Microarrays and Monoclonal Antibodies for a High-throughput Food Authentication Process

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Abstract

Population increase, climate change, greed. These are just some of the major factors that pose a continuing threat to food safety and sustainability. Food safety allows consumers to feel secure in the product chosen for consumption. Food fraud is one issue that threatens this. An age-old problem, food fraud is the act of intentionally deceiving a customer about aspects of the product including origin, ingredients, or quality: mostly an economically motivated act. In some cases, food fraud can have widespread effects, proving fatal at times. As a consequence, food authentication techniques have been developed to combat fraud. Authentication allows for verification that the product is what it claims. It is important that food safety is maintained, authentication processes are constantly evolving in line with developing fraud techniques. Current techniques used in the food industry include, amongst others, Nuclear Magnetic Resonance (NMR), DNA profiling, and microscopy.

This project focuses on how high-throughput microarray technology and molecular probes, specifically monoclonal antibodies, can be used in food authentication. Three products were tested, including whole grains, saffron and gluten. For whole grains and saffron, polysaccharide profiles were used to measure authenticity. The focus for whole grains was to develop a method that would distinguish the proportion of whole grains within a product and fraction by investigating its polysaccharide profile. For saffron, a polysaccharide profile was created using reference samples and compared against other market samples to see if it would be an appropriate technique, and how profiles could differ. For the gluten experiment, gliadin, a protein within gluten, was chosen as the target. The purpose of this was to develop a high-throughput method allowing for allergen detection in food products. Here it could be successful in identifying gliadin within food products in case of wheat allergies or even the auto-immune condition, celiac disease, whereby gluten would be a tell-tale sign of cross contamination.

These experiments highlight how microarray technology coupled with molecular probing could be used as a high-throughput food authentication technique.

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Chapter 1. Introduction

As consumers we have the right to feel secure in the foods that we eat. Body autonomy, or the rights over our own persons, is highly valued, and includes control over the diets we choose to follow and the foods we choose to consume. Societal attitudes to food have guided a change in diets to suit medical and moral needs, with consumption of low-carb, gluten free, vegetarian, and vegan diets amongst those that have seen an upwards trend across the globe over the years (Atkins, 1998; Melina et al., 2016; Newberry et al., 2017). Many take the quality and safety of food products at face value, believing food products to be safe, with a sense of trust in the providers, and that consumer rights are robust enough to provide protection.

This work is especially timely in view of the considerable pressure that food supply chains face. We are in the midst of an impending of food crisis (Brassesco et al., 2021). Multiple factors including war, climate change, and a pandemic have led us to witnessing increases in food prices, the reduction in the availability of staple products such as eggs and flour, and the threat of famine in certain areas such as Africa (Seife 2020; Azadi et al., 2021; Pryor and Dietz, 2022; Hellegers, 2022). Climate change has led to more drastic weather events including drought, flooding, storms, and temperature extremes, all of which have the ability to decimate crops, and add to the increasing uncertainty around food security (Asseng et al., 2015; Hasegawa et al., 2018). This may impact pricing of goods that may still be in high demand, but now low supply, as the UK has seen with staple items such as eggs during the COVID-19 pandemic, and more recently, the bird flu epidemic (Trollman et al., 2021; Haider et al., 2023). The disadvantage of this is that some may see it as an opportunity to dupe consumers into purchasing fraudulent goods that may be of lesser value, or even adulterated with other ingredients.

When considering food safety, thought is usually turned to how food is prepared, most naturally, will this make a consumer ill through cross contamination? However, attention may not necessarily be focussed on what is being eaten, in terms of finer ingredients and composition. At face value would it be possible to tell the difference between horsemeat and beef? Cod and hake? Olive oil and vegetable oil? These differences may be very hard to spot at face value, which is why it is so important to place focus on the authenticity of food products on sale, mainly as a safety measure

for the public. The focus of this project is the use of technology based on microarrays and molecular probes to help support food security and work against food fraud.

1.1 What is food fraud?

Food fraud is the act of knowingly deceiving customers about the status of their food whether it be product origin, organic status, or substitution with lower-value ingredients (European Commission, 2020 (a); European Commission, 2020 (b)). As will be explored throughout this project, food products can be subject to criminal acts, which have evolved in complexity over the years, therefore there is no 'one size fits all' solution to the problem. Whilst not all food fraud is equally harmful to public health, some may feel that all fraud related crime should be dealt with at the same severity, as it is an active intent to deceive both customer and the authorities.

The UK National Food Crimes Unit (NFCU) categorises food fraud as food crime, with an outright focus on 7 different sectors (Food Standards Agency, 2021 (a)):

- Document Fraud
- Adulteration
- Waste Diversion
- Substitution
- Illegal Processing
- Misrepresentation
- Theft

More detailed definitions of these categories can be found in Figure 1.1. Economically motivated adulteration (EMA) is a specific type of fraud which sees products being tampered with by ways such as substitution of ingredients with inferior ones or even addition of sub-par ingredients with the premise of 'bulking up' the item. One of the more prolific EMA cases of recent times is that of the 2013 UK horsemeat scandal whereby horsemeat had been found, undeclared, in beef products being sold in the UK (European Commission, 2014).

It could be considered that most, if not all, acts of fraud are economically motivated, with the most extreme of cases giving little thought as to the potential impact of the act(s) they are committing.

Food authentication entails the suite of processes used to verify food-products, through scientific analysis or checking documentation, ensuring that the product is what it claims to be (Danezis et al., 2016).

1.2 Punishment

Legal sanction for committing food fraud within the UK is uncommon. Within the UK there is no legally defined definition of what constitutes as fraud, for this reason it may be difficult to comprehend what would classify as a fraudulent act (Food Standards Agency, 2017). Another possible reason for low conviction rates could be that some may not see food fraud as a crime that would impose danger, especially consumers and so it is not seen as a pressing issue for most (Kendall et al., 2019). Cases such as document fraud could be seen as 'victimless', however it has been documented that products subjected to document fraud can also be fraudulent in other ways as described by the NFCU, as has been seen in some areas of the fish trade such as being caught without permits and illegal imports or exports (Pramod et al., 2014).

As was previously discussed, the NFCU defines seven categories that would constitute food fraud, and these are what they base their prosecutions upon. Punishments and their severity also differ depending upon where they occur: for instance, in China there have been cases where convicted individuals have been sentenced to death, whereas other countries would impose fines or even jail time depending upon the fraud committed and the resulting effect (Xiu and Klein, 2010; Falkheimer and Heide, 2015).

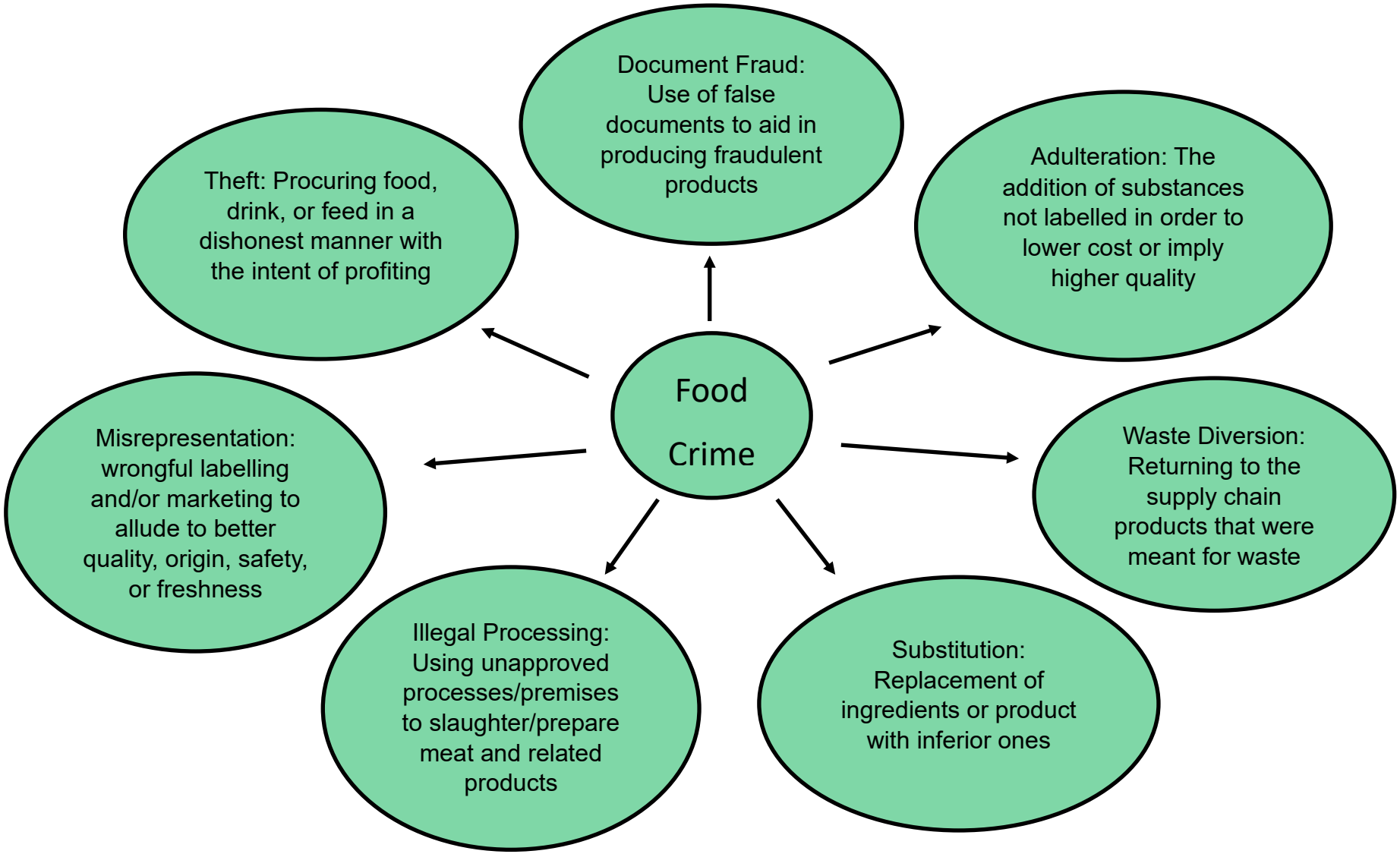


Figure 1.1. NFCU Food Crime Definitions: A schematic outlining the seven sectors the NFCU defines as 'Food Crime'. It can be seen that not all may pose a danger to consumer health, but all outline an instance of deceiving the customer (Food Standards Agency, 2021 (a)).

1.3 Who tackles food fraud?

With the existence of food fraud potentially threatening public safety, it is imperative that there are bodies and legislations in place to protect the health of the consumers in terms of food. Within the UK there are two main bodies, the Food Standards Agency (FSA) and Department for Environment, Food and Rural Affairs (Defra). As a response to the breakout of numerous foodborne illnesses, including salmonella in eggs, and bovine spongiform encephalopathy (BSE) in meat (Nathanson et al., 1997; Lane et al., 2014) the FSA was created in early 2000. An independent study by Philip James (1997) outlined the necessity for an independent body to oversee food standards within the UK. The FSA is a non-ministerial division and has four main goals to make sure that:

- 'Food is safe'
- 'Food is what it says it is'
- 'Consumers have access to affordable food'
- 'Consumers are able to make informed choices about what they eat'

And an overall mission statement:

'Food we can trust'

(Food Standards Agency, 2020)

The NFCU was created in response to the 2013 horsemeat scandal, whereby horsemeat was found to be in various meat products being sold within the UK. The NFCU was established as a law enforcement branch, essentially becoming an extension of the FSA. They perceive food fraud as a crime and have the power to uphold the law in relation to food safety and security, for example the suspension and removal of internet postings for the sale of illegal supplements (Sullivan and Davies, 2020; Food Standards Agency, 2021 (b)).

Defra is the ministerial body which aims to protect and improve the current environment. As a governmental department, Defra is able to create and implement policies with the aim of creating a sustainable environment, and for the protection of food, environment, and rural affairs. Their focus is to 'restore and enhance the environment for the next generation, leaving it in a better state than we found it' (National Audit Office, 2015; Department for Environment Food and Rural Affairs, 2021). Defra's priorities lie in ensuring the protection of the environment, with a large focus on the sustainability aspect of food production especially with regards to farming

practices. However, monitoring the safety of foods on the market mainly lies with the FSA.

Whilst these bodies aid in monitoring legislation and the creation of food laws, there are also protective definitions that can be designated to certain foods that allow a consumer to be aware of the quality and authenticity of the product (Table 1.1). These titles include:

- Protected Designation of Origin
- Protected Geographical Indication
- Traditional Specialities Guaranteed
- International Organisation for Standardisation

Although these protections exist and are legally enforced, it is not to be assumed that products utilising these titles would not be subject to fraud. The benefit to the producer subscribing to a protective title scheme is that they can market their products at a higher premium, citing authenticated quality, which in turn, makes these specific products prime targets for fraud.

Table 1.1. Listing of the protective categories that can be assigned to food and drink products, upon the understanding that certain, specific requirements are met. Due to the UK leaving the EU, any UK protections from 2021 onwards are not recognised by the EU and separate applications have to be made. N.B.: There is also a separate class called geographical indication (GI) for spirit drinks and aromatised wines. (European Commission, 2021).

Name:	Definition:	UK Examples:
Protected Designation of Origin (PDO)	'Every part of the production, processing and preparation must take place in the specified region'	• Cornish Native Fal Oysters • East Kent Goldings • Cornish Clotted Cream
Protected Geographical Indication (PGI)	'At least one stage of the production, processing or preparation takes place in the specified region'	• Melton Mowbray pork pie • Dorset Blue cheese • Ayrshire New Potatoes

Traditional Specialities Guaranteed (TSG)	Puts a focus on 'The traditional aspects, such as the way a product is made or its composition – not linked to geographical area'	• Traditionally farmed Gloucestershire Old Spots Pork • Traditionally Reared Pedigree Welsh Pork • Traditional farmfresh Turkey
International Organisation for Standardisation (ISO)	Allows for standardisation across a product by meeting specific requirements, such as taste or colour.	• Saffron – ISO 3632-1:2011 • Bee products – ISO/TC 34/SC 19 • Durum wheat semolina and alimentary pasta – ISO 7304-1:2016

1.4 Fraud through the ages

Food laws have existed for hundreds, and potentially thousands of years. Early English food laws often spoke about the pricing of foods, including what weights certain foods, such as meat, should be and for what price. Early laws often seemed to weigh in favour of the consumer, aiming to give them a fair deal for the products they seeked (Walford, 1880).

It would be reasonable to assume that where there is food, there is fraud. There have been documented cases of food fraud going back hundreds of years, with most consumers oblivious the ongoing struggle. Frederick Accum, during the 1820s, aimed to educate the British public on how to spot adulteration in various scenarios, including within food products, but also various household items – such as paint (Table 1.2). Whilst it is claimed that the publication made quite the impact, with rumoured lawsuits that followed, it is clear from public perception within the 21st century that the idea of food fraud, adulteration and subsequent authentication is not one that has remained at the forefront of consumer interest (Accum, 1820).

Table 1.2. Examples of common types of food fraud for certain food products, and home-style authentication techniques during the 1800s (Accum, 1820).

Product	Common Method of Adulteration	Method of Detection
Bread	Addition of alum to the mixture.	Pour a half pint of boiling distilled water on 2 ounces of sample. Boil the mixture and filter. Let ¾ of the mixture evaporate and add a solution of 'muriate of barytes'. If a white precipitate forms and doesn't dissolve with the addition of nitic acid then the presence of alum can be assumed.
Beer	Addition of vegetable substances.	Look for iron sulphate: Evaporate the beer sample, mix the residue with potassium chlorate and burn. The residue left may contain iron sulphate which can then be assayed.
Red Wine	Addition of beetroot, for colour.	Add lime water – this removes the colour added by the beetroot.
Cheese	Gloucestershire cheese contaminated with red lead.	Add a crushed portion of the cheese to a mixture of hydrogen sulphide, muriatic acid, and water. If lead is present, the cheese will turn black or brown.

Tea	Leaves of another plant being given instead.	Examine the leaves and compare them with pictures of tea leaves.

Whilst all of these methods of detection may seem primitive, they were the most suitable at the time. Today, analysis is often dependent upon the types of fraud or the food type, for example methods useful within meat authentication may not be as useful for wine authentication, and authentication techniques have become much more complex and modern in comparison to those from the 1800's (Table 1.3).

Table 1.3. Examples of modern authentication techniques that have been used on the food products outlined in Table 1.2.

Product	**Modern Authentication Techniques**
Bread	• Isotope Ratio Mass Spectrometry (Longobardi et al., 2015) • Quantitative Polymerase Chain Reaction (Pontonio et al., 2017) • Near-Infrared Spectroscopy (Duarte et al., 2022)
Beer	• Liquid Chromatography-Mass Spectrometry (Mattarucci et al., 2010) • Nuclear Magnetic Resonance (Kuballa et al., 2018) • Excitation-emmision Matrix Fluorescence Spectroscopy (Fang et al., 2022)
Red Wine	• Ultra-High Performance Liquid Chromatography (Rubert et al., 2014) • Ultraviolet-visible Spectroscopy (Geanâ et al., 2019) • Fourier-transform Infrared Spectroscopy (Geanâ et al., 2019)

Cheese	• Multiple-collector inductively coupled plasma mass spectrometry (Fortunato et al., 2004) • Gas Chromatography Mass Spectrometry (Caligiani et al., 2016) • Aroma Profiles – Flame Ionisation Detector (Štefániková et al., 2019)
Tea	• High Performance Liquid Chromatography-Diode Array Detector (Peng et al., 2021) • High Performance Liquid Chromatography – Fluorimetric Detector (Pons et al., 2021) • DNA Metabarcoding (Frigerio et al., 2021)

As science and technology progresses, it would be reasonable to assume that methods of fraud may also advance in order to elude detection. However not all fraud cases use complex methods, especially within the realm of adulteration.

The next four cases, from the 1800s through to more recent times highlight the devastation fraud has the potential to cause, and the need for stringent authentication testing.

1.4.1 Bradford humbug poisoning — 1858

Humbug sweets are a popular sweet in Britain, being widely stocked and sold. This was also true in 1858. The 1800s saw a large amounts of money raised from the sugar tax, because sugar was such a valued commodity at the time, but in limited supply. Due to the soaring costs, some turned to adulteration in order to turn a profit. A common adulteration method of the time was to mix in extraneous materials, such as limestone, in with their sugar to make an ingredient known as ‘daft’. During 1858 a Bradford market stall owner, William Hardaker, was one such supplier using daft in their products, humbug sweets. Hardaker sourced his daft, a usually harmless substance, from the local chemist. On one occasion when buying daft, Hardaker was

mistakenly supplied with arsenic instead. The sweets themselves were then made by another employee under Hardaker. With daft and arsenic being similar white powders, the mistake was not caught by the inexperienced employee. When sold to the public, over 200 people fell ill, with 20 casualties – reports at the time suggested that one humbug sweet contained 2 lethal doses. As a consequence of this scandal, the 1860 Adulteration of Food and Drink Bill was created, as well as the UK Pharmacy Act of 1868, which imposed tighter regulations for pharmacists. Due to the cause of fatalities, charges of manslaughter were brought about, but no conviction was made (Johnson, no date).

1.4.2 Toxic rapeseed oil — 1981

Oil is a key ingredient in many processes and is used all over the world, with Spanish cuisine being no exception. 1981 saw certain regions of Spain gripped by a mystery illness, that officially struck 20,643 residents and lead to the eventual deaths of over 300 as an initial estimate (Abaitua-Borda et al., 1993). The first indication of an incoming crisis was the hospital admittance of an 8-year-old child suffering respiratory complications, who died shortly after, and was one of 6 members of their family to experience the illness (Tabuenca, 1981).

It took a little over a month after the young child's death to establish cause. By this time hundreds had presented with symptoms and an investigation was launched. A breakthrough came when the Niño Jesús Childrens Hospital in Madrid decided to focus on a specific age group of those infected – infants aged between 6 and 12 months. Considerations that it could be an airborne illness were scrapped as no patients presented under 6 months old, so the thought process turned to food. The logic behind this was that a young infant's diet is more controlled and therefore it would be easier to pinpoint items consumed. It was discovered that each patient admitted to this particular hospital had consumed oil bought from a door-to-door salesman (Tabuenca, 1981).

During the investigatory phase, it was found that rapeseed oil was the causative agent. In line with Spanish law at the time, rapeseed oil was illegal when purchased or used for human consumption. The stated the requirement for any imported rapeseed oil to be denatured, one of these denaturing substances was aniline. It was common for these denatured oil products to be mixed with other oils, the resultant mixture would be refined and sold on (Posada de la Paz et al., 2001). The company at the centre of the outbreak had been found to have been mixing the denatured rapeseed oil with

others after the refinery process had taken place and so the aniline was never removed, and so this was being consumed with disastrous effects. It was this company that was found to be the root of the illness that came to be known as Toxic Oil Syndrome (TOS) (Posada de la Paz et al., 1996).

When it came to diagnosing TOS in patients, certain symptoms were required to be present (Table 1.4), these symptoms were categorised as major and minor. Initial guidance outlined by the Spanish Clinical Commissions specified that either 2 major symptoms were present or 1 major and 4 minor (Posada de la Paz et al., 2001).

Table 1.4. Listing of symptoms required for the diagnosis of TOS as outlined by the Spanish Clinical Commission (Posada de la Paz et al., 2001).

Major Category	**Minor Category**
Consumption of oil presumed toxic before onset of illness or occurrence of the illness in the nuclear family	Epidemic outbreak in the community
	Severe skin itching
	Rash or localised edema of the skin
Pulmonary pathology with radiologic findings of diffuse interstitial or alveolar interstitial infiltrates, with or without pleural effusion	Severe and persistent mouth dryness
	Neurologic pathology
	Minimal or moderate myalgias
Incapacitating myalgias with functional impairment	Abdominal pain
	Clinical or analytical signs of hepatic involvement
	Recent onset of exertional dyspnea (shortness of breath)
Eosinophil count greater than 500 eosinophils per mm^3	Recent onset of hypoxemia
	Pulmonary hypertension
	Cardiomyopathy
	Vascular thrombosis

Within 8 years of the incident, 839 deaths had been attributed to TOS, however this figure rose over the succeeding years. Close to 10 years later, the World Health Organisation (WHO) estimated that over 1600 of those who suffered from TOS

eventually succumbed to the illness, however it was stated that this could only be an approximate estimate of total deaths, whilst those involved with the production and selling of the oil were prosecuted (World Health Organisation, 1992).

1.4.3 Melamine milk scandal — 2008

Formula milk power is often used as a substitute for natural breast milk. Many parents opt to use formula over breast milk with the belief that it will provide sufficient nutritional value for their child's development. A 2008 publication in the Shanghai Daily reported the cases of 14 infants that had presented with kidney stones, all of whom had drank a specific brand of formula milk (Gossner et al., 2009). Instances of kidney stones and renal failure began to increase – with the consumption of the formula identified as the link between cases. In total 6 fatalities were linked and approximately 294,000 others were affected. It was found that, in order to make the protein content seem higher, a compound known as melamine was added intentionally. Melamine is commonly used in the production of plastics, with China being a top producer (Sharma and Paradakar, 2010).

After a full-scale investigation ensued, elevated levels of melamine were found to have been added to many other products such as cakes, biscuits, and liquid milk to name a few. As a consequence of this, approximately 68 countries reported that they would be applying restrictions on trade of food products from China. In terms of punishment, a number of those involved were convicted, with two of the culprits executed for their crimes (Gossner et al., 2009; BBC 2010).

1.4.4 Horsemeat scandal — 2013

One of the most high-profile cases of recent times bringing food fraud to the forefront of consumer attention, especially in the UK and the rest of Europe, was "The Horsemeat Scandal". In 2013 the Food Safety Authority of Ireland found the presence of horse DNA in multiple meat products, indicating that horsemeat was present. For example, testing of what should have been 100 % beef lasagne ready meals found a significant proportion of horsemeat present, triggering a wider investigation into other products whereby horsemeat was once again identified (Stanciu, 2015). The European Commission, (2014) announced that whilst fraudulent labelling occurred, this incident did not pose a risk to public health or food safety.

Whilst in this instance the fraudulent activity did not cause harm, the outcry may have been due to the consumers feeling duped into eating something they didn't agree to, effectively removing their choice and leaving them to feel vulnerable.

Whilst some of the most well-known, the above examples are only a slither of the cases that occur worldwide but show a variety of fraudulent techniques and the chaos that it can cause. It may be considered that in terms of food fraud, adulteration could be most harmful for consumers as the consumption of unknown substances can have potentially life changing impacts or even result in death. The techniques used by food fraudsters are constantly adapting as new technologies are implemented to try and outpace the development of authentication techniques.

The horsemeat scandal highlighted the importance of stringent food safety measures. Seeing how the scandal had such an impact on the food market, the Elliott Review was commissioned by Defra and the FSA. The review outlined 8 pillars of integrity that, when fully implemented together, would create a functioning system for tackling food fraud (Figure 1.2). (Elliott, 2014). It is the laboratory services pillar that encapsulates the focus of this thesis by exploring possible avenues for food authentication.

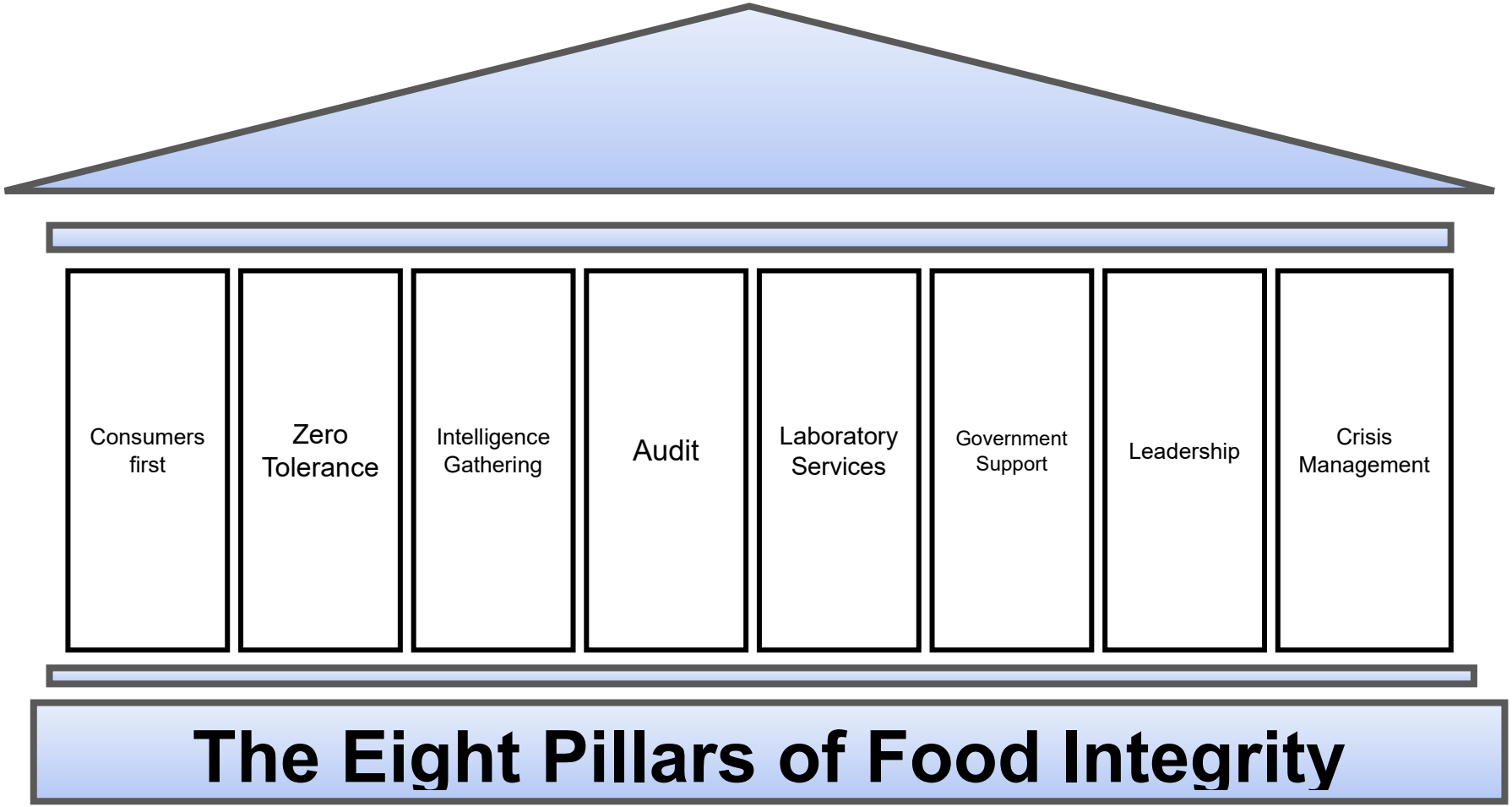


Figure 1.2. The Pillars of Food Integrity. These were outlined as the eight themes that would provide the backbone of a system geared towards monitoring and investigating food fraud (Elliott, 2014).

1.5 Techniques used within authentication procedures

Laboratory services are the lab-based procedures used when investigating food fraud. These methodologies should be standardised and have been proven to be effective in food testing (Elliott, 2014). There are a wide range of techniques used within UK testing and worldwide, and at times more than one technique may need to be applied in order to get a conclusive answer on the validity of a certain food product.

1.5.1 Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) has become a highly useful method when researching and investigating food authenticity. NMR focuses on the molecular structure of a compound by analysing how the nuclei react when exposed to a magnetic field (Kim et al., 2013). The benefit of using NMR within authentication is that the isotope ratio can be indicative of factors such as geographical origin, or even the climate conditions at time of growth. This could help in cases whereby a product claims to have PDO status and could be checked using NMR technology (Drivelos and Georgiou, 2012; Danezis et al., 2016).

NMR has been researched as an authentication tool when looking into coffee. In one experiment, NMR was used to distinguish between particular coffee types – Arabica and Robusta, as it was considered that Arabica could potentially be targeted for fraud due to its expense, with Robusta, a cheaper bean, blended in. The technique was considered a success with results showing that it was possible to distinguish Arabica from Robusta (Monakhova et al., 2015).

Another authentication experiment focusing on coffee looked into whether it would be possible to identify the degrees of roast. This could be a useful tool as roasting has been considered an important factor in how the coffee tastes, another factor that could determine price (Illy and Viani, 1996). The NMR process aimed to identify levels of various compounds, such as sucrose, present throughout the process. The analysis was deemed to be successful with suggestions that it could be utilised within authentication (Wei et al., 2012).

1.5.2 Polymerase chain reaction

Polymerase chain reaction (PCR) allows for the replication of specific DNA sections and can aid in identifying extraneous materials that have been added to a food product, such as an unknown meat sample or plant material from different species.

Quantitative PCR ((Q)PCR) in particular has been employed by the New Zealand government for use in tackling manuka honey fraud. This technique aims to detect and quantify DNA from manuka and differentiate this from any other DNA that may be present from other plants. The DNA is extracted and the manuka quantified, it should be noted that other plant species would be expected to be present due to the nature of bees, although manuka should be the dominant species (Ministry for Primary Industries, 2017). Guidance states that manuka honey can be deemed as such if it contains 70 % manuka (Moar, 1984).

Manuka honey is a popular export for New Zealand, often valued for its antibacterial activity. Research has found that manuka honey is often fraudulently blended with other honeys, some may add additional sugar, or document a false origin in order to make it look like the honey had originated from New Zealand (Loh et al., 2022). Knowing these methods of fraud, it is of clear importance to the New Zealand government to protect consumers from any fake manuka honey products by using a robust authentication technique.

NMR has also been researched as a manuka honey authentication measure, monitoring the levels of a specific compound within manuka honey, methylglyoxal (Donarski et al., 2010). This also highlights how methods can be used for a plethora of food products, enabling versatility within the area of food authentication.

1.5.3 Mass spectrometry

Mass spectrometry is considered a valuable tool within food authenticity, using the mass to charge ratio of molecules to analyse the ‘fingerprint’ of a sample. Mass spectrometry provides such a broad scope of techniques enabling it to be applied to a multitude of products from processed meats to truffles (Stachniuk et al., 2019; Segelke et al., 2020).

As previously noted, oils are a valued commodity within cuisine, with various types needed for certain dishes. One experiment focussed on the authenticity of seed and vegetable oils by using gas chromatography – mass spectrometry (GCMS) to establish the seed or vegetable constituents within various oil samples, as some oils may be adulterated with lesser value oils. It highlighted how the technique is effective as the technology is usually widely available within laboratories and relatively simple as an authentication measure (Mota et al., 2021).

The above outlined techniques have already been established as effectual tools for authentication. It should be noted, however, that this does not mean that new protocols cannot be established, just that any new methods should be standardised and produce accurate results (Elliot, 2014; Magnusson and Örnemark, 2014).

1.6 Molecular biomarkers in foods

Some food fraud can be tackled using standard investigative or accounting techniques that involve examination of appropriate paperwork. Often though, the only 'evidence' to examine is the food material itself. In these cases, regulatory authorities must rely on intrinsic biomarkers that provide information about the origin, quality, or composition of the food. As noted in relation to the horsemeat scandal, DNA is one example of a marker, although techniques for determining and interpreting nucleotide sequences can be complex and require expert knowledge. Foods typically contain very complex mixtures of other molecular components that can provide diagnostic signatures useful for fraud detection, as previously mentioned in cases such as honey. The focus of this thesis is on non-nucleotide biomarkers, namely polysaccharides and proteins.

1.6.1 Plant polysaccharides

Polysaccharides are the major components of plant-based foods and a major source of dietary fibre within grains (Harris and Smith, 2006). They have many roles in plant growth and development and can also be useful biomarkers for monitoring food quality. Polysaccharides, such as starch and fructans, are useful in facilitating storage for plants (Ottenhof and Farhat, 2004; Livingstone III et al., 2009) and construct the walls that surround almost all plant cells by forming matrices of pectins, cross-linking glycans, and cellulose microfibrils (McCann and Roberts, 1991; Carpita and Gibeaut, 1993). The fine structures of polysaccharides vary between plant species, organs, tissues and across developmental phases (Pettolino et al., 2012), and these differences could be exploited for food monitoring purposes. To do this though, the polysaccharides must first be extracted. Usually, this entails grinding the food material into a powder and then sequential extraction with solvents that promote the removal of polysaccharides from their cellular context. For example, cyclohexanediaminetetraacetic acid (CDTA) disrupts calcium-mediated pectin networks (Morris et al., 2009), and sodium hydroxide (NaOH) is used to extract hemicelluloses such and mannans, xylans and xyloglucans (Hamilton and Quimby, 1957). These extracts then allow for the polysaccharides to be profiled, as exhibited further within this thesis.

1.6.2 Polysaccharides and the cell wall

Polysaccharide-rich cell walls are a defining feature of plants and collectively the largest source of biomass on earth (Brandon and Scheller, 2020). A typical human diet contains very considerable amounts of plant cell wall material, delivered through plant-based foods such as fruit, vegetables, and grains. Although little or no nutrition is derived from these polysaccharides (since we have evolved the necessary enzymatic machinery to process them) they none the less are vital for gut health and maintaining a healthy microbiome (Song et al., 2021).

Cell walls have a variety of functions, they support the plant body, act as defensive barriers against biotic and abiotic stress and are a source of signalling molecules and developmental cues (Cosgrove, 2005; Voiniciuc et al., 2018). These diverse functions are reflected in diverse structures and as noted, considerable temporal and spatial variation.

The 'primary' walls that surround cells in growing tissues are constructed of a load-bearing matrix of cellulose microfibrils (β-1,4-linked glucan chains tethered together) enmeshed in a network of 'hemicelluloses' (Somerville, 2006; Keegstra, 2010; Houston et al., 2016). The term hemicellulose does not refer to a structural characteristic, but instead polysaccharides that can be extracted from the cell wall with the use of strong chaotropic solutions (Valent and Albersheim, 1974; Pauly et al., 2013). Hemicelluloses include xyloglucans, mannans, galactomannans, mixed-linkage glucans, glucans and xylans (Amos and Mohen, 2019). Some hemicellulose polysaccharides are able to form hydrogen bonds with cellulose microfibrils and some hemicelluloses are embedded within cellulose microfibrils (Keegstra, 2010). In primary walls, the cellulose/hemicellose network is embedded in a matrix of pectic polysaccharides. The primary cell walls of dicotyledonous plants contain approximately 35 % (by mass) of pectin although this level is substantially lower for graminaceous monocotyledons, such as wheat and maize (Fry, 1988; Voragen et al., 2009; Haas and Peaucelle, 2021). Pectins are based on backbone containing α-linked galacturonic acid and can be subdivided into a number of distinct structural forms including homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II (Figure 1.3) (Yapo, 2011; Willats et al., 2006; Vincken et al., 2003). As with other cell wall components, the fence structures of pectin vary widely according to species, organ tissue cell type, and developmental stress. The roles of pectins are equally diverse from involvement in cell wall porosity due to its influence on the production of gel formation, to functioning as a signalling

mechanism through the release of oligosaccharides when degraded by bacterial or fungal enzymes (McNeil et al., 1984; Rogers et al., 2000; Willats et al., 2001).

Figure 1.3. Structural sections within pectic polysaccharides. These diagrams highlight regions of homogalacturonan (a), rhamnogalacturonan II (b), and rhamnogalacturonan I (c). (Image sourced from Mohen, 2008).

The cells in mature parts of plants that have stopped growing, for example woody stems, are surrounded by 'secondary' walls whose primary function is structural support especially to bear compressive forces loaded by the weight of the plant above (Koch, 2004; Speck and Burgert, 2011; Cosgrove and Jarvis, 2012). Lignin is an important component of the secondary cell wall, however as a food quality biomarker lignin is a less attractive target than polysaccharide cell wall components since it is difficult to extract and isolate, and few molecular probes are available for its detection in comparison to polysaccharides (Mellerowicz and Sundberg, 2008; Achyuthan et al.,2010; Radotić and Mićić, 2016).

1.6.3 Proteins

The protein content of foods provides essential nutritional value, but proteins can also be problematic in terms of triggering allergic reactions (Table 1.5). Research from recent years has found that hospital admissions for anaphylaxis in children (under 14) has increased over the years, although deaths have decreased (Turner et al., 2015; Yu et al., 2016). It remains to be important that food is checked for possible cross contamination when it comes to allergens. There have been cases in the past whereby allergen inducing ingredients have made it into food products, as adulterants, cases of mislabelling, or cross-contamination, and these cases can have severe consequences.

With such importance placed on allergen testing, it would be appropriate that there are already established methods available. One such method commonly used is an Enzyme Linked Immunosorbent Assay (ELISA), which has proven to be successful in detecting gluten in food products by specifically targeting the gliadin protein. With the use of antibodies, a sample can be placed in a well. Binding of the antibody to the protein involved can induce a colorimetric response to inform if the allergen is present or not (Clark et al., 1986). Chapter 5 of this thesis will explore how antibodies could be used for allergy testing without the ELISA methods.

1.6.4 Antibody probing

Monoclonal antibodies have proven themselves to be highly beneficial within scientific research and medical applications, with their ability to be applied to multiple areas from cancer therapy, by targeting tumours, and inducing anti-tumour immune responses (Zahavi and Weiner, 2020), to being a possible preventative measure against malaria (Gaudinski et al., 2021).

Monoclonal antibodies (mAbs) are able to recognise specific epitopes, and therefore can specify if a certain structure is present, whereas polyclonal antibodies (pAbs) are able to recognise a multitude of epitopes which may be more relevant when not searching for specific structures or when wanting to assess a wider scope. The choice of which to use comes down to the specifics of the experiment being performed (Lipman et al., 2005).

Many studies have utilised antibodies as probes for targeting polysaccharides either to gather insight on the polysaccharides within a plant (Chateigner-Boutin et al., 2014; Wood et al., 2017; Cornuault et al., 2018), or a look at how polysaccharide profiles can

evolve during a food manufacturing process (Fangel et al., 2018). These experiments often use a wide range of mAbs to encapsulate the profile as best as possible.

As already mentioned, ELISA is one such technique that facilitates the use of both mono- and polyclonal antibodies. There are some ELISA tests that can be commercially bought to test for food allergies such as peanut or gluten, by targeting specific proteins associated with those food products (Table 1.5). Whilst it is beneficial to have these commercial tests, there is always an extra need for confirmed testing by medical professionals or certified laboratories. Using antibodies as a means of authentication testing is understandably an option when considering techniques for food authentication and safety.

Table 1.5. Common allergens. This table outlines some of the common food allergens and a selection of the proteins involved, as well as the family that the protein belongs to.

Food Product	Allergen Protein(s)	Protein Family
Peanut	Ara h 1 Ara h 3 Ara h 2 Ara h 6	Cupin Cupin Prolamin Prolamin (Mueller et al., 2014)
Milk	α-lactalbumin β-lactoglobulin Caseins Bovine serum albumin	Calcium metalloprotein Lipocalin Casein Albumin (Hiraoka et al., 1980; Shoormasti et al., 2016)
Wheat	ω-5 Gliadins Prolamins α-Amylase Inhibitors	Gliadin Prolamin α-Amylase inhibitors (Pasha et al., 2016)
Egg	Gal d 1 Gal d 2 Gal d 3 Gal d 4 Gal d 5 Phosvitin Apovitellenin	Serine protease inhibitor Serine protease inhibitor Transferrin Glycoside hydrolase Serum albumin Transferase Low density lipoprotein

		(Mine and Yang, 2008)

1.8 Thesis premise

The aim of this thesis was to create a high-throughput authentication technique focusing on using previously mentioned monoclonal antibody techniques with partially automated microarray technology. The overarching project focused on three different samples, focusing on specific targets for each (Table 1.6):

- Whole grains – Polysaccharide profile
- Saffron – Polysaccharide profile
- Gluten – Presence of gliadin protein

Chapters 3 and 4 utilise the uniqueness of polysaccharide profiles to look specifically into food authentication. Chapter 3 focuses on using mAb probing to look into specific profiles of different grains and its subsequent layers, as well as different types of whole grain products such as flour or semolina. Using this technique, it was possible to see differences in polysaccharide profiles of different grains as well as polysaccharide differences in layers of the same grain.

Chapter 4 focuses on analysis of saffron with the utilisation of polysaccharide profiles to investigate the authenticity of samples. Authenticity was questioned by comparing collected samples to reference samples of known quality. This chapter also uses fluorescence microscopy with fluorescent probes allowing for the visualisation of polysaccharide localisation within saffron strands.

Chapter 5 describes the development of a high-throughput screening process for allergens in products – specifically targeting proteins. This chapter focuses specifically on gluten, targeting the gliadin protein due to the commercial availability of gliadin specific antibodies and the presence of gliadin in gluten.

Whilst the three experimental chapters are stand-alone experiments, the methodologies used are consistent throughout. All three pertain to the development of a high-throughput technique to be used for food authentication, with the main method being microarray printing coupled with mAb probing.

Table 1.6. Proposed experiments. This table outlines the food products that will be focused on in this thesis. Also included are the targeted components of the products and the methods that will be used.

Focus of Chapter/ Chapter Number	**Target**	**Methods used**
Whole Grains (3)	Polysaccharides	• Polysaccharide extraction • Microarray printing • Probing with monoclonal antibodies
Saffron (4)	Polysaccharides	• Polysaccharide extraction • Microarray Printing • Probing with monoclonal antibodies • Fluorescent microscopy • Dye analysis
Gluten (5)	Gliadin protein	• Protein extraction • BCA protein assay • Dot blot • Microarray printing • Probing with monoclonal antibodies

Chapter 2: Methods

This chapter includes any methodologies that are used in more than one chapter (Figure 2.1).

2.1 Alcohol Insoluble Residue (AIR) preparation

Samples were not weighed as specific weights were not required at this point. The samples were ground by pestle and mortar in liquid nitrogen. The samples were then transferred to a microcentrifuge tube (1.5 mL), and mixed with 70 % ethanol (70 % ethanol, 30 % distilled water, 1mL). The samples were centrifuged (13000 rpm, 10 minutes) and the supernatant was discarded. The previous steps were repeated with methanol:chloroform (1:1 ratio, 1 mL), and acetone (100 %, 1 mL). The remaining pellet was left to dry overnight. Any sample not used after the AIR process was stored in an airtight container in a cool, dark place.

2.2 Comprehensive polysaccharide profiling process

Methods 2.2.1 – 2.2.3 outline the comprehensive polysaccharide profiling methodology as outlined by Moller et al., (2012) that were followed for these experiments, with minor alterations as noted.

2.2.1 Glycan extraction

For each sample triplicates were prepared separately. The AIR prepped samples (10 mg) were suspended in 30 µL/mg (300 µL) CDTA (50 mM, pH 7.5). Steel beads were added to the samples (5 mm), and the samples lysed using a tissue lyser (Qiagen II, 27 s^{-1}, 2 minutes, then 10 s^{-1}, 2 hrs). The samples were centrifuged (13,000 rpm, 10 minutes), and the supernatant decanted and stored at 4 °C. The remaining pellet was mixed with 30 µL/mg (300 µL) NaOH (4 M + 0.1 % $NaBH_4$) and lysed in the same conditions. The samples were centrifuged (13,000 rpm, 10 minutes) and supernatant stored at 4 °C the pellet was discarded. An alteration was made to the Moller method with the removal of the cadoxen extraction as it was deemed to be more abrasive for these experiments (Zaccaron et al., 2022). Any alterations for specific experiments regarding glycan extraction have been outlined in the relevant chapters.

2.2.2 Microarray printing

Post extraction, the samples were printed in preparation for probing. The samples were printed in 4 serial dilutions, being diluted with system buffer (540 mL of 86-89 % glycerol solution, 800 µL of 6 % Triton-100, 400 µL of Proclin, made up to 1 L with water), at a dilution series of 1:2, 1:10, 1:40 and 1:100, sample:buffer. The wells in the 384 well plate (Greiner Bio-one) were filled with 40 µL of the sample and dilution mixture. The printing was performed using an ArrayJet ink-jet microarray robot, at 20 °C and 50 % humidity. The printhead was automatically washed 4 times between sample prints to prevent contamination. All samples were printed onto a nitrocellulose membrane (0.2 µm, Amersham™ Protran) that would facilitate the monoclonal antibody probing.

2.2.3 Monoclonal antibody probing

The array was incubated in a milk solution for 1 hr (5 % w/v milk protein in TBST; 20 mM Tris-HCl, 140 mM NaCl, pH 7.5 and 0.1 % Tween-20, v/v) to block the membrane. The volume of milk solution used was dependent on the size of the well-used but should be enough to submerge the array. The array was incubated for 1.5 hrs in the milk blocking solution with a chosen primary antibody (Table 2.1) (1:10 dilution, or appropriate dilution according to antibody manufacturer suggestion). The array was washed thoroughly in TBST at least 3 times over the course of 30 minutes. The array was incubated for 1.5 hrs in the blocking solution and an appropriate secondary antibody (usually mouse (Anti-Mouse IgG (whole molecule)–Peroxidase antibody produced in rabbit, Sigma Aldrich) or rat (Anti-Rat IgG (whole molecule) −Peroxidase antibody produced in rabbit, Sigma Aldrich)) conjugated with alkaline phosphatase (1:1000 dilution). The array was washed thoroughly in water at least 3 times over the course of 30 minutes. An enzyme marker substrate was made according to Bio-Rad NBT-BCIP protocol (BioRad Laboratories, Online protocol), the membranes were left to develop in the solution for 20-30 minutes. The reaction was stopped by placing the array in water, then left to dry on filter paper until completely dry (around 1hr) before analysis.

Table 2.1. A list of the monoclonal antibodies used within these experiments, their specificity, and the experimental chapter they were used in.

Monoclonal Antibody Name	Antibody Specificities	Chapter(s) used	Reference
LM1	Extensin	3 4	Smallwood et al., (1995)
LM2	Arabinogalactan protein (AGP)	3 4	Smallwood et al., (1996)
LM5	(1 → 4)-β-D-galactan	3 4	Jones et al., (1997)
LM6-M	(1-5)-α-L-arabinan	3 4	Cornuault et al., (2017)
LM7	Homogalacturonan	3 4	Willats et al., (2001)
LM8	Xylogalacturonan	4	Willats et al., (2004)
LM9	Feruloylated-(1-4)-β-D-galactan	4	Clausen et al., (2004)
LM10	(1 → 4)-β-D-xylan	3 4	McCartney et al., (2005)
LM11	(1 → 4)-β-D-xylan/arabinoxylan	3 4	McCartney et al., (2005)
LM12	Feruloylated polymers	4	Pedersen et al., (2012)
LM13	Linear (1 → 5)-α-L-arabinan	3	Moller et al., (2007)
LM14	Arabinogalactan Protein	3 4	Moller et al., (2007)
LM15	Xyloglucan	3 4	Marcus et al., (2008)
LM18	Homogalacturonan	4	Verhertbruggen et al., (2009)
LM19	Homogalacturonan partially/de-esterified	3 4	Verhertbruggen et al., (2009)

LM20	Homogalacturonan	4	Verhertbruggen et al., (2009)
LM21	Heteromannan	3 4	Marcus et al., (2010)
LM22	(1 → 4)-β-D-(gluco)mannan	3 4	Marcus et al., (2010)
LM23	Xylosyl	3 4	Pedersen et al., (2012)
LM24	Xyloglucan	3 4	Pedersen et al., (2012)
LM25	Xyloglucan/unsubstituted β-D-glucan	3 4	Pedersen et al., (2012)
LM27	Grass xylan preparations	3 4	Cornuault et al., (2015)
LM28	Glucuronoxylan	3 4	Cornuault et al., (2015)
JIM5	Homogalacturonan	4	Knox et al., (1990); Clausen et al., (2003); Verhertbruggen et al., (2009)
JIM7	Homogalacturonan	4	Knox et al., (1991); Clausen et al., (2003); Verhertbruggen et al., (2009)
JIM8	Arabinogalactan protein	3 4	Pennell et al., (1991)
JIM11	Extensin	3 4	Smallwood et al., (1994)
JIM12	Extensin	3	Smallwood et al., (1994)
JIM13	Arabinogalactan protein	3 4	Knox et al., (1991)

INCH 1	Starch	3	Rydhal et al., (2017)
M139	Xylan	3	Pattathil et al., (2010)
M140	Xylan	3	Pattathil et al., (2010)
M143	Xylan	3	Pattathil et al., (2010)
M149	Xylan	3	Pattathil et al., (2010)
M151	Xylan	3	Pattathil et al., (2010)
M157	Xylan	3	Pattathil et al., (2010)
MLG	(1 → 3;1 → 4)-β-D-glucan	3 4	Meikle et al., (1994)
Anti-Gliadin antibody	Gliadin	5	Abcam - Anti-Gliadin antibody [14D5]

2.2.4 Microarray analysis

The array was attached to card, and scanned using a scanner (2400 dpi, greyscale), and TIFF file images were created. Once scanned the image was analysed using ArrayPro software to determine binding intensity. The resultant .txt files were imported into an excel macro file used for array analysis (Kračun, 2012). After processing the raw data with the analysis software, the triplicate samples were averaged, with one reading for each dilution. After that an average was taken of the four dilutions, leaving one number to represent each sample. The highest reading was given a value of 100, and all other samples were normalised to this amount using the following equation:

$$\frac{Relative\ abundance\ value\ of\ sample}{Highest\ relative\ abundance\ value}\ x\ 100$$

A colour scale was created to show the relative abundance of different polysaccharide epitopes for different monoclonal antibodies from lowest to highest.

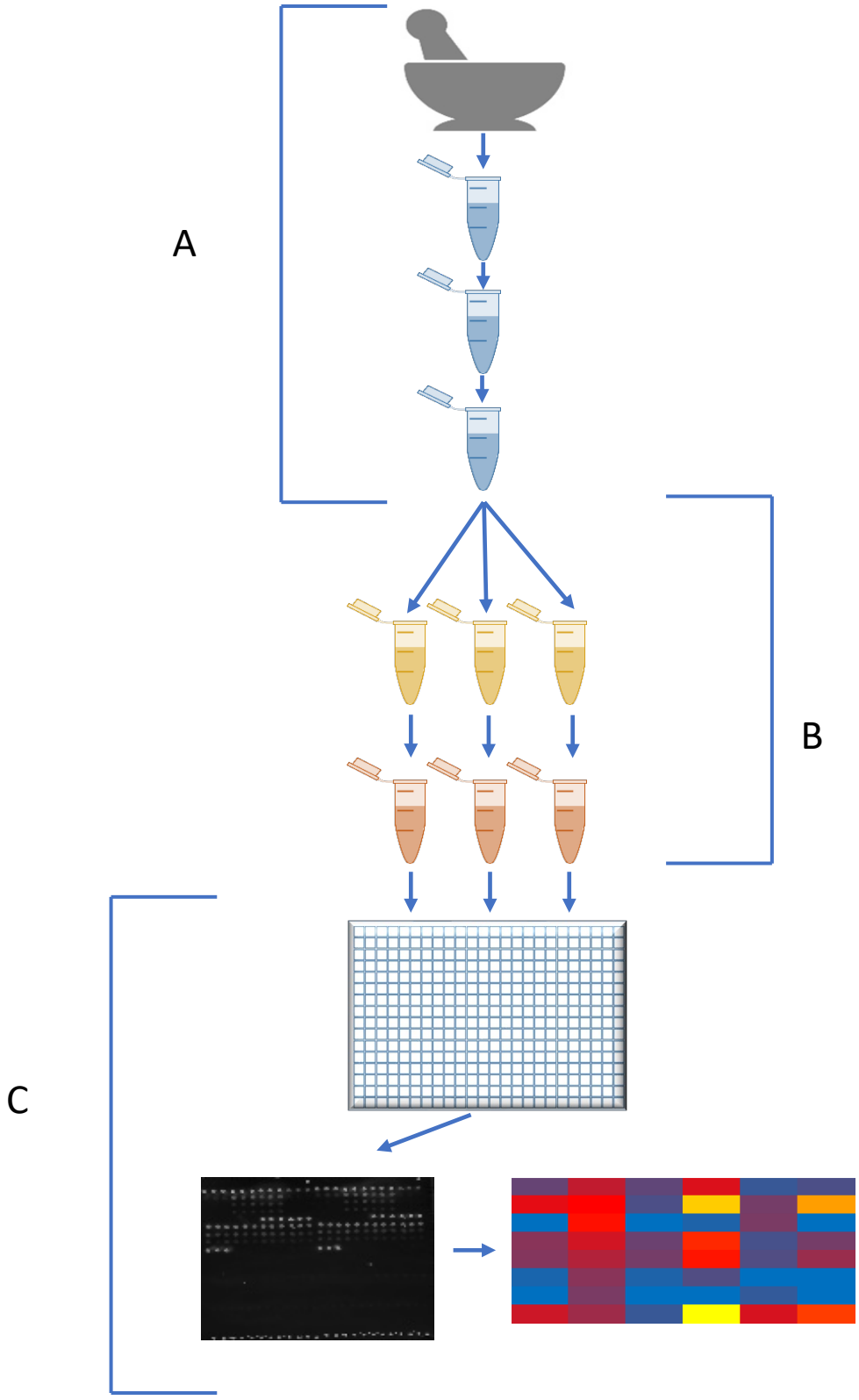


Figure 2.1. A schematic outlining the CoMPP process. Samples were all ground using a pestle and mortar until they resembled a fine powder (A). Alcohol insoluble residue (AIR) was prepared using; Ethanol (70 %), methanol, chloroform, and acetone. 3 biological replicates (10

mg) were taken from the AIR for the glycan extraction. A set of sequential extractions is performed (CDTA and NaOH) (B). The extracts were put into a 384 well plate and printed onto a nitrocellulose membrane prior to probing with selected monoclonal antibodies. Using the intensity readings, a heatmap was created to illustrate the diversity of extractable polysaccharides (C).

Table 2.2. The methods described within this chapter and which chapters they have been used in.

Methodology and paragraph reference	Chapters used
Alcohol insoluble residue preparation (2.1)	3,4
Glycan extraction (2.2.1)	3,4
Microarray printing (2.2.2)	3,4,5
Monoclonal antibody probing (2.2.3)	3,4,5
Microarray analysis	3,4

Chapter 3. Whole Grains

3.1 Introduction

Dietary scrutiny has increased over recent years, with health foods rising in popularity. Nutritional information displayed on the front of packaging, akin to a 'Traffic-light system', may be one factor that aids consumers in choosing healthier products. Social media may also play a role in the increasing popularity of healthy eating, as some 'influencers' have become 'health gurus' and in doing so amass a large following, notably with younger generations, based off their diet and exercise tips. Some studies have even suggested that the use of social media 'influencers' within healthy eating campaigns could be beneficial in terms of success (Folkvord et al., 2020).

Whole grains (WG), for thousands of years, have been a staple health food, and their health benefits are extensively documented via systematic reviews and studies involving large cohorts (Aune et al., 2013; Hullings et al., 2020; Prasadi and Joye, 2020; Seal et al., 2021). It is often noted that in some areas, grains contribute a considerable portion of daily calorie intake (Awika, 2011; Shiferaw et al., 2013) indicating the importance WGs have on most daily diets. The global market for WG products is rapidly expanding. Growing demand has elevated the need for a standardised monitoring system regarding WG product quality, with the main focus of this study being on wheat varieties.

The grain itself is formed of 3 distinct sections: bran (14-16% of composition), germ (2-3%), and endosperm (81-84%) (Pomeranz, 1988) (Figure 3.1). The Healthgrain forum considers the most accurate definition of a WG as being a grain consisting of 'the intact, ground, cracked or flaked kernel after the removal of inedible parts such as the hull and husk'. They also specify that once ground, the components should be 'present in the same relative proportions as they exist in the intact kernel' (Van der Kamp et al., 2014). It is important to note that this is not legislation but advice that could be easily adopted across the EU to give uniformity. The Healthgrain forum also decided upon what products could be considered as a whole grain, with a definitive list of cereals and pseudocereals (Table 3.1).

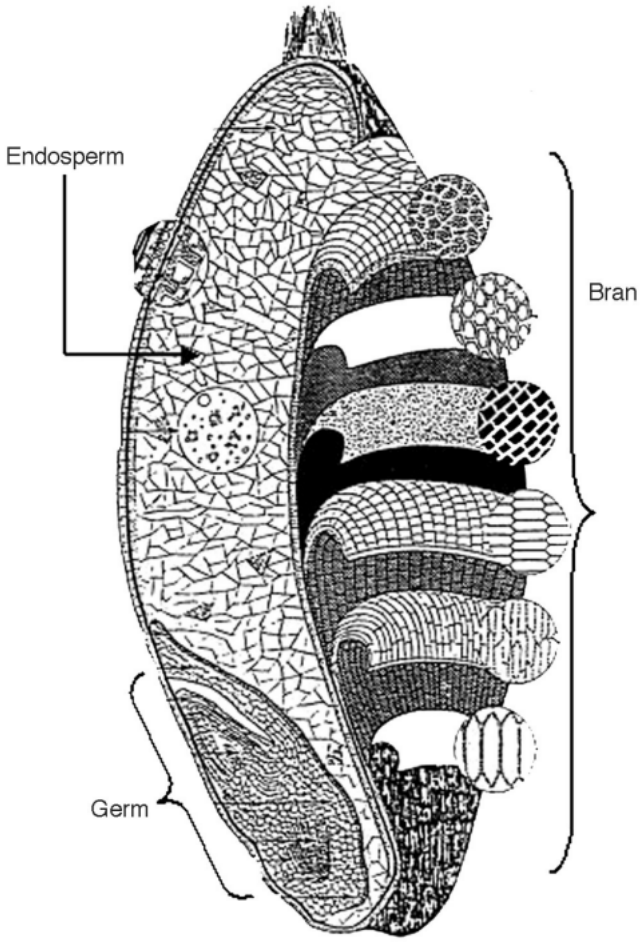


Figure 3.1. Anatomy of a whole grain. A diagram outlining the 3 distinct sections of a whole grain (Diagram sourced from Slavin, 2004).

Table 3.1. Classification of Whole grains. This table outlines the cereals that the Healthgrain forum deemed suitable for WG classification. Some grains may lose their WG status after certain milling processes (such as pearled barley). A cereal is defined as a type of grass whereby its grain is used for consumption. Adapted from Van der Kamp et al., (2014).

Cereal	**Scientific Name**
Wheat; spelt, emmer, faro, einkorn, khorasan wheat (Kamut), durums	*Triticum* spp.
Rice; brown, black, red, and others	*Oryza* spp.
Barley (not including pearled)	*Hordeum* spp.
Maize	*Zea mays*
Rye	*Secale* spp.

Oats, including hull-less or naked	*Avena* spp.
Millets	*Brachiaria* spp., *Pennisetum* spp., *Panicum* spp., *Setaria* spp., *Paspalum* spp., *Eleusine* spp., *Echinochola* spp.
Sorghum	*Sorghum* spp.
Teff	*Eragrotis* spp.
Triticale	*Triticale*
Canary seed	*Phalaris* canariensis
Job's tears	*Coizlacryma-jobi*
Fonio, black fonio, Asian millet	*Digitaria* spp.

3.1.2 Whole grain anatomy

Whole grains have 3 main components: bran, germ, and endosperm. Several studies have focused on the possible benefits of bran and the germ in particular as separate entities (Stevenson et al., 2012; Jefferson and Adolphus, 2019; Mohammadi et al., 2019; Salehi-Sahlabadi et al., 2022). When discussing the classification of WG products the bran is the most important.

The bran consists of several layers located at the outer region of the grain and is often considered the fraction of the grain with the largest benefits when consumed. This is due to the presence of various beneficial components such as fibre, in addition to a variety of B vitamins. Koh-Banerjee et al., (2004) focused on the health benefits of a bran enriched diet. This study found that increased bran consumption aided weight loss, with no significant correlation witnessed in diets involving refined grains or added germ intakes. Within wheat, dependent upon the chosen milling process, bran can account for between 14% and 16% of the overall composition.

Within the overall grain cell wall, the most predominant non-starch polysaccharides are β-Glucans, arabinoxylans and cellulose account for approximately 10% of the cell wall, and starch accounting for around 75% of the cell wall (Stone and Morell, 2009; Andersson et al., 2013). The levels of these polysaccharides fluctuate dependent upon species, but these can also differ due to growth conditions. In terms of wheat, studies show a higher level of arabinoxylans in comparison to barley and oats. Xyloglucans,

mannans and arabinogalactans are also present but in lesser amounts (Lafiandra et al., 2014).

3.1.3 Milling

The milling process allows for the production of various WG products, including semolina, middlings and flour. Archaeological evidence suggests that a primitive form of modern milling was developed around 28000 BC, likely developed in order to produce flour (Revedin et al., 2010). Milling allows for the separation of fractions, with the sections being reintroduced or certain portions missed out – in the case of 'white flours' whereby the bran is discarded (Table 3.2).

Table 3.2. Brief explanations as to the definitions of the milling fractions.

Milling Fraction	**Definition**
Grain	The whole, intact grain with only the inedible outer husk removed
Middlings	The fractions left over from the production of white flour
Wholemeal Flour	The intact grain (minus inedible husk) ground into a flour
Semolina	The endosperm fraction, not ground as finely as flour
White Flour	The endosperm fraction, ground into a fine powder
Bran	The outer layer of the grain, once the inedible husk has been removed
Pearled	The intact grain after the bran has been removed

3.1.4 Uses of whole grains

One of the benefits of WGs is it's use for multiple food products:

- Bread
- Pasta
- Semolina (dependent upon milling conditions)
- Flours
- Crackers

Not all products made with WGs however should be considered WG products. Products would need to conform with the Healthgrain Forums definition and keep the same proportions as the intact grain. For this reasoning, white flours, and their derivatives, are not considered WG products.

3.1.5 Health benefits of whole grain consumption

Over the years there have been many studies involving varying sized cohorts into the correlation between consumption of WG products and the diminishing risk of the development of numerous diseases. Research suggests a positive link between WGs and lowered risk of ailments such as coronary heart disease (CHD) and type 2 diabetes, hence it can be understood why the inclusion of WGs in our diets is so fundamental (Aune et al., 2016; McRae, 2017; Seal et al., 2021).

3.1.6 Modern vs ancient grain types

Increase in WG consumption has in turn, allowed the spotlight to be shone on ancient grains and their perceived benefits over modern highly cultivated grains. Examples of ancient grains are kamut, spelt, emmer, and einkorn, with the latter two documented as the first cultivated wheat species (Dubcovsky and Dvorak, 2007).

Ancient grains are those that have not been subjected to hybridisation or intense selective breeding, subsequently there are some who perceive ancient grains as more beneficial than modern grains. It could be believed that ancient grains are more nutritious due to the lack of selective breeding which led to modern larger, more carbohydrate rich grains, and therefore are more beneficial as a health food. Published data from trials that have been conducted are currently few and far between, however do look promising (Valli et al., 2018). The trials themselves are often conducted on small numbers of cohorts and the findings not conclusive enough to establish a clear verdict on which grouping is healthier (Dinu et al., 2018). If ancient grains were to be

considered more valuable, this could lead to cases of adulteration within whole grain products.

3.1.7 Popularity with current consumers

Market research conducted by the International Food Information Council (2021), found that of 1014 American consumers interviewed, 46% were trying to increase their intake of WG products. The awareness of the benefits of WG foods is there, and market demand continues to increase. During 2020, whole grain market value was estimated at $44.3 billion, with an expected increase to $57 billion by 2027. Whilst recent years have seen an elevated demand in the WG sector, consumption itself is still below the recommended levels for most consumers (International Food Information Council, 2021).

3.1.8 Authentication of whole grain products

Due to there being no definitive standard of whole grain classification, authentication of whole grain products in terms of monitoring levels of whole grains is redundant. However, there have been studies focusing on authenticating products that claim to include specific types of grain, or certain geographical origins.

One study proposed using isotopic composition as a means of authentication. The idea behind this was that isotopic composition should vary dependent upon geographical origin. By identifying location, this could help when grain products claim to have PGI status. Wheat samples were collected from various regions within India, and levels of δ15N and δ13C were measured using isotopic ratio mass spectrometry. The study concluded that whilst δ15N could not be used in a discriminatory capacity, δ13C was a viable option with significant differences reported between the samples as levels of δ13C can be influenced by geographical variances such as temperature, altitude, and latitude (Rashmi et al., 2017).

Another study investigated how to distinguish between different Triticum species. Durum wheat is known for incurring a higher market price than other wheat types, such as common wheat. Knowing this, direct analysis real time-high-resolution mass spectrometry was applied to over 60 samples of differing wheat species. Analysis showed the presence of 18 markers that would aid in distinguishing between species and could protect against fraud within grains (Miano et al., 2018).

These studies highlight some of the techniques employed in the area of whole grain product authentication. This chapter will demonstrate how microarray technology and monoclonal antibody probing can be applied to whole grain authentication.

3.1.9 The current issue

As previously discussed, there is no legislation dictating the required amount of WG for foodstuffs to be considered a 'Whole grain product'. It would be sensible to assume that most consumers are unaware of how a WG portion is defined and are consequently oblivious as to the amount of WGs in the product of choice.

Much of the current issue comes down to inconsistent standardisation between countries as well as a lack of guidance on what classifies as a WG product. Most countries have their own guidance on what a portion of WG should be considered as (Table 3.3).

In 2017, the Healthgrain Forum published guidance on how we should classify what a WG product is. The purpose of this was to aid in helping consumers make an informed decision, something which is important when aiming for a healthy diet. They recognised the lack in consistency when it came to definitions, and so produced advice that could hopefully be used by all to create a linear understanding that would be easy for companies and governments to adopt, as well as consumers to follow. The resulting verdict was a statement that declared:

> "… a food may be labelled as "whole grain" if it contains ≥30% whole-grain ingredients in the overall product and contains more whole grain than refined grain ingredients, both on a dry-weight basis" (Ross et al., 2017).

Table 3.3 This table outlines some of the varying recommendations for whole grain intake from around the globe (Mann et al., 2015; Whole Grains Council, 2021).

Country	**Recommendation**
Australia	6 servings (Adult aged 19-50) • 1 serving is equal to: ½ cup of porridge • 2/3 cup of cereal flakes • 1 slice of bread ½ cup of cooked rice, pasta, or noodles ¼ cup muesli
Denmark	Based on a diet of 2400 calories: • 75 g whole grains daily Based on a diet of 2000 calories: • 63 g whole grains daily
Mexico	'Consumption of cereals is recommended, preferably whole grains'
Netherlands	Recommendations for ages 9+ • Ages 9-13 or 70+, 4-5 slices of whole grain bread • Ages 51-70, 5-6 slices of whole grain bread • Ages 14-50, 6-7 slices of whole grain bread
United States	For ages 9+ • At least 3 servings of whole grains
United Kingdom	There is currently no official advice on whole grain consumption.

When choosing WG products, consumers are faced with varying categories relating to the whole grain composition itself. There are three main groupings: whole grain, refined grain (RG) and enriched grain (EG). Refined grains are renowned for yielding the least nutritional benefits due to the removal of the bran and germ. Enriched grains, also referred to as fortified, are commonly refined grains with select nutritional values added such as folic acid (Brent and Oakley, 2005). It is possible that consumers could be

deceived into believing a refined grain product is actually whole grain, leading to the need for a process that could distinguish different sections of a grain's anatomy.

There are schemes that aim at informing consumers that the product does contain at least one serving of WG per portion (by US standards). One such scheme has been trialled by the Whole Grains Council within the US, whereby labels are applied to WG products and correlate to the amount of WG in a product. The labels are:

- 100% - All of the ingredients are WG, with a minimum of 16g (one portion) within the product.
- 50+% - At least half of the composition of this product is WG, a minimum of 8g is required (half portion), not inclusive of refined grains.
- Basic – This product must contain at least 8g (half portion), however it may be composed of more refined grain.

It should be noted that as of 2021, the scheme has also been rolled out for various products within 22 countries, including the UK, using the standards as described above (Whole Grains Council, 2021). Schemes such as this may be beneficial in allowing the consumer to make an informed decision on which product may be best for them, although there is still a need for authentication tools to identify the validity of the claims made.

3.1.10 Aims

Combining the uniqueness of the polysaccharide profiles with monoclonal antibody probing, a robust authentication process for plant-based food products was formed. Moller et al., 2012, outlined an effective method for glycan extraction.

With the knowledge of high abundance of starch within grain samples (page 19), constituting between 65-75% of the whole grain (Stone and Morell, 2009), it was deemed suitable for a start-up experiment to be conducted. This would aid in determining the final process that would be applied within the analysis, in addition to concluding how the presence of starch would affect any polysaccharide profiles and so a select set of samples were subject to destarching measures prior to polysaccharide analysis. The cadoxen extraction was substituted with a cellulase extraction as it was deemed a more appropriate extraction technique for the aim of this experiment.

Articles have been published demonstrating the effectiveness of tracing polysaccharides through a food production process via the extraction and probing technique. Fangel et al., (2018), studied the polysaccharide profile of at different stages of beer production; from the grain, through the brewing process, to the final bottled product. A component of this experiment focused on the polysaccharide profile of the barley grain. This study proved the methodology for the analysis of grains through their polysaccharide profile, a process that would be further adaptable for use on a wider variety of whole grains.

The overall aim of this experiment is the development of a technique that could be used to identify wheat species, a group of crops that shared a close breeding history. In order to facilitate identification, it was idealised that specific sequences of mAb probes would be utilised to identify both specific grain species, in addition to their separate fractions, using probes for starch or arabinoxylans which are already known to be abundant (Lafiandra et al., 2014). By developing an adequate analysis protocol, it would pave the way for enabling the identification of whole grains and separate fractions present within a whole grain product. Whilst not a quantitative method, by profiling the grain as a whole, it can be determined how the ratio of the polysaccharides within different fractions of the grain should present. The aim is that this procedure would be beneficial in identifying WG levels within WG products.

The main aim for this experiment was:

- To develop a technique that could be used to identify whole grain species, specifically wheat

The grains chosen are wheat varieties in varying states of processing i.e., semolina. Some of these may not be considered as WG products if their milling processes meant that the resulting product was not in line with the Healthgrain Forum's recommendation.

3.2 Methods

3.2.1 Sample Acquisition

Grain samples were provided by two separate milling companies: Silvery Tweed and Gilchester. Whilst sample species varied, all were grain varieties (Table 3.4). The samples were used for different experiments, demonstrating that the technique is applicable to a wide range of samples. The samples were procured in various states,

from bran, to semolina and white flour. All samples were ground to the same consistency, a fine powdered form. An initial start-up experiment was performed to ascertain the suitability of choosing to study grains in this way.

Table 3.4 Grain samples. These tables represent the samples and milling fractions used within this chapter. The left-hand side shows the Silvery Tweed samples, and the right-hand side shows the Gilchester samples.

Silvery Tweed Species	**Milling Fraction**
Wheat	Pearled
Wheat	Heavy Fraction
Triticale	Pearled (1 min)
Triticale	Pearled (2min)
Waxy Barley	Heavy Fraction
Waxy Barley	Pearled
Spelt	Pearled
Spelt	Heavy Fraction
Barley	Pearled
Wheat	Flour
Waxy Barley	Grain
Barley	Flour

Gilchester Species	**Milling Fraction**
Emmer	Grain
Einkorn	Grain
Filderstolz	Grain
Oberkulmer	Grain
Modern Wheat	Middlings
Modern Wheat	Wholemeal Flour
Modern Wheat	Semolina (Fine)
Modern Wheat	White Flour
Modern Wheat	Grain
Modern Wheat	Bran
Heritage Wheat	Semolina (Fine)
Heritage Wheat	Wholemeal Flour
Heritage Wheat	Grain
Heritage Wheat	White Flour
Heritage Wheat	Semolina (Coarse)
Spelt	Bran

Spelt	White Flour
Spelt	Middlings
Spelt	Grain
Spelt	Wholemeal Flour
Emmer	Wholemeal Flour
Emmer	Grain
Einkorn	Wholemeal Flour
Einkorn	Grain

3.2.2 Work up experiment

Fangel et al., (2018) had already demonstrated the viability of using the CoMPP process (Chapter 2.2) on grain samples, this method was compared to one developed by Wood et al., (2017) to decide which process would be most appropriate for this experiment.

Select Silvery Tweed samples of varying milling fractions (Figure 3.1) were used for this experiment. AIR preparation (Chapter 2.1) was conducted on all samples prior to the glycan extractions taking place. The same selected samples were used for both protocols.

Samples were subjected to the glycan extraction process as outlined in chapter 2 (Chapter 2.2.2).

A fresh set of samples were subjected to the glycan extraction process as outlined by Wood et al., (2017), with minor alterations made for suitability purposes within this experiment – the extraction time was reduced to 6 hrs instead of 12 hrs. 10 mg of AIR prepped sample was added to a 1.5 mL Eppendorf and 200 µL of ammonium oxalate (50 mM) was added and extracted for 6 hrs at room temperature. The sample was centrifuged (13,000 rpm, 10 minutes), and the supernatant was stored (4 °C). The process was repeated on the left-over pellet with 3 more solvents: Na_2CO_3 (50 mM) + $NaBH_4$ (0.5 % wt/vol), 1 M KOH + $NaBH_4$ (1 % wt/vol) and 4 M KOH + $NaBH_4$ (1 % wt/vol).

Extractions from both protocols were printed onto the same microarray, before being probed and analysed (Chapter 2.2.2 – 2.2.4, Table 2.1, Figure 3.1). The resultant relative abundance values were normalised to the highest value achieved over both extraction protocols. After analysing the values, it was decided that the experiment would go ahead using the Moller et al., (2012) CoMPP protocol, this was mainly due to the compact nature of the process (4 hrs total extractions vs. 24 hrs) whilst still giving a descriptive look into the extractable polysaccharides.

3.2.3 Secondary experiment

Destarching

A small sample set of the Silvery Tweed samples were selected (Figure 3.3) for a trial experiment to investigate what effect destarching would have on the samples and if it would be a beneficial addition to the protocol.

Samples were de-starched via an α-amylase treatment (α-Amylase, from Bacillus amyloliquefaciens, Sigma), using 10 mg of AIR prepped material, 240 µL of α-amylase and 260 µL of phosphate buffer (10 mL 0.1 M disodium phosphate, 90 mL 0.1 M monosodium phosphate, pH 5.9) was added. The sample was incubated at 80 ºC on a heat block shaker for 24 hrs. 500 µL of ethanol was then added to the sample before being centrifuged (13000 rpm, 10 minutes). The supernatant was discarded, and the pellet was allowed to air dry. The process was repeated once more and again the supernatant discarded and the pellet left to dry. This process took place after AIR preparation but before CoMPP. Destarching was carried out on one set of samples, with a duplicate set not subjected to the treatment

Cellulase Treatment

The second experiment investigated 2 different protocol additions; destarching and cellulase treatment.. Both sample sets from the destarching experiment were treated with cellulase as a final step in the glycan extraction process (Chapter 2.2.1).

After the CDTA and NaOH extractions had occurred, the remaining pellet was subjected to cellulase treatment, Cellulase 5A (from Bacillus spp., Nzytech Lisbon, Portugal). The experiment was carried out following the protocol outlined by Hakamada et al. (1997). The samples were incubated at 45 ºC for 16 hrs, with the sample centrifuged (13,000 rpm, 5 minutes), and supernatant stored at 4 ºC once the treatment had ended.

Heatmap and data analysis

The destarched and non-destarched samples were printed (Chapter 2.2.2) and probed (Chapter 2.2.2, Table 2.1) before analysis (Chapter 2.2.4). The sample sets produced separate heatmaps, the destarched samples were normalised to their highest amount and the non-destarched were normalised to the highest reading for that protocol (Figure 3.3).

After analysis of both heatmaps, it was decided that the destarched and non-destarched samples provided different views that could not be discounted. Going forward there would be 2 sample sets; a destarched and a non-destarched. It was also found that cellulase was a beneficial addition and would be added to this process.

3.2.4 Final experiment

The process was finalised after deciding the parameters that would be used. For this experiment a larger sample set of the Gilchester's samples was used (Table 3.4). The samples were AIR prepped, and then the CoMPP process, with the addition of the cellulase extraction (Chapter 2.2.1-2.2.4) was duplicated for destarched and non-destarched samples, with additional monoclonal antibodies being used in the probing stage (Table 2.1). This process produced two separate heatmaps, with destarched and non-destarched samples being analysed separately and normalised within their own grouping.

3.2.5 Hierarchical clustering

Hierarchical clustering was performed on the non-destarched full data set after the heatmap had been created. Single-linkage cluster analysis was performed on heatmaps for each solvent separately using cluster analysis software and selecting cluster analysis, and single-linkage analysis (Genesis 1.8.1, Genomics & Bioinformatics Graz, Graz University of Technology).

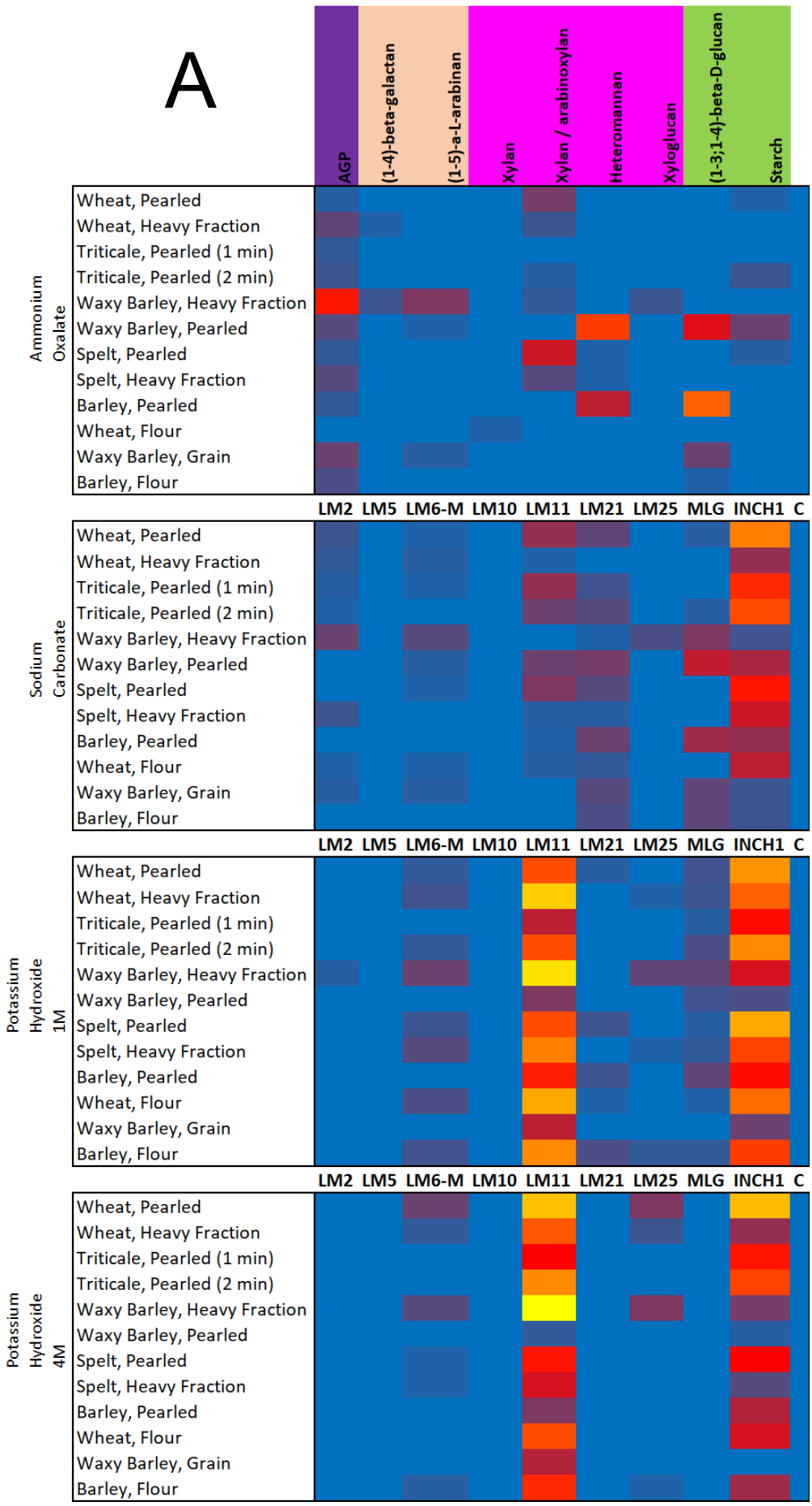
3.3 Results and Discussion

Compiling polysaccharide analyses of differing cereal fractions will enable for an eventual authentication protocol in terms of WG products. Developing a catalogue of profiles from various species will also enable scrutiny of products where it is suspected a lower value cereal has been fraudulently classified as a more expensive one. Whilst the majority of research into cereal cell wall composition has been conducted through techniques such as nuclear magnetic resonance (NMR) (Shewry et al., 2017) or thin layer chromatography (TLC), Fangel et al. (2018), studied the progress of

polysaccharides throughout the brewing phase, applying the CoMPP technique chosen for this process.

With the knowledge of how WGs react to the CoMPP technique developed by Moller et al., (2012), proof of concept had already been outlined as shown within the Fangel paper. In 2017, Wood et al., studied the plant cell wall, although used a different methodology for the glycan extraction. The methods used within this paper were trailed on a select set of grains (Figure 3.1), to see if it may have been a more feasible technique for WG analysis. It was ultimately decided that the Moller technique would be used as it required fewer steps as well as time, yet still allowed for sufficient data to create an informative polysaccharide profile.

The polysaccharide profile using the Wood method shows that most of the arabinoxylan (LM11) and starch (INCH1) was released within the potassium hydroxide fractions (1 M and 4 M), with these too being the most predominant polysaccharides for all samples over all extractions. In comparison to the Moller method, heteromannan (LM21) gave the highest reading for waxy barley in the CDTA extraction. MLG also gave increased abundance within the NaOH fraction for the Moller method, something that wasn't seen in the previous experiment. Seeing these results, arabinoxylan and starch seemed to dominate the results of the Wood experiment, whereas a broader view was given when following the Moller method.


A
AGP
(1-4)-beta-galactan
(1-5)-a-L-arabinan
Xylan
Xylan / arabinoxylan
Heteromannan
Xyloglucan
(1-3;1-4)-beta-D-glucan
Starch
Ammonium Oxalate
Sodium Carbonate
Potassium Hydroxide 1M
Potassium Hydroxide 4M
Wheat, Pearled
Wheat, Heavy Fraction
Triticale, Pearled (1 min)
Triticale, Pearled (2 min)
Waxy Barley, Heavy Fraction
Waxy Barley, Pearled
Spelt, Pearled
Spelt, Heavy Fraction
Barley, Pearled
Wheat, Flour
Waxy Barley, Grain
Barley, Flour
LM2 LM5 LM6-M LM10 LM11 LM21 LM25 MLG INCH1 C

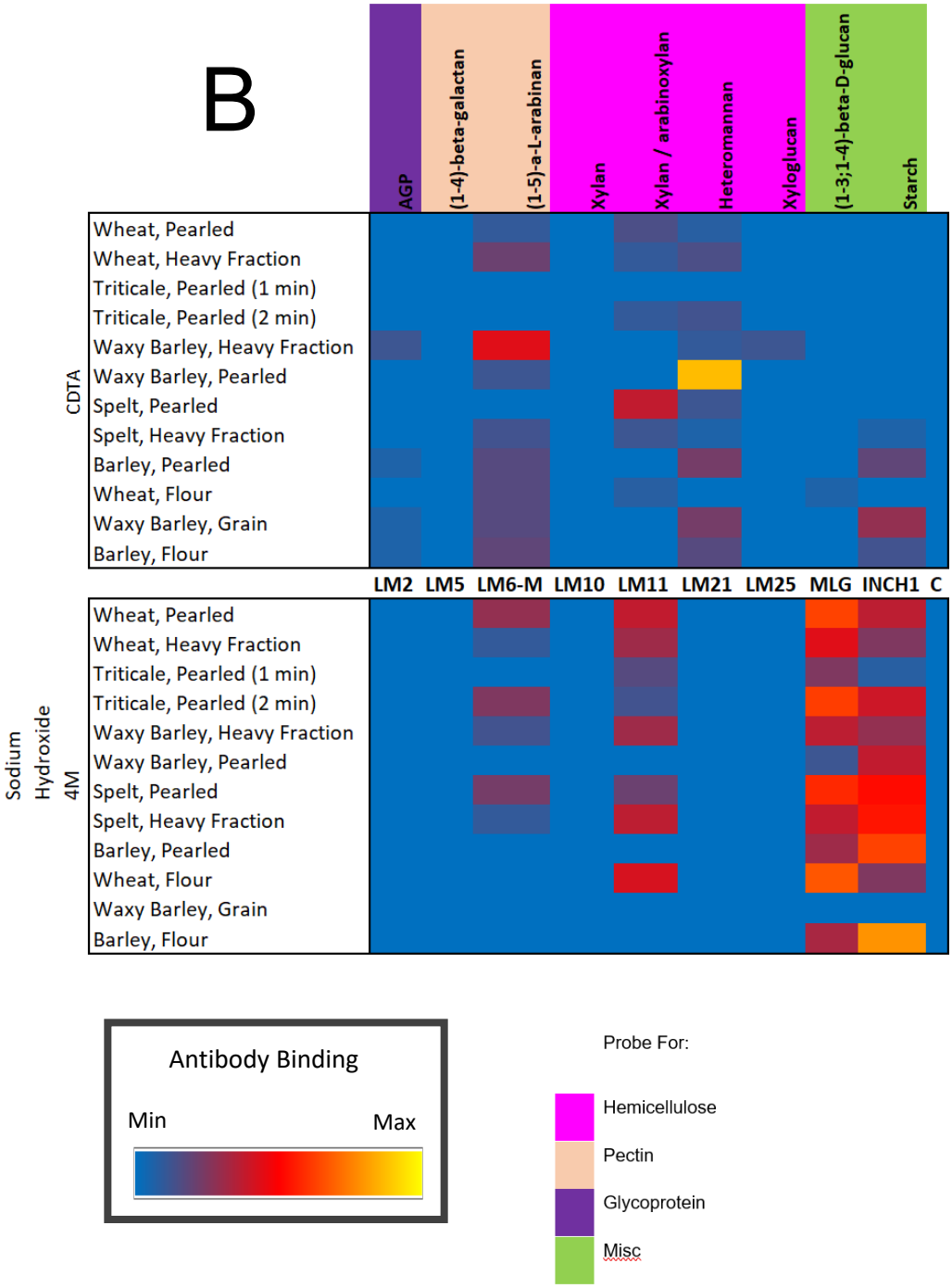


Figure 3.1 A trial experiment was conducted testing the Wood CoMPP method (A) against that used by Moller (B). A select set of Silvery Tweed samples with differing grain fractions were used, as well as a smaller array of mAb probes. Samples from both methods were printed on the same array, and a heatmap produced to compare values. The heatmap was normalised to one value with the other altered in comparison. Any readings below 5 were regarded as insignificant.

Shewry et al., (2017) explored the level of diversity between various cereal species including emmer and einkorn. Whilst not delving into the polysaccharide profiles, this study focused on genetic diversity, highlighting differences between species.

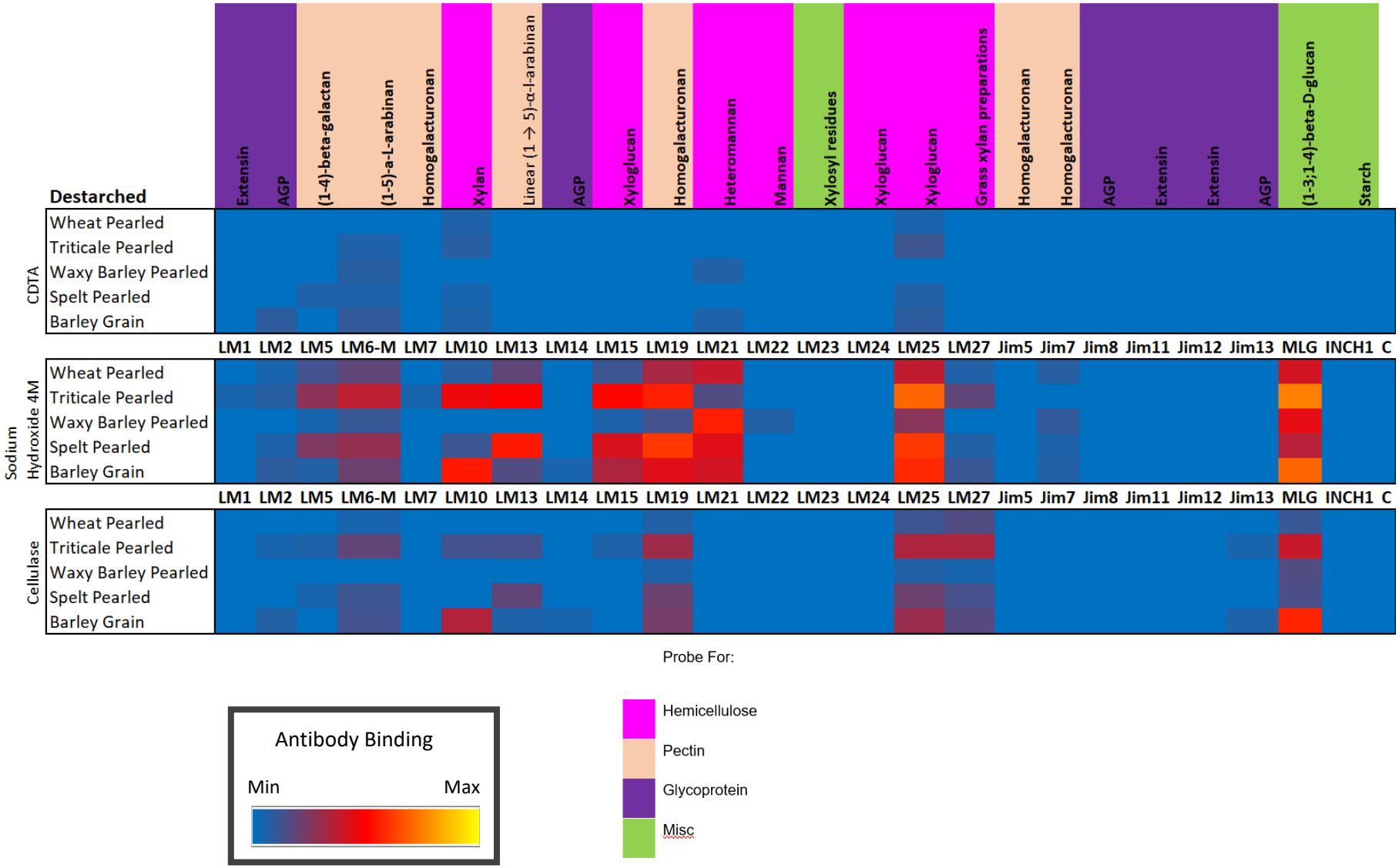
Starch has been identified as the most abundant polysaccharide within the plant cell wall (Shewry et al., 2013), and other research has indicated that it is possible for polysaccharides to be 'masked' by others (Marcus et al., 2008; Hervé et al., 2010). Due to starch's abundant presence within the cell wall, it was considered this masking phenomena would make developing a representative profile of all extractable polysaccharides impossible. Thus, a work-up experiment comparing a select sample of destarched and non-destarched cereal was conducted.

To ensure integrity of the collected results, a standard error profile was compiled (Figure 3.2) of a select set of samples within the work-up experiment. This highlighted consistency between replicates, indicative of a process that will gain concordant results. When analysing the results, it was noticed that LM11 had a higher result than other probes. This was also seen on the physical arrays, whereby after probing with LM11 the array would have a lot of background staining that could have been picked up during computer analysis. For this reason, it was decided that LM11 would no longer be used, and probes M139 -M157 would be used instead.

Dilution 1	**LM1**	**LM2**	**LM5**	**LM6-M**	**LM7**	**LM10**	**LM11**	**LM13**	**LM14**	**LM15**	**LM19**	**LM21**	**LM22**	**LM23**	**LM24**	**LM25**	**LM27**	**Jim5**	**Jim7**	**Jim8**	**Jim11**	**Jim12**	**Jim13**	**LM28**	**MLG**	**INCH-1**
Wheat Pearled	0	0	0	0	0	2	2	1	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	2	2	0
Triticale Pearled	0	0	1	1	0	2	3	1	0	1	1	1	0	0	0	2	1	0	0	0	0	0	0	1	2	0
Waxy Barley Pearled	0	0	0	0	0	0	1	0	0	0	1	2	0	0	0	2	0	0	0	0	0	0	0	1	1	0
Spelt Pearled	2	0	1	1	0	3	7	0	0	1	1	1	0	0	0	0	0	0	1	0	0	0	0	2	0	0
Barley Grain	0	0	0	0	0	1	6	1	0	1	1	2	0	0	0	1	0	0	0	0	0	0	0	2	1	0
Dilution 2	**LM1**	**LM2**	**LM5**	**LM6-M**	**LM7**	**LM10**	**LM11**	**LM13**	**LM14**	**LM15**	**LM19**	**LM21**	**LM22**	**LM23**	**LM24**	**LM25**	**LM27**	**Jim5**	**Jim7**	**Jim8**	**Jim11**	**Jim12**	**Jim13**	**LM28**	**MLG**	**INCH-1**
Wheat Pearled	0	0	2	1	0	0	4	2	0	2	1	0	0	0	0	3	0	0	0	0	0	0	0	1	1	0
Triticale Pearled	0	0	1	0	0	1	2	1	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	2	2	0
Waxy Barley Pearled	0	0	0	2	0	0	4	0	0	0	2	2	0	0	0	1	0	0	2	0	0	0	0	1	2	0
Spelt Pearled	0	0	0	1	0	0	4	1	0	1	1	1	0	0	0	2	0	0	0	0	0	0	0	4	1	0
Barley Grain	0	0	0	0	0	1	3	0	0	0	1	1	0	0	0	2	2	0	0	0	0	0	0	8	0	0
Dilution 3	**LM1**	**LM2**	**LM5**	**LM6-M**	**LM7**	**LM10**	**LM11**	**LM13**	**LM14**	**LM15**	**LM19**	**LM21**	**LM22**	**LM23**	**LM24**	**LM25**	**LM27**	**Jim5**	**Jim7**	**Jim8**	**Jim11**	**Jim12**	**Jim13**	**LM28**	**MLG**	**INCH-1**
Wheat Pearled	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	2	2	0
Triticale Pearled	0	0	2	2	0	2	4	2	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	2	2	0
Waxy Barley Pearled	0	0	0	0	0	0	5	0	0	0	0	1	0	0	0	3	0	0	0	0	0	0	0	1	0	0
Spelt Pearled	0	0	0	0	0	0	3	1	0	2	1	2	0	0	0	2	0	0	0	0	0	0	0	1	2	0
Barley Grain	0	0	0	0	0	2	10	0	0	2	2	0	0	0	0	2	0	0	0	0	0	0	0	5	1	0
Dilution 4	**LM1**	**LM2**	**LM5**	**LM6-M**	**LM7**	**LM10**	**LM11**	**LM13**	**LM14**	**LM15**	**LM19**	**LM21**	**LM22**	**LM23**	**LM24**	**LM25**	**LM27**	**Jim5**	**Jim7**	**Jim8**	**Jim11**	**Jim12**	**Jim13**	**LM28**	**MLG**	**INCH-1**
Wheat Pearled	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
Triticale Pearled	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	1	0	0
Waxy Barley Pearled	0	0	0	0	0	0	4	0	0	0	0	1	0	0	0	3	0	0	0	0	0	0	0	2	0	0
Spelt Pearled	0	0	0	0	0	0	3	2	0	0	2	0	0	0	0	1	0	0	0	0	0	0	0	3	0	0
Barley Grain	0	0	0	0	0	2	3	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	3	1	0

Figure 3.2. Standard error performed on a small sample set to highlight the consistency between samples when analysing them in triplicate. The standard error values were taken from the NaOH fraction of the destarched work-up samples (Figure 3.3). Due to the difference in results from LM11, considered to be due to strong background, it was decided that this probe would not be used in further experiments. The standard error was calculated prior to the heatmap being created.

Work-up experiments exploring the effect of masking, utilised enzymatic reactions to remove polysaccharides that impede the detection of less prevalent polysaccharides. α-amylase was used to remove the vast majority of starch from the wheat sample resulting in a ‘destarched’ sample that was compared against the ‘non-destarched’ version. INCH-1 was included as a probe due to its specificity to starch, therefore the presence or absence of starch could be identified. Figure 3.3 highlights the successful removal of starch from samples via α-amylase treatment.


Destarched
Extensin
AGP
(1-4)-beta-galactan
(1-5)-a-L-arabinan
Homogalacturonan
Xylan
Linear (1 → 5)-α-l-arabinan
AGP
Xyloglucan
Homogalacturonan
Heteromannan
Mannan
Xylosyl residues
Xyloglucan
Xyloglucan
Grass xylan preparations
Homogalacturonan
Homogalacturonan
AGP
Extensin
Extensin
AGP
(1-3;1-4)-beta-D-glucan
Starch
CDTA
Wheat Pearled
Triticale Pearled
Waxy Barley Pearled
Spelt Pearled
Barley Grain
LM1 LM2 LM5 LM6-M LM7 LM10 LM13 LM14 LM15 LM19 LM21 LM22 LM23 LM24 LM25 LM27 Jim5 Jim7 Jim8 Jim11 Jim12 Jim13 MLG INCH1 C
Sodium Hydroxide 4M
Wheat Pearled
Triticale Pearled
Waxy Barley Pearled
Spelt Pearled
Barley Grain
LM1 LM2 LM5 LM6-M LM7 LM10 LM13 LM14 LM15 LM19 LM21 LM22 LM23 LM24 LM25 LM27 Jim5 Jim7 Jim8 Jim11 Jim12 Jim13 MLG INCH1 C
Cellulase
Wheat Pearled
Triticale Pearled
Waxy Barley Pearled
Spelt Pearled
Barley Grain
Probe For:
Hemicellulose
Pectin
Glycoprotein
Misc
Antibody Binding
Min
Max

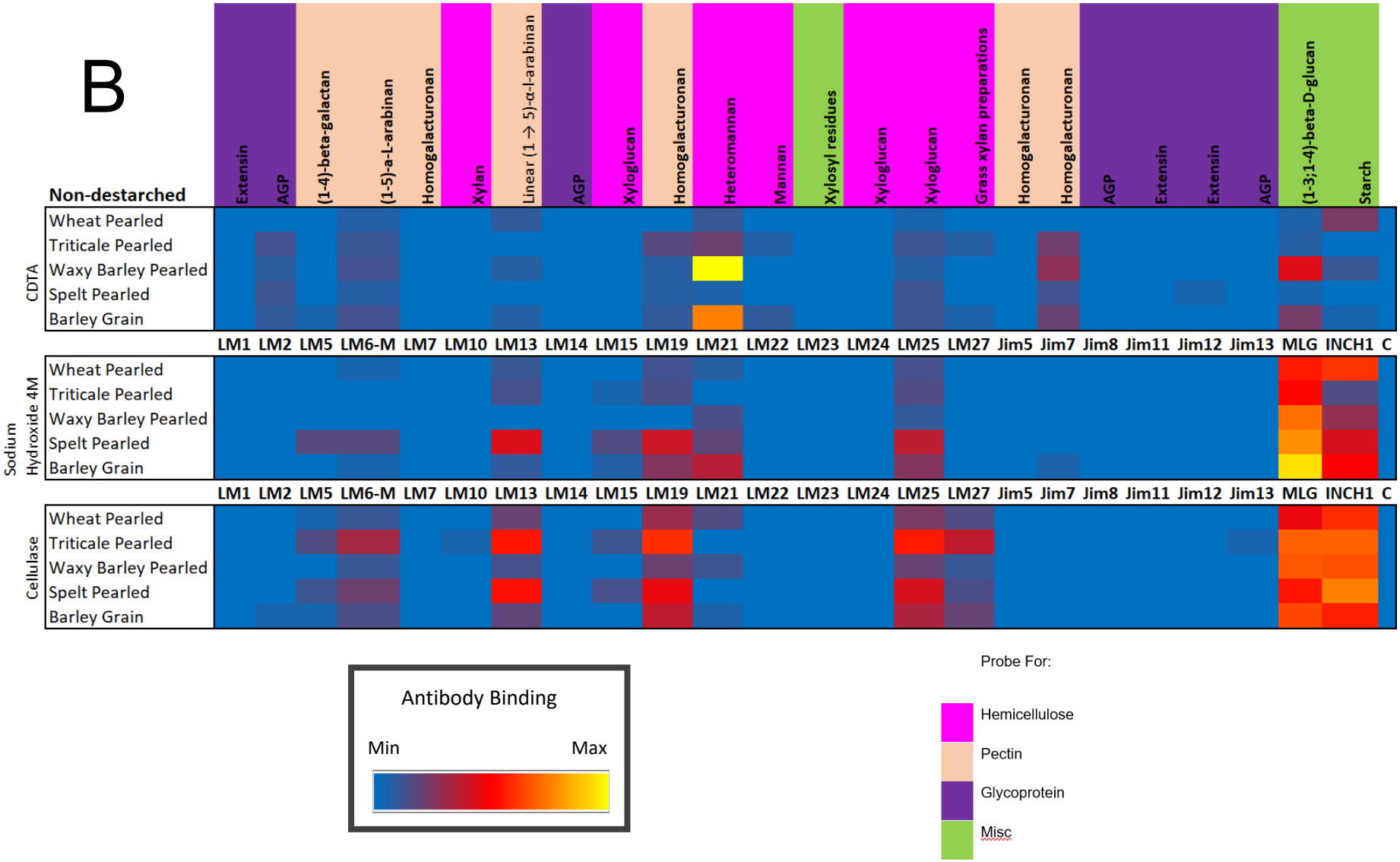
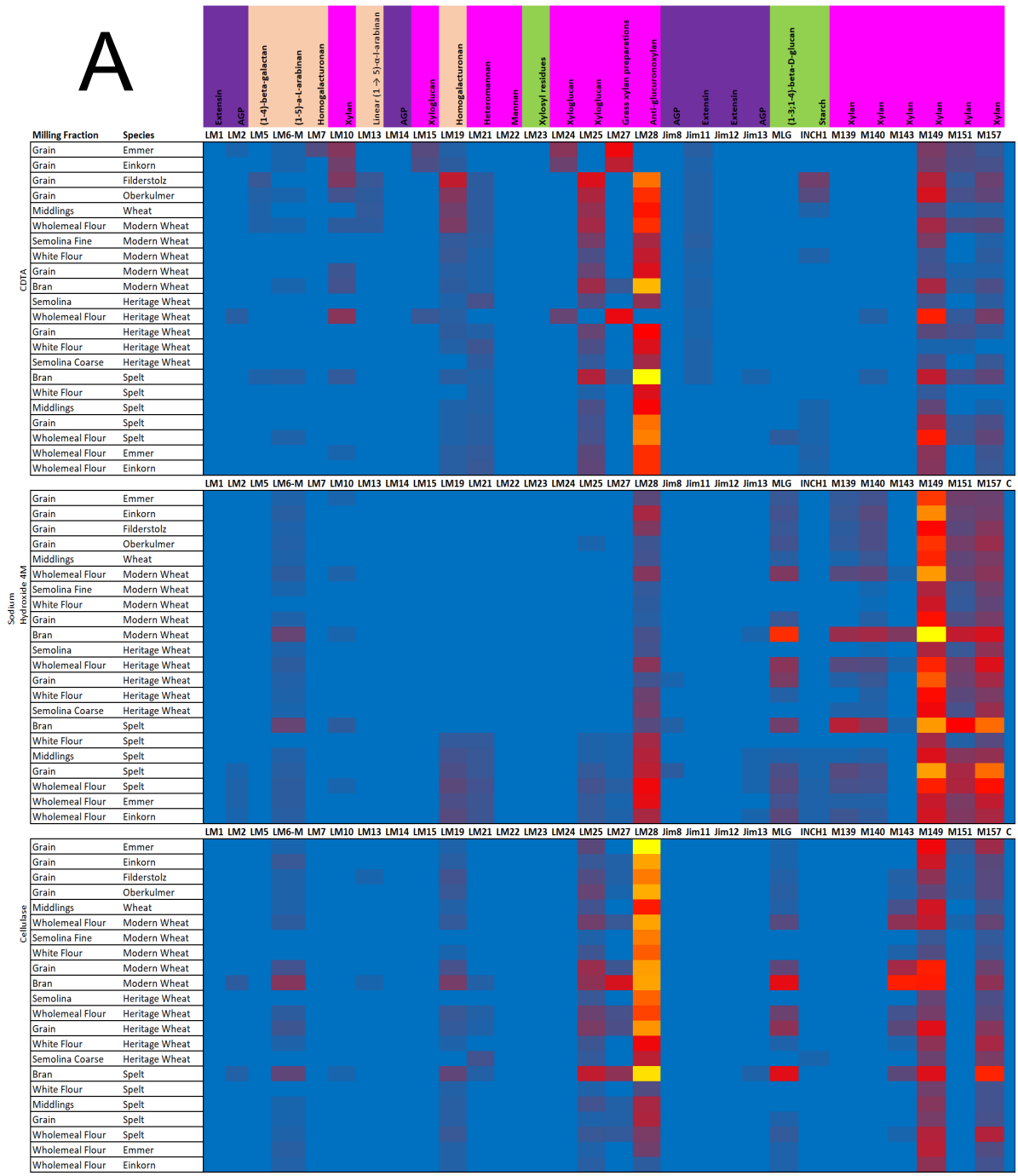

B
Non-destarched
Extensin
AGP
(1-4)-beta-galactan
(1-5)-a-L-arabinan
Homogalacturonan
Xylan
Linear (1 → 5)-α-l-arabinan
AGP
Xyloglucan
Homogalacturonan
Heteromannan
Mannan
Xylosyl residues
Xyloglucan
Xyloglucan
Grass xylan preparations
Homogalacturonan
Homogalacturonan
AGP
Extensin
Extensin
AGP
(1-3;1-4)-beta-D-glucan
Starch
CDTA
Wheat Pearled
Triticale Pearled
Waxy Barley Pearled
Spelt Pearled
Barley Grain
LM1 LM2 LM5 LM6-M LM7 LM10 LM13 LM14 LM15 LM19 LM21 LM22 LM23 LM24 LM25 LM27 Jim5 Jim7 Jim8 Jim11 Jim12 Jim13 MLG INCH1 C
Sodium Hydroxide 4M
Wheat Pearled
Triticale Pearled
Waxy Barley Pearled
Spelt Pearled
Barley Grain
LM1 LM2 LM5 LM6-M LM7 LM10 LM13 LM14 LM15 LM19 LM21 LM22 LM23 LM24 LM25 LM27 Jim5 Jim7 Jim8 Jim11 Jim12 Jim13 MLG INCH1 C
Cellulase
Wheat Pearled
Triticale Pearled
Waxy Barley Pearled
Spelt Pearled
Barley Grain
Antibody Binding
Min
Max
Probe For:
Hemicellulose
Pectin
Glycoprotein
Misc

Figure 3.3 Analysis resulting from solvent extraction of glycans and subsequent probing of a small sample of cereals provided by Silvery Tweed. The samples were analysed as two sets; destarched (A) and non-destarched (B). A range of monoclonal antibody probes were used, and the highest value changed to 100 and the other readings were normalised to this. Intensity readings below 5 were disregarded and changed to 0 and each sample set normalised within the solvent extract.

Initial analysis of the two data sets suggested to not pre-treat the samples with α-amylase and continue to conduct extractions in the standard manner (using non-destarched samples). This is due to the lower intensities of probes for polysaccharides (Figure 3.3, B) that had the potential to identify specific cereal species, such as LM19 (homogalacturonan) within the cellulase extraction, and LM21 (mannan) within the NaOH extraction. Further analysis of the data sets found that whilst some probes show diminished intensity, certain other probes displayed increased intensity. This meant that we would not be able to discount that when studying a larger sample set, destarching may aid in identifying specific cereal species and/or milling fractions. Therefore, it was decided to perform extractions on both destarched and non-destarched samples.

With a set experimental plan, a total of 22 new cereal samples with varying milling fractions were chosen for analysis (Figure 3.4). With the exception of modern wheat, all samples came from ‘ancient cereal’ species. Due to purported health benefits these ancient grain species are steadily increasing in popularity with consumers. This experiment utilised a broader range of mAbs than previous establishment experiments to gain a more in-depth polysaccharide profile. This larger net of detection improved the final heatmap profiles as they are only indicative of the polysaccharides that are extractable, and work on the relative abundance of the epitopes present within the extractions.


A
Extensin
AGP
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Grain Heritage Wheat
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Semolina Coarse Heritage Wheat
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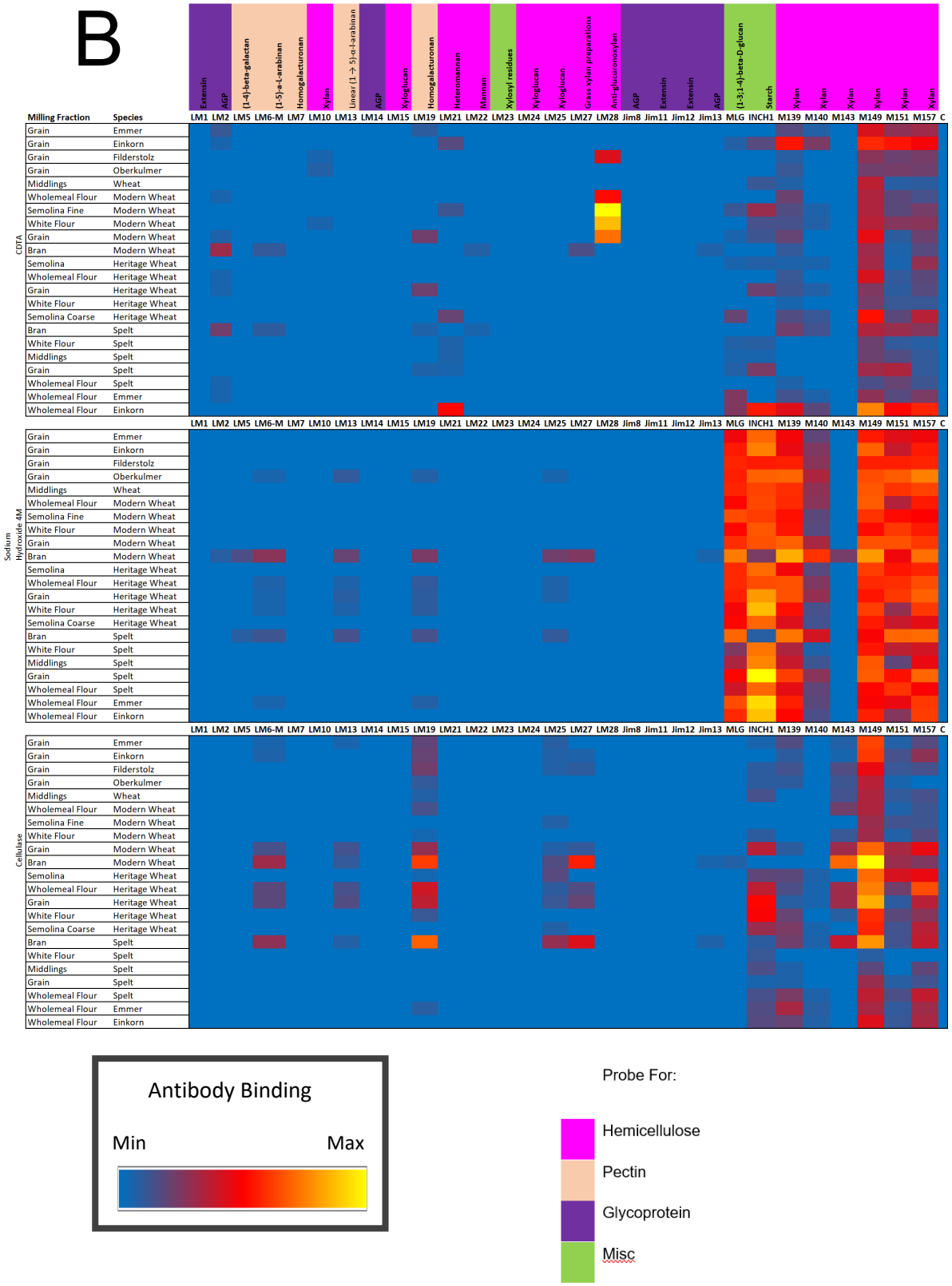
Grain Spelt
Wholemeal Flour Spelt
Wholemeal Flour Emmer
Wholemeal Flour Einkorn
Antibody
Min
Max
Probe For:
Hemicellulose
Pectin
Glycoprotein
Misc

B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Homogalacturonan
Heteromannan
Mannan
Xylosyl residues
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Grass xylan preparations
Anti-glucuronoxylan
AGP
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AGP
(1-3;1-4)-beta-D-glucan
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Semolina Coarse Heritage Wheat
Bran Spelt
White Flour Spelt
Middlings Spelt
Grain Spelt
Wholemeal Flour Spelt
Wholemeal Flour Emmer
Wholemeal Flour Einkorn
Antibody Binding
Min
Max
Probe For:
Hemicellulose
Pectin
Glycoprotein
Misc

Figure 3.4. Heatmaps detailing the intensity values gained from the solvent extractions of glycans from a wide range of cereal samples, with various milling fractions. The samples were analysed as two sets: destarched (A) and non-destarched (B). A range of monoclonal antibody probes were used, and the highest value changed to 100 and the other readings were normalised to this. Intensity readings below 5 were disregarded and changed to 0 and each sample set normalised within the solvent extract.

Within both the CDTA and cellulose extractions (Figure 3.4 A), glucuronoxylan (LM28) was viewed to elicit the most intense response, with xylan (M149) being the most predominant polysaccharide within the NaOH fraction. This would fall in line with published research documenting that xylans are the most abundant of the hemicellulose class within monocots i.e., cereals, whilst arabinoxylan specifically, is the most abundant hemicellulose within the cereal cell wall.

The primary phase of the experiment involved developing a polysaccharide profile of barley. The data described how (1,3) (1,4)-β-d-Glucan within the NaOH fraction was the most abundant of the polysaccharides for the selected set of probes, which falls in line with the non-destarched barley grain analysed within the work up experiment (Figure 4). This differs from wheat whereby (1,3) (1,4)-β-d-Glucan usually constitutes around 1% of cell walls where the predominant non-starch polysaccharides localised within wheat bran are; glucuronoarabinoxylans, cellulose and (1,3) (1,4)-β-d-Glucan. The intense binding of (1,3) (1,4)-β-d-Glucan (Figure 3.4 B) demonstrates this, with the bran fractions giving more intense readings than the others. The NaOH fraction showed a high relative abundance of xylans (M139, M149, M151 and M157) which fits in with previously discussed studies. Glucuronoxylan, starch, and xylans were the most predominant extractable polysaccharides for these profiles, which was to be expected, however that does not mean that other polysaccharides were not present, but that their abundance was much lower in relation and so not demonstrated as much on the heatmaps.

Milling Fraction	Abbreviation
Bran	B
Grain	G
Middlings	M
Semolina	S
Semolina coarse	SC
Semolina fine	SF
White flour	WF
Wholemeal flour	WMF

Figure 3.5. Hierarchical clustering of the non-destarched samples. Samples were clustered within their extractions: CDTA (A), NaOH (B), and Cellulase (C), the table shows the abbreviations relating to the sample milling fractions. Clusters created using Genesis 1.8.1, Genomics & Bioinformatics Graz, Graz University of Technology.

Having analysed the results of the full non-destarched sample set, clustering analysis was conducted to view similarities between the polysaccharide profiles (Figure 3.5). As the samples had not been destarched, it gave a clearer view into how similar the natural polysaccharide profiles of the samples were.

The clustering analysis highlighted relatedness of the polysaccharide profiles for the different samples and extraction solvents. As can be seen from the CDTA extractions (figure 3.5(A)), spelt WMF appears to be the least related to the other samples, this would be understandable considering its polysaccharide profile (figure 3.4B) doesn't highlight much abundance of any polysaccharides in comparison to the other samples, with low levels of xylan. Samples within the NaOH clustering (figure 3.5(B)) seem to be less clustered, with more singular branches, and the cellulase clustering shows smaller groupings. Overall, there does not seem to be any groupings that are distinctive and consistent within the analysis, this could highlight the close relatedness of the grains themselves.

3.4 Conclusion

Using the uniqueness of polysaccharide profiles within plant-based products, the technique described above could be highly beneficial in screening whole grain products at a high capacity, especially if legislation were to be introduced on what constitutes an appropriate level of WG. It would also be worthy to note that the technique developed could easily be applied to other plant products as an effective measure within food authentication at a high-throughput level. Future investigation into this area could include a definitive way of quantifying how much WG is in a product, going as far as to identify whether the correct proportions of WG is present.

Chapter 4. Saffron

4.1 Introduction

A pairing of common commodities that have enrichened our plates for millennia, herbs and spices allow us to bequeath dishes with varying aromas, colourings, and tastes, dependent upon which you choose. Venture into kitchens across the globe and there is a high certainty of finding a plethora of herbs and spices. In line with ISO676:1995(en) standards, herbs and spices are both collectively categorised as spices. The FAO currently uses the ISO definition of spices as 'Vegetable products or mixtures, free from extraneous matter, used for flavouring, seasoning or imparting aroma in foods', with herbs being a 'sub-sect of spices, derived from leaves of a plant – dried or fresh' (FAO, 2005).

Spices can be harvested from a variety of plant areas including, but not limited to, flowers, bark, leaves, roots, and stigma. The stigma provides us with the focus of this chapter, Saffron. Prior to modern times, spices were not as readily available. Global exploration and the subsequent spice trade made these 'exotic' ingredients accessible to areas where the spice was not native – mainly Europe. Whilst European cuisine in some areas may have favoured meat juices and local herbs over these 'tropical spices', these spices would serve another purpose for the Europeans, medicine (Freedman, 2012).

" Let food be thy medicine, and medicine be thy food"- Hippocrates

As previously mentioned, spices became widely popular for their perceived medicinal value. Throughout the ages we have been able to see how and where these spices were used, for example:

- Middle East (~3000BC) – poppy, mandrake, henbane (Petrovska, 2012; Kelly, 2010).
- China (2500BC) – Ginseng, cinnamon bark, camphor (Bottcher, 1965, Wiart, 2006).
- Middle Ages – Sage, anise, mint (Petrovska, 2012).
- Greece (500BC) – Garlic, parsley, celery, asparagus (Bojadzievski, 1992; Gorunovic and Lukic, 2001).

Prior to the discovery of modern medicine, spices were held in high regard, and high demand as can be seen with their mention throughout history as listed above.

Derived from the stigma of the *Crocus sativus*, Saffron is today colloquially deemed ‘Red Gold’, for its vibrant colour and title of most expensive spice (Figure 4.1). We are aware that saffron is an age-old spice, having been mentioned throughout the ages. It has been written that the Babylonians were frequent users of saffron (2700 years ago) and has been used throughout time in many different capacities, and still used today in dishes such as paella (Asimopoulos et al., 2013).

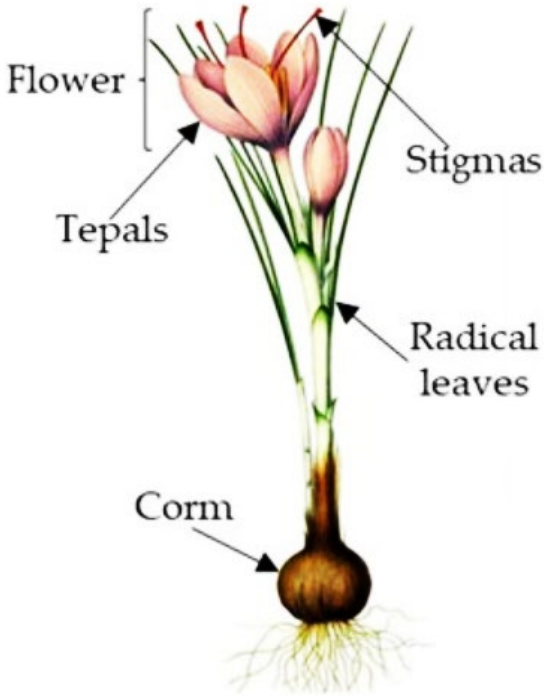


Figure 4.1. Image depicting different components of the *Crocus sativus* plant which produces the spice saffron, referred to as the stigmas in this diagram (Brandt et al., 1898).

Modern times have seen food availability blossom, with trade routes open to almost all corners of the planet. This means that we are able to consume delicacies that otherwise would be restricted to specific regions.

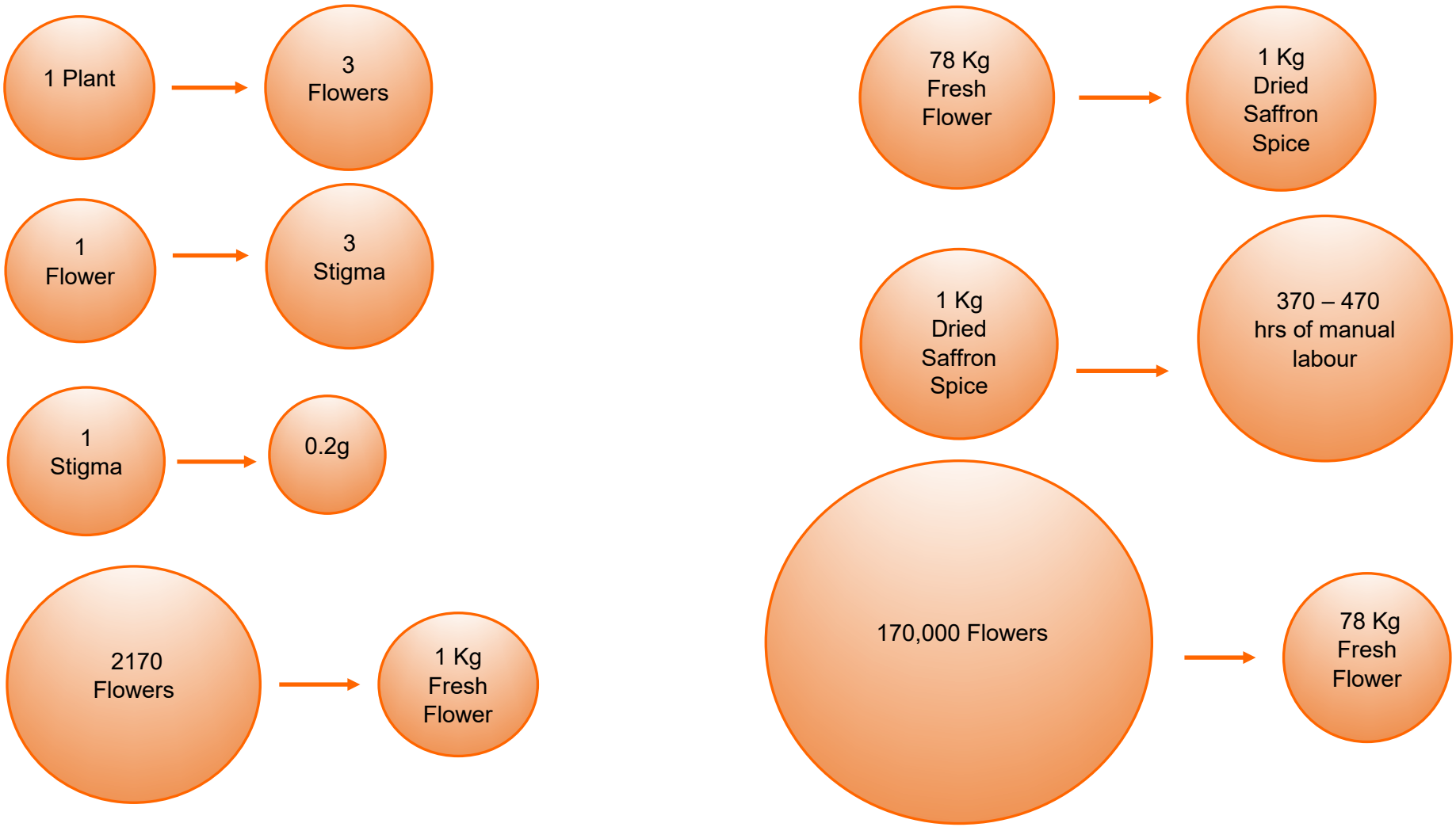


Figure 4.2. Schematic outlining some of the figures involved in the production and harvesting of saffron. It is well known that saffron is one of the most expensive spices, but not much consideration is put into why this is. Manual handling adds a lot of time, and cost, to the harvesting process, but this diagram outlines just how much is required for just one kilo of saffron (Asimopoulos et al., 2013).

4.1.2 Origins

We have seen that saffron has many mentions throughout history, although there is no agreement as to where saffron originated. There are theories of origins in the middle east, or southern Greek islands, but these are only theories. Saffron is unable to produce seeds, therefore reproduction is facilitated by its corms. Studies have shown that the closest relative of *C. sativus* is *C. cartwrightianus* (Nemati et al., 2018; Larsen et al., 2014). It is important to note that there have been instances of fraud whereby extraneous material from *C. sativus,* or other closely related species of crocus have been added (Cardone et al., 2020).

Although the precise origins are unknown, saffron is documented to have been grown in various regions worldwide. One such region is Saffron Walden, located in the county of Essex, southern England. It is known that saffron is not native to the UK and thought that the *C. sativus* was first introduced during the reign of Edward III (1327-1377). When *C. sativus* growth bloomed during the 1500s, the town adopted the name 'Saffron Walden' as it is still known today. Due to the availability of cheaper, imported saffron, production within the UK began to dwindle during the 1700s (Saffron Walden Town Council, 2021). Whilst still relatively rare, there are some areas in the UK beginning to harvest saffron again.

4.1.3 Harvesting

In modern times saffron has been grown in numerous regions; Spain, Iran, Afghanistan, and they all have their own versions. Part of the reasoning behind the expense of saffron comes down to growth. It is estimated that just one kilogram of saffron would need 78 kilograms of the crocus flower and approximately 370 – 470 hours of manpower as this includes carefully removing the saffron from the flower by hand (Figure 4.2) (Asimopoulos et al., 2013; Emadi and Yarlagadda, 2008). In addition to this, much of the harvesting process is still manual, however automated processes are being researched. Complications arise when trying to find a process that minimises damage during the cutting and collecting process. As well as difficulties with applying automation, the crocus plants struggle when in competition with weeds, which are often picked by hand and could be missed. In Iran, one of the highest exporters, it is reasonable to expect that at least 200 labourers would be needed per hectare for harvesting purposes, this would, understandably, induce a sizeable cost (Ghorbani, 2007).

It has been suggested that as well as good growth conditions, some areas are more favourable due to low labour costs which means a larger workforce, and less pressure is felt by the lack of automated harvesting.

Analysis of saffron quality has shown that various factors contribute to the overall value of the spice. Quality is mainly dictated by smell, taste, and colour, being influenced by factors such as:

- Storage conditions (Carmona et al., 2005; Priscila Del Campo et al., 2010; Magsoodi et al., 2012).
- Treatments applied post-harvest (peeling from flower, greasing etc.) (Anastasaki et al., 2010).
- Origin of the corm (Lage and Cantrell, 2009; Macchia et al., 2013; Cardone et al., 2019).
- Climate conditions during growth (Cardone et al., 2020).

This highlights that all areas from growth to harvest contribute to the characteristics of the saffron, with pressure to make the saffron as profitable as possible.

4.1.4 Market trends

Nearly all consumers use spices in their cooking, for this reason alone it would be sensible to assume that there is a lot of market interest. Figures show that during 2014 dried 'herbs and spices' accounted for £173 million worth of UK sales (Black et al., 2016). Saffron is marketed based on its grading. As would be expected higher graded saffron commands a higher price. Grading works on the quality of three features of saffron; taste, smell, and colour as outlined by ISO 3632 (2010).

In recent years (2010 – 2020) Iran and Spain have dominated the saffron market, accounting for over 70% of exports, with these figures only slightly fluctuating as the years go on. Other significant producer exporters include Hong Kong, Afghanistan, Greece, and Italy (Figure 4.3). On the other hand, there has been no clear leader of the imports market, although Spain and the United Arab Emirates (UAE) have often remained high (Figure 4.4). Trends tend to fluctuate over the years for the countries that import lower amounts (OEC, 2021). It may seem contradictory that some countries import masses when they produce their own saffron, however, as seen with the fall of English saffron, this may be due to cheaper sourcing from elsewhere. In addition to this, dependent upon the grading of the saffron being produced, the item being

imported may be of higher quality. For Spain in particular, most of the imported saffron comes from Iran, even though they themselves are a substantial producer of saffron. This is due to the higher quality of saffron, and Spain utilises saffron in national dishes such as paella (Mohammadi and Reed, 2020). With the high cost, and its lucrative market status, it is clear that saffron would be a prime target for food fraud.

4.1.5 Geographical distinctions

As previously discussed, saffron quality, and its subsequent price, can be dictated by its geographical origin in terms of growth location. Whilst not the only determining factor, it is clearly an important one. Due to this, it should be considered that geography may influence chemical composition. Research has been conducted into identifying geographical origin of saffron samples to aid with fraud. Priscila del Campo et al., (2009) targeted amino acids as a form of geographical indicator. Samples from Greece, Iran, Italy, and Spain were analysed with particular focus on ammino acid content. It was found that levels of certain amino acids were higher dependent upon location. Alanine was found to be highest in saffron samples from Iran, proline was found to be highest in Greek samples, and aspartic acid was found to be highest in Spanish samples. It was also noted that by measuring levels of free amino acids, this methodology was able to separate the Iranian samples from those originating from Europe.

In another experiment, a method was trialled aiming to distinguish between saffron from Iran and China. The focus of this experiment was on carbon and nitrogen content, as well as various stable isotopes within the samples; $\delta^{13}C$, $\delta^{2}H$, $\delta^{18}O$, and $\delta^{15}N$, with analysis via chemometrics. The findings suggested that quantifying $\delta^{13}C$ and $\delta^{2}H$ would aid in distinguishing Chinese saffron, and lower carbon and nitrogen was indicative of Iranian saffron (Nie et al., 2023).

Whilst experiments focusing on polysaccharide profiles in saffron samples are very limited, Zhang et al., (2019), used a phenol-sulfuric acid colorimetric method to look into total polysaccharide content of saffron samples. Samples originated from China, Nepal, Greece, Morocco, Spain, and Iran. Findings showed that the total polysaccharide content varied between the samples with a range of 52.65-67.18 mg/g. Analysis showed that Spain had the highest total polysaccharide content at 67.18 mg/g, with the lowest at 52.65 mg/g coming from Jiande in China. This experiment showed polysaccharide content differs amongst geographical origin, whilst it didn't focus on specific polysaccharides, it did look into monosaccharide content using FT-

IR, with the highest monosaccharide levels being galacturonic acid, galactose, and arabinose, within this the levels again differ between countries, highlighting how geographical origin could influence polysaccharide profiles.

The above are just a few experiments conducted on identifying geographical location. These show that there are multiple distinct markers capable of distinguishing origin and have the potential to aid in the authentication of saffron products if origin were to be questioned.

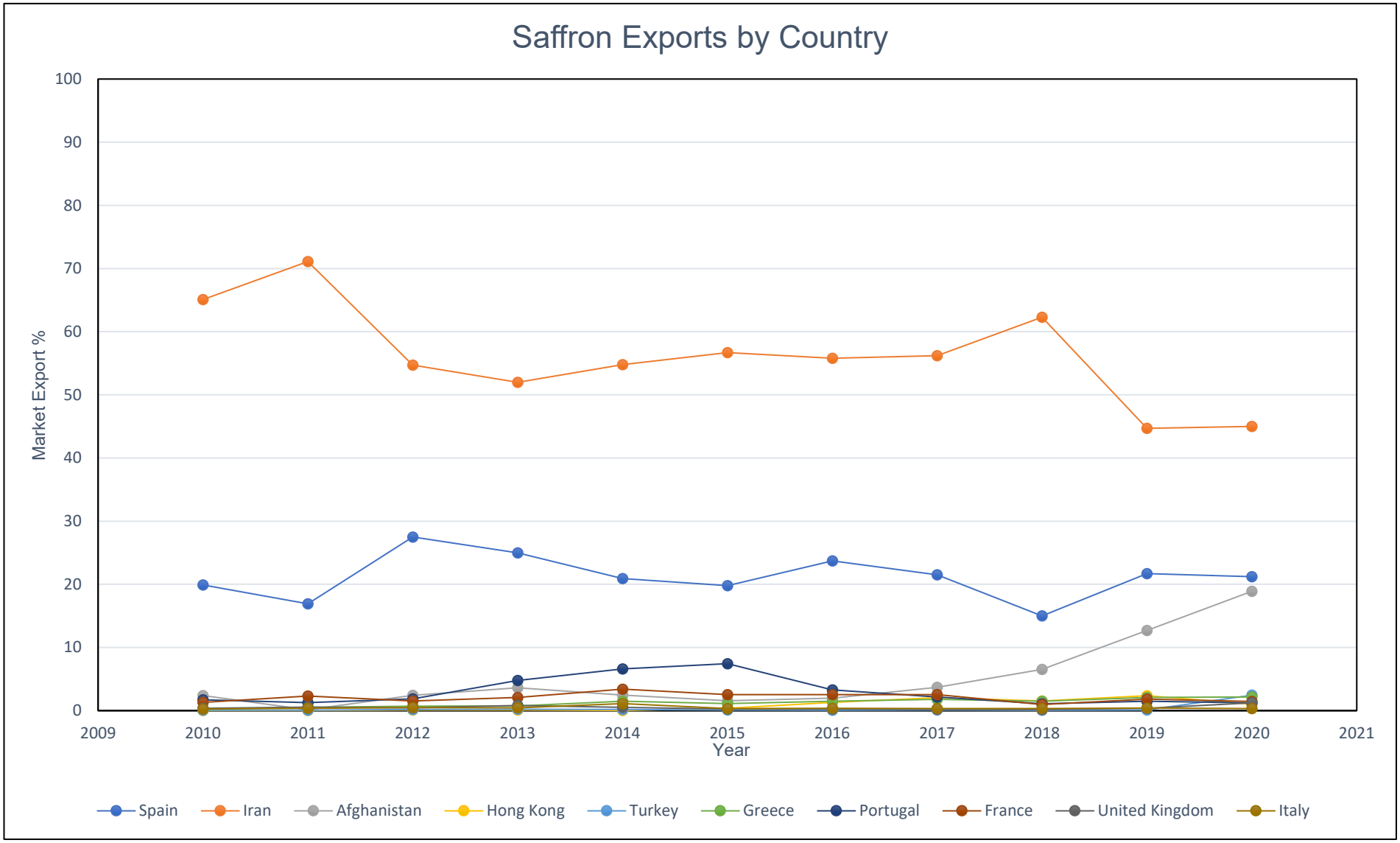


Figure 4.3. Graphical visualisation of selected countries saffron exports from 2010 - 2020. Values are represented as a percentage of the overall worldwide exports for that year. It can be seen that Iran has dominated the saffron market over these years (OEC, 2021).

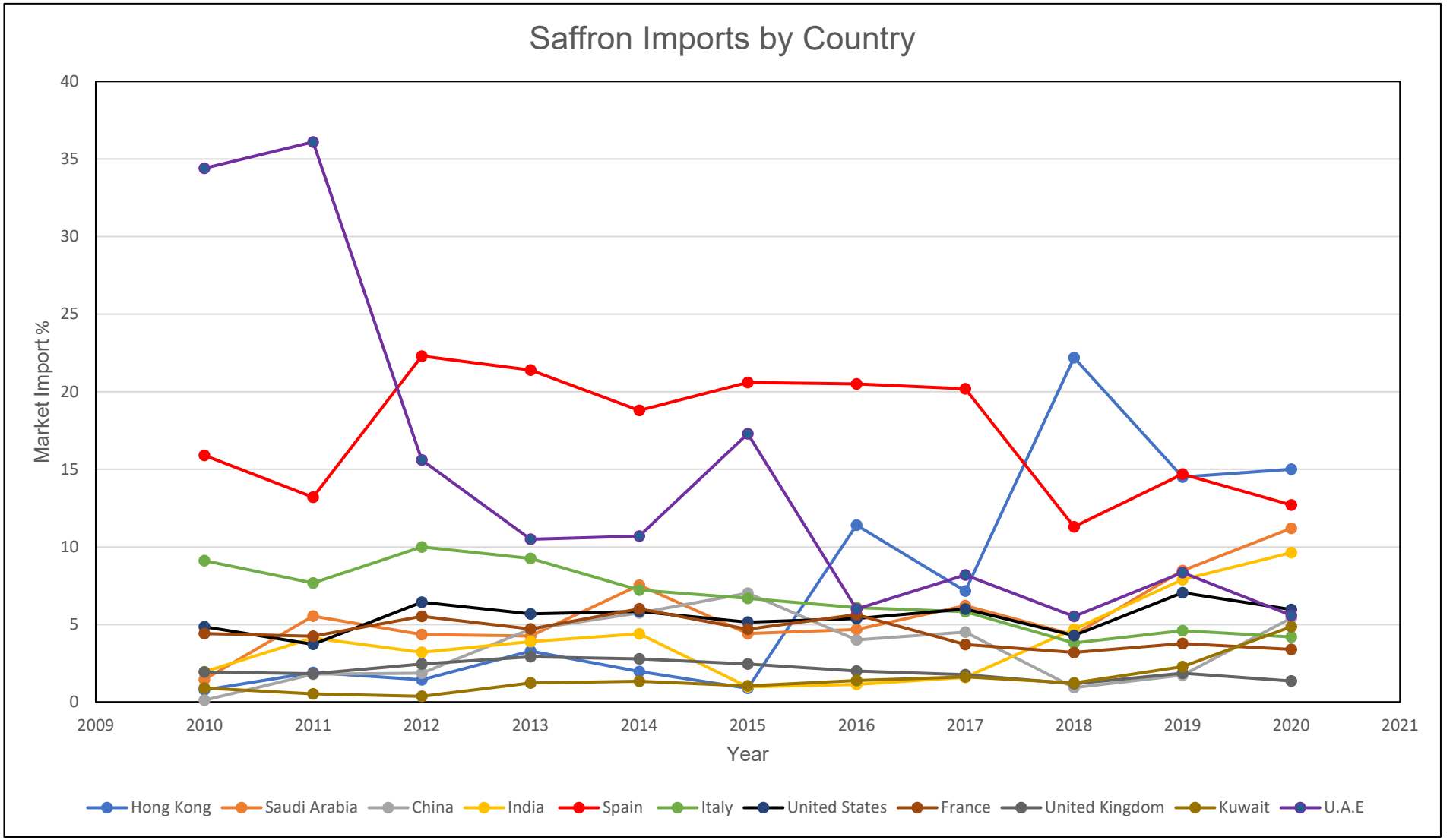


Figure 4.4. Graphical visualisation of saffron imports to selected countries form 2010 – 2020, represented as a percentage of worldwide imports. It can be seen that most countries import levels fluctuate over the decade (OEC, 2021).

4.1.6 Examples of fraud

As previously mentioned, the high value associated with saffron makes it a prime candidate for fraud. There are often media articles outlining the latest bust on fraudulent saffron.

One of the more common methods is adulteration with safflower, *Carthamus tinctorius*. This can be done by mixing safflower into the leaves. Often if the saffron is cut or ground it is much harder to identify fraudulent behaviours, this type of fraud can also occur with other parts of *C. sativus,* or other crocus species (Kafi et al., 2003; Javanmardi et al., 2011).

Most of the fraudulent methods could be seen as harmless, such as the horsemeat scandal, and purely for economic gain. This is not always the case. Sudan dyes are one such adulterant that have been known to be used and be potentially harmful to humans. The group of dyes are most commonly used to impart colour on things such as textiles, waxes, etc, and are also classed as carcinogens (IARC, 1975). Sudan dye has previously been used to dye an adulterant, such as leaves, to give a red colour – mimicking that of the saffron strands. The natural dye imparted by saffron when in liquids such as water, for which the compound crocin is responsible (Figure 4.5), is yellow and those dyed with Sudan dye would be red. Due to the red colour of saffron strands, the dye would only really be detectable if the saffron was placed in a liquid that would impart its colour, and not necessarily detectable at a surface glance (Petrakis et al., 2017).

4.1.7 Authentication techniques

As saffron is such a high value commodity, research into authentication techniques have found numerous ways of detecting fraud.

One way of detecting fraud focused specifically on identifying samples dyed with Sudan dyes, which as discussed before can be dangerous when consumed. Petrakis et al., (2017), used ^{1}H NMR in the detection of fraudulent samples, by quantifying the amount of dye present, and it was proposed that this technique would be a suitable authentication methodology. The benefit of using NMR techniques for authentication is that it could be used to detect more than one type of fraud. As mentioned before it has been used in research pertaining to dyed saffron produce, as well as distinguishing geographical origin (Maggi et al., 2011). Whilst this methodology could be considered

to be high-throughput, the length of time taken to prepare samples and subsequent analysis could be considered a more negative aspect, as well as the need for a larger sample in comparison to other techniques such as mass spectrometry, although many experiments still use multiple techniques within their methods (Elipe et al., 2003).

In addition to laboratory techniques, there is also a quick ‘at-home’ method for dye analysis. In advice aimed at guiding wary consumers, the saffron is ran under tap water and if the water runs a bright yellow, it can be reasonably assumed that the saffron has not been dyed. If the water runs red, it can be suspected that dye has been involved and the specimen may be fraudulent (Chen et al., 2022).

As has been previously mentioned, authentication techniques are in constant development as those committing fraud adapt their methods. With constant research, it can sometimes be discovered that an authentication technique that was once viable, may not always be the best method. UV-Vis spectrophometry was previously considered a beneficial method for analysing saffron colouring (Orfanou and Tsimidou, 1996). This methodology was used to analyse specific markers within saffron including safranal, picrocrocin, and crocin, responsible for odour, taste, and colour respectively (Figure 4.5). Studies have suggested that this may not be an appropriate methodology for authentication as the markers can fluctuate over time, or if the sample is not stored in specific conditions. In the case of safranal, levels were found to increase over time, in addition to this, it has been mentioned that, especially in the case of safranal, levels can be dependent upon the region in which they were grown and so should not be considered a viable measure of authenticity (Moraga et al., 2009; Sabatino et al, 2011; García-Rodríguez et al., 2017; Sereshti et al., 2018).

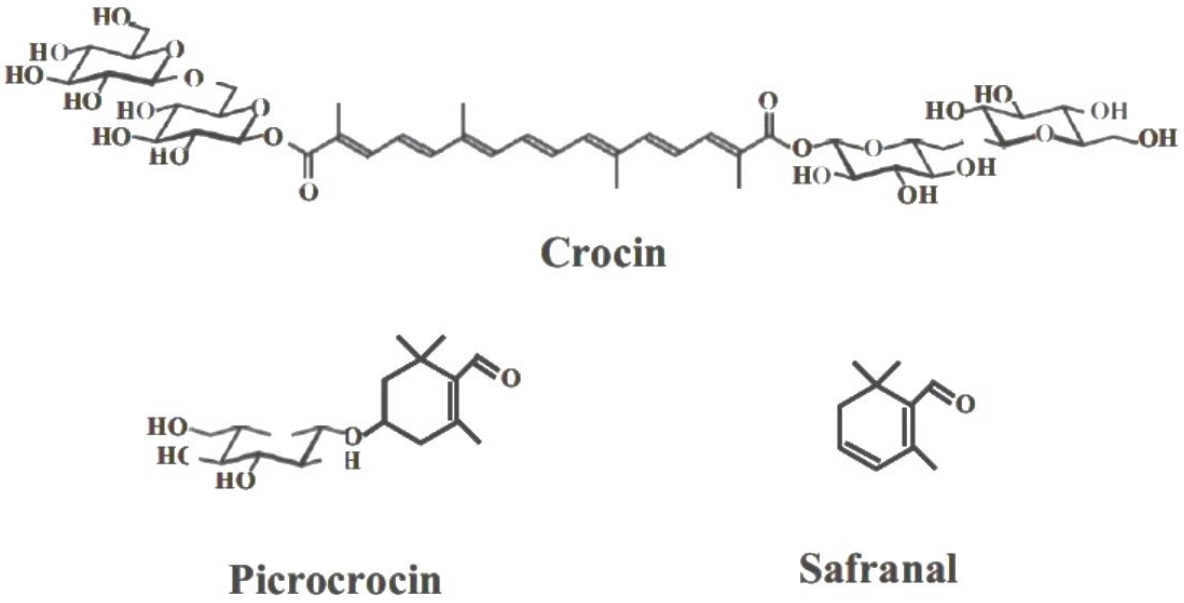


Figure 4.5. Structures of picrocrocin, crocin, and safranal, the compounds responsible for taste, colour, and odour respectively (Srivastava et al., 2010).

Whilst not an authentication technique itself, saffron can be protected by the International Organisation for Standardisation (ISO). This informs consumers that the saffron is of a particular standard, and as such many retailers may charge a higher price for a premium product. ISO 3632 was first introduced in 1975, as a way to ascertain quality and authenticity of saffron produce. Since 1975 there have been multiple revisions, with the last revision in 2011. Part of the ISO allows for the grading of saffron, from the highest at grade 1, and this is dependent on features attributed to specific components which should be present in certain amounts and analysed via UV-Vis spectrophotometry (Table 4.1) (Kumar et al., 2009; Giorgi et al., 2017).

Table 4.1. ISO guidance on the required levels of different compounds within saffron. Grading status is based on these values, higher grading can command higher sales prices (Giorgi et al., 2017; ISO, 2010).

Quality	Compound Responsible	UV-Vis spectrophotometry wavelength required (nm)	Absorbance Levels Required
Taste	Picrocrocin	257	Grade 1: >70 Grade 2: >55 Grade 3: >40
Odour	Safranal	330	Grade 1: 20-50 Grade 2: 20-50 Grade 3: 20-50
Colour	Crocetin esters	440	Grade 1: >200 Grade 2: >170 Grade 3: >120

In terms of authentication, ISO 3632 outlines exactly what methods should be used, this involves methods such as:

- Microscopy – allows for a more in depth look at the finer surface details of the sample
- UV-Vis spectrophotometry - allows for dye analysis to analyse levels of safranal, picrocrocin and crocin
- Identification with a magnifying glass – allows for preliminary identification before further investigation

4.1.8 The current issue

Saffron is still the most expensive spice on the market, and as so, sets itself up to become a prime candidate for food fraud. As there are numerous ways to defraud saffron from illegitimate documentation, to adulteration, there is a requirement for constant evolvement in terms of authentication techniques.

4.1.9 Aims

The focus of this chapter will be on creating a polysaccharide profile of saffron samples, to investigate the possibility of using polysaccharide profiling as a means of authentication for saffron produce. The main experiment will produce a polysaccharide profile, whilst supplementary experiments will be performed to compliment the data. The aims of this chapter are:

- To ascertain if polysaccharides are a good measure of authenticity for spices, particularly for saffron
- Create a polysaccharide profile for saffron
- Use supplementary data to back up the results

4.2 Methods

4.2.1 Sample acquisition

37 different sample types were used throughout the course of this experiment. Samples were sourced from various locations; UK supermarkets, McCormick, and a Turkish Market located in Turkey (Table 4.2). The samples acquired from the UK supermarkets did not specify a geographical origin, and so this wasn't able to be documented. The samples from McCormick outlined the origin, as well as the harvest year, something that wasn't available for the rest of the samples. Whilst working with these samples, all were stored in dark, cool conditions.

Table 4.2. Information pertaining to all the samples used within this experiment. The information includes what type of sample it was, where it was acquired and in which experiments it was used, with reference to the relevant results.

Sample Name	Sample Type	Acquired From	Experiments used in
Turmeric	Turmeric	UK supermarket sample	Spice suitability experiment (Figure 4.7)
Onion Granules	Onion Granules	UK supermarket sample	Spice suitability experiment (Figure 4.7)

Paprika	Paprika	UK supermarket sample	Spice suitability experiment (Figure 4.7)
Cumin	Cumin	UK supermarket sample	Spice suitability experiment (Figure 4.7)
Saffron	Saffron	UK supermarket sample	Spice suitability experiment (Figure 4.7)
Coriander	Coriander	UK supermarket sample	Spice suitability experiment (Figure 4.7)
Oregano	Oregano	UK supermarket sample	Spice suitability experiment (Figure 4.7)
Dill	Dill	UK supermarket sample	Spice suitability experiment (Figure 4.7)
Garlic	Garlic	UK supermarket sample	Spice suitability experiment (Figure 4.7)
Rosemary	Rosemary	UK supermarket sample	Spice suitability experiment (Figure 4.7)
Market 1	Saffron	UK supermarket sample	Select spice in-depth look (Figure 4.8), and Saffron expanded sample set (Figure 4.9)
Market 2	Saffron	UK supermarket sample	Select spice in-depth look (Figure 4.8), and Saffron expanded sample set (Figure 4.9)

Market 3	Saffron	UK supermarket sample	Select spice in-depth look (Figure 4.8), and Saffron expanded sample set (Figure 4.9)
Market 4	Saffron	UK supermarket sample	Select spice in-depth look (Figure 4.8), and Saffron expanded sample set (Figure 4.9)
Market 5	Saffron	UK supermarket sample	Select spice in-depth look (Figure 4.8), and Saffron expanded sample set (Figure 4.9)
Market 6	Saffron	UK supermarket sample	Select spice in-depth look (Figure 4.8), and Saffron expanded sample set (Figure 4.9)
Market 1	Oregano	UK supermarket sample	Select spice in-depth look (Figure 4.8)
Market 2	Oregano	UK supermarket sample	Select spice in-depth look (Figure 4.8)
Market 3	Oregano	UK supermarket sample	Select spice in-depth look (Figure 4.8)
Market 4	Oregano	UK supermarket sample	Select spice in-depth look (Figure 4.8)
Market 5	Oregano	UK supermarket sample	Select spice in-depth look (Figure 4.8)
Market 6	Oregano	UK supermarket sample	Select spice in-depth look (Figure 4.8)
Market 1	Paprika	UK supermarket sample	Select spice in-depth look (Figure 4.8)

Market 2	Paprika	UK supermarket sample	Select spice in-depth look (Figure 4.8)
Market 3	Paprika	UK supermarket sample	Select spice in-depth look (Figure 4.8)
Market 4	Paprika	UK supermarket sample	Select spice in-depth look (Figure 4.8)
Market 5	Paprika	UK supermarket sample	Select spice in-depth look (Figure 4.8)
Market 6	Paprika	UK supermarket sample	Select spice in-depth look (Figure 4.8)
Greece 2018	Saffron	McCormick	Saffron expanded sample set (Figure 4.9), visual analysis (Figure 4.10), and dye analysis (Figure 4.11)
Spain 2015	Saffron	McCormick	Saffron expanded sample set (Figure 4.9), visual analysis (Figure 4.10), and dye analysis (Figure 4.11)
Iran 2010	Saffron	McCormick	Saffron expanded sample set (Figure 4.9), visual analysis (Figure 4.10), and dye analysis (Figure 4.11)
Greece 2018 (2)	Saffron	McCormick	Saffron expanded sample set (Figure 4.9), visual analysis (Figure 4.10), and dye analysis (Figure 4.11)

India	Saffron	Turkish Market	Saffron expanded sample set (Figure 4.9), visual analysis (Figure 4.10), and dye analysis (Figure 4.11)
Turkish Light	Saffron	Turkish Market	Saffron expanded sample set (Figure 4.9), visual analysis (Figure 4.10), and dye analysis (Figure 4.11)
Turkish Dark	Saffron	Turkish Market	Saffron expanded sample set (Figure 4.9), visual analysis (Figure 4.10), and dye analysis (Figure 4.11)
Persian (Strands)	Saffron	Turkish Market	Saffron expanded sample set (Figure 4.9), visual analysis (Figure 4.10), and dye analysis (Figure 4.11)
Persian (Powder)	Saffron	Turkish Market	Saffron expanded sample set (Figure 4.9), visual analysis (Figure 4.10), and dye analysis (Figure 4.11)

The experiment was carried out in 3 parts: visual analysis, dye analysis, and polysaccharide profiling (Figure 4.6). Whilst each experiment was separate, the results

from the polysaccharide profiling dictated which spice would be focused on in more detail.

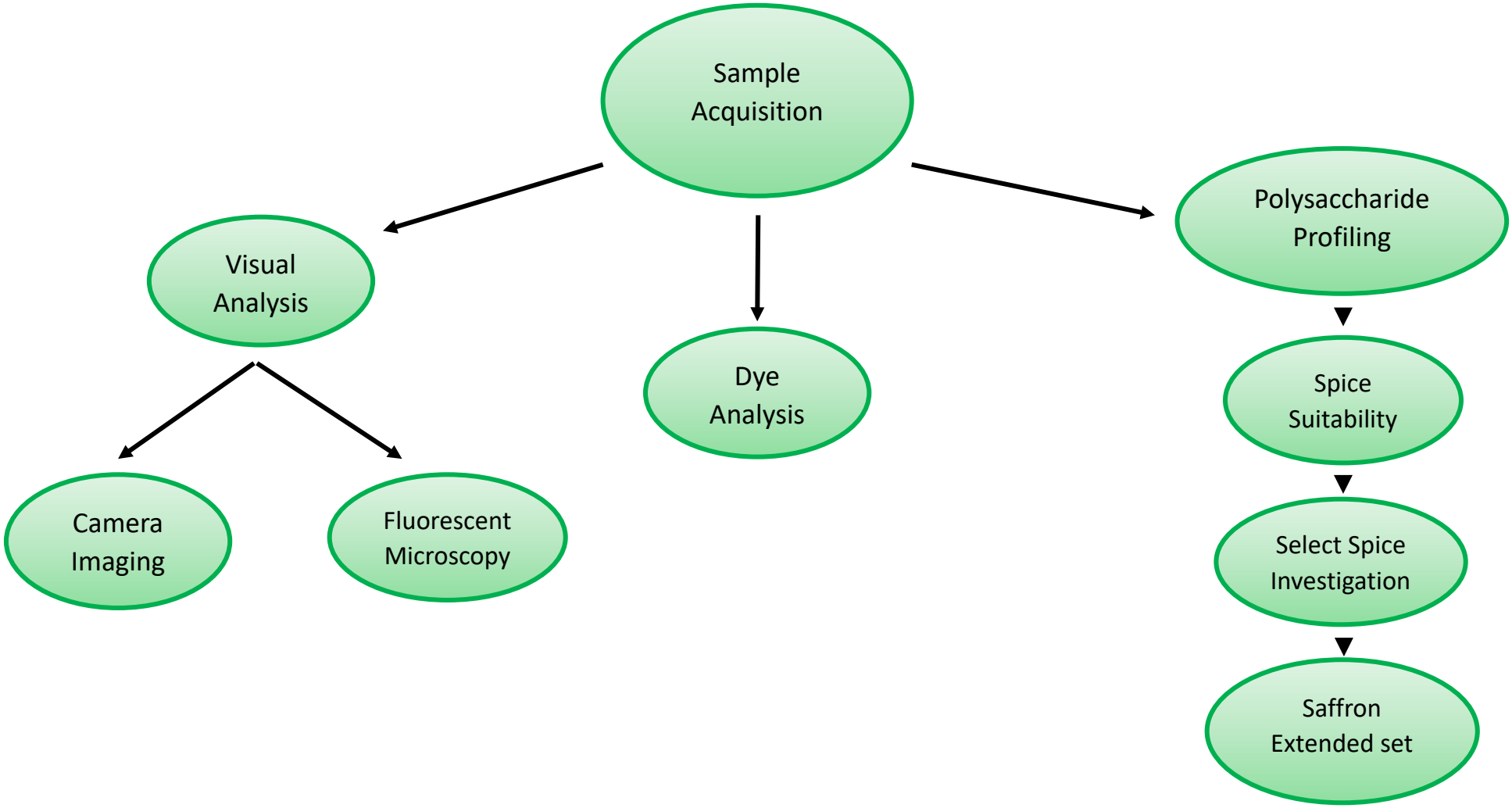


Figure 4.6. Schematic outlining the flow of experiments involved within this chapter. Three separate experiments were performed. Whilst all were standalone experiments, the results were complementary to each other, and reinforced the results of the other experiments.

4.2.2 Polysaccharide profiling

Spice suitability experiment

The first experiment was a suitability experiment to ascertain whether investigating polysaccharide abundance could be a viable authentication technique for spices. 10 common kitchen spices were chosen: turmeric, onion granules, paprika, cumin, saffron, coriander, oregano, dill, garlic, and rosemary. AIR preparation (Chapter 2.1) was carried out on each of the 10 samples, and the CoMPP methodology (Chapter 2.2.1 - 2.2.4) was followed to create a heatmap showcasing the relative abundance of polysaccharides within the samples, and the relative abundance values normalised to the highest value gained after the averages of each sample had been calculated (Figure 4.7). The polysaccharides chosen (Chapter 2, Table 2.1) were chosen to showcase a range of epitopes to give a wider view.

Select spice in-depth look

Following the results of the primary experiment (Figure 4.7), 3 spices were chosen: saffron, paprika, and oregano. Each showed to have distinctive profiles and were investigated further. 6 samples were collected for each of the 3 spices, sourced from UK supermarkets. AIR preparation was carried out on the samples (Chapter 2.1) and then CoMPP followed (Chapter 2.2.2 – 2.2.4) to create a heatmap. When creating the heatmap, each spice was normalised individually to the highest amount, and so the heatmaps are standalone for each spice (Figure 4.8)

Saffron expanded sample set

After analysing the 3 spices, saffron was selected as the spice that would be focussed on. In addition to the 6 market samples, 4 reference samples and 5 samples from a Turkish market were sourced. All of the samples went through AIR preparation (Chapter 2.1), before going through the CoMPP process (Chapter 2.2.2 – 2.2.4). As all the samples in this experiment were saffron samples, all values were normalised to the highest value reported after the samples were averaged from the raw data (Figure 4.9).

4.2.3 Dye visualisation

This experiment used the Turkish market samples and reference samples. 1 strand, or 0.5 g if in powdered form, of each sample was placed in a 1.5 mL eppendorf, and 1 mL of room temperature distilled water was added. The samples were left to incubate at room temperature for 10 minutes on a rotary wheel (20 rpm) before removing the sample and imaging the dye left in the tube with a mobile phone camera (Figure 4.10).

4.2.4 Visual analysis

Part 1: The Turkish market samples and reference samples were imaged using a mobile phone camera to capture what would be seen at eye level (Figure 4.11).

Part 2: For each sample there were 2 test parameters, a control sample that would allow for identification of natural fluorescence, and one that had been incubated with Jim5. 1 strand or 0.5 g of sample was used. All samples were incubated with 10 mL of milk solution (5 % w/v milk protein in TBST, 20 mM Tris-HCL, 140 mM NaCl, pH 7.5, and 0.1 % Tween 20 v/v). One set of samples was incubated with the Jim5 antibody at a dilution of 1:10 (1 mL). The samples were incubated at room temperature for 1.5 hrs. The samples were washed with TBST (TBS and 0.1 % Tween-20), rinsed 3 times over a half hour to ensure the solution had been cleared. The samples were incubated in the milk solution (10 mL) for 1.5 hrs, with a 1:1000 dilution (0.1 mL) of fluorescent secondary antibody (Goat anti-Rat IgG (H+L) cross-adsorbed secondary antibody conjugated with Alexa Fluor 488 (Invitrogen). The samples were incubated at room temperature but covered to protect from any light sources. After incubation the samples were washed in the TBST solution at least 3 times over a half hour, still kept in as dark conditions as possible. The samples were affixed to microscope slides using AF1 mounting solution (CitiFluor) before being imaged.

The samples were viewed using a Leica DM5000 microscope with camera attachment using the fluorescence setting, and a magnification level of x10. The images were created and analysed using LAS X to view the images (Figure 4.11).

4.3 Results and discussion

Spices are often targets of food fraud due to their constant use, so are seen as an easy and profitable target. As common components in cooking, it is important that their validity is monitored through authentication techniques. As fraudulent practices are constantly evolving, measures of authentication are also required to adapt. In this instance saffron has been pinpointed as a spice of interest.

The experiments explored three main areas: Polysaccharide profiling, fluorescent probing viewed by microscopy, and dye visualisation.

4.3.1 Polysaccharide profiling

The initial analysis during the spice suitability experiment (Figure 4.7) showed that the main polysaccharide was homogalacturonan (LM18 and LM19). The highest signal was given by LM19 binding to homogalacturonan within dill for the CDTA extraction. Other notable readings for homogalacturonan occurred for coriander, oregano, and dill (LM18), and oregano (LM19), again occurring within the CDTA extraction. Whilst homogalacturonan appeared high for some spices, for others it was on the lower end of the scale, particularly for cumin and rosemary. The highest levels of binding within the NaOH fraction occurred for; Oregano – xylan/arabinoxylan (LM11), onion granules and oregano - xyloglucan (LM25).

There were some mAbs that provided no/ insignificant readings. This does not mean that the polysaccharides were not present, but that they either were not extractable with this particular technique, or due to the relative abundance of other epitopes, the levels were deemed insignificant. In the spice suitability experiment, the epitopes with the least significant binding for the CDTA extraction were Xylan (LM10, LM11), xyloglucan (LM15), grass xylan preparations (LM27), glucuronoxylan (LM28), arabinogalactan protein (AGP) (Jim8), extensin (Jim11), and (1-3;1-4)-β-D-glucan (MLG). Heteromannan (LM21) also had minimal/ no binding for most of the samples apart from cumin which showed reasonably high levels of heteromannan in relation to the relative abundance.

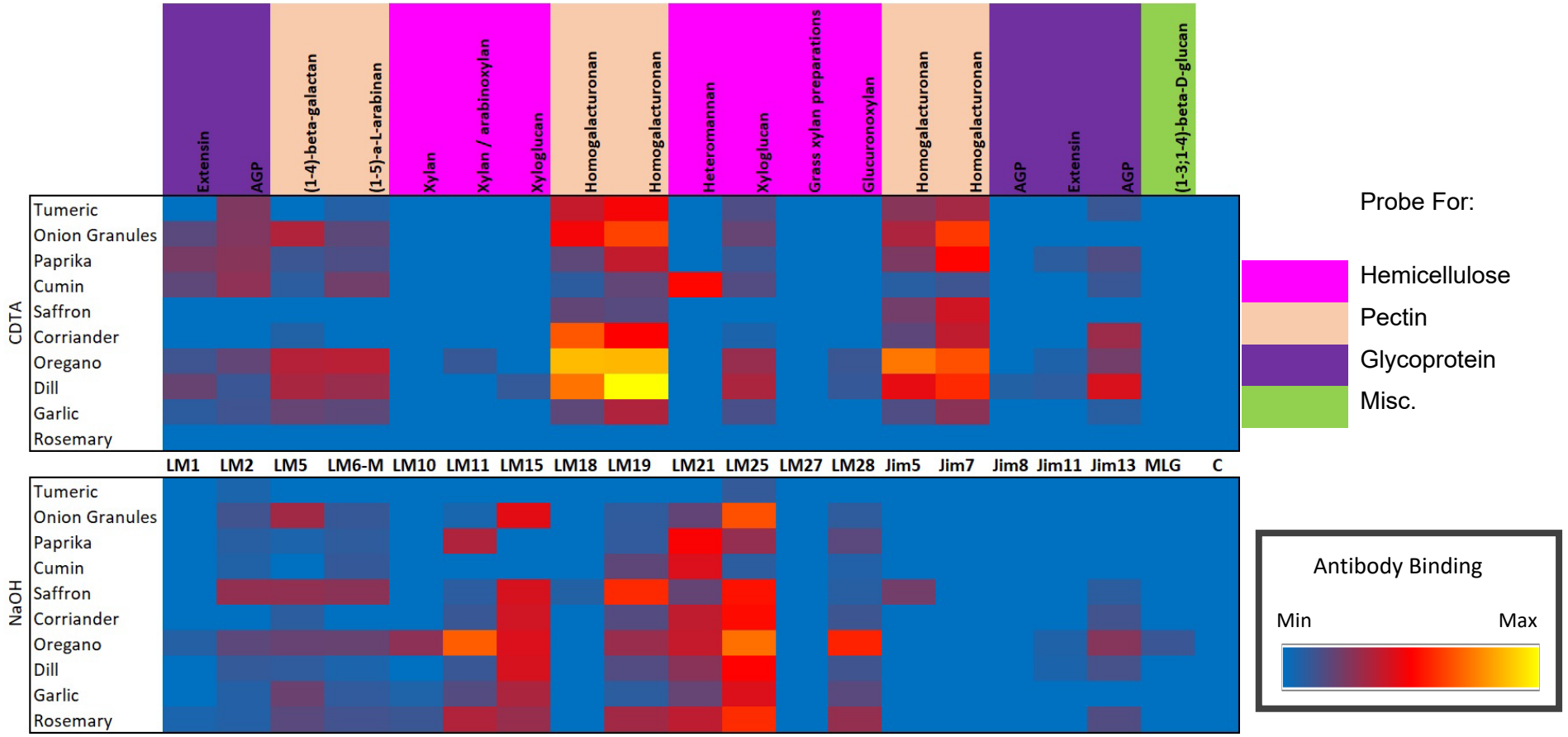


Figure 4.7. Spice suitability experiment. 10 spices were chosen and sourced from the same supermarket. Numerous mAbs were chosen to gain a broad look at which extractable polysaccharides may be present. The heatmap portrays the relative abundance of specific extractable polysaccharide epitopes within the cell wall. A control array was also probed only using the secondary antibody to ensure no contamination had occurred during the probing process. The whole heatmap was normalised to the highest intensity value, that value was then changed to 100 and other samples were normalised afterwards. Any intensity values below 5 were discounted. The numbers themselves do not provide any quantitative value, and so were hidden from view.

When analysing the NaOH extraction set, the epitopes that facilitated the least binding overall were; Extensin (LM1, Jim 11), homogalacturonan (LM18, Jim5, Jim7), grass xylan preparations (LM27), and (1-3;1-4)-β-D-glucan (MLG). It would not be unusual to see binding in one extraction set over the other due to the reasoning that CDTA targets pectins (Morris et al., 2009), and NaOH targets hemicelluloses (Hamilton et al., 1957). Rosemary provided no significant binding for the CDTA extraction, however there was intensity readings gathered for the NaOH extractions, with the highest relative abundance attributed to xyloglucan (LM25). This primary experiment showed that, for certain spices, looking into the relative abundance of polysaccharides could be an achievable venture. Figure 4.7 also showed that there is variability within the profiles of different spices, with each spice having its own unique profile, ensuring that spices could be distinguished from each other.

After the spice suitability experiment, 3 spices were chosen for further analysis: saffron, oregano, and paprika. These three were chosen specifically due to their popularity and the fact that they are also often targets for fraudulent production. The fraudulent issues surrounding saffron have already been covered within this chapter. As with saffron, oregano has an ISO standard attached to it (ISO 7925), however, as it is already known, this does not stop fraud from occurring. Black et al., (2016), conducted an experiment into identifying fraudulent oregano using FT-IR and LC-HRMS. During this experiment they analysed samples from the UK/Ireland (53 samples) and discovered that 13 of these (24.5%) being fraudulent produce, adulterated mainly with olive or myrtle leaves, some samples were adulterated by up to 70%, adulteration with other plant material being one of the more common methods. Many more studies have been done into oregano, using an array of techniques such as GC-MS, LC-MS/MS, and sequence-characterised amplified region markers (SCARs), amongst numerous others (Bononi et al., 2010; Bononi and Tateo, 2011; Marieschi et al., 2011; Wielogorska et al., 2018; Drabova et al., 2019).

As with saffron and oregano, paprika also has an ISO (ISO 7540) outlining what to expect from an authentic sample of saffron. There are also multiple regions that have a PDO for the paprika they produce. The most common methods concerning the fraud of paprika, are adulteration and document fraud, especially relating to origin. Paprika has also been subjected to many experiments focusing on authenticity. Techniques used have included Near-infrared spectroscopy, UHPLC-HRMS, HPLC-UV and ED-

XRF, amongst others (Ceto et al., 2020; Oliveira et al., 2020; Barbosa et al., 2020; Fiamegos et al., 2020).

Taking the above into account, it was reasonable that these three spices were chosen. After seeing the results of the spice suitability experiment, it was decided that several of the mAbs would be replaced. LM10 (xylan), LM11 (xylan/arabinoxylan), LM25 (xyloglucan), LM28 (glucuronoxylan), Jim8 (AGP), Jim11 (extensin), Jim 13 (AGP) and MLG (1-3;1-4)-β-D-glucan) were all removed. LM7 (homogalacturonan), LM8 (xylogalacturonan), LM9 (feruloylated-galactan), LM12 (feruloylated polymers), LM14 (AGP), LM20 (homogalacturonan), LM22 (mannan), LM23 (xylosyl residues), and LM24 (xyloglucan) were used instead.

For this experiment, each spice was treated separately and normalised internally for the creation of the heatmap (Figure 4.8). There was a six-sample set for each, all sourced from 6 popular UK supermarkets. It can be seen that each spice had a different epitope displaying the highest relative abundance. Homogalacturonan was the main polysaccharide for both saffron and oregano, although shown by different mAbs due to the targeting of different epitopes within this polysaccharide (LM19 NaOH, and Jim5 CDTA respectively). For paprika, mannan (LM21) NaOH extraction dominated the readings and provided the highest intensity value. This again shows variety in the polysaccharide profiles between different spices.

As with the spice suitability experiment, there were mAbs that did not provide significant binding. LM7 (homogalacturonan), LM8 (xylogalacturonan), LM9 (Feruloylated-galactan) and LM12 (Feruloylated polymers) gave minimal/ no binding for all spices in both the CDTA and NaOH extractions. Extensin (LM1) was found to be present in mid-range relative levels within paprika, however appeared to be absent, or present in much lower amounts for saffron and oregano. (1-4)-β-D-galactan (LM5) appeared relatively high within saffron (NaOH), giving readings indicative that in some of the samples it was second highest in abundance. These readings were not mimicked within the other spices. Homogalacturonan (LM20) appeared relatively high within saffron, and oregano (CDTA), however was on the lower end of the scale for paprika.

When analysing the results of the heatmap, it appears that the profiles of saffron and oregano are more similar than that of paprika, especially in terms of elevated levels of homogalacturonans, in comparison to the paprika samples.

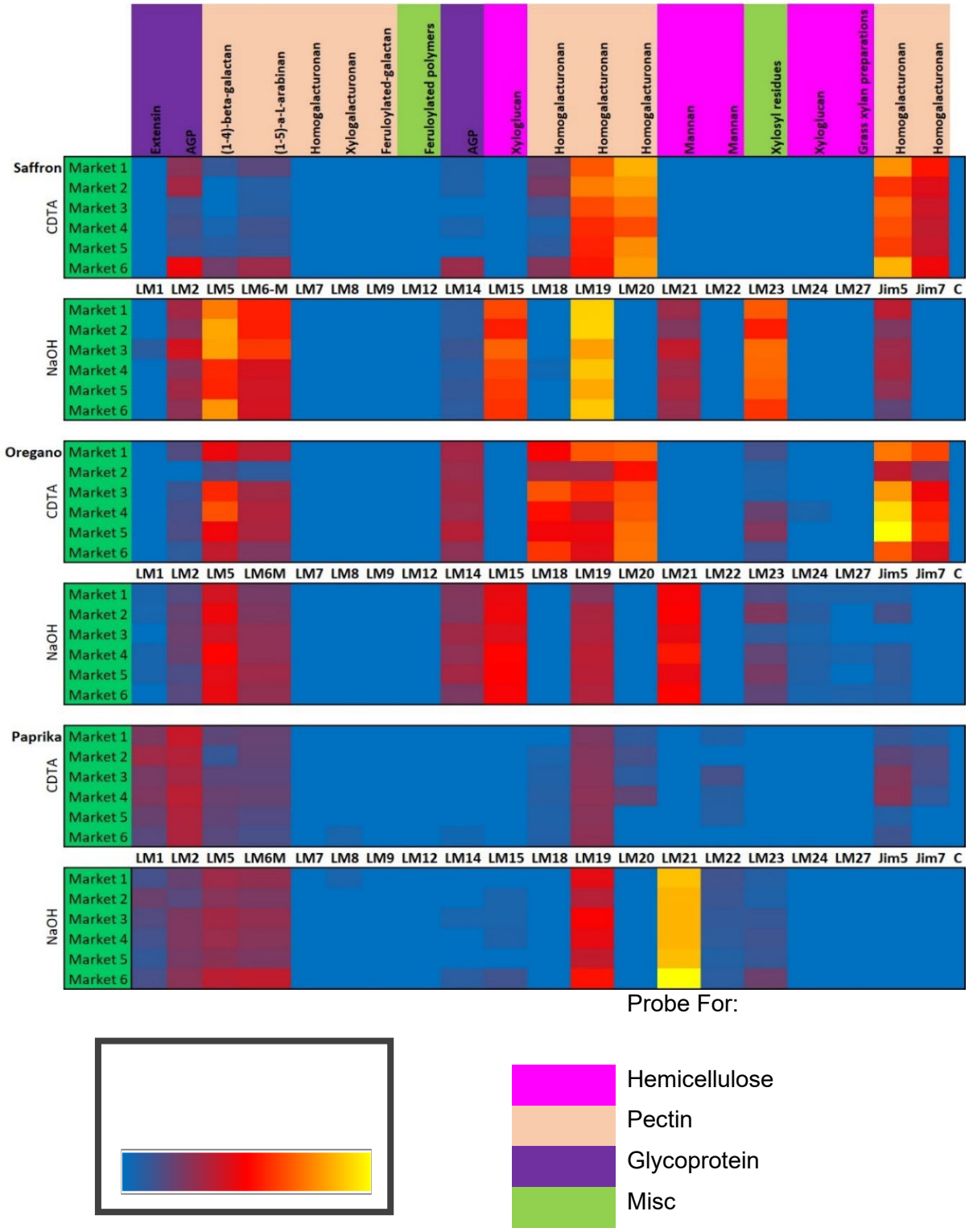


Figure 4.8. Heatmap portraying a more in-depth polysaccharide profile of three of the spices involved in the initial spice suitability experiment: Saffron, oregano, and paprika. The samples were sourced from 6 different popular UK supermarkets. The mAbs were selected based on the results of the first experiment (Figure 4.7). The heatmap displays the relative abundance of specific extractable polysaccharide epitopes within the cell wall. A control array was also probed only using the secondary antibody to ensure no contamination had occurred during the probing process. Each spice heatmap was created separately. Once the highest value had

been identified that value was then changed to 100 and other samples were normalised afterwards. Any intensity values below 5 were discounted. The numbers themselves do not provide any quantitative value, and so were hidden from view.

When analysing the results of this particular experiment, it can be seen that there are fluctuations within the polysaccharide levels of the same spice. This could be due to the origins of the samples themselves. The samples were not labelled with origin country, however Zhang et al., (2019), conducted research into polysaccharide contents of saffron samples of differing origins using multiple methods. Results suggested that polysaccharide contents do differ depending on the area of origin.

Following on from this experiment, it was decided that saffron would be the focus of a more in-depth look, involving more samples. This was due to the popularity of saffron and the fact that it is the most expensive spice in the world. For this extended sample list, 15 samples were used; the 6 supermarket samples, 4 reference samples from a reputable source, and 5 samples sourced from a Turkish market stall. The main aim of this experiment was to see how the samples compared to the reference samples, especially the ones bought from the Turkish stall. The aim was not to categorically declare if one was fraudulent, but to see if any could show reasonable suspicion from a polysaccharide profile (Figure 4.9).

Within this experiment, all samples were normalised to the highest amount recorded for relative abundance (Chapter 2.2.4), which in this case was for Turkish light saffron, homogalacturonan (LM19), CDTA extraction. When compared with the previous heatmap (Figure 4.8) it may look like the binding is not as strong considering homogalacturonan (LM19) NaOH was providing the highest intensity values. However due to the high readings from the Turkish light samples, this would have proportionally affected the relative abundance for this set of samples. As was seen previously, LM7 (homogalacturonan), LM8 (xylogalacturonan), LM9 (Feruloylated-galactan) and LM12 (Feruloylated polymers), LM24 (xyloglucan), and LM27 (grass xylan preparations), gave no/ minimal signal from the probing process. Within this set of samples, it was as much about epitopes that were present, than ones that seemingly weren't. It has already been shown that extensin (LM1) CDTA extraction was present in relatively small or negligent amounts for the saffron samples. Within this set, the relative abundance of the Turkish market samples was around mid-range or lower, with the exception of the Persian powder which did not register any significant readings. (1-4)-

β-galactan (LM2) and (1,5)-α-L-arabinan (LM6-M) also registered higher relative abundances for the Turkish market samples than the reference and supermarket samples. This would imply that these epitopes are of higher abundance in the Turkish market samples than the reference and UK supermarket samples, implying that the pairings of these probes and epitopes could be useful in identifying suspected fraudulent samples, with the probes having high specificity for the epitopes they are binding to, makes monoclonal antibodies, in this case, a more suitable solution than polyclonal antibodies which may not be as specific.

Homogalacturonan (Jim5 and Jim7, CDTA) can be seen to be in higher relative abundance for the reference and UK supermarket samples than that of the Turkish market samples. This would imply that this epitope is not as present at this point and could be seen as a probe/ epitope pairing of importance within the authentication process.

For the NaOH extractions, LM19 (homogalacturonan), LM23 (Xylosyl residues) and Jim5 (homogalacturonan) could all be identified as probes that may be beneficial in authentication measures. Both gave higher readings for the reference and UK market samples than the Turkish market samples. For the NaOH extraction, there were no intensity values, and subsequently no relative abundance for the Persian powder sample.

Whilst it would not be fair to outright declare a sample as fraudulent within this experiment, it would seem that the samples sourced from the Turkish market have significantly different profiles to those of the reference and those obtained from UK supermarket samples. As previously mentioned, polysaccharide content can differ in saffron due to origin, but the current question is how much of this divergence can be attributed to that?

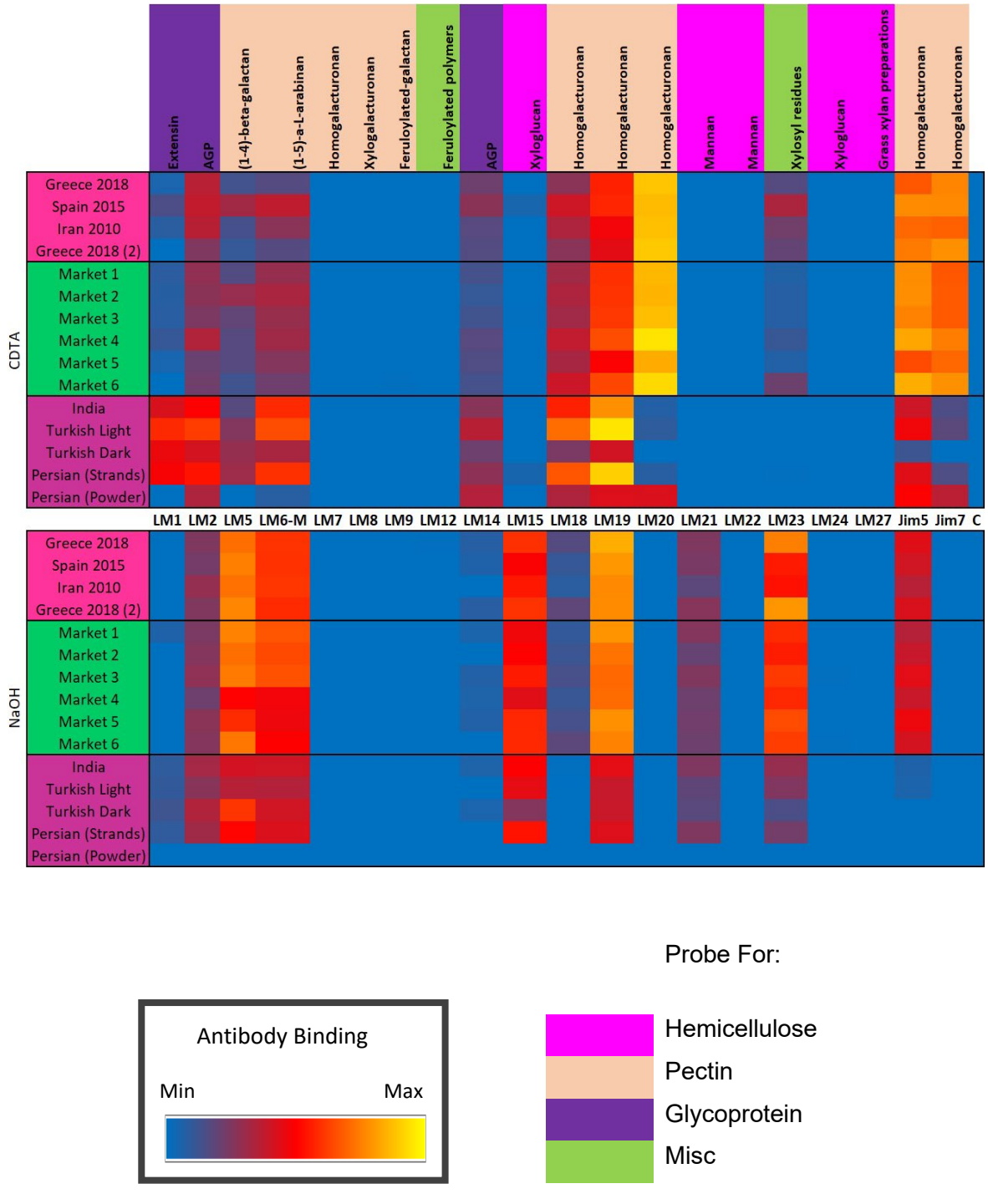


Figure 4.9. Heatmap portraying a polysaccharide profile of saffron samples. Saffron was chosen to be investigated further. The samples included: 4 reference samples from a reputable source (Pink), 6 supermarket samples (Green), and 5 samples bought from a local market in Turkey (Purple). The mAb selection remained the same as the previous experiment (Figure 4.8). The heatmap displays the relative abundance of specific extractable polysaccharide epitopes within the cell wall. The whole heatmap was normalised to the highest intensity value, that value was then changed to 100 and other samples were normalised afterwards. Any intensity values below 5 were discounted. The numbers themselves do not provide any quantitative value, and so were hidden from view.

Polysaccharide analysis of spices, especially via mAb probing, has not seen a wide interest in terms of research, including the area of saffron. This meant that comparison to published works was not as simple, therefore more experiments into different characteristics were conducted – visual authentication, using sight and fluorescent microscopy, as well as dye analysis – looking visually at the colour of the dye given off.

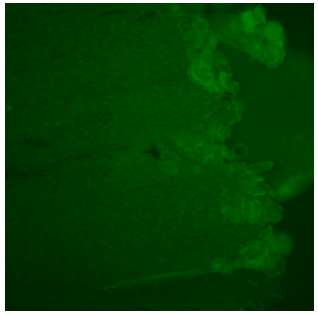
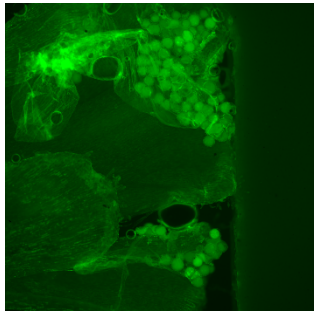
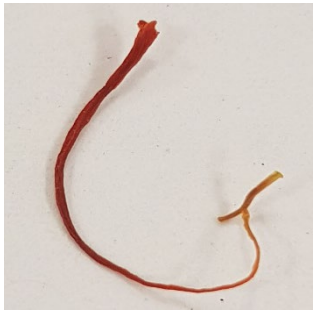
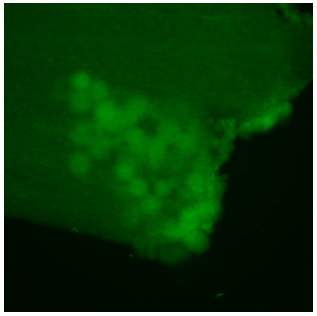
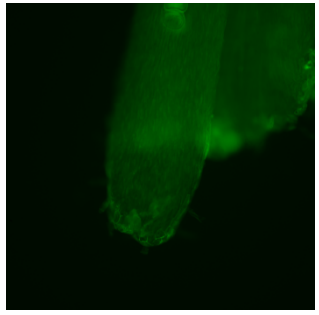
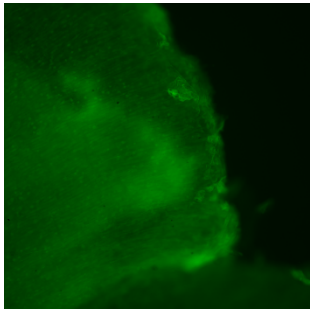
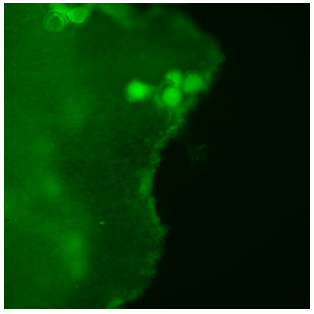
4.3.2 Visual analysis

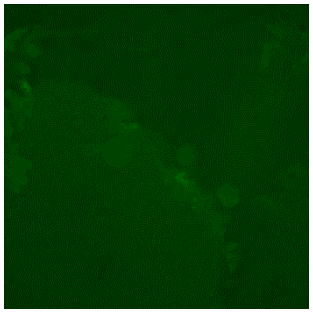
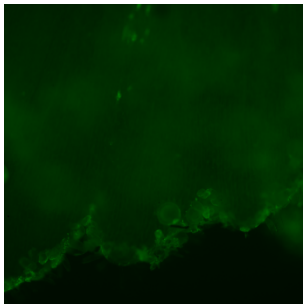
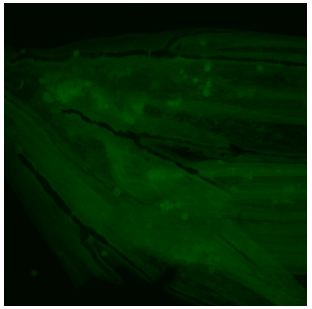
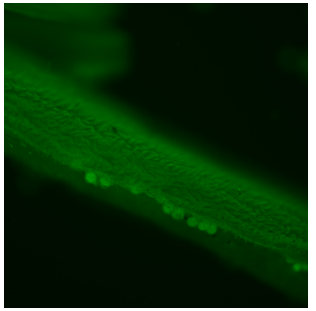
Images were taken of the 4 reference samples and the 5 Turkish market samples used during the saffron extended sample set experiment (Figure 4.9). This provided a visualisation, and it can be seen (Figure 4.10), that some of the samples do not have the typical saffron characteristics, primarily the Indian Saffron, Turkish light, Turkish dark, and Persian samples. It could be suspected that these are fraudulent samples, possibly another species of plant, such as safflower. Usually, you would expect to see dark red strands as seen in reference samples 1-4, however the other samples are more orange/yellow in colour, with a more bud-like structure rather than a long stand as seen with the reference samples. The powdered Persian saffron has been ground to a powder and so visual inspection at this level would not wield any beneficial results.

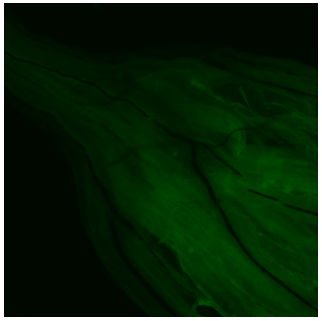
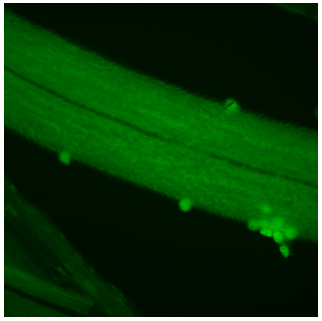
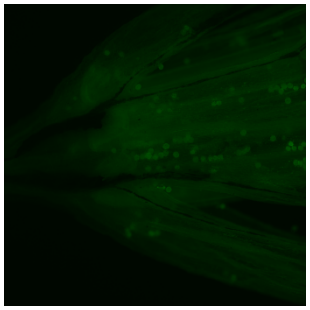
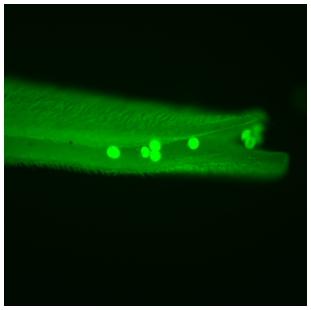
The fluorescent microscopy allows for a more intense look at the structures. Each of the images depicts the tip of the sample. As can be seen in Figure 4.10, the reference samples have very similar structures with small amounts of natural fluorescence highlighted within the control samples and circular structures collected on the tip – most likely pollen. When incubated with the Jim5 antibody, there was minimal amounts of fluorescence on the actual body of the strand, indicative of low levels of homogalacturonan within this area, although some was found on the circular structures. With the Indian Saffron, Turkish light, Turkish dark, and Persian samples, the control samples had minimal natural fluorescence as seen by the darker images, however, when probed with Jim5 the length of the structure shows presence of homogalacturonan, which wasn't seen in the reference samples. In addition to this, at 10x magnification, hair like structures can be seen on the body of the samples, something not present within the reference samples. The powdered Persian saffron showed no/ minimal binding in the Jim5 probed samples, alluding to the lack of surface homogalacturonan.

Jim5 was chosen as a probe after the results from the saffron expanded sample set polysaccharide profiling showed a distinct difference between the extractable levels of

homogalacturonan within the reference/UK supermarket samples, and the Turkish market samples. It was considered that Jim5 could be a potentially beneficial probe when looking at authentication and see how this would translate when probing the whole strand rather than just looking at extractable polysaccharides.

		Control	Jim5
Reference Sample 1:			
Reference Sample 2:			
Reference Sample 3:			

		Control	Jim5
Reference Sample 4:			
Indian Saffron:			

		Control	Jim5
Turkish Light Saffron:			
Turkish Dark Saffron:			

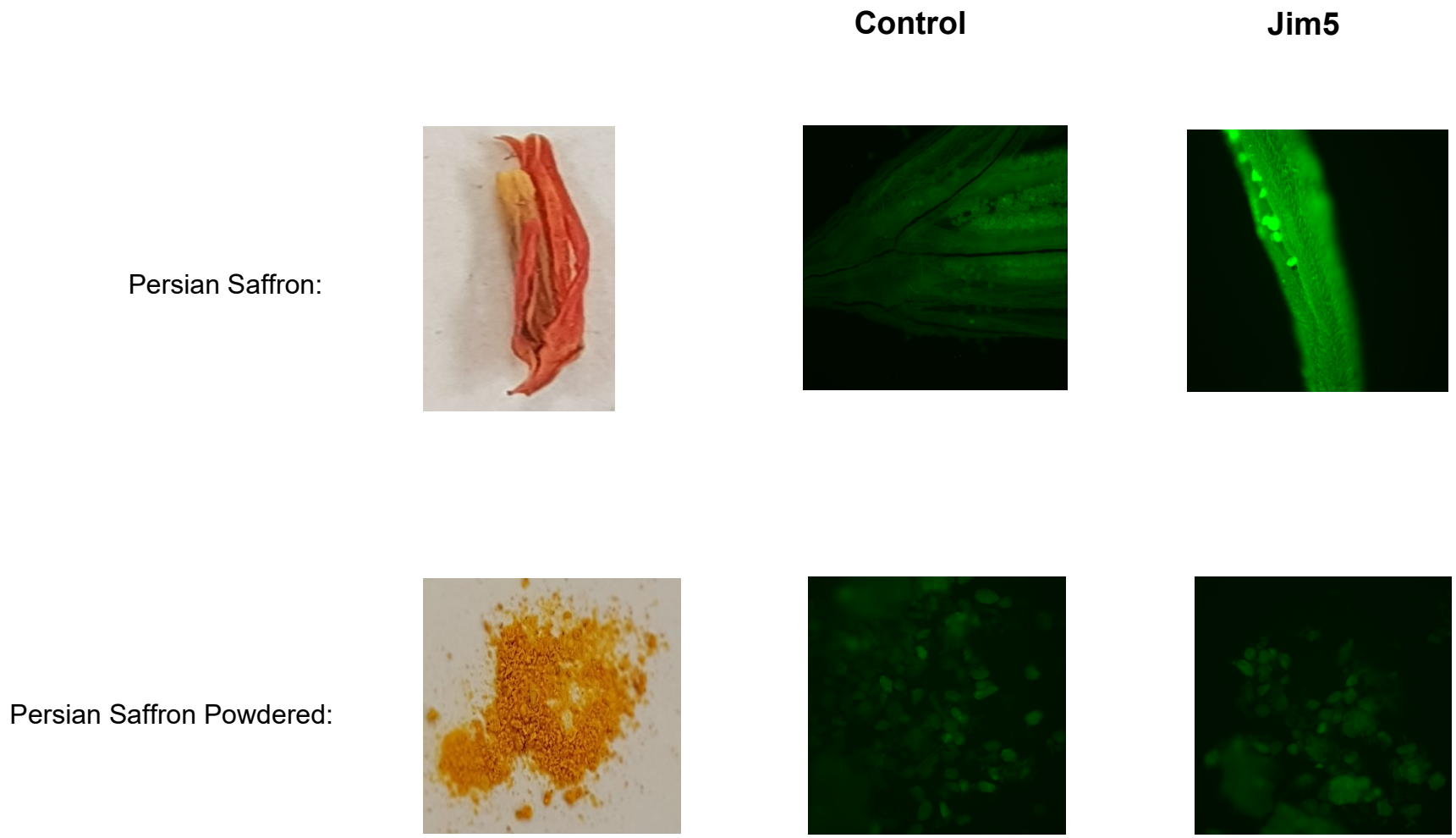


Figure 4.10. Visual analysis of the samples. The reference and Turkish market samples were imaged as well as fluroescently probed (Jim5 and a control sample) and visualised using a fluorescent microscope at x10 magnification. The images show drastic differences between structures in the samples.

4.3.3 Dye analysis

As outlined in ISO 3632, dye analysis may enable us to conclude whether a sample is fraudulent or not. In this case it would ascertain whether the sample has been dyed – possibly with a carcinogenic substance, as discussed before. The dye visualisation protocol was followed, as outlined previously, on the reference samples and the Turkish market samples (Figure 4.11). UV-Vis spectrophotometry analysis is still being debated as a good measure of authenticity due to concerns that the wavelengths used to identify safranal and picrocrocin (330 nm and 257 nm), are also wavelengths at which other compounds are absorbed meaning this technique may not be selective (The Food Integrity Project, 2018). Whilst there are reservations about the effectiveness of UV-Vis analysis, it remains to be a named method within ISO 3632. The water test is a crude test that cannot declare authenticity outright, but it could give a basis of an idea, and can be done at home by consumers if they had any suspicions.

UV-Vis spectrophotometry however is not the only way dyes have been analysed. Balbas et al., (2021), used Raman spectroscopy, and Petrakis et al., (2017), used ^{1}H NMR, so there are other viable options, although UV-Vis spectrophotometry is still recommended within the ISO 3632 protocol.

Whilst the above experiment is by no means an authentic measure of identifying fraud, it wielded an interesting result and could provide a basis or extra information to back up previous results especially when coupled with the images of the samples (Figure 4.11).

The top row of figure 4.11 contains the reference samples used for the extended saffron sample experiment. All 4 samples have produced a light-yellow dye, due to the presence of crocetin glycosyl esters (crocin) (Lech et al., 2009), when subjected to water treatment. It would be a reasonable assumption that these samples had not been dyed due to the presence of the yellow hued dye in conjunction with the knowledge that the samples were from a reputable source.

The bottom row contains the Turkish market samples (L-R) Indian saffron, Turkish Light Saffron, Turkish Dark Saffron, Persian Saffron and Persian Saffron (Powder). The Indian, Turkish Light, and Persian (powder) samples all have a light-yellow hue and would not arouse the suspicion of being dyed. The Persian saffron has a yellow-red tinge, nothing conclusive but could warrant further testing. The Turkish dark sample

displayed a red dye which would warrant further testing as it could be suspected to be fraudulent.

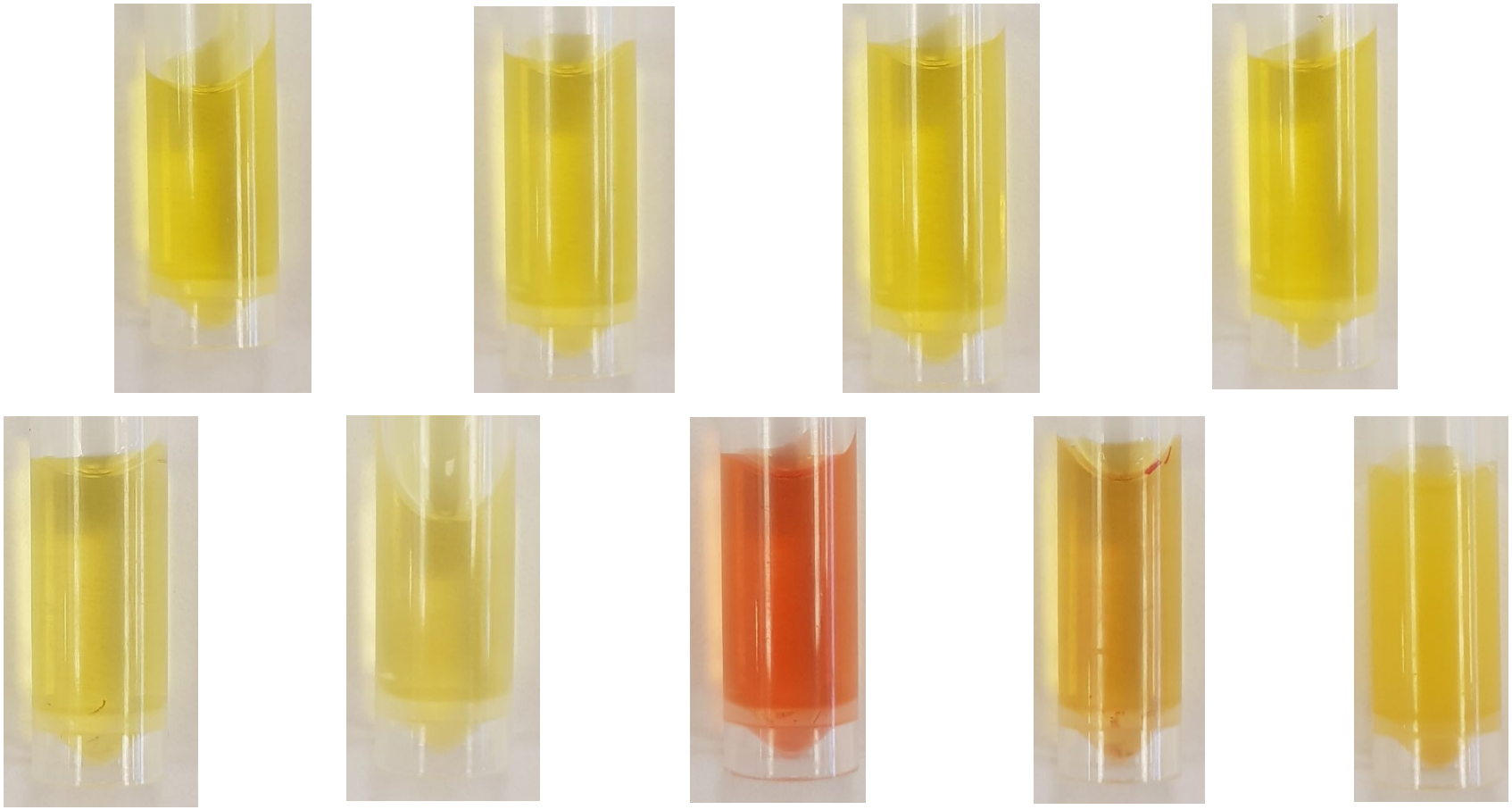


Figure 4.11. Visualisation of the dye of 9 saffron samples. 4 reference samples and 5 Turkish market samples were covered with water and the subsequent dye that was given off was imaged. Although saffron is a vibrant red colour, the dye given off when ran under water it yellow. The top row consists of the reference samples 1-4, respectively, and the bottom row shows the dye of the Turkish market samples (L-R) – Indian saffron, Turkish Light Saffron, Turkish Dark Saffron, Persian Saffron and Persian Saffron (Powder).

4.4 Conclusion

Saffron is a valued commodity within medicine, fragrance, and culinary areas. Due to its elevated cost and reputation, it is a prime target for fraud. With the constant threat of fraudulent produce, it is important to constantly monitor authenticity to protect consumers and limit economic loss. As previously mentioned, some economies are well supported by the saffron trade and pride themselves on the quality of produce. It is possible that the protocol described could be applied to saffron authentication. This would provide a high-throughput means of testing focusing on polysaccharide profiles, this can be seen by the significant differences highlighted in the profiles, and the further analysis from the microscopy and dye visualisation. Whilst saffron featured heavily within this chapter, it should be noted that the primary experiments included other spices and showed that polysaccharide profiling is an area to be further explored for spice authentication.

Chapter 5. Gluten

5.1 Introduction

For those with allergies, intolerances and food related autoimmune disorders, having confidence in the food that they eat is of the utmost importance. The UK has strict measures with regards to listing the major food allergens on packaging, however cross contamination may also occur whether that is accidental, or purposely for the reasons of economic gain. This means regular food testing on both large and small scales is imperative.

Attitudes to food within the UK have seen drastic changes since the turn of the millennium. Social media and easier access to a wider range of foods have allowed consumers more autonomy over what they consume, with many opting for limited diets such as vegetarian, 'carb-free' and gluten-free, with many of these diets being conducted without consulting a nutritional expert. This change in the approach to what we consume has led to an influx of foods that are 'free-from' (e.g. meat-free, sugar-free, gluten-free), however the majority of these categories are not fully legislated and so the claims are rarely substantiated.

5.1.1 Allergies

It is estimated that approximately 1-3% of adults worldwide, are currently living with a validated food allergy, with rates in children as high as 8% (Osterballe et al., 2005; Rona et al., 2007; Venter et al., 2008; Longo et al., 2013). Research conducted between 1998-2018 reported that whilst deaths caused by anaphylaxis due to food allergies have decreased within this period, hospitalisations have nearly doubled (Conrado et al., 2021). Instances of allergy related deaths have made for dramatic headlines in recent years. In 2017 a young male died after consuming a burger at a UK chain restaurant that he did not realise contained buttermilk. The fatal reaction occurred within an hour of ingestion. The resulting coroner's report made some suggestions that the death may have been facilitated by the lack of staff training in relation to allergies, as well as insufficient/unclear information regarding allergens that may be present within the product (Coroners report, 2019). Another case in 2016 involved a young female who ate a baguette sandwich and suffered a fatal reaction to sesame seeds that were inside, but not documented on the labelling. The coroner's inquest detailed that there were failings including not adequately or clearly stating the allergens, as well as a lack of a system documenting allergic reactions to the

companies produce, which would have helped in tracking shortfalls within the company (Coroners report, 2018). As a result of this case in particular, the government created Natasha's Law (Food Standards Agency, 2021 (c)). Both of these unfortunate cases occurred due to failings in clearly listing the allergens present, and as such would not necessarily be deemed a case of food fraud. However, this is not always the case.

In 2014, an adult male succumbed to anaphylactic shock after consuming a takeaway meal later found to contain peanuts. When bought to trial, the restaurant owner was charged with manslaughter and sentenced to jailtime. This was one of the first instances in the UK where such a sentence had been brought about due to death by anaphylactic shock caused by food allergies. It came to light that although the deceased had been assured there would be no peanuts present in his meal, the restaurant owner had resorted to cost-cutting measures and had substituted an almond mix for a ground nut powder found to contain peanuts. The core reason for this case was for financial gain, therefore the owner was charged with manslaughter due to gross negligence (BBC, 2016; Anaphylaxis Campaign, 2017). Whilst these three cases differ, it highlights the importance of food testing. Whilst not all allergies result in death it can still be a traumatic experience. Consumers have a right to feel secure about the food that they are eating.

Due to instances such as the cases previously outlined, the UK government brought in measures aimed at limiting the risk to those who suffer allergies when choosing to consume certain food produce. Natasha's Law, previously mentioned above, was officially introduced into UK law in 2021 (Food Standards Agency, 2021 (c)). It summarised the requirements for businesses to state any allergen that is present in their food produce even when packaged and sold at the same site – prepacked for direct sale (PPDS) (Food Standards Agency, 2021 (d)).

5.1.2 Intolerances

Whilst food allergies gain more recognition as seen from above, food intolerances can also have major effects on a persons' diet and day to day quality of life. The main difference between a food intolerance and a food allergy is that an intolerance does not involve the immune system (Bindslev-Jensen et al., 1994; Ortolani and Pastorello, 2006; Zopf et al., 2009). Dairy and gluten have been identified as the two main food

intolerances in the UK, with reports suggesting that up to 20% of the population suffers from a food intolerance, and whilst intolerances often cause discomfort, these symptoms are not life threatening, with those that suffer often experiencing mild bloating, headaches, nausea etc (Lomer, 2014).

5.1.3 Autoimmune disorders

Autoimmune disorders can also be triggered by certain foods. Some theories suggest diet and the consumption of certain foods may play a role in triggering disorders such as multiple sclerosis, due to the complex nature of internal systems (Haghikia et al., 2015). From current understanding only one autoimmune disorder has been directly associated with the consumption of a specific food – Celiac disease. Celiac disease is trigged by the consumption of food products that contain the protein gluten which induces symptoms such as bloating and abdominal distension.

The main focus of this chapter will be on gluten. This protein is present within some cereals such as wheat, and whilst not the direct trigger for all, is involved in the mechanisms of:

- wheat allergy
- celiac disease
- gluten ataxia
- dermatitis herpeteformis
- non-celiac gluten sensitivity,

amongst others (Bonciani et al., 2012; Nilsson et al., 2015; Cianferoni, 2016; Watkins and Zawahir, 2017; Leonard et al., 2017). With the current market value estimated to be around £835 million per year, the gluten-free market can understandably be viewed as a promising venture (Coeliac UK, website, 2022).

5.1.4 Gluten

For most of those able to consume it, gluten is ingested almost daily, and constitutes a portion of worldwide food staples such as leavened bread and pasta. Within the UK it is currently listed as one of 14 foods required by law to be listed on food packaging as ‘cereals containing gluten’ (Food Standards Agency, 2021 (d)). Whilst gluten may at times be hard to avoid, the gluten-free food market is on the increase, with many adopting a gluten-free diet (GFD) either through necessity or for their own reasons. When considering the legality of ‘free-from’ foods, gluten-free is the only protected

category, meaning it is governed by legislation to keep consumers from any possible harm caused by consumption (Food and Drink Federation, 2019). Current UK law defines a gluten-free product as one that contains less than 20 parts per million of gluten. Products that require information on the presence of gluten are those containing: wheat, barley, rye, and oats. It should be specified that ancient grain types, such as spelt, are also included within this list. Whilst oats themselves do not contain gluten, due to the processing procedures, oat products can often be contaminated as they are handled in the same facilities as gluten containing cereals and so cross-contamination is near impossible to avoid (Food and Drink Federation, 2019).

Prior to recent times, gluten was not considered an area of much scientific interest with regards to gluten related disorders (GRDs), however with the steady increase of GRD diagnoses as well as an influx of people participating in a GFD, the focus on gluten has increased as there is now more money involved in the gluten-free market. Wrigley and Bietz, (1988) categorised gluten as 'the largely proteinaceous mass which remains when a dough made from wheat flour and water is gently washed in an excess of water or dilute salt solution to remove most of the starch and soluble material'.

Gluten proteins are fundamental for progress within the primary stages of a plant's life, as they are involved in supporting germination and the subsequent development of the seedling (Shewry, 2019). Within the grain they are wholly located within the largest area, known as the starchy endosperm. Due to the elastic nature of dough made from wheat flour, we are able to manufacture staple food items such as leavened bread, pasta, noodles, and other various bakery produce which are not feasible with other cereal types due to their lack of gluten (Shewry, 2019).

Gluten has a plethora of properties that deem it desirable, such as the ability to retain moisture and improve areas such as texture or flavour. With these useful characteristics, gluten is often used in foods that may be unexpected including:

- ice-cream
- processed meats
- meat substitutes
- stuffing
- marinades

just to name a few (Biesiekierski, 2017). With these seemingly inconspicuous additions, it is of even more importance for those unable to consume gluten to be aware of the inclusion, and for testing regimens to be reliable and robust when looking for gluten.

Gluten makes up to 90% of the total protein within wheat and is primarily made of 2 major proteins: glutenin and gliadin (Biesiekierski, 2017). These gluten proteins can vary in composition as well as proportions of proteins between grain species. Glutenin and gliadin are categorised as prolamins, meaning that they are extractable by ethanol, however, they are insoluble in water. As prolamins, they are also known for being mainly constituted of glutamine (38%) and proline residues (20%) (Wieser, 2007). There are 4 classes of gliadin: alpha (α), beta (β), gamma (γ), and omega (ω). The primary structure of gliadins is dependent upon which class it belongs to, which is determined by factors such as molecular weight (Shewry and Lookhart, 2003; Biesiekierski, 2017). Gliadins and glutenins are present in different proportions dependent upon grain species. Due to these differences, the properties of gluten are effected – higher levels of gluten will affect the viscosity, whereas higher levels of glutenins are indicative of a stronger dough (Wieser, 2007).

This chapter will focus on gliadin as the target of the experiments due to the availability of the mAb probes used and the higher levels of gliadin in comparison to glutenin within gluten (Geisslitz et al., 2019).

5.1.5 Current industrial food testing

In line with the Association of European Coeliac Societies (AOECS, 2020), a gluten-free food product can be defined as such if at least one of the following criteria is met:

- The product must not contain ingredients of wheat (inc. species), rye, barley, oats. There must not be a higher level than 20 mg/kg of gluten. The gluten level within the product should be, proportionally, no higher than 20 mg/kg
- The product may include wheat (inc. species), rye, barley, oats if they are varieties whereby measures have been taken to remove the gluten – as above, the gluten content must be less than 20 mg/kg

Oats are included in this description as they are often produced within the same facilities with gluten-containing produce, meaning that cross contamination is almost always an issue as it is difficult to control.

Whilst there are at home kits available for those who wish to check gluten content within a product, although the results of these can vary between kits (Hochegger et al., 2015), it is also important for analysis to take place at an industrial level, with spot checks important for monitoring conformity.

The AOECS advises on the procedures that should be used for analysis.

- The general method of use is the R5 ELISA
- For produce such as flours (unprocessed), an ethanol extraction is advised

The ethanol extraction is a simplistic methodology that relies on the use of 60% ethanol to remove gluten from the samples (Melini and Melini, 2018), whereas the R5-sandwich-ELISA works by using the monoclonal antibody R5 which is specific to rye secalin (Hochegger et al., 2015). The sample is added to a well that is already coated with a 'capture' antibody and incubated. If the sample contains the specific antigen, then it will bind to the capture antibody. The wells are then washed to ensure the removal of any sample that has not bound. An enzyme conjugate is then added, which is specific to the antigen, creating a 'sandwich', the mixture is incubated before being washed to remove any excess. A chromogen is then added which binds to the enzyme conjugate, and if the specific antigen is present in the sample, a colorimetric response will occur, indicating the target is present within the sample (Hochegger et al., 2015). In this case, if gluten is present in the sample, a colorimetric response would be given, alerting to the presence of gluten.

Whilst it is important for spot-checks to be conducted, it is also important for the environment to maintain limited cross contamination. Current guidance outlines how GF produce should, where possible, be produced in its own space. If this is not the case then a sufficient cleaning and testing/sampling regimen should be performed in order to minimise risk of cross contamination (AOECS, 2019).

5.1.6 Celiac disease

First described in 1887 by Samuel Gee (Burki, 2019), Celiac disease (CD) prevents the effective digestion of gluten for those affected. Current estimations have the rates of CD at around 1% of the global population, with the chances increasing to 10% if a

close relative suffers from the disease (Ralbovsky and Lednev, 2021). The prevalence of CD is slowly increasing, which may be due to better testing or even better awareness although the actual cause is unknown (Catassi, 2014).

CD is categorised as an autoimmune disorder, and research suggests that there is a correlation between diagnosis age and the risk a patient has of developing further autoimmune diseases, linked to the often-prolonged exposure to gluten (Ventura and Greco, 1999).

It should also be noted that ancient grains should be avoided by those with CD. Research produced by Geisslitz et al., (2019) showed that when researching spelt, emmer, einkorn, and common wheat, wheat contained the lowest gluten content at 33.2 mg/g, in comparison to Oberkulmer (a spelt cultivar) at 111.6 mg/g.

Within the same experiment it was shown that spelt, emmer, and einkorn all contained higher levels of gliadin, rather than glutenin, within gluten in comparison to common wheat, although this was not the case for durum wheat, the gliadin contents were described as:

- Spelt – 70-83%
- Emmer – 75-92%
- Einkorn – 79-92%
- Common Wheat – 61-79%

The report also specified that gluten levels were influenced by species rather than factors such as growing conditions (Geisslitz et al., 2019). The findings of this study would indicate that those diagnosed with CD would not be able to consume ancient wheat varieties as a compromise. This study is not the only one to have been conducted on the viability of including ancient grains in the diet, others have reached the same conclusion that it would not be reasonable or safe to include ancient grains within a gluten-free diet (Malagoda et al., 2019; Colombo et al., 2021).

Whilst there is an estimated 1% worldwide prevalence, it is thought that cases of CD could be much higher due to those whose symptoms are not present and therefore may not be aware they are affected by the disease (Hischenhuber et al., 2006). Interestingly, the Saharawi people of the Western Sahara Desert, have a 5.6% presence of CD amongst them – much higher than the general population (Catassi et

al., 1999). Research has suggested that in some cases, CD could have been triggered by infection at a young age and so would not necessarily be linked to genetic factors (Bouziat et al., 2017; Brown et al., 2018).

Conducted studies have found that the most well-defined genetic factor for CD risk (35%), is the presence of major histocompatibility complex (MHC) class II proteins, this includes human leukocyte antigen (HLA) DQ2 and HLA-DQ8 (Romanos et al., 2009; Trynka et al., 2010). Of those with the genetic predisposition, 90% possess the HLA-DQ2 over the HLA-DQ8. Whilst a subject may possess a risk factor, of those that do, it is estimated that roughly 1-3% go on to develop CD (Sollid et al., 1989; Hoffenberg et al., 2003).

When ingested, gliadin is able to interact with the intestinal cells. The interaction causes the inter-enterocyte tight junctions to disassemble. As a consequence of this, the tight junctions cause an up-regulation of zonulin (a peptide involved in gut permeability). This allows for gliadin to enter intestinal cells via the epithelial barrier, T-lymphocytes which are located within the lamina propria are then activated by this (Parzanese et al., 2017).

These T-lymphocytes go on to excrete pro-inflammatory cytokines, which has the possibility of creating a ‘clonal expansion’ of B-lympocytes – these have the ability to tell the difference between plasma-cells that secrete anti-gliadin and anti-tissue-transglutaminase antibodies (Björck et al., 2015). Not all gliadin peptides are recognised by these antibodies, and so those that aren’t, are available to activate intestinal epithelial cells as well as antigen-presenting cells. This mechanism means that the mucosal surface is impaired causing nutrient absorption to be sub-par and the manifestations of symptoms such as bloating, fatigue and abdominal pain (Barker and Liu, 2008).

Currently, the main effective treatment for CD is a gluten-free diet, highlighting the importance for monitoring the lack of gluten in food produce, allowing consumers to be comfortable in the choices they make.

5.1.7 Wheat allergy

An allergy to wheat can be classified as IgE or non-IgE mediated. Non-IgE mediated are those such as CD, or non-celiac gluten sensitivity (NCGS), whereby there may be a delayed response after ingestion. IgE-mediated wheat allergy is one where the

immune system may respond to several proteins found within wheat within a short period of being exposed – this includes proteins found within gluten (Cabanillas, 2020).

IgE-mediated allergies can be triggered by either ingestion or inhalation of the product. For an IgE-mediated response to occur, T helper type 2 inflammation causes the production of IgE antibodies, from B cells, which are specific to certain produce. Another path is that this allows for a chronic cellular inflammation which can be recognised by the T-cells or eosinophils present (Cianferoni and Spergel, 2009).

Studies have shown that the highest prevalence of wheat allergy is within children, especially those under the age of 12, although it is known that the allergy is often outgrown, explaining the lower instances of cases within adults (Poole et al., 2006; Keet et al., 2009). Research has also produced observations of a negative correlation between wheat IgE levels and the proportion of those who outgrow the allergy; as IgE levels increase, the percentage of those who outgrow it decrease, although it should be stated that it is not impossible to outgrow just because there is a high presence of wheat IgE.

Current diagnosis for a wheat allergy is based on analysis of previous reactions, which are of interest especially if they occurred within a few hours of ingestion/inhalation. In conjunction with taking a history, the allergy can be confirmed via testing serum for IgE or a skin prick test (Keet et al., 2009).

Baker's asthma

A subsect of wheat allergy, Baker's Asthma (BA), mainly affects career bakers working in flour laden environments whereby clouds of flour 'dust' are able to form and is classified as an IgE-mediated allergy. Evidence has suggested that this affliction had been noticed as early as Roman times, with writings about how workers during these times would use cloth to protect their faces when working with flour. Although the Romans had noted the possible effects of working with flour, it wasn't until 1700 that these observations became more popular. Bernardo Ramazzini described how bakers and those working with flour would sometimes present with respiratory difficulties and the link was formed to working in close confines with flour (Brisman, 2002).

BA is now fully recognised within the UK as an occupational hazard, and the second leading cause of occupational asthma. Global studies put the prevalence of

sensitisation of those that work with flour at anywhere between 5-28% (Jeebhay and Baatjies, 2020). The inhalation of the dust formed by working with flour is the causative agent, and so it is imperative that measures are put in place to limit exposure. Health and Safety Executive (HSE) UK, (2021) outline ways in which BA can be avoided in the workplace. HSE advice is to employ the use of dust extractors or introduce the use of respiratory equipment, as well as working carefully to avoid the creation of dust clouds – this includes not sweeping or employing the use of compressed air.

Whilst the inhalation of flour dust may seem trivial, BA can have serious negative effects on the sufferer's day to day well-being. A case was brought to light whereby a school cook had developed BA after being required to work in an area with poor ventilation, this led to them being unable to sleep laying down and trouble breathing meant that they could no longer walk. The cook was required to produce dough from flour in an environment where no measures were put in place to limit dust. Despite complaints, no changes were taken, the council eventually took responsibility for the development of BA and the cook was awarded £200,000 (HSE, 2021).

After one study, gliadin was considered to be an 'inhalable allergen' after 33% of a tested cohort were found to react against gliadin (Bittner et al., 2008). In addition to BA, bakers may also experience dermatitis herpeteformis (DH) as a side effect of working with flour without adequate protections. DH presents itself as blisters upon the skin, and whilst it is normally associated with celiac disease it has also been reported in those working with flour and the resultant dust (Kárpáti, 2012).

5.1.8 Non-Celiac Gluten Sensitivity

A letter to the editor in an April 1978 edition of The Lancet outlined some of the adverse allergic reactions various patients had experienced with different foods. One of these patients was later described as suffering from a wheat allergy and recommended a gluten-free diet (Dickerson et al., 1978). A short while after, in a different edition, A. Ellis and B.D. Linaker (1978) mentioned this letter when their own was published. This letter described a patient they had been observing. A 43 year-old female had been suffering for 4 months with various symptoms including diarrhoea, abdominal pain, and abdominal distension. The patient underwent numerous tests ranging from x-rays to biopsies in order to rule out various ailments. All tests were inconclusive. The patient

continued to suffer this issue for the next 2 years until it was suggested that they try a gluten-free diet, and the symptoms began to clear after 4 days.

To ascertain if gluten was the problem, the patient was put back on a diet containing gluten. They displayed symptoms relating to an adverse effect to gluten and had a biopsy at the end. The biopsy returned a normal result. Ellis and Linaker concluded that from these tests, the patient must be suffering from a gluten sensitivity, rather than celiac disease or wheat allergy, and thus described one of the first documented cases of non-celiac gluten sensitivity (NCGS) (Ellis and Linaker 1978).

A 1980 article published in Gastroenterology shortly after the Ellis and Linaker letter described a study following 17 patients. 15 women and 2 men were studied, all of whom had not received a diagnosis for celiac, however had been suffering concerning symptoms. Having suffered from anywhere between 20 years to 7 months, some of the described symptoms included:

- Chronic diarrhoea – Suffered by all
- Colicky pain, reduced by defecation – Suffered by all
- Abdominal distension
- Weight loss
- Mouth ulcers

Whilst these patients were presenting with celiac type symptoms, tests were negative and so celiac disease was ruled out. As with the Ellis and Linaker study, the patients were given a GFD for 3 months. Of the 17 patients, 9 responded positively (neither of the male participants reacted positively) and symptoms ceased to continue but resumed upon consumption of gluten containing produce. Due to this the final conclusion was that the patients who reacted positively to the GFD were experiencing diarrhoea brought on by gluten sensitivity (Cooper et al., 1980).

NCGS is currently defined as a reaction to gluten whereby ‘allergic and autoimmune mechanisms have been ruled out’ (Sapone et al., 2012), with these criteria being used in the diagnostic process. In some articles NCGS is written as non-celiac wheat sensitivity (NCWS) due to the mystery surrounding its mechanisms, however some have issue with this nomenclature due to the exclusion of other cereals that contribute to the symptoms including rye and barley (Catassi et al., 2015). Since its description in 1978, research into NCGS has made few steps in terms of understanding its

mechanism, and diagnosis is only given on the basis of other disorders being ruled out. Some have suggested that NCGS may not even be a medical disorder, or that the issue itself may not be fully related to gluten but other proteins present in the offending foods (Skodje et al., 2018; Priyanka et al., 2018).

The difficulty of diagnosing NCGS presents itself due to the lack of a specific biomarker that would guarantee a diagnosis (Barbaro et al., 2018; Expósito-Miranda et al., 2022). Further complicating issues with some studies is the fact that many of those suffering from NCGS are self-diagnosed, and when participating in a trial, some claims are not upheld by the tests they undergo (Ruemmele, 2018). It has been documented that in some studies, the patients suffer a 'nocebo' effect, indicating that their symptoms appeared or even increased in response to a placebo (Molina-Infante and Carroccio, 2017), which further leads to questioning as to whether gluten is the culprit in these scenarios. Some have reasoned that gluten may not be a causative agent in all cases, and instead have theorised that diets low in fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAPs) may be the solution, although more in-depth research is needed in this area (Priyanka et al., 2018; Dieterich and Zopf, 2019, Dieterich et al., 2019).

A publication by the Salermo experts in 2015, attempted to outline a methodology for use in diagnosing NCGS. This involves a number of steps that are recommended for clinicians to follow in order to reach a diagnosis for NCGS once celiac disease and wheat allergy have been ruled out. The 2 steps are a clinical evaluation of the symptoms the patient is suffering, rated on a numeric scale from 1 (mild) to 10 (severe). The symptoms under analysis include, but are not limited to; dermatitis, fainting and foggy mind, with steps 1 and 2 looking into the same symptoms. For step 1, the patient is required to have carried on with a gluten containing diet for at least 6 weeks prior, this aids in gaining a baseline for the severity of the symptoms, which can then be compared whilst following a gluten-free diet. The purpose of step 2 is to further the findings of step 1 and allow for a diagnosis of NCGS to be made, dependent upon the results. Step 2 begins with the patient following a strict GFD for no shorter than 4 weeks in order to ascertain a baseline. The main premise of step 2 is a 'Double-Blind Placebo-Controlled Challenge' in research settings, as this is deemed effective in terms of gaining a diagnosis, or for clinical purposes a 'single blinded' trial will work. After the 4-week GFD, the trial is able to begin – with the advised amount of gluten

being 8g per day, however there is also a placebo which is identical in appearance and texture to the ‘gluten vehicle’. The trial begins with a weeklong challenge where their diet includes either the gluten vehicle, or placebo, followed by a strict 1-week GFD. The challenge is repeated for another 2 weeks with week 1 being the adverse to the first week-1 challenge. The questionnaire from step 1 is repeated and the recommendation is that is a 30% difference in answers between the placebo and gluten-based diet must be observed for a NCGS diagnosis to be made – this reduces the risk of a nocebo effect being diagnosed instead. If steps 1 and 2 prove to be inconclusive, then the clinician is advised to investigate other possibilities for illness (Catassi et al., 2015).

Although it was stated that until a reliable biomarker is found, diagnosis will continue to be a drawn-out process, however it was hoped that meeting these criteria will limit the number of patients that self-diagnose. Whilst gluten may not be the cause in NCGS, it would be reasonable to assume from studies that it would be contained in the foods causing an adverse reaction, and so is a beneficial marker to look for.

The current hope is that more understanding will be uncovered surrounding the mechanisms of NCGS, and a specific biomarker will one day be found, allowing for a concise test leading to a positive diagnosis. However, this will not eliminate the issue, and many will continue to be affected by gluten containing foods. Due to the rising cases, it has never been of more importance to carry on with the thorough testing of foods for those who cannot ingest gluten due to various ailments including celiac and NCGS.

As noted above, the ingestion of gluten can harbour serious effects for those with intolerances or allergies, therefore, the creation of a high-throughput printing system coupled with mAb probing may prove effective in screening products to ensure the absence of gluten and protect consumers. Whilst the ELISA methods are already established, this high-throughput methodology would allow for large scale food testing on an industrial level, especially whilst more consumers are adopting a GFD.

5.1.9 The Current Issue

As previously stated, ‘gluten-free’ products are increasing in popularity. This produce is highly popular with consumers who follow a medically advised GFD, as well as those who feel it benefits their diet. Whilst these products are legally protected, some may

see these produce as a potential target to adulterate and make a financial profit. A recently published article stated findings that of 794 ‘gluten-free’ products, 80 (10.3 %) were over the limit (20 mg/kg), and 24 (>3 %) of these products containing over 100 mg/kg, over five times the limit of gluten permitted within ‘gluten-free products’. This research occurred in India whereby gluten labelling is not legally required but shows how rigorous testing is needed to combat fraudulent claims (Mehtab et al., 2021). It is also important to monitor these products in the off chance of cross contamination.

5.1.10 Aims

This chapter will focus on the creation of a high-throughput method for screening samples containing gluten. Whilst work has already been conducted pertaining to grains in this thesis, it was considered that targeting polysaccharides would not be appropriate in this instance and instead chose to focus on proteins, more specifically, gliadin. The main aspects of the experiment were to:

- Choose a quick and effective method to crudely extract gliadin
- Perform dot-blots to make sure the method has worked
- Conduct microarrays as a form of printing the protein

With the popularity of gluten-free produce, the gluten-free market is thriving with more and more options becoming available. It is imperative to validate these products from both a food safety and food authentication aspect, as the gluten-free market may be susceptible to economically motivated adulteration which would put consumers at risk.

5.2 Methods

Two samples were involved within this experiment, wheat grain, and gluten powder (gluten from wheat, Merck). The wheat grain was ground using a pestle and mortar until it resembled a fine powder akin to that of the gluten powder.

The experiment was split into 3 phases:

- Phase 1 – Crude extraction of protein and subsequent dot blot
- Phase 2 – BCA assay to ascertain protein concentration
- Phase 3 – Microarray printing of protein

5.2.1 Protein extraction

Six different solutions were trialled for the crude extraction of gluten, it is already known that gluten is insoluble in water, yet extractable with ethanol, both of these were included to show this (Weiser, 2007). The selected extraction solvents being:

- Water
- 70% Ethanol, 30% water
- Methanol and Chloroform (1:1)
- Tris Buffer, trace NaCl (50 mM), pH 7.6
- Tris-HCl, pH 7.6

10 mL of the chosen extraction solution (listed above) was added to 1 g of sample with all samples being extracted in duplicate, if a protease inhibitor (Cytiva, protease inhibitor mix) was being used, it was added at this point (Table 5.1). The sample was left for 24 hours in a cold room on a rotary wheel (25 rpm). The samples were centrifuged (13,000 rpm, 10 minutes) and an acetone precipitation performed. The acetone was cooled (-20 °C), and 40 mL (4x the amount of extraction solution used) was added to the tube. The sample was vortexed and incubated (-20 °C, 60 minutes), before being centrifuged (13,000 rpm, 10 minutes). Without disturbing the pellet, the solution was decanted and disposed of, and the acetone was allowed to evaporate for roughly 30 minutes, with care taken not to let the sample fully dry.

After the extraction and evaporation phase, the samples were reconstituted. One sample from the pair was reconstituted in water (1 mL) and the other in system buffer (1 mL), (Chapter 2.2.2) and stored before use.

As this experiment was focussed on the eventual printing of the proteins, it was decided that a crude extraction protocol would be trialled first to garner suitability.

5.2.2 Dot blot

A grid of 0.5 x 0.5 cm squares was prepared on nitrocellulose (0.2 µm, Amersham™ Protran). The top row consisted of 1 µL of ink in each square as a reference point. Each column was dedicated to a different sample, with decreasing concentrations down the column, and the dilutions differed dependent upon the experiment (Table 5.1). 1 µL of each sample and dilution was pipetted into one of the squares. Where a control sample of gliadin protein (Recombinant Wheat Gliadin protein, abcam, >94%, 20 µg/mL) was included,1 µL was added at the base of the dot blot to show that the

probing was working. The samples were left to dry for 30 minutes before being probed (Chapter 2.2.3) and photographed to show results.

Table 5.1. Completed dot blot experiments for gluten analysis. An outline of all the dot blots performed for this experiment series, the samples used, and the relevant figure within the results section.

Dot Blot Experiment Number	Dot Blots performed	Solutions Used	Dilutions (same for all solutions)	Sample(s)	Dilution solution	Standard used (Yes/No)	Protease inhibitor (Yes/No)	Relevant Figure
1	1	Water 70% Ethanol Methanol:Chloroform Tris-HCL Tris	Pure sample 1:2 1:10	Wheat grain (odd numbered lanes) Gluten powder (even numbered lanes)	Water	No	No	5.1
2	2	A) Water 70% Ethanol Methanol:Chloroform Tris-HCL Tris	Pure sample 1:2 1:10	Wheat grain (odd numbered lanes) Gluten powder (even numbered lanes)	Water	No	No	5.2 A

		B) Water 70% Ethanol Methanol:Chloroform Tris-HCL Tris	Pure sample 1:2 1:10	Wheat grain (odd numbered lanes) Gluten powder (even numbered lanes)	System Buffer	No	No	5.2 B
3	2	A) Water 70% Ethanol Methanol:Chloroform Tris-HCL Tris	1:2 1:10 1:40 1:1000	Wheat grain	Water (Lanes 1-5) System buffer (Lanes 6-10)	Yes	No	5.3 A
		B) Water 70% Ethanol Methanol:Chloroform Tris-HCL Tris	1:2 1:10 1:40 1:1000	Gluten powder	Water (Lanes 1-5) System buffer (Lanes 6-10)	Yes	No	5.3 B
4	2	A) Water 70% Ethanol Methanol:Chloroform	1:2 1:10 1:40 1:1000	Wheat grain	Water (Lanes 1-5) System buffer (Lanes 6-10)	Yes	Yes	5.5 A

		Tris-HCL Tris						
		B) Water 70% Ethanol Methanol:Chloroform Tris-HCL Tris	1:2 1:10 1:40 1:1000	Gluten powder	Water (Lanes 1-5) System buffer (Lanes 6-10)	Yes	Yes	5.5 B

5.2.3 BCA assay

A BCA was performed on the samples after the protein extraction (5.2.1) to ascertain protein concentration (Bicinchonicic acid kit for protein determination, Sigma-Aldrich). BSA was used as the standard within this protocol. The samples (Figure 5.4) were prepared by mixing 0.1 mL with 2 mL of the protein determination solution (1 ml of Copper Sulfate Pentahyrate 4 % Solution to 49 mL of the Bicinchoninic Acid Solution). The samples were prepared in triplicate, and vortexed thoroughly before being incubated (37 °C, 30 minutes). The samples were transferred to a 384 well plate and read using a plate reader (Clariostar, 562 nm) to determine protein concentration. A standard curve was produced from the readings gathered using Microsoft excel software.

5.2.4 Microarray

After the dot blots were conducted, it was decided that a microarray would be trialled to look into whether the protein extracts could be printed. The BCA assay had already been performed and confirmed the presence of protein in the samples. The samples from both dot blots of the 4th experiment (Table 5.1), were printed onto the array (Chapter 2.2.3), along with the gliadin protein, and the microarray array probed post printing with an anti-gliadin antibody (Table 2.1, Chapter 2.2.3).

5.3 Results and Discussion

It is commonly known that, when consumed, gluten can be linked to multiple ailments for some. For this reason alone, it is important to monitor the authenticity of ‘gluten-free’ foods. This experiment focused on a high-throughput technique that could be used for mass testing.

The final aim for this experiment was to enable the printing of the extracted proteins. There are already documented uses of mAbs for the detection of gluten. Ellis et al. (1998) trialled an experiment to form a method for quantifying the amount of gluten within a product using mAbs when the EU declared that this would be a requirement.

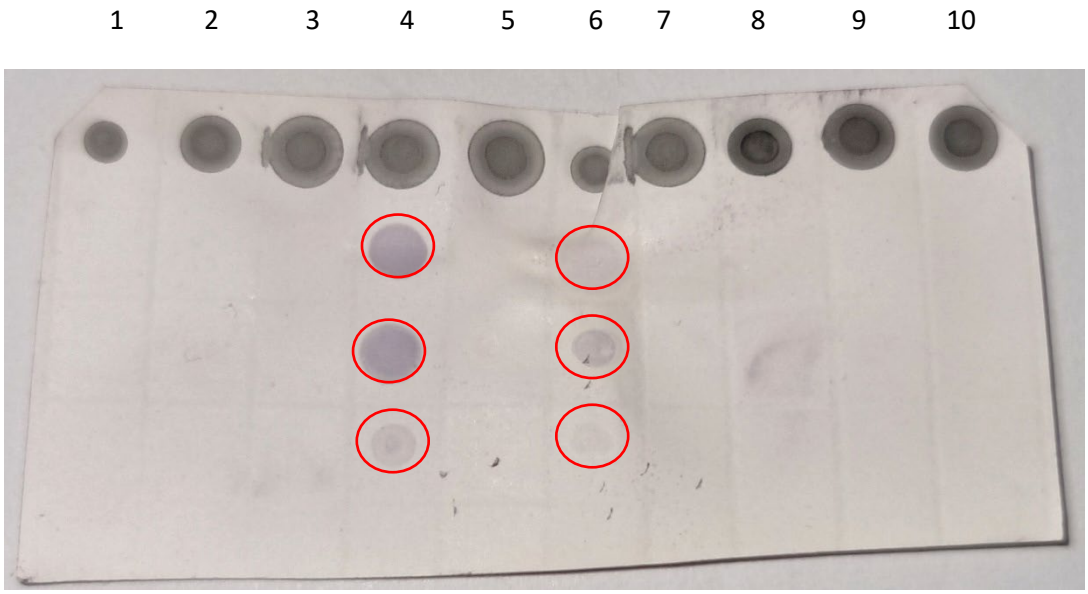
The investigation began with a trail looking into how best to crudely extract gluten from the product. Most of the common methodologies for gluten are time consuming, this experiment was mainly focused on the possibility of printing the proteins and so a crude extract was deemed suitable.

The first dot blot wielded limited results (Figure 5.1). As a control sample was not available at this time, it was not used.

3 different dilution sets were used for this experiment:

- Pure Sample
- 1:2
- 1:10

All extracts were reconstituted and diluted with water for this experiment. As can be seen (Figure 5.1), only 2 samples facilitated binding – both from the gluten powder extraction, the ethanol extraction and the methanol:chloroform extraction (lanes 4 and 6). This ties in with the knowledge that gliadin is extractable using 70% ethanol (Elzoghby et al., 2015), and protocols extracting wheat proteins using a methanol:chloroform solution (Thakare et al., 2008). It also makes sense that there is no binding when extracted with water, as gluten is a water-insoluble protein (Arnold et al., 1964). Binding occurred for all dilutions of the gluten powder extractions with ethanol, and all extractions for the gluten powder extractions with methanol:chloroform, although these gave much fainter spots. The highest intensity was from the gluten ethanol extraction for both pure sample and 1:2 dilution.

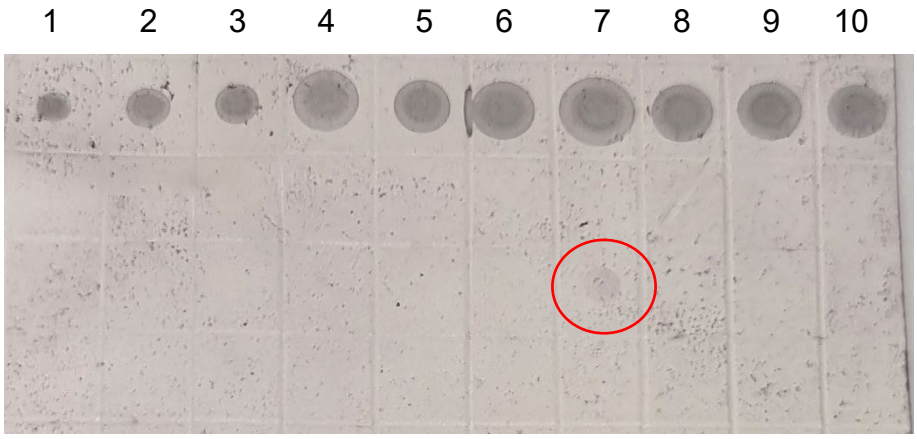


	Dilution	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10
Sample		Wheat	Gluten	Wheat	Gluten	Wheat	Gluten	Wheat	Gluten	Wheat	Gluten
Row 1		Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink
Row 2	Pure	Water	Water	70% Ethanol	70% Ethanol	Methanol:Chloroform	Methanol:Chloroform	Tris-HCL	Tris-HCL	Tris	Tris
Row 3	1:2	Water	Water	70% Ethanol	70% Ethanol	Methanol:Chloroform	Methanol:Chloroform	Tris-HCL	Tris-HCL	Tris	Tris
Row 4	1:10	Water	Water	70% Ethanol	70% Ethanol	Methanol:Chloroform	Methanol:Chloroform	Tris-HCL	Tris-HCL	Tris	Tris

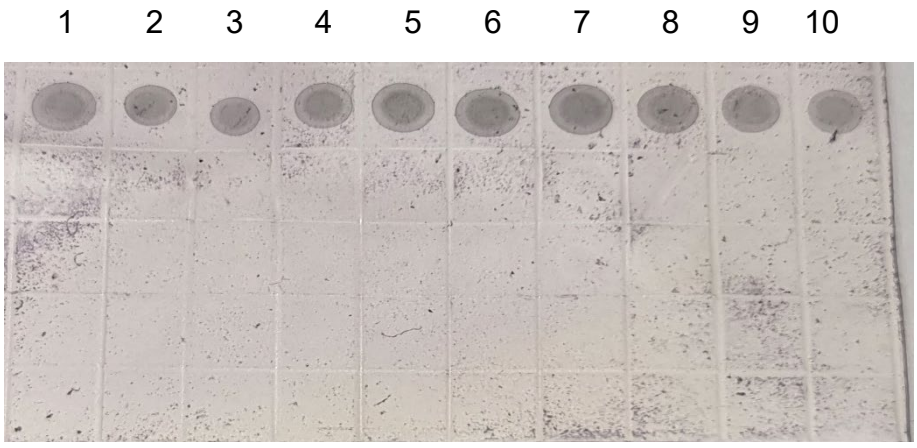
Figure 5.2. First dot blot experiment and table layout of samples. 5 extraction solutions were used; Water (lanes 1 and 2), 70% ethanol (lanes 3 and 4), methanol:chloroform (lanes 5 and 6), Tris-HCL (lanes 7 and 8), and Tris (lanes 9 and 10). The odd numbered lanes were extracts conducted on wheat grain, and the even numbered lanes were extractions from gluten-powder. The top row is ink spots for comparison, the subsequent rows are dilutions of; pure sample, 1:2, 1:10 – with the diluted samples being mixed with water. Any samples that produced a binding spot have been circled in the image, and the corresponding boxes have been highlighted within the table.

The second dot-blot experiment used factors that would potentially decide if printing would be viable. In order for the machine to print any samples, they must be at the correct viscosity and so are required to be mixed with a ‘system-buffer’ prior to printing. 2 dot blots were performed with the same parameters, and layout, as the first dot blot experiment (Figure 5.1). The difference with these dot blots is that when dilutions were performed, one set was done with water (Figure 5.2 A), and the other with system buffer (Figure 5.2 B) (Chapter 2.2.2). Again, no standards were used for this experiment (Figure 5.2).

A



B



A Water	Dilution	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10
Sample		Wheat	Gluten	Wheat	Gluten	Wheat	Gluten	Wheat	Gluten	Wheat	Gluten
Row 1		Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink
Row 2	Pure	Water	Water	70% Ethanol	70% Ethanol	Methanol:Chloroform	Methanol:Chloroform	Tris-HCL	Tris-HCL	Tris	Tris
Row 3	1:2	Water	Water	70% Ethanol	70% Ethanol	Methanol:Chloroform	Methanol:Chloroform	Tris-HCL	Tris-HCL	Tris	Tris
Row 4	1:10	Water	Water	70% Ethanol	70% Ethanol	Methanol:Chloroform	Methanol:Chloroform	Tris-HCL	Tris-HCL	Tris	Tris

B System buffer	Dilution	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10
Sample		Wheat	Gluten	Wheat	Gluten	Wheat	Gluten	Wheat	Gluten	Wheat	Gluten
Row 1		Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink
Row 2	Pure	Water	Water	70% Ethanol	70% Ethanol	Methanol:Chloroform	Methanol:Chloroform	Tris-HCL	Tris-HCL	Tris	Tris
Row 3	1:2	Water	Water	70% Ethanol	70% Ethanol	Methanol:Chloroform	Methanol:Chloroform	Tris-HCL	Tris-HCL	Tris	Tris
Row 4	1:10	Water	Water	70% Ethanol	70% Ethanol	Methanol:Chloroform	Methanol:Chloroform	Tris-HCL	Tris-HCL	Tris	Tris

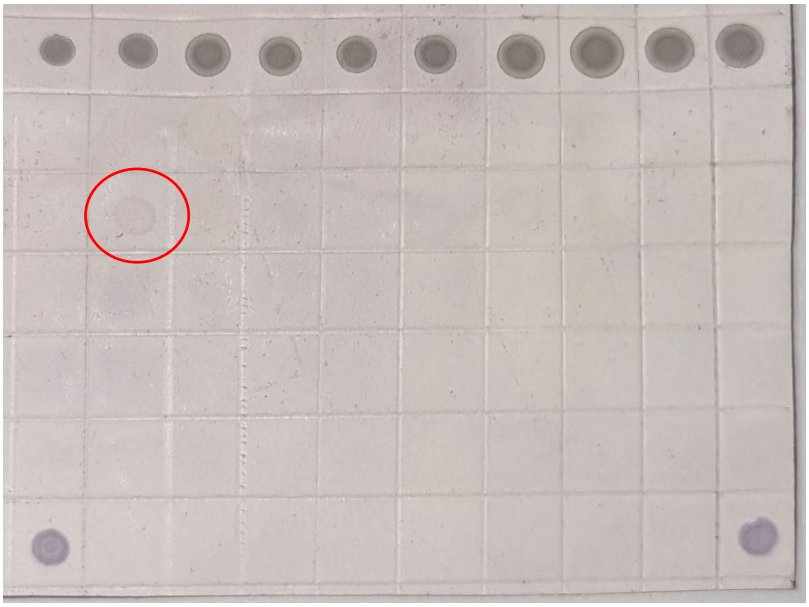
Figure 5.2. Dot blots with different dilution solutions and table layout of samples. Dot blot A samples were diluted with water, whilst dot blot B samples were diluted with system buffer. 5 extraction solutions were used; Water (lanes 1 and 2), 70% ethanol (lanes 3 and 4), Methanol:chloroform (lanes 5 and 6), Tris-HCL (lanes 7 and 8), and Tris (lanes 9 and 10). The odd numbered lanes were extracts conducted on wheat grain, and the even numbered lanes were conducted on gluten powder. The top row is ink spots for comparison, the subsequent rows are dilutions of; pure sample, 1:2, 1:10. Any sample that facilitated binding has been circled, and the corresponding boxes have been highlighted within the table.

The only sample from this experiment to produce an intensity spot was the wheat sample, extracted with Tris-HCL at a dilution of 1:2 and diluted with water. This was in contrast with the first experiment whereby multiple results were recorded for ethanol and methanol:chloroform.

The next set of dot blots focussed even further on the conditions of the microarray printing process. This time, the dilutions mimicked those of the microarray printing process; 1:2, 1:10, 1:40, 1:100. Wheat and grain extractions were conducted on separate dot blots (Figure 5.3). A control sample of gliadin was available for this experiment, located in the bottom corners, blotted on as a pure sample, with no dilution, or extraneous buffers.

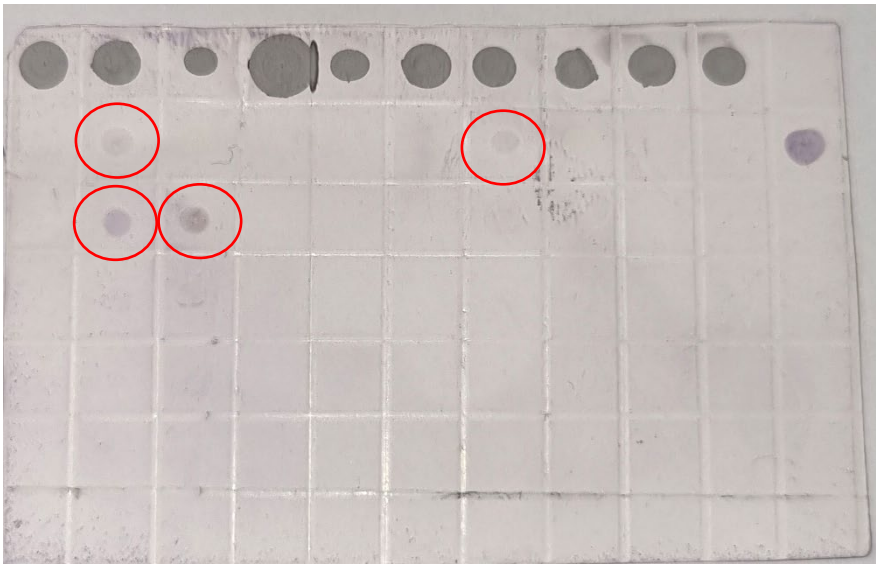
1 2 3 4 5 6 7 8 9 10

A



1 2 3 4 5 6 7 8 9 10

B



A Wheat	Dilution	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10
Buffer		Water	Water	Water	Water	Water	System Buffer	System Buffer	System Buffer	System Buffer	System Buffer
Row 1		Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink
Row 2	1:2	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris
Row 3	1:10	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris
Row 4	1:40	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris
Row 5	1:100	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris

B Gluten Powder	Dilution	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10
Buffer		Water	Water	Water	Water	Water	System Buffer	System Buffer	System Buffer	System Buffer	System Buffer
Row 1		Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink
Row 2	1:2	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris

Row 3	1:10	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris
Row 4	1:40	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris
Row 5	1:100	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris

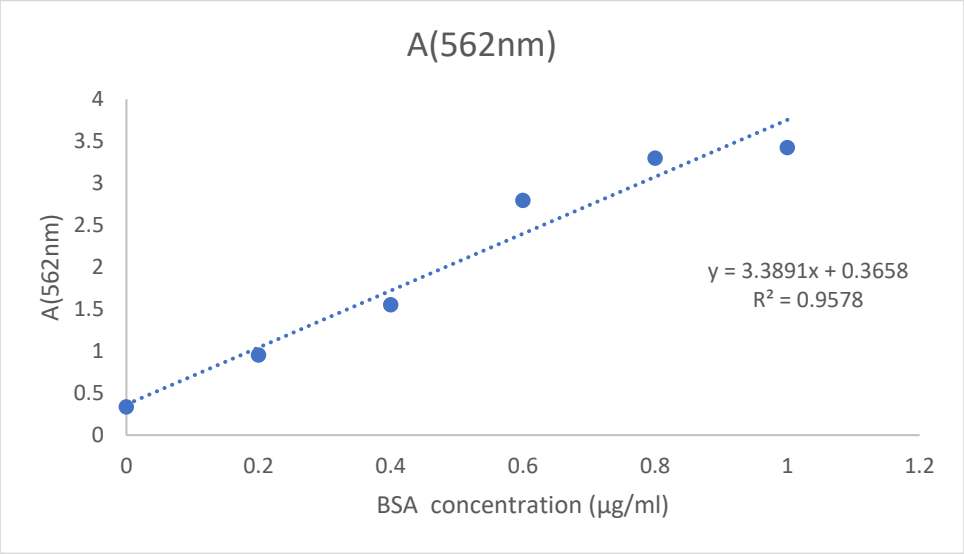
Figure 5.3 Dot blots conducted to microarray standards. Wheat samples (A) and gluten samples (B). Columns 1-5 are diluted with water where required, and 6-10 are diluted with system buffer. The solvent order is; water (lanes 1 and 6), ethanol 70% (lanes 2 and 7), methanol:chloroform (lanes 3 and 8), Tris-HCL (lanes 4 and 9) and Tris (lanes 5 and 10). The gliadin protein standard is located in the furthest column, on the right.

Some faint binding responses occurred with these dot blots, for blot A, very faint binding occurred with 70% ethanol, 1:10 water dilution (column 2). For blot B binding occurred for ethanol 70%, 1:2 and 1:10 dilution water, methanol:chloroform 1:10 dilution water and again with ethanol 1:2.

As this process seemed to be getting minimal binding, 2 experiments were conducted:

- BCA assay to ascertain protein concentration
- Introduction of a protease inhibitor

The BCA assay allowed for the concentration of protein within a sample. A standard curve was created, and the samples concentrations were figured out from there (Figure 5.4).



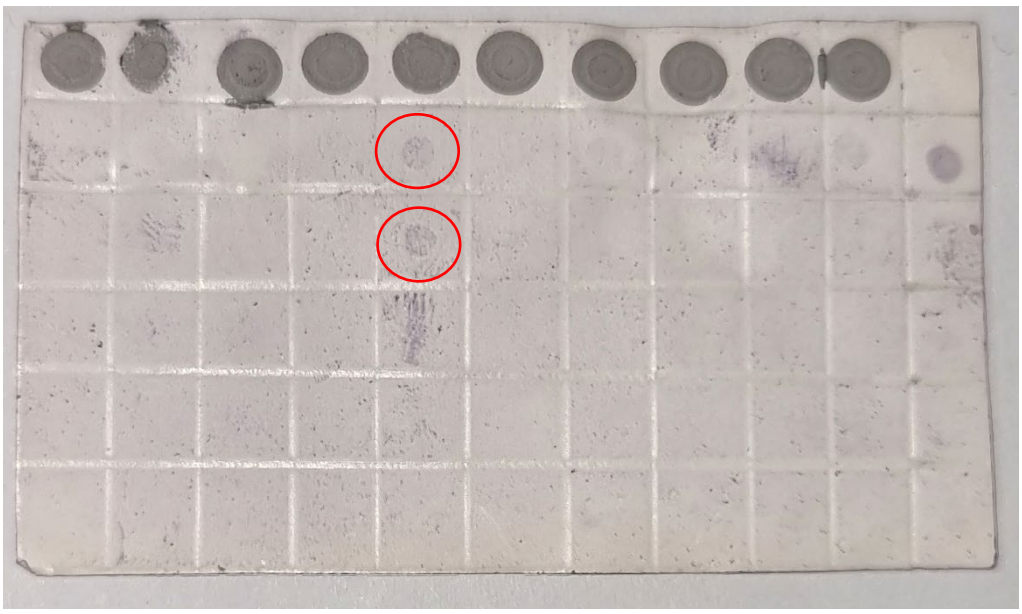
Extraction Solution	Sample Type	Concentration (µg/ml)	Inhibitor?
Water	Wheat Grain	0.9516	No protease inhibitor
Methanol	Wheat Grain	0.2665	No protease inhibitor
Tris	Wheat Grain	0.8894	No protease inhibitor
Ethanol	Wheat Grain	0.8242	No protease inhibitor
Tris-Hcl	Wheat Grain	0.9030	No protease inhibitor
Water	Wheat Grain	0.9351	Protease inhibitor
Methanol	Wheat Grain	0.5645	Protease inhibitor
Tris	Wheat Grain	0.9263	Protease inhibitor
Ethanol	Wheat Grain	0.9336	Protease inhibitor

Tris-Hcl	Wheat Grain	0.9888	Protease inhibitor
Water	Gluten	0.9788	No protease inhibitor
Methanol	Gluten	1.0065	No protease inhibitor
Tris	Gluten	1.0697	No protease inhibitor
Ethanol	Gluten	0.9974	No protease inhibitor
Tris-Hcl	Gluten	0.5114	No protease inhibitor
Water	Gluten	0.9915	Protease inhibitor
Methanol	Gluten	0.9696	Protease inhibitor
Tris	Gluten	1.0168	Protease inhibitor
Ethanol	Gluten	0.9513	Protease inhibitor
Tris-Hcl	Gluten	0.9443	Protease inhibitor

Figure 5.4. A BCA assay of all the samples. Each sample was plated in triplicate and the average taken. The concentrations were calculated from the graph above. It seemed that the protease inhibitor only had a minor effect on concentration.

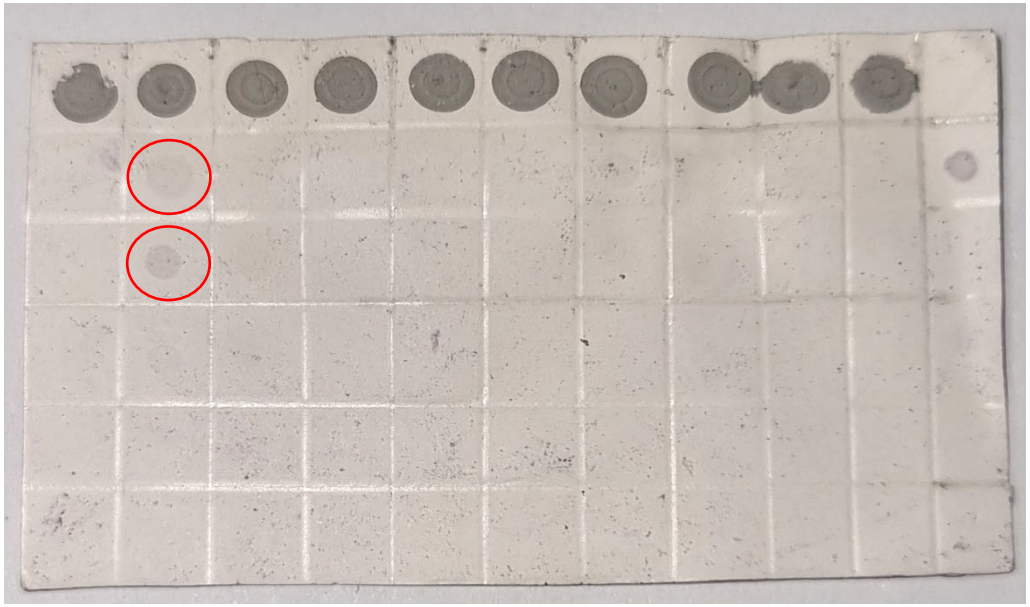
The next set of dot blots looked into how a protease inhibitor would affect the extractions (Figure 5.5), protein standards were used again to make sure there was no error with the probing process. The dot blots were conducted with the samples used for the BCA assay (Figure 5.4).

A



1 2 3 4 5 6 7 8 9 10

B



A Wheat	Dilution	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10
Buffer		Water	Water	Water	Water	Water	System Buffer	System Buffer	System Buffer	System Buffer	System Buffer
Row 1		Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink
Row 2	1:2	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris
Row 3	1:10	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris

Row 4	1:40	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris
Row 5	1:100	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris

B Gluten Powder	Dilution	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10
Buffer		Water	Water	Water	Water	Water	System Buffer	System Buffer	System Buffer	System Buffer	System Buffer
Row 1		Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink	Ink
Row 2	1:2	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris
Row 3	1:10	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris
Row 4	1:40	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris
Row 5	1:100	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris	Water	70% Ethanol	Methanol:Chloroform	Tris-HCL	Tris

Figure 5.5 Two dot blots with protease inhibitor used during extractions. The conditions remain the same as the previous experiment (Figure 5.3). Wheat samples (A), and gluten samples (B). Columns 1-5 are diluted with water where required, and 6-10 are diluted with system buffer. The solvent order is; water (lanes 1 and 6), ethanol 70% (2 and 7), methanol:chloroform (3 and 8), Tris-HCL (4 and 9) and Tris (5 and 10). The gliadin protein standard is located in the furthest column, on the right.

As can be seen from the dot blots (Figure 5.5), minimal binding occurred. For the wheat grain samples, blot (A), binding occurred in column 5, Tris extraction, 1:2 and 1:10 dilution, and column 10, tris extraction 1:2 dilution. For the gluten powder samples, blot (B), binding occurred in column 2, 70% ethanol at dilutions 1:2 and 1:10.

When comparing the BCA assay results with the dot blots (Figure 5.5 A and B), it can be seen that the protein concentration fluctuates dependent upon extraction buffer, however these concentrations don't mimic the results of the dot blots, this could be due to diluting the samples further with water or system buffer.

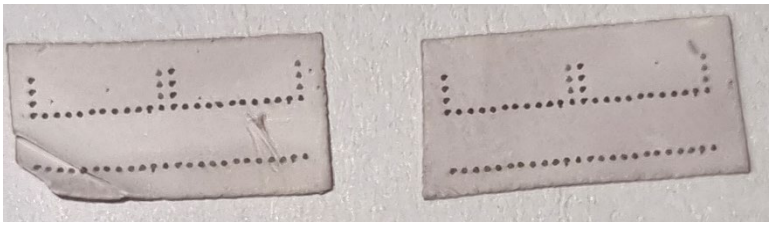


Figure 5.6 Probed microarrays of all the samples. Left is the array probed with a gliadin antibody; right is the control. No binding occurred.

Whilst it could be seen that the extractions were somewhat successful especially with the ethanol extractions. It was not certain that this would work for microarray printing. However, a trial was run, 2 arrays were produced and probed – one with the gliadin antibody and one a control (Figure 5.6). The microarrays were unsuccessful at this stage, however that does not mean that this could not happen in the future to be applied to high-throughput testing of food samples for the detection of gluten.

5.4 Conclusion

Whilst the methodology outlined within this chapter did not prove to be viable at this stage, there is huge potential for future work. It has already been established that antibodies are an effective tool – ELISA being a commonly used technique, and so this is a good starting point to develop upon. The use of high-throughput technology is vital for the mass screening of food produce and could wield promising results should research be further carried out, and could applied to a multitude of food products, not just gluten.

Chapter 6: General Discussion

Highlighting the prevalence of food fraud ensures that we are aware of the need for adaptation and evolution when it comes to authentication measures. The horsemeat scandal of 2013 brought food safety issues to public attention. At a time where many consumers are highly scrutinous over their diets and its contents, it is important that food safety is monitored, and consumers feel secure with the produce they are eating. Food fraud could occur with any product, in a multitude of ways from false documentation to adulteration as has previously been discussed. Consumer protection is somewhat reliant on a level of trust between consumer and producer, that the food products being delivered are what they say they are. As has been presented in earlier parts of this thesis, food fraud and subsequent authentication measures are in constant development, and it is still an issue that haunts the food supply chain.

The focus of this thesis was exploring the viability of using microarray technology and monoclonal antibody probing as an authentication technique for different food products. Whilst many techniques have already been established, such as NMR or PCR, the combination of microarrays and monoclonal antibodies allows for targeting different targets such as polysaccharides or proteins. Microarray technology also has the advantage of being high-throughput, with thousands of samples being able to be printed on small arrays and kept for prolonged periods of time. The ability to print such a large amount of samples would mean that food testing on a large scale would be more than feasible. With this reasoning, 3 foods were investigated using this technique:

- Whole grains – chapter 3
- Saffron – chapter 4
- Gluten – chapter 5

*6.*1 Whole grains

Chapter 3 focused on utilising polysaccharide profiles to determine the viability of applying microarray technology and monoclonal antibody probing to ascertain how whole grains could be profiled within whole grain products. Whilst the methods used were not quantitative, they highlighted the relative abundance of various polysaccharides within the grains. With whole grain content not currently measured and scrutinised in products, it was an area of food safety and authentication that could be explored.

Whole grain authentication within food products is still an expanding area. With lack of consistency and clarity surrounding recommended inclusion of whole grains in the diet, it is understandable that concern around levels of whole grain within a whole grain product is limited. The polysaccharide profiles showed that there were differences between grains, and grain fractions, although clustering analysis did reveal close relations, possibly due to the historical breeding and closeness of the selected wheat samples.

This method did not provide any qualitative results, which could be applied for further research and develop a methodology for quantifying whole grains within whole grain products, with further research into quantifying fraction amounts in these products.

6.2 Saffron

Chapter 4 moved onto monitoring authenticity within saffron. Being a highly regarded product, saffron is a prime target for economically motivated adulteration. Whilst the primary investigation focused on using polysaccharide profiling to establish ways of measuring authenticity, other techniques such as fluorescent microscopy and dye analysis were utilised to give a broader view as to the possible authenticity of the samples.

Many experiments focusing on saffron authenticity have been performed, looking at aspects from origin, to whether samples have been dyed to appear authentic. The first set of experiments in chapter 4 investigated the polysaccharide profile of various saffron samples. By analysing multiple different spices at first, it as shown that polysaccharide profiles of spices were varied enough to allow differentiation between profiles. Moving on from this, oregano, paprika, and saffron were given a further in-depth look before choosing to further explore saffron. In total 15 samples were collected; 6 supermarket samples, 4 reference samples, and 5 sourced from a Turkish market. Analysis of these samples showed that the profiles of the supermarket samples and reference samples harboured similar profiles, whereas the Turkish market samples could warrant further testing in order to ascertain authenticity.

These experiments combined gave a look into the suspected authenticity of various saffron samples. Whilst one methodology may be deemed enough, this experiment gave a broader view into how saffron can be authenticated. If this experiment were to be taken further, it could look into quantifying some of the polysaccharides present within saffron to use as markers of authenticity.

6.3 Gluten

Chapter 5 took a different direction by focusing on proteins. Still using microarray technology coupled with monoclonal antibody probing, this experiment looked into the functionality of using these techniques to detect gliadin, a protein within gluten. Understanding that gluten-free diets are imperative for some consumers, gluten was a suitable target to aim for.

Crude extractions of the gliadin protein were of relative success, even more so when these samples were diluted with the buffer that the machines require to print. By mimicking the printing parameters in terms of buffer and printing dilutions, gliadin was able to be blotted and probed with the appropriate antibody. Difficulty arose when transposing this method to the printers themselves. Whilst the samples could be blotted physically, printing via the robots did not present with any usable data at this time.

Whilst this experiment did not wield the desired result, further research could focus on improving the extraction and printing procedure. Once this has been achieved it would be reasonable to extend this methodology to other allergens such as peanut, as well as aiming to identify allergens within food products such as chocolate. The advantage of making this work on such a large scale is that one array could harbour thousands of samples from different food products, with only one antibody needed for each array.

As mentioned at the beginning of this chapter, authentication techniques are in constant need of evolution. One approach will not solve all food fraud issues, and so it is of high importance to explore every conceivable avenue available. The use of microarray technology throughout these experiments has shown that its capabilities are not limited to just one food product but has the potential to be applied to a plethora of products. The main advantage of utilising this process would be the ability to monitor thousands of samples via a high-throughput process.

Food fraud is not an issue that will disappear, and so the best defence is adaptation and innovation.

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Appendices

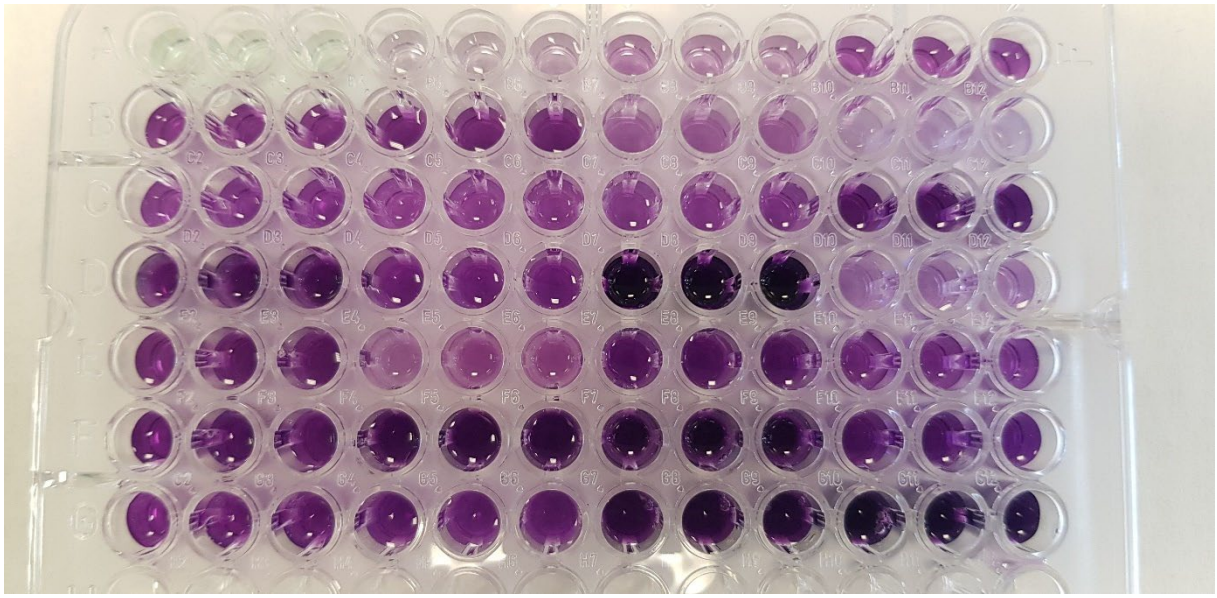


Figure 1. The 96 well plate used for the BCA assay. A deep purple is indicative of a higher protein concentration. Some of the samples congealed whilst in the well and this may have contributed to a lower reading value.

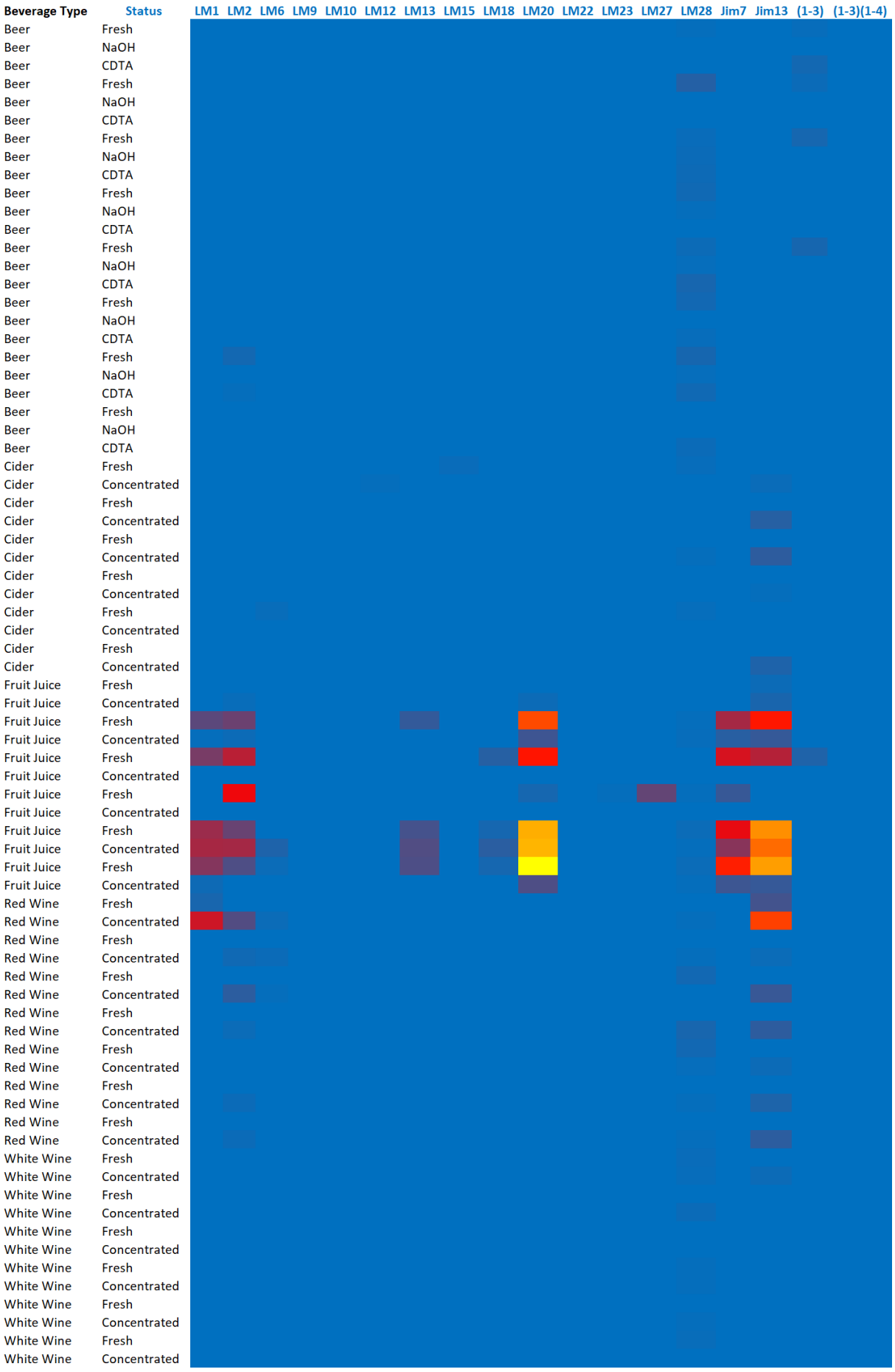
Table 1. Settings used for the BCA assay.

Basic settings	
Measurement type:	Absorbance
Microplate name:	GREINER 384

Endpoint settings	
No. of flashes per well:	15
Scan mode:	spiral scan
Scan diameter [mm]:	3

Optic settings	
Excitation:	562

General settings	
Top optic used	
Aperture spoon:	-
Injection needle holder type:	-
Reading direction:	bidirectional, horizontal left to right, top to bottom
Target temperature [°C]:	set off
Target concentration O2 [%]:	set off
Target concentration CO2 [%]:	set off
Absorbance path length correction volume [µl]:	100
Absorbance path length correction factor:	1.3699


Beverage Type
Status
LM1 LM2 LM6 LM9 LM10 LM12 LM13 LM15 LM18 LM20 LM22 LM23 LM27 LM28 Jim7 Jim13 (1-3) (1-3)(1-4)
Beer Fresh
Beer NaOH
Beer CDTA
Beer Fresh
Beer NaOH
Beer CDTA
Beer Fresh
Beer NaOH
Beer CDTA
Beer Fresh
Beer NaOH
Beer CDTA
Beer Fresh
Beer NaOH
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Beer NaOH
Beer CDTA
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Fruit Juice Fresh
Fruit Juice Concentrated
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Fruit Juice Fresh
Fruit Juice Concentrated
Red Wine Fresh
Red Wine Concentrated
Red Wine Fresh
Red Wine Concentrated
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Figure 2. A work-up experiment looking into beverages. Beverages are also at risk of food fraud, especially beers and wines. As can be seen a limited profile was achieved and it was decided to not be an appropriate venture at this time.