Elemental and Molecular Profiling of Illicit Tobacco

By

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Abstract

Tobacco has been labelled by the World Health Organisation as the largest preventable threat to modern day health, due to the tobacco plant being highly susceptible to bioavailable elements and the accumulation of over 4,000 different chemicals throughout cultivation. The risks of using tobacco are significantly heightened when the tobacco is illicit due to the use of poor grade unregulated tobacco and sub-standard delivery systems. This research that was conducted in collaboration with Lancashire Trading Standards and allows for the profiling of a diverse range of different tobaccos using 88 samples collected from Lancashire Trading Standards, various licit retailers in Preston, France and Sweden.

The aims of this research include; the elemental profiling of tobacco using X-ray Fluorescence Spectroscopy as a rapid handheld qualitative technique, the quantification and comparison of nicotine levels within licit, illicit and niche tobacco using Gas Chromatography – Mass Spectrometry, including a single strand extraction study to determine the possibility of the natural spatial distribution of nicotine along the tobacco leaf and finally, the molecular profiling of tobacco using Fourier Transform Infrared Spectroscopy aided by multivariate data analysis.

Through analysis of spectra collected using X-ray Fluorescence, we were able to determine elemental differences between dried leaf and treated tobacco using fluctuations in elements such as Potassium, Calcium and Iron. The most significant elemental difference between niche and licit tobacco was the presence of Chlorine found within the Snüs.

The extraction and quantification of nicotine using Gas Chromatography – Mass Spectrometry identified significantly higher levels of nicotine present within illicit tobacco when compared to that of a licit cigarette, supporting the theory that illicit

tobacco contains higher doses of nicotine leading to higher rates of addiction. Data collected from single stranded extractions identified similar high standard deviations (> 25%) to that as the main nicotine study, supporting the theory that nicotine has inconsistent spatial distribution across the tobacco leaf.

Data that was collected using Fourier Transform Infrared Spectroscopy was pre-processed and vector normalisation was applied. Variable ranking was used to determine the highest discriminative wavenumbers, highlighting spectral fingerprints within the spectra relative to each different type of tobacco, identifying absorptions in the regions of 1050-1150 cm^{-1}, 1350-1480 cm^{-1} and 1600 cm^{-1}.

This research compares and identifies differences between tobacco available on the licit and illicit market and establishing a platform for the full profiling of licit, illicit and niche tobacco and their constituents, where limited research has previously been conducted.

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List of Abbreviations

WHO	World Health Organisation
PAC	Public Accounts Committee
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
AAS	Atomic Absorption Spectroscopy
XRF	X-ray Fluorescence Spectroscopy
CNS	Central Nervous System
HPLC	High Performance Liquid Chromatography
GC-MS	Gas Chromatography Mass Spectrometry
FTIR	Fourier Transform Infrared Spectroscopy

Chapter 1

Introduction

1 Introduction

1.1 Tobacco

Nicotiana tabacum, more commonly known as the tobacco plant [1], the leaves of which are typically the consumable part of the plant and are harvested to be smoked, chewed or sniffed depending on the users preferred method of ingestion [2]. Tobacco plants are renowned for their ability to accumulate over 4,000 different chemicals throughout cultivation [3]. There is a delay from initial tobacco use and the first adverse physiological health effects. Prolonged exposure has been linked to the development of life threatening illnesses such as cancer, cardiovascular disease, strokes and chronic obstructive lung diseases. The effects of illicit tobacco are usually made more prominent due to poor grade unregulated tobacco and sub-standard delivery systems, dramatically increasing the long term impacts on user's health [4].

1.2 Illicit and Niche Tobacco

Illicit tobacco typically sold in the form of cigarettes or hand rolled tobacco, which is grouped by U.K. trading standards into two main groups; Counterfeit products or 'Cheap Whites'. Counterfeit tobacco products mimic licit brand packaging in an attempt to masquerade as licit products and contain low grade unregulated tobacco which is sold on to unsuspecting consumers. In comparison, 'Cheap Whites', which are usually cigarettes made with poor filters and low grade tobacco, are marketed under illicit brand names e.g. Jin Ling, New Line, that are purely targeted for sale to the U.K. illicit market. Niche tobacco has emerged in recent years from South America and South East Asia and has over time increased in popularity all over the world. Niche tobacco is effectively smokeless tobacco, where the product is able to be consumed without full pyrolysis of the product. The products vary drastically in content depending on the

desired flavour and required method of ingestion [5]. Niche tobacco is a branch of illicit tobacco that is not for sale on the U.K. market as it typically does not meet standards set out in U.K. or European legislation. Niche tobacco is usually a licit source of tobacco in another country, however, the main restrictions on niche tobacco are based around the limited knowledge of adverse health effects and the content information upon packaging, which does not usually state levels of components in English if they are not omitted completely [6]. Products can be used orally or nasally with absorption of the product occurring after it is introduced to a thin, wet, membrane allowing chemical substances, such as nicotine, to pass into the bloodstream with ease stimulating cholinergic receptors, allowing for the release of the hormone Dopamine. Effects last somewhere between 20 minutes to 6 hours with an almost instantaneous effect due to the quick transference into the bloodstream [7].

1.3 Tobacco Cessation

The global increase in tobacco use is being described by the World Health Organization (WHO) as an epidemic, declaring in 2013 tobacco as the sole biggest threat to modern day health. Tobacco use is related to a current annual death toll of six million deaths per year, which is set to rise to 8 million by 2030 if drastic action is not taken. The WHO enforced the 'Framework convention on tobacco control' in 2005, which has received over 160 international signatories in an attempt to introduce strategies that would reduce the enormous impact on human life. The primary method of deterrence introduced was a drastic 10% increase taxation. There was an expected reduction in consumption of 4%, with financial availability being deemed a key point in the continuation of tobacco use among young smokers [8]. The introduction of taxation increases was not necessarily a strategically advantageous act due to being undermined by a flourishing illicit trade, with numbers of loyal illicit smokers within the U.K. rising to 17%, debasing any form of financial restrictions set in place.

Lower income consumers are at a higher risk of purchasing illicit tobacco with the recent economic hardship sustained, particularly in financially deprived regions of the U.K. such as the North East and North West, causing smokers to be less brand loyal and more open to trying alternative cheaper illicit tobacco. The illicit tobacco trade does not solely affect those that have chosen to purchase illicit tobacco, it also effects regular consumers who believe they are purchasing the genuine article. In a recent study carried out in a high street of South East England, where questionnaires and samples were collected from participants at random, the results show of all tobacco collected 28% was illicit and only 13% of the participants actually knew that is was not a licit source of tobacco [9].

In recent years HMRC has come under media scrutiny from the Public Accounts Committee (PAC) regarding their effectiveness at tackling the issue, primarily due to the U.K. economy losing £1.9 billion per year to the illicit tobacco trade. Trading of illicit tobacco is not perceived to be an immediate serious issue in comparison to international drugs trafficking or money laundering but in reality it is more of a threat to the lives of millions than the two put together. With low weighted penalties served by the courts and easy payment of fines, criminals are able to make allowances for a predetermined financial risk within the profit margin of each shipment of tobacco. With severely limited information on minor offences and little coordination between police forces and HMRC at both regional and national level, it almost allows the market to continue trading without any serious implications. This generates an easily lucrative trade, attractive to terrorist funding operations and organised crime gangs [10].

1.4 Elemental Content

Many of the chemical substances that are associated with the tobacco plant are attributed to atmospheric depositions or the application of phosphate fertilizers and sewage sludge. Sources of heavy metal concentrations, in particular cadmium, which

has been found in illicit tobacco in excess of 500% when compared to a licit cigarette, decrease in the following order throughout the plant: Roots> Leaves> Fruits> Seeds [2,4]. Tobacco plants are susceptible to the accumulation of bioavailable elements such as Cadmium, Lead and Zinc by the preferential uptake of these elements, as the presence of one mobile element within the soil will stimulate the uptake of another. The Cations of Cd and Pb strongly bind to sulphur containing ligands. The interaction of Cd^{+} and Pb^{+} with sulphur-hydrogen bonded groups inactivate enzymes, disturbing the metabolic process. The high uptake of one of these heavy metals leaves a deficit within the soil over time, stimulating the uptake of the other in replacement of the soil deficit [11]. Unlike organic materials found in soil, inorganic impurities are not usually removed from a source by chemical or microbial degradation [12].

There is a growing need for a simple method of ascertaining inorganic trace elements within plant material, especially for those plants that are known to be heavily affected by the bioavailability of elements in soils high in fertilizers and atmospheric depositions.

Techniques such as Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and Atomic Absorption Spectroscopy (AAS) have been used to quantify elements found in tobacco products, however, their aggressive digestion methods do not necessarily allow for the full extraction of elements. These methods are also limited to quantifying mostly metals, whereas elements such as Chlorine and Bromine are outside the limit of their capabilities, although they are able to quantify low levels of toxic trace elements that are below the detection limits of X-Ray Fluorescence Spectroscopy (XRF) [13].

XRF is an economic, sensitive, rapid technique that can be used to determine the elemental composition of plant foliage, including tobacco. Previous attempts had poor sensitivity due to X-ray scattering caused by the high background levels of organic

matrices, which has only recently been overcome by the use of polarised X-ray sources using a primary photon beam scattered by a secondary target [12,13]. Research conducted by W.E. Stephens of the University of Saint Andrews using ICP-MS notes that as well as Cd being in excess of 500% and heightened levels of Se and Pb, analysis by W.E. Stephens using XRF highlighted that there were also increased levels of Fe, Ca, Ni, Mn, Cu, and Zn [4,12]. Research conducted by Dhaware *et al.* (2009) and Verma *et al.* (2010) into tobacco focuses on samples using Indian Niche smokeless products. These studies emphasize the importance of the presence of heavy metals, and the specific health risks associated with their use due to ingestion via mucosal membranes [19].

1.5 X-ray Fluorescence

1.5.1 Fundamentals of X-Ray Fluorescence

X-Ray Fluorescence Spectroscopy (XRF) is a highly sensitive non-destructive analytical technique used to detect elements with a greater atomic number than oxygen within the periodic table, that can be used qualitatively or quantitatively [14]. XRF offers an alternative multi-element analytical tool to other elemental analysis techniques such as Inductively Coupled Plasma Mass Spectrometry (ICP-MS) or Atomic Absorption Spectroscopy (AAS) which use aggressive digestion methods that do not necessarily fully recover elements contained within plant material [15,16,17].

XRF requires photons to be fired at a sample in order to excite the atoms causing them to fluoresce. The ionization of core electrons occurs and electrons from higher orbitals occupy the position of the ionized electron, occurring upon the exposure to short wavelength X-rays. Excess energy is then emitted in the form of a photon, typically within the X-ray region of the electromagnetic spectrum. The energy emitted is equal to the energy difference between the orbitals, giving each different element a characteristic X-ray emission spectrum which is displayed as a sharp set of peaks. The location of peaks for each element will differ due to the energy difference between the 2*p* and 1*s* electron shells. The main transitions observed in a spectrum are L->K, expressed as Kα,

M->K, expressed as Kβ [14].

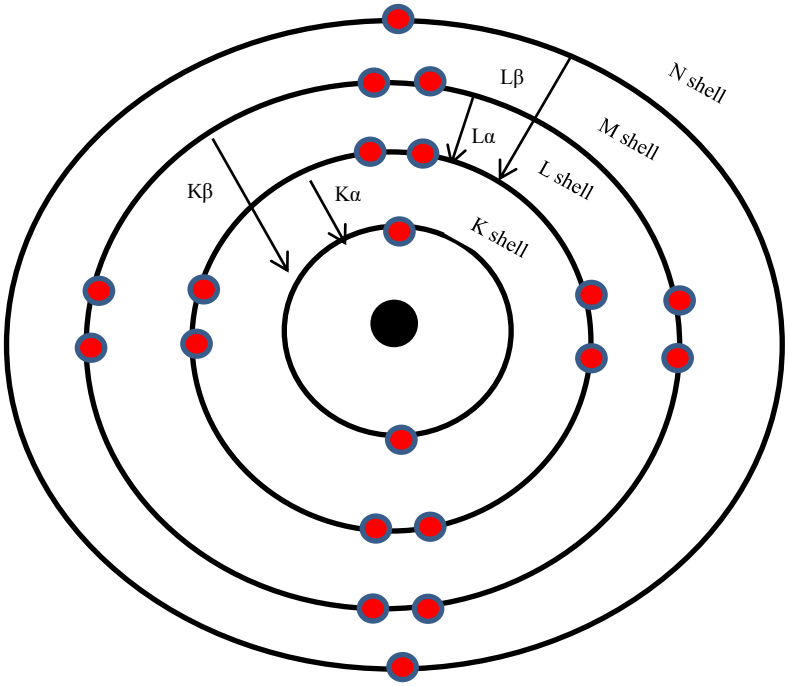


Figure. 1 A diagram expressing the energy transitions upon the application of X-rays, K and L Lines.

1.5.2 Applications of X-ray Fluorescence on the Elemental Analysis of Licit, Illicit and Niche Tobacco

Tobacco use has been named as a major source of heavy metals that have been found within the human body, in particular Cd [18]. A rapid non-destructive handheld tool for fast elemental analysis could potentially allow for the development of a tobacco classification system. This system based on the potential adverse health effects of the seized product by the determination of elemental profiles at the scene, could be utilised for increasing the weighting of prosecutions by HMRC.

1.6 Nicotine and Addiction

Nicotine, 3-(1-methyl-2-pyrroildinyl) pyridine is the major non-volatile highly toxic alkaloid found within the leaves of *Nicotiana tabacum*, representing 95% of the total

alkaloid fraction that are used as indicators of tobacco use (see figure. 2 below). Nicotine is extracted from tobacco as a pale yellow to colourless hygroscopic oily liquid and has an LD_{50} of 50-60mg. Increased levels of nicotine have been proportionally linked to a higher rate of addiction, leading to a higher nicotine dependency prolonging the use of tobacco [2,4,19,20].

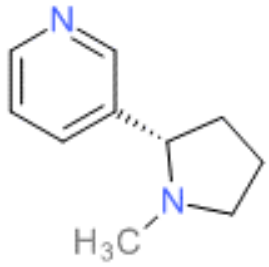


Figure. 2 Nicotine

Nicotine is able to activate and desensitize nicotinic acetylcholine receptors, ligand-gated ion channels that normally bind to acetylcholine, within the central nervous system (CNS). This stimulation releases a variety of neurotransmitters in the brain, the most relevant being dopamine, that signals a pleasureable experience, therefore, compelling and reinforcing the use of tobacco. Repeated exposure to nicotine creates a tolereance level due to desensitization amongst receptors, therefore as desensitization increases, neuroadaption reacts and increases the number of binding sites present on the nicotinic acetylcholine receptors. Withdrawl symptoms occur when desensitized receptors become responsive during a period of abstinence. The nicotine binding that occurs during ingestion of tobacco relieves cravings and withdrawl symptoms for a period of time. It is this cycle of gratification after withdrawl in relation to the dose of nicotine, that determines the amount of tobacco consumed by the user to satisfy withdrawl symptoms and the rate of success when attempting to abstain from using tobacco [21,22].

Research has been conducted optimising the extraction of nicotine from tobacco and the quantification of nicotine and its metabolites from blood serum or urine for use in the

detection and determination of tobacco metabolites upon ingestion. Analytical methods employed to conduct these previous quantification's of nicotine using serum and urine were typically High Performance – Liquid Chromatography (HPLC) and Gas Chromatography – Mass Spectrometry (GC-MS). The quantification of nicotine and its metabolites using these methods required multiple extractions over a period of 24 hours into an aqueous phase using high grade expensive solvents before quantification was possible [23,24].

Recent studies have shown that ultrasonic extraction maximises contact between the solvent and solute, due to the high amounts of energy released when ultrasonic waves cause acoustic cavitation and consequential collapse of bubbles that are formed within the extraction vial. GC-MS is a highly sensitive technique that when coupled with ultrasonic extraction, an optimal method for the extraction and quantification of nicotine has been developed. By quantifying and regulating the amount of nicotine made available in tobacco products, there is potential to prevent the user's transition from experimental smoking to addiction [25].

Research into the quantification of nicotine relating to addiction using niche and illicit tobacco focuses on investigations around cigarettes and shisha use, as these are the most commercially alluring types of tobacco. Shisha tobacco is smoked in larger quantities in a short space of time in comparison to cigarette smoking; therefore, the nicotine dose from a one hour water pipe session is significantly higher in comparison to the smoking of a packet of cigarettes over the course of a whole day.

There has been no comparative analysis of a diverse range of tobacco samples within a single study that has allowed for nicotine quantification and comparison. This makes it hard to determine precisely which types of tobacco pose greater risks relating to addiction and adverse health implications.

1.7 Fundamentals of Gas Chromatography – Mass Spectrometry

Gas Chromatography - Mass Spectrometry is a combined technique that uses a Gas Chromatograph to separate volatile substances coupled with Mass Spectrometer, allowing for the determination and quantification of complex organic samples.

1.7.1 Gas Chromatography

Gas-Chromatography is a technique of analyte separation using volatile samples held in the gaseous phase. Samples are typically dissolved or extracted into a solvent and vaporized in order to separate the analytes between the stationary phase and the mobile phase of the gas chromatograph column. The mobile phase is a chemically inert gas (typically nitrogen or hydrogen) which is preheated and filtered with a molecular sieve that carries the analyte through the heated column and has no other interaction or purpose within this technique [26,27].

The samples are typically injected through a sample port at the head of the column using a calibrated micro syringe, delivering typically 1µl of sample into a heated sample port. The sample is then vaporized due to the temperature of the port being held above the lowest boiling point of known volatile compounds within the sample. This port can be split or split less, depending on the quantity of sample needed for detection varying on the type of sample (headspace or liquid) and also on the column utilized, as using a split injector removes excess vaporized samples which are carried off into the waste [26,27].

There are a variety of columns available on the market, each of which is specific to the requirements of the type of samples being analysed, however those most commonly used are typically copper fused silica walled columns. The walls of the column are made by drawing up purified silica, allowing for column widths as small as 0.1mm and lengths as long as 100m. These columns are coated in order to withstand extreme heat, a decrease in the amount of sample quantities needed for analysis and increased

efficiency. The column resides inside an oven which is programmed to a specific heating method, optimizing separation of the samples analytes. This usually begins at a temperature exceeding 100°C and is held before a ramp to the peak temperature, at which point the temperature is held again, allowing the volatiles of different boiling points time to elute from the column over time, optimizing separation and sharpening detected peaks. Upon elution from the column the analytes hit a detector, which provides a quantitative measurement of the components within the mixture [26,27].

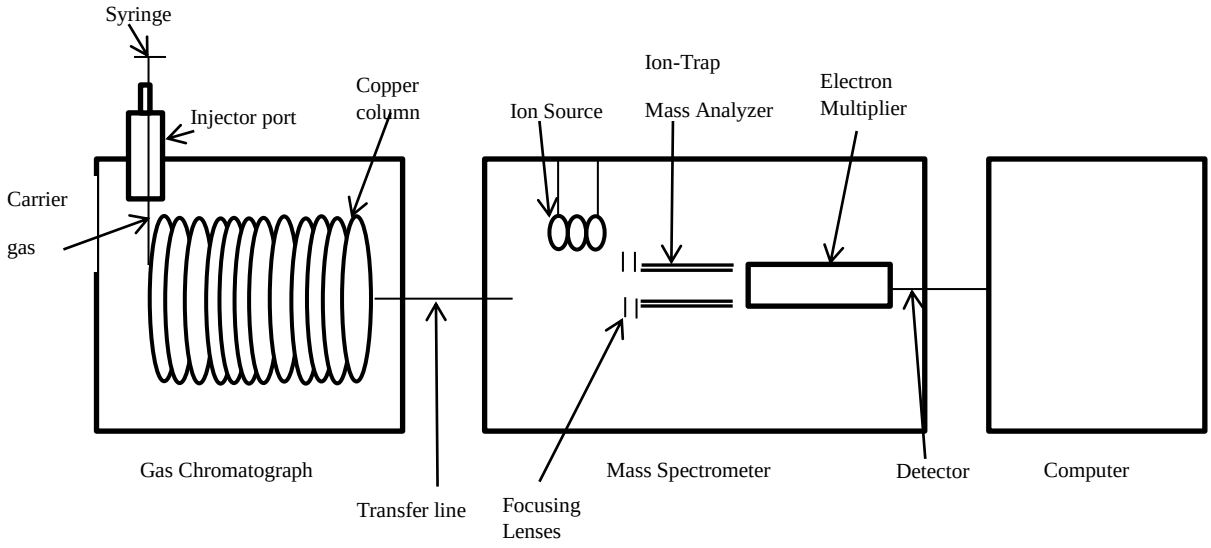


Figure.3 A typical GC-MS with quadrupole mass selector adapted from reference [27]

1.7.2 Mass Spectrometry

Samples that elute from the GC column are transferred directly into a Mass Spectrometer (MS) and compounds are ionized before detection based upon their mass to charge ratio. Upon introduction to the mass spectrometer, the vaporised sample molecules are subject to electron ionization and are bombarded with a high energy electron beam. This eliminates a valence electron from the molecule, forming an ion as denoted in the equation below. Due to the amount of energy lost due to this bombardment, the ion becomes unstable and hastily breaks down, fragmenting into smaller molecules which are positively charged or remain in a neutral state.

$$M + e^- \rightarrow M^{+\bullet} + 2e^-$$

Within the mass selector, neutral fragments are removed using a vacuum and positive cations and any remaining fragmented ions are accelerated towards the detector using a magnetic field which deflects the ions based on their mass to charge ratio, which are then quantified and sorted upon hitting the detector. For GC-MS analysis a quadrupole mass analyser is utilised to filter out non-resonant ions irrelevant to analysis by causing the ions to travel in a spiral determined by the molecular weight of the charged particles, giving added selectivity to the molecules of which a mass ratio can be obtained [28,29].

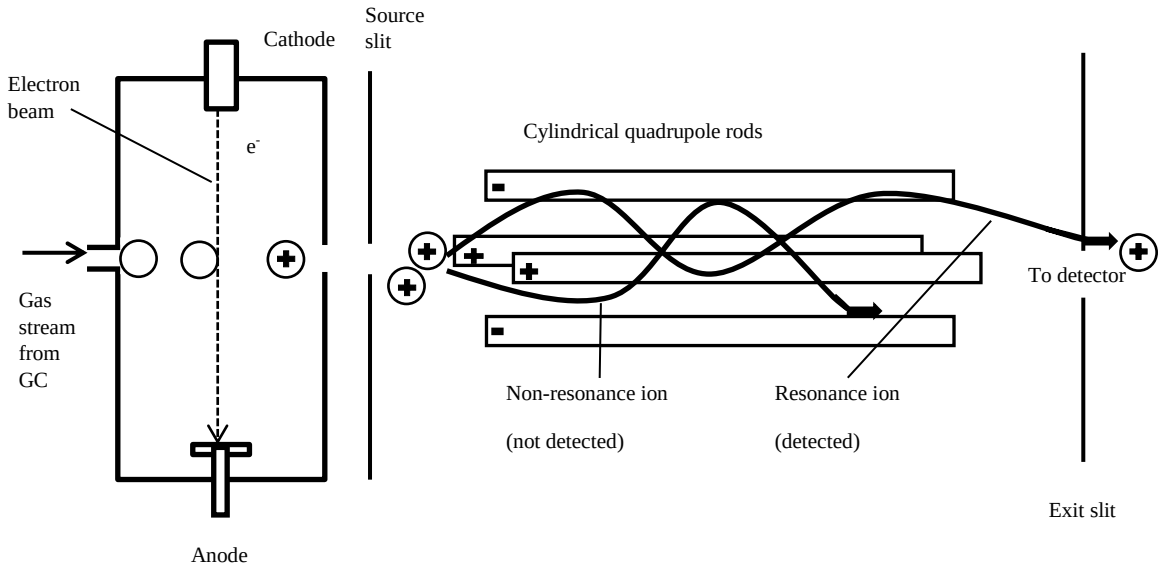


Figure. 4 Showing electron ionization within a Mass Spectrometer with Quadrupole mass selector adapted from reference [29].

1.8 Fundamentals of Infrared Spectroscopy

Infrared Spectroscopy (IR) is a widely used non-destructive analytical technique that allows for rapid determination of functional groups within most organic compounds and organic ions, by the detection of absorbances of light from the Infrared Region (4000-650 cm^{-1}) of the electromagnetic spectrum.

Infrared spectroscopy determines functional groups by measuring the bond vibration frequencies within a molecule, relating specific absorbances to a functional group upon

interaction with electromagnetic radiation. These bond vibrations are caused by the selective absorbance of IR radiation, causing a net change in the dipole moment of covalent bonds. In consequence, the vibrational energy level increases from ground to excited state, allowing the frequency of the absorption peaks to be determined due to the vibrational energy gap [31,32].

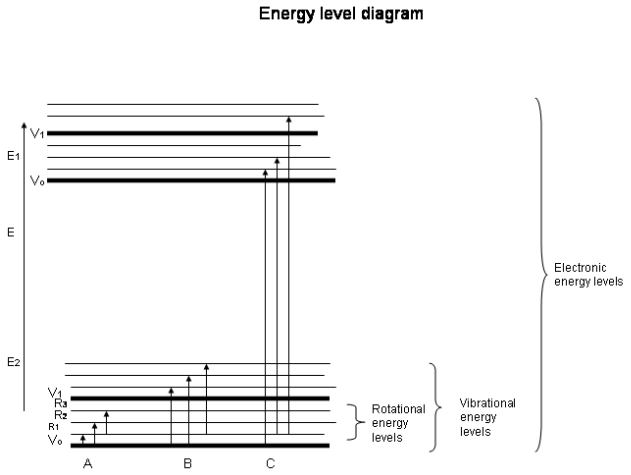


Figure. 5 Energy Level Diagram taken from reference [40].

The number of absorption peaks is determined by the number of degrees of freedom for each molecule. The intensity of each peak is relative to the transition between energy levels from ground to excited state upon change of dipole moment. IR radiation can only affect molecules which have an uneven dipole moment, therefore IR spectroscopy cannot detect diatomic molecules such as O_2. Diatomic molecules have no change in dipole moment due to the charge being shared equally, therefore, there can be no change in the rotation or vibration of these molecules.

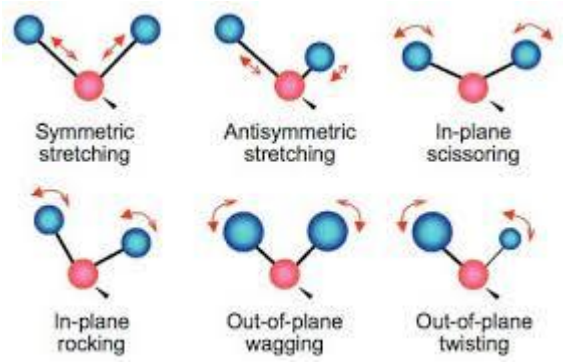


Figure. 6 A diagram of vibrational modes within molecules taken from reference [41].

Previous dispersive IR spectrometers only included a radiation source of infrared light, a monochromator and detector, with the monochromator being used to disperse broad beam spectrums into individual narrow frequencies. The most notable difference between early IR spectrometers and more modern Fourier Transform Infrared Spectrometers is the use of the Michelson interferometer [31,32].

1.8.1 Michelson Interferometer

The Michelson interferometer is made up of two perpendicular mirrors that are used to split beams of light into two so that the beam paths are different. The interferometer recombines the beams as they enter the detector allowing for the difference of intensity to be measured relative to "function paths" [31,32].

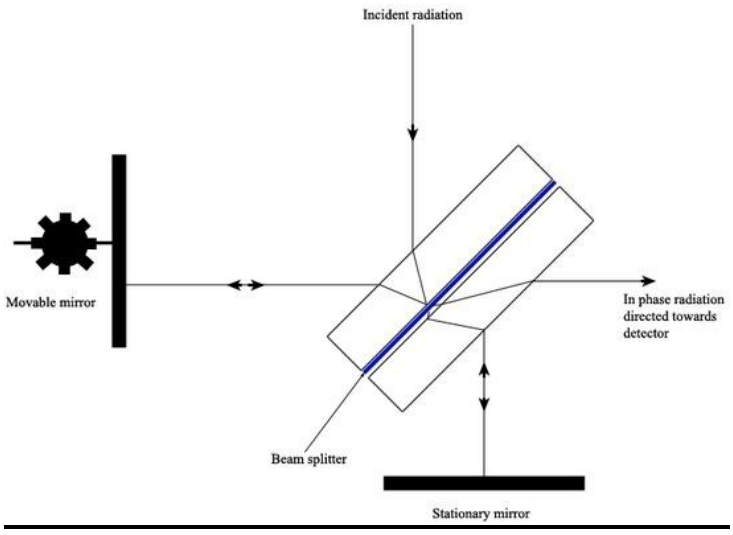


Figure. 7 Michelson Interferometer taken from reference [31].

1.8.2 Fourier Transform Infrared Spectroscopy (FTIR)

A typical FTIR requires a liquid or solid sample to be placed within or upon the sample compartment. The sample is then exposed to the source of broad beam infrared radiation which is split using an interferometer. These split beams take different routes, one through a fixed path that has no interaction with the sample and one that passes through the sample causing the bonds within the molecules to vibrate. The beam interferometer recombines the beams before hitting the detector. The signals that are produced are converted using an analogue to digital converter then extracted to a computer, where the Fourier Transform can be applied, allowing for a spectrum of either absorbance or transmission to be generated [31,32].

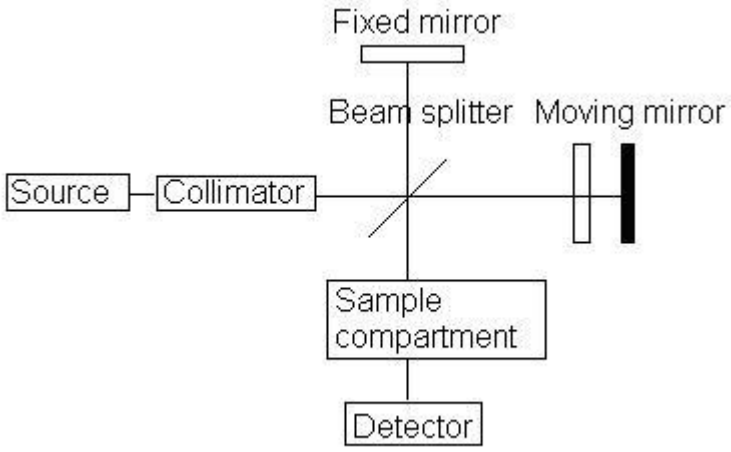


Figure. 8 Fourier Transform Infrared Spectrometer taken from reference [31].

The Fourier transform (below) is a mathematical correction which is applied to the data received from signals that hit the detector, which are converted through the analogue to digital converter before a spectrum is produced.

$$F(\omega) = \int_{-\infty}^{\infty} f(t) e^{-i\omega t} dt$$

$$f(t) = \frac{1}{2\pi} \int_{-\infty}^{\infty} F(\omega) e^{i\omega t} d\omega$$

This equation has had significant impacts on the sensitivity of infrared spectroscopy. The Fourier Transform allows for a wider scan range, higher wavenumber accuracy, higher resolution of peaks and a significantly higher signal to noise ratio which allows for the detection of subtle differences between spectra [31,32].

In some cases it is extremely hard to distinguish between licit and counterfeit tobaccos visually. A rapid, simple method of analysis could potentially establish a platform for the discrimination of licit tobacco from counterfeit products using spectroscopy. This would benefit and support work carried out by trading standards and customs at regional and national level. This could be established by using FTIR to develop a spectral library that discriminates between absorbances within the spectra of constituent compounds within each type of tobacco, this high sensitivity coupled with high discriminatory multivariate data analysis can allow for a comparative spectral analysis.

FTIR spectra is used to gather information about the constituent functional groups and provide a comparative analysis using the highest discriminative wavenumbers within a sample to identify regions of interest rather than detecting the presence of a single marker compound. Previous research using older methods of IR, used the ashed products of pyrolysis from plant foliage and licit tobacco to identify different spectral regions relating to plant constituents. This research was unable to detect a significant portion of minor alkaloids due the infrared wavelengths not spanning a great enough range, giving low resolution of peaks [33].

Due to new advances in the optimization of detection limits and resolution using FTIR, it has been suggested that the detection of minor changes in the alkaloid fractions of tobacco is possible, which has begun to be utilized by the tobacco cultivation industry in order to determine the onset of tobacco disease in the plants within the incubation period. However, it is not possible to definitively isolate a single absorption band and

attribute it to a specific plant constituent, such as chlorophyll or nicotine, when comparing different types of tobacco due to complex mixtures within plant foliage [34]. FTIR has been established an economical discriminatory analytical tool for quality control within the food regulation industry, in place of expensive chemical techniques, ensuring the product conforms to the description and product information as provided by the manufacturer [35]. IR has also been used to discriminate between the two species of coffee bean, *Coffea Arabica* and *Coffe Canephora Robusta* within instant coffee. Arabica beans are valued most highly by the coffee trade, as they produce a finer, highly desirable flavour. In recent years supermarkets have launched investigations into allegations suppliers are using the cheaper Robusta in some products previously thought to contain Arabica. IR analysis was able to identify the chemical origin of the coffee using multivariate data analysis and PCA loadings to discriminate between the two types [36]. This research has established a platform for the spectroscopic determinations of plant origins within the same species, potentially applicable to research on the different species of tobacco plants using FTIR.

1.9 Study Aims and Rationale

The primary aim of this research is to conduct a molecular and elemental profiling of licit, illicit and niche tobacco, where little previous research has been done to analyse a sample set that is representative of tobacco seizures of by trading standards. This investigation will focus on a comparison of licit, illicit and niche tobacco, using samples provided by Lancashire trading standards. The individual techniques to profile the tobacco are stated below:

- An investigation into the nicotine content of the tobacco samples will be carried out using GC-MS as the analytical tool to determine consistency and content of illicit and niche sources when compared against licit tobacco, giving insights into a potential increase in rates of addiction.

- A further investigation to support reproducibility examines the natural variation of nicotine along the leaf, quantifying single strand extractions of tobacco using GC-MS.
- An elemental analysis of licit, illicit and niche tobacco was conducted using XRF as a non-destructive quick and simple multi-element analytical tool which can be utilised to qualitatively determine potentially toxic elements contained within the samples.
- A full spectral analysis of licit, illicit and niche tobacco using ATR-FTIR to identify potential regions of interest and differences between the spectra in an attempt to find identify spectral fingerprints for each type of tobacco.

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Sample Origin

Samples were procured from Lancashire Trading Standards and a variety of licit sources around Preston, the United Kingdom, France and Sweden in November 2013. These samples are only for the use of this research and will be destroyed upon completion as per the contract set out by Lancashire Trading Standards (Appendix 8). The samples that were received varied between illicit, duty free and niche tobacco and were organised into category *via* brand and seizure type (See Appendix 1). Each sample was split into triplicate and ground into a fine powder prior to analysis unless stated otherwise.

Cigarettes

Cigarettes are the most common tobacco product available on the market, made up of flakes of tobacco leaf that have been rolled into a cylindrical shape using a filter and thin filter paper [39].

Miniature Cigars

Miniature cigars are roughly the same size as a cigarette, made using large tobacco flakes rolled in whole tobacco leaf to allowing for a slower rate of pyrolysis [39].

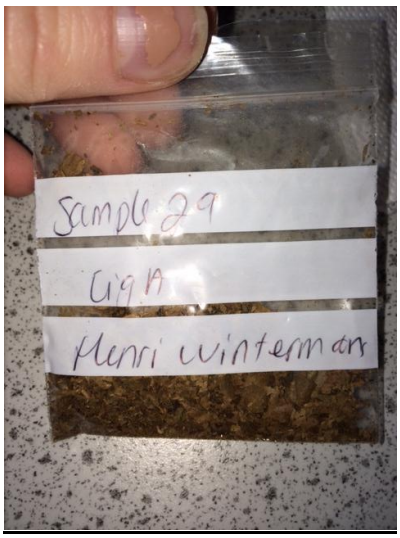


Figure. 9 Miniature Cigar tobacco

Hand Rolled tobacco

Hand rolled tobacco is usually a blend of several types of tobacco, with thin wiry strands that are rolled using filter paper into a cigarette form or alternatively smoked in a pipe [39].

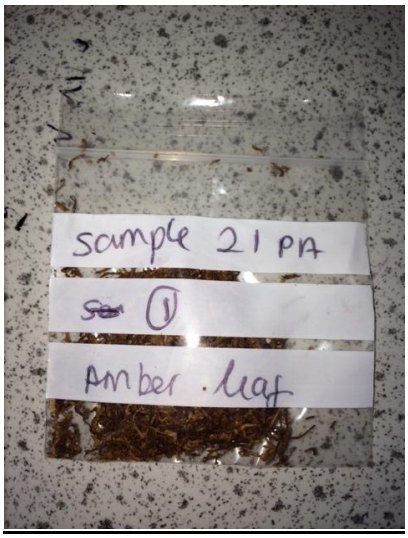


Figure. 10 Hand Rolled Tobacco

Khaini

Khaini has a predominantly male market within India and Pakistan. Consumed socially, the user combines the Khaini by pressing it into the form of a ball, then places it in the oral cavity where it is then held and sucked occasionally for 10-15 minutes. Khaini contains fragments of leaf material, tobacco, slaked limed paste and areca nut [39].

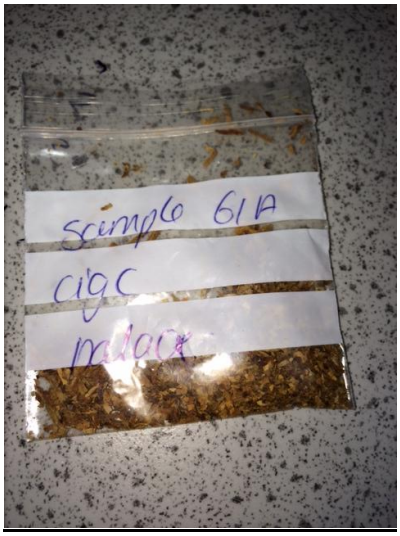


Figure. 11 Khaini tobacco

Gutkha

India and Pakistan are the main retailers and consumers of Gutkha products, with a target market of young men and boys. Gutkha is sucked, spat or chewed and typically contains betel nut, catechu, tobacco, lime, saffron and additional flavouring agents specific to the brand [39].

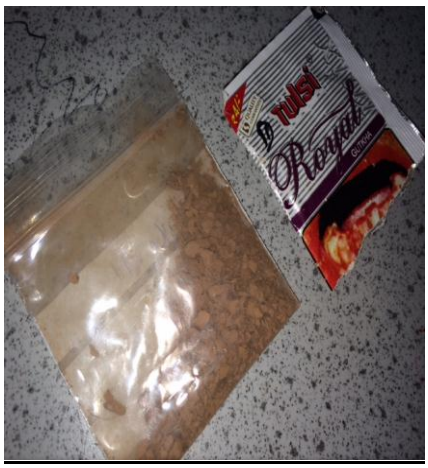


Figure.12 Gutkha tobacco

Snuff

Fire cured tobacco, more commonly known as snuff, is found in a dry powdered form with less than 10% moisture content which is then either sniffed or held in the oral cavity. Snuff is a product typically found within the U.K, USA, India and Sweden [39].

Figure.13 Snuff tobacco

Snüs

Predominantly produced in Sweden on a large scale, Snüs is found in either loose or pouched form with a typical portion being between 0.5g-1.0g. The pouch or loose tobacco is held in between the wet membrane of the gum and cheek to allow for rapid absorption of constituents into the blood stream. The tobacco is finely ground, dried and mixed with aromatic substances, salts, humidifying agents such as Sodium Chloride or Sodium Carbonate, additional nicotine and water [39].

Figure. 14 Snüs tobacco

Shisha

Shisha or Water pipe tobacco, the composition of which varies and is typically found to have thick almost bark like fragments of tobacco leaf mixed with artificial flavourings. Additional nicotine and aromatic compounds are present resulting in a sticky oily residue that is used to give a specific desired fragrance to the tobacco. Shisha tobacco is marketed based on flavour/fragrance and is typically produced in North African countries, Eastern Europe and Southern Asia [39].

Figure. 15 Shisha tobacco

2.2 X-Ray Fluorescence Materials and Methods

2.2.1 Materials

Ethanol (99% Sigma Aldrich) was used to clean the vessels before and after sampling. Mylar X-ray grade film was used to line the XRF sample vessels before 0.25g of tobacco sample was added. The Mylar film within each vessel was replaced between each sample. The sample vessel was then placed on the platform and using a handheld initiation button the X-rays were turned on, with a real time display of results feeding through to the computer, where the spectra was displayed and peak picked using the K line only.

Each sample was ran in triplicate and was ran at 25Kv, 35µA and then 40 Kv, 15µA.

2.2.2 Apparatus

The equipment used was Bruker Handheld XRF Tracer IV-SD.

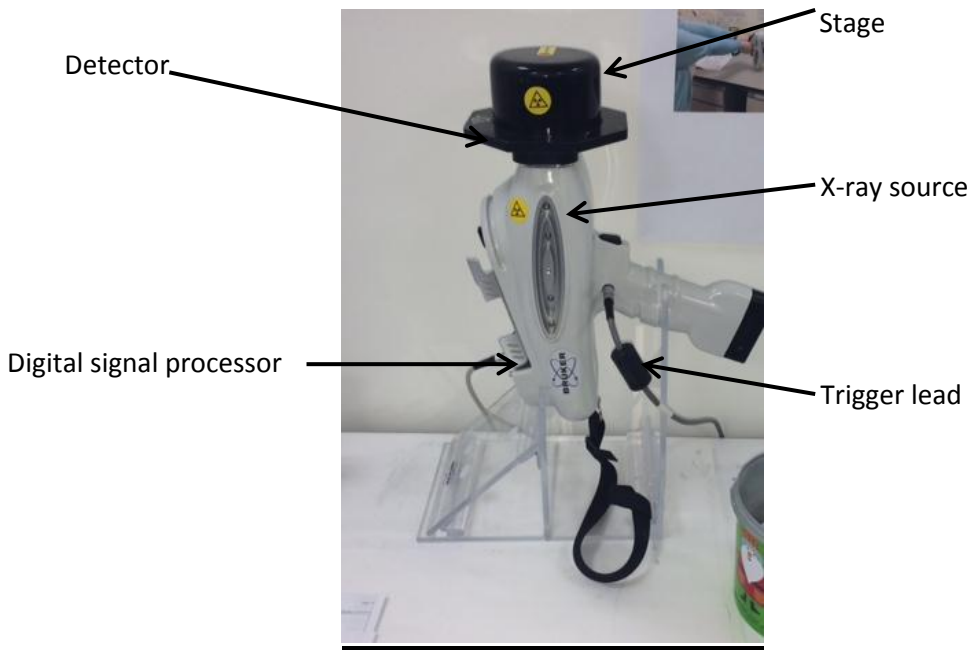


Figure. 16 Bruker Handheld XRF Tracer IV-SD

2.3 Gas Chromatography Mass Spectrometry

2.3.1 Apparatus

GC-MS measurements were carried out using a Thermo Scientific TriPlus TRH auto sampler alongside a Thermo Scientific Trace 1300 Gas Chromatograph equipped with a split/split less injector, coupled with a Thermo Scientific ITQ1700 Mass Spectrometer. The column utilised for analysis was a Thermo TG-SQC 30M, 0.25mm Internal Diameter, 0.25µl Silica packed column.

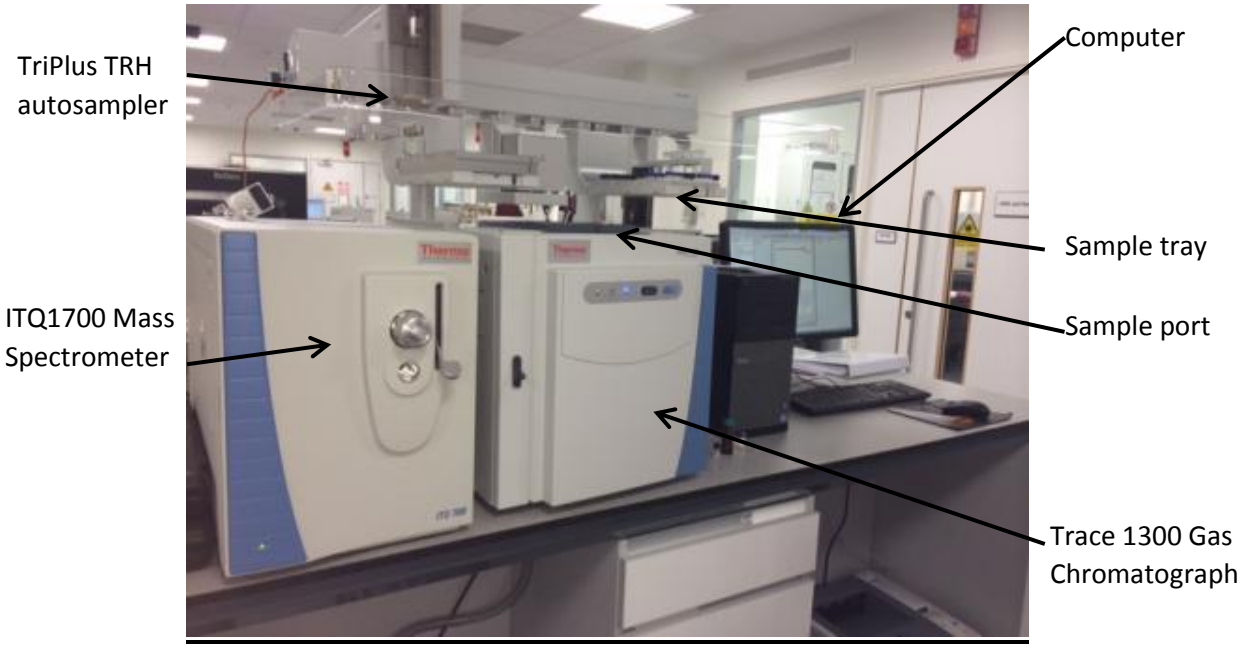


Figure. 17 Thermo Scientific GC-MS

2.3.2 Materials and Reagents

Analytical grade reagents were used unless stated otherwise. Nicotine at 99% purity (Fishers) was used as the standard, diluted with 98% methanol (Sigma Aldrich). Di-Ethyl ether 99% purity (Sigma Aldrich), 98% *n*-heptadecane (Fishers) and 5% Sodium hydroxide (made up using 99% solid sodium hydroxide pellets (Sigma Aldrich) and e-pure distilled water) utilised for the extraction of nicotine from tobacco.

2.3.3 Method of extraction

Each sample of tobacco was ground down to a fine powder and 0.25g of each sample weighed into a glass vial along with 5ml di-ethyl ether, 2.5ml 5% sodium hydroxide and 25µl of *n*-heptadecane (used as internal standard solution). After 2x15 minutes of ultra-

sonication with a standing time of 15 minutes before each round, the two layers were separated and the ether layer transferred into a vial. One microliter (µl) of the ether layer was injected into the GC-MS to be analysed in triplicate [25].

2.3.4 GC-MS Methods

Initial injection of samples used 1µl of the extracted ether layer into a split less injector with a constant carrier gas flow of nitrogen. The oven method began with an initial temperature of 120°C that was held for 10 minutes, before ramping to 350°C which was then held for 5 minutes, before reduction to 250°C over the space of 4 minutes.

2.3.5 Single strand extraction method

Each sample consisted of one whole tobacco flake, chopped into three equal sections which were then placed into individual small glass vials along with 0.5ml di-ethyl ether, 0.25ml 5% sodium hydroxide solution and 2.5µl of *n*-heptadecane. After 2x15 minutes of ultra-sonication with a standing time of 15 minutes before each round, the two layers were separated and one microliter of ether layer was injected into the GC-MS to be analysed in triplicate using the same GC-MS oven method as the bulk tobacco analysis [25].

2.3.6 Dilution Standards

Dilution standards were produced as in Table 1. to the following concentrations of Nicotine in 10ml of Methanol, using Nicotine at 99% and Methanol at 98% purity.

Table. 1 Dilution Standards of Nicotine

Concentration of Nicotine (ppm)	Amount of nicotine present in standard (µl)	Amount of methanol present in standard (µl)
1.218	0.01218	0.99999879
2.437	0.02437	0.99999757
4.875	0.04875	0.99999513
9.75	0.0975	0.99999025
19.5	0.195	0.9999805
39	0.39	0.999961
78	0.78	0.999922
156	1.56	0.999844
312	3.125	0.9996875
625	6.25	0.999375
1,250	12.5	0.99875
2,500	25	0.9975
5,000	50	0.995
10,000	100	0.99

2.3.7 Calibration

Calibration was performed by plotting the ratio of the average peak area for the standard dilutions of nicotine which were run in triplicate. This ratio is then used to obtain the concentration of nicotine from a calibration curve using a linear trend plot of

$y = mx + c$.

2.4 ATR-FTIR Materials & Methods

2.4.1 Method

A sample of tobacco was placed directly onto the ATR and crushed beneath the clamp to maximise contact. Before each sample the ATR stage was cleaned using Ethanol (99% Sigma Aldrich) ensuring the crystal was free from previous tobacco sample deposits. Samples were divided into three individual sample bags per tobacco, with three spectra collected from each single sample placed on the ATR. A background was ran in between the changing of samples with the ATR clamp open to establish that the FTIR was uncontaminated and in good working condition.

2.4.2 Apparatus

The equipment used was a Specac Golden Gate ATR and a Jasco FT/IR 410 recording absorbances between 650-4000cm- 1 with a resolution of 4cm^{-1}, 1 spectrum represents 64 added scans.

Figure.18 Jasco ATR-FTIR

2.4.3 Data Pre-processing and Multivariate Data Analysis

Pre-processing and multivariate data analysis was carried out on the raw data using MATLAB version 7.11.0 (R2010b) (The MathWorks, Inc., USA) using in house written software and the Spectroscopy Toolbox (University of Central Lancashire, University of Birmingham). Pre-processing of data was kept to a minimum to increase reproducibility and spectra was subjected to visual quality tests to ensure spectra was adequate before inclusion in data analysis.

Groups of data were compared to that of licit cigarettes as it is assumed that licit cigarettes will have higher levels of consistency between samples due to being from a controlled cultivation plant source. Licit hand rolled tobacco was only used for comparison against other hand rolled tobacco, as hand rolled tobacco typically contains 'blends' of different tobacco plants [38].

Vector normalisation was applied to all the grouped raw data using the Spectroscopy Toolbox. Samples of plant material, including tobacco, are naturally variable and incredibly sensitive to environmental factors, therefore vector normalization is applied to mathematically account for inconsistencies caused by natural variance [38].

Variable ranking was applied using the spectroscopy toolbox allowing for the retention of the top 30% highest discriminatory wavenumbers. From this 30%, the top 30 wavenumbers listed were used to determine functional groups with the most significant differences between the spectra, identifying a spectral fingerprint for each type of tobacco.

Chapter 3

X-Ray Fluorescence Results and Discussion

3 X-Ray Fluorescence Results and Discussion

This chapter allows for the elemental profiling of licit, illicit and niche tobacco using X-Ray Fluorescence (XRF) as a multi-element analytical tool. Results from this chapter have been accepted and presented as poster presentations at Sci X 2014, Reno and ASH Wales 2014, Cardiff (Appendices 3, 4, 5). XRF is typically utilized as a qualitative analytical technique for the detection of elements between Na and U. XRF is able to be utilized quantitatively using counts from the region of interest and a calibration spread sheet developed by the manufacturer specific to each machine. Quantitative analysis was not conducted for this analysis as the calibration spread sheet specific to the machine used was unavailable.

3.1 Optimization of Spectra

An initial sample optimization investigation was carried out using one sample from each of the different types of tobacco. The optimization investigation was carried out to identify the best voltage and filter combination that provided the best resolution of peaks, as well as to determine which of the transitions between electron shells would be best to pick the peaks.

It was found that when compared against spectra using 25Kv (Fig. 19), the spectra produced at 40Kv (Fig. 20) had better peak resolution and less noise, allowing for the detection of elements in low concentrations such as Ni, Cu and Zn which were previously considered to be background noise. When picking the peaks, it was determined that the alignments of the K line transitions were the best fit when picking the peaks. Although 40Kv allowed for better peak resolution of elements that were not

previously visible in the spectra, the higher increased the chance of seeing Sum Peaks, Escape peaks and Compton Scatter within the spectra.

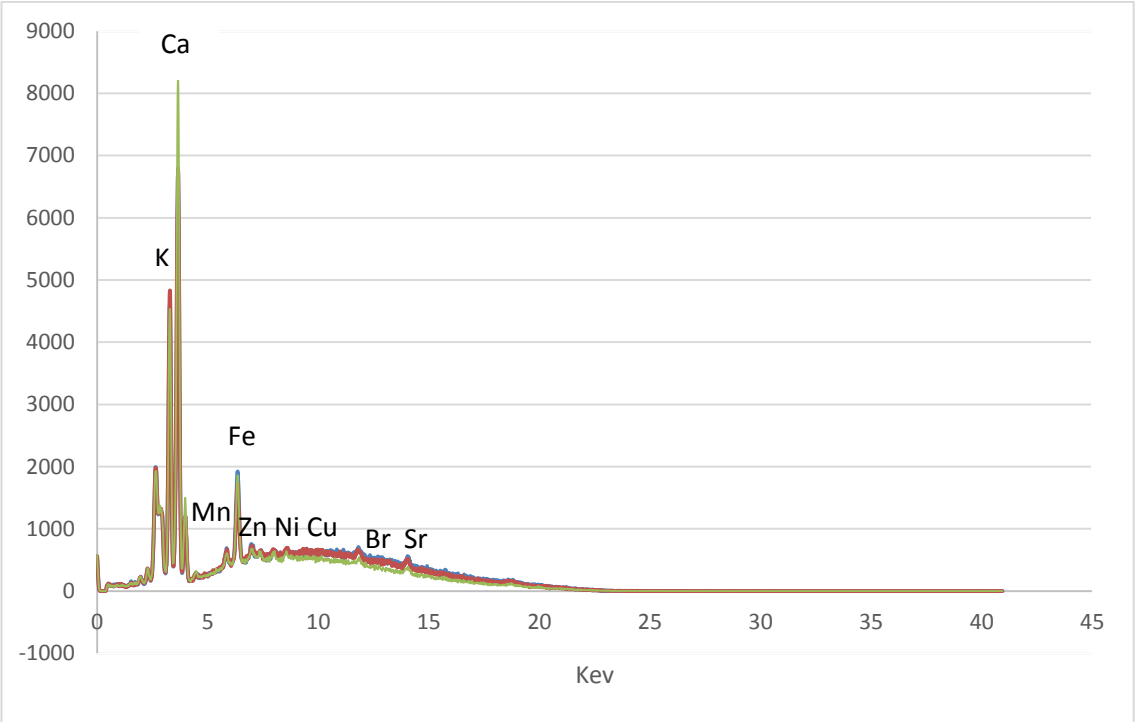


Figure. 19 Shows results for hand rolled Golden Virginia at 25Kv

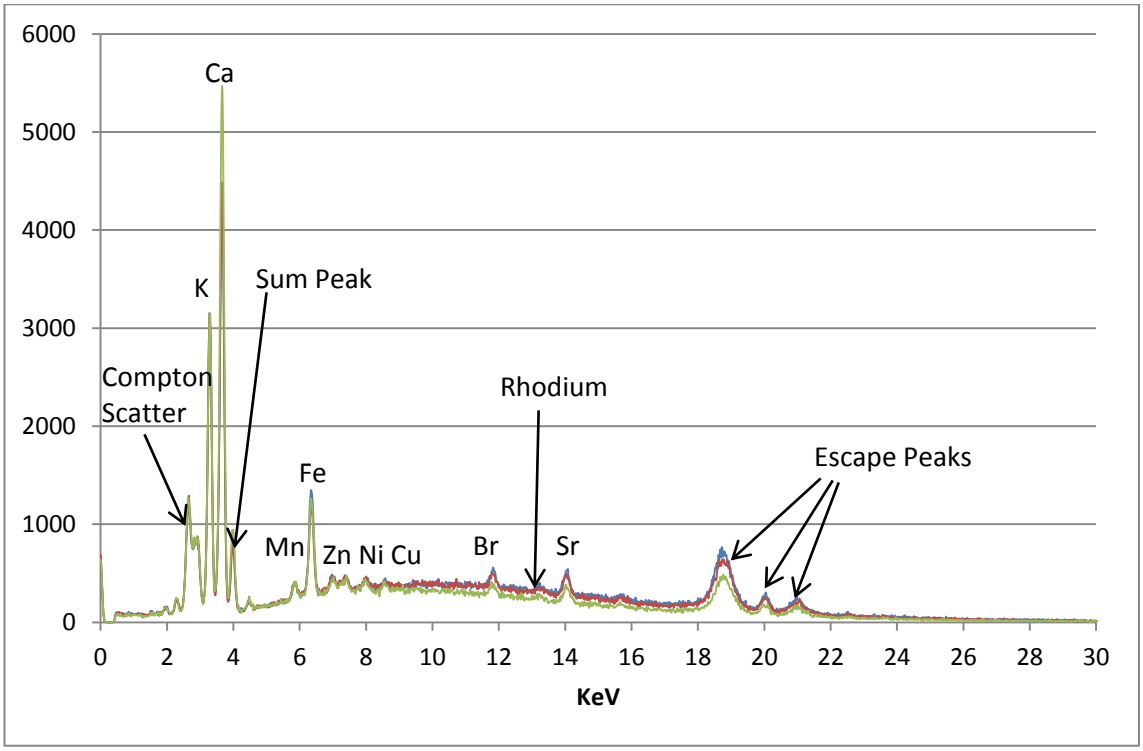


Figure. 20 Shows results for hand rolled licit Golden Virginia at 40Kv.

Sum peaks are attributed to the detection of two or more pulses as one within spectra, seen to the right of the Ca peak and is labelled above. X-rays from the Rhodium X-ray tube strike the sample promoting fluorescence within the spectra that shows a peak which is actually not present, in the case of this research the Cl peak is Compton Scatter unless stated as otherwise. Escape peaks, typically seen as three broad peaks, which are the last significant peaks on the right hand side of the spectra. As X-rays strike the sample promoting fluorescence, some of the Si fluorescence from the detector escapes and causes these peaks. These peaks appear consistently throughout the spectra and are ignored throughout the conclusions unless stated as otherwise.

Spectra were grouped into the following; Licit Cigarettes, Licit Hand Rolled, Licit miniature cigars, Duty Free cigarettes, Duty Free hand rolled, Illicit counterfeit cigarettes, Illicit 'Cheap Whites' Cigarettes, Illicit Counterfeit hand rolled, Snüs, Gutkha, Khaini, Shisha and Snuff. Elements typically associated with soil attributions such as K, Ca, Mn, Fe, Ni, Cu, Zn and Br were consistent throughout the results.

3.2 Licit, Illicit Counterfeit, Illicit 'Cheap Whites' and Duty Free Cigarettes

Levels of K, Ca and Fe fluctuated between the different types of samples (Fig. 21, 22, 23, 24), and within illicit cigarettes peaks were more prominent. The illicit 'Cheap White' cigarettes appeared to have higher, clearer defined levels of elements than the other cigarettes, although there was less consistency between the samples.

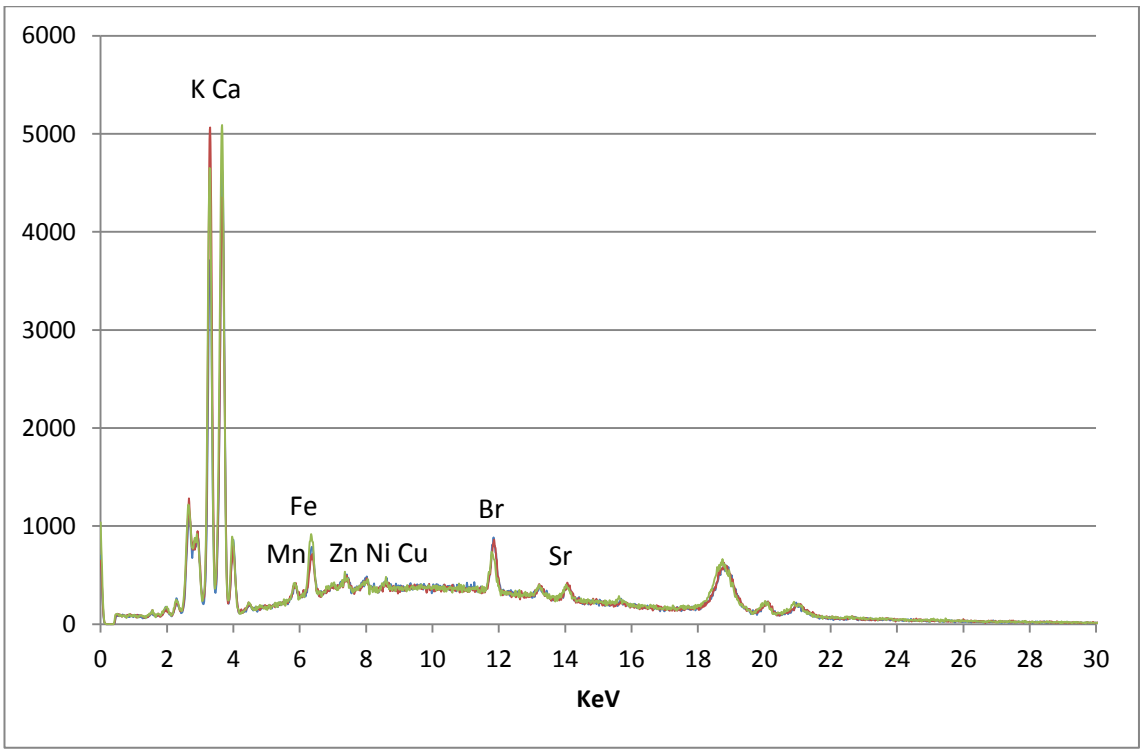


Fig. 21 Park Drive 40kv, Licit Cigarette

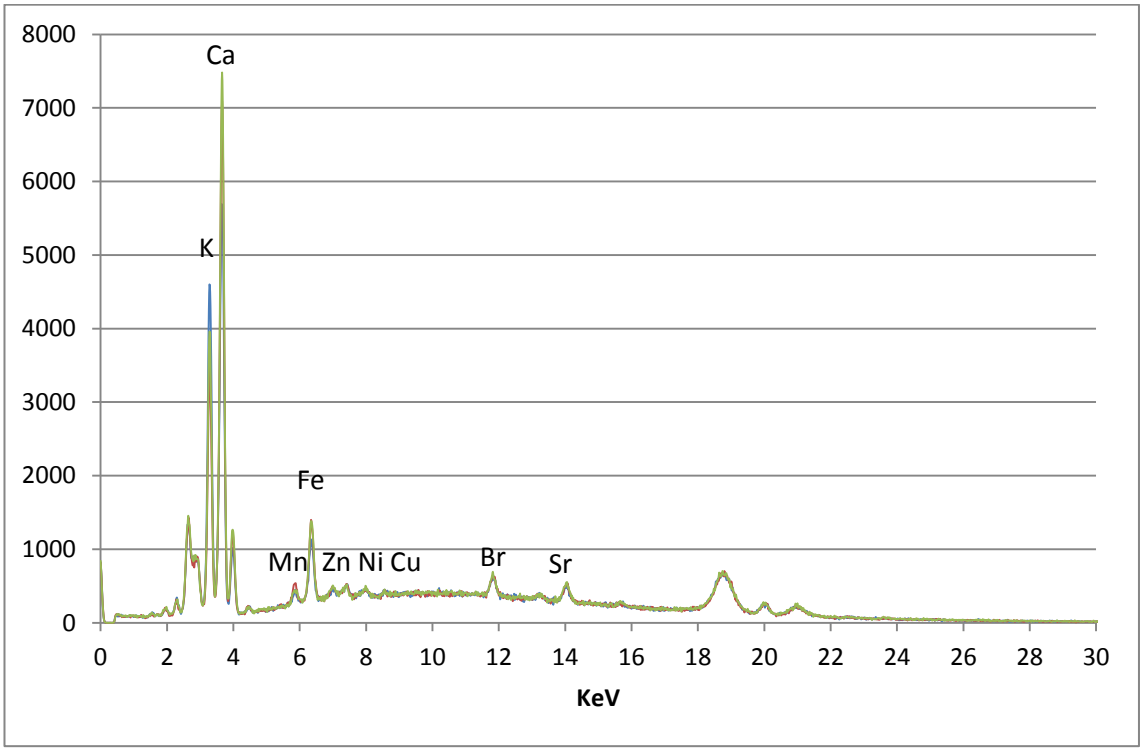


Figure. 22 Richmond, Illicit Counterfeit

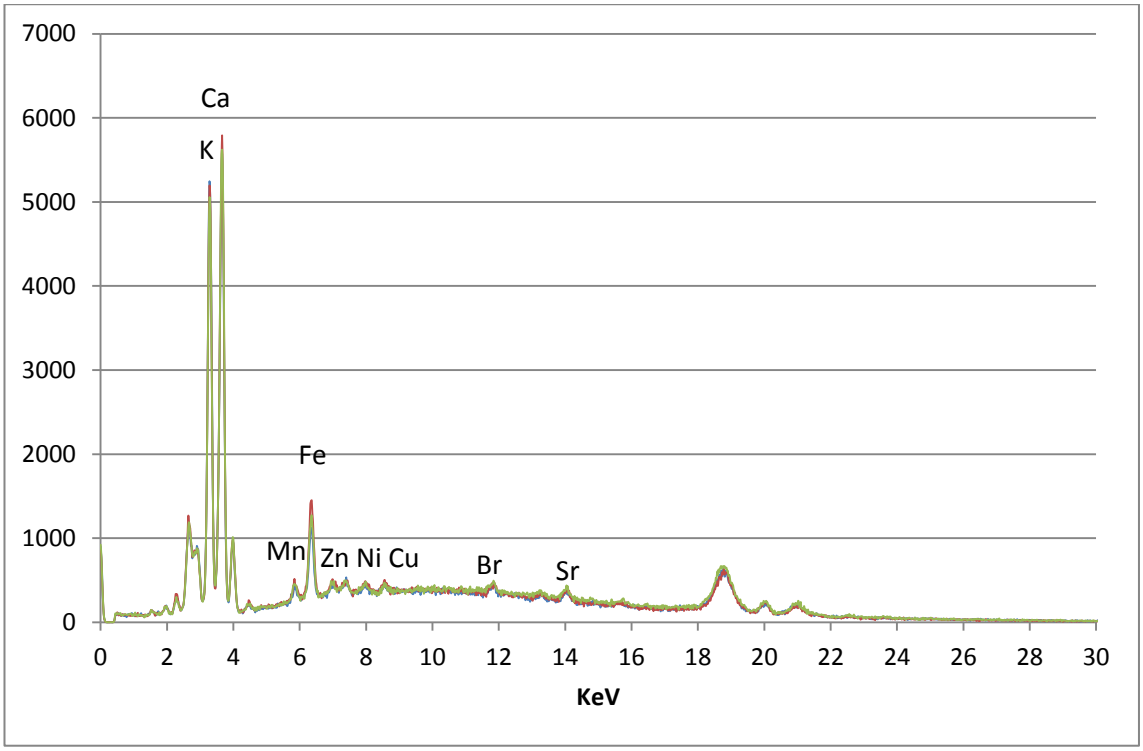


Figure. 23 Marlboro, Illicit Counterfeit Cigarette

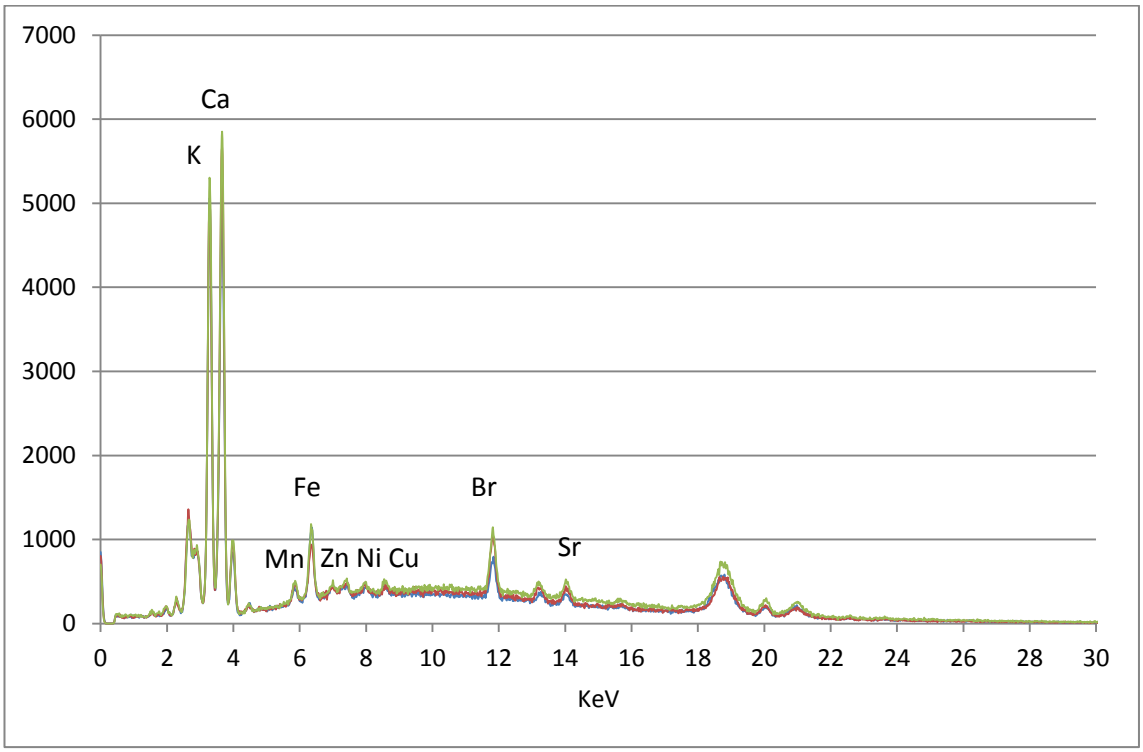


Figure. 24 Jin Ling, Illicit Cheap Whites Cigarettes

3.3 Licit, Illicit Counterfeit, Duty Free Hand rolled

There is little difference between the any of the types of hand rolled tobacco in comparison to the cigarette tobacco as above, other than the slight increase in Fe within the counterfeit hand rolled tobacco (Fig. 25, 26, 27).

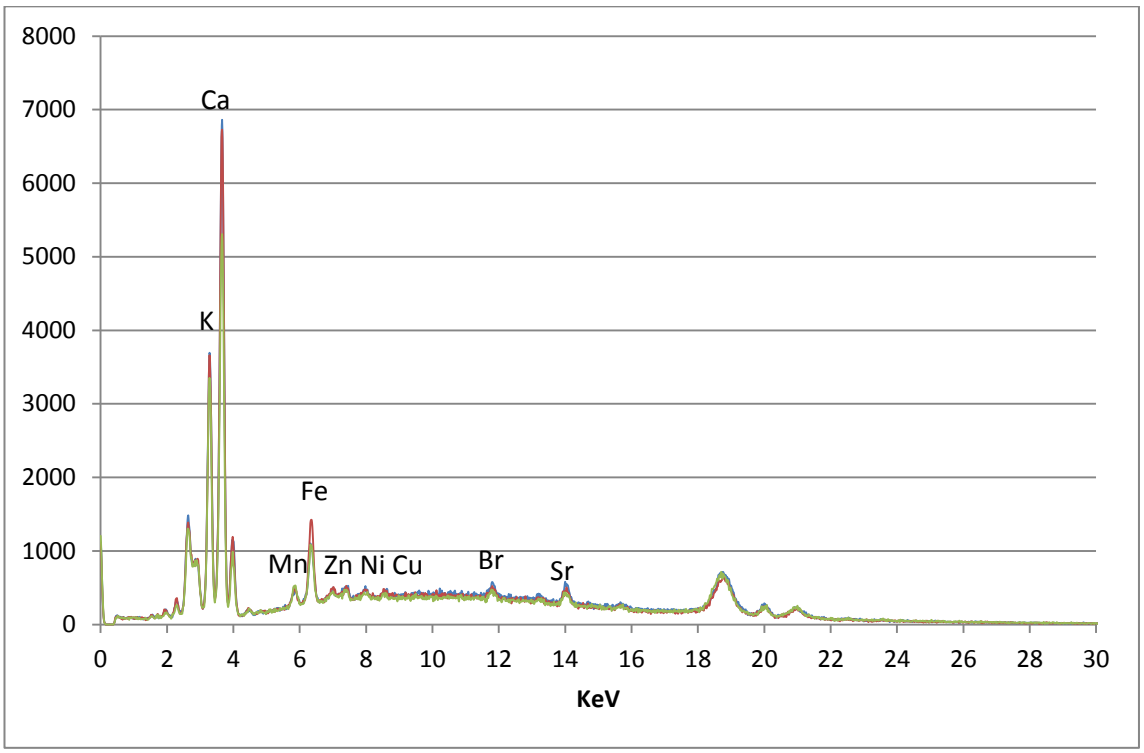


Figure. 25 Golden Virginia, Licit

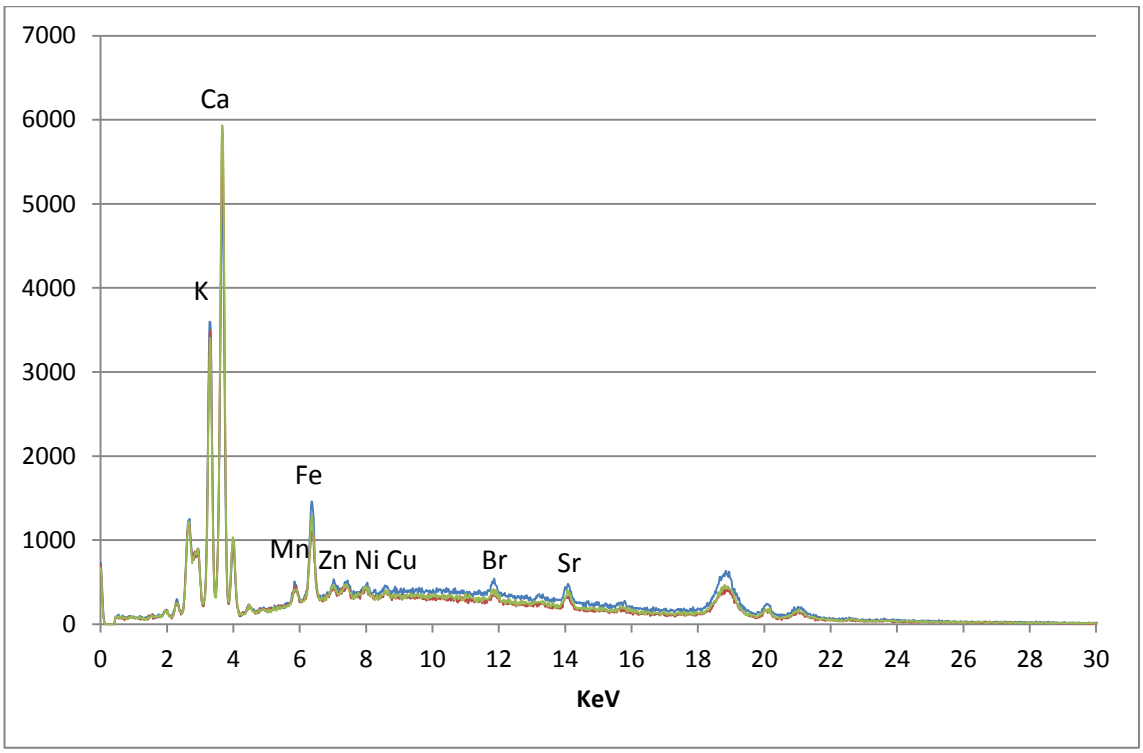


Figure. 26 Golden Virginia, Duty Free

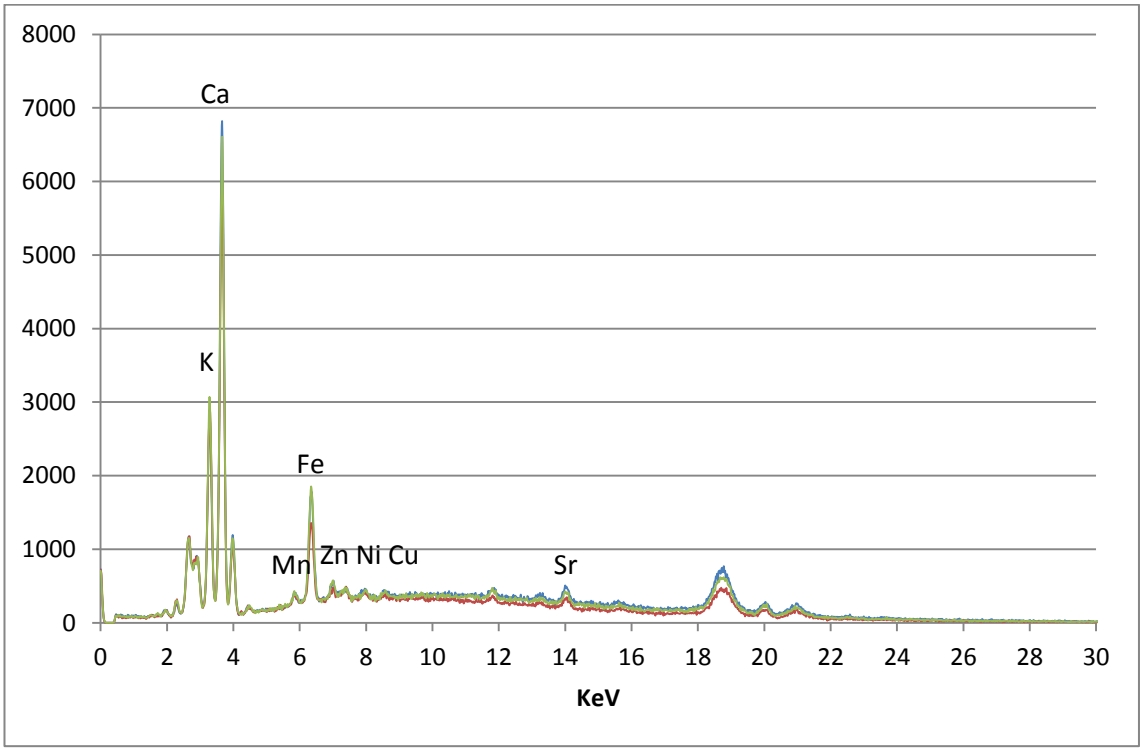


Figure. 27 Golden Virginia, Counterfeit

3.4 Miniature Cigars

The spectra of miniature cigars varies very little when compared to that of cigarettes and hand rolled tobacco, apart from the significant increase in K and Ca (Fig. 28).

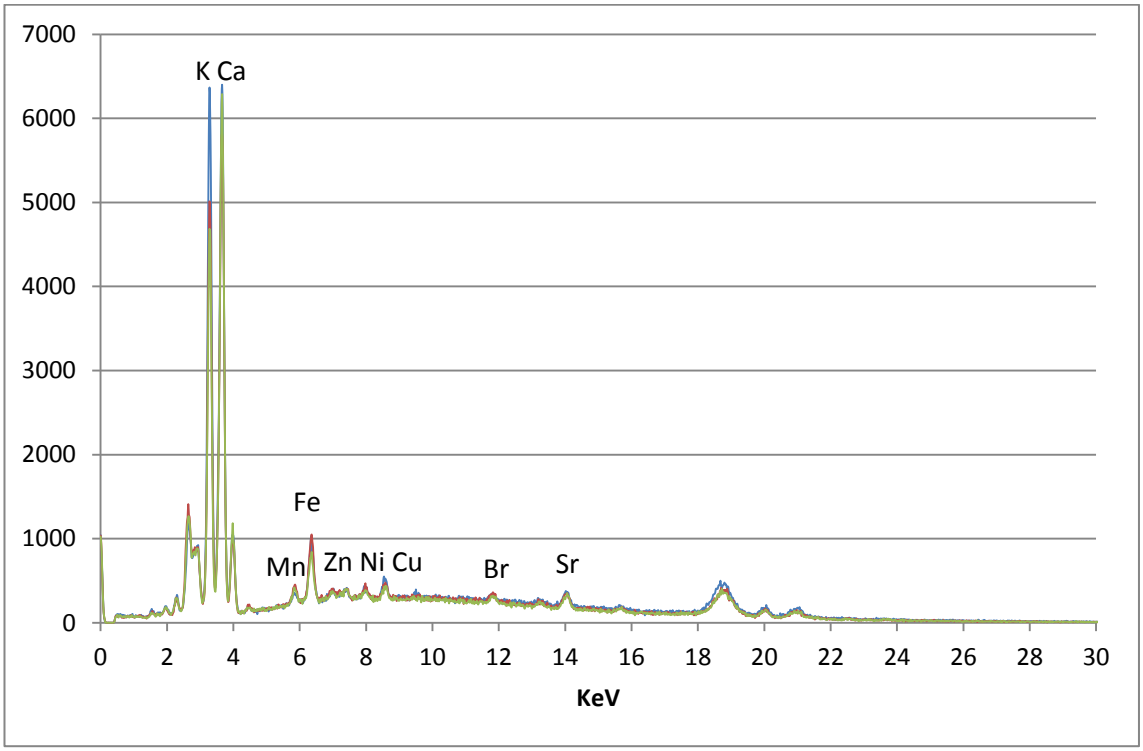


Figure. 28 Castella Miniatures, Licit Cigars

3.5 Niche tobacco

Niche tobacco samples have very little research conducted around their elemental profiles, however, it would not be expected to find a concentrated amount of any naturally occurring element attributed to soil depositions due to these being lost throughout the treatments and blends the tobacco is subject to during production.

Gutkha and Khaini show very similar low background spectra, with only significant peaks of Ca and Fe (Fig. 29, 30).

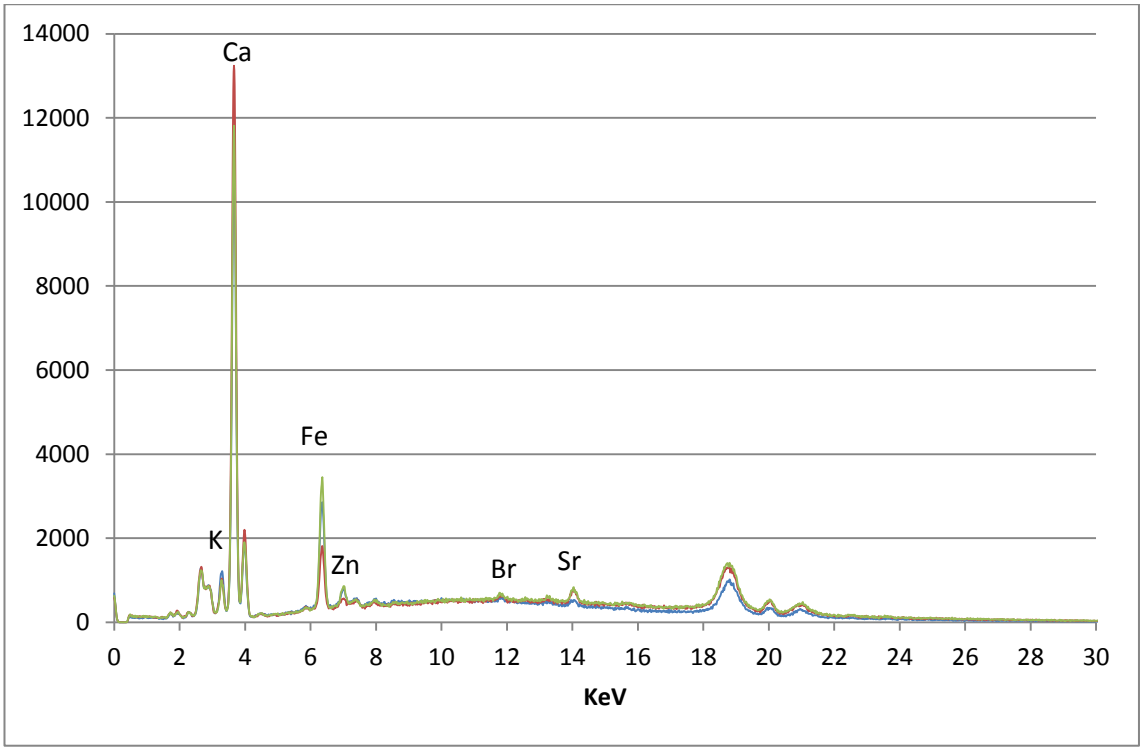


Figure. 29 Tulsi, Gutkha

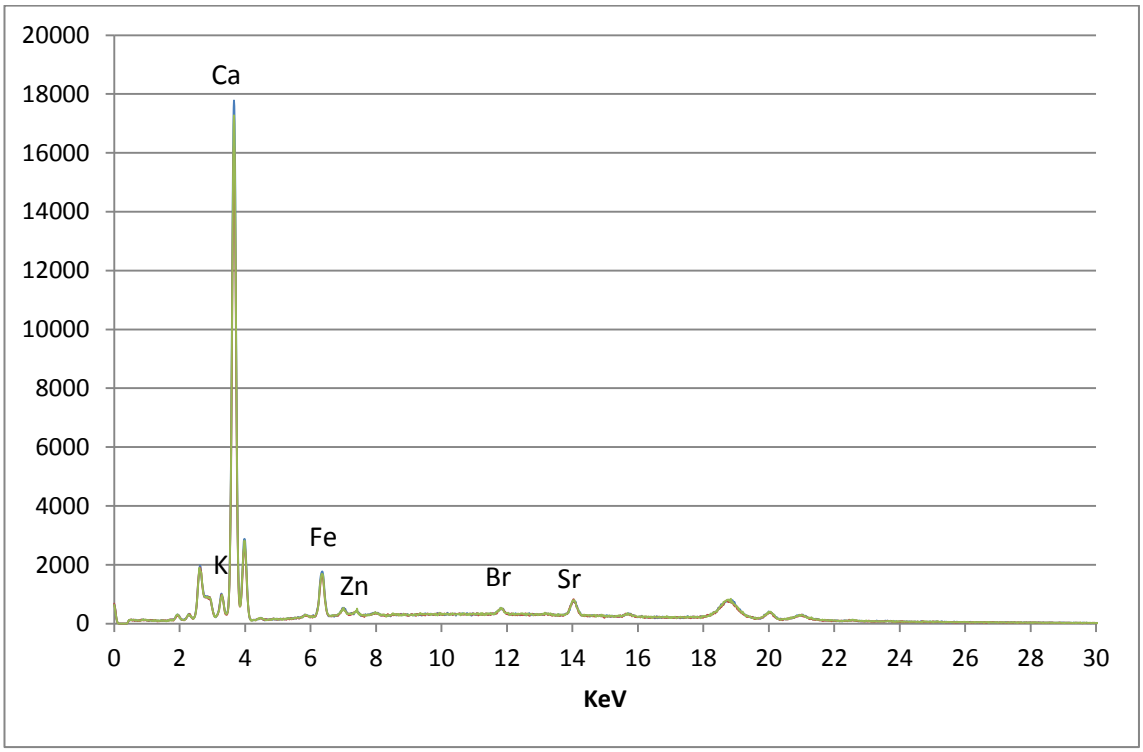


Figure. 30 Miraj, Khaini

Both Snuff samples had similar spectra to Gutkha and Khaini, which would be expected as they are similar in composition (Fig.31).

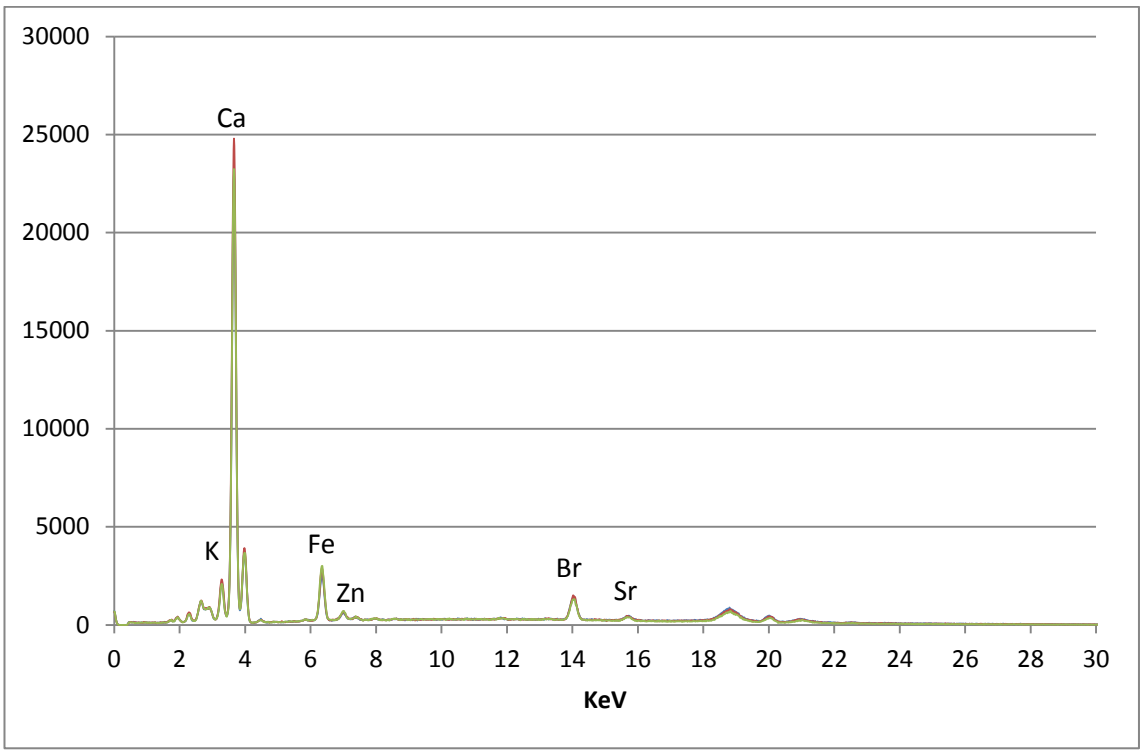


Figure. 31 Black Snuff, Snuff

Samples of Shisha all gave similar spectra with poor determination of elements (Fig. 32). These samples appeared to have high levels of background and an increased susceptibility to escape peaks, compton scatter and sum peaks which can possibly be attributed to the oily state of samples upon analysis.

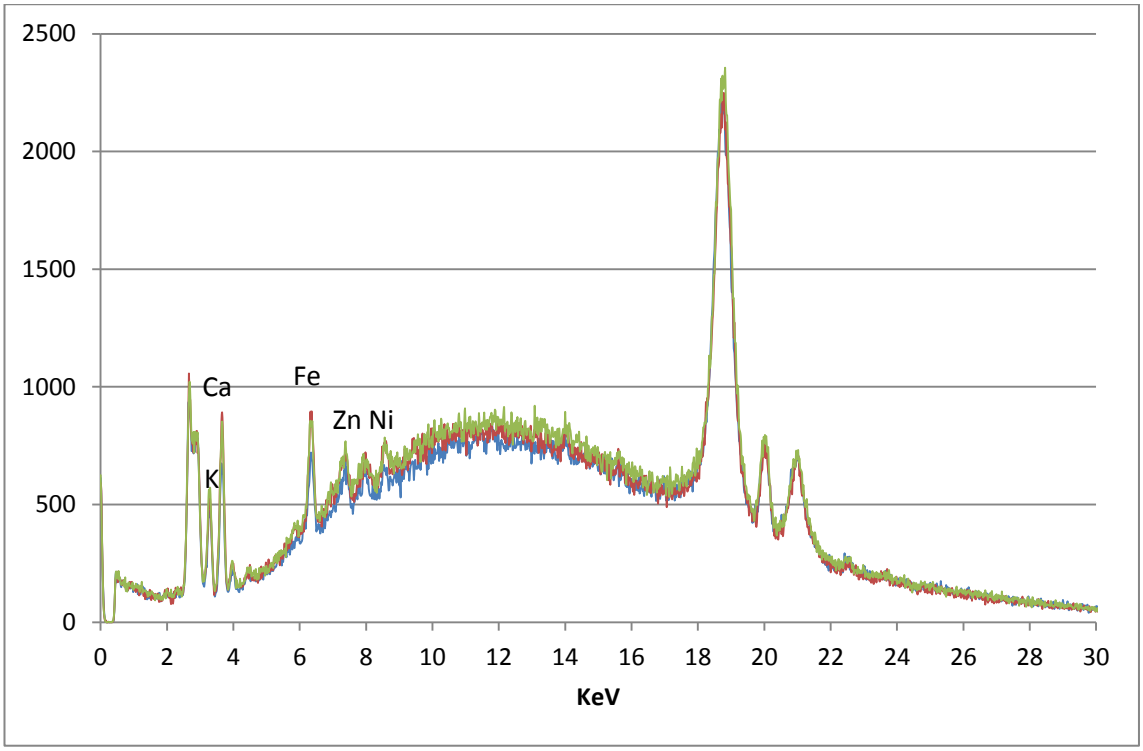


Figure. 32 Marharba Apple, Shisha

All samples of snüs were found to have significant Cl peaks in comparison to the other groupings of tobacco contained heightened element spectra similar to that of cheap whites (Fig. 33). Previous Cl peaks seen were attributed to fluorescence caused by the Rhodium X-ray tube. As with previous Shisha spectra (Fig. 32). the snüs samples were moist and had noticeable escape peaks and higher background levels in comparison to other types of dry tobacco.

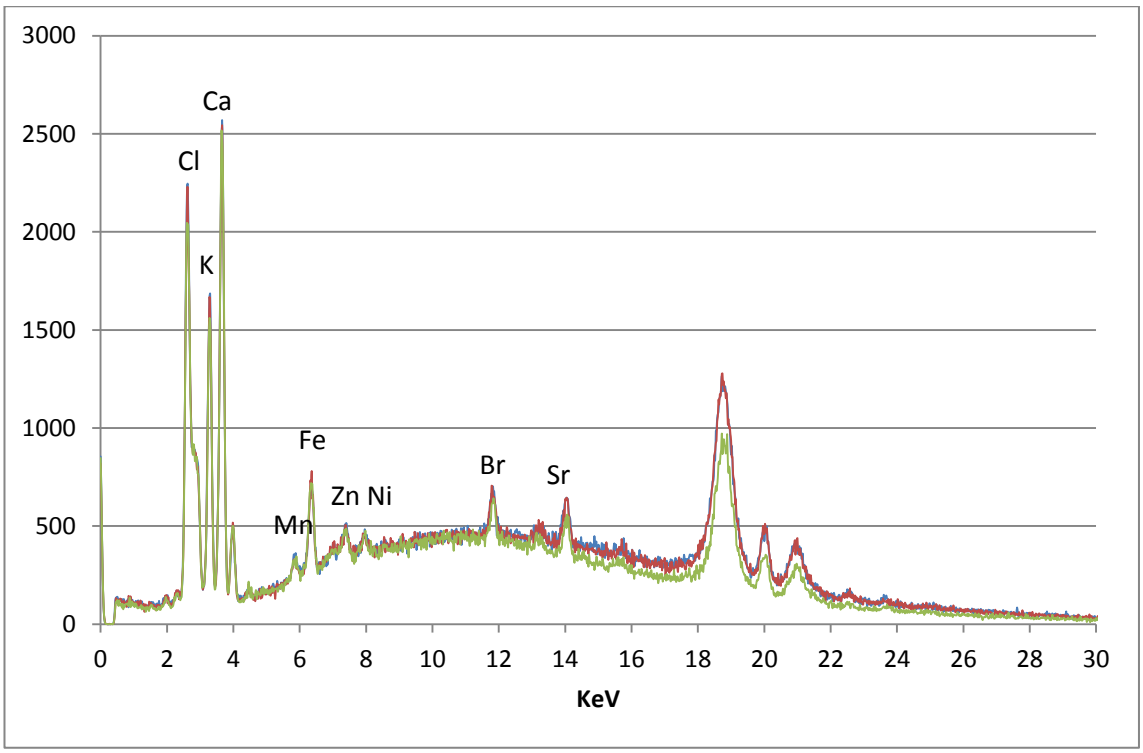


Figure. 33 Lossnüs Grov, Snüs

3.6 Conclusions

Samples were expected to contain elements typically associated with soil attributions such as K, Ca, Fe, and Br which were consistent throughout the results. Levels of Ni, Cu and Zn in comparison were noted in lower amounts within the cigarettes, hand rolled tobacco, cigars and snüs. There was no obvious qualitative indicator of the differences between licit and illicit tobacco other than slight fluctuations in K, Ca and Fe. Niche tobacco, specifically the shisha and snüs tobacco, which were moist upon analysis, provided the most distinguishable spectra from other tobaccos. Gutkha, Khaini and snuff tobaccos all had similar low background spectra, making it easy to determine peak identification. The shisha spectra had high background, potentially due to the oily nature of the tobacco, making it open to spectral problems such as larger escape, sum and compton peaks. The snüs tobacco, similar to shisha in that it has a higher background levels and heightened escape peaks, showed a significant chlorine peak, thought to be introduced to the tobacco throughout preparation. In previous spectra the

peak is attributed to the rhodium of the X-ray tube scattering causing a peak to appear. It is of concern that levels of toxic metals are not fully detectable using XRF as a quick easy method of detecting elemental trends within the tobaccos, however, this technique readily identifies differences between treated, and dried leaf tobaccos.

3.7 Future Work

As previously stated, many of the harmful trace elements found in tobacco that tobacco plants are susceptible to accumulating i.e. Lead, Cadmium etc. are found in levels below the detection limits of XRF. In future work, ICP-MS should be fully utilized as a tool for the determination and quantification of trace elements. ICP-MS is not able to detect or quantify elements within the halide series of the periodic table, therefore levels of Bromine and Chlorine need to be quantified using another analytical method sensitive to halides such as EDAX-SEM. Qualitatively XRF analysis is not comprehensive enough to determine the levels of elements needed for full elemental profiling of tobacco, however XRF could qualitatively discriminate between trace elemental patterns within different food groups, identifying origin and trade routes using a non-destructive analytical technique.

Chapter 4

Gas Chromatography – Mass Spectrometry Results and Discussion

4 GC-MS Results and Discussion

4.1 GC-MS Results Introduction

Packets of licit tobacco have to state nicotine content levels to meet UK trading standards requirements, with the average licit cigarette stated to contain 0.9mg per 1.0g cigarette tobacco. This research used 0.25g of tobacco per extraction therefore we expect to see values around 0.225mg for the nicotine content of licit tobacco. Anything over this value will be deemed as a high nicotine content. This investigation into the quantification of nicotine between licit, illicit and niche tobacco is to determine the following; whether licit tobacco contains around the same amount of nicotine as stated upon the packaging, to determine levels of nicotine in licit, illicit and niche tobacco and to make a comparison between the different types, to determine consistency of nicotine levels in illicit and niche tobacco. If samples have been omitted from the original sample list in Appendix 1, this is due to the sample being fully used up in previous analysis. Results from this chapter have been accepted and presented as poster presentations at Sci X 2014, Reno and ASH Wales 2014, Cardiff (Appendices 3, 4, 5). Heptadecane could not be used to assist calibration as the amount of internal standard was not consistent throughout the results in the first set of nicotine extraction samples.

4.2 Calibration Standard Results

The results of the calibration standards to determine the quantification of nicotine are stated in table 2.

Table 2. Nicotine calibration standards

Nicotine Standard ppm	Concentration (Peak Area)
1.218	48599
2.44	100265
4.88	209894
9.75	456496
39	967018
78	2070718
156	4488501
312	9592710
625	22308534
1250	55969684
2500	112776674

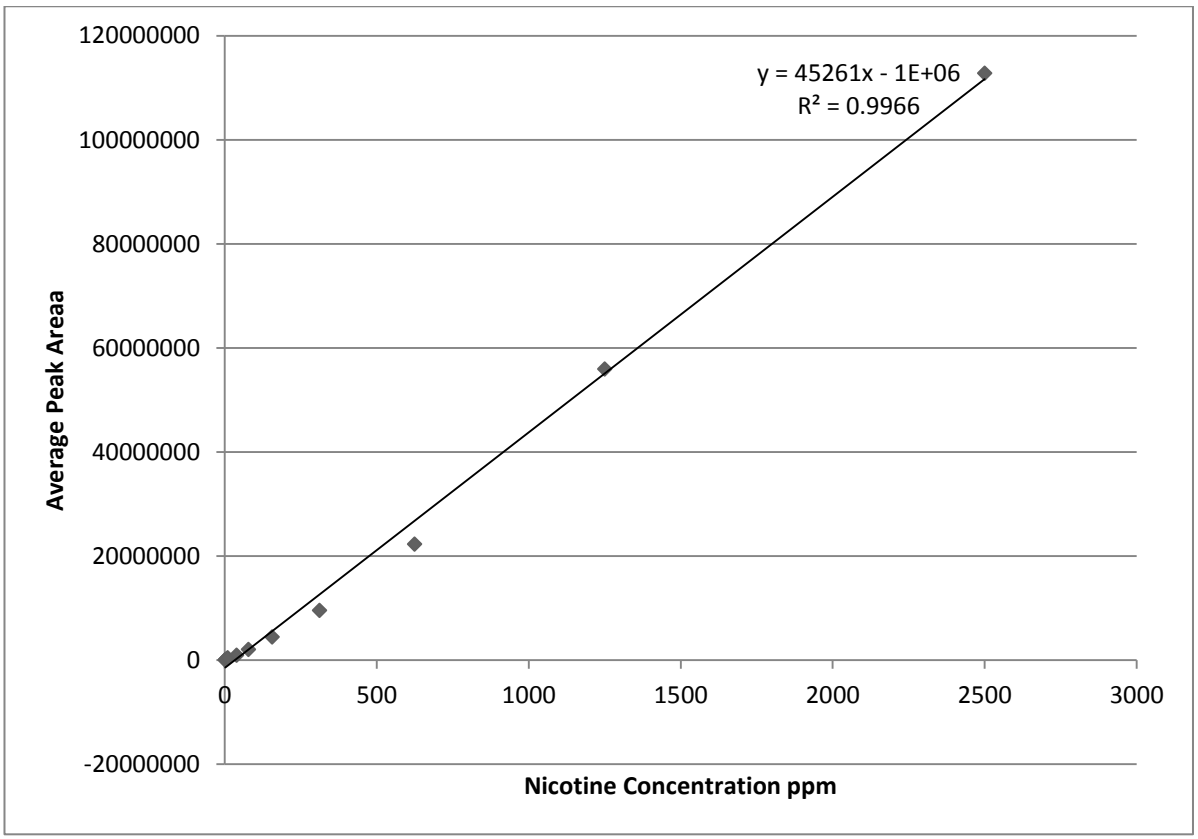


Figure. 34 Nicotine Standard Calibration graph

Concentration was calculated using a linear trend line plotted using the $y= mx+c$ equation. The equation was rearranged to $x= (y-c)/m$

x= unknown concentration

y= Peak area

m= Slope

c= Intercept

The intercept and slope used to calculate the following concentrations of nicotine are as stated below.

Slope = 45260.608 Intercept = -14846283.186

The peak for nicotine was visible upon elution from the column at 5.06 minutes (Fig. 35) The concentration was initially calculated in parts per million (ppm) then converted to mg/g. The initial sample run as seen in Appendix 5 had high standard deviation for many of the samples, potentially affecting the reproducibility of the research. In order to determine if this was a sample preparation method or continuity issue, any samples with a percentage standard deviation of >25% were re-ran under the same conditions, the results of which are shown in Appendix 6. Both runs were averaged and are available in Appendix 9.

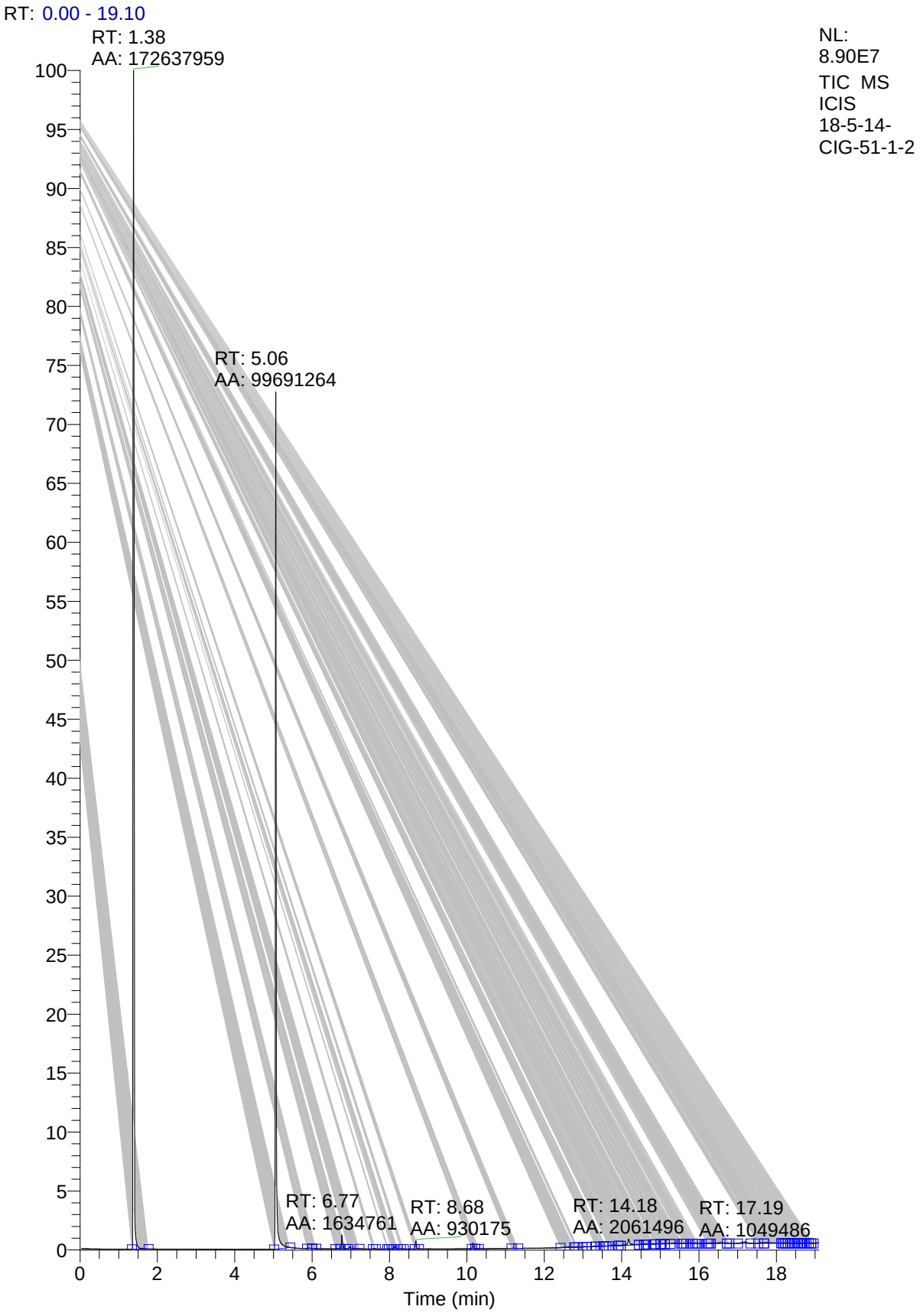


Figure. 35 Gas Chromatograph of typical tobacco sample identifying peaks for the elution from the column of Di-Ethyl ether at 1.38 and Nicotine at 5.06 minutes.

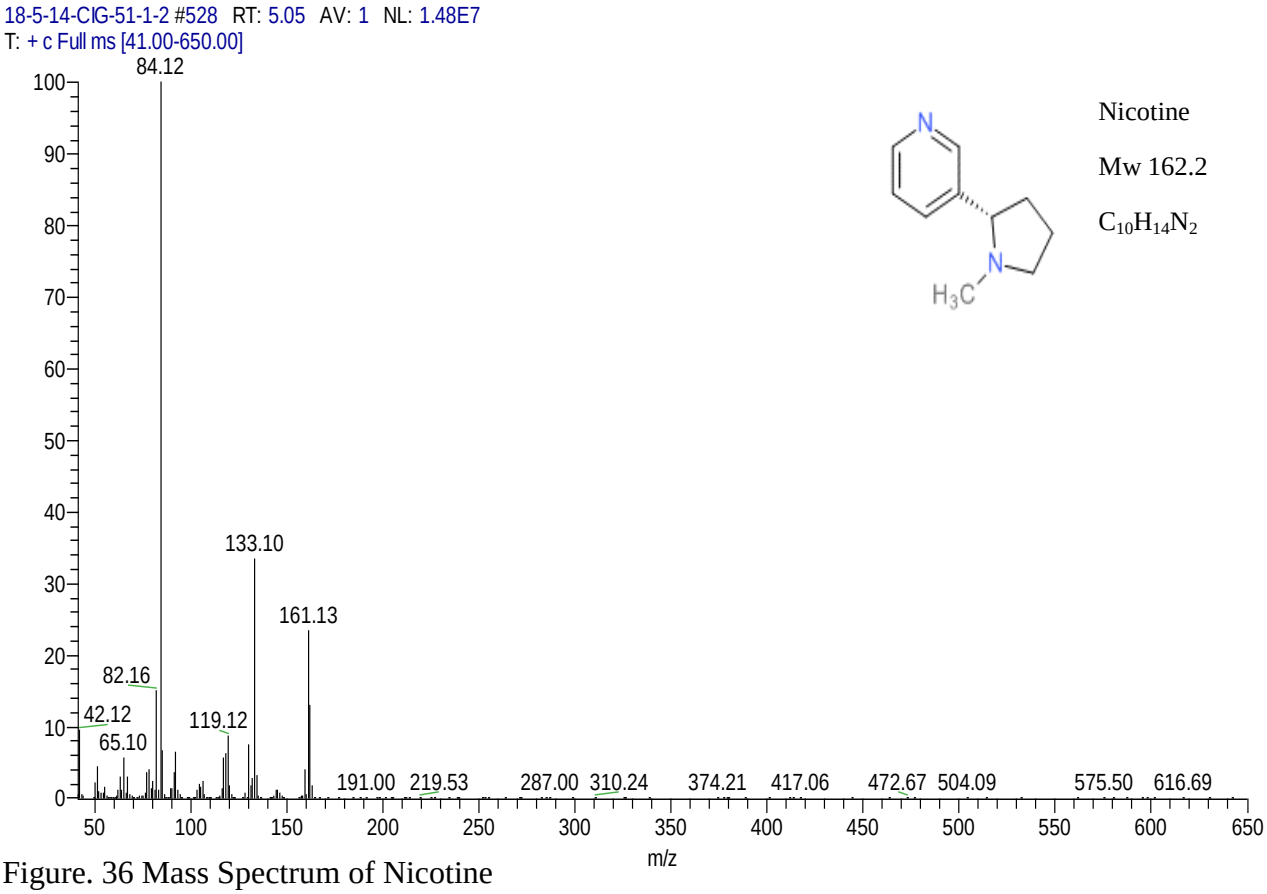
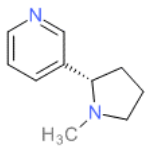
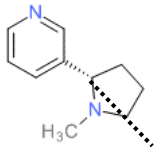
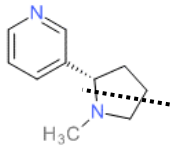
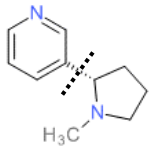
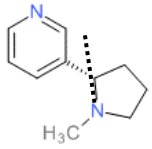
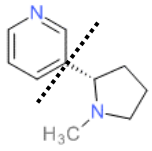
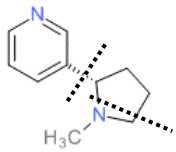


Figure. 36 Mass Spectrum of Nicotine

Table.3 A table denoting the fragmentation pattern of nicotine as detected by the Mass Spectrometer in Fig. 36.

Molecular Weight	Chemical Formula	Fragmentation pattern
161.30	$C_{10}H_{14}N_2$ [M – H^+]	
133.10	$C_9H_{11}N$ [M – H^+]	
119.12	C_8H_9N [M – H^+]	
84.12	$C_5H_{10}N$ [M – H^+]	
82.16	C_5H_8N [M – H^+]	
65.10	C_4H_3N [M – H^+]	
42.12	C_3H_6 [M – H^+]	

4.3 Licit Tobacco Results

It appears the extractions of licit cigarette tobaccos contain slightly higher levels of nicotine than the suggested 0.25mg per cigarette. The licit hand rolled tobacco nicotine content fluctuated slightly between brand, particularly between Amber leaf and Golden Virginia and has a similar nicotine content than the other cigarettes around 0.450 mg. The licit cigars nicotine content varies from around 0.06mg to 0.25mg, lower than licit cigarettes and hand rolled tobacco. There are some samples such as sample 50, Lambert and Butler, donated by Lancashire trading standards which was previously labelled as a licit tobacco sample, however, judging from a comparison between the licit sample 86 that was purchased in Preston of the same brand and type of cigarettes, we can now determine that this is a well-produced counterfeit sample due to the consistently high nicotine levels. Sample 50 exhibits all of the characteristics tobacco grown in uncontrolled conditions.

4.4 Illicit Tobacco Results

Illicit cigarettes have significantly higher levels of nicotine than licit tobacco, particularly within the ‘Cheap White’ tobacco samples with around twice the nicotine of a licit cigarette. This is not consistent throughout sample runs, possibly due to the lack of controlled features throughout the production and cultivation processes. Illicit Hand Rolled tobacco easily has the highest nicotine content consistently throughout the results with some concentrations being in excess of 2.195 mg, just under 10x more nicotine by weight of tobacco than a suggested licit cigarette.

4.5 Niche Tobacco Results

Khaini and Gutkha nicotine content levels did not vary drastically between samples although they were found to have around 0.06mg of nicotine per sample, around five times less than a regular licit cigarette. Shisha samples, even though they still contain treated tobacco leaf, were found to contain less than half of the licit cigarettes nicotine

content. Of the snuff samples, the snuff was found to contain almost half, if not less, the 0.25mg dose of nicotine. Most of the snüs samples contained similar levels of nicotine to that of previous niche tobacco.

4.6 Single Strand Extraction Results

A lower set of calibration standards was required for single strand extraction.

Using data from the standards within Table 4. the concentration was determined using the following.

Table.4 Low Concentration Nicotine Standards

Nicotine Standard ppm	Concentration (Peak Area)
1.23	48599
2.44	100265
4.88	209894
9.75	456496
39	967018
78	2070718
156	4488501
312	9592710

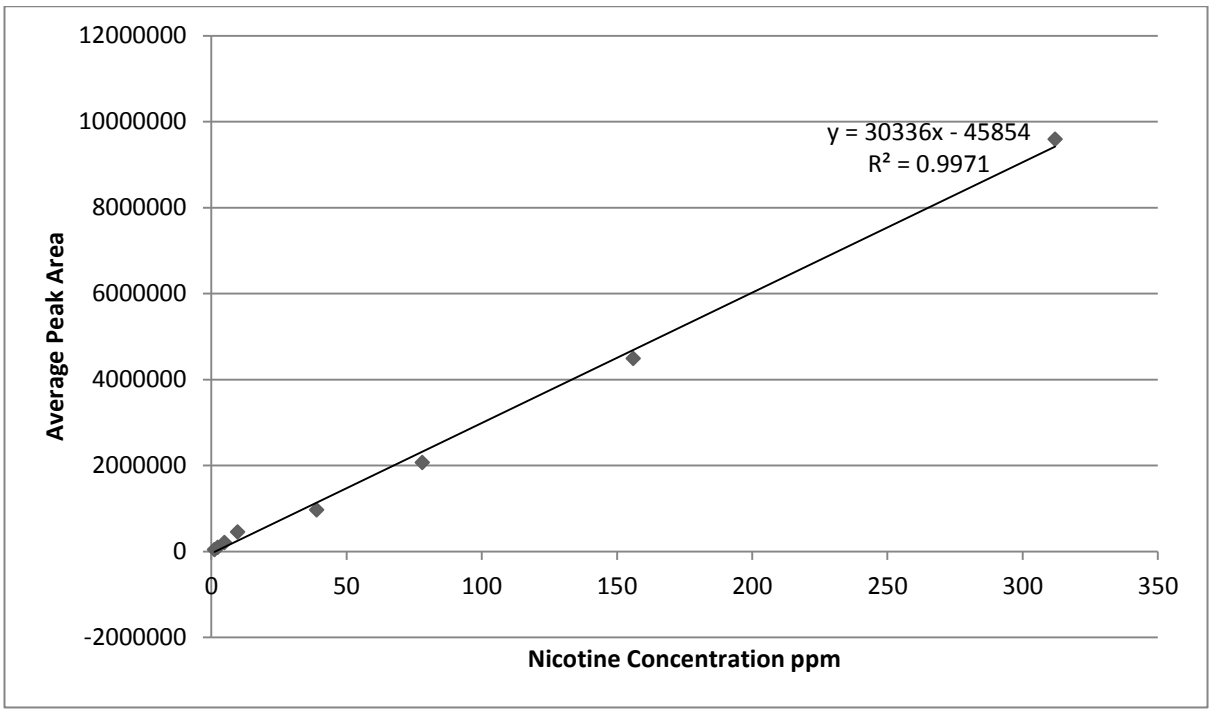


Figure. 37 Nicotine Lower Concentration Standard Calibration Graph

The linear trend line equation $y=mx+c$ was still used and rearranged to $x= (y-c)/m$, however the value for slope and intercept were altered as follows to reflect the lower concentration standards used:

Slope = 30335.86

Intercept = -45854.2

The single strand extraction results using licit tobacco show a natural variance in nicotine concentration along the tobacco leaf. Samples 87 and 88 have slight variations in low concentrations, in comparison to other samples which have higher nicotine concentrations with greater variance. The percentage standard deviation for each of the single strand results are greater than the 25% standard deviation previously used to determine sample reproducibility in the main sample runs, therefore we can deduce that levels of nicotine are not consistent along the tobacco leaf.

4.7 Conclusions

Quantification of nicotine levels within licit tobacco samples allowed for the determination that many of the licit cigarettes actually contained slightly more nicotine per cigarette than stated on the packaging. We have to account throughout the results for the fact that a greater volume of tobacco by weight in comparison to large flakes of tobacco and that nicotine may not have been extracted fully from the tobacco. Nicotine concentrations in hand rolled tobacco at single strand level still showed higher levels upon comparison. Illicit cigarettes, in particular the 'Cheap Whites' have a variety of nicotine levels, ranging from as high as 1.456mg to as low as 0.060mg. The variance may be due to inconsistencies between the tobacco plants cultivation or production process, or it can be attributed to little attention to plant generations with inconsistent levels of nicotine by producers, increasing greater variation of natural nicotine distribution along the leaf. Niche tobacco overall had less nicotine content per sample than a licit cigarette; however, we must remember that the nicotine levels in these niche samples are not necessarily representative of a single intake of nicotine. It is important to recognise that these types of tobacco, in particular shisha, are heavier and actually have less plant material to weight than cigarettes as they include other ingredients such as areca nut and slaked lime to create the desired product which are consumed in greater quantities over a shorter period of time.

With standard deviation of both runs still being high and the determination that the results are not due to a sample preparation problems, we opted to conduct a single strand extraction of nicotine, dicing a leaf into three sections and quantifying the nicotine in order to determine natural spatial nicotine distribution along the leaf. Standard deviation for the single strand extractions was in excess of 25% for each of the samples similarly to the main run data. The single strand extraction showed that the fine hand rolled tobacco even at single strand level, had higher levels of nicotine than the

large tobacco flakes of cigarettes. Each of the samples showed variation in nicotine concentration between each section of the leaf and the difference in variation altered slightly between the brands of tobacco.

4.8 Future work

Further research into nicotine content and illicit tobacco needs to be conducted in order to fully understand the risks posed by illicit tobacco and rates of addiction. Investigations into single strand extractions of nicotine need to be conducted, in order to determine natural spatial distribution along the leaf. These single strand extractions results should be supported by using Time of Flight Secondary Ion Mass Spectrometry (ToF SIMS) to produce a mass resolved image of the spatial distribution of nicotine.

Chapter 5

Fourier Transform Infrared Spectroscopy Results and Discussion

5 FTIR Results and Discussion

5.1 FTIR Results Introduction

The results chapter investigates the spectroscopic differences between different types of tobacco using Fourier Transform Infrared Spectroscopy. Spectra used in this chapter has been subject to grouping, pre-processing and multivariate data analysis using MATLAB version 7.11.0(R2010b) (The Math Works, Inc., USA) and the Spectroscopy Toolbox (University of Central Lancashire, University of Birmingham) before interpretation after vector normalisation and variable ranking had been applied. Results from this chapter have been accepted and presented as poster presentations at Sci X 2014, Reno and ASH Wales 2014, Cardiff (Appendices 3, 4, 5).

Samples were compared against their licit counterparts respectively, niche tobacco was compared against licit cigarettes due to having no licit counterpart. Typical licit spectra contained several absorbances that were consistent between both types of licit tobacco (Table. 5). Differences within the spectra are denoted by the red line, with any positive absorbances being attributed to the illicit or niche sample spectra, whereas any negative absorbances are attributed the licit sample spectra.

Table.5 Typical spectral absorbances related to tobacco.

Functional Group	Absorptions	Vibrational Mode
C-O	1050-1150 cm^{-1}	Carbonyl Stretch
-C-H	1350-1480 cm^{-1}	Alkane Bending
N-H	1600 cm^{-1}	Amide Bending
C-H	2850-3000 cm^{-1}	Alkane Stretch
O-H	3200-3600 cm^{-1}	Alcohol Stretch

As previously stated plant materials are largely composed of hydrogen, oxygen and nitrogen, therefore it is typical to see absorbances related to these functional groups.

5.2 Licit Cigarettes vs. Counterfeit and 'Cheap White' Cigarettes

Licit, 'Cheap Whites' and Counterfeit tobacco all have similar spectra with very few definitive spectral differences. Figure. 38 below shows some of the minor spectral differences between the two counterfeit tobaccos. For the majority of the spectra the illicit tobaccos have slightly lower absorbances except for absorbances at 1350-1480 cm^{-1} and 1600 cm^{-1}. These absorbance's, supported by information gained from variable ranking, are the highest variable peaks within the spectra even though absorbances are only minutely different but were still observed with the help of data pre-processing and variable ranking.

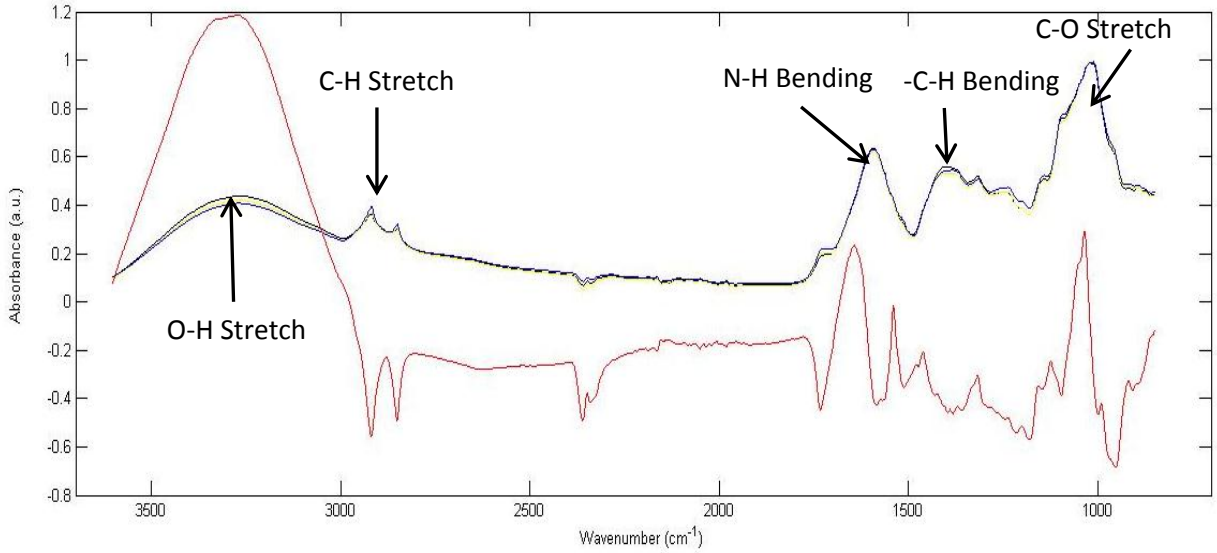


Figure. 38 Spectra denoting absorptions from Licit Cigarettes (Black), Counterfeit Cigarettes (Blue), 'Cheap White' Cigarettes (Yellow) and the highest discriminative wavenumbers (Red).

5.3 Licit Hand Rolled vs. Illicit Hand Rolled

For this analysis licit hand rolled tobacco was used to aid the comparison against counterfeit tobacco (Fig. 39). Similar absorbances were observed at 1050-1150 cm^{-1} and 2800-3000 cm^{-1}. Lower absorbances of counterfeit tobacco were observed at 1350-1480 cm^{-1} and 1600 cm^{-1}, along with a higher absorbance at 3200-3600 cm^{-1} provide the highest discriminative wavenumbers within this tobacco.

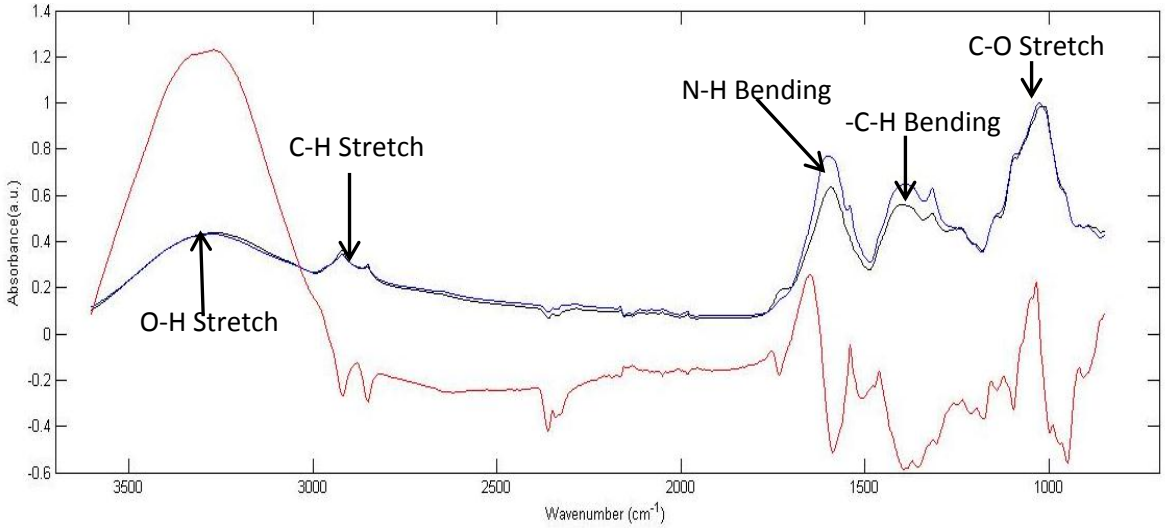


Figure. 39 Spectra denoting absorptions from Licit Hand Rolled tobacco (Black), Counterfeit Hand Rolled Tobacco (Blue).

5.4 Licit Cigarettes vs. Gutkha

When compared to licit cigarettes, Gutkha shows higher absorbances at 1050-1150 cm^{-1} and 1600 cm^{-1} (Fig. 40). Variable ranking establishes the highest discriminative wavenumbers to be related to the absorbances these wavenumbers. All other absorbances related to tobacco spectra were lower than licit tobacco.

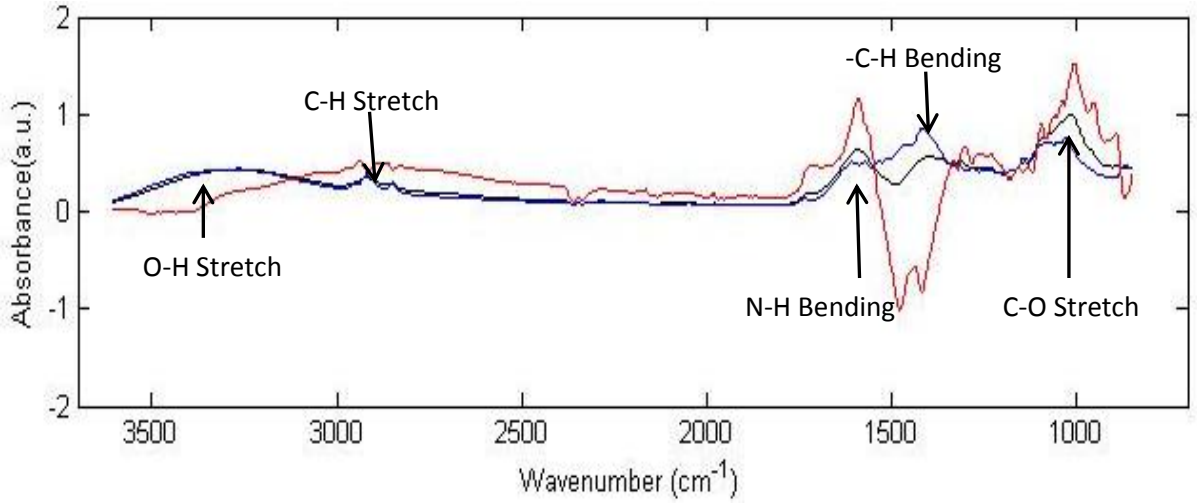


Figure. 40 Spectra denoting absorptions from Licit Cigarettes (Black), Niche Gutkha (Blue) and the highest discriminative wavenumbers (Red).

5.5 Licit Cigarettes Vs Khiani

Khiani appeared to have lower absorbances that licit tobacco at 1050-1150 cm^{-1} and 2850-3000 cm^{-1}. Significant absorbance's at 1350-14580 cm^{-1} and slightly increased absorbances at 3200-3600 cm^{-1} (Fig. 41). Variable ranking identifies 1050-1150 cm^{-1} and 1350-1480 cm^{-1} as the highest discriminative wavenumbers for this spectra.

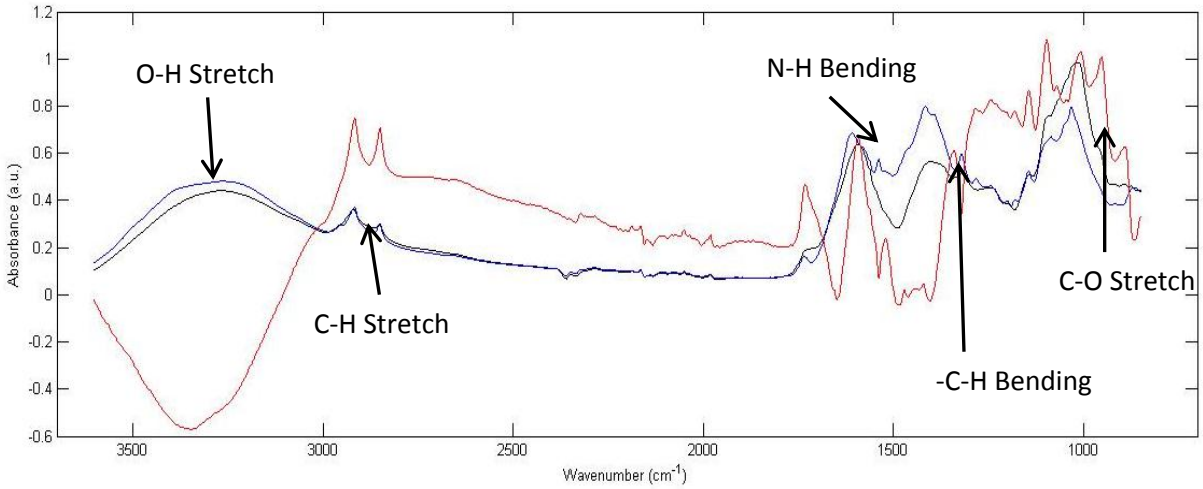


Figure. 41 Spectra denoting absorptions from Licit Cigarettes (Black), Niche Khiani (Blue) and the highest discriminative wavenumbers (Red).

5.6 Licit Cigarettes Vs Shisha

Spectra of shisha had slightly lower absorbances at 1050-1150 cm^{-1} and a slightly higher absorbance at 3200-3600 cm^{-1}. Absorbances 1350-1480 cm^{-1} and 1600 cm^{-1} were significantly lower than that of licit cigarettes. Using wavenumbers identified within variable ranking, we can identify these as the highest discriminative peaks for further analysis.

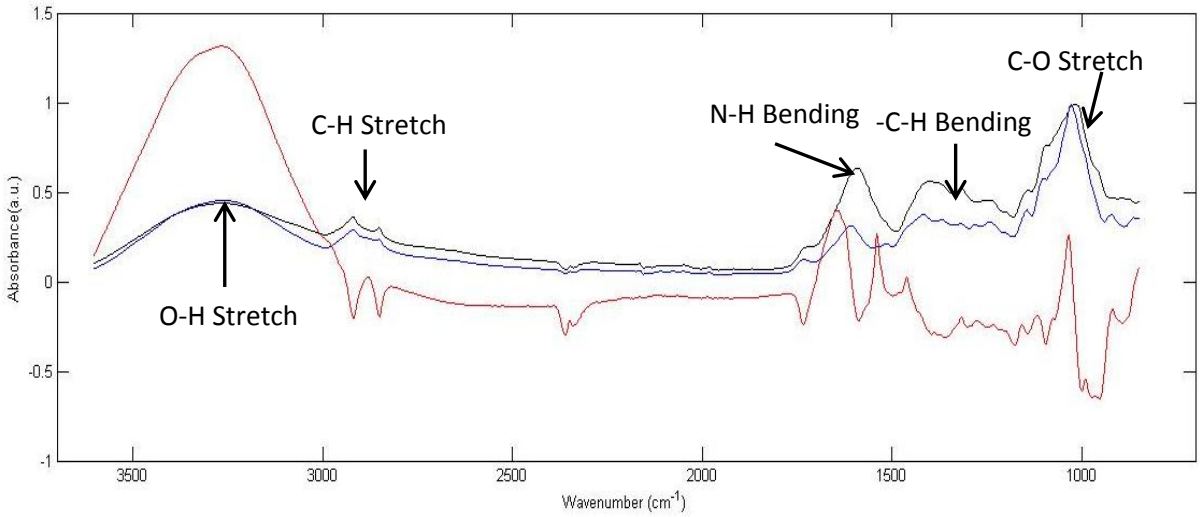


Figure. 42 Spectra denoting absorptions from Licit Cigarettes (Black), Niche Shisha (Blue) and the highest discriminative wavenumbers (Red).

5.7 Licit Cigarettes vs. Snuff

Overall the spectra of Snuff appears to have less significant absorptions than licit tobacco spectra, with significantly lower absorptions at 1050-1150 cm^{-1}, 1600 cm^{-1} and 2850-3000 cm^{-1}. Absorbance levels at 3200-3600 cm^{-1} were only slightly higher than that of licit tobacco, whereas Snuff absorbances at 13250-1480 cm^{-1} were significantly higher. Variable ranking identifies absorbances at 1600 cm^{-1} and 13250-1480 cm^{-1} as the highest discriminative peaks with the spectra.

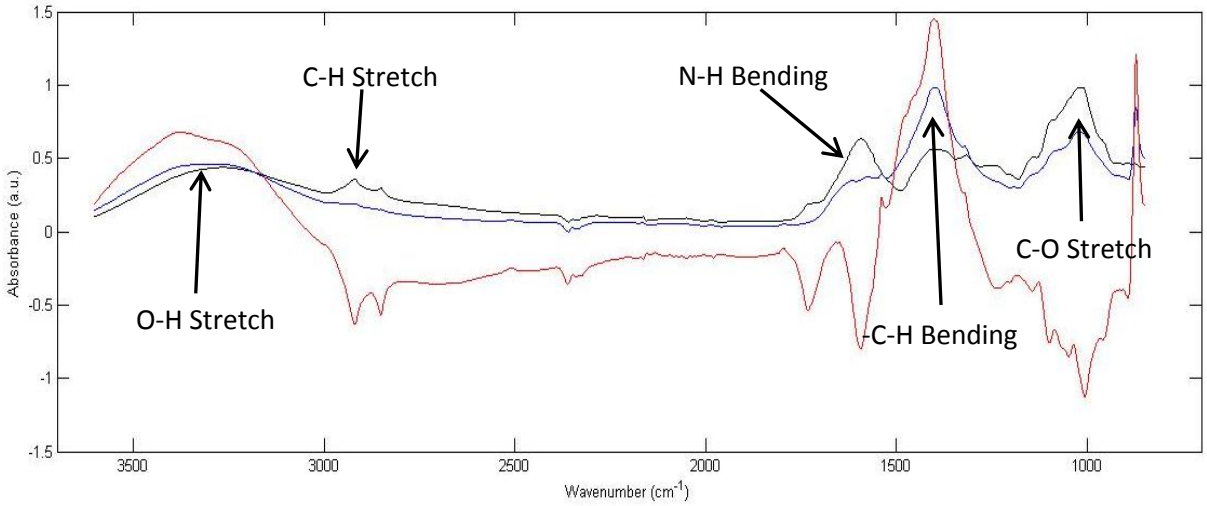


Figure. 43 Spectra denoting absorptions from Licit Cigarettes (Black), Niche Snuff (Blue) and the highest discriminative wavenumbers (Red).

5.8 Licit Cigarette vs. Snüs

Spectra of Snüs is highly similar to that of licit tobacco apart from the higher absorbance at 1350-1480 cm^{-1} and 1600 cm^{-1}. Variable ranking identifies these peaks as being the highest discriminative wavenumbers within the spectra.

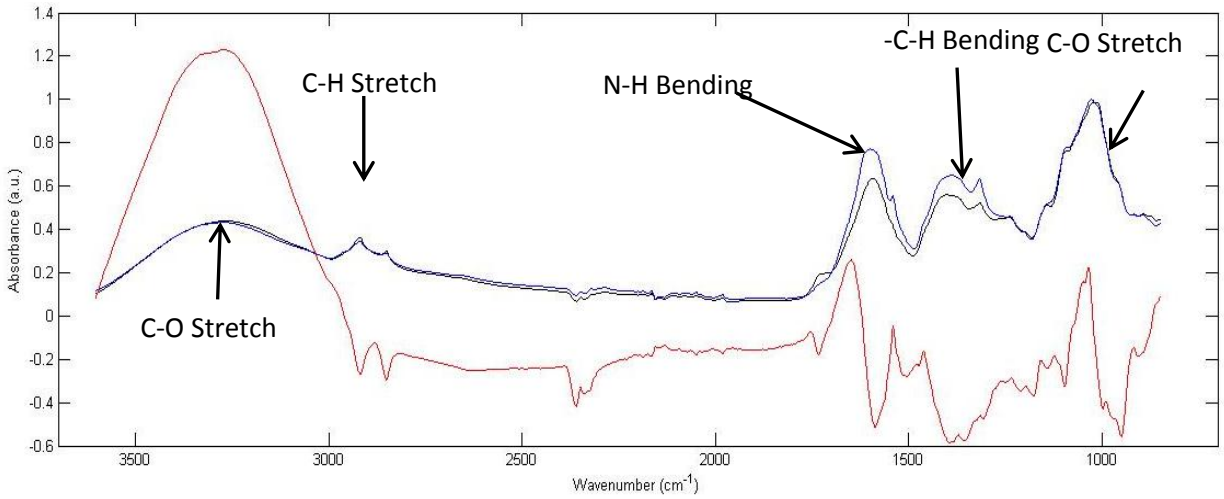


Figure. 44 Spectra denoting absorptions from Licit Cigarettes (Black), Niche Snüs (Blue) and the highest discriminative wavenumbers (Red).

5.9 Conclusions

The spectra were similar in the respect that absorbances were consistent at the same wavenumbers throughout the different tobaccos indicating these absorbances were typical of tobacco plant foliage, even in the treated niche tobaccos. Using data analysis software we were able to rank the highest discriminative wavenumbers to aid comparative analysis.

The most significant differences between absorbances of the different types of tobaccos were recorded to be within the regions of 1050-1150 cm^{-1}, 1350-1480 cm^{-1} and 1600 cm^{-1} and related to C-O, -C-H and N-H. These absorbances have no specific pattern between levels of absorbance, however, they can be used to discriminate between the different types of tobacco. The Licit Hand Rolled tobacco vs. Illicit Counterfeit tobacco is a prime example of using variable ranking to determine minor differences between

the spectra that may have previously been overlooked. Identifying regions of interest has provided a future platform for the spectroscopic determination of licit, illicit and niche tobacco.

5.10 Future Work

This research has established a platform for the spectroscopic determination of differences between different types of tobacco. Further research needs to be conducted using FTIR to determine if the slight differences observed within the spectra of Licit and Illicit tobacco in this research are applicable to a larger data, possibly including a blind sample set in order to determine if we can determine provenance of the samples using spectroscopy only. With so little previous research in this area, specifically in relation to niche tobacco, using FTIR there is potential to develop a spectral library of tobaccos to aid rapid identification of unknown samples.

Chapter 6

Conclusions

6 Conclusions

This research elementally profiles tobacco using X-ray Fluorescence, readily identifying elemental differences between the dried leaf and niche tobaccos. It is noted that trace elemental profiling is below the detection limits of X-ray Fluorescence (XRF), highlighting a need for further investigations in to quantification of trace elements. Spectral profiling of tobacco using Fourier Transform Infrared (FTIR) spectroscopy identifies wavenumbers within the regions of 1050-1150 cm^{-1}, 1350-1480 cm^{-1} and 1600 cm^{-1} related to C-O, -C-H and N-H. Using this information, there is potential for a spectral library of tobacco to be developed, which would readily identify differences between sources of tobacco. The adoption of FTIR as a handheld technique by trading standards and HMRC at seizures, could rapidly distinguish between licit and counterfeit samples. The quantification of nicotine after the extraction from tobacco samples using Gas Chromatography – Mass Spectrometry (GC-MS) identifies a significantly higher amount of nicotine found within illicit tobacco, in some cases 10X the levels of nicotine when compared to a licit cigarette, Higher levels were predominantly associated with Illicit Hand Rolled, Illicit Counterfeit and Illicit "Cheap White" tobacco. These higher levels of nicotine are associated with a higher rate of addiction, increasing the frequency of use. Therefore, tobacco products containing these levels of nicotine, expose users of illicit tobacco to higher levels of nicotine, heavy metals and carcinogens previously associated with tobacco use.

Single strand extractions of nicotine that were conducted in an effort to identify if levels of nicotine vary naturally along the leaf causing standard deviations in

excess of 25%. The results supported the theory of natural spatial distribution of nicotine along the leaf and explained the high levels of standard deviation within the main nicotine extraction work.

This research investigates the elemental and molecular profiling of licit, illicit and niche tobacco where little research has been done previously. Results from the methods used within this research profiling tobacco, provide a platform for the adoption of rapid specific detection methods of illicit tobacco by Trading Standards and HMRC, without outsourcing samples for analysis to the tobacco companies and private sector as is currently practiced.

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Appendix 1-

Sample	Brand	Legal Status
1	Baba	Niche, Pipe tobacco
2	Richman	Illicit , Counterfeit Cigarette
3	Marlboro	Illicit , Counterfeit Cigarette
4	New Line	Illicit, Cheap White Cigarette
5	Brendal	Illicit, Cheap White Cigarette
6	Jin Ling	Illicit, Cheap White Cigarette
7	Golden Virginia	Licit, Hand Rolled
8	Palace	Illicit, Cheap White Cigarette
9	Master	Illicit, Cheap White Cigarette
10	Marlason	Illicit, Cheap White Cigarette
11	Tulsi Royal Gutkha	Niche, Gutkha
12	Marlboro	Illicit , Counterfeit Cigarette
13	Marharba Molasses, Apple	Niche, Shisha
14	Dubai Tobacco, Cappucino	Niche, Shisha
15	Unlabelled Snuff	Niche, Snuff
16	Black Snuff	Niche, Snuff
17	Marble	Illicit, Cheap White Cigarette
18	Richmond	Illicit , Counterfeit Cigarette
19	Benson & Hedges	Illicit , Counterfeit Cigarette
20	John Player	Licit, Cigarette
21	Amber Leaf	Licit, Hand Rolled
22	Castella	Licit, Cigarette
23	Castella Classic	Licit, Cigarette
24	Richmond	Illicit , Counterfeit Cigarette
25	Beaumont	Licit, Cigarette
26	Park Drive	Licit, Cigarette
27	King Edward	Licit, Cigarette

28	Castella Miniatures	Licit, Miniature Cigar
29	Henri Wintermans	Licit, Miniature Cigar
30	Regal	Illicit , Counterfeit Cigarette
31	Regal	Illicit , Counterfeit Cigarette
32	Lambert & Butler	Licit, Cigarette
33	Richman	Illicit, Cheap White Cigarette
34	Royals	Licit, Cigarette
35	Consulate Menthol	Licit, Cigarette
36	Golden Virginia	Licit, Hand Rolled
37	Superkings	Illicit , Counterfeit Cigarette
38	Essentials	Illicit, Cheap White Cigarette
39	Bon International	Illicit, Cheap White Cigarette
40	Cyclone, Sugar cane	Illicit, Blunt – not used in any analysis
41	Cyclone, Supreme Nos +	Illicit, Blunt– not used in any analysis
42	Cyclone, Blueberry	Illicit, Blunt– not used in any analysis
43	Cyclone, Mango	Illicit, Blunt– not used in any analysis
44	Cyclone, Honey	Illicit, Blunt– not used in any analysis
45	Cyclone, Grape	Illicit, Blunt– not used in any analysis
46	Cyclone, Peach	Illicit, Blunt– not used in any analysis
47	Cyclone, Strawberry Kiwi	Illicit, Blunt– not used in any analysis
48	Raquel	Illicit, Cheap White Cigarette
49	Golden Virginia	Licit, Hand Rolled
50	Lambert & Butler	Licit, Cigarette
51	Silk Cut	Illicit , Counterfeit Cigarette
52	Silk Cut	Illicit , Counterfeit Cigarette
53	Amber Leaf	Licit, Hand Rolled
54	L&M Blue Label	Illicit , Counterfeit Cigarette
55	Amber Leaf	Illicit, Hand Rolled
56	Raquel	Illicit, Cheap White Cigarette
57	Jin Ling	Illicit, Cheap White Cigarette
58	Gold Classic	Illicit, Cheap White Cigarette

59	Match 444	Illicit, Cheap White Cigarette
60	Sovereign	Illicit, Cheap White Cigarette
61	Palace	Illicit, Cheap White Cigarette
62	Golden Virginia	Illicit, Hand Rolled
63	Golden Virginia	Licit, Hand Rolled
64	Golden Virginia	Licit, Hand Rolled
65	Lilja Mayaza- Apple	Niche, Shisha
66	Star Premium- Gutkha	Niche, Gutkha
67	Mirage – Lime Mixed	Niche, Khiani
68	RMD Gutkha	Niche, Gutkha
69	Goa 1000 Gutkha	Niche, Gutkha
70	Miraj	Niche, Khiani
71	Lossnus Grov	Niche, Snüs
72	L.D. Los	Niche, Snüs
73	G'R Gotebords rape	Niche, Snüs
74	General Classic White	Niche, Snüs
75	Kaliber Orginial	Niche, Snüs
76	Scaferlati Caporal	Licit, Hand Rolled
77	Gauloies Brunes	Licit, Hand Rolled
78	Old Holborn	Licit, Hand Rolled
79	Winston Classic	Licit, Hand Rolled
80	Amandis	Licit, Hand Rolled
81	Gouloises Brune Hand Rolled	Licit, Hand Rolled
82	Regal King Size	Licit, Cigarette
83	Amber Leaf	Licit, Hand Rolled
84	Marlboro	Licit, Cigarette
85	Silk Cut	Licit, Cigarette
86	Lambert & Butler	Licit, Cigarette
87	Benson & Hedges	Licit, Cigarette
88	Golden Virginia	Licit, Hand Rolled

Appendix 2

Abstract accepted to:-

- Ash Wales Conference 2014, Cardiff, Poster Presentation
- Sci X Conference 2014, Reno, Poster Presentation (Not present)

See the abstract below accepted to these conferences

ELEMENTAL AND MOLECULAR PROFILING OF LICIT, ILLICIT AND NICHE TOBACCO

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Nicotiana tabacum, more commonly known as the tobacco plant [1], the leaves of which are typically the consumable part of the plant, are harvested to be smoked, chewed or sniffed depending on the desired physiological response [2]. There is a delay from initial tobacco use and the first adverse physiological health effects. The effects of illicit tobacco are usually made more prominent due to poor grade unregulated tobacco and sub-standard delivery systems, dramatically increasing the long term impacts on user's health [3].

Tobacco plants are renowned for their ability to accumulate over 4,000 different chemicals throughout cultivation[4]. Many of the chemical substances that are associated with the tobacco plant are attributed to atmospheric depositions or the application of phosphate fertilizers and sewage sludge[2]. Previous research by W. E. Stephens *et. al*. identifies toxic cadmium as being in excess of 500% within illicit tobacco when compared against licit tobacco [3].

Nicotine, 3-(1-methyl-2-pyrroildinyl) pyridine is the major non-volatile highly toxic alkaloid found within the leaves of *Nicotiana tabacum* [5], representing 95% of the total alkaloid fraction that are used as indicators of tobacco (see fig.1) [6]. Nicotine is extracted from tobacco as a pale yellow to colourless hygroscopic oily liquid and has an LD_{50} of 50-60mg[7]. Increased levels of nicotine have been proportionally linked to a higher rate of addiction, leading to a higher nicotine dependency prolonging the use of tobacco[8].

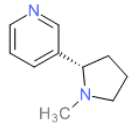

Fig.1 Nicotine

The work presented here investigates the elemental and molecular composition of illicit tobacco and the detrimental health effects incurred by its consumption. The quantification of nicotine and trace elemental analysis will be determined by using Gas Chromatography-Mass Spectrometry, Inductively Coupled Plasma – Mass Spectrometry and X-Ray Flourescence respectively.

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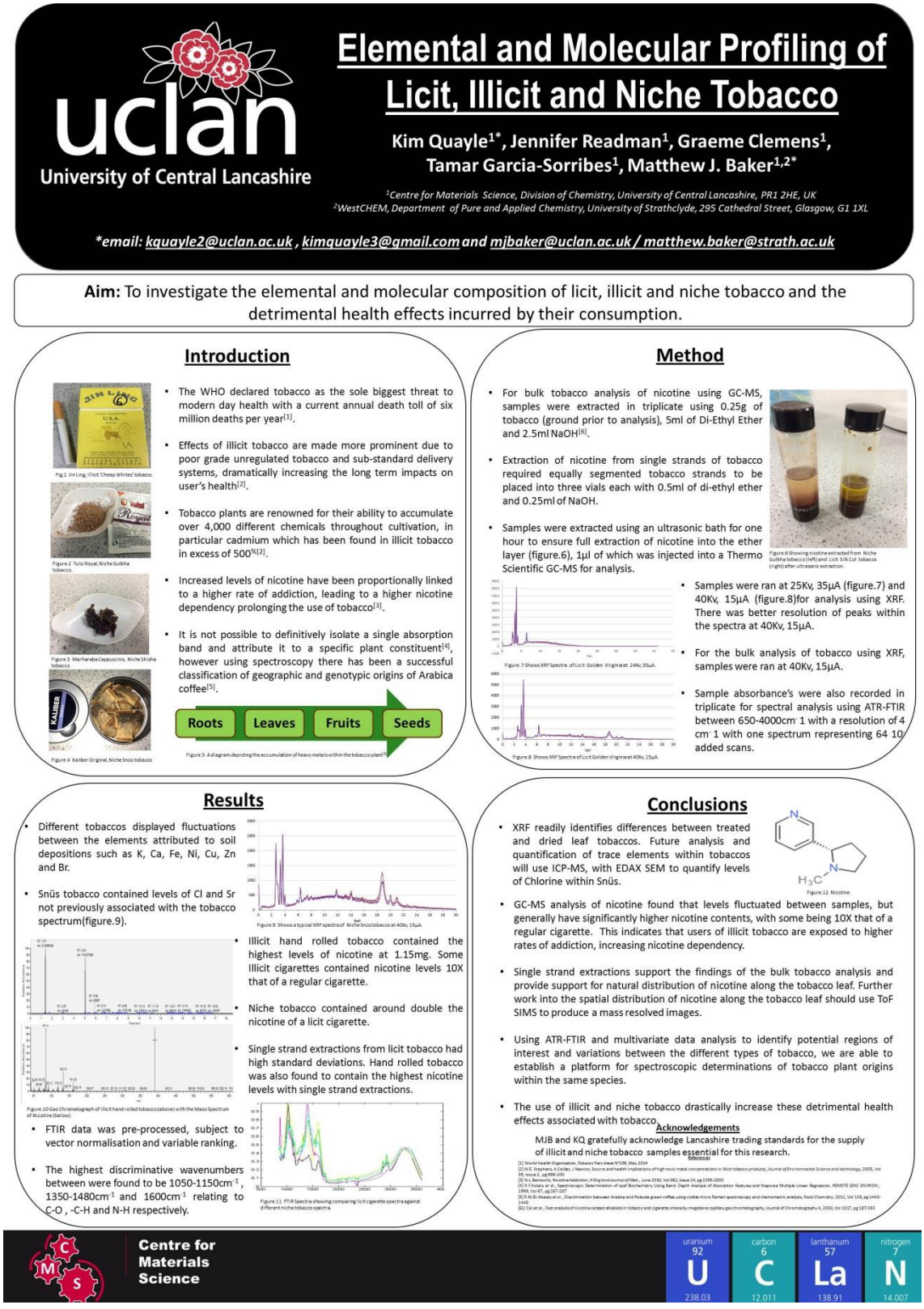
Appendix 3

A poster entitled "Elemental and Molecular profiling of Licit, Illicit and Niche tobacco" for ASH Wales 2014, Cardiff



Elemental and Molecular Profiling of Licit, Illicit and Niche Tobacco

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Aim: To investigate the elemental and molecular composition of licit, illicit and niche tobacco and the detrimental health effects incurred by their consumption.

Introduction

- The WHO declared tobacco as the sole biggest threat to modern day health with a current annual death toll of six million deaths per year[1].
- Effects of illicit tobacco are made more prominent due to poor grade unregulated tobacco and sub-standard delivery systems, dramatically increasing the long term impacts on user's health[2].
- Tobacco plants are renowned for their ability to accumulate over 4,000 different chemicals throughout cultivation, in particular cadmium which has been found in illicit tobacco in excess of 500%[2].

Figure.1 A diagram depicting the accumulation of heavy metals within the tobacco plant[2].

- Increased levels of nicotine have been proportionally linked to a higher rate of addiction, leading to a higher nicotine dependency prolonging the use of tobacco[3].
- It is not possible to definitively isolate a single absorption band and attribute it to a specific plant constituent[4], however using spectroscopy there has been a successful classification of geographic and genotypic origins of Arabica coffee[5].

Figure.2 Jin Ling, Illicit 'Cheap Whites' tobacco.

Figure.3 Tulsi Royal, Niche Gutkha tobacco.

Figure.4 Marharaba Cappuccino, Niche Shisha tobacco.

Figure.5 Kaliber Original, Niche Snüs tobacco.

Method for GC-MS, XRF and FTIR analysis

- For bulk tobacco analysis of nicotine using GC-MS, samples were extracted in triplicate using 0.25g of tobacco (ground prior to analysis), 5ml of Di-Ethyl Ether and 2.5ml NaOH[6].
- Extraction of nicotine from single strands of tobacco required equally segmented tobacco strands to be placed into three vials each with 0.5ml of di-ethyl ether and 0.25ml of NaOH.
- Samples were extracted using an ultrasonic bath for one hour to ensure full extraction of nicotine into the ether layer (figure.6), 1µl of which was injected into a Thermo Scientific GC-MS for analysis.

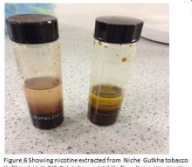

Figure.6 Showing nicotine extracted from Niche Gutkha tobacco (left) and Licit Silk Cut tobacco (right) after ultrasonic extraction.

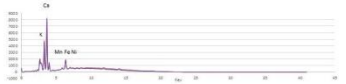

Figure. 7 Shows XRF Spectra of Licit Golden Virginia at 24Kv, 35µA.

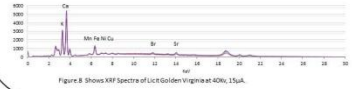

Figure.8 Shows XRF Spectra of Licit Golden Virginia at 40Kv, 15µA.

- Samples were ran at 25Kv, 35µA (figure.7) and 40Kv, 15µA (figure.8)for analysis using XRF. There was better resolution of peaks within the spectra at 40Kv, 15µA.
- For the bulk analysis of tobacco using XRF, samples were ran at 40Kv, 15µA.
- Sample absorbance's were also recorded in triplicate for spectral analysis using ATR-FTIR between 650-4000cm^{-1} with a resolution of 4 cm^{-1} with one spectrum representing 64 10^{-} added scans.

Results

- Different tobaccos displayed fluctuations between the elements attributed to soil depositions such as K, Ca, Fe, Ni, Cu, Zn and Br.
- Snüs tobacco contained levels of Cl and Sr not previously associated with the tobacco spectrum(figure.9).

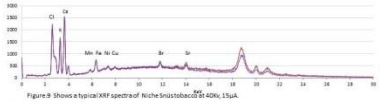

Figure.9 Shows a typical XRF spectra of Niche Snüs tobacco at 40Kv, 15µA.

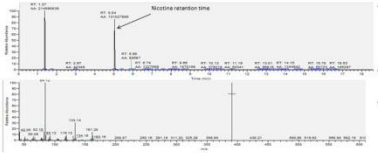

Figure.10 Gas Chromatograph of illicit hand rolled tobacco (above) with the Mass Spectrum of Nicotine (below).

- Illicit hand rolled tobacco contained the highest levels of nicotine at 1.15mg. Some Illicit cigarettes contained nicotine levels 10X that of a regular cigarette.
- Niche tobacco contained around double the nicotine of a licit cigarette.
- Single strand extractions from licit tobacco had high standard deviations. Hand rolled tobacco was also found to contain the highest nicotine levels with single strand extractions.
- FTIR data was pre-processed, subject to vector normalisation and variable ranking.
- The highest discriminative wavenumbers between were found to be 1050-1150cm^{-1} , 1350-1480cm^{-1} and 1600cm^{-1} relating to C-O , -C-H and N-H respectively.

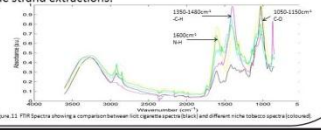

Figure.11 FTIR Spectra showing a comparison between licit cigarette spectra (black) and different niche tobacco spectra (coloured).

Conclusions

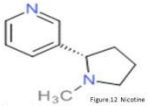

Figure.12 Nicotine

- XRF readily identifies differences between treated and dried leaf tobaccos. Future analysis and quantification of trace elements within tobaccos will use ICP-MS, with EDAX SEM to quantify levels of Chlorine within Snüs.
- GC-MS analysis of nicotine found that levels fluctuated between samples, but generally have significantly higher nicotine contents, with some being 10X that of a regular cigarette. This indicates that users of illicit tobacco are exposed to higher rates of addiction, increasing nicotine dependency.
- Single strand extractions support the findings of the bulk tobacco analysis and provide support for natural distribution of nicotine along the tobacco leaf. Further work into the spatial distribution of nicotine along the tobacco leaf should use ToF SIMS to produce a mass resolved images.
- Using ATR-FTIR and multivariate data analysis to identify potential regions of interest and variations between the different types of tobacco, we are able to establish a platform for spectroscopic determinations of tobacco plant origins within the same species.
- The use of illicit and niche tobacco drastically increase these detrimental health effects associated with tobacco.

Acknowledgements

MJB and KQ gratefully acknowledge Lancashire trading standards for the supply of illicit and niche tobacco samples essential for this research.

References

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uranium 92 U 238.03	carbon 6 C 12.011	lanthanum 57 La 138.91	nitrogen 7 N 14.007

Appendix 5

GC-MS results first run

Sample	Peak Area (Average)	Standard Deviation	Standard Deviation (%)	Concentration (ppm)	Concentration (mg/g)
2	2729787.3	207489.8	7.6	93.11	0.093
3	3005493	2563395.4	85.3	99.20	0.099
4	7469504.6	2357706.7	31.6	197.84	0.197
5	2594713.6	115342.1	4.4	90.13	0.090
6	5415729.6	1287756.4	23.7	152.46	0.152
7	8478208.3	2910119.7	34.3	220.12	0.220
8	2789173.6	487808.9	17.5	94.42	0.094
9	2067927	642841.8	31.1	78.49	0.078
11	81321.7	35543.5	43.7	34.59	0.035
13	134805	26940	19.9	35.78	0.036
14	149551.6	78154.3	52.3	36.10	0.036
15	2942292.3	1923584.7	65.4	97.82	0.098
16	1473618.3	658118	44.7	65.36	0.065
17	482493	10322.7	2.1	43.46	0.043

Sample	Peak Area (Average)	Standard Deviation	Standard Deviation (%)	Concentration (ppm)	Concentration (mg/g)
18	25600253	24991289.1	97.6	598.42	0.598
19	3946229.3	2635099.9	66.77	119.99	0.119
20	7883309.3	11148682.9	141.4	206.98	0.207
21	25133704.6	19078975.2	75.9	588.11	0.588
22	14591790.6	8758068.1	60	355.19	0.355
23	34230085.6	17493993.9	51.1	789.09	0.789
25	1303060.6	265541	20.4	61.59	0.062
27	7474546.6	8706655.2	116.5	197.94	0.198
28	9638317	5886707.2	61.1	245.75	0.246
29	573581.3	9492.6	1.7	45.47	0.045
30	1831776	196392.3	10.7	73.27	0.073
31	5267787	4439623.1	84.3	149.18	0.149
32	64430934.67	47730492.7	74.1	1456.37	1.456
33	9541095	10300103.6	107.9	243.60	0.244
34	2702121	1225831.7	45.4	92.5	0.093
35	2083687.3	4425803.4	21.2	78.84	0.079
36	3412224.3	1219283.3	35.7	108.19	0.109

Sample	Peak Area (Average)	Standard Deviation	Standard Deviation (%)	Concentration (ppm)	Concentration (mg/g)
37	2522859.6	1424381.5	56.5	88.54	0.089
38	371399	60793.5	16.4	41.01	0.041
39	510084.3	53829.7	10.6	44.08	0.044
48	3796475.3	3811798.9	100.4	116.68	0.117
49	3481239	1524112.1	43.8	109.7	0.110
50	117779508.3	104283409.5	88.5	2635.05	2.635
51	65258044.6	26036393.55	39.9	1474.63	1.475
52	178997631.3	31470983.8	17.6	3987.62	3.988
53	2202119.6	359045.5	16.3	81.46	0.081
54	5216568.3	2699869.5	51.7	148.06	0.148
55	5084760.3	3499885.7	68.8	145.15	0.145
56	33124715.3	13815465.4	41.7	764.67	0.765
57	35365702.7	33085055.1	93.6	814.18	0.814
58	9427468	3811537.8	40.4	241.09	0.241
59	8562095	6719663.5	78.5	221.974	0.222
60	7683466.3	2824648.5	36.8	202.56	0.203
61	23277926.6	2162075.3	9.3	547.11	0.547

Sample	Peak Area (Average)	Standard Deviation	Standard Deviation (%)	Concentration (ppm)	Concentration (mg/g)
63	103027.3	20574.9	19.9	35.08	0.035
64	22452419.3	12111450.9	53.9	528.87	0.529
65	443819.6	181120.3	40.8	42.61	0.043
66	1337543.3	425044.4	31.8	62.35	0.062
67	205784	94180.3	45.8	37.35	0.037
68	243401.6	145995.8	59.9	38.18	0.038
69	438237	186865.2	42.6	42.48	0.042
70	234456	67217.9	28.7	37.98	0.038
71	2541261.6	677474.5	26.7	88.95	0.089
72	1042358	18710.9	1.8	55.83	0.056
73	1477237	176460.2	11.9	65.44	0.065
74	1207666.3	107518.4	8.9	59.48	0.059
75	4894149.3	364449.7	7.4	140.93	0.141
77	800634	244486.6	30.5	50.49	0.051
79	1435781.3	172351.1	12	64.52	0.065
80	688282	33854.3	4.9	48.00	0.048
81	1016169	80202.7	7.9	55.25	0.055

Sample	Peak Area (Average)	Standard Deviation	Standard Deviation (%)	Concentration (ppm)	Concentration (mg/g)
82	1394803.6	144290.4	10.3	63.61	0.064
83	1375522.6	444235.6	32.2	63.19	0.063
84	3759179.3	1755172.9	46.7	115.86	0.116
85	1685698.3	319109.5	18.9	70.05	0.070
86	2370137.6	1514260.9	63.8	85.17	0.085
87	1210485	71930.6	5.94	59.55	0.060
88	28335373.3	782471.2	27.61	95.41	0.095
89	5224792.6	623313.6	11.9	148.24	0.148

Appendix 6

GC-MS Results re-runs

Sample	Peak Area Average	Standard Deviation	Standard Deviation (%)	Concentration ppm	Concentration mg/g
2	-	-	-	-	-
3	29557681	6917046.1	23.4	685.85	0.686
4	1566992268.3	13451902	85.7	379.51	0.380
5	-	-	-	-	-
6	9448792	5946058.9	62.9	241.56	0.242
7	5662795.7	586899.9	10.4	157.92	0.158
8	6286525	3364431.4	53.5	171.69	0.172
9	8041329.7	3379343.2	42	210.47	0.210
11	5942700.7	927945.9	15.6	164.10	0.164
13	1618080	506725.4	31.3	68.55	0.069
14	119488.3	111141.1	93	35.44	0.035
15	4486539	3676503.8	81.9	131.93	0.132
16	203304.3	278733.5	137.1	37.29	0.037
17	-	-	-	-	-
18	13720538.3	12487683	91	335.95	0.336

Sample	Peak Area (Average)	Standard Deviation	Standard Deviation (%)	Concentration (ppm)	Concentration (mg/g)
19	25217186.7	6592820.3	26.1	589.96	0.590
20	21526298.3	6680552.7	31	508.41	0.508
21	5016689.7	1788023.9	35.6	143.64	0.144
22	25258279.7	2714872.3	10.7	590.86	0.591
23	34230085.7	21425679	62.6	789.09	0.789
27	27625013	16667099	60.3	643.16	0.643
28	70319385.67	39855650	56.7	1586.47	1.586
29	-	-	-	-	-
30	-	-	-	-	-
31	3025497.3	1400340.3	46.3	99.65	0.099
32	64430934.7	58457676	90.7	1456.36	1.456
33	19759130	6015863.6	30.5	469.37	0.469
34	21409238	2526068.4	11.8	505.82	0.506
35	4213829.7	1208117.9	28.7	125.90	0.126
36	19759130	6015863.6	30.5	469.37	0.469
37	2519192.3	1041280.8	41.3	88.47	0.088
38	44977918.7	13832853	30.8	1026.56	1.027

Sample	Peak Area (Average)	Standard Deviation	Standard Deviation (%)	Concentration (ppm)	Concentration (mg/g)
39	-	-	-	-	-
48	31753077.3	13620767	42.9	734.36	0.734
49	17361491.7	4728192.7	27.2	416.39	0.416
50	117779508.3	127720571	108.5	2635.05	2.635
51	42281050.6	7871686	18.6	966.97	0.967
52	16690525.3	17498519	104.8	401.57	0.402
53	30939390.7	3354789.5	10.8	2635.05	2.635
54	5549582.3	2154240.3	38.8	966.97	0.967
55	28154489.3	6548799.8	23.2	401.57	0.402
56	4103743.7	3099856.6	75.5	716.38	0.716
57	3724381.7	2518870	67.6	155.42	0.155
58	7768753	3957265.6	50.9	204.45	0.204
59	16655012.7	12064939	72.4	400.78	0.401
60	35177832.3	16805493	47.8	810.03	0.810
61	-	-	-	-	-
63	3097958.7	756396.8	24.4	101.25	0.101
64	22515814.7	15645040	69.5	530.27	0.530

Sample	Peak Area (Average)	Standard Deviation	Standard Deviation (%)	Concentration (ppm)	Concentration (mg/g)
65	5488823.3	1021160	18.6	154.07	0.154
66	49297287	1170597.2	27.2	127.47	0.127
67	2321119	328875.5	14.1	84.09	0.084
68	3774885.7	1084009.8	28.7	116.21	0.116
69	4966196.7	5653943.1	113.8	142.53	0.143
70	16286228.3	9532749.4	58.5	392.63	0.393
71	9124172	3338488.4	36.6	234.39	0.234
72	-	-	-	-	-
73	-	-	-	-	-
74	-	-	-	-	-
75	-	-	-	-	-
77	79718235.3	44198507	55.4	1794.12	1.794
79	-	-	-	-	-
80	-	-	-	-	-
81	-	-	-	-	-
82	-	-	-	-	-
83	43418773.3	6315609.3	14.5	992.11	0.992

Sample	Peak Area (Average)	Standard Deviation	Standard Deviation (%)	Concentration (ppm)	Concentration (mg/g)
84	49754341.7	25480391	51.2	1132.08	1.132
85	28243868.3	3512485	12.4	656.83	0.657
86	36266414.3	24035584	66.2	834.08	0.834
87	-	-	-	-	-
88	33822799	10758221	31.8	780.09	0.780
89	-	-	-	-	-

Appendix 7

Nicotine variance along single tobacco strand results

Sample	Peak Area Average	Standard Deviation	Standard Deviation (%)	Conc ppm	Conc mg/g	Conc within 5ml
83-1	135261	78092.97	57.7	5.97	0.006	0.0006
83-2	301092.7	139648.8	46.4	11.43	0.011	0.0011
83-3	1164900	1063574	91.3	39.91	0.040	0.0040
84-1	82770.6	78022.4	94.3	4.24	0.004	0.0004
84-2	126806	136818.1	107.9	5.69	0.006	0.0006
84-3	787600.3	825448	104.8	27.47	0.027	0.0027
85-1	0	0	0	0	0	0
85-2	312784	180637.9	57.75	11.82	0.012	0.0012
85-3	198861.3	63474.02	31.91	8.06	0.009	0.0009
86-1	283414	236658	83.50	10.85	0.011	0.0011
86-2	83305	50481.54	60.59	4.26	0.004	0.0004
86-3	85959.3	22596.42	26.28	4.34	0.004	0.0004
87-1	37048	21389.6	57.75	2.73	0.003	0.0003
87-2	63805	36837.83	57.73	3.61	0.004	0.0004
87-3	98891	26571.36	26.87	4.77	0.005	0.0005
88-1	51846	15412.74	29.73	3.22	0.003	0.0003
88-2	130757.7	44253.03	33.84	5.82	0.006	0.0006
88-3	149324.7	81477.64	54.56	6.43	0.006	0.0006

Appendix 8

Memorandum of understanding between Lancashire Trading Standards and the University of Central Lancashire.

MEMORANDUM OF UNDERSTANDING

This is an agreement between Lancashire County Council Trading Standards Service and the University of Central Lancashire in relation to an MSc research project to be carried out by Kim Quayle and overseen by Doctor Matthew Baker.

The aim of the research is to analyse illicit tobacco products including niche and use this spectral database to provide a classification system based on the potential adverse effects of known harmful substances on the human body. Similarities will also be isolated between the tobaccos relating to geographical origin, which should identify areas of origin & potential trade routes.

- Samples of illicit and niche tobacco products including shisha will be made available to UCLAN where appropriate and available from Lancashire Trading Standards and possibly other authorities throughout the North West.

- The project will run from Oct 2013 to Oct 2014.

- All products given to UCLAN will be signed for including a statement detailing their use and destruction. All products will be stored securely by UCLAN.

- No funding will be taken at any time from the tobacco industry in line with UCLAN's Ethical Policy and Lancashire County Council's(LCC) policy.

- The results of the MSc research project will be published as a journal in line with UCLAN, including published online.

- Both parties will have awareness in regard to media enquiries and enquiries from smoking cessation campaigning bodies. Both parties will be made aware of any such enquiries. Any press releases shall be jointly agreed between UCLAN and Lancashire County Council Communications Service.

Signed .....

Of .....

Dated .....

Signed .....

Of .....

Dated .....

Appendix 9

Average nicotine run data

Sample	First Run Nicotine (mg)	Re Run Nicotine (mg)	Average Nicotine (mg)
2	0.093	-	0.093
3	0.099	0.686	0.442
4	0.197	0.380	0.286
5	0.090	-	0.090
6	0.152	0.242	0.273
7	0.220	0.158	0.299
8	0.094	0.172	0.133
9	0.078	0.210	0.144
11	0.035	0.164	0.100
13	0.036	0.069	0.053
14	0.036	0.035	0.034
15	0.098	0.132	0.164
16	0.065	0.037	0.051
17	0.043	-	0.043
18	0.598	0.336	0.467
19	0.119	0.590	0.355
20	0.207	0.508	0.358
21	0.588	0.144	0.366
22	0.355	0.591	0.473
23	0.789	0.789	0.789
25	0.062	0.643	0.353
27	0.198	1.586	0.892
28	0.246	-	0.246
29	0.045	-	0.045
30	0.073	-	0.073

31	0.149	0.099	0.124
32	1.456	1.456	1.456
33	0.244	0.469	0.357
34	0.093	0.506	0.300
35	0.079	0.126	0.103
36	0.109	0.469	0.289
37	0.089	0.088	0.088
38	0.041	1.027	0.555
39	0.044	-	0.044
48	0.117	0.734	0.426
49	0.110	0.416	0.263
50	2.635	2.635	2.635
51	1.475	0.967	1.221
52	3.988	0.402	2.195
53	0.081	2.635	1.358
54	0.148	0.967	0.558
55	0.145	0.402	0.274
56	0.765	0.716	0.741
57	0.814	0.155	0.485
58	0.241	0.204	0.225
59	0.222	0.401	0.312
60	0.203	0.810	0.507
61	0.547	-	0.547
63	0.035	0.101	0.068
64	0.529	0.530	0.529
65	0.043	0.154	0.099
66	0.062	0.127	0.095
67	0.037	0.084	0.061
68	0.038	0.116	0.077
69	0.042	0.143	0.0925

70	0.038	0.393	0.201
71	0.089	0.234	0.162
72	0.056	-	0.056
73	0.065	-	0.065
74	0.059	-	0.059
75	0.141	-	0.141
77	0.051	1.794	0.9225
79	0.065	-	0.065
80	0.048	-	0.048
81	0.055	-	0.055
82	0.064	0.992	0.528
83	0.063	1.132	0.598
84	0.116	0.657	0.3865
85	0.070	0.834	0.452
86	0.085	-	0.085
87	0.060	0.780	0.420
88	0.095	-	0.095
89	0.148	-	0.148