Identifying the component responsible for the intrinsic fluorescence of seminal fluid

by

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DECLARATION

"I certify that this thesis does not incorporate, without acknowledgment, any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another, except where due reference is made in the text."

Thomas Hensel

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SUMMARY

The intrinsic fluorescent property of seminal fluid is routinely used in forensic investigations for the location of seminal fluid stains especially in sexual assault cases. Although used, the compound that is responsible for this intrinsic fluorescent property is not known. There are several compounds in seminal fluid that are known to be fluorescent but none of these have been linked to the more unique fluorescent properties observed with seminal fluid. To potentially improve the current technique used and to gain a better understanding it would be beneficial to know what component is responsible for the fluorescence.

An issue that arises when looking at the fluorescence of seminal fluid is the quenching effect of water. This makes using some traditional methods of analysis difficult as they are wet techniques and the sample must be dry to properly observe the fluorescence. Because of this a reliable method, based on previous ones, for visualising the fluorescence when the sample is dry was developed that included a way of taking photographs to record the fluorescence.

Seminal fluid is also a very complicated sample and since little was known about the compound responsible, simplifying the sample matrix that the fluorescent component is in was not easily achievable. A method using a HPLC system with attached sample collector was used to produce a sample with a simplified matrix. This was further purified using SPE to produce a sample that could be analysed further.

This sample could be analysed using LCMS. The results from this analysis pointed towards the compound responsible being a polypeptide or protein. Following this, the sample was analysed using proteomic techniques, but the final identity of the compound was not determined. Some possible candidates were identified in the sample, but further work is required to confirm what is responsible for the fluorescence in seminal fluid.

A variation in the level of fluorescence was observed between samples from different donors. After removing the variable of the level hydration of the seminal

fluid from the samples, the variation of fluorescence between them was reduced but not eliminated. This suggests that the there is a relatively consistent amount of the fluorescent component in seminal fluid, in relation to other compounds excluding water, but there is still some natural variation.

Experiments have led to a much better understanding on using the Far-Infrared beamline at the Australian Synchrotron. Polytetrafluoroethylene was shown to be a suitable alternative to polyethylene, an established disc matrix, and for some compounds it was possible to get a spectrum from a disc of pure compound. The effects of concentration of sample in the disc were studied and it was shown that higher concentrations were better as more information could be obtained. For some compounds, it was shown that a disc that is made up of pure compound could be formed and a clear spectrum obtained. In addition to this, small changes to a molecules structure was shown to have a significant effect on the resultant spectrum.

PUBLICATIONS

Conference papers

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ABBREVIATIONS

ACN	acetonitrile
CSV	comma-separated values
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FLS	forensic light source
Far-IR	Far-Infrared
GC	gas chromatography
GE	gel electrophoresis
GFC	gel filtration chromatography
H_2O	water
HILIC	hydrophilic interaction liquid chromatography
HPLC	high performance liquid chromatography
HSA	human serum albumin
IR	infrared
KBr	potassium bromide
LC	liquid chromatography
LLE	liquid-liquid extraction
MALDI	matrix assisted laser desorption ionisation
MeOH	methanol
MS	mass spectrometry
PAGE	polyacrylamide gel electrophoresis

PE	polyethylene
PEG	polyethylene glycol
PIP	prolactin-induced protein
PTFE	polytetrafluoroethylene
SDS	sodium dodecyl sulphate
SEC	size-exclusion chromatography
SPE	solid phase extraction
TLC	thin layer chromatography
UV	ultra-violet
Vis	visible
ZAG	zinc-alpha-2-glycoprotein

UNITS

cm^{-1}	wavenumbers
g	grams
g/mol	grams per mole
kDa	kilodaltons
l	litres
M	moles per litre
mAU	milli absorbance units
mg	milligrams
min	minutes
ml	millilitres
mmol	millimole
MΩ	megohm
nm	nanometres
rpm	revolutions per minute
µm	micrometres
µl	microlitres
%	percent

CHAPTER 1 INTRODUCTION

1.1 AIM

The aim of this project is to identify the compound present in seminal fluid that is responsible for the intrinsic fluorescent observed. To attempt to so this several different techniques are required. Seminal fluid is a very complex matrix and comprised of a variety of different compounds. If the compound responsible cannot be identified the aim is to narrow down the list of potential suspects and leave us closer to knowing the identity of the compound, this may include narrowing it down to a class of compounds so that further research can be more focused from the outset.

Little is also known about the fluorescent compound meaning that targeted methods or techniques could not be used initially. The previous work conducted has provided so idea of where to start and continuing with these ideas was the best place to start. This was chiefly using HPLC analysis and conducting experiments using the synchrotron.

1.2 FORENSIC SCIENCE AND SEXUAL ASSAULT

A crime that unfortunately occurs frequently and that, depending on the severity, can require the collection of evidence is sexual assault. According to statistics released by the Australian Bureau of Statistics, an estimated 14.8% of women and 6.6% of men had experienced selected types of sexual harassment during a one year period[1]. The Australian Institute of Criminology released figures that show that there were 18,153 sexual assaults reported in 2012[2].

Depending on the nature of the sexual assault, such as type and location, there is the potential that the key evidence that will be found will be DNA. A common source of DNA from these sorts of crimes is from semen contained in seminal fluid.

1.3 SEMEN STAIN DETECTION

1.3.1 Intrinsic fluorescence

An intrinsic property of seminal fluid can be utilized in the location of stains. The property is that they are fluorescent when excited by light in the ultraviolet (UV)-

blue wavelength region[3, 4]. It is important to be able to effectively and efficiently locate these fluids without compromising the sample or scene. Utilising the fluorescent property is one such way. Techniques have been developed that use the fluorescence that allow for screening of evidence for seminal fluid.

The techniques that detect seminal fluid via fluorescence all require a light source capable of delivering light at selected wave bands. Many different sources of light are used from hand held portable devices that use wavelength specific LED (Light-Emitting Diodes) to larger, more powerful Xenon arc lamps such as a Polilight®[4, 5] which use filters to provide selected wave bands of light. These light sources can be given the generic name of Forensic Light Source (FLS) when used in forensic applications.

The light from a suitable FLS of the appropriate wavelength is shone onto the object or surface on which a stain is believed to be located. The light causes the stain to luminescence and become visible, the process behind this is discussed in detail later (1.6 Fluorescence). When the excitation light is in the visible region of the spectrum, a barrier filter is needed to exclude reflected incident light while allowing the fluorescent light through. Several different wavelengths of light and different filter combinations can be used which has led to research into optimising the technique.

Semen has a blue luminescence when exposed to UV light on non-photoluminescent backgrounds [6]. This is one of many different variations that can be used due to the broad excitation range of seminal fluid. Another established variation, that is important to this work, uses light that has a wavelength of approximately 450 nm. This is used in combination with orange goggles that act as a filter. This combination is good for viewing stains that are hidden on material that also has background fluorescence [3, 4]. When viewed under these conditions the seminal fluid appears as a yellow luminescence.

1.3.2 Development and improvement of the technique

There has been work done to improve the use of fluorescence in the detection of seminal fluid stains. This includes work done to improve detection of a stain when

the background material is photoluminescent. Work done by Stoilovic who identified the excitation and emission spectra of dried semen using a fluorescence spectrometer[6]. It was found that seminal fluid had a broad excitation range and subsequently a broad emission range. The excitation spectrum was found to have a range from 300 nm to 480 nm and the emission spectrum having a range from 400 nm to 700 nm depending on the excitation wavelength[6]. This can be seen in the spectrum in Figure 1. Given how broad these ranges are there is a high potential that there are multiple compounds contributing to the fluorescence.

Problems with fluorescence detection on some materials arise when the item being tested is highly light absorbent, dark in colour and/or they are photoluminescent [3, 5, 6]. Photoluminescent material can be a problem because it can cause background fluorescence under the same conditions as semen. This causes interference which can mask the stain and therefore make it difficult to locate and in some cases, may be completely masked. Some brighteners that are found in detergents and fabric conditioners can add to the background fluorescence of items[5].

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Figure 1: The excitation and emission spectra of dry untreated semen as determined by Stoilovic[6]

From experiments, Stoilovic recommended a procedure for the detection of semen stains when background photoluminescence is an issue. The procedure requires the user of a FLS to vary the settings of the light source, i.e. change to different

wavelengths, and to change the coloured goggles worn (or change the filter the stain is observed through) until the background is no longer fluorescent.

The work on the reduction of background photoluminescence interference was expanded upon by Kobus et al. [3]. It was demonstrated that, with the use of appropriate interference filters, semen stains not visible due to background interference could be made visible. The narrower band pass of interference filters compared to coloured googles removes the background photoluminescence while still allowing the seminal fluid fluorescence to be observed. When the background is found to be fluorescent it is recommended that various band pass interference filters could be used to remove the interference.

This is demonstrated in

Figure 2 in which a stain on a pink satin top is viewed under two different conditions. Both times an excitation wavelength of 450 nm was utilised, the difference being that the picture on the left is being viewed though orange goggles and the one of the right through a 530 nm interference filter. The addition of the narrower band pass interference filter has made the stain much more visible due to the exclusion of interfering substrate emission.

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Figure 2: Semen stain on a pink satin top; Left-450 nm excitation viewed through orange goggles; Right -450 nm excitation viewed through 530 nm interference filter from work conducted by Kobus et al. [3]

It should be noted that the using fluorescence to find seminal fluid stains is not a confirmatory test. Using the fluorescent properties is a highly effective method for locating stains but it is only a way of screening for potential stains and cannot confirm that a stain is seminal fluid. This is because other biological fluids, such as saliva and blood, can also fluoresce under the same conditions along with other chemicals. Work that was done by Vandenberg et al. [5] on the use of a FLS for the detection of seminal fluid, saliva, and bloodstains compared detection of semen stains and found that it was poor at distinguishing between the body fluids.

Detection of stains by FLS is sensitive, safe, simple, non-invasive and non-destructive [5]. But due to its inability to distinguish between bodily fluids, additional tests are required to confirm that a stain is seminal fluid. One such test involves testing for the presence of Seminal Acid Phosphatase [4] but there are other tests that can be done.

1.3.3 Development of new techniques in seminal fluid investigations

Recent work in this area has focused little on the detection/location of seminal fluid stains and has instead focused on the identification of bodily fluid stains. This includes the development of presumptive and also confirmatory tests as alternatives to current methods such as the test for prostate specific antigen (PSA)[4]. This is an overview of some of the research but because it is on identification of a stain rather that stain fluorescence it is not critical to this work.

A review done by Virkler et al. [4] covers body fluids including seminal fluid and their analysis. It states that an alternative light source (ALS) or FLS is commonly used for the location of stains and is a good technique for this. They go on to state that using a FLS as a confirmatory test would not be suitable as it is not specific enough. The other techniques that are then discussed are ones that are presumptive or confirmatory tests, both currently accepted techniques and new techniques that are being developed.

From the review by Virkler et al. most of the emerging techniques involve immunological markers. The detection of semen-specific genes by mRNA analysis is the main area of focus. These are tests that would confirm if a stain was in fact seminal fluid and could be an alternative to the PSA test. There has also been some work done using a portable X-ray fluorescence spectrometer [7] to see if it a viable method for identifying stains could be developed.

Since the review by Virkler et al. in 2000, work by Elkins[8] has used Attenuated Total Reflection Fourier Transform Infrared Spectroscopy to attempt to distinguish between different body fluids, including seminal fluid. In this work it is stated that that the use of a FLS is the primary technique for the location of body fluids to be then followed by analysis using a spectrometer to identify the stain.

Alternatives to the current methods for identifying body fluid stains could be useful in forensic investigations. If the technique developed is more accurate, simpler and cheaper, it would make sense to implement them. However, these methods still rely on the use of the fluorescent properties to first locate the stains.

1.3.4 Research on human seminal fluid fluorescence

Research that has been conducted on human seminal fluid fluorescence has not solely been on the identification of the fluorescent component. Studies have looked for a link between the fluorescence and the sperm quality parameters in both humans and animals. One study that involved human seminal fluid was conducted by Amano et al. [9] which was looking into the relationship between fluorescence of seminal fluid and sperm quality parameters. The results from this work showed that

there was a link between the concentration of sperm and the level of fluorescence. Research of literature failed to find any evidence that the seminal fluid from men who are infertile or who have had vasectomies is non-fluorescent.

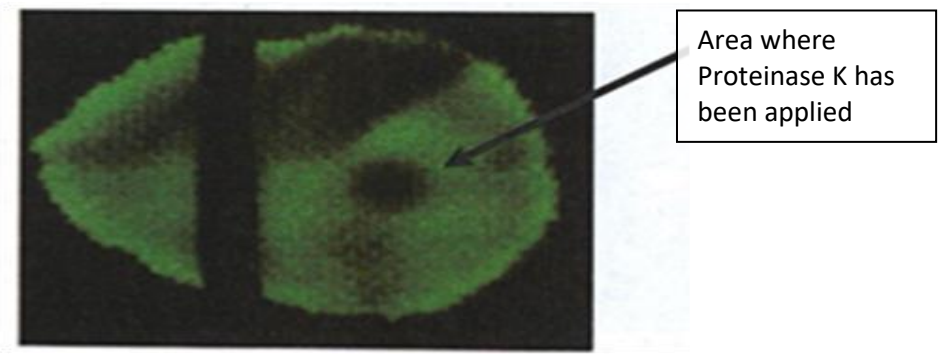

Figure 3: A fluorescing semen stain which has had a section treated with a Proteinase K solution [10]

While these results are interesting, there was no attempt to identify the cause of the fluorescence. The studies that were done on animal seminal fluid are the same and do not attempt to identify the cause of the fluorescence. Because of this and the fact they are on animal seminal fluid not human they were not included in this review.

In work conducted, as part of an honours project, at Flinders University and Forensic Science South Australia, by Camilleri et al. [10], Proteinase K was applied to a seminal fluid stain. Proteinase K is an enzyme that breaks down protein structures. In

Figure 3 the area where the enzyme was applied has become non-fluorescent. This would suggest that the proteinase K has broken down protein(s) that caused the fluorescence. It could also be possible that the addition of proteinase k in an aqueous solution caused a washing effect that simply moved the fluorescent component from the applied area. Camilleri also investigated how the fluorescence of a semen stain changed as it is exposed to air and allowed to dry. It was found that the intensity of the fluorescence increased the longer the semen was exposed, with maximum intensity occurring once the stain had dried seen in Figure 4.

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Figure 4: Results from the drying of seminal fluid done by Camilleri[10]

In further work, seminal fluid stains were treated with water. It was shown that water quenches the fluorescence after a stain has dried while also showing that the fluorescent component is water soluble. Synthetic seminal fluid solutions were made to try to replicate real seminal fluid and its fluorescent properties and it was found that the component that had the closest fluorescent properties to that of seminal fluid was that of Bovine Serum Albumin[10].

Carver[11], also in a honours project undertaken at Flinders University, analysed seminal fluid by liquid chromatography-mass spectrometry (LC-MS) attempting to separate and identify the components in seminal fluid. Identification of components detected was found to be difficult due to the complex nature of seminal fluid. Some were identified but only by comparison to results from synthetic seminal fluid samples that had been made from known components.

Further work by Carver attempted to separate out the components of semen by Gel Electrophoresis. There was some success with a series of compounds separated out on a gel. However, it could not be identified where the fluorescent component had

ended up on the gel. At some point during the preparation for the gel, or because of the separation on the gel, there had been a quenching of the fluorescence.

Church[12], like Caver[11] used LCMS to try to identify the fluorescent component. Separation of some compounds was achieved but no significant results were obtained, only known components known not to be responsible for the fluorescence were identified.

More recently, Tan[13] looked into separation of the components of seminal fluid using centrifugal based filtration devices. The size ranges explored were <3kDa, 3-10kDa, 10-30kDa, 30-50kDa, 50-100kDa and >100kDa. In addition to this, polyethylene glycol (PEG) fractionation was explored. From the fractionation work it was concluded that the fluorescence was from a larger molecule, likely to be a protein.

The results from the filtration devices showed that the fluorescence was being caused by a component or components larger than 100kDa in size. The results can be seen in Figure 5 with the more than 100kDa spot having the highest level of fluorescence along with some slight fluorescence in the 10-30kDa size fraction. However, it is possible that the device was blocked by the larger molecules and hence, nothing passed through the first filter; 100kDa was the first filter size used and had most the fluorescent material.

Work was also done by Tan using the Far-Infrared (Far-IR) beam of the Australian Synchrotron (http://www.synchrotron.org.au/) and using an infrared microscope. Each of the size fractions, from the filtration device work, were analysed using a Fourier Transform Infrared microscope. Each fraction exhibited similar peaks with the difference between them being the intensity of the peaks. The peaks in the IR spectra were deemed to be indicative of proteins.

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Figure 5: Fluorescence of the different size fractions from the filtration work done by Tan[13] (K = kDa)

The work done using Far-IR provided little insight, mostly due to issues stemming from properties of the sample. Getting the seminal fluid into a state suitable for analysis was found to be difficult. Polyethylene/dried seminal fluid discs were not uniform and too thick for analysis. When the sample was dried on a gold plate for grazing incidence angle analysis the surface was not uniform enough for successful analysis [13].

Several authors, reference in the paper by Amano et al. [9] suggest that riboflavins may cause the fluorescence but this is yet to be confirmed. One of the issues with this notion is that riboflavins are fluorescent in water, but water is known to reduce the fluorescence of seminal fluid. Counter to this is the possibility that the reduction in fluorescence is from an interaction with a second component not the water itself. A second issue is that riboflavins can be unstable and can break down under certain conditions such as exposure to light. Seminal fluid stains have been reported from case work to remain fluorescence for several years, but this is not enough evidence to completely dismiss them as the potential cause of the fluorescence.

None of the work so far has been able to give a definitive answer as to the identity of the fluorescent component but has provided some evidence as to its nature. There is evidence to suggest that the candidate is a protein or a group of proteins. Amano et al [9] also draws this conclusion in his work stating that protein plays an important role in the fluorescence, based on similarities in the fluorescence spectra to other body fluids. However, at this point there is not enough evidence to rule out other types of components.

1.4 SEMINAL FLUID

1.4.1 Composition of seminal fluid

Seminal fluid is made up of secretions from several different glands. The first contribution comes from the testes which release spermatozoa. The rest of the compounds that make up the seminal plasma, the liquid around the spermatozoa, are designed to assist in the spermatozoa successfully getting to the egg by aiding in sperm motility and providing protection and nourishment [14, 15]. These compounds come from the seminal vesicles (65% contribution), prostate (20%), epididymis (10% with testes), and the periurethral gland (minimal) [15].

There have been many studies done on seminal fluid, both human and non-human (animal). These have focused on aspects other than the source of the fluorescence. The work done has been more focused on whether there are relationships between concentrations of different compounds in semen and infertility or how fertile a man is and links to cancer. Therefore, these samples are from males that may not be representative of the total male population. The other aspect looked at is the function of different compounds in the seminal fluid.

A recent review (2009) done by Virkler et al. [4] into the analysis of body fluids for forensic purposes, listed the major components of each fluid including semen. In the article by Poiani [14] there is a more complete list of the seminal fluid components and their function in mammals. This showed that the many different substances can be separated into groups. The key groups to be considered are peptides, proteins,

amino acids, polyamides, sugars, salts, and metal ions. A paper reference by Poiani by Batruch et al. [15] identified over 2000 proteins in seminal fluid.

The review of literature conducted by Owen et al. [16] in 2005 was done to determine what was important to be included in synthetic seminal fluid. They looked at the chemical and physical properties of seminal fluid, such as pH, so that a formulation for a good representation of semen could be created. Some of the components of seminal fluid are also summarised in a paper by Alexander et al. [17] who were studying the immunology of semen.

1.5 SEMINAL FLUID ANALYSIS

Most analysis that has been conducted on seminal fluid has focused on identifying a single compound or single protein within the seminal fluid. In some cases, the target compound is not one normally present in seminal fluid such as a drug. Studies that have had a broader focus have looked at groups of compounds such as antioxidants [18] and proteins [15, 19].

1.5.1 Separation techniques in seminal fluid analysis

Three main techniques have been used as separation techniques when working with seminal fluid; high performance liquid chromatography (HPLC), Gel electrophoresis (GE), and gel filtration chromatography (GFC). The specific technique used depended on the nature of the target compound or compounds. There are other techniques, such as gas chromatography (GC) [20], that have also been used, but to a lesser extent. It should be noted that in most cases the identification of compounds was done using mass spectrometry.

When it came to the organic compounds, the favoured analytical technique was High Performance Liquid Chromatography HPLC. HLPC analysis was preceded by an extraction/clean up technique to simplify the matrix that the compound of interest was in. Examples of extraction/clean up techniques include Solid Phase Extraction (SPE), Liquid-Liquid Extraction (LLE), protein precipitation and some also used chemical reactions as part of the procedure. Examples are summarised below.

The following are examples of where SPE and HPLC have been used to identify an organic compound present in seminal fluid. While the techniques are specified they could potentially be adapted to either be used to either gain some understanding of the targets properties or used more specifically if the fluorescent compound/s is/are found to be a simpler organic molecule.

Hofer et al. [21] determined the levels of ribavirin, a drug, in seminal fluid. Doehl et al. [22] research was on E type prostaglandins and they were extracted from seminal fluid samples and separated using HPLC then detected with UV. Kato et al. [23] determined the levels of phthalate metabolites in seminal fluid and Teo et al. [24] analysed for thalidomide. Amoako et al. [25] made use of this combination for the quantitative analysis of anandamide and related acylethanolamides. Bisphenol A levels in semen was the interest of work by Inoueet al. [26].

Examples of where LLE has been used rather than SPE includes work by Avery et al. [27] and Kand'ar et al. [28] both of whom used LLE methods followed by HPLC analysis. Avery et al. used these techniques to look at Efavirenz, a drug, in seminal plasma. Retinol and alpha-tocopherol were the targets in the work by Kand'ar et al. Again, these techniques could be adapted to be suited to extracting the fluorescent compound/s if it is organic.

In a separate study Kand'ar et al. [29] looked at ascorbic acid and uric acid in seminal plasma. However, in this work a chemical reaction was used (reduction reaction using dithiothreitol) along with centrifugation to clean up the sample. Centrifugation was also used by Sharma et al. [30] when looking at the sidenafil citrate levels in seminal fluid. In this work an acetonitrile precipitation was used which was followed by the use of a centrifuge to assist in removing the precipitate. These techniques could be adapted to help simplify the matrix and give some idea as to the nature of the target fluorescent compound/s.

HPLC was also used in the previous honours work by Caver [11] and Church [12] to try to assist in identifying the fluorescent component in seminal fluid. Carver and Church were able to separate some compounds and mass spectra data was obtained using LCMS [11, 12].

When proteins and/or peptides were the main interest of the research into seminal fluid, the most common technique for the separation was gel electrophoresis. Work related to proteins is generally referred to as 'proteomics'. Work on proteins is a specialised area of research and identification can be difficult due to the complex nature of proteins. If the fluorescent compound/s were proteinaceous in nature, then gel electrophoresis would likely be a very useful technique. Some of the methods used in the following studies could be adapted to isolate the fluorescent compound/s.

Batruch et al. [15] used a different approach to gel electrophoresis. Instead HPLC was used to separated digested proteins and then identification was obtained via mass spectrometry. The work looked to identify as many proteins as possible to compare people who had had vasectomies with those who had not. If the fluorescent compound/s was protein based then digesting could disrupt the fluorescence, limiting the potential for this method.

Fung et al. [19] looked at peptides and proteins in seminal fluid. The research was also nonspecific but used 1D and 2D gel electrophoresis along with mass spectrometry to try to identify as many proteins and peptides as possible. 2D gel electrophoresis was also used in nonspecific protein identification work that was carried out by Zylbersztejn et al. [31] concerning varicoceles. Similar work was conducted by Hosseinifar et al. [32] again using 2D gel electrophoresis.

Starita-Geribaldi et al. [33] used 2D gel electrophoresis while looking to map seminal fluid proteins to see if there is a difference between men with normal or impaired spermatogenesis. Walkar [34] used a variation of gel electrophoresis, known as disc electrophoresis, to compare normal semen samples to abnormal samples which included the protein components.

Work focusing on prostate specific antigen in seminal fluid was conducted by Vegvari et al. [35]. They used 1D gel electrophoresis along with other methods. 1D and 2D gel electrophoresis was used by Qian et al. [36] who also looked at prostate specific antigen. 1D gel electrophoresis was also part of the research done by Finiet al. [37] looking into 5'-nucleotidase in seminal plasma.

Another separation technique that has been used with proteins is gel filtration chromatography (GFC). Ohman et al. [38] and Kooistra et al. [39] used GFC in work that looked at what was causing people to have allergic reactions to seminal fluid. Both studies used a gel filtration column to separate out the seminal fluid into fractions. Tests were then performed using the fractions such as tests to see if there were proteins present in each of the fractions.

Other studies have used gel filtration as part of a method to purify a specific protein or molecule from human seminal fluid. Samloff et al. [40] used gel filtration for purification of group II pepsinogens. Zhang et al. [41] worked on purification of different molecular forms of prostate-specific antigen and Skidgel et al. [42] the purification of basic carboxypeptidase.

Other studies involving human seminal fluid and gel filtration includes work done by Kavanagh [43], Panari et al. [44] and Friberg [45]. Kavanagh looked at the zinc binding properties of human seminal fluid along with prostatic tissue and prostatic secretion. Panari's studies looked at the polymorphism of A, B and H substances in seminal fluid and Friberg's work was to do with immunological studies and sperm-agglutinating in seminal fluid.

Since the size of the compound/s is not known it would be difficult to apply this technique. It is important to know the size of the molecule so that an appropriate separation media can be selected. Once more is known about the size or size range then this technique could become useful.

Another group of substances that are found in seminal fluid are metals. Common analytical techniques used for the analysis of metals are applied to the analysis of seminal fluid. The two main techniques that have been utilised are Atomic Absorption Spectroscopy [11, 46, 47] and more recently, Inductively Coupled Plasma Mass Spectrometry [48]. Some initial work has also been done on a High Resolution Multi Collector Inductively Couple Plasma Mass Spectrometry by Walker (unpublished results, personal communication, 2011). While these techniques would not be able to identify the compound/s it would be useful to identify the presence of metals if they play a role in enhancing fluorescence.

Another technique that is used for analysis of seminal fluid is Enzyme-linked Immunosorbent Assay [26, 49]. This technique is limited to the number of different compounds that are to be analysed and the compound/s that are to be determined need to be known. It is not a technique that is used for the identification of compounds.

Because the fluorescent compound/s could be simple and organic in nature or be large and proteinaceous in nature the techniques used initially in this research should be non-specific. Some of the techniques that have been discussed can be used in a general way to gain a better understanding as to the nature of the fluorescent compound/s.

1.6 FLUORESCENCE

1.6.1 The cause of fluorescence

At room temperature, most molecules exist in an electronic ground state which is usually a singlet state (S_0). In the singlet state, all the electrons in the molecule are paired. Within these pairs of electrons, each electron has a spin which is opposite to that of the other electron in the pair [50]. When exposed to light, many compounds are able to absorb photons; the absorption of this energy may lead to the electronic excitation of an electron to a higher singlet state (S_1 S_2...) [51]. Along with there being singlet states there are also vibrational states within the singlet states.

From this excited state, there are several processes that occur that lead to the return to the ground state, S_0. Two of the processes release the energy in the form of a photon, phosphorescence and fluorescence, also more generally known as photoluminescence. The other processes are vibrational relaxation, internal conversion and intersystem crossing [50-52]. Normally it is a combination of two or more of these processes that occurs. Which processes that do occur depend on how long each process takes with shorter times favoured. If a photon of light is not released, then all the energy gained is released as heat.

The processes that can occur are best shown in what is known as a Jablonski Diagram, which can be seen in Figure 6. For fluorescence, the first process that must

occur is the absorption of a photon. This is either the purple or blue line in the diagram. Once this has occurred processes known as internal conversion and vibrational relaxation (dashed line) can occur which lead to a loss of energy. In many cases, all the absorbed energy is lost this way as they are fast processes and fluorescence does not occur.

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Figure 6: A basic version of the Jablonski Diagram showing the processes involved in fluorescence [55]

Sometimes not all the energy is lost this way and the remaining energy is released as a photon (green line). The reason that fluorescent light is over a range of wavelengths is due to the various energy levels in each state that the electron can fall to and from (the horizontal lines). The energy that is lost to internal conversion and vibrational relaxation is what causes the released fluorescent light to be a different wavelength to the absorbed light. The wavelength is longer than that of the light that caused excitation. This is what is known as the Stokes shift[53].

It is because of this change in the wavelength that a filter can be used to help visualise fluorescence. The excitation light does not need to be seen by someone viewing the stain so a filter can be used to shield them from seeing this light but allow the fluorescent light to be seen due to the changed wavelength. This is useful as the light source being used to cause the excitation is much more intense than fluorescence and can mask it quite easy.

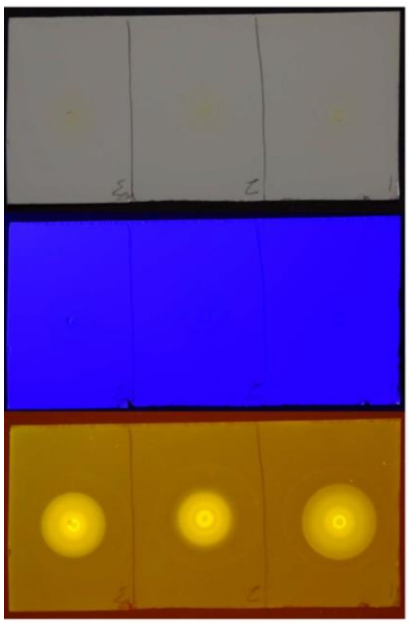


Figure 7: A silica TLC slide under natural light and no filter (top), under 450 nm light from a Polilight® with no filter (middle) and 450 nm light with orange filter (bottom)

In this work the excitation light is blue. It is much more intense and over powers the luminescence from the seminal fluid. Once an orange filter is used to block the blue light from reaching the viewer or the recording device, the seminal fluid luminescence becomes visible. This can be clearly being seen in Figure 7 which shows the same TLC silica slide under three different conditions. With the blue light on it with no filter, no fluorescence can be seen from the seminal fluid. This changes when a filter is added to the camera and the fluorescence can be seen clearly. With the filter on the camera lens the blue light coming from the Polilight® is removed and the yellow fluorescence can be captured.

1.6.2 Fluorescent centres or fluorophores

Not all compounds are fluorescent. Compounds that are fluorescent are referred to as fluorophores, a compound that can absorb light and then reemit it. The wavelengths of both the absorbed and emitted light are specific for different compounds. These wavelengths are dependent on many factors including the chemical structure and environment of the fluorophore [54]. It is important to note that almost all electronic excitations are in the pi bonds from the ground state to an excited state ($\pi \rightarrow \pi^*$) [50]. This means that for the most part, compounds that exhibit fluorescence have a conjugated pi bond system in their structure. Most

fluorescent compounds contain one or more aromatic functional groups which have many double bonds [50] and potential for conjugation.

Functional groups that are attached to the aromatic rings can influence fluorescence. In general, electron donating groups improve the fluorescence, whereas electron withdrawing groups have a negative effect [55]. Another structural property that affects fluorescence is rigidity; generally, the more rigid the structure is the more fluorescent it is [50]. The addition of metals ions can lead to a molecule being more rigid. The fluorescent compounds can complex to the metal ion leading to an increase in the intensity of the fluorescence. An example of this is the when zinc is added to fingermarks developed using ninhydrin [56].

External factors that can affect fluorescence include temperature, the solvent that the compound may be dissolved in and the pH of the solvent [50]. In most cases, increasing the temperature decreases the fluorescence. This is because increased temperature means that there are more collisions between molecules which can lead to an increase in the amount of external conversions occurring [53].

Increasing or decreasing the pH can lead to the removal or addition of a hydrogen ion (H^+) to the structure of some fluorescent molecules. This can have either a positive or negative effect on fluorescence depending on the structure of the molecule [50]. Changing the polarity of the solvent can also affect fluorescence. Polar solvents can stabilise the excited state which leads to there being a greater Stokes shift [53].

1.6.3 Potential fluorophores in seminal fluid

The two most likely candidates that are responsible for the fluorescence of interest are an organic molecule or a protein. Riboflavin and other similar compounds have been suggested to be the compound responsible. Many of these compounds are known to be fluorescent [57]. The structures all share a common cyclic backbone which is responsible for them being fluorescent.

Proteins are made up from polypeptide chains. The peptides that form these chains are not fluorescent or have very low fluorescence with the expectation of

tryptophan [58]. However, the fluorescence of tryptophan does not occur in the range that is of interest in this work. Some proteins are intrinsically fluorescent not due to tryptophan. For these proteins there are other structural reasons for the fluorescence [59, 60].

1.7 GEL ELECTROPHORESIS

Gel Electrophoresis (GE) is a method that can separate out analytes based on charge and/or size. It is the main method employed to separate out biological polymers (such as proteins or DNA) [61]. The basic set up is a thin gel slab, normally made from polyacrylamide or agarose gel [62], into which the analytes are loaded at one end. A voltage is then applied across the gel which causes the charged analytes to move, e.g. positively charged molecules move towards the negative end. The proteins are separated out based on the speed they travel through the gel, which is dependent on the charge of the protein which relates to the size/molecular weight. Therefore, proteins are separated based on molecular weight [63].

1.7.1 SDS-PAGE

The most commonly used gel technique is sodium dodecyl sulphate-polyacrylamide gel electrophoresis [63] (SDS-PAGE). In this method, the proteins to be separated are first treated with SDS along with other chemicals. This causes the proteins to denature and unfold so that they move through the gel better [63, 64]. Along with denaturing the proteins, the SDS also binds to the protein giving it a uniform negative charge. The amount of SDS that binds to a protein is proportional to the size [65]. Therefore, the larger the protein is the higher the overall negative charge.

The sample is then placed on the gel and a voltage is applied for a set time period causing separation of the proteins based on charge. Once the gel has reached completion it can be stained using various stains which cause the proteins to become visible. The most common stain is Coomassie Blue [64, 66]. Proteins appear as bands on the gel if they have been well separated. If all the proteins are of different sizes, then each separated band should contain only a single protein.

Since proteins are separated based on their charge, which is proportional to their molecular weight, the size of the proteins present can roughly be determined by comparison of bands to that of a calibration ladder. A ladder is a mixture of known sized reference proteins which separate out over the gel [64]. Since the sizes are known, the bands of the ladder can be assigned molecular weights and thus be compared to bands from the samples.

1.7.2 Protein identification by mass spectrometry

To gain more information and identify the proteins once they are separated a second technique is required. Mass spectrometry is the technique used to identify proteins but it generally requires some sample preparation before use [63, 67-69]. For proteins to be properly identified via mass spectrometry they most often need to be digested as mass spectrometry systems do not work well with the large masses. Digesting the protein breaks them up into smaller peptide fragments which are better suited for identification using a mass spectrometer [70, 71].

The most common method for digesting proteins is known as a tryptic digest [71, 72]. A serine proteinase, known as trypsin, breaks polypeptide chains in very specific places. It works by cleaving C-terminal (carboxyl side) to lysine and arginine [73] in a polypeptide chain. Due to this specificity, it will break down a protein into a specific set of fragments consistently. A mass spectrometer can be used to detect these fragments. Since each protein will be consistently broken down into its own unique set of fragments, data can be compared to a database to identify the protein.

Two different mass spectrometry systems are commonly used, the first being LC-MS and the other being MALDI-MS (Matrix-assisted Laser Desorption/Ionisation Mass Spectrometry) [63, 67-70]. Each has their advantages and disadvantages, but both can achieve the same goal of protein identification.

1.7.3 Native gel electrophoresis

In SDS-PAGE gels it is necessary to add SDS to unfold the proteins so that they run through the gel better, as discussed above. This does have a downside in that, because the proteins are denatured or unfolded, they lose some of their properties.

This is an issue when one of these properties is required for detection or for further work and/or analysis. A way around this is to do a native gel.

Native electrophoresis is a technique that does not denature the proteins before they are analysed [74, 75]. This means that the proteins keep their properties that would otherwise be disrupted by denaturing. The proteins are run on the gel after little-to-no modification to their structure [75]. A downside to this is that the proteins no longer go through the gel as they would in a SDS PADE gel and their size cannot be determined through this method.

In native electrophoresis, the separation of proteins is affected by their size and shape along with their charge. The size and shape of a protein is not always related to the molecular mass [76], which is what a SDS PAGE gel can separate proteins based on. Since proteins vary in charge, size and shape they can be separated using a native gel.

Once separation has been achieved then the proteins can be analysed as with SDS PAGE gels. The band or bands of interest can be cut from the gel and then be digested, cleaned up and analysed by mass spectrometry [75]. Since the structure is maintained, properties of the protein in its native state can be used to locate it on the gel which wouldn't be possible on a SDS PAGE gel. It also means that the protein can be extracted from the gel and its properties explored.

CHAPTER 2 METHODS AND MATERIALS

2.1 CHEMICALS

All water used was Type 1 (18.2MΩ) produced by a Barnstead E-pure system. Depending on the use for the water it was either collected fresh or used from a plastic storage container. The water was stored for no longer than two weeks in the container before being replaced. All chemicals used throughout the research are listed in Table 1.

Table 1: List of chemicals used along with supplier and grade/purity

Chemical	Supplier	Grade/Purity
Acetic acid	ChemSupply	Analytical (99.7%)
Acetonitrile	Merck	LiChroSolv
Ammonium acetate	ChemSupply	Analytical (97%)
Ammonium formate	ChemSupply	Analytical (97%)
Ethanol	ChemSupply	Analytical (95.5)
Ethyl Acetate	Optigen	Optigrade (99%)
Formic acid	ChemSupply	Analtical (99%)
Hexane	Scharlau	Analytical (96%)
Methanol	Merck	LiChroSolv
Polyethylene	Induchem AG	Inducos 13/1
Polytetrafluoroethylene	Sigma-Aldrich	Free flowing powder, 1µm particle size
Ascorbic Acid	UNILAB	Laboratory reagent
Ninhydrin	Sigma-Aldrich	ACS reagent
Riboflavin	Acros Organics	98%
Glycine	Sigma-Aldrich	ACS reagent (≥98.5%)
Zinc Chloride	Sigma-Aldrich	Reagent grade (≥98%)

2.1.1 HPLC

Acetonitrile, methanol and water were used unfiltered for HPLC analysis unless other chemicals were added such as buffer or formic acid.. Solvents were filtered through 0.45µm Nylon filter membranes from Sigma-Aldrich. Water was collected fresh when

required for HPLC solvent preparation. The buffer solutions, ammonium formate and ammonium acetate, were prepared as required from a stock solution.

2.2 CONSUMABLES

2.2.1 Spotting material

The five different materials used during the spotting material experiments are listed in Table 2 including the details of each. All materials were used "as is" with no cleaning. The fabric/textile was obtained from an item of cotton/polyester clothing that was available in the laboratory.

Table 2: List of materials used in spotting testing

Material	Manufacturer	Details
Silica TLC	Merck	TLC Silica Gel 60 F_{254} Aluminium sheet 20x20cm
Cellulose TLC	Merck	TLC PEI Cellulose F Plastic sheets 20x20cm
Glass	ProSciTech	Soda-lime glass slide 70x26x1mm
Filter Paper	Whatman	Grade 1 Qualitative 90mm circle
Fabric/Textile	-	White Cotton/Polyester blend 70/30

2.2.2 Size-exclusion chromatography

The SEC medium that was used was Sephadex LH-20 from GE Health Care and was purchased for this work. Methanol, acetonitrile and water were used, as stated in Table 1 with the method adapted from the manufactures recommendations.

2.2.3 Solid phase extraction

Table 3: Solid phase extraction cartridge details

Cartridge type	Maker	Details
Strata C18	Phenomenex	Strata C18-E 55µm 500mg/3ml
Strata X	Phenomenex	Strata-X 33µm Polymeric reverse phase 500mg/3ml
DSC-18	Supelco	Discovery DSC-18 500mg/3ml

The cartridges that were used for SPE work are listed in Table 3. Solvents used are as stated above in Table 1. The cartridges trialled were selected based on availability and their general applications according to the manufactures recommendations.

2.3 INSTRUMENTATION

2.3.1 HPLC

The HPLC used for this work was a 1200 series from Agilent Technologies, Santa Clara, California. The 1200 series has a modular design so that different interchangeable modules of each component can be easily selected to suit requirements. The details for each of the modules for this system are listed below in Table 4.

Table 4: Details of the HPLC system

Module	Model
Quaternary pump	G1311A
Solvent Degasser	G1322A
Auto sampler	G1329A
Thermostatted Column Compartment	G1316A
Diode array and multiple wavelength detector	G1315D
Fraction Collector	G1364C

Chromatograms from the HPLC work were saved as a CSV file and then processed using Origin®Pro (version 9) to produce images of the chromatograms reported in this work.

2.3.2 Columns

The C18 column used was a column made available to researchers by the Flinders University teaching laboratory. The HILIC (Hydrophilic Interaction Liquid Chromatography) column was purchased for the research group. The HILIC column was used with a Phenomenex Security Guard Column with a HILIC disc.

Table 5: Details of the two columns that were used in the HPLC analysis

Column	Manufacturer	Details	Size
C18	Phenomenex	PhenoSphere-Next 5u C18	150x4.6mm
HILIC	Phenomenex	Luna 5u HILIC 200A	150x3mm

2.3.3 Forensic light source

Both FLS's used were a Polilight® system which is a trade mark of Rofin-Sinar, Plymouth, Michigan. Two different Polilight® models were used throughout this work. The first was an older model, PL6 system, which was used for quick visualisation in the lab. The second was a PL500 model which was set up with camera for imaging.

2.3.4 Camera, lens and filter

The camera used to take the fluorescent photographs was a Nikon D5300 digital SLR. This was equipped with a Nikon AF-S Micro Nikkor 60 mm lens f/2.8 aperture. The lens allowed for the attachment of a 62mm filter. The barrier filter used was from Schneider Optics and was attached with an adaptor. The filter was a B+W 67 040 4x F-Pro. This is a yellow-orange filter that blocks light below 530 nm.

All fluorescent images recorded in this work were taken using 450 nm light for excitation and the yellow-orange filter.

2.3.5 Synchrotron

The detector that was attached to the Far-Infrared beamline at the Australian Synchrotron was a Bruker, Billerica Massachusetts USA, IFS 125HR spectrometer. The detector was controlled by Bruker's OPUS software. On initial visits, some of the processes were manually done. This included pumping down the sample chamber and opening the beamline. These eventually became computer controlled using in house developed software. Positioning of the samples in the beamline remained manually controlled.

2.4 ETHICS AND BIOSAFETY

Due to the nature of this work ethics was required before any work could be conducted. This was to cover both the collection of sample and the use of them in the lab. Biosafety was a potential issue but after discussions with the relevant committee it was confirmed that this work was suitable to be conducted under physical containment level 1 lab conditions.

2.4.1 Source

The first step towards getting ethics approval for this work was to find a reliable source of seminal fluid. This needed to be somewhere that ideally had a constant supply of potential samples which would give a good variety and volume of sample. It was decided that it would be better and much easier to obtain ethics approval if samples were not collected by the researchers.

A source was found in the Flinders Medical Centre. Flinders Fertility performs fertility testing in the medical centre and has a regular stream of patients seeking their services. The testing performed routinely by Flinders Fertility does not require the full amount of sample received and leftover sample is disposed of. The option taken was to use this excess sample for the research with permission from the patient.

An information page was written up for the patients to read explaining what the research was about and how their surplus samples would be used along with how their anonymity would be protected. There was also a form supplied to be signed to allow access to the sample. All samples were de-identified before being received with Flinders Fertility being the only people who know what sample belongs to whom.

2.4.2 Ethics approval

Approval to be able to access the samples and then work with them was sought from the Southern Adelaide Clinical Human Research Ethics Committee. The two previously mentioned forms along with the standard ethics form were submitted and after amendments were accepted and ethics approval granted on the 16th of January 2014 and assigned the number 514.13.

2.5 SAMPLES

Once ethics approval was granted, Flinders Fertility began providing patients with the documents. When a patient agreed to allow access to a sample it was kept by Flinders Fertility and arrangements made for the sample to be collected. In most cases these were collected the day that they were donated but in a couple of cases they were collected the following day. Patients would attend in the morning and the sample would be available in the afternoon. As discussed earlier, the samples were de-identified before being received. Samples were received with a simple label of S1 (for sample one).

Samples were received in a 10ml centrifuge tube with screw cap, weighed in the container and then placed in a freezer (-18°C) until required. Typically, each sample was around 1-2 ml in volume but naturally there was some variation in the volume. The samples were weighed to keep track of how much of each sample had been used.

Before use, samples were removed from the freezer and allowed to defrost by allowing them to warm to room temperature. The samples were vortexed immediately prior to taking a volume to ensure that any sample that was taken was homogeneous. In general, where appropriate, multiple samples were mixed for an experiment to reduce the possibility of a single sample affecting results.

A list of the samples that were received can be seen in Table 19 in Appendix A. Samples S6 and S7 were not received due to a mix up with paper work and the sample names were not later reassigned to different samples. In most cases the date received is the date that the sample was donated but is some cases there was a one-day delay where the sample was stored at 4°C until it was picked up.

2.6 SYNCHROTRON

2.6.1 Disc formation

Known masses of sample and disc matrix (polyethylene (PE), polytetrafluoroethylene (PTFE) or a mixture of both) were weighed out and then placed in a mortar and

pestle. These were ground together until the mixture was homogenous. An amount of this mixture was taken and placed into a KBr die set. The amount taken was based on judgment as to how much would be needed to form an even but thin disc. This was then pressed with 10 tons of force to form the disc.

2.6.2 Disc and data analysis

Discs were placed in a sample holder that clamped three samples in place for analysis. Once in the instrument the sample chamber was pumped down to remove the air. The beamline was then opened, and the sample holder adjusted so that the desired sample was in the path of the beam. A live reading of the amount of light being detected could be used to determine when the sample was in optimal position. Once in positon several spectra were recorded successively for each sample.

The recorded data was saved and edited later with the OPUS software. Frist the several spectra that were recorded for the one sample were averaged. Then a blank disc spectrum was used as a background spectrum and was subtracted from the sample spectrum. The final step was to convert the spectrum from transmission to absorbance.

2.6.3 Formation of Ruhemann's purple and zinc complex

Ruhemann's purple was formed from the reaction that occurs between ninhydrin and amino acids. Two parts ninhydrin was added to one-part glycine in water. The reaction was heated to boiling and then gently boiled for 5 minutes. During this period the solution turned purple. It was then allowed to cool. The water was removed under vacuum to get Ruhemann's purple for analysis.

For the metal complex, the reaction was as above but once cooled, instead of removing the water zinc chloride was added to the solution. This caused a dark red precipitate to form. The precipitate was collected by filtration and washed with water. The collected solid was dried completely in a desiccator.

2.6.4 Synthesis of lumichrome

This synthesis was modified from Bergstad et al. [77]

2.38g (14.87mmol) of alloxan monohydrate and 1g (16.17mmol) of boric acid were dissolved in hot acetic acid (95ml). The resulting solution was added to a stirred solution of 2.06g (15.12mmol) of 4,5-dimethyl-o-phenylenediamine in 25ml of acetic acid. The solution was stirred for a further 75 minutes at room temperature and the precipitate collected by filtration, then washed with acetic acid followed by ether. Drying gave a bright yellow powder. Product was confirmed by mass spectrometry (see Figure 57 in Appendix B). Expected mass 242g/mol ($C_{12}H_{10}N_4O_2$). Mass plus sodium from mass spectrometry 265g/mol ($C_{12}H_{10}N_4O_2Na$), 242g/mol minus sodium.

2.6.5 Synthesis of lumiflavin

The following procedure was done as reported by Imada et al. [78]

To a stirred solution of 4.00g (24.07mmol) of 4,5-dimethyl-2-nitroaniline in 50ml of conc. H_2SO_4, 50ml of formaldehyde (37%) was added over 3 hours. Once added the solution was heated to 65°C for three hours. The solution was then poured onto ice water and the precipitate collected by filtration. The solid was dissolved in ethyl acetate and washed with sat. $NaHCO_3$ and dried with $MgSO_4$ and solvent removed. Solid was dissolved in hot ethanol, allowed to cool and water added, precipitate was collected and dried giving N,4,5-trimethyl-2-nitroaniline.

The following procedure was modified from one published by Sichula et al.[79]

3.73g (20.70mmol) of N,4,5-trimethyl-2-nitroaniline was dissolved in conc. HCl (110ml) and 11.49g of tin powder was added portion wise and the reaction mixture heated to 100°C for 3 hours under nitrogen. The flask was cooled in an ice bath and sodium hydroxide (≈300ml, 3.5 M) added until the pH was approx. 10 causing precipitation of a white solid. The reaction flask was then heated to boil causing the white precipitate to turn grey and then the precipitate removed by filtration and rinsed with 300ml of boiling water. The filtrate was cooled in an ice bath and extracted with chloroform and dried with sodium sulphate under nitrogen and then

the solvent removed. The product, 1-N,4,5-trimethylbenzene-1,2-diamine was used immediately without purification in the next reaction.

1.52g (10.12mmol) of 1-N,4,5-trimethylbenzene-1,2-diamine was added to a solution of 2.01g (12.55mmol) of alloxan monohydrate and 0.70g (11.32mmol) of boric acid dissolved in acetic acid (30ml) under nitrogen and the mixture stirred overnight at room temperature. 60ml of water was added to the reaction and extracted with chloroform and dried with sodium sulphate and concentrated. Compound was purified on a column using chloroform/methanol (1:1), yield not recorded. Product confirmed by mass spectrometry (see Figure 58 in Appendix B). Expected mass 256g/mol ($C_{13}H_{12}N_4O_2$). Mass plus sodium from mass spectrometry 279g/mol ($C_{13}H_{12}N_4O_2Na$), 256g/mol minus sodium.

CHAPTER 3 SYNCHROTRON

3.1 OBJECTIVE

The main objective of the following work was to try and identify if the bond between zinc and another compound that results in a fluorescent compound was able to be observed in the Far-IR spectrum. The complex that forms form the addition of zinc to a compound known as Ruhemann's purple is known to be fluorescent, so this was selected to be analysed.

One issue that had to be overcome was that there was little precedence for analysis on such samples using this method. Because of this analysis on less complex samples as conducted to get a better understanding of the instrument, how to analyse the results and what techniques would be best. This included experiments with different sample preparation to make sure the best results were being obtained.

3.2 INTRODUCTION

The most recent research, Honours project at Flinders University [13], on the fluorescence of seminal fluid involved the use of the Far Infrared beam line at the Australian Synchrotron. The initial work undertaken for this thesis was a continuation on this.

A synchrotron is a source of very bright light that can be split into various wavelength regions, such as the Far-IR region (400-30cm^{-1} [50]). Due to the intensity of this light it can reduce the run time of experiments greatly. In addition to this, experiments that are not possible on standard laboratory equipment can be conducted [80]. The light produced is several orders of magnitude more intense than conventional sources [80] and therefore can be used on samples with very low concentration. It is used in conjunction with a high resolution spectrometer with a resolution of 0.001cm^{-1} [81].

Initial experiments done in the honours work were on neat seminal fluid [13]. Seminal fluid was initially analysed using a grazing incident angle. The beam was bounced off the surface of a sample on to a detector. For seminal fluid, this did not produce a spectrum different to the surface it was placed on. The other method trialled involved the use of a polyethylene disc. Seminal fluid was both dried and

pressed into the disk along with it being smeared onto a pre-pressed polyethylene disc and then dried. These experiments were unsuccessful and did not produce a spectrum with usable information.

One of the objectives was to see if anything could be linked to a metal ligand complex. These bonds can be detected in the Far-IR region and a zinc complex potentially has a role in the fluorescence in seminal fluid [11]. Due to the complexity of seminal fluid and some of the limitations of IR spectroscopy, clear results were going to be difficult to obtain. Experiments following this were therefore conducted on less complex samples. Once methods for sample preparation and analysis had be perfected then more complex samples could be introduced.

3.2 DISC PREPARATION

The sample holder used at the Australian Synchrotron required that samples be in a form that could cover a small opening (5mm diameter) which light (typically diameter of 3mm) was then directed through. The best way of doing this was to have the sample in a similar form to a potassium bromide (KBr) disc that is used in IR analysis. KBr could not be used in this work as it is not transparent enough in the Far-IR range. The matrix that was recommended to be used to form the disc was polyethylene (PE).

Disc preparation was like that of a KBr disc. The sample was weighed and placed into a mortar and pestle with a weighed amount of the disc matrix and ground until homogeneous. Once a homogeneous sample was achieved it was press into a disc that was as thin as possible. The disc needed to be thin so that the maximum amount of light would be able to be transmitted through to give a good quality spectrum.

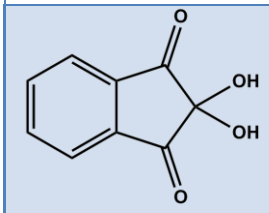
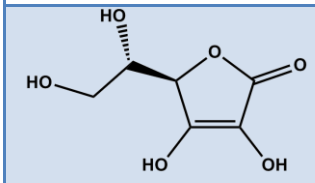
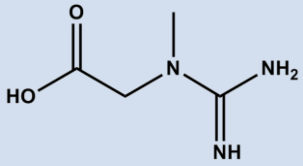
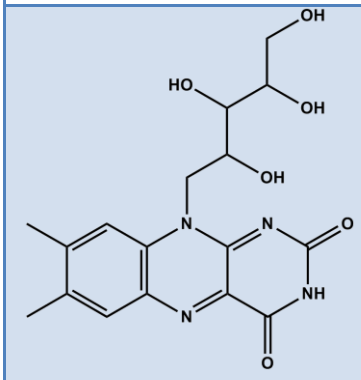
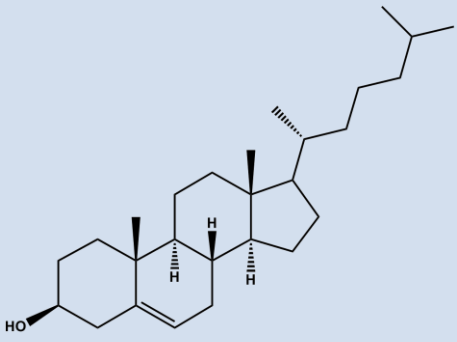
It was attempted, where possible and to the best of our abilities, to make sure the amount of matrix used was even between samples. The amount of compound and disc matrix in each sample was weighed to ensure that there was approximately an even concentration of sample between discs.

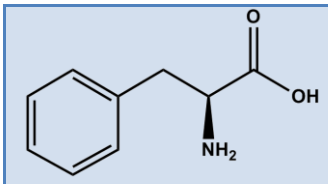
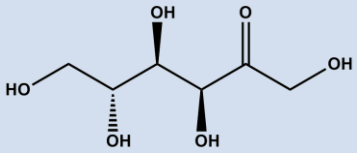
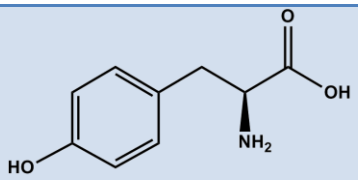
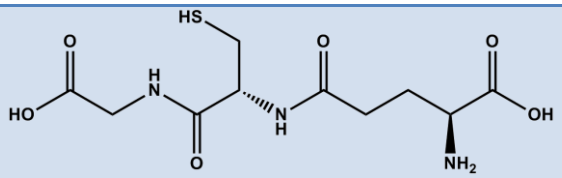
3.3 TESTING COMPOUNDS

The list of compounds that were selected to be tested were picked based on their; known presence in seminal fluid, solid form and availability. This was apart from ninhydrin which was chosen because it is known to form a fluorescent complex with zinc. This occurs in the development of fingermarks and could be used as a model fluorescent zinc complex.

The chemicals tested, and their structures can be seen in Table 6. The various compounds chosen had a variety of structures which would increase the chance of finding compounds that gave good quality Far-IR spectra.

Table 6: List of the compounds analysed using the synchrotron, (Albumin structure excluded due to its complexity)

Ninhydrin	Albumin
	Protein structure
Ascorbic acid	Creatine
	
Riboflavin	Cholesterol
	
L-Phenylalanine	Fructose

	
L-Tyrosine	L-Glutathione
	

All the chemicals were made up into discs using PE as the disc matrix. These were then all run on the synchrotron using established settings recommended by Australian Synchrotron staff. Regular blanks were run to monitor changes in the signal which happened due to how the beamline was created at the time.

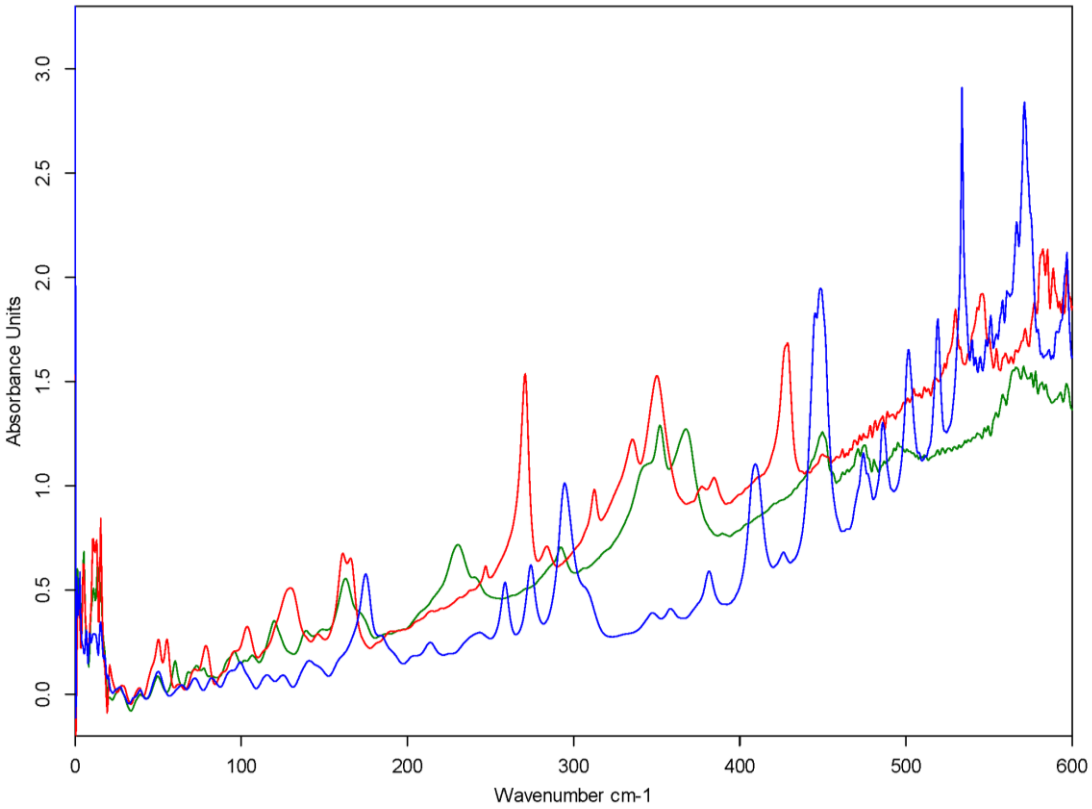


Figure 8: Overlay of the spectra from ascorbic acid (green), ninhydrin (red) and riboflavin (blue) all in PE

Only three of the ten compounds that were analysed resulted in good quality spectra. These were ninhydrin, ascorbic acid and riboflavin. An overlay of their spectra can be seen in Figure 8. In each of the samples several peaks can be seen

showing that they absorb in the Far-IR region. All other compounds did not absorb in the Far-IR region. Subsequently, ninhydrin, ascorbic acid and riboflavin were used for further experiments.

Figure 9 shows the spectrum produced by cholesterol against that of ninhydrin. Where the spectrum for ninhydrin has clear well defined peaks the spectrum from the cholesterol has no clear peaks. The peaks that can be seen in the spectrum are from background sources. This is determined by their uniformity and their presence makes it difficult to use the data.

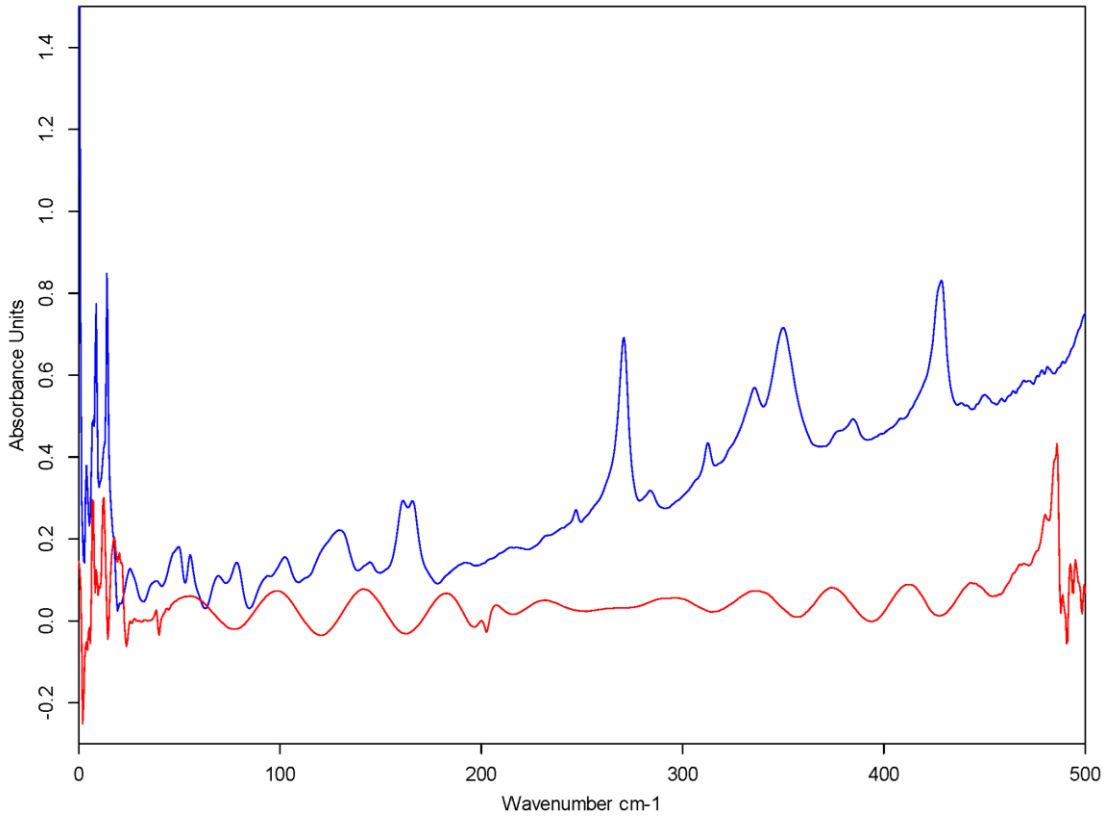


Figure 9: Spectrum showing ninhydrin (blue) which produced good spectrum versus cholesterol which did not give a good spectrum

3.4 ASCORBIC ACID AND RIBOFLAVIN VS. LITERATURE SPECTRA

The Far-IR spectra for ascorbic acid and riboflavin have previously been reported. Cao et al.[82] did studies on ascorbic acid using terahertz time domain spectroscopy. This technique gives a spectrum that is in the same region as Far-IR spectroscopy. In

this work the spectrum of ascorbic acid was recorded and compared to one that had been predicted using modelling software.

Figure 10 shows a comparison between the spectrum reported and a spectrum that was recorded at the synchrotron. The two spectra show good agreement. Both spectra have four peaks between 50 and 80cm^{-1}. The peaks appear weaker in the synchrotron spectrum, but this is due to the sample being less concentrated. The first peak occurs at 1.8THz which is 60.04cm^{-1} (1THz ≈ 33.36cm^{-1}) and matches the synchrotron peak at 60.13cm^{-1}.

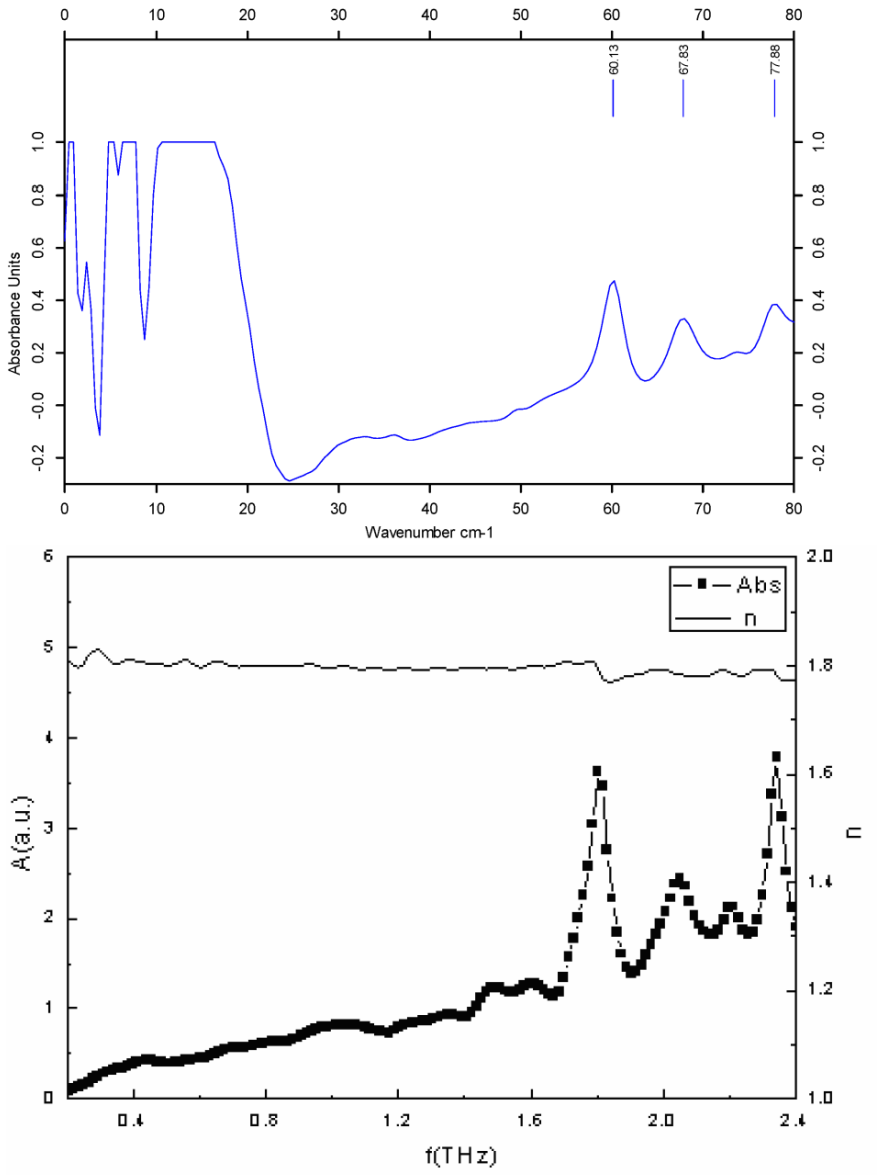


Figure 10: Comparison between the synchrotron spectrum of ascorbic acid (16 mg in PTFE, top) and the reported spectrum by Binghua et al.[82] (bottom)

Takahashi et al.[83] analysed riboflavin in the Far-IR region. The spectrum obtained can be seen in Figure 11 along with a spectrum of riboflavin from the synchrotron. There is again good agreement between the two spectra. The peaks occur at slightly different points, but the differences are less than a wavenumber.

The reported spectra from literature show good agreement with the synchrotron spectra. In each case the reported spectra are limited and do not cover the whole Far-IR region. The spectra that were obtained from the synchrotron go to much higher wavenumber and the spectra retain very good quality.

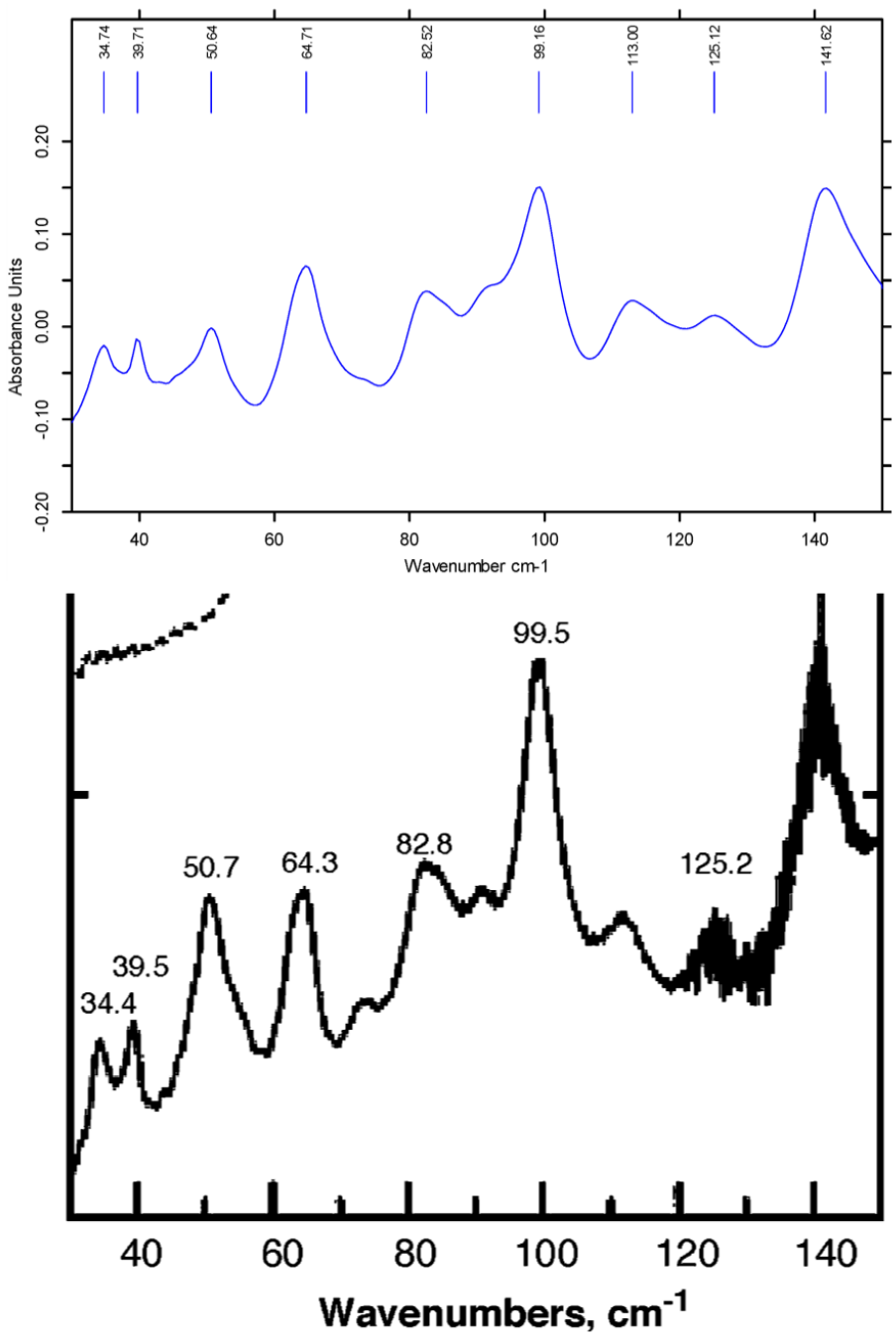


Figure 11: Comparison between the synchrotron spectrum of riboflavin (16mg in PE, top) and the reported spectrum by Takahashi et al.[83] (bottom)

3.5 DISC MATRIX

The initial analysis was done using PE as the disc matrix. There were some issues with this matrix. Since the sample preparation process was time consuming the discs had to be pressed in advance (to maximise the number of samples that could be

analysed in the time available) and then transported (by car and aeroplane) to the synchrotron. PE discs were found to be fragile and regularly did not make it intact. Other polymers may also be more transparent in the Far-IR region. A polymer that was chosen to be experimented with was polytetrafluoroethylene (PTFE).

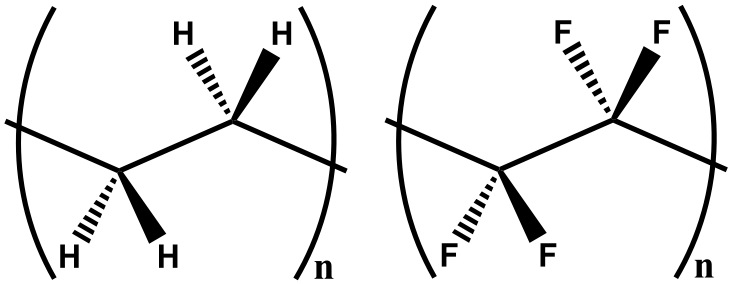


Figure 12: Chemical structures of PE (left) and PTFE (right)

PTFE has the same structure as PE except the hydrogen atoms are replaced with fluorine atoms. This produces a polymer with different properties that would hopefully lead to a more durable disc. The two polymers were tested to see which one was more durable during transport, had the best transparency and to see if there were any differences in the spectra produced by samples.

3.5.1 Disc matrix properties

PTFE discs were found to be opaquer to the naked eye than PE discs. It appeared that when being pressed the PTFE was forming a more plastic like disc. The PE discs were more like a tablet and still looked powdery. The PTFE discs were more flexible and small amounts of bending did not fracture the disc. They also survived transportation from Adelaide to Melbourne better along with loading into the sample holder.

One issue that was present for both matrixes but more prevalent in PTFE samples was static charge. Once ground it was found that the mixture would become statically charged and could become very difficult to work with. Attempting to get the sample from the mortar to the KBr die was hampered as the mixture would ‘flick’ away from the spatula. It would also stick to the wrong places of the die set.

Each of the matrixes had their good points and their bad points. Overall it would seem that PTFE was the better option if the discs needed to be transported. If

minimal transportation was required, then PE was a better option as it suffered less from static electricity effects.

3.5.2 Spectrum quality of the different disc matrixes

The first experiment with the new disc matrixes was test its transparency in the Far-IR region and compare it to PE. A disc of pure PTFE and one of pure PE were pressed and analysed. The resulting spectra from each of these samples can be seen in Figure 13. It should be noted that the spectra are of the transmission of light not absorbance spectra.

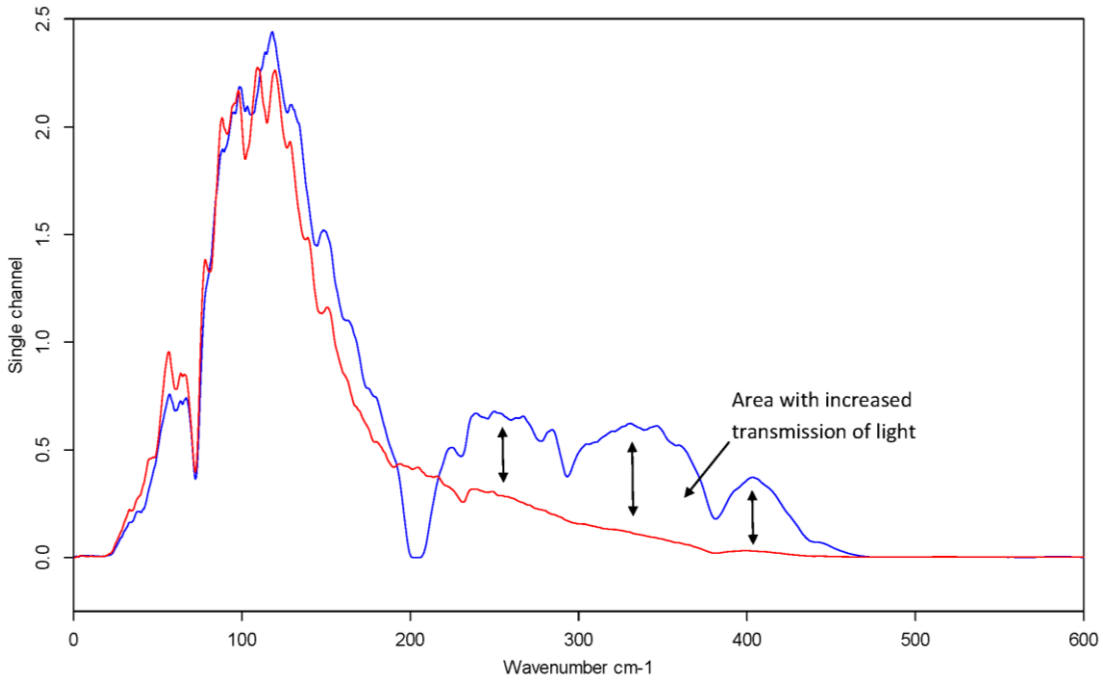


Figure 13: Synchrotron spectra produced for pure PE (red) and pure PTFE (blue) in transmission mode

The spectra are largely similar for the first part of the spectrum. Differences between the two become noticeable above 175cm^{-1}. The most obvious is the loss of transmission of light around the 200cm^{-1} area in the PTFE sample. This is thought to be due to the carbon to fluorine bond in the PTFE. There is the possibility of a loss of information in this region in the spectra of samples. While this could be a problem it could also serve as a calibration point between spectra as it should be very consistent.

Another difference occurs at the higher wavenumber end of the spectrum. From after where the PTFE is absorbing at 200cm^{-1} on to approximately 450cm^{-1}, more light is passing through the disc. This potentially means that a more detailed spectrum could be produced in this wavenumber region.

Since the PTFE appeared to allow good transmission of light in the Far-IR region the next step was to make discs with various compounds in them to test the quality of the spectra produced. The compounds that were used to test the spectrum quality for the disc matrixes were ninhydrin, ascorbic acid and riboflavin. Discs were made that had approximately the same concentration of the compound in them.

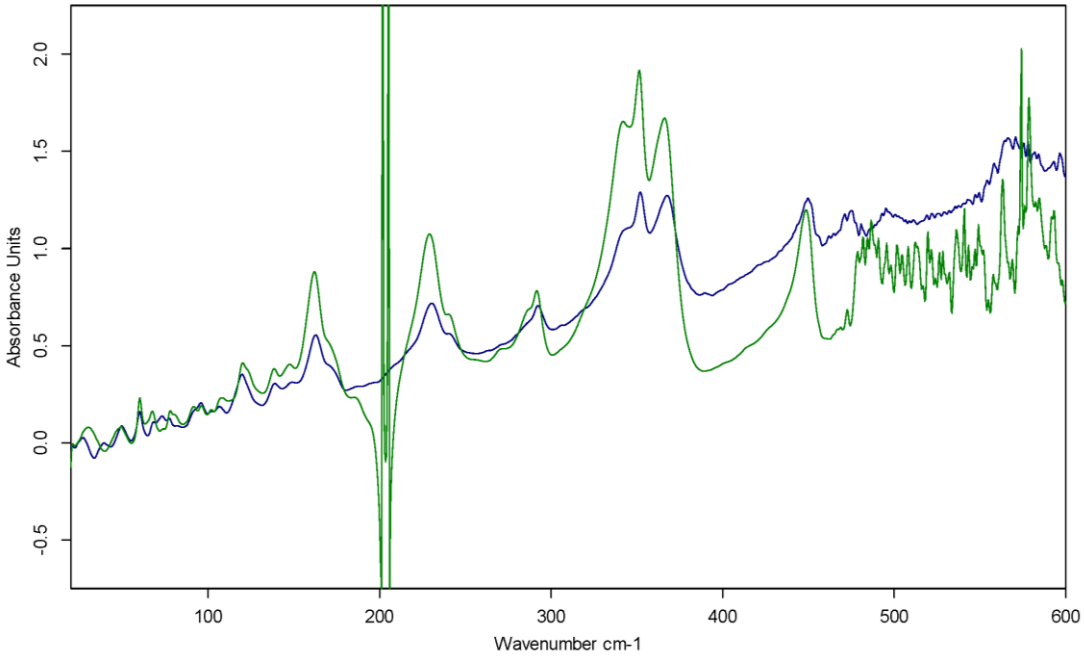


Figure 14: Absorbance spectra for ascorbic acid in PE (Blue) and PTFE (Green)

Both matrixes produced similar spectra for ascorbic acid, Figure 14. The key difference between the two is that the PTFE sample has a lower baseline above 200cm^{-1}. This meant that the peaks have better definition.

The trend of the sloping base line in the PE can be seen here and it has the effect of masking the peaks higher up. It is about at 450cm^{-1} that both spectra lose quality and become noisy. The PTFE also has a total absorption around the 200cm^{-1} mark from the characteristic peak causing a loss of information.

As was the case with ascorbic acid, there is the characteristic signal in the PTFE spectrum at 200cm^{-1} and sloping base line in the PE spectrum for ninhydrin. The peaks again occur at the same points but this time the intensity maximums are about the same for both matrixes. The peaks just appear smaller in the PE at higher wavenumbers due to the base line being higher. The peaks also have very similar shapes and definition which wasn't always the case for ascorbic acid where some of the peaks had more apparent difference.

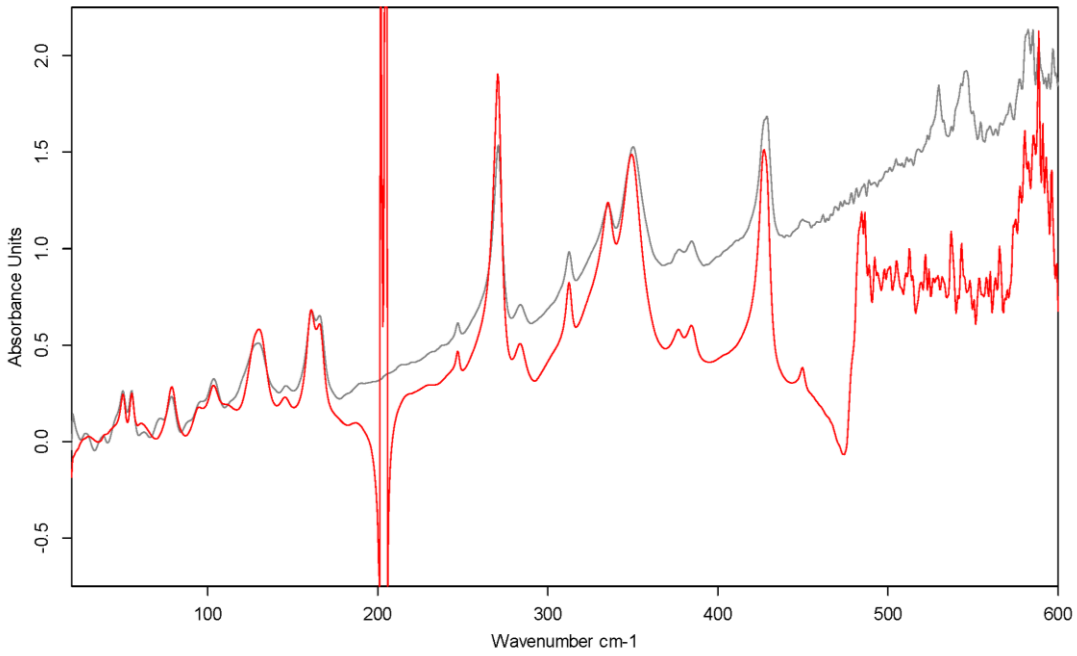


Figure 15: Absorbance spectra for ninhydrin in PE (Grey) and PTFE (Red)

The third compound tested was riboflavin and the spectra can be seen in Figure 16. The peak shape and intensities in these two spectra are very similar. The peak intensities are similar in the PE despite the increasing base line. The baseline rise in the PE spectrum appears to be less pronounced compared to the previous two compounds. There are a series of peaks that occur above 450cm^{-1} in the PE which cannot be seen in the PTFE spectrum.

There are some common trends with the spectra that were produced. The first is in the PTFE spectra at the 200cm^{-1}, where the peak was noted in the blank spectrum earlier. Over a small range (200cm^{-1} ± 10cm^{-1}) the spectra have large absorbance and

there is a loss of information. This is not desirable; if there is an important peak occurring here then it would be lost.

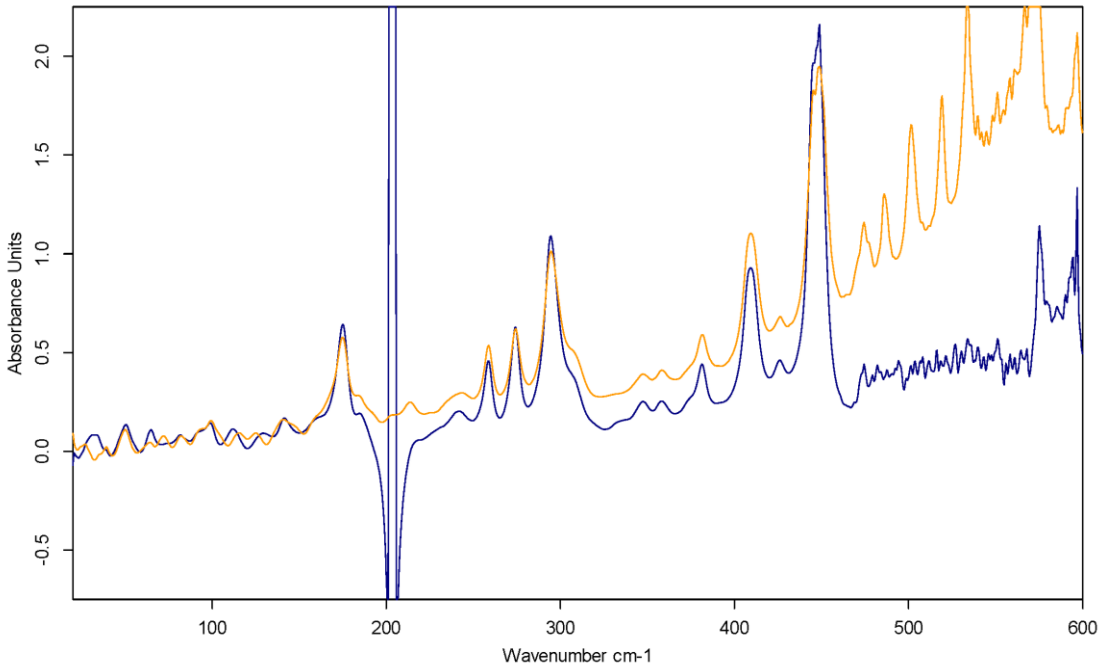


Figure 16: Absorbance spectra for riboflavin in PE (yellow) and PTFE (blue)

It can also be seen that the PTFE spectra have a better base line when compared to the PE spectra. The PE has a rising base line that becomes most evident above 400cm^{-1} wavenumber in the spectrum. In both the PTFE and PE the spectra become noisy above approximately 500cm^{-1} and no useful information can be extracted from this area of the spectra. The spectrum for riboflavin in PE is an exception with some clear peaks visible but in general there is not much to see. This isn't a huge issue as this is getting into the mid infrared range.

From this initial work, no clear standout disc matrix could be picked so both options were used for further work. Both appeared to have advantages and disadvantages. It was thought that maybe a mix of the two could perhaps be the best option. Three different mixtures of the disc matrixes were trialled to see if this would be better. The mixes were PE to PTFE in the following ratios; one to three, one to one and three to one. Each was run, and the best was decided to be the mix of three parts PE to one part PTFE. This gave increased stability of the disc with a smaller peak at 200cm^{-1} and did not have the noise around 500cm^{-1}.

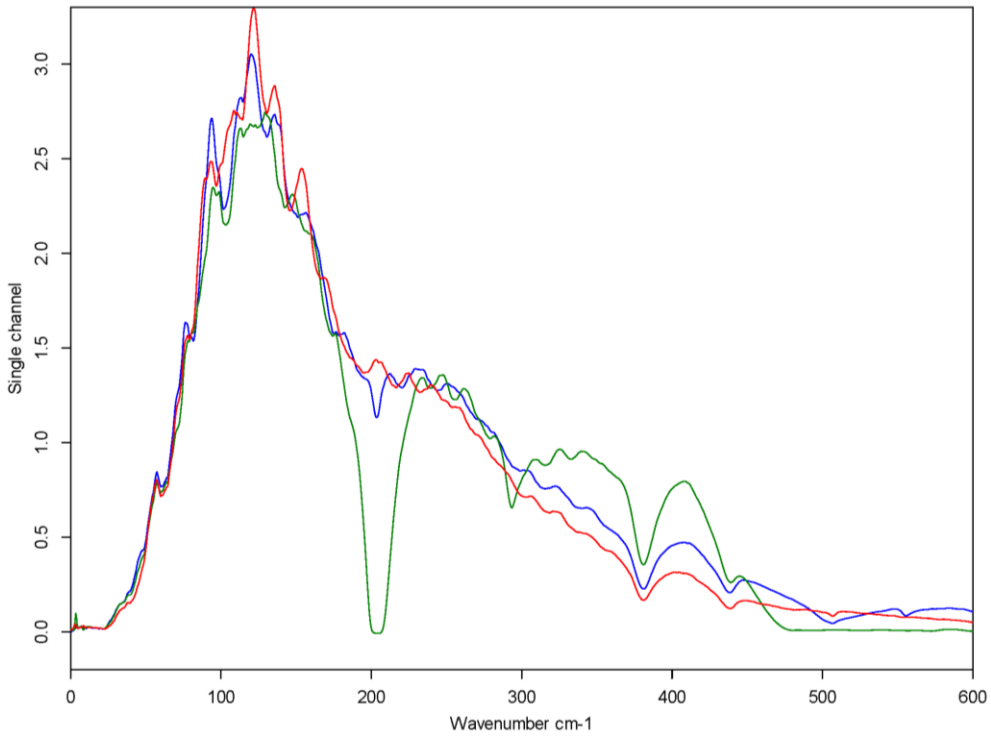


Figure 17: Overlay transmission spectra of PE (red) PTFE (Green) and 3:1 mix (blue)

The overlaid spectra for PE, PTFE and the 3:1 mix can be seen in Figure 17. As it would be expected, as the disc was mostly PE, the spectrum for the mix most closely resembles that of PE. The characteristic 200cm^{-1} peak in PTFE is greatly reduced in the mix but is still present and can act as a calibration peak. Since there would be a reduction in the peaks intensity it could lead to there being better transmission of light and there being no loss of information. This mix of the two matrixes did produce a disc that was robust, with the PTFE thought to be helping to hold the PE together better.

3.5.3 Pure sample discs

There was also work done on making the disc from pure compound with no binding disc matrix. Reasons for wanting to do this are there could be the potential for the disc matrix to interact with the sample and alter the resultant spectrum. It would also remove the matrix absorption. There would also be less sample preparation needed for the pure discs is some cases as the compound would not need to be mixed with the disc matrix. However, a greater mass of sample would be required to make the disc. It would need to be ground to a fine powder as this is better for

forming discs. This would have its limitations but could be useful for some samples.

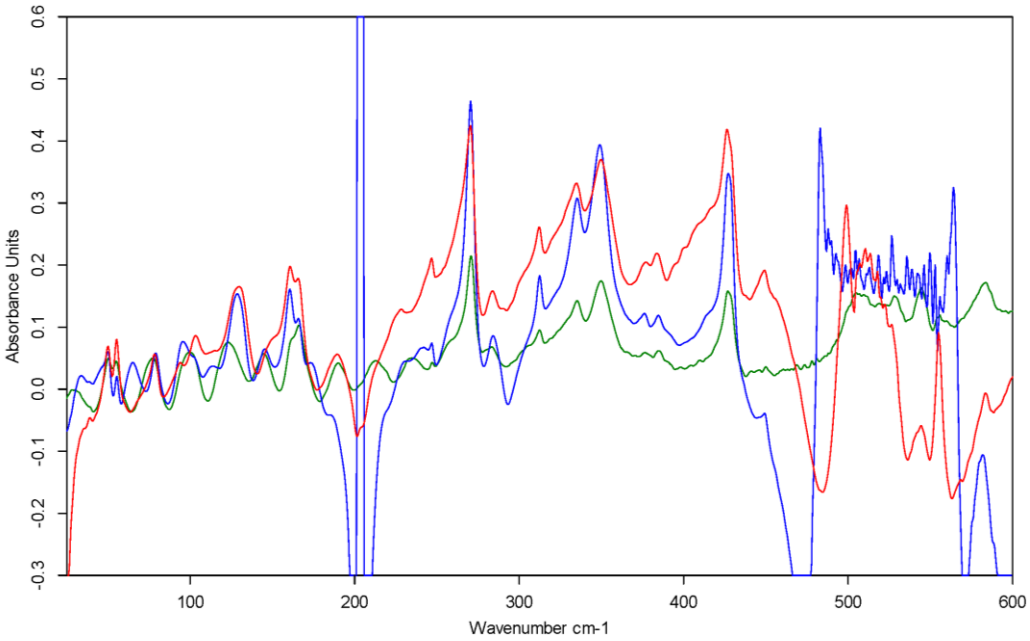


Figure 18: Overlay spectrum of ninhydrin in PE (green) PTFE (blue) and 3:1 (red)

The sample would need to be able to hold together by itself which some matrixes would not do but most should be able to be compressed into a disc, at least long enough to obtain a spectrum. Once a spectrum had been obtained then the sample could be removed from the washer and still be available for further testing. This would be a non-destructive form of analysis. But given that the disc is purely made from sample there could be a potential issue of not enough light passing through affecting the spectrum. Care would need to be taken so that the disc formed was as thin as possible to allow as much light through as possible.

The pure sample discs were much smaller than the normal ones. 3mm in diameter compared to the 10mm diameter of the samples mixed with disc matrix. So, while the amount of sample needed would be higher it would not be such a large increase. They were also formed using a commercially available hand operated KBr pellet press. The discs were pressed inside a steel washer and the formed disc would remain in the washer. This was a useful thing as trying to remove the sample from the washer as an intact disc was difficult but the disc itself would be too small to mount in the sample holder. By keeping it in the washer it would be safer and more convenient.

3.5.4 Pure ascorbic acid, ninhydrin and riboflavin

The pure disc experiments again were trialled on the three usual compounds, ascorbic acid, ninhydrin and riboflavin. The ninhydrin did require some grinding to make it a fine powder but the other two were fine enough powders that they could be pressed with no additional sample preparation. Each of the samples was successfully pressed into a disc and a spectrum was obtained for each.

Spectra were only successfully obtained for two of the samples. The spectrum for pure ascorbic acid can be seen in Figure 19 in which it is overlaid with a spectrum of ascorbic acid in PE. The PE sample was 20mg of ascorbic acid to 60mg of disc matrix.

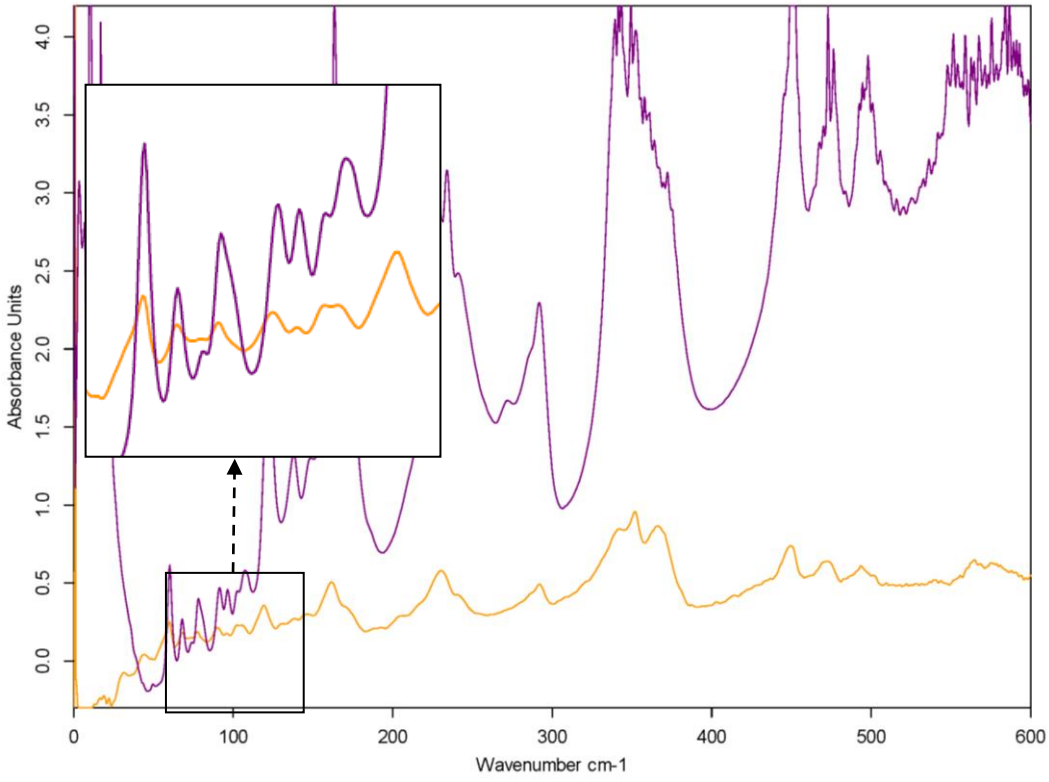


Figure 19: Spectra for pure ascorbic acid (purple) and ascorbic acid in PE (orange) overlaid with a zoomed view of an area highlighting the improved spectrum quality

What this shows is that the pure disc produced a much better spectrum compared to the PE. The peaks are larger, there are more of them and some are more clearly defined. One area where there is a large improvement is from 50-100cm^{-1}. In the pure disc there are a series of well-defined peaks which are much clearer than in the PE spectrum. There are some peaks that become distorted but for the most part the

spectrum is very clear. This shows that for ascorbic acid the concentration needs to be higher to get the detail.

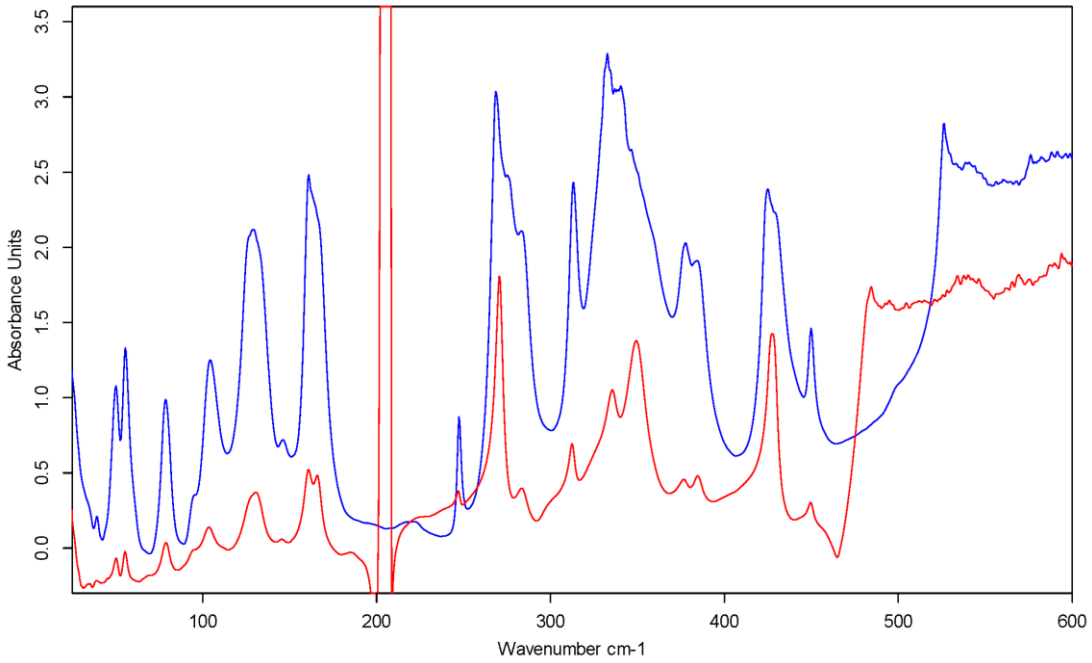


Figure 20: Spectra for pure ninhydrin (blue) and ninhydrin in PTFE (red) overlaid

Figure 20 shows the other compound that produced a spectrum from the pure disc. It is the pure ninhydrin overlaid with ninhydrin in PTFE at the same concentration as the ascorbic acid in Figure 19. The results from the ninhydrin are more mixed than those of ascorbic acid. In some cased there is an improvement in the peak detail with them being more prominent and defined. In other areas, there is a loss of detail and some peaks become distorted and less clear. The area between 300cm^{-1} and 350cm^{-1} is an example of this. In the PTFE spectrum, there are three clear peaks but this becomes less resolved in the pure spectrum. There is the appearance of shoulders on the peaks that do not match anything from the PTFE spectrum.

3.5.5 Other pure disc compounds

After the success of two of the initial compounds, further compounds were run as pure samples, and some could produce good spectra. An example of this can be seen in Figure 21 which is the pure disc spectrum and PE disc spectrum for 1,2-indanedione, a chemical used in fingermark enhancement. In this the pure spectrum is overlaid with the spectrum in PE. The spectra look similar with the pure samples

spectrum having more intense peaks. There appears to be some loss of detail at the higher wavenumber region of the spectrum for the pure disc.

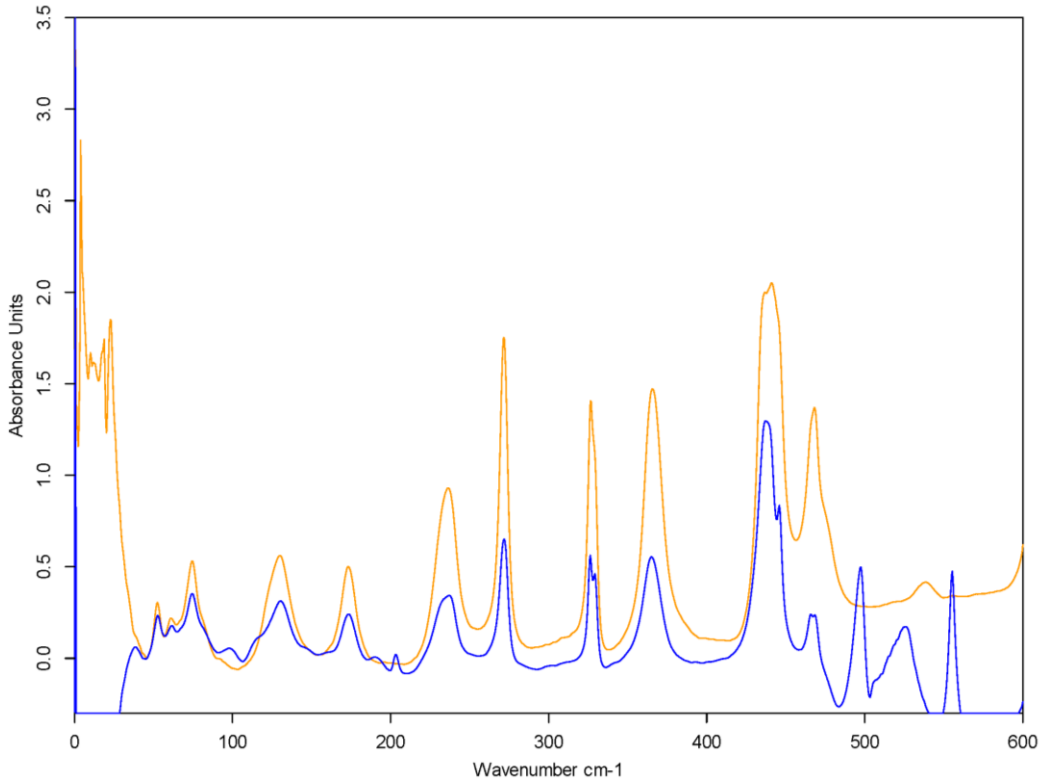


Figure 21: Pure compound disc spectrum for 1,2 indanedione (orange) along with the PE spectrum (blue)

3.6 CONCENTRATION

As with all spectrometric techniques a balance must be achieved between having enough sample present to get sufficient absorbance without overloading and getting total absorbance. Since there was little information available as to sample concentrations for synchrotron analysis, it was important to explore this. Each of the three test compounds was again used in these tests. Both disc matrixes, PE and PTFE, were used as earlier as the additional spectra for each might help show which was a better disc matrix.

Three different concentrations were run of the three compounds. The concentrations used were 8, 12 and 16mg of compound in 80mg of disc matrix. These were again made using the standard method with the amount of matrix used to make the disc kept as similar as possible for all. In hindsight, more even discs

could have been obtained by having the KBr die on a set of scales and weighing the amount of matrix used to make the disc. This would not have been completely accurate though because some of the matrix does stick to other places of the die due to static.

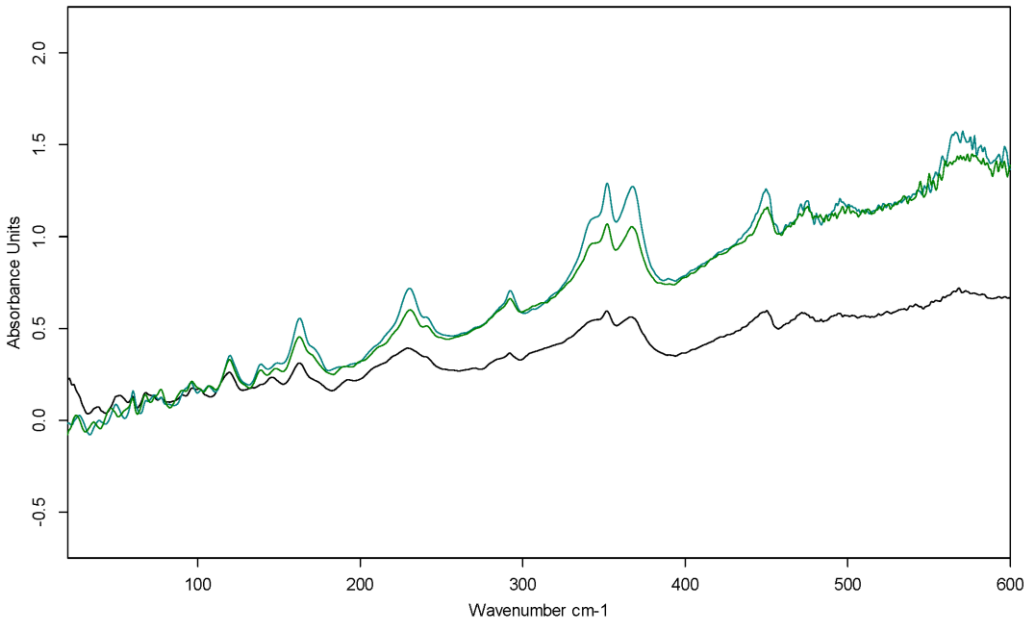


Figure 22: Overlaid spectra of the various concentrations of ascorbic acid in PE. Black = 8mg, green = 12mg and blue = 16mg

The spectra that were obtained from the analysis of the ascorbic acid samples can be seen in Figure 22 and Figure 23. For the sample in PE, there is not a significant change to the spectra as the concentration increased. There is difference with the 8mg spectrum and it is less intense than the others which are quite similar. There is a general increase in the intensity of the peaks but there is not much of an increase in information or peak quality.

A reason for the difference in the intensity between the 12 and 16mg samples not being as large as expected could be due to the disc thickness. If there is an increase in the thickness of the disc then there would be an increase in the amount of sample that the light travels through, raising the absorbance.

The PTFE spectra show an even increase in the intensities of the peaks as the concentration increases. There is no real change between the spectra with no new peaks appearing, but the peaks do become clearer and slightly more defined. There

is an increase in the baseline as the concentration increases but since the peaks are more intense it is not an issue. Increasing the concentration within this range has a positive effect but it does not increase the amount of information gathered.

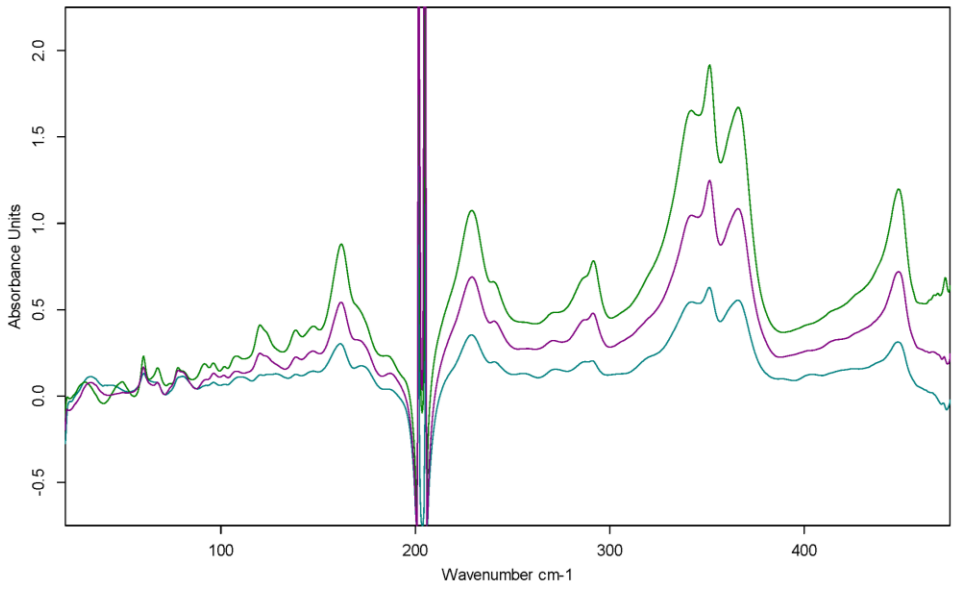


Figure 23: Overlaid spectra of the various concentrations of ascorbic acid in PTFE. Blue = 8mg, purple = 12mg and green = 16 mg

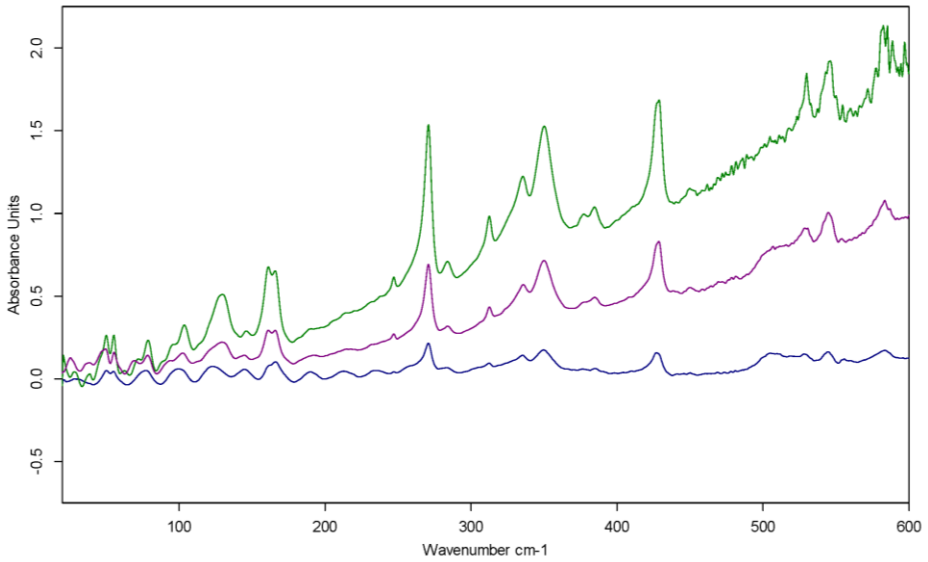


Figure 24: Overlaid spectra of various concentrations of ninhydrin in PE. Blue = 8 mg, purple = 12 mg and green = 16 mg

Figure 24 and Figure 25 are the spectra that were obtained for the various concentrations of ninhydrin. In the PE spectra, there was a noticeable increase in the base line as the concentration increases. There was also an increase in the intensity

of the peaks along with an increase in definition. Peaks in the lower wavenumber region of the spectrum become more distinct in the higher concentration spectra.

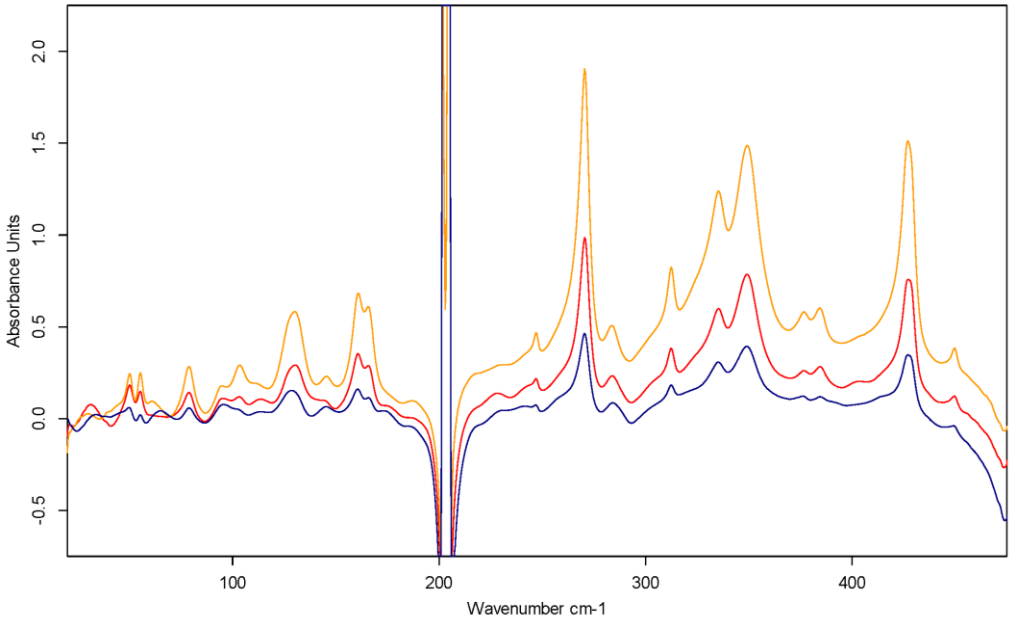


Figure 25: Overlaid spectra of various concentrations of ninhydrin in PTFE. Blue = 8 mg, red = 12 mg and orange = 16 mg

In the spectra for the PTFE, as it was in the PE samples, the two higher concentrations were clearer and had more peaks at the lower end of the spectrum. Some of the smaller peaks have become more visible without the larger peaks masking them.

The spectra for riboflavin are seen in Figure 26 and Figure 27. The results are like the previous two compounds. There is an increase in the peak intensities with increasing concentration along with an increase in the baseline. Some of the smaller peaks become much more noticeable but lower down in the spectrum there is still little to be seen.

There is one peak that is worth noting which occurs at approximately 450cm^{-1}. In the lower concentration, it appears as a single peak but as the concentration increases it begins to look as if there are in fact two overlapping peaks. By the highest concentration, it forms into a distinct peak rather than appearing as a shoulder.

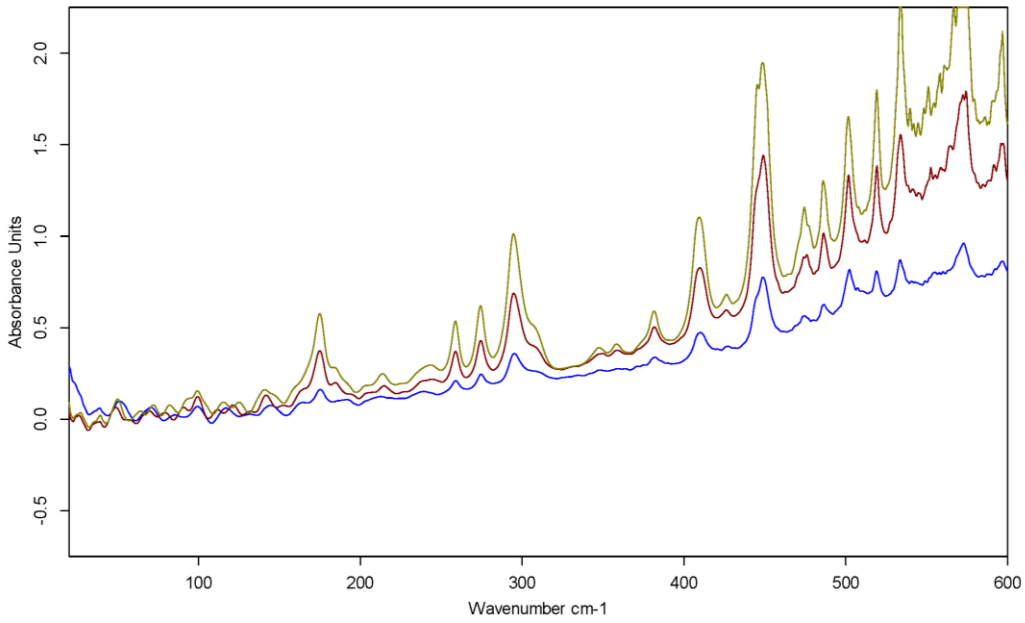


Figure 26: Overlaid spectra of various concentrations of riboflavin in PE. Blue = 8 mg, red = 12 mg and green/yellow = 16 mg

There is one peak that is worth noting which occurs at approximately 450cm^{-1}. In the lower concentration, it appears as a single peak but as the concentration increases it begins to look as if there are in fact two overlapping peaks. By the highest concentration, it forms into a distinct peak rather than appearing as a shoulder.

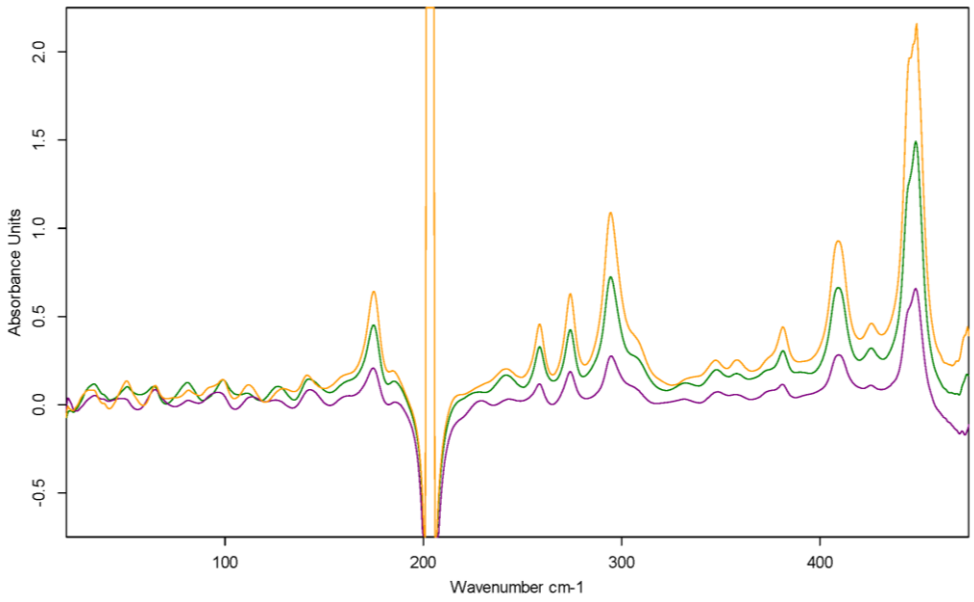


Figure 27: Overlaid spectra of various concentrations of riboflavin in PTFE. Purple = 8 mg, green = 12 mg and yellow = 16 mg

The results show that a high concentration of sample can be analysed. There is no apparent loss of signal and the spectra can potentially become more detailed. As discussed earlier, samples were run that were pure compound and they still allowed enough light through to produce spectra. This shows that it could be valuable to run samples at various concentrations and as a pure disc if possible to get the most accurate spectrum possible.

3.7 MIXED COMPOUND DISCS

Using the three test compounds, mixed compound discs were made and analysed to see what effect this would have and see if it is easy to identify what is present. There is the possibility for interactions between the compounds causing changes making identification difficult. If it is not possible to determine if a compound is present in a mixture, then this limits the types of samples that can be analysed.

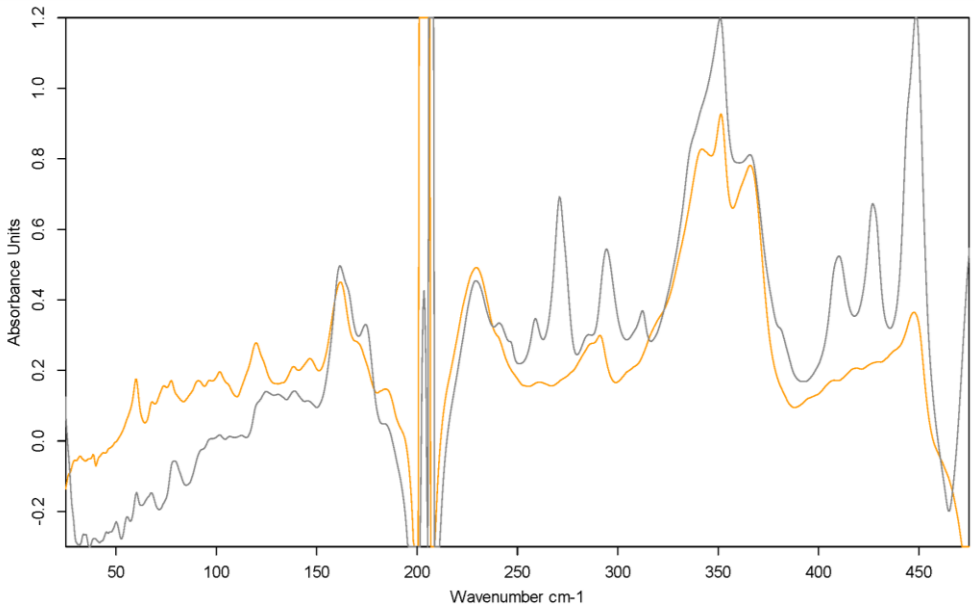


Figure 28: Spectra of ascorbic acid (orange) and of a mix of all three compounds (grey)

Figure 28 shows the comparison of ascorbic acid in PTFE against a mix of all three compounds in a PTFE disc. It is apparent that all the peaks that are present in ascorbic acid have an associated peak in the mixes spectrum. In Figure 8 which has all three compounds individual spectra overlaid, the only standout unique peak for

ascorbic acid is at approximately 225cm^{-1}. There is good correlation of this peak between the mix and the individual sample in the spectra in Figure 28.

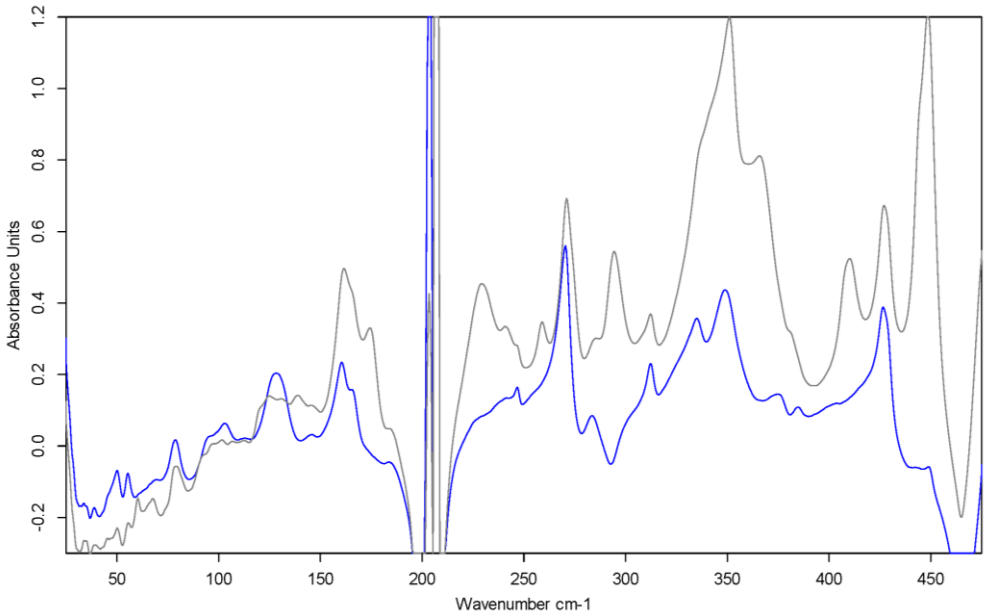


Figure 29: Spectra of ninhydrin (blue) and of a mix of all three compounds (grey)

For ninhydrin, there are a few spots where the mix spectrum appears to match and follow the trends of the ninhydrin spectrum, seen in Figure 29. Almost all the peaks of ninhydrin overlap with a peak from one of the other two compounds. At 130cm^{-1} there is a peak that is unique but there is no corresponding peak. The best correlation occurs at 260cm^{-1} where the peaks match quite well.

The easiest compound to see in the mix is riboflavin. This is mostly because the compound itself has the most peaks in its pure spectrum. Figure 30 is the overlay of the mix spectrum and that of riboflavin and there are many peaks that match. There are some in the riboflavin that are more intense, but this would be due to the riboflavin being higher in concentration in the non-mix sample.

What the mixed sample showed is that this technique can be used on mixed samples, but knowledge of what key components are there would be needed. Even knowing what to look for it can be difficult to see therefore it is best to work with pure samples.

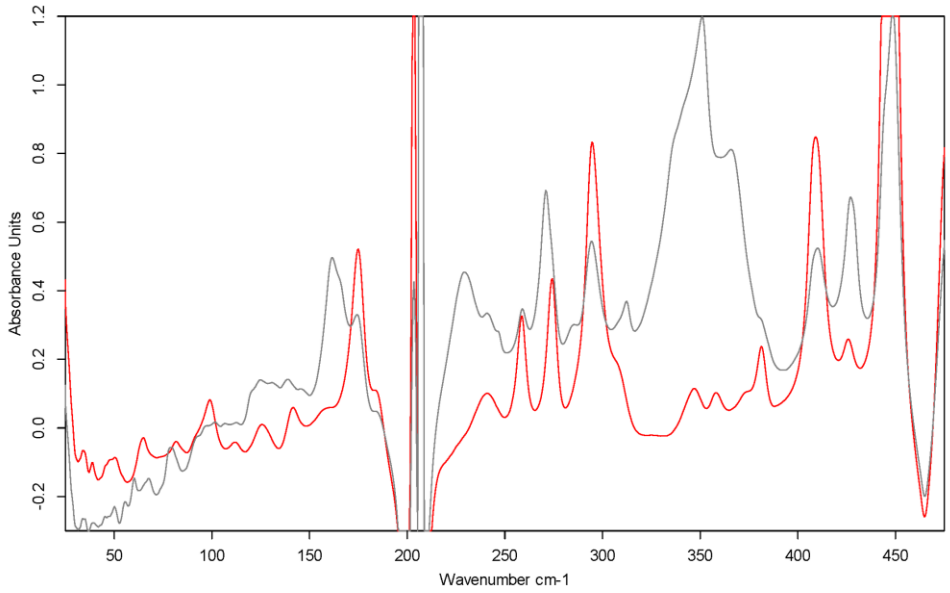


Figure 30: Spectra of riboflavin (red) and of a mix of all three compounds (grey)

3.8 MODEL FLUORESCENT COMPOUND

In unpublished work by Walker (personal communication, 2011) it was noted that there was a potential relationship between fluorescence and zinc concertation. Metals such as zinc can form complexes with organic compounds that are fluorescent. The bonds between metals and organic compounds are observable in the Far-IR region. To explore this potential zinc/organic compound interaction a model compound that was known to fluoresce due to an interaction with zinc was studied.

The complex chosen to be studied was the Ruhemann's purple zinc complex that occurs in fingermark enhancement[84]. This complex was selected mainly because it is based on ninhydrin which had already been shown to produce a good spectrum.

Ninhydrin is a chemical that is used to make latent fingermarks visible on porous surfaces in forensic investigations. The ninhydrin reacts with the amino acids in the fingermark to form a compound that is known as Ruhemann's purple[84]. However, this does not always give enough contrast and further enhancement can be achieved by the addition of zinc. The zinc forms a complex with the Ruhemann's purple which is fluorescent under the right conditions[56]. The structures of ninhydrin and of Ruhemann's purple can be seen in Figure 31.

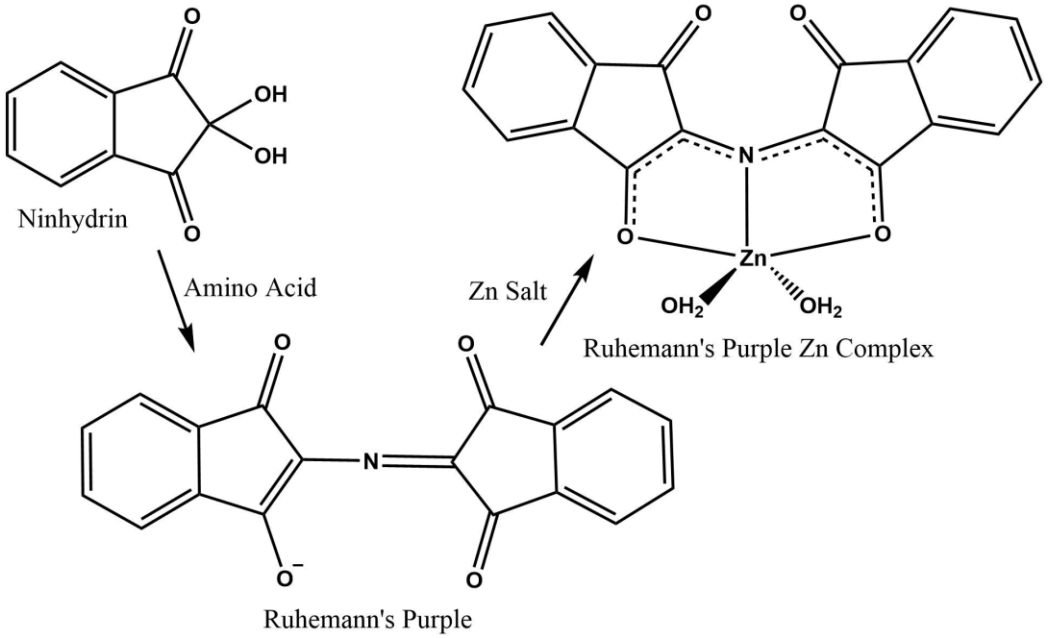


Figure 31: The structures of ninhydrin and Ruhemann's purple along with the zinc/Ruhemann's purple complex (adapted from reported structures by Davies et al.[85] and Champod et al.[86])

By synthesising the complex, it could be analysed to see if there is a unique peak from the interaction between the zinc and Ruhemann's purple. Since it is not fluorescent until the addition of zinc, the bond/s that are formed are important to the fluorescence. There was also the opportunity to see how the structural changes that occur through the synthesis affected the resultant spectra.

Glycine was chosen as the amino acid to react ninhydrin with to form Ruhemann's purple. The Ruhemann's purple was reacted with zinc sulphate heptahydrate to form the metal complex. Both glycine and the zinc sulphate heptahydrate were analysed individually so that any contribution they might have as impurities to the spectra could be accounted for. The spectra for both compounds showed that they did not produce any peaks in the Far-IR region.

When Ruhemann's purple was analysed the results were interesting. As can be seen above the structure is not too dissimilar to that of ninhydrin, sharing several commonalities, but the spectrum is very different. There is a complete loss of information with no real peaks appearing in the spectrum, seen in Figure 32. What this might suggest is that the two hydroxyl groups that are lost during the reaction have a lot to do with the peaks that are seen for ninhydrin.

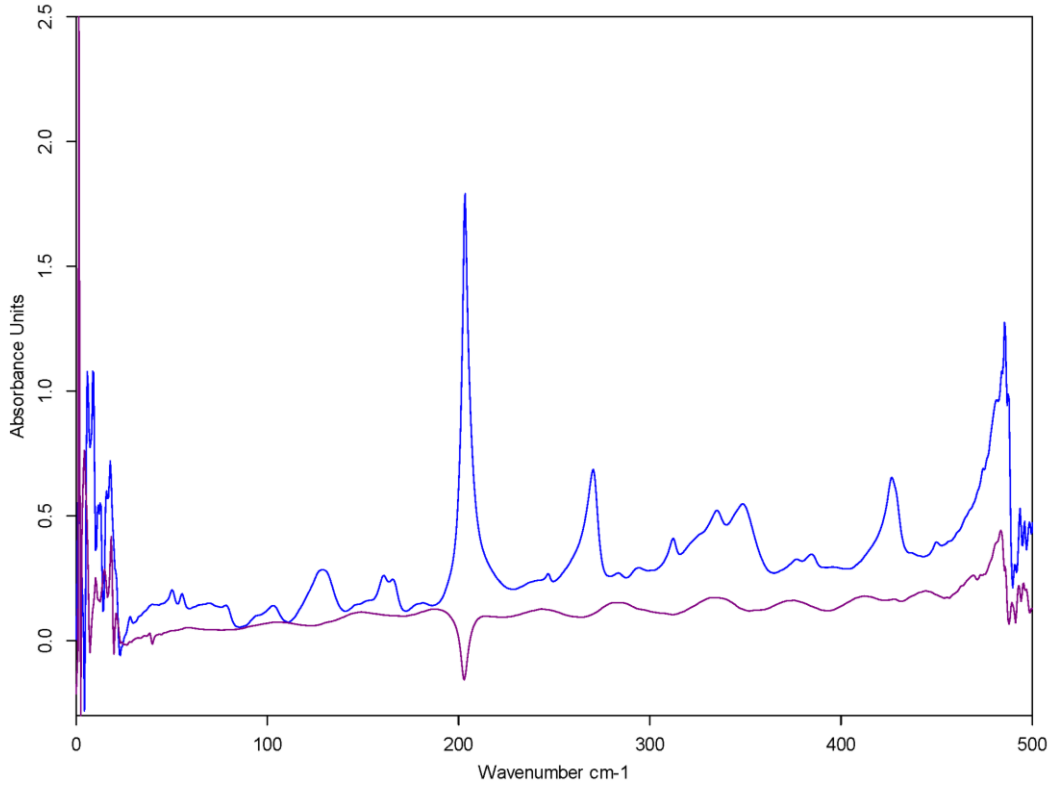


Figure 32: Ruhemann's purple (purple) and ninhydrin (blue) in PTFE

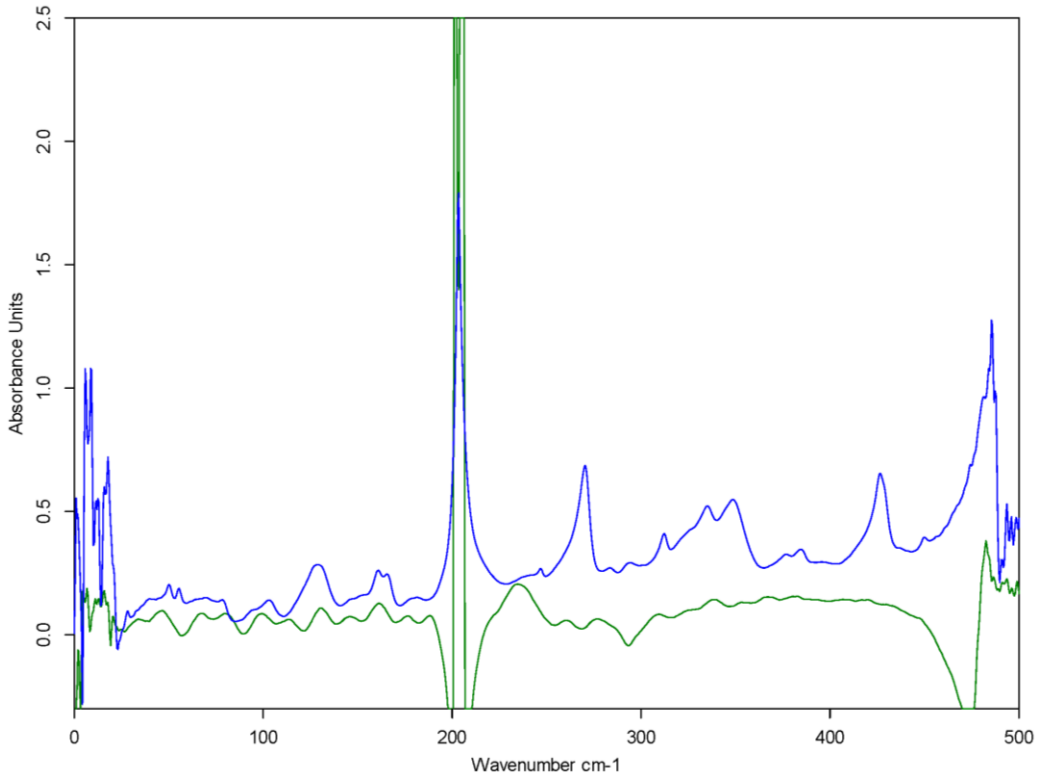


Figure 33: Ninhydrin (blue) and the spectrum for the zinc Ruhemann's purple complex (green) both in PTFE

The spectrum for the zinc Ruhemann's purple complex showed, like the Ruhemann's purple spectrum, very little information. It can be seen in Figure 33 along with ninhydrin. It is much the same as the Ruhemann's purple spectrum apart from a broad peak that occurs just after the 200cm^{-1} area of the spectrum. It seems unusual that the change from ninhydrin through to the complex would have these results. The basic structure does not alter that much but the resulting spectra is very different.

3.9 RIBOFLAVIN AND ITS DEGRADATION PRODUCTS

Riboflavin consistently gave good spectra in the above work. It is also known to degrade in ultraviolet light into two major products, lumiflavin and lumichrome. The two degradation products were analysed to see what differences would be observed in the spectra between the three compounds. Since the degradation products were synthesised in the laboratory the starting materials and some of the intermediates, where possible, were also analysed.

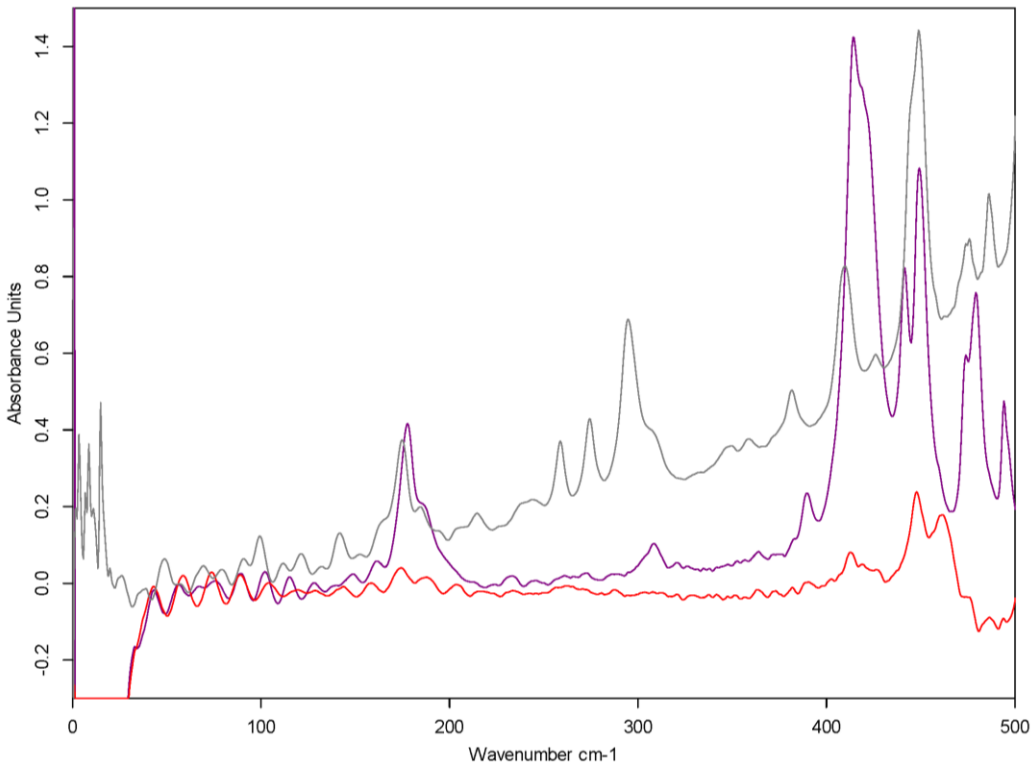


Figure 34: Far-IR spectra of Riboflavin (grey) Lumichrome (purple) and lumiflavin (red) overlaid

The overlaid spectrum in Figure 34 is of riboflavin and the two degradation products, lumiflavin and lumichrome. The structures of these three compounds can be seen in Figure 35. All three chemicals share the same aromatic ring backbone. The difference is in the side chain that is attached to the nitrogen. In riboflavin, this is a long carbon chain with four hydroxyl groups attached. In lumichrome there is nothing attached to the nitrogen as the ring bonding is slightly altered, with a hydrogen atom being present on a different nitrogen in the structure. For lumiflavin there is a methyl group on the nitrogen.

The spectrum for lumiflavin has little information in it and therefore has little correlation to the other two spectra. There are no real peaks until higher up in the mid IR region. Lumichrome did have some peaks in its spectrum. The two main peaks occurred at 190cm^{-1} and 310cm^{-1}. These two peaks both correlate to a peak in the spectrum for riboflavin. The spectrum for riboflavin has more detail compared to the other two compounds. As was the case with ninhydrin and Ruhemann's purple, the absence of hydroxyl groups has reduced the number of peaks.

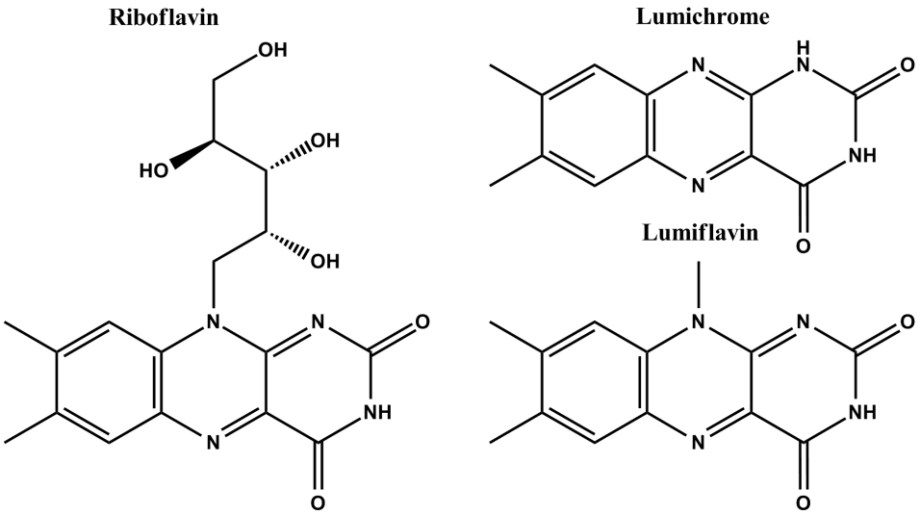


Figure 35: Chemical structures of riboflavin and two of its degradation products lumichrome and lumiflavin

The synthetic pathway for lumichrome can be seen in Figure 36. The two starting materials are alloxan and 4,5-dimethyl-o-phenylenediamine. The two adjacent ketones on the alloxan react with the amines to form the structure of lumichrome. All three of these compounds, the two starting materials and the final product were analysed at the synchrotron in PE, seen overlaid in Figure 37.

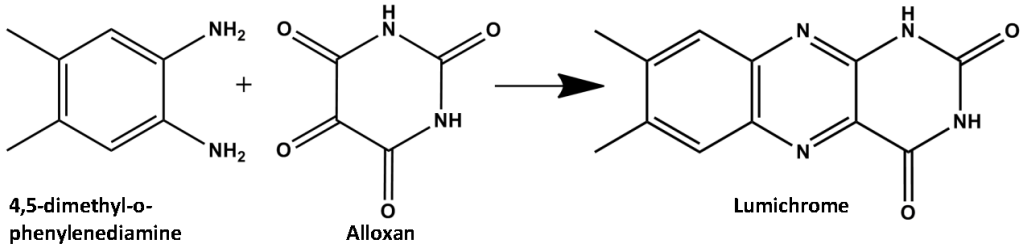


Figure 36: Synthetic pathway for lumichrome showing starting material and product structures

There seems to be little in the way of correlation between the spectra for each of the starting materials and the final product. There is a peak that occurs at roughly the same point in all three just below 200cm^{-1}. All the compounds have peaks that occur higher up in the spectrum close to the mid-infrared region. Given that lumichrome has the most peaks in this area it would appear they are to do with the aromatic rings. Below 400cm^{-1} the compound that has the most peaks is alloxan. Alloxan has the highest number of ketone groups so the peaks could be related to the ketone groups.

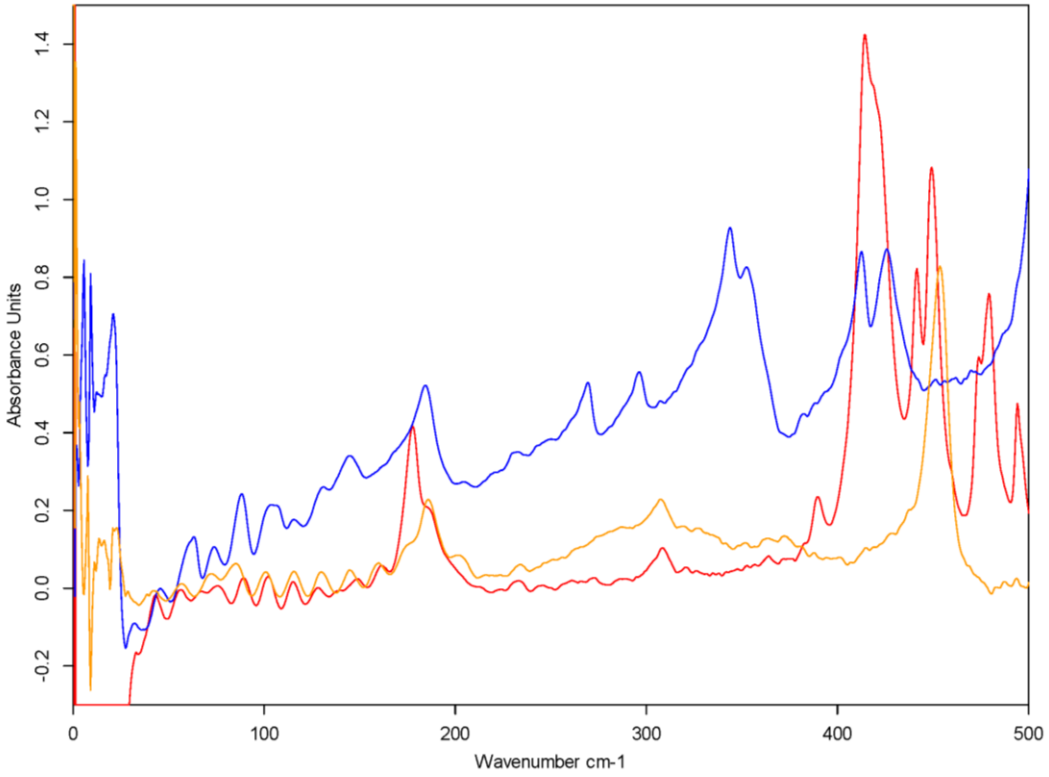


Figure 37: Overlaid spectra of Lumichrome (red) Alloxan (Blue) 4-5 di methyl (orange)

The structures of main compounds from the more complicated synthetic pathway for lumiflavin can be seen in Figure 38. All but the reduced product after

methylation, N,4,5-trimethyl-o-phenylenediamine, were analysed as this compound was unstable.

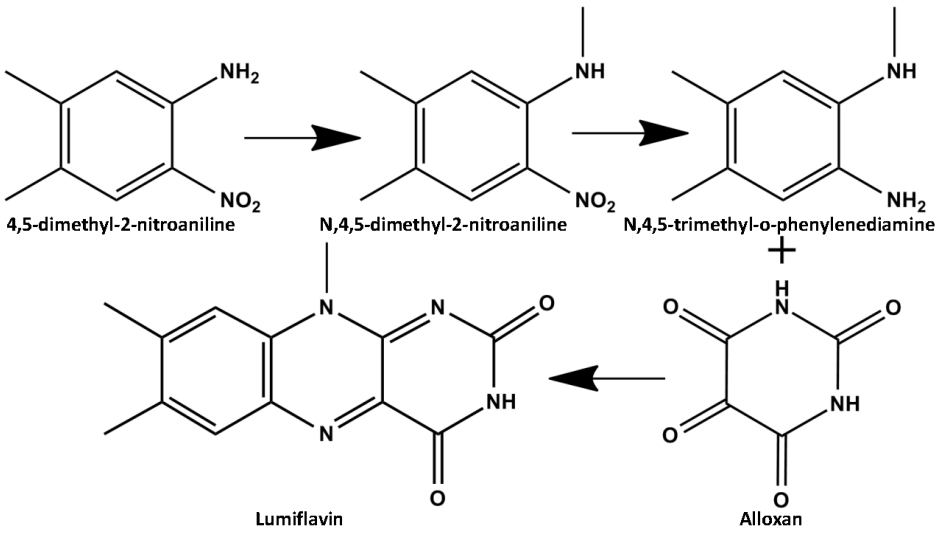


Figure 38: Synthetic pathway for lumiflavin showing structures of starting materials, intermediates and the final product

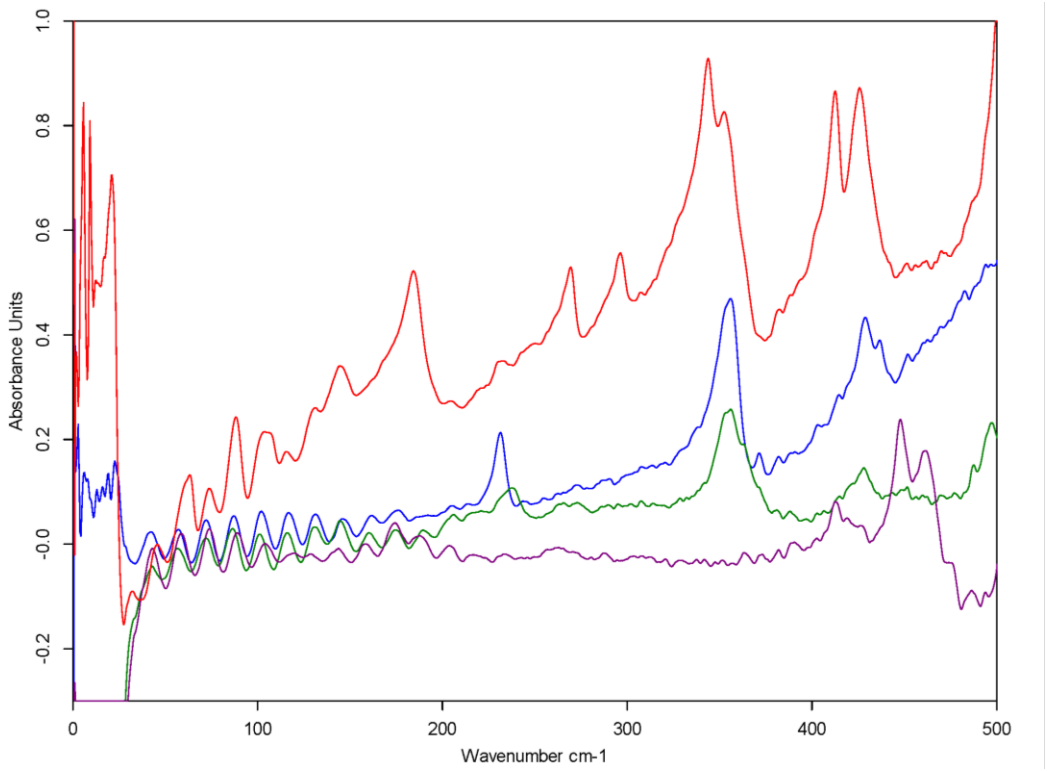


Figure 39: Lumiflavin (Purple) alloxane (red) tri methyl (green) di methyl (blue)

The resulting spectra can be seen overlaid in Figure 39. There is little in common between the spectra of the starting materials and the final product. For all but

alloxane there is the presence of 'ringing' up until 200cm^{-1}. It is also alloxan that again has the most peaks. The spectrum of lumiflavin shows very little in the way of peaks at the lower end. The only real peaks that can be seen occur in the mid IR region. There are similarities between the spectra of the di-methyl and try-methyl compounds. Both have peaks occurring around 225cm^{-1} and 350cm^{-1}. These two compounds uniquely have amine groups which could be the cause of these peaks.

3.10 CONCLUSION

The initial goal for the synchrotron work was to try and establish if the bond that forms between zinc and another molecule that results in a fluorescent compound would produce a peak in the Far-IR region. Seminal fluid was to be analysed to see if a similar peak was present which would add evidence to the idea that the zinc present in seminal fluid has a role in the fluorescence observed. Unfortunately, there was no peak that was observed in the analysis of the model compound. There were however some other interesting results from the work conducted.

When the model compound was analysed there was a surprising about of change in the spectra between ninhydrin and Ruhemann's purple. This was also observed when a second set of similar compounds were analysed. While the structures were not changed by a large amount there was a large variation in the spectra.

Some very interesting results were obtained regarding the use of different materials in the formation of a disc for analysis. PTFE was found to be a suitable, and at times better, alternative to PE. While there was a loss of spectrum quality when PTFE was used, the region where this occurred was consistent, so it could be used a point of reference when comparing spectra. In some cases, it also leads to a more detailed spectrum in areas when compared to PE.

It was also found that in some cases pure sample could be analysed without there being a loss of quality in the resulting spectra. Using pure sample could be advantageous since the sample used could easily be recovered as it would not be mixed with another compound. It does however require more sample in the first place.

CHAPTER 4 INITIAL FLUORESCENCE EXPERIMENTATION

4.1 FLUORESCENCE VISUALISATION

4.1.1 Introduction

The first requirement, once samples were received, was to have a reliable method for the for the visualisation of the fluorescence. It would be advantageous if the method that was developed was simple and could include a way to concentrate samples to increase fluorescence. Matrix simplification methods could lead to a diluted sample and it could also produce a high number of samples to be tested.

Methods for testing for the presence of fluorescence involved using a fluorescence spectrometer, usually with a solid sample probe, or applying seminal fluid to some material and then visualising as is done in case work (light source and filters). A fluorescence spectrometer was not readily available, and the sample preparation and analysis would have been time consuming. Samples could only be analysed one at a time. Spotting on to some material would be a rapid and reliable method.

Squares of cotton material have been used previously when testing samples for fluorescence. It was not seen as the only or best option in this work as it could potentially be inconsistent and depending on treatments it can be fluorescent. The thickness of the cotton material could also affect the levels of fluorescence observed. Different fabrics or materials may offer a better surface for visualisation.

4.1.2 Illumination and filtering of Light

A common combination of excitation wavelength and barrier filter in forensic investigations is approximately 450 nm for excitation (blue light) with an orange filter (550 nm with 35mm bandwidth) as previously discussed. These were the conditions that were selected to be used to visualise the seminal fluid fluorescence in this work. If the same conditions are used in the lab as case work, then it can be said that the same fluorescent compound was being targeted.

A common forensic light source in forensic laboratories, and one that was selected for this work, is a Polilight® from Rofin. These instruments can produce intense light over a broad range of wavelengths, from ultraviolet through the visible spectrum to

infrared light. Filters are then used to select light with a narrower waveband. The range of the light is dependent on the model and the wavelength selected. The blue light produced by the model used was 450 nm with a band width of ±50nm[87].

The most convenient way to view objects through a barrier filter is to use coloured goggles, in this case orange goggles. These are often used in case work. The sample can be illuminated using a FLS and the emitted fluorescence viewed through the goggles. The goggles exclude light below approximately 550nm, therefore the incident 450 nm light from the FLS is filtered out while still allowing the fluorescent light through.

A feature of the FLS instrument used in this work is that the light produced is passed down an optic fibre that is inside a flexible tube making it possible to direct the light at the sample without moving the main part of the instrument. The light produced is also able to illuminate an area of up to 20cm^2 which is useful for illuminating multiple spots at one time.

4.1.3 Photography of fluorescence

To capture the fluorescence a camera was used. The camera was attach securely to a stand that allowed for fine adjustments of the distance from the sample. A separate stand had a clamp that held the light source in place. This allowed for reproducible conditions when taking images.

As standard, the lens on the camera allowed for the attachment of a filter. In this case, it was a yellow-orange photography filter that blocked light below 530 nm. The setup was in a dark room so that there would be no interference from external light sources.

Samples were initially placed onto a white bench to be photographed but the initial photographs did not show the fluorescence as clearly as planned. Subsequent photographs were taken with a black background. The dark background made the pictures clearer and they had better contrast, demonstrated in Figure 40. The black background caused the camera to increase the exposure since it tries to produce an image with a neutral grey exposure.

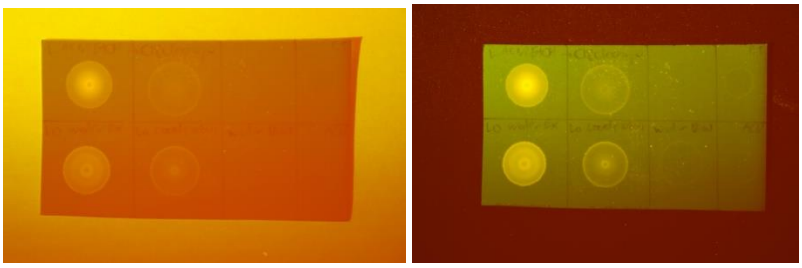


Figure 40: Photographs of the same TLC plate, left is with light background and right with black card

Most of the settings for the camera were left in auto mode apart from the focusing. It was found that the camera could have trouble focusing when there were no sharp edges. For each set of photographs, the camera was focused manually and then the photograph taken with the camera deciding the exposure time and aperture setting.

Where possible, samples that were to be compared for levels of fluorescence were placed on the one piece of material and photographed at the same time to avoid variation from image to image. This is because the camera will adjust the exposure time and aperture for each image leading to variation. When the fluorescence is low the camera will artificially brighten the image, making it seem more fluorescent. If the samples are in the one image, then the exposure settings will be consistent and trace variations in fluorescent intensity detected.

4.1.4 Spotting material

Five different materials were selected to be tested for their suitability. The first two materials selected were Silica Gel on aluminium and Cellulose on aluminium. These are both used for thin layer chromatography and are both absorbent medias. A glass slide was also trialled as a non-absorbent material to see how it would compare to the others. A cotton/polyester blend fabric (comparable to the cotton fabric used in other work) and filter paper (which has also been used on occasion in previous work) were both compared against the other materials.

To each of the materials, 10µl of a tenfold dilution of seminal fluid in water and 10µl of water (both type 1) was applied on opposite sides. These were allowed to air dry completely. The quickest materials to dry were the silica, filter paper and cellulose.

Figure 41 shows the image of four of the five materials tested excluding cellulose. The fabric tested is a good example of how background fluorescence can mask the fluorescent properties of seminal fluid. Any fluorescence from the seminal fluid has been completely masked by the background fluorescence of the fabric. The fabric was quickly eliminated as an option along with the paper. The filter paper contains a water soluble fluorescent compound, appearing as a ring of fluorescence on the water side. This makes it indistinguishable from the seminal fluid sample and not suitable.

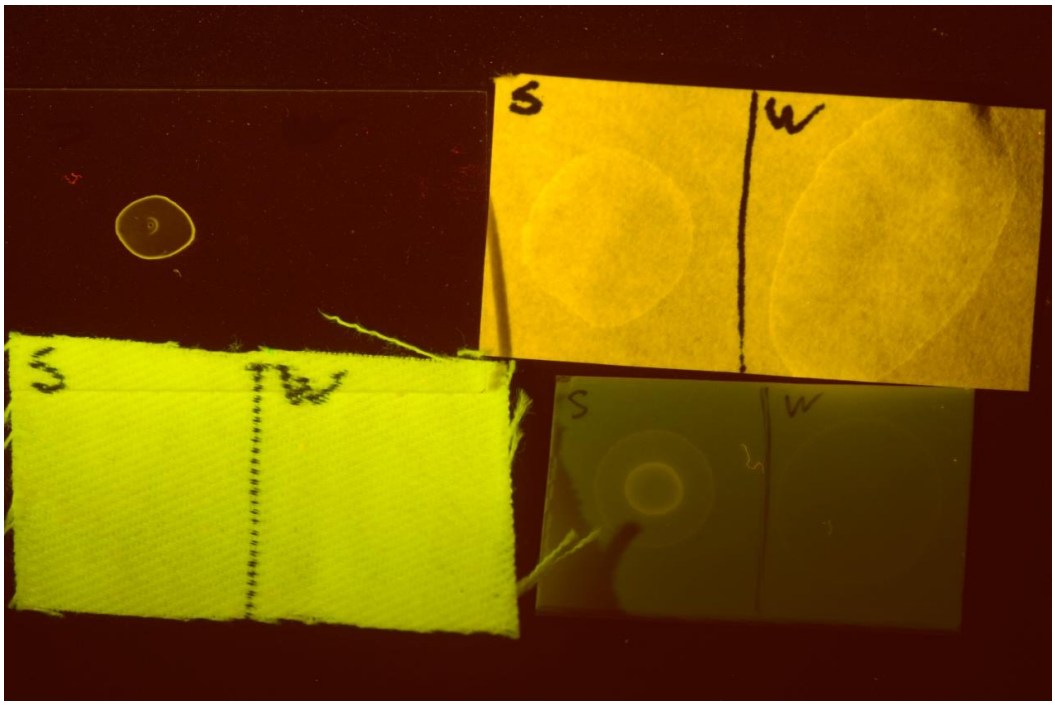


Figure 41: Four of the five materials tested for the visualisation of fluorescence. Top left is the glass slide, top right is the filter paper, bottom left is the fabric and bottom right is the silica TLC plate. Not shown is the cellulose

The cellulose plate is not shown in Figure 41. The same experiment was conducted as with the other samples. It was however another example of where background fluorescence caused too much interference to be able to visualize the seminal fluid fluorescence. As was the case with the filter paper the responsible component appeared to be water soluble and caused a ring of fluorescence.

The two remaining materials, the glass slide and silica TLC plate, both gave good clear results. Distinguishing between just water and the seminal fluid sample is quite easy. The difference between the two materials was that the silica TLC plate could

absorb the liquid making it easier to work with. This made the drying time much shorter.

4.1.5 Heat assisted vs air drying

To test if a hairdryer could be used to increase the rate of drying without damaging and reducing the fluorescence, a sample was selected and 10µl of a tenfold dilution in water was spotted on two separate silica TLC plates. One was allowed to air dry, the other was dried with a hair dryer. Visual comparison of the fluorescence of the two samples showed that there was no obvious difference in the intensity.

4.1.6 Sample spotting

There were two methods in which the sample was spotted onto the TLC plate. The method that was use depended on whether direct comparison between samples was required or not. If a sample needed to be visualized quickly a capillary tube was used. Using a capillary tube also made it possible to effectively concentrate samples. Sample could be applied to the same spot multiple times and since the fluorescent compound/s did not move on the plate it would effectively concentrate the fluorescence.

When comparison between samples was need then a known, consistent volume of sample was required. The method for this used a micropipette to control the volume. The sample was not ejected from the pipette as that cause spreading of the fluorescent compound/s. Instead, the tip was removed carefully, and the sample allowed to absorb out like when applying with a capillary tube. The volumes that were typically used when using a micropipette were between 10µl and 20µl.

4.1.7 Silica plate preparation

It was found that for the volumes that were being used, each sample tested required a section of Silica TLC plate approximately 2cm by 2cm in size. If multiple samples were being tested or direct comparison was required, it was best if the Silica was kept as one large sheet. Having all the samples on one sheet removed the variation that could come from having separate photos taken. Water was used as a blank and was done on the same piece of silica.

4.1.8 Final method

A volume of sample was taken using a micropipette or a capillary tube. This is then spotted into the marked area on a Silica Gel plate. If the sample needs concentrating, multiple volumes were applied to the same spot. This was then allowed to dry which was assisted using a hair dryer. Once dry, the sample has visualized using 450 nm excitation and viewed through orange goggles or photographed with an orange filter on the camera lens.

CHAPTER 5 FLUORESCENT COMPARISON

5.1 INTRODUCTION

A unique opportunity arose for comparison from the number of samples that were collected and how they were collected. Since all had been collected in the same way and had all been treated the same it was a good opportunity to compare the levels of fluorescence from one sample to another. It would be interesting to see how much variation there was between individuals.

Two ways of comparing the levels of fluorescence were chosen. The first was to compare by equal wet mass and the other by equal dry mass. The equal wet mass would basically be a direct comparison of the samples as received, just diluted to better suit the spotting and visualisation method. Through this method any variation could potentially be from variations in the amount of the fluorescent component and be affected by the hydration or water content of the seminal fluid.

The dry mass method would remove the variability that could potentially come from the hydration. Any variation in the fluorescence should mostly be due to changes in the amount of the fluorescent component compared to the other constituents that are present in seminal fluid excluding water such as other proteins.

5.2 SAMPLE PREPARATION

5.2.1 Wet mass

The research plan was to pipette known volumes of sample and dilute them. It was thought that accurate volumes could be pipetted. However, after weighing of the tubes empty and then once the sample had been added, it was seen that there was a significant amount of variation. To confirm that the factor was the pipetting itself, the same sample was pipetted four times and weighed each time. It was seen that there was variation, so the samples were diluted based on wet mass rather than assuming all were the same volume.

The volume that was selected to be pipetted for the wet mass was 25µl. Each sample was taken from the freezer and allowed to warm to room temperature. Samples were then vortexed for one minute immediately prior to the sample being taken to

ensure homogeneity. The 25µl was pipetted into pre-weighed Eppendorf tubes which were reweighed after to find the mass pipetted. Typically, the weights varied from 23mg to 27mg.

The samples were originally going to be diluted tenfold with water based on volume. This was changed so that the dilution would be based on the weight of the seminal fluid. The mass of water added was ten times the mass of the seminal fluid; using the assumption that 1µl of water weighed 1mg. Once the water had been added the samples were vortexed to evenly mix the seminal fluid through the sample and then were stored at 4°C until spotted.

5.2.2 Dry mass

For the dry mass samples, 25µl of sample was pipetted into pre-weighted Eppendorf tubes. The tubes had previously been placed in a desiccator to remove any moisture that could affect the weighs of the tubes. Each sample was vortexed immediately prior to a volume being taken to again ensure sample homogeneity. Once all the samples had been prepared they were placed in desiccators to dry. After four days four random samples were weighed and placed back in the desiccator. The same four samples were weighed on a regular basis to see if there was any variation in weight. This was repeated until the mass of the samples no longer changed indicating samples were dry.

Once dry, the samples were removed, and the lids placed on the tubes to keep the sample from absorbing moisture from the atmosphere. They were then weighed, and the mass of dry sample calculated. To get a similar volume and therefore dilution as the wet mass work, 100 times the mass of the sample was added as water since the dry masses were about $1/10^{th}$ the mass of the wet ones. This resulted in samples that have an equal dry mass for volume.

5.3 SPOTTING

Before the samples could all be spotted and compared, a volume had to be selected that was appropriate. It needed to be enough to be able to see the fluorescence

once spotted but also not so much that it was flooded with fluorescence and any differences would be masked.

To determine the most appropriate amount, four random samples were chosen to be spotted at three different volumes. The samples that were use were S4, S18, S27 and S39. The volumes selected were five, ten and fifteen microliters. These volumes were selected because they are a good volume for spotting. They are around the right amount of liquid to give a good spot.

In all the samples the fluorescence was quite intense, especially in the dry mass samples. The fluorescence was too intense to be able to observe any variation. This was partly due to the spotting technique which, as previously discussed, has the effect of concentrating samples. To try and make any variation more visible either the samples needed to be made more dilute or the spotting technique changed.

Diluting the samples was the better option so a sample was chosen and diluted for each of the dry and wet samples. Sample S39 was selected as it had the highest volume and therefore more sample to experiment with. For both the wet sample and the dry sample the first dilution trialled was 1:1 with water. 5µl of each sample was spotted and the fluorescence visualised. In the case of the wet sample this proved to be enough of a dilution. The sample was no longer highly intense and did not appear to be saturated with the level of fluorescence.

The 1:1 dilution of the dry sample was not enough, and the fluorescent spot was still too bright. 1:2 and 1:3 were also too fluorescent but a 1:4 dilution appeared to be much better. The fluorescence no longer appeared to be too intense.

For each of the wet samples, 50µl of sample was taken and 50µl of water was added to produce a new diluted sample ready to be spotted. In the case of the wet samples, 20µl of sample was taken and then 80µl of water added. These new samples were then spotted onto two large silica gel sheets, one for the wet samples and one for the dry samples.

The fluorescence pictures were taken as per normal. Once set up, each gel was photographed in four sections with only the gel sheet being moved to keep variables

to a minimum. A picture of the whole sheet was also taken of each after adjusting the distance the camera was from the sheet.

5.4 RESULTS

The first result of note is that all samples were fluorescent. This is not surprising but shows that using the fluorescence to detect seminal fluid is effective. The samples were from a fertility clinic so there is potential for some to be from azoospermic males. As discussed earlier there is no evidence that this causes a complete loss of fluorescence and the results potentially support this. There is, however, variation in the levels of fluorescence from person to person but the variation that was observed was not large.

It was in the wet mass samples where the variation of the fluorescence appeared to be the highest. Figure 60 in Appendix D shows all the wet mass samples and some variation can be seen between some of the samples. Some of the variation is due to the spotting and some due to different levels of light but when comparing to similar shaped spots of the nearest neighbours, there are differences to be seen.

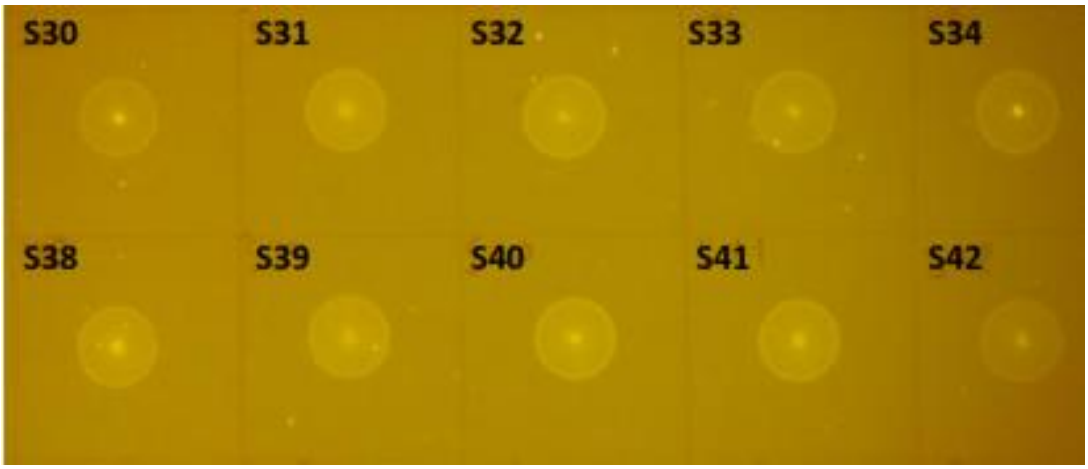


Figure 42: Selection of the fluorescence image of the wet sample spots taken from Figure 60 in Appendix D

A good example of the variation can be seen in Figure 42 which is a small section of the larger image. The four samples on the right of the image are similar in terms of the size of the spot but the levels of fluorescence appear to vary. In the image S34 appears to be more intense than the closest two samples in S33 and S42. Another to note is S30 which also appears to be more intensely fluorescent than S31.

For the dry mass samples, the variation between the samples was less than that seen in the wet mass samples. All the dry mass samples can be seen in Figure 61 in Appendix D. There appeared to more consistency in the level of fluorescence. This is more clearly seen in Figure 43 which is the same selection of samples as Figure 42. There is more consistency in the levels of fluorescence between samples. In the wet mass photograph S34 was noted for standing out but in this photograph, it does not stand out as much especially compared to S42.

The variation that is seen in the wet mass samples is possibly due to two main reasons. The first would be that there would be natural variation from person to person as to the actual level of the fluorescent component in seminal fluid. The work by Amano [9] et al showed that there was a correlation between sperm concentration and fluorescence. This is possibly responsible for the variation seen in these results.

Another reason for the variation could be due to differing levels of hydration. As the dry mass results showed, the removal of this variable did reduce the variation. Hydration levels would vary from person to person and would vary from sample to sample from the same individual.

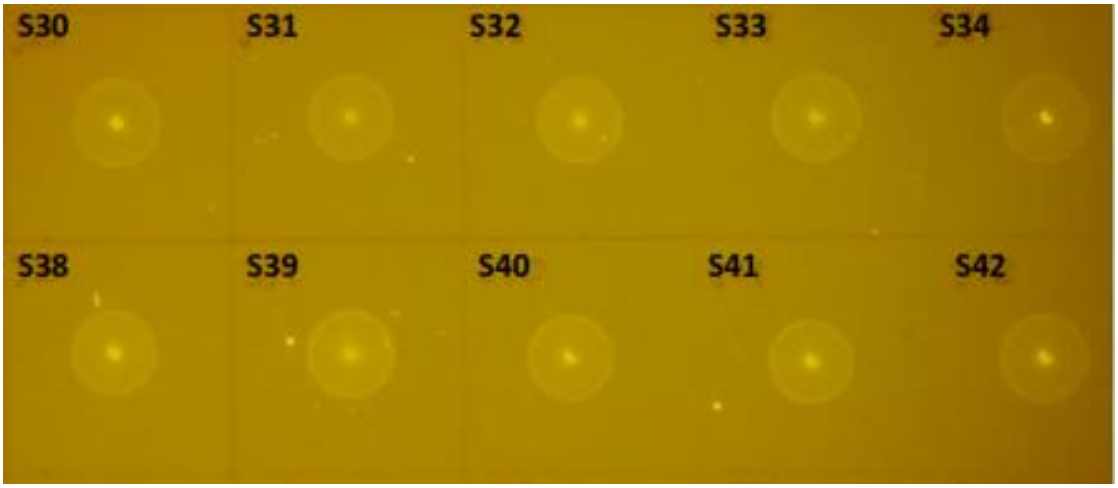


Figure 43: Selection of the fluorescence image of the dry sample spots take from Figure 61 in Appendix D

By drying the samples, the effect of the amount of water present was removed and it led to there being less variation between samples. This can be seen by comparing Figure 42 and Figure 43. This suggests that the water content of seminal fluid does

affect the level of fluorescence; this is not at all surprising. The fact that there is still remaining variation shows that each person does produce varying amounts of the fluorescent component in relation to the rest of the non-water (and other volatiles) constituents.

CHAPTER 6 MATRIX SIMPLIFICATION AND HPLC

6.1 MATRIX SIMPLIFICATION

Seminal fluid is a complex mixture of components which makes identifying the fluorescent compound/s difficult. Simplifying the matrix would make any further analysis on the sample easier. The technique selected for further analysis was liquid chromatography mass spectrometry (LC-MS). A simplified matrix would make achieving good separation on a high-performance liquid chromatography (HPLC) system easier. The developed method could then be applied to a LC-MS system to identify compounds present.

6.1.1 Liquid extraction

A literature search was conducted to find research which had used liquid extraction on seminal fluid as part of their methods. A variety of methods were chosen as they all targeted different types of compounds. While all were liquid extraction techniques, some were effectively acting as protein precipitation methods. The methods that were selected were used as a guide to select solvents and not strictly followed.

In work done by Oliw [88], two parts ethanol and two parts acetonitrile were added to one part seminal fluid and the proteins removed by centrifugation. Kand'ár [28] et al. added two parts of cold ethanol and five parts of n-hexane to a sample and the n-hexane layer collected after vortexing. Avery [27] et al. used a 1:1 ratio of hexane and ethyl acetate with a ammonium formate buffer. The two liquids were added to a dried sample in a ratio of two to three of mix to buffer. Sharma [30] et al. added acetonitrile to seminal fluid in eight parts to one.

From these, simple extraction methods were developed to be trialled along with a general methanol water system since methanol can be used to precipitate proteins. The solvent systems and the volumes of each solvent used can be seen in Table 7. Sample along with the extraction solvent/s were added to an Eppendorf tube and they were vortexed for two minutes each. After this the samples were centrifuged at 3200rpm for 5 minutes. In the hexane trials the hexane layer was spotted onto a TLC plate. In the other trials the supernatant was spotted without disrupting the precipitate.

Table 7: The different solvent systems trailed for extraction along with the volumes used of each solvent in each extraction

Solvent system	Solvent 1 Volume (µl)	Solvent 2 Volume (µl)	Plasma Volume (µl)
Acetonitrile	1600	0	200
(Hexane/Ethyl acetate)/Water	500	500	200
Ethanol/Hexane	200	500	200
Ethanol/Acetonitrile	400	400	200
Methanol/Water	400	200	200

Only three of the above solvent systems showed any extraction of the fluorescent component. These were the acetonitrile, ethanol/acetonitrile and methanol/water with the latter two appearing more effective, potentially due to the presence of the alcohol. The image of the fluorescence can be seen in . The fluorescence levels for the samples was low with the camera making them appear more intense.

Testing of the two more effective methods showed that the ethanol/acetonitrile extractions appeared to be more fluorescent. Further testing on this system, by rehydrating the precipitate and visualising the fluorescence, showed that while it did extract some of the fluorescent component it did not do so efficiently. When it was compared to a similarly diluted sample of seminal fluid with water the fluorescence was much lower. The rehydrated precipitate had levels of fluorescence similar to the diluted seminal fluid.

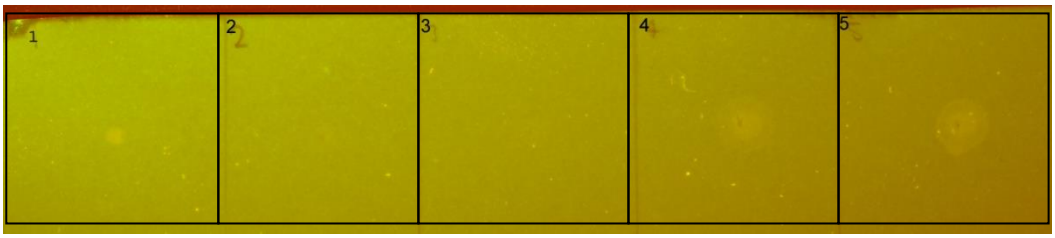


Figure 44: Picture of the spot testing for the five extraction trials. Spots in the same order as the listed trials in Table 7

The results show that the compound/s responsible for the fluorescence are highly water soluble with little to no solubility in organic solvents. This would suggest that the compound/s that cause the fluorescence are polar. While organic compounds cannot be ruled out it is less likely they are the cause based on these results, especially those soluble in organic solvents. Since these methods acted as protein

precipitation methods it adds evidence that a protein or proteins is responsible for the fluorescence.

One of the methods used above could be used to simplify the sample matrix but it was unclear how effective this would really be. Since seminal fluid is water based to begin with it is likely that few compounds would be removed by this step and the sample matrix would probably still be highly complex.

6.1.2 Solid phase extraction

Since the liquid extraction methods did not provide a reliable clean up method, Solid Phase Extraction (SPE) was performed to see if better simplification of the matrix could be achieved. Three different SPE cartridges that were readily available were tested. Two were common C18 style cartridges and the third was a polymeric based cartridge. The three cartridges were a Strata C18-E and a Strata X polymeric from Phenomenex® and a Discovery DSC-18 cartridge.

The general method used was as follows in Table 8. This method was based on a recommended general method to be used with the Strata C18-E column. Since there were many unknowns a more specific method was not considered.

Table 8: The SPE method used in the trial clean up

Step	Solvent
Condition 1	2mL Methanol
Condition 2	2mL Water
Loading buffer	2mL Water (4-fold dilution of sample)
Wash	Water
Elution solvent	Methanol/Acetonitile (50:50)

The load and wash were collected as one sample. The elute, a mixture of methanol and acetonitrile, was collected separately from the other solvents and concentrated by removing solvent under vacuum and then tested for fluorescence. For all three the fluorescence was found in the load and wash solvents meaning that it was not retained on the column.

A review of the previous work undertaken on HPLC systems showed that the samples analysed had not had any prior sample clean up. For the initial HPLC analysis it was decided that this could be done until more information about the fluorescent compound was obtained. Results from HPLC analysis could be used to better tailor a SPE method for the sample.

6.2 HPLC SAMPLE PREPARATION

As it was decided that the initial HPLC analysis should follow the previous work closely [11, 12], the sample preparation was fairly basic. The method that was used had some slight variations to that of the previous work but was largely the same.

The seminal fluid was first diluted with three parts water. This was then vortexed for one minute to ensure a homogeneous sample was produced. Samples were then centrifuged for five minutes at 3,200 rpm. This caused the larger components such as cells, spermatozoa and cell debris to form a solid pellet at the bottom of the tube. Some of the fluorescence component was lost in this pellet but a majority was found to be in the supernatant following a visual fluorescence comparison.

The supernatant was taken and filtered through a 0.45µm filter to produce the sample that would be used in HPLC analysis. In some of the previous work the samples were not filtered before analysis. It was difficult to filter the samples even after being centrifuged. A typical 1.5mL sample, the size of the HPLC sample vials, required the use of two filters as the first would get blocked. All samples were tested for fluorescence after preparation and remained highly fluorescent with no discernible drop in fluorescence observed.

The seminal fluid used to produce HPLC samples was always a mixture of several different received samples. This was to remove errors that could occur from using just one sample. The various samples that were made and used can be seen in Table 9.

The names generally had some relevance to the seminal fluid samples that were used to make them up or related to when they were made. The volumes of each

sample used generally related to how much sample was available. A higher volume was used from samples there were more of such as S13.

Table 9: HPLC samples that were prepared and what was used to make them

Sample name	Seminal samples used	Volume each (µl)
M101	S1, S2, S3, S4, S5	300
M201	S3, S4, S8, S9	(S3,S8, S9) 100 (S4) 200
M235	S2, S3, S5	150
M123	S1, S2, S3, S4, S5	90
M348	S3, S4, S8, S13, S14	100
M543	S13, S14, S15	500
M533	S3, S10, S13, S14, S15	300
M340*	S13, S14	1000
M313*	S8, S11, S13, S16, S18	(S8, S11, S13, S16, S18) 200 (S13) 1200

*multiple vials of this sample made

6.3 HPLC

HPLC was selected as the best approach towards identifying the fluorescent component. In theory, a method should be able to be developed that can separate out the various components in seminal fluid and then identifying them by mass spectrometry.

Several different methods were trialled to try and develop a method that separated out the fluorescent component from the bulk of the mixture. Some were based on the previously used method while others were more general solvent combination that is commonly used. A hydrophilic interaction liquid chromatography (HILIC) column was purchased and experimented with.

6.3.1 Previous HPLC work

Three of the four previous honours projects that were conducted at Flinders University that investigated seminal fluid used HPLC. The first was Camilleri [10] in 2005 who referred to work that had previously been conducted at Forensic Science

South Australia by Scharnberg et al. [89] Sample preparation was not clearly discussed.

The HPLC conditions used in this work were taken from the previous work by Scharnberg et al. The column used was a C4 column. Two solvent systems were selected which both were comprised of methanol and phosphate buffer pH 3.2 in different ratios. The first was 20:80 methanol to buffer and the second 90:10. The two solvents were pre-mixed as the HPLC system that was used was not capable of performing gradient runs. The first solvent was run until no more peaks eluted followed by the second solvent, again until no more peaks eluted.

Following this was work done the next year by Church [12]. Sample preparation was described in this work as follows; 500µl of seminal fluid was taken and centrifuged at 3,000 rpm for ten minutes. 300µl of the supernatant was taken and 900µl of water was added to this. 20µl of this diluted sample was injected. A C4 column was again used in this work along with the same solvents as used by Camilleri. Fractions were collected and dried to visualise fluorescence, but this was unsuccessful.

Some work was also done using a LC-MS system in this study which substituted the phosphate buffer for a formate buffer at pH 3.5. No compounds were identified in this work after attempts were made to match the found masses with those of known components in seminal fluid.

The third honours project, by Carver [11] in 2008, used different solvents and a different column. The solvents used in the optimised method were water and acetonitrile, both with 0.1% fomic acid. The column used in this work was an Agilent ZORBAX 300Extend C18 column rather than a C4 column. No significant results were reported from this work.

6.4 METHOD DEVELOPMENT

6.4.1 Solvent systems

Two key solvent systems were used in this work. The first was water/acetonitrile and the second was buffer/methanol. These two were trialled both as standard reverse

phase techniques. There was also a trial with the organic solvent being the main solvent at the beginning of the run. While not conventional, the compound was shown to be poorly soluble in organic solvents and some separation may be achieved. Given the poor organic solubility seen in the LLE experiments there could be some separation in a HPLC system. An acetonitrile/methanol system was also explored since some fluorescence was extracted using a combination of acetonitrile and an alcohol. All methods were run as gradients.

6.4.2 Other HPLC settings

Table 10: Details of some of the HPLC run settings

Variable	Setting
Column	Phenonomex PhenoSphere-Next 5u C18
Column temperature	25°C
Injection Volume	50µl
Flow rate	1ml/min
Detector Settings	200 nm, 254nm, 280 nm
Samples	M101, M201

The column that was selected for this work was a C18 column. This is a commonly used column that is robust and often used in many different methods. Although C18 SPE cartridges were unable to retain the fluorescent component the use of different solvent systems and solvent gradients can change this. In the previous work (by Camilleri [10], Scharnberg [89] et al. and Church [12]) a C4 column was used on a couple of occasions but it was thought that enough variation could be achieved using different solvent systems that a C18 column would be suitable. The effect of changing the temperature was thought to be minimal for this work so was held constant at 25°C.

For all the runs the injection volume was set to 50µl. This volume set to ensure as many components were at a detectable level as possible while not overloading the column. If too much was injected onto the column, then the separation would be affected. The flow rate was set at 1ml/min as this a common setting.

Three different wavelengths, 200 nm, 254nm and 280 nm, were selected for the UV-Vis detector on the instrument. These three wavelengths were selected as they are commonly used and most compounds absorb at these wavelengths. A fluorescence detector was not used at this initial stage. Given the difficulties introduced by the quenching effect of water, it was decided that using fluorescence detection would be introduced later.

Only two different samples were used throughout this work to reduce the variables. Both were prepared using the same method and were confirmed to be fluorescent. Similar seminal fluid samples were used to prepare the samples to reduce variables.

Some of the initial methods that were preformed were quite long to ensure all material was being eluted from the column. This ensures, along with a column flush time between runs, that one run does not have any effect on the next one. Method run times in previous work were generally quite lengthy.

6.4.3 Methods

The first method that was run was the combination of acetonitrile and methanol. In the LLE extraction experiments more of the fluorescent compound was extracted in the presence of the alcohol so it was used as the solvent that had the gradient from low concentration to high. The conditions can be seen in Table 11.

Table 11: HPLC mobile phase composition of the run of method A

Time (min)	ACN	MeOH
0	95	5
10	95	5
20	75	25
25	75	25
35	50	50
40	50	50
55	25	75
65	25	75
80	0	100

Looking at the chromatograph for this method there is little to no separation of the peaks. There is an initial peak where a large portion of the compounds have eluted immediately after the void volume and have not been retained on the column.

The next notable feature is a second peak at approximately ten minutes. This shows that a group of compounds were retained on the column, but they were not resolved. There is also a single peak towards the end of the chromatogram that was retained on the column for a long time.

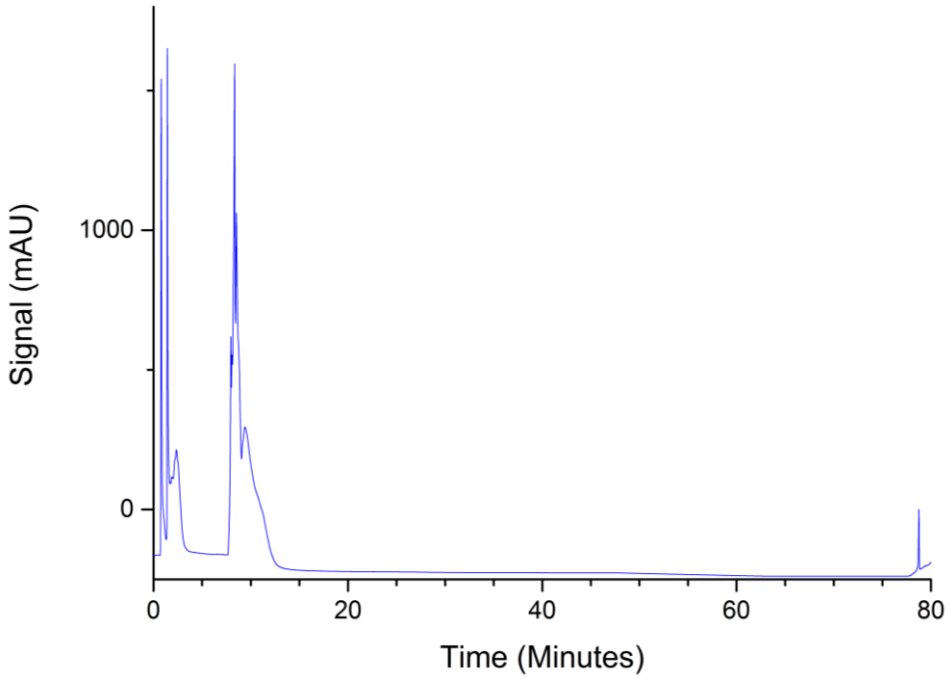


Figure 45: 200 nm UV-Vis chromatogram of sample M101 run using method A

The next two methods used acetonitrile and water. Compared to the previous method the run time was shorter. For the first method, there was an initial period where the solvents were isocratic. After five minutes a gradient change occurs that continues through to the end of the method.

Table 12: HPLC mobile phase composition of the run of method B

Time (min)	ACN	H_2O
0	10	90
5	10	90
10	25	75
20	90	10
25	100	0

Figure 46 shows the resultant chromatogram for method B. Again, there is the common feature of a large peak towards the beginning of the chromatogram where several compounds are eluting from the column. This occurs over most of the five-minute isocratic period of the method.

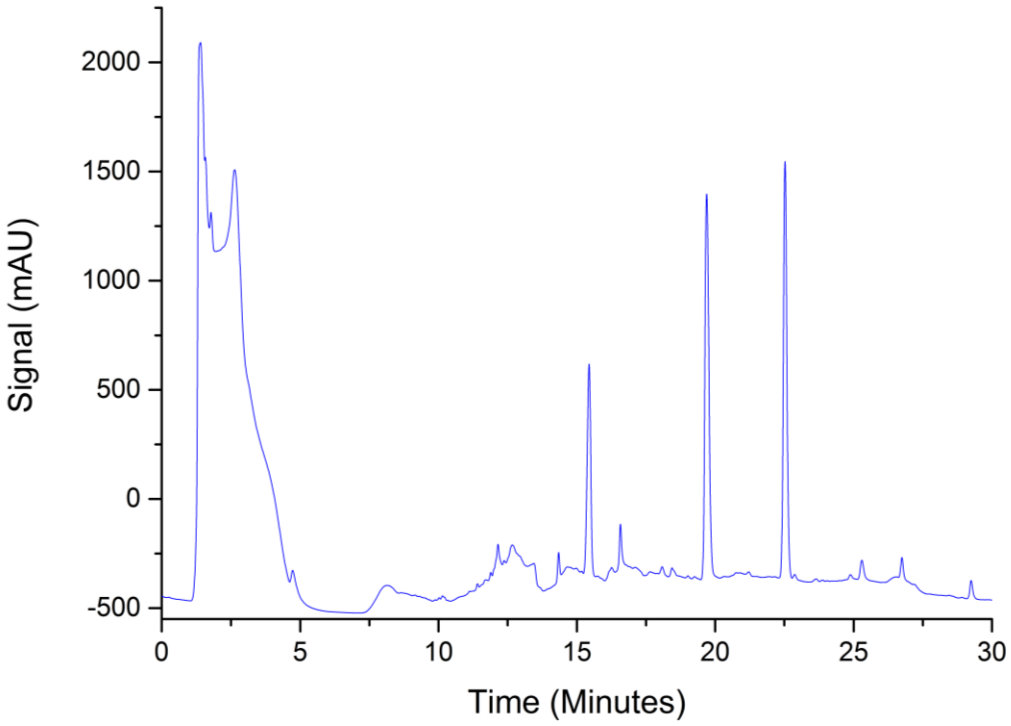


Figure 46: 200 nm UV-Vis chromatogram of sample M101 run using method B

Unlike the previous method, there have been several compounds that were retained and that have been resolved in this method. There are several smaller peaks along with three more notable peaks occurring after ten minutes into the run. The three larger peaks occurred at 15.5, 19.5 and 22.5 minutes.

Method C is a slight variation on the previous method. The initial portion of the run in this method is now a gradient followed by an isocratic period. This was done to see what effect it would have on the initial large peak that occurs. After this the gradient change resumes but is more rapid than the previous method. The last five minutes of both runs are the same.

Table 13: HPLC mobile phase composition of the run of method C

Time (min)	ACN	H_2O
0	10	90
5	25	75
10	25	75
15	90	10
20	90	10
25	100	0
30	100	0

The gradient change at the beginning did not lead to better resolution of the initial peak and only caused it to elute slightly quicker. After this the change is more noticeable. The resolution of peaks in method C is much poorer with only two broad peaks occurring after the initial peak. The poor resolution occurs around the period where the solvents were isocratic for the second time.

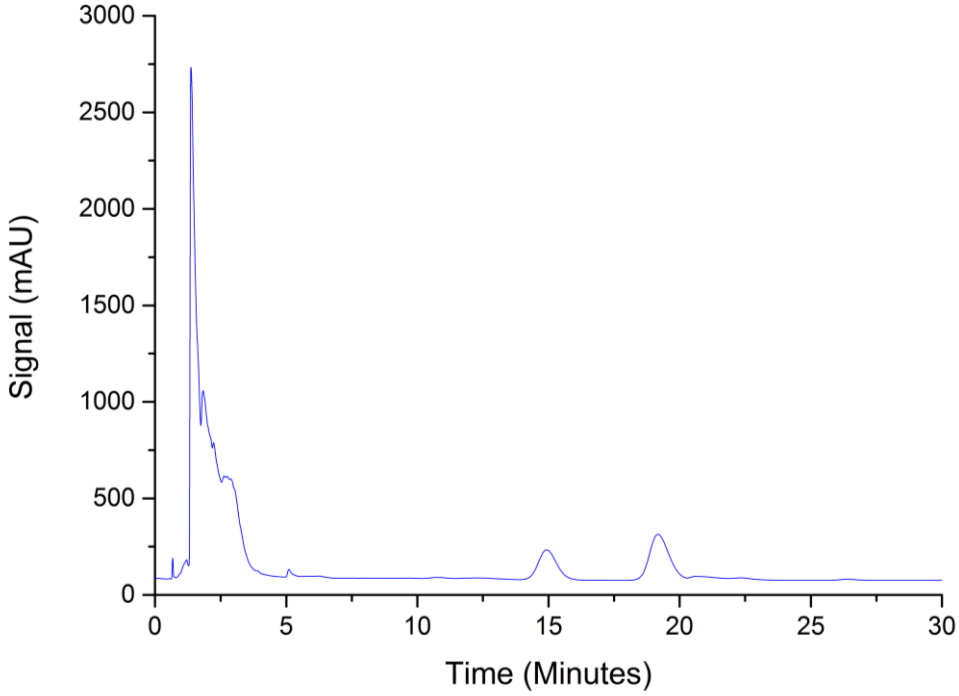


Figure 47: 200 nm UV-Vis chromatogram of sample M201 run using method C

The final method that was trialled used an ammonium formate buffer and methanol as the solvents. Methanol was used as opposed to acetonitrile as methanol was used in the previous work by Camilleri, Scharnberg et al. and Church. The main difference between this and the other methods is the addition of a buffer which alters the pH

of aqueous portion of the mobile phase. Altering the pH of the mobile phase changes how certain compounds are retained on a column.

The method began with a short initial isocratic period then switched to a gradient. The run was of a similar length to methods B and C. The results show a big improvement in the separation of compounds from the previous two methods.

The chromatogram for method D can be seen in Figure 48. Like what was seen in methods C and D, there is a large peak at the beginning from un-retained compounds, but it appears to be slightly better resolved. It is after five minutes that the chromatogram differs from others. Over the next ten minutes of the chromatogram there are several peaks that occur. These peaks are not completely resolved but can be seen clearly.

Table 14: HPLC mobile phase composition of the run of method D

Time (min)	Buffer	MeOH
0	80	20
2	80	20
20	10	90
23	10	90
28	0	100
30	0	100

Figure 49 more clearly shows the section of the chromatogram where most of the retained compounds have eluted. Several peaks can be seen with many of them having some overlap with another. The change from acetonitrile to methanol would not have had much of an effect on the retention of compounds. The biggest effect would have been from the buffer changing the pH of the water. This has caused more compounds to be better retained.

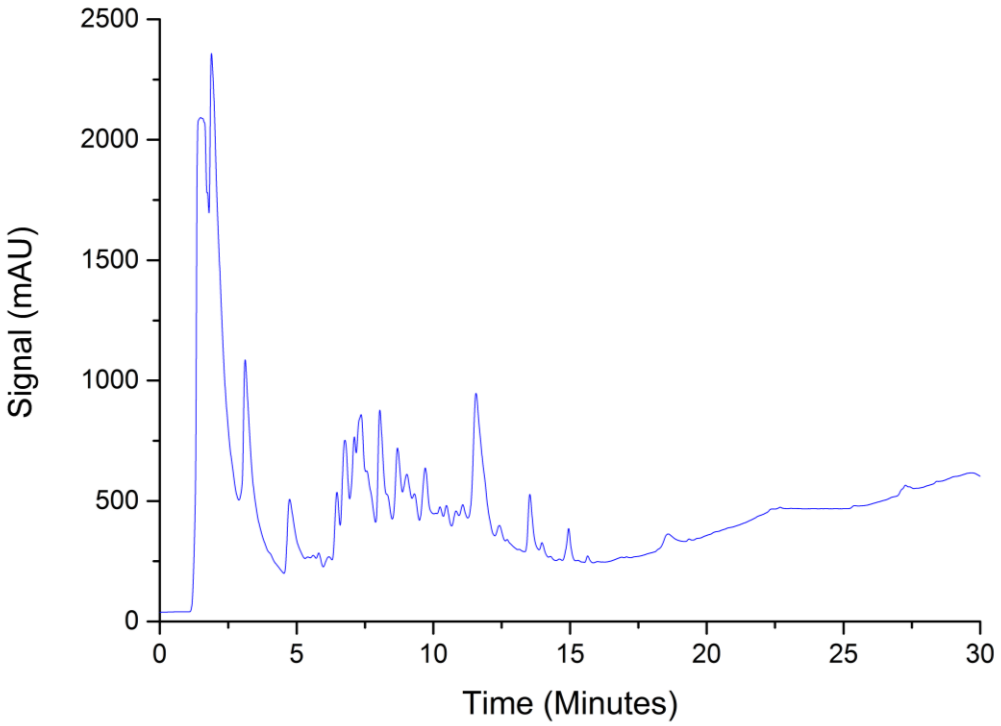


Figure 48: 200 nm UV-Vis chromatogram of sample M201 run using method D

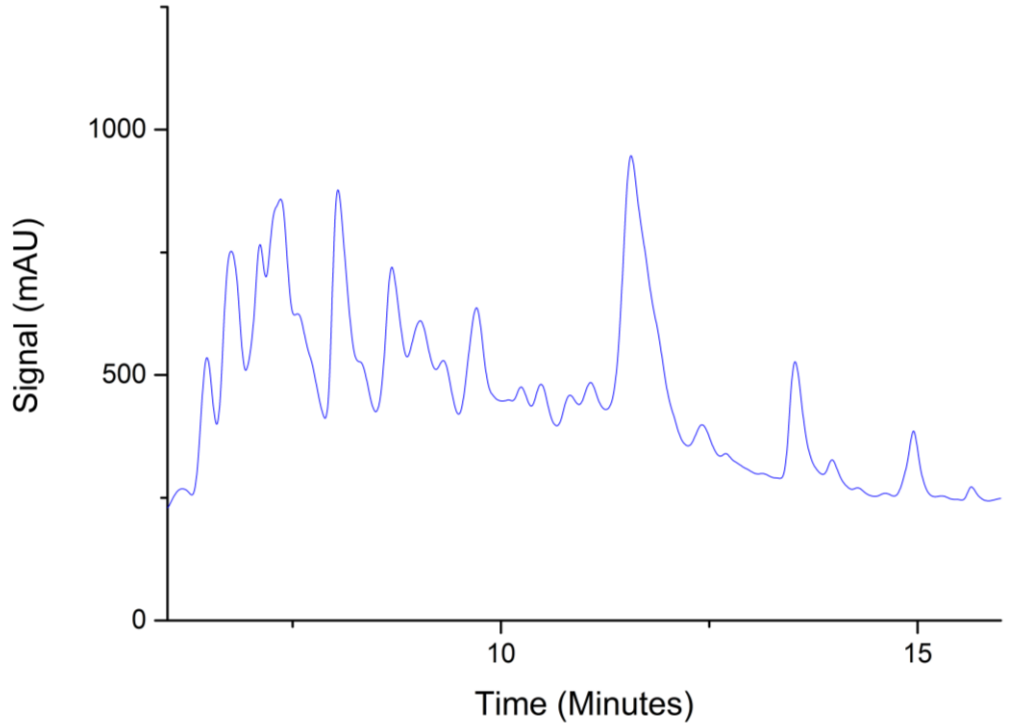


Figure 49: 6 to 16-minute section of the 200 nm UV-Vis chromatogram for method D

6.5 HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY

The results seen in the previous HPLC runs show that there are several compounds that are not being very well retained in the reverse phase methods. A common reason for poor retention is that the compounds are highly polar. Reverse phase HPLC methods rely on compounds interacting with a non-polar stationary phase to lead to separation.

One method that has been developed that is good for separating polar molecules using a HPLC system uses a column know as a Hydrophilic Interaction Liquid Chromatography (HILIC) column. The stationary phase in HILIC columns is more polar that in traditional reverse phase columns so it is better at retaining polar compounds[90].

The column that was available was from Phenomenex and was a Luna HILIC column. It came with a suggested trial method that should be done with two different buffers to see if there is any retention of the target compound/s. One of the buffer systems is the same as previous methods, ammonium formate, while the second uses an ammonium acetate buffer. The pH of the formate buffer is as it was for the previous methods and the acetate buffer has a pH of 5.8. Unlike in the previous buffered method the buffer is in both solvents not just the water.

Table 15: Solvent conditions for the HILIC test run

Time (min)	ACN	H_2O
0	90	10
2.5	90	10
10	50	50
12.5	50	50

Table 15 shows the solvent conditions throughout the runs. There are two isocratic periods with a gradient period in between. It begins with a 9:1 ratio of acetonitrile to water and then ends with a ratio of 1:1. It is stated by the manufacture that if these conditions do not give any sort of meaningful separation then it is unlikely that it can be achieved.

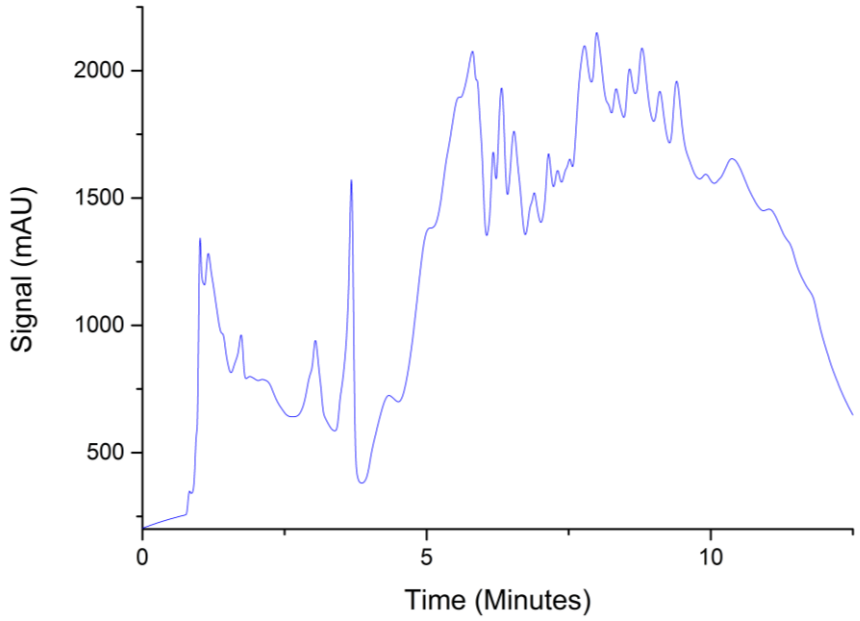


Figure 50: 200 nm UV-Vis Chromatogram for sample M201 with the HILIC column using the formate buffer

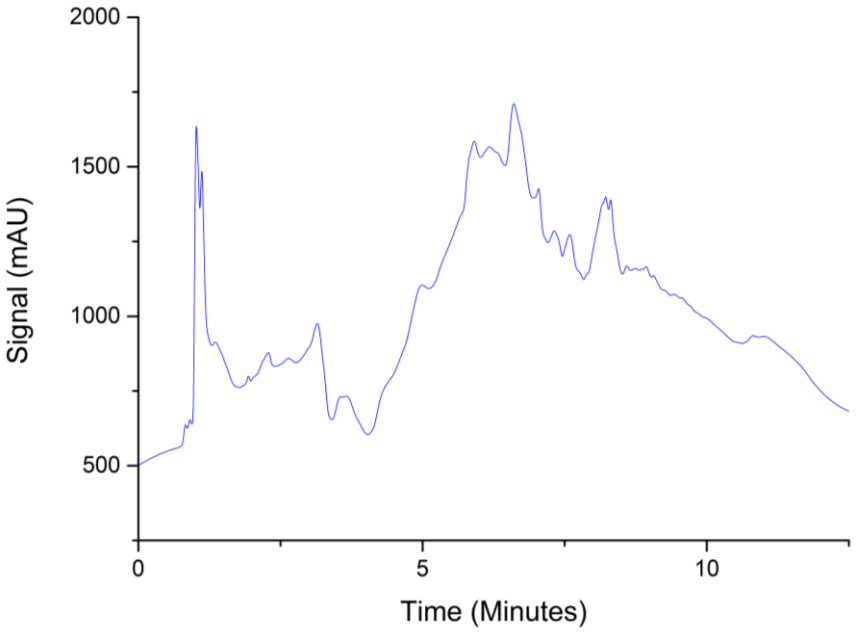


Figure 51: 200 nm UV-Vis Chromatogram for sample M201 with the HILIC column using the acetate buffer

The results from the two different buffers can be seen in Figure 50 (formate buffer) and Figure 51 (acetate buffer). Both appear to show retention of several compounds. This was more evident in the method that uses the formate buffer. There are several peaks throughout the chromatogram. While the peaks are not all well resolved, this

could be improved with some adjustments to the run conditions. This would only be done if the fluorescent compound is being retained and not eluting quickly.

6.6 FRACTION COLLECTING

A method for being able to determine where the fluorescent component was eluting was developed. Since using a fluorescence detector was not seen as a good option yet, the best option remaining was fraction collecting. The main reason for not using fluorescence detection was due to the known quenching effects of water so a method where this effect was removed would be the best option.

Fraction collecting was picked as the best way of determining where the fluorescent compound/s were eluting. Fractions from a HPLC run could be collected and then the solvent could be removed to concentrate the compounds in each fraction. The concentrated fraction could then be spotted to determine which is fluorescent. Repeating the separation several times and combining the fraction of interest could make observation of the fluorescence easier.

The methods that were selected for fraction collecting were the three reverse phase methods, B, C and D, along with the two HILIC methods. Fractions were collected from two runs of the same method and combined to increase the amount of fluorescent compound/s present. For the longer HPLC methods were broken up into five-minute fractions while for the shorter HILIC methods this was changed to 3 minutes.

The solvent was removed from the combined duplicate fractions under vacuum. Samples were then redissolved in 250µl of water. Once redissolved, equal portions of each was spotted onto silica gel plates. If the fluorescent component had eluted in the first fraction, there would be no point in further investigating the method as it would be difficult to better resolution of the initial peak. Only the first fraction from each method was tested and if it was not fluorescent then the other fractions were tested.

After testing the first fraction for each method, the only one in which the fluorescent component did not elute in the first fraction was method D. The fractions that were

collected from five to twenty-five minutes for this method were dried, redissolved and spotted. The fraction sections can be seen in Figure 52.

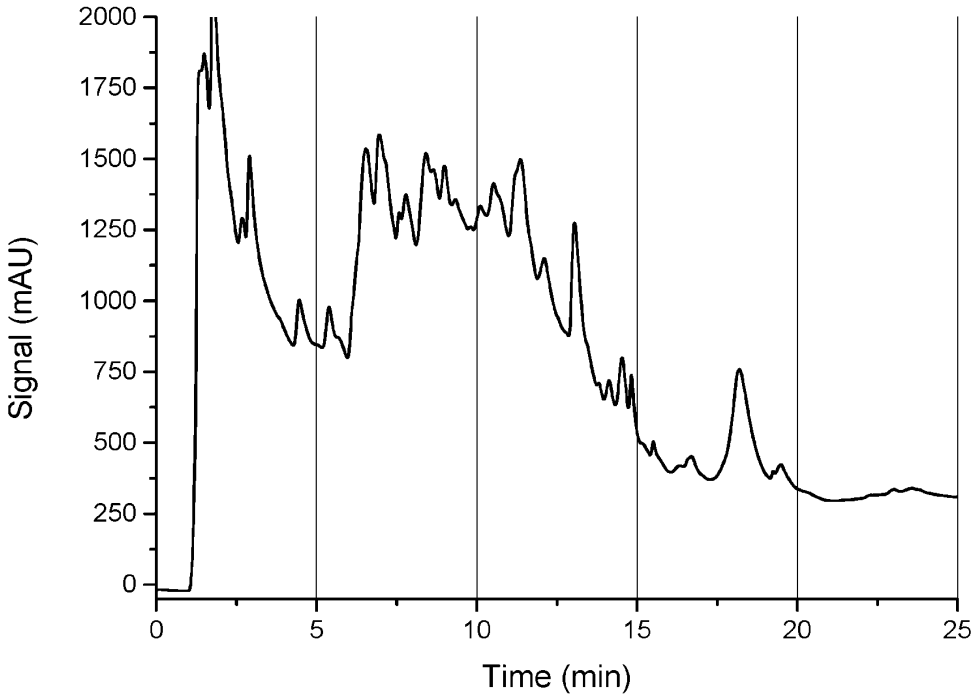


Figure 52: The 200-nm chromatogram produced for method D with the fraction ranges shown over 25 minutes

The fluorescence image of the five collected fractions can be seen in Figure 53. One fraction clearly appears to be fluorescent and is the ten to fifteen-minute fraction. This means that most, if not all, of the fluorescent compound/s are eluting in this fraction. Looking at the chromatogram over the associated period several peaks can be seen. There are two larger peaks but there is no way to identify from this which is responsible for the fluorescence, or if any of the visible peaks are the target.

This method was repeated six times to ensure that this same result was achieved. Different samples were also used to make sure it was not just the two initial samples that this worked for. In each case the ten to fifteen-minute fraction was always found to contain the fluorescent component. This successful method was then used for further experiments.

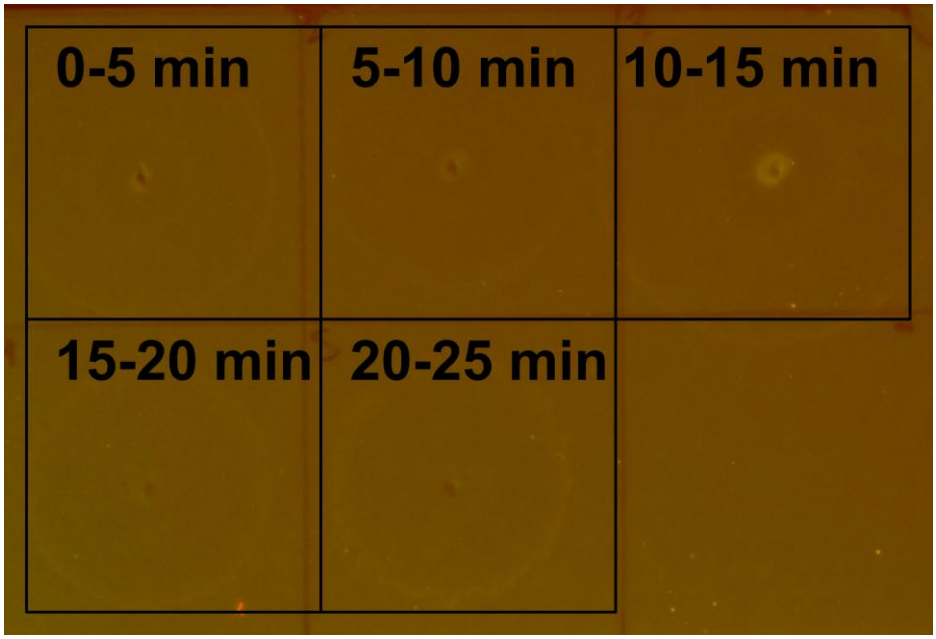


Figure 53: Photograph of the spot testing for method D showing the fluorescence occurring in the 10-15-minute fraction

6.7 HPLC TO SIMPLIFY THE SAMPLE MATRIX

Since the HPLC method D could retain the fluorescent compound/s, and the fluorescence was appearing in a single fraction, it was seen that a simplified sample could be produced that could then be used for further experimentation.

The method was run several times and the ten to fifteen-minute fraction collected. Initially, seven different samples were used in a total of twenty runs. The twenty fractions were combined. The combined fractions were taken to dryness and then redissolved to produce a new sample. To produce a 1.5ml sample (after drying and reconstitution in water) twenty fractions were combined.

The new sample was then taken and re-run on the HPLC system to see if better separation of the components in that fraction could be achieved. Advice received (D. Jardine 2014, pers. comm.) suggested that the concentrating of the buffer salt from the combination of the fractions should have little effect on any subsequent HPLC runs.

The focus with the new sample was to see if method D could be modified so that better separation could be achieved. By changing the rate of which the gradient changed the separation should be improved. Several experiments where conducted

were the gradient was slowed by various levels. The change that occurred over the five-minute period initially was eventually extended to over a ten-minute period. This did lead to better separation of the visible peaks.

Fraction collecting was again used to determine when the fluorescent compound/s were eluting. The fractions collection periods were shortened to two minutes and as before, multiple runs were combined. Spotting of these fractions revealed that the fluorescent compound/s were eluting over an extended period. Three of the fractions were found to be fluorescent.

There were three main conclusions as to why the fluorescence was eluting over an extended period:

- The responsible compound is being poorly resolved and the visible peaks are not related to the fluorescent compound/s.
- There are multiple compounds that are responsible which are very similar but have differences leading to small changes in retention on the column.
- The responsible compound has variations to is structure or size which result in varied elution times.

A different method of detection would be required to determine which of the above was correct.

6.8 FLUORESCENCE DETECTOR

Because a method had been established where it was known over what period the fluorescent component was eluting, it was decided that the fluorescence detector should be used to see if the compound/s fluorescence was detectable. The initial conditions for the detector were set to replicate what was being used in the fluorescence imaging, excitation wavelength of 450 nm and emission detection at 580 nm.

The fraction collection sample and two of the original samples were run with the fluorescent detector. Each sample was run two times at different injection volumes. The results from all the samples that were run were not very conclusive. It could not

clearly be seen in any chromatogram where the fluorescent component was eluting. The excitation and emission wavelength were varied with no improvement in the results. The broad elution time of the fluorescent compound from the column may have affected this.

More time with the instrument to fine tune the settings may have led to better results. Before this it was decided that more information about what was present in the sample could be achieved using different techniques. This is discussed in the subsequent chapter – LCMS and Proteomics.

CHAPTER 7 LCMS AND PROTEOMICS

7.1 INITIAL LCMS

The Agilent 1200 series HPLC system that was available did have its limitations. Since the fluorescence could not be detected using the fluorescence detector a different detection method was used to gather more information about what was present in the sample. It was decided that the sample should be submitted for analysis via Liquid Chromatography Mass Spectrometry (LCMS).

The bulk of work using the LCMS was undertaken by Dr Daniel Jardine in Flinders Analytical a part of Flinders University. Since the established methods on the HPLC system could simply be applied to LCMS, the methods and solvents were suppled for the samples to be run with a quadrupole mass spectrometry as the detector. The samples used for LCMS analysis were produced from fraction collecting.

The inclusion of the mass spectral analysis was hoped to give more information on the new sample. LCMS can produce a chromatogram that is based on the total ion count. What this means is that potentially more compounds can be detected than with a UV-Vis detector which only picks up UV-Vis active compounds. It can also be more sensitive than other detectors and can reveal compounds that may have been masked by co-elution.

The initial runs that were performed used the same solvents as the HPLC/UV-Vis detection analysis. The column was also a C18 column but not the same one that was used for the fraction work. Because the sample that was analysed was a combination of eluent from HPLC runs it had a high buffer salt concentration. This would also have caused any impurities to become more concentrated given the large volume of solvent collected to make the new sample.

The initial analysis of the sample using the method conditions established on the HPLC was not successful. There were too many different compounds co-eluting to be able to accurately determine what compounds were present. There also appeared to be impurities that added to the problem. These appeared to originate in one of the HPLC solvents used. Not only did they cause interference across the whole run they were also concentrated in the sample from preparation.

The solvent system was changed from the buffer/methanol solvent system to a water/acetonitrile solvent system, both of which were acidified with 0.1% formic acid v/v. This was done because these solvents were known to be pure. These solvents are used often for LCMS analysis and the water would have a similar pH compared to the buffer. The addition of acid to the organic component of the solvent system could assist in improving the separation of the compounds present in the sample. The methods that were trialled were like method D with the acidified water as the initial main solvent which changed by gradient to acidified acetonitrile.

After a few different methods were trialled, it appeared that some improved separation was being achieved. The method that appeared to give the best separation was looked at further. From manipulation of the mass spectral data from the method, it was noticed that there were some larger mass compounds in the sample that were eluting over an approximate three-minute period of one of the methods. A number of the peaks that were appearing in the chromatogram had associated mass spectra that appeared to be associated with peptides or polypeptides. Figure 59 in appendix C is an example mass spectrum that was being produced showing the masses of the peaks along with the associated charge. It shows that there are several compounds present, some with masses over 3000g/mol. Not much more could be determined about these peaks due to instrument limitations. The analysis of peptides and, more so, polypeptides can be difficult and has its own specific field of analysis, proteomics.

Since no fraction collector was available a rudimentary method was used to collect fractions over the period where these peaks were appearing. This was done by looking at previous runs and collecting fractions manually over a period where the compounds of interest eluted. Thirty second fractions were collected over a three-minute time period from the whole twenty-minute method, fractions were not collected over the entirety of the run.

The collected fractions were taken to dryness, a small amount of water was added and then were spotted onto silica gel. Several of the fractions appeared to have a small amount of fluorescence once spotted and dried. However, it was not enough

to be confident that they were fluorescent. The fractions that appeared to have fluorescence, five of the six collected over three minutes, were combined and concentrated. The resulting sample was more clearly fluorescent. During the three-minute period where these fractions were collected no other compounds could be identified in the mass spectral data. All that was known from mass spectra analysis was that there were polypeptides present.

Identification of any of the peaks that appeared was not possible due to the complex nature of identifying polypeptides. This did however provide information giving a better idea of the potential cause of the fluorescence so that methods could become more focused, essentially ones used with peptides and polypeptides.

7.2 SPE CLEAN UP

The initial attempts at SPE proved unsuccessful but since more had been learnt about the compound a more specific method could be applied. Since it was now suspected that the fluorescent component was either a polypeptide or protein, a SPE method which was designed for these molecules could be done. If the fluorescent compound could be purified using such methods, then this would further suggest that it is indeed a polypeptide or protein. This process would also remove the buffer salt and other contaminants that had hindered the LCMS analysis.

A general protein desalting method was selected to see if it would trap the component and clean up the sample. The method was adapted from one available from the Central Proteomics Facility on the Dunn School of Pathology website, part of the University of Oxford[91]. It was seen as an appropriate method to follow as it would remove the buffer salt from the sample while trapping proteins.

The SPE method chosen used a C18 SPE cartridge which relates to the HPLC column that was used. The solvents, including the sample, were also acidified like the buffer in the HPLC method using 0.1% formic acid. The composition of each of the different solvents used for each step and the volumes used can be seen in Table 16.

The load and wash solvents were collected as one and concentrated. This was to test whether the fluorescent compound was being trapped on the cartridge. Spotting of

the concentrated sample on silica gel did reveal some fluorescence but it was minimal. The intensity was much less than that of the original sample.

Table 16: Solvents and volumes used for SPE method

Step	Solvent
Condition 1	2mL acidified acetonitrile
Condition 2	2mL acidified water
Loading buffer	1.5 acidified water
Wash	Acidified water
Elution solvent	70/30 acidified acetonitrile/acidified water

The elute solvent from the method was also collected and concentrated to a volume that was similar to that of the original sample and tested for fluorescence. It was shown to be fluorescent but there was a reduction in the intensity compared to the original sample. Along with what was not trapped some may have remained on the column. Since the SPE method was not optimised there was likely to be a loss. Since the SPE method chosen is targeted at proteins and that the method successfully trapped then eluted the fluorescent component at the right times adds more evidence to the idea of a protein being responsible for the fluorescence.

7.3 PROTEOMICS

The LCMS analysis and the following SPE clean up on the sample made a strong case that the compound of interest was a protein. Since proteomic analysis can be highly complex and specified field, it was thought it would be best to get the sample analysed by an established proteomics facility. The facility that was chosen was the Flinders Proteomic Facility and Laboratory, which is part of the Flinders Medical Centre.

The LCMS results that were obtained were taken and discussed with Dr Tim Chataway of the proteomics facility. This lead to a sample being submitted for initial analysis. The sample analysed was the one resulting from the SPE clean-up. Since it was still unknown how complex the sample was the logical first step was to run the

sample on a SDS-PAGE gel. This would separate out the proteins that are present in the sample and give us more of an idea as to the nature of the sample.

Protein concentration tests on the initial sample showed it to be too low. Therefore, the sample had to be concentrated before it was run on the gel. The sample was concentrated to two different levels. The two different concentrations were done to give the best chance of getting good separation. These two concentrations were run on the gel along with a standard containing a ladder so that the approximate sizes of any proteins present could be determined.

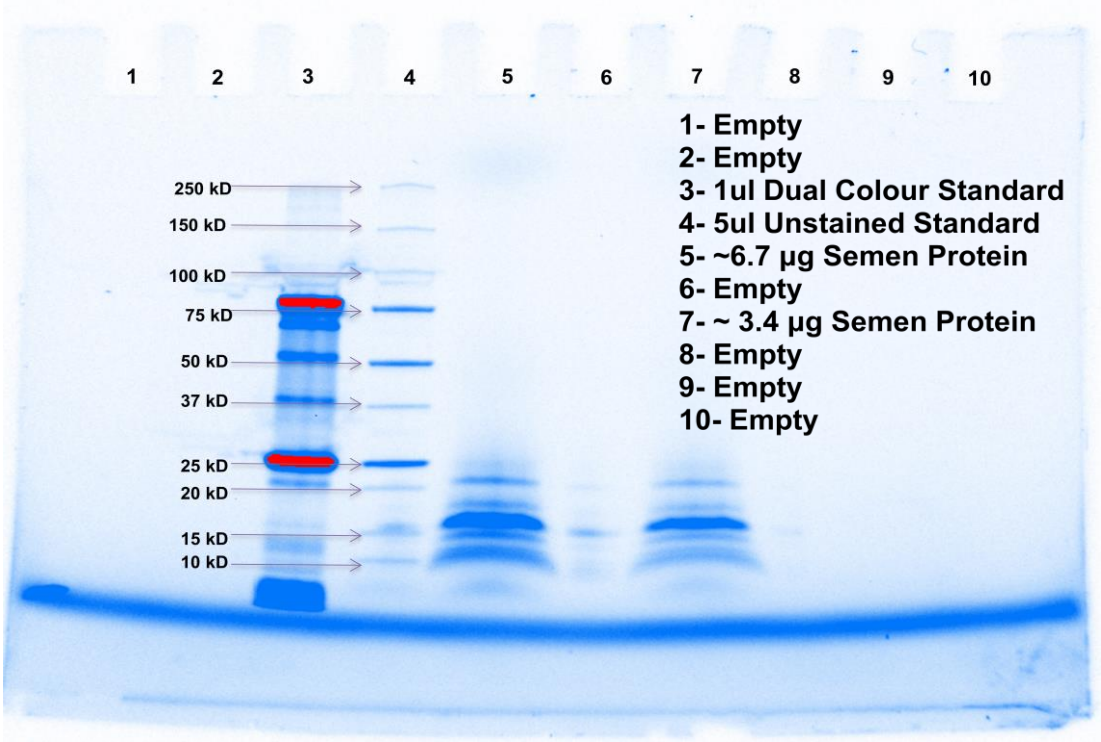


Figure 54: Photograph of the stained SDS PAGE gel. Lanes 5 and 7 contain the seminal fluid sample at different protein concentrations

The resulting gel from the analysis can be seen in Figure 54. Lanes four, five and seven are the lanes of most importance. The bands in lane three are from a coloured standard that was used so that the gel could be stopped before proteins potentially ran off the gel. Lane four is the one which has the standard with the ladder. Bands could clearly be seen and the associated sizes of each band are labelled.

The two sample lanes, five and seven, both show several bands in the low molecular mass area. All bands that can be seen are below the 25kD size band in the ladder. In

total, there are seven bands for each of the samples. However, two of these bands are weak and after discussions further analysis was focused on the five strongest bands in the gel.

Before any of the bands were analysed further a fluorescence image of the gel was taken. If one of the bands was found to be fluorescence, then the others could be ruled out. The image was taken using an Amersham ImageMaster VDS-CL at Flinders Proteomics Facility using similar settings to the method previously used. This was not successful and no fluorescence was seen which could have been due to several factors. Firstly, the gel was not dried for the imaging process so there could have been quenching from the solvents present.

Secondly, part of the sample preparation process for the gel involves the denaturing of the proteins using sodium dodecyl sulfate (SDS). In order for the proteins to run through the gel efficiently they must be unravelled or unfolded. If the fluorescence was dependant on the structure of the protein, then the denaturing process would cause quenching.

Finally, it may not just be a protein by itself that is responsible for the fluorescence. The fluorescence could arise from an interaction between the protein and a second molecule or ion, e.g. Zn^{2+}. The addition of the chemicals in the denaturing process or the separation on the gel could disrupt this interaction and cause quenching. If a metal ion is important to the fluorescence, then denaturing and adding EDTA (which is part of the process) could remove the ion.

Since none of the bands could be identified as fluorescent all five were analysed by mass spectrometry. Each band was excised from the gel and then individually treated using standard techniques to remove the protein from the gel. This was then followed by a tryptic digest before being mass spectral analysis.

The mass spectral results from the bands showed that each band contained multiple proteins or protein fragments. This is not ideal but it did lead to a list of proteins. The list of proteins from the analysis can be seen in Table 17. In total, there were fifteen different proteins identified. Some were in low concentrations and have a lower

chance of causing the fluorescence. Most of the proteins did not appear to stand out as they seem to be quite common or are common contaminants. There were however a couple that were interesting.

A protein that does stand out in the list is zinc-alpha-2-glycoprotein. The thing that makes it stand out is the fact that it contains zinc. Previous work by Walker (unpublished) measuring the zinc concentration across a semen stain by Laser Ablation High Resolution Inductively Coupled Plasma Mass Spectrometry indicated that as the fluorescence of a stain increases, the concentration of zinc also increased. While this is not definitive it does make this a protein of interest.

Table 17: List of proteins from mass spec analysis of gel

Proteins Detected		
Semenogelin 1	Semenogelin 2	Fibronectin
Histone H2B	Clusterin	Ribonuclease pancreatic
Antileukoproteinase	Zinc-alpha-2-glycoprotein	Polyubiquitin-C
Epididymal secretory protein E1	Far upstream element-binding protein 1	Far upstream element-binding protein 2
Prolacitn-inducible protein	Actin, cytoplasmic 1	Apolipoprotein D

The most common protein was Semenogelin, 1 and 2. This is not surprising as they are also the most prevalent proteins in seminal fluid. The main roll of these proteins is in the formation of a gel matrix. They also bind to the zinc that is present in seminal fluid.

It is unlikely that these proteins are the cause of the fluorescence seen as they are present in such a high concentration, it would be expected that the fluorescence would be more intense. Interestingly, they may still play a role in some way if an interaction with another compound leads to the fluorescence.

7.4 NATIVE GEL

As discussed above, one of the potential reasons for the fluorescence not being visible in the SDS PAGE gel was the fact that the proteins are denatured before being

run on the gel. After further discussions with Dr Tim Chataway, it was decided that it could be beneficial to do a native gel.

In native gels the proteins are not denatured before they are run on the gel. This does have the disadvantage that the size of proteins cannot be determined through this method. Since the proteins keep their structure they no longer run through the gel as easily so other properties more greatly influence the separation process. However, the big advantage is that the proteins structures are relatively unchanged. If the fluorescence is structurally related, then fluorescent compounds should be more likely to be visible in the gel as they may retain their fluorescence.

The results from the native gel were mixed. On the positive side the fluorescence was retained using this method and it could be visualised on the gel. There was a single band on the gel that showed fluorescence. The single fluorescent band appeared just above the die front in the gel. This band that did appear to be fluorescent was cut from the gel and analysed by mass spectrometry following trypsin digestion as had been done for the other gel.

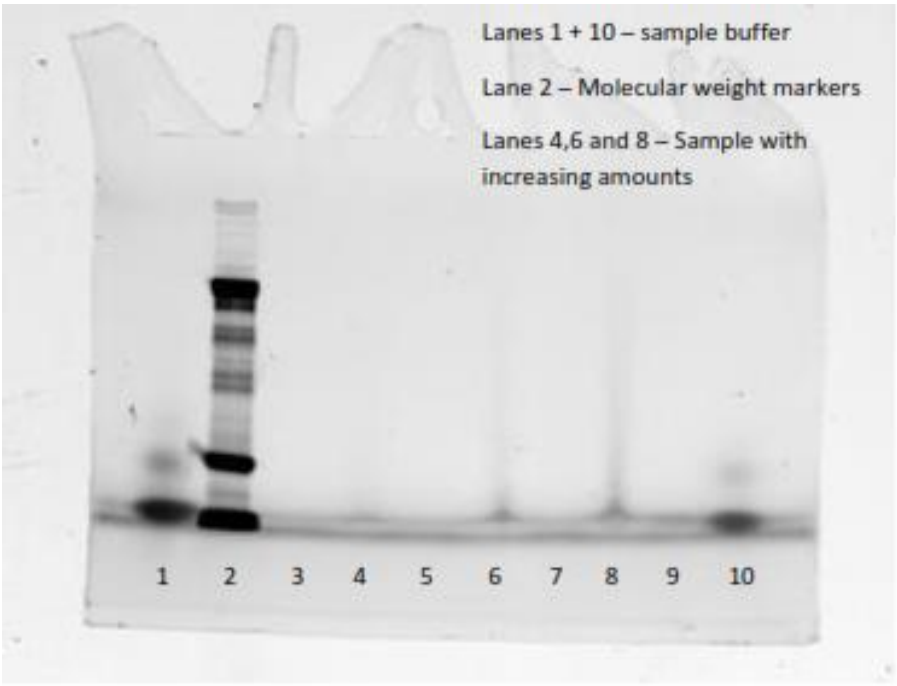


Figure 55 Fluorescence image (488nm excitation, 610 emission) of the native gel

A single protein identified would have provided strong evidence as to what is responsible for the fluorescence seen in seminal fluid. The results received from the

mass spectrometry analysis were not so simple. What was returned was a large list of proteins that were found in the band. Some of these were typical impurities but there were still a very large number of other proteins that were identified.

7.5 IDENTIFIED PROTEINS

Since there were so many proteins that were identified, it was decided that two main groups should be focused on. These were proteins that appeared in higher amounts and proteins that were identified from the previous gel. Most of the proteins from the previous gel were also in this band so there was not much narrowing of potential suspects for the fluorescence.

Table 18: Proteins identified in the Gel and descriptions of function

Protein	Descriptor/Function
Semenogelin	Participates in the formation of the gel matrix during coagulation and other roles [92]
Fibronectin	A glycoprotein that forms extracellular matrixes [93]
Zinc-alpha-2-glycoprotein	Has a major role in the lipolysis of adipose tissue [94]
Histone	Found in the chromatin all eukaryotic cells, DNA-binding protein [95]
Prolactin-inducible protein	Binds to several different proteins, exact role not clearly known [96]
Actin	In most eukaryotic cells it is the most abundant protein, participates in protein-protein interactions [97]
Ribonuclease pancreatic	RNA cleavage catalyst[98]
Epididymal secretory protein	Cholesterol-binding/transporting protein [99]

Semenogelin was found in both gels and one of the biggest contributors which as discussed earlier this is not surprising as it is the most common protein in seminal fluid. A process that occurs in seminal fluid is that it congeals and then liquefies. Semenogelin is one of the proteins that assists with the congealing process. Other proteins that are present then cause the liquefaction by breaking down the congealed proteins which would result in there being various sizes of semenogelin, explaining why it would appear in several bands in the initial gel. It may also be a case that as the semonogelin protein causes congealing, it binds to other proteins and or chemicals present. This binding could lead to the formation of a stable, large compound that could have fluorescent centres.

Two other proteins in both gels were prolactin-inducible protein (PIP) and zinc-alpha-2-glycoprotein (ZAG). The later was discussed as interesting above as it contained zinc which has a potential link to the fluorescence. While looking into these proteins it was found that there is evidence that they bond together forming a single structure[94].

Not only does PIP form bonds with ZAG but also with other proteins present in seminal fluid. PIP has been reported to bind to actin and also to human serum albumin which were identified in at least one of the gels. As noted in the table, PIP is known to bind to several proteins.

The bonding of PIP to other proteins could potentially affect the structure on one or both proteins. Structural changes could lead to changes in intrinsic fluorescence or the formation of new fluorescent properties. There is also the possibility that the bonding that occurs between the proteins could affect the fluorescent properties.

Histone was also found in both gels. There is evidence for histone being fluorescent [100] but the fluorescent spectrum does not match that seen with seminal fluid. The excitation and emission spectra for histone occur at lower wavelengths. The fluorescence spectra are more in line with what is seen from tryptophan which does not appear to be responsible for the fluorescence of interest.

7.5.1 Proteins present in single gel

There is the potential that some proteins did not show up in the bands of the first gel but they could still have been present. They may have been in a concentration that was too low or may have not run properly for some reason. Another option is that during the liquefaction process that occurs they were cut into small segments that were too small to move on the gel. Since the proteins are digested to be run through the mass spectrometer they could still be identified from the smaller fragments.

One of these proteins is lysozyme which was only identified in the second gel. Research into this protein and its potential fluorescence did turn up some interesting results. A paper [101] that looked at lysozyme from hen egg white contained a fluorescent spectrum that shows some similarity to that of seminal fluid. It is not the same but its excitation and emission bands are broad, but they are not over the same wavelength, seen in Figure 56. The excitation and emission ranges are, however, closer to that seen in seminal fluid than other proteins.

Images removed due to copyright restriction.

Figure 56: Fluorescence spectrum of hen egg-white lysozyme, Chan[101] et al Figure 4 A, left, and the fluorescence spectrum of seminal fluid from Stolvic[6] Figure 1

Human serum albumin (HSA) was identified in the second gel and has been explored previously as a potential candidate for the fluorescent properties in seminal fluid. Similarities between the fluorescence of seminal fluid and that of blood plasma have been noted previously and HSA could be the reason, as it is found in both fluids. But it is not the only protein in this category, lysozyme is also present in blood plasma.

7.5.2 Amyloid fibrils

Through research of the identified proteins an interesting molecule emerged known as an amyloid fibril. An amyloid fibril is a protein or peptide that has miss-folded and formed a new structure containing a high amount of β-sheets [101, 102]. Even though the structure of the original proteins may be quite varied the resulting amyloid fibrils are very similar in their structure [102]. Amyloid fibril formation has been linked to several diseases including Alzheimer's [101]. They are also noted to be poorly soluble and is a reason they form deposits [102].

As discussed earlier, Chan et al. [101] looked at the fluorescence of lysozyme from hen egg white. The fluorescence that was being explored in this work was from an amyloid fibril and was its intrinsic fluorescence. Since the fluorescence spectrum that is being observed is intrinsic to the lysozyme amyloid fibrils and lysozyme is present in seminal fluid [103], then there is potential that an amyloid fibrils could be responsible for, or contribute to, the fluorescence properties of seminal fluid.

Lysozyme is not the only protein that is present in seminal fluid that forms amyloid fibrils. Prostatic acid phosphatase [104-106] and fragments of semenogelin [107] are also known to form amyloid fibrils. These have received attention due to their potential to enhance the infection by viruses such as human immunodeficiency virus.

Since there are several different proteins that can form fibrils then there is a chance that these could contribute to the fluorescence seen in seminal fluid. It can also be seen that fibrils from different proteins have intrinsic fluorescence that is similar [108, 109] further adding evidence to the idea of the cause of the fluorescence.

CHAPTER 8 CONCLUSIONS AND FUTURE DIRECTIONS

8.1 Synchrotron

Seminal fluid was not able to be analysed directly any further in this work due to its complexity but useful information for future users of this technique was gathered. Data was successfully gathered in the Far-IR region using PTFE as an alternative to the more traditional PE. Furthermore, some spectra were successfully gathered using a pure sample disc with no binding matrix. This could be very useful since no matrix is added to the sample it is easily recovered.

Studies into the concentration of the sample in the disc proved that increasing the concentration can lead to a more detailed spectrum. Care would have to be taken to not have a concentration too high but as shown by the pure sample discs this will not be an issue for all samples. Running samples as pure discs may give the most detailed spectrum.

The small amount of work that was done on the mixed sample containing ascorbic acid, ninhydrin and riboflavin showed that even just three compounds mixed can make it very difficult to interpret the spectrum. This also showed that doing analysis on a sample as complex as seminal fluid would be very difficult.

It was found that relatively small changes to a molecule can have a huge effect on the resulting spectrum. The difference between the spectra for ninhydrin to Ruhemann's purple was quite interesting. The difference between their structures is not a large one, the latter effectively being two ninhydrin molecules joined by a nitrogen. Because of this it was surprising to see such a difference in the spectra that were produced. Ninhydrin gave a very detailed spectrum but Ruhemann's purple did not show anything significant. The symmetry that exists in Ruhemann's purple could be a factor in this.

8.2 Fluorescence visualisation

Using established techniques an easy and reliable method was developed to be able to visualise the intrinsic fluorescence of seminal fluid. It is a method that can be used to quickly identify if a liquid sample still contains the fluorescence seen in seminal fluid when doing separation techniques.

There is a natural variation in fluorescence that occurs between individuals. Most of this variation is due to hydration as removing this as a variable reduced the observed variation. The remaining variation was most likely from differences in the amount of the fluorescent compound/s present. Natural variation between individuals is to be expected but other factors like diet and health may also have an effect.

8.3 Separation and Identification

A HPLC method was also developed that could separate out the fluorescent component from some of the other compounds in seminal fluid. This leads to the ability to produce a simpler sample that further work can be performed on.

Mass spectral analysis along with fraction collecting pointed towards the fluorescence compound/s being a polypeptide of some description. A SPE method that was designed for protein clean-up could trap and elute the fluorescence adding further support to a protein or proteins being responsible for the fluorescence.

Proteomic analysis of the sample identified several different proteins present. Two different gel electrophoresis techniques were used to try and identify the fluorescent compound/s, standard SDS PAGE and native gel electrophoresis. Insufficient separation of proteins was achieved to be able to identify the cause of the fluorescence but some candidates were identified.

Research into the proteins that were identified from the proteomic work that was performed has shown that there are potentially some complex interactions and processes occurring in seminal fluid. The cause of the intrinsic fluorescent property of seminal fluid was not identified but several possible causes have been discussed. Amyloid fibrils formed by lysozyme were identified as having a similar absorption and emission spectra to that of seminal fluid.

8.4 Future Directions

The new methods developed for sample preparation for analysis using the synchrotron could be applied to different samples. Some samples that may have not been able to be analysed could potentially now be analysed. Being able to do the

analysis with minimal loss of sample may make it a more attractive and viable technique.

Further work is required to identify what in seminal fluid gives it its fluorescent properties. It is not possible to completely rule out the fluorescence is originating from small organic compound/s. The results from this research indicate that the most likely candidate is a protein or several proteins.

Further proteomic analysis would be the logical next step. This could initially include work using spin tubes or some other form of size separation. Most of these techniques are non-destructive and would lead to a less complex sample to analyse. This may also reveal if there are multiple proteins responsible or if the protein that is responsible varies in size.

Additional analysis using native gels may yield a method that can separate out all the proteins present in a sample while also retaining the fluorescent properties. If better separation can be achieved while still being able to visualise the fluorescence, then there is a good chance the fluorescent compound/s could be identified. A way of achieving better separation is by doing a two-dimensional gel.

APPENDIX A – TABLE OF SEMINAL FLUID SAMPLES RECEIVED

Table 19: Samples received from Flinders Fertility with information

Semen sample	Date received	Total weight (g)	Semen sample	Date received	Total weight (g)
S1	28/03/2014	10.76	S26	10/10/2014	9.40
S2	31/03/2014	11.92	S27	10/10/2014	10.57
S3	14/04/2014	12.02	S28	20/10/2014	11.99
S4	14/04/2014	12.54	S29	20/10/2014	9.11
S5	14/04/2014	10.61	S30	24/10/2014	8.24
S6	not received	-	S31	27/10/2014	11.27
S7	not received	-	S32	31/10/2014	9.99
S8	29/04/2014	10.73	S33	14/11/2014	9.09
S9	29/04/2014	9.93	S34	17/11/2014	10.77
S10	29/04/2014	10.50	S35	20/11/2014	11.82
S11	8/05/2014	9.71	S36	20/11/2014	10.76
S12	15/05/2014	9.51	S37	21/11/2014	9.39
S13	20/06/2014	16.76	S38	27/11/2014	9.79
S14	20/06/2014	12.62	S39	27/11/2014	8.98
S15	4/07/2014	12.20	S40	5/12/2014	10.05
S16	9/07/2014	10.56	S41	5/12/2014	10.62
S17	18/07/2014	10.41	S42	12/01/2015	10.62
S18	21/07/2014	11.44	S43	15/01/2015	10.84
S19	7/08/2014	8.40	S44	19/01/2015	13.60
S20	8/08/2014	9.22	S45	21/01/2015	9.37
S21	17/08/2014	9.53	S46	28/01/2015	8.80
S22	8/09/2014	12.86	S47	30/01/2015	9.15
S23	8/09/2014	8.11	S48	3/02/2015	10.32
S24	12/09/2014	8.91	S49	3/02/2015	9.50
S25	18/09/2014	8.70	-	-	-

APPENDIX B – MASS SPECTRAL DATA LUMICHROME AND LUMIFLAVIN

Flinders Analytical

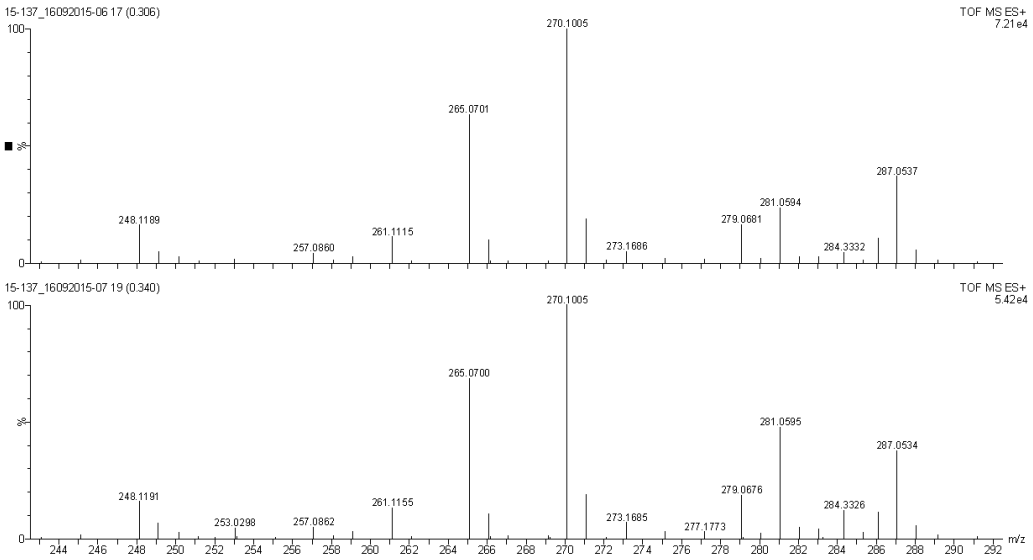
Accurate Mass Analysis

Date: 17-09-2015

Flinders Analytical Job Number: 15-137

Sample: Lumichrome

High Resolution Spectra
Positive ion



Accurate Mass Data

Observed Mass	Formula $[M+Na]^+$	Calculated mass	Difference (ppm)	iFiT (norm)
265.0700	C12H10N4O2Na	265.0701	-0.4	4.3
265.0701	C12H10N4O2Na	265.0701	0.0	4.3

The peak at the nominal m/z value of 270 is carry over from a previous sample

Dr. Daniel Jardine
Flinders Analytical, Flinders University, South Australia

Figure 57: Accurate mass analysis data for lumichrome

Flinders Analytical

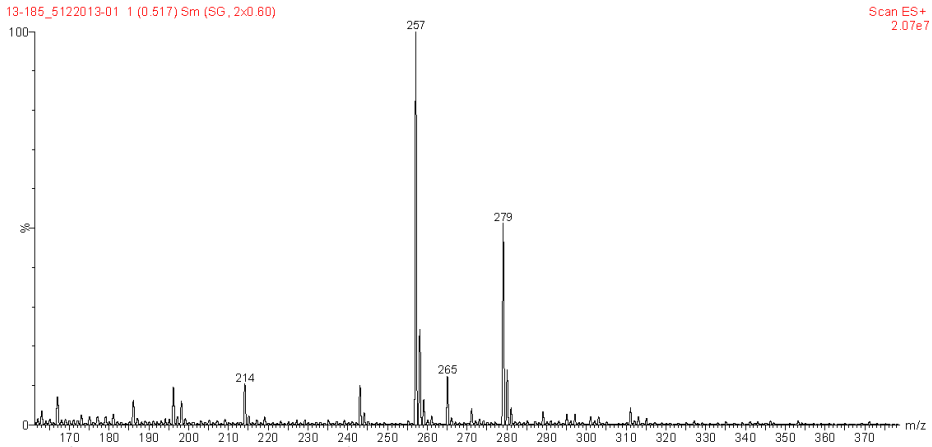
ESI Mass Analysis

Date: 5-12-2013

Flinders Analytical Job Number: 13-185

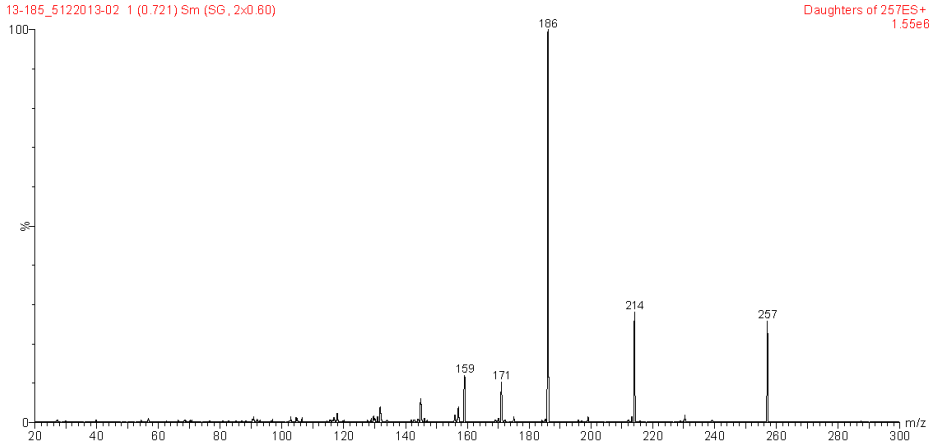
Sample: Lumiflavin

Electrospray mass spectrum, positive ion.



$M+H]^+$ ion at m/z 257, $[M+Na]^+$ ion at m/z 279.

MS/MS spectrum of the peak at m/z 257



Dr. Daniel Jardine
Flinders Analytical, Flinders University, South Australia

Figure 58: Accurate mass analysis data for lumiflavin

APPENDIX C – MASS SPECTRUM FROM LCMS ANALYSIS

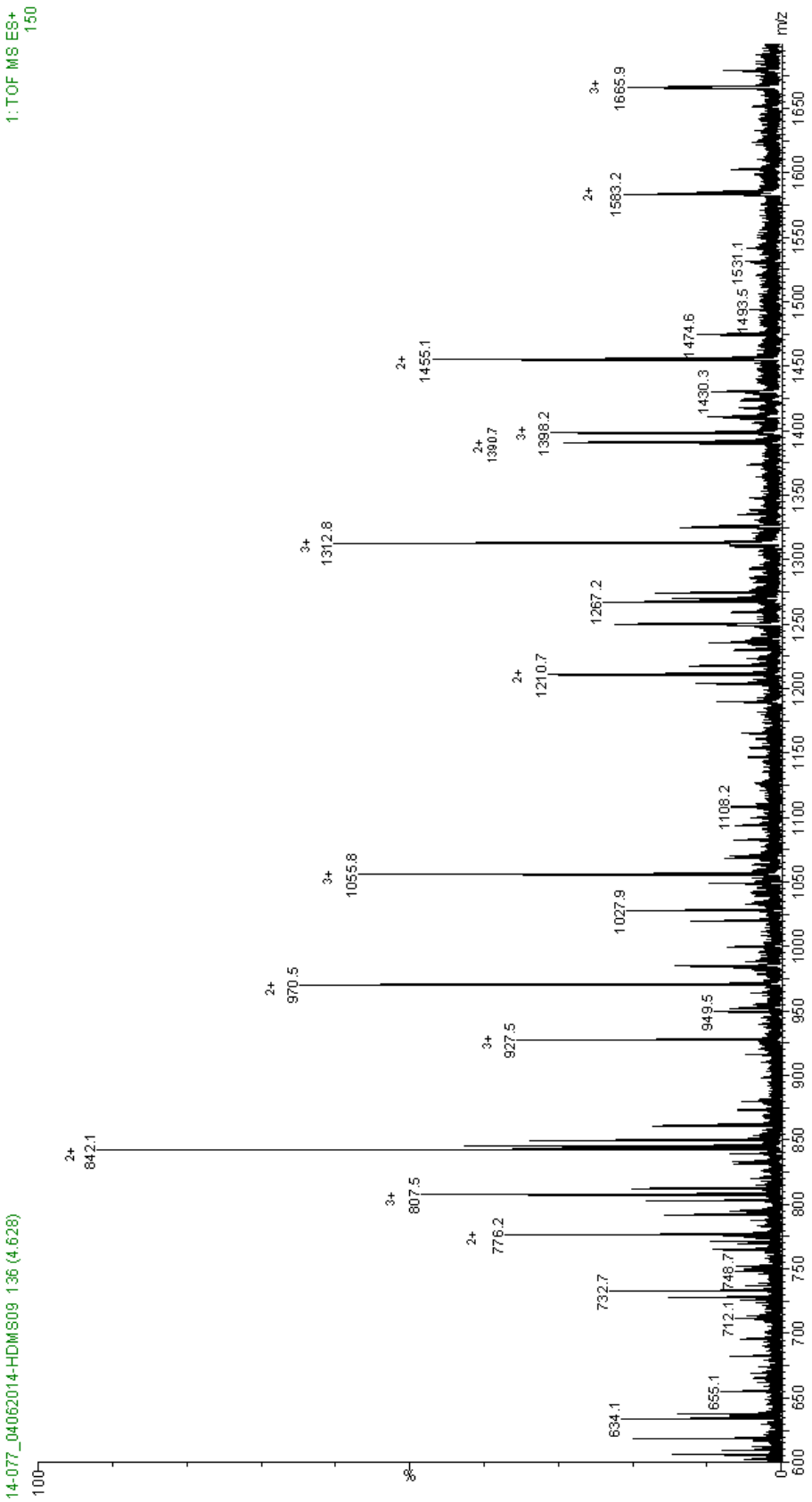


Figure 59: A TOFMS ES+ mass spectrum from the LCMS analysis, of the post HPLC clean up seminal fluid sample, seen during the period that the fluorescence was eluting from the column

APPENDIX D – FULL IMAGES FROM FLUORESCENCE COMPARISON

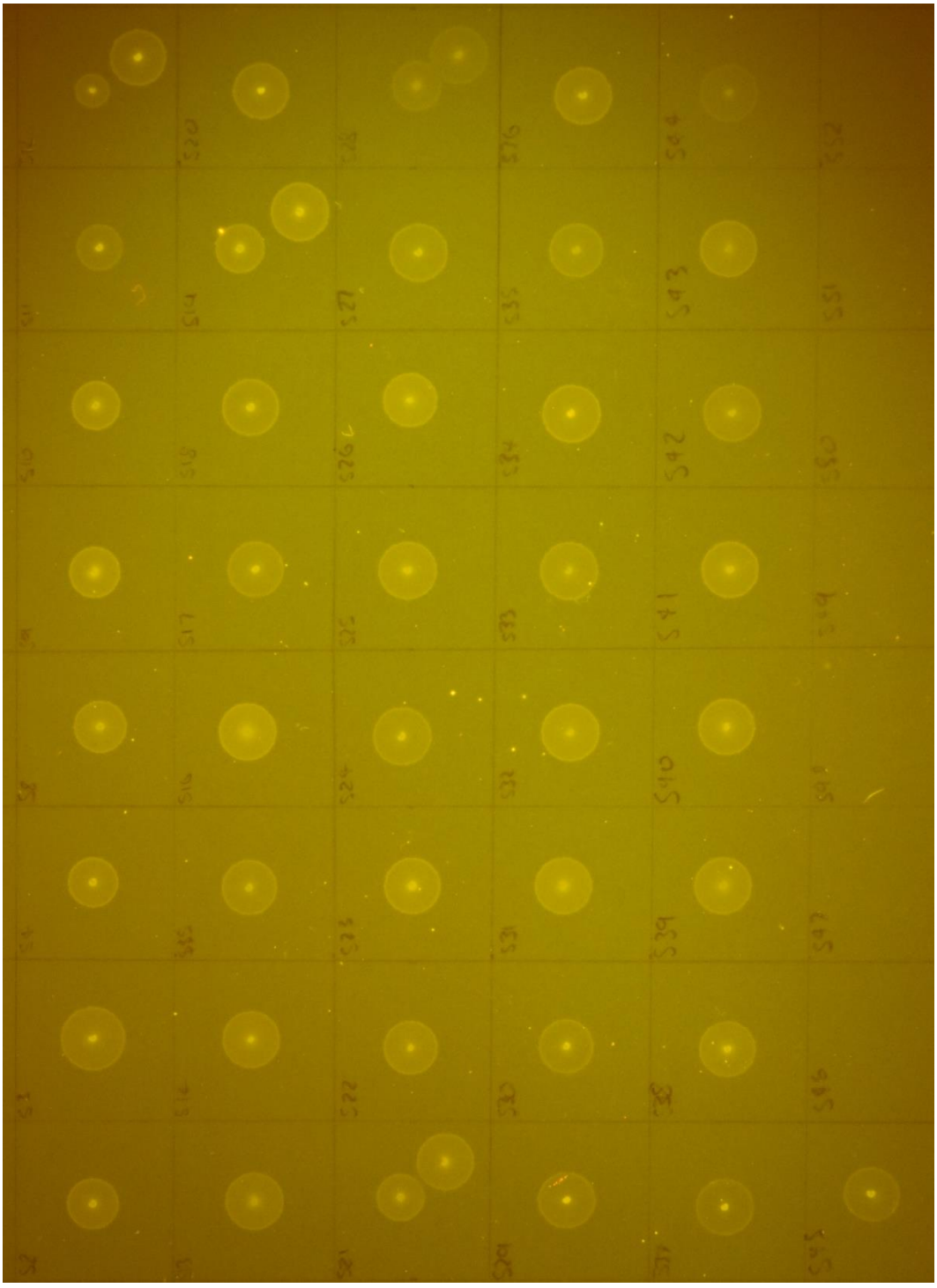


Figure 60: Fluorescence image of all the spotted wet mass samples

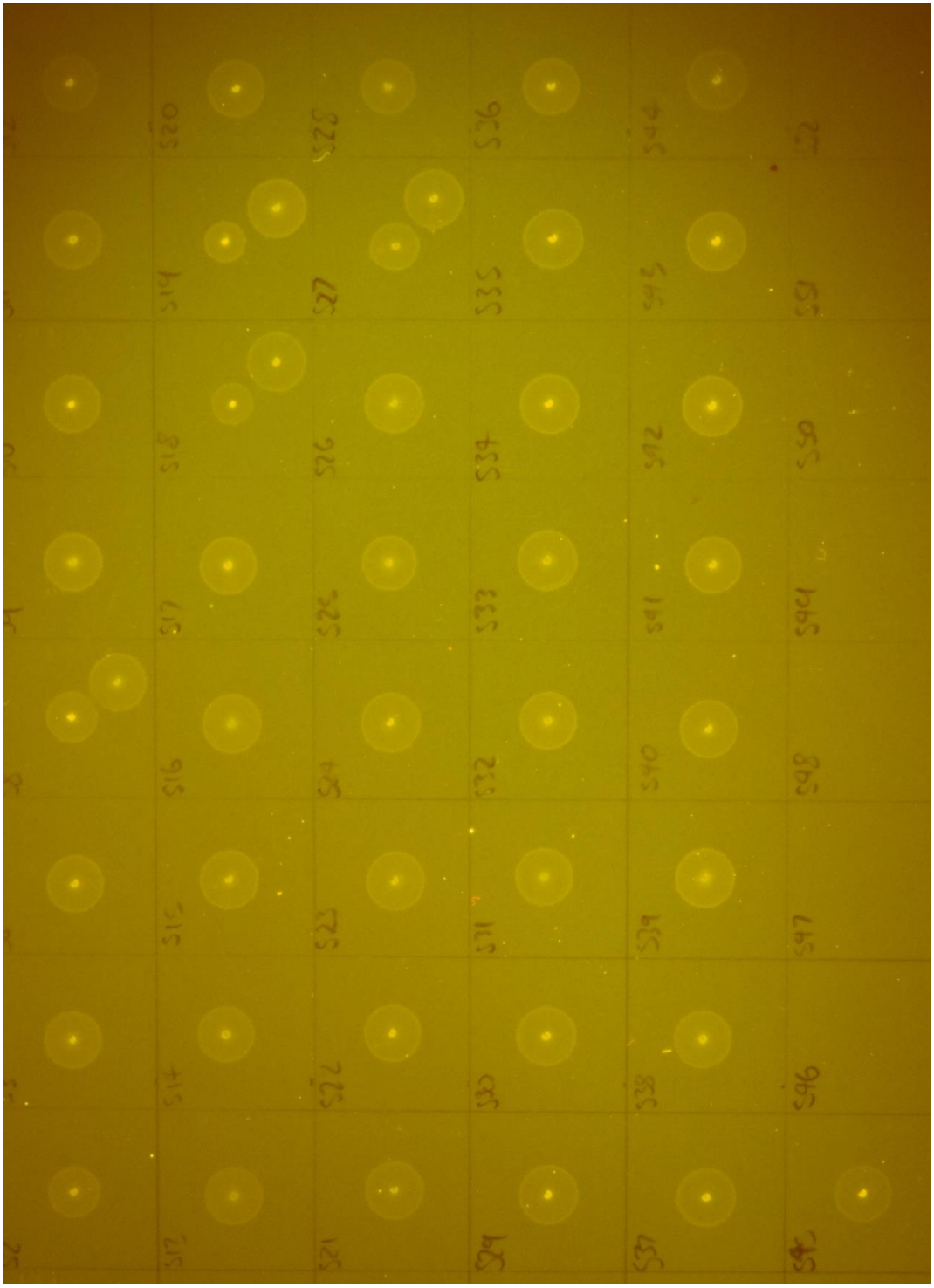


Figure 61: Fluorescence image of all the spotted dry mass samples

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