University of
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EXTRACTION, CHARACTERISATION AND PROPERTIES OF POLYSACCHARIDES FROM NOVEL SOURCES

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ABSTRACT

In many foods, pectins are used as thickeners, gelling agents, texturizers, emulsifiers and stabilizers; these functional properties rely on their physio-chemical properties and therefore on their extraction and growing conditions or the pectin source including genetic variants.

Pectins were isolated using aqueous extraction at pH 6.0 from okra pods of different genetic varieties and from different geographical origin. Furthermore, pectins from pumpkin were extracted at different pHs, times and temperatures. An isolation protocol and a statistical experimental approach were designed to extract pectin and to study the influence of a number of factors on the physicochemical properties of pectins from natural sources. After the successful isolation full characterization via an array of analytical techniques was carried out. By high-performance anion exchange chromatography HPAEC and nuclear magnetic resonance (NMR), it was determined that the carbohydrates were most likely pectin based which is due to the presence of galacturonic acid. Also, extracted polysaccharides were assessed using size exclusion chromatography (SEC). An Fourier Transform infrared spectroscopy (FT-IR) method was developed to measure the degree of esterification (DE) of pectin samples. The properties and stability of the resulting pectin were examined by means of ζ-potential measurements and capillary viscometry, as pectin extracted from both pumpkin and okra were negatively charged polyelectrolytes. NMR spectroscopy was used to further elucidate the chemical structure of the pectin. An ultraviolet visible (UV-Vis) spectrophotometer was also applied to determine the acetyl content, protein content, uronic acid content and the free radical content for antioxidant activity. The present investigation for okra and pumpkin pectins show that: okra genotypes had pectin yields of 11.3–14.6 % while the pumpkin pectin produced in quantities between 2.8 % - 8.0 % of alcohol insoluble residue (AIR). The greatest total amount of pumpkin pectin yield 8.0 % on a dry basis was found for 1h of extraction and at 80 ºC, pH 2 for pumpkin pectin and pH is the most important factor in the extraction of pumpkin pectins. Total polysaccharide and protein content for okra pectins ranging from 58 % –70.1 % and 8.0 –15.1 % respectively, whereas pumpkin varied from 30.9 to 72.2 % total polysaccharides and 1.8 –14.3 % protein content. Galacturonic acid was the main component for okra and pumpkin samples and both had similar range between 43.7 to

72.6 % mol % and 41.7 to 72.6 % mol % galacturonic acid respectively, where is it important to note that for commercial purposes pectins are required to be at least 65 % galacturonic acid. The degree of esterification, calculated using Infra-red spectroscopy (IR), the highest value was 96 % for pumpkin pectin whilst for okra pectin was 39 %. The degree of acetylation for both was high and in the same range varied from 30.3 % to 76.2 %. Monosaccharide composition analysis showed that both pectins extracted contained particularly, rhamnose, arabinose, galactose, glucose and xylose. The intrinsic viscosities were high for both polysaccharide values solutions. This was attributed to the okra polysaccharides having a relatively large molecular mass which was further supported by using SEC. Furthermore, most of the zeta potentials obtained in this study for okra and pumpkin pectins were highly negative. ^{1}H NMR spectroscopy confirmed the presence of uronic acids in the free and methyl ester forms. The antioxidant activity for both pectins was estimated using 1,1-diphenyl -2- picrylhydrazl and hydroxyl radical assays. A new combined hydrodynamic approach was used to estimate the conformation and flexibility of the pectin solution. In terms of the persistence length, L_p of the equivalent worm-like chain model, the chain flexibility was then investigated. Equivalent radii and ratios of radii from solution properties were the two main factors that were used as indicators for macromolecular conformation, shape, and flexibility. In addition, both the average number and length of side chains on a pectin molecule was estimated using the physicochemical data coupled with the conformational data. For the first time (to our knowledge) this data from the conformational analysis was combined with structural information from the chemical analysis of pectins to obtain information on the degree and the length of branching in the “hairy” RG-I regions. Principal component analysis (PCA) was used for both pectins to show global differencessimilarities between samples using all the primary data determined.

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LIST OF ABBREVIATIONS

Ara	Arabinose
DA	Degree of Acetylation
DE	Degree of Esterification
D_2O	Deuterium oxide
DM	Degree of Methylation
DMSO	Dimethyl Sulfoxide
FTIR	Fourier Transform Infrared spectroscopy
Fuc	Fucose
Gal	Galactose
GalA	Galacturonic acid
GC-MS	Gas Chromatography-Mass Spectrometry
Glc	Glucose
GlcA	Glucuronic acid
HMP	High Methoxyl Pectin
$[\eta]$	Intrinsic Viscosity
η_{rel}	Relative Viscosity
η_{sp}	Specific Viscosity
HPAEC-PAD	High-Performance Anion – Exchange Chromatography with Pulsed Amperometric Detection
i.d	Inside/inner diameter
LMP	Low Methoxyl Pectin
M_w	Weight-average Molecular Weight
$NaBD_4$	Sodium borodeuteride
NMR	Nuclear Magnetic Resonance
PCA	Principal Component Analysis
PMAA	Partially Methylated Alditol Acetates
Rha	Rhamnose
SEC-MALLS	Size – Exclusion Chromatography coupled to Multi-Angle Laser LightScattering
TFA	trifluoroacetic acid
Xyl	Xylose
ζ	Zeta potential
HPLC	High Performance Liquid Chromatography

Chapter 1

General Introduction

1 GENERAL INTRODUCTION

This chapter provides an inclusive review of the relevant literature. This will include an overview of carbohydrates, including the chemical and physical properties of the major carbohydrates utilized during the thesis. This chapter will discuss the basic facts relating to polysaccharides such as their structural, physical and chemical properties and pharmaceutical applications also the bioactivity.

1.1 Carbohydrates:

In general, the term 'carbohydrate' comprises monosaccharides, oligosaccharides and polysaccharides. Carbohydrates are simple organic compounds consisting of aldehydes and ketones and which also contain many hydroxyl groups, commonly one on each carbon atom. 'Sugar' is a term is often used to describe monosaccharides and short-chain oligosaccharides. It is notable that about 3 % of the compounds recorded by the Chemical Abstracts Service (more than 360 000) are classified as carbohydrates (McNaught, 1997).

Carbohydrates come by their name due to the generic formula which is $C_n\,(H_2O)_m$ where n is equal to or greater than three, they are one of the most plentiful organic compounds on earth and they demonstrate a large number of functions such as the molecules of energy storage, construction blocks, in addition to bacterial and viral recognition targets, therefore carbohydrates are considered as very important molecules. Some of them have a part in medical applications, for example oligosaccharides which can be used in influenza virus treatment (heparin, Tamiflu and different types of vaccines) (Azofra et al., 2014).

'Sugar' is a term is often used to describe monosaccharides and short-chain oligosaccharides. It is notable that about 3 % of the compounds recorded by the Chemical Abstracts Service (more than 360 000) are classified as carbohydrates (McNaught, 1997).

1.1.1 Classification of carbohydrates:

Carbohydrates can be classified by chain length as monosaccharides, oligosaccharides and polysaccharides.

1.1.2 Monosaccharides

Monosaccharides are the most basic units of carbohydrates which can be divided into two groups aldoses: (*e.g* glucose, ribose and galactose) and ketoses: (*e.g* fructose and ribulose) as shown in **Figure 1.1.** Glucose and fructose are widely found in nature and are considered to be the most common monosaccharides (Moynihan, 1998). Glucose is a basic energy source for almost all forms of life (Berg, 2012), it is found in the free form in the blood of animals, as well as in plants, fruits and honey (Roberts and Caserio, 1977) while fructose is another significant monosaccharide which is present in corn syrup, fruits and honey (Seager and Slabaugh, 2013).

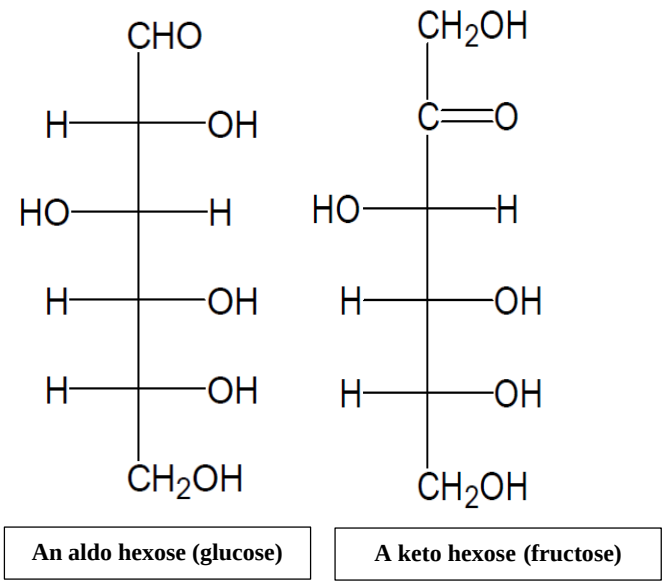


Figure 1.1: Example aldose and ketose monosaccharides (Seager and Slabaugh, 2013).

Monosaccharides are the simplest sugars which form the building blocks of carbohydrate chemistry, they are colourless and have sweet taste, they dissolve easily in water, but they do not dissolve quickly in polar liquids such as ethanol (Nelson et al., 2008). Monosaccharides in contrast to oligosaccharides or polysaccharides signify a single unit, therefore without glycosidic linkages to other units (McNaught, 1997). It is according to the length of the carbon chain and the nature of the carboxyl group that monosaccharides are classified (Dyke and Dyke, 1960).

1.1.3 Disaccharides and oligosaccharides:

Disaccharides are two carbohydrate molecules linked together (Moynihan, 1998). For example, sucrose (table sugar) which is the most important disaccharide formed from glucose and fructose see **Figure 1.2.** It is plentiful in sugar beet and sugar cane also about 65 % sucrose is found in Maple syrup (Seager and Slabaugh, 2013). Maltose (malt sugar) which is formed from two glucose molecules; it is abundant in germinating grain such as barley (Seager and Slabaugh, 2013). Also, lactose (milk sugar) which is formed from glucose and galactose where about 5 % of cow's milk and 7 % of human milk is lactose (Moynihan, 1998, Seager and Slabaugh, 2013).

Oligosaccharides are carbohydrates with short chains consisting of three to ten monosaccharide units (Roberts and Caserio, 1977). Raffinose is an example of an oligosaccharide which is plentiful in beans and peas (Seager and Slabaugh, 2013). According to (Nelson and Cox, 2008) oligosaccharides are short chains of monomers that are linked by glycosidic bonds.

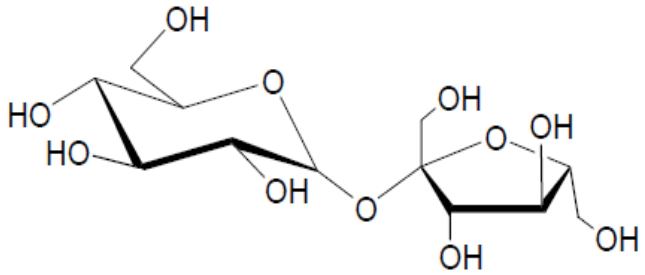


Figure 1.2*:* Structure of sucrose (Seager and Slabaugh, 2013).

1.1.4 Polysaccharides:

Polysaccharides are carbohydrates polymers consisting of more than 10 sugar units linked together, of which there is a vast variety for example :energy storage polysaccharides (starch and glycogen), structural polysaccharides (cellulose and chitin), heteropolysaccharides: polysaccharides that consist of more than one type of monosaccharide units *e.g.* pectin and arabinoxylan and homopolysaccharides: polysaccharides that consist of from the same type of monosaccharide units, include: plant polysaccharides *e.g.* starch and glycogen, animal polysaccharide *e.g.* chitin and glycogen and bacterial polysaccharides *e.g.* xanthan gum, gellan gum, dextran and curdlan (Simi, 2010, Khowala et al., 2008).

Polysaccarides are cheap and can consist of many different structures with many useful industrial properties. In addition they are highly stable, non-toxic, not harmful and biodegradable (Sinha and Kumria, 2001). Polysaccharides are classified as shown in **Figure 1.3:**

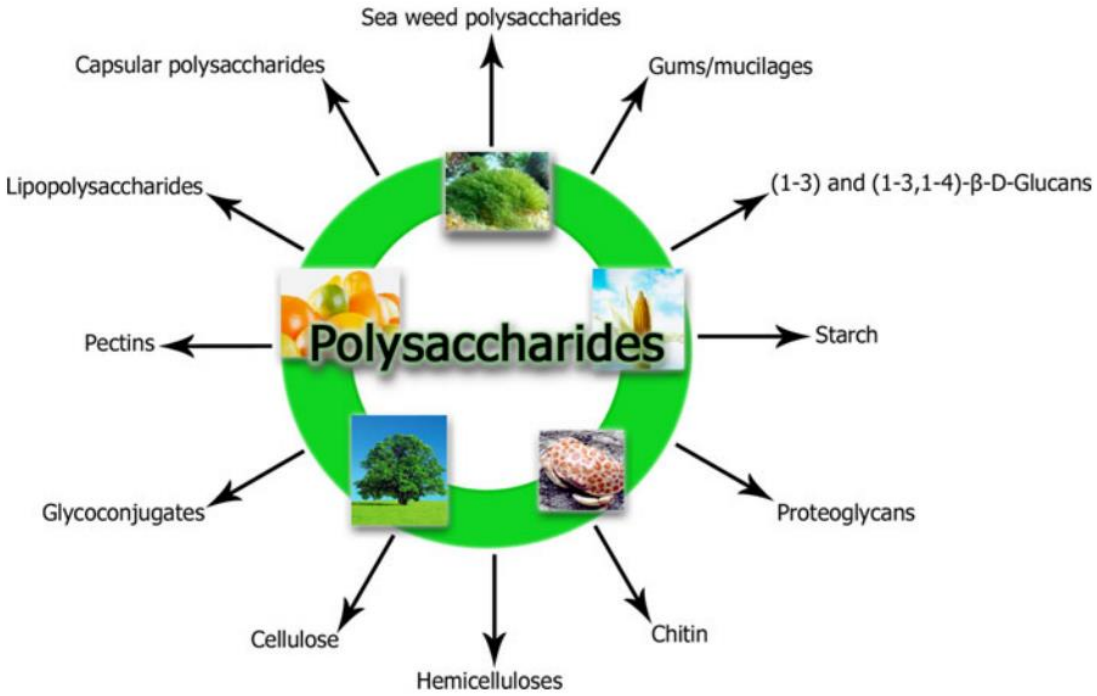


Figure 1.3: Molecular varieties of polysaccharides (Cunha and Gandini, 2010)**.**

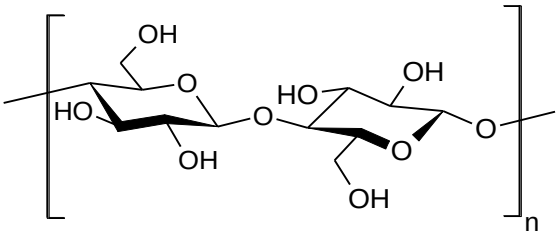
Some of the polysaccharides commonly seen in plants include: cellulose, starch, xyloglucans, galactomannans and pectin are discussed in some detail below:

1.2 Some important polysaccharides:

1.2.1 Cellulose:

Cellulose is the most abundant polysaccharide, it is found in cell walls of both plants and fungi and also can be synthesised by some bacteria. Structurally, it is made up of β glucopyranose molecules joined together with (1→ 4) linkages, see **Figure 1.4**. This polysaccharide is organized in a long ribbon formation and has a high molecular weight with each residue in the chain rotated 180° from the one before. There are two kinds of cellulose which are the most significant in commercial materials; cellulose esters and cellulose ethers, however, native cellulose are insoluble in water (Cui, 2005). As this

polysaccharide is insoluble in water it will not be in the aqueous fraction recovered in the extraction of crude polysaccharides.



Cellulose

Figure 1.4: Linear chain structure of cellulose (Simi, 2010).

1.2.2 Starch:

Starch is a material that in particular found in energy storage areas of, for example seeds, fruit and root tubers of the majority of plants (Lehmann, 1998). Starch comprises three types: one found in cereals (A-starch), the second from tubers which is a less common type (B-starch), the last one C-starch (a mixture of A-starch and B-starch). The two types have very similar polysaccharide chains but are packaged differently (Rao, 1998). Starch is made up of two structurally similar homopolysaccharides; amylose and amylopectin whereby both of these are made up of glucose chains in varying length and branching depending on the plant species. Amylose is unbranched and is made up of glucose molecules with α- 1,4 bonds whereas amylopectin is branched and contains α- 1,6 bonds and α- 1,4 bonds in roughly 1:30 ratio see **Figure 1.5** (Berg, 2007).

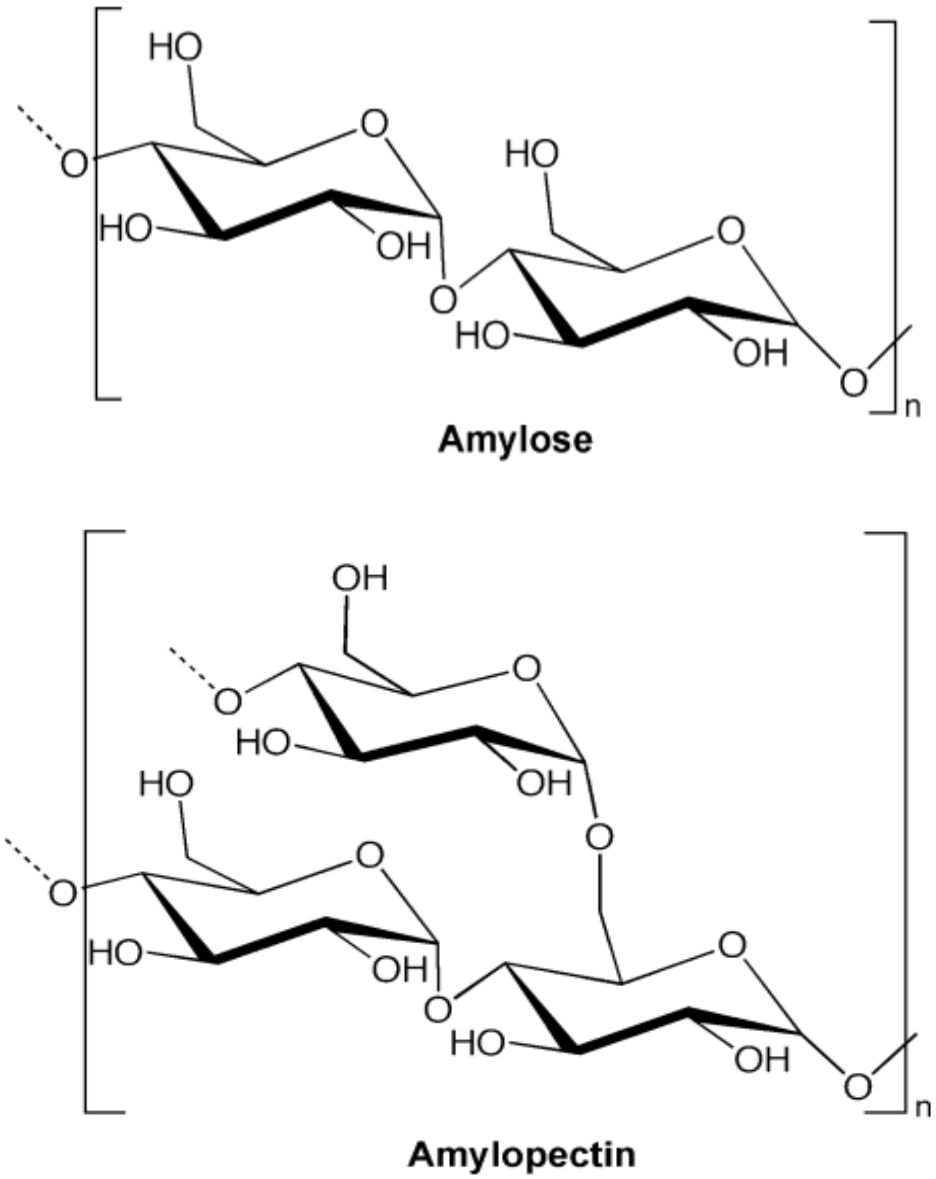


Figure 1.5: Linear and branched structures that make up starch (Cunha and Gandini, 2010).

1.2.3 Xyloglucans:

Xyloglucans are storage polysaccharides (hemicellulosic polysaccharides) which are present in the cell walls of higher plants in linear structure with side chains (Nishinari et al., 2007). They have a common backbone structure which is the same as cellulose, consisting of β (1→4)-linked D-glucan molecules, which are substituted every three or four glucose residues by α-D-xylose or galactosyl residues in position O6. This is the general structure of all xyloglucan and fine structure depends on the source. Additional residues are attached to xylose, the differences in side chain residues results in the

extraction of different xyloglucans from different species see **Figure 1.6**. The branching pattern, depends on plant species *e.g.*, the repeating unit of *Lactuca sativa, Tanacetum ptarmiciflorum*, and *Daucus carota* has XXXG-type (*) branching pattern, which is the most typical xyloglucan repeating unit, containing α-D-Xyl*p*,β-D-Gal*p*-(1→2)-α-D-Xyl*p*, and α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→2)-α-D-Xyl*p* side chains (Picout et al., 2003, Nishinari et al., 2007). The most common xyloglucan studied particularly in terms of the rheological behaviour for different applications is called tamarind seed xyloglucan (TSX) (Nishinari et al., 2007). In addition, xyloglucan has many uses in the food industry for example as a thickener, stabilizer, gelling agent and starch modifier (Yoshimura et al., 1999). Besides, it is used in pharmaceutical industries whereby it can be used in mucoadhesive drug delivery systems due to its hydrophilic and mucoadhesive properties (Burgalassi et al., 2000). Furthermore, it is used in cosmetic industries as an ultraviolet protective agent when it in combination with other ultraviolet protective agent (Simi, 2009).

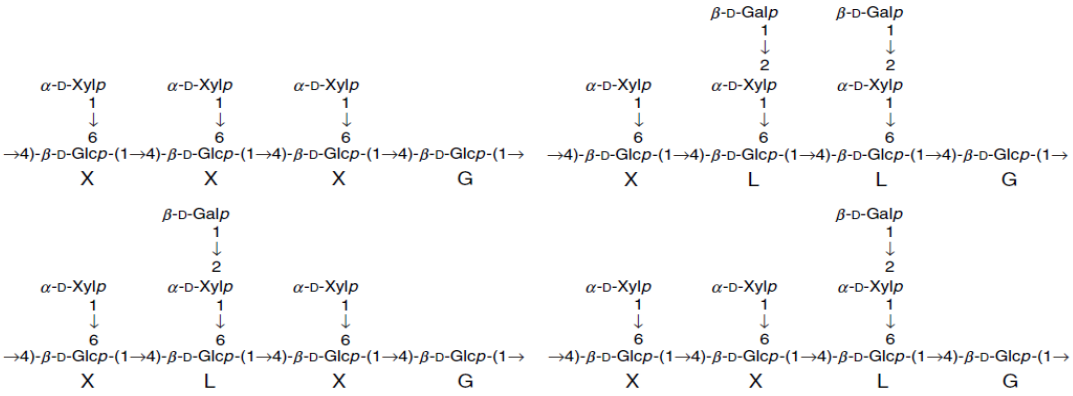


Figure 1.6: The chemical structure of the xyloglucan repeat unit in (Nishinari et al., 2007).

*Abbreviations such as X and G are shown in **Figure 1.6** above.

1.2.4 Galactomannans:

Galactomannans are normally identified as seed gums (Dea and Morrison, 1975). They are heterogeneous polysaccharides which are vastly spread in nature. However, they have attracted a lot of academic attention and industrial interest, this is due to their properties in aqueous media such as thickening and gelling (Nishinari et al., 2007). Their backbones consist of a β-(1→4)-D-mannan to which is attached a single D-galactose branch linked α-(1→ 6) **Figure 1.7.** The differences between each other can be seen through the ratio of mannose/galactose (M/G) which varies with plant origin (Cerqueira et al., 2011, Srivastava and Kapoor, 2005). Nevertheless, the main sources of galactomannans are: locust bean (*Ceratonia siliqua*), guar (*Cyamopsis tetragonoloba*), tara (*Caesalpinina spinosa Kuntze*) and fenugreek (*Trigonella foenum-graecumL.*) (Prajapati et al., 2013). Galactomannan's average molecular weight ranges from 1 to 2 $\times 10^6$ g/mol, but will depend on extraction/ solubilisation conditions.

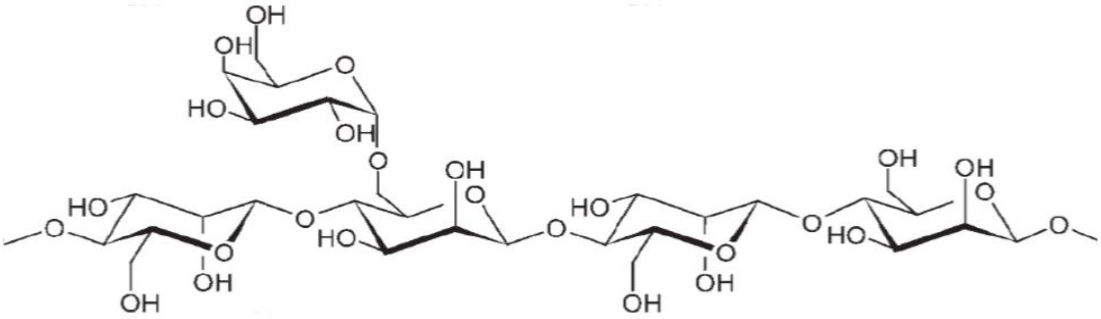


Figure 1.7: Primary structure of galactomannan (Ebringerová, 2005).

Galactomannans are extensively used in industry. Mostly in the food industry (dairy products) such as coffee whiteners, bakery, powdered products, fruit-based water gels, seasonings, sauces and soups, baby milk formulations, tinned meats and frozen and cured meat foods. This is due to the suitable functional properties of galactomannans such as binding, thickening, and stabilizing abilities. Besides, galactomannans are multilateral materials which can be used in a lot of applications: they are excellent stabilizers and stiffeners of emulsions and can often be used in different forms for human consumption. Also, galactomannans are utilized in the textile, pharmaceutical, biomedical and cosmetics industries because they are less toxic polysaccharides (Vieira et al., 2007, Prajapati et al., 2013, Stephen, 1995).

1.2.5 Pectin:

Pectins are a family of biopolymers which occur in nature (Mishra et al., 2012). In the last few years they have become more and more important in terms of their potential applications. They have been extensively studied and published on but it is problematic to characterize them as a model system due to the heterogeneous nature of the polymer. However, pectins are commercially extracted from citrus peels and apple pomace under mildly acidic conditions (Sriamornsak, 2003). Therefore, they are considered to be one of the most widespread hydrocolloids, this is because pectins have been used successfully for many years in the food as a colloidal stabilizer, gelling agent and thickening agent for its capability to control texture. (Sriamornsak, 2003). Pectin is a substance that is able to bind water and to form gels at low concentrations (Ciriminna et al., 2016). Commercially, the form of pectin produced is a white to light brown powder (Srivastava and Malviya, 2011) see **Figure 1.8**. Pectins are ionic polysaccharides, due to the polyelectrolyte behaviour they have a number of useful features for various applications (Cardoso et al., 2003a).

Figure 1.8: Pectin in powder form after extraction (Ciriminna et al., 2016).

Generally, a number of studies mentioned that pectin polysaccharides can be classified in three types based on D-galacturonic acid content (Valdés et al., 2015) as shown in **Figure 1.9**, and/or also the classification of pectin can be according to its procedure of extraction from plant cell walls (Yuliarti, 2011): water-soluble pectin is extractable with water or dilutes salt solutions, chelator soluble pectin is extractable with calcium-chelating-agent solutions (*i.e.* ethylene diamino tetraacetic acid (EDTA), cyclohexane diamino tetraacetic acid (CDTA) or hexametaphosphate and protopectin: is extractable

with alkali or hot dilute acid solutions (Van Buren, 1991). Protopectin is the native pectin fraction in the cell wall that cannot be isolated without some degardation (Wang et al., 2002).

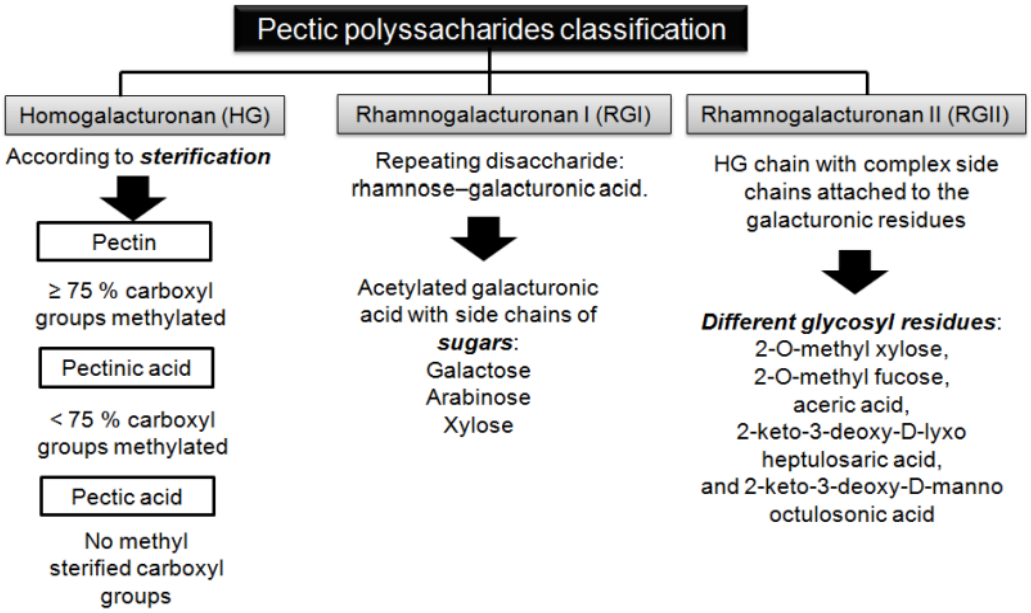


Figure 1.9: Classification of pectic polysaccharides based on D-galacturonic acid (Valdés et al., 2015).

Pectins are the polysaccharide which are the most interested in and the next chapter will discuss their structure, extraction, properties and applications in more details.

1.3 Aims, objectives and hypothesis:

1.3.1 Aims:

- To determine the influence of different extraction conditions on the physicochemical properties of pectins extracted from pumpkin using factorial design.
- To ascertain the influence of genetic variation and growth conditions on pectins extracted from okra.
- To evaluate the effect of genotype on the yield and physicochemical properties of okra pectins and their potential impact on specific applications.

1.3.2 Objectives:

The main objectives of this study were to:

- Investigate a number of factors on the physicochemical properties of pectins from natural sources *e.g.* pumpkin (chapter 5) and okra (chapter 6). Factors which may be of interest are extractions in the case of pumpkin *e.g.* pH, temperature, time, *etc*, and in the case of okra genetic variation of source materials. Successfully extract and isolate carbohydrates.
- Purify the extracted samples using both dialysis and freeze-drying to remove salts formed in the extraction process.
- Analyse the purified polysaccharide sample using a range of different analytical techniques.
- For example, the use of HPAEC-PAD to identify and quantify the constituent sugars, which form the polysaccharide chains. Also using size-exclusion chromatography to identify the length of those polysaccharide chains; this will be combined using a global hydrodynamic conformational analysis to estimate the number and length of branches. In addition, infra-red spectroscopy and NMR will carry out to confirm the structures of the polysaccharides identified. ζ-potential measurements and capillary viscometry also to examine the properties and stability of the resulting pectin.

1.3.3 The hypothesis:

For the hypothesis of this study, extraction conditions and/or genetic variation can be chosen to design tunable pectins with desired properties.

1.4 Thesis structure:

This thesis includes the extraction, characterisation and modification of pectins from pumpkin and okra plants. The first chapter (1) shows the general information about carbohydrates and their classification. The second chapter (2) includes the structure, extraction, applications and physio-chemical properties of the materials utilized in this study (pectins). The third chapter (3) provides background information about the main instruments used in this study for characterisation. The fourth chapter (4) explains the general experimental and methodology that applied in results chapters (chapter 5 and 6). The fifth chapter (5) discusses the results obtained from the physio-chemical characterisation of the pectin extracted from the pumpkin. The sixth chapter (6) presents and discusses the results obtained from the physicochemical characterisation of the pectin isolated from different genotypes of okra. The seventh chapter (7) concludes the results obtained in this thesis together with recommendations for future perspectives. The eighth chapter (8) which is the final chapter, which provides a list of the literature cited which have been read in the design, interpretation and writing of this thesis.

Chapter 2

Structure, Extraction, Properties and Applications of Pectin

2 STRUCTURE, EXTRACTION, PROPERTIES AND APPLICATIONS OF PECTINS

2.1 Pectin chemical structure:

Pectins are negatively charged, hydrophilic biopolymers (Tsai et al., 2014). The structure, composition and physiological properties of pectins depend on certain factors whereas these factors make them different from one to another for example: the plant source, the type of tissue, geographical location, growth or metabolic stage, the ecological state, conditions of extraction, polymer conformation, and flexibility of polymer chains (Round et al., 2010, Kpodo et al., 2017). Besides, the plant source, genotype, stage of ripening and extraction methods are factors for pectins which can show heterogeneity in their macromolecular characteristics, which thus affect their functional properties (Alba and Kontogiorgos, 2017). Pectins are acidic heteropolysaccharides (anionic polysaccharides) as has been described wherein they mainly consist of α- (1→4) linked α-D-galacturonic acid (GalA) residues **Figure 2.1**. However, in biology, pectin has specific functions depending on its location and molecular structure (Chan et al., 2017).

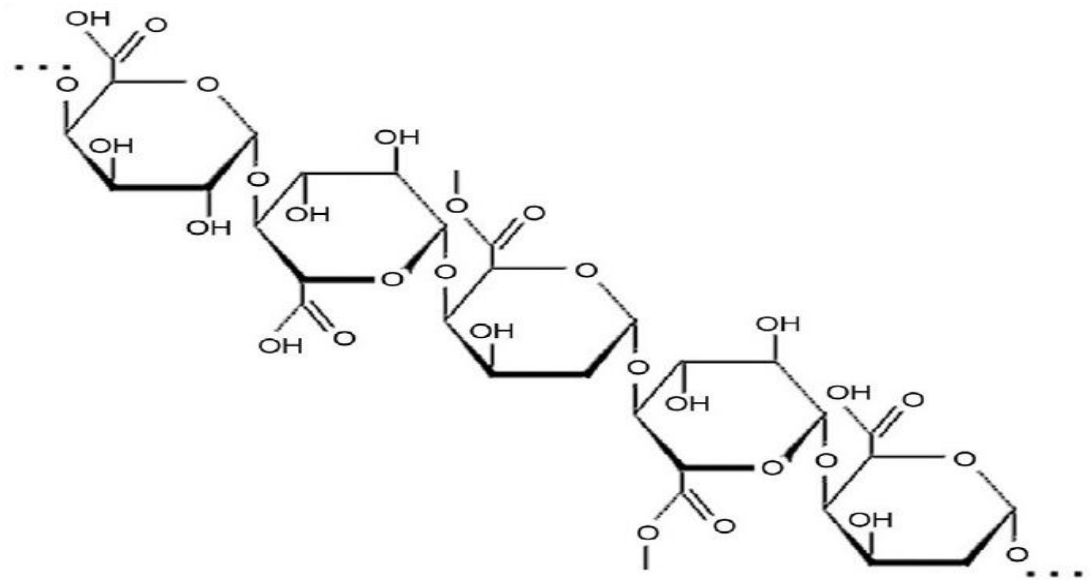


Figure 2.1: Structure of pectin adapted from (Rajpurohit et al., 2010).

Though pectin was discovered over 200 years ago until now the structure and composition of pectin are still not totally understood (Sriamornsak, 2003). The structure

of pectin is very difficult to determine because pectin can change during isolation from plants, storage, and processing of plant material (Novosel'skaya et al., 2000). As for most natural polysaccharides, pectins are characterized by structural and molecular weight heterogeneity and polydispersity (Stephen and Phillips, 2010). Structurally there are two main regions for pectin, hairy (branched) region and smooth (linear) region (Mishra et al., 2012) which are shown in **Figure 2.2** below:

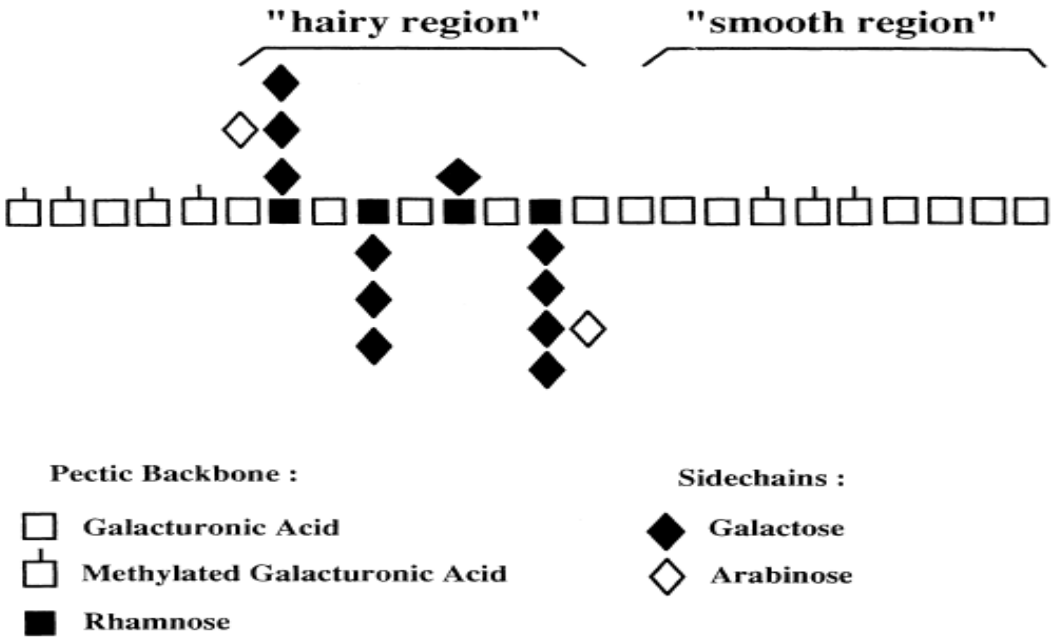
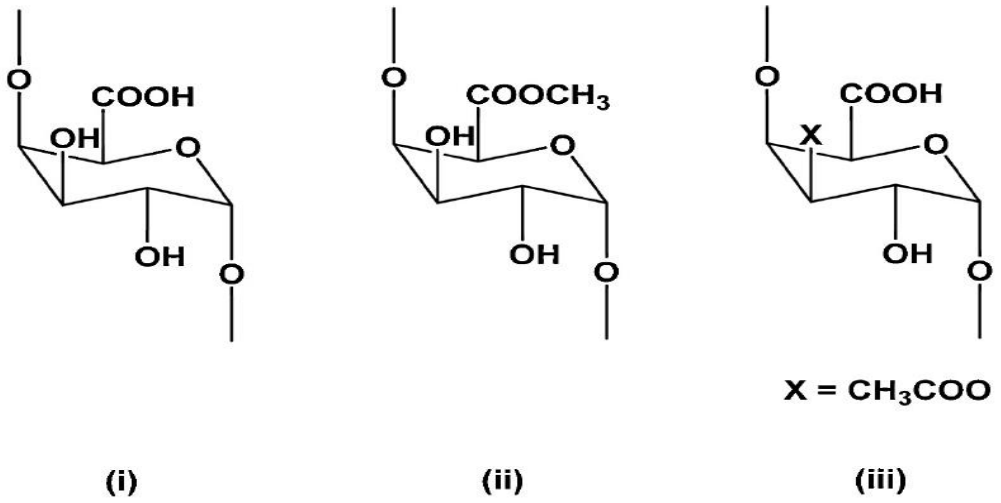
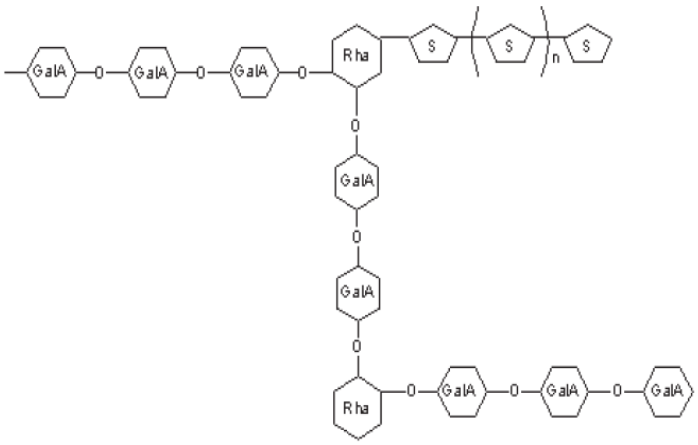


Figure 2.2: A simple schematic drawing of pectin showing the smooth and hairy regions (Catoire *et al.*, 1998).

Pectins are polysaccharides rich in galacturonic acid **Figure (2.3A)** (i) (Mohnen, 2008). They have a complex structure named pectic polysaccharides. Roughly 70 % of pectin and all the pectic polysaccharides contain galacturonic acid linked at the O-1 and the O-4 positions (Mohnen, 2008). Because of pectin's structural complexity, they are associated with many enzymes, including lyases, hydrolases, and esterases (Bonnin et al., 2014). Basically, the pectic polysaccharides are characterised by different structural domains, which can be composed of up to 17 distinct monosaccharides bearing carboxylic groups, some of them methyl esterified **Figure (2.3A)** (ii) or bearing acetyl groups **Figure (2.3A)** (iii). O-acetyl-esterification exists mainly at the O-3 position and sometimes at the O-2 position. Besides, L-arabinose, D-galactose, L-rhamnose and others can be interrelated through 20 different linkages with the GalA units, see **Figure (2.3B)** (Ridley et al., 2001, Kaya et al., 2014).



(A)



(**B**)

Figure 2.3: (A) Forms of galacturonic acids (GalA) found in pectin: (i) GalA, (ii) Methylated GalA and (iii) O-Acetylated GalA. (B) Schematic diagram showing how rhamnose (Rha) insertions cause linking of galacturonic acid (GalA) chain; S = neutral sugars. Adapted from (Sriamornsak, 2003).

Some early studies discovered that regular, individual pectic polysaccharides have long branches. Over the years, these pectic structural domains have been described (Round et al., 2010) which are described in the following sections.

2.1.1 Homogalacturonan (HGA):

Homogalacturonan (HGA): is a homopolymer of α-1→4-linked galacturonic acid residues that are regularly methyl esterified on C6. However, on C2 or C3 of the GalA residues, HGA can be acetylated but the degree of acetylation varies a lot between species, see **Figure 2.4** (Scheller et al., 2007, Ridley et al., 2001, Gullón et al., 2013). . HGA is the most plentiful pectic polysaccharide subfraction and has been shown to be present in stretches of approximately 100 GalA residues in length that comprises 65 % of pectin (Khodaei and Karboune, 2013). However, there are two types of HGAs depending on the degree of methyl esterification whereby HGs with >50 % methyl-esterification of GalpA residues are defined as high methyl-esterified HGs while those with <50 % are described as low methyl esterified HGs. Besides, the industrial applicability (gelling ability, for example) of pectin is determined by the degree of methyl esterification of linear HG units, taking into account the distribution of methyl groups on the HG backbone and the amount of methyl-esterification (Alba, 2015). Pectins from different sources can be dissimilar in their composition. This may be due to the location of esterified GalA which could be random or non-random along the backbone chain (Oakenfull, 1991); non-random esterification can also be achieved via enzymatic extraction procedures.

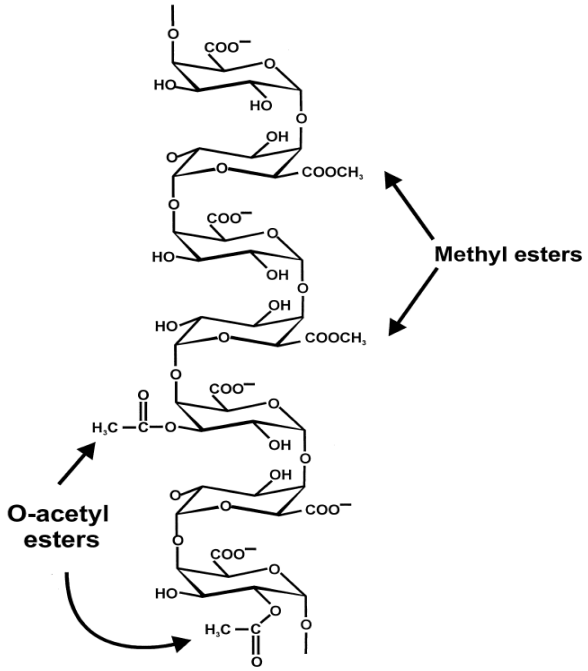
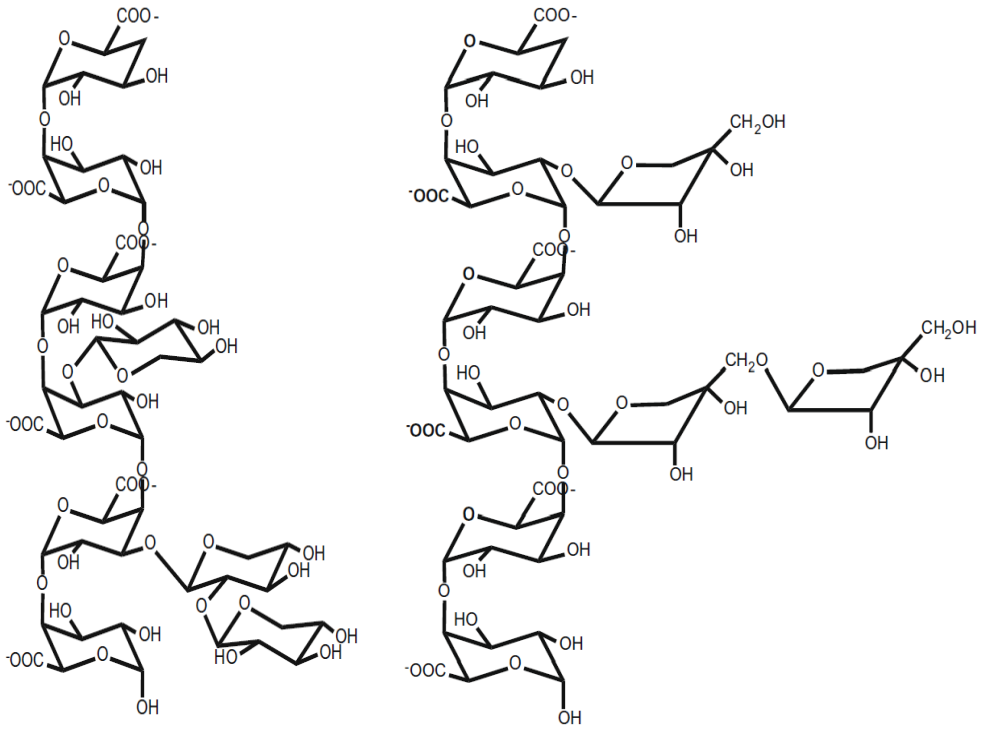


Figure 2.4: Theprimary structure of homogalacturonan one of the major pectic polysaccharides (Ridley et al., 2001).

2.1.2 Xylogacturoanan and Apiogacturonan:

Xylogalacturonan (XGA) is similar to homogalacturonan (HGA) residues, replaced with single β-(1→ 3) -Xyl residues see **Figure 2.5a**. XGA has been stated mainly in reproductive tissues, but this type of pectin possibly exists in all tissues, at least in Arabidopsis (Scheller et al., 2007, Albersheim et al., 1996). However, ithas been found in the cell walls of cotton seeds, watermelons, apples, marine seagrasses, soybeans and peas (Zandleven et al., 2007). XGA proposed as a side chain of RG-I of pectin (Vincken et al., 2003a).

Apiogalacturonan (AGA) was detected in the walls of aquatic plants like duckweeds (*Lemnaceae*) and marine seagrasses (*Zosteraceae*) with D-apiose residues 2,3-linked to homogalacturonan see **Figure 2.5b** (Caffall and Mohnen, 2009).



a **b**

Figure 2.5: The substituted galacturonans (a) xylogalacturonan and (b) apiogalacturonan (Caffall and Mohnen, 2009).

2.1.3 Rhamnogalacturonan I (RG-I):

Rhamnogalacturonan type I (RG-I) is another constituent polysaccharide of pectin, alternative units of rhamnose (Rha) and GalA branched mainly with galactan, arabinan

and arabinogalactan,see **Figure 2.6** (Gullón et al., 2013). However, in the plant cell wall RG-I represents about 20 – 35 % of pectin (Mohnen, 2008, Øbro et al., 2004). Furthermore, RG-I is a pectic subfraction that comprises a backbone of the disaccharide repeat [1,4-α-D-GalA-1,2-α-L-Rha-]$_n$ where *n* can be larger than 100. The backbone of RG-I is partly replaced at O-4 and/or O-3 positions of α-L-Rhap residues with side-chains often composed of α-(1,5)-L arabinans and β-(1,4)-D galactans, arabinogalactans I (AG-I), arabinogalactans II (AG-II) and galacto-arabinans (Mohnen, 2008). There are two types of side chain: (i) a single unit such as [*β*-D-Gal*p*-(1→4)] or (ii) polymeric, as arabinan and arabinogalactan I (AG-I). The backbone of arabinans comprised of a 1,5-linked *α*-L-Ara*f*, can be replaced with *α*-L-Ara*f*-(1→2)-, *α*-L-Ara*f*-(1→3)- and/or *α*-L-Ara*f*-(1→3)-*α*-L-Ara*f*-(1→3)-, side chains depending on the pectin source for example: sugar beet, soybean (Ridley et al., 2001). In the side chains of RG I the galactan and arabinan are considered as the most mobile parts of the pectin molecule with the higher degree of mobility (flexibility) presented by arabinan (Sinnott, 2007). In addition, depending on the source of the polysaccharide, the proportion and distribution of branched Rha*p* residues usually vary in the range of 20-80 % (Visser and Voragen, 1996). This in turn creates the heterogeneous structure of RG-I arabinan and galactan side-chains from source to source, which has been observed for pectic polysaccharides from various cell walls such as soybean, potato, sugar beet, apple and persimmon (Huisman et al., 2001, Duan et al., 2003, Øbro et al., 2004, Sakamoto and Sakai, 1995, Schols and Voragen, 1996). Nevertheless, unbranched RG-I molecules have been reported in seed mucilages (Western et al., 2004). Also, RG-I backbone at position (O-3 of Rha*p*) or at (O-2 and/or O-3-GalpA) residues can be acetylated depending on the plant species (Sengkhamparn et al., 2009b, Vincken et al., 2003b).

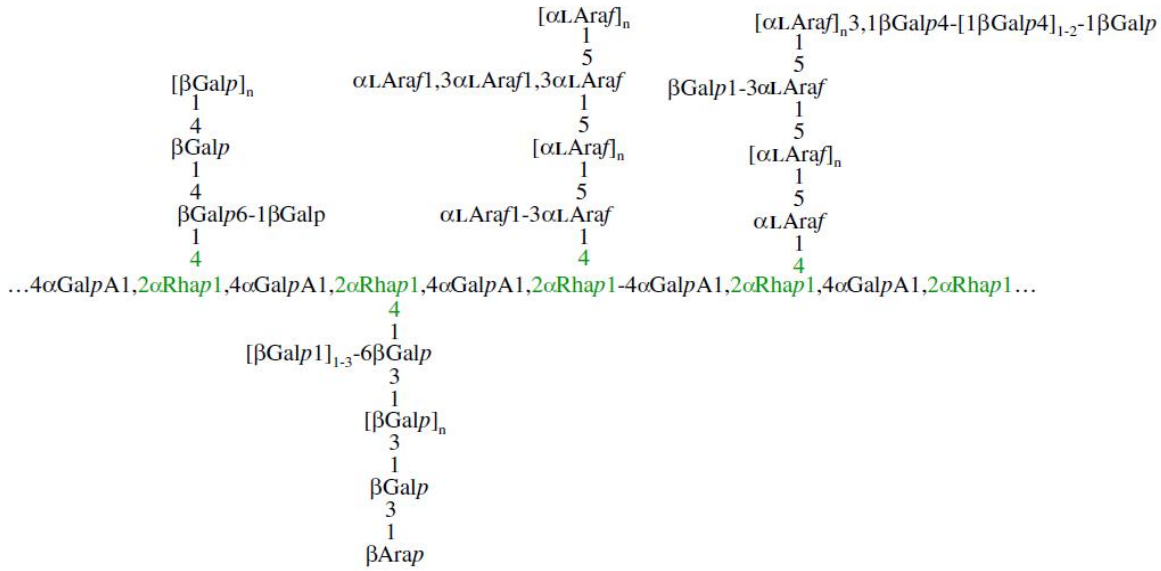


Figure 2.6: The structure of rhamnogalacturonan I (Caffall and Mohnen, 2009).

2.1.4 Rhamnogalacturonan II (RG-II)

Rhamnogalacturonan type II (RG-II) a polymer which is covalently linked to RG-I to form a macromolecular pectin complex (Ishii and Matsunaga, 1996). Also, it consists of GalA, Rha, Gal and some unusual sugars, for example, 2-O-methyl xylose, 2-O-methyl-fucose, aceric acid, 2-keto-3-deoxy-D-lyxoheptulosaric acid (Dha) and 2-keto-3-deoxy-D-manno octulosonic acid (Kdo) (Stevenson et al., 1988, York et al., 1985, Ochoa-Villarreal et al., 2012). RG-II is believed to play a key role in the architecture of the cell wall despite being present as a quantitatively minor pectic subunit with high structural complexity (Ishii and Matsunaga, 2001). So, with 12 different types of sugars and over 20 different linkages, the structure of RG-II is highly complex (Caffall and Mohnen, 2009). Furthermore, (RG-II) domains are more complex in structure than HG but despite this they have lower molecular weights (Fukuda, 2014). The tissues of the cell walls of the following edible plants: such as apple, kiwi, carrot, tomato and radish have been identified as the most common sources of RG-II (Cui, 2005). RG-II is defined as a stretch of HG backbone, around 7 to 9 (1,4-linked *α*-D-GalA*p*) residues attached with four of variable length side-chains (marked as A-D, **Figure 2.7**)(Caffall and Mohnen, 2009).

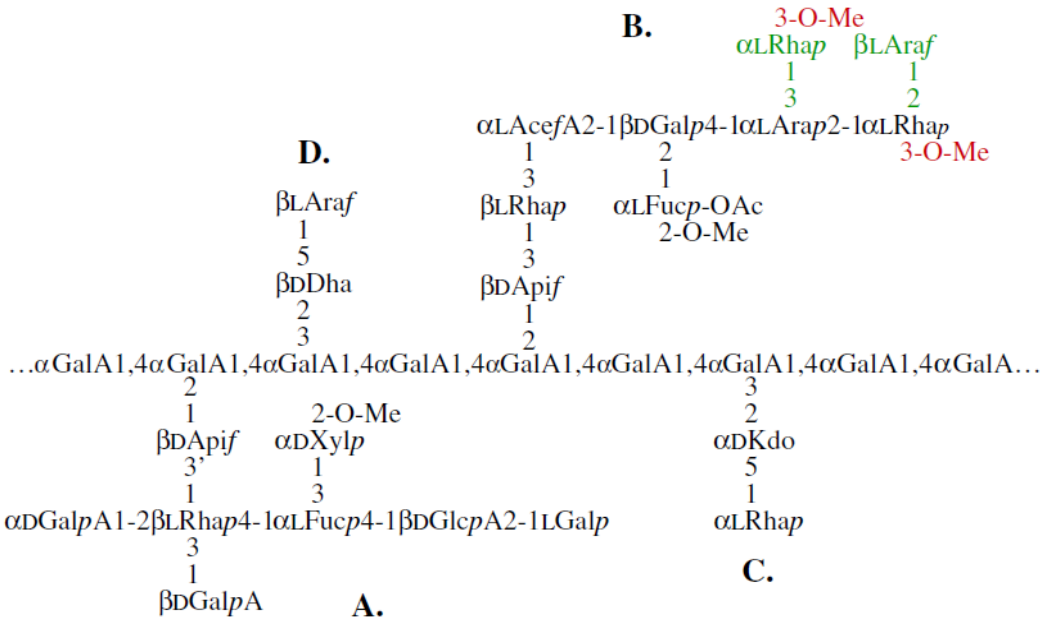


Figure 2.7: The primary structure of rhamnogalacturonan II (Caffall and Mohnen, 2009).

The structure of the four major pectic subfractions is shown in **Figure 2.8.**

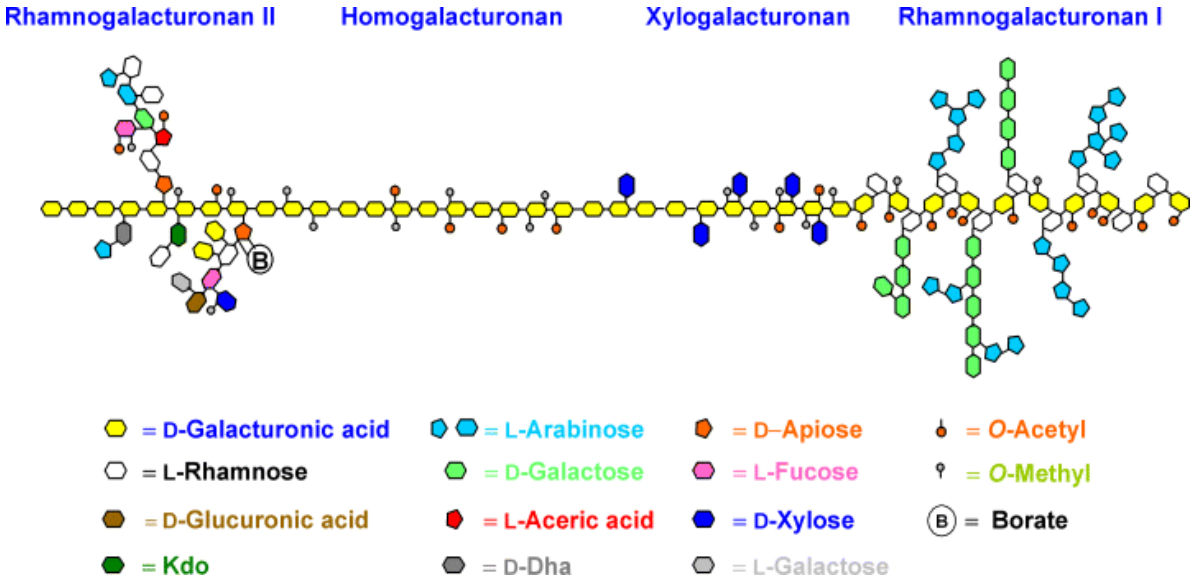
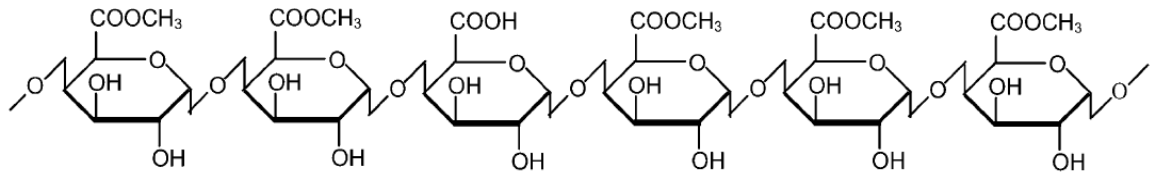
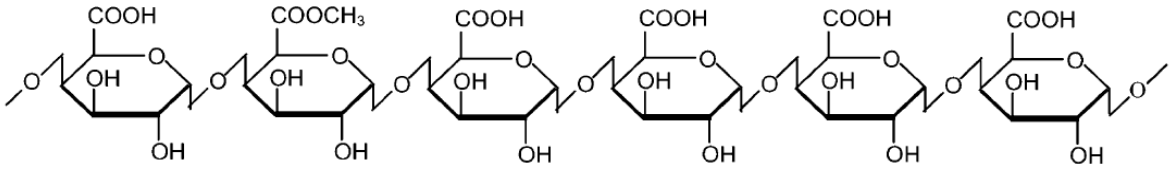


Figure 2.8: Schematic structure of pectin showing the four main pectic subfractions: homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) adapted from (Scheller et al., 2007). *N.B.* the relative proportions of each subfraction are shown for visualisation purposes and in reality, HG and RG-I are much more abundant.

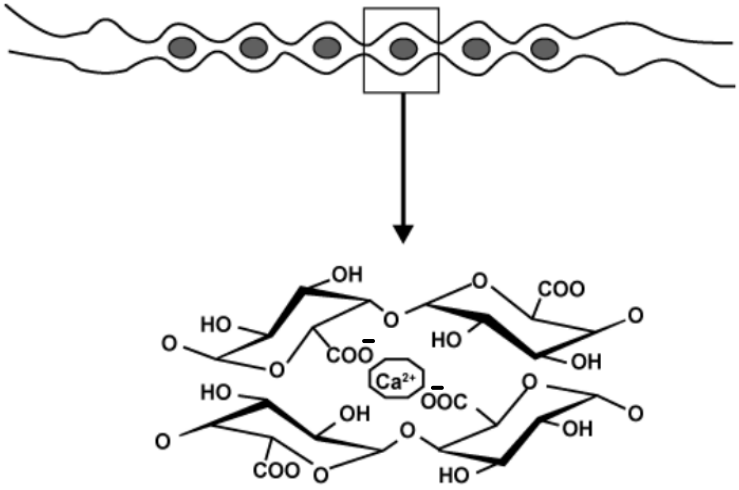
According to the degree of methylation pectins are divided into two main groups: (i) high methoxyl pectin (HMP) having DM > 50 % see **Figure 2.9a**, which, can form a gel in case of high concentrations of sugar about 55 % and pH lower than 3.5. (ii) Low methoxyl pectin (LMP) having DM < 50 % see **Figure 2.9b**, wherein its gel forms by reacting with divalent cations, in particular, Ca $^{2+}$ according to the "egg box" model. The model comprises extended collections of site-bound Ca^{2+} between (1→4) linked GalA chains in a highly buckled two-fold conformation (Jarvis and Apperley, 1995) see **Figure 2.9c**. Typically, DE values range from 60-75 % and 20-40 % for HM-pectins and LM-pectins respectively (Sriamornsak, 2003). This gelling ability has been used for a variety of foods (Liang et al., 2012a). Wherein, HMP is mostly used as a gelling agent in the manufacture of jams and fruit preservatives especially in fruit-based products, such low-calorie jams and jellies, confectionery jelly products. while LMPmeanwhile is used to formulate gels with a minimized level of dissolved solids, and is of great importance because of their lower caloric value (Seixas et al., 2014b). LMP gels can be also utilized in jellies and bakery jams for retorting, glazing, baking, microwaving and pasteurization or sterilization the heat reversibility (Pagán et al., 2001). HMP and LMP have different gelation mechanisms, although the gel characteristics are governed by the same macromolecular properties such as the size, composition and conformation of the polymers (Axelos and Thibault, 1991).



(a)



(b)



(c)

Figure 2.9: Structure of (a) high methoxyl pectin (b) low methoxyl pectin (c) LM-pectin's gelation mechanism is based on the egg-box model adapted from Tharanathan, (2003), Sundar Raj et al., (2012).

Technically, there are several analytical tools for exploring the fine structures and functionalities of the pectin macromolecules, they are listed in **Table 2.1**, whereby combining of these techniques with enzymatic finger-printing is a strong approach to clarify the complex structure of pectin (Daas et al., 1998, Daas et al., 1999, Daas et al., 2000, Limberg et al., 2000).

Table 2.1: Some of the qualified analytical techniques of exploring the fine structures and functionalities of the pectin macromolecule reported by (Chan et al., 2017)**.**

Technique	References
Fourier transform infrared spectroscopy (FT-IR)	(Coimbra et al., 1998)
FT-Raman spectroscopy	(Bichara et al., 2016)
Gas chromatography (GC)	(Huisman et al., 2004, Walter and Sherman, 1983)
High-performance liquid chromatography (HPLC)	(Levigne et al., 2002b, Voragen et al., 1986)
Gas chromatography-mass spectrometry (GC-MS)	(Savary and Nuñez, 2003)
Electrospry-ionization mass spectrometry (ESIMS)	(Ishii et al., 2002)
Ion exchange chromatography (IEC)	(Bonnin et al., 2002, Kravtchenko et al., 1992, Ralet et al., 2001)
Polysaccharide analysis using carbohydrate gel electrophoresis (PACE)	(Goubet et al., 2005)
Capillary electrophoresis (CE)	(Ström et al., 2005, Zhong et al., 1998)
Nuclear magnetic resonance NMR spectroscopy	(Cardoso et al., 2002)

2.2 Occurrence, sources and production of pectin:

Pectin has been studied widely by scientists from various branches of science (Chan et al., 2017). Pectin is a multifunctional polymer whereby the highest concentration of pectin is situated mainly between cells in the middle lamella in higher plant tissues which is often related with other cell wall components such as cellulose, lignin and hemicellulose, which is a very significant raw material for food and pharmaceutical products (McNaught, 1997, Stephen and Phillips, 2010, Yuliarti, 2011) see **Figure 2.10.**

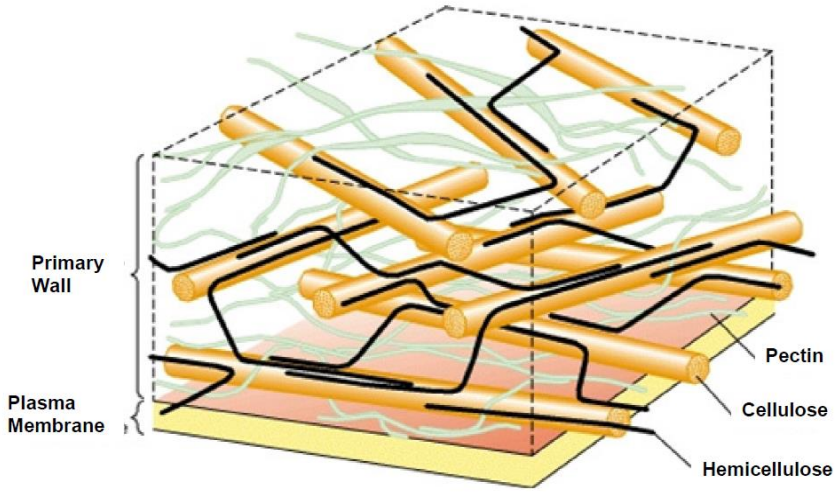


Figure 2.10: The structure of the cell wall. Pectins are regularly linked with other cell wall components, for instance, cellulose or hemicellulose in plant tissues. Adapted from (Yuliarti, 2011).

Generally, pectin is most plentiful in the middle lamella layers between neighbouring plant cells. This is followed by the primary cell walls of plants which also contain a high amount of pectin. Then the amount of pectin is very low or even absent starting from the secondary cell walls of plants towards the plasma membranes of plant cells see **Figure 2.11** (Chan et al., 2017).

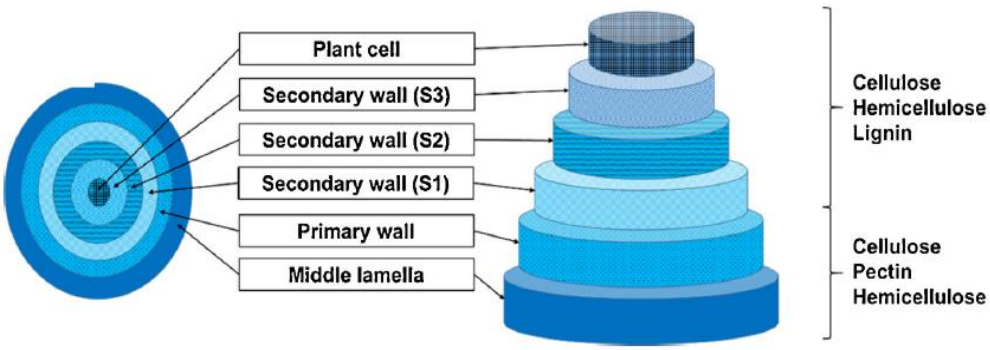


Figure 2.11: The presence of pectin in the middle lamella and primary cell walls, while small quantities present in secondary cell walls towards the plasma membrane (Chan et al., 2017).

In spite of pectin availability in a lot of plant species, many sources that can be utilized for the commercial production of pectins are very limited, due to variations in some parameters such as the molecular size and degree of esterification (DE) which are critical for gel formation., Pectin from various sources does not have similar gelling ability due to variations in these parameters.(Sriamornsak, 2003). Traditionally, it has also been shown that the main sources of pectin are apple pomace and sugar-beet pulp, then recently there has been an increasing use of citrus peel (Kar and Arslan, 1999b, Brejnholt, 2010, Willats et al., 2006). However, the most industrial pectins are found in apple pomace and citrus peels (Dixon, 2008). Also, pectin is present in limited amounts in cereal (Seixas et al., 2014b). In addition, there are some considered sources for pectin which are residues from the seed heads of sunflowers and sugar beet (Thakur et al., 1997). Generally, in dicotyledonous plants pectin constitutes about 35 % of the polymeric composition of primary cell walls (Coenen, 2007). In addition, in general, due to pectin's ability to control surface and sensitive properties by means of the gel characteristics, it is considered as one of the greatest dispersed hydrocolloid used in the food industry (Migliori et al., 2010). Furthermore, in several pumpkin species, pectin components are considered as potential sources, and were demonstrated to show useful functional properties and significant biological effects (Adams et al., 2011, Caili et al., 2006, Caili et al., 2007, Yadav et al., 2010). In continuation of searches for novel pectin resources, Košťálová et al (2013) estimated the biomass of fruit using an orchardman plant which is economically significant in Austria and the neighbouring countries by the production of oil from the seeds of the Styrian oil-pumpkin *Cucurbita pepo* var. Styriaca. Previous papers reported the gross composition of this biomass (Košťálová

et al., 2013). Pectins are essentially found in many fruits and vegetables, some are presented in **Table 2.2** as previously reported by (Searle-van Leeuwen et al., 1992, Kertesz, 1951, Padival et al., 1979, Abdel-Rahman, 2002, El-Shafie, 1981, Ciriminna et al., 2016, Srivastava and Malviya, 2011, Grassino et al., 2016, Alba, 2015, Alba et al., 2015, Kpodo et al., 2017). Various plant classes have different pectin content and the pectins isolated from each plant classes have different physicochemical properties.

Table 2.2: Some sources of pectin from different fruits and vegetables as previously reported:

Raw Materials Producing Pectins	Pectin content %
Grapes	0.2 – 1.0
Apples	0.5 – 1.6
Lemon	3 – 4
Lemon Seeds	~6
Grapefruit	1.6 – 4.5
Sugar beet pulp	~30
Fresh pear	0.5 – 0.7
Fresh tomatoes	0.1 – 0.5
Bannana	0.7 – 1.2
Lime peel	15 – 30
Mango pulp	0.35 – 0.78
Fresh pumpkin	~0.5
Citrus Peels	~85
Oranges	~13
Carrots	~1.4
Cherries	~0.4
Tomato peel	14.9 – 83.5
Okra Pods	11 – 15

Recent work in our group (Adams et al., 2011, Simpson and Morris, 2014, Ghori et al., 2014, Alba et al., 2015, Kpodo et al., 2017) has demonstrated that two novel sources of pectin: pumpkin and okra may be of particular interest.

2.2.1 Pumpkin

it is one of the summer vegetable and fruit crops which composed of 118 genera and around 825 species. Protein and carbohydrates and up to 90 % water are considered its basic components (Aliu et al., 2012, Avinash and RavishankarRai, 2013). In general, this family is considered to include some of the more common harvest vegetables: squash, cucumber, watermelon, muskmelon and pumpkin (Roura et al., 2007, Adams et al., 2011). This study will be focussing on the pumpkin species *Cucurbita maxima* (the pumpkin commonly used to make Jack O' Lantern decorations at Halloween) and also will be focussing on okra pods (known as the *"ladies fingers"* in section 2.2.2.
Pumpkin is one of the more common vegetable crops which belong to Cucurbitaceae family (Roura et al., 2007). Pumpkin is grown in many countries such as China, Argentina, India, Mexico, Brazil and America (Caili et al., 2006). In China and Korea pumpkin growth becomes more widespread where farmers consume it as the basic nutritional and agricultural product (Yang et al., 2007). Besides, pumpkin is a kind of dietary fibre, it has a high amount of pectin, which it has been claimed can be used to control glycaemic levels and reduce the need for insulin when fibre-rich foods are consumed by patients with diabetes (Adams et al., 2011).

One main anxiety is that a large amount of work has been carried out on crude material and not on highly purified polysaccharide components (Paterson, 2008) a feature which does not sit well with western pharmaceutical practices. Furthermore as with all natural polysaccharide products the chemical composition and molar mass will vary depending on the season and geographical origin (Dong et al., 2003). As well storage over time will have an effect on their properties. An enormous number of substantial physiological properties including tumour growth inhibition, wound healing, immunomodulating and hypoglycemic effect were exhibited in many reports of traditional medicinal polysaccharides. In the current study, we are particularly interested in plant polysaccharides with a proposed anti-oxidant effect. It has been proposed that approximately 200 plant species have the ability to reduce blood glucose levels (Jia et al., 2003) many of which have one or more active polysaccharide component *e.g.* pumpkin polysaccharides (Caili et al., 2006). However many of the proposed medicinal properties have been overemphasized and not backed up by scientific data.

Food and pharmaceutical industries in Africa and other developing areas rely on difficulty on petrochemicals and imported up to about 90 % of raw materials. This leads to high prices and slow growth of local industries that are very important for the majority of the population. This is the biggest shock when it is considered that these countries are wealthy in sustainable sources of raw materials (*e.g.* okra, pumpkins, *etc*.) appropriate for use in both the food and pharmaceutical industries. When pumpkin has an orange colour, it has many uses in the field of cooking as one of the ingredients in pies, soups, stews, bread and other types of cooking (Guiné et al., 2011a).. It includes a succulent stem and seeds, the seeds of pumpkin are considered as an important source of β-carotene, amino acids (**Figure 2.12**) and the elements K, P, Fe Mg, Zn, Cu, Mo and Se, *etc*. (Caili et al., 2006, Yadav et al., 2010). Pumpkins seeds are also useful for the hair, nails, nerves and teeth whereas these seeds might be eaten whole and can be used in nutrition in numerous forms (Aliu et al., 2012).

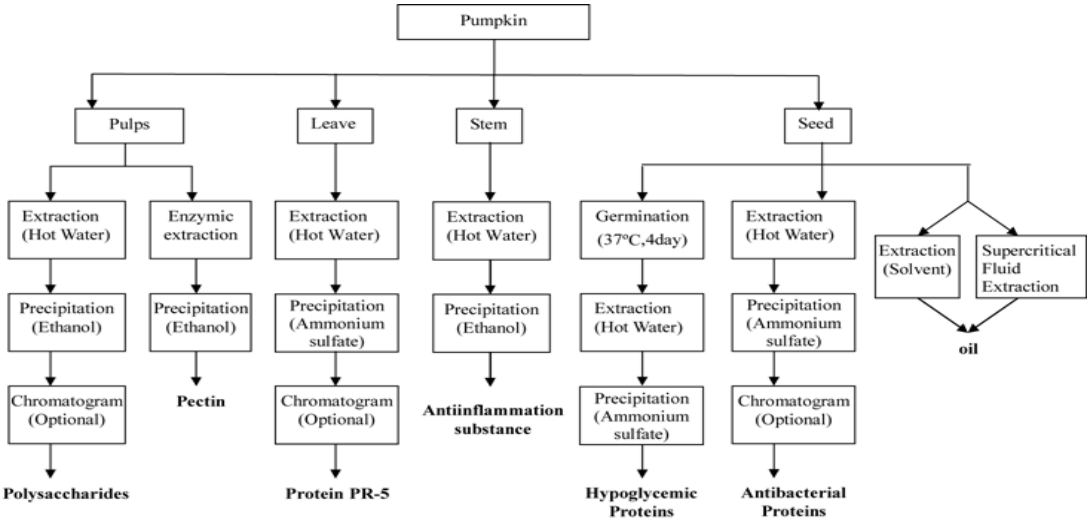


Figure 2.12: Recovery of pumpkin bioactive materials (Caili et al., 2006).

Nutritionally, pumpkin has good benefits with balanced calories where it is thought it is an important source of carotenoids. Furthermore, pumpkin is one of a number of varieties including *Cucurbita pepo*, *Cucurbita mixta*, and *Cucurbita maxima* which have good storage capacity, high efficiency in production and nutritive value (El-Hamed and Elwan, 2011). The health benefits of pumpkin can be summarised in **Figure 2.13.**

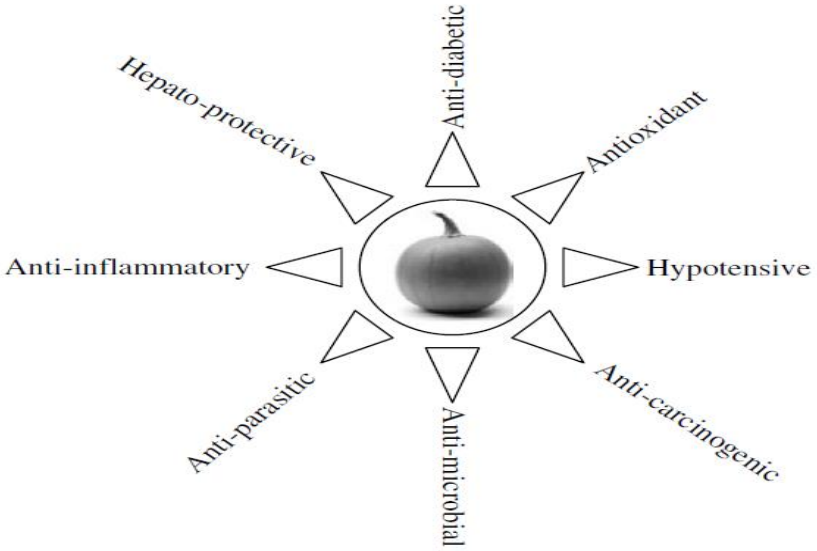


Figure 2.13: The reported medicinal properties of pumpkins (Yadav et al., 2010).

In the diet, different kinds of Cucurbitaceae plants have many known health benefits due to their carbohydrate, vitamin and mineral contents (Roura et al., 2007).In starters and in salads roots, shoots, seeds, leaves, flowers and fruits of the cucurbits can be used (Avinash and RavishankarRai, 2013). The seeds of cucurbits are deemed as an important source of proteins and lipids (Aliu et al., 2012); they have different size, shape and structure (Nerson, 2007). Worldwide, cucurbits play a significant role in many areas such as utensils, fuels, ornamentals, sponges, boxes, and musical instruments. Medical applications include constipation treatment and purification of the blood, also as a diuretic, epilepsy treatment and prevention of bleeding from internal organs (Rahman et al., 2008).

In many countries such as China, Argentina, India, Mexico, Brazil and America pumpkin is grown because of its significant benefits and they have been used in their traditional medicine (Caili et al., 2006). Cucurbits has risen and has been reported a number of useful properties in terms to assist human health (Kelly et al., 2005). There has been a lot of research into the medicinal properties of the *Cucurbitaceae* family and most of the traditional uses have been found to have a grounding in scientific fact. Pumpkins have antibacterial, hypocholesterolemic, immune system modulating, cancer preventing, anti-oxidant and anti-mutagenic properties. Research suggests that these properties are due to their polysaccharides (Adams et al., 2011). Pumpkins have anti-

diabetic properties as has been displayed many times. Powdered pumpkin from the species *Cucurbita moshata duch* has hypoglycemic properties in human type 2 diabetes sufferers which were shown to be due to polysaccharide components. Previously, it has been proved that protein-bound polysaccharides have the ability to reduce blood glucose concentrations, increase the serum insulin levels and improve the glucose tolerance in rats which have been treated with alloxan which breaks down their β cells and thus leads to diabetes, this maybe due to the antioxidant nature of the polysaccharide protecting the pancreatic β cells (Simpson and Morris, 2014). In addition, a lot of cucurbits have different impacts on the fasting blood of alloxan-induced diabetic rats over time. To determine the extent of the hypoglycemic effect for the diabetic rats they have been given the polysaccharides extracted from pumpkins which proved that they have a significant effect on their blood glucose levels (Quanhong et al., 2005, Simpson and Morris, 2014).

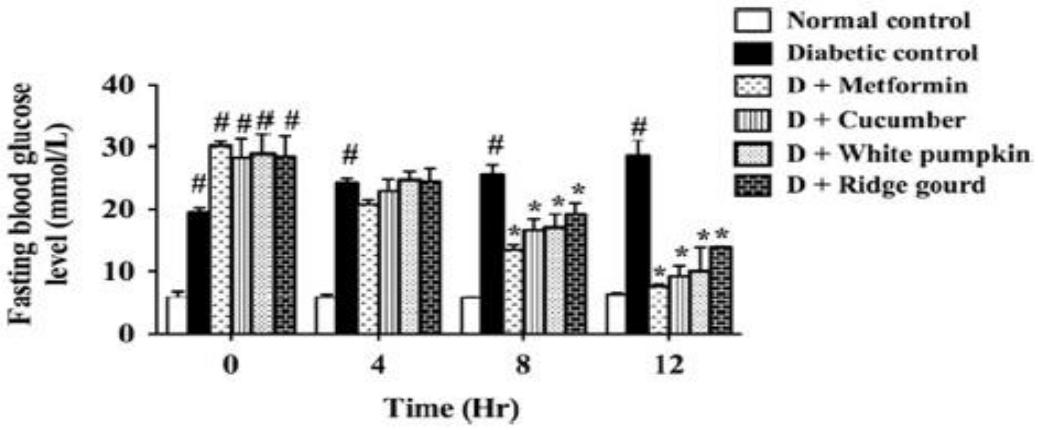


Figure 2.14: shows a significant decrease in blood glucose in alloxan-induced diabetic rats when treated with cucurbit extracts (Sharmin et al., 2012), where # describes an important increase in blood glucose levels when compared to the healthy control, * describes an important decrease compared to after treatment and metformin is diabetes medicine help to control blood sugar levels (Group, 1998) .

Figure 2.14: above shows the highest hyperglycemic potency which is in the cucumber then the white pumpkin then the lowest is the ridge gourd; nonetheless all three had important antihyperglycemic impacts (Sharmin et al., 2012).

2.2.2 Okra:

Okra (*Abelmoschus esculentus L.)*, is a flowering, annual plant ofAfrican origin which belongs to the Malvaceae family (Georgiadis et al., 2011, Alba, 2015). Okra has many names in different parts of the world whereby in England, is known as lady's fingers, while in USA and India called gumbo and bhindi respectively. However, it is one of the plant species cultivated for over 2000 years. Tropical and subtropical areas such as India, the Middle East, Southern USA and West Africa are the primary cultivation areas for the okra plant (Karakoltsidis and Constantinides, 1975, Alba, 2015). The global production of okra is evaluated to be around 8.7 million tonnes, India is the largest producer of okra in the world where about 3.24 million tonnes are produced per year the followed by Nigeria produced about 1 million tonnes and Sudan 0.3 million tonnes(Sahoo and Srivastava, 2002, FAOSTA, 2013, Kaplan et al., 1987, Wittgren and Wahlund, 1997, Alba, 2015). Okra is considered as one of the latest sources of natural polysaccharides which have a lot of uses such as texture/viscosity modifiers, thickening agents and gelling agents (Alba et al., 2015). it is also used as an egg white and fat substitute in cookies, chocolate bars, fruit drinks and dairy products. Further in the human diet, okra plays an important role whereby it is high in vitamin C, minerals (*e.g*., Ca, Mg and K) and fibre. Okra isan attractive resource for industrial applications sinceextracts acquired from fresh okra pods which grow naturally, are cheap and are not related to toxic species (Ghori et al., 2014). The high fibre content and polysaccharides present in okra have been the topic of important research of their applications in food and pharmaceutical industries (Alba, 2015). Some studies revealed that flour possesses antioxidant activity, where with roasting okra this activity increases (Adelakun et al., 2009), suggesting their uses in food industry applications during storage as inhibitors of lipid oxidation and microbial activity in meat products (Arapitsas, 2008). Besides, okra seeds are deemed as a coffee substitute and also as a high-protein oilseed crop that can be utilized to supplement other protein sources (Martin, 1982).

The polysaccharide content (pectin) is responsible for the slimy texture of okra extracts and is of main technological interest for food, non-food and pharmaceutical/medicinal applications (Kpodo et al., 2017, Alba et al., 2015). Industrially, in food and non-food applications okra is a probable source of natural polysaccharides which can be exploited as functional ingredients (Georgiadis et al., 2011). In the production of commercial

polysaccharides, the use of natural gums and mucilages is preferred because they are cheap, readily available and less toxic (Alamri et al., 2012). Mucilage polysaccharides are commonly referred to as hydrocolloids due to their varied range of functional properties which have positive technological applications (Archana et al., 2013). Different varieties of okra are likely to have different mucilage yield and composition. Okra pectins have been isolated on a laboratory scale and evaluated for various food and non-food applications (Ndjouenkeu et al., 1996). A number of solvent protocols have been used by research studies for the extraction of okra polysaccharides which comprise water, sequential buffer systems, ethanol, methanol, acetone, phosphate buffer systems and mixtures of solvent extraction systems (Alba et al., 2015, Zheng et al., 2014, Alba et al., 2013, Archana et al., 2013, Samavati, 2013, Alamri et al., 2012, Kontogiorgos et al., 2012, Georgiadis et al., 2011, Ameena et al., 2010, Sengkhamparn et al., 2010, Ndjouenkeu et al., 1996, Woolfe et al., 1977). Besides, isolation of polysaccharides has also been done on a laboratory scale using sequential solvent treatments (Sengkhamparn et al., 2009a, Georgiadis et al., 2011, Kontogiorgos et al., 2012, Alba et al., 2013). However, the solvent used for extraction, pH and time duration of extraction have been identified as factors that affect the yield and composition of polysaccharides isolated (Alba et al., 2015). The functional and rheological or physicochemical properties of food polysaccharides are important factors to consider in food processing; formulation of new food products or hydrocolloid systems. Different cultivars/genotypes of a plant material (and in this case, okra) may have different physiochemical, functional and rheological characteristics. To our knowledge, okra pectins from different genotypes have not been systematically characterised for their potential applications.

Okra pods and pumpkin are both used as a vegetables and they have many uses in the field of cooking as a thickening agents for soups and stews (Sengkhamparn et al., 2009a, Guiné et al., 2011a). Also, they have been used in traditional medicine as a treatment agent for dental diseases this is due to their polysaccharide content (Sengkhamparn et al., 2010, Sali et al., 2012). Additionally, in Turkey, the leaves of okra after its preparation are used to minimize inflammation (Sahoo and Srivastava, 2002). Whereas pumpkin has a significant effect on blood glucose levels in alloxan-induced diabetic rats (Simpson and Morris, 2014), okra has been shown to lower plasma cholesterol levels in rats (Sengkhamparn et al., 2009a).

In the present study we are predominantly interested in plant polysaccharides proposed anti-oxidant activities. Furthermore, other constituents of plant extracts such as ascorbic acid, β-carotene, tocopherol, tannins, flavonoids, and anthocyanins have been presented to have antioxidant activities. Moreover, it has been stated that plant polysaccharides from different sources have strong antioxidant properties and can be explored as novel potential antioxidants (Chen et al., 2008b, Tseng et al., 2008, Hu et al., 2003, Wang and Luo, 2007, Kong et al., 2010, Zha et al., 2009), however it is expected that as pectin structure and molecular weight depends on extraction conditions it is expected this may also be the case for anti-oxidant activities (Levigne et al., 2002a, Denman and Morris, 2014).

2.3 Pectin extraction:

Extraction of pectin is a multistage process that involves the physical-chemical hydrolysis and extraction of pectin macromolecules from plant tissue (Seixas et al., 2014b) under the influence of different factors, mainly temperature, pH and time duration which allows their solubilisation take place (Pagán et al., 2001). this is followed by the isolation of the extracted pectin and purification, followed by the drying process (Joye and Luzio, 2000). Pectin has been extracted from various resources such as apple pomace, orange peel, lemon and sugar beet pulp under mildly acidic conditions. Apple pomace and orange peel are particularly plentiful and contain high levels of pectic polysaccharides (Guo et al., 2012, Mishra et al., 2012). Besides, various studies described extraction procedures applied to the isolation of pectin from pumpkin fruit and peel (Shkodina et al., 1998, Jun et al., 2006). The isolated pectic polysaccharides were characterized by composition and physicochemical properties (Zhemerichkin and Ptitchkina, 1995).

There are several methods that are usually used for the extraction of pectin including, isolation with enzymes (*e.g*, polymethylgalacturonases, polygalacturonases, polygalacturonate lyases),electromagnetic induction heating, direct boiling and microwave and ultrasound associated isolation (Bagherian et al., 2011, Kashyap et al., 2001, Wang et al., 2007, Mishra et al., 2012). The isolation of polysaccharides can be carried out on a laboratory scale (conventional methods) for extraction of cell wall

material, requiring the use of chelating agents (*i.e.* potassium-oxalate), dilute acids (*i.e.* HCl) or dilute sodium hydroxide solutions, cold and /or hot aqueous and buffers (Levigne et al., 2002a, Alba, 2015). The extraction with chelating agents has a disadvantage in that it is difficult to remove the residual chelates, also alkaline extraction facilitates reduction of chain length and in the degree of acetylation and methylation by β-elimination (Rombouts and Thibault, 1986). In general the highest yield of pectic substances are obtained by the most suitable method for industrial extraction of pectin which has been recently proposed and performed until now is hot acid extractions (nitric acid, sulfuric acid and hydrochloric acid). one of the advantages of the extraction method with high temperature around 70 – 90 ºC, pH 1.5 – 2.5 and the time depending on the raw material (May, 1990, Pagan et al., 1999, Alba, 2015). Acid extraction has another advantage which is that the pectin obtained is ordinarily enriched with GalA units (Da Silva and Rao, 2006). The pectin raw extract is then separated by a filtration or centrifugation process. Pectin can then be separated from the purified extract by precipitation with alcohol (methanol, ethanol or isopropanol) or by precipitation with an insoluble salt by addition of aluminium, for example. The precipitate obtained is washed with alcohol and pressed to remove soluble contaminants, and finally dried and milled to get powdered pectin (Stephen, 1995).

It is also reported in earlier studies the temperature, pH and time duration could modify the quantity as well as the quality of the extracted pectins (Levigne et al., 2002a). Additionally, in previous studies such as Kliemann et al., (2009), Kumar and Chauhan, (2010), Pagan et al., (1999), Samavati, (2013) and Sudhakar and Maini, (2000) on isolation of pectin from apple and peach pomace, mango peel, okra pods and passionfruit researchers reported the noticeable impact of extraction temperature, time duration, pH, kind of acid, number of extraction cycles, the ratio of water to raw material and volumes of organic solvent on the yield and chemical composition of pectin from different plant sources. Pectins have been extracted from different food industry by-products and, considering that food processing is often faced with large amounts of waste material, these types of extraction processes could represent an efficient and environmentally friendly recovery method for the production of functional compounds (Kroyer, 1995).

2.4 Pectin conformation:

2.4.1 Flory-Huggins Theory:

When polymers are dissolved in a solvent, dissolution takes place in two stages (i) the solvent will diffuse through the polymer to form a hydrated gel, (ii) the gel will break down and individual polymer molecules will be dispersed in a "true" solution, although the formation of a "true" solution is not always possible (Su, 2013). Flory-Huggins Theory describes the thermodynamics of dissolving a polymer solution in a solvent, whilst taking into account that polymer molecules are usually much greater in size than solvent molecules. During dissolution the energy of the polymer-solvent system (ΔG_{mix}) will be lowered when the enthalpy (ΔH_{mix}) decreases or the product of temperature and entropy change ($T\Delta S_{mix}$), due increased polymer chain mobility upon mixing is large enough to compensate for any increase in enthalpy, where:

$$\Delta G_{mix} = \Delta H_{mix} - T\Delta S_{mix} \qquad 2.1$$

This this theory uses the lattice model (**Figure**) to represent and arrange polymer and solvent molecules. In **Figure 2.15** the polymer molecules occupy the grey sites in the lattice and the solvent molecules the white sites (Teraoka and Teraoka, 2002).

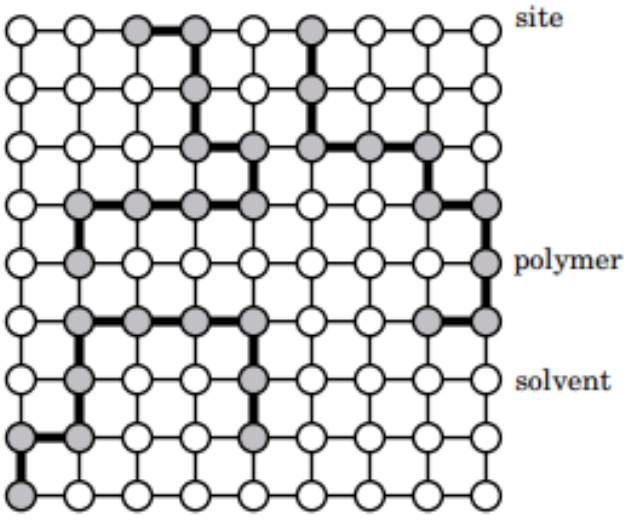


Figure 2.15: Lattice model for a polymer solution. Grey sites are occupied by polymer chains, and white sites are occupied by solvent molecules adapted from (Teraoka and Teraoka, 2002).

As the probability of polymer segment (or solvent molecule) sitting on any given lattice position is random the entropy of mixing can be written as follows:

$$\Delta S_{mix} = -k\,(N_1 \ln\phi_1 + N_{2r} \ln\phi_2) \qquad 2.2$$

where k is the Boltzmann constant (1.381×10^{-23} JK^{-1}) and N_1 and N_2 are the respective number of solvent and polymer molecules, ϕ_1 and ϕ_2 are the respective volume fractions of each component.

Similarly, the enthalpy of mixing can be defined as:

$$\Delta H = kTN_1\phi_2\chi_{12} \qquad 2.3$$

where k, $N_{solvent}$ and $\phi_{polymer}$ are the same as in equation 2 and T is the temperature in K. χ_{12} is polymer-solvent (Flory-Huggins) interaction parameter and it defined in equation 2.4:

$$\chi_{12} = \frac{V_1}{RT}(\delta_1 - \delta_2)^2 \qquad 2.4$$

where V_1 is the molar volume of the solvent, R is the gas constant (8.314 $Jmol^{-1}$) and δ_1 and δ_2 are solubility parameters which should be of similar magnitudes. The solubility parameter for the solvent is related to it latent heat of vaporisation and the solubility parameter for the polymer can be derived from the attraction constants of its functional groups, the polymer density and molecular weight as follows:

$$\delta_2 = \frac{d\Sigma G}{M} \qquad 2.5$$

where different monomer units have different values of G (see for example **Table 2.1** in (Su, 2013). Although it should be noted that this doesn't take into account hydrogen bonding for example. This is then related to the hydrodynamic volume (or radius) of the polymer which is in turn related to chain conformation (see **section 3.12.1**). Therefore, if polymer-solvent interactions are strong (compared to polymer-polymer or

solvent-solvent interactions) the polymer will have larger hydrodynamic volume (**Figure 2.16**).

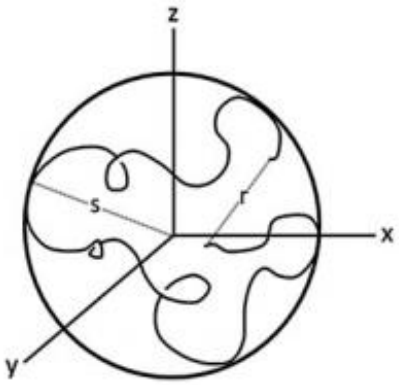


Figure 2.16: Schematic representation of a molecular coil, r = end-to-end distance and s = radius of gyration (R_g). Adapted from (Su, 2013).

Conversely if polymer-solvent interactions are decreased and therefore polymer-polymer or solvent-solvent interactions become more important the hydrodynamic volume will decrease. This leads to the concept of unperturbed dimensions (r_0 or s_0) which represent the size of the polymer in the absence of solvent effects and the expansion factor α where:

$$\bar{s}^2 = s_0^2 \alpha^2 \qquad 2.6a$$

$$\bar{r}^2 = r_0^2 \alpha^2 \qquad 2.6b$$

and therefore

$$\alpha = \frac{\left(s^2\right)^{1/2}}{\left(s_0^2\right)^{1/2}} = \frac{\left(r^2\right)^{1/2}}{\left(r_0^2\right)^{1/2}} \qquad 2.7$$

this leads to the observation that the greater the value of α the better the solvent and as the value of α is temperature dependent the minimum temperature for which $\alpha = 1$ is called the theta (θ) or Flory temperature and the polymer behaves as an ideal coil (Su, 2013). In terms of the physico-chemical characterisation of polymers this relates to the

intrinsic viscosity of a polymer via the Flory-Fox equation for a polymer under theta conditions (Flory and Fox, 1951).

$$[\eta] = \frac{\Phi\left(\bar{r}_0^2 \alpha^2\right)^{3/2}}{M} \qquad 2.8$$

where θ is the Flory-Fox constant which a value of ~3 x 10^{24} mol^{-1} for non-draining Gaussian coils (Bohdanecky, 1983b, Garcia Bernal et al., 1991) and finally through re-arrangement of equation 8 such that a constant K = $\phi(\bar{r}_0^2 M^{-1})^{3/2}$ in theta conditions where α = 1 (Su, 2013).

Then equation 2.8 reduces to:

$$[\eta] = K\bar{M}^{1/2} \qquad 2.9a$$

which is just a special case of the Mark-Houwink-Kuhn-Sakurada equation (2.9b):

$$[\eta] = K\bar{M}^{a} \qquad 2.9b$$

where K and a can be obtained from the intercept and slope of the double log plot of [η] vs. M. The value of "a" can then be used as an estimation of gross macromolecular conformation (see **section 3.12.1**) and hence "a" exponent values of ~0 correspond to spheres, 0.5 - 0.8 to random coils, and up to 1.8 to rigid rods see, *e*.g. Therefore, the value of the Mark-Houwink-Kuhn-Sakurada exponent is a measure of solvent quality, in a "good" solvent where the heat of mixing is favourable the molecule becomes more extended and the "a" exponent is high for example >0.75, whereas in a "poor" solvent which a less favourable heat of mixing the polymer chains attract one another and hence the Mark-Houwink-Kuhn-Sakurada exponent "a" and the intrinsic viscosity are reduce (Masuelli, 2014).

As with other biopolymers, the molecular weight and conformation are two factors on which the functional properties of pectins are highly dependent. Pectins show high

heterogeneity in molecular weight as for other natural polysaccharides. The average molecular weight and the molecular weight distribution differ with the stage of ripening, plant source, and extraction methodologies. Also, pectins that have the same molecular weight may have dissimilar hydrodynamic volumes, this is because the differences in neutral sugar content, branching and degree of esterification which makes their solution behaviour even more complex (Morris et al., 2000, Morris and Ralet, 2012a). In dilute solution, pectin chains are stiff and extended molecules, then a change in conformation from an extended (twofold) structure to a more compact (threefold). Conformation has been proposed for polygalacturonate in dilute solution, in response to a decrease in pH at fixed temperatures (Stephen, 1995).

Biopolymers, while usually utilized in the form of solutions exhibit changing physical properties as a result of the effect of Brownian motion. This is in turn causes the modification in the dimensions and shapes of the molecule (Cui, 2005). A change in conformation of the biopolymer, occurs continuously due to the collisions between solvent molecules with individual monomer units. Biopolymers might occur in one of several various conformations (extended, compact, random rod and sphere) and their occurrence is limited by the chemical bonds in the molecule. The molecule may have a number of structures, yet, only certain conformations are probably due to the interference and restrictions imposed by rotational angles under certain conditions. The global conformation in polysaccharides chiefly depends on alternation about the glycosidic linkages due to the limited flexibility of the pyranose ring and small impact of the pendant groups on the conformational space of the polysaccharide (Pérez et al., 2000). Thus, the basic source of polymer flexibility arises from the differences of torsion angles of the glycosidic linkages. In polysaccharides, there are two torsion angles (ϕ, ψ) by which monosaccharide units rotate around the glycosidic linkages: Angle ϕ which exists between the anomeric carbon and the oxygen of the glycosidic linkage of the first monomer, and ψ which exists between the oxygen of the glycosidic linkage and the non-anomeric carbon of the second monomer **Figure 2.17**. An extra torsion angle (ω) around C-5 and C-6 bond are introduced by the formation of a (1→6) glycosidic linkage between two monosaccharides (Kontogiorgos, 2014). The most stable conformation, which is usually the one with the lowest energy can be adapted by those angles when they increase the freedom of a molecule, this stable conformation is indicated as secondary structure (*e.g.*, ribbon or helix). In branched RG-I

polysaccharide case, (ϕ, ψ, ω) are observed for arabinan side-chains making them the most mobile parts of the pectin molecule **Figure 2.17** (Sinnott, 2007, Alba, 2015). Nevertheless, the impact of *α*-L-rhamnosyl residues, methyl and acetyl groups on the total conformation of the pectic backbone has not been investigated (Pérez et al., 2000).

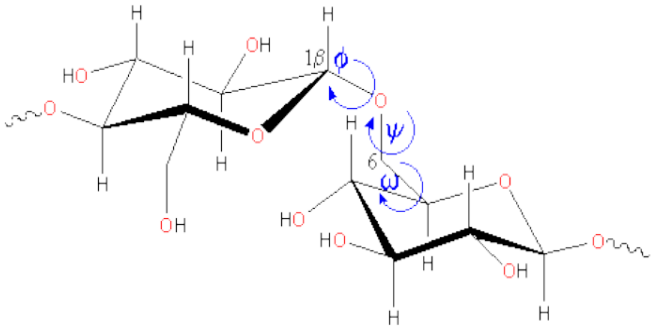


Figure 2.17: Torsion angles (*ϕ, ψ* and *ω*) model in a polygalacturonate chain (Alba, 2015).

In addition, there are another two conformations can be adapted by polysaccharides: ordered or disordered conformations. Ordered conformations are characterized by the constant values of torsion angles due to the complicated interactions between sugar residues (Cui, 2005). In general, using the geometrical relationship within each monosaccharide unit will help to dictate the shape of a polysaccharide chain for example:

- β-(1→4) and β-(1→3) are ribbon-like (*e.g.*, cellulose, xylan, mannan).
- α-(1→4) is a hollow helix (*e.g.*, amylose, curdlan).
- β-(1→2) is a crumpled ribbon (*e.g.*, pectin RG-I sequences) species of conformations.

Furthermore, a poly-α-D-galacturonic acid sequences in pectin which is one of the anionics (1→4)-linked polysaccharides can adopt a buckled ribbon conformation. This kind of chain organizing may leave spaces when they pack together that are usually stabilized by absorbing metal cations (*e.g*, Ca^{2+})(Cui, 2005, Alba, 2015). The ribbon-ribbon association which is related to this type is described as the so-named egg-box model and some polysaccharides, like alginates and low-methoxylated pectins, form

ordered structures (for example gels) by means of this model (Jarvis and Apperley, 1995).

There are several hydrodynamic techniques that have been used to assess polysaccharides stiffness and flexibility (conformation), these techniques include: sedimentation velocity, sedimentation equilibrium, size exclusion chromatography coupled to multi-angle laser light scattering (SEC-MALLS), laser Doppler micro-electrophoresis and viscometry (Wyatt, 1993, Harding, 1997, Morris et al., 2008, Morris et al., 2014). Their results have shown that polysaccharides span a vast range of conformational flexibilities and sizes with large hydrated volumes which are significant in relation to polysaccharide structure-function relationships. Polysaccharides and their derivatives attract a great attention in the food, cosmetic and pharmaceutical industries, wherein in these applications physiochemical characterisation of polysaccharides is important. Thus, knowledge of polysaccharide conformations and of their solution behaviour can give us an insight into the complex mechanisms and help in predicting best conditions to extract pectins from complex matrices (as it can be seen in Chapter 5 and 6).

Most previous studies focused on the yield, properties and structure of different pectic fractions and only some attention has been given to the conformation analysis but not the degree of branching of plant cell wall material after extraction. Observation of the degree of branching may also be very interesting and useful, which may help scientists to understand the length of the pectin if is it linear (smooth) or how many branches are in the pectin if it is branched (hairy).

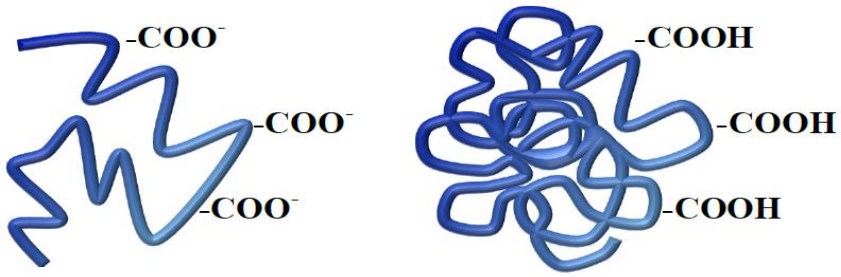
2.5 Pectin functional properties:

Pectin is a nontoxic biopolymer (Tsai et al., 2014). It is a water-soluble polysaccharide (WSP) that is added to food systems to promote their functional properties (Belitz and Grosch 2009). Water-binding ability and viscosity enhancement are both the most important functional properties of WSPs (Wang and Cui, 2005). Pectin has a range of singular properties that have allowed it to be utilized as a matrix for the trapping and/or provision of a variety of medications, cells and proteins (Sriamornsak, 2003). Properties of pectin are a function of their structure, which is that of a linear polyanion

(polycarboxylate). Some information on the solubilisation, viscosity and gelation functional properties of pectin are described briefly below to clarify the role of pectin in the food system.

2.5.1 Solubility:

The two most important parameters that determine polysaccharide solubility are molecular architecture and molecular weight (Belitz et al., 2009). Pectin is soluble in pure water easily; some alkali metals (monovalent cation) are usually soluble in water and may form salts with pectin (*e.g.*, sodium and potassium pectates) and pectic acids some are weakly soluble or insoluble including di- and trivalent cations salts. The heterogeneous structure (HG, RG-I, RG-II) is the main reason which gives pectic polysaccharides their high solubility. In addition, the presence of hydrophilic carboxyl and hydroxyl groups interact with water via hydrogen bonding (Sriamornsak, 2003). Pectin solubility is affected by their degree of polymerisation (DP) also a degree of methylation (DM) and the branching of side chains. Generally, pectin molecules with a higher degree of esterification and lower molecular weight dissolve more easily. In addition, there are some factors or conditions which have an influence on the solubility of pectins such as pH, temperature and the solution concentration (Thakur et al., 1997). The ionization of carboxyl groups in pectin always occurs at high pH values (pH > pKa). Thus, due to the electrostatic repulsions between the carboxylate anions, biopolymer chains will highly hydrated and extended see **Figure 2.18a**. Furthermore, the solubility is affacted by the degree of methyl esterification and degree of blockiness (DB) with methyl groups generally involved in determining the number and distribution of negative charges. On the other hand, with (pH < pKa) the ionization of carboxylic groups decreases and consequently enhance chain-to-chain combinations see **Figure 2.18b**. Hence, the absence of repulsion results in a higher viscosity of pectin solutions. The adding of salts of monovalent cations to pectin solutions results in viscosity reduction due to the suppression of electrostatic repulsions (Thakur et al., 1997).

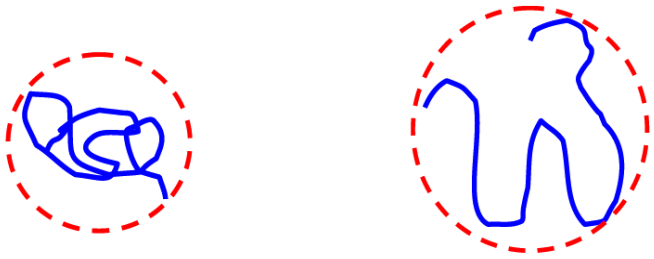


(A) (B)

Figure 2.18: Conformations of pectin chain (A) extended (pH > p*K*a), (B) compact (pH < p*K*a) (Alba, 2015).

2.5.2 Viscosity:

In general, "the ratio of applied shearing stress to the shear rate" is the definition of the viscosity (Wang and Cui, 2005). Viscosity in solution is generated as follows: linear polysaccharide molecules gyrate and pleat, sweeping out a big space and repeatedly colliding with each other, thus friction is created and energy consumed. Whereas, highly branched polysaccharides spread out over less space, collide less thus the generation of viscosity will be less in branched polysaccharides than in linear polysaccharide (Yuliarti, 2011). Pectin is considered to be a semi-flexible coil polysaccharide (Morris et al., 2008, Morris et al., 2000). There are two kinds of polymer random coil shapes: (a) flexible and (b) semi-flexible see **Figure 2.19.**



(a) Flexible (b) Semi-flexible

Figure 2.19: Random coiled polysaccharide molecules flexibility (Yuliarti, 2011).

There are some factors that greatly affect the viscosity of pectin solution including: the concentration of the solute, DE, temperature, pH, molecular weight, presence of counterions in the solution, the shape, flexibility and configuration of the polysaccharide molecules (Sriamornsak, 2003, Bourne, 2002, Li and Chang, 1997). Mostly, the viscosity of pectin under constant conditions increases with increasing DE,

pectin concentration and M_w. For example, at a constant temperature increasing solute concentration will increase the pectin solution viscosity thence a direct linear relationship can be seen (Bourne, 2002, Da Silva and Rao, 2006). Viscosity and temperature are highly correlated with each other. In general, there is an inverse relationship between them, where increasing the solution temperature the viscosity of the solution will decrease. This could be attributed to the effect of the increased temperature on the intermolecular chain association of solution (Yuliarti, 2011).

2.5.3 Gelation:

Gelling happens when a three-dimensional (3D) network of polymer chains are formed with solvent and solutes trapped within (Loh et al., 2014, Loh et al., 2011, Nguyen et al., 2011). The ability to form gels is a characteristic property and the most unique and outstanding property of pectin in the presence of Ca^{2+} ions or sugar and acid, wherein this property makes pectin an important ingredient of many food products (Thakur et al., 1997). Gelation of pectin is attributed to its solubility and viscosity, which are a measures of its molecular weight (Kar and Arslan, 1999b). The gel-forming ability depending on the degree of esterification (DE) and the molecular size (Sriamornsak, 2003). Pectin gelation mechanism depends on the degree of methoxylation (Wehr et al., 2004). There are some mechanisms proposed in several papers to show how gelation occurs (Walkinshaw and Arnott, 1981, Oakenfull and Scott, 1984). The simplest mechanism of pectin gelation was obtained by (Walkinshaw and Arnott, 1981) see **Figure 2.20.** The mechanism (gelation of HMP in presence of sucrose with low pH almost below 3.5) involves non-covalent polymer cross-linking in junction zones, which are primarily hydrogen bonds and hydrophobic interaction between methyl ester groups. Accordingly, hydrogen bonding and hydrophobic interactions between pectin molecules are attributed to high methoxyl pectin gels. While, in the presence of di- and polyvalent cations which crosslink and neutralise the negative charges of the pectin molecule low methoxyl pectin gels formed (Cardoso et al., 2003b). In general, solubility, viscosity and gelation are associated. For instance, an increase in the tendency to gel is attributed to factors that increase gel strength so for example, increases viscosity, and decreased solubility. (Sriamornsak, 2003).

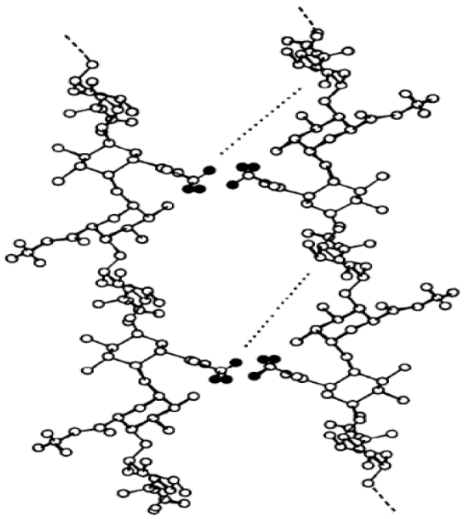


Figure 2.20: The junction zones structure in an HMP gel: (…..) hydrogen bonds; (●) hydrophobic interactions of the methyl ester groups (Walkinshaw and Arnott, 1981).

2.5.4 Antioxidant activity:

In biology, some physicochemical properties of carbohydrates such as types and ratios of constituent monosaccharides, the size of the molecule and features of glycosidic linkages (*i.e*. a sequence of monosaccharides, formation and place of glycosidic linkages) are really related to the biologic activities (Li et al., 2013). Antioxidants perform a substantial role as health protecting agents. They reduce the risk for chronic diseases such as cancer and heart disease as suggested by some researchers. In nature, the primary sources of antioxidants are fruits, vegetables and all grains. Furthermore, polysaccharides, carotenes, phenolic acids, vitamin C, vitamin E, *etc.* which have been known as having the potential to decrease disease risk are considered as the main plant source for antioxidants (Tailor and Goyal, 2014).

There are many ways to define the antioxidant but this is depending on the methods used to determine the antioxidant activity wherein changing the method, will change the observed antioxidant activity. As reported in (Gutteridge and Halliwell, (2010) an antioxidant is defined as ‘‘any substance that delays, prevents or removes oxidative damage to a target molecule”(Alam et al., 2013).

Several methods are utilized to examine the antioxidant property of different samples (plants, foods extracts, commercial antioxidants *etc*.). These methods were classified into two types as reported by Alam et al., (2013):

Table 2.3: *in vitro* and *in vivo* methods used to determine the antioxidant activity adapted from (Alam et al., 2013).

***In vitro* methods**		***In vivo* models**
DPPH scavenging activity	Oxygen radical absorbance capacity (ORAC)	The ferric reducing ability of plasma
Hydrogen peroxide scavenging (H_2O_2) assay	Reducing power (RP)	Reduced glutathione (GSH) estimation
Nitric oxide scavenging activity	Phosphomolybdenum	Glutathione peroxidase (GSHPx) estimation
Peroxynitrite radical scavenging activity	Ferric thiocyanate (FTC)	Glutathione-S-transferase (GSt)
Trolox equivalent antioxidant capacity (TEAC) method/ ABTS radical cation decolorization assay	Thiobarbituric acid (TBA) method	Superoxide dismutase (SOD)
Total radical-trapping antioxidant parameter (TRAP)	DMPD(N,N-dimethyl-p-phenylene diamine dihydrochloride)	Catalase (CAT)
Ferric reducing-antioxidant power (FRAP) assay	β-carotene linoleic acid method/conjugated diene assay.	γ-Glutamyl transpeptidase activity (GGT) assay
Superoxide radical scavenging activity (SOD)	Xanthine oxidase	Glutathione reductase (GR) assay
Hydroxyl radical scavenging activity.	Cupric ion reducing antioxidant capacity (CUPRAC)	Lipid peroxidation (LPO) assay
Hydroxyl radical averting capacity (HORAC)	Metal chelating activity	LDL (low-density lipoproteins) assay

There are two common approaches which can be used to evaluate free radical scavenging activity:spectrophotometric and electron spin resonance (ESR)

spectrometry methods. Spectrophotometric methods are commonly applied to study free radical scavenging capacities of antioxidants using stable radicals and trapping agents (Yu et al., 2002). Determination of antioxidant capacity of food involves the use of a rapid, simple and cheap method called free radical, 2, 2-Diphenyl-1- picrylhydrazyl (DPPH) which is used to test the ability of compounds to act as free radical scavengers or hydrogen donors (Tailor and Goyal, 2014). In addition, it is predominantly used to classify the scavenging activity of phenolic compounds (AH) (Nenadis and Tsimidou, 2002). Furthermore, it has been used in our current study.

Antioxidants may be of major advantage in refining the quality of life by stopping or deferring the commencement of degenerative diseases. Likewise, they have a possibility for considerable savings in the cost of health care delivery and they are deemed as important nutraceuticals with several health benefits (Alam et al., 2013, Dröge, 2002, Sharma and Bhat, 2009, Mimica-Dukic et al., 2004). In addition, antioxidants can be used to improve food quality and stability when they are added during food processing (Yu et al., 2002).

It has been noted by Balasundram et al., (2006) that antioxidant activity relies not only on the content but also composition of the phenolics, however, it may be influenced by the presence of other non-carbohydrate components and/or the structural and molecular properties of the polysaccharides (Yang et al., 2008). Furthermore, phenolic compounds play a big role as antioxidants because of the presence of hydroxyl substituents and their aromatic structure, which allows them to scavenge free radicals (Villano et al., 2007).

Various natural compounds extracted from plants (*i.e* polysaccharides) have shown biological activities (Mimica-Dukic et al., 2004, Li et al., 2006). Some published data obtained by Chen et al., (2012), Capek et al., (2009), Luo et al., (2010), Garde et al., (2001), Kong et al., (2010), Li et al., (2006), Liu et al., (2010), Xu et al., (2009) and Chen et al., 2008b , showed that polysaccharides isolated from plants and from other natural resources inclusive of carbohydrate polymers (crude or purified extracts) have certain antioxidant activity on free radicals and can be explored as novel potential antioxidants. Thus, it is still important to identify active natural compounds with probable antioxidant and/or radical scavenger properties (Capek et al., 2009). Besides,

a number of studies indicated that isolated pure polysaccharides are more effective antioxidants *in vitro* than crude polysaccharides (Xu et al., 2009). These polysaccharides have antioxidant activity as indicated by previous studies (Chen et al., 2008a, Wang and Luo, 2007) which might widely relate to structural characteristics and chemical properties of these substances. for the Cucurbitaceae family. There has been a lot of research into their medicinal properties and it was indicated that pumpkins have anti-oxidant properties. Research suggests that these properties are because of their polysaccharide contents (Adams et al., 2011, Qingsheng et al., 2000, Makni et al., 2008, Li and Xiang, 2006).

2.6 Applications of pectin:

Pectin is extensively used in a variety of food products pectin is extensively used, but the amount used in the pharmaceutical industry is much smaller compared to the food uses (Brejnholt, 2010). In pharmaceutical applications pectin is used in tablets, films, hydrogels, pellets and beads (Ghaffari et al., 2006, Ghaffari et al., 2007). It used as a stabilizer and gel-forming agent (Laurent and Boulenguer, 2003), and can act as a stabilizer, emulsifier, thickener, cation-binding agent, *etc* (Ptichkina et al., 2008, Gullón et al., 2013). Besides, it is also an important source of dietary fibre. Pectin has a variety of applications in the bakery industry where it used to retain moisture and to improve volume, flexibility and softness in bread (Brejnholt, 2010). Pectin is used in various food products such as tomato ketchup, salad dressing, foams, mayonnaise and beverages because of its stabilizing and thickening properties (Pilnik and Voragen, 1992). Pectin is often used as a fat replacer in low-fat foods (Min et al., 2010). It is also used in yoghurts for different purposes depending on the sort of product; as a water binder in stirred yoghurts, as a thickener in yoghurts and as emulsifier and to provide fat-like mouthfeel in low-fat yoghurts (May, 1990, Voragen et al., 1995). In addition, pectin can be used to stabilize acidic protein drinks such as a fat substitute in baked goods and drinking yoghurt (Srivastava and Malviya, 2011).

In medicine, because increase of viscosity and volume of stool, pectin is used against constipation and diarrhoea. It has also been shown to have use in medical adhesives such as colostomy devices, used in wound healing preparations and in throat lozenges as a demulcent as well (Srivastava and Malviya, 2011). Further, it has also been shown

that pectin can reduce glucose levels, cholesterol levels in the serum of diabetic and obese patients and reduce heart disease and gallstones and may have anticancer activities as well (Mishra et al., 2012, Coenen, 2007). It is also used in the dental, health promotion and cosmetic industries for its gelling properties (Pagán et al., 2001, Sriamornsak, 2003). Pectin has many positive effects on human health, for example, stimulating the immune response (Mohnen, 2008, Inngjerdingen et al., 2007). Furthermore, pectin has important role in manufacturing of a variety of products comprising paper substitutes, adhesives, foams and plasticizers, materials for biomedical implantation, surface modifiers for medical devices, edible and biodegradable films and the delivery of drugs nasally, orally and vaginally (Mohnen, 2008) and for development of implantable and prosthetic devices (Mishra et al., 2012). Derivatives of pectin have been employed in vaccine manufacture (Gullón et al., 2013).

Chapter 3

Important Methods in Polysaccharide Characterisation

3 IMPORTANT METHODS IN POLYSACCHARIDE CHARACTERISATION

In order to collect adequate data to allow for the full characterization of the polysaccharides under investigation a wide range of analytical techniques were required, these techniques are highlighted below. Therefore, this chapter provides an overview of these techniques, including theory and popular terminologies, regarding the physicochemical characterisation of pumpkin and okra pectins.

3.1 High-Performance Anion-Exchange Chromatography (HPAEC):

HPAEC is a method which was developed specifically to analyse the sugar composition of polysaccharides and depends on the separation of monosaccharides in their anionic form (Corradini et al., 2012). At high pH, polysaccharides will be completely or partly ionize depending on the pKa of the monosaccharides as shown in **Table 3.1** which presents dissociation constants of some common carbohydrates (in water at 25 ºC) (Bhattacharyya and Rohrer, 2012). Also, HPAEC is regularly used for determinations of sialic acid, mannose-6-phosphate and oligosaccharide contents of glycoproteins; although the first use of HPAEC in carbohydrate analysis began in 1983, the first applied to glycoprotein analysis was in the late 1980s (Rohrer et al., 2013).

In the past, carbohydrate characterisation and detection were done using GC-MS and other techniques which can take a long time, especially in terms of sample preparation. However, nowadays HPAEC in conjunction with a highly efficient and popular method named Pulsed amperometric detection (PAD) makes HPAEC one of the most popular methods for the identification and quantitation of carbohydrates in food products and natural samples where it is increasing the technique specificity and sensitivity. Also, HPAEC - PAD has a number of advantages, which include the ability to measure picomole levels of carbohydrates samples (Rohrer et al., 2013, Guignard et al., 2005). HPAEC contains a general-purpose column, CarboPac1 PA1, consisting of a polystyrene/divinylbenzene substrate agglomerated with a Microbead^{TM} quaternary amine functionalized latex, having an ion-exchange capacity equal to 100 µeq per 250 × 4 mm i.d. This was designed by Dionex to use for monosaccharides, disaccharides

and some oligosaccharides. However, there is another column designed particularly for analysis of mono- and disaccharide called CarboPac PA10 which is quite similar to PA1 but holds a higher percentage of divinylbenzene which is used in this study. Comparing HPAEC with other methods such as HPLC and GC that are used to separate mixtures of multi-component samples containing (mono, di and oligo) saccharides, this method is the most superior (Cataldi et al., 2000, Cataldi et al., 1998). In addition, Tomiya et al., (2001) (Tomiya et al., 2001) reported in their study about the determination of nucleotides and sugar nucleotides, HPAEC gave an excellent and reproducible separation with high sensitivity and reproducibility compared to HPLC. HPAEC was used in this study for the separation of carbohydrate molecules and then for identifying monosaccharide units within a complex carbohydrate molecule.

Table 3.1: pKa values for some monosaccharides and disaccharides at 25 ℃ (Bhattacharyya and Rohrer, 2012).

Compound	**pKa at 25 ºC**
Glucuronic acid	3.20
Galacturonic acid	3.48
Glucose -6- phosphate	0.94, 6.11
Mannose	12.08
Xylose	12.15
Glucose	12.28
Galactose	12.39
Arabinose	12.43
Sucrose	12.62
Raffinose	12.74
Fructose	12.03
Lactose	11.98
Maltose	11.94

3.2 Fourier Transform Infrared Spectroscopy (FT-IR):

IR is a direct spectroscopy method used for determination of methylesterification degree (MED) of pectins (Manrique and Lajolo, 2002). Also it is a sensitive, rapid powerful analytical tool for quantitative analysis and investigation of the plant cell

walls, including pectin which is one of the most widely studied polysaccharides by IR spectroscopy and is considered as cheap technique (Synytsya et al., 2003b, Kacurakova et al., 2000, Kačuráková and Wilson, 2001). It is a technique appropriate for monitoring chemical changes in the cell walls and in particular, the changes in the degree of esterification of pectins (Chatjigakis et al., 1998). Therefore, during the different stages of plant development, the chemical changes in the cell wall components can be monitored using FT-IR (Manrique and Lajolo, 2002). Measuring the degree of esterification (DE) is a routine procedure in analytical analysis pectin. DE can be expressed as a percentage of total methyl esterified carboxyl groups to the total of free carboxyl groups (Gnanasambandam and Proctor, 2000). IR spectroscopy was used for the determination of the degree of esterification of the extracted polysaccharides in order to determine further structural information.

In addition, FT-IR is used for measuring the methylation level of different pectin fractions isolated from pumpkin and okra where all the samples used were in the solid state. The area under each peak for both the esterified carbonyl peak and the free carbonyl peak values were calculated from the absorbance spectra of the solid samples using a relationship involving absorbance intensities for 1635 and 1730 cm^{-1} bands (Manrique and Lajolo, 2002) using equation 3.1. However, DE can also be calculated by the colourimetric determination from the ratio of methanol divided by total unoric acid (Chatjigakis et al., 1998). The degree of esterification (DE) is an essential parameter which will help to define the conformational and the rheological characteristics of pectins (Kar and Arslan, 1999a).

The degree of esterification was calculated:

$$x = \left(\frac{\text{Area of esterified peak}}{\text{Area of esterified peak+Area of free carboxyl peak}}\right) \times 100 \qquad 3.1$$

3.3 Nuclear Magnetic Resonance (NMR):

In NMR spectroscopy the nuclei of atoms possess a magnetic property which is named spin this is the essential foundation upon which it is based (Stephen and Phillips, 2006). As it is well known each nucleus has a spin but in NMR studies ^{1}H, ^{13}C, ^{19}F, ^{31}P and ^{15}N are used. ^{1}H and ^{13}C are regularly used in the structural analysis of polysaccharides.

Comparison of their NMR signals found that ^{1}H is much more sensitive than ^{13}C (^{13}C at least three times less sensitive than ^{1}H) this is due to the abundance of ^{13}C (Mulloy, 1996). In addition, in the analysis of polysaccharides ^{13}C-NMR has important benefits over ^{1}H-NMR spectroscopy *e.g*. the chemical shifts in the ^{13}C-NMR spectra is extensively spread, which helps overcome the difficulties related with the proton spectrum (Agbenorhevi, 2011).

NMR is an influential, multipurpose and non-destructive analytical tool, which allows for the identification of the overall structure of chemical compounds including polymers (Kehlbeck et al., 2014, Marcon et al., 2005). Also, it has been used to characterise the composition and sequence of polysaccharide units so it is considered as an informative method (Zhu et al., 2014). Besides, this technique can be used to confirm if there are any polysaccharides in foods. NMR can be used to show the number of nuclei of specific atoms for instance, in order to identify how many hydrogen atoms are in a carbohydrate. It is possible to identify the nature of the environment around each of the nuclei too (Pavia et al., 2008). In NMR spectroscopy the amount of sample required is small yet the technique yields good structural data and this makes it relatively sensitive (Duus et al., 2000, Marcon et al., 2005). From the functional side, it is not easy to prepare the pectin solution for NMR spectroscopy due to its viscosity which complicates the applications of NMR analysis despite the ease of its solubility in the water. Nevertheless, up to the present, the conformation of pectin macromolecule chains in solid and gel states has been identified using NMR (Zhu et al., 2014, Synytsya et al., 2003a).

3.4 Capillary viscometry:

The principle of the capillary viscometer is simple, it measures the time for a constant volume (solution or solvent) to flow through the capillary which is vertically aligned; this measurement is made for the solvent, and then the solution in one or more concentrations (Harding, 1997) **Figure 3.1**.

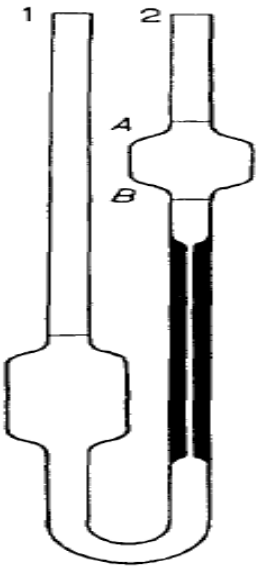


Figure 3.1: Schematic representation of an Ostwald viscometer (adapted from ref. (Harding, 1997)).

The flow time is directly proportional to the viscosity and is inversely proportional to the density (Agbenorhevi, 2011, Harding, 1997) as shown in equations 3.2 and 3.3.

$$t_{solvent} = \frac{\eta_{solvent}}{\rho_{solvent}} \qquad 3.2$$

$$t_{solution} = \frac{\eta_{solution}}{\rho_{solution}} \qquad 3.3$$

The relative viscosity is defined as the ratio $\eta_{solution} / \eta_{solvent}$ and for most polymer solutions at the concentrations of interest $\rho_{solution}$ and $\rho_{solvent} \approx 1$. Thus the relative viscosity is a simple time ratio by using the approximation:

$$\eta_{rel} = t_{solution} / t_{solvent} \qquad 3.4$$

Whereas, the fractional variation in viscosity upon addition of polymer is defined as the specific viscosity (Agbenorhevi, 2011).

Both relative η_{rel} and specific η_{sp} viscosities were calculated from the following equations:

$$\eta_{rel} = \left(\frac{t}{t_\circ}\right)\left(\frac{\rho}{\rho_\circ}\right) \quad 3.5$$

$$\eta_{sp} = \eta_{rel} - 1 \quad 3.6$$

Where (t) indicates the flow time of the pectin solution at each concentration, (t_0) is the flow time for the solvent (water) and ρ/ρ_0 was assumed to be unity (Morris et al., 2000).

The concentration of viscosity approaches zero to the limit which is reduced describe the meaning of the intrinsic viscosity, to extrapolate the reduced viscosity to its value at zero solute concentration the determination of the magnitude of intrinsic viscosity is considered as a basic method (Kar and Arslan, 1999b).

$$[\eta] = \lim_{c \to 0} \frac{\eta_{sp}}{C} \quad 3.7$$

Where c is polymer solution concentration (g/mL), η_{sp} the specific viscosity, η_{red} the reduced viscosity (mL/g) and [η] is the intrinsic viscosity (mL/g).

Two methods can be used for the plot of specific viscosity **Figure 3.2**; these two equations usually remain almost linear if measurements are limited to relative viscosities lower than ~ 2 (Evageliou et al., 2005). The first method is the Huggins plot (Huggins, 1942) which can be used to calculate intrinsic viscosity from the intercept and the Huggins parameter K_H from the gradient of the line **Eq 3.8**.

$$\frac{\eta_{sp}}{C} = [\eta] + [\eta]^2 K_H \, C \quad 3.8$$

The other method which can be used to calculate the intrinsic viscosity is the Kraemer plot (Kraemer, 1938). This plots the natural log value of relative viscosity (ln η_{rel} often called the inherent viscosity η_{inh}) against solution concentration **Eq 3.9** (Migliori et al., 2010).

$$\frac{\ln \eta_{rel}}{C} = [\eta] + [\eta]^2 K_K \, C \quad 3.9$$

For polysaccharide solutions, the intrinsic viscosity ([η]) is considered as a characteristic property (Han et al., 2011) and is related to both molecular weight and conformation.

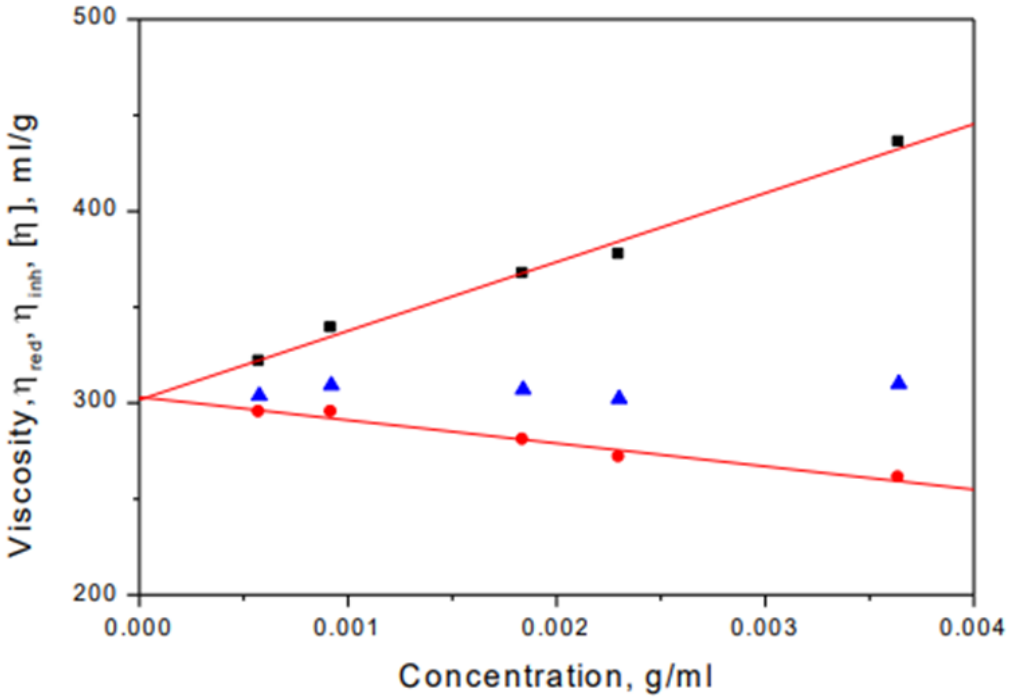


Figure 3.2: The Huggins (Black Square), Kraemer (Red circle) and Solomon-Ciuta (Blue triangle) plots for the low methoxy pectin HL 7192 adapted from (Morris, 2001) where the mean intercept of the Huggins and Kraemer plots is the intrinsic viscosity.

3.5 Laser Doppler micro-electrophoresis:

Laser Doppler micro-electrophoresis is a measuring method that is used to determine the zeta potential (ζ) of hydrocolloids by estimation of the electrophoretic mobility (Hunter, 2013). In an applied field, electrophoretic mobility (*e.g.* direction and speed) of the particles depend on some factors which are solvent viscosity, solvent dielectric constant, zeta potential and the strength of the applied electric field (Streng, 2012). Many years ago, zeta potential played an important role in the treatment of the water industry to help determine coagulant dosages (Nobbmann et al., 2002). Zeta potential is defined as the measurement of the electrostatic or charge repulsion/attraction between particles. Zeta potential is the potential presents at the boundaries of the outer diffuse layer surrounding charged particles . From **Figure 3.3**, the electrical double layer that surrounds each particle is divided into two layers; the Stern layer where the ions are

attached strongly while the ions that are less associated are known as the diffuse layer. Also, there is a boundary known as the slipping plane where the ions of the diffuse layer and particles build a stable unit and ions do not move with the particle. The potential at this boundary is known as the zeta potential (Kumar and Kumbhat, 2016).

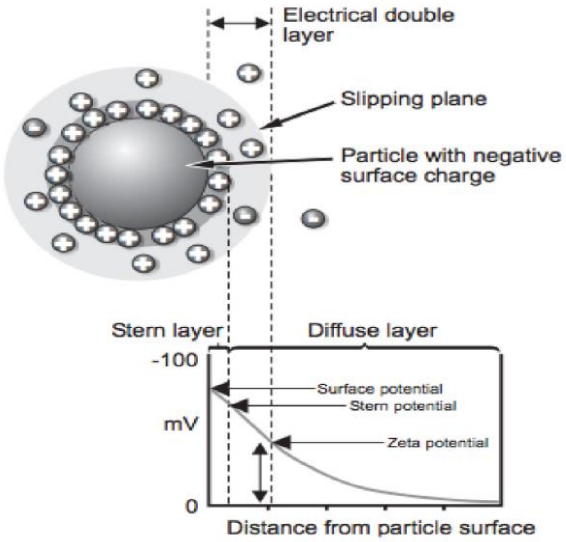


Figure 3.3: Schematic representation of the zeta potential: ionic concentration and potential differences as a function of distance from the charged surface of a particle suspended in a medium (Kumar and Kumbhat, 2016).

The particles in the solution present in two cases when zeta potential impacts on their stability: dispersion case (stable particle) or aggregation case (unstable particle) as shown in **Figure 3.4.** Usually, between stable and unstable suspensions there is a boundary of +30 mV or -30 mV; particles with zeta potentials less positive than +30 mV or less negative than -30 mV are in general deemed unstable (Schmidt et al., 2012, Abodinar, 2016).

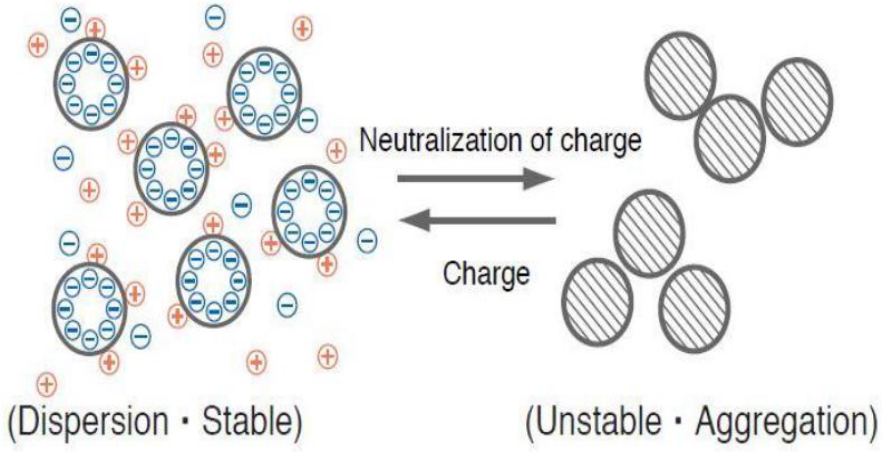


Figure 3.4: Diagram shows how particle stability in solution is affected by the value of zeta potential.

In many applications, zeta potential is considered as a significant parameter mainly in the characterization of biomedical polymers, membrane efficiency, microfluidics and electrokinetic transport of particles or blood cells as well (Sze et al., 2003).

3.6 Size Exclusion Chromatography- Multi-Angle Laser Light Scattering (SEC-MALLS)

Size-exclusion chromatography (SEC) is also known as gel permeation chromatography (GPC). It is a generic process, which is widely used for the measuring of molecular weights of molecules in a sample. This process involves the separation of molecules according to size (which assuming there is no change in conformation can be simplified to the molecular mass) when a solution flows through a packed bed of porous packing rather than interaction process, therefore, SEC differs from all other chromatographic techniques (Rounds and Gregor, 2003). In order to achieve separation a liquid the sample is passed through the column carried by a solvent (eluent); the large molecules are excluded whilst the smaller molecules have access to more pores than larger molecules. Therefore, the smaller molecules take a longer route through the column (entering the pores on the beads) which means that they take a longer time to elute from the column. (Kostanski et al., 2004, Wyatt, 1991). In general, GPC-MALLS it used to evaluate the average molar masses (M_w, weight average molar mass; M_n, number-average molar mass).

Average molecular weights and the degree of polydispersity of polysaccharides are determined rapidly by using high-performance size-exclusion chromatography (HPSEC) coupled with a laser light scattering detector, thus directly the molecular weight distribution of polysaccharides can be also determined (Agbenorhevi, 2011). Following size exclusion chromatography, the light scattering (MALLS) detector permits the determination of molecular weight distributions whilst not being completely affected by the composition or structure of the eluting polysaccharides. The laser light is directed from multiple angles towards the flow of molecules, the scattering is then recorded by photodiodes (Bean and Lookhart, 2001). The light scattering signals from the sample polysaccharides are determined at 18 angles simultaneously, then, each constituent is measured by a refractive index detector with the result being linked to the molecular weight of each fraction. The radii of gyration may also be obtained from the light scattering data at each angle and since they are dependent on the conformations and branching of the polysaccharides sample, additional structural information can then be obtained (Tackx and Bosscher, 1997).

3.7 Gas Chromatography-Mass Spectrometry GC-MS

Gas chromatography with mass spectrometry is often used in polysaccharide analysis as it is one of the few techniques which has the capability of identifying the linkages which form the polysaccharide chain (Sparkman et al., 2011, Carpita and Shea, 1989). In studies such as this, GC-MS is the preferred method of analysis of polysaccharide linkages since it requires less starting material than other techniques *e.g.* NMR (Lindhorst, 2000). One method to determine the different monosaccharide components in the samples is using GC. This method provides data on the monosaccharide units' linkage as in what manner are sequence they are bonded. To prepare the sample for linkage analysis, methylation and acetylation are used to identify where monosaccharides were linked to one another and where there were no bonds.

By carrying out methylation prior to the hydrolysis into the constituent monosaccharides which form the polysaccharide, and performing acetylation after hydrolysis, methyl groups will substitute hydroxyls not involved in linkage and acetyl groups will be found on the hydroxyl groups which were bound to another monosaccharide. Through detecting the location of these groups on the

monosaccharides using GC-MS it will be possible to identify the types of links in the chain (*i.e*. it will be possible to tell if a hexose is 1-4 linked or part of an end terminal *etc*.) (Walker and Rapley, 2008).

Additionally, GC-MS can be used in the determination of glycosidic linkages in polysaccharides. This analysis is done by methylating all the free OH groups in the sample and then using acid hydrolysis to break the glycosidic bonds. Partially methylated alditol acetates (PMAAs) are then produced by reducing and acetylating the methylated sugars. The volatility of PMAAs make them analysable by the GC-MS method; the reactions are shown in **Figure 3.5**. The locations of the acetyl groups on the molecules disclose carbon atoms that are part of glycosidic linkages in the polysaccharide. An examination of the fragmentation patterns in the mass spectra can be used to identify the ring size and linkages of each monosaccharide.

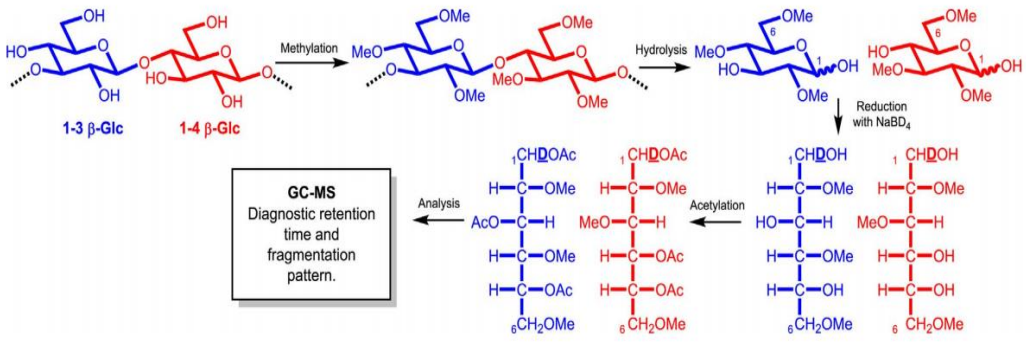


Figure 3.5: Preparation of PMAAs (Sims et al., 2018).

3.8 Colourimetric spectrophotometry:

Colourimetric analysis (colourimetry) is an analytical technique that has been previously used for a long time. Traditionally, it is a subset of visible spectroscopy. Colourimetry has several advantages including fast analysis; small sample size and versatility. A spectrophotometer is an instrument utilised in biological and chemical sciences. It works by radiation of light, filtered to a particular wavelength (or quite narrow range of wavelengths) **Figure 3.6**. UV/Vis is a simple, sensitive and cheap instrument, however, it is beneficial for both identification and quantitative analysis of compounds (Biswas et al., 2011, Jones, 1985).

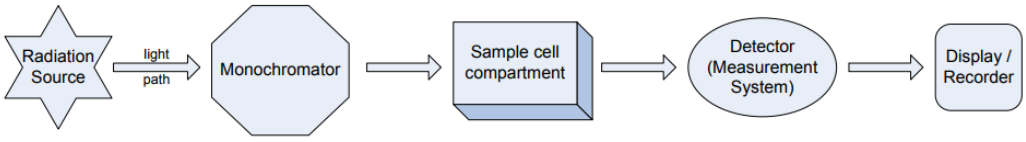


Figure 3.6: The basic components of a spectrophotometer (Dixon, 2008).

Though construction of a calibration curve UV-vis spectroscopy technique can be used for estimating concentration. Furthermore, UV-vis spectrometers are available in all laboratories, the analysis does not change the sample, and the recording of the spectrum does not take long (Amendola and Meneghetti, 2009). For pectins, total GalA content is a very important measure (Moraru et al., 2004). A number of colourimetric methods that were considered in this study as follows: first described by (DuBois et al., 1956b), phenol-sulphuric acid has been used as a colourimetric reagent in the determination of total carbohydrate content. Filisetti-Cozzi and Carpita, (1991) developed the *m*-hydroxydiphenyl method to determine total GalA content in pectin. When uronic acid is heated in concentrated sulphuric acid/tetraborate and further treated with meta-hydroxydiphenyl, a chromogen develops. This chromogen greatly reduces interferences of neutral sugars prevalent in other colourimetric methods. In addition, specificity and increased sensitivity are enhanced versus other listed colourimetric methods. A common reagent, Bradford is used to determining total protein content in polysaccharides. When the reagent is added to any protein co-ectracted with the pectin, a coloured complex is produced suitable for spectroscopic analysis. Absorbance is measured at 595 nm. Developed by McComb and McCready, (1957), hydroxylamine has been used for many years as a colourimetric reagent in the determination of acetyl content. For this reaction, a concentrated aqueous solution of hydroxylamine hydrochloride converts esters to hydroxamic acids. Red complex formation of hydroxamic acid is measured at 510 nm. A number of anti-oxidant assays work on similar principles.

3.9 Principal Component Analysis PCA:

PCA is a technique used to emphasise differences and bring out strong patterns in a dataset. It is often used to make data easy to explore and visualise. The PCA objectives are: (a) to extract the most important information from a data table, (b) compressing the size of the data set, keeping only the relevant information, (c) to simplify the description

of the data set, and (d) to analyse the observations and variable structure. PCA finds a new coordinate system in which every point has a new (x, y) value. The axes don't actually mean anything physical; they are combinations of a different variables called "principal components" that are chosen to give one axis lots of variation (Abdi and Williams, 2010, Richardson, 2009). Principal Component Analysis is a method that resolves multivariate datasets into principal components (PCs) using loadings and scores. So, it is considered as a descriptive chemometric method. **Figure 3.7** provides a summary of the various goals one can have for analysing a data matrix (Wold et al., 1987, Hotelling, 1933).

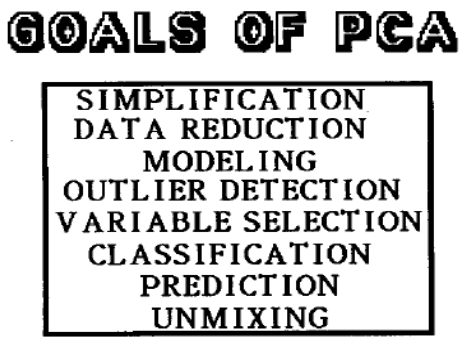


Figure 3.7: Many goals of principal component analysis on a data matrix (Wold et al., 1987).

The purpose of using PCA is to gain a small number of principal components (PC), which explain the variation of the data. Thus, it produces an orthogonal conversion of the raw data, which is more simple to interpret than original data (Lo et al., 2007). Normally, a low number of Principal Components is sufficient to express the measured variance of a (chemical) system.

3.10 Conformational analysis

3.10.1 Estimation of persistence length

For polysaccharides, the conformation of the polymer chains is the key to all their properties (Ralet et al., 2008). Where the global analysis method (HYDFIT) is used;, linear flexibility of the polymer chains can be quantitatively represented in terms of the persistence length, L_p of equivalent *worm-like chains* (Kratky and Porod, 1949). Here the persistence length is defined as the average projection length along the initial direction of the polymer chain (Morris et al., 2010b). the multi-Hydfit program was

created by Ortega and García de la Torre, (2007) to combine analysis from one or more physical property. This software considered estimation of the persistence length, L_p and mass per unit length, M_L, by the use of data sets of intrinsic viscosity and molar mass. Next the best values of M_L and L_p were found by using a minimisation process as reported in (Ortega and García de la Torre, 2007) satisfying the equation of the Bushin–Bohdanecky method (Bohdanecky, 1983a). Where the knowledge is available from the compositional analysis, for example, the mass per unit length (M_L) may be fixed. This method is considered as one of the most common methods for estimating chain persistence lengths mainly for semi-flexible polymers (Ralet et al., 2008).

$$\left(\frac{Mw^2}{[\eta]}\right)^{1/3} = A_o M_L \emptyset^{-1/3} + B_O \quad \emptyset^{-1/3} \left(\frac{2Lp}{ML}\right)^{-1/2} Mw^{1/2} \qquad 3.10$$

$$M_L = \frac{M}{L} \qquad 3.11$$

Whereby, in equation 3.10 $\emptyset$ is the Flory-Fox constant (~2.86 x 10^{23} mol^{-1}) and A_0 and B_0 are tabulated coefficients. However in equation 3.11: M is the average molar mass of a monomer while L is the length of the average monomeric unit which is ~ 0.5 nm (Bohdanecky, 1983a).

Therefore, the utilisation of these techniques will enable the full characterisation of pectins from both pumpkin (chapter 5) and okra (chapter 6), and can potentially give an insight into how pectin functionality, for example anti-oxidant activities, depends on structure, composition, molecular weight and conformation.

3.10.2 Conformation zoning (Normalised scaling relations)

Conformation zoning or normalised scaling relations is used to represent semi-empirically the conformation of a polymer based on a series of hydrodynamic measurements. For example, in Sedimentation Conformation Zoning (Pavlov et al., 1997, Pavlov et al., 1999) a plot of ksM_L versus $[s]/M_L$ is used to estimate the "overall" solution conformation of a polymer in solution ranging from Zone A (extra rigid rod) to Zone E (globular or branched) - see **Figure 3.8a.** The parameter ks (ml/g) is the sedimentation concentration dependence or ''Gralén'' coefficient and [s] is related to

the sedimentation coefficient (Morris et al., 2008). Pavlov, et. al. (1999) have described a further procedure for representing the conformation of polymer in solution based on the relationship between their molar mass, intrinsic viscosity and mass per unit length, M_L (**Figure 3.8b**). The latter procedure was performed in this study.

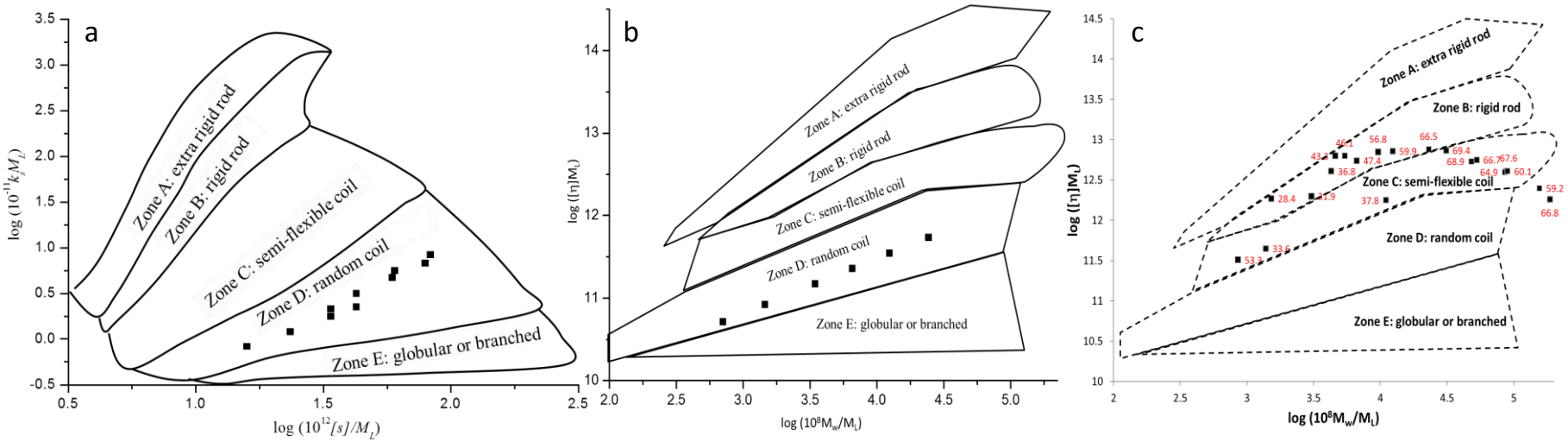


Figure 3.8: Idealised conformation zoning plots (adapted from (Pavlov et al., 1997, Pavlov et al., 1999). Zone A: extra-rigid rod; Zone B: rigid rod; Zone C: semi-flexible; Zone D: random coil and Zone E: globular or branched: a – sedimentation conformation zoning and b – viscometric conformation zoning. Data are shown for pullulan adapted from (Nishinari et al., 1991, Kawahara et al., 1984, Kato et al., 1984, Pavlov et al., 1999, Pavlov et al., 2003). c –an example plot for sugar beet pectin's, where the numbers (in red) represent the neutral sugar content (Morris and Ralet, 2012b).

Chapter 4

Materials and Methods

4 MATERIALS AND METHODS

In this chapter, the experimental details of the different materials and methods (as described in the previous chapter) used in the analysis of both pumpkin and okra pectins will be outlined. Colourimetric assays using Ultraviolet-Visible Spectroscopy (UV/Vis) was used to determine total carbohydrates, galacturonic acid content, acetyl content, and protein content. Neutral sugars :glucose (Glc), arabinose (Ara), *etc*, amino sugars: glucosamine (GlcN), galactosamine (GalN) and uronic acids: galacturonic acid (GalA), glucuronic acid (GlcA) composition were analysed using High-Performance Anion-Exchange Chromatography (HPAEC). Intrinsic viscosity was determined using the capillary viscometry and zeta potential was measured using thelaser Doppler micro-electrophoresis . The degree of methyl esterification (DE) of pectins was determined by the Fourier Transform Infrared Spectroscopy (FT-IR) method (Gnanasambandam and Proctor, 2000). The weight-average molecular weight (M_W) of polysaccharides was estimated by using Size Exclusion Chromatography coupled to Multi-Angle Laser Light Scattering (SEC-MALLS). Gas Chromatography-Mass Spectrometry, GC-MS was the method used in the analysis of polysaccharide linkages. Nuclear Magnetic Resonance (NMR) was used to confirm where possible the structure of polysaccharides.

4.1 Materials and reagents

The material to be analysed consisted of pumpkins and okra pods. Two whole fresh pumpkins, weighing about 2 kg, were purchased from the local supermarket and used to prepare the samples while the okra extracts were provided by Dr. Jacob Agbenorhevi, from the Department of Food Science and Technology at Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana. Dialysis tubing (Mw cut-off 12-14 000 g mol^{-1}) was purchased from Sigma-Aldrich (Gillingham, UK).

Reagents used in these analyses were purchased from Sigma-Aldrich (Gillingham, UK): sodium tetraborate (Borax) 99.0 %, trifluoroacetic acid > 99.0 %; sodium sulphate (anhydrous) ≥ 98.0% ; dimethyl sulfoxide (anhydrous) ≥ 99.9 %; methyl iodide ≥ 99.5 %; sodium azide ≥ 99.5 %; phenol ≥ 99.0 % ;deuterium oxide 99.9 % 85 %; sulfamic acid 99.3 %; ethyl acetate (anhydrous) 99.8 %; bovine serum albumin (BSA) 99 %; Bradford reagent; 1-diphenyl-2-picrylhydrazyl (DPPH) ≥ 85 %; ferrous sulphate ≥ 99.0

%; hydrogen peroxide >30 % w/v; potassium sulfamate ; ≥ 99.0 % salicylic acid ≥ 99.0 %; m-hydroxydiphenyl 85 %; hydroxylamine hydrochloride 97 % and ferric perchlorate (perchloric acid 60 % w/w) (all analytical grade reagents). The standards glucose, galactose, rhamnose, arabinose, xylose, and galacturonic acid (all >99 %) were all also obtained from Sigma-Aldrich (Gillingham, UK). While concentrated sulphuric acid, ethanol (96% w/w), hydrochloric acid 37 %, sodium hydroxide 2M, glacial acetic acid 100 %, potassium hydroxide 85 %, ice, methanol ≥99.9 %and acetone ≥99.9 % were obtained from Chemical Stores at University of Huddersfield and deionised water was used throughout all the experiments.

4.2 Experimental design

4.2.1 Pumpkin pectin extraction:

Different conditions for the extraction of pumpkin pectin were investigated using a composite factorial design see **Table 4.1** (Mesbahi et al., 2005).

Table 4.1: Full factorial design using different extraction conditions (pH, temperature and time).

Sample	pH	T (ºC)	t (h)
1	4	60	1
2	4	60	2
3	4	60	4
4	2	60	1
5	2	60	2
6	2	60	4
7	4	80	1
8	4	80	2
9	4	80	4
10	2	80	1
11	2	80	2
12	2	80	4
13	3	70	1.5
14	3	70	3

4.2.2 Okra pectin extraction:

Pectins from okra pods were isolated by Dr Jacob Agbenorhevi (Department of Food Science and Technology at Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana) using the experimental protocol shown in **Figure 4.2**. The extraction protocol resulted in the isolation of 10 pectin samples; the names of A-J okra samples for isolates extracted at pH 6 as indicated in **Table 4.2**.

Table 4.2: Names of A-J okra samples isolated at pH 6

Sample	Full Name	Abbreviation
A	AGBAGOMA	AGB
B	AGBAGOMA deep green fresh	AGB (F)
C	PEMKRUMAH (TECHI)	PEM (T)
D	PEMKRUMAH (KEN)	PEM (K)
E	KPOMG (TAMELE)	KPO (T)
F	ASONTEM (KEN)	ASO (K)
G	AGBAGOMA (AG)	AGB (AG)
H	SENGEVI	SEN
I	ASHA	ASHA
J	AISHA FRESH	ASHA (F)

4.3 Extraction and isolation of pumpkin pectin

4.3.1 Isolation of alcohol-insoluble remainder of pumpkin

Two fresh pumpkins weighing 2 kg were purchased from the local vegetable supermarket, the outer and damaged skin of the pumpkins was removed as well as the seeds. The residual skin and flesh were cut into small pieces then grated. The grated pieces were soaked in ethanol (methylated spirit: 99% ethanol, 1% methanol) for 24 h then filtered prior to being soaked in acetone for 24 h then filtered. The filtered grated pieces were then left to dry at 50 ºC for three days to leave the alcohol -insoluble residue (AIR) 49.07 g see **Figure 4.1**.

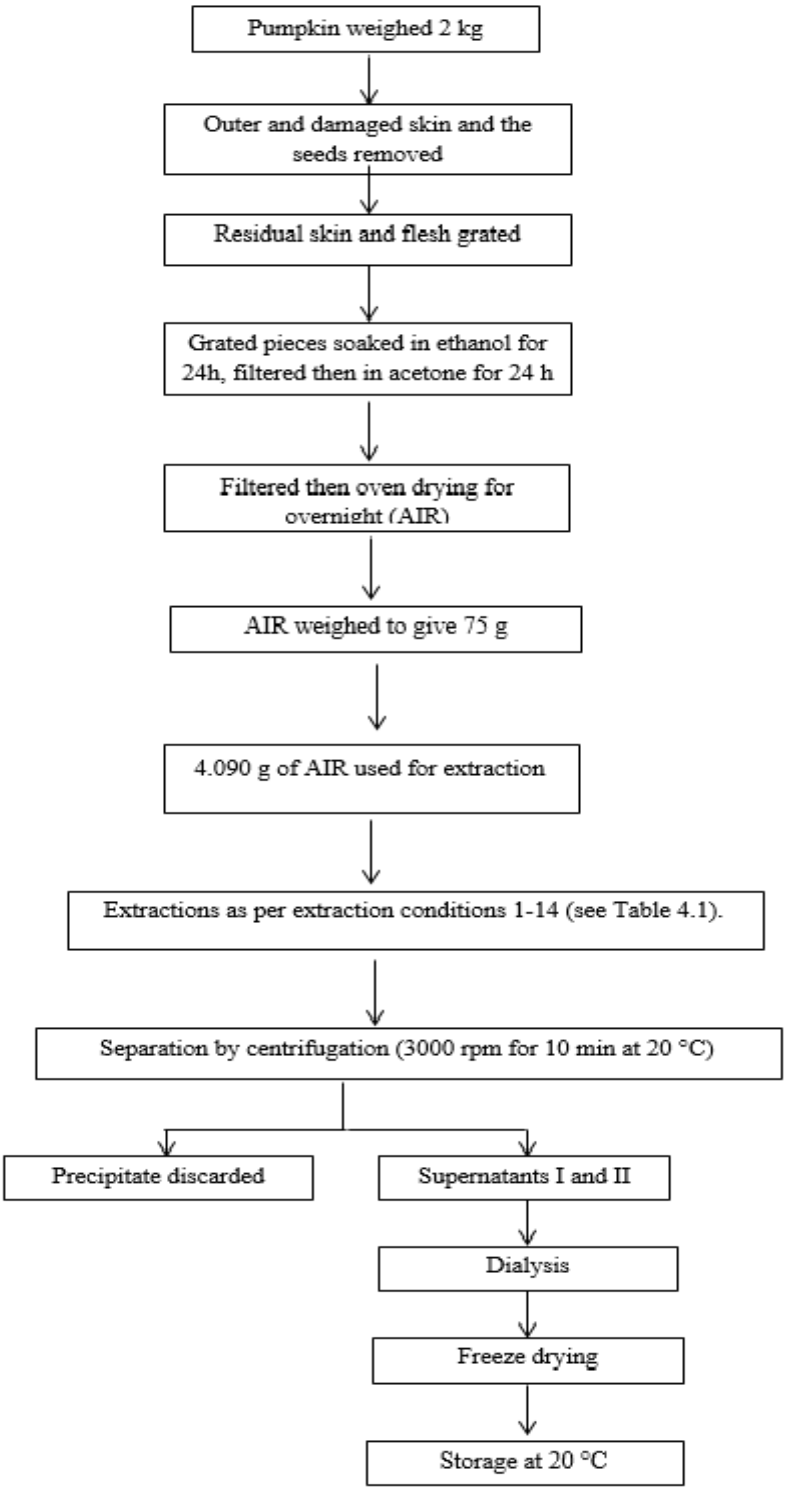


Figure 4.1: Diagram for the methodology of the extraction of polysaccharides from the pumpkin.

4.3.2 Pectin extraction:

4.09 g of 49.07 g of the (AIR) of dried pumpkin powder was used for the extraction process of 14 samples . This amount of the sample (4.09) g was mixed with 120 ml of deionised water. The pumpkin pieces were then extracted using three extraction parameters (pH, temperature and time) as shown in **Table 4.1** and the pH of the mixture was then adjusted to pH 2, 3 or 4 using concentrated HCl. Then the mixture was held at a constant temperature 60, 70 or 80 ºC for a specific time 1, 2, 3 or 4 h, heated on a hot plate with magnetic stirring, the mixture was then adjusted to pH 5 using NaOH solution (2 M), then centrifuged at 20 ºC for 10 min at 5000 rpm in a controlled temperature centrifuge. After centrifugation, the pectin precipitate and the supernatant were separated to get a "cleaner" pectin extract which was then ready for dialysis (Mesbahi et al., 2005), see **Figure 4.1.**

4.3.3 Dialysis

The extract samples were then dialysed using cellulose membrane dialysis tubing with a 12 - 14 000 g mol^{-1} cut-off (Sigma-Aldrich Gillingham, UK) for dialysis against deionised water. The samples were dialysed for 24 h and the water was changed four to five times over this period, the dialysed sample solution was then placed in a freezer for 24h in preparation for freeze-drying.

4.3.4 Dehydration

After the dialysis process, samples were then collected in vials where they were then frozen and freeze-dried for 24 h in order to remove all of the water and other solvents. These dry solids were then weighed and stored prior to further analyses.

4.4 Extraction and isolation of okra pectin

The isolation protocol followed in this study to extract pectin was designed as previously reported by (Alba et al., 2015). Seeds and calyces of okra pods were removed; the remaining material was then freeze dried. The pods were then ground to reduce size their size after which they were defatted with petroleum ether (bp 25 ºC) using a rotary shaker. The lipid-free material, 1 g was then extracted twice using 0.1 M phosphate buffer pH 6.0 at 80 ºC; insoluble particles were removed by centrifugation

and the supernatants were evaporated at 80 ºC, the polysaccharides were precipitated with ethanol 96 %. An aliquot of the precipitate was then frozen and freeze-dried, see **Figure 4.2** for the full protocol. *N.B.* okra samples were not dialysed.

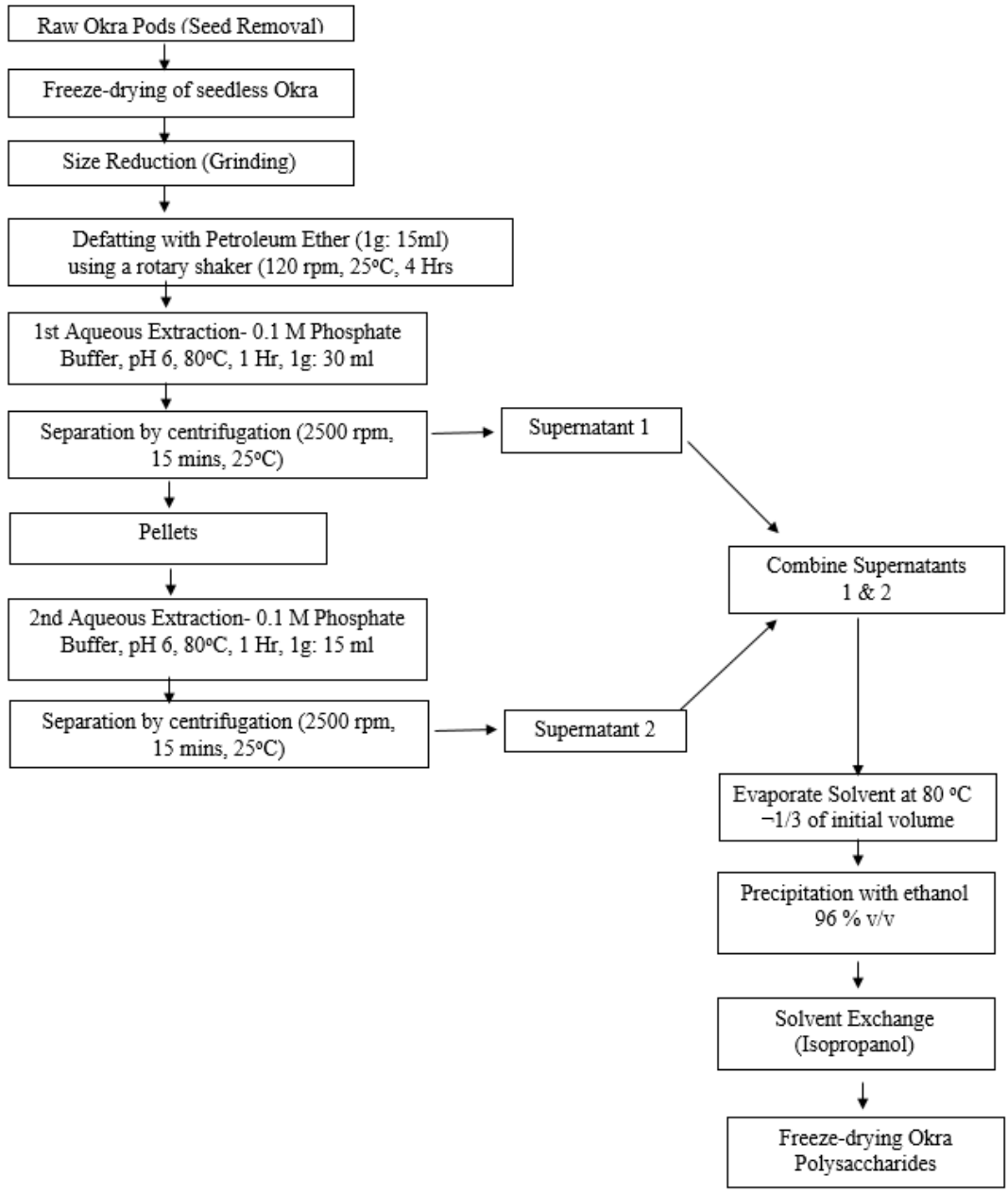


Figure 4.2: Protocol for isolating polysaccharides from okra pods.

4.5 Physicochemical characterisation

After the extracted samples were purified using centrifugation, freeze-drying and dialysis (pumpkin only) procedures they were ready for characterization by the following methods (Yapo et al., 2007).

4.5.1 Ultraviolet-Visible (UV-Vis) Spectroscopy/ Colorimetric Assays

Total carbohydrates were determined by the phenol-sulfuric acid method (Dubois et al., 1956a), using D-Gal as a standard. The galacturonic acid content of pectins was determined colourimetrically by *m*-hydroxydiphenyl method (Filisetti-Cozzi and Carpita, 1991) using D-GalA as a standard. Acetyl content was determined by the hydroxamic acid reaction as described by McComb and McCready, (1957). Protein content was assessed by Bradford's method with bovine serum albumin as a standard (Bradford, 1976). Small quartz (1 cm) cuvettes were used for all UV measurements.

4.5.1.1 Determination of total carbohydrates using the phenol-sulphuric acid assay

In the presence of phenol and sulphuric acid, mono-, oligo- and polysaccharides react and turn to an orange-yellow colour. The degree of colour change, determined by the amount of light absorbed at 490 nm, in a set time can be used to give an approximation of the total concentration of carbohydrate in a sample. This procedure was carried out using a Shimadzu UV–160A UV–Vis spectrophotometer in order to estimate the total concentration of carbohydrate in the prepared samples. Standards of galactose solutions at known concentrations (0, 10, 20, 40, 60, 80 and 100 ppm) were prepared, the extracted samples (0.5 mg) were also dissolved in (5 ml) water to give a nominal 100 ppm solution. All the samples and standard solutions (500 µl) were then mixed with 0.5 ml of 5 % phenol in 0.1 M hydrochloric acid followed by 2.5 ml of concentrated sulphuric acid. After 10 minutes of room temperature incubation, the standards were placed in a Shimadzu UV-160A UV-Vis spectrophotometer and the light absorbance at 490 nm were measured using 1 cm path length plastic cuvettes and using 0.5 ml of 5 % phenol with 2.5 ml concentrated sulphuric acid as blank to zero the instrument. All measurements were made in triplicate. A plot of absorbance against concentration of the standard solutions with linear least-squares regression was produced. The

regression equation of the calibration graph was used to calculate the total carbohydrate concentration in the sample solution (as galactose equivalents) **see Figure 4.3.**

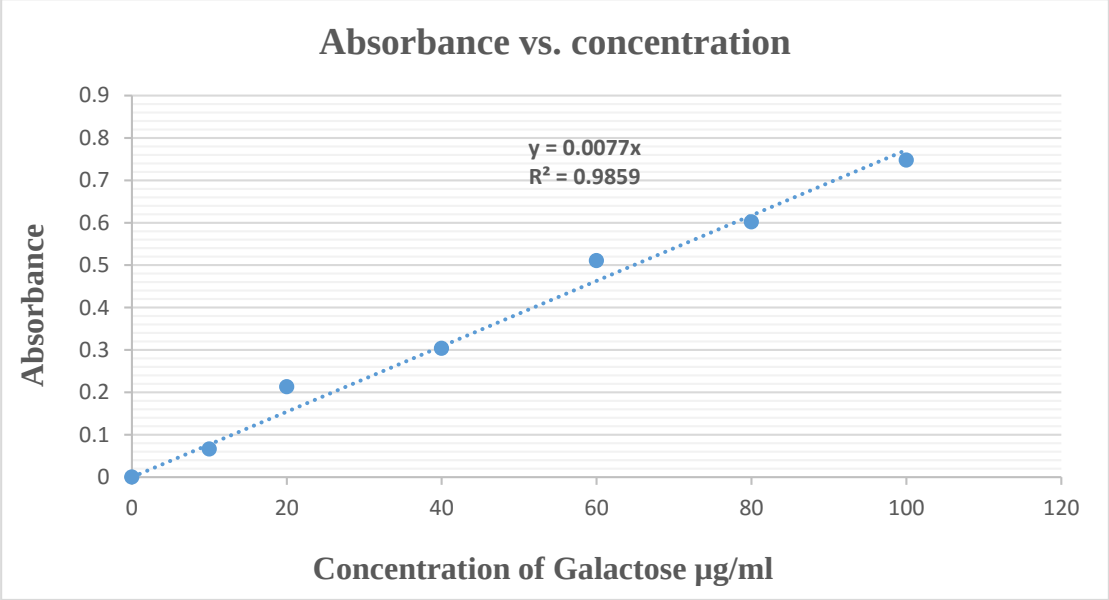


Figure 4.3. Calibration curve of absorbance vs. Concentration for galactose as standard at 490 nm.

4.5.1.2 Determination of uronic acid content using a colourimetric assay

To determine the presence of uronic acids the colourimetric *m*-hydroxyphenyl method was used (Filisetti-Cozzi and Carpita, 1991). A calibration curve was made using standards containing galacturonic acid at 0, 20, 40, 60, 80, and 100 ppm; they were made up to equal volumes using deionised water. The samples were prepared in the same way with a nominal 1000 ppm of the sample in place of galacturonic acid. Aliquots (500 µl) of standards and pectin solutions were placed in 15 ml screw-capped glass tubes; 1 ml of concentrated sulphuric acid was added to all samples, including a control, containing only 1 ml of concentrated sulphuric acid. Samples were stirred using spin bars in an ice bath for 5 min. A further 1 ml of concentrated sulphuric acid was added to all samples with stirring on ice for another 5 min. Afterwards, 0.5 ml of deionised water was added with stirring for 5 min in ice again; another 0.5 ml water was added with stirring for 5 further minutes. All solutions were diluted with water to 10 ml in 10 ml volumetric flasks. The solutions were then transferred to 15 ml centrifuge tubes and centrifuged for 10 min at 2000 rpm at room temperature to remove any non-hydrolysed material. After centrifugation, the upper layers of the solutions

were collected, 500 µl of each were transferred into three test tubes A, B and C. Potassium sulfamate solution (40 µl, 4 M and pH 1.6) was added to all tubes and using a fixed speed vortex mixer all tubes contents mixed for 5 seconds. Sodium tetraborate (2.4 ml of 75 mM) in sulphuric acid solution was added to all tubes prior to heating in a water bath at 100 ºC for 20 min. All tubes had glass marbles placed on top of them to prevent contamination of the samples by condensation, the tubes were then cooled using an ice bath for 10 min. After cooling, 80 µl of *m*-hydroxydiphenyl solution was added to tubes A and B (sample and reagent control tubes) and 80 µl of 0.5 % w/v sodium hydroxide was added to tube C which is marked as sample control. All tubes were vortex mixed for 15s, then recordings were made of absorbance at 525 nm. A calibration curve was drawn and the reading from the sample minus the non-specific reaction was read to give the amount of uronic acid in the sample.

4.5.1.3 Determination of total protein content using Bradford assay

The Bradford assay is a method which is used in the determination of protein content based on the binding of Coomassie Brilliant Blue G-250 dye to proteins (Bradford, 1976). This dye has three forms: cationic (red), neutral (green) and anionic (blue) and at maximum absorbance 470 nm under acidic conditions, the dye will be mostly in the two-fold protonated cationic form (red). When the dye binds to a protein, it is transformed into a stable unprotonated blue form. This complex is detected by following this assay using a spectrophotometer (Agbenorhevi, 2011). Aqueous standard solutions of 40, 80, 100, 200, 400 and 800 ppm BSA (Bovine Serum Albumin) was prepared and analyzed alongside a nominal 100 ppm aqueous solution of freeze-dried pumpkin and okra pectin samples. 100 µl of either sample or standard solutions including a blank (distilled water) were transferred to correctly labelled test tubes and 5 ml of Bradford reagent was added to each tube prior to being vortexed. The tubes were incubated at room temperature for 10 min; after 2 min the absorbance was read using a UV-visible spectrophotometer at 595 nm.

4.5.1.4 Determination of acetyl content:

The acetyl content was estimated using the method described by McComb and McCready, (1957). In order to produce a calibration curve, a freshly prepared 1:1

mixture of sodium hydroxide and hydroxylamine hydrochloride was prepared. 2 ml of this solution was pipetted into four 25 ml volumetric flasks after which 5 ml of diluted standard glucose pentaacetate solutions containing 120, 240, 300 and 420 μg of acetyl was added with stirring. These solutions were left to stand for 30 min and then 5 ml of acidic methanol solution was added with stirring. This solution was diluted to volume by adding ferric perchlorate in small increments with mixing after each addition. The standards were then left for five minutes to allow the colour to develop. For sample analysis 0.01 g of the pectin sample was weighed into a small beaker then 2.5 ml of hydroxylamine solution was added to the sample drop by drop over a period 3-5 min followed by adding 2.5 ml of sodium hydroxide solution with stirring by using bar stirrer, this mixture was then covered until the sample was completely solubilised. 2 ml of the solution was then transferred into 25 ml volumetric flasks then 5 ml of deionised water and 5 ml of acid-methanol were added with mixing. Ferric perchlorate was then added drop by drop. After 5 min the solution was filtered using 0.45 μm membrane filters and after 15 min the absorbance of each sample was determined by using a Shimadzu UV–160A UV–Vis spectrophotometer at 510 nm equipped with a 1 cm cuvette.

The degree of acetylation (DA) was calculated from the following equation using the values for galacturonic acid and acetyl content that were determined above.

$$\text{DA } \% = \frac{176 \times \text{acetyl content}\left(\% \frac{\text{w}}{\text{w}}\right)}{43 \times \text{GalA content}\left(\% \frac{\text{w}}{\text{w}}\right)} \times 100 \qquad 4.1$$

176 and 43 g/mol are related to the molecular weight for anhydrous galacturonic acid and acetyl content respectively.

4.5.2 Free radicals scavenging effects of pumpkin and okra pectin:

4.5.2.1 DPPH assay:

The scavenging influence of pumpkin pectin on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical was measured according to the method of (Li et al., 2008) with some modification. A sample solution at 5 mg/5 ml was prepared, from this solution 2 ml

was transferred into small vials in duplicate. 0.1 mM DPPH in absolute ethanol was also prepared and after preparation, 2 ml of this DPPH solution was added to each of the small vials which contained the sample solutions. These mixtures were shaken then left for 30 min to incubate at 25 ºC, the absorbance of each solution was read at 517 nm. The blank used was DPPH solution 2 ml, the scavenging influence was calculated from the following equation:

DPPH scavenging activity (%) =

[(blank absorbance – sample absorbance)/blank absorbance] × 100 % 4.2

4.5.2.2 Hydroxyl radical assay

A hydroxyl radical assay was carried out following the same approach described in Chen et al., (2013). Concentrations of 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 and 2.0 mg/ml aqueous pectin sample solutions were prepared. A mixture (0.2 ml) of each sample solution and (2.0 ml) deionized water were mixed before adding ferrous sulfate $FeSO_4$ (9.0 mM, 0.2 ml), salicylic acid (9.0 mM, 0.2 ml) and H_2O_2 (8.8 mM, 0.2 ml). The resulting mixture obtained after adding of the last solution was incubated at 37 ºC for 30 min. Using an Agilent Cary 60 UV-Vis spectrophotometer using 1 cm path length glass cuvettes and using deionised water as zero, the absorbance of each solution was measured at 510 nm. This process was performed in duplicate. By using the following equation, the percentage of hydroxyl radical inhibition was calculated:

$$\text{Inhibition (\%)} = \frac{A_0 - (A_1 - A_2)}{A_2} \times 100 \qquad 4.3$$

Where A_0 is the absorbance of the control containing deionised water, $FeSO_4$, salicylic acid, and H_2O_2, A_1 is the absorbance of the test sample and A_2 is the absorbance of the test sample without H_2O_2.

4.5.3 Determination of intrinsic viscosity and zeta potential

A stock solution of the dried pumpkin or okra pectin was prepared by dissolving about 120 mg of each sample in 60 ml of deionised water. Samples were continuously stirred at room temperature than from these stock solutions of different concentrations were

prepared at a concentration of solutes ranging from 0.00025–0.002 g/ml in order to measure viscosity and zeta potential as a function of the solution concentration.

4.5.3.1 Capillary viscometry:

The intrinsic viscosity is a measure of the hydrodynamic volume occupied by macromolecules under the same conditions of chain inflexibility and solvent types (Han et al., 2011).

Sample analysis was performed using a 15 ml Schott-Geräte Oswald viscometer that is as yet still the most widespread viscometer see **Figure 3.1**. For viscosity measurements about 10 ml of pectin solutions were pipetted into the capillary viscometer. The viscosities of the solutions measurement were performed in a water bath under exact temperature control (25.00 ± 0.01 ºC). It is necessary to control the temperature in the water bath using accurate temperature measurement, for example, using an accurately calibrated platinum resistance thermometer. The time for the samples to flow from one indicator to other, known as flow time was recorded for each sample with a stopwatch with a precision of ± 0.1 s (Kar and Arslan, 1999a). The samples were measured in triplicate. Prior to measurement the sample solutions and solvent were filtered using 0.45 μm membrane filters to remove dust and insoluble impurities (Besson et al., 2014). See **section 3.4** for equations used to calculate all types of viscosity.

4.5.3.2 Zeta-potential (ζ)

The measurements of zeta potential were made by using laser Doppler micro-electrophoresis at 25 ºC (Zetasizer 2000, Malvern Instruments Ltd., Malvern, UK (Smistad et al., 2012)). This technique is fairly automated; in the software a standard operating procedure can be included where it just needs small amount of the sample and the data collection takes a few minutes (Nobbmann et al., 2002). Hence the samples were measured in triplicate in disposable cuvette. The sample solutions and solvent were filtered using 0.45 μm membrane filters before measurement to remove dust and insoluble impurities (Hwang et al., 1998). The zeta potential was calculated from the electrophoretic mobility using the Smoluchowski equation (4.5) (Kirby et al., 2003),

where, the electrophoretic mobility, the speed of a particle in an electric field depends upon the strength of the electric field or applied voltage gradient (Abbas, 2010).

$$\zeta = \frac{\eta\sigma}{\varepsilon\varepsilon_{\circ}}\frac{\Delta V}{\Delta P} \qquad 4.5$$

Where η is the viscosity, σ the conductivity, ε is the permittivity, which is linearly related to the pressure-voltage, ΔV is the changing voltage and ΔP is the pressure difference across the channel.

4.5.4 Neutral sugars concentration determination using High-Performance Anion-Exchange Chromatography (HPAEC)

Neutral sugars (Glc, Gal, Rha, Ara, Fuc, Man and Xyl), amino sugars (GlcN, GalN) and uronic acids (GalA and GlcA) were determined using a Dionex ICS-5000 HPAEC-PAD system (Thermo Fisher, Loughborough, UK). A concentration of 10 mM NaOH and 0.5 ml,/min flow rate was used the first 12 min, this was then followed by a 0.05 min step to change from 0-17 % 1 M sodium acetate in 150 mM NaOH. This was then kept constant at 17 % 1 M sodium acetate in 150 mM NaOH for the remainder of the analysis. The run was continued to elute any uronic acids present. A pre-run equilibration step of 10 min using 200 mM NaOH followed by 20 min of 10 mM NaOH was used to regenerate the column prior to each injection.

2 ml of 2M trifluoroacetic acid was added to the sample (2 mg) in a pressure tube to prepare it for hydrolysis, this was then heated at 121 ºC for 2 h. After the 2 h period had elapsed the sample was cooled to room temperature and the cap of the pressure tube was removed. The solution evaporated to dryness under a constant stream of nitrogen at 65 ºC to give monomers which were dissolved in 2 ml deionised water, filtered using 0.45 μm membrane filters to remove dust and insoluble impurities and injected directly in HPAEC at a nominal concentration of 1000 ppm.

Monosaccharide composition can be used to calculate the sugar ratios (Houben et al., 2011) which can be used to detect information related to the pectin structure **Table 5.12**. Also, by using the two following equations (4.7) and (4.8) which were described

by (Msakni et al., 2006), the molar percentage for homogalacturonan (HG) and rhamnogalacturonan-I (RG-I) can be calculated.

HG (mol %) = GalA (mol %) – Rha (mol %) 4.7

RG-I (mol %) = 2Rha (mol %) + Ara (mol %) + Gal (mol %) 4.8

4.5.5 Fourier Transform Infrared Spectroscopy (FT-IR) determination

FT-IR spectra were obtained by applying a small amounts of dry pectin samples to the instrument and the spectra were measured between 4000 and 400 cm^{-1} wavenumbers in Attenuated Total Reflection (ATR) mode at a resolution of 4 cm^{-1} using 128 scans (Nicolet 380, Thermo Scientific, UK). Spectral smoothing was applied using instrument software (OMNIC 3.1).

4.5.5.1 The degree of esterification:

There are many methods that have been previously suggested for determination of degree of esterification (DE) obtained by (Wood and Siddiqui, (1971), Voragen et al., (1983), McFeeters and Armstrong, (1984), Kim and Carpita, (1992), Mizote et al., (1975), Maness et al., (1990), Voragen et al., (1986). In the current study DE in the carbohydrate was calculated according to Chatjigakis et al., (1998) after the areas of the esterified carbonyl peak at 1730 cm^{-1} (A_{1730}) and free carbonyl peak 1635 cm^{-1} (A_{1635}) has been determined to see **equation 4.6**.

$$DE = \left(\frac{\text{Area of esterified peak}}{\text{Area of esterified peak+Area of free carboxyl peak}}\right) \times 100 \quad 4.6$$

4.5.6 Molecular Weight (M_w) determination

The molecular weight (M_W) of polysaccharides were estimated by using Size Exclusion Chromatography coupled to Multi-Angle Light Laser Scattering (SEC-MALLS) at 25 ºC. Pectin samples were solubilised in 0.1 M sodium chloride (NaCl) solution (4 mg/2 ml) at ambient room temperature with stirring overnight, filtered over 0.45 µm membrane filters. Samples were injected onto SEC columns: PL aquagel guard column which was linked in series with PL aquagel-OH 60, PL aquagel-OH 50 and PL aquagel-

OH 40 (15 µm particle size, 25 cm 4 mm, Agilent, Oxford, UK) and were eluted with distilled water at a flow rate of 0.7 ml/min. The eluent was detected online with an RI detector (Optilab rEX, Wyatt Technology, Santa Barbara, USA) and a multi-angle laser light scattering (MALLS) detector (DAWN EOS, Wyatt Technology, Santa Barbara, USA). The differential index of refraction increment (*dn/dc)* was was taken to be 0.147 mL/g which is typical for pectin (Alba, 2015, Morris et al., 2000). Molecular parameters (M_w, M_n, R_g, and M_w/M_n) were then estimated (Alba et al., 2015).

4.5.7 Nuclear Magnetic Resonance (NMR) structural determination

For all the samples, ^{1}H NMR was conducted at 70 ºC with 19000 scans by applying 12800 pulses with a delay time of 2 s and 30 degrees' pulse angle. NMR spectroscopy was applied with a Bruker AV 500 spectrometer (Bruker Co., Switzerland) at 500 MHz 1H and 125.76 MHz ^{13}C using a 5mm probe . and deuterium oxide D_2O (99.9%) was used as the solvent. 2 mg of each of the samples were dissolved in 2 ml of deuterium oxide (D_2O) then freeze-dried. The freeze-dried samples were re-dissolved in 600 µl D_2O, and finally, they were placed in NMR tubes to run them for ^{1}H, whereas, no beneficial information was obtained from the ^{13}C NMR.

Chapter 5

Characterisation of pumpkin pectins

5 CHARACTERISATION OF PUMPKIN PECTINS

The aim of this study was to investigate the influence of different extraction conditions on the molecular characteristics of pectic polysaccharides extracted from fresh pumpkin. Extraction with dilute HCl (pH 2, pH 3 or pH 4) was undertaken at different times (1, 1.5, 2, 3 or 4 hours) and different temperatures (60, 70 and 80 °C) using a factorial design. The physicochemical characterisation of pumpkin pectins (yield, protein content, DAc, DE, GalA content, total sugars, neutral sugars, sugars ratios, viscosity, zeta potential, NMR determination, antioxidant activity and conformational analyses) are shown in the tables and figures of this chapter. During the current study, 14 physicochemical characteristics were determined and the analyses were performed as per previously published procedures (**Table 5.1**). The chapter will be separated into four distinction sections: chemical characterisation (**5.1**), physical characterisation (**5.2**), conformational analyses (**5.3**) and antioxidant activities (**5.4**). The full graphs for the FT–IR, constituent sugars composition, intrinsic viscosity, zeta potential and molecular weight distributions such as (light scattering, reflective index) are shown in appendices (**9.1.1**, **9.1.2**, **9.1.3**, **9.1.4** and **9.1.5)** respectively in **chapter 9**.

Table 5.1: List of procedures adopted for the analysis of physicochemical characterisation of pectin samples used in this study.

Parameter	Methods followed
1. Yield (w/w on a dry weight basis)	(Samavati, 2013)
2. Total carbohydrates	(Dubois et al., 1956a)
3. Protein content	(Bradford, 1976)
4. Uronic acid	(Filisetti-Cozzi and Carpita, 1991)
5. The degree of methylesterification (DM)	(Alba et al., 2015)
6. Acetyl content (DAc)	(McComb and McCready, 1957)
7. Monosaccharides composition	(Denman and Morris, 2014)
8. Compositional ratios	(Houben et al., 2011)
9. Structural determination	(Alba et al., 2015)
10. Intrinsic viscosity ([η])	(Harding, 1997)
11. Zeta (ζ)-potential	(Abodinar et al., 2014)
12. Molecular weight	(Alba et al., 2015)
13. Antioxidant activity	(Li et al., 2008, Chen et al., 2013)
14. Conformational analyses	(Morris et al., 2014)

5.1 Chemical characterisation of pumpkin pectin:

In this section, the chemical composition of pumpkin pectin will be determined; this will include the following: total carbohydrates, protein content, galacturonic acid and acetyl content. In addition, neutral sugars (Glc, Ara, *etc*) and uronic acid (GalA, GlcA) composition will be analysed. Then, the degree of methyl esterification (DE) of pectins will be determined and Nuclear Magnetic Resonance (NMR) used to identify the structure of polysaccharides. The results will be compared with previous studies on the pumpkin and other polysaccharides from similar sources, for example, apple pomace, peach, *etc*.

5.1.1 Determination of yield (%)

The yield of alcohol insoluble residue (AIR) from ~2 kg fresh pumpkin tissue and skin which weighed 49.07 g was 2.4 %. The AIR was divided into 12 equal portions with an extra two portions used as centre points using more pumpkin sample as per the experimental design and extracted (**Table 4.1**). The yield of pectin extracted ranged between 2.8 % - 8.0 % of acid extracted pectin (**Table 5.2)**. The results showed that acidic extractions gave high pectin yields (**Figure 5.1**) compared to those documented in the literature (Micard and Thibault, 1999a). The highest extraction yield (8.0 %) was obtained for sample 10 when the dried pumpkin was treated at pH 2, 80 °C and extraction time of 1 h, this yield value is the same as that estimated in Garna et al., (2007) under the same conditions pH 2, 80 °C and 1 h extraction time. This was similar to those reported in (Urias-Orona et al., (2010) for chickpea husk pectin 8 %. Besides, it appears to be in a similar region to other values for example 7.9 % at pH 1, 60 °C and 2 h reported in Denman and Morris, (2014) for melon pectins and in good agreement with yield values reported in Matora et al., (1995) for pectin sugar beet.

Furthermore, compared to the literature data, the pectin yields of 3.4 % at pH 4, 60 °C and 4 h were close to those of (Denman and Morris, 2014) from *Cucumis melo* pectin: 3.4 % at pH 3, 80 °C and 4 h. But, they were higher than those reported in (Koubala et al., 2008) for pectin extracted from ambarella peels which ranged from 0.8 - 2.97 %. Conversely, the yield values obtained appear lower than those reported in Levigne et al., (2002b) for pectin extracted from fresh sugar beet and lower than those obtained by Matora et al., (1995) for pectin extracted from pumpkin which ranged from 9 - 22 %. Yields were also lower than those determined in Khodaei and Karboune, (2013) for potato cell wall (22 - 55 %) and passion fruit peel (Seixas et al., 2014a) which ranged from 9 - 30 %.

Table 5.2: Experimental values for a yield of pectins extracted according to a full factorial design.

Sample	pH	T (°C)	t (h)	Yield %
1	4	60	1	$3.0 \pm 0.3^{d,e}$
2	4	60	2	2.8 ± 0.6^{e}
3	4	60	4	$3.4 \pm 0.3^{d,e}$
4	2	60	1	$3.3 \pm 0.4^{d,e}$
5	2	60	2	$4.3 \pm 0.3^{c,d}$
6	2	60	4	$5.4 \pm 0.4^{b,c}$
7	4	80	1	$4.4 \pm 0.2^{c,d}$
8	4	80	2	$4.3 \pm 0.4^{c,d}$
9	4	80	4	$3.3 \pm 0.4^{d,e}$
10	2	80	1	8.0 ± 0.6^{a}
11	2	80	2	6.4 ± 0.4^{b}
12	2	80	4	$4.9 \pm 0.6^{c,d}$
13	3	70	1.5	$4.1 \pm 0.6^{c,d}$
14	3	70	3	$4.6 \pm 0.5^{c,d}$

Values were given as means of triplicate ± SD. Means with different superscript letters within a column are significantly different (Tukey test, $p < 0.05$). SD = Standard deviation of the mean.

Furthermore, the lowest extraction yield was 2.8 % in pH 4 at 60 °C and an extraction time of 2 h, it is very low compared to those obtained by Levigne et al., (2002a) from fresh sugar beet but in agreement with the results of yield achieved for cinnamon apple pomace at pH 2 (2.8 %) (Besson et al., 2014). In terms of temperature, the higher the temperature the higher the extraction yield (Marcon et al., 2005). The table obviously confirmed that the extraction yield of pectin increased with the highest extracted temperature 80 °C while there was a slight decrease in the extraction yield of pectin at the lowest extracted temperature 60 °C (**Figure 5.1**). This could be clarified by the solubility of pectin extracted which increases with increasing the temperature thus giving high yield for pectin (Guo et al., 2012).

In general, acids have a strong effect on the yield of extracted pectin, therefore, low pH is considered as the strongest extracting agent (Yapo, 2009a) and this is consistent with the information in **Figure 5.1**.

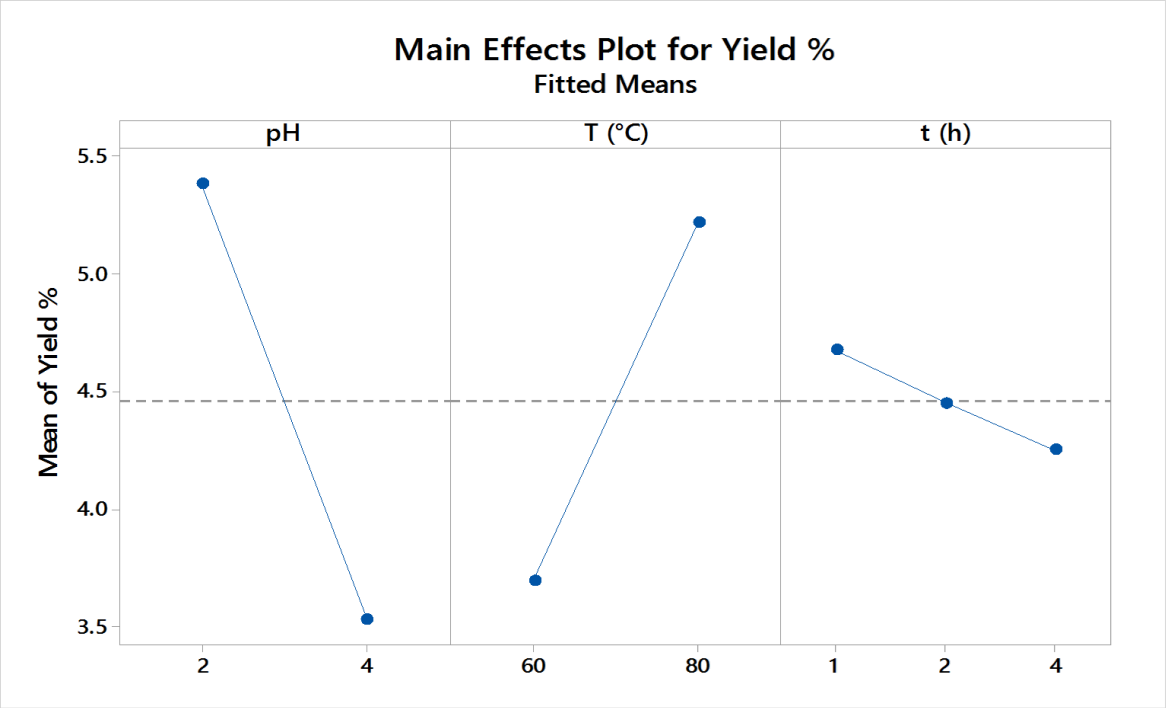


Figure 5.1: The main effect plots for yield (%): pH, temperature and time. The overall mean (~4.46 %) is shown as a dotted line and the steeper the slope the greater the effect of a particular parameter.

An experimental design approach was undertaken it is possible to gain some further understanding of the influence of each extraction condition and how they interact with one another. Therefore, the main (the largest) effect on yield is pH (**Figure 5.1**). The yield increases as when pH moves from a high level (pH 4) to lower level (pH 2). However, the main effects plots also indicate that temperature has a similar effect.

Mesbahi et al., (2005) reported that, to extract the pectin for food application experiments the extraction conditions of 80 °C, pH 2 for 1 h should be used. In fact, in the present study, at constant pH, pectin yields obtained at 60 ºC for the extraction were lower than those of 80 °C, this is exactly the case with the values of the yield for pectin from fresh peach pomace (Pagan and Ibarz, 1999). Furthermore, the yields of pectins extracted at pH 2 were higher than those extracted at pH 4 (Happi Emaga et al., 2008). From previous studies, it would be expected that the yield at pH 1.5 would be higher than those at pH 2 (Garna et al., 2007). Interpretation of

Figure 5.1 shows that the pH was the main parameter which influences the extraction performance in terms of yield (Yoo et al., 2012b). In general, the performance of yield increase with decreasing pH of the solvent was reported before (Levigne et al., 2002a, Besson et al., 2014, Garna et al., 2007). Therefore, from **Figure 5.2**the extracting conditions using pH 2 was optimal in isolating a higher yield (5.4 ± 1.7 %) of pectin compared to 3.5 ± 0.7 % at pH 4, indicating that stronger acid conditions were desirable for the extraction, from pumpkin of an amount (>5 %) of pectins considered to be commercially usable (Mohamed and Hasan, 1995). However, it is important to take into account the interactions with other extraction conditions (**Figure 5.2**).

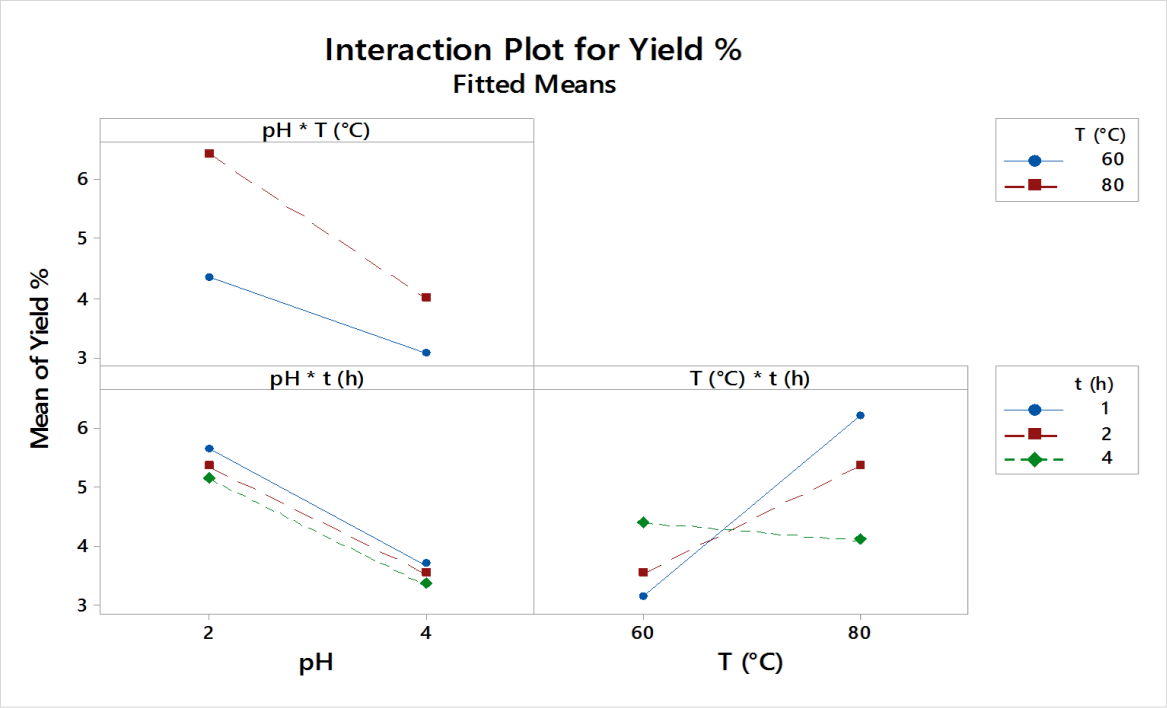


Figure 5.2: The interaction plots for yield %. To visualize these effects, the Y-axis scale is always the same for each combination of factors. When the lines are parallel, interaction effects are zero.

In brief, an interaction plot basically reveals whether there is an interaction between two different extraction conditions for a certain response to the yield (Sanjuán and Argiz, 2016). When the lines are parallel, interaction effects are zero as is the case for pH and time. The more different the slopes, the more influence the interaction effect has on the results (Israel et al., 2014). In **Figure 5.2** the slopes for the pH*Temperature and Temperature*time are non-parallel indicating there are interactions between the different extraction conditions, however, the interaction between temperature and time is the largest. The 2-factor interactions are 1.02, 0.02

and 5.73 for pH*temperature, pH*time and temperature*time, respectively. The 3-way (pH*temperature*time) interaction is 1.83.

Based on all these interpretations resulting from the factorial design, it is possible to say that under these specific conditions that the parameters at pH 2, temperature 80 ºC and an extraction time of 1 hour to give the highest/ optimum yield under the examined conditions.

5.1.2 Determination of total carbohydrates:

Total carbohydrate values of 14 pumpkin pectins are detailed in **Table 5.3** varied from 30.9 to 72.2 %. The pectin extract obtained at pH 2.0, 60 ºC and 1h extraction time (sample 4) was remarkably rich in total carbohydrate (72.2 %). As can be seen, the recovery for total carbohydrate does not equate to 100 %; this may because the use of galactose as standards and also as the response to the assay varies with different monosaccharides (DuBois et al., 1956b, Bath, 1958). The results are comparable to those found for pectins from pumpkin (Guiné et al., 2011b), cacao pod husk flour (Vriesmann et al., 2011), *Citrus depressa* (De Ruiter et al., 1992) and *cupuassu* pulp (Vriesmann and de Oliveira Petkowicz, 2009). However, values are slightly lower than those obtained from of *Citrus depressa* (Tamaki et al., 2008), potato cell wall (Fissore et al., 2007) and from pumpkin (Sharma and Rao, 2013, Saeleaw and Schleining, 2011, Song et al., 2013). In contrast, they are higher than those described in the literature for Ambarella and lime pectins (Koubala et al., 2008).

Table 5.3. Shows the total carbohydrates percentage of pumpkin pectins under different extraction conditions.

Sample	pH	T (°C)	t (h)	Total carbohydrate %
1	4	60	1	$55.8 \pm 6.3^{c,d}$
2	4	60	2	$68.1 \pm 2.4^{a,b}$
3	4	60	4	$55.7 \pm 1.1^{c,d}$
4	2	60	1	72.2 ± 3.7^{a}
5	2	60	2	$62.1 \pm 2.2^{b,c}$
6	2	60	4	39.5 ± 0.4^{g}
7	4	80	1	61.7 ± 1.2^{c}
8	4	80	2	$46.5 \pm 4.1^{e,f}$
9	4	80	4	$36.3 \pm 0.1^{g,h}$
10	2	80	1	$41.1 \pm 1.5^{f,g}$
11	2	80	2	$57.1 \pm 1.1^{c,d}$
12	2	80	4	30.9 ± 6.2^{h}
13	3	70	1.5	59.5 ± 0.2^{c}
14	3	70	3	$51.6 \pm 0.1^{d,e}$

Values were given as means of triplicate ± SD. Means with different superscript letters within a column are significantly different (Tukey test, $p < 0.05$). SD = Standard deviation of the mean.

An experimental design approach allows some further understanding of the influence of each extraction condition and how they interact with one another. In this case, the main (the largest) effect on total carbohydrate is time (**Figure 5.3**). The total carbohydrate content decreases moving from low level (1 hour) to high level (4 hours). However, the main effects plots also indicate that temperature has a similar effect.

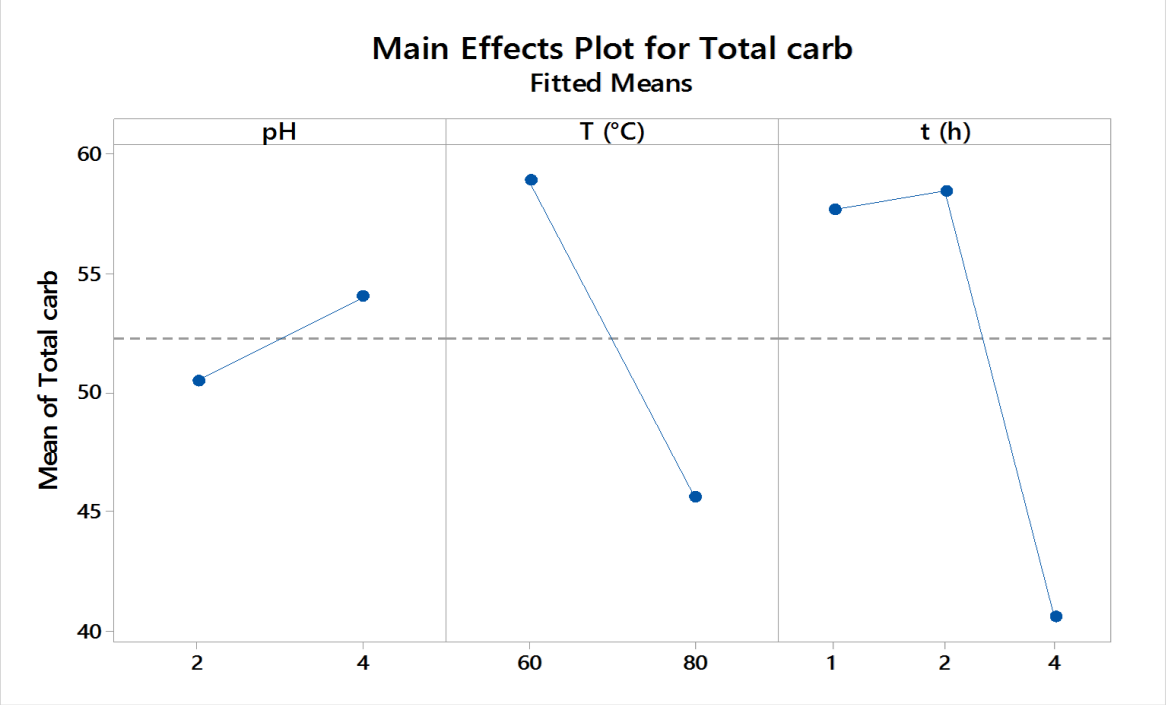


Figure 5.3: The main effect plots for total carbohydrate (%): pH, temperature and time. The overall mean (~52.2 %) is shown as a dotted line and the steeper the slope the greater the effect of a particular parameter.

The interaction plot reveals whether there is an interaction between two different extraction conditions for a certain response on the total carbohydrate content. When the lines are parallel, interaction effects are zero which is essentially the case for temperature and time (**Figure 5.4**). The more different the slopes, the more influence the interaction effect has on the results (Israel et al., 2014). In **Figure 5.4** the slopes for the pH*Temperature and pH*time are non-parallel indicating there are interactions between the different extraction conditions, however, the interaction between pH and time is the largest. The 2-factor interactions are 7.68, 88.89 and 0.98 for pH*temperature, pH*time and temperature*time, respectively. The 3-way (pH*temperature*time) interaction is 432.62 and is therefore very important.

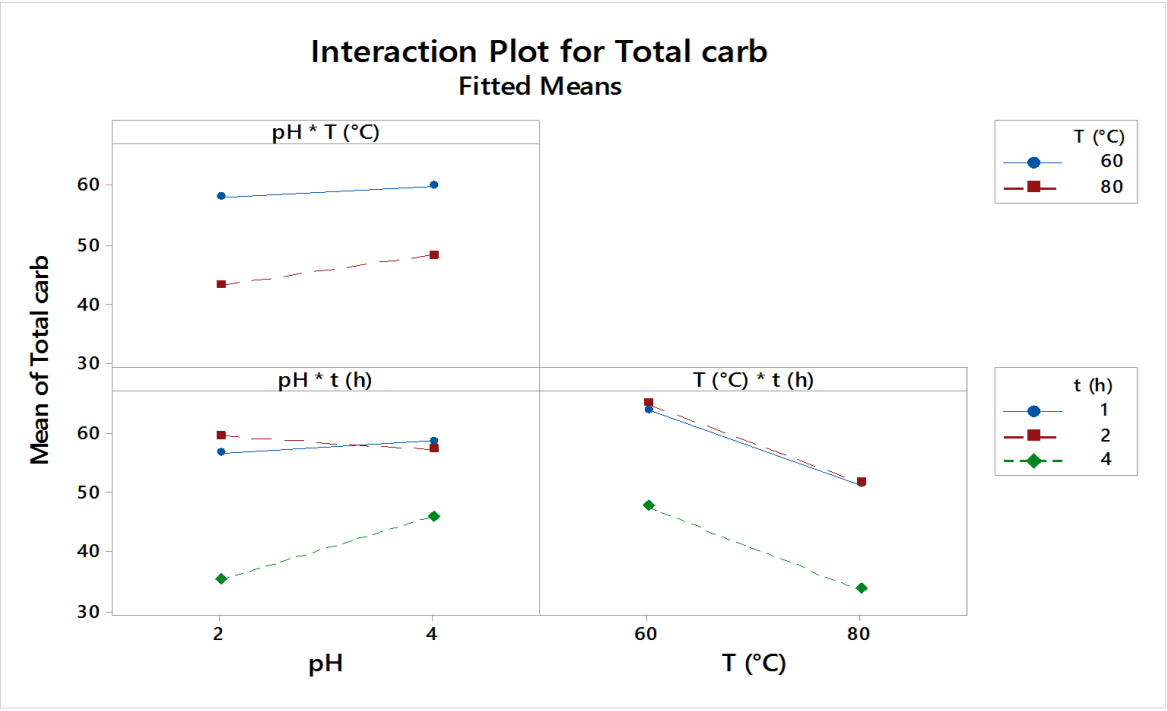


Figure 5.4: The interaction plots for total carbohydrate (%). To visualize these effects, the Y-axis scale is always the same for each combination of factors. When the lines are parallel, interaction effects are zero.

Based on all these interpretations resulting from the factorial design, it is possible to say that under these specific conditions that the parameters at pH 2, temperature 60 ºC and an extraction time of 1 hour give the highest/ optimum total carbohydrate content under the examined conditions. This is of course with the considration the response to the assay varies with different monosaccharides (DuBois et al., 1956b, Bath, 1958). This may be consistent with more than one or more other processes taking place alongside extraction, for example, pectin degradation. This is perhaps consistent with a two-phase mechanism for the extraction of pectin which has previously been proposed for both from fresh peach pomace (Pagan and Ibarz, 1999) and melon (Denman and Morris, 2014); the first step being acid solubilisation of the pectin followed by a second hydrolysis reaction where the pectin is degraded, lowering the yield (or galacturonic acid content) at longer extraction times, and once all the "extractable" pectin has been exhausted the hydrolysis predominates.

5.1.3 Determination of protein content:

The presence of nitrogenous products such as proteins in the pectin extracts was determined by the Bradford assay using bovine serum albumin (BSA) as a standard protein (Bradford, 1976).

Values varied with the extraction conditions and the results are presented in **Table 5.4**. These results presented indicated slightly higher protein values compared with those reported in Garna et al., (2007) for pectin extracted from apple pomace using the same experimental design. Also, much higher than those in Urias-Orona et al., (2010) for chickpea pectin which contained only very trace amounts of protein. However, pectins extracted at pH 3 (14.3 %) contain more protein than those extracted at pH 2 and 4. Besides, these results seem closer to the results published in (Košťálová et al., 2013) for pectins isolated from the fruits of Styrian oil-pumpkin (*Cucurbita pepo var. Styria*). Likewise, they were in good agreement with the protein content values reported in (Saeleaw and Schleining (2011) for pumpkin powder.

Table 5.4: Indicates the protein content of pumpkin pectins using different extraction conditions.

Sample	pH	T (°C)	t (h)	Protein content %
1	4	60	1	$9.1 \pm 1.5^{b,c}$
2	4	60	2	$6.7 \pm 0.4^{c,d}$
3	4	60	4	$2.5 \pm 0.4^{d,e}$
4	2	60	1	$4.8 \pm 4.8^{c,d,e}$
5	2	60	2	5.3 ± 1.9^{e}
6	2	60	4	1.8 ± 0.6^{e}
7	4	80	1	$3.8 \pm 0.3^{d,e}$
8	4	80	2	$2.9 \pm 2.2^{d,e}$
9	4	80	4	$3.1 \pm 0.9^{d,e}$
10	2	80	1	$3.9 \pm 2.9^{d,e}$
11	2	80	2	8.9 ± 1.6^{c}
12	2	80	4	$13.0 \pm 2.8^{a,b}$
13	3	70	1.5	14.3 ± 0.4^{a}
14	3	70	3	13.4 ± 1.4^{a}

Values were given as means of triplicate ± SD. Means with different superscript letters within a column are significantly different (Tukey test, $p < 0.05$). SD = Standard deviation of the mean.

The highest amount of protein was for pectin 13 which was higher than the other pectin samples and also higher than the protein content of water extracted pectin WEP, chelating agent extracted pectin ChEP, and acid extracted pectin HEP (0.17 %, 0.28 % and 3.23 %, respectively) of pectins isolated from creeping fig seeds (*Ficus pumila* Linn.)(Liang et al., 2012b). On the contrary, the protein content for these pectins was lower than those extracted from pumpkin seeds (Karanja et al., 2013) which ranged from 14.05 - 33.29 %. It was also lower than pectins extracted from yellow passion fruit rind (Yapo and Koffi, 2006) using different extraction protocols. Besides, these results are also very low when compared with those obtained by Guiné et al., (2011b) for fresh and dehydrated pumpkin. In contrast, the protein values present in this study is similar to those reported in (Sharma et al., 2006) for a number of pectin extracts and to the protein content estimated in pumpkin pectin fraction A (Cui and Chang, 2014). The strong acid (HCl) used in this extraction process would perhaps be expected to "cosolubilize" more protein residues than those of water or weak acid, for example, oxalate. Higher acid concentration (lower pH) and higher temperatures also increased co-extraction of protein (**Figure 5.5**).

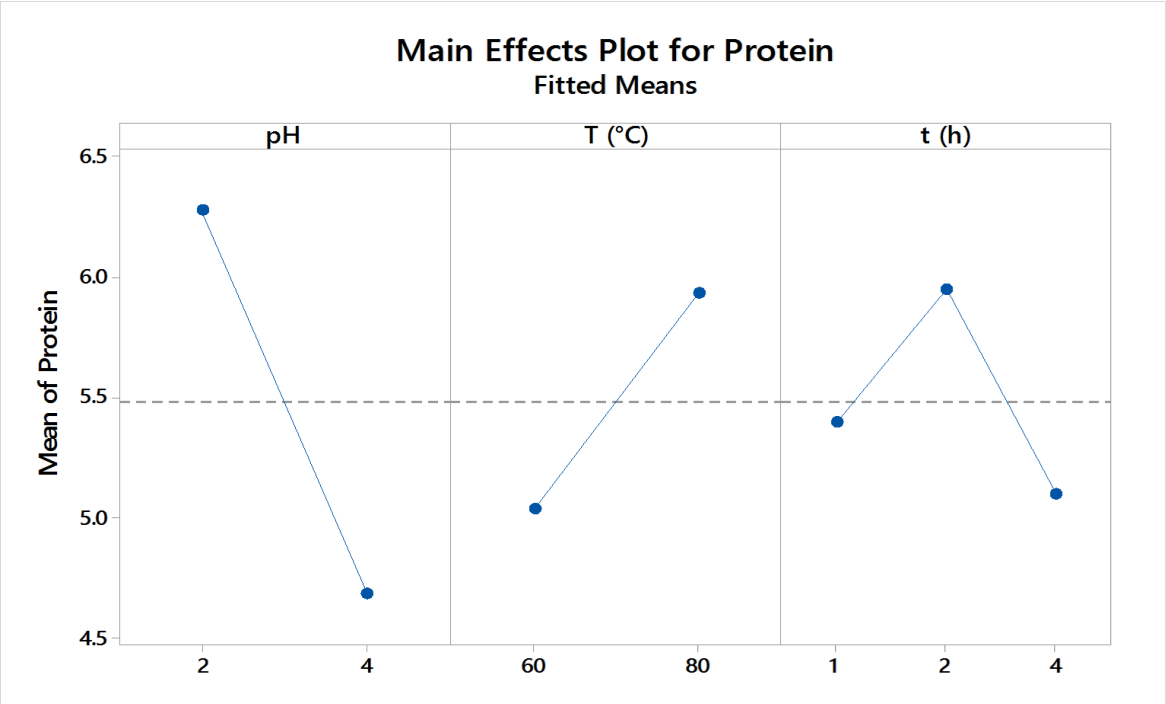


Figure 5.5: The main effect plots for protein content (%): pH, temperature and time. The overall mean (~5.48 %) is shown as a dotted line and the steeper the slope the greater the effect of a particular parameter.

The experimental design enables a further understanding of the influence of each extraction condition and how they interact with one another. Therefore, the main (the largest) effect on protein content is pH (**Figure 5.5**). The protein content decreases moving from a low level (pH 2) to a high level (pH 4), temperature also has an important effect.

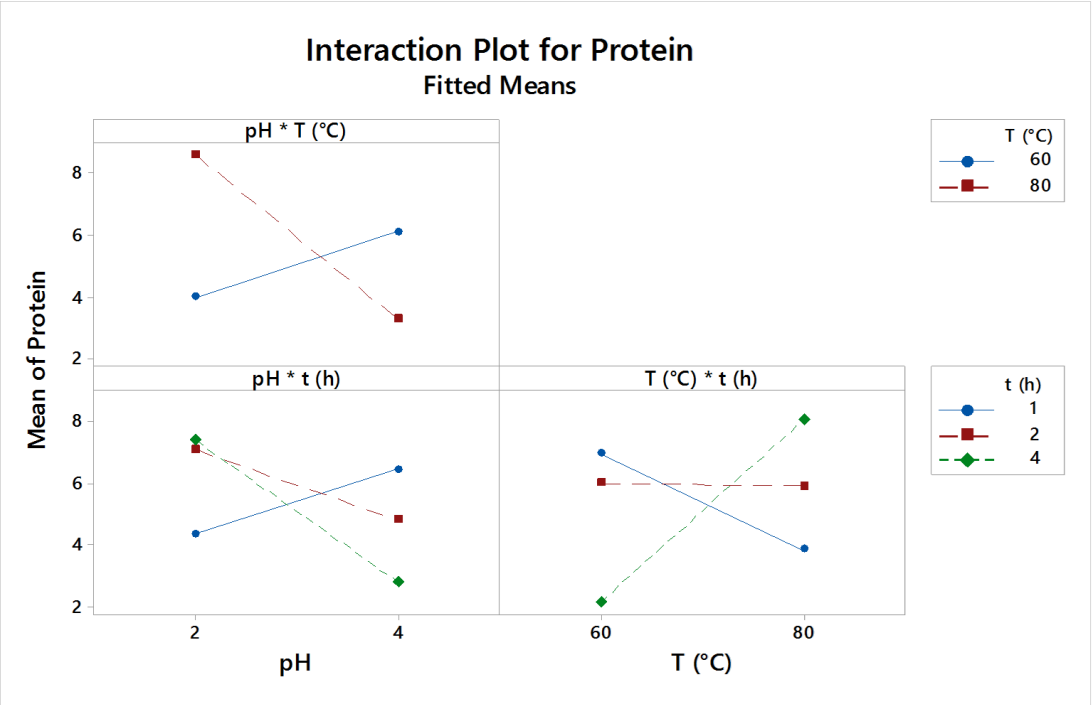


Figure 5.6: The interaction plots for protein content (%). To visualize these effects, the Y-axis scale is always the same for each combination of factors. When the lines are parallel, interaction effects are zero.

In **Figure 5.6** the slopes for all three are non-parallel indicating there are interactions between the different extraction conditions, however, the interaction between pH and time and temperature and time are the largest. The 2-factor interactions are 41.81, 23.18 and 42.00 for pH*temperature, pH*time and temperature*time, respectively. The 3-way (pH*temperature*time) interaction, in this case, is very small (4.81).

Based on all these interpretations resulting from the factorial design, it is possible to say that under these specific conditions that the parameters at pH 2, temperature 60 ºC and an extraction time of 4 hours to give the lowest/ optimum protein content under the examined conditions.

5.1.4 Determination of uronic acid:

The GalA content of pumpkin pectin samples varied from 41.7 - 72.6 % shown in **Table 5.5**. The highest uronic acid content was for sample 10 (72.6 %) also samples 5 and 3 (65.6 % and 66.4 %) respectively, this was in an agreement with the Food and Agriculture organisation (FAO) and European Union (EU) which mentions that 'pectin' must consist of at least 65 % GalA (Willats et al., 2006). Samples with > 65 % GalA have been highlighted in **bold** in **Table 5.5**. The other pectin samples contain lower uronic acid content, below the required standards. These results are consistent with published data for galacturonic acid content extracted from passion fruit peel *(Passiflora edulis f. flavicarpa*) using acetic acid and tartaric acid: 62.5 and 58.5 % respectively (Seixas et al., 2014a). Furthermore, they were in agreement with galacturonic content extracted from okra pods OP2 and OP6: 46.8 and 56.9 % respectively (Alba et al., 2015). Values are also similar to those estimated for pectins extracted from yellow passion fruit and pumpkin (Yapo, 2009b, Yoo et al., 2012b). However, uronic acid levels in this study are higher than those published in (Jun et al., 2006) for pumpkin peel. On the other hand, these results seem slightly lower than those obtained by (Cui and Chang, 2014) for pumpkin pectin which was extracted enzymatically and likewise lower than those extracted from the endocarp of Citrus *depressa* (Tamaki et al., 2008).

Table 5.5: Indicates the percentage of galacturonic acid in the pumpkin pectins using different extraction conditions.

Sample	pH	T (°C)	t (h)	GalA %
1	4	60	1	55.3 ± 0.1^{g}
2	4	60	2	58.7 ± 0.2^{e}
3	4	60	4	$\mathbf{66.4 \pm 0.1}^{b}$
4	2	60	1	56.2 ± 0.1^{f}
5	2	60	2	$\mathbf{65.6 \pm 0.4}^{c}$
6	2	60	4	51.7 ± 0.2^{h}
7	4	80	1	43.7 ± 0.2^{j}
8	4	80	2	55.5 ± 0.1^{g}
9	4	80	4	60.4 ± 0.4^{d}
10	2	80	1	$\mathbf{72.6 \pm 0.4}^{a}$
11	2	80	2	60.8 ± 0.2^{d}
12	2	80	4	50.7 ± 0.2^{i}
13	3	70	1.5	41.7 ± 0.2^{k}
14	3	70	3	52.1 ± 0.2^{h}

Values were given as means of triplicate ± SD. Means with different superscript letters within a column are significantly different (Tukey test, $p < 0.05$). SD = Standard deviation of the mean.

Through the use of an experimental design approach it is possible to gain some further understanding of the influence of each extraction condition and how they interact with one another. Therefore, the main (the largest) effect on galacturonic acid content is pH (**Figure 5.7**). The galacturonic acid content decreases moving from low level (pH 2) to high level (pH 4), in this case, the overall effect of time is almost identical, the pattern is more complex and may be consistent with more than one or more other processes taking place alongside extraction, for example, pectin degradation as previously mentioned in section **5.1.2**.

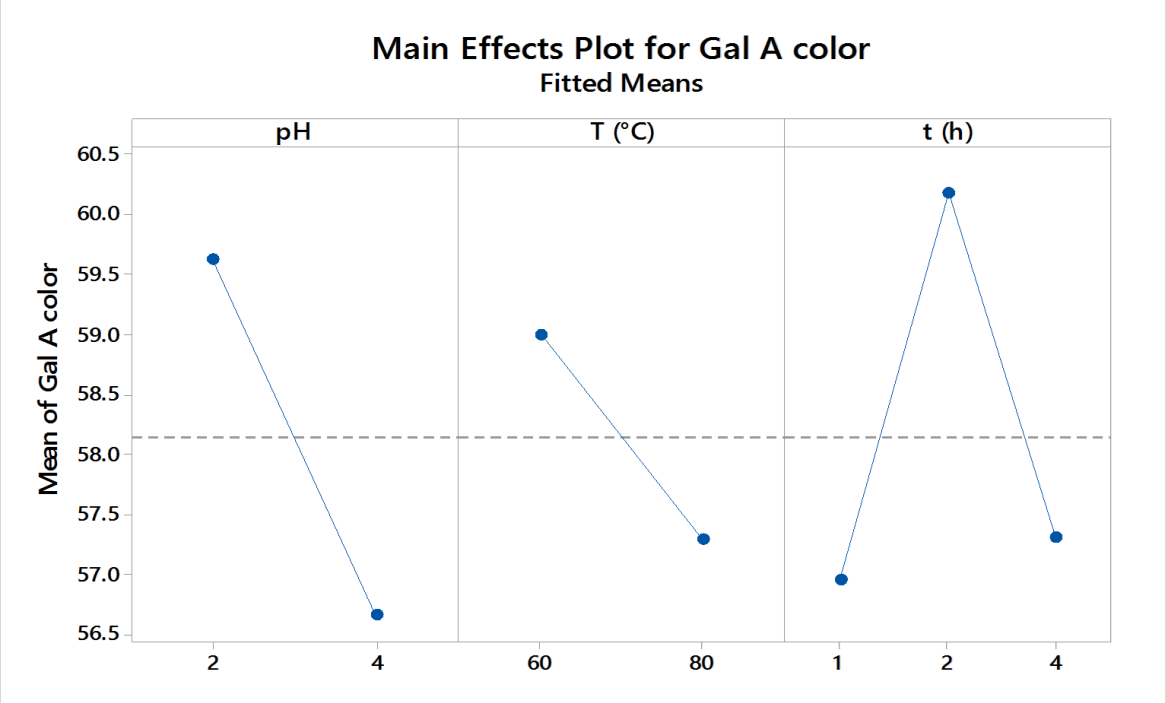


Figure 5.7: The main effect plots for galacturonic acid content (%) using a colourimetric assay: pH, temperature and time. The overall mean (~58.1 %) is shown as a dotted line and the steeper the slope the greater the effect of a particular parameter.

In **Figure 5.8** the slopes for all three are non-parallel indicating there are interactions between the different extraction conditions, however, the interaction between pH and time is the largest. The 2-factor interactions are 81.64, 382.57 and 25.57 for pH*temperature, pH*time and temperature*time, respectively. The 3-way (pH*temperature*time) interaction is 121.33.

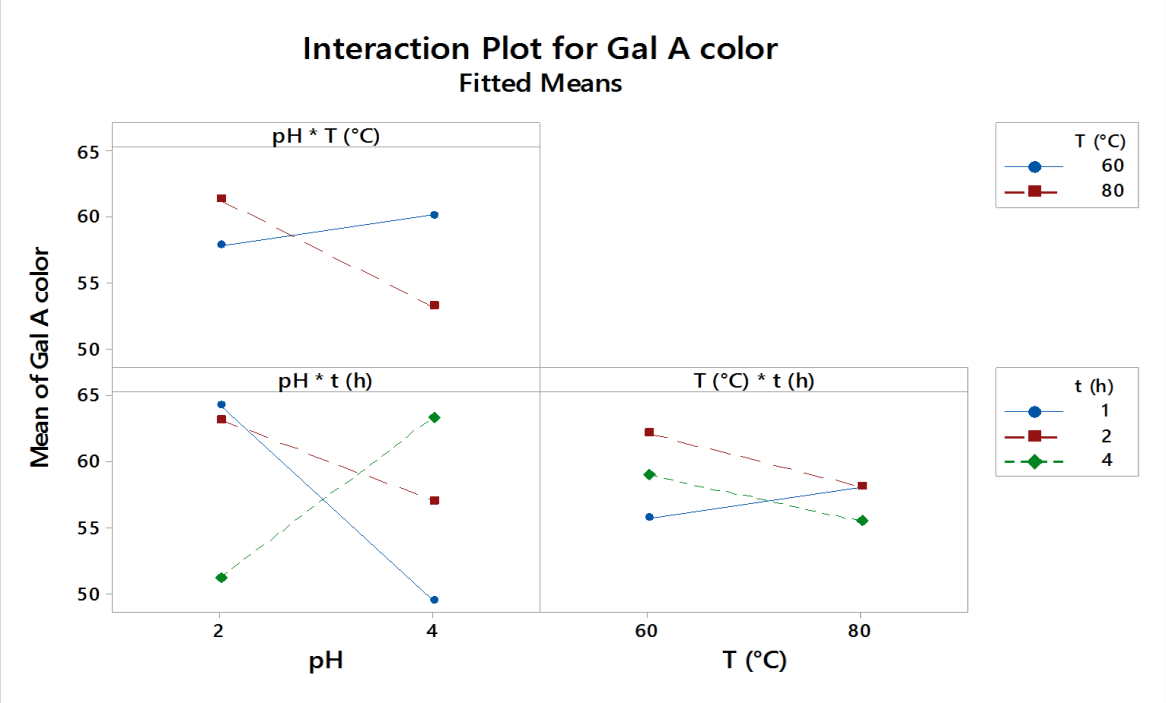


Figure 5.8: The interactions plots for galacturonic acid content (%) using a colourimetric assay. To visualize these effects, the Y-axis scale is always the same for each combination of factors. When the lines are parallel, interaction effects are zero.

Based on all these interpretations resulting from the factorial design, it is possible to say that under these specific conditions that the parameters at pH 2, temperature 80 ºC and an extraction time of 1 hour to give the highest/ optimum galacturonic acid content under the examined conditions.

5.1.5 Determination FT-IR spectra:

Infrared has been known as a strong analytical technique in the food industry for many years (Van de Voort, 1992). Furthermore, FT-IR is a fast, non-destructive tool, very appropriate for the study of edible oils, fats and carbohydrates requiring minimum sample preparation (Saucedo-Hernández et al., 2011).

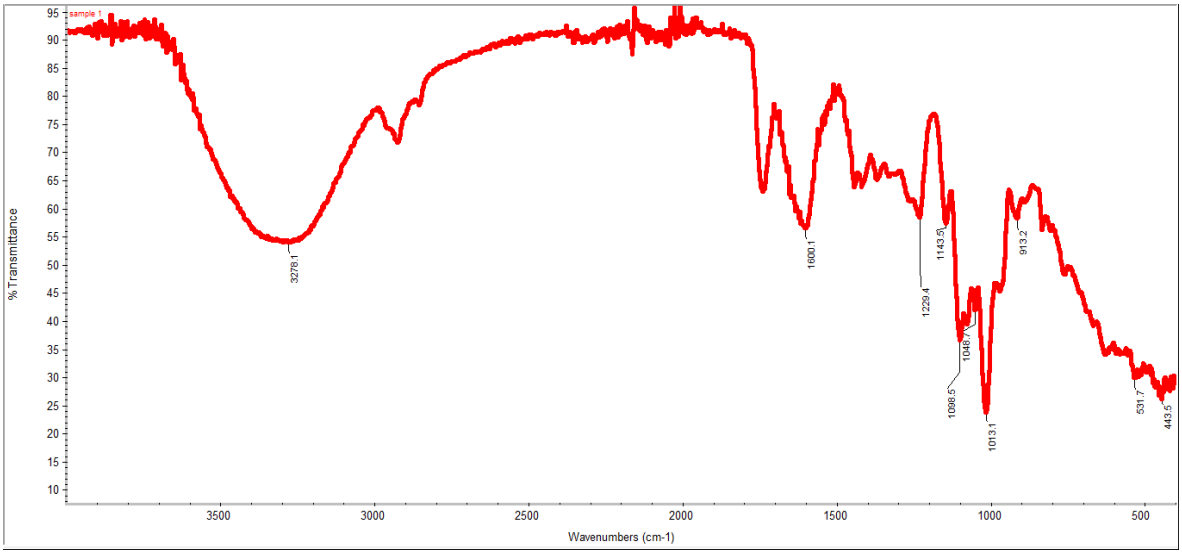
Example FT-IR spectra of pumpkin samples (1 and 13) are presented in **Figure 5.9.** The spectra for the pumpkin samples were examined and compared with spectral data from IR studies of pectins and other plant polysaccharides (Gnanasambandam and Proctor, 2000). However, the spectra of this pectin sample were close to those obtained by (Kamnev et al., (1998) for dry pulp pumpkin. **Table 5.6** indicates typical peaks for a number of functional groups expected to be present in pectins as shown in Jittra, (2004), Alba et al., (2015), Kamnev et al., (1998), Synytsya et al., (2003b). The broad, strong peak between 3500 and 2500 cm^{-1} refers to O-H stretching absorption and bands around 2950 cm^{-1} (3000-2800 cm^{-1}) refer to C-H absorption. Furthermore, the band at 1630 cm^{-1} corresponds to vibrations of the structure O=C−O while the band that appears at about 1740 cm^{-1} can be assigned to C=O stretching vibration of methyl esterified carboxylic group similarly as reported earlier (Gnanasambandam and Proctor, 2000, Jittra, 2004). All the absorption bands listed above were characteristic FTIR peaks of carbohydrate polymer and primarily indicated the polysaccharide nature of pumpkin.

Table 5.6: Frequencies and intensities of functional groups present on commercial pectin samples analysed by diffuse reflectance fourier transform infrared spectroscopy adapted from (Gnanasambandam and Proctor, 2000).

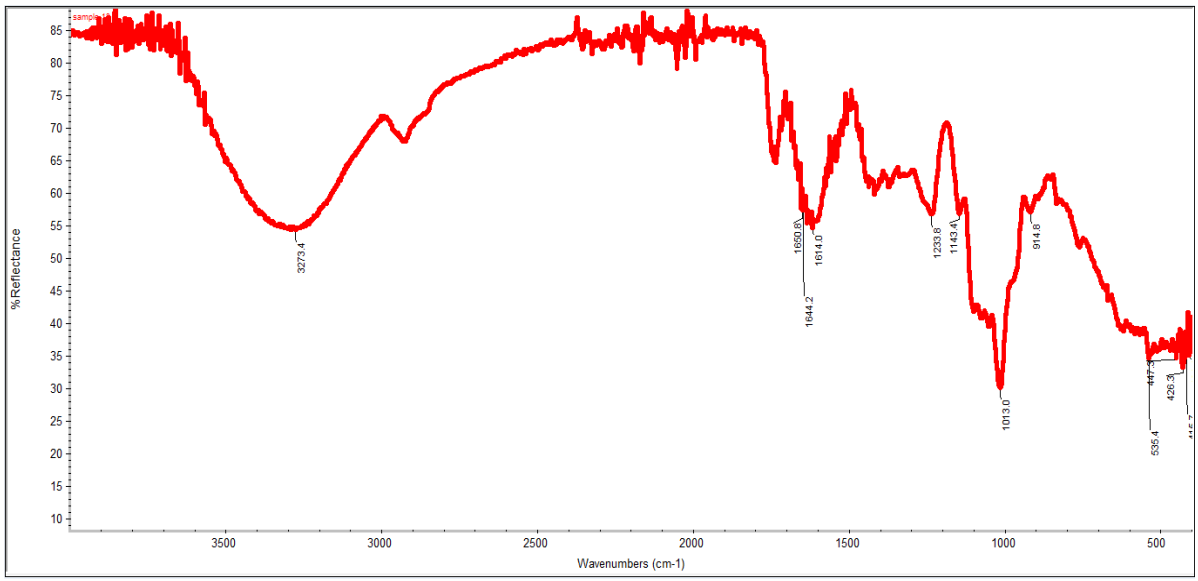
Frequency (wave number) cm^{-1}.	**Functional groups**	**Intensity**
1760 - 1745	C=O esterified	Strong
1640 - 1620	COO^- asymmetric stretching	Strong
3500 - 2500	O-H stretching	Broad, strong
3000-2800	C-H stretching, symmetric, asymmetric	Sharp, occasionally double overlapping with O-H

The full spectra are shown in **appendix 9.1.1** which exhibits the FT-IR spectra of pectin samples extracted under different conditions, where these spectra indicated that they are

similar. The FT-IR spectra contained several identifiable peaks as previously mentioned however now it is known that samples contain a larger proportion of GalA (**Table 5.5**), it is the peaks at 1630 and 1740 cm^{-1} which are of most interest for this study as they can be used to estimate the degree of esterification.



Sample (1)



Sample (13)

Figure 5.9: FT-IR spectra of some pumpkin pectin samples extracted under different extraction conditions *i.e.* samples (1) and (13).

5.1.5.1 Determination of degree of esterification:

DE is defined as " the amount of methyl-ester (mol) present per 100 mol of total galacturonic acids (free galacturonic acid and substituted ones)" (Guillotin et al., 2007). In theory, it can differ from 0 % to 100 % (Pilgrim et al., 1991).

Table 5.7: Indicates the percentage of the degree of esterification of pumpkin pectins using different extraction conditions.

Sample	pH	T (°C)	t (h)	DE %
1	4	60	1	$94 \pm 0.7^{a,b}$
2	4	60	2	92 ± 0.7^{b}
3	4	60	4	65 ± 0.7^{f}
4	2	60	1	87 ± 0.7^{c}
5	2	60	2	82 ± 0.7^{d}
6	2	60	4	87 ± 0.7^{c}
7	4	80	1	83 ± 0.7^{d}
8	4	80	2	86 ± 0.7^{c}
9	4	80	4	95 ± 0.7^{a}
10	2	80	1	96 ± 0.7^{a}
11	2	80	2	75 ± 0.7^{e}
12	2	80	4	61 ± 0.7^{g}
13	3	70	1.5	63 ± 0.7^{g}
14	3	70	3	67 ± 0.7^{f}

Values were given as means of triplicate ± SD. Means with different superscript letters within a column are significantly different (Tukey test, $p < 0.05$). SD = Standard deviation of the mean.

The results of triplicate FT-IR measurements to determine the DE in the sample polysaccharides are presented in **Table 5.7.** The degree of esterification was calculated from solid samples using IR spectroscopy using stretching bands centred on 1635 $cm^{-1}$and 1730 cm^{-1}.The degree of esterification (DE) was calculated using infra-red spectroscopy as the molar ratio of methanol (MeOH) and acetic acid (HAc) to galacturonic acid (GalA) (Levigne et al.,

2002). The degree of acetylation and methylation are both used to measure the degree of esterification where the degree of methylation (DM) is equal to the percentage of carboxyl groups esterified with methanol. Whereas, the degree of acetylation (DA) is a percentage of residues esterified with an acetyl group assuming that only the hydroxyl groups of residues were acetylated (Voragen et al., 1986).

The degree of esterification of pumpkin pectins was high when compared to citrus and beet pulp pectin (Morris et al., 2008, Mesbahi et al., 2005) and melon pectin (Denman and Morris, 2014). Moreover, they were higher than the values that already published (Lim et al., 2012) for Yuza pectin, and higher than those obtained by Urias-Orona et al., (2010) for commercial apple pectin 57 % and for chickpea husk pectin 10 %. The highest value of DE for pumpkin pectin was 96 % (this was high when compared with Levigne et al., (2002). This high value was found at pH 2 at high temperature 80 ºC and short extraction time 1 h. However, the lowest DE 61 % was obtained at pH 2 for 4 h at 80 ºC. These values were similar to those obtained for apple pomace pectin extracted at pH 2 at 80 ºC for 1 h (Garna et al., 2007). In addition, the DE values for pectin samples are close to the DE values for pectin samples extracted from peach pomace (Pagan et al., 1999).

. From data determined it is safe to conclude that there is a very high degree of methyl esterification of the galacturonic acid backbone in the pectin molecules that are present in the extracted samples. This would support the data previously measured colourimetrically for GalA content whereby there was a high concentration of galacturonic acid in all extracted samples. According to this data, it shows that the degree of esterification decreased significantly with increasing extraction times (**Figure 5.10**). The reason for this is thought to be the longer extraction time of the pectin during the extraction process contributes to de-esterification (Joye and Luzio, 2000), which also in agreement with results for pectin obtained from apple pomace (Marcon et al., 2005).

The experimental design approach allows a further understanding of the influence of each extraction condition and how they interact with one another. Therefore, the main (the largest) effect on the degree of esterification is time (**Figure 5.10**). The DE decreases moving from low level (1 hour) to high level (4 hours).

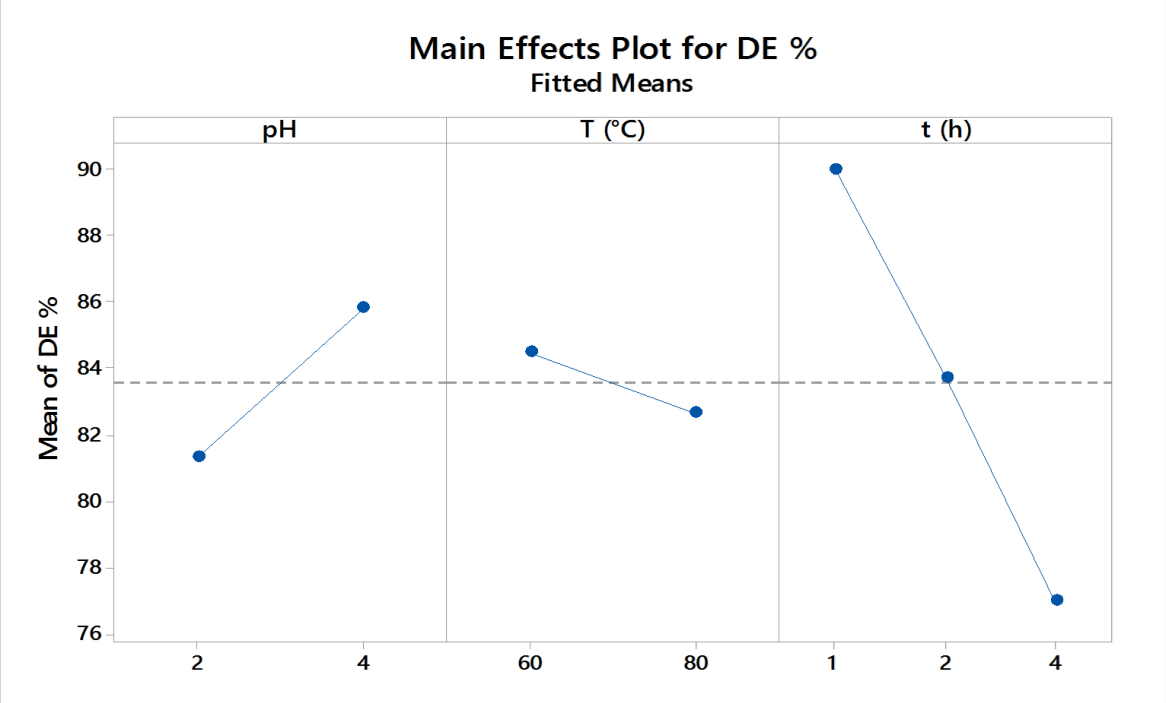


Figure 5.10: The main effect plots for the degree of esterification (%): pH, temperature and time. The overall mean (~83.6 %) is shown as a dotted line and the steeper the slope the greater the effect of a particular parameter.

In **Figure 5.11** the slopes for all three are non-parallel indicating there are interactions between the different extraction conditions, however, the interaction between pH and temperature and pH and time are the largest. The 2-factor interactions are 114.08, 94.50 and 37.17 for pH* temperature, pH*time and temperature*time, respectively. The 3-way (pH* temperature*time) interaction is 770.17 and is therefore very important.

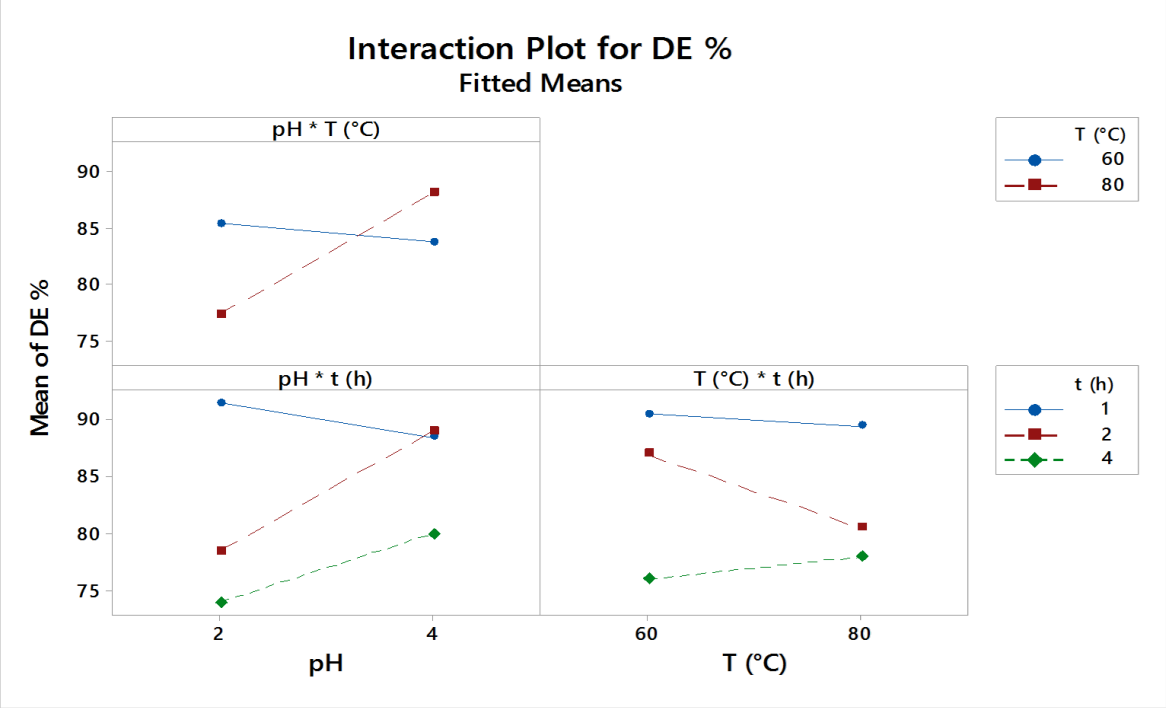


Figure 5.11: The interaction plots for the degree of esterification (%). To visualize these effects, the Y-axis scale is always the same for each combination of factors. When the lines are parallel, interaction effects are zero.

Based on all these interpretations resulting from the factorial design, it is possible to say that under these specific conditions that the parameters at pH 2, temperature 80 ºC and an extraction time of 1 hour to give the highest/ optimum DE under the examined conditions.

5.1.6 Determination of degree of acetylation:

The degree of acetylation of the samples is detailed in **Table 5.8.** It can be seen that the samples had very high degrees of acetylation which varied from 30.3 to 76.2 %. The highest value was obtained at pH = 4, 80 ºC and 1 h which is in agreement with previous studies on sugar beet and apple pomace pectins (Levigne et al., 2002a, Garna et al., 2007). These results can be explained by the hydrolysis of acetic acid groups from galacturonic acids under harsher extraction conditions.

Table 5.8: Displays the percentage degree of acetylation of pumpkin pectins using various extraction conditions.

Sample	pH	T (°C)	t (h)	DAc %
1	4	60	1	55.9 ± 0.1^{e}
2	4	60	2	48.4 ± 0.1^{h}
3	4	60	4	45.6 ± 0.3^{i}
4	2	60	1	34.7 ± 0.2^{l}
5	2	60	2	32.4 ± 0.5^{m}
6	2	60	4	$30.3 \pm .0.1^{n}$
7	4	80	1	76.2 ± 0.1^{a}
8	4	80	2	61.7 ± 0.6^{d}
9	4	80	4	53.9 ± 0.1^{f}
10	2	80	1	36.6 ± 0.1^{k}
11	2	80	2	38.6 ± 0.2^{j}
12	2	80	4	66.4 ± 0.7^{c}
13	3	70	1.5	68.8 ± 0.7^{b}
14	3	70	3	51.2 ± 0.3^{g}

Values were given as means of triplicate ± SD. Means with different superscript letters within a column are significantly different (Tukey test, $p < 0.05$). SD = Standard deviation of the mean.

DA values in this study are in agreement with DA values for pectin obtained by Voragen et al., (1986) and by Levigne et al., (2002a) using similar experimental designs. Results reported in studies on other pectins from, for example, Ambarella (4.2-5.5 %) and lime (1.2-1.6 %) apple

(5.0 %), lemon (1.4 %), cinnamon apple pomace and pumpkin pectins were lower when compared with DA values reported in this study (Koubala et al., 2008).

All pectin samples in this study have been found to be quite highly acetylated. In general, the degree of acetylation has a deep influence on the functional properties of pectin such as gelling or colloidal stabilizing ability and thus determine the possible applications in food formulations. Wherein, the acetyl group in pectin substances have a significant role on account of their effect on the gel-forming ability (Abdel-Rahman, 2002).

A previous study for okra pectin/calcium matrix at pH 3.0 (Alba, 2015) indicated that okra pectin is a non-gelling pectin this causes its inability for structuring which was due to the presence of a high amount of acetyl groups that intercept the formation of hydrogen bonds between the carboxyl groups of galacturonic acid and calcium ions. In addition, it has been reported that non-gelling acetylated pectin from sugar beet displays interfacial activity and could be used in colloidal dispersions as an emulsifying agent (Alba, 2015).

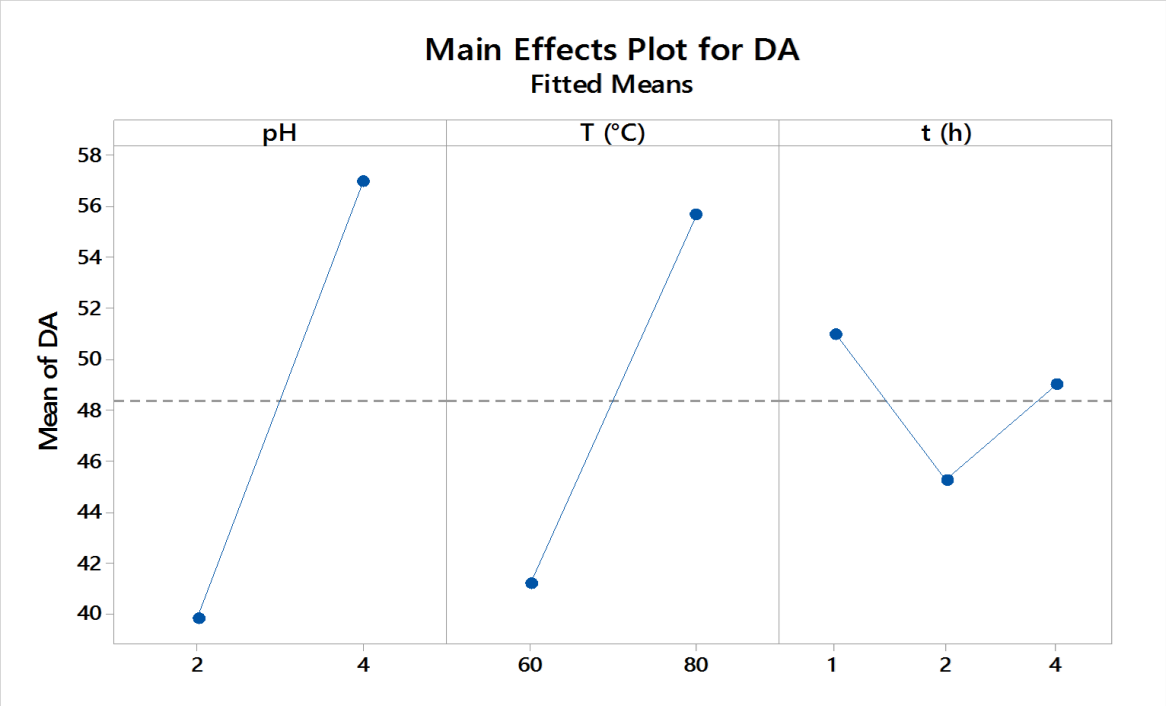


Figure 5.12: The main effect plots for the degree of acetylation (%): pH, temperature and time. The overall mean (~ 48.4 %) is shown as a dotted line and the steeper the slope the greater the effect of a particular parameter.

Again, through the experimental design approach, it is possible to gain some further understanding of the influence of each extraction condition and how they interact with one another. Therefore, the main (the largest) effect on the degree of acetylation is pH (**Figure 5.12**). The DA increases moving from a low level (pH 2) to high level (pH 4); temperature also has an important effect.

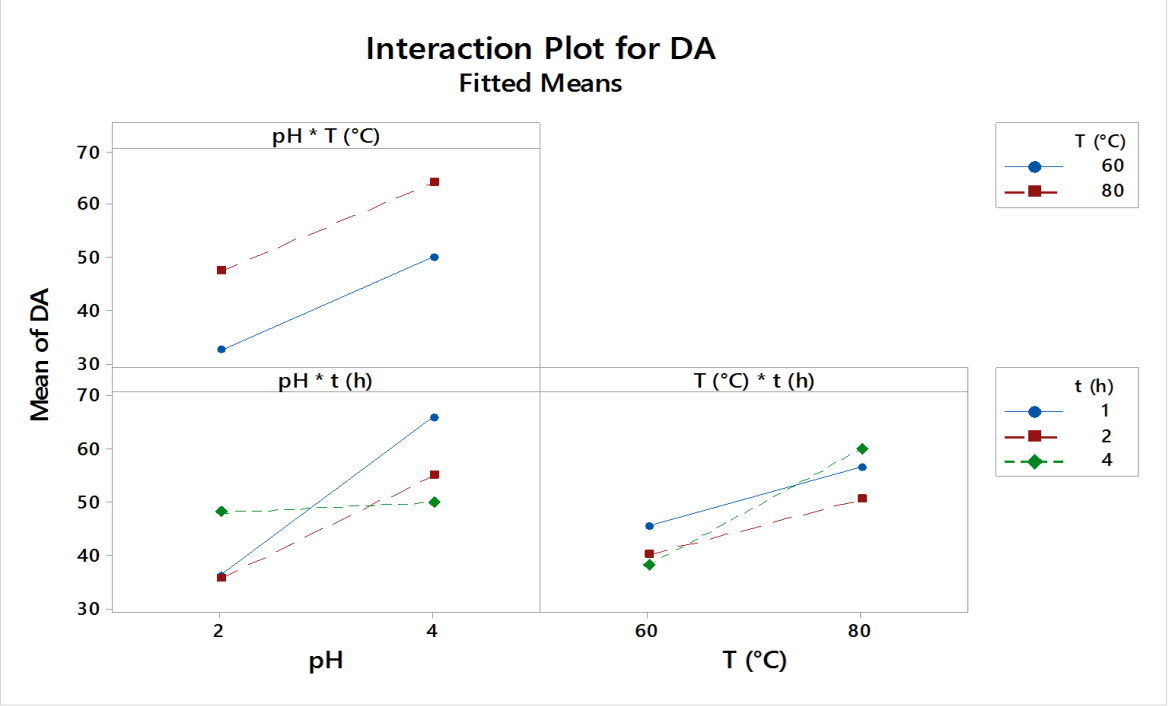


Figure 5.13: The interaction plots for the degree of acetylation (%). To visualize these effects, the Y-axis scale is always the same for each combination of factors. When the lines are parallel, interaction effects are zero.

Furthermore, the interaction plot reveals whether there is an interaction between two different extraction conditions for a certain response on the degree of acetylation. When the lines are parallel, interaction effects are zero as is the case for pH* temperature. The more different the slopes, the more influence the interaction effect has on the results (Israel et al., 2014). In **Figure 5.13** the slopes for pH*time and temperature*time are non-parallel indicating there are interactions between the different extraction conditions, however, the interaction between pH and time is the largest. The 2-factor interactions are 0.75, 200.08 and 84.50 for pH* temperature, pH*time and temperature*time, respectively. The 3-way (pH* temperature*time) interaction is 288.50 and is therefore also important.

Based on all these interpretations resulting from the factorial design, it is possible to say that under these specific conditions that the parameters at pH 4, temperature 80 ºC and an extraction time of 1 hour to give the highest/ optimum DA under the examined conditions.

5.1.7 Determination of constituent sugars composition:

The constituent sugars of the pumpkin samples were determined by HPAEC. The glycosidic bonds were hydrolysed and the samples compared to the appropriate standards. Standards were used based on literature for the most common saccharides present in the Cucurbitaceae family as shown in **Table 5.9** (in order of elution); rhamnose, arabinose, galactose, glucose, xylose and galacturonic acid. The procedure was performed in triplicate to reduce errors in analysis and to calculate relative response factors for the different monosaccharides. The result was used to determine molar percentages (mol %) for the different monosaccharides from the relative percentage peak areas as shown in **Table 5.9** below. The mean molar percentages (mol %) of the monosaccharides is shown in **Table 5.10**.

Table 5.9: Retention times and relative response factors (relative to xylose) for monosaccharide standards.

Standard	R.t Ave min	Area Ave nC*min	Relative response factor
Rha	4.3	5.2	0.20
Ara	4.6	22.6	0.86
Gal	5.6	17.0	0.65
Glc	6.2	22.7	0.86
Xyl	7.0	26.4	1.00
GalA	17.7	2.2	0.08

Table 5.10: Calculated concentrations of the constituent sugars mol % in pumpkin extracts.

Sample	Rha mol %	Ara mol %	Gal mol %	Glc mol %	Xyl mol %	GalA mol %
1	6 ± 0.1^{c}	4 ± 0.2^{e}	12 ± 0.2^{e}	6 ± 0.1^{c}	1 ± 0.1^{d}	$\mathbf{68 \pm 0.2^{d}}$
2	5 ± 0.2^{d}	9 ± 0.3^{a}	16 ± 0.1^{b}	6 ± 0.2^{c}	2 ± 0.2^{c}	63 ± 0.1^{i}
3	6 ± 0.1^{c}	4 ± 0.1^{e}	13 ± 0.3^{d}	7 ± 0.1^{b}	1 ± 0.3^{d}	$\mathbf{69 \pm 0.3^{c}}$
4	5 ± 0.2^{d}	2 ± 0.3^{f}	15 ± 0.3^{c}	5 ± 0.3^{d}	1 ± 0.2^{d}	$\mathbf{70 \pm 0.1^{b}}$
5	7 ± 0.2^{b}	2 ± 0.2^{f}	12 ± 0.1^{e}	6 ± 0.2^{c}	2 ± 0.1^{c}	$\mathbf{70 \pm 0.1^{b}}$
6	7 ± 0.1^{b}	2 ± 0.3^{f}	17 ± 0.3^{a}	8 ± 0.2^{a}	4 ± 0.2^{a}	62 ± 0.2^{j}
7	3 ± 0.2^{f}	9 ± 0.3^{a}	15 ± 0.2^{c}	8 ± 0.3^{a}	1 ± 0.3^{d}	64 ± 0.3^{h}
8	3 ± 0.2^{f}	7 ± 0.1^{c}	12 ± 0.2^{e}	7 ± 0.3^{b}	1 ± 0.1^{d}	$\mathbf{70 \pm 0.1^{b}}$
9	3 ± 0.1^{f}	6 ± 0.2^{d}	12 ± 0.1^{e}	6 ± 0.2^{c}	2 ± 0.2^{c}	$\mathbf{71 \pm 0.2^{a}}$
10	4 ± 0.3^{e}	8 ± 0.3^{b}	13 ± 0.2^{d}	8 ± 0.1^{a}	1 ± 0.3^{d}	$\mathbf{66 \pm 0.2^{f}}$
11	6 ± 0.1^{c}	2 ± 0.2^{f}	18 ± 0.1^{a}	5 ± 0.2^{d}	3 ± 0.2^{b}	$\mathbf{67 \pm 0.3^{e}}$
12	8 ± 0.2^{a}	6 ± 0.2^{d}	12 ± 0.3^{e}	8 ± 0.1^{a}	1 ± 0.1^{d}	$\mathbf{65 \pm 0.1^{g}}$
13	4 ± 0.1^{e}	8 ± 0.3^{b}	10 ± 0.3^{f}	6 ± 0.3^{c}	1 ± 0.3^{d}	$\mathbf{70 \pm 0.2^{b}}$
14	4 ± 0.3^{e}	6 ± 0.1^{d}	15 ± 0.2^{c}	8 ± 0.1^{a}	2 ± 0.1^{c}	$\mathbf{65 \pm 0.3^{g}}$

Values were given as means of triplicate ± SD. Means with different superscript letters within a column are significantly different (Tukey test, $p < 0.05$). SD = Standard deviation of the mean.

Galacturonic acid was the main component of the pectin, this was in an agreement with the FAO and EU which mentions that 'pectin' must consist of at least 65 % GalA (Willats et al., 2006), samples with > 65 % GalA have been highlighted in **bold** in **Table 5.10**. There are 11 of the 14 different extraction conditions which contain ≥ 65 % demonstrating that pumpkins are a good potential source of pectin. As well as GalA other important pectin monosaccharides (**Figure 2.8**) including Rha, Ara, Gal and Xyl were present together with some glucose which was present in low amounts (≤ 8 %) and was hence expected to be a ''contaminant'' from co-extracted starch and/or cellulose. **Table 5.5** shows the results from a colourimetric method, which gives a good indication that pectins are present, but the estimate of GalA will be influenced by the presence of interferences including neutral sugars.

It can be seen in **Table 5.10** that the pectins obtained in the present work had a medium to high galacturonic acid content which varied from 62 to 71 % which were similar to those extracted from yellow passion fruit (Yapo, 2009b). The highest value was obtained for sample 9 (71 %) which was extracted at pH 4 and 80 °C for 4 h, whereas the lowest value was obtained for sample **6** (62 %) which was extracted at pH 2 and 60 °C for 4h. The value of the latter is similar to that of pectin extracted from passion fruit peels using acetic acid: 62.5 % (Seixas et al., 2014b). The values for all pumpkin samples are lower than those for pectin from creeping fig: (77 – 87 %) (Liang et al., 2012a), pectins extracted from lime pectin (77.6 %) using HCl as the extraction solvent (Koubala et al., 2008), pectin extracted from passion fruit peels (Seixas et al., 2014b) using nitric acid (82 %), sugar beet pectin (Matora et al., 1995) and of commercial citrus pectin (75 %). In contrast, the values are higher than those published for pectins extracted from pumpkin (50 - 60 %) (Yoo et al., 2012a), sugar beet pectin. (Levigne et al., 2002a), ambarella pectin (55.7 %) using HCl as extraction method (Koubala et al., 2008) and pectins extracted from *Cucumis melo* (24 - 46 %) (Denman and Morris, 2014). However, they were in line with the finding for apple, citrus and sugar beet pectins (Ptitchkina et al., 1994, Savary and Nuñez, 2003) who reported (73.5 %), (72.1 %) and (64-72 %) respectivly. They are also comparable to those reported for pumpkin pectin (60 %) using hydrochloric acid as an extraction agent (Shkodina et al., 1998), grapefruit peel pectin (68.3 %) (Wang et al., 2016), cinnamon apple pectin (67 %) cinnamon apple pectin (67 %) and for chickpea husk pectin (67 %) (Urias-Orona et al., 2010).

An experimental design approach has the potential for further understanding of the influence of each extraction condition and how they interact with one another. Therefore, the main (the largest) effect on galacturonic acid content is pH (**Figure 5.14**). The GalA content increases moving from a low level (pH 2) to a high level (pH 4), although in this case, all the main effects are relatively small.

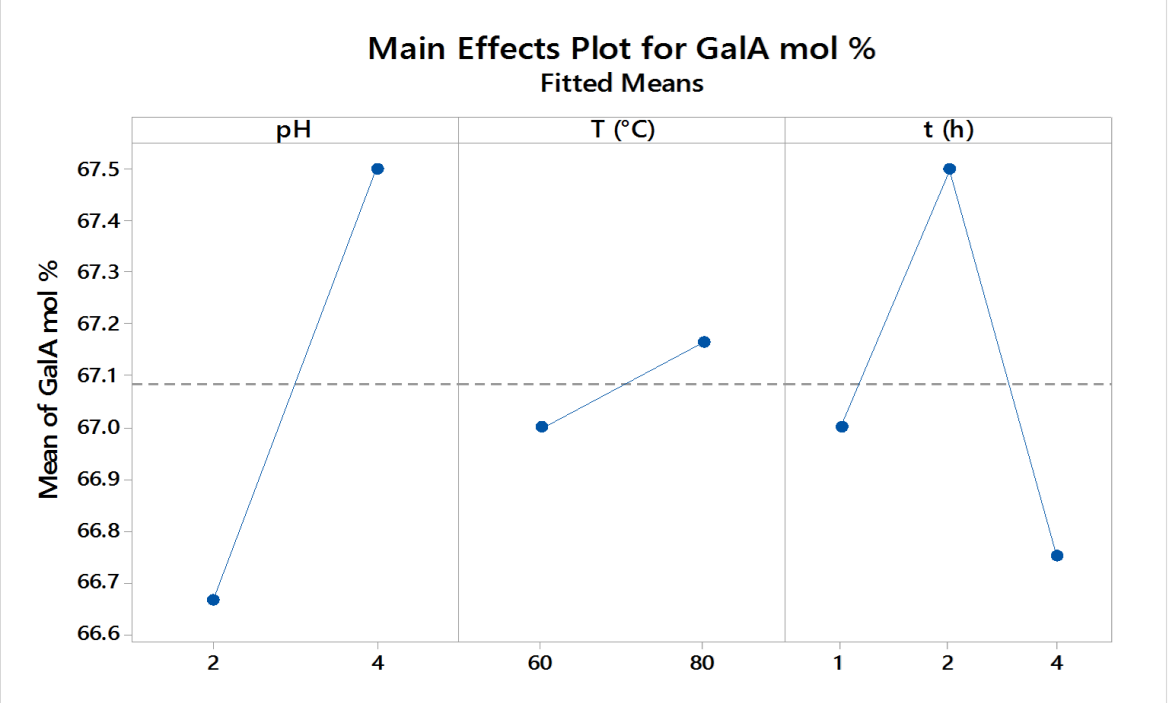


Figure 5.14: The main effect plots for galacturonic acid content (mol %): pH, temperature and time. The overall mean (~67.1 %) is shown as a dotted line and the steeper the slope the greater the effect of a particular parameter.

In **Figure 5.15** all the slopes are non-parallel indicating there are interactions between the different extraction conditions, however, the interaction pH and time is the largest. The 2-factor interactions are 6.75, 48.17 and 26.17 for pH* temperature, pH*time and temperature*time, respectively. The 3-way (pH* temperature*time) interaction is 18.50.

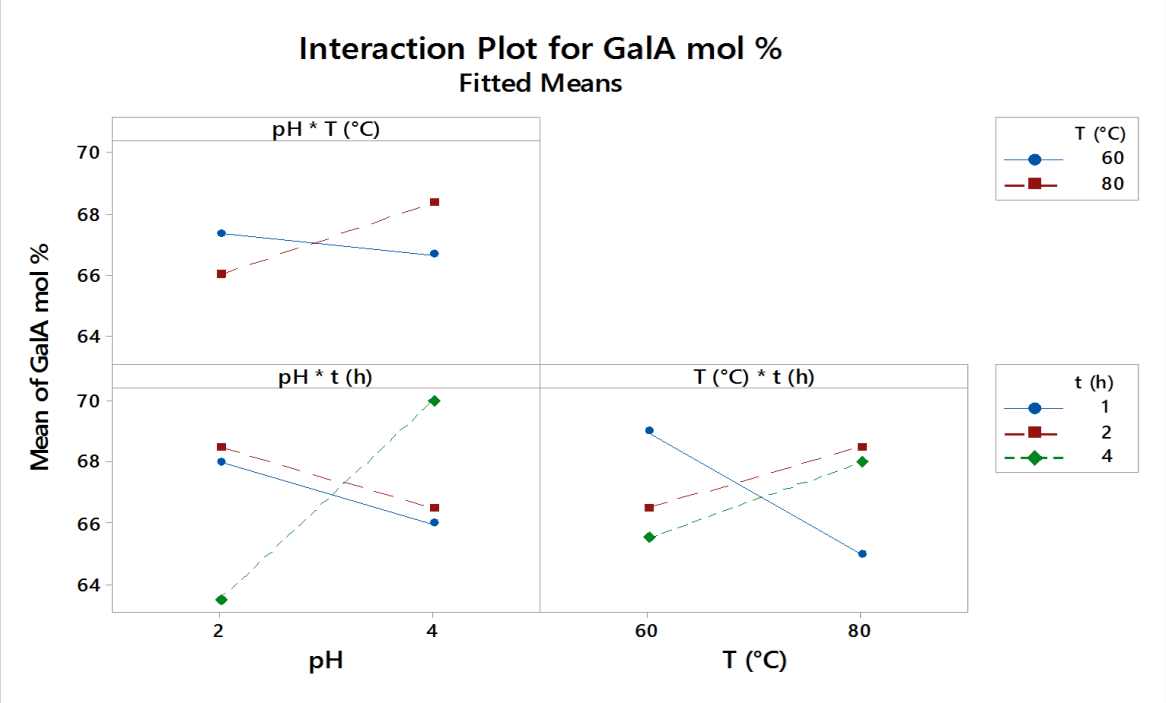


Figure 5.15: The interaction plots for galacturonic acid content (mol %). To visualize these effects, the Y-axis scale is always the same for each combination of factors. When the lines are parallel, interaction effects are zero.

Based on all these interpretations resulting from the factorial design, it is possible to say that under these specific conditions that the parameters at pH 4, temperature 80 ºC and an extraction time of 4 hours give the highest/ optimum GalA content under the examined conditions.

Furthermore, five more neutral sugars besides GalA are displayed in **Table 5.10** for all pumpkin pectin samples used in this study.

1. Rhamnose content: In this study the Rha contents ranged from 3 – 8 %;, these values of Rha are similar to those for pectin extracted from *Cucumis melo* (Denman and Morris, 2014), pumpkin and sugar beet pectin (Zhemerichkin and Ptitchkina, 1995). However, they are higher than in another study for pumpkin pectins using different extractive agents (Yoo et al., 2012b). The values are close to those for commercial citrus pectin (Shkodina et al., 1998) and grapefruit peel (Wang et al., 2016). The highest value of Rha content was obtained for sample 12 (8 %) when the AIR was treated at pH 2 and 80 °C for 4 h while the lowest amounts of Rha where obtained when the pectin treated at pH 4 and 80 °C irrespective of extraction time. This might suggest that a high pH (low acid concentration) the extraction medium is not able to solubilise

rhamnose rich fractions. Rha content for some pumpkin samples at pH 2 higher than at pH 4, this has been suggested before in the extraction of *Cucumis melo* pectin (Denman and Morris, 2014) also in the extraction of sugar beet pectin (Levigne et al., 2002a). Hence, the samples extracted at pH 2 are rich in rhamnogalacturonan whereas those extracted at pH 3 and 4 are rich in homogalacturonan (Denman and Morris, 2014).

Using an experimental design approach enables some further understanding of the influence of each extraction condition and how they interact with one another. Therefore, the main (the largest) effect on rhamnose content is pH (**Figure 5.16**). The Rha content decreases moving from a low level (pH 2) to a high level (pH 4).

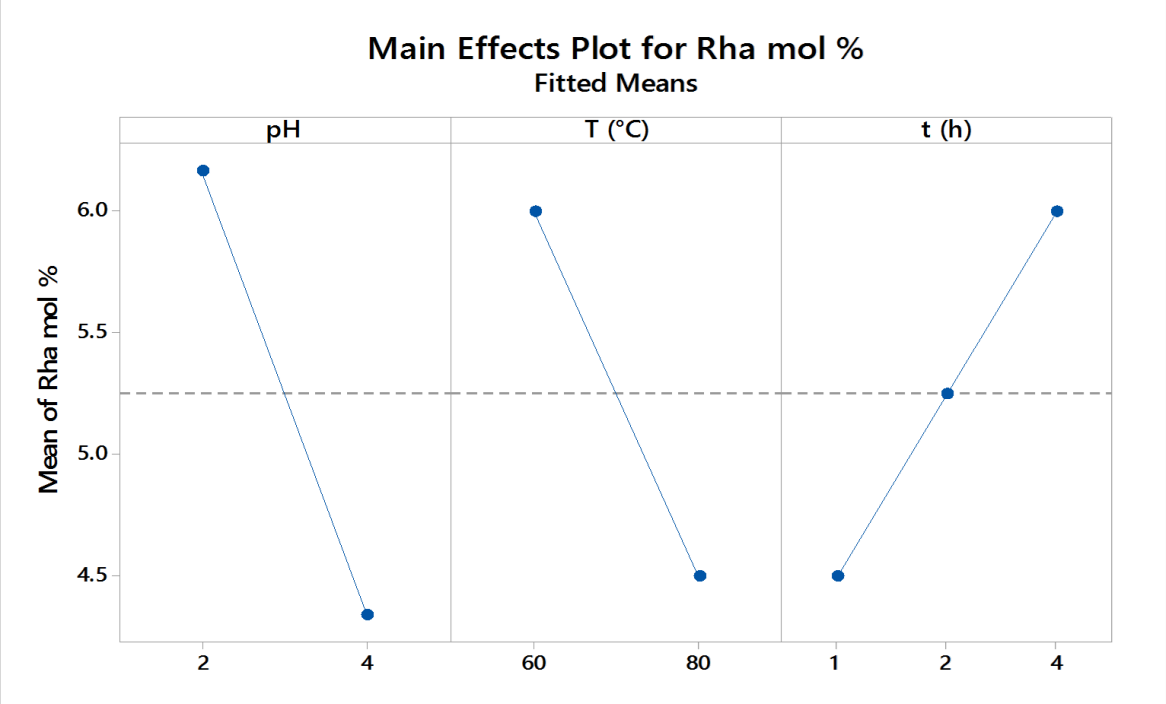


Figure 5.16: The main effect plots for rhamnose content (mol %): pH, temperature and time. The overall mean (~5.3 %) is shown as a dotted line and the steeper the slope the greater the effect of a particular parameter.

In **Figure 5.17** the slopes for pH*Temperature and pH*time are non-parallel indicating there are interactions between the different extraction conditions, however, the interaction pH and time is the largest. The 2-factor interactions are 4.08, 5.17 and 0.5 for pH* temperature, pH*time and temperature*time, respectively. The 3-way (pH* temperature*time) interaction is 1.17.

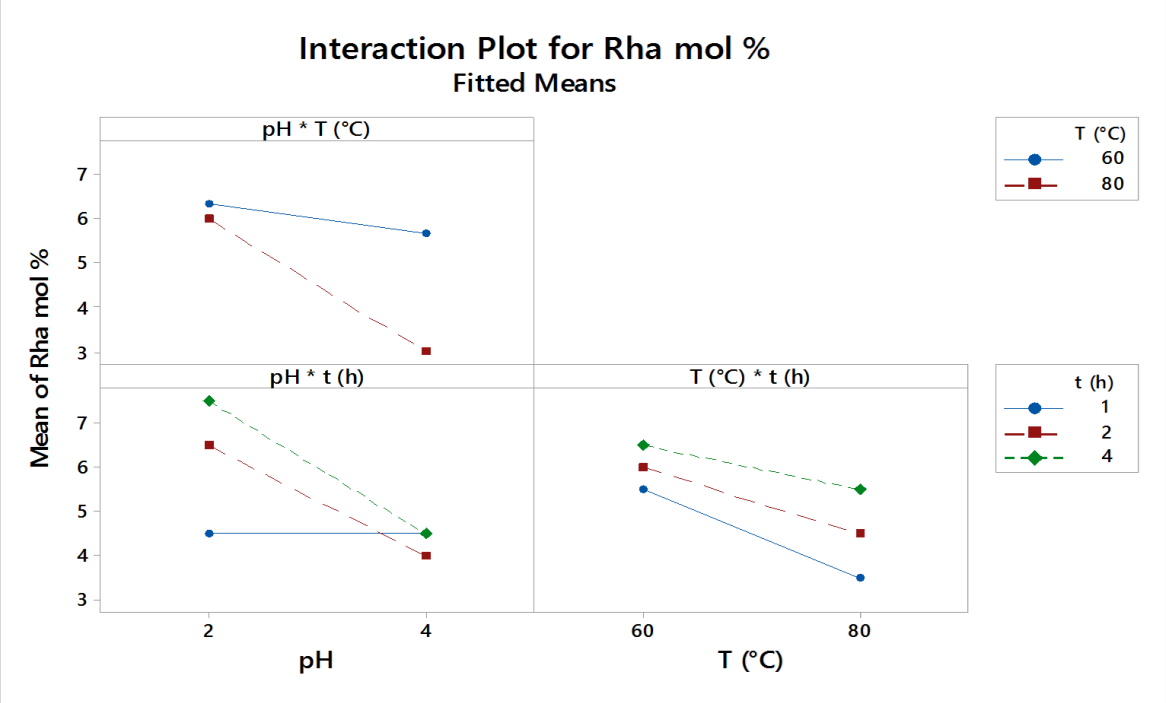


Figure 5.17: The interaction plots for rhamnose content (mol %). To visualize these effects, the Y-axis scale is always the same for each combination of factors. When the lines are parallel, interaction effects are zero.

2. Arabinose content, which is varied from 2 to 9 % of AIR apparently in the same range of Rha content, it is higher than the content of Ara that previously published for pumpkin pectin using different extraction methods (Shkodina et al., 1998). The highest Ara content value 9 % was obtained at pH 4. signifying arabinose content of pectin increases with pH increase. This is indicative of different populations of pectins being extracted under different conditions (Denman and Morris, 2014). In comparison with time and temperature, there is some variation in the content of the Ara for all pumpkin samples. These values are generally lower than those obtained for pectin extracted from *Cucumis melo* (Denman and Morris, 2014). Nevertheless Ara content appeared in similar to the Ara content that reported in (Zhemerichkin and Ptitchkina, 1995).

Experimental design approaches enable a further understanding of the influence of each extraction condition and how they interact with one another. Therefore, the main (the largest) effect on arabinose content is pH (**Figure 5.18**), although the temperature is also important. The Ara content increases moving from a low level (pH 2) to a high level (pH 4) this is consistent with Ara being an acid labile monosaccharide (Denman and Morris, 2014).

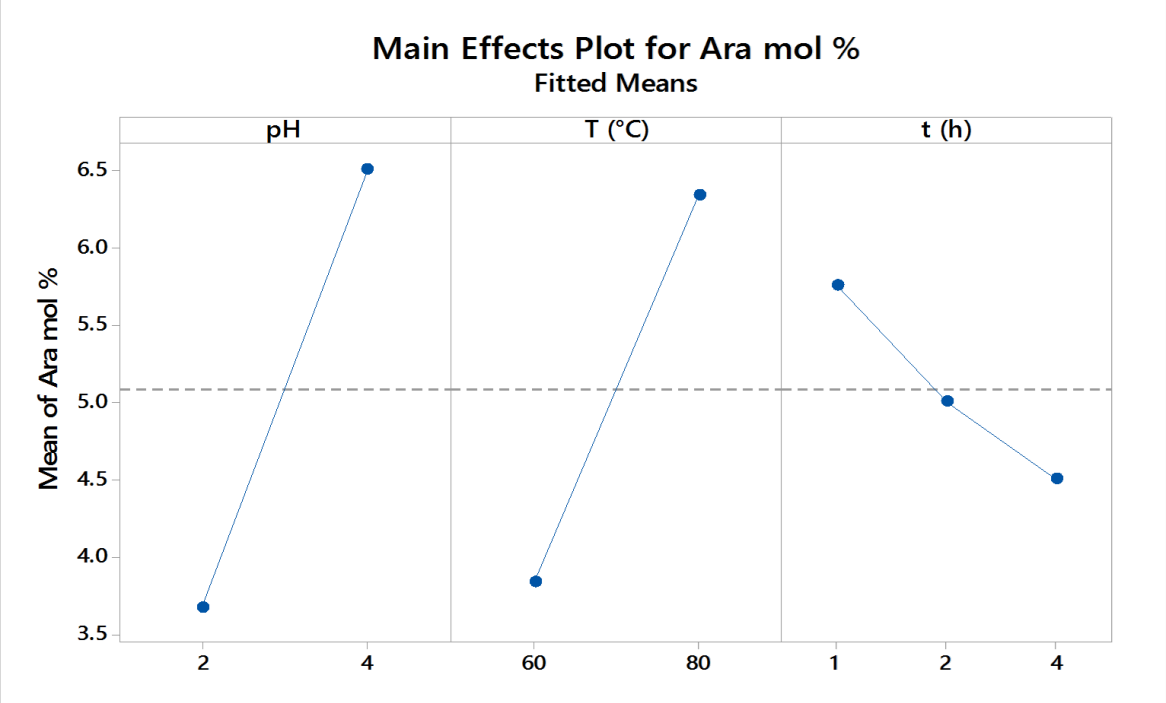


Figure 5.18: The main effect plots for arabinose content (mol %): pH, temperature and time. The overall mean (~5.1 %) is shown as a dotted line and the steeper the slope the greater the effect of a particular parameter.

In **Figure 5.19** the slopes for all the plots are non-parallel indicating there are interactions between the different extraction conditions, however, the interaction temperature and time is the largest. The 2-factor interactions are 2.08, 15.17 and 21.50 for pH* temperature, pH*time and temperature*time, respectively. The 3-way (pH* temperature*time) interaction is very small (0.17).

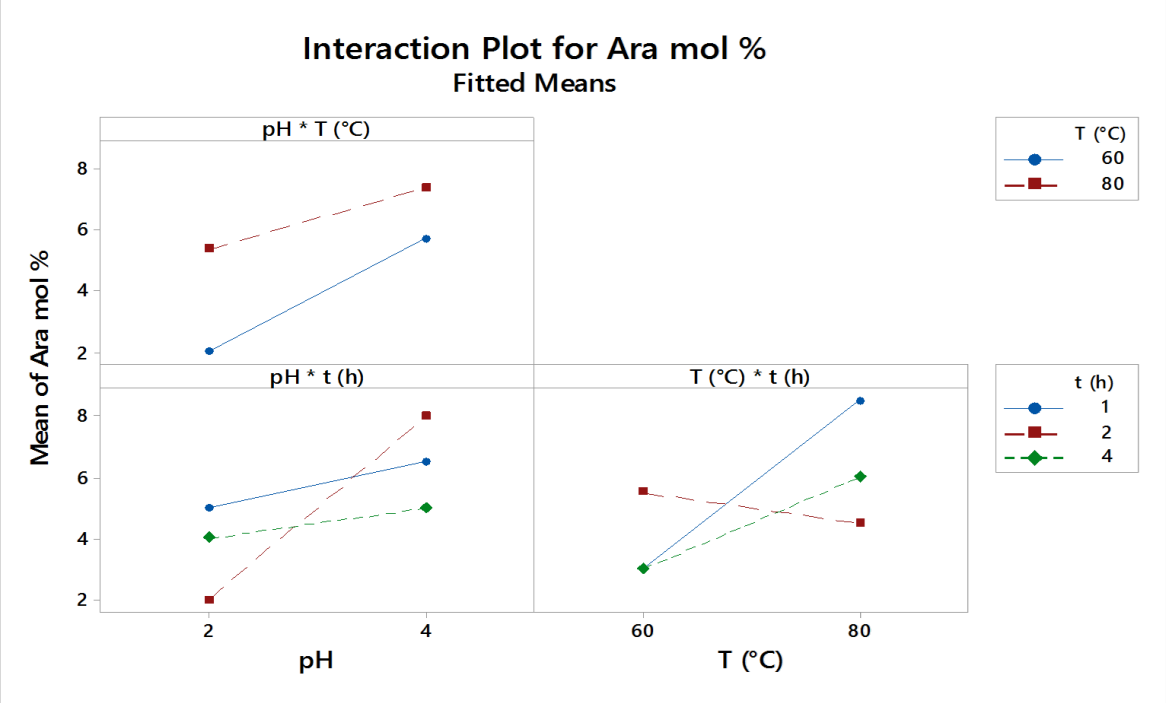


Figure 5.19: The interactions plots for arabinose content (mol %). To visualize these effects, the Y-axis scale is always the same for each combination of factors. When the lines are parallel, interaction effects are zero.

3. Galactose content - according to **Table 5.10** the galactose values range from 10 to 18 %; the highest value was obtained when the pectin was extracted at pH 2 and 80 ˚C for 2 h, while the lowest value was obtained at pH 3 and 70 ˚C for 1.5 h. These appear to be similar to values for pumpkin using hydrochloric acid as an extractive agent (Shkodina et al., 1998) and sugar beet pectins (Zhemerichkin and Ptitchkina, 1995). These values for galactose are much higher than for pumpkin pectin using different extraction methods (3-5 %) reported in (Yoo et al., 2012b) and for commercial citrus pectin (4 %) (Shkodina et al., 1998). In comparison to the values obtained for pectin extracted from *Cucumis melo* (16 to 40 %) (Denman and Morris, 2014) there are some samples (D and H) which are close to those reported in this study (sample 2, sample 11). However, the other samples in this study seem much lower in galactose content than those in Denman and Morris, (2014). The levels of arabinose and galactose suggest the presence of galactans, arabinans, arabinogalactans and/or rhamnogalacturonan side chains (Urias-Orona et al., 2010).

Adoption of an experimental design approach enables further understanding of the influence of each extraction condition and how they interact with one another. Therefore, the main (the

largest) effect on galactose content is pH (**Figure 5.20**). The Gal content decreases moving from a low level (pH 2) to a high level (pH 4).

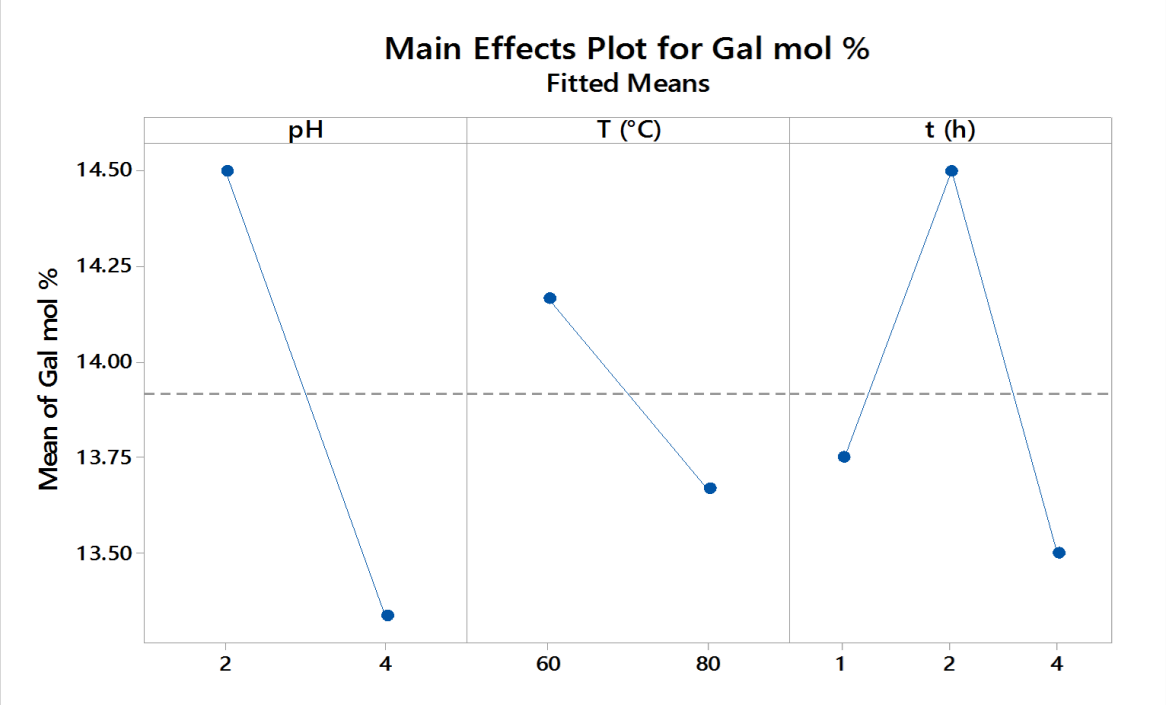


Figure 5.20: The main effect plots for galactose content (mol %): pH, temperature and time. The overall mean (~13.9 %) is shown as a dotted line and the steeper the slope the greater the effect of a particular parameter.

In **Figure 5.21** the slopes for pH*time and temperature*time are non-parallel indicating there are interactions between the different extraction conditions, however, the interaction between temperature and time is the largest. The 2-factor interactions are 0.08, 1.17 and 9.50 for pH* temperature, pH*time and temperature*time, respectively. The 3-way (pH* temperature*time) interaction is large (35.17).

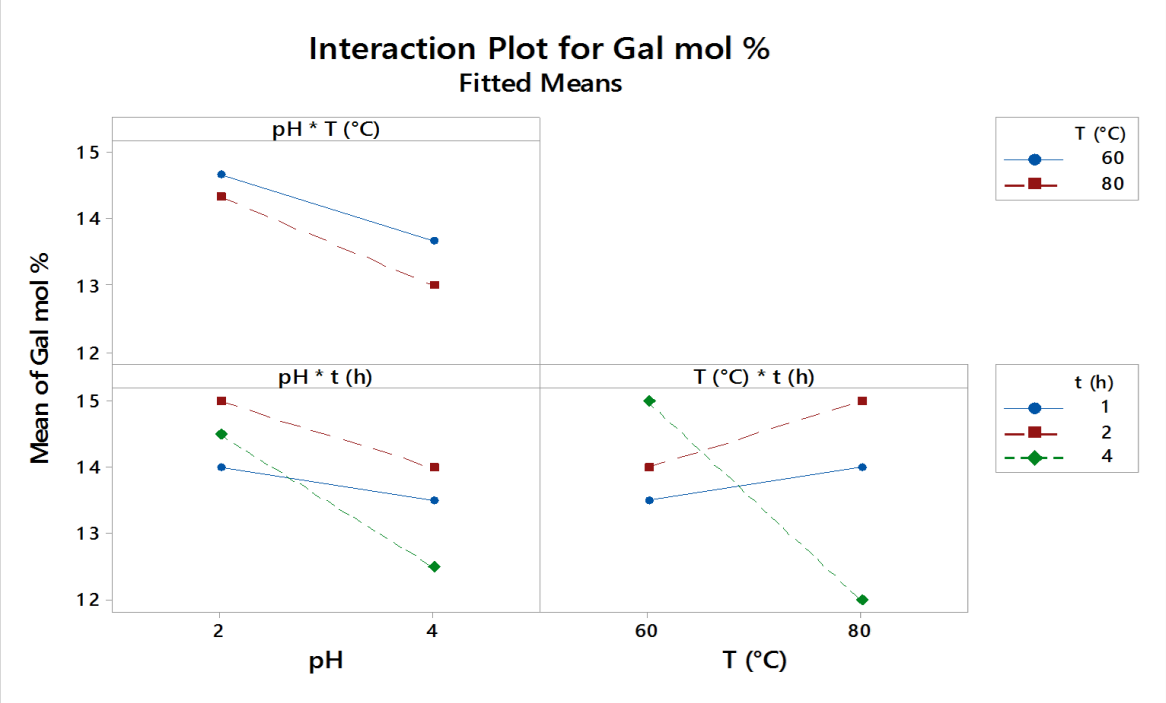


Figure 5.21: The interaction plots for galactose content (mol %). To visualize these effects, the Y-axis scale is always the same for each combination of factors. When the lines are parallel, interaction effects are zero.

4. Glucose content varied from 5 to 8 %, these values are higher than the glucose content reported for commercial citrus pectin (Shkodina et al., 1998). Furthermore, the values were generally lower than those obtained for pectins extracted from pumpkin and melon (Denman and Morris, 2014, Yoo et al., 2012b, Dos-Santos et al., 2011).

Employment of an experimental design approach facilitates further understanding of the influence of each extraction condition and how they interact with one another. Therefore, the main (the largest) effect on glucose content is time (**Figure 5.22**). In general, the Glc content increases moving from low level (1 hour) to high level (4 hours).

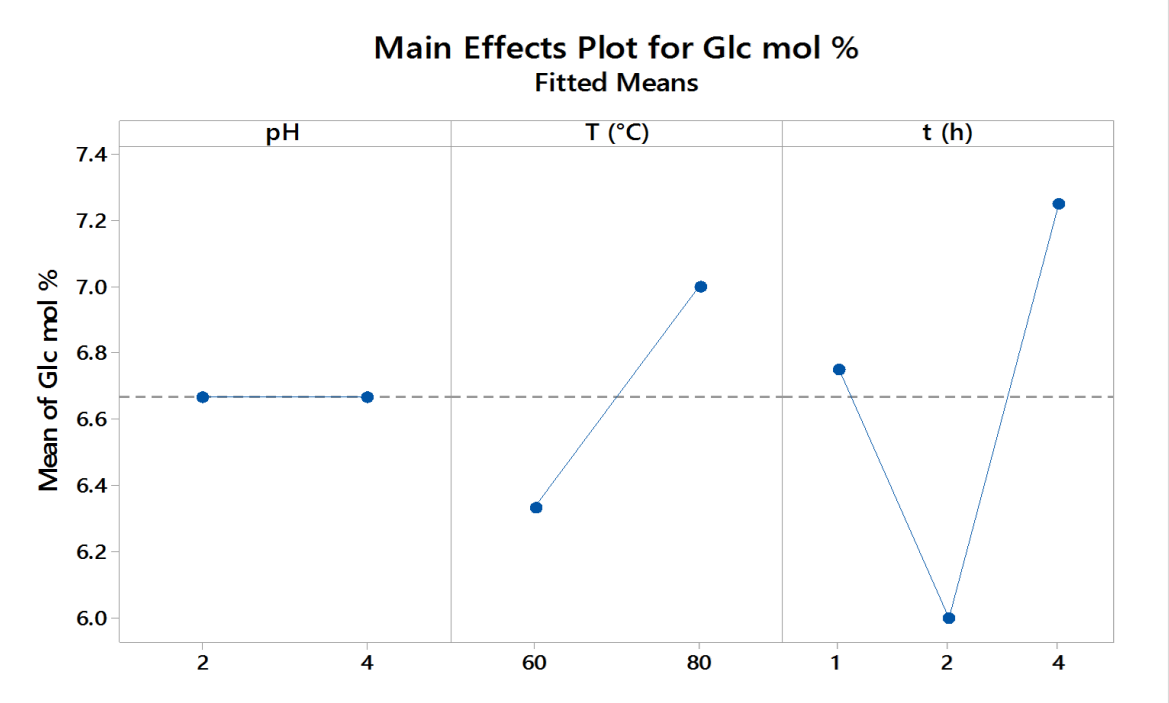


Figure 5.22: The main effect plots for glucose content (mol %): pH, temperature and time. The overall mean (~6.7 %) is shown as a dotted line and the steeper the slope the greater the effect of a particular parameter.

In **Figure 5.23** the slopes for pH*time and temperature*time are non-parallel indicating there are interactions between the different extraction conditions, however, the interaction between temperature and time is the largest. The 2-factor interactions are 0.00, 3.50 and 5.17 for pH* temperature, pH*time and temperature*time, respectively. The 3-way (pH* temperature*time) interaction is 1.50.

Based on all these interpretations resulting from the factorial design, it is possible to say that under these specific conditions that the parameters at pH 2, temperature 80 ºC and an extraction time of 2 hours to give the lowest/ optimum Glc content. (Glucose is a contaminant probably co-extracted starch and/or cellulose and therefore it would be beneficial to minimise the amount under the examined conditions).

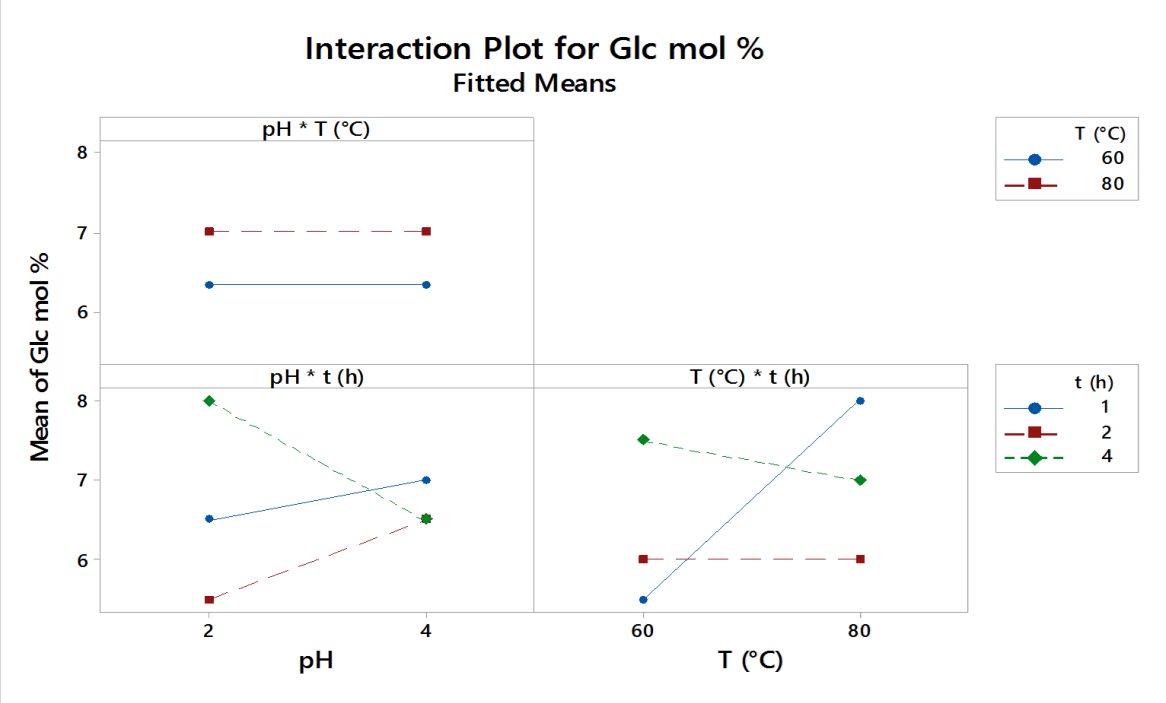


Figure 5.23: The interactions plots for glucose content (mol %). To visualize these effects, the Y-axis scale is always the same for each combination of factors. When the lines are parallel, interaction effects are zero.

5. Another constituent sugar of the side chain of pectin was also identified - **x**ylose which was present in low amounts (1 - 4 %), the lowest of all the extracts. By comparison with other studies (Yoo et al., 2012a) and (Denman and Morris, 2014) it is quite high, but lower than those estimated by Dos-Santos et al., (2011). Values are in the same range as those from pectins from pumpkins and sugar beets (Zhemerichkin and Ptitchkina, 1995).

The undertaking of an experimental design approach further understanding of the influence of each extraction condition and how they interact with one another. Therefore, the main (the largest) effect on xylose content was time (**Figure 5.24**). In general, the xylose content increased moving from low level (1 hour) to high level (4 hours), although there is little change after 2 hours.

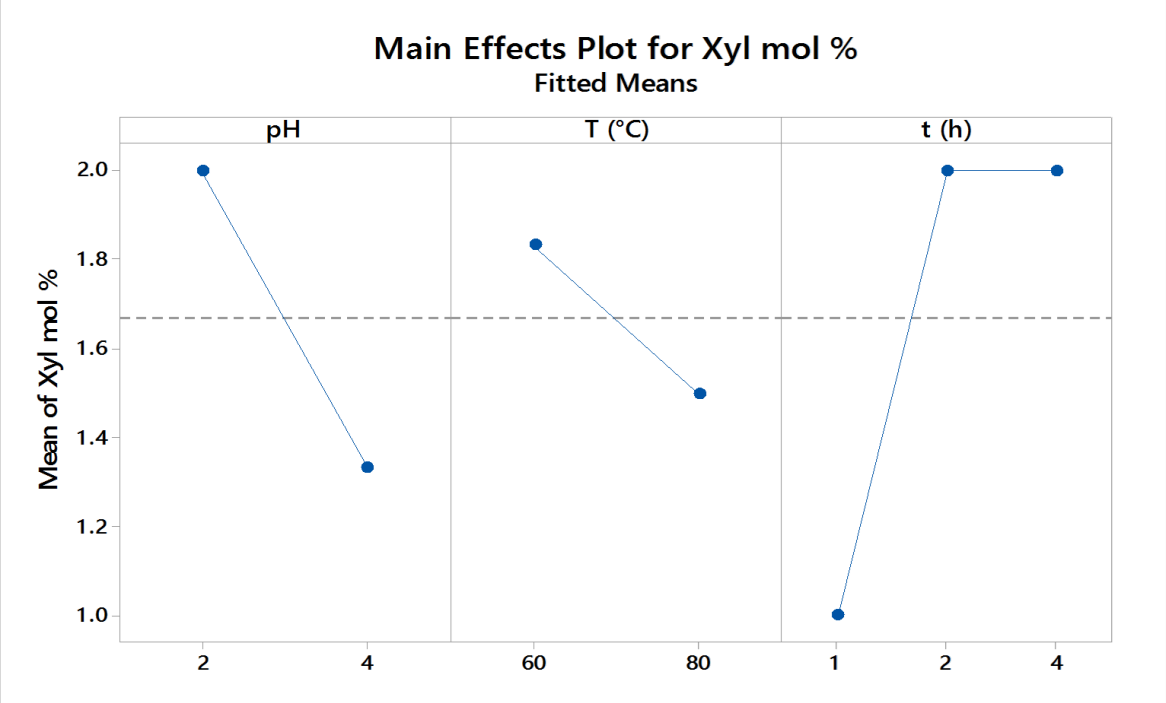


Figure 5.24: The main effect plots for xylose content (mol %): pH, temperature and time. The overall mean (~1.7 %) is shown as a dotted line and the steeper the slope the greater the effect of a particular parameter.

In **Figure 5.25** the 2-factor interaction for all the factors are less than 1. The 3-way (pH* temperature*time) interaction is 4.67.

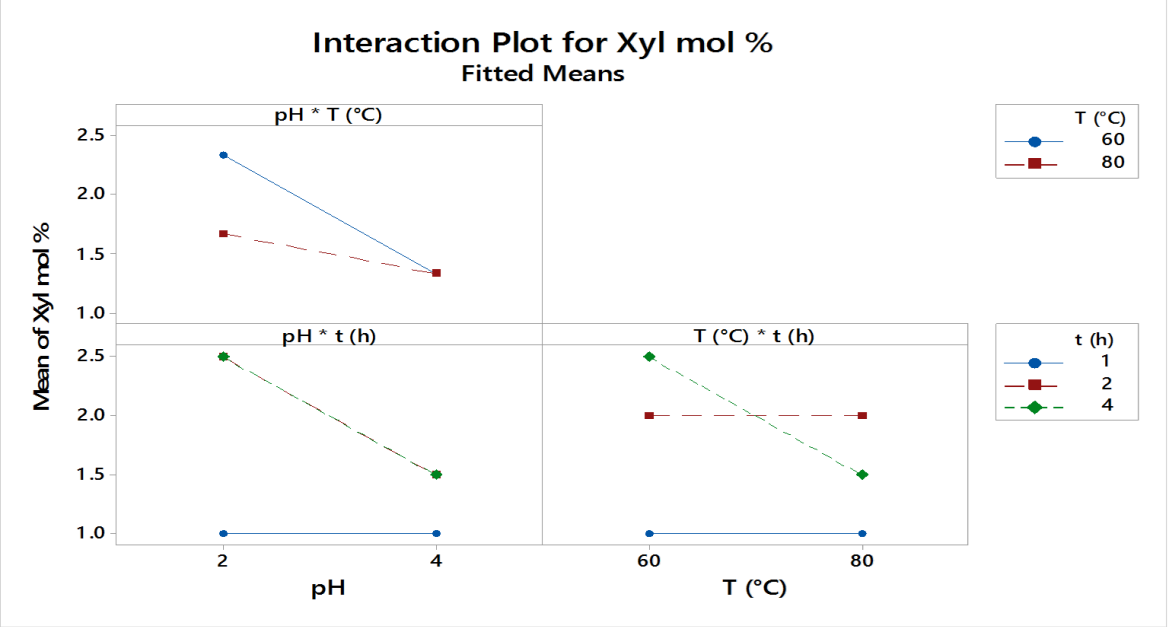
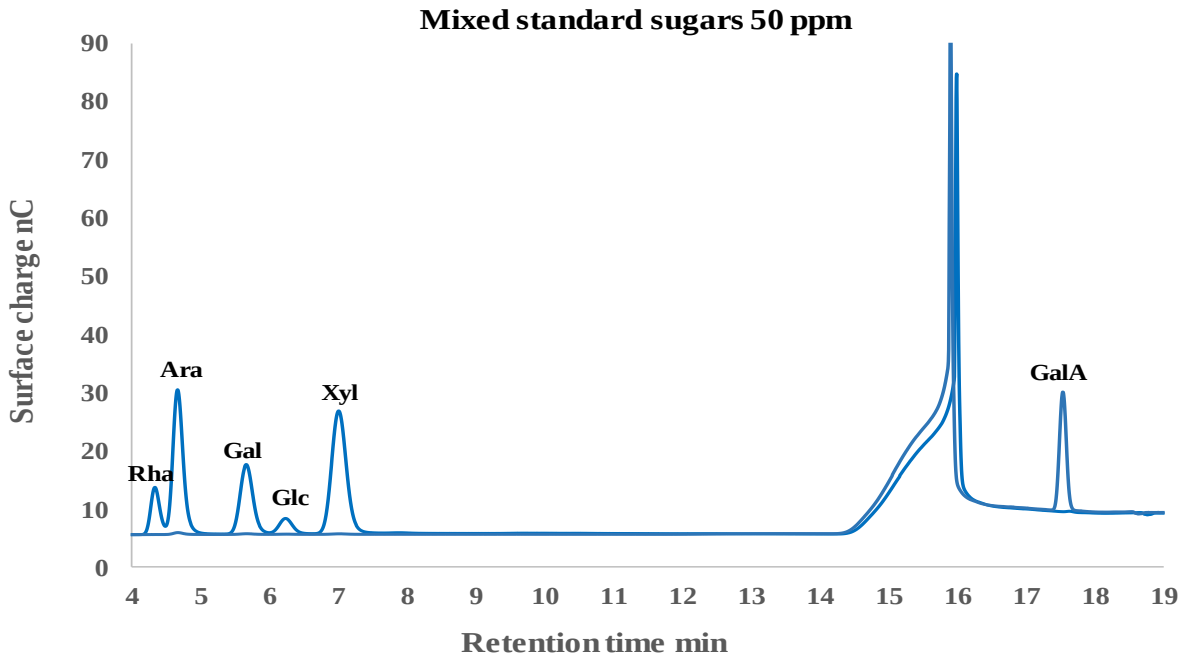


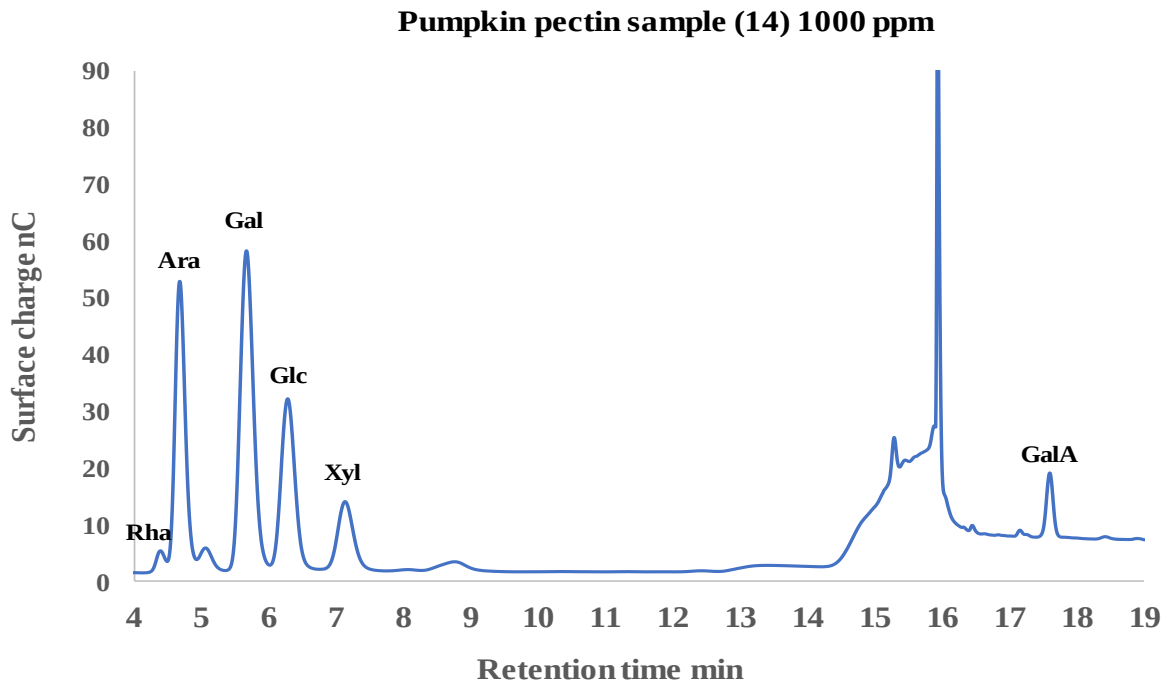
Figure 5.25: The interaction plots for glucose content (mol %). To visualize these effects, the Y-axis scale is always the same for each combination of factors. When the lines are parallel, interaction effects are zero.

The presence of rhamnose, galactose, and arabinose in the pumpkin pectin fractions is indicative of the presence of rhamnogalacturonan I with side chains, such as arabinans, galactans, and arabinogalactans (Tamaki et al., 2008, Wu et al., 2013, Cui and Chang, 2014). The presence of glucose and xylose in the pumpkin pectin fractions could explain the presence of xyloglucan as one of the side chains and the presence of xylogalacturonan (Fissore et al., 2007, Košťálová et al., 2013, Vincken et al., 2003b, Cui and Chang, 2014).

In this study, high content of galacturonic acid and a low proportion of neutral sugars of the polysaccharide strongly suggests that the extracted pectins may have the unique gelling capability (Liang et al., 2012a). Each constituent sugar gave individual peaks on the chromatogram due to their differing retention times (**Figure 5.26**).



Mixture of standards



Pumpkin pectin sample (14)

Figure 5.26: Peaks for a mixture of standards and for each monosaccharide present in some pumpkin samples *i.e*. sample (14) using HPAEC.

5.1.8 Calculating ratios between certain component monosaccharides:

The ratios between the composition sugars can be used to gain information on the polymeric level, which could be useful as an interpretation for the sugar composition data. In this study, eight 'sugar ratios' were defined to utilize as a term for the appearance and properties of conformed cell wall polysaccharides from sugar composition data, see **Table 5.11** in (Houben et al., 2011).

Ratio No	Sugar ratio	Property
1	$\frac{\text{GalA}}{\text{Rha} + \text{Ara} + \text{Gal}}$	A larger value is indicative of more linear/less branched pectins.
2	$\frac{\text{Rha}}{\text{GalA}}$	A smaller value is indicative of more linear/less branched pectins
3	$\frac{\text{Ara} + \text{Gal}}{\text{Rha}}$	A larger value is indicative of the larger average size of the branching side chains.
4	$\frac{\text{Gal}}{\text{Rha}}$	A larger value is indicative of the larger average size of the branching side chains excluding arabinose.
5	$\frac{\text{Rha} + \text{Ara} + \text{Gal} + \text{GalA}}{\text{Glc}}$	A larger value is indicative of a "more pure" pectin extract.
6-8	$\frac{\text{GalA}}{\text{Ara}}, \frac{\text{Gal}}{\text{Ara}}, \frac{\text{Rha}}{\text{Ara}}$	A larger value is indicative of more severe extraction conditions and loss of arabinofuranoside (Ara*f*) residues.

Table 5.11: Sugar ratios and property based on sugar composition data. For more details see for example (Alba, 2015, Denman and Morris, 2014).

The monosaccharide composition determined in this study can be used to calculate the sugar ratios of pectin (Houben et al., 2011) to detect information concerning the pectin structure **Table 5.12**.

Table 5.12: Composition ratios and pectin region % based on the mol % quantifiable neutral sugars and galacturonic acid.

Sample	Ratio 1	Ratio 2	Ratio 3	Ratio 4	Ratio 5	Ratio 6	Ratio 7	Ratio 8	% HG	% RG-I	HG: RG-I
	Linearity of pectin	Contribution of RG	Branching of RG		Co-extractants	Severity of extraction					
1	$2.9 \pm 0.3^{a,b}$	$0.09 \pm 0.1^{c,d,e}$	2.7 ± 0.1^{g}	$2.0 \pm 0.1^{e,f}$	$15.0 \pm 0.2^{b}15$	17.0 ± 0.2^{d}	3.0 ± 0.1^{d}	1.5 ± 0.1^{d}	62 ± 0.6^{c}	$28 \pm 0.2^{e,f}$	$2.2 \pm 0.1^{d,e}$
2	1.9 ± 0.1^{c}	$0.08 \pm 0.2^{d,e,f}$	5.0 ± 0.1^{c}	$3.2 \pm 0.2^{c,d}$	15.8 ± 0.1^{b}	7.0 ± 0.3^{h}	$1.8 \pm 0.1^{f,g}$	$0.6 \pm 0.1^{e,f}$	58 ± 0.5^{d}	35 ± 0.1^{a}	1.6 ± 0.2^{f}
3	$2.8 \pm 0.2^{a,b}$	$0.09 \pm 0.1^{c,d,e}$	$2.8 \pm 0.1^{f,g}$	$2.2 \pm 0.1^{e,f}$	13.1 ± 0.3^{c}	17.3 ± 0.2^{d}	3.3 ± 0.2^{d}	1.5 ± 0.1^{d}	63 ± 0.9^{c}	$29.0 \pm 0.1^{d,e}$	$2.1 \pm 0.1^{d,e}$
4	$3.0 \pm 0.1^{a,b}$	$0.07 \pm 0.1^{d,e,f}$	3.4 ± 0.1^{e}	3.0 ± 0.1^{d}	18.4 ± 0.2^{a}	35.0 ± 0.2^{a}	7.5 ± 0.2^{b}	2.5 ± 0.1^{c}	56 ± 0.5^{b}	$27 \pm 0.2^{e,f,g}$	$2.4 \pm 0.2^{b,c}$
5	$3.0 \pm 0.1^{a,b}$	$0.10 \pm 0.1^{a,b,c,d}$	2.0 ± 0.1^{h}	$1.7 \pm 0.1^{f,g}$	15.1 ± 0.2^{b}	35.0 ± 0.2^{a}	6.0 ± 0.1^{c}	3.5 ± 0.1^{a}	63 ± 0.9^{c}	$28 \pm 0.1^{e,f,g}$	$2.2 \pm 0.1^{c,d}$
6	$2.1 \pm 0.3^{a,b,c}$	$0.11 \pm 0.2^{a,b,c}$	2.7 ± 0.1^{g}	2.4 ± 0.1^{e}	11.0 ± 01^{d}	31.0 ± 0.4^{c}	8.5 ± 0.2^{a}	3.5 ± 0.1^{a}	55 ± 0.5^{d}	$33 \pm 0.3^{b,c}$	1.6 ± 0.1^{f}
7	$2.3 \pm 0.4^{a,b,c}$	$0.05 \pm 0.1^{e,f}$	8.0 ± 0.1^{a}	5.0 ± 0.2^{a}	11.4 ± 0.2^{d}	7.1 ± 0.3^{h}	$1.7 \pm 0.1^{f,g,h}$	0.3 ± 0.1^{g}	61 ± 0.5^{c}	$30 \pm 0.1^{c,d}$	$2.0 \pm 0.2^{d,e}$
8	$3.0 \pm 0.1^{a,b}$	0.04 ± 0.2^{f}	6.3 ± 0.4^{b}	4.0 ± 0.1^{b}	13.4 ± 0.4^{c}	10.0 ± 0.2^{f}	$1.7 \pm 0.1^{f,g,h}$	$0.4 \pm 0.1^{e,f}$	$67 \pm 0.9^{a,b}$	$25 \pm 0.1^{g,h}$	$2.6 \pm 0.2^{a,b}$
9	3.1 ± 0.3^{a}	0.04 ± 0.2^{f}	6.0 ± 0.1^{b}	4.0 ± 0.1^{b}	15.3 ± 0.2^{b}	11.0 ± 0.2^{e}	$2.0 \pm 0.1^{e,f,g}$	$0.5 \pm 0.1^{e,f,g}$	68 ± 0.5^{a}	24 ± 0.2^{h}	2.8 ± 0.1^{a}
10	$2.5 \pm 0.4^{a,b,c}$	$0.06 \pm 0.2^{e,f}$	5.3 ± 0.2^{c}	3.3 ± 0.1^{d}	11.4 ± 0.2^{d}	8.3 ± 0.3^{g}	$1.6 \pm 0.1^{h,g}$	$0.5 \pm 0.1^{e,f,g}$	62 ± 0.5^{c}	$29 \pm 0.2^{d,e,f}$	$2.1 \pm 0.1^{c,d}$
11	$2.3 \pm 0.2^{b,c}$	$0.09 \pm 0.1^{c,d,e}$	$3.3 \pm 0.1^{e,f}$	1.3 ± 0.2^{g}	18.6 ± 0.3^{a}	33.5 ± 0.2^{b}	9.0 ± 0.1^{a}	3.0 ± 0.1^{b}	61 ± 0.5^{c}	$32 \pm 0.1^{b,c}$	1.9 ± 0.1^{e}
12	$2.4 \pm 0.2^{b,c}$	$0.12 \pm 0.1^{a,b}$	2.3 ± 0.2^{g}	3.0 ± 0.1^{d}	15.4 ± 0.4^{d}	$10.8 \pm 0.4^{e,f}$	$2.0 \pm 0.2^{e,f}$	1.3 ± 0.1^{d}	57 ± 0.5^{d}	$34 \pm 0.2^{a,b}$	1.6 ± 0.2^{f}
13	$3.0 \pm 0.1^{a,b}$	$0.06 \pm 0.2^{e,f}$	4.5 ± 0.3^{d}	2.5 ± 0.2^{e}	15.3 ± 0.2^{b}	8.8 ± 0.1^{g}	1.3 ± 0.2^{h}	$0.5 \pm 0.1^{e,f,g}$	$66 \pm 0.5^{a,b}$	$26 \pm 0.1^{f,g,h}$	2.5 ± 0.1^{b}
14	$2.4 \pm 0.2^{b,c}$	$0.04 \pm 0.2^{e,f}$	5.3 ± 0.2^{c}	$3.8 \pm 0.1^{b,c}$	15.3 ± 0.4^{d}	$10.8 \pm 0.1^{e,f}$	2.5 ± 0.1^{e}	0.7 ± 0.1^{e}	61 ± 0.5^{c}	$29 \pm 0.1^{d,e}$	$2.1 \pm 0.2^{d,e}$

Values were given as means of triplicate ± SD. Means with different superscript letters within a column are significantly different (Tukey test, $p < 0.05$). SD = Standard deviation of the mean.

Ratio (**1**) is a useful indication of the linearity of pectin which relates the backbone GalA sugar to the neutral sugars involved in side chains. Whereas, ratio **2** is related to the proportion of Rha to GalA and is therefore indicative of the contribution of RG-I to the entire pectin population. The RG-I backbone is typically composed of alternating units of rhamnose and galacturonic acid. Lower values of the ratio are indicative of less branched pectin. However, Ratios **3** and **4** compare the amount of side-chain sugars to Rha as an estimate of the length of RG-I branching. Ratio **4** helps to establish the length of the galactose side chains present in the RG region. Ratio **5**, the ratio of typical pectin components to any co-extractants. The severity of the extraction conditions (ratios **6**-**8**) was assessed by comparing the presence of acid labile arabinose to galacturonic acid, galactose and rhamnose respectively. The sugar ratios for the different cell wall extracts, calculated based on the sugar content determined experimentally, are displayed in **Table 5.12**. From this table, it can be seen high values of ratio 1 indicate that these pectins have a great linearity and there is not a large variation in the values of ratios. The values of this ratio are higher than those previously published for *Cucumis melo Inodorus* which ranged from 0.4 to 1.4 (Denman and Morris, 2014), and for those reported for okra pectin: 1.1 and 1.3 for PO2 and PO6 respectively (Alba et al., 2013) and for tomato fruit (Houben et al., 2011). The ratio 1 values in this study were higher than those for grapefruit peel pectin extracted by ultrasound-assisted extraction (Wang et al., 2016). On the other hand, they are lower than those obtained from *piel de sapo* (5.29) (Dos-Santos et al., 2011) and for broccoli florets (Houben et al., 2011). However, these values were in line with the finding of (Houben et al., 2011) who reported 3.1, 3.6 and 3.5, 2.9, particularly for water-soluble fraction WSF and chelate-soluble fraction CSF for broccoli stem and carrot root respectively. In addition, they were close to data for grapefruit peel pectin extracted by conventional heating extraction (Wang et al., 2016).

For ratio 2 the results achieved in this analysis indicate low amounts of RG-I regions which mean less branched structures. These differ from those suggested for the heavily-branched structures of *Cucumis melo* pectin (A, B, C, E, F and G) but close to D and H pectins extracted under the same extraction conditions (Denman and Morris, 2014). Also, the pectins show a rather high degree of linearity and a very low proportion of RG-I compared to okra pectin (Alba et al., 2015); this is likely due to the use of different extraction methods. However, values for this ratio in this study are similar to those for the water-soluble pectins from broccoli floret and broccoli steam of 0.04 - 0.09 but very different to other pectins extracted under the same aqueous conditions, having values of 0.02, 0.03 and 0.10 (Houben et al., 2011). Similarly,

samples 1 and 12 have ratio values very close to those of grapefruit peel pectin (Wang et al., 2016).

In the case of ratio 3 which indicates the degree of branching of RG-I segments, as shown in **Table 5.12** it can be seen that the RG-I regions have a low degree of branching compared to those found for *Cucumis melo* (3.6 - 10.6) and for *piel de sapo* (9.8 - 10.3) (Denman and Morris, 2014, Dos-Santos et al., 2011). They also appear to be less branched than the extracts from broccoli and carrot (Houben et al., 2011). Contrariwise, they are similar to pectins from grapefruit peel (Wang et al., 2016). Therefore, pumpkin pectin appears to have shorter side chains on their RG-I regions.

Ratio 4 distinguishes itself in that it assists in determining the length of the galactose side chains located in the RG-I region. Results for this ratio are lower than those ratios already published by Denman and Morris, (2014) for *Cucumis melo*.

The values for ratio 5 demonstrate that the great majority of the pumpkin sample was composed of pectin (**Table 5.12**). The proportion of pectin is higher than either of the *piel de sapo* samples reported in Dos-Santos et al., (2011), and also for a melon (*Cucumis melo*) (Denman and Morris, 2014).

For the last three ratios (**6 - 8**) the highest ratio values were estimated for samples 4, 5 and 11 as shown in **Table 5.12** which in each case demonstrate the loss of arabinose relative to GalA, Gal and Rha. Thus, this is related to more severe extraction conditions (low pH, higher temperature or longer times) and loss of arabinofuranoside (Ara*f*) residues (Denman and Morris, 2014).

HG and RG-I molar percentages can be calculated from mol % of neutral sugars and GalA using the following equations (Msakni et al., 2006).

$$\text{HG (mol \%)} = \text{GalA (mol \%)} - \text{Rha (mol \%)} \quad (5.1)$$

$$\text{RG-I (mol \%)} = 2\text{Rha (mol \%)} + \text{Gal (mol \%)} + \text{Ara (mol \%)} \quad (5.2)$$

In the present study, the results achieved show a higher proportion of HG in the pumpkin samples than RG-I, with 55 – 68 % HG and 24 – 35 % RG-I. % HG values are higher by

comparison with those obtained by Denman and Morris, (2014) for melon but lower than those for okra in Alba et al., (2015) and for *piel de sapo* pectin (Dos-Santos et al., 2011). However, the degree of branching in the RG-I was slightly higher in pumpkin pectin than in the *piel de sapo* (Dos-Santos et al., 2011). The RG-I fraction is higher than for the pectins isolated from apple or sugar beet, where RG-I segments made up ~16.2 or ~31.9 % of the pectin population, respectively (Leroux et al., 2003). On the other hand, the RG-I fraction was lower than those in okra and melon (Alba et al., 2015, Denman and Morris, 2014), though the levels of branching are still comparable and the minor difference may well be due to the cultivar. The simple acid extraction used on the pumpkin would be better able to extract the linear HG molecules than the branched RG-I molecules. **Table 5.12** also gives us some information about the ratio of HG: RG-I, which varied from 1.6 to 2.8, signifying the presence of approximate proportions of HG and RG-I segments. These ratios were higher than those obtained by Denman and Morris, (2014) for a melon (0.3-1.1) and likewise with those obtained by Alba et al., (2013) for okra PO2 and PO6: 0.9 and 0.7 respectively.

An experimental design approach enables further understanding of the influence of each extraction condition and how they interact with one another. Therefore, the main (the largest) effect on the HG: RG-I ratio is temperature (**Figure 5.27**), although all the factors have only a small effect. In general the HG: RG-I ratio increases moving from low level (60 °C) to high level (80 °C).

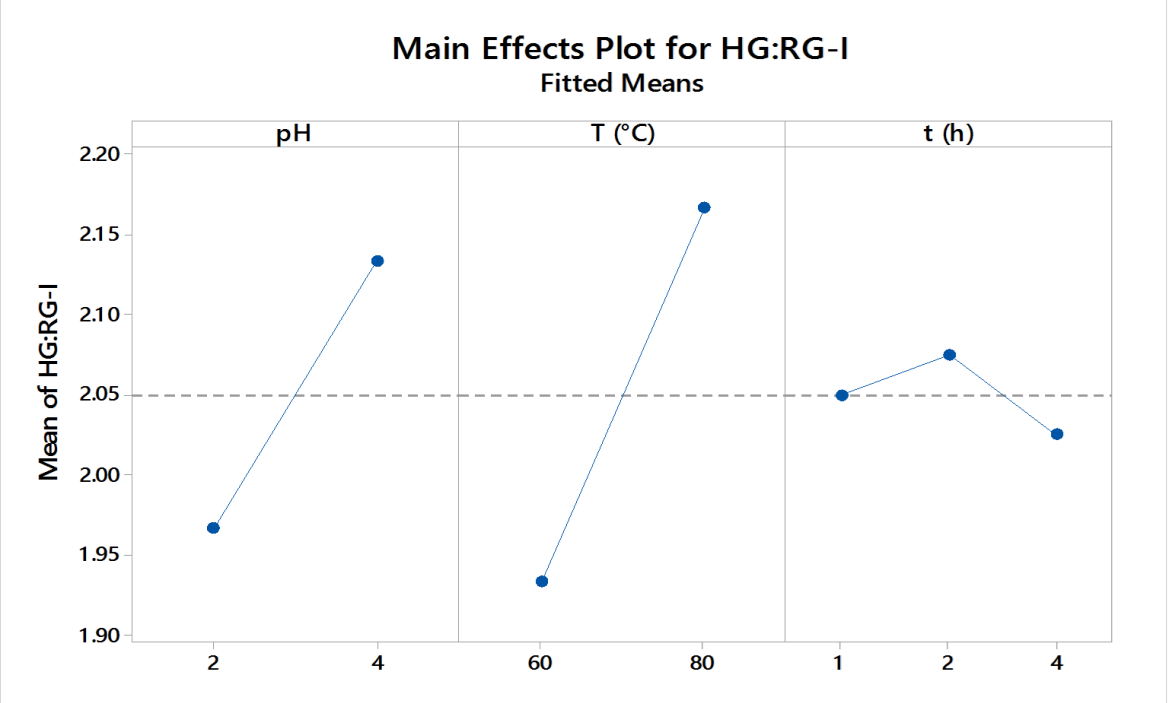


Figure 5.27: The main effect plots for HG: RG-I ratio: pH, temperature and time. The overall mean (~2.05 %) is shown as a dotted line and the steeper the slope the greater the effect of a particular parameter.

In **Figure 5.28** the 2-factor interaction for all the factors are less than 1. The 3-way (pH*Temperature*time) interaction is 0.07.

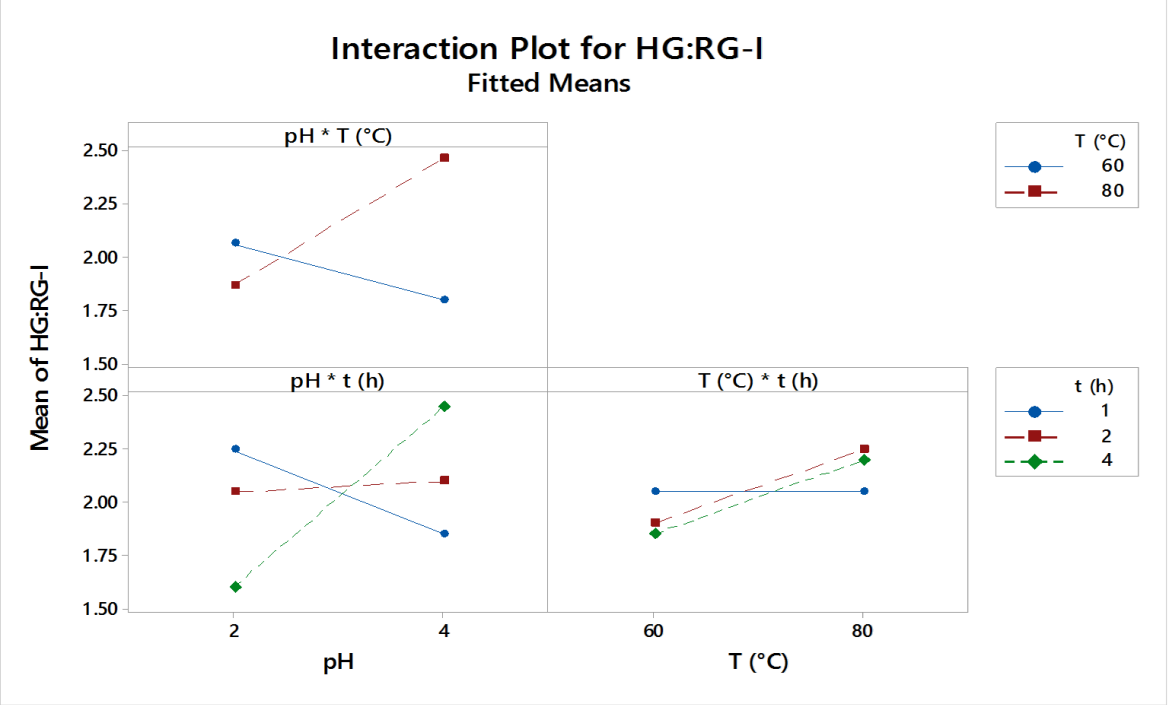


Figure 5.28: The interaction plots for HG: RG-I ratio. To visualize these effects, the Y-axis scale is always the same for each combination of factors. When the lines are parallel, interaction effects are zero.

Determination of structure using NMR:

The poor solubility of the long chain polysaccharides that were extracted from the prepared pumpkin samples made the usefulness of Nuclear Magnetic Resonance rather limited in this project even though NMR is a very powerful analytical technique for structural determination. The samples were dissolved in D_2O over 24 hours. The poor solubility the samples presented other limitations such as the obscuring of a large proportion of the peaks in the ^1H spectra by very large HOD solvent peak at around 4.7 ppm. An example of this is shown in the spectrum for the acid extracted for some samples in **Figure 5.29,** other samples weren't possible due to lack of sample and/or poor solubility. From **Figure 5.29** it can be seen that there are two signals at 1.21 ppm and 1.35 ppm they were derived from methyl groups of L-rhamnose and were assigned to O-2 linked rhamnose and to the O-2,4 linked rhamnose respectively (Voragen et al., 1986, Tamaki et al., 2008, Cui and Chang, 2014). Around 2 ppm and 2.1 ppm, there were two signals derived from acetyl groups binding at 2-O and 3-O of GalA (Tamaki et al., 2008, Perrone et al., 2002). The acetyl group was identified by comparison with the observed spectrum that already obtained by Košťálová et al., (2013), Perrone et al., (2002). However, a very large signal at 3.71 ppm was derived from methyl groups binding to carboxyl groups of GalA (Winning et al., 2007, Tamaki et al., 2008, Košťálová et al., 2013). Besides, more signals

observed in the spectrum of the pumpkin sample: H-1, 5.12 ppm; H-3, 3.9 ppm; H-4, 4.4 ppm; H-5, 4.96 ppm which related to D-galacturonic acid they were close to those previously obtained by (Souza et al., 2012, Tamaki et al., 2008) from pumpkin and endocarp of *Citrus depressa* pectins, also reported in Marcon et al., (2005)for pectin from apple pomace. These signals were detected for protons found when the polysaccharide was de-esterified as reported in (Mukhiddinov et al., 2000).

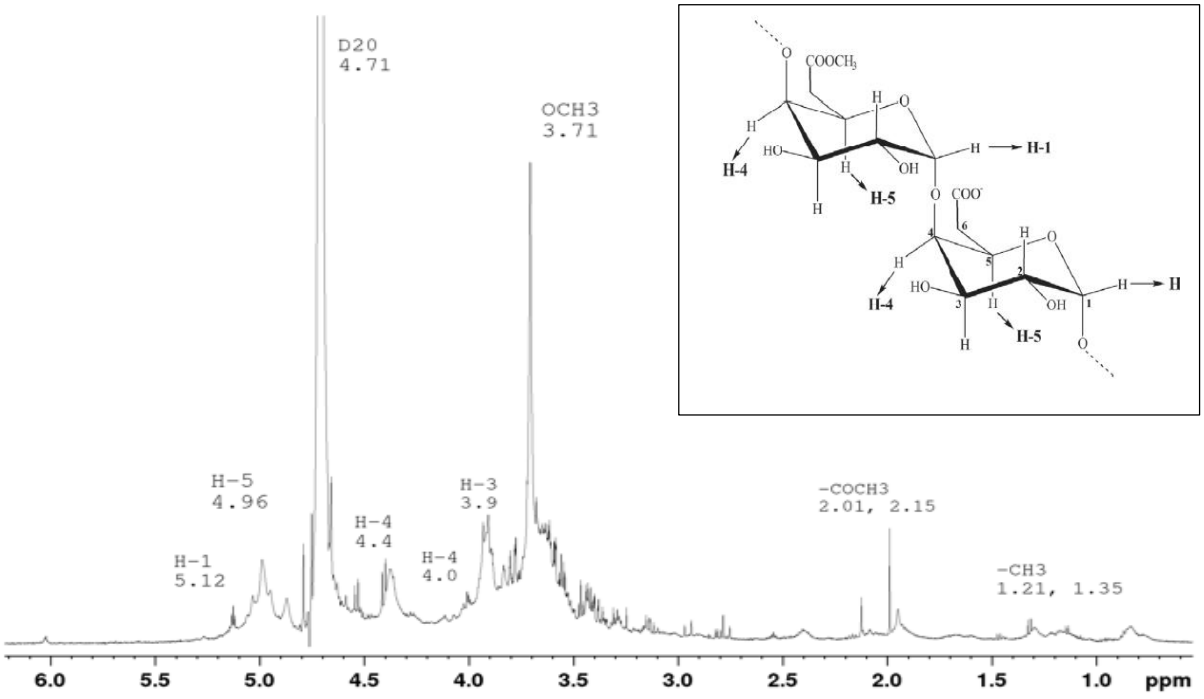


Figure 5.29: ^{1}H NMR spectrum of pumpkin pectin extracted under different conditions.

5.2 Physical Characterisation of pumpkin pectin:

In this section the physical characterisation of pumpkin, pectin will be determined. This will include intrinsic viscosity, zeta potential, and molecular weight (M_W) determination of pumpkin pectins.

5.2.1 Intrinsic viscosity:

From Huggins (♦) and Kraemer (▄) plots the intrinsic viscosity for some of the pumpkin pectins extracted under different conditions are calculated see **Figure 5.30**.

Intrinsic viscosities were high for all pectin samples extracted from pumpkin pectin, the values varied from 260 to 1342 mL/g **Table 5.13**. These values which were obtained are much higher

than those reported in Levine et al., (2002a) for pectins extracted from fresh sugar beet and pumpkin (Arslan, 1995, Shkodina et al., 1998).

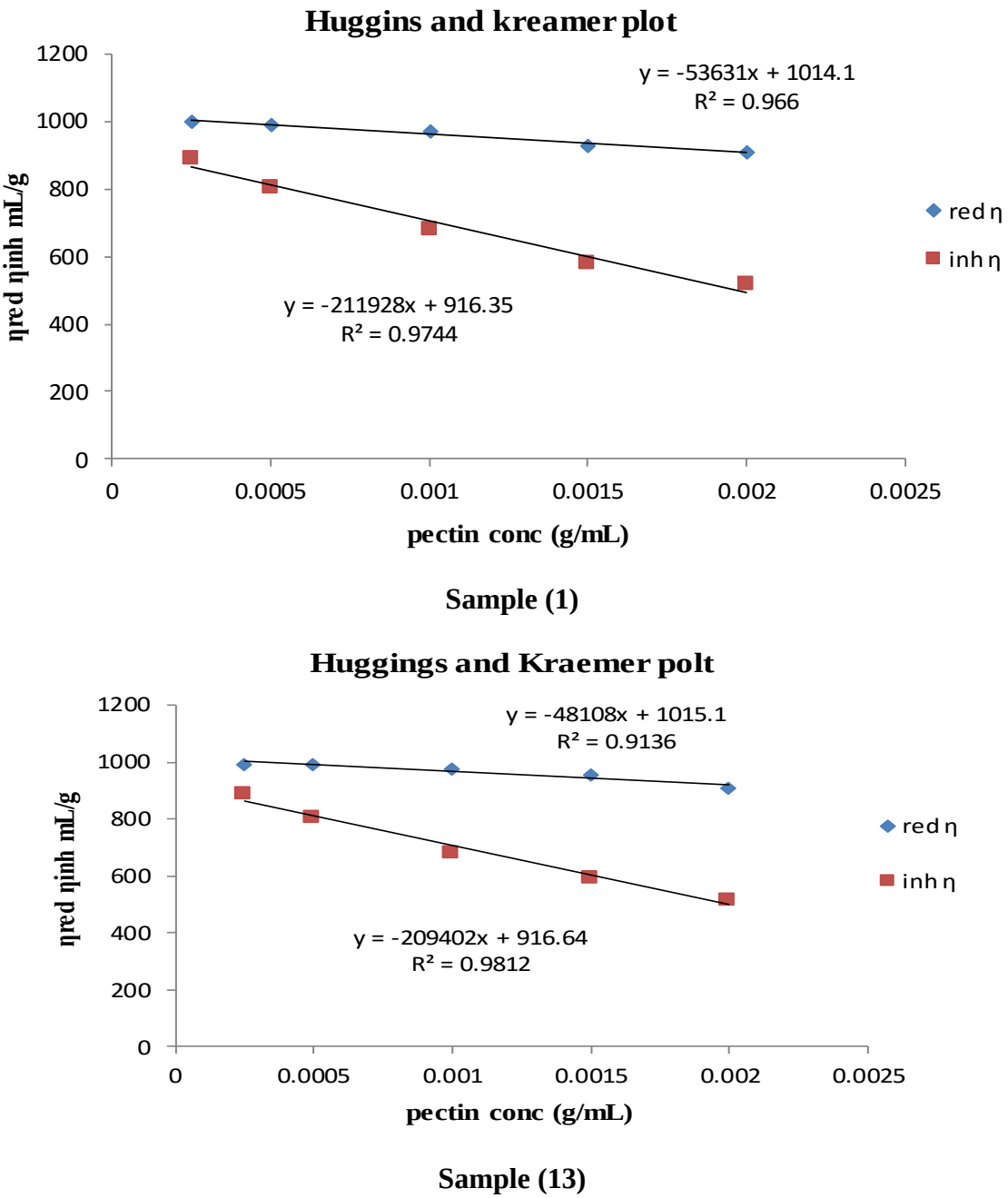


Figure 5.30: Huggins (♦) and Kraemer (■) plots for some of the pumpkin pectins extracted under different conditions *i.e.* samples (1) and (13), ♦ red η and ■ inh η represent the reduced and inherent viscosity respectively.

Table 5.13: Indicates the values of intrinsic viscosity [η] for pumpkin pectin extracted under different extraction conditions.

Sample	pH	T (°C)	t (h)	[η] mL/g
1	4	60	1	880 ± 18^{a}
2	4	60	2	947 ± 19^{c}
3	4	60	4	830 ± 17^{f}
4	2	60	1	537 ± 11^{c}
5	2	60	2	299 ± 6^{e}
6	2	60	4	260 ± 5^{e}
7	4	80	1	932 ± 19^{h}
8	4	80	2	1342 ± 27^{i}
9	4	80	4	810 ± 16^{h}
10	2	80	1	640 ± 13^{g}
11	2	80	2	503 ± 10^{d}
12	2	80	4	608 ± 12^{f}
13	3	70	1.5	966 ± 19^{a}
14	3	70	3	904 ± 18^{b}

Values were given as means of triplicate ± SD. Means with different superscript letters within a column are significantly different (Tukey test, $p < 0.05$). SD = Standard deviation of the mean.

The highest value (1342 ml/g) was obtained at pH 4 at 80 ºC for 2 h extraction time, while the lowest value (260 ml/g) was obtained at pH 2 at 60 ºC for 4h extraction time. Moreover, it can be observed that the highest value for viscosity was obtained at the highest temperature, pH 80 ºC, 4 and extraction time 2 h respectively.

Table 5.13 clearly confirmed that the extraction viscosity of pectin was significantly increased with the pH (**Figure 5.31**). In all cases, the intrinsic viscosity of the samples from pumpkin was notably higher at pH 4 than that at pH 2. In this report, pH had the most prominent influence on the viscosity of extracted pectin.

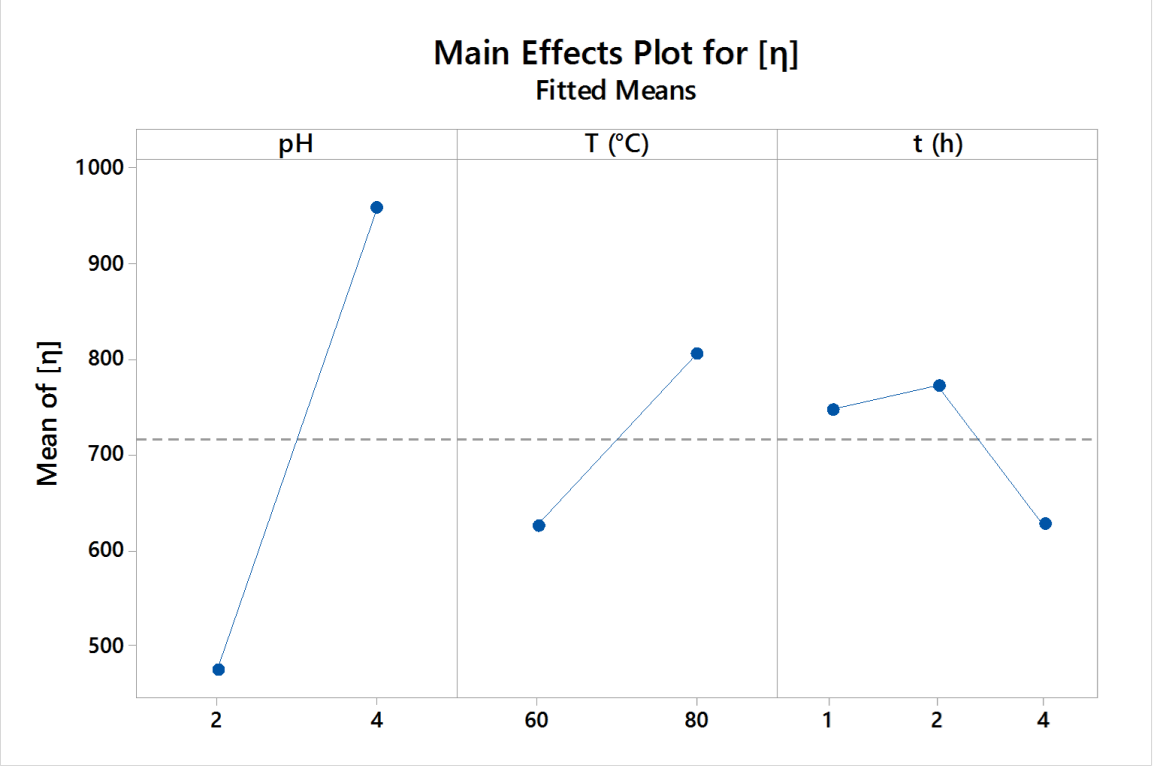


Figure 5.30: The main effect plots for intrinsic viscosity: pH, temperature and time. The overall mean (715 ml/g) is shown as a dotted line and the steeper the slope the greater the effect of a particular parameter.

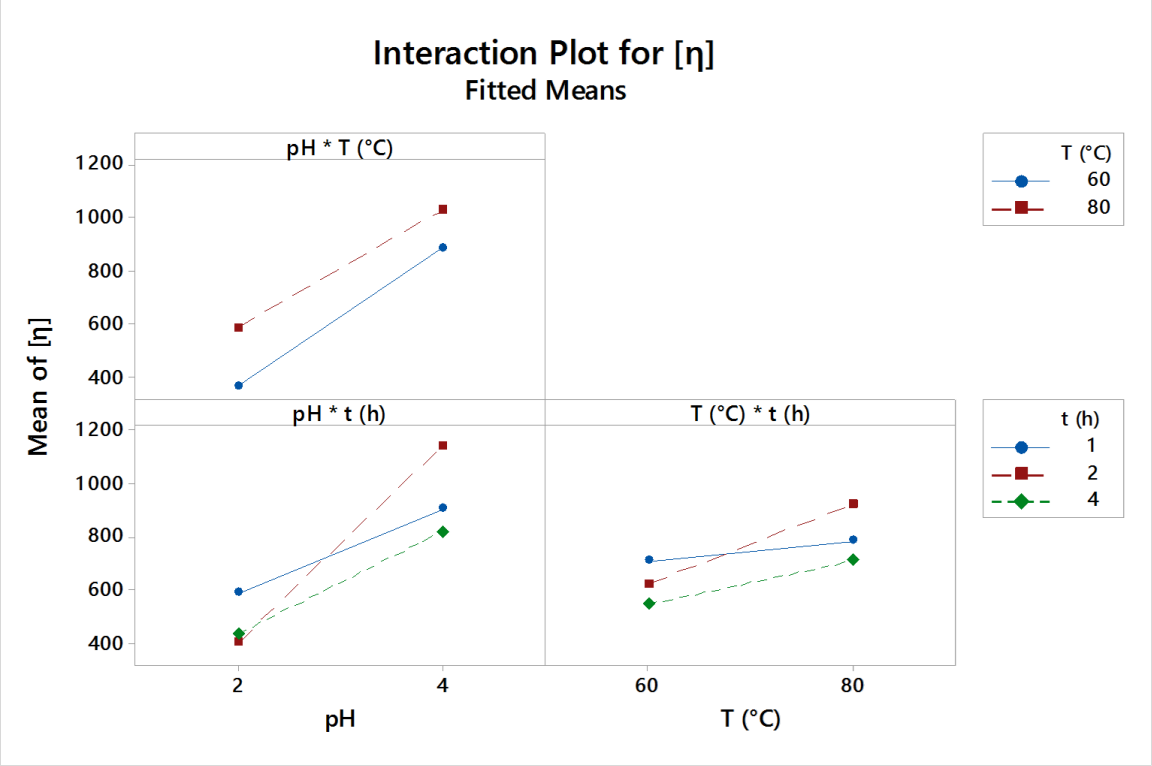


Figure 5.31: The interaction plots for intrinsic viscosity. To visualize these effects, the Y-axis scale is always the same for each combination of factors. When the lines are parallel, interaction effects are zero.

In Figure **5.32** the slopes for pH*time and temperature*time are non-parallel indicating there are interactions between the different extraction conditions, however, the interaction between pH and time is the largest. The 2-factor interactions are 0.04, 1.04 and 0.04 x 10^5 for pH* temperature, pH*time and temperature*time, respectively. The 3-way (pH* temperature*time) interaction is 0.39 x 10^5.

Based on all these interpretations resulting from the factorial design, it is possible to say that under these specific conditions that the parameters at pH 4, temperature 80 ºC and an extraction time of 2 hours to give the highest/ optimum intrinsic viscosity.

5.2.2 Zeta potential:

Measurement of ζ-potential (electrophoretic mobility) is a useful method to characterize the surface charge properties of pectin. ζ-potential values were negative (-34 to -25 mV) due to the ionization of the carboxyl groups of the pectin. Although looking at the main effects plot (**Figure 5.33**) none of the extraction conditions had a very large influence on ζ-potential, the temperature was the largest. The ζ-potential decreases (becomes more negative) moving from low level (60 ºC) to high level (80 ºC).

Table 5.14: Indicates the values of zeta potential ζ for pumpkin pectin extracted under different extraction conditions.

Sample	pH	T (°C)	t (h)	ζ mV
1	4	60	1	$-29 \pm 0.4^{c,d}$
2	4	60	2	$-31 \pm 0.2^{e,f}$
3	4	60	4	-27 ± 0.3^{b}
4	2	60	1	-32 ± 0.2^{f}
5	2	60	2	-25 ± 0.1^{a}
6	2	60	4	$-29 \pm 0.4^{c,d}$
7	4	80	1	$-28 \pm 0.3^{b,c}$
8	4	80	2	-27 ± 0.4^{b}
9	4	80	4	-32 ± 0.2^{f}
10	2	80	1	$-30 \pm 0.5^{d,e}$
11	2	80	2	-34 ± 0.3^{g}
12	2	80	4	$-28 \pm 0.2^{b,c}$
13	3	70	1.5	-32 ± 0.7^{f}
14	3	70	3	$-31 \pm 0.4^{e,f}$

Values were given as means of triplicate ± SD. Means with different superscript letters within a column are significantly different (Tukey test, $p < 0.05$). SD = Standard deviation of the mean.

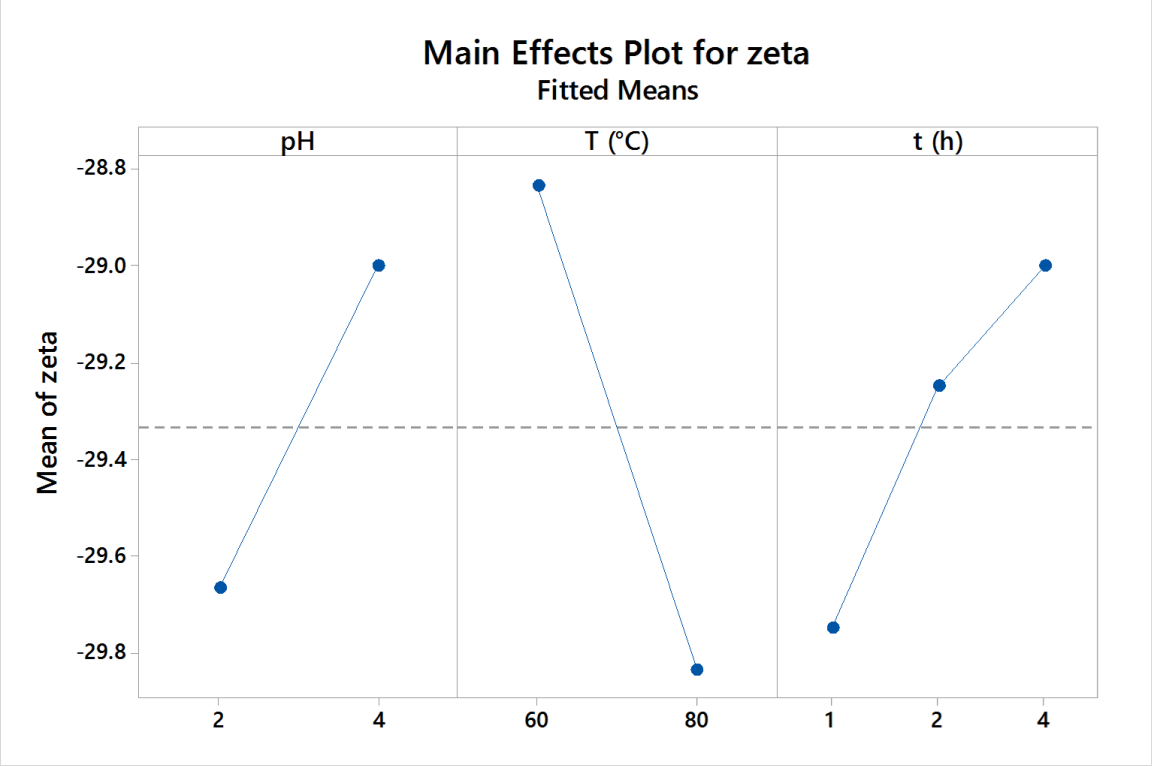


Figure 5.32: The main effect plots for ζ-potential: pH, temperature and time. The overall mean (-29.3 mV) is shown as a dotted line and the steeper the slope the greater the effect of a particular parameter.

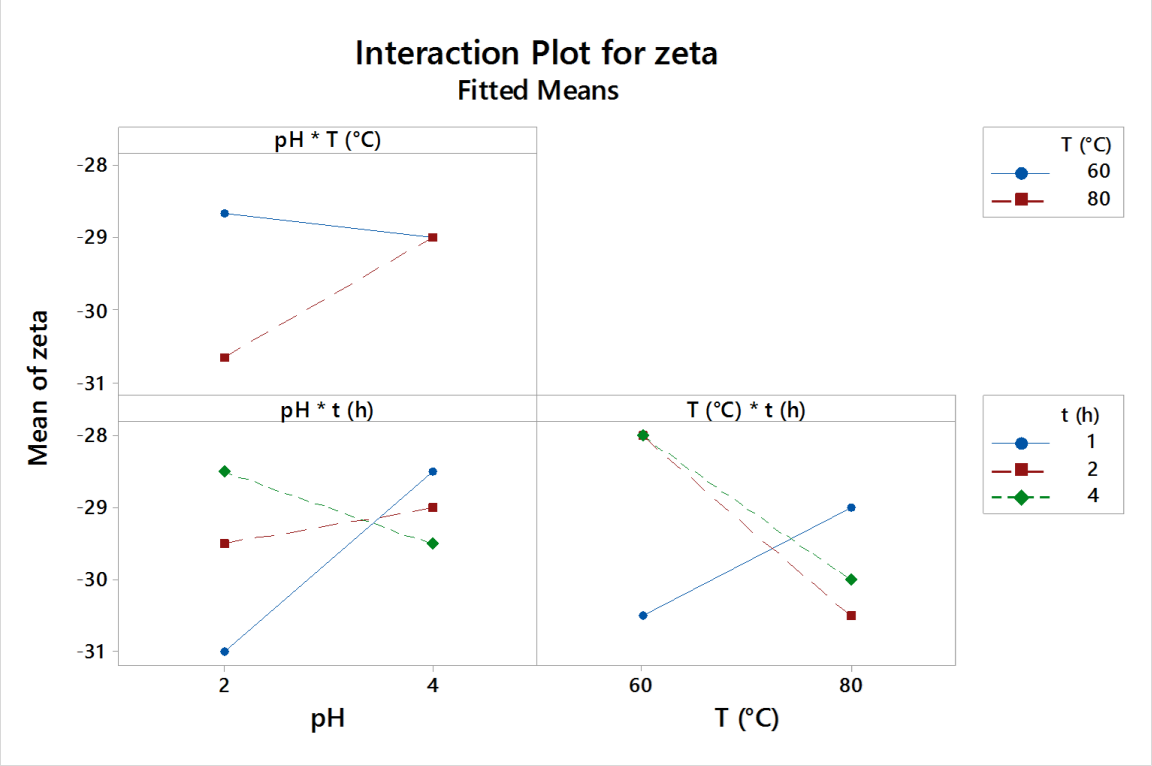
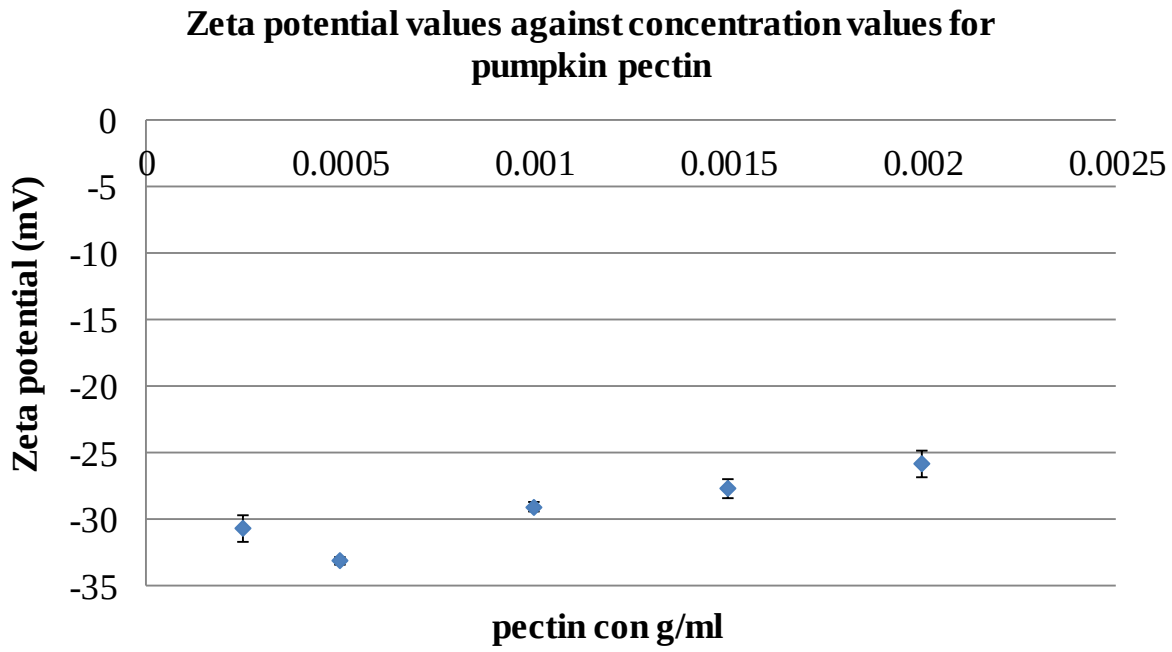


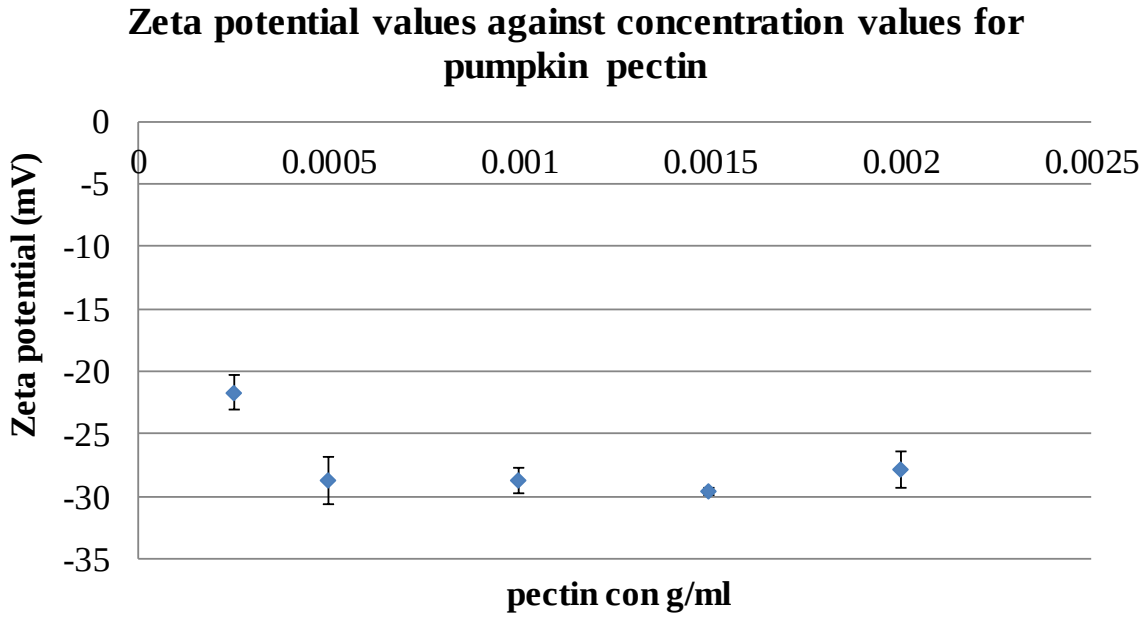
Figure 5.33: The interaction plots for ζ-potential. To visualize these effects, the Y-axis scale is always the same for each combination of factors. When the lines are parallel, interaction effects are zero.

In Figure **5.34** the slopes for pH* temperature, pH*time and temperature*time are non-parallel indicating there are interactions between the different extraction conditions, however, the interaction between temperature and time is the largest. The 2-factor interactions are 3.00, 6.17 and 9.50 for pH* temperature, pH*time and temperature*time, respectively. The 3-way (pH* temperature*time) interaction is 48.50 and is therefore very important and demonstrates that the charge on pectins after extraction is difficult to control.

Based on all these interpretations resulting from the factorial design, it is possible to say that under these specific conditions that the parameters at pH 2, Temperature 80 ºC and an extraction time of 2 hours to give the lowest (most negative)/ optimum ζ-potential.



Sample (1)



Sample (3)

Figure 5.34: The effect of concentration on ζ-potential for some of the pumpkin pectins extracted under different conditions, *i.e.* samples (1) and (3).

5.2.3 Determination of pectin molecular weight:

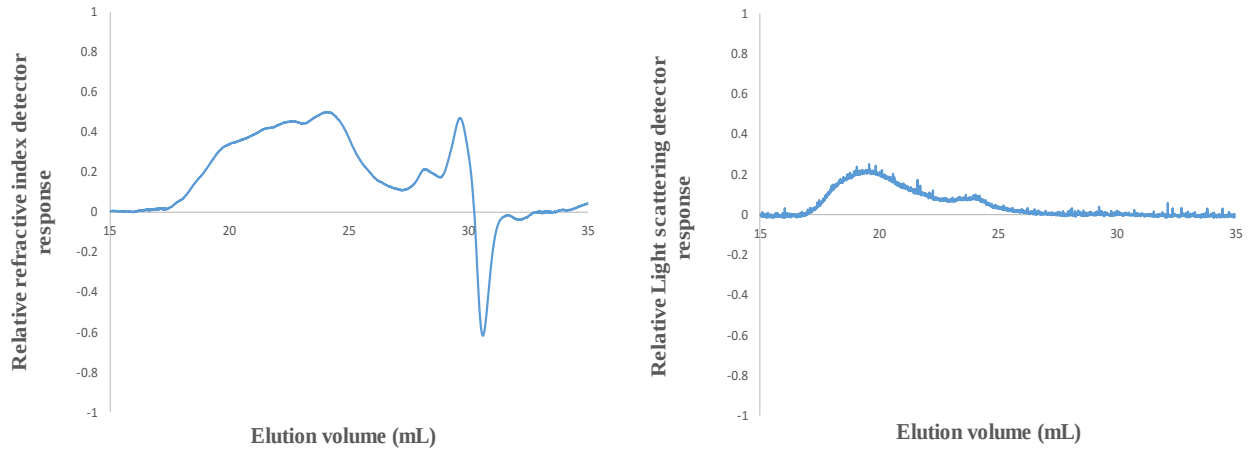
Size exclusion chromatography (SEC) coupled to multi-angle laser light scattering (MALLS) has been used to determine pectin weight average (M_w), number average (M_n) molecular weights, z-average radius of gyration ($R_{g,z}$) and polydispersity index (PI = Mw/Mn); these estimated molecular characteristics are shown in **Table 5.15**. From this table it can be seen the molar mass of pumpkin pectins ranged from 120 000 to 283 000 g/mol, all have high M_w which was considerably higher than those obtained by Pagan et al., (1999), Urias-Orona et al., (2010) and Wang et al., (2016).

Table 5.15: Molecular characteristics of pumpkin pectins. Weight average molecular weight (M_w), number average molecular weight (M_n), the z-average radius of gyration ($R_{g,z}$) and polydispersity index (PI = M_w/M_n).

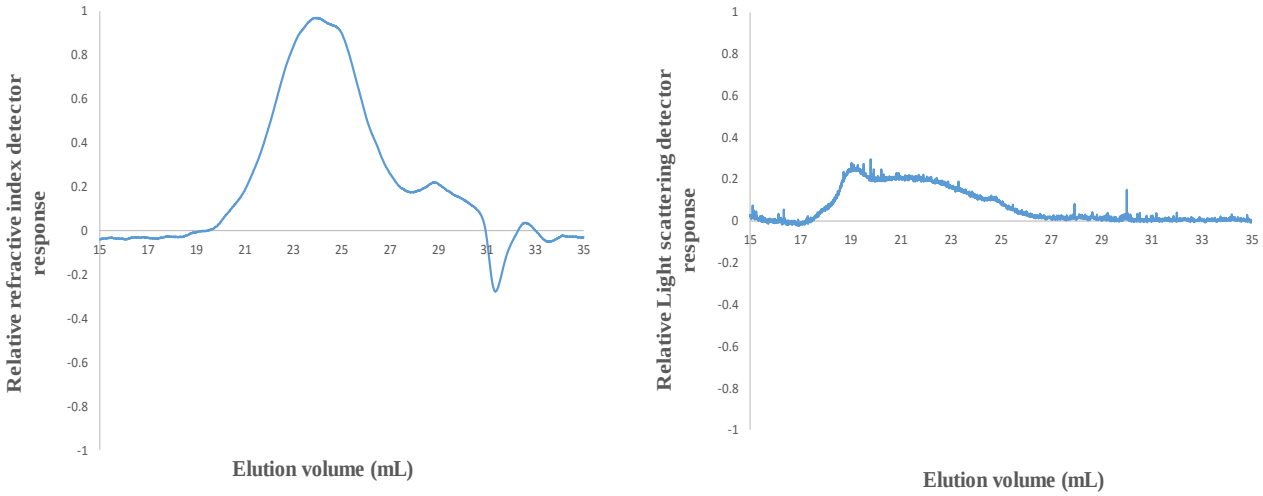
Sample	$M_w \times 10^5$ (g / mol)	$M_n \times 10^4$ (g / mol)	$R_{g,z}$ (nm)	PI = (M_w/M_n)
1	1.33 ± 0.1^{h}	6.16 ± 1.33	37.4 ± 1.0	2.16 ± 0.23
2	2.21 ± 0.2^{d}	15.00 ± 0.08	45.2 ± 0.1	1.47± 0.07
3	1.85 ± 0.1^{e}	7.89 ± 0.76	36.7 ± 0.1	2.34 ± 0.10
4	2.42 ± 0.3^{c}	10.80 ± 0.05	27.9 ± 0.1	2.24 ± 0.06
5	2.43 ± 0.1^{c}	7.62 ± 1.05	32.6 ± 0.2	3.19 ± 0.14
6	2.23 ± 0.1^{d}	5.17 ± 1.44	41.9 ± 0.2	4.31 ± 0.29
7	2.53 ± 0.2^{b}	9.64 ± 0.52	36.8 ± 0.2	2.62 ± 0.06
8	1.83 ± 0.2^{e}	5.85 ± 0.67	29.1 ± 0.1	3.13 ± 0.12
9	1.46 ± 0.1^{g}	5.98 ± 0.87	34.1 ± 0.2	2.43 ± 0.15
10	1.39 ± 0.2^{h}	5.27 ± 0.67	44.4 ± 0.1	2.63 ± 0.14
11	1.66 ± 0.1^{f}	7.17 ± 0.38	38.2 ± 0.1	2.32 ± 0.06
12	1.20 ± 0.1^{i}	2.85 ± 0.87	30.8 ± 0.3	4.21 ± 0.31
13	2.83 ± 0.2^{a}	6.20 ± 1.56	38.9 ± 0.1	4.57 ± 0.25
14	1.37 ± 0.1^{h}	6.63 ± 1.46	33.3 ± 0.2	2.06 ± 0.23

Values were given as means of triplicate ± SD. Means with different superscript letters within a column are significantly different (Tukey test, $p < 0.05$). SD = Standard deviation of the mean.

Such high values may be explained by the presence of molecular aggregates in the pectin solutions which may be due to the extraction of RG-I cross-linked with RG-II (Ridley et al., 2001). On the contrary, they seem lower than those reported in Seixas et al., (2014a) for passion fruit peel pectin. However, on the whole, they are consistent with typical pectin M_w which is expected to be in the range 10^4 - 10^5 g/ mol (Corredig et al., 2000). **Figure 5.36** represents the reflective index and light scattering plots for some the pumpkin pectins, an example has been given for sample 1 and sample 8.



Sample 1



Sample 8

Figure 5.36: The realative reflective index and light scattering plots for some of pumpkin pectins (*i.e.* samples 1 amd 8).

The use of an experimental design approach has allowed the further understanding of the influence of each extraction condition and how they interact with one another. Therefore, the main (the largest) effect on weight average molecular weight is time (**Figure 5.36**). In general, the molecular weight decreases moving from low level (1 hour) to high level (4 hours), although there is an initial increase after 2 hours. This again may be related to a 2-stage process of solubilisation and degradation.

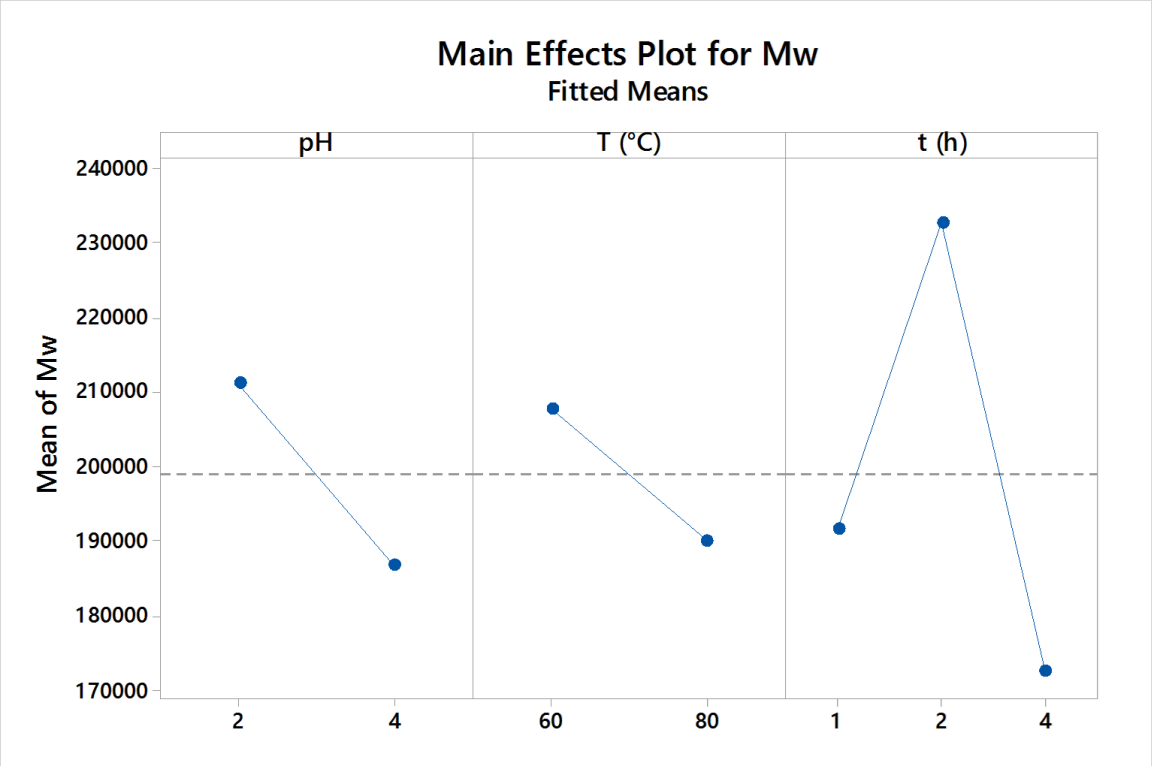


Figure 5.35: The main effect plots for molecular weight: pH, temperature and time. The overall mean (199 000 g/mol) is shown as a dotted line and the steeper the slope the greater the effect of a particular parameter.

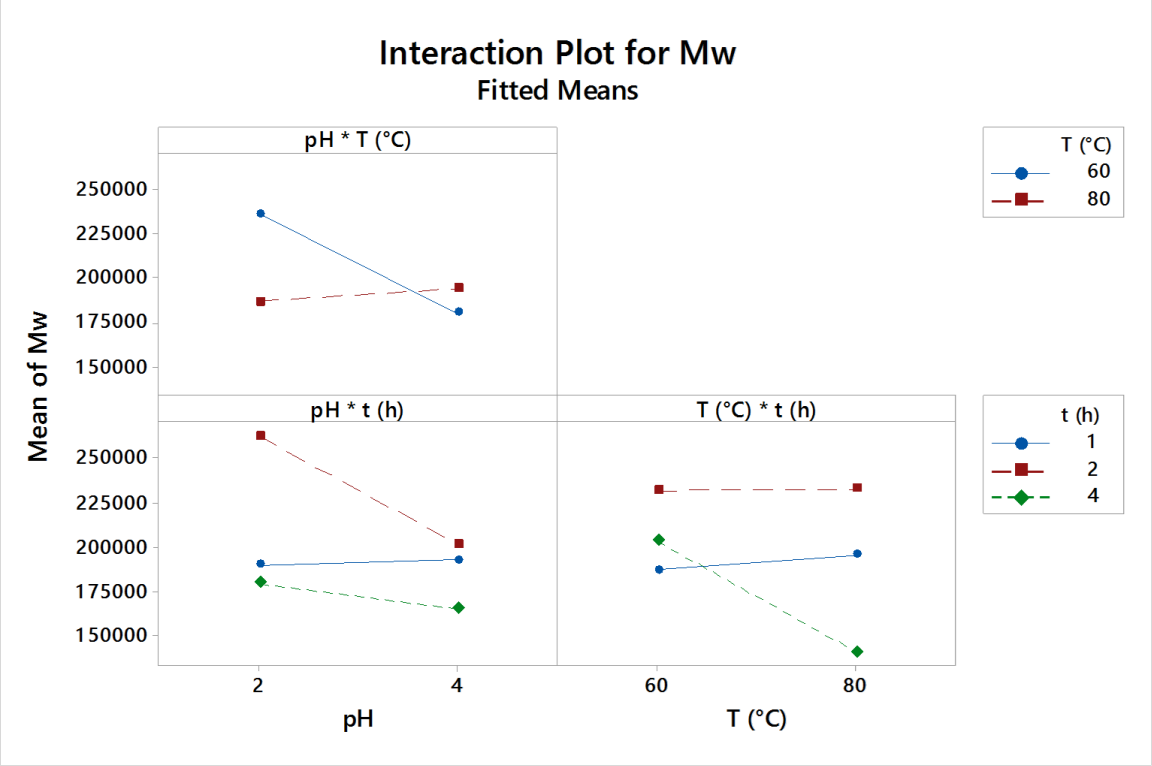


Figure 5.36: The interaction plots for molecular weight. To visualize these effects, the Y-axis scale is always the same for each combination of factors. When the lines are parallel, interaction effects are zero.

In Figure **5.37** the slopes for pH*time and temperature*time are non-parallel indicating there are interactions between the different extraction conditions, however, the interaction between pH and time and temperature and time are the largest. The 2-factor interactions are (3.03, 2.15 and 3.03) x 10^9 for pH* temperature, pH*time and temperature*time, respectively. The 3-way (pH* temperature*time) interaction is 1.14 x 10^{10} and is therefore very important and demonstrates that the molecular weight of pectins after extraction is difficult to control.

Based on all these interpretations resulting from the factorial design, it is possible to say that under these specific conditions that the parameters at pH 2, temperature 80 ºC and an extraction time of 2 hours to give the highest/optimum molecular weight.

In addition, pumpkin samples are polydisperse with M_w/M_n ratios ranging from 1.47 to 4.57, which were lower than those already published for grapefruit peel pectin (Wang et al., 2016). Accurate determination of the M_w distribution is extremely difficult because of the heterogeneous nature of pectins such as the presence of smooth and hairy regions, and the

varying inter-and intramolecular distribution of methyl esters (Kim et al., 2005). A radius of gyration of 45.2 nm which was the highest estimated for sample 2 that was extracted at pH 4, 60 °C under 2 h extraction time; other samples had lower $R_{g,z}$. Radius of gyration values of this study were lower than those for xyloglucans obtained byPatel et al., (2008).

These results indicate the variance in molar mass of pumpkin pectins according to the extraction conditions used, or the type of method used for the determination of molecular parameters, whose values depend, not only on the extraction process used but also on the plant material used (Kratchanova et al., 1991, Levigne et al., 2002a).

5.3 Conformational analysis:

The physicochemical properties of polysaccharides naturally depend on both the chemical composition (**section 5.1**) and their physical characteristics (**section 5.2**), however, a third important property which impacts on their functionality is conformation (*i.e.* their 3D arrangement in solution) (Ralet et al., 2008). This can be perhaps best demonstrated in **Figure 5.38**.

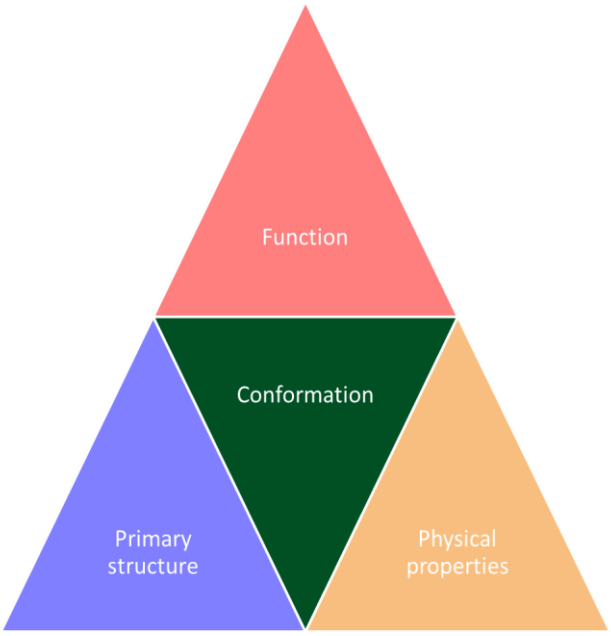


Figure 5.37: Structure-function triangle demonstrating how primary structure, physical properties and conformation underpin functionality of polysaccharides.

5.3.1 Global analysis method (HYDFIT)

For the pumpkin pectins two possible scenarios were considered (Patel et al., 2008):

1. Only the chain diameter, d was fixed at 0.8 nm: this is a typical value for a monosaccharide and extensive simulations (Ortega and García de la Torre, 2007) have shown that estimates for L_p and M_L are relatively insensitive to the value of the chain diameter. Minima of the target function are shown in **Figure 5.39** (indicated with an a).

2. The chain diameter, d was fixed at 0.8 nm and the mass per unit length, M_L was fixed using previous knowledge of the HG: RG-I ratio and the degree of esterification (**Table 5.12**). Minima of the target function are shown in **Figure 5.39** (indicated with a b).

The Multi-HYDFIT program then freely floats the variable parameters (L_p and M_L in case 1; L_p in case 2) in order to find a minimum of the target function.

Flexibility can also be estimated from the ratio of L_p/M_L (nm^2mol/g) which increases with increasing stiffness (Patel et al., 2008, Morris et al., 2010b).

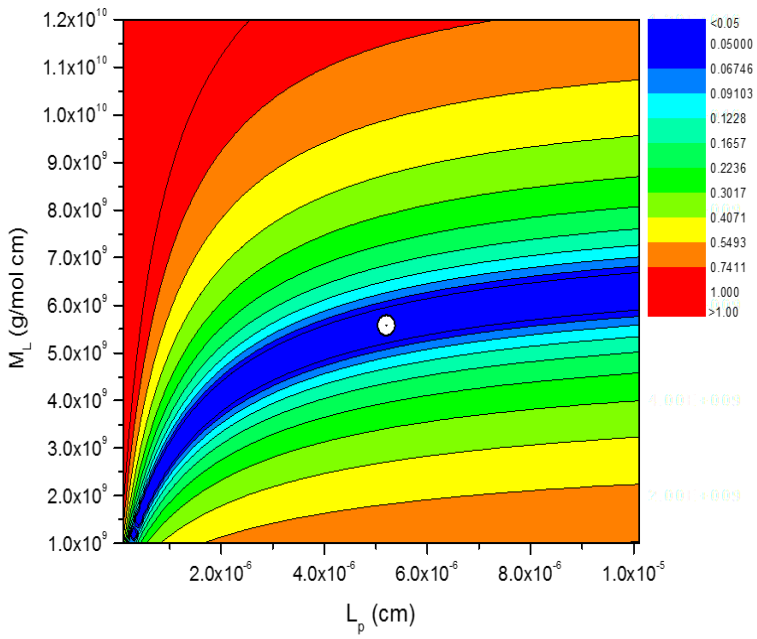
Table 5.16: Mass per unit length M_L and chain flexibility (L_p) estimations from combining molecular weight and intrinsic viscosity data through HYDFIT for pumpkin pectin when the mass per unit length has been allowed to float freely.

Sample	[η] (g/mL)	Mw × 10^5 g/mol	L_p (nm)	M_L (g/(mol nm))	L_p / M_L (nm^2 mol/g)
1	880	1.33	52^{a}	$558^{a,b,c}$	0.093^{a}
2	947	2.21	38^{a}	$650^{a,b,c}$	0.058^{a}
3	830	1.85	98^{a}	$778^{a,b,c}$	0.126^{a}
4	537	2.42	78^{a}	1052^{a}	0.074^{a}
5	299	2.43	2^{a}	210^{c}	0.010^{a}
6	260	2.23	32^{a}	1135^{a}	0.028^{a}
7	932	2.53	98^{a}	$888^{a,b}$	0.110^{a}
8	1342	1.83	90^{a}	$622^{a,b,c}$	0.145^{a}
9	810	1.46	60^{a}	$640^{a,b,c}$	0.094^{a}
10	640	1.39	10^{a}	$347^{b,c}$	0.029^{a}
11	503	1.66	32^{a}	$714^{a,b,c}$	0.045^{a}
12	608	1.20	19^{a}	$457^{b,c}$	0.042^{a}
13	966	2.83	26^{a}	$631^{a,b,c}$	0.041^{a}
14	904	1.37	59^{a}	$576^{a,b,c}$	0.102^{a}

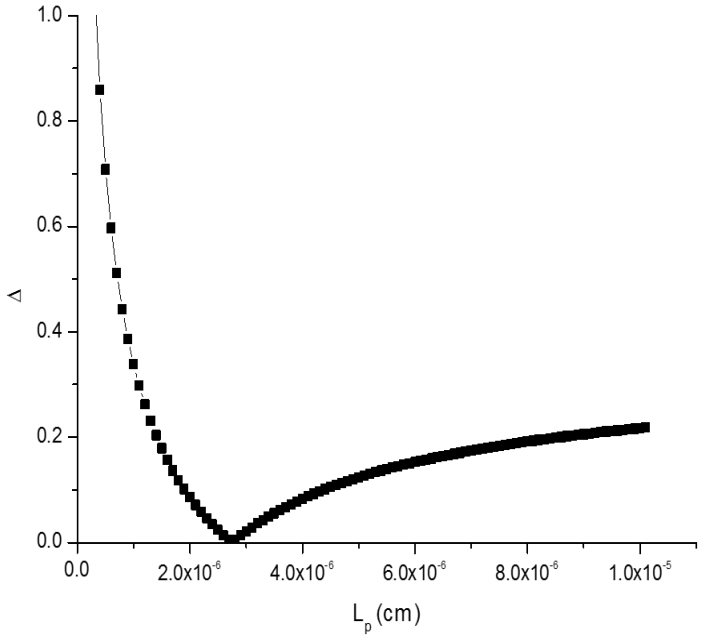
Values were given as means of triplicate ± SD. Means with different superscript letters within a column are significantly different (Tukey test, $p < 0.05$). SD = Standard deviation of the mean.

In **Table 5.16** above, we can see that the persistence lengths L_p for pumpkin pectin used in this study are large, the largest values were obtained for samples 3 and 7 which were extracted at pH 4, although for different times and temperatures this would suggest a more rigid conformation for these two pectins compared with the other pectins, while the lowest persistence length value was obtained for sample 5 at pH 2, 60 °C and 2h extraction time. These results are higher than those for sugar beet (*Beta vulgaris*) pectins (7.3, 10) nm persistence length and (330, 620) g/mol nm mass per unit length which it has been suggested have the flexible structure (Morris et al., 2010b).

Besides, the mass per unit length values M_L as shown in **Table 5.16** indicated that some of the samples (*e.g.* 4, 6 and 7) are highly branched (hairy) while other samples would appear to be as less branched, this in contrast with values obtained by (Morris et al., (2010b) for sugar beet (*Beta vulgaris*) pectins which were lower. Values for these samples are lower than those reported inRalet et al., (2008). However, the mass per unit length value for sample 13 was similar to those demonstrated in the literature (Ralet et al., 2008). From **Table 5.16** above it can be seen that the samples with less flexibility/ highest stiffness (*e.g.* 3, 7 and 8) have the highest ratios of L_p/M_L. Therefore, L_p/M_L (nm^2 mol/g) ratio can be used to estimate the overall flexibility whereby it decreases with increasing flexibility (Patel et al., 2008).

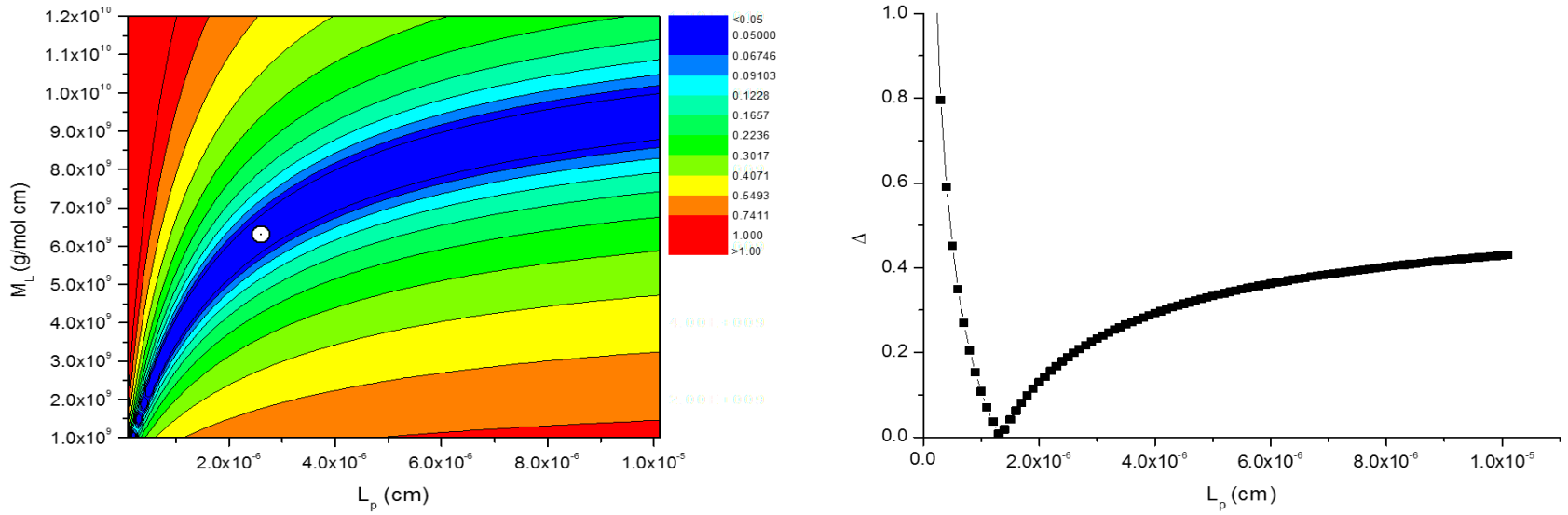

1.2x10^10
1.1x10^10
1.0x10^10
9.0x10^9
8.0x10^9
7.0x10^9
6.0x10^9
5.0x10^9
4.0x10^9
3.0x10^9
2.0x10^9
1.0x10^9
M_L (g/mol cm)
2.0x10^-6
4.0x10^-6
6.0x10^-6
8.0x10^-6
1.0x10^-5
L_p (cm)
<0.05
0.05000
0.06746
0.09103
0.1228
0.1657
0.2236
0.3017
0.4071
0.5493
0.7411
1.000
>1.00

a


1.0
0.8
0.6
0.4
0.2
0.0
Δ
0.0
2.0x10^-6
4.0x10^-6
6.0x10^-6
8.0x10^-6
1.0x10^-5
L_p (cm)

b

Sample (1)



a **b**

Sample (13)

Figure 5.38: HYDFIT analysis for pectic polysaccharides. A solution of the Bohdanecky (1983) and Yamakawa-Fujii (1973) relations for L_p with M_L allowed to float. The x-axis and y-axis represent L_p (nm) and M_L (g/ (mol nm)) respectively. The target function, Δ is calculated over a range of values for M_L and Lp. In these representations, the values of Δ function are represented by the full-colour spectrum, from blue ($\Delta \leq 0.05$) to red ($\Delta \geq 1$). The global minima are indicated (○). Samples designated with an "a". HYDFIT analysis for pectic polysaccharides. A solution of the Bohdanecky (1983) and Yamakawa-Fujii (1973) relations for Lp (for a known mass per unit length M_L as indicated in Table 5.17. The plot of target function (Δ) vs. persistence length for samples designated with a "b". An example has been given for samples 1, 13 and see appendices 9.1.5 for all other samples.

Adoption of an experimental design approach allows further understanding of the influence of each extraction condition and how they interact with one another. Therefore, the main (the largest) effect on persistence length is pH (**Figure 5.40**). In general, the persistence length increases moving from a low level (pH 2) to a high level (pH 4).

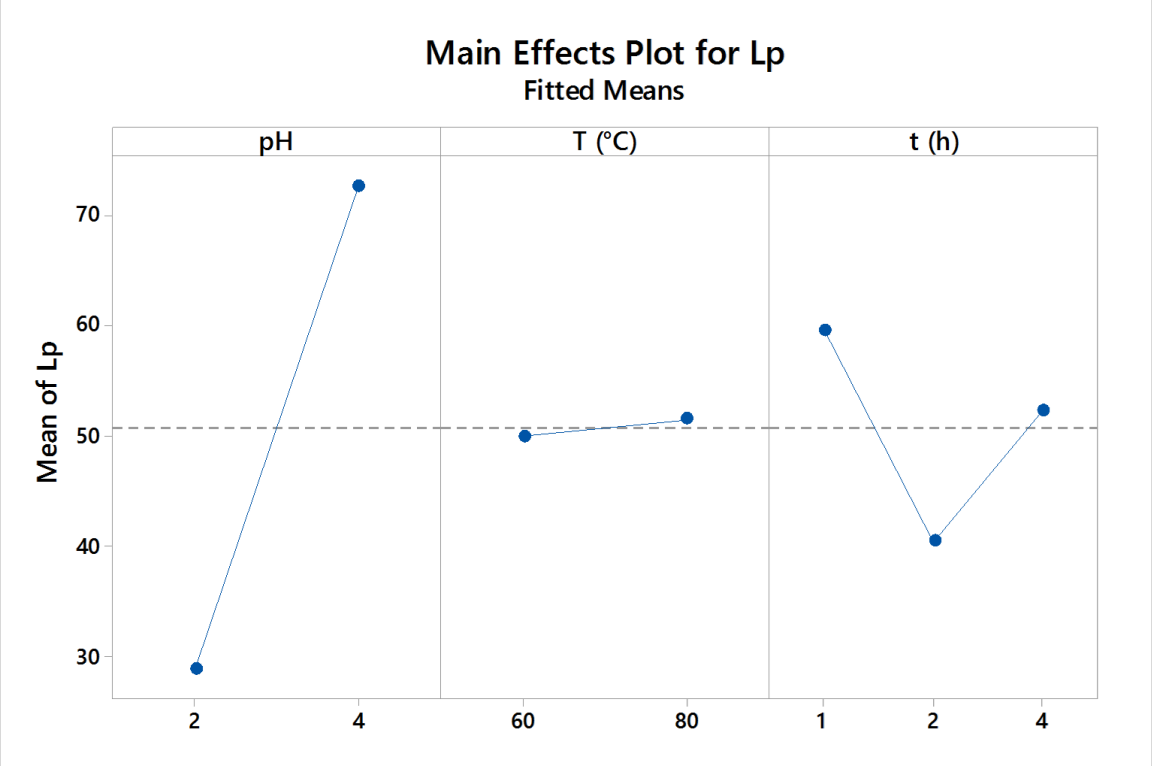


Figure 5.39: The main effect plots for persistence length: pH, temperature and time. The overall mean (51 nm) is shown as a dotted line and the steeper the slope the greater the effect of a particular parameter.

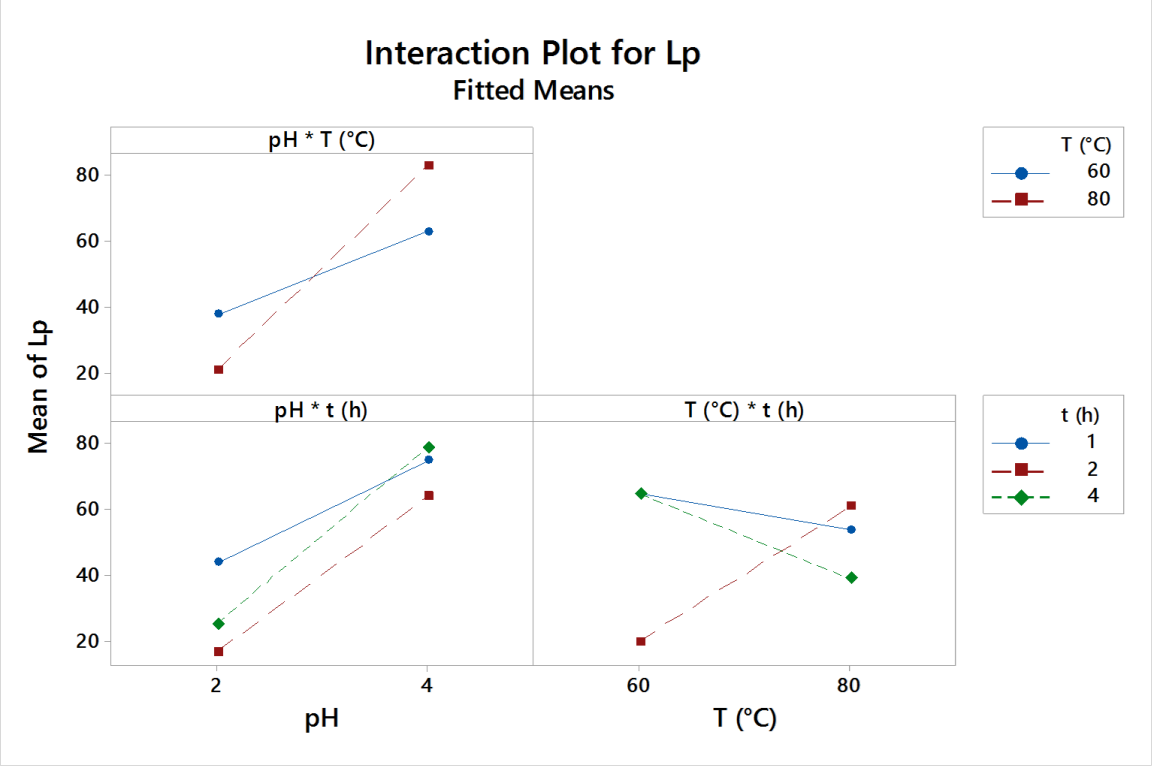


Figure 5.40: The interaction plots for persistence length. To visualize these effects, the Y-axis scale is always the same for each combination of factors. When the lines are parallel, interaction effects are zero.

In Figure **5.41** the slopes for all the two-factor interactions are non-parallel indicating there are interactions between the different extraction conditions, however, the interaction between temperature and time is the largest. The 2-factor interactions are 1027, 268 and 2446 for pH* temperature, pH*time and temperature*time, respectively. The 3-way (pH* temperature*time) interaction is 2500 and is therefore very important and demonstrates that the persistence length of pectins after extraction is difficult to control. Persistence length is also influenced by mass-per-unit length so, therefore, the effect of the different factors on this has been analysed in **Figures 5.42** and **5.43**.

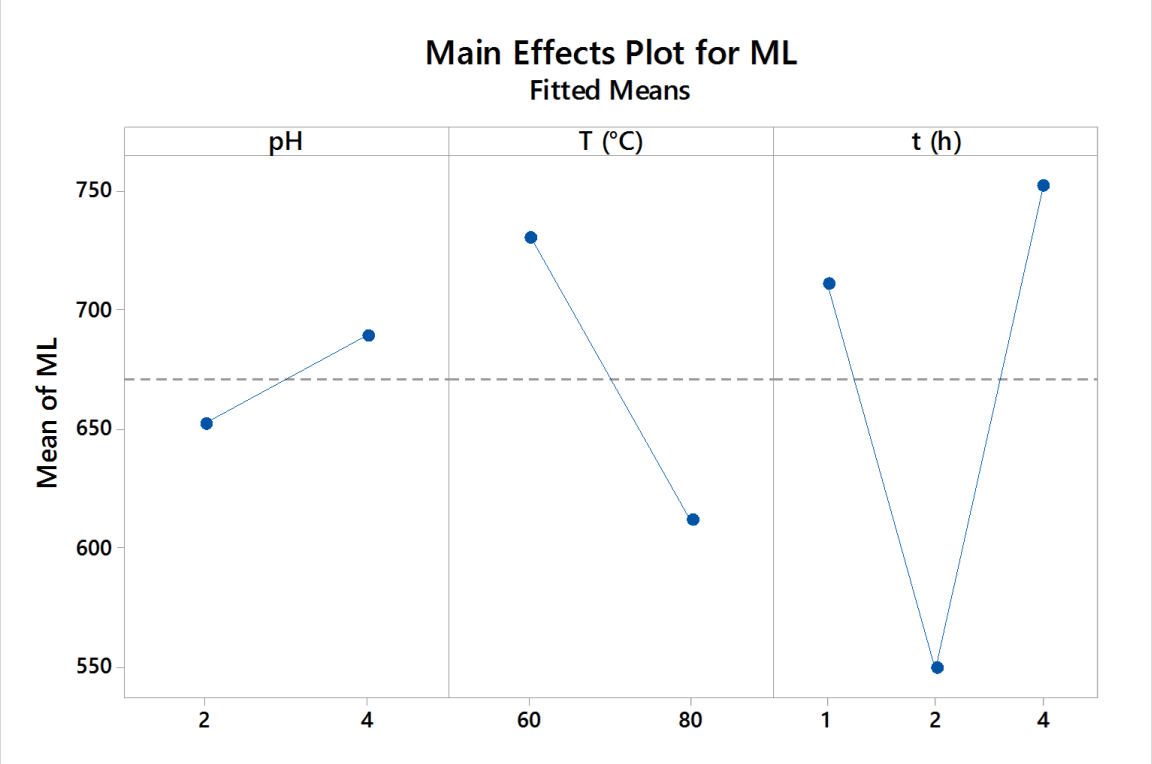


Figure 5.41: The main effect plots for mass per unit length: pH, temperature and time. The overall mean (671 g/ (mol nm)) is shown as a dotted line and the steeper the slope the greater the effect of a particular parameter.

Therefore, the main (the largest) effect on a mass per unit length is time (**Figure 5.42**), although in this case, the influence is complex.

In Figure **5.43** the slopes for all the two-factor interactions are non-parallel indicating there are interactions between the different extraction conditions, however, the interaction between temperature and time is the largest. The 2-factor interactions are 91000, 34000 and 216000 for pH* temperature, pH*time and temperature*time, respectively. The 3-way (pH* temperature*time) interaction is 321000 and is therefore very important and demonstrates that the mass per unit length of pectins after extraction is difficult to control.

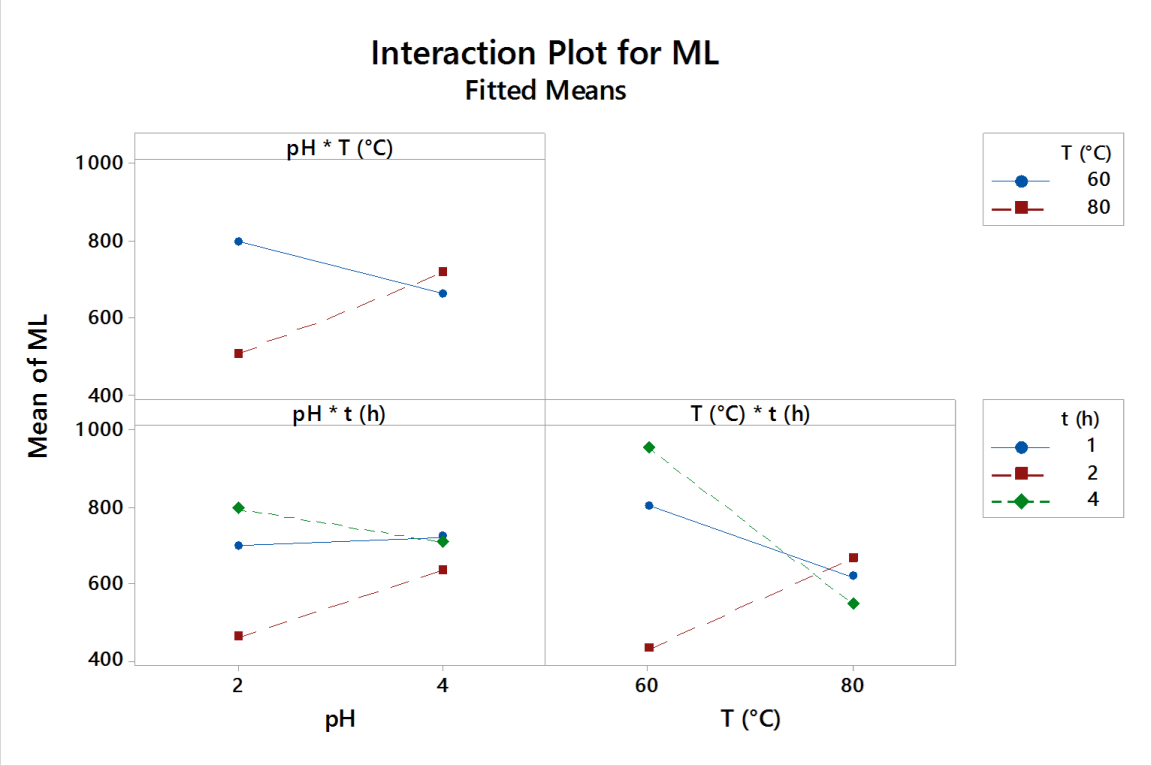


Figure 5.42: The interaction plots for mass per unit length. To visualize these effects, the Y-axis scale is always the same for each combination of factors. When the lines are parallel, interaction effects are zero.

As it difficult to see any meaningful trends from either persistence length or mass per unit length, the ratio of the two L_p/M_L may be more informative in this case (**Figures 5.44** and **5.45**).

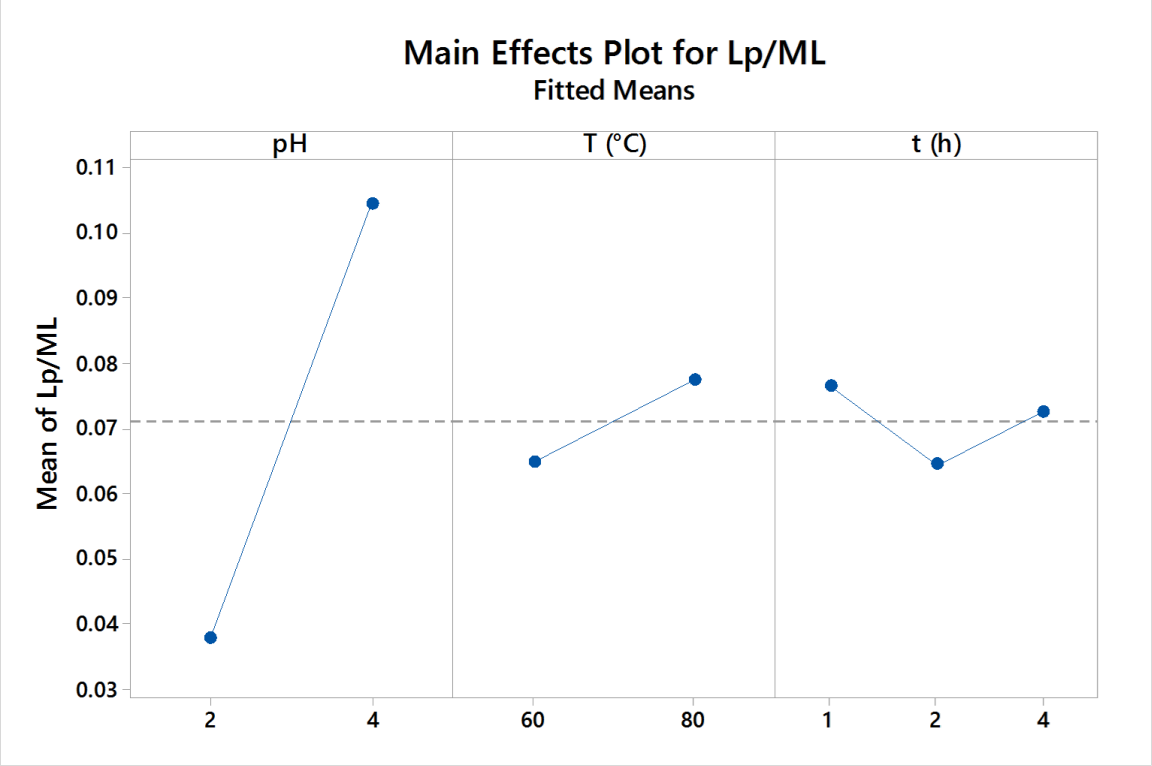


Figure 5.43: The main effect plots for L_p/M_L: pH, temperature and time. The overall mean (0.071 nm2 mol/g) is shown as a dotted line and the steeper the slope the greater the effect of a particular parameter.

The undertaking of an experimental design approach enables further understanding of the influence of each extraction condition and how they interact with one another. Therefore, the main (the largest) effect on L_p/M_L is pH (**Figure 5.44**). In general, the L_p/M_L increases moving from a low level (pH 2) to a high level (pH 4), indicating that pectins become stiffer (less flexible).

In **Figure 5.45** the slopes for all the two-factor interactions are parallel indicating there are little interactions between the different extraction conditions, however, the interaction between temperature and time is the largest. The 2-factor interactions are 3.85×10^{-4}, 4.01×10^{-4} and 3.52×10^{-3} for pH* temperature, pH*time and temperature*time, respectively.

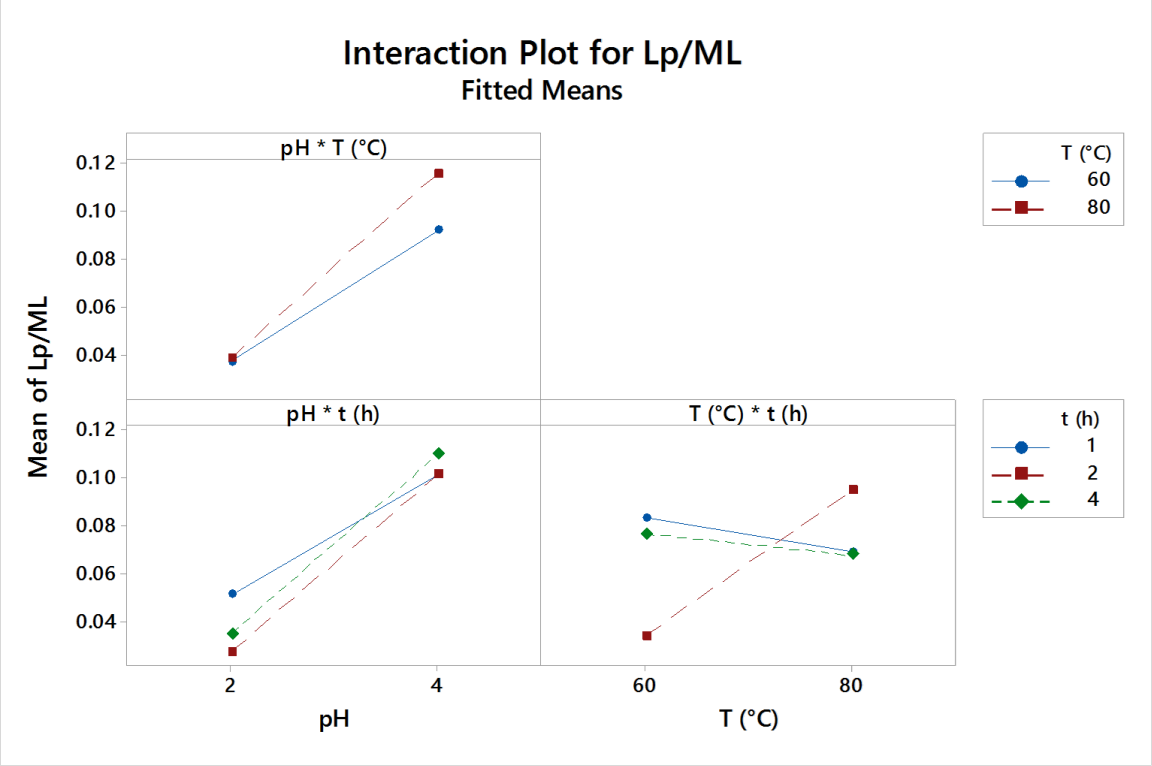


Figure 5.44: The interaction plots for L_p/M_L. To visualize these effects, the Y-axis scale is always the same for each combination of factors. When the lines are parallel, interaction effects are zero.

These estimates for persistence length and mass per unit length are solely derived from physical measurements (molecular weight and intrinsic viscosity), however as the chemical composition of these pectins has also been characterised the mass per unit length can also be estimated from the chemical composition, and persistence length estimated based on these measurements (**Table 5.17**).

From the data in **Table 5.17**, it is apparent that in most cases the persistence length is lower than when mass per unit length has been allowed to float and the values are now more typical of those for other pectins (Morris et al., 2008, Morris et al., 2010b). One important point that this does, however, highlight which is often overlooked is that when mass per unit length is allowed to float there are a number of possible conformations (see **Figure 5.39**) which are all quite close to the global minimum and in most cases they are essentially identical within experimental error. In real terms what this means is that perhaps Lp/M_L is a better indicator of stiffness/ flexibility as it mitigates for this problem.

Table 5.17: Mass per unit length M_L and persistence length (L_p) estimations from combining molecular weight and intrinsic viscosity data through HYDFIT for pumpkin pectin when the

Sample	[η] (g/ml)	Mw $\times 10^5$ g/mol	L_p (nm)	M_L (g/(mol nm))	L_p / M_L (nm^2 mol/g)
1	880	1.33	$27^{a,b}$	462	$0.0584^{a,b}$
2	947	2.21	$17^{c,d}$	461	$0.0369^{b,c,d,e}$
3	830	1.85	$16^{c,d,e}$	446	$0.0359^{b,c,d,e}$
4	537	2.42	$9^{e,f}$	442	$0.0204^{e,f}$
5	299	2.43	5^{f}	443	0.0113^{f}
6	260	2.23	5^{f}	455	0.0110^{f}
7	932	2.53	$15^{c,d,e}$	462	$0.0325^{c,d,e,f}$
8	1342	1.83	29^{a}	449	0.0646^{a}
9	810	1.46	$20^{b,c}$	446	$0.0449^{a,b,c,d}$
10	640	1.39	$16^{c,d,e}$	449	$0.0357^{b,c,d,e}$
11	503	1.66	$11^{d,e,f}$	449	$0.0245^{d,e,f}$
12	608	1.20	$20^{b,c}$	462	$0.0433^{a,b,c,d,e}$
13	966	2.83	$13^{c,d,e}$	448	$0.0290^{d,e,f}$
14	904	1.37	$25^{a,b}$	448	$0.0558^{a,b,c}$

mass per unit length has been fixed depending on the monosaccharide composition.

Values were given as means of triplicate ± SD. Means with different superscript letters within a column are significantly different (Tukey test, $p < 0.05$). SD = Standard deviation of the mean.

5.3.2 Conformation zoning (Normalised scaling relations)

Pavlov, Harding & Rowe (1999) developed a protocol which semi-empirically estimates the conformation of polymers in solution based on the relationship between their molar mass, intrinsic viscosity and mass per unit length, M_L. In this case, use has been made of the mass per unit length calculated previously using the HYDFIT algorithm (Ortega and García de la Torre, 2007) and from the chemical composition. As with estimates of persistence length, the normalised scaling plot is a useful tool for semi-quantitatively estimating polymer stiffness, however, it is very highly reliant on the quality of the determination of the mass per unit length as it is used in the estimation of both the x- and y-axes values. As seen in **Tables 5.16** and **5.17** in almost all cases the mass per unit length from the HYDFIT procedure is typically higher (a notable exception being sample 5) and therefore stiffness estimates from floated data will have a larger y-value and a lower x-value and therefore more stiff using this procedure. This can be seen clearly in **Figure 5.46**, again with the notable exception of sample 5. That being said the conformations estimated for these pectin samples are consistent with persistence length estimates in **Tables 5.16** and **5.17** and are in general agreement with previous studies on similar pectins (Morris et al., 2008, Morris et al., 2010b). See for example **Figure 3.8c**.

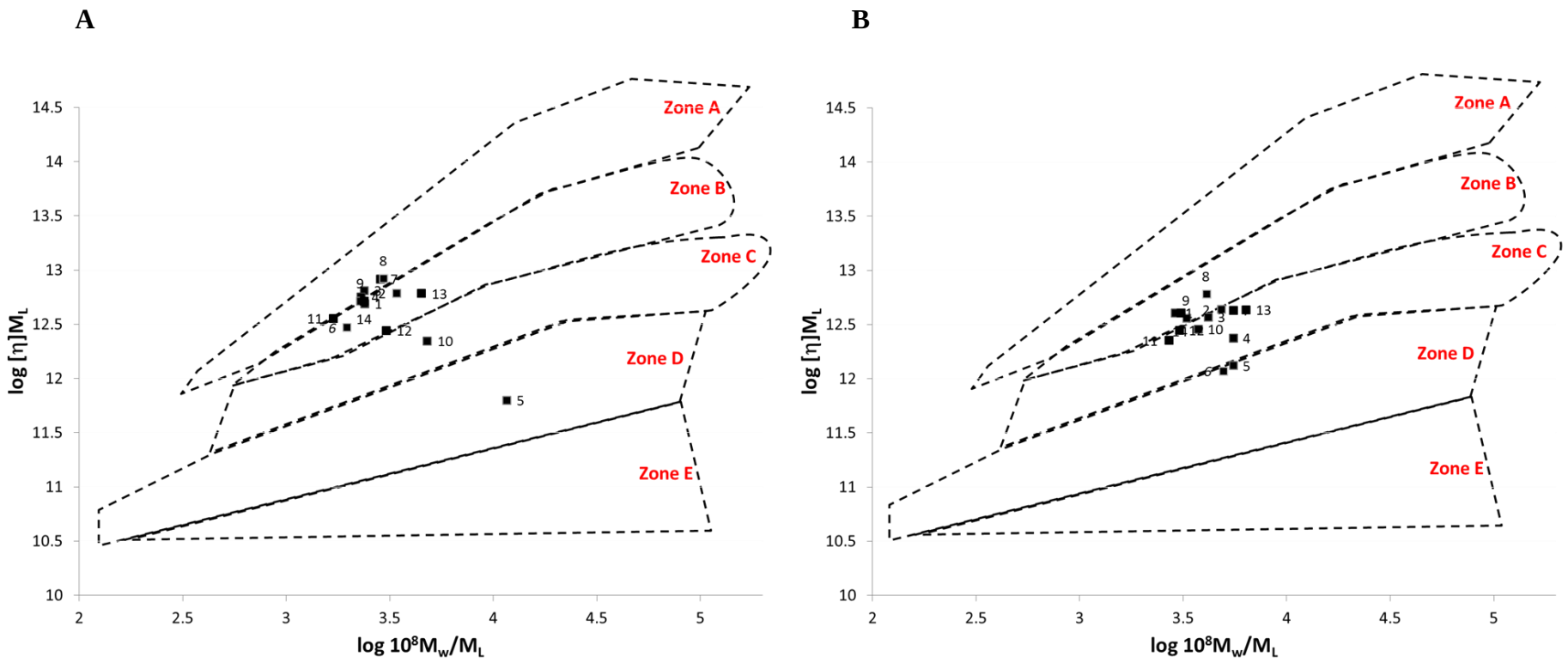


Figure 5.45: Normalised scaling plot of $[\eta]M_L$ versus M_w/M_L adapted from Pavlov et al., (1999) where the neutral sugar content for each pumpkin pectin was used. Zone A: extra rigid rod; Zone B: rigid rod; Zone C: semi-flexible; Zone D: random coil and Zone E: globular or branched. A: when mass per unit length has been fitted form physical data using HYDFIT (**Table 5.16**) and B: when mass per unit length was been calculated from chemical composition (**Table 5.17**). For clarity, ■ represents the pectin's samples which have been named 1-14 as per **Table 5.17**.

5.3.3 Degree of branching

From the physicochemical data (**5.1** & **5.2**) coupled with the conformational data (**5.3**), there is the potential to estimate both the average number and length of side chains on a pectin molecule. Again, this can be done using the mass per unit length calculated from the freely floated HYDFIT procedure (**Table 5.16**). Which then with knowledge of the HG: RG-I ratio enables the estimation of the mass per unit length of both the HG and RG-I sub-fractions. In order to partially verify this method, an example with previously well characterised HG and RG-I regions from sugar beet (Morris et al., 2010b) where the average side chain length for an HG region should be zero has been included.

It is believed that , this is the first time this type of analysis has been used to estimate the number and length of pectin side chains. Clearly, there is a large difference in the results of the model for the different pectin samples, but what can be observed is that in general, these pectins have a large number of relatively short side chains. These side chains are large when compared to those from sugar beet pectin analysed using the same technique, but short when compared to those visualised by atomic force microscopy (Paniagua et al., 2014), which had a size of ~ approximately 30 – 170 nm (~60 – 340 monosaccharide units assuming a typical monomer is 0.5 nm). Furthermore, there is no apparent correlation between this parameter and the chemical composition, although it does appear that the more rigid molecules have a larger number of shorter side chains.

Table 5.18: Average number and degree of branching for pumpkin pectin when the mass per unit length was allowed to float[a]

Sample	Average side chain length (number of monosaccharides)	Average number of side chains
1	4	81
2	6	101
3	9	43
4	16	29
5	n.d.	n.d.
6	14	40
7	11	52
8	7	52
9	7	35
10	n.d.	n.d.
11	7	37
12	2	156
13	7	81
14	5	59
HG	n.a.	0
RG-I	3	346

[a]in order to determine the degree of number and degree of branching, knowledge of the mass per unit length, HG: RG-I ratio, weight average molecular weight and the average mass of side chain sugar ratio which is, in this case, the weighted average mass of neutral sugar residues is required.

5.4 Antioxidant activity of pumpkin pectin

There are several assays using various substrates, reaction kinetics, and analytical methods can be used to estimate antioxidant activity (Mahattanatawee et al., 2006).

5.4.1 DPPH radical-scavenging activity:

The radical-scavenging activity is a significant characteristic of antioxidants (Ornano et al., 2013). DPPH is a free radical compound, which is commonly used as a method to estimate the free radical scavenging ability of natural compounds. DPPH is one of the compounds that possessed a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers (Yamaguchi et al., 1998). In addition, DPPH assay is the most viable for hydrophobic antioxidant evaluation (Wang et al., 2016).

DPPH is a constant free radical that indicates maximum absorption at 517 nm in methanol. Whereby in the test, stable DPPH radicals and hence the absorbance at 517 nm can be reduced by the antioxidants (Jao and Ko, 2002). The impact of antioxidants on DPPH radical scavenging was clearly due to their hydrogen donating ability. Azizah et al., (2009) reported that all fresh and cooked pumpkins are able to minimize the stable, purple-coloured radical DPPH into the yellow coloured DPPH-H reaching 50 % of reduction. The antioxidant activity of a substance can be expressed as its ability in scavenging the DPPH free radical (Wang et al., 2014, Fan et al., 2009). Total DPPH scavenging effects of all samples at different extraction conditions were measured and the results were depicted in **Table 5.19**.

Table 5.19: Indicates DPPH radical-scavenging activity of a range of pumpkin sample solutions

Sample	Free radicals scavenging effects DPPH (%)
1	68 ± 0.6^{d}
2	70 ± 0.6^{c}
3	73 ± 0.6^{b}
4	$69 \pm 0.6^{c,d}$
5	78 ± 0.6^{a}
6	37 ± 0.6^{k}
7	40 ± 0.6^{j}
8	39 ± 0.6^{j}
9	45 ± 0.6^{i}
10	49 ± 0.6^{g}
11	54 ± 0.6^{e}
12	47 ± 0.6^{h}
13	52 ± 0.6^{f}
14	30 ± 0.6^{l}

Values were given as means of triplicate ± SD. Means with different superscript letters within a column are significantly different (Tukey test, $p < 0.05$). SD = Standard deviation of the mean.

The scavenging ability ranged from 30 % to 78 %. It is clear that the samples display considerable ability to scavenge DPPH radicals. Sample 5 which was extracted at pH 2, 60 ℃ under 2 h extraction time exhibited the highest DPPH radical-scavenging activity of 78 %, which is much higher than some of the other pumpkin samples. Meanwhile, the DPPH free radical-scavenging activity of samples 2 and 3: 70 %, 73% respectively were in agreement with those obtained by (Kanatt et al., (2007), Murcia et al., (2004). In addition, the highest value found (sample 5) is close to the results for fresh pumpkin: 87.4 % and also to those boiled for a few minutes which ranged from 81 - 94 % (Azizah et al., 2009). This is true even though the pumpkin samples used in the present study were kept during the extraction for hours rather than minutes.

Pumpkin samples also have similar DPPH activity in comparison to chickpea protein hydrolysate (CPH) (Li et al., 2008).

These values of DPPH radical scavenging are lower than those reported by Fan et al., (2009), Mau et al., (2005a), Huang et al., (2002) and Dalonso and de Oliveira Petkowicz, (2012). Also, lower than mung bean extract MB1and butylated hydroxyanisole BHA which were examined in Lai et al., (2010) whereas sample 2 is similar with mung bean extract MB2 in the same study. In addition, Mau et al., (2005a) found that at 5 mg/ml, scavenging abilities of the hot water extracts from mature and baby Ling Chih, mycelia and filtrate were 71.9, 67.1, 59.6 and 50.0 %, respectively, which are comparable with those presented in this study. These results show that the purified polysaccharide especially at temperature 60 °C has a remarkable impact on scavenging free radicals. In total, in this study all tested pumpkin varieties showed significant scavenging activities against DPPH radicals. The antioxidant mechanisms of pumpkin extracts may be attributed to strong hydrogen donating ability, and their effectiveness as scavengers of free radicals (Que et al., 2008).

In general, the extraction and isolation methods with many factors including chemical components, molecular mass, structure and conformation have a significant influence on the bioactivities of polysaccharides (Yuan et al., 2008), although from **Figure 5.47** pH of extraction has no effect on DPPH activity.

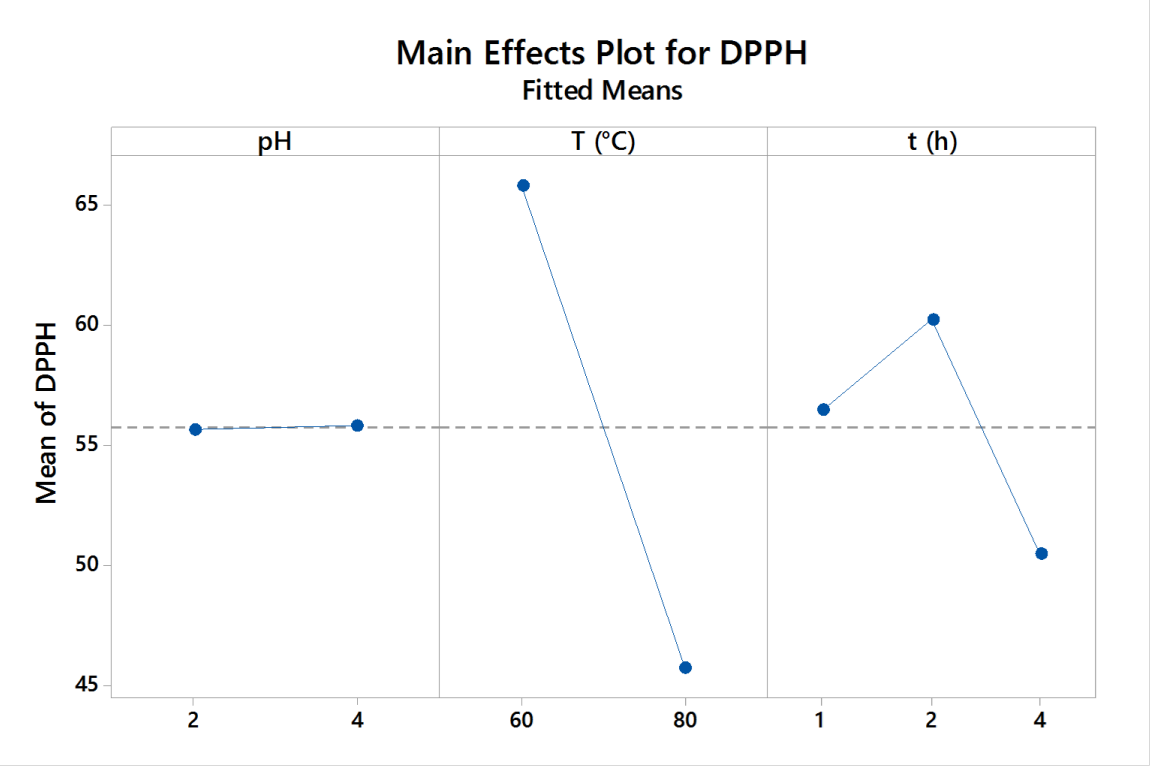


Figure 5.46: The main effect plots for the DPPH assay: pH, temperature and time. The overall mean (~56 %) is shown as a dotted line and the steeper the slope the greater the effect of a particular parameter.

The experimental design approach employed allows further understanding of the influence of each extraction condition and how they interact with one another. Therefore, the main (the largest) effect on DPPH is temperature (**Figure 5.47**). In general the DPPH activity decreases moving from low level (60 ºC) to high level (80 ºC), indicating that pectins have less DPPH activity when they are extracted at higher temperatures, this is clearly important from a processing point of view, although the 2- and 3-factor interactions need to be taken into consideration.

In **Figure 5.48** the slopes for all the two-factor interactions are non-parallel indicating there are interactions between the different extraction conditions, however, the interaction between pH and time is the largest. The 2-factor interactions are 234, 446 and 193 for pH* temperature, pH*time and temperature*time, respectively. The 3-way (pH* temperature*time) interaction is 155. This further demonstrates the importance of extraction conditions on the functional properties of pectins and polysaccharides in general.

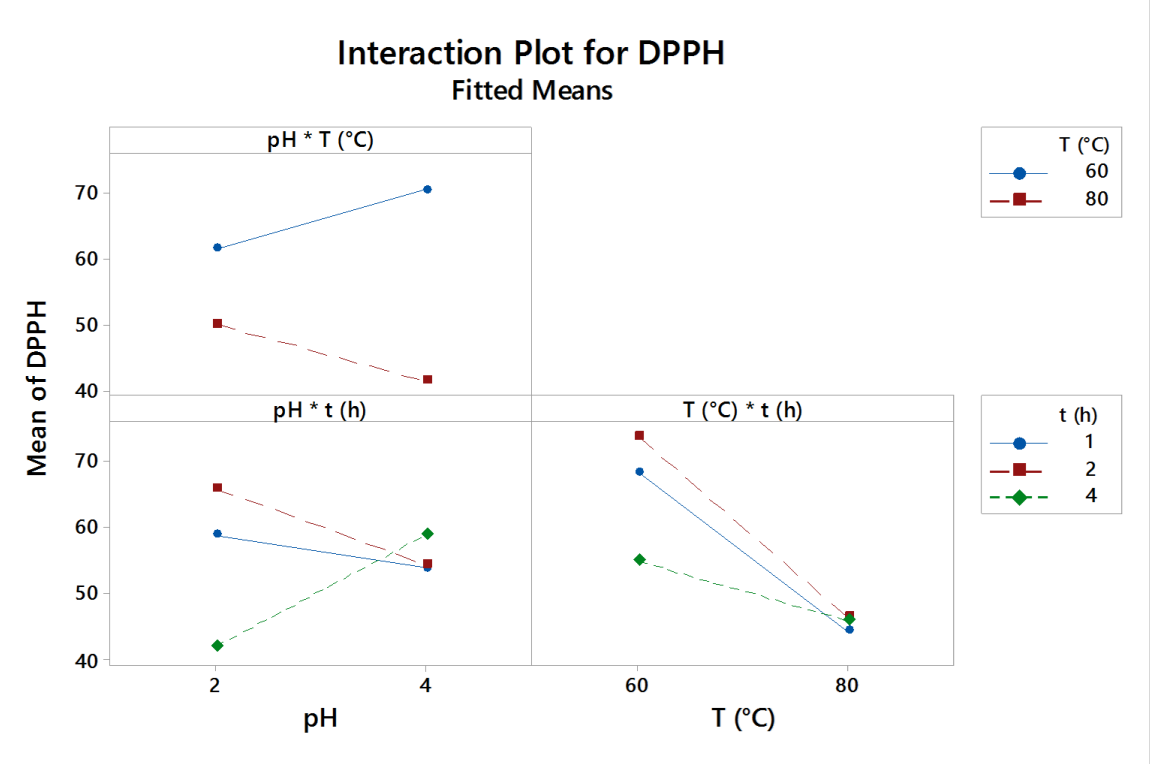


Figure 5.47: The interactions plots for the DPPH assay. To visualize these effects, the Y-axis scale is always the same for each combination of factors. When the lines are parallel, interaction effects are zero.

5.4.2 Hydroxyl radical inhibition assay:

The hydroxyl radical is the most reactive in chemistry and it can react with all macromolecules in living cells which leads to intense harm to the neighbouring macromolecules (Zhang et al., 2013). In addition, it can be formed from superoxide anion and hydrogen peroxide, in the presence of metal–ions, such as copper or iron (Li et al., 2008).

The pumpkin samples were analysed for hydroxyl radical-scavenging activity to better examine their antioxidant properties. **Table 5.20** shows the percentage hydroxyl scavenging effects of purified pumpkin samples extracted under different conditions. The data showed that the effects of scavenging hydroxyl radicals were high for all these samples. However, there was no significant difference in scavenging activity between all pumpkin samples; they are similar to each other. This may due to the amount of GalA acid in the sample which is previously suggested as an effective indicator of the antioxidant activity of the samples (Li et al., 2011). The inhibition of the pumpkin

samples was much higher than those reported by Yuan et al., (2008), Mau et al., (2001), Mau et al., (2005a) and Li et al., (2008).Values were also higher than those obtained by (Zha et al., 2009) for polysaccharide precipitated with 60% ethanol (PW2) and 80% ethanol (PW3) but are similar to that precipitated with 40% ethanol (PW1). In contrast, the results observed for pumpkin pectin were lower than those reported by Dalonso and de Oliveira Petkowicz, (2012) and Lai et al., (2010) . Nevertheless, these results clearly showed that pumpkin pectin under different extraction conditions has the high potential antioxidant ability to scavenge hydroxyl radicals. Antioxidants may be able to inhibit the peroxidation reaction, which leads to the destruction of β-cells in diabetes patients, and therefore pectins with these structural motifs may provide protection against this. In this case, as the hydroxyl assay shows little or no effect of extraction conditions on the percentage inhibition it is unnecessary to show the effects and interactions plots.

Table 5.20: indicates the hydroxyl radical inhibition of pumpkin pectins using different extraction condition.

Sample	Hydroxyl radical inhibition %
1	$97 \pm 0.6^{a,b}$
2	$97 \pm 0.6^{a,b}$
3	$95 \pm 0.6^{b,c}$
4	94 ± 0.6^{c}
5	$95 \pm 0.6^{b,c}$
6	$97 \pm 0.6^{a,b}$
7	$97 \pm 0.6^{a,b}$
8	$97 \pm 0.6^{a,b}$
9	$97 \pm 0.6^{a,b}$
10	$97 \pm 0.6^{a,b}$
11	$97 \pm 0.6^{a,b}$
12	98 ± 0.6^{a}
13	$97 \pm 0.6^{a,b}$
14	$97 \pm 0.6^{a,b}$

Values were given as means of triplicate ± SD. Means with different superscript letters within a column are significantly different (Tukey test, $p < 0.05$). SD = Standard deviation of the mean.

5.5 Summary of results for pumpkin pectins

In this chapter, the physicochemical properties of pectin were studied under different extraction conditions (pH, temperature and time). Pectin samples contained some protein with high DAc and high total carbohydrate content, which were determined experimentally using a spectrophotometric method. The HPAEC data showed the concentrations of the different constituent sugars that were in each sample and all showed the presence of the major pectin monomeric units rhamnose, arabinose, galactose and galacturonic acid in high concentrations. These monosaccharide compositions indicate that the extracted polysaccharides were as expected mostly pectins, with 24-35 % being RG-I and 55-68 % and being HG. The remaining polysaccharides are most likely cellulose and hemicelluloses. This demonstrates that

the structures are linear with rhamnose as the linking branching unit. This data was confirmed by NMR and GC-MS data (not shown) as the methylated samples that were run through the GC-MS were less successful and so full linkage analysis of the pectins could not be given. The structural features of extracted pectin samples and molecular weights (M_w) were characterized by ^1H NMR spectroscopy and high-performance size-exclusion chromatography. The SEC-MALLS data showed that all pumpkin samples have high molecular weights that could be due to the extraction conditions that were carried out for each sample. The number average molecular weight of each sample was sensible for that of a pectin-based polysaccharide. Thus, the intrinsic viscosity of the pumpkin pectin solutions was high. The measurements of zeta potential for pectins featured ζ values representations negative surface charge. The FT-IR data shows that there is a degree of methyl esterification which can only occur if there is galacturonic acid present in the sample. The high degree of esterification helps to support the fact that there is a high concentration of galacturonic acid present in the samples, therefore; this pectin is high methoxy pectin, HMP. Besides, it is also expected that these extraction conditions will have an influence on conformation and any potential bioactivity. Whereby, using DPPH and hydroxyl radicals were positively good for assessment of the sample's antioxidant properties. Principal component analysis (PCA) plot for pumpkin pectins was used to emphasise variation in samples by using some information determined for the pectins to give a global picture of pectin structure and quality. Moreover, the global analysis method (HYDFIT) was employed to explore the pectin’s conformation.

5.5.1 PCA (principal component analysis):

A principal component analysis (PCA) plot for pumpkin pectins using different extraction conditions is shown in **Figure 5.49** (see information beside each point for extraction pH, temperature and time). The PCA plot can be used to emphasise variation in samples and bring out strong patterns or similarities in a dataset. In this case, some features of pumpkin pectin determined above has been used to give a global picture of pectin structure and quality. In the PCA the first component is positively correlated with molecular weight and negatively with intrinsic viscosity (see **Figure 5.50**) which may be reflective of different pectins with different molecular weights and conformations being extracted under different conditions or again with the concept of a two-stage extraction process of solubilisation and depolymerisation, probably due to the loss of arabinose for example. Factors which influence the second component are yield and galactose content for example, whereas galacturonic acid content is negatively correlated which would suggest that higher yield would, unfortunately, go hand-in-hand with lower quality (lower GalA) pectins.

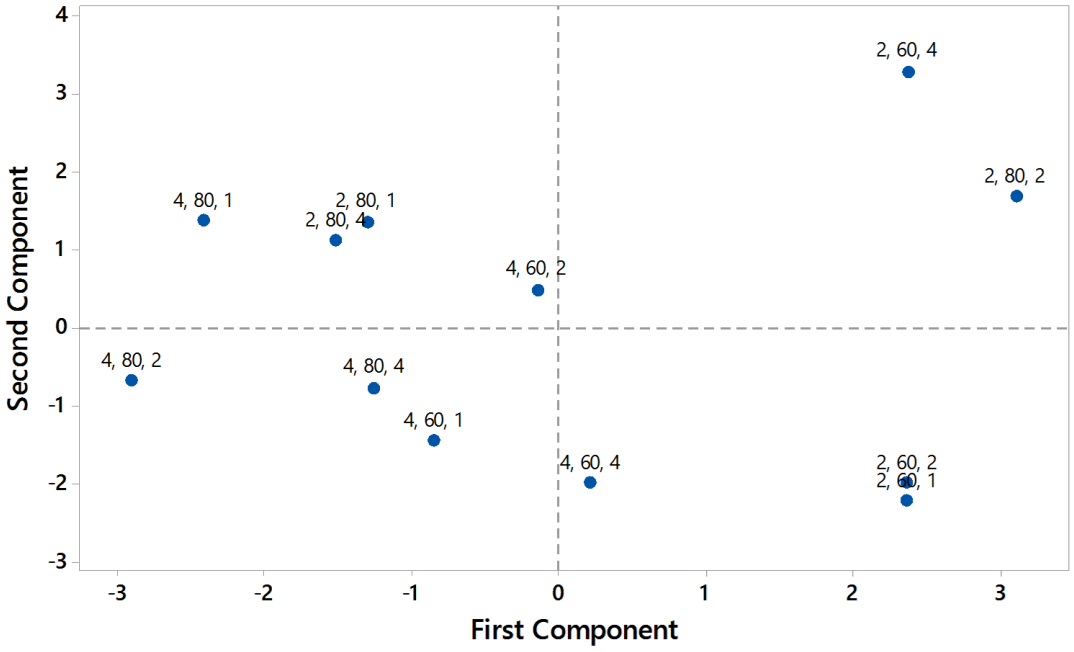


Figure 5.48: A principal component analysis (PCA) plot for pumpkin pectins using different extraction conditions (see information beside each point for extraction pH, temperature and time).

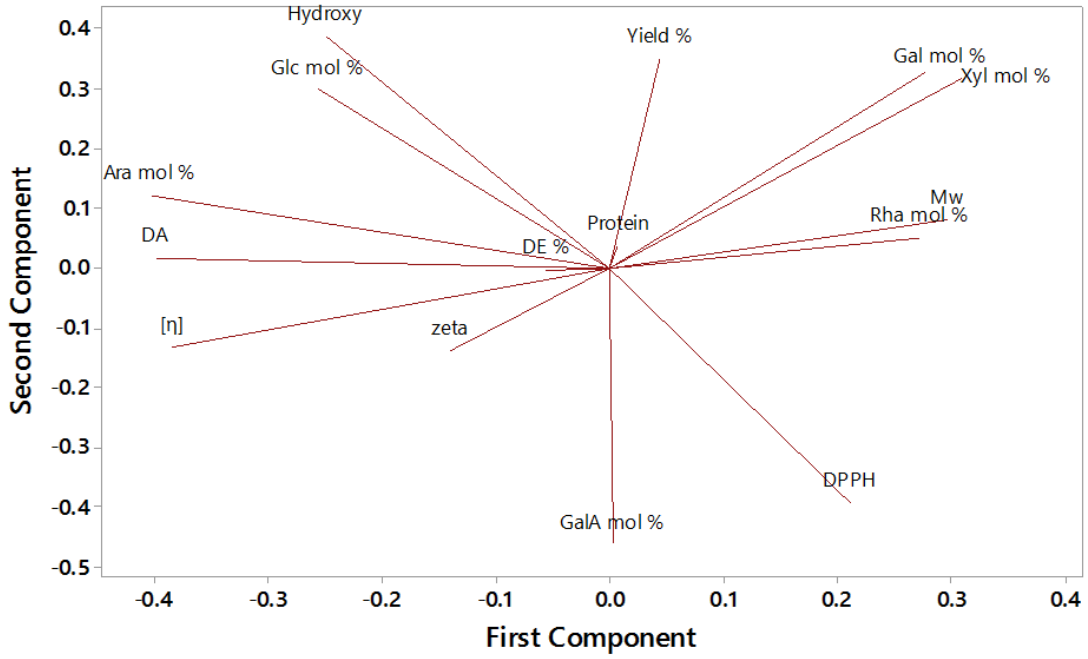


Figure 5.49: loading biplot for pumpkin pectins using different extraction conditions.

The loading biplot also shows the following:

- Galactose, xylose, rhamnose, and molecular weight have large positive loadings on component 1. Therefore, this component focuses on the size of the pectin and the RG-I fraction.
- Galacturonic acid and DPPH have large negative loadings on component 2. Therefore, this component demonstrates that these properties could be related (Li et al., 2011).

5.6 Conclusions

Pumpkin pectins have been characterised in terms of their chemical composition (**5.1**), physical properties (**5.2**), solution conformation (**5.3**) and their antioxidant activities (**5.4**). These properties have then been related to the extraction conditions (pH, temperature and time); this can now enable the estimation of optimum extraction conditions for specific properties. For example, if for a specific application a pectin of high galacturonic acid, high molecular weight, high DPPH activity, but low intrinsic viscosity is required the optimum extraction conditions would be pH 2 at 60 ºC for 2 hours.

Chapter 6

Characterisation of okra pectins

6 CHARACTERISATION OF OKRA PECTIN

To study the influence of different genetic variation on the molecular characteristics of pectic substances extracted from fresh okra pods an isolation protocol was designed. The first step **Figure 4.2**, the extraction with petroleum ether (25 ºC) was completed to obtain a lipid-free material, which was then used in aqueous extraction at pH 6.0 with 0.1 mM phosphate buffer. The physiochemical characterization of okra pectins (yield, protein content, DAc, DE, GalA content, total sugars, neutral sugars, sugar ratios, viscosity, zeta potential, NMR determination, antioxidant activity and conformational analysis are shown in the tables and figures of this chapter. During the current examination, a total of 12 physiochemical characteristics were determined and the analysis was undertaken as per standard procedures (**Table 5.1**). The results will be compared with previous studies on okra and other polysaccharides from similar sources, for example, apple pomace, peach, *etc*.

6.1 Chemical Characterisation of okra pectin:

6.1.1 Determination of yield

The percentage yield was calculated based on the amount of dry powder sample used for the extraction process and the amount of dry soluble mucilage polysaccharide obtained after extraction (Archana et al., 2013, Samavati, 2013). The percentage yield (w/w) was calculated using the formula:

$$\text{Percentage extraction yield} = \frac{\text{The weight of dried mucilage obtained}}{\text{the weight of powder taken}} \; x \; 100 \qquad 6.1$$

Table 6.1: Pectin yield of okra genotypes.

Sample	Short name	Yield %
AGBAGOMA	AGB	14.2 ± 0.1^{a}
AGBAGOMA deep green fresh	AGB (F)	n.d
PEMKRUMAH (TECHI)	PEM (T)	11.3 ± 0.1^{b}
PEMKRUMAH (KEN)	PEM (K)	14.4 ± 0.1^{a}
KPOMG (TAMELE)	KPO (T)	14.4 ± 0.1^{a}
ASONTEM (KEN)	ASO (K)	13.2 ± 0.1^{a}
AGBAGOMA (AG)	AGB (AG)	n.d
SENGEVI	SEN	11.3 ± 0.1^{a}
ASHA	ASHA	14.6 ± 1.2^{a}
AISHA FRESH	ASHA (F)	n.d

Values were given as means of triplicate ± SD. Means with different superscript letters within a column are significantly different (Tukey test, $p < 0.05$). SD = Standard deviation of the mean.

The okra genotypes had pectin yields of 11.3–14.6 % of the crude polysaccharide extract, which is probably not too surprising as they were all extracted under the same extraction conditions [*N.B.* there was not sufficient information available to calculate the yield for samples AGB (F), AGB (AG) and ASHA (F)]. The pectin yield (%) of the crude extracts from the okra genotypes studied were higher than yields reported for extracts using acetone (8.6 %), methanol (0.28 %), and distilled water containing 1% sodium metabisulphite (1.46 %) (Archana et al., 2013, Noorlaila et al., 2015), but comparable with yields previously obtained by Alba et al., (2015) and Levigne et al., (2002a) for okra and sugar beet pectins respectively. Likewise, they are in good agreement with those obtained by Samavati, (2013) who stated that temperature has an important influence on the extraction yield of okra polysaccharides. The current okra polysaccharides isolation was performed at 80 ºC in order to facilitate the solubilisation of insoluble pectic substances (protopectin) easily. From **Table 6.1** samples AGB, PEM (K), KPO (T) and ASHA had yield values similar to each other at ~ 14 % those values of yield were close to those for sugar beet pectin (Micard and Thibault, 1999b). Besides, they were in close to those in (Mesbahi et al., 2005) for pectin from sugar beet pulp at pH 1, 80 ºC under 1 h extraction time. In addition, samples PEM (T) and SEN were similar as well at 11 %, these latest two samples and sample ASO (K) were in good agreement with those obtained by

Sengkhamparn et al., (2009b) for hot buffer soluble solids HBSS and dilute alkaline soluble solid DASS of okra polysaccharides 11.2 % and 13.2 % respectively.

Although the temperature used in this extraction was 80 ºC this is in contrast with that of Chen et al., (2014) who stated that yield decreased with a higher temperature which may be due to the partial degradation of pectin. However, in general the yields are consistent with other sources of pectins (Noorlaila et al., 2015, Archana et al., 2013, Samavati, 2013, Alba et al., 2015, Levigne et al., 2002a, Mesbahi et al., 2005, Sengkhamparn et al., 2009b, Chen et al., 2014).

6.1.2 Determination of total carbohydrates:

Table 6.2: Shows the total polysaccharides % for different okra genotypes.

Sample	Short name	Total carbohydrate (%)
AGBAGOMA	AGB	70.2 ± 0.1^{e}
AGBAGOMA deep green fresh	AGB (F)	68.2 ± 0.1^{d}
PEMKRUMAH (TECHI)	PEM (T)	58.4 ± 0.01^{a}
PEMKRUMAH (KEN)	PEM (K)	59.2 ± 0.1^{a}
KPOMG (TAMELE)	KPO (T)	68.3 ± 0.1^{d}
ASONTEM (KEN)	ASO (K)	63.0 ± 0.1^{b}
AGBAGOMA (AG)	AGB (AG)	64.5 ± 0.1^{c}
SENGEVI	SEN	59.2 ± 0.1^{a}
ASHA	ASHA	68.2 ± 0.1^{d}
AISHA FRESH	ASHA (F)	63.2 ± 0.1^{b}

Values are mean (SD) of at least triplicate determinations. Mean values in a column with different letters are significantly different ($p < 0.05$).

An estimation of the crude polysaccharides extracts had total polysaccharide ranging from (58.4 % –70.2 %) **Table 6.2**. The total carbohydrate levels for all crude okra pectins studied in the present work were close to each other and was lower than those for extracts obtained by Alba et al., (2015): 70 – 81.8 %. Furthermore, they were significantly lower than the total okra polysaccharides found by Sengkhamparn et al., (2010), Sengkhamparn et al., (2009b). In contrast, they were higher than the total polysaccharides content estimated by Xia et al., (2015) for okra pods (38.65 %). Besides, they were higher than those obtained by Sato et al., (2011)

for apple pomace pectin (39.13 %). It is of note that as different monosaccharides have different responses to the total carbohydrate assay (Dubois et al., 1956a, Masuko et al., 2005) therefore the total estimate will depending on the monosaccharide composition of the pectin and on the standard used.

6.1.3 Determination of protein content:

Table 6.3: Protein content of pectin isolated from different okra genotypes.

Sample	Short name	Protein content %
AGBAGOMA	AGB	8.0 ± 0.1^{a}
AGBAGOMA deep green fresh	AGB (F)	10.1 ± 0.1^{b}
PEMKRUMAH (TECHI)	PEM (T)	13.6 ± 0.1^{d}
PEMKRUMAH (KEN)	PEM (K)	14.7 ± 0.1^{e}
KPOMG (TAMELE)	KPO (T)	11.1 ± 0.1^{c}
ASONTEM (KEN)	ASO (K)	14.8 ± 0.1^{e}
AGBAGOMA (AG)	AGB (AG)	15.1 ± 0.1^{e}
SENGEVI	SEN	9.5 ± 0.1^{b}
ASHA	ASHA	8.0 ± 0.1^{a}
AISHA FRESH	ASHA (F)	10.1 ± 0.1^{b}

Values are mean (SD) of at least triplicate determinations. Mean values in a column with different letters are significantly different ($p < 0.05$).

An evaluation of the protein level of the crude polysaccharide extract had a protein content between 8.0 –15.1 %. The protein levels of the pectins studied were lower than those of okra extracts obtained by Alba et al., (2015): 4.3-6.3 % but higher than those in Alba et al., (2013), Happi Emaga et al., (2008) and Mesbahi et al., (2005). This is possible because the crude pectins obtained were not further purified by exhaustive dialysis. However, protein amounts in the present study were close to data already published for okra by (Sengkhamparn et al., 2009b) for chelating-agent soluble solids CHSS: 10.5 % and concentrated alkaline soluble solids CASS: 13.2 % also to those obtained for okra by Georgiadis et al., (2011) for dilute alkaline soluble solids DASS: 11.4 %. In addition, the protein content for pectin AGB (AG) was similar to that reported for hot buffer soluble solids (HBSS) pectin by Thöle et al., (2015). Any differences may be attributed to the difference in composition between various cultivars of okra and geographical variations (Georgiadis et al., 2011).

6.1.4 Determination of uronic acid:

Table 6.4: Indicates GalA % of okra genotype using the same extraction method.

Sample	Short name	GalA %
AGBAGOMA	AGB	55.3 ± 0.1^{g}
AGBAGOMA deep green fresh	AGB (F)	58.7 ± 0.2^{e}
PEMKRUMAH (TECHI)	PEM (T)	$\mathbf{66.4 \pm 0.1^{b}}$
PEMKRUMAH (KEN)	PEM (K)	56.2 ± 0.07^{f}
KPOMG (TAMELE)	KPO (T)	$\mathbf{65.6 \pm 0.4^{c}}$
ASONTEM (KEN)	ASO (K)	51.7 ± 0.2^{h}
AGBAGOMA (AG)	AGB (AG)	43.7 ± 0.2^{j}
SENGEVI	SEN	55.5 ± 0.1^{g}
ASHA	ASHA	60.4 ± 0.4^{d}
AISHA FRESH	ASHA (F)	$\mathbf{72.6 \pm 0.4^{a}}$

Values are mean (SD) of at least triplicate determinations. Mean values in a column with different letters are significantly different ($p < 0.05$).

In the present work, the group of ten genotypes had GalA content with a minimum of 43.7 % and a maximum of 72.6 % see **Table 6.4.** From a commercial point of view samples PEM (T), KPO (T) and ASHA (F) are greater than the minimum 65 % galacturonic acid content required for pectins (highlighted in bold) whilst a number of other samples would also meet this specification after protein removal. These values of GalA were close to those previously determined by Alba et al., (2015) for okra: 46.8 % and 56.9 % and also close to those in sugar beet and apple pomace (Levigne et al., 2002a, Sato et al., 2011). In contrast, they were significantly higher than those obtained by Sengkhamparn et al., (2009b) (Sengkhamparn et al., 2009b) for okra hot buffer soluble solids HBSS 35% but in good agreement with dilute alkaline soluble solids (DASS 48 %) and chelating-agent soluble solids (CHSS 63 %) in the same study.

6.1.5 Determination FT-IR spectra:

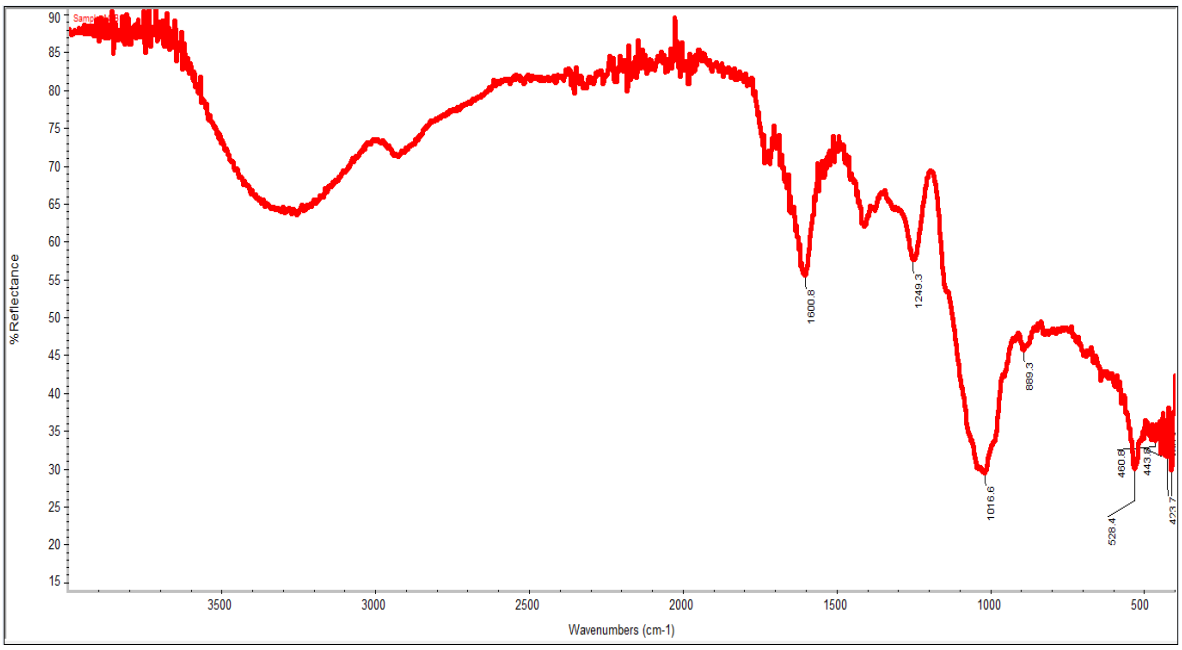
Polysaccharides extracted from okra pods were analysed using FT-IR spectroscopy in the region 900 - 4000 cm^{-1}. **Figure 6.1** indicates spectra for ten different genotypes of okra pectins. It was found that all the FT-IR spectra of okra polysaccharides presented a comparable

common pattern and showed similarities of the absorption patterns, although, they were from different varieties. This in agreement with Sato et al., (2011) who reported that samples from the same source/origin can be expected to have lower/different FT-IR spectral variations, where variations of this study were totally different from those obtained by (Alba et al., 2015) for okra pods. The frequencies and the peak assignments of surface functional groups of pectins are shown in **Table 6.5.** In pectin samples, the O−H stretching and bending vibrations can be seen which range from 2500 to 3600 cm^{-1} due to inter- and intramolecular hydrogen bonds. Whereby, in the pectin samples absorption in the O−H region is due to inter and intramolecular hydrogen bonding of the galacturonic acid polymer. However, bands at 2800-3000 cm^{-1} refer to C - H absorption which includes CH, CH_2, and CH_3 stretching and bending vibrations (Kamnev et al., 1998, Gnanasambandam and Proctor, 2000, Jittra, 2004). In addition, there is a small band at 1760 cm^{-1} which can be related to the C=O stretching vibration of methyl esterified to acetyl esters. Besides, the band at 1635cm^{-1} is corresponding to vibrations of the structure O=C−O; both would be important in the identification and quantitation of pectin samples. There are bands that are commonly problematic to interpret between 1500 and 800 cm^{-1} referred to as the "fingerprint" region that is unique to a compound. Other bands in pectin samples can be seen but less important; C- H bending that occur at 1380 cm^{-1}, and C-O stretching placing at 1300-1000 cm^{-1} (Gnanasambandam and Proctor, 2000, Jittra, 2004). The IR band at 1250 cm^{-1} has been previously assigned to the presence of an acetyl group (Synytsya et al., 2003b, Jittra, 2004).

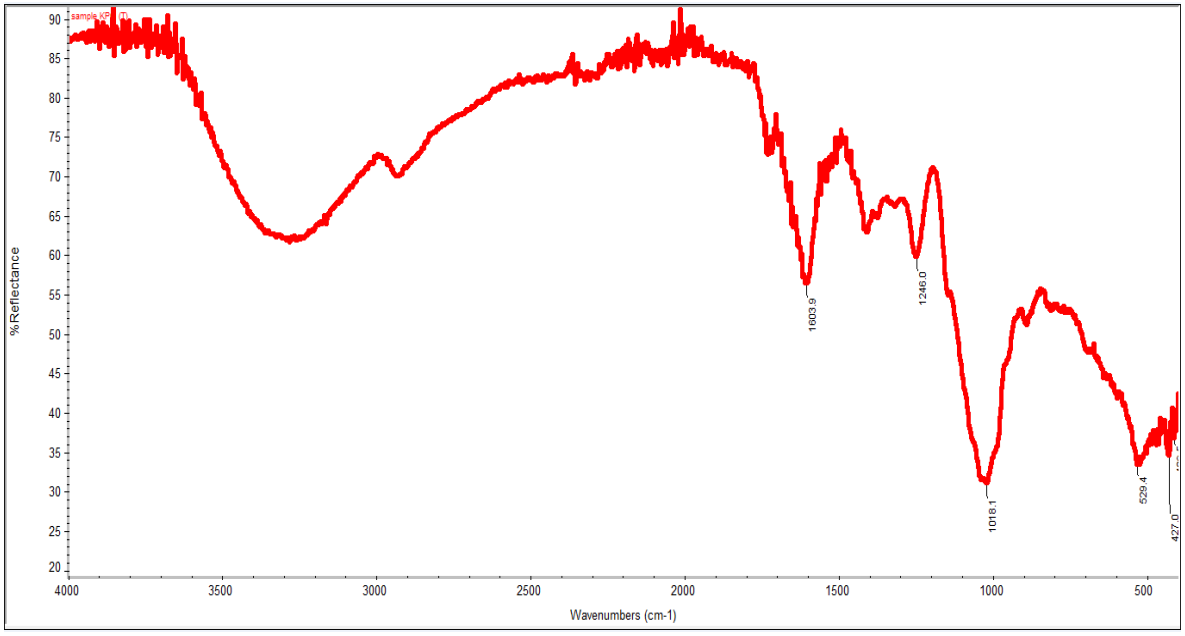
Table 6.5: Frequencies and intensities of functional groups present on pectin samples analyzed by diffuse reflectance Fourier transform infrared spectroscopy.

Frequency (wave number) cm^{-1}.	**Functional groups**	**intensity**
3600 - 3000	O- H stretching	Broad, strong
3000-2800	C-H stretching, symmetric, asymmetric	Sharp, occasionally double overlapping with O-H
1760 - 1730	C=O esterified	strong
1640 - 1620	COO- asymmetric stretching	strong
1400	COO- asymmetric stretching	weak
1380	C-H bending	weak
1300 - 1000	C=O stretching	weak

See for example (Alba et al., 2015, Sato et al., 2011, Kamnev et al., 1998, Gnanasambandam and Proctor, 2000, Synytsya et al., 2003b).



Sample AGB



Sample KPO (T)

Figure 6.1: IR spectra for some okra genotypes pectin *i.e.* AGB and KPO (T)

6.1.1 Determination of degree of esterification:

Table 6.6: Displays the degree of esterification of okra pods pectins.

Sample name	Short name	DE %
AGBAGOMA	AGB	31 ± 0.7^{b}
AGBAGOMA deep green fresh	AGB (F)	31 ± 0.7^{b}
PEMKRUMAH (TECHI)	PEM (T)	$29 \pm 0.7^{b,c}$
PEMKRUMAH (KEN)	PEM (K)	30 ± 0.7^{b}
KPOMG (TAMELE)	KPO (T)	37 ± 0.7^{a}
ASONTEM (KEN)	ASO (K)	$28 \pm 0.7^{c,d}$
AGBAGOMA (AG)	AGB (AG)	23 ± 0.7^{e}
SENGEVI	SEN	26 ± 0.7^{d}
ASHA	ASHA	14 ± 0.7^{f}
AISHA FRESH	ASHA (F)	39 ± 0.7^{a}

Values are mean (SD) of at least triplicate determinations. Mean values in a column with different letters are significantly different ($p < 0.05$).

The degree of esterification (DE) is a key factor which determines the conformational and rheological properties of pectins (Hwang et al., 1992). The DE for pectins extracted was estimated using FT-IR. DE was calculated from the ratio of the area of the band at 1740 cm^{-1} (corresponding to the number of esterified carboxylic groups) over the sum of the areas of the bands at 1740 and 1630 cm^{-1} (corresponding to the number of total carboxylic groups)

Table 6.6 shows the values of the degree of esterification (DE) of pectins isolated from okra pods at pH 6, 80 ℃ and 1h, which were in the range of 14 % to 39 %. The DE was lower than 50 % in all samples and therefore all the pectins are LM pectins. These results for the DE had the same tendency as those observed for the DM (Alba et al., 2015) for OP6 using the same conditions are also for the DM observed by Senghkamparn et al., (2009b) for hot buffer soluble solids HBSS: 24 %. On the other hand, these values of DE appeared lower than those obtained by Garna and others (2007) who extracted apple pomace pectin using the same time and temperature (80 ℃ for 1 h) but lower pH (pH 2): (68.8 to 88.2 %). In addition, DE values were lower than DE values determined for dried pomace apple pectin with an average 72.29 %. These values of DE for okra pectins were also lower than those published for peach pectin

Pagan et al., (1999) which varied from 72.1% to 95.4%. Pectin samples used in this study were obtained from different genetic variants grown in different regions of Ghana. Differences in sample source/origin and processing variation might account for part of the variation in their chemical composition. Therefore even small differences in the structure and constitution of a molecule results could in significant changes in the physicochemical properties (Gnanasambandam and Proctor, 2000).

6.1.1 Determination of degree of acetylation:

Table 6.7: The degree of acetylation content for different okra genotypes.

Sample	Short name	DA %
AGBAGOMA	AGB	55.9 ± 0.1^{e}
AGBAGOMA deep green fresh	AGB (F)	48.4 ± 0.1^{h}
PEMKRUMAH (TECHI)	PEM (T)	45.6 ± 0.3^{i}
PEMKRUMAH (KEN)	PEM (K)	34.7 ± 0.2^{l}
KPOMG (TAMELE)	KPO (T)	32.4 ± 0.5^{m}
ASONTEM (KEN)	ASO (K)	$30.3 \pm .0.1^{n}$
AGBAGOMA (AG)	AGB (AG)	76.2 ± 0.1^{a}
SENGEVI	SEN	61.7 ± 0.6^{d}
ASHA	ASHA	53.9 ± 0.1^{f}
AISHA FRESH	ASHA (F)	36.6 ± 0.1^{k}

Values are mean (SD) of at least triplicate determinations. Mean values in a column with different letters are significantly different ($p < 0.05$).

DA of ten okra pectins from different genetic variants varied from 30.3 % to 76.2 %. The acetyl content (DA) of these pectins was calculated as outlined in chapter 3 section 3.5.5. Eq (3.1). The highest DA value was obtained for sample AGB (AG) while the lowest value was obtained for sample ASO (K) see **Table 6.7.** These values of DA were close to data already published for okra pectin (Alba et al., 2015, Sengkhamparn et al., 2009b) and also to DA values that obtained by (Levigne et al., 2002a) of fresh sugar beet pectin. This uncommonly high degree of acetylation compared to previous studies should be attributed to differences in the origin of raw materials and the extraction conditions applied. The degree of acetylation values for samples AGB (F) and PEM (T) are close to each other whereas samples PEM (K), KPO (T),

ASO (K) and ASHA (F) are closer to each other as well. In addition, samples AGB A and ASHA are in the same range of DA.

6.1.1 Determination of constituent sugars composition:

The main neutral sugars determined in isolated okra pectin by using HPAEC technique are represented below in **Table 6.8.**

Table 6.8: Indicates the main sugars (mol %) of isolated okra pectin using HPAEC.

Sample	Short name	Rha	Ara	Gal	Glc	Xyl	GalA
AGBAGOMA	AGB	7 ± 0.1^{b}	9 ± 0.1^{a}	10 ± 0.2^{g}	5 ± 0.3^{b}	5 ± 0.3^{b}	$\mathbf{66 \pm 0.2^{c}}$
AGBAGOMA deep green fresh	AGB (F)	6 ± 0.2^{c}	4 ± 0.2^{e}	14 ± 0.1^{d}	2 ± 0.2^{c}	2 ± 0.2^{e}	$\mathbf{66 \pm 0.1^{c}}$
PEMKRUMAH (TECHI)	PEM (T)	6 ± 0.3^{c}	4 ± 0.2^{e}	21 ± 0.2^{a}	3 ± 0.1^{f}	3 ± 0.2^{d}	63 ± 0.2^{e}
PEMKRUMAH (KEN)	PEM (K)	7 ± 0.2^{b}	4 ± 0.2^{e}	11 ± 0.1^{f}	1 ± 0.1^{g}	1 ± 0.1^{f}	$\mathbf{77 \pm 0.2^{b}}$
KPOMG (TAMELE)	KPO (T)	5 ± 0.2^{d}	2 ± 0.1^{f}	10 ± 0.1^{g}	4 ± 0.2^{e}	6 ± 0.2^{a}	$\mathbf{79 \pm 0.1^{a}}$
ASONTEM (KEN)	ASO (K)	8 ± 0.2^{a}	2 ± 0.1^{f}	20 ± 0.2^{b}	10 ± 0.1^{b}	6 ± 0.2^{a}	55 ± 0.2^{g}
AGBAGOMA (AG)	AGB (AG)	4 ± 0.1^{e}	5 ± 0.2^{d}	14 ± 0.1^{d}	7 ± 0.2^{d}	4 ± 0.1^{c}	$\mathbf{65 \pm 0.3^{d}}$
SENGEVI	SEN	8 ± 0.2^{a}	7 ± 0.1^{c}	14 ± 0.2^{d}	4 ± 0.1^{e}	4 ± 0.2^{c}	63 ± 0.1^{e}
ASHA	ASHA	7 ± 0.1^{b}	9 ± 0.2^{a}	13 ± 0.2^{e}	8 ± 0.2^{c}	1 ± 0.1^{f}	63 ± 0.2^{e}
AISHA FRESH	ASHA (F)	6 ± 0.2^{c}	8 ± 0.1^{b}	16 ± 0.1^{c}	11 ± 0.2^{a}	1 ± 0.1^{f}	59 ± 0.3^{f}

Values are mean (SD) of at least triplicate determinations. Mean values in a column with different letters are significantly different ($p < 0.05$).

From **Table 6.8** it can be seen that galacturonic acid was the main sugar of the total sugars where it accounted for about 55 to 79 % for all ten okra pectins. These contents of GalA were in line with the study conducted previously for okra pectin at pH 6 (56.9 %) by Alba et al., (2015) and also close to those reported for chelating-agent soluble solid CHSS (63 %) by Sengkhamparn et al., (2009b) .Additionally they were similar to those published by Arslan, (1995) and Michel et al., (1985) of about 65 %, GalA contents ≥ 65 % have been highlighted in bold, but they were significantly higher than those found for okra hot buffer soluble solid HBSS (35 %) (Sengkhamparn et al., 2009b) and higher than GalA content obtained by (Levigne et al., 2002a) for sugar beet (29.5-52.8 %). The GalA contents estimated by this method are in line with those estimated by a colourimetric method which may due to using the same extraction conditions see **Table 6.4**. The main neutral sugar determined was galactose which was comprised of about 10 to 21%. These values of Gal content are comparable to those previously found for okra by Alba et al., (2015) of 17 and 26.1 % for PO2 and PO6 respectively. In addition, values were close to galactose content obtained for okra CHSS of 17 % (Sengkhamparn et al., 2009b) but lower than those achieved for okra HBSS of 34 %, in the same study. However, these values were higher than Gal contents obtained for sugar beet pectin by Levigne et al., (2002a). Rhamnose and arabinose content varied from 4 to 8 % and 2 to 9 % respectively. These values of Rha and Ara were in good agreement with those previously determined for okra pods by Alba et al., (2015). Nevertheless, they were lower than those reported for sugar beet by Levigne et al., (2002a). On the other hand, okra HBSS and CHSS had contents of Rha which were higher than the present study but lower in Ara contents (Sengkhamparn et al., 2009a). Glucose amounts varied from 1 to 11 % and were in similar with those found for okra by Alba et al., (2015) and Sengkhamparn et al., (2009a). Xylose was in low level compared with other neutral sugars and ranged from 1 to 6 % which was close to xylose levels estimated for OP2 (2 %) by Alba et al., (2015). However they were higher than the levels of xylose obtained for HBSS and CHSS by Sengkhamparn et al., (2009a) but lower than those obtained for CASS (27 %) in the same study.

Comparing the ten genotypes polysaccharide obtained from the sequential extraction protocol, as it can be seen from **Table 6.8** the highest values of GalA acid were obtained for samples PEM (K) and KPO (T)E while the lowest was obtained for sample ASO (K). Samples AGB and AGB (F) were close to each other also samples PEM (T), SEN and ASHA were close to each other. In addition, the highest amounts of Rha were obtained for samples ASO (K) and SEN where they had the same amount whereas sample AGB (AG) had the lowest amount of

Rha. Samples AGB (F), PEM (T) and ASHA (F) were close and samples AGB, PEM (K) and ASHA were close to each other as well. For Arabinose the highest amounts obtained for samples AGB and ASHA which had the same amount whilst KPO (T) and ASO (K) had the lowest amount and they were close as well. Samples AGB (F), PEM (T) and PEM (K) had the same amount of Ara. Galactose was the main neutral sugar: the highest level found for sample PEM (T) whereas the lowest was for sample AGB The highest value for glucose was reported for sample ASHA (F) while the lowest was for sample PEM (K). samples AGB (F) and PEM (T) had the same amount also samples, ASO (K) and SEN had the same amount. Samples ASO (K) and KPO (T) had the highest level of xylose while sampling PEM (K), ASHA and ASHA (F) had the lowest amount of Xyl and they were in same range. Sample AGB (AG) and SEN had a similar amount of Xyl.

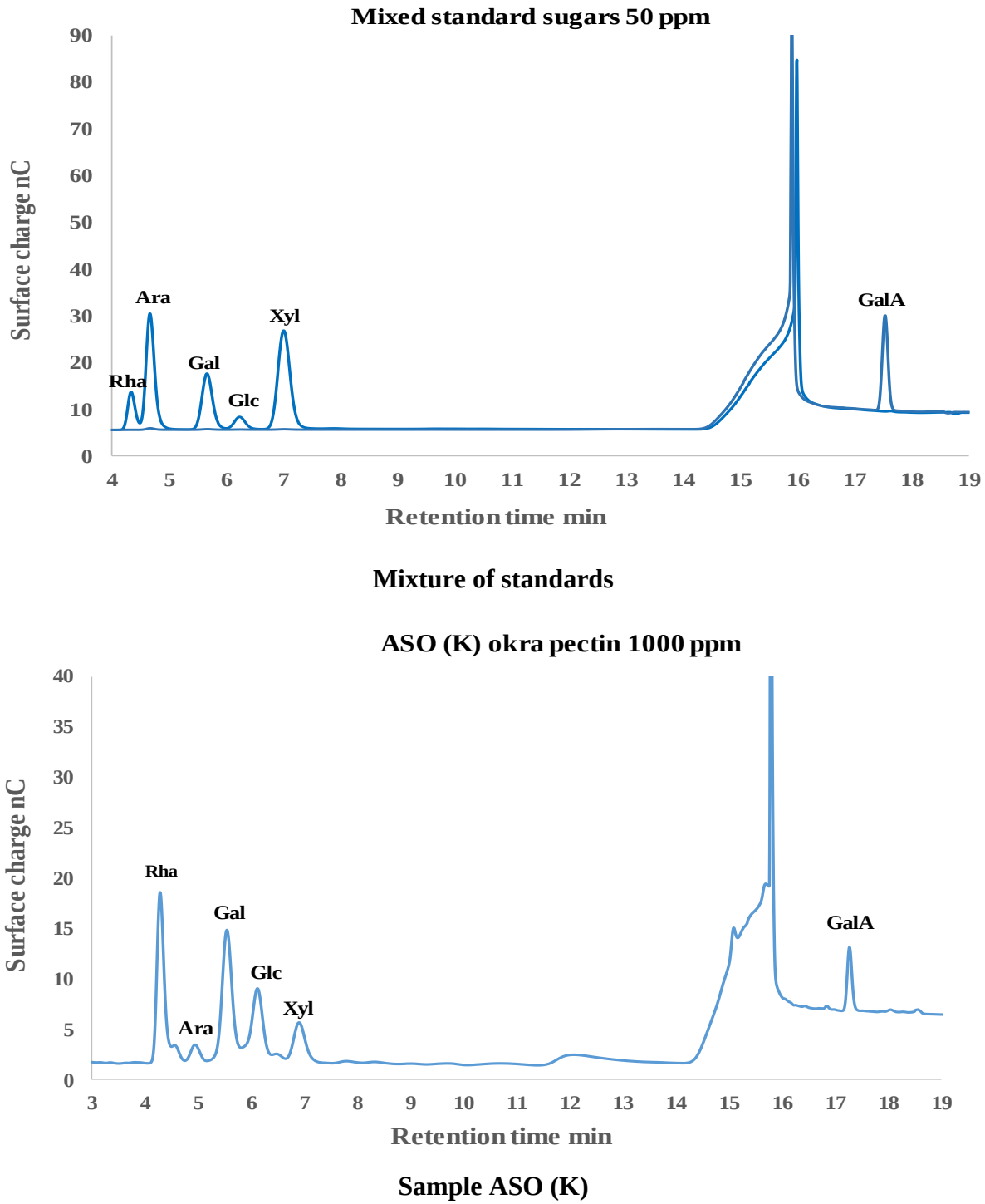


Figure 6.2: Peaks for a mixture of standards and for each monosaccharide present in okra samples *i.e.* ASO (K) using HPAEC.

6.1.2 Calculating ratios between certain component monosaccharides:

The sugar ratios for the different cell wall extracts, calculated based on the sugar content determined experimentally are displayed in **Table 6.9**. Sugar ratios properties from 1 to 8 for pectin are described in section 5.1.8 **Table 5.11.**

Table 6.9: Composition ratios and pectin region % based on the mol % quantifiable neutral sugars and galacturonic acid.

Pectin short name	Ratio 1	Ratio 2	Ratio 3	Ratio 4	Ratio 5	Ratio 6	Ratio 7	Ratio 8	% HG GalA-Rha	% RG-I 2Rha+Ara+ Gal	HG : RG-I
	Linearity of pectin	Contribution of RG	Branching of RG		Co-extractants	Severity of extraction					
AGB	$2.1 \pm 0.1^{e,f}$	$0.11 \pm 0.1^{b,c}$	$2.7 \pm 0.1^{d,e}$	1.4 ± 0.1^{f}	18.4 ± 0.4^{e}	7.3 ± 0.1^{h}	1.1 ± 0.1^{f}	0.7 ± 0.1^{f}	$59 \pm 0.1^{c,d}$	33 ± 0.1^{b}	1.7 ± 0.1^{d}
AGB (F)	2.5 ± 0.1^{c}	$0.09 \pm 0.1^{c,d}$	$3.0 \pm 0.2^{c,d}$	$2.3 \pm 0.1^{c,d}$	45 ± 0.5^{b}	16.5 ± 0.2^{d}	3.5 ± 0.2^{c}	1.5 ± 0.2^{d}	60 ± 0.2^{c}	$30 \pm 0.1^{b,c}$	2.0 ± 0.3^{c}
PEM (T)	$1.8 \pm 0.1^{f,g}$	$0.09 \pm 0.1^{c,d}$	4.2 ± 0.1^{b}	3.5 ± 0.2^{a}	31.3 ± 0.4^{c}	15.8 ± 0.3^{e}	5.2 ± 0.1^{b}	1.5 ± 0.2^{d}	$57 \pm 0.1^{d,e}$	37 ± 0.2^{a}	1.5 ± 0.1^{e}
PEM (K)	3.3 ± 0.2^{b}	$0.09 \pm 0.1^{c,d}$	2.1 ± 0.1^{f}	1.5 ± 0.1^{f}	99 ± 0.5^{a}	19.3 ± 0.2^{c}	$2.7 \pm 0.2^{d,e}$	1.7 ± 0.1^{c}	70 ± 0.1^{b}	29 ± 0.3^{c}	2.4 ± 0.2^{b}
KPO (T)	4.3 ± 0.1^{a}	0.06 ± 0.2^{d}	2.4 ± 0.3^{f}	2.0 ± 0.1^{d}	24 ± 0.5^{d}	39.5 ± 0.2^{a}	5.0 ± 0.1^{b}	2.5 ± 0.2^{b}	54 ± 0.1^{a}	22 ± 0.1^{e}	3.3 ± 0.1^{a}
ASO (K)	1.5 ± 0.1^{g}	0.15 ± 0.1^{a}	$2.8 \pm 0.1^{d,e}$	$2.5 \pm 0.1^{b,c}$	18.5 ± 0.4^{g}	27.5 ± 0.3^{b}	10 ± 0.2^{a}	4.0 ± 0.1^{a}	47 ± 0.2^{g}	38 ± 0.2^{a}	1.2 ± 0.1^{f}
AGB (AG)	$2.4 \pm 0.2^{c,d}$	0.06 ± 0.2^{d}	4.8 ± 0.2^{a}	3.5 ± 0.2^{a}	12.6 ± 0.8^{f}	13.0 ± 0.5^{f}	2.8 ± 0.1^{d}	0.8 ± 0.1^{f}	61 ± 0.1^{c}	27 ± 0.1^{d}	2.2 ± 0.1^{c}
SEN	$1.9 \pm 0.1^{f,g}$	0.13 ± 0.1^{b}	2.6 ± 0.1^{e}	$1.7 \pm 0.1^{e,f}$	23 ± 0.7^{d}	9.0 ± 0.6^{g}	2.0 ± 0.1^{e}	1.1 ± 0.2^{e}	55 ± 0.2^{e}	37 ± 0.4^{a}	$1.4 \pm 0.1^{e,f}$
ASHA	$2.1 \pm 0.1^{d,e}$	$0.11 \pm 0.1^{b,c}$	3.1 ± 0.1^{c}	1.8 ± 0.1^{e}	11.5 ± 0.2^{f}	7.0 ± 0.6^{h}	1.4 ± 0.1^{f}	0.7 ± 0.1^{f}	56 ± 0.2^{e}	36 ± 0.4^{a}	$1.5 \pm 0.2^{d,e}$
ASHA (F)	$1.9 \pm 0.1^{e,f,g}$	$0.10 \pm 0.1^{b,c}$	4.0 ± 0.1^{b}	2.6 ± 0.2^{b}	8.1 ± 0.1^{g}	7.3 ± 0.1^{h}	2.0 ± 0.1^{e}	0.8 ± 0.1^{f}	53 ± 0.1^{f}	36 ± 0.4^{a}	$1.4 \pm 0.1^{e,f}$

Values were given as means of triplicate ± SD. Means with different superscript letters within a column are significantly different (Tukey test, $p < 0.05$). SD = Standard deviation of the mean.

Interestingly the values for **ratio 1** which indicates the linearity of pectin are higher than those for pectins extracted from *Cucumis melo* (Denman and Morris, 2014) also than those obtained for okra pods (Alba et al., 2015) which were 0.4 – 1.4 and 1.1 – 1.3 respectively. On the other hand, they were similar to those for the water-soluble pectins from broccoli stem, carrot root and tomato fruit (Houben et al., 2011). This indicates that pectins extracted from these different okra genotypes are on average more linear (less branched) than melon pectin (Denman and Morris, 2014), also than okra pectins previously extracted using the same protocol (Alba et al., 2015), but of a similar structure to broccoli, carrot and tomato pectins (Houben et al., 2011). With respect to **ratio 2** lower values of this ratio are indicative of less branched pectins. These values were lower than those found previously for okra pectin (Alba et al., 2015), but some of the results are in line with a study conducted by (Denman and Morris, 2014) for *Cucumis melo* pectin extracted under different conditions. These results are also similar to those of water-soluble pectin (Houben et al., 2011) specifically for a sodium carbonate-soluble fraction (NSF) of broccoli florets, broccoli stem and carrot root. **Ratios 3** and **4** are indicative of a low level of branched structures. Suggesting that the length of the side chains attached to Rha are short in the RG-I backbone. Generally, okra pectins exhibited a higher degree of branching of side chains than was previously reported for okra polysaccharides obtained by sequential extractions (Sengkhamparn et al., 2009b). on the contrary, values obtained here in are lower than those obtained by (Houben et al., 2011) in the study of other potentially novel pectin sources. On the other hand, the values obtained are close to those previously obtained for okra by Alba et al., (2015) and to some of those obtained for *Cucumis melo* pectin (Denman and Morris, 2014), particularly those extracted under "harsh" extraction condition of low pH, higher temperature and longer times. **Ratio 4** helps to establish the length of the galactose side chains present in the RG region; specifically lower values signify shorter sugar chain and *vice versa*. These ratios was lower than those obtained by Denman and Morris, (2014) indicating that the okra side chains are of a lower than average size. For sugar **ratio 5** where a larger value indicates more pure pectin extract **Table 6.9** reveals that the ratios appeared very high compared to the ratios obtained for pectin extracted from *Cucumis melo* (Denman and Morris, 2014). The highest ratio value was obtained for sample PEM (K)99 which has the highest purity of pectin; however, it should be noted that this relates only to the carbohydrate fraction and does not include any protein impurities (**Table 6.3**), where incidentally pectin PEM (K) has the highest protein content. Regarding **ratios 6-8,** large values indicate a very low level of arabinofuranoside (Ara*f*) residues, which may due to relatively "harsh" extraction conditions and/or that okra pectin are naturally low in arabinose. The HG: RG-I ratio for these okra pectins

can be calculated from HG and RG-I % from equations (**4.7** and **4.8**) that have been previously reported in section **4.5.7** using the mol % of neutral sugars and GalA. From **Table 5.9**, we can see that okra pectins in this study have higher values of HG region (more linear) than RG-I region (more branched) which are similar to those obtained by Sengkhamparn et al., (2009b) for okra CHSS-extracted pectin which was about 24 % for RG-I. Values, were in very good agreement with those found by (Msakni et al., 2006). This contrasts contrary with a previous study of okra and *Cucmis melo* pectins (Alba et al., 2015, Denman and Morris, 2014) where their pectins extracted are high in RG-I and low in HG, the same with okra HBSS found by (Sengkhamparn et al., 2009b) where RG-I was about 85 % . The HG: RG-I ratios varied from 1.2 to 3.3. These values show difference in the structure of pectin firstly between each other depending on genetic variant and also with those obtained by Denman and Morris, (2014) and Alba et al., (2015) for okra and *Cucmis melo* pectins, which were more branched. Whereas, on the other hand, they are less branched than pectins isolated from apple or sugar beet (Leroux et al., 2003). Furthermore, even taking into account that okra pectins are rich in HG regions this does not appear to fully explain the low values of ratio 2. One possible explanation could be as the RG-I backbone is typically composed of alternating units of rhamnose and galacturonic acid, it would be expected that the molar ratio of Rha: GalA in the RG-I domain to be nearly 1:1 (Yapo, 2011). Since the values of Rha: GalA ratio are less than 1 this indicated that oligogalacturonic acid units might be present in the RG-I domain. All of the information in **Table 6.10** and the other results discussed in this section further illustrates the importance of source, genetic variation and extraction conditions on the chemical structures of pectins.

6.1.3 Determination of structure using NMR:

NMR spectroscopy was applied to investigate the structure of pectin. In the current study, ^{1}H NMR spectra of all okra genotypes isolated revealed similar resonance patterns suggesting similarities in compositional characteristics of all pectins A comparison of ^{1}H-NMR spectra of okra isolated shows a high similarity between all spectra **appendices 9.2.5** and the important structural features are indicated in **Figure 6.3** for AGB okra pectin. A signal at 4.16 ppm derived from methyl groups connecting to carboxyl groups of GalA (Alba et al., 2015). However, the signal at 2.50 ppm indicates the presence of *O*-acetyl substituent, which is similar to that reported in a previous study for okra pods (2.10 ppm) and is consistent with a degree of acetylation (**Table 6.6**). It is obvious that the samples contain both unbranched α -1, 2-linked rhamnose (1.62 ppm) and branched α - 1, 2, 4-linked rhamnose (1.85 ppm); similar to the (hot

buffer soluble solids) HBSS sample, also for PG treated CHSS (chelating agent soluble solids) 1, 2-linked- , 1, 2, 4-linked-rhamnosyl units were found (Sengkhamparn et al., 2009b). In the down field region at 5.4 ppm and 5.7 ppm, there are two signals seen on the okra pectins spectrum which are comparable with the spectrum obtained from okra pectin (Alba et al., 2015) but contrasted with the spectrum obtained from flax pectin (Bédouet et al., 2003). ^{1}H NMR of all okra polysaccharides were very similar to the spectrum of okra polysaccharides previously isolated (Alba et al., 2015) and also, with those isolated from flax stems, pumpkin and citrus plant (Bédouet et al., 2003, Cozzolino et al., 2006, Košťálová et al., 2013). Additionally the NMR spectra of okra pectins in this study were similar to the ^{1}H NMR spectra of pumpkin pectins that reported in this study see **Figure 5.28** in section **5.1.9.**

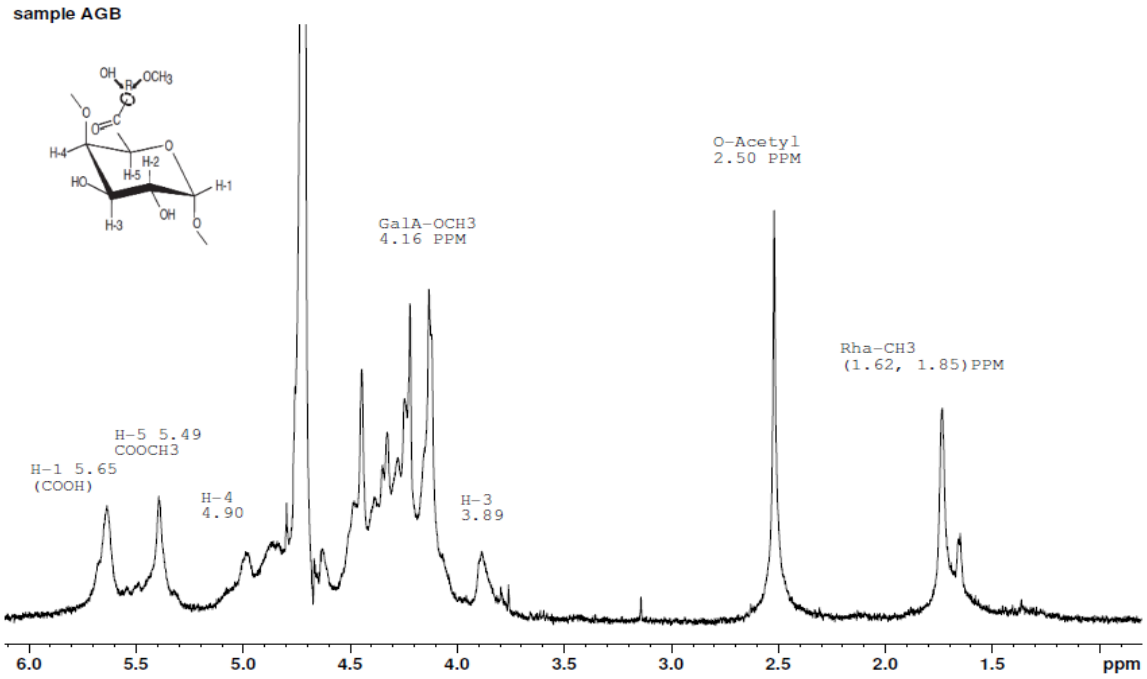


Figure 6.3: ^{1}H NMR spectra for AGB okra pectin sample in D_2O at 70 ˚C.

6.2 Physical Characterisation of okra pectin:

6.2.1 Intrinsic viscosity:

Huggins and keramer plot are shown in **Figure 6.4** below which have been used to calculate the intrinsic viscosity for okra pectin solutions.

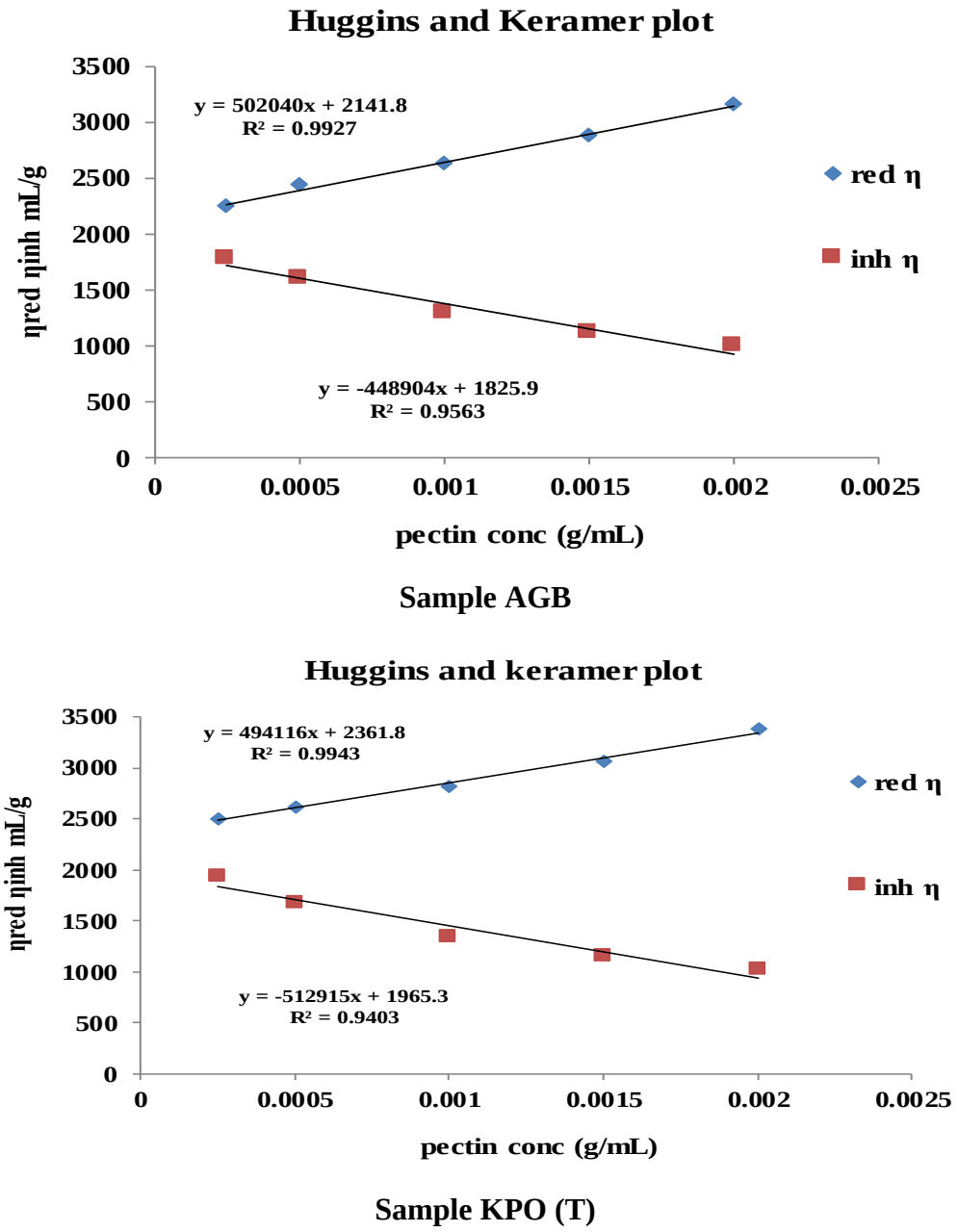


Figure 6.4: Huggins (♦) and Kraemer (■) plots for some okra pectins, an example: AGB and KPO (T), ♦ red η and ■ inh η represent the reduced and inherent viscosity respectively.

Table 6.10: Intrinsic viscosity of pectin isolated from different sources of okra pods.

Sample full name	Sample short name	[η] mL/g
AGBAGOMA	AGB	$1985 \pm 40^{b,c}$
AGBAGOMA deep green fresh	AGB (F)	1630 ± 30^{f}
PEMKRUMAH (TECHI)	PEM (T)	920 ± 18^{h}
PEMKRUMAH (KEN)	PEM (K)	2052 ± 41^{b}
KPOMG (TAMELE)	KPO (T)	2165 ± 43^{a}
ASONTEM (KEN)	ASO (K)	1499 ± 30^{g}
AGBAGOMA (AG)	AGB (AG)	965 ± 19^{h}
SENGEVI	SEN	1890 ± 38^{d}
ASHA	ASHA	$1946 \pm 39^{c,d}$
AISHA FRESH	ASHA (F)	1779 ± 36^{e}

Values are mean (SD) of at least triplicate determinations. Mean values in a column with different letters are significantly different ($p < 0.05$).

The intrinsic viscosities of the pectin samples are detailed in **Table 6.10** and varied from 920 to 2165 mL/g. All the samples had high viscosities obtained at pH 6, 80 ˚C and 1h extraction time. The highest value was obtained for pectin KPO (T) (2165 g/ml) while the lowest value obtained for sample PEM (T) (920 ml/g). The result indicated that pectin samples had [η] much higher than the values of ~ 400 ml/g obtained by (Alba et al., 2015). In this study, the pH had a huge effect on the intrinsic viscosity for samples extracted at the same conditions. Also, these values were higher than those obtained for pectin extracted from fresh sugar beet (Levigne et al., 2002a) 172 to 493 ml/g. Furthermore, they are much higher than that found in the literature (Emaga et al., 2008) for pectin extracted from banana peels in similar conditions 50 to 180 ml/g. in addition, higher than those obtained by Kontogiorgos et al., (2012) for okra pectin. The viscosity values reported here for our okra samples are rather high compared to other pectins from different sources such as pectin obtained by Morris et al., (2010b), Evageliou et al., (2005), Morris et al., (2010a) and Koubala et al., (2008) from sugar beet (*Beta vulgaris*), pumpkin biopectin, CP Kelco and ambarella peel (*Spondias cytherea*) pectins which indicate higher molecular weight and/ or very extended conformations. These discrepancies with present data could be attributed to the origin of the initial material and the extraction conditions applied or to any seasonal variations in pectin structure/ molecular weight/conformation.

6.2.2 Zeta potential:

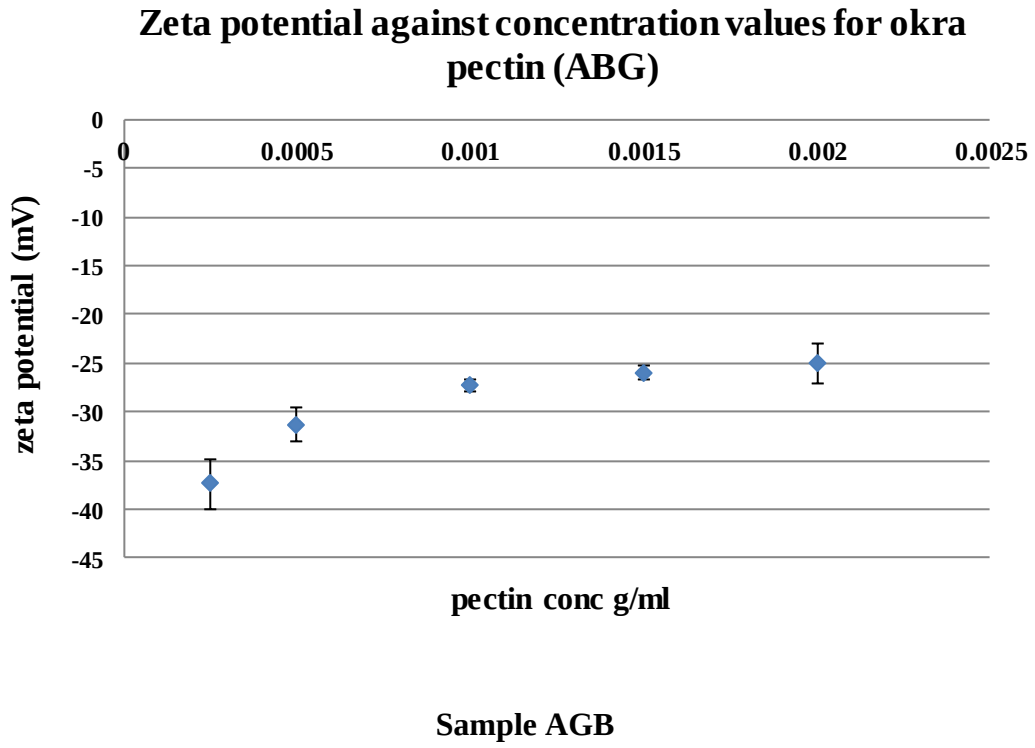
Table 6.11: Zeta potential of pectin isolated from different sources of okra pods.

Sample full name	Sample short name	ζ mV
AGBAGOMA	AGB	-29.4 ± 0.1^{f}
AGBAGOMA deep green fresh	AGB (F)	-26.7 ± 0.1^{c}
PEMKRUMAH (TECHI)	PEM (T)	-22.3 ± 0.1^{a}
PEMKRUMAH (KEN)	PEM (K)	-26.4 ± 0.1^{c}
KPOMG (TAMELE)	KPO (T)	-28.9 ± 0.1^{e}
ASONTEM (KEN)	ASO (K)	-26.6 ± 0.1^{b}
AGBAGOMA (AG)	AGB (AG)	-28.4 ± 0.1^{d}
SENGEVI	SEN	-23.3 ± 0.1^{b}
ASHA	ASHA	-33.2 ± 0.1^{h}
AISHA FRESH	ASHA (F)	-30.7 ± 0.1^{g}

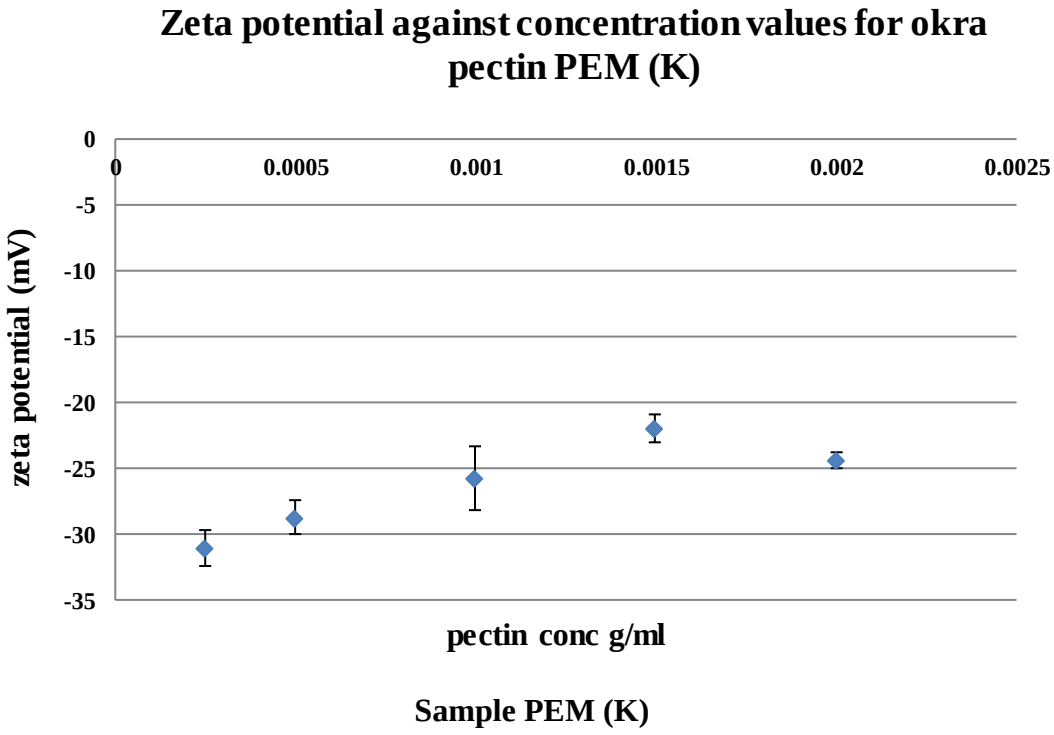
Values are mean (SD) of at least triplicate determinations. Mean values in a column with different letters are significantly different ($p < 0.05$).

The zeta potential values for the pectin samples are represented in **Table 6.11**. The zeta potentials obtained in this study are highly negative within the range of −33.2 to −22.3 mV. The negative value of ζ-potential was attributed to the presence of anionic polysaccharides in all okra extracts; these values were higher in comparison to the values obtained in Tello et al., (2015) for pectin solution at pH 3, which is consistent with all pectins having a low degree of methyl esterification (section 5.15.1). Pectins in our study have high electronegativity at pH 6, where the electronegativity of the pectin solution increased with increasing pH. On the other hand, zeta potential becomes more positive / gets closer to zero. It can be observed that by comparing the electronegativity of our pectin solution at pH 6 (*i.e.* -33.2 mV) with pectin solution reported in (Tello et al., 2015) at pH 3 (-19.2 mV) and (-51.6 mV) at pH 7. Similar trends were observed for the zeta potential of alginate with negative zeta potential values of -36.1mV to -74.5 mV at pH 3 and pH 7 respectively. So, the zeta potential values obtained for both pectin solutions were less electronegative than those obtained for the alginate solution reported in the literature (Tello et al., 2015). Zeta potential of alginate solutions decreases with increasing pH *i.e.* -19.2 mV, -33.2 mV and -51.6 mV at pH 3, 6 and 7 respectively. The same

results have been stated in (Harnsilawat et al., 2006) the zeta potential of alginate solutions decreased from - 8.7 to - 68.4 mV when the pH was increased from 2 to 8. In the current work as shown in **Table 6.11**, the highest negative charge for zeta potential was obtained for sample ASHA and ASHA (F), which would appear to be in line with them being relatively high in GalA and low DE pectins. Samples AGB, AGB (F), PEM (K), KPO (T) and AGB (AG) were less charged, while particles PEM (T) and SEN had an even lower absolute zeta potential value. The zeta potential values were lower than those reported by (Georgiadis et al., 2011, Alba et al., 2013) for okra. The full graphs for zeta potential are shown in **Figure 6.5.**



Sample AGB



Sample PEM (K)

Figure 6.5: Graphical representation of zeta potential for some okra pectin samples solutions, an example: AGB and PEM(K).

6.2.3 Determination of pectin molecular weight

The pectins were analysed by high-performance size-exclusion chromatography (HPSEC) equipped with multi-angle laser light scattering (MALLS) and refractive index (RI) detectors. The weight-average molar mass values of various okra pectins ranged widely from 0.32×10^6 to 7.6×10^6 g/mol (**Table 6.12**) which were higher, the presence of high-M_W population may be due to the extraction of RG I cross-linked with RG II and remaining HG (Khodaei and Karboune, 2013).

Table 6.12: Molecular weight of extracted pectic polysaccharides from okra pods.

Sample full name	Sample short name	$M_w \times 10^6$ (g/mol)	$R_{g,z}$ (nm)
AGBAGOMA	AGB	7.6 ± 0.1^{a}	138 ± 15
AGBAGOMA deep green fresh	AGB (F)	0.34 ± 0.1^{e}	102 ± 15
PEMKRUMAH (TECHI)	PEM (T)	$1.1 \pm 0.1^{b,c,d}$	133 ± 12
PEMKRUMAH (KEN)	PEM (K)	1.4 ± 0.1^{b}	140 ± 14
KPOMG (TAMELE)	KPO (T)	$1.3 \pm 0.1^{b,c}$	137 ± 16
ASONTEM (KEN)	ASO (K)	$0.83 \pm 0.3^{d,e}$	138 ± 17
AGBAGOMA (AG)	AGB (AG)	0.42 ± 0.1^{e}	134 ± 11
SENGEVI	SEN	0.32 ± 0.1^{e}	130 ± 13
ASHA	ASHA	0.47 ± 0.1^{e}	138 ± 18
AISHA FRESH	ASHA (F)	$0.79 \pm 0.1^{c,d,e}$	136 ± 20

Values are mean (SD) of at least triplicate determinations. Mean values in a column with different letters are significantly different ($p < 0.05$).

The molecular weight of pectin is one of the most important factors to determining its quality because low molecular weight could result in undesirable weak gelling properties for example. In general, the molecular weights of pectins are difficult to measure accurately because of the presence of heterogeneous groups along with branched and smooth regions (Yoo et al., 2012a). In this study, the highest M_w values were found for pectin AGB 7 600 000 g/mol which had a substantially higher molar mass than the others in spite of using the same extraction method, so is probably due to differences in origin of materials. However, the large values could in part be due to dissolved components within the samples such as co-extracted cellulose or starch that

may have remained after extraction, this is particularly likely for samples with high glucose contents. The other pectins had weight average molecular weights in the range $3 \times 10^5 – 1.5 \times 10^6$ g/mol which are in agreement with those extracted under similar conditions 6.4×10^5 and 7.6×10^5 g/mol for OP2 and OP6 respectively (Alba et al., 2015). The molecular weights were also similar to those in the higher range of those extracted using different extraction conditions (Sengkhamparn et al., 2009b), andalso in the same range as pectins extracted from lime, passion fruit peel, sugar beet and citrus fruits (Levigne et al., 2002a, Leroux et al., 2003, Fishman et al., 2006, Seixas et al., 2014a). Molecular weight values are also consistent with the high intrinsic viscosities and radii of gyration (**Tables 6.10** and **6.11**). The data in Tables (**6.10 - 6.12**) suggest that together with different physicochemical properties okra pectins extracted from different genetic variants will also adopt different conformations in solution and therefore we have used a number of different conformational analyses to probe these differences see for example (Morris et al., 2014).

6.3 Conformational analysis:

6.3.1 Global analysis method (HYDFIT)

The linear flexibility of polymer chains is estimated quantitatively in terms of the persistence length, L_p "where the persistence length is defined as the average projection length along the initial direction of the polymer chain. In the case of a theoretical perfect random coil $L_p = 0$ and for the equivalent extra-rigid rod $L_p = \infty$, although in practice limits of ~ 1 nm for random coils (e.g. pullulan) and 200 nm for an extra-rigid rod (e.g. xanthan) are more appropriate" (Harding, 1997). The persistence length, L_p and mass per unit length, M_L can be estimated using the Multi-HYDFIT program (Ortega and García de la Torre, 2007) which considers datasets of intrinsic viscosity and molar mass. It then performs a minimisation procedure see (Ortega and García de la Torre, 2007) finding the best values of M_L and L_p satisfying the Bushin-Bohdanecky (Bushin et al., 1981, Bohdanecky, 1983a) see equations 3.10, 3.11

where Φ is the Flory-Fox constant (2.86×10^{23} mol^{-1}), A_0, B_0 are tabulated coefficients (Bohdanecky, 1983a), m is the molar mass of the average monomeric unit and l is the length of the average monomeric unit 0.5 nm.

For the okra pectins two possible scenarios were considered that as the same was employed in section **5.3.1**.

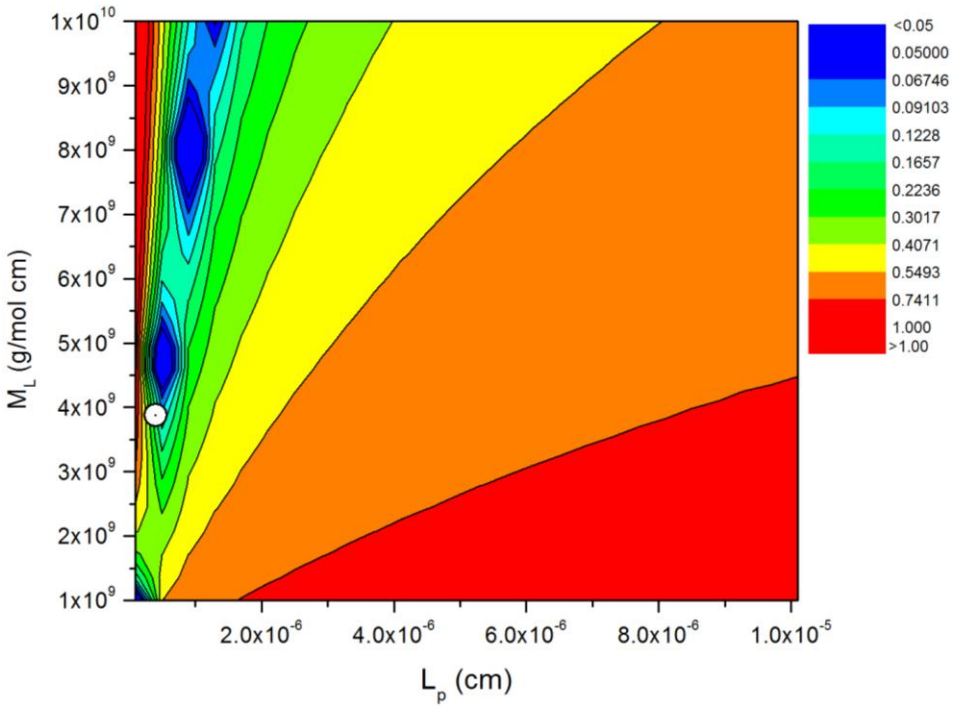
Table 6.13: Mass per unit length M_L and chain flexibility (L_p) estimations from combining molecular weight and intrinsic viscosity data through HYDFIT for okra pectin when the mass per unit length has been allowed to float freely.

Sample	Short name	[η] (g/ml)	$M_w \times 10^6$ g/mol	L_p (nm)	M_L (g/(mol nm))	L_p / M_L (nm^2 mol/g)
A. AGBAGOMA	AGB	1985	7.6	4^b	$388^{a,b}$	0.0103^a
B. AGBAGOMA deep green fresh	AGB (F)	1630	0.34	78^a	$783^{a,b}$	0.0996^a
C. PEMKRUMAH (TECHI)	PEM (T)	920	1.1	10^b	$675^{a,b}$	0.0222^a
D. PEMKRUMAH (KEN)	PEM (K)	2052	1.4	11^b	$496^{a,b}$	0.0330^a
E. KPOMG (TAMELE)	KPO (T)	2165	1.3	$30^{a,b}$	$909^{a,b}$	0.0360^a
F. ASONTEM (KEN)	ASO (K)	1499	0.83	$35^{a,b}$	972^a	0.0174^a
G. AGBAGOMA (AG)	AGB (AG)	965	0.42	3^b	172^b	0.1111^a
H. SENGEVI	SEN	1890	0.32	79^a	$711^{a,b}$	0.0185^a
I. ASHA	ASHA	1946	0.47	$47^{a,b}$	$729^{a,b}$	0.0645^a
J. AISHA FRESH	ASHA (F)	1779	0.79	$63^{a,b}$	1110^a	0.0568^a

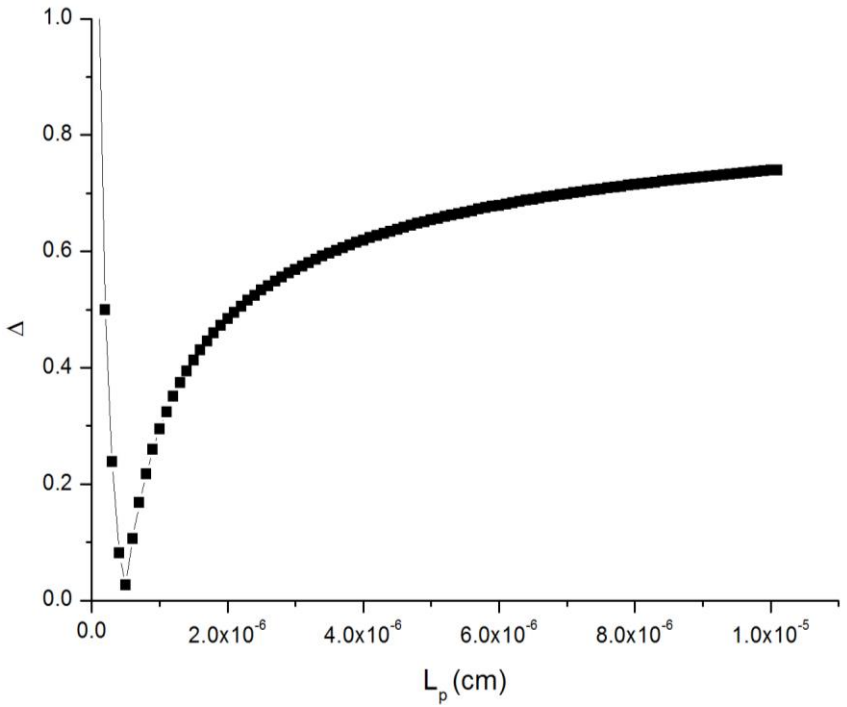
Values are mean (SD) of at least triplicate determinations. Mean values in a column with different letters are significantly different ($p < 0.05$).

Looking at **Table 6.13** it is clear that there is a wide range of different average conformations adopted by okra pectins, generally higher persistence lengths (L_p) would indicate more flexible conformations whereas lower values of persistence length would be consistent with a more compact structure. Whereas the mass per unit length (M_L) is an indication of the average side chain length. However, as you can see from **Figures 6.6** the minima in the target functions are actually quite large when you take into account experimental errors. Therefore, when considering these plots the ratio of L_p/M_L is often a better indication of overall flexibility (Patel et al., 2008, Morris et al., 2010b). Perhaps surprisingly the values of L_p/M_L do not correlate particularly with HG: RG-I ratio or the degree of esterification (DM or DAc). The predominant influence on flexibility appears to be molecular weight and from the data above, with exception of sample AGB (AG), the higher the molecular weight the less rigid/ more compact the pectin. This phenomenon has been shown previously for amylopectin and is due to the fact that larger molecules have conformations that are more flexible (Burchard, 1992). However, the chemical

analyses in section 6.1 enable the possibility to fix an average M_L and therefore look at flexibility in terms of L_p without the additional complication of simultaneously fitting mass per unit length (**Table 6.14**).

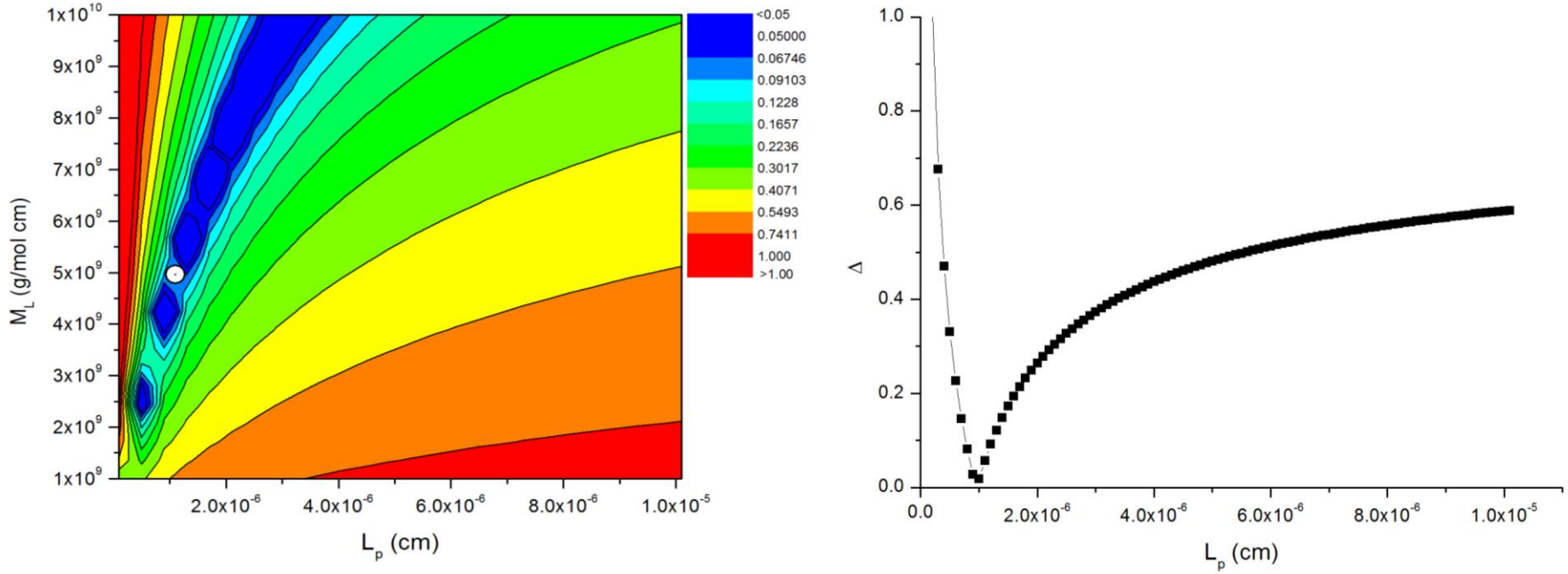


a



b

Sample (AGB)



a **b**

Sample PEM (K)

Figure 6.6: HYDFIT analysis for pectic polysaccharides. A solution of the Bohdanecky (1983) and Yamakawa-Fujii (1973) relations for L_p with M_L allowed to float. The x-axis and y-axis represent L_p (nm) and M_L (g/ (mol nm)) respectively. The target function, Δ is calculated over a range of values for M_L and Lp. In these representations, the values of Δ function are represented by the full-colour spectrum, from blue ($\Delta \leq 0.05$) to red ($\Delta \geq 1$). The global minima are indicated (○). HYDFIT analysis for pectic polysaccharides (left). A solution of the Bohdanecky (1983) and Yamakawa-Fujii (1973) relations for Lp (for a known mass per unit length M_L as indicated in **Table 6.14**. The plot of target function (Δ) vs. persistence length (right). An example has been given for samples AGB, PME (K) and see **appendices 9.2.6** for all other okra samples.

Table 6.14: Mass per unit length M_L and persistence length (L_p) estimations from combining molecular weight and intrinsic viscosity data through HYDFIT for okra pectin when the mass per unit length has been fixed depending on the monosaccharide composition.

Sample	Short name	[η] (g/ml)	$M_w \times 10^6$ g/mol	L_p (nm)	^aM_L (g/(mol nm))	L_p / M_L (nm^2 mol/g)
A. AGBAGOMA	AGB	1985	7.6	5[e]	451	0.0111[e]
B. AGBAGOMA deep green fresh	AGB (F)	1630	0.34	19[b]	442	0.0430[b]
C. PEMKRUMAH (TECHI)	PEM (T)	920	1.1	6[e]	452	0.0133[d,e]
D. PEMKRUMAH (KEN)	PEM (K)	2052	1.4	10[c,d]	441	0.0227[c]
E. KPOMG (TAMELE)	KPO (T)	2165	1.3	9[d]	420	0.0214[c,d]
F. ASONTEM (KEN)	ASO (K)	1499	0.83	10[c,d]	457	0.0219[c,d]
G. AGBAGOMA (AG)	AGB (AG)	965	0.42	10[c,d]	447	0.0224[c]
H. SENGEVI	SEN	1890	0.32	25[a]	460	0.0544[a]
I. ASHA	ASHA	1946	0.47	18[b]	452	0.0398[b]
J. AISHA FRESH	ASHA (F)	1779	0.79	12[c]	454	0.0264[c]

[a] calculated from the HG: RG-I ratio in **Table 6.9** and the degree of methylation and acetylation in **Tables 6.6** and **6.7**respectively. The mass per unit length of an average RG-I monomeric unit was taken as 555 g/mol nm (Morris et al., 2010b). Values are mean (SD) of at least triplicate determinations. Mean values in a column with different letters are significantly different ($p < 0.05$).

Estimates for the persistence lengths (**Table 6.14**) are mostly in the range of values which have been found previously for pectins of ~ 10 – 20 nm (Morris et al., 2008). Again, looking at the results in **Table 6.14** there is no evident influence of charge or degree of esterification on the chain stiffness of pectin chains and it again molecular weight, which has the most important

influence. This agrees with results on citrus pectin of differing degrees of methyl esterification (Morris et al., 2010a) which concluded that it was not the only charge, which dictated the rigidity of a pectin chain.

6.3.2 Conformation zoning (Normalised scaling relations)

(Pavlov et al., 1999) developed a procedure to estimate the conformation of polymers in solution based on the relationship between their molar mass, intrinsic viscosity and mass per unit length, M_L. In this case, the mass per unit length calculated previously using the chemical composition has been used (**Table 6.14**) as well as from the HYDFIT calculation (**Table 6.13**). In the first case, we have taken the mass per unit length calculated previously using the HYDFIT algorithm (Ortega and García de la Torre, 2007). As can be seen from **Figure 6.7** okra pectins have conformations which fall in either the extra-rigid rod, rigid rod, semi-flexible coil or random zones (Zones A-D); which is in general agreement with findings on citrus pectins (Morris et al., 2008) and sugar beet pectins (Morris et al., 2010b). Again, it should be noted that high or low values of M_L can lead to an overestimation or underestimation of the rigidity (Morris et al., 2010b). As M_L is included in the calculation of both the x- and y-coordinates conformation zoning sensitive to this. Therefore, as in the previous section the values for mass per unit length from the chemical analysis have also been used (Figure 5.16). In this scenario, all the pectins have conformations which fall in the rigid rod (Zone B), or semi-flexible coil (Zone C); this is in much closer agreement with findings on citrus pectins (Morris et al., 2008) and sugar beet pectins (Morris et al., 2010b). Furthermore, in both cases, the estimates of pectin rigidity are in good general agreement with those using the HYDFIT algorithm (**Tables 6.13 and 6.14**).

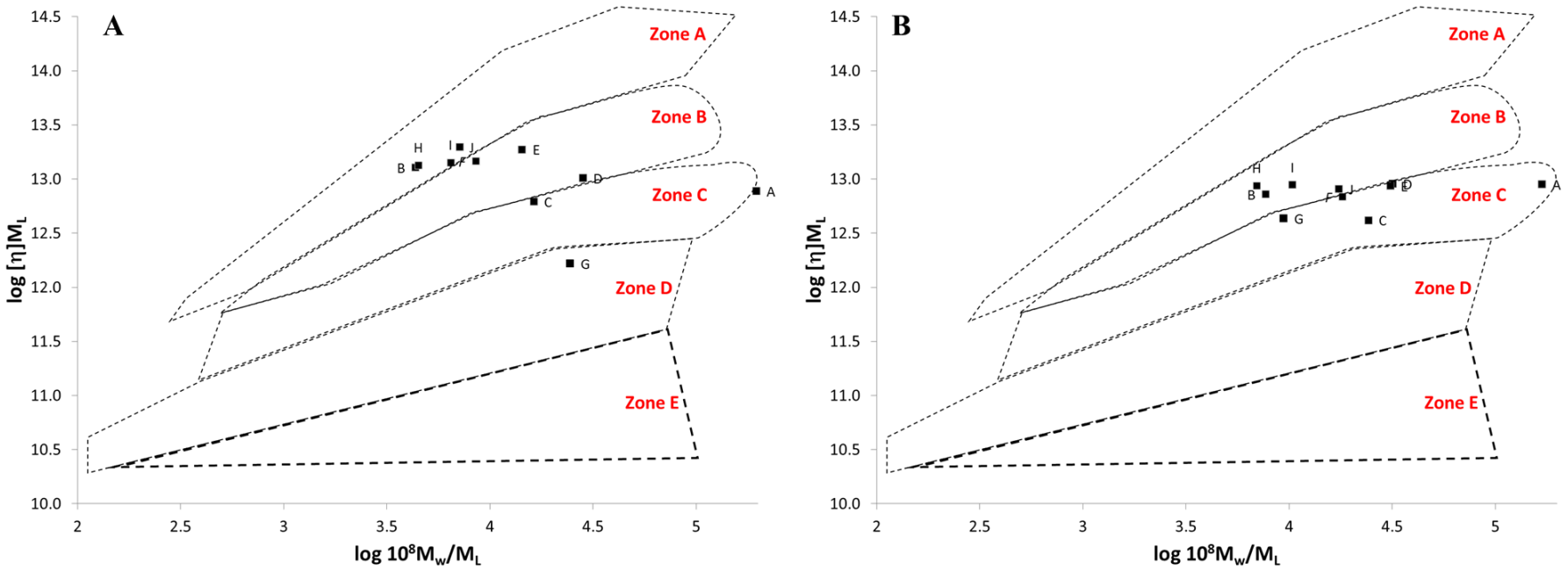


Figure 6.7: Normalised scaling plot of $[\eta]M_L$ versus M_w/M_L adopted from Pavlov et al., (1999) for each okra pectin. Zone A: extra rigid rod; Zone B: rigid rod; Zone C: semi-flexible; Zone D: random coil and Zone E: globular or branched. A: when mass per unit length has been fitted from physical data using HYDFIT (**Table 6.13**) and B: when mass per unit length has been calculated from chemical composition (**Table 6.14**). For clarity, ■ represents the pectin's samples which have been named A-J as per **Table 6.14**.

6.3.3 The degree of branching:

From the physicochemical data (**6.1** & **6.2**) coupled with the conformational data (**6.3**), there is the potential to estimate both the average number and length of side chains on a pectin molecule. Again, this can be done using the mass per unit length calculated from the freely-floated HYDFIT procedure (**Table 6.15**). Which then with knowledge of the HG: RG-I ratio enables the estimation of the mass per unit length of both the HG and RG-I sub-fractions. In order to partially verify this method, previously well characterised HG and RG-I regions from sugar beet has included as an example (Morris et al., 2010b) where the average side chain length for an HG region should be zero.

Table 6.15: Average number and degree of branching for okra pectin when the mass per unit length was allowed to float[a]

Sample	Short name	Average side chain length (number of monosaccharides)	Average number of side chains
AGBAGOMA	AGB	1	14800
AGBAGOMA deep green fresh	AGB (F)	9	80
PEMKRUMAH (TECHI)	PEM (T)	6	480
PEMKRUMAH (KEN)	PEM (K)	4	760
KPOMG (TAMELE)	KPO (T)	16	120
ASONTEM (KEN)	ASO (K)	10	250
AGBAGOMA (AG)	AGB (AG)	n.d.	n.d.
SENGEVI	SEN	6	140
ASHA	ASHA	7	180
AISHA FRESH	ASHA (F)	13	170
HG	/	n/a	0
RG-I	/	3	346

[a]in order to determine the number of side chains and degree of branching knowledge of the mass per unit length, HG: RG-I ratio, weight average molecular weight and the average mass of side chain sugar ratio(which is, in this case, the weighted average mass of neutral sugar residues) is required.

it is believed that, this is the first time this type of analysis has been used to estimate the number and length of pectin side chains. Clearly, there is a large difference in the results of the model for the different pectin samples, but can be observed is that in general, these pectins have a large number of relatively short side chains. These side chains are large when compared to those from sugar beet pectin analysed using the same technique, but short when compared to those visualised by atomic force microscopy (Paniagua et al., 2014), which had a size of ~ approximately 30 – 170 nm (~60 – 340 monosaccharide units assuming a typical monomer is 0.5 nm). Furthermore, there is no apparent correlation between this parameter and the chemical composition, although it does appear that the more rigid molecules have a larger number of shorter side chains.

6.4 Antioxidant activity of okra pectin:

The antioxidant activity of ten pectins isolated from okra pods was detected by two methods, including 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging and hydroxyl radical inhibition assay. Naturally, fruits, vegetables and grains delay disease onset and help maintain human health because they are both containing antioxidants and low in calories (Gutteridge and Halliwell, 2010). Some pharmacological studies detected that okra possesses antioxidant and anti-fatigue activities (Tongjaroenbuangam et al., 2011, Yang et al., 2012, Xia et al., 2015).

6.4.1 DPPH radical-scavenging activity:

Table 6.16: DPPH radical scavenging activity for ten genotypes of okra.

Sample full name	Sample short name	Free radicals scavenging effects DPPH (%)
AGBAGOMA	AGB	61 ± 0.6^{b}
AGBAGOMA deep green fresh	AGB (F)	48 ± 0.7^{e}
PEMKRUMAH (TECHI)	PEM (T)	67 ± 0.7^{a}
PEMKRUMAH (KEN)	PEM (K)	51 ± 0.6^{d}
KPOMG (TAMELE)	KPO (T)	51 ± 0.6^{d}
ASONTEM (KEN)	ASO (K)	56 ± 0.5^{c}
AGBAGOMA (AG)	AGB (AG)	41 ± 0.6^{f}
SENGEVI	SEN	62 ± 0.7^{b}
ASHA	ASHA	56 ± 0.5^{c}
AISHA FRESH	ASHA (F)	34 ± 0.7^{g}

Values are mean (SD) of at least triplicate determinations. Mean values in a column with different letters are significantly different ($p < 0.05$).

The DPPH radical is one of the stable radical sources which is widely used to estimate free radical scavenging activity and antioxidant electron-donating ability. In natural compounds, it has been accepted as a good tool to evaluate this activities (Wu et al., 2014). In the present study as shown in **Table 6.16**, the results indicated that pectins isolated from okra pods have some anti-oxidant activity at the concentration assayed. These DPPH percentages are less than those obtained for okra which was around 80 % (Ansari et al., 2005),and also lower than those published by Xia et al., (2015), Doreddula et al., (2014) and Li et al., (2011). On the other hand, they were higher than the antioxidant activity obtained by Ahiakpa, (2013) using aqueous extract from different okra collected from eight geographical regions. Again, they were higher than those published by Ye et al., (2008)for crude polysaccharides from the brown seaweed *Sargassum pallidum* which ranged from 10.2% to 19.1%. However, they were in line with those obtained by the same study using an ethanol extract. They were in good agreement with those reported for Brown crude polysaccharide fractions from the fruiting bodies of *Zizyphus Jujuba cv. Jinsixiaozao* CZS4P and ZS3P 42.4 % and 53.6 % respectively by Li et al., (2011). In **Table 6.17,** it can be seen that sample PEM (T)

showed much higher antioxidant activity than other samples (67 %). Thus, sample PEM (T) was the active okra genotype for antioxidant activity. In addition, samples AGB and SEN had high antioxidant activity. However, sample ASHA (F) presented weak influence in DPPH test (34 %). Based on the data acquired from the current study, these okra genotypes might be deemed as strong free radical scavengers, indicating their aptitude to limit free radical damage happening in the human body. Taking into account the properties discussed in the previous sections in this chapter it looks as though samples with lower HG: RG-I ratios perform the best in this assay.

6.4.2 Hydroxyl radical inhibition assay:

Table 6.17: Indicates the hydroxyl radical activity for okra pods from different varieties.

Sample full name	Sample short name	Hydroxyl radical inhibition %
AGBAGOMA	AGB	$72 \pm 0.7^{d,e}$
AGBAGOMA deep green fresh	AGB (F)	71 ± 0.5^{e}
PEMKRUMAH (TECHI)	PEM (T)	70 ± 0.7^{e}
PEMKRUMAH (KEN)	PEM (K)	85 ± 0.6^{a}
KPOMG (TAMELE)	KPO (T)	$84 \pm 0.7^{a,b}$
ASONTEM (KEN)	ASO (K)	$74 \pm 0.7^{c,d}$
AGBAGOMA (AG)	AGB (AG)	82 ± 0.7^{b}
SENGEVI	SEN	75 ± 0.6^{c}
ASHA	ASHA	66 ± 0.5^{f}
AISHA FRESH	ASHA (F)	$83 \pm 0.7^{a,b}$

Values are mean (SD) of at least triplicate determinations. Mean values in a column with different letters are significantly different ($p < 0.05$).

The hydroxyl radical is another of the reactive oxygen species which is considered as the most active free radical (Wu et al., 2014). The results of hydroxyl radical scavenging activity of okra genotypes were displayed in **Table 6.17**. The scavenging activity of okra pectins on inhibition of the hydroxyl radical was effective, it varied from 66 to 85 %. The highest values obtained for sample PEM (K) while the lowest was obtained for

sample ASHA. Samples PEM (K), KPO (T), AGB (AG) and ASHA (F) had the highest values of hydroxyl radical scavenging activity which may relate to the presence of some other phytochemicals (*i.e.* tocopherol and pigments) in the crude pectin (Li et al., 2011). In addition, activity of samples AGB, AGB (F), PEM (T), ASO (K) and SEN was also high but a bit lower than the above samples see **Table 6.18**. These results were in good agreement with those reported by (Li et al., 2011) for Brown crude polysaccharides (CZSP) about 77.6 %. But, they were lower than those obtained by Wang et al., (2015), Mau et al., (2001) and Mau et al., (2005b).

Generally, a molecular weight which is known at one of the most significant structural features for polysaccharides, has an important role in antioxidant activity of polysaccharides, whereby several reports stated that polysaccharides with low molecular weight have higher antioxidant activity (Liu et al., 2010, Wang et al., 2015). Furthermore, the presence of a high number of acetyl groups probably can result in high antioxidant activity as the highly acetylated polysaccharides showed stronger antioxidant activity (Chen et al., 2014). However, in this case, there is no correlation between the degree of acetylation and activity, the most obvious correlation appears to be with higher HG: RG-I ratios, which is contrary to the DPPH assay. However it should be noted that these assays are not measuring the same mechanism of antioxidation, this further illustrates the point that the degree of branching (HG: RG-I ratio) has a quite complex influence on bioactivity (Inngjerdingen et al., 2007).

6.5 Summary:

To summaries , 10 different genotypes of pectin extracted from okra pods from Ghana were characterised. In this chapter, the study of physicochemical properties of these different genetic okra pod pectins have been investigated. It revealed that these types of pectins had yields close to each other, and contained: carbohydrate (Gal, Glc, Rha, Ara, Xyl, GalN and GlcN). Protein and glucose contents were high for okra genotypes this is because okra pectins used in this study were not further purfied by dialysis. DEs was less than 50 % for all okra genotypes. Irrespective of the genotype, extracted okra pectin had a high degree of acetylation. Despite these common structural similarities, sugar molar ratios and variability in the HG and RG-I domains revealed a range of structures. All okra pectin isolates contained a high amount of linear HG segments as

indicated by the ratio of rhamnose to galacturonic acid. The structural features of isolated okra samples were characterised by ^{1}H NMR spectroscopy which was identical and typical for all pectins. Molecular weight values are consistent with the high intrinsic viscosities for all okra genotypes. Also, zeta potentials indicated a negative charge for okra pectins. DPPH and hydroxyl radical assays were applied for antioxidant activity of okra genotypes. Results of the current work display that with a selection of the suitable okra genotype there potential to tailor the properties of pectin depending on the required functionality.

Overall, the data would lead to the summary that different genetic variations have significantly affected the properties of okra extracts controlling the physicochemical behaviour of the resulting pectin. In addition, there is a high concentration of polysaccharides present in each of the aqueous extracts and so the okra plants themselves are expected to be rich in these polysaccharides.

6.5.1 PCA (Principal Component Analysis):

If all the primary data (*i.e.* those other than protein content which has been measured directly) are taken, the information can be used to show global difference/ similarities between samples with the use of a principal component analysis (PCA) plot (**Figure 6.8**).

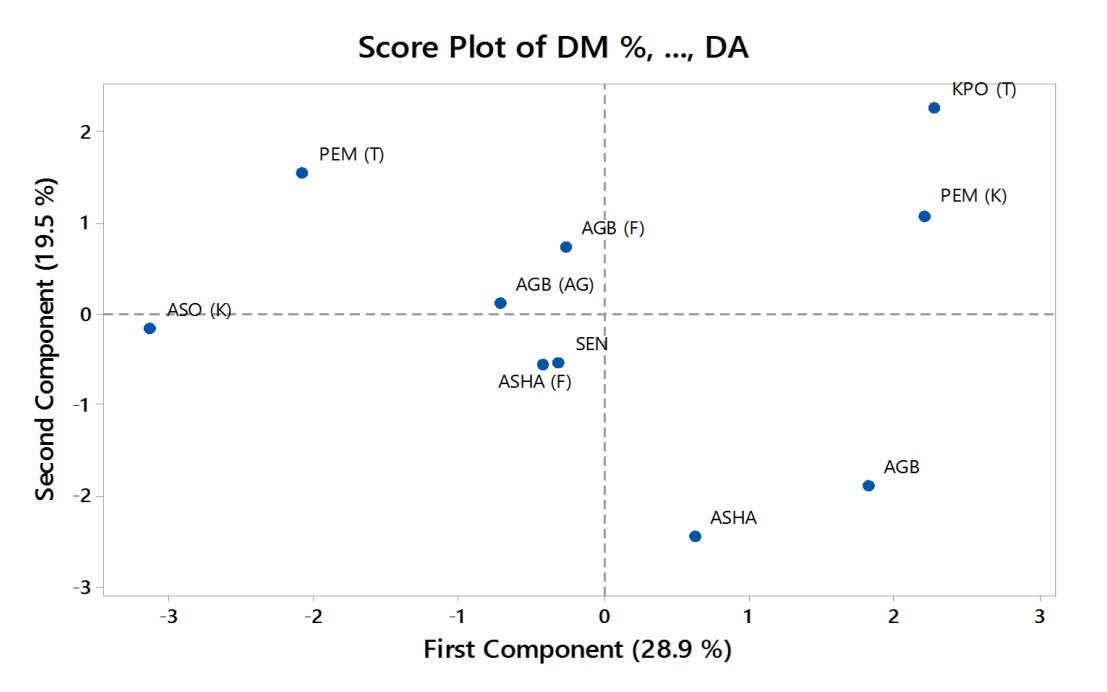


Figure 6.8: A principal component analysis (PCA) plot for okra pectins of different genetic variations and different growing conditions.

In **Figure 6.8,** the first component (PC1) is positively correlated with the degree of methylation, arabinose, galacturonic acid, intrinsic viscosity, molecular weight and degree of acetylation; whereas it is negatively correlated with the other parameters. The second component (PC2) is positively correlated with the degree of methylation, galactose, galacturonic acid and zeta potential whereas it is negatively correlated with the other parameters (**Figure 6.9**).

Therefore, sample KPOMG that has the highest degree of methylation together with high molecular weight and intrinsic viscosity is in the top right of the plot, whilst sample AGB which is low in galactose and galacturonic acid together with high molecular weight and intrinsic viscosity is in the bottom right of the plot. However, it is important to note that PC1 and PC2 are only explaining 48.4 % of the variation in these samples. In general PC1 seems to correlate well with the HG: RG-I ratio with the smoother pectins on the right of the plot and the hairier to the left. The second component (PC2) seems to be associated with an overall charge on the pectin as zeta potential and degree of methylation are important parameters. PC3 (not shown for clarity) appears to be associated with "purity" of the pectin as it is positively correlated with glucose content, whereas PC4 and PC5 are influenced strongly by rhamnose contents. Although

somewhat surprised there is no apparent correlation with the phenotype of the okra cultivar.

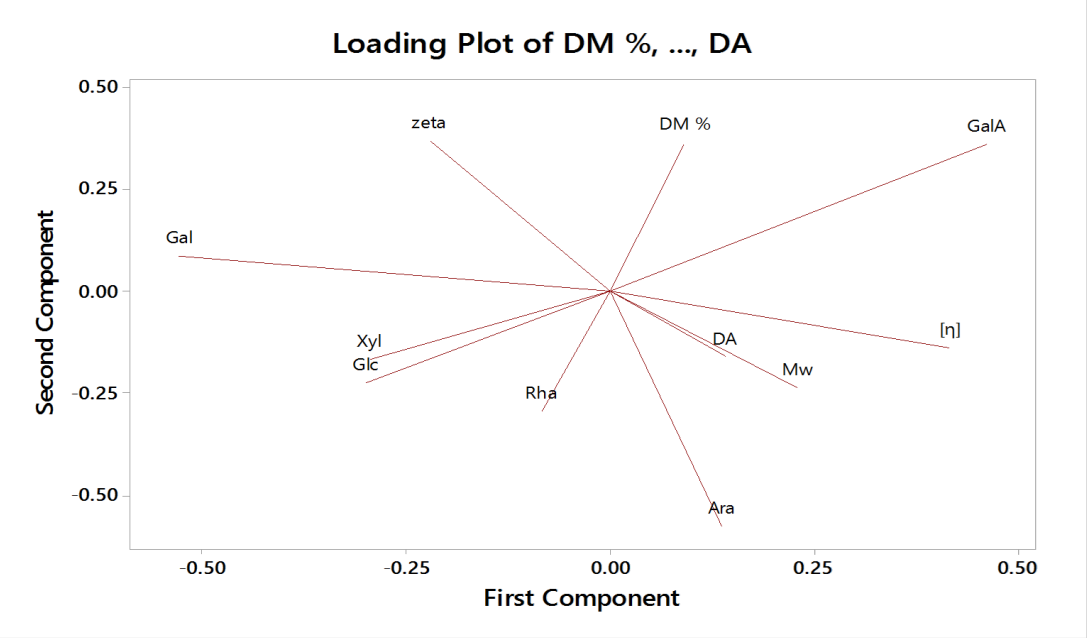


Figure 6.9: Loading biplot for okra pectins from different genetic genotypes.

6.6 Conclusions

Okra pectins have been characterised in terms of their chemical composition (**6.1**), physical properties (**6.2**), solution conformation (**6.3**) and their antioxidant activities (**6.4**). These properties have then been related to their genetic variant, therefore this may lead to a further understanding as to how the relationship between phenotype and pectin properties which could allow specific genetic varieties to be used depending on the desired physicochemical and functional properties. This work was instrumental in obtaining funding to further explore this principle (Robert S. McNamara Fellowships Program (RSM) Award and the George Weston Ltd-ACU Titular Fellowship 2016 Award).

Chapter 7

General conclusions and future work

7 GENERAL CONCLUSIONS AND FUTURE WORK

7.1 General conclusions:

From a scientific point of view; pectins are used as gelling agents and thickening agents or stabilizers in food industries. The aim of this study was to successfully investigate a number of factors (extraction conditions, genetic variation) on the physicochemical properties of pectins from natural sources. The degree of esterification, galacturonic acid, neutral sugar content and chemical structure of pectins rely on these properties, also they are influenced by the mode of extraction and purification. The effects of the pH, temperature and time were further investigated using aqueous extraction. The monosaccharides identified by the HPAEC with PAD were galacturonic acid, rhamnose, arabinose, galactose, glucose and xylose for both pumpkin and okra. This was in part verified by the GC-MS data which showed similar concentrations of monosaccharides, however, the GC-MS results of methylated samples were inconclusive. Therefore, full linkage analysis of the pectins could not be provided, most likely due to the difficulty in the methylation of sugar acids e.g. galacturonic acid. However, it seems likely that the relative proportions of the linkages detected were influenced by factors such as incomplete hydrolysis of the polysaccharide and loss of analytes. The SEC-MALLS data showed that there was polysaccharide present in each sample with high molecular weights with some additional low molecular weight material. The results from the FT-IR indicate a degree of methyl esterification, even if only in a low concentration, proving the presence of galacturonic acid in the sample. The lower proportion of galacturonic acid present in the samples is confirmed by the low degree of esterification and indicates a highly branched ‘hairy’ pectin structure; such structures are common in the gourd family. Finally, by measuring zeta potential both pumpkin and okra pectins have highly negative charge, and the intrinsic viscosity of the okra polysaccharide solutions was much higher than that of pumpkin polysaccharides because of the comparatively large molecular mass of the okra polysaccharides. Furthermore, by estimated the bioactivity both pectins are antioxidant. Considerations such as the time, temperature, pH, and type of acid used in the extraction procedure can significantly affect the properties of the extracted polysaccharides.

Overall, the data would lead to the conclusion that different genetic variations have significantly affected the properties of okra extracts controlling the physicochemical behaviour of the resulting pectin. In addition, since there is a high concentration of polysaccharides present in each of the aqueous extracts, the okra plants themselves are expected to be rich in these polysaccharides. The effects of the growth conditions, maturity and cultivar of the okra on the structures and potentially medicinal properties of its polysaccharides could also be studied.

7.2 Further work

There are many aspects in this study that can be subject to future work, while many characterizations were determined, there are still more needed studies to fully assign a structure to the pectins that were extracted from the pumpkin and okra plants. A first task would be to repeat any analysis with inconclusive results; so, to overcome the solubility issue new NMR samples would have to be prepared in a different way to give them more solubilisation, which could be done by using a changing the solvent or using differing concentrations of the sample solution. It would also be possible the new NMR instrument due to be online later in 2018, would allow for higher resolution. Also, the GC-MS analysis needs to be repeated since methylation data was inconclusive; this information is necessary for conjunction with NMR for a full linkage investigation. The arrangement of the monosaccharide units could be determined and a clear identification to the specific structures of the pectins could be given by using a successful methylation method and analysis, although the novel branching analysis proposed in this thesis is an interesting new approach in the estimation of branching in polysaccharides.

The SEC-MALLS result suggests the presence of a large amount of low Mw material. Therefore, it is suggested to dialyse the sample or do some preparative SEC on the okra pectin preparation, which has now been done as part of an ongoing collaborative project with the Kwame Nkrumah University of Science and Technology, (Ghana) and has been funded by the Robert S. McNamara Fellowships Program (RSM) and the George Weston Ltd-ACU Titular Fellowship 2016 Awards. More assays to determine antioxidant activity may be possible whereby there are many assays which have not

been applied, or to test other functional properties, for example, emulsifying activity. In addition, more Minitab work could be undertaken regarding the extraction of different polysaccharides within the plant. Similar extraction and characterization methods, as in this study, could be performed on plants grown in different geographical environments and at different stages of ripeness to see how the pectin structures are affected over time by the biological processes of the plant; these studies could be carried out along the same line of studies done on other plants outside the gourd family (Dong et al., 2003).

Chapter 8

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Chapter 9

Appendices

9 APPENDICES

9.1 For pumpkin:

9.1.1 FT-IR spectra:

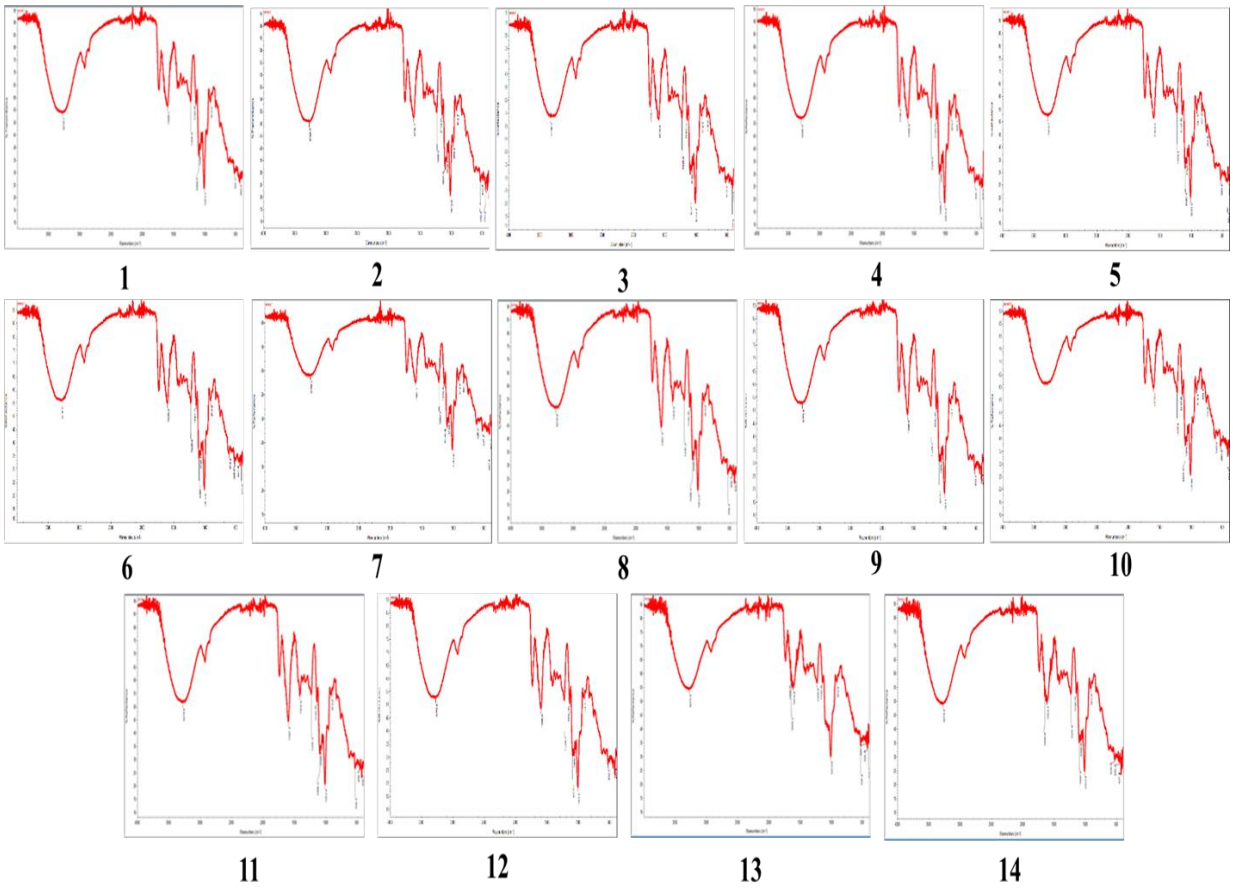


Figure 9.1: FTIR spectrum of pumpkin pectins extracted under different extraction conditions with a frequency range of 400 – 4000 cm^{-1}.

9.1.2 Constituent sugars composition:

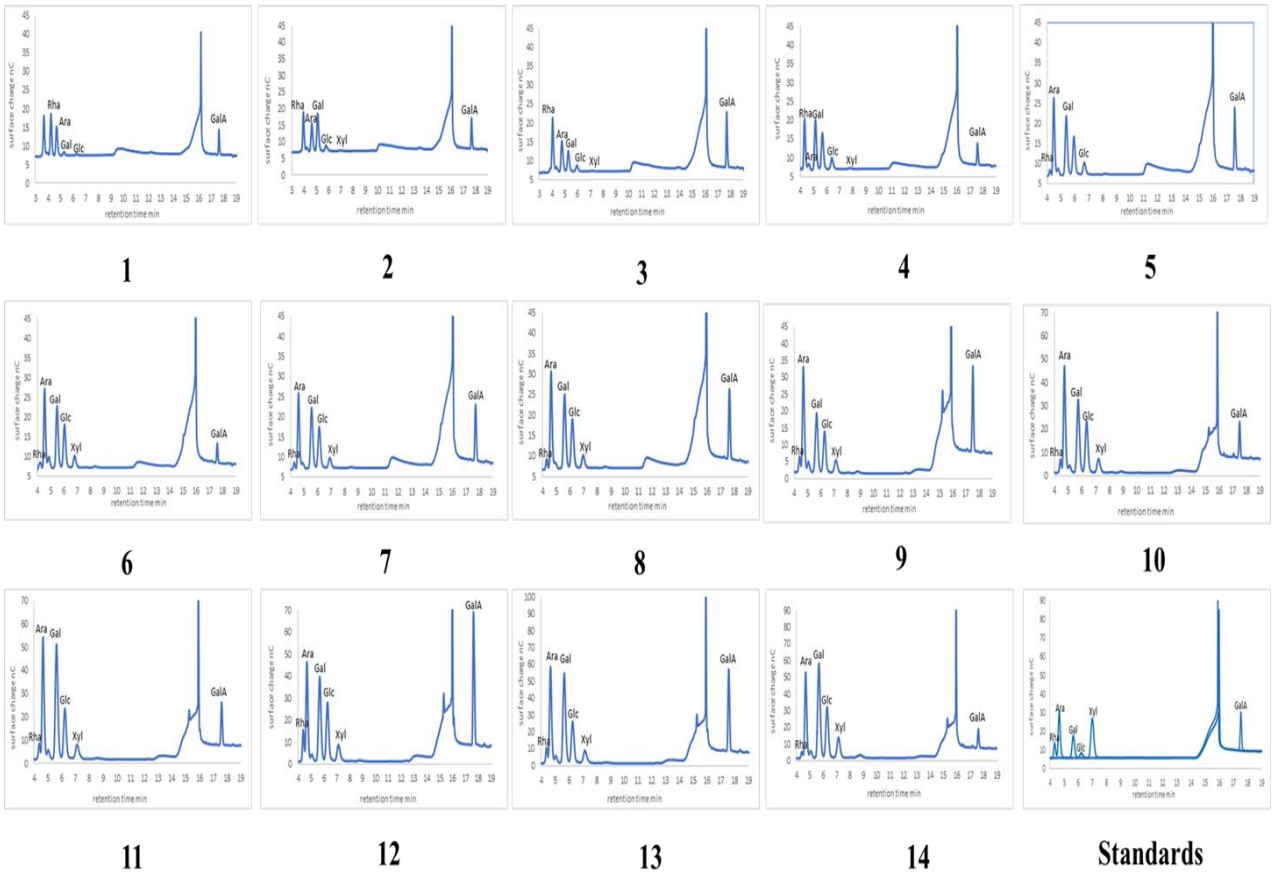


Figure 9.2: Peaks for a mixture of standards and for each monosaccharide present in pumpkin pectin samples using HPAEC.

9.1.3 Intrinsic viscosity:

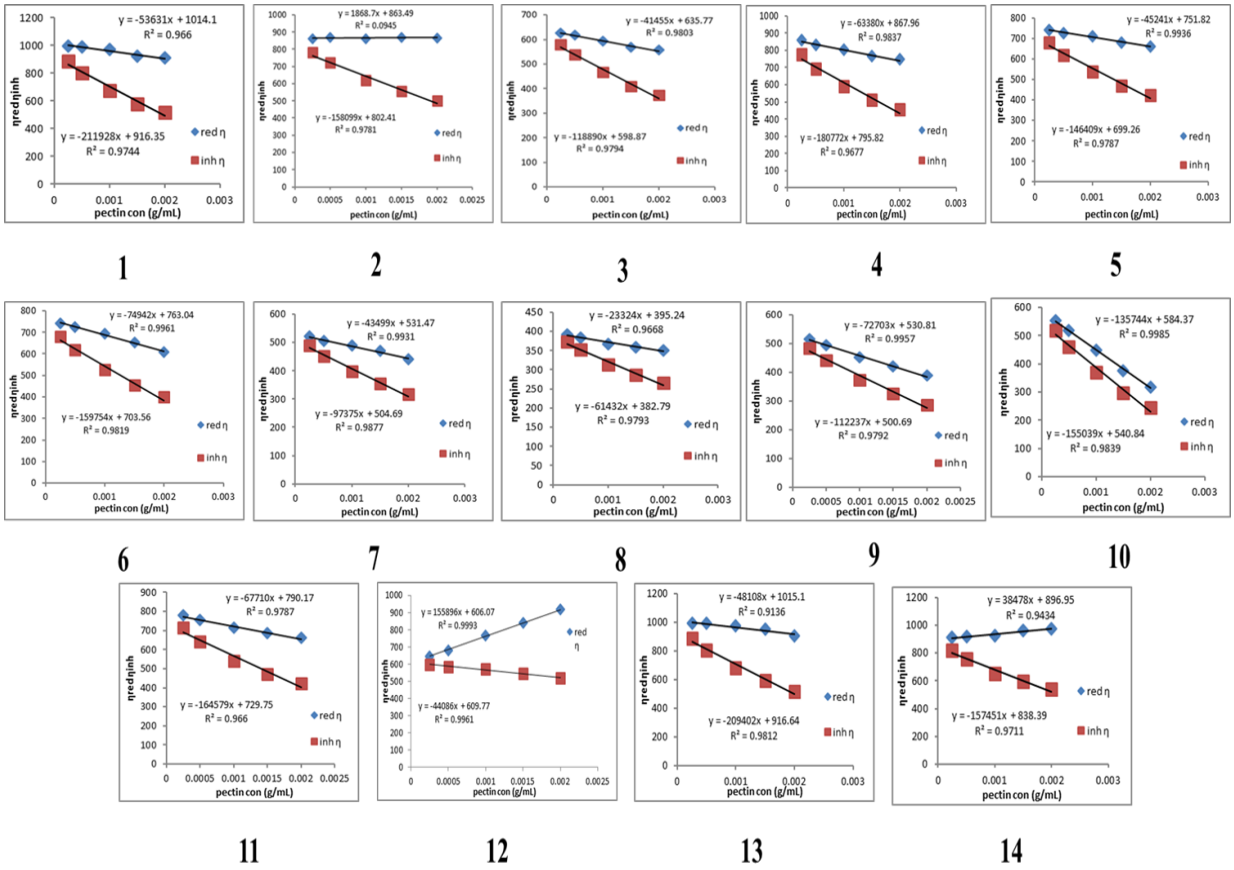


Figure 9.3: Huggins (♦) and Kraemer (■) plots for all pumpkin pectins extracted under different conditions.

9.1.4 Zeta potential:

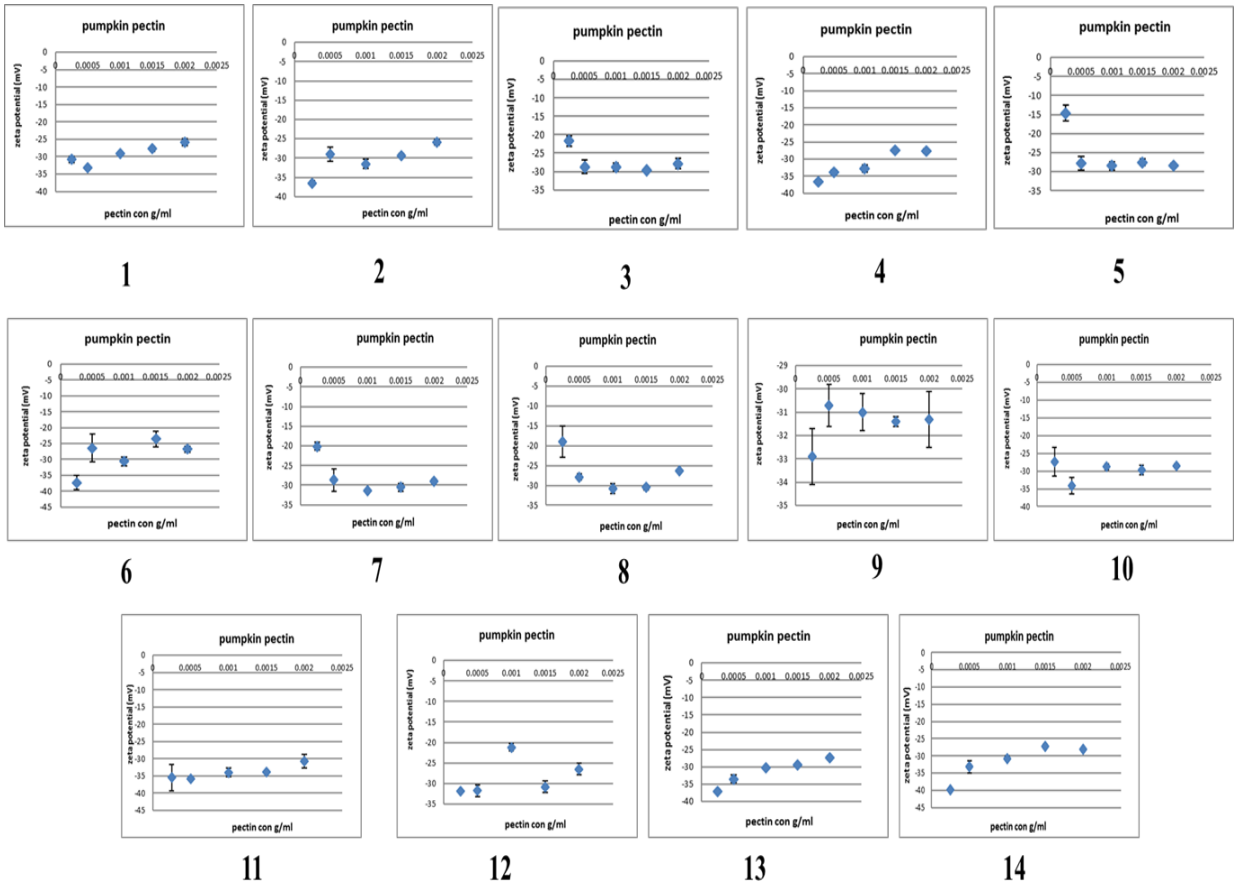
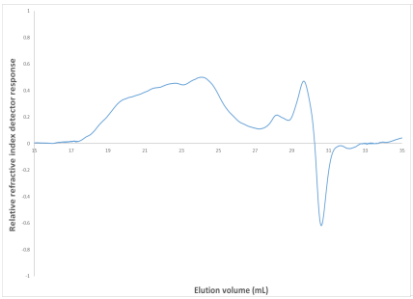


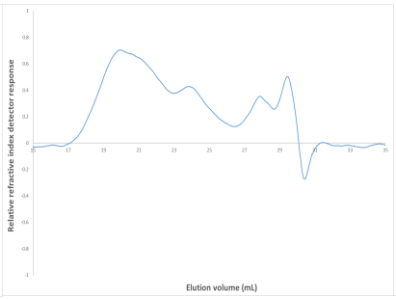
Figure 9.4: The effect of concentration on ζ-potential for all pumpkin pectins extracted under different conditions.

9.1.5 SEC-MALLS:

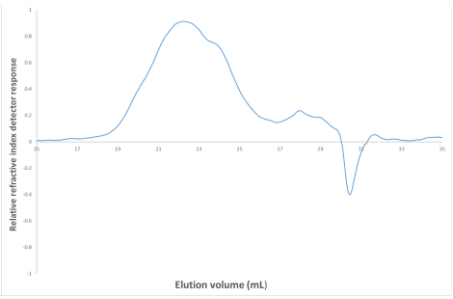
9.1.5.1 Reflective index:



1



2



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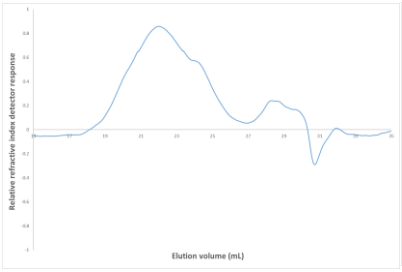

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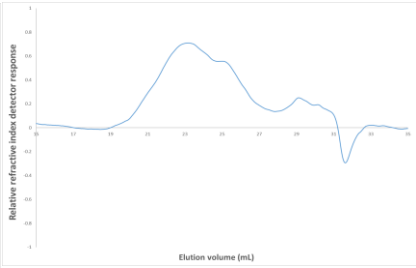

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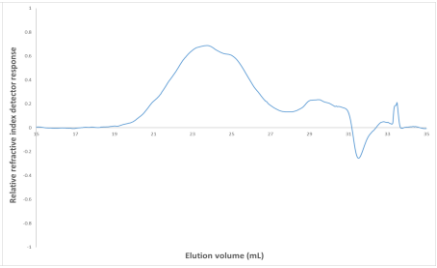

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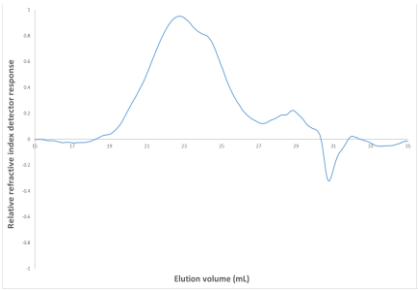

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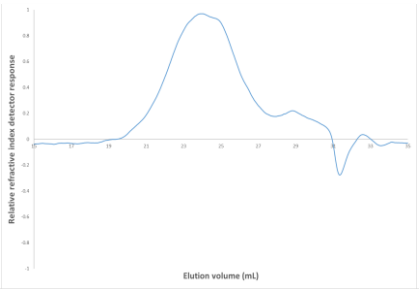

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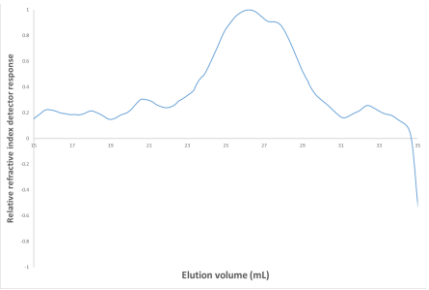

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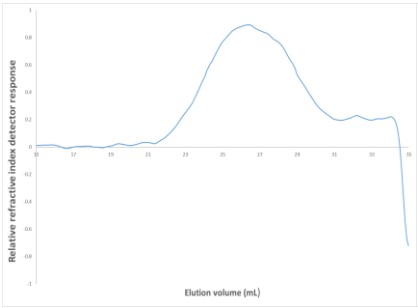

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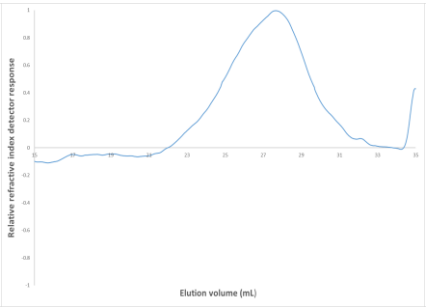

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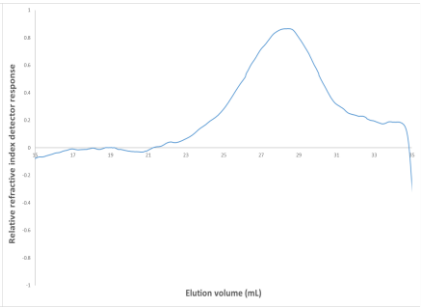

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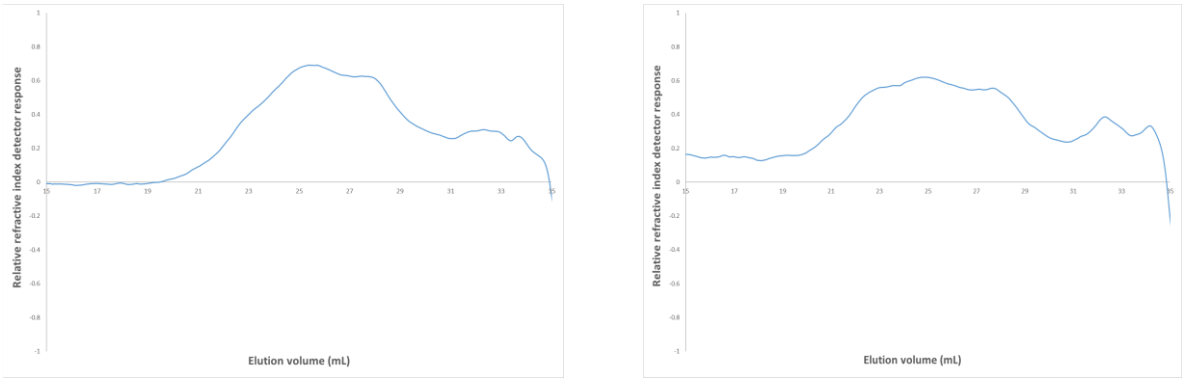
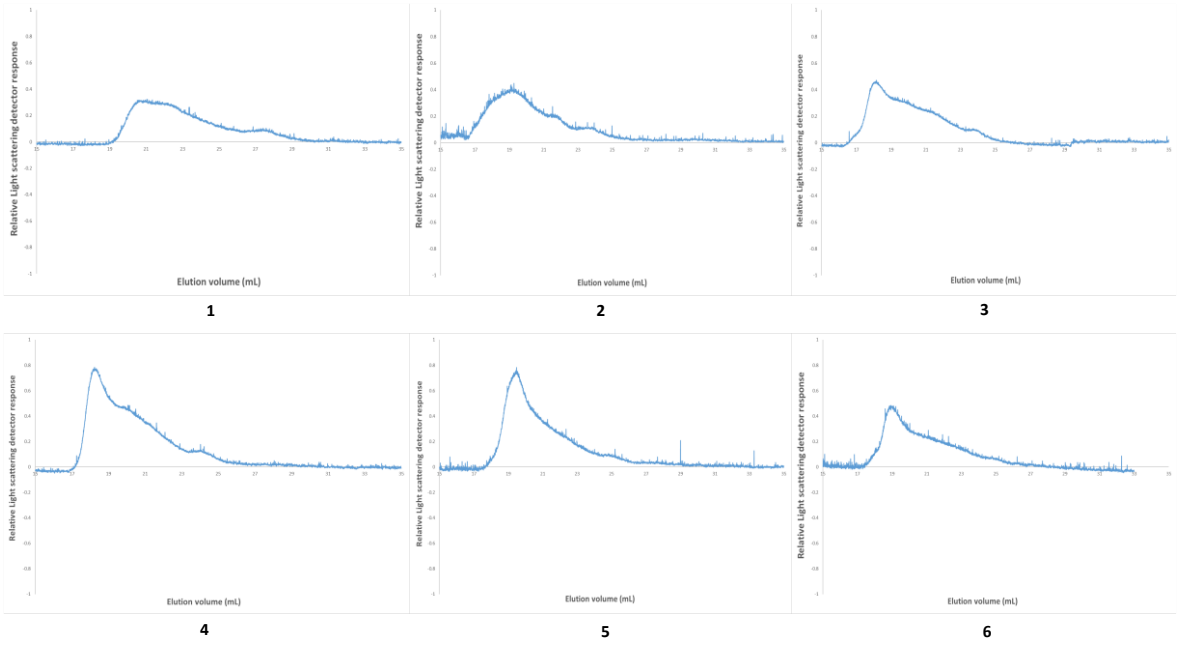


Figure 9.5: The realative reflective index plots for all pumpkin pectin samples extracted under different extraction conditions using SEC-MALLS.

9.1.5.2 Light scattering LS:



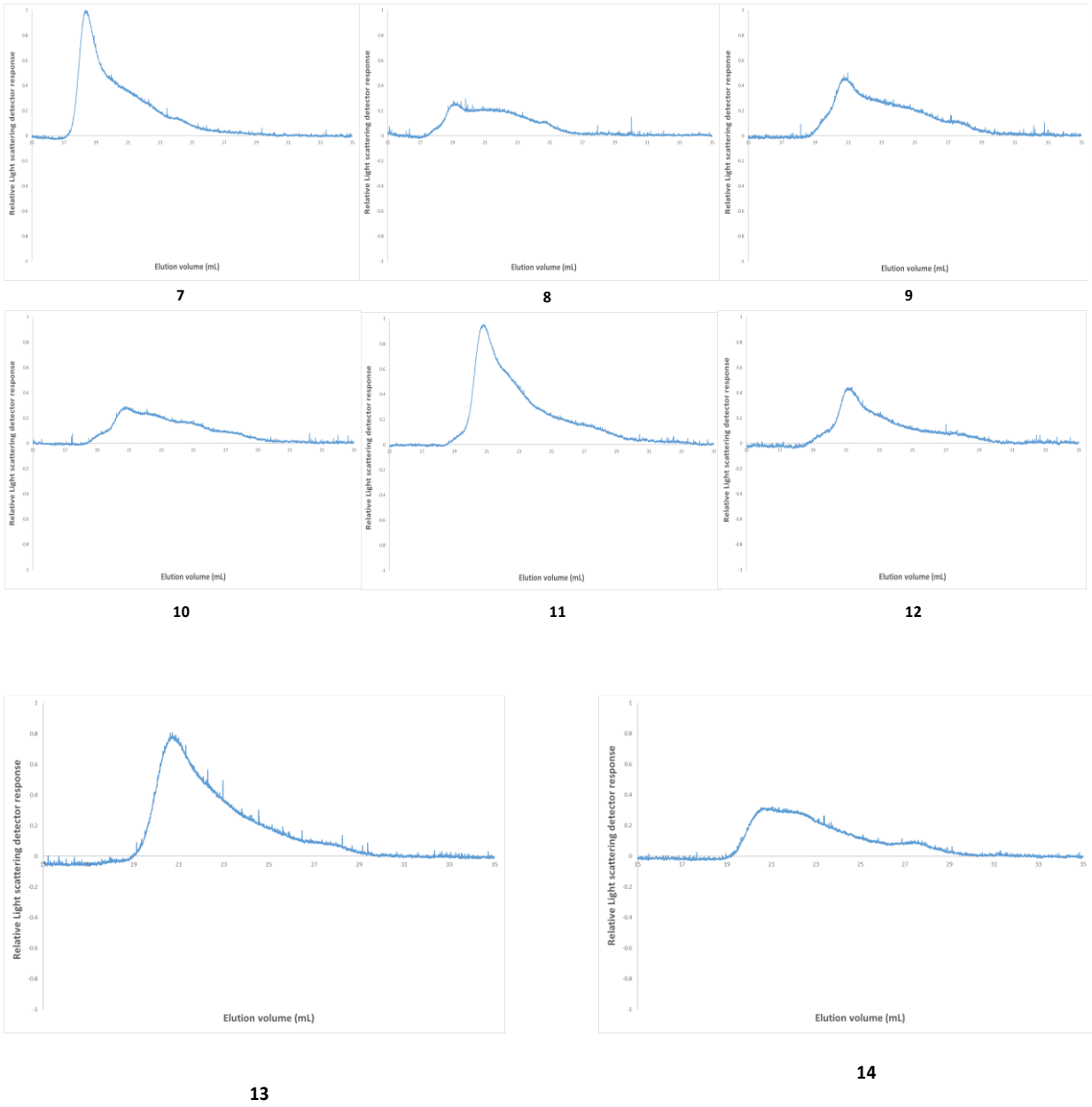
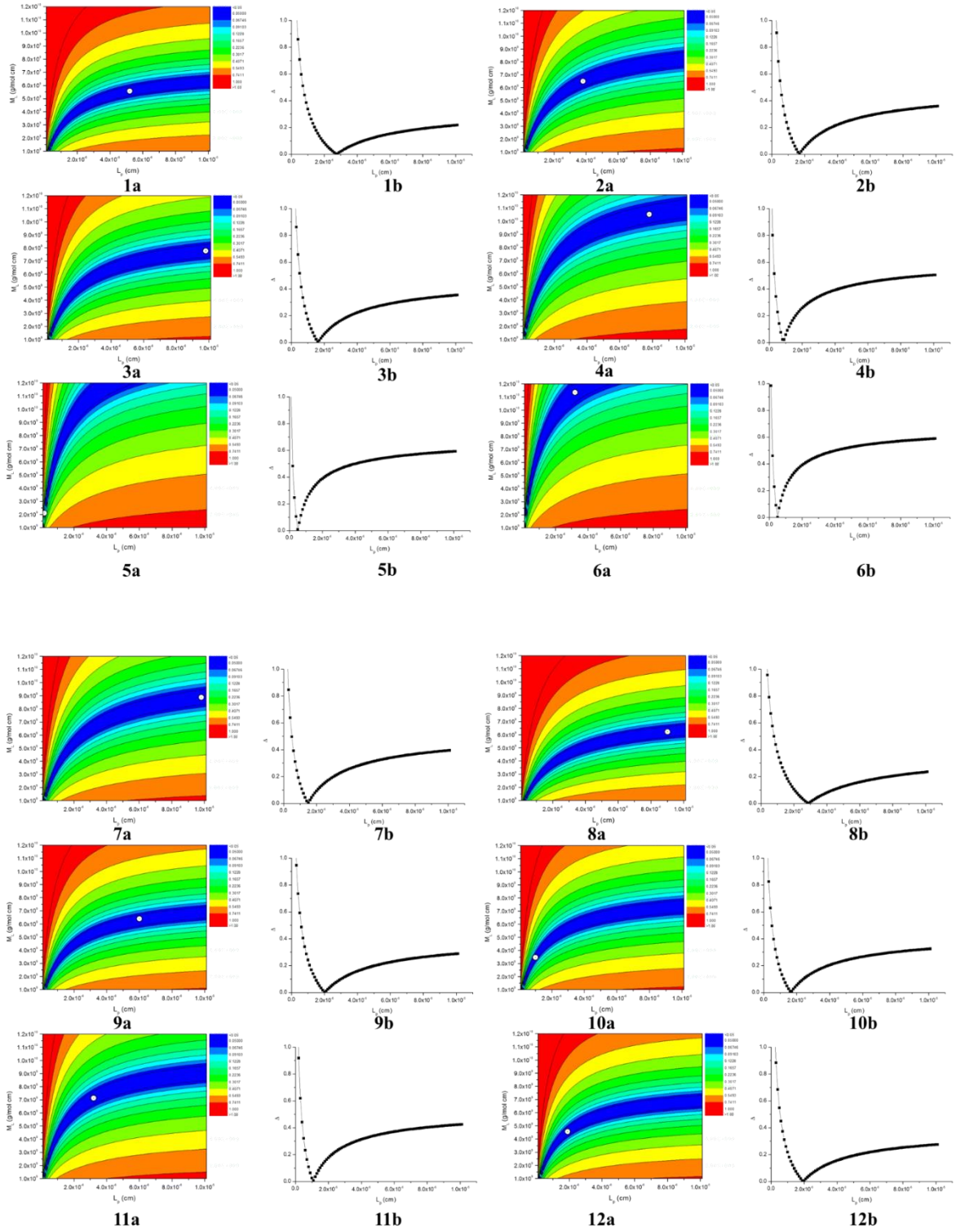


Figure 9.6: The light scattering plots for all pumpkin pectin samples extracted under different extraction conditions using SEC-MALLS.

9.1.6 HYDFIT analysis for pumpkin pectic polysaccharides:



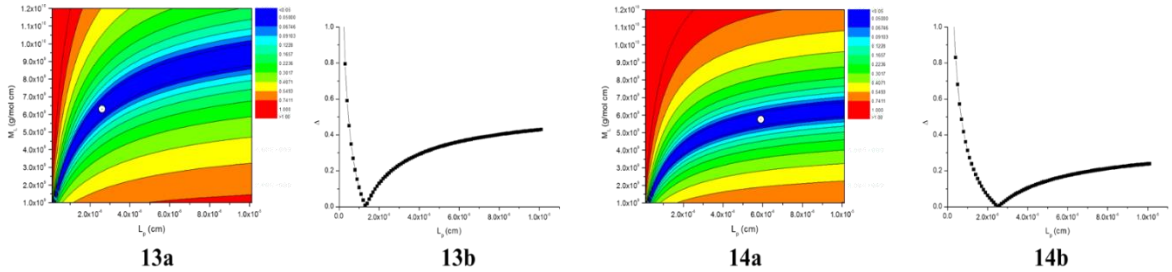


Figure 9.7: HYDFIT analysis for pectic polysaccharides. A solution of the Bohdanecky (1983) and Yamakawa-Fujii (1973) relations for L_p with M_L allowed to float. The x-axis and y-axis represent L_p (nm) and M_L (g/ (mol nm)) respectively. The target function, Δ is calculated over a range of values for M_L and Lp. In these representations, the values of Δ function are represented by the full-colour spectrum, from blue ($\Delta \leq 0.05$) to red ($\Delta \geq 1$). The global minima are indicated (○). Samples designated with an "a". HYDFIT analysis for pectic polysaccharides. A solution of the Bohdanecky (1983) and Yamakawa-Fujii (1973) relations for Lp (for a known mass per unit length M_L as indicated in Table 5.17. The plot of target function (Δ) vs. persistence length for samples designated with a "b".

9.2 For okra:

9.2.1 FT-IR spectra

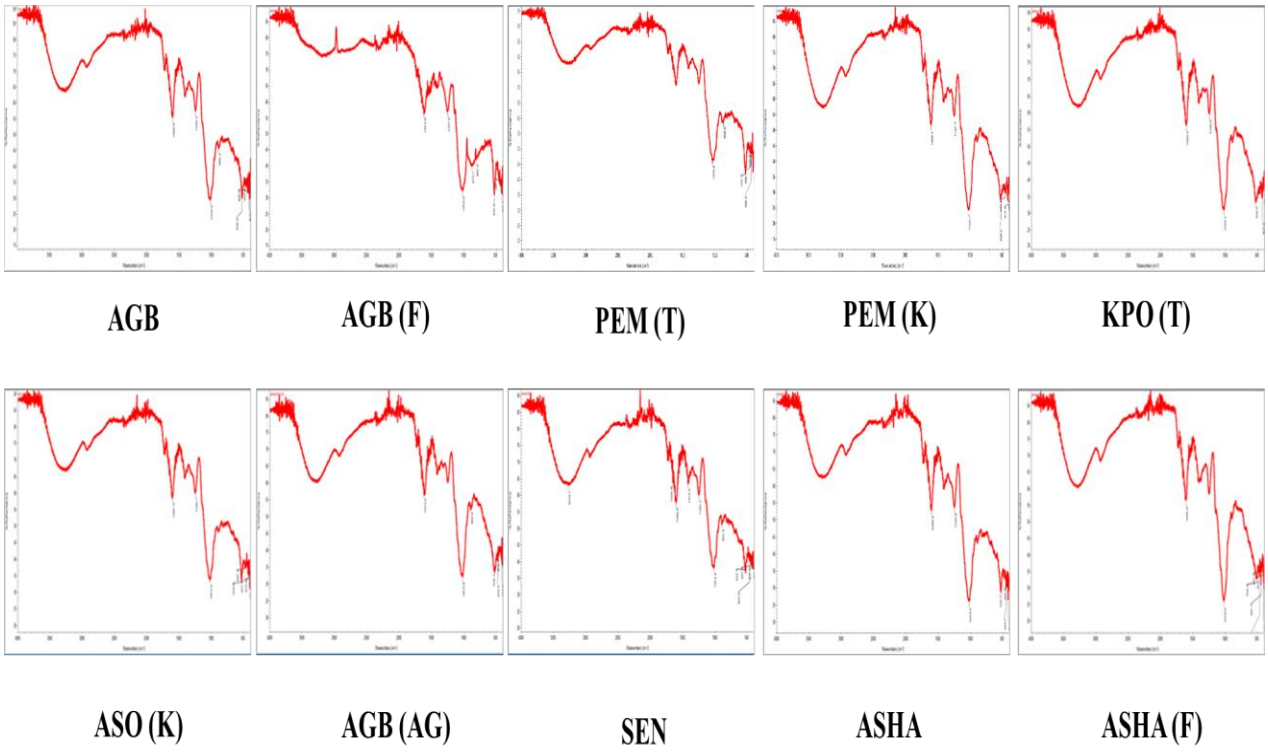


Figure 9.8: FTIR spectrum of okra pectins with a frequency range of 400 – 4000 cm^{-1}

9.2.2 Constituent sugars composition:

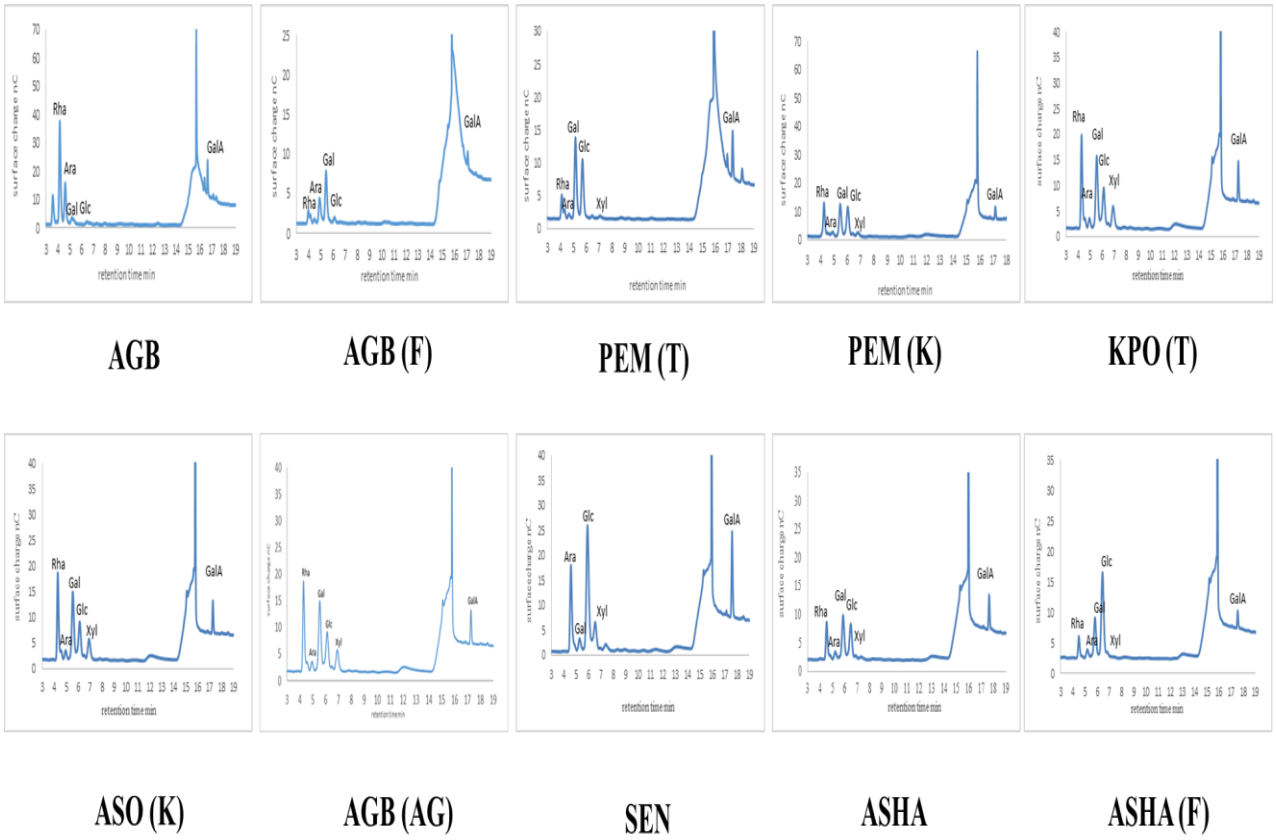


Figure 9.9: Peaks for a mixture of standards and for each monosaccharide present in okra pectin samples using HPAEC.

9.2.3 .NMR spectra:

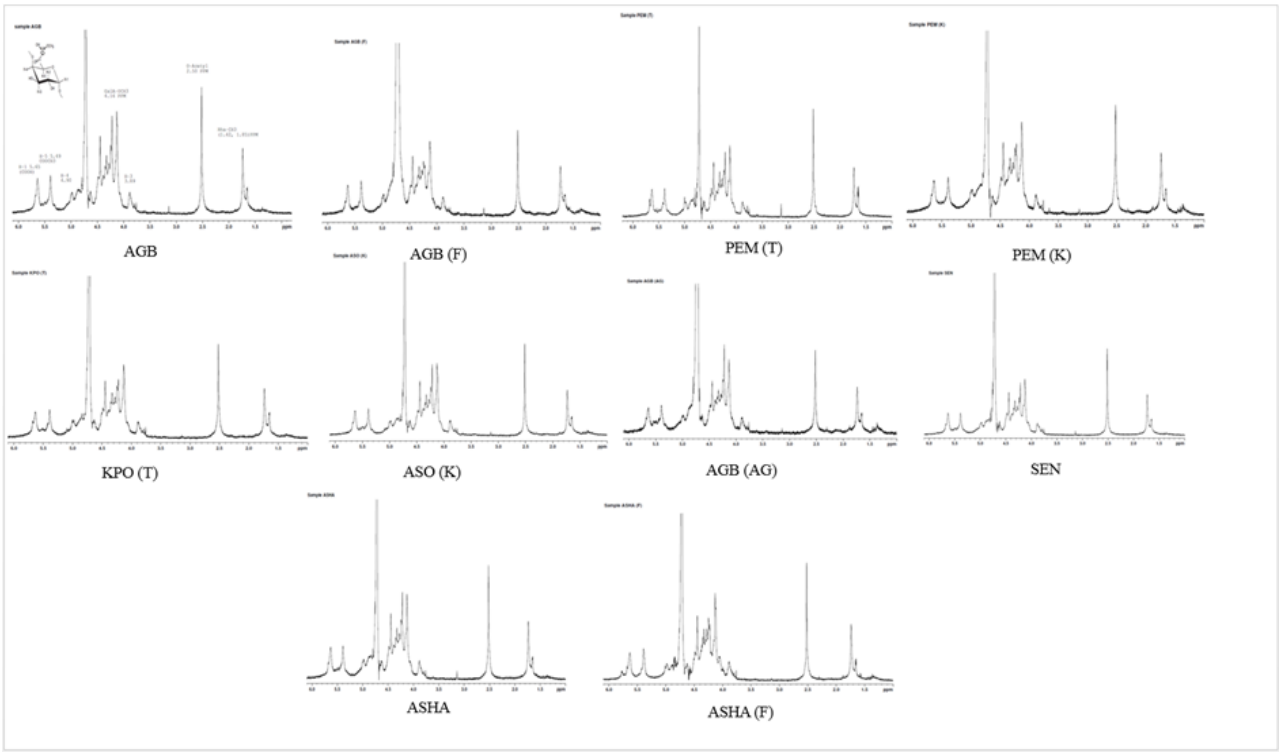


Figure 9.10: comparative of ^{1}H NMR spectra for all okra pectin samples in D_2O at 70 ˚C.

9.2.4 Intrinsic viscosity:

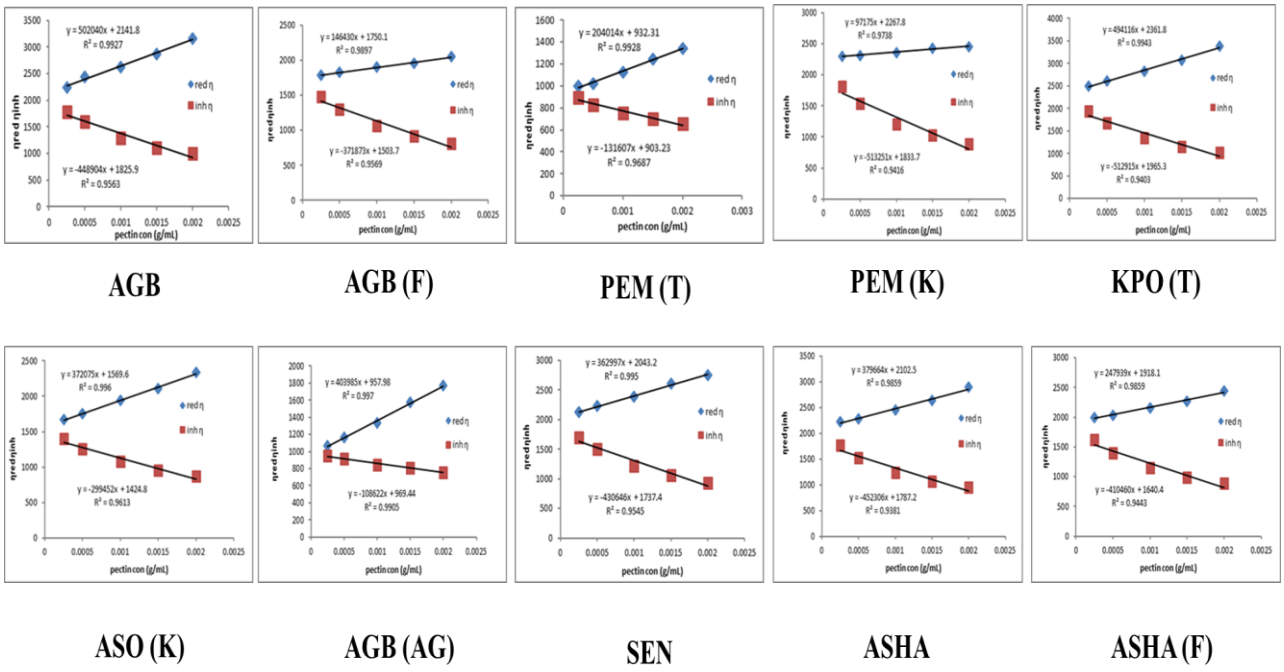


Figure 9.11: Huggins (♦) and Kraemer (■) plots for all okra pectins samples.

9.2.5 Zeta potential:

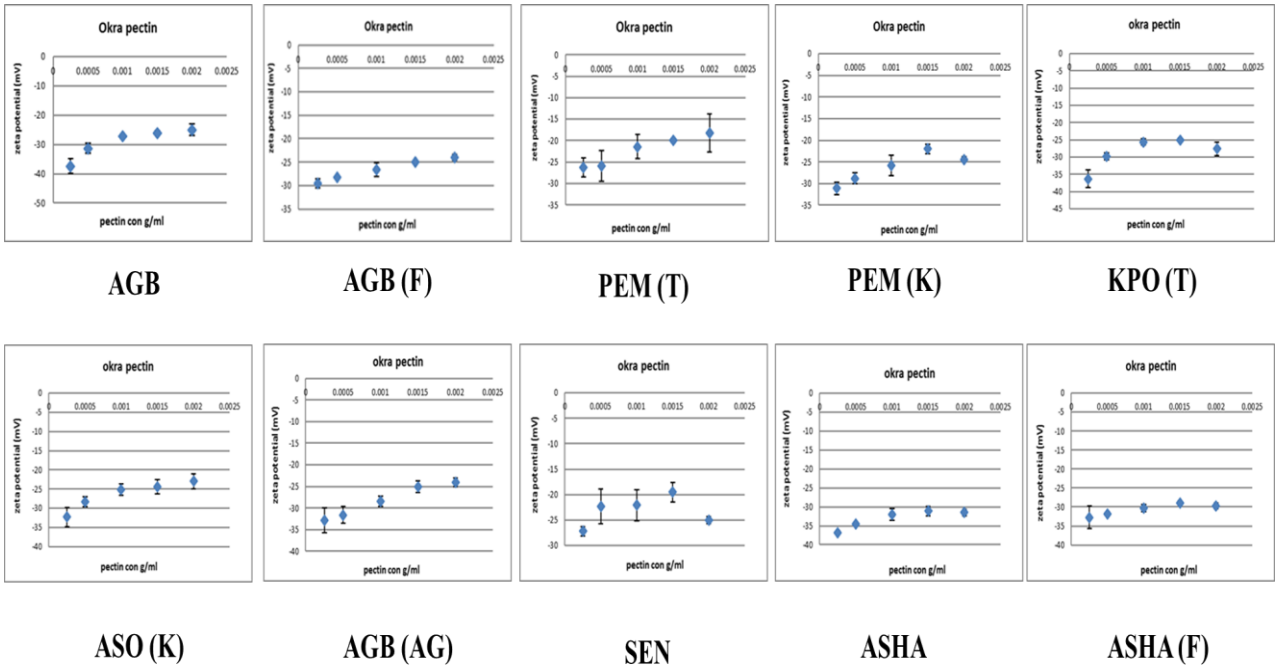
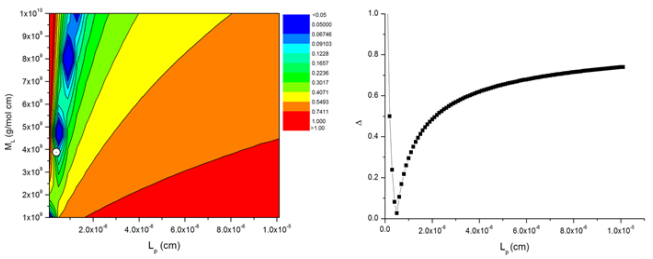
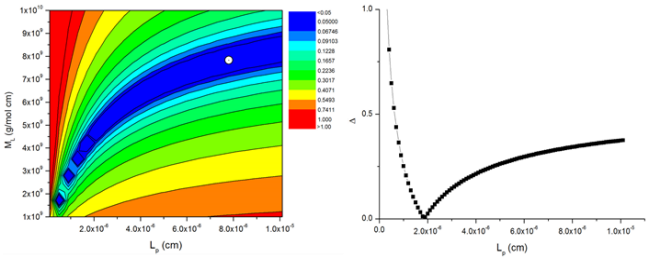


Figure 9.12: Graphical representation of zeta potential for okra pectin samples solutions.

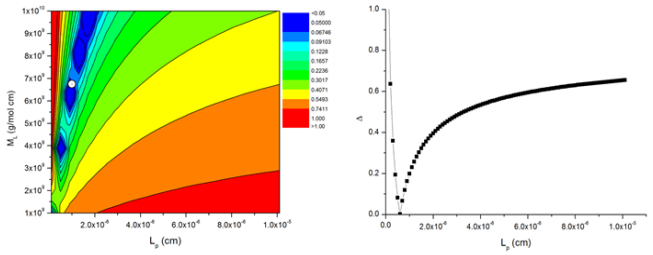
9.2.6 HYDFIT analysis for okra pectic polysaccharides:



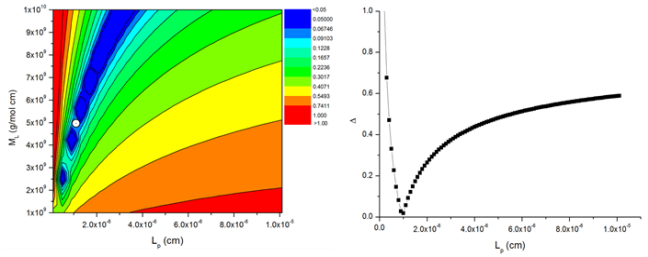
AGB



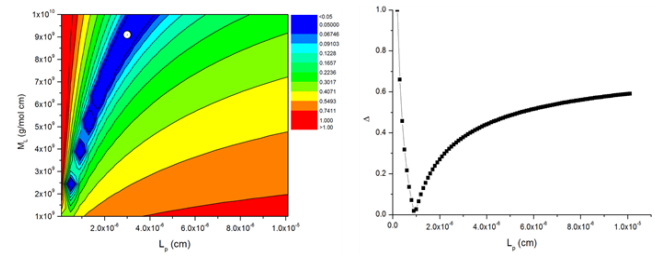
AGB (F)



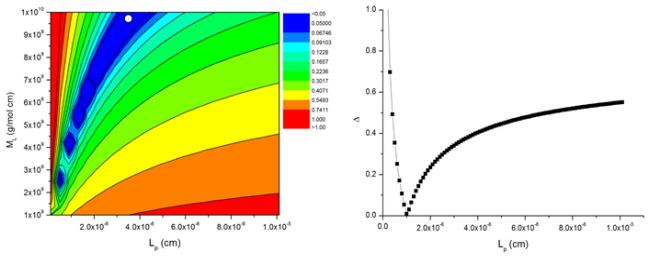
PEM (T)



PEM (K)



KPO (T)



ASO (K)

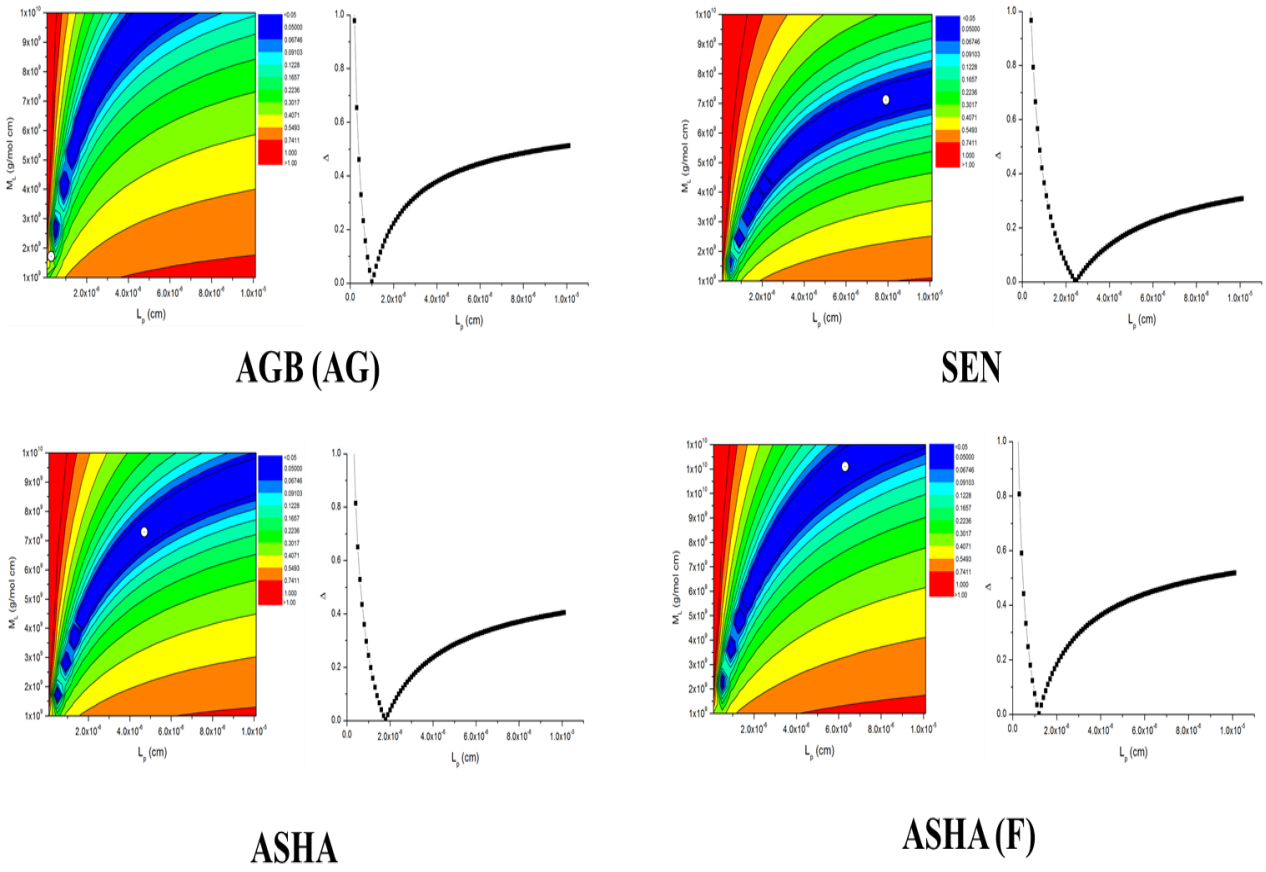


Figure 9.13: HYDFIT analysis for pectic polysaccharides. A solution of the Bohdanecky (1983) and Yamakawa-Fujii (1973) relations for L_p with M_L allowed to float. The x-axis and y-axis represent L_p (nm) and M_L (g/ (mol nm)) respectively. The target function, Δ is calculated over a range of values for M_L and Lp. In these representations, the values of Δ function are represented by the full-colour spectrum, from blue ($\Delta \leq 0.05$) to red ($\Delta \geq 1$).