric metalloporphyrin complex with the metal cation at its centre surrounded by "four Isoindole units connected by four nitrogen atoms that form together in an internal 16 member ring of alternate carbon and nitrogen atoms" [42]. Although available as dyes, phthalocyanines tend to be used more in their pigmented form [41] to impart brilliant intense blue and green colours for a wide variety of applications [42]. They possess superior technical properties in terms of their fastness to light and their resistance to heat, water, solvents, acids and alkalis [42]. Figure 1.4 shows CI Pigment Blue 15, otherwise known as copper phthalocyanine, the most significant of the phthalocyanine pigments [35], known to be used in both gel inks [13, 27] and in its β–polymorph form as the predominant colorant for cyan toners [35].

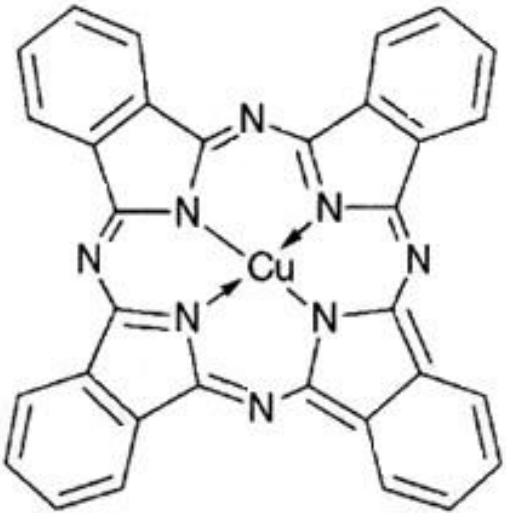


Figure 1.4: Copper Phthalocyanine (CI Pigment Blue 15) (reproduced from [42])

1.4.3.3 Triarylmethanes and Azine Colorants

Triarylmethanes, an arylcarbonium ion colorant, and azines are structurally similar to the polymethine chemical class [43]. Triarylmethanes (also known as triarylmethines and triphenylmethanes) consist of two or three aromatic ring systems linked together by a central carbon atom [43], whilst azines are heterocyclic compounds where one or more of the methine groups (-CH=) on the polymethine chain has been substituted for an aza

nitrogen (-N=) group [44]. Both classes of colorant can exist in cationic, anionic or neutral structural forms [43, 44]. Historically, azine dyes have been used in black and blue writing ink formulations (Nigrosine dyes) [12], whilst triarylmethanes have provided a full range of intense bright colours [43]. However, these classes of dyes possess poor light fastness and resistance properties (i.e. heat, water, solvent) in comparison to the azo and phthalocyanine chemical classes, and as such are of less commercial importance than they once were [43]. However, some triarylmethane pigments still find use in some printing ink applications due to their high brilliance and transparency [43], whilst triarylmethane and azine dyes may be used in the preparation of ballpoint inks [45].

1.4.3.4 Pigments

Pigments are classified as either organic or inorganic, each class providing their own specific set of properties [35]. The azo and phthalocyanine pigments described above are organic, which are recognised for their range of intense bright colour and high transparency, which is of particular benefit to coloured toners and printing inks since high transparency prevents one colour from obscuring another [35]. However, organic pigments (with the exception of the phthalocyanines and recently developed high performance (azo) pigments) tend to be technically inferior in comparison to inorganic pigments which offer excellent fastness and resistance properties, but not the brightness, intensity and transparency of the organic pigments [35]. An important inorganic pigment is carbon black [35], i.e. CI Pigment Black 6 and 7, which is used in the formulation of black gel inks [13].

1.5 Scientific Examination of Inks

1.5.1 History and Development

Historically, prior to the introduction and widespread distribution of ballpoint pens and the subsequent development of more contemporary writing instruments, ink analysis depended largely on a combination of physical and chemical examinations using light of different wavelengths and relatively simple micro-chemical spot tests [2, 5, 6, 46-48]. The physical examinations involved the use of infrared (IR) photography or photoscopy (particularly useful for detecting obliterations and mechanical erasures) and Ultra Violet (UV) fluorescence photography (useful for detecting chemical erasures) [2, 6, 46, 47]. Since IR and UV use light of very different wavelengths, certain classes and colours of ink would

exhibit different behaviours under these lighting conditions, providing a means of differentiating inks on paper. Simple chemical spot tests involved adding, either in situ [2, 6] or by removal of a part of the ink line to microscope slide [47, 48], micro-droplets of chemical reagents such as 2% dilute sodium hydroxide solution [2]. Any resulting colour change was observed through a magnifying lens or microscope and used as a characteristic feature to determine a particular class of writing ink, e.g. fountain pen or ballpoint [2, 5, 47, 48]. These kinds of tests were essentially non-destructive and required little or no scientific expertise to perform [46]. However, they were not suitable for identifying specific ink formulations within a class and were therefore of limited evidential value [46]. During the 1950's and 1960's ink analysis underwent a revolution in response to the increasing popularity of writing inks containing synthetic dyestuffs available on the consumer market. Several studies were published that detailed the findings from the investigation of paper chromatography [49-52] and electrophoresis [53, 54] as a means by which to separate out the individual dye components of an ink formulation for comparison to other ink samples and possible identification of brand formulation. Initially electrophoresis was favoured over paper chromatography due to problems arising with poorly resolved dye bands in the latter technique [50]. However, in 1966, Tholl (as referenced in [46]) published his study describing the efficient separation of dye and non-visible ink components from micro-quantities of ballpoint ink using the technique of Thin Layer Chromatography (TLC). It was this significant work combined with developments in the use of dichroic filters, IR luminescence and UV photography suggested by Brunelle and Reed [46], that has since formed the foundation of modern day ink analysis.

1.5.2 Methods Used in the Examination of Inks

The purpose of an examination of writing ink on a questioned document is to [11, 55]:

- Identify the type of pen and ink used
- Compare similarities and/or differences exhibited by ink samples under certain analytical conditions to determine if:
    - Any additions and/or alterations have been made to the document
    - Two or more ink entries may have common origin, i.e. made using the same pen
- Estimate the age of an ink entry

Any examination performed must take into account the limited amount of ink available on the document for analysis. For this reason, and for reasons of preserving evidence, an overwhelming consideration in questioned document analysis has been to prioritise non-destructive examinations over destructive ones [11, 55]. In the 1970's attempts were made to standardise the analytical approach to the examination, comparison and identification of writing inks [56, 57]. This has led to the approach widely accepted and used in forensic laboratories worldwide, and involves a non-destructive visual examination by microscopy followed by filtered light examination, and then if required, destructive analysis by TLC.

1.5.2.1 Microscopy

The first step in the examination of ink on paper is to view it under low power magnification (x 10 – x 100) with the aid of a stereomicroscope [37]. This serves to provide an indication of the type of writing instrument (pen class) and colour (shade and depth) of ink used to produce the document [11, 37]. Features present in the line morphology and the general appearance of the ink line can be used to determine the type of pen used, examples of which are given in Table 1.3.

This information can be used to provide the forensic scientist with an early indication as to whether two or more ink entries may have a common origin [37]. Clearly, if the ink used is a different colour and can be shown to have come from a different class of pen, then no further examinations are required. However, caution should be urged when making an assessment of the depth of colour of two ink entries on the same document by low power microscopy as the intensity of ink lines produced by the same pen may vary, presumably as a consequence of varied pressure applied during writing [11].

The general consensus amongst questioned document examiners appears to be that the writing or marks made by a ballpoint pen are readily distinguishable from that of a non-ballpoint pen [5, 11, 46]. The former contains a paste like ink that, when deposited onto paper, appears as a glossy layer on the surface of the fibres since it is only partially absorbed [11]. In contrast, non-ballpoint pens use water (or solvent) based ink that is completely absorbed into the paper fibres and will diffuse into the fibres adjacent to the main writing line, effectively dyeing the fibres [11, 37]. However, distinguishing between non-ballpoint pen classes appears to be much more complicated due to an overlap in the

identifying characteristics between the different pen types. Hilton [5], claimed that as well as being able to distinguish between the work of a porous tip and rollerball pen based on the presence or absence of a groove in the paper under the writing line, it was possible to distinguish the work of a fountain pen from the other non-ballpoint pen classes owing to the presence of a tracking effect produced when pressure is applied causing the two points that make up a fountain pen nib to separate. The effect on the line morphology is to create a lightly inked centre portion with a darker line either side not seen in the work of a porous tip or rollerball pen, although this feature may be less obvious in modern fountain pens which possess less flexibility in their nibs. It is worth noting that this observation was made prior to the introduction of the gel pen, and that recent observations have commented on a similar tracking effect associated with the writing of such an instrument [19, 37]. However, Brunelle and Crawford [37] suggested that the work of a fountain pen and a gel pen are easily distinguished on the basis that the former exhibits a dual track in the writing line whereas the latter exhibits only a single track arising from the rolling ball mechanism which appears to push the gel ink out from the centre of the line to the edges [19]. It has been suggested that this feature maybe used to distinguish the work of a gel pen from that of the other non-ballpoint pen classes [19].

Where a non-ballpoint pen class has been identified from microscopic examination of the writing line, it is generally accepted that it is rarely possible to identify a specific pen as having been responsible for producing it unless the mark produced by the pen exhibits some feature attributable to a defect unique to the pen in question [5, 11, 46]. However, it may be possible to exclude a pen as a potential source of the mark as a result of morphological examination, for example a fine porous tip versus a broad porous tip will produce ink lines of significantly different width [5, 19]. It may be possible to differentiate one ballpoint pen from another based on the colour of the ink, the size of the rotating ball (associated with the width of the ink line) and any defects associated with individual pens that may have resulted in features such as striations in the writing stroke [19]. However, it is clear that microscopy alone is not sufficient for distinguishing between different pen types and identifying a particular writing instrument.

	Pen Class	Characteristic Features
Ballpoint Ink	Ballpoint	• Partial absorption of ink into paper fibres creates a glossy paste like appearance [11] • A build-up of dirt in the ball housing can cause imperfections on the surface of the rotating ball that manifest itself in the form of striations, i.e. blank lines moving out from the centre of the pen line to the outer edges in the direction of the writing line [37], or short gaps in the writing line known as skipping [19] • Heavy deposits of ink known as gooping may appear in the writing line where the direction of pen movement has changed [11, 37], i.e. in individual letters with loops or when connecting letters to form a word [19] • The nature of the ballpoint pen requires relatively heavy pressure to produce writing which causes a groove or indentation in the paper surface in the centre of the ink line [5, 11] visualised in oblique lighting [37] • No diffusion of ink into paper fibres parallel to the writing line [37]
Non-ballpoint Ink (Fluid or Gel Ink)	Fountain	• Characteristics of writing line will differ according to the width and flexibility and/or hardness of the nib [5, 37] and the presence required to write efficiently • Dual track marks in the writing line arising from the nib design [5] • Ink diffusion into paper fibres parallel to the writing line [37] • Ink flow back apparent as a darkened area to the end of the writing stroke [37] • Width of ink line may vary significantly between up and down strokes and horizontal movement of pen due to separation of the dual points in the pen nib from applied pressure, and is known as shading [37] – more pronounced in older flexible nibs than modern stiff nibs [5, 19]
	Porous Tip	• Strong intensity of colour to the ink [5] • Variable tip widths will determine the width of the ink line [5], i.e. fine or broad, but essentially the ink line may be ribbon like in appearance [19] • As little pressure is required for fibre-tips the ink line may appear streaked and/or brush like [19] • Heavier pressure may be required to write with a hard porous tip resulting in a groove in the paper [37] • Diffusion of ink into the paper fibres parallel to the ink line [37] • Prolonged use of a fibre-tip may result in damage to the stylus in the form of frayed fibres which can appear as the fine ink lines running parallel to the main writing line [37] • Fibre-tips used for calligraphy with bevelled tips may produce shading in the writing line and/or may exhibit ink flow back [37]
	Rollerball	• Writing stroke similar in appearance to porous tip [5] • Groove in paper may be present arising from pressure applied during writing, however depth of groove may be shallow due to only light pressure required and the nature of the water based ink can differentiate it from paste like ballpoint ink [5, 37] • Lack of striations, skipping, or gooping [19, 37] • Possible diffusion of ink into paper fibres parallel to ink line [37] • Possible ink flow back apparent as darkened area at end of writing stroke [19]
	Gel	• Heavier ink tracks at outer edges and lighter appearance to centre of the ink line possibly due to heavy pressure applied during writing and rolling ball pushing gel ink out from the centre of the line to the edges [19, 37] • Lack of gooping and/or striations [37] • Possible groove to paper dependent on pressure applied [37] • Stable intensity and darkness of colour to ink line [37]

Table 1.3: Characteristic microscopic features of ink lines produced by the five modern types of writing pens (based on information acquired from [5, 11, 19, 37]

1.5.2.1.1 Microscopy of Gel Inks

In terms of further research into characterising gel inks by microscopy, Gernandt and Urlaub [17] performed a visual examination of a limited number of Japanese brands of gel ink using low power microscopy. They commented on features such as the absorption of each of the four brands of gel ink examined into paper fibres as well as the quality of the written line. Attempts were made to characterise each of the brands on the basis of variations in both the shade of colour (i.e. for the blue and black inks) and in writing line quality, which appeared to be dependent upon the quality of both the ink formulation and design of the rolling ball mechanism. Furthermore, ink absorption by the paper fibres appeared to be much less than for other classes of non-ballpoint ink, although no direct comparison with other such ink appears to have been undertaken in this preliminary study.

Mazella and Khammy-Vital [15] investigated the evidential value of blue gel inks and commented that examination of 33 different gel pen ink lines on office paper by the naked eye and low power microscopy revealed an initial classification based on visual examination. This resulted in three classes of gel pen ink distinguished on the basis of their visual appearance – milky, metallic and normal. The latter group were subjected to further analyses by filtered light, Raman Spectroscopy and Scanning Electron Microscopy (SEM), the findings of which will be discussed later.

Wilson *et al* [26] also commented on the use of low power microscopy to examine the morphology of the ink line on filter paper in their study of 29 black gel ink pens. Differences in line thickness (or line width) were observed, but were discounted as a discriminating feature since external factors such as applied pressure and the type of substrate upon which ink was deposited could influence the thickness of the resultant line. Interestingly, two inks exhibited a glittery appearance, whilst others appeared matt black, though it is not clear if the former were gel ink pens specifically marketed as glitter gels. Florence *et al* [13] also examined the microscopic appearance of gel ink lines on filter paper in an attempt to characterise and discriminate between eight brands of various colours, and other non-gel based writing ink. The inks were examined using low power microscopy (x 7.5 – x 64 magnifications) and Polarising Light Microscopy ((PLM) x 50 – x 500 magnifications). Greater detail of the ink lines was observed using higher magnifications, but lower magnifications were found to provide sufficient detail for comparison and

discrimination. Florence *et al* [13] describe the visual characteristics of gel inks and draw reference to the similarities and differences with other pen types, in particular rollerball pens. Features such as the presence of metal or glitter particles were considered unique to gel inks, though were not always found to be present depending on the nature of the ink formulation. Attention was drawn to sudden variations in line width, skipping of ink deposition within the line and gooping of heavy deposits of pigment arising from changes in writing direction and writing pressure. These features were all seen in the gel inks studied to varying degrees, which the authors suggested could be useful as discriminating features, contradicting the view of Wilson *et al* [26] in respect of line thickness. Interestingly, no gooping was observed to the ink produced by the rollerball pens studied, although the number of such pens examined was not provided. Depositions of dried ink between paper fibres was observed for both rollerball and gel inks however, and discrimination was possible based on the abundance of wicking seen in the former. Florence *et al* [13] suggested that, although gel inks tend to exhibit less wicking due to their viscous nature, the development of hybrid gel inks may limit the value of this feature for discriminating between gel and rollerball ink. Finally, the tracking effect highlighted earlier as a possible feature for discriminating a gel ink from other inks was found to be influenced significantly by variations in writing pressure, even with the same pen, which the authors suggest would also limit its value as a discriminating characteristic. Overall, Florence *et al* [13] concluded that it may not be possible to discriminate between rollerball ink from gel inks by low power microscopy in some cases.

Polarising light microscopy was suggested as a means of facilitating the observation of morphological characteristics such as colour, size and shape of large metallic and glitter particles [20]. However, PLM was of less benefit in characterising coloured pigment particles other than to comment on their presence or absence. Additional information concerning refractive index and birefringence may also be acquired using PLM [20]. It is worth noting that further to examination by low power and polarising light microscopy, Mazella and Khammy-Vital [15] were the first to study the morphology of gel ink lines on office paper at high magnifications (x 1000 – x 10,000) using scanning electron microscopy (SEM). Discrimination of the majority of 11 pigmented inks was achieved from a total of 17 inks analysed including discrimination of inks from the same brand, however only one of the remaining six dye based gel inks could be distinguished.

1.5.2.2 Filtered Light Examination (FLE)

The term Filtered Light Examination (FLE) refers to the examination of inks under different lighting conditions, namely IR absorption/reflectance, IR luminescence/fluorescence and UV fluorescence. These examinations have been performed for several decades, but technological developments have meant that the use of IR and UV photography to perform such examinations have been replaced in most laboratories by specialist equipment such as the Video Spectral Comparator (VSC) produced by Foster and Freeman Ltd [37], the latest version of which (VSC 6000/HS) incorporates both digital technology and Hyperspectral Imaging (HSI) capabilities [58]. In these cases, an image of the questioned document placed on a stage inside the VSC is provided via a charge coupled device (CCD) onto either a self-contained visual display unit in older systems or onto a computer screen via a connected PC workstation in the latest versions [58]. The ink present on the document can be viewed under different lighting conditions using a system of in-built illumination sources, filters (i.e. coloured, dichroic, and interference filters), lenses and sensitive tubes [11, 37].

1.5.2.2.1 Infrared Absorption/Reflectance (IRR)

Inks may or may not absorb IR radiation, and for those that do, the wavelengths in the IR region of the electromagnetic spectrum (~780 – 1000 nm) at which they absorb or cease to absorb will vary dependent upon the chemical nature of the colorant(s) used in their formulation. When an ink on a document is subjected to light of a wavelength in the near-IR region (NIR), its behaviour can be visualised on the viewing monitor as a change in appearance. For example, an ink that does not absorb any IR radiation, but only visible radiation, will appear white on screen as light in the NIR (~730 nm) is directed upon it [11], effectively making it invisible against the background of the paper substrate [11, 46]. However, if the ink completely absorbs IR radiation as the wavelength of light moves out of the visible region into the NIR region it will appear black [37]. The less IR radiation the ink absorbs as the wavelength moves further into the IR region, the lighter the shade of grey the ink line will appear until eventually it disappears on screen when it stops absorbing IR radiation [37]. A series of filters corresponding to different wavelengths of IR radiation can be placed into the path of the illumination source to determine at what wavelength an ink stops absorbing into the IR region thereby providing a useful means for comparing and discriminating inks [11].

1.5.2.2.2 Infrared Luminescence/Fluorescence (IRL)

Complimentary to IRR, is the phenomena of IR luminescence (IRL) or fluorescence which can be very useful for added discrimination since different inks will luminesce at variable intensity and at different wavelengths [11]. This involves directing light of a specific wavelength in the visible region onto the ink to promote the emission of fluorescence at a longer wavelength in either the visible or IR region. Visible light is normally passed through a blue-green filter to provide the excitation wavelength (650 – 900 nm) in order to determine which corresponds to the luminescence of the ink under study [11]. To the examiner, a luminescent ink line will appear brilliant white on the VDU screen, varying in intensity between different inks, whilst a non-luminescent ink will appear black [46].

1.5.2.2.3 Disadvantages of IRR and IRL

Although both IRR and IRL can be very useful in ink examination, there are several potential issues that an examiner needs to consider in order to make a correct interpretation of whether two or more inks are, in fact, different. Ellen [11] highlights the significance of ink line thickness in the interpretation of IRR. It is possible that two separate ink samples on the same document written with the same pen could be misinterpreted as having been written in two different inks, if there is a significant difference in their line thickness since a thicker line will exhibit a more intense IRR response than a thinner line. This is also a consideration for IRL examinations [37], which can also be influenced by effects from different paper substrates and contact solvents [11, 37]. The paper upon which the ink being examined is deposited may also exhibit luminescence at the same wavelength as the ink. If the luminescence of the paper is more intense than that of the ink then the fluorescence from the latter may be masked, making it appear as if the ink is non-luminescent at that wavelength. This is more of a consideration when an ink on different paper substrates needs to be compared, than for ink on the same substrate [37]. Additionally, IRL of an ink maybe affected by the colour of the paper it is written on, since the excitation wavelength may be absorbed by the dyes in some coloured papers [37]. The final consideration is that of quenching of any IRL by an ink that may contain different compounds that in combination both absorb and emit fluorescence at the same excitation wavelength. Such compounds can be separated from each other on the paper as a result of contact between the ink and a solvent such as sweat arising from handling of the document [11, 37]. This may have the effect of making an ink entry written in the same ink as the rest

of the document exhibit a difference in IRL which may be erroneously interpreted as the document having been written with two different inks.

1.5.2.2.4 UV Fluorescence

UV fluorescence uses both short (265 nm) and long (365 nm) wavelengths in the UV region to excite the dye molecules in the ink thus promoting the emission of fluorescence of a wavelength in the visible region [11]. UV fluorescence has limited use in the examination of everyday writing inks as the majority of blue and black inks, the most common colours encountered in forensic casework [59, 60], absorb radiation in the UV region and therefore do not fluoresce under these conditions [11]. However, red inks do tend to exhibit a variable degree of fluorescence which can assist in their comparison and discrimination [11, 46]. Brunelle and Reed [46] also comment that UV fluorescence lighting is useful for modern synthetic dyestuff used in fountain pen inks, though it must be stressed that these types of inks are no longer routinely encountered in everyday writings, but tend to be reserved for documents of a specialist nature such as official documents requiring a signature [12].

1.5.2.2.5 Hyperspectral Imaging (HSI)

In recent years, Hyperspectral Imaging (HSI) has emerged as a promising technique for the analysis of a variety of forensic samples, including writing inks [61-64]. HSI is a combination of two techniques, Digital Imaging and Reflectance or Fluorescence Spectroscopy [62]. Through the use of liquid crystal tuneable filter (LCTF) technology [63, 64], or a combination of a 100 W halogen spot light and continuously variable band pass filter [65], it is possible to view the ink on a document over a series of finely tuned wavelengths across the visible-NIR range. For example, in the VSC 6000/HS, it is possible to examine a document across a range of 400 - 1000 nm at incremental steps of between 1 – 20 nm in order to produce a digitally stored image cube [66]. An operator can playback the images as a real time movie to determine how two or more inks behave across the wavelength range, making it easier to visualise differences in their appearance under filtered light. In addition, the intensity of each pixel in each image is plotted as a function of wavelength, allowing the operator to produce a reflectance or fluorescence spectrum of the ink at any given wavelength in order to detect subtle spectral differences between chemically similar inks [64]. The potential for enhanced discrimination together with the non-destructive nature of this technique and

minimal sample preparation means HSI has considerable benefits over existing non-destructive filtered light examination techniques [62-64].

1.5.2.3 Thin Layer Chromatography (TLC)

For some forensic examinations of ink on questioned documents, the combination of visual assessment by eye with the aid of a low power microscope and filtered light examination using a VSC is sufficient to distinguish between two inks of similar appearance, and thus no further examinations are required. However, if after these non-destructive examinations have been performed, the two entries in question remain indistinguishable, it may be necessary to perform further destructive chemical analysis of the ink by a technique such as thin-layer chromatography (TLC) [11, 46, 55].

TLC is a type of planar chromatography, and is a simple technique that can be used to separate out the dye and other non-visible organic components present in an ink formulation [11, 46, 55]. A qualitative comparison of the dye components present for two or more ink samples chromatographed together under the same conditions can be made to determine if they could be of the same formulation [11, 46, 55]. Moreover, it may be possible to identify the ink formulation by comparison to a known standard [55]. A quantitative assessment of the relative concentration of each dye component may also be made by Densitometry [11, 46, 55]. Numerous standardised procedures for describing the TLC analysis of different writing inks can be found in the literature [46, 55, 67-69], but they all involve the same general procedure [70].

1.5.2.3.1 Stationary and Mobile Phases

The stationary phase is a thin layer of micro particulate sorbent material (~250 µm), such as silica, held on the surface of a flat medium, such as aluminium. Often, a soluble fluorescent reagent is included in the stationary phase material that acts to combine with the solutes to make component bands visible under UV light. This is particularly useful for visualising components that are otherwise colourless under white light. Wide arrays of TLC plates are commercially available. These vary in size (5 – 20 cm square) and stationary phase, from which the most suitable to the required application can be selected.

The mobile phase can either be a single solvent or, more commonly, a mixture. The latter is preferred because it is possible to vary the proportions of individual solvents within the mixture to influence overall eluting power of the mobile phase and maximise resolution of the component bands. The R_f values of the component bands are influenced by the overall polarity of the mobile phase, which will determine the rate at which component solutes migrate. A typical mobile phase for inks is n-butanol or ethanol with water.

1.5.2.3.2 Retention Factor (R_f) Values

The term retention is used to describe the "interaction of solutes with stationary phase slows down the migration relative to the velocity of the mobile phase" [70]. After separation, a numerical value (R_f value) for each component band can be calculated using Equation 1.4. An example of a developed TLC plate together with associated R_f values for separated component spots is presented in Figure 1.5.

$$R_f = \frac{\text{Distance spot travelled from baseline}}{\text{Distance between solvent front and baseline [32]}} \qquad \text{Equation 1.4}$$

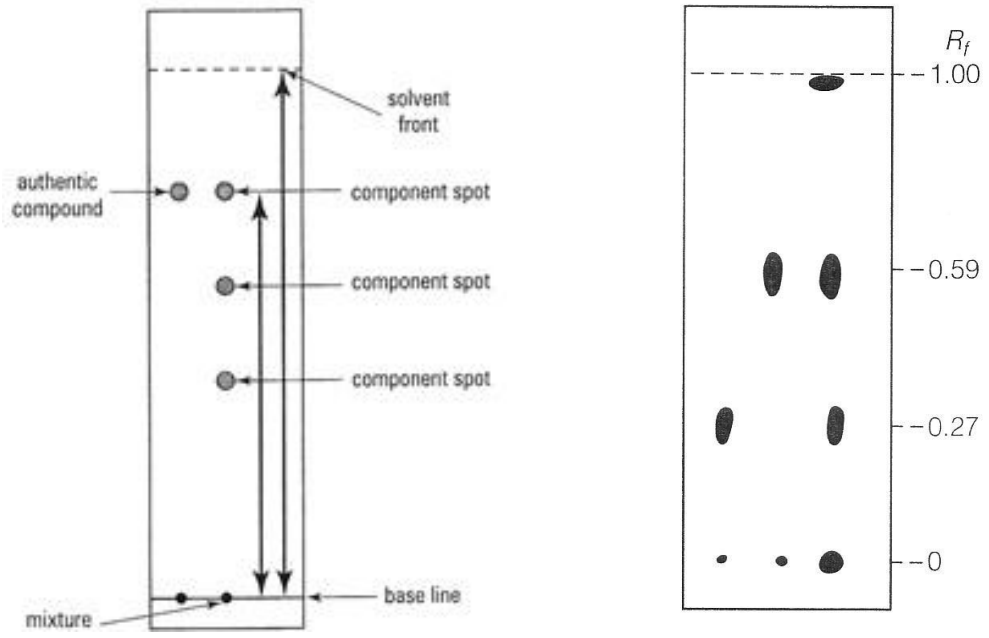


Figure 1.5: Example of a developed TLC plate with associated R_f values for component spots (reproduced from [70, 71])

An array of alternative analytical techniques have been applied to the analysis of writing inks, predominantly ballpoint inks due to their popularity, with varying degrees of success.

These have mostly focussed on the analysis of colorants, but some have analysed other ink components, and have included:

- Chromatographic Techniques: High Performance Thin-Layer Chromatography (HPTLC) [72-77], High Performance-Liquid Chromatography (HPLC) [78-83],

- Electrophoretic Techniques: Capillary Electrophoresis [84-87]

- Mass Spectrometric Techniques: Gas Chromatography-Mass Spectrometry (GCMS) [73, 88, 89], Electro-Spray Ionisation Mass Spectrometry (ESI-MS) [90], Inductively Coupled-Mass Spectrometry (ICP-MS) [91], Field Desorption-Mass Spectrometry (FD-MS) [92], Direct Analysis in Real Time (DART) [93], Laser Desorption-Mass Spectrometry (LD-MS) [94-102], Matrix Assisted Laser Desorption-Mass Spectrometry (MALDI-MS) [103, 104], Time of Flight-Secondary Ion Mass Spectrometry (ToF-SIMS) [105] and Laser Ablation-Inductively Coupled Plasma-Mass Spectrometry (LA-ICP-MS) [106].

- Other Spectroscopic Techniques: Microspectrophotometry (MSP) [74, 107-113], Fourier Transform Infrared Spectroscopy (FTIR) [83, 114-120], Raman Spectroscopy [121-125], Inductively Coupled-Atomic Emission Spectroscopy (ICP-AES) [126] and Laser Induced Breakdown Spectroscopy (LIBS) [125, 127].

Despite the availability and emergence of alternative analytical techniques, TLC has remained popular as the method of choice for further discrimination of writing inks due to its ability to provide a rapid, cost effective means of discrimination from only a small quantity of ink. With the development and increasing popularity of gel inks however, a problem with the traditional method of ink examination has arisen. Since gel inks predominantly use insoluble pigments to provide colour rather than soluble dyes, TLC is ineffective for the discrimination of many gel inks other than to confirm their pigmented nature. To that end, there is the need for a new analytical approach to be developed for their examination. Spectroscopic analysis offers great potential for the successful examination of pigment based gel inks, and together with the traditional methods of Filtered Light Examination (FLE) and TLC, forms the main focus of this thesis. In particular,

Visible-Microspectrophotometry (Vis-MSP), Fourier Transform Infrared Spectroscopy-Attenuated Total Reflectance (FTIR-ATR) and Raman Spectroscopy have been investigated.

1.5.2.4 Spectroscopic Techniques

1.5.2.4.1 Visible-Microspectrophotometry (Vis-MSP) [32]

The human eye detects visible radiation sending signals to the brain which are then interpreted in terms of both what colour the eye perceives and how bright that colour is. It is believed the human visual system has the ability to distinguish up to six million different shades of colour. However, occasions arise frequently where two or more colours of ink when viewed under identical lighting conditions appear to be visually indistinguishable, yet their chemical composition may be different due to the manufacturing process. Microspectrophotometry (MSP) can be used to investigate these compositional differences without the need to solubilise the sample.

1.5.2.4.1.1 Principles of Microspectrophotometry [32]

MSP is a spectroscopic method that involves directing UV-Vis electromagnetic radiation upon a coloured sample to induce transitions between electronic energy levels of chemical bonds within the molecule under analysis [36]. An electronic transition will only occur where the energy of the incident radiation corresponds exactly to the energy required to excite an electronic transition within the molecule [36]. When this is the case, some of the incident radiation is absorbed by the molecule, and an electron in the HIGHEST OCCUPIED MOLECULAR ORBITAL (HOM0) becomes excited and is promoted to the LOWEST UNOCCUPIED MOLECULAR ORBITAL (LUMO) [128]. Only two types of electronic transition associated specifically with the π and n valency electrons surrounding the chemical structure of the colourant, i.e. chromophore and auxochrome, can be excited by energies associated with the UV-Vis range (200 – 800 nm) [32, 128]. Visible radiation is absorbed when π valency electrons surrounding conjugated double and triple bonds found in the chromophore are excited (causing $\pi - \pi^*$ electronic transitions), the ease of which determined by the greater the number of conjugated bonds present [32, 128]. Absorption of UV-Vis radiation occurs when n electrons present in both the chromophore and auxochrome groups become excited (causing $n - \pi^*$ electronic transitions) [32, 128]. A spectrometer coupled to a computer generates the final output, an absorption spectrum, which provides a graphical representation of the amount of radiation absorbed by the

colourant and at which wavelengths the absorption occurs (Absorbance 'v' Wavelength) [128]. The λ_{max} position on the spectrum, i.e. the apex of the tallest peak, represents the wavelength at which maximum absorption occurs and corresponds to the energy required to excite the electronic transition. The λ_{max} therefore provides a means by which to identify the chromophore responsible for the absorption [36]. Since colour and its depth is determined by the amount of radiation absorbed by the chromophore and auxochrome groups respectively, the absorption spectrum represents an objective means by which to compare the colour of two or more inks [32].

1.5.2.4.1.2 Instrumentation [32]

The basic instrumental components include a microscope, spectrophotometer and a computer with specialist software installed, in order to control spectral acquisition and perform data processing. A printer is often also attached via the computer, in order to produce print-outs of the spectra to enable direct comparison by eye. The sample is placed onto the microscope XYZ translation stage and brought into focus using the focus controls, either through the monocular eyepiece, or via the video image on the computer screen provided through a camera attached to the microscope. The spectrophotometer comprises three essential components: a light source, a monochromator and a photomultiplier tube. The light source, typically a tungsten or xenon lamp, provides the electromagnetic radiation required to excite the electrons, either visible or UV respectively. A grating monochromator is positioned before the sample to convert light from the source into monochromatic light of the desired spectral range, i.e. 400 – 700 nm. A photomultiplier tube is positioned after the sample to convert light within a range of 200 – 800 nm into electrical energy. The computer and specialist software, will convert this electrical energy into an absorption spectrum. In addition, the software may be capable of providing an objective interpretation of the colour observed via the use of (complimentary) chromaticity co-ordinates (see Colorimetry). The instrument can be operated in several modes including transmission, absorption, and reflectance, the latter being desirable when it is not possible to transmit light through a sample because of its physical nature, i.e. opaque paint flakes.

1.5.2.4.1.3 Interpretation [32]

MSP is a technique used for the identification and comparison of colour only. It is not able to identify a particular colorant. Comparison of spectra from two or more different (ink)

samples would traditionally be achieved by overlaying print outs of spectra on top of one another whilst holding up to a source of natural light. Features for comparison include the wavelength at which absorption is at a maximum (λ_{max}) and a minimum (λ_{min}), the shape of the spectral curvatures and any shoulders present, points of inflection and relative peak intensities. Software can be used to compare spectra on-screen from two or more samples on the same axis, as well as providing normalisation and smoothing functions to aid direct comparison and remove any noise. A positive match is one where the spectrum from Sample A fits strictly within the range of those spectra obtained from Sample B. Furthermore, where several spectra for a given sample have been acquired and substantial sample variation observed, at least one spectrum from Sample A should have no significant differences from at least one spectrum in Sample B. This does introduce a subjective element to an otherwise objective means to interpretation when spectra appear similar. However, mathematical and/or statistical algorithms can be applied to transform and compare spectral data to enable possible further discrimination. For example, the first derivative spectral data can be used to highlight subtle areas of difference in spectra from two or more samples that may not be obvious from the raw spectral data [129, 130]. The first derivative method is based on co-ordinate geometry, where the slope of the curves in a spectrum is measured to provide an alternative spectral output. Using Equation 1.5, the slope of the line (M) between two points (x_1, y_1) and (x_2, y_2) can be calculated.

$$M = (y_2 - y_1)/(X_2 - X_1)\ [129] \qquad \text{Equation 1.5}$$

The slope, expressed in Abs/nm (absorbance/wavelength), represents the rate of incline or decline of the curve. The first derivative spectrum therefore is a plot of slope (M), or absorbance, against wavelength. Whilst the first derivative spectrum can reveal subtle differences in spectral response from two or more samples, it should always be used in tandem with the original raw spectral data to reach a conclusion as to whether two or more samples are chemically indistinguishable in terms of their colour. An example of a raw spectrum and the associated first derivative for three microscopically similar blue acrylic fibres is presented in Figure 1.6, highlighting subtle chemical differences. This method has been used widely in the forensic examination of textile fibres over the past decade [129-134], but has courted some recent controversy due to subjective interpretation [135, 136].

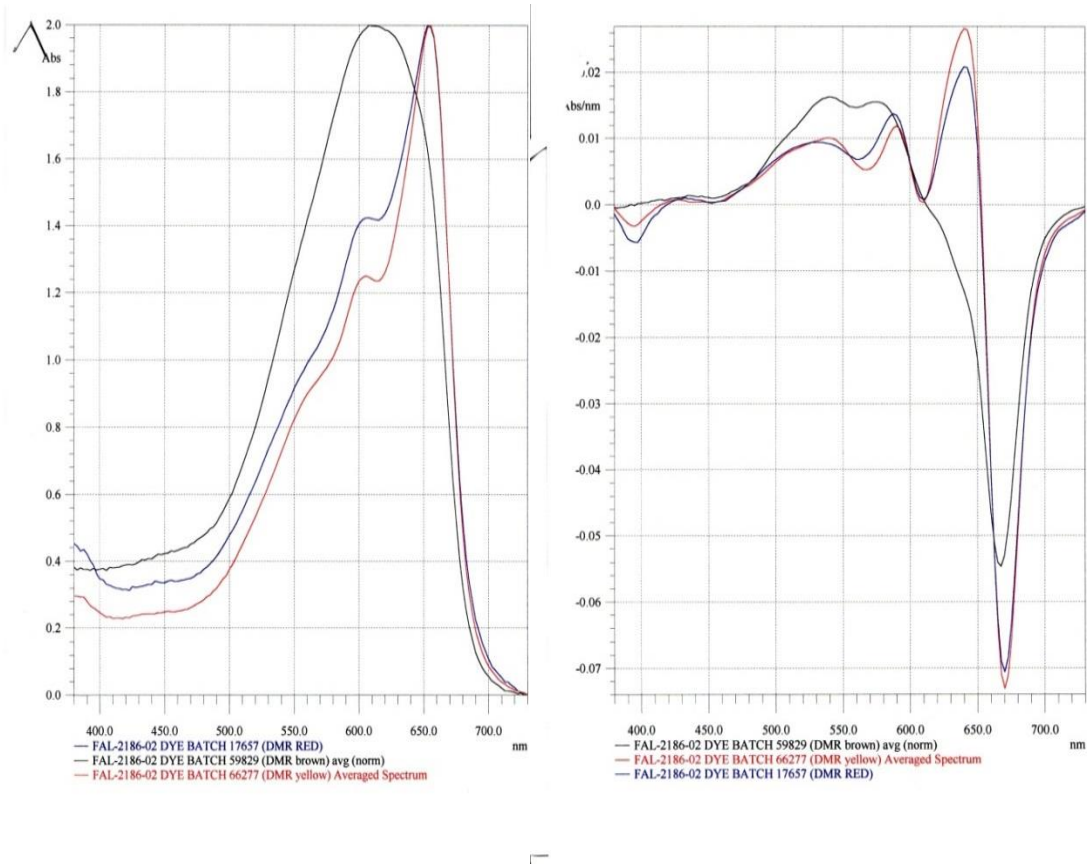


Figure 1.6: Example of Vis-MSP spectra from three blue acrylic fibres taken from three different dye batches. MSP spectra (left) show fibre denoted by black spectral line to be clearly different to the other two which appear indistinguishable. First derivative spectra (right) show a subtle difference around 500 – 600 nm between the fibres represented by blue and red spectral lines suggesting all three batches can be discriminated (reproduced from [32])

1.5.2.4.2 Infrared and Raman Spectroscopic Analysis

IR Absorption and Raman Spectroscopy are two different analytical techniques that both use electromagnetic radiation to excite vibrational transitions within a molecule to provide information concerning the chemical structure of a compound [137]. This information is presented in the form of an IR absorption spectrum or Raman spectrum respectively. These spectra are characteristic for the compound under study that can be used for identification and comparison purposes as well as quantitative determination of the chemical components present [137]. The two techniques provide different, but complimentary, information about the molecular structure of the compound under study, and for this reason are frequently employed in tandem.

The fundamental principle of IR Absorption and Raman Spectroscopy holds its roots in the different properties of electromagnetic radiation. Two theories are used to explain the

different properties of electromagnetic radiation: the wave theory and the particle theory [138]. The particle, or quantum theory, is the most important for explaining the principle of both techniques as it provides an explanation for the interaction of electromagnetic radiation with a molecule in terms of energy and its relationship with the frequency of radiation [137]. It is important to acknowledge the relationship between frequency (v) and wavelength (λ) which is represented in Equation 1.6:

$$\lambda = c/v \quad [138] \qquad \text{Equation 1.6}$$

Where c is the velocity of light in a vacuum (3.00×10^{8} m/s)

Quantum theory suggests that "electromagnetic radiation is a stream of discrete particles or wave packets of energy" [138]. Each of these packets of energy, called quanta or photons, possess energy (E) that is proportional to the frequency of radiation as demonstrated by Equation 1.7:

$$E = hv \quad [138] \qquad \text{Equation 1.7}$$

Where h is Planck's constant (6.63×10^{-34} J/s)

The relationship between energy and wavenumber ($\tilde{v}$ (cm^{-1})) is represented in Equation 1.8:

$$E = hc/\lambda = hc\tilde{v} \quad [138] \qquad \text{Equation 1.8}$$

Thus wavenumber is proportional to the frequency of vibration, or the reciprocal of wavelength, which in turn is proportional to energy, represented in Equation 1.9:

$$\tilde{v} = v/c = 1/\lambda \quad [137, 138] \qquad \text{Equation 1.9}$$

1.5.2.4.2.1 Vibrational Transitions [32]

Quantum theory proposes that each molecule possesses its own specific set of energy states, which combined contribute to its overall energy [138]. The term ground state is used to describe the lowest of these energy states or levels and the majority of molecules will occupy the ground state at room temperature [138]. When a molecule is in contact with either UV, Visible or IR radiation it becomes excited by transfer of energy from the

photon to the molecule, promoting it from ground state to a higher energy excited state [138]. There are three possible energy transition states that can occur within a molecule when this is the case:

- Electronic transitions
- Vibrational transitions
- Rotational transitions

Electronic transitions are concerned with the absorption of UV and visible radiation, whilst vibrational and rotational transitions can be induced by interaction with visible and IR radiation [138]. IR and Raman Spectroscopy are concerned specifically with vibrational transitions which relate to the chemical bond structure within the molecule [137]. Within the chemical bonds, there are many energy levels called vibrational states. Chemical bonds may vibrate in several ways when radiation interacts with the molecule. Stretching of the bonds may occur, either symmetrically or asymmetrically; or the bond may bend by rocking, scissoring, wagging and twisting [138]. A classic example is the triatomic molecule of carbon dioxide, whose bonds exhibit both symmetric and asymmetric stretching as well as a bending vibration, shown in Figure 1.7 [137]. The energies associated with each of the vibrational states are different, and for each electronic state within a given molecule there are a number of different vibrational energy levels [138].

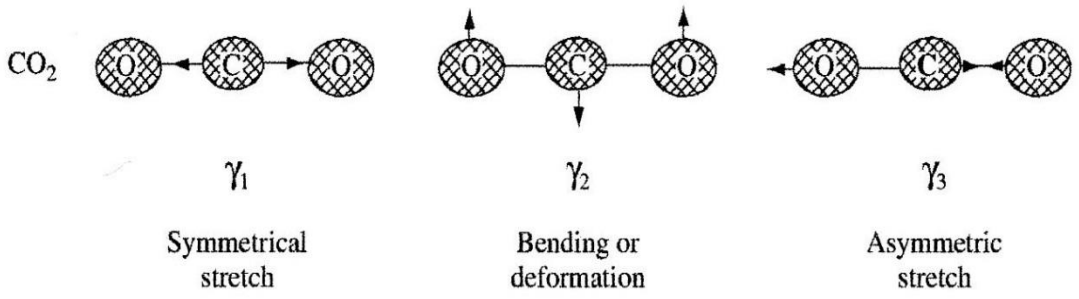


Figure 1.7: Stretching of chemical bonds in a carbon dioxide molecule (reproduced from [137])

1.5.2.4.2.2 Infrared Spectroscopy: Absorption of Radiation [32]

The IR region of the electromagnetic spectrum is divided into three sub-regions,: Near Infrared (NIR (12820 cm^{-1} – 3333 cm^{-1}), the Mid Infrared (3333 cm^{-1} – 333 cm^{-1}) and Far Infrared region (333 cm^{-1} – 33 cm^{-1}). IR Absorption Spectroscopy is concerned with

absorption of radiation by molecules in the Mid-IR region only, because its energy corresponds to the difference in energy between the vibrational transition states of organic molecules. Absorption is the process whereby a "chemical species in transparent medium selectively attenuates certain frequencies of electromagnetic radiation" [138]. This means that when a material, such as ink, absorbs radiation, its chemical nature will decrease the intensity of certain frequencies of radiation.

If a photon with an amount of energy that precisely corresponds to the energy difference between the ground state and higher energy state of a particle passes close to that particle, then absorption of radiation will occur. Energy is passed from the photon to the particle promoting the particle to a higher energy level. The particle is then in an excited state and will remain so for a period of 10^{-6} – 10^{-9} seconds (s) before returning to ground state. Excess energy is subsequently passed to other particles within the sample. Because a particle is in its excited state for only a brief moment, the concentration at any given time is so small that it will not have any detrimental effect on the sample under analysis. In addition, undetectable amounts of thermal energy are released upon return to ground state.

An absorption spectrum is generated that is a "plot of some function of attenuation of beam radiation 'v' wavelength, frequency or wavenumber" [138]. The terms Transmittance (T) and Absorbance (A) are used to describe the amount of radiation being transmitted or absorbed by certain chemical species. Transmittance is defined as "the fraction of incident radiation emerging from the sample once it has passed through it" [32]. Absorbance is related to transmittance by the relationship shown in Equation 1.10.

$$A = Log\ (\frac{1}{T})$$ [138] Equation 1.10

Absorbance basically describes the amount of radiation that is absorbed or is not transmitted through the sample under analysis. Typically an IR spectrum will be constructed by plotting wavenumber (cm^{-1}) along the x-axis and % Transmittance along the y-axis. However, in forensic science, it is not uncommon for the IR spectrum to be presented in units of Absorbance as demonstrated in Figure 1.8. For this reason, the latter approach has been adopted for the presentation and discussion of FTIR-ATR in this thesis.

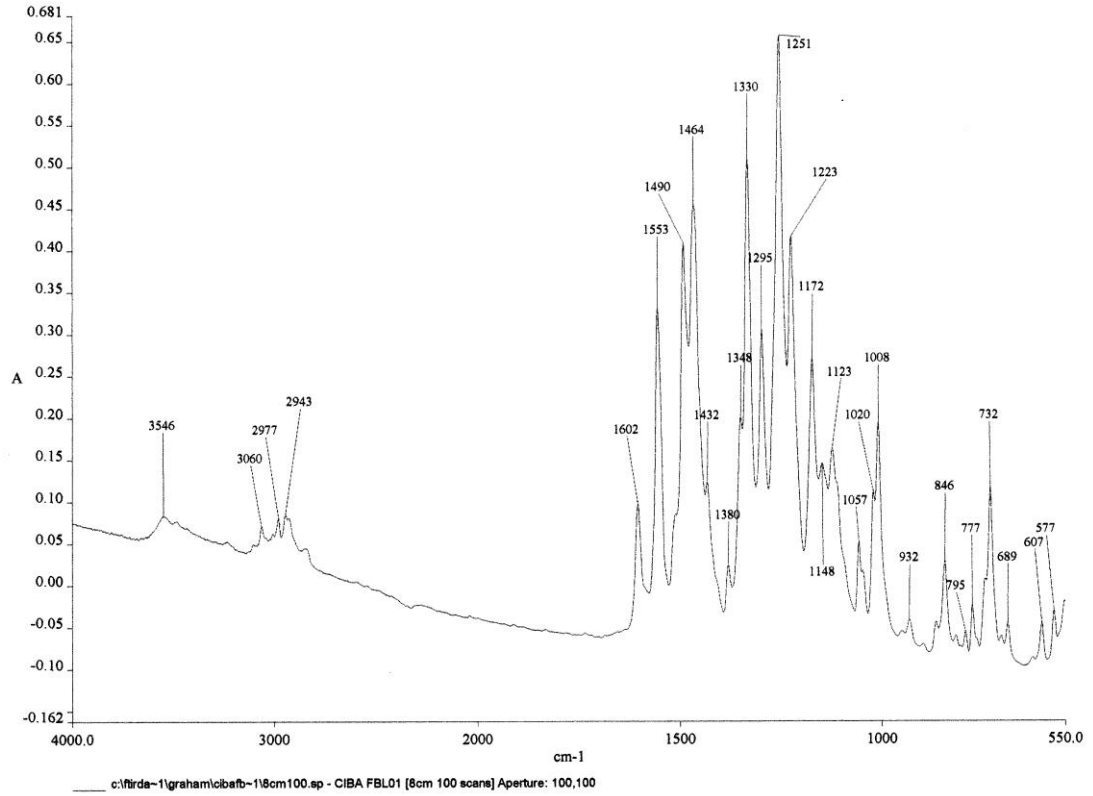


Figure 1.8: Example of an IR absorption spectrum of a Ciba Maxilon Black FBL-01 dye with key peak absorptions labelled [8 cm^{-1} resolution, 100 scans]

1.5.2.4.2.3 Raman Spectroscopy: Scattering of Radiation

Raman Spectroscopy uses a single wavelength (or frequency of radiation) of visible or NIR electromagnetic radiation to change the shape of the electron cloud surrounding the nuclei in the molecule. This change of shape or distortion of the electron cloud reflects a change in polarization of the molecule and may result in nuclear motion, i.e. a change in distance between the nuclei of the molecule [137, 139]. The energy provided by the incident photons is enough to briefly excite the molecule to a temporary virtual state, resulting in the emission of scattered radiation in all directions as the molecule falls back to another energy level as shown in Figure 1.9 [137, 140]. This scattered radiation can be in the form of Rayleigh scattered and Raman scattered radiation [137]. In Rayleigh scattered radiation, (elastic scattering) the more dominant process, a distortion of the electron cloud occurs, but with no accompanying change in distance between the nuclei of the molecule. The result is the scattering of radiation with the same wavelength as that of the incident radiation and is of no analytical value [139]. With Raman scattering (inelastic scattering),

nuclear motion is induced as a result of the change in polarization, giving rise to two Raman scattering mechanisms: Stokes and Anti-Stokes scattering [137].

Stokes scattered radiation occurs when energy is transferred from the incident photon to the molecule promoting it from its ground vibrational state (m) to an excited state (n) with higher energy. This occurs via a virtual state whose energy is determined by the frequency of the incident laser [137]. Since the majority of molecules exist in the ground state at room temperature, Stokes scattered radiation is the dominant Raman Scattering mechanism [137]. However, a small proportion of molecules will already exist in an excited state as a result of thermal energy, the exact number determined by the Boltzmann Equation [137]. Anti-Stokes scattered radiation occurs as a result of energy transfer from the molecule to the scattered photon, demoting the molecule from an excited state to its ground vibrational state via the virtual state [137]. Since the number of molecules in the ground state is far greater than those already in an excited state prior to interaction with the laser, Stokes scattering is more frequently used as the basis of Raman Spectroscopy [137]. The energy difference between the lowest excited state (n) and the ground state (m) is detected as the difference between the incident and scattered radiation which corresponds to one vibrational unit [137]. This shift in energy is presented in the form of a Raman spectrum with wavenumber (cm^{-1}) along the x-axis, normally spanning a range between 3600 – 200 cm^{-1} [137], and the intensity (arbitrary units) of the scattered radiation along the y-axis. Figure 1.10 provides an example of a Raman spectrum.

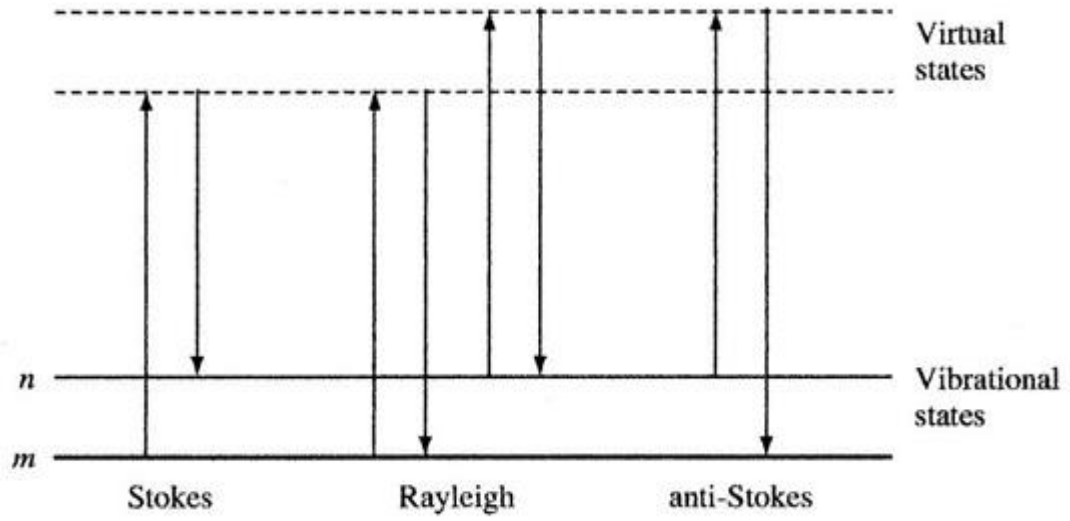


Figure 1.9: Excitation processes in Rayleigh and Raman scattered radiation (reproduced from [137])

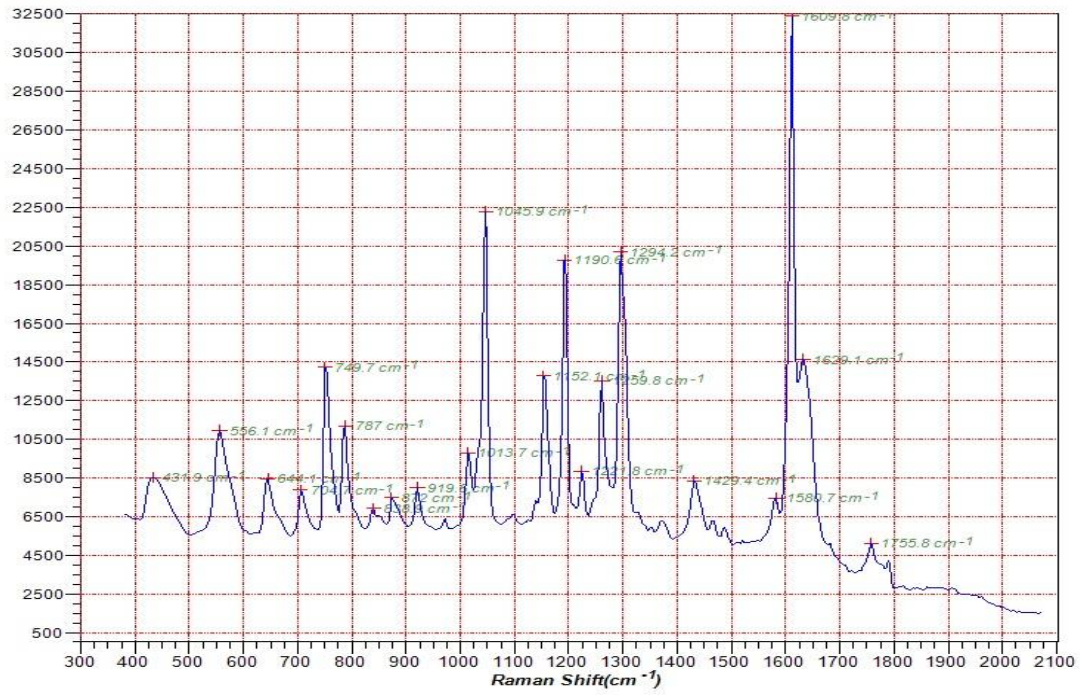


Figure 1.10: Example of a Raman Spectrum of an aspirin tablet taken at 785 nm excitation wavelength with key peaks labelled

1.5.2.4.2.4 IR Absorption Spectroscopy in Comparison with Raman Spectroscopy

A distinction between Raman Spectroscopy and IR Absorption Spectroscopy can be drawn. In Raman Spectroscopy the sample is exposed to a single wavelength of high energy radiation and the resulting scattered radiation is used to determine structural information about the molecule [137]. However, in IR Absorption Spectroscopy, the sample is irradiated by a range of wavelengths (or frequencies) to ensure the frequency corresponding to the exact energy required to excite vibrational transition is absorbed and detected as a loss in frequency of radiation from the incident beam transmitted through the sample to provide structural information [137].

Not all vibrational transitions can be excited by IR Absorption and Raman Spectroscopy, since not all will absorb or scatter radiation respectively [137]. Both techniques rely on a change in the different properties of the molecule arising from its interaction with electromagnetic radiation [141]. Whether a vibrational transition will give rise to an intense peak or band in an IR or Raman spectrum will depend upon whether it is IR active or Raman active respectively [137]. For a vibrational transition, and thus the molecule, to be IR active and absorb radiation, the interaction with incident electromagnetic radiation must cause a change in the dipole moment of the molecule during the vibration [137]. For

the vibrational transition, and thus the molecule, to be Raman active and scatter radiation, the incident electromagnetic radiation must cause a change in polarization of the molecule during the vibration [137].

As highlighted earlier, molecules can vibrate in a number of ways. These vibrations are also termed as the vibration mode of a molecule [137]. Some of the modes of vibration give rise to intense Raman scattering, for example symmetric stretching, whilst others result in an intense absorption of radiation, such as bending vibrations [137]. Returning to the example of carbon dioxide, this triatomic molecule exhibits a symmetric stretch, asymmetric stretch and a bending vibration when irradiated [137, 141]. Of these three vibration modes, only the symmetric stretch is Raman active since it causes a change in polarization of the molecule sufficient to cause nuclear motion and hence intense Raman scattering [137, 141]. There is no change in dipole moment for this vibration mode and therefore it is IR inactive, unlike the asymmetric stretch and bending vibration modes which do exhibit a change in dipole moment, but no change in polarization and therefore strongly absorb IR radiation making them both IR active, but Raman inactive respectively [137, 141]. In terms of how these vibrations are presented in the resulting spectra, all three vibration modes will be represented as peaks or bands in the IR and Raman spectrum of carbon dioxide, yet their spectra will not be identical [140], because those vibration modes that are Raman active will appear much more intensely in the Raman spectrum than in the IR spectrum. Likewise, those vibration modes which are IR active will be much stronger in the IR spectrum than in the Raman spectrum [142].

Whether a molecule is IR active, Raman active or both will therefore depend on the nature of the vibrations induced in them by interaction with electromagnetic radiation, which ultimately will be determined by the type of chemical bond present in the structure of the molecule [140]. Compounds containing bonds with a dipole moment, i.e. C-O, C-N, N-H, O-H, etc. will yield more information from IR Absorption Spectroscopy, whilst compounds containing non-polar bonds, such as the double and triple bonds found in dye and pigment molecules will provide more information in Raman Spectroscopy [140]. Since the spectra produced by both techniques provide different information about the vibrational state of the molecule, they are considered to provide complimentary information about the chemical structure of the compound under study [137, 140]. This is illustrated by the ability

to distinguish between different isomers of the centrosymmetric molecule such as copper phthalocyanine, which do not possess bonds that can be both IR and Raman active [137].

For complex molecules such as dyes and pigments, the number of possible vibrations may be large. The number of vibrations for a given molecule can be determined by considering its total energy as a number of degrees of freedom used to describe its spatial arrangement [137]. For non-linear molecules, the number of vibrational transitions and hence the number of bands in a Raman spectrum associated with those transitions can be calculated from Equation 1.11, whilst for linear molecules they can be determined from Equation 1.12 [137]. The number of atoms in the molecule is denoted by the letter N, whilst the number of degrees of freedom associated with the translational and rotational movement of the molecule is denoted by the subtraction factor (i.e. 6 for non-linear molecules).

$$3N - 6 \text{ (non-linear molecules) [137]} \qquad \text{Equation 1.11}$$

$$3N - 5 \text{ (linear molecules) [137]} \qquad \text{Equation 1.12}$$

Clearly for a complex molecule with a large number of atoms, the number of possible vibrations and potential corresponding bands in a spectrum would be large making it impossible to identify all the vibrational states of the molecule under study, and a simpler approach is required [137, 142]. Individual vibrations can be combined to represent groups of atoms connected by chemical bonds of similar energy and of close proximity to each other so as they interact [137].

These combinations of individual vibrations are called group frequencies and as a consequence of spatial arrangement within the molecule can represent different types of vibration, and hence can be distinguished from each other on the basis of sufficient differences in bond vibrational energies and/or distance between bonds [137]. These group frequencies are represented as individual peaks or Raman bands in the spectrum, and therefore it is possible to derive information about the type of chemical bond or functional group involved on the basis of its wavenumber position and relative intensity [137]. The wavenumber position for a group frequency will be dependent upon the relationship between the mass of an atom, its bond strength and vibrational frequency as

described by Hooke's Law [137]. However, a general rule is that higher frequencies of scattered radiation will be associated with strong bonds and light atoms, whilst weak bonds and heavy atoms will give rise to low frequencies of scattered radiation [137]. Group frequency tables can be used as a means to interpret the chemical structure of the molecule from the wavenumber position (Raman Shift (cm^{-1})) of the Raman bands [137]. For example, many of the functional groups associated with dyes or pigment molecules can be determined from the Raman Shift of peaks in a spectrum representing characteristic group frequencies. Similar frequency tables are available for IR Absorption Spectroscopy.

1.5.2.4.2.5 Instrumentation

1.5.2.4.2.5.1 IR Absorption Spectroscopy [32]

There are various different forms of IR instrumentation commercially available. However, two basic types of spectrometer are common, the dispersive and interferometric instruments. The essential components of each are basically the same. Each instrument must contain a source of radiation to provide a light beam in the Mid-IR region. The sample is placed in the path of the light beam, and as the light emerges from the sample it is collected by a monochromator, typically a diffraction grating, which acts to disperse radiation into its component wavelengths. A thermal detector connected to a data processor then detects each of these wavelengths, converting the signal into an IR (absorption) spectrum.

1.5.2.4.2.5.1.1 Dispersive Spectrometers [32]

Dispersive Spectrometers may be either single beam (Figure 1.11) or double beam (Figure 1.12) instruments, both of which possess a single source of radiation, typically an oxide coated ceramic rod, which is heated to a high temperature (~1500°C). What happens to the emitted radiation beam is dependent upon the type of instrument. For single beam instruments, the sample is placed in front of the beam allowing it to pass through the detector where it is converted into a sample spectrum. The sample is then removed and the beam allowed to either pass through air or a reference cell containing pure solvent (in which the sample has been prepared) to the detector to generate a background or reference spectrum respectively. The background spectrum is then subtracted from the sample spectrum to give an IR spectrum of the sample alone, however this is time consuming. By contrast, double beam instruments are capable of recording both sample

and background beams simultaneously. The radiation beam from the source is split into two beams of equal intensity by a beam splitter. A system of mirrors directs one beam through the sample (sample beam), whilst the other is directed through air or pure solvent (reference beam). The two emerging beams are detected by one or two identical photodetectors, and the difference in intensity of the two beams is measured and represented as a peak in the IR spectrum occurring at wavenumber positions characteristic of the functional groups from which they have originated.

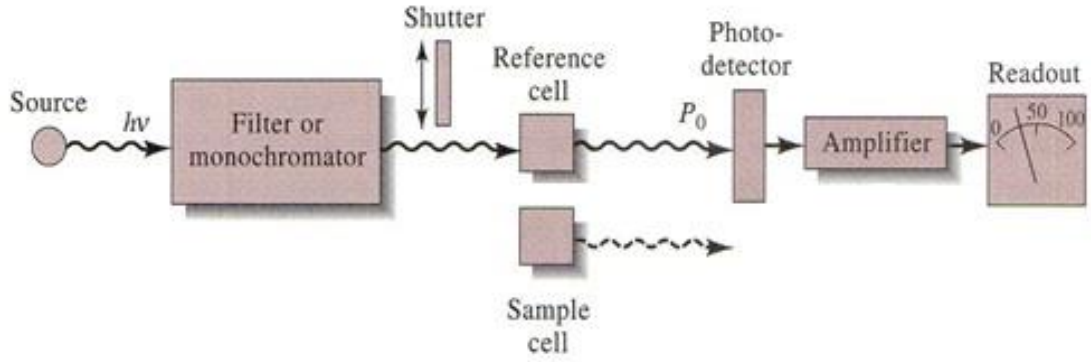


Figure 1.11: Single beam dispersive IR Spectrometer schematic diagram (reproduced from [138])

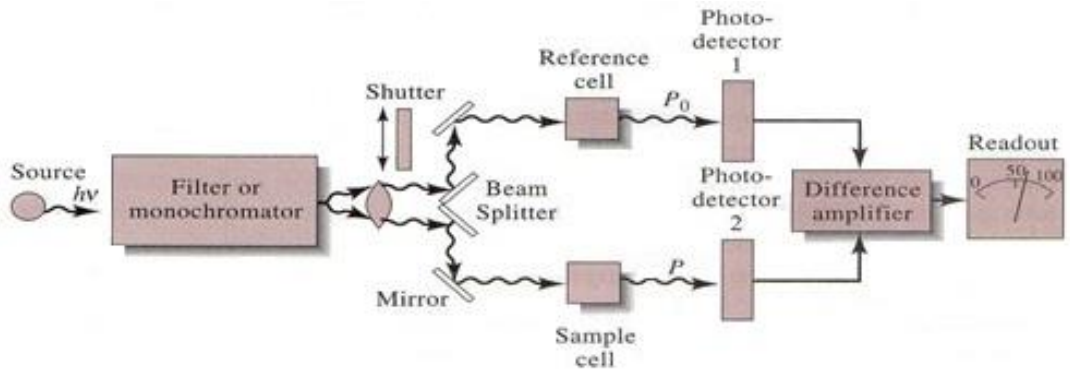


Figure 1.12: Double beam dispersive IR Spectrometer schematic diagram (reproduced from [138])

1.5.2.4.2.5.1.2 Interferometric Spectrometers [32]

Interferometric Spectrometers similar to that shown in Figure 1.13 are referred to as Fourier Transform Spectrometers (FTIR), so called because of the mathematical conversion process used to transform detected signals into an IR spectrum. The radiation source, typically polychromatic, generates a beam of radiation composed of the complete range of frequencies within the IR region. Similar to the double beam instrument, the beam is split in two, but in contrast one beam is directed to a fixed mirror (fixed optical path length) and

the other to a moving mirror (variable optical path length). Both beams reflect back to the beam splitter and recombine into a single beam in which "for a given wavelength, there will be either constructive interference (more light energy) or destructive interference (less light energy)" [143]. The recombined beam composed of a mixture of modulated frequencies is directed through the sample to the detector. The response is amplified and digitised into an interferogram, which is a function of the optical path difference. The interferogram is then converted to an IR spectrum by a Fourier Transformation algorithm using a reference wavelength supplied by a helium-neon laser. The instrument is controlled using specialist computer software and can also be used to manipulate the IR spectrum to perform background subtraction to give a clearer spectrum of the sample allowing closer inspection of specific wavenumber regions. FTIR has crucial advantages over dispersive instruments in the way of superior sensitivity, rapid scanning, (typically a good spectrum can be generated in 10 s), and simple operation, however, such instruments are generally more expensive.

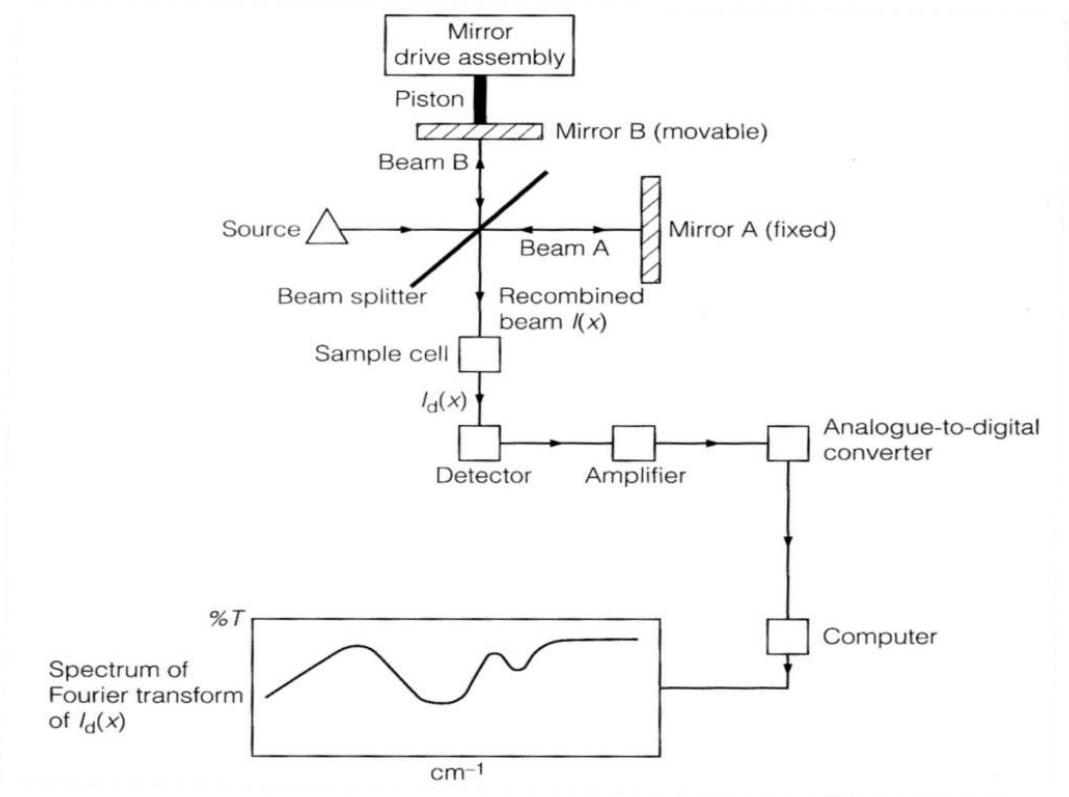


Figure 1.13: Schematic diagram of the components of an FTIR Spectrometer (reproduced from [141])

FTIR instruments also offer better resolution and control of the signal to noise ratio of the spectra. The Signal (S) defines the "average value of output of an electronic device" [138] whilst noise (N) refers to "fluctuations in the output of an analytical instrument" [138]. The standard deviation of the signal is a measure of the noise. The signal to noise ratio (S:N) is "the ratio of the average value of the output signal to its standard deviation" [138]. The resolution (R) of a spectrometer is related to the signal by Equation 1.13 where ΔS is the "smallest difference between two signals" [138].

$$R = S/\Delta S \text{ [138]} \quad \text{Equation 1.13}$$

Simply, the greater the S:N ratio the better the quality of the spectrum. Therefore, the spectrum will exhibit little noise and clear well defined, resolved peaks. If the S:N ratio is reduced, the spectral quality deteriorates making interpretation difficult.

1.5.2.4.2.5.1.3 Attenuated Total-Reflectance (ATR)

FTIR is capable of analysing a wide variety of gaseous, liquid and solid samples, but sample type and instrument set-up will determine the sampling technique used. Traditionally, liquids were sampled as thin films held between Sodium Chloride (NaCl) plates and solids ground into a fine powder mixed with Nujol (paraffin oil) to form a paste or with Potassium Bromide (KBr) to form a disc after pressure applied with a hydraulic press. Both of these sampling techniques are time consuming and suffer from reproducibility issues [144]. However, liquid and solid samples can also be analysed in-situ and quickly by a microscope attached to a spectrometer system (IR microscope) [140]. Furthermore, Attenuated Total Reflectance (ATR) accessories can be fitted to IR microscopes or included in portable spectrometer systems to provide a superior sampling technique that is rapid, simple and non-destructive, making it particularly well suited to forensic samples [144, 145].

ATR technology works on the principle of changes in energy to a totally internally reflected IR beam once it has been in contact with a sample. An ATR accessory consists of a horizontally placed optically dense crystal material with high refractive index (RI, typically 2.38 – 4.01). Typical ATR crystal materials include Germanium, Zinc Selenide and Diamond, with the latter being the best due to its robustness [144]. As Figure 1.14 illustrates, an IR beam is directed upon the diamond ATR crystal at an angle, so that the beam becomes

internally reflected within the crystal in the form of an evanescent wave. This evanescent wave will extend beyond the crystals surface by a few microns (5 µ max), and by placing a sample onto the crystals surface, the IR wave will be in contact with the sample. The wave will be attenuated as a result of absorption of IR by the sample and the energy change in the IR beam detected by the spectrometer as it emerges from the opposite side of the crystal. This energy change will be represented in the form on an IR absorption spectrum generated using the instrument software supplied as normal [144].

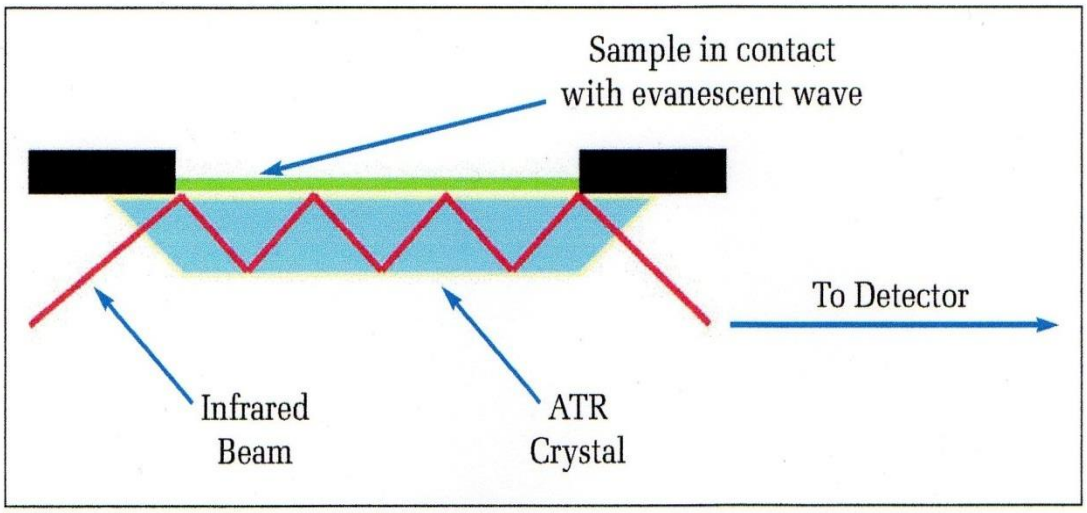


Figure 1.14: ATR sample interface schematic diagram (reproduced from [144])

It is important that the sample, especially for solids, is in direct contact with the ATR crystal so as to ensure that the IR evanescent wave comes into contact with it. Many ATR accessories therefore include high pressure clamps (capable of delivering up to 30, 000 psi) to hold the sample in position and provide good optical contact with the crystal [144, 146]. Furthermore, recent developments have included video microscope viewing capabilities integrated into the ATR accessory to provide images of the sampling area up to magnification of x 110 [145]. Such technology makes it easier to ensure that the IR absorption spectrum acquired is actually from the desired sample area, and is of particular value for forensic samples. In addition, internal reflectance of the IR beam will only occur where the RI of the sample is significantly lower than that of the ATR crystal. Since most solids and liquids possess RI much lower than 2, ATR is therefore well suited to these particular sample types [144].

1.5.2.4.2.5.2 Raman Spectrometers

To excite vibrational transitions in a molecule to include Raman scattering, suitable excitation sources with sufficient energy equivalent to that of the difference between the energy levels of the molecule under study must be used [137]. This is provided by visible and/or NIR laser excitation sources [147]. Typically, Dispersive Spectrometer systems are used in conjunction with visible lasers and Fourier Transform Spectrometers with NIR laser systems (i.e. 1064 nm), though it should be noted that some Dispersive Spectrometers use NIR lasers operating at excitation wavelengths between 790 – 850 nm, extending the range of visible instruments [147].

Developments in instrumentation mean that both types of spectrometer can be coupled to a microscope set up in order to focus on extremely small samples or a small area of a larger sample in situ [147]. For example, a sample may be placed on to the microscope stage and the area of interest placed in the path of the laser beam either by manually operating the XYZ translation stage or automatically operating it via computer software [147]. A simplified diagram of a visible Raman Microscope Spectrometer system is presented in Figure 1.15.

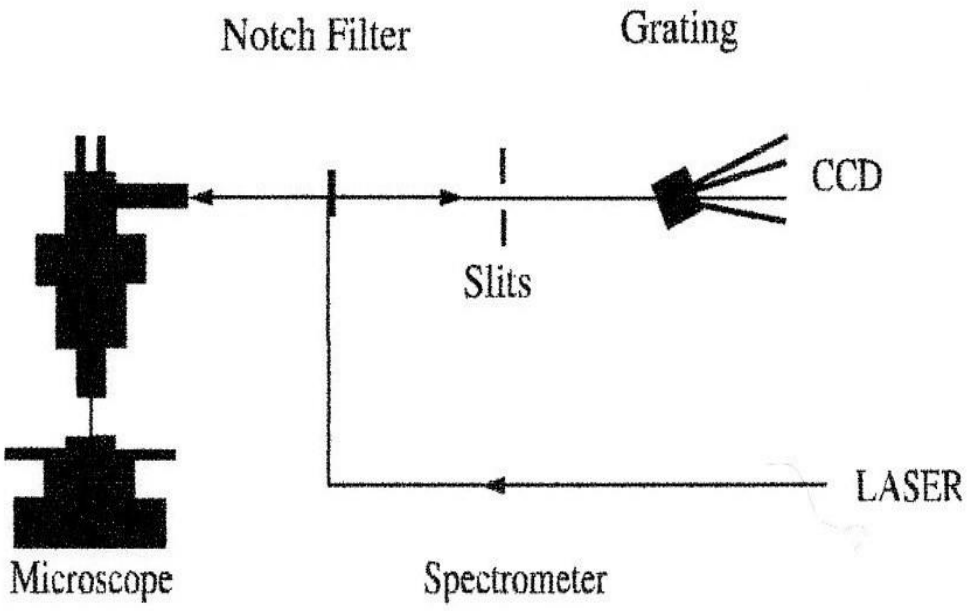


Figure 1.15: Schematic diagram of a Raman Microscope Spectrometer system (reproduced from [147])

The monochromatic laser beam which is directed through a pinhole aperture and expanded to fill the optical system of the microscope is then passed through a plasma filter to ensure

that only radiation of the exact excitation wavelength reaches the sample [147]. The filtered laser radiation is focussed upon the sample via a collection lens through an appropriate microscope objective (obj.), and is concentrated as a high power density spot with a spatial resolution of a few μm dependent upon the excitation wavelength and objective [147]. The scattered radiation from the sample is collected at a 180° angle via a collection lens and in dispersive instruments directed towards a notch or edge filter via a mirror [147]. The notch filter, the most common, is an interference filter which removes the dominant Rayleigh scattered and less intense Anti-Stokes scattered radiation, allowing only the Stokes scattered radiation to pass through to a monochromator, where it is dispersed into its different frequencies of radiation, detected by a CCD and converted by computer into a Raman Spectrum [147]. In FT Spectrometers, the scattered radiation is passed through an interferometer and detected by an FT program to convert it into a Raman Spectrum [147].

1.5.2.4.2.6 Fluorescence

Raman Spectroscopy has a number of advantages as a technique for providing structural information about a sample that are of particular interest. In particular samples can be analysed in situ with no sample preparation using a Raman microscope, and analysis times are relatively quick, typically a few seconds [147]. However, the Raman scattering is weak in comparison to Rayleigh scattering, with only one in 10^6 – 10^8 scattered photons being Raman scattered and is dependent on the 4^{th} power of the frequency of incident radiation [147]. Therefore in order to detect weak Raman signals from the sample, the excitation laser must be focussed as a small spot with a high power density on the sample [147]. The problem with this is that the sample or any impurities present in the sample may induce an intense fluorescence effect that can effectively mask the weak Raman signals [147]. In addition, such intense power may burn the sample causing its thermal degradation [147].

Fluorescence is a relaxation process, illustrated in Figure 1.16, that occurs at low energies and results in the emission of a photon when an excited molecule returns to its ground state [148]. For fluorescence to occur, molecules must contain certain structural groups such as aromatic rings and conjugated double bond systems typically found in organic dye and pigment molecules [148]. Fluorescence is a greater problem for systems using a high energy visible excitation source rather than a lower energy NIR excitation source, since in

the latter only a small number of molecules will be lower in energy sufficient to cause fluorescence relaxation [147]. However, by the same token, Raman signals will be even weaker when induced by low energy NIR excitation lasers. NIR lasers have been used to extend the visible excitation wavelength range in some instruments. Although this has meant fluorescence is less of a problem at wavelengths between approximately 790 – 850 nm, since these wavelengths are towards the upper detection limit for most CCD detectors the sensitivity of such instruments towards higher frequency vibrations is reduced [147].

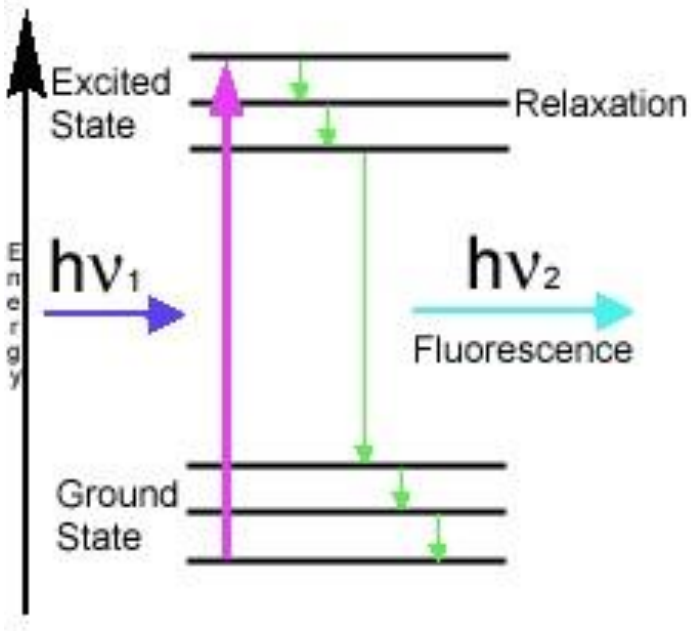


Figure 1.16: Jablonski energy diagram of fluorescence (reproduced from [149])

Fluorescence is visualised in a Raman spectrum as a broad band or high background which may partially or fully obscure the Raman signal peaks detected from the sample, as demonstrated by Figure 1.17. It may originate from the sample itself, or from impurities present within it [147]. It is normally possible to determine if the fluorescence arises from an impurity by repeating the spectral measurement on another location within the sample to see if the high background fluorescence appears in the second spectrum [150]. If not, then the fluorescence is from an impurity, but if yes, then the fluorescence is inherent to the sample when irradiated at that particular excitation wavelength. There are several ways in which a fluorescence background from visible excitation may be reduced to enhance the weak Raman signals from the sample [150].

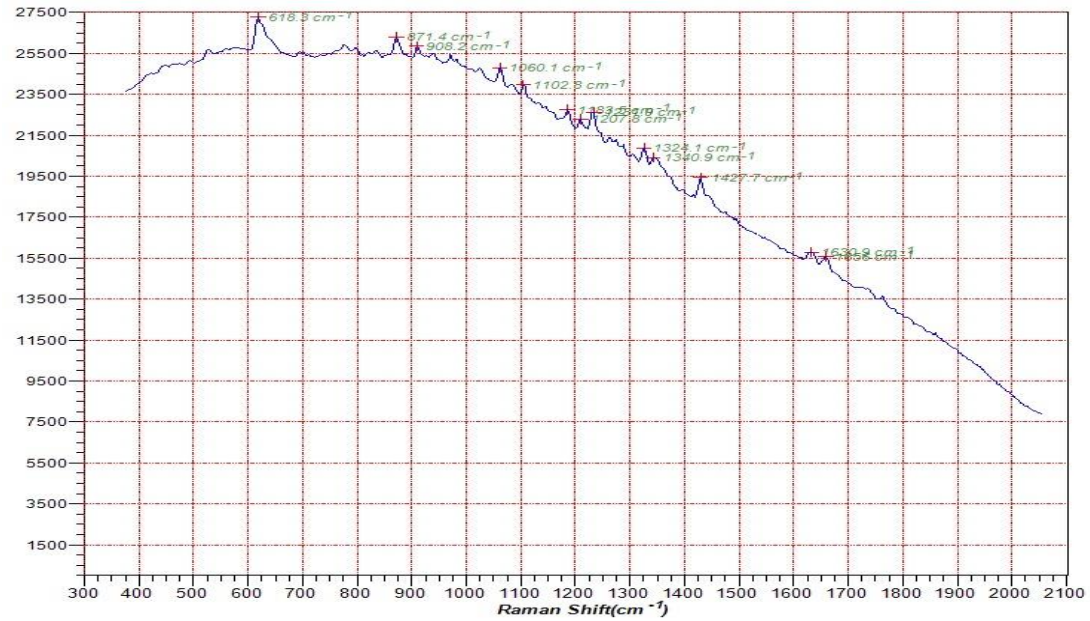


Figure 1.17: Example of high fluorescence background obscuring Raman signal (labelled peaks) from a heroin (Diamorphine) drug sample analysed at 785 nm excitation wavelength

1.5.2.4.2.6.1 Quenching

If the sample is irradiated by the visible excitation laser for a set period of time prior to spectral acquisition, it may have the effect of burning out or quenching the fluorescence resulting in a reduced fluorescent background [147, 150]. The success of this technique and the amount of time required to quench the fluorescence, which can be anywhere between a few minutes to several hours, is dependent upon the nature of the sample, and will not work for all sample types [147, 150]. Where quenching is successful it is because "there is a specific absorption of the light into the fluorophore so that it is potentially degraded" [147]. However, this technique can result in damage to the sample by burning which may influence any subsequent spectra. This is especially true for coloured samples [147], therefore quenching is preferred only for reducing fluorescence caused by impurities [147].

1.5.2.4.2.6.2 Confocality

It is possible to operate a Raman microscope in confocal mode, which may reduce fluorescence background [121, 147, 150]. Essentially operating the system in confocal mode involves focussing a sharp laser spot onto the sample via a pinhole, so that only scattered light from a small focussed sample volume is collected [147]. The pinhole prevents the majority of unfocussed light, for example fluorescence from the entire sample matrix, from re-entering the microscope system and thus swamping the Raman signal

[147]. The pinhole may be created either by a pinhole aperture located within the focal plane or by the crossing over of two slits in the optical path, one in the focal plane positioned at 90° to the other located in the spectrometer [147]. Fully automated microscope systems will allow operation in the confocal mode by simply ticking the appropriate box on the software menu, but for other systems slit width may also need to be adjusted by hand [150].

Two other means by which fluorescence in visible systems may be reduced are through changing the laser power intensity at the sample interface and/or changing the excitation wavelength, since both are related to the intensity of the Raman scattering effect [147].

1.5.2.4.2.6.3 Laser Power Intensity

The intensity of the laser power on the sample may be controlled by the instrument software, to allow a percentage (%) proportion of the maximum power output of the laser excitation source to reach the sample. This may enhance the Raman signal relative to any background signal, but not always [150], since it will depend upon the absorption maximum of the compound ultimately determined by its chemical structure [148].

1.5.2.4.2.6.4 Changing Excitation Wavelength

Intensity of the Raman signal is related to the fourth power of the frequency of incident radiation, consequently, changing to a higher excitation wavelength to increase the frequency of radiation may enhance the Raman signal relative to the background [147].

1.5.2.4.2.6.5 Surface Enhanced Resonance Raman Spectroscopy (SERRS)

Surface Enhanced Resonance Raman Spectroscopy (SERRS), a combination of two techniques, can be used to both enhance Raman signal intensity and reduce fluorescence simultaneously. SERRS utilises the principle that subjecting a sample to irradiation using a laser with the same or closely similar excitation wavelength corresponding to that of the absorption maximum (or electronic transition) of the colorant in the compound enhances the intensity of the Raman signals [139]. This is known as Resonance Raman Spectroscopy, which has the advantage of being more selective than non-resonance Raman Spectroscopy. However, the former is more likely to induce fluorescence owing to the greater likelihood of absorption resulting from using incident radiation close to that of a molecules electronic

transition, as demonstrated in Figure 1.18 [151]. By coating the sample in a silver or gold colloid solution containing an aggregating agent (i.e. Poly-L-Lysine), any fluorescence can be dramatically reduced or even eliminated, thus further enhancing the Raman signal from the sample [139]. It has been reported that the sensitivity of the SERRS technique can enhance intensity 10 fold relative to normal Raman Spectroscopy [122, 139].

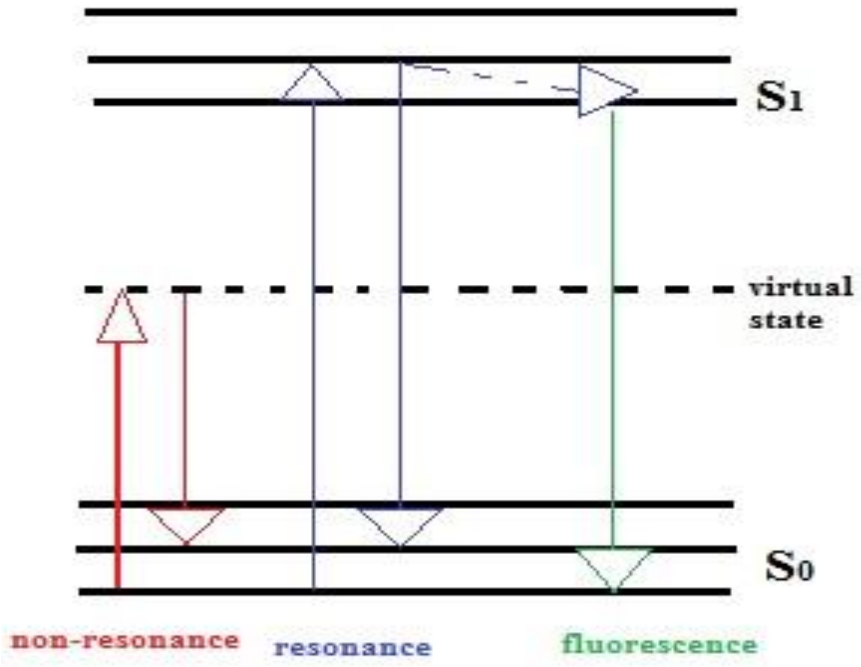


Figure 1.18: The different excitation processes for non-resonance and resonance Raman Spectroscopy, illustrating the higher likelihood of encountering fluorescence in conjunction with resonance Raman Spectroscopy (reproduced from [151])

1.5.2.4.2.7 Interpretation of Raman Spectra

Once spectral measurements have been taken, the corresponding Raman spectra generated will need to be analysed and interpreted. The type of interpretation required will depend upon the purpose for which the data has been generated. Raman Spectroscopy is predominantly used for qualitative analysis, and as such interpretation may involve simple pattern recognition for the purposes of identification and comparison, or a more complex band interpretation in order to deduce the structure of the molecule of the compound under study [147]. Quantitative interpretation of Raman spectra may be possible in order to determine the concentrations of different components within the compound mixture [147]. However, this relies on absolute rather than relative band

intensities, which are influenced by a number of factors including sampling, instrumental effects, type of vibration, orientation and laser power, and as such is not an area until relatively recently which has received much attention [137, 147].

1.5.2.4.2.7.1 Scale and Normalisation

The intensity of a Raman spectrum is given in arbitrary units and the software will automatically scale the spectrum to the tallest peak [147]. It may appear therefore that several spectra taken from different regions of the same sample exhibit the same intensity, but this may not be the case. It is important to observe the y-scale value in this instance as it may be that the intensity for one spectrum is significantly weaker than the other. This could be as a result of a number of factors including "instrument effects, sample mounting, diluents" [147] or "the sample may just be a poor Raman scatterer" [147] suggesting that any intensity difference could be as a result of an impurity with a strong Raman effect. To ensure such information is not overlooked, it is possible to normalise the spectra to the tallest peak, given a y-value of 1, to allow direct comparison of relative peak intensities between spectra from the same sample or different samples [121, 147].

1.5.2.4.2.7.2 Baseline Correction

The slope of background fluorescence that could not be avoided by the methods previously discussed may be removed from a spectrum to make the baseline appear flat using the baseline correction function [121, 147]. This function is only useful where there is "sufficient Raman signal on top of the sloping background" [150] and cannot be used to remove a fluorescence slope that has completely masked any Raman signal from the sample. Care should be taken when using this function to ensure that no peaks are mistakenly removed from or added to the spectrum which could have a negative effect on any subsequent interpretation [152].

1.5.2.4.2.7.3 Cosmic Rays and Emission Lines

Some Raman Microscope Spectrometer systems may be prone to interference from ambient light, such as overhead strip lighting, in the laboratory in which they are situated, [147]. This interference is known as the cosmic ray emission line effect and is visualised as intensely strong sharp and narrow bands, which through automatic scaling can have the effect of masking any weak Raman signals [147]. Stray bands arising from cosmic rays and

emission lines are readily identifiable in the spectrum because of their strong intensity, and as such can be removed using a software function called Zapping [150]. Prior to running the zap function, it is important to repeat the measurement to ensure that the stray band is due to cosmic rays and not some other contaminant. Upon removing the spurious band, the spectrum will automatically rescale accordingly to the tallest Raman peak. The effects of cosmic rays and emission lines can be reduced by performing spectral measurements in low level lighting [152] or operating the Raman microscope in an enclosure [147]. Other software functions such as smoothing and spectral subtraction may also be performed to improve S:N ratio or to identify pure components from a mixture [150, 153]. However, none of these functions should be used in preference to performing a better measurement by varying parameters such as excitation wavelength, laser power intensity, exposure time and the number of accumulation scans [150].

1.5.2.4.2.7.4 Pattern Recognition in Spectroscopic Analysis

A Raman spectrum consists of a number of peaks or bands occurring at particular wavenumber positions and intensities, whilst an IR absorption spectrum consists of a similar set of peaks exhibiting different levels of absorbance. In either case, since a spectrum is based on the chemical composition of the sample, it provides a "fingerprint" characteristic of the compound under study [137]. Spectra from known compounds can be compared to a spectrum from an unknown sample for the purposes of identification, or the spectra from two or more unknown samples can be compared in order to determine if they have the same or similar chemical composition. Computer based spectral libraries can make pattern recognition a relatively quick process compared to attempting to identify a compound by trawling through hard copy reference collections of known spectra [147]. However, in the case of Raman Spectroscopy at least, only a limited number of commercial electronic reference collections for different types of compounds are currently available [147]. It is preferable for individual laboratories to build their own Raman or IR reference collections specific to their own needs. Some laboratories who have taken this approach have made their libraries available on the internet [147].

1.5.2.4.2.7.5 Band Interpretation

As previously mentioned, wavenumber positions and relative intensities of Raman bands can be used to determine the types of structural groups present within the compound

being studied on the basis of their characteristic group frequencies. In comparison to pattern recognition, this approach to interpretation is more complex and requires both knowledge and experience of the chemistry of the types of compounds being studied [147]. Band interpretation involves the use of frequency range tables in specialist text books, a simplified example of which is shown in Figure 1.19, which give details of the energy ranges (wavenumber region) of characteristic group frequencies and their expected relative intensities, normally considered in terms of strong, medium or weak for the purposes of qualitative interpretation [147]. As a general rule, asymmetric stretch vibrations occur at high wavenumber ranges, bending vibrations at low wavenumber ranges and symmetric stretch vibrations in the mid-wavenumber ranges spanning the overall wavenumber range between 4000 – 200 cm^{-1}. It is worth noting the 1500 – 600 cm^{-1} region is called the fingerprint region since this is the region which provides information specific to the molecule being analysed [137, 147]. Knowledge of the types of structural features present in the compound under study and those expected for that sample type can be used to suggest possible molecular structures [147]. Confirmation of molecular structure is achieved by cross-referencing the unknown spectrum with an appropriate reference spectrum [147].

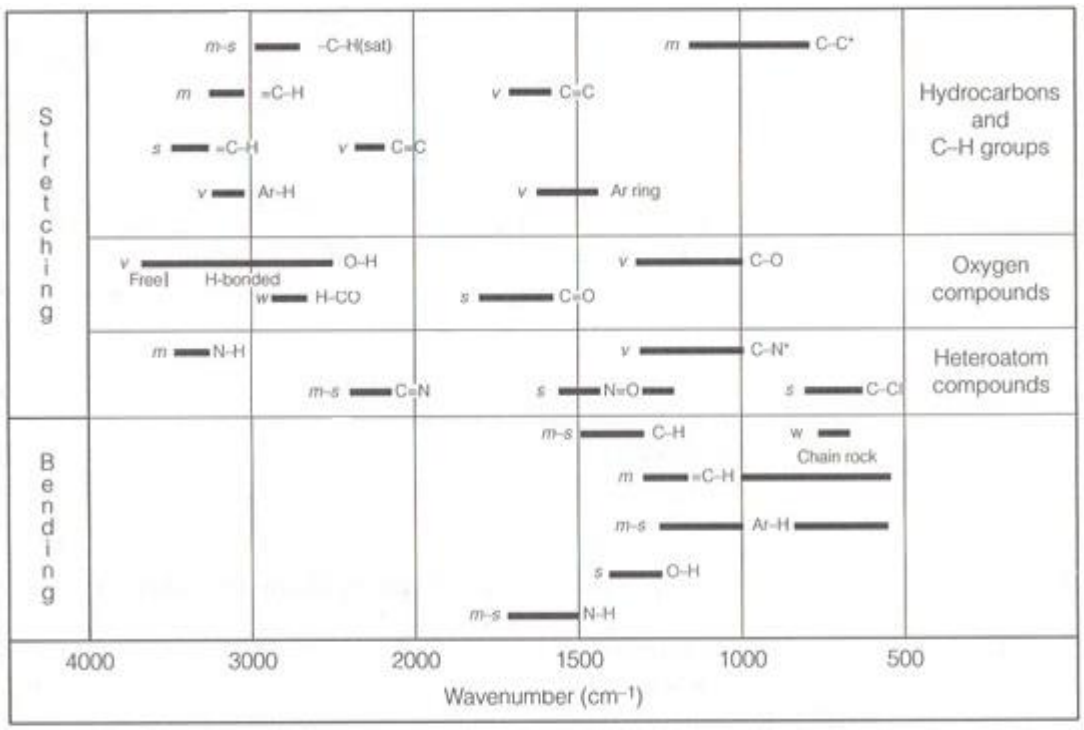


Figure 1.19: Chart of infrared characteristic group frequencies showing relationship between different functional groups and their corresponding wavenumbers (reproduced from [141])

1.6 Data Analysis

1.6.1 Discriminating Power

In 1973, Smalldon & Moffat introduced the concept of Discriminating Power (DP) within a forensic science context [154]. The DP of an analytical technique or combination of analytical techniques serves as "a measure of their probability of discriminating two samples selected at random from the population of interest" [154]. This probability value between 0.0 – 1.0, sometimes described as a percentage value, provides an indication of how effective a particular analytical protocol maybe for discriminating certain forensic evidence types. It can therefore be useful for deciding which analytical techniques should be adopted for routine use in operational laboratories for certain evidence types against other considerations such as cost, laboratory space, caseload and sampling requirements. Smalldon & Moffat originally described discriminating power in the Equation 1.14 [154]:

$$DP_k = \frac{1-2M}{[N(N-1)]} \qquad \text{Equation 1.14}$$

Where DP_k = discriminating power, M = total number of matching pairs, and N = total number of samples. It has been simplified by some researchers [15] into Equation 1.15:

$$DP = \frac{Number\ of\ discriminated\ sample\ pairs}{Number\ of\ possible\ pairs} \qquad \text{Equation 1.15}$$

The discriminating power has been used to describe the ability of certain analytical techniques to discriminate a wide range of samples of forensic interest including glass [155], fibres [134], paint [156, 157], soil [158], paper [159] and writing inks [15].

1.6.2 Multivariate Statistics

Visual pattern recognition has traditionally been used to compare and discriminate between spectra from different samples. However, this is a subjective process, and where spectra may be highly similar, a different conclusion may be reached by different analysts. The use of a multivariate statistical approach to spectral data classification would reduce this subjectivity. In forensic science, multivariate statistical methods in the form of Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) are the most

common [160, 161], however, more recently the use of Artificial Neural Networks (ANN) has demonstrated great potential [75-77], in particular the application of Self-Organising Feature Maps (SOFM) [161-166]. All three of these techniques are described as unsupervised methods of pattern recognition, that is, they are able to detect relationships amongst large complex datasets without any prior knowledge to identify groups or clusters of samples with similar attributes [167]. Furthermore, they all provide a visual output of the clustering to allow the forensic scientist to see clearly the grouping of the samples. These groups can then be validated by comparison to the observations made through visual pattern recognition. Cluster Analysis is an exploratory investigative technique and it may be necessary to analyse a dataset using several different methods, often with some form of pre-processing transformation, to achieve the desired outcome [160, 167].

1.6.2.1 Principal Component Analysis (PCA)

PCA reduces the dimensionality of the original data set by calculating a new set of variables called the Principal Components [161]. These Principal Components (PC's) are calculated from normalised linear combinations of the original variables, organised according to the amount of variance in the original data set that they explain, and are uncorrelated to one another. Typically, only the first two PC's are required to explain the majority (80 – 90%) of the variance in the original dataset, and are used to generate a graphical output called a score plot [161, 163, 165]. In the score plot, an example of which is illustrated in Figure 1.20, the samples are arranged in space relative to each other according to their first two PC scores. Those samples with similar scores will occupy a similar position, whilst those with dissimilar scores will be positioned some distance away, thus allowing the forensic scientist to identify any clusters.

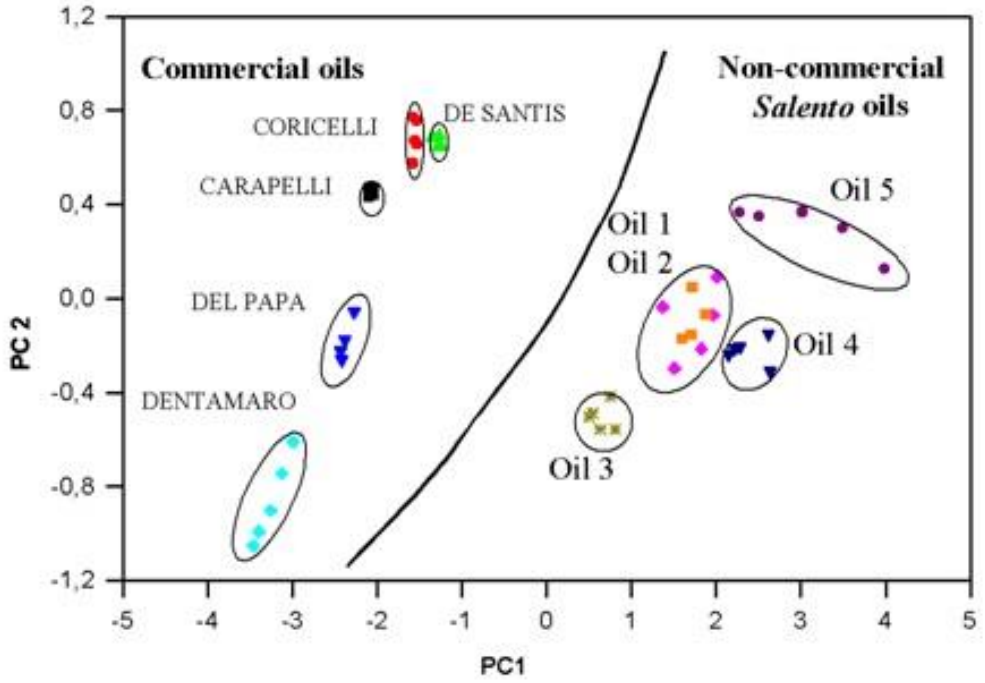


Figure 1.20: "PCA score plot related to the sensor array response to the volatiles of some commercial and non-commercial local olive oils from Salento province (Italy)" (reproduced from *Capone et al* [168])

1.6.2.2 Hierarchical Cluster Analysis (HCA)

HCA orders a large complex data set into clusters which are graphically displayed as a tree diagram called a dendrogram that indicates the level of similarity (via a similarity index (%)) between individual samples and groups of samples relative to the entire dataset [161, 167]. The dendrogram, shown in Figure 1.21 is produced typically using agglomerative methods where clustering starts with individual samples and proceeds sequentially until all samples are linked together. Several agglomerative methods of combining clusters and distance measures exist, but two of the most common are Single Linkage (where the "distance between two clusters is the minimum distance between a variable in one cluster and a variable in the other cluster", also known as nearest neighbour [169]) used to calculate the distance between clusters, and Euclidean Distance (the "square root of the sum of squared differences" [169]) used to calculate the distance between samples respectively [167]. Different methods may result in different outcomes, and the chosen method for a particular dataset is commonly based on a trial and error process. The ideal outcome is a dendrogram which exhibits clusters with a relatively large similarity index and comparatively small distances between connected clusters.

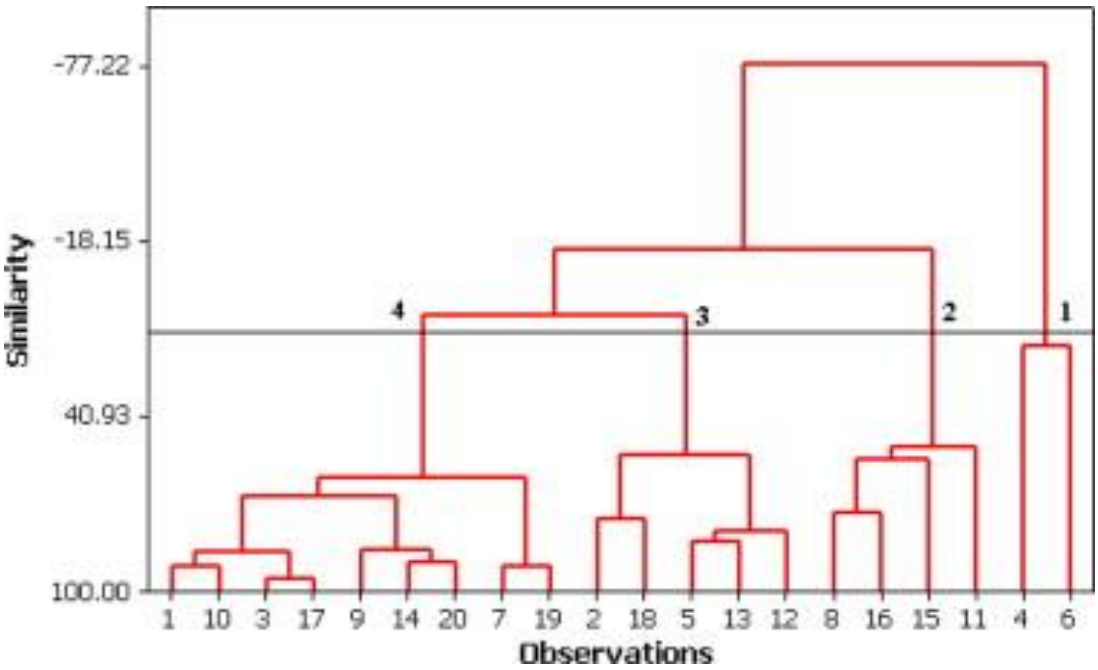


Figure 1.21: "Hierarchical dendrogram from the HCA for the 20 bottled water brands" (reproduced from *Kermanshahi et al* [170])

1.6.2.3 Artificial Neural Network (ANN) Analysis: Self-Organising Feature Maps (SOFM)

SOFM, or Kohonen Neural Networks, are an unsupervised learning ANN that can classify large complex datasets in a similar way to how the human brain would classify spectral data through visual pattern recognition [161, 163, 165]. The arrangement of an SOFM consists of an input layer, where the variables describing the data or input pattern is entered, and an output layer, upon which the input pattern is mapped. The output layer is a grid of neurons, each of which is connected to each of the neurons in the input layer and has an associated weighting. Once the input pattern has been entered, a competitive training process begins, whereby neurons compete against one another to be stimulated by the input pattern. Through each training cycle, winning neurons, or Best Matching Units (BMU's), are identified on the basis of similarity between the weight vector of the output neuron and the input vector using the Euclidean Distance. As the training process continues, adjustments are made to the weights of the BMU's and their closest neighbour neurons to make them closer to the input vector. The SOFM organises itself so that input vectors with similar weightings are mapped onto similar output neurons, and once the training process is complete, reveals clustering of samples through a colour coded visual output map. Each cluster in the SOFM is represented by a different colour to aid

discriminations between different groups. Furthermore, where samples within a group are considered highly similar, i.e. share common variables, but distinguishable, i.e. differ by at least one variable, this is represented as a change in intensity or hue of the colour representing the entire cluster [161-163, 165]. Figure 1.22 presents an SOFM produced from a simple dataset partially based on an example first provided by Kohonen [171] and described in Dzulkiflee [165].

Figure 1.22: Example of a Self-Organising Feature Map (SOFM) demonstrating clustering within a data set of 13 animals based on similarities across 12 physical and physiological attributes

The dataset describes 13 animals by a series of 12 different physical and physiological attributes (small, medium, big, two legs, four legs, hair, mane, feathers, hunt, run, fly and swim). The data matrix or input pattern consists of 14 rows x 13 columns. When an attribute for a given animal is present, it is assigned a "1" value; if absent, it is assigned a "0" value. The resulting output map places each of the 13 animals into one of three colour coded clusters that appear to correspond to the animal family to which they belong, i.e. cat family (red cluster), dog family (yellow cluster) and bird family (blue cluster). Furthermore, animals with two legs, feathers and the ability to fly occupy the right side of the output

map, whilst those with four legs, hair and are unable to fly populate the left side, demonstrating that SOFM describe the distribution of subjects (i.e. animals) across the output map area according to similarities in their attributes. Within each cluster, the segments representing each animal show variations in colour hue suggesting strong similarity with other animals within the cluster, but some subtle difference that distinguishes them in some way. For example, the segment representing the Lion in the red cluster is darker in hue to that of the Tiger. Both animals belong to the cat family, are big in size, have four legs, can run and hunt, and possess hair. Neither possesses the remaining attributes with one exception. The Lion has a mane, whereas the Tiger does not. This difference in physical appearance is reflected by the difference in colour hue of the segments representing these two animals. SOFM software permits the user to determine which particular attribute is responsible for subtle differences within clusters in the output map by generating individual component maps representing each variable present within the data set being studied, highlighting which samples share that attribute or variable and which do not.

PCA, HCA and SOFM all have two aims in common; (a) to create a dataset of similarity and dissimilarity measures between samples from an original dataset (either in its raw form or pre-processed format); and (b) to cluster similar samples together with minimal distance between them and to provide maximum distance between different clusters [167]. In this way patterns or trends amongst large multivariate data sets can be readily identified.

Chapter Two

Solubility Testing, Thin Layer Chromatography and % Reflectance Microspectrophotometry

2.1 Introduction

Numerous papers have discussed TLC analysis of writing inks over the past 50 years or more, predominantly focussing upon ballpoint inks [49-53, 56, 57, 72, 73, 112, 172-180]. Unsurprisingly, a limited number have explored TLC of gel inks [13, 17, 26, 181, 182]. Gernandt and Urlaub in their preliminary observations of gel ink analysis, suggested the pigmented nature of gel inks could be a useful means by which to discriminate them from other inks since they would not separate out into their individual colorant components [17]. However, as Florence *et al* [13] highlights, this study was conducted prior to widespread availability of dye based and hybrid gel inks. This prompted Wilson *et al* [26] to include TLC as part of their study of 29 black gel based inks, including nine dye based gel inks. TLC analysis, using a single solvent system of ethyl acetate:ethanol:water (75:35:30), of 20 pigment based gel inks, did not provide any useful means of discrimination as expected. Interestingly however, two inks, both branded as erasable, did make a unique spot at the point of origin on the TLC plate, which could be distinguished between on the basis of their appearance. For nine dye based inks already classified into five groups by a combination of microscopy and filtered light examination, TLC permitted further discrimination into eight groups.

Jasuja *et al* [181] investigated the solubility of 98 gel inks of various colour. Five solvents (distilled water, acetone, methanol, chloroform and pyridine) were tested against gel inks deposited on bond paper, of which 58 were insoluble in at least one solvent tested and many more soluble in more than one solvent. Two solvents were identified as most useful for solvating gel inks, methanol (27 inks) and acetone (25 inks). It would appear that 40 gel inks were insoluble in the five solvents tested, suggesting they were pigment based, though not verified. It is interesting to note that Mazella and Buzzini [27] used a simple solubility test using methanol to classify blue gel inks as dye or pigment based. On the basis of Jasuja

et al [181] however, it would suggest that performing a solubility test for this purpose using just methanol is not reliable, since this study demonstrated some brands of gel ink which were not soluble in methanol were soluble in other solvents, e.g. chloroform.

Jasuja *et al* [181] performed analysis of a large number of gel ink samples using 13 different solvent systems, two of which were "useful in differentiating the soluble gel inks": butanol:ethanol:water:acetic acid (60:20:20:0.5) and butanol:ethanol:water (50:25:25). The number and colour of spots visualised under white light and UV light (short and long wavelength) as well as R_f values for both solvent systems were reported as shown in Table 2.1. Four brands exhibited different R_f values for the same spot when examined using two different solvent systems.

Massonnet and Stoecklein [183] recently proposed a method for the extraction and analysis of highly pigmented paints that could potentially be applied for the successful extraction of non-soluble organic pigments from gel inks. Since most of these pigments contain oxygen or nitrogen, these atoms can be protonated by a strong acid such as hydrochloric acid (HCl) and converted into anionic compounds suitable for TLC. In Fernandez [182], six brands each of blue and black gel inks were extracted using a range of solvents/conditions and subsequently analysed with three different solvent systems to determine the optimal conditions for successful separation. Heating the samples with the aid of a sand bath for 5 minutes assisted the extraction of brands not immediately soluble in solution. Acetic acid and methanol were considered to be the most suitable solvents for the extraction of blue and black gel inks respectively, with successful extraction of four brands achieved (Pentel, Pilot, WH Smiths and Papermate), confirming their dye based nature. The remaining two brands, Zebra and Uniball, were considered to be pigment based, yet Fernandez [182] claimed they could be successfully extracted by the addition of HCl, with successful separation achieved using a solvent system of ethyl acetate:ethanol:water (75:35:30).

Ink	Colour of Ink	White Light	254 nm	365 nm	R_f Value
Wright-A-One	Red	Orange	Bright Yellow	Bright Yellow	0.98 (0.82)
Montex Hycute	Blue	Purple Blue			0.57 (0.61) 0.34 (0.29)
Montex Hyspeed	Blue	Purple Blue			0.55 (0.61) 0.32 (0.44)
Montex	Green	Blue			0.22 (0.36)
Montex	Blue	Blue Green Blue			0.79 0.70
Add Gel	Black	Blue (Grey) (Dark grey)			0.75 (0.87) (0.84) (0.64)
Add Gel	Blue	Pink Blue	Pink	Pink	0.65 (0.41) 0.31 (0.32)
Add Gel	Blue	Pink Blue	Pink	Pink	0.42 (0.39) 0.27 (0.33)
Add Gel	Blue	Blue	 Pink	 Pink	0.44 (0.34) 0.54 (0.43)
Add Gel	Blue	Blue	 Pink	 Pink	0.35 0.50
Add Gel	Blue	Blue (Purple)	 (Pink)		0.74 (0.29) (0.39) (0.82)
Add Gel	Blue	Blue			0.79 (0.84)
Add Gel	Blue	Blue Purple			0.35 (0.38) 0.56 (0.85)
Rotomac	Blue	Purple Blue (Blue)			0.53 0.34 (0.39)
Uniball	Silver-Violet	Pink Pink Blue	Pink Pink	Pink Pink	0.73 0.59 0.46
Uniball	Silver-Orange	Orange			0.80
Uniball	Silver-Green	Blue			0.34
Uniball	Silver-Blue	Blue			0.32
Zebra	Blue	Blue			0.30
Zebra	Copper	Pink			0.86
Zebra	Blue-Green	Green			0.31
BIC	Metallic Pink	Pink			0.73
Pilot	Green	Blue			0.30
Pilot	Blue	Blue			0.79
Zebra	Pink	Pink			0.64
Zebra	Purple	Purple			0.50
Pentel	Red	Pink	Orange	Orange	0.51
Papermate	Red	Pink			0.68
Pentel	Purple	Purple			0.62
Sanford	Red	Pink			0.70
Pilot	Red	Pink Pink	Orange Yellow	Orange Yellow	0.84 0.72
Papermate	Purple	Purple			0.58

Table 2.1:"TLC results using solvent system 1 (butanol:ethanol:water:acetic acid (60:20:20:0.5) and solvent system 2 (butanol:ethanol:water (50:25:25). The results of system 2 that differed from system 1 are shown in parenthesis" (Reproduced from Jasuja *et al [181]*)

2.2 Experimental

2.2.1 Sample Collection

An array of gel ink pen samples representing 19 different brands and 27 models available in seven different countries were purchased from High Street retailers and online suppliers. The source countries included the UK, USA (US), South America (SA), Australia (AUS), Japan (JAP), Hong Kong (HK), and Malaysia (MAL). Samples purchased within the UK will be referred to as UK acquired, whilst those purchased abroad will be referred to as INTL acquired. Brands were chosen to reflect a mixture of well-known stationary brands (i.e. BIC, Uniball, etc.) and some lesser known and/or discount brands (i.e. Poundland and The Works discount stores). Where possible, six pens of each brand/model combination for each of two colours, blue and red were acquired. These colours were chosen to reflect some of the most common colour of ink encountered in forensic casework. A summary of the various pens acquired is presented in Tables 2.2 and 2.3.

Colour	Number of Pens	Number of Brands	Number of Models	Number of UK Acquired Brand/Models	Number of International Acquired Brand/Models
Blue	163	19	26	15	14
Red	128	16	23	13	12

Table 2.2: Summary of number of pens, brands, models and source of general origin for each colour group

No.	Brand and Model	Country of Purchase	Colour, No. and Ref. Code of Pens					
			Blue	Full Ref. Code	Short Ref. Code	Red	Full Ref. Code	Short Ref. Code
1	BIC Reaction Gel	UK	6	BICBLU01 - 06	BIC UK #01 - #06	6	BICRED01 – 06	BIC UK #01 - #06
2	BIC Velocity Gel	US	6	BICBLU07 - 12	BIC US #07 - #12	0	-	-
3	Papermate Gel 2020	UK	6	PPMBLU01 - 06	PPM UK #01 - #06	6	PPMRED01 – 06	PPM UK #01 - #06
4	Parker Reflex Gel	UK	6	PKRBLU01 – 06	PKR UK #01 - #06	0	-	-
5	Pentel Hybrid Gel DX Rollerball	UK	6	PTLBLU01 - 06	PTL UK #01 - #06	6	PTLRED01 – 06	PTL UK #01 - #06
6	Pentel Slicci Gel	Japan	6	PTLBLU07 - 12	PTL JAP #07 - #12	6	PTLRED07 – 12	PTL JAP #07 - #12
7	Pilot G2-07 Retractable Gel	UK	6	PLTBLU01 - 06	PLT UK #01 - #06	6	PLTRED01 – 06	PLT UK #01 - #06
8	Pilot G2-07 Retractable Gel	US	6	PLTBLU07 - 12	PLT US #07 - #12	6	PLTRED07 – 12	PLT US #07 - #12
9	Stabilo Point Visco	UK	6	STBBLU01 - 06	STB UK #01 - #06	6	STBRED01 – 06	STB UK #01 - #06
10	Staedtler Triplus Gel	UK	6	STDBLU01 - 06	STD UK #01 – 06	6	STDRED01 – 06	STD UK #01 - #06
11	Uniball Gel Impact UM-153S	UK	6	UNIBLU01 - 06	UNI UK #01 - #06	6	UNIRED01 – 06	UNI UK #01 - #06
12	Uniball Signo RT	Japan	6	UNIBLU13 - 18	UNI JAP #13 - #18	6	UNIRED07 – 12	UNI JAP #07 - #12
13	Uniball Signo Gel Stick UM-170	Australia	6	UNIBLU19 - 24	UNI AUS #19 - #24	6	UNIRED19 – 24	UNI AUS #19 - #24
14	Uniball Signo DX UM-151	Hong Kong	6	UNIBLU25 - 30	UNI HK #25 - #31	4	UNIRED25 – 28	UNI HK #25 - #28
15	Uniball Signo Bit UM-201	Hong Kong	0	-	-	2	UNIRED29 – 30	UNI HK #29 - #30
16	Uniball Signo DX UM-151	Hong Kong	6	UNIBLU31 - 36	UNI HK #31 - #36	0	-	-
17	Uniball Signo	South America	3	UNIBLU37 - 39	UNI SA #37 - #39	3	UNIRED31 - 33	UNI SA #31 - #33
18	Zebra Jimnie Gel	UK	6	ZBRBLU01 - 06	ZBR UK #01 - #06	6	ZBRRED01 – 06	ZBR UK #01 - #06
19	Zebra Sarasa	Japan	6	ZBRBLU07 - 12	ZBR JAP #07 - #12	6	ZBRRED07 - 12	ZBR JAP #07 - #12
20	Great Expressions Gel	UK	6	GREBLU01 – 06	GRE UK #01 - #06	6	GRERED01 – 06	GRE UK #01 - #06
21	Inoxchrom Short Roller Gel	UK	6	ICMBLU01 – 06	ICM UK #01 - #06	6	ICMRED01 – 06	ICM UK #01 - #06
22	Partners Broad Gel	UK	6	PTNBLU01 – 06	PTN UK #01 - #06	6	PTNRED01 – 06	PTN UK #01 - #06
23	Staples Sonix Gel	UK	6	STPBLU01 – 06	STP UK #01 -#06	6	STPRED01 – 06	STP UK #01 - #06
24	Staples Sonix Gel	US	3 (different shades)	STPBLU07 - 09	STP US #07 - #09	2 (different shades)	STPRED07 – 08	STP US #07 - #08
25	WH Smiths Gel	UK	6	WHSBLU01 – 06	WHS UK #01 - #06	6	WHSRED01 – 06	WHS UK #01 - #06
26	Works Essentials Gel	UK	6	WKEBLU01 – 06	WKE UK #01 - #06	0	-	-
27	Corner Office Retractable Gel	US	6	COFBLU01 – 06	COF US #01 - #06	0	-	-
28	Faber Castell True Gel Rollerpoint	Malaysia	4	FBCBLU01 – 04	FBC MAL #01 - #04	3	FBCRED01 – 03	FBC MAL #01 - #03
29	M & G R3 Retractable Gel Pen	Malaysia	6	MGBLU01 – 06	MG MAL #01 - #06	3	MGRED01 – 03	MG MAL #01 - #03
30	G'Soft GS2 Gel Pen	Malaysia	3	GSFBLU01 - 03	GSF MAL #01 - #03	3	GSFRED01 - 03	GSF MAL #01 - #03

Table 2.3: Summary of brand/model combinations, number of each colour and source of origin

The same pens were used for all experimental work presented throughout this thesis. However, during the course of the three year project some pens were mislaid or stopped working, so it was not always possible to analyse them by every technique. Table 2.4 provides details of those pens that were mislaid and those pens where the ink dried out before the completion of the project.

Reason for Exclusion	Blue Group		Red Group	
	UK	INTL	UK	INTL
Missing	BIC UK #03 PPM UK #06 STP UK #03 WHS UK #04	BIC US #10 UNI HK #34 UNI SA #38 COF US #05		
Ink Dried	PTN UK #01	UNI HK #25 STP UK #08 STP UK #09		STP US #08

Table 2.4: Pens mislaid throughout the course of the project or whose ink dried out before completion of the practical work

In the within brand variation study, all available pens within a brand/model combination set were used. In the between brand variation study, the first pen in a brand/model combination set was used, i.e. PTNBLU01 (PTN UK blue pen #01). Where this was not possible for reasons described above, the next pen in the sequence was used, i.e. PTNBLU02 (PTN UK blue pen #02). Where either of these scenarios was encountered it is described in the experimental section of the relevant Chapter.

2.2.2 Ink Sample Preparation on Paper

Sample deposition was made on A4 white office paper from the same pack of 500 sheets (80 gsm) throughout the study to ensure samples were analysed on the same substrate irrespective of technique.

2.2.3 Solubility Study

Solubility testing was undertaken to determine the most appropriate solvent for the extraction of blue and red gel inks from paper in order to perform subsequent TLC analysis. In addition, the method for protonation of organic pigments in paint using a strong acid [183] was explored for its potential application to the analysis of pigmented gel inks.

2.2.3.1 Sample Preparation

Ink spots (~5 mm diameter) from a single pen within a brand/model combination set were deposited onto a piece of A4 white office paper. No within brand variation of solubility was performed since the aim was to determine the most suitable solvent for each colour group as a whole. Brand/model combination pens not tested due to unavailability or ink dry out included: PTN UK #01, UNI HK #25 and STP US #09 (blue ink group); and STP US #08 (red ink group). Underlined samples indicate brand/model combinations of a particular colour represented by a single pen only.

Individual ink spots were cut out from the paper and placed in small glass vials labelled with the appropriate brand/model identity. Approximately 0.5 – 1 mL of solvent was transferred, using a Pasteur pipette, to each vial. Each vial was shaken briefly by hand and allowed to stand in solution for 5 minutes before noting any colour change indicating a successful extraction. The vials were then transferred to a sand bath pre-heated to 400°C, heated for a minimum of 15 minutes and a maximum of 60 minutes dependent upon colour group and solvent combination. Vials were removed, shaken briefly again, and any change in colour indicating a successful extraction noted. In addition to the ink samples, extracts from an un-inked paper sample and a solvent blank were also prepared and treated under the same conditions to monitor for any components attributable to the paper or solvent respectively.

All ink samples were left to air dry for at least 15 minutes prior to extraction and analysed within 120 hours (5 days) of deposition. Samples were left on an open bench exposed to natural light during the day and room temperature overnight.

2.2.3.2 Solvents

With the aforementioned exceptions, samples from all brand/model combinations within both colour groups were tested for their solubility once in each of the following solvents: acetone (puriss ≥99% (GC)), acetic acid (≥99.8% (GC)), methanol (MeOH) (puriss ≥99.7% (GC)), ethanol (EtOH) (puriss ≥99.8% (GC)), ethyl acetate (EtAc) (puriss ≥99.5% (GC), chloroform (puriss 99 – 99.4% (GC)), hydrochloric acid (HCl) (37% AR grade) (Sigma-Aldrich), xylene (VWR International), butanol (Lab Reagent Grade, Fisher Scientific), distilled water (H_2O), ethanol:water (1:1), and ethyl acetate:ethanol:water (75:35:30).

2.2.4 TLC Study

TLC was performed on extracts of blue and red gel inks using the three different solvent systems identified in the literature described previously as being suitable for TLC of gel inks in order to compare their separation and discrimination ability.

2.2.4.1 Sample Preparation

2.2.4.1.1 Repeatability and Reproducibility

Six ink spots from each of the six pens within the PTL JAP brand/model combination (PTL JAP #07 - #12) and six ink spots from a single pen (PTL JAP #07) were deposited onto a piece of A4 white office paper as samples for a study of repeatability and reproducibility respectively. This particular brand/model combination was chosen because it was identified as having multiple coloured components considered adequate to permit assessment of repeatability and reproducibility. TLC was performed once on a single set of extracts and was sufficient to provide separation suitable for comparison of all three solvent systems.

2.2.4.1.2 Within Brand Variation

Ink spots from all pens within three brand/model combinations for each colour group listed below, were deposited onto a piece of A4 white office paper:

- PKR UK, ICM UK and COF US (blue ink group);
- BIC UK, **WHS UK** and PLT US (red ink group)

These particular brand/model combinations were selected at random, with the exception of one highlighted in bold, because it had demonstrated evidence of within brand variation by another analytical technique, i.e. Raman Spectroscopy.

TLC was performed once on a single set of extracts for each colour group.

2.2.4.1.3 Between Brand Variation

With the aforementioned exceptions, ink spots from all brand/model combinations across both colour groups were deposited onto a piece of A4 white office paper. This consisted of

31 and 25 brand/model combinations for the blue and red ink groups respectively. All ink samples were allowed to air dry at room temperature for at least 15 minutes prior to extraction and analysed within 48 hours of deposition. Ink samples were stored at room temperature in a laboratory notebook overnight. TLC was performed once for the blue ink group samples, and was sufficient to achieve separation suitable for comparison and discrimination. For the red ink group however, the number of TLC runs performed, per extract set and solvent system, varied from 1 – 2 in order to achieve successful separation.

2.2.4.1.4 Extraction

Extraction was performed using the most appropriate solvent for a given colour group identified from the solubility study and following a similar procedure to that described previously. For the blue ink group samples, the repeatability/reproducibility and within brand samples were heated for up to 30 minutes at 400°C. The red ink group samples were extracted under slightly different conditions, where a complete set of the red ink group samples were heated for each of 30, 45 and 90 minutes respectively. For all studies, one set of blue ink group samples were extracted. For the red ink group within brand variation study, two sets of samples were extracted under the same conditions. For the red ink group between brand studies, two sets of samples were extracted under the 30 and 90 minute heating conditions, and a single set was extracted under the 45 minute heating conditions.

For all studies, extractions of a piece of un-inked paper and a vial containing only the appropriate solvent for each colour group were also undertaken to identify any component spots which could be attributed to the substrate and/or solvent.

2.2.4.1.5 Solvent Systems

Three solvent systems were studied: ethyl acetate:ethanol:water (75:35:30) [Solvent System 1], butanol:ethanol:water (50:25:25) [Solvent System 2], and butanol:ethanol:water:acetic acid (60:20:20:0.5) [Solvent System 3]. Mobile phases were freshly prepared at the beginning of each day. Three large TLC chambers were each lined with filter paper and allowed to equilibrate for at least two hours prior to TLC being performed, and given a 30 minute re-equilibration time between TLC runs.

2.2.4.1.6 Plate Preparation

Method development involved conducting several TLC runs for both colour groups to fine tune the conditions required to achieve good separation and an informative TLC analysis. Based on this, TLC was performed using Merck 20 cm x 20 cm silica gel 60 pre-coated plates with fluorescent indicator UV_{254}. The baseline was drawn in pencil 1 cm from the bottom edge of each plate and samples spotted at 1 cm indented positions from either outside edge, and at 1 cm intervals along its length. Each ink extract was spotted onto the plate using a (pulled) fine capillary tube (1 mm diameter) between 3 – 6 times depending on the extract strength and allowed to air dry at room temperature between each application. Once a TLC run was complete, the plates were removed, dried with the aid of a hair dryer and the solvent front marked with pencil. Any visible spots were recorded. TLC plates were examined under UV_{254} and UV_{365} for the presence on any invisible component spots.

2.2.5 % Reflectance Microspectrophotometry of Components on TLC Plate

% Reflectance spectra were acquired from dye component bands present on the TLC plates developed in solvent system 1 only, for both colour groups, with one exception.

2.2.5.1 Repeatability and Reproducibility

A set of six spectra were acquired from the same area of a single blue component spot to test for instrumental variation (repeatability). Six spectra were also acquired from different areas within each component spot for one blue brand/model combination (COF US) to detect any sample variation (reproducibility).

2.2.5.2 Within and Between Sample Variation

A single spectrum was recorded from each component spot of the dye containing gel inks developed in solvent system 1 for both colour groups, with one exception. A red component in the STP US #08 blue ink group sample was more pronounced under solvent system 2, therefore a % reflectance spectrum was taken from this sample. Some component spots were very pale which created difficulties in detecting them via the video camera screen when positioning the sample slit. To resolve this, the component spot was circled in pencil as described by Fuller [108] and spectral measurements taken from within

this boundary. Three spectra from different areas of each TLC plate were acquired to monitor for background reflectance interference.

2.2.5.3 Instrumentation

All spectra were acquired using an Olympus BX41 microscope fitted with a video camera, and attached to a J & M Tidas Microspectrophotometer. The microscope was fitted with an MPLAN N (UIS 2) x 10 objective (obj.) and a UPLAN FL (Olympus) x 40 obj. The MSP system was connected to a PC workstation with Onyx Software (version 1.9.0.0, Cavendish Instruments Ltd © 2003 – 2006) installed. The measurement slit dimensions were 4.185 µm x 5.115 µm (width x height) and the full scan range was 361 – 781 nm. All spectra were acquired in % reflectance scan type mode using reflected light supplied by an Olympus TH4-200 light source. All spectra were normalised for ease of comparison and smoothed by 35 points using the Savitsky-Golay algorithm [130] to improve signal to noise (S:N) ratio.

2.3 Results and Discussion

2.3.1 Solubility Study

The inks could be classified into dye containing (i.e. dye based or hybrid) or pigment based on the basis of their solubility. This colorant type classification is referred to throughout the thesis in order to assess the ability of the other analytical techniques studied for discriminating samples by their colorant class.

2.3.1.1 Blue Ink Group

In total 31 blue brand/model combinations were analysed. These contained 10 dye containing and 21 pigment based inks. The results of the extractions in each solvent tested are presented in Table 2.5.

	Sample	Acetic Acid		Xylene		MeOH		Acetone		EtOH		EtAC		Distilled Water		EtAC:EtOH:H_2O		EtOH:H_2O		Butanol		Chloroform				Hydrochloric Acid		
Time (mins)		5	15	5	15	5	15	5	15	5	15	5	15	5	15	5	15	5	15	5	15	5	15	30	60	5	15	60
Dye or Dye and Pigment	PTL JAP	*	*	-	-	*	*	-	*	*	*	-	-	*	*	*	*	*	*	*	*		*	*	*	*	*	*
	ICM UK	*	*	-	-	*	*	*	*	*	*	-	-	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	PLT UK	*	*	-	-	*	*	*	*	*	*	-	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	STB UK	*	*	-	-	*	*	-	-	*	*	-	-	*	*	*	*	*	*	-	-	-	-	-	-	-	-	*
	PKR UK	*	*	-	-	*	*	-	-	*	*	-	-	*	*	-	*	*	*	-	*	-	-	-	-	*	*	*
	COF US	*	*	-	-	*	*	*	*	*	*	-	-	*	*	*	*	*	*	*	*	-	-	-	*	*	*	*
	PLT US	*	*	-	-	*	*	*	*	*	*	-	-	*	*	*	*	*	*	*	*	*	*	*	*	*	-	*
	STP US #07	*	*	-	-	*	*	*	*	*	*	-	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	STP US #08	*	*	-	-	*	*	-	-	*	*	-	-	*	*	*	*	*	*	-	-	-	-	-	-	*	*	*
	STP US #09	*	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*
Pigment	PTN UK	-	-	-	-	-	-	-	-	-	-	-	-	*	*	-	-	-	-	-	-	-	*	*	*	*	*	*
	BIC UK	-	-	-	-	-	-	-	-	-	-	-	-	*	*	-	-	-	-	-	-	-	*	*	*	*	*	*
	BIC US	-	-	-	-	-	-	-	-	-	-	-	-	*	*	-	-	-	-	-	-	-	*	*	*	*	*	*
	PTL UK	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-	-	*	*	*	-	*	*
	WKE UK	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-	-	*	*	*	-	*	*
	GRE UK	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-	-	-	*	*	-	*	*
	UNI SA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	-	*	*
	UNI JAP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	-	*	*
	UNI UK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	-	*	*
	UNI HK #25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	-	*	*
	UNI HK #31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	-	-	*
	ZBR JAP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	-	*	*
	UNI AUS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	-	-	*
	ZBR UK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	-	*	*
	WHS UK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	-	-	*
	STD UK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	-	-	*
	STP UK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	-	-	*
	PPM UK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*
	MG MAL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	-	-	*
	FBC MAL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	-	-	*
	GSF MAL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	-	-	*

Table 2.5: Solubility table for the blue ink group. Purple highlighted areas indicate the most suitable solvent/conditions for extracting the soluble dye containing blue gel inks. Yellow highlighted areas indicate samples that were initially soluble in those solvents but subsequently determined to be insoluble. * denotes successful extraction; - denotes unsuccessful extraction; NB STP US #09 was only available for testing in acetic acid, xylene, acetone and hydrochloric acid

The following conclusions drawn from the solubility study of both colour groups exclude the findings in relation to the hydrochloric acid extracts. The findings in relation to these are discussed separately at the end of the solubility section and provide an explanation as to why they are considered in isolation.

2.3.1.1.1 Dye Containing Samples

Acetic acid, highlighted in purple in Table 2.5, was considered the most suitable solvent for the blue ink group, providing strong extractions from ten brand/model combinations: PTL JAP, ICM UK, PLT UK, STB UK, PKR UK, COF US, PLT US, STP US #07, STP US #08 and STP US #09. These samples extracted readily after 5 minutes in solvation without heating, in agreement with Fernandez [182]. Five other solvents (methanol, ethanol, distilled water, ethyl acetate:ethanol:water (75:35:30) and ethanol:water (1:1)) also extracted the same samples, but with varying degrees of extraction strength assessed by the intensity of the extraction colour. Several samples appeared weakly extracted, even after heating for 15 minutes. Butanol and acetone extracted only some of these dye containing samples, whilst two solvents, xylene and ethyl acetate, provided no extraction of the blue ink group samples at all.

2.3.1.1.2 Pigment Based Samples

Interestingly, as highlighted in yellow in Table 2.5, both distilled water and chloroform appeared to extract some of the pigment based inks especially after heating for 15 minutes. For chloroform, heating the extracts for longer appeared to increase the number of successful extractions from the pigmented group. This initially seemed to support Jasuja *et al* [181] who claimed several brands in their study were also soluble in chloroform, including some used in this research (i.e. BIC, Zebra, Pentel, Papermate), although specific models were not identified. However, when extractions in distilled water and chloroform were repeated during TLC method development prior to the within and between brand variation studies, only the dye component samples successfully extracted, and only four extracted in chloroform in particular: ICM UK, PLT UK, PLT US and STP US #07. These four samples were the only dye component samples that extracted in chloroform without heating from the initial solubility study performed. TLC of all these extracts confirmed that component spots were only observed for the dye containing samples that had visibly extracted. It was believed this discrepancy was due to the ink not being completely dry on

the paper when solvated. In the initial solubility study, ink samples used with these two solvents had been prepared on the day and had only been deposited on paper for 15 minutes prior to extraction. Whereas the extractions performed during TLC method development, used ink samples that had been prepared approximately 72 hours beforehand, giving sufficient time to dry completely.

In addition, in a study by Fernandez [182], xylene successfully extracted two pigmented blue gel inks (Zebra and Uniball) in less than 5 minutes of heating in a sand bath. By contrast, in this solubility study none of the 31 blue brand/model combinations were extracted in xylene, even after 15 minutes heating.

Neither Jasuja *et al* [181] or Fernandez [182] comment as to how long the ink had been on the paper substrate prior to extraction. It seems likely therefore, that in light of the observations made during this research, the findings in relation to solubility from these two previous studies be treated with caution since it cannot be verified if the extractions in chloroform or xylene were genuine or as a result of a still wet ink sample at time of solvation. The extracted samples are presented in Figure 2.1.

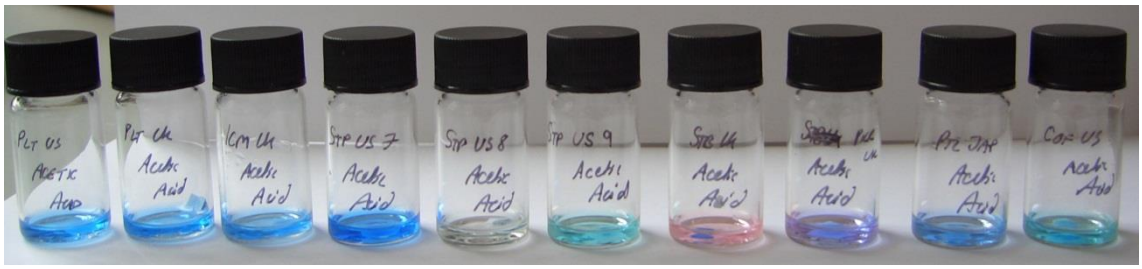


Figure 2.1: Ten blue ink group dye component samples exhibiting visible extraction in acetic acid

2.3.1.2 Red Ink Group

In total 26 red inks representing 25 brand/model combinations were analysed. These contained 24 dye containing and 2 pigment based inks. The results of the extractions in each solvent tested are presented in Table 2.6.

	Sample	Acetic Acid		Xylene		MeOH		Acetone		EtOH		EtAC		Distilled Water		EtAC:EtOH:H_2O		EtOH:H_2O		Butanol		Chloroform		Hydrochloric Acid		
Time (mins)		5	15	5	15	5	15	5	15	5	15	5	15	5	15	5	15	5	15	5	15	5	15	5	15	60
Dye or Dye and Pigment (P)	ZBR UK	*	*	-	*	-	-	-	*	-	-	-	*	-	-	-	-	-	-	-	-	*	*		*	*
	ICM UK	*	*	-	*	*	*	-	*	*	*	-	-	*	*	*	*	*	*	*	*	-	-	*	*	*
	BIC UK	*	*	-	-	-	*	-	*	-	*	-	*	-	-	-	*	-	-	-	*	-	*	*	*	*
	PTN UK	-	-	*	*	-	-	*	*	-	-	*	*	-	-	-	-	-	-	-	-	*	*	-	*	*
	WHS UK #01	*	*	-	-	-	*	*	*	-	*	*	*	-	-	-	-	-	-	-	*	*	*	-	*	*
	WHS UK #04	*	*	*	*	-	*	*	*	-	*	*	*	-	-	-	-	-	-	-	*	*	*	-	*	*
	PPM UK	*	*	*	*	-	*	*	*	-	*	-	*	-	-	-	-	-	-	-	*	*	*	-	*	*
	STD UK	*	*	*	*	-	*	-	*	-	*	*	*	-	-	-	*	-	-	-	*	*	*	*	*	*
	PTL UK	*	*	*	*	-	-	*	*	-	*	*	*	-	-	-	*	-	-	-	*	*	*	-	*	*
	GRE UK	-	-	-	-	-	-	*	*	-	*	*	*	-	-	-	-	-	-	-	*	*	*	-	*	*
	UNI UK	*	*	-	-	-	*	*	*	-	*	*	*	-	-	-	*	-	-	-	-	*	*	-	*	*
	STP UK	*	*	-	*	-	*	*	*	-	*	*	*	-	-	-	*	-	-	-	-	*	*	-	*	*
	PLT UK	*	*	*	*	*	*	*	*	*	*	-	-	*	*	*	*	*	*	*	*	-	-	*	*	*
	UNI SA	*	*	*	*	-	*	*	*	-	*	*	*	-	-	-	*	-	-	-	-	*	*	-	*	*
	UNI AUS	*	*	*	*	-	*	*	*	-	-	*	*	-	-	-	*	-	-	-	-	*	*	-	*	*
	STP US #07	-	-	-	*	-	*	*	*	-	*	*	*	-	-	-	*	-	-	-	-	*	*	-	*	*
	PLT US	*	*	*	*	*	*	*	*	*	*	-	-	*	*	*	*	*	*	*	*	-	-	*	*	*
	ZBR JAP	-	-	-	-	-	-	-	*	-	-	-	*	-	-	-	*	-	-	-	-	*	*	-	*	*
	PTL JAP	-	-	-	*	*	*	*	*	*	*	-	-	*	*	*	*	*	*	*	-	-	-	*	*	*
	UNI HK #25	*	*	-	-	-	*	*	*	-	*	*	*	-	-	-	*	-	-	-	*	*	*	-	*	*
	UNI HK #29	-	-	-	-	-	*	*	*	-	*	*	*	-	-	-	-	-	-	-	*	*	*	-	*	*
	FBC MAL	*	*	*	*	-	*	*	*	-	*	*	*	-	-	-	*	-	-	-	*	*	*	-	*	*
	MG MAL	*	*	-	-	-	*	*	*	-	*	*	*	-	-	-	*	-	-	-	*	*	*	-	*	*
	GSF MAL	-	-	-	-	-	*	*	*	-	*	*	*	-	-	-	*	-	-	-	*	*	*	-	*	*
P	STB UK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*
	UNI JAP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*

Table 2.6: Solubility table for the red ink group. Purple highlighted areas indicate the most suitable solvent/conditions for extracting the dye containing red gel inks. * denotes successful extraction; - denotes unsuccessful extraction; NB STP US #08 was excluded due to ink dry out

Acetone, highlighted in purple in Table 2.6, provided extracts from the majority of the red ink group samples (24) and was therefore considered the most suitable solvent of those tested. Before heating, 19 samples extracted after 5 minutes, but this increased after heating for 15 minutes. The majority of extracts appeared strong in intensity. It was noted however, that heating for 15 minutes appeared to improve extraction strength for certain samples suggesting a longer heating time may improve extraction strength further. Only two samples showed no visible signs of extraction, STB UK and UNI JAP, confirming these as pigment based gel inks. The large number of successful extractions was surprising, and suggested that red gel inks are either primarily dye based, or perhaps contain a combination of dye and pigment colorants, such as in hybrid gel ink pens. Excluding HCl, all other solvents extracted only some brand/model combinations under certain conditions with varying degrees of extraction strength. The extracted samples are presented in Figure 2.2 and Figure 2.3.

Figure 2.2: Fourteen UK acquired red ink group samples exhibiting visible extraction in acetone, except for STB UK

Figure 2.3: Thirteen INTL acquired red ink group samples exhibiting visible extraction in acetone, except for UNI JAP. NB STP US #08 was excluded from this study

2.3.1.3 Hydrochloric Acid Extraction – Protonation of Pigmented Gel Inks

The solubility study demonstrated that with the application of heat, all ink samples (deposited >24 hours) irrespective of colour extracted in hydrochloric acid. For the red ink group, this was achieved after only 15 minutes of heating, whilst for the blue ink group 60 minutes of heating was required for all 31 samples to be extracted. To confirm HCl as a suitable extraction solvent, TLC analysis was performed on all available ink samples.

2.3.1.3.1 TLC Analysis of Hydrochloric Acid Extracts

Hydrochloric acid extracts of all samples within both colour groups were prepared for TLC using all three solvent systems. All samples within a group were spotted onto a single TLC plate using a pulled capillary tube with 0.5 cm intervals between samples. None of the red ink group samples exhibited any component spots. For the blue ink group, a total of seven dye component samples across all three TLC plates exhibited either one or two coloured component spots. These brands were PLT US, PLT UK, PKR UK, STP US #07, STP US #08, STP US #09, STB UK and PTL JAP. However, these spots were very diffuse in appearance. To ensure insufficient sample spotting was not the reason for lack of separation, TLC was repeated on 12 samples, six from each colour group, on new TLC plates. These samples were spotted with un-pulled capillaries and with 1 cm intervals to ensure no interference between samples. Coloured sample spots were clearly visible on the baseline for all samples. After TLC, coloured areas were observed about 1 cm above the baseline, though not always clear or sharp. Four of the blue ink group samples exhibited the clearest blue "component spots", all of which were dye component inks. Furthermore, under UV it was noted that the acid appeared to have severely burnt the TLC plate around the baseline and for a few centimetres (cm) above it.

Given lack of separation in the pigmented samples, the fact that dye component samples could be extracted in other solvents, and time constraints, attention was focussed on the extraction of dye component samples only using the most appropriate solvents identified, and their subsequent TLC analysis.

2.3.2 TLC Study

Exemplar photographs of a TLC plate relevant to each study and for each colour group are shown throughout the Chapter.

2.3.2.1 Repeatability and Reproducibility

The PTL JAP blue brand/model combination set was selected for a repeatability and reproducibility study to ensure all three solvent systems were working effectively.

Visually, component spots were sharp and well resolved, with a few minor exceptions for solvent system 2. Within and between plates, the number and colour of component spots was

indistinguishable. Four dye components were observed: dark blue (furthest from baseline), pink, light blue and pale light blue. Under UV, the pink spot fluoresced orange with greatest intensity observed at UV_{365}. No further spots were observed under UV light. No component bands were observed in the paper or solvent blanks. Figure 2.4 shows an example of a TLC plate illustrating good repeatability and reproducibility.

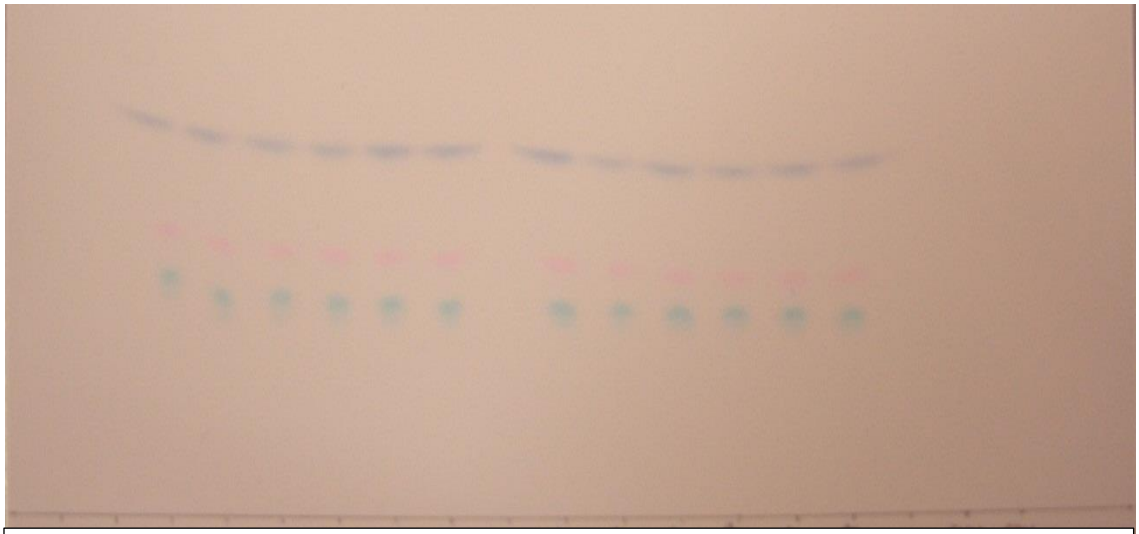


Figure 2.4: Multiple extracts from PTL JAP #07 blue gel pen (left) and six extracts from each of PTL JAP #07 - #12 blue gel pen (right) developed in solvent system 1 (ethyl acetate:ethanol:water (75:35:30)) illustrating an example of the TLC plates developed for a study of reproducibility and repeatability respectively

The repeatability study showed that irrespective of component spot, but with the exception of solvent system 1, % RSD for six samples of the same extract from a single pen was below 5.0%. Edge effects influenced % RSD for solvent system 1 where for all component bands it was below 7.0%.

When the reproducibility of separation of single extracts from all six pens within the brand/model combination set was studied, % RSD was below 5.0% regardless of component band and solvent system. This confirmed that all three solvent systems gave good repeatability and reproducibility of separation. These results are presented in Table 2.7.

Repeatability												
	Solvent System 1				Solvent System 2				Solvent System 3			
Extract	**Spot #1**	**Spot #2**	**Spot #3**	**Spot #4**	**Spot #1**	**Spot #2**	**Spot #3**	**Spot #4**	**Spot #1**	**Spot #2**	**Spot #3**	**Spot #4**
	Dk. Blue	Pink	Lt. Blue	Pale Blue	Dk. Blue	Pink	Lt. Blue	Pale Blue	Dk. Blue	Pink	Lt. Blue	Pale Blue
PTLBLU07 #1	0.56	0.42	0.35	0.33	0.80	0.44	0.42	0.40	0.77	0.34	0.28	0.27
PTLBLU07 #2	0.55	0.40	0.32	0.30	0.81	0.43	0.37	0.40	0.77	0.33	0.28	0.27
PTLBLU07 #3	0.53	0.39	0.32	0.30	0.81	0.42	0.40	0.39	0.77	0.33	0.28	0.27
PTLBLU07 #4	0.53	0.38	0.31	0.29	0.81	0.42	0.40	0.40	0.77	0.33	0.28	0.27
PTLBLU07 #5	0.52	0.38	0.31	0.29	0.80	0.44	0.42	0.40	0.77	0.33	0.28	0.27
PTLBLU07 #6	0.52	0.38	0.31	0.29	0.79	0.44	0.42	0.40	0.76	0.33	0.28	0.27
Mean	**0.54**	**0.39**	**0.32**	**0.30**	**0.80**	**0.43**	**0.40**	**0.40**	**0.77**	**0.33**	**0.28**	**0.27**
Std. Dev.	**0.02**	**0.02**	**0.02**	**0.02**	**0.01**	**0.01**	**0.02**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
% RSD	**3.7**	**5.1**	**6.3**	**6.7**	**1.3**	**2.3**	**5.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
Reproducibility												
Extract	**Spot #1**	**Spot #2**	**Spot #3**	**Spot #4**	**Spot #1**	**Spot #2**	**Spot #3**	**Spot #4**	**Spot #1**	**Spot #2**	**Spot #3**	**Spot #4**
	Dk. Blue	Pink	Lt. Blue	Pale Blue	Dk. Blue	Pink	Lt. Blue	Pale Blue	Dk. Blue	Pink	Lt. Blue	Pale Blue
PTLBLU07	0.51	0.36	0.30	0.28	0.79	0.45	0.42	0.41	0.76	0.33	0.28	0.27
PTLBLU08	0.50	0.35	0.30	0.28	0.79	0.44	0.42	0.40	0.77	0.32	0.28	0.27
PTLBLU09	0.49	0.35	0.29	0.28	0.80	0.43	0.41	0.40	0.77	0.32	0.28	0.27
PTLBLU10	0.49	0.34	0.29	0.28	0.79	0.43	0.41	0.40	0.77	0.32	0.28	0.27
PTLBLU11	0.49	0.34	0.29	0.28	0.79	0.44	0.41	0.40	0.77	0.32	0.27	0.26
PTLBLU12	0.50	0.35	0.29	0.27	0.78	0.44	0.42	0.40	0.76	0.32	0.28	0.26
Mean	**0.50**	**0.35**	**0.29**	**0.27**	**0.79**	**0.44**	**0.41**	**0.41**	**0.77**	**0.32**	**0.28**	**0.27**
Std. Dev.	**0.01**	**0.01**	**0.01**	**0.00**	**0.01**	**0.01**	**0.00**	**0.00**	**0.01**	**0.00**	**0.00**	**0.01**
% RSD	**2.0**	**2.9**	**3.4**	**0.0**	**1.3**	**2.3**	**0.0**	**0.0**	**1.3**	**0.0**	**0.0**	**3.7**

Table 2.7: Summary table of component spot R_f values and % RSD for the repeatability and reproducibility of PTL JAP blue gel ink across all three solvent systems

2.3.2.2 Within Brand Variation

2.3.2.2.1 Blue Ink Group

Three dye containing brand/model combinations, PKR UK, ICM UK and COF US, were extracted and analysed to see if any exhibited within brand variation. Three component spots were observed for both the PKR UK and ICM UK samples, whilst six component spots were observed for the COF US samples as illustrated in Figure 2.5. This was consistent across the plates developed in all three solvent systems. Under UV light, the pink component fluoresced orange with the greatest intensity under UV_{365}.

In general, all three solvent systems exhibited good separation and resolution between component spots. However, using solvent system 1, a blue spot visible in three samples, was not observed in the remaining two samples of the COF US brand. Given this blue spot was observed for all samples on plates developed in the other two solvent systems, this was considered to be a separation issue rather than evidence of within brand variation. Some bearding was observed in the pink component of COF US, but only on the plate developed from solvent system 2. With these exceptions, no evidence of within brand variation was observed in these three brands from the blue ink group, and the three brands could be distinguished from each other quite readily. The R_f data derived from the TLC analysis is provided in Table 2.8. No component bands were observed in the paper or solvent blanks.

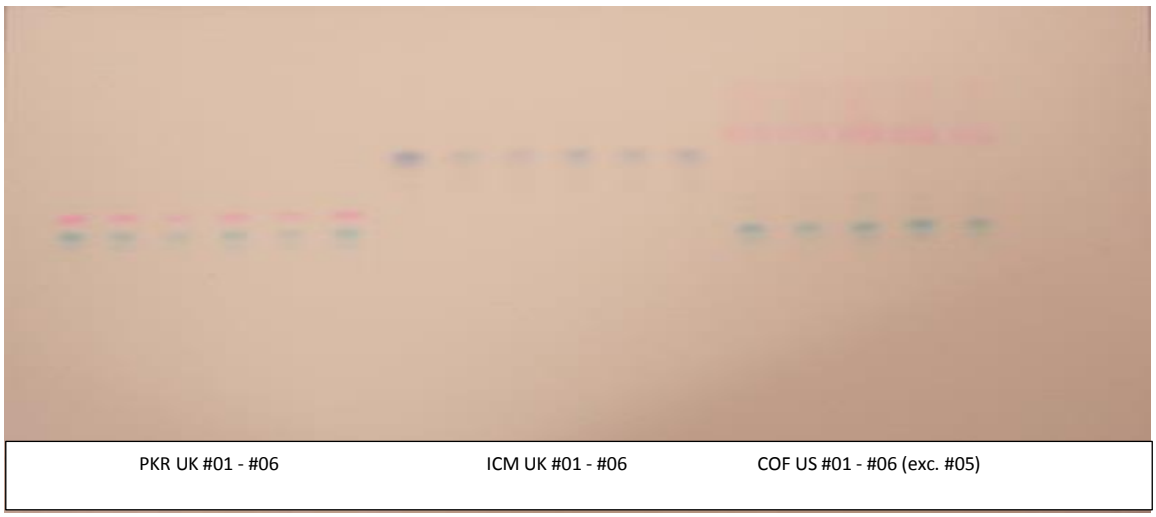


Figure 2.5: Extracts from all pens within three brand/model combinations of blue gel ink (PKR UK, ICM UK and COF US) separated in solvent system 2 (butanol:ethanol:water (50:25:25)) illustrating an example of the TLC plates developed for a study of within brand variation

Sample	Spot Colour	SS.1	SS.2	SS.3	Sample	Spot Colour	SS.1	SS.2	SS.3	Sample	Spot Colour	SS.1	SS.2	SS.3
PKRBLU01	Pink	0.60	0.52	0.45	PKRBLU01	Lt. Blue	0.56	0.48	0.39	PKRBLU01	Pale Lt. Blue	0.52	0.47	0.38
PKRBLU02	Pink	0.57	0.52	0.43	PKRBLU02	Lt. Blue	0.52	0.48	0.38	PKRBLU02	Pale Lt. Blue	0.48	0.47	0.35
PKRBLU03	Pink	0.57	0.52	0.42	PKRBLU03	Lt. Blue	0.51	0.48	0.36	PKRBLU03	Pale Lt. Blue	0.48	0.47	0.35
PKRBLU04	Pink	0.56	0.53	0.42	PKRBLU04	Lt. Blue	0.51	0.49	0.36	PKRBLU04	Pale Lt. Blue	0.47	0.47	0.35
PKRBLU05	Pink	0.57	0.53	0.42	PKRBLU05	Lt. Blue	0.51	0.49	0.36	PKRBLU05	Pale Lt. Blue	0.47	0.47	0.35
PKRBLU06	Pink	0.57	0.53	0.42	PKRBLU06	Lt. Blue	0.51	0.49	0.36	PKRBLU06	Pale Lt. Blue	0.48	0.47	0.35
	Mean	**0.58**	**0.52**	**0.43**		**Mean**	**0.52**	**0.49**	**0.37**		**Mean**	**0.48**	**0.47**	**0.36**
	Std. Dev.	**0.01**	**0.00**	**0.01**		**Std. Dev.**	**0.02**	**0.00**	**0.01**		**Std. Dev.**	**0.02**	**0.00**	**0.01**
	% RSD	**1.7**	**0.0**	**2.3**		**% RSD**	**3.8**	**0.0**	**2.7**		**% RSD**	**4.2**	**0.0**	**2.8**
ICMBLU01	Dk. Blue	0.77	0.65	0.56	ICMBLU01	Pale Dk. Blue	0.76	0.64	0.54	ICMBLU01	Very Pale Dk. Blue	0.71	0.59	0.48
ICMBLU02	Dk. Blue	0.77	0.66	0.56	ICMBLU02	Pale Dk. Blue	0.76	0.64	0.54	ICMBLU02	Very Pale Dk. Blue	0.71	0.59	0.48
ICMBLU03	Dk. Blue	0.78	0.66	0.56	ICMBLU03	Pale Dk. Blue	0.77	0.64	0.54	ICMBLU03	Very Pale Dk. Blue	0.71	0.59	0.48
ICMBLU04	Dk. Blue	0.78	0.66	0.56	ICMBLU04	Pale Dk. Blue	0.77	0.64	0.54	ICMBLU04	Very Pale Dk. Blue	0.72	0.59	0.48
ICMBLU05	Dk. Blue	0.78	0.66	0.55	ICMBLU05	Pale Dk. Blue	0.77	0.65	0.54	ICMBLU05	Very Pale Dk. Blue	0.72	0.59	0.48
ICMBLU06	Dk. Blue	0.78	0.66	0.55	ICMBLU06	Pale Dk. Blue	0.77	0.64	0.54	ICMBLU06	Very Pale Dk. Blue	0.72	0.59	0.48
	Mean	**0.77**	**0.66**	**0.56**		**Mean**	**0.77**	**0.66**	**0.54**		**Mean**	**0.72**	**0.59**	**0.48**
	Std. Dev.	**0.00**	**0.01**	**0.00**		**Std. Dev.**	**0.00**	**0.00**	**0.00**		**Std. Dev.**	**0.01**	**0.00**	**0.00**
	% RSD	**0.0**	**1.5**	**0.0**		**% RSD**	**0.0**	**0.0**	**0.0**		**% RSD**	**1.4**	**0.0**	**0.0**
COFBLU01	Pink	0.97	0.80	0.75	COFBLU01	Pink	0.84	0.70	0.62	COFBLU01	Very Pale Lt. Blue	0.64	0.56	0.45
COFBLU02	Pink	0.98	0.80	0.75	COFBLU02	Pink	0.84	0.70	0.62	COFBLU02	Very Pale Lt. Blue	0.64	0.56	0.44
COFBLU03	Pink	0.98	0.80	0.75	COFBLU03	Pink	0.85	0.70	0.61	COFBLU03	Very Pale Lt. Blue	0.64	0.56	0.44
COFBLU04	Pink	0.99	0.80	0.75	COFBLU04	Pink	0.85	0.70	0.60	COFBLU04	Very Pale Lt. Blue	0.64	0.56	0.42
COFBLU06	Pink	0.99	0.80	0.75	COFBLU06	Pink	0.85	0.69	0.59	COFBLU06	Very Pale Lt. Blue	0.64	0.56	0.40
	Mean	**0.98**	**0.80**	**0.75**		**Mean**	**0.85**	**0.70**	**0.61**		**Mean**	**0.64**	**0.56**	**0.43**
	Std. Dev.	**0.01**	**0.00**	**0.00**		**Std. Dev.**	**0.01**	**0.01**	**0.02**		**Std. Dev.**	**0.00**	**0.00**	**0.02**
	% RSD	**1.0**	**0.0**	**0.0**		**% RSD**	**1.2**	**1.4**	**3.3**		**% RSD**	**0.0**	**0.0**	**4.7**
COFBLU01	Very Pale Lt. Blue	0.60	0.54	0.39	COFBLU01	Cyan/Lt. Blue	0.53	0.50	0.36	COFBLU01	Pale Lt. Blue	0.49	0.47	0.35
COFBLU02	Very Pale Lt. Blue	0.59	0.53	0.39	COFBLU02	Cyan/Lt. Blue	0.53	0.50	0.36	COFBLU02	Pale Lt. Blue	0.49	0.47	0.35
COFBLU03	Very Pale Lt. Blue	0.59	0.53	0.38	COFBLU03	Cyan/Lt. Blue	0.53	0.50	0.35	COFBLU03	Pale Lt. Blue	0.49	0.48	0.35
COFBLU04	Very Pale Lt. Blue	0.60	0.54	0.36	COFBLU04	Cyan/Lt. Blue	0.53	0.50	0.35	COFBLU04	Pale Lt. Blue	0.49	0.48	N/A
COFBLU06	Very Pale Lt. Blue	0.59	0.54	0.35	COFBLU06	Cyan/Lt. Blue	0.53	0.50	0.34	COFBLU06	Pale Lt. Blue	0.49	0.48	N/A
	Mean	**0.60**	**0.54**	**0.38**		**Mean**	**0.53**	**0.50**	**0.35**		**Mean**	**0.49**	**0.48**	**0.35**
	Std. Dev.	**0.00**	**0.00**	**0.02**		**Std. Dev.**	**0.00**	**0.00**	**0.01**		**Std. Dev.**	**0.00**	**0.00**	**0.00**
	% RSD	**0.0**	**0.0**	**5.3**		**% RSD**	**0.0**	**0.0**	**2.9**		**% RSD**	**0.0**	**0..0**	**0..0**

Table 2.8: Within brand variation study: summary table of component spot R_f values and % RSD for three brand/model combinations (PKR UK, ICM UK and COF US) of blue gel ink separated by all three solvent systems; NB pen COFBLU05 was unavailable for testing

2.3.2.2.2 Red Ink Group

To assess within brand variation, three brand/model combinations, BIC UK, WHS UK and PLT US, were extracted and analysed. Both the BIC UK and WHS UK samples exhibited a single red component spot, whilst the PLT US samples exhibited a dark pink and pale pink spot as illustrated in Figure 2.6. This was consistent across all three solvent systems. No additional spots were visualised under UV. However, the PLT US component spots did fluoresce, with the pale pink spots exhibiting an intense fluorescent orange under UV_{365}, and the dark pink spots a fluorescent yellow. Under UV_{254}, both pink spots fluoresced orange.

Solvent system 1 appeared to produce the best TLC results with relatively sharp and well resolved spots. For solvent system 2, although the spots were well defined, separation between the two PLT US components was poor compared to that for the other two solvent systems. Spots exhibited the poorest definition under solvent system 3 by comparison, and for some samples the component spot was relatively pale, i.e. BIC UK #01 - #03 and WHS UK #01 – #03. Interestingly, for an unknown reason, the BIC UK #04 - #06 component spots appeared to have travelled a slightly greater distance up the plate to that of BIC UK #01 - #03, but were in a similar position to the six component spots representing the WHS UK samples. This gave the impression of within brand variation however this observation was not the case for the plates developed under the other two solvent systems. A repeat run of these samples also showed a small but obvious difference in distance travelled between all six red components of the BIC UK samples compared to the WHS UK samples. The R_f data derived from the TLC analysis is provided in Table 2.9. No component bands were observed in the paper or solvent blanks.

Figure 2.6: Extracts from all pens within three brand/model combinations of red gel ink (BIC UK, WHS UK and PLT US) separated in solvent system 1 (ethyl acetate:ethanol:water (75:35:30)) illustrating an example of the TLC plates developed for a study of within brand variation

Sample	Spot Colour	SS.1	SS.2	SS.3	Sample	Spot Colour	SS.1	SS.2	SS.3
BICRED01	Red	0.92	0.79	0.73	WHSRED01	Red	0.96	0.83	0.75
BICRED02	Red	0.92	0.80	0.72	WHSRED02	Red	0.96	0.83	0.75
BICRED03	Red	0.92	0.80	0.72	WHSRED03	Red	0.96	0.83	0.75
BICRED04	Red	0.92	0.80	0.75	WHSRED04	Red	0.95	0.83	0.75
BICRED05	Red	0.92	0.80	0.75	WHSRED05	Red	0.95	0.83	0.75
BICRED06	Red	0.92	0.80	0.75	WHSRED06	Red	0.95	0.83	0.74
	Mean	**0.92**	**0.80**	**0.74**		**Mean**	**0.96**	**0.83**	**0.75**
	Std. Dev.	**0.00**	**0.01**	**0.02**		**Std. Dev.**	**0.00**	**0.00**	**0.00**
	% RSD	**0.0**	**1.25**	**2.7**		**% RSD**	**0.0**	**0.0**	**0.0**
PLTRED07	Dk. Pink	0.70	0.73	0.56	PLTRED07	Pale Pink	0.81	0.75	0.65
PLTRED08	Dk. Pink	0.70	0.73	0.57	PLTRED08	Pale Pink	0.81	0.75	0.65
PLTRED09	Dk. Pink	0.70	0.73	0.57	PLTRED09	Pale Pink	0.81	0.75	0.66
PLTRED10	Dk. Pink	0.70	0.73	0.58	PLTRED10	Pale Pink	0.81	0.75	0.67
PLTRED11	Dk. Pink	0.70	0.73	0.58	PLTRED11	Pale Pink	0.81	0.75	0.68
PLTRED12	Dk. Pink	0.70	0.73	0.59	PLTRED12	Pale Pink	0.81	0.75	0.69
	Mean	**0.70**	**0.73**	**0.58**		**Mean**	**0.81**	**0.75**	**0.67**
	Std. Dev.	**0.00**	**0.00**	**0.01**		**Std. Dev.**	**0.00**	**0.00**	**0.01**
	% RSD	**0.0**	**0.0**	**1.7**		**% RSD**	**0.0**	**0.0**	**1.5**

Table 2.9: Within brand variation study summary table of component spot R_f values and % RSD for three brands of red gel ink (BIC UK, WHS UK and PLT US) separated in all three solvent systems

2.3.2.3 Between Brand Variation

2.3.2.3.1 Blue Ink Group

Generally, all ten blue dye containing samples exhibited fairly sharp and well resolved bands across all solvent systems investigated. The PTL JAP sample exhibited two additional pale blue spots on the plates run in solvent systems 2 and 3, which were not seen in the repeatability/reproducibility study. These spots were barely visible, so it is unclear if these were genuine or simply carry-over from another component. Also for solvent system 3, an additional blue spot was observed in four samples: PLT UK, PLT US, ICM UK and STP US #07, giving a total of four spots per sample compared to only three in solvent systems 1 and 2. Solvent system 2 and 3 gave better resolution for these indistinguishable samples than solvent system 1. A single additional pale blue spot was also observed in the COF US and STP US #08 samples using solvent system 1. A distinctive red spot observed in the STP US #08 sample was more intense on the plates from solvent system 2 and 3, but was present in all three. This red component distinguished STP US #08 from the #09 sample. Under UV, all pink spots fluoresced with greatest intensity under UV_{365}. The PKR UK pink spot appeared a more intense orange than other pink spots under UV_{254}, whilst under UV_{365}, the COF US pink spot fluoresced yellow when all others appeared orange. No component bands were observed in the paper or solvent blanks. Figure 2.7 shows the TLC plate developed in solvent system 2, whilst Table 2.10 summarises the component spots and R_f values.

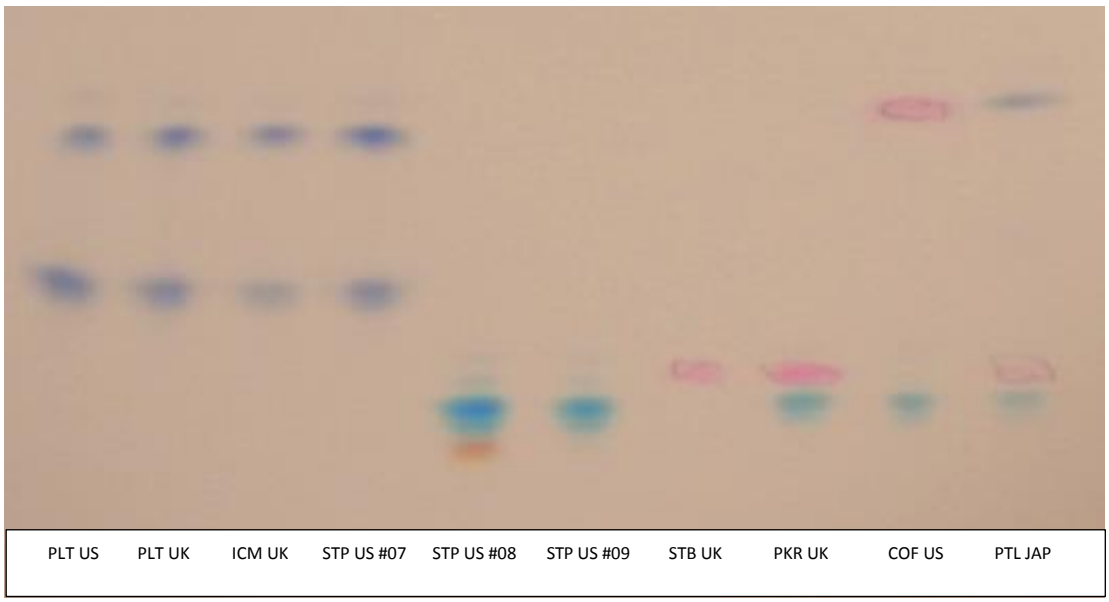


Figure 2.7: Ten dye containing brand/model combinations of blue gel ink separated by solvent system 2 (butanol:ethanol:water (50:25:25)) illustrating an example of the TLC plates developed for a study of between brand variation

Sample	Spot Colour	Solvent Sys. 1	Solvent Sys. 2	Solvent Sys. 3	Group
PLT US	Pale Dk. Blue	0.77	0.78	0.78	1
	Dk. Blue	0.70	0.74	0.74	
	Dk. Blue	-	0.56	0.55	
	Pale Dk. Blue	0.69	-	0.53	
PLT UK	Pale Dk. Blue	0.75	0.77	0.78	
	Dk. Blue	0.69	0.74	0.74	
	Dk. Blue	-	0.56	0.52	
	Pale Dk. Blue	0.67	-	0.49	
ICM UK	Pale Dk. Blue	0.74	0.77	0.77	
	Dk. Blue	0.67	0.74	0.74	
	Dk. Blue	-	0.55	0.52	
	Pale Dk. Blue	0.66	-	0.49	
STP US #07	Pale Dk. Blue	0.73	0.74	0.77	
	Dk. Blue	0.66	0.74	0.73	
	Dk. Blue	-	0.55	0.51	
	Pale Dk. Blue	0.65	-	0.48	
STP US #08	Pale Lt. Blue	0.53	0.47	0.40	2
	Pale Lt. Blue	0.48	0.44	0.34	
	Cyan	0.40	0.42	0.29	
	Lt. Blue	0.36	0.40	0.26	
	Red	0.34	0.37	0.24	
	Pale Lt. Blue	0.32	-	-	
STP US #09	Pale Lt. Blue	0.53	0.47	0.40	3
	Pale Lt. Blue	0.48	0.44	0.34	
	Cyan	0.40	0.42	0.29	
	Lt. Blue	0.35	0.40	0.26	
	Pale Lt. Blue	0.32	0.38	0.24	
STB UK	Pink	0.50	0.45	0.37	4
PKR UK	Very Pale Blue	0.49	0.48	0.40	5
	Pink	0.49	0.45	0.36	
	Lt. Blue/Cyan	0.40	0.45	0.36	
	Pale Lt. Blue	0.35	0.42	0.29	
COF US	Pink	0.94	0.76	0.75	6
	Very Pale Blue	0.53 0.48	0.48	0.40	
	Pale Blue	0.39	0.45	0.34	
	Pale Lt. Blue	0.35	0.42	0.29	
	Pale Lt. Blue	0.33	0.40	0.26	
PTL JAP	Very Pale Blue	-	0.81	-	7
	Dk. Blue	0.72	0.77	0.74	
	Very Pale Blue	-	0.62	0.57	
	Pink	0.49	0.45	0.36	
	Lt. Blue/Cyan	0.39	0.42	0.29	
	Pale Lt. Blue	0.35	0.41	0.27	
	Very Pale Blue	-	-	0.79	
All Other Remaining 21 Brands	Non-Soluble				8

Table 2.10: Summary of component spot R_f values for ten blue ink group dye component samples

Given the occurrence of additional spots in some samples observed on plates from different solvent systems it was difficult to determine which solvent system performed

best. Regardless, for all three solvent systems, it was clearly possible to classify the samples into the same groups on the basis of the number, colour and position of component spots. The dye component samples were classified into seven groups. When the non-soluble samples are included then the blue ink group could be classified into a total of eight groups.

2.3.2.3.2 Red Ink Group

During method development, where several TLC runs of the red ink group samples were performed, it was apparent that a large number exhibited indistinguishable component spots. It was decided to spot these samples onto one plate and the remaining samples onto a second plate. In addition, two samples (ZBR UK and ZBR JAP) spotted on plate 1 were spotted onto plate 2 as well in order to enable a comparison of spot distance to the other samples. For plate 1, which contained 19 of the red ink group samples, solvent systems 2 and 3 were found to give sharper spots than for solvent system 1. For all three solvent systems, only a single red band occurring at approximately the same position, a few millimetres below the solvent front in solvent system 1 was observed for all 19 samples. Some samples exhibited a paler spot than others, but nonetheless all were visible. No additional spots were observed under UV light. For plate 2, which contained the remaining seven red samples, as well as two samples also on plate 1, the definition of the component spots varied within and between sample and solvent system. However, all were visible and distinct. Considering the observations of both plates, it was difficult to suggest which solvent system offered the best performance for the red ink group. Interestingly, the BIC UK sample exhibited a single red band that occurred at a slightly lower position than the single red band exhibited by the two ZBR samples. This observation was consistent throughout several TLC runs of the red samples carried out during method development, suggesting that the BIC UK sample contained a different dye component to that of the 19 samples on plate 1. As expected, neither of the non-soluble red ink samples, UNI JAP and STB UK, exhibited any component spots, thus confirming their pigmented nature. Figure 2.8 shows the TLC plates developed in solvent system 3, whilst Table 2.11 summarises the component spots and R_f values for each ink sample.

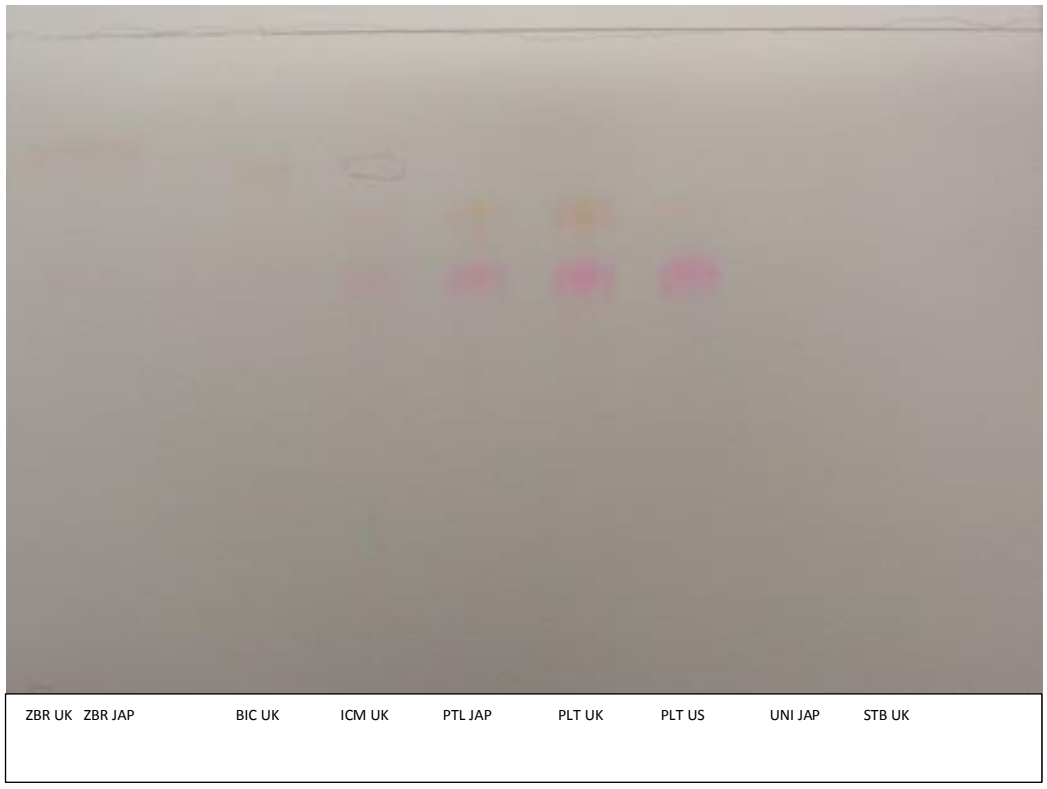


Figure 2.8: Extracts from 25 brand/model combinations (26 samples) of red gel ink split between plate 1 (top) and plate 2 (bottom) separated in solvent system 3 (butanol:ethanol:water:acetic acid (50:25:25:0.5) illustrating an example of the TLC plates developed for a study of between brand variation

Plate	Sample	Spot Colour	Solvent Sys. 1	Solvent Sys. 2	Solvent Sys. 3	Group
1	GRE UK	Red	0.97	0.79	0.83	1
	STD UK	Red	0.98	0.79	0.84	
	STP UK	Red	0.98	0.79	0.84	
	PTL UK	Red	0.98	0.80	0.84	
	PPM UK	Red	0.98	0.80	0.83	
	PTN UK	Red	0.98	0.80	0.83	
	ZBR UK	Red	0.97	0.80	0.83	
	WHS UK #01	Red	0.98	0.80	0.82	
	WHS UK #04	Red	0.98	0.80	0.83	
	MG MAL	Red	0.98	0.81	0.83	
	GSF MAL	Red	0.98	0.81	0.83	
	FBC MAL	Red	0.98	0.81	0.83	
	UNI UK	Red	0.98	0.81	0.83	
	ZBR JAP	Red	0.98	0.81	0.82	
	UNI HK #25	Red	0.98	0.82	0.83	
	UNI HK #29	Red	0.98	0.83	0.83	
	STP US #07	Red	0.99	0.83	0.83	
	UNI AUS	Red	0.99	0.83	0.83	
	UNI SA	Red	0.99	0.83	0.83	
2	ZBR UK	Red	0.98	0.83	0.83	For Comparison Only
	ZBR JAP	Red	0.98	0.83	0.83	
	BIC UK	Red	0.93	0.79	0.79	2
	ICM UK	Pale Pink	-	0.83	0.80	3
		Pale Pink	0.83	0.76	0.72	
		Dk. Pink	0.73	0.73	0.63	
	PTL JAP	Pale Pink	0.82	0.74	0.72	
		Dk. Pink	0.72	0.71	0.63	
	PLT UK	Yellow	0.85	0.80	0.74	4
		Pale Pink	0.82	0.75	0.72	
		(Small Pink)	0.79	-	0.69	
		Dk. Pink	0.72	0.73	0.63	
	PLT US	Yellow	0.85	0.80	0.74	
		Pale Pink	0.82	0.75	0.72	
		(Small Pink)	0.79	-	0.70	
		Dk. Pink	0.72	0.73	0.63	
	UNI JAP	Non-Soluble				5
	STB UK					

Table 2.11: Summary of component spot R_f values for red ink group samples (based on 30 minute heating time)

The remaining four samples: ICM UK, PTL JAP, PLT UK and PLT US, proved to be quite problematic in their discrimination. When extracts of these four brand/model combinations were prepared under different heating times and analysed by TLC, discrepancies in the number and colour of component bands for each sample were observed. Table 2.12 shows the number and colour of component spots for each of these four brand/model combinations extracted under 30, 45 and 90 minutes of heating time. The pale and dark pink highlighted squares indicate the dye component bands that were

similar in appearance and position across all four brand/model combinations and were therefore considered reproducible. The yellow highlighted squares indicate a dye component seen consistently in the second set of PLT sample extracts heated for 30 and 90 minutes, but not in the first set or those extracts heated for 45 minutes. Table 2.12 clearly illustrates additional non-reproducible banding in the samples across the different conditions and within making it difficult to reliably discriminate these four inks. With one exception only, no component bands were observed in the paper or solvent blanks. For extract set #01 (90 mins, 400°C), a single violet band visible only under UV_{365} was observed in the solvent blank, similar to that observed in the PLT UK sample. In both cases, this component band was only observed on the TLC plate developed in solvent system 1, and not observed in all samples on that plate. This suggests that it most likely arose from a contaminant in the extraction vials used for these two samples rather than from contamination of the solvent itself, which is entirely possible since the extraction vials were washed and reused each day. It is possible that all additional banding seen in the red ink group samples could be attributable to contamination from the extraction vials. However, problems with additional banding were not encountered for the blue ink group samples, suggesting this problem was restricted to the red ink group samples only, making it more likely an issue with these four red gel inks specifically.

Despite these inconsistencies, certain components, as highlighted in Table 2.12, were consistently seen across the different extracts. A pale pink and dark pink component spot was observed at similar positions on all TLC plates for all four brand/model combinations. Both the PLT UK and PLT US samples could arguably be differentiated from the ICM UK and PTL JAP samples by an additional yellow component spot that was considered reproducible dependent upon extract/heating time combination. All pale pink spots fluoresced intensely orange under UV_{365}, whilst all dark pink spots fluoresced yellow. Under UV_{254}, both pink spots for all four samples fluoresced orange. The ICM UK and PTL JAP samples were considered indistinguishable to each other as were the PLT UK and PLT US samples. Classification of red ink group samples was based on results from TLC of extract set #02 (30 mins, 400°C), and in light of the above, was made on the basis of those component spots considered reproducible within that particular set of conditions and observed across all three solvent systems. Therefore the red samples could be classified into five groups.

However, it should be noted that the additional yellow spot observed for both PLT brand/model combinations during the between brand study, and used as the basis of discrimination from the ICM UK and PTL JAP samples, was not observed in the PLT US samples analysed in the within brand study. This conflicting result in addition to those described above perhaps suggest that the PLT brand/model combinations cannot be reliably discriminated from the ICM UK and PTL JAP samples by TLC alone and that these four inks should be considered indistinguishable until shown otherwise.

Sample	Heating Time/Extract Set/Solvent System															
	30 Minutes						45 Minutes			90 Minutes						
	Extract Set #01			Extract Set #02			Extract Set #01			Extract Set #01			Extract Set #02			
	SS1	SS2	SS3	SS1	SS2	SS3	SS1	SS2	SS3	SS1	SS2	SS3	SS1	SS2	SS3	
ICM UK	All component bands travelled at solvent front - excluded	Poor band resolution - excluded	(dull orange UV_{365})	-	feint pink	feint pink	Extraction Unsuccessful			(dull orange UV_{365})	-	-	-	-	-	
			-	-	-	-				pale red	(intense violet UV_{365})	(intense violet UV_{365})	pale pink (pale yellow UV_{365})	pale pink (pale yellow (UV_{365})	pale pink (pale yellow UV_{365})	
			red (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})				red (orange UV_{254}/yellow UV_{365})	red (orange UV_{254}/yellow UV_{365})	red (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})	
			pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})				pink (orange UV_{254} and UV_{365})	pink (orange UV_{254} and UV_{365})	pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	
PTL JAP			(dull orange UV_{365})	-	-	-	(dull yellow UV_{365})	(dull yellow UV_{365})	(dull yellow UV_{365})	(dark band UV_{254})	-	-	(dark band UV_{254})	(dark band UV_{254})	(dark band UV_{254})	
			red (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})	-	-	-	red (orange UV_{254}/yellow UV_{365})	red (orange UV_{254}/yellow UV_{365})	red (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})	
			pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	pink (orange UV_{254} and UV_{365})	pink (orange UV_{254} and UV_{365})	pink (orange UV_{254} and UV_{365})	pink (orange UV_{254} and UV_{365})	pink (orange UV_{254} and UV_{365})	pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	
PLT UK			-	-	-	-	-	-	-	(violet UV_{365})	-	-				
			(dull orange UV_{365})	yellow	yellow	yellow	-	-	-	(dark band UV_{254})	-	-	yellow	yellow	yellow	
			-	-	-	-	-	-	-	small pink	-	-	-	-	-	
			red (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})	feint pink/red (orange UV_{254}/yellow UV_{365})	feint pink/red (orange UV_{254}/yellow UV_{365})	feint pink/red (orange UV_{254}/yellow UV_{365})	red (orange UV_{254}/yellow UV_{365})	red (orange UV_{254}/yellow UV_{365})	red (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})	
			-	small pale pink	-	small pale pink	-	-	-	-	-	-	small pale pink	(dark band UV_{254})	(dark band UV_{254})	
			pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	pink (orange UV_{254} and UV_{365})	pink (orange UV_{254} and UV_{365})	pink (orange UV_{254} and UV_{365})	pink (orange UV_{254} and UV_{365})	pink (orange UV_{254} and UV_{365})	pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	
PLT US			(dull orange UV_{365})	yellow	yellow	yellow	-	-	-	(dark band UV_{254})			yellow	yellow	yellow	
			-	-	-	-	-	-	-	small red			-	-	-	
			red (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})	feint pink/red (orange UV_{254}/yellow UV_{365})	feint pink/red (orange UV_{254}/yellow UV_{365})	feint pink/red (orange UV_{254}/yellow UV_{365})	red (orange UV_{254}/yellow UV_{365})	red (orange UV_{254}/yellow UV_{365})	red (orange UV_{254}/yellow UV_{365})	pale pink (orange UV_{254}/yellow UV_{365})	(dark band UV_{254})	pale pink (orange UV_{254}/yellow UV_{365})	
			-	small pale pink	-	small pale pink	-	-	-	-	-	-	small pale pink	pale pink (orange UV_{254}/yellow UV_{365})	(dark UV_{254})	
			pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	pink (orange UV_{254} and UV_{365})	pink (orange UV_{254} and UV_{365})	pink (orange UV_{254} and UV_{365})	pink (orange UV_{254} and UV_{365})	pink (orange UV_{254} and UV_{365})	pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	dark pink (orange UV_{254} and UV_{365})	

Table 2.12: Summary of component bands observed in four brand/model combination extracts heated for 30, 45 and 90 minutes at 400°C and separated by solvent systems 1 – 3 illustrating the presence of reproducible and non-reproducible components highlighting the difficulty in discriminating these particular samples. Pale and dark pink highlighted squares indicate the dye component bands that were similar in appearance and position across all four brand/model combinations and were therefore considered reproducible. Yellow highlighted squares indicate a dye component seen consistently in the second set of PLT sample extracts heated for 30 and 90 minutes, but not in the first set or those extracts heated for 45 minutes

2.3.3 % Reflectance Microspectrophotometry of Components on TLC Plate

2.3.3.1 Repeatability and Reproducibility

A set of six spectra acquired from the same and different areas of each component spot within the COF US blue extract separated in TLC solvent system 1, exhibited excellent repeatability and reproducibility as illustrated in Figure 2.9 and Figure 2.10 respectively.

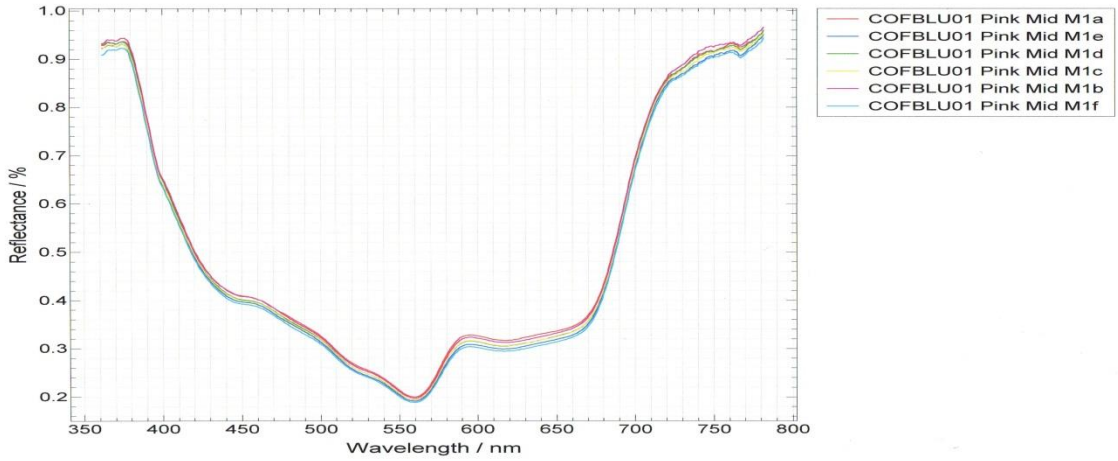


Figure 2.9: Six % reflectance spectra acquired from the same position on the pink component spot of the COF US blue extract separated in TLC solvent system 1 (ethyl acetate:ethanol:water (75:35:30)) illustrating excellent repeatability

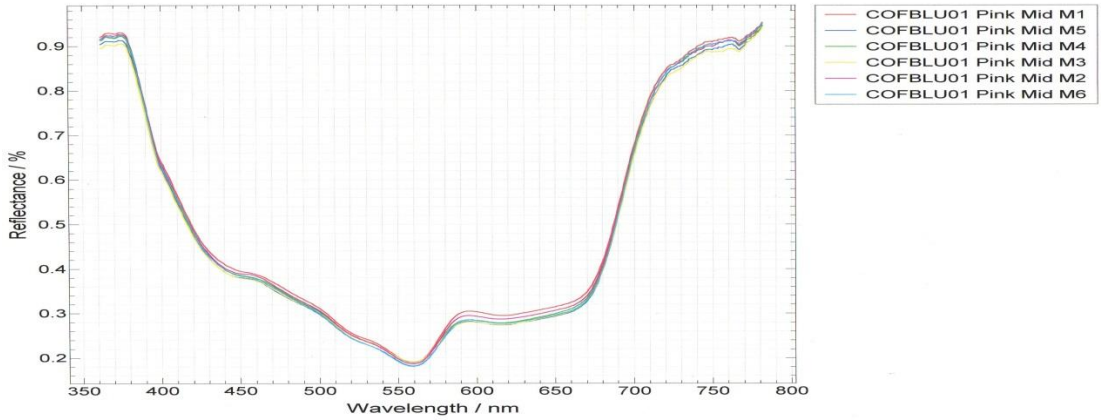


Figure 2.10: Six % reflectance spectra acquired from different areas of the same pink component spot in the COF US blue extract separated in solvent system 1 (ethyl acetate:ethanol:water (75:35:30) illustrating excellent reproducibility

Previous research has demonstrated that % reflectance spectra taken of component spots on TLC plates by Vis-MSP can offer a further possible means of discrimination [108, 182]. To test this, single spectra were taken from each component spot within each dye containing sample across both colour groups, and compared.

2.3.3.2 Blue Ink Group

With the exception of the red component spot visible for STP US #08, all other spots observed within the blue ink group were either blue or pink. The COF US sample exhibited both, with five blue spots of varying intensity and a single pink spot. Two of the palest blue spots closest to the solvent front were so pale, that it was difficult to detect them on the video camera when positioning the sample slit. Circling the component spot in pencil as described by Fuller [108] and taking measurements within this boundary did not assist since spectra were similar to that from the blank TLC plate suggesting that these component bands were too pale to be detected by reflectance Vis-MSP. Therefore no spectra were recorded from these components (highlighted in blue in Table 2.13). However, spectra were recorded for each of the three remaining blue and single pink component bands, shown in Figure 2.11.

Spectra from the three blue component bands did reveal some spectral difference with the presence of trough occurring between 600 nm – 650 nm, differentiating the bands from the spectra of the TLC plate and from each other. The depth of the trough appeared slightly greater for the mid-pale blue spot than for the bottom pale blue spot closest to the baseline, otherwise spectral shape was considered indistinguishable. The spectrum from the pink component could clearly be distinguished from that of the blue spots and the TLC plate by a deep trough followed immediately by a small peak between 550 nm – 600 nm. Similar observations were made for the PKR UK and PTL JAP samples which also contained mixed coloured components, the STB UK sample which exhibited a single pink spot and the STP US #08 and STP US #09 samples which exhibited several blue components. In particular, the STP US #09 sample illustrated the variation in depth of the trough between 600 nm – 650 nm for its six blue components of varying intensity as demonstrated in Figure 2.12. The red component spot of the STP US #08 sample, similar to the blue components, exhibited a slight difference in spectral shape around 600 – 650 nm due to a slight, but pronounced trough distinguishing it from the TLC plate spectrum illustrated later.

Interestingly for the ICM UK, STP US #07, PLT UK and PLT US samples, which exhibited three indistinguishable blue component spots, spectral response from each component was considered no different to that of the TLC plate, thus providing no further information for discrimination. This is clearly presented in Figure 2.13.

Sample	Spot Colour	Solvent Sys. 1	Group
PLT US	Pale Dk. Blue	0.77	1
	Dk. Blue	0.70	
	Dk. Blue	-	
	Pale Dk. Blue	0.69	
PLT UK	Pale Dk. Blue	0.75	
	Dk. Blue	0.69	
	Dk. Blue	-	
	Pale Dk. Blue	0.67	
ICM UK	Pale Dk. Blue	0.74	
	Dk. Blue	0.67	
	Dk. Blue	-	
	Pale Dk. Blue	0.66	
STP US #07	Pale Dk. Blue	0.73	
	Dk. Blue	0.66	
	Dk. Blue	-	
	Pale Dk. Blue	0.65	
STP US #08	Pale Lt. Blue	0.53	2
	Pale Lt. Blue	0.48	
	Cyan	0.40	
	Lt. Blue	0.36	
	Red	0.34	
	Pale Lt. Blue	0.32	
STP US #09	Pale Lt. Blue	0.53	3
	Pale Lt. Blue	0.48	
	Cyan	0.40	
	Lt. Blue	0.35	
	Pale Lt. Blue	0.32	
STB UK	Pink	0.50	4
PKR UK	Very Pale Blue	0.49	5
	Pink	0.49	
	Lt. Blue/Cyan	0.40	
	Pale Lt. Blue	0.35	
COF US	Pink	0.94	6
	Very Pale Blue	0.53 0.48	
	Pale Blue	0.39	
	Pale Lt. Blue	0.35	
	Pale Lt. Blue	0.33	
PTL JAP	Very Pale Blue	-	7
	Dk. Blue	0.72	
	Very Pale Blue	-	
	Pink	0.49	
	Lt. Blue/Cyan	0.39	
	Pale Lt. Blue	0.35	
	Very Pale Blue	-	

Table 2.13: Summary table of component spot R_f values for ten blue ink group dye component samples separated in solvent system 1 (ethyl acetate:ethanol:water (75:35:30). Blue highlighted square indicates component spots that were too pale to be detected by % reflectance Microspectrophotometry

Blue Ink Group: COF US

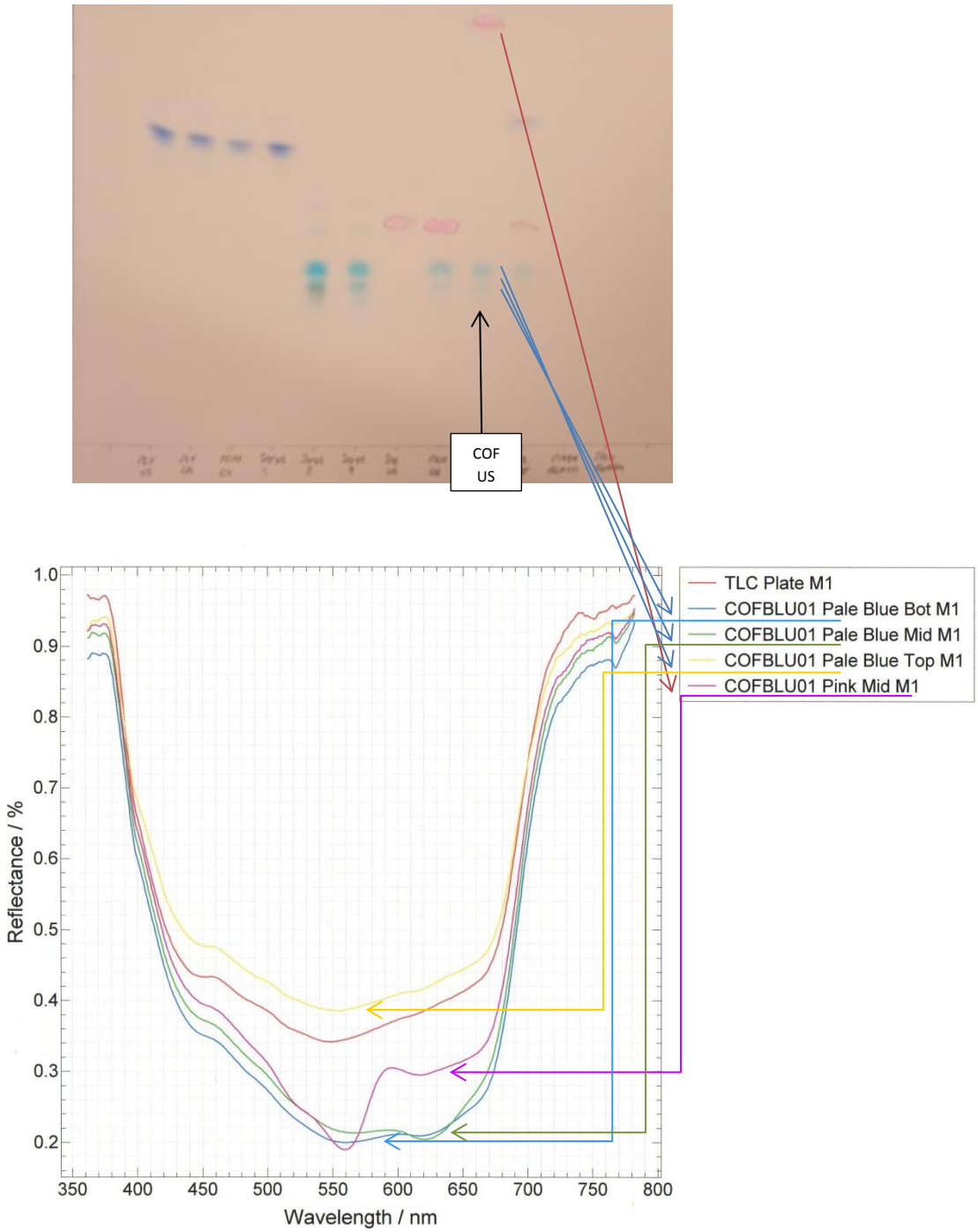


Figure 2.11: % Reflectance spectra from three blue and one pink component spot in COF US blue gel ink extract separated on a TLC plate developed in solvent system 1 (ethyl acetate:ethanol:water (75:35:30)) illustrating the spectral differences between and within the coloured components and the blank TLC plate

Blue Ink Group: STP US #09

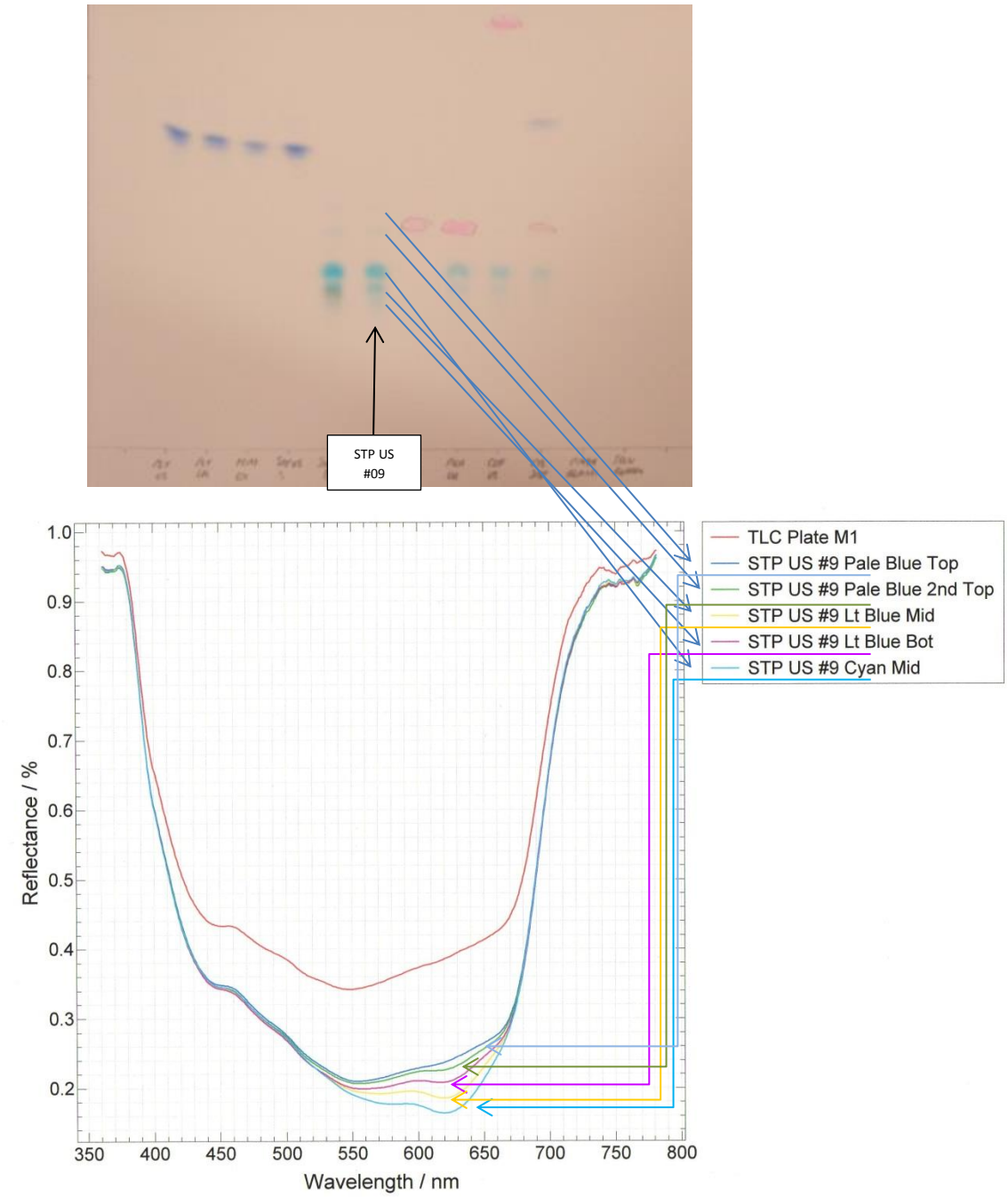


Figure 2.12: % Reflectance spectra from five blue component spots in STP US #09 blue gel ink extract on a TLC plate developed in solvent system 1 (ethyl acetate:ethanol:water (75:35:30)) illustrating the increasing depth of the trough around 600 – 650 nm in spectra from each blue component

Blue Ink Group: PLT UK, PLT US, ICM UK and STP US #07

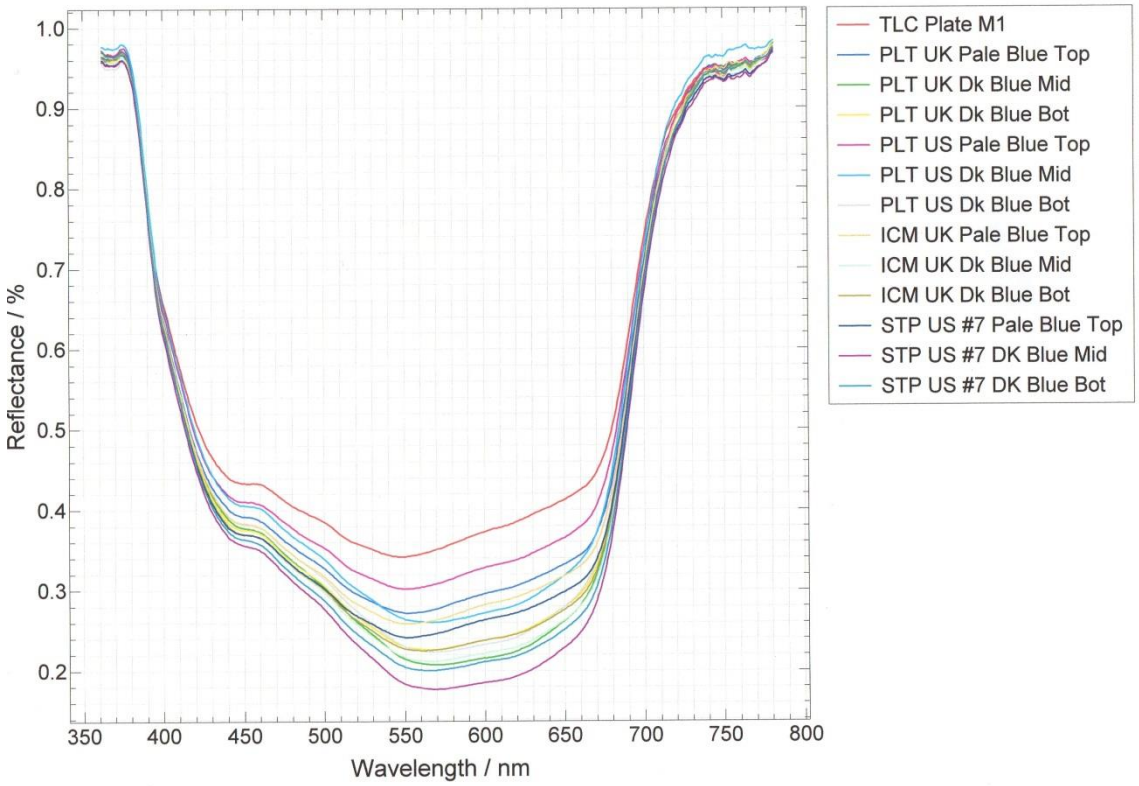


Figure 2.13: % Reflectance spectra from the blue component spots on a TLC plate developed under solvent system 1 (ethyl acetate:ethanol:water (75:35:30) of four brand/model combinations of blue gel ink (considered indistinguishable by TLC) together with % reflectance spectra from the TLC plate itself illustrating the inability to distinguish between them

In terms of discriminating similar coloured components between samples, this was achievable only on the basis of % reflectance at certain spectral points. For the blue components (Figure 2.14) that could be distinguished from the TLC plate spectrum, the % reflectance or depth of trough around 600 – 650 nm exhibited differences. For the pink components (Figure 2.15), the depth of trough around 560 nm also showed differences that could distinguish between similar coloured components from different samples. Interestingly, the red component of STP US #08 could be distinguished from the pink component by the lack of a trough at ~560 nm, but did exhibit a trough around 600 – 650 nm, also seen at varied % reflectance in the pink component spectra, but as mentioned previously, not the spectrum of the TLC plate.

Blue Ink Group: Blue Component Spots Across All Ink Samples

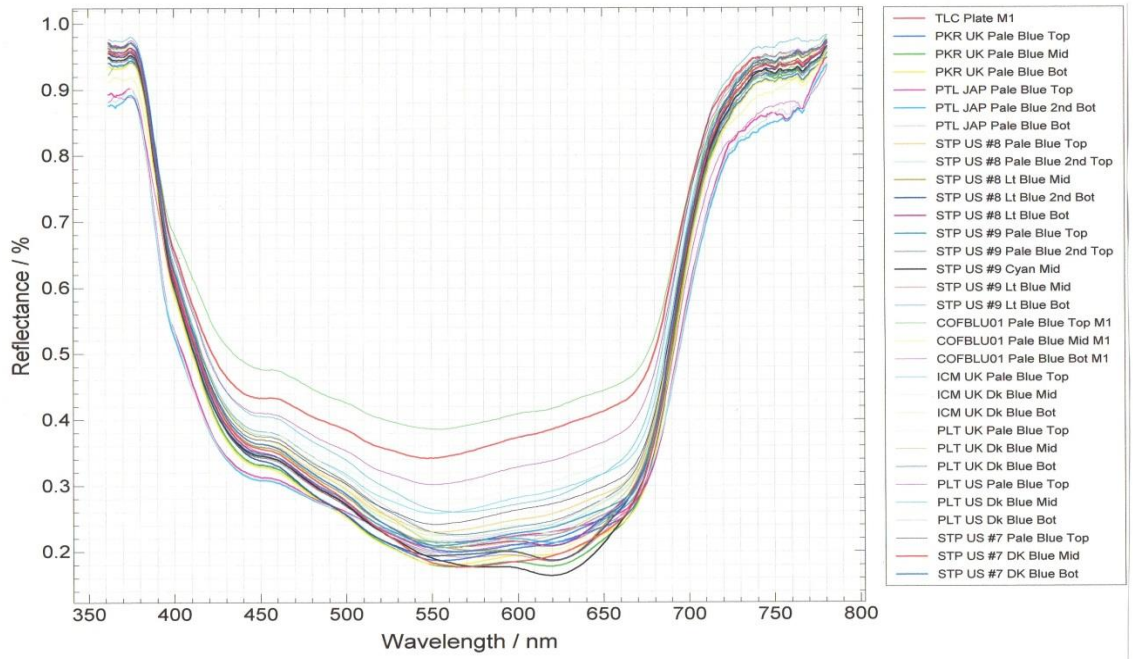


Figure 2.14: % Reflectance spectra from blue component spots of all samples in the blue ink group and from the TLC plate developed in solvent system 1 (ethyl acetate:ethanol:water (75:35:30) illustrating the variation in % reflectance (depth of trough) around 600 – 650 nm

Blue Ink Group: Pink and Red Component Spots Across All Ink Samples

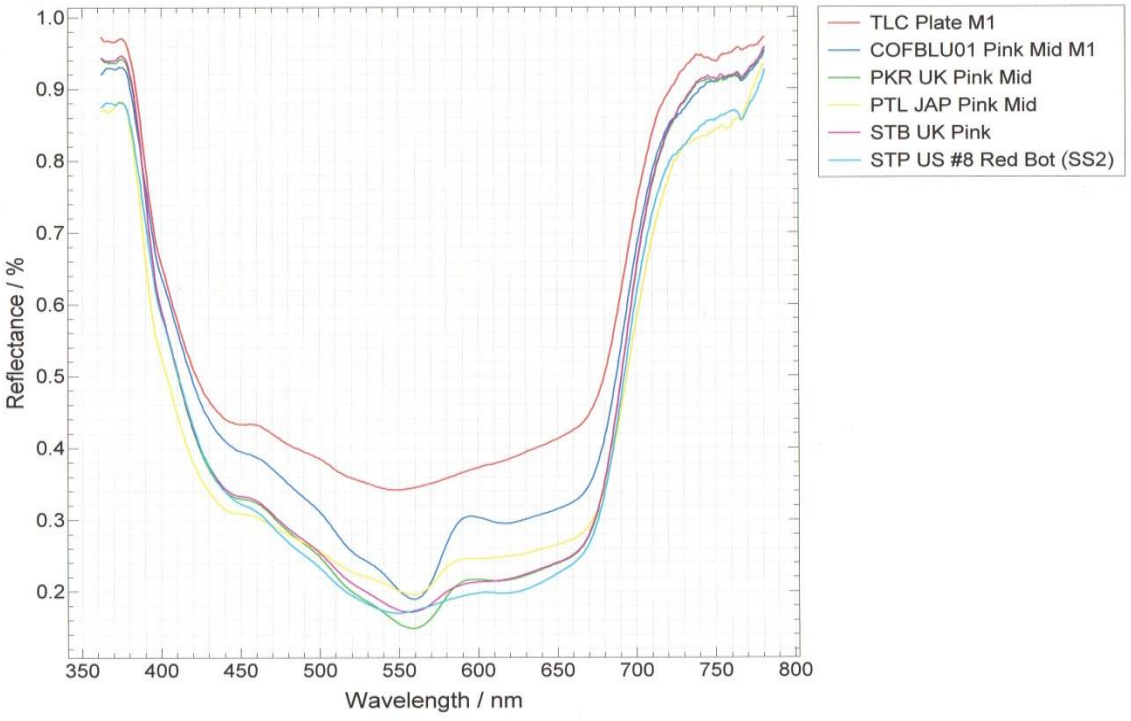


Figure 2.15: % Reflectance spectra from the pink and red component spots in all samples of the blue group and TLC plate developed in solvent system 1 (ethyl acetate:ethanol:water (75:35:30) illustrating variations in % reflectance (depth of trough) around 560 nm and 600 – 650 nm discriminating the red component from the pink component, and both from the TLC plate

2.3.3.3 Red Ink Group

No spectral difference was observed between any of the spectra recorded from the red component for the 19 brands of red ink on plate 1 to each other or to the TLC plate. Likewise, on Plate 2, no spectral difference was observed for the single red component of the BIC UK sample against these other single component samples. Interestingly, the % reflectance spectra from these single red component spots also could not be discriminated from that of the TLC plate suggesting they were too pale to be detected. Figure 2.16 shows spectra from all 20 single component red group samples together with spectra from the TLC plate to illustrate the lack of discrimination.

Red Ink Group: Red Component Spots Across All Single Dye Component Ink Samples

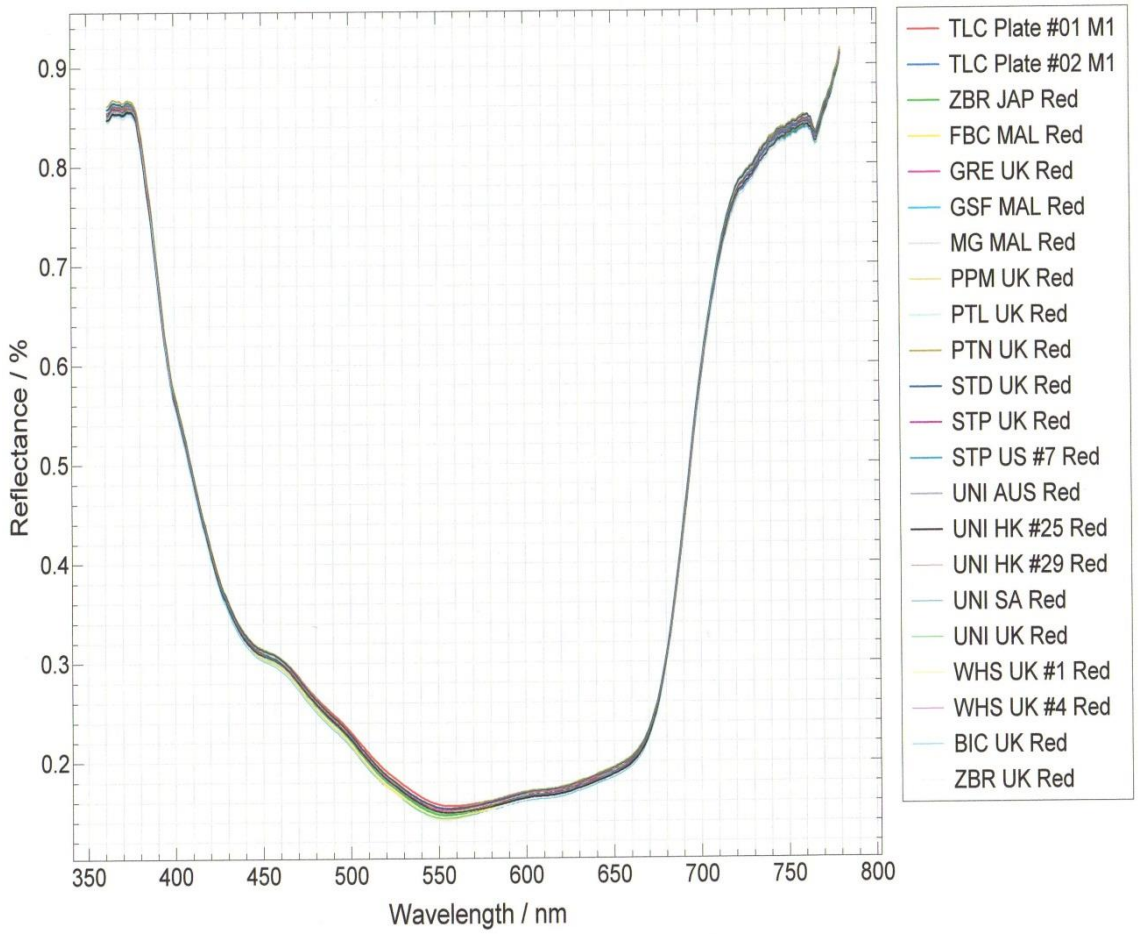


Figure 2.16: % Reflectance spectra from the single red component on a TLC plate developed under solvent system 1 (ethyl acetate:ethanol:water (75:35:30) of 20 brand/model combinations of red gel ink together with % reflectance spectra from the TLC plate itself illustrating the inability to distinguish between them

The ICM UK, PTL JAP, PLT UK and PLT US samples contained at least two dye components, including an upper pale pink and a lower dark pink spot that were considered indistinguishable between these four brand/model combinations but distinguishable from the other red pens. The pale pink spot closest to the solvent front, exhibited spectra that had a λ_{min} around 530 nm – 540 nm, whilst the single red component of the other 20 samples exhibited a λ_{min} around 550m – 560 nm. The spectra recorded from the dark pink spot closest to the baseline exhibited spectra with a λ_{min} also around 550 nm and overall spectral shape appeared similar to that of the single component samples. However, the λ_{min} exhibited a notably deeper trough in these four multi-component samples than for the single component samples which appeared quite flat. Spectra recorded from each of the two component spots of these four multi-component samples were considered indistinguishable, suggesting that they contained the same dye components. Figure 2.17 and Figure 2.18 demonstrate these spectral similarities and differences for the upper pale pink and lower dark pink component spots respectively.

Red Ink Group: Upper Pale Pink Component Band in ICM UK, PTL JAP, PLT UK and PLT US

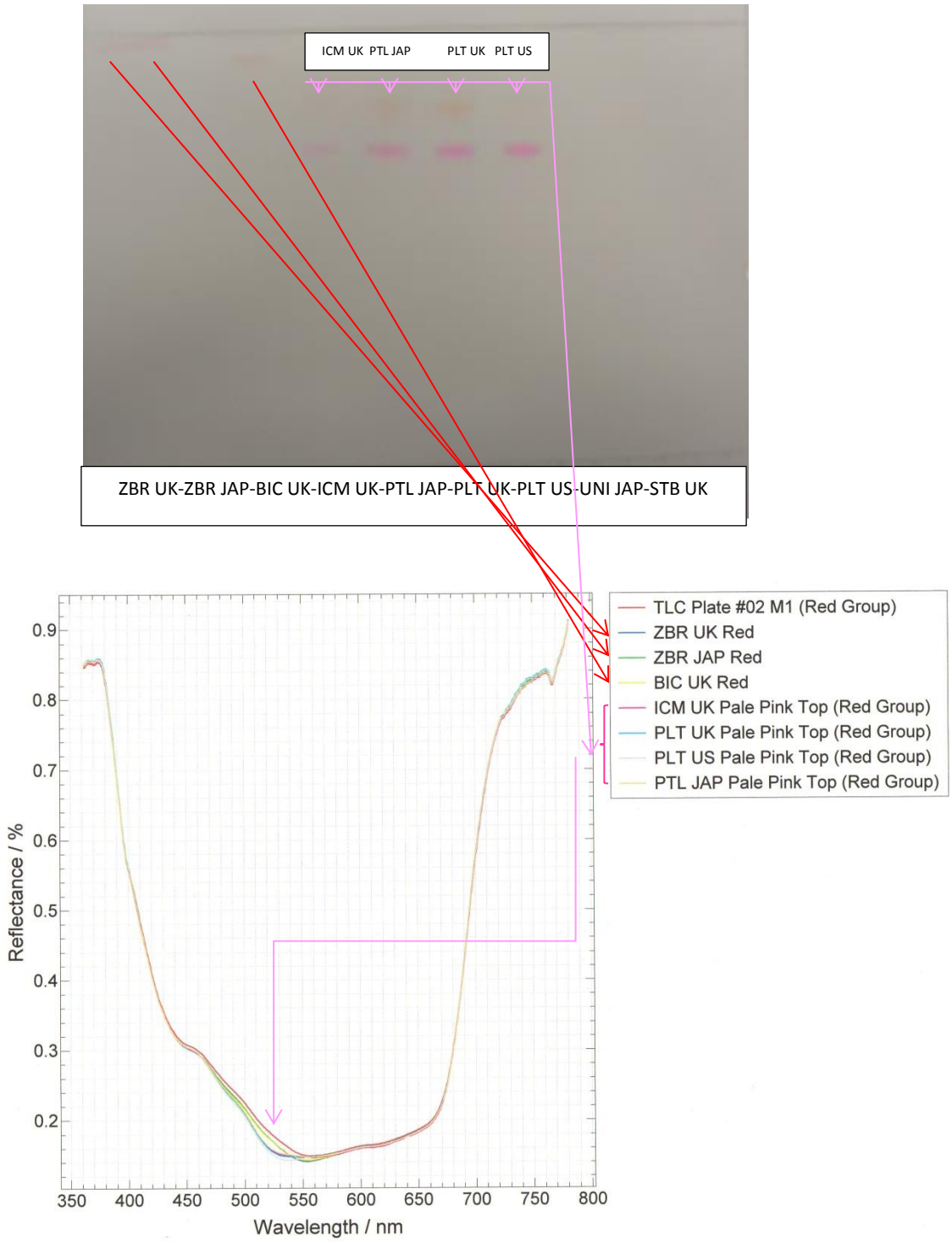


Figure 2.17: % Reflectance spectra from a single red component spot observed in several samples against the upper pale pink component spot in the four dual component sample extracts separated on a TLC plate developed in solvent system 1 (ethyl acetate:ethanol:water (75:35:30)) illustrating the slight shift in λ_{min} between 530 – 560 nm discriminating the former and the latter brand/model combination component spot

Red Ink Group: Lower Dark Pink Component Band in ICM UK, PTL JAP, PLT UK and PLT US

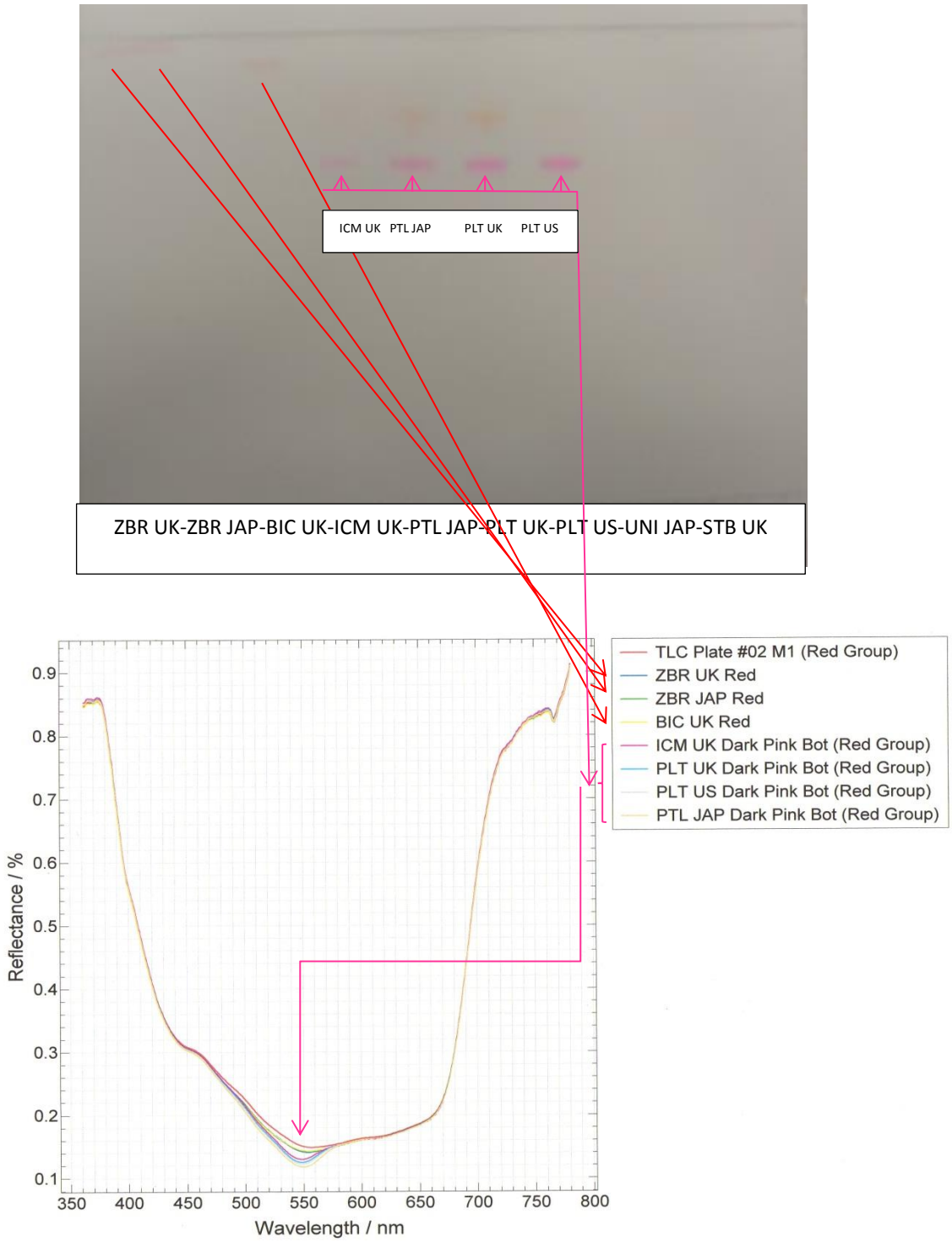


Figure 2.18: % Reflectance spectra from single red component spot observed in several samples against the lower dark pink component spot in the four dual component sample extracts separated on a TLC plate developed in solvent system 1 (ethyl acetate:ethanol:water (75:35:30)) illustrating the increased depth of the λ_{min} trough around 550 nm discriminating the dual component brand/model combination component spot from the single component samples

2.4 Conclusion

On the basis of solubility, it was possible to preliminarily class the inks as being either containing a dye component and/or pigments. The blue ink group could be split into 10 dye component gels and 21 pigmented inks. The red ink group however, was the most interesting, with only two inks identified as solely pigmented. The remaining 24 must all have contained dyes either alone or in combination with pigments as in hybrid gel ink formulations.

In terms of suitable solvents, of the 12 studied, acetic acid was confirmed as being the most appropriate for blue gel inks, providing good extraction without heating. Whilst acetone was the most appropriate for the red ink group in combination with heating in a sand bath for at least 15 minutes. Heating for longer may improve the extraction strength of certain brand/model combinations when in solution, but may risk degradation of some dye components.

The protonation method of extraction using HCl in combination with heating between 15 – 60 minutes dependent upon colour group initially appeared to result in visible extraction of ALL gel ink samples irrespective of colorant. However, subsequent TLC analysis resulted in very poor discrimination of the samples. This method clearly requires further development, perhaps with the use of a different extraction solvent such as dimethylformamide (DMF) which has been shown to extract some slightly soluble phthalocyanine pigments [178].

With regards to TLC all three solvent systems exhibited good repeatability and reproducibility. No obvious within brand variation was observed across the brand/model combinations analysed. However, the WHS UK red samples did appear to exhibit subtle differences in terms of the intensity and resolution of the single red component band. This may suggest that pen #01 - #03 were chemically different to pen #04 – #06, but requires confirmation from another analytical technique.

With regards to the between brand variation study, no single solvent system was identified as giving better performance than the others and it was possible to discriminate the dye containing samples for each colour group into the same groups irrespective of solvent system used. In their entirety, the blue ink group samples could be discriminated into eight

groups and the red ink group into five groups. For the red ink group, it was noted that 19 of the 26 samples analysed exhibited a single red component in a similar R_f position. The BIC UK sample also exhibited a single red component band, but at a slightly lower R_f position. Two samples, UNI JAP and STB UK did not extract and therefore did not yield any component spots confirming their pigmented nature. The remaining four samples (ICM UK, PTL JAP, PLT UK and PLT US) proved highly difficult to discriminate with certainty by TLC alone due to non-reproducible component bands visualised between TLC runs of different extract sets heated for different periods of time. However, arguably, there was sufficient evidence to suggest that it may be possible to discriminate the PLT samples from the other two samples, but not from each other.

Comparison of Vis-MSP % reflectance spectra from dye components separated on a TLC plate offered no further discriminatory value for gel ink samples within a colour group.

Chapter Three

Video Spectral Comparison and Hyperspectral Imaging

3.1 Introduction

The application of filtered light to the examination of writing inks, in particular ballpoint inks, is well documented [184-195]. The application of filtered light examination to gel ink samples has occurred more recently. Gernandt and Urlaub [17] analysed four Japanese brands of gel ink pen available on the US market using the infrared (IR) and Ultra Violet (UV) functions of a Video Spectral Comparator (VSC-1). Three brands were available in black and blue, and one in red, all of which the authors claimed exhibited no UV fluorescence or IR fluorescence/luminescence (IRL) when viewed under the VSC-1. Two brands were available in several colours and exhibited a range of fluorescence, though the authors highlighted these were specifically manufactured to have fluorescent properties. Giles [16] further commented on their work after conducting similar examinations on a selection of the limited gel ink pens available on the British market during the mid-1990's. Using a VSC-4, Giles [16] claimed that a "faint but distinct" fluorescence was observed (between 440 nm – 600 nm using 830 nm viewing camera filter) for the blue gel ink of one of the same brands examined by the previous authors who had been unable to detect any fluorescence using the VSC-1. Giles [16] queried whether this anomaly was due to a real difference between UK and US pens or simply a lack of sensitivity of the older instrument.

Mazella and Khammy-Vital [15] examined a larger number of blue gel ink samples available on the European market analysed by IR absorption/reflectance (IRR) and IR fluorescence/luminescence (IRL) using a Projectina Docucenter 3000. In an attempt to reduce the influence of factors such as aging, environmental conditions, the amount of ink, and type of paper, on the interpretation of results from these filtered light examinations, the authors adopted an "objective" approach to classification. All IRR examinations were classified on whether the ink appeared to "absorb" or "reflect" the incident radiation when viewed through 780 nm barrier filter. Whilst IRL examinations were classified according to whether the ink exhibited luminescence, absorption or a combination of both when

illuminated between 380 – 570 nm and 590 – 620 nm and viewed through a 780 nm barrier filter. For the IRR examinations six gel inks were found to "absorb" radiation whilst 11 were found to "reflect". With regards to the IRL examinations at 380 – 570 nm, four gel inks exhibited absorption, 11 exhibited luminescence and two exhibited a combination of both. For the IRL examinations at 590 – 620 nm, six gel inks exhibited absorption, eight exhibited luminescence and three exhibited a combination of both. Interestingly, all six dye based gel inks reflected under IRR conditions and exhibited luminescence under IRL conditions, whilst pigmented gel inks varied in their response, suggesting the preliminary work by Gernandt and Urlaub [17] may well have been affected by the sensitivity of the instrument used as suggested by Giles [16]. Overall, combining the filtered light examination results, the 11 pigmented gel inks could be classified into four groups whilst the six dye based gel inks could be classified into two groups. Despite their objective approach, the authors still urged caution when considering their findings in relation to filtered light examinations presumably due to its inherent subjective nature.

To assess the influence of paper substrate on the interpretation of filtered light examinations Mazella and Khammy-Vital [15] examined marks made by 17 blue gel inks on ten randomly chosen different plain white paper substrates. In all instances, the paper substrate was found not to influence the filtered light classification rating given of a gel ink. Wilson *et al* [26] used a VSC 2000/HR to discriminate between 20 pigmented black gel and nine dye based black gel inks under visible and Near Infrared (NIR) reflectance (400 – 1000 nm) and IRL conditions (400 – 580 nm band pass filter/610 nm cut off filter and 480 – 620 nm band pass filter/665 nm cut off filter).

Jasuja *et al* [181] examined 98 gel ink pens representing 13 colours (including blue and red) from 20 different manufacturers available in India, Europe and the United States. As well as examining different brands and samples, Jasuja *et al* [181] also examined "multiple samples of a single colour" from the same brand and samples of metallic gel inks. Ink writings on white bond paper were examined visually by low power microscopy (x 10), UV light (short (254 nm) and long (365 nm) wavelength), and video based IR reflectance and luminescence using a Wratten 87 filter and Schott B9-18 excitation filter amongst other techniques. Visual observations based on low power microscopy are not commented on by the authors making it unclear whether this technique was used just to assess the response of the inks to UV light or if observations as to their general appearance under normal white

light was also undertaken. Details of the video based IR reflectance and luminescence examination were not discussed in depth, but illustrated the vast majority did not exhibit IR fluorescence/luminescence. The authors drew attention to the fact that from their examinations by filtered light, different brands of the same colour could be distinguished. Examination by UV light was found to be of limited use with little response from most of the ink samples. Of the limited inks that did fluoresce under UV, most were bright or unusual colours including pink, orange and green amongst others. These may well therefore have contained specific fluorescent components for their particular application.

3.2 Experimental

Gel inks within both colour groups were examined using IR absorption/reflectance (IRR) and IR fluorescence/luminescence (IRL) in order to assess the discriminating capabilities of the VSC 6000/HS for within and between brand variability. A preliminary investigation into the Hyperspectral Imaging (HSI) capabilities for discrimination of the UK acquired samples only representing both colour groups was also conducted.

3.2.1 Video Spectral Comparison (VSC)

3.2.1.1 Sample Preparation

3.2.1.1.1 Within Brand Variation

Five brand/model combinations of gel ink from each of the two colour groups were selected for examination as follows:

- BIC US, PTL UK, STB UK, STD UK and UNI AUS (blue ink group);
- **WHS UK**, ICM UK, PLT US, PPM UK, UNI UK (red ink group).

These brand/model combinations were selected mostly at random or in one case, highlighted in bold type, because of evidence of within brand variation by another technique, i.e. Raman Spectroscopy. They were chosen to reflect as many different brand/model combinations possible to highlight the variety within and between each colour group.

A sheet of 15 sample grids, measuring 7 x 7 squares, was prepared on a single sheet of A4 squared white paper. Each sample grid was prepared so that the ink from each pen within a brand/model combination would be compared to the ink from all other pens within that brand/model combination. Prior to the addition of the ink samples, the sample sheet was photocopied onto sheets of plain white A4 office paper (80 gsm). Ink lines from the relevant pens were then added to the appropriate sample squares within a grid. In each sample square the ink line on the left represents the pen number given in the column, whilst the ink line on the right represents the pen number given in that row. An example of the sample grid is illustrated in Figure 3.1.

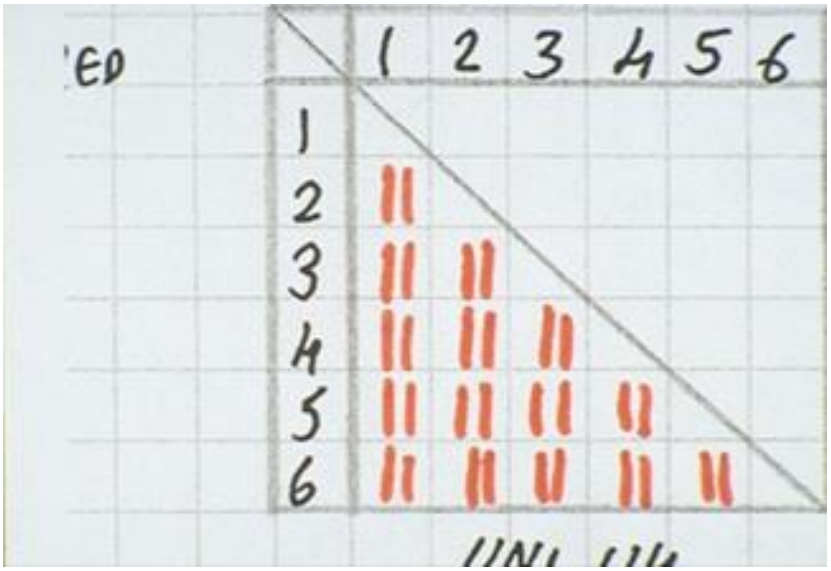


Figure 3.1: Example of a within brand variation sample grid containing ink lines from all six pens within the UNI UK red ink group brand/model combination. Ink lines on the left in each square correspond to the pen # labelled for that column, whilst ink lines on the right correspond to the pen # labelled for that row

3.2.1.1.2 Between Brand Variation

In total, four test sheets were prepared on A4 squared white paper with 18 sample grids per sheet. The dimensions of each grid measured 11 x 6 squares (66 squares). Since the purpose of each grid was to contain ink lines from a single brand/model combination of gel ink compared to ink lines from all other brand/model combinations within that colour group, each grid needed to be prepared with the relevant information. For example, the PTL UK blue brand/model combination was compared to the other 30 brand/model combinations of blue gel ink in a pairwise comparison fashion. Thus, a downward ink line from the PTL UK sample was drawn into each of 30 squares within the grid. In each of the

squares, a second ink line to the right of the PTL UK ink was drawn using one of the other blue gel ink brand/model combinations. The grids were prepared so that each row of squares containing ink samples were separated by a row of squares containing information about the brand/model combination in the corresponding square.

As before, prior to any ink samples being added to the grids, each sample sheet was then photocopied onto a sheet of plain A4 white office paper. Ink lines (~1 cm) were then written in the appropriate squares within the sample grids. Ink lines from only one pen per brand/model combination were examined due to available instrument access time and to provide a manageable number of pairwise comparisons for determination of the discriminating power. An example of a sample grid is illustrated in Figure 3.2.

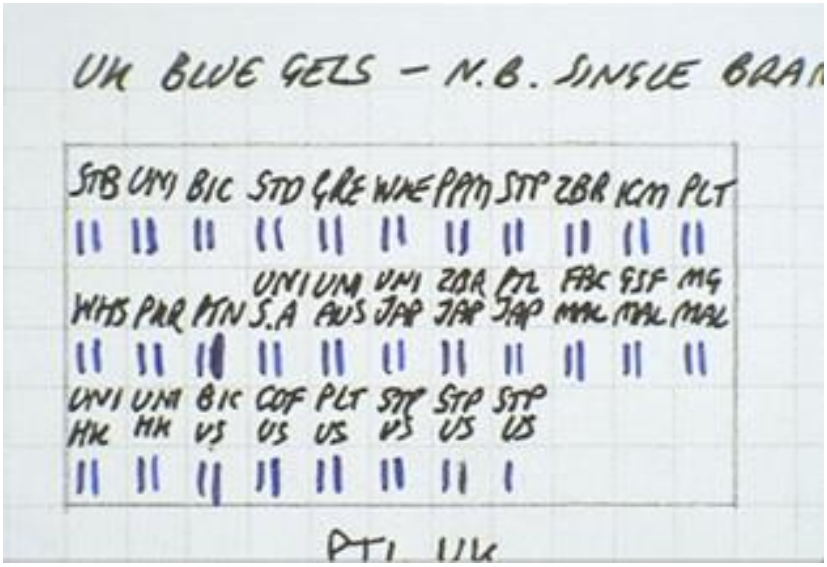


Figure 3.2: Example of a between brand variation sample grid containing all 31 brand/model combinations in the blue ink group (except STP US #09). Ink lines on the left in each square were from the same PTL UK pen, whilst ink lines on the right correspond to the brand/model combination labelled in the square above

Images were also taken from blank areas of all paper sheets upon which ink samples were deposited using all relevant IRR camera and IRL optical filters for a given colour group.

3.2.2 Hyperspectral Imaging (HSI)

3.2.2.1 Sample Preparation

Two sample grids, measuring 8 x 8 squares, were drawn onto a single sheet of white A4 squared paper. Each grid represented the UK acquired samples for each of the colour

groups. Relevant brand/model combination information was added to the grids so that all appropriate samples for a given source of origin and colour were in the relevant grids. The sample sheet was again photocopied onto a single sheet of white A4 office paper. Ink samples, from the first pen in a brand/model combination set were drawn in the form of the numerals “10” and “58” into each of the corresponding blank squares within a sample grid. This was done to provide a good coverage of ink for effective imaging. An example of the sample grid is illustrated in Figure 3.3.

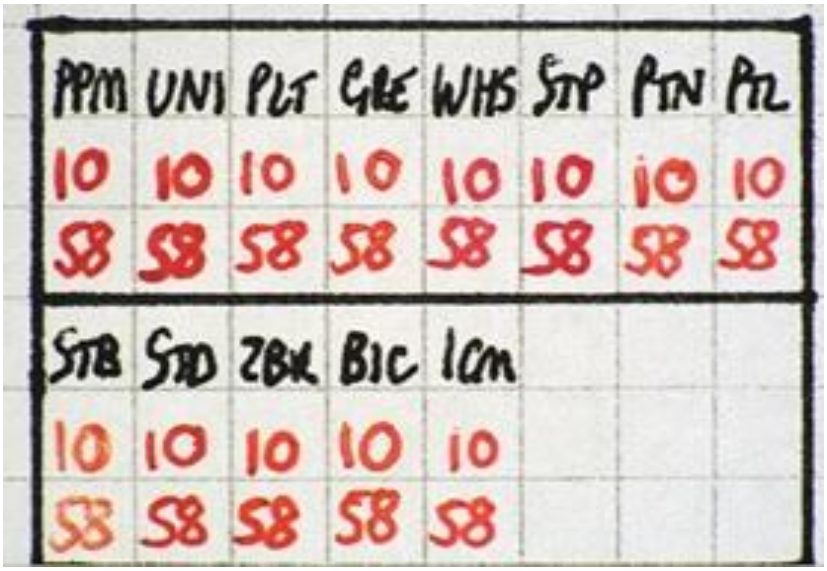


Figure 3.3: Example of HSI sample grid containing ink samples from 13 brand/model combinations of UK acquired red gel ink

For all aforementioned studies, ink lines were allowed to air dry at room temperature for at least 15 minutes prior to examination and were examined within 72 hours of deposition. Samples were stored at room temperature in a laboratory notebook overnight.

The dimensions of all sample grids were designed to ensure that all ink samples were exposed to the light source within the VSC 6000/HS permitting successful examination by IR absorption/reflectance and IR fluorescence/luminescence.

3.2.3 Instrumentation

All filtered light examinations were performed using a VSC 6000/HS, a PC based document imaging system consisting of a main unit, PC system and bespoke VSC software. The VSC main unit contains a video camera, various light sources, optical filters and a high resolution

grating spectrometer. The hardware associated with the VSC 6000/HS as described in the manufacturers hardware and software manuals is illustrated in Figure 3.4 [161, 162].

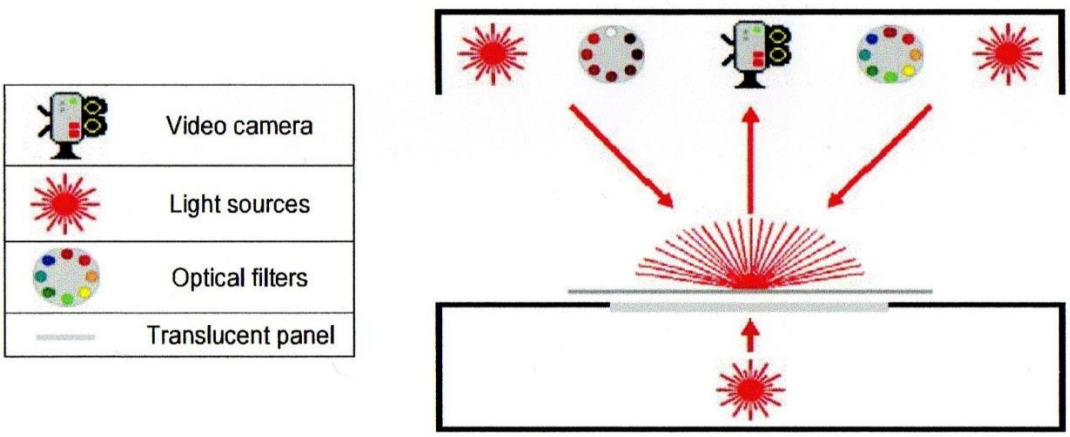


Figure 3.4: Basic hardware component set up inside the VSC 6000/HS [161, 162]

The document is placed on the document platen within the main unit, and brought into view on the PC monitor via the video camera positioned directly above it. The video camera uses both a motorised zoom lens and high magnification lens to give a maximum resolution of 2584 x 1292 pixels.

Flood illumination for IR absorption conditions is provided by incandescent filament lamps: which produce white (visible) light and infrared radiation to provide an overall range of 400 – 1000 nm. White light emitting diodes (LED's) are used to white balance the image and long pass filters in front of the camera are used to view the image in the infrared. For HSI mode a 100W halogen spot light (capable of producing 400 – 1000 nm) is used together with a continuously variable band pass filter placed in front of the light source.

To control the wavelengths of illuminating light, optical filters are placed into the path of the optical system. For IRR examination, long pass filters are used to allow longer and shorter wavelengths of light rather than a specific cut off wavelength value to illuminate the document, whilst, band pass filters are used to allow a defined narrow range or band of wavelengths between a long and short cut off wavelength value.

The operation of the main unit is controlled using the VSC software, with each mode of operation associated with one of several working screens. For example, live and stored

images of the document can be viewed via the main screen, which also controls the VSC settings, whilst spectral and chromaticity data can be viewed via the spectrum screen. Table 3.1 describes each of the viewing settings, their function in relation to the live camera image and whether they can be controlled automatically by the software.

	Setting	Function	Auto-Controlled
Exposure	Brightness	Controls brightness of image across a range of 0 – 100 (default value 60)	Yes
	Gamma Correction	Can be applied to brighten darker areas within the image to reveal hidden detail	
	Iris	Controls the amount of light reaching the camera across a range of 0 – 100%	Yes (Auto Exposure)
	Integration Time	Controls the time permitted for integration of the camera signal	
Focus	Magnification	Controls the optical zoom magnification	Yes (Auto Focus)
	Zoom		

Table 3.1: Summary table of viewing settings and their respective functions [162]

The type of light source used is controlled by an illumination panel. For IRR and IRL examinations, only flood and spot (fluorescence) lighting are relevant. The former provides broad band illumination, whilst the latter provides high intensity narrow band illumination to stimulate fluorescence. The choice of optical filter is determined from the filter control panel. For IRR examinations, there is a choice of 16 filters: visible light is provided by a short pass camera filter, whilst light of long and short wavelength is provided by 15 long pass camera filters identified by the wavelength cut-off value of that particular filter. For IRL examinations, ten default optical filters identified by the wavelength range or corresponding colour of light within the visible spectrum they permit, are available. In addition, an optical filter allowing all wavelengths in the visible region to pass through the optical path is available. It is also possible to manually adjust the long and short cut-off values of each band pass filter to tailor the wavelength range.

The VSC 6000/HS incorporates Hyperspectral Imaging (HSI) technology, whereby it is possible to record images of a document using reflected light at specific well defined wavelengths across a spectral range of 400 – 1000 nm. Up to a maximum of 150 images can be taken and stored as an image cube, which can be replayed to view the wavelengths at which differences occur. A band pass filter step width between 5 – 10 nm can be selected to generate sufficient imagery data across the entire spectral range. Furthermore,

spectral data from any given pixel in the image can be generated to compare any spectral differences within the document.

3.2.3.1 Instrumental Conditions

3.2.3.1.1 Video Spectral Comparison (VSC)

The VSC 6000/HS was operated in auto exposure and auto focus mode, and the default brightness value of (60) used for viewing each sample sheet. Illumination was provided by incandescent filament bulbs and operated in flood and spot fluorescence mode for IRR and IRL examinations respectively. In addition to a short pass camera filter for visible light illumination, 15 other camera filters were available for initial IRR method development and ten default band pass filters. No manual adjustments to the long and short cut-off wavelength values for the default band pass filters were made.

3.2.3.1.1.1 Method Development

The camera and optical filters which provided the best levels of visual discrimination for a particular colour group of ink examined using IRR and IRL respectively were determined prior to examining all samples.

3.2.3.1.1.1.1 IR Absorption (IRR)

For both colour groups, the sample grid that contained ALL brand/model combinations within a given colour group was examined using ALL 15 camera filters available, i.e. the first grid in a colour group sequence. Table 3.2 summarises the selected camera filters for each colour group. For each colour group, four camera filters were selected for their discrimination ability. These camera filters were different for each colour group. The ability of each camera filter to discriminate within a given colour group was based on visual assessment by eye, and those selected exhibited the greatest variations in absorbance.

3.2.3.1.1.1.2 IR Fluorescence (IRL)

A similar approach to that described above was adopted to select the most suitable optical filters for discrimination by IRR. Each sample grid representing all brand/model combinations within a colour group was examined under ALL ten default optical filters. Table 3.2 summarises the selected optical filters and their corresponding colour for each colour group. Four optical filters were selected for discrimination of the blue ink group,

compared to three for the red ink group. In addition, the white light filter was also found to provide a good level of discrimination for both colour groups.

Colour Group	IR Absorption/Reflectance (IRR)	IR Fluorescence/Luminescence (IRL)	
	Camera Filters (nm)	Optical Filters (nm)	Colour
Blue Group	645	485 – 610	Yellow
	695	515 – 640	Light Orange
	780	545 – 675	Orange
	1000	585 – 720	Red
		380 – 800	White
Red Group	530	400 – 485	Blue
	610	400 – 535	Cyan
	695	445 – 570	Green
	1000	380 – 800	White

Table 3.2: Summary of camera and optical filters selected for the IR absorption/reflectance (IRR) and IR fluorescence/luminescence (IRL) examination respectively of blue and red ink group samples by VSC 6000/HS

All sample grids within a colour group were subsequently examined using the selected camera and optical filters for that group, to assess their IRR and IRL behaviour, and provide pairwise comparisons in order to calculate the discriminating power, discussed in **Chapter Seven**. These settings were also applied to the investigation of within brand variation within the colour groups.

3.2.3.1.2 Hyperspectral Imaging (HSI)

3.2.3.1.2.1 Imaging

For HSI, the instrument was calibrated for the entire spectral range (400 – 1000 nm) permitted by the instrument, against a piece of A4 white office paper. Flood lighting was used to provide reflected light for IR absorption conditions. Auto exposure (integration times 1.6 – 2.0 ms (dependent upon colour examined), iris (81%) and auto focus modes (magnification 7.10) were switched on with the default brightness value (60) used for viewing each sample grid. This provided an image width of 45.63 mm, sufficient to incorporate a sample grid in its entirety on the video screen. Gamma mode was switched off. A band pass filter step width of 5 nm was selected, to provide 121 images from a possible 150 maximum. Images recorded for all examinations were electronically stored for subsequent assessment at a later date.

3.2.3.1.2.2 Spectral Acquisition

Spectral data was acquired in reflectance mode. To check repeatability and assess instrumental variation, a set of ten spectra were acquired from the same area of one blue brand/model combination (UNI UK). To check reproducibility and assess sample variation, a set of ten spectra were acquired from different areas of all brand/model combinations. Spectra were acquired from well inked areas of all four numbers in an ink sample. To monitor for possible interference from the paper substrate, spectra were also acquired from ten different un-inked areas within a sample grid representing each colour group.

3.3 Results and Discussion

3.3.1 Video Spectral Comparison (VSC)

3.3.1.1 Within Brand Variation

Five brand/model combinations for each colour group were selected, these were:

- BIC US, PTL UK, STB UK, STD UK, UNI AUS (blue ink group);
- **WHS UK**, ICM UK, PLT US, PPM UK, UNI UK (red ink group).

With only one exception, no within brand variation was observed for both IRR and IRL. The exception was the WHS UK red brand/model combination under IRL where very clear differences were observed under all four IRL optical filters. The WHS UK red samples could be split into two groups, pen #01 – 03 and pen #04 – 06, the latter exhibiting a more intense luminescent appearance illustrated in Figure 3.5.

These differences were also observed by IR and Raman Spectroscopy (**Chapter Five** and **Six** respectively) and clearly suggests that within this brand/model, a change in formulation has occurred at some point. These two groups within the same brand had been purchased within five days of each other from different branches of WH Smiths Stationers within a one mile radius of Glasgow City Centre in Scotland, UK.

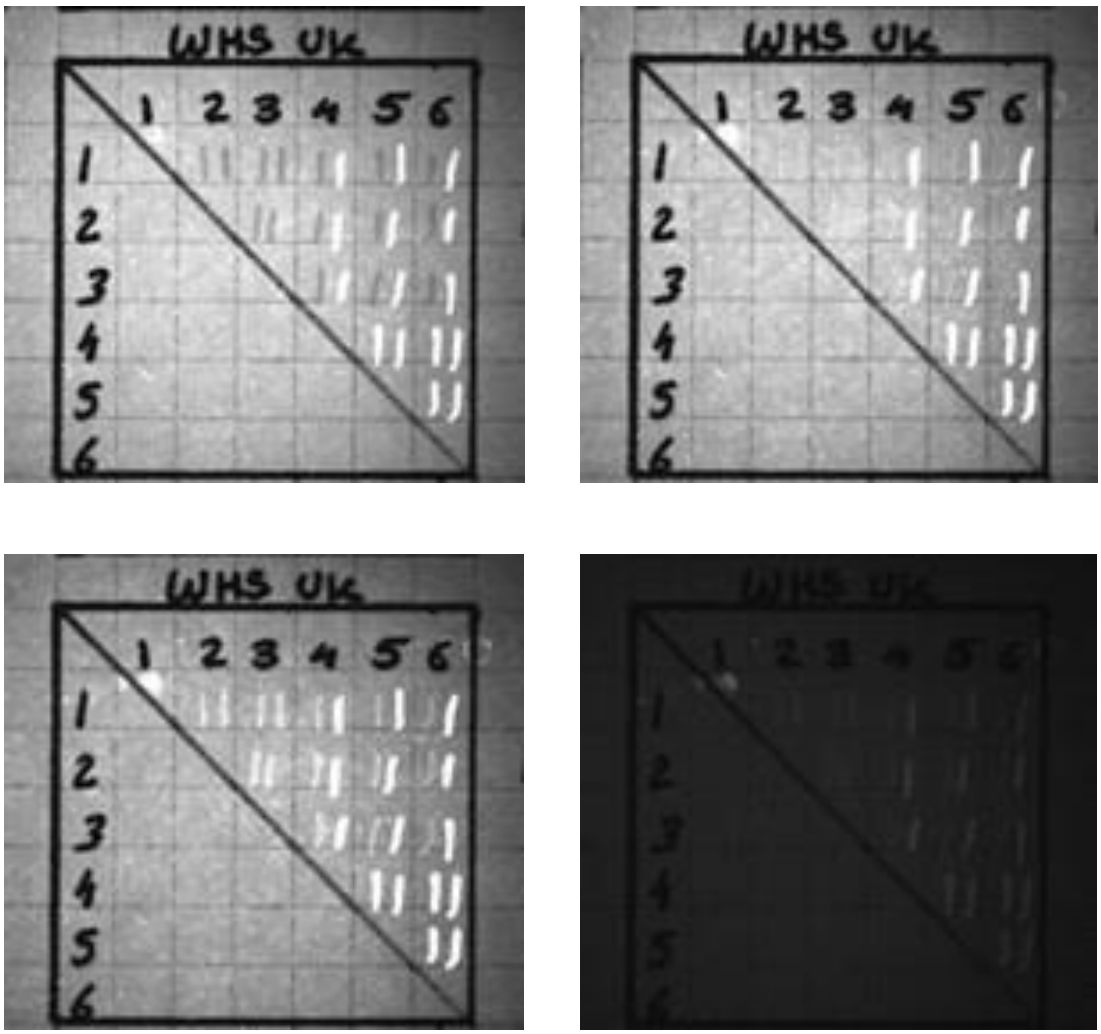


Figure 3.5: IRL images for within brand variation of the WHS UK red gel ink samples: 400 – 485 nm (top left), 400 – 535 nm (top right), 445 – 570 nm (bottom left) and 380 – 800 nm (bottom right) illustrating differences between pen #01 – #03 and pen #04 - #06

3.3.1.2 Between Brand Variation

A summary of the classification groupings based on IR absorption/reflectance and IR fluorescence/luminescence examination of the blue and red ink groups are presented in Table 3.3.

3.3.1.2.1 Blue Ink Group

3.3.1.2.1.1 IR Absorbance (IRR)

At 645 nm, all ink samples were absorbing strongly and no obvious differences in intensity were observed. In addition, at 1000 nm, the inks were considered to be fully reflecting and therefore invisible. However, at 695 nm and 780 nm the inks exhibited varying degrees of

absorbance, enabling visual discrimination by eye. At both wavelengths it was possible to discriminate the inks into three groups. Two of the groups contained either a dye/hybrid or a pigment containing ink only as identified by the Solubility and TLC study, whilst the third contained a combination of both.

3.3.1.2.1.2 IR Fluorescence (IRL)

It was possible to discriminate the inks into groups for each of the five optical filters used based on differences in their luminescent behaviour, but the number of groups varied across the filters. When the results were combined, it was possible to discriminate the inks into at least ten groups, which could be broadly split into four dye containing or hybrid groups and six pigment based groups. Using the white light filter, it was possible to tentatively provide some further discrimination based on intensity of absorbance however this was considered to be too subjective to be completely reliable.

No further discrimination was possible considering both IRR and IRL examinations in combination. Images captured under each selected camera and optical filter demonstrating the IR absorbance/reflectance and IR fluorescence/luminescence behaviour of the blue ink group samples are presented in Figure 3.6.

3.3.1.2.2 Red Ink Group

3.3.1.2.2.1 IR Absorbance (IRR)

Absorbance of the red inks only occurred at 530 nm, but no obvious intensity differences were noted. At all other camera filter cut-off wavelengths, all the red inks were considered to be fully reflecting and thus invisible. Therefore, it was only possible to discriminate the red inks into a single group containing all brand/model combinations.

3.3.1.2.2.2 IR Fluorescence (IRL)

It was possible to discriminate the red inks into 12 groups using the combination of results obtained across the four individual optical filters. In contrast to the blue ink group, the pens were not distinguished depending only on whether they contained dyes or pigments and the two brands known to be pigment based (UNI JAP and STB UK) could be discriminated from each other. Images captured under the selected camera and optical

filters demonstrating the IR absorbance/reflectance and IR fluorescence/luminescence behaviour of the red ink group samples are presented in Figure 3.7.

Fluorescence arising from the paper substrate was problematic when attempting to distinguish the blue and particularly the red gel inks under certain optical filters during IRL examination. For the red ink group, viewing the inks using the white light optical filter resolved these discrimination difficulties. For the blue ink group, however, discrimination of a small number of samples under these conditions had to rely solely on opinion since examination using all filters did not resolve the issue. Given this problem, possible influences from line thickness [11] and the inherent subjective nature of this technique, the aforementioned classifications and subsequent discriminating powers described in **Chapter Seven**, should be treated with caution.

Blue Ink Group

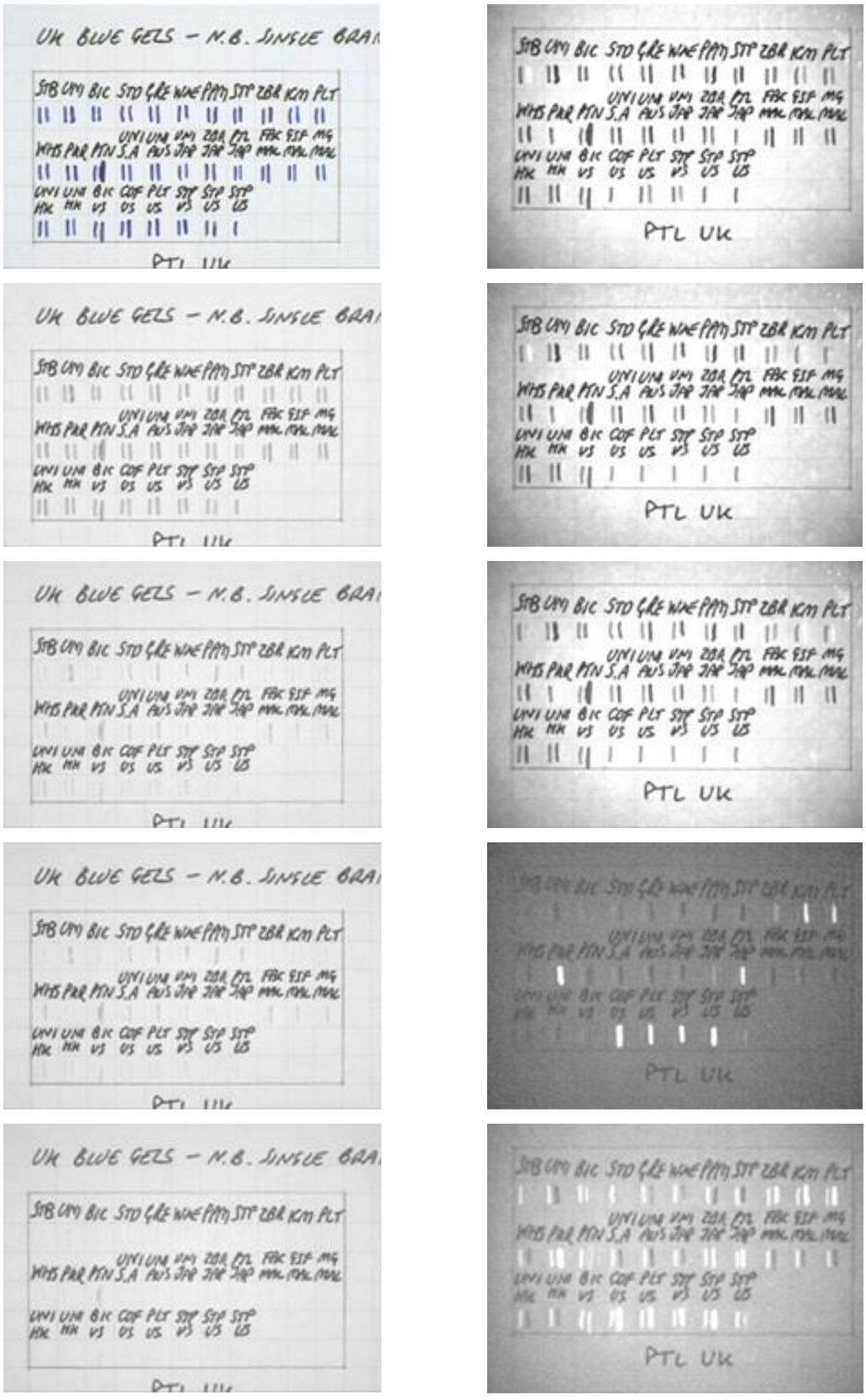


Figure 3.6: IRR images (left): Vis (top), 645 nm, 695 nm, 780 nm and 1000 nm (bottom); IRL images (right): 485 – 610 nm (top), 515 – 640 nm, 545 – 675 nm, 585 – 720 nm and 380 – 800 nm (bottom)

Red Ink Group

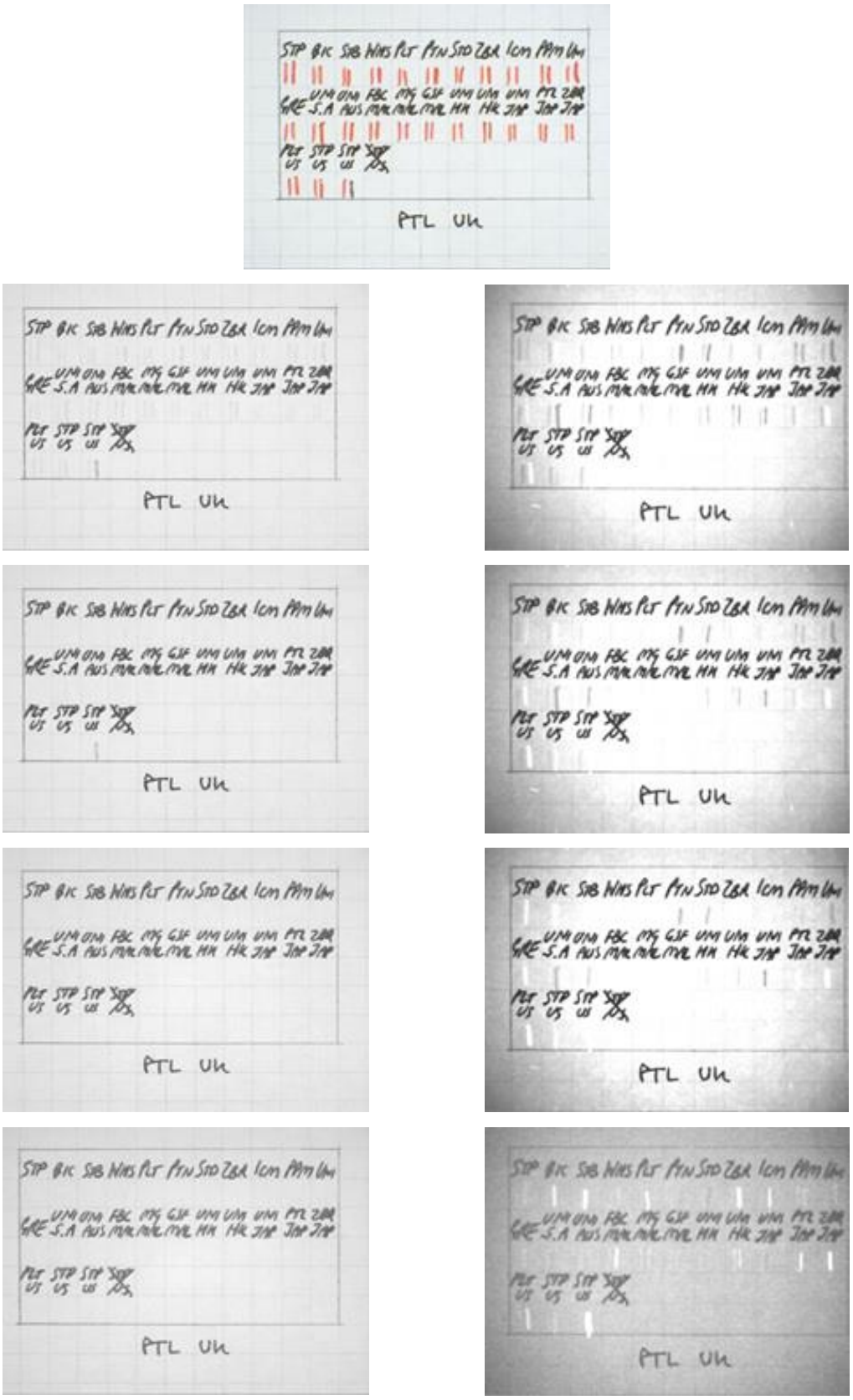


Figure 3.7: Vis (top-mid); IRR images (left): 530 nm (top), 610 nm, 695 nm and 1000 nm (bottom); IRL images (right): 400 – 485 nm (top), 400 – 535 nm, 445 – 570 nm and 380 – 800 nm (bottom)

Colour Group	Infrared Absorption/Reflectance (IRR)			Infrared Fluorescence/Luminescence (IRL)		
	Sample	Class Group	Colorant	Sample	Class Group	Colorant
Blue	PKR UK: PTL JAP: COF US: STP US #08	1	Dye or Both (Hybrid)	STB UK	1	Dye or Both (Hybrid)
				ICM UK; STP US #07	2	
	UNI UK; STD UK; GRE UK; WKE UK; PPM UK; STP UK; WHS UK; PTN UK; UNI SA; UNI AUS; UNI JAP; FBC; MAL; GSF MAL; MG MAL; UNI HK #25; UNI HK #31	2	Pigment	PLT UK; PLT US	3	
				PKR UK; PTL JAP; COF US; STP US #08	4	
				PTL UK	5	Pigment
				BIC UK; BIC US	6	
	PTL UK; STB UK; BIC UK; ZBR UK; ICM UK*; PLT UK*; ZBR JAP; BIC US; PLT US*; STP US #07*	3	Dye* or Pigment or Both (Hybrid)	ZBR UK; ZBR JAP	7	
				STD UK	8	
				PTN UK	9	
				UNI UK; PPM UK; STP UK; UNI SA; UNI AUS; UNI JAP; WHS UK; FBC MAL; GSF MAL; MG MAL; GRE UK; WKE UK; UNI HK #25; UNI HK#31	10	
Red	BIC UK; PLT US; ICM UK; PTL JAP; ZBR JAP; GRE UK; STP US #07; STP UK PTN UK; STD UK; UNI JAP UNI UK; UNI SA; UNI AUS; UNI HK #25; UNI HK #29 PPM UK; PTL UK FBC MAL; GSF MAL; MG MAL; WHS UK #01 ZBR UK; PLT UK; STB UK	1	Dye or Pigment or Both (Hybrid)	BIC UK; PLT US; ICM UK; PTL JAP	1	Dye or Pigment or Both (Hybrid)
				ZBR JAP	2	
				GRE UK	3	
				STP US #07	4	
				STP UK	5	
				PTN UK; STD UK; UNI JAP	6	
				UNI UK; UNI SA; UNI AUS; UNI HK #25	7	
				UNI HK #29	8	
				PPM UK; PTL UK	9	
				FBC MAL	10	
				GSF MAL; MG MAL; WHS UK #01	11	
				ZBR UK; PLT UK; STB UK	12	

Table 3.3: Summary of classification groupings by colour group for IRR and IRL examinations in isolation and in combination with each other

3.3.2 Hyperspectral Imaging (HSI)

Sample grids containing the UK brand/model combinations of gel ink representing both colour groups were prepared for a preliminary study of Hyperspectral Imaging (HSI). Images of the sample grids under reflected light of specific well defined wavelengths across a spectral range of 400 – 1000 nm were recorded. A total of 121 images out of a maximum 150 were acquired and stored as an image cube, replayed in real time, to view and record the specific wavelengths at which differences in the behaviour of the inks under IR absorption/reflectance could be observed, thus permitting discrimination. A band pass filter step width of 5 nm was selected to generate sufficient imagery data across the entire spectral range. Furthermore, spectral data from any given pixel within an image was generated to provide a % reflectance spectrum of the inks enabling comparison of spectral differences between the different brand/model combinations, providing a second means of discrimination. Discrimination of brand/model combinations within a colour group was made solely on the basis of imagery data or spectral data alone, and on both sets of data combined, as summarised in Table 3.4.

3.3.2.1 Background Measurements

Spectra taken from un-inked areas of the paper substrate exhibited some variation. It was suggested that peaks in some of the paper spectra were artefacts arising from spectral measurements taken using a three chip RGB camera. These peaks appeared to arise at cross-over points between the three RGB channels, e.g. around 800 nm, an example of which is demonstrated in Figure 3.8. Similar peaks were also observed in some, but not all, of the ink spectra across the two colour groups. If the % reflectance spectra produced by the VSC 6000/HS is to be relied upon, this issue requires further investigation.

3.3.2.2 Repeatability and Reproducibility

A set of ten spectral measurements acquired from the same area of a UNI UK blue ink sample exhibited excellent repeatability and no instrumental variation as demonstrated in Figure 3.9. A set of ten spectral measurements were also acquired from each of the brand/model combinations analysed across both colour groups. For all samples, spectra within a brand/model combination set exhibited good reproducibility in terms of spectral shape with only minor spectral variations, an example of which is illustrated in Figure 3.10.

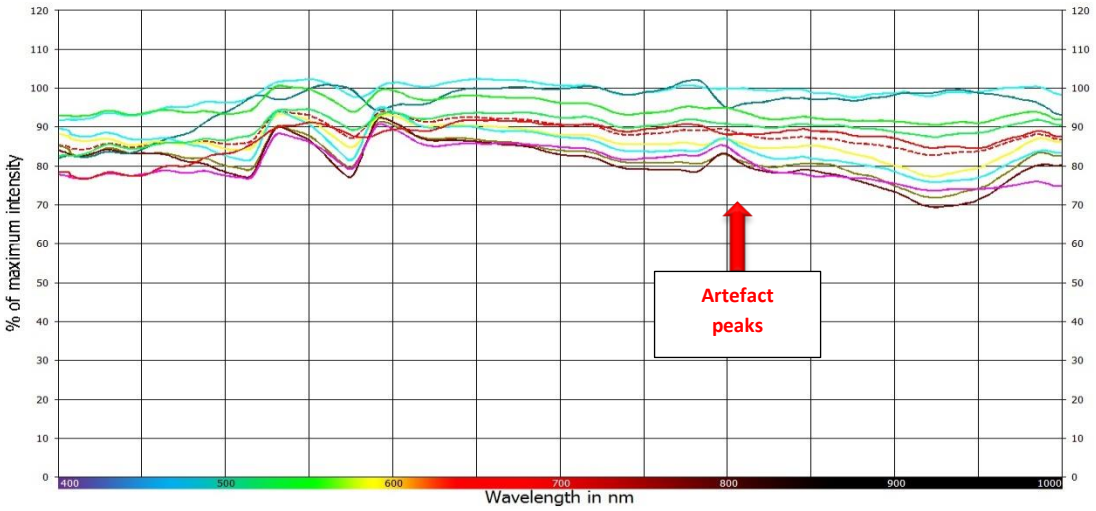


Figure 3.8: A set of ten spectra acquired from different areas of the un-inked paper illustrating artefact peaks around 800 nm attributable to the cross-over points of the three RGB channels of the camera

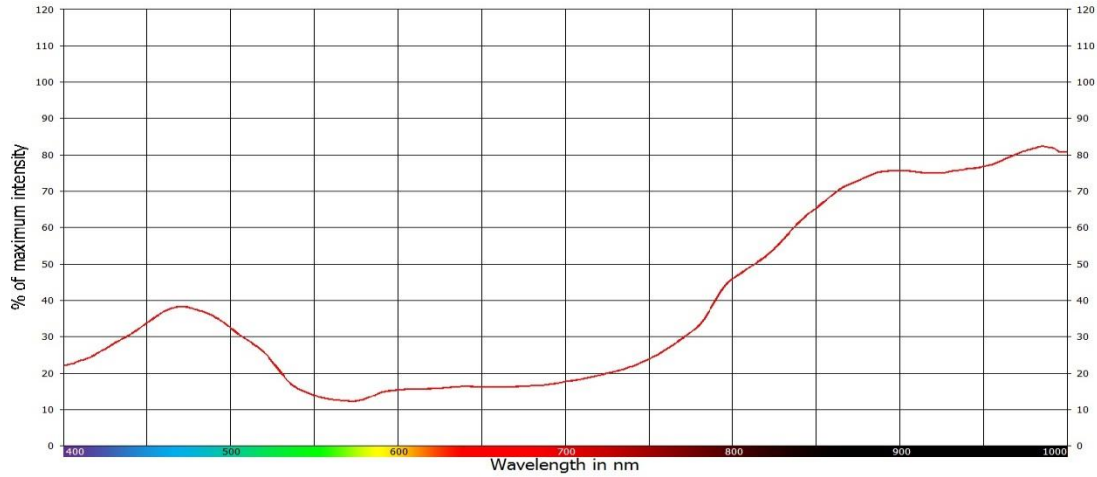


Figure 3.9: A set of ten spectra acquired from the same area within a UNI UK blue ink sample demonstrating excellent repeatability and thus no instrumental variation

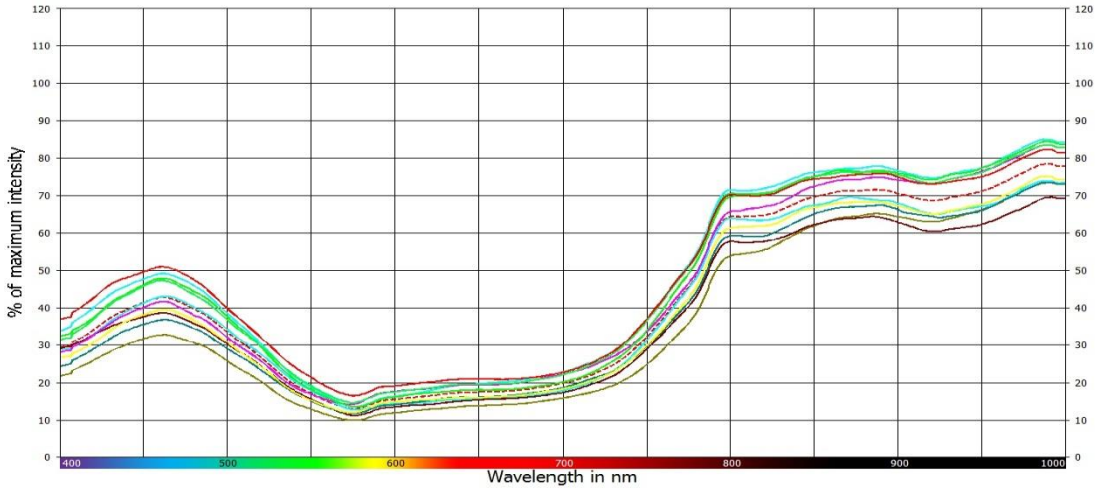


Figure 3.10: A set of ten spectra acquired from different areas of the STD UK blue ink sample illustrating good reproducibility

3.3.2.3 Blue Ink Group

Two spectral regions within the entire range exhibited the greatest variation between the inks: 400 – 500 nm and 700 – 900 nm. Between 500 – 700 nm, all inks were absorbing strongly, and beyond 900 nm all inks were considered to be fully reflecting. Based on the imagery data alone it was considered possible to discriminate the 15 brand/model combinations into six groups. Three samples stood out from the others: PKR UK notably started to fade at 700 nm and by 750 nm was completely reflecting, whilst all others exhibited some degree of absorbance. The STB UK and ICM UK samples exhibited notable fading between 400 – 500 nm, whilst the other samples did not. This enabled them to be discriminated from the other samples even though between 750 – 800 nm they exhibited similar absorbance/reflectance behaviour to several other samples. Five brand/model combinations (PTL UK; PPM UK; ZBR UK; UNI UK and WHS UK) exhibited strong absorbance between 750 – 800 nm, whilst several others (PLT UK; GRE UK; WKE UK; PTN UK; STD UK; and BIC UK) exhibited signs of fading. By 800 – 850 nm, it was possible to differentiate these into four groups. The PPM UK and UNI UK samples could be discriminated from the others as they remained strongly absorbing until after 850 nm. The PTL UK and ZBR UK samples could be discriminated because they were fully reflecting by 800 nm, as were PLT UK, BIC UK and PTN UK, but unlike the latter, the former were still strongly absorbing at 750 nm. The remaining samples (Group 4) were still weakly absorbing at 800 nm, only fully reflecting by 900 nm. Images of the blue ink group samples under 450 nm, 700 nm, 750 nm, 800 nm, 850 nm and 900 nm are displayed in Figure 3.11 illustrating the discrimination behaviour described above.

At first glance, individually, the % reflectance spectra for all 15 samples appeared very similar. However, on closer inspection, it was possible to discriminate the samples into seven groups based on subtle differences in spectral shape, slope and/or curve, and λ_{max} positions. Figure 3.12 demonstrates the variability in spectral shape between all 15 brand/model combinations, whilst Figure 3.13 shows sets of spectra acquired from three different brand/model combinations considered distinguishable. Interestingly, these groups closely resemble those from the imagery data, but an additional spectral group was established, which contained two brand/model combinations: BIC UK and STD UK, from Group 3 and Group 4 of the imagery data respectively. Therefore, considering the HSI data in full, the UK blue ink group could be discriminated into eight groups overall.

Blue Ink Group

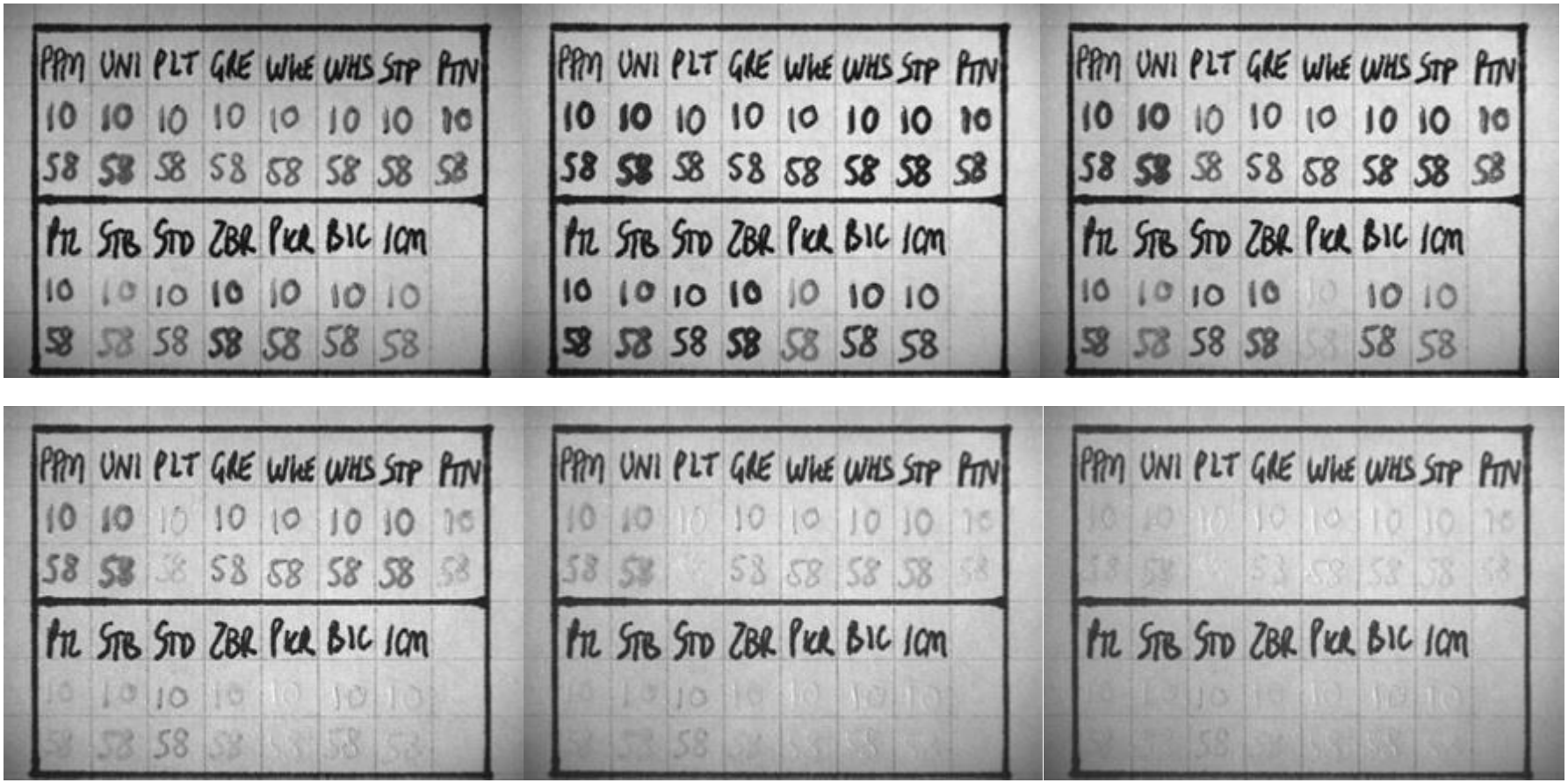


Figure 3.11: Imagery data of UK acquired blue gel inks on paper under IR absorption/reflectance illumination in sequence at 450 nm, 700 nm, 750 nm, 800 nm, 850 nm and 900 nm illustrating the discrimination behaviour of STB UK and ICM UK brand/model combinations around 450 nm, the PKR UK brand/model combination between 700 – 750 nm and all other brand/model combinations between 750 – 900 nm

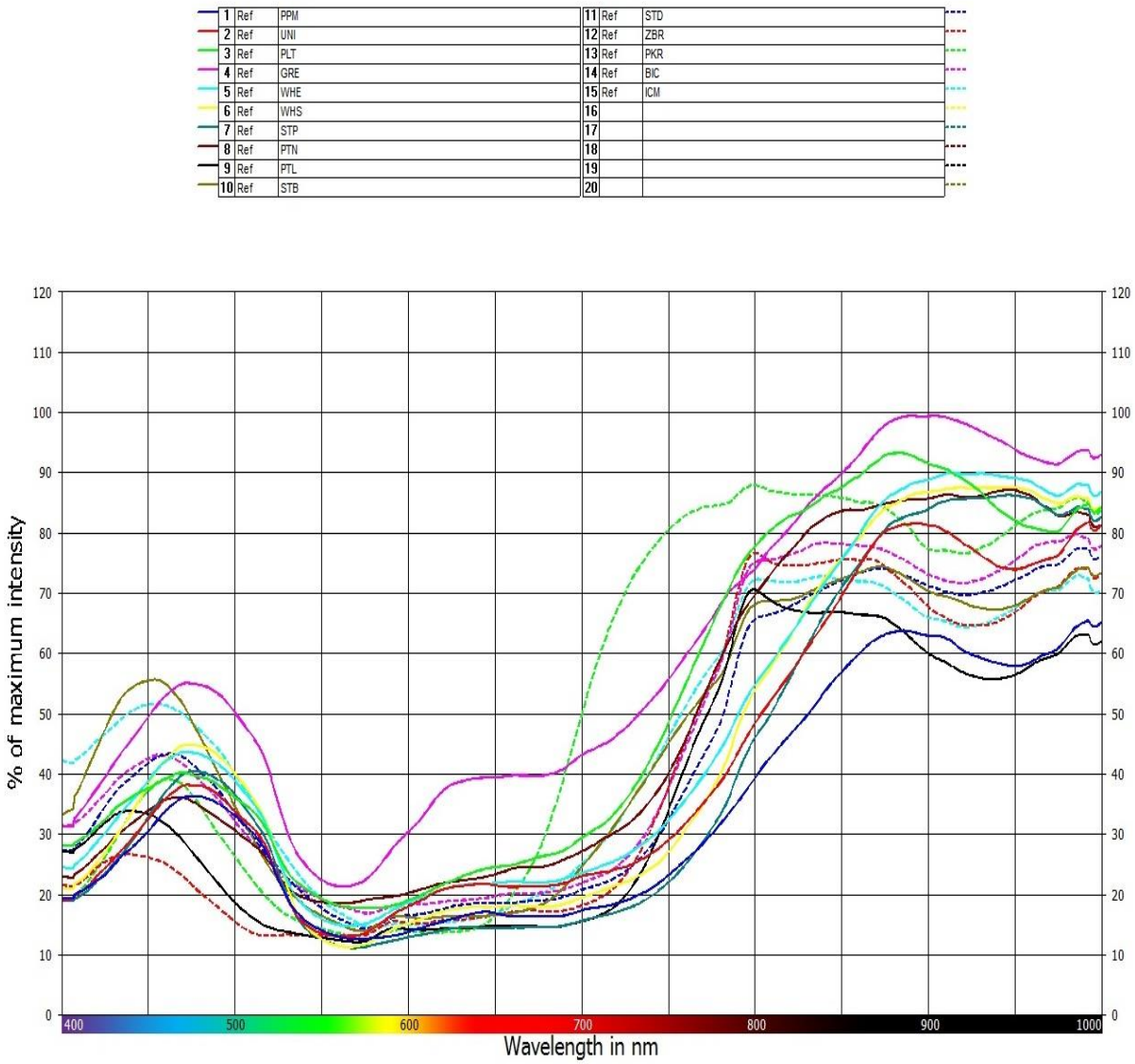


Figure 3.12: A single spectrum from each of 15 brand/model combinations of UK acquired blue gel ink on the same axis illustrating the similarity and differences between them

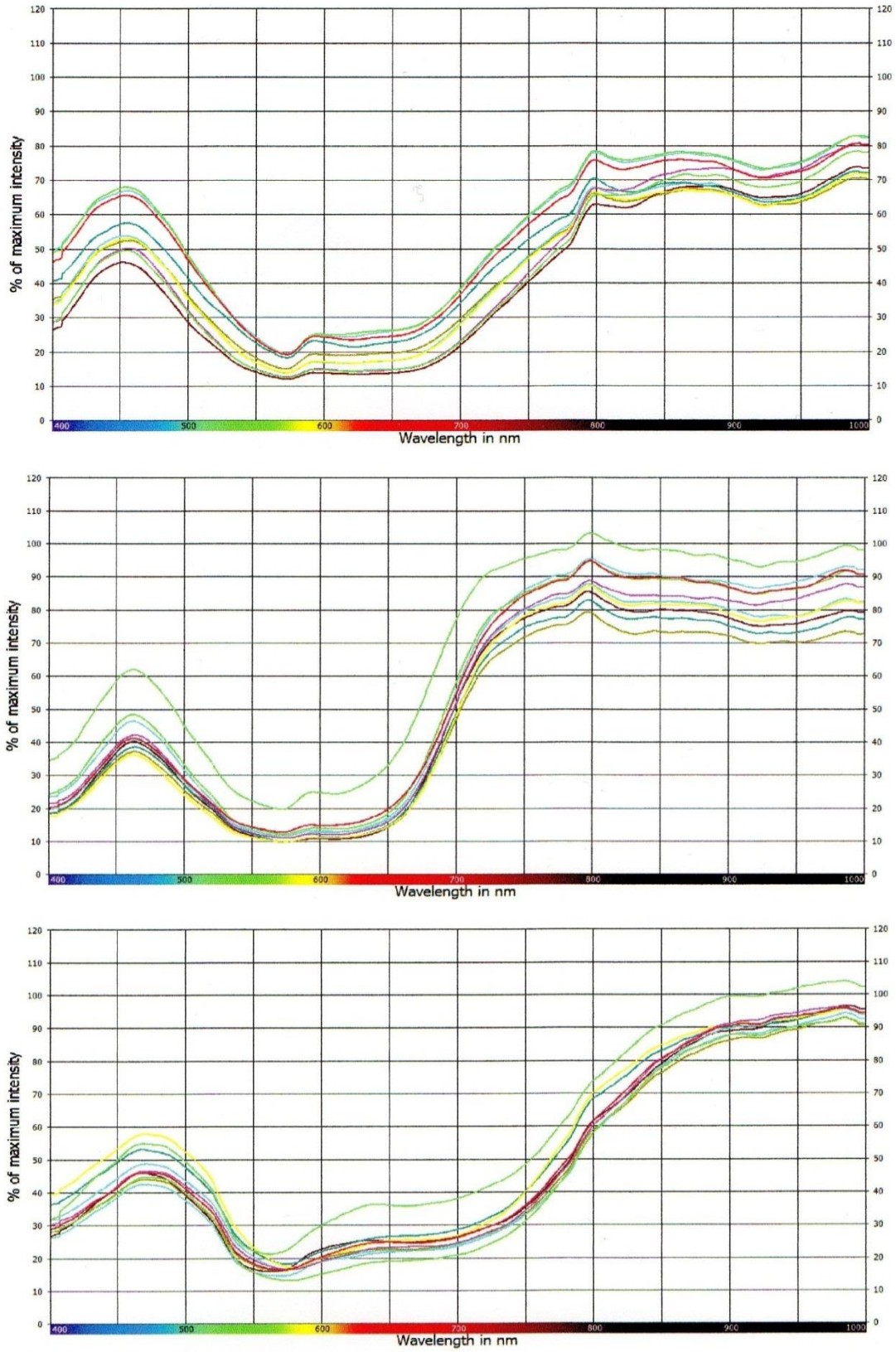


Figure 3.13: A set of % reflectance spectra of STB UK (top), PKR UK and GRE UK (bottom) blue gel inks on paper, representing Group 1, Group 2 and Group 5 based on spectral data alone. The spectra were taken from 10 different areas of the same ink sample

3.3.2.4 Red Ink Group

Discrimination of ten of the 13 brand/model combinations based on imagery data was considered possible within a narrow spectral region: 550 – 650 nm. Between 400 – 550 nm, with the exception of the STB UK sample which exhibited signs of fading from 400 nm, all other samples were absorbing comparatively strongly. Beyond 650 nm, all samples were fully reflecting. The STB UK sample was discriminated from all others at 450 nm since it appeared to be absorbing less. At 560 nm, a further three brand/model combinations (BIC UK; GRE UK and PTN UK) started to fade, whilst another four started to fade at 570 nm. By 590 nm all samples were exhibiting signs of fading, with STB UK almost fully reflecting: ICM UK; BIC UK; PTN UK and PTL UK appeared to be absorbing less than all others. At 610 nm ICM UK; BIC UK; PTN UK; PTL UK; PPM UK; PLT UK; GRE UK and WHS UK #01 were almost fully reflecting, whilst UNI UK; STP UK; STD UK and ZBR UK were still weakly absorbing. By 620 nm, all but two samples (UNI UK and STP UK) were fully reflecting. Images of the red ink group samples under 450 nm, 560 nm, 570 nm, 590 nm, 610 nm and 620 nm are shown in Figure 3.14 illustrating the discrimination behaviour described above.

Close inspection of red ink group spectra revealed subtle differences in spectral shape and slope. It was therefore considered possible to discriminate the red gel pens into six groups based on spectral data alone. Figure 3.15 shows the variability in spectral shape between all 13 brand/model combinations, whilst Figure 3.16 shows a set of spectra from three different brand/model combinations considered distinguishable. The STB UK spectra exhibited a squared rather than curved spectral shape around 550 - 600 nm which distinguished it from all others. The ICM UK and PLT UK spectra exhibited a notable trough between 500 – 600 nm, not observed elsewhere. However these two inks could be distinguished by the gradient of the spectral curve around 600 – 650 nm, which was more gradually curved in the PLT UK spectra. Group 4 could be distinguished from Group 5 and 6 on the basis of spectral shape around 900 – 1000 nm, with the latter two groups exhibiting a small trough, whilst the former was more flat. Group 5 and 6 could be discriminated by the overall shape and gradient of curve around 600 – 650 nm, although this difference was considered subtle and therefore based on individual subjective opinion.

Considering all HSI data combined, it was thus considered possible to discriminate all 13 samples into 13 groups, effectively giving 100% discriminating power using this technique.

Red Ink Group

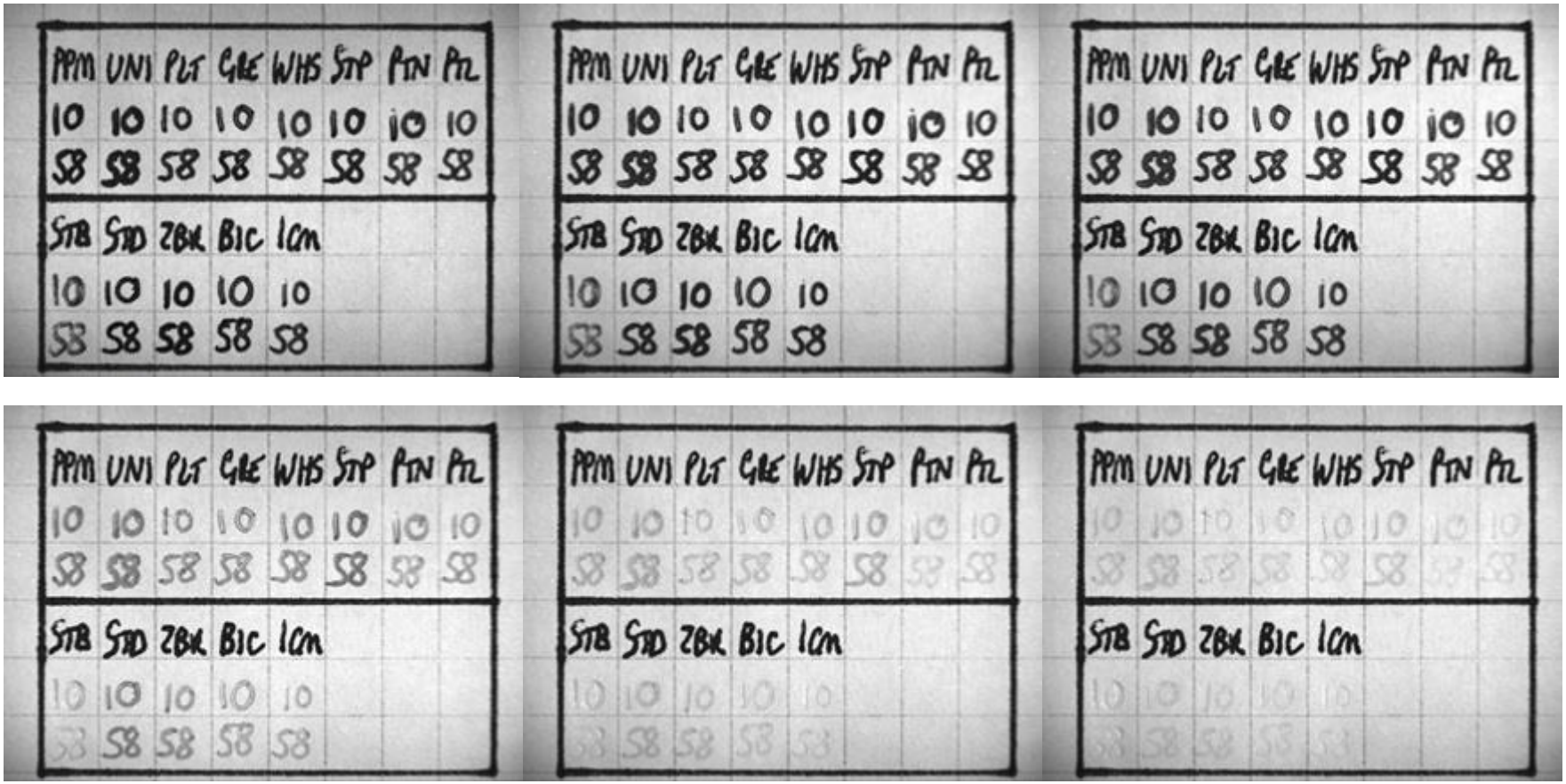


Figure 3.14: Imagery data of UK acquired red gel inks on paper under IR absorbance/reflectance illumination in sequence at 450 nm, 560 nm, 570 nm, 590 nm, 610 nm and 620 nm illustrating the discrimination behaviour of the STB UK brand/model combination around 450 nm, and for all other brand/model combinations between 560 – 620 nm

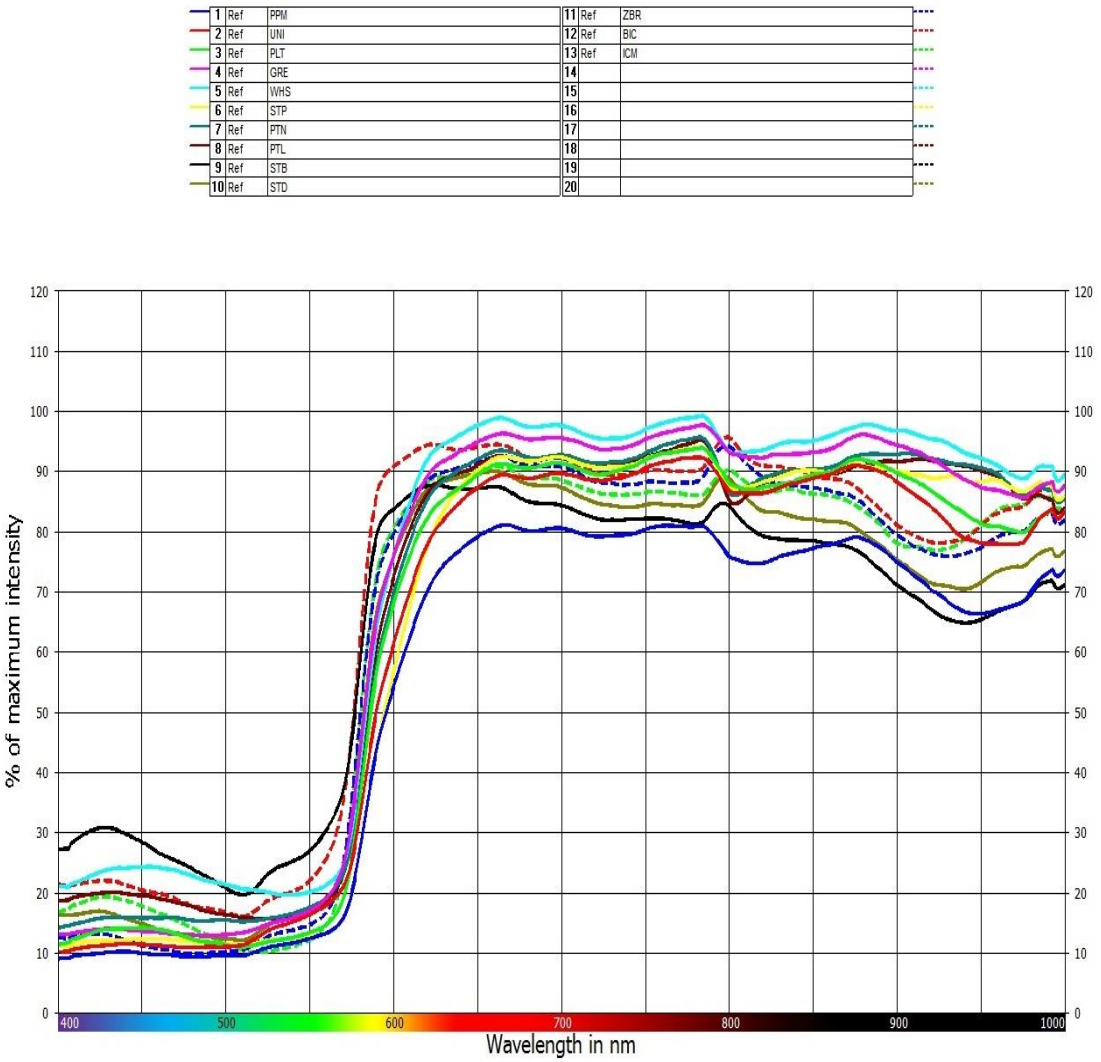
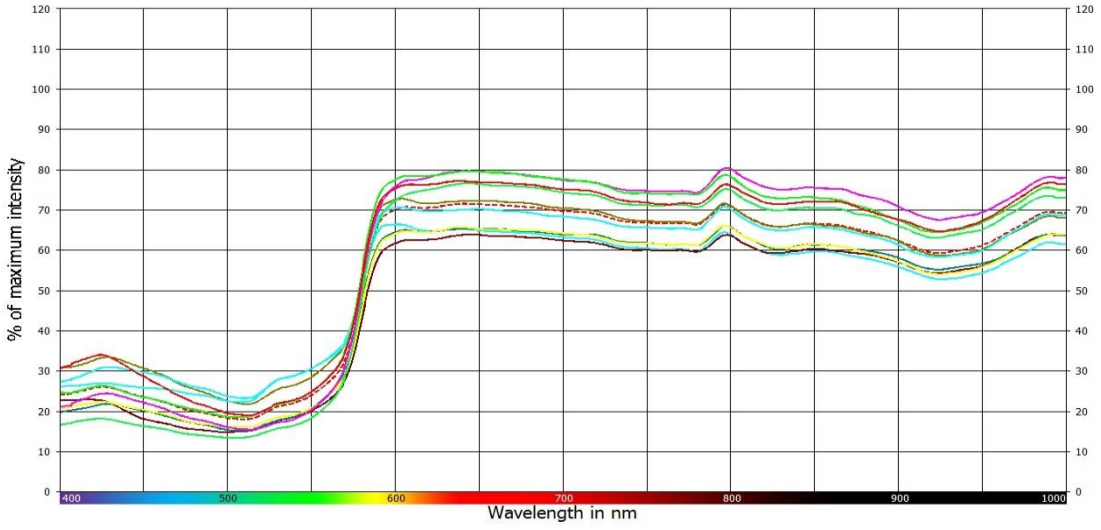
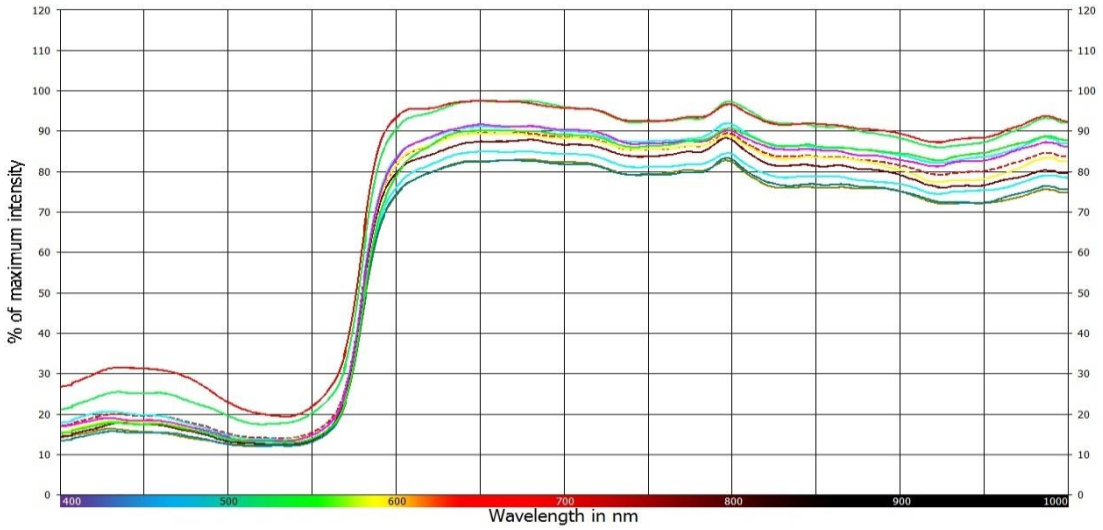
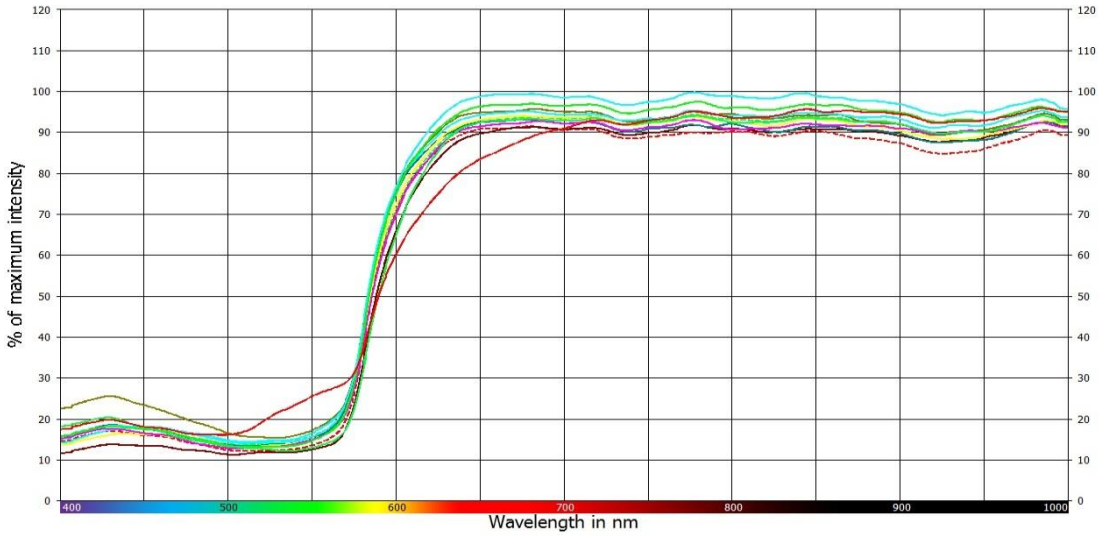


Figure 3.15: A single spectrum from each of 13 brand/model combinations of UK acquired red gel ink illustrating the similarity and differences between them







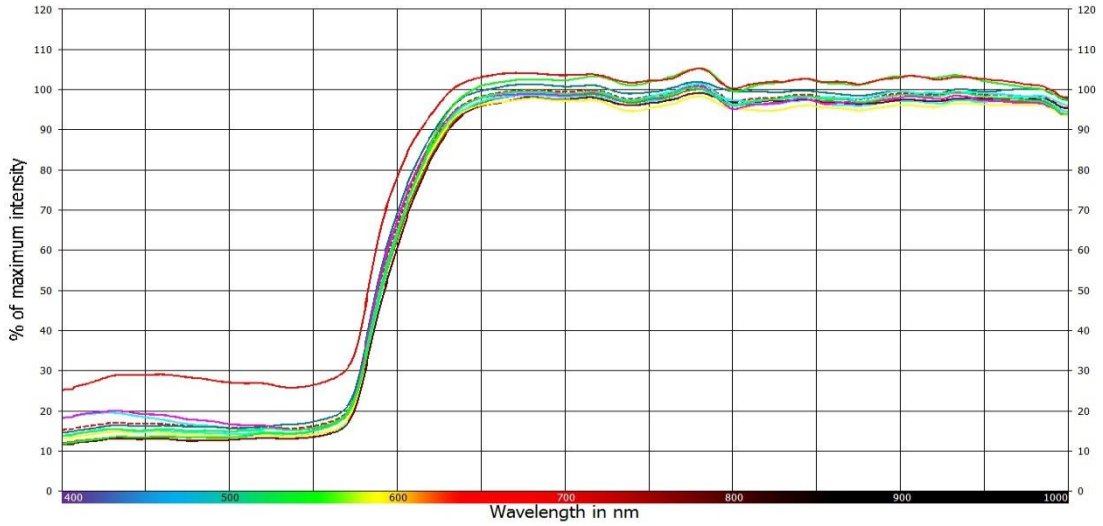


Figure 3.16: A set of % reflectance spectra of STB UK (previous page)), ICM UK (top of this page), PLT UK and STP UK (bottom of this page) red gel inks on paper representing Group 1, Group 2, Group 3 and Group 9 based on spectral data alone

Colour Group	Imagery Data			Spectral Data			Combined HSI Data		
	Sample	**Class Group**	**Colorant**	**Sample**	**Class Group**	**Colorant**	**Sample**	**Class Group**	**Colorant**
Blue	STB UK; ICM UK	1	Dye or Both (Hybrid)	STB UK; ICM UK	1	Dye or Both (Hybrid)	STB UK; ICM UK	1	Dye or Both (Hybrid)
	PKR UK	2		PKR UK	2		PKR UK	2	
	PLT UK*; PTN UK; BIC UK	3	Dye* and/or Pigment	PLT UK*; PTN UK	3	Dye* and/or Pigment	PLT UK*; PTN UK	3	Dye* and/or Pigment
				BIC UK; STD UK	4	Pigment	BIC UK	4	Pigment
	GRE UK; WKE UK; WHS UK; STP UK; STD UK	4	Pigment	GRE UK; WKE UK; WHS UK; STP UK	5		STD UK	5	
							GRE UK; WKE UK; WHS UK; STP UK	6	
	PTL UK; ZBR UK	5		PTL UK; ZBR UK	6		PTL UK; ZBR UK	7	
	PPM UK; UNI UK	6		PPM UK; UNI UK	7		PPM UK; UNI UK	8	
Red	STB UK	1	Pigment	STB UK	1	Pigment	STB UK	1	Pigment
	BIC UK; PTN UK	2	Dye or Pigment or Both (Hybrid)	ICM UK	2	Dye or Pigment or Both (Hybrid)	ICM UK	2	Dye or Pigment or Both (Hybrid)
				PLT UK	3		PLT UK	3	
	GRE UK	3		PTL UK; PTN UK; STP UK; WHS UK; GRE UK	4		BIC UK	4	
	PLT UK; WHS UK	4					STD UK	5	
	PTL UK	5		ZBR UK; BIC UK; STD UK	5		PTN UK	6	
	STD UK	6					GRE UK	7	
	ICM UK	7					WHS UK	8	
	PPM UK	8		PPM UK: UNI UK	6		STP UK	9	
	UNI UK; STP UK	9					ZBR UK	10	
							STD UK	11	
							UNI UK	12	
	ZBR UK	10					PPM UK	13	

Table 3.4: Summary of classification groupings by colour group from the preliminary study of HSI of UK acquired ink samples

3.4 Conclusion

Based on the camera and optical filters selected for IR absorption/reflectance (IRR) and IR fluorescence/luminescence (IRL) in this study, within brand variation was observed in a single brand/model combination only, of those examined; the WHS UK red gel ink. Based on IRL examinations alone, six pens could be split into two groups representing pens #01 – #03 and pens #04 – #06. This finding strongly suggests a change in chemical formulation for this particular brand/model combination of gel ink has occurred during manufacture.

In terms of between brand variations, the inks within each of the two colour groups could be discriminated into several groups by IRR and IRL examinations, both individually and in combination. Considering both sets of examinations combined, the blue and red ink groups could be discriminated into 10 and 12 groups respectively. Furthermore, for the blue ink group only, these groups contained either dye containing or pigment based samples only. IRL examinations provided a better level of discrimination between samples across both colour groups than IRR examinations, but problems associated with fluorescence from the paper substrate affected discrimination. These problems could be partly overcome by including observations from the white light filter (380 – 800 nm) during such examinations. However, owing to these and other difficulties associated with the inherent nature of the technique, all subsequent classification groupings should be treated with caution.

A study of the HSI capabilities of the VSC 6000/HS enabled discrimination of the UK acquired blue gel pens into eight groups and the red gel pens into 13 groups representing a 100% discrimination power in the latter. Spectral reproducibility within a brand/model combination was considered good, but spectral differences used to discriminate between samples were subtle requiring intensive examination by eye. In this study, discrimination of samples was achieved by comparing spectral printouts side by side with the naked eye since access to the instrument software was limited. Using the instrument software to compare spectra on the same axis may have aided comparison. Furthermore, an objective statistical method of comparison incorporated into the instrument software could be of great benefit for the discrimination of such samples by this technique. Artefact peaks at ~800 nm were observed in spectra of the ink on paper and un-inked paper, attributable to a crossover point between the three RGB channels. This requires further investigation if the HSI capabilities of the instrument are to be relied upon with full confidence.

Chapter Four

Visible-Microspectrophotometry

4.1 Introduction

During the 1980's and 1990's a small body of research formed around the use of Vis-MSP for questioned document examination in response to the need for a more objective approach to colour comparison. Pfeffferli [111] introduced the Nanospec 10s Microspectrophotometer and its potential for discrimination of ink based on % reflectance spectra (Vis-MSP: 380 – 740 nm). Ballpoint, fibre-tip and fountain pen inks were the samples of choice, and multiple spectra taken from across the same ink line exhibited "fairly good" reproducibility with only minor variations in overall shape of % reflectance curve observed. The number of peaks, wavelengths at which peaks hit maximum % reflectance and relative peak intensities were all identified as features to be considered when comparing spectra from two or more inks. Practical considerations were made with regards to sampling, including the need to keep the document flat during spectral acquisition, sampling an area of ink less than 1 mm in diameter and adjusting the measurement slit to provide maximum aperture in order to counterbalance the effect of colour density changes arising from the inhomogeneous distribution of ink on paper. An ideal measurement slit area of ~130 μm^2 was recommended. Whilst Pfeffferli [111] identified % reflectance spectra as preferable over transmission spectra for this particular application, interference from glossed areas of the ink could affect % reflectance values and thus avoiding these during spectral acquisition was recommended. Vis-MSP was found to be a rapid, non-destructive technique capable of providing improved discrimination, over traditional chemical analysis by TLC, however, it was recommended as a complimentary technique only rather than a replacement.

Laing and Isaacs [109], investigated the use of both reflectance and transmission operational modes of the Nanospec 10s MSP (390 – 790 nm). A large number of blue and black ballpoint and fibre-tip pen inks were analysed to assess the effectiveness of Vis-MSP for ink analysis applications. Reproducibility of % reflectance spectra was negatively influenced by glossy areas in the ink line. This phenomenon was identified as "bronzing",

where a “reddish metallic sheen” is observed in “small uneven distributed patches” across the ink line and can result in shifts in absorbance maxima (λ_{max}). Furthermore, heavier ink strokes were found to be more susceptible to bronzing than lighter ink strokes which in turn were shown to provide distinguishable spectra from the same ink sample. Transmission spectra acquired from individual ink stained paper fibres distributed on a glass slide in a mounting medium however did not suffer from bronzing effects or interference from the paper fibres suggesting this should be the method of choice. Furthermore, no interference from paper of different types and colours was observed. Transmission spectra were found to be reproducible with only minimal shifts in λ_{max} observed, i.e. >10 nm. A high level of discrimination between inks of same type and colour were observed, with discrimination powers ranging between 0.85 – 0.92 achieved.

Fuller [108] investigated the application of the Nanospec 10s MSP in reflectance mode (380 – 900 nm) to the analysis of dye component bands on TLC plates from a limited number of ballpoint and fibre-tip pen ink samples, amongst other samples of forensic interest. Discrimination was largely achievable from TLC alone, but further discrimination was possible based on weak spectral responses from the individual component spots. Practical difficulties associated with locating faint or lightly coloured component bands using the monocular eye piece were encountered, and % reflectance was found to be lower from areas within component spots of strong intensity. Possible identification of dyes by comparison to a spectral library was mooted, but required a comprehensive reference collection to be effective. A large number of paint pigments, i.e. monoazo pigments (red, orange and yellow) and phthalocyanines (blue and green) were also analysed. Most monoazo pigments were found to be soluble in dichloromethane, whilst the phthalocyanine pigments were slightly soluble in 1-methylnapthalene, i.e. Pigment Blue 15, permitting their analysis by TLC. Several spectral types characterised the red and orange monoazo pigments, whilst the yellow monoazo and blue phthalocyanine pigments could not be distinguished within their colour group from their % reflectance spectra.

Totty *et al* [74] followed the method described by Laing and Isaacs [109] to compare transmission Vis-MSP (390 – 790 nm) with High Performance Thin Layer Chromatography (HPTLC) to assess the effectiveness of both techniques for discrimination of blue and black aqueous inks, i.e. fibre-tip and rollerball. HPTLC was more effective than transmission Vis-

MSP, with discriminating powers ranging between 0.95 – 0.98 and 0.84 – 0.86 achieved respectively. This advantage of HPTLC over transmission Vis-MSP was attributed to its ability to detect minor dye components within an ink sample. Only one pair considered indistinguishable by HPTLC could be distinguished by transmission Vis-MSP. Reproducibility of spectra was better for the blue inks, but fewer spectral features for discrimination were observed compared to the black inks. Overall, it was concluded that transmission Vis-MSP was a valuable addition rather than alternative technique for ink analysis.

Olson [110] utilised the Nanospec 10s MSP in reflectance mode for the discrimination of printing and stamp pad inks on travel and identity documents. A number of practical considerations were identified including the recommendations of orienting the measurement slit in a north-south position in line with the orientation of the monochromator, spectral acquisition from between 3 – 5 different areas within a sample to account for sample variation and the comparison of features such as peak and trough positions, and relative peak heights for discrimination. Further research into spectral refinement to achieve better discrimination was recommended, but as with previous studies [74, 111], its value as a quick, non-destructive additional technique was concluded.

Zeichner *et al* [113] conducted an in depth investigation of the value of % reflectance and transmission spectra in terms of quantitative comparison, focussing in particular on the influences of scattering and bronzing effects on the spectra of ink on paper, although an alternative MSP instrument to the Nanospec 10s was used, the Docuspec TM/1 MSP. Spectra from ink deposits on glass slides were found to obey Beer-Lambert Absorption Law, whilst deviations attributable to scattering and bronzing interferences were observed in spectra from ink on paper. As with previous studies [109, 111], bronzing was found to preclude the use of % reflectance spectra over transmission spectra due to absorption maxima reproducibility issues. The destructive method of acquiring transmission spectra used by Laing and Isaacs [109] was compared to that from a non-destructive method, simply acquiring transmission spectra from the ink on paper in situ. It was found the latter reduced the ability to acquire reproducible spectra, whilst the former exhibited poor reproducibility compared to the corresponding ink on glass slide spectra in terms of Beer-Lambert Absorption Law. Paper opacity variations, as well as scattering were suggested as the reasons for poor spectral reproducibility observed for the non-destructive method over

the destructive method. Zeichner *et al* [113] recommended therefore that comparison of transmission spectra should be based on distinct spectral features rather than on the basis of Beer-Lambert Law, i.e. a qualitative rather than quantitative approach.

Roux *et al* [112] used Vis-MSP in reflectance mode (380 – 880 nm) in combination with filtered light and TLC to discriminate 49 blue and 42 black ballpoint inks available on the Australian market. Discrimination powers <0.99 were achieved for both colour groups using all three techniques combined, but TLC was the most individual discriminating technique and MSP the least. A computerised approach to spectral comparison based on a representative average spectrum for each brand/model combination and a % match threshold value was adopted in the first instance to distinguish between pairs. Where pairs were considered indistinguishable, further comparison by eye was undertaken taking into consideration overall spectral shape, λ_{max} and λ_{min} positions and distinct spectral features such as shoulders or plateaus. Despite care in avoiding bronzed areas of the ink line, spectral reproducibility with regards to % reflectance values rather than shifts in λ_{max} and λ_{min} positions were observed. The combined approach using these three specific techniques focussed on different characteristics of the writing inks and was found to be highly effective for between brand and to a certain extent within brand discrimination.

To date, there appears to be only one study investigating the use of MSP applied to gel inks in the literature. Martin & Lyter [28] investigated the use of Craic Technologies Questioned Document System II (QDSII) Microspectrophotometer in diffuse reflectance and fluorescence mode to examine seven different black gel and two blue gel inks, representing six manufacturers, as lines drawn on office paper. Diffuse reflectance spectra representing within (five) and between (four) line measurements of the same sample of ink exhibited excellent reproducibility and satisfactory signal to noise (S:N) ratio when analysed over 390 – 850 nm wavelength range. Two black gel inks could be distinguished from the other five in the sample set which were all considered indistinguishable to each other, whilst both blue inks could be distinguished from each other. The authors highlighted that for one brand/model of each colour analysed, the NIR region of the spectrum was essentially the same. When the inks were examined by Fluorescence Microspectroscopy at 365 nm, 405 nm and 436 nm, mostly they were found not to fluoresce, but it was possible to differentiate between the two blue gel inks. It was concluded that the QDSII system was

able to differentiate black and blue gel inks that other techniques had been unable to do, with excellent reproducibility, reasonable S:N ratio and with little sample preparation. However, given the limited sample set, it was not possible to investigate the full benefits of the system. Although the authors commented that its ability to scan across the full visible spectral range suggested it may be useful for discriminating between other colours of gel ink by manufacturer, of which work was underway to investigate at the time of publication, but has yet to appear in the literature.

The work presented here was performed in an attempt to address the deficit in the literature with regards to the MSP of gel inks. The suitability and level of discrimination achievable using Vis-MSP in transmitted light mode for the analysis of gel inks on paper representing both colour groups was investigated both in terms of within and between brand variability. The use of the first derivative method [130] which has been widely used in the forensic examination of dyed fibre samples [131-134] was also assessed as an aid to further discrimination of gel ink samples. Spectral data was acquired with a view to further evaluation using a multivariate statistical approach.

4.2 Experimental

4.2.1 Sample Preparation

Strips of paper (~3.5 cm x 1.5 cm) from a single sheet of A4 white office paper (80 gsm) were cut out and secured to glass microscope slides with sellotape. For a within brand study, three brand/model combinations for each colour group were selected, mostly at random, except for two highlighted in bold below, which were found to display evidence of within brand variation by another technique, i.e. Raman Spectroscopy. These brand/model combinations were:

- ICM UK, PKR UK and **ZBR UK** (blue ink group);
- BIC UK, ICM UK and **WHS UK** (red ink group).

For a between brand variation study, ink lines representing all brand/model combinations were drawn onto the paper strips. All UK acquired samples for a given colour group were drawn onto a single slide, whilst all international (INTL) acquired samples for a given colour

group were drawn onto another slide. All samples were allowed to air dry at room temperature for at least 15 minutes prior to analysis and were analysed within 24 hours of deposition. Samples were stored at room temperature in a laboratory notebook overnight.

4.2.2 Instrumentation

All spectra were acquired using an Olympus BX41 microscope fitted with a video camera, and attached to a J & M Tidas Microspectrophotometer. The microscope was fitted with an MPLAN N (UIS 2) x 10 objective (obj.) and a UPLAN FL (Olympus) x 40 obj. The MSP system was connected to a PC workstation with Onyx Software (version 1.9.0.0, Cavendish Instruments Ltd © 2003 – 2006) installed. The measurement slit dimensions were 4.185 µm x 5.115 µm (width x height) and the full scan range was 361 – 781 nm. All spectral measurements of ink on paper were performed using transmitted light in the intensity/counts scan type mode. All spectra were normalised for ease of comparison and smoothed by 35 points using the Savitsky-Golay algorithm [130] to improve S:N ratio. Spectral data was then transferred to Microsoft Excel 2007 (version 12.0.4518.1014, part of MS Office Basic 2007) or 2010 (version 14.0.6112.5000 (32 bit), part of MS Office Home and Student 2010) for subsequent data analysis.

4.2.3 Spectral Acquisition

The sample slide was placed on the XYZ translation stage of the microscope and an ink line brought into focus via the camera screen using the x 10 obj. The x 40 obj. was used to provide greater magnification of a sample area and for acquiring spectra. Before the instrument was used each day, a dark spectrum was acquired to ensure no stray light was entering the optical system. In addition, a background spectrum taken from an area close to the sample was taken. A set of ten spectra from the same area of a single ink line were taken each day to check for instrumental variation. Sample variation was checked by taking between 6 - 10 spectra from different areas of the same ink line. In addition, a single spectrum from an un-inked area of the paper close to each ink line was taken to monitor for spectral interference from the paper substrate.

4.3 Results & Discussion

4.3.1 Instrumental Variation

Variations in spectral response were assessed by recording a set of ten spectra from the same area of a single ink line. Figure 4.1 illustrates a typical set of results and reveals no appreciable differences between spectral responses.

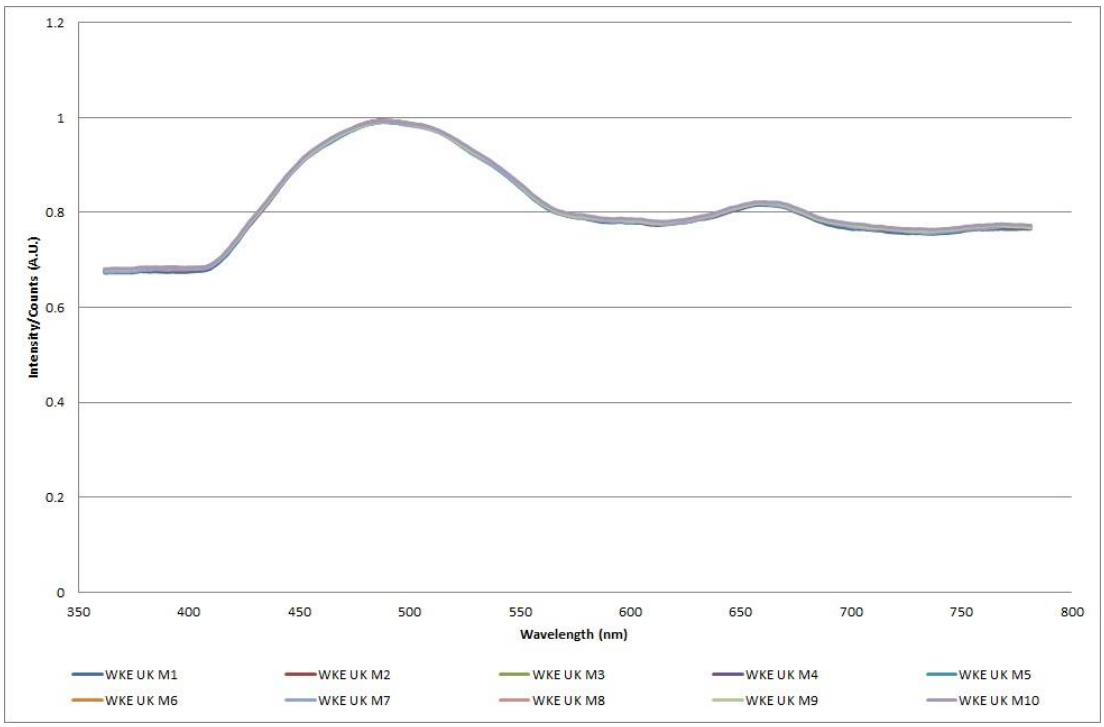


Figure 4.1: A set of 10 spectra from the WKE UK blue gel ink demonstrating no instrumental variation (M = Measurement)

Similar instrumental checks were taken each day to monitor instrumental variation. None was observed.

4.3.2 Spectral Interference

Spectral interference from the paper substrate was revealed as a spectrum with poor S:N ratio. This noise could easily be removed by smoothing the spectrum by 35 points using the Onyx software without loss of overall spectral shape. In terms of spectral shape, Figure 4.2 shows little to no difference was observed between multiple (n = 6) spectra taken from the glass slide and the paper attached to the glass slide suggesting that no contribution from the paper would be observed in spectra taken from an ink deposited on paper.

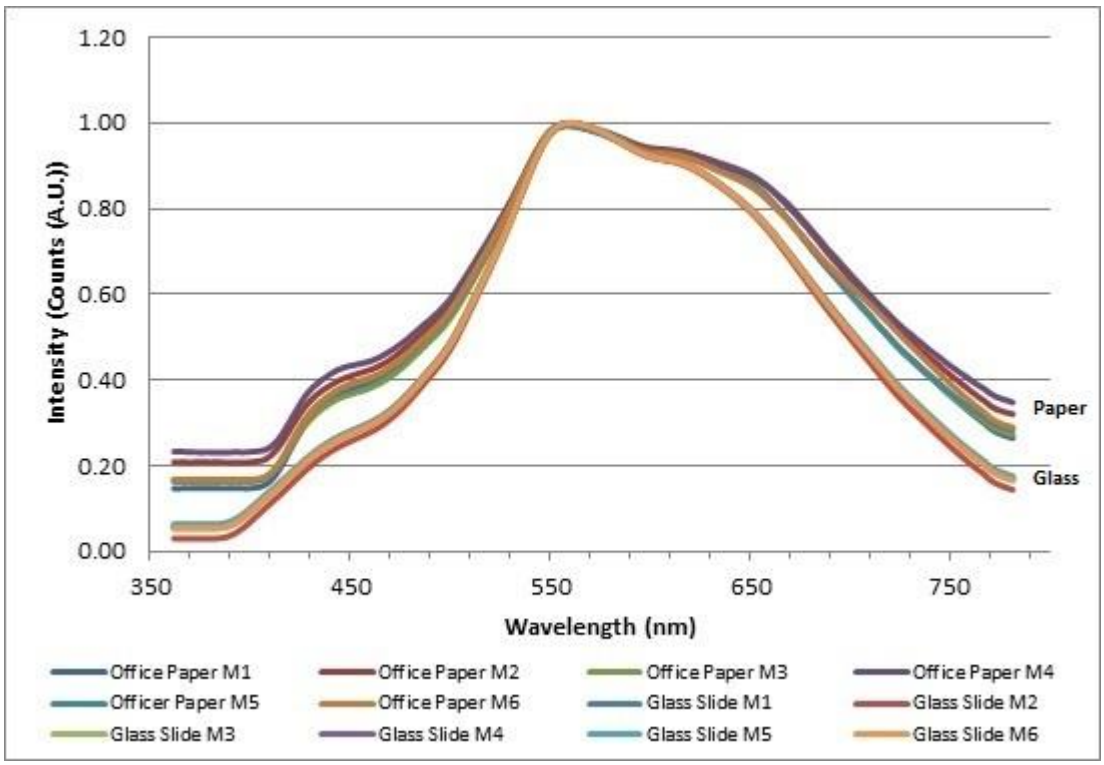


Figure 4.2: A set of six spectra (normalised and smoothed (35 points)) from each of a glass slide and paper secured to glass slide demonstrating little to no spectral interference arising from the paper substrate

4.3.3 Within Brand Variation

4.3.3.1 Blue Ink Group

The ICM UK, PKR UK and ZBR UK samples were analysed. The ICM UK samples exhibited a very uniform spectral response, the only differences detected being in the λ_{max} position which occurred across a range of 475 nm – 510 nm. For both the PKR UK and ZBR UK samples, spectral shape within a brand/model combination was very similar, however some spectral variation in terms of shape and intensity was observed. Examination of six spectra within a single pen ink line for all six pens per brand/model showed only some minor variation in spectral shape as illustrated in Figure 4.3. Therefore it was considered that no notable within brand variation was observed within the three blue ink group samples.

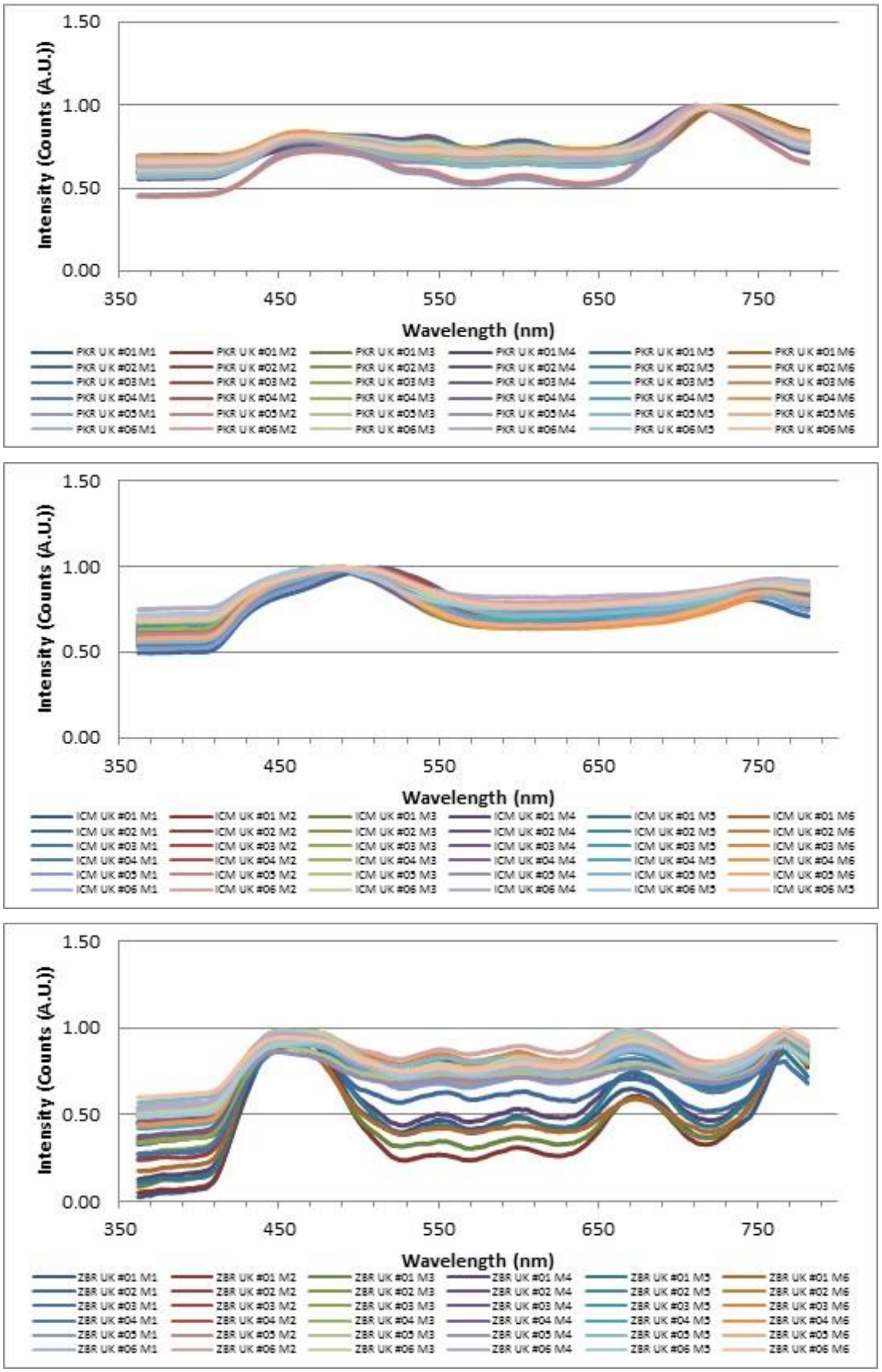


Figure 4.3: Spectra representing three brand/model combinations of blue gel ink (PKR UK; ICM UK and ZBR UK) on paper demonstrating no within brand variation

4.3.3.2 Red Ink Group

The BIC UK, ICM UK and WHS UK samples were analysed. For the BIC UK and ICM UK samples, multiple (n = 6) spectra across all six pens were considered indistinguishable, exhibiting no notable within brand variation. For the six WHS UK pens, the VSC 6000/HS study clearly indicated a difference in luminescent behaviour between pens #01 – #03 and pens #04 - #06 highly suggestive of a change in formulation during manufacture. The Vis-MSP spectra of all six pens exhibited a characteristic large peak above 600 nm, and below that a comparatively featureless spectrum. However, a small reproducible well defined peak at ~550 nm was observed in the spectra from pens #01 - #03 (Figure 4.4) that was not as notable in the spectra from pens #04 - #06 (Figure 4.5). This difference, highlighted in Figure 4.6, may be considered evidence of within brand variation, and is supported by the findings from the VSC 6000/HS study. However, in isolation, it is difficult to say with certainty this subtle spectral difference is due to a real chemical difference, and therefore, in the author's opinion, cannot be relied upon as evidence of within brand variation.

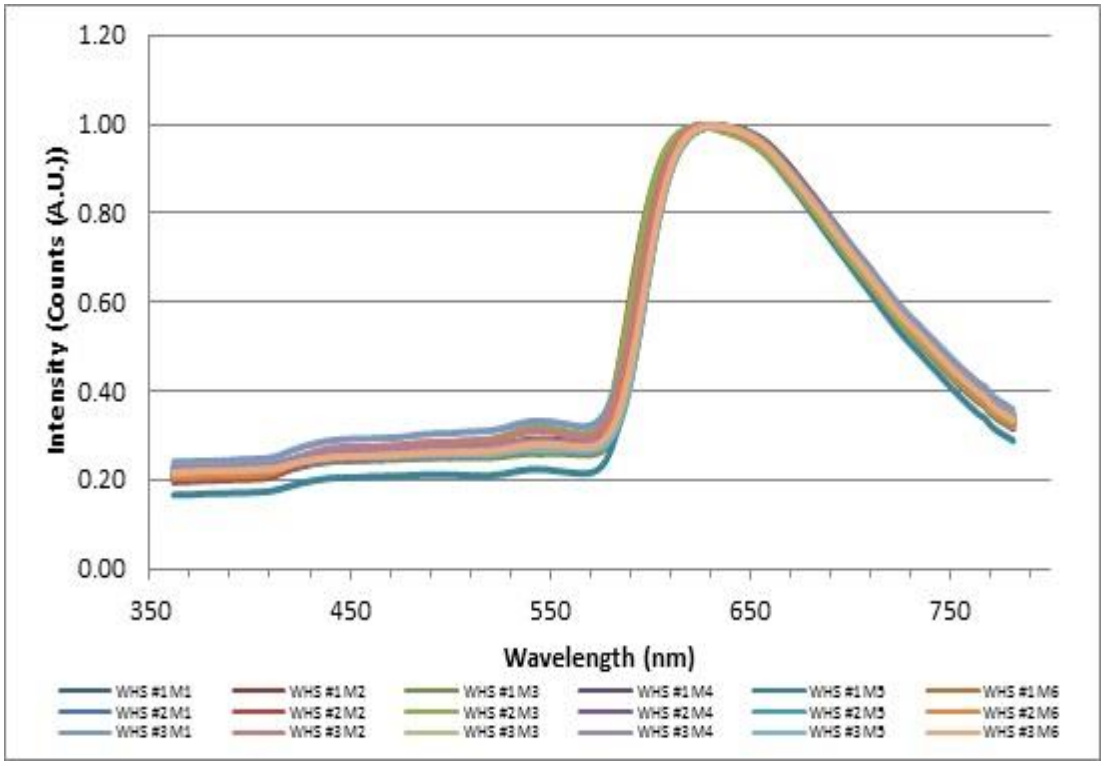


Figure 4.4: A set of six spectra from each of WHS UK red gel ink pens #01 - #03

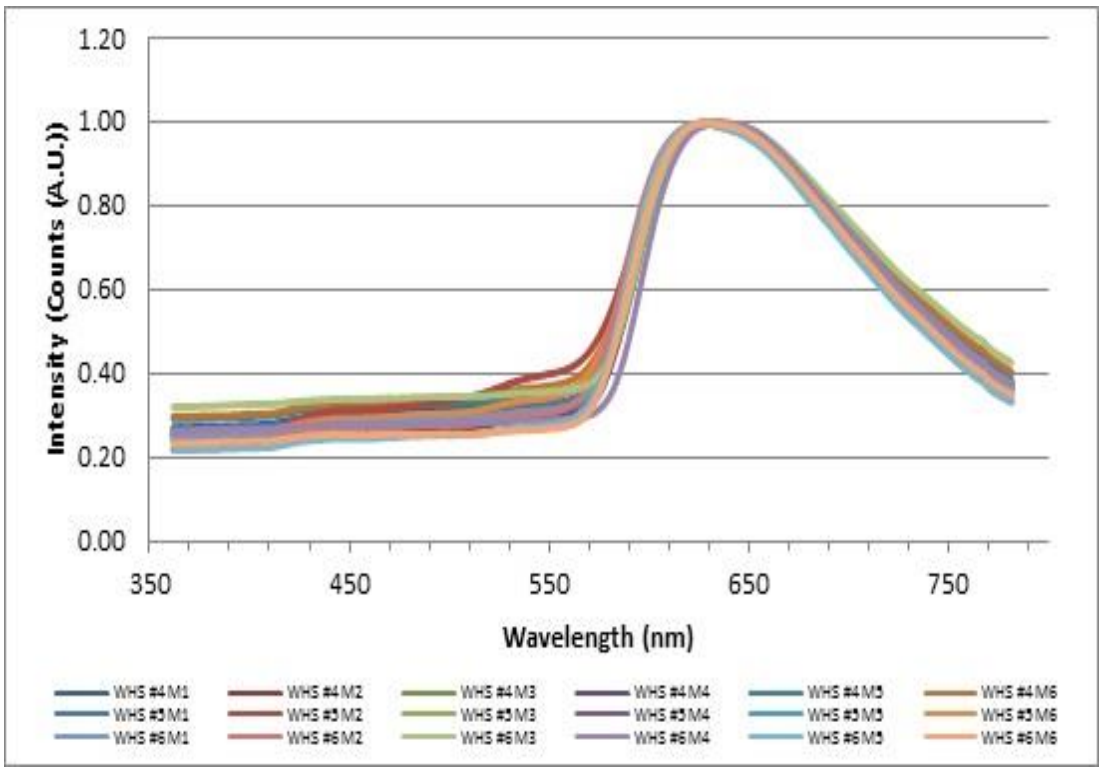


Figure 4.5: A set of six spectra from each of WHS UK red gel ink pens #04 - #06

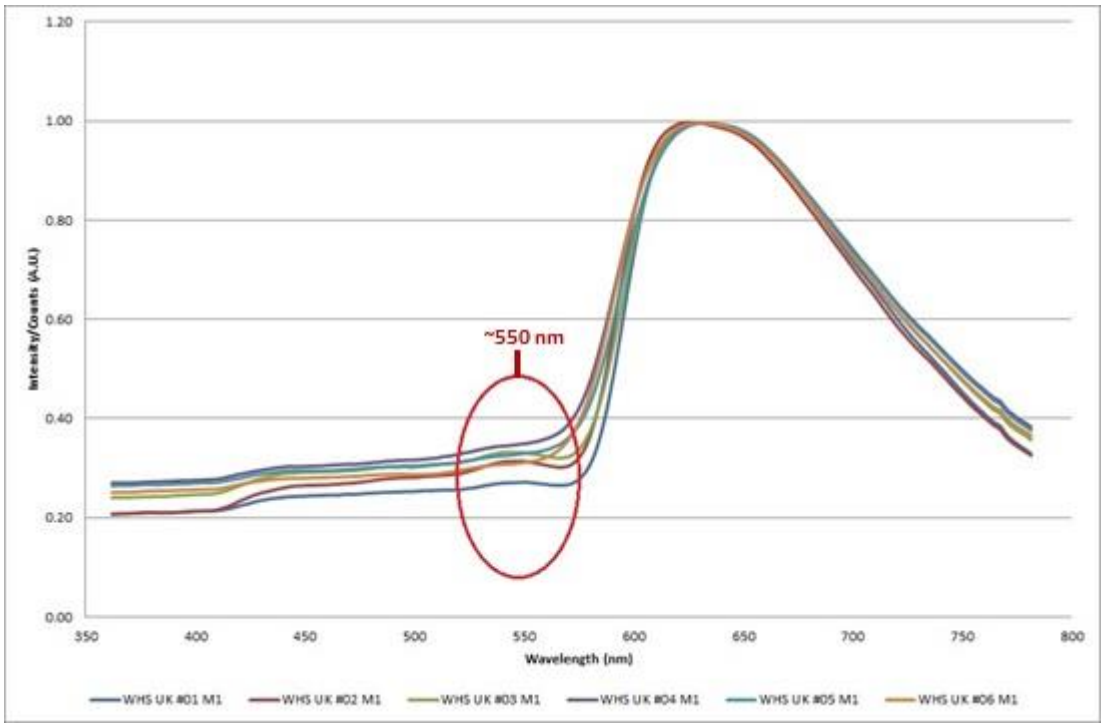


Figure 4.6: An example spectrum from each of WHS UK red gel ink pens #01 - #06 illustrating a small peak at ~550 nm (highlighted within the red circle) more pronounced within the spectra representing pens #01 - #03 than in those representing pens #04 - #06, possibly providing evidence of detectable within brand variation

4.3.4 Between Brand Variation

4.3.4.1 Blue Ink Group Variability

The Vis-MSP data derived from the blue ink group displayed a wide variety of different spectral patterns suggesting that a good level of discrimination between blue gel inks may be afforded by Vis-MSP in transmitted light mode.

4.3.4.1.1 Within Sample Variability

Little or no instrumental variation was observed between repeat measurements of the same ink area for a given sample. However, some sample variation was observed in a set of repeat measurements taken from different areas of a single ink line for a given sample and this appeared to vary according to the brand/model combination under study as illustrated in Figure 4.7. For some brand/models (i.e. PKR UK), excellent reproducibility of spectra was observed. For most however, (i.e. PPM UK), minor variability in spectral shape was observed between a set of ten spectra, but the general spectral shape was consistent. Four brand/model combinations, in particular, appeared to exhibit wide variability within their spectral response, both in terms of spectral shape and intensity: BIC UK, BIC US, STD UK and PTL JAP. This wide sample variation posed a difficulty when considering how best to classify these brand/model combinations based on their spectral response.

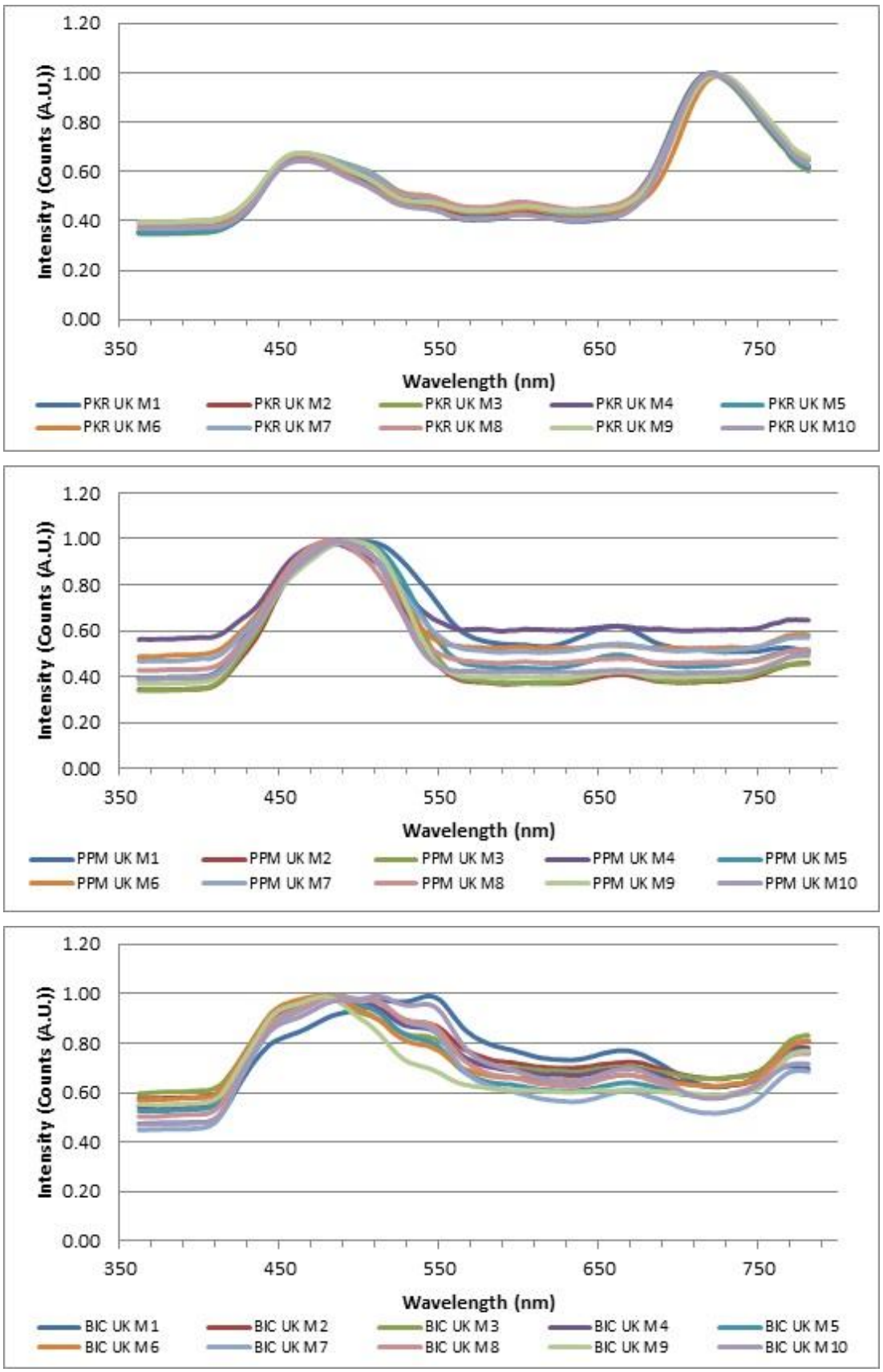


Figure 4.7: Spectra representing three brand/model combinations of blue gel ink (PKR UK; PPM UK and BIC UK) on paper illustrating little, minor and wide sample variation respectively

Repeat measurements (n = 6) were taken using the same sample position and as expected minimal spectral variation was observed. Repeat analysis (n = 10) of the BIC UK, BIC US, STD UK and PTL JAP samples, however, confirmed that these four brand/model combinations appeared to exhibit a wide variation in their spectral response according to where along the ink line the spectral measurement was taken from. During the selection of the sampling sight for these four brand/model combinations, it was noted that some areas of the ink line appeared to exhibit a darker more intense appearance than other areas which appeared much lighter. This observation is illustrated in Figure 4.8. It was postulated that this may account for the difference in spectral response from the same ink line. To test this hypothesis, each of these four samples were analysed again. This time, six spectra were taken from a single dark area of the ink line, and six from a single light area of the ink line. The resulting spectra from all four samples, presented in Figure 4.9, clearly showed that the spectral response differed between the light and dark areas of the ink line.

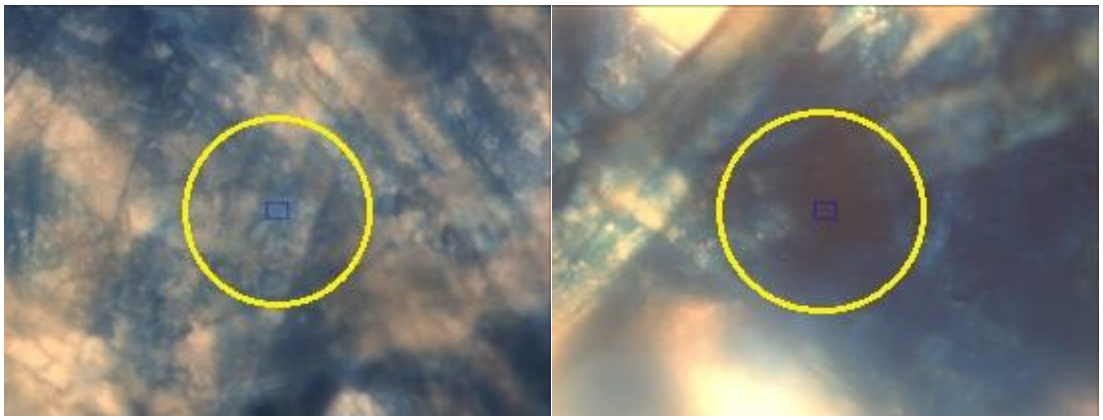


Figure 4.8: Images (x 40 obj.) illustrating a light (left) and dark (right) area, highlighted by a yellow circle, within a single BIC UK blue gel ink line on paper from which spectra were acquired

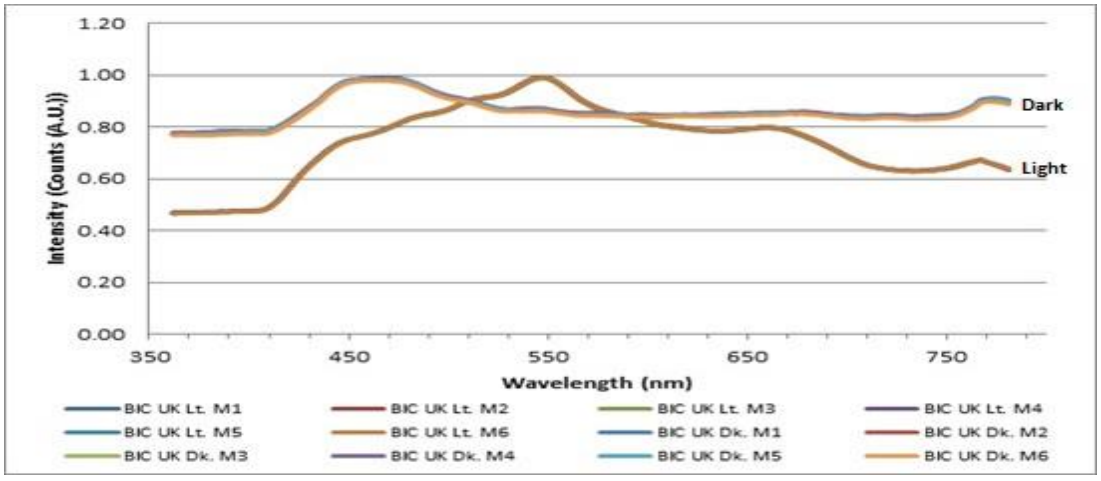


Figure 4.9: A set of six spectra from each of a light and dark area within a BIC UK blue gel ink line on paper

This finding shows that in practice, consideration needs to be given to where from within the ink sample spectra should be acquired, since distribution of the ink on the paper appears, in some cases at least, to yield highly variable spectra for the same sample. A similar phenomenon is seen in the Vis-MSP analysis of dyes in textile fibres [33]. In natural fibres, i.e. cotton or wool, distribution of the dye within the fibre can be highly variable thus resulting in wide spectral variation. In manmade fibres, i.e. polyester or nylon, however, the uniform morphology of the fibre structure results in a more even distribution of dye throughout thus resulting in spectra with minimal spectral variation. It is not known why some brand/model combinations of blue gel ink exhibit less spectral variation than others, but it may be related to the bronzing effects observed in other types of ink described by other researchers [109, 111, 113].

4.3.4.1.2 Between Sample Variability

In an attempt to classify samples on the basis of similarity in their spectral patterns, spectra acquired from the repeat analysis were compared and classification groupings identified. These classification groupings were confirmed by cross-referencing against spectra acquired from the initial analysis to ensure that any within sample variation identified in that study would not result in any misclassification. Furthermore, where a group contained more than one sample, the first derivative spectra for those samples were compared to each other to see if any further discrimination within that group was possible. Initial assessment of the spectral data enabled discrimination between the dye containing and pigment based samples, confirmed by the findings from the Solubility and TLC study.

The dye containing group could be split into seven groups which corresponded to the classification groups identified from the TLC study. Two samples, STB UK and PTL JAP, exhibited spectral patterns shown in Figure 4.10 and Figure 4.11 respectively that were both clearly distinguishable from all others, thus making classification of these samples relatively simple. For the remaining eight dye containing samples, initial classification into three groups was possible followed by further discrimination only after closer scrutiny of the spectra. Table 4.1 provides a summary of the classification groupings based on the original spectra only and further discrimination when considered in combination with the corresponding first derivative.

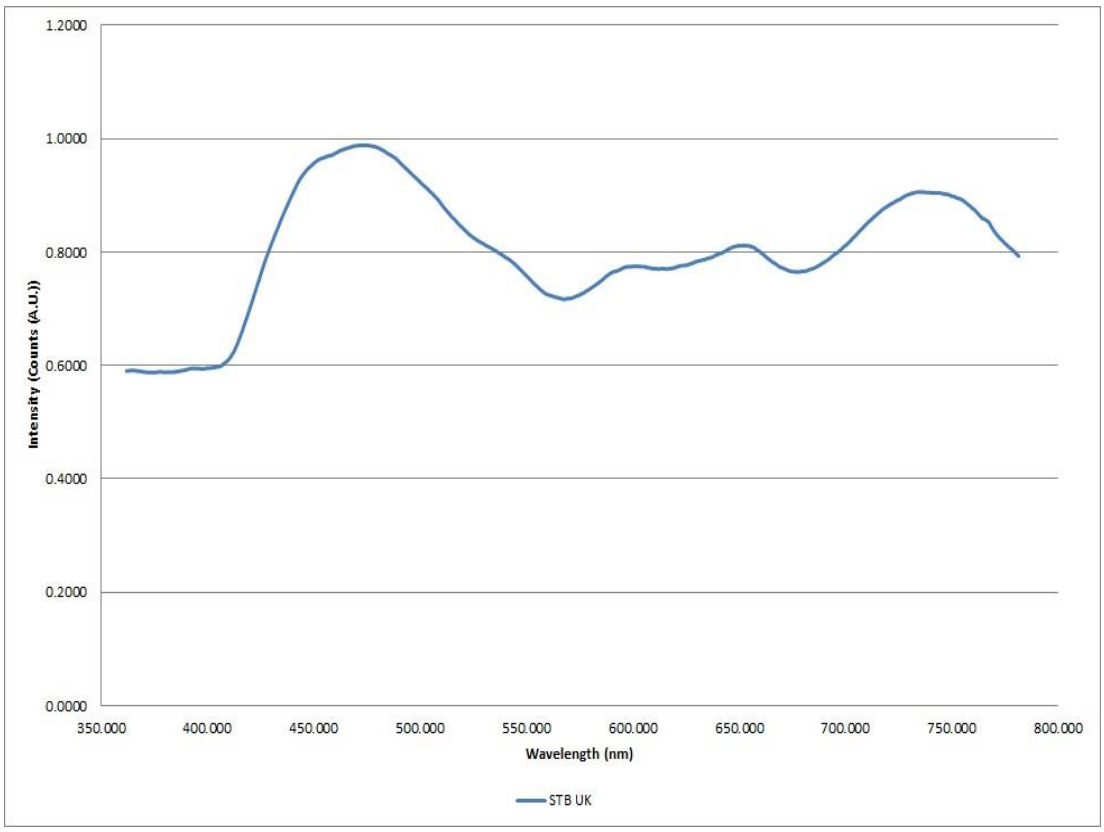


Figure 4.10: Example spectrum from STB UK blue gel ink line on paper (Group 1)

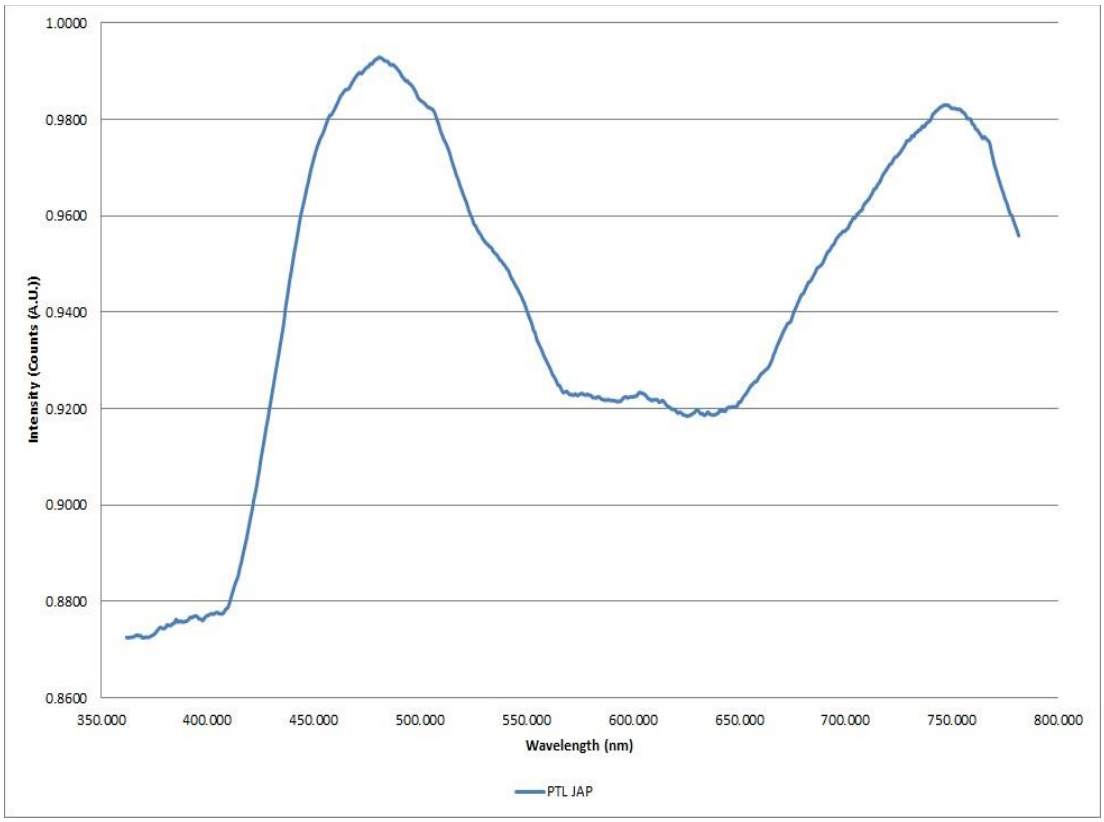


Figure 4.11: Example spectrum from PTL JAP blue gel ink line on paper (Group 2)

Colorant	Sample	λ_{max} (nm)	Classification (Original Spectra)	Further Classification (First Derivative)
Dye or Both (Hybrid)	STB UK	475	1	1
	PTL JAP	485	2	2
	COF US	710 – 720	3	3
	PKR UK	720 – 730	4	4
	STP US #08	695 – 700	5	5
	STP US #09	700 - 720	6	6
	PLT UK	475 – 510	7	7
	ICM UK	475 – 510		
	PLT US	475 – 530		8
	STP US #07	460 - 500		9
Pigment	GRE UK	480 – 510	8	10
	PPM UK			
	STP UK			
	WHS UK			
	WKE UK			
	UNI UK			
	UNI AUS			
	FBC MAL			11
	GSF MAL			
	MG MAL			
	UNI SA			
	UNI JAP			12
	UNI HK #25			
	UNI HK #31			
	BIC UK	475	9	13
	BIC US			
	STD UK			
	PTN UK			14
	ZBR UK	460	10	15
	ZBR JAP			
	PTL UK			16

Table 4.1: Summary of classification groups for blue gel ink based on comparison of original spectra alone (λ_{max} indicated), and in combination with the corresponding first derivative

4.3.4.1.2.1 Dye Containing Samples

Initially, the STP US #08 and STP US #09 samples appeared to possess a similar spectral pattern which at first glance could be considered indistinguishable. However, closer inspection revealed that the peak and trough positions varied across the two samples. For the STP US #08 sample, the peak positions occurred between ~555 – 560 nm (Peak #1) and ~695 – 700 nm (Peak #2), whilst for STP US #09 they occurred at ~540 – 550 nm and ~700 – 720 nm respectively. Additionally, the trough between these two peaks occurred at ~635

nm in STP US #08, but ~650 – 660 nm in STP US #09. Within a set of spectra, it was noted that the STP US #08 brand/model exhibited minimal shift between peak and trough positions, i.e. 5 – 10 nm, whereas for STP US #09 the shifts ranged between 10 – 20 nm and were particularly notable for peak 2. These differences distinguished these two samples from one another, but the similarity in spectral shape suggested that they contained similar dye components. This was also supported by the TLC study which showed that the key difference between these two samples was the presence of a single red component in STP US #08 not seen in STP US #09. Unfortunately, the STP US #09 brand/model combination was not available for the VSC 6000/HS study and thus could not be compared to the STP US #08 sample to determine any differences in their IR reflectance or IR luminescent behaviour. Figure 4.12 shows an example of the spectrum from each of the STP US #08 and STP US #09 ink samples illustrating the differences described.

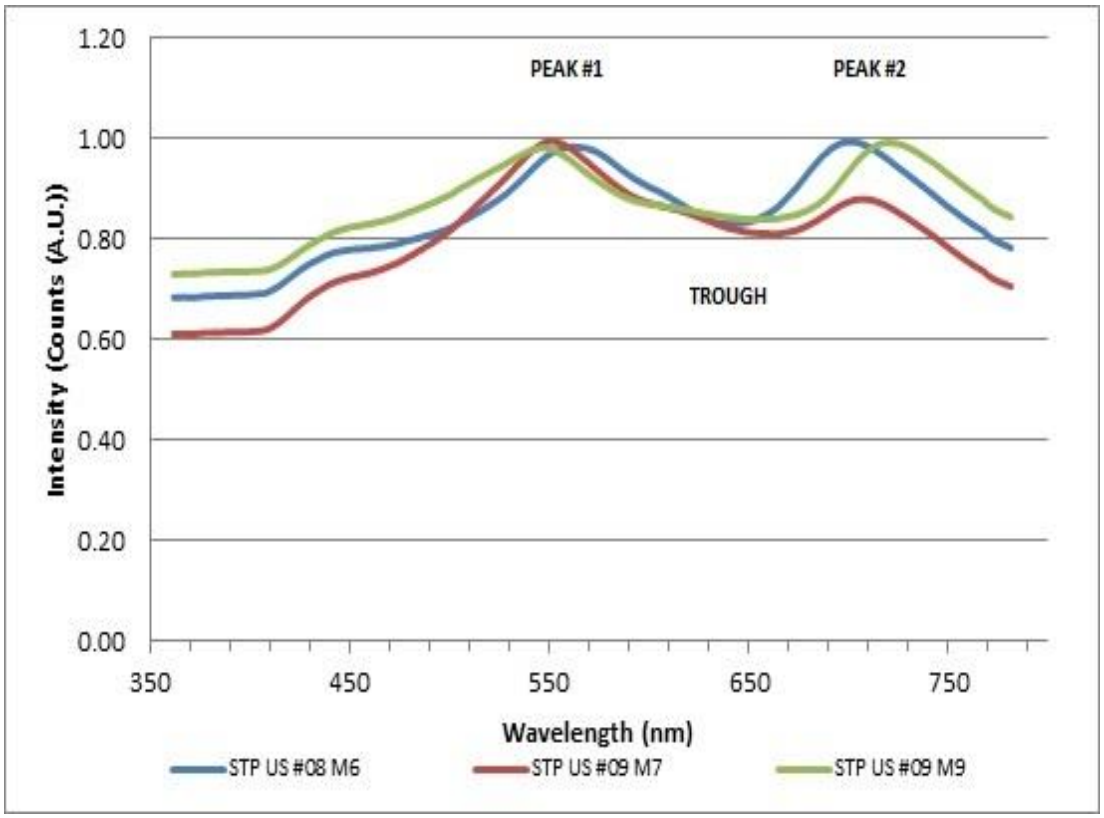


Figure 4.12: Example spectra from each of STP US #08 and STP US #09 blue gel ink lines on paper, the latter taking into account the wider variation in peak and trough positions noted within a set of ten spectra

Spectral pattern similarity was also observed for the COF US and PKR UK samples, but as with the STP US #08 and STP US #09 samples, they could be distinguished on the basis of

differences in peak position. The spectra for these two samples exhibited four peaks, occurring at ~450 nm (Peak #1), ~525 nm (Peak #2), ~570 – 580 nm (Peak #3) and ~710 – 720 nm (Peak #4) for the COF US sample compared to ~460 – 470 nm, ~540 nm, ~595 – 610 nm and ~720 – 730 nm for the PKR UK sample. Comparing a set of six spectra acquired from each sample ink line on the same axis, these differences in peak position are obvious whilst demonstrating the similarity in spectral shape. It should be noted that initial attempts to classify these samples based on a visual assessment of spectra on printouts resulted in them being grouped together. Only by placing the spectra on the same axis were the two samples discriminated from each other, as demonstrated in Figure 4.13.

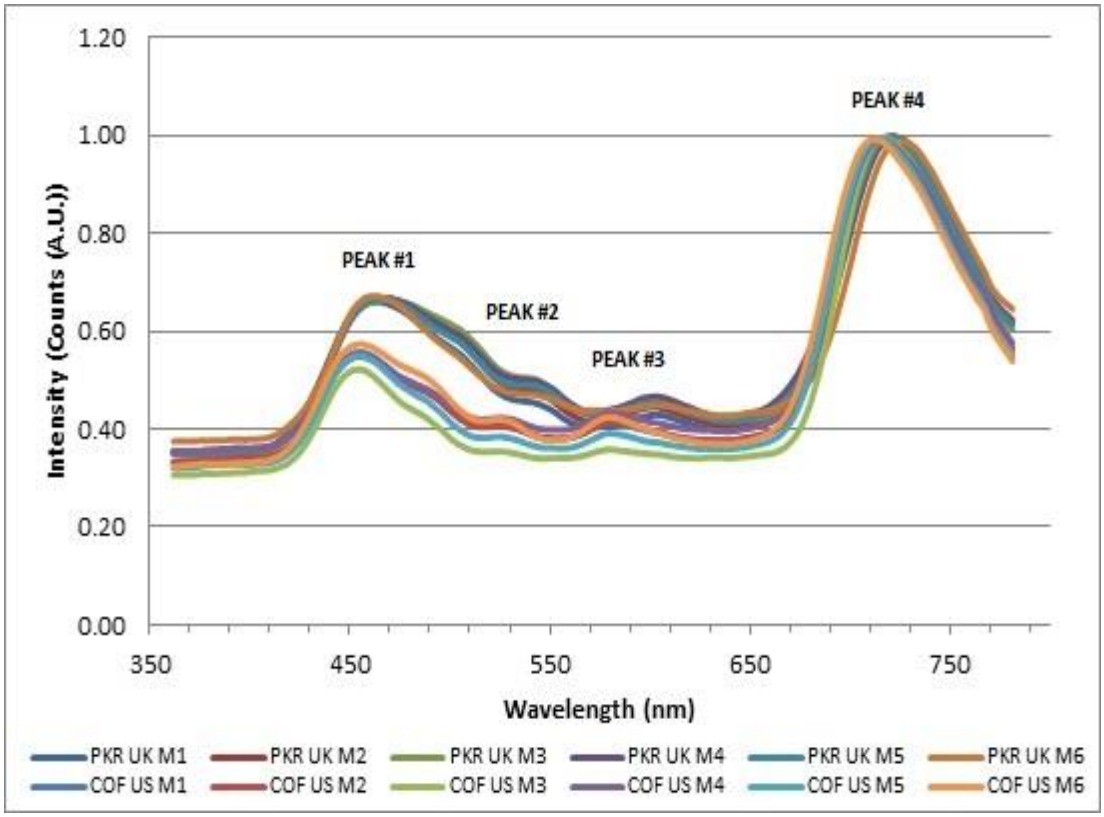


Figure 4.13: A comparison of a set of six spectra from each of the COF US and PKR UK blue gel ink lines on paper highlighting a similar, yet distinguishable spectral pattern on the basis of differences in peak position

The remaining four dye based samples proved harder to discriminate into a definitive group or number of groups. Based on data from the repeat analysis of these samples to obtain a representative spectrum for each, the spectra appeared similar in terms of spectral shape, although minor differences in peak position were observed, as shown in Figure 4.14.

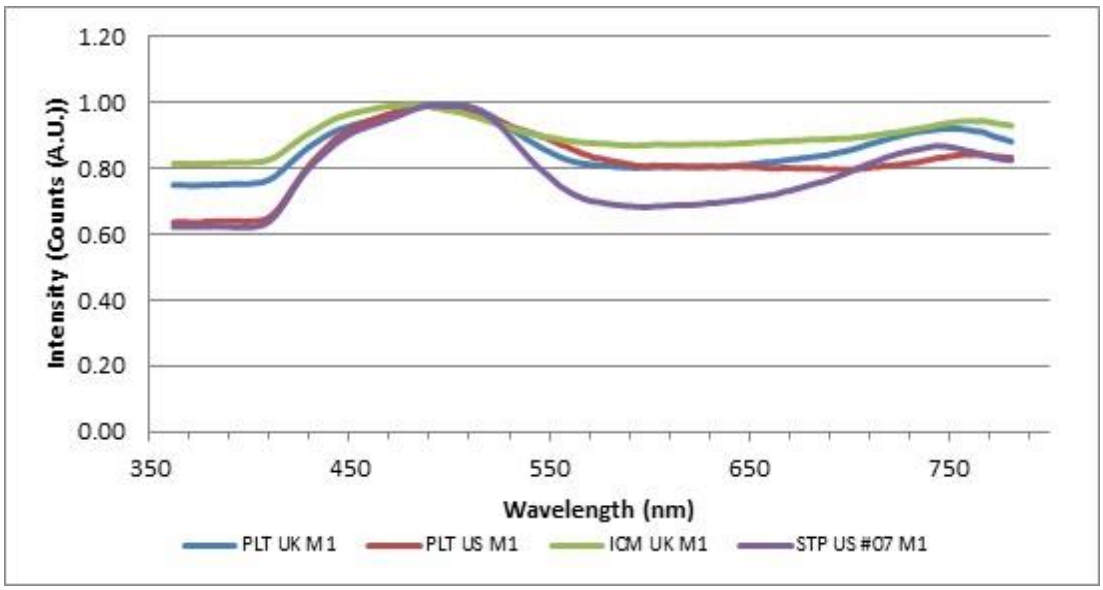


Figure 4.14: Example spectra representing four dye containing brand/model combinations (PLT UK; PLT US; ICM UK and STP US #07) of blue gel ink lines on paper demonstrating a similar spectral pattern, but with minor differences in peak position

An example of the first derivative spectrum for each of these four brand/model combinations is presented in Figure 4.15. There appeared to be little difference between the PLT UK and ICM UK samples and as such their spectra were considered indistinguishable, as highlighted in Figure 4.16. However, these two samples could be distinguished from the remaining two, PLT US and STP US #07 which were also distinguishable from each other, the latter point illustrated by Figure 4.17.

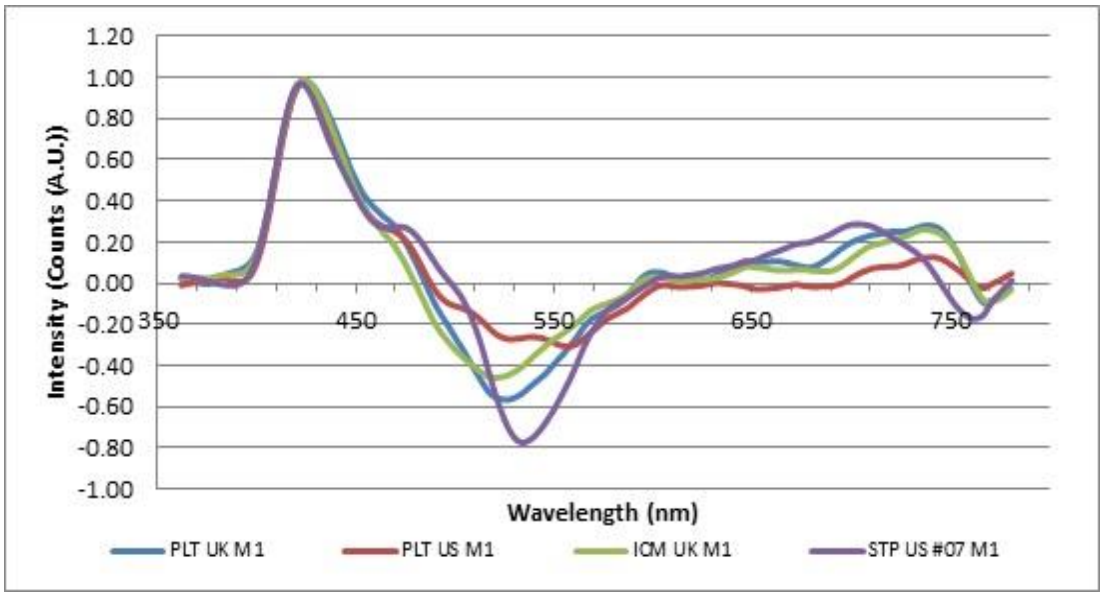


Figure 4.15: Example first derivative spectra representing four dye containing brand/model combinations (PLT UK; PLT US; ICM UK and STP US #07) of blue gel ink lines on paper highlighting subtle spectral differences permitting some discrimination

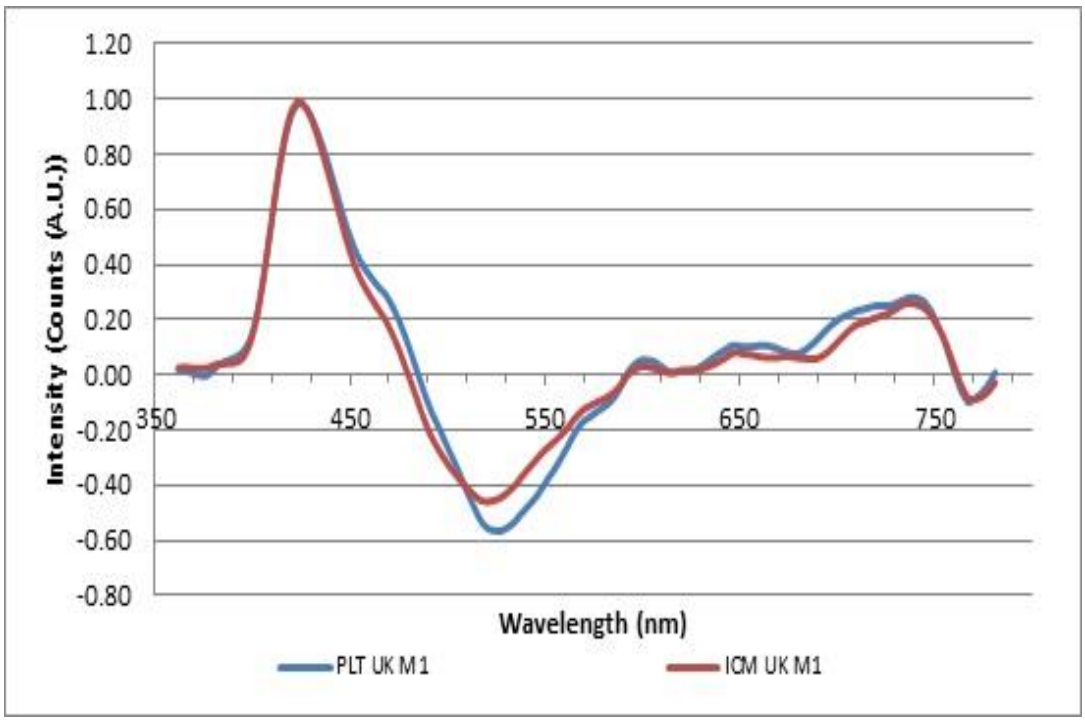


Figure 4.16: Comparison of indistinguishable first derivative spectra representing PLT UK and ICM UK blue gel ink on paper

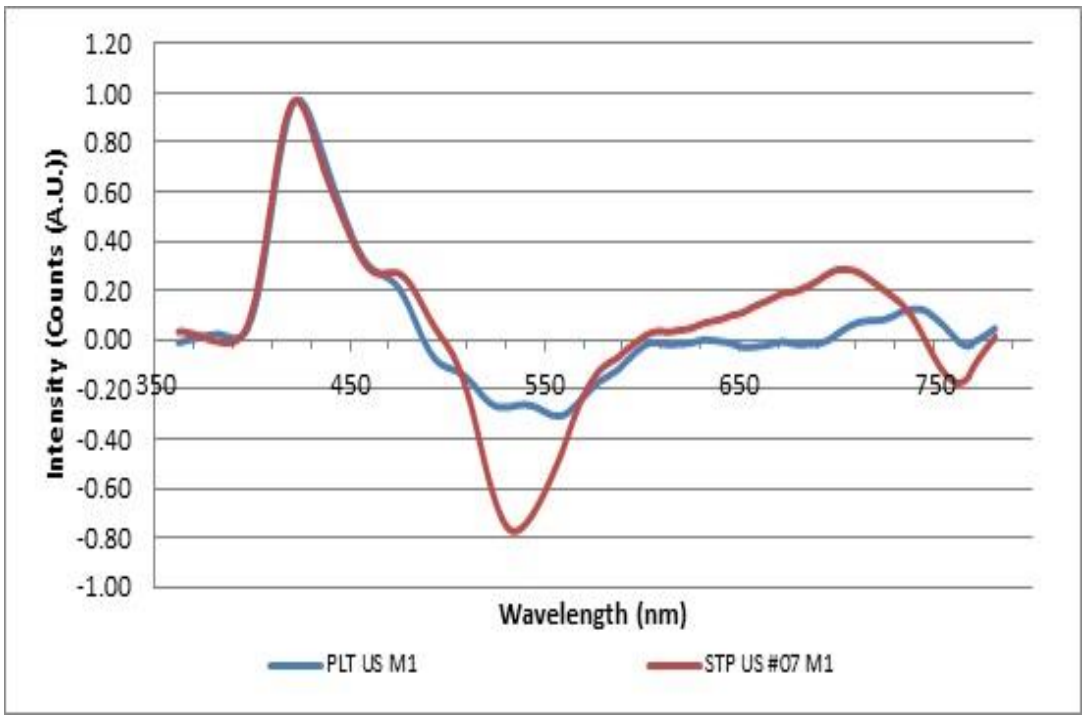


Figure 4.17: Comparison of distinguishable first derivative spectra representing PLT US and STP US #07 blue gel ink on paper

Interestingly, these four samples produced indistinguishable results under the TLC study and were classified as a single group using that technique. The VSC 6000/HS study also suggested that the PLT UK and PLT US samples were indistinguishable, and that the ICM UK

and STP US #07 samples were also indistinguishable, but that these two groups were distinguishable from each other. These observations contradict those of the Vis-MSP study creating confusion as to how much discrimination can be afforded between these samples.

In an attempt to clarify discrimination, the set of ten spectra taken from these four samples during the initial sample variation study were examined. For all four samples, spectral shape was similar and showed minimal variation. The characteristic large peak for the ICM UK and PLT UK samples yielded a λ_{max} between 475 – 510 nm, whilst for the PLT US and STP US #07 samples the λ_{max} occurred between 475 – 530 nm and 460 – 500 nm respectively. Although the λ_{max} for all four samples occurred in a similar region it was notable that the PLT US sample exhibited a wider range extending beyond that of the other three samples, whilst the STP US #07 sample exhibited a range extending below that of the other samples, as illustrated in Figure 4.18. It is not clear if this is simply down to a sampling issue or if perhaps it is an indication that these four samples contain a similar dye composition but due to subtle differences in the ink batch formulations can be discriminated by Vis-MSP. This uncertainty highlights the subjective nature of the interpretation of data from both the filtered light and Vis-MSP techniques and demonstrates the need for a more definitive and reliable method of discrimination between such samples.

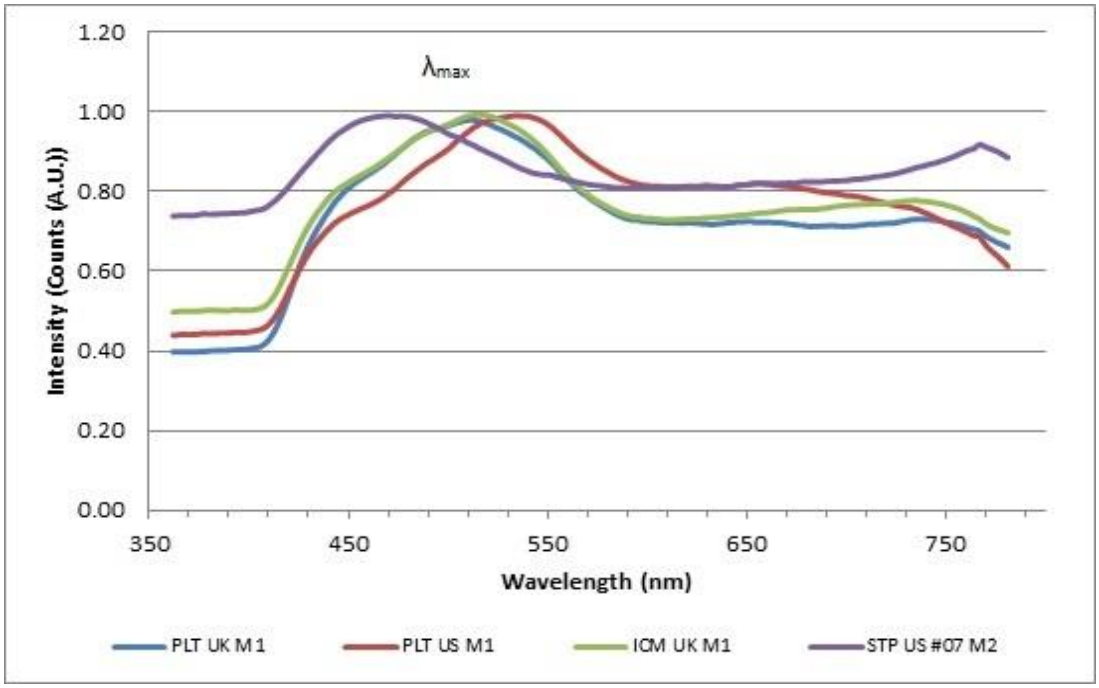


Figure 4.18: Example spectra representing four dye containing brand/model combinations (PLT UK; PLT US; ICM UK and STP US #07) of blue gel ink lines on paper demonstrating similar spectral shape, but different λ_{max} position

4.3.4.1.2.2 Pigmented Samples

For the pigment based group, which consisted of 21 different brand/model combinations, the majority (14) could be classified into a single group (Group 8, according to original spectra classification groupings summarised in Table 4.1) based on the repeat study data. This group, shown in Figure 4.19, exhibited a characteristic large peak with a λ_{max} occurring between 480 – 510 nm (Peak #1). A second much smaller peak was observed between 650 – 660 nm (Peak #2). The original sample variation study revealed that within a given sample, minimal variation in spectral shape was observed, except for variations in the intensity of the second peak. The λ_{max} position did vary within a sample set over a range of between 15 – 35 nm dependent upon brand/model, but all 14 samples exhibited a λ_{max} between 470 – 515 nm.

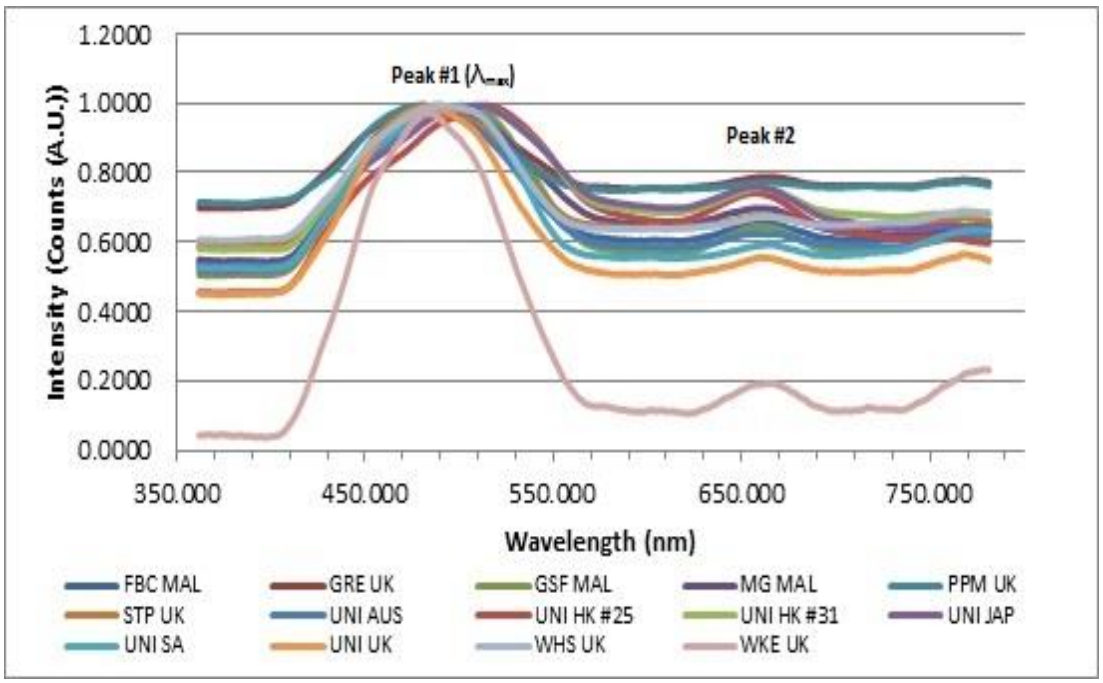


Figure 4.19: Example spectra representing each of 14 pigment based blue gel ink (Group 8) lines on paper

First derivative (FD) spectra, however, suggests that further discrimination within this group may be possible based upon λ_{min} and variations in spectral shape. Whilst spectral shape generally appears similar, as demonstrated in Figure 4.20, there appears to be three groups (FD Groups 10 – 12, according to first derivative spectral classification groupings summarised in Table 4.1) that can be distinguished based on an obvious difference in λ_{min} position.

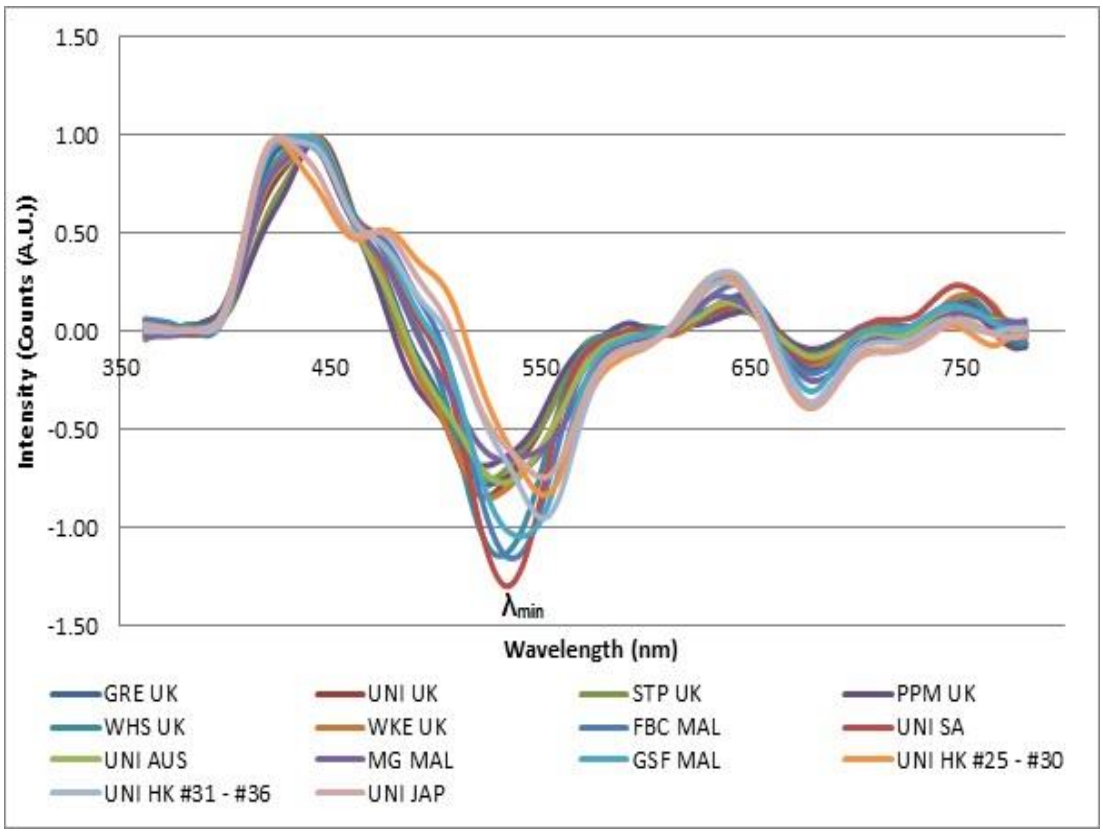


Figure 4.20: Example first derivative spectra representing each of 14 pigment based (Group 8) blue gel ink lines on paper highlighting subtle spectral differences in λ_{min} positions enabling further discrimination into three groups (FD Groups 10 – 12)

The first of these groups (FD Group 10) consists of seven samples (GRE UK, PPM UK, STP UK, WHS UK, WKE UK, UNI UK and UNI AUS), the second (FD Group 11) four samples (FBC MAL, GSF MAL, MG MAL and UNI SA), and the third (FD Group 12) three samples (UNI JAP, UNI HK #25 and UNI HK #31 (Asian UNI samples)). FD Group 10 exhibits a λ_{min} between 518 – 528 nm compared to 528 – 542 nm for FD Group 11, whilst FD Group 12 is distinct from both with a λ_{min} around 548 nm. Furthermore, FD Group 12 exhibits a slight change in spectral shape between 460 - 490 nm compared to the other two groups and is most notable in UNI JAP and UNI HK#25 sample spectra. Again these spectral differences are very subtle, and it is unclear if they are indications of a real detectable difference within these samples or simply a sampling issue. Given that the original spectral data exhibited a wide range in the λ_{max} positions within a sample, it could be argued that this variation between samples in λ_{min} in the first derivative is not indicative of a real difference. However, the difference in spectral shape observed in FD Group 12 could be. An example of the first derivative spectrum for each sample representing the three groups (FD Groups 10 - 12) is presented in Figure 4.21.

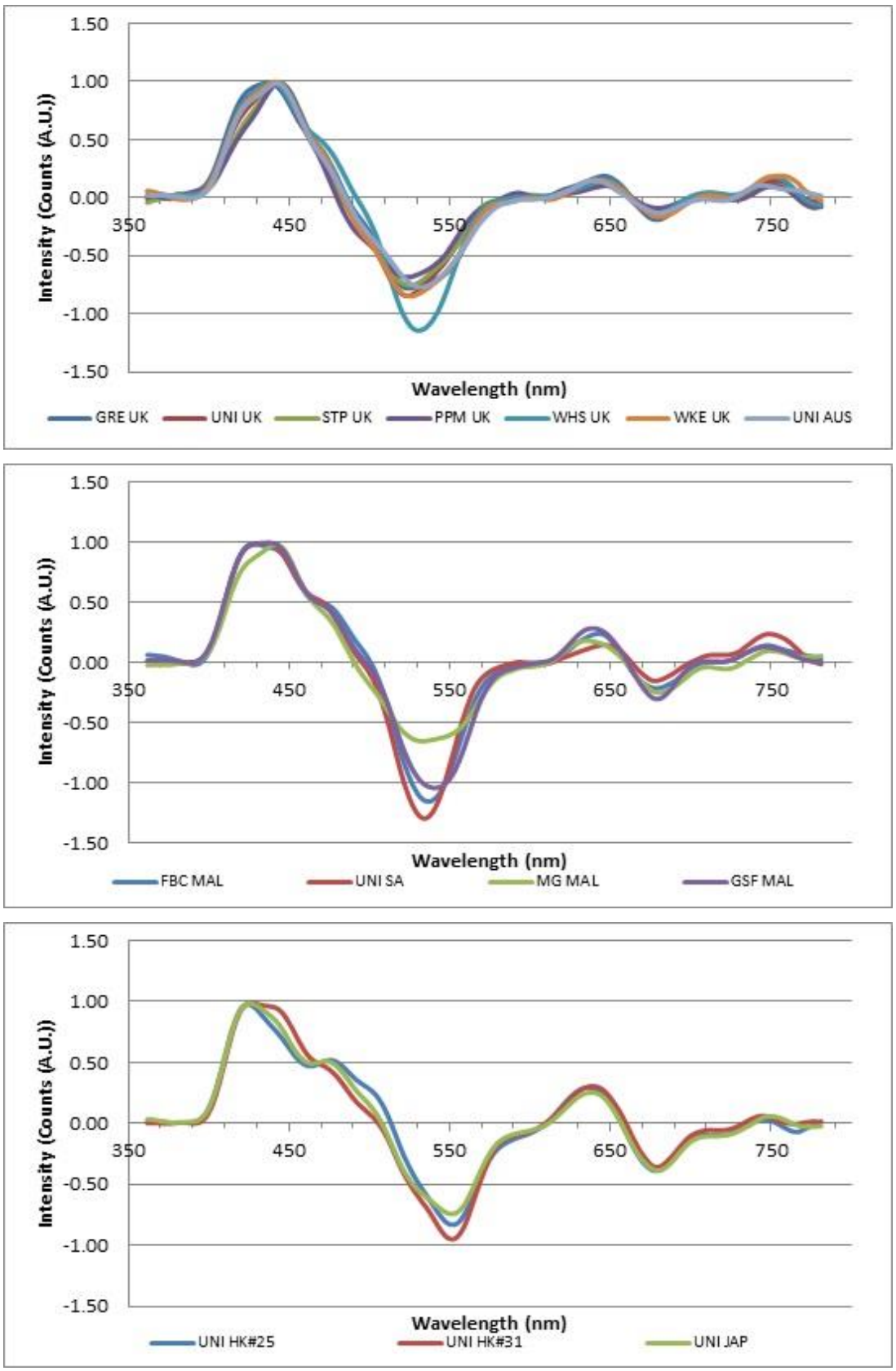
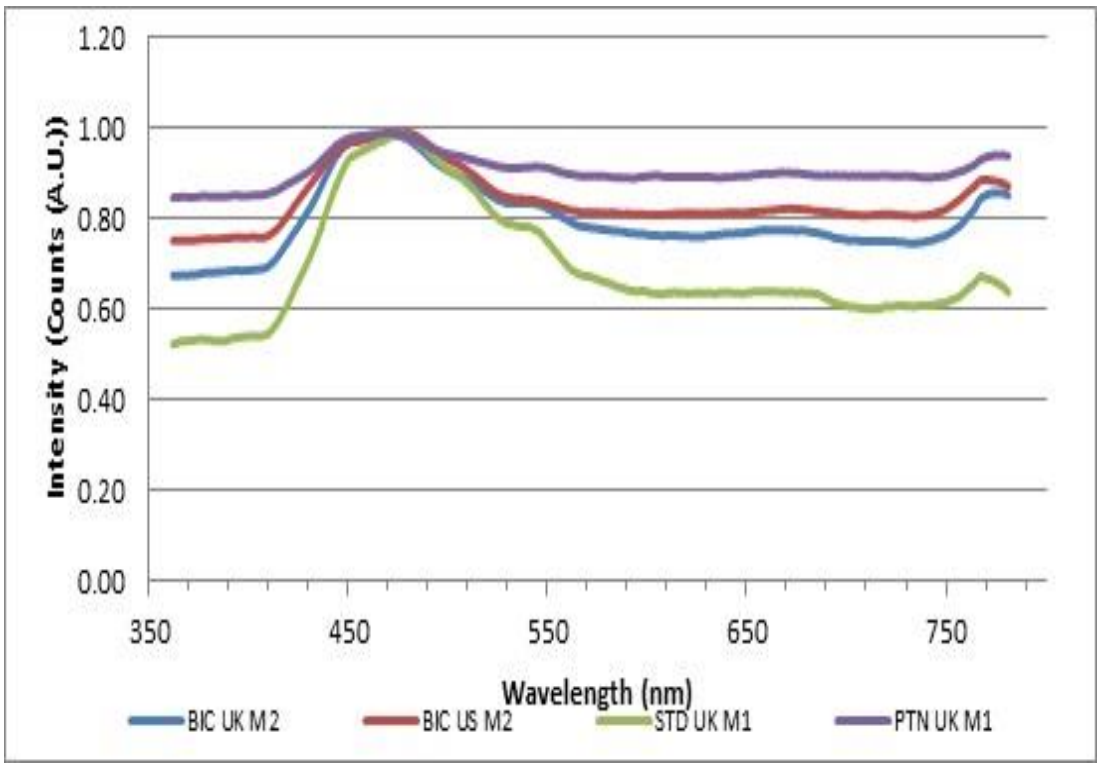


Figure 4.21: Example first derivative (FD) spectra representing 14 pigment based (Group 8) blue gel ink samples further classified into three groups: FD Group 10 (top), 11 and 12 (bottom)

The remaining seven samples could be broadly classified into two groups. The first of these consisted of four samples: BIC UK, BIC US, STD UK and PTL UK (Group 9, according to original spectral classification groupings summarised in Table 4.1). As discussed, three of these brand/model combinations exhibited wide sample variation out of ten spectral measurements that appeared to be related to where in the sample line the measurement was acquired from. The fourth sample, PTN UK, exhibited minimal sample variation in terms of spectral shape. This observation alone could be suggestive that the PTN UK sample can be discriminated from the other three samples. The first derivative spectra were examined to see if they could reveal any subtle differences to further discriminate between these samples. As expected the spectra were very similar, but there appeared to be a difference in the spectral pattern around ~490 – 500 nm where the BIC UK and PTN UK samples could be distinguished from the BIC US and STD UK samples on the basis of the former exhibiting a sharp and deep trough followed by a shoulder compared to a shallow trough followed by a second shallow trough. Apart from this difference, there was very little difference between the BIC US, BIC US and STD UK samples. The PTN UK spectrum did display other areas of difference, namely a peak at ~590 nm and ~645 nm as well as a trough at ~675 nm. An example of the original and corresponding first derivative spectrum for each of these four brand/model combinations is presented in Figure 4.22 to illustrate the points discussed.

It is worth noting that the VSC 6000/HS study indicated that both the STD UK and PTN UK samples could be discriminated from the BIC UK and BIC US samples, which were also indistinguishable to each other, under IR luminescence, most notably under the 380 – 800 nm optical filter. The combination of the VSC 6000/HS and Vis-MSP spectral observations would at least suggest that the PTN UK and STD UK samples can be distinguished from the BIC UK and BIC US samples, suggesting that these four samples can be further discriminated into three groups overall.



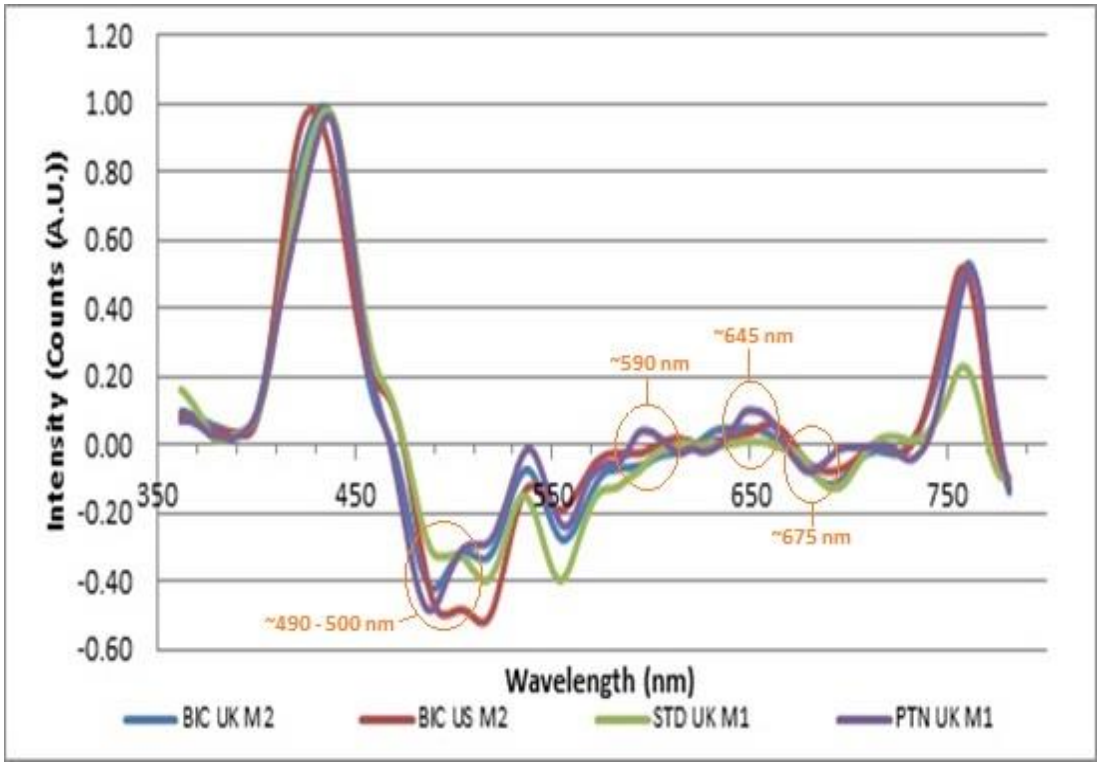
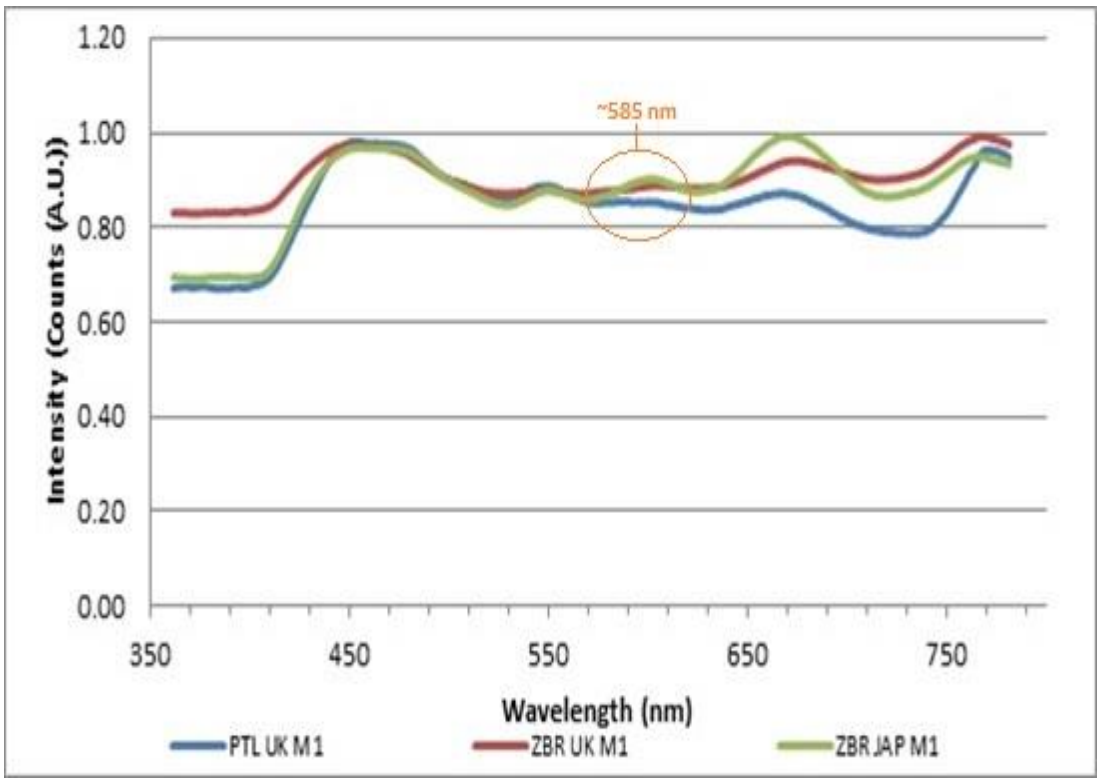


Figure 4.22: Example spectra (original (top) and corresponding first derivative (bottom)) representing four pigment based (Group 9) blue gel ink lines on paper. The first derivative (FD) provides further discrimination between the samples (FD Group 13 – 14), highlighted within the orange circles

The final three samples (ZBR UK, ZBR JAP, and PTL UK (Group 10, according to the original spectral classification groupings summarised in Table 4.1)) all exhibited similar spectral patterns, however, the PTL UK sample appeared to show a difference at ~585 nm where both the ZBR UK and ZBR JAP samples exhibit a peak, but the PTL UK sample remains flat before developing into a trough. This was a reproducible difference observed across the set of ten spectra from all three samples, although it was noted that the ZBR samples did exhibit variation in the intensity of this peak, but the peak was always visible. Examination of the first derivative spectra showed differences at ~510 nm and ~717 nm in the form of a shoulder in the PTL UK spectra not observed in that of the ZBR spectra. The findings of the VSC 6000/HS study support the view that PTL UK is distinguishable from both ZBR samples. Figure 4.23 shows an example of the original and corresponding first derivative spectrum for each of these three brand/model combinations to highlight the points raised.



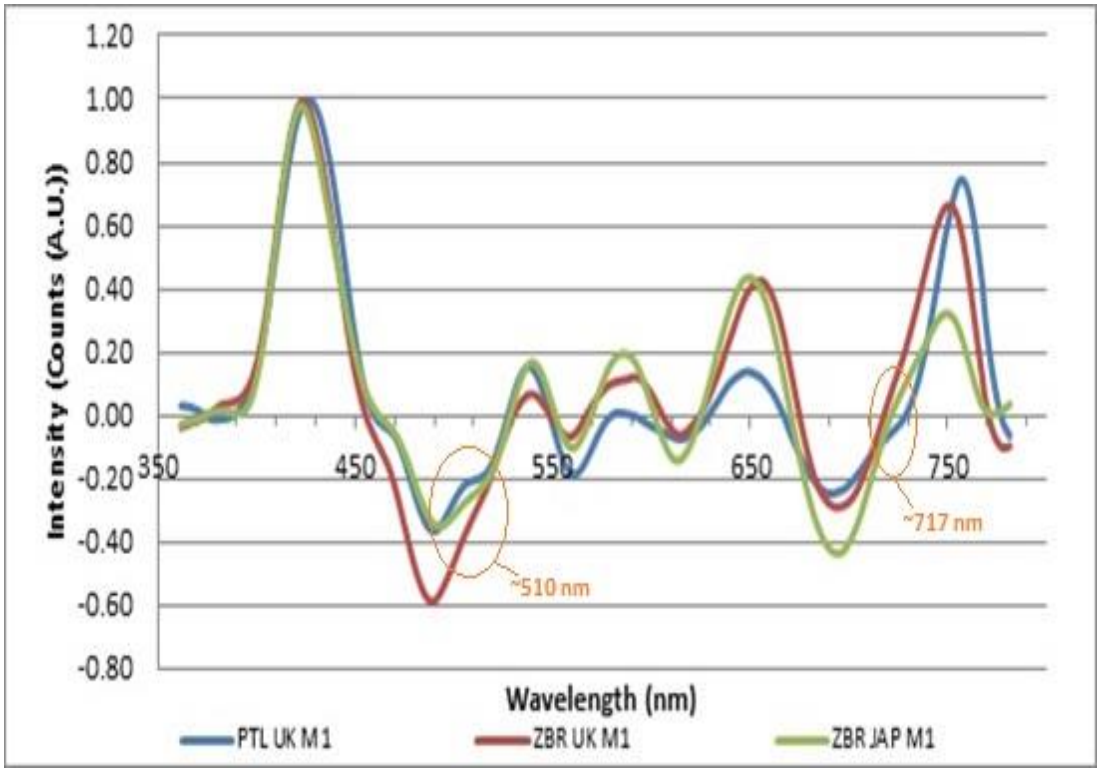


Figure 4.23: Example spectra (original (top) and corresponding first derivative (bottom)) of three pigment based (Group 10) blue gel ink lines on paper with areas of difference highlighted within orange circles. The first derivative (FD) provides further discrimination between the samples (FD Groups 15 – 16)

4.3.4.2 Red Ink Group

4.3.4.2.1 Reproducibility

Overall, good reproducibility was achieved for spectra within a brand/model combination of the red ink group. Minor variations in spectral shape were observed below 600 nm, and the λ_{max} varied within a narrow range of approximately 20 nm for some samples. Examples of these observations are given in Figure 4.24.

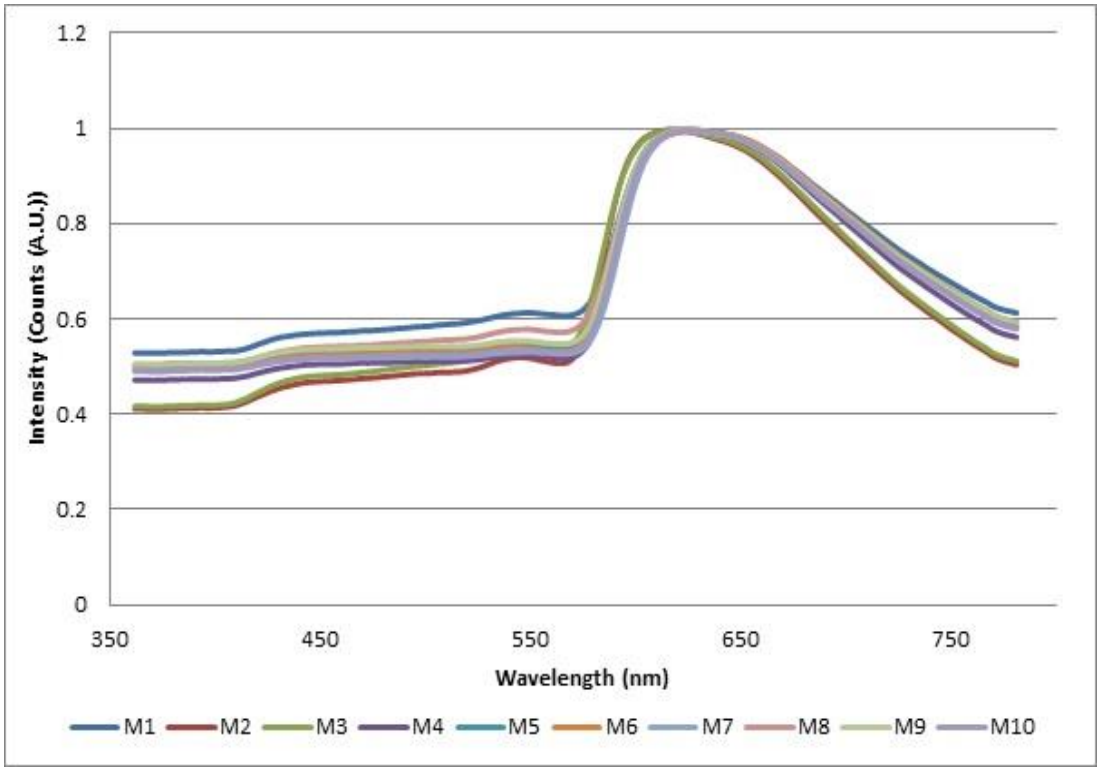


Figure 4.24: Example of a set of reproducibility measurements (n = 10) from the MG MAL red brand/model combination illustrating minor differences in spectral shape below 600 nm and a slight shift in λ_{max} position

4.3.4.2.2 Between Sample Variability

The spectra produced by the red ink samples were very similar and were characterised by a large broad peak occurring between ~580 nm – 780 nm, with a λ_{max} between 600 nm – 650 nm as illustrated in Figure 4.25. Below 580 nm, spectra were relatively flat with some small peaks observed in some samples. Considering the sample variation within this region and the characteristic presence of a single large peak, spectra from all 26 samples analysed were considered so similar, as to be of little value for discrimination of the red gel inks.

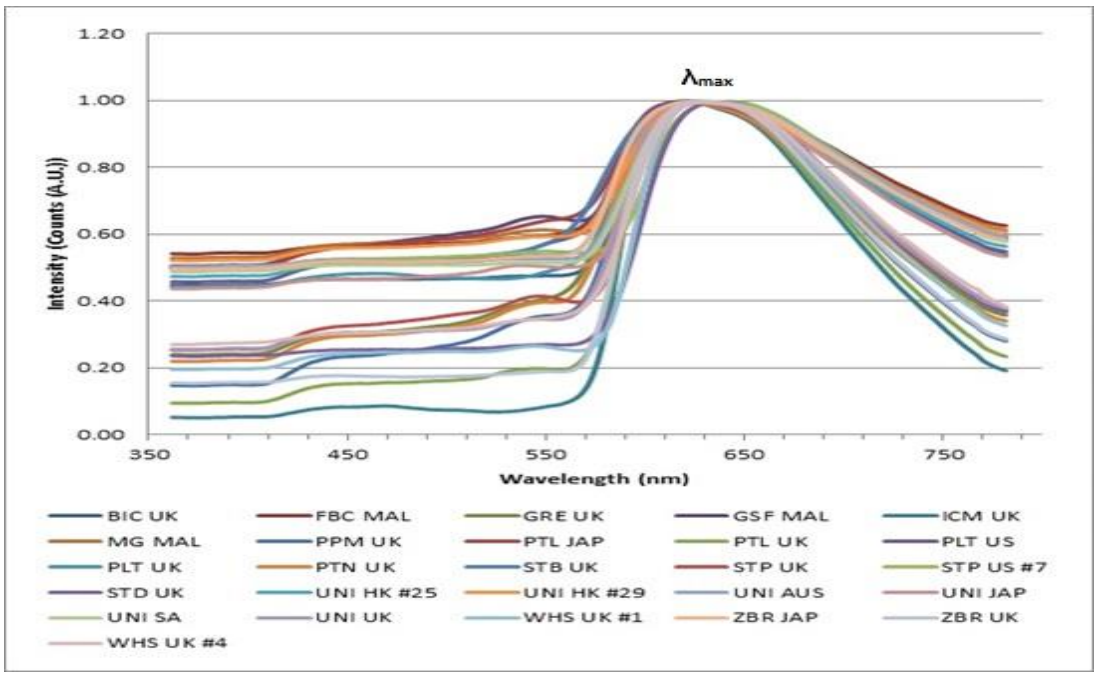


Figure 4.25: Example spectra representing each of 25 samples (representing 26 brand/model combinations) of red gel ink lines on paper providing little to no discriminatory value

4.4 Conclusion

Only one brand/model combination, the WHS UK red group ink, exhibited evidence of possible within brand variation in the form of a subtle spectral difference between pens #01 - #03 and pens #04 - #06. This observation was supported by the findings of the VSC 6000/HS within brand variation study, but in isolation was not considered sufficiently reliable for discrimination of the within brand samples.

In terms of between brand variation due to the lack of spectral features exhibited in the red ink group, little to no discrimination of original spectra was considered possible within this colour group. However, a wide selection of spectral patterns was observed for the blue ink group samples, thus permitting a high degree of discrimination. Reproducibility within a brand/model combination set varied, but was generally considered good or excellent. However, four brand/model combinations in particular exhibited a comparatively large amount of spectral variation, which appeared to be linked to the location along the ink line at which a spectrum was acquired. Therefore, sample location and the number of spectra acquired clearly needs to be carefully considered when acquiring spectra representative of an ink sample. Spectral interference from the paper substrate was not considered to be a problem for qualitative comparison and no instrumental variation was observed.

The solubility and TLC study enabled the 31 blue ink group brand/model combinations to be categorised into dye containing and pigment based samples. The Vis-MSP study confirmed that the dye containing samples could be discriminated from the pigment based samples. Additionally, further discrimination within these two categories was possible from examination and comparison of the original spectral data. The ten dye containing samples could be discriminated into a further seven groups that corresponded with the TLC classifications. The remaining 21 pigment based samples could be discriminated into a further three groups. The blue ink group samples could therefore be discriminated into at least ten groups based on the original spectra only. Combining comparison of the original spectra together with the corresponding first derivative provided support for the view that further discrimination within all three pigment based groups and one dye containing group was possible, permitting maximum discrimination into 16 groups for the blue ink group samples. Discrimination was based on features such as overall spectral shape, λ_{max} and λ_{min} positions and peak intensities. Comparing highly similar spectra from two or more brand/model combinations on the same axis was found to be beneficial in aiding discrimination between samples exhibiting similar spectral patterns.

The four samples in the dye containing group arguably could be discriminated into three further groups consisting of PLT UK/ICM UK, PLT US, and STP US #07 based on spectral differences. Whilst the TLC study confirmed that these four samples were indistinguishable to each other, but distinguishable to all other dye containing samples, the VSC 6000/HS study suggested that discrimination into a further two groups was possible. However, these groups suggested that the PLT UK/PLT US and ICM UK/STP US #07 samples were indistinguishable. It is not possible based on the VSC 6000/HS and Vis-MSP data alone to say with certainty if these different classification groups are evidence that these samples can be discriminated on the basis of very subtle chemical differences in the gel ink formulations, or whether they are as a result of the highly subjective nature of these two techniques. Either way it is clear that there is a need for a more objective method by which to discriminate samples either by filtered light or Vis-MSP. Given the numerical based nature of the latter technique, a multivariate statistical approach to discrimination may provide that objective determination.

Chapter Five

Fourier Transform Infrared – Attenuated Total Reflectance Spectroscopy

5.1 Introduction

Several researchers have investigated Infrared (IR) Spectroscopy, in its different forms, to the forensic analysis of (writing) inks. This has involved mostly ballpoint inks, but more recently gel inks have received some attention.

Trzcinska 1990 [116] suggested that IR Spectroscopy could be useful for the discrimination of ballpoint inks with similar formulations. Spectra were acquired from extracts and pure ink samples on KBr pellets from several batches of ball pen paste from the same manufacturer. Differences in the presence and intensities of absorption bands attributable to the solvent base were noted in spectra from the extracts compared to that of the corresponding pure sample.

After unsuccessful attempts by Harris (in [115]) to analyse inks by Diamond Cell Transmission and Micro-reflectance Spectroscopy, Merrill and Bartick [115] explored the use of reflectance and diffuse reflectance FTIR microscopy for the analysis of ink on paper. This too was considered unsuccessful due to strong spectral interference from the paper substrate masking any peaks that may have arisen from the ink itself. Furthermore in line with Trzcinska, [116] spectral differences between the ink on paper and the corresponding pure ink were noted by Harris (in [115]) lending further support to the view that identification of written ink by comparison to pure ink spectra could not be relied upon. However, Merrill and Bartick [115] did have success analysing a large number of extracted ballpoint inks on KBr windows by Diffuse Reflectance IR Micro-Spectrometry (DRIFTS) and investigated the usefulness of spectral libraries generated from different sample forms of the same ink for identification purposes, e.g. pure ink, extracted ink, etc.

Good spectral reproducibility with only minor variations was observed within and between ink extracts, but wide variability in paper spectra was observed due to its heterogeneous nature suggesting background measurements needed to be taken close to the ink sample site to minimise interference from background spectral differences. Most importantly, large spectral variation between different brands of ink was observed, suggesting potential discrimination. An example of 18 black BIC inks were grouped into three classes based on formulations corresponding to different geographical regions. The identification of certain resins in a number of samples on the basis of the presence of certain absorption bands as well as spectral changes arising from heating of the ink extracts was noted. Successful identification of 29 extracted ballpoint inks was achieved by performing spectral subtraction of the paper background from the ink spectrum and searching the wavenumber region below 2000 cm^{-1} against a spectral library of the extracted ink samples. Most samples matched within the first five suggested identifications with 86% matching the first hit, whilst all samples were identified within the first ten suggestions, highlighting the importance of examiner inspection to ensure correct identification.

A classification method was described by Wang *et al* [119] for the analysis of over 100 blue ballpoint inks analysed directly on KBr plates using FTIR Micro-Spectroscopy. Based on the presence or absence of strong carbonyl absorptions at around 2000 - 1700 cm^{-1}, the inks were initially classified into two groups. Pattern recognition analysis was used to analyse absorption band position and intensities, with further classification into 35 sub-groups achieved using the Correlation Co-efficient. Group 1 was further discriminated into six sub-groups whilst Group 2 was split into 29 subgroups on this basis. Wang *et al* [119] emphasised the importance of acquiring sufficient spectra from a given sample to achieve the best Correlation Co-efficient, as well as setting the correct weighting value. It was suggested that this classification method should be tested using ink samples on paper. The authors also attempted spectral interpretation by attributing a number of absorption bands to certain chemical components within the inks analysed.

Wilkinson *et al* [120] further suggested the use of Synchotron Radiation Based IR Spectromicroscopy for the successful in-situ analysis of ink on paper, something which to date had proved to be quite problematic. Ink on paper strips were analysed and the resulting spectra compared with that of the corresponding pure ink analysed on a silver

coated microscope slide. The complexities of band interpretation for writing inks were discussed and the fingerprint region (~1700 – 800 cm^{-1}) was suggested as the region where many of the ink components would exhibit absorption bands, and perhaps overlap. The authors suggested the superior sensitivity and resolution of this form of IR Spectroscopy over traditional IR Spectroscopy maybe better suited to ink analysis. Wilkinson *et al* [120] also supported the findings of Merrill and Bartick [115] that the heterogeneous nature of paper may result in spectral variation across a single sheet as well as within and between sheets of different batches, and this should therefore be considered before performing spectral subtraction of the paper background. In addition, they suggested the ratio of ink to paper within a measurement area will vary and therefore measurements should be taken along several locations of the ink line to account for this.

Kher *et al* [83] analysed eight blue ballpoint pens by HPLC and IR Spectroscopy investigating Chemometric analysis as a means by which to discriminate between different formulations. The authors initially investigated DRIFTS and Grazing Angle NIR sampling methods to obtain useable spectra from ink on laser print paper, but without success. However, the use of an ATR microscope accessory did yield a strong IR signal in the 4000 – 650 cm^{-1} wavenumber region (Mid-IR region) from the ink in-situ. Kher *et al* commented that spectral variability was mostly concentrated in the fingerprint region (1800 – 650 cm^{-1}), whilst spectral variability between 4000 – 2000 cm^{-1} could be attributed to atmospheric water vapour and carbon dioxide (CO_2), and therefore for comparison and discrimination the region between 2000 – 650 cm^{-1} should be used. In particular, attention was drawn to the region between 800 – 650 cm^{-1} which the authors proposed represented aromatic ring vibrations attributable to dye components within the inks. Furthermore, absorption bands between 1580 – 1500 cm^{-1} could be attributable to carboxylate acid salts and polymeric non-dye components, i.e. resinous materials used as lubricants, viscosity adjusters, corrosion inhibitors and wetting agents, whilst polyamides could account for variations between 1680 – 1670 cm^{-1}. Practical difficulties encountered using FTIR-ATR included acquiring spectra from a single ink line and strong spectral interference from the paper substrate which could not be removed by spectral subtraction. To overcome this and acquire spectra suitable for discrimination, the paper sample sheet had to be completely covered with ink to ensure total contact with the ATR sampling crystal. Analysis by HPLC did not suffer from these difficulties, and better discrimination was achieved using the HPLC data. However,

reasonable discrimination and classification of the FTIR-ATR spectra was achieved, and Kher *et al* [83] suggested that further development of an IR spectroscopic method for the analysis of ink on paper coupled with Chemometric analysis could provide a powerful forensic tool for the discrimination and classification of writing inks.

Jones and Wolstenholme [196] used three non-destructive spectroscopic techniques, including FTIR-ATR for the analysis and discrimination of blue and black ballpoint and gel pen inks. Strong interference from paper background was observed in spectra of ink lines which could not be removed easily by spectral subtraction, concurring with the view of Kher *et al* [83]. However, the fingerprint region between 1600 – 650 cm^{-1} exhibited relatively large absorption bands attributable to the ink that could be used for discrimination on the basis of peak position and relative intensities.

Fuestal and Briggs [145] used a Gladi-ATR vision accessory coupled to an FTIR Spectrometer to analyse three black ballpoint pen ink lines on paper. Viewing capabilities via the integrated x 110 mag video camera positioned under the diamond ATR sampling interface ensured ink was in contact with the crystal. Good reproducibility was observed in triplicate measurements from different areas of both the un-inked and inked paper. Expected strong interference from the paper was observed, but the 1400 - 800 cm^{-1} fingerprint region did appear to display relatively small absorption peaks from the ink, i.e. 695 cm^{-1}, 1366 cm^{-1}, 1584 cm^{-1} (carboxylate acid salts and polymeric non-dye constituents, e.g. resin, as described by Kher *et al*) and 1725 cm^{-1}. Discrimination between the three inks based on the presence/absence of these absorptions, albeit with weak intensity, was possible. Averaged spectra with no additional spectral manipulation were used for comparison, but the authors suggested that a statistical application such as discriminate analysis could extract spectral differences to further enhance discriminating capabilities of the technique.

Zieba-Palus and Kunicki [197] analysed a combination of 70 blue and black ballpoint and gel ink pens using a range of spectrometric techniques including micro-FTIR. In contrast to other studies [115, 116, 118], no qualitative difference between ink extracted from paper and the corresponding pure ink was observed, and it was suggested that strong peak absorbencies could all be attributed to dye components, whilst components such as pigments, binders and other additives could be attributed to peak absorbencies with

weaker intensities. On the basis of peak position and relative intensities the authors were able to discriminate the inks into several groups using the IR spectra alone. However, Causin *et al* [114], in their study of 33 extracts from blue and black ballpoint pens using DRIFTS, used mainly peak presence or absence since relative peak intensities could be affected by the age of the ink sample as demonstrated by Humecki and Becker *et al* described in Brunelle and Cantu [198]. Relative peak intensities were only used as a feature of discrimination in this study where the differences between adjacent peaks were large. A recent study by Dirwono *et al* [199] using FTIR-ATR again used both peak position and relative intensities to discriminate between different brands of red seal ink with some success.

While the literature reflects a growing body of work investigating the potential of FTIR-ATR to discriminate inks in general, only a few studies have focussed on gel pen inks [196, 197]. The exploration of the effectiveness of FTIR-ATR for the discrimination of gel inks within each of the two colour groups in terms of both within and between brand variability is addressed in this Chapter. Sampling requirements have been explored together with the use of the first derivative, used previously in the analysis of printing inks and photocopier toners [200], as an aid to spectral interpretation. Peak positions attributable to the ink are identified and listed by colour group. Spectral data was acquired with a view to further evaluation using a multivariate statistical approach outlined in **Chapter 7**.

5.2 Experimental

5.2.1 Sample Preparation

5.2.1.1 Within Brand Variation

Three brand/model combinations per colour group, identified below, were analysed. The brand/model combinations were chosen at random except for two, highlighted in bold, which were found to exhibit evidence of within brand variation using Raman Spectroscopy.

- ICM UK, PKR UK and **ZBR UK** (blue ink group);
- ICM UK, UNI UK and **WHS UK** (red ink group).

Ink spots (~1 cm diameter) were drawn directly onto squares (~6 x 5 cm) of white office paper (80 gsm) cut from a single A4 sheet. A set of six spectra from different areas within each ink spot to monitor for sample variation, were acquired under the instrumental conditions described below. Spectra were also acquired from an un-inked area of the paper to monitor for spectral interference from the substrate.

All samples were allowed to air dry for at least 25 minutes prior to analysis and analysed within a seven day period. Samples were stored at room temperature in a laboratory notebook overnight.

5.2.1.2 Between Brand Variation

Ink spots (~1 cm diameter) were drawn directly onto squares (~6 x 5 cm) of white office paper cut from a single A4 sheet. All inks were examined and the UK acquired samples of the same colour group were drawn onto one sample sheet whilst the international (INTL) acquired samples were drawn onto another.

Six spectra were acquired from different areas within each ink spot to monitor for any sample variation. In addition, ten spectral measurements were taken from different un-inked areas of each sample sheet to monitor for spectral interference from the paper substrate.

All samples were allowed to air dry at room temperature for at least 25 minutes prior to analysis. All samples were analysed on the day of preparation.

5.2.2 Instrumentation

All spectra were acquired using an A_2 Technologies ML Bench-top FTIR Spectrometer which utilises an incorporated diamond ATR sampling interface and a high pressure clamp [146]. The diamond ATR sampling interface was wiped clean with an ethanol soaked tissue before and after each sample was analysed. A reference spectrum of air was taken at the start of each day prior to any sample measurements being taken to ensure that the instrumentation was working correctly. Spectral measurements were taken over a spectral range of 4000 cm^{-1} – 650 cm^{-1} with a resolution of 4 cm^{-1}.

All spectra were recorded using Panorama software (v3.0.24.0 Lab Cognition Analytical Software GmbH & CoKG). For a given set of measurements, spectra were normalised to ease comparison for an assessment of reproducibility. For classification purposes an average spectrum for each brand/model combination was generated together with its corresponding first derivative spectrum. Unless otherwise stated, all original spectra were smoothed to 11 points using the Savitsky-Golay algorithm [130] to improve the signal to noise (S:N) ratio. Spectral data were processed using MS Excel 2003 (v 11.8169.8192.SP3 part of MS Office Professional Edition) and MS Excel 2010 (v14.0.6112.5000 (32-bit) part of MS Office Home and Student 2010) in preparation for statistical pre-processing.

5.2.2.1 Selection of Instrumental Parameters

Studies were undertaken to investigate the best instrumental parameters in terms of peak smoothing, number of scans, and background subtraction. The instrumental parameters selected were validated for a selection of inks under test by recording six repetitive spectra. The % Relative Standard Deviation (% RSD) of the peak intensities at various wavenumbers were calculated as a measure of repeatability and reproducibility.

5.2.3 Sampling Method Development

Preliminary studies were conducted in order to determine the answer to the following questions. Five brand/model combinations from each colour group were randomly selected and spectra recorded as described previously.

a) Was it possible to acquire a spectrum from a forensic sized sample, i.e. single ink line, using this particular instrument?
b) Would the spectrum from an ink line compare well to that from an ink spot, and could both be differentiated from that of un-inked paper?
c) Was discrimination between brand/model within a colour group on the basis of peak position and relative intensities possible?

5.3 Results and Discussion

5.3.1 Selection of Instrumental Parameters:

5.3.1.1 Spectral Smoothing

Overall, spectral quality was considered noisy and spectral lines were quite "ragged" in places which was considered a potential flaw when comparing and discriminating spectra from a large number of samples. The Panorama software permits the smoothing of spectra to remove unwanted noise giving a clearer spectrum for comparison. Spectra can be smoothed using the Savitsky-Golay algorithm [130] from a selection of nine pre-determined numerical points, i.e. 5, 7, 9, 11, 13, 15, 17, 19 or 21 points. However, smoothing runs the risk of losing spectral information and should be used with caution since it needs to be effectively balanced against spectral quality and spectral information. Since band interpretation was not a primary aim in this work, but comparison and discrimination was, it was decided the smoothing algorithm should be applied.

A spectrum from the ZBR UK red ink sample was chosen for an assessment of the smoothing function since it appeared to contain a large number of peaks believed attributable to the ink. A spectrum of the ink was smoothed to each of the nine smoothing points available, and all spectra were compared by eye to determine the effect on noise reduction and spectral detail. It was concluded that smoothing to 11 points appeared to give the best balance between improving spectral quality and losing spectral detail. Spectral lines were smooth and the spectrum appeared clearer for comparison. It was noted that some spectral detail had been changed, namely in the form of split peaks being resolved and an overall reduction in peak intensity making some smaller peaks in the unsmoothed spectrum into shoulders in the smoothed spectrum. However, no peaks were considered to have been lost since shoulders were to be considered as a discriminating feature. Below 11 points, some noise was still observed, and beyond 11 points, the spectral pattern began to change significantly with the loss of some peaks. For the within and between brand variation studies, the smoothing algorithm was applied to 11 points and therefore the results should be considered in light of that spectral manipulation. The spectra are presented in Figure 5.1.

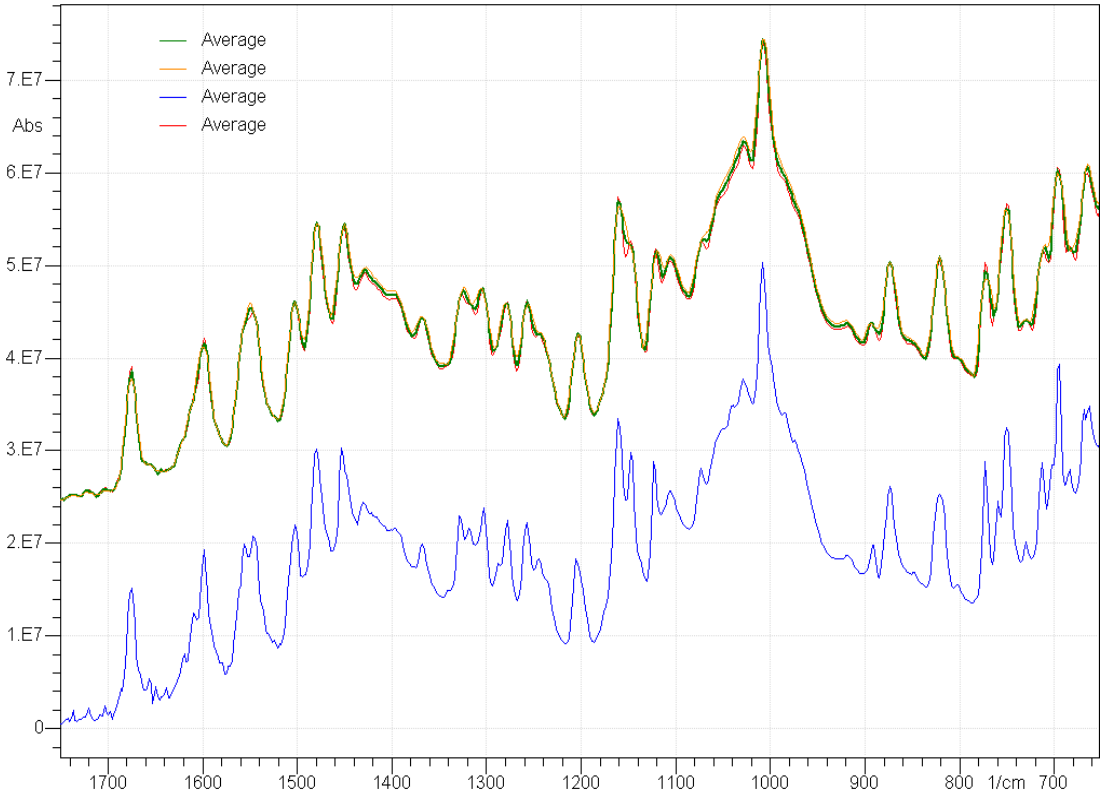


Figure 5.1: Example of the ZBR UK red gel ink normalised (and offset) averaged spectra with and without a smoothing algorithm applied. Unsmoothed spectrum (blue); 9 pts smoothed spectrum (red); 11 pts smoothed spectrum (green); and 13 pts smoothed spectrum (orange)

5.3.1.2 Increasing Spectral Scans

Increasing the number of scans taken to generate a spectrum can also have an effect on improving S:N ratio by increasing the intensity of peak absorbencies, but this also increases acquisition time, which could be an issue when analysing a large number of samples. A comparison of spectra from a sample of STD UK red ink, presented in Figure 5.2, which also exhibited a number of peaks believed attributable to the ink, acquired using 16, 120 and 240 scans was conducted to see if this would also improve spectral quality. Whilst the overall spectral intensity increased with increasing number of scans, no significant difference in spectral pattern was observed. Therefore, the default option of 16 scans remained in place throughout the acquisition of all subsequent spectra.

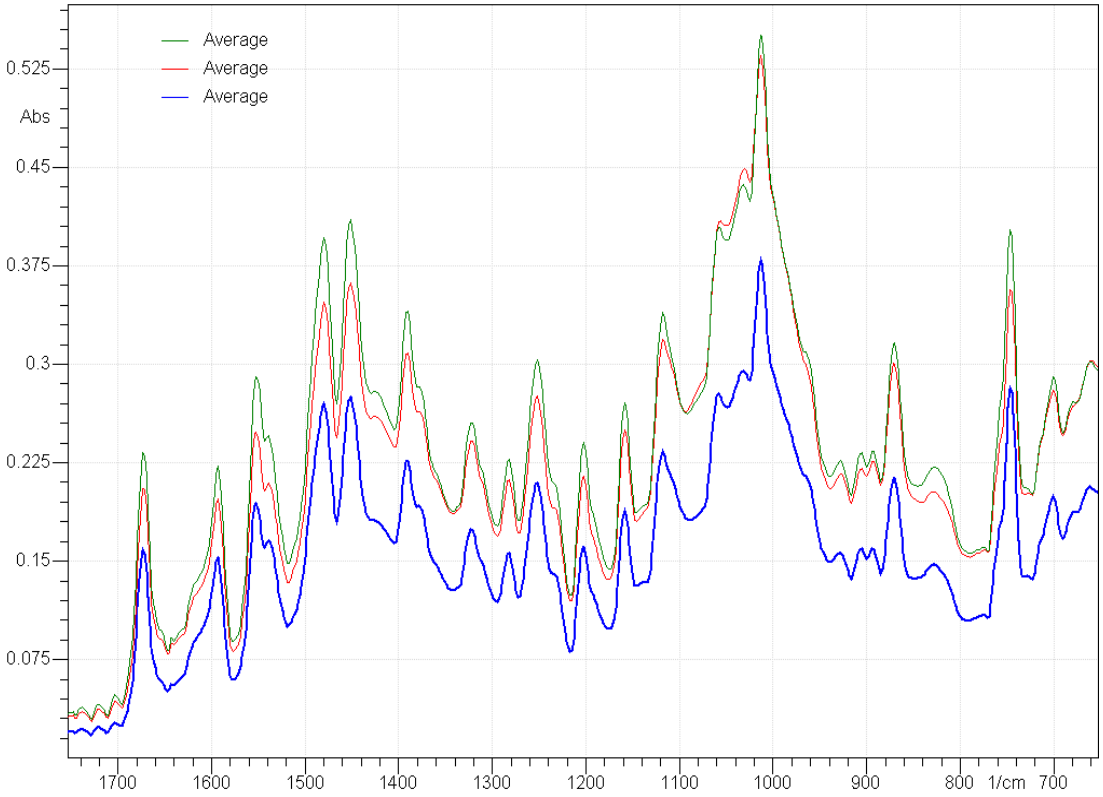


Figure 5.2: Example of the STD UK red gel ink averaged smoothed (11 pts) spectra acquired using 16 (blue), 120 (red), and 240 (green) scans illustrating an increase in spectral intensity in line with increasing scans, but no change in spectral pattern

5.3.1.3 Influence of Paper and Background Subtraction

As expected, significant spectral contribution from the paper was observed in the spectra of an ink on paper. Mansfield *et al* [201] attributed peaks between 1200 – 1000 cm^{-1} to the cellulose and hemi-cellulose components of paper. The large absorption around 1050 – 1045 cm^{-1} was attributed to hemi-cellulose, whilst peaks at 1106 cm^{-1} and 1170 – 1160 cm^{-1} could be attributed to the anti-symmetric ring stretch and bridge stretching of C-O-C groups in cellulose and hemicellulose respectively. Bands at 811 cm^{-1}, 895 cm^{-1}, 1250 cm^{-1} and 1460 cm^{-1} could also be attributed to hemi-cellulose, whilst bands around 1317 – 1315 cm^{-1} were assigned to CH_2 wagging vibrations in both cellulose and hemi-cellulose.

The use of spectral subtraction to reduce the influence from the paper substrate has been advocated by some [115, 118], but discouraged by others [83, 120, 196]. To investigate if it would be of benefit in this study, an averaged spectrum of the paper was subtracted from the averaged spectrum of a ZBR UK red ink on paper spectrum. The resulting spectrum (presented in Figure 5.3) was compared against the averaged spectrum of the ink on paper.

Spectral subtraction removes the intensity of the background [202], but did not appear to remove peaks attributable to the paper substrate. As a result, little difference in spectral pattern was observed between the corrected and uncorrected averaged spectra. Therefore, spectral subtraction was considered to be of little benefit in this work.

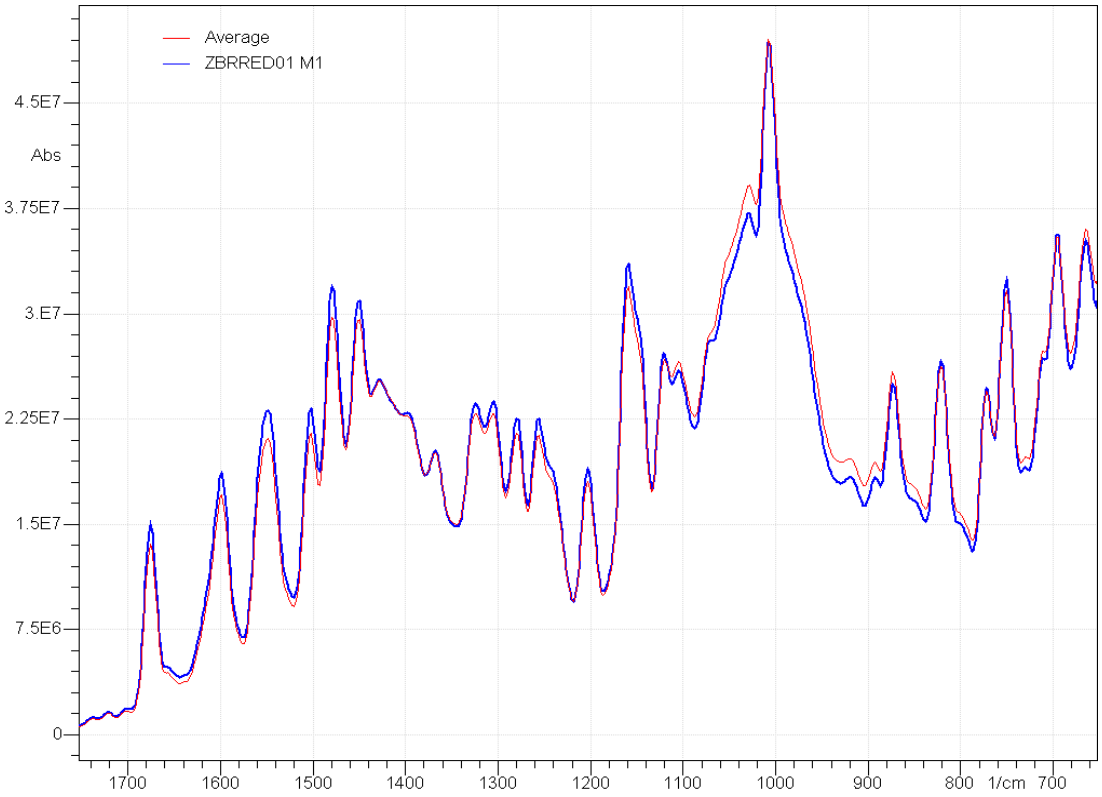


Figure 5.3: Comparison of an averaged spectrum (red) and background subtracted averaged spectrum (blue) of ZBR UK red gel ink (normalised and smoothed (11 pts)) demonstrating no appreciable difference in spectral pattern (M = Measurement)

5.3.2 Validation

The repeatability and reproducibility of spectral measurements were assessed for each colour group using a single brand/model combination from each. These were the GRE UK (blue ink group) and the ZBR UK (red ink group) samples. The wavenumber positions for a series of notable peaks (between 14 – 25 peaks dependent on colour group) present in the spectra representing each colour group were recorded and % RSD calculated. A set of six spectra taken from the same area within a sample were used to confirm repeatability (instrumental variation), whilst a set of six spectra taken from different areas within a sample were used to confirm reproducibility (sample variation) of the method.

The % RSD of repeatability for both colour groups was no greater than 0.4% whilst the % RSD for reproducibility was no greater than 0.2% indicating that the method exhibited excellent repeatability and reproducibility demonstrated in Figures 5.4 and 5.5 respectively, and Tables 5.1 and 5.2.

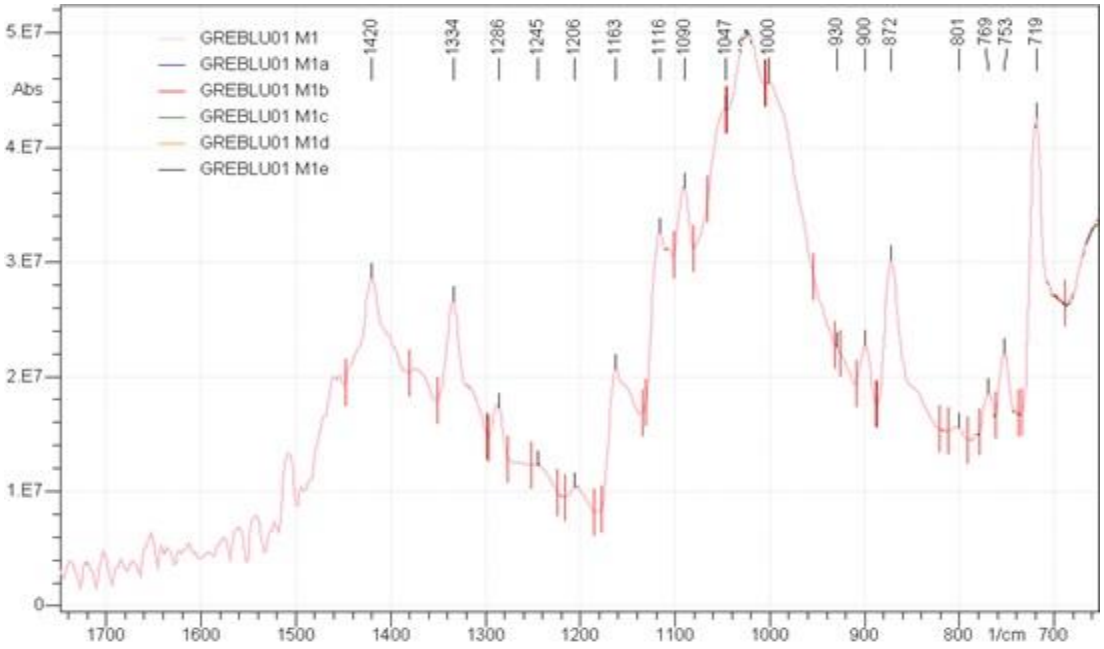


Figure 5.4: A set of spectra (n = 6) from the GRE UK blue gel ink on paper demonstrating excellent repeatability (normalised and smoothed (11 pts))

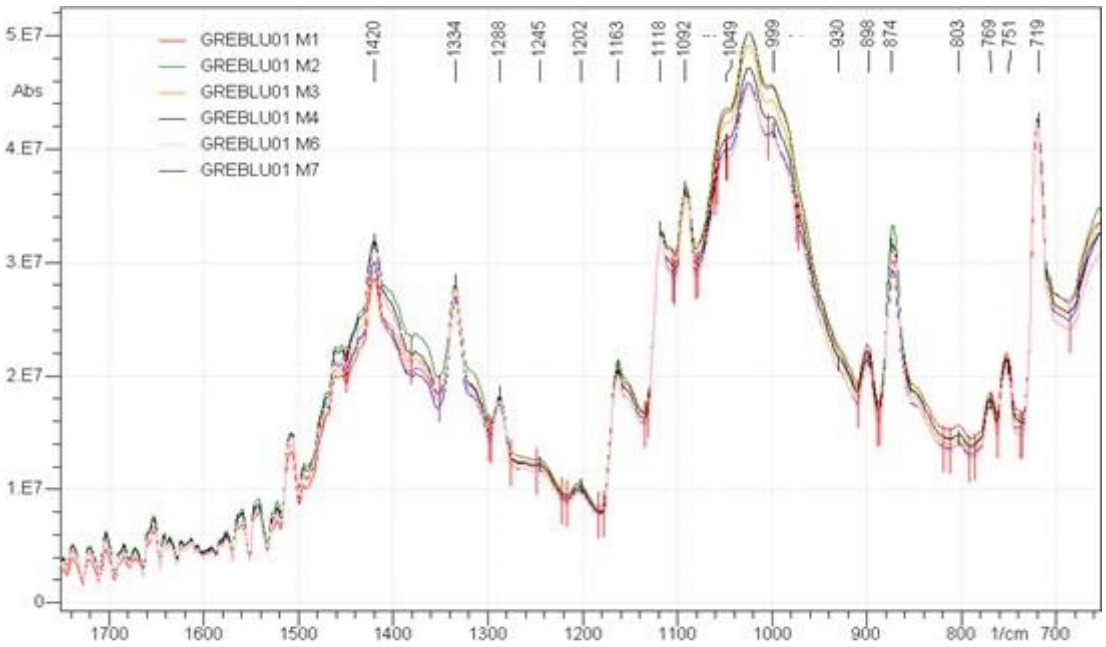


Figure 5.5: A set of spectra (n = 6) of the GRE UK blue gel ink on paper demonstrating excellent reproducibility (normalised and smoothed (11 pts))

Repeatability (Instrumental Variation)																
Sample	**Wavenumber Position (cm^{-1})**															
GRE UK M1A	719	753	767	801	872	898	1025	1049	1092	1114	1163	1202	1241	1286	1334	1420
GRE UK M1B	719	753	767	803	872	898	1025	1049	1090	1116	1163	1204	1243	1286	1334	1418
GRE UK M1C	719	753	767	803	872	900	1025	1049	1092	1116	1163	1204	1241	1288	1334	1420
GRE UK M1D	719	753	769	803	870	900	1025	1045	1090	1118	1163	1202	1243	1288	1334	1418
GRE UK M1E	719	753	767	803	872	898	1023	1047	1090	1116	1163	1204	1243	1288	1336	1420
GRE UK M1F	719	754	769	803	870	900	1023	1043	1092	1116	1163	1204	1245	1288	1332	1420
Mean	**719**	**753**	**768**	**803**	**871**	**899**	**1024**	**1047**	**1091**	**1116**	**1163**	**1203**	**1243**	**1287**	**1334**	**1419**
Std. Dev.	**0.00**	**0.41**	**1.03**	**0.82**	**1.03**	**1.10**	**1.03**	**2.53**	**1.10**	**1.26**	**0.00**	**1.03**	**1.51**	**1.03**	**1.26**	**1.03**
% RSD	**0.0**	**0.1**	**0.1**	**0.1**	**0.1**	**0.1**	**0.1**	**0.2**	**0.1**	**0.1**	**0.0**	**0.1**	**0.1**	**0.1**	**0.1**	**0.1**
Reproducibility (Sample Variation)																
Sample	**Wavenumber Position (cm^{-1})**															
GRE UK M1	719	751	769	803	870	898	1025	1049	1092	1116	1163	1202	1243	1288	1332	1420
GRE UK M2	719	751	769	801	874	898	1027	1043	1092	1116	1163	1202	1245	1288	1336	1420
GRE UK M3	719	753	769	801	872	900	1025	1047	1092	1116	1163	1204	1243	1288	1334	1420
GRE UK M4	719	753	769	801	872	900	1025	1049	1090	1116	1163	1204	1243	1288	1334	1420
GRE UK M5	721	753	769	801	872	900	1025	1047	1092	1118	1163	1204	1243	1288	1334	1420
GRE UK M6	719	753	769	801	872	898	1025	1047	1090	1116	1163	1202	1243	1288	1332	1420
Mean	**719**	**752**	**769**	**801**	**872**	**899**	**1025**	**1047**	**1091**	**1116**	**1163**	**1203**	**1243**	**1288**	**1334**	**1420**
Std. Dev.	**0.82**	**1.03**	**0.00**	**0.82**	**1.26**	**1.10**	**0.82**	**2.19**	**1.03**	**0.82**	**0.00**	**1.10**	**0.82**	**0.00**	**1.51**	**0.00**
% RSD	**0.1**	**0.1**	**0.0**	**0.1**	**0.2**	**0.1**	**0.1**	**0.2**	**0.1**	**0.1**	**0.0**	**0.1**	**0.1**	**0.0**	**0.1**	**0.0**

Table 5.1: A selection of 16 peaks representing a blue gel ink (GRE UK) used to determine % RSD across six measurements for repeatability and reproducibility

Colour Group	**% RSD**	
	Repeatability	**Reproducibility**
Blue	0.0 – 0.2	0.0 – 0.2
Red	0.0 – 0.4	0.0 – 0.2

Table 5.2: Summary of % RSD by colour group for repeatability and reproducibility

5.3.3 Sampling Method Development

For some brand/model combinations reproducibility of the spectra from an ink line was problematic. Within a set of six spectra from an ink line, differences in terms of peak presence/absence and relative intensities were observed. Furthermore, when compared to the corresponding set of spectra from an ink spot, peaks observed in the latter were not always observed in the former, suggesting that sufficient contact between the ink line and ATR sampling surface had not been achieved and this is illustrated in Figure 5.6. The use of a camera system incorporated into the ATR sampling interface as described in Fuestal and Briggs [145] could resolve this issue.

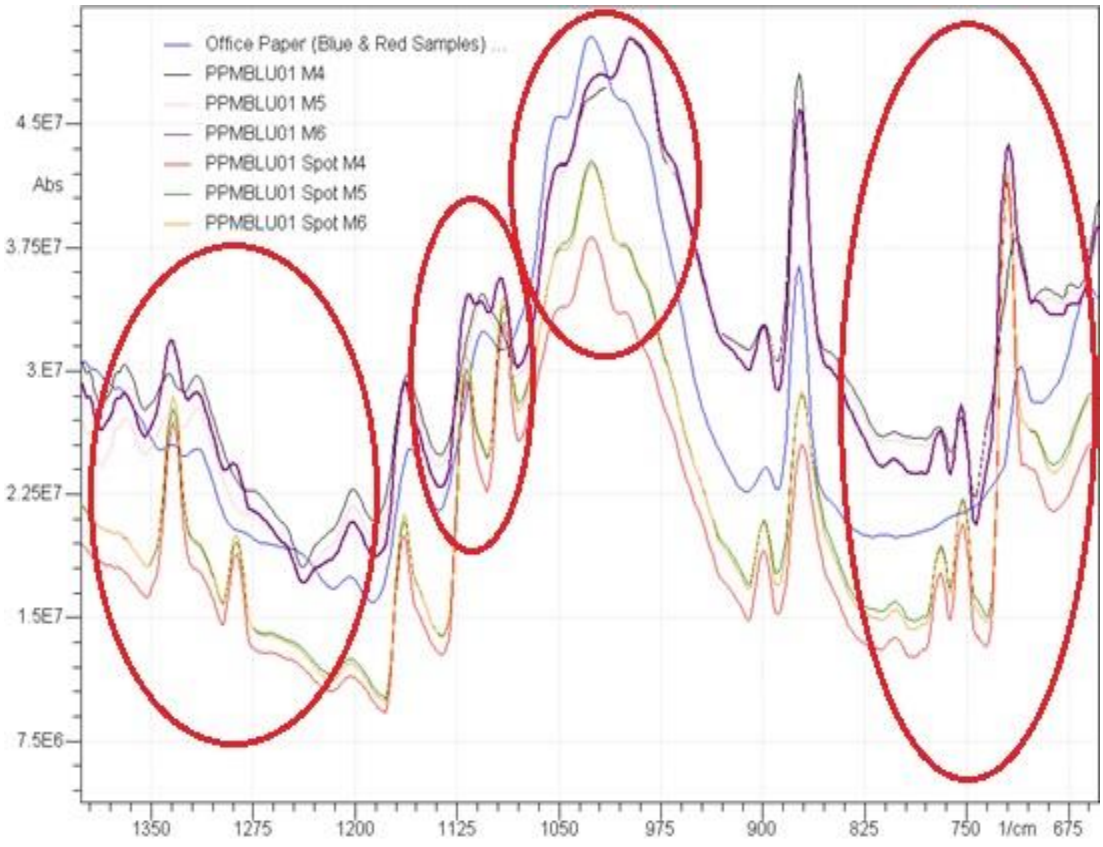


Figure 5.6: A set of three spectra representing each of a PPM UK blue gel ink spot and ink line on paper highlighting (in red circles) spectral differences in terms of peaks present and absent, intensity and overall reproducibility, compared against a spectrum from an un-inked area of the paper to highlight peaks attributable to the ink (normalised and smoothed (11 pts.))

Spectra from the same area of the un-inked paper exhibited excellent reproducibility despite its heterogeneous nature, supporting the view of Fuestal and Briggs [145]. For the blue and red ink group spectra, when compared to the spectra from un-inked paper, as illustrated in Figure 5.7, peaks believed attributable to the ink were observed in both ink

line and ink spot spectra. It was also possible to differentiate between brand/model combinations within these colour groups based mainly on the presence/absence of certain peaks, suggesting that discrimination of gels inks using this particular instrument was achievable.

It was noted that for the blue ink group, peaks attributable to the ink occurred predominantly in the region 1400 – 650 cm^{-1}, whilst for the red ink group they largely occurred in the region 1750 – 650 cm^{-1}. This region corresponds to the fingerprint region and concurs with previous observations described in the literature [83, 115, 118-120, 145, 196]. In the region 4000 – 2000 cm^{-1} any spectral variation can be attributed to atmospheric water vapour and CO_2 as described by Kher *et al* [83], thus providing little information for discrimination.

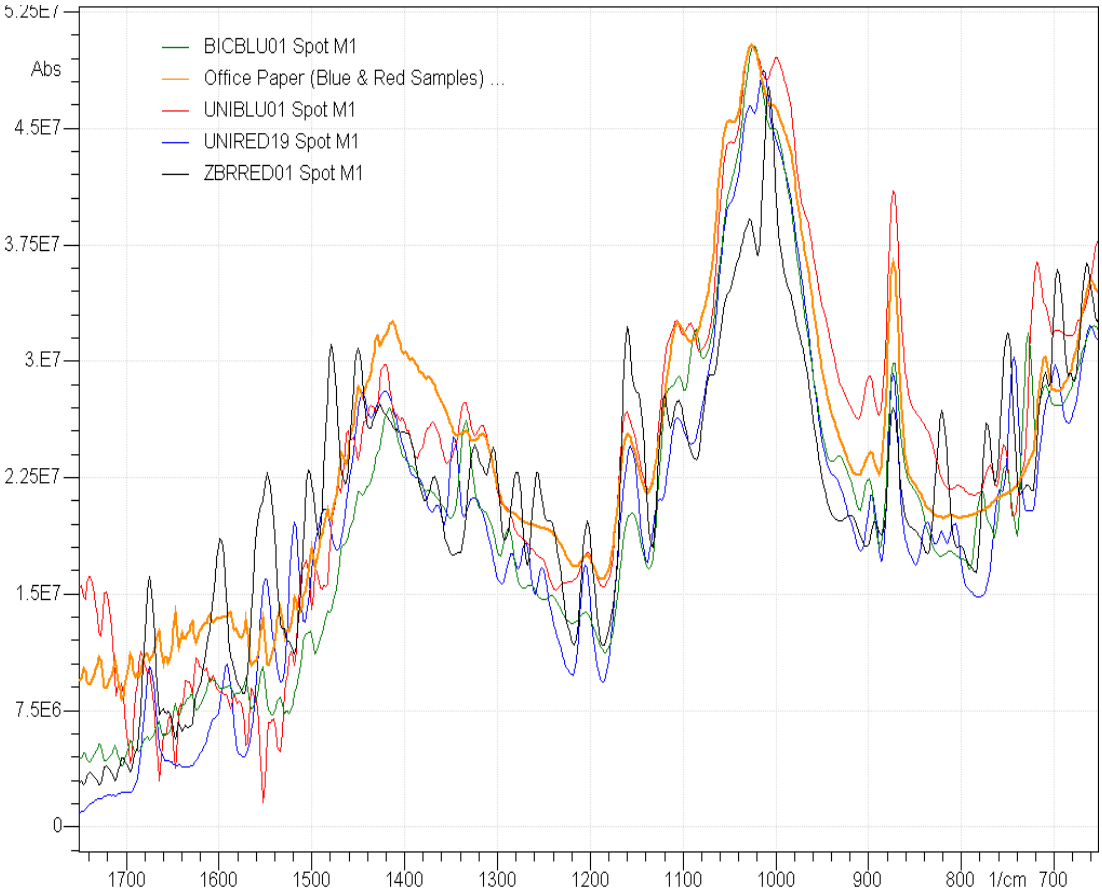


Figure 5.7: Examples of blue (UNI UK and BIC UK) and red (UNI AUS and ZBR UK) gel ink spectra against an un-inked paper spectrum demonstrating potential for discrimination within and between colour groups (normalised and smoothed (11 pts))

5.3.4 Within Brand Variation

Three brand/model combinations per colour group were analysed for an assessment of within brand variation, these were:

- ICM UK, PKR UK and **ZBR UK** (blue ink group);
- ICM UK, UNI UK and **WHS UK** (red ink group).

The two brand/model combinations highlighted in bold were found to exhibit evidence of within brand variation using Raman Spectroscopy.

No within brand variation was noted in the blue ink group, however in the ZBR UK (blue) spectral set, highlighted in Figure 5.8, a slight variation in spectral shape was observed in the region around 1100 – 1000 cm^{-1}. This region corresponded to the wavenumber region where interference from the paper was at its strongest due to cellulose and hemicellulose components. It therefore was considered more likely this difference was the result of an ink/paper interaction rather than a real chemical difference between the inks.

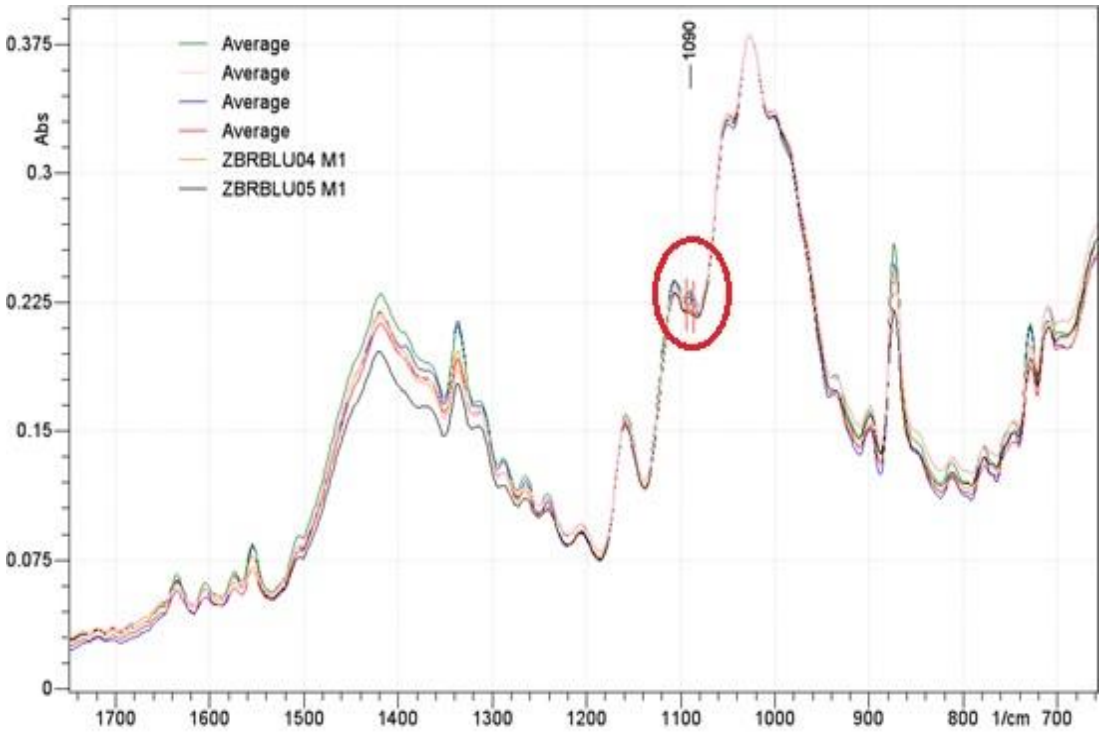


Figure 5.8: Averaged spectra representing six ZBR UK blue gel ink pens of the same brand/model combination illustrating (in the red circle) minor spectral variation between 1100 cm^{-1} – 1000 cm^{-1} considered more likely attributable to interference from the paper substrate rather than evidence of within brand variation

No within brand variation was observed in the brand/model combinations within the red ink group apart from the six WHS UK pens. WHS UK pens #01 – #03 exhibited a spectral pattern considered to be indistinguishable from each other. Pen #06 also exhibited a spectral pattern similar to that of pens #01 - #03 except that the overall intensity of the spectrum was notably stronger emphasising the intensity of some peaks, i.e. 830 – 800 cm^{-1} region. It seems likely that this may have been the result of a better contact between the ink and the ATR crystal rather than a real chemical difference. That said, peaks at 1446 cm^{-1} and 1485 cm^{-1} were observed in the spectra from pen #06 that did not appear to be present in that of pens #01 – #03. Whether these were present as a result of an overall intensity increase or as a result of a genuine chemical difference is unclear. However, the spectra from pens #04 and #05, whilst very similar to each other, did exhibit some notable differences to those of the other pen spectra as illustrated in Figure 5.9. These differences were centred on the presence or absence of certain peaks around the following wavenumber positions: 743 cm^{-1}, 786 cm^{-1}, 807 cm^{-1}, 1258 cm^{-1}, 1347 cm^{-1} and 1519 cm^{-1}. These observations provided further evidence that pens #04 and #05 at least were chemically different from that of pens #01 - #03, and that pen #06 may also have a slightly different chemical formulation.

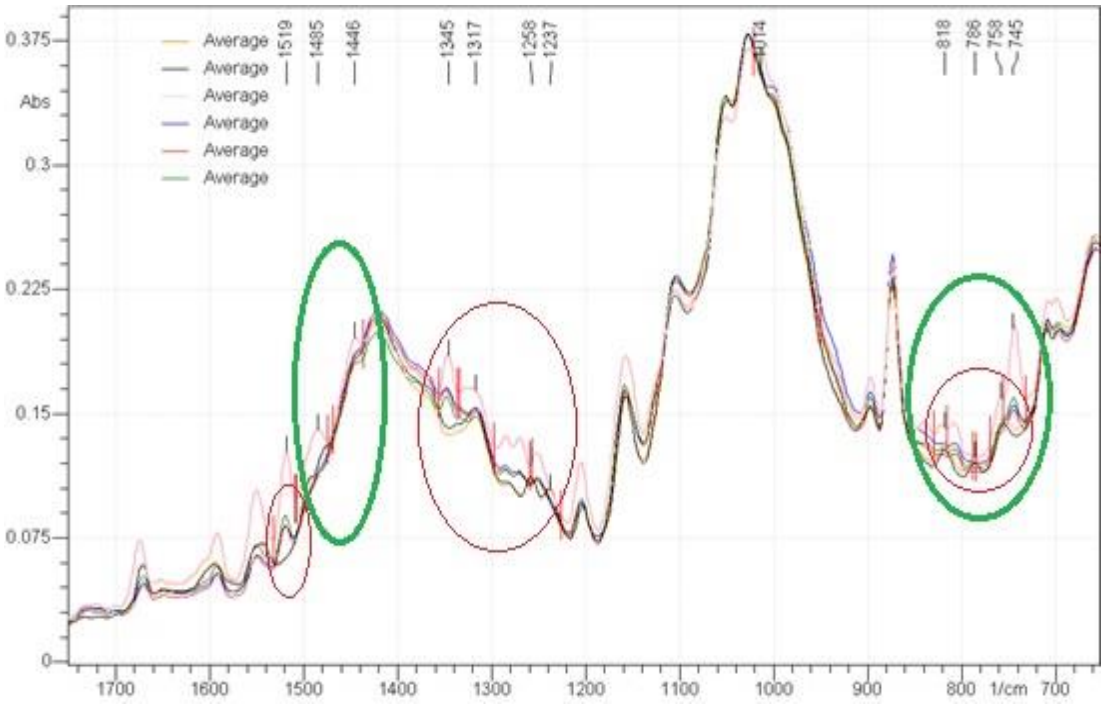


Figure 5.9: Averaged spectra representing each of six WHS UK red gel ink pens (Pens #01 - #06). Areas highlighted within green circles relate to spectral differences associated with pen #06 (pink spectral line), whilst areas highlighted within red circles relate to spectral differences associated with pens #04 (black spectral line) and #05 (gold spectral line)

5.3.5 Between Brand Variation

As expected, the reproducibility of spectra taken from each ink spot sample across both colour groups was good; therefore an averaged spectrum representative of each ink sample was used. Spectral comparison was focussed on the regions 1400 – 650 cm^{-1} and 1750 – 650 cm^{-1} for the blue and red ink groups respectively. In order to identify peaks attributable to the ink, a comparison of each sample against an averaged un-inked paper spectrum was conducted and peaks of interest labelled. For each colour group, peak tables detailing the peak positions attributable to each ink sample were compiled and interrogated for patterns in recurring peak positions. Obvious differences in relative peak intensities (RPI) were also noted. In this way it was possible to discriminate samples within each colour group into groups of similar spectral patterns based on peak presence or absence, and differences in relative peak intensities. However, this method of discrimination was not straightforward, since some peaks exhibited relatively weak absorbency, and as such were difficult to distinguish between as a real chemical difference or simply background noise. This difficulty questioned the validity of using such peaks as a reliable feature of discrimination. Therefore, within each colour group, final discrimination of ink samples using original spectra only was provided through a tiered system, with higher tier levels corresponding to a greater number of discriminations made using progressively weaker peak absorbencies. The presence or absence of peaks and differences in relative peak intensities was also applied to the corresponding first derivative spectra for each sample in both colour groups and considered in combination with the original spectra to see if any further discrimination was possible.

Figures illustrating the spectral groups for both colour groups based on the original spectra, and in a few cases, the first derivative, are provided below. Due to a limitation in the instrument software, it was not possible to label individual averaged spectral lines with the corresponding brand/model combination reference code. To overcome this, the spectra have been reproduced in Excel to permit identification of individual spectral lines. Where appropriate, both the instrument Panorama software and Excel versions of a spectral group have been incorporated within a single Figure. Spectral differences described have been highlighted, as necessary, within red and green circle markers in the Panorama version, and individually labelled within the corresponding Excel version. It is hoped this will assist the reader in understanding the discriminating features described.

The peak positions for all brand/model combinations within a given class were examined, and were found to be within the ± 4 cm^{-1} resolution of a central wavenumber position for the instrument illustrating that the wavenumbers presented are not absolute and are subject to slight variations.

Peaks occurring at the following approximate wavenumber positions were attributed to the paper spectrum: 874 cm^{-1}, 894 cm^{-1}, 1027 cm^{-1}, 1051 cm^{-1}, 1105 cm^{-1}, 1165 cm^{-1}, 1204 cm^{-1}, 1340 cm^{-1} and 1418 cm^{-1}.

5.3.5.1 Blue Ink Group

Using the original FTIR spectra only, the blue ink group samples could be broadly classified into pigment based and dye containing on the basis of the absence of certain peaks in the latter occurring within three wavenumber regions: 1290 – 1260 cm^{-1}, 1130 – 1080 cm^{-1} and 900 – 700 cm^{-1}. Discrimination of all 31 ink samples was split between four Tier levels (T1 – T4). T1 and T2 were considered more reliable than T3 and T4 as discrimination was based on overall relatively stronger peak intensities in the former, i.e. the peaks attributed to the ink were of relatively strong intensity for the T1 and T2 level discriminations than the T3 and T4 level discriminations where the peaks exhibited relatively weak intensity, and could not therefore be excluded as background noise. Discrimination into a potential maximum of 17 distinct groups (T4) and a minimum of three groups (T1) was possible as illustrated in Table 5.3. Where notable differences in relative peak intensities (RPI) have been used as a feature of discrimination, this is stipulated in the Table.

Colorant	Sample	T1		T2		T3		T4	
		Group	Peaks (cm^{-1})	Group	Peaks (cm^{-1})	Group	Peaks (cm^{-1})	Group	Peaks (cm^{-1})
Pigment	PPM UK	1	**717** **753** **769** 803 **1090** **1288**	1	**1118**	1	698 shoulder	1	
	STP UK								
	WHS UK								
	FBC MAL								
	GSF MAL								
	UNI AUS								
	UNI SA								
	GRE UK					2		2	
	WKE UK					3	1261 and weaker RPI at 1114	3	
	UNI UK					4	698 shoulder and weaker RPI at 1118	4	
	MG MAL								
	UNI JAP			2	Weaker RPI	5		5	
	UNI HK #25								
	UNI HK #31								
	BIC UK	2	**728** **753** **777** 812 933shoulder **1088** 1244 1263 **1288**	3	1116 peak or shoulder	6		6	
	BIC US								
	PTN UK								
	STD UK					7	698	7	
	ZBR UK			4	900 **1241** and **1263** stronger RPI	8		8	
	ZBR JAP								
	PTL UK					9	697 797 812 weaker RPI	9	
Dye or Hybrid	COF US	3	1243	5	915 peak or shoulder	10		10	
	PKR UK					11	1276	11	
	PTL JAP					12	918 and 1245 weaker RPI	12	
	STP US #08							13	754
	STP US #09							14	691 754
	STB UK			6		13		15	
	ICM UK								
	STP US #07					14	691 805	16	747
	PLT UK							17	782
	PLT US								

Table 5.3: Summary of discriminating peak positions for the blue ink group based on original data. Bold type indicates a strong relative peak intensity (RPI)

5.3.5.1.1 Pigment Based Samples

Initial discrimination into two groups was possible on the basis of presence or absence of certain peaks. Group 1 (T1), shown in Figure 5.10, contained 14 samples and was characterised by key peak positions (highlighted within red circles) at ~**717 cm^{-1}**, ~**753 cm^{-1}**, ~**769 cm^{-1}**, ~803 cm^{-1}, ~**1090 cm^{-1}**, and ~**1288 cm^{-1}** observed in all samples (bold type indicates relatively strong peak intensity). Further discrimination within this group *could also be* considered possible.

The absence of a peak at ~**1118 cm^{-1}** (highlighted within the red circle) together with an overall decrease in relative peak intensities distinguished the UNI JAP, UNI HK #25 and UNI HK #31 (Asian UNI samples (Group 2 (T2), shown in Figure 5.11) from the other 11 samples (Group 1 (T2), shown in Figure 5.12) in the group which all exhibited comparatively stronger peak intensities. The absence of a small shoulder at ~698 cm^{-1} distinguished the GRE UK and WKE UK samples from the remaining 11. On the basis of a small peak at ~1261 cm^{-1} and a weaker relative intensity at ~1114 cm^{-1} it was possible to discriminate the WKE UK sample from the GRE UK sample, to form Group 3 (T3/T4) and Group 2 (T3/T4) respectively as shown in Figure 5.13. For the remaining nine samples, the UNI UK and MG MAL samples were discriminated from the others on the basis of a weaker relative intensity to the peak at ~1114 cm^{-1}, to form Group 4 (T3/T4), illustrated in Figure 5.14.

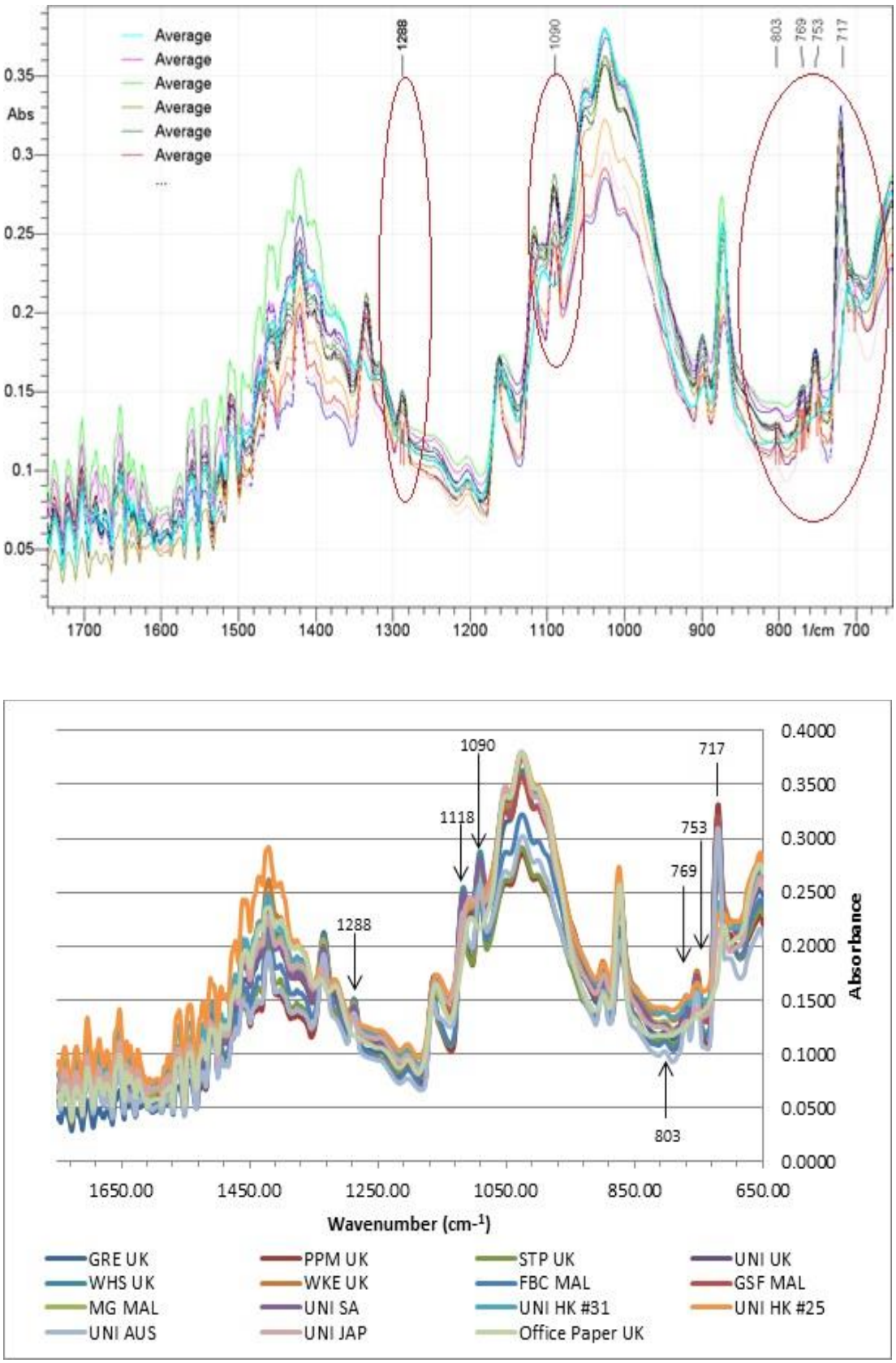


Figure 5.10: Averaged spectra representing the 14 brand/model combinations of blue gel ink forming Group 1 (T1). Panorama software chart version (top) and Excel version (bottom)

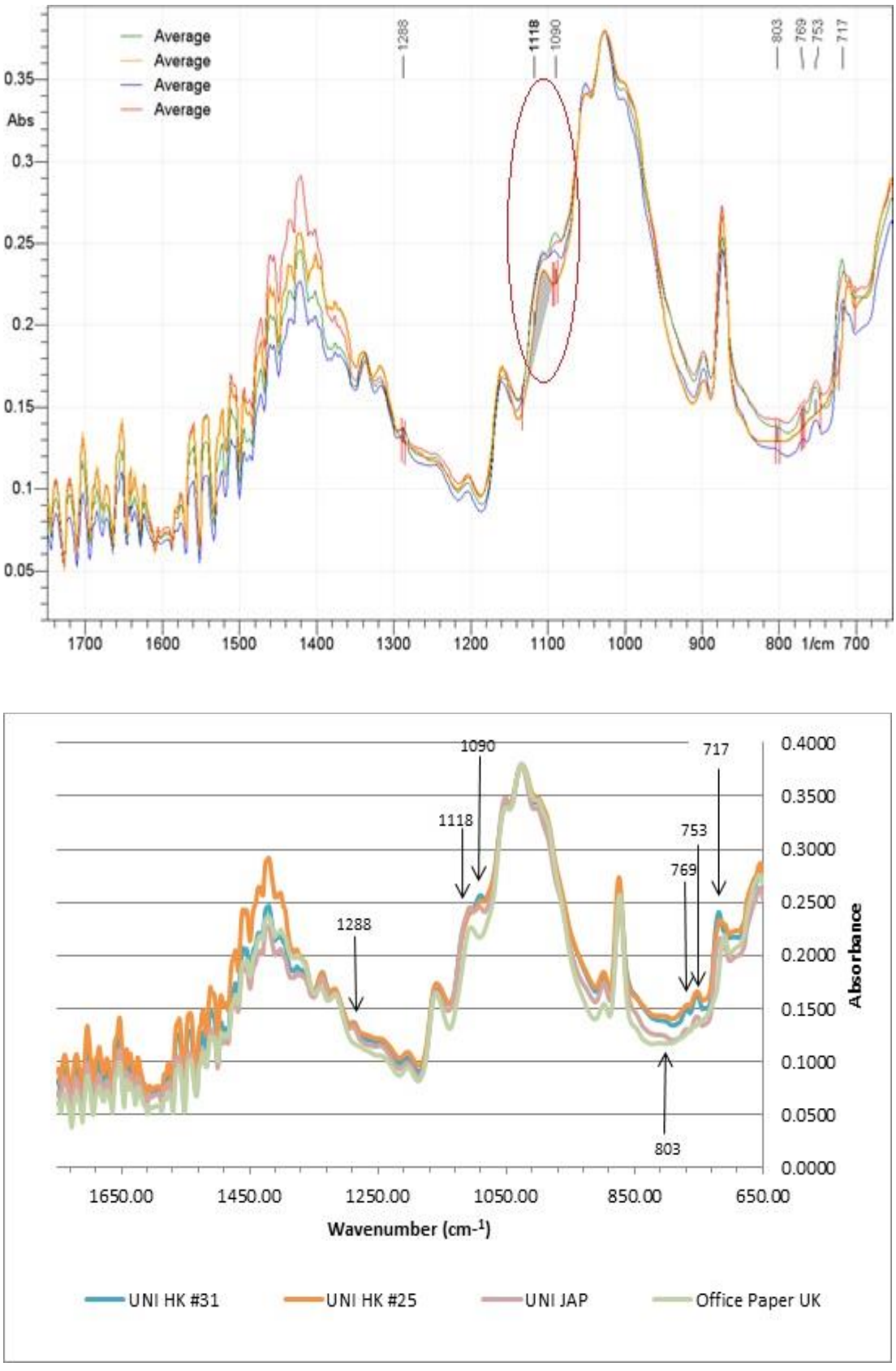


Figure 5.11: Averaged spectra representing three Asian Uniball (Asian UNI) brand/model combinations of pigment based blue gel ink forming Group 2 (T2). Panorama software chart version (top) and Excel version (bottom)

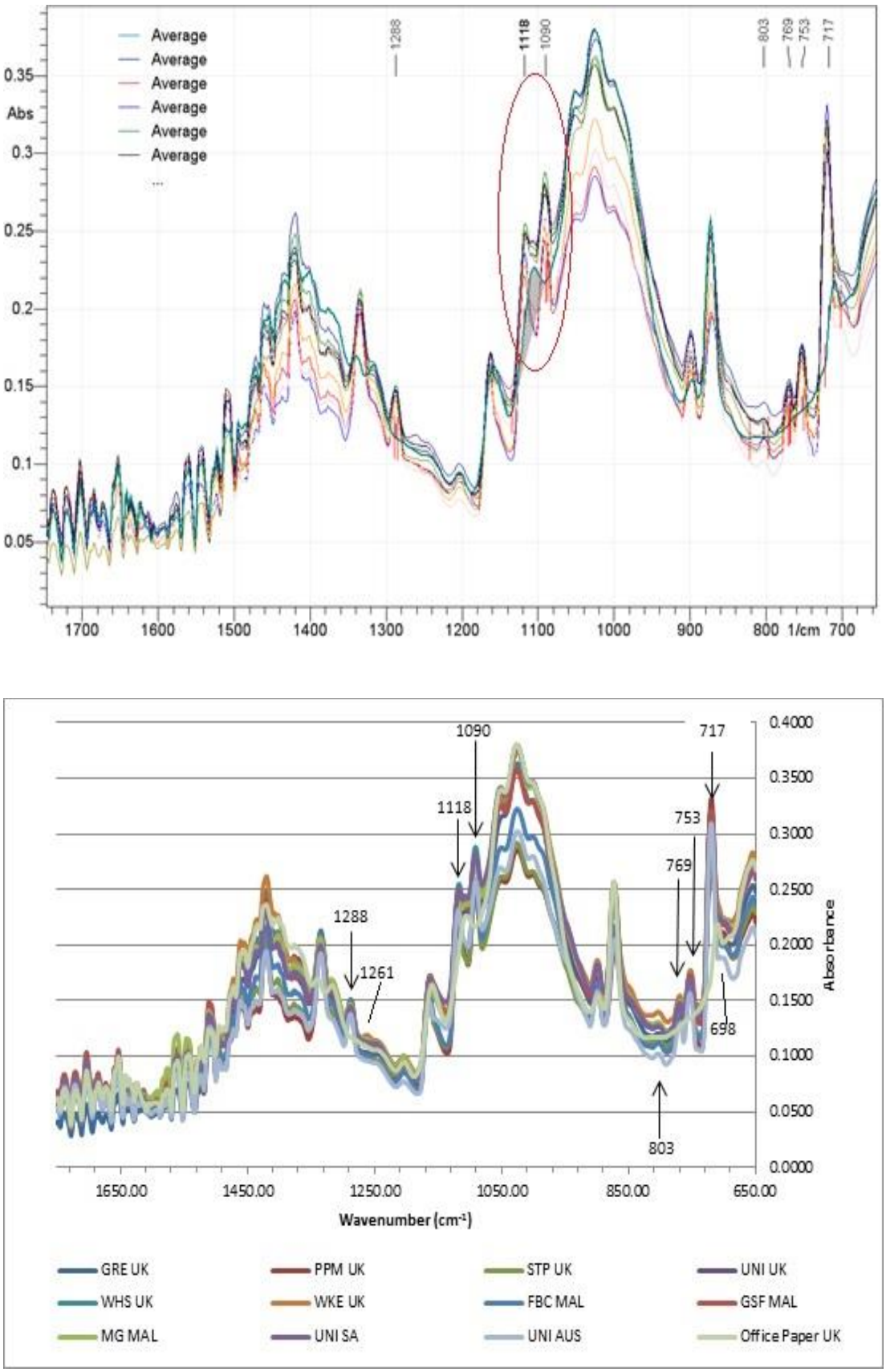


Figure 5.12: Averaged spectra representing the 11 brand/model combinations of pigment based blue gel ink forming Group 1 (T2). Panorama software chart version (top) and Excel version (bottom)

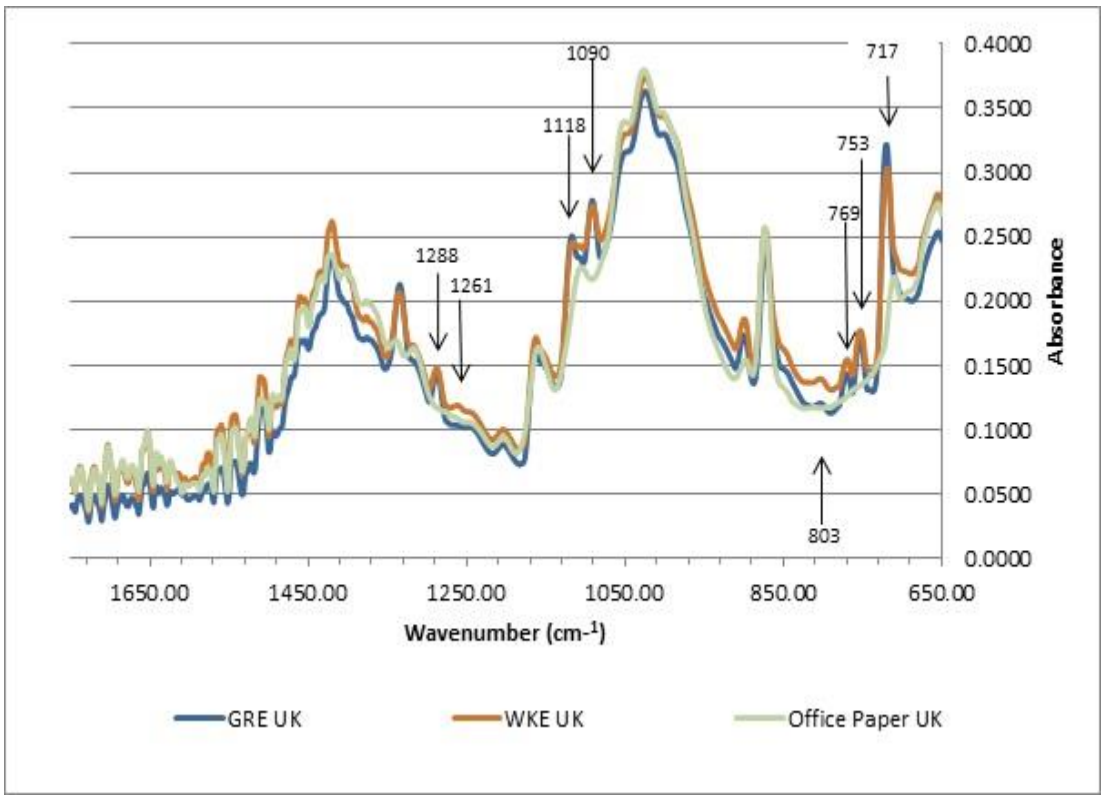


Figure 5.13: Averaged spectra representing the two brand/model combinations of pigment based blue gel ink forming Group 2 and 3 (T3/T4)

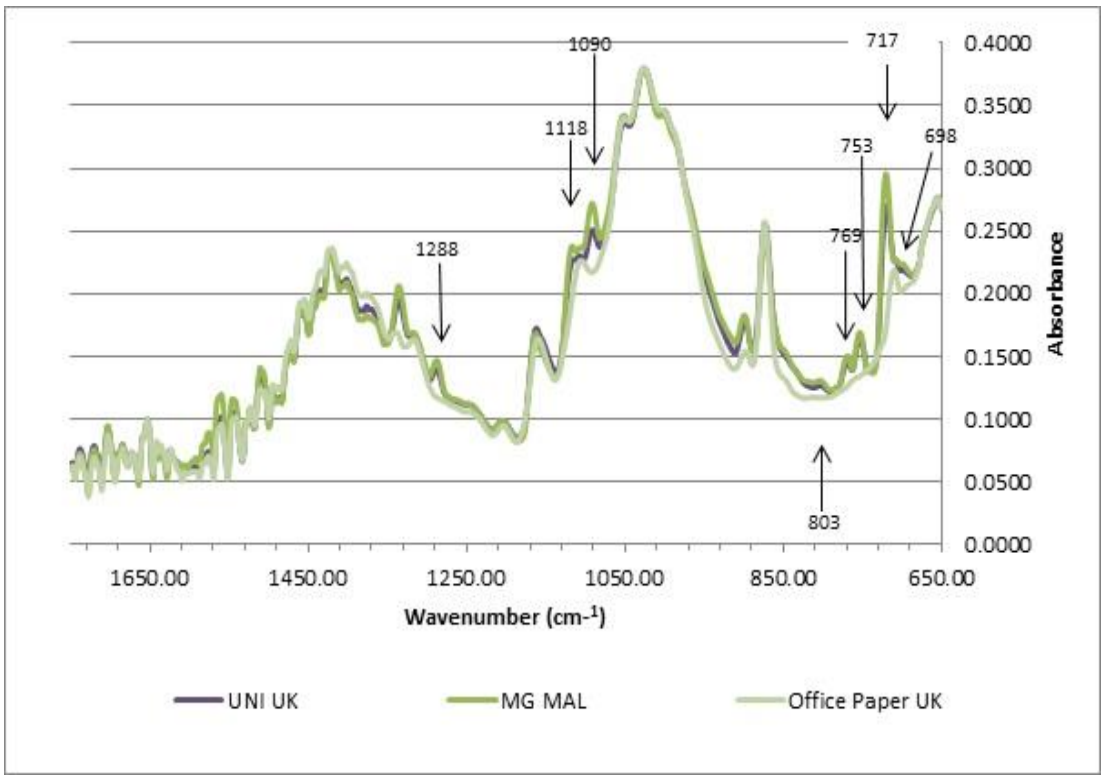


Figure 5.14: Averaged spectra representing the two brand/model combinations of pigment based blue gel ink forming Group 4 (T3/T4)

Group 2 (T1), shown in Figure 5.15, was characterised by peak positions at ~**728 cm^{-1}**, ~**753 cm^{-1}**, ~**777 cm^{-1}**, ~812 cm^{-1}, ~933 cm^{-1} (shoulder), ~**1088 cm^{-1}**, ~1244 cm^{-1}, ~1263 cm^{-1}, and ~**1288 cm^{-1}**, again observed in all samples. Group 2 (T1) could be clearly distinguished from Group 1 (T1) by differences in peak positions in the 700 – 820 cm^{-1} region as well as the presence of a shoulder at ~933 cm^{-1} and a peak at ~1244 cm^{-1}. Further discrimination within this group *could also be* considered possible.

Further discrimination into at least two sub-groups by the presence or absence of a peak or shoulder at ~1116 cm^{-1} and ~900 cm^{-1} as well as a difference in the relative intensity of the peaks at ~1241 cm^{-1} and ~1263 cm^{-1} was possible. Furthermore the STD UK sample could be distinguished from the other three pens in Group 3 ((T2); BIC UK, BIC US and PTN UK, shown in Figure 5.16) and the PTL UK sample from the other two in Group 4 ((T2); ZBR UK and ZBR JAP, shown in Figure 5.17) on the basis of a small shoulder at ~698 cm^{-1}. The presence of a small peak at ~797 cm^{-1} and a weaker intensity to the peak at ~812 cm^{-1} supported the discrimination of the PTL UK sample from the two ZBR samples. These further discriminations formed Groups 6 – 9 (T3/T4) as shown in Table 5.3. The spectral differences described are highlighted within the red circles.

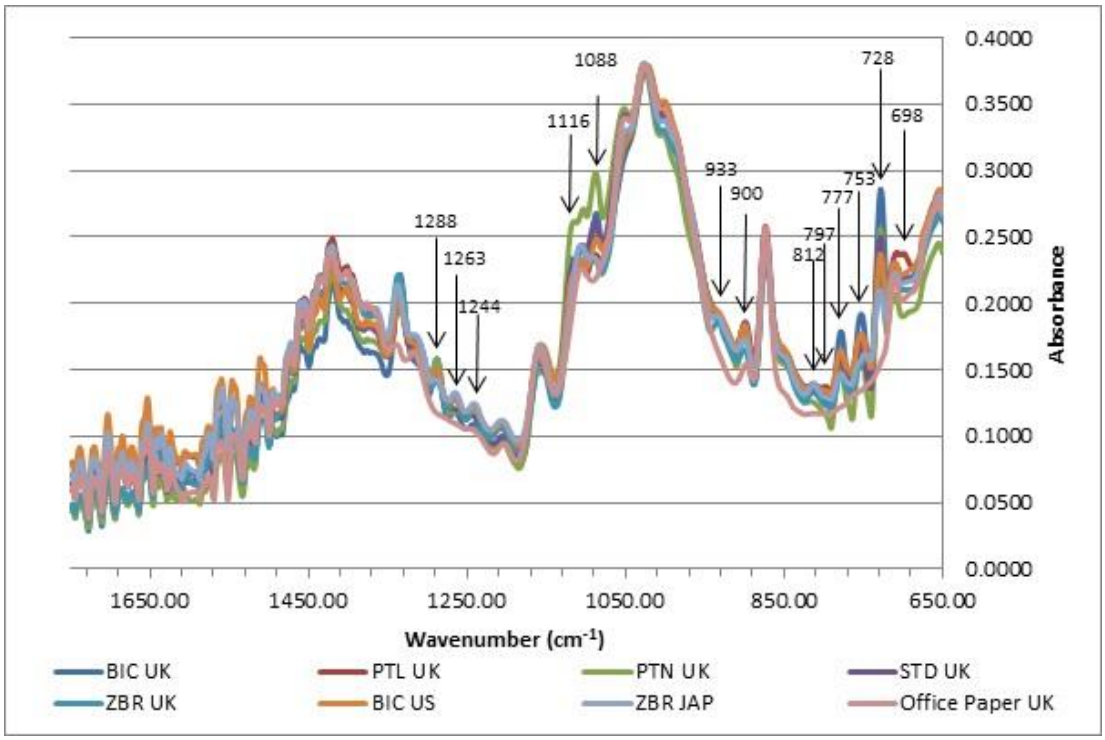


Figure 5.15: Averaged spectra representing the seven brand/model combinations of pigment based blue gel ink forming Group 2 (T1)

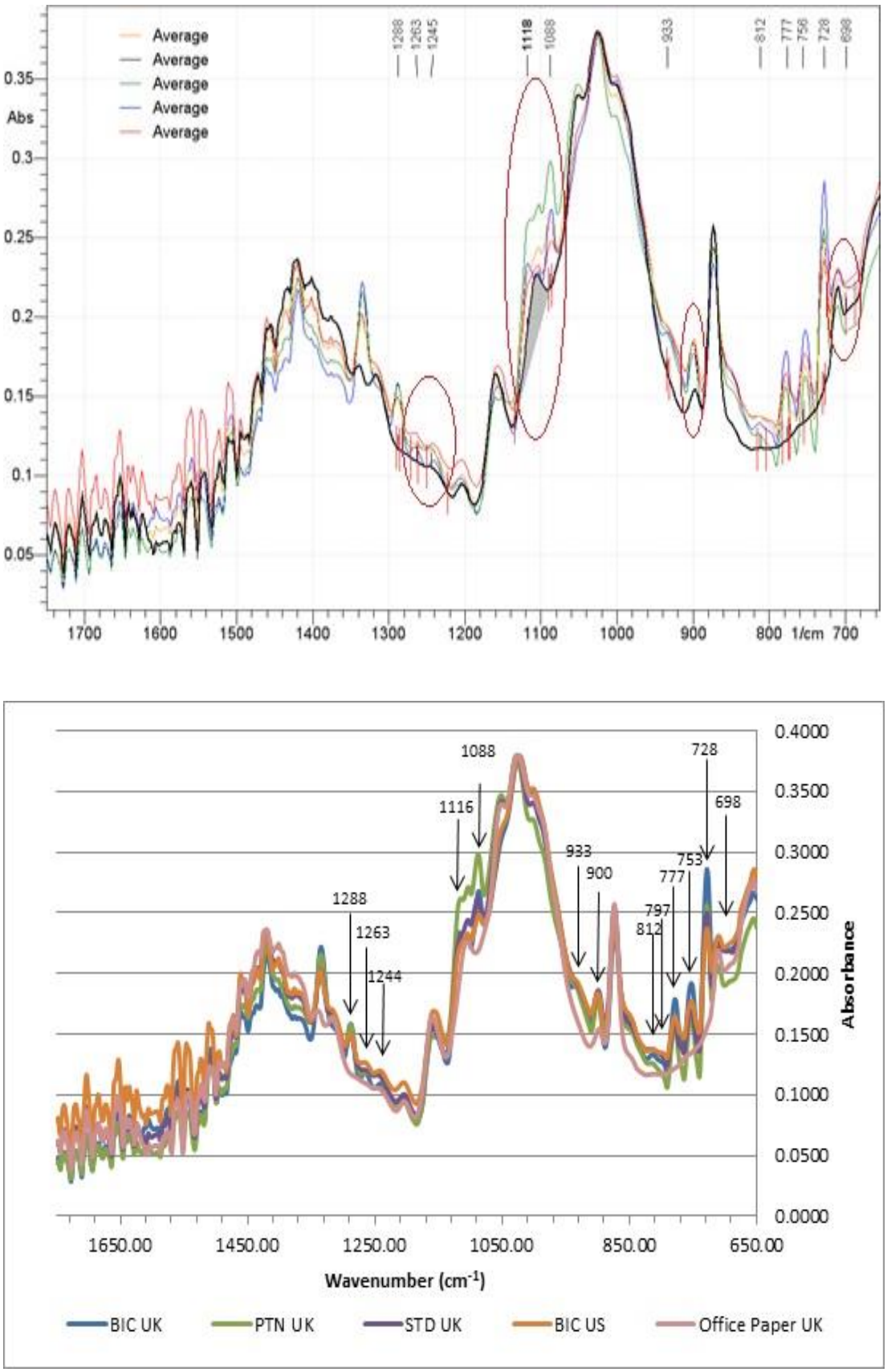


Figure 5.16: Averaged spectra representing the four brand/model combinations forming Group 3 (T2). Panorama software chart version (top) and Excel version (bottom)

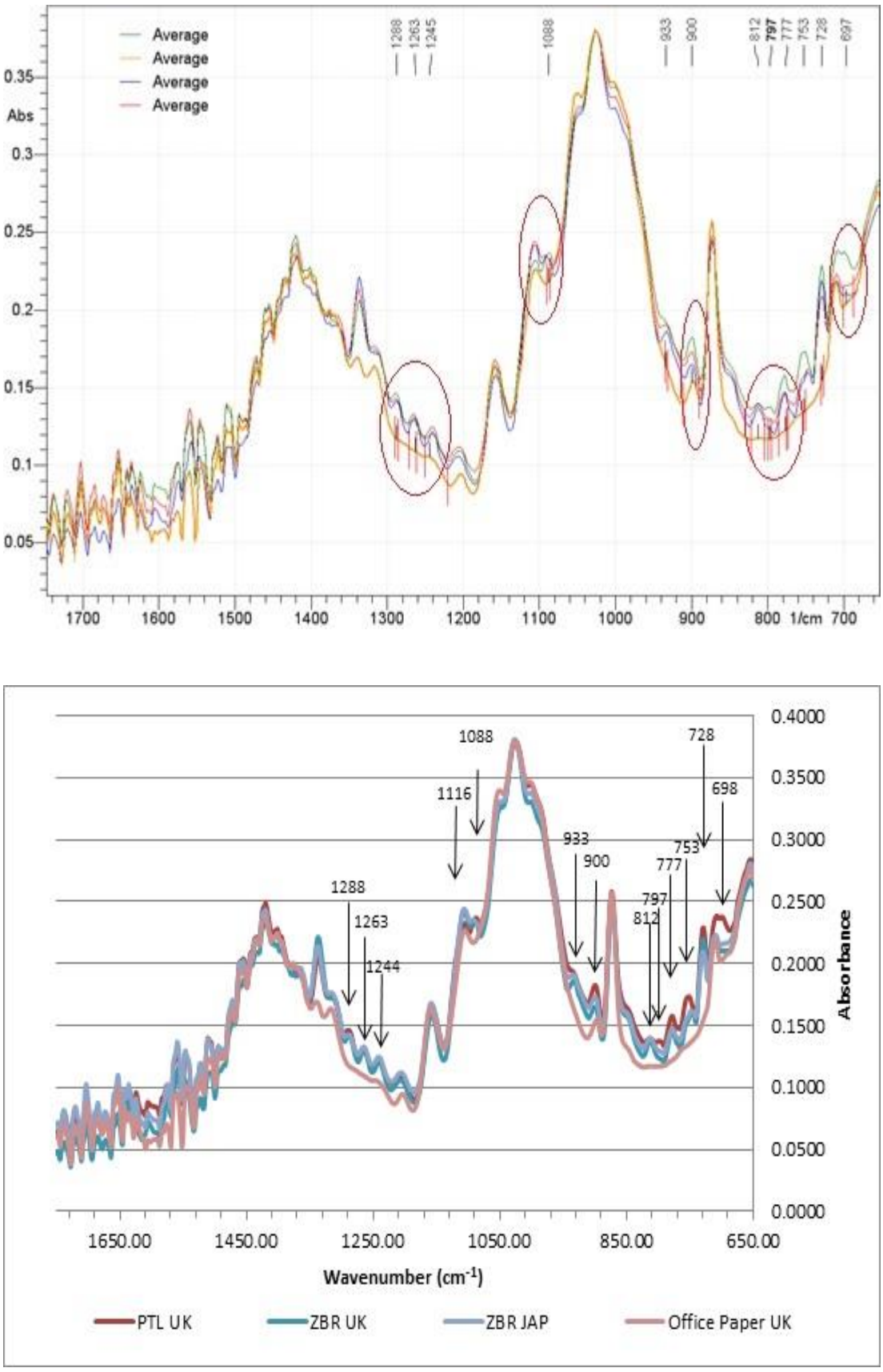


Figure 5.17: Averaged spectra representing the three brand/model combinations forming Group 4 (T2). Panorama software chart version (top) and Excel version (bottom)

5.3.5.1.2 Dye Containing Samples

The dye component group generally exhibited fewer peaks than the pigment based groups. However, based primarily on very small peaks or shoulders, some discrimination within this group was considered possible. All ten dye containing samples exhibited a notable peak at ~1243 cm^{-1}, varying in relative intensity. The presence or absence of a small peak or shoulder at ~915 cm^{-1} split the group into two sub-groups containing five samples each (Group 5 (T2): PKR UK; COF US; PTL JAP; STP US #08 and STP US #09, shown in Figure 5.18: and Group 6 (T2): STB UK; ICM UK; STP US #07; PLT UK and PLT US, shown in Figure 5.19). Within Group 5 (T2), the presence of a peak at ~1276 cm^{-1} distinguished the PKR UK sample, whilst weaker relative intensities of peaks at ~918 cm^{-1} and ~1243 cm^{-1} distinguished the COF US sample. The presence of a small peak at ~754 cm^{-1} distinguished the STP US #08 and #09 samples from the PTL JAP sample, whilst a small shoulder at ~691 cm^{-1} discriminates STP US #09 from STP US #08. These spectral differences are highlighted within the red circles.

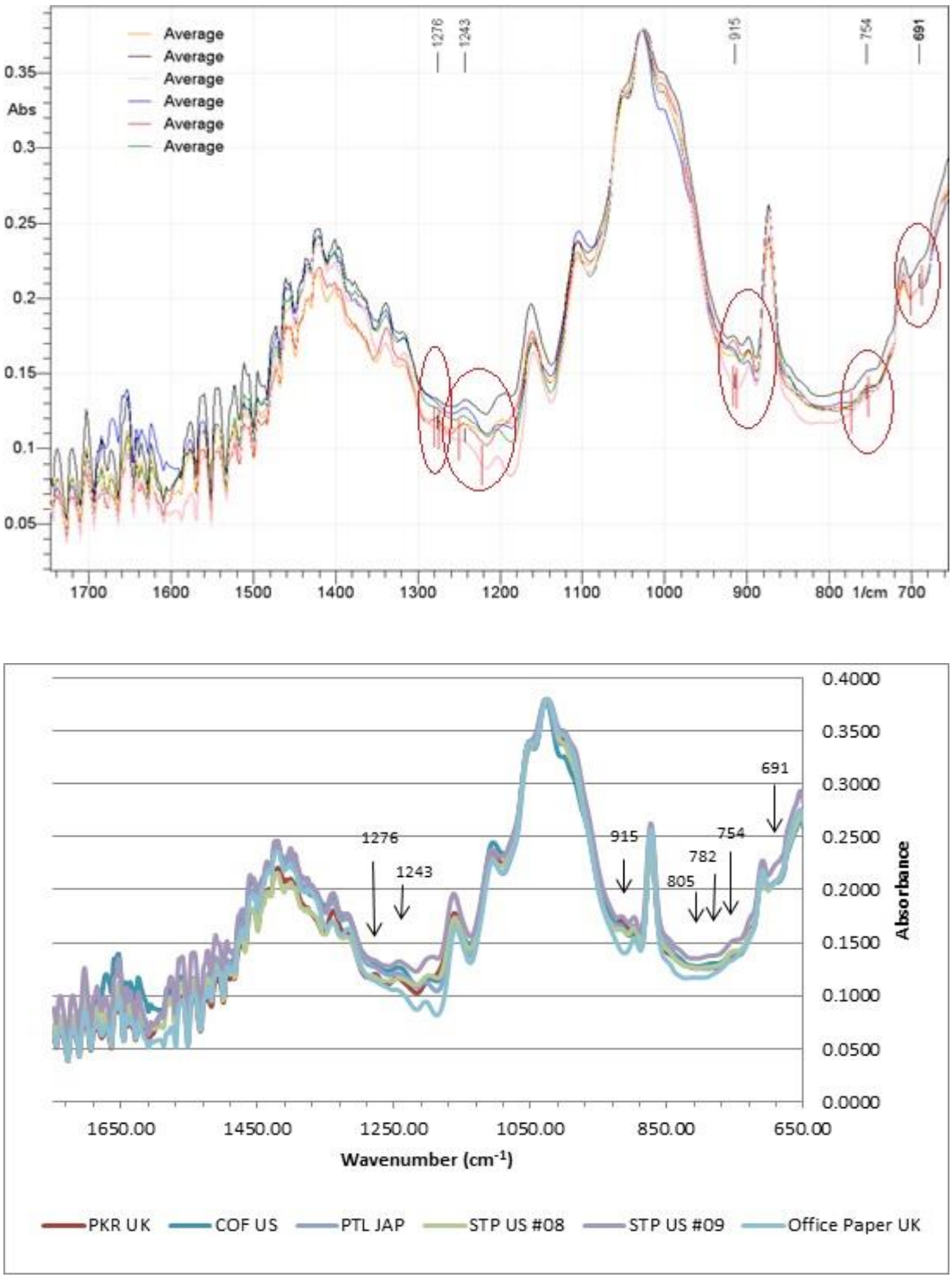


Figure 5.18: Averaged spectra representing the five brand/model combinations of dye containing blue gel ink forming Group 5 (T2). Panorama software chart version (top) and Excel version (bottom)

In Group 6 (T2), illustrated in Figure 5.19, the ICM UK and STB UK samples were very similar to the paper background spectrum with the exception of the peak at ~1243 cm^{-1}. The PLT UK, PLT US and STP US #07 samples exhibited stronger peak intensities at ~1243 cm^{-1} to

these samples. In addition all three samples exhibited small peaks at ~691 cm^{-1} and ~805 cm^{-1}. However the STP US #07 could be discriminated from the PLT samples by the presence of small peaks at ~747 cm^{-1} and ~782 cm^{-1}. These spectral differences are highlighted within the red circles.

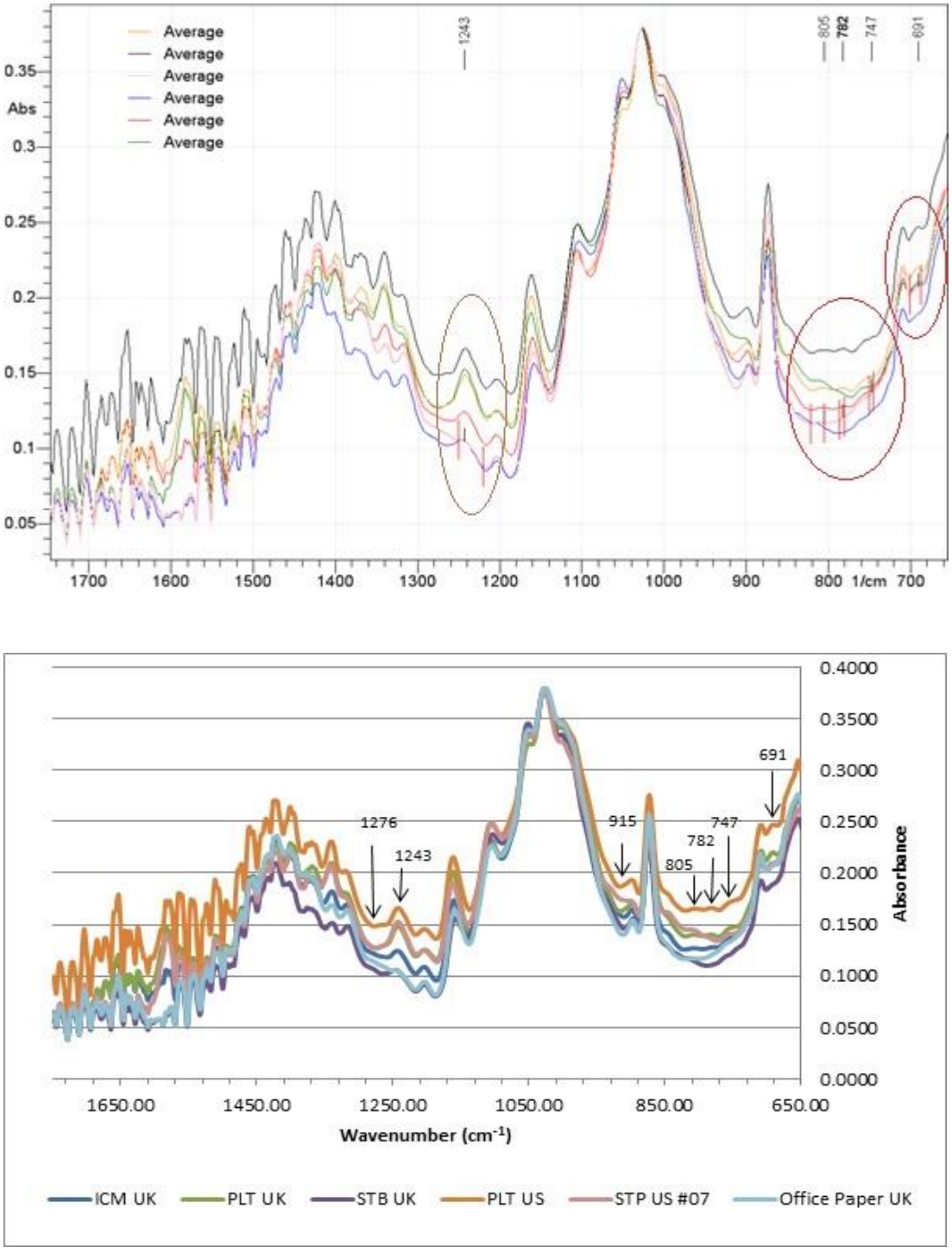


Figure 5.19: Averaged spectra representing the five brand/model combinations of dye containing blue gel ink forming Group 6 (T2). Panorama software chart version (top) and Excel version (bottom)

A similar study of the first derivative of the averaged spectra confirmed the blue ink group could be confidently classified into at least three groups. These consisted of two pigment based groups and one dye component group corresponding to the initial classification based on the original FTIR spectra. Further discrimination of both pigment based groups was possible using the first derivative (FD) spectra. Fourteen samples in FD Group 1 presented in Figure 5.20 revealed a peak at ~797 cm^{-1} (highlighted within the red circle) in the first derivative spectra, which was of weaker intensity in the three Asian UNI samples. Furthermore, a peak observed at ~1114 cm^{-1} (highlighted in the green circle) was not present in the spectra from the Asian UNI samples and was also absent in the UNI UK spectra. It was not possible to further discriminate the MG MAL, GRE UK and WKE UK samples using the first derivative spectra even though these were distinguished using the original spectral data.

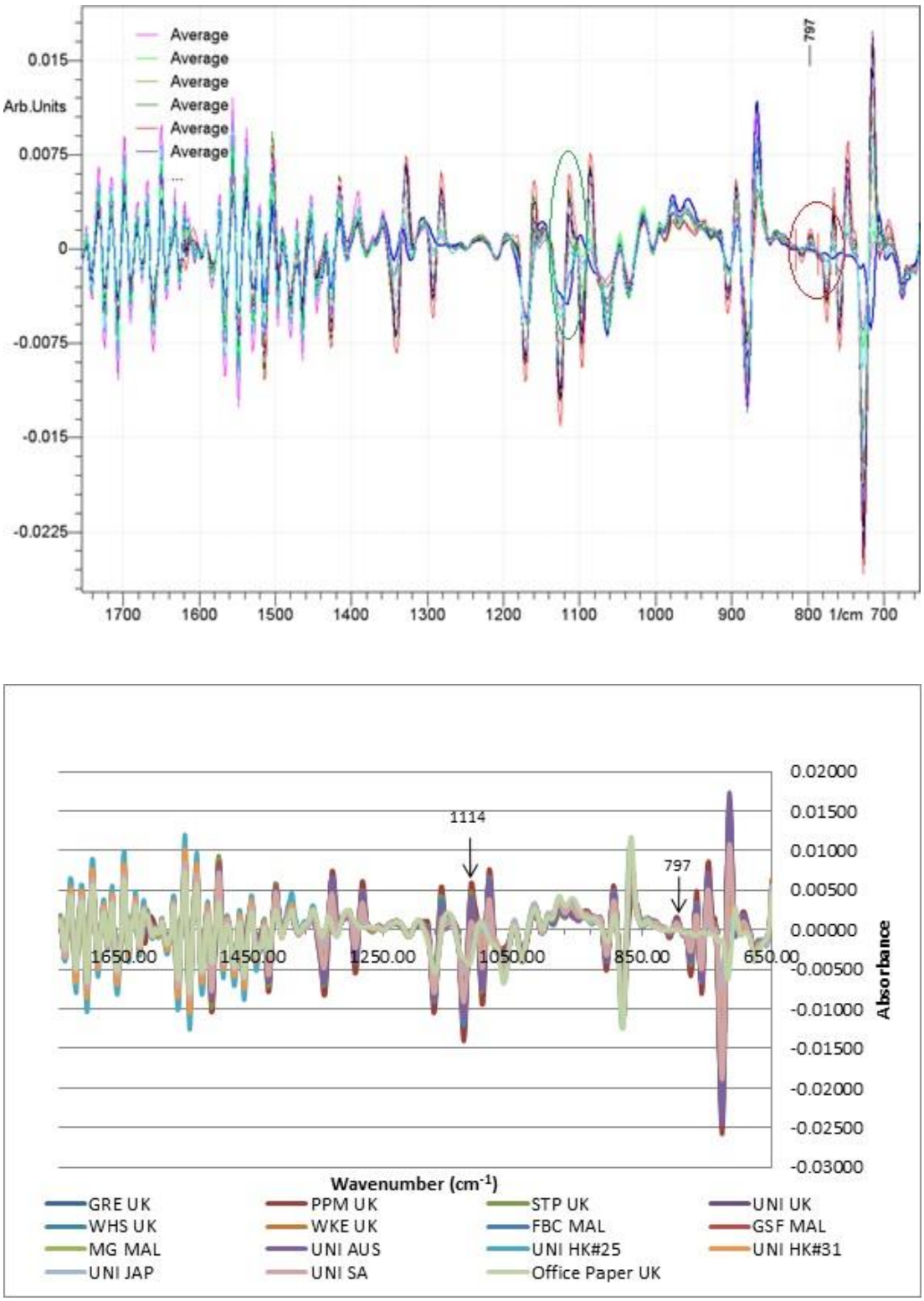


Figure 5.20: Averaged first derivative (FD) spectra representing the 14 brand/model combinations of pigment based blue gel ink forming FD Group 1. Panorama software chart version (top) and Excel version (bottom)

FD Group 2 was differentiated into two sub-groups (FD Group 2a and 2b, shown in Figure 5.21 and 5.22 respectively) on the basis of the presence or absence of a peak at ~1112 cm^{-1}.

The samples within these sub-groups also corresponded to the classification made from the original FTIR spectra. The STD UK sample could also be discriminated from the PTN UK, BIC UK and BIC US samples by the presence of a small peak at ~693 cm^{-1}, as could the PTL UK sample from the ZBR samples, again consistent with the original classifications.

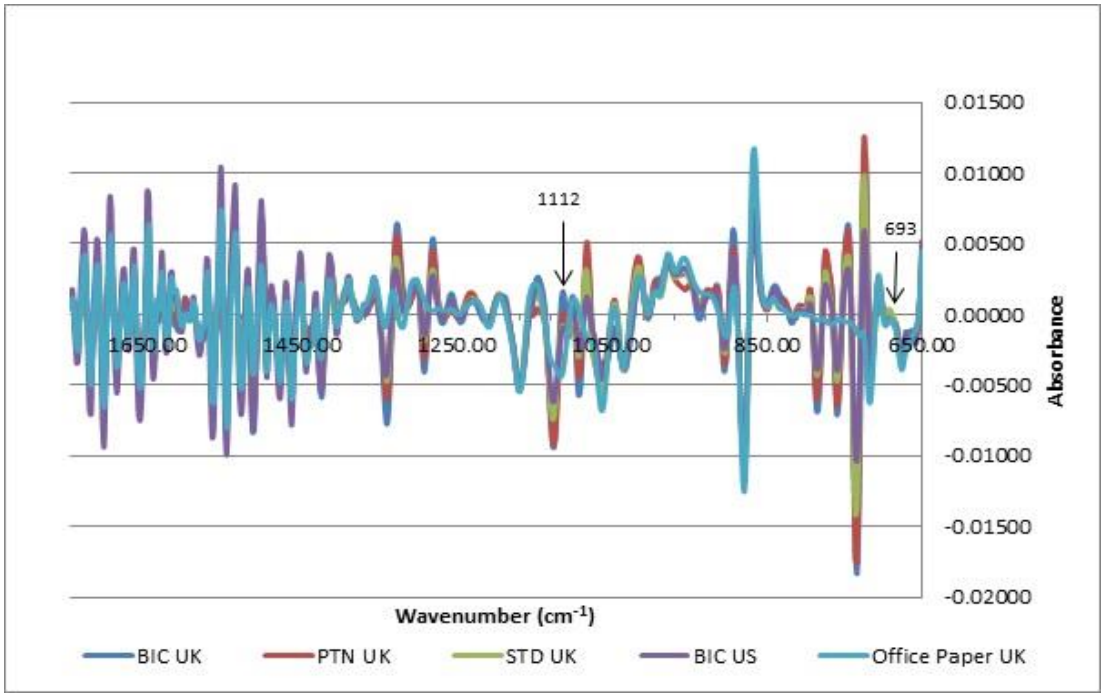


Figure 5.21: Averaged first derivative (FD) spectra representing the four blue gel ink samples forming FD Group 2a

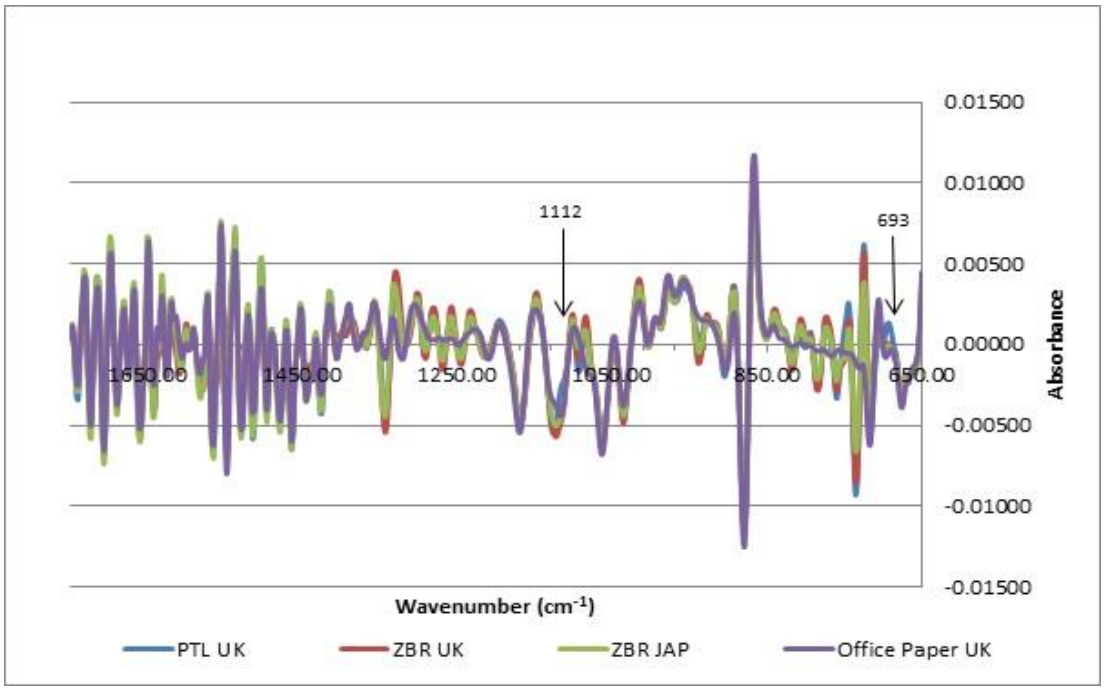


Figure 5.22: Averaged first derivative (FD) spectra representing the four blue gel ink samples forming FD Group 2b

FD Group 3, containing the dye component samples could be discriminated into two sub-groups (FD Group 3a and 3b, shown in Figure 5.23 and 5.24 respectively) by the presence or absence of a peak at ~913 cm^{-1}. In FD Group 3a the PKR UK sample was differentiated by a peak at ~1269 cm^{-1}, but unlike for the original FTIR spectra further discrimination of the remaining four samples in this group was not considered possible.

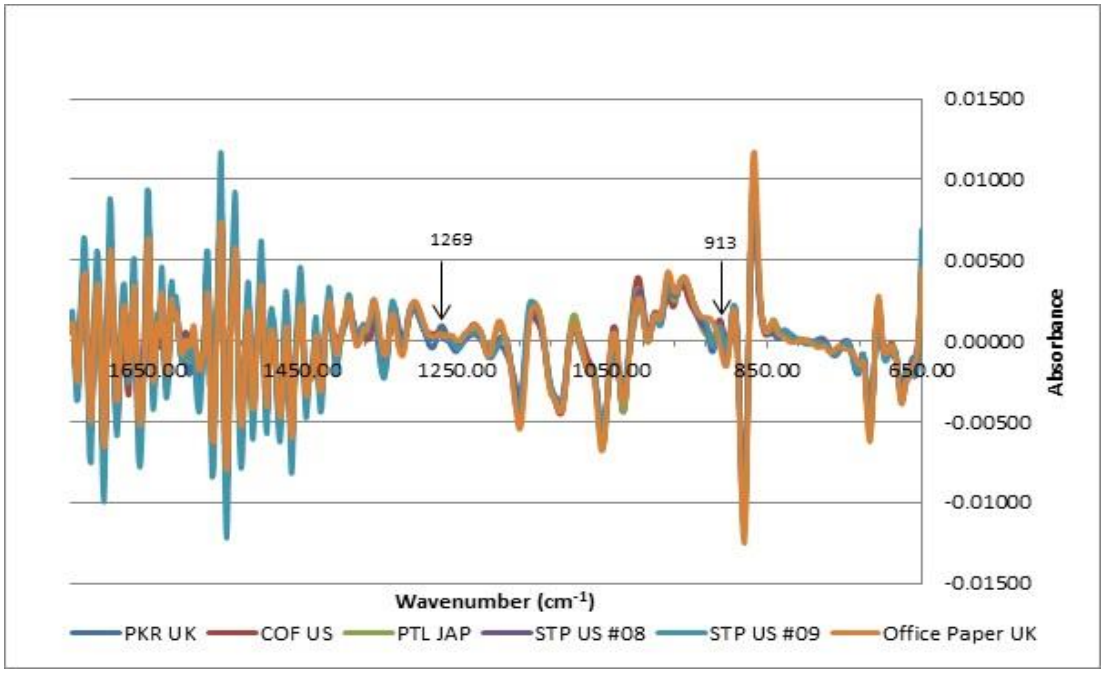


Figure 5.23: Averaged first derivative (FD) spectra of the five brands forming FD Group 3a

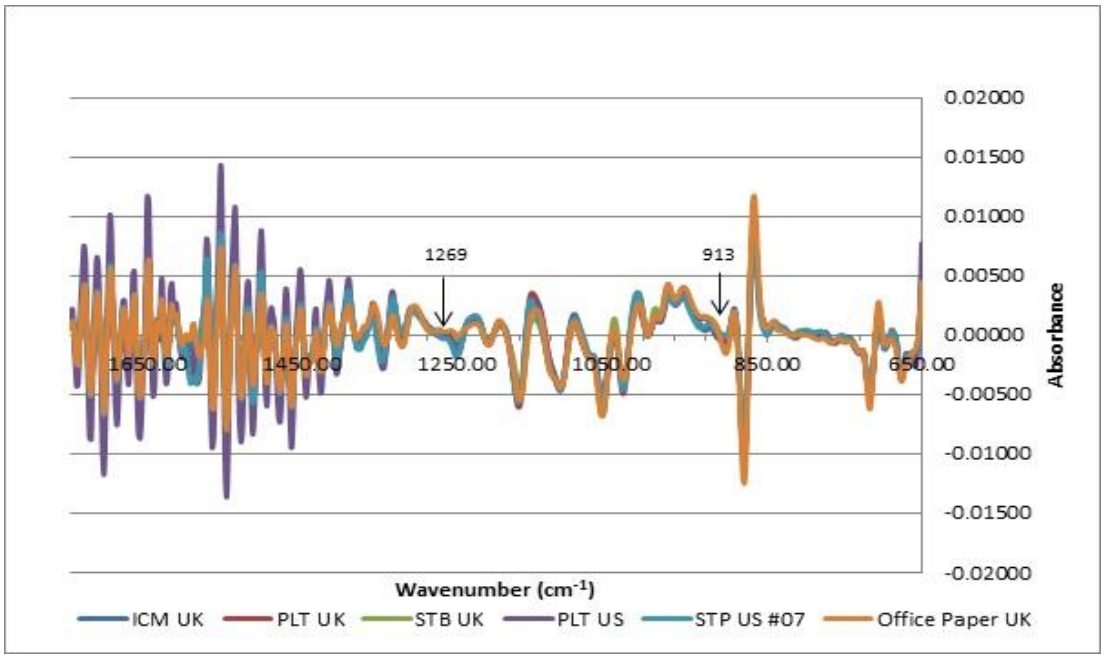


Figure 5.24: Averaged first derivative (FD) spectra of the five brands forming FD Group 3b

5.3.5.2 Red Ink Group

Using the original FTIR spectra only, discrimination of the 26 red ink group samples was split between two Tier levels. T1 discrimination was based solely on the presence or absence of certain peak absorbencies, whilst T2 considered differences in relative peak intensities. Discrimination into a minimum of 14 (T1) and a maximum of 24 distinct groups (T2) was possible as illustrated in Table 5.4.

Colorant	Sample	T1		T2	
		Group	Peaks (cm^{-1})		Peaks (cm^{-1})
Dye Only or Dye & Pigment (Hybrid)	GSF MAL	1	698; **743** 760 shoulder; 807; 820; 840 933 shoulder: 1014: 1127 peak or shoulder: 1252:1269:1286: **1347**: 1364: 1388: 1446: 1487: **1519**: **1550**: **1591**: **1675**	1	RPI of 698: 807: 820: 840: 1014: 1252: 1269: 1286: 1364: 1446: 1487
	UNI AUS			2	
	UNI SA			3	
	STP US #07			4	
	PTL UK			5	
	WHS UK #01			6	
	PPM UK			7	
	UNI UK			8	
	UNI HK #25			9	
	UNI HK #29			10	
	ZBR UK	2	**665**: **697**: 730: **749**: **773**: **822**: 894: 917: **1008**: 1073 shoulder: **1120**: 1148 shoulder: 1243 shoulder: **1256**: **1280**: **1304**: **1368**: 1394 shoulder: **1450**: **1480**: **1502**: **1549**: **1597**: **1675**	11	
	ZBR JAP				
	PLT UK	3	758: **1239**: **1351**: 1534	12	
	PLT US				
	MG MAL	4	698: 747: 760 shoulder: 808: 1250: 1273: 1284: 1349: **1519**: **1552**: **1593**: 1670	13	RPI of 747 & 1349
	STP UK			14	
	FBC MAL	5	695: **754**: **786**: **818**: **1239**: **1258**: 1282: 1444: **1478**: 1494: **1549**: **1595**: **1672**	15	
	STD UK	6	680 shoulder: **700**: **747**: **829**: 905: **928**: 963 shoulder: **1012**: **1062**: **1118**: 1234 shoulder: **1252**: **1282**: 1375s: **1390**: **1450**: **1480**: **1539**: **1552**: **1593**: **1673**	16	
	BIC UK	7	691: **753**: **769**: **816**: 930: 1014:**1082**: **1256**: **1284**: 1440: 1480: 1508: **1552**: **1606**: **1666**	17	
	GRE UK	8	756 shoulder: 797: 816: 928 shoulder: **1248**: 1269: 1284: 1349: 1522: 1543: 1558: 1597: 1672	18	
	ICM UK	9	762 shoulder: 924 shoulder: **1239**	19	
	PTL JAP	10	734 shoulder: 762 shoulder: 924 shoulder: **1237**: 1355: 1452 shoulder: 1552: **1612**	20	
	PTN UK	11	747: 928 shoulder: 1239: **1250**	21	
	WHS UK #04	12	695: **754**: **786**: **818**: **1237**: **1258**: **1543**: **1593**: **1672**	22	
Pigment	UNI JAP	13	**756**: 807 shoulder: **823**: **844**: **1090**: **1142**: **1196**: 1247: 1282 shoulder: **1325**: 1446 shoulder: **1498**: 1554: **1606**: **1644**	23	
	STB UK	14	1247	24	

Table 5.4: Summary of discriminating peaks for the red ink group samples based on original spectra. Bold type indicates strong relative peak intensity (RPI); underlined entries indicate variable intensity across a sample set

The peak positions in bold gave consistently strong peak intensity across all samples, whist those underlined exhibited different intensities across the sample set. If relative peak intensity is considered, then it is possible to discriminate all ten samples within Group 1 (T1), shown in Figure 5.25.

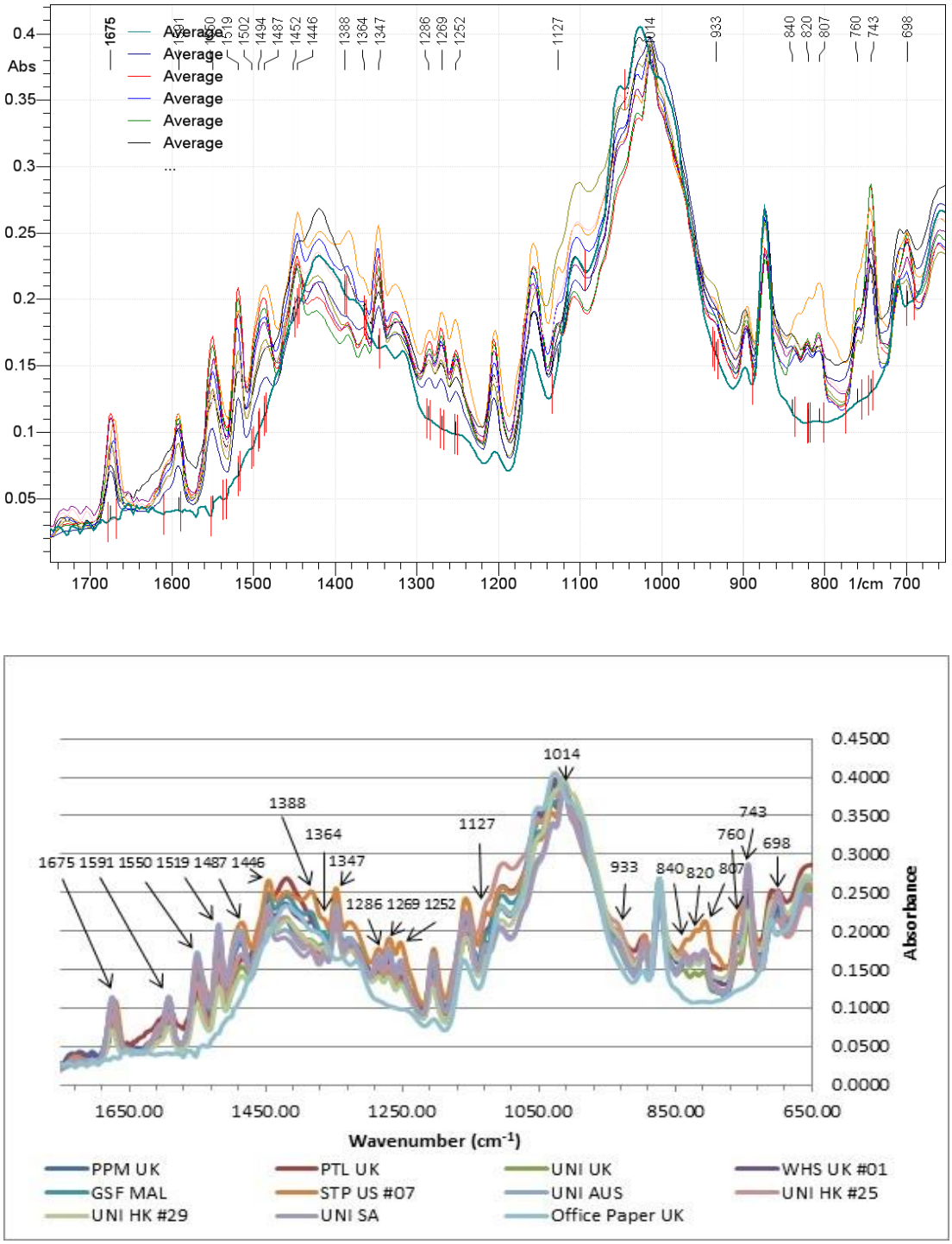


Figure 5.25: Averaged spectra representing the 10 brand/model combinations of red gel ink forming Group 1 (T1). Panorama software chart version (top) and Excel version (bottom)

Group 2 (T1), presented in Figure 5.26, consisted of both ZBR samples, both of which exhibited similar relative peak intensities and as a consequence further discrimination was impossible.

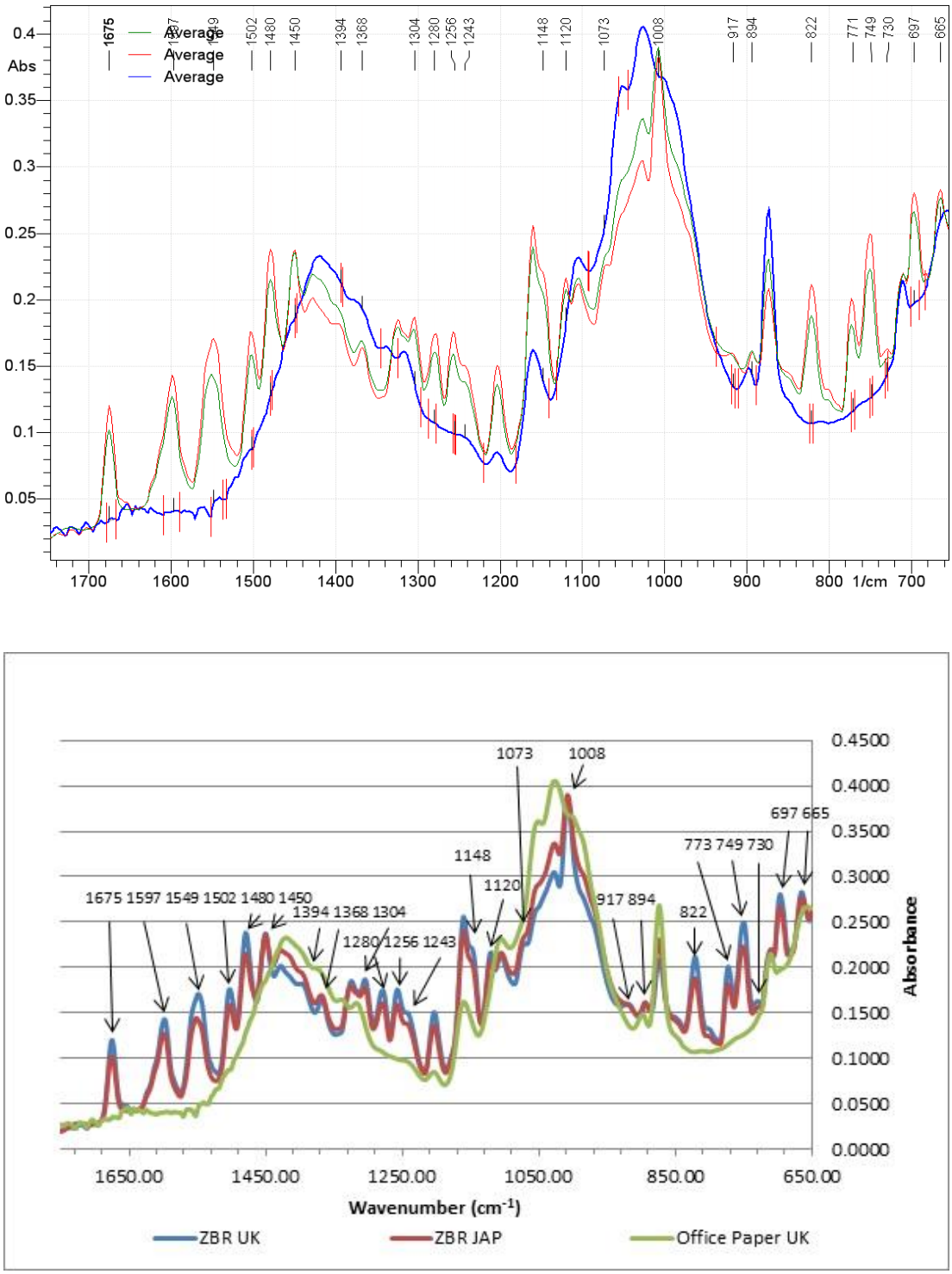


Figure 5.26: Averaged spectra representing the two brand/model combinations (ZBR UK (red spectral line) and ZBR JAP (green spectral line)) of red gel ink forming Group 2 (T1). Panorama software chart version (top) and Excel version (bottom)

Group 3 (T1) contained both PLT samples (presented in Figure 5.27) and revealed two key peaks (~**1239 cm^{-1}** and ~**1351 cm^{-1}**, highlighted within the red circle) which could discriminate the samples from the un-inked paper spectrum.

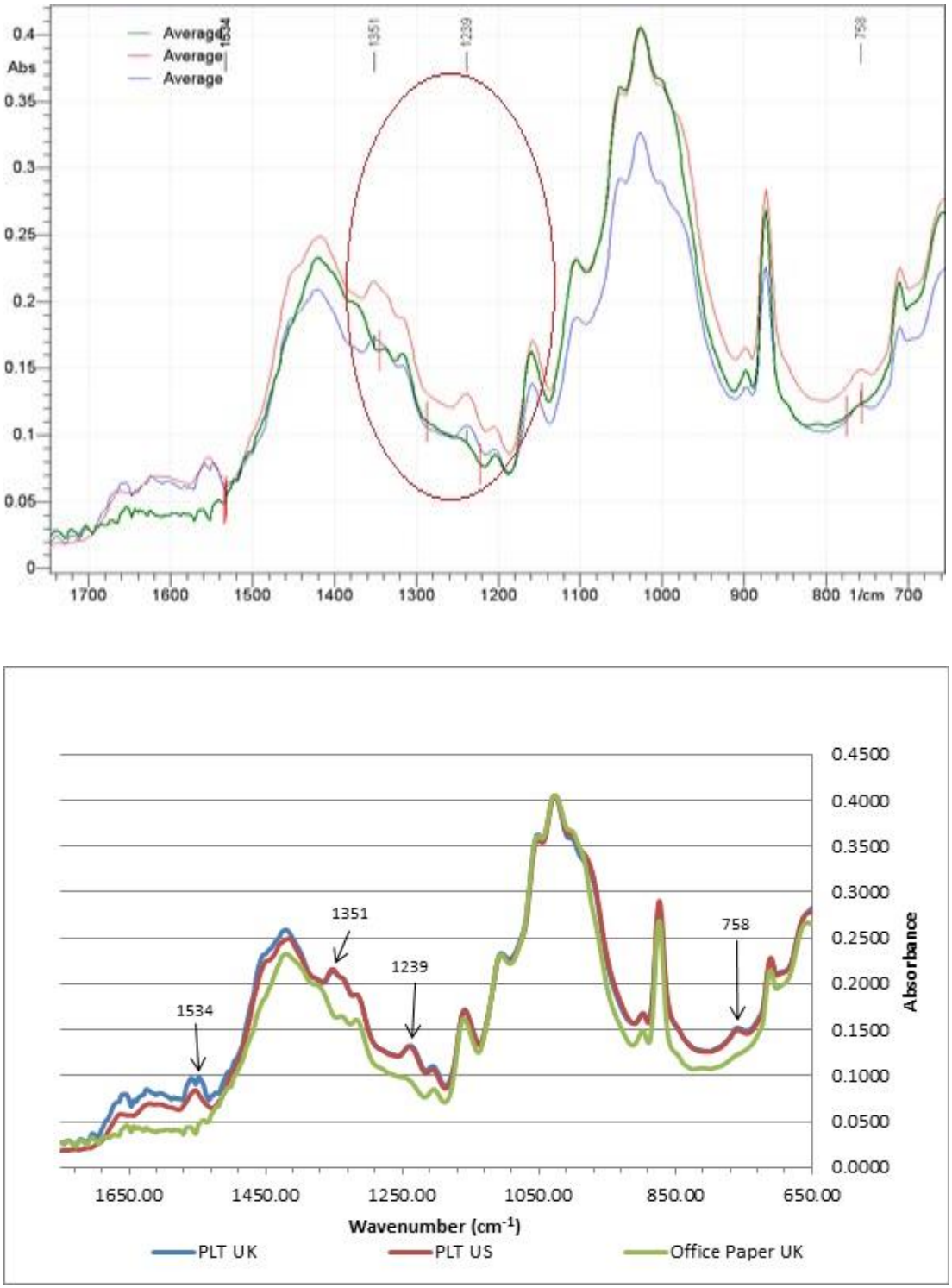


Figure 5.27: Averaged spectra representing the two brand/model combinations (PLT UK (pink spectral line) and PLT US (blue spectral line)) of red gel ink forming Group 3 (T1). Panorama software chart version (top) and Excel version (bottom)

Group 4 (T1), shown in Figure 5.28, consisted of the MG MAL and STP UK samples characterised by peaks at ~698 cm^{-1}, ~<u>747 cm^{-1}</u>, ~760 cm^{-1} (shoulder), ~808 cm^{-1}, ~1250 cm^{-1}, ~1273 cm^{-1}, ~1284 cm^{-1}, ~<u>1349 cm^{-1}</u>, **~1519 cm^{-1}**, **~1552 cm^{-1}**, and **~1593 cm^{-1}** (~1673 cm^{-1}). Discrimination on the basis of differences in relative peak intensities at those peak positions underlined was possible. Groups 5 – 14 (T1) inclusive represented in Figure 5.29, each contained single samples only and were discriminated from all other groups.

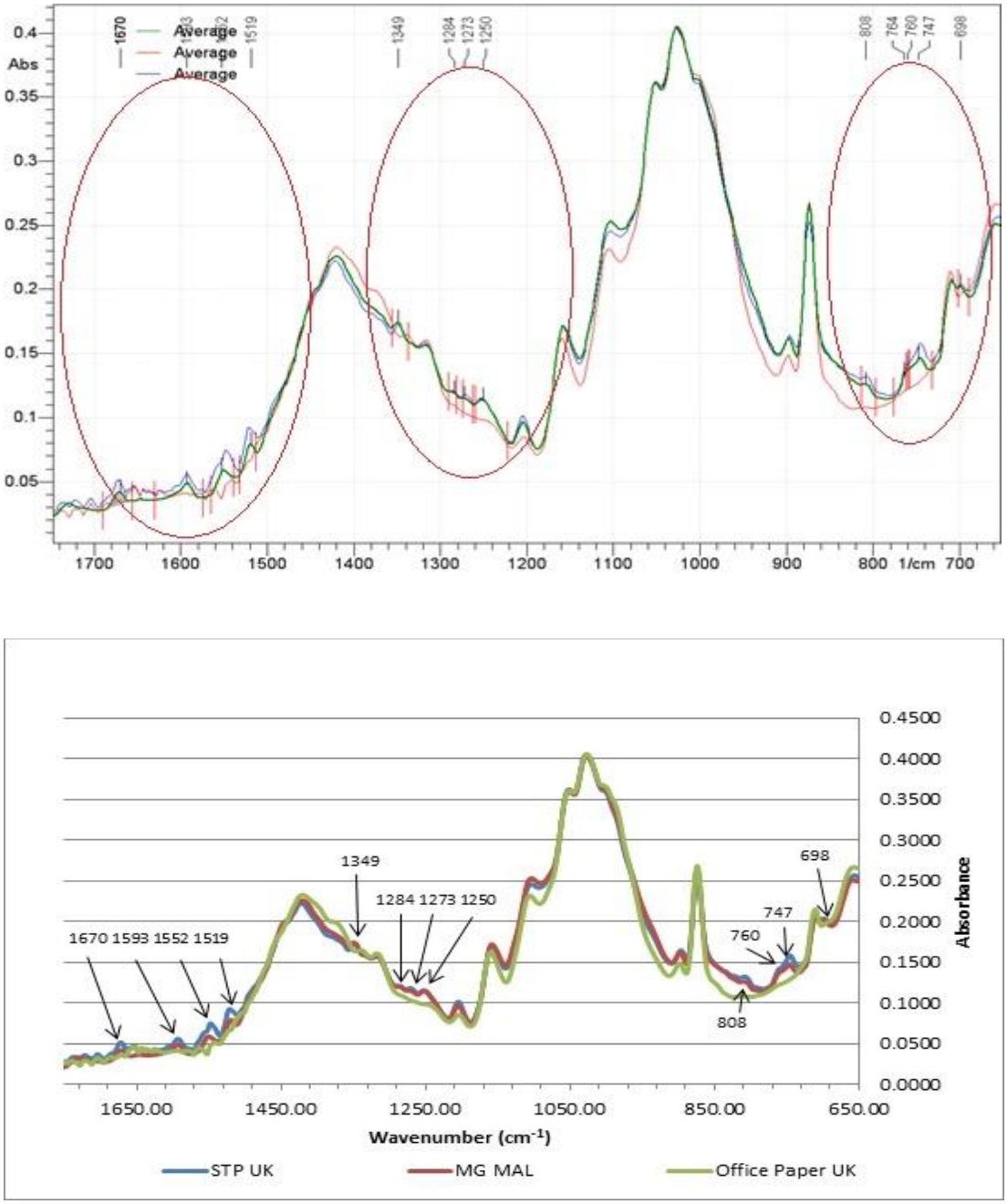


Figure 5.28: Averaged spectra representing the two brand/model combinations (MG MAL (green spectral line) and STP UK (blue spectral line)) of red gel ink forming Group 4 (T1). Panorama software chart version (top) and Excel version (bottom)

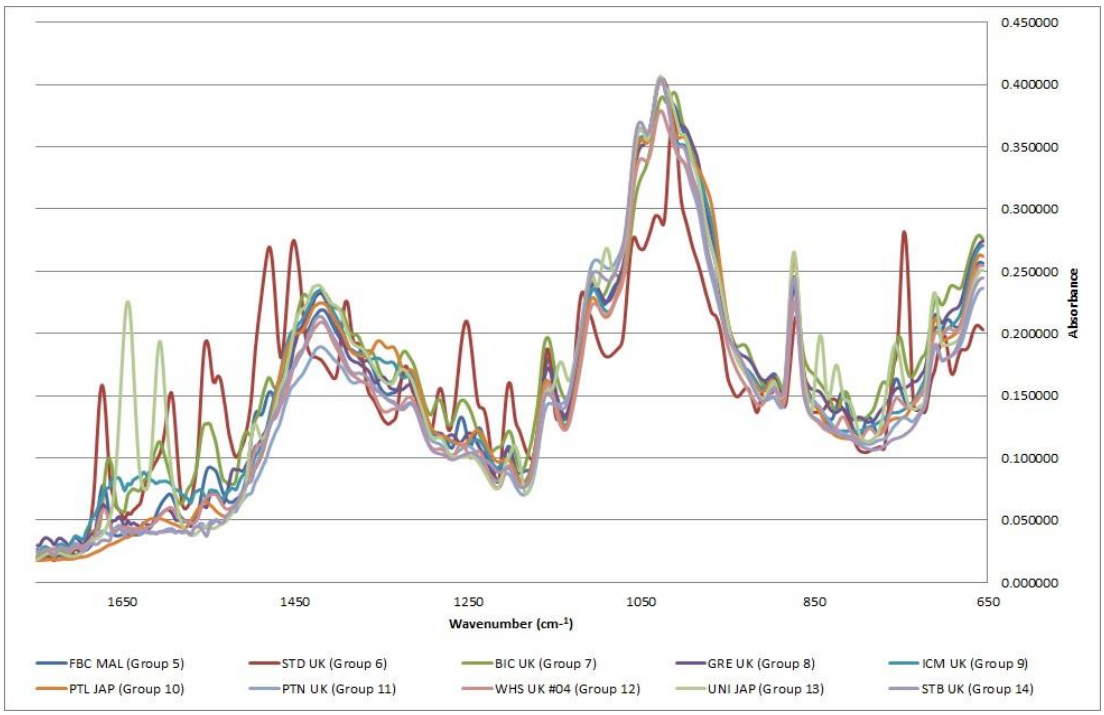


Figure 5.29: Averaged spectra representing the 10 brand/model combinations forming Groups 5 – 14 (T1)

The Solubility and TLC study of the red ink group indicated that only two brand/model combinations were solely pigment based: UNI JAP and STB UK. Both could be clearly discriminated from each other by FTIR-ATR as well as from all other samples in the group. The STB UK sample only exhibited one relatively weak peak at ~1247 cm^{-1} which discriminated the sample from the un-inked paper spectra. The UNI JAP spectrum in comparison to that of the other UNI samples in the red ink group was quite different, as illustrated in Figure 5.30, confirming the findings of the TLC study. The greatest region of difference occurred between 1750 - 1400 cm^{-1} where the UNI JAP brand/model combinations exhibited two absorption peaks of strong intensity at ~1644 cm^{-1} and ~1606 cm^{-1} not seen in the other UNI samples. Several other areas of difference were apparent throughout the fingerprint region.

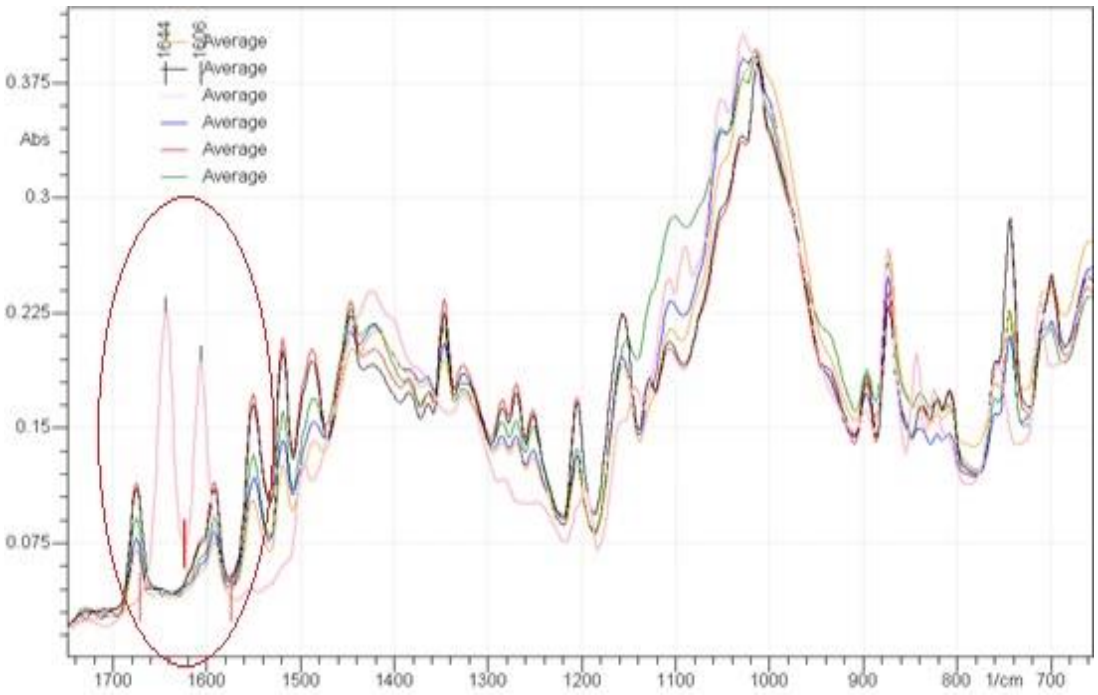


Figure 5.30: Comparison of averaged spectra representing all Uniball red gel ink brand/model combinations demonstrating an obvious spectral difference at ~1644 cm^{-1} and ~1606 cm^{-1} (highlighted within the red circle) between the UNI JAP (pink spectral line) ink sample against all others

Analysis of the first derivative of the averaged spectra permitted classification into the same groups identified from the original FTIR spectra. However small peak differences also provided some discrimination and as such shouldn't be overlooked.

5.4 Conclusion

Use of the A2 Technologies ML Bench-top FTIR-ATR Spectrometer for the analysis and discrimination of gel inks on paper was viable, but only when comparatively large ink spots were used as the sample rather than single ink lines due to reproducibility difficulties experienced with the latter. No instrumental variation was observed, but as expected interference from the paper substrate contributed heavily to the IR spectrum for any given sample. However, peaks attributable to the ink could be identified when individual spectra were compared to that of the un-inked paper. Furthermore, clear spectral differences based on the presence or absence of certain peak absorbencies as well as relative peak intensities predominantly within the fingerprint region permitted discrimination of the samples within each colour group. Good reproducibility permitted the use of an average

spectrum to represent an ink sample, and the use of an 11 point smoothing function enhanced spectral quality with minimal loss of spectral detail to aid comparison.

With one exception, no within brand variation was detected in the samples studied across both colour groups. However, the WHS UK red ink group brand/model combination did show evidence of within brand variation based on the presence or absence of peak absorbencies. Spectra from pens #04 and #05 were considered indistinguishable to each other but distinguishable from that of pens #01, #02, #03 and #06 which were also considered indistinguishable with the arguable exception of pen #06 whose spectral profile was highly similar, but exhibited a significantly higher overall intensity. These within brand differences were corroborated by analysis using other techniques.

In terms of the between brand variation studies, the blue ink group could be discriminated into a minimum of three groups (two pigment based, and one dye containing group (T1)), but up to six groups (T2) with confidence based on the original FTIR spectra. However, further discrimination into a maximum of 17 groups (T4) was considered possible based on weak peak absorbencies. Analysis of the first derivative spectra permitted discrimination of the blue ink group into 6 - 9 groups. Further discrimination within (sub) groups is based on certain weak peak absorbencies that Zieba-Palus and Kunicki [197] suggest are attributable to binders and other additives. Although a smoothing algorithm has been applied, given their relatively weak intensities these peaks cannot be excluded as being attributable to background signal noise. However, these small features were reproducible and as such it seems more likely that they are indicative of real detectable chemical differences between the samples. Without comparison to spectra acquired from the corresponding pure ink sample, it is difficult to say with certainty which is the case and thus further classification based on these weak peak absorbencies should be treated with caution.

The red ink group could be confidently discriminated into at least 14 groups (T1) based on peak presence or absence in the original spectra. Further discrimination into 24 groups (T2) was possible when differences in relative peak intensities are considered. Analysis of the first derivative spectra supports the initial classification into 14 groups, and furthermore discriminated between the MG MAL and STP UK samples. However, the findings of the first derivative in relation to the largest group of (ten) pigmented samples contradicted that of

the original FTIR spectra when difference in relative peak intensities were considered. Based on the latter all ten pigmented samples could be discriminated from each other, when peak presence or absence in the first derivative spectra was considered they could only be further discriminated into five groups to give a maximum number of 20 groups for the red ink group. This highlights the difficulties associated with visual pattern recognition when relatively minor spectral differences are used as an aid to discrimination. The question has to be at what point does a Scientist consider a peak as a significant feature of discrimination or an insignificant spectral difference? Clearly a more objective approach to discrimination and classification of FTIR-ATR spectra may greatly assist in this matter.

Chapter Six

Raman Spectroscopy

6.1 Introduction

The application of Raman Spectroscopy for the analysis of inks, was first suggested by Claybourn and Ansell [121] who focused on the ability to discriminate between black ballpoint inks using a Renishaw system equipped with two excitation wavelengths, 514 nm and 782 nm. Ideal operating conditions, i.e. objective (obj.) magnification, laser power, confocality and sample positioning, were described, and under these, 514 nm was proposed as the optimal excitation wavelength, although the use of multiple wavelengths was advocated. The possibilities of a fully comprehensive spectral library to aid identification and comparison were suggested. Spectral interference from the paper substrate was an issue only at 782 nm, with peaks at ~1092 cm^{-1}, ~1117 cm^{-1}, ~1334 cm^{-1} and ~1377 cm^{-1} attributable to cellulosic components observed in the ink spectra. Caution in the use of spectral processing features, e.g. baseline correction, was urged, although perhaps of only minimal concern due to fluorescence and Raman signals occurring at significantly different scales of spectral resolution.

Raman Spectroscopy for the analysis of gel inks in particular was originally proposed by White [122] who suggested that Surface Enhanced Resonance Raman Spectroscopy (SERRS) could be used for their identification and comparison. Some discrimination was observed between a limited sample of ballpoint and gel pen ink spectra using visual examination and comparison, and where two or more spectra were similar, the relative intensity of the component Raman signal bands provided added discrimination. Whilst ideal excitation wavelengths for the analysis of black (514.4 nm) and blue (632.5 nm) ballpoint inks were suggested, only comments on the difference between spectra at 514.4 nm was discussed with respect to blue gel inks. Likewise, whilst reproducibility of spectra for ballpoint inks was considered good, no mention as to reproducibility for gel inks was provided.

Andermann [203] used a combination of traditional Raman Spectroscopy and SERRS to analyse a larger sample set of different types and colour of ink, including a limited number

of gel inks. Two Raman Spectrometer systems, LabRam Infinity and a Foster & Freeman FORAM 685, combined, equipped with four excitation wavelengths (514 nm, 633 nm, 685 nm and 785 nm) were used to analyse ink lines on white office paper. Two SERRS methods were also employed to assess the discrimination power of SERRS over traditional Raman Spectroscopy. The use of SERRS at 685 nm improved the discrimination power (DP) for some inks studied from ~0.47 using Raman Spectroscopy to ~0.80 using SERRS reagents poly-L-Lysine solution and silver colloid as previously described by White [122]. Andermann [203] also demonstrated that for gel inks, both instruments provided comparable spectra that could be used to discriminate between the inks. Furthermore, it was demonstrated that the use of SERRS reagents poly-L-lysine solution and silver colloid with ascorbic acid solution improved the ability to discriminate between three black gel inks using the FORAM 685. The authors concluded that Raman Spectroscopy when used in conjunction with other analytical techniques was useful for the discrimination of inks. However, they were unable to recommend any specific sample technique or excitation wavelength, suggesting that using a combination of all available wavelengths be used to gather as much spectral information as possible upon which to base the discrimination. In addition, the spectral range of an instrument was highlighted as an important point to consider, since a greater wavenumber range may offer more molecular information to aid discrimination. Andermann [203] also advocated the potential for a spectral database of inks based on either pure ink or dye standards for identification purposes.

Mazella and Khammy-Vital [15] were the first to attempt to suggest a potential analytical protocol for gel inks, using a combination of Raman Spectroscopy, Filtered Light Examination (FLE) and Scanning Electron Microscopy (SEM) to analyse 33 blue gel inks available on the European market, to assess their evidential value and discriminating power. Using visual examination by eye and low power microscopy, the 33 gel inks were initially discriminated into three groups, including one containing 17 blue inks. This group was subjected to FLE, SEM and Raman Spectroscopy. A Renishaw RM1000 Spectrometer system equipped with x 50 obj. and two excitation wavelengths (514.4 nm and 830 nm) was used. The authors found that six inks did not yield a spectrum, which they suggested may have been because they contained dyestuffs rather than pigments, which was subsequently confirmed by Thin Layer Chromatography (TLC). The remaining 11 gel inks were discriminated into groups based on their Raman spectra, the number of which was

dependent upon the excitation wavelength used. The greatest number of groups, five, was provided by using a combination of both wavelengths, thus providing supporting evidence for Andermann's [203] suggested multi-wavelength approach. In isolation, Raman Spectroscopy provided a discriminating power of 0.76, but the best discriminating power, 0.91, was achieved using all three techniques together, again highlighting the advantage of using a combined analytical sequence (individual discriminating power for FLE (0.72) and SEM (0.88)). Mazella and Khammy-Vital [15] constructed a preliminary ink database using the Raman spectra produced, leading to correct identification of a small number of pens. In addition, they expanded further on the possibility of a spectral database, discussing its feasibility and limitations. They concluded it would be impossible to maintain a fully comprehensive database of all the globally produced gel ink formulations, therefore the evidential value of any identification from such a library search would be limited.

Jones and Wolstenholme [196] analysed a selection of blue and black gel inks available on the UK market, using Infrared-Attenuated Total Reflectance (IR-ATR), Raman Spectroscopy and SERRS. The results do not appear in the literature however, it was observed that despite spectral interference from the paper substrate, a strong spectrum from the ink with minimal interference was observed in the fingerprint region (650 cm^{-1} – 1600 cm^{-1}). The authors suggested that this was sufficient to be able to compare and discriminate between spectra based on peak position and relative peak intensity.

Florence *et al* [13] investigated the combined use of Raman Spectroscopy and elemental analysis by Energy Dispersive X-Ray Spectrometry (EDS) to analyse eight brands of gel inks representing various colours, including blue and red. Unlike previous studies, samples were analysed as ink lines on filter paper to reduce the risk of spectral interference from the paper substrate. An excitation wavelength of 514 nm was used. No comment was made on discrimination of the blue gel inks, other than the predominant pigment was identified as copper phthalocyanine on the basis of several key Raman peaks (distinct peaks at ~1528 cm^{-1}, ~1450 cm^{-1} and ~1339 cm^{-1}, as well as peaks of variable intensity at ~1142 cm^{-1}, ~680 cm^{-1} and ~593 cm^{-1}). Red gel inks were swamped by fluorescence, and could therefore not be discriminated. Florence *et al* [13] concluded that an approach using multiple techniques was "*most helpful*" and that the techniques of Raman Spectroscopy and EDS provided complimentary information.

Mazella and Buzzini [27] further investigated Raman Spectroscopy for the analysis of 55 blue gel inks from a variety of different brands and models available on the global market. Methanol was used to perform an initial solubility test to distinguish between dye based and pigment based gel inks. The remaining 36 pigment based gel inks were analysed using a Renishaw Raman RM1000 Spectrometer System, equipped with two excitation wavelengths (514.4 nm and 830 nm) and x 50 obj. The authors suggested that TLC maybe suitable to analyse the 19 dye based gel inks, although this was not performed. The discriminating power for the 514 nm excitation wavelength was 0.62 compared to 0.55 for the 830 nm excitation wavelength. These two wavelengths combined gave a discriminating power of 0.68, which was lower than a previously published discriminating power of 0.76 [15]. In agreement with Andermann [203] and others [13, 15, 181], the authors recommended Raman Spectroscopy should be used in conjunction with other analytical techniques for the discrimination of gel inks.

Mazella and Buzzini [27] also created preliminary databases of five known pigment standards and of the Raman Spectra generated for their samples to attempt identification of the pigment composition and the ink brand/model itself. As expected, copper phthalocyanine (Pigment Blue 15) was confirmed as one of the main pigment components, but Pigment Violet 23 was also identified and its Raman bands reported. The 514 nm excitation wavelength was better suited to identification of pigment mixtures than the 830 nm excitation wavelength, which was only capable of identifying Pigment Blue 15. This demonstrated the importance of running library searches using spectral data acquired from two or more excitation wavelengths. In line with a previous study [15], correct identification of a small number of pens was achieved using the ink database.

Unlike Jones and Wolstenholme [196] no significant spectral interference arising from the paper substrate was observed in the spectra taken in Mazella and Buzzini [27], although only a limited number of paper types were used. It was also noted in this study that for gel ink pens of the same brand and model purchased in different geographical locations, Raman spectra were the same, indicating that subtle differences in ink formulations tailored for the end user climate may not be detectable by this technique.

Zieba-Palus and Kunicki [197] used a combination of Raman Spectroscopy, Micro-FTIR and X-Ray Fluorescence (XRF) to analyse 69 ink samples including a small number of blue and black gel inks. A FORAM 685-2 was used, but for 27 ink samples fluorescence obscured the Raman spectrum, which was a particular problem for the gel inks. It is possible that these gel inks contained dyes rather than pigments which could explain the fluorescence problem. It was not clear if any attempt had been made to vary laser power intensity at which these gel inks were analysed, but it may be that doing so could have significant impact on spectral quality. The remaining 42 ink samples all provided good quality Raman spectra. The authors suggested that as well as peak position and relative peak intensity, the course and shape of the background curve should also be taken into account. Good discrimination was possible using spectral data from Micro-FTIR and Raman Spectroscopy combined, with further discrimination possible using the elemental data from XRF. When the data from Micro-FTIR and Raman Spectroscopy were combined the discriminating power of the limited number of gel inks studied was calculated to be ~0.90 dependent upon colour.

Zieba-Palus *et al* [204] also investigated a prototype instrument (Praxis) capable of performing both μ-Raman and μ-XRF analyses of ink on paper. Whilst Raman Spectroscopy has been investigated in depth, elemental analysis of gel inks is a comparatively under explored area, although is starting to receive more attention [127], but will not be discussed further here. Automobile paint, fibres, plastics as well as inks were analysed using this dual mode instrument. In terms of ink, 80 ballpoint and gel ink pens in blue, red and black were examined using 633 nm excitation wavelength. Fluorescence was a significant problem with spectra from around half of the samples obscured. However, for the remaining samples, discrimination of the blue gels (~70% of samples) and red inks (~80% of samples) by visual comparison of peak positions and relative peak intensities was possible. Spectral interference from the paper substrate was not observed, and Raman Spectroscopic analysis was found to perform better for ballpoint inks than gel inks supporting the view of previous studies [27, 197]. Zieba-Palus *et al* [204] suggested that spectra were comparable to those from other instruments or perhaps even slightly better owing to better spot resolution and focussing. Using a pigment spectral database, phthalocyanines were again confirmed as the main pigment component of blue gel inks. The use of fluorescence as a feature of discrimination was highlighted in cases where two

samples give a markedly different spectral response. In addition, it was suggested that where fluorescence background is high, difficulties maybe encountered when attempting to identify the pigment from a library search. The authors suggested that the addition of one or more excitation laser wavelengths to the Praxis instrument may improve its analytical capabilities with respect to Raman Spectroscopy since it has been observed that fluorescence of a sample is influenced by the incident laser radiation upon it. Excitation wavelengths of 514 nm, 780 nm and 830 nm were suggested, with special attention focussed on 780 nm, which the authors pointed out had already been demonstrated to provide better spectra to that of a 632 nm excitation wavelength for dyes contained within fibre samples [196]. In addition, Zieba-Palus *et al* [204] promoted the idea of a combined analytical approach using Raman Spectroscopy and XRF in conjunction with IR Spectroscopy to provide information on colorant, elemental, and resinous composition respectively.

The overall aim of this study was to investigate further the potential of Raman Spectroscopy for the discrimination of gel inks within each of the two colour groups, as well as its ability to detect within brand variation. Optimum instrumental conditions in terms of laser power intensity and objective magnification for a given colour group at each of five specific excitation wavelengths (514.5 nm, 632.5 nm, 685 nm, 785 nm and 830 nm) were determined, as well as the development of a validated sampling and analysis protocol. The appropriate conditions were then applied to all samples within a colour group to assess discriminating capabilities, both by visual pattern recognition and multivariate profiling. Furthermore, a comparison of two different Raman Spectrometer systems using a 785 nm excitation wavelength on both colour group samples was made, together with an attempt to identify their pigment component(s) using an in-house purpose built spectral library composed of a large number of known pigment standards.

6.2 Part A – Method Development

6.2.1 Experimental

Raman spectra were recorded using a variety of instruments from two different manufacturers. In total, spectra were recorded across five excitation wavelengths which facilitated both an investigation of different wavelengths within a single instrument manufacturer and the direct comparison of different manufactured instruments at the

same wavelength (785 nm). Spectral data from all instruments was copy and pasted into Microsoft Excel 2007 or 2010 for presentation purposes and/or subsequent pre-processing prior to multivariate statistical profiling.

6.2.1.1 Instrumental Conditions

6.2.1.1.1 Renishaw Instruments

Spectral acquisition was performed using two Raman Spectrometer systems; a Renishaw InVia single wavelength (λ) system providing an excitation at 514.5 nm coupled to a research grade Leica DM/LM microscope fitted with a UIS x 20 (Numerical Aperture (N.A.) 0.40) and x 50 (N.A. 0.75) obj. where dielectric edge filters were used to reject Rayleigh scattered light; and, a Renishaw InVia multi-wavelength system equipped with three laser excitation sources coupled to a research grade Leica DMI 5000M Inverted microscope fitted with a Renishaw H117 inverted prior stage. All spectral measurements were performed using a Leica x 50 (N.A 0.50) or Leica x 100 (N.A. 0.75) obj. Dielectric edge filters were used to reject Rayleigh scattered light.

Table 6.1 gives details of each of the laser sources used in the Renishaw InVia single and multi-wavelength instruments.

Instrumental	Laser Source	Maximum Power Output at Sample (mW)	Excitation Wavelength (nm)
Single λ	Ar+	9.8	514.5
Multi λ	HeNe	13	632.5
	NIR diode	150	785
	NIR diode	160	830

Table 6.1: Summary of laser sources, maximum power output at sample and excitation wavelength details for Renishaw InVia Single and Multi-wavelength systems

Measurements were recorded either over the full spectral range (100 – 3200 cm^{-1}) or a manually selected spectral range (100 – 2000 cm^{-1}) of the instrument in extended scan mode. Spectra were acquired using the default settings of 10 second exposure time, 1 accumulation and the instruments operated in standard (non-confocal) mode unless

otherwise stated. Fixed laser power settings of 100%, 50%, 10% and 1% were used to acquire spectra.

Performance checks were conducted prior to any sample measurements each day by recording the spectrum of a silicon (Si) standard to ensure an expected sharp peak at 520 cm^{-1} was observed. If required, the instrument was re-calibrated against the Si standard using a "Quick Calibration" function.

All spectral measurements were initially recorded using Renishaw WIRE 2.0™ or WIRE 3.0™ software for excitation wavelengths 514.5 nm and all others respectively. Spectral processing, i.e. baseline correction, normalisation and/or curve fitting was performed using Thermo Galactic GRAMS AI 7.00™ software.

6.2.1.1.2 Foster and Freeman Instruments

The spectral measurements were performed on one of two Raman Spectrometer systems, a FORAM 685-2 and a FORAM 785.

Both systems were equipped with x 5, x 10 and x 20 objectives, the former recommended for sample positioning only [205]. The FORAM 785 was also equipped with an optional x 50 obj., not currently supplied as standard by the manufacturers. All measurements were recorded over the full spectral range (400 – 2000 cm^{-1}) of the instrument using an average of six spectral counts to improve signal to noise (S:N) ratio. The integration time was either manually selected, usually the 1.0s default scan time, or automatically detected using the Auto Exposure function to provide optimal signal intensity for a given sample. Fixed laser power settings of 100%, 25% and 10% were used to acquire spectra. The specific details of the laser sources used in the FORAM instruments are presented in Table 6.2.

Instrument	Laser Source	Maximum Power Output at Sample (mW)	Excitation Wavelength (nm)
FORAM 685-2	Ondax Single Mode Diode	4.5	685
FORAM 785		2.5	785

Table 6.2: Summary of laser source, maximum power output at sample and excitation wavelength details for the FORAM 685-2 and 785

Prior to recording spectral measurements each day, the instrument was calibrated using a polystyrene bead. All spectra were initially recorded using the FORAM software supplied with the instrument, as were all baseline correction and smoothing functions performed.

6.2.1.1.3 Stray Peaks

Occasionally stray peaks were observed in a spectrum that were attributable to either stray light entering the spectrometer system, or the laser hitting a contaminant in the sample during spectral acquisition. These stray peaks were removed from the Renishaw InVia spectra in one of two ways. In the first approach, spectral measurements for those samples that exhibited a stray peak were repeated. The y-axis co-ordinates for these spectral measurements were copy and pasted into the original data set to enable comparison of all spectral measurements for a given brand/model combination on the same axis. A slight shift (i.e. <1 cm^{-1}) in wavenumber position (x-axis co-ordinates) was observed. In the second approach, stray peaks were removed using the "zap" function incorporated into the WIRE 2.0™ instrument software. This was done either during spectral acquisition or subsequently when observed. Both approaches were used for the blue ink group at 785 nm and the first approach only at 830 nm. The second approach only was adopted for the red ink group at 785 nm. Stray peaks could not be removed from FORAM spectra, but where appropriate spectral measurements were repeated at original time of analysis.

6.2.1.2 Sample Preparation

All method development and validation work was performed using a Pentel Hybrid Gel DX Rollerball (PTL UK) ink pen, representing both colour groups, chosen specifically on the basis that it is marketed as a "pigmented gel ink" and would therefore be expected to give a Raman response. Samples of ink were analysed on paper and glass substrates, the latter to give an indication of the spectrum expected for the pure ink free from any potential interference from a paper substrate.

6.2.1.2.1 Ink on Glass

Samples of ink on glass were deposited either as single lines, concentrated spots or smears (~5 – 8 mm diameter) onto a microscope slide. The line or spot were drawn directly onto the surface of the slide, whilst the smear was transferred indirectly via a semi-gloss paper medium. An ink spot was drawn directly onto the paper which was then pressed down

firmly onto the surface of the slide and pressure applied by hand for ~10 seconds to achieve sufficient transfer of ink to generate a Raman signal suitable for comparison.

6.2.1.2.2 Ink on Paper

Samples of ink on paper were presented either as single lines (~2 – 5 cm long), dots (~1 mm diameter) or spots (~5 mm diameter) drawn directly onto commonly encountered white A4 office paper (80 gsm). Due to differences in instrument design, sample sheets required further preparation to achieve correct sample positioning in order to avoid de-focussing of the laser beam during spectral acquisition. This was achieved either by cutting the paper into strips (~2 – 3 cm long) and securing it to microscope slide with sellotape (Renishaw), or by cutting the sheet in half and placing it directly onto the XYZ translation stage of the instrument, securing it in place with two magnetic strips (FORAM) supplied by the manufacturers.

All samples were allowed to air dry at room temperature for a minimum of 15 minutes prior to analysis. All ink samples were stored in the dark prior to analysis and were analysed within 72 hours (i.e. 3 days) of deposition on the substrate.

A set of spectral measurements (reproducibility measurements) were acquired from six different areas of the un-inked paper and/or glass slide to assess spectral interference from the substrate for all experimental conditions.

6.2.1.3 Method Development for Renishaw InVia

Initial method development was performed using the single wavelength system, i.e. 514.5 nm excitation wavelength.

6.2.1.3.1 Expected Ink Spectrum

To gain an appreciation of the spectrum expected for each of the two PTL UK ink colours, a set of reproducibility measurements (n = 6) from ink spots on a glass slide representing both colour groups and analysed at full laser power intensity (100%) under x 20 obj. were acquired. Lower laser power settings of 50% and 10% were also applied to the red ink spot as single spectral measurements.

6.2.1.3.2 Influence of Laser Power Intensity

Fluorescence masking weak Raman signals is an inherent problem associated with this technique, but controlling the amount of radiation hitting the sample can reduce its effects. To assess the influence of varied laser power on spectral quality (S:N ratio) and reproducibility for a particular colour group, six measurements from different areas of a PTL UK ink line on paper, representing both colour groups, at three laser power settings (100%, 50% and 10%) were acquired under the x 20 obj. using the single wavelength system. Based on these findings, a systematic study of varied laser power intensity was performed using the multi-wavelength system. Ink lines on paper and ink spots on a glass slide representing both colour groups were analysed six times at each wavelength (632.5 nm, 785 nm and 830 nm) using three laser power settings (100%, 50% and 10%) under x 50 obj.

6.2.1.3.3 Further Investigations of the PTL UK Blue Ink

6.2.1.3.3.1 Influence of Objective Magnification, Confocality and Sample Size

Based on the preliminary findings of varying laser power intensity using the single wavelength system, studies into the influence of objective magnification, Confocality and sample size on spectral quality and reproducibility were conducted on the PTL UK blue gel ink at 10% laser power intensity. A set of reproducibility measurements (n = 6) for an ink line on paper and ink spot on glass under both x 20 and x 50 obj. were recorded to study the influence of objective magnification. A set of reproducibility measurements (n = 6) for an ink line on paper under the x 50 obj., operating the instrument in both standard and high confocal mode, were recorded. The x 50 obj. was chosen since Confocality is known to work better under higher magnifications [121]. For the sample size study, a set of reproducibility measurements (n = 6) under x 20 obj. were recorded for each of an ink line on paper, and an ink line, spot and smear on glass. In addition, for an ink dot on paper, a single measurement from each of six dots was recorded due to the limited available sample size.

6.2.1.3.3.2 Further Investigation of 632.5 nm Excitation Wavelength

Based on the preliminary findings of varying laser power intensity using the multi-wavelength system, further investigation of the suitability of the 632.5 nm excitation wavelength for the discrimination of blue gel inks was undertaken. Ink lines on paper from a single pen representing 27 of the 31 blue ink group brand/model combinations were

analysed twice along the length using 10% laser power and x 50 obj. The following four brand/model combinations, all dye containing, were not analysed due to ink dry out: PLT US, STP US #07, STP US #08 and STP US #09.

6.2.1.3.4 Further Investigations of the PTL UK Red Ink

Based on the preliminary findings of varying laser power intensity using the single wavelength system, further investigation of the suitability of the single excitation wavelength system for the discrimination of red gel inks was undertaken. Ink lines from a single pen representing each of 13 UK red gel ink brand/model combinations were analysed once each using 10% and 1% laser power and x 20 obj.

6.2.1.3.4.1 Influence of Objective Magnification – Red Ink 785 nm Only

Based on the preliminary findings of varying laser power intensity using the multi-wavelength system, further investigation of using a higher powered objective magnification in an attempt to enhance the Raman signal from red gel inks at 785 nm, and hence improve the overall Raman spectrum obtained, was undertaken. Ink lines from a single pen from each of three red gel ink brand/model combinations (PTL UK; PLT UK and ICM UK) were analysed six times at different points along the length using 100% laser power and x 100 obj.

6.2.1.4 Validation of Renishaw InVia Instruments

6.2.1.4.1 Single Wavelength System (514.5 nm Excitation Wavelength)

Initial validation of the analysis of blue gel ink on paper was performed using the spectral data acquired from the ink line and dot on paper as well as the ink line, spot and smear on the glass slide using the 514.5 nm excitation wavelength. Each set of spectral data was baseline corrected and normalised with respect to the tallest peak observed. A curve fitting function was used to calculate the peak centre wavenumber position (i.e. the Raman Shift) for eight randomly selected peaks. These peak wavenumber positions were used to calculate the % Relative Standard Deviation (% RSD) using Equation 6.1 for each of the eight peaks in a set of spectral measurements for each sample size (i.e. ink line on paper).

$$\% RSD = \frac{s}{\overline{x}} \times 100$$ Equation 6.1

Where s is standard deviation and $\overline{x}$ is mean value

6.2.1.4.2 Multi-Wavelength System (632.5 nm/785 nm/830 nm Excitation Wavelength)

A revised approach to validation of the method for the analysis of blue (785 nm and 830 nm) and red (785 nm) gel ink lines on paper was adopted. This revised approach excluded baseline correction and normalisation, and used an automatic peak picking function to determine wavenumber position. This approach was compared against the original approach using the blue ink data at 785 nm. The % RSD values of eight randomly selected peaks for both approaches were calculated and compared.

6.2.1.5 Method Development for Foster and Freeman FORAM

To determine the ideal conditions for acquiring good quality reproducible spectra for a particular colour of ink, an investigation of the influence of varied laser power intensity and objective magnification was carried out using both FORAM instruments. The influence of integration time through a comparison of manually (default 1.0s scan time) and automatically (Auto Exposure mode) selected integration times on Raman signal intensity was also considered.

6.2.1.5.1 Influence of Laser Power Intensity

A set of reproducibility measurements (n = 6) from PTL UK ink lines on paper and ink spots on glass slide representing both colour groups were acquired using both x 10 and x 20 objectives and all three laser power settings (100%, 25% and 10%). In addition, for the FORAM 785 only, a set of reproducibility measurements (n = 6) from ink lines on paper representing both colour groups were acquired under x 50 obj. and all three laser power settings. All spectra were recorded using the 1.0s default manual integration time.

6.2.1.5.2 Influence of Higher Objective and/or Auto Exposure

Based on the findings in relation to laser power/obj. combination, the influence of integration time, i.e. Auto Exposure, on Raman signal intensity was investigated.

6.2.1.5.3 FORAM 685-2

A set of two measurements were taken from different areas within a PTL UK ink line on paper representing both colour groups. For the PTL UK blue ink line, spectra were recorded using 10% laser power and x 20 obj., whilst for the PTL UK red ink lines spectra were recorded using 25% laser power and x 20 obj.

6.2.1.5.4 FORAM 785

A set of reproducibility measurements (n = 6) were taken from different areas within a PTL UK ink line on paper representing both colour groups. All spectra were recorded using full (100%) laser power under both x 20 and x 50 obj.

Both instruments were operated in Auto Exposure mode to provide optimum signal intensity for a given ink sample. Spectra were compared to the corresponding spectra acquired under 1.0s default manual integration time.

6.2.1.6 Validation of Foster and Freeman FORAM Instruments

Validation for the analysis of PTL UK blue gel ink lines on paper using FORAM 785 was performed. The blue ink group method development data set was baseline corrected and smoothed using the FORAM software. The labelling function was used to determine the peak position of eight randomly selected peaks. These values were used to calculate the % RSD of the peaks.

6.2.2 Results & Discussion

6.2.2.1 Renishaw InVia

6.2.2.1.1 Single Wavelength System (514.5 nm Excitation Wavelength)

6.2.2.1.1.1 Ink on Glass Slide – Expected Ink Spectrum

The PTL UK blue ink spot exhibited a detailed Raman spectrum with several obvious Raman bands spanning the wavenumber region 200 cm^{-1} – 1700 cm^{-1} as illustrated in Figure 6.1. Good signal to noise (S:N) ratio and reproducibility was observed.

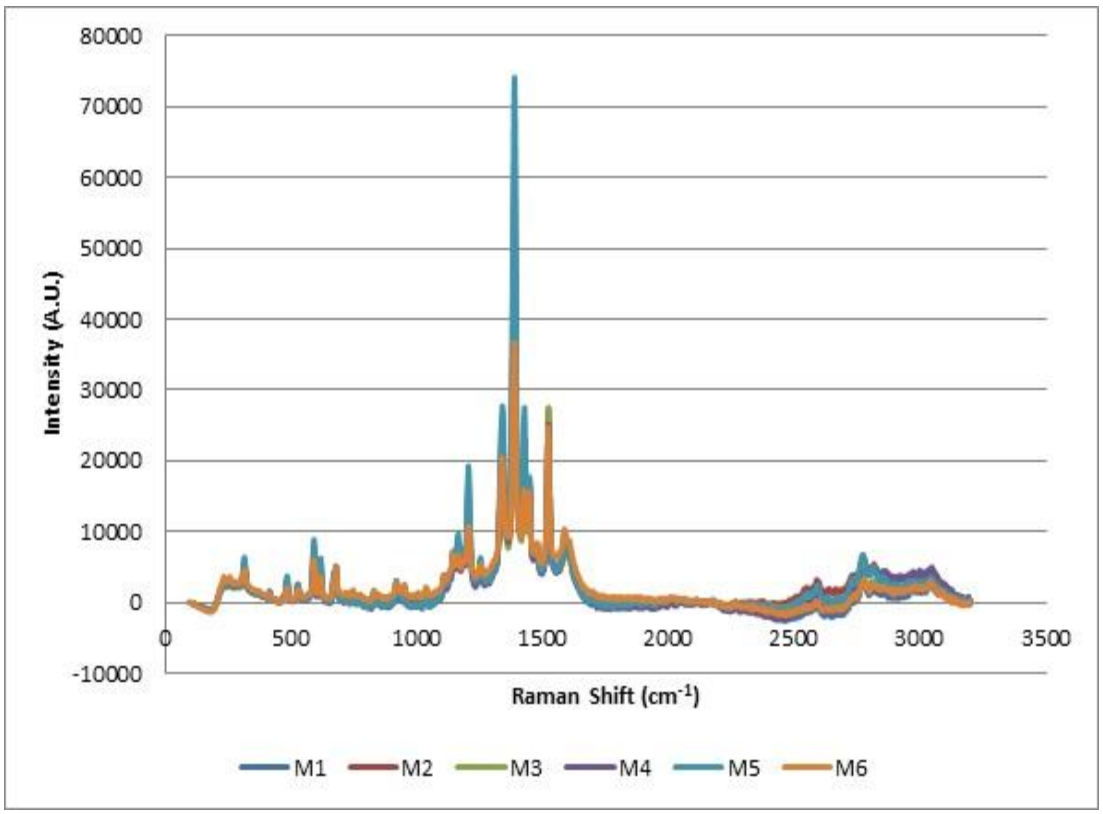


Figure 6.1: Reproducibility measurements (n = 6) taken from different areas of a PTL UK blue gel ink spot on glass slide demonstrating good reproducibility and S:N ratio (Renishaw InVia 514.5 nm 100% laser power/x 20 obj. [M = Measurement])

At full laser power intensity, the PTL UK red ink spot spectrum was fully obscured by a combination of fluorescence and detector overload (i.e. too much scattered light saturating the detector). Reducing laser power to 50% and 10% did not resolve this issue, suggesting that this excitation wavelength may not be suitable for the analysis of red gel inks in line with previous observations [13].

6.2.2.1.1.2 Further Investigations of the PTL UK Blue Gel Ink

6.2.2.1.1.2.1 Ink on Paper - Influence of Laser Power Intensity

The 10% laser power setting was found to yield a spectrum of the blue ink line exhibiting optimal S:N ratio and reproducibility, with no spectral interference from either substrate.

6.2.2.1.1.2.2 Comparison of Ink on Paper against Ink on Slide

Only the blue sample exhibited similar spectra between substrates, and by visual comparison was considered indistinguishable in terms of peak position. Baseline correction

and normalisation of the spectra revealed that relative peak intensities could also be considered indistinguishable as illustrated in Figure 6.2.

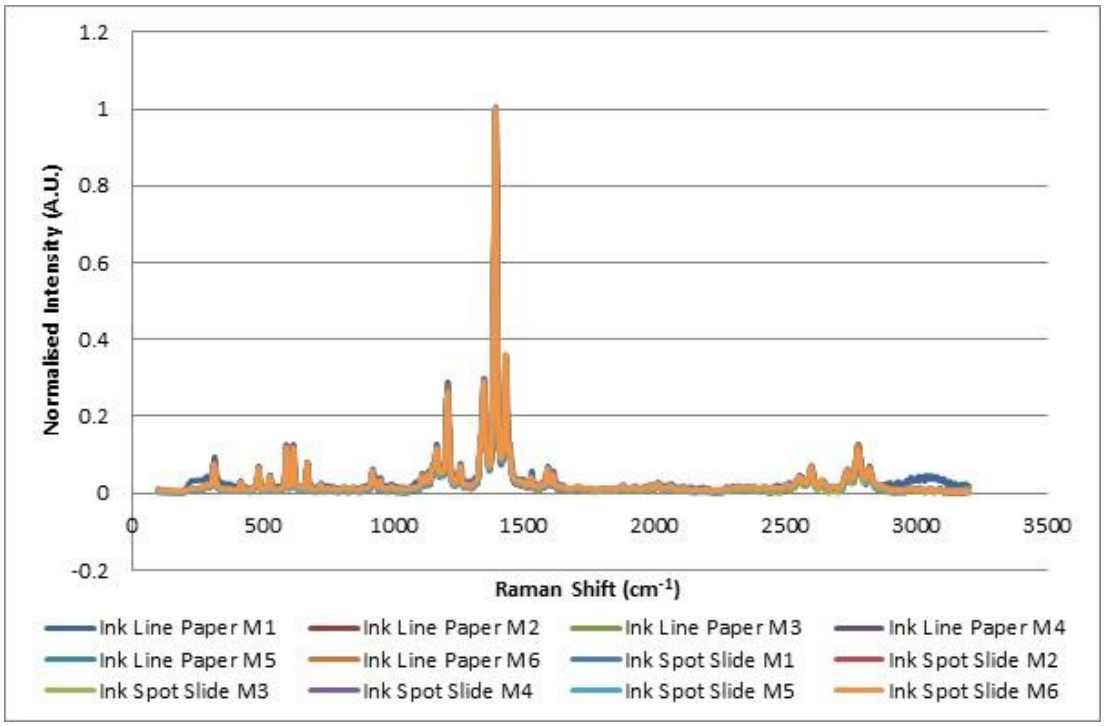


Figure 6.2: Comparison of a set of reproducibility measurements (n = 6) from each of a PTL UK blue gel ink line on paper and a blue ink spot on glass slide (Renishaw InVia 514.5 nm 10% laser power/x 20 obj., baseline corrected and normalised)

6.2.2.1.1.2.3 Influence of Objective

The literature [15, 27] suggests that typically a x 50 obj. is used for Raman Spectroscopy of inks, which is suggested allows "*spatial resolution of paper fibres and location of ink on fibres*" [121]. Since the Renishaw InVia single wavelength system was equipped with both x 20 and x 50 objectives, it was decided to investigate what influence objective magnification would have on spectral quality.

Spectra of the blue ink on both substrates taken under both objectives were reproducible, exhibited good S:N ratio and were considered highly similar in terms of peak position and is presented in Figure 6.3. Minimal fluorescence background was observed under both objectives, with a clear Raman signal present on top. The only notable difference was a slight overall spectral pattern intensity increase, including background fluorescence, observed in spectra acquired using the x 50 obj., which one might expect due to an accompanying decrease in laser spot size directed upon the sample.

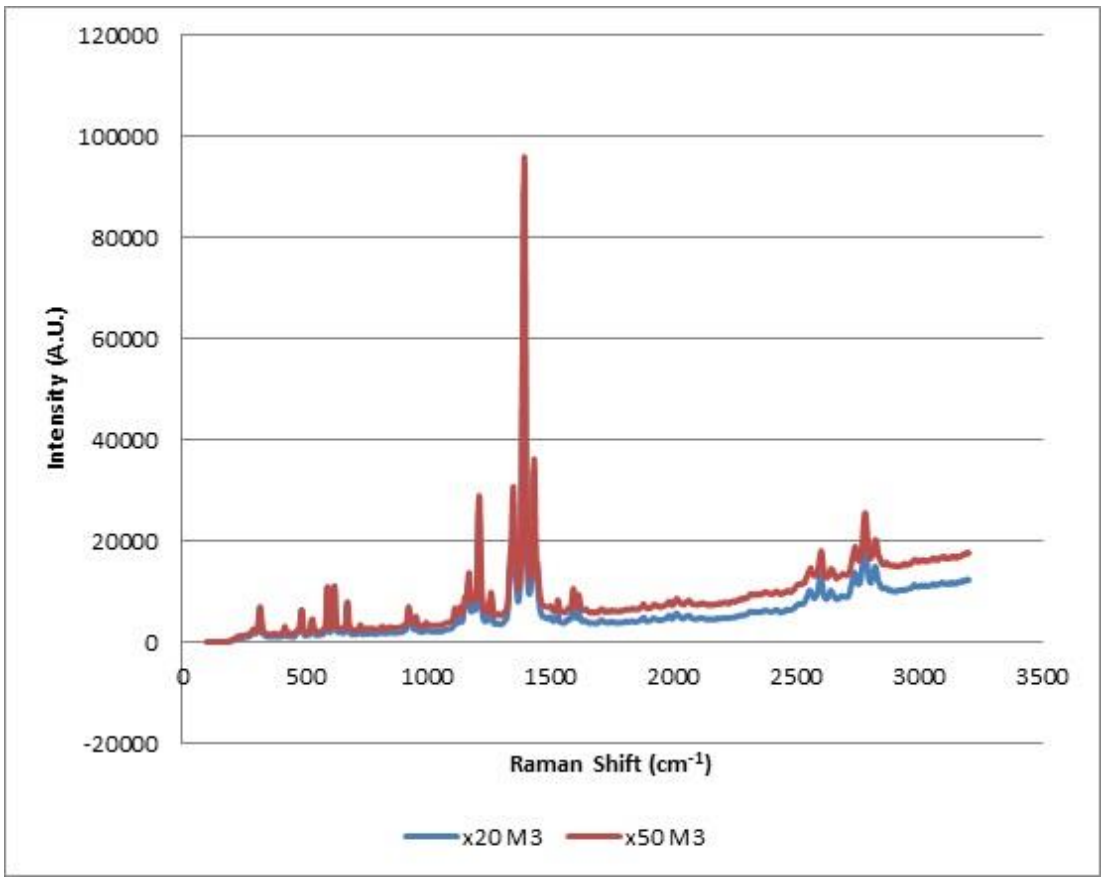


Figure 6.3: Example spectra of PTL UK blue gel ink line on paper acquired using two different objectives (Renishaw InVia 514.5 nm 10% laser power/both x 20 and x 50 obj.)

6.2.2.1.1.2.4 Influence of Confocality

Operation in confocal mode can reduce fluorescence background since only a small area of the sample is exposed to the incident laser. Furthermore, it is suggested that it ensures "the response was from the ink layer only and not from paper beneath" [121]. An investigation of the influence on spectra acquired, when operating in both confocal (high confocal) and non-confocal (standard confocal) modes was conducted.

A comparison of spectra (blue ink line on paper) acquired from both modes revealed good reproducibility and S:N ratio, with minimal fluorescence background above 2000 cm^{-1} as shown in Figure 6.4. Little difference in the peaks present was observed, but a weaker intensity in the Raman peaks of some spectra acquired in confocal mode was noted. This made it more difficult to resolve by eye some of the smaller peaks in comparison to spectra acquired in non-confocal mode. In fact, overall spectral intensity appeared stronger when the non-confocal mode of operation was used.

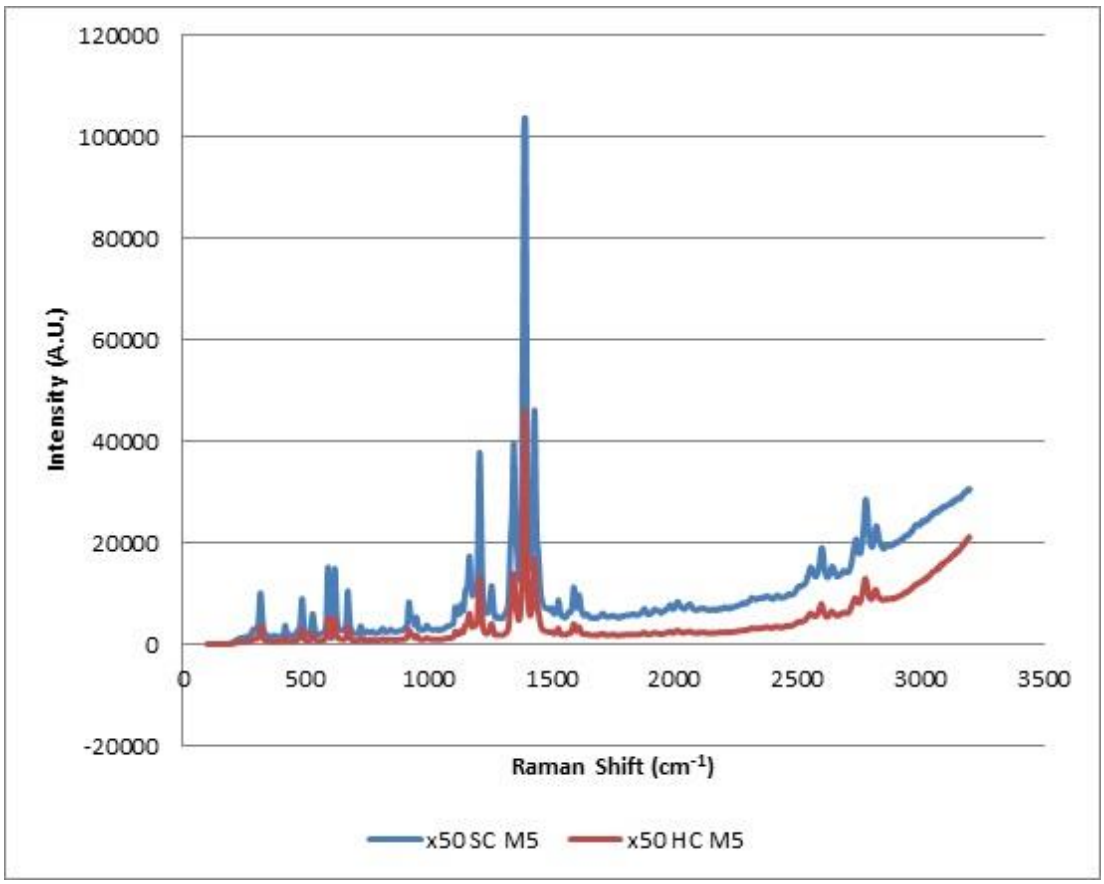


Figure 6.4: Example spectra of PTL UK blue gel ink line on paper acquired in standard (SC) and high-confocal (HC) operating modes (Renishaw InVia 514.5 nm 10% laser power/x 50 obj.)

6.2.2.1.1.2.5 Influence of Sample Size

6.2.2.1.1.2.5.1 Ink on Paper

Spectra acquired from a blue ink (PTL UK) line, spot and smear on glass slide, as well as from a blue ink line and dot on paper were of good S:N ratio, reproducible and indistinguishable to one another. This confirmed, only a small sample of ink the approximate width of an ink line (~1 mm) was sufficient to acquire spectra suitable for comparison and discrimination.

6.2.2.1.1.3 Further Investigation of the PTL UK Red Ink

6.2.2.1.1.3.1 Ink on Paper – Influence of Laser Power Intensity

For the PTL UK red ink line, only fluorescence and/or detector overload were observed irrespective of laser power setting used. Since no useful spectra of the PTL UK red ink was acquired from the ink spot on glass slide either, this suggested the 514.5 nm excitation wavelength is unsuitable for the analysis of red gel ink. Of 13 brand/model combinations of

red gel ink analysed on paper at 10% laser power, no useful spectra for comparison and discrimination were obtained due to a combination of fluorescence and detector overload. Reducing laser power further to 1% did not resolve this. These findings are consistent with those from a previous study in relation to red gel inks [13], supporting the view that this excitation wavelength is of no value for the analysis of red gel inks.

6.2.2.1.2 Multi-Wavelength System (632.5 nm Excitation Wavelength)

6.2.2.1.2.1 Ink on Glass Slide – Expected Ink Spectrum

The best S:N ratio and reproducible spectrum from the PTL UK blue ink spot was achieved using full (100%) laser power. The spectrum exhibited Raman bands on top of a steep background fluorescence with some detector overload above 1900 cm^{-1}. In contrast, a low laser power setting of 10% provided the best quality reproducible spectra from the PTL UK red ink spot, although the Raman signal was weak on top of a fluorescence background.

6.2.2.1.2.2 Ink on Paper

A combination of fluorescence and detector overload obscured any Raman signal for both colour groups irrespective of laser power setting used. As presented in Figure 6.5, only the PTL UK blue ink line provided albeit weak Raman signal on a fluorescence background at 10% laser power. On this basis it was preliminarily concluded that the 632.5 nm excitation wavelength was unsuitable for the analysis of gel inks representing both colour groups, but that further investigation in relation to blue gel ink was required.

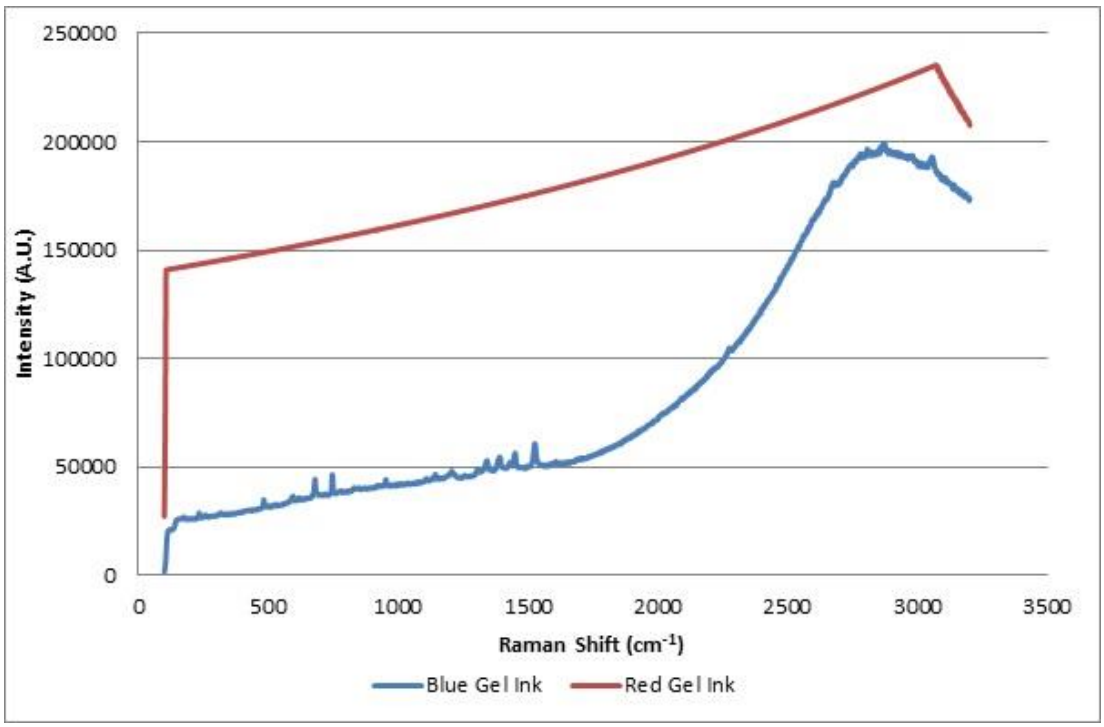


Figure 6.5: Example spectra taken of a PTL UK blue and red gel ink line on paper (Renishaw InVia 632.5 nm 10% laser power/x 50 obj.)

6.2.2.1.2.2.1 Further Investigation of the 632.5 nm Excitation Wavelength - Blue Ink Only

A previous study by Zieba-Palus [204] which included the analysis of blue and red gel inks using 633 nm, suggested that fluorescence was a particular problem associated with this excitation wavelength. Certainly, the findings from method development would support this. However, since a weak Raman response from only the PTL UK blue ink line on paper was observed, further investigation of the suitability of the 632.5 nm excitation wavelength for the analysis of blue gel inks only was undertaken.

Of the six dye containing ink samples analysed, only two exhibited a Raman signal. The PTL JAP brand/model combination exhibited a relatively strong signal intensity on a fluorescence background, whilst the STB UK brand/model combination exhibited a Raman spectrum obscured by fluorescence below ~800 cm^{-1}, losing some spectral detail. The remaining four dye containing samples gave a spectrum swamped by fluorescence and/or detector overload.

For the 21 pigment based brand/model combinations analysed the spectral response was generally weak and obscured by fluorescence. Furthermore, several samples

(approximately half) exhibited spectral patterns where fluorescence background shape differed between spectra within the same brand/model combination. Therefore the reproducibility of spectra for these brand/model combinations was poor. No spectral interference in the ink spectra arising from paper substrate was observed, although fluorescence from the paper itself rather than or in combination with, the ink was responsible for the poor reproducibility and spectral response at this particular wavelength.

These findings further support those of Zieba-Palus *et al* [204] in relation to fluorescence problems encountered using 633 nm. Despite these problems however, Zieba-Palus *et al* [204] claimed ~70% of blue gel ink samples analysed could be discriminated. In contrast, the findings described above suggested the fluorescence problem prevented little reliable discrimination between blue gel inks in this study. Furthermore, the initial method development findings in relation to the PTL UK red ink suggested similar problems with fluorescence would likely be encountered with the red ink group samples, despite Zieba-Palus *et al* [204] claiming ~80% of red inks analysed using 633 nm were differentiable. Based on our own experimental findings it was therefore decided to exclude the 632.5 nm excitation wavelength from the within and between brand studies described in *Part B*.

6.2.2.1.3 Multi-Wavelength System (785 nm Excitation Wavelength)

6.2.2.1.3.1 Ink on Glass Slide – Expected Ink Spectrum

A 10% laser power setting yielded the best quality and reproducible spectra from the PTL UK blue ink spot, with minimal background fluorescence observed between 200 – 2000 cm^{-1}. For the PTL UK red ink spot, in contrast, full (100%) laser power provided good quality, reproducible spectra.

No spectral interference was observed in the PTL UK blue ink spectra as demonstrated in Figure 6.6. However, a broad fluorescence band between 1000 – 2000 cm^{-1} was observed in the spectrum of the PTL UK red ink spot under all three laser power settings. This interference was attributed to the glass substrate, and confirmed by analysis of an un-inked area of the slide. Figure 6.7 shows the influence of spectral interference from the glass substrate on the PTL UK red ink spectra under all three laser powers.

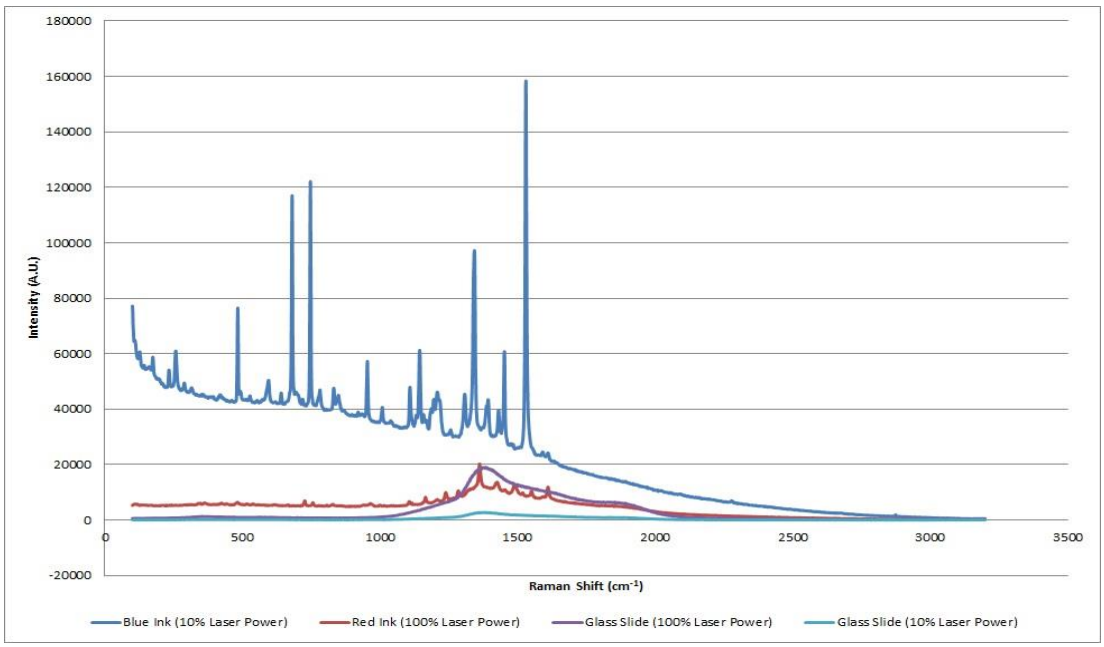


Figure 6.6: Raman spectra of pure blue and red gel ink spots on glass slide compared to spectra from an un-inked glass slide to demonstrate spectral interference from substrate in red ink spectra but not blue ink spectra (Renishaw InVia 785 nm 100% and 10% laser power/x 50 obj.)

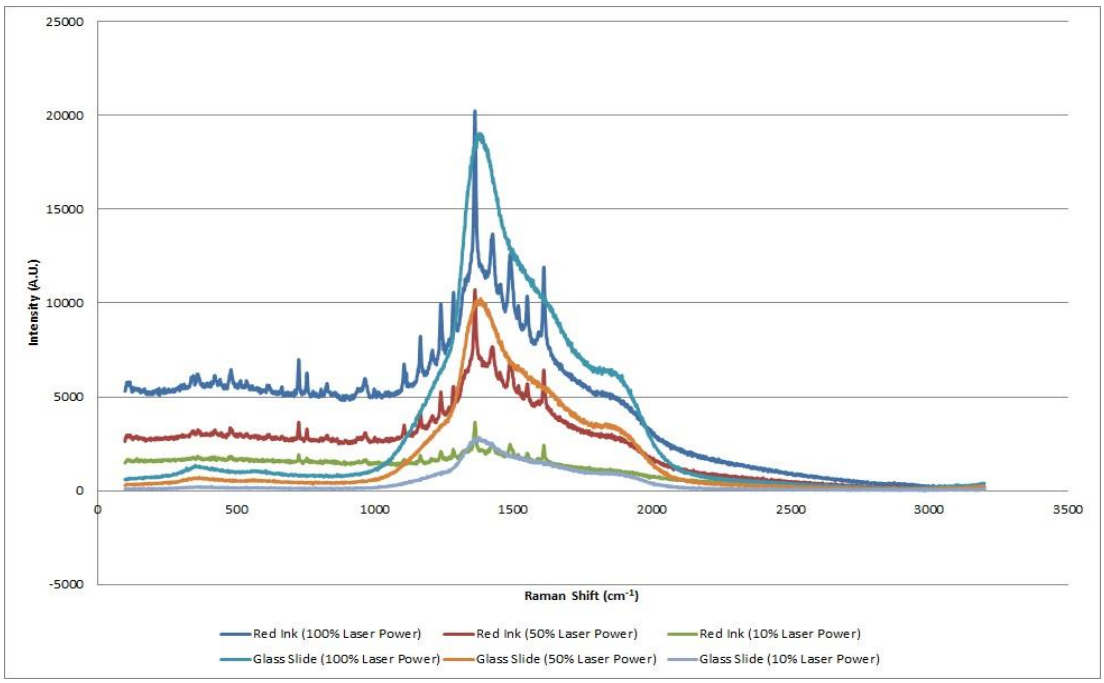


Figure 6.7: Raman spectra of pure red gel ink spots on glass slide compared to spectra from an un-inked glass slide to demonstrate spectral interference from substrate at all three laser power settings (100%; 50% and 10%)

6.2.2.1.3.2 Ink on Paper

A 10% laser power achieved good quality and reproducible spectra for the PTL UK blue ink line, which was considered indistinguishable to the corresponding spectra acquired from

the same PTL UK blue ink deposited on glass slide. In contrast, a 100% laser power setting achieved the best spectrum for the PTL UK red ink line although strong Raman bands were observed at all three laser powers. Spectral interference in the form of a peak at ~1094 cm^{-1} was observed. Taking into consideration interference from the glass and paper substrate, above 1200 cm^{-1}, spectra of the red ink acquired from both substrates compared well. Below 1200 cm^{-1} however, some discrepancies were observed where the Raman signal was obscured by interference and is illustrated in Figure 6.8.

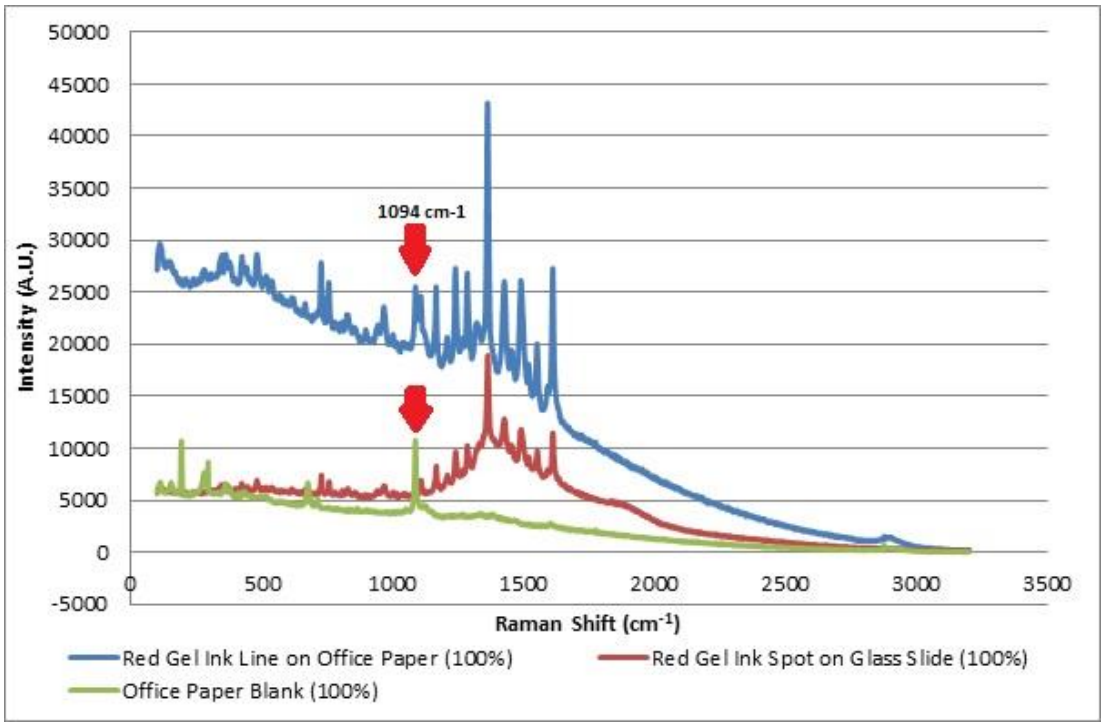


Figure 6.8: Comparison of spectra taken of a PTL UK red gel ink line on paper, an un-inked area of the paper itself and red gel ink spot on glass microscope slide (Renishaw InVia 785 nm 100% laser power/x 50 obj.)

6.2.2.1.3.2.1 Influence of Higher Objective Magnification (x 100) – Red Ink Only

Given the variable intensity response of the Raman spectra from the red ink group taken using x 50 obj., the use of a higher objective magnification (x 100) was investigated to see if it would enhance spectral intensity further and improve discrimination. Figure 6.9 and Figure 6.10 show example comparisons for the PTL UK brand/model combination using both objectives. No improvement in intensity of the resulting spectra using the x 100 obj. was observed, suggesting that a higher objective magnification will not necessarily provide a stronger Raman signal. This was true for the other two brand/model combinations analysed under the x 100 obj.

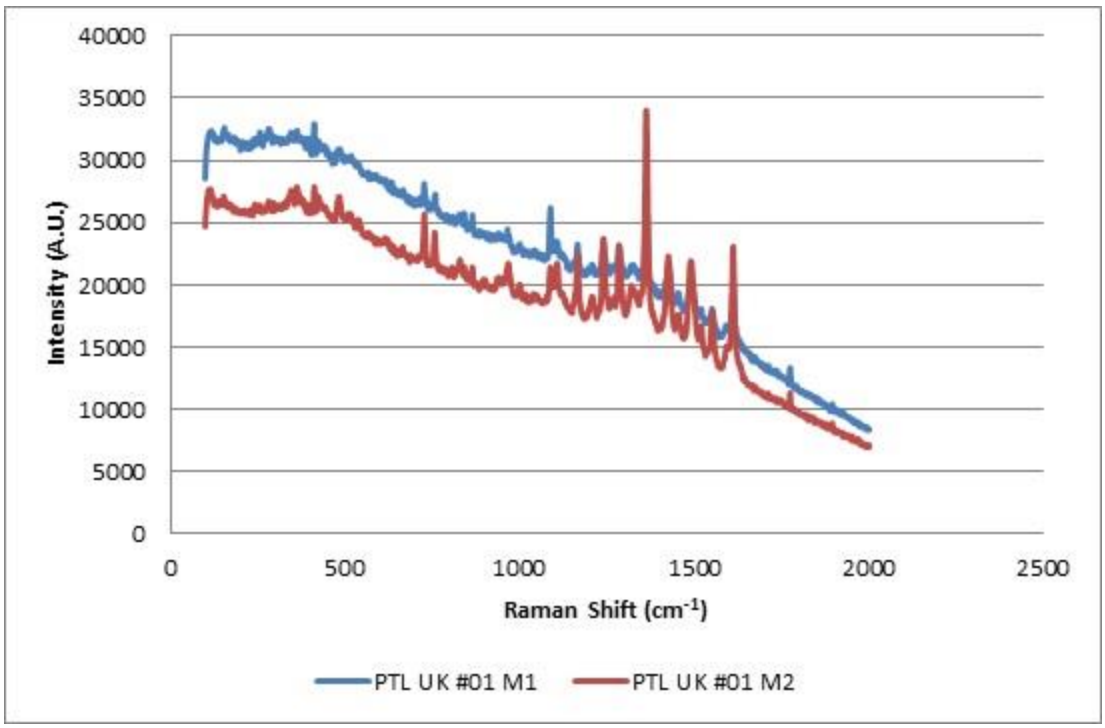


Figure 6.9: Duplicate spectra of PTL UK red gel ink on paper (Renishaw InVia 785 nm 100% laser power/x 50 obj.)

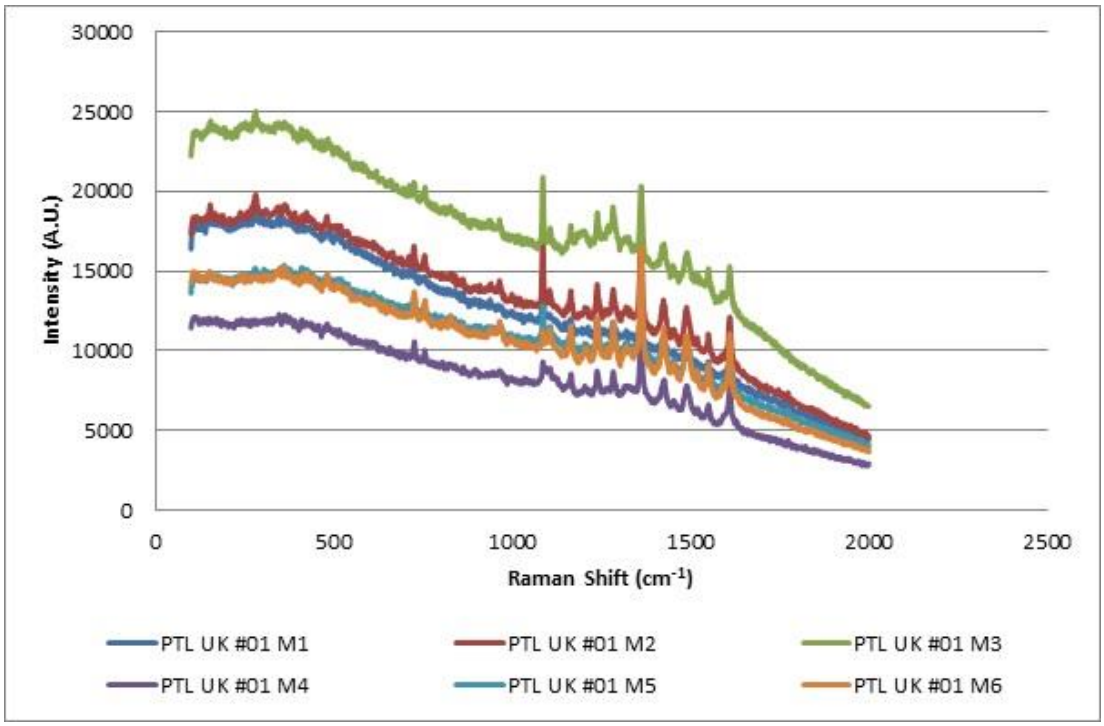


Figure 6.10: Set of reproducibility measurements (n = 6) of PTL UK red gel ink on paper (Renishaw InVia 785 nm 100% laser power/x 100 obj.)

6.2.2.1.4 Multi-Wavelength System (830 nm Excitation Wavelength)

6.2.2.1.4.1 Ink on Glass Slide – Expected Ink Spectrum

The best spectrum in terms of S:N ratio and reproducibility from the PTL UK blue and red ink spots were achieved using full (100%) laser power. However, interference from the glass in the form of a broad fluorescence band was observed in the wavenumber region 500 – 1500 cm^{-1}. For the PTL UK blue ink spectra the Raman peaks were still relatively strong on top of the fluorescence band, but for the PTL UK red ink spectra they were comparatively weaker. Figure 6.11 demonstrates the influence of interference from the glass on the PTL UK blue and red ink spectra.

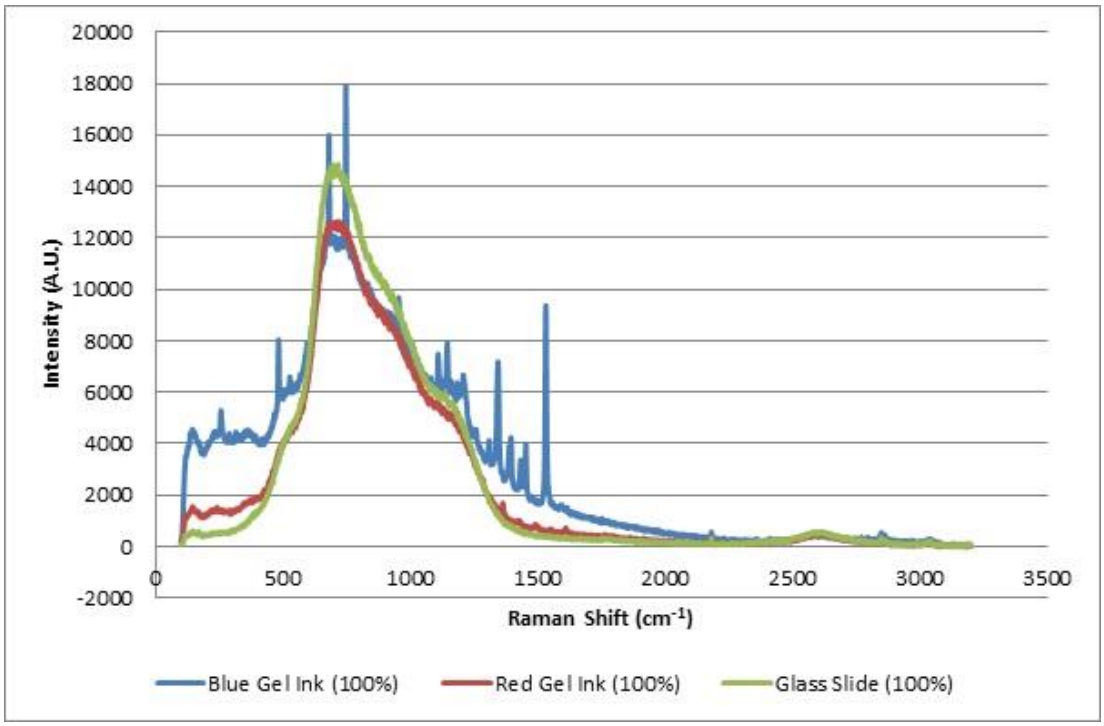


Figure 6.11: Example spectra taken of a PTL UK blue and red gel ink spot on glass slide, and of the slide itself demonstrating spectral interference from the substrate (Renishaw InVia 830 nm 100% laser power/x 50 obj.)

6.2.2.1.4.2 Ink on Paper

For the PTL UK blue ink line, a distinctive Raman spectral pattern was observed using all three laser power settings, but full (100%) laser power yielded the best spectrum. This was also true for the PTL UK red ink line, but unlike for the PTL UK blue ink spectra, Raman peaks were relatively weak in contrast to the fluorescence background which raised doubts about the suitability of 830 nm for the analysis of red gel ink on paper. Spectral interference from the paper substrate was observed in both the PTL UK blue and red ink

spectra. Taking into account interference from the glass and paper substrates, the PTL UK blue ink spectra acquired from both substrates compared well, but some Raman peaks were masked by fluorescence in the former. For PTL UK red ink spectra, where the signal strength permitted, spectra acquired from the glass and paper also compared well (i.e. 1200 – 1600 cm^{-1}).

6.2.2.1.5 Foster & Freeman FORAM

6.2.2.1.5.1 FORAM 685-2 (685 nm Excitation Wavelength)

6.2.2.1.5.1.1 Ink on Glass Slide – Expected Ink Spectrum

For the PTL UK blue ink spot, a reproducible spectral pattern of weak Raman peaks on a fluorescence background was observed using all laser power/obj. combinations. However, a combination of 10% laser power and x 20 obj. provided the better spectral response in terms of reproducibility. Similar observations were noted for the PTL UK red ink spot, although a combination of 100% laser power and x 20 obj. was considered the most suitable for this particular colour group.

Inconsistent but frequent appearance of a series of three peaks occurring between 500 – 600 cm^{-1} was noted in some spectra acquired from the PTL UK red ink spot at all laser power/obj. combinations, illustrated for the former in Figure 6.12. A similar set of peaks was also observed in some spectra taken from an un-inked area of the glass slide when a combination of full laser power and x 10 obj. was used. The presence of these peaks in spectra from the glass substrate and an ink spectrum suggested some form of spectral interference from the glass slide. Although stray light entering the spectrometer system was a possible explanation, it was considered more likely these peaks may have arisen from a contaminant on the surface of the glass such as a fingerprint since they were not consistent between measurements of the same ink spot and only appeared in the PTL UK red ink spectral data.

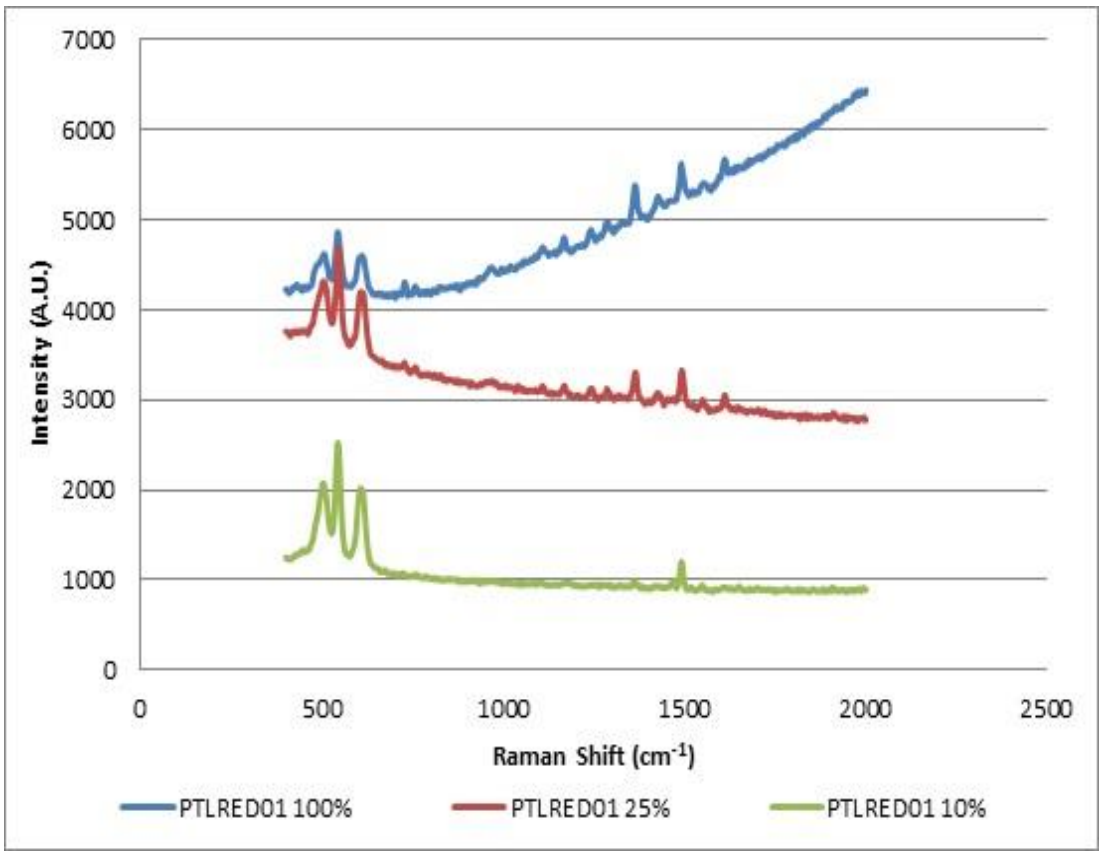


Figure 6.12: Example spectra of PTL UK red gel ink spot on glass slide exhibiting a series of three peaks between 500 – 600 cm^{-1} possibly arising from a contaminant on the glass surface or stray light (FORAM 685-2 varied laser power/x 20 obj., 1.0s default scan time)

6.2.2.1.5.1.2 Ink on Paper

For the PTL UK blue ink line a spectral response of weak peaks on fluorescence background was observed, with a combination of low (10%) laser power and x 20 obj. offering the best S:N ratio and reproducibility, illustrated in Figure 6.13. For the PTL UK red ink line using all laser power/obj. combinations, spectra were swamped by fluorescence and/or detector overload, but the least noise was observed at mid (25%) laser power. No spectral interference from the paper substrate was observed.

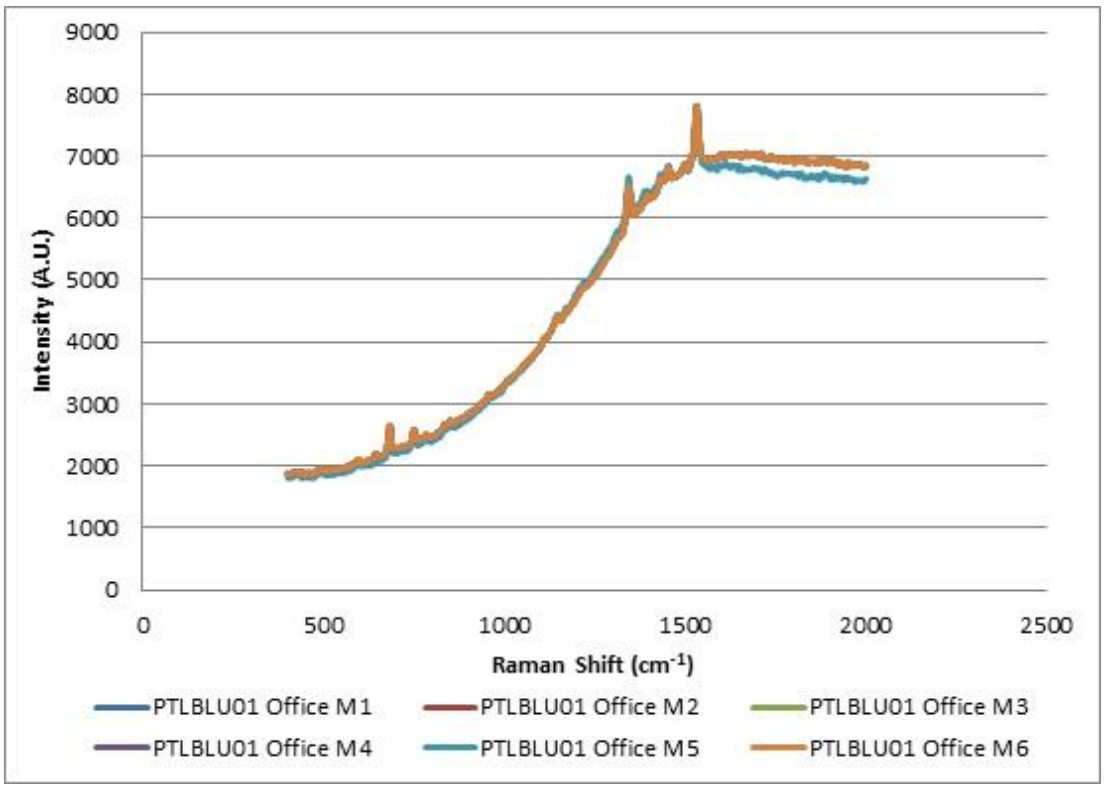


Figure 6.13: A set of reproducibility measurements (n = 6) taken of a PTL UK blue gel ink line on paper (FORAM 685-2 10% laser power/x 20 obj.)

A comparison of spectra, by colour group, acquired using both manually and automatically selected integration times under the most appropriate laser power/obj. combinations described, was undertaken to assess the influence of operating the FORAM 685-2 in Auto Exposure mode. With respect to the blue ink sample, Auto Exposure increased the overall intensity of the Raman spectrum raising it from a maximum of ~8, 000 (A.U.) in manual mode to ~20, 000 (A.U.), thus providing a stronger spectrum of the PTL UK blue ink. No loss in spectral detail or reproducibility was observed, and no spectral interference from the paper substrate identified. Integration times selected in Auto Exposure mode differed only slightly to the 1.0s default manual integration time, i.e. 1.0 – 2.0s. This was similar for the red ink sample, where automatic and manual integration times were the same at 1.0s. In contrast however, using Auto Exposure provided no further value in acquiring a Raman signal from the PTL UK red ink. It was therefore concluded based on this, that the 685 nm excitation wavelength was unsuitable for the red ink group samples. Based on these findings, the benefit of operating the FORAM 685-2 in Auto Exposure mode during spectral acquisition of blue gel inks was evident and applicable to the spectral acquisition of these colour group samples described in *Part B – Within and Between Brand Variation*.

6.2.2.1.5.2 FORAM 785 (785 nm Excitation Wavelength)

6.2.2.1.5.2.1 Ink on Glass Slide – Expected Ink Spectrum

For the PTL UK blue ink spot, the best S:N ratio and reproducibility was achieved using a combination of full (100%) laser power and x 20 obj. This was also true for the PTL UK red ink spot. Spectral interference from the glass was problematic for both ink colour spectra, illustrated in Figure 6.14. A fluorescence band between 1300 – 1800 cm^{-1} was observed. The intensity of this band was reduced under lower laser power settings, but also had a negative impact on spectral quality. Similar observations were made using the x 10 obj.

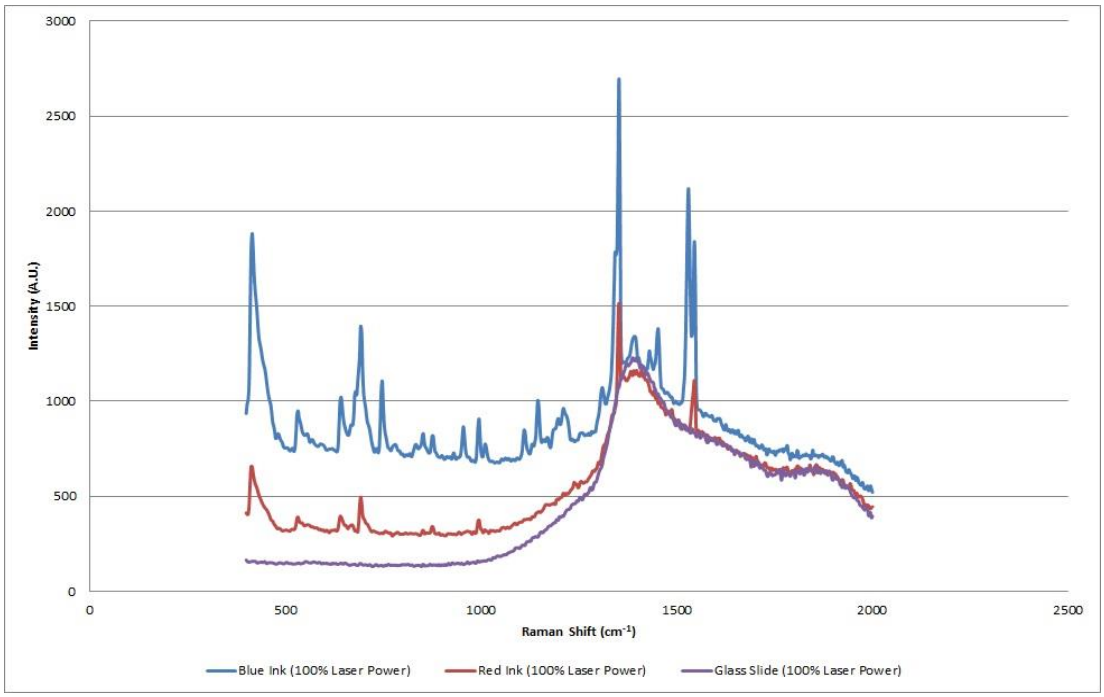


Figure 6.14: Raman spectra of PTL UK blue and red ink spots on glass slide compared to a spectrum from an un-inked area of glass slide demonstrating spectral interference from the substrate [FORAM 785, 100% laser power/x 20 obj., 1.0s default scan time]

It was noted the spectral pattern across both colour groups bore some resemblance. These similarities may have arisen from stray light entering the spectrometer system. The x 10 and x 20 objectives were fitted with a light collar that was twisted down to prevent ambient light from entering the spectrometer during spectral acquisition. However, given the thickness (~2 - 3 mm) of the glass slides, these could not be fully closed thus providing an opportunity for stray light to enter the system. Comparison with spectra acquired for the PTL UK red ink spot taken using the x 5 obj. which is not fitted with a light collar showed similarity to the corresponding spectra acquired using higher laser power and higher

objective combinations. Figure 6.15 and Figure 6.16 illustrate this point. This provided evidence supporting the view that stray light was interfering with spectral acquisition of gel inks deposited on glass slides.

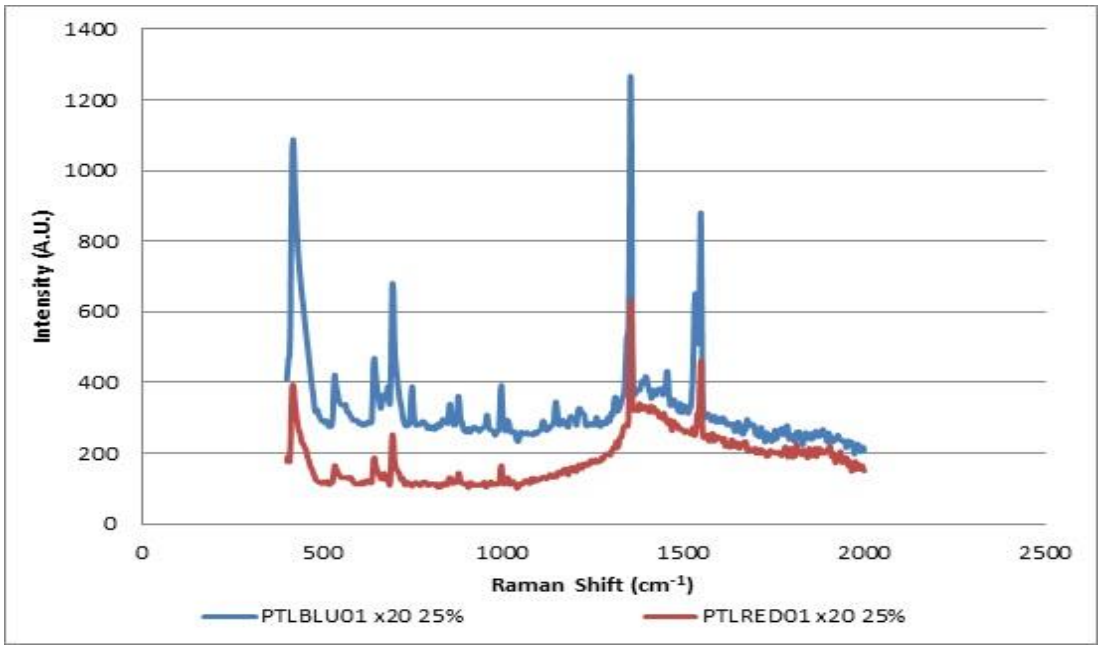


Figure 6.15: Comparison of spectra from PTL UK blue and red gel ink spot on paper demonstrating surprising similarity in spectral pattern (FORAM 785 25% laser power/x 20 obj., 1.0s default scan time)

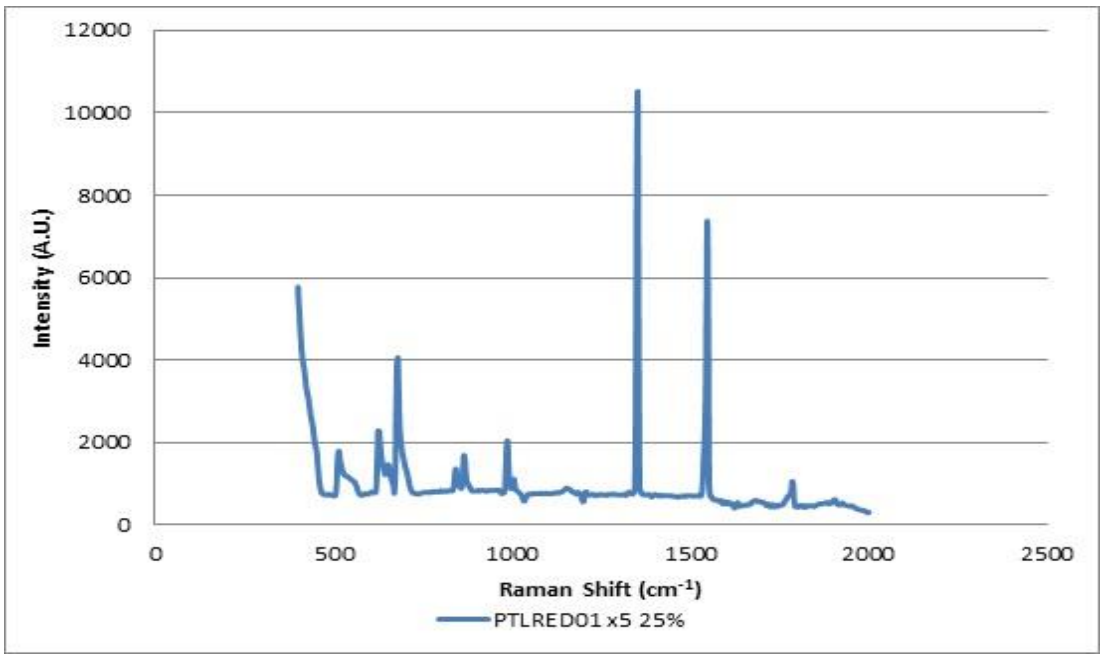


Figure 6.16: Example spectra of a PTL UK red gel ink spot on glass slide (FORAM 785 25% laser power/x 5 obj., 1.0s default scan time (no light collar))

6.2.2.1.5.2.2 Ink on Paper

For the PTL UK blue and red ink line, a combination of full (100%) laser power and x 20 obj. provided the best S:N ratio and reproducibility, displaying strong Raman bands on top of a slight fluorescence background slope for the PTL UK blue ink, but with variable overall spectral intensity between measurements. As presented in Figure 6.17, the PTL UK red ink line produced a spectral pattern of weak Raman peaks on a fluorescence background. The intensity of the Raman peaks was considered even weaker when the x 10 obj. was used irrespective of laser power setting. Some spectral interference arising from the substrate was observed, in particular a peak at ~1094 cm^{-1} was observed. Additional peaks at ~1400 cm^{-1} and ~1600 cm^{-1} were also observed in the PTL UK red ink spectra.

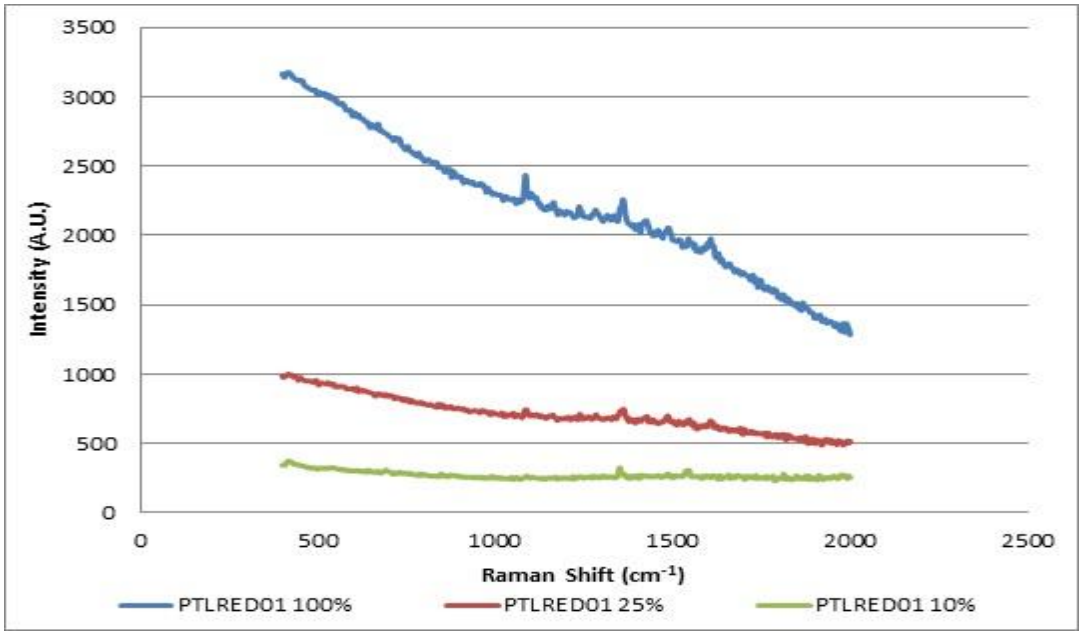


Figure 6.17: Example spectra of a PTL UK red gel ink line on office paper (FORAM 785 varied laser power/x 20 obj., 1.0s default scan time)

As for the FORAM 685-2 method development, a comparison of spectra, by colour group, acquired using manual and automatic integration times was undertaken. Additionally, a comparison of spectra taken under both x 20 and x 50 objectives was also performed.

For the blue ink sample, under x 20 obj., only a slight improvement in overall signal intensity of the Raman spectrum was observed, increasing from ~16, 000 (A.U.) in manual mode to ~18, 000 (A.U.). However, under x 50 obj., a greater difference was noted, increasing from ~16, 000 (A.U.) in manual mode to ~25, 000 (A.U.) in Auto Exposure mode.

This suggests that when used in combination, a higher objective magnification and Auto Exposure mode provide the best spectral response from the PTL UK blue ink. No loss in spectral detail or reproducibility was observed irrespective of objective and use of Auto Exposure. Likewise, integration times were either similar or only slightly greater than the 1.0s default manual integration time, i.e. ~1.0s – 2.0s. However, spectral interference from the paper substrate in the form of a small peak at ~1094 cm^{-1} was observed in all spectra.

For the red ink sample, using Auto Exposure greatly improved the overall signal intensity of the Raman spectra irrespective of objective, increasing from ~2, 500 (A.U.) in manual mode to ~18, 000 (A.U.). In contrast to the blue ink sample, this suggests that a higher objective magnification may not necessarily provide an improved spectrum, but that operation in Auto Exposure mode is crucial. Integration times were higher in Auto Exposure mode, between 4.0s – 8.0s, than the 1.0s manual integration time. No loss of spectral detail or reproducibility was observed other than slight differences in some peak intensities acquired in Auto Exposure mode. Spectral interference from the paper substrate was more problematic in manual integration time conditions, with peaks observed at ~1094 cm^{-1}, ~1400 cm^{-1} and ~1600 cm^{-1} attributable to the paper observed, compared to only at ~1094 cm^{-1} in Auto Exposure spectra.

Based on these findings, spectral acquisition from the blue and red ink group samples was performed operating the FORAM 785 in Auto Exposure mode in combination with x 50 obj. This permitted a like for like comparison between the spectral data acquired using both the Renishaw InVia and FORAM at the 785 nm excitation wavelength as described in *Part B – Within and Between Brand Variation*.

6.2.2.1.6 Validation of Renishaw InVia Instruments

6.2.2.1.6.1 Single Wavelength System (514.5 nm Excitation Wavelength)

The % RSD values of peak centre position for the eight selected peaks from all five sample size sets in PTL UK blue ink (i.e. ink line, spot, smear etc.) ranged from between 0.0 – 0.3%. In particular, for the PTL UK blue ink line on paper, % RSD values were <0.1%. This demonstrated excellent reproducibility of Raman spectra irrespective of sample size. Table 6.3 provides a breakdown and summary of the calculated % RSD values for each sample size tested on paper and glass slide.

Substrate	Sample Type	Peak No.	Spectral Measurement						Basic Statistics			
			M1	M2	M3	M4	M5	M6	Mean	St. Dev.	% RSD	% RSD Range
Standard White Office Paper	Ink Line	1	1431.37	1429.94	1429.47	1429.53	1429.94	1429.54	1429.97	0.72	**0.1**	
		2	1390.84	1389.81	1389.41	1389.50	1389.85	1389.45	1389.81	0.54	**0.0**	
		3	1345.52	1344.61	1344.23	1344.41	1344.56	1344.25	1344.60	0.48	**0.0**	
		4	1208.02	1206.65	1206.31	1206.37	1206.77	1206.43	1206.76	0.64	**0.1**	
		5	1164.36	1163.85	1163.76	1163.87	1164.22	1163.71	1163.96	0.26	**0.0**	**0.0 - 0.1**
		6	618.46	617.36	617.24	616.91	617.27	617.54	617.46	0.53	**0.1**	
		7	590.69	589.70	589.63	589.32	589.64	589.94	589.82	0.47	**0.1**	
		8	315.48	314.74	314.44	314.36	314.73	31470	314.74	0.40	**0.1**	
	Ink Spot	1	1430.42	1428.89	1428.20	1428.69	1427.51	1431.07	1429.13	1.35	**0.1**	
		2	1390.30	1388.39	1387.99	1388.62	1387.49	1390.84	1388.94	1.33	**0.1**	
		3	1345.12	1343.02	1342.77	1343.19	1342.06	1345.58	1343.62	1.40	**0.1**	
		4	1207.08	1205.76	1205.09	1205.33	1204.56	1207.81	1205.94	1.25	**0.1**	**0.1 - 0.3**
		5	1164.26	1162.35	1162.09	1162.74	1162.23	1164.50	1163.03	1.07	**0.1**	
		6	617.43	616.51	616.16	616.33	616.075	618.43	616.82	0.93	**0.2**	
		7	589.78	588.92	588.62	588.81	588.70	590.80	589.27	0.86	**0.1**	
		8	314.75	313.66	313.21	313.53	313.27	315.66	314.01	0.98	**0.3**	
Glass Microscope Slide	Ink Spot	1	1429.21	1429.23	1429.12	1429.04	1428.62	1428.56	1428.96	0.30	**0.0**	
		2	1389.27	1389.30	1389.20	1389.11	1388.74	1388.66	1389.05	0.28	**0.0**	
		3	1343.92	1344.09	1343.87	1343.91	1343.31	1343.23	1343.72	0.36	**0.0**	
		4	1206.24	1206.25	1206.17	1206.00	1205.74	1205.75	1206.03	0.23	**0.0**	**0.0 - 0.1**
		5	1163.33	1163.05	1163.08	1163.60	1163.33	1163.16	1163.26	0.21	**0.0**	
		6	617.17	617.25	617.29	617.08	616.89	616.92	617.10	0.17	**0.0**	
		7	589.73	589.83	589.86	589.63	589.48	589.51	589.67	0.16	**0.0**	
		8	314.38	314.50	314.50	314.22	314.07	314.12	314.30	0.19	**0.1**	
	Ink Line	1	1428.51	1428.20	1428.08	1428.63	1429.01	1428.38	1428.47	0.33	**0.0**	
		2	1388.59	1388.34	1388.19	1388.71	1389.01	1388.49	1388.56	0.29	**0.0**	
		3	1343.22	1343.04	1342.61	1343.29	1343.72	1343.14	1343.17	0.36	**0.0**	
		4	1205.56	1205.37	1205.26	1205.66	1206.00	1205.49	1205.56	0.26	**0.0**	**0.0 - 0.1**
		5	1164.00	1162.81	1163.12	1163.20	1163.86	1162.53	1163.25	0.58	**0.0**	
		6	616.73	616.72	616.66	616.91	616.99	616.76	616.80	0.13	**0.0**	
		7	589.28	589.32	589.26	589.55	589.53	589.35	589.38	0.13	**0.0**	
		8	313.84	313.81	313.79	314.13	314.22	313.85	313.94	0.19	**0.1**	
	Ink Smear	1	1429.95	1429.96	1430.46	1430.00	1428.92	1429.85	1429.86	0.51	**0.0**	
		2	1389.94	1389.95	1390.36	1389.98	1389.02	1389.87	1389.85	0.44	**0.0**	
		3	1344.61	1344.67	1345.21	1344.66	1343.62	1344.54	1344.55	0.52	**0.0**	
		4	1206.74	1206.81	1207.26	1206.98	1206.01	1206.71	1206.75	0.42	**0.0**	**0.0 - 0.1**
		5	1164.36	1164.05	1165.02	1164.05	1163.54	1164.15	1164.20	0.49	**0.0**	
		6	617.29	617.47	617.57	617.57	617.14	617.30	617.39	0.17	**0.0**	
		7	589.83	589.92	589.92	590.01	589.76	589.79	589.87	0.09	**0.0**	
		8	314.70	314.83	315.03	314.84	314.37	314.75	314.75	0.22	**0.1**	

Table 6.3: % RSD for the peak centre position of eight randomly selected peaks in spectra of a PTL UK blue gel ink on paper and glass slide deposited in various sample sizes

6.2.2.1.6.2 Multi-Wavelength System (632.5 nm/785 nm/830 nm Excitation Wavelength)

For PTL UK blue gel ink lines on office paper (785 nm), % RSD of eight randomly selected peaks following the revised validation approach was found to be 0.0%. For the original validation approach, % RSD of the same eight peaks ranged between 0.0 – 0.1%, comparable to the revised approach. The revised approach was applied to PTL UK red ink samples on office paper (785 nm) and PTL UK blue ink samples on office paper (830 nm) recorded under the most appropriate conditions identified through method development. In both instances, % RSD of eight randomly selected peaks was found to be 0.0%. These findings again demonstrated excellent reproducibility of Raman spectra.

6.2.2.1.7 Validation of Foster & Freeman FORAM Instruments

6.2.2.1.7.1 685 nm and 785 nm Excitation Wavelength

For the PTL UK blue gel ink lines on office paper using FORAM 785, % RSD values of peak position for eight randomly selected peaks ranged between 0.0 – 0.1%, confirming excellent reproducibility. Figure 6.18 shows an example of eight randomly selected peaks and Table 6.4 shows the corresponding peak positions for each of six spectral measurements in a method development data set together with calculated % RSD values.

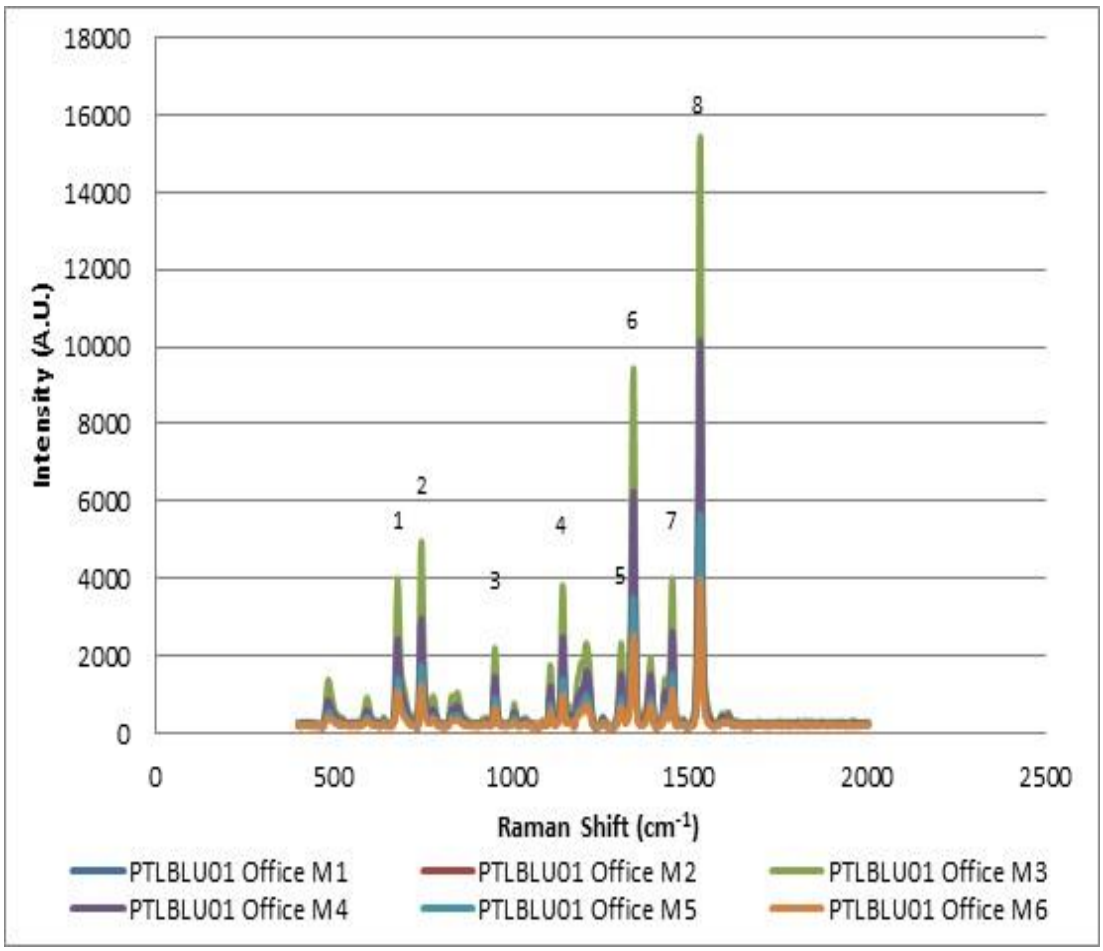


Figure 6.18: Eight randomly selected peaks used to calculate % RSD for validation to analyse blue gel ink lines on office paper (FORAM 785 100% laser power/x 20 obj., 1.0s default scan time, baseline corrected and smoothed)

Blue 785 nm 100% Laser Power/x 20 obj.									
Peak No.	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**	**Mean**	**St. Dev.**	**% RSD**
1	678.7	678.7	678.7	678.7	678.7	678.7	678.7	0.0	0.0
2	746.7	746.7	746.7	746.7	746.7	746.7	746.7	0.0	0.0
3	953.6	953.6	953.6	953.6	953.6	953.6	953.6	0.0	0.0
4	1142.7	1142.7	1142.7	1142.7	1142.7	1142.7	1142.7	0.0	0.0
5	1308.4	1308.4	1308.4	1308.4	1308.4	1308.4	1308.4	0.0	0.0
6	1342.2	1342.2	1342.2	1342.2	1342.2	1342.2	1342.2	0.0	0.0
7	1452.3	1452.3	1452.3	1452.3	1452.3	1452.3	1452.3	0.0	0.0
8	1530.8	1530.8	1527.5	1530.8	1530.8	1530.8	1530.3	1.4	0.1

Table 6.4: % RSD of peak centre position for eight randomly selected peaks from spectra of PTL UK blue gel ink line on paper (FORAM 785)

Validation for the analysis of red gel ink lines on paper using FORAM 785 and the analysis of blue ink lines on paper using the FORAM 685-2 was not considered possible due to the weak Raman signal exhibited in comparison to the strong Raman signal observed for the blue ink using FORAM 785. Baseline correction could have been performed to remove the background fluorescence and enhance the Raman signal sufficiently to perform validation in the way described above. However, the literature suggests that baseline correction should only be used when there is "sufficient Raman signal on top of the sloping background" [150, 206] and cannot be used to remove a fluorescence slope that has completely masked any Raman signal from the sample. Incorrect use can result in the addition or removal of peaks which in turn can negatively impact subsequent interpretation [152]. Given that the original Raman response for the red gel ink at 785 nm and the blue gel ink at 685 nm was largely obscured by fluorescence, the use of baseline correction in this instance was considered unsuitable.

6.2.3 Conclusion: Method Development

6.2.3.1 Renishaw InVia Instruments

6.2.3.1.1 Single Wavelength System (514.5 nm Excitation Wavelength)

6.2.3.1.1.1 Blue Gel Ink

Preliminary conclusions drawn from the investigation of the influence of varied laser power intensity on signal to noise (S:N) ratio and reproducibility suggested the Renishaw InVia 514.5 nm excitation wavelength was suitable only for the analysis of blue gel ink on paper at low (10%) laser power. Further investigations into the influence of objective magnification, confocality and sample size were therefore focused on blue gel ink.

6.2.3.1.1.1.1 Influence of Objective Magnification

It was concluded that whilst higher objective magnification power increased overall spectral intensity, little difference in spectral detail over the lower power objective magnification was observed. Therefore, the x 20 obj. was considered adequate for the examination, comparison and discrimination of blue gel ink on paper at 514.5 nm.

6.2.3.1.1.1.2 Influence of Confocality

Operating the Renishaw InVia instrument in high confocal mode appeared to offer no additional benefit over operation in non-confocal mode. However, this conclusion should be treated with caution since it was not clear at the time of experimentation whether the instrument was correctly set up for confocal mode operation. Taking this into consideration, all further spectral measurements conducted using the Renishaw InVia instruments were taken in non-confocal mode of operation.

6.2.3.1.1.1.3 Influence of Sample Size

Little or no difference was observed between spectra from blue ink acquired from paper and/or glass irrespective of whether the ink sample took the form of a line, dot, spot or smear. This also demonstrated that an ink line (~1 mm) is sufficient for providing a detailed Raman spectrum suitable for comparison and discrimination confirming the view of Jones and Wolstenholme [196].

6.2.3.1.1.2 Red Gel Ink

Fluorescence obscured spectra from red gel ink at 514.5 nm, supporting the findings from a previous study [13].

6.2.3.1.2 Multi-Wavelength System (632.5 nm/785 nm/830 nm Excitation Wavelength)

Preliminary conclusions from an investigation of the influence of varied laser power intensity suggested the 632.5 nm excitation wavelength was unsuitable for the analysis of both colour groups owing to problems with fluorescence. Based on the examination of two different colours representing a single brand/model combination of gel ink (PTL UK), the best, albeit very weak Raman response, was seen from the blue gel ink only (10% laser power/x 50 obj.). Extending the study to a larger range of blue gel ink brand/model combinations confirmed that fluorescence was a particular problem associated with this excitation wavelength, affecting reproducibility and Raman signal intensity. On this basis the 632.5 nm excitation wavelength was excluded from the within and between brand variation studies described in *Part B*. In contrast, the 785 nm and 830 nm excitation wavelength permitted acquisition of good quality reproducible spectra from blue gel ink on paper when using low (10%) and high (100%) laser power respectively. Furthermore, the

785 nm excitation wavelength also produced good quality reproducible spectra from red gel ink on paper using full (100%) laser power intensity.

6.2.3.2 Foster and Freeman FORAM Instruments

6.2.3.2.1 685 nm and 785 nm Excitation Wavelength

From an investigation of laser power/obj. combination for the 685 nm and 785 nm excitation wavelengths using the FORAM instruments, both appeared to be suitable for the analysis of blue gel inks on paper. Using a combination of 10% laser power intensity with x 20 obj. provided the best spectral response at 685 nm excitation wavelength, whilst a combination of 100% laser power with x 50 obj. was the most suitable for the 785 nm excitation wavelength. Using full laser power and x 50 obj. provided a reasonable spectrum for red ink on paper at 785 nm excitation wavelength, albeit consisting of weak Raman peaks on a fluorescence background. Using a higher powered objective (x 100) did not enhance the Raman signal intensity for the red gel ink. In all instances, operating the FORAM instruments in Auto Exposure mode enhanced overall spectral intensity. Spectral acquisition under these conditions was quick for both colour groups, typically only a few seconds. The 685 nm excitation wavelength was not suitable for the analysis of red gel inks on paper due to fluorescence masking the Raman signal. Operating the instrument in Auto Exposure mode provided no further improvement.

Table 6.5 provides a summary of the instrumental settings identified as most suitable for a particular colour of gel ink and excitation wavelength combination applied to the within and between brand variation studies described in *Part B*.

6.2.3.3 Spectral Interference from the Substrate

No spectral interference was observed from either the paper or the glass slide at lower laser power intensity. At higher excitation wavelengths (785 nm and 830 nm) minimal spectral interference from the paper was observed particularly at ~1094 cm^{-1}, ~1400 cm^{-1} and ~1600 cm^{-1}, although this was dependent upon instrument and colour group combination. Likewise, at higher excitation wavelengths, interference from glass in the form of a broad fluorescence band obscuring the Raman signal from the ink was observed. In addition, the design of the FORAM led to interference arising from stray light being an

issue at the 785 nm excitation wavelength. It was concluded that given the observations from the examination of gel ink on glass, the use of the microscope slide sampling method was not considered a suitable means by which to acquire spectra from pure ink for the purposes of creating a spectral database to aid identification of unknown ink samples.

6.2.3.4 Validation Studies

% RSD values <0.3% demonstrated excellent reproducibility in terms of peak position for all validation approaches, instruments and excitation wavelengths (where calculated). Sample size appeared to have little or no influence on the reproducibility of spectral data, and reproducibility of spectral measurements for an ink line on office paper were shown to be excellent. Successful validation for the analysis of blue ink group samples on office paper using 514.5 nm, 785 nm (Renishaw and FORAM) and 830 nm excitation wavelengths was achieved, as it was also for the analysis of red ink group samples on office paper using 785 nm excitation wavelengths (Renishaw only).

Colour Group	Experimental Conditions																	
	Renishaw InVia (10 s Integration Time/1 Accumulation)												FORAM (Auto Exposure/Six Counts)					
	514.5 nm			632.5 nm			785 nm			830 nm			685 nm			785 nm		
	Laser Power		Objective	Laser Power		Objective	Laser Power		Objective	Laser Power		Objective	Laser Power		Objective	Laser Power		Objective
	%	mW		%	mW		%	mW		%	mW		%	mW		%	mW	
Blue	**10**	**0.98**	**x 20**				**10**	**15**	**x 50**	**100**	**160**	**x 50**	**10**	**0.45**	**x 20**	**100**	**2.5**	**x 50**
Red	Fluorescence			Fluorescence			**100**	**150**	**x 50**	Fluorescence and Paper Interference			Fluorescence and Detector Overload			**100**	**2.5**	**x 50**

Table 6.5: Summary of laser power/obj. combinations by excitation wavelength and instrument that provided best spectral quality and reproducibility for a particular colour of ink on paper; laser power/obj. combinations highlighted in **bold** **blue** and **red** denote the conditions applied to the respective colour groups described in *Part B – Within and Between Brand Variation*

6.3 Part B – Within and Between Brand Variation

6.3.1 Experimental

6.3.1.1 Gel Ink Pen Samples

To reiterate from **Chapter Two** an array of gel ink pen samples representing 19 different brands and 27 models available in seven different countries were purchased from High Street retailers and online suppliers. The source countries included the UK, USA (US), South America (SA), Australia (AUS), Japan (JAP), Hong Kong (HK), and Malaysia (MAL). Henceforth, those samples purchased within the UK will be referred to as UK acquired, whilst those purchased abroad will be referred to as INTL acquired. Brands were chosen to reflect a mixture of well-known stationary brands (i.e. BIC, Uniball, etc.) and some lesser known and/or discount brands (i.e. Poundland and The Works discount stores). Where possible, six pens of each brand/model combination for two colour groups blue and red were acquired. A summary of the various pens acquired is presented in Tables 2.2 and 2.3 in **Chapter Two**.

6.3.1.2 Within and Between Brand Variation

For all of the proceeding studies, all pens within a brand/model combination set, where possible, were analysed under the conditions described. This permitted an assessment of both within and between brand variations, to be made for both colour groups under the appropriate excitation wavelengths identified from method development.

6.3.1.2.1 Blue Ink Group

Of the five excitation wavelengths investigated during method development, four key excitation wavelengths were found to be particularly suited to blue gel ink: 514 nm; 685 nm; 785 nm and 830 nm. The fifth excitation wavelength, 632.5 nm, suffered from problems associated with fluorescence and consequently reproducibility and was therefore not used in the investigation of within and between brand variations of the blue ink group samples.

6.3.1.2.1.1 Renishaw InVia

Ink lines from all pens within 31 blue ink group brand/model combinations were prepared as previously outlined. Each line was analysed at two different points along the length

using the laser power/obj. combination described in Table 6.6 for each of three different excitation wavelengths associated with the Renishaw InVia.

Excitation Wavelength (nm)	Laser Power Intensity		Objective
	%	mW	
514.5	10	0.98	x 20
785	10	15	x 50
830	100	160	x 50

Table 6.6: Summary of instrumental conditions (laser power/obj. combination) used for analysing blue ink group samples using the Renishaw InVia at three different excitation wavelengths

6.3.1.2.1.2 Foster & Freeman FORAM

Findings from method development showed operating the FORAM instruments in Auto Exposure mode provided a greater overall spectral intensity. Each ink line was therefore analysed twice at different points along the length using the laser power/obj. combinations and scan time conditions described in Table 6.7 for both excitation wavelengths.

Excitation Wavelength (nm)	Laser Power Intensity		Objective	Auto Exposure (s)
	%	mW		
685[1]	10	0.45	x 20	*[2]
785	100	2.5	x 50	*[3]

Table 6.7: Summary of instrumental conditions (laser power/obj. combination) used for analysing blue ink group samples using the FORAM at two different excitation wavelengths [1] Pens not analysed due to unavailability or ink dry out, BIC UK #03, PPM UK #06, BIC US #10, COF US #05, UNI SA #38, STP UK #03, WHS UK #04, UNI HK #34, WKE UK #03, PTN UK #02 and #03; [2] maximum integration time 16.0s; [3] maximum integration time 1.0s

To permit a like for like comparison between the Renishaw and FORAM instruments at 785 nm excitation wavelength, the FORAM 785 instrument was operated in Auto Exposure mode using the x 50 obj.

6.3.1.2.2 Red Ink Group

Method development showed the 785 nm excitation wavelength to be best suited to the analysis of red gel inks, therefore the red ink group samples were only analysed in full at

this excitation wavelength using both the Renishaw and FORAM instruments under conditions summarised below.

6.3.1.2.2.1 Renishaw InVia

Ink lines from all red gel ink pens representing 25 different brand/model combinations (consisting of 26 ink samples) were analysed at two different points along the length using full (100%) laser power and x 50 obj.

6.3.1.2.2.2 Foster and Freeman FORAM

As described above, ink lines were analysed under full (100%) laser power using the FORAM 785. To enable a like for like comparison between the Renishaw and FORAM data sets, x 50 obj. was used and the FORAM instrument operated in Auto Exposure mode.

6.3.1.3 Pigment Spectral Library

Access to 657 known pigment standards that had previously been characterised by Raman Spectroscopy using 532 nm and 780 nm excitation wavelengths was available [207]. A short list of 221 pigment standards composed of a variety of pigment colours, including blue, violet, green, red, orange and yellow, amongst others provided good spectra at 780 nm and were further examined.

A small quantity (~5 mg) of pigment powder was deposited onto a clean dimpled glass microscope slide. The slide was placed on the XYZ translation stage under x 20 obj. and analysed using the FORAM 785 at full laser power intensity (100%) in Auto Exposure mode. After the analysis, the pigment powder was returned to the storage container and the slide washed with acetone. All spectra were saved, and collated into a spectral database using the instrument software. Of the 221 shortlisted pigments, 200 were included in the library. The outstanding pigments were excluded because they did not yield a good quality spectrum at 785 nm.

A representative spectrum for each identified spectral group of the blue and red gel ink groups was searched against the pigment spectral library to identify any possible match. The software returns a shortlist of possible matches with an associated % match value indicating how similar the sample and library spectra are. Only those pigments with a %

match above 70% were compared manually against the sample spectra to confirm or exclude identification. A search could be performed against the database using sample spectra in its uncorrected or baseline corrected format without affecting the search results.

6.3.2 Results and Discussion

6.3.2.1 Within Brand Variation

All available pens representing the 31 brand/model combinations in the blue ink group were analysed using four excitation wavelengths: 514.5 nm, 685 nm, 785 nm and 830 nm; whilst all pens representing the 25 brand/model combinations in the red ink group were analysed using a single excitation wavelength: 785 nm.

6.3.2.1.1 Blue Ink Group

No within brand variation was observed at lower excitation wavelengths (514.5 nm and 685 nm) however some evidence was identified at higher excitation wavelengths (785 nm and 830 nm).

Based on findings from the FORAM 785, two of the ZBR UK pens (#02 and #03) whist exhibiting an indistinguishable spectral pattern to all other pens within the set, exhibited a different, but reproducible, background fluorescence shape as illustrated in Figure 6.19. The spectra from the other four pens were all indistinguishable to one another in terms of background fluorescence shape. A difference in spectral response between these two pens and the others in the set was also detected using the Renishaw InVia at 785 nm and 830 nm. At these excitation wavelengths, the spectra from these two pens were partially obscured by a combination of fluorescence and detector overload, whilst a reproducible spectral pattern was observed for the other four pens within the set despite a fluorescence background. This is illustrated in Figure 6.20 at 785 nm. In contrast, using the 514.5 nm and (FORAM) 685 nm excitation wavelengths, all six pens exhibited indistinguishable spectra with a Raman signal as illustrated for the former in Figure 6.21. It was possible therefore that the composition of the ink in pen #02 and #03 differed slightly in some way from that of pen #01, #04, #05 and #06. It is worth noting, that at 514.5 nm, the spectral pattern for all ZBR UK samples was of strong Raman peaks on minimal background

fluorescence. At all other excitation wavelengths, fluorescence background interference was a greater issue in general, but especially so for pen #02 and #03.

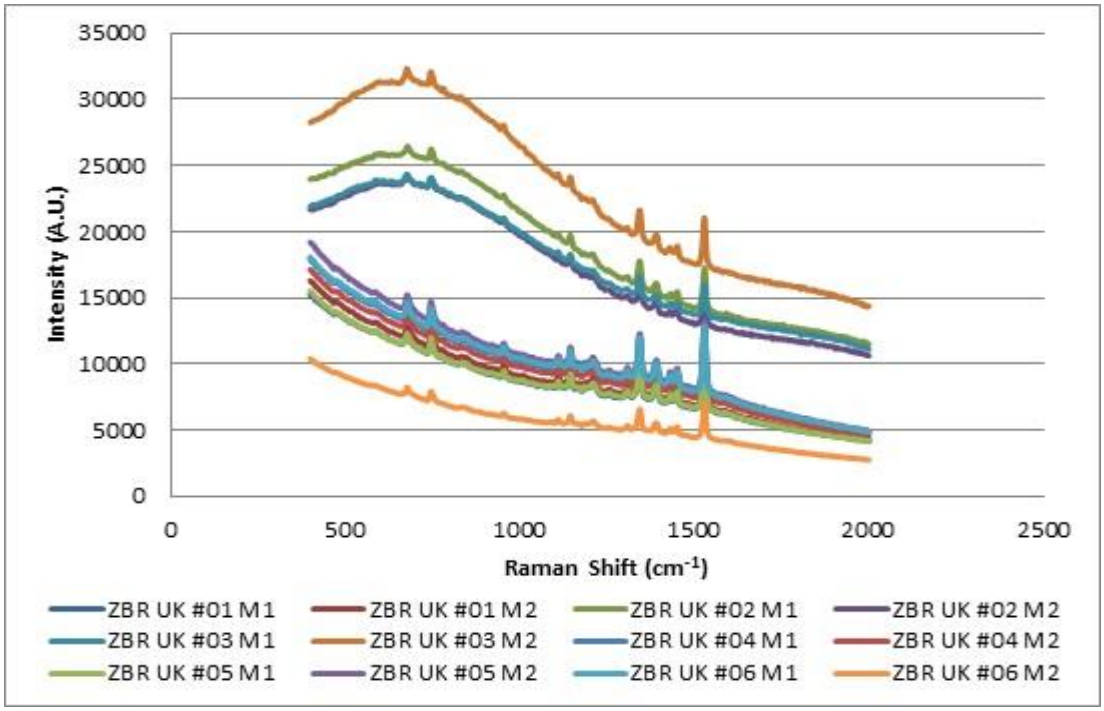


Figure 6.19: Duplicate spectra taken from each of blue ZBR UK pens #01 - #06 on paper demonstrating reproducible differences in background fluorescence shape suggestive of within brand variation (FORAM 785 100% laser power/x 20 obj., Auto Exposure)

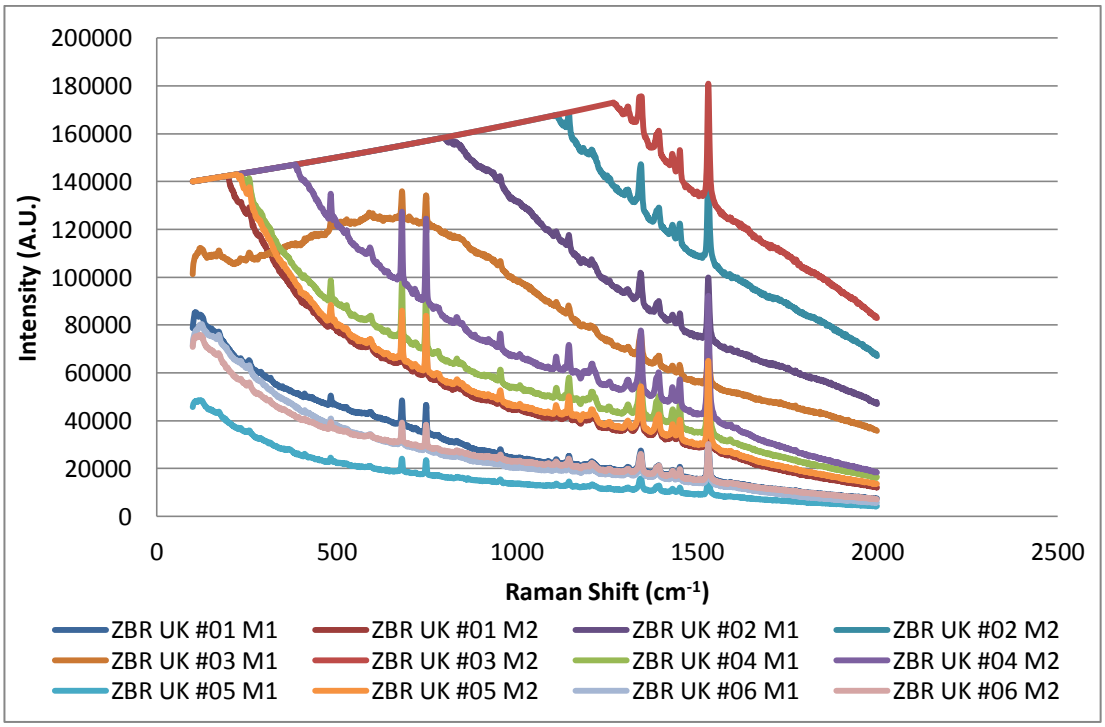


Figure 6.20: Duplicate spectra from each of blue ZBR UK pens #01 – 06 demonstrating some evidence of within brand variation (Renishaw InVia 785 nm 10% laser power/x 50 obj.)

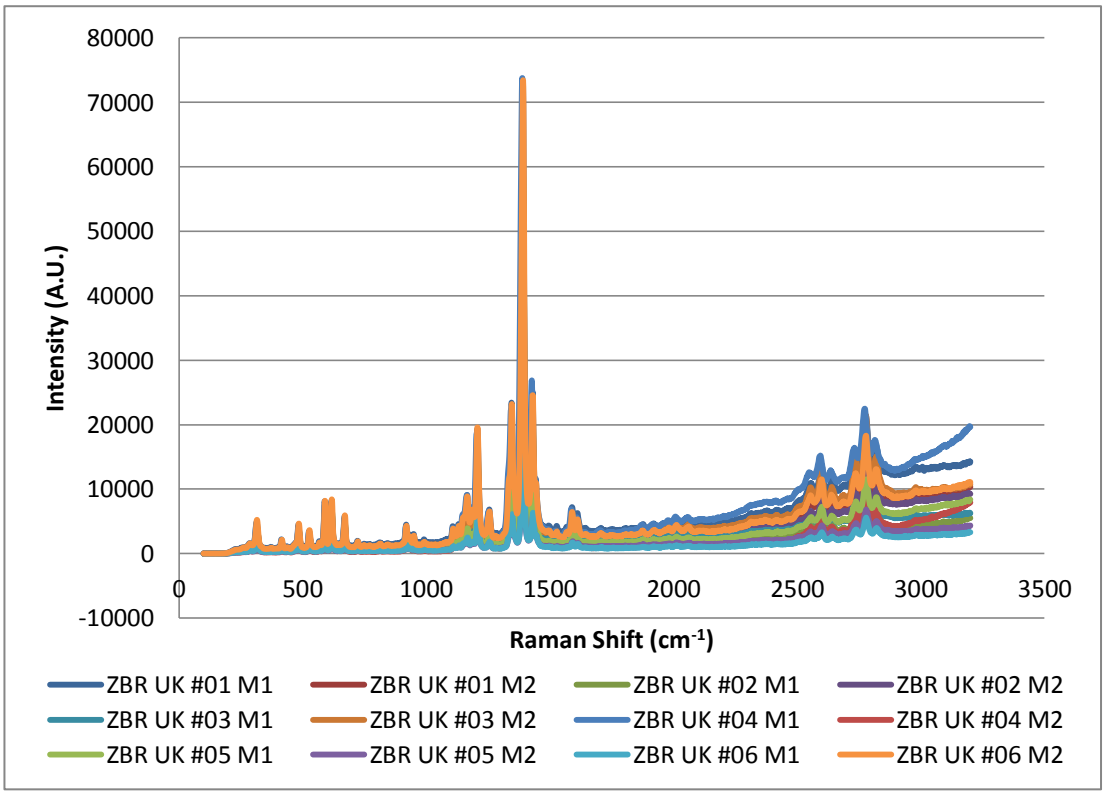


Figure 6.21: Spectra from ZBR UK pens #01 – 06 demonstrating no evidence of within brand variation, contradicting the findings at 785 nm (Renishaw InVia 514.5 nm 10% laser power/x 20 obj.)

As presented in Figure 6.22, the COF US brand/model combination, pen #06, exhibited a notably different spectral pattern to that of the other five pens within the set. Given at other wavelengths, the Raman signal for all COF US samples was obscured by fluorescence and/or detector overload, this within brand variation was only detected at 830 nm. This was interesting since the six COF US pens were acquired from two different packs containing four pens each, suggesting that in at least one pack, the pens may have contained gel ink of two different formulations.

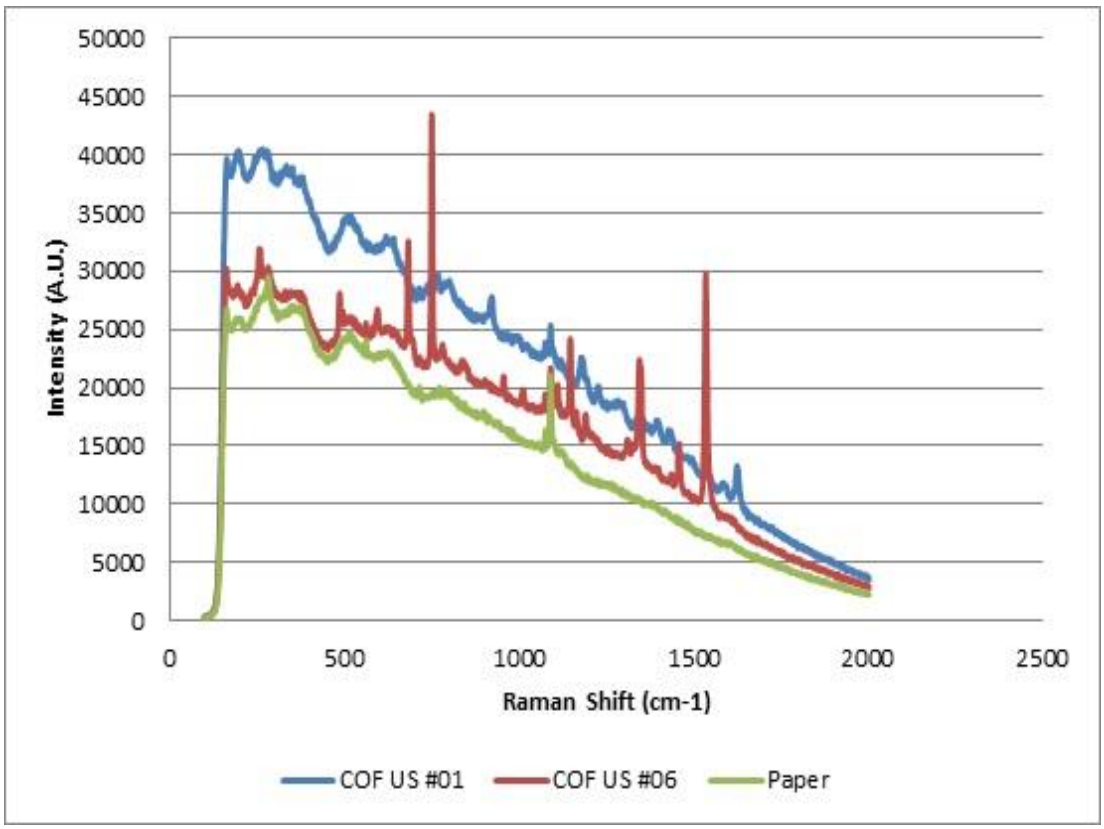


Figure 6.22: Example of within brand variation observed in COF US brand/model combination, as well as spectral interference from the paper substrate (Renishaw InVia 830 nm 10% laser power/x 50 obj.)

No further within brand variation was observed amongst the blue ink group samples across all four excitation wavelengths studied in depth.

6.3.2.1.2 Red Ink Group

The WHS UK brand/model combination could be discriminated into two different groups (Group 1 & 2) based on clear spectral differences, i.e. peak presence or absence. Pen #01 – #03 exhibited spectra that were all indistinguishable to each other, but clearly distinguishable from that of pen #04 – #06. For this particular brand/model combination, the ink composition for these two sub-groups of pen differed sufficiently to be detected by Raman Spectroscopy, using both Renishaw InVia and the FORAM, thus providing evidence of within brand variation. An example of this difference is shown in Figure 6.23 representing the Renishaw InVia spectra of WHS UK #01 and WHS UK #04. Specifically spectral differences, highlighted with the green circles, can be observed in the region 1590 – 1500 cm^{-1}, 1051 - 970 cm^{-1} and at ~792 cm^{-1}, ~569 cm^{-1} and ~337 cm^{-1}. No further within brand variation was observed amongst the red ink group samples at 785 nm.

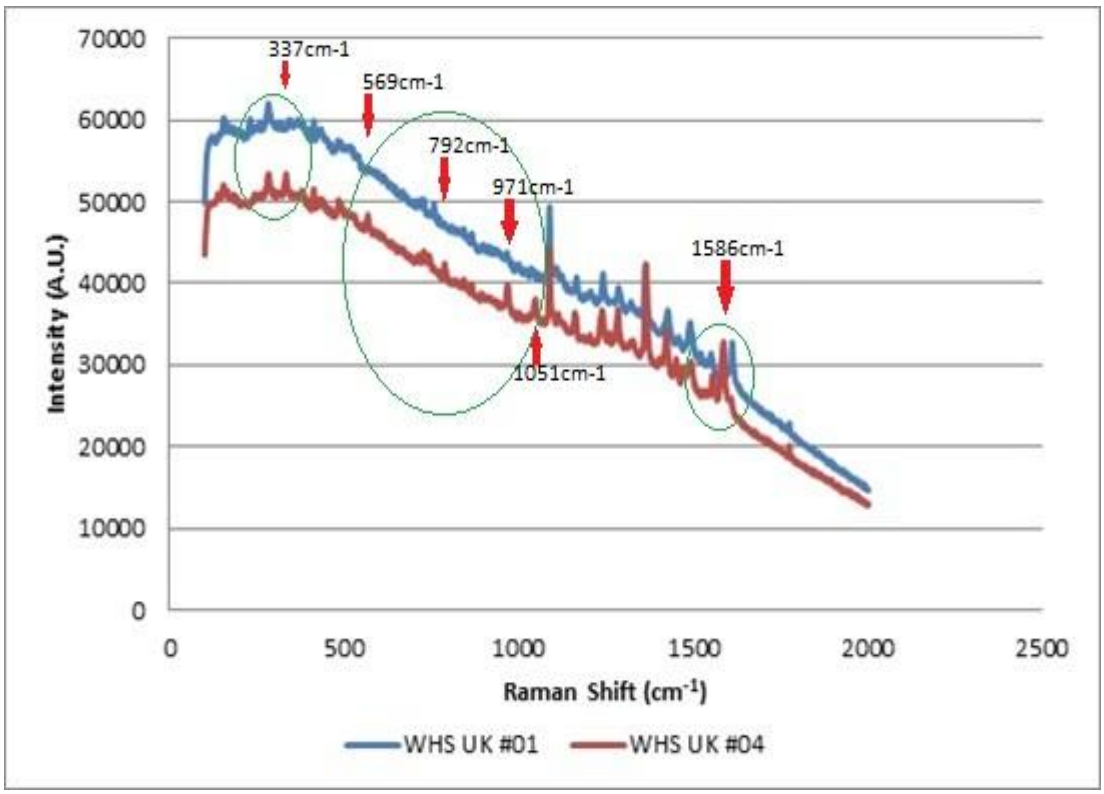


Figure 6.23: Comparison of spectra from WHS UK #01 'v' WHS UK #04 red gel ink on paper demonstrating within brand variation (Renishaw InVia 785 nm 100% laser power/x 50 obj.). Regions of difference highlighted within green circles with specific wavenumber positions identified by red arrows

6.3.2.2 Between Brand Variation

6.3.2.2.1 Blue Ink Group

All pens representing the 31 blue ink group brand/model combinations were analysed using four excitation wavelengths: 514.5 nm, 685 nm, 785 nm and 830 nm to assess between brand variation.

6.3.2.2.1.1 514.5 nm Excitation Wavelength

6.3.2.2.1.1.1 Spectral Quality and Reproducibility

Twenty five brand/model combinations provided spectra with a strong Raman signal whilst the remaining six (dye containing) exhibited spectra swamped by fluorescence and/or detector overload. All spectra within a brand/model combination exhibited good S:N ratio and reproducibility, an example of which is provided in Figure 6.24. No spectral interference from the paper substrate or stray peaks was observed. For those samples which exhibited a Raman signal, only minimal background fluorescence was seen.

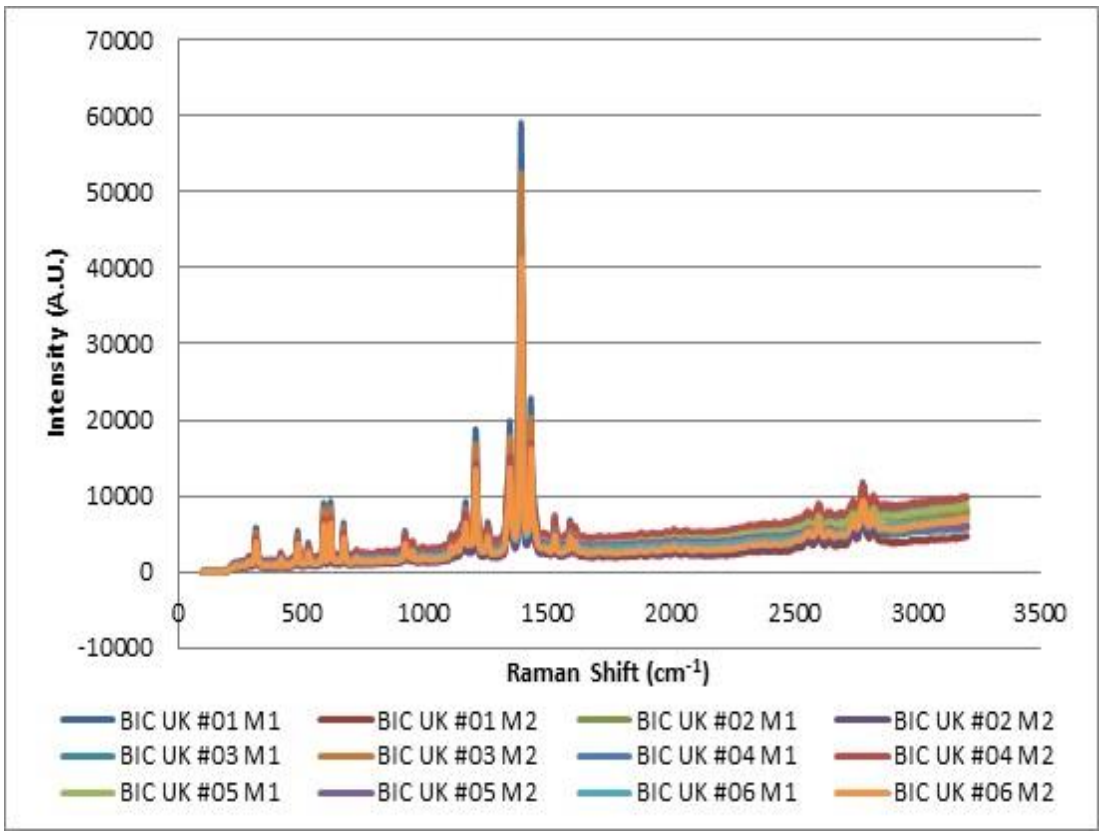


Figure 6.24: Comparison of duplicate spectra from six BIC UK blue gel ink pen lines on paper demonstrating good reproducibility and strong Raman signals (Renishaw InVia 514.5 nm 10% laser power/x 20 obj.)

6.3.2.2.1.1.2 Classification

Discrimination of the 31 brand/model combinations representing the blue ink group at a particular excitation wavelength was achieved by visual pattern recognition based on the presence or absence of peaks and relative peak intensities.

All brand/model combination spectra could be discriminated into six groups shown in Figures 6.25 – 6.30. These consisted of four pigment based groups (Group 1 (nine samples), Group 2 (seven samples), Group 3 (three samples) and Group 4 (two samples)) and two dye containing groups (Group 5 (four samples) and Group 6 (six samples)).

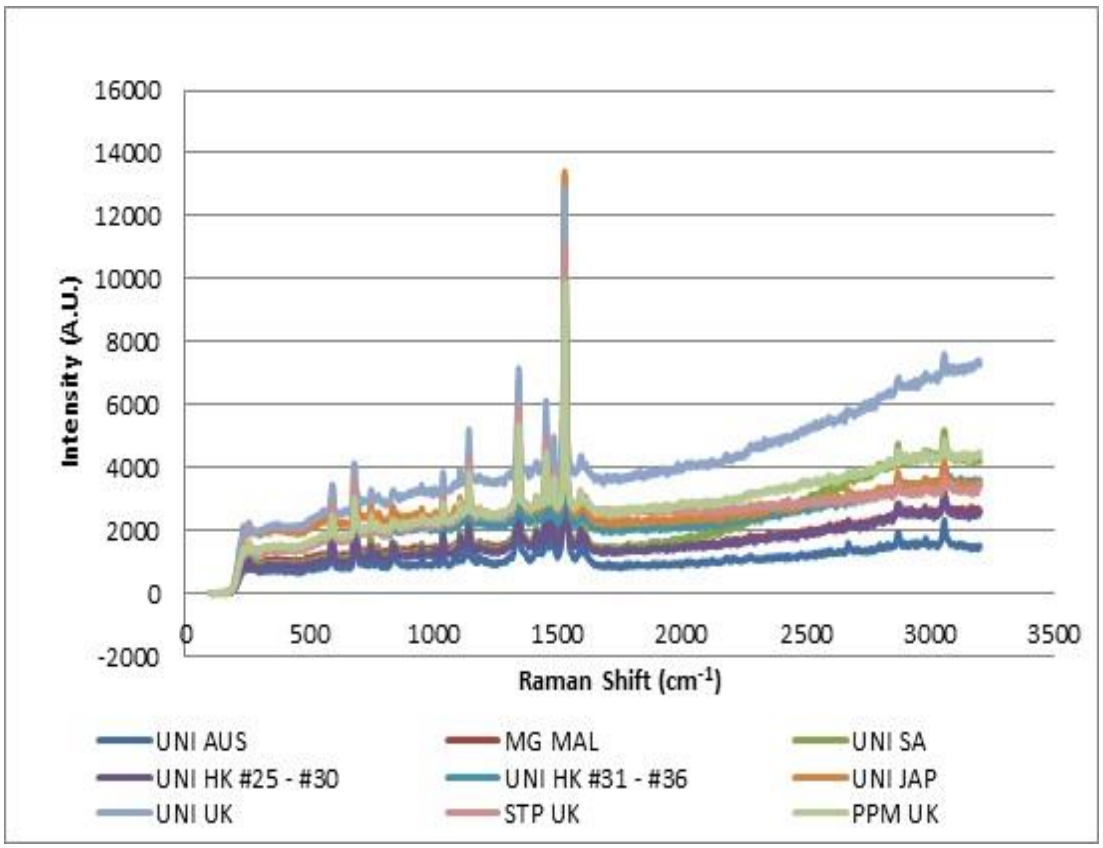


Figure 6.25: Brand/model combinations of blue gel ink forming Group 1 (Renishaw InVia 514.5 nm 10% laser power/x 20 obj.)

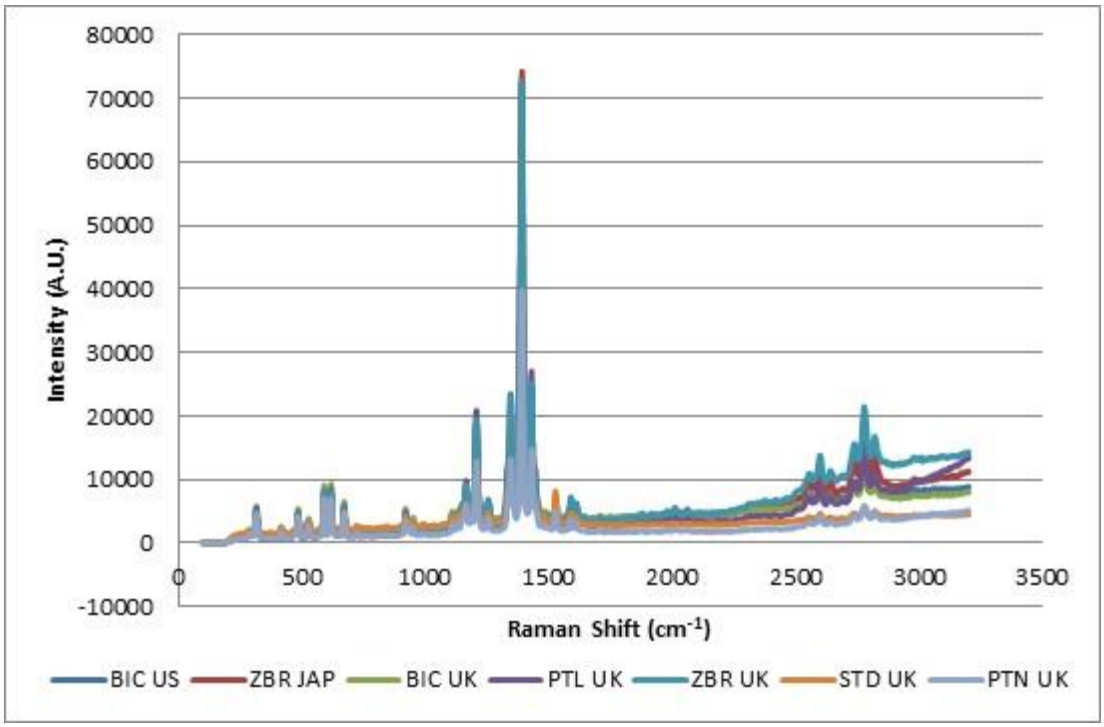


Figure 6.26: Brand/model combinations of blue gel ink forming Group 2 (Renishaw InVia 514.5 nm 10% laser power/x 20 obj.)

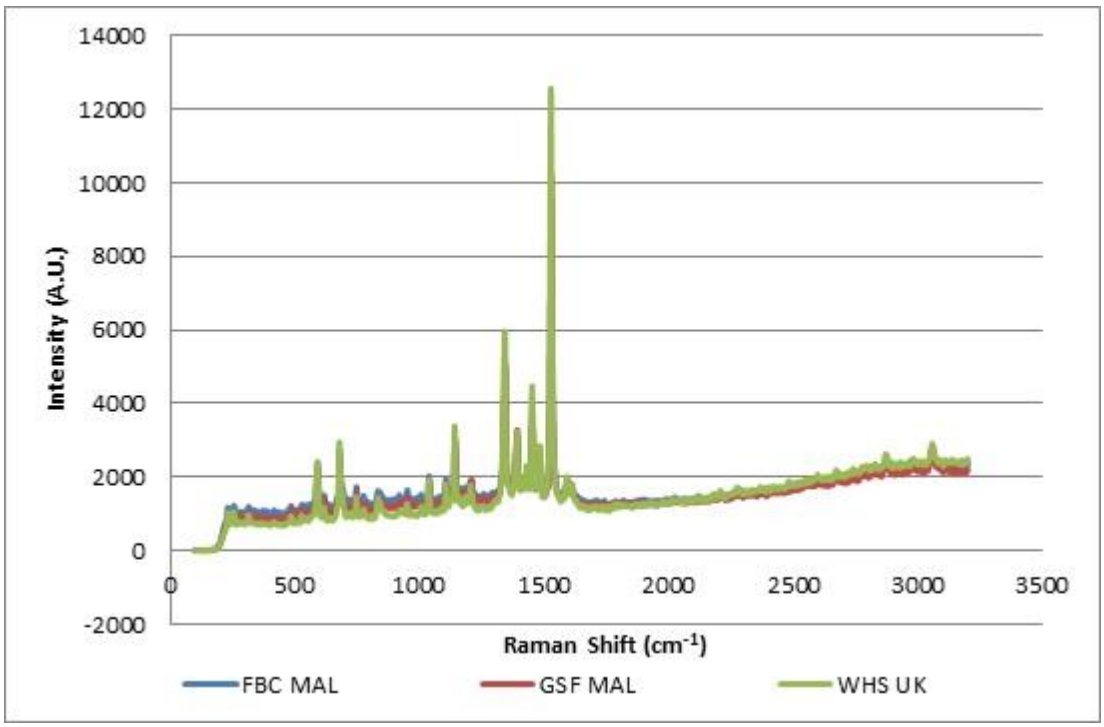


Figure 6.27: Brand/model combinations of blue gel ink forming Group 3 (Renishaw InVia 514.5 nm 10% laser power/x 20 obj.)

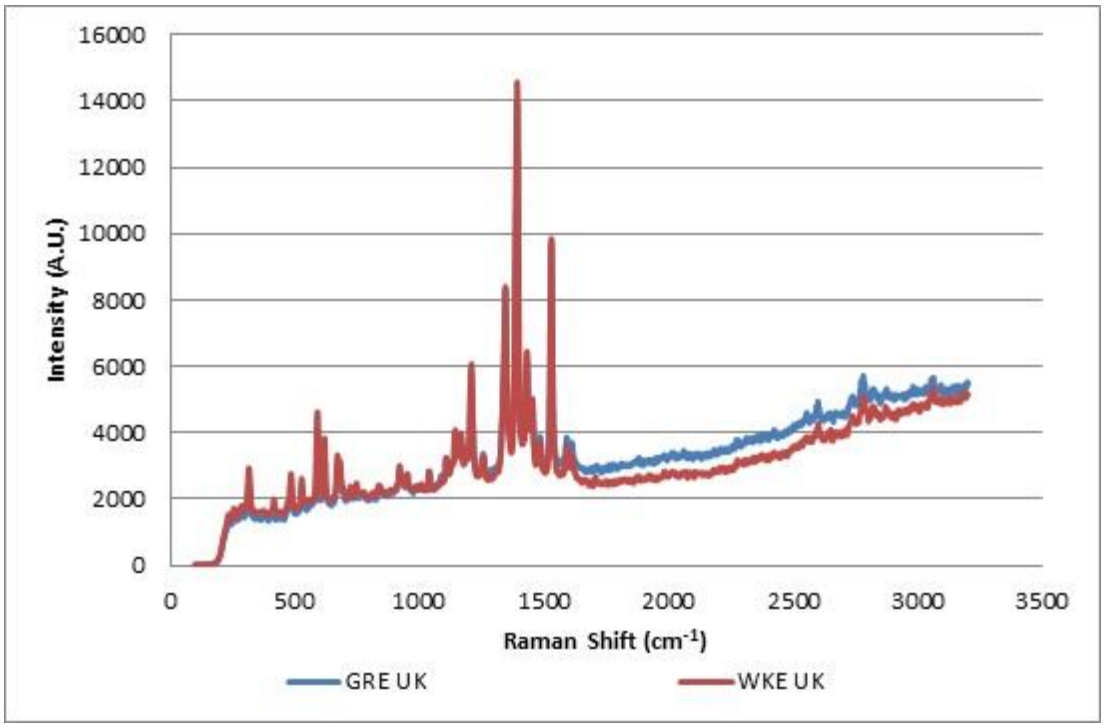


Figure 6.28: Brand/model combinations of blue gel ink forming Group 4 (Renishaw InVia 514.5 nm 10% laser power/x 20 obj.)

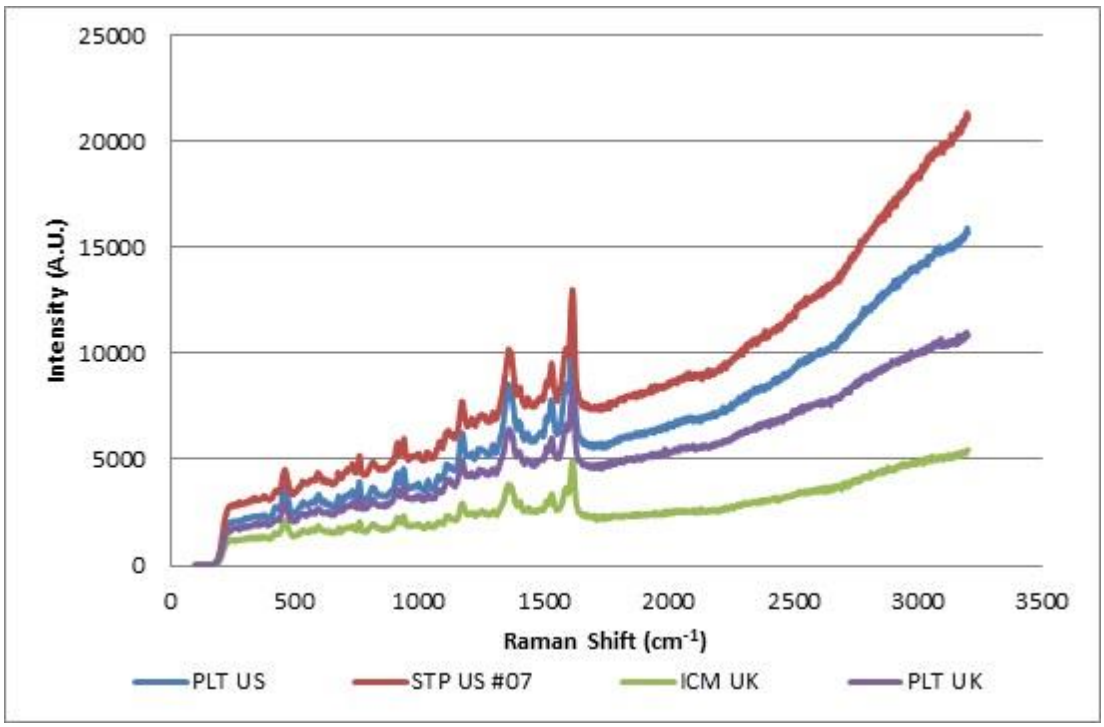


Figure 6.29: Brand/model combinations of blue gel ink forming Group 5 (Renishaw InVia 514.5 nm 10% laser power/x 20 obj.)

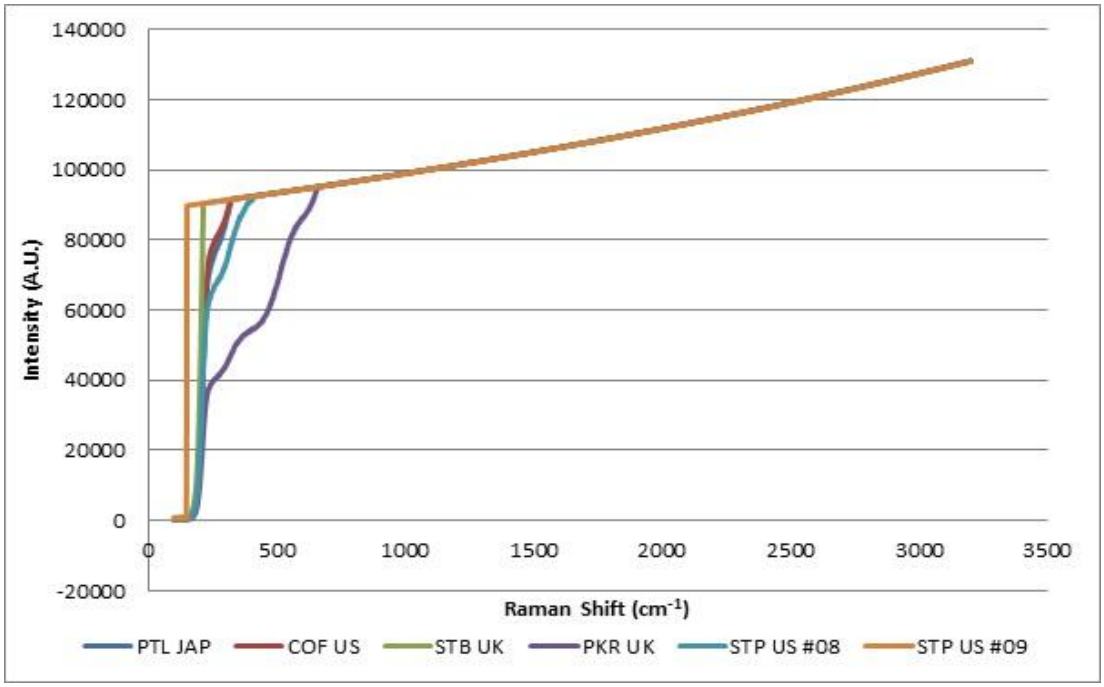


Figure 6.30: Brand/model combinations of blue gel ink forming Group 6 (Renishaw InVia 514.5 nm 10% laser power/x 20 obj.)

Group 1 and 3 spectra, shown in Figure 6.31, were very similar, but peaks at ~1391 cm^{-1} and ~1205 cm^{-1}, as well as several smaller peaks below ~590 cm^{-1} differentiated the latter from the former. All remaining groups were clearly distinguishable from all others.

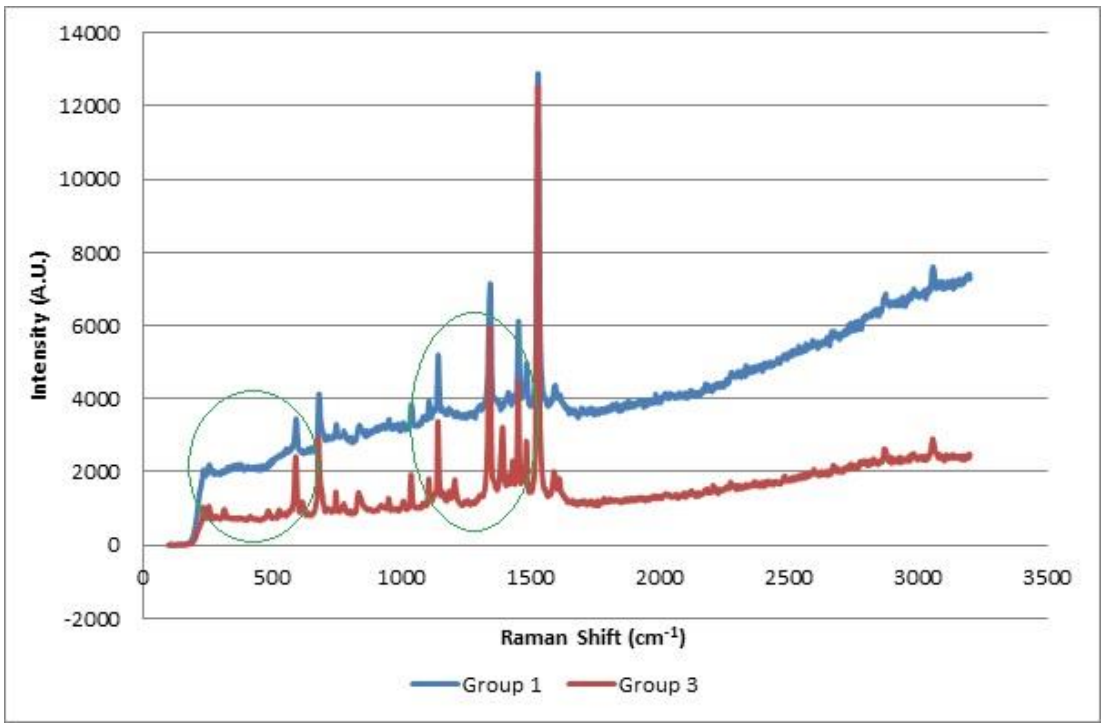


Figure 6.31: Comparison of Group 1 and Group 3 spectra for blue gel ink samples, spectral differences highlighted within green circles (Renishaw InVia 514.5 nm 10% laser power/x 20 obj.)

6.3.2.2.1.2 685 nm Excitation Wavelength

6.3.2.2.1.2.1 Spectral Quality and Reproducibility

Twenty two brand/model combinations provided a Raman signal, whilst nine dye containing brand/model combinations yielded spectra swamped by fluorescence and/or detector overload. Spectra exhibited a good S:N ratio and reproducibility within a brand/model combination, with only very minor variations in fluorescence background shape observed as demonstrated in Figure 6.32. No spectral interference from the paper substrate or stray peaks was observed. Integration times selected by Auto Exposure ranged between 0.01s – 16.0s, providing an overall spectral intensity generally >20, 000 (A.U.).

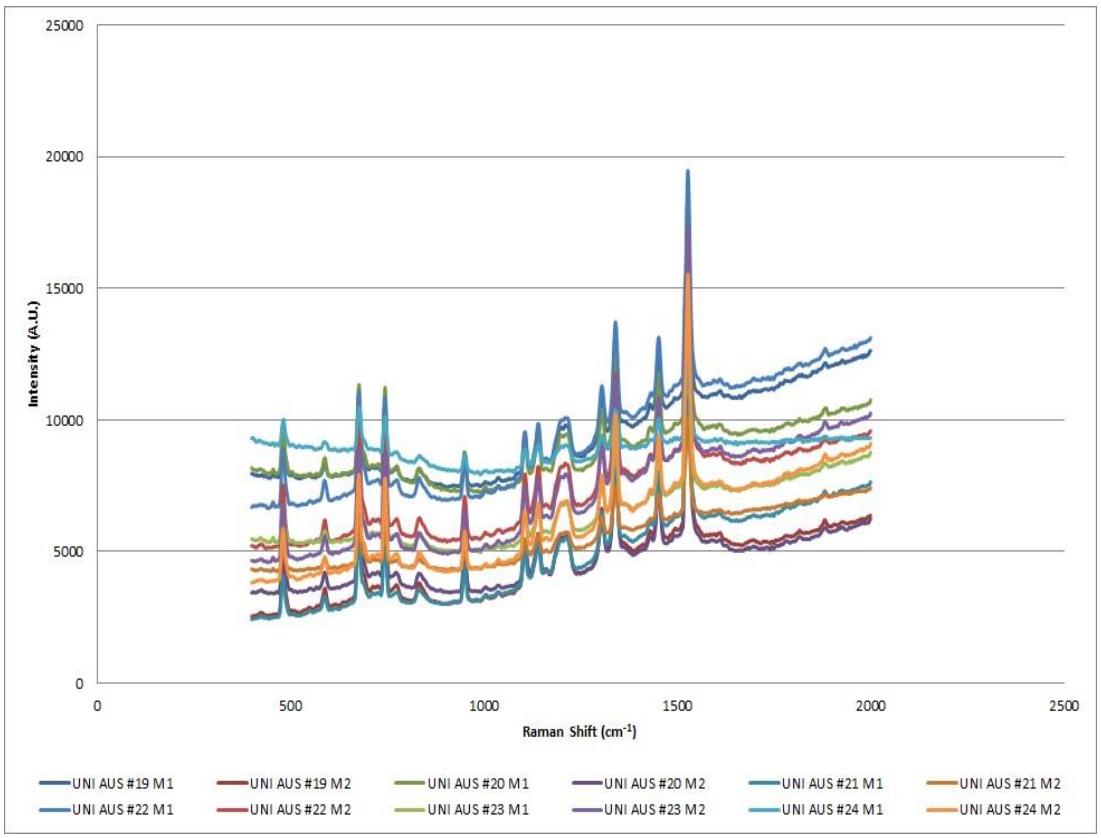


Figure 6.32: Duplicate spectra from each of blue UNI AUS pens #19 – #24 demonstrating good reproducibility and S:N ratio with only minor variation in background fluorescence shape (FORAM 685-2 10% laser power/x 20 obj., Auto Exposure)

6.3.2.2.1.2.2 Classification

All brand/model combination spectra could be discriminated into four groups as shown in Figures 6.33 – 6.36. These consisted of two pigment based groups (Group 1 (14 samples) and Group 2 (seven samples)) and two dye containing groups (Group 3 (one sample) and Group 4 (nine samples)).

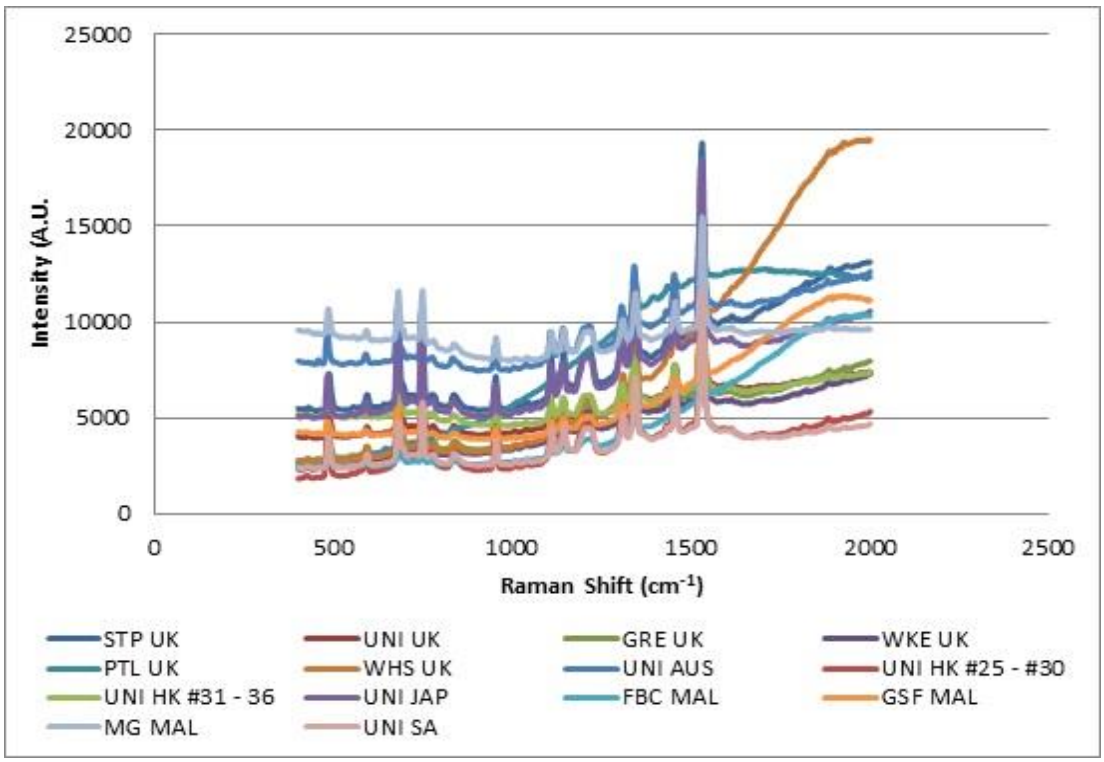


Figure 6.33: Brand/model combinations of blue gel ink forming Group 1 (FORAM 685-2 10% laser power/x 20 obj., Auto Exposure)

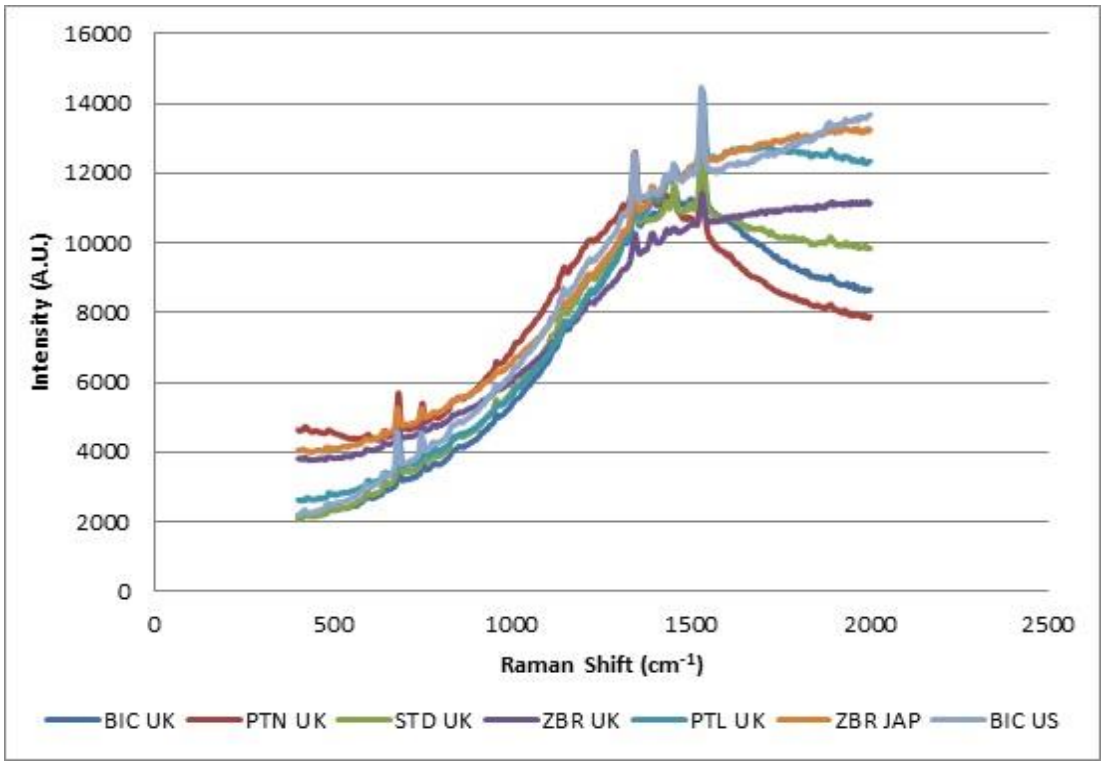


Figure 6.34: Brand/model combinations of blue gel ink forming Group 2 (FORAM 685-2 10% laser power/x 20 obj., Auto Exposure)

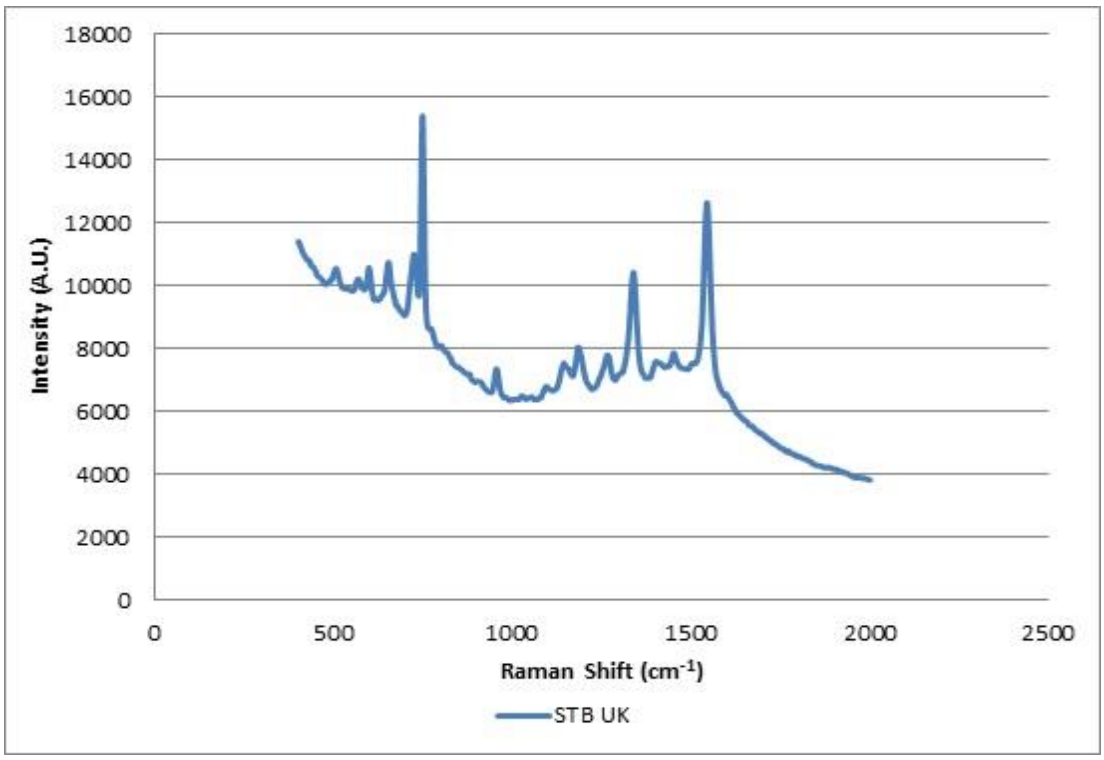


Figure 6.35: Brand/model combination of blue gel ink forming Group 3 (FORAM 685-2 10% laser power/x 20 obj., Auto Exposure)

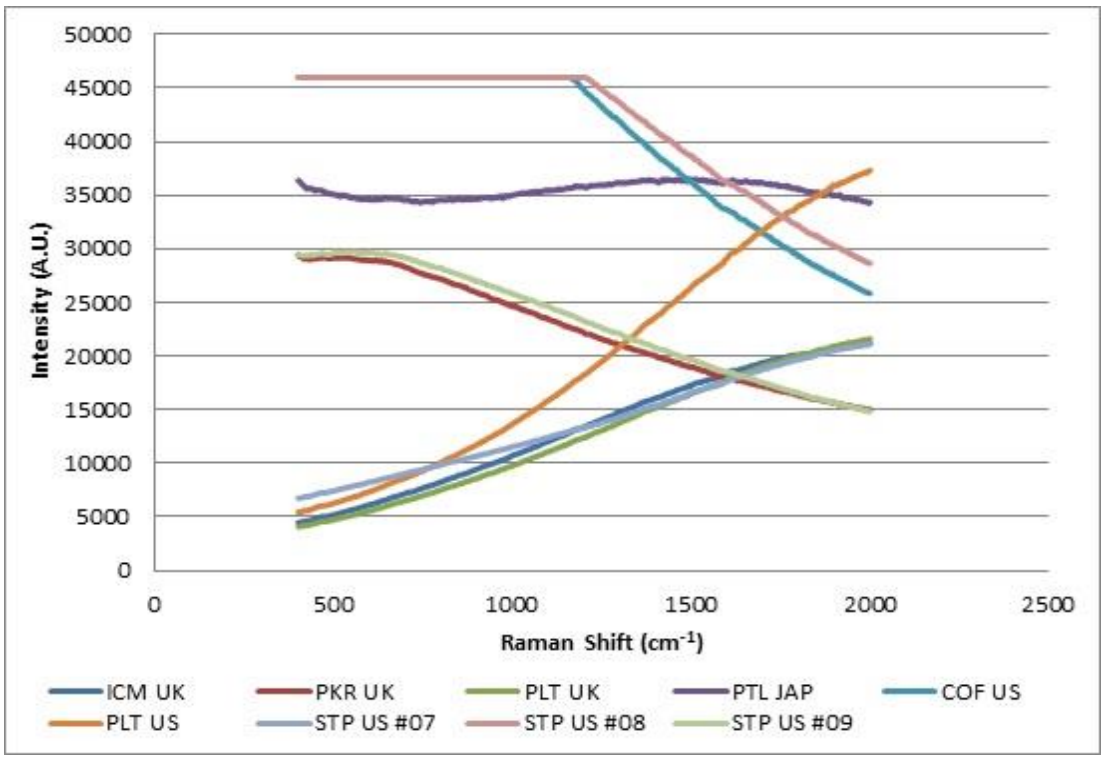


Figure 6.36: Brand/model combinations of blue gel ink forming Group 4 (FORAM 685-2 10% laser power/x 20 obj., Auto Exposure)

6.3.2.2.1.3 785 nm Excitation Wavelength

6.3.2.2.1.3.1 Spectral Quality and Reproducibility

6.3.2.2.1.3.1.1 Foster & Freeman FORAM

Twenty two brand/model combinations provided a strong Raman signal, whilst nine dye containing brand/model combinations yielded spectra swamped by fluorescence and/or detector overload. With the exception of the ZBR UK sample set (see *Within Brand Variation*), spectra exhibited good reproducibility within a brand/model combination, with only minor variations in fluorescence background shape observed. Good S:N ratio was also observed. Spectral interference from the paper substrate was minimal with only a peak at ~1094 cm^{-1} noted. Integration times selected by Auto Exposure ranged between 0.05s – 16.0s, with the majority recorded at 1.0s, the same as the default manual integration time. An overall spectral intensity range between 18, 000 – 40, 000 (A.U.) was observed, with the majority of spectra recording between 20, 000 – 25, 000 (A.U).

6.3.2.2.1.3.1.2 Renishaw InVia

Twenty two brand/model combinations exhibited spectra with a strong Raman signal. The remaining nine dye containing brand/model combinations provided spectra obscured by fluorescence and/or detector overload. Good reproducibility within a brand/model combination was observed, with only minor spectral variation observed in four samples (UNI UK; GRE UK; STB UK and UNI HK #31 – #36) due to S:N ratio. An example of this is presented in Figure 6.37. The background fluorescence shape was generally reproducible within a brand/model combination set. However, for several samples, some minor variation, predominantly in the form of varied steepness to the fluorescence curve below 1000 cm^{-1} was observed (e.g. PPM UK; UNI UK; GRE UK, WKE UK, ZBR UK; UNI HK #31 – #36; UNI JAP; ZBR JAP and UNI SA). An example of this is provided in Figure 6.38. For three brand/model combinations (WHS UK; FBC MAL and BIC US), partial detector overload was observed between 0 – 1000 cm^{-1} in some spectra within a set as illustrated by Figure 6.39. A difference in spectral response from two pens within the ZBR UK brand/model combination set, also detected using the FORAM 785 was noted (See *Within Brand Variation*). Peaks attributable to the substrate identified in the un-inked paper spectra occurred at ~400 cm^{-1}, ~900 cm^{-1}, ~1094 cm^{-1}, ~1800 cm^{-1}, and ~2900 cm^{-1}. However, only the peak at ~1094 cm^{-1} was observed in the majority of ink spectra and at only a very low intensity as demonstrated in Figure 6.40. The peaks at ~400 cm^{-1}, ~900 cm^{-1}, ~1800 cm^{-1}

and ~2900 cm^{-1} were only visible at low intensity in a few ink spectra. Therefore, spectral interference from the paper was minimal, but is recommended as a consideration when comparing and/or identifying ink spectra.

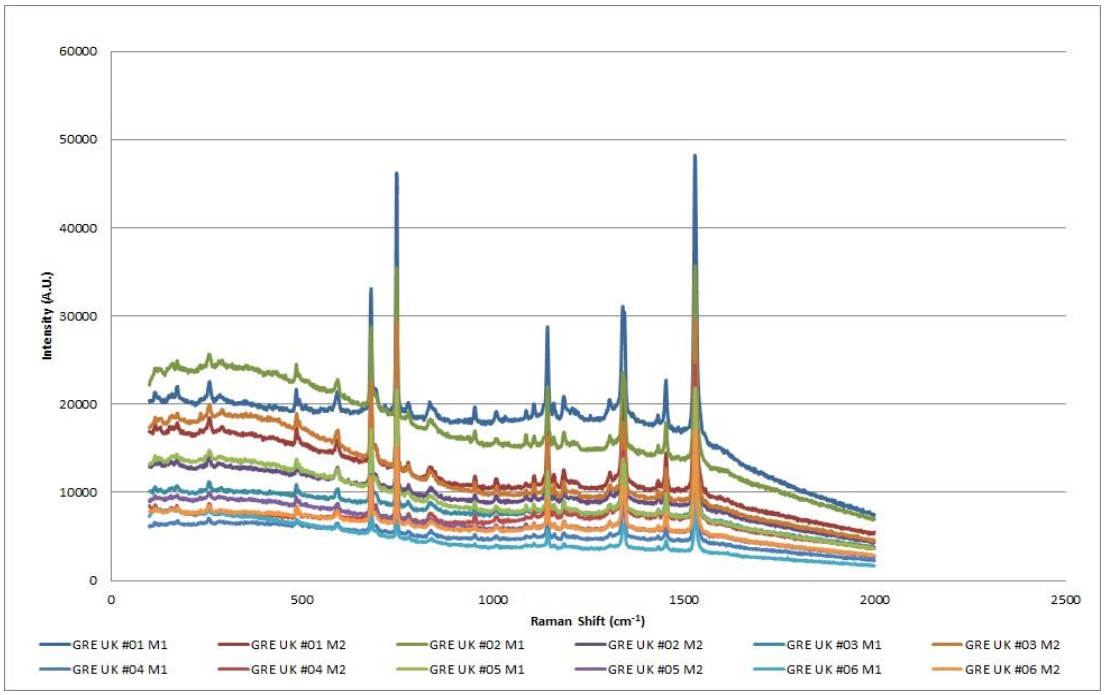


Figure 6.37: Set of 12 spectra from GRE UK blue gel ink illustrating good reproducibility with only minor spectral variation due to S:N ratio (Renishaw InVia 785 nm 10% laser power/x 50 obj.)

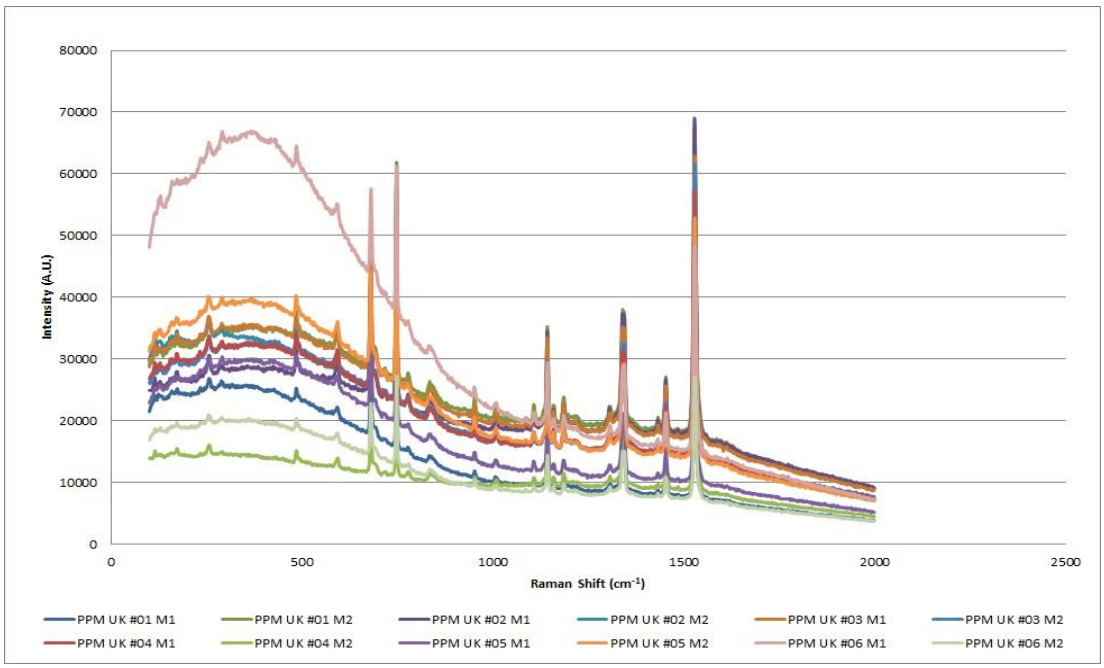


Figure 6.38: Set of 12 spectra from PPM UK blue gel ink illustrating minor variation in background fluorescence shape (Renishaw InVia 785 nm 10% laser power/x 50 obj.)

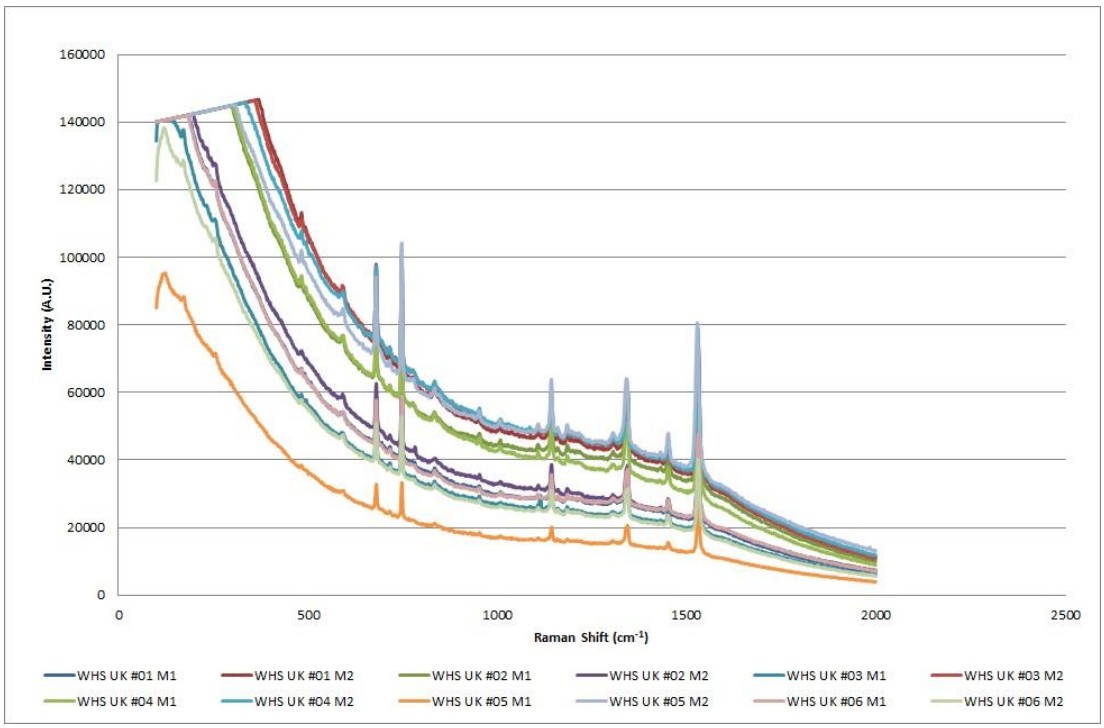


Figure 6.39: Set of 12 spectra from WHS UK blue gel ink illustrating partial detector overload in several measurements (Renishaw InVia 785 nm 10% laser power/x 50 obj.)

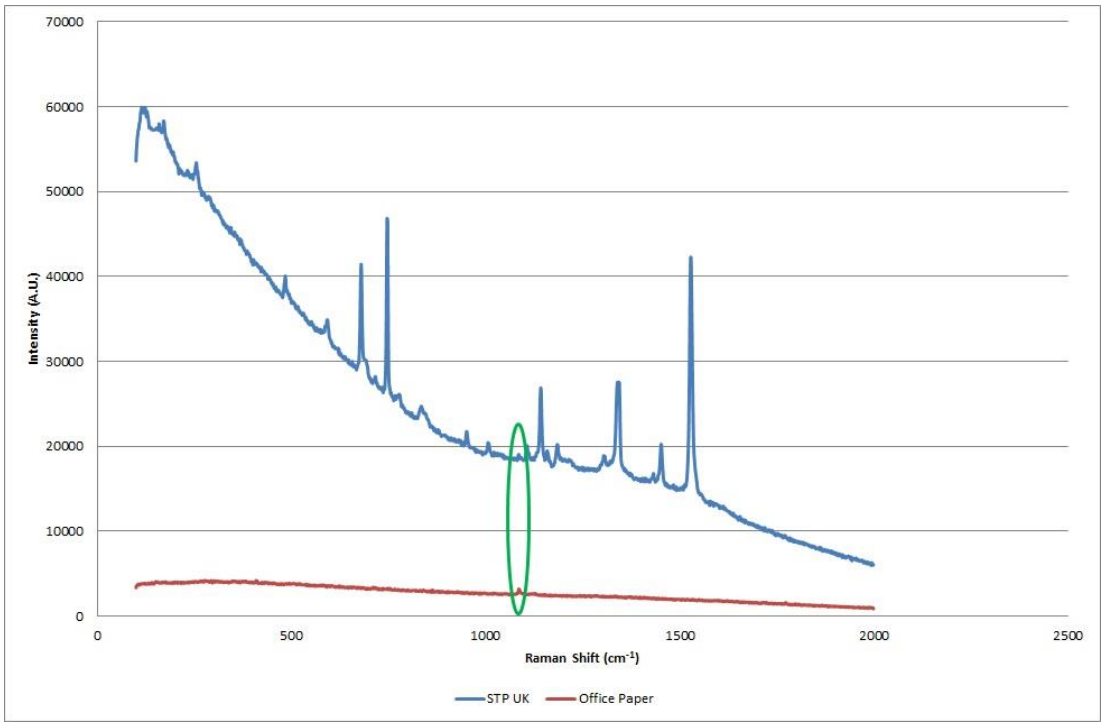


Figure 6.40: An example spectrum of STP UK blue gel ink on paper compared to a spectrum of the un-inked paper illustrating minimal spectral interference from the paper substrate except for a minor peak at ~1094 cm^{-1} highlighted within the green circle (Renishaw InVia 785 nm 10% laser power/x 50 obj.)

6.3.2.2.1.3.2 Classification

6.3.2.2.1.3.2.1 Foster and Freeman FORAM

All brand/model combination spectra could be discriminated into the same four groups identified using the FORAM 685 irrespective of the instrumental conditions used and are shown in Figures 6.41 – 6.44.

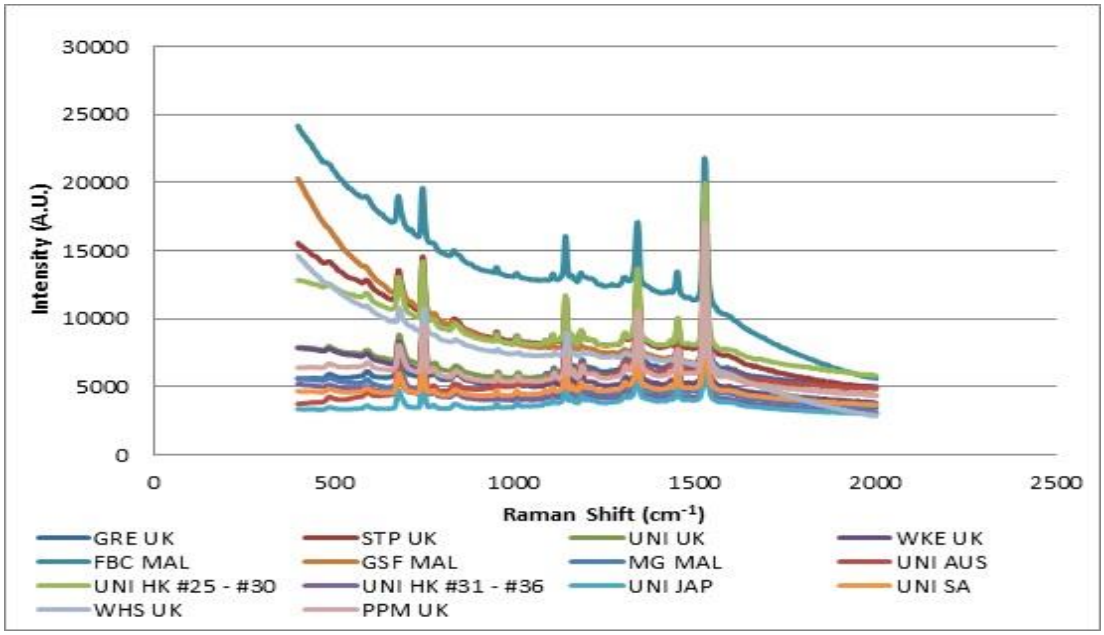


Figure 6.41: Brand/model combinations of blue gel ink forming Group 1 (FORAM 785 100% laser power/x 50 obj., Auto Exposure)

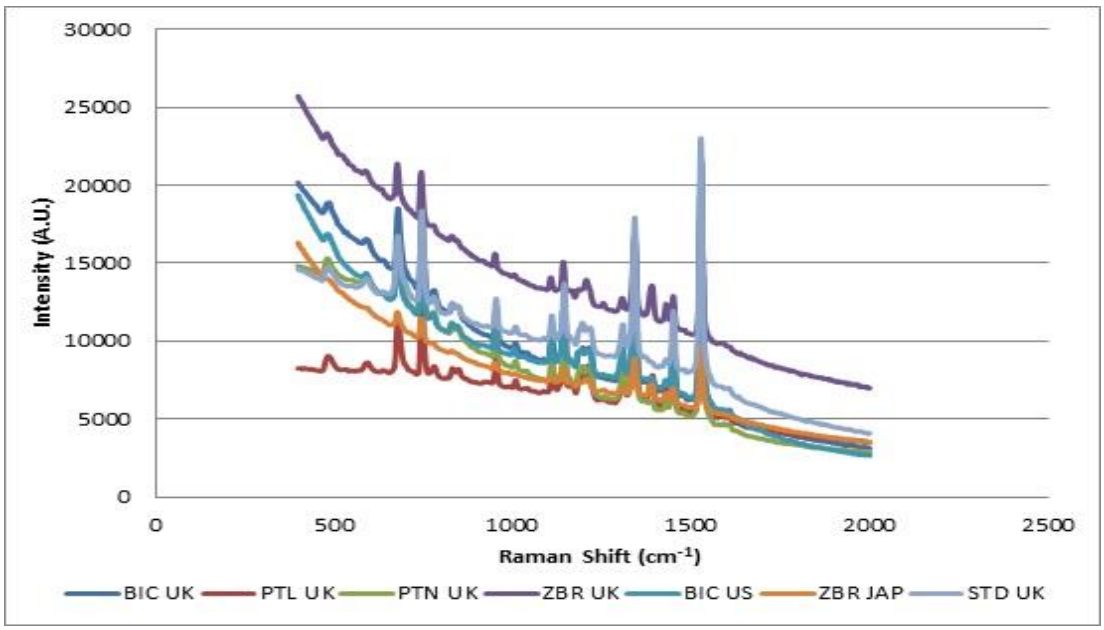


Figure 6.42: Brand/model combinations of blue gel ink forming Group 2 (FORAM 785 100% laser power/x 50 obj., Auto Exposure)

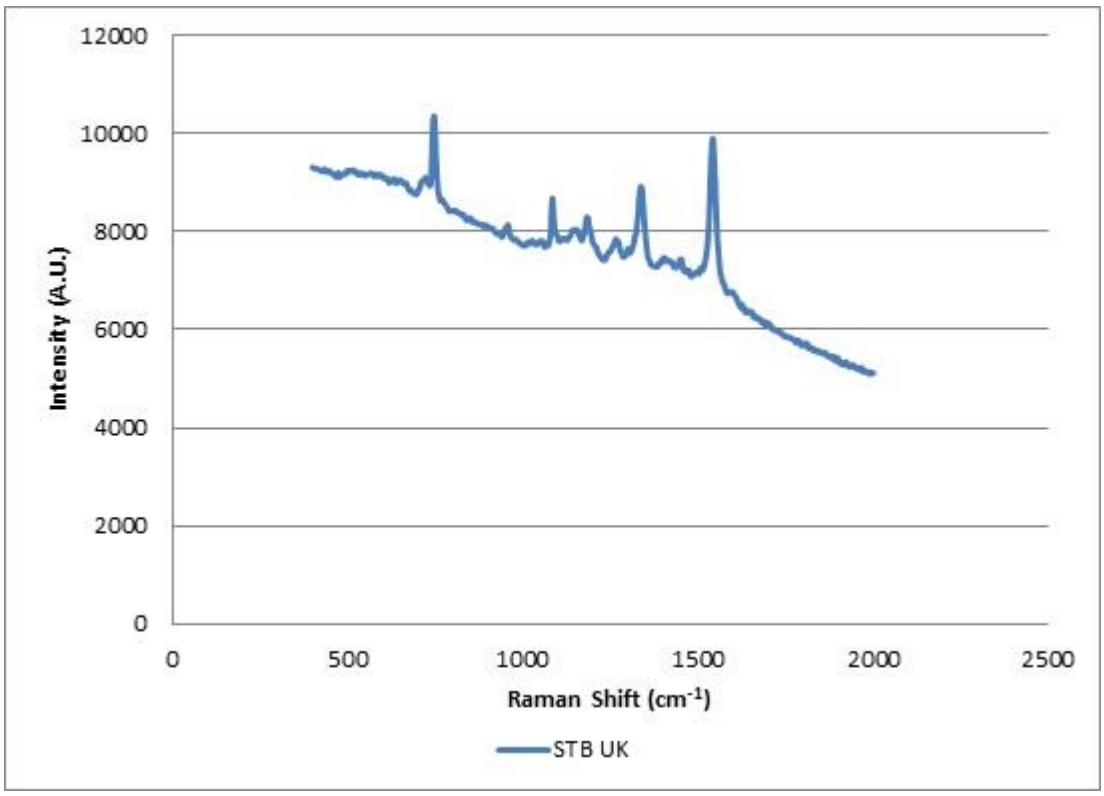


Figure 6.43: Brand/model combinations of blue gel ink forming Group 3 (FORAM 785 100% laser power/x 50 obj., Auto Exposure)

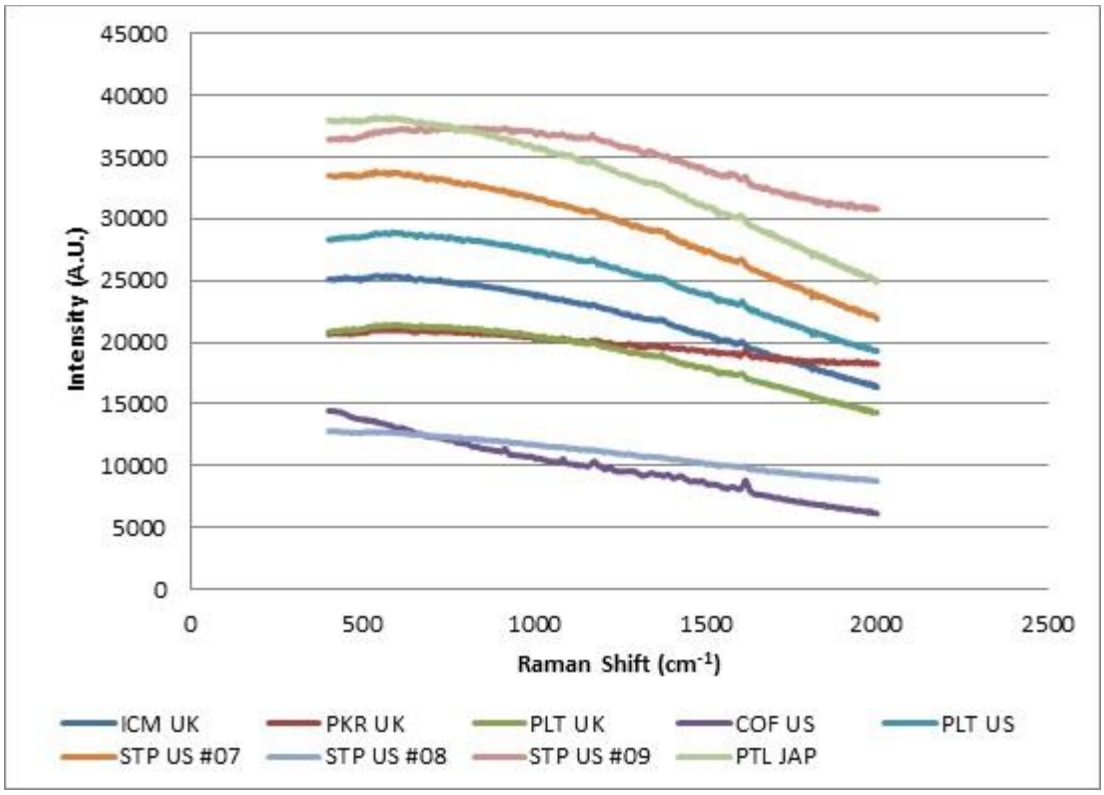


Figure 6.44: Brand/model combinations of blue gel ink forming Group 4 (FORAM 785 100% laser power/x 50 obj., Auto Exposure)

6.3.2.2.1.3.2.2 Renishaw InVia

All brand/model combination spectra could be discriminated into the same four groups identified using both the FORAM 685-2 and FORAM 785. Since the samples were analysed over several days, and the Raman Spectrometer re-calibrated at the beginning of each day, a slight shift (<1 cm^{-1}) in wavenumber position was observed between similar spectral measurements taken on different days. To compare spectra from samples analysed on different days, only one set of x-axis co-ordinates was used. Furthermore, a difference in the total number of x-axis co-ordinates recorded for all INTL acquired samples, prevented spectra from these being plotted on the same axis as the UK acquired samples for a single group as they gave the appearance of being distinguishable. When plotted on separate charts and compared side by side however, the spectral patterns were considered indistinguishable. Therefore the Renishaw classification groups, shown in Figure 6.45 – Figure 6.51, are presented individually as INTL and/or UK samples only, rather than as INTL and UK sample spectra for one group on the same axis.

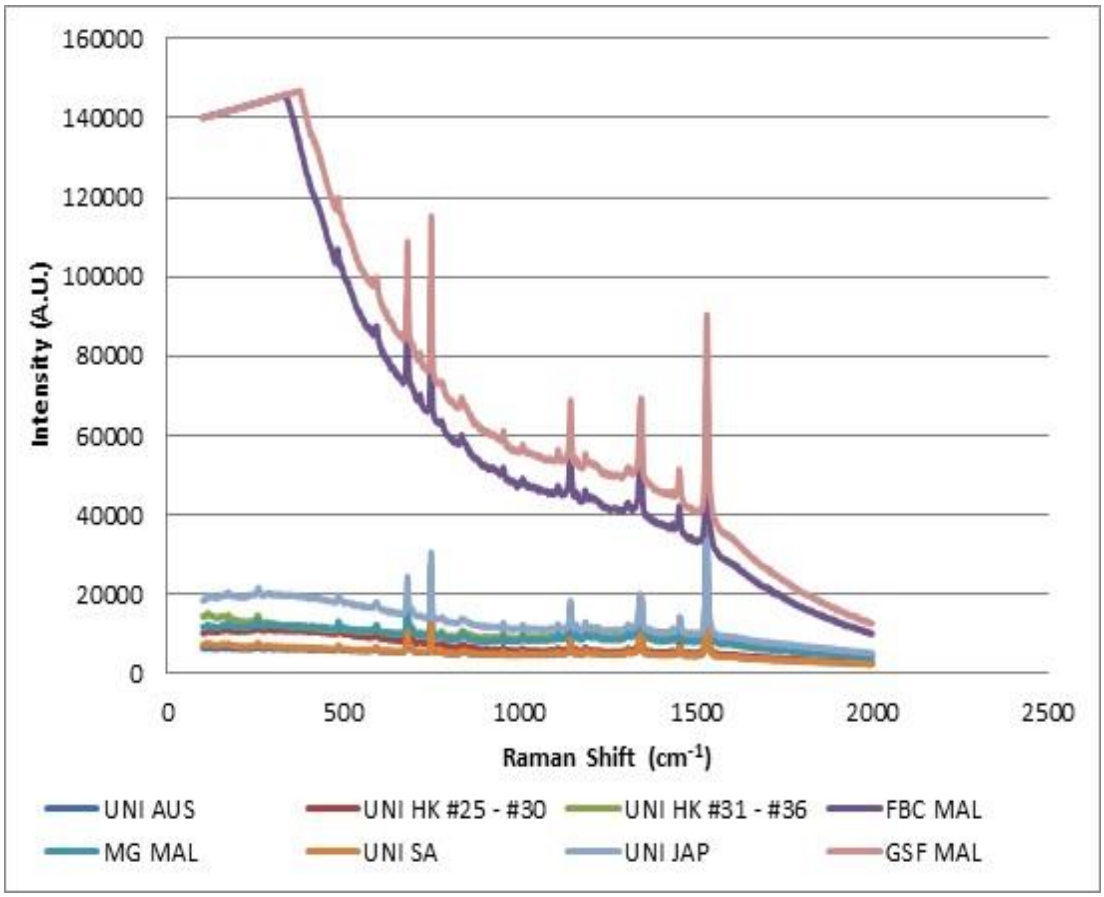


Figure 6.45: INTL brand/model combinations of blue gel ink forming Group 1 (Renishaw InVia 785 nm 10% laser power/x 50 obj.)

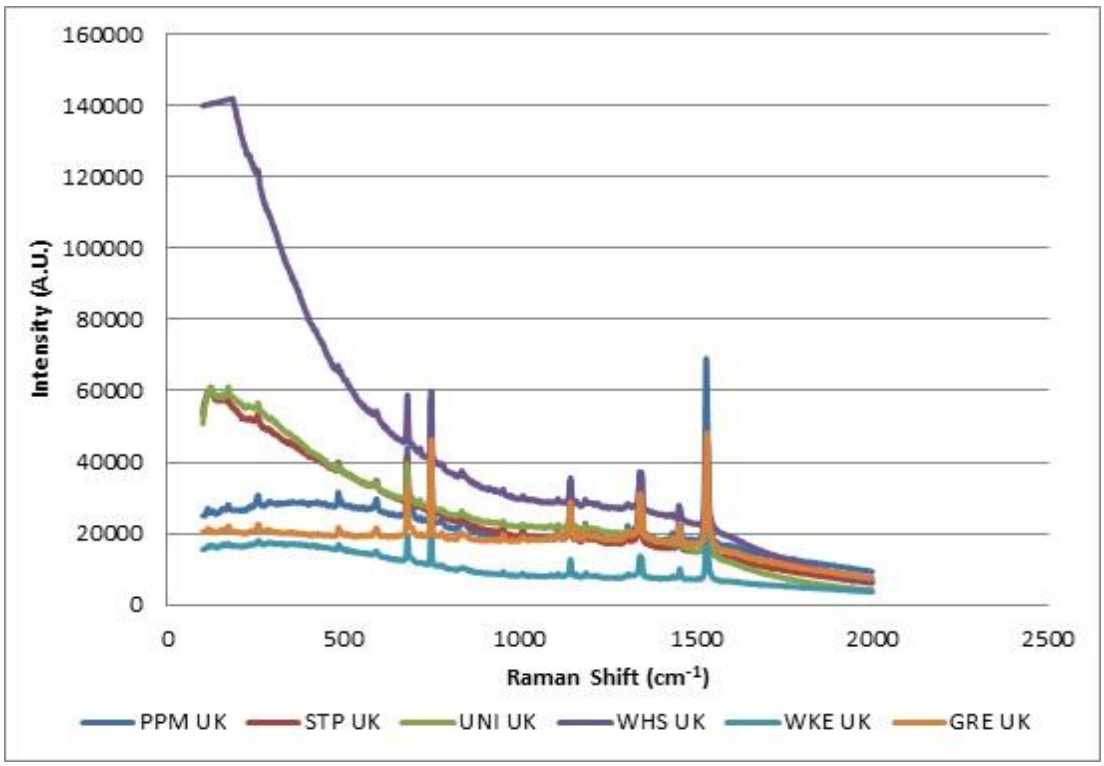


Figure 6.46: UK brand/model combinations of blue gel ink forming Group 1 (Renishaw InVia 785 nm 10% laser power/x 50 obj.)

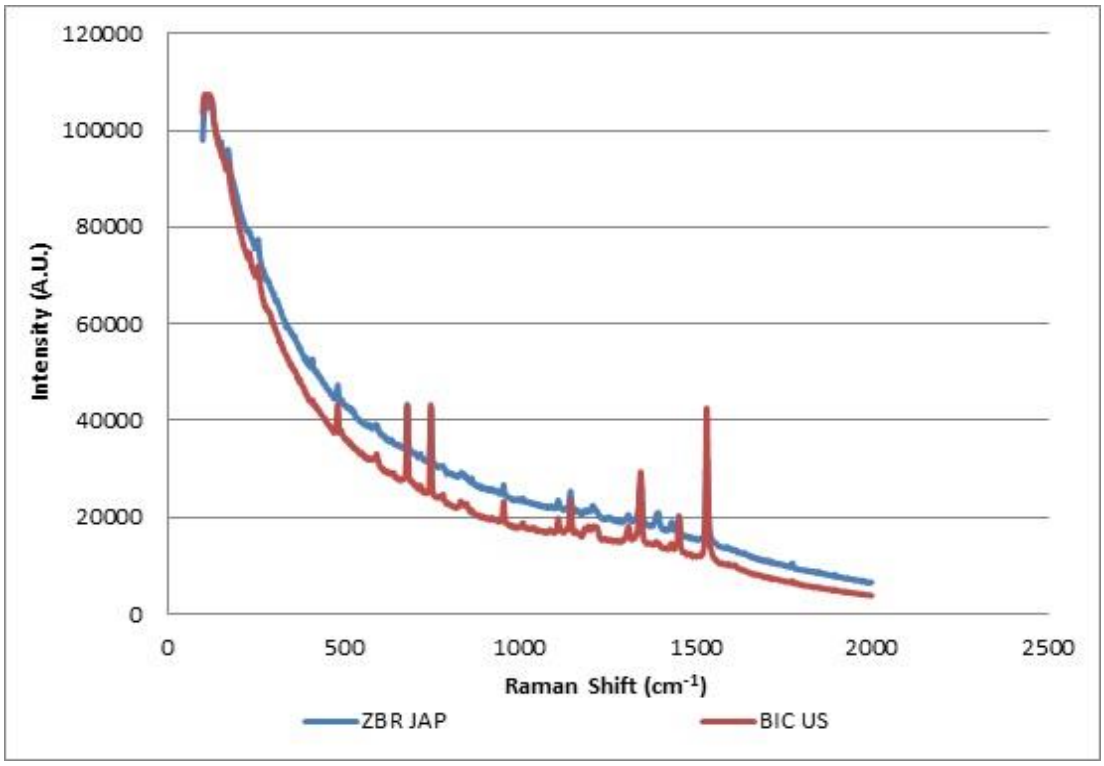


Figure 6.47: INTL brand/model combinations of blue gel ink forming Group 2 (Renishaw InVia 785 nm 10% laser power/x 50 obj.)

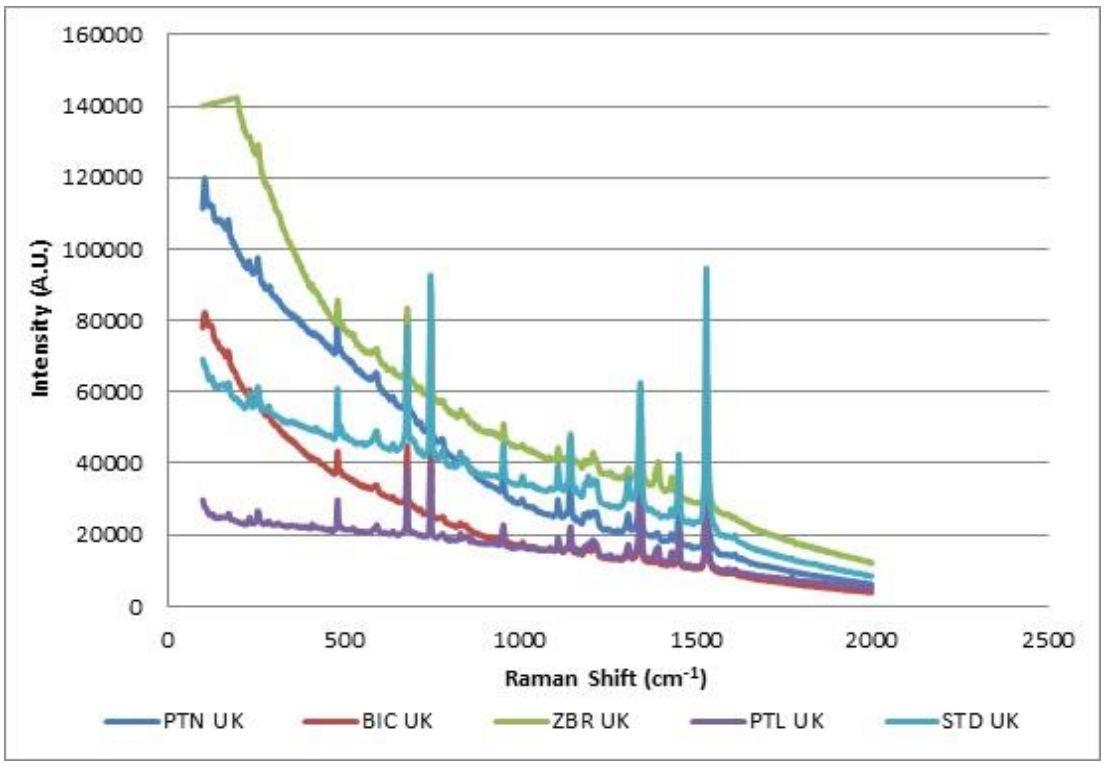


Figure 6.48: UK brand/model combinations of blue gel ink forming Group 2 (Renishaw InVia 785 nm 10% laser power/x 50 obj.)

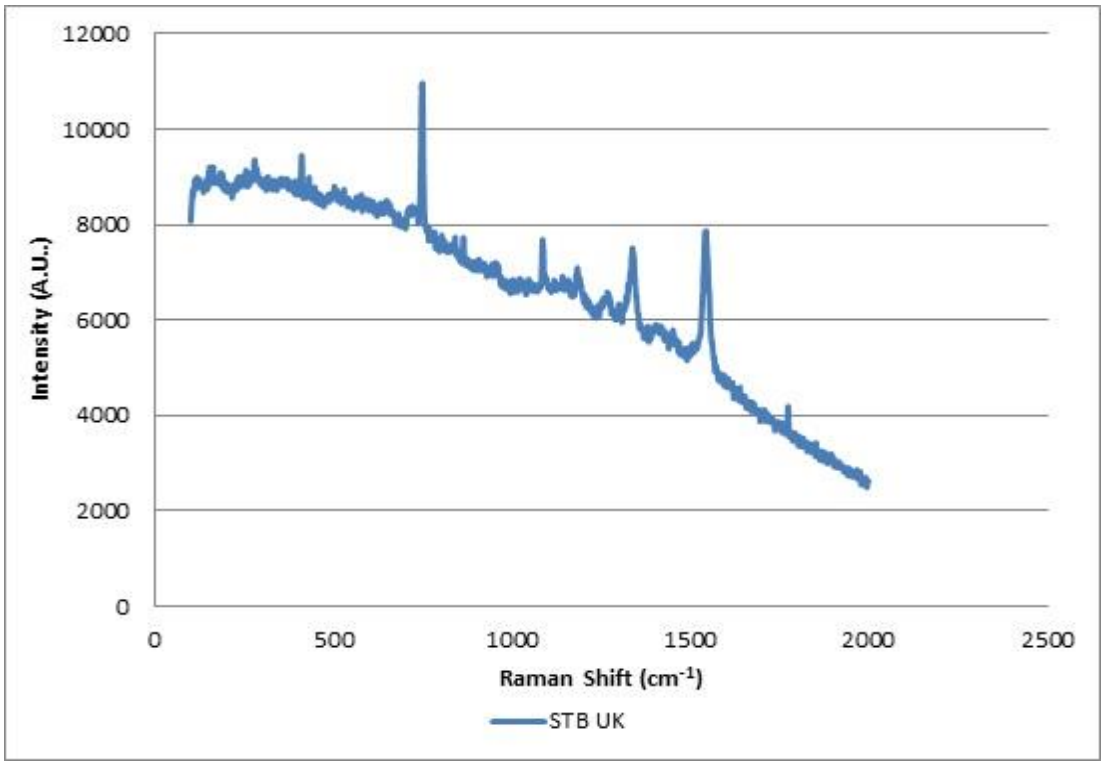


Figure 6.49: UK brand/model combinations of blue gel ink forming Group 3 (Renishaw InVia 785 nm 10% laser power/x 50 obj.)

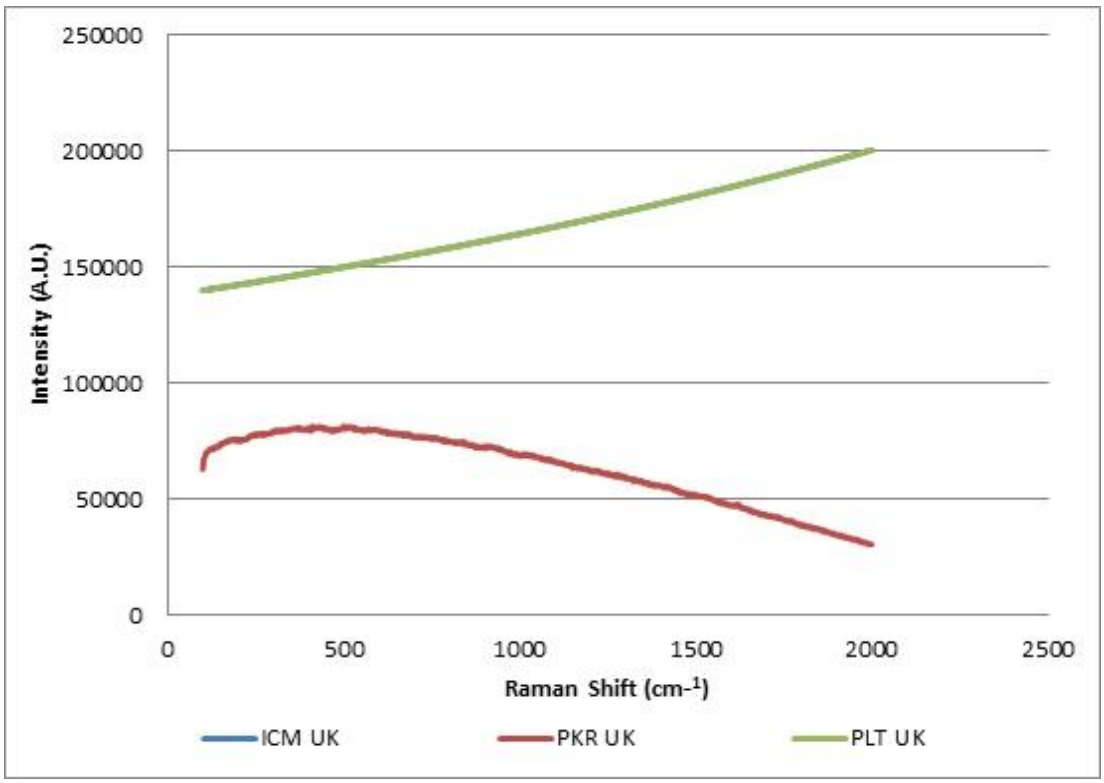


Figure 6.50: INTL brand/model combinations of blue gel ink forming Group 4 (Renishaw InVia 785 nm 10% laser power/x 50 obj.)

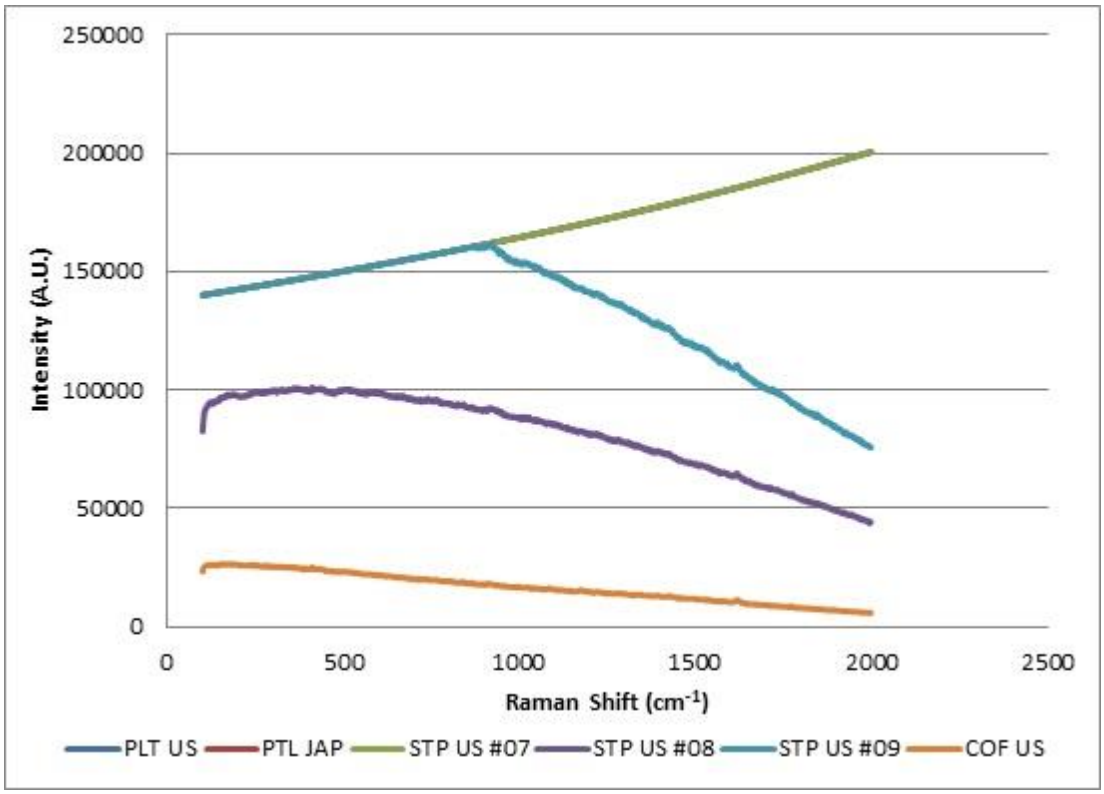


Figure 6.51: UK brand/model combinations of blue gel ink forming Group 4 (Renishaw InVia 785 nm 10% laser power/x 50 obj.)

Group 1 spectral pattern was very similar to Group 2, but distinguishable on the basis of a reproducible difference in the shape of peaks in the region of ~840 cm^{-1} and ~1200 cm^{-1}, as well as the presence of a small peak at ~1145 cm^{-1} observed in Group 1. These subtle spectral differences are highlighted within the green circles in Figure 6.52. All remaining groups were clearly distinguishable from all others.

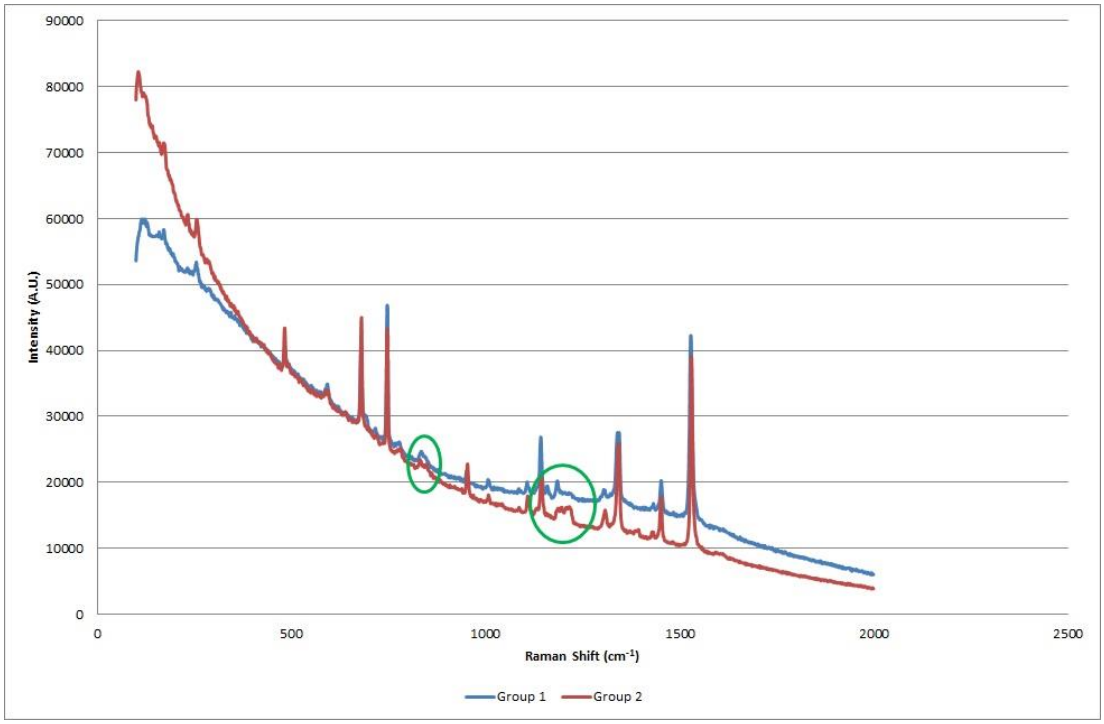


Figure 6.52: Example spectra representing Group 1 and Group 2 highlighting (within the green circles) subtle spectral variations permitting discrimination between the two (Renishaw InVia 785 nm 10% laser power/x 50 obj.)

6.3.2.2.1.4 830 nm Excitation Wavelength

6.3.2.2.1.4.1 Spectral Quality and Reproducibility

Twenty three brand/model combinations provided a strong Raman signal, whilst eight dye containing brand/model combinations exhibited spectra completely obscured by fluorescence and/or detector overload. For nine brand/model combinations (MG MAL; WHS UK; WKE UK; PTL UK; UNI UK; UNI AUS; UNI JAP; UNI HK #25 – 30 and UNI HK #31 – 36) reproducibility within a set was considered excellent. However, for the remaining brand/model combinations that gave a strong Raman signal, reproducibility was affected by a number of factors including stray peaks; fluorescence, detector overload and/or excessive spectral intensity (see *Discussion*). This was the only excitation wavelength of the four studied in depth, where reproducibility was an issue.

Twelve brand/model combinations (UNI UK; GRE UK; STP UK; WHS UK; BIC UK; BIC US; PPM UK; UNI AUS; ZBR UK; ZBR JAP; GSF MAL and STB UK) contained one or two spectra within the set that exhibited a non-reproducible stray peak. Three brand/model combinations (STB UK; FBC MAL and GSF MAL) exhibited a single spectrum within the set that was fully obscured by fluorescence. Three brand/model combinations (ZBR JAP; PTN UK and PPM UK) exhibited a spectrum from at least one pen within the set partially obscured by detector overload and/or excessively high spectral intensity. For the ZBR JAP and PTN UK sample sets, only one spectral measurement was affected in each. For the PPM UK sample set however, as illustrated in Figure 6.53, a single spectrum from four different pen inks within the set of six were affected. In all the aforementioned, only one spectrum of the two taken for any particular pen was affected by this issue. Furthermore, the second spectrum taken for that pen was considered indistinguishable to all others within the set that were also not affected. Taking this into consideration, reproducibility of the spectral measurements as a whole was considered satisfactory to proceed with reliable discrimination and classification without the need for repeat work.

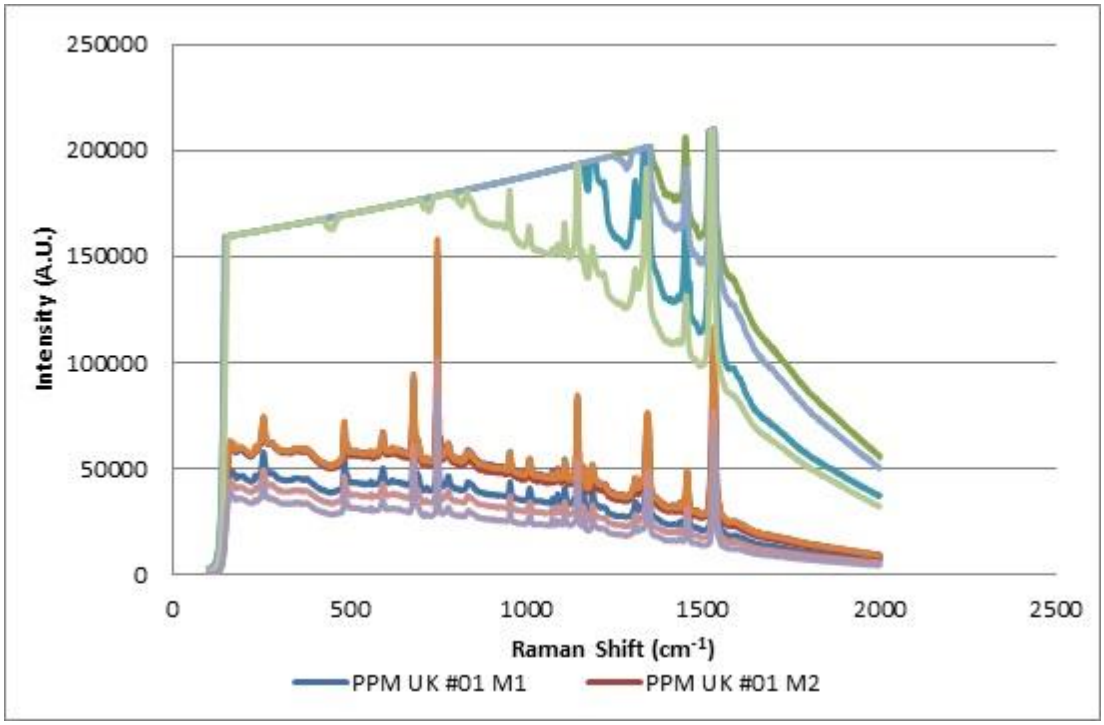


Figure 6.53: Example of a spectrum exhibiting detector overload and/or an unusually high spectral intensity in comparison to other spectra for the blue PPM UK brand/model combination (Renishaw InVia 830 nm 10% laser power/x 50 obj.)

A double peak attributable to the paper substrate of notable intensity was observed at ~1094 cm^{-1} in all ink spectra. Further peaks of low intensity attributable to the substrate were observed at ~164 cm^{-1}, ~285 cm^{-1}, ~563 cm^{-1} and ~711 cm^{-1} in some, but not all ink spectra, and were not always seen together in a single spectrum. The background fluorescence shape of the ink spectra was similar to that of the un-inked paper spectra suggesting fluorescence interference originating from the substrate. This was most obvious in the STB UK and COF US sample spectra, but for all brand/model combinations, Raman signals on top of the fluorescence background were visible. In general, spectral interference from the paper was minimal, but as for the 785 nm excitation wavelength, is recommended for consideration when comparing and/or identifying ink spectra.

6.3.2.2.1.4.2 Classification

Despite the reproducibility issues affecting this excitation wavelength, all brand/model combinations could be discriminated into six groups as shown in Figures 6.54 – 6.59. These consisted of three pigment based groups (Group 1 (14 samples), Group 2 (four samples) and Group 3 (three samples)) and three dye containing groups (Group 4 (one sample), Group 5 (one sample) and Group 6 (eight samples)).

Prior to classification, spectral measurements exhibiting stray peaks considered an issue were repeated and added into the original spectral data set as previously described. Additionally, during pre-sample acquisition testing of the brand/model combinations, no peaks were observed above 2000 cm^{-1} other than non-reproducible stray peaks and/or peaks attributable to the paper. Therefore, all spectra were acquired over the manually selected scan range of 100 – 2000 cm^{-1}.

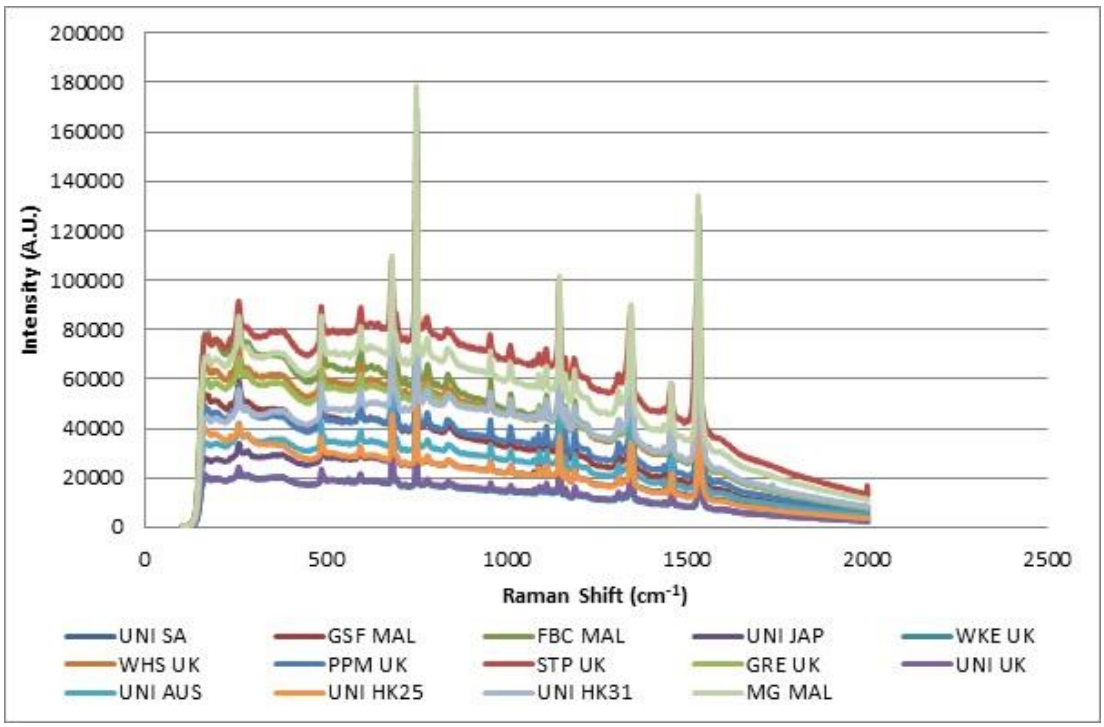


Figure 6.54: Brand/model combinations of blue gel ink forming Group 1 (Renishaw InVia 830 nm 10% laser power/x 50 obj.)

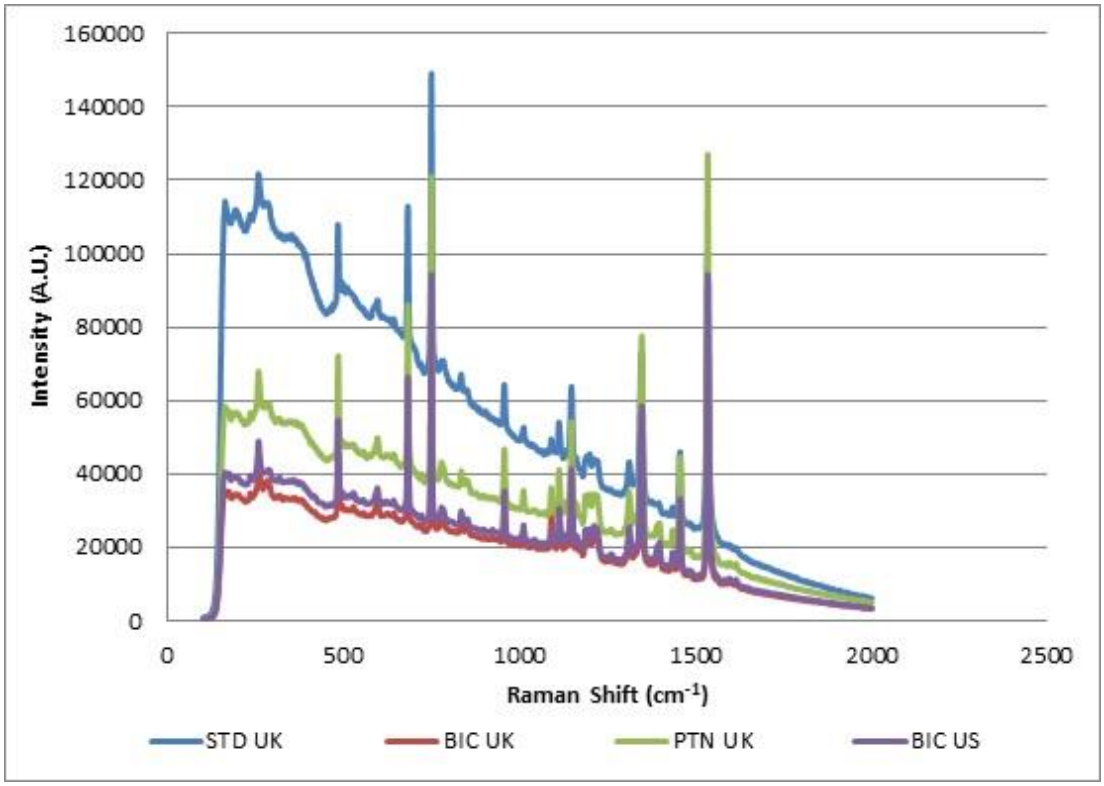


Figure 6.55: Brand/model combinations of blue gel ink forming Group 2 (Renishaw InVia 830 nm 10% laser power/x 50 obj.)

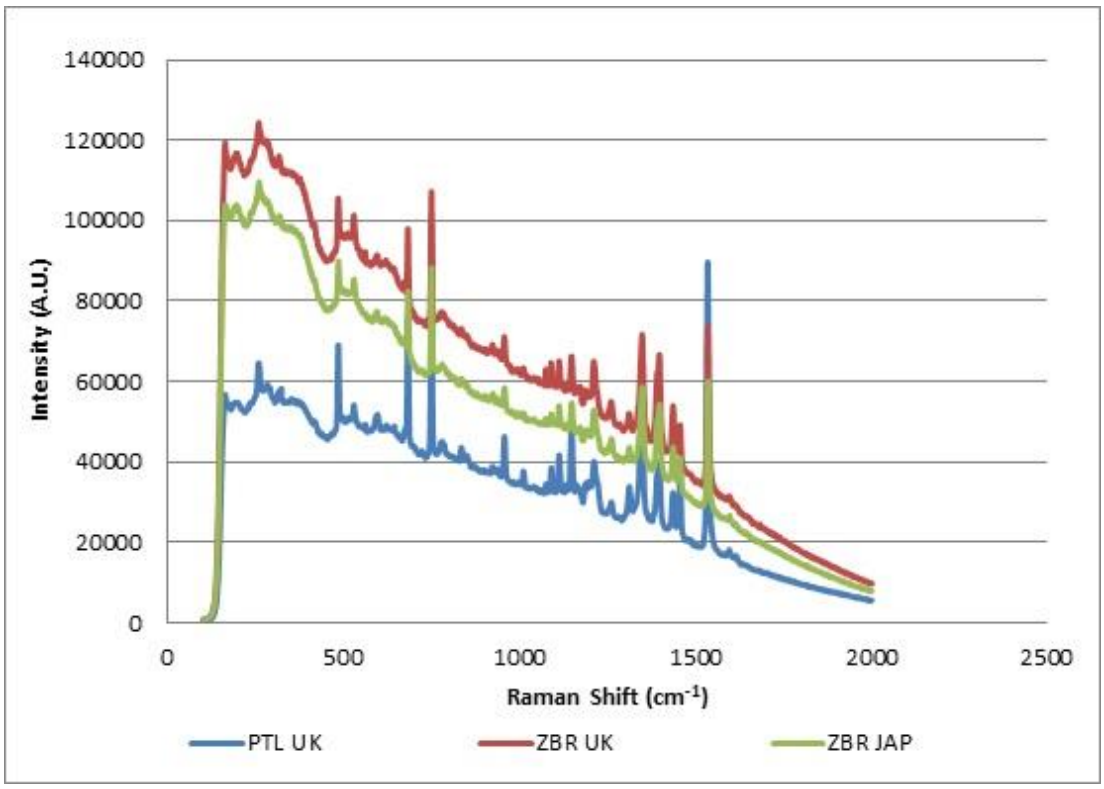


Figure 6.56: Brand/model combinations of blue gel ink forming Group 3 (Renishaw InVia 830 nm 10% laser power/x 50 obj.)

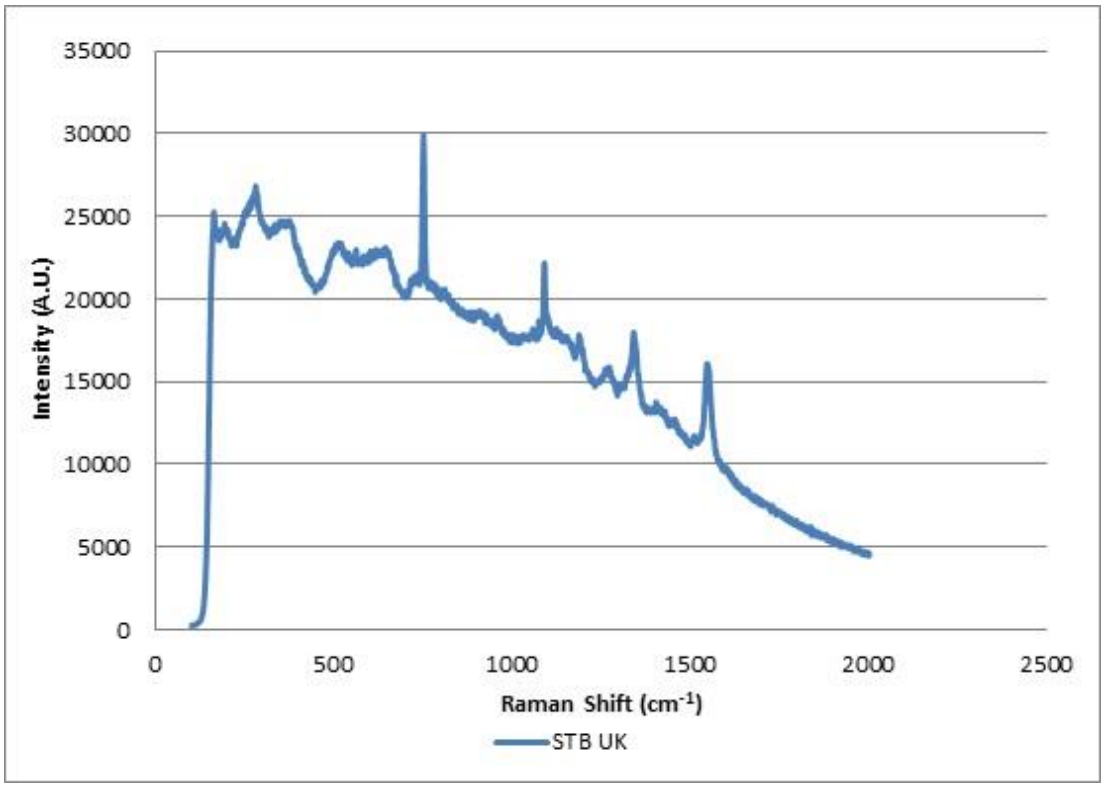


Figure 6.57: Brand/model combinations of blue gel ink forming Group 4 (Renishaw InVia 830 nm 10% laser power/x 50 obj.)

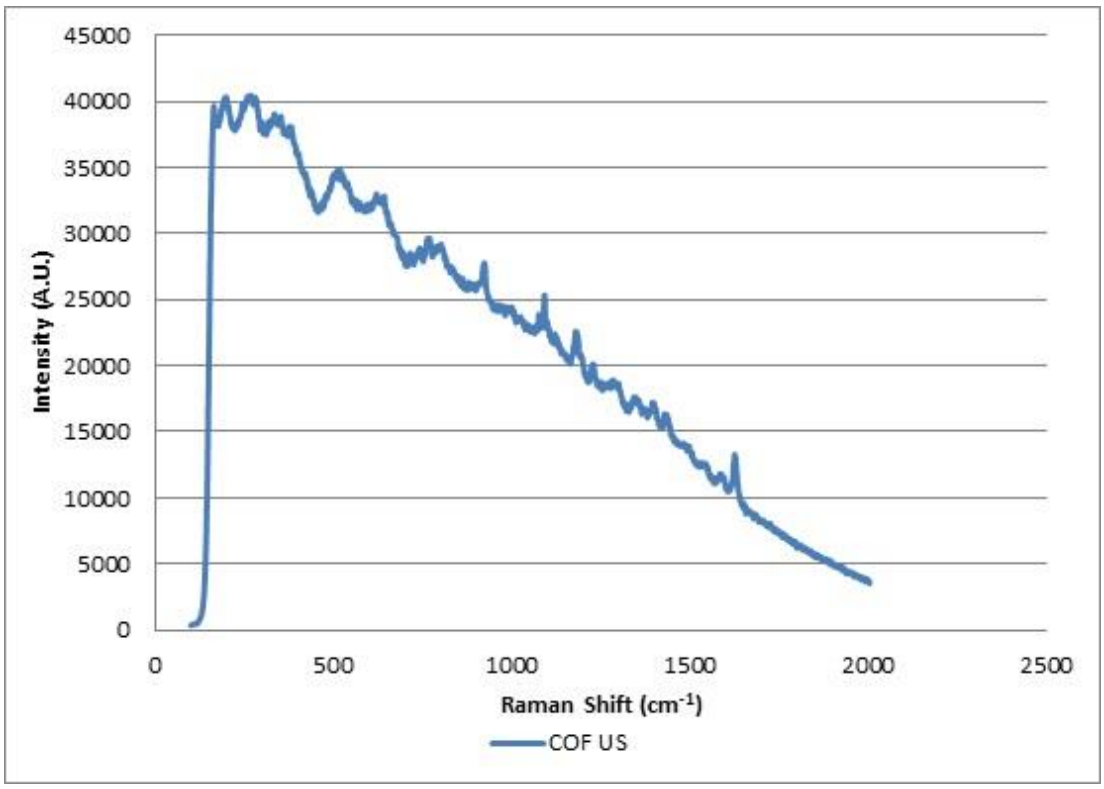


Figure 6.58: Brand/model combinations of blue gel ink forming Group 5 (Renishaw InVia 830 nm 10% laser power/x 50 obj.)

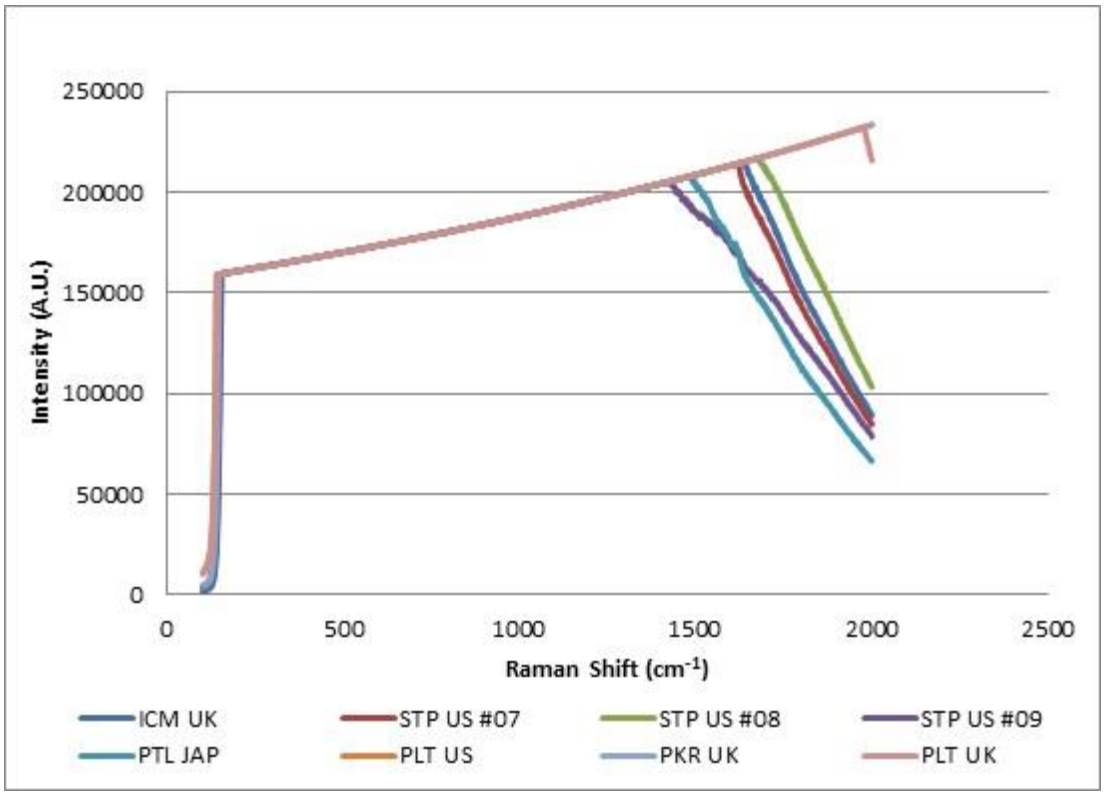


Figure 6.59: Brand/model combinations of blue gel ink forming Group 6 (Renishaw InVia 830 nm 10% laser power/x 50 obj.)

6.3.2.2.1.5 Discussion

Several observations were noted from this study of blue gel inks by Raman Spectroscopy.

6.3.2.2.1.5.1 Using Background Fluorescence as a Feature of Discrimination

Previous studies by Zieba-Palus *et al* [197, 204] have suggested "the use of fluorescence as a feature of discrimination". In this study, the FORAM 785 spectra within Group 1 exhibited the same Raman peaks, but the shape of the fluorescence background for three brand/model combinations (FBC MAL; GSF MAL and WHS UK) differed slightly from all others within the group. The shape of this background fluorescence was reproducible within these three brand/model combination sets. Furthermore, the shape of the background fluorescence for the other 28 brand/model combinations was also reproducible. Therefore, in line with the views of Zieba-Palus *et al* [197, 204] this marked difference could be used as a feature of discrimination, thus enabling Group 1 to be further discriminated. By the same principle, two brand/model combinations within Group 2 (PTL UK and STD UK) also exhibited a slightly different reproducible shape to their background fluorescence to the other brand/model combinations within that group.

With respect to the Renishaw InVia 785 nm spectra, a similar observation for Group 1 described above was made. The three brand/model combinations (WHS UK, FBC MAL and GSF MAL) exhibited a slightly different background fluorescence shape in the form of a steeper curve below 1000 cm^{-1}, but in terms of peak presence were otherwise considered indistinguishable. Again, since this difference was reproducible within the brand/model combination, Group 1 could arguably be further discriminated (into two sub-groups) on this basis. However, in contrast to the FORAM 785 spectra some variation in shape of background fluorescence was observed within several other brand/model combination sets associated with this group, therefore suggesting these differences may simply have arisen from a sampling location issue rather than a real chemical difference, and therefore are not a reliable feature of discrimination. Furthermore, if spectra within Group 1 and 2 are baseline corrected and smoothed, as shown in Figure 6.60 for the former, the background fluorescence is removed to exhibit indistinguishable spectra in terms of peak pattern within the respective groups. Therefore, it is recommended using the difference in background fluorescence shape as a feature of discrimination should be done so with caution, and

interpretation should primarily focus upon peak presence or absence and relative peak intensities.

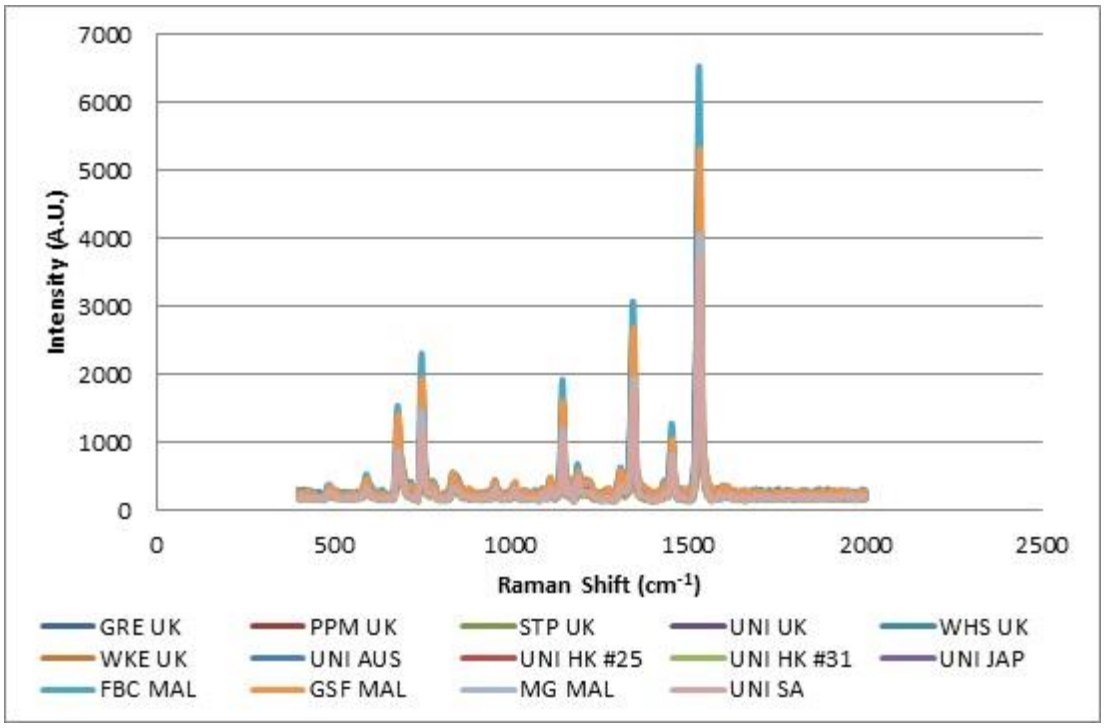


Figure 6.60: Brand/model combinations of blue gel ink forming Group 1 showing an indistinguishable spectral pattern after baseline correction and smoothing applied (FORAM 785 100% laser power/x 20 obj., 1.0s default scan time)

6.3.2.2.1.5.2 Relative Peak Intensities as a Feature of Further Discrimination

Under the 514.5 nm and 785 nm wavelengths, seven pigment based brand/model combinations occupied the same single group (Group 2), but could be further discriminated into two sub-groups (Group 2a: BIC UK; BIC US; STD UK; PTN UK and Group 2b: ZBR UK, ZBR JAP; PTL UK) based upon a reproducible difference in relative peak intensity at ~1550 cm^{-1} (Figures 6.61 and 6.62) and ~1390 cm^{-1} (Figures 6.63 and 6.64) respectively. Interestingly, for both BIC and ZBR brands, the two different models for each can be grouped into the same sub-group. Given this was the only difference observed for all seven samples they were treated as a single group at these excitation wavelengths, distinguishable into two sub-groups, rather than two individual groups in their own right. However, under 830 nm, these same seven brand/model combinations could be split into two individual groups (Group 2 and 3) based on several areas of difference within their spectra. These spectral differences included a change in spectral shape in the 1183 – 1220 cm^{-1} region, and relative

peak intensity differences at ~348 cm^{-1}, ~533 cm^{-1}, ~1266 cm^{-1}, ~1399 cm^{-1} and ~1438 cm^{-1}. This provided further confirmation that these particular brand/model combinations were distinguishable by a real chemical difference in their composition.

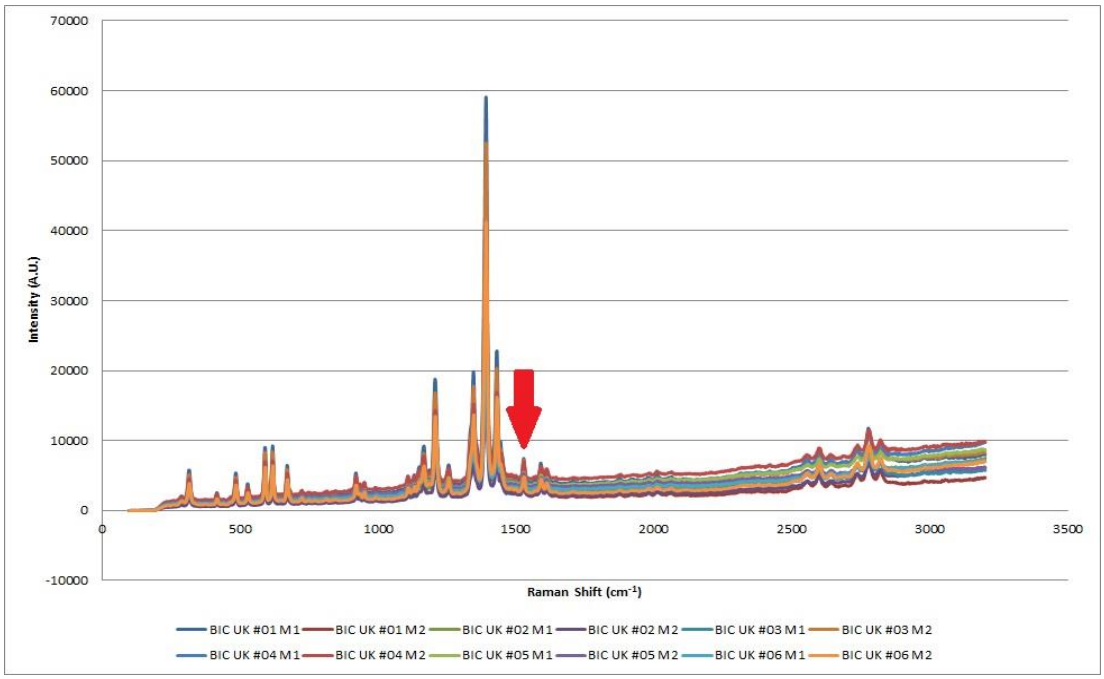


Figure 6.61: Example of a brand/model combination (BIC UK) in Group 2a exhibiting a reproducible spectral pattern with an obvious peak at ~1550 cm^{-1} highlighted by the red arrow (Renishaw InVia 514.5 nm 10% laser power/x 20 obj.)

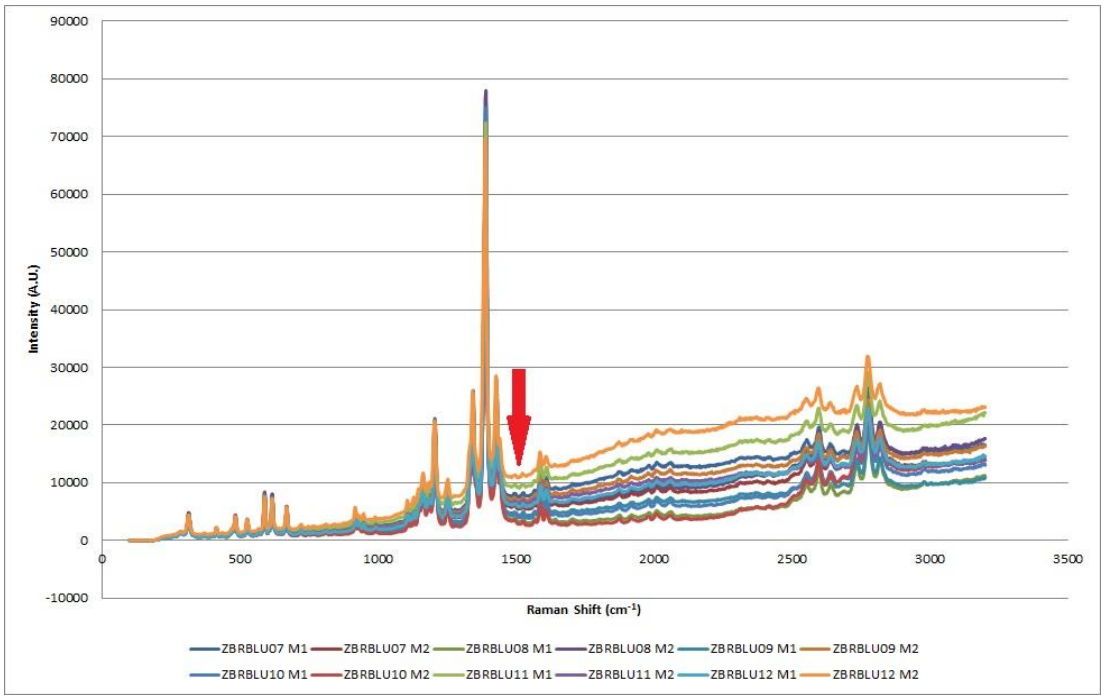


Figure 6.62: Example of a brand/model combination (ZBR JAP) in Group 2b exhibiting a reproducible spectral pattern without an obvious peak at ~1550 cm^{-1} highlighted by the red arrow (Renishaw InVia 514.5 nm 10% laser power/x 20 obj.)

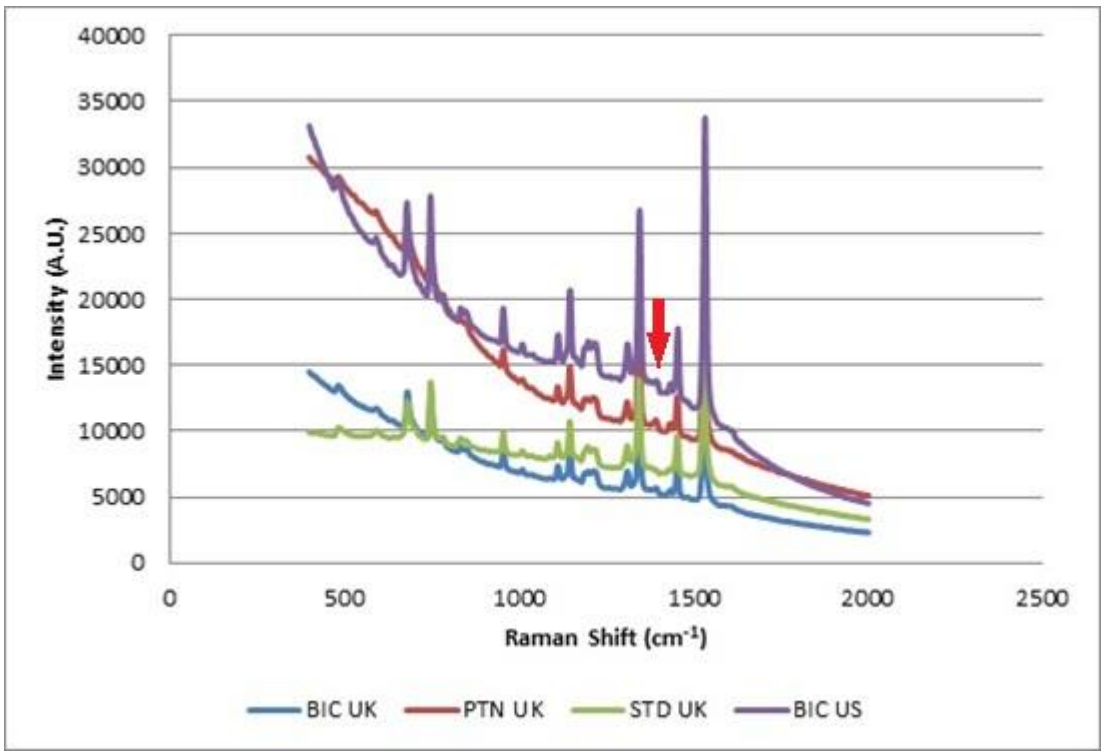


Figure 6.63: Brand/model combinations of blue gel ink forming Group 2a based on relative peak intensity at ~1390 cm^{-1} highlighted by the red arrow (FORAM 785 100% laser power/x 50 obj., Auto Exposure)

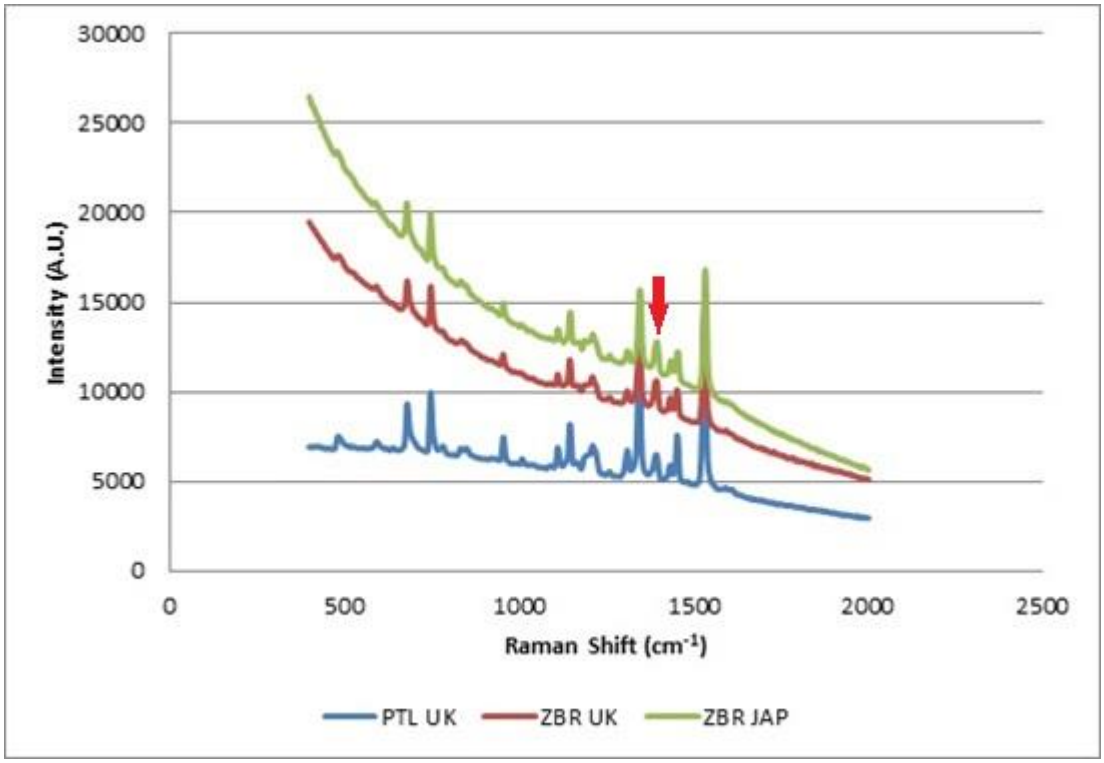


Figure 6.64: Brand/model combinations of blue gel ink forming Group 2b based on relative peak intensity at ~1390 cm^{-1} highlighted by the red arrow (FORAM 785 100% laser power/x 50 obj., Auto Exposure)

6.3.2.2.1.5.3 Red Sheen Appearance and Laser Sample Positioning

During analysis of the BIC US and PTN UK ink lines under 785 nm (Renishaw InVia only), it was observed that some areas of the blue ink exhibited a red sheen, similar to the bronzing effect discussed in **Chapter Four**. When spectra were acquired from these areas, the spectral response was very intense, with peaks exhibiting excessively high intensity and some partial detector overload. When spectra were acquired away from these red sheen areas, and only from the blue areas, the spectral response was far less intense, exhibiting no detector overload, and only lower intensity peaks on a fluorescence curve. These observations are illustrated in Figure 6.65. During spectral acquisition of all samples care was taken to avoid acquiring spectra from areas that exhibited this red sheen or where it was minimal, to ensure a full spectrum devoid of detector overload was recorded. This may also explain the excessive spectral intensity observed in spectra from three brand/model combinations that affected reproducibility at 830 nm.

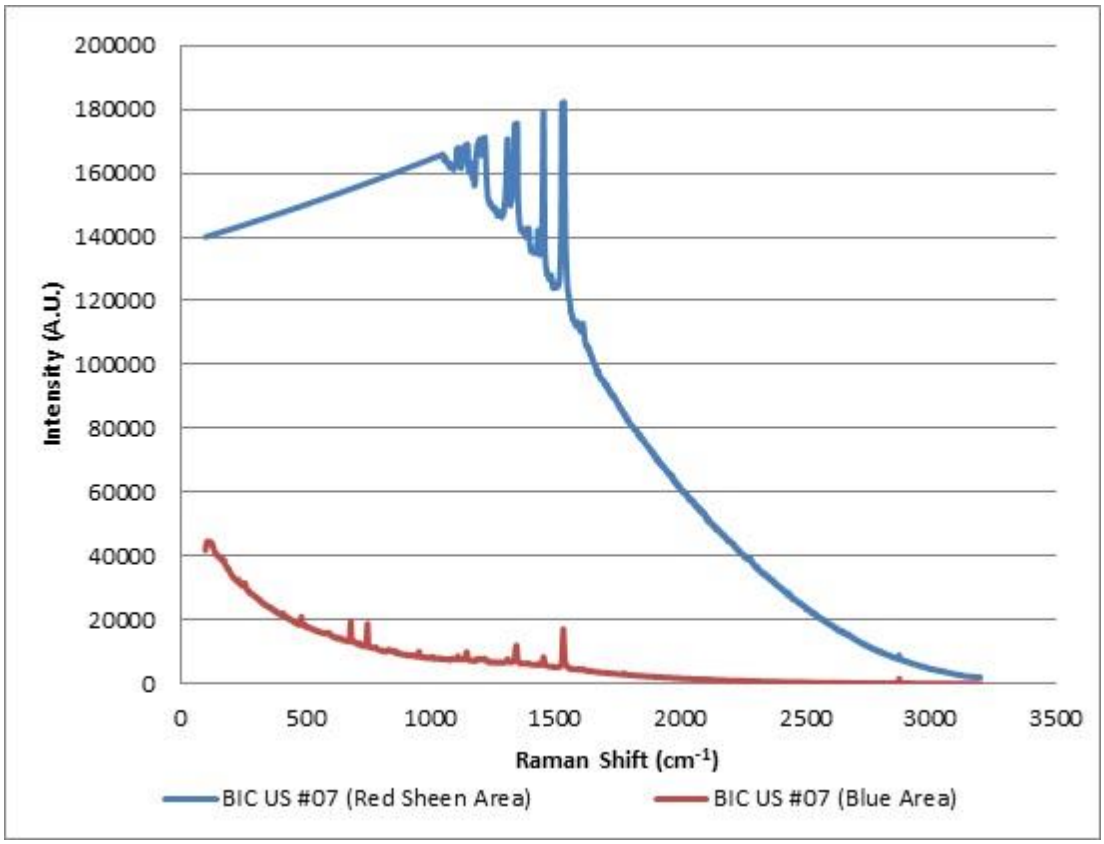


Figure 6.65: Spectra of BIC US blue gel ink on paper illustrating an excessive spectral intensity from the red sheen area of the blue ink line (Renishaw InVia 785 nm 100% laser power/x 50 obj.)

6.3.2.2.1.5.4 Problems with Fluorescence at 685 nm Excitation Wavelength

Previous studies by Zieba-Palus *et al* [197, 204] have shown fluorescence was a particular problem for (blue) gel inks analysed under both 633 nm and 685 nm. Indeed, fluorescence badly affected spectral reproducibility during method development of the 632.5 nm excitation wavelength with respect to blue gel ink in this study. However, whilst fluorescence was observed in spectra acquired under 685 nm in this study, it did not prevent useful discrimination. The majority of dye containing samples (9) did not provide a useful spectrum, but this was expected and consistent with previous observations that Raman Spectroscopy is "inefficient" for dye based inks [15, 27]. The remaining 22 ink samples provided a good reproducible spectrum. Fluorescence obscured the Raman signal for seven pigment based samples, but the shape and curve of the fluorescence in this instance added discriminatory value in comparison to the other 15 samples, which provided a strong Raman signal.

A tentative comparison of spectra acquired during method development under 632.5 nm against corresponding spectra acquired under 685 nm was undertaken. Given the two sets of spectra were acquired under different objectives this cannot be considered a true like for like comparison. However, it does provide an indication of the performance of these two excitation wavelengths with respect to blue gel inks.

Of the six dye containing brand/model combinations analysed under both excitation wavelengths, four (COF US; PLT UK; ICM UK and PKR UK) were swamped by fluorescence under both 632.5 nm and 685 nm. STB UK was the only dye containing brand/model combination that yielded a Raman spectrum under both excitation wavelengths. However, whilst the spectra were highly similar, the region below ~800 cm^{-1} was obscured by fluorescence in the 632.5 nm spectra losing some spectral information. The PTL JAP dye containing brand/model combination provided no useful spectrum under 685 nm, yet at 632.5 nm a relatively strong Raman signal on a fluorescence background was observed. In contrast, the two BIC pigment based brand/model combinations did not provide a useful spectrum under 632.5 nm, but a relatively strong Raman signal on a fluorescence background under 685 nm was produced. These findings suggest one excitation wavelength over another may provide more information about a particular brand/model combination than the other.

As described previously, fluorescence was a problem for the 632.5 nm excitation wavelength, affecting reproducibility and signal intensity. This was not considered a problem for the 685 nm wavelength in this study. No spectral interference from the paper substrate was encountered at either wavelength, although it was considered that perhaps the 632.5 nm excitation wavelength was more prone to fluorescence interference from the paper (and the ink) accounting for the poorer spectral response.

Overall, the 685 nm excitation wavelength appears better suited to the analysis of blue gel inks than the 632.5 nm, providing greater information about the samples. Some discriminatory value may be provided by the 632.5 nm excitation wavelength, but fluorescence was particularly problematic affecting reliability.

6.3.2.2.1.5.5 Differences in Formulation Composition

Several observations relating to similarities and differences in certain ink formulations were made irrespective of excitation wavelength used.

All Uniball brand/model combinations exhibited the same spectral pattern and were considered a single spectral group. This suggests the colorant composition of the Uniball ink formulations is the same across the globe irrespective of model. However, discrimination of the three Asian UNI brand/model combinations (UNI JAP; UNI HK #25 – 30 and UNI HK #31 – 36) from all others was possible using Vis-MSP and FTIR-ATR. This suggests subtle differences in colour and additive composition may exist between different geographic locations, and that these are not detectable by Raman Spectroscopy alone. This supports an earlier finding by Mazella and Buzzini [27] that subtle differences in ink formulations (for the same brand and model of pen) tailored to end user climates may not be detectable by Raman Spectroscopy.

Spectra from the Pentel brand/model combinations were different, indicating one was pigment based (PTL UK) and the other contained a dye component (PTL JAP), confirmed from solubility testing. This suggested variation in colorant composition across the globe for this particular brand, although since they represented two different models, this may have been model dependent. The two Staples brand/model combinations, which were the same model (Staples Sonix), also exhibited a distinguishable spectral pattern, again

indicating one was pigment based (STP UK) and the other dye containing (STP US #07 - #09). This suggested colorant composition for this brand/model combination varied by location of purchase and/or manufacture. This was not true for the Pilot G2-07 brand/model combination (PLT UK and PLT US), also the same model, since they could be grouped together in the same group and were found to be dye containing. These findings suggest that some pen manufacturers may prefer to use dyes rather than (or in addition to) pigments for the same model of pen used in different locations, whilst others may not. This may be because of technical or financial reasons.

6.3.2.2.1.5.6 Hybrid Gel Ink Formulations

The Solubility/TLC study identified ten dye containing blue gel inks, which were discriminated into seven groups, all of which with one exception contained a lone brand/model combination. Of these ten, Raman spectra were acquired from six brand/model combinations analysed under the four main excitation wavelengths studied in depth. At 514.5 nm, four dye containing ink samples exhibited an indistinguishable spectrum (PLT UK; PLT US; ICM UK and STP US #07); at 685 nm, 785 nm and 830 nm the STB UK samples provided a strong Raman signal, whilst at 830 nm only, the COF US sample also yielded a Raman spectrum. Spectra for all other dye containing brand/model combinations at the respective excitation wavelengths were swamped by fluorescence. Since previous studies [15, 27] have suggested that Raman Spectroscopy is “inefficient” for dye based gel inks, this finding suggests that these six brand/model combinations contain both dyes and pigments and are therefore hybrid gel ink formulations. It is possible the PTL JAP dye containing brand/model combination is also a hybrid formulation since it provided a Raman spectrum at 632.5 nm during method development.

6.3.2.2.1.6 Summary

The 31 blue ink group brand/model combinations could be discriminated into four groups at both, 685 nm and 785 nm excitation wavelengths, the latter irrespective of instrument used. At both 514.5 nm and 830 nm, discrimination into six groups was possible. Table 6.8 summarises the blue ink group samples belonging to each classification group by all four excitation wavelengths studied.

Group #	Colorant/Sub-Group/Excitation Wavelength (nm)									
	Colorant	Sub-Group	514.5	Colorant	685	Colorant	Sub-Group	785	Colorant	830
1	Pigment		UNI AUS UNI HK #25-30 UNI UK MG MAL UNI HK #31-36 STP UK UNI SA UNI JAP PPM UK	Pigment	GRE UK PPM UK STP UK UNI UK WHS UK WKE UK UNI AUS UNI SA UNI HK#25-30 UNI HK#31-36 UNI JAP FBC MAL GSF MAL MG MAL	Pigment		GRE UK PPM UK STP UK UNI UK WHS UK WKE UK UNI AUS UNI SA UNI HK #25–30 UNI HK #31–36 UNI JAP FBC MAL GSF MAL MG MAL	Pigment	GRE UK PPM UK STP UK UNI UK WHS UK WKE UK UNI AUS UNI SA UNI HK #25–30 UNI HK #31–36 UNI JAP FBC MAL GSF MAL MG MAL
2		2a	BIC UK BIC US STD UK PTN UK		BIC UK BIC US STD UK PTN UK		2a	BIC UK BIC US STD UK PTN UK		BIC UK BIC US STD UK PTN UK
		2b	ZBR UK ZBR JAP PTL UK		ZBR UK ZBR JAP PTL UK		2b	ZBR UK ZBR JAP PTL UK		
3			FBC MAL GSF MAL WHS UK	Dye or Dye and Pigment	STB UK	Dye or Dye and Pigment		STB UK		ZBR UK ZBR JAP PTL UK
4			GRE UK WKE UK		ICM UK PLT UK COF US STP US #07 STP US #08 STP US #09 PKR UK PTL JAP PLT US			ICM UK PLT UK COF US STP US #07 STP US #08 STP US #09 PKR UK PTL JAP PLT US	Dye or Dye and Pigment	STB UK
5	Dye or Dye and Pigment		PLT UK PLT US ICM UK STP US #07							COF US
6			PTL JAP COF US STB UK PKR UK STP US #08 STP US #09							ICM UK STP US #07 STP US #08 STP US #09 PTL JAP PLT US PKR UK PLT UK

Table 6.8: Summary of blue ink group classifications for all four excitation wavelengths studied

6.3.2.2.2 Red Ink Group

6.3.2.2.2.1 Spectral Quality and Reproducibility

6.3.2.2.2.1.1 FORAM 785

Of the 25 brand/model combinations of red gel ink analysed, the majority produced a spectrum consisting of relatively weak peaks on a strong fluorescence background, although some brand/model combinations were stronger than others, i.e. STD UK Reproducibility within a set was considered good as was S:N ratio, although some minor spectral variation arising from peak intensity in a couple of spectra was observed (e.g. FBC MAL, MG MAL, UNI HK #25, PLT UK and PTL UK) as demonstrated in Figure 6.66. The only spectral interference from the paper substrate observed, was a small peak at ~1094 cm^{-1}, highlighted by the red arrow.

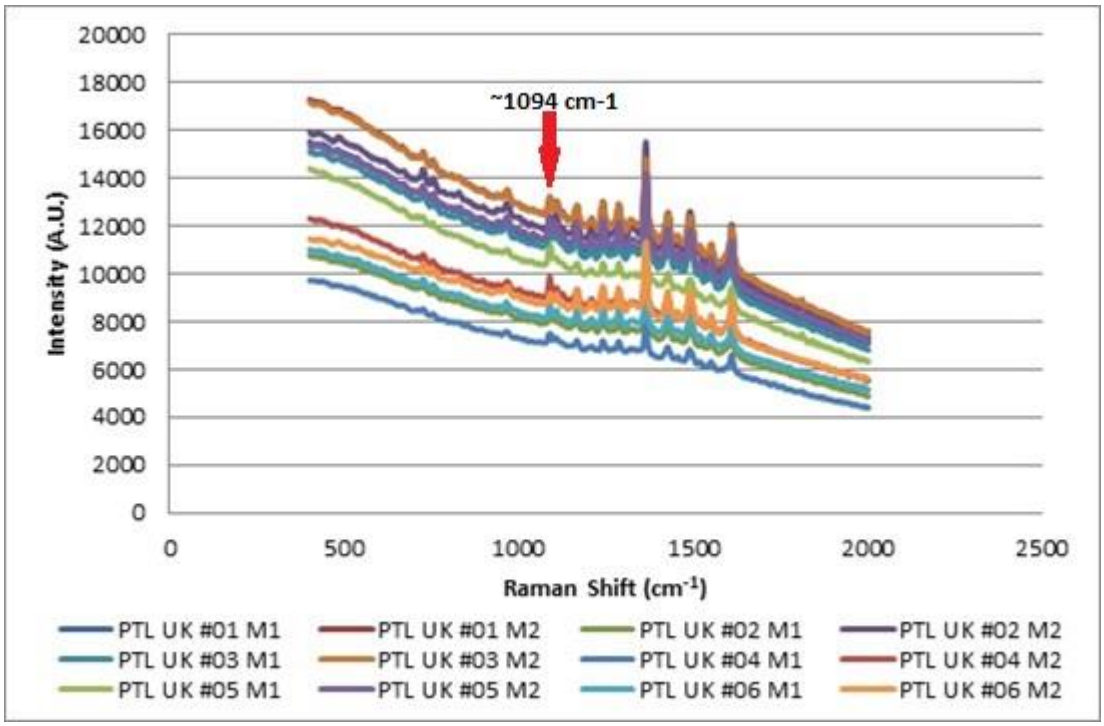


Figure 6.66: Spectra from PTL UK red gel ink on paper demonstrating good reproducibility but some peak intensity variation (FORAM 785 100% laser power/x 50 obj., Auto Exposure). A peak attributable to the paper substrate at ~1094 cm^{-1} highlighted by the red arrow

The integration times selected by Auto Exposure ranged between 1.0 – 16.0s. An overall spectral intensity range between 16, 000 – 30, 000 (A.U.) was observed, with the majority of spectra recorded between 18, 000 – 20, 000 (A.U.) The majority of samples required a higher integration time than the manual default 1.0s scan time to achieve optimal signal intensity.

6.3.2.2.2.1.2 Renishaw InVia (785 nm)

Of the 25 brand/model combinations analysed, all 25 brand/model combination sets analysed provided a Raman response, although the quality of the response varied between different brand/model combinations. Generally, high background fluorescence in all brand/model combinations, was observed, which in contrast to the blue ink group samples, influenced the quality of the resulting spectra. A few brand/model combinations exhibited excellent spectra with strong peaks and good reproducibility; however, the majority exhibited some variation in the intensity of the Raman response between spectral measurements within a brand/model combination set, which affected overall reproducibility when compared on the same axis. Figure 6.67 shows an example of a brand/model combination that exhibited a strong spectrum with good reproducibility, whilst Figure 6.68 shows a brand/model combination that exhibited a comparatively weak spectrum. Minimal spectral interference from the paper substrate in the form of a small peak at ~1094 cm^{-1} was observed in the majority of brand/model combination spectra, as highlighted by the red arrow.

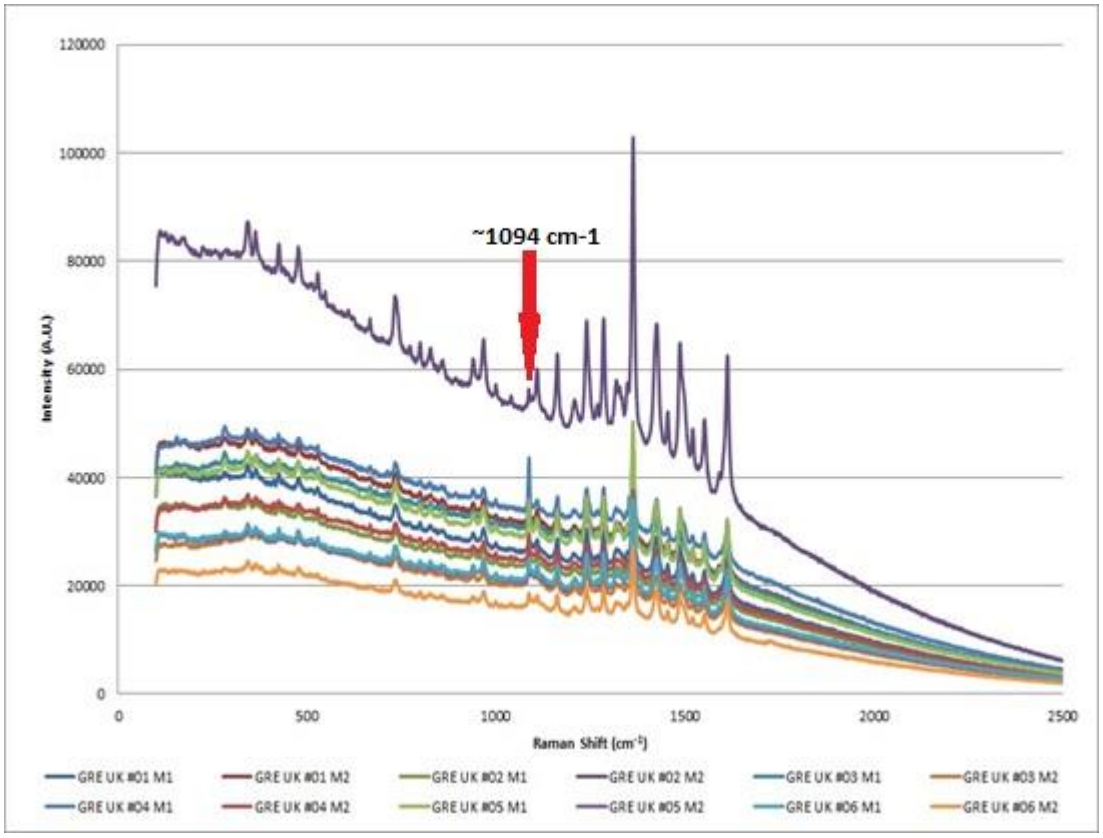


Figure 6.67: Spectra of GRE UK pens #01 – #06 red gel ink on paper demonstrating strong signal intensity and good reproducibility (Renishaw InVia 785 nm 100% laser power/x 50 obj.). A peak attributable to the paper substrate at ~1094 cm^{-1} highlighted by the red arrow

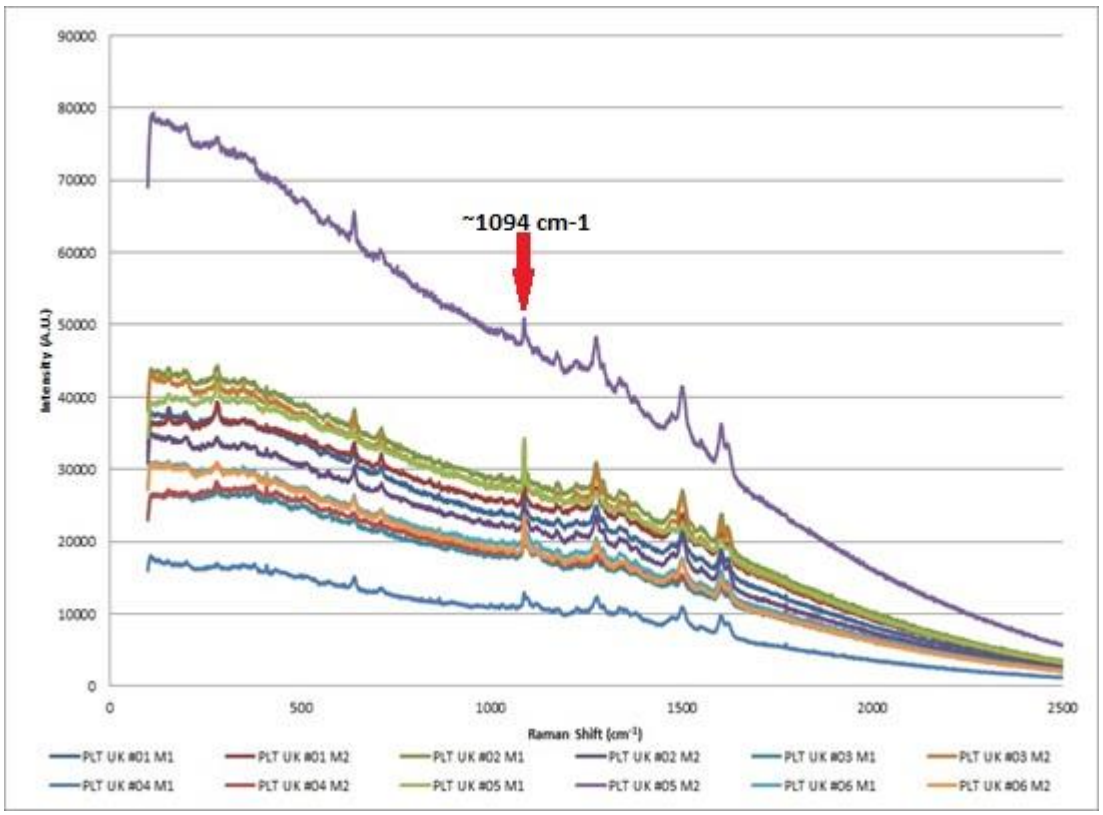


Figure 6.68: Spectra of PLT UK pens #01 - #06 red gel ink on paper demonstrating weak signal intensity (Renishaw InVia 785 nm 100% laser power/x 50 obj.). A peak attributable to the paper substrate at ~1094 cm^{-1} highlighted by the red arrow

Partial detector overload was observed in some of the spectra of the UNI HK #29 - #30 sample set. Reducing laser power to 50% and 10% to try and eliminate this was successful however it did not provide any further spectral information.

It was noted, that generally, when the laser was focussed on a lightly inked area, the resulting spectra were weaker in intensity than the spectra acquired from a heavily inked area. This was not observed for the blue ink group samples, which all appeared to give a good strong spectrum irrespective of laser positioning over the sample. Figure 6.69 shows a red gel ink line under x 20 obj. demonstrating light and heavy inked areas indicated within blue and green circles respectively.

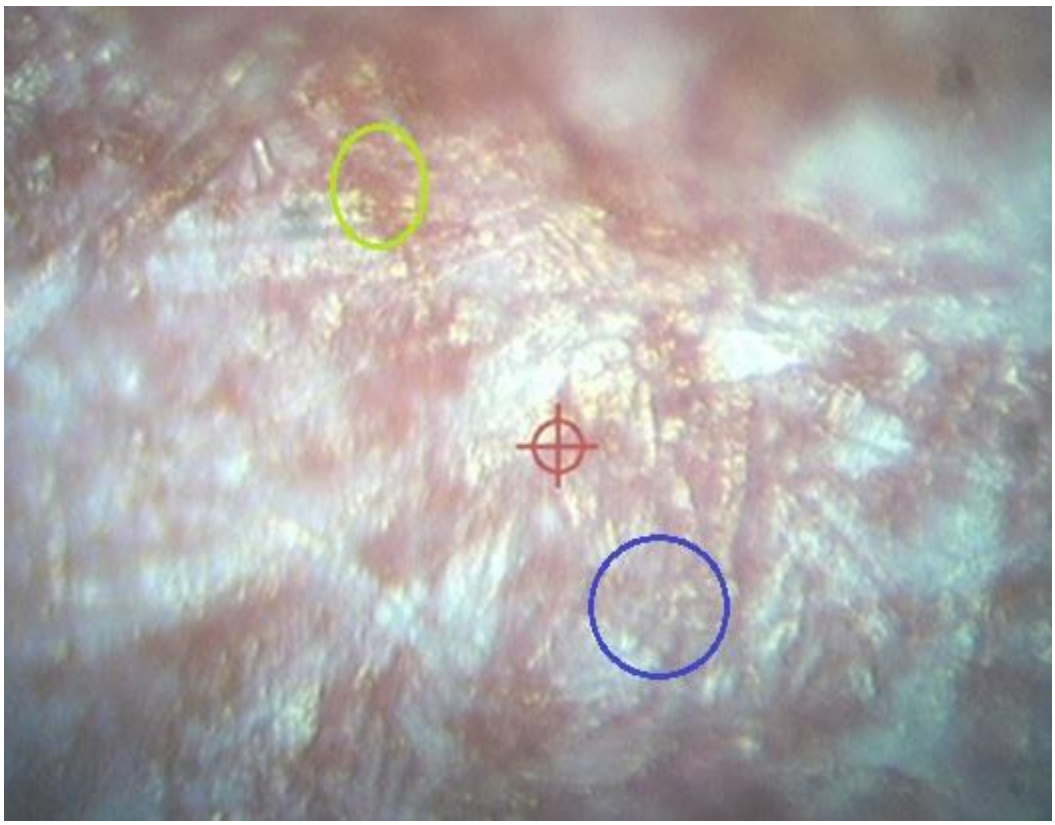


Figure 6.69: Red gel ink on office paper showing light (blue circle) and heavy (green circle) inked areas (FORAM 785 x 20 obj.)

6.3.2.2.2.2 Classification

The 25 red ink group brand/model combinations were discriminated into ten groups based on visual pattern recognition of FORAM 785 spectral data as illustrated in Figures 6.70 – 6.79. Discrimination into ten groups, containing the same brand/model combinations, based on Renishaw InVia 785 nm spectral data was also achievable, and are shown in Figures 6.80 – 6.89.

6.3.2.2.2.2.1 FORAM 785

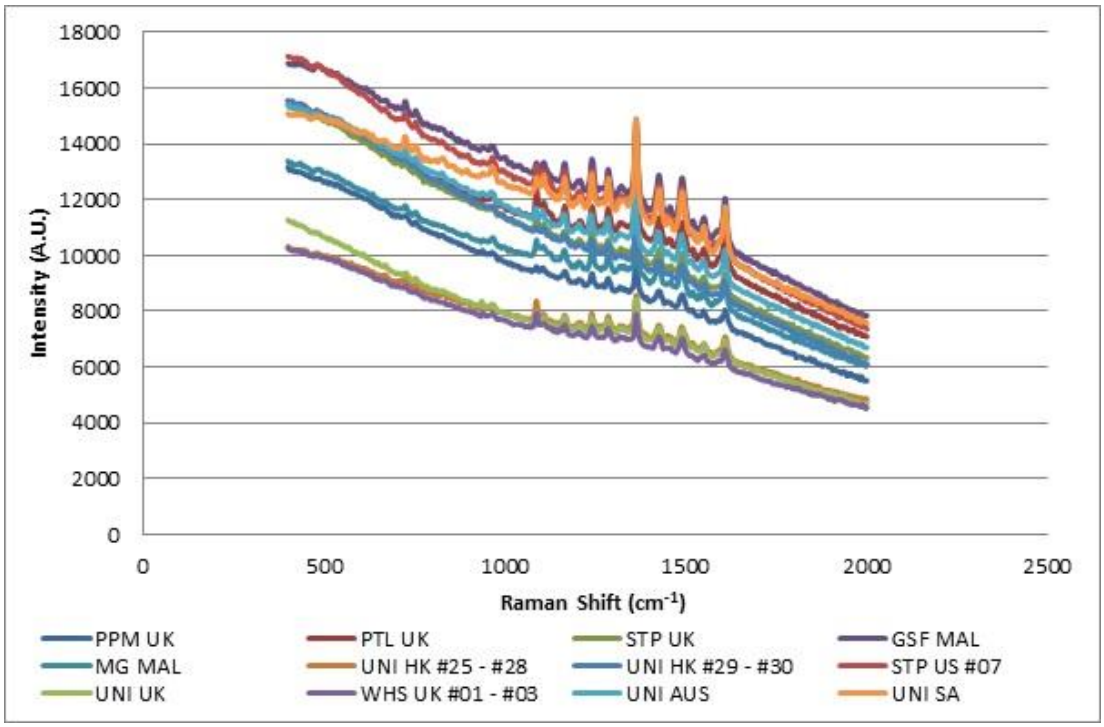


Figure 6.70: Brand/model combinations of red gel ink forming Group 1 (FORAM 100% laser power/x 50 obj., Auto Exposure)

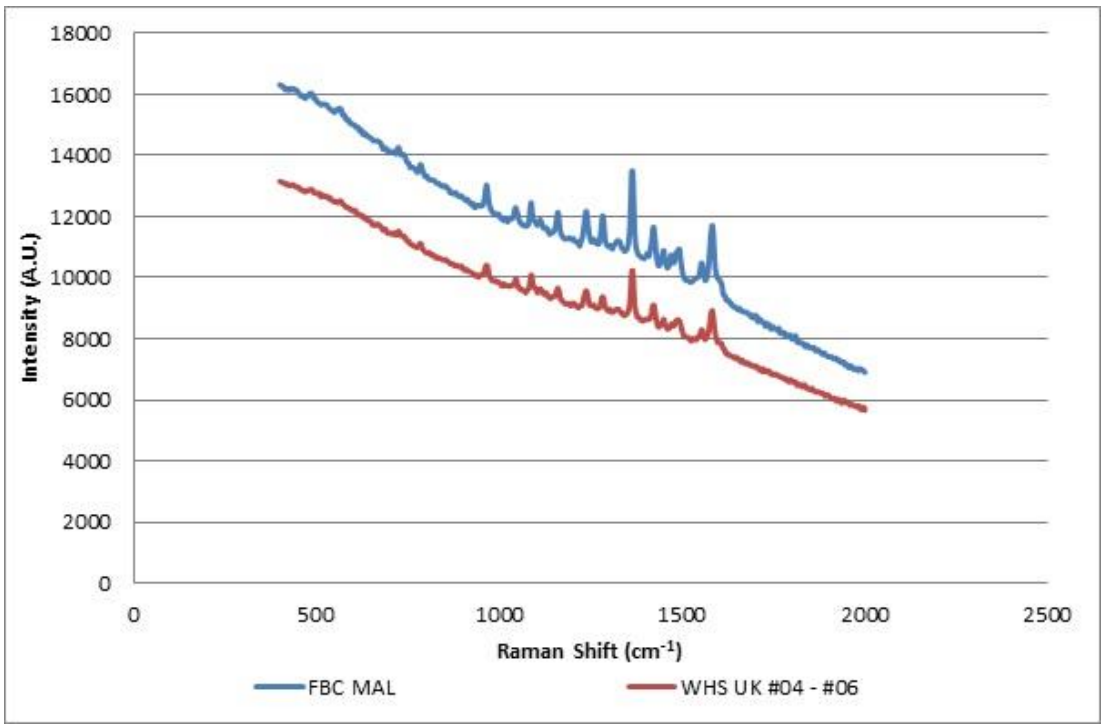


Figure 6.71: Brand/model combinations of red gel ink forming Group 2 (FORAM 785 100% laser power/x 50 obj., Auto Exposure)

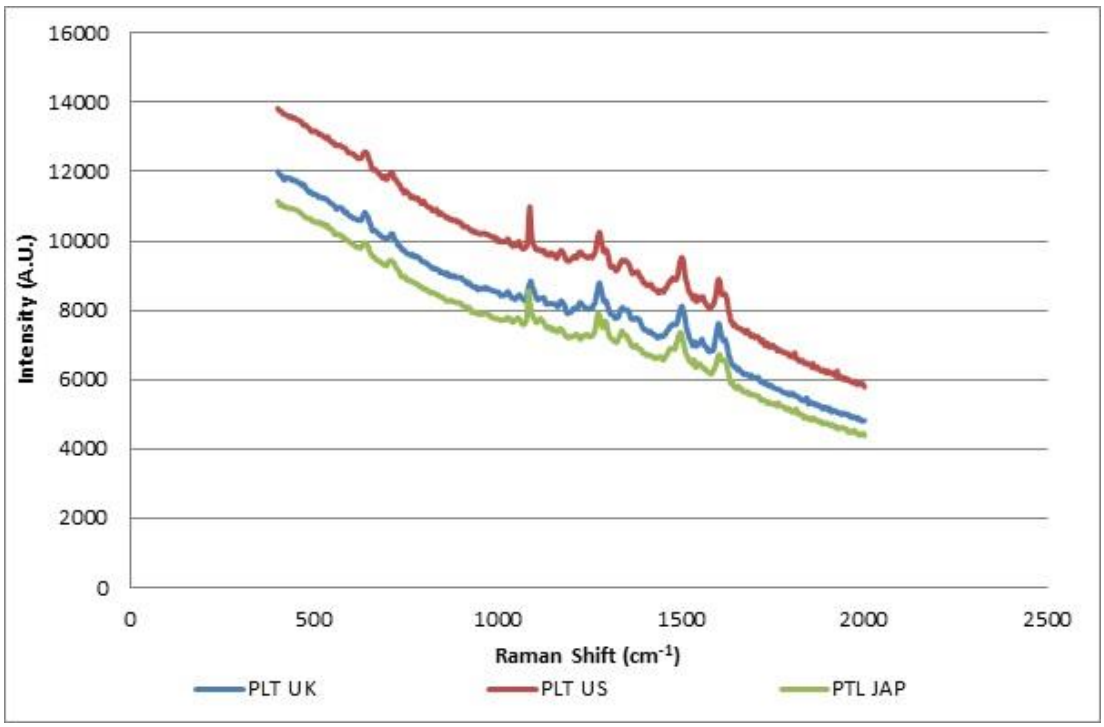


Figure 6.72: Brand/model combinations of red gel ink forming Group 3 (FORAM 785 100% laser power/x 50 obj., Auto Exposure)

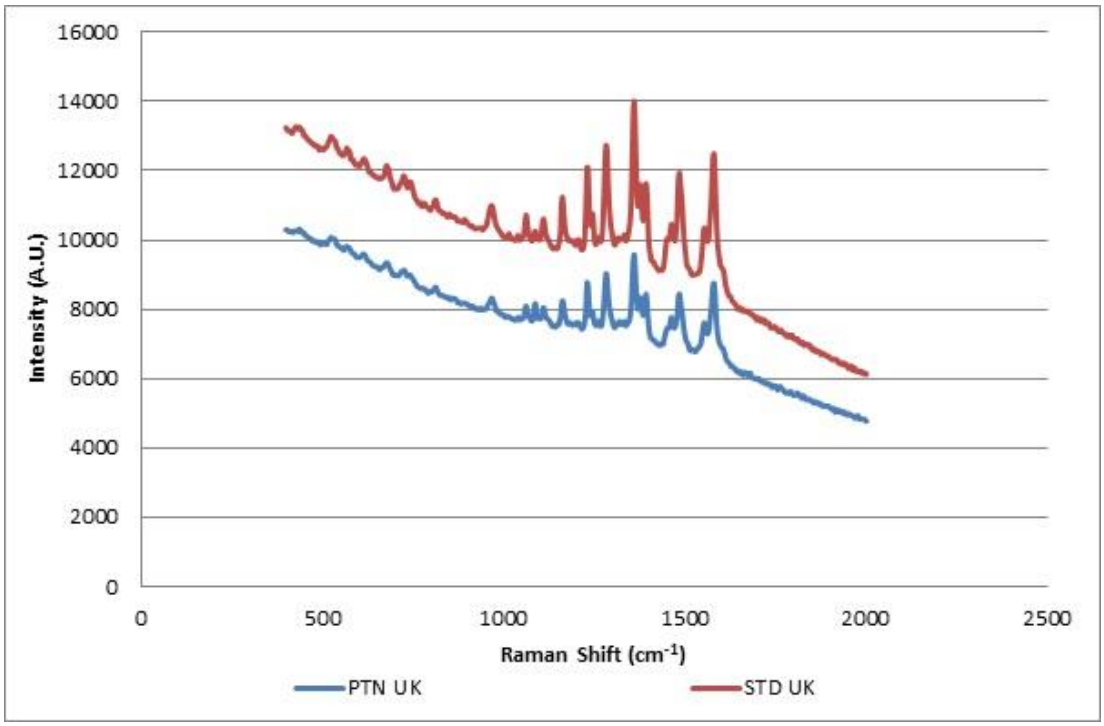


Figure 6.73: Brand/model combinations of red gel ink forming Group 4 (FORAM 785 100% laser power/x 50 obj., Auto Exposure)

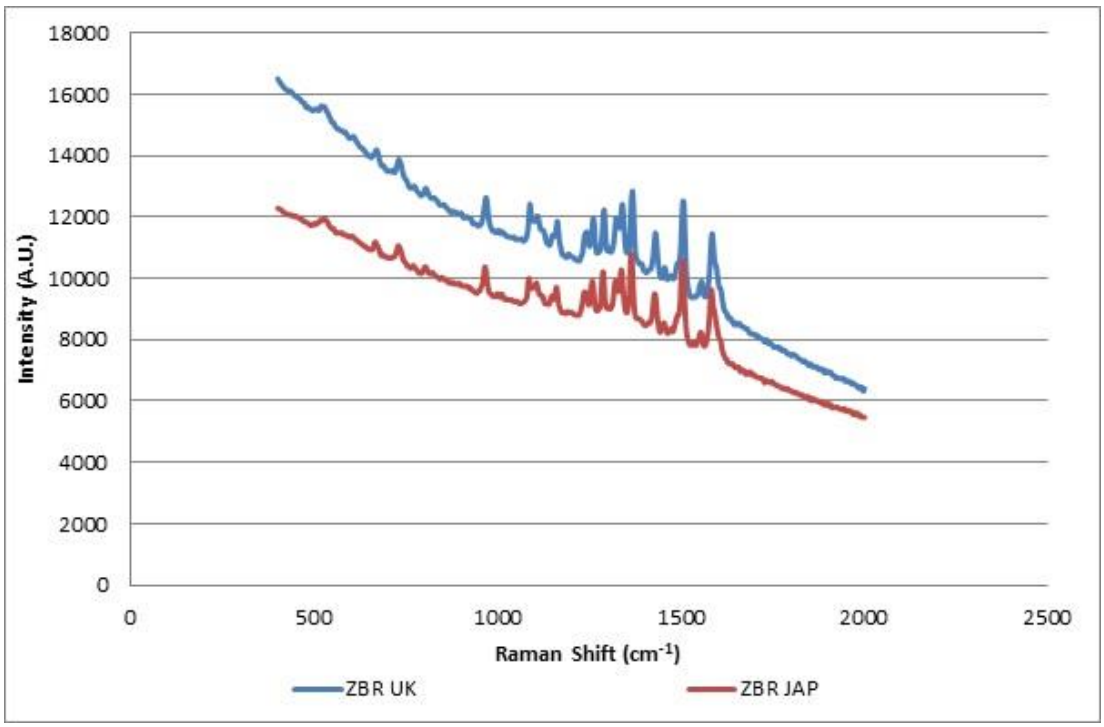


Figure 6.74: Brand/model combinations of red gel ink forming Group 5 (FORAM 785 100% laser power/x 50 obj., Auto Exposure)

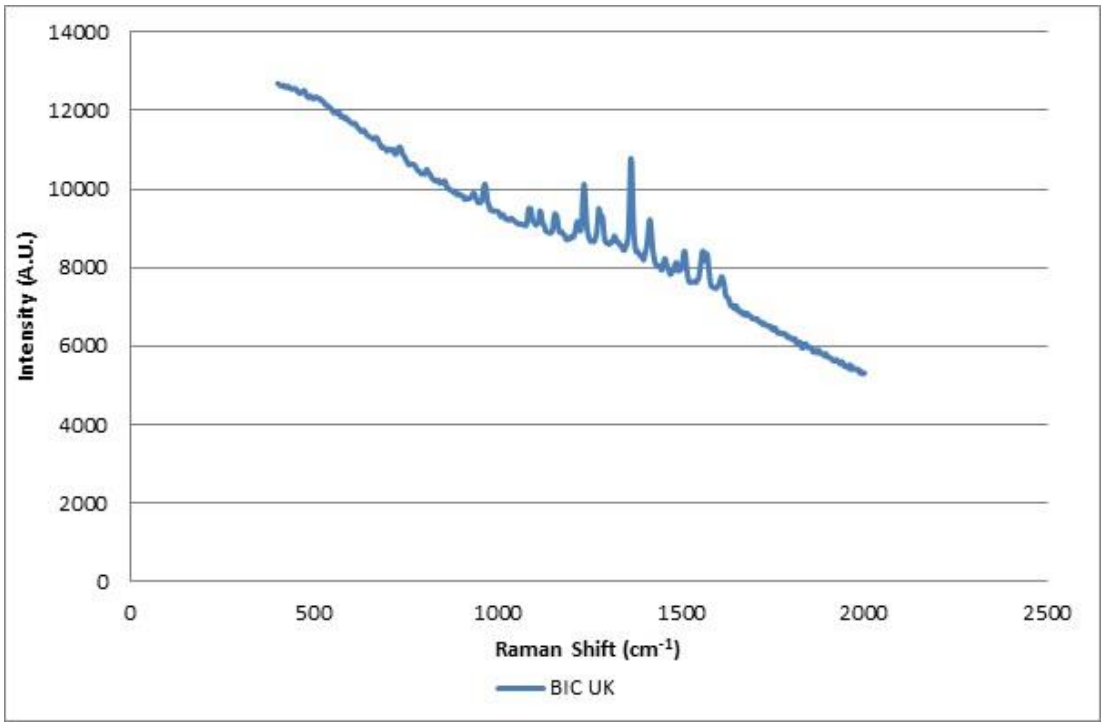


Figure 6.75: Brand/model combination of red gel ink forming Group 6 (FORAM 785 100% laser power/x 50 obj., Auto Exposure)

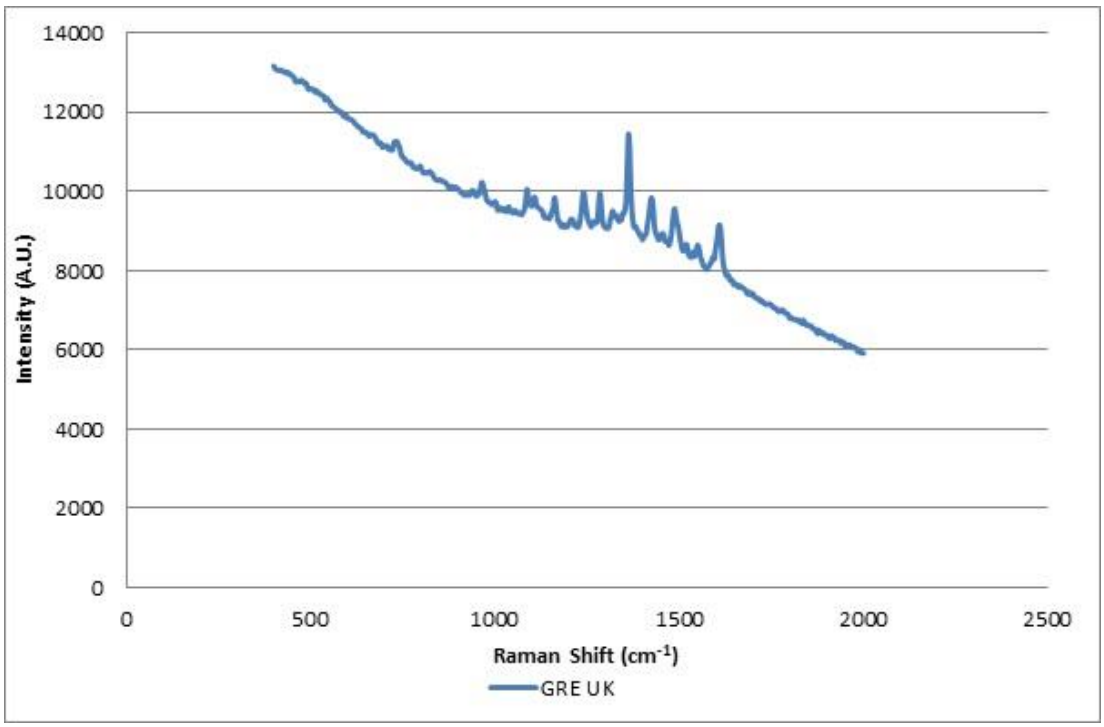


Figure 6.76: Brand/model combination of red gel ink forming Group 7 (FORAM 785 100% laser power/x 50 obj., Auto Exposure)

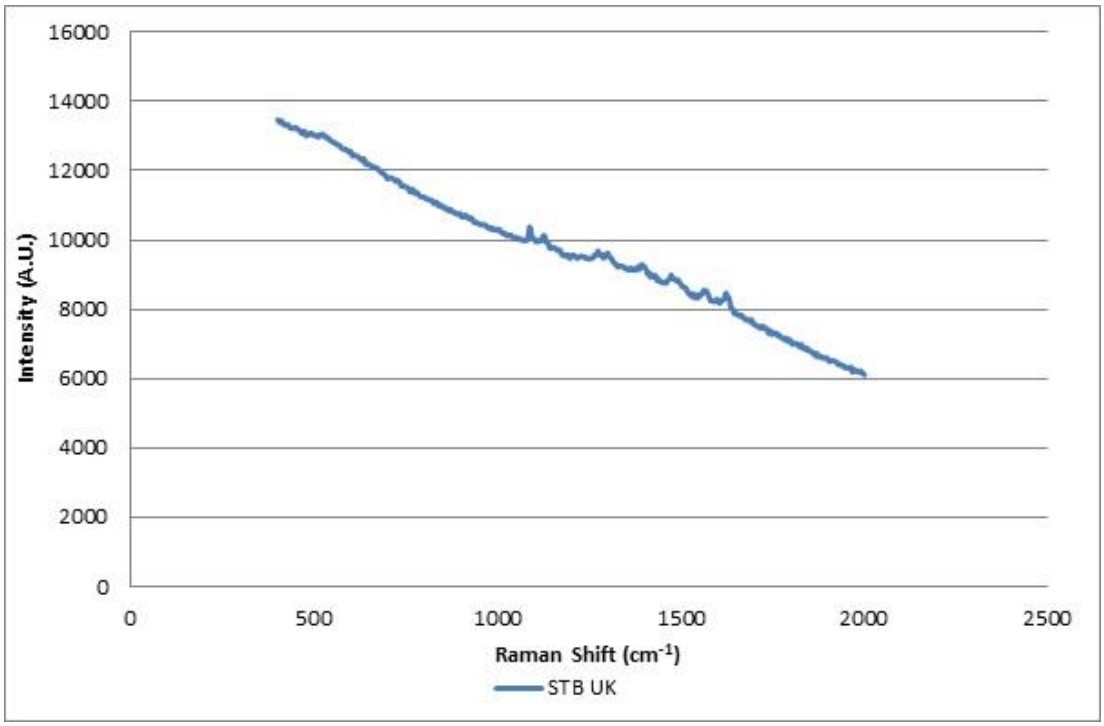


Figure 6.77: Brand/model combination of red gel ink forming Group 8 (FORAM 785 100% laser power/x 50 obj., Auto Exposure)

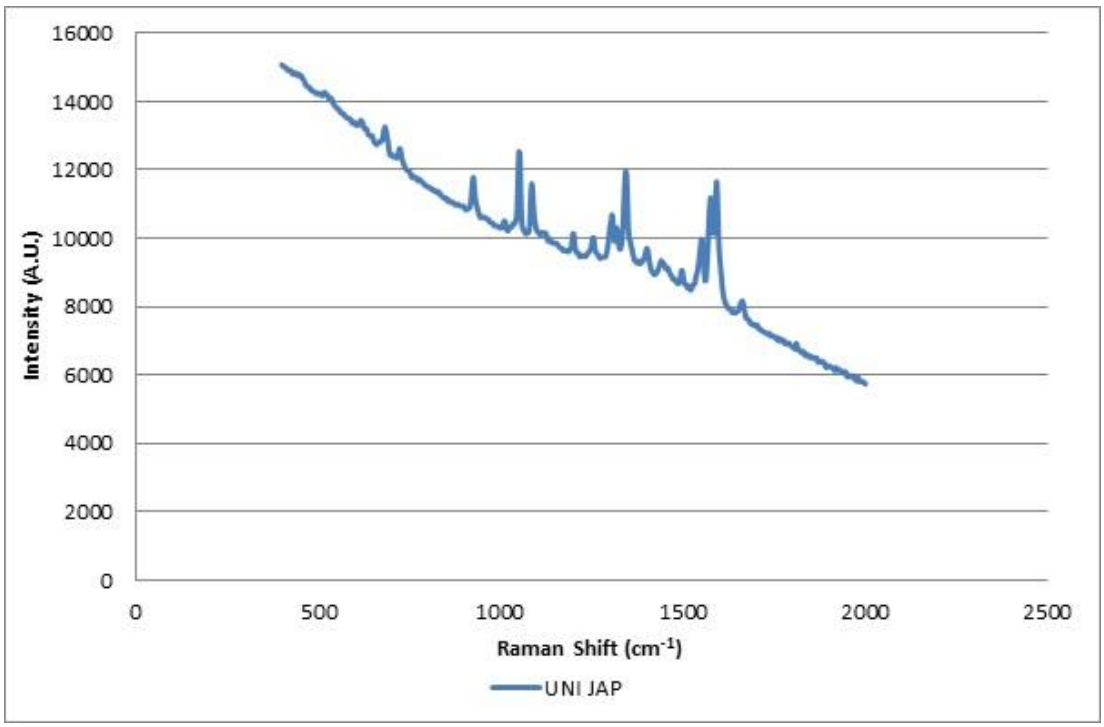


Figure 6.78: Brand/model combination of red gel ink forming Group 9 (FORAM 785 100% laser power/x 50 obj., Auto Exposure)

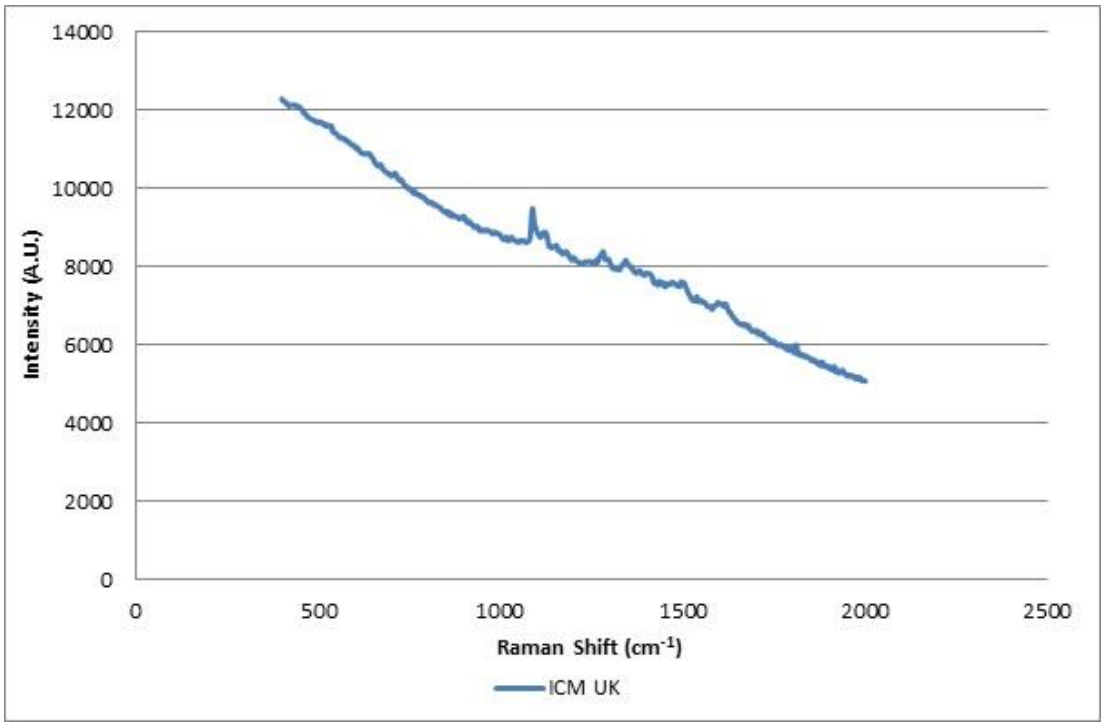


Figure 6.79: Brand/model combination of red gel ink forming Group 10 (FORAM 785 100% laser power/x 50 obj., Auto Exposure)

6.3.2.2.2.2.2 Renishaw InVia (785 nm)

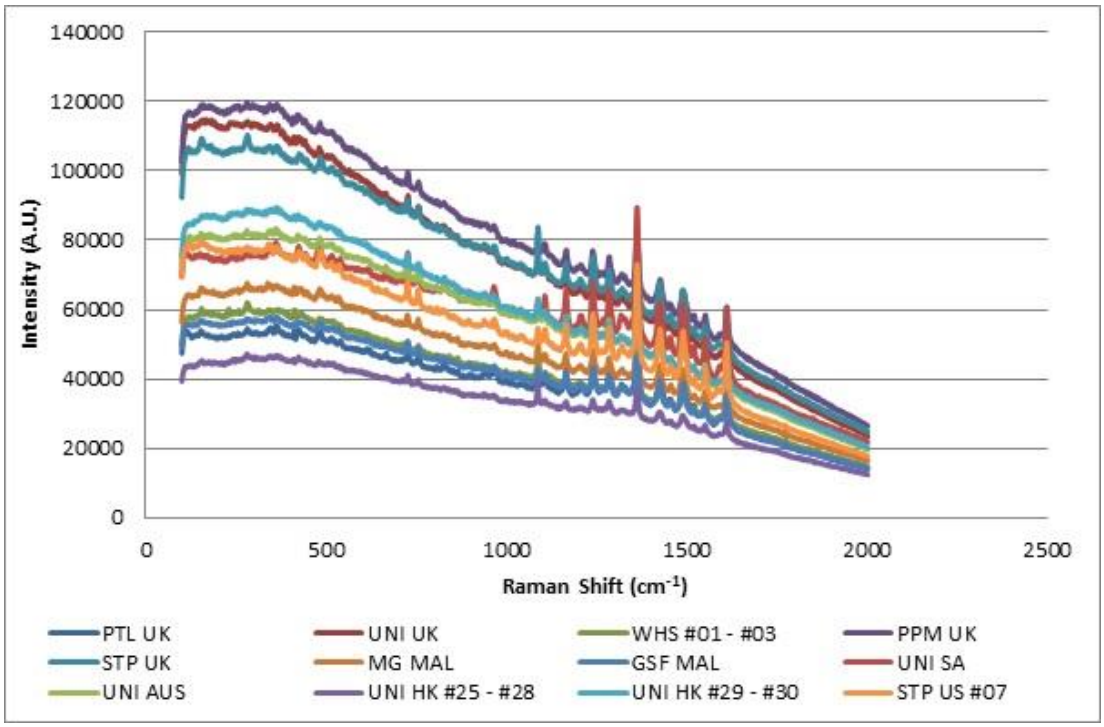


Figure 6.80: Brand/model combinations of red gel ink forming Group 1 (Renishaw InVia 785 nm 100% laser power/x 50 obj.)

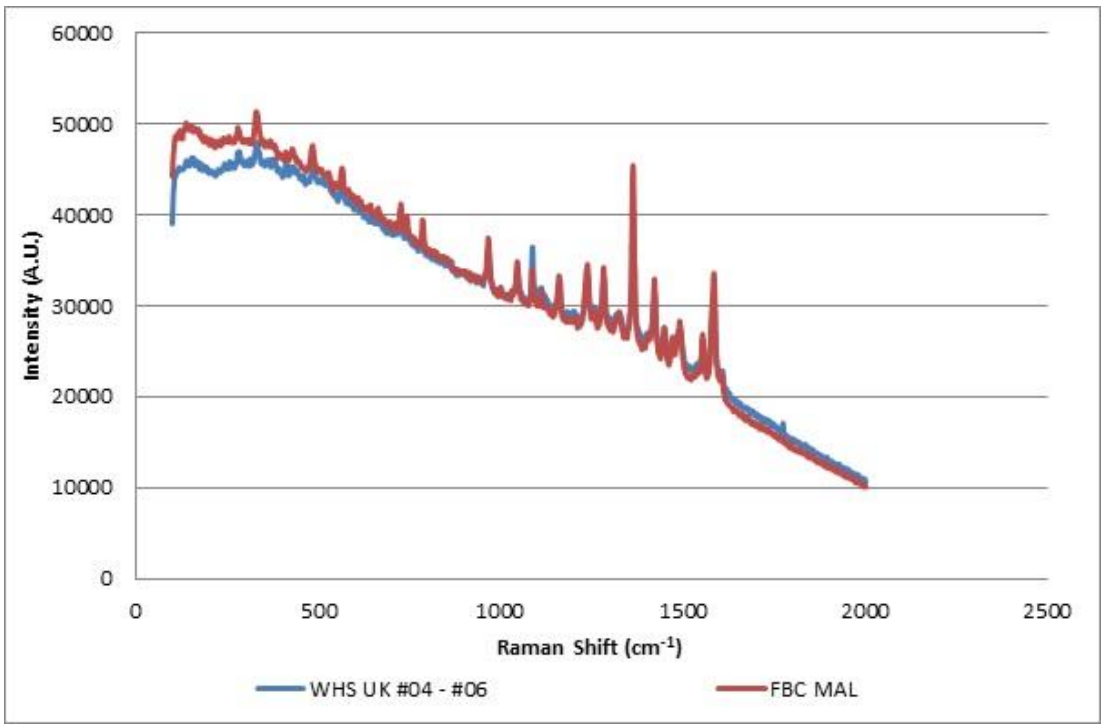


Figure 6.81: Brand/model combinations of red gel ink forming Group 2 (Renishaw InVia 785 nm 100% laser power/x 50 obj.)

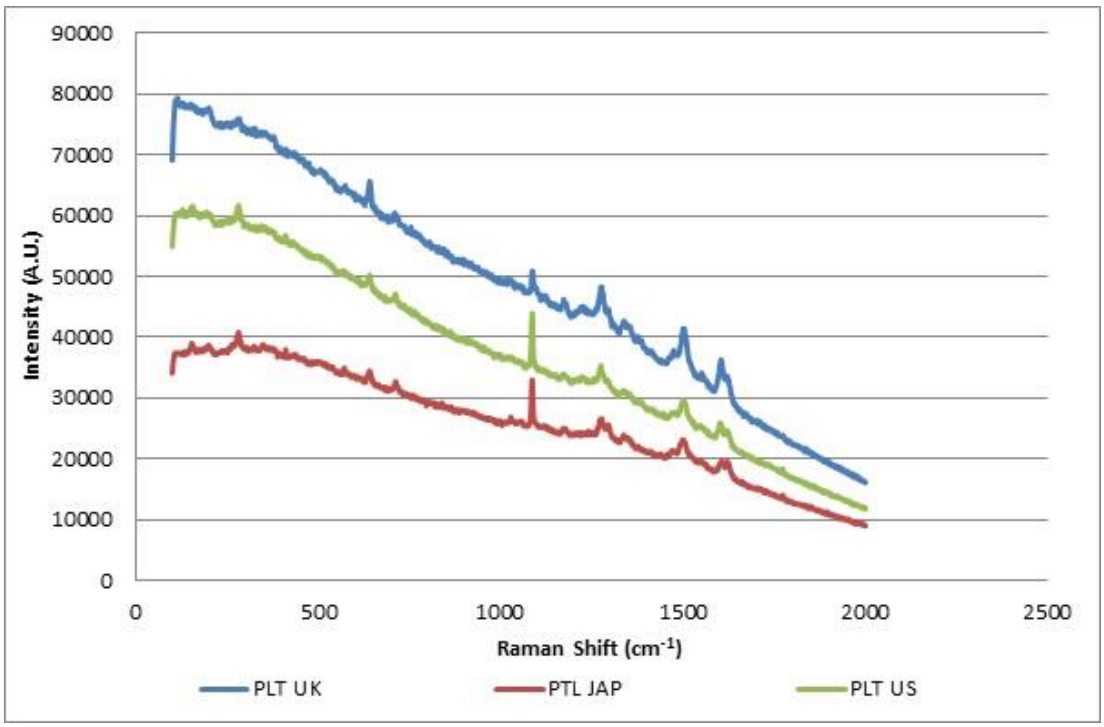


Figure 6.82: Brand/model combinations of red gel ink forming Group 3 (Renishaw InVia 785 nm 100% laser power/x 50 obj.)

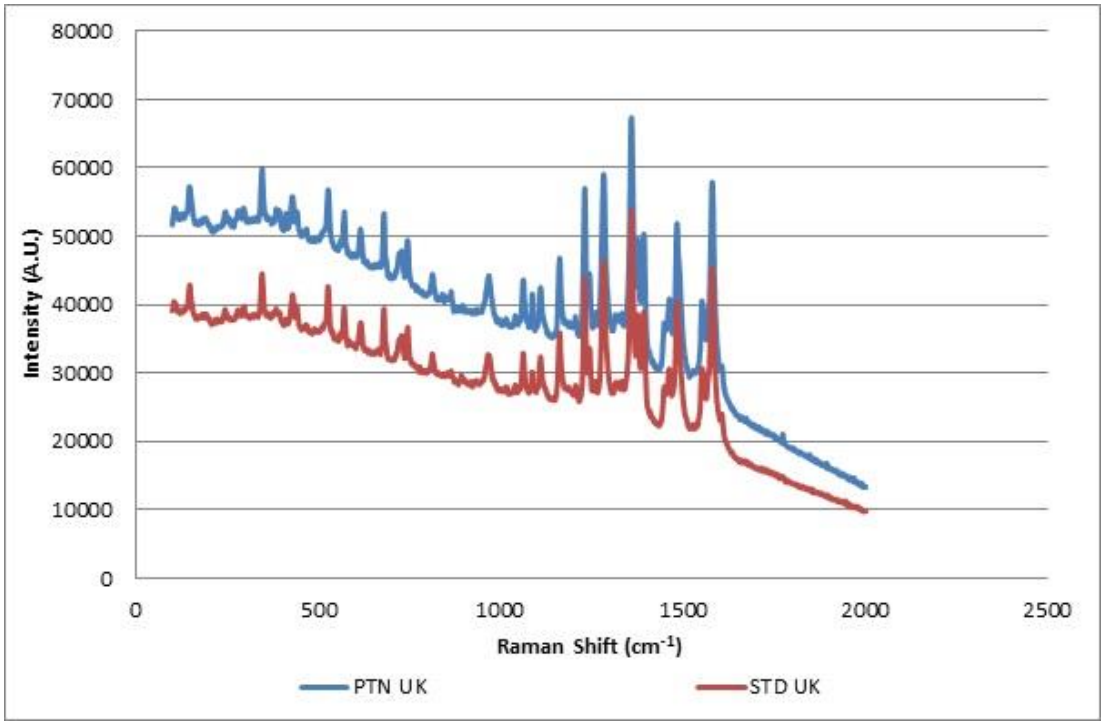


Figure 6.83: Brand/model combinations of red gel ink forming Group 4 (Renishaw InVia 785 nm 100% laser power/x 50 obj.)

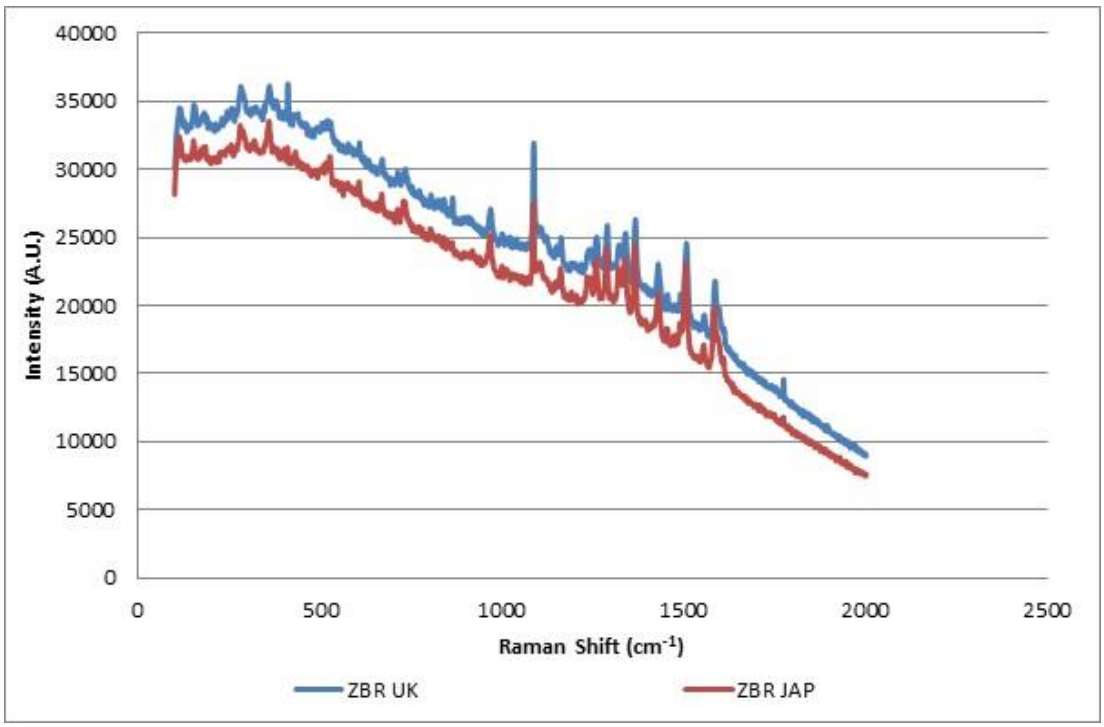


Figure 6.84: Brand/model combinations of red gel ink forming Group 5 (Renishaw InVia 785 nm 100% laser power/x 50 obj.)

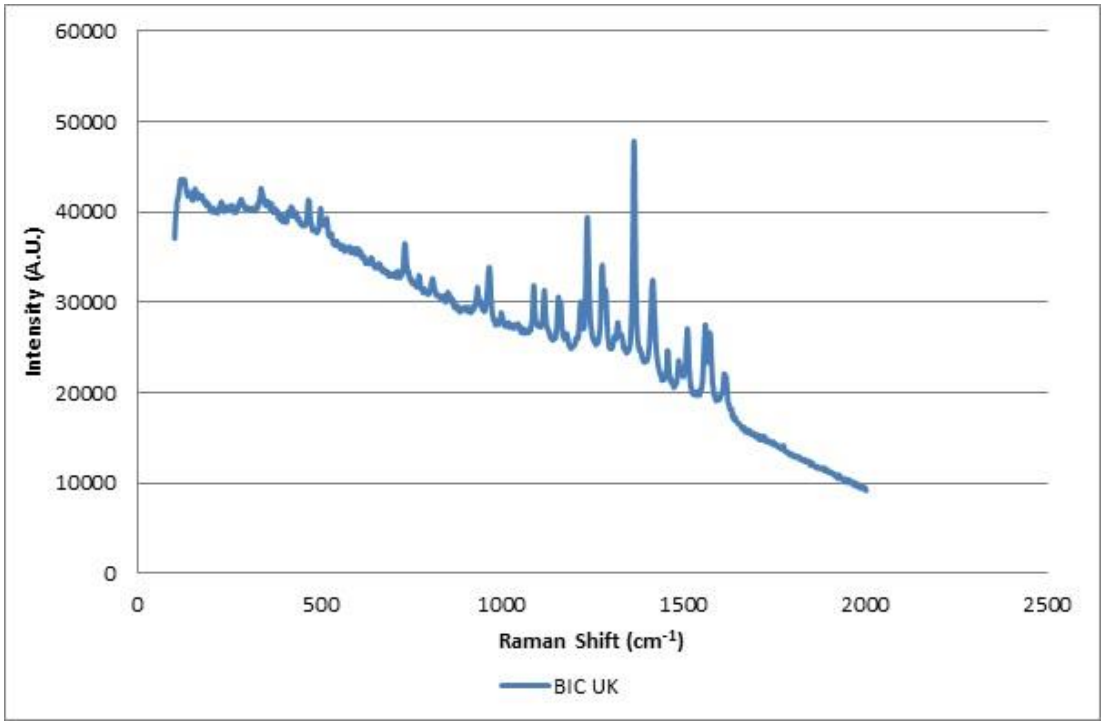


Figure 6.85: Brand/model combination of red gel ink forming Group 6 (Renishaw InVia 785 nm 100% laser power/x 50 obj.)

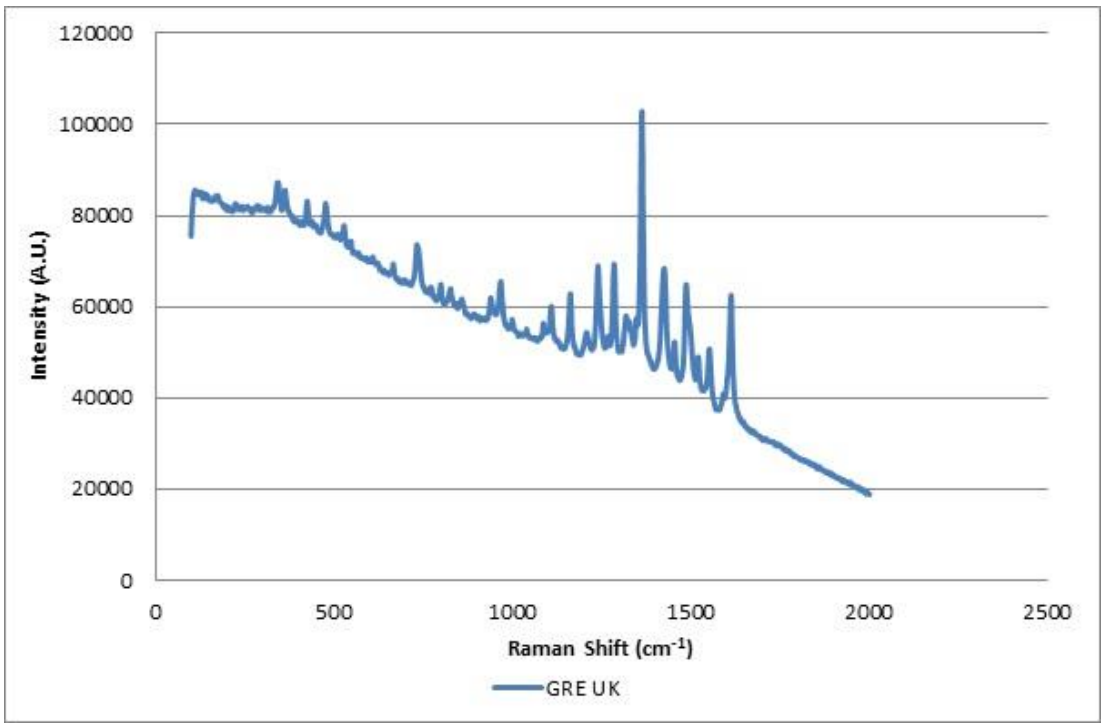


Figure 6.86: Brand/model combination of red gel ink forming Group 7 (Renishaw InVia 785 nm 100% laser power/x 50 obj.)

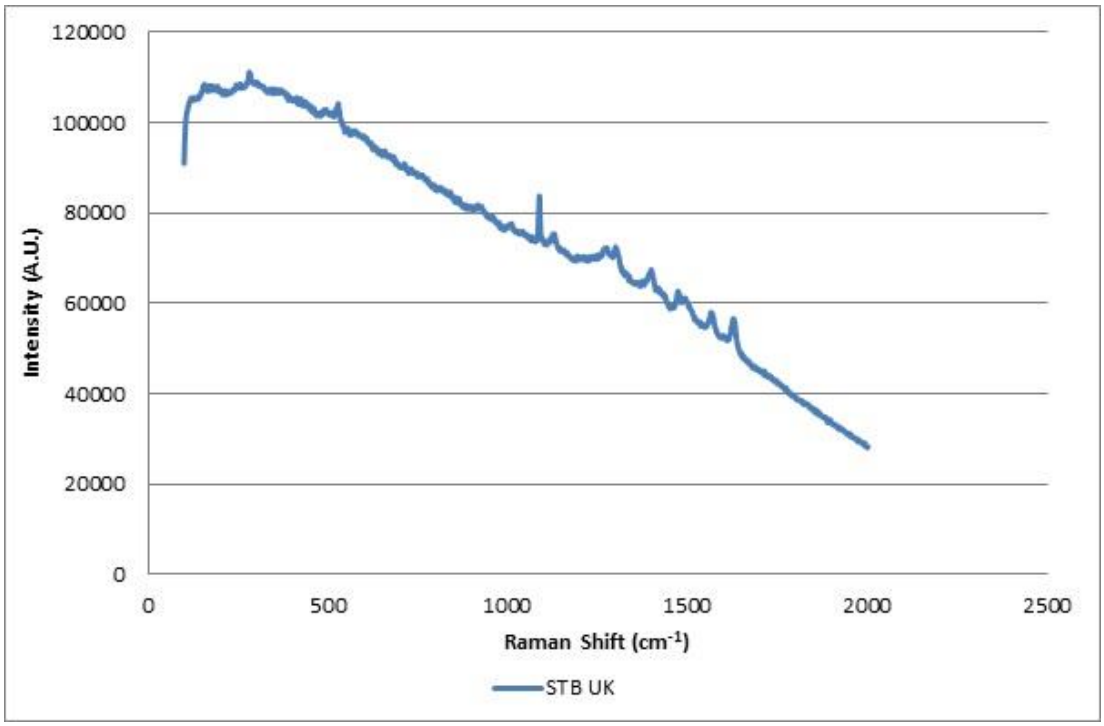


Figure 6.87: Brand/model combination of red gel ink forming Group 8 (Renishaw InVia 785 nm 100% laser power/x 50 obj.)

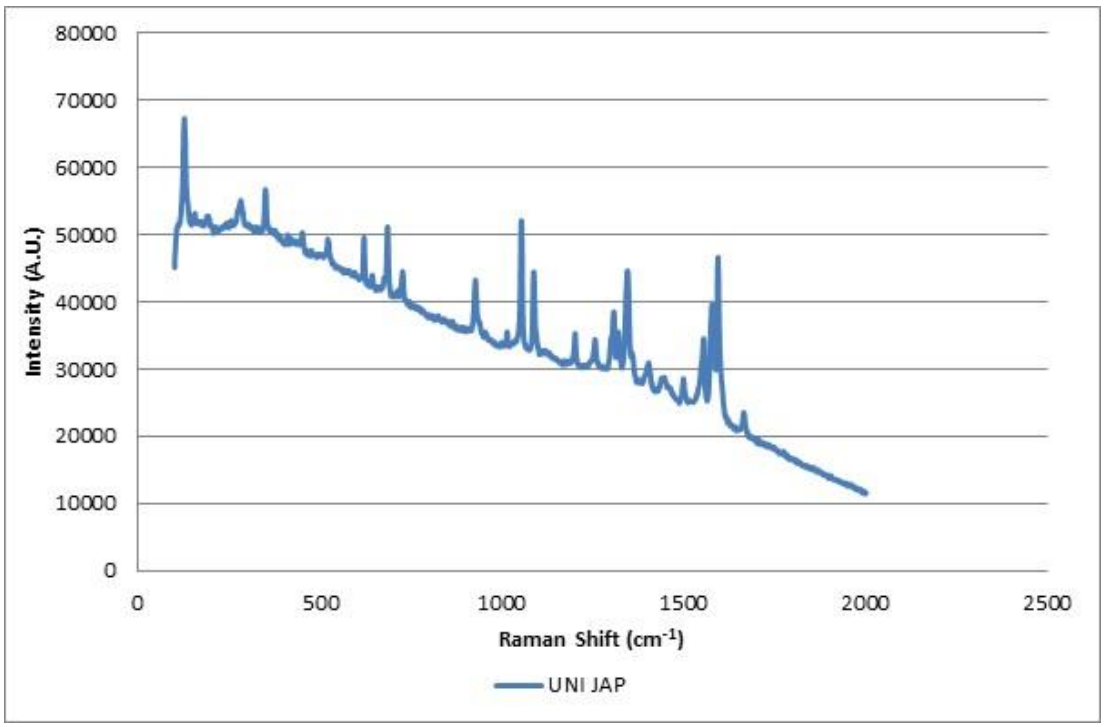


Figure 6.88: Brand/model combination of red gel ink forming Group 9 (Renishaw InVia 785 nm 100% laser power/x 50 obj.)

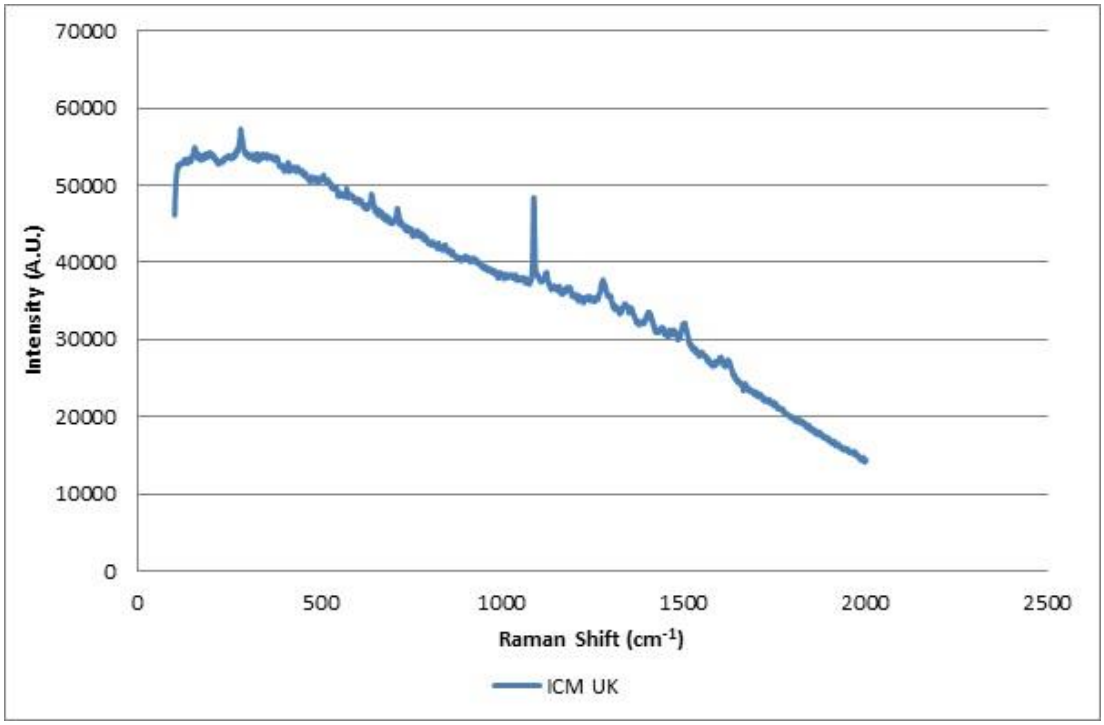


Figure 6.89: Brand/model combination of red gel ink forming Group 10 (Renishaw InVia 785 nm 100% laser power/x 50 obj.)

Table 6.9 summarises the red ink samples associated with each group by instrument.

Group	FORAM 785	Renishaw InVia 785 nm
1	PTL UK STP UK UNI AUS UNI UK MG MAL UNI HK #25 - #28 UNI HK #29 - #30 WHS UK #01 - #03 GSF MAL PPM UK UNI SA STP US #07	PTL UK STP UK UNI AUS UNI UK MG MAL UNI HK #25 - #28 UNI HK #29 - #30 WHS UK #01 - #03 GSF MAL PPM UK UNI SA STP US #07
2	WHS UK #04 – #06 FBC MAL	WHS UK #04 - #06 FBC MAL
3	PLT UK PLT US PTL JAP	PLT UK PLT US PTL JAP
4	PTN UK STD UK	PTN UK STD UK
5	ZBR UK ZBR JAP	ZBR UK ZBR JAP
6	BIC UK	BIC UK
7	GRE UK	GRE UK
8	STB UK	STB UK
9	UNI JAP	UNI JAP
10	ICM UK	ICM UK

Table 6.9: Summary of red ink group classification groupings based on FORAM 785 and Renishaw InVia 785 nm spectral data

Spectral patterns characterising each group were generally easy to visually distinguish between. However, Group 7 spectral pattern (GRE UK) was highly similar to that of Group 1 (representing multiple brand/model combinations), distinguishable only on the basis of an additional peak at ~720 nm observed in the former as highlighted within the green circle in Figure 6.90. Furthermore, the spectral pattern representing Group 3 (PLT UK, PLT US and PTL JAP), Group 8 (STB UK) and Group 10 (ICM UK) red gel ink samples consisted of relatively weak Raman peaks on high background fluorescence. Nonetheless, there was sufficient spectral detail to permit effective discrimination between these brand/model combinations. In comparison, spectral Groups 1, 2, 4, 5, 6, 7 and 9 consisted of relatively strong Raman peaks on high background fluorescence, and with the aforementioned exception, were readily distinguishable.

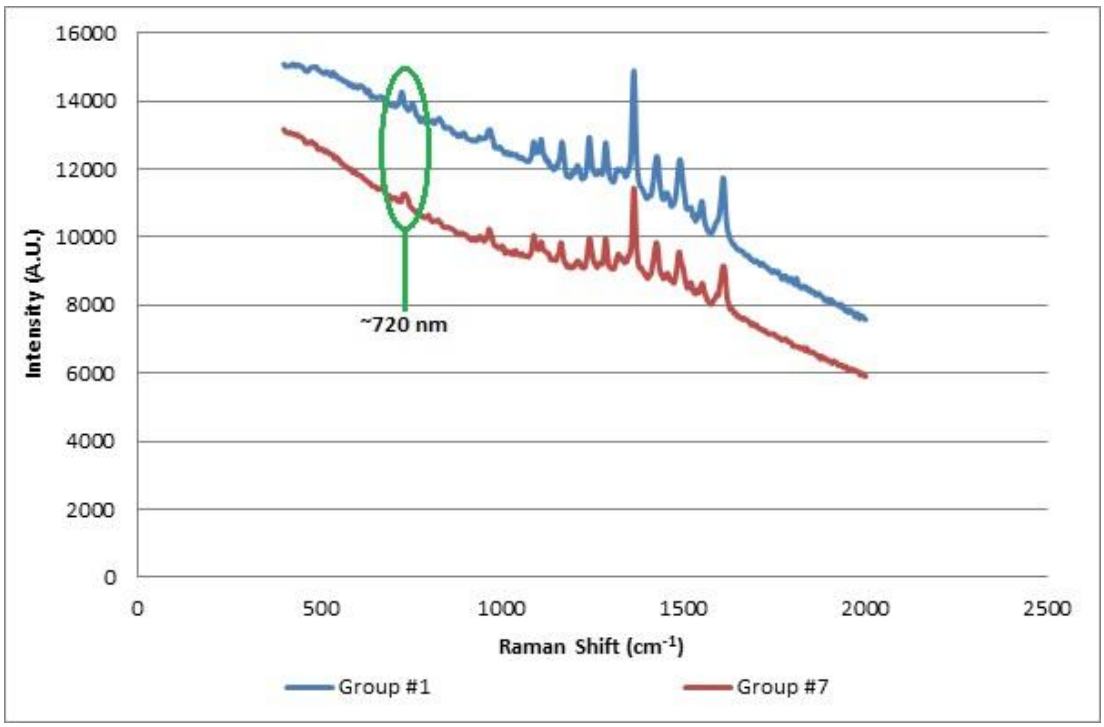


Figure 6.90: Highly similar spectral pattern representing Group 1 and Group 7 red gel ink brand/model combinations distinguishable by an additional peak at ~720 nm observed in Group 1 spectra (FORAM 785 100% laser power/x 50 obj., Auto Exposure)

6.3.2.3 Comparison of Renishaw InVia and FORAM 785

6.3.2.3.1 Blue Ink Group

A like for like comparison between the Renishaw InVia and FORAM at the 785 nm excitation wavelength for their ability to analyse and discriminate between brand/model combinations of blue and red gel inks on paper (See *Red ink group*) was conducted.

When comparing spectra produced from both instruments, with one exception, the Renishaw InVia spectral data exhibited a greater overall maximum spectral intensity. This was likely attributable to the use of a more powerful laser source used in the Renishaw InVia Spectrometer (maximum power output at source 500 mW). The effect of this upon spectral pattern meant some peaks appeared more intense in the Renishaw spectra than in the corresponding FORAM spectra. This is illustrated in Figures 6.91 and 6.92 for the STD UK sample analysed under the Renishaw and FORAM instruments respectively. For the STB UK sample, as presented in Figure 6.93, the FORAM spectra exhibited a slightly greater overall spectral intensity and better S:N ratio, but the intensity of the peaks compared to those in the Renishaw spectra, shown in Figure 6.94 appeared similar. For all brand/model

combinations, the spectral pattern exhibited by both instruments was indistinguishable from 500 cm^{-1} – 2000 cm^{-1}, therefore no difference in classification groupings between instruments was observed. Furthermore, in the Renishaw InVia spectra, no peaks above 2000 cm^{-1} were observed other than single non-reproducible stray peaks or a peak attributable to the paper. Therefore, Renishaw spectral data could be manually reduced to cover a wavenumber range of 100 – 2000 cm^{-1}, similar to that of the FORAM 785 to ease comparison of spectra between instruments. For those brand/model combinations that were swamped by detector overload in the Renishaw spectra, this was eliminated in the FORAM spectra which simply exhibited fluorescence. For the ZBR UK brand/model combination, pen #02 and #03 which exhibited substantial detector overload in the Renishaw spectra, exhibited a reproducible background fluorescence shape in the FORAM spectra that was clearly distinguishable from the spectra of the other pens in the set, even though in terms of peak presence all spectra were indistinguishable (See *Within Brand Variation*).

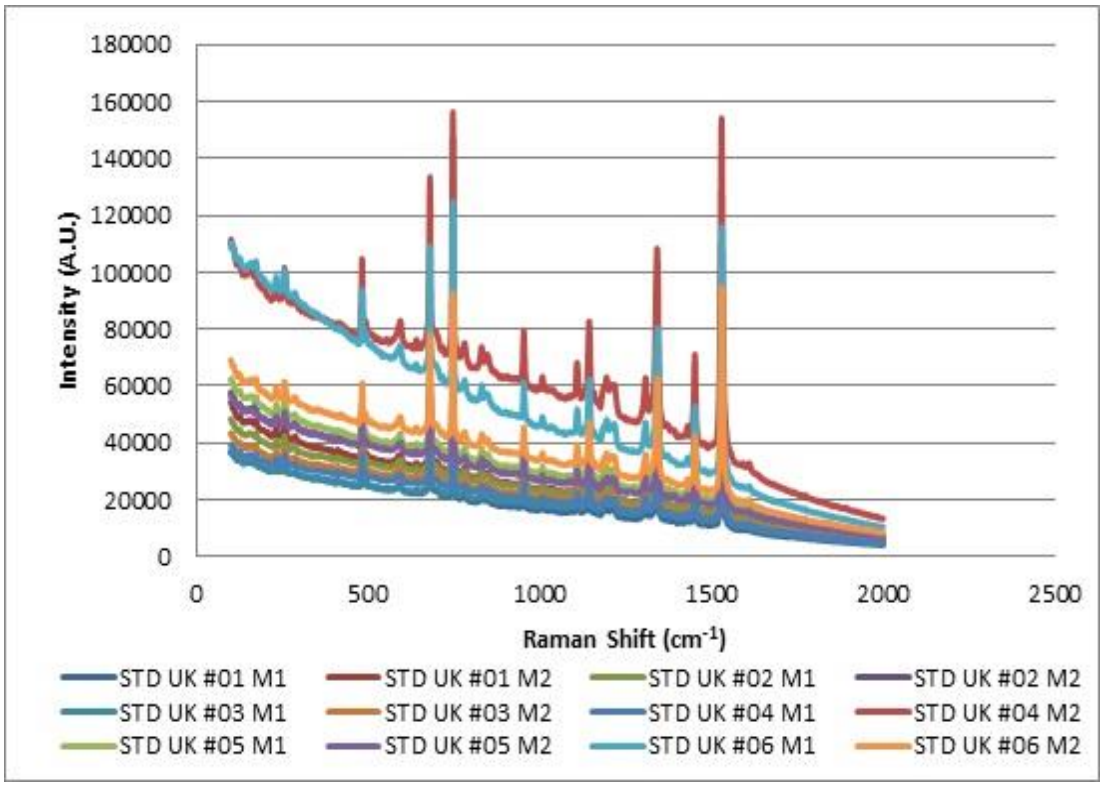


Figure 6.91: Duplicate spectra of STD UK pens #01 - #06 blue gel ink on paper (Renishaw InVia 785 nm 10% laser power/x 50 obj.)

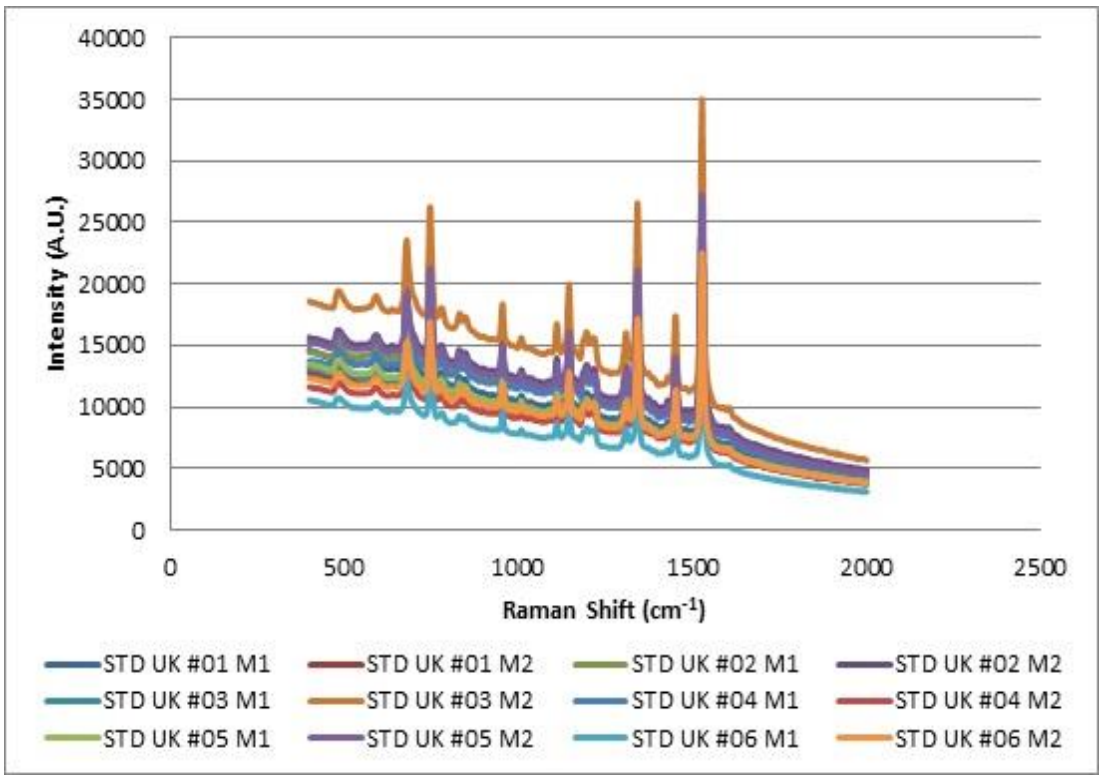


Figure 6.92: Duplicate spectra of STD UK pens #01 – #06 blue gel ink on paper (FORAM 785 10% laser power/x 50 obj., Auto Exposure)

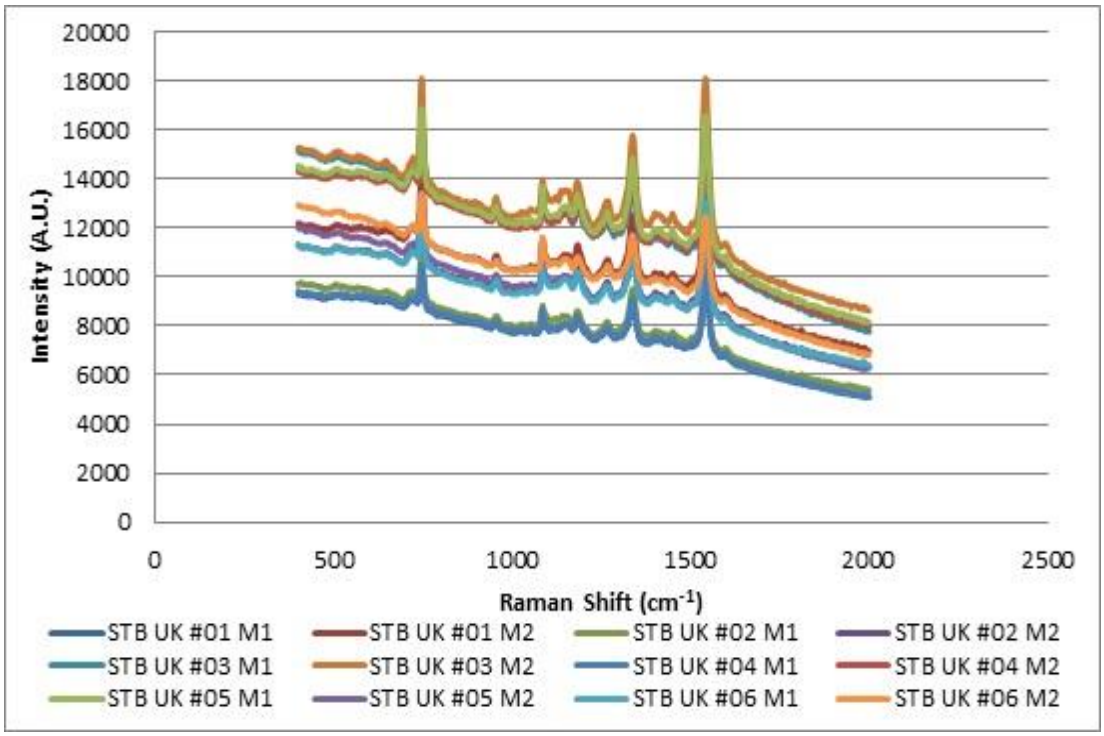


Figure 6.93: Duplicate spectra of STB UK pen #01- #06 blue gel ink on paper (FORAM 785 100% laser power/x 50 obj., Auto Exposure)

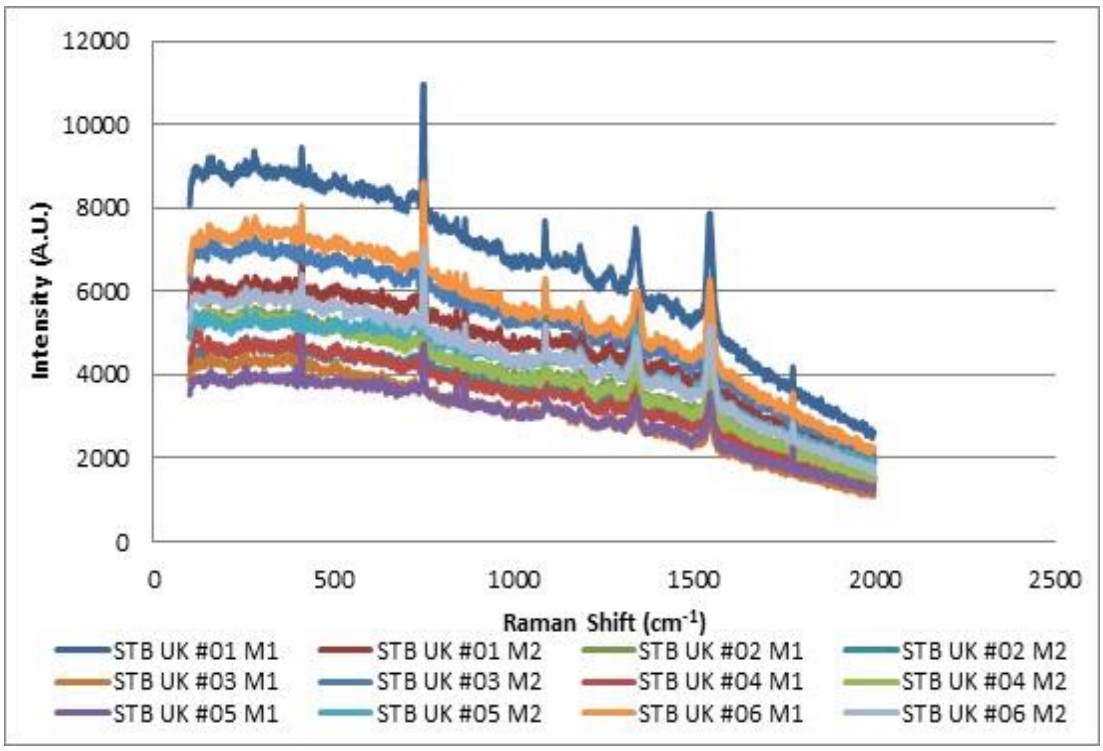


Figure 6.94: Duplicate spectra of STB UK pens #01 - #06 blue gel ink on paper (Renishaw InVia 785 nm 10% laser power/x 50 obj.)

6.3.2.3.2 Red Ink Group

Similarly to the blue ink group, the overall maximum spectral intensity was greater in the Renishaw spectra than the FORAM 785. This made the intensity of the peaks in the FORAM spectra appear weaker than in the corresponding Renishaw spectra, making comparison of spectra from the same brand/model combination between instruments difficult, since some peaks which appeared relatively strong in the Renishaw spectra were almost undetectable in the FORAM spectra. An example of this is demonstrated in Figure 6.95 – Figure 6.96. However, where direct comparison was possible, the spectrum for the same brand/model combination between instruments was indistinguishable as shown in Figure 6.97 – Figure 6.98. As for the blue ink group, the red gel ink brand/model combinations were discriminated into the same spectral groups irrespective of instrument.

The Solubility/TLC study identified only two pigment based samples from the 25 brand/model combinations analysed, STB UK and UNI JAP. The findings from Raman Spectroscopy show that all brand/model combinations contain a pigment component, suggesting the vast majority of red gel inks, at least in this study, are hybrid formulations, thus affording a high level of potential discrimination.

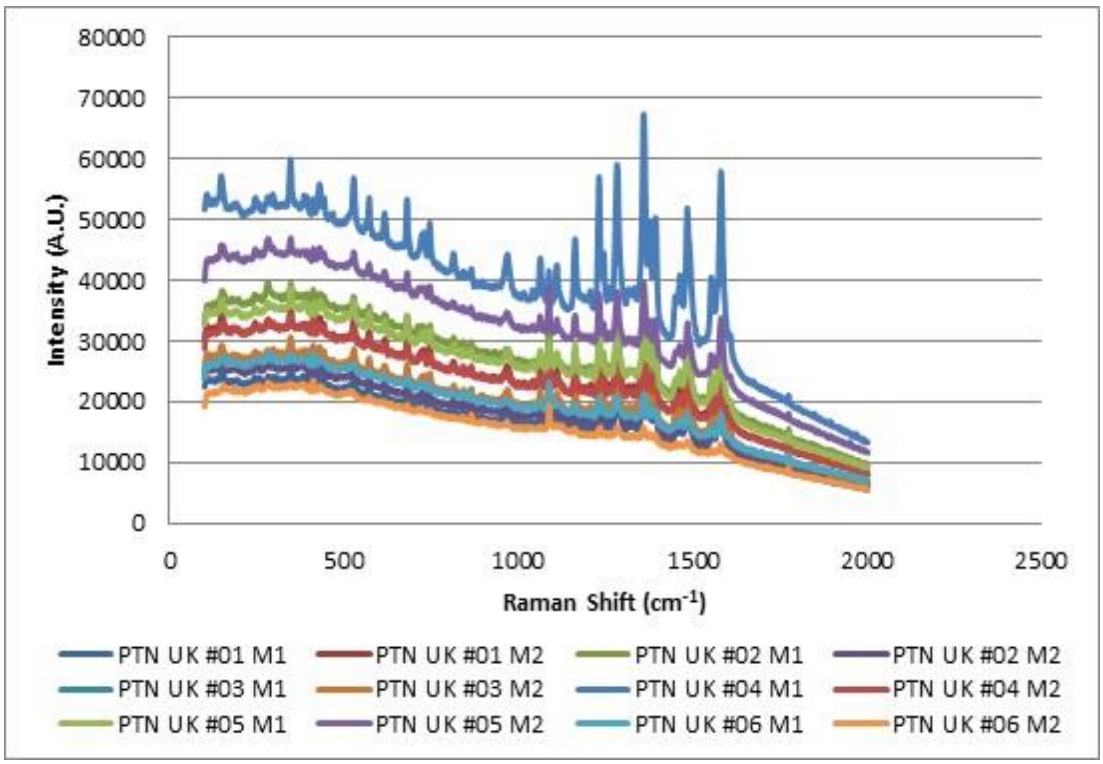


Figure 6.95: Duplicate spectra of PTN UK pens #01 – #06 red gel ink on paper (Renishaw InVia 785 nm 100% laser power/x 50 obj.)

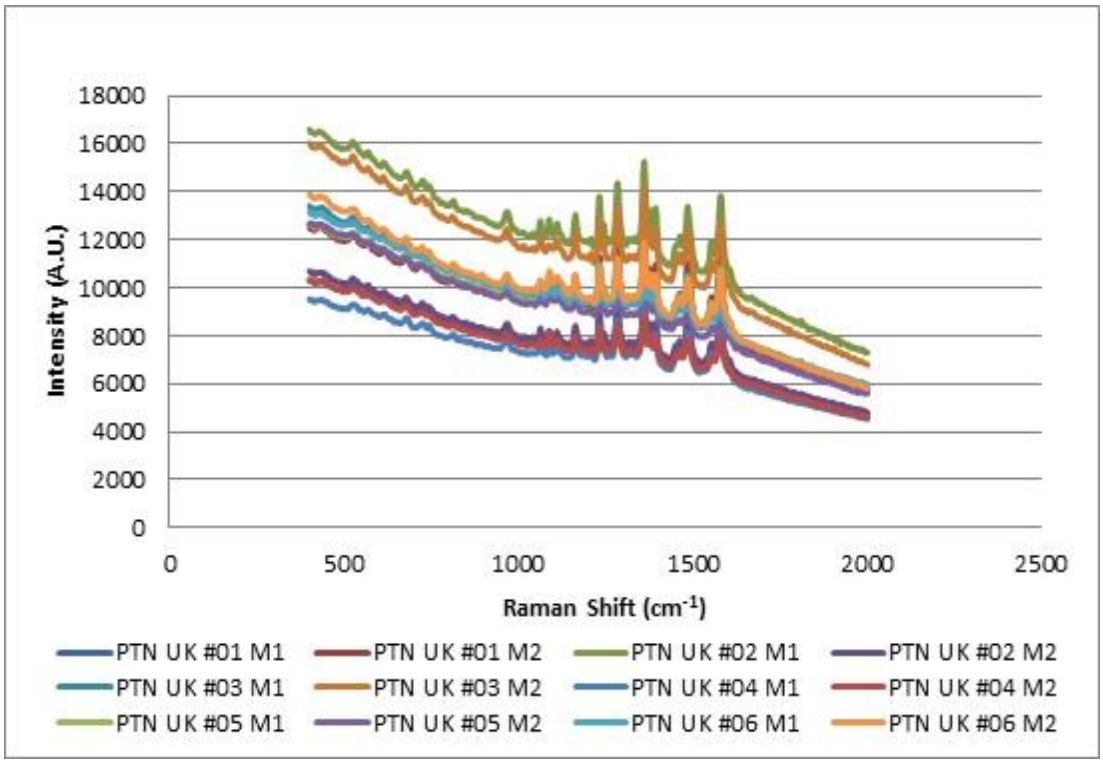


Figure 6.96: Duplicate spectra of PTN UK pens #01 - #06 red gel ink on paper (FORAM 785 100% laser power/x 50 obj., Auto Exposure)

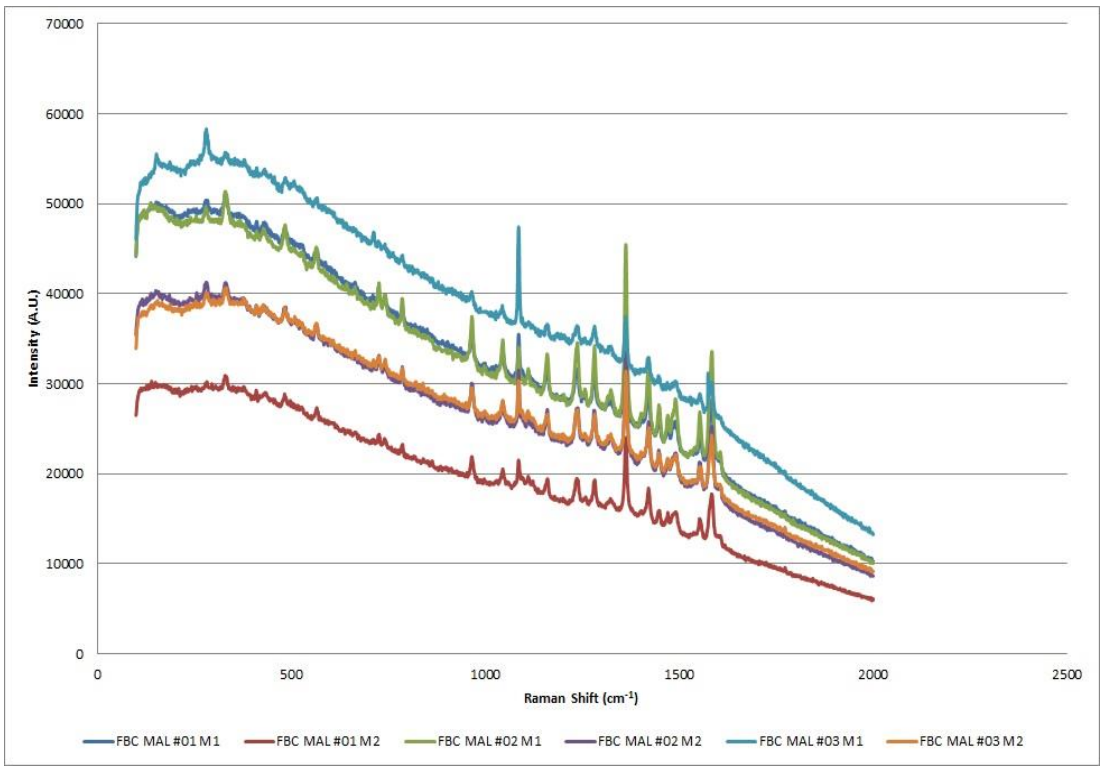


Figure 6.97: Duplicate spectra of FBC MAL pens #01 - #03 red gel ink on paper (Renishaw InVia 785 nm 100% laser power/x 50 obj.)

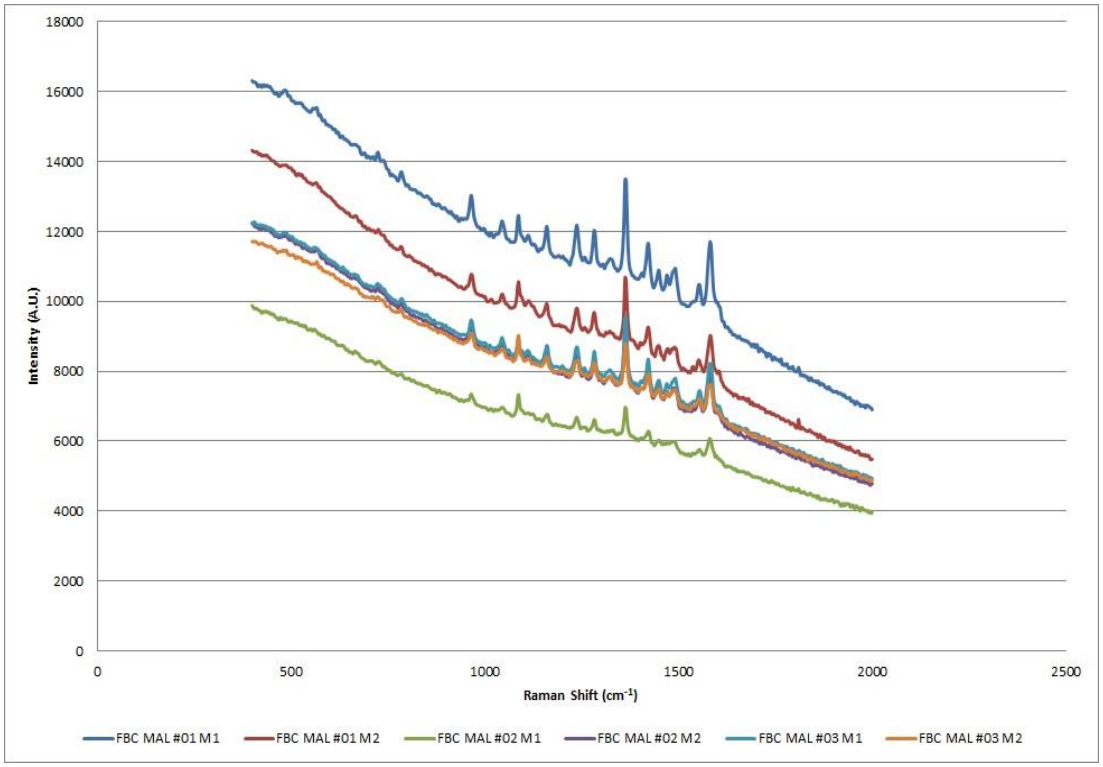


Figure 6.98: Duplicate spectra of FBC MAL pens #01 - #03 red gel ink on paper (FORAM 785 100% laser power/x 50 obj., Auto Exposure)

6.3.2.3.3 Stray Peaks

During spectral acquisition several Renishaw spectra exhibited stray peaks. Eighteen brand/model combinations (PTL UK; UNI UK; ZBR UK; BIC UK; PPM UK; STP UK; STD UK; MG MAL; FBC MAL; UNI HK #25 - #28; UNI HK #29 - #30; UNI AUS; PTL JAP; ZBR JAP; ICM UK; UNI SA; PLT UK; PLT US), exhibited at least one spectrum with a stray peak, and never more than three spectra within a brand/model combination set. An example of this is presented in Figure 6.99. Given their non-reproducible nature, they were considered irrelevant and removed using the instrument software (See *Materials and Methods*).

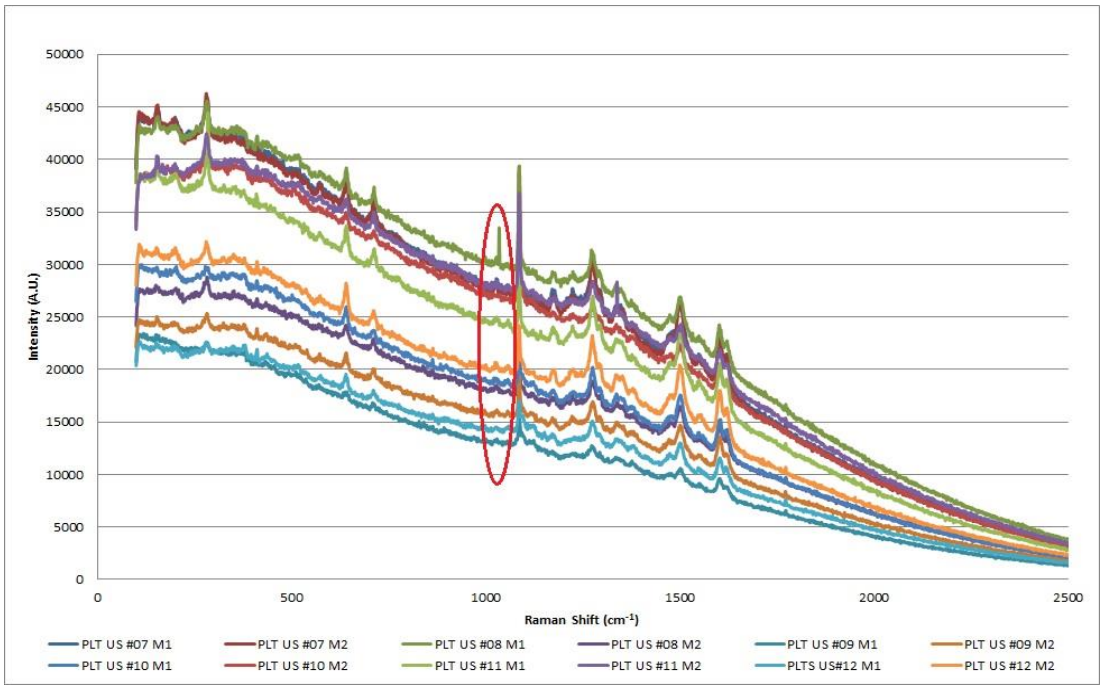


Figure 6.99: Spectra of PLT US pens #07 - #12 red gel ink on paper exhibiting a stray peak at ~1035 cm^{-1} highlighted in the red circle (Renishaw InVia 785 nm 100% laser power/x 50 obj.)

6.3.2.4 Pigment Spectral Library

6.3.2.4.1 Blue Ink Group

The blue ink group samples were discriminated into four groups using FORAM 785. As expected for Group 3 and Group 4, both dye containing, no pigment identifications were made. However, possible pigment identifications were made for Group 1 and Group 2 ink samples. The representative spectrum for Group 1 (UNI UK) was searched against the library, and returned seven possible ID matches above the 70% threshold criteria. These included both blue and green pigment standards, including three phthalocyanine blue

pigments. Interestingly the top three suggestions were Fluorescent Pigment Blue (56050 – CI Unidentified), Cadmium Green Light (44500) and Cadmium Green Dark (44510) with match values of 97.4%, 95.6% and 94.7% respectively. Whilst their spectra were highly similar to the ink spectrum, they could be excluded by the presence or absence of certain peaks, differences in the shape of certain peaks and/or relative peak intensities. A possible identification was made against the seventh suggestion, Phthalo Blue Primary (23050 – CI Pigment Blue 15:1) with a 76.2% match value, highlighting the importance of manual comparison to confirm identifications made by a computer library. Figure 6.100 shows a comparison between the ink spectrum, against the most likely pigment match spectra.

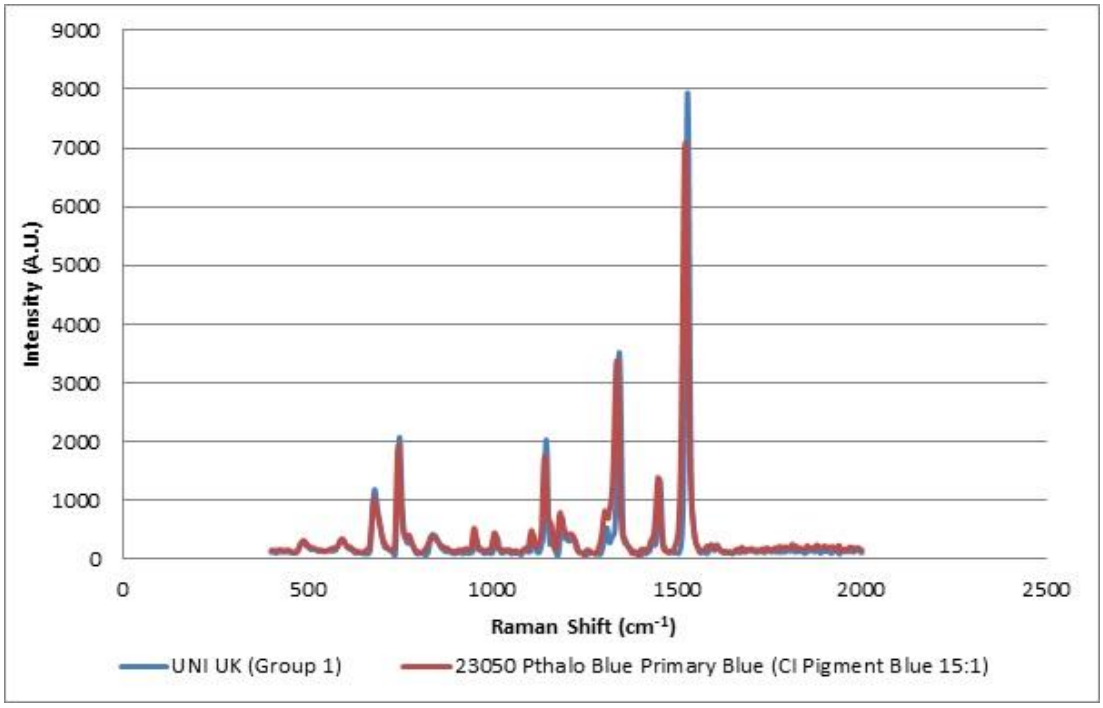


Figure 6.100: Blue Ink Group 1, suggested pigment identification of Phthalo Blue Primary Blue (CI Pigment Blue 15:1) (FORAM 785)

For Group 2, representative spectra were selected to account for the possible sub-group classification of samples (BIC UK and ZBR UK). For Group 2a (BIC UK), five suggestions above the 70% match value were returned. The top suggestion, with a 99.4% match value was Fluorescent Pigment Blue, and was confirmed as a possible identification by visual comparison. Likewise, the Cadmium Green Light and Dark pigments were also high on the list along with two other green pigments, but all could be excluded. Just on or below the % match threshold value, three phthalocyanine blue pigments were listed. Of these, Phthalo

Blue Royal (23060 – CI Pigment Blue 15:3) was also found to be similar to that of the ink spectra and was therefore suggested as a second possible ID despite being just below the 70% threshold criteria (69.7% match). The other two phthalocyanine blue pigments could be excluded on the basis of differences in the shape of some of the peaks particularly around 1190 – 1200 cm^{-1} and ~1380 cm^{-1}. This highlights the similarity in spectral response between pigments of similar chemical composition, and again the importance of manual comparison to confirm pigment identifications. Figure 6.101 shows a comparison between the ink spectrum, against the two most likely pigment match spectra. For Group 2b (ZBR UK), eight suggested hits above the threshold were listed including both Cadmium Green Light and Dark pigments and Fluorescent Pigment Blue in the top three, as well as the three phthalocyanine blue pigments. All could be excluded by the absence of peaks at ~1390 cm^{-1} and ~1430 cm^{-1}, suggesting the pigment concerned was not contained within the database. This demonstrates the importance of a complete database for identification purposes.

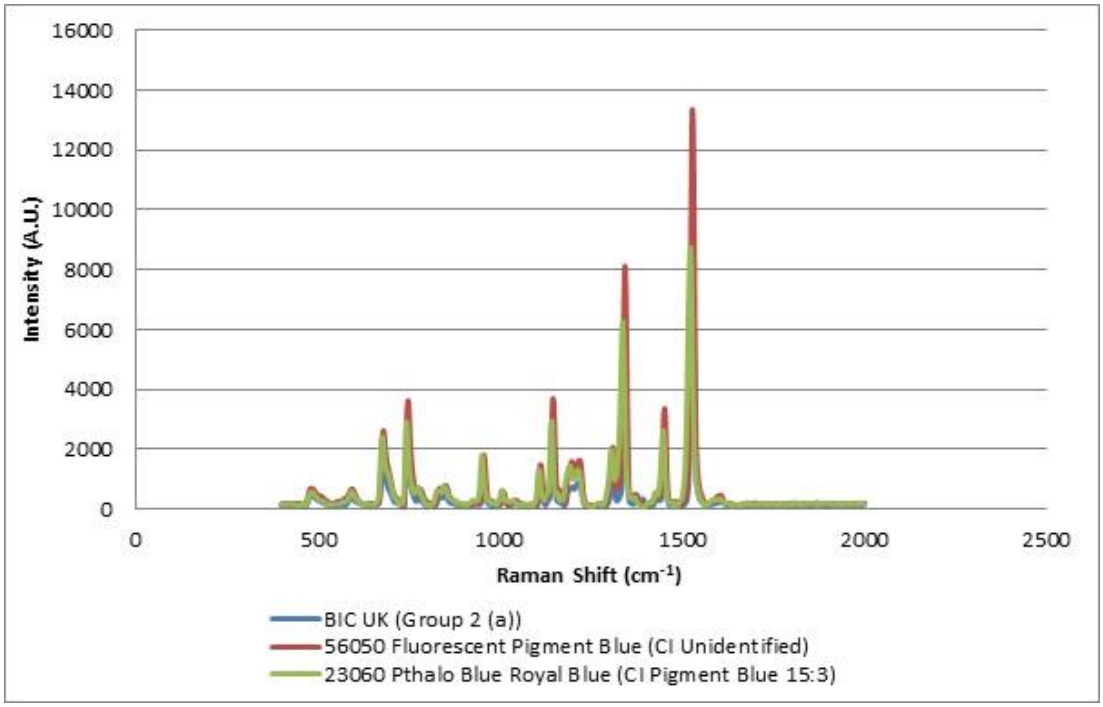


Figure 6.101: Blue Ink Group 2, suggested pigment identifications of Phthalo Blue Royal (CI Pigment Blue 15:3) and Fluorescent Pigment Blue (CI Unidentified) (FORAM 785)

Both Pigment Blue 15:1 and Pigment Blue 15:3 are bright blue copper phthalocyanine pigments known to be used for writing inks, with the latter notably greener and brighter than the former and used to provide maximum brightness in cyan blue ink [38]. These identifications are consistent with those suggested in the literature [13, 27]. No

information other than its known use in art and restoration was found for Fluorescent Pigment Blue, and its identification as a pigment known to be used in blue gel ink has not been acknowledged before.

6.3.2.4.2 Red Ink Group

The red ink group was discriminated into ten groups by the FORAM 785, of which only two provided a hit against the pigment spectral library. Group 4, containing the PTN UK and STD UK samples, provided a 94.6% match against XSL Poppy Red (26308 – CI Pigment Red 112) and are shown in Figure 6.102. Group 9, containing the UNI JAP sample, provided a 97.4% match against Irgazine Red DPP BO (23180 – CI Pigment Red 254) and is shown in Figure 6.103. Both hits were the first suggestion amongst several listed. Furthermore, on manual inspection a peak at ~1094 cm^{-1} seen in the ink spectrum, but not in that of the pigment, could be attributed to the paper substrate. This is illustrated in Figure 6.102. Excluding this peak, both pigment matches provided highly likely identifications of the pigment components within the gel ink. Once again, this highlights the importance of manual comparison to confirm identifications made by a computer library.

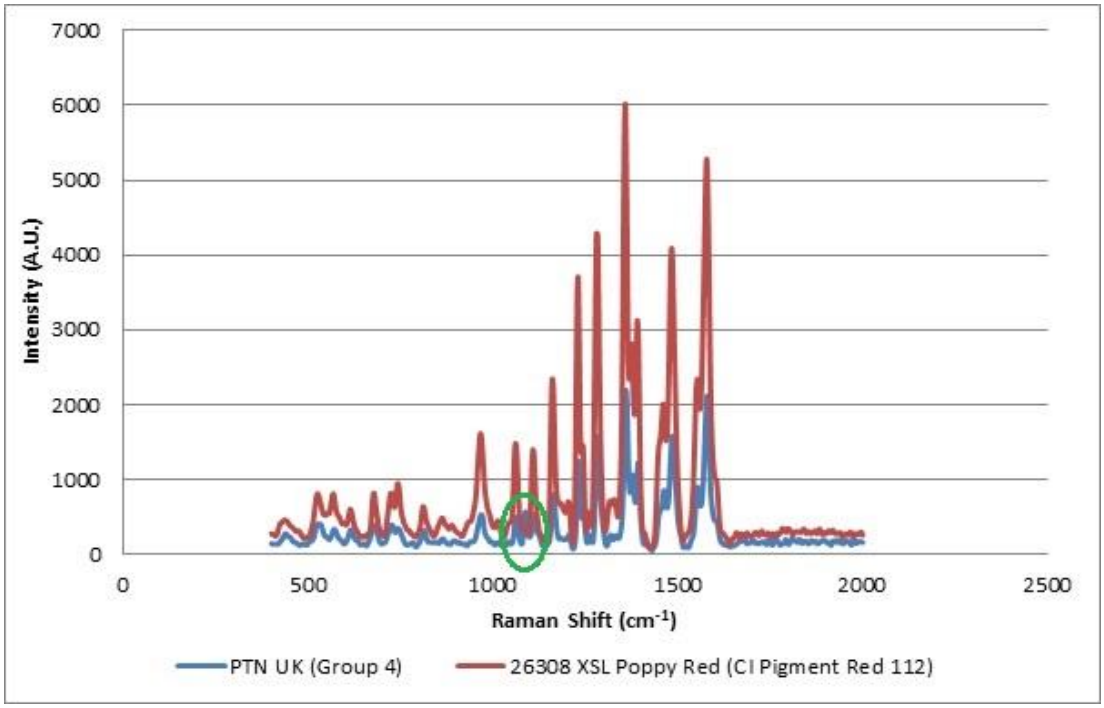


Figure 6.102: Red Ink Group 4, suggested pigment identification of XSL Poppy Red (CI Pigment Red 112) (FORAM 785). A peak at ~1094 cm^{-1} attributable to the paper substrate and observed in the ink spectrum, but not the pigment spectrum is highlighted within the green circle.

Pigment Red 112 is described as a Monoazo group pigment and is known to be used for inks amongst other applications [38]. Other than known to be used in art and restoration, no further information concerning Pigment Red 254 was available. The findings from the TLC, FTIR-ATR and Renishaw Raman Spectroscopy analysis of the UNI JAP sample confirmed that it was distinguishable from the other Uniball brand/model combinations studied and was one of only two red samples to contain a pigment component only.

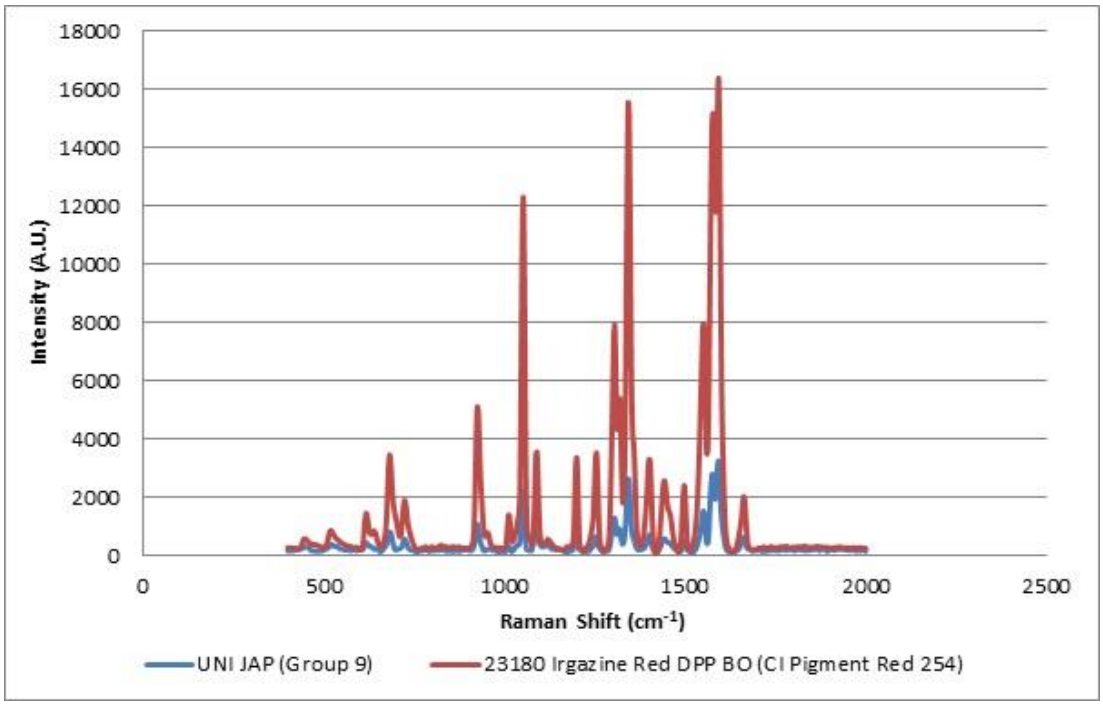


Figure 6.103: Red Ink Group 9, suggested pigment identification of Irgazine Red DPP BO (CI Pigment Red 254) (FORAM 785). NB Auto-scaling of the two sample spectra on the same axis gives a false impression of spectral differences, when spectra compared side by side a high level of similarity is observed

6.3.3 Conclusion: Within and Between Brand Variation

6.3.3.1 Within Brand Variation

6.3.3.1.1 Blue Ink Group

Some evidence of within brand variation was observed in two brand/model combinations, perhaps suggestive of an additional and/or different colorant component present in the formulation, although for one brand/model combination a change in additives could not be excluded. Interestingly, this within brand variation only appeared to be detectable at higher excitation wavelengths (785 nm and 830 nm).

6.3.3.1.2 Red Ink Group

Within brand variation was detected in only one brand/model combination (WHS UK) where clearly a change in formulation with respect to the colorant composition at least had occurred at some point during the manufacturing process.

6.3.3.2 Between Brand Variation

6.3.3.2.1 Blue Ink Group

For 514.5 nm, 685 nm and 785 nm, spectral reproducibility and S:N ratio was generally good. For the 830 nm excitation wavelength however, reproducibility was problematic affected by stray peaks, fluorescence, detector overload and/or excessively high spectral intensity. Spectral interference from the paper substrate appeared minimal at lower excitation wavelengths (514.5 nm and 685 nm), but slightly more problematic at higher excitation wavelengths. Specifically a small peak at ~1094 cm^{-1} was frequently observed in the ink spectra, along with others seen less frequently. At 830 nm, background fluorescence also appeared to be influenced by the substrate. These issues should be considered when comparing and/or identifying blue gel inks by Raman Spectroscopy.

Stray peaks were also an issue at higher wavelengths, in particular with the Renishaw InVia. Performing spectral acquisition in low ambient light alleviated this problem to an extent, but did not eliminate it completely. The "zap" function in the Renishaw WIRE2.0/3.0™ software was valuable in removing stray peaks; a feature the FORAM software would benefit from. Where stray peaks do occur however, repeating spectral acquisition is preferred, although not always practical in this study due to restricted instrument access.

The blue ink group samples were discriminated on the basis of presence or absence of certain Raman peaks and relative peak intensities into 4 – 6 groups dependent upon excitation wavelength. The 514.5 nm and 830 nm wavelengths offered the greatest discrimination, with samples grouped into six different groups respectively, whilst both 685 nm and 785 nm enabled discrimination of samples into the same four groups. Irrespective of wavelength, groups represented either pigment based or dye containing samples. The 514.5 nm wavelength was particularly useful for discrimination of pigment based blue gel inks. It was evident however, discrimination based on spectral information from all four wavelengths studied in depth proved the most informative.

Several samples confirmed as dye containing by solubility testing, may also contain a pigment component, indicative of a hybrid gel ink formulation since they only exhibited a Raman signal under certain wavelengths. Furthermore, discrimination of several pigment based samples required spectral information from at least two excitation wavelengths to provide confirmation of a real chemical difference, i.e. 514.5 nm and 830 nm. A tentative comparison between spectra recorded under 685 nm and 632.5 nm suggested one wavelength may be more informative for a particular brand/model combination than the other. All of these findings promote the value of a multi-wavelength system.

There was evidence to suggest, for some pigment based samples within a group at 785 nm, further discrimination based on reproducible differences in background fluorescence shape may be possible, but should be treated with caution. In other samples, differences in background fluorescence shape within a set of spectra from the same brand/model combination were observed. Furthermore, when baseline corrected, an indistinguishable spectral pattern for all samples within a group, irrespective of background fluorescence shape, was revealed. Since the samples which exhibited these differences were identified as insoluble it seems unlikely they are attributable to a dye component, indicating a non-colorant component may be responsible for the fluorescence effect.

With respect to formulation differences, there was evidence to suggest that some manufacturers, i.e. Uniball, use the same colorant for a range of models within the brand across the globe, whilst for others, i.e. Pentel, different colorants are used for different models in different geographical locations. There was also evidence suggesting that some manufacturers, i.e. Staples, may use different colorants for the same model sold in different geographical locations, whilst others may not, i.e. Pilot. With the Staples Sonix brand/model combinations for example, a pigment colorant only is used in the UK model, but in the US version dyes appear to be the primary colorant, with one exception where a combination of both types appear to have been used. Subtle variations in colour and additive composition are not detectable by Raman Spectroscopy alone.

6.3.3.2.2 Red Ink Group

The 785 nm excitation wavelength was particularly suited to the analysis and discrimination of red gel inks. In general reproducibility was considered good, but fluorescence weakened

the strength of the Raman signal for many samples. Despite this, spectra suitable for comparison and discrimination were acquired from all 25 brand/model combinations analysed. Similar observations to those seen in the blue ink group with regards to spectral interference from the paper substrate and stray peaks were observed. Discrimination into ten groups was possible, and together with the findings from solubility testing suggested 92% of brand/model combinations analysed, were likely hybrid gel ink formulations.

6.3.3.3 Comparison of Instruments

6.3.3.3.1 Blue Ink Group

In terms of comparison between instruments at the 785 nm excitation wavelength, the overall signal intensity was far greater in the Renishaw spectra than the FORAM 785 spectra. It was considered likely this was due to a more powerful laser source used in the Renishaw instrument. However, this created an issue with sample burning not seen with the FORAM. Furthermore, the Renishaw system was more prone to detector overload than the FORAM. Operating the FORAM in Auto Exposure mode generally enhanced signal intensity and S:N ratio without loss of spectral detail. Spectra for the same brand/model combination were considered indistinguishable between the two instruments. The greater scan range of the Renishaw InVia provided no further spectral information over the FORAM, with all Raman peaks occurring below 2000 cm^{-1} at higher excitation wavelengths. However, Raman peaks above 2000 cm^{-1} were observed in the 514.5 nm spectra, and thus scan range remains an important consideration for preventing loss of spectral information.

6.3.3.3.2 Red Ink Group

In terms of spectral pattern, reproducibility was considered good for both instruments although in the Renishaw InVia spectra signal intensity was found to vary markedly within a brand/model combination set. Fluorescence background was particularly high at the 785 nm excitation wavelength and colour group, but was more of a problem for the FORAM where the Raman signal was weaker in comparison to the Renishaw InVia. Even so, a Raman signal from all 25 brand/model combinations could be detected on top of the fluorescence background for both instruments. Similar observations to those seen in the blue ink group with regards to spectral interference arising from the paper substrate and stray peaks were also observed in the red ink group. Also similar to the blue ink group, the use of Auto Exposure mode enhanced signal intensity in the FORAM 785 spectra. Poor S:N

ratio exhibited in the FORAM spectra, meant it wasn't always possible to adequately compare spectra from the same brand/model combination acquired using both instruments, but when it was, spectra were considered indistinguishable. Brand/model combinations were discriminated into the same spectral groups irrespective of instrument.

6.3.3.4 Pigment Identification

6.3.3.4.1 Blue Ink Group

Comparison of spectra acquired using the FORAM 785 to an in-house pigment spectral library identified possible pigment components for two pigment based groups. Phthalocyanine pigments (Pigment Blue 15:1 and 15:3) were identified as the most likely components in 86% of the pigment based inks, although a fluorescent blue pigment could not be excluded as the possible component of the Partners Broad (PTN UK), Staedtler Triplus (STD UK), BIC Reaction (BIC UK) and BIC Velocity (BIC US) pigmented blue inks.

6.3.3.4.2 Red Ink Group

The pigment component for three brand/model combinations was identified. XSL Poppy Red (Pigment Red 112) appears likely to have been used in the manufacture of the Partners Broad (PTN UK) and Staedtler Triplus (STD UK) red gel inks, whilst for the Uniball Signo RT red gel ink Irgazine Red DPP BO (Pigment Red 254) was a likely colour component..

6.3.3.5 Multivariate Profiling

The spectral data sets representing between brand variation studies were subjected to multivariate profiling. For the blue ink group, these were those representing ink samples on paper acquired at all four excitation wavelengths: 514.5 nm; 685 nm; 785 nm and 830 nm, whilst for the red ink group, spectral data sets from both instrument systems acquired under 785 nm only were interrogated. Clustering of samples by Principal Component Analysis (PCA), Hierarchical Cluster Analysis (HCA) and Self-Organising Feature Maps (SOFM) were all explored and are described in **Chapter Seven** together with the multivariate profiling of the FTIR-ATR spectral data.

Chapter Seven

Discriminating Power and Multivariate Analysis

7.1 Introduction

7.1.1 Discriminating Power

In 1973, Smalldon and Moffat introduced the concept of Discriminating Power (DP) to the forensic community providing a probability of discriminating two samples selected at random from the population of interest [154]. The discriminating power has been used to describe the ability of certain analytical techniques to discriminate a wide range of samples of forensic interest including trace materials, i.e. glass [155], fibres [134], paint [156, 157], soil [158], paper [159] and writing inks [15].

Smalldon and Moffat originally described discriminating power in equation 7.1 [154]:

$$DP_k = \frac{1-2M}{[N(N-1)]} \qquad \text{Equation 7.1}$$

Where DP_k = discriminating power, M = total number of matching pairs, and N = total number of samples. It has since been simplified by some researchers [15] into Equation 7.2:

$$DP = \frac{Number\ of\ discriminated\ sample\ pairs}{Number\ of\ possible\ pairs} \qquad \text{Equation 7.2}$$

7.1.2 Multivariate Analysis

Spectroscopic methods consist of a suite of modern analytical techniques used in forensic investigations that generate data of high dimensionality. Vis-MSP, FTIR-ATR and Raman Spectroscopy yield spectra composed of a wide array of spectral intensities at several hundred wavelengths or wavenumber positions. Traditionally a forensic scientist would examine and compare spectra by eye to identify features for discrimination of two or more samples. This qualitative approach to pattern recognition relies heavily on an individual examiners experience and as such is highly subjective making it open to conflicting

interpretation. Whilst obvious similarities or differences such as the presence or absence of certain peaks or relative intensities may be easy to detect, more subtle differences may be missed if obscured by background noise or fluorescence, or if large numbers of samples are being compared. Advances in computation and availability of modern statistical software packages incorporating multivariate Chemometric capabilities make it viable for such data to be interrogated with relative ease in order to identify objectively, patterns or trends that have potential to increase the discriminating power of an analytical technique.

Kher *et al* [82] appear to have been the first to apply Chemometrics to the discrimination of writing inks, using Principal Component Analysis (PCA) to classify eight pens each of blue and black ballpoint ink analysed by High Performance Liquid Chromatography (HPLC) with Photodiode Array Detection. The statistical based classifications were compared to those made using classification flow charts based on the presence or absence of specific peaks at certain wavelengths. Discrimination for the black inks was based on both peak area and retention time at a single wavelength, whilst better overall discrimination for the blue inks was achieved simultaneously using data from four selected wavelengths with cross-validated PCA and visual pattern matching. A normalisation pre-processing step was required for the black inks data matrix, whilst Mean Centre and Standardisation (subtraction of the mean followed by division using the standard deviation) was performed on the blue ink data matrix to allow a comparison within each of the four wavelengths. Using PCA alone resulted in fewer number of classification groups for both ink sets compared to classification by the flow charts demonstrating that where different manufacturers have used highly similar ink formulations, i.e. from the same source, PCA could not distinguish between them. Use of the classifier flow charts however, in contrast to PCA, could not be used to distinguish between pens from the same manufacturer. The potential for an ink classification database using this combined analytical and Chemometric approach was put forward.

Thanasoulias *et al* [208] used a multivariate approach incorporating Cluster Analysis (CA), PCA and Discriminant Analysis (DA) for the full discrimination of 50 blue ballpoint inks analysed by Vis-Spectroscopy (400 – 750 nm). Differences in peak shape and relative intensities observed in the spectra from ethanolic extracts of the ink on paper and the pen itself were used as the basis for discrimination. The approach avoided common multi-

colinearity difficulties associated with broad absorption bands by systematically reducing the number of variables to consider for discrimination. Although 50 pens were studied, these represented only five brands which could easily be discriminated by eye on the presence or absence of a second peak into two groups. As such the authors suggested further work should be conducted on a larger sample set, within brand variability, and reflectance spectral data acquired from ink on paper to improve objectivity.

Kher *et al* [83] investigated the application of Self Independent Modelling of Class Analogies (SIMCA) and Linear Discriminant Analysis (LDA) to HPLC and FTIR-ATR data from eight different varieties of blue ballpoint inks. Better discrimination was achieved using HPLC (~0.98 by LDA) as opposed to FTIR-ATR in combination with Chemometrics (0.63 by LDA). Data pre-treatment steps in the form of data reduction by PCA using the Covariance method and normalisation was required for the IR data (2000 – 650 cm^{-1}) only to reduce the number of variables for discrimination and the influence of line thickness respectively. The application of LDA in combination with PCA for the IR data resulted in some pens correctly classified, but others incompletely resolved and some misclassified. Despite these limitations, Kher *et al* [83] suggested the application of Chemometrics gave better discrimination over highly difficult visual pattern recognition. It was suggested that improvements in spectral quality may enhance the method further to provide a valuable tool for the discrimination of writing inks. A second approach to data reduction was also explored by selection of certain discrete wavenumber regions where maximum variability attributable to the ink was observed (i.e. 800 – 680 cm^{-1} and 1700 – 1500 cm^{-1}), but this resulted in a poorer level of discrimination compared to the PCA approach.

Adam *et al* [209] further explored PCA to discriminate 25 black ballpoint inks by the UV-Vis spectra of pure ink and ink extracted from paper. Although the full scan range was 200 – 800 nm, only the region 300 – 700 nm was statistically interrogated since features for discrimination outside of this were considered either unreliable (200 – 300 nm) or non-existent (700 – 800 nm). Most of the sample variance (99.2%) was accounted for by the first three Principal Components (PC's), and a loading plot of PC 3 against PC 1 was sufficient to visually discriminate the samples into five clusters. This was confirmed by TLC analysis, and further individualisation of pens within a group was considered possible. The use of a two-step PCA approach in combination with a spectral database for ink

identification and classification to overcome computational limitations of such a database was discussed. Again, the application of a Chemometric approach to reflectance spectra from ink on paper was highlighted for investigation, especially since in this study the extraction process was found to influence the spectral data enough to discriminate the extracted and corresponding pure ink samples.

Indeed, Adam [210] further investigated a Chemometric approach to the discrimination of black ballpoint inks on paper using Luminescence Spectroscopy in combination with PCA and Linear Regression. PCA was used for data reduction in order to produce linear regression lines where gradient differences represented differences in the luminescence intensities of two inks. Further pairwise comparison by the t-test was then performed to determine if two inks were statistically indistinguishable. Discriminating power was found to be lower in handwritten samples (0.64 with 95% confidence interval) compared to calibrated samples (0.87 with 95% confidence interval), attributable to poor spectral quality and increased sample inhomogeneity in the former. Nevertheless, the ten pen ink samples studied were all considered indistinguishable using traditional FLE highlighting the potential of this approach for improved discrimination of ballpoint inks.

In a study by Denman *et al* [211], both the organic and inorganic content of 24 blue ballpoint pen inks on paper were analysed by Time of Flight Secondary Ion Mass Spectrometry (ToF-SIMS) in combination with PCA. A selection of peaks for each mass spectrum were integrated and normalised with respect to the secondary ion intensity and an average spectrum from a set of ten for each pen sample was produced giving a correlation matrix which was then subjected to PCA analysis. The results were compared to classification of the mass spectra by visual pattern recognition. In the latter, 38 of 45 possible pairs were distinguishable whilst in the former, 41 pairs were discriminated, highlighting the advantage of a multivariate approach for discrimination. A score plot of the first two PC's was sufficient for visual discrimination. Denman *et al* [211] commented that PCA was more successful because it would have considered subtle intensity differences in small peaks that may have been overlooked by visual comparison thus increasing confidence in the reliability of the discrimination. It was noted in this study that within a brand, different models of pen use highly similar ink in terms of their organic dye composition, but that subtle variations in the inorganic content arising from additives

appear to provide an additional feature of discrimination. Furthermore, the same formulations between brands also appear to be used suggesting that different manufacturers use the same source of ink for their products.

Recently the techniques of Laser Desorption Mass Spectrometry (LDMS) and Matrix Assisted Laser Desorption Ionisation – Mass Spectrometry (MALDI-MS) have been highlighted as techniques suitable for the detection and identification of pigments and dyes respectively in gel ink pens [101, 104]. Both techniques use UV radiation of a single wavelength (i.e. 337 nm) delivered by a pulsed nitrogen laser to cause desorption/ionization of the colorant molecules in a sample by absorption. For LDMS, a small portion of the ink sample on paper can be removed from the document and placed directly in the path of the laser beam [104]. For MALDI-MS, samples of the ink on paper are coated in small quantities of a MALDI matrix solution containing additives [104]. A sufficient quantity of dye is extracted out into the MALDI matrix, and once dried form a layer of MALDI target crystals on the surface of the paper which can be analysed by LDMS to enhance detection of a dye analyte [104].

Weyermann *et al* [95] used LDI-MS to analyse 33 blue gel inks on paper, describing a statistical methodology based on Pearson Correlation Co-efficient and Euclidean Distance algorithms for their discrimination. The variables chosen for discrimination were selected relative peak areas and subjected to PCA using the Correlation method. Statistical evaluation of both peak dense regions in the positive and negative mode mass spectra as well as the entire spectrum were conducted. Prior to applying Euclidean Distance, normalisation of the variables was carried out to reduce the influence of scaling variations. This data pre-processing step was not required before conducting Pearson Correlation. However, other pre-processing steps in the form of Square Root and Standardisation were applied. Overall, Pearson Correlation Co-efficient was found to perform better than the Euclidean Distance algorithm. For positive mode mass spectral data, Standardisation as a data pre-treatment method yielded better discrimination in combination with Pearson Correlation, whilst Square Root was more suited for the negative mode data. The best discrimination, 0.92, was achieved using the entire spectral range in negative mode and subjecting it to Standardisation followed by Pearson Correlation. However, good discrimination was also achieved using a reduced data set. Based on variables from a

selected spectral range, a discriminating power of 0.77 in negative mode and 0.69 in positive mode were achieved, the latter similar to that for Raman analysis of the same sample set analysed in a previous study. Weyermann *et al* [95] highlighted the importance of the choice of variables for achieving a good level of discrimination suggesting that reproducibility and high sample variation were a necessity. The same statistical methodology of Standardisation followed by Pearson Correlation was applied to 30 black gel inks in a subsequent study by Weyermann *et al* [96]. When the entire sample set were considered, a combination of both positive and negative mode mass spectral data yielded a discriminating power of 0.85. When a small number of dye containing samples were excluded, a discriminating power of 0.82 was achieved. This showed great improvement over the discriminating power of traditional techniques such as VSC (0.49) and MSP (0.74) conducted on the same sample set in a previous study.

Neumann and Margot presented a series of three papers describing a "generic methodology to provide ink identification and comparison with better objectivity, reliability, efficiency and to maximise the information obtained" from analysis of ballpoint inks performed using High Performance Thin Layer Chromatography (HPTLC) [75-77]. The second paper in the series was concerned with the development and comparison of the performance of six algorithms for the discrimination of inks under a variety of conditions [76]. The algorithms were based on the popular Euclidean Distance and Pearson Correlation Co-efficient, as well as the lesser known concept of Artificial Neural Networks (ANNs). It was concluded that certain algorithms perform best under certain conditions, but that overall the ANN algorithms were superior since they could be specifically trained for the task at hand. The training procedure, as well as the data set used, was highlighted as two "critical" considerations for developing a suitable ANN algorithm. Since the application of ANNs to the multivariate analysis of forensic samples is a relatively new, but promising concept [161, 162], further development and optimisation of such algorithms for particular tasks relating to ink discrimination were recommended.

7.2 Experimental

7.2.1 Discriminating Power

To assist in the calculation of Discriminating Power (DP) for a given set of conditions, analytical technique and/or colour group, pairwise discrimination tables were constructed. The complete set of samples for a given colour group were listed in sequence along the first row and down the first column of each table. If two inks could be discriminated by a particular condition/analytical technique, then a "Y" for "Yes" was inserted in the relevant square. If not, then an "N" for "NO" was inserted. The maximum number of possible pairs was determined, and the discriminating power calculated. Combined or overall discrimination powers for a given set of conditions were calculated by adding together the total number of discriminated and maximum possible pairs. For example, the four selected camera filters in IRR mode used to analyse the blue ink group yielded four individual DP's based on 465 possible pairs each. The calculation of the combined DP for IRR mode was therefore based on adding 465 + 465 + 465 + 465 to give the total number of possible pairs, and adding together the number of discriminated pairs for each of the four filters. Where classifications were made using original and first derivative spectra, i.e. Vis-MSP, the combined DP for the two sets of spectral data has been based on the overall ability to discriminate the samples using the two methods in combination, i.e. 465 possible pairs total rather than 2 x 465 possible pairs based on individual original and first derivative classifications. Likewise, when an overall DP for a combined analytical sequence has been quoted, this has been based on the overall ability to discriminate samples within a given colour group taking into consideration the data for all relevant techniques.

7.2.2 Multivariate Analysis

A statistical methodology incorporating PCA, HCA and SOFM for the classification and discrimination of gel ink on paper was developed and applied to both the Raman and FTIR-ATR data. This methodology was based on combined data reduction, binning and normalisation pre-processing steps similar to those described in Tan *et al* [212] and Burger *et al* [213]. All pre-processing steps were performed in Microsoft Excel 2007 or 2010, whilst PCA and HCA analysis was performed in Minitab 15 or 16, and SOFM analysis performed in Viscovery SOMINE. It is worth noting that although Excel 2007 or 2010 was used, to be able to analyse data matrices in Viscovery SOMINE, they had to be saved in Excel 1997 – 2003

compatibility mode. To date, attempts to develop a statistical methodology for the Vis-MSP data have been unsuccessful and would benefit from further investigation.

7.2.2.1 Raman Spectroscopy

7.2.2.1.1 Data Preparation

To avoid overcrowding of graphical outputs hindering discrimination and since reproducibility of Raman spectra was generally considered good; data matrices were prepared from a single set of measurements representing each ink sample. To reduce the data set further, only data from identifiable peaks within a spectrum representing each individual sample was considered. Non-baseline corrected data was used, since early development work suggested no additional benefit to using baseline corrected data. Intensity values at fixed wavenumber positions (i.e. peak maximum) were highlighted and all other data discarded. It is worth noting that only those intensity values identified at wavenumber positions within the group to which a given sample belonged were included for that sample.

Tan *et al* [212] and Burger *et al* [213] describe a pre-processing methodology where data output from GCMS and Electropherogram was reduced by dividing the profile into a series of data regions called BINS. The signal output, i.e. peak area, within each BIN is summed to give a total BIN value, which is then subsequently normalised with respect to the highest BIN value to account for instrumental variations. It was decided to adopt a similar approach to reduce the Raman Spectroscopy data matrices further.

Data binning was undertaken by dividing the entire scan range of the Raman Spectrum into a series of data regions or data BINS. The scan range for FORAM was 400 – 2000 cm^{-1} while that for the Renishaw InVia Spectrometer was wider at 100 – 3200 cm^{-1}. Data BINS were generated for each data set from both instruments so that each BIN represented a 100 cm^{-1} increment. This meant that more data BINS were generated for the Renishaw data than the FORAM data. For each individual sample, the intensity values corresponding to each selected fixed wavenumber position falling within a given BIN region were summed, giving a total value for each individual BIN. BINS which contained no data were excluded from the final data matrix due to software limitations. Finally, the BIN values for each sample were normalised with respect to the highest BIN value for that sample.

Data matrices were prepared for all relevant colour group and excitation wavelength combinations. For the blue ink group, data matrices were prepared for the four key excitation wavelengths studied: 514.5 nm, 685 nm, 785 nm and 830 nm. For the red ink group, data matrices were prepared for the 785 nm excitation wavelength only. For both the blue and red ink groups at 785 nm, data matrices were prepared from both the FORAM and Renishaw InVia spectra.

7.2.2.1.2 Statistical Methods

7.2.2.1.2.1 Principal Component Analysis (PCA)

PCA was performed according to the Correlation method and clustering identified by eye from a score plot. Clusters were highlighted manually by drawing colour coded circles around groups of samples with similar attributes.

7.2.2.1.2.2 Hierarchical Cluster Analysis (HCA)

HCA was performed using Single Linkage and Euclidean Distance algorithms since they are amongst the most common algorithms chosen for HCA and have been shown to provide effective clustering of other types of forensic samples [163, 164] The influence of standardising the variables on the HCA was also investigated. In addition, the number of expected clusters was also inputted into the software for it to be able to assign the samples to a particular cluster identified by colour coding.

7.2.2.1.2.3 Artificial Neural Network (ANN) Analysis

The data matrix was imported into the Viscovery SOMINE software to generate an SOFM and again the number of expected groups was inputted into the software.

Data sets were interrogated by all three aforementioned statistical treatments to enable evaluation of the associated graphical outputs for correct clustering of samples.

7.2.2.2 FTIR-ATR

7.2.2.2.1 Data Preparation

A similar profiling methodology based on data reduction, binning and normalisation of selected peak absorbencies was applied to the FTIR-ATR data sets for each colour group.

The selected peaks were those identified in Tables 5.3 and 5.4 for each colour group shown in **Chapter Five**, as they were considered attributable to the ink only.

7.2.2.2.1.1 Blue Ink Group

Visual pattern recognition of the blue ink group samples resulted in four Tier levels of discrimination (T1 - T4) based on the presence or absence of peak absorbencies and relative peak intensities (RPI).

A data matrix was prepared where all discriminating peaks identified across all four tiers for the samples within a given group were included. For example, in T1 the PLT samples occupy Group 3 along with eight other dye containing samples. However, in T4, the PLT samples can be discriminated visually from the other samples by the presence of a small peak absorbency around 782 cm^{-1}. The data matrix was thus prepared so that for all ten samples within Group 1 at T1 the absorbance value at 782 cm^{-1} for all ten samples was included, even though this was a discriminating peak for the PLT samples only.

Since the peaks of interest only occurred in the wavenumber region 1450 - 650 cm^{-1}, only this region was selected for binning. From this initial data matrix, three further data matrices comprised of 8 BINS (100 cm^{-1} increment), 16 BINS (50 cm^{-1} increment) and 32 BINS (25 cm^{-1} increment) were created to investigate if increasing the number of BINS, thus improving the sensitivity of the method, would have an effect on clustering. The data matrices were normalised with respect to the BINS with the highest value for a given sample.

Other data matrices reflecting only the T2, T3 and T4 classification groupings were prepared. These were prepared by incorporating the absorbance values corresponding to all identified discriminating peaks for a given sample, but for that sample only, irrespective of the group it belonged to in T1. For example, in preparation of the T4 data matrix: the PLT samples were discriminated from all other dye containing samples in T4 by a small peak at around 782 cm^{-1}, therefore, the absorbance value corresponding to this peak position for the two PLT samples only was included in addition to all other peak positions and corresponding absorbance values used to discriminate the PLT samples at other tiers. The data matrices were prepared in a 32 BIN format, and normalised.

A 32 BIN data matrix was prepared from the first derivative data set in a similar fashion. However, the methodology differed slightly in that an additional pre-processing step was introduced to account for some negative values associated with the first derivative data. These negative values yielded some negative total BIN values. To achieve normalisation of these negative BIN values, all BIN values had to be squared to eliminate the negative sign.

7.2.2.2.1.2 Red Ink Group

The red ink group was discriminated into two Tier levels (T1 and T2) based on the original spectra. T1 discrimination was based solely on the presence or absence of peak absorbencies, whilst T2 was based solely on differences in RPI.

Peaks attributable to ink for the red ink group occurred in the region 1750 - 650 cm^{-1}, so only this region was included in the binning procedure. A data matrix was prepared from the characteristic peaks representing T1, divided into 44 BINS (25 cm^{-1} increment), and normalised with respect to the BIN with the highest value for a given sample. Since T2 was based solely on RPI differences in the peaks that represented T1, the same data matrix was used to assess the ability of the statistical treatments to classify the samples in line with T2 visual comparisons.

The first derivative spectral data was prepared in a 44 BIN format in a similar fashion to that described for the blue ink group.

All aforementioned FTIR-ATR data sets were interrogated by PCA, HCA and SOFM. With respect to HCA, an investigation of all possible algorithm combinations provided by the software, with or without Standardisation of variables, was also performed.

7.3 Results and Discussion

7.3.1 Discriminating Power

7.3.1.1 Thin Layer Chromatography (TLC)

To recap, the Solubility and TLC study revealed that 10 of 31 blue inks and 24 of 26 red inks contained a dye component. The Discriminating Power (DP) of TLC for the analysis of gel inks can be viewed in terms of either the complete sample set for a given colour group, or

just in terms of the sub-set containing a dye component only. When all samples are considered, the DP of the technique is at its highest for the blue ink group, 0.54. The red ink group yields a DP a little lower at 0.45. When non-soluble pigment based samples are taken into consideration, the overall DP of the technique was calculated to be 0.50. However, focussing on the dye containing samples only, significantly increases the DP for the blue ink (0.87) group, comparing well to other studies of blue dye component inks quoting similar values [114]. The red ink group yields a DP that is only a little lower at 0.36 than when all samples are considered. This is because of the low number of pigment only samples and the high number of samples with an indistinguishable dye component composition within this particular colour group. This approach has the effect of reducing the overall DP of the technique calculated to be 0.43. Unsurprisingly TLC was found to only be useful for the discrimination of gel inks containing a dye component.

7.3.1.2 VSC 6000/HS

Discussion of the DP for Filtered Light Examination (FLE) is quite complex because of the number of illumination type and camera or filter combinations to be considered for each individual colour group as well as for the technique as a whole. Furthermore, the inherent subjectivity of the technique makes interpretation of the results difficult. Mazella and Khammy-Vital [15] recognised this in their study of blue gel inks, and formulated an approach to interpretation of FLE results designed to give “maximum objectivity”. This approach excluded discriminations between pairs of inks based on intensity differences, but rather focussed on a simplified approach of whether or not the inks were absorbing or reflecting (appeared black or white) under IRR conditions, or luminescing (appear white), absorbing (appear black) or a combination of the two (appear similar to the background) under infrared luminescence (IRL) conditions. This objective approach reduces examiner subjectivity and the effect of external influences on interpretation of the results. It can however, result in some misclassifications which could negatively impact on the calculated DP. For example, under infrared reflection (IRR) conditions, two different brands of ink may both appear to be reflecting, but to very different intensities. Using the objective approach described, these two inks would then be classed as indistinguishable to one another when in fact they are clearly distinguishable either due to a sampling issue or because of a very real difference in their chemical formulation. This is where the experience of an examiner is essential to the interpretation of FLE’s, and should not be underestimated despite its

subjective nature. For this reason, it was decided to interpret pairwise comparisons of the FLE's in this study using both the objective and subjective approach. The two approaches are compared in terms of their DP under IRR, IRL and IRR/IRL conditions, both for individual and combined camera/optical filters per colour group and for the technique as a whole. These findings will be discussed in sequence of objective approach followed by subjective approach for each of the two colour groups.

7.3.1.2.1 Blue Ink Group

Using the objective approach, the four selected camera filters used in the IRR mode could be ordered in the following sequence from highest to lowest DP: 780 nm (0.26) > 695 nm (0.20) > 1000 nm (0.02) > 645 nm (0.00), giving an overall DP for IRR of 0.12. In contrast, the five optical filters used in the IRL mode could be ordered as: 380 – 800 nm (0.67) > 585 – 720 nm (0.59) > 485 – 610 nm and 545 – 675 nm (0.52) > 515 – 640 nm (0.51), giving an overall DP for IRL of 0.56. These results gave a combined IRR/IRL DP of 0.37 for the blue ink group when the objective approach was followed.

Following the subjective approach, the four camera filters used in IRR mode could be ordered as: 780 nm (0.72) > 695 nm (0.71) > 645 nm (0.64) > 1000 nm (0.24), giving an overall DP for IRR of 0.58, significantly higher than the corresponding value using the objective approach. It is interesting to note that using the objective approach the 645 nm camera filter yielded a 0.00 DP, yet when the subjective approach was used the DP was calculated to be 0.64, highlighting the drawback of the objective approach to interpretation. In IRL mode, the five optical filters could be ordered as: 380 – 800 nm and 515 – 640 nm (0.73) > 545 – 675 nm (0.69) > 485 – 610 nm (0.67) > 585 – 720 nm (0.63), giving an overall DP for IRL of 0.69, which although higher than the IRR DP, exhibits a narrower difference between the two than for the corresponding values using the objective approach. The combined IRR/IRL DP for the blue ink group following the subjective approach was calculated to be 0.64, nearly double that of the corresponding value using the objective approach. Surprisingly this DP is lower than the 0.72 quoted by Mazella and Khammy-Vital [15] in their FLE examination of 19 blue gel inks using a combination of IRR/IRL and an objective approach to interpretation.

7.3.1.2.2 Red Ink Group

For both the objective and subjective approaches, it was not considered possible to discriminate between any of the red ink group samples under IRR conditions using the four chosen camera filters, and as such the DP for both was calculated to be 0.00. However, discrimination was considered possible using both approaches under IRL conditions.

Following the objective approach, the four selected optical filters in IRL mode could be ordered as: 400 – 535 nm (0.60) > 445 – 570 nm (0.56) > 380 – 800 nm (0.55) > 400 – 485 nm (0.54), giving an overall IRL DP of 0.57. However, using the subjective approach, the optical filters could be ordered in the following sequence: 400 – 485 nm (0.75) > 400 – 535 nm and 380 – 800 nm (0.70) > 445 – 570 nm (0.65), giving an overall IRL DP of 0.70. This translates into a combined IRR/IRL DP for the red ink group of 0.35 following the subjective approach, compared to only a slightly lower 0.31 following the objective approach. Clearly, this demonstrates that IRL conditions alone are suitable for the discrimination of red gel inks, and that irrespective of the interpretational approach taken, a reasonable level of discrimination is possible.

When the results for both colour groups are considered together, the overall DP for the technique was calculated to be 0.34 following the objective approach compared to a higher 0.53 using the subjective approach. This demonstrates how the DP of an analytical technique maybe under-estimated (or over-estimated) depending on the way in which the data is interpreted. Clearly the VSC 6000/HS was most useful for discrimination of blue gel inks on paper, providing a moderate to moderately strong level of discrimination depending on interpretational approach used (IRR/IRL combined 0.37 - 0.64), with a moderate to limited discrimination afforded for the red (0.31 – 0.35) gel inks.

7.3.1.3 Vis-MSP

For the red ink group, no discrimination between samples was considered possible on the basis of visible spectra since the spectral pattern was so similar as to be deemed indistinguishable, and as such the DP was calculated to be 0.00. Since the original spectra for the red ink group exhibited few features for discrimination, it was not considered worthwhile investigating whether further differentiation was possible using the first derivative method. However, for the blue ink group, wide variability in spectral shape was

observed between samples, and therefore both the original and first derivative spectra were used as the basis for discrimination. Based on the original spectral classifications only, the DP was calculated to be 0.77, demonstrating a very strong level of discrimination amongst the blue ink group by Vis-MSP. When the first derivative was considered in combination with the original spectra, the DP was calculated to be even higher at 0.92, providing an extremely strong level of discrimination. Overall, the DP for the analytical technique was calculated to be between 0.45 – 0.54, comparable to that for the VSC 6000/HS. However, it is clear that the overall DP has been lowered as a result of relatively low discriminating powers for the red ink group. For blue gel inks on paper, using Vis-MSP in transmitted light mode, with or without use of the first derivative, offers a very satisfactory level of discrimination.

7.3.1.4 FTIR-ATR

Visual discrimination of the spectra into groups with similar spectral patterns based on peak presence or absence and relative peak intensities was found to be quite difficult. This was due to the level of confidence that could be attributed to peaks with very weak absorbencies as a feature of discrimination, and as a result, discrimination within a colour group was based on a tiered system. For example, classification within the blue ink group was based on four Tier levels (T1 – T4), where T1 and T2 were considered more reliable than T3 and T4 as the discrimination level was based on relatively strong peak absorbencies in the former. With this approach in mind, the blue ink group DP for T1 and T4 ranged between 0.65 – 0.93. T2 and T3 yielded DP's at 0.81 and 0.92 respectively. The red was discriminated on the basis of two tiers yielding DP's between 0.85 – 0.99. Clearly, when all peak differences are considered for discrimination, the DP for the FTIR-ATR technique is extremely strong. Even when only strong peak absorbencies are considered, the DP for the blue and red ink group is relatively strong. The overall DP for the analytical technique using only original spectra was calculated to be between 0.74 – 0.96 dependent upon tier level. When the first derivative is considered in combination with corresponding original spectra the DP was calculated to be 0.94 for the blue ink group and 0.99 for the red ink group, maintaining an overall DP for the technique at 0.96. Although there is no information concerning the DP of FTIR-ATR in relation to gel inks on paper described in the literature, Causin *et al* [114] investigated the application of FTIR for the analysis of blue and black ballpoint ink extracts quoting DP's of up to 95% for the former. In this study, discrimination

was based predominantly on a qualitative comparison of the presence or absence of peaks, with relative intensity only considered as a feature of discrimination when differences were large. These DP values compare well to those calculated for this study, and highlight the strong potential of FTIR-ATR for the discrimination of gel and other writing inks. It is worth noting however, that despite a generally strong overall agreement in the classification groupings when original or first derivative spectra were considered in isolation, some differences were noted. This again raises questions as to the validity of using minor peak absorbencies as a feature of discrimination, but also the definition of what constitutes a small peak absorbency. It also strengthens the need for a more objective approach to discrimination of FTIR-ATR spectra.

7.3.1.5 Raman Spectroscopy

Unlike its complimentary technique FTIR-ATR, discriminating the Raman spectra into classification groupings for each colour group by visual pattern recognition was less problematic. For the four key excitation wavelengths applied to the blue ink group, they could be ordered from highest to lowest DP as follows: 514.5 nm (0.79) > 830 nm (0.72) > 785 nm (0.71) > 685 nm (0.68). When the results from all four wavelengths are combined, the overall DP of the technique for the blue ink group was calculated to be 0.73. These values compare well to the values of 0.76 and 0.68 quoted in previous studies by Mazella *et al* [15, 27] for the analysis of blue gel inks on paper using a combination of 514.4 nm and 830 nm excitation wavelengths. Interestingly the DP for the two excitation wavelengths combined for this study was calculated to be notably higher than in the previous studies, at 0.87.

Discrimination within the red ink group was found to only be possible using the 785 nm excitation wavelength, but despite this also gave a very strong DP of 0.78, irrespective of instrument used. These values suggest that Raman Spectroscopy offers a good level of discrimination for red and blue gel inks on paper. With this in mind, the overall DP for the analytical technique was calculated to be between 0.72 - 0.73.

Table 7.1 summarises the discriminating powers associated with each analytical technique investigated, both by individual colour group and in combination to give an overall discriminating power for each technique.

VSC 6000/HS												
Colour	**Lighting Type**	**Objective Approach**					**Subjective Approach**					**Maximum No. of Pairs**
		Camera or Optical Filter (nm)	**DP**	**Combined DP**	**Combined IRR & IRL DP**	**Overall DP**	**Camera or Optical Filter (nm)**	**DP**	**Combined DP**	**Combined IRR & IRL DP**	**Overall DP**	
Blue	IRR	645	0.00	0.12	0.37	0.34	645	0.64	0.58	0.64	0.53	435
		695	0.20				695	0.71				
		780	**0.26**				**780**	**0.72**				
		1000	0.02				1000	0.24				
	IRL	**380 – 800**	**0.67**	0.56			**380 – 800**	**0.73**	0.69			
		485 – 610	0.52				485 – 610	0.67				
		515 – 640	0.51				**515 – 640**	**0.73**				
		545 – 675	0.52				545 – 675	0.69				
		585 – 720	0.59				585 – 720	0.63				
Red	IRR	530	0.00	0.00	0.31		530	0.00	0.00	0.35		300
		610	0.00				610	0.00				
		695	0.00				695	0.00				
		1000	0.00				1000	0.00				
	IRL	380 – 800	0.55	0.57			380 – 800	0.70	0.70			
		400 – 485	0.54				**400 – 485**	**0.75**				
		400 – 535	**0.60**				400 – 535	0.70				
		445 – 570	0.56				445 – 570	0.65				

TLC						
Colour	**DP (all samples)**	**Overall DP**	**Maximum No. of Pairs**	**DP (dye only)**	**Overall DP**	**Maximum No. of Pairs**
Blue	0.54	0.50	465	0.87	0.43	45
Red	0.45		325	0.36		276

<table>
<tr><th colspan="10">Vis-MSP</th></tr>
<tr><th>Colour</th><th colspan="3">DP (Original Spectra Only)</th><th>Overall DP</th><th colspan="3">DP (Original & First Derivative Spectra)</th><th>Overall DP</th><th>Max No. of Pairs</th></tr>
<tr><td>Blue</td><td colspan="3">0.77</td><td rowspan="2">0.45</td><td colspan="3">0.92</td><td rowspan="2">0.54</td><td>465</td></tr>
<tr><td>Red</td><td colspan="3">0.00</td><td colspan="3">N/A</td><td>325</td></tr>
<tr><th colspan="10">FTIR-ATR</th></tr>
<tr><th>Colour</th><th>Tier</th><th>DP (Original)</th><th>Overall DP</th><th>DP (First Derivative)</th><th>Overall DP</th><th>DP (Original & FD)</th><th>Overall DP</th><th colspan="2">Max No. of Pairs</th></tr>
<tr><td rowspan="4">Blue</td><td>1</td><td>0.66</td><td rowspan="6">0.74 (min)

0.96 (max)</td><td rowspan="4">0.85</td><td rowspan="6">0.90</td><td rowspan="4">0.94</td><td rowspan="6">0.96</td><td colspan="2" rowspan="4">465</td></tr>
<tr><td>2</td><td>0.81</td></tr>
<tr><td>3</td><td>0.92</td></tr>
<tr><td>4</td><td>0.93</td></tr>
<tr><td rowspan="2">Red</td><td>1</td><td>0.85</td><td rowspan="2">0.97</td><td rowspan="2">0.99</td><td colspan="2" rowspan="2">325</td></tr>
<tr><td>2</td><td>0.99</td></tr>
<tr><th colspan="10">Raman Spectroscopy</th></tr>
<tr><th>Colour</th><th>Excitation λ (nm)</th><th>DP (Exc. Sub-Group)</th><th>DP by Colour</th><th>Overall DP</th><th>DP (Inc. Sub-Group)</th><th>DP by Colour</th><th>Overall DP</th><th colspan="2">Max No. of Pairs</th></tr>
<tr><td>Blue</td><td>514</td><td>0.77</td><td rowspan="4">0.71</td><td rowspan="5">0.72</td><td>0.79</td><td rowspan="4">0.73</td><td rowspan="5">0.73</td><td colspan="2">465</td></tr>
<tr><td></td><td>685</td><td>0.68</td><td>N/A</td><td colspan="2">465</td></tr>
<tr><td></td><td>785</td><td>0.68</td><td>0.71</td><td colspan="2">465</td></tr>
<tr><td></td><td>830</td><td>0.72</td><td>N/A</td><td colspan="2">465</td></tr>
<tr><td>Red</td><td>785</td><td>0.78</td><td>0.78</td><td>N/A</td><td>N/A</td><td colspan="2">325</td></tr>
</table>

Table 7.1: Summary of Discriminating Power (DP) calculated for different conditions by each analytical technique (entries in red denote the optical or camera filter providing the highest DP within a given colour group and set of illuminating conditions for filtered light examination)

7.3.1.6 Conclusion: Discriminating Power

Taking into consideration all the analytical techniques shown to be useful for a given colour group, a combined analytical sequence DP range can be suggested for qualitative discrimination of gel ink samples. The minimum value takes into account classifications made by TLC for dye containing samples, the minimum number of classifications made by both Vis-MSP and FTIR-ATR (original spectra only), classifications made by Raman Spectroscopy at all appropriate excitation wavelengths, and classifications determined by VSC 6000/HS (IRR and/or IRL). The maximum value considers all of the aforementioned, but for both Vis-MSP and FTIR-ATR, instead of the minimum number of classifications, the maximum number (original and corresponding first derivative spectra combined) has been used. Based on this approach, the DP for the blue ink group was calculated between 0.93 – 0.98 and for the red ink group 0.98 – 1.00. For both the blue and red ink groups, even at the lowest level of qualitative discrimination, the DP of the combined analytical approach is extremely strong.

For blue ink group samples, as expected, TLC was sufficient for discriminating dye containing samples. For all samples, discrimination using original Vis-MSP and FTIR-ATR spectra was good. For the latter, the level of discrimination varied over a tiered range dependent upon the peak absorbencies selected, whilst for the former, use of the first derivative spectra enhanced the discriminating capability of the technique. The 514.5 nm and 830 nm excitation wavelengths were found to offer the greatest level of discrimination by Raman Spectroscopy, either individually or combined, whilst 785 nm was found to be slightly more discriminating than the 685 nm excitation wavelength. As expected VSC 6000/HS, offered a satisfactory level of discrimination, with IRL preferred over IRR.

In terms of the red ink group, TLC offered a limited level of discrimination due to lack of variation in the dye component(s) of the samples, whilst IRR by VSC 6000/HS and Vis-MSP offered no discrimination between samples. In contrast, IRL provided a good level of discrimination, as did Raman Spectroscopy, but only at the 785 nm excitation wavelength. Consideration of the original spectra produced by FTIR-ATR also offered a good level of discrimination, which was slightly enhanced when the first derivative spectra were also considered, although some contradiction in classification of samples between the two data sets was noted.

7.3.2 Multivariate Analysis

7.3.2.1 Raman Spectroscopy

7.3.2.1.1 Blue Ink Group

The blue ink group samples were analysed at four key excitation wavelengths: 514.5 nm, 685 nm, 785 nm (Foster and Freeman FORAM 785 and Renishaw InVia) and 830 nm and the respective data sets interrogated by PCA, HCA and SOFM (ANN).

7.3.2.1.1.1 514.5 nm

7.3.2.1.1.1.1 Principal Component Analysis (PCA)

Visual pattern recognition of the Raman spectra represented by this data set classified the samples into six groups. The PCA score plot shown in Figure 7.1 supports this, demonstrating six well resolved clusters. Most of these, show tight clustering of samples within each group, although the samples representing Group 1 (blue circle) and Group 3 (yellow circle) appear more widely spaced out in comparison. One could argue therefore, that unless prior knowledge of how many clusters would be expected and which samples belonged to which cluster, misclassification based on PCA alone could occur.

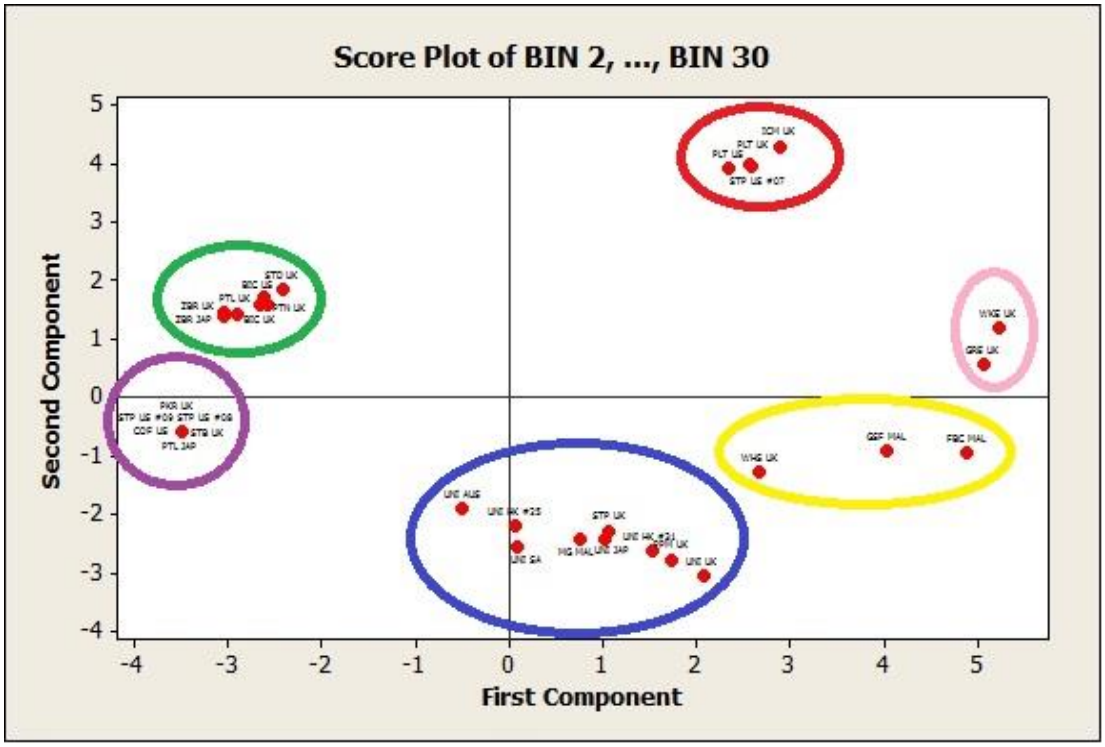


Figure 7.1: PCA score plot of blue ink group samples analysed by Renishaw InVia 514.5 nm demonstrating successful discrimination into six clusters

Group 2 (green circle) and Group 6 (purple circle) were discriminated from all other groups by the influence of PC 1, whilst PC 2 was most influential in discriminating Group 1 (blue circle), Group 3 (yellow circle) and Group 6 from all others. Interestingly both Group 5 (red circle) and Group 6 ink samples contained a dye, with the former also found to contain a pigment, i.e. hybrid. The Raman data had the specific ability to distinguish the hybrid gel ink samples from the dye based gel ink samples.

7.3.2.1.1.1.2 Hierarchical Cluster Analysis (HCA)

From the dendrogram shown in Figure 7.2 it can be clearly seen that the expected six clusters have been correctly identified using HCA. The similarity index on the y-axis which can be viewed in terms of a percentage (%) value demonstrates that each of the six groups can be distinguished from one another with a reasonable level of dissimilarity. Furthermore, within a group, the samples show a higher level of similarity, suggesting less of a difference between their Raman spectra. Interestingly, Group 2 (green), which could arguably be divided into two sub-groups by eye based on a difference in relative peak intensity for a single peak at ~1550 cm^{-1}, was also divided into two sub-groups by HCA consistent with those identified by visual pattern recognition. However, the level of similarity between these two sub-groups was so strong, that HCA was not able to detect sufficient difference between them to characterise them as two different clusters, even when the number of expected clusters was increased to seven. Standardisation of the variables did not change the classification groupings, and was therefore not a considered a necessity for this statistical approach.

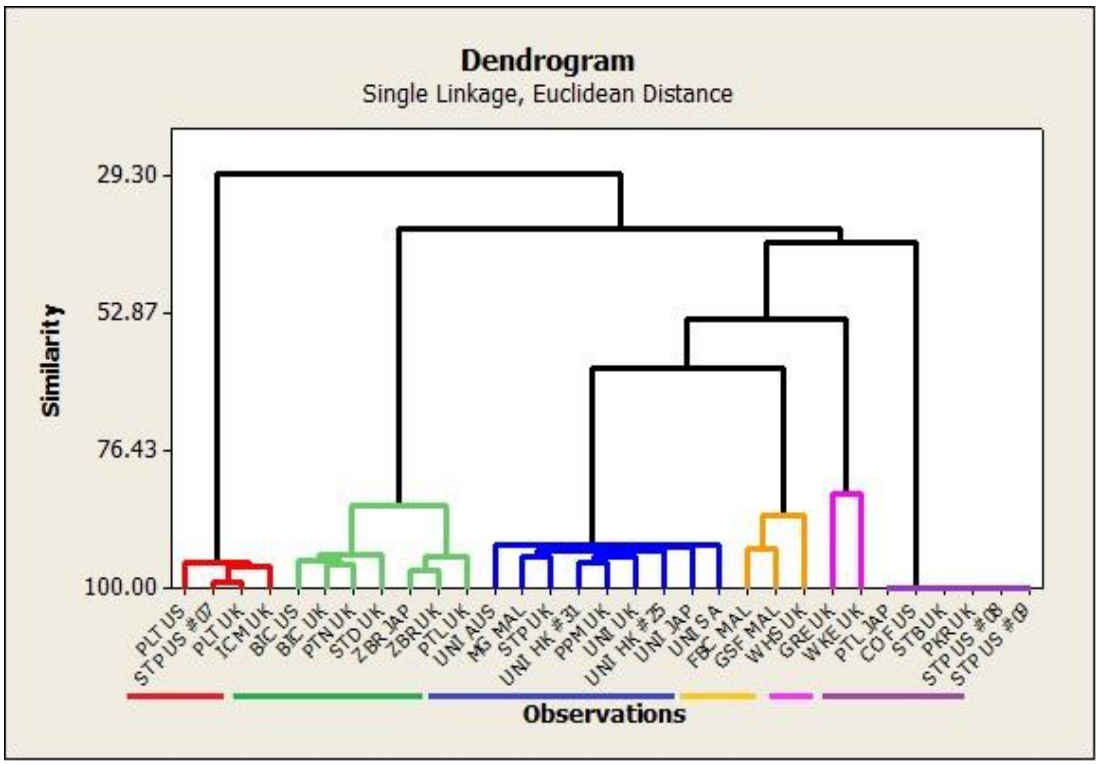


Figure 7.2: HCA dendrogram of blue ink group samples analysed by Renishaw InVia 514.5 nm demonstrating successful discrimination into six clusters

7.3.2.1.1.1.3 Artificial Neural Network (ANN) Analysis

Correct classification into six groups by SOFM was also achieved, highlighted in Figure 7.3. The six colour coded clusters represent the spectral groups distinguished by visual pattern recognition largely on the basis of presence or absence of specific peaks. Within a cluster, variations in colour hue can be observed. This reflects subtle differences attributable to the combined relative peak intensities for the selected wavenumbers representing those ink samples within a particular group. Group 2 (red cluster), for example, shows variation amongst the seven samples represented by segmentation of the samples into at least three different colour hues. When discriminating on the basis of visual pattern recognition, this particular spectral group was distinguishable into two sub-groups based on a reproducible difference in relative peak intensity for the peak at ~1550 cm^{-1}. This sub-classification is not reflected by the variation in colour hue within the red cluster representing Group 2 ink samples. This is likely due to relative peak intensities at several other wavenumber positions characterising ink samples in this particular spectral group also being considered. Furthermore, when the number of expected clusters is increased to seven, the sub-classification of Group 2 is still not recognised. Instead, Group 1 (blue cluster) samples are

split into two separate clusters. This demonstrates the subtle difference between the two sub-groups within Group 2 alone, was insufficient to be considered statistically significant, emphasising the high level of similarity between the seven ink samples composing Group 2.

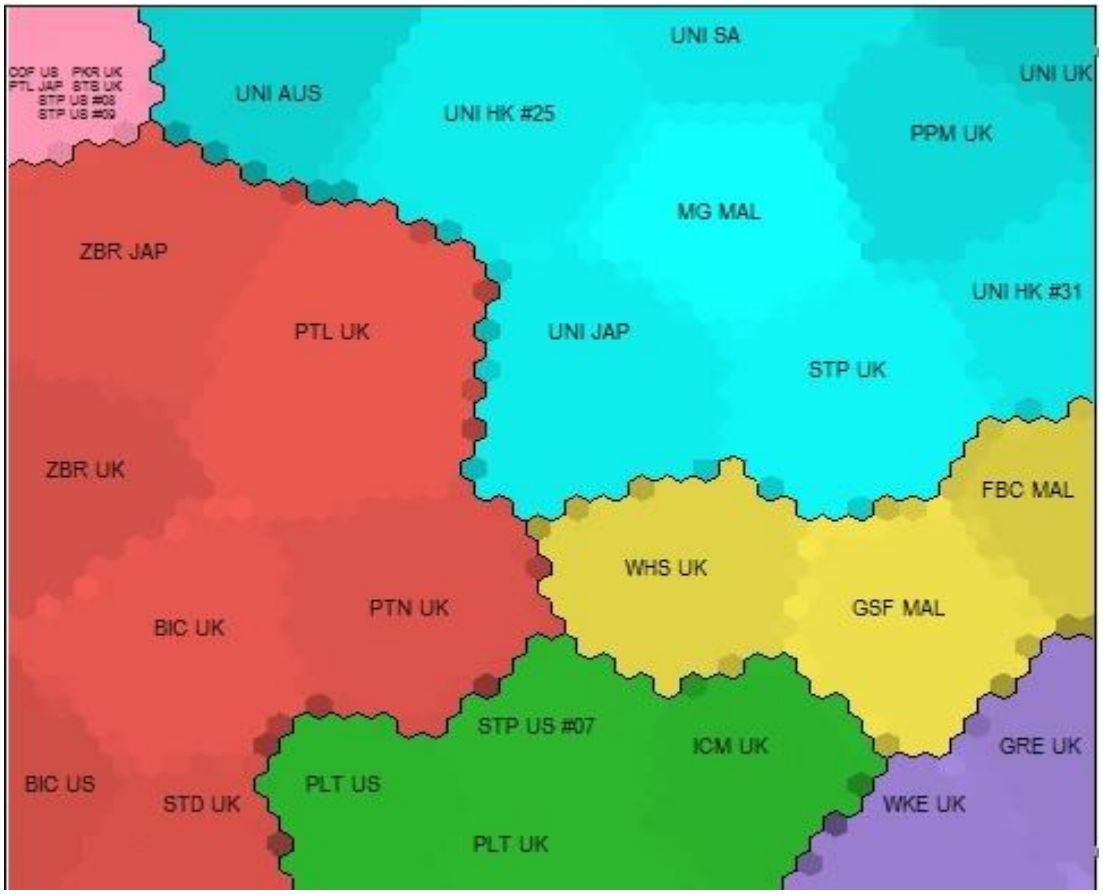


Figure 7.3: SOFM of blue ink group samples analysed by Renishaw InVia 514.5 nm demonstrating successful discrimination into six clusters, with subtle variations between samples within a cluster represented by a change in colour hue

7.3.2.1.1.2 685 nm

7.3.2.1.1.2.1 Principal Component Analysis (PCA)

The PCA score plot shown in Figure 7.4 represents four clusters that correspond to the four classification groupings determined from visual pattern recognition of this spectral data set. All four clusters show clear separation between one another, with Group 3 (blue circle) the furthest distance from all other clusters. All samples within a cluster are tightly grouped, with the exception of Group 1 (red circle) where samples are more linearly spread over a comparatively larger area. Without prior knowledge of the number of expected clusters, one would still be able to identify at least four groups from the PCA score plot alone.

Arguably, the pattern of spread amongst samples in Group 1 could lead to an erroneous fifth cluster being identified if classification was based on PCA alone.

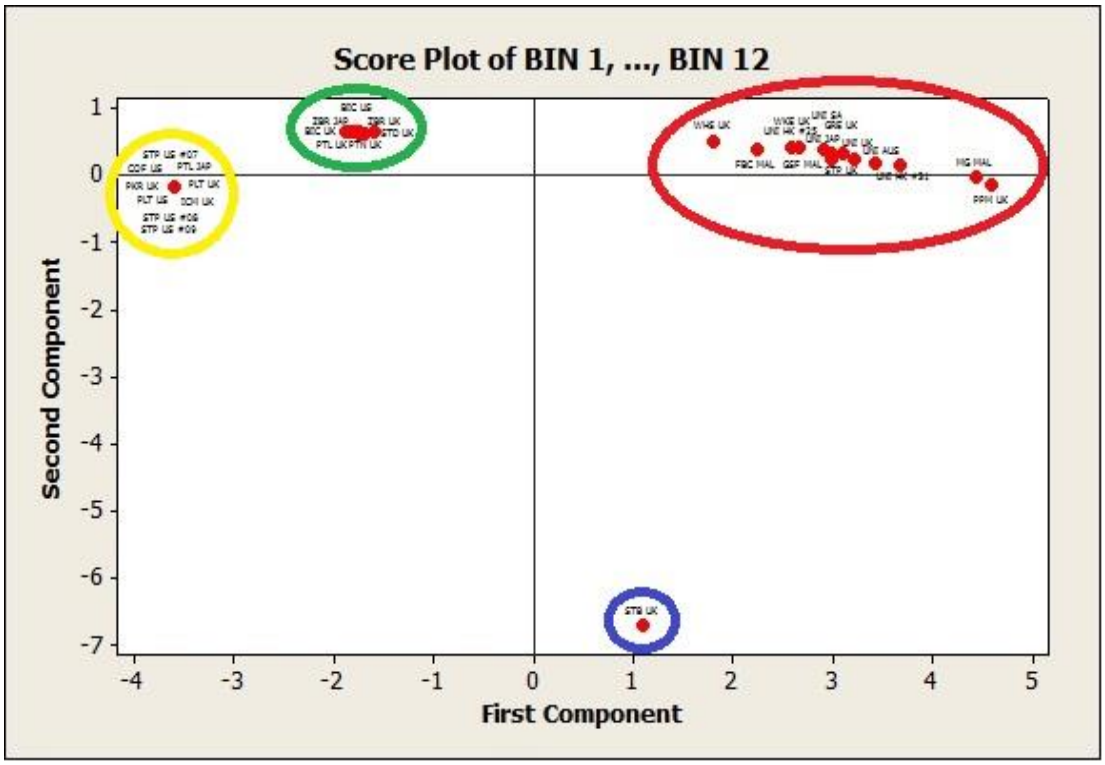


Figure 7.4: PCA score plot of blue ink group samples analysed by FORAM 685 demonstrating successful discrimination into four clusters

PC 1 appeared to have significant influence over Group 2 (green circle) and Group 4 (yellow circle) clearly distinguishing them from the other two groups, whilst PC 2 had a significant influence over distinguishing Group 3 (blue circle) from all others. Both Group 3 and Group 4 represented samples containing a dye, with the former also containing a pigment, suggesting that once again Raman data has specific ability for distinguishing hybrid gel ink formulations from dye based formulations.

7.3.2.1.1.2.2 Hierarchical Cluster Analysis (HCA)

All four clusters were correctly classified by HCA in line with the visual pattern recognition findings, shown in Figure 7.5. A high level of dissimilarity was observed between clusters compared to greater similarity observed within a cluster. Group 3 (blue) representing a hybrid gel ink formulation can be clearly distinguished from all other groups, which was also reflected by the notable difference in spectral pattern when compared visually with the

other Raman spectra. Standardisation of the variables was of no further value to discrimination.

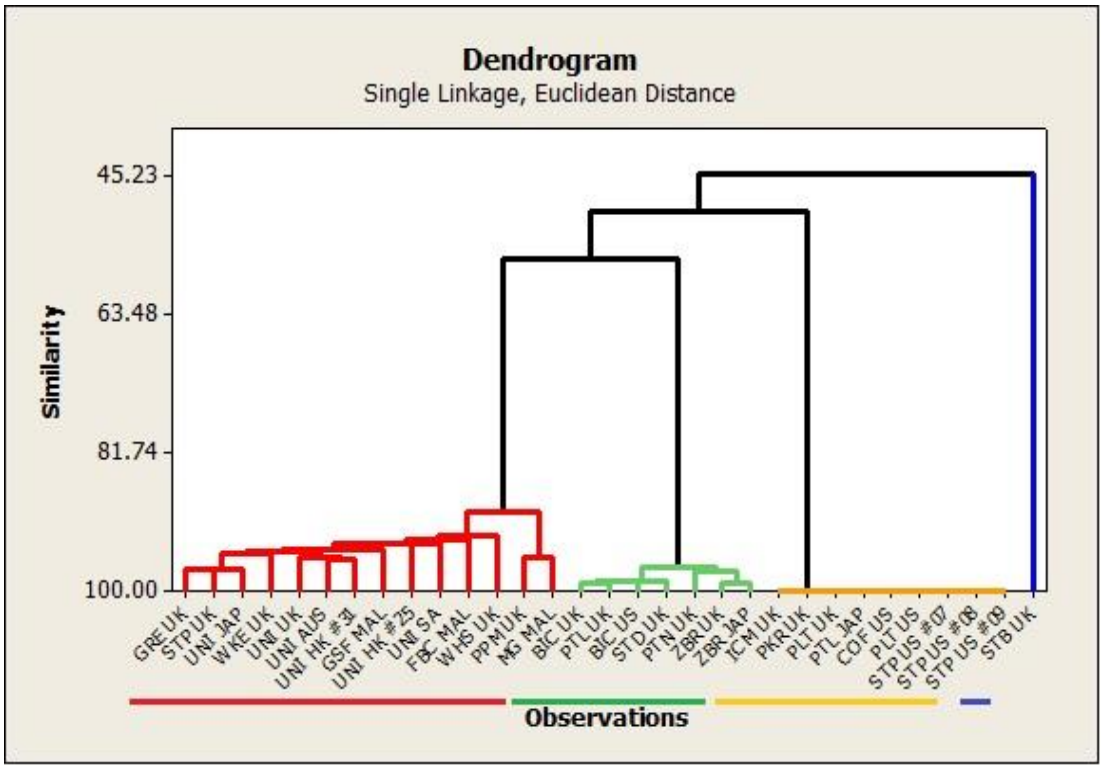


Figure 7.5: HCA dendrogram of blue ink group samples analysed by FORAM 685 demonstrating successful discrimination into four clusters

7.3.2.1.1.2.3 Artificial Neural Network (ANN) Analysis

In line with the other two statistical tests, SOFM correctly classified the samples into four groups corresponding to those identified from visual comparison demonstrated by Figure 7.6. Variations in colour hue, most notable within Group 1 (blue cluster), but also observed in Group 2 (red cluster), highlight subtle variations in combined relative peak intensities for the wavenumbers characterising the ink samples in those two spectral groups. This demonstrates a high degree of similarity in spectral pattern amongst the ink samples contained within these groups, but suggests subtle differences not necessarily obvious from visual pattern recognition can be detected by computer analysis. These differences are similar to the differences in similarity index highlighted amongst ink samples clustered together within a HCA dendrogram.

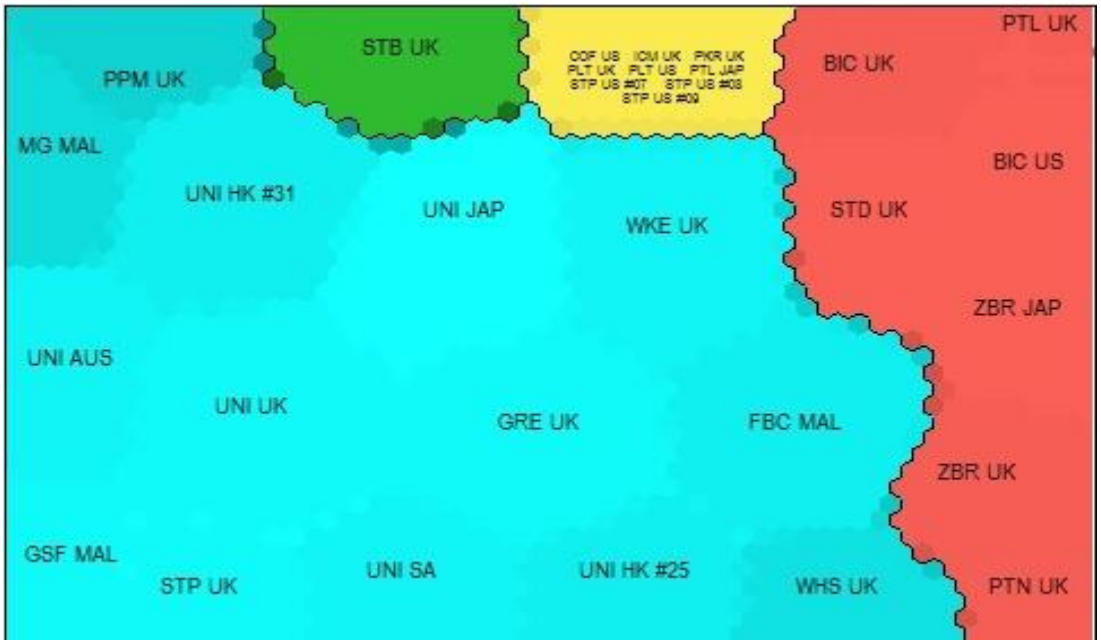


Figure 7.6: SOFM of blue ink group samples analysed by FORAM 685 demonstrating successful discrimination into four clusters. Variation in colour hue within the blue and red clusters demonstrate subtle differences in combined relative peak intensities of the wavenumbers characterising ink samples in those particular spectral groups

7.3.2.1.1.3 785 nm

7.3.2.1.1.3.1 FORAM

7.3.2.1.1.3.1.1 Principal Component Analysis (PCA)

A PCA score plot of the first two PC's using the Correlation method is presented in Figure 7.7. Four clear clusters corresponding to the four groups identified through visual pattern recognition for this particular data set can be observed, with good separation between them. The samples within Group 1 (red circle) and Group 4 (yellow circle) appear quite tightly clustered, whilst good separation is observed between the two groups. Good separation is also observed between Group 2 (green circle) and Group 3 (blue circle – sample STB UK), whilst the seven samples within the former are more spaced out from one another in comparison to those in Group 1 and Group 4. It could be argued that unless the correct classifications were known, distinguishing the eight samples that make up Group 2 and Group 3 by PCA alone could be difficult.

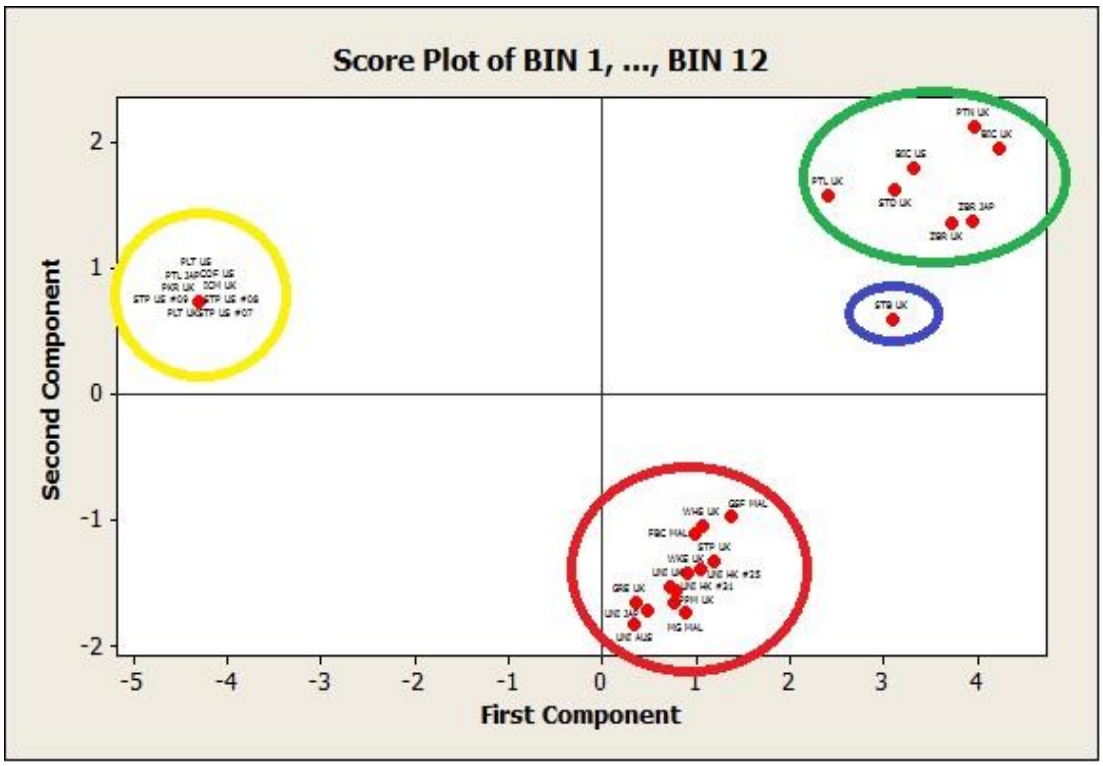


Figure 7.7: PCA score plot of blue ink group samples analysed by FORAM 785 demonstrating successful discrimination into four clusters

PC 1 had a much greater influence on Group 4 (yellow), distinguishing them completely from all other samples. The influence of the second Principal Component on this group was much less. PC 2 did have a significant effect however on Group 1 (red). This suggests that the Raman data has specific abilities in distinguishing the Group 1 and Group 4 samples from each other, which would stand to reason since the Group 4 samples were identified as dye containing and as such yielded spectra swamped by fluorescence and/or detector overload. Although one might also have expected PC 2 to have had a similar influence on Group 2 (green circle) and Group 3 (blue circle) since they did yield a good Raman spectrum.

7.3.2.1.1.3.1.2 Hierarchical Cluster Analysis (HCA)

It can be clearly seen in the dendrogram shown in Figure 7.8 that the expected four groups have been correctly identified using HCA, with the STB UK sample in Group 3 (blue) clearly distinguished from the seven samples that make up Group 2 (green). The similarity index on the y-axis, which can be viewed in terms of a percentage value, demonstrates that each of the four groups can be distinguished from one another with a reasonable level of

dissimilarity. Furthermore, within a group, the samples show a higher level of similarity, suggesting less of a difference between their Raman Spectra. Interestingly, Group 2 (green) which could arguably be divided into two sub-classes by eye based on a difference in relative peak intensity for a single peak at ~1390 cm^{-1}, could not be distinguished using this statistical approach even if the number of expected clusters is increased to five. This is in contrast to the sub-classification of Group 2 observed in the 514.5 nm data set, where HCA successfully identified two sub-groups of high similarity within the same cluster. Standardisation of the variables did not change the classification groupings, providing no further discriminatory value.

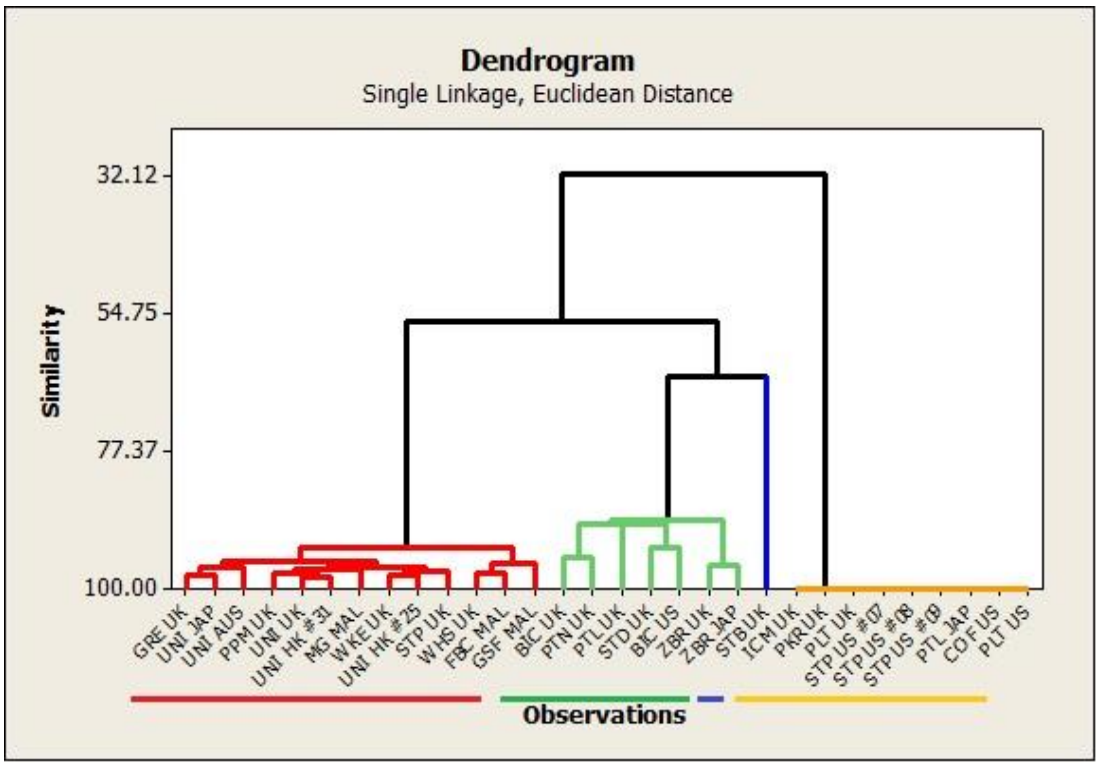


Figure 7.8: HCA dendrogram of blue ink group samples analysed by FORAM 785 demonstrating successful discrimination into four clusters

Again, as with the PCA analysis, HCA specifically suggests that the Raman data has specific abilities in distinguishing the Group 4 samples from all others. In this case the discrimination of the Group 4 samples is less specific. HCA provides further information relating to the nature of the link between the other groups but which mirrors that of the PCA data.

7.3.2.1.1.3.1.3 Artificial Neural Network (ANN) Analysis

The SOFM is shown in Figure 7.9, and once again correct organisation of the various brands of gel ink within the four blue ink groups has been demonstrated. As for the 514.5 nm data set, increasing the number of expected groups to five did not provide a discrimination of the two possible sub-groups, based on a reproducible difference in relative peak intensity at 1390 cm^{-1}, associated with Group 2 (red cluster). Furthermore, variation in colour hue within the red cluster also did not reflect the sub-classification of Group 2 ink samples.

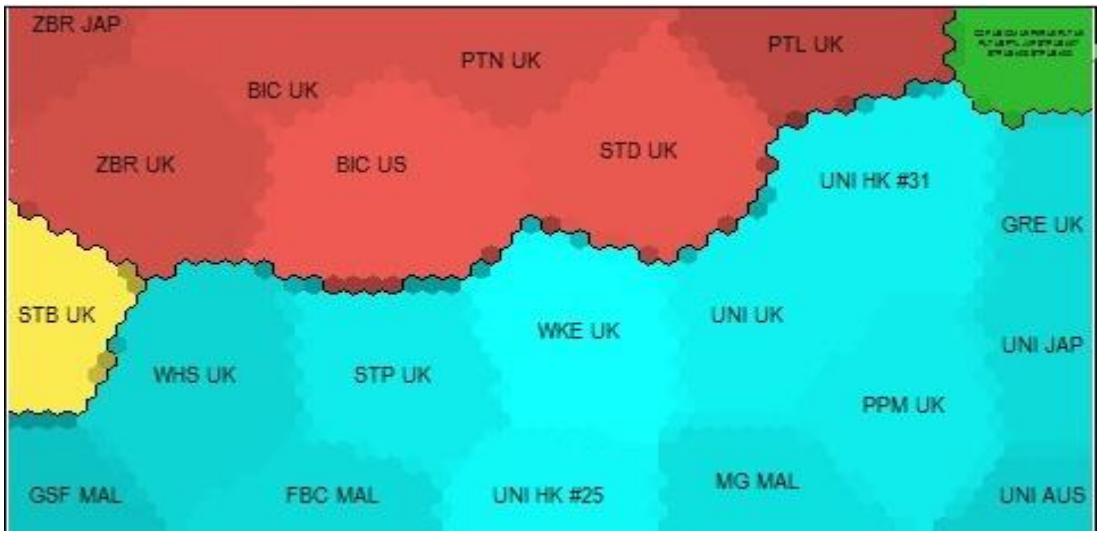


Figure 7.9: SOFM of blue ink group samples analysed by FORAM 785 demonstrating successful discrimination into four clusters, with variations in colour hue within the blue and red clusters highlighting subtle differences in combined relative peak intensities associated with the wavenumbers characterising the ink samples within the two spectral groups

7.3.2.1.1.3.2 Renishaw InVia

The Renishaw 785 nm blue ink group data set produced similar observations as those made of the FORAM 785 data set. Correct classification of samples was achieved for all three techniques, with good separation between clusters observed for both the PCA and HCA as illustrated in Figure 7.10 and Figure 7.11 respectively.

With respect to PCA, PC 1 had significant influence over Group 4 (yellow circle), distinguishing them from the other groups. PC 2 had significant influence over Group 1 (red circle), Group 2 (green circle) and Group 4, consequently, clearly distinguishing them from the remaining Group 3 (blue circle). Both Group 3 and Group 4 represent dye containing samples, with the former representing a hybrid sample. This once again supports the view that Raman data can distinguish between hybrid and dye based gel ink formulations.

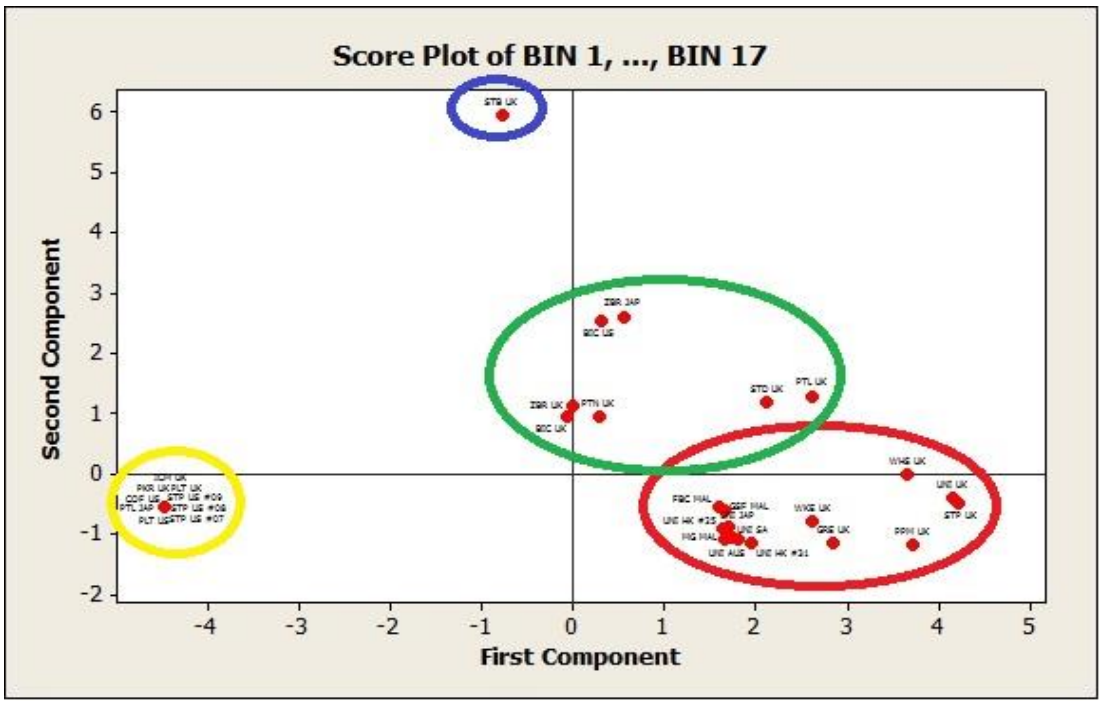


Figure 7.10: PCA score plot of blue ink group samples analysed by Renishaw InVia 785 nm demonstrating successful discrimination into four clusters

A notable difference was that for the Renishaw data set, the similarity index between samples within a HCA cluster was spread over a wider range than that for the FORAM data.

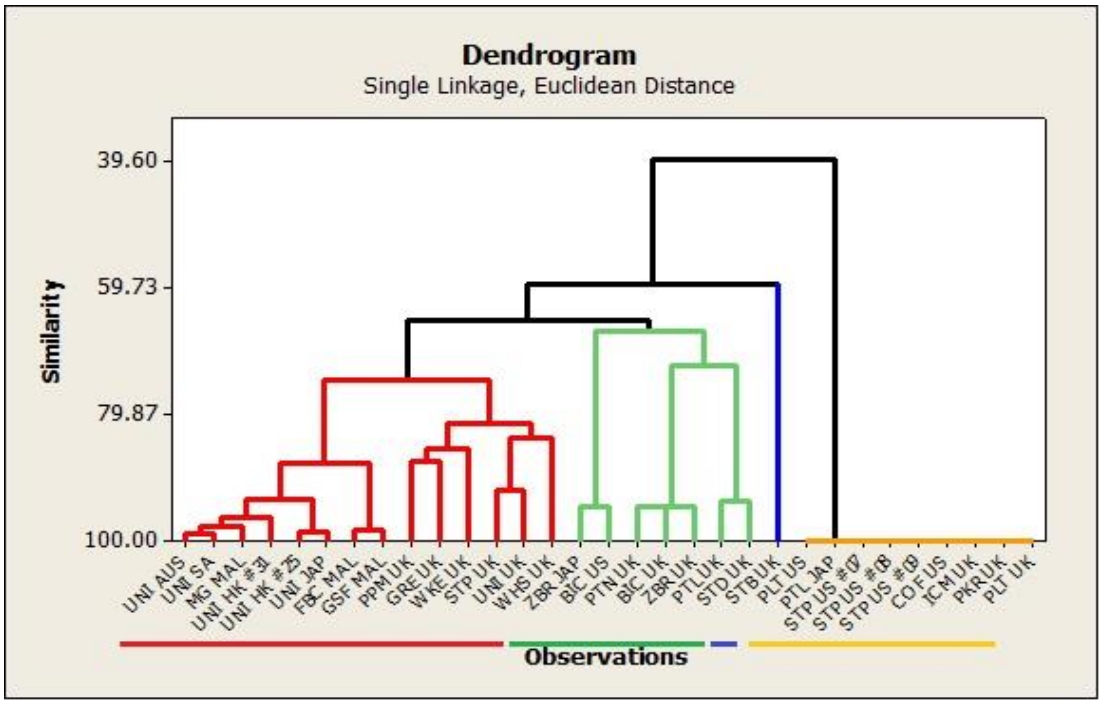


Figure 7.11: HCA dendrogram of blue ink group samples analysed by Renishaw 785 nm demonstrating successful discrimination into four clusters and illustrating a wider similarity index than for the same samples analysed by FORAM 785

7.3.2.1.1.4 830 nm

7.3.2.1.1.4.1 Principal Component Analysis (PCA)

Six groups were identified from visual comparison of spectra from this data set, and as shown in Figure 7.12, this is supported by PCA. Group 1 (red circle) and Group 6 (purple circle) are well resolved from each other and from the other four clusters. Samples within these two clusters are reasonably close together highlighting their similarity. Samples representing the remaining four clusters however, are more widely spaced out, which if relying on PCA alone could lead to misclassification of samples without visual comparison of their spectra. Furthermore, most of the samples representing Group 2 (green circle) and Group 3 (blue circle) are relatively close to one another, which again, without physically comparing spectra by eye, could lead to erroneous classifications.

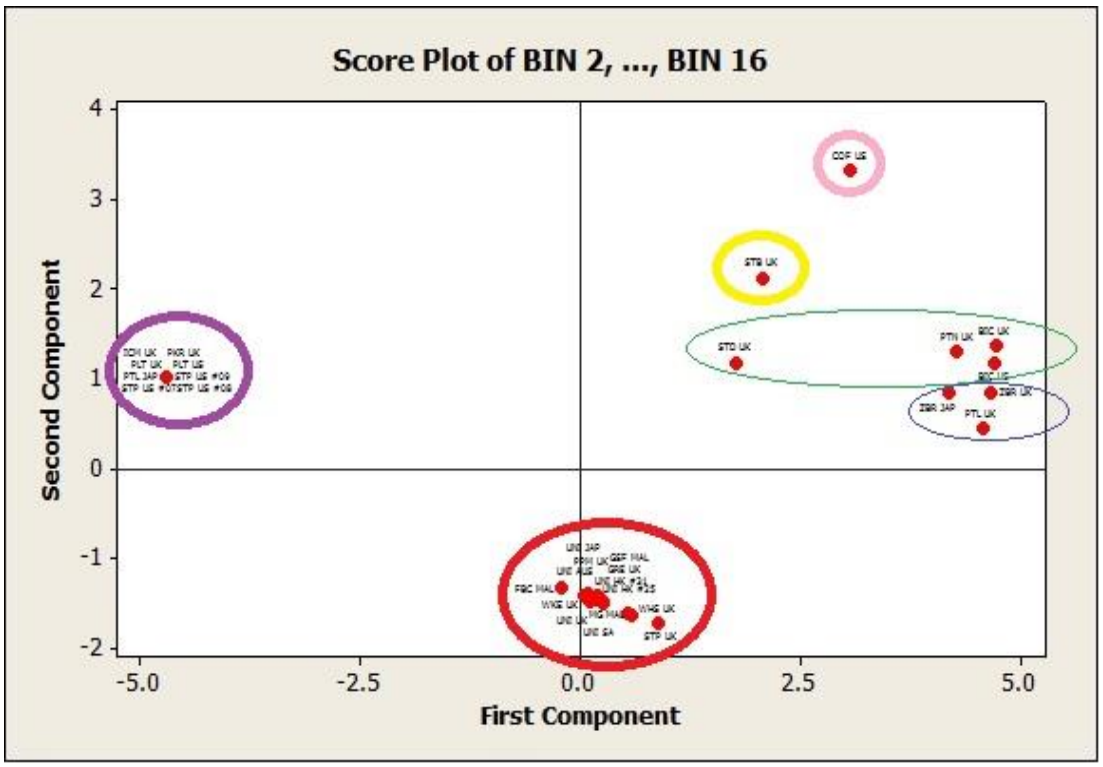


Figure 7.12: PCA score plot of blue ink group samples analysed by Renishaw InVia 830 nm demonstrating discrimination into six clusters, some well resolved with others quite close together potentially leading to erroneous clustering

PC 1 had a significant influence over Group 6 (purple circle) clearly distinguishing it from the remaining five groups, whilst PC 2 had a significant influence over Group 1 (red circle) with similar effect. This suggests that Raman data has specific abilities to distinguish Group 1 and Group 6 samples. Group 6 represents dye based samples, whilst Group 1 represents

pigment based samples. Both Group 4 (yellow circle) and Group 5 (pink circle) represent hybrid formulations, containing both a dye and pigment component, demonstrating again the ability of Raman Spectroscopy to distinguish hybrid formulations from dye only ones.

7.3.2.1.1.4.2 Hierarchical Cluster Analysis (HCA)

Clustering into six groups by HCA, as demonstrated in Figure 7.13, was in line with the classifications made by visual comparison of Raman spectra. Interestingly, Group 2 (green) and Group 3 (blue) representing seven ink samples in total are clearly discriminated from one another. These seven samples when analysed at 514.5 nm and 785 nm represented a single group, that could only be distinguished into two sub-groups on the basis of a relative peak intensity difference for a single peak, at ~1550 cm^{-1} and ~1390 cm^{-1} respectively. HCA analysis of these two data sets could not find sufficient difference between them to class them as two separate groups. This demonstrates that analysis of these particular seven ink samples at 830 nm excitation wavelength was necessary in order to be able to confirm discrimination into two separate groups, which was only hinted at by the lower excitation wavelengths.

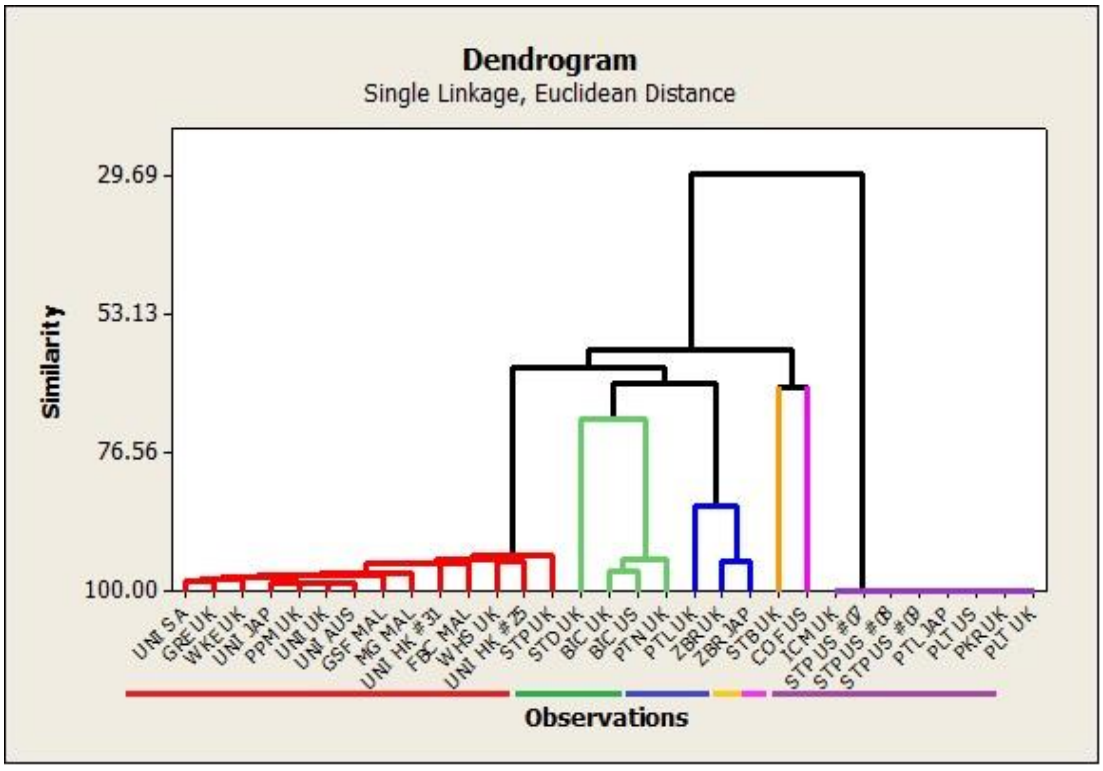


Figure 7.13: HCA dendrogram of blue ink group samples analysed by Renishaw InVia 830 nm demonstrating successful discrimination into six clusters

7.3.2.1.1.4.3 Artificial Neural Network (ANN) Analysis

The SOFM shown in Figure 7.14 illustrates successful classification of the ink samples into six groups corresponding to those made by visual pattern recognition. Similarly to HCA, Group 2 (yellow cluster) and Group 3 (red cluster) ink samples are clearly distinguished at 830 nm, something which was not achieved for the same samples at 514.5 nm and 785 nm. Subtle differences in combined relative peak intensities of the wavenumber positions characterising the ink samples within Group 1 (blue cluster), Group 2 and Group 3 can be observed. This is most notably for the STD UK ink sample within Group 2, which appears a much darker hue of yellow compared to the other three samples suggesting it exhibits a greater degree of difference within the cluster.

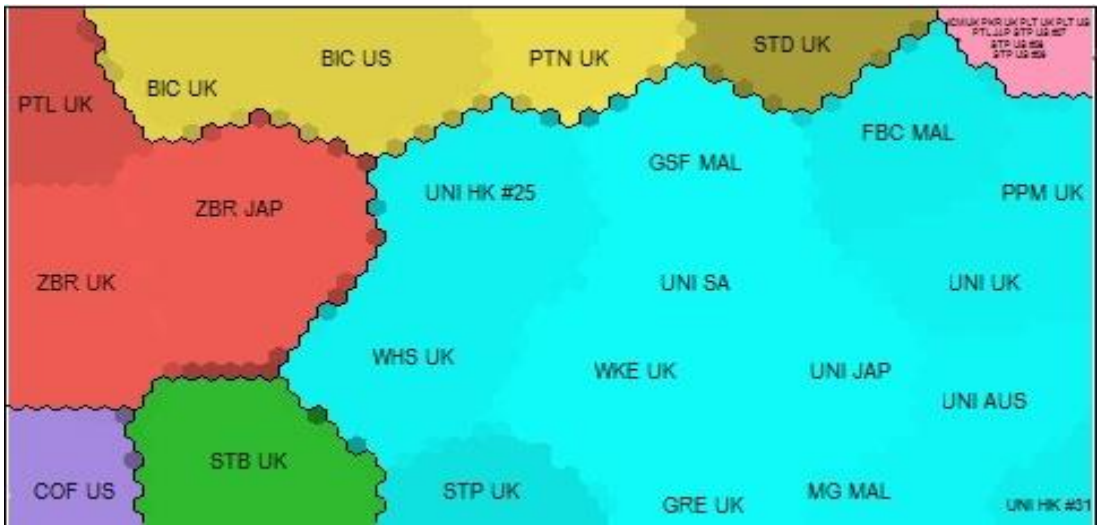


Figure 7.14: SOFM of blue ink group samples analysed by Renishaw InVia 830 nm demonstrating successful discrimination into six clusters. Variation in hue of coloured segments within a cluster indicative of subtle differences in the combined relative peak intensities for the wavenumbers characterising the ink samples within that cluster, i.e. STD UK exhibits a much greater difference to the other three ink samples within the yellow cluster representing Group 2

7.3.2.1.1.5 Conclusion

7.3.2.1.1.5.1 Blue Ink Group

The blue ink group samples were analysed at four key excitation wavelengths: 514.5 nm, 685 nm, 785 nm (Foster and Freeman FORAM and Renishaw InVia) and 830 nm. Correct classification for the blue ink group at all excitation wavelengths was achieved using all three statistical techniques. With respect to PCA, whilst all clusters could be identified and resolved, in practice prior knowledge of which samples belonged to which clusters were required to avoid misclassification. The HCA and SOFM approach correctly classified the

samples for each of the excitation wavelengths however, prior knowledge of the number of expected clusters, as opposed to providing information relating to which sample should group together, was required, suggesting an examiner would have to base their final conclusions on a combination of visual pattern recognition and statistical classification.

In terms of HCA, clear differences were observed between groups at all excitation wavelengths, whilst within a group of inks classed together a high level of similarity was revealed. It is worth noting for one group of seven samples (Group 2) analysed using 514.5 nm and 785 nm excitation wavelengths, visual discrimination into two sub-groups on the basis of a difference in relative peak intensity at ~1550 cm^{-1} and ~1390 cm^{-1} respectively ((Group 2a) BIC UK, BIC US, PTN UK and STD UK, and (Group 2b) ZBR UK, ZBR JAP and PTL UK) was possible. This further discrimination was confirmed using the 830 nm excitation wavelengths where a number of differences permitted the samples in these two sub-groups to be classified into two groups in their own right. When the number of expected clusters was increased by one to account for this, in the 514.5 nm and 785 nm data sets, the software failed to classify the samples correctly. Changing the linkage algorithm to the Ward Linkage ("the distance between two clusters is the sum of squared deviations from points to centroids" [169] (without Standardisation of the variables)) did result in a correct classification of these two sub-groups in the 514.5 nm data set. This change of linkage algorithm did not work for the 785 nm data set. Sub-classification within Group 2 in the 514.5 nm or 785 nm data sets was also not detected by SOFM, either by a change in colour hue within the relevant cluster or by increasing the number of expected clusters.

7.3.2.1.2 Red Ink Group

Visual pattern recognition resulted in discrimination of the red gel inks into 10 spectral groups containing the same samples irrespective of instrument used to provide the 785 nm.

7.3.2.1.2.1 Principal Component Analysis (PCA)

PCA resulted in correct classifications by instrument being identified. Clusters were generally well resolved, and for groups containing multiple inks, samples were generally clustered tightly together reducing the risk of erroneous classifications. However, prior knowledge of which samples belonged to each cluster was still necessary to avoid any misclassification of the ink samples occurring. As demonstrated in Figure 7.15,

representing the FORAM data set, the influence of PC 1 on Group 3 (blue circle) was particularly notable, clearly separating this Group from all others which were concentrated on the right side of the score plot. For the Renishaw data set however, shown in Figure 7.16, clusters were distributed fairly evenly across the entire score plot area.

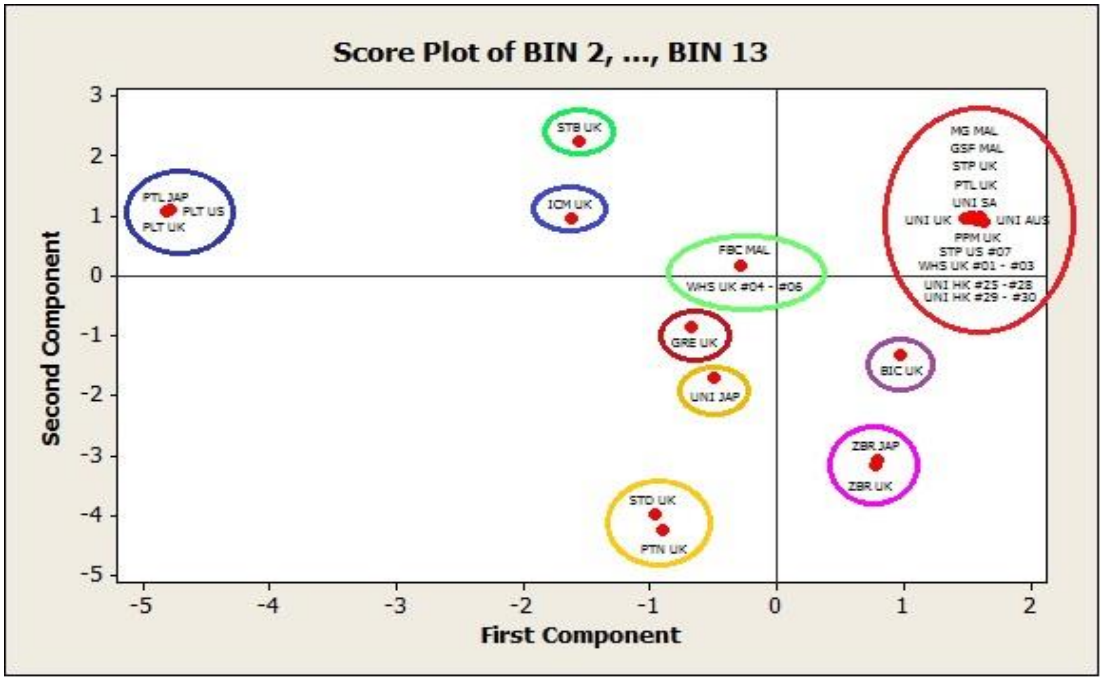


Figure 7.15: PCA score plot of red ink group samples analysed by FORAM 785 demonstrating successful discrimination into 10 clusters

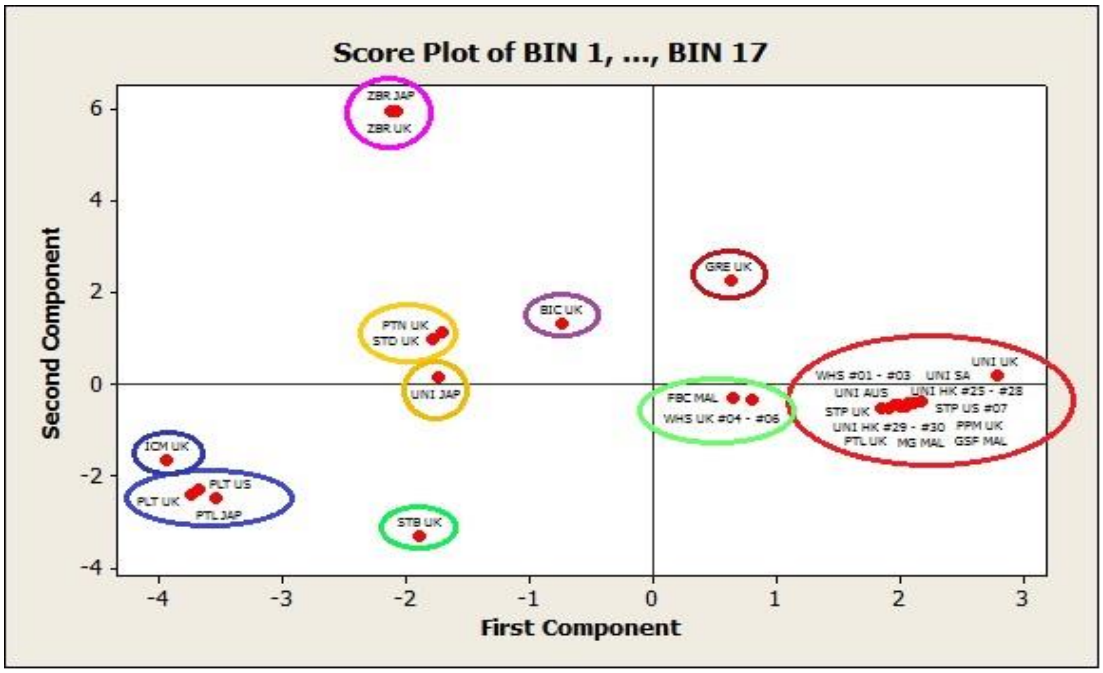


Figure 7.16: PCA score plot of red ink group samples analysed by Renishaw InVia 785 nm demonstrating discrimination into 10 clusters, some quite close together

7.3.2.1.2.2 Hierarchical Cluster Analysis (HCA)

Correct classification of ink samples by instrument was also achieved using HCA, even when spectra exhibited a high level of similarity. As illustrated in Figures 7.17 and 7.18, a high level of dissimilarity was observed in both data sets between Group 1 (red) and all others.

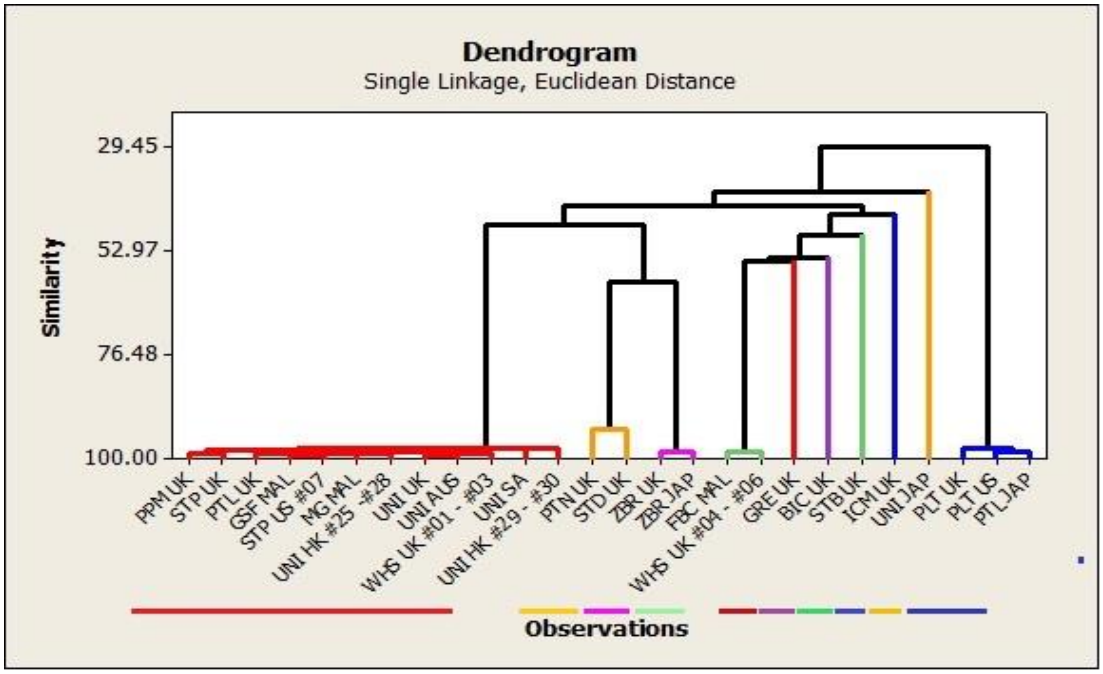


Figure 7.17: HCA dendrogram of red ink group samples analysed by FORAM 785 demonstrating successful discrimination into 10 clusters

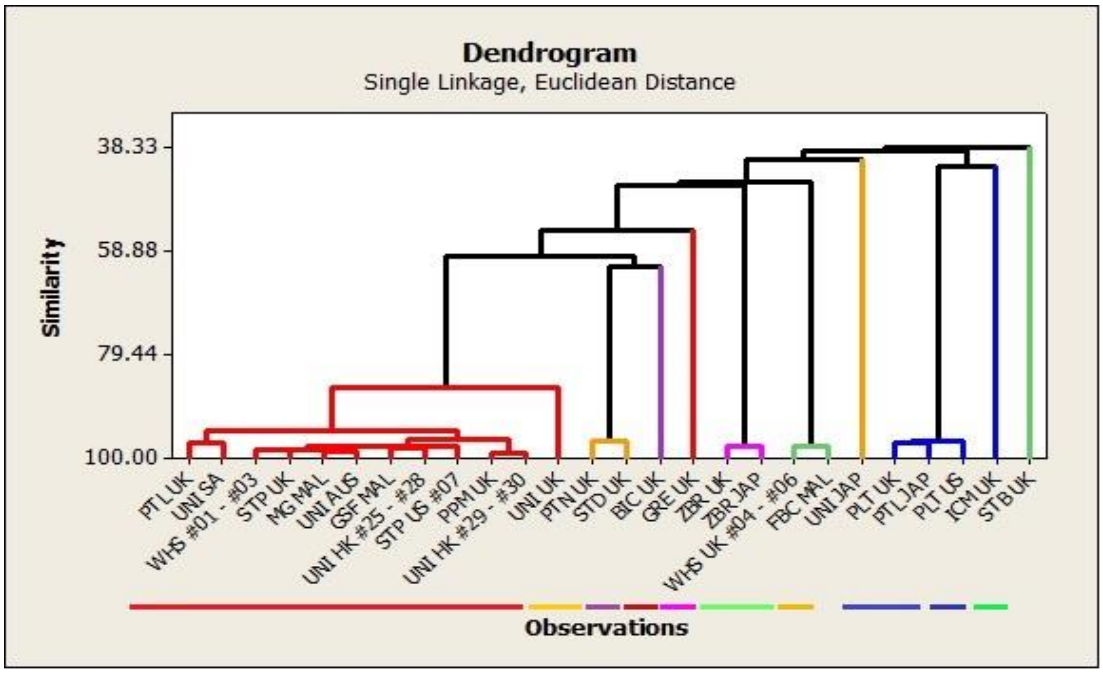


Figure 7.18: HCA dendrogram of red ink group samples analysed by Renishaw 785 nm demonstrating successful discrimination into 10 clusters

7.3.2.1.2.3 Artificial Neural Network (ANN) Analysis

Correct classification by instrument was observed in the SOFM, in line with the visual pattern recognition observations, as demonstrated in Figure 7.19 and Figure 7.20 for the FORAM and Renishaw data sets respectively. With the exception of Group 1 (blue cluster), minimal variation in colour hue within clusters containing two or more ink samples was observed in the SOFM's from both data sets. Group 1, containing the greatest number of ink samples (12) demonstrated the greatest variation in colour hue amongst those samples, which was more notable in the SOFM representing the Renishaw data set. This may be due to stronger spectral intensity arising from the use of a more powerful laser source within this instrument compared to the FORAM 785 thus revealing greater spectral detail. This notwithstanding, the differences in colour hue within this cluster and others are very subtle demonstrating a high level of similarity within groups containing multiple ink samples.

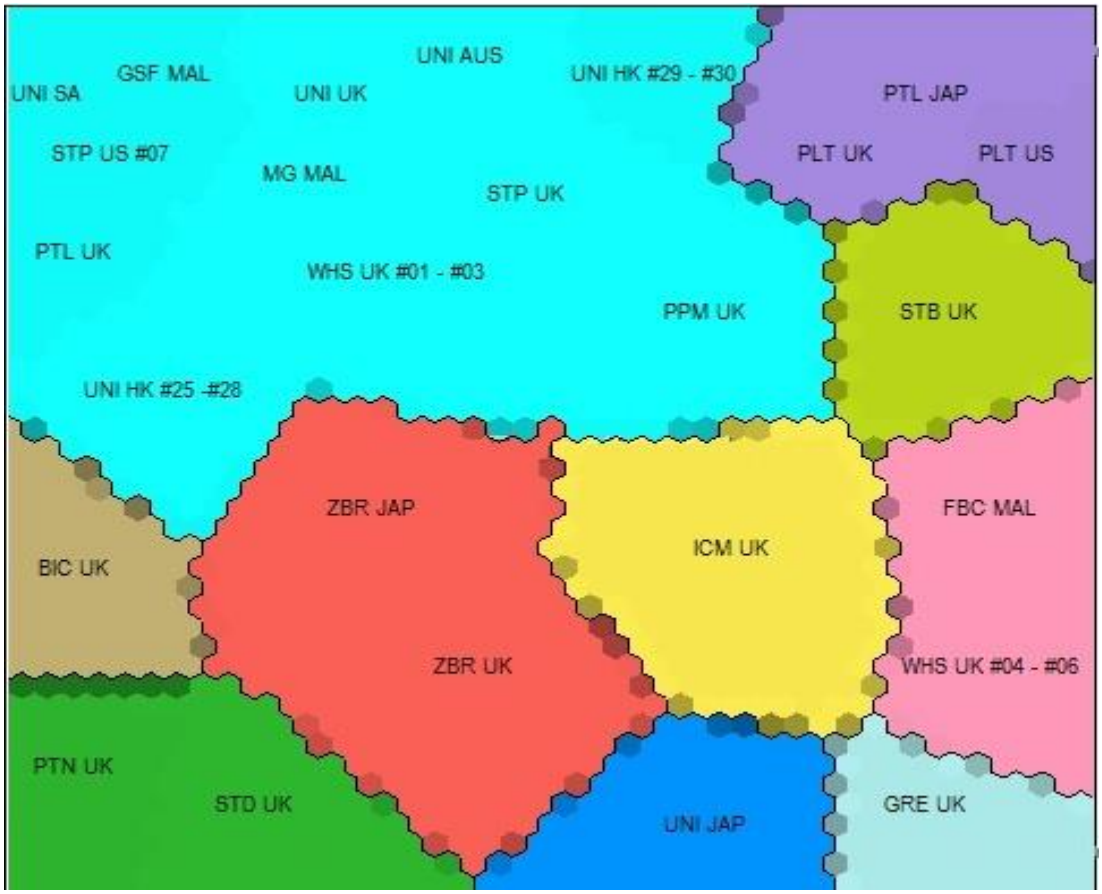


Figure 7.19: SOFM of red ink group samples analysed by FORAM 785 demonstrating successful discrimination into 10 clusters. Minimal variation in colour hue is observed within clusters containing multiple ink samples

Figure 7.20: SOFM of red ink group samples analysed by Renishaw 785 nm demonstrating successful discrimination into 10 clusters. Minimal variation in colour hue within clusters containing multiple samples demonstrate a high degree of similarity between ink samples contained within them, with Group 1 (blue cluster) exhibiting the greatest degree of variation

7.3.2.1.2.4 Conclusion

7.3.2.1.2.4.1 Red Ink Group

Classification of the 785 nm data from two different instruments by visual pattern recognition resulted in discrimination of the red gel inks into the same 10 spectral groups. For both instrumental data sets, the correct classifications were achieved using all three statistical treatments. Prior knowledge of which ink samples belonged to which group was required in order to achieve successful clustering by PCA, primarily where distinguishable ink samples were positioned close together. This knowledge was not required for successful classification by HCA or SOFM, however, the number of expected clusters had to be known in order to instruct the software to discriminate the samples accordingly.

7.3.2.2 FTIR-ATR

7.3.2.2.1 Blue Ink Group

7.3.2.2.1.1 T1 – T4 Inclusive Data Matrices (8, 16 and 32 BINS)

Based on the 8 BIN data matrix, the PCA score plot clearly showed three well separated clusters corresponding to the three classification groupings made for the blue ink group at T1, i.e. two pigment based groups and one dye containing group.

The HCA and SOFM output also resulted in correct classifications based on T1 when the number of expected clusters was given as three. However, when the expected number of clusters was increased in line with T2, T3 and T4 visual classifications, i.e. 6, 14 and 17 clusters respectively, then for both techniques misclassification of samples occurred.

Similar observations were made for the 16 BIN data matrix in respect of all three techniques. In addition, with regards to HCA of the 8 BIN and 16 BIN data matrices, Standardisation of the variables provided similar results to the corresponding non-standardised HCA dendrograms.

It appeared therefore, using the data matrix in 8 and 16 BIN format, with all three techniques, only the minimum level of classification groupings, i.e. T1, could be achieved, i.e. three groups. This is illustrated by the example of the PCA score plot representing the 16 BIN data matrix in Figure 7.21.

A data matrix consisting of 32 BINS was prepared in an attempt to improve the sensitivity of the method and thus hopefully provide further discrimination. Similar observations as for the 8 and 16 BIN data matrices were observed for all three statistical techniques, with only T1 level classification being successfully achieved as illustrated by the PCA score plot shown in Figure 7.22.

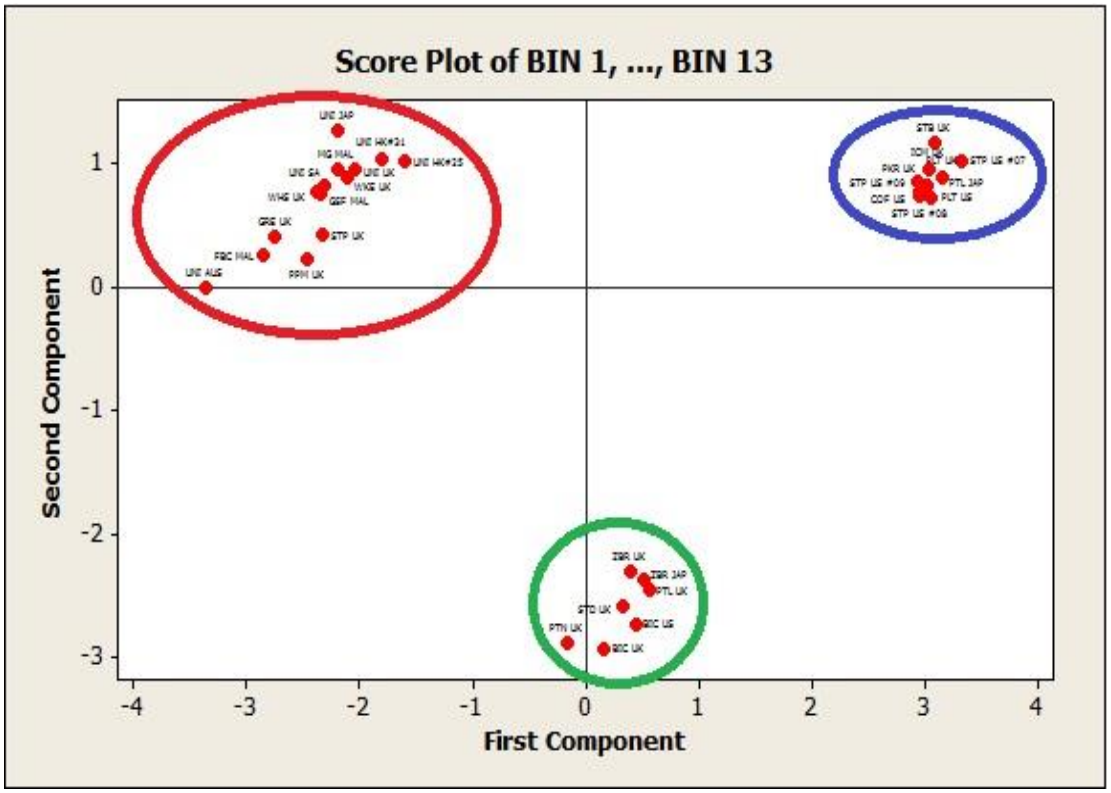


Figure 7.21: PCA score plot of blue ink group samples analysed by FTIR-ATR presented in a 16 BIN data matrix format demonstrating three clearly resolved clusters in line with T1 level visual pattern recognition groups

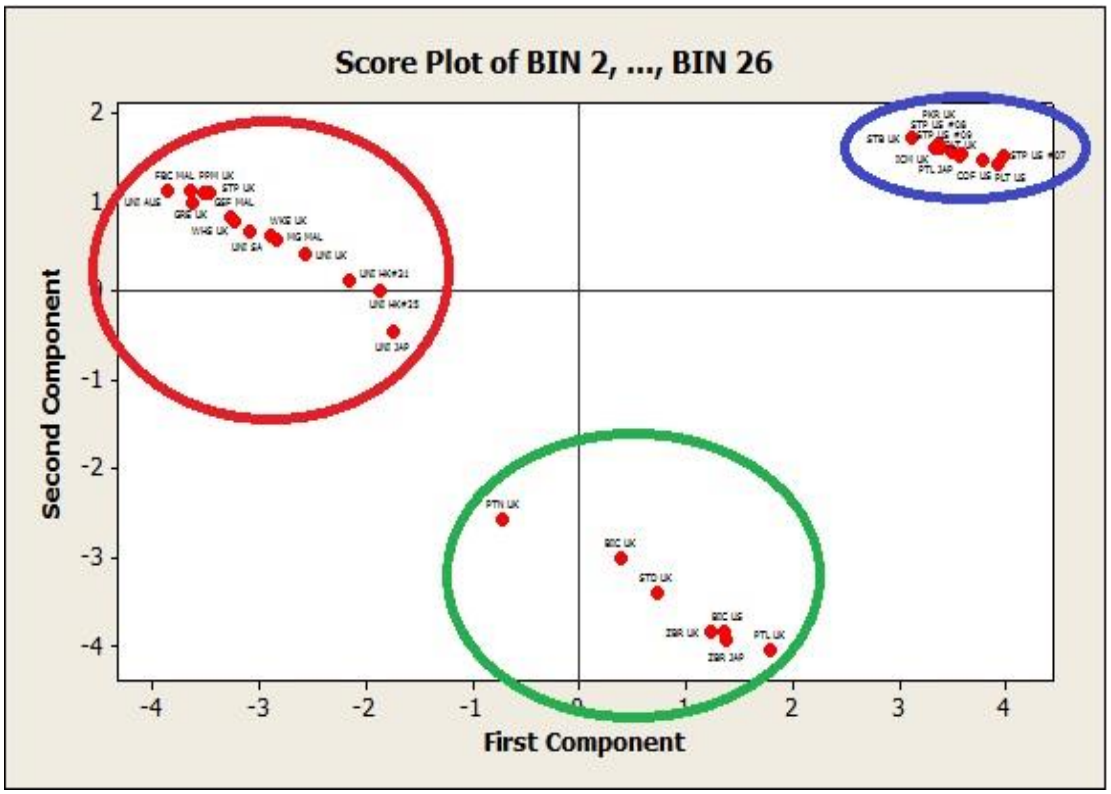


Figure 7.22: PCA score plot of blue ink group samples analysed by FTIR-ATR presented in a 32 BIN data matrix format demonstrating three clearly well resolved clusters in line with T1 level visual pattern recognition groups

A different approach to constructing the data matrix was investigated to try and improve the statistical discrimination of the samples in line with the visual classifications. This approach involved creating data matrices that incorporated all relevant peak information that characterised a particular Tier level of discrimination, i.e. T4 (greatest number of visual classification groupings), T3 and T2.

7.3.2.2.1.2 T4 Data Matrix (32 BINS)

7.3.2.2.1.2.1 Principal Component Analysis (PCA)

As demonstrated in Figure 7.23, PCA identified 11 clusters, differing from the 17 expected for T4 based on visual discrimination.

The 14 pigment based samples that initially made up Group 1 in T1, were further discriminated visually into five groups in T4. However, these 14 samples were clustered tightly together in the PCA score plot and as such were considered too close to one another to be considered anything other than a single cluster.

The seven pigment based samples that initially made up Group 2 in T1 were visually discriminated into two groups in T2 and subsequently four groups in T4. It was possible to cluster these seven samples on the PCA score plot into the two groups corresponding to T2 with good separation between them, and arguably into the four groups observed in T4, although with some crossover observed between two of the latter clusters.

For the remaining ten dye containing samples, which initially constituted Group 3 in T1, and further discriminated into eight groups in T4, clustering into six groups could be achieved from PCA. The PKR UK, STP US #08 and STP US #09 samples could all be isolated from the other seven samples, whilst ICM UK and STB UK clustered together tightly. However, it was not possible to resolve the COF US and PTL JAP samples into two clusters, nor was it possible to separate the STP US #07 sample from the PLT samples. All ten dye containing samples occupied the same general area of the score plot, so were clustered reasonably close together, but were differentiable with prior knowledge of clustering acquired from visual pattern recognition.

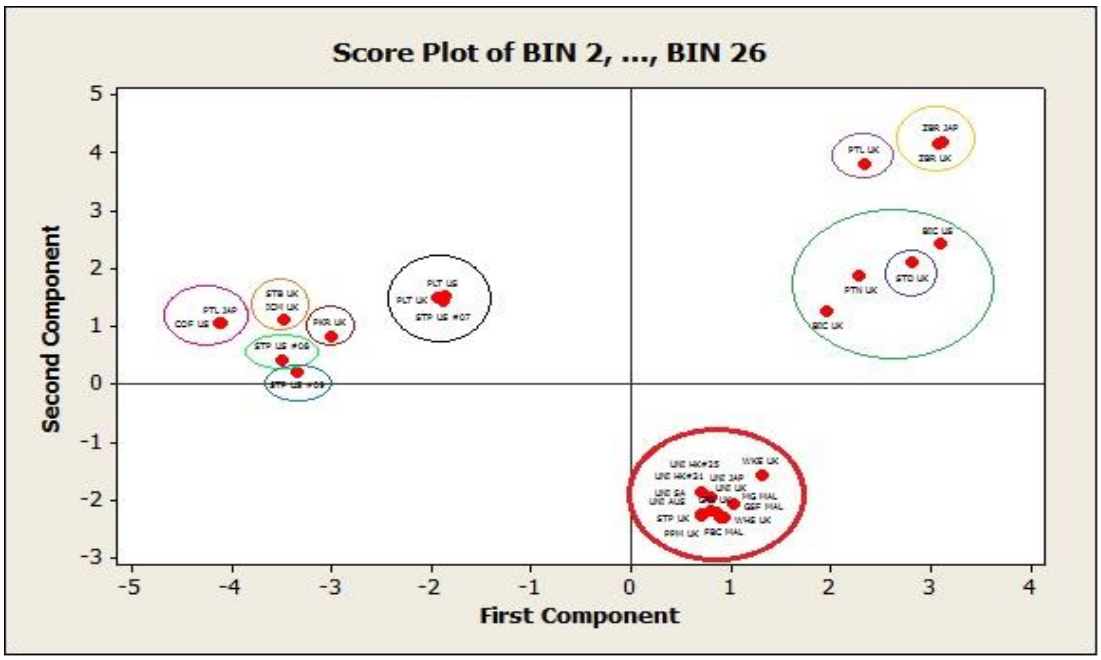


Figure 7.23: PCA score plot of blue ink group samples analysed by FTIR-ATR presented in a 32 BIN data matrix format reflecting T4 level visual pattern recognition groups. PCA clearly shows 11 clusters inconsistent with the 17 clusters expected

7.3.2.2.1.2.2 Hierarchical Cluster Analysis (HCA)

When 17 clusters were expected as per T4 visual discrimination, the HCA clustering did not wholly reflect this, with some misclassification observed as shown in Figure 7.24.

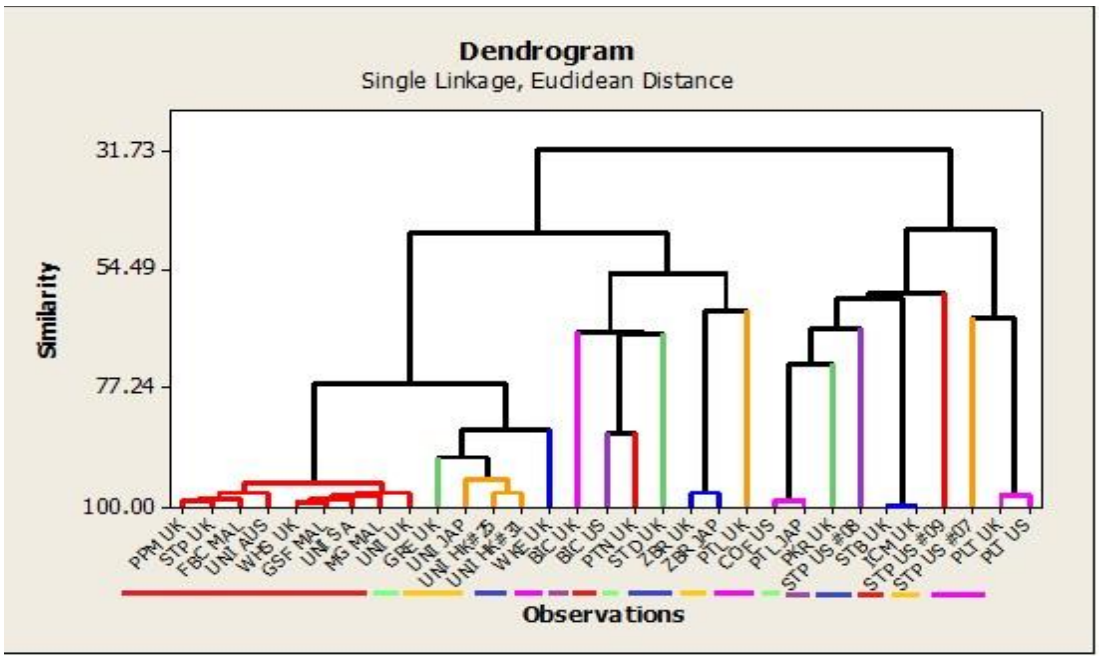


Figure 7.24: HCA dendrogram of blue ink group samples analysed by FTIR-ATR presented in a 32 BIN data matrix format reflecting T4 level visual pattern recognition groups. HCA resulted in misclassification of samples, and hence was inconsistent with the expected 17 clusters distinguished by visual pattern recognition

The 14 pigment based samples initially represented by Group 1 in T1 were clustered into four groups instead of the expected five for T4. This discrepancy was attributed to the UNI UK and MG MAL samples that were not isolated into a fifth cluster.

The seven samples initially representing Group 2 in T1 were classified into six clusters instead of the expected four from T4. Interestingly, whilst the PTL UK, STD UK and ZBR samples occupied three different clusters, the remaining three samples (BIC UK, BIC US and PTN UK) were all clustered individually rather than as one.

The ten dye based samples that initially represented Group 3 in T1, were classified correctly into the seven clusters expected from T4 with the exception of the COF US and PTL JAP samples that occupied the same cluster.

When the number of expected clusters was reduced to three in line with T1 visual classification, the samples were incorrectly classified, with Group 1 and Group 2 clustered as one and Group 3 samples separated into two clusters that bore no resemblance to the two clusters associated with this group at T2. Standardisation of variables added no further discriminatory value.

7.3.2.2.1.2.2.1 Investigation of Different Algorithm Combinations for HCA

An investigation of different algorithm combinations with and without Standardisation of variables was performed on both the T1 - T4 inclusive 32 BIN and T4 32 BIN data matrices.

For the T1 – T4 inclusive 32 BIN data matrix, clustering into three correctly classified groups in line with T1 visual discrimination was achievable using all algorithm combinations irrespective of whether the variables were standardised or not. If however, the number of expected clusters was increased in line with higher Tiers, i.e. T2, T3 and T4, then misclassification of samples occurred.

For the T4 32 BIN data matrix, misclassification of samples, similar to that described above using Single Linkage and Euclidean Distance, was observed for all algorithm combinations. Interestingly when the expected number of clusters was reduced to three, in line with T1 visual discrimination, all algorithm combinations, with the exception of Single Linkage and

Euclidean Distance, resulted in correct classification. Standardisation of variables yielded similar results. Increasing the number of expected clusters in line with T2 and T3 visual discrimination, i.e. 6 and 14, resulted in misclassification irrespective of algorithm combination used.

Despite the findings described in relation to the T4 data matrix, some similarity was observed between the statistical and visual classifications for T4. For this reason it was decided to interrogate data matrices reflecting both T3 and T2 visual classification to determine if either of these was validated by multivariate profiling.

7.3.2.2.1.2.3 Artificial Neural Network (ANN) Analysis

In contrast, SOFM analysis provided correct classification when the number of expected clusters was three (T1), but when increased to 17 clusters (T4), similar misclassifications as for HCA were observed. Furthermore, the GRE UK sample could not be isolated from the Asian UNI samples (even on the basis of colour hue variation), and the UNI AUS, FBC MAL, STP UK and PPM UK samples unexpectedly occupied a cluster of their own. Figure 7.25 shows the SOFM generated for the expected 17 T4 clusters. Minimal variation in colour hue within some clusters also demonstrates the high degree of similarity between samples.

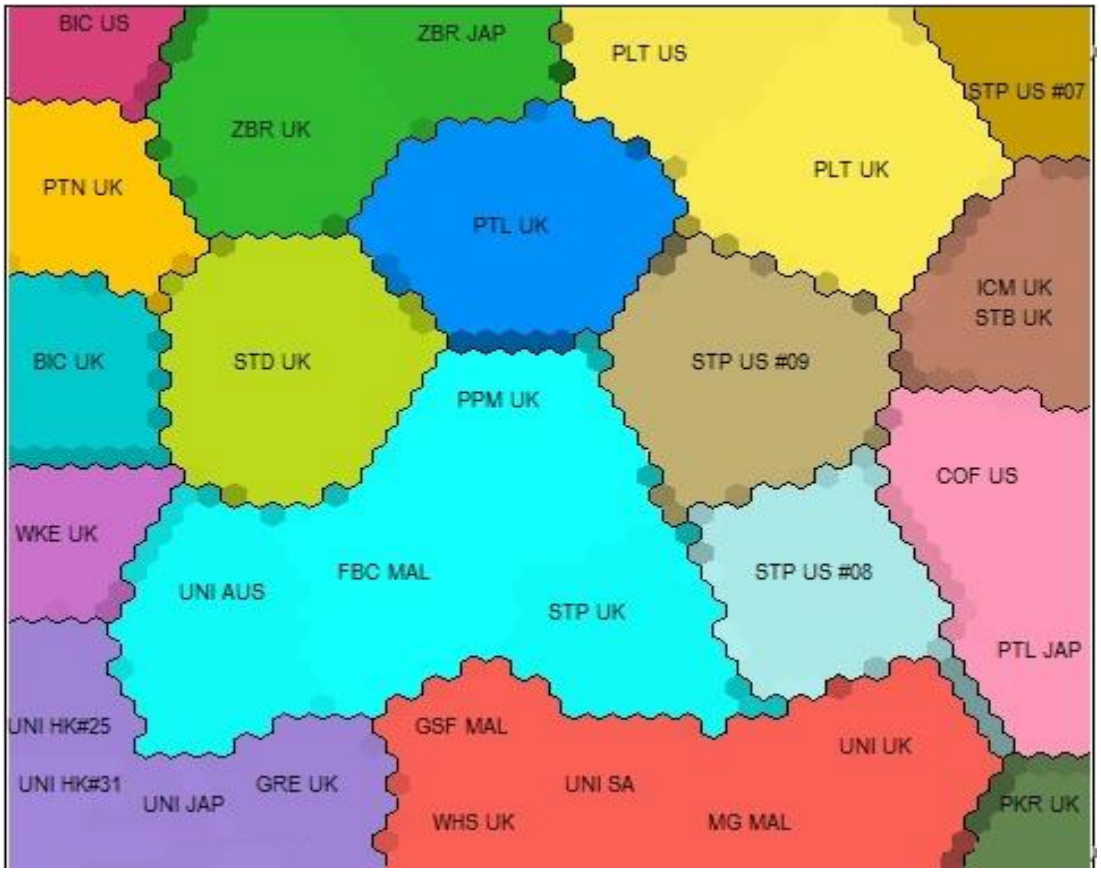


Figure 7.25: SOFM of blue ink group samples analysed by FTIR-ATR presented in a 32 BIN data matrix format reflecting T4 level visual pattern recognition groups. SOFM resulted in misclassification of samples inconsistent with the expected 17 clusters distinguished by visual pattern recognition

For both HCA and SOFM, increasing or decreasing the number of clusters did not resolve the misclassifications. Furthermore, inputting the expected number of clusters in line with T2 and T3 did not give fully correct classification either.

7.3.2.2.1.3 T3 Data Matrix (32 BINS)

7.3.2.2.1.3.1 Principal Component Analysis (PCA)

The expected number of classification groupings based on T3 visual pattern recognition was 14. However, only nine clusters were identified by PCA as illustrated by the score plot in Figure 7.26.

The 14 pigmented samples originally representing Group 1 in T1 were clustered too close together to be able to resolve them into the expected five clusters from T3 visual discrimination. Similar observations were noted in the T4 data matrix as described earlier.

The seven samples originally represented by Group 2 in T1 could be resolved into the expected four clusters for T3. Again, similar observations were noted in the T4 data matrix described above.

The ten dye containing samples representing Group 3 at T1 could be grouped into four clusters with only the inability to isolate the COF US sample from the PTL JAP and STP US #08 and #09 samples differing from the T3 visual classification.

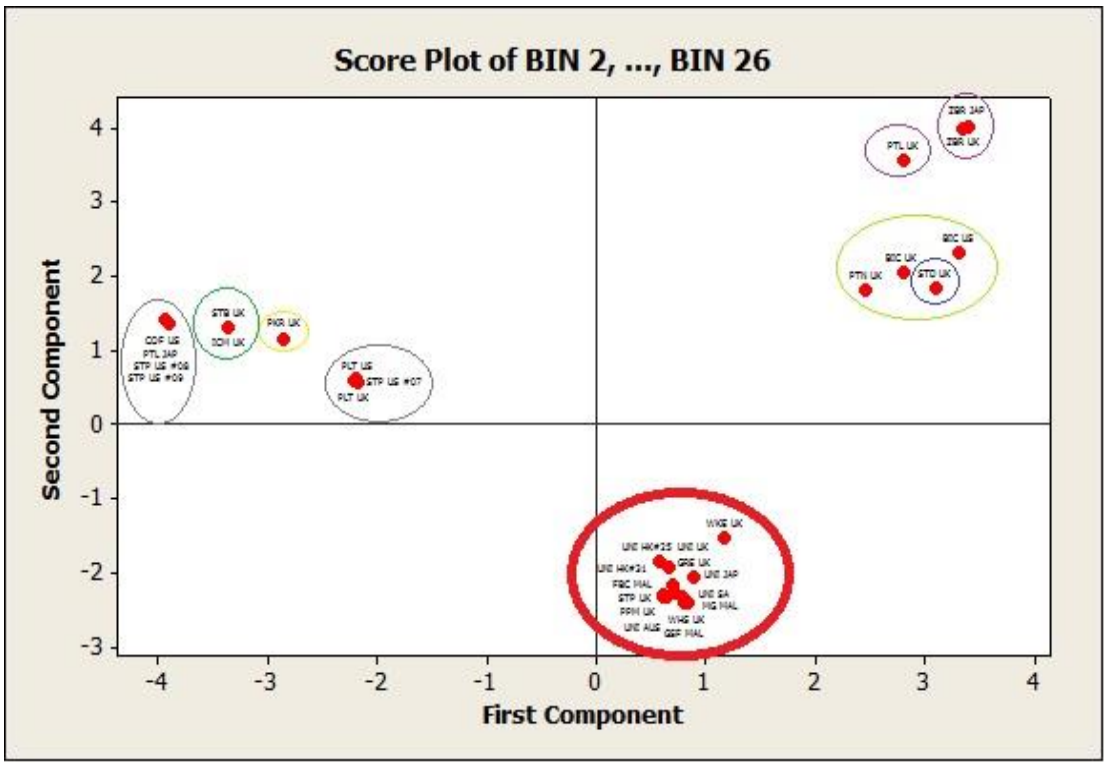


Figure 7.26: PCA score plot of blue ink group samples analysed by FTIR-ATR presented in a 32 BIN data matrix format reflecting T3 level visual pattern recognition groups. PCA discrimination produced 9 clusters inconsistent with the expected 14 clusters distinguished by visual pattern recognition

7.3.2.2.1.3.2 Hierarchical Cluster Analysis (HCA)

Similarly to the T4 HCA dendrogram, some similarity and differences were observed in the HCA dendrogram of the T3 data matrix when compared to the T3 visual classifications. Figure 7.27 shows the T3 HCA dendrogram.

All 21 pigmented samples were clustered in the same groups as that observed in the T4 HCA dendrogram. The only difference between the T3 and T4 HCA dendrograms was in the clustering of the ten dye containing samples (originally representing Group 1 in T1) which in

the former were grouped in a similar fashion to the PCA clustering of the T3 data matrix. Standardisation of variables provided no further discriminatory value. The application of all other algorithm combinations with or without Standardisation of variables to the T3 32 BIN data matrix only resulted in misclassification of samples.

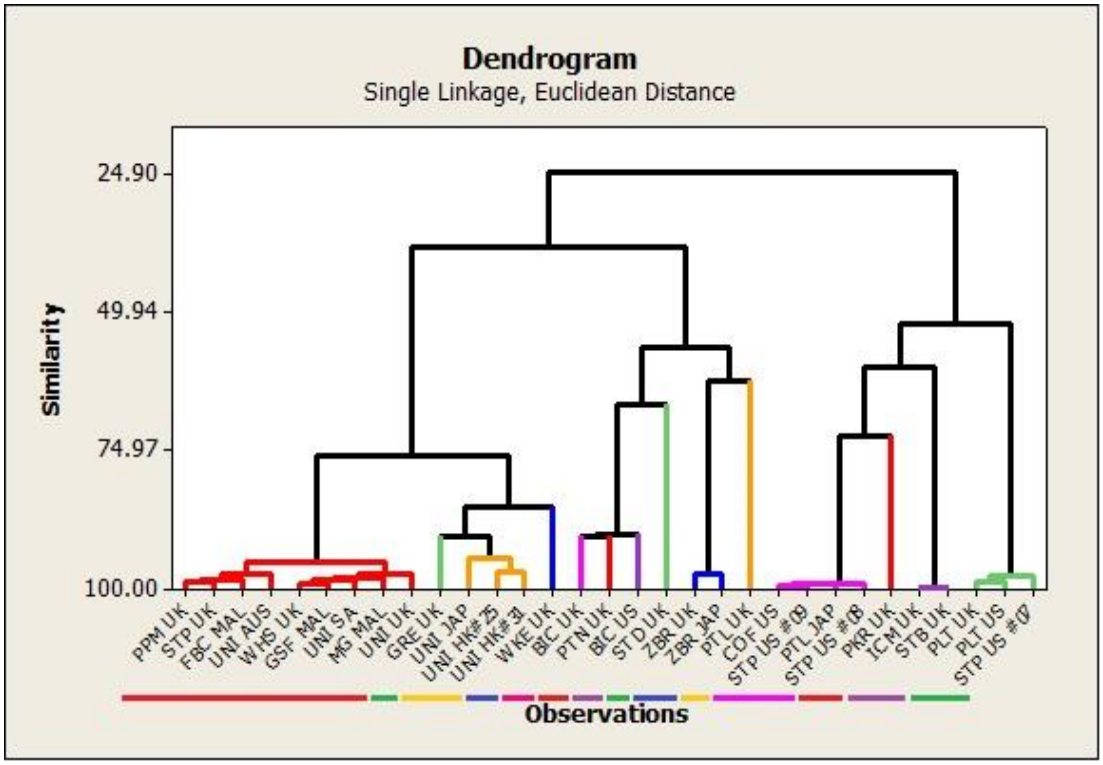


Figure 7.27: HCA dendrogram of blue ink group samples analysed by FTIR-ATR presented in a 32 BIN data matrix format reflecting T3 level visual pattern recognition groups. HCA resulted in misclassification of samples, and hence was inconsistent with the expected 14 clusters distinguished by visual pattern recognition

7.3.2.2.1.3.3 Artificial Neural Network (ANN) Analysis

SOFM analysis revealed similar observations to HCA, with the exception of the BIC US sample occupying a different cluster to the BIC UK and PTN UK samples. In addition, the UNI AUS, FBC MAL, STP UK and PPM UK samples were clustered separately in a single group as illustrated in Figure 7.28. Some similarity between the visual and statistical clustering was observed when a lower number of expected clusters were inputted into the software, as illustrated in Figure 7.29, but it was not possible to statistically confirm the visual clustering fully. Taking into consideration variations in colour hue intensity within a cluster provided no further meaningful discrimination.

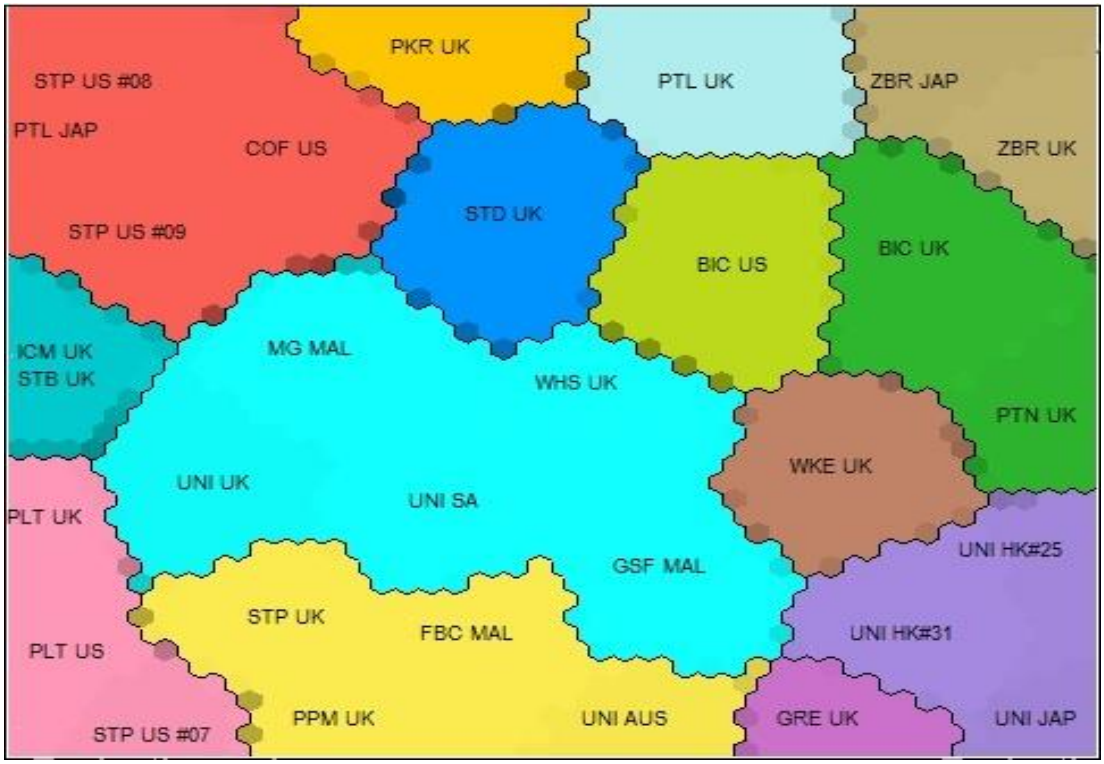


Figure 7.28: SOFM of blue ink group samples analysed by FTIR-ATR presented in a 32 BIN data matrix format reflecting T3 level visual pattern recognition groups. SOFM resulted in misclassification of some samples inconsistent with the expected 14 clusters distinguished by visual pattern recognition

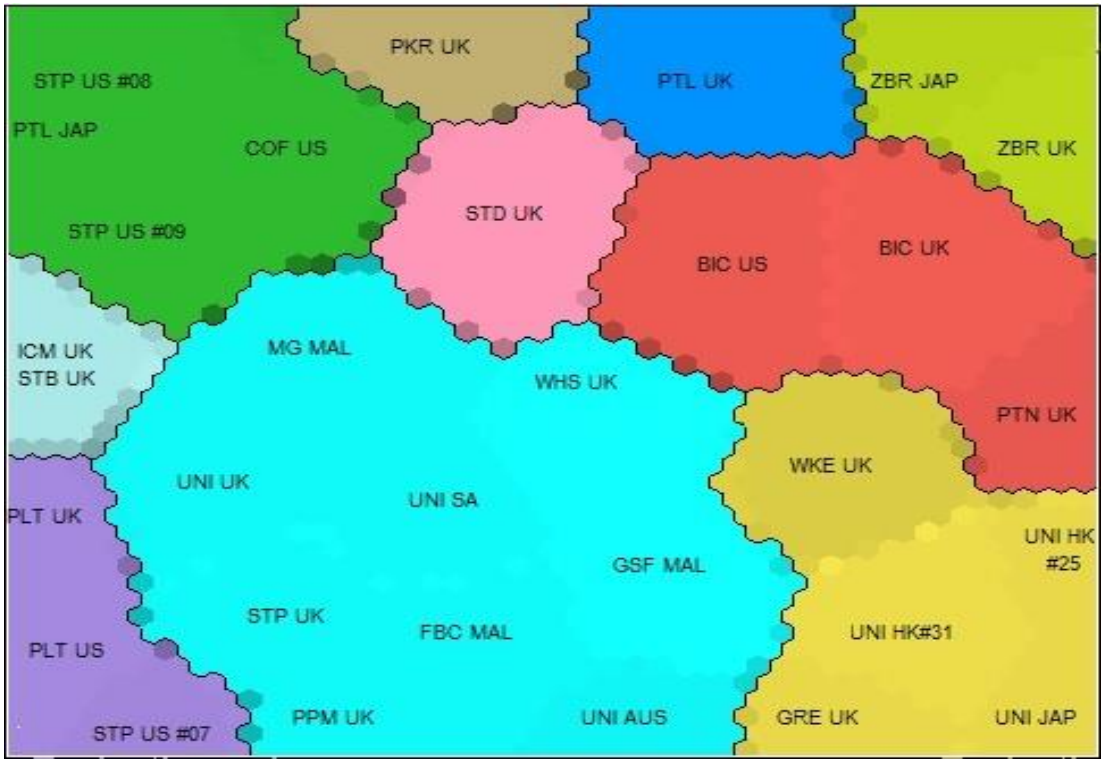


Figure 7.29: SOFM of blue ink group samples analysed by FTIR-ATR presented in a 32 BIN data matrix format reflecting T3 level visual pattern recognition groups. Inputting the expected number of clusters as 10 resulted in an SOFM demonstrating some, but not full, similarity to the expected 14 clusters distinguished by visual pattern recognition

7.3.2.2.1.4 T2 Data Matrix (32 BINS)

Statistical interrogation of the T2 32 BIN data matrix provided the most interesting observations. A T1 data matrix was not prepared since it was considered that if the T2 data matrix yielded the correct classifications then a T1 data matrix would also by default, since the differences between the two tiers were simply in the number of discriminating peaks and relative peak intensities.

7.3.2.2.1.4.1 Principal Component Analysis (PCA)

Five of the expected six clusters for T2 visual discrimination were identified, and were well resolved as illustrated in Figure 7.30. This discrepancy related to the three Asian UNI samples, which according to T2 visual classification could be discriminated from several other samples that originally formed Group 1 in T1 on the basis of differences in RPI. The PCA showed the Asian UNI samples were too closely clustered to the other similar samples to be considered a separate cluster.

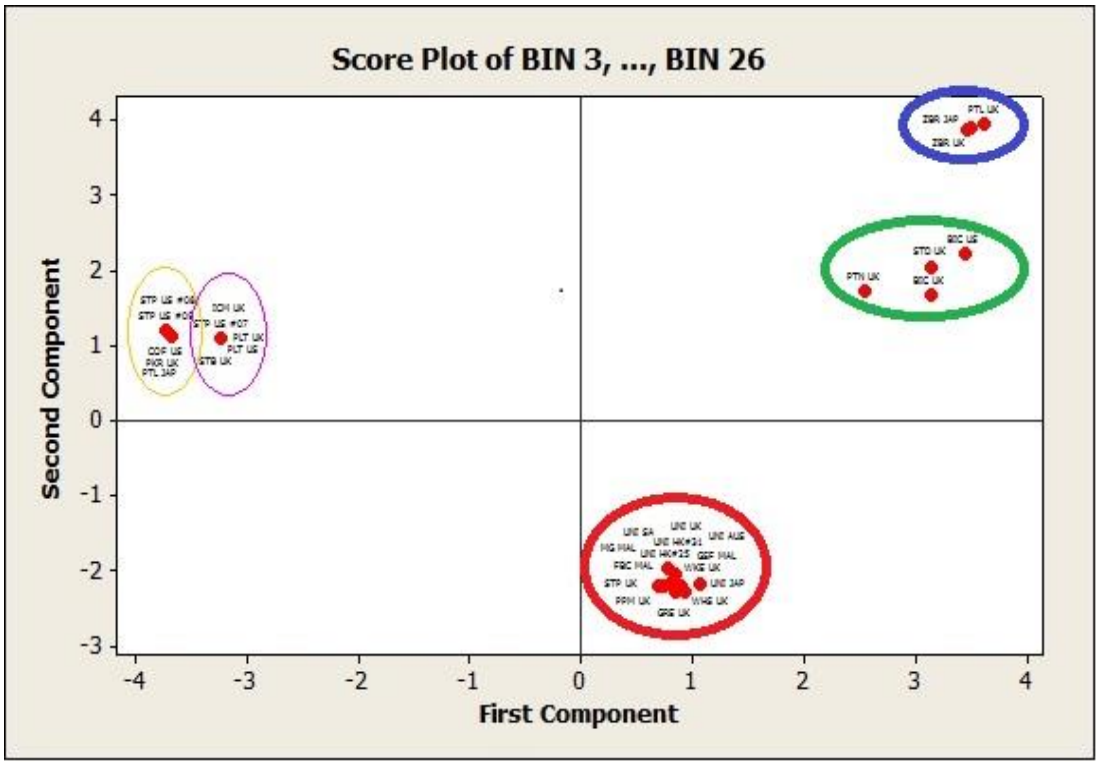


Figure 7.30: PCA score plot of blue ink group samples analysed by FTIR-ATR presented in a 32 BIN data matrix format reflecting T2 level visual pattern recognition groups. PCA discrimination resulted in only five clusters albeit highly similar to the expected six clusters distinguished by visual pattern recognition

7.3.2.2.1.4.2 Hierarchical Cluster Analysis (HCA)

Similarly, for HCA, the same five clusters were identified as illustrated in Figure 7.31. Increasing the number of expected clusters to six simply resulted in a misclassification of the samples, rather than the isolation of the Asian UNI samples as desired. Standardisation of variables did not resolve this.

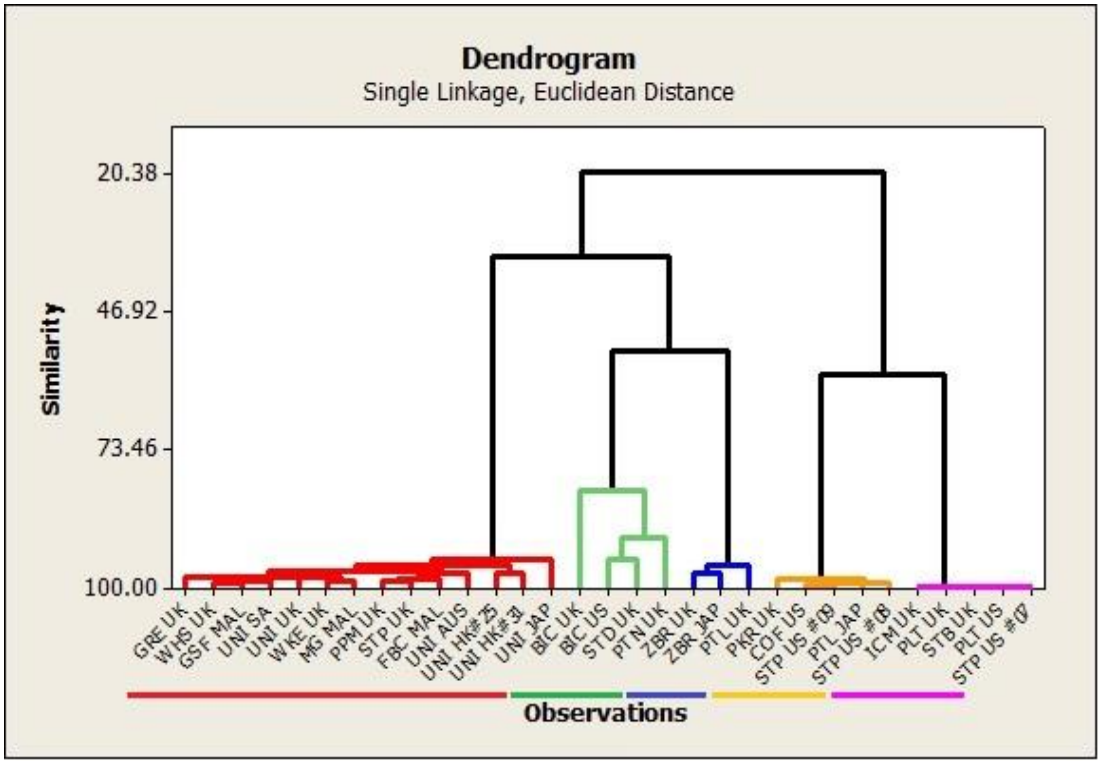


Figure 7.31: HCA dendrogram of blue ink group samples analysed by FTIR-ATR presented in a 32 BIN data matrix format reflecting T2 level visual pattern recognition groups. HCA discrimination resulted in only five clusters albeit highly similar to the expected six clusters distinguished by visual pattern recognition

7.3.2.2.1.4.2.1 Investigation of Different Algorithm Combinations for HCA

When different algorithm combinations were investigated, a change to Ward Linkage in combination with Euclidean Distance did result in correct classification of the six expected T2 groups as illustrated in Figure 7.32. This was also achievable using Pearson Distance (the "square root of the sum of square distances divided by variances" [169]), which in addition, when used in combination with Single Linkage also yielded the same five clusters identified initially using Euclidean Distance. Standardisation of variables yielded the same sets of classifications. The application of all other algorithm combinations with or without Standardisation of variables to both T2 and T3 32 BIN data matrix resulted in misclassification.

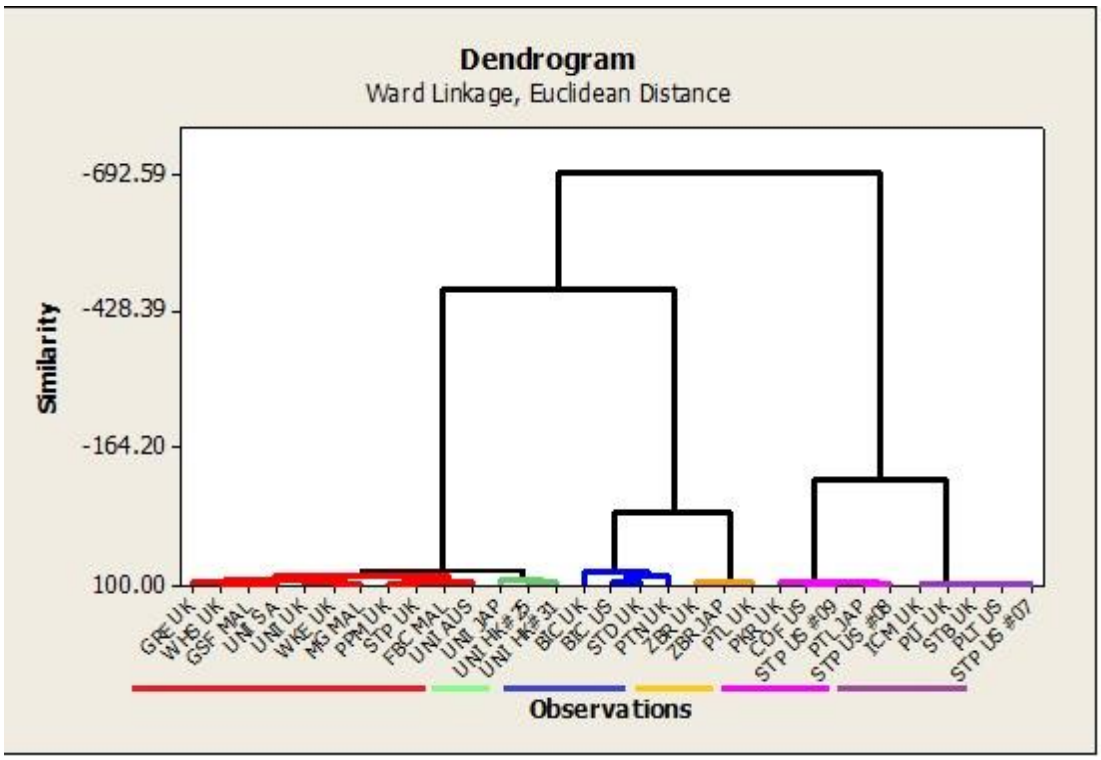


Figure 7.32: HCA dendrogram of blue ink group samples analysed by FTIR-ATR presented in a 32 BIN data matrix format reflecting T2 level visual pattern recognition groups. HCA resulted in successful discrimination into six clusters in line with T2 level visual discrimination when using a combination of Ward Linkage and Euclidean Distance

7.3.2.2.1.4.3 Artificial Neural Network (ANN) Analysis

SOFM analysis resulted in the same five clusters being identified, as per the other techniques, of the T2 32 BIN data matrix demonstrated in Figure 7.33. In similar fashion to HCA, increasing the number of expected clusters did not provide the desired isolation of the three Asian UNI samples and only resulted in conflict with the T2 visual classification groupings. Variation in colour hue of the segments representing the Asian UNI samples also failed to reflect their isolated discrimination from the other samples within the blue cluster.

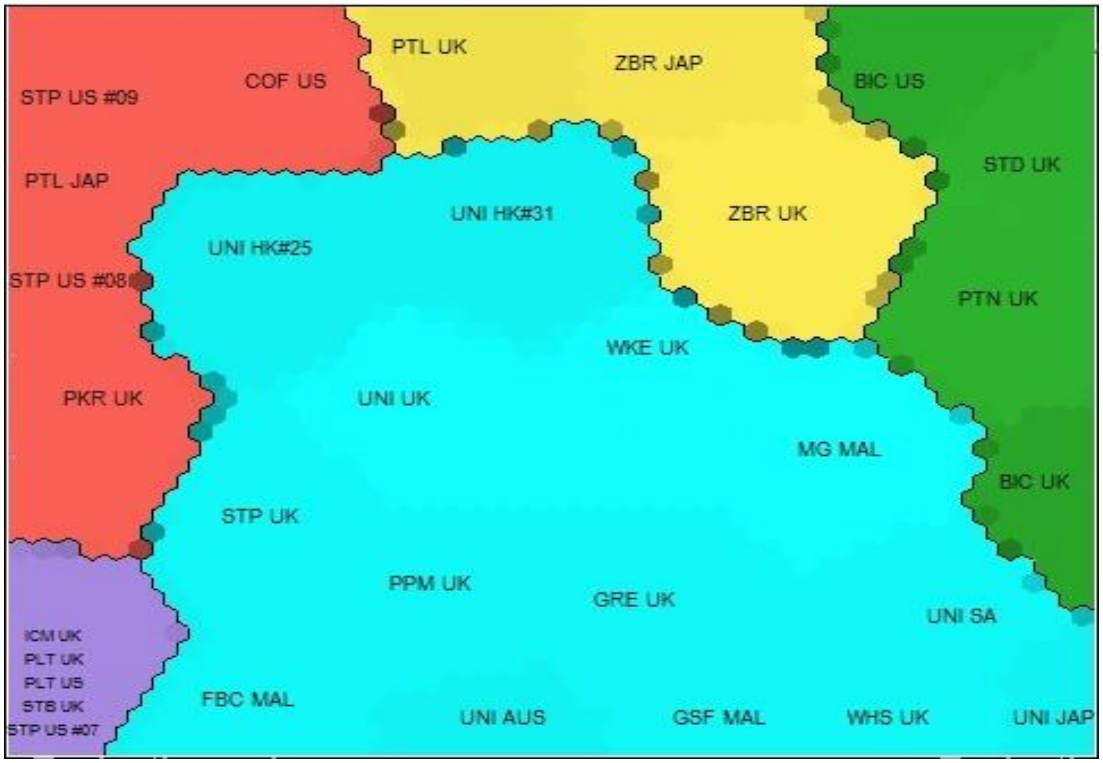


Figure 7.33: SOFM of blue ink group samples analysed by FTIR-ATR presented in a 32 BIN data matrix format reflecting T2 level visual pattern recognition groups. SOFM discrimination resulted in only five clusters, albeit highly similar to the expected six clusters identified by visual pattern recognition

7.3.2.2.1.5 First Derivative (32 BINS)

When subjected to all three statistical techniques successful clustering in line with the nine identified visual classifications based on the first derivative spectral data set, was unachievable. With respect to HCA, an investigation of all possible algorithm combinations with or without Standardisation of variables resulted in a high level of misclassification when compared to the visual pattern recognition groups as highlighted in Figure 7.34.

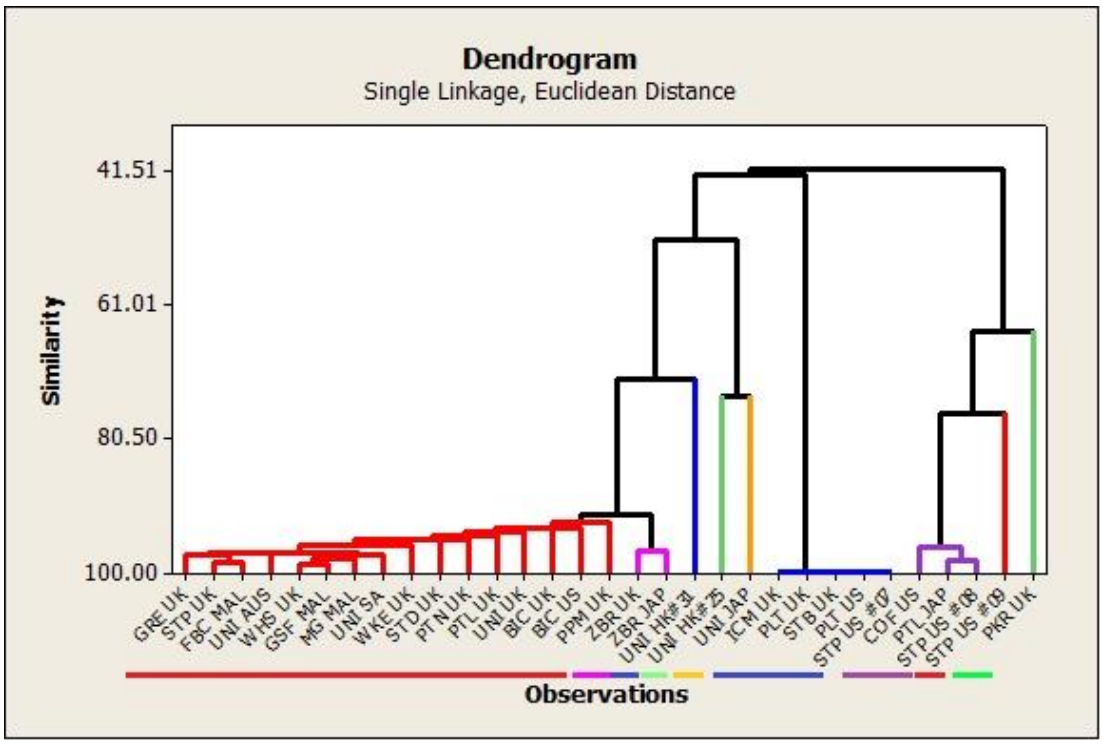


Figure 7.34: HCA dendrogram of blue ink group samples analysed by FTIR-ATR representing the first derivative data set and illustrating misclassification of samples within the expected nine clusters distinguished by visual pattern recognition

7.3.2.2.1.5.1 Influence of Relative Peak Intensity (RPI)

An interesting observation from this study of the blue ink group is that of the possible influence of relative peak intensities (RPI) upon the HCA discrimination of samples. This is best observed in the T3 32 BIN HCA dendrogram, where some samples were correctly classified in accordance with the visual classifications whilst others were not.

Some samples in T3 were only distinguishable visually on the basis of differences in (RPI). For example, based on visual classification, the COF US sample could only be distinguished from the PTL JAP, STP US #08 and STP US #09 samples on the basis of a weaker RPI in peak absorbencies around 918 cm^{-1} and 1245 cm^{-1} observed in the latter four samples. Likewise, the UNI UK and MG MAL samples were discriminated from several other pigmented samples on the basis of a shoulder at 698 cm^{-1} and a weaker RPI of the peak around 1118 cm^{-1}.

These differences were not detected in the HCA, yet the three Asian UNI samples, which were discriminated into a single cluster visually on the basis of a notably weaker RPI at 1118 cm^{-1}, were detected. This suggests that the difference in RPI for some samples was

insufficient to be able to statistically discriminate them by HCA, whilst for others samples the difference was sufficient to be recognised by HCA. Several other samples were also visually discriminated partially on the basis of RPI differences, and these classifications have been confirmed by HCA. However, the presence or absence of additional peak absorbencies was also a feature of discrimination for these samples, and as such it is difficult to assess how much influence RPI has on their statistical discrimination.

Interestingly for the T2 32 BIN data matrix, where only the three Asian UNI samples were visually discriminated on the basis of RPI differences, a change of algorithm combination was required to detect this difference by HCA. For T3 and T4 32 BIN data matrices, a change in algorithm combination did not further discriminate the samples named above into the correct visual classifications.

7.3.2.2.2 Red Ink Group

7.3.2.2.2.1 T1 – T2 Data Matrices

Since T1 visual classification was based on the presence or absence of peak absorbencies and T2 was based on relative peak intensities (RPI). This provided an opportunity to explore the influence of RPI on statistical discrimination further.

7.3.2.2.2.1.1 Principal Component Analysis (PCA)

The 26 red ink group samples were discriminated into 14 groups by visual pattern recognition for T1, and 24 groups for T2. Based on PCA, the samples were clustered into 13 groups, with only the PTN UK sample unresolved from the two PLT samples thus accounting for the discrepancy in the number of expected clusters. This is illustrated in Figure 7.35.

Since many of the samples were classified individually as single groups, separation between clusters was generally considered good. For Group 1 (red circle) clustering was quite tight, but arguably samples were grouped into three sub-clusters which did not reflect the T2 visual classification, suggesting that RPI differences were insufficient for discrimination. Arguably, the STP UK and MG MAL samples (Group 4 (orange circle)) which were positioned next to one another in the PCA score plot, could be separated into two individual clusters which would support the view that they could be discriminated.

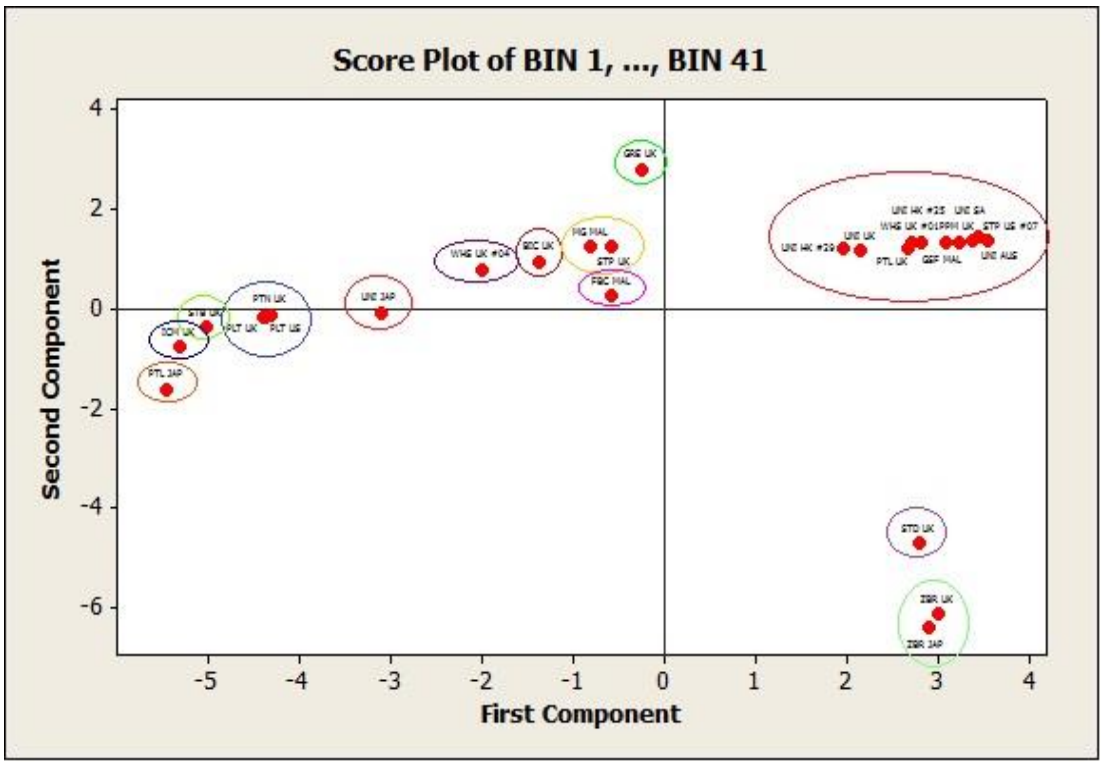


Figure 7.35: PCA score plot of red ink group samples analysed using FTIR-ATR demonstrating clustering into 13 groups differing to the expected 14 groups discriminated by visual pattern recognition due to poor resolution between PTN UK and the PLT samples

7.3.2.2.2.1.2 Hierarchical Cluster Analysis (HCA)

When the expected number of clusters was inputted as 14, in line with T1 visual discrimination, correct classification was achieved with or without Standardisation of variables. An example of the HCA is presented in Figure 7.36. However, when the expected number of clusters was increased to 15, misclassification occurred. One would have expected the MG MAL and STP UK samples to be resolved from one another, since T2 visual discrimination suggested that they were distinguishable on the basis of an RPI difference, this was not the case. Standardisation of variables also resulted in misclassification of samples.

For T2, the expected number of clusters was 24 however, a number of incorrect classifications were observed. For example, the MG MAL and STP UK samples were not discriminated from each other, whilst, the two ZBR samples were unexpectedly resolved from one another. In addition, for the ten samples associated with Group 1 in T1, discrimination into nine groups rather than the expected ten was observed. Standardisation of variables provided no further discrimination.

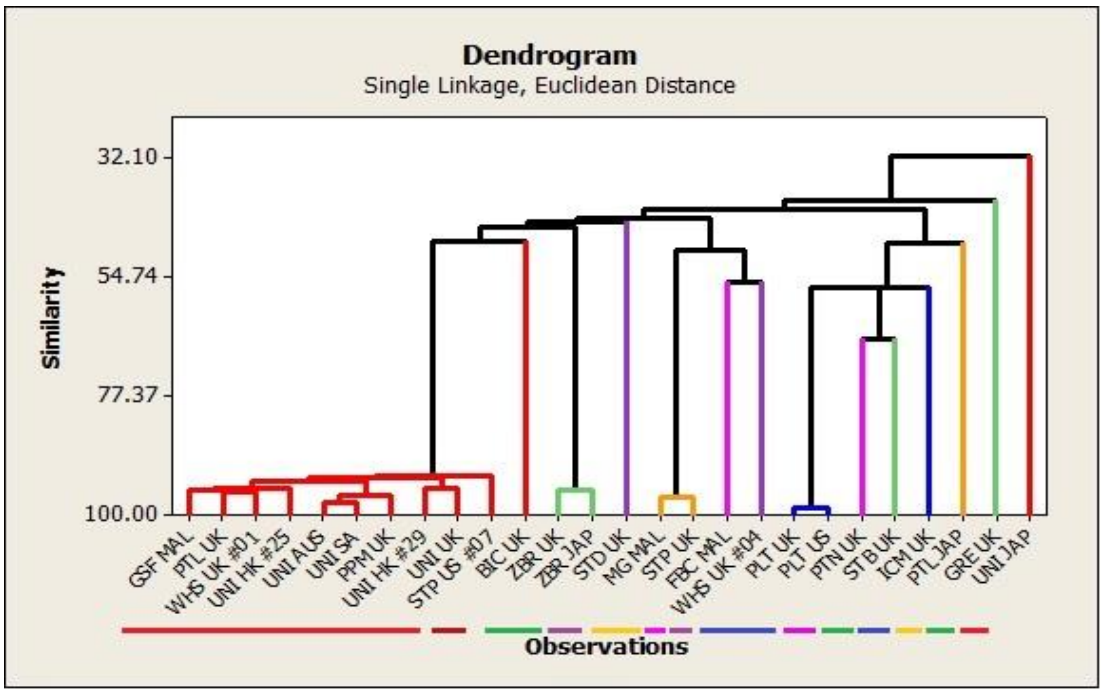


Figure 7.36: HCA dendrogram of red ink group samples analysed by FTIR-ATR demonstrating successful discrimination into 14 clusters in line with T1 level visual pattern recognition groups

An investigation of various algorithm combinations to explore further the visual classifications obtained using the T1 and T2 criteria was undertaken in order to test whether or not the mathematical methods confirmed or otherwise the visual classification.

For T1, correct classification of 14 clusters was achieved without Standardisation of variables using all algorithm combinations with the exception of Ward Linkage/Manhattan Distance ("the sum of absolute distances" [169]) and Complete Linkage ("the distance between two clusters is the maximum distance between an observation in one cluster and an observation in the other cluster" also known as furthest neighbour [169])/Manhattan Distance. Standardisation made no appreciable difference. Furthermore, increasing the number of expected clusters to 15 to account for a possible difference in RPI between STP UK and MG MAL samples was not supported by HCA using any of the available algorithm combinations with or without Standardisation. The same was true when the expected number of clusters was increased to 24 in line with T2 visual classification groupings. An example of the HCA generated using Single Linkage and Euclidean Distance when the expected number of clusters was inputted as 15 and 24 is presented in Figure 7.37 and Figure 7.38 respectively.

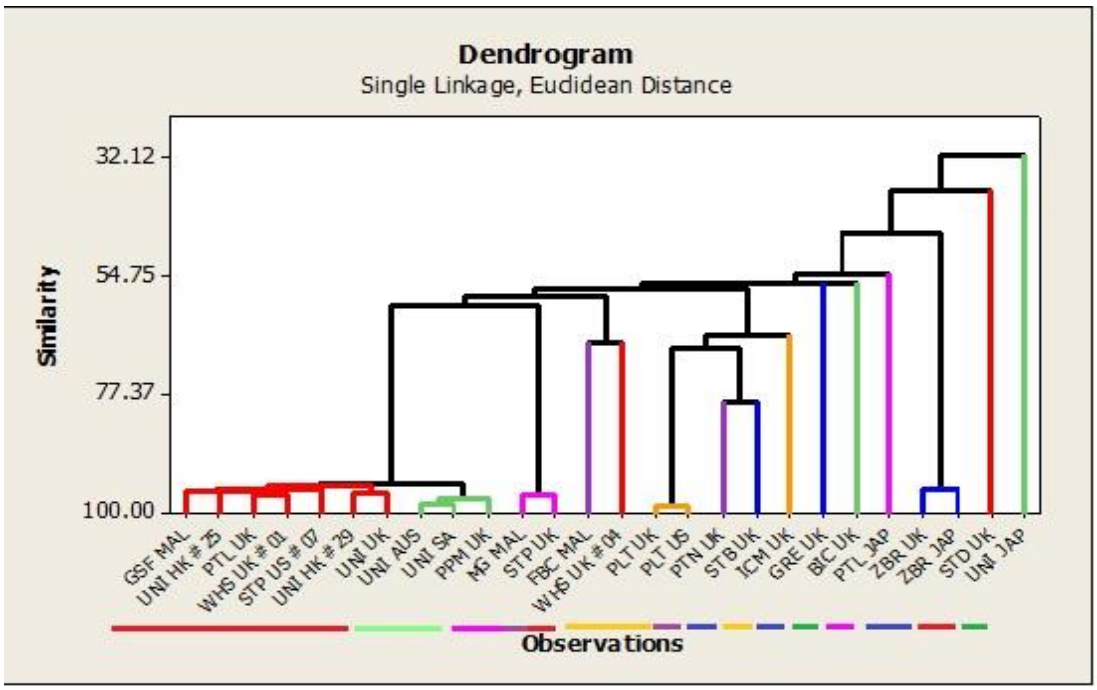


Figure 7.37: HCA dendrogram of red ink group samples analysed by FTIR-ATR demonstrating discrimination into 15 clusters. The expected discrimination between STP UK and MG MAL samples is not observed, instead sub-division of the largest group (n = 10) occurs. This demonstrates inconsistency between multivariate profiling and visual pattern recognition observations suggesting relative peak intensity differences used to discriminate the STP UK and MG MAL samples could not be validated

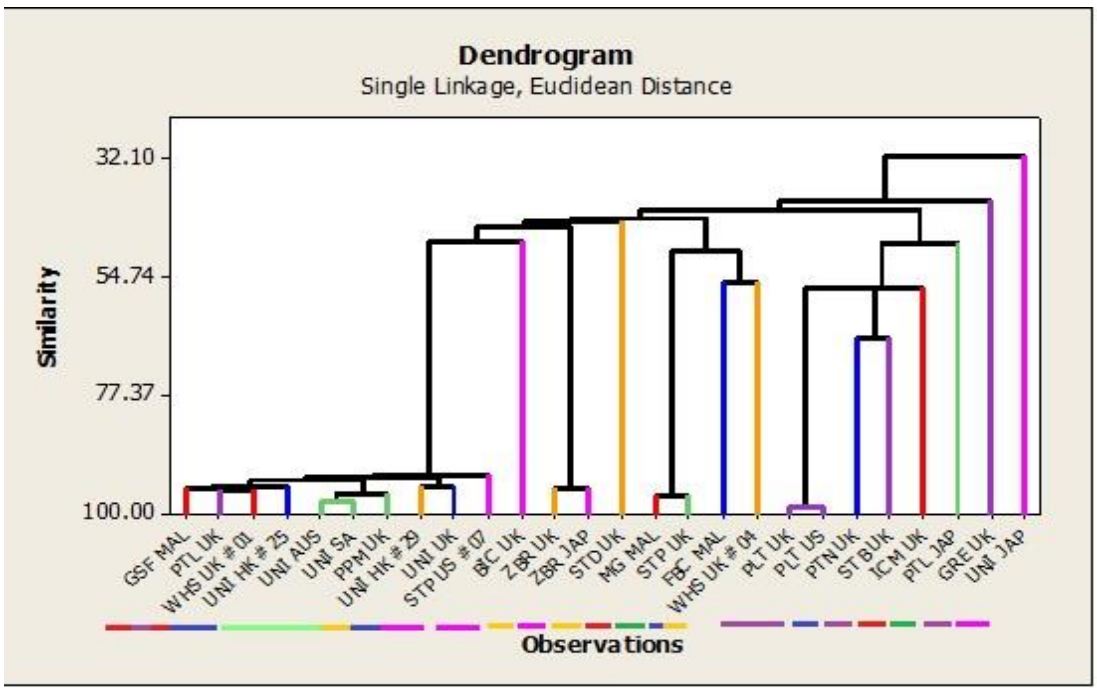


Figure 7.38: HCA dendrogram of red ink group samples analysed by FTIR-ATR demonstrating discrimination into 24 clusters inconsistent with T2 level visual pattern recognition groups

7.3.2.2.2.1.3 Artificial Neural Network (ANN) Analysis

SOFM, as shown in Figure 7.39, resulted in correct classification of samples when the expected number of clusters was given as 14 in line with T1 visual comparison. If more clusters were suggested, misclassification of samples was observed, demonstrated by the SOFM presented in Figure 7.40 representing 24 T2 clusters. These findings suggest that for the red ink group, the statistical methodology in terms of HCA and SOFM in particular, supports the minimum number of classifications made by visual pattern recognition, i.e. T1, but refutes any further meaningful discrimination based on RPI differences alone, i.e. T2.

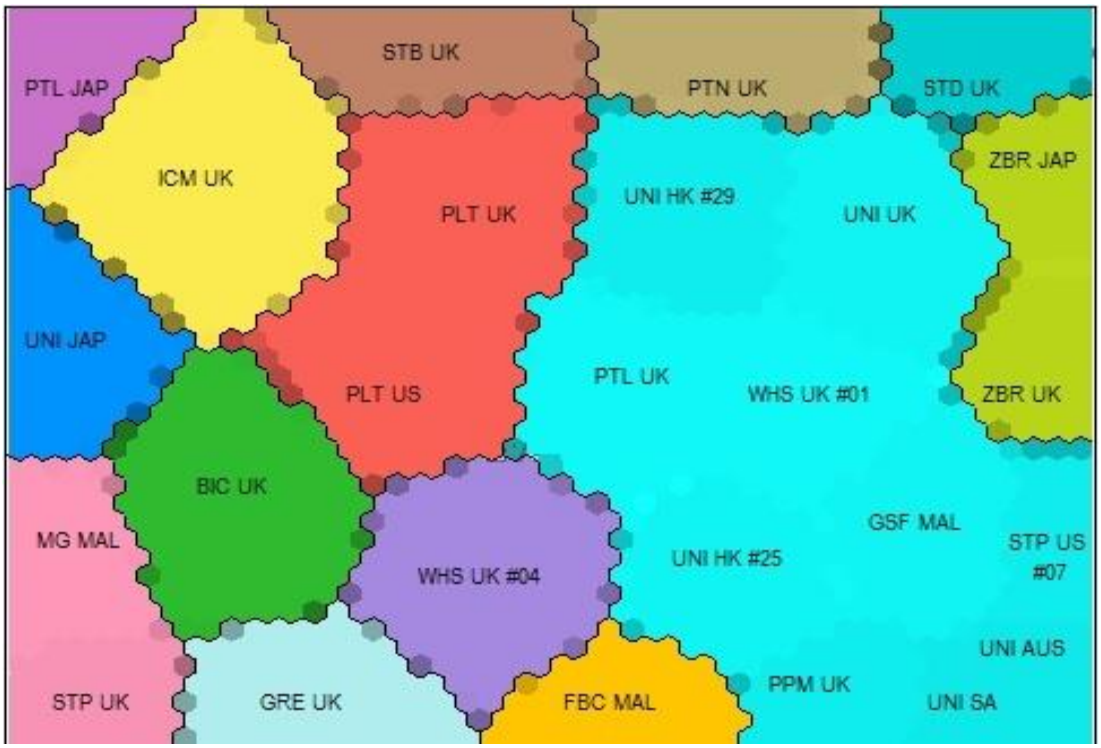


Figure 7.39: SOFM of red ink group samples analysed by FTIR-ATR demonstrating successful discrimination into 14 clusters in line with T1 level visual pattern recognition groups. Minimal variation in colour hue within a cluster containing multiple ink samples demonstrates a high degree of similarity between samples within that cluster

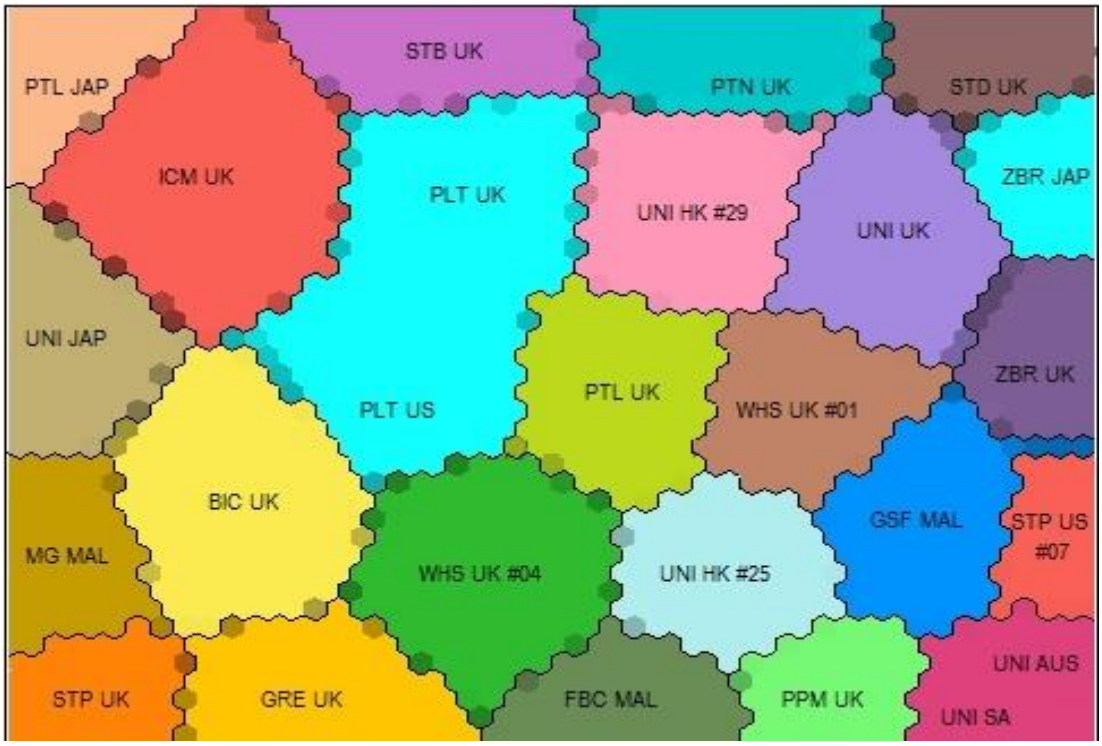


Figure 7.40: SOFM of red ink group samples analysed by FTIR-ATR demonstrating discrimination into 24 clusters inconsistent with T2 level visual pattern recognition groups

7.3.2.2.2.2 First Derivative

Visual pattern recognition resulted in the red ink group being discriminated into 20 groups based on first derivative spectral data. These largely corresponded to those made based on the original spectra, with the only difference being that the ten samples making up Group 1 in T1 formed five sub-groups rather than ten individual groups. The first derivative also supported the view that the STP UK and MG MAL samples were distinguishable.

Successful classification into the 20 expected clusters was not achieved via any of the three statistical treatments. Furthermore, with respect to HCA, correct classification could not be achieved through any of the algorithm combinations with or without Standardisation of variables.

7.3.2.3 Conclusion: Multivariate Analysis

A statistical methodology using PCA, HCA and SOFM for the successful discrimination of gel inks on paper was developed for both Raman and FTIR-ATR spectroscopic data. The

statistical methodology is based on three key data pre-processing steps of data reduction, binning and normalisation.

For the Raman spectral data, successful classification of both colour groups was achieved irrespective of the excitation wavelength and/or instrument used. In general, sub-classification within a group was not detected. However, dependent upon excitation wavelength, a change of algorithm combination, in respect of HCA, could detect sub-groups. The methodology can be applied successfully to both uncorrected and baseline corrected spectral data.

For the FTIR-ATR spectral data, pre-treatment and subsequent successful classification of samples proved to be more complex. For both colour groups, discrimination of the samples based on visual pattern recognition led to a number of tiers and samples were classified into groups based on the presence or absence of certain peak absorbencies and differences in relative peak intensities. For the red ink group, samples were correctly classified only in accordance with the lowest Tier level (i.e. T1 = 14 groups). The blue ink group could be reliably classified into five groups instead of the expected six (for T2) unless a change of algorithm combination was instigated. These findings suggest that differences in relative peak intensities are a less reliable discriminator than the presence or absence of peak absorbencies. In addition, smaller peak absorbencies were a less reliable discriminator than stronger peak absorbencies, although to an extent some contradiction on this point was noted from the findings of the blue ink group. The statistical methodology applied was found to only be suitable for original spectral data and not the corresponding data in its first derivative form.

All three statistical techniques were found to be useful, although in general better clustering was achieved using HCA or SOFM rather than PCA. For the latter, although good clustering was achieved, resolution between clusters was sometimes an issue and prior knowledge of which samples belonged to which cluster was essential. For the former, only prior knowledge of the expected number of clusters was required to achieve correct classification.

For HCA, an algorithm combination of Single Linkage with Euclidean Distance was generally successful, although for both instrumental techniques a combination of Ward Linkage with Euclidean Distance was sometimes found to achieve the desired outcome when the former could not, e.g. sub-classification. The Pearson Distance was also found to yield the same result when used in conjunction with Euclidean Distance under certain circumstances.

In terms of pre-processing, all three steps of data reduction, binning and normalisation were essential in achieving anything close to the desired or expected outcome. Furthermore, it was determined that if a greater number of BINS were used to reduce the data set, the sensitivity of the multivariate analysis improved, thus increasing the opportunity of achieving the desired outcome.

The proposed methodology confirmed the classification groupings made by visual pattern recognition of the Raman spectral data with 100% accuracy, but for the FTIR-ATR spectral data it highlighted similarities and differences between the subjective and objective classifications. For this reason, it is therefore recommended that at present, if used in operational casework, final conclusions drawn by an examiner should be based on the consideration of both subjective opinion and objective classification of the data. This would reduce the likelihood of erroneously favouring one approach over the other. Further development of a multivariate profiling approach may improve this situation, eventually leading to total reliance on multivariate profiling.

Taking into consideration both the multivariate profiling and qualitative discriminations described above, a minimum DP for the combined analytical sequence for a given colour group was determined. For the blue ink group, this value was 0.93, corresponding to the minimum value described earlier. If further discrimination of the Asian UNI samples by FTIR-ATR is also taken to be valid as suggested by a change of algorithm combination above, then the DP would increase to 0.96. For the red ink group the minimum DP of 0.98 also stands. Clearly these findings suggest that the combined analytical approach described in this research offers a high level of discrimination for both blue and red gel inks, which in part is supported by an objective multivariate statistical assessment of Raman and IR spectral data.

CHAPTER EIGHT

Conclusions and Further Work

8.1 Blue Ink Group

8.1.1 Within Brand Variation

No within brand variation was detected amongst the blue ink group brand/model combination sets studied using TLC, VSC 6000/HS (IRR and IRL), Vis-MSP or FTIR-ATR. However, using Raman Spectroscopy at high excitation wavelengths, some evidence of within brand variation in two brand/model combinations was observed.

Ink from pen #02 and #03 of the ZBR UK sample set exhibited a reproducible Raman response at 785 nm and 830 nm that differed to that of the other four ZBR UK pens in the set in that the spectrum was partially obscured by fluorescence. No within brand differences were observed at the other Raman excitation wavelengths, or by any other technique studied for this brand/model combination set.

Ink from pen #06 in the COF US sample set exhibited a reproducible spectrum that differed from the other COF US pens in terms of peaks present at 830 nm. At other excitation wavelengths, this sample set exhibited spectra fully obscured by fluorescence and/or detector overload indicating the presence of a dye component, suggesting a hybrid gel ink formulation. No observable differences were evident within the COF US set of pens for analysis using TLC, Vis-MSP and FTIR-ATR suggesting the potential of Raman to provide the only discriminating evidence in some cases.

8.1.2 Between Brand Variation

Solubility testing permitted the 31 brand/model combinations of blue gel ink to be discriminated into 10 dye containing and 21 pigment based samples. Acetic acid was the most effective solvent of those tested for the solvation of the dye containing samples, and worked well even without the application of heat. Protonation of the pigment based samples in hydrochloric acid appeared to be ineffective for subsequent successful TLC, but

perhaps further testing using a solvent such as dimethylformamide (DMF), known to extract slightly soluble phthalocyanine pigments [178], should be investigated.

All three solvent systems tested worked well for the separation of the 10 dye containing samples, permitting discrimination into eight groups based on the number and colour of component bands visualised. Only four brand/model combinations could not be distinguished by TLC alone, and no further discrimination was possible by comparison of the % reflectance spectra of the component bands.

Based on the camera and optical filters selected for IR absorption (IRR) and IR luminescence (IRL) examination, the blue ink group samples were discriminated into a maximum of ten groups, representing either the dye or pigment based samples only. IR luminescence was more discriminating than IR absorption, and incorporating the white light (380 – 800 nm) optical filter into the former partially resolved interference from the paper substrate affecting discrimination between samples at the other wavelengths studied.

A wide array of different Vis-MSP spectral patterns from the blue ink group samples were observed, permitting discrimination into at least ten groups based on original spectra that again reflected either dye or pigment based samples. Further discrimination by additional comparison of the first derivative spectra for each brand/model combination into a maximum of 16 groups was considered possible. Discrimination was based upon features such as overall spectral shape, λ_{max} and λ_{min} positions and relative peak intensities. Reproducibility of spectra within a brand/model combination set was generally good apart from four brand/model combinations where wide spectral variation was observed, apparently linked to the distribution of the ink on paper. This emphasised the importance of taking spectra from several areas of the same ink line to take into account sample variation when conducting casework examinations to avoid erroneous discrimination. For qualitative comparison, spectral interference from the paper substrate was not considered an issue. Normalisation and smoothing (35 pts) of the spectra using the Savitsky-Golay [130] algorithm within the instruments software aided spectral comparison within and between samples, as did comparison of highly similar spectra from different brand/model combinations on the same axis.

Reproducibility of FTIR-ATR spectra within a sample set was good only when ink spots (approximately 1 cm diameter) were used as opposed to ink lines, the latter being more in keeping with those encountered in forensic casework. The use of an imaging system incorporated into the FTIR-ATR instrument may be able to resolve the reproducibility issues associated with smaller sample sizes permitting accurate contact between the ink and the ATR crystal. The use of average spectra to represent a brand/model combination was possible and an 11 point smoothing function using the Savitsky-Golay algorithm aided spectral comparison between samples with minimal loss of detail.

Clear evidence of spectral difference between brand/model combinations could be observed in the original spectra generated by FTIR-ATR, in spite of heavy spectral interference from the paper substrate. Peaks attributable to the ink could be identified, permitting the construction of peak tables used to discriminate between samples. Using the original spectra, the blue ink group could be discriminated using a four-Tier system (T1 – T4) based on the presence or absence of peak absorbencies and relative peak intensities occurring in the fingerprint region (1400 – 650 cm^{-1}). The samples were separated into a minimum of three (T1) and maximum of six (T2) groups using peaks with strong absorbencies and further discrimination into a minimum of 11 (T3) and maximum of 17 (T4) groups based on the presence or absence of relatively weak peak absorbencies. Use of the first derivative spectra either supported or added minimal additional discriminatory value, giving a minimum of six and maximum of nine separate groups. All classification groupings reflected either dye or pigment based samples. Discrimination based on relatively strong peak absorbencies was considered to be more reliable, however the usefulness of weaker absorbing peaks may be resolved if the pure ink samples from the 31 brand/model combinations were deposited directly onto the ATR crystal, removing any potential interference from the paper substrate.

With respect to Raman Spectroscopy, spectral interference from the paper substrate was observed to a greater extent at higher excitation wavelengths, (785 nm and 830 nm), than lower excitation wavelengths, (514.5 nm and 685 nm), but was considered minimal with only a peak at ~1094 cm^{-1} seen frequently. Stray peaks were also an issue at higher excitation wavelengths, in particular with the Renishaw InVia instrument. Performing spectral acquisition in low ambient light reduced this problem, but did not resolve it

entirely. A “zap” function provided in the Renishaw software assisted greatly in the removal of stray peaks, and the FORAM software could benefit from a similar function.

Spectra of sufficient quality suitable for comparison and discrimination was acquired from ink lines at the four main excitation wavelengths studied: 514.5 nm, 685 nm, 785 nm and 830 nm. Reproducibility of spectra was generally good, with the exception of some acquired at 830 nm, where stray peaks, fluorescence and/or detector overload and excessively high spectral intensity were observed. Despite this, based on the presence or absence of peaks and relative peak intensities, the blue ink group samples could be discriminated into between 4 - 6 groups dependent upon the excitation wavelength used. The greatest level of discrimination was achieved using a combination of 514.5 nm and 830 nm, distinguishing the 31 blue inks into a maximum of 9 spectral groups. As for previous techniques all groupings reflected either dye or pigment based samples with the 514.5 nm wavelength particularly useful for the discrimination of pigmented gel inks.

Using all excitation wavelengths in combination provided the greatest amount of information about the blue ink group samples, emphasising the importance of adopting a multi-wavelength approach. Several pigment based samples exhibiting very similar, spectra at one excitation wavelength, i.e. 514.5 nm or 785 nm, could be clearly discriminated on the basis of several differences in spectra acquired at 830 nm. Of the ten dye containing samples identified by TLC, Raman spectra suggested seven also contained a pigment component, indicative of a hybrid gel ink formulation. Comparison of spectra acquired to a purpose built in-house spectral library composed of 200 pigment standards analysed using the FORAM 785, confirmed phthalocyanine pigments as the primary colorant in at least 14 of the 21 pigmented gel inks, whilst a fluorescent blue pigment was identified as a possible primary colorant in four other samples.

At 785 nm, reproducible differences in background fluorescence shape observed for some brand/model combinations could arguably be used as an additional feature for further discrimination within a group. However, for other brand/model combinations, fluorescence background shape was not always found to be reproducible, and furthermore, baseline correction could be used to remove fluorescence to present indistinguishable

spectra within a group. As such, fluorescence background shape as an additional feature for discrimination should be treated with caution.

Raman Spectroscopy demonstrated that some pen manufacturers appeared to use different colorants for the same model of pen sold in different geographical locations, whilst others did not. For example, Pilot appeared to use the same colorant in the same pen model (G2-07 Retractable) purchased from two different geographical locations (UK and US), whilst Staples used different colorants (dyes and/or pigments) for the same model (Sonix) also purchased from two different geographical locations (UK and US). Furthermore, other manufacturers, i.e. Uniball, appeared to use the same colorant across a wide range of different models irrespective of geographical location.

8.1.3 Discriminating Power and Multivariate Analysis

Based on qualitative discrimination of the blue ink group samples using all five analytical techniques, a range of discriminating powers were calculated. Taking a conservative approach, based on classifications made using TLC (dye containing samples only), VSC 6000/HS (IRR and IRL combined), the lowest number of classifications made using original spectral data generated by Vis-MSP and FTIR-ATR (T1 level), and classifications made using all four main excitation wavelengths studied in Raman Spectroscopy, the discriminating power of the combined analytical approach was calculated to be at least 0.93.

A statistical methodology based on PCA, HCA, and SOFM was successfully applied to all Raman Spectroscopic data acquired at all four main excitation wavelengths and for the FTIR-ATR data. However, further investigation of a method applicable to Vis-MSP spectral data is needed. Data pre-treatment in the form of data reduction, binning and normalisation were essential in achieving successful objective mathematical classification in line with those made by subjective visual pattern recognition.

Classification groupings based on qualitative comparison of spectra from Raman and FTIR-ATR (only to T2 level) were confirmed by multivariate profiling, increasing the discriminating power of the combined analytical approach reliably to 0.96. Further discrimination using original IR spectra based on weak peak absorbencies was not supported by an objective statistical approach, suggesting these small peaks were less

reliable discriminators. Furthermore, the developed methodology did not support classifications made by comparison of the first derivative IR spectra.

For successful classification of the samples prior knowledge of the expected number of clusters was required. With respect to HCA, the most effective distance algorithm of those studied was found to be Euclidean Distance, although the Pearson Distance also achieved the desired outcome under some circumstances. The most effective linkage method was Single Linkage, which when used in combination with Euclidean Distance, usually provided the desired outcome. However, when this combination was ineffective, Ward Linkage was found to be of value, especially in confirming sub-classification groupings.

8.1.4 Summary

Solubility testing was effective at distinguishing the pigmented inks from those containing a dye. TLC was effective for discriminating ten dye containing samples into seven groups, with four brand/model combinations considered indistinguishable: PLT UK, PLT US, ICM UK and STP US #07. These samples were also considered indistinguishable by comparison of Vis-MSP and FTIR-ATR original spectra, and Raman spectra acquired by all four main excitation wavelengths studied. Analysis at 514.5 nm identified a pigment component in these samples suggesting hybrid formulations. IR luminescence permitted subjective discrimination into two groups: PLT UK/PLT US and ICM UK/STP US #07. The 21 pigment based samples were discriminated into at least nine groups when filtered light observations and original spectral data were considered in combination and this was supported by multivariate profiling of the IR and Raman spectral data. All four of the dye containing samples (PLT UK, PLT US, ICM UK and STP US #07) and several other pigment based samples could be distinguished if first derivative spectral data from Vis-MSP and weak peak absorbencies in FTIR-ATR original spectra were considered, but this was not supported by multivariate profiling.

Figure 8.1 provides a summary of the discrimination of the blue ink group samples based on the combined analytical approach described, and following the conservative view supported by multivariate profiling.

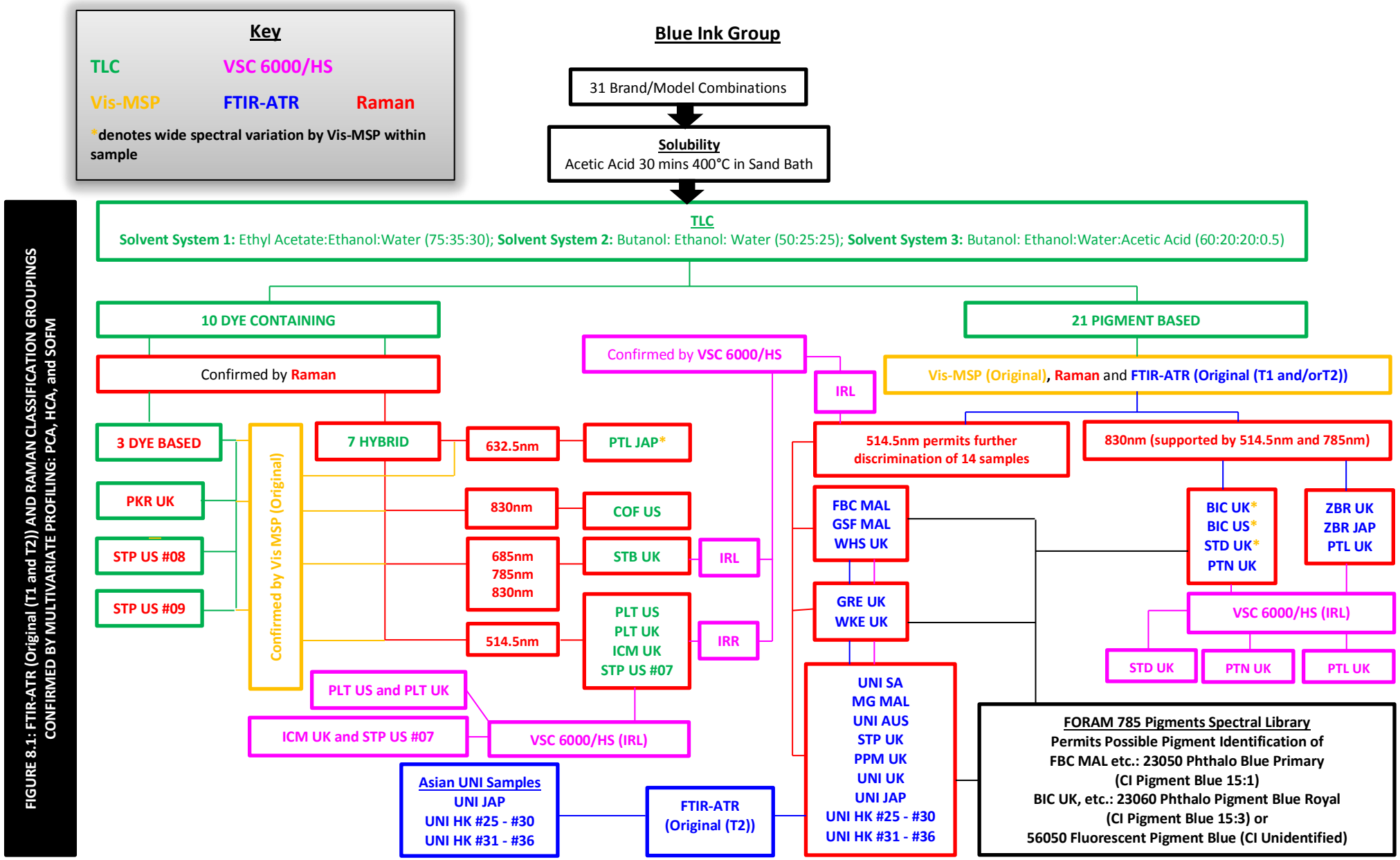


FIGURE 8.1: FTIR-ATR (Original (T1 and T2)) AND RAMAN CLASSIFICATION GROUPINGS CONFIRMED BY MULTIVARIATE PROFILING: PCA, HCA, and SOFM

8.2 Red Ink Group

8.2.1 Within Brand Variation

Of the brand/model combination sets studied, only one exhibited evidence of within brand variation across the techniques employed. TLC analysis suggested a difference between the WHS UK pens #01 - #03 and pen #04 - #06 samples demonstrated by a slight intensity difference in the hue of a red dye component. However, since only a single dye component with similar R_f value was extracted from all six pens, TLC alone was not sufficient to demonstrate within brand variation. A subtle difference in spectral shape using Vis-MSP also suggested this within brand variation, but, again in isolation was not considered definitive. VSC 6000/HS (IRL) and Raman Spectroscopy (785 nm) both highlighted clear and distinct differences between the two WHS UK sample sub-sets in terms of their IR luminescent and spectral appearance respectively. This confirmed at the very least, a change in colorant within the formulation of this brand/model combination had taken place during the manufacturing process. Furthermore, FTIR-ATR indicated that the ink from at least two pens (#04 and #05) likely contained a different additive composition to that of the other pens within the sample set, providing further evidence of within brand variation.

8.2.2 Between Brand Variation

Solubility testing revealed 23 of the 25 red ink group brand/model combinations examined contained a dye component. Acetone in combination with heating in a sand bath for between 15 – 30 minutes at 400°C was most effective for extracting the red inks. Heating for slightly longer may improve the extraction of some brand/model combinations, but risks degradation of some dye components. This warrants further investigation in order to refine the TLC protocol for red gel inks. The protonation extraction method appeared ineffective for subsequent TLC of the two pigmented samples (UNI JAP and STB UK).

The red ink group samples could be discriminated into five groups based on TLC, irrespective of the solvent system used. The majority of brand/model combinations (19) appeared to contain a single red dye component that with one exception (BIC UK) was indistinguishable across the samples. TLC confirmed two brand/model combinations as pigment based. The remaining four brand/model combinations, all contained at least two dye components, dark pink and pale pink, and were difficult to discriminate by TLC due to

the visualisation of non-reproducible component bands between TLC runs of different extract sets prepared under different heating times. This supported the view that longer heating times may result in degradation of the dye components. Comparison of % reflectance spectra from the TLC bands was of no value in further discriminating these samples. Tentative discrimination into two groups (PLT UK/PLT US and ICM UK/PTL JAP) was therefore made only on the basis of those component bands considered reproducible.

Examination by IR absorption/reflectance using the selected camera filters of the VSC 6000/HS was ineffective for the discrimination of all red ink group samples. This was also true for Vis-MSP examination, where all red ink group samples were characterised by a single broad peak between 600 nm – 780 nm, and a relatively flat featureless spectrum below 600 nm. Filtered light examination using the selected optical filters for IR luminescence however, offered a high level of subjective discrimination separating the inks into 12 groups. Interestingly, the two pigmented samples, although distinguishable from each other, could not be distinguished from at least two dye containing brand/model combinations by this technique. Fluorescence interference from the paper substrate was also encountered but as before, could be partially overcome by including discriminations under the white light filter (380 – 800 nm). Given the subjective nature of this technique, the classification groupings for both colour groups should be treated with caution.

Both FTIR-ATR and Raman Spectroscopy offered a high level of comparatively objective discrimination. Based on the presence or absence of peak absorbencies in the region 1750 – 650 cm^{-1} of the original IR spectra, discrimination into at least 14 groups (T1 level) was achieved. If relative peak intensities were also considered, then discrimination into a maximum of 24 groups (T2 level) was considered possible, although some contradiction in classification was encountered when first derivative spectral data was also considered.

Raman Spectroscopy demonstrated all 25 brand/model combinations contained a pigment component, which when considered in combination with the findings from TLC suggested the vast majority were hybrid gel ink formulations, thus providing a high level of potential discrimination. Indeed, discrimination into 10 spectral groups was possible based on Raman Spectroscopy alone, but only under 785 nm, using either the FORAM or Renishaw InVia instruments. A search against the pigment Raman spectral library revealed the

primary pigment component of the PTN UK and STD UK brand/model combinations was likely to be 26308 XSL Poppy Red (CI Pigment Red 112), whilst for UNI JAP, 23180 Irgazine Red DPP 8 O (CI Pigment Red 254) was a good candidate.

8.2.3 Discriminating Power and Multivariate Analysis

Based on qualitative discrimination of the red ink group samples using TLC, IR luminescence, FTIR-ATR (T1 level) and Raman Spectroscopy (785 nm), the discriminating power of this combined approach was calculated to be 0.98 This was supported by the multivariate profiling of the FTIR-ATR and Raman spectroscopic data.

The same statistical methodology used for the blue ink group was applied to the red ink group samples, with similar observations made in respect of clustering efficiency, suitable algorithms and data pre-treatment. The lowest number of classification groupings identified from original FTIR-ATR spectra (T1 level) were confirmed by multivariate profiling, but the classifications based on differences in relative peak intensities were not supported statistically suggesting they were a less reliable feature of discrimination than the presence or absence of peak absorbencies. The multivariate profiling methodology developed did not work successfully on the first derivative of the original IR spectral data.

8.2.4 Summary

TLC provided limited discrimination due to lack of variation amongst dye components, whilst no discrimination was possible on the basis of IR absorption/reflectance or Vis-MSP. In contrast, IR Luminescence, FTIR-ATR (original spectra) and Raman Spectroscopy (785 nm) afforded a high level of discrimination, between 10 – 14 groups, dependent upon technique. Consideration of relative peak intensities and first derivative FTIR-ATR spectra provided further discrimination, but contradicted some classifications made on the basis of peak presence or absence within the original spectra and therefore should be treated with caution. Classifications based on visual pattern recognition of the IR (original T1 level) and Raman Spectra were supported by multivariate profiling using PCA, HCA and SOFM.

Four dye containing brand/model combinations of red gel ink were particularly difficult to discriminate: PLT UK, PLT US, ICM UK and PTL JAP. TLC proved to be an unreliable

discriminator of these samples owing to reproducibility issues associated with some additional component bands. Despite this, tentative discrimination into two groups was possible based on those component bands present could be made: PLT UK/PLTUS and ICM UK/PTL JAP. Examination by IR luminescence suggested that the PLT UK sample was distinguishable from the PLT US sample, whilst comparison of original IR spectra and Raman spectra (785 nm) confirmed that the PTL JAP sample was distinguishable from the ICM UK sample, effectively discriminating all four samples from each other. Multivariate profiling of the IR and Raman Spectroscopic data supported the classification groupings made by visual pattern recognition. The BIC UK brand/model combination could be discriminated from all other samples examined using TLC, FTIR-ATR or Raman Spectroscopy, but interestingly, under IR luminescence was indistinguishable to the PLT US, PTL JAP and ICM UK samples. Two pigment based brand/model combinations, UNI JAP and STB UK could be discriminated from each other using IR luminescence, FTIR-ATR or Raman Spectroscopy. Interestingly the UNI JAP sample exhibited a notably different IR spectrum to that of the other Uniball brand/model combinations studied, which all appeared indistinguishable to each other.

Some similarity was observed between the classification groupings made by FTIR-ATR and Raman Spectroscopy, but also some differences, which proved useful where two or more brand/model combinations could not be distinguished by one technique but could by the other. For example, the FBC MAL and WHS UK #04 -#06 samples were considered indistinguishable using Raman Spectroscopy, but clearly different under FTIR-ATR. This was also true for the PTN UK and STD UK samples.

The group containing the largest number of brand/model combinations identified by Raman Spectroscopy (12 samples), largely corresponded with the largest group (ten samples) identified by FTIR-ATR. Only the STP UK and MG MAL ink samples could be discriminated into a separate group by FTIR-ATR. All of these findings highlight the important complimentary nature of these two spectroscopic techniques. Further discrimination using IR luminescence was also possible.

Figure 8.2 provides a summary of the discrimination of the red ink group samples based on the combined analytical approach described supported by multivariate profiling.

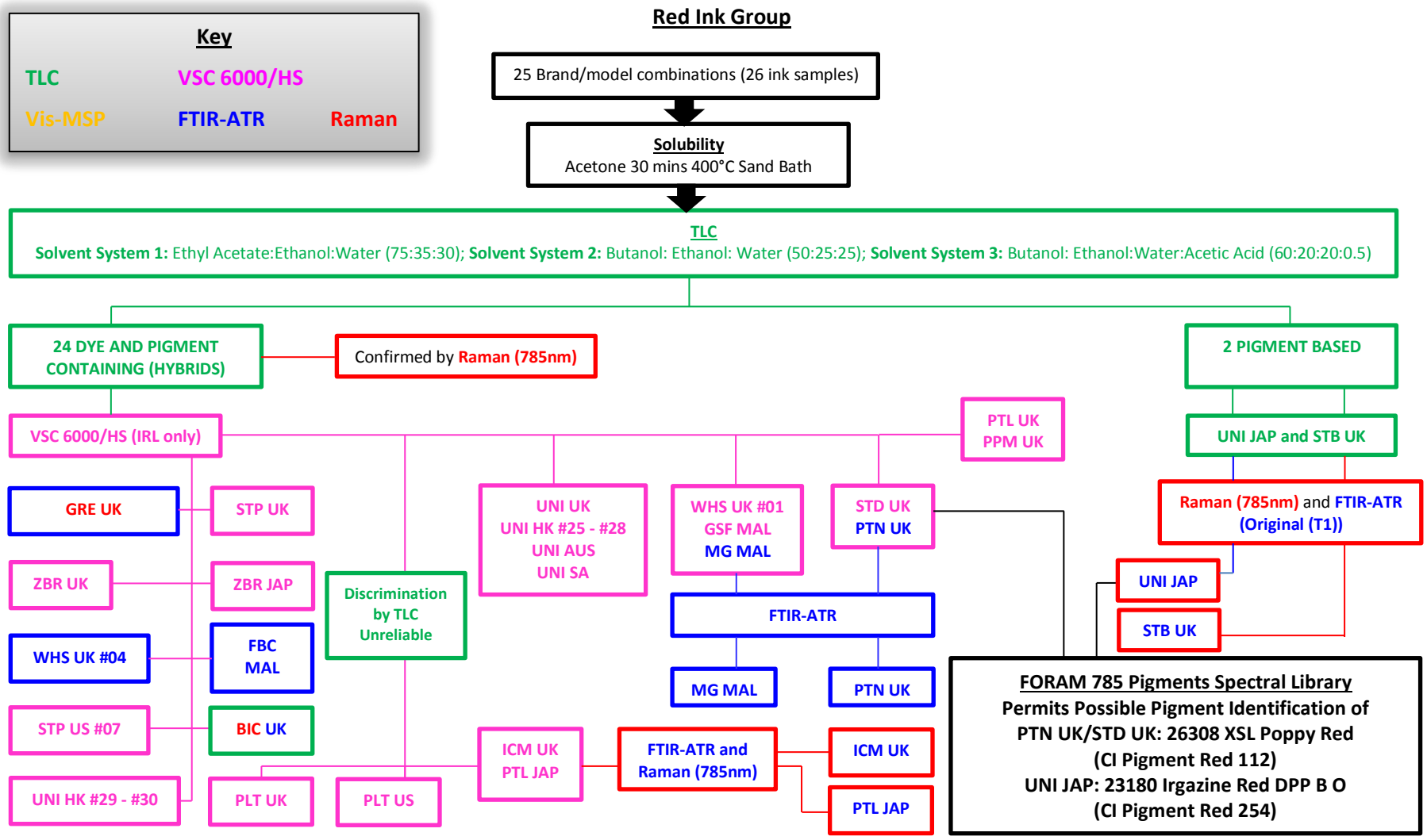


FIGURE 8.2: BETWEEN 10 – 14 GROUPS IDENTIFIED BY FTIR-ATR AND RAMAN SPECTROSCOPY (785 nm) DEPENDENT UPON TECHNIQUE CLASSIFICATION GROUPINGS CONFIRMED BY MULTIVARIATE PROFILING: PCA, HCA and SOFM

8.3 Comparison of Renishaw InVia and FORAM 785 Raman Spectrometer Systems

Since both the Renishaw InVia and FORAM 785 were equipped with a 785 nm excitation wavelength, a comparison of instrument performance with respect to the analysis of blue and red ink group samples was possible. In general, spectra acquired from the Renishaw InVia exhibited much greater spectral intensities than the corresponding spectra from the FORAM 785. This advantage of the Renishaw instrument was attributed to the use of a more powerful laser source, but led to drawbacks of sample burning and a tendency towards detector overload. In contrast, an Auto Exposure function used in the FORAM 785 optimised signal intensity for a given sample automatically without loss of spectral detail, and in some circumstances provided more spectral information than when operated under default scan time conditions. Both instruments were fitted with x 50 objectives (obj.), allowing a like for like comparison, although this was not fitted as standard in the FORAM 785. In the FORAM 785, spectral intensity was not always enhanced through the use of x 50 obj. over x 20 obj., and no difference in spectral pattern was observed. Spectral reproducibility for both instruments at this excitation wavelength was considered good, with minimal spectral interference from the paper observed. However, for the red ink group spectra acquired using the Renishaw InVia, signal intensity varied markedly within a sample set. Interference from stray peaks arising either as a result of a contaminant within or on the ink samples or ambient light entering the spectrometer system was a problem at this excitation wavelength using the Renishaw InVia. Interestingly, positioning of the laser over a heavily inked area rather than a lightly inked area for the red ink group samples only appeared to have an influence on the quality of spectrum produced.

With respect to the blue ink group, spectra representing the same brand/model combination acquired using both instruments was considered indistinguishable for all 31 samples. In contrast, within the red ink group, a combination of high fluorescence background and weak Raman signal meant that the FORAM spectra could not always be adequately compared to the corresponding spectra from the Renishaw InVia, even though a Raman spectrum was generated for all 25 brand/model combinations studied. When adequate comparison was possible, corresponding spectra between the instruments was considered indistinguishable. Classification groupings for the blue and red ink groups were

the same between both instruments. The developed multivariate profiling methodology provided an objective method which confirmed the classification groupings made for both the blue and red ink group samples by visual pattern recognition, and for each instrument. At 785 nm, the greater scan range of the Renishaw InVia (100 – 3200 cm^{-1}) provided no further spectral information for either colour group, confirming that the 400 – 2000 cm^{-1} scan range of the FORAM 785 was adequate at this excitation wavelength. However, at lower excitation wavelengths, i.e. 514.5 nm, the risk of losing some spectral detail appeared greater, and would therefore need consideration in any future instrumental development.

8.4 Emerging Techniques

A preliminary study into the Hyperspectral Imaging (HSI) capabilities of the VSC 6000/HS showed great potential for the discrimination of gel inks representing the blue and red ink groups, particularly for the latter. Based on classification made from visual comparison through imagery and spectral data combined, 15 UK blue groups and 13 UK red groups could be discriminated into 8 and 13 groups respectively. This gave discriminating powers of 0.90 for the blue ink group and complete discrimination of the red ink group samples. Discrimination using the imagery data was easier than by spectral data, owing to subtle variability in the latter. The system could therefore benefit from some form of statistical feature incorporated into the software to provide an objective interpretation of the results. Furthermore, improvements in the software and/or hardware are required to eliminate spectral interference associated with cross-over points between the RGB channels. Despite this, HSI should be considered as an emerging technique of great value to gel ink analysis.

As part of this research, samples from each colour group were analysed on paper using the Foster and Freeman LIBS based system, ECCO. At present, the system incorporates a database of 35 elements for identification of trace inorganic constituents. Whilst reproducibility of spectra was considered good, on the face of it, very little discrimination between samples within a colour group was achieved. Furthermore, many peaks were unidentifiable from a database search suggesting more elements need to be included for satisfactory elemental profiling of inks. Amongst the elements that were identified, copper, attributable to copper phthalocyanine pigment, characterised the blue ink group samples, whilst evidence of manganese and chromium were observed in the red ink group samples. Discrimination potential maybe improved by focussing on small peak differences, but a

comprehensive elemental database would be required to distinguish these from instrumental noise. In light of these findings, the benefits of these techniques including minimal sample destruction, rapid sample analysis, and multi-element capabilities for superior discrimination, warrant further investigation of their potential for gel ink analysis.

8.5 Suggestions for Further Work

As a consequence of the research undertaken within this project the following suggestions for further work can be made:

TLC

- Investigation of alternative solvents, i.e. trifluoroacetic acid (94%) and dimethylformamide (DMF), for their suitability for extracting pigments such as slightly soluble phthalocyanine pigments from gel inks
- Further development of the extraction and TLC methods for the analysis of red ink group samples, e.g. investigation of heating time on extraction and dye component degradation

VSC 6000/HS

- Development of a statistical method to aid discrimination of (gel) ink samples based on pixelated images from IR absorption/reflectance and/or IR luminescence examination
- Development and incorporation of a multivariate statistical profiling method to aid discrimination of % reflectance spectra recorded using the HSI feature of the VSC 6000/HS

Vis-MSP

- Investigation of % reflectance spectra against transmittance for discrimination
- Development of a multivariate statistical profiling method for Vis-MSP transmittance spectra

FTIR-ATR

- Further investigation of the Gladi-ATR accessory with incorporated imaging system for the FTIR analysis of (gel) ink lines on paper to resolve reproducibility issues

- Analysis of blue and red ink group pure ink deposits using the A2 Technologies ML bench top FTIR-ATR to compare spectra against corresponding ink on paper
- Analysis of pigment standards and/or other additives known to be used in (gel) ink formulations by FTIR-ATR to aid identification of (gel) ink components

Raman Spectroscopy

- Further investigation of background fluorescence shape as an additional feature of discrimination
- Given the claim by Zieba-Palus *et al* [204] that discrimination of red gel inks using 633 nm was possible, it may be beneficial to analyse a wider range of brand/model combinations of red gel inks, to confirm or refute the suitability of this excitation wavelength for that application
- Investigation of other excitation wavelengths for the analysis of blue and red ink group samples, i.e. 1064 nm

Alternative and Emerging Techniques

- Investigation of silver colloid for use with the FORAM 685-2 SERRS application kit
- Further development and investigation of the Foster and Freeman ECCO (LIBS) system for the elemental profiling of the blue and red ink group samples, perhaps in comparison with LA-ICP-MS
- Investigation of other destructive techniques, i.e. SEM, GCMS and HPLC, for the discrimination of the blue and red ink group samples.

It should be noted that as part of this research, a third colour group containing black gel inks was also investigated for within and between brand variations using all analytical techniques described. No evidence of within brand variation, and limited between brand discrimination was achieved hence further details of these studies have been excluded from presentation within this thesis. Some of the suggestions for further work described above could therefore equally be applied to black gel inks as well as other less commonly encountered colours, i.e. green, purple, etc.

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