

**HYDRODYNAMIC CHARACTERISATION OF
THERAPEUTIC GLYCAN AND GLYCAN-LIKE
COMPLEXES**

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Abstract

Natural resources and plant extracts with therapeutic properties are considered important sources not only for food products, but also for the treatment of disease. Among edible herbal medicines are the gourd family or *Cucurbitaceae*, beta glucans, *Nigella sativa* and lignin. It is worth noting that after many research studies using a variety of screening, characterization and isolation methods that these glycan and glycan-like complexes have therapeutic components which have the ability to reduce the risk of many metabolic diseases. The properties of glycan and glycan-like complexes and the use as anti-diabetics were indicated. Although there have been great efforts made toward revealing the bioactive substances present in these materials, and several research studies have been undertaken, the bioactive components responsible are still not fully explored. The aim of this study is to explore further anti-diabetic properties and the hydrodynamic characterisation of glycan and glycan-like complexes obtained from Analytical Ultracentrifugation (AUC), viscometry, Dynamic Light Scattering (DLS) and Fourier Transform Infrared Spectroscopy (FTIR). Eliminating and analysis of fatty acids was performed in order to separate and purify protein-polysaccharide complexes from the family *Cucurbitaceae* and the complexes were also the target of investigation in this study. The value of sedimentation coefficient and a corresponding molecular weight yields a value for the frictional ratio ~ 13 , suggesting a very high degree of hydration and asymmetry for the complexes in the family of *Cucurbitaceae*. The results of gas chromatography (GC) analysis indicate that polyunsaturated fatty acids (PUFAs) formed approximately 60% of the total oil from family of *Cucurbitaceae* which it is of high value in the human diet. The protein-like sedimentation profile of lipase adopts a polysaccharide like broad profile and sedimenting at a significantly faster rate suggesting all the lipase has bound to the beta-glucan. A plate shape structure for all three lignins with aspect ratio $\sim 30:1$ seems to be probable.

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Abbreviations

| | |
|----------------------------------|--|
| ADA | American Diabetes Association |
| AUC | Analytical Ultracentrifugation |
| $c(s)$ | Distribution function (corrected for diffusion) of sedimentation coefficients |
| $D, D^{0}_{20,w}$ | Translational diffusion coefficient, Translational diffusion corrected for non-ideality, temperature and buffer conditions ($\text{cm}^2 \text{s}^{-1}$) |
| Da | Daltons |
| DLS | Dynamic Light Scattering |
| DM | Diabetes Mellitus |
| dn/dc | Refractive index increment (ml/g) |
| HDL | High-density lipoproteins |
| FTIR | Fourier Transform Infrared Spectroscopy |
| η | Dynamic viscosity (mPa s) |
| η_r, η_{sp} | Relative, specific viscosity |
| $\eta_{red}, \eta_{inh}, [\eta]$ | Reduced, inherent, intrinsic viscosity (ml/g) |
| k_B | Boltzmann constant (1.381×10^{-16} erg/K) |
| k_H, k_K | Huggins, Kraemer constant |
| k_s | Gralén coefficient/concentration dependence of sedimentation coefficient (ml/g) |

| | |
|-----------------------------------|---|
| ls-g*(s) | least square Gaussian apparent fit of sedimentation coefficients |
| LDL | low-density lipoprotein |
| M | Molarity, Molar concentration (mol/l) |
| MHKS | Mark Houwink Kuhn Sakurada coefficients a, b, -ε (from power law scaling relationships between M, with [η], s or D respectively) |
| M _{n,w,z} | Number average, weight average, Z average molar mass (Daltons) |
| N _A | Avagadro's constant (6.022x10 ²³ mol ⁻¹) |
| <i>N. sativa</i> | <i>Nigella sativa</i> |
| PBS | Phosphate buffered saline |
| r | Radius (cm) |
| ρ | Density (g/ml) |
| R | Gas constant (8.314x10 ⁷ erg/(K mol)) |
| R _g | Radius of gyration (nm, cm) |
| r _H | Radius of hydration (nm) |
| s, s ⁰ _{20,w} | Sedimentation coefficient, corrected for non-ideality, temperature and buffer conditions (1S = 1 Svedberg = 1 x10 ⁻¹³ sec) |
| TC | Total cholesterol |

| | |
|-----------|--------------------------------|
| TQ | Thymoquinone |
| \bar{v} | Partial specific volume (ml/g) |
| VLDL | Very-low-density lipoprotein |
| ω | Angular velocity (rad/s) |

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Chapter 1: Introduction

1.1 Scope of thesis: hydrodynamic characterisation of glycans and related glycan molecules against serious disease

This thesis aims to show how hydrodynamic and associated methods are making inroads into our understanding of the use - or potential use - of glycan (polysaccharide) and glycan related materials (lignins) being used in the fight against *Diabetes Mellitus* and other serious diseases. The main focus is on the use of polysaccharide and polysaccharide complexes from cucurbits and its effect on *Diabetes Mellitus*. We will also consider the properties of beta glucans from oat being considered for use as cholesterol lowering agents, and their behaviour in the presence of lipases. Finally, we consider the properties of a group of molecules, which are often associated with polysaccharides, namely lignins and three in particular: Alcell, Kraft and Soda.

1.2 Introduction to *Diabetes Mellitus* (DM)

Diabetes Mellitus (DM) is currently one of the most globally prevalent epidemics, and is increasing rapidly worldwide, with the prediction that approximately 400 million people will present with this disease by 2025 (Park *et al.*, 2012; Makni *et al.*, 2011; Marić, 2010). *Diabetes Mellitus* (DM) is not only a chronic disease, but the spread of the disease will impact financially on all countries, especially with the microvascular and macrovascular consequences of the disease (Adams *et al.*, 2011; Kavishankar *et al.*, 2011) such as eyes, kidney and heart (Yoshinari *et al.*, 2009; Sedigheh *et al.*, 2011; Teugwa *et al.*, 2013).

This type of disease has long been known to man, and the word Diabetes comes from the Greek word which means "to pass through" as it was first described by Aretaeus of Capadocia more than nineteen centuries ago (Alexiou and Demopoulos, 2010; Patel *et al.*, 2012). The word Mellitus, which means "honey" in Latin-Greek language, was first added by John Rollo in order to differentiate *Diabetes Mellitus* from polyurinic diseases with the absence of sugar in the urea (Alexiou and Demopoulos, 2010). However, Grover *et al.* (2002) reported that DM was identified and distinguished in two types in India in approximately 500 BC. As can be clearly seen from the name of this disease, *Diabetes Mellitus* (DM) is linked with the occurrence of sugar in the body, and in fact this disorder is seen through abnormal sugar levels in the blood stream.

Although it may not at first appear to be a crucial task to master this disease, the main concept of the disease remains ambiguous, and while extensive investigation has been made over decades to reveal the causes and the effects of DM, this is still subject to considerable challenge (Makni *et al.*, 2011; Li *et al.*, 2004; Adams *et al.*, 2011; American Diabetes Association ADA, 2012). It is worth noting before discussion of the various types of DM that one of the disease's major causes is an effective reduction in insulin, which is a protein hormone that is secreted by the pancreas and in particular by beta cells of the islets of Langerhans (Adams *et al.*, 2011; Schulze and Hu, 2005; Maiti *et al.*, 2004; Raj *et al.*, 2010). In terms of its complex structure, insulin in general is a protein composed of 51 amino acids, arranged in two polypeptide hormone chains, which are linked by disulphide bonds (chain A with 21 amino acids and chain B with 30 amino acids) (Hadaruga *et al.*, 2009; Joshi *et al.*, 2007; Varshosaz, 2007; Ruiz *et al.*, 2012). Interestingly, insulin can be found as a monomer or dimer, or also as a hexamer (Varshosaz, 2007). In a healthy body where Diabetes is not present, the level of blood glucose increases after consuming a meal, which stimulates insulin (in monomer form), and this insulin plays a key role in reducing the blood glucose level by binding to insulin

receptors on the cell, allowing the cell to take in the glucose from the blood stream. The consequence of this action is that it reduces the glucose to a normal level, which is followed by a decreasing in insulin to the normal level, normal for person without diabetes: 70–99 mg/dl (Adams *et al.*, 2011; Yadav *et al.*, 2009; Jarald *et al.*, 2008).

DM is a heterogeneous clinical and chronic endocrine metabolic disorder defined by the inability of the body to respond to the elevated level of glucose in the bloodstream (hyperglycaemia) and the abnormality of carbohydrate metabolism by the cause of either the absence or the non-activity of insulin (Adams *et al.*, 2011; American Diabetes Association ADA, 2012; Makni *et al.*, 2011; Saleem, 2010; Schulze and Hu, 2005; Teugwa *et al.*, 2013; Sedigheh *et al.*, 2011; Andrade-Cetto and Heinrich, 2005; Park *et al.*, 2012; Kavishankar *et al.*, 2011; Varshosaz, 2007; Card and Magnuso, 2011; Raj *et al.*, 2010; Patel *et al.*, 2012; Gu *et al.*, 2013; Yadav *et al.*, 2009; World Health Organization, 1999). However, non-insulin secretion and lack of insulin are not the only causes of DM, as there are many causes, which can include patho-physiological and pathological changes (Saleem, 2010). For instance, at the cellular level there are many significant proteins that support insulin to permeate into the cell, such as: Calpain-10 (CAPN-10) proteins, which help to disintegrate fat; Ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP-1), which carries insulin into the cell; and Insulin receptor substrate (IRS protein), which receives and phosphorylates insulin and is used to activate Glucose transporter type 4 (GLUT-4,) which allows glucose to enter the cell (Skrobuk, 2012). Hence, if there is any failure in the performance of these elements, the outcome is DM.

With regard to categorisation of Diabetes, there are four classes of DM as denominated by The American Diabetic Association (ADA), which are; two forms of Idiopathic Diabetes, also known as type 1 and type 2, and two other types, known as gestational diabetes and diabetes associated with other specific

conditions: the latter two types are also known as secondary Diabetes (ADA, 2012; Adams *et al.*, 2011; Teugwa *et al.*, 2013; Alexiou and Demopoulos, 2010; WHO, 1999; Yadav *et al.*, 2012; Adams *et al.*, 2013).

Type 1 DM, which is known as insulin-dependent diabetes mellitus (IDDM), juvenile-onset and diabetes β -cell destruction, is of unrecognised aetiology resulting from a disorder of insulin secretion or the absence or complete deficiency of insulin, which is usually seen as an auto-immune disease, as the beta-cell is attacked by the immune system (ADA, 2012; Coppieters *et al.*, 2012; Teugwa *et al.*, 2013). At the time of writing this thesis, the logic of this attack is not fully understood. However, Schulze and Hu (2005) claim that there are minor adjustable environmental risk factors that produce type 1 DM. This type of DM usually occurs in childhood, and as the name insulin-dependent diabetes mellitus (IDDM) indicates, a patient depends on external insulin: otherwise, his or her life is at risk (Yadav *et al.*, 2009; Varshosaz, 2007; Gu *et al.*, 2013).

The second type of DM is type 2 DM is known as non-insulin-independent diabetes mellitus (NIDDM) or adult-onset diabetes, and also can be described as relative insulin deficiency (Teugwa *et al.*, 2013; Alexiou and Demopoulos, 2010). Although insulin in this case is produced at a normal rate or even a higher rate than normal, the blood glucose level is found to be remarkably high and the pancreas loses its ability to produce insulin (Adams *et al.*, 2011). Type 2 DM is the most frequently-occurring or major form of DM, with approximately 90% of all patients who are suffering from DM. This type of Diabetes is associated with young adulthood (Yadav *et al.*, 2009; Alexiou and Demopoulos, 2010). As mentioned above, the causes of DM are unclear, but there are many potential contributors to the development of the disease. For example, type 2 is found to be linked with obesity, as this disease leads to insulin resistance, which is one of the symptoms of type 2 DM (ADA, 2012). Therefore, any impairment in the metabolism of fat might lead to DM (Teugwa *et al.*, 2013). The remaining types of DM, which are gestational diabetes

and diabetes associated with other specific conditions, do not account for a large proportion of diabetic patients, according to ADA (2012) and WHO (1999). Further, both the WHO (1999) and ADA (2012) reported that the diabetes associated with other specific conditions varies from one patient to another in terms of causes, which include genetic disorder of the b-cell or insulin function, drug induced disease, and infectious diseases which attack the exocrine pancreas.

In concluding this overview of *Diabetes Mellitus*, it is worth pointing out that regardless of the type of DM, controlling the blood glucose level is a compulsory target for both patients and scientists, in order to maintain health (Jarald *et al.*, 2008).

1.3 The use of *Cucurbita* seeds in treatment of diabetes

As previously stated, *Diabetes Mellitus* is the most common disorder of the endocrine system in the world with 170 million people worldwide in the year 2004 (Kaushik *et al.*, 2010) with a predicted 350 million by 2030; according to current global epidemic statistics, Mexico is predicted to have the highest number of persons with diabetes by the year 2030 (Mootosamy and Fawz- Mahomoodally, 2014). According to a Mexican health service report, diabetes was among the first causes of morbidity and mortality among its population. Diabetes is the common cause of chronic morbidities and disabilities among the productive population because of the associated retinopathy, heart diseases, renal diseases, and neuropathy (Preedy *et al.*, 2011).

With *Diabetes Mellitus* being a metabolic disorder and associated with many etiologies, it is marked by chronic hyperglycemia levels and accompanying disturbance of fat, carbohydrate, and protein metabolism. All these aberrations result from: an abnormality in insulin secretion, the functions of insulin or both

(Bajpai *et al.*, 2012). The main causes of type 2 *Diabetes Mellitus* are either predominant secretory defects of insulin or insulin resistance (Yeager, 2008).

In most cases, the patients are diagnosed in primary health care facilities and the medical practitioners at the centers normally prescribe appropriate antidiabetic medications such as Biguanides, Sulfonylureas and Meglitinides. The diagnosed patient can and does often seek the services of traditional healers, or herbalist (Yeager, 2008). This is, therefore, a disease in which many traditional treatments have been developed over the past decades by herbal healers. Some plant compounds in the USA have been used along with other herbal remedies and other medications. In some cases, the diabetes patients used these compounds instead of the conventional diabetes medications (Yeager, 2008). Even though, hypoglycemic and insulin drugs are the constituents of the treatment regimen for *Diabetes Mellitus*, use of appropriate nutritional food and other plant products is on the increase in some regions of the world (Wang *et al.*, 2011).

1.3.1 Background

A pumpkin that belongs to the *Cucurbitaceae* family is a perennial plant that is traditionally consumed in a variety of foods that include cooked or fresh vegetables. In addition, it can be stored in the frozen or canned form (Mootoosamy and Fawzi-Mahomoodally, 2014). The seeds of pumpkins are in great demand throughout the world because of their pharmacological inputs on the health of a person, for instance the diabetes patient (Borokini *et al.*, 2013). The oil extracted from the seeds in addition, confers many benefits to the health of an individual (Lim, 2012). The seed is undoubtedly beneficial to the health of an individual, but further tapping of its potential benefits is yet to be explored. A human depends on plants and its products in the acquisition of essential nutrients and compounds that foster healthy existence. It is prominently important to his wellbeing from the

time human races began. The commercialization and the use of the pumpkin seed products have not only influenced the economic life of an individual but also the social aspects of life as well. Similar to the other plants, pumpkin seed have importance to the health of an individual.

1.3.2 The biological description of pumpkin

The *Cucurbita mixta* belong to the family of *Cucurbitaceae* and is a gourd squash. The family is comprised of nearly eighty genera and above 800 species. It has a climbing stem of nearly 12 meters in length. They are further classified under some of the following, *Cucurbita moschata*, *Cucurbita mixta*, *Cucurbita pepo*, and *Cucurbita maxima* (Vibhute *et al.*, 2013). The classification system is based on both the texture and the shape of their stems. They typically have green leaves, thick yellow or orange shell and creasy nature from the bottom to the top of the stem. The pumpkins are either runners on the surface of the soil or the hardy creepers. Given the presence of a long climbing stem, it can climb to wherever they can find support (Wang *et al.*, 2011).

There are three types of species of pumpkins, which are readily available in the market. These species include *C. moshata*, *C. pepo*, and *C. maxima*. The three species are cultivated throughout the worldwide by numerous communities. The seed of the pumpkin is versatile in its use and usually dried and stored for future use (Borokini *et al.*, 2013). Most parts of the pumpkin are fleshy and edible. The edible parts include the yellow or orange shell, leaves, seeds, and sometimes the flowers. The ripe fruit of the pumpkin can be boiled, steamed, baked or roasted (Hechtman 2012).

The pumpkin can be consumed in different ways that include stored canned or frozen forms, as well as cooked or fresh vegetables. It can also be processed to

produce flour that has a longer shelf life as compared to other forms (Saxena, 2010). The pumpkin flour can also be used given its extremely desirable flavor, deep yellow, orange appearance, and its sweetness. According to some study reports, the flour of pumpkin has been used in the supplementation of cereal flour by bakery companies. It has also been used to supplement sauces, spices, instant noodles and soups. It has been a coloring agent in flour mixes, pasta and deserts, as well (Saxena, 2010).

The pumpkin seeds are encased in a husk that is yellow-white. They are also flat in nature and oval-like in their appearance. The family has chemicals that include saponins, fibers, tetracyclic triterpenes, minerals and polysaccharides. The examples of minerals present include zinc, copper, iron and manganese (Vibhute *et al.*, 2013). Pectin (which is a major constituent of the cell wall of plants) is found in pumpkins in abundance. The seeds have vital health benefits and are a rich source of fatty acids, phenol compounds and antioxidant vitamins. The examples of present, fatty acids include oleic acids, linoleic acids, stearic acid and palmitic acid. The antioxidant vitamins present includes tocopherols and carotenoids (Coulson *et al.*, 2013). To this end, some pharmacological feature of the pumpkin seeds has been postulated for the different species, including the antioxidant effects, the lipid-lowering ability, and the hepato-protective nature. In addition, it has anti-microbial and anti-carcinogenic properties (Harman, 2007). More importantly, it has the antidiabetic properties for the human body.

The seeds of the pumpkin are consumed as a snack food in many cultures globally. According to the report Teugwa *et al.*, (2013) the nutritional value of pumpkin seeds is increased through roasting. Roasting of the seeds increases the vitamin E and sterol contents. They are also rich in proteins. A study carried out on the oil content reported that the oil content is high and ranges from 40-60% of the whole seed content. Out of this content level, nearly 61% is contributed by fatty acids. The levels of specific fatty acids are as follows: oleic acid is close to 47%, and

linoleic acid content is nearly 40.5%, with the rest approximately 17% (Wang, *et al.*, 2011). The ratio of mono-saturated to poly-saturated is 0.6 to 0.75 grams. The pectin content is about 30 percent. The virgin oil can be obtained through roasting the seeds of pumpkins at about 100 degrees Celsius before processing the seed. The cold oil can be obtained by pressing the non-roasted seeds by use of the screw press and the temperature should not exceed 50 degrees centigrade (Wang, *et al.*, 2011).

1.3.3 The composition of *Cucurbita* seeds

The seed of the pumpkin is nutritionally beneficial given its constituents that are essential to the health integrity of an individual. They are fried, salted or cooked for consumption. In Korea, the fleshy part of the pumpkin is eaten in the form of soups, and juices. It is also incorporated into various types of food (El-Demery, 2011). It has been used as medicine in various countries of the world that include Argentina, Brazil, India, Mexico and Korea. The reason it has been used as medicine is the health value of its content of antioxidant vitamins, minerals, and fat lowering ability (El-Demery, 2011).

The seeds and the oil extracts are good repositories of alternative traditional medical properties. The oil is thick, brown to dark brown in color and extremely rich in linoleic acid. The abundant oleic and linoleic acids reduce the plasma cholesterol levels significantly. It lowers the low-density lipids and raises the levels of high-density lipids (Kwon *et al.*, 2007). Therefore, it serves nowadays in the fight against cardiovascular disease. The pumpkin seed oil is sold in most reputable US health stores. It is formulated into capsules that contain 1 gram of oil. In addition, the oil obtained from the pumpkin seeds have been hypothesized to have some pharmacological activities, which include antifungal, antidiabetic, and anti-inflammatory properties (Rahim *et al.*, 2013).

The pumpkin oil has been used as an antidiabetic drug in most regions of the world. The oil extracted from pumpkin has hypoglycemic effects in alloxan-induced diabetic rats and the normal animals. Pectin is the constituent of the seed that is responsible for the hypoglycemic effects of the oil (Lim, 2012). In addition, the pumpkin seed oil contains elevated levels of other polysaccharides, which may be another component responsible for its hypoglycemic actions. According to Li *et al.*, (2001) the oil obtained from un-germinated pumpkin seeds has hypoglycemic ability. Research reports have also deduced that the protein contained in the seeds with a molecular weight of less than 3-kilo and over 60-kilo Daltons increases the plasma insulin levels. Furthermore, the oil from the germinated seeds improves the blood glucose tolerance (Kwon *et al.*, 2007). However, the protein components obtained from ungerminated pumpkin seeds do not possess any hypoglycemic abilities (Caili *et al.*, 2007). Many studies postulate that the compounds found in pumpkins are useful in the management of blood levels of insulin and the risk for diabetes (Nawfal, 2011).

1.3.4 Toxicity of the pumpkin seed and oil

The seeds of the pumpkin are considered when taken at levels that produce the medicinal effects and in addition, when taken in food amounts. However, there is potential for an individual allergic reaction to the pumpkin and pumpkin products (Díaz-Flores *et al.*, 2012). The medicinal amount of pumpkin is contraindicated during pregnancy or the lactation period. Some studies have described the anti-nutrients ability of seeds and the leaves of the pumpkin. According to the current available literature, there are no identified contraindications or side effects associated with consumption of pumpkin seeds (Wang *et al.*, 2012).

With regards to the antidiabetic activity in diabetic induced rats, an experiment was carried out, which used white male Wistar rats with the body weights ranging

from 180 to 220 grams. The animals were all kept under standardized conditions with free access to food and water (Sedigheh *et al.*, 2011). A two-week period was allowed to elapse before the commencement of the study. This was aimed at allowing the animals to adapt to the new environment. Type 1 *Diabetes Mellitus* was induced by use of alloxan monohydrate via intraperitoneal route. The amount of alloxan monohydrate injected in most of the experiments was 120 milligrams per kilogram of body weight. The compound was dissolved in normal saline prior to administration (Andrade-Cetto and Heinrich, 2005). The diabetic disorder was then confirmed three days after injection through the measurement of fasting blood glucose levels. The ascertained glucose levels were over 130 milligrams per deciliter of blood, and therefore considered diabetic (Rahim *et al.*, 2013).

The rats were then divided into one experiment groups and two control groups. The serum glucose levels were significantly elevated in the diabetic control groups as compared to the normal group of rats. The consumption of 1 to 2 grams per kilogram of the pumpkin powder reduced levels of sugars in the plasma of diabetic rats (Sedigheh *et al.*, 2011). This was a similar effect to that ascertained with the use of the Glibenclamide drug. The insulin levels were found to be lower among the diabetic group as opposed to the normal control group (Díaz-Flores *et al.*, 2012).

The histological analysis shows significant variation in diameter and the quantity of the Langerhans islet cells between the normal control groups and the diabetic experiment groups (Ragab *et al.*, 2013). This suggests that the alloxan monohydrate compound selectively destroys the beta cells of the pancreas, which are responsible for the production of insulin. This, therefore, categorically induces type 1 *Diabetes Mellitus*, thus making the study feasible. Its structural likeness to glucose enables the compound to penetrate the cells easily and produce an effect soon after its administration.

Given that the pumpkin contains some components that are biologically active, it has the ability of lowering the blood glucose levels tremendously. Some of these biologically active compounds include para-aminobenzoic acid, sterols, peptides, polysaccharides, and amino butyric acids (Ragab *et al.*, 2013). According to Ranganathan *et al.*, (2013) pumpkin has antioxidant activity in the body cells. This antioxidant ability protects the cell membranes and other structures of the organism against the damage by the oxidative compounds. These antioxidant compounds found in pumpkins have an input in the enhancement of the antidiabetic activity of pumpkin seeds (Amin and Thakur, 2013). The antidiabetic effect is achieved through the activity of antioxidant compounds in increasing the number of beta cells of the pancreas. It increases the number of beta cells through enhancement of cellular restoration and repair usually witnessed in type 1 diabetes (Borokini *et al.*, 2013). Numerous studies have postulated that with the administration of the antioxidant compounds to rats and rabbits the number of the viable beta cells in diabetic pancreas is increased. Therefore, the protective property of the pumpkin to the pancreas and its accompanying anti-hyperglycemic activity are attributed to its antioxidant compounds components (Sedigheh, *et al.*, 2011).

It was found that the saponin extract of pumpkin has the highest hypoglycemic effects (El-Demery, 2011). Moreover, the pectin present in the pumpkin was ascertained to have another additional antidiabetic effect on a diabetic study animal. In addition, the presence of elevated polysaccharide levels in pumpkin is further postulated to be associated with the hypoglycemic properties of pumpkin seeds.

The 'health for all' is a goal in which humans share and strive to achieve. However, the current state of modern pharmaceuticals has proved that this goal is not achievable for the larger proportion of human beings. (Makni *et al.*, 2011). Consequently, traditional medicines are now the best alternative options to help

bridge the gap left by the modern pharmaceuticals. This has been appreciated as the valuable source of human knowledge that is tapped to bring tremendous health benefits. Herbal medicines are now regarded as important, though still underutilized in most cases. These are the medical tools believed to be highly compatible with human wellbeing and have lesser side effects as compared to modern drugs (El-Demery, 2011). Western populations are now looking for safer natural drugs that are effective. This is done in regard to the profound adverse effects associated with synthetic drugs.

India is the leading producer of traditional medicinal herbs in the world (Amin and Thakur, 2013). In addition, it is rich in traditional medicine heritage that constitute various components (Vibhute *et al.*, 2013). There are fifty varieties of *Cucurbita* all over the world. The functions of the *Cucurbitaceae* are purgative action, anti-inflammatory effects, intramural, cytotoxic, and antifertility in the mice (Coulson *et al.*, 2013). They can also play other biological roles that include growth regulation and anti-feeding effects on insects, although the exact mechanism for this is unknown.

Standardization and Quality Control are currently needed in ensuring that pumpkin oil is placed on a proper pharmacological footing. Some of the raw materials introduced in the market are usually adulterated with less potent, cheaper and spurious materials than the pumpkin seed oil extracts. This material used when adulterating poses a danger to the health of the public (Kaushik *et al.*, 2010).

Medical and dietary oils have been a core target by the chemist over the past decades. Their pharmacological applications paved the way for the chemists to carry out their studies in relation to the diseases targeted. Refractive index is one of the preferred methods used to ascertain different values of value extracts (Bajpai *et al.*, 2012). The key advantages of the technique include the simplicity

of determining the value, rapidity, and the selectivity (Saxena, 2010). Despite many studies on *Cucurbita maxima* extracts, the accurate information on the components and the value of the extracts are unclear. Given that the pH of a human is in a constant range, effective determination of the pH of the extract is vital. Few test equations have been generated to be used to determine different constant values of the extract. In addition, the equations help in giving scientific and systematic data that are useful in the maintenance of uniformity and the quality of the oil extract (Nawfal, 2011).

Acids, molecular weight, refractive index, free fatty acid content, apparent density, degree of unsaturation values are all vital in the determination of the potency and the shelf life of the extract, given that fatty acids have high medicinal value. Many researchers, therefore, propose a move to setting up of a system that ensures that every packet of herbal medicine is sold at the correct amount that will help induce the desired therapeutic effects. This is a system called *standardization*. These herbs can be regarded as an example of a synthetic laboratory that produces products that contain chemical compounds. These compounds are associated with the medicinal properties of pumpkin extracts (Nawfal, 2011). A complete investigation of phytochemicals of most of the available herbs is critical for the standardization and determination of the right therapeutic doses of the herbal drugs. In addition, there should be a quality control system in place that carries out the test of the entire herbal preparations to ensure the best quality of the drugs (Hechtman, 2012). Nearly all the traditional medical systems have their own way of ensuring proper standardization that assures the products of the highest quality (Kunyanga *et al.*, 2012).

1.3.5 Extraction of the drug from the seeds

From the study that has been carried out by Makni *et al.*, (2011) the seeds were first powdered and later used to extract the desired drugs. The compound used during the extraction process was petroleum ether. In addition, ethanol was used to extract the crude drug from the powder of pumpkin seeds, as well. Different parameters were used to determine the purity levels of the crude drug. The parameters measured included moisture content and phytochemical levels (Makni *et al.*, 2011). Several phytochemical studies have been used to identify various constituents of the pumpkin powder with an aim of extracting a crude drug with anti-diabetic effects.

Glibenclamide has been among the common drugs used in the management of diabetes with the aim of stimulating insulin secretion by the pancreatic beta cells. The current data hypothesized that various extracts of *Cucurbita maxima* significantly reduced the blood sugar levels in a person with diabetes (Borokini, 2013). According to Harman, (2007) the maximum results are obtained from the alcoholic-based extracts of *Cucurbita maxima*. Lipids play a role in the pathogenic process of *Diabetes Mellitus*. The serum lipid levels are occasionally elevated in a person suffering from *Diabetes Mellitus*. Given the elevated levels of lipids in plasma, the diabetic patient stands at a greater risk for coronary heart diseases. Major risk factors for coronary heart diseases include elevated level of low-density lipids and increased total plasma cholesterol (Jacobo-Valenzuela *et al.*, 2011). The resulting elevation of plasma levels of lipids in a diabetic person is due to increased metabolism of the free fatty acids stored at the peripheral fat depots. This is a secondary effect of the reduced plasma levels of insulin for insulin usually inhibits sensitivity to lipase enzyme (Makni *et al.*, 2010). The initial effect of acute insulin deficiency is elevated levels of the free fatty acid metabolism from the fat stores. This is the reason the hypertriglyceridemia and hypercholesterolemia in the chronic diabetics are profound. It is a vital measure during the management of

the diabetic patient to lower levels of blood lipids (Saxena, 2010). This helps in the prevention of complication associated with high sugar levels, such as ischemic and atherosclerotic conditions (Mootoosamy and Fawzi Mahomoodally, 2014).

1.3.6 Ability to prevent associated diabetic complications

In addition to the ability to raise insulin secretion levels; the prevention of complications is another dimension that the extracts of the *Cucurbita maxima* aid (or can potentially aid) in the management of diabetic patients (Kwon *et al.*, 2007, p. 9). Numerous *in vitro* studies suggested that the extract of pumpkin seeds resulted in a profound reduction, in the levels of plasma lipids. These lipids include very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), total cholesterol (TC), (Andrade-Cetto and Heinrich, 2005). It reduced these lipid products in the experimental animals to nearly the normal levels. Subsequently, the pumpkin extract resulted in the elevation of HDL (High density lipoproteins) and this desirable feature. This grants the extract the *antilipidemic* property. According to Kaushik *et al.*, (2010) the alcohol-based extracts of *Cucurbita maxima* showed reduction in the levels of very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), total cholesterol (TC), and triglycerides (TG'S) with the use of three different extracts.

Furthermore, serum levels of hepatic enzymes are elevated in a diabetes patient. This is a reflection of the damages exerted in the liver or hepatocellular inflammatory disorders. The majorly elevated enzymes include alkaline phosphate (ALP), and the glutamic pyruvic transaminase (SGPT). In addition, bilirubin levels are also elevated. The different extracts of pumpkin seeds cause a significant reduction in the activities of several hepatic enzymes that included SGOT, ALP, Bilirubin, and SGPT to normal levels (Amin and Thakur, 2013). This strongly suggests *Cucurbita maxima* extracts have protective abilities. Many researchers

suggest the effect as a consequent of the abilities to stabilize the cell membranes, and the rapid repair levels of the damaged hepatic cell perpetrated by streptozotocin, as well. The reduction effect of *Cucurbita maxima* extracts on ALP and bilirubin suggest an improvement in the liver secretory mechanisms (Amin and Thakur, 2013). Several pharmacological and phytochemical studies indicate that different extracts of pumpkin seeds contain flavonoids, carbohydrates, phenolics and saponin. According to Mootosamy and Fawzi-Mahomoodally, (2014) saponin appears to be strongly associated with stimulation beta cells of the pancreas and resulting increased secretion levels of insulin.

From the present available data, it can be extrapolated the oral intake of *Cucurbita maxima* extracts from the seeds has significant antidiabetic ability in the control of blood glucose levels. Furthermore, it has antihyperlipidemic effects that help lower levels of both triglycerides and cholesterol. In addition, it helps in increasing the levels of HDL-cholesterol. This is essential in the move towards preventing complications associated with increased levels of free fatty acids in diabetes people. Other studies are currently underway that are aimed at isolating and characterizing the active compound in the extract and the further determination of the mechanism behind its antidiabetic effect.

Globally, people have been looking for new sources of nutritional products that have proved to be completely healthy food. Therefore, there should be a move to air the nutritional values associated with *Cucurbita* seeds and specifically the antidiabetic ability. This will foster realization nutritional requirements of conscious populations throughout the world. The current literature supports the idea of conducting more research studies to investigate the other potentials of pumpkin seeds that are not yet explored. The *Cucurbita maxima* contribute a lot to human life that include diet, boost food security, culinary diversification, and nutrition. This is applicable in the management of diabetes since it is highly accompanied by numerous dietary restrictions. Some of the diabetic patients may

not be able to adhere to the recommended dietary content because of their costs and availability, at times.

The versatile nature of the *Cucurbita maxima* proves its ability to be cultivated under any climatic conditions. This enables many communities globally to grow and utilize the crop. Their input might be guided in the way to cultivate, and appropriate measures to ensure quality yield from the crop.

Herbal medicine may prove unsafe to human life because of the lack of proper standardization and quality control. There should be an immediate move towards researching on the safe standards of herbal drugs before licensing their commercialization. It is also vital for drug manufacturing companies to try to extract the crude drug from the herbal powder and to refine it appropriately. A standardizing policy should then be put in place.

1.4 The *Cucurbitaceae*

Natural resources with therapeutic properties are considered one of the most important sources not only for edibles but also in terms of the treatment of disease (Jacobo-Valenzuela *et al.*, 2011). Arguably, herbal medicine was practised as the only healthcare application for an extended period of time without any understanding of the functionality of these resources (Li *et al.*, 2004; Patel *et al.*, 2012). Since the time of the scientific and industrial revolutions however, there has been an increase in the knowledge and understanding of disease and its causes, and chemical drugs have come to be used as effective agents for managing a large range of diseases. Regardless of the benefits of using this type of treatment, such as the provision of pure substances and more effective elements, there has recently been increased attention focused on the use of natural plants as a substitute treatment or possible cure for many diseases. This

has occurred for several reasons, as Rates (2001) indicated, such as the misuse of chemical drugs and the side effects of consuming this kind of treatment. In addition, Patel *et al.* (2012) report that use of naturally sourced treatments is recommended by The World Health Organization (WHO), as these may represent harmless components with highly effective properties, which are for use in oral drug delivery.

Diabetes Mellitus (DM) is one of the diseases for which a cure has been investigated in terms of therapeutic elements obtained from plants, as the complications of this disease can result in a significant death rate (Li *et al.*, 2004). Patel *et al.* (2012) suggest that there are more than 780 plants which might contain bioactive components with the ability to treat DM (Park *et al.*, 2012). Therefore, fruits and vegetables which are rich in beneficial nutrients and have the bioactive materials which might eventually reduce the risk of DM have attracted the attention of many scientific researchers from a variety of fields and many of these species have been elucidated (Grover *et al.*, 2002; Kavishankar *et al.*, 2011; Bhushan *et al.*, 2010; Park *et al.*, 2012). Among these is the gourd family or *Cucurbitaceae*. This family contains more than 70 genera and its species number approximately 800, including species such as *Cucurbita maxima*, *Cucurbita pepo*, *Cucurbita moschata*, *Cucurbita mixta*, and *Cucurbita ficifolia* (Yadav *et al.*, 2012). The name *Cucurbitaceae* comes from the chemical components found in these species known as cucurbitacins, which are oxygenated tetracyclic triterpenoids (Samuel *et al.*, 1995). Interestingly, all parts of these fruit-bearing plants, such as oils, pulp, vines, leaves and seeds, are suitable for consumption. Fruits of this family are also used as a traditional way of celebrating, as seen in Halloween carvings (Raman and Lau, 1996; Patel, 2013). These fruits have also been frequently used in treating many diseases in several countries, including India, China, America, Canada, Mexico, Brazil, Argentina, Yugoslavia, Italy, Iran, Nigeria, Guatemala and Cameroon (Jacobo-Valenzuela *et al.*, 2011;

Patel, 2013; Kreft et al., 2002; Teugwa et al., 2013; Ranganathan et al., 2013). It is worth noting that after many research studies (for example Adams et al., 2011; González et al., 2001; Caili et al., 2007; Andjelkovic et al., 2010; Nkosi et al., 2006; Makni et al., 2008; Makni et al., 2010; Teugwa et al., 2013; Gossell-Williams et al., 2008; Kreft et al., 2002; Jiang and Du, 2011; Veronezi and Jorge, 2012; Roman-Ramos et al., 2012; Jafarian et al., 2012; El-Fiky et al., 1996; Chaturvedi et al., 2004; Maiti et al., 2004; Yoshinari et al., 2009; Alarcon-Aguilar et al., 2002; Hsiao et al., 2013; Aguilar et al., 2011; Mohamed et al., 2009; Ranganathan et al., 2013; Adams et al., 2012) using a variety of screening, characterization and isolation methods such as antimicrobial assay, animal models, Analytical Ultracentrifugation (AUC), gel filtration, ion exchange chromatography, sodium dodecylsulfate (SDS) gel electrophoresis, and high performance liquid chromatography–mass spectrometry (HPLC–MS), that this family has therapeutic components which have the ability to reduce the risk of many diseases.

Cucurbita pepo, or sometimes, *Cucurbita maxima*, which is known as pumpkin in English, *calbaza* in Spanish, *kabocha* in Japanese and *huiioy* in Guatemala, is one of the remarkable fruits that belongs to the family *Cucurbitaceae*, and has been used world-wide as an active medicine and edible fruit (Caili et al., 2006; Adepoju and Adebajo 2011; Bnouham et al., 2006). Pumpkin has been described (Amin and Thakur, 2013) as a gourd squash plant that has three commercial types; *Cucurbita pepo*, *Cucurbita maxima* and *Cucurbita moschata*. These species are all known as pumpkins, with the differences between them being found in the shape of stem (proximal) and structure (Xanthopoulou et al., 2009; Sedigheh et al., 2011; Kikuchi et al., 2013). However, these species vary in their acclimation and consequently differ across the world (Paris and Brown, 2005). All parts of the pumpkin, including pulp, seeds and peel, have been studied and investigated to reveal their therapeutic aspects, and the following tables will summarize the

findings of the major studies conducted regarding the medicinal functions of the pumpkin and its phytochemical components.

As can be seen from Tables 1.1, 1.2 and 1.3, the fruit of the pumpkin has multi-therapeutic purposes, which have attracted scientists from a variety of fields, and several reviews and studies have been described to reveal and characterise all parts of the pumpkin. According to Quanhong *et al.*, (2005) pumpkin has, in general, different components which involve proteins, polysaccharides (e.g. starch), vitamins (e.g. vitamin A and B) and fibrin. Also, studies report several phytochemical components within pumpkin, such as phenols, α -tocopherol, flavonoids, β -carotene, tetrasaccharide glyceroglycolipids, tetrasaccharide glyceroglycolipids (QGMG-3 and QGMG-2), pectin, trigonelline and nicotinic acid (Jiang and Du, 2011; Dini *et al.*, 2013; Jun *et al.*, 2006; Yoshinari *et al.*, 2009). Moreover, a protein-bound polysaccharide has been isolated from pumpkin in this study, although the type isolated is not revealed. In addition, Bang *et al.* (2002) studied the leaves of the pumpkin, and in this study, unsaturated hydroxy fatty acid (13-Hydroxy-9Z, 11E, 15E-octadecatrienoic acid) was isolated. The seed of the pumpkin is utilized as a snack world-wide, and it has been characterized in terms of the benefits of its phytochemical components such as proteins, mono- and polyunsaturated fatty acids, polysaccharides, zinc and other substances which have not been described (Adams *et al.*, 2011; Alfawaz, 2004; Glew *et al.*, 2006; Veronezi and Jorge 2012; Patel, 2013; Caili *et al.*, 2007; Maknia *et al.*, 2011; Rezig *et al.*, 2012).

Table 1.1: List of Pumpkin seeds having therapeutics activity.

| Components (phytochemical informat) | Function | Reference(s) |
|--|---|---------------------------------------|
| 1. Protein from the seed. | 1. Replacement for animal protein in traditional foods. | 1. (Atuonwu and Akobundu, 2010) |
| 2. Flax and pumpkin seeds. | 2. To prevent diabetes. | 2. (Makni <i>et al.</i> , 2010) |
| 3. Seed from pumpkin. | 3. Immunosuppressive activity. | 3. (Winkler <i>et al.</i> , 2005) |
| 4. Seed from pumpkin. | 4. Modulate humeral immune response. | 4. (Ranganathan <i>et al.</i> , 2013) |
| 5. Protein from the seed. | 5. Hepatoprotective and hypolipidemic activities. | 5. (Mohamed <i>et al.</i> , 2009). |
| 6. Protein from the seed. | 6. Reducing the effect of Certain Plasma Enzymes in CCl4- (liver damage). | 6. (Nkosi <i>et al.</i> , 2006). |
| 7. Phenolics, tocopherols and sterols from the seed. | 7. Protection against oxidative stress. | 7. (Rezig <i>et al.</i> , 2012). |
| 8. Protein from the seed. | 8. Nephro- and hepato-protective ability. | 8. (Adepoju and Adebajo, 2011). |

Table 1.2: List of additional Pumpkin seeds having therapeutics activity.

| Components (phytochemical informat) | Function | Reference(s) |
|---|--|--|
| <p>9. Oil from the seed.</p> <p>10. Oil from the seed.</p> <p>11. Protein from the seed (cucurmoschin).</p> <p>12. Pumpkin seed with flax seed.</p> <p>13. Phenolic content from the seed.</p> <p>14. Protein from the seed (globulins).</p> <p>15. Seed from pumpkin.</p> <p>16. Three multiflorane-type triterpenes from the seed of the pumpkin.</p> | <p>9. Antioxidant activity.</p> <p>10. Antioxidant potential (reduce the risk of cardiovascular).</p> <p>11. Antifungal properties.</p> <p>12. Anti-atherogenic activity.</p> <p>13. Antioxidant activity.</p> <p>14. Anti-hyperglycaemic activity.</p> <p>15. Anti-diabetic properties.</p> <p>16. Anticancer activity.</p> | <p>9. (Nawirska-Olszańska <i>et al.</i>, 2013).</p> <p>10. (Gossell-Williams <i>et al.</i>, 2008).</p> <p>11. (Wang and Ng, 2003).</p> <p>12. (Makni <i>et al.</i>, 2008).</p> <p>13. (Xanthopoulou <i>et al.</i>, 2009).</p> <p>14. (Teugwa <i>et al.</i>, 2013).</p> <p>15. (Sharma <i>et al.</i>, 2013).</p> <p>16. Kikuchi <i>et al.</i> 2013)</p> |

Table 1.3: List of Pumpkin pulps and peel having therapeutics activity.

| Components (phytochemical informat) | Function | Reference(s) |
|---|--|---|
| <ol style="list-style-type: none">1. Protein-Bound Polysaccharide.2. Phenolic compounds.3. Acidic protein-bound polysaccharide.4. Tetrasaccharide glyceroglycolipids, QGMG-3 and QGMG-2.5. Alkali soluble pectic substance from peel.6. Trigonelline and Nicotinic Acid (Pumpkin paste). | <ol style="list-style-type: none">1. To prevent diabetes (prevented the destruction of β cells of islets in the pancreas).2. Antioxidant activity.3. Hypoglycaemic activity.4. Anti-diabetic properties.5. Antimicrobial properties.6. Anti-diabetic properties. | <ol style="list-style-type: none">1. (Quanhong <i>et al.</i>, 2005).2. (Dini <i>et al.</i>, 2013).3. (Caili <i>et al.</i>, 2007).4. (Jiang and Du, 2011).5. (Jun <i>et al.</i>, 2006).6. (Yoshinari <i>et al.</i>, 2009) |

Diabetes Mellitus (DM) is described by Yadav *et al.* (2009), as one of the leading causes of mortality. Consequently, it is imperative to find a solution to this disease as soon as means are available. Therefore, various studies have been conducted regarding natural plants and the bioactive substances within them which have therapeutic effects on DM (Rates, 2001). One of these plants is pumpkin, which offers one of the most critical health benefits protecting against DM (as will be discussed below), but at the time of writing, all the data and results that have been reported are still to be described as a primary study. Interestingly, all substances from the pumpkin, including polysaccharides, proteins and fatty acids, have been reported to be anti-diabetes. As the protocols of the studies that have

been published are generally similar in terms of characterization and the screening methods, the following will only highlight in general the use of pumpkins as hypoglycaemic active components for diabetic patients reported during the period 2003-2013, using one study as an example. It is worth noting that several studies are not included in the review as either they were challenging to obtain, or due to language barriers. As the protocols of the studies that have been published are generally similar in terms of characterization and the screening methods, the following will only highlight in general the use of *Cucurbita* as hypoglycaemic active components for diabetic patients reported during the period 2003-2013, using one study as an example and highlighting the important of studying the protein-polysaccharides complex.

Shi *et al.* (2003) report that polysaccharide granules from pumpkin have the ability to control glycaemia in type 2 *Diabetes Mellitus*. This clinical study was carried out on a treatment group who had been diagnosed with diabetes. The group were fed with polysaccharide granules from pumpkin and the results were compared with a control group treated with Xiaoke pills (Xiaoke is the terminology for Diabetes within Chinese medicine, and the name of an anti-diabetic medicine used in China). The comparison shows that polysaccharide granules from pumpkin have a similar effect to Xiaoke pills in reducing plasma glucose and urination. Caili *et al.* (2006), in their review, report that a study carried out by Zhang (2004) in diabetic rats which were induced by alloxan showed that polysaccharides (water-soluble) from pumpkin control glycaemia in diabetic rats. In 2005, a study was carried out by Quanhong *et al.* showing that protein-bound polysaccharides from pumpkin reduce blood glucose levels by preventing the destruction of β -cells of islets in the pancreas. Nishimura *et al.* (2006) have suggested that polyamines, which can be found in pumpkin seeds, might have a role in preventing pancreatitis and may help to reduce hypoglycaemia. In 2007, acidic protein-bound polysaccharide from pumpkin was isolated and investigated and the results

showed that the amino acid alanine is the main amino acid, and that the complex of protein-bound polysaccharides has hypoglycaemic activity (Caili *et al.*, 2007). Interestingly, in this study the protein from fresh pumpkin seeds was shown not to be anti-diabetic, whereas the results of Quanhong *et al.* (2003) showed that the protein from germinant pumpkin seeds has anti-diabetic activity. Moreover, Adams *et al.* (2011), in their review, report that Gu and Li (2008) had found the same effect of protein from germinant pumpkin seeds as found by Quanhong *et al.* (2003).

In 2009, the anti-diabetic properties of pumpkin pastes were investigated by Yoshinari *et al.* (2009), and the results showed that trigonelline and nicotinic acid from pumpkin could improve insulin resistance and help with diabetes. Makni *et al.* (2010) suggest that a flax and pumpkin seeds mixture might prevent diabetes. A further study was carried out by Makni *et al.* (2011) on a flax and pumpkin seed mixture, the results of which suggest that the seed oil of the pumpkin might help to prevent diabetes by increasing the level of plasma enzymes. Further, Rezig *et al.* (2012) report that the pumpkin seed oil, with its bioactive components such as phenolics, tocopherols and sterols, might offer protection against oxidative stress, and thus help protect against diabetes. The anti-diabetic activity of proteins in pumpkin has been evaluated by Teugwa *et al.* (2013) and the results show that globulin is the major storage protein and might have the ability to reduce blood glucose levels.

It is noteworthy that although there have been great efforts made toward revealing the bioactive substances present in the family *Cucurbitaceae*, and several research studies have been done, the bioactive components are still not fully explored. However, Table 1.4 and 1.5 represent some of the possible bioactive components. The question remains at the end of each study of what are the bioactive components of *Cucurbita* (Tables 1.4 and 1.5) which have the ability to reduce blood glucose levels, and what is the mechanism of these substances.

Also to be explored is the question of whether it would be possible to provide these components in sufficient amounts by manipulating the plant to produce them, and what effects are gained by consuming these bioactive substances orally.

Table 1.4: Bioactive components which have been reported as an anti-diabetic drug (Bhushan *et al.*, 2010).

| Bioactive components | Mechanism | Function | Ref |
|-----------------------------|--|--------------------|-----------------------------|
| Alkaloids | Inhibit alpha glucosidase and decrease glucose transport through the intestinal epithelium. | anti-diabetic drug | (Pan <i>et al.</i> , 2003) |
| Dietary fibres | Effectively adsorbs glucose, retards glucose diffusion and inhibits the activity of alpha-amylase, and may be responsible for decreasing the rate of glucose absorption and concentration of postprandial serum glucose. | anti-diabetic drug | (Chau <i>et al.</i> , 2003) |

Table 1.5: Additional bioactive components which have been reported as an anti-diabetic drug (Bhushan *et al.*, 2010).

| Bioactive components | Mechanism | Function | Ref |
|-----------------------------|--|--------------------|---------------------------------|
| Flavonoids | Suppress the glucose level, reduce plasma cholesterol and triglycerides significantly and increase hepatic glucokinase activity, probably by enhancing the insulin release from pancreatic islets. | anti-diabetic drug | (Bhushan <i>et al.</i> , 2010) |
| Imidazoline | stimulate insulin secretion in a glucose-dependent manner | anti-diabetic drug | (Bhushan <i>et al.</i> , 2010) |
| Polysaccharides | Increase the level of serum insulin, reduce the blood glucose level and enhance tolerance to glucose. | anti-diabetic drug | (Quanhong <i>et al.</i> , 2005) |
| Saponin | stimulates the release of insulin and blocks the formation of glucose in the bloodstream | anti-diabetic drug | (Ng <i>et al.</i> , 1986) |

1.5 Beta-glucan and cholesterol lowering

About 60% of the population have higher than recommended blood cholesterol levels, which is an important risk factor for cardiovascular disease, which is responsible for over 150,000 deaths annually in the UK, costing the NHS an estimated £30 billion/year. Water soluble beta glucan (beta glucan) is effective at reducing plasma cholesterol concentrations, a factor that is recognised by the European Foods Standard Agency EFSA. The claim in the UK is that: The inclusion of oats as part of a diet low in saturated fat and a healthy lifestyle can help reduce blood cholesterol (May 6, 2004). Claims relate to whole oat flour, rolled oats and oat bran but not isolated oat beta glucan (June 2, 2006). However, the recommended intake currently requires large quantities of cereals such as oats or barley to be consumed daily, so that compliance is a real barrier to its effectiveness. The main problem is that processed beta glucan are less successful at reducing plasma cholesterol and because the mechanisms of action are not clear, more effective, acceptable forms of dietary beta glucan rich foods are not currently being produced.

Beta glucan is found principally in the endosperm cell walls of cereal grains such as barley and oats, but also in their bran more precisely in the aleurone and sub-aleurone layer. It is a linear polymer of glucose units with mixed β -(1→4) and β -(1→3) glucosidic bonds. The glucose residues are mainly (>90%) organised into cellotriosyl and cellotetraosyl units separated by single (1→3)- β -linkages. The ratio of trisaccharides to tetrasaccharides is specific to the cereal: 2:1 for oat and 3:1 for barley beta glucan. It is a polydisperse polysaccharide with reported values of (weight) average molecular weight (M_w) between ~0.1 – 3.0 million Da. This structural variability together with the modifications resulting from the initial stages of processing (i.e. dehulling and milling) will have a direct impact on some of the properties of beta glucan. For instance, manipulating the M_w and the particle size of oat/barley particles will lead to materials with different solubility and

viscosity. Cooking has also been shown to promote beta glucan depolymerisation as demonstrated in bread (Trogh *et al*, 2004; Tiwari and Cummins, 2011; Tosh *et al*, 2010).

Beta glucans have been shown to have beneficial effects on health, in particular reducing plasma cholesterol concentrations. Numerous *in vivo* studies have looked at the effects of beta glucans (and related soluble dietary fibre molecules) One crucial observation is that beta glucan seems more effective in reducing total and LDL cholesterol when consumed in its original unprocessed form (i.e. barley groat, rolled oats, oat bran) rather than when it is processed in various food products (i.e. bread, muffin, soup or juice) (Fillery-Travis *et al*, 1997).

Beta glucan depolymerisation occurring during food preparation/processing (i.e. milling, cooking, interaction with other ingredients) is likely to reduce the potential health benefits associated with their consumption, as observed with bread and other derived products made from oat and barley (Kerckhoffs *et al*, 2003; Tosh *et al*, 2010). Moreover, storage - including freezing - is known to modify the viscosity of beta glucan; an important factor here is probably a reduction in the solubility of the polymer (Wolever *et al*, 2010). A better understanding of the physical characteristics in response to processing is called for, and also in its behaviour with lipase: does beta glucan interact with lipase, helping to prevent its action in helping to degrading fats to cholesterol?. Or can we rule out this interaction as part of the action? It is important for Researchers and Clinicians to know.

1.6 *Nigella sativa* - its medicinal properties

Nigella sativa, with its dynamic medicinal properties has the potential to be utilized as an effective means for treatment of various ailments. Most importantly, the extracts of the seed of the plant has various bioactive components that are

antidiabetic. Thymoquinone (TQ) has the highest potential in controlling and maintenance of blood glucose as seen with various supporting literature. Other unsaturated fatty acids such as linoleic acid and oleic acid are known to promote insulin production or at least prevent the production of compounds that can compromise insulin secretion in healthy individuals. However, most of the literature available on antidiabetic properties of the seed of *N. sativa* is based on the whole seed extract or powdered seed. Analysis of individual biochemicals on its efficacy in prevention or management of diabetes is yet to be investigated. Whatever the case, *N. sativa* seed is an efficient traditional medicine in the management of *Diabetes Mellitus*.

1.7 Lignins

Lignins represent a class of biopolymer that are closely associated with glycans/polysaccharides, and found together in the cell walls of plants. Although the function of lignin in plants is not fully understood (DeMartino, 2005), lignin provides biomechanical support to plants, facilitates transport of nutrients and water, and protects plants from damaging enzymes and insects (Vanholme *et al.*, 2010; Hatfield and Vermerris, 2001).

One of the most common areas where lignin has been used is in the pulp and paper industry (Brebun and Vasile, 2010) although in the pharmaceutical industry, lignin is being considered for use in the fight against colorectal cancer (Harris and Smith, 2006). In addition, lignin with starch can be used as drug delivery system (Calgeris *et al.*, 2012). Lignin has also been reported to have multiple properties such as antimicrobial and antioxidant effect (by the hunting action of phenolic structures on oxygen-containing reactive free radicals) (Boeriu *et al.* 2004, Salanti *et al.*, 2010; Blomhoff, 2010; Raghuraman *et al.*, 2005). It was surprising that lignin has the ability to inhibit the expression of HIV-1 gene as it has been reported for the first time by Mitsuhashi *et al.*, (2008). Lignin might therefore provide

another major macromolecule in the fight against diabetes based on its antioxidant activity as diabetes is generally associated with decreased in antioxidant defences (McLennan *et al.*, 1991) and is worthy of further study (Pan *et al.*, 2009; Mukai *et al.*, 2011). Critical to its application in Biopharma is an understanding of its physical properties. We focus on three different lignins from wood: alcell, kraft and soda.

The main aim of the investigation is to use hydrodynamics and other related techniques to assess the structural and conformational properties of the macromolecules considered above. By increasing the level of understanding of these systems, it might promote greater knowledge and, in turn, better pharmaceutical and pharmacological therapeutics for the treatment of disease. In the following chapter we consider the methods used in the investigation.

Chapter 2: Analytical Methods

2.1 Analytical Ultracentrifugation

Analytical Ultracentrifugation (AUC) is a method for separating macromolecular components without any need for a matrix or column. Molecules are separated with respect to their shape and size within a centrifugal field. The particles of larger size will sediment faster than those of smaller size. Equally, globular particles (spheres, spheroids) will sediment faster than rods or coils due to the lower friction forces more compact shapes experience.

2.1.1 Mechanical systems

2.1.1.1 Analytical Ultracentrifuges

Two Beckman Optima XL-I analytical ultracentrifuges were used as a part of these examinations (see for example (Furst, 1997)). Ultracentrifuges have a top speed of 60k rotations per minute (RPM). Under vacuum (<0.7 Pa), the rotor is spun and held at a constant temperature to within $\pm 0.1^\circ\text{C}$. A centrifugal field of up to 100,000g is delivered by the rotation of the rotor. A Proteome Lab 5.7 was used to procure scans (Beckman, Indianapolis, US).

2.1.1.2 Cells

Two types of analytical ultracentrifuge cell were used. The first incorporated a 12mm aluminum epoxy resin centerpiece as this is suitable for centrifugation of aqueous, biological materials of pH 3 to 10.

The sapphire end windows are contained inside aluminum window housings, secured with gaskets. The parts which include windows and centerpieces are set into the cell housing and fixed with an aluminum screw ring, sealed to 13.6 - 15.8 nm. The cells were adjusted in connection to the focal point which means the central rotation. The centerpiece septa are radially arranged such that sedimenting particles do not accumulate against the sides as they sediment.

2.1.1.3 Rotors

8 hole (An-50Ti) or 4 hole (An-60Ti) titanium rotors were used for holding one counterbalance and 7/3 analytical cells respectively.

2.1.2 Optical systems

During rotation, the monochromator (UV absorption) and camera (Rayleigh Interference) perform precisely-timed scans at high speed through the cells. Two optical systems were used in these investigations: Rayleigh Interference and Absorbance.

2.1.2.1 Rayleigh Interference

A monochromatic laser beam is split into 2 beams which pass through the solution and reference sectors of each cell and then are recombined by an optical system resulting in the production of fringes or "signal". The signal transformed by a Fourier Transform (FT) to convert the image into an ASCII format (Furst, 1997). Fringes are displaced when there is a difference in refractive index, due to a change of concentration in the solution cell, during or after a sedimentation

process. The relative fringe increment (ΔJ) is related to mass concentration (c) through:

$$\Delta J = c \frac{dn}{dc} \frac{l}{\lambda} \quad (2.1)$$

where (λ) is the wavelength of monochromatic light, (l) is the path-length the cell and the refractive index increment is denoted by (dn/dc). The wavelength of light from the AUC in this investigation (Beckman Optima XL-I) was 655nm and 675nm for the (Beckman Optima XL-G).

2.1.2.2 Absorbance

UV absorbance is helpful for systems containing nucleic acids or protein. However, it is ineffective for molecules that do not absorb, for instance polysaccharides. Absorbance scans take approximately two minutes, contingent upon choose resolution, because of the development of the optical framework beside the outspread size or the length of the cell. However, a newer model of Beckman centrifuge is about to come to market, which can deliver scans in approximately 6 seconds.

An ASCII record is made (marked as a ". RA" file) with position (cm), Absorbance (Optical Density, OD) and standard error. Absorbance is related to mass concentration through the Lambert-Beer Law:

$$\left(\ln \frac{I}{I_0}\right) = \epsilon_{280nm} l c \quad (2.2)$$

where I is the light intensity after passing through the solution, I_0 is the light intensity through the solvent (assumed to be non-absorbing), ϵ_{280} (ml. g⁻¹ cm⁻¹)

the extinction coefficient at 280nm, l is the path length (cm) and c is the concentration (g. ml⁻¹).

2.1.3 Sedimentation velocity

2.1.3.1 Theory

High speeds are used to obtain the sedimentation coefficient, s (rate of movement of the sedimenting boundary per unit centrifugal field), according to the classical Svedberg equation (Svedberg and Pedersen, 1940):

$$s = \frac{v}{\omega^2 r} = \frac{M(1-\bar{v}\rho)}{N_A f} \quad (2.3)$$

v denotes the terminal velocity (cm. s⁻¹), r is the distance (cm) from the center of rotation, ρ the solvent density (g. ml⁻¹), ω is the angular velocity (rad. s⁻¹), N_A is Avogadro's constant (mol⁻¹), M is the molar mass (g mol⁻¹), f the friction coefficient and \bar{v} the partial specific volume (ml. g⁻¹). The units of the sedimentation coefficient s are seconds, s or Svedberg units S , where $1S = 10^{-13}$ s.

During the sedimentation process the boundary will spread due to diffusion broadening. The Svedberg equation (2.3) is complemented by the Stokes-Einstein equation for the diffusion coefficient D (units cm². s⁻¹)

$$D = \frac{k_B T}{6\pi\eta r_H} \quad (2.4)$$

where k_B is the Boltzmann constant, r_H is the hydrodynamic radius (cm) and:

$$D = \frac{k_B T}{f} \quad (2.5)$$

Combining eq. 2.3 with eq. 2.5 we obtain:

$$s = \frac{M(1-\bar{v}\rho)D}{N_A k_B T} \quad (2.6)$$

Current software such as SEDFIT (see for instance Schuck (2000)) depends on the Lamm equation (Lamm, 1929):

$$\frac{dc}{dt} = D \left[\left(\frac{d^2c}{dr^2} + \frac{1}{r} \left(\frac{dc}{dr} \right) \right) \right] - s\omega^2 \left[r \left(\frac{dc}{dr} \right) + 2c \right] \quad (2.7)$$

Due to non-ideality effects (solvent backflow during sedimentation and charge effects) a sedimentation coefficient measured at a concentration c will be an underestimate. This can be corrected for by working at very low concentrations (generally 0.3 mg/ml or less) or making measurements at different concentrations and extrapolating back to zero concentration. The commonly used extrapolation equation of Gralen (1944) is often used:

$$1/s = \{1/s^0\} \cdot \{1 + k_s \cdot c\} \quad (2.8)$$

where k_s (ml g⁻¹) is the Gralen coefficient. Once obtained, k_s is also interesting in that the Wales-van Holde ratio, $k_s/[\eta]$, where $[\eta]$ is the intrinsic viscosity (see below) is a measure of macromolecular shape (Wales and Van Holde, 1954). For example,

$k_s/[\eta]=1.6$ for a spherical particle or randomly coiled particle, but much lower for a rod, approaching a limit of ~ 0.15 . For comparison purposes, s (measured at a temperature T in a buffer b) is corrected for the density and viscosity of water at 20.0°C to give $s_{20,w}$.

$$s_{20,w} = \frac{(1-\bar{v}\rho)_{20,w}}{(1-\bar{v}\rho)_{T,b}} \frac{\eta_{T,b}}{\eta_{20,w}} S_{T,b} \quad (2.9)$$

This correction is performed using either the routine SEDNTERP (Hayes *et al.*, 1995) or the routine SEDFIT (Schuck, 2000). SEDFIT also provides the framework for sedimentation coefficient distribution analysis, $g(s)$ vs s (or $g(s)$ vs $s_{20,w}$). SEDFIT analyses in this study were performed by SEDFIT v12.4 or later.

SEDFIT utilizes two primary methods for sedimentation velocity examination. The first gives the sedimentation coefficient distribution $g(s)$ vs s , not corrected for diffusion broadening (this is not so important for large polysaccharides where diffusive effects are relatively small) or $c(s)$ vs s where the sedimentation coefficient distribution is corrected for diffusion broadening. The latter is based on an approximation that all the molecules in a distribution have the same friction coefficient f .

2.1.3.2 The frictional ratio

The frictional ratio is a useful parameter as it relates to macromolecular shape and hydration

The frictional ratio is defined as

f/f_0 = frictional coefficient of a macromolecule / frictional coefficient of a dry spherical particle of the same mass

It can be calculated from the sedimentation coefficient by:

$$f/f_0 = \frac{M(1-\bar{v}\rho)}{N_A 6\pi\eta_0} \left(\frac{4\pi N_A}{3\bar{v}M} \right)^{1/3} \frac{1}{s} \quad (2.10)$$

where η_0 is the solvent viscosity. (f/f_0) is also related with the diffusion coefficient:

$$f/f_0 = \frac{k_B T}{6\pi\eta_0} \left(\frac{4\pi N_A}{3\bar{v}M} \right)^{1/3} \frac{1}{D} \quad (2.11)$$

2.1.3.4 Extended Fujita method

The sedimentation coefficient distribution $g(s)$ vs s can be converted to a molecular weight distribution using a method known as the "Extended Fujita" method (Harding *et al.*, 2011). This applies the following "power-law" relationship (Harding *et al.*, (1991) see (2.12).

$$s = \kappa_s M^b \quad (2.12)$$

The shape coefficient b lies between 0.2 (rod) and 0.67 (sphere). A random coil has a b value of between 0.4 and 0.5. κ_s can be calculated if b is known and if a value of s is known for one value of M for that material (e.g. the weight average s and the weight average M – from sedimentation equilibrium). The method builds on a method first given by Fujita (Fujita, 1962) for random coils only.

The transformation is given by

$$f(M) = g(s) \left(\frac{ds}{dM} \right) \quad (2.13)$$

$$\frac{ds}{dM} = b\kappa_s^{1/b} s^{(b-1)/b} \quad (2.14)$$

This routine has been built into the SEDFIT package. To minimize non-ideality effects, the $g(s)$ vs s profile being transformed must be obtained at a low enough concentration that non-ideality effects are negligible.

2.1.4 Sedimentation equilibrium

2.1.4.1 Theory

In sedimentation equilibrium analysis, lower speeds are chosen so that the sedimentation forces on a particle become much lower, and become comparable with diffusion forces in the opposite direction. After a period of time (usually > 24h) these forces come to equilibrium and this is the state of sedimentation equilibrium. In this state, the final pattern depends only on the molecular weight and not on the frictional coefficient or shape.

$$\frac{d \ln(c)}{dr^2} = \sigma = \frac{\omega^2 M(1-\bar{v}\rho_0)}{2RT} \quad (2.15)$$

2.1.4.2 Analysis: MSTAR

A popular way of analyzing the data at sedimentation equilibrium is the MSTAR algorithm, based on the M^* function of Creeth and Harding (1982) for evaluating

the weight average molecular weight M_w of the distribution of particles in solution.

The M^* function is defined (Creeth and Harding, 1982) by

$$M^*(r) = \frac{(c(r)-c(a))}{kc(a)(r^2-a^2)+2k \int_a^r r [c(r)-c(a)] dr} \quad (2.16)$$

where

$$k = \frac{\omega^2 M(1-\bar{v}\rho_0)}{RT} \quad (2.17)$$

$c(r)$ is the concentration at radial position r , a is the radial position at the meniscus.

The weight average molecular weight over the whole distribution M_w is obtained from the identity shown by Creeth and Harding (1982) that $M^*(b) = M_w$, where $M^*(b)$ is the value of M^* extrapolated to the cell base ($r=b$).

2.1.4.3 SEDFIT-MSTAR

The M^* procedure has recently been built into the SEDFIT suite of algorithms as the program SEDFIT-MSTAR (Schuck *et al.*, 2014) and we use the release SEDFIT-MSTAR v1. SEDFIT-MSTAR also provides a method for obtaining local estimates of the weight average molecular weight $M_w(r)$ as a function of radial position (r) (Schuck *et al.*, 2014). This gives an idea of the spread or distribution of molecular weights in a material.

2.2 Viscometry

2.2.1 Theory

The intrinsic viscosity $[\eta]$ (ml/g) is a measure of the hydrodynamic volume occupied by macromolecules in solution and thus a reflection of its size and shape. It can be determined by measuring the relative viscosity η_r . The relative viscosity is defined as the ratio of the viscosity of a solution η to that of the solvent in which it is dissolved η_o :

$$\eta_r = \frac{\eta}{\eta_o} = \left(\frac{t}{t_o} \cdot \frac{\rho}{\rho_o} \right) \quad (2.18)$$

Equation 2.18 shows how η_r can be measured from the ratio of the flow time of solution t to that of solvent t_o . There is also a correction for the ratio of the density of solution ρ to that of solvent ρ_o but generally at the low concentrations used to study polysaccharides (<0.2 mg/ml) this ratio is usually ~ 1 . At higher concentrations the correction is necessary. Also for polysaccharide solutions – particularly at higher concentration, corrections for Non-Newtonian behavior may be necessary. Although conventional U-tube Ostwald viscometers do not allow this, the new generation “rolling ball” viscometers do permit a correction (or alternatively rheometers can be used).

Experimentally $[\eta]$ it is obtained from measurements of relative viscosity of solutions with different polymer concentrations. By the division of specific viscosity with the concentration (c) the reduced viscosity is calculated:

$$\eta_{red} = \frac{\eta_{sp}}{c} \quad (2.19)$$

where the specific viscosity is $(\eta_r - 1)$. The units of η_{red} are ml/g.

Another useful parameter is the inherent viscosity:

$$\eta_{inh} = \frac{\ln(\eta_r)}{c} \quad (2.20)$$

whose units are also ml/g. In an ideal system, the reduced viscosity and inherent viscosity would be equal, and represent the intrinsic viscosity (Harding, 1997). Due to non-ideality this is usually not the case and plots of η_{red} versus concentration (Huggins, 1942), or inherent viscosity against concentration (Kraemer, 1938) are necessary. Extrapolations to zero concentration yield $[\eta]$ in both cases. If the plots are linear, the plots yield the Huggins (K_H) and Kraemer (K_k) constants respectively.

$$\eta_{red} = [\eta](1 + [\eta]K_H c), \quad \eta_{inh} = [\eta](1 - [\eta]K_k c) \quad (2.21)$$

and

$$\lim_{c \rightarrow 0}(\eta_{red}) = \lim_{c \rightarrow 0}(\eta_{inh}) = [\eta] \quad (2.22)$$

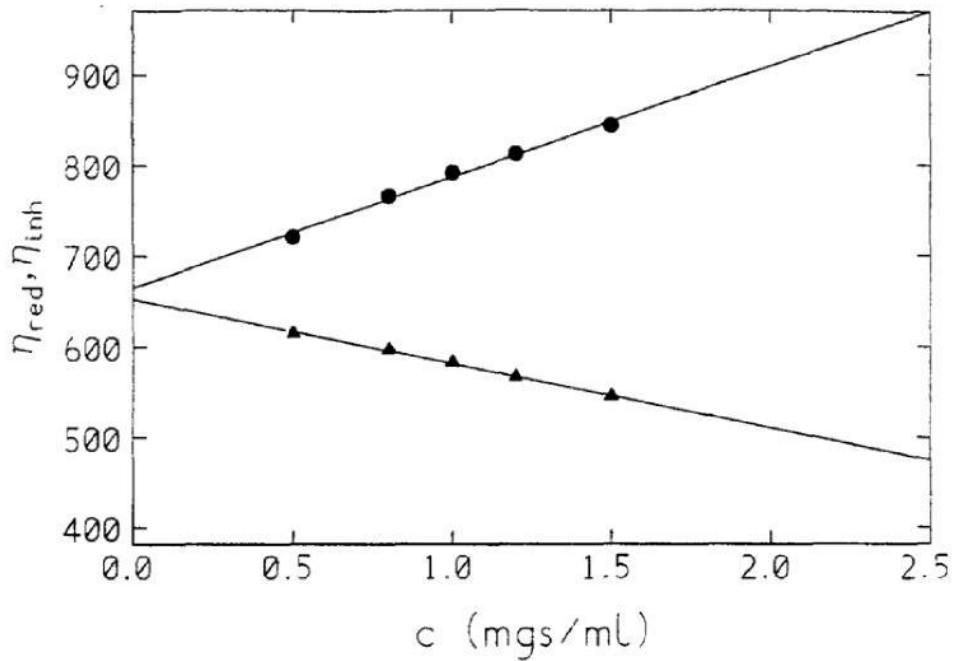


Figure 2.1: Illustration of the extrapolation of the inherent (triangles) and reduced (circles) viscosities. At infinite dilution, the intrinsic viscosity is yielded (Harding, 1997, Jumel, 1994).

2.2.2 Dependence on size and shape

The macromolecular intrinsic viscosity is determined by its shape and size (molecular weight). The equation of Mark-Houwink-Kuhn-Sakurada (MHKS) (see, Tombs and Harding, 1998) has provided the basis for this:

$$[\eta] = KM^a \quad (2.23)$$

where (K) a parameter which depends on the solvent conditions and the "MHKS coefficient" a depends on the conformation (see for example Harding *et al.* (1991)). For a perfect sphere, $a=0$, for a non-draining random coil structure for $a=0.5-0.6$ and for a rigid rod $a=1.8$.

2.2.3 Apparatus

2.2.3.1 Ostwald U-tube capillary

This records the flow times under gravity alone (see for instance Harding, 1997; Serdyuk *et al.* 2007). Temperature control is crucial, and in this study the temperature was controlled through a Schott-Geräte water bath (Schott/SI analytics GmbH, Germany) supplemented by a coolant system (Haake, Thermo Fisher Scientific, MA, USA).

The Ostwald viscometers (of capacity 1-2ml) were obtained from (Schott/SI analytics GmbH, Germany).

2.3 Refractometry

Concentrations of macromolecular solutions in this study were determined by the Jencons Atago differential refractometer. Light is refracted by a solution depending on the concentration, c (g/ml) and the specific refractive increment dn/dc (ml/g). Theisen *et al.* (2000) give an extensive list of dn/dc values, and this list was used in this study.

After the reference solvent was measured, 2ml of a macromolecular solution was injected and a "BRIX" (% w/v) value obtained, relative to a sucrose calibration solution of known dn/dc (0.150 ml/g) at 20.0°C) Concentrations were converted to mg/ml using the relation

$$conc, = BRIX(\%) * \left(\frac{(dn/dc)_{molecule}}{(dn/dc)_{sucrose}} \right) * 10 \left(\frac{mg}{ml} \right) \quad (2.24)$$

2.4 Dynamic Light Scattering (DLS)

Dynamic light scattering was employed to assess the wider distribution of sizes: in many cases, besides forming solutions, larger supramolecular particles were present, and, unlike the analytical ultracentrifuge, DLS provided a convenient way of exploring the complete size range.

The movement of larger sized particles is slower than particles of small size: this means the diffusion coefficients will be smaller for larger particles (see equation 2.4). Dynamic light scattering provides a very rapid and useful indicator of the size distribution. It is based on the fact that laser light impinging on a solution of particles will be scattered by the particles – the scattered intensity will fluctuate because of the Brownian motion of the particles – small particles moving or diffusing faster than the larger, slower diffusing particles. The intensity will fluctuate with time, and a DLS instrument records these fluctuations and “correlates” the changes in intensity with time.

By measuring how a “correlation function” $g^{(2)}(t)$ (or how the logarithm of $g^{(2)}(t)$ changes with time t), the translational diffusion coefficient D can be estimated and from that the hydrodynamic or “Stokes” radius r_h using equation 2.4. An excellent description of the process applied to polysaccharides is given in (Burchard, 1992).

Current software based on the CONTIN (Provencher, 1992) and similar algorithms generates a distribution of diffusion coefficient (and corresponding distribution of size $g(r_h)$ vs r_h) similar to how SEDFIT produces a distribution of sedimentation coefficients. DLS, similar to all light scattering techniques, is extremely sensitive to the presence of dust and contaminants, so vessels have to be scrupulously clean, and solutions filtered through $0.45\mu\text{m}$ Millipore filters (or pore sizes less than that) before meaningful results can be obtained (Serdyuk *et al*, 2007).

2.5 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectrometry (FTIR) is a very versatile tool and can be used to detect glycan (polysaccharide) and glycan related materials, by-products of sample degradation and additives. It provides a spectrum of reflection of the bands of the functional groups of both the inorganic and organic substances, for which it is possible to perform an identification of the materials (Gajjar *et al.*, 2013). The equipment is equipped with a probe with fibre optics which allows the direct analysis of the surface of the object of study.

FTIR is one of the most popular tools in liquid analysis laboratories (Mayers *et al.*, 2013, Gajjar, *et al.*, 2013).

The basic principle behind molecular spectrometry is that molecules absorb energy from light at specific wavelengths, known as their resonance (vibration) frequencies. The similarity of functional groups creates a problem with FTIR. For example, if a sample is analysed and an absorption of light is recorded in the region of 3600-3400 cm.

FTIR has a performance advantages over dispersive instrumentation due to the fact that FTIR has a system, which is called the interferometer, allowable entry larger than the gap spectral element dispersing detector with the same resolution. Therefore, Fourier spectrometer (FS) compared them with the gain in Aperture (Mayers *et al.*, 2013). This reduces the time of registration spectra, reduce the signal noise, improve resolution and reduce the spot size on the detector. The presence of a computer in the device allows, in addition to the calculation of the spectrum, produce transaction processing of the experimental material, manage and control the operation of the device.

One of the major limitations of the differential spectrum procedure is that it is often impractical to send a new sample to the used sample whenever such a test

is required. In order to make the results more precise, the new reference sample must not only be of the same type, brand and degree of viscosity, but must come from the same manufacturing batch.

The manufacturers of FTIR equipment are aware of this limitation and many have introduced the concept of self-reference in an effort to resolve this situation (Mayers *et al.*, 2013). Self-referencing consists of analysing a new sample of each type of lubricant in the laboratory and storing the spectrum of that new materials in a database, to be used as a reference for future samples whenever used samples are sent to their analysis by FTIR.

The basis of Fourier spectrometer is a Michelson interferometer, which uses a source of monochromatic light emission. One of the interferometer mirrors can move forward. Complex hardware that performs these operations is called a Fourier spectrometer (FS). It has usually addition of two-beam interferometer lighter, detector, reference system, amplifier, analog-to-digital converter and a computer (electronic computer) built into the device or installed in the computer centre. Difficulty in obtaining spectra on FS offset its advantages over other spectral instruments.

Chapter 3: Extraction, Isolation and Characterization of Protein-Polysaccharide Complexes from the Flowers, Pulps and Seeds of the *Cucurbita*: Butternut Squash (NJBT2), Zucchini (NJZI4) and Pumpkin (NJPN1)

3.1 Introduction

Natural products with therapeutic properties are considered to be one of the most important sources not only for nutrition but also in terms of the treatment of disease (Jacobo-Valenzuela *et al.*, 2011). For centuries traditional herbal medicine practices with natural products had been practised for healthcare application without any understanding of the functionality of these resources (Li *et al.*, 2004; Patel *et al.*, 2012). Since the time of the scientific and industrial revolutions however, there has been an increase in the knowledge and understanding of disease and its causes, and chemical drugs have come to be used as effective agents for managing a large range of diseases. Regardless of the benefits of using this type of treatment, such as the provision of pure substances and more effective elements, there has recently been increased attention focused on the use of natural plants as a substitute treatment or possible cure for many diseases. This has occurred for several reasons. For example, Rates (2001) has indicated the misuse of chemical drugs and the side effects of consuming this kind of treatment. In addition, Patel *et al.* (2012) have reported that use of naturally sourced treatments is recommended by The World Health Organization (WHO), as these may represent harmless components with highly effective properties, which are for use in oral drug delivery.

As it has been stressed throughout this thesis, *Diabetes Mellitus* (DM) is one of the diseases for which a cure has been investigated in terms of therapeutic elements obtained from plants, as the complications of this disease can result in a significant death rate (Li *et al.*, 2004). Patel *et al.* (2012) suggest that there are more than 780 plants which might contain bioactive components with the ability to treat DM (Park *et al.*, 2012). Therefore, fruits and vegetables which are rich in beneficial nutrients and have the bioactive materials which might eventually reduce the risk of DM have attracted the attention of many scientific researchers from a variety of fields and many of these species have been elucidated (Grover *et al.*, 2002; Kavishankar *et al.*, 2011; Bhushan *et al.*, 2010; Park *et al.*, 2012). In this Chapter the hydrodynamic characterization of the soluble protein-polysaccharide components of *Cucurbita* are described.

3.1.1 *Cucurbita* as hypoglycaemic active components

Diabetes Mellitus (DM) has been described by Yadav *et al.* (2009), as one of the leading causes of mortality. Consequently, it is imperative to find a solution to eliminating or controlling this disease as soon as means are available. Therefore, various studies have been conducted regarding natural plants and the bioactive substances within them which have therapeutic effects on DM (Rates, 2001). One of these plants is pumpkin, which offers one of the most critical health benefits protecting against DM (as will be discussed below), but at the time of writing, all the data and results that have been reported are still to be described as a primary study. Interestingly, all substances from the pumpkin, including polysaccharides, proteins and fatty acids, have been reported to be anti-diabetic. In this chapter, as a step in our understanding of these phenomena protein-polysaccharide complexes from pumpkins complex are investigated. Fatty acids will be considered in the following chapter (Chapter 4).

Extracts containing protein and polysaccharide from 3 species of *Cucurbita*, namely Butternut Squash (NJBT2), Zucchini (NJZI4) and Pumpkin (NJPN1) were isolated and characterised from the flowers (f), pulps (p) and seeds (s). The hydrodynamic methods of sedimentation velocity, sedimentation equilibrium, viscometry and dynamic light scattering are used to characterize the components that be primarily responsible for the *Cucurbita*'s hypoglycaemic effect.

3.2 Materials and Methods

3.2.1 Materials

Sodium chloride, potassium dihydrogen phosphate, disodium dihydrogen phosphate, all were purchased from Sigma. Samples of Butternut Squash (NJBT2), Zucchini (NJZI4) and Pumpkin (NJPN1) were purchased from the local market.

3.2.2 Preparation from *Cucurbita* flowers, pulps and seeds

The experimental model by Li *et al.*, (2005) for extraction protein-polysaccharides complex was used. The ripe fruits (NJBT2, NJZI4 and NJPN1) were cut into small pieces 1cm and dried at 45°C for 24 hours and the seeds were separated. The separated seeds were washed and left to dry for 5 days in 50°C. The dried flowers, pulps and seeds were then ground to a powder using electric grinder (Kenwood Blender (Model BL 650) then they have been weighted. The powders were dissolved in deionized water (1g/20 ml) at 45°C for 3 hours with constant stirring. The samples were centrifuged (Beckman centrifuge Model J2-21M) at 4800rpm for 25 min at 20°C. The supernatant were concentrated at 45°C for 17 hours. The concentrated samples were filtered using 90 mm filter paper. 95% chilled ethanol was added to the filtered contraction in the ration 1:3 (v/v). The samples were centrifuged twice at 4800 rpm for 25 min at 4°C to get the pellet. Absolute ethanol was used to wash the pellet then freeze at -20°C. The samples were left for 24

hours at -20°C but they did not freeze it may due to the present of ethanol in the samples. The powder was thoroughly mixed, stored at 4°C , and used as the stock seed samples for further analyses.

3.2.3 Gel Permeation Chromatography (GPC)

For Gel Chromatography the methodology of (Jiwani, 2016) was followed. A column of Sephacryl 400 (2.1 x 50cm) was prepared using 0.02M EDTA 0.1M sodium acetate pH 6.5 buffer. 0.7ml/min was adjusted as flow rate. The column in this experiment was packed based on the available literature (Seymour and Harding, 1987). Approximately 50mg/ml of each protein-polysaccharide solution was loaded onto the column and 2.5 ml of fractions were collected. The eluent was tested for the presence of protein-polysaccharide using the Biuret assay and spectrophotometry. Fractions rich in protein-polysaccharides were selected for further analysis.

3.2.4 Spectrophotometry of *Cucurbita*

Fractioned samples from 3.2.3. were collected and an Ultrospec 2100Pro spectrophotometer used to determine the uv absorbance characteristics. The buffer (EDTA and acetate acid) was used as the blank sample. A series of fractions (~2-102) were examined at absorbances of 280nm and 230nm for detecting protein (absorbance from tyrosine and tryptophan for 280nm, and the peptide bond for 230nm).

3.2.5 The Phenol Sulphuric Acid Test for polysaccharides

Fractionated samples were tested using 1.0 ml 2.5% phenol and 2.5ml sulphuric acid. A golden brown colour indicates the presence of polysaccharides and this was visibly observed between fraction numbers 80 to 99. The absorbance of fractionated samples from fraction number 75-102 were measured at a wavelength of 490nm.

3.2.6 The Biuret Test for proteins

2 ml of the fractions were added to a dry test tube. 2 ml of the Biuret Test Reagent were added to three tubes. Distilled water was added as a negative control in the first tube. Samples were added to the second tube. Albumin was added as a positive control in the third tube. The samples were left to allow the mixture to stand for 5 min. The colour changes were observed.

3.2.7 Refractometry

BioDesignDialysis Tubing (D006) (Spectra, MWCO: 350) was pre-wetted with 0.1 M PBS buffer. The sample was then loaded into the dialysis tubing and dialysed against the buffer which was changed every 5 hours. The sample of NJBT2 was dialysed overnight against 0.1 M PBS buffer. Refractometry using an Atago (Fairfax, Canada) DD-5 refractometer was used to determine the concentration of dialysed samples by first of all injecting the buffer into both blank and sample cells. The instrument had previously been calibrated with standard glucose solutions. The indicator was set for zero-ing the instrument, and then sample was injected into the sample cell. A refractive index increment (dn/dc) = 0.150 ml/g

(Alfawaz, 2004) was used. Measurements were made on solutions in the concentration range 0.5-2.5 mg/ml. Measurements were repeated for all samples.

3.2.8 Sedimentation velocity of *Cucurbita* in the analytical ultracentrifuge

Sedimentation velocity experiments were carried out using a Beckman instruments (Palo Alto, California, U.S.A.) Optima XL-I ultracentrifuge. Samples (~395 μ l) with the concentration range 0.25, 0.50, 1.00, 1.25, 2.50, and 3.00 mg/ml and PBS buffer dialysate (400 μ l) at pH 7.0 and ionic strength 0.1M were injected into sample and reference channels respectively of double sector 12 mm optical path length cells. The balanced cells were then loaded into an analytical 8-hole titanium rotor An50-Ti and placed in AUC. The Rayleigh interference and absorbance optics systems were used for recording concentration profiles and the movement of the sedimentation boundary in the analytical ultracentrifuge cell (see e.g. Harding, 2005). A rotor speed of 40000 rpm was selected. The standard conditions of density and viscosity of water at 20⁰C were used for adjustment of sedimentation coefficients to $s_{20,w}^0$ (Schachman, 1992). Scans were taken at 2 min intervals for a run time of ~ 24 hours. The data was analysed using the "least squares ls-g*(s) model" SEDFIT algorithm in terms of distributions of sedimentation coefficient distribution g(s) vs s (see e.g. Dam and Schuck, 2003; Harding, 2005) to provide an assessment of sample polydispersity. The data was also analysed using the continuous distribution c(s) Lamm equation model of SEDFIT c(s) vs s. This is a modified form of g(s) vs s, that partially corrects for diffusive broadening (Dam and Schuck, 2003; Harding, 2005).

3.2.9 Sedimentation equilibrium of *Cucurbita* in the analytical ultracentrifuge

Sedimentation equilibrium experiments were also carried out using the Beckman Optima XL-I ultracentrifuge. Samples ($\sim 80\mu\text{l}$) at the concentration range 0.25, 0.50, 1.00, 1.25, 2.50, and 3.00 mg/ml and PBS buffer dialysate ($90\mu\text{l}$) at pH 7.0 and ionic strength 0.1M were injected into sample and reference channels respectively of double sector 12 mm optical path length cells. The balanced cells were then loaded into an analytical 8-hole titanium rotor An50-Ti and placed in AUC. The Rayleigh interference optical system was used for recording concentration profiles and the movement of the sedimentation boundary in the analytical ultracentrifuge cell (see e.g. Harding, 2005). A rotor speed of 10000 rpm was selected. Scans were taken at 2 min intervals for a run time of ~ 72 hours. The data was analysed using the SEDFIT- MSTAR program (Schuck *et al.*, 2014) to obtain weight average molecular weights, M_w , as explained in Chapter 2.

3.2.10 Capillary viscometry of *Cucurbita*

The reduced viscosity of a series solutions ranging in concentration 0.50-1.30 mg.ml⁻¹ were measured using an Ostwald viscometer (Chapter 2). The U-tube viscometer was suspended in an accurate temperature regulated water bath. The temperature was kept constant at $(20.00\pm 0.05)^\circ\text{C}$ throughout. The amount of each solution injected into the viscometer was 2ml and the solution flow time t determined manually (particularly for highly viscous solution) using a precision clock timer. From the flow time of the buffer (t_0) the ratio t/t_0 of the solution was estimated. Because of the low concentrations no density correction was necessary (Harding, 1997).

3.2.11 Dynamic light scattering (DLS) of *Cucurbita*

The hydrodynamic radius r_h of samples was determined by Dynamic light scattering (DLS) (see e.g. Harding *et al.*, 1992) using the Zetasizer Nano-ZS apparatus (Malvern, UK). A stock solution of each sample was prepared in 20 ml PBS at concentrations of ~ 2.5 mg/ml. All solutions were made up to have 0.1M salt content and to be pH 7. All the solutions were centrifuged for 20 min to remove all impurities and air bubbles then filtered using a 0.45 μm pore size sterile filter. 1 ml of solution was placed in a glass cuvette which was then placed in a temperature controlled sample environment. The translational diffusion coefficient D was measured at an angle of 13° (low enough so that rotational effects were negligible) at temperatures of 20.0°C . Five measurements, consisting of 60 sub-runs each, were made for each sample. The hydrodynamic radius r_h was then obtained from D according to the following Stokes-Einstein equation (see Chapter 2 equation (2.4)).

3.2.12 Fourier Transform Infrared Spectroscopy (FTIR) of *Cucurbita*

The dry samples were prepared as described in section 3.2.2. Characterization was carried out using FTIR (IRAffinity-1S, Shimadzu, Japan), scanning range was 4500 to 400cm^{-1} with a resolution of 4cm^{-1} and 20 replicate spectra were obtained for each sample. The shifts in the respective IR spectra bands were observed and reported, as described by (Mayers *et al.*, 2013). FTIR analysis of functional groups were analysed.

3.3 Results

3.3.1 Spectrophotometry of *Cucurbita* and the Biuret test for proteins from NJBT2

UV absorbance profiles for the extracted substances in fractions numbered 80-120 from Butternut squash from the seeds, NJBT2-s and pulps, NJBT2-p are shown in (Fig. 3.1) and pulps (Fig. 3.2) respectively.

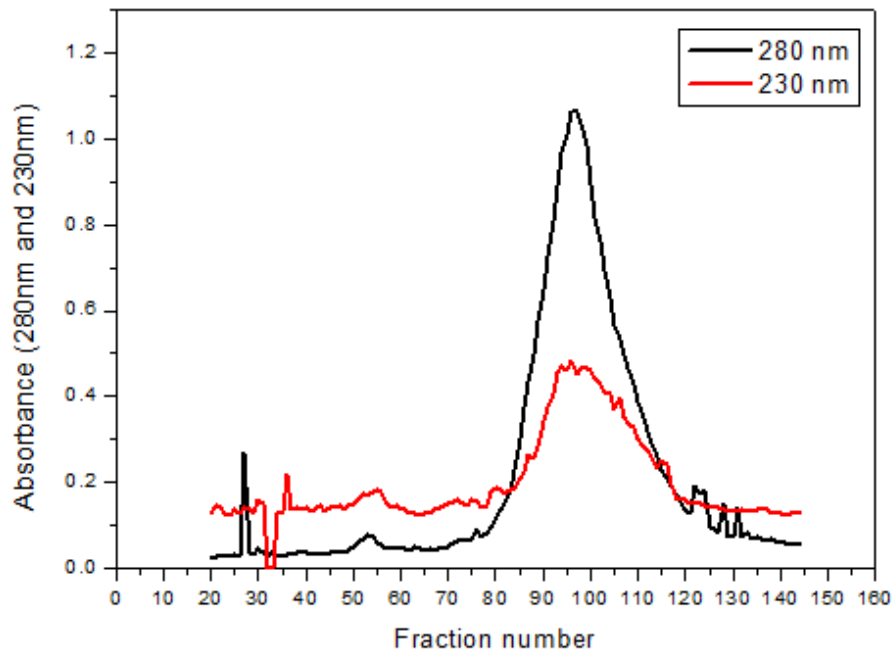


Figure 3.1: UV absorbance profiles at 280nm (detecting aromatic residues) and 230 nm (detecting the peptide bond) for extracts from seeds of Butternut squash, NJBT2 -s.

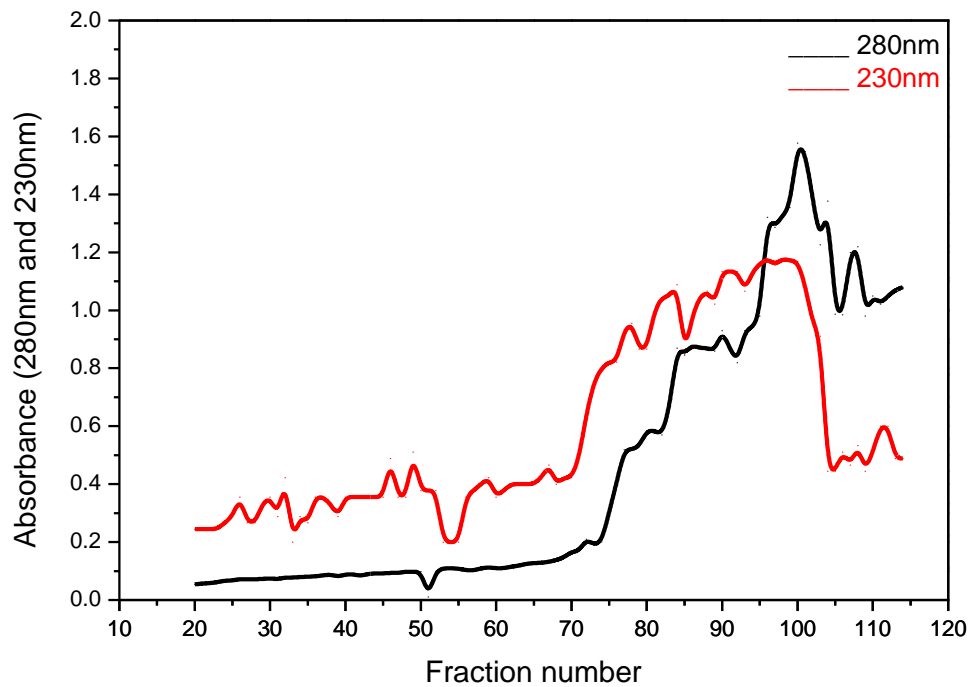


Figure 3.2: UV absorbance profiles at 280nm (detecting aromatic residues) and 230 nm (detecting the peptide bond) for extracts from pulps of Butternut squash, NJBT2-p.

The 230nm detection of the peptide bond is problematic due to the contributions of the amino acid side chains and buffer salts. A more reliable method is the Biuret method and Fig. 3.3 shows the Biuret method to determine the presence of peptide bonds in protein. From the left Biuret Reagent with de-ionized water (negative control), Biuret Reagent with NJBT2 and Biuret Reagent with BSA (positive control).

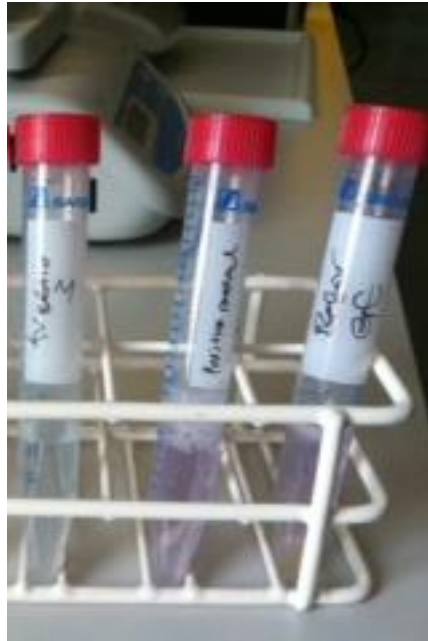


Figure 3.3: From the left Biuret Reagent with de-ionized water (negative control), Biuret Reagent with NJBT2 and Biuret Reagent with BSA (positive control).

Table 3.2: Biuret determination of the presence of peptide bonds in protein.

| Observations | Interpretation |
|--|-------------------------------|
| No change | Proteins are not present |
| The solution turns from blue to violet | (purple) Proteins are present |
| The solution turns from blue to pink | Peptides are present |

3.3.2 Sedimentation velocity: use of both refractometric (interference) and uv-absorption optics

Fig. 3.4 shows the sedimentation coefficient distribution $c(s)$ plotted versus s from SEDFIT for NJBT2-f. Fig. 3.4 is an example for the use of both interference optics

(which detects protein and polysaccharide present) and absorbance optical records at a wavelength of 280nm (which detects only the protein component) for 3 concentrations (2.0, 1.5 and 1.0 mg/ml). Interestingly, the two optical systems give corresponding profiles for each concentration, but the absorption profiles are broader. This suggests that the polysaccharide (if present) and protein components are not sedimenting independently (i.e. they may be complexed). The broader profiles from absorption maybe a feature of the much longer scan times required (uv absorption – a few minutes; interference – instantaneous). These plots also illustrate how heterogeneous the macromolecular extracts from the *Cucurbita* are.

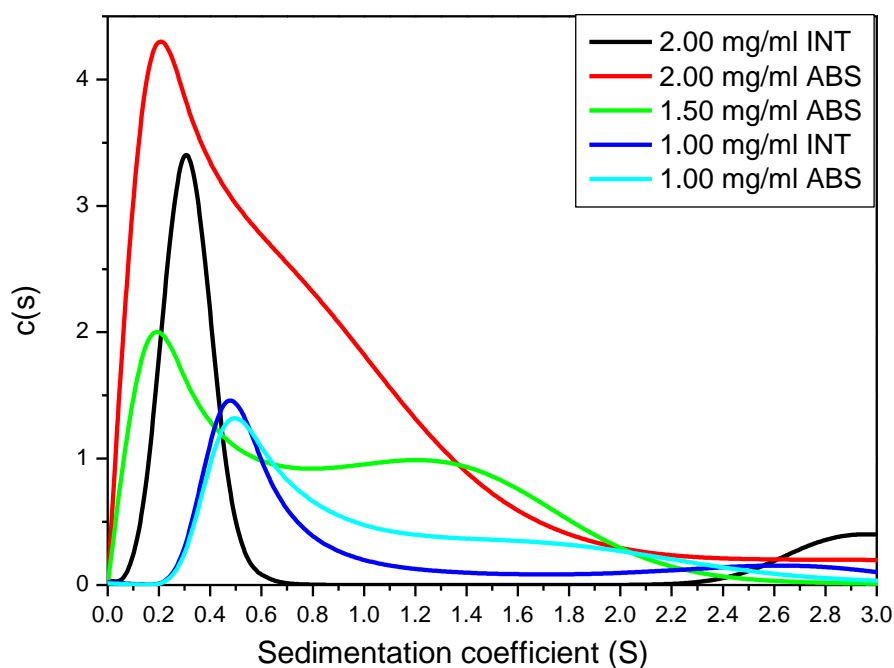


Figure 3.4: Sedimentation velocity of NJBT2-f. Sedimentation coefficient distribution plots $c(s)$ versus s for 5 loading concentrations. The plot compares profiles from absorption at 280nm (which detects protein) and interference (which detects protein and polysaccharide), for 3 concentrations. NB. The “concentrations” are nominal concentrations and the profiles correspond to the soluble parts.

We now compare interference and absorption optical sedimentation coefficient distribution plots for the various cucurbit extracts. Due to problems of solubility it was not possible to compare all the samples: this was true of all the hydrodynamic measurements with the exception of dynamic light scattering which was suitable for analysis of the very large supramolecular particles (section 3.3.9 below).

3.3.3 Sedimentation velocity in the analytical ultracentrifuge of flowers from *Cucurbita*

The sedimentation coefficient $c(s)$ versus s distributions for NJBT2-f for 6 concentrations are now shown separately (for clarity) for each optical system in Fig. 3.5 and Fig. 3.6. For further comparison the least squares sedimentation coefficient distribution plots, $ls-g^*(s)$ vs s (see for example, Fig. 3.7 from NJBT2-f), confirmed the heterogeneity of these samples.

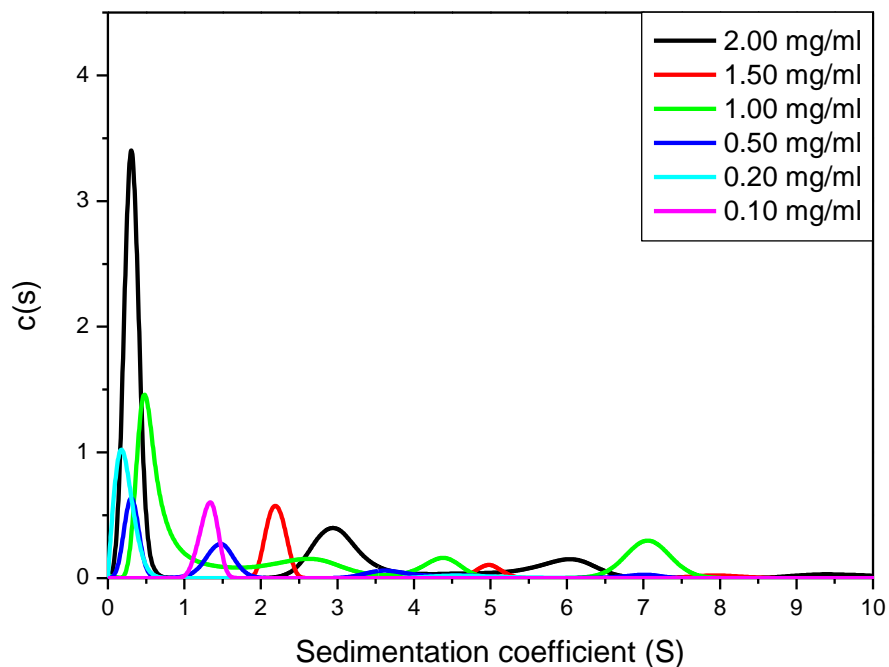


Figure 3.5: Sedimentation velocity sedimentation coefficient $c(s)$ vs s distribution plots obtained with interference optical for Butternut Squash flowers (NJBT2 – f).

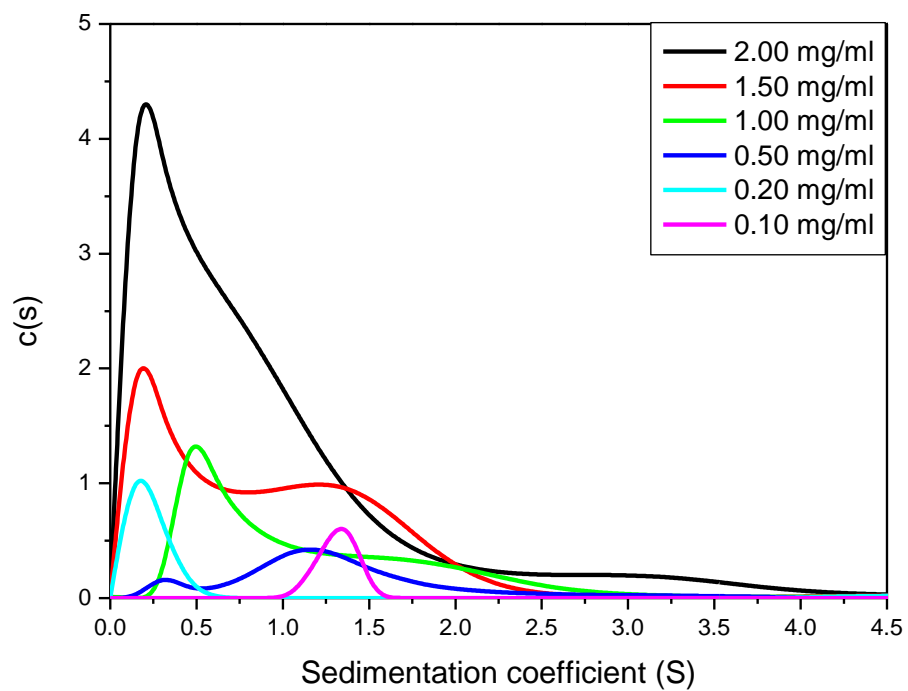


Figure 3.6: Sedimentation velocity $c(s)$ vs s distribution plots with absorbance optics of extracts from the flowers of Butternut Squash (NJBT2-f).

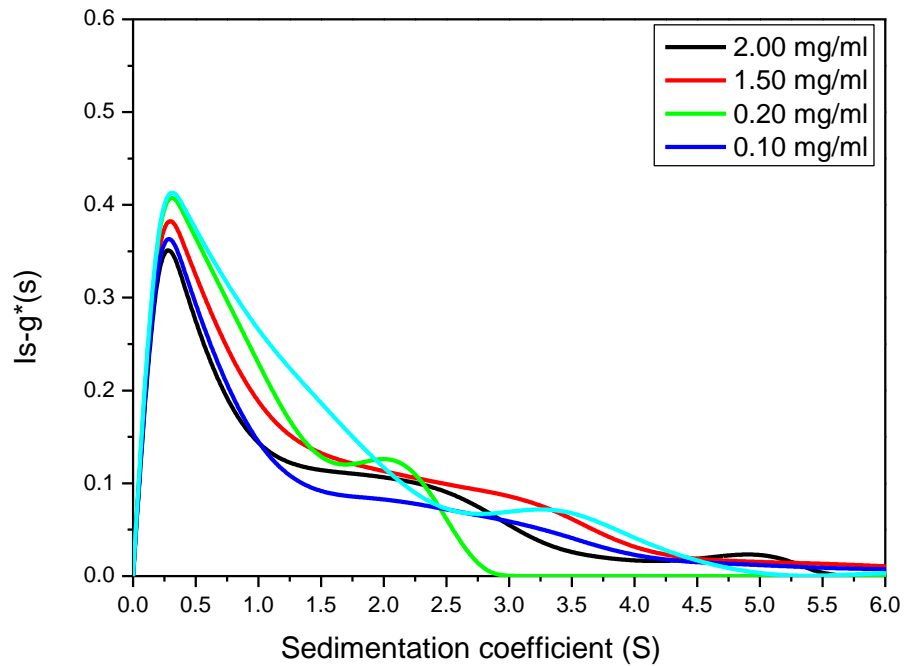


Figure 3.7: Sedimentation velocity $g(s)$ vs s distribution plots from the interference optical system for extracts from the flowers of Butternut Squash (NJBT2-f).

3.3.4 Sedimentation velocity of pulps from *Cucurbita*

The sedimentation coefficient $c(s)$ versus s distributions at the different concentrations are shown in Fig. 3.8 for interference optics for NJBT2-p, again showing large heterogeneity.

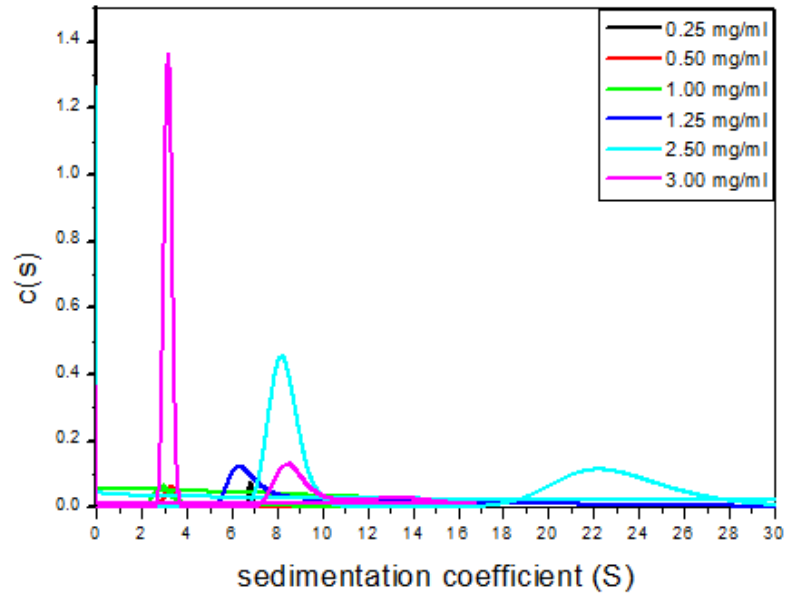


Figure 3.8: Sedimentation velocity with interference optics for NJBT2-p. Sedimentation coefficient distribution plots, $c(s)$ versus s from SEDFIT.

Fig. 3.9 shows the $c(s)$ vs s interference optics data for NJZI4. Similar to Butternut squash the Zucchini extract shows a greater proportion of the lower $-s$ species, and this is confirmed by the $g(s)$ vs s plot (Fig 3.10).

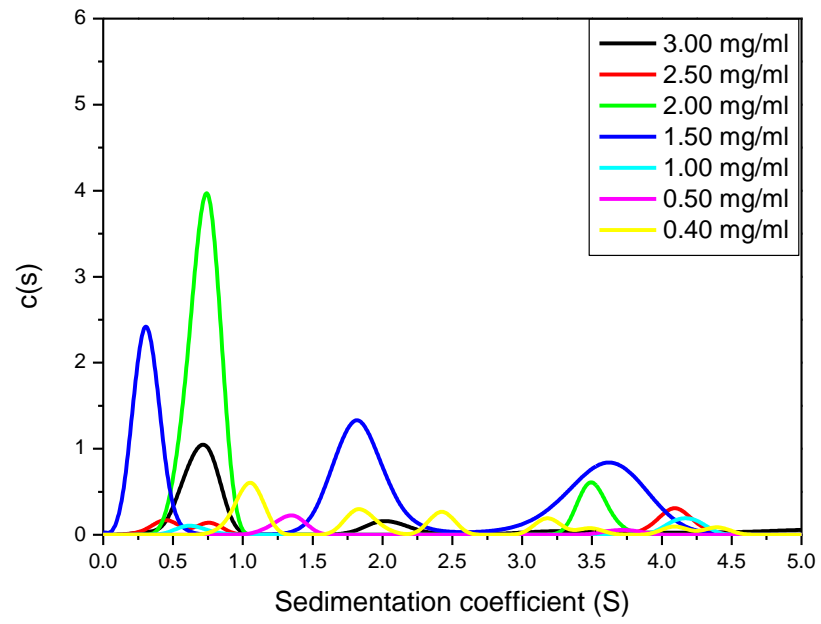


Figure 3.9: Sedimentation velocity with interference optics for Zucchini extract NJZI4-p. Sedimentation coefficient distribution plots, $c(s)$ versus s from SEDFIT.

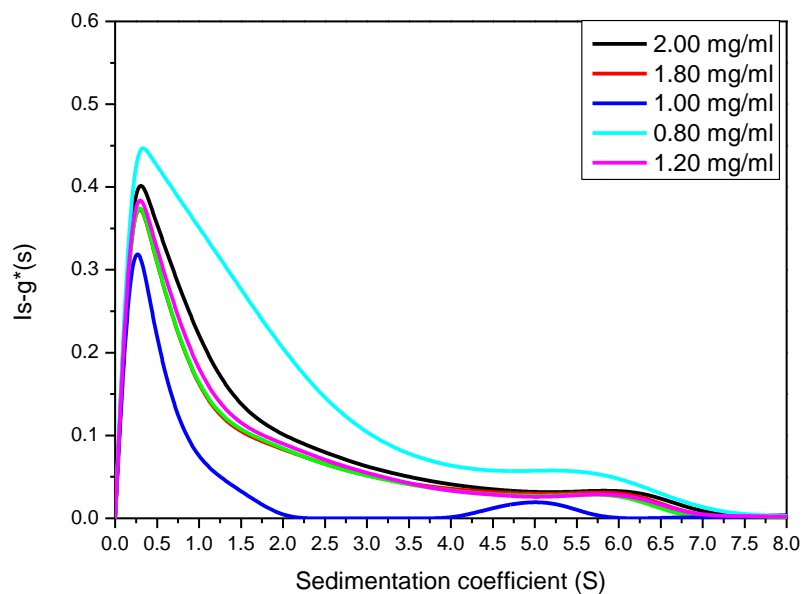


Figure 3.10: Sedimentation velocity with interference optical of NJZI4. Sedimentation coefficient distribution plots, $g(s)$ versus s from SEDFIT for NJZI4-p.

3.3.5 Sedimentation velocity of seeds from *Cucurbita* in the analytical ultracentrifuge.

The sedimentation coefficient $c(s)$ versus s distributions at the different concentrations for pumpkin NJPN1-s are shown in Fig. 3.11 using interference optics and Fig. 3.12 with uv-absorption optics.

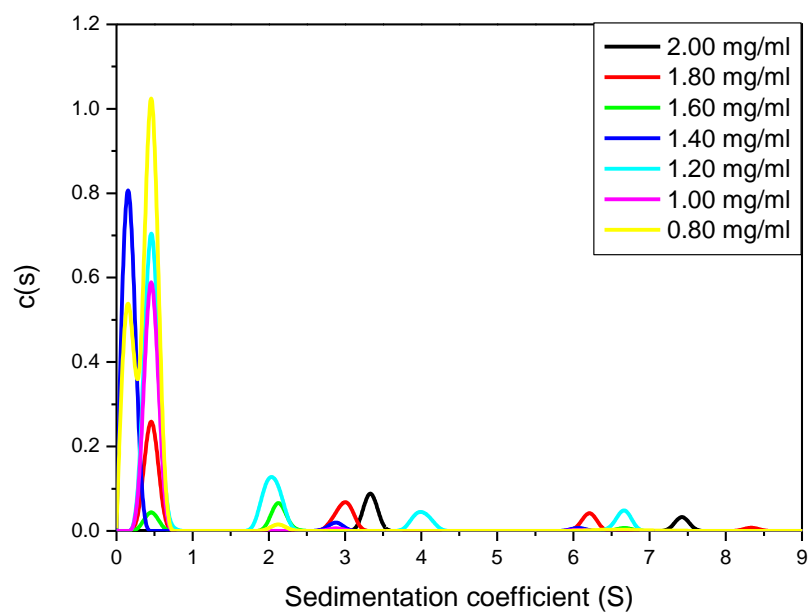


Figure 3.11: Sedimentation coefficient distribution plots (AUC with interference optics), $c(s)$ versus s from SEDFIT for NJPN1-s.

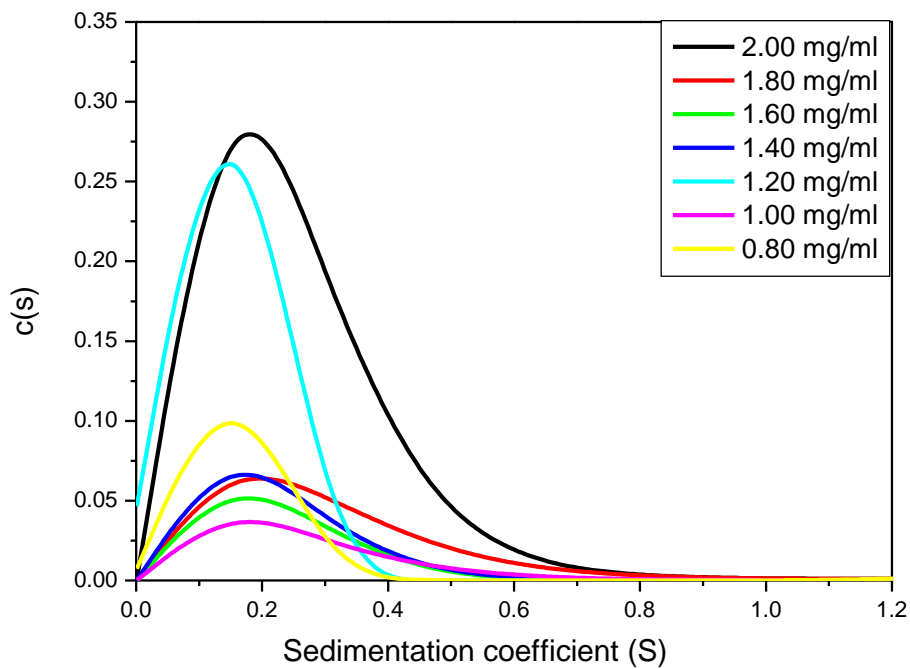


Figure 3.12: Sedimentation velocity with absorbance optics of protein NJPN1-s. Sedimentation coefficient distribution plots, $c(s)$ versus s from SEDFIT.

3.3.6 Comparison of sedimentation coefficients for the main components in each extract.

We now consider a comparison of the sedimentation coefficients of the main components from each of the extracts (and again using both interference and absorption optics). To facilitate the comparison we follow the normal convention (see Harding, 2005) of standardizing to $s_{20,w}$ values (normalising to the density and viscosity of water at 20.0°C), and also to eliminate or minimize the effects of non-ideality (through co-exclusion, charge or backflow effects) by extrapolating $s_{20,w}$ values to zero concentration (to give $s_{20,w}^0$ values) or if this is not possible, to take the $s_{20,w}$ value at a low concentration and approximate $s_{20,w}^0 = s_{20,w}$. For the extrapolation, we chose either $s_{20,w}$ vs c or a reciprocal plot $1/s_{20,w}$ vs c , depending on which gives the better fit to the data.

Figs. 3.13 – Fig 3.16 gives some examples.

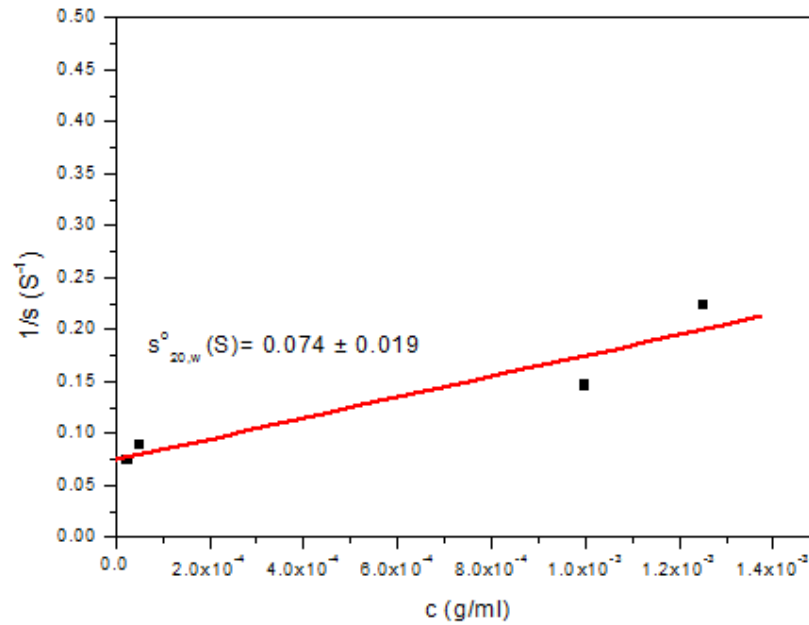


Figure 3.13: Concentration dependence of the reciprocal sedimentation coefficient (s) for NJBT2.

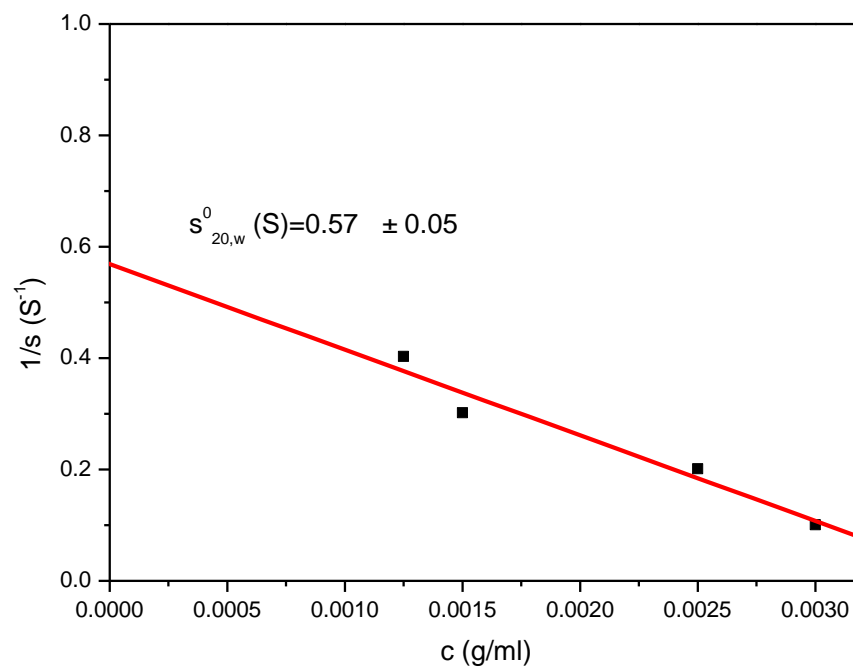


Figure 3.14: Concentration dependence of the sedimentation coefficient (s) for NJBT2-s using uv-absorbance.

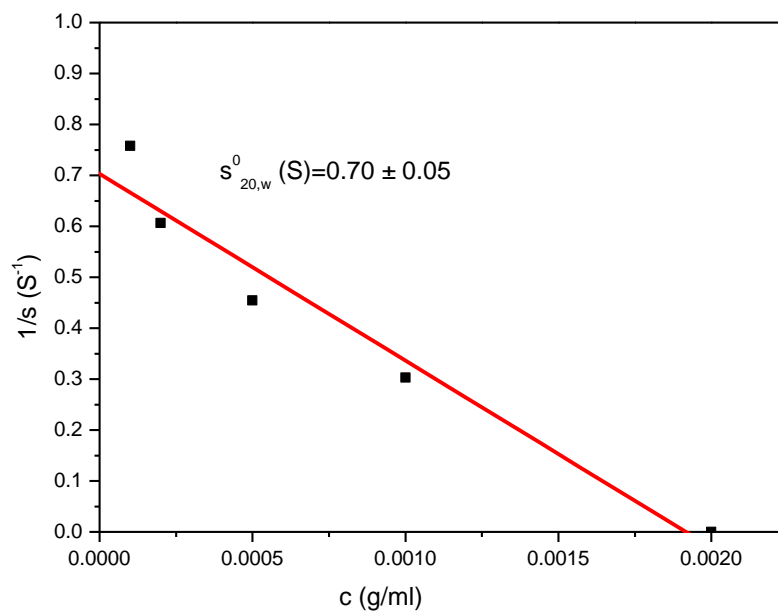


Figure 3.15: Concentration dependence of the sedimentation coefficient (s) for NJBT2-f, using uv-absorbance.

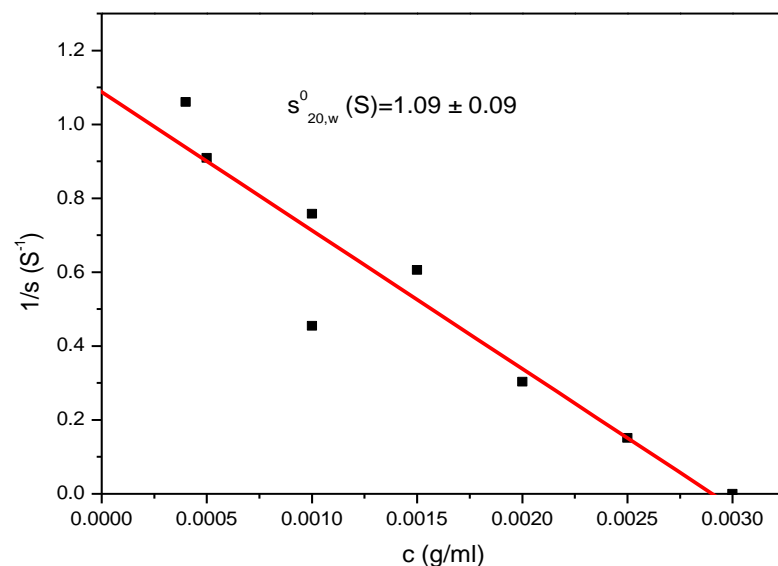


Figure 3.16: Concentration dependence of the sedimentation coefficient (s) for NJZI4.

Table 3.3 shows the summary of sedimentation coefficient, normalized to standard conditions – density and viscosity of water at 20.0°C ($s^{\circ}_{20,w}$) obtained from AUC for the *Cucurbita*. As explained above, not all values were available due to solubility issues or very high heterogeneity.

Table 3.3: Summary of sedimentation coefficients $s^{\circ}_{20,w}$ (in Svedbergs, S) obtained from AUC for the main components in the *Cucurbita* extracts. Due to solubility or high heterogeneity issues, not all samples could be evaluated.

| <i>Cucurbita</i> | $s^{\circ}_{20,w}$ (S) Interference optics | $s^{\circ}_{20,w}$ (S) Absorbance optics |
|------------------------|--|--|
| NJBT2-p pulps | (2.0±0.2) | ND |
| NJBT2-s seeds | ND | (0.6±0.1) |
| NJPN1-s seeds | (0.5±0.1) | (0.4±0.0) |
| NJZI4-p pulps | (1.1±0.1) | (0.4±0.0) |
| NJZI4-f flowers | (0.7±0.1) | (0.5±0.0) |

3.3.7 Sedimentation equilibrium in the analytical ultracentrifuge

The s (or $s_{20,w}^0$ values) depend on the conformation of the macromolecules as well as the molecular weight. Sedimentation equilibrium gives an absolute measure of molecular weight, in terms of the weight average molecular weight M_w . As with sedimentation velocity, measurements can be affected by thermodynamic non-ideality (through co-exclusion and charge repulsion effects), so what we actually measure (Chapter 2) is the apparent weight average molecular weight $M_{w,app}$. However by working at low concentration M_w is approximately $M_{w,app}$, i.e. $M_w \sim M_{w,app}$. Measurements were done by SEDFIT-MSTAR (Schuck *et al*, 2014) as described in Chapter 2. The results are given in Fig. 3.17, 3.18 and 3.19 for NJBT2-s, NJPN1-s and NJZI4-s respectively. Because of solubility issues we were only able to analyse one of the extracts for each.

The raw $c(r)$ data vs r in 3.17A, 3.18A and 3.19A show an upward curve $c(r)$ data vs r plot. In Fig. 3.17B, 3.18B and 3.19B log concentration $\ln c(r)$ vs r^2 plot show the best fit straight line (red line) with data. The M^* extrapolation to the base of the cell in Fig. 3.17C, 3.18C and 3.19C gives the weight average molecular weight $M_{w,app}$ over the whole distribution (red line arrow). The local apparent or point weight average molecular weight $M_{w,app}(r)$ at radial position r plotted against radial positions in Fig 3.17D and 3.19D. Because of the heterogeneity the estimated error in the values quoted are $\pm 10\%$. As stated above, because of the low loading concentrations used, non-ideality effects are assumed to negligible and $M_{w,app} = M_w$.

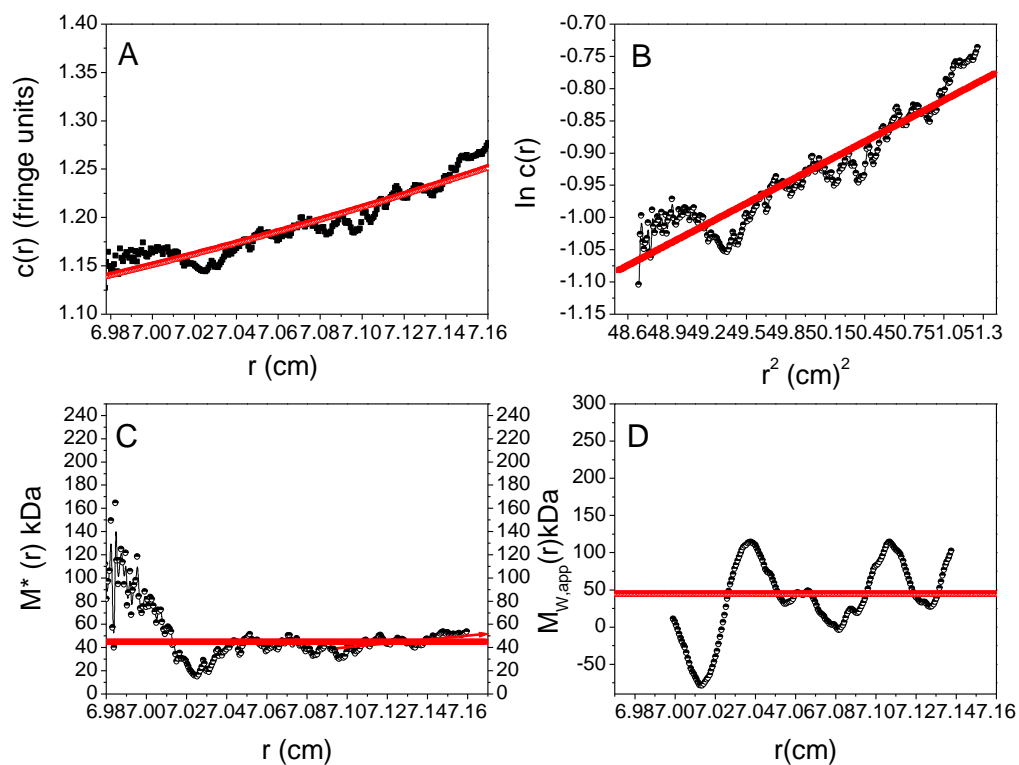


Figure 3.17: SEDFIT-MSTAR output for NJBT2. (A): the raw $c(r)$ data vs r (B): log concentration $\ln c(r)$ vs r^2 plot (C): M^* vs r plot with $M_{w,app} = (53 \pm 5)$ kDa. (D): local apparent or point weight average molecular weight $M_{w,app}(r)$ at radial position r plotted against concentration for different radial positions. The red line in all plots represents the fit.

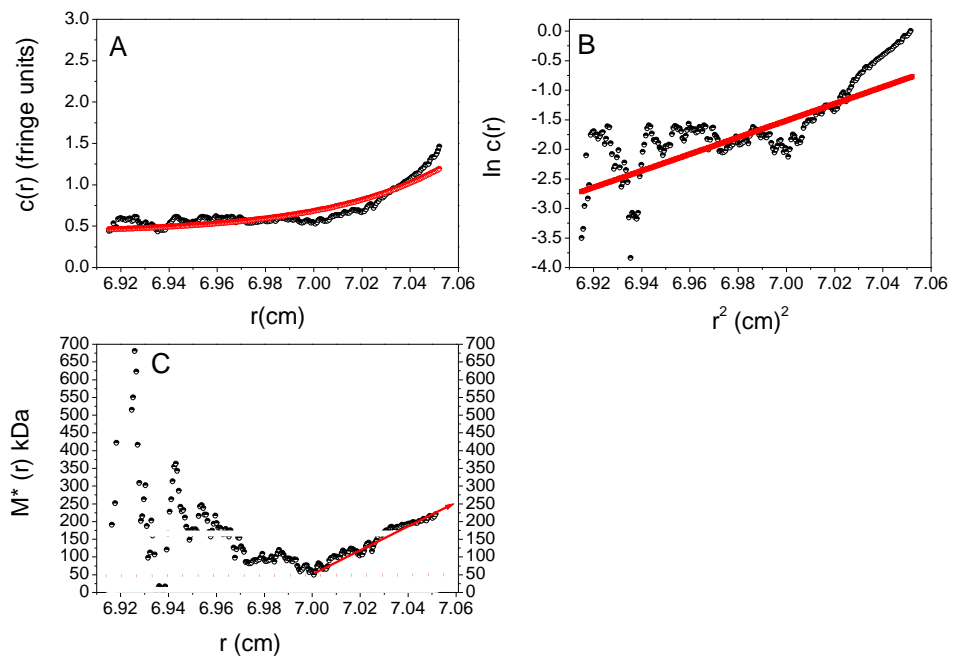


Figure 3.18: SEDFIT-MSTAR output for NJPN1 (A): the raw $c(r)$ data vs r (B): log concentration $\ln c(r)$ vs r^2 plot (C): M^* vs r plot with $M_{w,app} = (250 \pm 20)$ kDa. NB. If the upturn at higher radial positions is ignored (influenced by the very large supramolecular material) a value of $\sim (50 \pm 20)$ kDa is obtained.

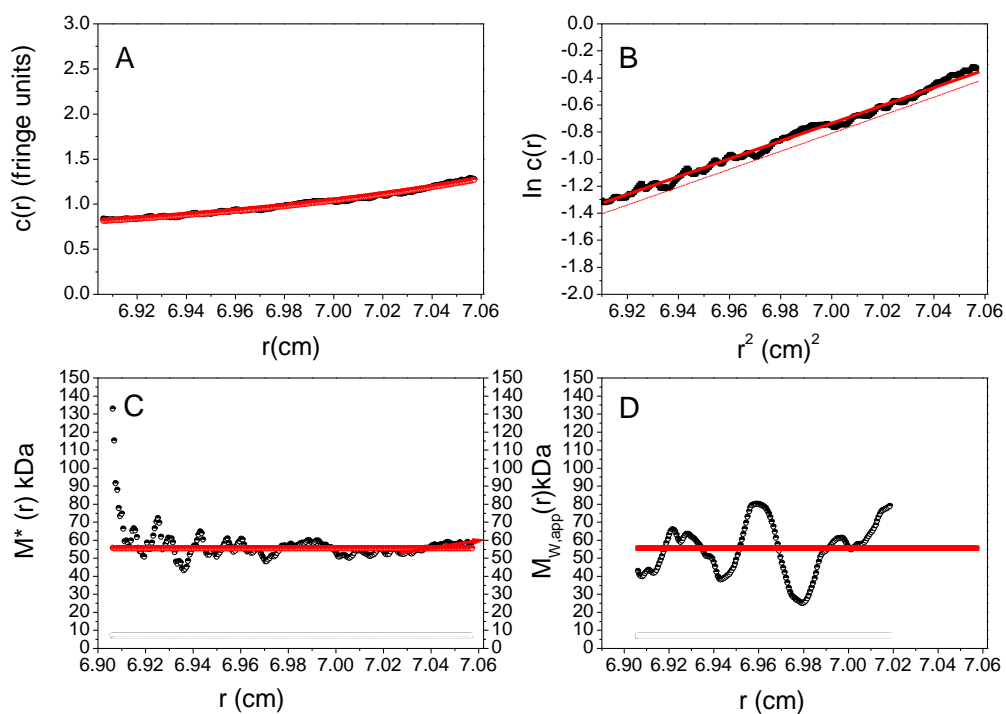


Figure 3.19: SEDFIT-MSTAR output for NJZI4 (A): the raw $c(r)$ data vs r (B): log concentration $\ln c(r)$ vs r^2 plot (C): M^* vs r plot, with $M_{w,app} = (60 \pm 6)$ kDa. (D): local apparent or point weight average molecular weight $M_{w,app}(r)$ at radial position r plotted against concentration for different radial positions. The red line in all plots represents the fit.

Table 3.4. shows the summary of molecular weight (kDa) obtained from sedimentation equilibrium AUC for NJBT2, NJPN1 and NJZI4.

Table 3.4: Summary of molecular weight (kDa) obtained from sedimentation equilibrium AUC for *Cucurbita*.

| <i>Cucurbita</i> | M _w kDa |
|------------------|--------------------|
| NJBT2-s | 53±5 |
| NJPN1-s | 250±20 50±20* |
| NJZI4-p | 60±6 |

*ignoring supramolecular material near the cell base

3.3.8 Capillary viscometry

Again, because of solubility issues, only one representative extract from each of the *Cucurbita* could be analysed. The Huggins and Kraemer extrapolations for the intrinsic viscosity $[\eta]$ are shown in Fig. 3.20 and 3.21 for NJBT2, and they extrapolate to a common intercept $[\eta] = (66.2 \pm 3.5)$ ml/g. It is possible also to comment on the approximate conformation of the monomer species. The

approximate aspect ratio of the monomeric species can be estimated however from both the sedimentation and viscosity data.

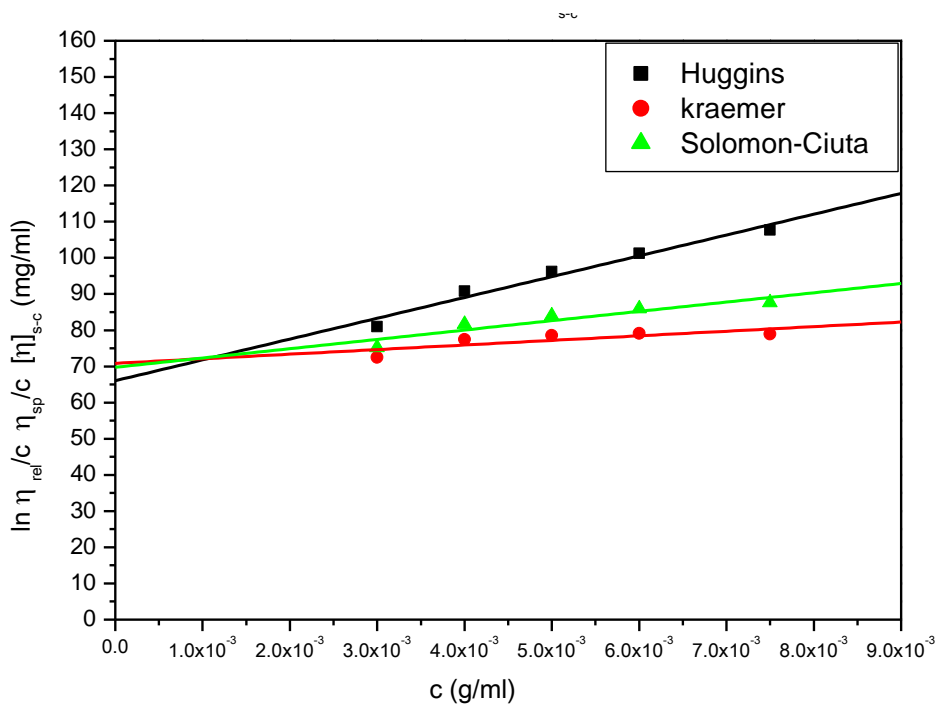


Figure 3.20: Plots of reduced viscosity (black data points) and inherent viscosity (red data points) from the rolling ball viscometer at 20°C for NJBT2-s.

Reduced viscosities fitted to the Huggins equation and inherent viscosities to the Kraemer equation, yielding an intrinsic viscosity $[\eta]$ of (66.20 ± 3.47) ml/g. (For interpretation of the references to colour in this figure legend).

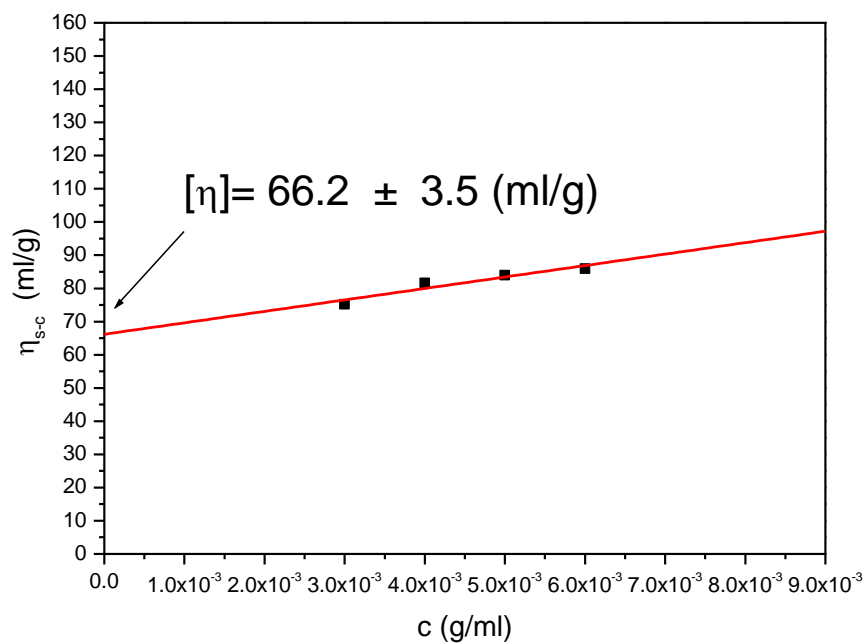


Figure 3.21: Intrinsic viscosity estimates at different concentrations for NJBT2-s.

The Huggins and Kraemer extrapolations for the intrinsic viscosity $[\eta]$ for NJZI4-p are shown in Fig. 3.22 and 3.23, and they extrapolate to a common intercept $[\eta] = (102.7 \pm 2.2)$ ml/g which is higher than the intrinsic viscosity from NJBT2-s.

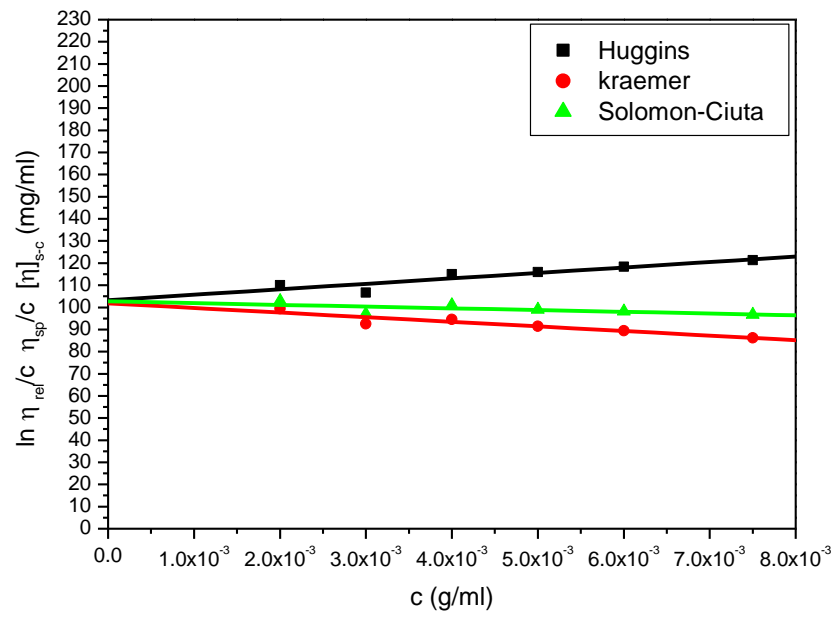


Figure 3.22: Plots of reduced viscosity (black data points) inherent viscosity (red data points) and Solomon-Ciuta (green) from the rolling ball viscometer at 20.0°C for NJZI4-p.

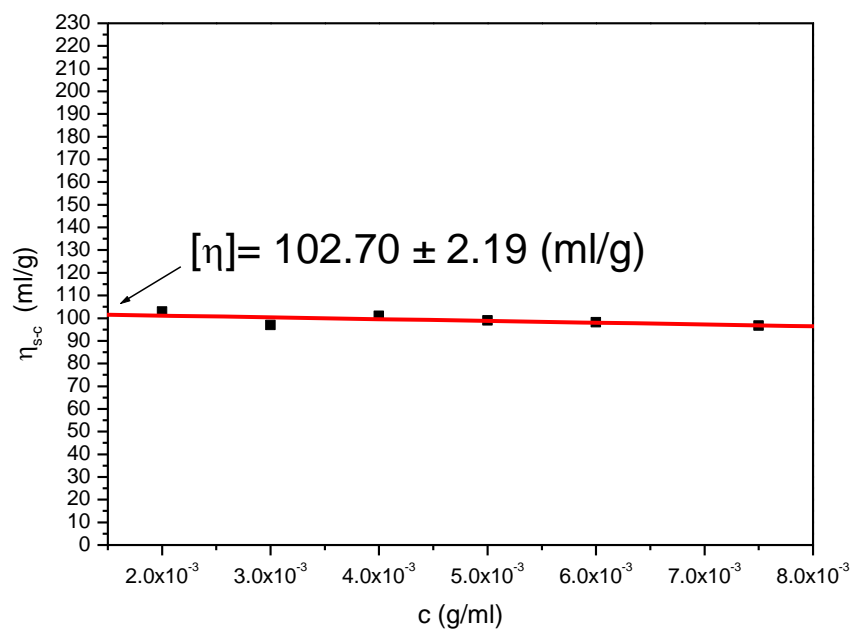


Figure 3.23: Reduced viscosity plot at different concentrations for NJZI4-p.

The Huggins and Kraemer extrapolations for the intrinsic viscosity $[\eta]$ for NJPN1-s are shown in Fig. 3.24 and 3.25, and they extrapolate to a common intercept $[\eta] = (99 \pm 11)$ ml/g.

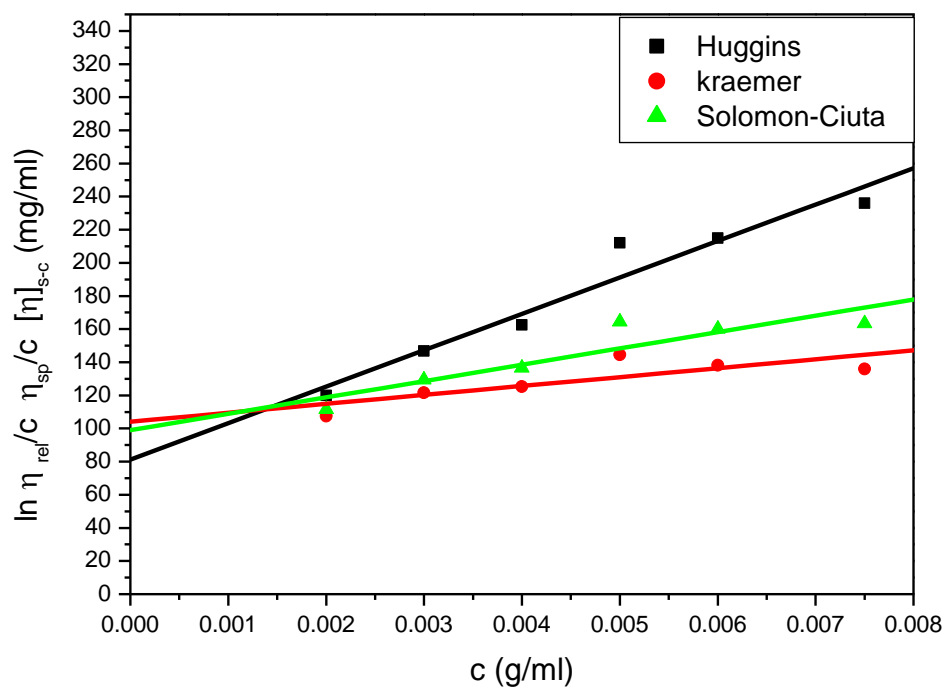


Figure 3.24: Plots of reduced viscosity (black data points) and inherent viscosity (red data points) from the rolling ball viscometer for NJPN1-s at 20.0°C.

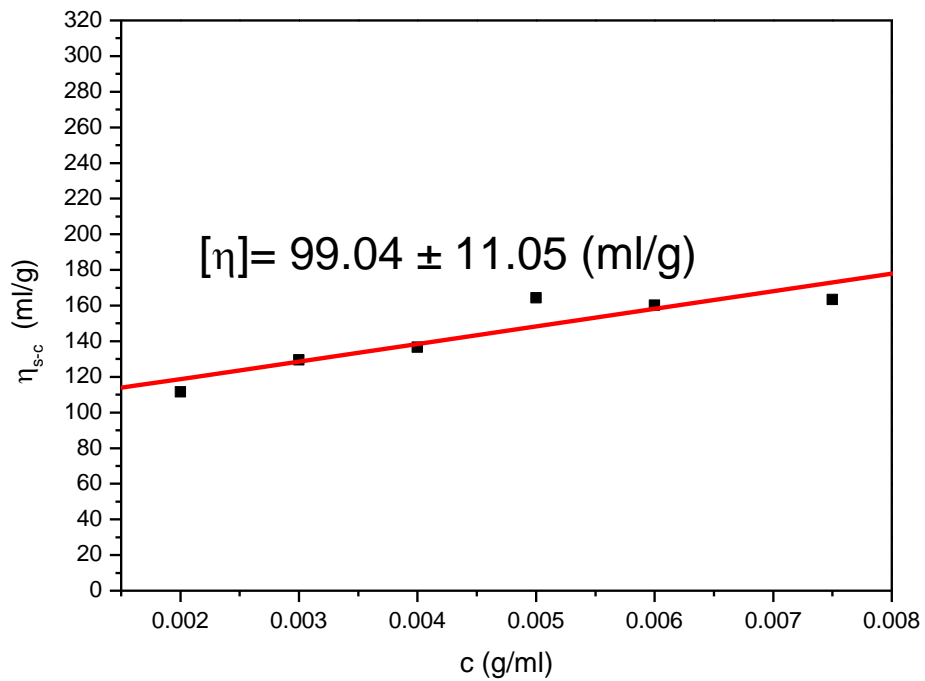


Figure 3.25: Intrinsic viscosity estimates at different concentrations for NJPN1.

Table 3.5 shows the summary of the intrinsic viscosity $[\eta]$ obtained from Capillary viscometry for *Cucurbita*. As it can be seen from the table the results of NJBT2 pulps, NJPN1 pulps and NJZI4 seeds were not obtained due to the lack resources of NJBT2 pulps and NJPN1 pulps and the difficulties of spreading the seed from the pulps in NJZI4.

Table 3.5: Summary of the intrinsic viscosity $[\eta]$ obtained from for *Cucurbita*

| <i>Cucurbita</i> Sample | $[\eta]$ (ml/g) |
|----------------------------|-----------------|
| NJBT2-s | 66 ± 4 |
| NJPN1-s | 99 ± 11 |
| NJZI4-p | 103 ± 2 |

3.3.9 Dynamic light scattering (DLS) of *Cucurbita*

Dynamic light scattering is sensitive to the large particles in a system, and this proved ideal for providing information on the supramolecular (larger than $r_h = 100\text{nm}$, where r is the hydrodynamic radius) parts of the extracts. The following two figures show the output from the Zetasizer NanoS 6.20 software (Malvern UK). The plot of volume % vs size r_h for seed NJBT2 at 13° shown in Fig. 3.26. Concentrations given are the loading concentrations. In Fig. 3.26 the corresponding (translational) z-average diffusion coefficient of NJBT2 $D_{20,w}$ is $(2.3 \pm 0.7) \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$.

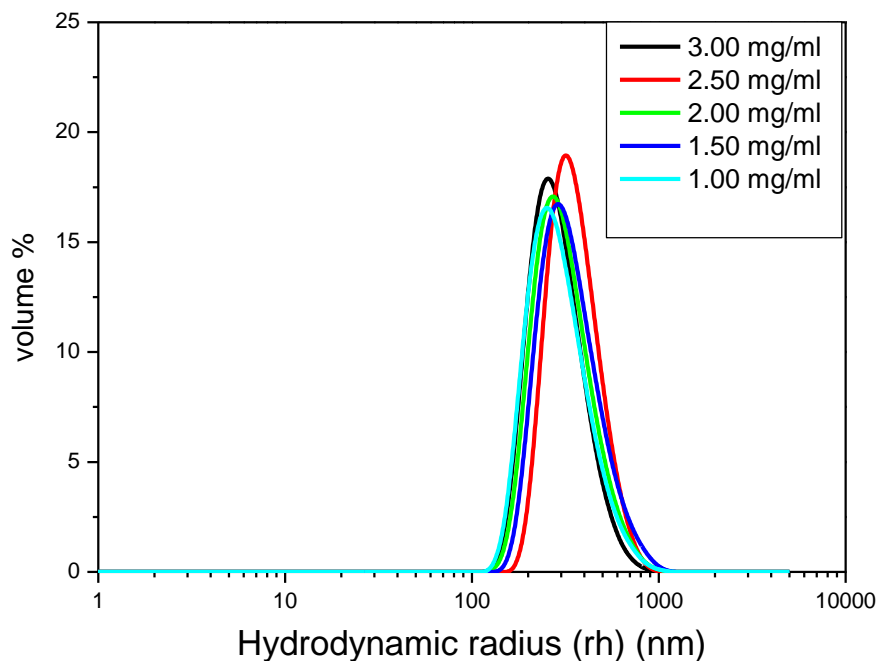


Figure 3.26: Plot of volume% vs size or hydrodynamic radius r . (nm) for seed NJBT2 at a scattering angle of 13° .

The plot of volume (%) vs size, r .(nm) for NJZI4 at a low scattering angle of 13° (to minimize complications through rotational diffusion effects) is shown in Fig. 3.27 which shows a similar Stoke radius $r_h \sim$ at 250 (nm), to that of NJBT2. For Fig. 3.28 the Diffusion coefficient of NJBT2-s is about $(2.3 \pm 0.7) \times 10^{-9} \text{cm}^2/\text{sec}^{-1}$. However, in Fig. 3.29 the Diffusion coefficient of NJZI4-p is about $(2.6 \pm 1.1) \times 10^{-8} \text{cm}^2/\text{sec}^{-1}$.

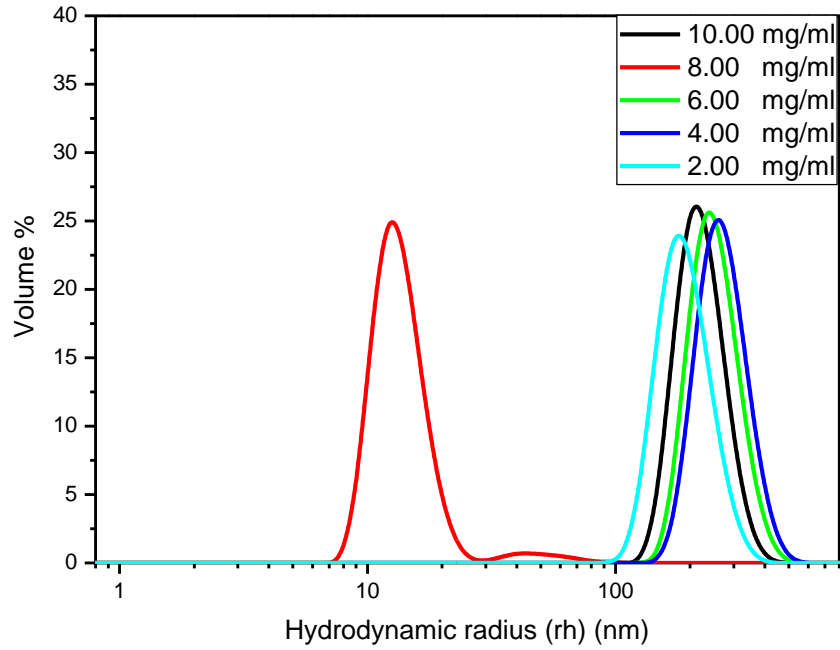


Figure 3.27: Plot of volume% vs size, r (nm) for NJZI4 at 13°.

Both figures show clearly the peaks at approximately the same diffusion coefficient, with the “molecular” component of $r_h \sim 12$ nm also appearing in Fig. 3.27.

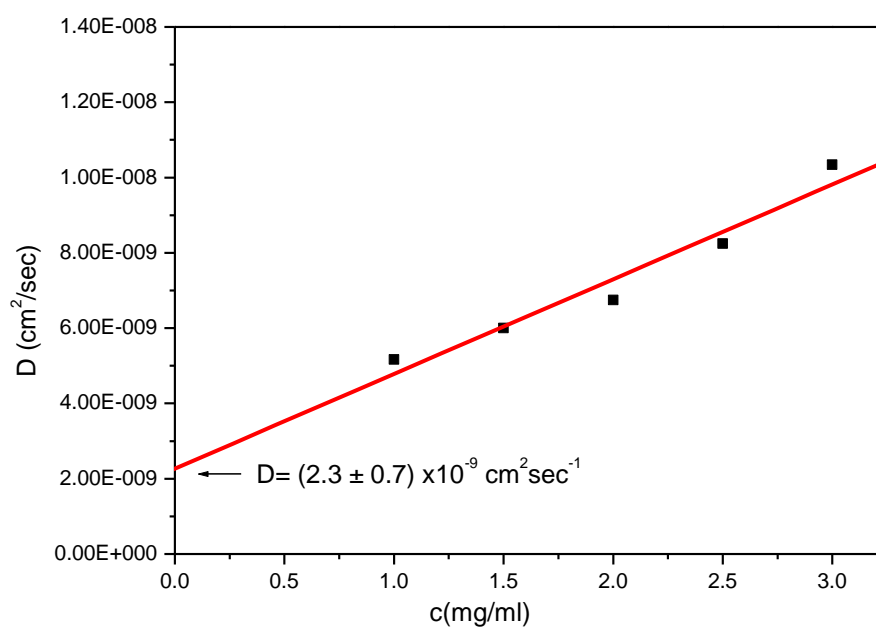


Figure 3.28: Diffusion coefficient of seed NJBT2-s at 13° .

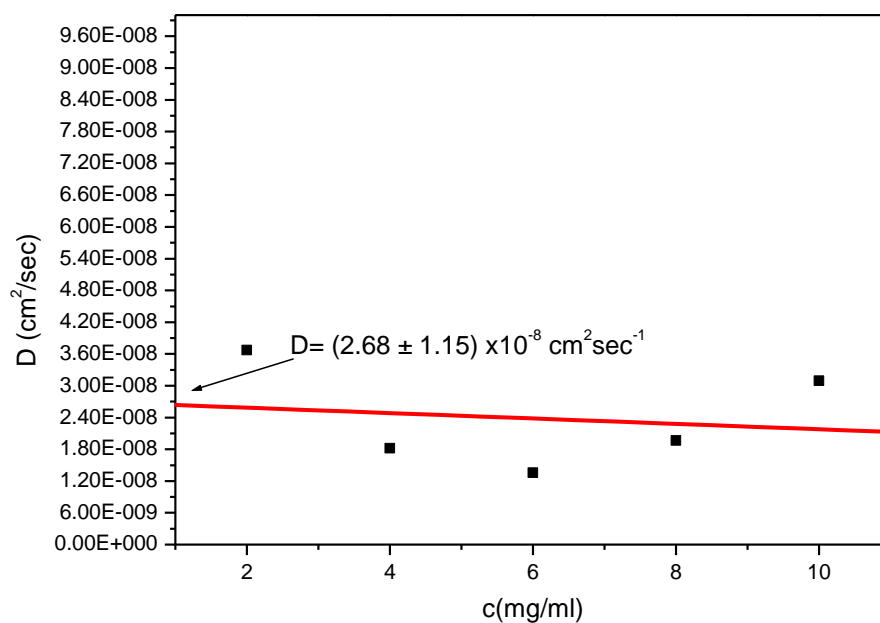


Figure 3.29: Diffusion coefficient of NJZI4-p at a scattering angle of 13° .

Table 3.6. shows the summary of sedimentation coefficient (s), molecular weight (kDa), Stoke radius r_h and the intrinsic viscosity $[\eta]$ obtained from DLS, AUC and viscometry for *Cucurbit* in this chapter. The DLS provides very much the information about the sizes of the supramolecular particles in the suspension, complementing the macromolecular information from ultracentrifugation and viscometry.

Table 3.6: Summary of sedimentation coefficient (s), molecular weight (kDa), the intrinsic viscosity $[\eta]$ (ml/g), Diffusion coefficient $D^{0}_{20,w}$ (cm²/sec) and Stoke radius r_h (nm) obtained from AUC, viscometry and DLS for *Cucurbit*.

| <i>Cucurbita</i> Sample | $s^{0}_{20,w}$ (S) Interference | $s^{0}_{20,w}$ (S) Absorbance | M_w kDa (from SE) | $[\eta]$ (ml/g) | $D^{0}_{20,w}$ (cm ² /s) | r_h (nm) |
|----------------------------|------------------------------------|----------------------------------|---------------------------|--------------------|--|------------|
| NJBT2-p | 2.0±0.2 | ND | ND | ND | ND | ND |
| NJBT2-s | ND | 0.6±0.1 | 52±10 | 66±4 | (2.3 ± 0.7) x10 ⁻⁹ | 250 |
| NJPN1-s | 0.5±0.1 | 0.4±0.1 | 250±20 50±20* | 99±11 | ND | ND |
| NJZI4-p | 1.1±0.1 | 0.4±0.1 | 60±20 | 103±2 | (2.6 ± 1.2) x10 ⁻⁸ | 250 |
| NJZI4-f | 0.7±0.1 | 0.5±0.1 | ND | ND | ND | ND |

*ignoring supramolecular components, ND: Not Determined

3.3.10 FTIR Analysis

3.3.10.1 FTIR Analysis of NJBT2, NJPN1 and NJZI4 pulps

FTIR spectra of NJBT2, NJPN1 and NJZI4 pulps are shown in Fig. 3.30. In Fig. 3.30 the mean broad band is about 3400 cm^{-1} (N-H and O-H bond) and the narrow bands at 1600 cm^{-1} (C=O bond), 1500 cm^{-1} (C-O bond) and 1100 cm^{-1} . The results in Fig. 3.30 show that the functional group are in five regions of wave numbers as it can be seen in Table 3.7.

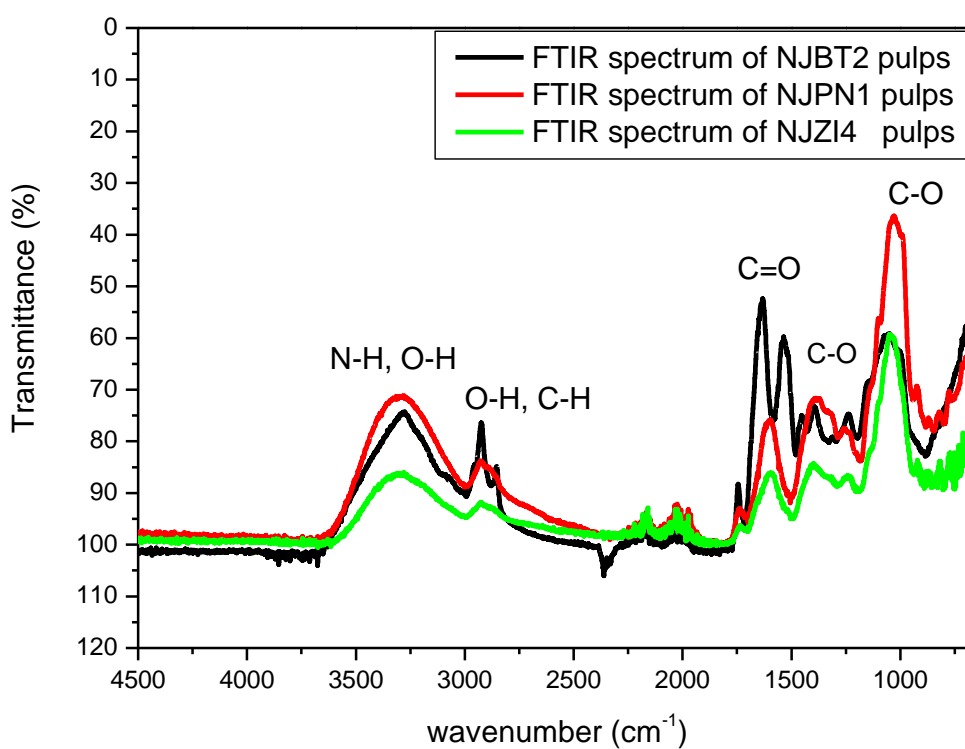


Figure 3.30: FTIR spectrum of NJBT2, NJPN1 and NJZI4 pulps.

Table 3.7: The functional groups Region in IR spectra of NJBT2, NJPN1 and NJZI4 pulps (Szymanska-Chargot and Zdunek, 2013; Lima *et al.*, 2012).

| The functional groups Region | Spectral region |
|--|----------------------------|
| The carbohydrates (C-O bond) | 900-1200 cm^{-1} |
| Mixed region of fatty acid and bending proteins (C-O bond) | 1200-1500 cm^{-1} |
| Proteins and polysaccharide (C=O bond) | 1500-1700 cm^{-1} |
| The fatty acid (O-H and C-H bond) | 2800-3000 cm^{-1} |
| Mixed region of fatty acid and bending proteins (N-H and O-H bond) | 3000-3600 cm^{-1} |

3.4 Discussion

Spectrophotometry of *Cucurbita* and the Biuret Test

The absorbances at 230nm, 280nm proved conclusively the presence of protein in the samples analyzed, together with the sedimentation velocity records at 280nm. The Biuret Reagent Method Fig. 3.3 was used to confirm the presence of peptide bonds in protein. A mixture of distilled water with Biuret Reagent gives a blue colour as a negative control. However, a mixture of with Biuret Reagent gives a violet colour as a positive control proving the presence of proteins. The samples with Biuret Reagent gives a violet colour proving the similar result with positive control that shows the presence of proteins as the colour changed were observed.

Heterogeneity: sedimentation velocity

The sedimentation velocity records all showed a high degree of heterogeneity for all samples studied, but most showing a main component at 0.4-1.1S. The data also showed a strong compliance between the sedimentation coefficient distributions obtained using either interference or absorption optics indicating a significant complexation/ attachment of the protein component with the polysaccharide that was present.

Sedimentation equilibrium in the analytical ultracentrifuge

This strong heterogeneity was observed also in the sedimentation equilibrium analyses. Fortunately, representatives for each of the *Cucurbita* were soluble enough to allow a comparison from the sedimentation equilibrium records. SEDFIT-MSTAR analysis showed that NJZI4 and NJBN2 were very similar with weight average molecular weights $M_w \sim 50\text{-}60\text{kDa}$. The pumpkin NJPN1 showed a much higher weight average of $(250 \pm 20)\text{ kDa}$. However, if the very large material influencing the higher radial positions were not included in the analysis a value of M_w of $\sim 50 \pm 20\text{ kDa}$ is obtained, although this estimate is more approximate, it is consistent with the values obtained for the extracts from Butternut Squash and Zucchini.

Frictional ratio f/f_0 estimate

Since we have an estimate for the sedimentation coefficient and the weight average molecular weight for some of the cucurbits, it is possible to also estimate the frictional ratio f/f_0 (ratio of the friction coefficient of a macromolecule to a spherical particle of the same molar mass). If we take an $s_{20,w}^0$ value of $\sim 0.5\text{S}$, and a corresponding molecular weight of 50kDa , this yields a value for the frictional ratio (see Harding, 2005) of ~ 13 , suggesting a very high degree of

hydration and asymmetry for the complexes. The value of f/f_0 for a dry, spherical particle is ~ 1 . Proteins generally have frictional ratios 1-1.3. The very high value is more typical of polysaccharides and this suggests that the hydrodynamic properties of the protein-polysaccharide complexes are most strongly influenced by the polysaccharide component.

Capillary viscometry

The polysaccharide-like behaviour of the complexes is also borne out in the viscosity behaviour. Globular proteins generally have intrinsic viscosities 3-5 ml/g. The intrinsic viscosities determined from all 3 Cucurbits were commensurate with polysaccharides:

$[\eta] = (66 \pm 4)$ ml/g for the Butternut Squash extract, $[\eta] = (99 \pm 11)$ ml/g for the pumpkin and $[\eta] = (103 \pm 2)$ ml/g for the Zucchini. Jiwani (2016) showed that the polysaccharide components were in the range of 20-79 ml/g for Zucchini and Song *et al.*, (2015) showed that heteropolysaccharide extracted from pumpkin (Song *et al.*, 2015) was in a similar range.

Dynamic light scattering

This was dominated by the supramolecular components, and successful distributions in the hundreds of nm range were obtained for extracts from all three *Cucurbita*. This data supplemented well the "molecular" information generated by the sedimentation and viscosity data.

FTIR Analysis

The presence of the functional components among other factors such as the hydrodynamic structure provide information on the ability of other compounds to bind to the natural plant products. FTIR allowed the characterisation of the sample by comparison between the sample and similar functional group that have similar wavenumber patterns. Infrared (IR) bands can be classified as strong, medium, or weak depending on their relative intensities in the infrared spectrum. For NJBT2 pulps, NJPN1 pulps and NJZI4 in Fig. 3.30 the mean broad band is about 3400 cm^{-1} (Carbonyl C=O stretching bond) and the narrow bands at 1600 cm^{-1} (High content in saturated fatty acids), 1500 cm^{-1} and 1100 cm^{-1} (Triglycerides) (see for example Siddiqui and Ahmad, 2013). The FTIR spectral region result show five regions of wave numbers as shown in Table 3.7. NJPN1 has the highest intensities of carbohydrates (C-O bond) region. However, NJBT2 and NJZI4 have the similar intensities of carbohydrates region. For the mixed region of fatty acid and bending proteins (C-O bond) region, NJBT2, NJPN1 have approximately similar intensities, whereas: NJZI4 has less intensities in this region. NJBT2 has the higher intensities of proteins and polysaccharide (C=O bond) region followed by NJPN1 and NJZI4, respectively. Moreover, NJBT2 has the higher intensities of the fatty acid (O-H and C-H bond) region followed by NJPN1 and NJZI4, respectively.

The spectra for the NJBT2 pulps, NJPN1 pulps and NJZI4 and the functional groups were compared in Fig. 3.30 and they showed that there were in most of the wavenumber with the mean broad band is about 3400 cm^{-1} and the narrow bands at 1600 , 1500 and 1100 cm^{-1} . Despite the similarity in wavenumber in the samples NJBT2 pulps, NJPN1 pulps and NJZI4 the transmittance (%) shows the difference between the functional group due to the differences of the relative intensities in the infrared spectrum.

Chapter 4: Fatty Acids Composition of *Cucurbita* seeds (NJBT2) using Gas Chromatography

4.1 Introduction

Fatty acids are molecules of long chain lipid-carboxylic acid commonly available in fats and oils. They are also found in cell membranes associated with other compounds and available as phospholipids and glycolipids. Both available in animal and plant fats and oils, they play important roles in the physiological function of the body and can be broadly classified into essential and non-essential fatty acids on the basis of their dietary requirement. The essential fatty acids are polyunsaturated fatty acids (PUFA), which cannot be synthesized in the human body and so are required in our diets. They are available in plants and oily fish. Both butternut squash, as well as pumpkin, are rich sources of essential and non-essential fatty acids and their dietary value in terms of fatty acids are explored in this chapter. Hence, the aim of the present study was to analyse the fatty acid content of butternut squash seed oil and to compare the result with the fatty acids of pumpkin seed oil.

4.1.1 Fatty acids of Butternut Squash

Butternut squash seed oil consists of different types of fatty acids—saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acids (PUFA). The SFAs of butternut squash constitute palmitic acid (25% of total fat content-TFC) and stearic acid (3%TFC). The MUFA oleic acid is present in butternut squash at 10% of TFC. The PUFAs consist of linoleic acid, which is also known as omega-6 fatty acid, (23%) and linolenic acid, which is also known as omega-3 fatty acid, (36%) (Neelamma *et al.*, 2016).

4.1.2 Fatty acids of Pumpkin

Pumpkin seed oil has a high content of essential as well as non-essential fatty acids (see Table 4.2). Some of the fatty acids are Lauric acid, Myristic acid, Palmitic acid, Stearic acid as SFAs; Palmitoleic acid, oleic acid as MUFAs; and linoleic acid and linolenic acid as PUFAs (Nutritiondata.self.com, 2015b).

4.1.3 Biological Function of constituent fatty acids

SFAs are vital in the biological function of our body. They are required for the production and storage of energy, synthesis of hormones and cell membranes as well as mechanical functions like shock absorption and thermal insulation in vital organs of our body. Moreover, in its biochemical functions, SFAs are used in some signalling pathways that are required for homeostasis. With regard to fatty acid metabolism, free fatty acids in the body are re-esterified to triacylglycerols in the intestinal mucosa cells and then transported as part of chylomicrons and transported bound to albumin in the circulation. After that they are taken up by protein carriers into the cells. Inside the cell, free fatty acids are activated to acyl-CoA and the formation of energy as ATP and heat in mitochondria. In terms of pharmaceutical aspects, fatty acids have been used as lipid formulations to carry the active particle (Rustan and Drevon, 2005; Gurr and Harwood, 1991).

Signalling pathways involving G-protein receptors require such group of fatty acids. Palmitic acid, Myristic acid and Lauric acid, which are 16, 14 and 12 carbon compounds respectively are important in such signalling pathways. SFAs like palmitic acid can be synthesized by the body or can be obtained from external source like vegetable oils or animal fats (Legrand and Rioux, 2010).

Depriving an organism of SFAs can have unwanted outcomes such as disruption of cellular functions due to unavailability of certain growth factors since signalling pathway by G-protein receptors requires presence of lipids.

Apart from signalling, SFAs are required for function of white blood cells. SFAs are also essential for maintenance of stability of proteins as in acylation of proteins, which means the attachment of functional groups through acyl linkages is important for the folding of the protein (Rustan and Drevon, 2005). Lauric acid is an antimicrobial fatty acid and functions as a protein stabilizer like palmitic and Myristic acid (Enig, 2015).

4.1.3.1 Monounsaturated fatty acids

Among all the dietary fats MUFAs are the healthiest type of fats (Schwingshackl and Hoffmann, 2012). Due to its chemical nature MUFAs tends to remain stable during cooking and have a very low chance of being hydrogenated to form SFAs. MUFAs reduce the risk of heart disease by decreasing the low density lipoproteins (LDL) (Schwingshackl and Hoffmann, 2012) and also provide the body with vitamin E (Valk and Hornstra, 2000). Fat soluble vitamins like vitamin A, D, E and K are absorbed and metabolized through MUFAs (Brousseau *et al.*, 1993).

By increasing the high density lipoproteins (HDL) and lowering LDL with MUFAs they provide a healthy function for the heart and blood vessels. MUFAs are also required for normal development of the brain and nervous system in children.

4.1.3.2 Polyunsaturated fatty acids

PUFAs are a common constituent of lipid membranes. PUFAs play an important role in the development of the nervous system and its proper functioning. As seen with MUFAs, PUFAs have the potential to reduce LDL and increase the concentration of HDL in our blood thereby promoting a healthy cardiovascular system (Schwingshackl and Hoffmann, 2012). PUFAs constitute essential fatty acids like linoleic and linolenic acids. These fatty acids are major components of our cell membrane synthesis (Gill and Valivety, 1997).

Essential fatty acids are essential component of our cell membrane and also play various important biological functions such as regulation of blood pressure as well as inciting inflammatory response (Abbott *et al.*, 2012). There are claims that linoleic acid can reduce the risk of cancer (Zock and Katan, 1998) as well as diabetes (Yary *et al.*, 2016).

4.1.4 Repository of medicinal properties of pumpkin seeds and its oil extracts

The seeds and the oil extracts are good repositories of alternative traditional medical properties. The oil is thick, brown to dark brown in colour and extremely rich in linoleic acid. The abundant oleic and linoleic acids reduce the plasma cholesterol levels significantly. It lowers LDL and raises HDL (Kwon *et al.*, 2007). Therefore, it serves in fighting cardiovascular diseases. Pumpkin seed oil is sold in most reputable US health stores. It is formulated into capsules that contain 1 gram of oil. In addition, the oil obtained from the pumpkin seeds have been hypothesized to have some pharmacological activities, which include antifungal, antidiabetic, and anti-inflammatory activities (Rahim *et al.*, 2013).

Pumpkin oil has been used as an antidiabetic drug in most regions of the world. The oil extracted from pumpkin has hypoglycemic effects in alloxan-induced diabetic rats and the normal animals. Pectin is the constituent of the seed that is responsible for the hypoglycemic effects of the oil as fatty acids and glucose absorption decreased in the presence of pectin (Lim, 2012). In addition, pumpkin seed oil contains elevated levels of polysaccharides, which may be another component responsible for its hypoglycemic actions. According to Li *et al.*, (2001) the oil obtained from un-germinated pumpkin seeds has hypoglycemic properties. Research reports have also deduced that the protein contained in the seeds with the molecular weight of between 3-60 kilo Daltons increases the plasma insulin levels. Furthermore, the oil from the germinated seeds improves blood glucose

tolerance (Kwon *et al.*, 2007). However, the protein components obtained from ungerminated pumpkin seeds do not possess any hypoglycemic abilities (Caili *et al.*, 2007). Many studies postulate that the compounds found in pumpkins are useful in the management of blood levels of insulin and the risk for diabetes (Nawfal, 2011).

4.1.5 Toxicity of the pumpkin seed and oil

As previously stated in chapter 1 (1.3.4) according to the current available literature, there are no identified contraindications or side effects associated with consumption of pumpkin seeds (Wang *et al.*, 2012).

4.2 Materials and Methods

The *Cucurbita* related materials which have been used have been considered in the previous chapter. The experimental aspects are presented below.

4.2.1 Materials

4.2.1.1 Chemical analyses

Crude oil was determined by using a Soxhlet extractor and petroleum ether was used as a solvent.

Petroleum ether, Acetone, potassium hydroxide, methanol, sulphuric acid, hexane, 11cm qualitative grade filter paper all were purchased from Sigma Aldrich (Gillingham, UK) and Fisher Scientific (Loughborough, UK). Samples of Butternut Squash (NJBT2) were purchased from the local supermarket.

4.2.2 Methods

4.2.2.1 Preparation of *Cucurbita* seeds

The ripe fruits (NJBT2) were cut and the seeds were separated. The separated seeds were washed and left to dry for 5 days in 50°C. The dried seeds were then ground to a powder using electric grinder then they were weighed. The powder was thoroughly mixed, stored at 4°C, and used as the stock seed sample for further analyses.

4.2.2.2 Fourier Transform Infrared Spectroscopy (FTIR) of *Cucurbita*

The dry samples were prepared as in the previous chapter (3.2.2). Characterization was carried out using FTIR (IRAffinity-1S, Shimadzu, Japan)

equipped with an Attenuated Total Reflectance module (ATR) with a diamond surface. Dry powder samples were placed onto the diamond and clamped down. Scanning range was 4500 to 400 cm^{-1} resolution of 4 cm^{-1} and 20 replicate spectra were obtained. The shifts in the respective IR spectra bands were observed and reported. FTIR analysis of functional groups were analysed.

4.2.2.3 Soxhlet extractor

The Soxhlet (Soxtherm, Gerhardt, Brackley, UK, described in Fig. 4.1) was used to extract fatty acids from the stock seed sample. 1.0293 g of *Cucurbita* seeds was weighed using a digital precision balance. Then the sample was placed onto an 11cm grade filter paper. After that the sample was placed into a thimble and was covered using a plug of cotton wool. As the apparatus heated up, 6 flasks were weighed and placed on a rack and 162 ml of petroleum ether was added to each flask. The thimble was placed in a thimble holder in each flask. The flasks were installed on the Soxtherm and extraction started. After four hours, it was ensured that the solvent was depleted and the thimble and holders were taken out to drain. The flasks were weighed again and the crude oil percentage was calculated using the following equation:

$$\text{Fat\%} = (\text{Weight (Wt) of extracted fat} \times 100\%) / \text{sample Weight (Wt)} \quad (4.1)$$

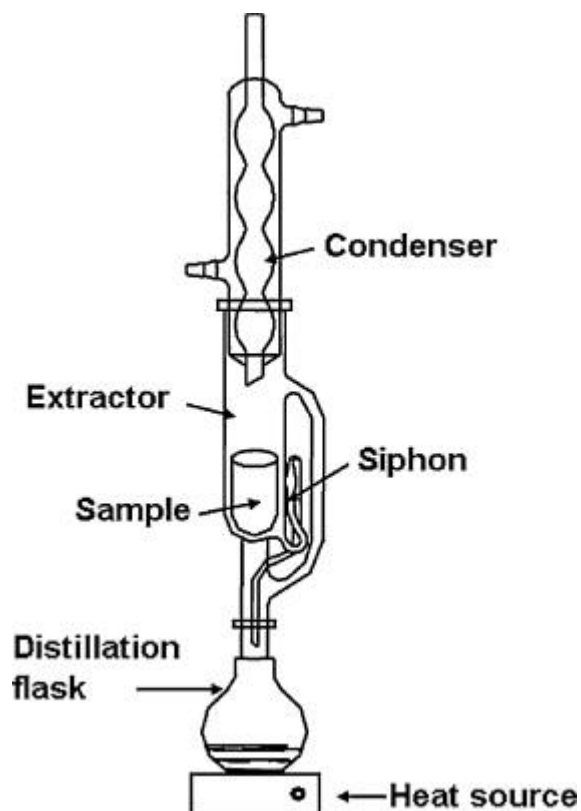


Figure 4.1: Conventional Soxhlet extractor (De Castro and Priego-Capote, 2010).

4.2.2.4 Preparation of fatty acid methyl ester (FAME)

Each sample plus 0.7 ml 10M potassium hydroxide and 5.3 ml methanol were placed in screw topped methylation tubes. Then samples were incubated in a water bath at 55°C for 90 minutes, with a 5 second vortex mix every 20 minutes. The samples were then cooled down in cold water for 10 minutes. 0.58 ml 12M sulphuric acid was added and the samples were mixed and incubated as before. Then the samples were cooled using the water bath followed by the addition of 3 ml hexane, then vortex mixed for 30 seconds. The samples were then centrifuged for 5 minutes at 2000 rpm. The hexane layer was transferred to a solvent resistant LP4 tube and stored at 30°C and kept until further analyses.

4.2.2.5 Analyses of fatty acid using Gas Chromatography

Gas Chromatography was used to determine the fatty acid content in the oil samples. The samples were analysed using Gas Chromatography (Hewlett-Packard) equipped with a flame ionization detector and integrator using helium as the carrier gas at flow rate of 0.7ml/min. The upper layer was collected for gas chromatography analysis and the oven temperature was maintained at 198°C, the injector at 280°C and detector at 280°C. The fatty acids were identified by comparing their retention times with those of standards from Sigma. This analysis was kindly provided by Chris Essex from the Division of Nutritional Sciences, University of Nottingham.

4.3 Results

4.3.1 The approximate oil composition of the whole seeds (NJBT2)

Removal and analysis of fatty acids was performed in order to analyse the fatty acids from family *Cucurbitaceae*. The crude oil percentage was calculated, as mentioned before.

Table 4.1 shows the proportion of crude oil from NJBT2 and the result indicate that this type of family has the high percentage of oil and this type of oil is highly nutritious (Alfawaz, 2004).

Table 4.1- The proportion of crude *Cucurbita* seed oils

| Sample/ replicate | Sample weight (g) | Flask weight (g) | Flask + fat weight (g) | Fat weight (g) | Sample fat content (%) | Mean fat content (%) |
|------------------------------|----------------------------------|---------------------------------|---|-------------------------------|---|-------------------------------------|
| 1 | 0.4923 | 137.34 | 137.52 | 0.18 | 36.5 | 36.3 |
| 2 | 0.5370 | 143.42 | 143.61 | 0.19 | 36.0 | |

4.3.2 FTIR Analysis of NJBT2 and NJPN1 seed

With the results from NJBT2 and NJPN1 seed in Fig. 4.2 it can be seen the similar peaks as in NJBT2 and NJPN1 not defatted seed at about 3400 cm^{-1} (N-H & O-H bond) and the narrow bands at 1600 cm^{-1} (C=O bond), 1500 cm^{-1} and 1100 cm^{-1} (C-O bond). The result of FTIR spectral region can be classified into five regions of wave numbers as mentioned previously in chapter 3 area (3.3.10). Fig. 4.2 shows the similarity of NJBT2 and NJPN1 seed in FTIR spectral region with a slight more intensities of NJPN1 seed in the carbohydrates (C-O bond) region.

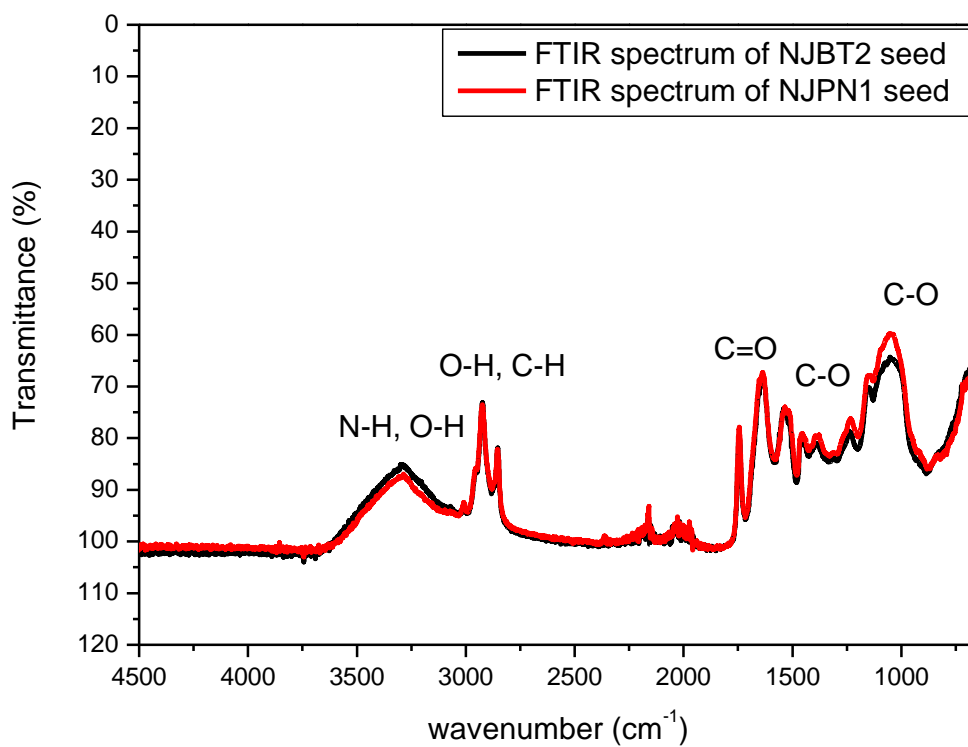


Figure 4.2: FTIR spectrum of NJBT2 (Butternut-Squash seed) not defatted and NJPN1 (Pumpkin-seed) not defatted.

Margarine was the result of presence of the oil in *Cucurbita* seeds using FTIR and Table 4.2 shows the total fat in pumpkin seeds and Margarine (Soft vegetable fat spreads).

Table 4.2: Fatty acid profile of Pumpkin Seed Oil and Margarine (Nutritiondata.self.com, 2015b)

| Types of fatty acids | Total fat in pumpkin % | Total fat in Margarine % |
|-----------------------------|-------------------------------|---------------------------------|
| Lauric acid | 1.2 | 0 |
| Linoleic acid | 2.5 | 37.8 |
| Linolenic acid | 3.5 | 0 |
| Myristic acid | 7 | 0 |
| Oleic acid | 7 | 21.9 |
| Palmitic acid | 42 | 17.7 |
| Palmitoleic acid | 7 | 0.2 |
| Stearic acid | 3 | 6.9 |

4.3.3 FTIR Analysis of NJBT2 not defatted and defatted seed

Similar to the results shown in 4.3.2 the mean broad band is about 3400 cm^{-1} (N-H & O-H bond) and the narrow bands at 1600 cm^{-1} (C=O bond), 1500 cm^{-1} and 1100 cm^{-1} (C-O bond) for the sample from NJBT2 not defatted and defatted seed in Fig. 4.3. However, it can be seen clearly the absence of margarine group after Soxhlet extractor (from the wavelength of $3000\text{--}2800\text{ cm}^{-1}$ spectral region which is the fatty acid region).

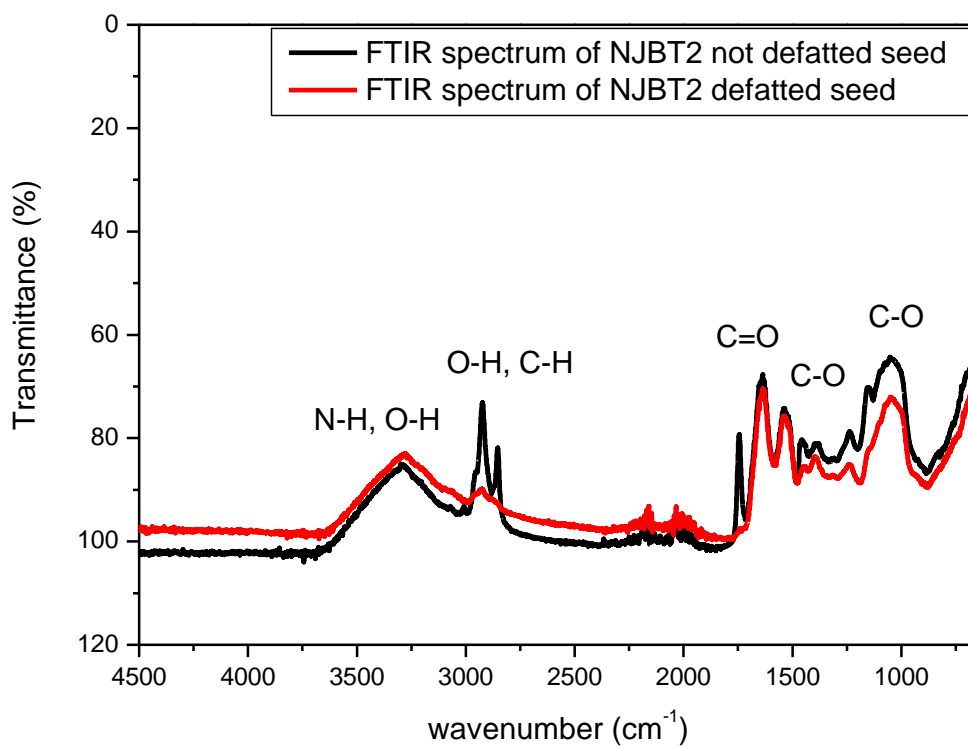


Figure 4.3: FTIR spectrum of NJBT2 (Butternut squash) not defatted and defatted seed.

4.3.4 The fatty acid composition of the whole seeds (NJBT2)

Gas Chromatography (GC) analysis

Table 4.3 shows the fatty acid composition of oils from the butternut squash seed and the mean values with standard deviations and Figure 4.4 a gas chromatogram of the extracted fatty acid sample. The results of gas chromatography (GC) analysis indicate that PUFAs formed approximately 60% of the total oil. Linoleic acid (18:2) was the major fatty acid component, at a concentration of 29.7%, followed by Oleic acid (18:1 cis) at a concentration of 27.4%. Because of the higher amount of PUFA in these seeds, it is of high value in the human diet, as well as other organisms. However, total SFA was almost 30% of the total oil. Palmitic acid (16:0) was present at a concentration of 14.3%, whereas Stearic acid (18:0) had the lowest fatty acid concentration, with approximately 6.1% and the rest of about 10% was lost during the classical procedures.

Table 4.3- Fatty acid composition of crude *Cucurbita* seed oils

| Sample | Palmitic acid (16:0) | Stearic acid (18:0) | Oleic acid(18:1 cis) | Linoleic acid(18:2) |
|------------------------|-----------------------------|----------------------------|-----------------------------|----------------------------|
| | % | % | % | % |
| Fatty acid type | Saturated | Saturated | Monounsaturated | Polyunsaturated |
| NJBT2 (1) | 14.08 | 6.14 | 26.97 | 27.68 |
| NJBT2 (2) | 13.85 | 6.02 | 26.64 | 28.05 |
| NJBT2 (3) | 14.68 | 6.30 | 28.26 | 31.58 |
| NJBT2 (4) | 14.75 | 6.10 | 27.84 | 31.43 |
| Mean % | 14.34±0.44 | 6.14±0.12 | 27.43±0.75 | 29.69±2.11 |

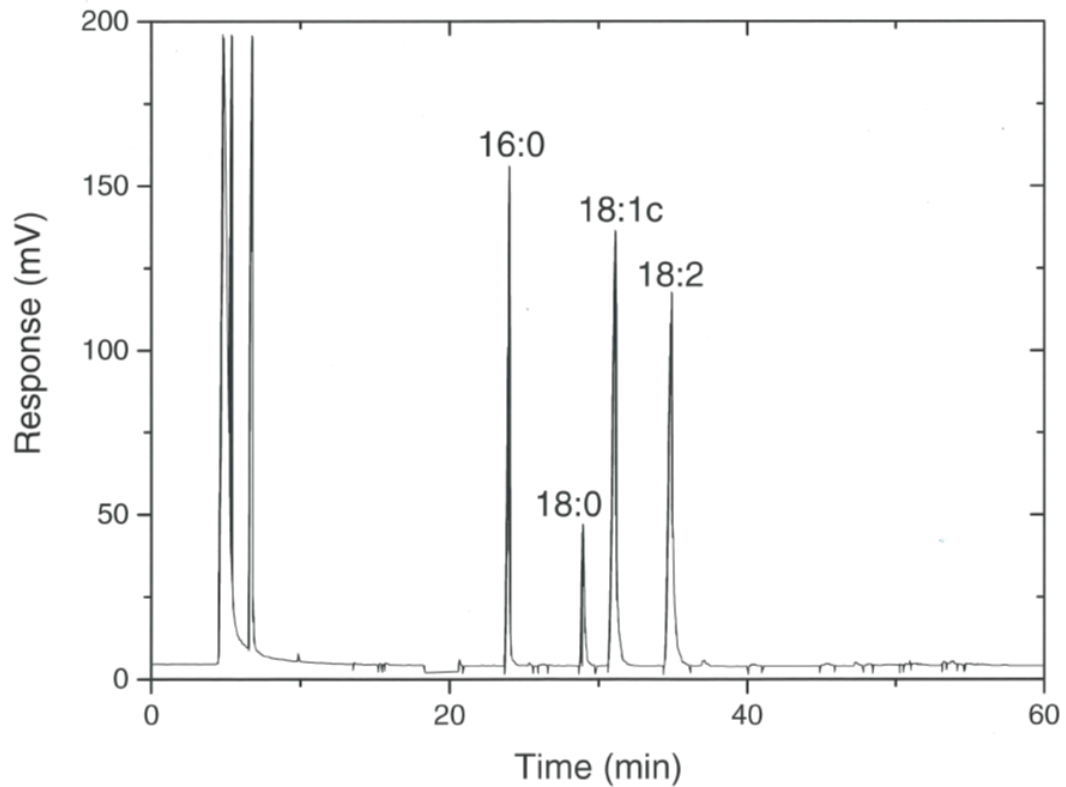


Figure 4.4:The retention times of NJBT2 (butternut squash) fatty acid sample in gas chromatography.

4.4 Discussion

As mentioned herein, that all parts of the *Cucurbitaceae* including pulp, seeds and peel, have been studied and investigated to reveal their therapeutic aspects. Butternut squash fatty acids were extracted from the seeds this was performed using Soxhlet section 4.3.1. This was confirmed using FTIR (section 4.3.2) before extraction the powder was recognised to contain similar spectra to Margarine. This

step is not indicating that the samples contain margarine, however it does confirm the presence of fat.

FTIR is not sensitive enough to distinguish between fatty acids, which is the reason that GC was later employed to elucidate the composition. After extraction, FTIR indicates that the powder was recognised to contain protein. Although the libraries matched the spectra of lysozyme, soybean protein etc, this step is not indicating that the samples actually contain these exact components. However, FTIR is sensitive enough to look at conformational changes in proteins but, in such a heterogeneous sample, these differences would be difficult to deconvolute. It does, however, confirm that the fat has been extracted leaving protein left over.

Although the seeds of butternut squash and pumpkin are rich in oil and protein, limited detailed studies have been claimed out on their composition and the properties of their oil (Alfawaz, 2004). Hence, as mentioned before the aim of the present study was to analyse the fatty acid content of butternut squash seed oil and to compare the result with the fatty acids of pumpkin seed oil. The fatty acid pattern in this study of Butternut Squash seed oils appears to be very similar to that of pumpkin seed oils, which consists mainly of PUFAs (Fruhirth and Hermetter, 2007). However, the present result shows that Linoleic acid (18:2) is the major fatty acid in butternut squash seed oil, whereas pumpkin seed oil has oleic acid as the major fatty acid, as per results obtained by Mitra *et al.* (2009), Nyam *et al.* (2009) and El-Adawy and Taha (2001) (Table 4.4, Figure 4.5). As shown in Fig. 4.4, the fatty acid profile, i.e. chromatograms of all NJBT2 sample, revealed an identical distribution of fatty acid. The most abundant from this sample was Linoleic acid (18:2) ranging from 27.68% to 31.43% of the total fatty acid content. The second most abundant was Oleic acid (18:1 cis) ranging from 26.64 % to 28.26% of the total fatty acid. The remaining contents were saturated fatty acid palmitic acid (16:0) and Stearic acid (18:0) ranging from 6.02% to 6.30% and from 13.85% to 14.75%, respectively. The fatty acids were identified

by comparing their retention times Fig. 4.4 with those of standards from Sigma. However, the rest of 22.4% of the fatty acids might be lost during the classical procedures or the step of evaporating the hexane phase extensively.

For NJBT2 and NJPN1 seed in Fig. 4.2 and NJBT2 not defatted and defatted in Fig. 4.3 the mean broad band is about 3400 cm^{-1} (Carbonyl C=O stretching bond) and the narrow bands at 1600 cm^{-1} (High content in saturated fatty acids), 1500 cm^{-1} and 1100 cm^{-1} (Triglycerides) (see for example Siddiqui and Ahmad, 2013). The infrared spectrum shows at about 3400 cm^{-1} and the narrow bands at 1600, 1500, and 1100 cm^{-1} and a narrow peak at about 2900 cm^{-1} . These results show that despite the similarity in the wavenumber the whole structure is depends on the intensity of the peak so it differs the suggested components. It was similar to Fig. 3.40, however, the peak at 2900 cm^{-1} is stronger than the one in Fig. 3.40 as it has more relative intensity. Therefore, the margarine group in NJBT2 and NJPN1 represent the presence of fatty acid (see for example Table 4.1). The result of NJBT2 and NJPN1 seed in Fig. 4.2 and NJBT2 not defatted in Fig. 4.3 are similar to the result that was obtained by Lankmayr *et al.*, (2004). Soxhlet extractor was performed and to investigate that fatty acid was eliminated, FTIR was used with the defatted seed and the result show in Fig. Similar to Fig.4.2 Infrared (IR) bands the relative intensities in the infrared spectrum shows the mean broad band is about 3400 cm^{-1} and the narrow bands at 1600, 1500 and 1100 cm^{-1} and the result show the absence of margarine group after Soxhlet extractor from the wavelength of $3000\text{--}2800\text{ cm}^{-1}$ spectral region which is the fatty acid region.

It is worth noting that the chemical structure and function of these fatty acids have been widely studied. The following table, Table 4.4 describes the chemical structure and function of the fatty acids that have been found in butternut squash seed oils.

Table 4.4: The chemical structure and function of the fatty acids from the seeds of butternut squash

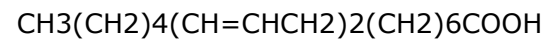
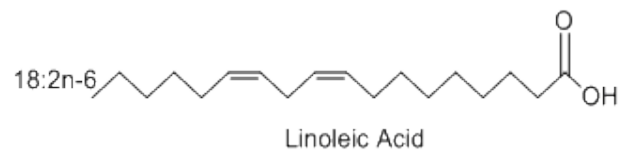
| Fatty acid | Density (g/ml) | Function | Causes | Stability | References |
|--|---------------------------|---|--------------------------------|--------------------------|--|
| Linoleic acid (18:2) ω-6 | 0.9 | “Regulates blood pressure, blood clotting, blood lipid levels. Reduces the risk of coronary heart disease” (citation). Treatment of postmenopausal symptoms (citation). | LIKELY SAFE for most adults | susceptible to oxidation | (See for example (Belury, 2002); (Ramsden <i>et al.</i> , 2013); (Parhizkar and Latiff, 2013); |
| Oleic acid (18:1 cis) ω-9 | 0.895 | Fatty acid storage, long-term source of energy, layer of insulation. ↓ LDL, ↑ HDL. | LIKELY SAFE for most adults | susceptible to oxidation | (Benjamin and Spener, 2009). |

| | | | | | |
|---------------------------------|------------------|------------|-------------------------------|--------|--|
| Stearic acid (18:0) | 0.847 (70°C) | ↑LDL, ↓HDL | Coronary artery disease | Stable | |
| Palmitic acid (16:0) | 0.853 (62 °C) | ↑LDL, ↓HDL | Coronary artery disease | Stable | |

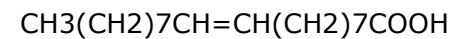
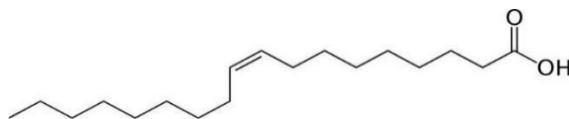
LDL: Low density lipoproteins carry cholesterol from liver to rest of body.

HDL: High density lipoproteins carry cholesterol back to the liver.

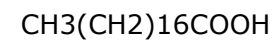
Linoleic
acid



Oleic acid



Stearic acid



Palmitic
acid

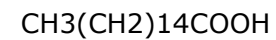
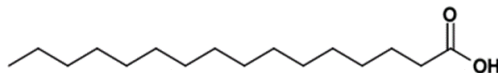


Figure 4.5: Structure of Linoleic acid, oleic acid, stearic acid and Palmitic acid (Rustan and Drevon, 2005).

4.4 Conclusion

In conclusion it should be noted that fats and oils are essential components of our diet and should be consumed in a balanced manner to promote a healthy lifestyle. SFAs though are not essential fatty acids as they can be synthesized by the body through metabolism of carbohydrates and proteins. Limiting the intake of the SFA can have severe side effects but a higher intake too is not recommended. MUFAs too have a big role in proper functioning of the body to maintain homeostasis. Most importantly, the essential fatty acids which are PUFAs can only be obtained through the diet. Plants and fish being rich in such fatty acids have an important role as a dietary component in balanced diet. Linoleic and Linolenic acids are known constituent of the plasma membrane and consumption of the two essential fatty acids in a balanced manner makes the body healthier in general.

Pumpkin and Butternut squash have the essential as well as non-essential fatty acids and can be considered a rich source of Linoleic and Linolenic acids. The study on protein-polysaccharide complexes were performed in previous chapter and in this chapter the study of fatty acids was performed so as to separate protein-polysaccharide complexes and further studies on the complexes will be investigated.

The work reported in this chapter was designed to evaluate and establish the fatty acid content in *Cucurbita* seeds. FTIR was performed over the range of wavenumber 4500–400 cm^{-1} . It was found that the functional groups present in of NJBT2 defatted seed, NJBT2 seed and NJNP1 seed were approximately the same as the wavenumber was approximately similar in these samples. However, they were found at different functional groups due to the presence of FTIR detailed information or proof of molecular formula or structure. It provides information on molecular fragments, specifically functional groups. It is worth noting here to point out that despite the similarity in wavenumber in the samples NJBT2 seed and

NJPN1 seed the transmittance (%) shows the difference between the functional group due to the differences of the relative intensities in the infrared spectrum.

Chapter 5: Hydrodynamic studies on beta glucan

5.1 Introduction

In the UK, 7 million people are suffering from cardiovascular disease (CVD) and almost 155,000 people die due to one of the many possible disorders of the cardiovascular system (British Heart Foundation, 2015). The annual financial burden of these health issues lies on the National Health Service and the annual cost is estimated at more than £26 billion (British Heart Foundation, 2015). The disorders that are described under CVD are related to heart attacks and circulatory issues such as peripheral vascular disease, coronary heart disease (CHD) and stroke (British Heart Foundation, 2015). The partial or complete blockage of one or more coronary arteries that supply blood to the muscular walls of the heart (the myocardium) is considered an indicator of CHD (Frayn, 2010). The main cause of this medical condition is atherosclerosis, which is a disorder due to the accumulation of fatty deposits on the innermost membrane of the arterial walls. Atherosclerosis can cause blockage in prime arteries of the body and restrict the blood flow to those body parts in which atherosclerosis has developed. However, if the brain or heart suffers from sudden blockage, it may result in the death of the person. The first symptom of CHD is called angina. During angina, the patient experiences severe chest pain, which often spreads to the neck, shoulders and arms; it is the result of inadequate blood flow to the heart. At the time of exertion, stress or excitement, a person can experience angina. Unfortunately, in most of the cases, the symptoms of angina do not develop, and the person is not able to detect that he is experiencing CHD that is myocardial infarction or heart attack. It is considered as a severe clinical event that has a high mortality rate (Frayn, 2010).

Accumulation of cholesterol on the arterial walls is identified as the main event of atherosclerosis. The relationship between CHD and increased cholesterol in the blood is considered as the major reason of this disease in few countries. The connection became more convincing when it was observed that statins, which are used to lower blood cholesterol, has its impact on the condition of CHD. This drug is used for those people, who are more vulnerable to CHD, and it helps to decrease the mortality rate due to CHD (Frayn, 2010). Therefore, it can be stated that the deaths, due to CVD, can be controlled by restricting the formation of LDL cholesterol and serum cholesterol in the blood. In fact, the population can be encouraged to consume such a diet, which is rich in these agents that can help to control or reduce the production of cholesterol and facilitate them to maintain good health. This solution is cheaper in comparison to the consumption of chemical agents to control the cholesterol level (Kumar *et al.*, 2012).

The European Food Safety Authority (EFSA) claims that the cholesterol concentration in the blood can be reduced by consuming oat beta glucan, which is efficient in reducing cholesterol from the blood and assists in minimising the risk of CVD. This statement suggests that the daily diet must be a combination of such food items that contain at least three grams of oat beta glucan. The EFSA approves those food items that have high oat beta glucan such as bran of oats and other food items, which consist of bran of oats (EFSA, 2010). However, the claim made by EFSA does not refer to consuming pure beta glucan. The effects of beta glucan as a polysaccharide, over lowering the cholesterol levels in the blood, are still debated but they are more effective when beta glucans are not manufactured. It was reported that the processing of food and storage conditions resulted in reducing the health benefits of beta glucans. The increase in lumen viscosity because of the beta glucans in the diet helps to protect the heart from cholesterol congestions (Lazaridou and Biliaderis, 2007). Further research needs to be investigated on the beta glucans to reveal their hydrodynamic properties in

terms of sedimentation coefficient behaviour and molecular weight. How the presence of lipase changes the sedimentation coefficient behaviour is examined, and we also look at the effect of processing on the intrinsic viscosity of the beta glucan.

5.1.1 Physical Properties and Molecular Structure of beta glucans

The polymers of glucose are called glucans, and they can be attached by either alpha or beta bonds. The beta glucans are one of the examples of non-starch polysaccharides that have a monomeric unit of D-glucose with beta-glycosidic bonds. Enzymatic breakdown of beta-glycosidic bonds is not possible in small vertebral intestines. Thus, beta glucans can be categorised as dietary fibres (Kumar *et al.*, 2012). The polysaccharide's macromolecular structure depends on the method of isolation and its origin (Khoury *et al.*, 2012). Cereal grains such as barley, wheat and oats are good sources of beta glucans. It has been observed that beta glucan ranges from 1.8 to 5.5% of the dry weight and sometimes it varies to 7% as per the quality of cultivation (Miller *et al.*, 1993), (Decker *et al.*, 2014). The beta glucan in oats is found in the innermost region of the cell walls of aleurone, starchy endosperm and subaleurone layers (Khoury *et al.*, 2012). The oat beta glucan is an example of a linear polymer of D-glucose units with β -(1 \rightarrow 4) and β -(1 \rightarrow 3) glycosidic bonds (Fig. 5.1) (Wang and Ellis, 2014). Most of the glucose substances are of cellotriosyl and cellotetraosyl units, which are connected with β -(1 \rightarrow 3). The quantities of trisaccharides to tetrasaccharides in β -glucans differs as per the cereal crop. An example of it could be, 2:1 is the ratio in oats whereas, in barley and wheat, it is 3:1 and 4:1 respectively (Cui *et al.*, 2000).

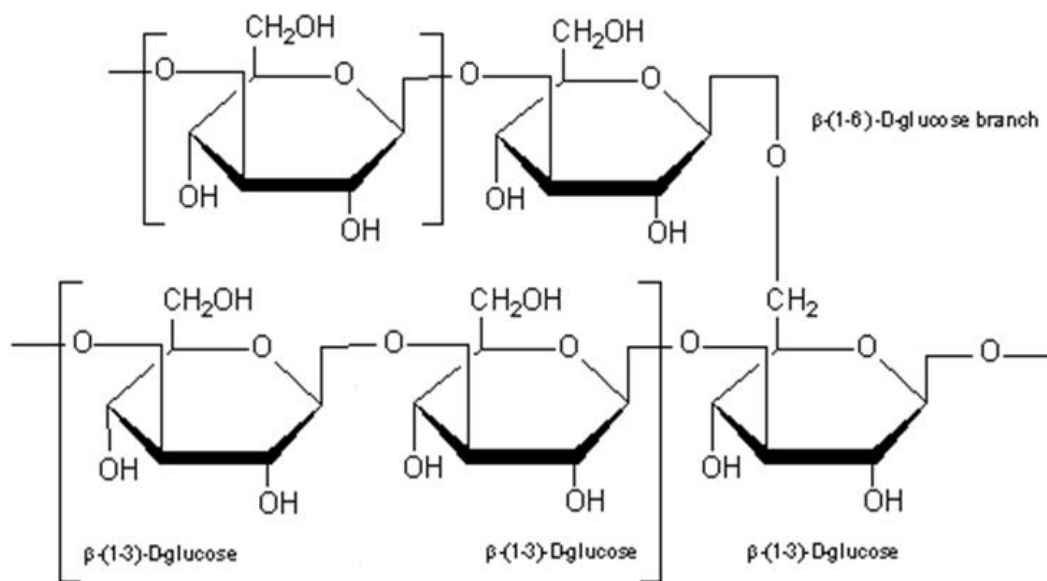


Figure 5.1. Beta glucan formation (Rahar *et al.*, 2011).

The molecular weight of the molecules and the biochemical structure of beta glucans affect the key physical characteristics, namely size, shape, viscosity and solubility. The size of the molecule is represented by the molecular weight whereas its shape is defined by its conformation. The literature shows that the conformation of beta glucan derived from oats has an approximately random coil structure when it is in an aqueous environment (Wang and Ellis, 2014). In terms of molecular weight, different techniques can give different types of information – different averages (weight, M_w , and number M_n , and distributions) (Tombs and Harding, 1998). The molecular weights of the molecules of separated oat beta glucans can be as high as 2×10^6 to 3×10^6 Da (Wang and Ellis, 2014). The environmental conditions and variety of the oats can cause the variation in the range (Andersson and Börjesdotter, 2011; Ajithkumar *et al.*, 2005). Another determinant that has been observed, is the action of endogenous hydrolytic enzymes, which acts at the time of storing and processing. The removal of the enzymes to confront depolymerisation promotes the process of extracting beta glucans of higher molecular weight (Wood, 1986; Doehlert *et al.*, 1997). Hence

the particular process of purification and isolation used influences the molecular weight (Wang and Ellis, 2014).

The extractability and solubility to give uniform dispersions depends on the environmental conditions as well as the molecular weight. A higher amount of extraction of the polymer can be achieved by reducing the molecular weight. The actions of temperature, enzymes and pH level are influencing elements for dissolving beta glucans. Some of the methods of extraction use techniques which generate a similar environment as inside the human stomach. By contrast some use more rigorous treatments such as water heated at 100°C (Wang and Ellis, 2014).

Improved solubility of the polymers can be achieved with the help of hot-water process of extraction and increases the dissolvability of beta glucans. Thus, it was realised that the higher level of solubility of the polymer depends on the temperature and source from which it has been extracted (Johansson *et al.*, 2007). Beer *et al.*, (1997) performed laboratory tests on samples of oat bran and rolled oats to know which of these have a higher proportion of beta glucans. The results of the tests clarified that rolled oats had a higher percentage of beta glucan in comparison to oats bran (Beer *et al.*, 1997). The availability of beta glucan in rolled oats proved that extraction of beta glucan was difficult as the layer of beta glucan is thin in the subaleurone layer, which is covered with a thick insoluble layer of hemicellulosic and cellulosic material (Miller and Fulcher, 2011; Wood and Fulcher, 1978). The dissolvability of beta glucan is also influenced by food processing. The process of extracting beta glucan from oat flakes was encouraged while fermentation and cooking (Johansson *et al.*, 2007; Zhang *et al.*, 2011; Degutyte-Fomins *et al.*, 2002). Besides this, Beer *et al.* (1997) had observed that the solubility of beta glucan decreases when oat muffins are stored in a cool environment. In order to support this statement, it has been highlighted that the

beta glucans are not extracted because the hydrogen bonding the chains of beta glucan is stabilised and it results in a more organised structure.

In terms of the viscosity property of beta glucan solutions, Lazaridou and Biliaderis (2007) have reported that the intrinsic viscosity of cereals beta glucans ranges between 28 and 960 ml/g. The optimum level of viscosity required to attain the cardio-protective beta glucans' impact completely is currently under investigation.

5.1.2 Impact of beta glucans in Lowering the Blood-Cholesterol

A recent study conducted on 126 humans, and their meta-analysis appears to show that the beta glucan from oats is potent for decreasing the level of total serum cholesterol and LDL. The physiological effects of beta glucan can be noticed if 3g of beta glucans of oats is consumed on a daily basis (Tiwari and Cummins, 2011). However, the literature shows a variability of conclusions with regards the beneficial effects. For example, Lovegrove *et al.* (2000) have shown that for healthy individuals no visible variations are noted in the level of LDL cholesterol and serum cholesterol in the people studied (using wheat or oats). Panahi *et al.* (1997) demonstrated that the drinks of a higher level of viscosity, which contains more of beta glucans, are able to affect physiology positively whereas the low-viscosity beta glucans have inverse effects. Further, Kerchkhoffs *et al.* (2003) have stated that solid food items made with oats beta glucans are less effective in bringing down the level of blood cholesterol in comparison to those liquids, which are efficient in controlling the level of cholesterol in the blood. The examples of food items are solids such as bread and cookies that have beta glucans, but they are not efficient in lowering the level of cholesterol in the blood whereas drinks such as oat smoothies, are rich in beta glucan and they are more effective.

The discrepancies can be explained, as when the oat beta glucans are processed a depolymerisation occurs that changes the nature of the molecules in terms of weight and solubility, and finally, results in a change in the level of viscosity.

In this Chapter we examine three aspects of the hydrodynamic properties of oat beta glucan:

- Sedimentation coefficient distribution (from sedimentation velocity) and molecular weight (from sedimentation equilibrium).
- How the presence of lipase changes the sedimentation coefficient behaviour, and assessing whether this indicates an interaction or not.
- Using intrinsic viscosity, to establish if processing (freezing/storage/autoclaving) has an effect on the size/shape of the beta glucans, and using dynamic light scattering (DLS) to assess the presence of any increased aggregate formation.

5.2 Materials and Methods

5.2.2 Materials

5.2.2.1 Chemicals

Sodium chloride, potassium dihydrogen phosphate, disodium dihydrogen phosphate, were all purchased from Fisher Scientific (Loughborough, UK).

OatWell ® oat beta glucan was kindly provided by King's College, London (KCL) (Swedish Oat fibre, Sweden). In the experiment, deionised distilled water was used to dissolve beta glucan at 80.0°C, and it was stirred continuously for 2 hours. The solution of oat beta glucans was dialysed at pH 7.0 level against phosphate buffered saline and ionic strength of 0.1M. The ATAGO DD-7 Differential Refractometer was used to measure the concentration level in the sample (Jencons Scientific, UK). The refractive increment value used for this was 0.151ml/g (Theisen *et al.*, 1999).

5.2.3 Methods

5.2.3.1 Preparation of beta glucan

The solution of beta glucans was split into four parts and labelled as BG1, BG2, BG3 and BG4. BG1 was left unprocessed as a control. The autoclaving of BG2 was accomplished at 121°C for 15 minutes with a Phoenix Autoclave (Rodwell Scientific Instruments, Essex, UK). Sample BG3 was kept at -20°C for 1 week in frozen storage whereas BG4 was stored at room temperature at approximately 21°C for 2 weeks.

5.2.3.2 Preparation of lipase solutions for interaction with beta glucan

Lipase (Type VI), with colipase, solutions were kindly provided by Dr Myriam Grundy, King's College London.

BG1 and Lipase solution were mixed in PBS for at 25°C at 0.6mg/ml:0.5mg/ml, 0.5mg/ml:0.5mg/ml, 0.4m/ml:0.5mg/ml, 0.3mg/ml:0.5mg/ml and 0.2mg/ml:0.5mg/ml.

5.2.3.3 Sedimentation velocity of beta glucan BG1 and lipase in the analytical ultracentrifuge

A Beckman XL-I ultracentrifuge was used as considered in section 2.3.4. Beta glucan samples (~395µl) at the concentrations 0.40, 1.00, 2.00, 3.00 and 4.50 mg/ml and PBS buffer dialysate (400µl) at pH 7.0 and ionic strength 0.1M were injected into sample and reference channels respectively of double sector 12 mm optical path length cell. The balanced cells were then loaded into an analytical 8-hole titanium rotor An50-Ti and placed in the AUC. As before, Rayleigh interference optical system and absorbance optics were used for recording concentration profiles and the movement of the sedimentation boundary in the analytical ultracentrifuge cell (Harding, 2005). An initial low rotor speed of 3000 rpm was adjusted to a rotor speed of 40000 rpm. The standard conditions of density and viscosity of water at 20°C were used for adjustment of sedimentation coefficients (Schachman, 1992). Scans were taken at 2 minute intervals for a run time of ~ 24 hours. The data were analysed using continuous distribution $c(s)$ Lamm equation model SEDFIT algorithm in terms of distributions of sedimentation coefficient distribution $c(s)$ vs s (see e.g. Dam and Schuck 2003; Harding, 2005).

A similar procedure was followed for the analysis of the lipase solution and mixtures of BG1 with lipase.

5.2.2.4 Sedimentation equilibrium of beta glucan BG1 in the analytical ultracentrifuge

Sedimentation equilibrium experiments were carried out using a Beckman instruments (Palo Alto, California, U.S.A.) Optima XL-I ultracentrifuge. Beta glucan samples ($\sim 80\mu\text{l}$) at the concentration range 0.25, 0.50, 1.00, 1.25, 2.50, and 3.00 mg/ml and PBS buffer dialysate ($90\mu\text{l}$) at pH 7.0 and ionic strength 0.1M were injected into sample and reference channels respectively of double sector 12 mm optical path length cells. The balanced cells were then loaded into an analytical 8-hole titanium rotor An50-Ti and placed in AUC. The Rayleigh interference optical system was used for recording concentration profiles and the movement of the sedimentation boundary in the analytical ultracentrifuge cell (see e.g. Harding, 2005). The rotor speed was 10000 rpm. Scans were taken at 1 hour intervals for a run time of ~ 72 hours. The data was analysed using the SEDFIT-MSTAR program (Schuck *et al.*, 2014) to provide an assessment of molecular weight.

5.2.3.5 Capillary viscometry of beta glucans BG1-BG4

The viscosity of a series of solutions for each beta glucan (BG1-4) ranging in concentration 0.50, 0.70, 0.90, 1.1, 1.3 mg/ml were measured using an Ostwald viscometer. The U-tube viscometer was suspended in an accurate temperature regulated water bath. The temperature was kept constant at 20.00°C throughout. The amount of each solution injected into the viscometer was 2ml and the solution flow time (t) determined manually (particularly for highly viscous solution) using

a clock timer. From the flow time of the buffer (t_0) the ratio t/t_0 of the solution was estimated. 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg/ml are six different concentrations in which the samples were diluted before the process of analysis. Whenever required, more concentrations of 0.8 and 1 mg/ml were added to the samples for analysis. It is notable that the concentration level of beta glucans was lower than the C^* value. The C^* represents the level of concentration at which change in the behaviour of rheological and interpenetration of the chains of the molecule are ascertained (Tombs and Harding, 1998).

5.2.3.6 Dynamic light scattering (DLS) of BG1-BG4

The hydrodynamic radius r_H of BG1-BG4 was determined by Dynamic light scattering (DLS) (see e.g. Harding *et al.*, 1992) using the Zetasizer Nano-ZS apparatus (Malvern, UK), to assess if there is a change in aggregation products on processing. A stock solution of each sample was prepared in 20 mL PBS at concentrations of ~ 2.5 mg/ml. All solutions were made up to have 0.1M salt content and to be pH 7. All the solutions were centrifuged for 20 minutes to remove all large, insoluble impurities and air bubbles then filtered using a 0.2 μm pore size sterile filter (Whatman, UK). 1 ml of solution was placed in a plastic, capped cuvette which was then placed in a temperature controlled sample environment. The translational diffusion coefficient (D) was measured at a scattering angle of 173° , at a temperature of 20.0°C . Five measurements, consisting of 60 sub-runs each, were made for each sample, BG1-4. The hydrodynamic radius r_H was then obtained from D according to the following Stokes-Einstein equation (see Chapter 2 equation (2.4)).

5.3 Results

5.3.1 Sedimentation velocity in the analytical ultracentrifuge

Fig.5.2 shows the sedimentation coefficient interference and absorbance distribution plots, $c(s)$ versus s of beta glucan (sample BG1) only. Clearly little absorbance is visible showing the purity of the polysaccharide. The polysaccharide is quite heterogeneous with main peaks at $\sim 1.4S$, $2.5S$ and $4.5S$.

In Fig. 5.3, showing the sedimentation coefficient distributions of the lipase solution, there are also three peaks. Peak one shows sedimentation coefficient at $1.8S$ with lower relative absorbance which represents colipase. However, peak two represents the lipase at sedimentation coefficient of $3.5S$. Peak three shows the high sedimentation coefficient at about $5S$ which represents lipase dimer.

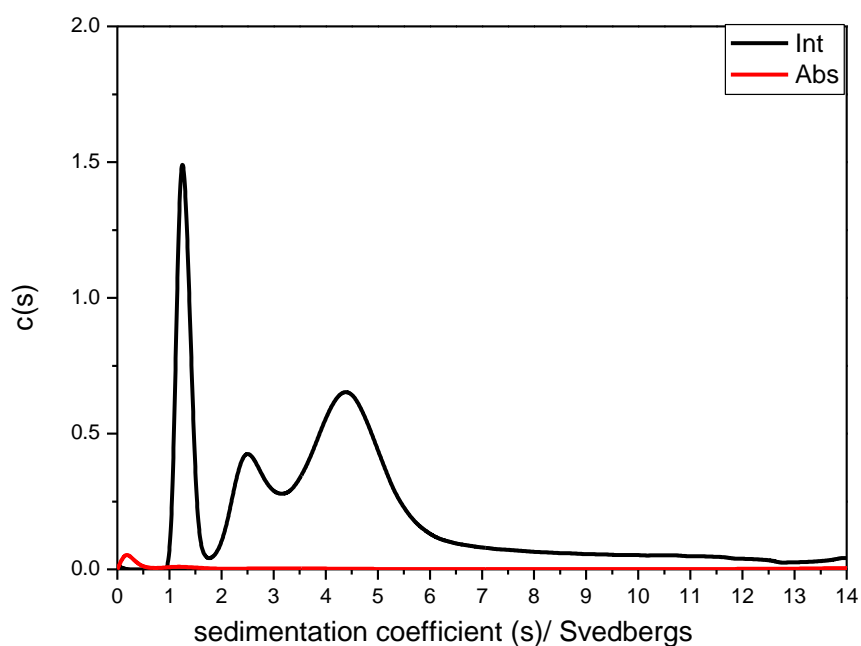


Figure 5.2: Sedimentation velocity of beta glucan BG1. Sedimentation coefficient distribution plots, $c(s)$ versus s from SEDFIT for beta glucan for interference and absorbance optics.

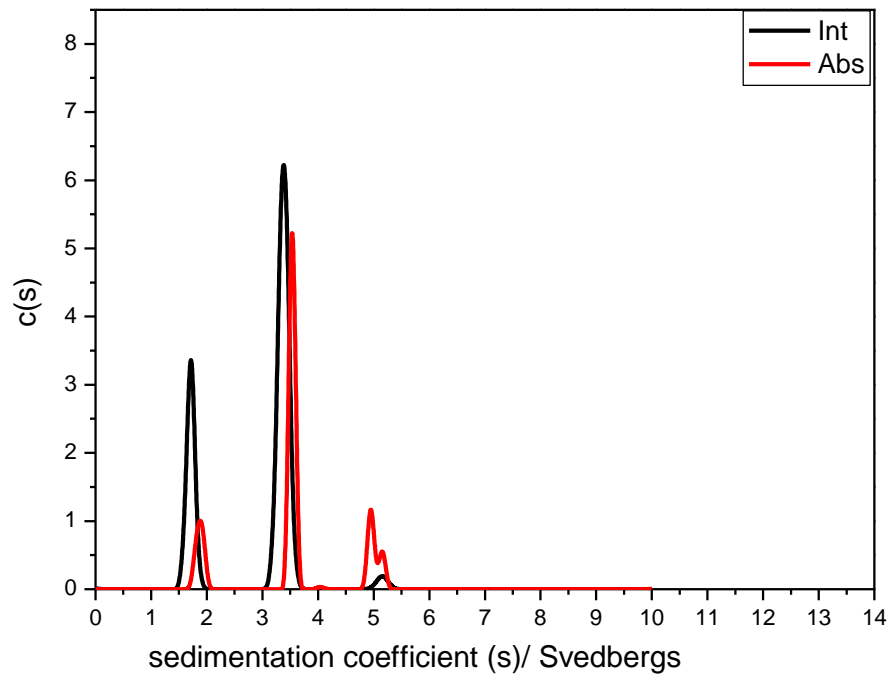


Figure 5.3: Sedimentation coefficient distribution plots, $c(s)$ versus s from SEDFIT for lipase solution of interference and absorbance optics.

The interaction between beta glucan and lipase was investigated using sedimentation velocity with different concentrations as it can be seen in Figs. 5.4, 5.5, 5.6, 5.7 and 5.8.

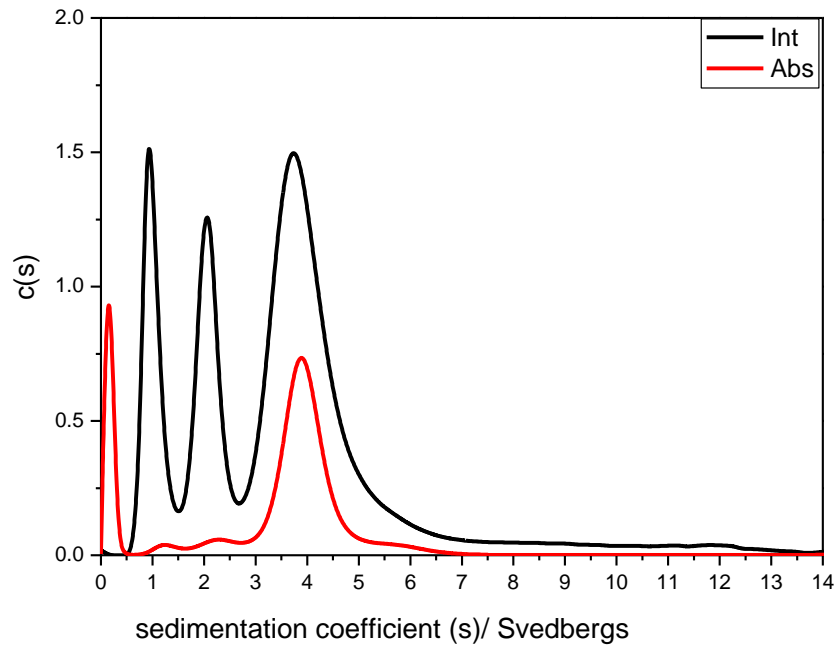


Figure 5.4: Sedimentation velocity of 0.5 mg/ml lipase + 0.6 mg/ml beta glucan BG1. Sedimentation coefficient distribution plots, $c(s)$ versus s from SEDFIT for lipase.

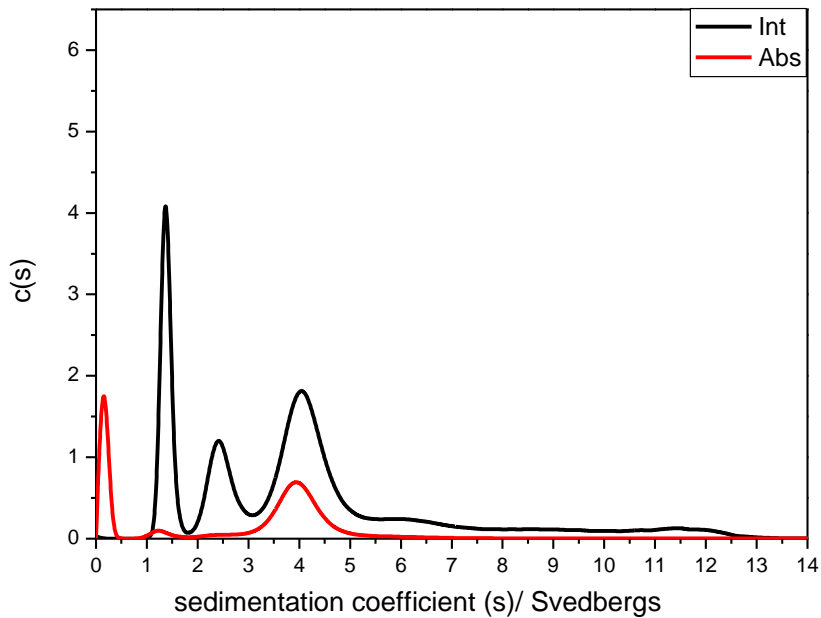


Figure 5.5: Sedimentation velocity 0.5 mg/ml lipase + 0.5 mg/ml beta glucan BG1. Sedimentation coefficient distribution plots, $c(s)$ versus s from SEDFIT for lipase.

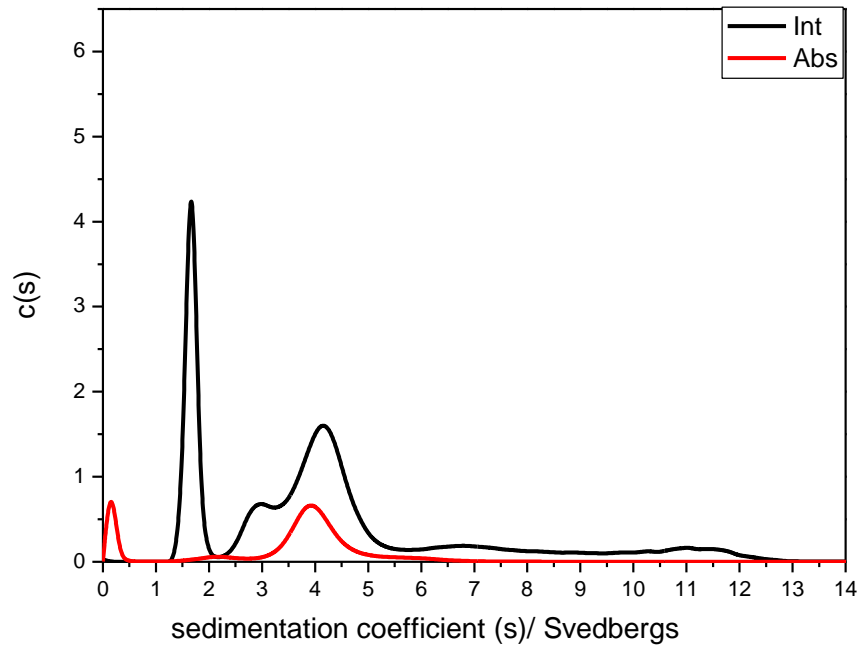


Figure 5.6: Sedimentation velocity of 0.5 mg/ml lipase + 0.4 mg/ml beta glucan BG1. Sedimentation coefficient distribution plots, $c(s)$ versus s from SEDFIT for lipase.

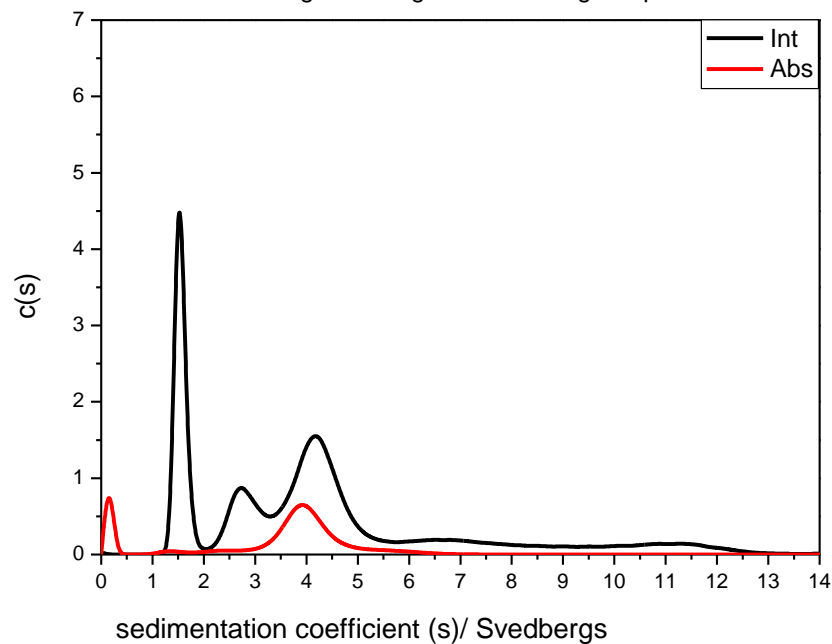


Figure 5.7: Sedimentation velocity of 0.5 mg/ml lipase + 0.3 mg/ml beta glucan BG1. Sedimentation coefficient distribution plots, $c(s)$ versus s from SEDFIT for lipase.

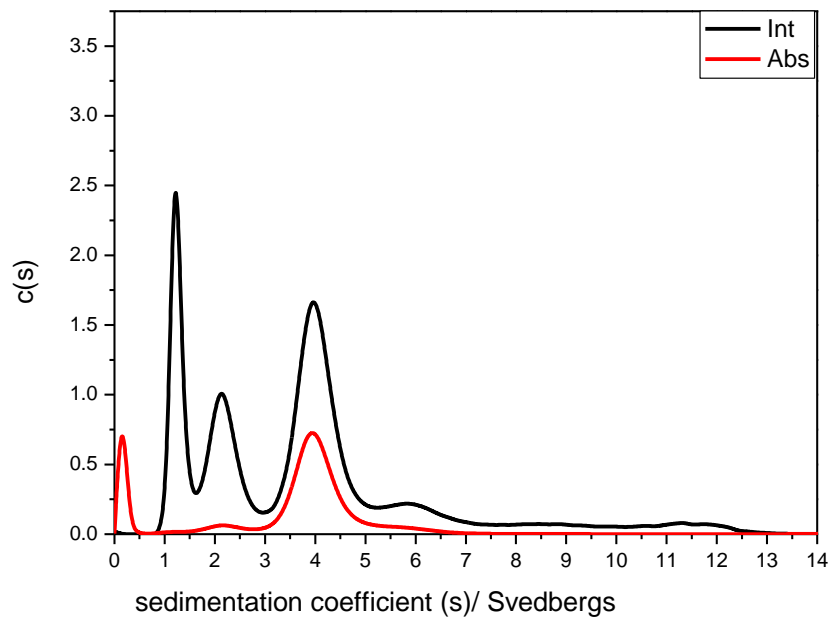


Figure 5.8: Sedimentation velocity of 0.5 mg/ml lipase + 0.2 mg/ml beta glucan BG1. Sedimentation coefficient distribution plots, $c(s)$ versus s from SEDFIT for lipase.

The composite comparison between sedimentation coefficient distributions from lipase and beta glucan is given in Fig. 5.9. From the plots there is little evidence of material with sedimentation coefficients higher than that of the beta glucan control, showing little effect on the beta glucan. However, there is a notable shift in the sedimentation coefficient of material absorbing in the UV at 280 nm (i.e. lipase) from $\sim 3.5S$ to $\sim 4.0S$, suggesting there may be an interaction, under the conditions studied. This interaction become clearer in Fig. 5.10 whereby we combine the uv absorption scans for the beta glucan (which shows no absorption), lipase and the lipase- beta glucan mixtures. The reason why an interaction may show a greater effect on the lipase rather than the beta-glucan properties is that beta-glucan has a much larger molecular weight. Lipases (depending on the

source) have a (weight average) molecular weight M_w generally $\sim 40\text{kDa}$ (Simpkin *et al*, 1991) whereas the beta glucan is $\sim 10\text{x}$ larger, as we will now explore.

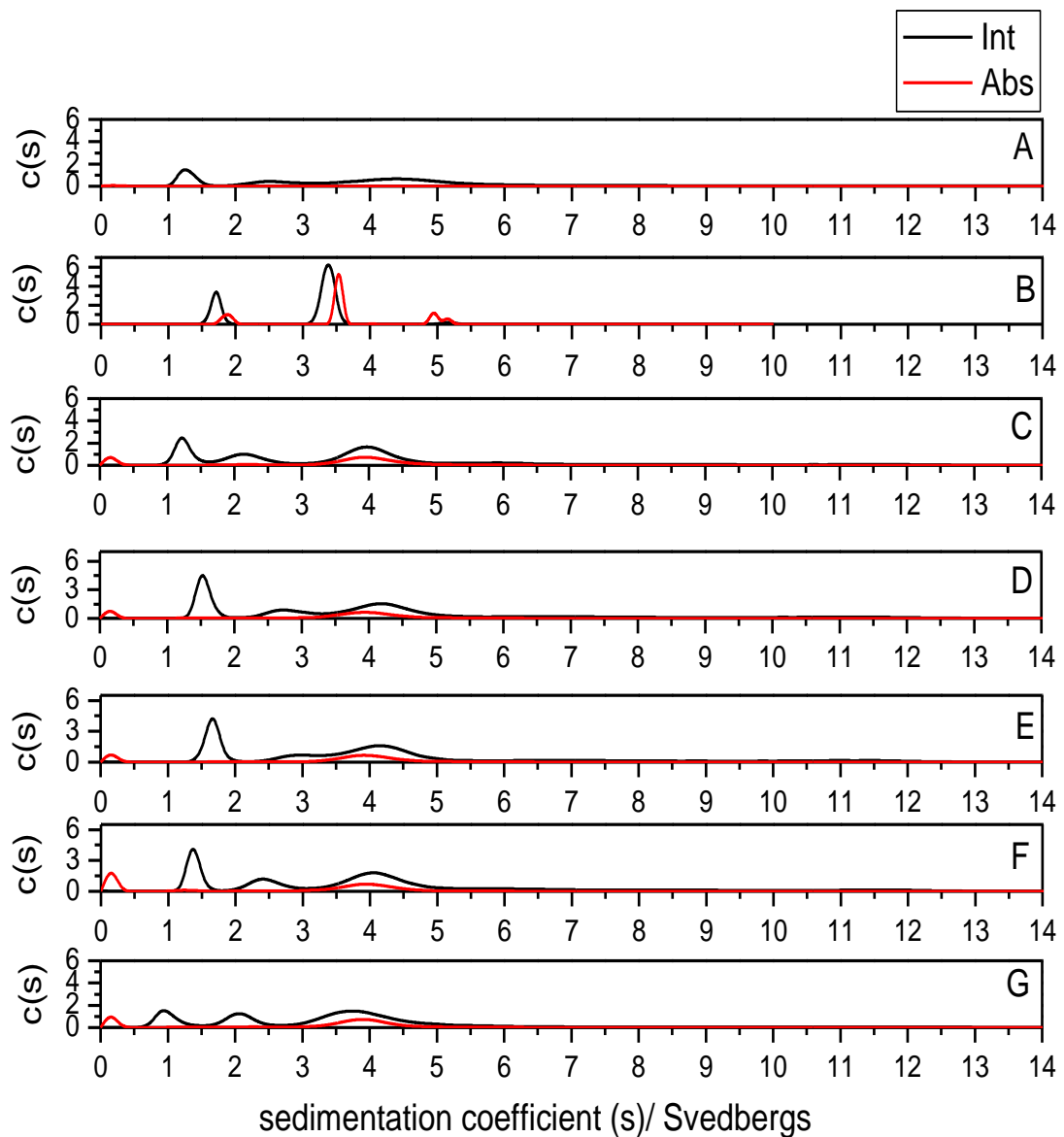


Figure 5.9: Sedimentation coefficient distribution plots, $c(s)$ versus s of
A: beta glucan only, B: Lipase only, C: 0.5 mg/ml lipase + 0.6 mg/ml beta
glucan BG1, D: 0.5 mg/ml lipase + 0.5 mg/ml beta glucan, E: 0.5 mg/ml
lipase + 0.4 mg/ml beta glucan, F: 0.5 mg/ml lipase + 0.3 mg/ml beta
glucan, G: 0.5 mg/ml lipase + 0.2 mg/ml beta glucan.

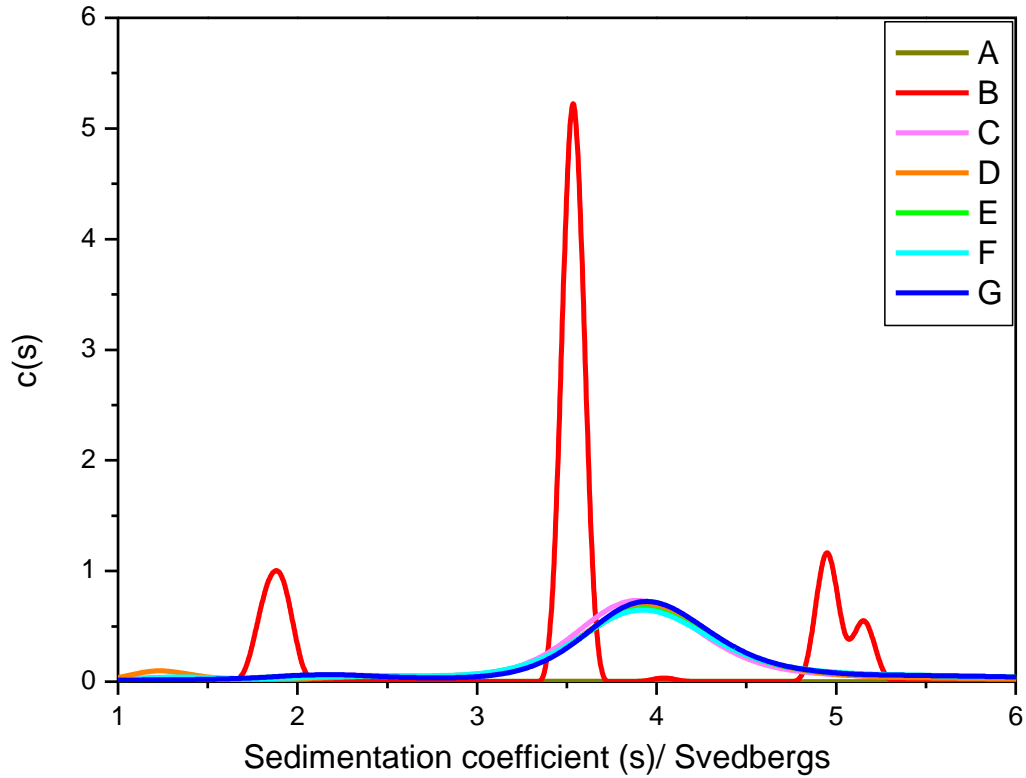


Figure 5.10: Sedimentation coefficient distribution plots of the absorbance profiles, $c(s)$ versus s of A: beta glucan only, B: Lipase only, C: 0.5 mg/ml lipase + 0.6 mg/ml beta glucan BG1, D: 0.5 mg/ml lipase + 0.5 mg/ml beta glucan, E: 0.5 mg/ml lipase + 0.4 mg/ml beta glucan, F: 0.5 mg/ml lipase + 0.3 mg/ml beta glucan, G: 0.5 mg/ml lipase + 0.2 mg/ml beta glucan.

5.3.2 Sedimentation equilibrium in the analytical ultracentrifuge

Sedimentation equilibrium was performed for beta glucan and apparent weight average molecular weights were obtained using SEDFIT-MSTAR as it can be seen in Fig. 5.11. The raw $c(r)$ data vs r in 5.11A. log concentration $\ln c(r)$ vs r^2 plot in Fig.5.11B show the best fit straight line (red line) with data. The M^* extrapolation to the base of the cell in Fig. 5.11C shows the molecular weight (red line arrow). M^* extrapolated to the cell base at $r=7.10\text{cm} = M_w$, the weight average molecular weight) = (420 ± 10) kDa. The local apparent or point weight average molecular weight $M_w(r)$ at individual radial positions r plotted against radius (red line) in Fig 5.11D shows values approximately similar to the value from M^* plot which gives the molecular weight over the whole distribution.

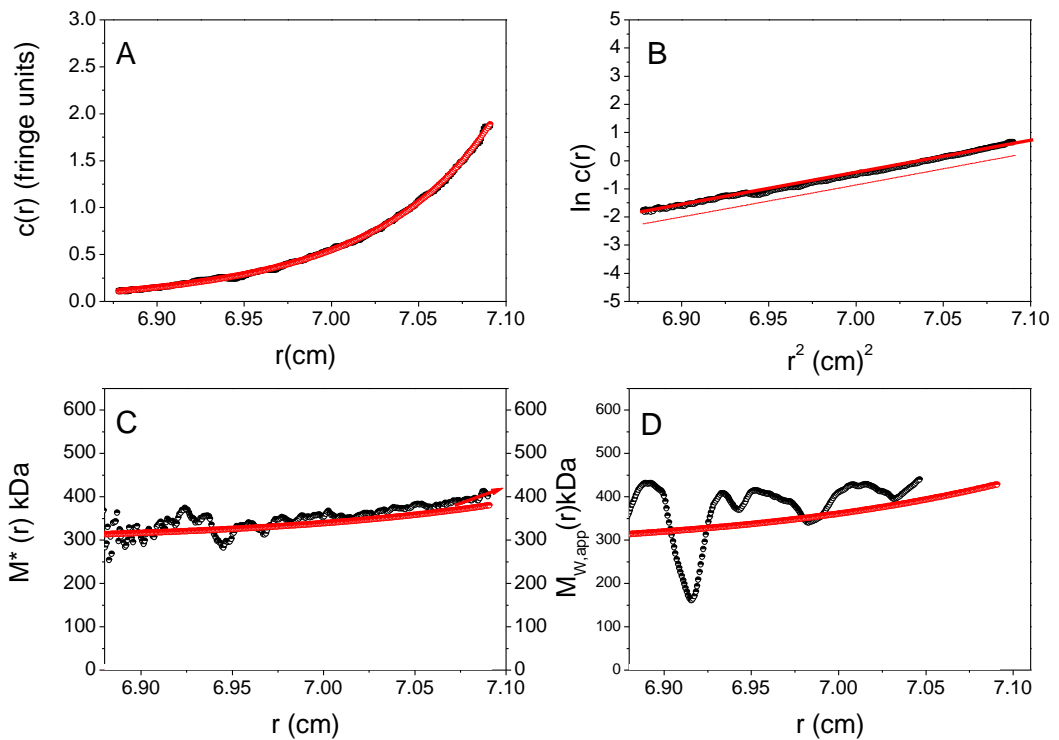


Figure 5.11: SEDFIT-MSTAR output for beta glucan BG1. (A): the raw $c(r)$ data vs r . (B): log concentration $\ln c(r)$ vs r^2 plot. (C): M^* vs r plot. The weight average molecular weight for the whole distribution $M_w = M^*$ (at the cell base, $r=7.10\text{cm}$) = (420 ± 10) kDa (D): local apparent or point weight average molecular weight $M_w(r)$ at radial positions r plotted against radial position (distance from the centre of rotation).

The red line in all plots represents the fit.

5.3.3 Capillary viscometry

The Huggins, Kraemer and Solomon-Ciuta are used to derive the intrinsic viscosities $[\eta]$ of the four solutions of beta glucans (BG1, BG2, BG3, BG4), to see if the processing of the thermal (BG2)/ freezing (BG3)/ storage (BG4) processing had an effect on the beta glucans. Intrinsic viscosity is a measure of the molecular weight, conformation and volume of the beta-glucan macromolecules (see Harding, 1997). Fig. 5.12 shows the values that are retrieved from the analysis of these solutions. Reduced viscosities fitted to the Huggins equation and inherent viscosities to the Kraemer equation, yielding an intrinsic viscosity $[\eta]$ in ml/g. (For interpretation of the references to colour in this figure legend) and the intrinsic viscosities of four samples are as follows, BG1 = (509 ± 19) ml/g, BG2 = (543 ± 33) ml/g, BG3 = (509 ± 10) ml/g and BG4 = (734 ± 49) ml/g. The autoclave process was used to acquire the values from the beta glucan. The samples that were kept at room temperature projected a gain in the intrinsic viscosity whereas a lesser level of intrinsic viscosity was noticed in the samples of frozen storage of the native beta glucans as in Fig. 5.12.

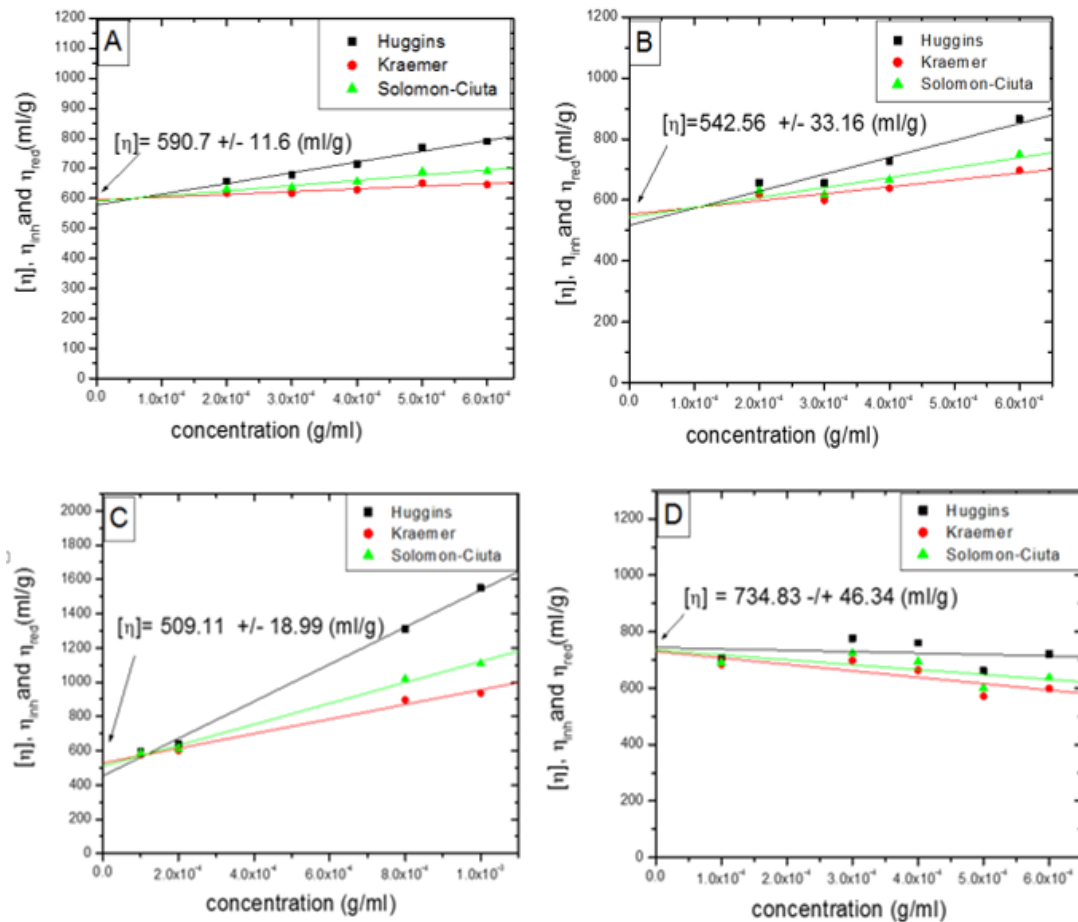


Figure 5.12: Plots of reduced viscosity (black data points) and inherent viscosity (red data points) from the U-tube viscometer at 20°C. A) BG1, B) BG2, C) BG3 and D) BG4.

Thermal processing (BG2) and Freezing (BG3) showed a ~ 10% lowering of $[\eta]$, corresponding to a reduction in molecular weight and volume of the beta-glucan. Storage seemed to have the opposite effect, leading to an aggregation (increase in $[\eta]$ of ~ 20%).

We finish this study by seeing if there is a change in the large aggregates, using dynamic light scattering, providing complementary information to viscosity.

5.3.4 Dynamic light scattering (DLS) of beta glucan

The DLS measurements were performed at a scattering angle of 173° to monitor for the presence/ change of *larger molecular weight aggregates* due to the processing of the thermal (BG2)/ freezing (BG3)/ storage (BG4) processing of the beta glucans. Beta glucan solutions were used at 0.6 mg/ml. The values of hydrodynamic radius r_H are displayed in the Figure 5.13, which shows that they are in similar range.

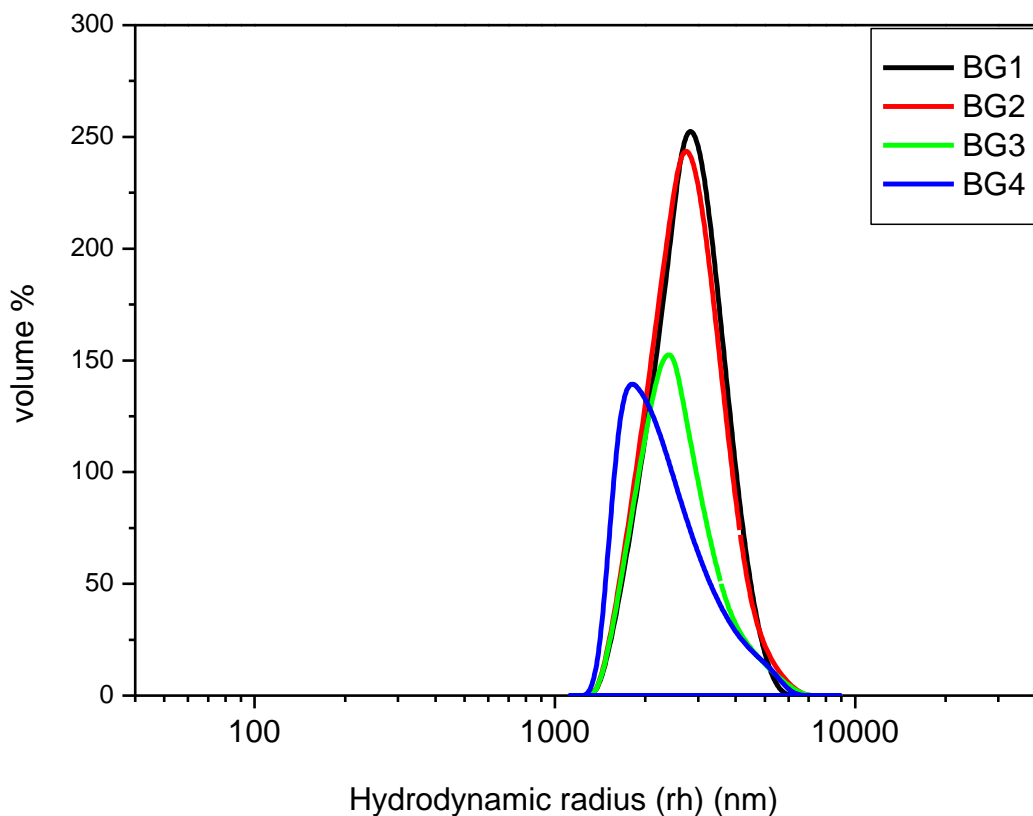


Figure 5.13: DLS of the large particulates of the beta glucans as a function of processing conditions. Plot of volume% vs size (r_h) for the beta glucans. No noticeable difference is seen

5.4 Discussion

In this chapter we have shown that:

The beta glucan (BG1) has a broad sedimentation coefficient profile, with sedimentation coefficients of $\sim 1.3S$, $\sim 2.55S$ and $4.5S$ with a broad tail from $6S$ is representing polydispersity.

With the sedimentation velocity study on the mixtures of BG1 with lipase at different mixing ratios, there was no noticeable effect on the sedimentation of the beta-glucan. However, the lipases – and whatever was bound to them – consistently showed an increase of $\sim 10\%$, which indicates an interaction.

Sedimentation equilibrium showed that BG1 has a weight average molecular weight of (420 ± 10) kDa, from the M^* analysis methods, a result consistent with the point average (apparent values $M_{w,app}(r)$ values as a function of radial position, r . Note that the result of (420 ± 10) kDa has not been corrected for non-ideality, we have assumed that at the concentration used non-ideality is relatively small. $[\eta] = 591 \pm 2$ (ml/g) for this beta glucan BG1. The intrinsic viscosity decreased by $\sim 10\%$ for BG2 (thermally processed) and BG3 (freezing processed) but actually increased $\sim 20\%$ for BG4 (storage processing), showing that thermal processing and freezing appears to decrease the molecular weight and/or volume of the macromolecules, whereas the reverse occurs on storage.

No noticeable change in the large aggregate profiles were seen from DLS for processing, with BG1, BG2, BG3 and BG4 all showing similar distributions.

According to the application of intrinsic viscosity in the industry of food, autoclaving process is used more often for sterilising the oat beta glucans. This result in highlighting the effect on the polysaccharide's hydrodynamic characteristics, which is needed to be investigated attentively (Wang, 2001). In addition to this, the study has included the samples of beta glucans that are autoclaved and direct towards a minor deduction in the reports of the intrinsic viscosity $[\eta]$. The research study has been in favour of close agreement and

supported with the available literature (Wang, 2001). In the condition of a little reduction in intrinsic viscosity, the result in the reality that, contrary to other water-soluble polysaccharides for example κ -carrageenan and guar galactomannan, beta glucans resist heat treatment (Kök *et al.*, 1999; Lai, 2000).

The reason associated with the decrease in the intrinsic viscosity is due to a partial depolymerisation of the beta glucan molecule. It also leads to the reduction in the molecular weight. It can be better understood with the help of an example. As per the viewpoint of Wang *et al.*, (2001), the research study has explored the concept and observed that there is a reduction in oat beta glucan's intrinsic viscosities $[\eta]$ and samples following autoclaving is because of polymer degradation. According to Dongowski (2005), a study conducted on the heat treatment found that the beta glucans' molecular weight has been infused from the flour and the intrinsic viscosity reduces due to heat treatment. The intrinsic viscosity $[\eta]$ helps in measuring the hydrodynamic volume of the solute molecules. In case of many polymers, the intrinsic viscosity might be related empirically to the molecular weight by the Mark-Houwink relation. The determination of decrease in the intrinsic viscosity and featured to the happening on the oxidative reactions during the treatment by heat. Autoclaving is affecting the oxidised functional groups. According to the viewpoint of Kivela (2012), it has been seen from the information that autoclaving can be the reason of formation of functional group's oxidation. It could result in the formation of carbonyl groups, which is oxidised functional groups (alkane) along with cleavage of backbone (Kivela, 2012).

The data findings derived from the research study is in accordance with the findings of previous research (Moriarty *et al.*, 2010) and others. According to Lazaridou and Biliaderis (2004), frozen storage of food items at a temperature of -4°C helps in increasing the rapid formation of small ice crystals (Lazaridou and Biliaderis, 2004). The sample of food has been cold stored at a temperature of -4°C so that its time of creation into small ice crystal can be identified. The structure

has assisted in dividing the molecular bonds and also helps in decreasing the molecular weight of the solutions of beta glucans. In addition to this, there is direct relationship in between the solubility of the molecules of beta glucans which reduces due to the deduction in the intrinsic viscosity of the samples (Moriartey *et al.*, 2010). So our current intrinsic viscosity results appear consistent with this finding.

Beta-glucans are also susceptible to the formation of aggregates. According to Lazaridou *et al.*, (2003), it can be seen that, at many times, a tiny modification in the concentration or the macromolecular size can result to the significant enhancement in the intrinsic viscosity (Lazaridou, *et al.*, 2003). Thus our observation of an increase in the intrinsic viscosity with storage time is not surprising.

Chapter 6: Hydrodynamic studies on *Nigella sativa*

6.1 Introduction

Nigella sativa, a widely used medicinal plant belonging to the family *Ranunculaceae*, is a common ingredient in many types of traditional medicines around the world. Ancient medicinal practices like Unani, Siddha or Ayurveda have mentioned the medicinal properties and uses of the plant. Also known as black cumin, the seeds of the plant yield oil which is linked to history of traditional medicine. Some of the medicinal uses of *Nigella sativa* are against hypertension, diuretics, and appetizers with additional antimicrobial properties. Although *Nigella sativa* seeds appears to have various medicinal properties, these remain under continued and thorough investigation (Mathur *et al.*, 2011).

Nigella sativa, an annual flowering plant, which grows to an average height of 20–30 cm and bears delicate white or pale flowers, is commonly found in Southern Europe, Southwest Asia as well as North Africa. Apart from its native habitat, the plant is cultivated worldwide in various countries due to its therapeutic properties. However, the true origin of the plant seems to be the Middle-East countries, where it is known as *gizah* or *haba-al-barakah*. Apart from its medicinal properties, the seed of the plant is also used as flavoring agents in foods such as pickles (Sativa, 2014).

6.1.1 Biochemical composition of *Nigella sativa* seeds

There are various compounds that have been isolated from the seed of the plant. Among the biochemical compounds, the one with the highest bioactivity is thymoquinone (see Fig. 6.1) with approximately 30–40%. Other compounds isolated and identified include thymohydroquinone, dithymoquinone and p-

cymene accounting for approximately 7–15%. Other compounds also include 4-terpineol, t-anethol, sesquiterpene longifolene, α -pinene and thymol. As with many other plants with medicinal properties, the black cumin/black seed contains alkaloids—isoquinoline alkaloids like nigellicimine as well as pyrazole alkaloids which include nigellicine and nigellidine (Nickavar *et al.*, 2003).

In addition, black cumin is a rich source of protein, fat and carbohydrate as well as crude fiber amounting to approximately 27%, 29%, 25% and 8.5%, respectively. Vitamins and minerals such as Cu, P, Zn and Fe are also present in the seeds. Fats can be further classified into saturated and unsaturated fatty acids. Unsaturated fatty acids include linoleic acid, oleic acid, eicodadienoic acid and dihomolinoleic acid. The saturated fatty acids are palmitic and stearic acids with trace compounds such as glycosides, terpenoids, oleic acids also found in the seed of *N. sativa* (Nickavar *et al.*, 2003).

6.1.2 Antidiabetic properties of *Nigella sativa* seeds

Nigella sativa seeds are used in various ailments including diabetes. The potential of *N. sativa* seeds to control blood glucose level has been previously assessed (Bamosa *et al.*, 2010). Ninety-four patients suffering from *Diabetes Mellitus* (DM) were selected randomly and distributed into three groups which received different amount of *Nigella sativa* seed containing capsules. The ability of *N. sativa* to control blood glucose levels were assessed using measurements of fasting blood glucose (FBS) two hours after meal. Apart from FBS levels, glycosylated hemoglobin (HbA1c) was also assessed. Changes in body weight and serum C-peptide were taken into account for the assessment of efficacy of the black seed in diabetes management. Using the homeostatic model assessment (HOMA2) the case of insulin resistance and β -cells function were also determined by Bamosa *et*

al., (2010). It was observed that the study group receiving *N. sativa* at a daily dose of 2g was seen to have a lower blood glucose level compared to the controls. Moreover, there was not much change in body weight along with reduction in HbA1c. The interesting part is that function of β -cells increased significantly followed by reduction in insulin resistance. Subsequent increase in dosage did not show much change in the observed parameters different from the 2g/day dose (Bamosa *et al.*, 2010).

6.1.3 Thymoquinone of *Nigella sativa* is an antidiabetic

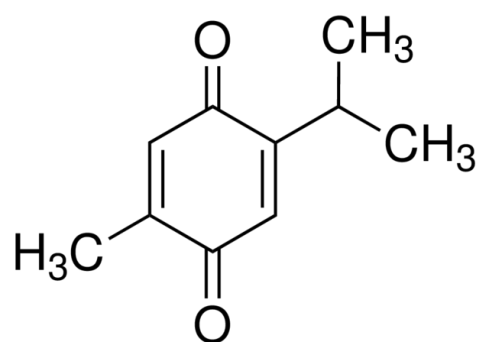


Figure 6.1: The biochemical structure of thymoquinone (Gali-Muhtasib *et al.*, 2006).

A study in streptozotocin (STZ) induced diabetic rats indicated that thymoquinone (TQ) has a potential as an antidiabetic drug. In the study, aqueous extracts of *N. sativa*, oil extract and TQ were tested for their efficacy in controlling blood sugar. Post STZ examination, electron microscope observation showed subcellular changes were observed in rats treated with *N. sativa* extract or thymoquinone with a significant reduction in diabetes-induced tissue pancreatic tissue malodialdehyde (MDA), serum glucose concentration and increased circulating

insulin as well as superoxide dismutase (SOD). Electron microscope observations showed that TQ prevented many aspects of cellular impairment such as DNA damage and mitochondrial membrane compromization induced by STZ. The study concluded that TQ has the potential to maintain β -cell integrity and decrease oxidative stress (Abdelmeguid *et al.*, 2010).

A study carried out by Pari and Sankaranarayanan determined the antidiabetic potential of TQ in STZ-induced diabetic mice, where TQ different dosages were administered orally. Results indicated that serum glucose level were significantly reduced in diabetic mice, where TQ was administered (Pari and Sankaranarayanan, 2009).

6.1.4 Oleic acid and linoleic acid as antidiabetic components

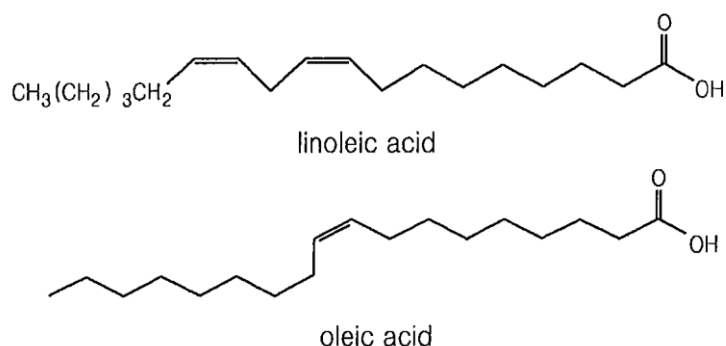


Figure 6.2: The biochemical structure of oleic acid and linoleic acid.

The seeds of *N. sativa* have a high content of oleic acid as well as linoleic acid. The antidiabetic properties of plant seeds are purported to support the positive effects in the management of diabetes as a result of the numerous unsaturated

fatty acids present. It is a well-known fact that obesity is a risk factor for developing non-insulin dependent diabetes mellitus. Adipose tissue on the other hand is known to produce large amount of tumor necrosis factor alpha (TNF- α). TNF- α is shown to inhibit production of insulin secretion as well as promote insulin resistance. In a study conducted to determine the effects of unsaturated fatty acid oleic acid on insulin production in presence of TNF- α , Vassiliou and colleagues observed that the unsaturated fatty acid had the ability to reverse the effects of TNF- α and promote insulin production (Vassiliou *et al.*, 2009).

Linoleic acid, one of the major component of seed of black cumin, is also a known to have antidiabetic properties. The mode of action of oleic acid, as with linoleic acid, seems to be anti-inflammatory with regards to adipose tissue, which prevents the secretion of TNF that can inhibit insulin secretion (Moloney *et al.*, 2007).

The aim of this chapter was to assess the hydrodynamic characteristics of *Nigella sativa*.

6.2 Materials and Methods

6.2.1 Materials

6.2.1.1 Chemicals

Sodium chloride, potassium dihydrogen phosphate, disodium dihydrogen phosphate, all were purchased from Sigma.

6.2.2 Methods

6.2.2.1 Preparation of *Nigella sativa* solutions

The *Nigella sativa* seeds were ground to a powder using electric grinder (Kenwood Blender (Model BL 650) then they have been weighted. The powders were dissolved in deionized water (1g/20 ml) at 45°C for 3 hours with constant stirring. The samples were centrifuged (Beckman centrifuge Model J2-21M) at 4800rpm for 20 min at 20°C. The supernatant were concentrated at 45°C for 17 hours. The concentrated samples were filtered using 90 mm filter paper. For each sample 10 ml of 5mg/ml solution were dissolved in buffer solution at pH=7.0 and ionic strength 0.1M. The solutions were left to hydrate on a roller mixer at room temperature overnight. The final dissolved was used for all subsequent dilutions.

6.2.2.2 Sedimentation velocity of *Nigella sativa* in the analytical ultracentrifuge

A Beckman XL-I ultracentrifuge was used as considered in section 2.3.4. Samples (~395µl) of concentrations 0.40, 1.00, 2.00, 3.00 and 4.50 mg/ml and PBS buffer dialysate (400µl) at pH 7.0 and ionic strength 0.1M were injected into sample and reference channels respectively of double sector 12 mm optical path length cell.

The balanced cells were then loaded into an analytical 8-hole titanium rotor An50-Ti and placed in the AUC. As before the Rayleigh interference optical system was used for recording concentration profiles and the movement of the sedimentation boundary in the analytical ultracentrifuge cell (Harding, 2005). An initial low rotor speed of 3000 rpm was adjusted to a rotor speed of 40000 rpm. The standard conditions of density and viscosity of water at 20°C were used for adjustment of sedimentation coefficients s (Schachman, 1992). Scans were taken at 2 min intervals for a run time of \sim 24 hours. The data was analysed using the "least squares $l_s-g^*(s)$ model" SEDFIT algorithm in terms of distributions of sedimentation coefficient distribution $l_s-g^*(s)$ vs s (see e.g. Dam and Schuck, 2003; Harding, 2005) to provide an assessment of sample polydispersity.

6.2.2.3 Capillary viscometry of *Nigella sativa*

The viscosity of series solutions ranging in concentration 0.50, 0.70, 0.90, 1.1, 1.3 mg.ml⁻¹ were measured using an Ostwald viscometer. The U-tube viscometer was suspended in an accurate temperature regulated water bath. The temperature was kept constant at 20°C throughout. The amount of each solution injected into the viscometer was 2ml and the solution flow time t determined manually (particularly for highly viscous solution) using clock timer. From the flow time of the buffer (t_0) the ratio t/t_0 of the solution was estimated. The relative viscosity η_r was then estimated as t/t_0 where t_0 is the flow time of the buffer (Harding, 1997). Viscosities were also checked with a rolling ball viscometer.

6.2.2.4 Dynamic light scattering (DLS) of *Nigella sativa*

The hydrodynamic radius r_h of samples was estimated by Dynamic light scattering (DLS) (see e.g. Harding *et al.*, 1992) using the Zetasizer Nano-ZS apparatus (Malvern, UK). A stock solution of each sample was prepared in 20 ml PBS at concentrations of ~ 2.5 mg/ml. All solutions were made up to have 0.1M salt content and to be pH 7. All the solutions were centrifuged for 20 min to remove all impurities and air bubbles then filtered using a 0.2 μm pore size sterile filter. 1 ml of solution was placed in a glass cuvette which was then placed in a temperature controlled sample environment. The (Z-average) translational diffusion coefficient D was measured at a low angle of 13° (to minimise complications through translational diffusion- Burchard, 1992), at temperatures of 20°C . Five measurements, consisting of 60 sub-runs each, were made for each sample. The hydrodynamic radius r_h was then obtained from D according to the following Stokes-Einstein equation:

$$D = \frac{k_B T}{6\pi\eta r_H} \quad (6.1)$$

The molecular weight can then be calculated by combination with the sedimentation coefficient from sedimentation velocity.

$$M = \frac{RTs}{D(1-\bar{v}\rho)} \quad (6.2)$$

Where: k_B is the Boltzmann constant, T is the absolute temperature, η_0 is the viscosity of the solvent. (see Harding *et al.*, 1992).

6.2.2.5 Fourier Transform Infrared Spectroscopy (FTIR) of *Nigella sativa*

The dry samples were prepared as in (6.2.2.1). Characterization was carried out using FTIR (IRAffinity-1S Shimadzu model), scanning range was 4500 to 400 cm^{-1} with a resolution of 4 cm^{-1} and 20 spectra were obtained. The shifts in the respective IR spectra bands were observed and reported. FTIR analysis of functional groups were analysed.

6.3 Results

6.3.1 Sedimentation velocity in the analytical ultracentrifuge

The sedimentation coefficient distribution for *Nigella sativa* (Fig. 6.4) showed a main peak with a very low sedimentation coefficient (within the range 0.2-0.4 S). The samples in Fig. 6.3 by contrast showed a homogeneous only a single significant peak at almost 0.3S.

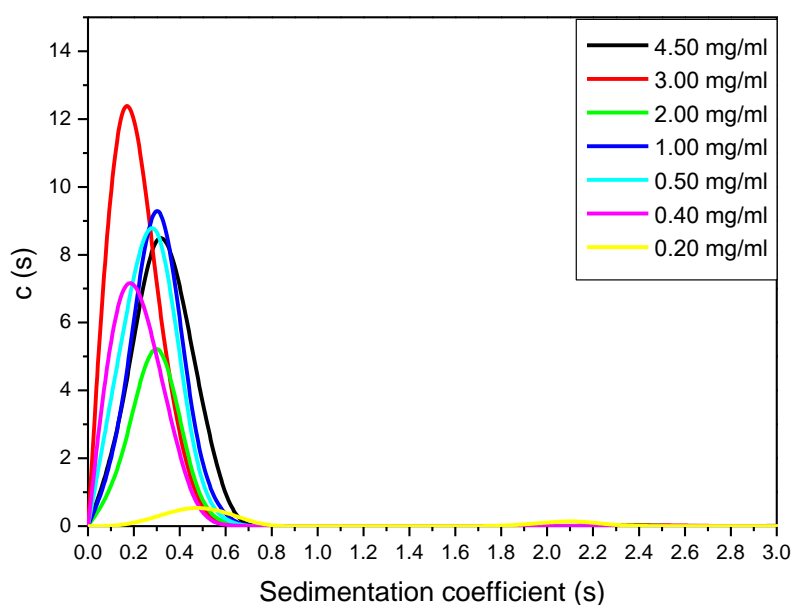


Figure 6.3: Sedimentation velocity of *N. sativa*. Sedimentation coefficient distribution plots, $c(s)$ versus s from SEDFIT for *N. sativa*. Rayleigh interference optics used, which records all macromolecule components.

Fig. 6.4 shows strong unimodality with one major peak for each concentration studied as a homogeneous molecular species at ~ 0.2 S.

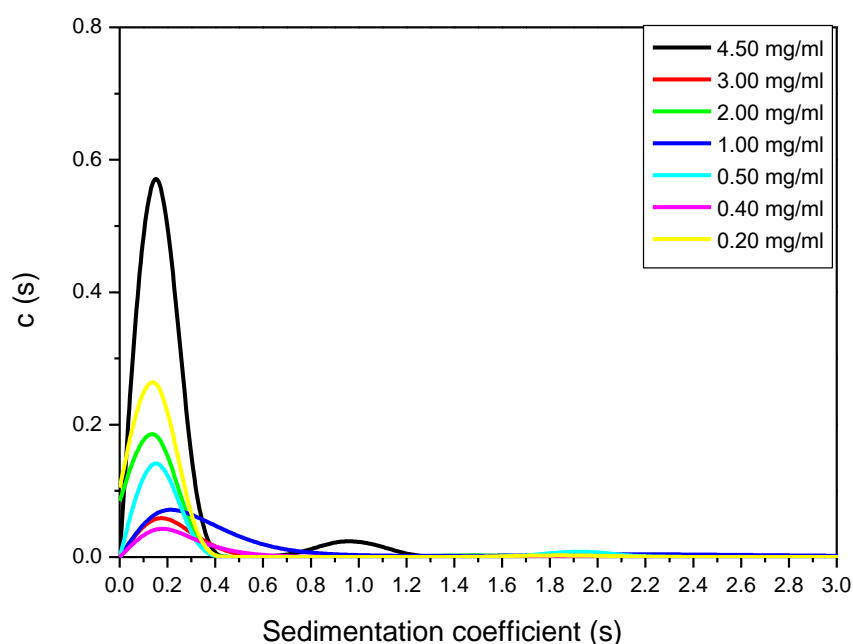


Figure 6.4: Sedimentation velocity of protein *N. sativa*. Sedimentation coefficient distribution plots, $c(s)$ versus s from SEDFIT for *N. sativa* Absorption optics at 280nm used, which records the sedimentation of proteins plus any other components bound to the proteins.

6.3.2 Sedimentation equilibrium in the analytical ultracentrifuge

Sedimentation equilibrium was performed for *N. sativa* and apparent weight average molecular weights were obtained using SEDFIT-MSTAR as it can be seen in Fig. 6.5. The raw $c(r)$ data vs r in 6.5A. Log concentration $\ln c(r)$ vs r^2 plot in Fig.6.5B show the best fit straight line (red line) with data. The M^* extrapolation to the base of the cell in Fig. 6.5C shows the molecular weight (red line arrow). This gives a reliable estimate for the weight average molecular weight over the distribution (M^* at the cell base = M_w – see Schuck et al, 2014), of (230 ± 20) kDa.

The local apparent or point weight average molecular weight $M_w(r)$ at radial position r plotted against concentration for different radial positions (red line) in Fig 6.5D shows the estimate of M_{wapp} . Despite the noise the data is at least consistent with the value of M_w from Fig 6.7C.

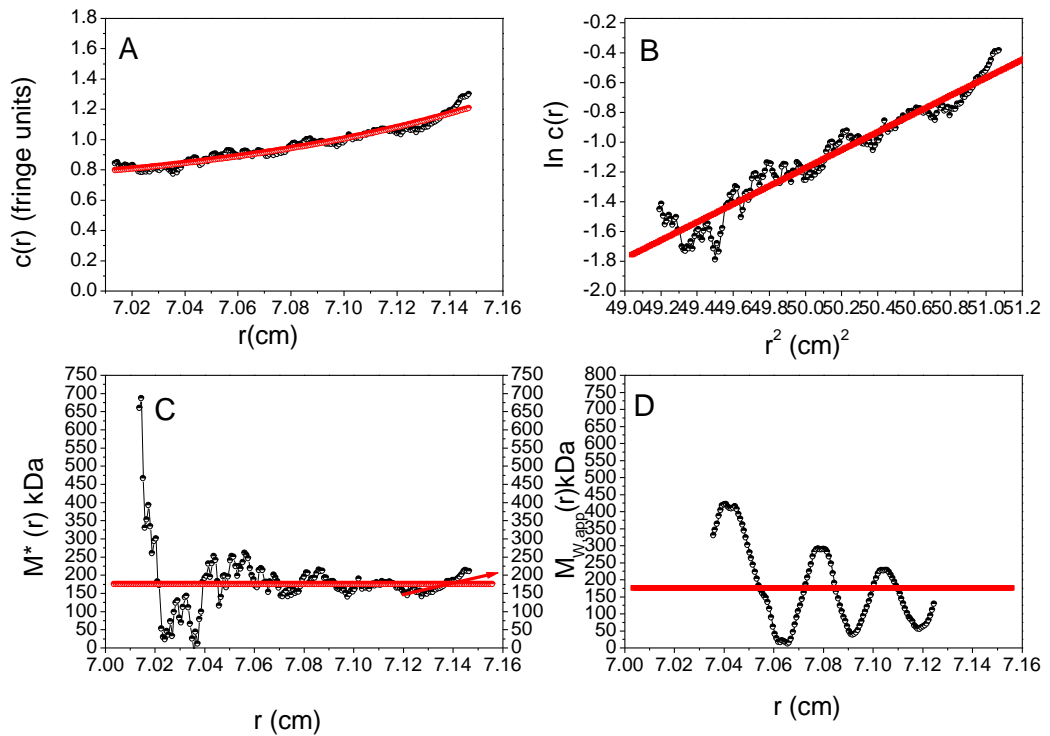


Figure 6.5: SEDFIT-MSTAR output for *N. Sativa* from M*-cell base $M_w = (230 \pm 20)$ kDa. (A): the raw $c(r)$ data vs r (B): log concentration $\ln c(r)$ vs r^2 plot (C): M^* vs r plot (D): local apparent or point weight average molecular weight $M_w(r)$ at radial position r plotted against concentration for different radial positions. The red line in all plots represents the fit.

6.3.3 Capillary viscometry

The intrinsic viscosity $[\eta]$ are shown in Fig. 6.6 they extrapolate to a common intercept $[\eta] = (86.5 \pm 5.9) \text{ ml/g}$.

Reduced viscosities fitted to the Huggins equation and inherent viscosities to the Kraemer equation, yielding an intrinsic viscosity $[\eta]$ of $(86.5 \pm 5.9) \text{ ml/g}$ in Fig. 6.6. Similar results were obtained from the rolling ball viscometer.

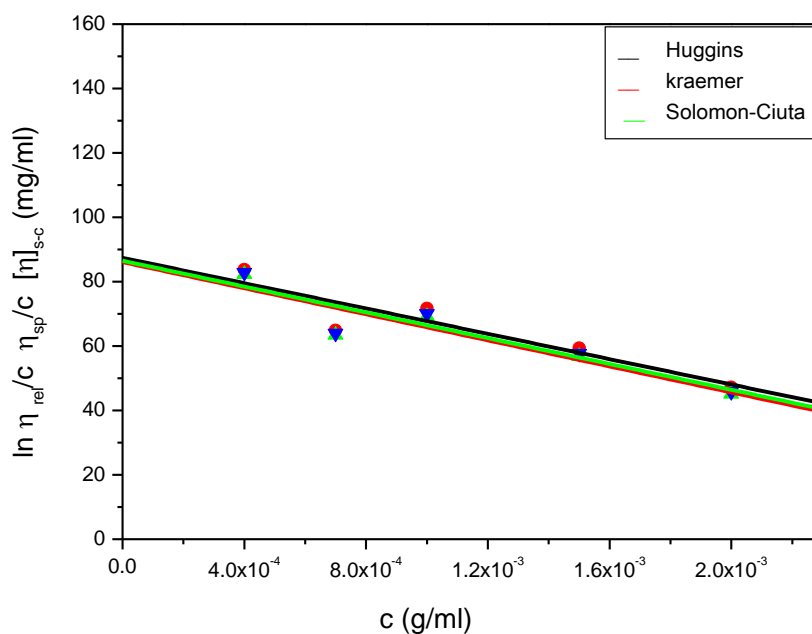


Figure 6.6: Plots of reduced viscosity (black data points) and inherent viscosity (red data points) from the rolling ball viscometer at 20.0°C.

6.3.4 Dynamic light scattering (DLS) of *Nigella sativa*

The size distribution (by volume) from *Nigella sativa* samples is shown in Fig. 6.7. The results showed differences between the different concentrations. *Nigella sativa* in this study was a homogenous sample showed a single peak and the radius of (r_h) is shown in Fig. 6.7 with a mean size of approximately 20-80 (nm).

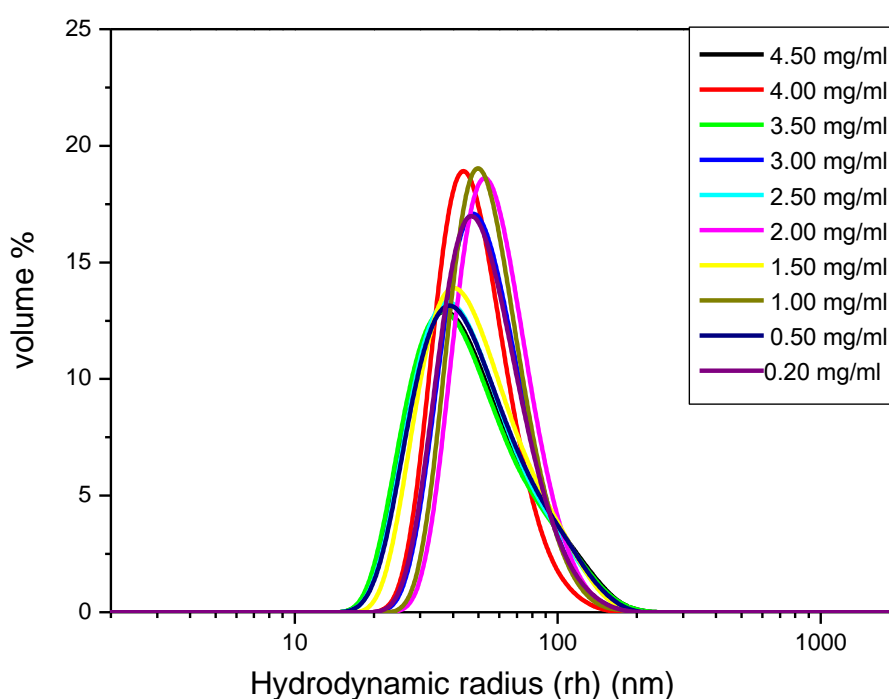


Figure 6.7: Plot of volume% vs size r_h (nm) for *Nigella sativa* at a scattering angle of 13° .

6.3.5 FTIR Analysis

FTIR spectra of *Nigella sativa* is shown in Fig. 6.8. The mean broad band is about 3400 cm^{-1} (N-H & O-H bond) and the narrow bands at 1600 cm^{-1} (C=O bond),

1500 cm^{-1} and 1100 cm^{-1} (C-O bond). Also in Fig. 6.8 it has the narrow peak at about 2900 cm^{-1} (Characteristic of the symmetric and asymmetric vibrations) with a strong band (Nurrulhidayah *et al.*, 2011).

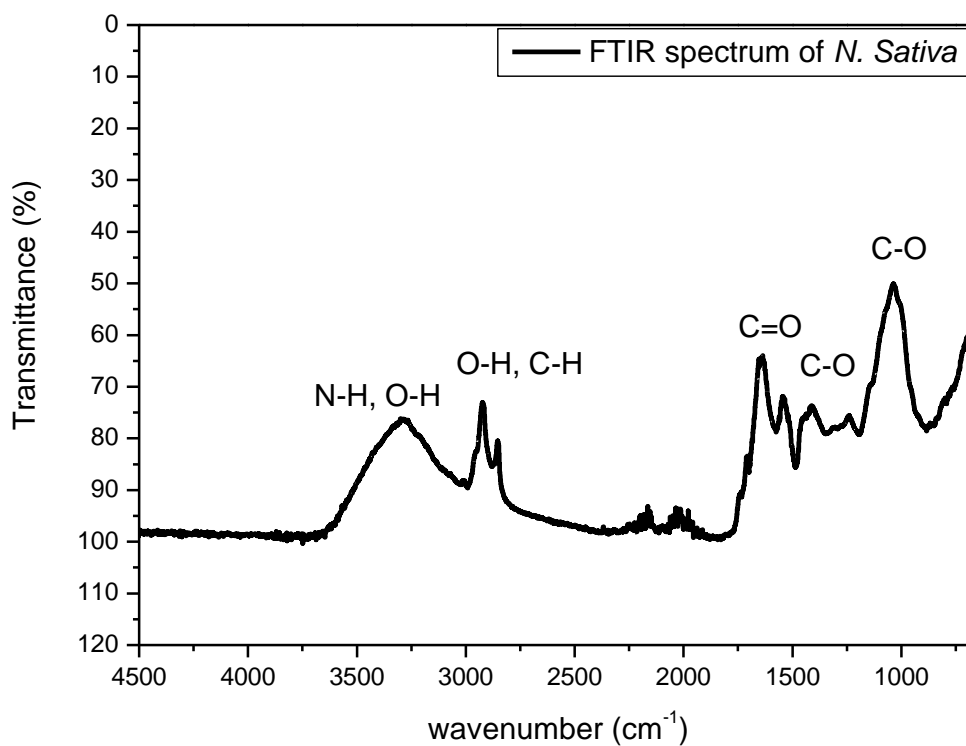


Figure 6.8: FTIR spectrum of *N. sativa*.

Table 6.1. Summary of sedimentation coefficient (s), weight average molecular weight (M_w), the intrinsic viscosity $[\eta]$ and Stoke radius r_h (nm) obtained from AUC, viscometry and DLS for *N. sativa*

| <i>N.sativa</i> Sample | $s^{\circ}_{20,w}$ (S) Interference | $s^{\circ}_{20,w}$ (S) Absorbance | M_w kDa (from SE) | $[\eta]$ (ml/g) | r_h (nm) |
|---------------------------|--|--------------------------------------|---------------------------|--------------------|------------|
| <i>N.sativa</i> | 0.3 | 0.2 | 230±20 | 86.5±5.9 | 60 |

6.4 Discussion

Heterogeneity and sedimentation coefficients

Sedimentation velocity in the analytical ultracentrifuge for *N. sativa* in Fig. 6.3 showed a homogeneous only a single significant peak at almost 0.3S. Fig. by contrast, Fig. 6.4 showed samples had a distribution of sedimentation coefficients for *Nigella sativa* showed a main peak with a very low sedimentation coefficient (within the range 0.2-0.4 S) and two clear minor peaks at higher sedimentation coefficient seemed to be present as a heterogeneous. The major component of *N. Sativa* in this study proved to be a homogeneous protein in Fig. 6.4. However, the high concentration at 4.50 mg/ml shows a heterogeneous sample with the main peak at 0.2 S and the other peak at around 1 S.

Sedimentation equilibrium in the analytical ultracentrifuge

From the plot in 6.5C it shows that *N. sativa* has a molecular weight of (230 ± 20) kDa. This value represents the whole macromolecular components (protein + carbohydrate), and is, not surprisingly, higher than the estimates given by the protein component alone (94-100 kDa), using the technique of sodium dodecyl sulfate polyacrylamide gel electrophoresis (see Forouzanfar *et al.*, (2014) M= 94 to 100 kDa).

Capillary viscometry

The intrinsic viscosity $[\eta]$ of *N. sativa* are shown in Fig. 6.6 they extrapolate to a common intercept $[\eta] = (86.51 \pm 5.85 \text{ ml/g})$, typical for a polysaccharide or protein-polysaccharide complex (Harding, 1997).

Dynamic light scattering (DLS) of *Nigella sativa*

The size distribution analysis by DLS is sensitive to the presence of large supramolecular particles and this explains the broad and large distribution we found. This information supplements the information we have obtained about the macromolecular components from the sedimentation and viscosity analysis.

FTIR Analysis

The result of functional groups in *N. sativa* in Fig. 6.8 shows the similarity with NJBT2 and NJPN1 in chapter 3 as they have the mean broad band is about 3400

cm⁻¹ (Carbonyl C=O stretching bond) and the narrow bands at 1600 cm⁻¹ (High content in saturated fatty acids), 1500 cm⁻¹ and 1100 cm⁻¹ (Triglycerides). These results show that despite the similarity in the wavenumber the whole structure is depends on the intensity of the peak so it differs the suggested components. *Nigella sativa* has anticancer activity (Agbaria *et al.*, 2015) and bleomycin is an anticancer agent (El-Mahdy *et al.*, 2005). Therefore, IRs Pharmaceuticals library was used to search for matching distributions and the results indicate the presence of anticancer agent in *N. sativa* which might be bleomycin or similar anticancer agent.

6.5 Conclusion

The sedimentation coefficient (s), Molecular weight (M_w), Stoke radius r_h (nm) and the intrinsic viscosity $[\eta]$ obtained from AUC, DLS and viscometry for *N. sativa* were studied in this chapter. The sedimentation coefficient of *N. sativa* shows the homogeneity of the sample. Moreover, the native state of a protein from *N. Sativa* is a monomer. The analysis of FTIR of functional groups in *N. sativa* include Bleomycin Hydrochloride, Bleomycin Sulfate and D_protein2 (Soy Bean Powder) respectively.

Nigella sativa, with its dynamic medicinal properties has the potential to be utilized as an effective means for treatment of various ailments. Most importantly, the extracts of the seed of the plant has various bioactive components that are anti-diabetic. TQ has the highest potential in controlling and maintenance of blood glucose as seen with various supporting literature. Other unsaturated fatty acids such as linoleic acid and oleic acid are known to promote insulin production or at least prevent the production of compounds that can compromise insulin secretion in healthy individuals. However, most of the literature available on antidiabetic properties of the seed of *N. sativa* is based on the whole seed extract or powdered seed. Analysis of individual biochemicals on its efficacy in prevention or

management of diabetes is yet to be investigated. Whatever be the case, *Nigella sativa* seed is an efficient traditional medicine in the management of *Diabetes Mellitus* a considerable (and previously unreported) degree of extension for all the samples. The fundamental hydrodynamic parameters measured here should hopefully prove useful in understating these molecules for biomedical applications.

Chapter 7: Hydrodynamic study on Lignin

7.1 Introduction

Lignin is a class of natural, highly branched phenylpropanoid macromolecules. It has a random and amorphous, three-dimensional structure, which is cross-linked, hydrophobic, heterogeneous and highly polydisperse. It possesses a complex, highly stable aromatic structure with a higher carbon content than the carbohydrates (Fig. 7.1) (Hatfield and Vermerris, 2001; Brebu and Vasile, 2010; Kubo and Kadla, 2004; Dong and Fircke, 1995; Mousavioun and Doherty, 2010; DeMartino, 2005; Malherbe and Cloete, 2002; Arora *et al.*, 2002; Hatakka, 2001; Brunow, 2001; Fang *et al.*, 2008; Harkin, 1969).

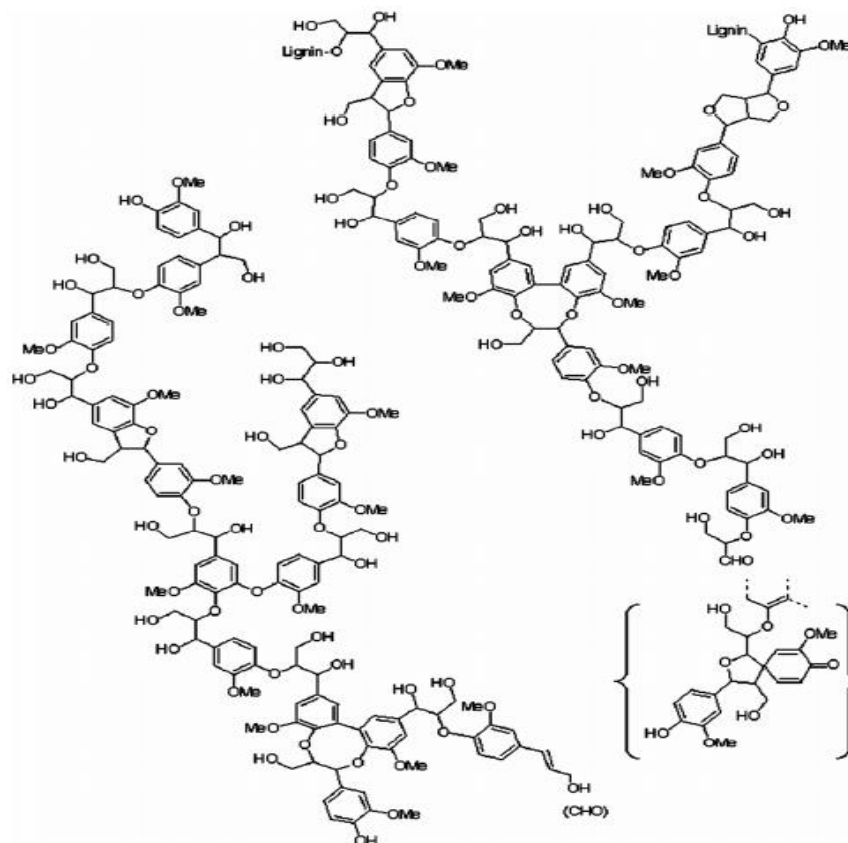


Figure 7.1: Complex, highly stable aromatic structure of lignin (Reprinted, with permission, from DeMartino, 2005).

In terms of its composite molecular structure lignin in general is a mixture of three monolignols, namely *p*-coumaryl alcohol "H-lignin", coniferyl alcohol "G-lignin" and sinapyl alcohol "S-lignin" (Boerjan *et al.*, 2003; Bonawitz and Chapple, 2010; Vanholme *et al.*, 2010; Mousavioun and Doherty, 2010; Zhong and Ye, 2007; Fang *et al.*, 2008). Moreover, lignin contains functional chemical groups such as phenolic hydroxyl, methoxyl and aldehyde and can be found in different types of plants.

Lignins from different sources differ in terms of the relative proportions of these units: for example, lignin from hardwood (angiosperm) primarily contains guaiacyl and syringyl units (coniferyl and sinapyl alcohol), whereas lignin from softwood (gymnosperm) contains primarily guaiacyl units (coniferyl alcohol units), and

lignin from grass contains guaiacyl units, syringyl units and *p*-hydroxyphenyl (*p*-coumaryl alcohol units) (Brebu and Vasile, 2010; Raiskila, 2008).

The chemical composition and molecular weight distributions of lignin, have been claimed to differ between cell types, tissue types and different plants (Raiskila, 2008). Commercially available lignins are usually available in two forms, firstly lignin as alkaline aqueous solution, and secondly as an isolated powder form (Käuper, 2004). "Kraft" (alkali), "Lignosulphonates" (sulphite), "Alcell" (organosolv) and "Soda" are four of the commonly used types (DeMartino, 2005). These four types differ according to the process of extracting lignin from biomass (Garver and Callaghan, 1991; De Martino, 2005), although Kubo and Kadla (2004) point out that the relationship between the structure and the process has been not considered in much detail. Kraft lignin is extracted mainly from wood and is treated with hydrous sodium sulphide or sodium hydroxide (Kubo and Kadla, 2004). In contrast, Alcell lignin is liberated by acidic ethanol-water and has a more oxidized structure and phenolic hydroxyl groups than other types (Kubo and Kadla, 2004; Lallave *et al.*, 2007). Soda lignin is produced by acid precipitation and is extracted from black liquors obtained by soda pulping of non-wood lignocellulosics (Käuper, 2004).

Although the function of lignin in plants is not fully understood (DeMartino, 2005), lignin provides biomechanical support to plants, facilitates transport of nutrients and water, and protects plants from damaging enzymes and insects (Vanholme *et al.*, 2010; Hatfield and Vermerris, 2001). In order to liberate lignin from plant tissues, the phenylpropane β -aryl linkage should be broken (Mousavioun and Doherty, 2010). The properties of lignin and its functions have attracted many scientists from a variety of fields. Its beneficial role in the soil, when it is degraded by microorganisms, has attracted microbiologists, plant pathologists and soil scientists, and its complex structure has attracted and frustrated the organic chemist (Harkin, 1969).

One of the most common areas where lignin has been used is in the pulp and paper industry, as substrate (Brebu and Vasile, 2010; Dong and Fircke, 1995). Also, it is studied as lignin-based plastics and lignin-based carbon fibres (Dong and Fircke, 1995), as a component for coal, and stabilizing agent for foam (Harkin, 1969). Moreover, in the pharmaceutical industry, lignin might be used against colorectal cancer (Harris and Smith, 2006). In addition, lignin with starch can be used as drug delivery system (Calgeris *et al.*, 2012). Lignin has also been reported to have multiple properties such as antimicrobial and antioxidant effect (by the hunting action of phenolic structures on oxygen-containing reactive free radicals) (Boeriu *et al.* 2004, Salanti *et al.*, 2010; Blomhoff, 2010; Raghuraman *et al.*, 2005). It was surprising that lignin has the ability to inhibit the expression of HIV-1 gene as it has been reported for the first time by Mitsuhashi *et al.*, 2008). Arguably lignin might be the new promising development of diabetic cure by the reason of the antioxidant activity as diabetes is generally associated with decreased in antioxidant defences (McLennan *et al.*, 1991). Although the previous results showed the antioxidant activity of lignin, the mechanism underlying the effects of lignin as the promising treatment on diabetes should be more investigated in future studies (Pan *et al.*, 2009; Mukai *et al.* 2011).

Critical to its consideration for application in diabetes or any application in biotechnology is an understanding of its physical properties. The molecular weight distribution principally varies from one lignin to another based on the type of chemical processing, and is not easily measured as it is a highly polydisperse substance (Kubo and Kadla, 2004; Hatakka, 2001) – and mostly insoluble in aqueous solvent. Generally, the molecular weight has been quoted to be in the range of 1,000 Da to 20,000 Da (Mousavioun and Doherty, 2010). In terms of gross conformation, lignin has been reported to have a compact spherical structure in dimethylformamide (DMF), although Sarkanen *et al.*, (1982) have claimed that lignin has a random coil structure (Dong and Fircke, 1995).

While extensive investigation has been made over decades to reveal the molecular structure of lignin this is still subject to considerable challenge (Ralph, 1999; Brunow, 2001; Hatakka, 2001) and there is still uncertainty about the size, heterogeneity and conformation of lignin. Progress has recently been made in attempts to standardize chromatographic based methods for determining relative molecular weight distributions of lignins, based on calibration relative to (sulphonated) polystyrene standards (Baumberger *et al.*, 2007, Gosselink *et al.*, 2010) although problems due to adsorption/non-inertness of column materials still cause difficulties in interpretation – and the values obtained are relative to the (sulphonated) polystyrene standards employed, which may have a different conformation than the lignin samples.

One technique which has been successfully used in the past (Goring, 1962), but has largely been neglected due to difficulties in implementation or unavailability of instrumentation has been analytical ultracentrifugation, a technique which includes both separation and analyses without the need for separation columns or membranes. Progress with analytical ultracentrifuge based methods for heterogeneity analysis (Harding 2005, Harding *et al.*, 2011) and molecular weight and molecular weight distributions for polymers and the development of the new SEDFIT-MSTAR (Schuck *et al.*, 2014) and MultiSig (Gillis *et al.*, 2013) now render it possible to obtain heterogeneity profiles and accurate absolute molecular weights (i.e. not requiring calibration standards) on a fairly routine basis. The aim of the study (Alzahrani *et al.*, 2016) has been to study three lignins from different sources solubilised in dimethyl sulfoxide with regards to their absolute molecular weights, molecular weight distributions, oligomeric state/ heterogeneity profiles and also with regards to their conformation in solution.

7.2 Materials and Methods

7.2.1 Materials

7.2.1.1 Chemicals

Lignins. Samples of lignin (Kraft, Soda, and Organosolv) were used Kraft softwood lignin, Indulin AT, Meadwestvaco (US), Soda mixed wheat straw/Sarkanda grass lignin, P1000, Greenvalue (CH), and Organosolv mixed hardwoods lignin from Repap Technology (Canada).

7.2.2 Methods

7.2.2.1 Preparation of lignins solutions

For each lignin sample 10 ml of a 5mg/ml solution were dissolved in 90% dimethyl sulfoxide (DMSO) and 10% phosphate-chloride buffer pH=7.0 and ionic strength 0.1M (prior to mixing with DMSO). The solutions were left to hydrate on a roller mixer at room temperature overnight. Undissolved material (approximately 10%) was removed by centrifugation at 12000 rpm. Final concentrations after clarification were assessed by an Atago DD-7 Differential Refractometer and a refractive index increment of 0.218 (Gupta and Goring, 1960).

7.2.2.2 Sedimentation velocity of lignins in the analytical ultracentrifuge

A Beckman XL-I ultracentrifuge was used. Lignin samples (~400µl) at concentrations ranging from 0.5 – 1.3 mg/ml, and 90% DMSO were injected into sample and reference channels respectively of double sector 12 mm optical path length cells. The balanced cells were then loaded into an analytical 8-hole titanium rotor An50-Ti and placed in the AUC. The Rayleigh interference optical system was

used for recording concentration profiles and the movement of the sedimentation boundary in the analytical ultracentrifuge cell (Harding, 2005). An initial low rotor speed of 3000 rpm to check for the presence of any aggregates was adjusted to a final rotor speed of 45000 rpm. The standard conditions of density and viscosity of water at 20 °C were used for adjustment of sedimentation coefficients s to $s_{20,w}$ values (Schachman, 1992). Scans were taken at 2 min intervals for a run time of ~ 24 hours. The data was analysed using the “least squares $ls-g^*(s)$ model” SEDFIT algorithm in terms of distributions of sedimentation coefficient distribution $g(s)$ vs s (Dam and Schuck, 2003; Harding, 2005) to provide an assessment of sample polydispersity. A value for the partial specific volume v of 0.61 ml/g was used (Rubio *et al*, 1979). Sedimentation coefficients $s_{20,w}$ were extrapolated to zero concentration to eliminate the effects of non-ideality, according to the relation of Gralen (1948):

$$(1/s_c) = (1/s^o)(1+k_sc) \quad (7.1)$$

7.2.2.3 Sedimentation equilibrium of lignins in the analytical ultracentrifuge

Molecular weights were determined by low speed sedimentation equilibrium in the analytical ultracentrifuge (Svedberg and Pedersen, 1940; Creeth and Pain, 1967), at a rotor speed of 25000 rpm. Lignin solutions ($\sim 100\mu\text{l}$) and the 90% DMSO reference solvent were injected into sample and reference channels respectively of double sector 12 mm optical path length cells. Rayleigh interference optics were again used. Initial scans (an average of 5) were taken as soon as 25000 rpm was attained, and these were then subtracted from the final equilibrium scans (an average of 5) to correct for anomalies through any window distortion etc. The final equilibrium concentration versus radial displacement profiles were then analysed using SEDFIT-MSTAR (Schuck *et al.*, 2014) which yields (i) an accurate

estimate for the apparent weight average molecular weight $M_{w,app}$ for the whole distribution using the M^* function of Creeth and Harding (1982) and the hinge point method (ii) a measure of how $M_{w,app}$ changes with radial position r (cm) and concentration $c(r)$ in the ultracentrifuge cell. Low loading concentrations (0.5mg/ml) were employed to minimize the effects of non-ideality ($M_{w,app} \sim M_w$).

7.2.2.4 Capillary viscometry of lignins

Relative viscosity measurements η_{rel} were estimated from flow time measurements of solution and solvent at solute concentrations ranging from 0.9 - 1.3 mg/ml using a Schott-Geräte Ostwald viscometer (see, e.g., Harding, 1997) at a constant temperature of $(20.00 \pm 0.01)^\circ\text{C}$ throughout. Because of the low concentrations a density correction to the flow time measurements was not necessary. Intrinsic viscosities $[\eta]$ were estimated at each concentration, c (g/ml) using the Solomon-Ciuta (1963) equation

$$[\eta] \simeq (1/c) \cdot [2\{\eta_{sp} - \ln(\eta_{rel})\}]^{1/2} \quad (7.2)$$

and a mean value taken as the consensus $[\eta]$.

7.3 Results and Discussion

7.3.1 Sedimentation velocity in the analytical ultracentrifuge

Heterogeneity and sedimentation coefficients

Sedimentation velocity in the analytical ultracentrifuge in Fig. 7.2 showed that all three lignin samples had a distribution of sedimentation coefficients, broad for Alcell and Soda, but narrow for Kraft. Alcell and Soda showed a main peak with a very low sedimentation coefficient (within the range 0.35-0.55S) and two clear minor peaks at higher sedimentation coefficient (Table 7.1). The Kraft samples by contrast showed only a single significant peak.

7.3.2 Sedimentation equilibrium in the analytical ultracentrifuge

Weight average molecular weights

Sedimentation equilibrium was then used to ascertain weight average molecular weights. Sedimentation equilibrium has the advantage over sedimentation velocity in providing estimates for molecular weight on an absolute basis (i.e. not requiring calibration using standards or assumptions over conformation) but is not as resolving for multicomponent.

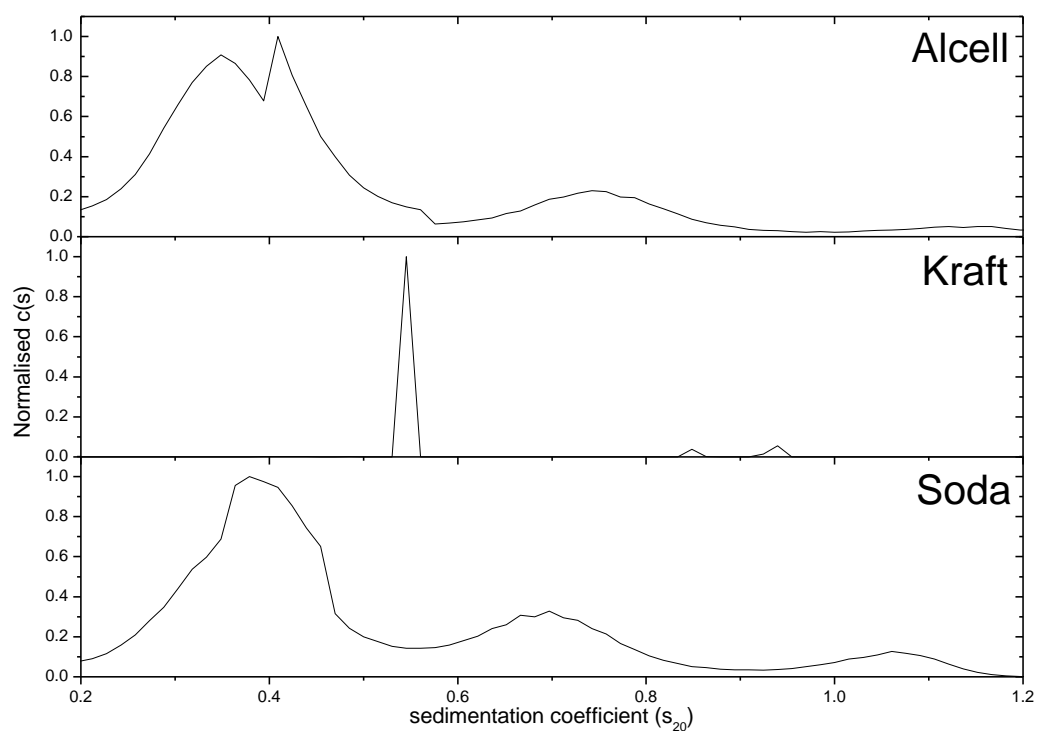


Figure 7.2: Sedimentation velocity of wood lignins. Sedimentation coefficient distribution plots, $c(s)$ versus s from SEDFIT for Alcell lignin (top), Kraft lignin (middle) and Soda lignin (lower) in 90% DMSO. At the concentrations used (~ 0.2 mg/ml), non-ideality effects can be assumed to be negligible.

Table 7.1: Hydrodynamic data for Alcell, Kraft and Soda lignins in 90% DMSO

| Lignin | ^a M_w kDa | ^b M_w kDa | ^c M_w kDa | ^d $S_{20,w}$ S | ^e $S_{20,w}$ S | ^f $S_{20,w}$ S | $[\eta]$ ml g ⁻¹ |
|---------------|---------------------------|---------------------------|---------------------------|------------------------------|------------------------------|------------------------------|--------------------------------|
| Alcell | 19.3±1.0 | 16.5 | 50.7 | 0.95 | 1.95 | 2.30 | 23.6±2.0 |
| Kraft | 24.9±1.0 | 27.2 | - | 1.25 | - | - | 22.7±0.8 |
| Soda | 15.4±0.7 | 10.3 | 30.2 | 0.91 | 1.66 | 2.20 | 22.0±0.3 |

a: from sedimentation equilibrium M^* extrapolation method in SEDFIT-MStar; b: main component (from MultiSig/M_INVEQ); c: minor component (from MultiSig/M_INVEQ). d: main component; e: 2nd component; f: 3rd component

systems. $M_{w,app}$ was obtained using the SEDFIT-MStar (Schuck *et al*, 2014) and the MultiSig/M_INVEQ (Gillis *et al*, 2013) procedures.

Because of the very low concentrations used, non-ideality was negligible and $M_w \sim M_{w,app}$. Similar behaviour is also seen with the sedimentation equilibrium data (Fig. 7.3). SEDFIT-MStar reveals a near-linear plot of $\ln(c_r)$ versus r^2 for Kraft but evidence of upward curvature for Alcell and Soda. In all cases the corresponding M^* plots converge into an accurate value for the whole distribution weight average from the identity $M_w = M^*(r=b)$. Despite the noise (deriving from the low fringe

increments because of the low concentrations employed), plots of point average $M_w(r)$ versus concentration (lower set of plots in Fig. 7.3) – derived from local slopes along the $d(\ln(c_r))$ versus r^2 curves – appear to show a characteristic increase in $M_{w,r}$ with c_r for Alcell and Soda, consistent with the clear existence of more than one significant component in sedimentation velocity (Fig. 7.3), whereas Kraft shows no concentration dependence consistent with its near-unimodal nature from sedimentation velocity and MultiSig analysis.

To explore this behaviour further we can apply more refined analyses of the sedimentation equilibrium data to provide quantitative information about the presence of different components (rather than averaging over everything with SEDFIT-MStar), using the routines MultiSig and M_INVEQ, described above (and gives us a more direct comparison with the sedimentation coefficient distribution profiles shown in Fig. 7.2). In Fig. 7.4 the results from these two modes of analysis are plotted on common axes. Application of the "MultiSig_radius" routine (Gillis *et al*, 2013) to give the three principal point average values over a range of radial positions was successful, and confirms (Fig. 7.5) that for Alcell and Soda, but not for Kraft, the average molecular weight values increase steadily over the column, implying certain polydispersity, and possible (but not confirmed) interactions. For Kraft, the average values are close to being constant, which can only arise from a very narrow weight distribution.

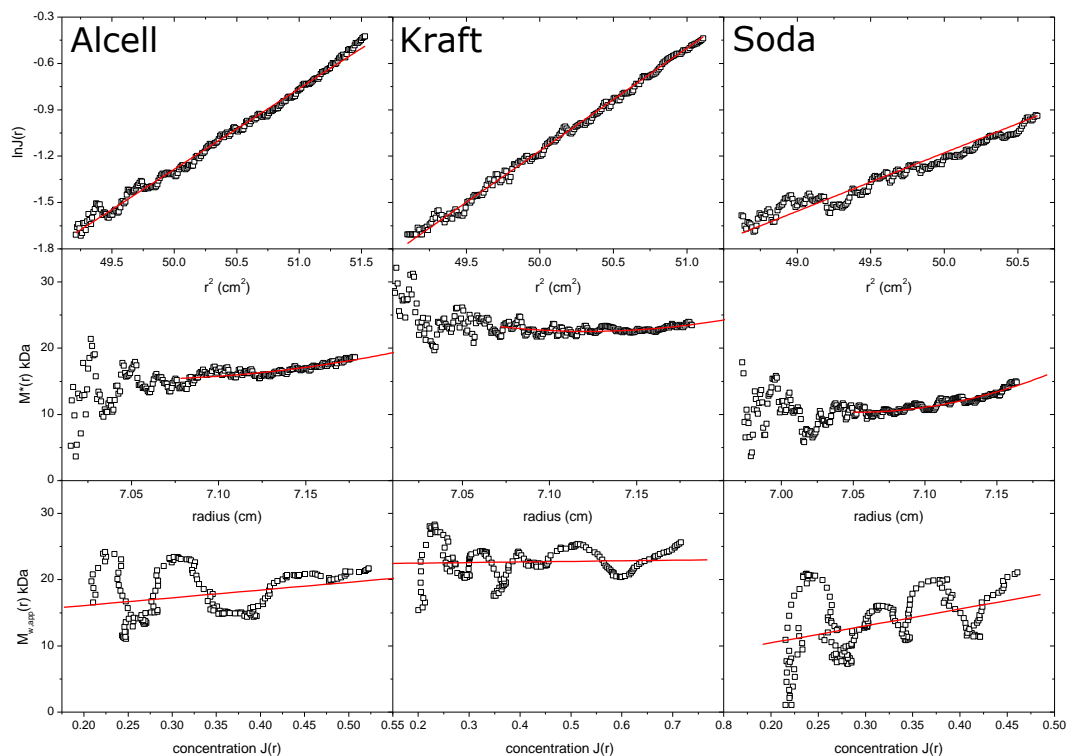


Figure 7.3: SEDFIT-MSTAR sedimentation equilibrium plots for Alcell (left column), Kraft (middle column) and Soda (right column) lignins in 90% DMSO at a loading concentration of ~ 0.2 mg/ml.

Top row: log concentration, in fringe displacement units, $J(r)$, versus the square of the radial displacement from the axis of rotation plot r^2 , where r (cm) is the radial distance from the centre of rotation (open squares);

Middle row: M^* versus r plot (open squares) and fit. The value of M^* extrapolated to the cell base = $M_{w,app}$, the apparent weight average molecular weight for the whole distribution.

Bottom row: point or local apparent weight average molecular weight $M_{w,app}(r)$ at radial position r (open squares) plotted against the local concentration $c(r)$ in the ultracentrifuge cell.

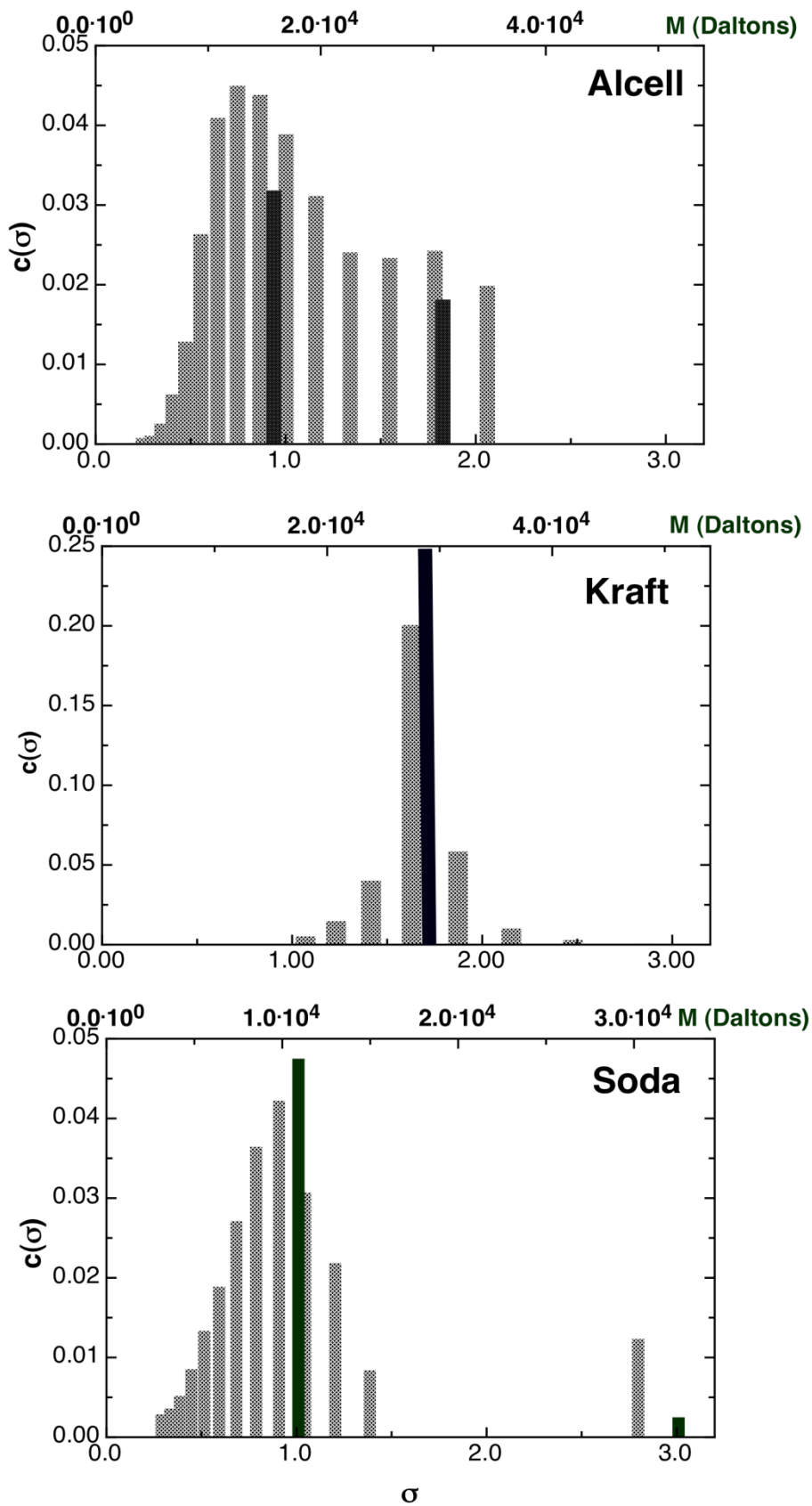


Figure 7.4: The distribution of molecular weight values as yielded by MultiSig analysis (grey) and 2-component M_INVEQ fit (black).

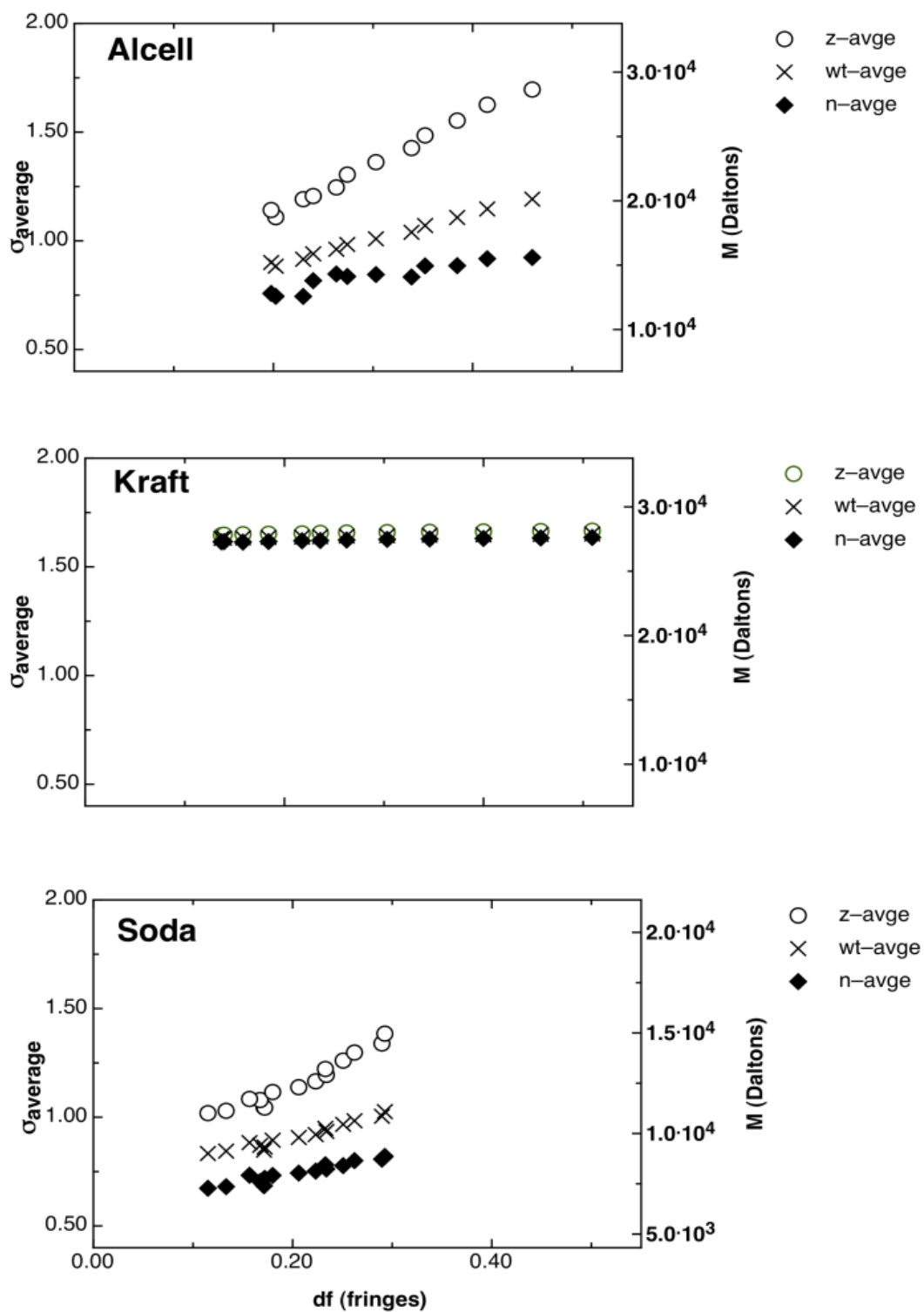


Figure 7.5: Plots of the number-, weight and z-average values from MultiSig as a function of concentration (in fringe values) for the three samples. No line has been fitted through these values, there being no hypothesis to support any particular model.

Summarising results from sedimentation equilibrium, we find that a consistent pattern emerges across them (Table 7.2). Soda has the lowest molecular weight value, around 10 kDa, with the presence of higher molecular weight material resulting in a weight average molecular weight for the whole distribution (from SEDFIT-MStar analysis) of ~ 15 kDa) of Alcell shows a main component of weight 17 kDa, and a lesser but not negligible component of approximately double that weight, leading to a weight average molecular weight for the whole distribution of ~ 19 kDa. By contrast Kraft is of very narrow distribution, and of higher weight average molecular weight (~ 25 kDa). These values are consistent with the sedimentation velocity $c(s)$ results.

7.3.3 Capillary viscometry

Intrinsic viscosities were also measured to assess the overall conformation of the lignins, and Fig. 7.6a-c compares the 3 samples. Broad agreement is observed with values between 22-24 ml g⁻¹. These values are higher than those obtained by Dong and Fricke (1995) of ~ 8 ml g⁻¹, although their value was obtained under quite different solvent conditions (dimethyl formaldehyde at a temperature of 45.0°C).

It is possible to estimate the extension or aspect ratio of the lignin molecules from the relation (Harding 1997):

$$[\eta] = v \cdot v_s \quad (7.3)$$

where v_s (ml g⁻¹) is the swollen specific volume (which takes into account solvation) and v is the Einstein-Simha shape parameter (Simha, 1940; Harding *et*

al., 1992). In Table 7.2 we have estimated v for 3 plausible values of v_s (0.6, 1.0 and 1.4 ml g⁻¹) and then used accurate relations linking this parameter with aspect ratio (equivalent prolate ellipsoid) to estimate the aspect ratio. The algorithm used was ELLIPS1 (Harding *et al.*, 1997). Figure 7.6d-f shows the corresponding 3D shapes of the extended molecules (based on a v_s of 1 ml g⁻¹) the arrows illustrating the effect of different values for v_s (0.6 – 1.4 ml g⁻¹).

An extended conformation is also consistent with the way the sedimentation coefficient changes with molecular weight. Although we only have 3 points the sedimentation coefficient values (for the major component) of Table 7.1 increase with molecular weight M_w with a value for a the Mark-Houwink-Kuhn-Sakurada parameter of ~ 0.26 , consistent with an extended structure.

Table 7.2 Viscosity increment for Alcell, Kraft and Soda lignins in 90% DMSO for different values of the hydration or v_s value (ml g⁻¹)

| <i>Lignin</i> | v (for $v_s = 0.6\text{ml g}^{-1}$) | v (for $v_s = 1.0\text{ml g}^{-1}$) | v (for $v_s = 1.4\text{ml g}^{-1}$) | a/b (prolate model) |
|---------------|--|--|--|---------------------|
| <i>Alcell</i> | 39.3 | 23.6 | 16.9 | 14.2 \pm 4.0 |
| <i>kraft</i> | 33.8 | 22.7 | 16.2 | 13.8 \pm 4.0 |
| <i>soda</i> | 36.7 | 22.0 | 15.7 | 13.6 \pm 4.0 |

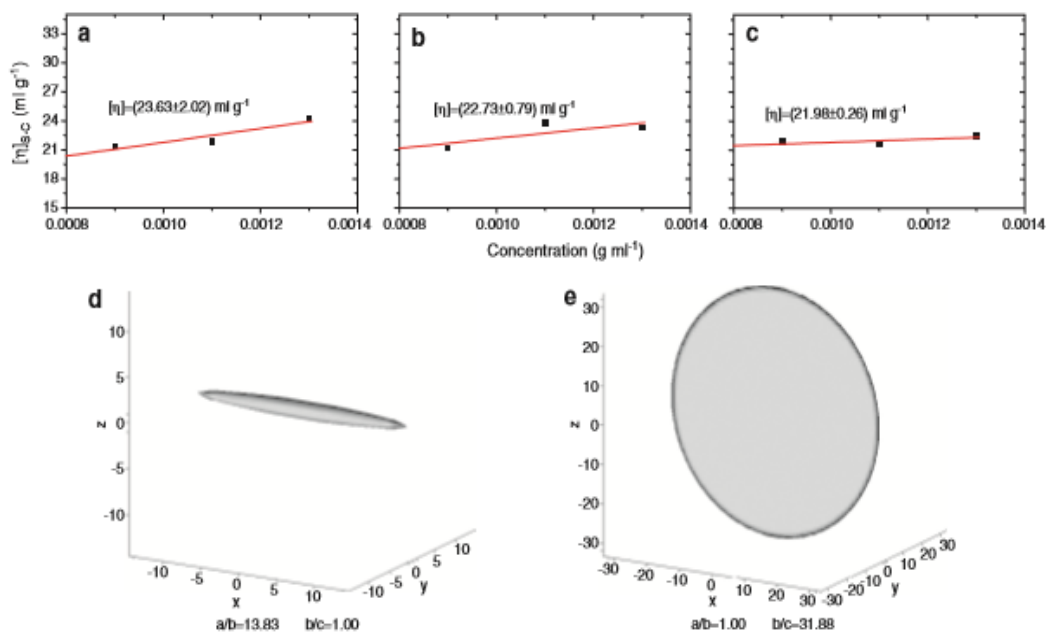


Figure 7.6: Intrinsic viscosity [from Eq. (2)] estimates at different concentrations for (a) Alcell lignin, (b) kraft lignin, and (c) soda lignin. (d) Corresponding prolate ellipsoid representation from ELLIPS1 for all three lignins and (e) corresponding oblate ellipsoid representation for all three lignins.

7.4 Conclusion

Implementation of sedimentation velocity and sedimentation equilibrium in the analytical ultracentrifuge provides an alternative to SEC-based procedures for the analysis of the heterogeneity and molecular weight of solubilised lignins. The limitation in the present research is, however, that only 90% of the lignins were soluble in DMSO. On the other hand, no calibration standards are required and there are no problems related to no inertness of columns as in case of SEC. The MULTISIG and M_INVEQ analyses show the presence of two clear components (major and minor) for Alcell L and (to a lesser extent) Soda L. For all three lignins investigated in this study, the properties lie within a plausible range, but the MW data obtained by analytical ultracentrifugation are significantly higher than the SEC-based values. A plate shape structure for all three lignins with aspect ratio $\sim 30:1$ seems to be probable. While the SEC approach is useful in routine work for comparative purposes, modern ultracentrifuge approaches for molecular weight and heterogeneity characterisation for lignins should be considered more in theoretical and practical lignin research.

Chapter 8: Conclusions and future work

This thesis aimed to show how hydrodynamic and associated methods are making inroads into our understanding of the use - or potential use - of glycan (polysaccharide) and glycan related materials (lignins) being selected and used in the fight against *Diabetes Mellitus* and other serious diseases. The main focus has been on the extraction, isolation and characterization of the protein-polysaccharides from flowers, pulps and seeds of *Cucurbita* (Butternut Squash (NJBT2), Zucchini (NJZI4) and Pumpkin (NJPN1)) and the use of polysaccharide and polysaccharide complexes from cucurbits and its suggested ways in which their consumption may influence on *Diabetes Mellitus*. The properties of fatty acids from *Cucurbita* were also investigated. Beta glucans from oat was considered for use as a cholesterol lowering agent, and their behaviour in the presence of lipases. The properties of *Nigella sativa* were investigated as it has medical uses against hypertension, diuretics, and appetizers with additional antimicrobial properties. Although *Nigella sativa* seed appears to have various medicinal properties, these remain under continued and thorough investigation. Finally, the properties of a group of molecules, which are often associated with polysaccharides, namely lignins were considered.

It would obviously appear that the answers to the questions that we have raised in the corresponding chapters for each study are yet to be established. These questions primarily include: what are the bioactive components that have the ability to reduce the blood glucose level? What is the mechanism of these substances? Would it be possible to provide these components in sufficient amounts by manipulating the plant to present these? What is the probability of consuming the bioactive substances orally? Nonetheless it is hoped that the fundamental hydrodynamic data provided in this study will help provide the basis

for these questions to be answered in the future. It is worth summarising what the results were found.

8.1 Extraction and isolation of the protein-polysaccharide complex

The first step was extraction and isolation of the protein-polysaccharide complex from the fruits and seeds of different species of *Cucurbitaceae*, and glycan (polysaccharide) and glycan related materials (lignins). There are different ways to separate, isolate and purify each sample: this includes using precipitation methods, which mean adding reagents which will precipitate protein. The second method is use of chromatography to isolate the protein: for instance, using gel chromatography (GC) to separate the sample according to the shape and the size of the sample. Centrifugation is the third method that is used to isolate the sample. Soxhlet extraction is a solid-liquid extraction (Jensen, 2007), and was used to extract lipids from the sample, using for example, petroleum ether as a solvent. Before this, the sample needed to be in a solid condition as a powder, and for that freeze drying was used. This method is suitable for the complex from both seeds and pulp. However, liquid extraction from seeds was investigated. It is noteworthy that the fundamental problem in obtaining a pure sample from plant material is the presence of insoluble components in the cell wall that surrounds the plant cell. Breaking down the cell wall releases various metabolic products, and without fractionating the sample, it would not be pure. For example, cellulose cannot be dissolved by an organic solvent, which might be used in any extraction protocols. Also, it should be pointed out that the activity of proteins might be affected by the increase in temperature as proteins degrade under these conditions: especially when extracting protein from the dried seeds. Hence, this is a significant step to be evaluated.

Once the bioactive components had been extracted, the contents were estimated using a variety of methods. For example, the Biuret Test and spectrophotometry were used to estimate the bioactive components (proteins in this case). Gel chromatography was used to separate the complex, and the fractions were estimated using spectrophotometry.

The final and important step, once a pure bioactive molecule has been obtained, was structural elucidation or physicochemical characterisation, which was performed using a variety of hydrodynamic based methods. In order to determine the physical properties (intrinsic viscosity, sedimentation coefficient distribution and the molecular weight) of the bioactive molecule, viscometry, Analytical Ultracentrifugation (AUC) and Dynamic Light Scattering (DLS) were performed. Also, the hydrodynamic shape of the bioactive components was determined – in the case of the lignins - using ellipsoidal modelling ELLIPS1 (this type of modelling is only applicable to molecules with limited or no flexibility). All of these methods are required in order to understand the structure of the bioactive molecule.

Pumpkins (genus; Cucurbita) belong to the family of Cucurbitaceae. It contains numerous seeds which are considered to be beneficial for health in a number of ways. They have been reported to show antioxidant activities (Wu *et al.*, 2008), lipid lowering, hepatoprotective (Makni *et al.*, 2008), anti-carcinogenic (Pan *et al.*, 2005), antimicrobial (Jiang *et al.*, 2005) and anti-diabetic effects (Jiang & Du, 2011; Yoshinari *et al.*, 2009). In an attempt by a research group to test hypoglycaemic activity of pumpkin extract, they isolated protein-bound polysaccharide by standard methods and found that it consisted mainly of polysaccharide (approximately 41.21%) and protein (approximately 10.13%). This fruit extract containing protein-bound polysaccharides, were found to increase levels of serum insulin and reduce blood glucose levels thereby improving glucose tolerance when administered in alloxan diabetic rats at different doses (Quanhong *et al.*, 2005; Quanhong *et al.*, 2003). Another research group

identified that hypoglycaemic polysaccharide contained various monosaccharides such as glucose, arabinose, galactose and rhamnose (Jin *et al.*, 2013). However, the mechanism of anti-diabetic action of pumpkin polysaccharide has not been identified yet. The Chinese research group also demonstrated that polysaccharide from pumpkin can reduce blood glucose level of diabetic rats along with decrease in production of Malondialdehyde (MDA) and Nitric oxide (NO) and improvement in ultrastructural morphology of injured islet cells of pancreas (Zhu *et al.*, 2007; Hong-Yan *et al.*, 2015). Similar studies were carried out on alloxan induced diabetic rabbits and it was found that when water soluble pumpkin polysaccharide was injected in diabetic rabbits, blood glucose, serum lipid and glycosylated haemoglobin levels were controlled. It was also observed that regeneration damaged islet cells was enhanced by pumpkin polysaccharide thereby reducing plasma glucose levels (Zhang *et al.*, 2013).

Hypolipidemic activity of polysaccharides from pumpkin has also been investigated. The study found out that oral dose of pumpkin polysaccharide in rats decreased the levels of triacylglycerol, total cholesterol, and low-density lipoprotein cholesterol in plasma (Zhao *et al.*, 2014).

Pumpkin polysaccharide was also found to reduce lipid and glucose levels along with oxidative stress in diabetic rats (Song, 2015). The antioxidant properties of polysaccharides from pumpkin were found to be enhanced in their phosphorylated derivatives when investigated in either *in vitro* or *in vivo* systems. Also, rat thymic lymphocytes were found to be protected from H₂O₂-induced oxidative damages when phosphorylated derivative were used (Song *et al.*, 2015). Polysaccharides extracted from *Cucurbita moschata* were shown to exhibit high reducing power and positive radical scavenging activity in an *in vitro* study (Wu *et al.*, 2014).

Some isolated reports have shown antibacterial properties of water soluble polysaccharides extract from pumpkin family. Cellulose-assisted extracted

pumpkin polysaccharide showed high antibacterial activity against various bacterial strains such as *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* (Qian, 2014). Besides, evidence from the literature also show anti carcinogenic properties of pumpkin polysaccharides. Another study revealed that purified white pumpkin polysaccharides induced apoptosis in hepatocellular carcinoma cell line HepG2, which involved a caspase-3-mediated mitochondrial pathway.

The current literature supports the idea of conducting more research studies to investigate the other potentials of pumpkin seeds that are not yet explored. The *Cucurbita* maxima contribute a lot to human life that include diet, boost food security, culinary diversification, and nutrition. This is applicable in the management of diabetes since it is accompanied by numerous dietary restrictions. Some of the diabetic patients may not be able to adhere to the recommended dietary content because of their costs and availability.

Despite the difficulties and challenges that *Cucurbita* present with regards molecular characterization this thesis was able to make a measurable comparison of protein-polysaccharides from Butternut Squash (NJBT2), Zucchini (NJZI4) and Pumpkin (NJPN1). The major component of flowers protein NJBT2 in this study proved to be a heterogeneous protein. This can be seen from the results of velocity experiment. Sedimentation velocity have been used to show that the similarity in Sedimentation coefficient of *Cucurbita* from different species. Sedimentation coefficient of *Cucurbita* verifying that the protein-polysaccharides complex are entirely heterogeneous as the samples contain a very broad range of sizes.

The sedimentation equilibrium was performed and apparent weight average molecular weights were obtained using SEDFIT-MSTAR and the result showed that NJPN1 had the higher molecular weight than NJBT2 and NJZI4, which were $248.4 \pm 20\text{kDa}$, $52.7 \pm 10\text{kDa}$ and $59.5 \pm 20\text{kDa}$ respectively. From the results of

velocity experiment and the width of the size distribution in this study for *Cucurbita* indicates that there is more than a single species presents in solution.

The work reported in this study was designed to evaluate and establish the efficiency of functional groups in *Cucurbita*. FTIR study was performed over the range of wavenumbers 4500–400 cm^{-1} by a FTIR (IRAffinity-1S Shimadzu model). It was found that the functional groups present in of NJBT2 pulps, NJNP1 pulps, and NJZI4 were approximately the same as would be expected. However, they were found at different functional groups due to the presence of FTIR detailed information or proof of molecular formula or structure. It provides information on molecular fragments, specifically functional groups. It is worth noting that despite the similarity in wavenumber in the samples NJBT2 pulps, NJPN1 pulps and NJZI4 the transmittance (%) shows the difference between the functional group due to the differences of the relative intensities in the infrared spectrum.

This is essential in the move towards preventing complication associated with an increased level of free fatty acids in diabetic people. There are other studies underway that are aimed at isolating and characterizing the active compound in the extract and further determine the mechanism behind its antidiabetic.

This study has demonstrated the applicability of a set of AUC-base approaches to successfully defining basic parameters for *Cucurbita* samples on a basis free of prior assumptions. Additionally, viscometric analysis has enabled us to provide estimates for molecular shape, indicating a considerable (and previously unreported) degree of extension for all the samples.

To summarize, *Cucurbita* polysaccharides display a number of activities beneficial to health. Various studies have explored their anti-diabetic, Hypolipidemic, anti-oxidant, anti-proliferative and anti-bacterial roles in different models. These studies definitely suggest that these extracts from *Cucurbita* family has the

potential to be used as a therapeutic agent against diabetes, hyperlipidaemia, cancer and some bacterial infections.

8.2 Beta-Glucan Lipase Interactions

Beta glucans are polysaccharides present in the cell walls of bacteria, fungi, yeasts, algae, lichens, oats and barley. The medicinal properties of beta glucans have been investigated by many research groups (Chen & Seviour, 2007; McIntosh *et al.*, 2005). Beta glucans are beneficial to health as they are known to stimulate immunity and used in the treatment of various infectious diseases and also cancer (Chen & Seviour, 2007). Beta glucans have also been implicated for the treatment of *Diabetes Mellitus* and associated cardiovascular diseases. *Diabetes Mellitus* is a chronic disease characterised by hyperglycaemia caused by either less secretion of insulin or lack of insulin action. It has been reported that oral administration of beta glucans from oats and fungi resulted in lowered blood glucose levels in animals and clinical trials (Lo *et al.*, 2006).

A number of studies were carried out using crude-impure beta glucan. When fruiting bodies and polysaccharides from *Tremella mesenterica* and *T. aurantia* were administered orally in diabetic rats, blood glucose concentration was reduced. Similar effects were observed when exopolysaccharide produced from submerged mycelial cultures of *Phellinus baummi* was used (Tadashi *et al.*, 2000; Tadashi *et al.*, 1995). Another study reported, when whole mushroom powder and its derivative fractions were administered orally in transgenic diabetic mice, it increased insulin sensitivity (Mayell, 2001). A hot water beta glucan extract of *Agaricus blazei basidiocarps* also showed anti hyperglycaemic effect in diabetic rats (Kim *et al.*, 2005). This group also showed that derivatives of crude beta glucans are more effective agents. Beta glucans from oats have been shown to

reduce postprandial glucose levels in clinical trials (Jenkins *et al.*, 2002; Tappy *et al.*, 1996). Oat beta glucan was shown to have a strong hypoglycaemic effect in diabetic mice by increasing insulin secretion, decreasing insulin resistance. Also, pancreatic tissue structures were found to be repaired and integrity of islet β -cell seemed to be improved (Liu *et al.*, 2016).

Research was also carried out by Tadashi *et al.*, (1995) to delineate the mechanisms by which beta glucans might be involved in anti-diabetic role. It was found out that beta-glucans might be possibly delaying clearing of stomach so that absorption of glucose is slowed down (Tadashi *et al.*, 1995). Thus, the feeling of hunger is reduced and therefore, it was suggested that beta-glucans might function by decreasing appetite and thus, food intake gets reduced.

Signalling pathways have a pivotal role in the pathogenesis of diabetes. PI3K/Akt signalling pathway is disturbed in patients with diabetes. It seems that beta glucan function to reduce blood glucose level through PI3K/Akt activation. It has been shown that beta glucan increase PI3K/Akt activation through several receptors (Chen & Seviour, 2007).

Dyslipidaemia caused by insulin resistance is a major risk factor for diabetes associated cardiovascular disease (CVD) (Turner *et al.*, 1998). Beta glucans are shown to decrease Low Density Lipopolysaccharide (LDL) and increase High Density Lipopolysaccharide (HDL), which improved dyslipidaemia and reduced CVD (Anderson, 1995; Kapur *et al.*, 2008). Cholesterol-lowering effect was first found in oats where beta glucan was the active component (Kerckhoffs *et al.*, 2002; Othman *et al.*, 2011).

In Chapter 5, the hydrodynamic properties of beta glucan and their interaction between beta glucan and lipase was described. It can be seen that there were larger species being formed when lipase and beta glucan were mixed which means there is interaction between them. The results shows that using high lipase

concentrations resulted in complete co-sedimentation with beta glucan and that this strong interaction occurred with multiple lipase/ beta glucan concentrations and ratios.

The sedimentation coefficient distribution of beta glucan shows three peaks sedimentation coefficient of 1.3S, 2.55S and 4.5S respectively. However, the broad tail from 6S indicates polydispersity. Whereas lipase in this study exists as mostly as monomer with a small amount of dimer. Sedimentation equilibrium was performed for beta glucan and apparent weight average molecular weights were obtained using SEDFIT-MSTAR and it shows that beta glucan has a molecular weight of 420 +/-10 KDa.

It has been concluded that there is a significant impact of different storage and processing conditions on the hydrodynamic features of solutions of oat beta glucan. The presence of both, frozen storage and autoclaving, helps in reducing the intrinsic viscosity of the solutions of oat beta glucan. Thus, it is found that the impact on polysaccharides on cholesterol reduces. The storage of sample at the temperature of a room enables intrinsic viscosity to increase that is not supported by the literature. The plot of volume% vs size (r.nm) for beta glucan at 173^o shows the similar Stoke radius RH (r.nm) for the four samples at the range of 2000 to 4000 (n.m). The outcome generated has observed that future experiments should repeat this method and perform a comparative analysis of the data. Moreover, in the study, more hydrodynamic techniques should be employed so that more beneficial health effects of oat beta glucans can be characterised. In the end, there is a scope for future studies in food processes such as extrusion, to be investigated.

Overall, beta glucan can prove to be potential therapeutic agents for treatment of diabetes and CVD, however, there is a need to investigate molecular mechanisms associated with their action.

8.3 *Nigella sativa*

Diabetes Mellitus is a lifelong disease characterized by high blood sugar (hyperglycaemia). It is a metabolic disorder developed mainly when enough insulin is not produced by the pancreas (type 1) or when cells becomes insensitive to the insulin (type 2) (Wu *et al.*, 2014). About 90% of diabetic patients are affected by Type 2 or non-Insulin-independent Diabetes Mellitus (NIDDM) (Chen *et al.*, 2012). Type 2 diabetes is also known to be associated with poor cardiac health. Over the years, research has been carried out to reveal the efficacy of plant based diet in reducing risk of chronic diseases. Due to the side effects of the synthetic drugs, WHO (1999) has suggested the evaluation of plants effective in different diseases. Medicinal plants of the Indian origin have been found to be helpful in treating diabetes. The seeds and oil of *Nigella sativa* (black cumin) are known to have medicinal properties all over the world. The volatile oil, extracted from *N. sativa* demonstrated hypoglycaemic activity, however, mechanism of its needs to be elucidated (Kaleem *et al.*, 2006).

The results in Chapter 6, show that *N. sativa* has a molecular weight of (230 ±20) kDa. This value represents the whole macromolecular components (protein + carbohydrate), and is, not surprisingly, higher than the estimates given by the protein component alone (94-100 kDa), using the technique of sodium dodecyl sulfate polyacrylamide gel electrophoresis (see Forouzanfar *et al.*, (2014) M= 94 to 100 kDa). The high molecular weight of *N. sativa* and the intrinsic viscosity of 86.5+5.9 ml/g are typical for a polysaccharide or protein-polysaccharide complex which may represent the bioactive component that have physiological effects of consuming *N. sativa*.

Due to their antioxidant properties, plant drugs (antioxidants, flavonoids and tannins) proved to be having the beneficial effects (Demir *et al.*, 2006). *Nigella sativa* seeds have many properties such as antibacterial, bronchodilator, diuretic,

hypotensive and immunopotentiating. El Tahir *et al.* (1993) showed that *N. sativa* seeds contain thymoquinone and many monoterpenes such as p-cymene and alpha-pinene. The study by Kantar *et al.*, (2003) suggested that there is a hypoglycaemic activity of *Nigella sativa* on streptozotocin induced diabetic rats. *N. sativa* seed extracts help in the prevention of muscle wasting that occurs in diabetes (Kantar *et al.*, 2003). The levels of glucose were decreased and insulin increased in the alloxan diabetic rabbits whose diets were supplemented with the oil extracted from *Nigella sativa* seeds.

Lipid lowering effects of *N. sativa* seeds have also been investigated in various studies. Although, eggs are an excellent dietary source of biomolecules such as amino acids, fatty acids, vitamins and minerals, however, due to their high cholesterol content consumption of eggs has declined in most developed countries (Dahri *et al.*, 2005; Sahebkar *et al.*, 2016). Dietary introduction of *N. sativa* seeds in albino rats considerably decreased serum low density lipoprotein cholesterol level, and increased serum high density lipoprotein cholesterol levels (Dahri *et al.*, 2005). A recent review and meta-analysis has also revealed the positive impact of *N. sativa* on plasma lipid concentrations, leading to lower triglycerides, total cholesterol, Low Density Lipoprotein (LDL) levels while increasing high density lipoprotein (Sahebkar *et al.*, 2016). Another report suggests that *N. sativa* supplementation in the poultry diet decreases the serum triacylglycerol and serum total cholesterol in eggs (El Bagir *et al.*, 2006). Further, El Bagir *et al.*, (2006) showed that dietary *N. sativa* reduced the total lipid and cholesterol from egg yolk. The actual mode of action in decreasing the egg yolk cholesterol is still need to be determined.

To summarize, antidiabetic and lipid lowering effects of *Nigella sativa* seeds are marked and need to be further explored for therapeutic purposes.

8.4 Lignin

As reported in Chapter 7, it is well accepted that lignin is a phenylpropanoid macromolecule. Consequently, it belongs to the bioactive components known as phenolic compounds (Kris-Etherton *et al.*, 2002). Thus, lignin has been reported to have multiple properties such as antimicrobial and antioxidant (by the action of phenolic structures on oxygen-containing reactive free radicals) effect (Salanti *et al.*, 2010; Blomhoff, 2010; Raghuraman *et al.*, 2005).

Plants contain biologically active compounds known as phytochemicals which has been shown to be effective in prevention of these diseases through both in vitro and in vivo experiments, and clinical trials (Steinmetz and Potter, 1991). Many classes of phytochemicals from plant based diets such as carotenoids and phenolic compounds (flavonoids, lignin) were identified by Steinmetz and Potter (1991), Ceriello & Testa, (2009) and Chaturvedi, (2007). Lignans are fibrous constituents of lignin in plant cell wall (Barceló, 1997). Lignans have antioxidant properties and act as phytonutrients. Lignans are very rich source of fibre in diet and are known to activate immune system and balance hormones.

Recently, accumulating evidence in literature have revealed the protective role of phytoestrogens (for example, lignans) against a number of chronic diseases, including cancers, dyslipidemia, hyperglycemia and cardiovascular dystrophy (CVD) (Duncan *et al.*, 2003). Phytoestrogens are structurally and functionally similar to lignin (Burton and Wells, 2002). Phytoestrogens helps in improving serum lipids and modifying LDL oxidation, the basal metabolic rate, and insulin stimulated glucose oxidation. These observations suggest, that the consumption of foods rich in phytoestrogens has a beneficial effect on obesity and diabetes (Bhathena, 2000).

The aim of this project was to provide more information about the physical properties of these polysaccharide related substances: three different commercial

types of lignin (Soda, Kraft and Alcell) by using dynamic light scattering (DLS) and viscometry and analytical ultracentrifugation (AUC). The properties of lignin were studied in dimethyl sulfoxide. Although their sedimentation behaviour and lignin is quite different the result show that the intrinsic viscosities of lignins are, coincidentally, approximately the same, at 20-23 ml/g.

To summarise, plant based lignins seem to have positive health effects and prove to be effective against diabetes and hypercholesterolemia. However, further insight into mechanism of their action is required to enhance their therapeutic effects.

8.5 Future work

Globally, people have been looking for new sources of nutritional products that have proved to be completely healthy food. Therefore, there should be a move to air the nutritional values associated with the materials we have focused on in this study - Cucurbita seeds extracts, beta glucan, *Nigella sativa* extracts and (edible) lignin with respect to antidiabetic and other therapeutic/health benefits. The current literature supports the idea of conducting further studies to investigate the other potentials of these natural products. The glycan (polysaccharide) and glycan related materials (lignins), contribute to human life that include diet, boost food security, culinary diversification, and nutrition. This is applicable in the management of diabetes since it is highly accompanied by numerous dietary restrictions. Some of the diabetic patients may not be able to adhere to the recommended dietary content because of their costs and availability, at times.

Any change in the environment of solution such as salt concentration, temperature and pH used can alter their properties. Therefore, the effect of different environmental conditions could be used to determine the stability of all the

materials we have considered here, extending the work we have already done on beta glucans.

Screened for the bioactivity is an essential step after extraction and isolation, the bioactive molecules will be screened for the bioactivity of each fraction. Wang *et al.* (2011) report that there are three types of screening for the bioactive substances from the plant, and use of these types depends on the desired compounds. The first type is screening bioactive material, which has been extracted from various sources, whereas the second type is screening the bioactive materials from one source using organic solvent to isolate the fraction, and the last type is screening of the pure identified and isolated compounds. In this study the bioactive material will be first extracted and isolated, then screened for bioactivity, and will be identified and characterised to reveal the structure of the components. As mentioned previously, because the *Cucubitaceae* family has been used world-wide as an active medicine and edible fruit, the bioactivity should be screened only for anti-diabetes components, and a series of concentrations will be investigated to find the suitable dosage. Therefore, two types of screening could be used in such a follow-up project, the first of which will use a cell model, as this will mimic the physio-pathological state of diabetes (Wang *et al.*, 2011). Another screening could be prepared using the bioactive molecules for drug delivery, and a series of concentrations would then be investigated to find the appropriate amounts for use with human models.

Since the answers to these questions appear to be the most significant within previous studies, it would seem to be crucial to uncover the bioactive components so as to be the initial study. It has been postulated that the bioactive contents might be found in the protein-polysaccharide complex, as suggested by Atuonwu and Akobundu (2010), Mohamed *et al.* (2009), Adepoju and Adebajo (2011), Teugwa *et al.* (2013), Quanhong *et al.* (2005), Caili *et al.* (2007) and Adams *et al.* (2011). In light of this, glycan (polysaccharide) and glycan related materials

(lignins) from the *Cucurbitaceae*, beta glucans, *Nigella sativa* and lignin were the object of this study. This study will hopefully provide underpinning knowledge useful for scientists/clinicians and others wishing to use these. A major challenge will be to use these substances for drug delivery.

It was also noted that these studies do not provide information regarding the nature of the interaction (i.e. whether there is a specific interaction between the beta glucan and lipase, or if the sedimentation of the beta glucan 'matrix' would co-sediment any protein) and that this would require further work. Also investigating the effect of the lipase- beta glucan interaction on the enzyme activity would be crucial.

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Appendix 1: Published/Submitted work

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