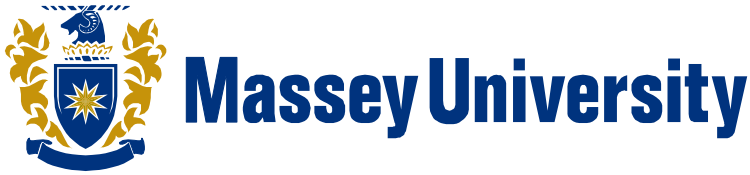
Isolation, Characterisation and Functional Properties of Pectin from Gold Kiwifruit (*Actinidia chinensis* cv. Hort16A)



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Abstract

This research was concerned with the isolation, physicochemical characterisation and functional properties of pectin from gold kiwifruit cv. Hort16A. The process of extracting pectin from gold kiwifruit was developed by evaluating three different techniques (acid, water and enzyme), four different conditions (time, temperature, puree to solvent ratio and enzyme concentration) and fruit of two different maturities (early-season and main-season fruit). The effects of the extraction techniques and conditions on the physicochemical properties and functional properties of pectin were studied in detail. The effects of the extraction techniques and the fruit maturity on the functional properties of pectin were also investigated. The chemical compositions, physical features and rheological behaviours of the extracted pectins were determined, underlining the importance of these features to the functional properties of gold kiwifruit pectin.

The total non-starch polysaccharide composition, sugar composition, protein and ash contents, degree of esterification (DE) and molecular properties (weight-average molecular weight (M_w), polydispersity index and root mean square radius) of pectin are influenced by the extraction techniques, the extraction conditions and the degree of maturity. Pectin from early-harvested fruit (EHF) (less mature fruit) is more difficult to extract than pectin from main-harvested fruit (MHF) (more mature fruit) when using extraction methods that have been developed for MHF. This is probably because the cell wall network of less mature fruit is more compact/dense and the pectin could still be insoluble, whereas the cell wall network of more mature fruit is less tightly bound because of physiological changes during maturation. Purified pectin from EHF is characterised by a lower yield (1.52 versus 3.64% w/w), a higher DE (90 versus 84%), a lower galacturonic acid (GalA) content (40.21 versus 55.50% w/w), more branching chains (a side chain every 47–57 GalA residues versus every 50−97 GalA residues), a higher protein content (25.94 versus 13.82% w/w), a lower M_w (9.7×10^5 versus 2.52×10^6 g/mol) and a higher viscosity than purified pectin from MHF. Overall, the physicochemical properties of gold kiwifruit pectin are consistent with its rheological behaviour.

Pectins extracted from fruit of different maturities and using different methods have different physicochemical properties. MHF pectin extracted using a water method is characterised by an M_w of 3.75 x 10^6 g/mol, a GalA content of 51.87% w/w, a degree of branching of 50 and a DE of 84%. MHF pectin extracted using an enzymatic method has a lower M_w (1.65 x 10^6 g/mol), similar to that of enzyme-extracted EHF pectin (0.21 x 10^6 g/mol). In contrast, EHF pectin extracted using a water method has low M_w (1.03 x 10^6 g/mol) and GalA content (42.88%), suggesting that gold kiwifruit pectin with high M_w is recovered from more mature fruit.

The rheological properties of the extracted pectin are affected by its M_w and DE. Purified pectin extracted by enzymatic treatment from both EHF and MHF exhibits the lowest viscosity and M_w. Purified EHF pectin obtained by water treatment exhibits a higher viscosity even though the pectin is characterised by a lower M_w (1.03 x 10^6 g/mol) with a higher DE (90%) compared to acid-extracted EHF pectin (a higher M_w: 1.66 x 10^6 g/mol and lower DE: 88%). This is because of low electrostatic repulsion of high DE pectin, which reduces intra- and/or intermolecular distances, resulting in greater molecular association, and the greater degree of branching, which leads to more chain−chain associations, therefore exhibits higher viscosity. A similar trend is observed for MHF pectin. Water-extracted MHF pectin, with a high M_w (3.75 x 10^6 g/mol) and a low DE (84%), exhibits a lower viscosity than EHF pectin (a low M_w but a high DE, *i.e.* 90%). The viscoelastic properties of the pectin are consistent with its physical properties.

Gold kiwifruit pomace, a by-product from gold kiwifruit juice manufacture, may be a new source of pectin. The physicochemical properties of pomace pectin are quite different from those of whole fruit pectin. Purified pomace pectin is characterised by a higher GalA content (64–68% w/w) but a lower M_w (6.7–8.4 x 10^5 g/mol) and exhibits a lower viscosity.

In the work on the functional properties of gold kiwifruit pectin, its gelation was investigated and was compared with that of other commercial pectins (apple and citrus). Gold kiwifruit pectin, which is classified as a high methoxyl pectin (HMP), has “weak gel” properties and gels at high temperature. Because of low electrostatic repulsion among the

pectin chains, the gelation properties are not markedly affected by varying the gelation conditions such as pH and sugar concentration. However, increasing the concentration of pectin leads to an increase in the gel strength. The viscoelasticity of the gel is strongly influenced by the DE; the gel strength increases as the DE decreases, because high charge density (or low DE) pectins have numerous active sites with high probability for hydrogen bonding, and sufficient hydrophobic interactions, thus resulting in more stabilised molecular networks.

A common defect in acidified milk drinks (AMDs) is sedimentation caused by protein aggregation. HMP is commonly added to prevent this separation. In this part of the work, the influence of gold kiwifruit pectins (DE 84, 85 and 90%) on the stability of AMDs (10% w/w low heat skim milk powder) was evaluated and was compared with that of pectins from other sources (apple and citrus). The stabilities, in terms of serum separation, amount of adsorbed and non-adsorbed pectin, viscosity and particle size, were compared at different pectin concentrations (0.1–1.0% w/w). High M_w gold kiwifruit pectin (DE 84%) stabilises an AMD at lower concentrations (0.3% w/w) than other commercial HMPs. In addition, HMPs with very low charge density and very low M_w do not have the ability to stabilise AMDs at the pectin concentrations used in this study. These results suggest that M_w has a very important role in stabilising an AMD. In addition, the viscosity effect created by high M_w apparently supports the dispersion of casein–pectin complexes in the drink.

Table of Contents

CHAPTER 2 Techniques

CHAPTER 3 Characterisation of Gold Kiwifruit Pectin Isolated by Acid and Water Treatment

CHAPTER 4 Characterisation of Pectin Isolated by Enzymatic Treatment

CHAPTER 6 Gelation Properties of Gold Kiwifruit Pectin

CHAPTER 7 Stabilisation of Acidified Milk Drinks: Addition of Gold Kiwifruit Pectin

List of Figures

List of Tables

List of Abbreviations

AMD	:	Acidified Milk Drink
ANOVA	:	Analysis of Variance
AOAC	:	Association of Official Analytical Chemists
AR	:	Arabinase
Ara	:	Arabinose
CA	:	Citric Acid
CCP	:	Colloid Calcium Phosphate
CE	:	Capillary Electrophoresis
CDTA	:	Cyclohexane Diamino Tetra Acetic Acid
CMC	:	Carboxymethylcellulose
CM	:	Carboxymethylcellulase
Cv	:	Cultivar
GalA	:	Galacturonic Acid
Gal	:	Galactose
GCP	:	Grinsted Citrus Pectin
GDL	:	Glucono-δ-Lactose
GKP	:	Gold Kiwifruit Pectin
GLC	:	Gas Liquid Chromatography
Glc	:	Glucose
GOPOD	:	Glucose Oxidase Peroxidase Colour Reagent
DF	:	Dietary Fiber
DE	:	Degree of Esterification
DLS	:	Dynamic Light Scattering
DM	:	Dry Matter
DMSO	:	Dimethyl Sulfoxide
DMP	:	3-5-dimethylphenol
DRI	:	Differential Refractive Index

Dwb	:	Dry Weight Basis
EDTA	:	Ethylene Diamino Tetra Acetic Acid
EH-GKP	:	Early-Harvested Gold Kiwifruit
E-MHGKP	:	Enzyme-Extracted Pectin from Main Harvested Fruit
Fuc	:	Fucose
HMP	:	High Methoxyl Pectin
HPLC	:	High Performance Liquid Chromatography
IU	:	International Unit
IEC	:	Ion-Exchanged Chromatography
LA	:	Amidated Low Ester Pectin
LC	:	Conventional Low Ester Pectin
LHSMP	:	Low Heat Skim Milk Powder
LMP	:	Low Methoxyl Pectin
LS	:	Light Scattering
MALLS	:	Multi-Angle Laser Light Scattering
Man	:	Mannose
M_w	:	Molecular Weight
NI	:	No Information
NSP	:	Non-Starch Polysaccharide
PAW	:	Phenol:Acetic:Acid:Water
PGalA	:	Polygalacturonic Acid
PG	:	Polygalacturonase
PME	:	Pectin Methyl Esterase
PMEI	:	Pectin Methyl Esterase Inhibitor
PS	:	Pure Serum
Rha	:	Rhamnose
RMS	:	Root Mean Square
RS	:	Resistant Starch
SEC	:	Size-Exclusion Chromatography
SI	:	System International
TPA	:	Texture Profile Analysis

TSS	:	Total Soluble Solid
USDA	:	United State Department of Agriculture
UV	:	Ultraviolet
XTH	:	Xyloglucan Endotransglucosylase/Hydrolase
Xyl	:	Xylose
W-EHGKP	:	Water-Extracted Pectin from Early Harvested Fruit
W-MHGKP	:	Water-Extracted Pectin from Main Harvested Fruit
WSP	:	Water Soluble Polysaccharide
Wwb	:	Wet Weight Basis

CHAPTER 1 Introduction

1.1 Research Background and Objectives

Pectin is the main polysaccharide of the cell walls of growing plants. Pectin can be obtained in soluble form and is well known for its ability to improve food quality through its influence on texture. The outstanding physicochemical and functional properties of pectin have ensured that it is used widely as a thickener and stabiliser. Apple pomace and citrus peel are currently the two main sources of commercially available pectin, although some other sources are being studied intensively. Pectins from other plants have been reported to vary in their functional properties or to perform differently in food systems and are generally not yet equal to citrus peel pectin and apple pomace pectin in their utility. However, the provision of alternative pectin sources is still an interesting research objective because of the increasingly diverse range of food functionalities that are of interest to food manufacturers.

Gold kiwifruit (*Actinidia chinensis* cultivar (cv.) "Hort16A") is one of the most important horticultural crops in New Zealand, where it has been grown widely and commercially successfully since 1992. This fruit is both exported and sold to the domestic market primarily as fresh whole fruit. Strict quality standards are applied by the marketers to maintain premium prices and this results in sound fruit being rejected (Lodge & Robertson, 1990). The fruit that cannot meet the market standard must be processed into alternative products; examples include: semi-processed fruit products (*e.g.* syrups) as ingredients or components used in ice cream, yoghurt, cakes and juice blending (Cassano, Figoli, Tagarelli, Sindona, & Drioli, 2006), or as juices; purees, leathers, canned or dried fruit and fruit pulp; wines, as for green kiwifruit (Heatherbell, Struebi, Eschenbruch, & Withy, 1980; Wilson & Burns, 1983). Other innovative alternative processing and product options are needed for kiwifruit because of the increasing quantities of fruit that are available for processing.

The dietary fibre content of kiwifruit has been reported to be higher than that of other fresh fruit, and is in the range 2.25−3.12 g/100 g (Lintas, Adorisio, Cappelloni, & Monastra, 1991). In addition, pectin has been found to be abundant (40−50%) in the cell walls of green kiwifruit (Redgwell & Percy, 1992). Therefore, gold kiwifruit could potentially be a good source of pectin and might offer different functional properties that may be useful in the food industry.

A major challenge in developing any new pectin product is the selection of the isolation procedures to be used and the influence of the method and the isolation conditions on the properties of the pectin. It is generally desirable to be able to preserve the physicochemical characteristics of pectin as these properties will contribute to its functionality.

The isolation of pectin can be approached in different ways: chemically, physically and enzymatically (Panouille, Thibault, & Bonnin, 2006). Chemical extraction using acid solutions is a method that is commonly employed in pectin manufacture. However non-conventional methods are also of interest, *e.g.* some specific enzymes may be used (Wang, Pagan, & Shi, 2002). Such alternative methods of extraction may be preferred over acidic extraction because acidic extraction results in highly polluting effluents of low pH. Both consumers and food manufacturers are increasingly demanding "green" ingredients and products.

This research will focus on gold kiwifruit (cv. Hort16A) because information on methods for the extraction of its water-soluble polysaccharides (WSPs) and their physicochemical characteristics is currently very limited. In this work, pectin was extracted from gold kiwifruit of two different maturities and by three different methods. The physicochemical characterisation of gold kiwifruit pectin and its relationship to the functional properties of the pectin products obtained, as a result of differences in maturity and extraction methods, were the main concerns of this research.

This study was undertaken with the following objectives.

1. To evaluate the effects of the extraction technique (acid and water) and conditions (time and temperature) on the yield and physicochemical composition of crude pectin from main-harvested gold kiwifruit (see Chapter **3**).
2. To evaluate the effects of an enzymatic extraction technique and its conditions (time, temperature and enzyme concentration) on the yield and physicochemical composition of crude and purified pectin from main-harvested gold kiwifruit (see Chapter **4**).
3. To evaluate the effect of a pectin extraction method that had been optimised for main-harvested fruit on fruit of different maturity (early-harvested fruit), in terms of the yield of purified pectin and its physicochemical composition (see Chapter **5**).
4. To compare the yield and the physicochemical composition of purified pectin from whole gold kiwifruit with those of pectin from gold kiwifruit pulp (see Appendix **E**).
5. To characterise the gelation properties of the purified pectin from whole gold kiwifruit of two different maturities that had been extracted using a water technique (see Chapter **6**).
6. To determine the effect of adding gold kiwifruit purified pectin on the stability and rheological properties of an acidified milk drink (see Chapter **7**).

1.2 Kiwifruit: Botanical and Production Information

Kiwifruit is not a crop that is native to New Zealand. The seed was brought to New Zealand from the Yangtze Valley in Southern China in 1904 (Ferguson, 2004). Kiwifruit was first grown in the Bay of Plenty near the city of Tauranga and commercial production commenced in 1930. From 1970, *Actinidia deliciosa* (*A. deliciosa)* cv. Hayward developed as the most important new fruit crop in the New Zealand horticultural industry (Ferguson, 1999, 2004), because the fruits were better sized, withstood long term storage better and offered improved flavour compared with fruit of other species. The key growing regions were primarily in northern and eastern parts of the North Island and in the northern part of the South Island (Hall, Kenny, Austin, & McPherson, 2001).

Actinidia chinensis (*A. chinensis*) has also become very important in New Zealand. It was introduced in the late 1970s from China and, after an 11-year process of natural cross pollination, one seedling was identified in 1992 as having particularly good fruit with a different appearance from that of cv. Hayward (Zespri International Ltd, 2008). This fruit is known as cv. Hort16A and is being marketed successfully as **Zespri® gold kiwifruit**.

Kiwifruit belongs to the genus *Actinidia* Lindl, which comprises about 60 species (Ferguson, 1999). These species differ markedly in size, shape, flesh colour, flavour, texture and composition. Figure **1.1** shows the diversity of *Actinidia.* The fruit of *A. chinensis* and *A. deliciosa* are noticeably different. *A. chinensis* fruit are smaller, more rounded and less cylindrical (Figure **1.2**) and, when the fruit ripens, the fruit hair is usually shorter than that of *A. deliciosa*. As the skin of *A. chinensis* fruit is very sensitive and easily damaged, it needs to be handled differently from *A. deliciosa* fruit during and post harvest (Ferguson, 1999).

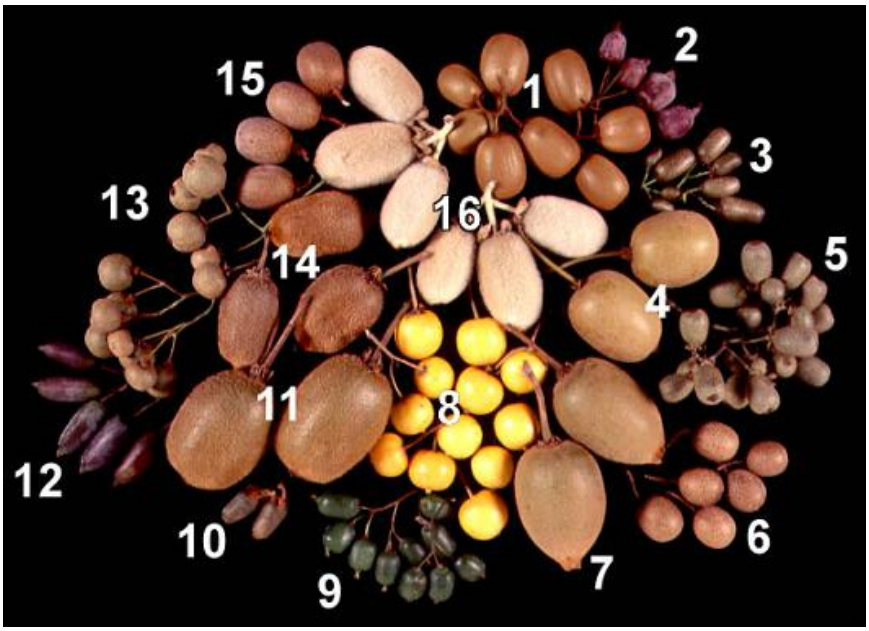

Figure 1.1 The diversity of kiwifruit (Ferguson, 1999).
(1) *A. rufa.* (2) *A. melanandra*. (3) *A. glaucophylla*. (4) *A. chinensis.* (5) *A. latifolia.* (6) *A. indochinensis.* (7) *A. chinensis* cv. Hort16A. (8) *A. macrosperma.* (9) *A. arguta.* (10) *A. fulvicoma.* (11) *A. deliciosa* cv. Hayward. (12) *A. arguta* var. *purpurea.* (13) *A. guilinensis.* (14) *A. setosa.* (15) *A. chrysantha.* (16) *A. eriantha.*

Figure 1.2 Kiwifruit: *Actinidia chinensis* (gold) (right) and *Actinidia deliciosa* (green) (left) (Ferguson, 1999).

The flesh of *A. chinensis* fruit varies in its colour, depending on its maturity. Immature fruit has a pale green flesh colour whereas full ripe fruit can have a quite deep and bright golden colour, so that it is known as "gold kiwifruit" (Figure **1.3a**). As the aroma of *A. chinensis* fruit is similar to that of some subtropical fruit and the taste is sweeter than that of *A. deliciosa* fruit (Ferguson, 1999), gold kiwifruit is preferred for processing and performs better during processing than *A. deliciosa* fruit in terms of colour and flavour (Huang & Ferguson, 2001). *A. deliciosa* fruit is known as green kiwifruit; the flesh has a green colour (Figure **1.3b**) because of its high and stable (at ambient and storage temperatures) chlorophyll content (Huang & Ferguson, 2001). It has brown-coloured skin and the surface is more hairy than that of *A. chinensis* fruit.

Figure 1.3 Kiwifruit: (a) *Actinidia chinensis* golden flesh; (b) *Actinidia deliciosa* green flesh (Ferguson, 1999).

1.3 Structure and Chemical Composition of Kiwifruit

1.3.1 Fruit Structure

As there is limited information in the literature on the structure and physiology of cv. Hort16A, the data provided in this thesis are mainly those for cv. Hayward unless otherwise stated. Figure **1.4** shows the cross-section of a kiwifruit. Kiwifruit generally exhibits four different structural regions (White, de Silva, Requejo-Tapia, & Harker, 2005): the outer pericarp; the inner pericarp with multiple locules, which contain the seeds; the columella (core), which is a lighter green than the pericarp tissues; the surface skin or epicarp. The outer and inner pericarps and the core are the edible parts of kiwifruit, whereas the skin is typically discarded during consumption (Jackson & Harker, 1997).

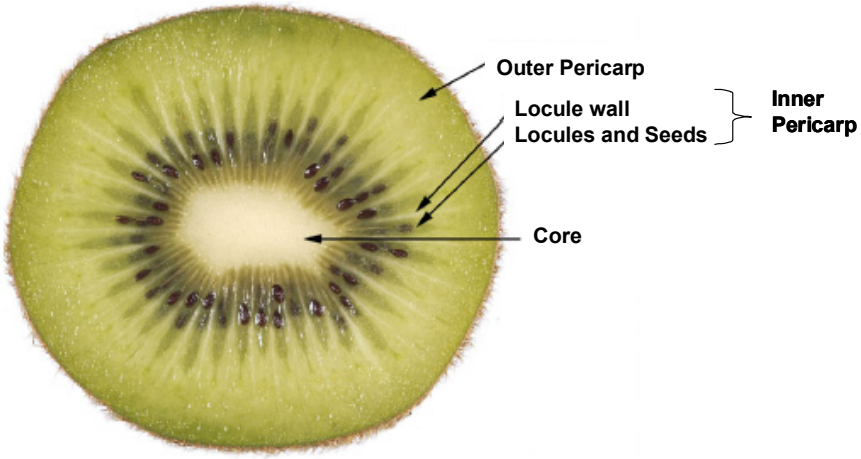

Figure 1.4 Cross-section of kiwifruit tissue zones (Schröder & Atkinson, 2006).

The core of kiwifruit contributes 7% w/w of the whole fruit fresh weight, and the inner and outer pericarps comprise 36% w/w and 57% w/w respectively (MacRae, Lallu, Searle, & Bowen, 1989b). The proportion of the fruit represented by the skin varies among species (commonly in the range 4.5−6.2 g) and a mean value of 4.7% w/w has been reported (Selman, 1983).

In the core, the cells are tightly packed with little air space between them. They are mostly spherical/ellipsoidal in shape with a cross-sectional dimension of between 0.1 and 0.2 mm and the tissue is comparatively uniform. The inner pericarp consists of two regions, the locules and the locule wall. The seeds are located in the locule area and the cells are radially elongated and thin walled; the locule wall consists of smaller thicker-walled radially elongated cells. The outer pericarp is surrounded by dead skin tissue on its outer side (Hallett, MacRae, & Wegrzyn, 1992).

The cross-section of mature unripe *A. deliciosa* skin comprises 2−4 layers of radially flattened cells with thickened cell walls and consisting mostly of suberin. These cells are dense and show no living cells (Hallett & Sutherland, 2005). The cells that are close to the dead cells contain mainly starch and phenolic compounds. Although *A. deliciosa* has a similar skin structure to *A. chinensis*, the skin of *A. chinensis* is much thicker and is made up of six or more layers of compressed cells (Hallett & Sutherland, 2005). Crisosto and Kader (1999) have stated that there are no stomata on the surface of kiwifruit but trichomes (large and small hairs) are found. Most of the small hairs on the surface are removed during the harvesting and post-harvesting operations.

When harvested, kiwifruit have various degrees of seed maturity and seed numbers. cv. Hayward has seed numbers in the range 1000−1100, with ~ 19 seeds per carpel, whereas cv. Hort16A has seed numbers in the range 400–600 (Patterson, Burdon, & Lallu, 2003). The seeds are ellipsoidal, with size approximately 2.5 mm x 1.6 mm (length x width). They are soft and white initially and then start to harden and darken in colour, until they reach full size (10 weeks after anthesis), and turn dark brown as the fruit reaches maturity (MacRae, *et al*., 1989b).

1.3.2 Chemical Composition

The parts of a kiwifruit (both green and gold) that are consumed are the outer and inner pericarps and the core. These parts are very different in their chemical compositions and thus have dissimilar taste qualities (Rossiter, 2000). Table **1.1** shows the typical

composition of fresh ripe cv. Hayward and cv. Hort16A respectively. Water is the largest constituent of both kiwifruits, followed by carbohydrate. The carbohydrate content of fresh green kiwifruit is about 17.5% w/w whereas that of gold kiwifruit is lower, at about 11.3% w/w on a wet weight basis (wwb). The carbohydrate content may vary depending on the maturity of the fruit and the variety. For example, the major sugars in Italian gold kiwifruit are reported to be fructose (4.7% w/w) and glucose (4.3% w/w); sucrose is present in only a small amount (Castaldo, Lovoi, Trifiro, & Gherardi, 1992). Glucose and fructose are also the main carbohydrates of cv. Hayward, and are present in equal amounts (3−5% w/w), whereas sucrose is present in smaller amounts (0.7–1.5% w/w) (Sanz, Villamiel, & Martinez-Castro, 2004). Similar to cv. Hayward, cv. Hort16A contains mainly glucose (5.2% w/w) and fructose (5.9% w/w) and no sucrose has been detected (Zespri International Ltd, 2006).

Although cv. Hort16A has a higher vitamin C content than cv. Hayward, both green kiwifruit and gold kiwifruit contain a significant amount of vitamin C compared with other fruits (McGhie & Ainge, 2002). Vitamin E is also higher in gold kiwifruit (2.2 mg/100 g of fresh fruit) than in green kiwifruit (1.46 mg/100 g of fresh fruit). Other vitamins found in kiwifruit are folic acid, niacin, vitamin A and vitamin B, including riboflavin and pantothenic acid.

Kiwifruit contains a wide range of minerals, with the most abundant being potassium. As shown in Table **1.1**, gold kiwifruit contains approximately half (299.6 mg/100 g of fresh fruit) the maximum potassium concentration in green kiwifruit (185−576 mg/100 g of fresh fruit), although the latter is subject to much uncertainty. In addition to potassium, kiwifruit contains significant quantities of other minerals such as calcium, nitrogen, iron, magnesium, sodium and copper.

The seeds contain oil, which contributes to the fat content of the fruit; however, overall, the oil contributes less than 1 g to the mass of a typical fruit (~ 100 g). The seed oil is presumed to be low in saturated fat and high in polyunsaturated fat, with no cholesterol being found. Green kiwifruit has been reported (United State Department of Agriculture (USDA), 2009) to be higher in polyunsaturated fatty acids (0.287% w/w of fresh fruit) than

gold kiwifruit (0.207% w/w of fresh fruit). Green kiwifruit is also observed to be slightly higher than gold kiwifruit in fat, with compositions of 0.07−0.90% and 0.60% w/w wwb respectively.

Table 1.1 Chemical compositions of fresh ripe cv. Hayward kiwifruit (Adapted from Beever & Hopkirk, 1999) and cv. Hort16A kiwifruit (Zespri International Ltd, 2006)

Component	cv. Hayward (Green)	cv. Hort16A (Gold)
Energy value (kJ/100 g)	205−276	226.8
Water (%)	80−88	83.4
Protein (%)	0.11−1.20	1.30
Lipid (%)	0.07−0.90	0.60
Ash (%)	0.45−0.74	0.90
Fibre (%)	1.10−3.30	1.40
Carbohydrate (%)	17.50	11.30
Vitamins (mg/100 g, unless stated)		
Ascorbic acid (vitamin C)	80−120	108.9
Vitamin A (IU/100 g)	175	72*
Thiamin	0.014−0.02	0.03
Riboflavin	0.01−0.05	0.05
Niacin	0−0.5	0.30
Pyridoxine (vitamin B_6)	0.15	0.10
Vitamin E	1.46*	2.20
Minerals (mg/100 g)		
Calcium	16−51	21.40
Magnesium	10−32	14.50
Nitrogen	93−163	NI
Phosphorus	22−67	28.7
Potassium	185−576	299.6
Iron	0.2−1.2	0.40
Sodium	2.8−4.7	3.20
Chloride	39−65	NI
Manganese	0.07−2.30	0.615
Zinc	0.08−0.32	0.10
Copper	0.06−0.16	0.10

*USDA (2009). NI: No information.

Kiwifruit is an excellent source of dietary fibre (DF). DF is "as a complex group of plant substances that are resistance to mammalian digestive enzymes" (de Man, 1999). It composes of insoluble components such as hemicellulose, cellulose, lignin, cutin, minerals and soluble fibre (pectin). Green kiwifruit contains about 1.1−3.3% w/w DF and gold kiwifruit contains approximately 1.4% w/w. The DF content of green kiwifruit has been found to be greater than that of many other fruits (Table **1.2**). Kiwifruit is comparable in soluble DF to apple and banana, however orange, is the richest in soluble DF. Similar to the carbohydrate content, the DF content of kiwifruit varies, depending on its maturity.

Table 1.2 Dietary fibre composition (% w/w wwb) of some fruits (adapted from Schakel, Pettit & Himes, 2001)

Fruit	Total DF	Insoluble DF	Soluble DF
Apple with skin	2.70	2.00	0.70
Kiwifruit	3.41	2.61	0.80
Orange	2.40	1.00	1.40
Banana	2.40	1.80	0.60

Kiwifruit is also well known for its allergenic properties. The consumption of kiwifruit may cause an allergic reaction that is characterised by a broad range of symptoms such as: local oral mucosa reactions, laryngeal oedema, urticaria, vomiting, asthma, cardiovascular collapse and life-threatening anaphylaxis (Lucas, Lewis, & Hourihane, 2003). Several food allergens have been identified in kiwifruit including actinidin, kiwellin, phytocystatin and many others. Gold kiwifruit has been shown to contain lesser amounts of these food allergens than green kiwifruit. Kiwellin is the major allergen in gold kiwifruit and actinidin is found in trace amounts (Ciardiello, *et al*., 2009; Takamasu & Kurihara, 2009). These researchers have also revealed that the concentration of these allergens increases as ripening progresses. As actinidin has been found to be denatured by thermal treatments under acidic condition (~ pH < 3.5), its quantity will depend on the extent of fruit processing (Bublin, *et al.*, 2008).

1.4 Physiology of Kiwifruit Cell Wall

The physiology of the kiwifruit cell wall that is relevant to the present study includes the cell wall materials and changes in their composition through the maturation process. As this study focused on the physicochemical characterisation of the pectin from gold kiwifruit of two different maturities, the term of maturation and the physiological changes that occur during maturation are discussed below. In addition, the term of ripening is also described.

Maturation is defined as "the stage of development leading to the attainment of physiological or horticultural maturity", where physiological maturity is "the stage of development when a plant or plant part will continue ontogeny even if detached" and horticultural maturity is "the stage of development when a plant or plant part possesses the prerequisites for utilization by consumers for particular purpose" (Watada, Herner, Kader, Romani, & Staby, 1984). This study is concerned with the extraction of pectin from two distinct physiological maturities of gold kiwifruit according to their harvest season namely "early-season" and "main-season fruit".

Ripening is considered to start soon after the stage of maturation (Faurobert, *et al*., 2007). Several physical and chemical changes occur when ripening is initiated. These changes can convert a fruit from physiologically mature but unpalatable form to suitable for eating (Reid, Heatherbell, & Pratt, 1982).

Kiwifruit is harvested when it is still unripe, at a certain stage of maturity such that it will continue to develop physiologically (physiological maturity) until it is suitable for consumption. If kiwifruit is harvested too early, it will never develop the full flavour and aroma that satisfy consumers (Beever & Hopkirk, 1990).

1.4.1 Cell Wall Contents

In general, the growth of kiwifruit is separated into two stages, namely enlargement (0−18 weeks after anthesis) and maturation (19 weeks onwards) (Li, Nakagawa, Nevins, & Sakurai, 2006). Anthesis is defined as "the stage when two sepals were separated from the petals" (Bredmose, 2000). The weight of fresh cv. Hayward fruit increases rapidly after pollination during enlargement. When the fruit reaches approximately 50% of its final weight (during the second month), the growth rate declines slowly until the sixth month, when the fruit is harvested (Gallego & Zarra, 1997). Physical and compositional changes occurring during growth and maturation are illustrated in Figure **1.5**, and will be described in the following sections.

Gallego and Zarra (1997) have reported that the cell wall content of kiwifruit (*i.e.* grams of cell wall per fruit) increases sharply during the first 3 months after pollination and then declines. In immature kiwifruit, cellulose, hemicelluloses and pectin are the primary cell wall polysaccharides in the pericarp and xyloglucan is present at only a very low level.

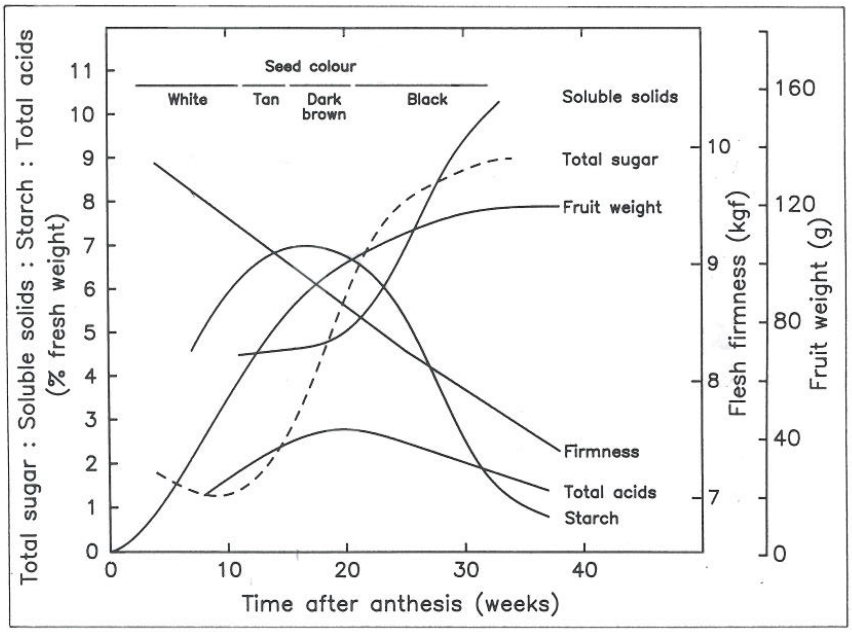

Figure 1.5 Physical and compositional changes occurring during growth and maturation (Beever and Hopkirk, 1990).

1.4.2 Cell Wall Changes

In this section, changes in the cell wall as a result of on-vine softening, such as pectin solubilisation, galactose loss and cell wall swelling, are briefly described. Redgwell and Percy (1992) have shown that the same processes occur in the cell walls of both green kiwifruit softened on the vine and green kiwifruit ripened by treatment with ethylene. Hence, these processes appear to be general features of the changes in the cell wall during the softening of kiwifruit.

An overview of physiological changes during ripening as described by MacRae & Redgwell (1992) will also be discussed in this section. These authors described the ripening process in green kiwifruit as comprising three phases (beginning, middle and final) of cell wall disintegration. In these phases, there are four key events: pectin solubilisation, cell wall swelling, galactose loss of side chains, and degradation of pectin and xyloglucan.

1.4.2.1 Softening

The cell walls of all plants are connected co-operatively by a bonding agent or matrix that is composed predominantly of pectin. It provides a mechanical structure with rigidity and strength (Roberts, Elliott, & Gonzalez-Carranza, 2002). In kiwifruit, the contents and the structure of the cell wall correlate with the firmness of the fruit. Gallego and Zarra (1997) have reported that changes in the firmness of the fruit tissue involve alterations to the structure of the cell walls. During development, kiwifruit is initially very hard but the firmness decreases gradually during the later phases and, once harvested, the flesh undergoes softening very quickly (depending on the storage temperature) (Beever & Hopkirk, 1990).

Regardless of the maturity at harvest or the post-harvest treatment applied, both the core and the flesh of kiwifruit will soften as the fruit ripens (MacRae, Bowen, & Stec, 1989a). The core of kiwifruit will always soften after the flesh, because the core has a higher starch content than the other tissues. The flesh of cv. Hort16A is much softer (~ 4−5 kgf) than that

of cv. Hayward (~ 6−9 kgf) at the point of commercial maturity (Beever & Hopkirk, 1990; Patterson, *et al*., 2003).

1.4.2.2 Compositional Changes

1.4.2.2.1 Starch

The starch content of the kiwifruit cell wall increases until 20 weeks after anthesis (reaching a maximum of 1.58 mg/g) and then decreases slightly to 1.42 mg/g at 25 weeks after anthesis (Beever & Hopkirk, 1990; Li, *et al*., 2006). Some studies (MacRae, *et al*., 1989a; MacRae & Redgwell, 1992) have shown that the starch and total carbohydrate contents in kiwifruit decrease in the first phase during softening because the starch is converted to soluble sugars, *i.e.* glucose. Consequently, the concentrations of fructose, glucose and sucrose and the sugar/acid ratio all increase. The loss of starch correlates well with the rapid loss of firmness during softening of the fruit.

1.4.2.2.2 Pectin and Other Cell Wall Fractions

The amount of pectin increases gradually from 8 to 16 weeks after enlargement, reaches a maximum at the 20th week after anthesis and then decreases gradually (Li, *et al*., 2006). Redgwell and Percy (1992) believe that the decrease in pectin is due to pectin solubilisation, which is the main event in kiwifruit softening. Redgwell, Melton and Brasch (1992) have observed that the process of pectin solubilisation is the change from a tightly bound pectin fraction (galactan pectin) to a less tightly bound fraction (extractable in Na_2CO_3) and ultimately the pectin becomes soluble in water.

In the first phase of softening (from 8.3 to 5.7 kgf) of on-vine kiwifruit, no pectin solubilisation is observed. As softening continues (< 5.7 kgf), the solubilisation of pectin is more obvious, as indicated by the increasing total uronic acid content in phenol:acetic acid:water (PAW)-soluble fractions. This could indicate that pectin changes from an insoluble form with a relatively rigid (solid-like) state to a more mobile (liquid-like) state (Newman & Redgwell, 2002).

The neutral sugars (arabinose, rhamnose and galactose) in the pectin fraction reach their maximum levels during fruit development and then decrease as the fruit matures. These sugars are present in only small amounts (~ 2.8 mg/g wwb) at the beginning of fruit enlargement, increase from 12 to 16 weeks after anthesis (~ 4.5 mg/g wwb) and thereafter decrease (to ~ 2 mg/g wwb) (Li, *et al*., 2006). Glucose, galactose and xylose are the main sugars in the hemicellulose fraction, with fucose found at only very low concentrations. In the cellulose fraction, glucose increases constantly through maturity and reaches maximum levels of 40% of the total cell wall material at harvest (Gallego & Zarra, 1997).

Galactose has been reported to be lost from the kiwifruit cell wall after 3 months of development. The decrease in galactose starts immediately after the fruit begins to soften, with approximately 80% lost at a firmness of 5.7 kgf. The loss of galactose has been reported to be due to enzyme hydrolysis of the galactose side chains of the cell-wall-bound polysaccharides.

1.4.2.2.3 Organic Acids

The main non-volatile organic acids in kiwifruit are citric, quinic and malic (Heatherbell, 1975; Harman & McDonald, 1989). The most abundant is citric acid, followed by quinic acid and malic acid. The acid composition of kiwifruit varies in total amount and in the ratio of one acid to another according to maturity, which will affect its pH and titratable acidity (MacRae, *et al*., 1989b).

1.4.2.2.4 Soluble Solids Concentration

In New Zealand, kiwifruit is harvested when it has reached a minimum maturity index of 6.2% w/v soluble solids concentration for cv. Hayward (Crisosto & Crisosto, 2001) and greater than 10% for cv. Hort16A (Patterson, *et al.*, 2003). If fruit is harvested with a soluble solids concentration of less than 6.2% or 10%, it is considered to be immature and does not develop a suitable flavour and texture after long term storage. The soluble solids concentration of cv. Hayward for eating ripeness is 13.0−14.5% (Lallu, Searle, & MacRae, 1989) and for customer satisfaction is at least 12.5% (Crisosto, Garmer, & Saez, 1999).

1.4.2.3 Enzymatic Changes

Polygalacturonase (PG), pectin methylesterase (PME), β-galactosidase, xyloglucan endotransglucosylase/hydrolase (XTH) and mannan transglycosylase are enzymes that are commonly involved in the softening of kiwifruit during ripening. PG is mostly involved in pectin degradation, but not in solubilisation, because this enzyme is detected when pectin degradation occurs, which is at the end of middle phase and at the final phase of the ripening process. Wegrzyn and MacRae (1992) reported that the activity of PME in ethylene-treated green kiwifruit gradually decreased as the fruit ripens. This decrease maybe due to the presence of a protein inhibitor of PME (PMEI) (Balestrieri, Castaldo, Giovane, Quagliuolo, & Servillo, 1990). However, the role of PME and PMEI on kiwifruit cell wall *in vivo* is still unclear (Schröder & Atkinson, 2006).

1.4.2.4 Cell Wall Swelling

Morphological studies of the cell walls have indicated that the softening of kiwifruit is associated with swelling of the cell walls. This phenomenon has been related to movement of water into the voids left in the cell wall of the cellulose–hemicellulose network by solubilised pectin (Schröder & Atkinson, 2006). The cell walls of unripe kiwifruit are compact and thin, and swell and become thicker as ripening proceeds (Schröder & Atkinson, 2006); they are three or four times thicker in ripe fruit than at harvest (Hallett, *et al*., 1992). The phenomenon of the swelling of the cell walls is observed not only during the ripening process but also in on-vine softened fruit. The cell walls start to swell when the fruit firmness is ~ 5.7 kgf, showing that cell wall swelling is accompanied by pectin solubilisation (Redgwell & Percy, 1992).

1.4.2.5 Changes on Weight-Average Molecular Weight (M_w) of Pectin Fractions

In general, the M_w of galacturonic acid (GalA)-rich polysaccharides decreases markedly during kiwifruit development. In each month of fruit development, the amount of GalA-rich polysaccharides with lower M_w increases. The GalA-rich polysaccharide fractions are high in M_w during the first 5 months of fruit development, but are lower in M_w by 6 months.

However, at the 6 months, the fruit has less neutral sugar content. A decrease in the high M_w fraction of GalA-rich polysaccharides is detected during pectin solubilisation; some GalA is degraded because of in vitro autolytic activity during the later months of fruit development (Gallego & Zarra, 1997). As reported by Brummell (2006), the solubilisation of pectin in the ripening process is accompanied by pectin depolymerisation, which becomes more obvious in the middle of the ripening process and is dominant in the late ripening stages.

1.5 Pectin

The primary cell wall of dicotyledons is composed of various polysaccharides: cellulose, pectin, xyloglucan and also protein. These polysaccharides are associated together and form an entangled network (cellulose/xyloglucan network) (illustrated in Figure **1.6**), which is embedded in the pectin matrix together with protein (Vincken, Dekeizer, Beldman, & Voragen, 1995; Carpita & McCann, 2000). The side chains of pectin and cellulose interact in their networks, and the type of neutral sugars bound to the cellulose microfibril differs from plant to plant (Oechslin, Lutz, & Amado, 2003; Zykwinska, Ralet, Garnier, & Thibault, 2005). Cellulose interacts with the galactan side chains in apple cell walls (Oechslin, *et al*., 2003) and with the arabinan side chains of cactus spine fibres (Vignon, Heux, Malainine, & Mahrouz, 2004). In addition, cellulose−hemicellulose complexes have a strong association with arabinan-rich pectins (Iwai, Ishii, & Satoh, 2001).

Pectin constitutes up to half of the polymeric component of the cell wall of kiwifruit (Brummell, 2006). It is located predominantly in the middle lamella of the cell wall and decreases in amount through the primary cell wall towards the plasma membranes (Darvill, McNeil, Darvill, & Albersheim, 1980).

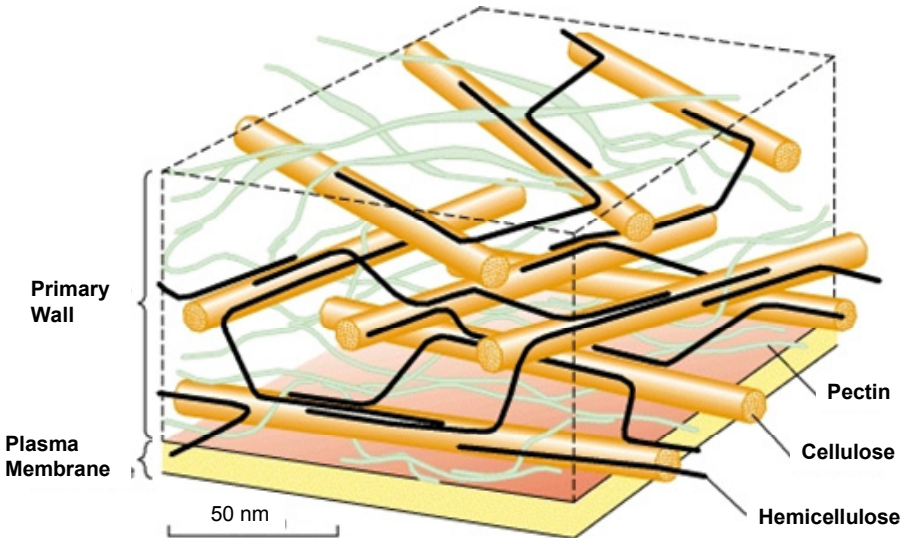

Figure 1.6 Schematic representation of the cell wall of an onion (McCann & Roberts, 1991).

1.5.1 Structure and Chemical Composition

Pectin is a linear chain polymer that is composed mainly of α-D-galacturonic acid residues joined by 1→4 linkages (Belizt, Grosch & Schieberle, 2009). Pectins are characterised by three families (Ridley, O'Neill, & Mohnen, 2000): homogalacturonan, rhamnogalacturonan type I and substituted galacturonan. Homogalacturonan (or smooth regions, Figure **1.7** and **1.9**) has a backbone with a linear chain of α-(1→4)-linked D-galactopyranosyluronic acid, with some of the carboxyl groups (–COOH) being methyl esterified (–$COOCH_3$) or partially O-acetylated at C-3 or C-2 in some plants (Figure **1.8**). As the location of esterified GalA can be random or non-random along the backbone chain, pectins from different sources can be dissimilar in their compositions (Oakenfull, 1991).

Rhamnogalacturonan I (branched rhamnogalacturonans or hairy regions, Figure **1.9**) has a backbone of α-(1→2)-linked L-rhamnosyl and α-(1→4)-linked D-galacturonosyl acid residues, with neutral sugars such as arabinans, galactans and arabinogalactans as the side chains. Rhamnose residues are inserted into the pectin backbone, which results in a kinked

chain rather than a linear chain (Axelos & Thibault, 1991). The relative proportion and the length of the neutral sugar chain attached depend on the type of isolation used and the plant source (Darvill, *et al*., 1993).

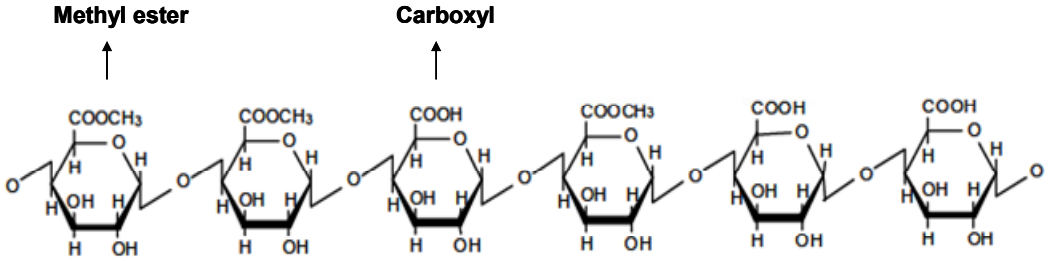

Figure 1.7 Homogalacturonan, which is composed of α-1,4-linked D-galacturonic acids partially esterified by methyl esters (modified from Guillotin, 2005).

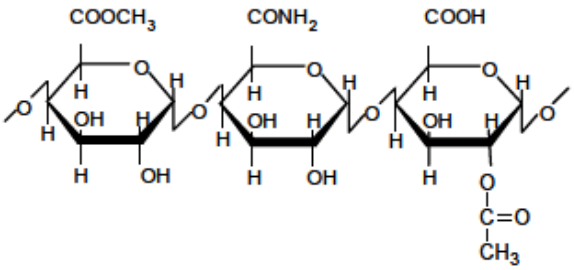

Figure 1.8 Representation of the different substitutions potentially present in commercial pectins, *i.e.* methyl ester, amide group and acetyl group respectively (Guillotin, 2005).

The substituted galacturonans are a group of polysaccharides that consist of α-(1→4)-linked D-galactosyluronic acid residues as the backbone (O'Neill, Albersheim & Darvil, 1990), such as rhamnogalacturonan II and xylogalacturonan. Rhamnogalacturonan II (Figure **1.9**) is composed of at least seven GalA backbone residues with four different structures of oligosaccharides attached as side chains (Ridley, *et al.*, 2000). Xylogalacturonan contains xylose as side chains, attached to the C-3 GalA backbone (O'Neill, *et al*., 1990). Another family of pectins that is recognised as substituted galacturonans has been reported by Cheng and Kindel (1997); apiogalacturonan is present in the cell walls of some aquatic

monocotyledons. This group has apiofuranosyl attached to the C-2 and C-3 GalA backbone.

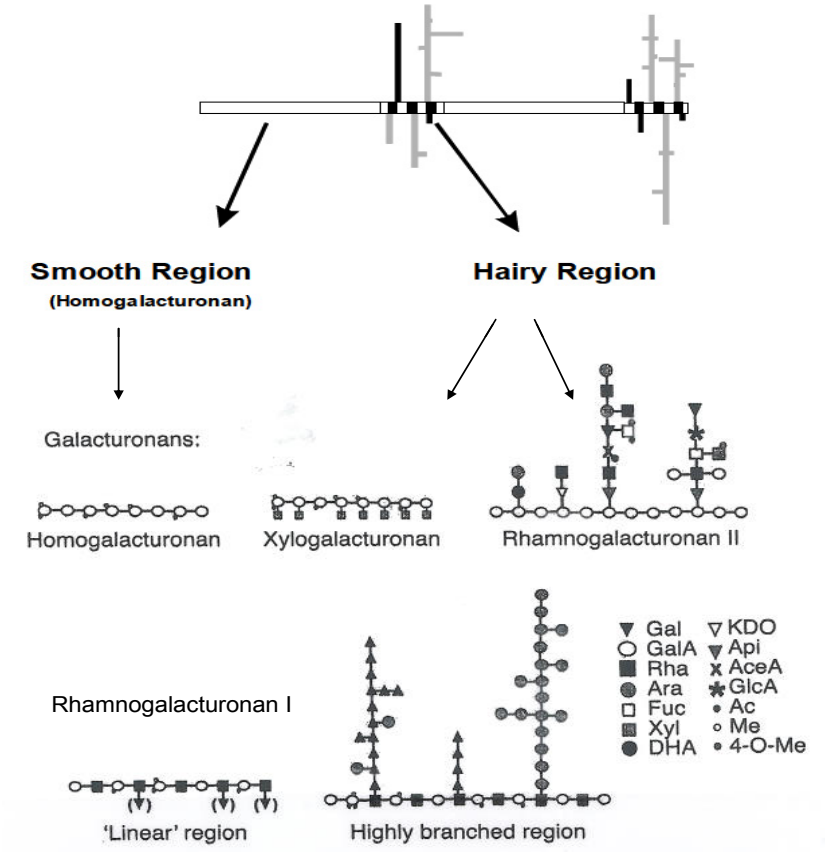

Figure 1.9 Structural elements of pectin (consisting of homogalacturonan and hairy/branching regions) (Voragen, Beldman & Schols, 2001).

Pectins are grouped according to their degree of esterification (DE). The DE is defined as "the amount of methyl-ester (mol) present per 100 mol of total galacturonic acids (free galaturonic acid and substituted ones)" (Guillotin, Bakx, Boulenguer, Schols, & Voragen, 2007). Theoretically, the DE can vary from 0% to 100% (Pilgrim, Walter & Oakenfull, 1991) and, based on this percentage, pectin is classified by its DE into high (methyl) ester pectin or HMP and low (methyl) ester pectin or LMP (Rolin & de Vries, 1990) (Figures **1.10a** and **1.10b**). HMP has a relatively high quantity of carboxyl groups, which occur as methyl esters, and a low proportion of carboxylic acid groups, which are present as the free acid or as ammonium, potassium, calcium or sodium salts. In contrast, LMP has less than

50% carboxyl groups that are present as methyl esters. There are two sub-groups of low ester pectin: conventional low ester (LC) pectin and amidated low ester (LA) pectin (Figure **1.10c**). Amidated pectin is obtained by further hydrolysis using ammonia, which results in the conversion of some of the ester groups to amide groups (May, 1990).

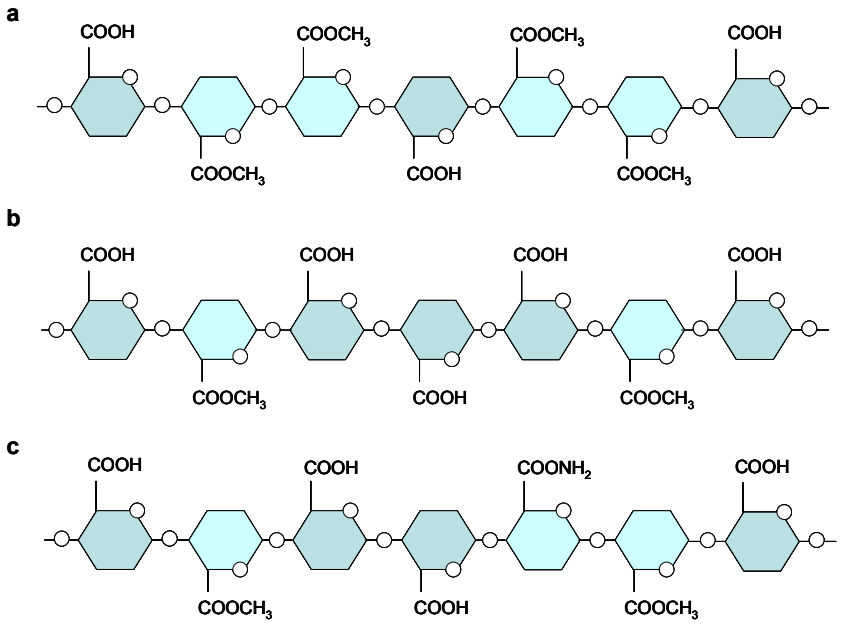

Figure 1.10 Different types of pectin: (a) HMP (DE 70%), (b) LMP (DE 40%), (c) LM amidated pectin (DE 40%).

1.5.2 Functionality: Viscosity, Gelation, Stabilisation

Pectin is a WSP that is deliberately added to food systems to enhance their functional properties. Its stability is highest at pH 3−4 (Belizt, *et al*., 2009). The most significant functional properties of WSPs are their water binding capacity and their viscosity enhancement. WSPs are widely used as stabilisers and viscosifiers to control the texture of semi-solid foods (Wang & Cui, 2005). Thus, pectin, in general, has three functional properties: viscosity build-up, gel formation and protein stabilisation. Information on the viscosity, gelation and stabilisation functional properties of pectin are described briefly below to illustrate the role of pectin in food systems.

1.5.2.1 Viscosity Build-up

Viscosity (η) is generally defined as "the ratio of applied shearing stress (τ) to the shear rate ($\dot{\gamma}$)" (Wang & Cui, 2005) or resistance against flow.

In solution, linear polysaccharide molecules gyrate and flex, sweeping out a large space, and frequently collide with each other, creating friction, consuming energy and thus generating viscosity (BeMiller & Whistler, 1996). Although highly branched polysaccharides will spread out over less space than linear polysaccharides with the same M_w, they will collide less frequently and therefore will produce less viscosity than linear polysaccharides (BeMiller & Whistler, 1996; Belizt, *et al*., 2009). Pectin is considered to be a random coil polysaccharide. Figure **1.11** illustrates two type of polymer random coil shapes; flexible and semi flexible.

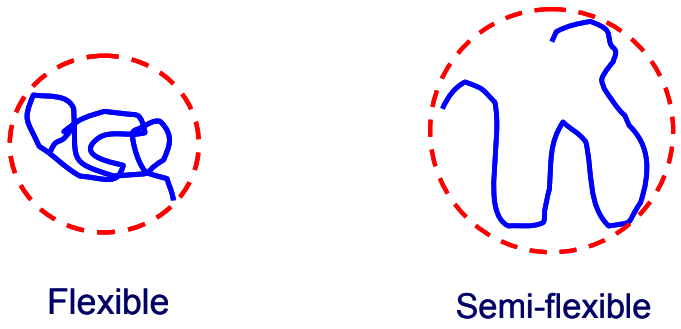

Figure 1. 11 The flexibility of random coiled polysaccharide molecules

According to BeMiller and Whistler (1996), the viscosity of a polymer solution is a function of the size and shape of its molecules and of the conformation they adopt in the solvent. In solution, the shape of a polysaccharide molecule is a function of the oscillations around the bonds of the glycosidic linkages; therefore, the larger the internal freedom at each bond, the greater is the number of conformations available to each individual segment.

A dilute pectin solution up to 1% is considered to be a Newtonian fluid. However, at concentrations greater than 1%, pectin demonstrates non-Newtonian behaviour with pseudoplastic flow. In the presence of calcium, calcium sensitive pectin has a more than

80-fold increase in viscosity than non-calcium sensitive, because this pectin is able to interact with calcium even forming a gel (Terpstra, Lapre, de Vries, & Beynen, 1998). Figure **1.12** shows both pseudoplastic flow (**1.12a**) and Newtonian flow (**1.12b**). Figure **1.12a** shows that the apparent viscosity of a pseudoplastic liquid is dependent on the shear rate; the viscosity decreases with increasing shear rate.

Pectin has been used widely to enhance the viscosity of food systems such as juices, sauces and soft drinks. High methoxyl citrus pectin has been added to stabilise the cloud sedimentation of mixed pineapple and passion fruit juice by improving the serum viscosity of the juice, as developed by Herbstreith and Fox Corporate Group Ltd.

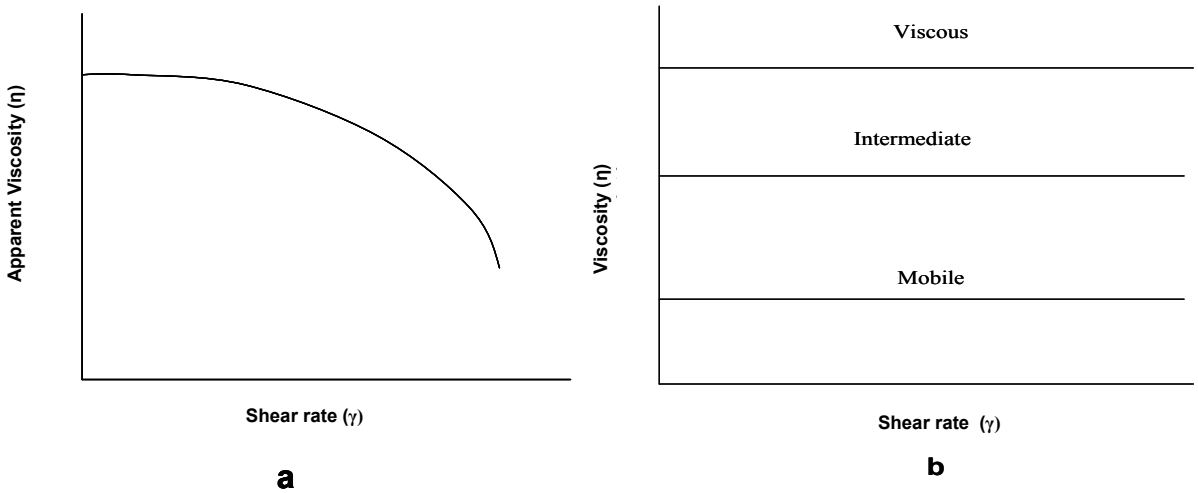

Figure 1.12 (a) Pseudoplastic flow: apparent viscosity versus shear rate. (b) Newtonian flow: viscosity versus shear rate (Bourne, 2002).

Temperature, the concentration of the solute, the M_w of the solute, the suspended matter (impurities) and the shape, flexibility and configuration of the polysaccharide molecules (Li & Chang, 1997; Bourne, 2002) greatly affect the viscosity of pectin. Generally, under constant conditions, the viscosity of pectin will increase with increasing DE, M_w and pectin concentration (da Silva & Rao, 2006). At constant temperature, increasing solute concentration will increase the pectin solution viscosity, thus exhibits a direct linear relationship (Bourne, 2002).

There is high correlation between viscosity and temperature. They generally have an inverse relationship, where an increase in temperature will decrease the solution viscosity. This could be because increasing the temperature will affect the intermolecular chain association of the solution. The viscosity of sunflower pectin has been shown to be affected by temperature and the pH of the buffer solution. At higher pH (3, 4 and 6), an increase in the heating temperature slightly decreases the viscosity; however, the opposite trend is obtained at pH 2 (Li & Chang, 1997).

1.5.2.2 Gelation

Gels are defined by Oakenfull, Pearce and Burley (1997) as "a form of matter intermediate between a solid and a liquid". Their properties are net results of the complex interaction between the solvent and the molecular network. Water as a solvent affects the arrangement and the extent of the intermolecular forces that maintain the integrity of the polymer network. The polymer network holds the water molecules and consequently keeps them in the network. Thus, the polymer molecules are aggregated into one three-dimensional structure that fills the space. Figure **1.13** illustrates the junction zones (Walkinshaw & Arnott, 1981) of a gel network.

Based on its gelling ability, pectin is classified into two main types that are ruled mainly by their DE, as described in section **1.5.1**. HMP promotes gelation mainly in an acidic environment (low pH) and in the presence of sugars, whereas LMP promotes gelation in the presence of calcium. Chapter **6** reports on the gelation properties of gold kiwifruit and other commercial pectin from different origin materials. To understand the gelation mechanism of HMP, the roles of sugar, pectin and their interactions are described below.

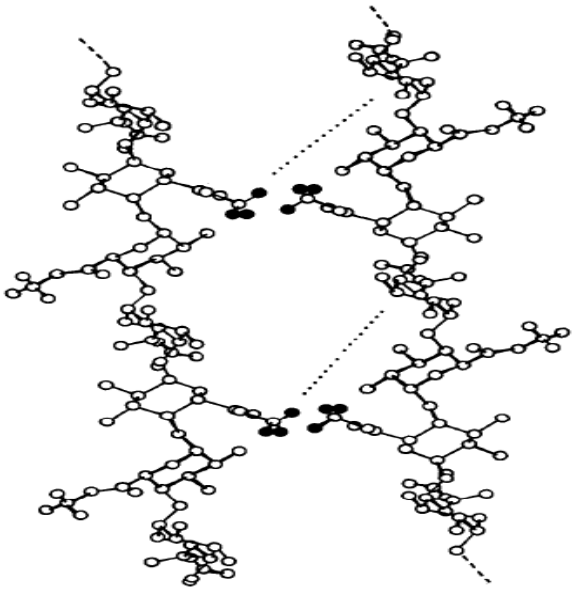

Figure 1.13 Structure of the junction zones in an HMP gel, based on X-ray diffraction studies: (·····) hydrogen bonds; (●) hydrophobic interactions of the methyl ester groups (Walkinshow & Arnott, 1981).

1.5.2.2.1 Sugar as a Co-solute in Pectin Gelation

The presence of a co-solute in the gelation process of HMP is very important. Many researchers have shown that the presence of a co-solute such as sugar can stabilise the hydrophobic interactions between the methyl ester groups and moreover that HMP will not gel if the concentration of sucrose as the co-solute is less than 55% (Oakenfull & Scott, 1984). The co-solute supports the formation of the network by competing with pectin for hydrogen bonding to water, by lowering the water activity and by creating conditions under which hydrophobic interactions are also likely to occur. Chang (2000) reported that water solutions containing mono- or disaccharides have a lower vapour pressure and therefore a lower water activity than pure water.

Sucrose has commonly been used to promote the gelation of HMP in food systems (Evageliou, Richardson, & Morris, 2000). Partial or complete substitution of sucrose with other co-solutes such as fructose, glucose syrup, high fructose syrup, alcohol and polyols can change the gelation time, the rheological properties of some gels, the water activity of

the gel system and the hydrophobic interactions (May & Stainsby, 1986; Baker, Berry, Hui & Barret, 2005).

Sucrose, as a disaccharide, consists of two monosaccharide units, namely glucose and fructose. It has eight hydroxyl groups, three hydrophilic oxygen atoms and 14 hydrogen atoms. Disaccharides are mostly stable at high pH, but will be hydrolysed to their monosaccharide forms during heating under acidic conditions. Most sucrose reactions are preceded by its conversion to its components−glucose and fructose (BeMiller & Whistler, 1996).

Because of its high solubility sucrose can form highly concentrated solutions. Its solubility is significantly affected by temperature: it will increase with increasing temperature. About 67% (w/v) dissolves at room temperature, rising to ~ 83% (w/v) at 100°C (Bubnik & Kadlec, 1995). The sugar used in the gelation studies in this thesis is mostly sucrose but, to achieve higher sucrose solubility at room temperature, a mixture of sucrose and glucose will be used. It has been reported that a mixture containing 70% sucrose and 30% glucose increases the solubility of sucrose to ~ 76% w/v (Nicol, 1979). Bhandari and Hartel (2002) have shown that the crystallisation of highly concentrated sucrose is inhibited by the presence of other sugars such as glucose.

1.5.2.2.2 Mechanism of HMP Gelation

In general, gels are formed from dissolved pectin as a result of physical or chemical changes that decrease the solubility of the pectin molecules (Turquois, Rinaudo, Taravel, & Heyraud, 1999). The mechanism of how gelation occurs has been studied and is well summarised in many papers (*e.g.* Walkinshaw & Arnott, 1981; Oakenfull & Scott, 1984). The simple mechanism of pectin gelation is shown in Figure **1.13**. The mechanism of the gelation of HMP in the presence of sucrose with low pH (below ~ 3.5) involves non-covalent polymer cross-linking in junction zones, which are primarily hydrogen bonds and hydrophobic interactions between methyl ester groups. According to Oakenfull and Scott (1984), there are about 18−250 GalA units involved in the formation of junction zones.

The lower the DE, the greater are the negative charges, thus strengthening repulsion by carboxyl ions (Wang, *et al*., 2002). At low pH (acidic conditions), carboxyl ions are converted to unionised (─COOH) carboxylic acid groups, thus reducing the repulsion between inter or/and intra pectin chains (Rascon-Chu, *et al*., 2009). This condition supports the formation of hydrogen bonds, which is why acidic conditions are essential for HMP to gel. To achieve sufficient hydrophobic interactions for gelation to occur, the water activity should be reduced, and this can be achieved by adding sucrose.

Hydrogen bonding and hydrophobic interactions are common interactions that take place during pectin gelation. A hydrogen bond is formed by the attractive forces between a pair of electrons on an electronegative atom (such as oxygen) and a hydrogen atom (partial positive charge) of another group ($O{-}H^{\delta+} \ldots O^{\delta-}$). Hydrogen bonds are stronger than other dipole−dipole interactions because hydrogen atoms have a strong tendency to become positively polarised and have a small radius (McClements, 2005).

Hydrophobic interactions are strong attractive forces that are created by non-polar groups. These groups come into contact with one another, separated by water. According to Israelachvilli (1992), the origin of this interaction is the ability of water molecules to form hydrogen bonds with other water molecules, whereas non-polar molecules can form relatively weak van der Waals' bonds. In pectin gelation, there is hydrophobic interaction between the methyl ester groups. The contribution of this interaction to the free energy of formation of junction zones has been reported by Oakenfull and Scott (1984) to be half of that from hydrogen bonding. However, hydrophobic interactions are considered to be essential interactions because hydrogen bonding alone is not enough to stabilise the interaction.

1.5.2.3 Protein Stabilisation

Several studies on the mechanism of pectin in the stabilisation of acidified milk drinks (AMDs) have been conducted. The ability of pectin to act as a stabilizer preventing serum separation in the drink is due to its electrostatic nature (Galazka, Dickinson, & Ledward, 1999). Under acidic conditions, the pectin polysaccharide is negatively charged whereas

casein is positively charged below its isoelectric point (pH 4.6) (de Kruif, 1998). When negatively charged pectin is added to positively charged casein, complexation between them takes place. Chapter **7** reports on the interaction between gold kiwifruit pectin and some commercial pectins and milk protein. For a greater understanding of this chapter and the complexes formed, the properties of milk proteins, acidification and the interaction between pectin and casein are described briefly.

1.5.2.3.1 Casein Micelles

Milk proteins are grouped into two types based on their solubility under acidic conditions (pH 4.6), namely caseins (insoluble proteins) and whey proteins or serum proteins (soluble proteins). Whey protein itself consists of two main types of protein, *i.e.* globulin, known as β-lactoglobulin, and albumin, or α-lactalbumin (Fox, 2003).

The casein in milk is present as colloidal particles that are commonly referred to as casein micelles. They are spherical in shape, with diameter between approximately 50 and 500 nm and with the M_w ranging from 10^6 to 3 x 10^9 Da. Casein consists primarily of α_{s1}-, α_{s2}-, β- and κ-caseins, in the approximate proportions 4:1:4:1. Figure **1.14** shows a schematic model of the cross-section of a casein micelle, as described by Walstra (1999). Basically, a casein micelle is built up from many sub-micelles of 10−15 nm diameter that are linked collectively by colloidal calcium phosphate (CCP). On the surface, the sub-micelles are covered with the C-terminal ends of κ-casein, which protrude from the micelle surface, creating hairy layers. These layers prevent the aggregation of micelles by steric and electrostatic repulsions, and stabilise the micelles through a zeta potential of ~ 20 mV (Fox, 2003). According to Walstra, Wouters and Geurts (2006), the hairs are hydrophilic and negatively charged. The thickness of the hairy layer is approximately 7 nm.

The question of how the individual sub-micelles are attracted to each other was answered by Horne (2003) with his dual-binding model. In this model, the caseins (α_{s1}-, α_{s2}-, β- and κ-caseins) are held together by hydrophobic interactions, leaving the hydrophilic parts free (creating a very porous structure) and with the hydrophilic regions of κ-casein extending into the aqueous phase.

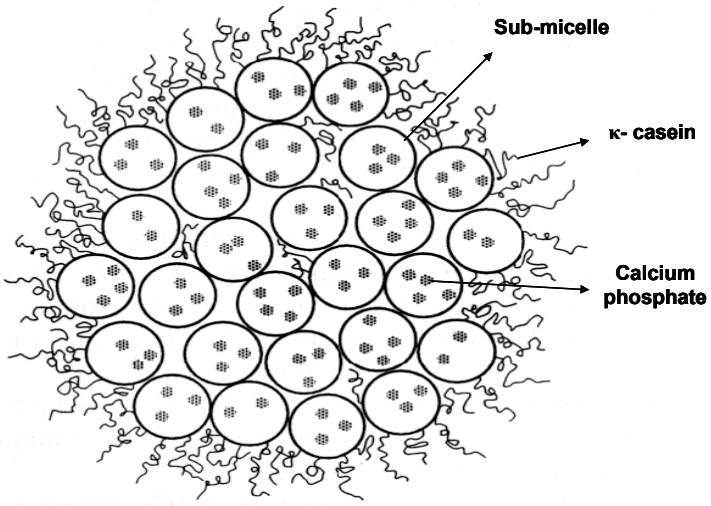

Figure 1.14 Model of the cross-section of a casein micelle (Walstra, 1999).

1.5.2.3.2 Milk Acidification

AMD such as yogurt drinks is produced by diluting and homogenizing fermented skim milk gels or by addition of fruit preparations and/or acid into skim milk. Acidification of milk is the basic principle of producing an AMD. Generally, the acidification process in the manufacture of an AMD can be achieved by fermenting the lactose to lactic acid by adding suitable bacterial cultures, which causes the pH to decrease (Amice-Quemeneur, Haluk, Hardy, & Kravtchenko, 1995). However, because irreproducible rheological and physical properties are obtained with uncontrolled bacterial fermentations, most research on AMDs has employed glucono-δ-lactose (GDL) for acidification (Lucey, Munro, & Singh, 1999). The discrepancy is due to the different rates in acidification during the critical stage of aggregation of the casein micelles and also to the physicochemical changes among the casein particles themselves (Lucey, Tamehana, Singh, & Munro, 1998). Amice-Quemeneur *et al.* (1995) have revealed that the decrease in pH with GDL addition is very rapid initially and then slowly declines, whereas a more gradual process is shown by lactic acid bacteria because they need a lag period as an environmental adjustment.

Casein micelles are physically stable at neutral pH (~ 6.7) and aggregation takes place when the pH is lowered to close to their isoelectric point (pH ~ 4.6). Aggregation of casein occurs if the hairy layer formed by κ-casein loses most of its negative charge. According to Walstra *et al*. (2006), decreasing the pH will decrease the absolute value of the zeta potential, and the casein eventually becomes positively charged. This is caused by increasing association of hydrogen ions (H^+) with acidic and basic groups of the protein and increasing calcium ion activity.

1.5.2.3.3 Pectin and Casein Interaction

Although the mechanism of how HMP stabilises an AMD has been widely studied, it is still not clear (Maroziene & de Kruif, 2000; Tuinier, Rolin, & de Kruif, 2002). It has been revealed that the stabilisation effect is related to the interfacial interaction between pectin and casein micelles (da Silva & Rao, 2006). However, Haylock, Towler and Hewitt (1995) initially proposed that the precipitation of protein is prevented in an AMD because the unbound pectin in the serum increases the serum viscosity. It has been reported that, throughout the acidification, HMP adsorbs on to casein micelles by electrostatic interaction (Tuinier, *et al.*, 2002). The adsorbed HMP prevents more aggregation and sedimentation of casein micelles mainly via *steric stabilisation* (Liu, Nakamura, & Corredig, 2006). Steric stabilisation is the stability conferred by polymer chains adsorbed on to the surface of colloidal particles (Dickinson, 1992). In this case, the interaction of adsorbed layers can induce two effects if the particles come close together: an *osmotic effect* (an increase in the local concentration of adsorbed species between the two particles) and an *entropic* or *volume restriction* effect (a loss of certain degree of freedom of the polymer due to crowding). Because of these effects, the interaction of the adsorbed layers results in repulsive interactions between the protein particles coated with pectin.

According to Tromp, de Kruif, van Eijk and Rolin (2004), (see Figure **1.15**) the adsorption of pectin chain on to the casein micelle surface occurs only at the charged blocks of the pectin due to the distribution of charges along its chain. The remaining uncharged pectin chain will extend into the solution to form an entropy-rich loop that causes repulsive

interaction between the micelles in an acidic solution. These loops act in the same way as κ-casein chains at the natural pH of milk (6.7).

The interaction between pectin and protein is driven mainly by their different charges, *i.e.* it is an electrostatic interaction that can lead to the formation of pectin−protein complexes (da Silva & Rao, 2006). As pectin is a negatively charged polysaccharide whereas protein is positively charged under acidic conditions, pectin and protein are attracted towards one another. The main class of positively charged residues in most proteins is the $-NH_3^+$ groups and the negatively charged residue of pectin is $-COO^-$. In an acidified milk beverage system, this interaction occurs between pectin and protein and leads to a stabilised system. This interaction is largely influenced by physicochemical factors such as pH, ionic strength, ratio of pectin to protein, the pectin and protein charges, and M_w and DE of the pectin (Syrbe, Bauer & Klostermeyer, 1998; da Silva & Rao, 2006).

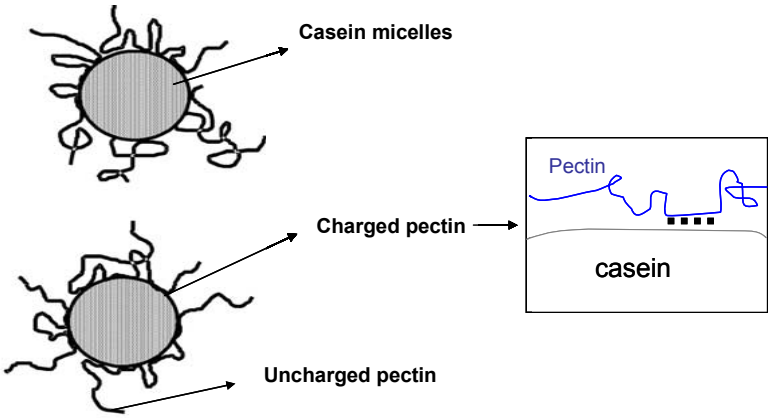

Figure 1.15 Casein micelles coated with adsorbed pectin molecules (modified from Tromp, *et al*., 2004).

1.5.3 Techniques for Pectin Extraction

The extraction of commercial pectin can be done chemically, physically and enzymatically (Panouille, *et al.*, 2006) and also by combinations of these methods. This research dealt with the extraction techniques commonly carried out in pectin manufacture, such as acidic and enzymatic extraction methods. A water extraction method was also included, for

comparison with the acidic and enzymatic extraction methods. To understand the type of pectin extracted and the extraction method applied, a brief overview of the pectin classes and of the extraction techniques is necessary.

1.5.3.1 Pectin Class

In general, pectin is classified into three classes according to its procedure of extraction from the plant cell wall: water-soluble pectin, which is extractable with water or dilute salt solutions; chelator-soluble pectin, which is extractable with calcium-chelating-agent solutions such as ethylene diamino tetraacetic acid (EDTA), cyclohexane diamino tetraacetic acid (CDTA) or hexametaphosphate; protopectin, which is extractable with alkali or hot dilute acid solutions (van Buren, 1991). The term protopectin describes the native pectin fraction in the cell wall that cannot be isolated without some degradation (Wang, *et al*., 2002).

Most water-soluble pectin is located in the middle lamella of the cell wall, and its availability decreases gradually through the primary cell wall towards the plasma membrane (Wang, *et al*., 2002). In addition, most chelator-soluble pectin is also found in the middle lamella of the cell wall. These pectins are composed predominantly of GalA residues, with rhamnose and neutral sugar contents of approximately 2% and 10−20% respectively. The composition of commercial pectin is relatively similar to those of water-soluble and chelator-soluble pectins, with low amounts of neutral sugars. However, it is possible that they are protopectins because their neutral sugars could be removed by hydrolysis during extraction (van Buren, 1991).

Protopectin or insoluble pectin is reported to be strongly bound in the cell wall of parenchymatous tissues (Renard, Voragen, Thibault, & Pilnik, 1990), where it is likely that the pectin chain is embedded in the cell wall with the rest expanding into the middle lamella (Renard, *et al*., 1990). According to these researchers, this pectin is extracted effectively only by hot dilute alkalis or acid. Isolating this type of pectin is difficult probably because it has strong linkages with other polysaccharides and is positioned in the primary cell wall.

Plant tissues vary in their amount and types of pectin (van Buren, 1991). For example, apple tissue contains mostly protopectin (O'Beirne, van Buren, & Mattick, 1982), whereas other fruit tissues, such as ripe freestone peach tissue, contain predominantly water-soluble pectin (Postlmayr, Luh, & Leonard, 1956).

1.5.3.2 Acidic Extraction

Commercially, the extraction of pectin from citrus peel and apple pomace is carried out in hot acidified water of pH 1−3 (Rolin, 2002). The extraction conditions such as time and temperature vary with the raw materials and the type of pectin desired, and normally range from 50 to 100ºC for 0.5−10 h (Wang, *et al*., 2002; Canteri-Schemin, Fertonani, Waszczynskyj, & Wosiacki, 2005). This process is carefully controlled to minimise the extent of hydrolysis of GalA and methyl ester in the pectin structure.

The basic principle of acidic extraction is acidic hydrolysis of protopectin (Minkov, Minchev, & Paev, 1996; Ptitchkina, Markina, & Runlyantseva, 2008). Principally, the pectin extraction involves three stages (Figure **1.16**): (*i*) hydrolysis of protopectin, which occurs in the solid phase in an acid solution; (*ii*) internal diffusion of the dissolved pectin after hydrolysis in the solid phase pores; (*iii*) external diffusion of the dissolved pectin through the liquid boundary layer. In these steps, some of the recovered pectin is degraded into low molecular weight components, which are undesirable in the extraction process (Panchev, Kirchev, & Kratchanov, 1988).

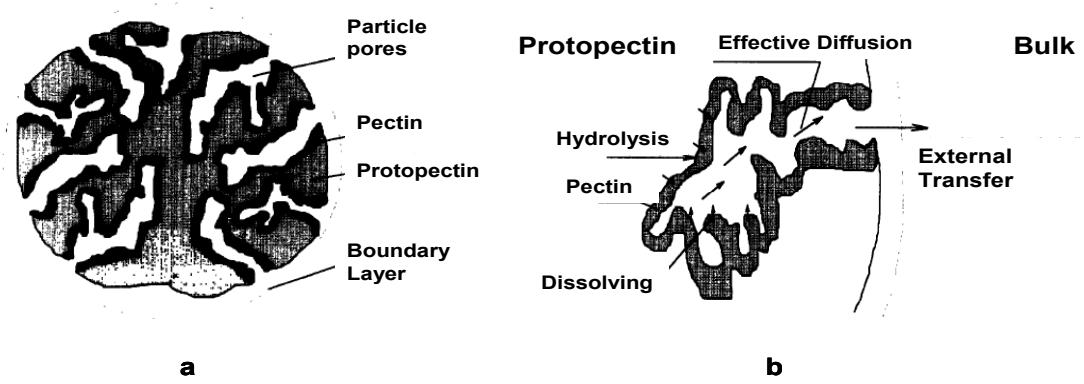

Figure 1.16 Illustration of (a) a porous particle and (b) the mechanism of pectin hydrolysis and transport of the pectin to the particle surface (Minkov, *et al*., 1996).

The hydrolysis of pectin glycosidic linkages is greater in strong acidic solution, whereas both glycosidic and methyl ester linkages are hydrolysed to similar extents in medium alkaline solution. This *β*-elimination by alkali occurs mostly with GalA units having an esterified carboxyl group, because the hydrogen (H) atom on C-5 is more acidic than in residues having a free carboxyl group (Belizt, *et al*., 2009). This is also why LMP is more stable than HMP at higher pH. Figure **1.17** shows the hydrolysis of pectin and the *β*-elimination process.

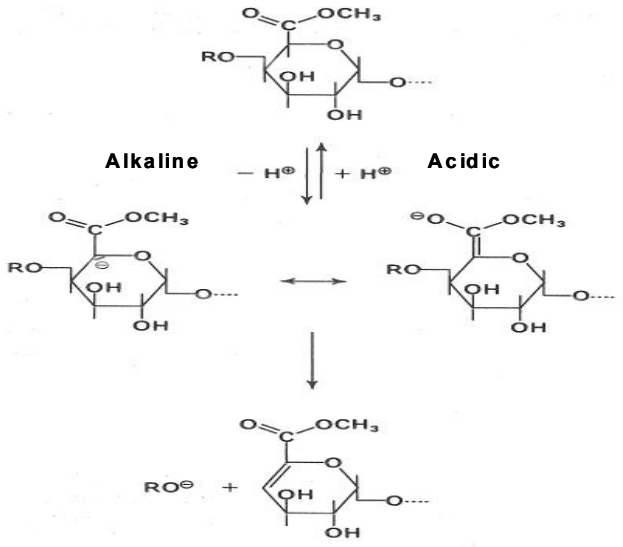

Figure 1.17 Hydrolysis of pectin and the *β*-elimination process (Belizt, *et al*., 2009).

1.5.3.3 Enzymatic Extraction

The most common method for extracting pectin without further degradation is to use enzymes. Compared with acid solution, commercial enzymes are preferred despite their cost because only a little effluent is found in the filtrate and because of consumer demands for green products. However, the mechanism of enzymatic extraction is still not fully understood (Panouille, *et al.*, 2006). This research considered the use of a commercial cellulase preparation such as Celluclast 1.5L to promote the release of pectic substances.

Celluclast 1.5L contains predominantly cellulases (endo-glucanase units) and has been found to be effective in extracting pectin from plant cell walls. It breaks down the cellulosic materials in chicory cell walls and converts them to glucose. Endo-glucanase hydrolyses the cellulose chain internally and produces oligosaccharides, cellobiose and glucose (Karlsson, *et al*., 2002). Cellulose is a water-insoluble linear polymer of β-($\rightarrow$4) linked D-glucopyranosyl units (BeMiller & Whistler, 1996). Panouille *et al*. (2006) showed that using Celluclast 1.5L together with protease (Neutrease) and Cellulyve TR400 increases the cellulose solubilisation to glucose to 80% and thus improves the pectin yield. It has also been found that the use of Celluclast 1.5L for pectin extraction increases the pectin yield, because there is a direct interaction between cellulose and the galactan side chain of pectin.

1.5.3.4 Water Extraction

Some of the pectin in the plant cell wall is available as water-soluble pectin; consequently, this type of pectin can be easily extracted with water. Although the most conventional and the easiest means of extracting pectin is to use hot water, this method is not applicable in industry because long times and high temperatures are required to isolate all the available pectin. These two factors will lead to degradation of the pectin during extraction and will be relatively costly because of the high energy requirement (Simpson, Egyankor, & Martin, 1984).

1.5.3.5 Precipitation of Pectin

The pectin extract is separated from the solid residue as efficiently as possible to avoid losing the extract. This step is fairly difficult because of the viscosity characteristic of the pectin, which increases as the concentration and the M_w increase. The pectin extract may be further clarified by filtration through a filter aid such as kieselguhr (a diatomaceous earth) (May, 1990). The addition of this filter aid is also aimed to increase the filter rate during the separation of ethanol-precipitated pectin.

The precipitation of pectin is usually accomplished with an alcohol (ethanol or methanol) or aluminium hydroxide. With aluminium hydroxide, the pectin separates as a greenish-yellow floc that tends to float on the surface of the solvent. Although this procedure is superior to the alcohol method, highly esterified pectin (DE above 70%) does not precipitate well with aluminium (de Luca & Joslyn, 1957) and the overall recovery is not as good as with the alcohol method (May, 1990). In addition, this method is time consuming because of the pH adjustment and the series of washing and rinsing steps that are required to remove the remaining effluents. Further literature data on the extraction conditions and their effect on the pectin obtained are presented in the introductions to Chapters **3**, **4** and **5**. Figure **1.18** describes the extraction method that is commonly applied in pectin manufacture (Rolin & de Vries, 1990).

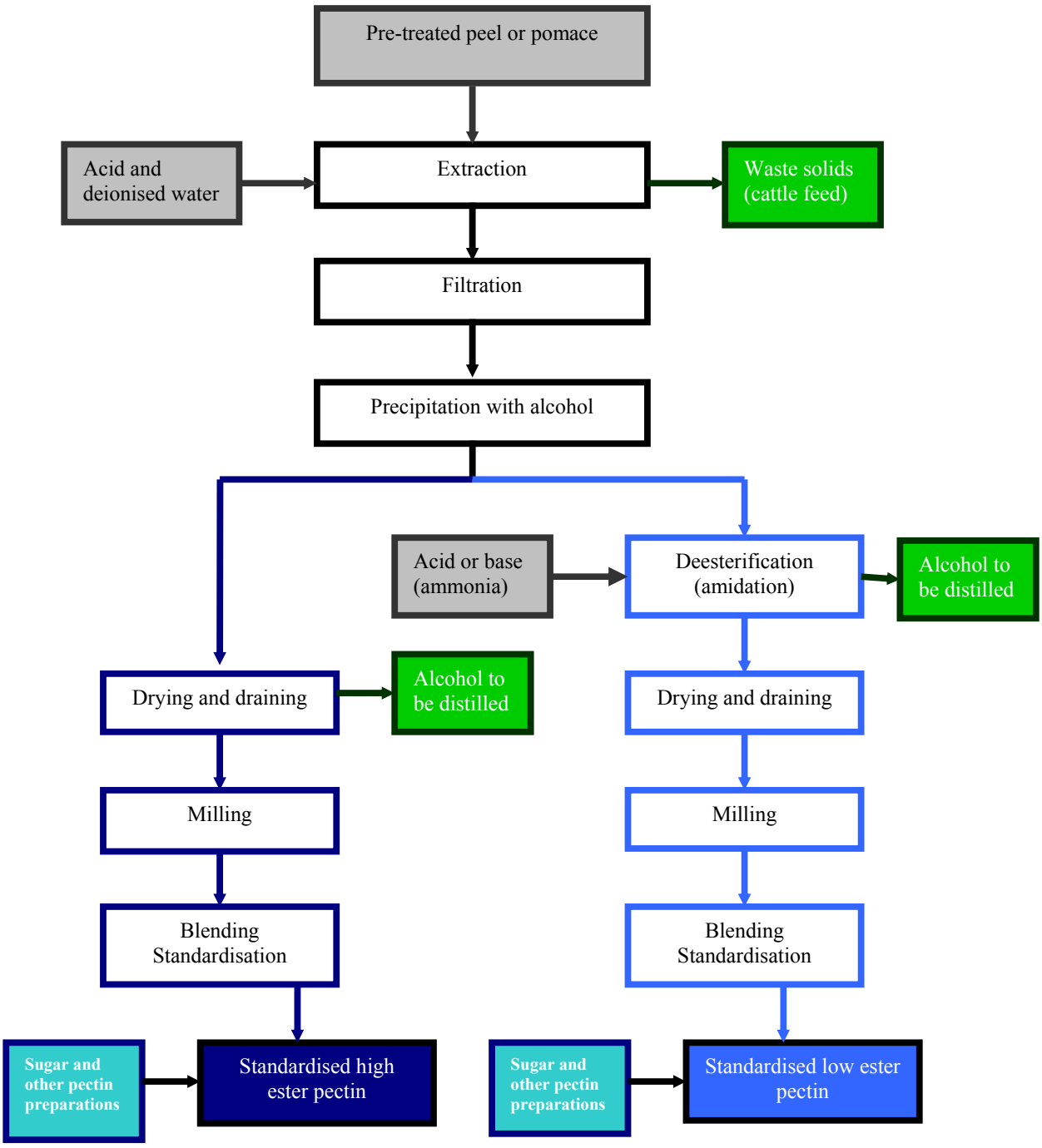

Figure 1.18 Commercial pectin production processes (Rolin & de Vries, 1990).

CHAPTER 2 Techniques

This chapter provides an overview of the techniques used, including theory and common terminologies, relating to gold kiwifruit pectin physicochemical characterisation.

2.1 Chemical Composition Determination

2.1.1 Total Non-Starch Polysaccharide and Monosaccharide Composition

Dietary fibre consists of polysaccharides, which are mainly cellulose, hemicellulose and pectic substances, suitably referred to as non-starch polysaccharides (NSP) (Englyst, Quigley, & Hudson, 2000). The total-NSP contains rhamnose, arabinose, xylose, mannose, glucose, galactose and GalA residues, as its main constituent sugars. The determination of dietary fibre as NSP, in this study, was carried out by the Englyst method.

2.1.1.1 Principles of Measurement

The Englyst procedure involves four primary principles: (*i*) removal of starch from polysaccharides, by enzymatic hydrolysis, (*ii*) NSP precipitation in acidified ethanol, (*iii*) dispersion and hydrolysis of precipitated NSP, with sulphuric acid and (*iv*) finally, the measurement of the released constituent sugars by gas liquid chromatography (GLC), high performance liquid chromatography or colorimetric (Englyst, *et al*., 2000). In this study, the NSP analysis was carried out by using GLC. This method identifies and quantifies the individual constituent sugars, as alditol acetates derivates. The basic procedure, for each step involved, is outlined in the following sections.

2.1.1.1.1 Starch Hydrolysis

Starch removal is achieved by enzymatic hydrolysis, followed by subsequent ethanol precipitation of NSP. The addition of heat-stable amylase, together with pancreatin (containing amylase, lipase and protease) and pullulanase (de-branching enzyme), improves the starch hydrolysis to glucose. Englyst, Quigley, Hudson and Cummings (1992) reported that the starch was completely hydrolysed after a 50 min incubation, without losing its NSP constituents. The presence of resistant starch (RS) used to be a significant issue, when determining NSP constituents, due to the fact that the presence of RS hinders the complete starch removal from the sample. The term RS was defined by Cummings and Englyst (1991) as all forms of starch that are resistant to digestion. However, in this method, RS is completely removed by dispersing the sample, using dimethyl sulfoxide (DMSO), which is a very highly polar solvent (Vignes, 2000) and therefore RS is not included in the NSP calculation.

The NSP precipitation, by acidified 80% ethanol (containing 1 mL of 5 M HCl per litre of ethanol, pH ~ 2.3), eliminates all available oligosaccharides and free GalA, since they remain in the sample. The precipitation of these sugars, together with NSP, could lead to a false value of NSP content in a sample (Englyst, *et al*., 1992). These authors have reported that oligosaccharides could be linked to polysaccharides, by non-covalent bonds forming complexes, which are insoluble in 80% ethanol.

2.1.1.1.2 NSP Hydrolysis

In order to hydrolyse cellulose and non-cellulosic polysaccharides present in the sample, the treatment by 12 M sulphuric acid (H_2SO_4), for 30 min at 35ºC, followed by treatment with 1 M H_2SO_4, for 1 h at 100ºC was carried out. These treatments have shown a complete NSP dispersion and hydrolysis, after the samples had been treated to de-starching and washing steps (Englyst, Quigley, & Hudson, 1994). A test on depolymerisation completeness was undertaken, in order to detect any remaining oligosaccharides in the solution, after the acid hydrolysis steps and no residual oligomers were detected, thus suggesting a complete hydrolysis of NSP (Englyst, *et al*., 2000).

2.1.1.1.3 Measurement of Sugars as Alditol Acetates

The monosaccharides released from NSP hydrolysis were determined as alditol acetates derivates, using GLC. These *alditol acetates* are volatile and they can be identified and quantified by GLC, hence a gas chromatogram is very important for this procedure (Melton & Smith, 2001). According to Sawardek and Sloneker (1965), the quantification of monosaccharide as *alditol* and the *alditol derivatives* separation have been the most widely used, since *alditol acetates* are relatively more polar and they show better resolution and (as consequence) this procedure would eliminate the problem of multiple peaks. Therefore, the resulting chromatograms are relatively simple to be analysed, since they give a single peak for each sugar (Melton & Smith, 2001; Brunton, Gormley, & Murray, 2007). Preparation of *alditol acetates* involves reduction with sodium borohydride and acetylation with acetic anhydride, using N-methylimidazole as a catalyst. Figures **2.1** and **2.2** illustrate the reaction scheme for the formation of alditol acetates and the GLC chromatogram of sugar standards.

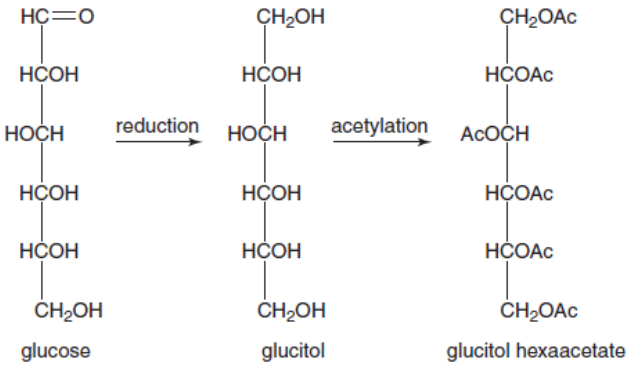

Figure 2.1 The reaction scheme of alditol acetates formation (Melton & Smith, 2001).

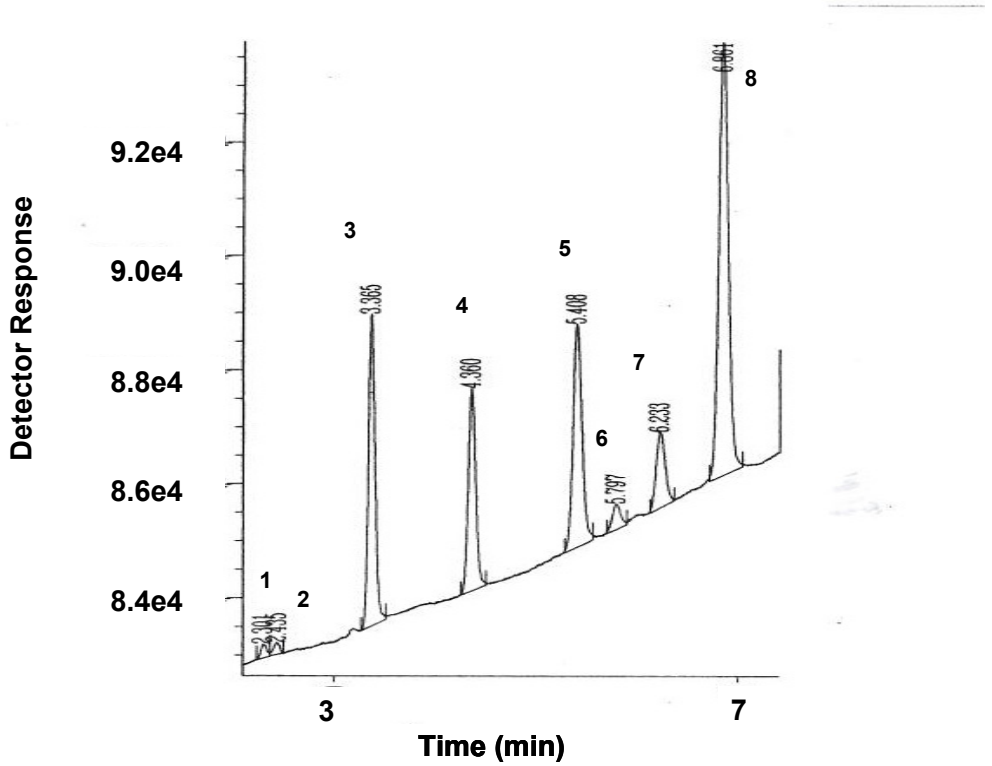

Figure 2.2 GLC chromatograms of alditol acetates derivates: a mixture of sugar standards 1. rhamnose; 2. fucose; 3. arabinose; 4. xylose; 5. allose; 6. mannose; 7. galactose; 8. glucose.

2.1.2 Colorimetric GalA Determination

The colorimetric determination of GalA, in this thesis, was carried out as described by Scott (1979), in conjunction with the GLC determination of total-NSP. This method is more specific and selective than other methods, such as m-hydroxydiphenyl, since the *chromophore* formed from GalA, by the addition of 3-5-dimethylphenol (DMP), is different from the one formed by neutral sugars. According to Luzio (2004), the neutral sugars which react with DMP do not form a similar *chromophore,* in the presence of chloride ion, as the one formed by GalA, and therefore corrections for browning caused by neutral sugars can be easily made. Whereas, in the m-hydroxydiphenyl method, the browning produced, during the first stage of heating, has to be corrected for the presence of neutral sugars.

2.1.2.1 Principles of Measurement

The principle of GalA measurement in this section is mainly taken from Scott (1979). The basic principle of this method is the selectivity of a colorimetric reagent, which is DMP for a chromogen named *5-formyl-2-furancarboxylic acid* (5FF). This substance is formed from GalA in concentrated H_2SO_4 at 70ºC and it is measured at 450 nm. The interference from other neutral sugars (such us rhamnose, arabinose, xylose, mannose, glucose, galactose) and lignin is minimised, by subtracting the value with the absorbance, at 400 nm.

The colorimetric determination of GalA is based on the total hydrolysis of pectic substances to free GalA, in concentrated sulphuric acid solution. However other sugars, such us hexoses and pentoses, are also hydrolysed. GalA reacts with H_2SO_4, in the presence of sodium chloride-boric acid solution, thus producing 5FF, whereas hexoses and pentoses form *5-hydroxymethyl-2-furancarboxal* and *2-furancarboxalaldehydes*, respectively.

The rate and extent of 5FF formation depends on the concentration of H_2SO_4 and the configuration of GalA, thus, in order to minimise the variability, the addition of H_2SO_4 to the sample, should be carried out quickly with an immediate mixing process, in order to achieve a uniform colour formation. Furthermore, the addition of borate as a catalyst in the form of H_3BO_3, also increases the rate of 5FF formation.

Generally, the interference with neutral sugars products is minimised by two ways: firstly by the addition of chloride (NaCl) and secondly by using the 450–400 nm absorbance differences to measure GalA. The presence of NaCl, during the procedure, is a primary factor in reducing the absorbance produced from glucose products, by 60%, whilst there is only a small loss in sensitivity to the absorbance of GalA. The *5-hydroxymethyl* derivatives will form *5-chloromethyl-2-furancarboxaldehyde,* with the addition of NaCl, which reduces the amount of color-producing degradation products.

2.1.3 Capillary Electrophoresis

Capillary electrophoresis (CE) is a technique, employed in this study, to determine the degree of esterification (DE) of the obtained pectins extracted from gold kiwifruit. Generally, this technique is used to separate a variety of compounds based on a separation method, which is performed in a fused-silica capillary (25–100 μm). The separation is driven by an electric field of a high voltage, and this further generates an electroosmotic and electrophoretic flow, which drags all analytes one way, within the capillary.

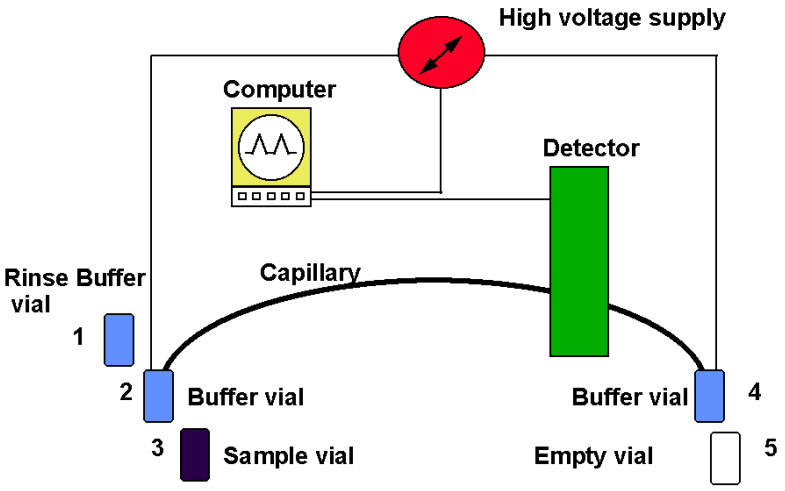

Figure 2.3 CE configuration

Figure **2.3** illustrates the basic instrumental configuration of CE. It consists of a fused-silica capillary; a controllable high voltage power supply; two electrodes assemblies; two buffer reservoirs; and an ultraviolet (UV) detector.

2.1.3.1 Principles of Measurement

The principle of the measurement is the separation of the compounds, based on their charge density, size and hydrophobicity. Electroosmosis in CE is the result of the surface charge of the capillary wall, which has ionisable silinol groups. When the capillary is filled up with buffer, the negatively charged wall attracts the positively charged ions from the buffer, thus generating an electrical double layer. As soon as the voltage is applied across the capillary,

the cations in the diffused portion of the double layer flow towards the cathode—the negative electrode — thus creating a net flow of buffer solution.

Electrophoretic mobility (μ) is measured from the migration time (t) of the injected sample analysed and the migration time (t_0) of a neutral marker, and it is expressed by the following equation (Williams, Buffet, & Foster, 2002):

$$\mu = \mu_{obs} - \mu_{eo} = \left(\frac{lL}{V}\right)\left(\frac{1}{t} - \frac{1}{to}\right) \qquad (2.1)$$

where

L is the total length of the capillary

l is the distance from inlet to detector

V is the applied voltage

μ_{obs} is the observed mobility

μ_{eo} is the mobility of the electroosmotic flow

Pectin is a negatively charged molecule at neutral pH, and its charge density varies with the degree of esterification (DE) (Christensen, 1986). CE can be used to measure the DE of a pectin sample, given the fact that there is a linear relationship between the electrophoretic mobility and the average charge, per residue, found in pectin (Zhong, Williams, Keenan, Goodall, & Rolin, 1997; Zhong, Williams, Goodall, & Hansen, 1998). This method has some advantages, compared to other methods, such as the ion-exchanged chromatography and size exclusion chromatography (IEC-SEC) method (Williams, *et al*., 2002; Williams, Foster, & Schols, 2003): (*i*) The separation quality is inherent, regardless of the size of the polymer this will elute, according to its charge density, (*ii*) The electrophoretic mobility of pectin has been reported to be not significantly affected by random or block wise intermolecular methyl ester distributions, (*iii*) This technique provides the possibility of quantifying the DE distribution, which reflects the intermolecular methyl ester distribution of a pectin sample (as shown in Figure **2.4**, the electropherograms obtained indicate the distribution of DE amongst pectin chains), and (*iv*) The DE analysis by CE is fast since the

analysis can be undertaken in only two hours including sample preparation and calibration, whereas IEC-SEC takes two days.

The first pectin analyses by CE were carried out by Zhong *et al*. (1997). These authors have indicated that the migration time of pectin, in the CE, is a function of DE. Jiang, Liu, Wu, Chang and Chang (2005) also investigated the relationship between the migration times and various DEs of alkaline de-esterified pectins. They observed that pectin with higher DE exhibited shorter migration time, than lower DE pectin, without considering the capillary length. This can be explained, based on equation (**2.1**): the observed mobility μ_{obs} is the vector sum of μ_{eo} and μ, and since the μ is negative and smaller magnitude than μ_{eo} the anions having the most negative mobility have the smallest μ_{obs} and thus the longest migration times (Williams, *et al*., 2002).

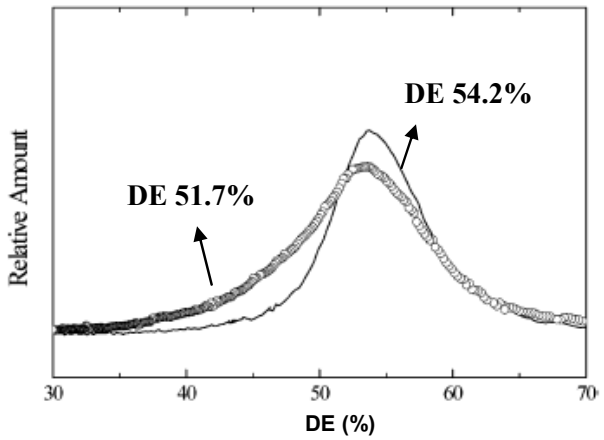

Figure 2.4 A typical electropherogram of intermolecular DE distribution of pectin samples (modified from Williams, *et al*., 2003).

2.2 Physical Analysis

2.2.1 Static Light Scattering

The basic principle of static light scattering (SLS) is schematically presented in Figure **2.5**. When a beam or light passes through a small particle, the incident light continues in its original direction, but a small fraction is scattered in different directions (Wyatt, 1998).

Light scattering is based on two basic principles: (*i*) the amount of light scattered is directly proportional to the M_w and the solute concentration (assuming that there is no absorbance or fluorescence from the particle); and (*ii*) the angular variation of scattered light is directly related to the size of the molecules. These two principles are described by Zimm (1948), as the Reyleigh-Debye-Gans light scattering model given in equation **2.2**.

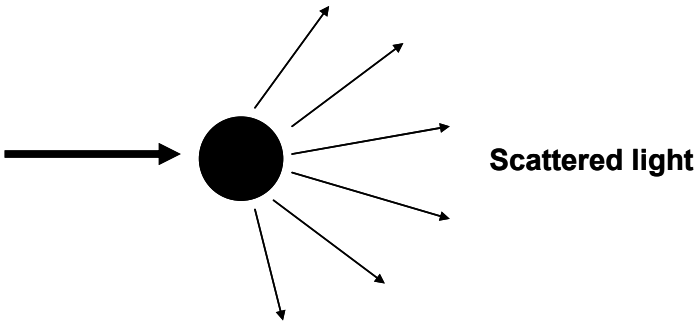

Figure 2.5 Basic principle of light scattering.

$$\frac{R_{(\theta)}}{Kc} = M_w P_{(\theta)} - 2A_2 c M_w^2 P^2{}_{(\theta)} \qquad (2.2)$$

where:

$R_{(\theta)}$ is the Rayleigh excess scattering, the excess intensity of light scattered at angle θ

K is an optical constant

c is the concentration of solutes (g/ml)

M_w is the weight average molar weight,

$P_{(\theta)}$ is the scattering function

A_2 is the second viral coefficient

K, the optical constant is derived from factor:

$$K = 4\pi^2 n_o \left[\frac{dn}{dc}\right]^2 \lambda^{-4} N a^{-1} \qquad (2.3)$$

where

dn/dc is the differential refractive index increment of the solvent-solute solution with respect to a change in solution concentration

n_o is the solvent refractive index at λ

λ is the incident wavelength in vacuum

Na is the Avogadro's number equal to 6.022 x 10^{23}

The Rayleigh excess scattering ($R_{(\theta)}$) is represented by:

$$R_{(\theta)} = \left(\frac{I_\theta r^2}{I_o V} \right) \qquad (2.4)$$

where

I_θ is scatter intensity

I_0 is incident beam intensity

V is volume of scattering medium

r is distance between the scattering volume and detector

$P_{(\theta)}$ is scattering function derived from:

$$P(\theta) = 1 + \left(\frac{16\pi 2 n_o^2}{3\lambda^2} \right) r_g^2 Sin^2 \left(\frac{\theta}{2} \right) \qquad (2.5)$$

where

n_o is the solvent refractive index

r_g is the radius of gyration

Equation **2.2** can be expressed in three different mathematical methods, namely, the Zimm, Debye and Berry methods. In the Zimm plot method, Kc/R_θ is plotted on the ordinate versus Sin² (θ/2), as equation **2.6:**

$$\frac{Kc}{R_{(\theta)}} = \frac{1}{M_w P_{(\theta)}} + 2A_2\ c \qquad (2.6)$$

For Debye Plot, *i.e* a plot of $Sin^2\left(\frac{\theta}{2}\right)$ versus $\frac{R_{(\theta)}}{Kc}$, to obtain the $\theta \rightarrow 0$, and $P_{(\theta)}$ approaches 1, the zero angle limit is:

$$\lim_{\theta->0} \frac{R_{(\theta)}}{Kc} = M_w - 2A_2 c M_w^2 \qquad (2.7)$$

Calculation of the Berry Method is performed by plotting square root of $\sqrt{\frac{R_{(\theta)}}{Kc}}$ versus $Sin^2\left(\frac{\theta}{2}\right)$ as below equation:

$$\sqrt{\frac{Kc}{R_{(\theta)}}} = \frac{1}{\sqrt{M_w} P_\theta} + A_2 c \sqrt{M_w} P_{(\theta)} \qquad (2.8)$$

These three plot methods are illustrated in Figure **2.6**. The plots are calculated for random coil polymers, with a root mean square (RMS) radius of 50, 150 and 250 nm and with the dots representing the 16 multiple scattering angles (Andersson, Wittgren, & Wahlund, 2003).

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

In this study, the MALLS photometer from Wyatt Technology (DAWN DSP, Santa Barbara, USA), was used to determine the pectin M_w, based on the Reyleigh-Debye-Gans light scattering model. In this system, a laser with a wavelength of 632.8 nm is used as a light source, in order to supply a monochromatic and well-collimated beam. The laser beam passes through the solution and the photometer simultaneously measures the intensity of the scattered light, at approximately sixteen angular locations, ranging from 22.2° to 157.3°.

The multi-angle detectors measure the intensity of scattered light and the signals are used in the calculation for molar mass determination.

Light scattering (LS) can be applied in either batch or chromatography mode, however, the later is generally more useful since the polysaccharide molecules can be separated, based on their sizes and the absolute weight-average molar masses for different fractions (Jumel, Browne, & Kennedy, 1991).

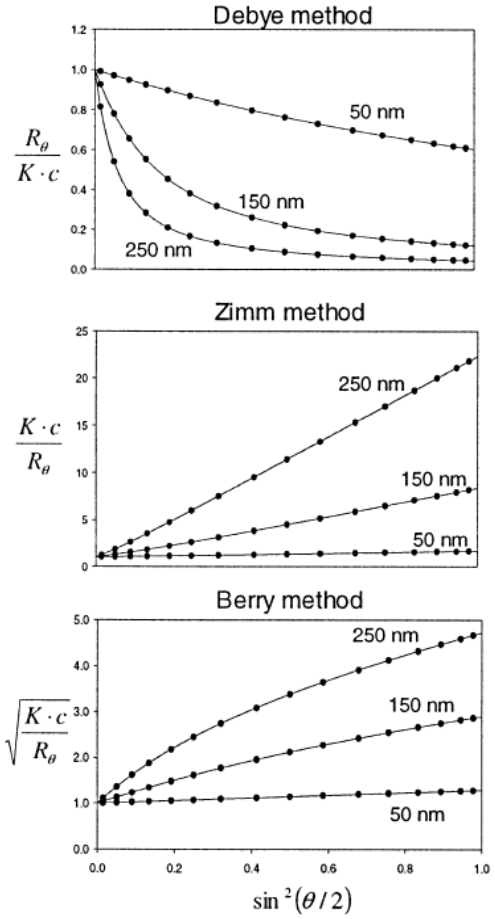

Figure 2.6 Three different plotting methods of Debye plot: Debye, Zimm and Berry (Andersson, *et al.*, 2003).

SEC-MALLS, in combination with concentration detectors, such as ultraviolet (UV), LS and a differential refractive index (DRI) system, is able to calculate the weight-average molecular weight (M_w) and Z-average root mean square radius (R_z) and the concentration of material eluting in small, individual slices of the SEC chromatogram (Wang & Lucey,

2003). A typical set-up of such a system (and the chromatograms produced) is shown in Figure **2.7** and **2.8**, respectively. In this system, the RI signal indicates the concentration of the polysaccharides, the UV signal correlates to the presence of protein in the sample whereas the LS signal depends on the size, M_w and concentration of the polymer molecules eluted through the SEC column.

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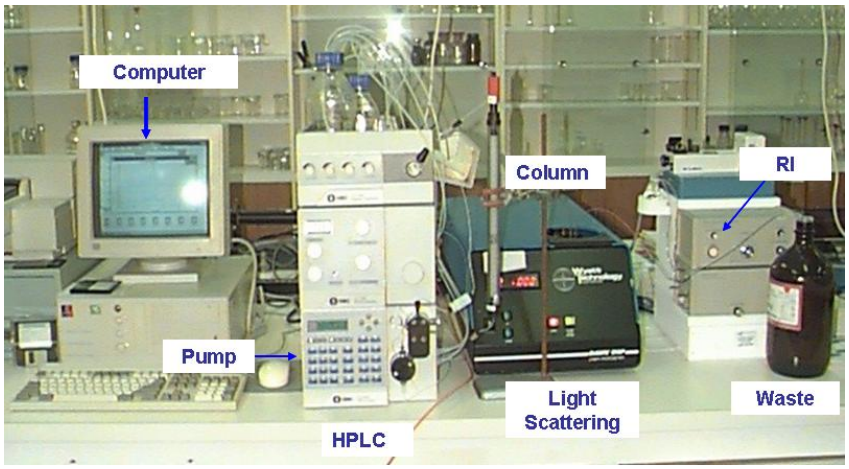


Figure 2.7 A photograph of the SEC-MALLS system connected in a series to HPLC, UV and DRI detectors, at Massey University, Palmerston North.

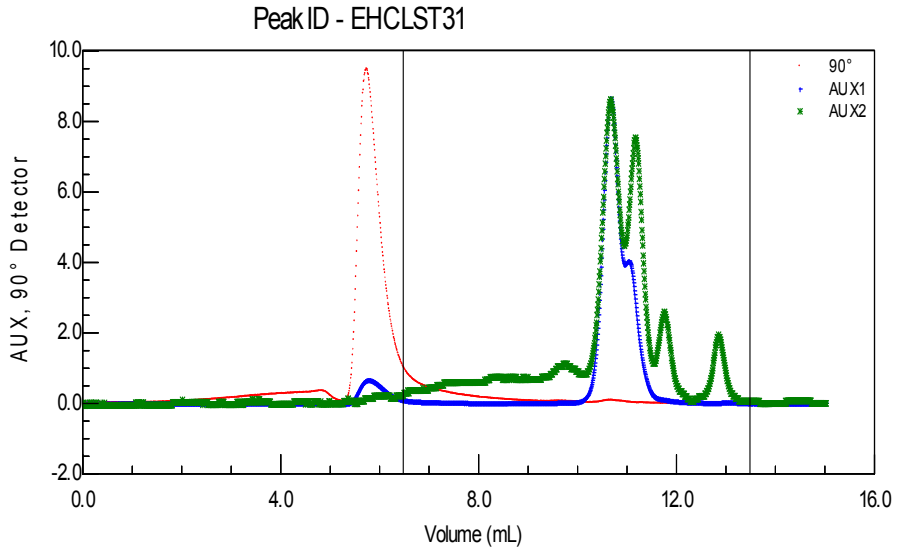


Figure 2.8 Typical example of chromatograms (RI, UV and LS signals) obtained from the SEC-MALLS system.

2.2.1.2 Molecular Weight Distribution

The molecular weight of a compound is defined as the sum of the atomic masses of the constituent atoms, in the molecular formula that form this compound (Boyd & Phillips, 1996). Molecular weight determination is one of the most essential parameters to characterise a macromolecule, whether it exists naturally or it has been synthetically formed (Harding, Varum, Stokke, & Smidsrod, 1991). Knowledge relating to the molecular weight of polysaccharides and their distribution are important, in order to understand the polysaccharides' functional properties and their application (Harding, *et al*., 1991). However, the precise determination of the molecular weight of a macromolecule, including polysaccharides, is very difficult (Harding, *et al*., 1991). This is due to the fact that they are polydisperse in nature, since they are comprised of species with heterogeneity in molecular weight and also because of the tendency of some polysaccharides to aggregate or self associate in solution, especially at high concentration. The molecular weight is commonly expressed as either a weight average (M_w) or a number average (M_n), or *Z-average* (M_z). The weight average of molecular weight (M_w) which was used in this study, is expressed as the equation below:

$$M_w = \frac{\sum\left(c_i / M_i\right)}{\sum c_i} \qquad (2.9)$$

The number-average molecular weight (M_n) and the *Z-average* molecular weight (M_z) are obtained by the equations below:

$$M_n = \frac{\sum c_i}{\sum\left(c_i / M_i\right)} \qquad (2.10)$$

$$M_z = \frac{\sum\left(c_i / M_i^2\right)}{\sum\left(c_i M_i\right)} \qquad (2.11)$$

where M_i and c_i is molecular weight and the concentration of the i^{th} slice.

2.2.1.3 Polydispersity Index

Polydispersity is defined by the presence of non-interacting components of different molecular weight and compositions (Harding, *et al*., 1991). The polydispersity index is basically a measurement of the degree of molecular weight distribution in a given polymer (Flory, 1953). It is calculated by the ratio of M_w/M_n. Generally, most polymers have a polydispersity index of approximately 2.0, given the fact that M_w is always greater that M_n, unless the polymer is monodisperse and then it has a polydispersity index equal to unity (Azapagic, Emsley, & Hamerton, 2003).

2.2.1.4 Specific Refractive Index Increment (*dn/dc*)

The specific refractive index increment (or *dn/dc)* is defined as how much the refractive index of solution changes for a given increment in concentration and it is expressed as mL/g (Wyatt, 1993). A *dn/dc* value is required for the determination of molecular weight by light scattering. Di Primo and Lebars (2007) reported that the refractive index increments could differ significantly, from one type of molecule to another. Therefore, accurate values of *dn/dc* are important, in order to obtain the correct molecular weight calculation.

2.2.3 Dynamic Light Scattering

In this study, particle size distributions of casein–pectin complexes were measured, using a Zetasizer (Malvern Instruments Ltd, Malvern, Worcs, UK), based on the principles of *dynamic light scattering* (DLS). DLS, also known as *Quasi Elastic Light Scattering* (QELS) or Photon Correlation Spectroscopy (PCS) is one of the most common methods used to determine particle size, within the sub-micron region. In this method, the scattering intensity is measured in time intervals as short as 10^{-7}s and therefore the experiment duration is relatively short (Burchard, 1994). This instrumentation consists of the following main components, shown in Figure **2.9** (Malvern Instruments Ltd, 2004): (*i*) a laser source (a red light source with 633 nm to illuminate the particle); (*ii*) a detector, positioned at a scattering angle 90º (it measures the intensity of the scattered light); (*iii*) an attenuator (to reduce the intensity of scattering); (*iv*) a correlator (this compares the scattering intensity at

consecutive time intervals, in order to obtain the rate at which the intensity is varying); and (*v*) a computer (the software analyses the data and derives the size information).

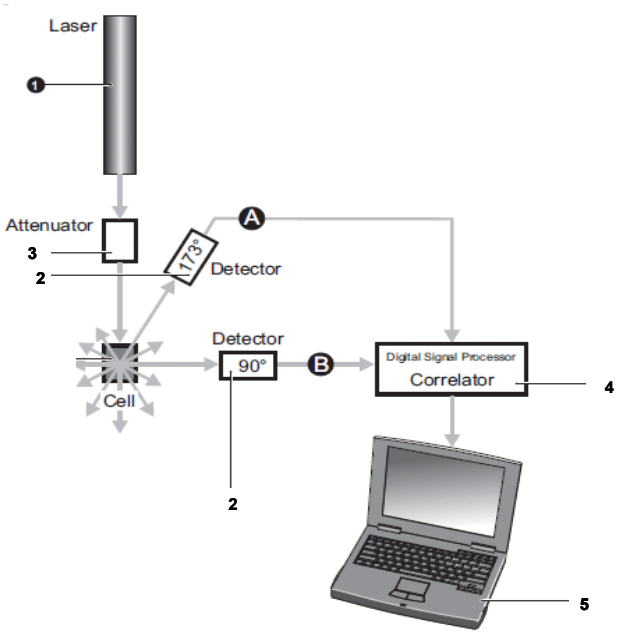


Figure 2. 9 Illustration of a Zetasizer operation system and components (Malvern Instruments Ltd, 2004).

2.2.3.1 Principles of the Measurement

This instrument measures *Brownian motion,* which relates to the size of the particle, by illuminating the particles with a laser and analysing the intensity fluctuations of the scattered light—*Brownian motion* is described as the random movement of colloidal particles, due to bombardment by the solvent molecules that surround them (Mazo, 2002). These fluctuations, in the intensity of the scattered light, are related to the rate of diffusion of molecules, in and out of the region being studied (Brownian motion) and the data can be analysed to directly give the diffusion coefficients of the particles creating the scattering. As long as the particles remain suspended in the fluid—the speed of the particle movements undergoing Brownian motion, is related to the size of the particles, as defined by the Stokes-Einstein equation (Equation **2.12**):

$$D = \frac{k_B T}{6\pi\eta R} \tag{2.12}$$

where

D is the diffusion coefficient

k_B is the Boltzmann constant

T is the temperature

η is the viscosity of the suspending liquid

R is the radius of the particle

As shown in Figure **2.10**, the small particles cause the intensity to fluctuate more rapidly, than the large ones, due to the higher translational diffusion speed of the small particles. The fluctuation in signal intensity is then fed to a digital correlator, in order to determine the rate of time intensity fluctuations, based on an autocorrelation function. The autocorrelation function relates to the particle size: considering the fact that larger particles move slowly, compared to smaller particles, which results in different intensity patterns. After the correlation function has been determined, this information can then be used to calculate the particle size distribution. A mean particle size, known as "*Z-average*" (or "cumulants mean"), can then be calculated, based on this intensity–size distribution.

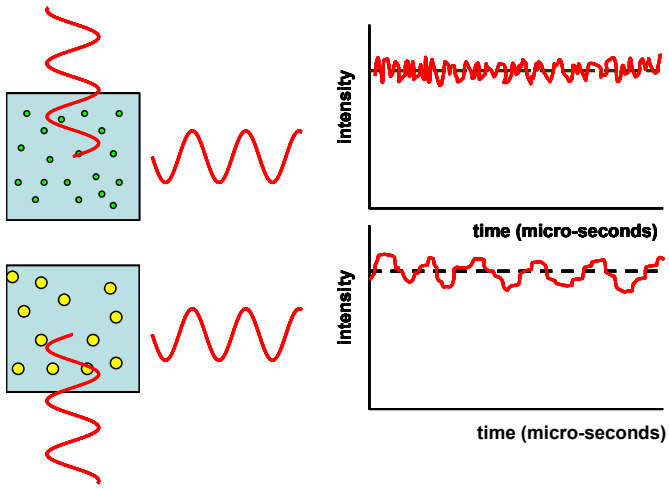


Figure 2.10 Intensity fluctuations over time from small and big particles detected in DLS.

2.2.4 Rheological Characterisations

Rheology is defined by Rao (2007), as the study of deformation and flow of matter. The rheological properties of food materials are based on their flow and deformation responses, when subjected to normal and tangential stresses. In this study, the principles of rheological measurements of food fluids and gels are the main focus.

2.2.4.1 Principles of Flow Properties Measurements

An ideal fluid deforms and continues to deform, so long as a load is applied. The material will not recover from its deformation, when the load is removed. This response is called *viscous.* The flow of simple viscous materials is described by *Newton's law,* which constitutes a direct proportionality between the shear stress (τ) (in SI units of Pa) and the rate of deformation or *shear rate* ($\dot{\gamma}$). The shear rate has dimensions of reciprocal time (s^{-1}). The proportionality constant is called the *shear viscosity* (η) and it has units of Pa.s (Equation 2.13) (Wang & Cui, 2005).

$$\eta = \frac{\tau}{\dot{\gamma}} \tag{2.13}$$

As shown in Figure **2.11**, the shear stress is the force applied per unit area and shear rate is the velocity gradient developed in the fluid, as a result of the applied shear stress (Bourne, 2002). Both shear stress and shear rate are expressed in equation **2.14** and **2.15**, respectively.

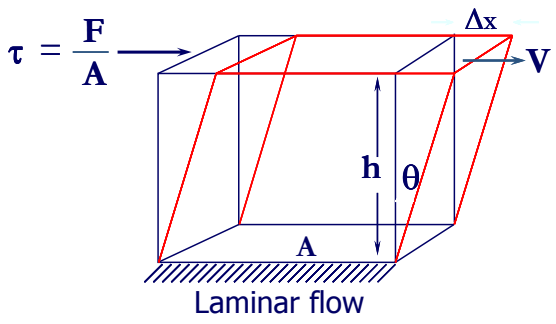


Figure 2. 11 Flow of fluid between two parallel plates separated by a distance (h).

$$\textit{Shear stress} \qquad \tau = \frac{F}{A} \tag{2.14}$$

$$\textit{Shear rate} \qquad \dot{\gamma} = \frac{V}{h} \tag{2.15}$$

where

F is (shearing) force (N) with1 N = 1 kg·m/s²

A is (shearing) area (m²)

V is velocity or speed (m/s)

h is plate distance or gap (m)

A flow curve describes the relationship between the shear stress and the shear rate. A *Newtonian fluid,* therefore, presents a constant viscosity independent of shear rate, so the relationship between shear stress and shear rate is linear. However, most food hydrocolloids dispersions are non-Newtonian fluids, above certain concentration. Their flow curves are nonlinear and they can also be time-dependent. In these cases, where a constant viscosity cannot be defined, the ratio between shear stress and shear strain is called *apparent viscosity*. *Non Newtonian fluids* can be divided into four types: *Pseudoplastic* (shear thinning, time independent); *Dilatant* (shear thickening, time independent); *Thixotropic* (shear thinning, time dependent); and *Rheopectic* (shear thickening, time dependent) (Mezger, 1998). Time dependent fluids are those where the change of the apparent viscosity, brought about by shearing, is irreversible and the sample does not recover its original characteristics, or it needs a certain period of time before recovery. Shear thinning is defined as behaviour where the apparent viscosity of the fluid decreases, when increasing shear rate. This behaviour can be observed in pectin solutions, depending upon polysaccharide concentration and molecular characteristics. In this study, shear viscosity curves will be plotted: this is, shear viscosity η (on the y-axis) plotted, versus shear rate $\dot{\gamma}$ (on the x-axis), although these curves could also be illustrated as η (y-axis), versus τ (x-axis) (Mezger, 1998).

The apparent viscosity of the pectin solutions and acidified milk drink was investigated by using a controlled-stress rheometer (Paar Physica MCR 301, Anton-Paar, GmbH, Germany). All rotational measurements, to characterise viscosity, were performed, using a cone and plate measuring system (CP 4/40, gap = 49 μm). This geometry was chosen, due to the relatively high viscosity of some samples and also due to the sample quantity limitations. Figure **2.12** shows a schematic illustration of a controlled-stress rheometer (Ross-Murphy, 1994) and the schematic diagram of a cone and plate geometry, used in the present study (Mezger, 1998). The principle of this measurement is the application of stresses, in the form of controlled torque and the recording of the strain induced in the sample, by measuring angular velocities, using a radial position transducer attached to the shaft. Torque values can be directly transformed into *stresses* —and angular velocities into *strains*. As shown in Figure **2.12**, the apex of a cone is brought into close proximity, but not into contact with a horizontal plate, where the minimum gap is normally of the order of 50 μm. In this analysis, only a small amount of sample is needed (~ 1−2 mL), since the gap between the cone and the plate is small (Rao, 2007).

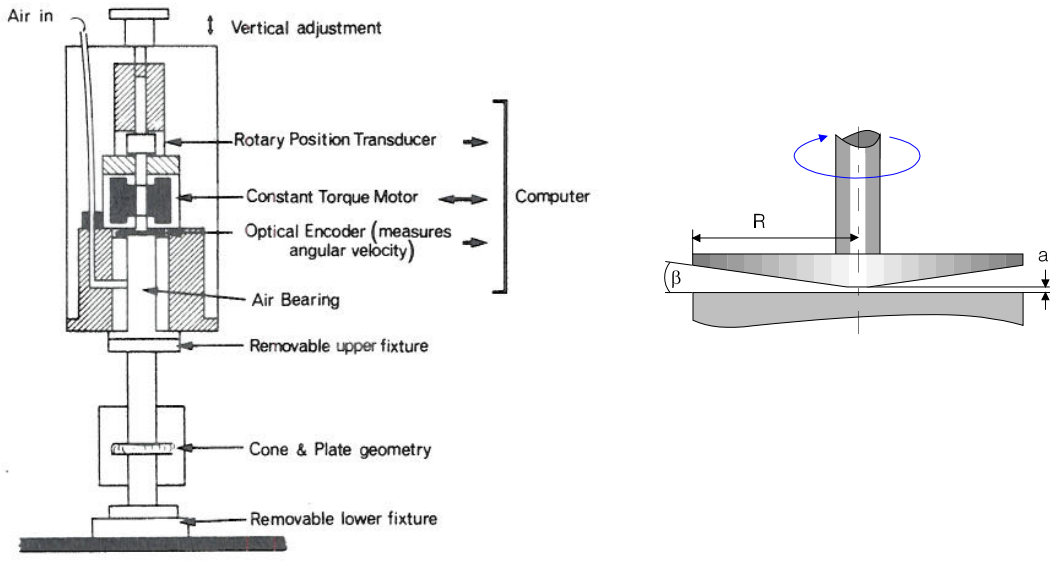


Figure 2.12 A schematic illustration of a controlled-stress rheometer (Ross-Murphy, 1994) and a cone and plate geometry (Mezger, 1998).

2.2.4.2 Principles of Viscoelasticity Measurements

In this study, the gel physical properties were investigated, by looking at the correlation between gel texture measured at large deformation (*i.e* gel hardness) and gel viscoelasticity measured at small deformation (*i.e* gel strength). The gel texture analysis was carried out by using a TA.XTplus texture analyser and this will be described in the next section. Dynamic oscillatory rheological testing was chosen, in order to measure gel viscoelasticity. This is an appropriate technique for observing the gelation process and for obtaining knowledge on the gel structure, due to the following (Rao, 2007): (*i)* the test is non-destructive and it does not interfere with the gelling process, (*ii*) there is a short testing time, and (*iii*) the results are comprehensive and easier to relate to gel structure. These oscillatory measurements were also carried out in a controlled-stress rheometer (Paar Physica MCR 301, Anton-Paar, GmbH, Germany), using concentric cylinders or a "bob and cup" measuring system (C-CC27/T200 Cup, B-CC27/Q1 Bob). Figure **2.13** shows the schematic illustration of this geometry. A cylinder (bob) is placed concentrically (coaxially) inside a cup, which contains a certain volume of tested sample (Rao, 2007).

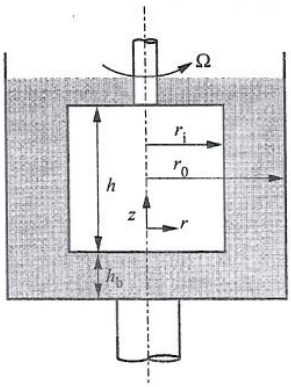


Figure 2.13 A schematic illustration of a bob and cup geometry (Rao, 2007).

Viscoelastic materials are described by Bourne (2002), as materials which simultaneously exhibit some of the plastic properties of an ideal solid and some of the flow properties of an ideal liquid. The viscoelastic behaviour of a gel comes about from the combination of the properties of a solid (elastic deformation) and a liquid (viscous flow), depending upon the

magnitude and time-scale of external forces (Barnes, Hutton**,** & Walters*,* 1998). Figure **2.14** shows a schematic illustration of the response of elastic, viscous and viscoelastic materials, to the application and removal of a stress (Bourne, 2002).

An ideal solid material responds to an applied load, by deforming finitely and recovering that deformation upon removal of the load. Such a response is called *elastic*. In contrast, an ideal fluid deforms and continues to deform, as long as the load is applied. This is the *viscous* response described earlier. The last response, shown in the illustration, above is known as *viscoelastic*, where the material behaves partially as a solid and partially as a liquid.

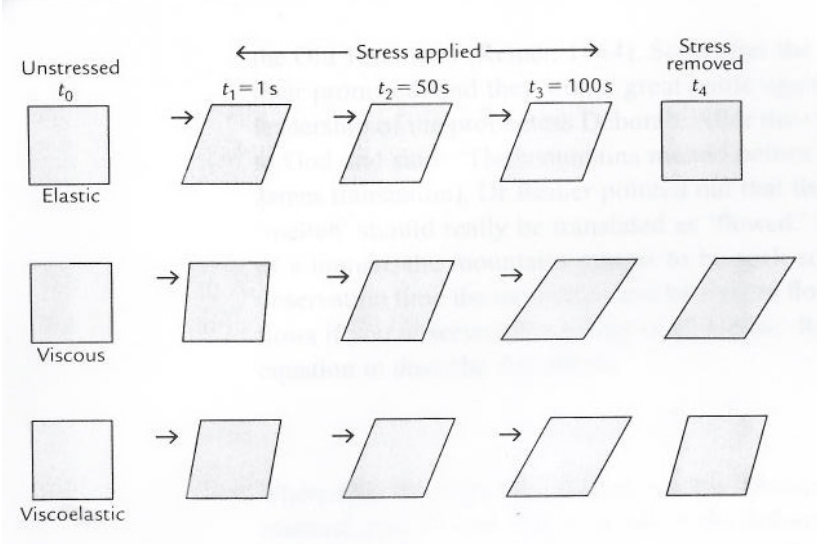


Figure 2.14 A schematic illustration of response of elastic, viscous and viscoelastic materials to the application and removal of a stress (Bourne, 2002).

In the *oscillatory rheological* method used here, to separate out these solid and liquid-like characteristics of pectin gels, an oscillating stress is applied and the viscoelastic parameters are determined, by comparing the stress with the resulting oscillating strain. This principle is illustrated in Figure **2.15** (Rao, 2007). When a sinusoidal stress wave is applied to a perfect *elastic solid*, the resulting oscillating strain is exactly *in phase* with the stress. In contrast, for a purely *viscous liquid,* the strain is 90º *out of phase* with the stress. Therefore, due to the viscoelastic response of gels, there is a *phase shift* (δ) between stress and strain

($0^o < \delta < 90^o$), whose value depends on the relative amounts of elastic and viscous behaviour, at the frequency of oscillation. The total resistance of the sample, against the applied stress is represented by the *shear complex modulus G**, which is defined as the ratio of the applied shear stress to shear strain ($G^* = \tau_o / \gamma_o$). The measured complex shear modulus can be treated as a vector quantity ($G^* = G' + iG''$) and decomposed into its two components, in terms of trigonometric functions. These are the in-phase component *G'*, known as the *elastic* or *storage* modulus (Equation **2.16**) which reflects the solid-like properties of a gel and the out-of-phase component *G''*, called the *viscous* or *loss* modulus (Equation **2.17**), which relates to the viscous character. Another commonly used dynamic viscoelastic property is the ratio of the two moduli, *G'' / G',* defined as the tangent of the phase angle, or *loss tangent* (Equation **2.18**). Thus, when viscous properties dominate over elastic, tan δ is greater than 1 and the phase angle is greater than 45º. Conversely, when the sample is a gel and the elasticity dominates, phase angles of less than 45º are recorded (Bourne, 2002; Ross-Murphy, 1994).

$$G' = G^* \cos\delta = \tau_0 / \gamma_0 \cos\delta \qquad (2.16)$$

$$G'' = G^* \sin\delta = \tau_0 / \gamma_0 \sin\delta \qquad (2.17)$$

$$\tan\delta = \frac{G''}{G'} \qquad (2.18)$$

Therefore, if *G*' value is much greater than *G''*, the material will behave more like a solid and the deformation will be more elastic, however, when the *G''* value is greater than *G'*, the energy used to deform the material will be dissipated viscously and thus, the material will behave like a liquid (Rao, 2007).

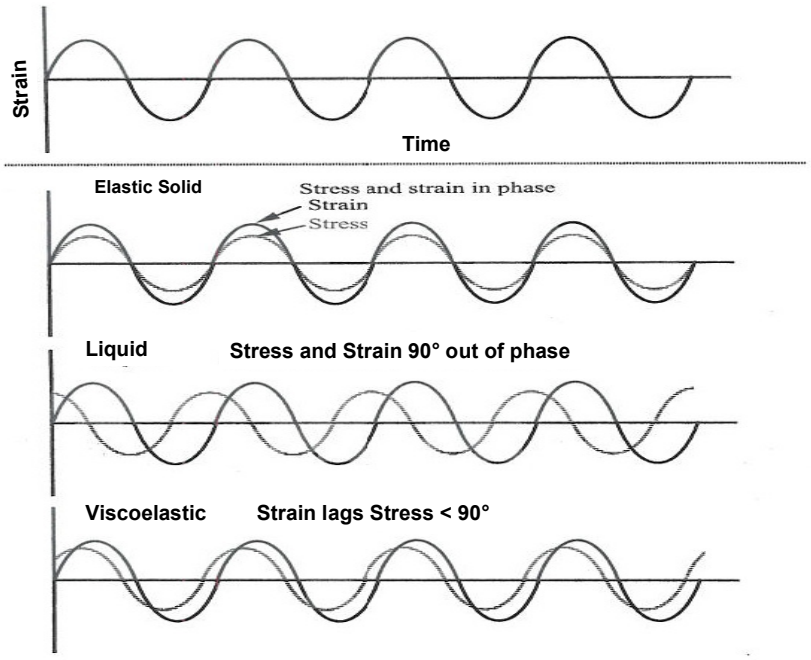


Figure 2.15 Stress versus strain response of a Newtonian liquid, a Viscoelastic liquid and Elastic solid in dynamic test (Rao, 2007).

2.2.4.2.1. Temperature Sweep Test (Small Deformation Test)

In this test, the value of *G'* and *G"* are determined, as a function of temperature at a fixed oscillatory frequency and deformation. In the present work, as described in section **6.2.7**, this test was carried out within the linear viscoelastic region, where the sample undergoes a small deformation, without breaking the gel network. The sample was held at low strain (0.5%) and at a frequency of 1 Hz. This test was chosen, in order to study the gels' characterisation, since it provides information on the gel's formation during cooling, gelation temperature and gelation time. According to Endress, Doschl-Volle and Dengler (1996), the gelation processes are not only temperature dependent, but also time dependent. Generally, gels with higher setting temperature will have a shorter setting time.

2.2.4.2.2 Time Sweep Test (Small Deformation Test)

This type of test is suitable for investigating the structure development/re-arrangements in physical gels, where *G'* and *G"* values are determined as a function of time, at a constant oscillatory frequency, deformation and temperature. In this thesis, the formed gels were held for 13 h, in order to observe their differences, in terms of gel strength (or viscoelastic values achieved). This experiment was conducted at a frequency of 1 Hz, at a constant temperature of 4ºC, and 0.5% strain, within the linear viscoelastic region.

2.2.4.2.3 Amplitude Sweep Test (Large Deformation Test)

The amplitude sweep test is a typical deformation test (from the linear to the non-linear viscoelastic region), where a food sample is subjected to increasing deformation or strain (*i.e.,* up to 1000%), in order to obtain information about its fracture and yielding behaviour, where the non-linear viscoelastic region begins. This is an important mark of quality, affecting aspects, such as (*i*) eating quality; (*ii*) food properties, such as ease of cutting and spreading; (*iii*) handling properties during storage and/or further processing, in connection with, *i.e* shape retention and pumping characteristics (van Vliet, Luyten & Walstra, 1991).

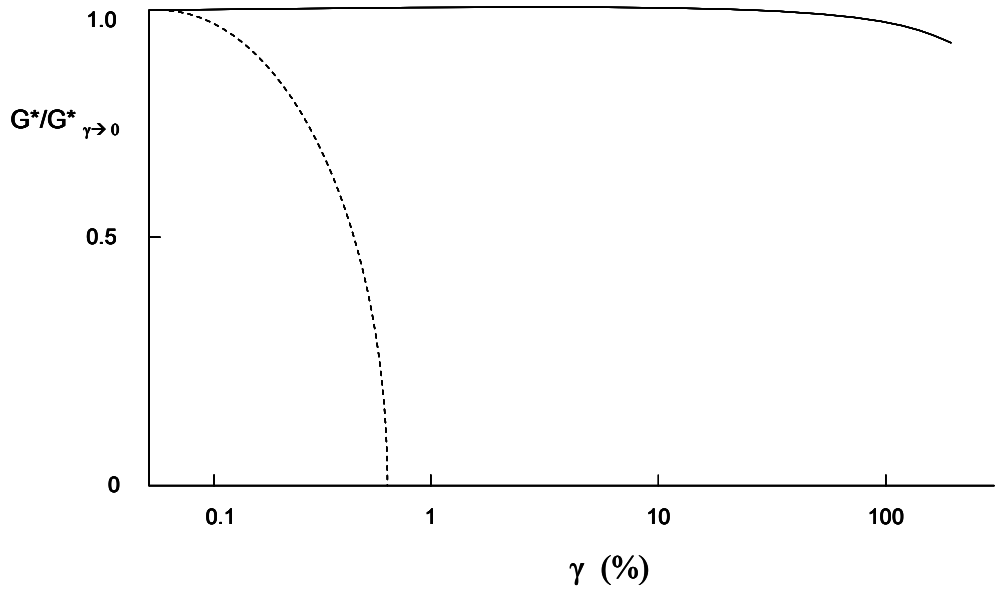


Figure 2.16 Typical modulus strain response for a weak gel (- - -) and strong gel (—) (Ross-Murphy, 1994).

In this study, the resistance of gels to deformation (complex modulus, G^*) was measured. Weak gels or many particle gels, such as yoghurts, are known to have a more pronounced strain dependency and short viscoelastic linear regions. However, if the gels are independent of strain up to 100% or more, they are characterised as strong gels, with a long viscoelastic linear region (Ross-Murphy, 1994). Figure **2.16** illustrates a typical standardised complex modulus response, versus strain for a weak gel (- - -) and a strong gel, or a viscoelastic fluid (—).

2.2.5 Gel Texture Analysis

In order to determine the gel's hardness and fracture properties, a texture profile analysis (TPA) was performed in this study, to characterise the rupture of gold kiwifruit pectin gels, by using a TA-XTplus texture analysis instrument. This test is designed to imitate the masticating action in the human mouth (acting as a jaw). It consists of a unit of plate supported by a flexible arm, which is attached to a strain gauge and a plunger that works on the food sample (Pons & Fiszman, 1996). Figures **2.17a** and **2.17b** show the TA-XTplus texture analysis instrument and a typical curve of a TPA, respectively.

The measurement principle of this instrument is the application of a compression force, which is perpendicular to the food sample in two cycles, as in a double bite. When the sample is compressed at a certain speed and distance, the strain gauge detects the generated force, which is recorded in a computer (Pons & Fiszman, 1996). A plot of force, as a function of time, is created and it can provide information about seven textural parameters: hardness, cohesiveness, springiness, resilience, chewiness, gumminess and adhesiveness. This thesis is concerned with the hardness parameter, which provides information about gel strength and it may correlate to the gel's rheological properties. Hardness is defined by Szczesniak (1963) as "the force needed to attain a given deformation", which is obtained in this case, as the maximum force obtained, during the first compression cycle (first bite).

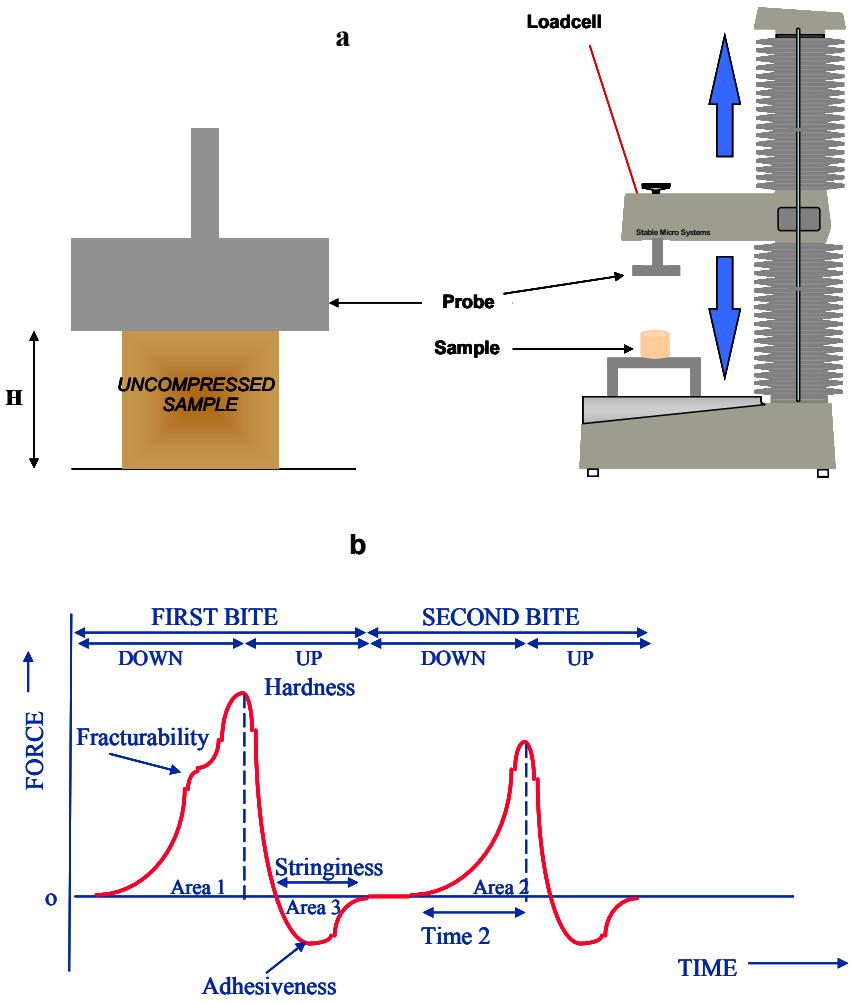


Figure 2.17 (a) TA-XTplus texture analysis instrument and (b) a typical curve of TPA (modified from Stable Micro Systems Ltd, 2005).

The testing conditions of TPA analysis, such as sample size and shape, the extent of deformation and cross head speed, are very important factors, which can influence the results. Pons and Fiszman (1996) reported that, if the tested sample is a gel, the shape and size are normally cubes or cylinders, made by setting the gel into a mould of a standard diameter and height. Also the compression devices are generally larger than the sample size, in order to generally gain the registered forces, due to unaxial compression forces.

The selection of the extent of deformation depends on the purpose of the experiment. In this study, the purpose was to imitate the destruction process of mastication in the mouth, so therefore, the deformation values were aimed to break the gel system, as previously described. Deformation values lower than 50% have been reported by Pons and Fiszman (1996) to be slightly weak and not causing gel breakage. A deformation value of 75% was applied here, for the compression tests (Chapter **6**), in order to observe the different gel hardness, which is generated by various pectins, in different conditions.

CHAPTER 3 Characterisation of Gold Kiwifruit Pectin Isolated by Acid and Water Treatment

3.1 Introduction

The isolation of pectin from fruits and vegetables has received much attention in the last few decades because pectin, which is a hydrocolloid, can impart important physical and functional properties in food systems. Pectin interacts with water and is capable of altering the rheological and textural attributes of food products (Uresti, Lopez-Arias, Ramirez, & Vazquez, 2003). Currently, pectin is extracted from different plant sources including citrus peel (May, 1990; Kurita, Fujiwara, & Yamazaki, 2008), apple pomace (Garna, *et al*., 2007) and sugar beet (Levigne, Ralet, & Thibault, 2002). In recent years, pectin fractions isolated from other fruits and vegetables including banana (Emaga, Ronkart, Robert, Wathelet, & Paquot, 2008b), chicory roots (Panouille, *et al*., 2006) and peach pomace (Pagan, Ibarz, Llorca, Pagan, & Barbosa-Canovas, 2001) have also been studied.

Pectin has been extracted from cell wall materials using water, buffer solutions (*e.g*. sodium acetate buffer) with chelating agents such as ethylene diamino tetraacetic acid (EDTA) or cyclohexane diamino tetraacetic acid (CDTA), hot dilute acids, sodium hydroxide or enzymes (Rombouts & Thibault, 1986; Panouille, *et al*., 2006). On an industrial scale, pectin is usually extracted using hot dilute acid. In cases where chelating agents are used, a residual amount of the chelating agents will remain in the final pectin sample, which may influence its functionality (Garna, *et al*., 2007). Extraction by alkali can cause a decrease in the degree of esterification (DE) and can reduce the length of the pectin chains by *β*-elimination (May, 1990). The addition of ammonia to pectin at low temperature can result in the conversion of some ester groups into amide groups (amidated pectins). The hydrolysis and *β*-elimination pectin with alkali and acid are described briefly in Chapter **1**, section **1.5.3.2.**

The acid extraction method is commonly used because it gives a good yield of pectin. Citric acid, nitric acid or hydrochloric acid of pH 1−3, with the extraction temperature and time ranging from 50 to 100ºC for 0.5 to 10 h, is commonly applied in the pectin extraction process (Wang, *et al*., 2002; Rolin, 2002; Canteri-Schemin, *et al*., 2005). However, the exact length of time and the exact temperature vary with the origin of the materials and the type of pectin desired (May, 1990). Another benefit of using the acid extraction method is the ability of acid to hydrolyse the methyl ester. This means that the DE can be modified to the desired functionality for different food applications.

The conditions used during pectin isolation are critical as they can alter the chemical structure of pectin, which could influence its physical and functional properties. Recent studies on pectin isolation from fruits have indicated that extraction processes that cause a high degree of pectin degradation can lead to a low recovery yield and loss of pectin functionality (Pagan, *et al*., 2001). Extraction time, temperature, pH and ratio of acid solution to the materials being extracted are factors that can greatly affect polysaccharide extraction from cell walls (Norziah, Fang, & Abd Karim, 2000; Virk & Sogi, 2004). These factors have been shown to influence the yield of pectin and its physicochemical properties. In addition, different sources of pectin may be affected differently by the processing conditions. Therefore, the extraction regime (temperature, pH and time) used should be properly selected and evaluated.

In this current study, the isolation of pectin from gold kiwifruit by acid- and water-based methods, and the extraction conditions (time and temperature) and the ratio of puree to extraction solvent were investigated. The overall objective was to identify the conditions that could be employed in the commercialisation of kiwifruit pectin. To that end, the effects of extraction methods and extraction conditions were explored and compared in terms of the yield of pectin, the composition of the polysaccharide fraction and the flow properties of the pectin in solution.

3.2 Materials and Methods

3.2.1 Whole Kiwifruit Puree Preparation

The gold kiwifruit (cv. Hort16A) used in this study was obtained from Zespri International Ltd (Hastings, New Zealand). The fruit was main-harvested fruit (main season fruit, approximately 20 weeks after pollination), with 3.3 kgf firmness, supplied from the store of a commercial processing plant in July 2007. The fresh fruit was analysed for its total soluble solids, protein, ash and moisture contents, dry matter, galacturonic acid (GalA) concentration, total non-starch polysaccharide (total-NSP) composition and neutral sugar composition as described in section **3.2.3**. The fruit specification is presented in Table **3.1**. The fruit was sorted (e.g. bruises) immediately after reaching Massey University and was stored at −20°C until required for analysis. It is noted that there is some possibility that disruption of the cell wall through freezing may have influenced the extraction behaviour; however, this was an effective method of retarding biochemical changes in the fruit as a result of the ripening process during storage. Prior to extraction, the gold kiwifruit was thawed for approximately 18 h at 4°C. The puree (Figure **3.1**) was obtained by homogenising 5 kg of randomly selected fruit for 2 min in a food processor (Compact 3100 Automatic Multicuve Magimix, France) at room temperature. The skin and seeds of the fruit were included in the puree preparation. The pH of the puree ranged from 3.5 to 3.6.

Figure 3.1 Gold kiwifruit puree (prepared by homogenising the fruit for 2 min).

3.2.2 Methodology for Pectin Isolation from Gold Kiwifruit by Acid and Water Methods

In the preliminary stages, isolation of the pectin was carried out by extraction using different acids (0.05 M hydrochloric acid (HCl) and 1.0% w/v citric acid (CA)), times (30 and 60 min), temperatures (50 and 90°C), extraction stages (the puree was subjected to three extraction stages) and puree to solvent ratios (1:1, 1:2, 1:3, 1:4 and 1:6 w/v). These factors were studied to determine their effects on the yield of pectin, its composition and its rheological properties. The viscosity of the pectin solutions was the key parameter employed in evaluating the functionality of the pectin. The conditions that gave isolated pectin with the highest viscosity were further investigated in the subsequent stage of evaluation. The viscosity parameter was selected as it is commonly used to infer the physicochemical properties of pectin (Bourne, 2002). From the preliminary study, 1.0% CA at 25 and 50°C for 30 and 60 min, an extraction puree to solvent ratio of 1:2 w/v and a one-stage extraction process were the extraction conditions selected for further investigation.

The use of water (instead of CA) as a solvent for the extraction method was also evaluated in the preliminary study. The conditions investigated were different extraction times (30 and 60 min), temperatures (50 and 90°C) and puree to water ratios (1:1, 1:2, 1:3, 1:4 and 1:6 w/v) and three extraction stages. From the preliminary study, extraction temperatures of 25 and 50°C for 30 and 60 min, a puree to solvent ratio of 1:2 w/v and a one-stage extraction process were the conditions selected for further investigation.

3.2.2.1 Evaluation of Extraction Methods, Times and Temperatures Using Acid and Water

Gold kiwifruit puree was extracted using either 1.0% (w/v) (pH 2.2 ± 0.01) CA (Hawkins Watts Ltd, New Zealand) solution or water, according to a method slightly modified from that of Virk and Sogi (2004). The CA solution was prepared by diluting 1 g of CA into 100 mL of deionised water (RO water). The puree to solvent ratio used was 1:2 (w/v). The pH values were ~ 3.0 and ~ 3.6 for the puree mixtures in CA solution and water respectively. The extraction was carried out in a temperature-controlled water bath. The temperatures

and times of extraction were 25 and 50°C for 30 and 60 min. Immediately after extraction, the mixture was cooled rapidly to approximately 3−4ºC by immersion in a bath of crushed ice for 20 min and centrifuged (Centra, MP4R, rotor 224, International Equipment Company, USA) at 3310 *g* at 4°C for 20 min. Because of the viscous nature of the extract, some insoluble particles remained in the supernatant. The supernatant was filtered through four layers of cheese cloth to remove the fine insoluble particles that remained. Water (at either 25 or 50°C) was added to the pellet at a ratio of 1:1 (w/v) and was stirred for 30 min to recover any soluble polysaccharide fractions that had been trapped in the pellet. The mixture was centrifuged again as before. All supernatants and filtrates were combined and precipitated with ethanol as is commonly carried out in pectin production (May, 1990). Figures **3.2a** and **3.2b** show the gold kiwifruit extract after filtration and after its precipitation with ethanol.

Ethanol Precipitation. Ethanol (95%) was added to the supernatant in a 5.3:1 (v/v) ratio to achieve 80% v/v ethanol. This concentration was chosen because monosaccharides and oligosaccharides have been reported to be soluble in 80% ethanol, whereas polysaccharides have been reported to be insoluble (Englyst, *et al*., 1992). The mixture of ethanol and extract was stirred using a magnetic stirrer for about 10 min at room temperature to obtain a uniform mixture (Canteri-Schemin, *et al*., 2005) and was then kept at 4°C (Li, Ding, & Ding, 2007) for 4 h to allow the polysaccharides to precipitate (Faravash & Ashtiani, 2007).

Drying the Ethanol-precipitated Pectin. To separate the polysaccharide precipitate from the solvent, the mixture was centrifuged (3310 *g*, 10 min, 4°C). The pellet was washed twice with 95% ethanol (1:1, w/v) and then centrifuged again as before. The pellet from the ethanol precipitation was vacuum dried (Eyela, Vacuum Oven, Voc-300 SD, Science Technique Ltd, New Zealand) at 58 ± 3°C, 65 cm Hg, for approximately 7−10 h until a constant weight was achieved. The vacuum-dried sample was ground and was identified as ***crude pectin I*** (see Figure **3.3a**). The vacuum-dried sample was dispersed in Milli-Q water (1.0% w/w) and stirred overnight (~ 15 h) in a 4°C chiller. The dispersion was then centrifuged at 30,000 *g* for 60 min (4°C) to separate out the insoluble fraction. The supernatant was freeze dried (FD18, Cuddon, Blenheim, New Zealand) for 3 days and the

recovered fraction was identified as either ***acid-extracted*** or ***water-extracted crude pectin*** (see Figure **3.3b**). The amount of crude pectin recovered was used to calculate the yield, as described by Ptitchkina *et al.* (2008) (see Equation **3.1**). All extraction experiments in this study were performed in duplicate. The extraction procedures are summarised as a flow diagram in Figure **3.4**.

$$D = 100 \left(\frac{m_{pectin}}{m_{kp}} \right) \tag{3.1}$$

where D is the percentage yield of purified pectin (%), m_{pectin} is the mass of recovered pectin (g) and m_{kp} is the mass of kiwifruit puree (g) used in the extraction.

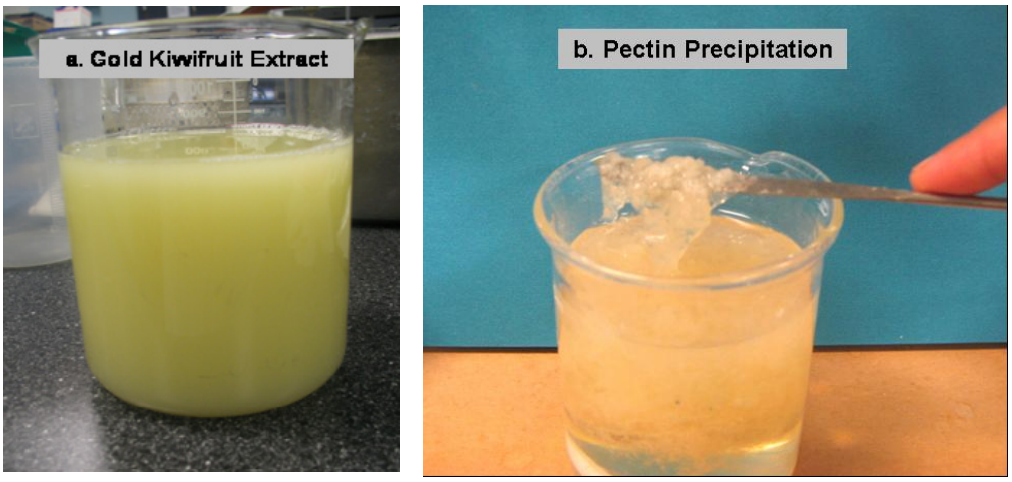


Figure 3.2 Extraction and isolation of gold kiwifruit pectin using an acid extraction method (50°C for 60 min, puree:acid solution ratio 1:2 (w/v)). (a) extract after centrifugation and filtration; (b) pectin precipitated using ethanol.

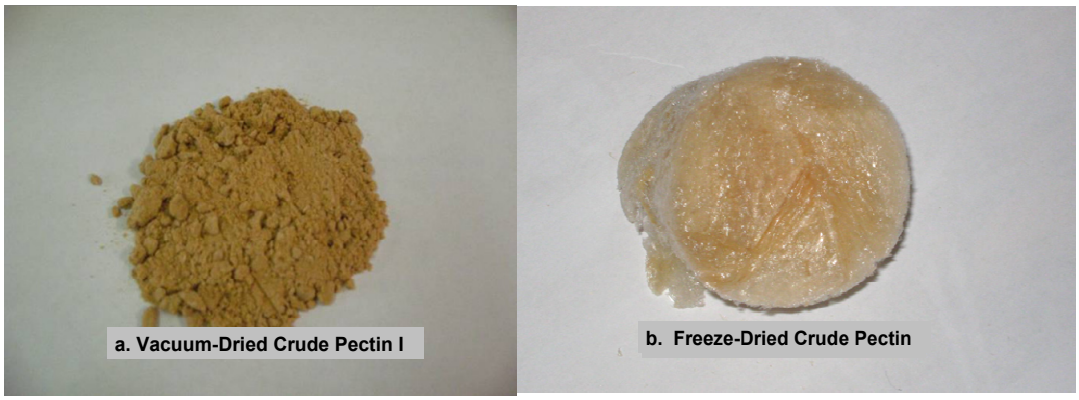


Figure 3.3 Pectin sample obtained by acid extraction at 50°C for 60 min, puree:acid solvent ratio 1:2 (w/v). The sample was (a) vacuum dried and (b) re-dispersed in water, centrifuged and freeze dried.

3.2.3 Chemical Analysis of Isolated Pectin Fractions

The recovered freeze-dried crude pectin was evaluated for its yield, total-NSP, neutral sugar compositions, GalA concentration, protein and ash contents and viscosity. All analyses were carried out at least in duplicate.

3.2.3.1 Total Soluble Solids (TSS) Determination

The TSS content of the gold kiwifruit was determined by placing approximately 0.5 mL of the fresh kiwifruit juice in a digital refractometer (RFM330 Refractometer, Bellingham and Stanley Ltd, UK). The measurement was carried out immediately on receipt of the fruit at Massey University, before being frozen for storage.

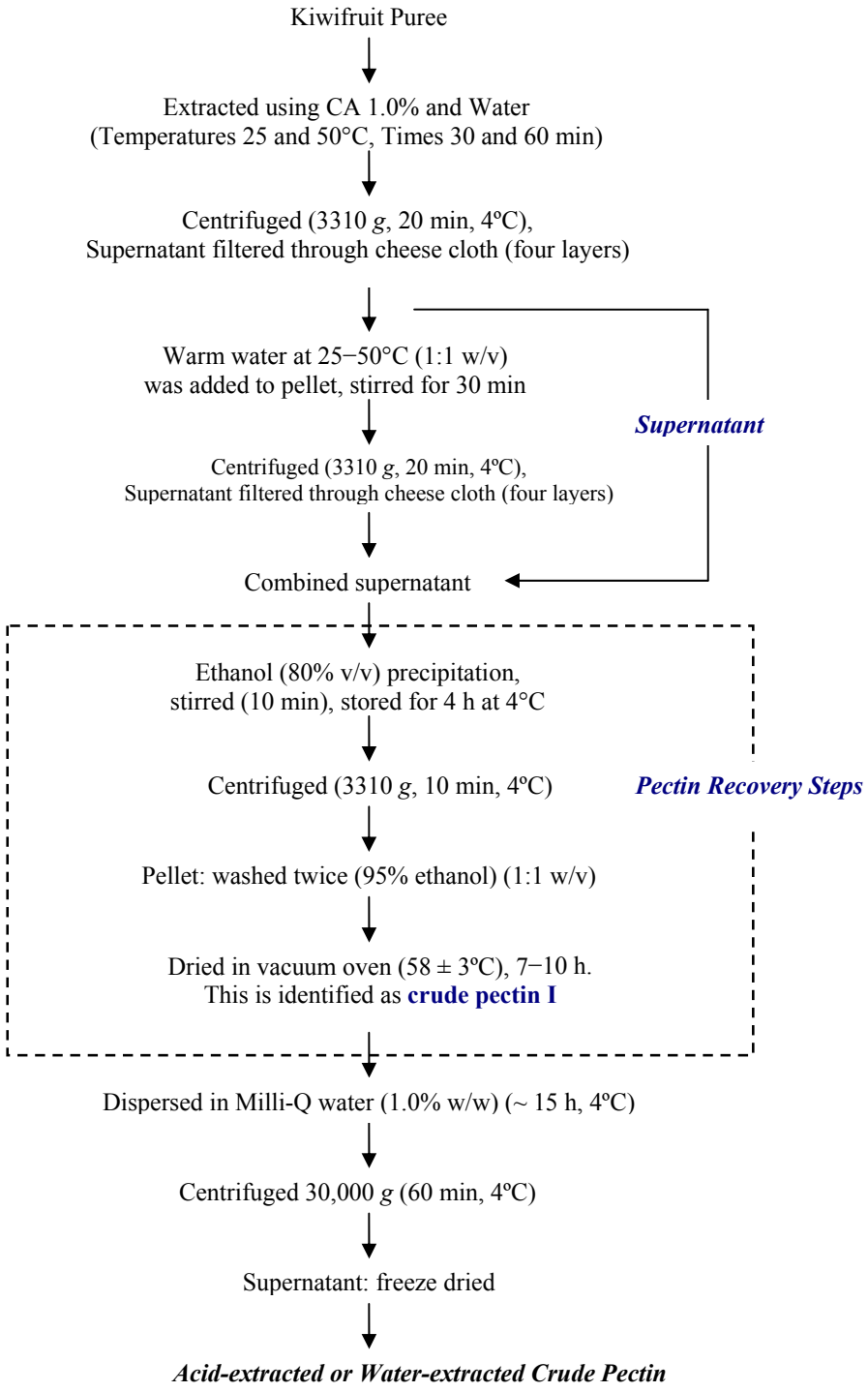


Figure 3.4 Flow chart of 1.0% CA or water extraction methods.

3.2.3.2 Dry Matter and Moisture Determination

The dry matter (DM) content was measured by calculating the weight difference after drying the sample, following AOAC method no. 964.22 (AOAC, 1990). Kiwifruit puree (2 g) was weighed into a dry pre-weighed aluminium dish, and the dish was then placed in an oven (Contherm Oven 240V, Auckland, New Zealand) at 105ºC for 4 h. After drying, the sample was cooled in a desiccator for approximately 2 h. The DM content was calculated as shown in Equation **3.2**. The moisture content was determined as 100−% DM.

$$\%DM = \frac{(m_{ds} + m_{ad}) - m_{ad}}{(m_{fs} + m_{ad}) - m_{ad}} \times 100 \qquad (3.2)$$

where m_{ds} is the mass of dry sample, m_{ad} is the mass of the aluminium dish and m_{fs} is the mass of fresh sample.

3.2.3.3 Ash Content Determination

Ash was analysed gravimetrically according to AOAC method no. 945.46 (AOAC, 1990). Two grams of freeze-dried whole kiwifruit was weighed in a crucible and placed in a muffle furnace (500ºC) for about 17 h. The sample was weighed after 2 h in a desiccator. The ash content was expressed on a % dry weight basis (dwb), as shown in Equation **3.3**:

$$\%Ash = \frac{(m_a + m_c) - m_c}{(m_{fs} + m_c) - m_c} \times 100 \qquad (3.3)$$

where m_a is the mass of ash, m_c is the mass of the crucible and m_{fs} is the mass of fresh sample.

3.2.3.4 Crude Protein Content Determination

The crude protein content was determined on the basis of the nitrogen content (%N x 5.18) by the Kjeldahl method, *i.e.* AOAC method no. 954.01 (AOAC, 1990). To verify the results, the sample was also sent to the Nutrition Laboratory, Massey University, Palmerston North. A freeze-dried whole kiwifruit sample (0.75 g) was placed in a digestion tube with 15 mL of concentrated sulphuric acid (H_2SO_4) and two Kjeltabs (each containing 3.5 g of K_2SO_4 and 0.0035 g of selenium as metallic catalyst). The Kjeldahl digestion was carried out at 420ºC for 1.5 h until the sample had been completely decomposed, as indicated by a clear/colourless solution. As the tube was cooling, 70 mL of hot water was added and thoroughly mixed; the solution was then distilled in a Foss Tecator Kjeltec apparatus (Denmark) with 6 mL of 0.1 M sodium hydroxide (NaOH), which converts ammonium salts to ammonia, in the presence of 25 mL of 4% boric acid solution. The amount of ammonium nitrogen present in the sample was determined by titration using 0.1 M HCl. For analysis of the protein content of the fruit, a correction factor of 5.18 was used; as most fruit protein consists of approximately 19.31% nitrogen, the factor used for converting nitrogen to protein should be 100/19.31 or 5.18 (Sikorski & Piotrowska, 2007). The percentage of nitrogen (%N) and hence the protein content were calculated using Equations **3.4** and **3.5** respectively, where 14.01 is the atomic weight of nitrogen, V_{HCl} is the volume of HCl used (mL), C_{HCl} is the concentration of HCl and S_w is the sample weight (g). The percentage was expressed on a dwb.

$$\%N = \frac{(C_{HCl} \times V_{HCl}) \times 14.01 \times 100}{1000 \times S_w} \tag{3.4}$$

$$\%\ Protein = \%N \text{ x } 5.18 \tag{3.5}$$

3.2.3.5 Total Starch Concentration Determination

The total starch content of the kiwifruit was analysed enzymatically using a Megazyme Assay Kit (Megazyme, Co. Wicklow, Ireland), based on the heat-stable α-amylase and amyloglucosidase method (McCleary, Gibson, & Mugford, 1997). The kit information and reagent preparations are shown in Appendix **A.1–A.2**. In this method, starch was hydrolysed in two phases:

Phase 1. In this phase, starch was hydrolysed to maltodextrins. The freeze-dried whole kiwifruit sample (~ 100 mg) was pre-treated with 0.2 mL of 80% (v/v) ethanol and 2 mL of dimethyl sulphoxide (DMSO). The sample was then treated with 3 mL of heat-stable α-amylase solution, and placed in a boiling water bath (100ºC) for 6 min (the mixture was vortex mixed at 2 and 4 min during incubation).

Phase 2. In this phase, the maltodextrin was hydrolysed to glucose by the addition of amyloglucosidase. In this step, 3 mL of 0.2 M sodium acetate buffer (pH 4.5) and 0.1 mL of amyloglucosidase were added to the (~ 5.3 mL) sample from phase 1. The sample was incubated for 30 min at 50°C, then transferred to a 100 mL volumetric flask and diluted with ~ 91.6 mL of RO water. The sample was centrifuged at 1500 *g* for 10 min. A 0.1 mL aliquot of the sample supernatant, a blank and a sugar standard were each placed in a clean tube. To determine the amount of glucose, 3.0 mL of GOPOD reagent buffer was added to each tube and the tubes were incubated at 50ºC for 20 min. The blank was prepared from 0.1 mL of RO water with 3.0 mL of GOPOD reagent buffer, whereas the sugar standard consisted of 0.1 mL of glucose standard solution and 3.0 mL of GOPOD reagent buffer. GOPOD is the commercial name of the enzyme buffer solution that is specific for glucose analysis. The composition of the GOPOD buffer is shown in Appendix **A.1**. GO stands for glucose oxidase, PO stands for peroxidase and D stands for a colour reagent.

The sample absorbance was read against the blank at 510 nm using an ultraviolet (UV)-160A spectrophotometer. Total starch was based on Equation **3.6**, where ΔA is the sample absorbance, F is the conversion from absorbance to μg (D-glucose in μg) (Equation **3.7**), FV

is the final volume, 100 is based on 100 mL of sample volume, 0.1 is the volume of sample analysed, 1/1000 is the conversion from μg to mg, 100/W is a factor to express "starch" as a percentage of flour weight, 162/180 is an adjustment from free D-glucose to anhydro D-glucose and ΔD is the absorbance for 100 μg of D-glucose. The percentage was expressed on a dwb.

$$\%\ Starch = \Delta A \text{ x } F \text{ x } \frac{FV}{0.1} \text{ x } \frac{1}{1000} \text{ x } \frac{100}{W} \text{ x } \frac{162}{180} \qquad (3.6)$$

$$F = \frac{100 \quad (\mu\text{g of D-glucose})}{\Delta D} \qquad (3.7)$$

3.2.3.6 Total-NSP Composition, Neutral Sugar Composition and GalA Concentration Determination

The neutral sugar composition was determined by gas liquid chromatography (GLC, BPX-70 column), in the form of alditol acetate derivatives as described by Englyst *et al*. (1994). This method was carried out using an Englyst Kit for NSP determination (Englyst Carbohydrate Ltd, UK). To verify the results, the samples were sent to Englyst Carbohydrate Ltd, UK. Unless otherwise stated, the analyses of total-NSP composition, neutral sugar composition and GalA concentration shown in this chapter were performed by Englyst Carbohydrate Ltd, UK. Information on the Englyst Kit and the reagent preparations are given in Appendix **A.3–A.4**. The procedures followed are described briefly below.

1. Dispersion and Starch Hydrolysis

Enzyme Solution I: 2.5 mL of heat-stable α-amylase was made up to 200 mL with 0.1 M sodium acetate buffer (pH 5.2) and was maintained at 50ºC in a water bath.

Enzyme Solution II: 1.2 g of pancreatin was dissolved in 12 mL of RO water, and then vortex mixed and stirred magnetically and continuously for 10 min. The solution was centrifuged at 50 *g* for 10 min. Ten millilitres of the supernatant was taken from the centrifuged mixture and was mixed with 2.5 mL of pullulanase. This solution was kept at room temperature.

Each freeze-dried pectin sample (60 mg) was dispersed in 2 mL of DMSO. A blank (without sample) was also included and was treated identically to the other samples. The mixture was vortex mixed for 5 min to ensure that it was uniformly dispersed (*i.e.* showed no lumps) and then placed in a boiling water bath (100ºC). It was vortex mixed after 20 s and returned to the boiling water bath for 30 min. Eight millilitres of enzyme solution I was added to the sample and mixed thoroughly and the sample was then placed back in a boiling water bath for 10 min. The sample was transferred to a 50ºC water bath and, after 3 min, 0.5 mL of enzyme solution II was added. The sample was mixed and left in a water bath for 30 min with continuous mixing at 10, 20 and 30 min; then, as the final step, it was transferred to a boiling water bath for 10 min. For total-NSP measurement, the sample was treated as described in step **2**, whereas, for insoluble-NSP measurement, the sample was treated as described in step **3**.

2. Precipitation and Washing of the Residue for Total-NSP Measurement

From the hot water bath, the sample was placed in a bath of crushed ice for 10 min. This was followed by the addition of 0.15 mL of 5 M HCl and the mixture was vortex mixed. Then 40 mL of acidified absolute ethanol (acidified by adding 1 mL of 5 M HCl to 1 L of absolute ethanol, pH ~ 1.5) was added and mixed, and the sample was left in an ice bath for 30 min to flocculate the precipitate. The sample was centrifuged at 1500 *g* for 10 min at 20°C.

The supernatant was discarded and 10 mL of acidified 80% ethanol (acidified by adding 1 mL of 5 M HCl to 1 L of 80% ethanol, pH ~ 2.3) was added to the residue and mixed, and the volume was made up to 50 mL with acidified 80% ethanol. The mixture was mixed thoroughly and centrifuged as before. The washing steps were repeated using 50 mL of absolute ethanol. At the final stage of washing, 30 mL of acetone was added to the sample residue, mixed and centrifuged as before. To remove the remaining acetone, the sample was placed and swirled in a 75ºC water bath. The final acetone-washed powder was considered to be NSP.

3. Extraction and Washing of the Residue for Insoluble-NSP Measurement

Insoluble-NSP determination was conducted after the starch hydrolysis step (step **1**). To each sample (~ 10.5 mL), 40 mL of sodium phosphate buffer (0.2 M, pH 7) was added, and the sample was placed in a boiling water bath for 30 min. It was mixed thoroughly during this period, and then cooled to room temperature by placing into a 20°C water bath for 5 min. The sample was centrifuged at 1500 *g* for 10 min. The supernatant was discarded and the pellet was re-dispersed in 50 mL of RO water and centrifuged as before. Similar washing steps using absolute ethanol and acetone were carried out as for the above procedure for total-NSP to obtain a final residue, which was considered to be insoluble NSP.

4. Acid Hydrolysis of Residues

The following step was applied to the total and insoluble NSP fractions. This step was designed to hydrolyse the recovered NSP fractions to their sugar constituents. To each of the NSP samples, 5 mL of 12 M H_2SO_4 was added and was mixed to disperse the sample. In order to disperse the cellulose, the sample was placed in a 35ºC water bath (30 min) and was mixed vigorously at 5, 10 and 20 min intervals to disperse it uniformly, facilitating cellulose hydrolysis. Then 25 mL of RO water was added to dilute the sample concentration to 1 M H_2SO_4 and the sample was further placed in a boiling water bath for 1 h. The sample was then removed and cooled to room temperature by placing into a 20ºC water bath.

5. Determination of Constituent Sugars by GLC

For GLC measurement of the sugars, the sample was prepared in the form of alditol acetate derivative compounds. As a reference and for calibration, a standard sugar mixture (SSM) was prepared by mixing 1.0 mL of GLC stock sugar solution and 5 mL of 2.4 M H_2SO_4. The preparation of alditol acetate derivatives consisted of two reaction steps: reduction of monosaccharides to alditols; and acetylation of alditols as briefly described in Chapter **2**, section **2.1.1.**

Reduction. The sample aliquot (1.0 mL) obtained from the acid hydrolysis (step **4)** and 1.0 mL of SSM were mixed with 0.50 mL of GLC internal standard (IS) (which contained 1 mg/mL of allose). The sample was placed in an ice bath and 0.4 mL of 12 M ammonium solution was added to neutralise it, followed by 0.1 mL of freshly prepared ammonium-sodium borohydride ($NaBH_4$) solution. The sample was then placed in a 40ºC water bath (30 min) and 0.2 mL of glacial acetic acid was added to eliminate residual borohydride.

Acetylation. The aliquot from the reduction process (0.5 mL) was transferred into a 30 mL glass tube and was mixed with 0.5 mL of 1-methylimidazole and 5 mL of acetic anhydride. As the reaction was exothermic, the sample was left for 10 min to cool. This was followed by the addition of absolute ethanol (0.9 mL) to form ethyl acetate, and the sample was left for 5 min. It was then mixed with 10 mL of RO water, and within 5 min, 0.5 mL of bromophenol blue solution (0.04 g/100 mL) was added as a colour indicator. The sample was placed in an ice bath, and 5 mL of 7.5 M potassium hydroxide (KOH) was added; after 5 min, another 5 mL of 7.5 M KOH was added as the final stage and the solution was mixed thoroughly. The sample was left so that the phases could separate (~ 15 min). The upper phase, indicated by a colourless solution, was considered to be alditol acetate derivatives and this solution was transferred to an Eppendorf tube.

6. GLC Conditions

The sample (~ 0.5 μL) was injected manually into a GLC system, Hewlett Packard (HP) 5890 with a BPX-70 wide-bore capillary column (25 m x 0.33 mm internal diameter). The calibration was carried out by running several samples of the SSM to obtain reproducible response factors (RFs). The RF is the response value of each sugar reference obtained by running the SSM through a GLC system; this can be calculated from the areas under the peaks for each alditol acetate compound relative to the area under the peak for alditol acetate. The peaks for these sugars and the RF value of each alditol are shown in Appendix **A.5–A.6**. The results obtained were integrated using HP ChemStation software. The injector temperature was set to 180ºC and the column temperature was 220ºC. Hydrogen with a flow rate of 8 mL/min was used as the carrier gas and the total run time per sample was 8 min.

7. Calculation of Neutral Sugars

The calculation of neutral sugars is shown in Equation **3.8**. The value was expressed as grams of polysaccharide per 100 grams of sample on a dwb.

$$\%Sugar = \frac{A(t) \times W(i) \times 100 \times R(f) \times cv}{A(i) \times W(t)} \tag{3.8}$$

where $A(t)$ and $A(i)$ are the peak areas of the sample and the IS respectively. $W(i)$ is the mass of the IS (15 mg, obtained from 30 mL of total hydrolysates x 0.5 mg allose), $W(t)$ is the mass (mg) of the sample, $R(f)$ is the RF for the individual sugar obtained from the calibration of the SSM and IS and finally, cv is the factor for converting monosaccharide values to polysaccharides (*i.e.* 0.89 for rhamnose and fucose, 0.88 for arabinose and xylose and 0.90 for mannose, glucose and galactose). Total-NSP, soluble-NSP and insoluble-NSP (g/100 g sample) were calculated based on Equation **3.9**.

$$\begin{aligned} Total\text{-}NSP &= T_{ns} + T_{GalA} \\ Insoluble\text{-}NSP &= I_{ns} + I_{GalA} \\ Soluble\text{-}NSP &= T_{nsp} - I_{nsp} \end{aligned} \tag{3.9}$$

where T_{ns} is total neutral sugars, T_{GalA} is total GalA, I_{ns} is insoluble neutral sugars, I_{GalA} is insoluble GalA, T_{nsp} is total-NSP and I_{nsp} is insoluble-NSP.

8. GalA Concentration Determination

GalA was determined by a colorimetric method using an UV-160A spectrophotometer (Shimadzu, Douglas Scientific, Auckland), in which the difference in absorbance of a sample at 400 nm and at 450 nm was measured against a blank consisting of 2 M H_2SO_4. The method has been described by Scott (1979). In this analysis, the sample (acid hydrolysates, step **4**) was further diluted (1:30 v/v) with 2 M H_2SO_4 to contain no more than 150 μg/mL of GalA. For calibration purposes, the standard sample was prepared by mixing

the GLC SSM in different amounts (0.5, 2.0 and 3.0 mL) into 10 mL of 2 M H_2SO_4, to give standards of 25, 100 and 150 μg/mL of GalA. For test preparation, a 0.3 mL aliquot from each tube (2 M H_2SO_4 as a blank, sugar standards and diluted samples) was mixed with 0.3 mL of sodium chloride−boric acid solution and this was followed by the fast addition of 5 mL of concentrated H_2SO_4 . The mixture was vortex mixed thoroughly to obtain a uniform concentration of H_2SO_4. The sample was then heated in a 70ºC water bath for 40 min. It was cooled for 2 min (by placing in a 20ºC water bath) before adding 0.2 mL of 3-5-dimethylphenol solution. GalA was calculated using Equation **3.10**. The GalA value was expressed as grams of polysaccharide per 100 grams of sample.

$$\%GalA = \frac{A(t) \times V(t) \times D \times C \times 100 \times 0.91}{A(s) \times W(t)} \qquad (3.10)$$

where $A(t)$ is the difference in absorbance of the sample solution, $V(t)$ is the total volume of sample solution (mL, *i.e.* 30 mL), D is the dilution factor, C is the concentration of the standard (0.1 mg/mL), $A(s)$ is the difference in standard absorbance (100 μg/mL), $W(t)$ is the sample mass and 0.91 is the factor for converting monosaccharide values to polysaccharides.

In this study, the degree of branching was calculated by dividing the amount of GalA by the rhamnose concentration (in mol%), as described by Parkar *et al*. (2010).

3.2.3.7 Viscosity Determination

Freeze-dried crude pectin samples (4% w/w) were re-dispersed in fresh Milli-Q water under continuous stirring at room temperature for 15 min, followed by stirring for 15 min at 60ºC. Note that the concentration was not based on the same polysaccharide concentration but on a dwb. The samples were de-gassed in an ultrasonic water bath (for a few seconds only) to release the bubbles in the solution, and the pH was adjusted to 3.5 ± 0.01 by the slow addition of 0.5 M HCl prior to the viscosity measurements. For reference purposes, a

commercial citrus pectin sample (P9135, Sigma-Aldrich, Cheme GmbH, Germany) was analysed at a similar concentration. Viscosity curves were obtained using a controlled-stress rheometer (Paar Physica MCR 301; Anton-Paar, GmbH, Germany) with a cone and plate measuring system (CP 4/40) at 20 ± 0.1°C and at shear rates between 1 and 1000 s^{-1}. Viscosity data at a shear rate of 53 s^{-1} were used for comparative purposes, *i.e.* a shear rate close to that of the mastication process (~ 50 s^{-1}) (Sherman, 1976).

3.2.4 Data Analysis

The experiment was a 2 x 2 x 2 factorial design, where the treatments extraction method (acid and water), temperature (25 and 50°C) and time (30 and 60 min) were combined to evaluate the significance of the main effects (temperature and time) and the interactions. Significant differences among the treatments were determined by Duncan's multiple range test ($P \leq 0.05$). The data were analysed by three-way analysis of variance (ANOVA) using the SAS program (version 9.1).

3.3 Results and Discussion

3.3.1 Gold Kiwifruit Composition

The measured composition of fresh main-harvested fruit is shown in Table **3.1**. The fruit was relatively mature, as indicated by the low starch content (1.50 ± 0.08%) and the high TSS content (16.08 ± 0.10%). In New Zealand, harvest is recommended to start when cv. Hayward (green kiwifruit) has at least 6.2% TSS (Crisosto & Crisosto, 2001) and cv. Hort16A (gold kiwifruit) has a percentage greater than 10% TSS (Patterson, *et al*., 2003). However, for consumption, a TSS content for green kiwifruit of at least 12.5% should be reached for consumer satisfaction. Proximate analysis of the whole fresh kiwifruit demonstrated that it contained 4.24 ± 0.07% protein and 3.94 ± 0.09% ash, which were relatively high compared with the protein and ash contents of fresh apple pomace for pectin production reported by Gullon, Falque, Alonso and Parajo (2007). These authors reported

that the fresh apple pomace by-product from juice manufacture has approximately 1.50% ash and 3.63% protein.

The neutral sugar (particularly arabinose and galactose) and GalA concentrations of the fresh fruit suggested that pectin was the most dominant water-soluble polysaccharide in main-harvested gold kiwifruit. The GalA content (2.87 ± 0.03%), together with the arabinose and galactose contents, indicated a value of approximately 4.03% pectin in this gold kiwifruit. No rhamnose and fucose was detected in this season's kiwifruit. Compared with the GalA concentration (3.55% w/w dwb) in fresh apple fruit (golden delicious) (Lal Kaushal & Sharma, 1995), the gold kiwifruit GalA concentration was slightly lower. The fresh fruit was relatively high in glucose (5.20 ± 0.03%) and xylose (1.73 ± 0.03%), which could indicate the presence of cellulose and hemicellulose as xyloglucan. However, no fucose was detected and only a small amount of mannose (0.29 ± 0.03%) was observed. A similar finding for green kiwifruit cv. Hayward was reported by Gallego and Zarra (1997); xylose and glucose were the dominant sugars at harvest, and xyloglucan was found as part of the hemicellulosic polysaccharide.

Table 3.1 Composition of fresh gold kiwifruit, including the skin, seeds and core.

Component	Concentration
Total soluble solids (%)	16.08 ± 0.10
Dry matter (%)	19.01 ± 0.14
Protein (% dwb)	4.24 ± 0.07
Ash (% dwb)	3.94 ± 0.09
pH	3.50 ± 0.05
Starch (% dwb)	1.50 ± 0.08
Total-NSP (% dwb)	11.07 ± 0.01
Sugar composition of NSP (% dwb)	
Arabinose	0.29 ± 0.03
Xylose	1.73 ± 0.03
Mannose	0.29 ± 0.03
Galactose	0.87 ± 0.03
Glucose	5.20 ± 0.03
GalA	2.87 ± 0.03

Total-NSP and sugar compositions were analysed by Englyst Carbohydrate Ltd, UK.
Mean ± standard error (n = 2).

3.3.2 Effect of Extraction Time and Temperature

3.3.2.1 Crude Pectin Yield

The percentage yield of crude pectin obtained by varying the extraction temperature (25, 50°C) and the extraction time (30, 60 min) and using either acid extraction or water extraction is shown in Table **3.2**. The yield is expressed as w/w on a dry matter basis. There was no significant effect of the extraction conditions (time and temperature) and the extraction methods on the yield of pectin ($P > 0.05$). The percentage yield ranged from 5.31 to 6.70%. Although the yields were not significantly different, further analyses showed that the GalA and total-NSP contents were quite different. This is discussed further in section **3.3.2.2**.

Table 3.2 Effect of extraction method, time and temperature on crude pectin yield (% w/w dwb).

Extraction Method	Temperature (ºC)	Time (min)	Crude Pectin Yield (% w/w)
Acid	25	30	6.68
		60	6.70
	50	30	6.30
		60	6.00
Water	25	30	5.31
		60	5.85
	50	30	6.69
		60	5.87
SEM[1]			**0.344**
Probabilities, *P* ≤			
Extraction Method			NS
Temperature			NS
Time			NS
Extraction Method x Temperature x Time			NS

NS: not significant.
[1]Pooled standard error of mean.
Each value represents the mean of two replicates.

The crude pectin yield obtained in this study was relatively high compared with the pectin yield from fresh apple peel (3.14% w/w dwb) reported by Virk and Sogi (2004) with a similar extraction acidulant (using 1.0% CA, 100°C for 25 min). However, the yield of gold kiwifruit pectin was observed to be lower than that of pectin extracted from dried apple

pomace (9.37% w/w dwb, extracted using 6.2% CA, 95°C for 30 min) reported by Canteri-Schemin *et al*. (2005). It is well established that factors such as the source of materials and the extraction conditions used are responsible for these differences.

3.3.2.2 Total-NSP Composition, Sugar Composition and Protein Content

Table **3.3** shows the total-NSP and monosaccharide compositions of gold kiwifruit pectin extracted using different methods, temperatures and times. The value is expressed as w/w on a dry weight basis. Overall, the total-NSP composition was dominated by GalA, comprising about 94% of the total-NSP. The occurrence of GalA together with rhamnose, galactose and arabinose in all extracts could imply that the soluble polysaccharide fraction was indeed pectin because pectin is reported to consist of a linear chain polymer of α-D-galacturonic acid residues with rhamnose residues inserted into the pectin backbone and with side chains of galactose and arabinose in the form of arabinans, galactans and arabinogalactans (Ridley, *et al*., 2000). Redgwell, Fischer, Kendal and MacRae (1997) reported that arabinogalactans are more likely to form part of a larger pectin molecule of kiwifruit, because the isolated arabinogalactans from green kiwifruit contained approximately 7% GalA. In addition, Parkar *et al*. (2010) revealed that pectin extracted from green kiwifruit cv. Hayward using different solvents (EDTA, monopotassium phosphate, CA and sodium lauryl sulphate) carried side chains at every 25−51 GalA residues, with arabinose and galactose as the dominant sugars.

The GalA concentration ranged from 21.75 to 28.40% based on the pectin dry weight. There was no interaction effect among the extraction methods, times and temperatures for the GalA content ($P > 0.05$). The GalA concentration was influenced mostly by the extraction method ($P < 0.05$). The GalA concentrations in gold kiwifruit pectin were generally lower than those in banana peel pectin (42−69.10% w/w dwb, extracted using water at 60°C for 2 h, Emaga, *et al*., 2008b) and apple pomace pectin (33.40–42.50% w/w dwb, extracted using 5% CA, Marcon, Vriesmann, Wosiacki, Beleski-Carneiro & Petkowicz, 2005). Acid-extracted pectin had lower GalA content (23.59%) than water-extracted pectin (26.54%), which could suggest that the water-extracted pectin was closer to

its native form than the acid-extracted pectin. This was possibly due to degradation of solubilised pectin into small molecular weight fractions by the CA during the extraction process. The smaller molecular weight fractions were not precipitated by ethanol, resulting in the lower GalA concentration from the acid extraction method compared with the water extraction method. Polysaccharides such as pectin are very prone to hydrolytic and non-hydrolytic cleavage. Hydrolysis of the glycosidic bonds joining monosaccharide units can be catalysed by either acid (H^+) or enzymes. The extent of hydrolysis is markedly influenced by the acid strength, the time and temperature of extraction used and the structure of the polysaccharides (BeMiller & Whistler, 1996).

Table 3.3 Effect of extraction method (Mtd.), time and temperature (Tmp.) on crude pectin total-NSP composition and sugar composition (% w/w dwb).

Extraction Method	Tmp. (°C)	Time	Rha	Ara	Xyl	Man	Gal	Glc	GalA	Total -NSP
Acid	25	30	0.20	0.60	0.30	0.25	1.00^{d}	0.95	21.85	25.10
		60	0.20	0.70	0.30	0.15	1.25^{d}	1.10	21.75	25.45
	50	30	0.30	0.95	0.30	0.20	1.70^{c}	1.40	25.65	30.40
		60	0.25	0.80	0.30	0.30	1.65^{c}	1.40	25.10	29.80
Water	25	30	0.60	1.55	0.30	0.15	2.65^{a}	1.65	28.40	35.30
		60	0.40	1.15	0.30	0.15	2.25^{b}	1.40	24.35	30.15
	50	30	0.55	1.60	0.30	0.15	2.50^{ab}	1.70	26.70	33.45
		60	0.55	1.45	0.30	0.05	2.50^{ab}	1.55	26.70	33.00
SEM [1]			**0.059**	**0.106**	**0.000**	**0.043**	**0.112**	**0.196**	**1.476**	**1.777**
Main Effect										
Extraction Mtd.										
Acid			0.24^{b}	0.76^{b}	0.3	0.23^{b}	1.40	1.21^{b}	23.59^{b}	27.69^{b}
Water			0.53^{a}	1.44^{a}	0.3	0.13^{a}	2.48	1.58^{a}	26.54^{a}	32.98^{a}
Tmp. (°C)										
25			0.35	1.00^{a}	0.3	0.18	1.79	1.28	24.09	29.00
50			0.41	1.20^{b}	0.3	0.18	2.09	1.51	26.04	31.66
Time (min)										
30			0.41	1.17	0.3	0.19	1.96	1.43	25.65	31.06
60			0.35	1.03	0.3	0.16	1.91	1.36	24.48	29.60
Probabilities, $P \leq$										
Extraction Mtd.			***	***	NS	*	***	*	*	**
Tmp.			NS	*	NS	NS	**	NS	NS	NS
Time			NS	NS	NS	NS	NS	NS	NS	NS
Extraction Mtd. x Tmp.x Time			NS	NS	NS	NS	*	NS	NS	NS

[a, b, c,d] Means in a column with different superscripts differ significantly ($P < 0.05$).
[1]Pooled standard error of mean. NS: not significant; ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$.
Total-NSP and sugar compositions were analysed by Englyst Carbohydrate Ltd, UK.
Each value represents the mean of two replicates.
Rha: rhamnose; Fuc: fucose; Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; GalA: galacturonic acid.

The results showed no significant difference in the total-NSP content when the extraction temperatures and times were varied. However, water-extracted pectin samples were significantly higher in total-NSP (32.98%) than acid-extracted pectin samples (27.69%) ($P < 0.01$). The GalA content followed a similar trend to the total-NSP content. Water-extracted pectin was also significantly higher in rhamnose (0.53%), arabinose (1.44%) and galactose (2.48%) content than acid-extracted pectin (0.24, 0.76 and 1.40% respectively for rhamnose, arabinose and galactose), possibly due to partial hydrolysis of hairy regions of the pectin, such as arabinogalactans or galactans, under acidic conditions. Garna *et al.* (2007) revealed that arabinofuranosyl linkages (linkages in the arabinan) were the linkages that were most labile to acid hydrolysis, followed by the linkages between rhamnose and GalA, and between GalA residues.

Considering the ratio of the GalA to rhamnose contents (degree of branching), the crude pectin extracted from gold kiwifruit in this study was less branched, carrying side chains every ~ 62 GalA residues, whereas apple pomace (Garna, *et al*., 2007) and sugar beet pectin (Wang & Chang, 1994) carried side chains every ~ 46 and 29 GalA respectively. These differences are probably due to the differences in the composition of the starting raw materials, the extraction methods and conditions used.

Xylose, mannose and glucose were also detected in the crude pectin for both acid- and water-extracted samples. The occurrence of these sugars might indicate that other cell wall polymers such as hemicelluloses and cellulose were also isolated during the extraction of gold kiwifruit. Some studies (Yapo, Robert, Etienne, Wathelet, & Paquot, 2007; Zhao, Liu, & Tu, 2008) have reported a similar finding, *i.e.* the presence of xylose, mannose and glucose in sugar beet pectin pulp. They regarded these sugars as contaminants from hemicellulose and sugar materials.

Table **3.4** shows the protein content of the extracted crude pectin. Interestingly, the protein content of acid-extracted pectin increased with increasing extraction temperature and time. This indicated the tendency of protein to be released from the cell wall networks and/or the fruit cells at a higher temperature and over a longer period of exposure time. A similar

observation was made by Marcon *et al*. (2005) for apple pomace pectin (extracted with 5% CA, protein content 6.0–21.60%), where the protein content was higher under the harsher extraction conditions of 100°C, 80 min (21.60% w/w dwb) than at 75°C, 55 min (6.0% w/w dwb). Protein is often regarded as a contaminant because proteins can affect the functionality of pectin. It has been shown that the protein can be linked to pectin or can exist in free form (Kravtchenko, Voragen, & Pilnik, 1992).

Table 3.4 Protein (% w/w dwb) composition of crude pectin extracted by different methods at different times and temperatures.

Extraction Method	Temperature (ºC)	Time (min)	Protein
Acid	25	30	5.05[g]
		60	5.50[f]
	50	30	7.20[c]
		60	7.40[b]
Water	25	30	7.50[a]
		60	6.30[d]
	50	30	6.00[e]
		60	6.30[d]
SEM[1]			**0.018**
Main Effect			
Extraction Method			
Acid			6.28
Water			6.53
Temperature (ºC)			
25			6.73
50			6.09
Time (min)			
30			6.43
60			6.38
Probabilities, $P \leq$			
Extraction Method			***
Temperature			***
Time			***
Extraction Method x Temperature x Time			***

[a,b,c,d,e,f,g] Means in a column with different superscripts differ significantly ($P < 0.05$).
[1]Pooled standard error of mean. NS: not significant; ***: $P < 0.001$.
Each value represents the mean of two replicates.

3.3.2.3 Viscosity

The viscosity properties of acid-extracted and water-extracted pectins (based on 4% w/w dry weight), extracted at different temperatures and times, are presented in Figure **3.5**. In this figure, the viscosities of both pectins were plotted at a shear rate of 53 s^{-1}, which is close to the shear rate of the mastication process (~ 50 s^{-1}). The pectin viscosity was also plotted as a function of shear rate, and this is shown in Appendix **A.7**. In general, all extracts demonstrated shear thinning behaviour (the viscosity of the pectin solution decreased with increasing shear rate), which is typical behaviour of a pectin solution. This is attributed to individual polymer chains being stretched out and elongated in the direction of flow, therefore having less resistance to flow (Dickinson, 1992). In this study, ANOVA of the effect of extraction techniques and conditions on pectin viscosity showed that water-extracted pectin exhibited higher viscosity ($P < 0.001$) than acid-extracted pectin under similar extraction conditions. For example, at 25°C for 30 min, water-extracted pectin exhibited higher viscosity (~ 63.2 mPa.s) than acid-extracted pectin (~ 31.7 mPa.s). Similar patterns were apparent for the other extraction conditions.

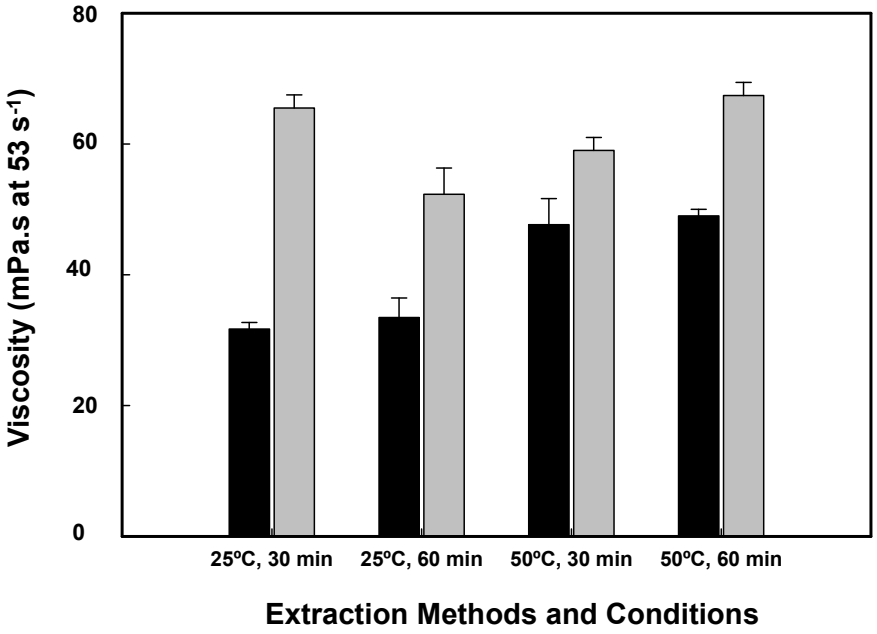


Figure 3.5 Viscosity properties of acid- (black bars) and water-extracted pectin (grey bars) (based on 4% w/w dwb, pH 3.50 ± 0.01) extracted at different temperatures (°C) and times (min). The viscosity was plotted at a shear rate of 53 s^{-1}.

The viscosity of acid-extracted pectin was higher ($P < 0.001$) when the pectin was extracted at a higher temperature (50°C). However, the extraction time did not affect the viscosity. The highest viscosity (~ 55.6 mPa.s) was obtained at an extraction temperature of 50°C, whereas the lowest viscosity (~ 45.3 mPa.s) was obtained at 25°C. A higher temperature appeared to be necessary to increase the solubility of the pectin in the cell wall of the kiwifruit, especially in acid-extracted pectin. The viscosity of gold kiwifruit pectin was relatively low, compared with the viscosity of commercial citrus pectin from Sigma P9135 (680 mPa.s, Figure **3.6a**) at a similar total solids concentration (4% w/w), possibly because the GalA concentration (~ 79.1%) of the commercial citrus pectin was higher than that of gold kiwifruit pectin.

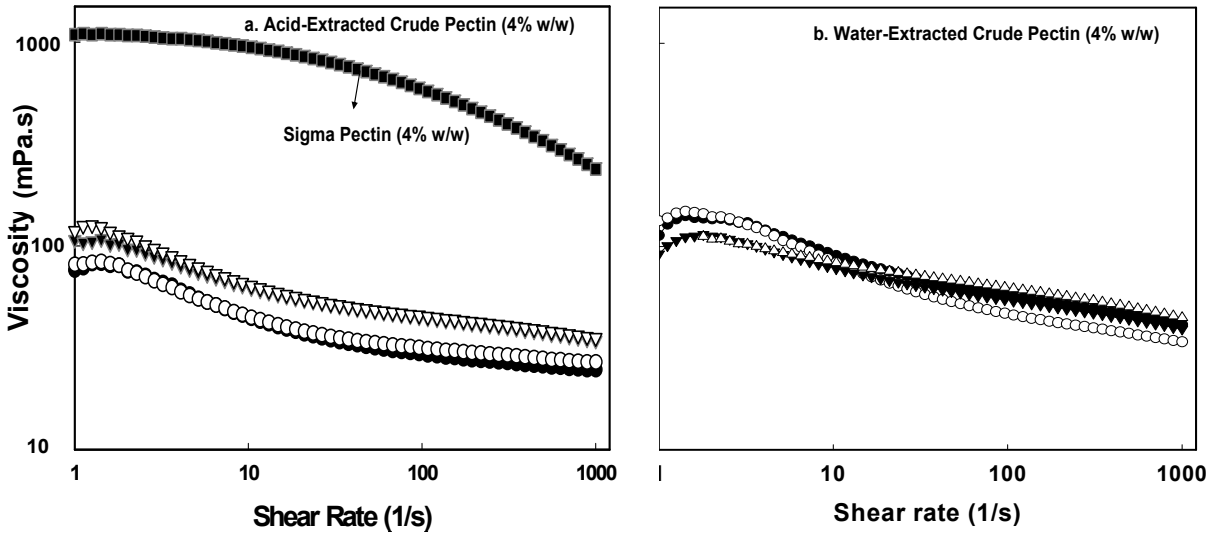


Figure 3.6 Viscosity curves of freeze-dried crude gold kiwifruit pectin solution from (a) acid extraction and (b) water extraction at different temperatures and times: (●) 25°C, 30 min; (○) 25°C, 60 min; (Δ) 50°C, 30 min; (▼) 50°C, 60 min with 1:2 (w/v) puree to water ratio, compared with (■) commercial citrus pectin (Sigma, P9135) at 4% w/w dwb in Milli-Q water, pH 3.50 ± 0.01.

The viscosity appeared to be strongly correlated with the GalA content of the extract (R^2 = 0.86, Appendix **A.8**). This GalA−viscosity correlation was plotted at a shear rate of 53 s^{-1}. In general, water-extracted pectin was notably higher in GalA (26.54%) than acid-extracted pectin (23.59%). This is consistent with the finding of Bourne (2002) that the viscosity of polysaccharides depends on their concentration, where the viscosity increases with

increasing polysaccharide concentration. The higher viscosity value for water-extracted pectin than for acid-extracted pectin could also be attributed to longer pectin chains (hence, higher molar mass) obtained by the water extraction method. A polymer molecule with a longer chain length implies a higher hydrodynamic volume in solution, which results in a higher viscosity. The viscosity data for a study on the effect of the ratio of puree to extraction solution are shown in Appendix **A.9**. The results suggested that the optimum ratios of puree to CA solution and puree to water were 1:6 and 1:4 respectively. Further study to determine the molar mass of pectin isolated using acid and water extraction methods in relation to the viscosity behaviours is described in Chapter **5**.

3.4 Conclusions

The crude water-soluble polysaccharide extracted from gold kiwifruit in this study consisted mainly of GalA. The presence of GalA together with neutral sugars that are characteristic of pectin side chains (rhamnose, arabinose and galactose) confirmed that the soluble polysaccharide fraction was pectin, as commonly found in fruits. There was no significant effect of the extraction methods and the extraction conditions on the yield. However, water-extracted pectin was significantly richer in GalA and neutral sugar content than acid-extracted pectin, indicating that extraction by acid affected the native pectin molecules isolated using water only. The GalA content correlated well with the pectin viscosity, where the pectin viscosity increased with increasing GalA content. Considering the physicochemical characteristics of the pectin, extraction conditions of 50ºC for 60 min with a puree to acid solution ratio 1:6 w/v are conditions that can be recommended for gold kiwifruit pectin extraction by acidic treatment. Extraction conditions of 25ºC for 30 min and a puree to water ratio of 1:4 w/v are recommended for water extraction. Determination of the molar mass of pectin fractions isolated by different methods and its correlation with the rheological behaviours were studied and are discussed in Chapter **5**. In Chapter **4**, further evaluation of pectin isolation using a commercial cellulase preparation is reported.

CHAPTER 4 Characterisation of Pectin Isolated by Enzymatic Treatment

4.1 Introduction

Enzyme preparations have been widely used for optimising the yield of processed fruits and vegetables (Voragen, 1990). For example, enzymes are used for mash treatment in juice manufacture to facilitate the pressing process and to improve the yield of fruit juice (Mihalev, Schieber, Mollov, & Carlet, 2004). In the olive oil industry, enzymes are used for breaking down the fruit polysaccharide cell wall, thus facilitating pressing and increasing the yield of oil (Vierhuis, Korver, Schols, & Voragen, 2003). The use of enzymes to recover certain components in food is considered to be energy efficient. Enzymatic extraction has been shown to achieve higher recovery of pectin than other extraction methods (Panouille, *et al.*, 2006; Ptitchkina, *et al*., 2008). Moreover, enzymatic extraction is regarded as an environmentally safe technique because: (*i*) the effluents produced generally pose fewer problems with respect to treatment and disposal; (*ii*) enzymes are organic materials; (*iii*) low concentrations are used during the treatment of food materials. Unfortunately, enzymatic extraction can be more expensive than other extraction methods, such as acid extraction, which normally uses mineral acids such as hydrochloric acid or nitric acid.

To reduce the cost of manufacture, "crude" commercial enzyme preparations rather than purified enzymes may be used (Panouille, *et al*., 2006). However, crude enzyme preparations always contain non-specific enzymes, which can influence the final properties of the material recovered. Therefore, the selection of commercial enzymes, the method of extraction and the extraction conditions should all be taken into consideration to ensure that the desired properties of the material recovered are intact. An example of such an application is in the recovery of pectin from plants and fruits such as chicory roots, cauliflower, pumpkin and apple. It is important to note that the physicochemical properties

of the pectin recovered may be altered by residual enzymes present in the crude preparations.

The cell walls of many plants consist mainly of hemicelluloses, pectic substances, cellulose and protein. In order to be able to isolate the pectic substances, the cell wall matrix needs to be disrupted. Many studies have shown that the use of different enzymes can improve the recovery of pectin from plant materials. For instance, the combination of cellulase and protease in the isolation of pectin from chicory roots and cauliflower has been studied by Panouille *et al*. (2006). These researchers indicated that this combination was effective in hydrolysing the cellulose and the protein respectively. This aided the release of pectin from the cell wall. However, this application in turn led to modification of the physicochemical properties of the pectin. Ptitchkina *et al*. (2008) studied the recovery of pumpkin pectin and demonstrated that the use of an enzyme (prepared from the fungus *Aspergillus awamori*) with higher cellulase activity (2000 international units (IU)/g) gave higher pectin recovery than enzymes prepared from fungi such as *Bacillus macerans* (0 IU/g) and *Trichoderma viride* (400 IU/g) with lower cellulase activity. However, these enzyme preparations also contained some residual activities such as pectinesterase and polygacturonase, which could alter the physicochemical properties of pectin. Technical information on cellulase is described briefly in Appendix **B.1.**

The extraction of pectin from whole gold kiwifruit using commercial enzymes has not been investigated to date. Therefore, the aim was to study the extraction of pectin from gold kiwifruit puree using commercial enzyme preparations. In this chapter, the effect of extraction time, extraction temperature and the concentration of enzymes on the yield of pectin and the physicochemical properties of the pectin extracted from gold kiwifruit are discussed.

4.2 Materials and Methods

4.2.1 Whole Kiwifruit Puree Preparation

In this study, similar batches of main-harvested gold kiwifruit obtained from Zespri International Ltd (Hastings, New Zealand) and the puree samples described in Chapter **3**, section **3.2.1**, were used. The chemical composition (protein, ash, dry matter, total starch, total non-starch polysaccharide (total-NSP), neutral sugar and galacturonic acid (GalA) concentrations) of the kiwifruit is presented in Chapter **3**, section **3.3.1**.

4.2.2 Preliminary Studies and Evaluation of Enzymatic Activities

In the preliminary studies, three different types of commercial enzyme preparations were used (Celluclast 1.5L, Cellulyve TR 400 and Cytolase CL) to evaluate the isolation of pectin from gold kiwifruit. Detailed specifications for these enzymes are shown in Appendix **B.1**. These enzymes were used because they contain mainly endo-glucanases (note that commercial enzymes are not entirely pure and often possess other enzymatic activities as well). All enzymes were evaluated at different incubation temperatures (25, 40 and 50ºC), as described in the following paragraphs. These temperature ranges were chosen to minimise pectin hydrolysis at high temperature.

The enzyme preparations were added to three commercial polysaccharide samples [carboxymethylcellulose (CMC) (BDH), polygalacturonic acid (PGalA) (Sigma) and arabinan (Sigma)] to determine the cellulase, polygalacturonase (PG) and arabinase (AR) activities respectively. The test was carried out using the method reported by Panouille *et al.* (2006).

In this method, the polysaccharide solutions (CMC, PGalA and arabinan) were prepared by dissolving 10 mg in 1 mL of 0.05 M sodium acetate buffer (pH 3.50 ± 0.01). This pH was chosen because the pH of the gold kiwifruit puree was ~ 3.5. Powdered Cellulyve TR 400 enzyme was prepared by adding 200 mg to 20 mL of sodium acetate buffer to obtain a liquid stock preparation of 1.0% (w/v). No dilution was carried out for the liquid

commercial enzymes. For each test, 450 μL of polysaccharide solution was placed in a test tube and 50 μL of enzyme sample was subsequently added. For the blank sample, 50 μL of sodium acetate buffer was added to the substrate solution to replace the enzyme. The samples (in duplicate) were incubated at 25, 40 or 50ºC for 10 min. In this analysis, the incubation temperature was varied to determine the optimum temperature that gave high cellulase activity but low PG and AR activities.

The reaction of the enzymes on the substrate was halted by heating the samples at 100ºC for 5 min. The quantity of reducing ends released by the enzyme hydrolysis was calculated using the Nelson−Somogyi method (Nelson, 1944) as shown in Appendix **B.2**. Reducing ends are disaccharides or polysaccharides that contain free anomeric carbons at the end of their structure (Garrett & Grisham, 2008). These sugars are easily oxidised by metal ions such as Ag^{+}, Fe^{3+} and Cu^{2+} and form a red-coloured precipitate when they react with Nelson−Somogyi reagents. Standard sugar curves (glucose, GalA and arabinose obtained from Sigma) were also plotted for each polymer (see Appendix **B.3**). The determination of enzyme activities was based on the amount of reducing sugars. The enzyme activity value was expressed in *n*katals, where one *n*katal is defined as the enzymatic degradation of one nmole of substrate per second (Malarczyk, Jarosz-Wilkolazka, & Kochmanska-Rdest, 2003). One IU of enzyme activity corresponds to 16.67 *n*katals.

Equation **4.1** was used to calculate the enzyme activities, where OD_t is the absorbance value of the sample at 520 nm and OD_b is the absorbance value of the blank sample at 520 nm. D is dilution factor (if the enzyme was diluted). T_{MV} is the total mixture volume (μL), E_V is the enzyme volume (μL) and I_{Tm} is the incubation time (s). The value was converted from micromoles to nanomoles by multiplying the value by 1000. The slope (S_{slope}) values were obtained from the standard sugar curves.

$$n\text{katals/mL} = \frac{\left(OD_t - OD_b\right)}{S_{slope}} \text{ x } \frac{T_{MV}}{E_V} \text{ x } \frac{1000}{I_{Tm}} \text{ x } D \qquad (4.1)$$

In this preliminary study, based on the enzyme activity (see Appendix **B.41**), Celluclast 1.5L (Novozymes, Copenhagen, Denmark) and incubation temperatures of 25 and 40ºC were adopted for subsequent extraction trials.

4.2.3 Methodology of Pectin Isolation from Gold Kiwifruit using Enzymatic Treatment

The basic steps used for the isolation of pectin from gold kiwifruit by an enzymatic method were similar to those used for the acid and water techniques (Chapter **3**, section **3.2.2.1**). Celluclast 1.5L was used, based on the results of a preliminary study (see Appendix **B.41** and **B.5**). Two main stages were involved in determining the optimum conditions for enzymatic treatment. The first stage involved the selection of the best time and temperature for pectin extraction using Celluclast 1.5L. The second stage involved investigating the optimum concentration of Celluclast 1.5L. The procedures and the criteria for selecting the extraction conditions are described in the following sections. A flow chart of the enzymatic extraction of gold kiwifruit is shown in Figure **4.1**.

4.2.3.1 Evaluation of Extraction Time and Temperature

This section describes the steps used to investigate the effects of the extraction time and temperature for Celluclast 1.5L based on the yield, total-NSP composition, sugar composition and viscosity of ***enzyme-extracted crude pectin.***

Gold kiwifruit puree (200 g) was mixed with Celluclast 1.5L (medium concentration, 1.05 mL/kg) based on the recommended concentration, as shown in Table **4.1**. The puree samples were heated to 25 and 40ºC before adding Celluclast 1.5L. The mixture (puree plus enzyme) was dispensed into glassware (500 mL), covered with aluminium foil and incubated at 25 and 40°C in a temperature-controlled water bath for 30 and 60 min with continuous stirring. The mixture was immediately cooled to approximately 3−4ºC in a bath of crushed ice for 20 min to slow further enzyme reactions and was then centrifuged (3310 *g*, 20 min, 4°C) (Centra, MP4R, rotor 224, International Equipment Company, USA) to separate the insoluble fraction. Because of the viscous nature of the extract, fine insoluble

particles were observed in the supernatant. The supernatant was filtered through four layers of cheese cloth to remove these particles. The filtrate was kept at 4°C until further analysis. Tepid water (25 or 40°C according to the extraction temperature) was added to the pellet, in the ratio 1:1 (w/v), and was stirred for 30 min to recover the remaining soluble polysaccharides trapped in the pellet. The mixture was centrifuged again as before. All supernatants and filtrates were combined and precipitated with ethanol. The procedures for pectin recovery by ethanol precipitation and the yield calculation (see Equation **3.1**) were similar to those described in Chapter **3**, section **3.2.2.1**. The sample is denoted as ***enzyme-extracted crude pectin.***

4.2.3.2 Evaluation of Enzyme Concentration

This section describes the method used to evaluate the effect of the concentration of Celluclast 1.5L based on the yield, total-NSP composition, sugar composition and viscosity of the ***enzyme-extracted crude pectin***. The extraction was done at 25ºC for 30 min, as developed in section **4.2.3.1.**

The Celluclast 1.5L concentration was varied at three levels (low, medium and high) according to the recommended optimum concentrations, as shown in Table **4.1**. The extraction and the isolation of pectin were carried out based on the same procedures as described earlier in section **4.2.3.1**, except that the temperature used was 25°C and the extraction time was 30 min. This time and this temperature were chosen because these were the conditions under which the pectin obtained exhibited the highest viscosity. The amount of crude pectin recovered was used to calculate the yield, as described by Ptitchkina *et al.* (2008) (see Equation **3.1**).

Table 4.1 Commercial enzyme preparation concentrations

Enzyme Commercial Name	**Celluclast 1.5L** (0.1−2.0 mL/kg*)
Low	0.1
Medium	1.05
High	2.0

*Optimum ranges of concentration (0.1−2.0 mL/kg) were obtained from enzyme technical bulletins.

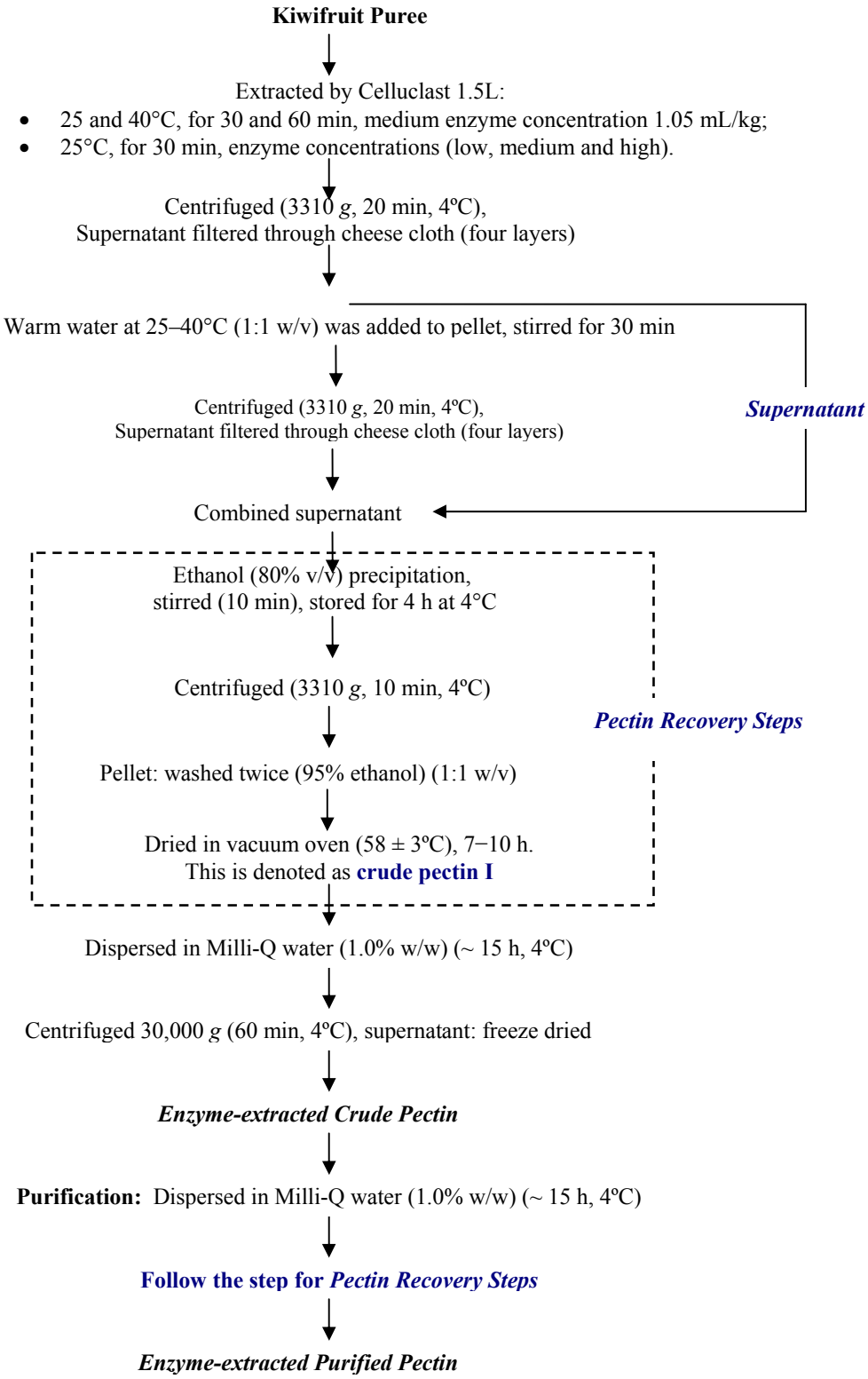


Figure 4.1 Flow chart of enzymatic extraction method for gold kiwifruit.

4.2.3.2.1 Purification

The ***enzyme-extracted crude pectin*** obtained from the evaluation of the enzyme concentration (section **4.2.3.2**) was purified for further pectin characterisation. The purification was carried out by dispersing the crude pectin in Milli-Q water (1.0% w/w) and stirring overnight (~ 15 h) at 4°C. The pectin in the solution was recovered by ethanol precipitation (80%), centrifuged, ethanol washed and vacuum dried, as described in Chapter **3**, section **3.2.2.1** and expressed as ***enzyme-extracted purified pectin***.

4.2.4 Chemical Analyses of Isolated Pectin Fraction

The ***enzyme-extracted crude pectin*** powder was analysed for total-NSP composition, neutral sugar composition, GalA concentration (see section **3.2.3.6**) and viscosity (see section **3.2.3.7**). Because of the limited sample of ***enzyme-extracted purified pectin*** obtained after purification, only the weight-average molecular weight (M_w), polydispersity index (M_w/M_n) and root mean square (RMS) radius were determined. To validate the total-NSP composition, neutral sugar composition and GalA values, some samples were analysed by the accredited commercial laboratory (Englyst Carbohydrate Ltd, UK). All analyses were carried out in duplicate.

4.2.4.1 M_w Determination Using SEC-MALLS

The M_w, M_w/M_n and RMS radius of vacuum-dried purified pectin were determined by size exclusion chromatography (SEC) coupled to a multi-angle laser light scattering (MALLS) system (Mini Dawn, Wyatt Technology Corp., Santa Barbara, CA, USA). This system consisted of a high performance liquid chromatography (HPLC) system (GBC Scientific Equipment Ltd, Victoria, Australia), which comprised an HPLC pump (model LC 1150), an ultraviolet (UV) detector (model LC 1200), a system organiser (model LC 1440) and a differential refractive index (DRI) detector (Waters, model R401, Milford, MA, USA).

The eluant was prepared by dissolving 0.02% w/v sodium azide and 0.1 M sodium chloride (NaCl) in Milli-Q water. The solution was filtered through a 0.22 μm membrane filter (Millipore Corp., Bedford, MA, USA) followed by a 0.025 μm membrane filter (Millipore) and was degassed prior to use. The pectin samples (0.35% w/w) were dispersed in 0.1 M NaCl solution. The samples were filtered through a 0.22 μm filter prior to sample loading. Two commercial polysaccharide samples, namely dextran (Sigma, $M_w \sim 6 \times 10^3$ g/mol) and citrus pectin (Sigma, P9135, unknown M_w) were also prepared for comparative purposes.

Separation of the molecular fraction was accomplished using a Shodex SB-805 column as a size-exclusion column connected to a guard column SB-6 (Shodex, Tokyo, Japan). The eluant was continuously gassed with helium and was pumped through the HPLC system to the SEC column at a flow rate of 0.50 mL/min at 20 psi. The eluant from the SEC column flowed through the UV detector at 280 nm, the MALLS detector and the DRI detector. The pectin sample (~ 100 μL) was loaded into the column through an injection port and was separated at 35ºC over an elution time of approximately 45 min. For the molar mass calculation, the specific refractive index increment (*dn/dc*) of the pectin fraction was determined (section below). The data were analysed using Astra software (version 4.50, Wyatt Technology Corp., Santa Barbara, CA, USA) and the Zimm plot method to determine the M_w, M_w/M_n and RMS radius of the pectin fraction.

4.2.4.2 Specific Refractive Index Increment (*dn/dc*)

NaCl solutions at various concentrations (0.025, 0.0625, 0.125, 0.1875 and 0.25% w/w) were prepared and measured using the DRI detector to obtain a plot of DRI voltage (V) versus NaCl concentration. The measurement was conducted at 35ºC. The DRI response factor (*dV/dc*) was obtained from the gradient of the plot of DRI as a function of NaCl concentration. The gradient (*dV/dc*) was divided by the known *dn/dc* value of NaCl (1.74 mL/g) to obtain *dV/dn*. Different concentrations of purified pectin sample (1, 0.50, 0.33, 0.25 and 0.2% w/w) were prepared by dissolving the pectin (based on GalA concentration) in the same solvent as for the M_w analysis (0.1 M NaCl, 0.02% sodium azide). The slope of the pectin sample (*dV/dc*) was divided by the factor (*dV/dn*) of the calibration slope to obtain the *dn/dc* value of the pectin. Because of the limited quantity of purified pectin

sample, determination of the *dn/dc* value was carried out for pectin extracted using Celluclast 1.5L at medium and low concentrations only. Determination of the *dn/dc* value was carried out in duplicate.

4.2.5 Data Analysis

The experiment in section **4.2.3.1** was based on a 2 x 2 factorial design, where the treatments extraction temperature (25 and 40°C) and extraction time (30 and 60 min) were combined to evaluate the significance of the main effects (temperature and time) or the interaction effect. The experimental design for section **4.2.3.2** was a completely randomised design (CRD), where enzyme concentration was varied (low, medium and high) at constant time (30 min) and temperature (25ºC). The data were analysed by two-way and one-way analysis of variance (ANOVA) respectively for the 2 x 2 and CRD using the SAS program (version 9.1). Significant differences among the treatments were determined by Duncan's multiple range test ($P \leq 0.05$).

4.3 Results and Discussion

4.3.1 Evaluation of Enzymatic Activities

Table **4.2** shows the activities of Celluclast 1.5L at pH 3.5 and temperatures of 25, 40 and 50ºC on three polysaccharides (CMC, PGalA and arabinan) for an incubation time of 10 min. The objective of this evaluation was to determine the best conditions for maximum cellulase activity while the other side activities (PG and AR) were minimised. Some studies have succeeded in isolating pectin using a commercial cellulase preparation at very low PG activities (Matora, *et al.*, 1995; Shkodina, Zeltser, Selivanov, & Ignatov, 1998). For example, the isolation of pectin from sugar beet and pumpkin with a cellulase preparation obtained from *Bacillus polymyxa* was carried out under conditions with minimal PG activity (Matora, *et al*., 1995).

Table 4.2 Activities of Celluclast 1.5L at 25, 40 and 50ºC (pH 3.50 ± 0.01)

Type of Enzymatic Activity	Activity Value (*n*katals/mL)		
	25ºC	40ºC	50ºC
Carboxymethylcellulase	50.81 ± 2.37	63.26 ± 1.44	28.73 ± 2.16
Polygalacturonase (PG)	40.82 ± 6.49	17.51 ± 2.69	91.88 ± 7.20
Arabinase (AR)	42.42 ± 0.24	26.79 ± 2.67	24.28 ± 0.67

Generally, cellulase (represented by carboxymethylcellulase) was observed to be the main activity of Celluclast 1.5L. The cellulase activity varied with temperature (~ 28.73−63.26 *n*katals/mL). It was highest (~ 63 *n*katals/mL) at an incubation temperature of 40ºC and lowest (~ 29 *n*katals/mL) at an incubation temperature of 50ºC. The results also show that Celluclast 1.5L possessed side activities, such as PG and AR, apart from cellulase. The different types and levels of enzymatic activity at different temperatures indicated that the dominant enzymatic activity could be selected by choosing the appropriate incubation temperature. For example, at 25ºC, Celluclast 1.5L showed PG activity of ~ 41 *n*katals/mL and AR activity of ~ 42 *n*katals/mL. However, at 40ºC, the activities of PG and AR were only ~18 and 27 *n*katals/mL respectively.

In the case of PG, the highest activity (~ 92 *n*katals/mL) was observed at 50ºC and the lowest activity was observed at 40ºC (~ 18 *n*katals/mL). In the case of AR, the lowest activity was obtained at 50ºC (~ 24 *n*katals/mL) and the highest activity was obtained at 25ºC (~ 42 *n*katals/mL). In a study conducted by Panouille *et al*. (2006) on Celluclast 1.5L activity, the cellulase (990 *n*katals/mL), PG (4 *n*katals/mL) and AR (1 *n*katal/mL) activity values were quite different. The disagreement in the values could have been due to the incubation pH (pH 4 instead of pH 3.5) because enzymatic activities are dependent on pH as well as temperature. From the overall trend, incubation temperatures of 25 and 40ºC were the conditions chosen for further evaluation of the enzymatic extraction of pectin from gold kiwifruit.

4.3.2 Effect of Extraction Time and Temperature

4.3.2.1 Enzyme-extracted Crude Pectin Yield

Crude pectin was obtained by the treatment of gold kiwifruit puree with Celluclast 1.5L enzyme at different times (30 and 60 min) and temperatures (25 and 40ºC) and a concentration of 1.05 mL/kg. The significant effect ($P < 0.05$) of temperature and time on the yield of crude pectin extracted using Celluclast 1.5L is shown in Table **4.3**.

Table 4.3 Effect of time and temperature on the yield (% w/w dry matter basis) of *enzyme-extracted crude pectin* using Celluclast 1.5L at a concentration of 1.05 mL/kg

Temperature (ºC)	Time (min)	Crude Pectin Yield (% w/w)
25	30	8.08 [b]
	60	7.78 [b]
40	30	8.13 [b]
	60	9.19 [a]
SEM[1]		**0.244**
Main Effect		
Temperature (ºC)		
25		7.93
40		8.66
Time (min)		
30		8.10
60		8.48
Probabilities, $P \leq$		
Temperature		*
Time		NS
Temperature x Time		*

[a,b]Means in a column with different superscripts differ significantly ($P < 0.05$).
[1]Pooled standard error of mean. NS: not significant; *: $P < 0.05$.
Each value represents the mean of two replicates.

The results show a significant interaction effect ($P < 0.05$) between extraction time and extraction temperature on crude pectin yield. The yield ranged from 7.78 to 9.19% based on the dry weight of the whole fruit. The highest yield (~ 9.19%) was obtained for an incubation regime of 40ºC and 60 min. The high yield at 40ºC could have been due to the high cellulase activity (~ 63 *n*katals/mL) and the low PG and AR activities (~ 18 and 27 *n*katals/mL respectively) compared with those at 25ºC (~ 51, 41 and 42 *n*katals/mL for

cellulase, PG and AR respectively). The higher cellulase activity could suggest that more efficient hydrolysis of cellulose occurred, which in turn favoured the release of pectin from the cell wall network.

4.3.2.2 Total-NSP and Sugar Compositions

Table **4.4** shows the total-NSP and sugar compositions of crude pectin extracted using Celluclast 1.5L at different times and temperatures. The value is expressed as w/w on a dry weight basis. In general, GalA was the main sugar component of crude pectin, confirming that the crude water-soluble polysaccharides (WSPs) extracted using Celluclast 1.5L were dominated by pectin. The extracted pectin also contained neutral sugars such as rhamnose, arabinose and galactose. These sugars are believed to form side chains along the GalA backbone. The amounts of GalA ranged from 35.17 to 53.66%; the amounts of galactose ranged from 1.3 to 1.7%; the amounts of arabinose ranged from 0.9 to 1.3%; and the amounts of rhamnose ranged from 0.5 to 0.7%. Other sugars such as fucose, xylose, mannose and glucose were also present but in small amounts (≤ 0.50%). The presence of these sugars could suggest that the crude pectin fraction also contained other polysaccharides (in addition to pectin), possibly arising from the hemicellulose fractions of the cell wall of gold kiwifruit, as mentioned earlier.

There was a significant interaction effect ($P < 0.05$) between extraction time and extraction temperature for GalA content, but not for the neutral sugar side chains such as rhamnose, galactose and arabinose ($P > 0.05$). This could suggest that the pectin side chains (possibly rhamnogalacturonan, galactan and/or arobinogalactan) were not affected by varying the extraction time and the extraction temperature. In addition, the degree of branching (ratio GalA to rhamnose) showed that enzyme-extracted pectin is not too branched; with side chains are inserted every ~ 78 GalA residues.

Increasing the extraction temperature from 25 to 40ºC unexpectedly lowered the GalA content from 53.05 to 44.60%. The GalA content was further reduced to 35.17% at a longer extraction time (60 min). The GalA results appeared to contradict the enzymatic activity

data discussed earlier because the PG activity was previously observed to be lower at 40ºC than at 25ºC. A possible explanation could be that the pectin chains of main-harvested gold kiwifruit were susceptible to hydrolysis at a higher temperature (40ºC) at pH 3.5. The use of an enzyme for extracting pectin could be highly dependent on the pectin structure from different plant sources as well as on the conditions employed during the extraction process.

Table 4.4 Effect of time and temperature on the total-NSP and sugar compositions (% w/w dwb) of *enzyme-extracted crude pectin* using a Celluclast 1.5L concentration of 1.05 mL/kg

Temperature (ºC)	Time (min)	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	Total -NSP
25	30	0.57	0.11	1.25	0.21	0.19[a]	1.68	0.29	53.05[a]	57.34[a]
	60	0.67	0.12	0.94	0.24	0.28[ab]	1.38	0.50	53.66[a]	57.75[a]
40	30	0.57	0.08	1.05	0.16	0.35[b]	1.52	0.43	44.60[b]	48.74[b]
	60	0.52	0.09	0.95	0.17	0.21[b]	1.33	0.46	35.17[c]	38.88[c]
SEM[1]		**0.069**	**0.018**	**0.074**	**0.016**	**0.025**	**0.060**	**0.047**	**1.181**	**1.320**
Main Effect										
Temperature (ºC)										
25		0.62	0.11	1.10	0.22[a]	0.24	1.53	0.39	53.35	57.55
40		0.54	0.08	1.00	0.16[b]	0.28	1.42	0.44	39.89	43.81
Time (min)										
30		0.57	0.09	1.15[a]	0.18	0.27	1.60[a]	0.36	48.83	53.04
60		0.59	0.10	0.94[b]	0.20	0.25	1.35[b]	0.48	44.41	48.31
Probabilities, $P \leq$										
Temperature		NS	NS	NS	*	NS	NS	NS	***	***
Time		NS	NS	*	NS	NS	*	NS	*	*
Temperature x Time		NS	NS	NS	NS	*	NS	NS	*	*

[a,b,c]Means in a column with different superscripts differ significantly ($P < 0.05$).
[1]Pooled standard error of mean. NS: not significant; ***: $P < 0.001$; *: $P < 0.05$.
Each value represents the mean of two replicates.
The total-NSP and sugar compositions were analysed by Englyst Carbohydrate Ltd, UK.
Rha: rhamnose; Fuc: fucose; Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; GalA: galacturonic acid.

The GalA content was observed to be lowest (35.17%) at extraction conditions of 40°C and 60 min, compared with the crude pectin yield (highest, at 9.19%). A higher pectin yield does not necessarily correlate with a high GalA content. In this case, such high recovery

may be related to the presence of contaminants such protein and ash, which could also be isolated during the extraction process of the crude pectin fraction.

4.3.2.3 Viscosity

Figure **4.2** shows a plot of viscosity as a function of shear rate for 4% w/w crude pectin solutions. Pectin extracted at 25ºC for 30 min demonstrated slightly higher viscosity (~ 46 mPa.s at 53 s^{-1}) than the other three pectin samples (~ 32 mPa.s at 53 s^{-1}). No noticeable differences in the viscosity curves were observed for pectin samples extracted at 25ºC (60 min) and at 40ºC (30 and 60 min). The higher viscosity for the sample extracted at 25ºC for 30 min could partially be explained by the high GalA concentration (53.05%). However, the viscosity of the crude pectin sample obtained using an extraction regime of 25ºC for 60 min was lower even though it had a similar GalA concentration (53.66%). In order to explain the results, the molar mass of the pectin is required. The M_w of the extract is discussed in section **4.3.3.4**.

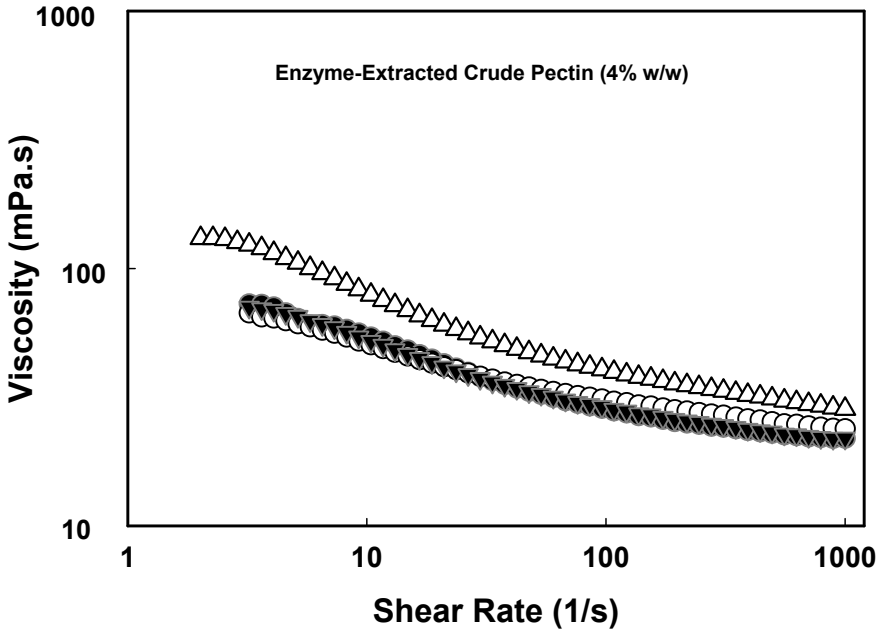


Figure 4.2 Viscosity curves of *enzyme-extracted crude pectin* samples (4% w/w dry weight in Milli-Q water, pH 3.50 ± 0.01). Crude pectin samples were extracted using Celluclast 1.5L (medium enzyme concentration, 1.05 mL/kg) at different temperatures and times: (Δ) 25ºC, 30 min; (○) 25ºC, 60 min; (▼) 40ºC, 30 min; (●) 40ºC, 60 min.

4.3.3 Effect of Enzyme Concentration

4.3.3.1 Enzyme-extracted Crude Pectin Yield

In this section, the effect of enzyme concentration on the yield of pectin is discussed. Table **4.5** shows the yields of crude pectin extracted using three enzyme concentrations at 25°C for 30 min. The yield is expressed as % w/w on a dry matter basis. A significant change ($P < 0.05$) in crude pectin yield was observed when the Celluclast 1.5L concentration was varied. The yield ranged from 6.58 to 8.08%. An enzyme concentration of 1.05 mL/kg showed the highest yield. In contrast, the use of either a low level or a high level of enzyme showed lower pectin yields. The high enzyme concentration used could have resulted in greater pectin hydrolysis whereas the low enzyme concentration could have resulted in a low yield because insufficient enzyme was used. Compared with other plant sources, the yield of pectin from gold kiwifruit was lower than that from dried pumpkin pulp (9−14.0% w/w) and from an alcohol-insoluble residue of chicory roots (~ 34.6% w/w) (Panouille, *et al*., 2006; Ptitchkina, *et al*., 2008). These differences were attributed to the source of the raw material, the purity of the enzyme and the extraction conditions employed.

Table 4.5 Effect of Celluclast 1.5L concentration on the crude pectin yield (% w/w dry matter basis) with an extraction regime of 25°C and 30 min

Commercial Enzyme Concentration	Crude Pectin Yield (% w/w)
Low	6.58[b]
Medium	8.08[a]
High	7.01[b]
SEM [1]	**0.235**
Probability, $P \leq$	*

[a,b]Means in a column with different superscripts differ significantly ($P < 0.05$).
[1]Pooled standard error of mean. *: $P < 0.05$.
Each value represents the mean of two replicates.
Low (0.1 mL/kg), medium (1.05 mL/kg) and high (2.0 mL/kg)

4.3.3.2 Total-NSP and Sugar Compositions

Table **4.6** shows the total-NSP and sugar compositions of the crude pectin extracted from gold kiwifruit using different Celluclast 1.5L concentrations. As described earlier in section **4.3.2.2**, GalA dominated the sugar composition of the extract. There was no significant effect of varying the Celluclast 1.5L concentration on the total-NSP and sugar compositions with the exception of rhamnose and fucose. Rhamnose was significantly influenced ($P < 0.05$) by enzyme concentration. The addition of a high level of Celluclast 1.5L (2 mL/kg) resulted in a lower rhamnose content (0.47%). This could suggest hydrolysis of rhamnogalacturonan chains induced by higher enzyme concentrations.

Table 4.6 Total-NSP and sugar compositions (% w/w dry weight) of crude pectin extracted from gold kiwifruit using Celluclast 1.5L at different enzyme concentrations (25ºC and 30 min)

Enzyme Concentration	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	Total-NSP
Low	0.52[a]	0.21[a]	1.06	0.27	0.12	1.55	0.36	52.43	56.53
Medium	0.57[a]	0.11[b]	1.25	0.21	0.19	1.68	0.29	53.05	57.34
High	0.47[b]	0.16[b]	1.08	0.25	0.16	1.76	0.35	50.4	54.62
SEM[1]	**0.047**	**0.013**	**0.108**	**0.019**	**0.026**	**0.095**	**0.042**	**0.926**	**1.113**
Probabilities, $P \leq$	*	*	NS	NS	NS	NS	NS	NS	NS

[a,b]Means in a column with different superscripts differ significantly ($P < 0.05$).
[1]Pooled standard error of mean. NS: not significant; *: $P < 0.05$.
Each value represents the mean of two replicates.
Rha: rhamnose; Fuc: fucose; Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; GalA: galacturonic acid.
Low (0.1 mL/kg), medium (1.05 mL/kg) and high (2.0 mL/kg)

4.3.3.3 Viscosity

Figure **4.3** illustrates the viscosity curves of ***enzyme-extracted crude pectin*** extracted at 25ºC for 30 min using different enzyme concentrations. The samples were prepared based on a dry weight basis of 4% w/w. The results show that pectin extracted with the medium Celluclast 1.5L concentration exhibited the highest viscosity (~ 46 mPa.s at 53 s^{-1}) compared with the other concentrations (low and high levels). There was a slight difference in viscosity (~ 30 mPa.s at 53 s^{-1}) for pectin samples isolated at the low and high Celluclast

1.5L concentrations. Pereyra *et al*. (1997) reported that the M_w differences of high methoxyl pectins (HMPs) probably account for the differences in apparent viscosity. The molar mass results for these samples are described in section **4.3.3.4.**

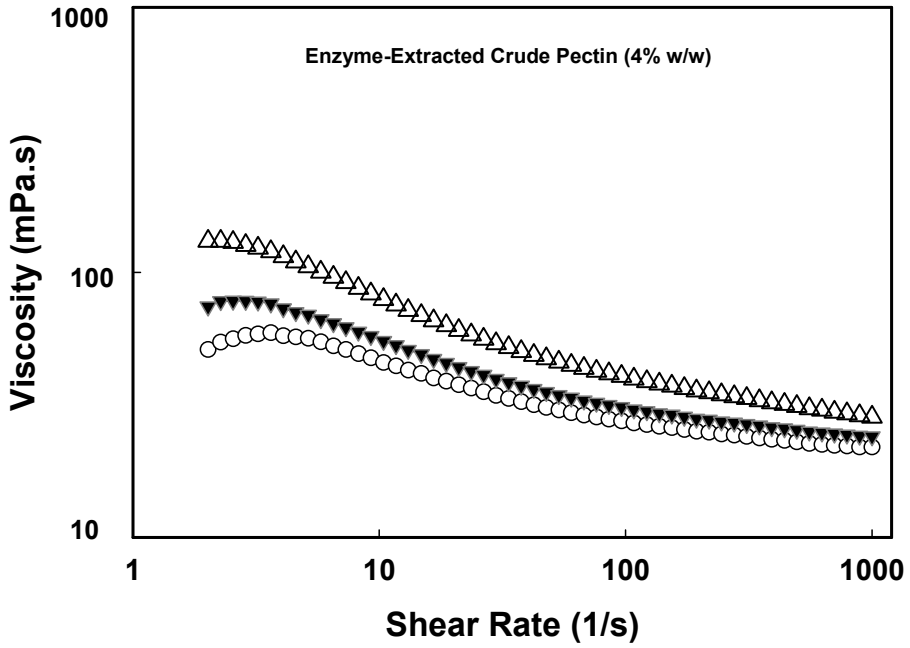


Figure 4.3 Viscosity curves of crude pectin (4% w/w dry weight in Milli-Q water, pH 3.50 ± 0.01) extracted using Celluclast 1.5L under extraction conditions of 25ºC and 30 min and at different concentrations: (○) high, (Δ) medium and (▼) low.

4.3.3.4 The *dn/dc* and M_w of Pectin Samples

dn/dc is the *specific refractive index increment* value, which depends on the type of polysaccharide (because different types of molecule may have different *dn/dc* values), the solvent and the wavelength (Harding, 1994). The *dn/dc* values obtained for pectin extracted using Celluclast 1.5 (medium and low concentrations) were relatively similar (0.185−0.191 mL/g). The average *dn/dc* value of 0.189 ± 0.002 mL/g was used in the determination of the M_w. The *dn/dc* value of gold kiwifruit pectin was higher than the *dn/dc* value of pectin from other fruits (0.146 mL/g for citrus pectin by Fishman, Chau, Kolpak, & Brady, 2001; 0.132 mL/g for citrus pectin by Cameron, Savary, Hotchkiss, & Fishman, 2005). However, Corredig, Kerr and Wicker (2000) reported a *dn/dc* value for HMP from citrus of 0.183 mL/g. These authors attributed the high *dn/dc* value to the greater number of methoxyl

groups present in the polysaccharide chains. Based on the *dn/dc* value determined in this study, the M_w of pectin samples was determined.

Determination of the M_w of pectin was based on the SEC-MALLS technique described in Goh, Hemar and Singh (2005). Figures **4.4a−4.4c** show chromatograms of the light scattering response at 90º (LS), ultraviolet (UV) and DRI signals as a function of the elution volume (mL) from samples obtained using the different enzymatic concentrations described in section **4.2.4.1**. The DRI signal is proportional to the concentration of the polymer, the UV signal correlates to the presence of protein in the sample, whereas the LS signal depends on the size, M_w and concentration of the polymer molecules eluted through the SEC column.

The DRI profiles showed multiple peaks, indicating that the purified pectin samples consisted of more than one polymer fraction. Low DRI signals indicate a low polymer concentration. At the initial elution volume (6 mL), a small DRI signal was observed but this was accompanied by large LS and UV signals. This could have been due to the presence of a small amount of pectin aggregates ($M_w \sim 10^7-10^8$ g/mol) that eluted first.

A second wider DRI peak was observed at an elution volume of 7−12 mL. This range consisted of about four peaks with the size depending on the concentration of enzymes used in the extraction process. This signal range is believed to be due to the pectin fraction and was used to determine the M_w. The LS, UV and DRI profiles of gold kiwifruit pectin were comparable with those of a commercial citrus pectin (see Figure **4.5**). The presence of aggregates at the initial volume appeared to be due to the nature of the pectin solution.

A small DRI peak that eluted after 12 mL was believed to be caused by low M_w species, such as proteins, oligosaccharides and salts, which might be eluted last. The molecular fractions at the start of the elution and at the end of the elution were not considered in the M_w determination. Therefore, only the LS data at an elution volume from 7 to 12 mL were used to determine the M_ws of the pectin samples. The results for the M_w and the polydispersity index are given in Table **4.7**.

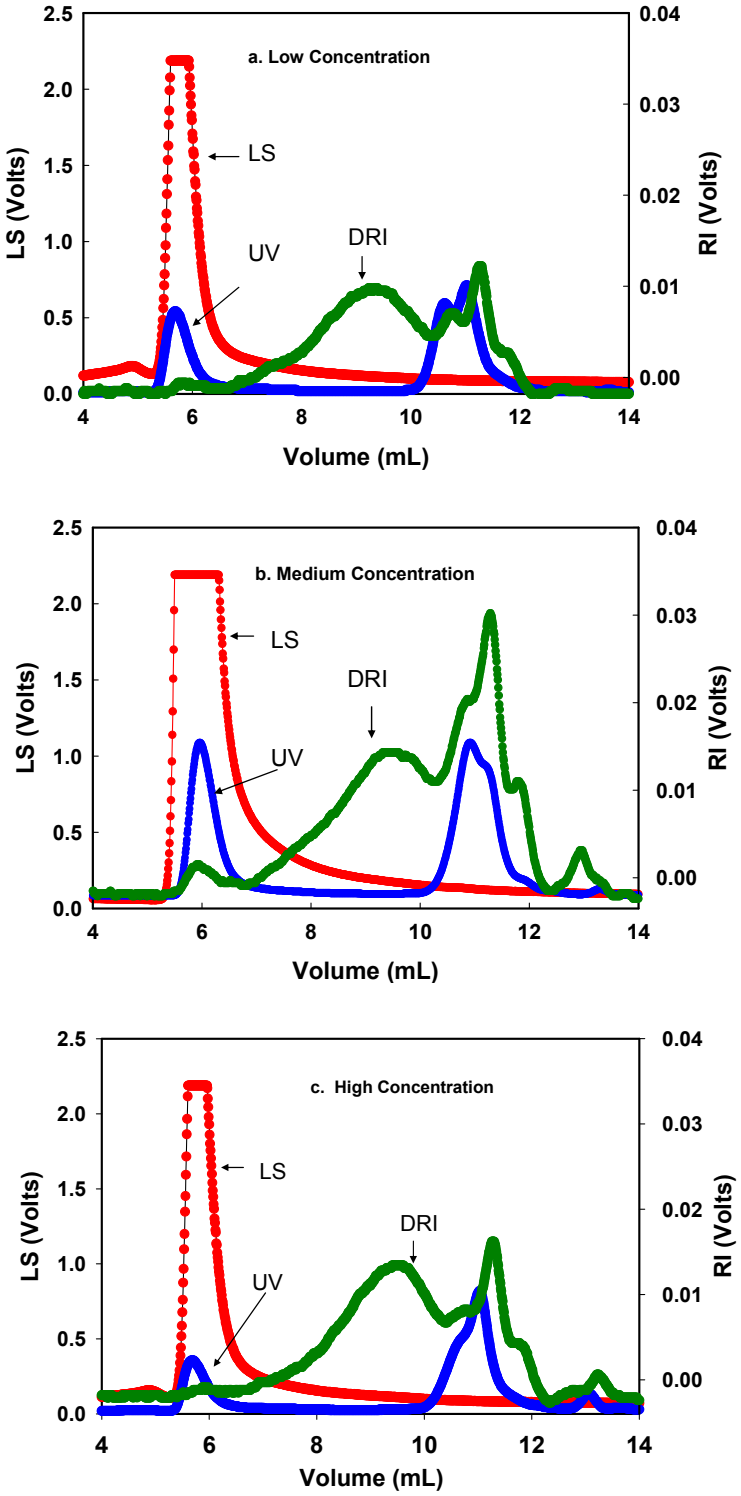


Figure 4.4 Light scattering (LS), ultraviolet (UV) and differential refractive index (DRI) signals of purified pectin extracted using different enzyme concentrations: (a) low; (b) medium; (c) high (based on 0.35% w/w GalA concentration, prepared in 0.1 M NaCl, 0.02% sodium azide).

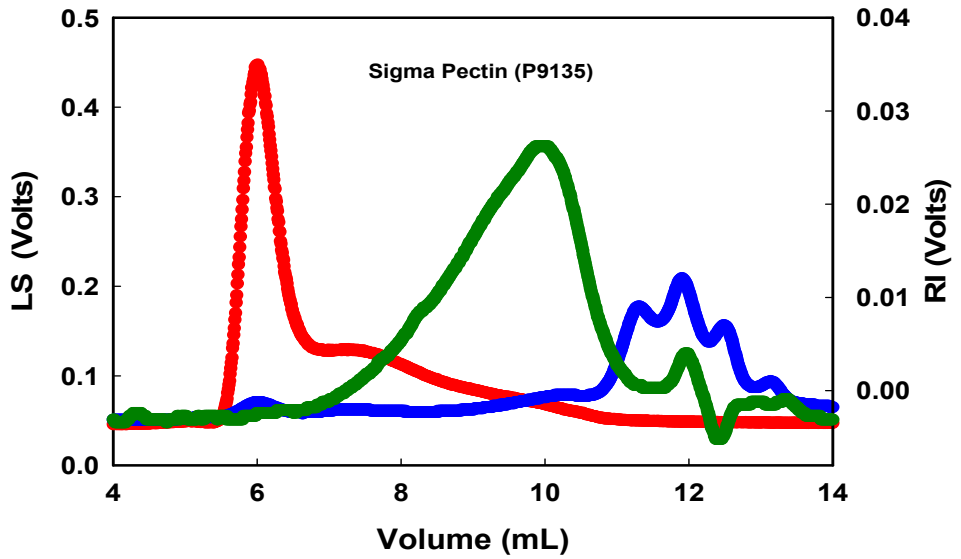


Figure 4.5 Light scattering (LS), ultraviolet (UV) and differential refractive index (DRI) signals of commercial citrus pectin (Sigma, P9135) based on 0.35% w/w GalA concentration, prepared in 0.1 M NaCl, 0.02% sodium azide.

Table 4.7 Average-weight molecular weight and polydispersity index of purified pectin extracted using different enzyme concentrations

Enzyme Concentration	M_w (x 10^5 g/mol)	Polydispersity Index (M_w/M_n)
Low	3.72 ± 0.01	2.42 ± 0.10
Medium	16.50 ± 0.37	2.49 ± 0.06
High	2.44 ± 0.01	2.29 ± 0.17
Sigma Pectin (P9135)	1.66 ± 0.55	4.82 ± 0.70

Mean ± standard error (n = 2).
Low (0.1 mL/kg), medium (1.05 mL/kg) and high (2.0 mL/kg)

The M_w results (Table **4.7**) showed that, at medium enzyme concentration, the extracted pectin had the highest M_w (~ 16.5 x 10^5 g/mol). At low and high enzyme concentrations, the M_ws were 3.72 x 10^5 and 2.44 x 10^5 g/mol respectively. At high enzyme concentration, the pectin molecular chains were smaller, which could have been due to the side pectinolytic activities of Celluclast 1.5L. The molar mass of pectin was also lower at the low enzyme concentration than at the medium enzyme concentration. It could be possible that there was insufficient enzyme to hydrolyse the cellulose network in which the larger molar mass fraction was trapped.

Generally, the M_w of pectin is expected to be in the range 10^4–10^5 g/mol (Corredig, *et al*., 2000). The M_w of commercial citrus pectin determined in this study was 1.66 x 10^5 g/mol. This value is in close agreement with data obtained from studies on citrus pectin (Mesbahi, Jamalian, & Farahnaky, 2005; Morris, *et al*., 2008). However, a value close to that of kiwifruit pectin, of the order of 10^6 g/mol, has been reported for cider apple pomace pectin extracted using CDTA and Na_2CO_3 (Chapman, Morris, Selvendran, & Oneill, 1987).

To explain the viscosity data described in section **4.3.3.3**, a linear regression between viscosity at 53 s^{-1} and M_w was plotted and a strong correlation (R^2 = 0.99) between viscosity and molar mass was obtained. The graph is presented in Appendix **B.6**. The molecular chains of pectin were influenced by the enzyme concentration, hence giving different viscosity readings.

4.4 Conclusions

From this work, Celluclast 1.5L was selected from among other commercial enzyme preparations for the extraction of gold kiwifruit pectin. The extraction time and the extraction temperature had a significant influence on the yield of pectin and the total-NSP and sugar compositions. The viscosity of the pectin solution was also affected by the extraction time and the extraction temperature. The conditions that produced a high pectin yield did not correspond to high pectin viscosity.

The optimum concentration of Celluclast 1.5L was also explored by isolating gold kiwifruit pectin at three different enzyme concentrations. The enzyme concentration had no significant effect on the yield of pectin and the sugar composition. However, the enzyme concentration could influence the molar mass, as shown by the different viscosities.

Considering the chemical composition and the physicochemical characteristics of the pectin, a Celluclast 1.5L concentration (medium) of 1.05 mL/kg using the extraction conditions of 25ºC (30 min) were recommended for the extraction of gold kiwifruit pectin by enzymatic treatment. A subsequent experiment on the enzymatic extraction of pectin from gold

kiwifruit of different maturities is described in Chapter **5**. The physicochemical properties of the pectin obtained were compared with those of the pectin obtained using acid and water extraction methods.

CHAPTER 5 Characterisation of Gold Kiwifruit Pectin from Fruit of Different Maturities

5.1 Introduction

Kiwifruits are usually harvested when they reach physiological maturity. This means that the fruits are mature but unripe with a minimum total soluble solids content of 6.2% for cv. Hayward (Beever & Hopkirk, 1990) and 10% for cv. Hort16A (Patterson, *et al*., 2003). Several protocols are employed for harvesting gold kiwifruits at different stages of maturity. Fruits can be harvested several weeks earlier (early-season fruits) than main-harvested fruits to compete with the world gold kiwifruit market and to achieve better financial returns (Schotsmans, Nicholson, MacKay, & Mawson, 2005). The fruits are usually stored at a certain temperature to allow the flesh to develop a golden colour before they are sold or exported. Normally, a storage temperature of 3°C for 12 weeks followed by 1.5°C for 6 weeks successfully reduces the level of chilling injuries during storage (Maguire, Amos, & Kelly, 2005). Main-harvested kiwifruits are left on the vine for approximately for certain months to increase their volume and dry matter content.

Compared with other plant cell walls, the cell walls of kiwifruits contain a large amount of polysaccharide. Redgwell, Melton and Brasch (1988) have reported that the cell wall material of green kiwifruit (cv. Hayward) at harvest is composed mainly of heterogeneous pectin−galactan mixtures (40–50%) and hemicellulose (15–25%), which is mostly in the form of xyloglucans. The concentration of these polysaccharides is different at each stage of maturity because of the physiological changes that occur mostly during the maturation process (Redgwell & Percy, 1992). These changes (Femenia, *et al*., 2009) and further processing applied during the extraction process (Femenia, Garcia-Pascual, Simal, & Rossello, 2003) could lead to significant differences in the functional properties of the polysaccharides of kiwifruit.

To date, there has been no research investigating the effect of different extraction methods on the properties of pectin isolated from gold kiwifruit at different stages of maturity. Thus, the objectives of this study were to determine the physicochemical characteristics of purified pectin extracted at different stages of maturity using the extraction methods that were evaluated for main-harvested kiwifruit, as described in Chapters **3** and **4**.

5.2 Materials and Methods

5.2.1 Whole Fruit Puree Preparation

In this study, two batches of gold kiwifruit at different maturities, *i.e.* main-harvested fruit (MHF) and early-harvested fruit (EHF) were used. The fruits were obtained from Zespri International Ltd (Hastings, New Zealand). The EHF were harvested in May 2008 (approximately 12 weeks after pollination) presenting a firmness of 5.3 kgf, and were 8 weeks less mature than the MHF. The MHF were from the same batch as that for all experiments described in Chapters **3** and **4**. The chemical composition (protein, ash, dry matter, total starch, total non-starch polysaccharide (total-NSP), neutral sugars and galacturonic acid (GalA) concentrations) of the EHF samples was determined using the procedures described in section **3.2.3** of Chapter **3.** All kiwifruits were frozen at –20°C after harvest and were thawed (at 4°C for approximately 18 h) prior to extraction. The puree was prepared as described in Chapter **3**, section **3.2.1**. The pH values of both puree samples were measured at room temperature.

5.2.2 Pectin Extraction

Three extraction techniques were applied to both batches of puree: acid, water and enzyme. The best extraction conditions for each method were used, based on the viscosity data for pectin isolated from MHF reported in Chapter **3** (for acid and water extractions) and Chapter **4** (for enzymatic extraction). The conditions for each method were as follows.

- Acid extraction: at 50ºC for 60 min with a puree to citric acid (CA) solution ratio of 1:6 (w/v).

- Water extraction: at 25ºC for 30 min with a puree to water ratio of 1:4 (w/v).
- Enzymatic extraction: at 25ºC for 30 min with a medium Celluclast 1.5L concentration (1.05 mL/kg).

5.2.2.1 Acid Extraction

Gold kiwifruit puree (200 g) was extracted with 1200 mL (1:6 (w/v) puree to CA solution ratio) of 1.0% (w/v) CA solution (pH 2.20 ± 0.01) and the mixture (pH 2.80 ± 0.01 for MHF, and pH 2.70 ± 0.01 for EHF) was heated in a 50°C water bath for 60 min under continuous stirring. Deionised water (RO water) was used throughout the study. The container was covered with aluminium foil to avoid moisture loss due to evaporation. After extraction, the mixture was cooled to approximately 20°C in a bath of crushed ice and then centrifuged at 3310 *g* for 20 min at 4°C. The supernatant was filtered through four layers of cheese cloth to remove the pulp and seeds that were not separated during centrifugation. Tepid water at 50°C was added to the pellet, in a 1:1 (w/v) ratio, and the mixture was stirred for 30 min (to remove any remaining soluble polysaccharides) and centrifuged again as described above. All extracts were combined and precipitated with ethanol as described in Chapter **3**, section **3.2.2.1**, until freeze-dried ***crude EHF-extracted pectin*** and ***crude MHF-extracted pectin*** were obtained. The flow chart in Figure **5.1** provides an overview of the pectin extraction procedures using the different extraction methods (*i.e.* water, acid and enzyme).

For purification, the freeze-dried ***crude EHF-extracted pectin*** and ***crude MHF-extracted pectin*** were dispersed in Milli-Q water (1.0% w/w) and stirred overnight (~ 15 h) at 4°C. The pectin in the solution was recovered by ethanol precipitation (80%), centrifuged, ethanol washed and vacuum oven dried as described earlier. The pectin yield was calculated as in Equation **3.1** and was expressed as ***purified EHF-extracted pectin*** and ***purified MHF-extracted pectin***. Figure **5.2** shows a pectin gel precipitated with ethanol. EHF had less pectin precipitate than MHF.

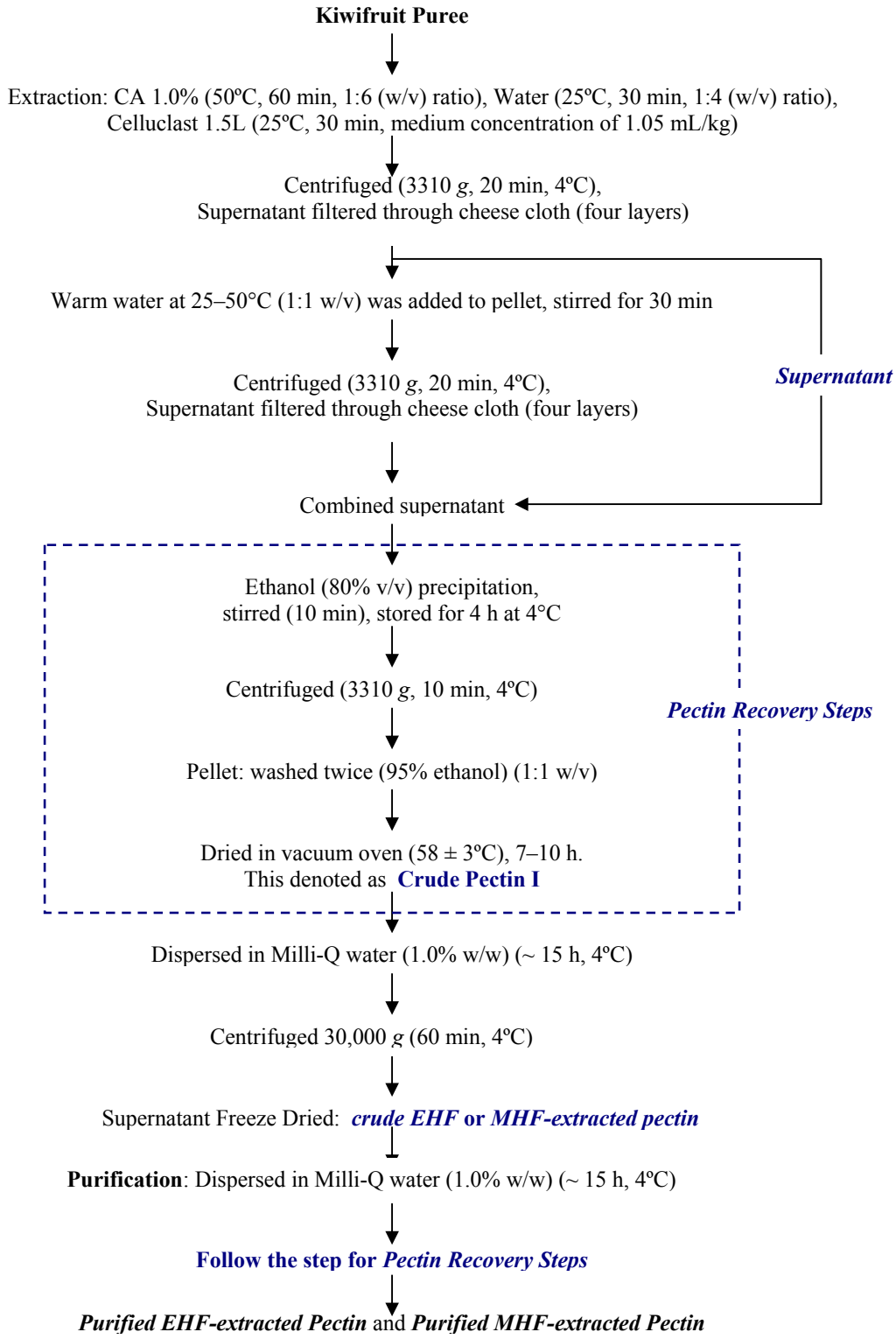


Figure 5.1 Flow chart of acid, water and enzymatic extractions of gold kiwifruit pectin from EHF and MHF.

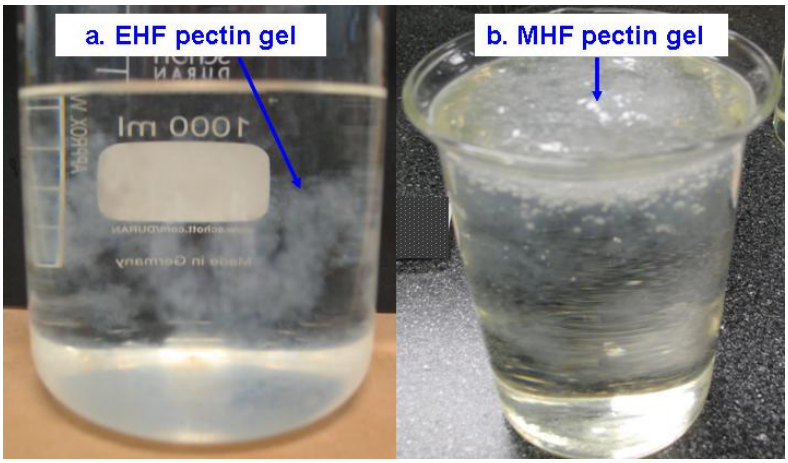


Figure 5.2 Gold kiwifruit pectin (1.0% w/w freeze-dried crude pectin) precipitated with 80% ethanol after storage at 4ºC for 4 h: (a) EHF; (b) MHF.

5.2.2.2 Water Extraction

The water extraction method was similar to the acid extraction method, except that RO water was used instead of CA solution. The extraction was carried out in a 25ºC water bath for 30 min with a puree to water ratio of 1:4 (w/v) (*i.e.* 200 g of puree mixed with 800 mL of RO water). The mixture pHs were 3.60 ± 0.01 for MHF and 3.50 ± 0.01 for EHF. Purification of the pectin was based on the procedures described for the acid extraction method.

5.2.2.3 Enzymatic Extraction

The pectin isolation and purification steps for the enzymatic extraction method were similar to those described in section **4.2.3.1** of Chapter **4**. The extraction was conducted at 25ºC for 30 min with a medium Celluclast 1.5L concentration (1.05 mL/kg). In contrast to the acid and water extraction methods, the puree was not diluted with water during the extraction process. The pHs of the MHF and EHF purees were 3.50 ± 0.05 and 3.20 ± 0.05 respectively.

5.2.3 Analysis of Purified Pectin Samples

The total-NSP composition, neutral sugar composition and GalA concentration of the purified pectins were determined based on the methods described earlier (see section **3.2.3.6**). Protein content (see section **3.2.3.4**), ash content (see section **3.2.3.3**), weight average molecular weight (M_w) (see section **4.2.4.1**), viscosity (see section **3.2.3.7**) and degree of esterification (DE) were also determined for the purified pectins from fruit of both maturities. All analyses were carried out in duplicate.

Further rheological analysis was carried out to confirm the viscosity results for the pectins from fruit of both maturities. The small deformation behaviours (viscoelasticity) of water-extracted MHF pectin and water-extracted EHF pectin (1.0% w/w based on the GalA concentration, in Milli-Q water, pH 3.50 ± 0.01) were studied using a cone and plate measuring system (CP 4/40, Paar Physica MCR 301; Anton-Paar, GmbH, Germany) at a temperature of 20 ± 0.1°C. The rheological tests were performed in the frequency range 0.01–100 Hz (strain of 0.5%).

5.2.3.1 Determination of the DE of Purified Pectin

The DE of purified pectin was determined using an automated capillary electrophoresis system (HD 3D) as described by Strom, Ralet, Thibault and Williams (2005). The method is based on the electrophoretic mobility (EM) (μ) of pectin molecules through a capillary. A linear relationship between the EM and the average charge per residue is obtained (Zhong, *et al*., 1998). In this measurement, the EM is related to the migration time of the samples (t) relative to a neutral marker zone (t_0), based on Equation **2.1** as described by Williams *et al*. (2002). The term "neutral marker" refers to a non-charged compound that can be used to measure the electroosmotic flow of the solvent.

Phosphate buffer (0.09 M, pH 7.0) was used as the capillary electrophoresis background electrolyte (BGE) and was prepared by mixing 39 mL of 0.2 M Na_2HPO_4 and 61 mL of 0.2 M NaH_2PO_4, until 0.09 M phosphate buffer was obtained. Prior to the measurement, the new capillary was conditioned by rinsing with 1 M sodium hydroxide (NaOH) for 30 min

followed 0.1 M NaOH solution for 30 min. The capillary was then rinsed with water for 15 min and finally with BGE for 30 min. Between runs, the capillary was washed for 2 min with 1 M NaOH, 2 min with 0.1 M NaOH, 1 min with Milli-Q water and 2 min with BGE.

The sample solutions were prepared by dissolving the pectin in Milli-Q water (2.5 mg/mL) at 60ºC for 30 min. The sample was injected hydrodynamically at 5000 Pa (an injected volume of about 10 nL) and electrophoresed across a potential of 25 kV. The measurement was carried out in a fused silica capillary of internal diameter 50 μm and total length 46.5 cm (40 cm from inlet to detector) obtained from Composite Metals, Hallow, UK. The capillary included an extended light-path detection window (150 mm) and was thermostatically controlled at 25ºC. The detection was carried out using an ultraviolet absorbance of 191 nm with a bandwidth of 2 nm.

5.2.4 Data Analysis

The experiment was based on a 2 x 3 factorial design, where the treatments of maturity (EHF and MHF) and extraction method (acid, water and enzyme) were combined to evaluate the significance of the main effects (maturity and extraction method) or interactions of maturity and extraction method. The data were analysed by two-way analysis of variance (ANOVA) using SAS (version 9.1). The significance of differences among the treatments was determined by Duncan's multiple range test ($P \leq 0.05$).

5.3 Results and Discussion

5.3.1 Compositions of EHF and MHF

The compositions of kiwifruits of both maturities are given in Table **5.1**. In general, the EHF, which were less mature than the MHF, had a lower total soluble solids content. The EHF also had lower protein content (~ 3.70%) and dry matter content (~ 16.02%) but higher ash content (~ 4.68%). The EHF were considerably richer in starch (~ 8.55%), which is typical of less mature gold kiwifruits (Femenia, *et al*., 2009). Li *et al*. (2006) have reported that the starch located in the cell wall of immature green kiwifruits appears to associate

strongly with pectin and hemicellulose. Less starch was observed in the MHF (1.50%) because starch was converted to soluble sugars during the maturation process. A similar result has been reported for green kiwifruits; the starch content decreases when the fruits on the vine mature (Reid, *et al*., 1982). The total-NSP composition of EHF (~ 12%) was slightly higher than that of MHF (~ 10%). The insoluble-NSP fraction of EHF (~ 9%) was also greater than that of MHF (~ 7%); however, the soluble-NSP fraction of EHF (~ 3%) was less than that of MHF (~ 4%). This could indicate physiological changes to the pectin during the maturation process.

Table 5.1 Composition of early-harvested and main-harvested gold kiwifruits

Fresh Fruit Composition	EHF 2008	MHF 2007
Total Soluble Solids (%)	13.02 ± 0.39	16.08 ± 0.10
Protein (% dwb)	3.70 ± 0.05	4.24 ± 0.07
Dry Matter (%)	16.02 ± 0.14	19.01 ± 0.14
Ash (% dwb)	4.68 ± 0.01	3.94 ± 0.09
Starch (% dwb)	8.55 ± 0.07	1.50 ± 0.08
pH	3.20 ± 0.05	3.50 ± 0.05
Total-NSP (% dwb)	11.54 ± 0.42	10.43 ± 0.34
Soluble	2.50 ± 0.40	3.52 ± 0.24
Insoluble	9.04 ± 0.43	6.91 ± 0.43

Mean ± standard error (n = 2).
The detailed sugar composition of the NSP in the fruit is shown in Appendix **C.1.**

5.3.2 Effect of Extraction Methods on the Purified Soluble Pectin Yield

The percentage yields of purified pectin from MHF and EHF obtained using different extraction methods are shown in Table **5.2**. Depending on the extraction method, the yield varied from 1.01 to 2.14% and from 3.27 to 4.39% for EHF and MHF respectively. The yield of pectin was lower than those from apple pomace (2.9–8.9%) (Garna, *et al*., 2007), chicory roots (28–35%) (Panouille, *et al*., 2006) and sugar beet pulp (4.1–16.2%) (Yapo, *et al*., 2007). The yield differences were probably influenced by the source of the pectin, the extraction method and the extent of purification.

Table 5.2 Yield of purified pectin (% w/w dry matter basis) extracted from gold kiwifruit of two different maturities using different extraction methods

Maturity	Extraction Method	Purified Pectin Yield (% w/w)
Early-harvested Fruits	Acid	1.43
	Water	1.01
	Enzyme	2.14
Main-harvested Fruits	Acid	3.27
	Water	3.27
	Enzyme	4.39
SEM[1]		**0.198**
Main Effect		
Fruit Maturity		
Early-harvested Fruits		1.52[a]
Main-harvested Fruits		3.64[b]
Extraction Method		
Acid		2.35[a]
Water		2.14[a]
Enzyme		3.26[b]
Probabilities, *P* ≤		
Fruit Maturity		***
Extraction Method		**
Fruit Maturity x Extraction Method		NS

[a,b,c]Means in a column with different superscripts differ significantly ($P < 0.05$).
[1]Pooled standard error of mean. NS: not significant; ***: $P < 0.001$; **: $P < 0.01$.
Each value represents the mean of two replicates.

Based on statistical analysis, both fruit maturity and extraction method had a significant influence on the yield of pectin. The pectin yield obtained from MHF (3.64%) was significantly higher than that obtained from EHF (1.52%). This was consistent with the monosaccharide composition data (see Table **5.1**), which showed more total soluble-NSP in MHF (~ 3.52%) than in EHF (~ 2.50%). In EHF, the pectin appeared to be strongly bound to the insoluble cellulose and hemicellulose fractions.

Comparison of the extraction methods showed that the highest pectin yield was obtained by enzymatic extraction (3.26%), suggesting that the pectin molecules in EHF were bound to the cellulose and hemicellulose of the cell wall. Consequently, Celluclast 1.5L hydrolysed and disrupted the cellulose network, which facilitated pectin release. This result was similar to those of studies conducted by Shkodina *et al*. (1998), Panouille *et al.* (2006) and Ptitchkina *et al.* (2008); pectin recovery by enzyme was superior to acid extraction.

5.3.3 Ash and Protein Contents of Purified Pectin

Table **5.3** shows the ash and protein contents of purified gold kiwifruit pectin extracted from fruit of two different maturities using different extraction methods. The value is expressed as % w/w on a dry weight basis. The amounts of ash and protein in the purified pectin samples were significantly affected by the fruit maturity and the extraction method used. Protein and ash constituted approximately 14.74–42.49% of the purified pectin samples. The ash content varied between 1.05 and 12.87%, whereas the protein content varied from 9.63 to 29.62%. The highest ash and protein contents were obtained from purified pectin samples extracted from EHF by the enzymatic method. The enzymatic method could potentially hydrolyse the cell wall material, resulting in the release of more non-pectin fractions. These non-pectin fractions, including proteins, were precipitated with the polysaccharides during the ethanol precipitation step.

Table 5.3 Ash and protein contents (% w/w dry weight basis) of pectin extracted from gold kiwifruit of two different maturities using different extraction methods

Maturity	Extraction Method	Ash	Protein
Early-harvested Fruits	Acid	1.05[a]	25.85[a]
	Water	4.07[b]	20.55[b]
	Enzyme	12.87[c]	29.62[c]
Main-harvested Fruits	Acid	2.42[d]	17.98[b]
	Water	5.11[e]	9.63[d]
	Enzyme	7.09[f]	13.85[e]
SEM [1]		**0.237**	**0.937**
Main Effect			
Fruit Maturity			
Early-harvested Fruits		5.99	25.94
Main-harvested Fruits		4.87	13.82
Extraction Method			
Citric Acid		1.74	22.82
Water		4.59	15.09
Enzyme		9.98	21.73
Probabilities, $P \leq$			
Fruit Maturity		**	***
Extraction Method		***	***
Fruit Maturity x Extraction Method		***	*

[a,b,c]Means in a column with different superscripts differ significantly ($P < 0.05$).
[1]Pooled standard error of mean. ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$.
Each value represents the mean of two replicates.

In general, EHF had a slightly higher ash content than MHF. This perhaps relates to the higher calcium content of less mature fruit. Preliminary studies indicated that water-extracted EHF pectin has ~ 3.0% w/w dwb calcium, whereas MHF pectin obtained using a similar extraction method has ~ 0.95% w/w dwb calcium content. A similar result has been reported by Galego and Zarra (1997) for the calcium content of green kiwifruits during growth. Calcium has an important effect on the firmness and membrane stability and hence delays softening of the fruit (Hopkirk, Harker, & Harman, 1990). The cell wall of less mature fruit is generally compact, which contributes to its firmness (Virk & Cleland, 1990). Calcium binds with GalA residues through calcium bridges (Jarvis, 1984) and could result in the formation of a gel network. The binding of GalA by calcium ions could contribute to the lower amount of soluble pectin. The use of harsher reagents such as more concentrated acid solution or alkali has been suggested by van Buren (1991) for the isolation of pectin from such a compact cell wall network. However, this could lead to pectin degradation, which could affect the yield and could alter the functional properties of the isolated pectin.

The lowest ash and protein contents were obtained in water-extracted MHF pectin (14.74%), indicating that this pectin was more pure than those obtained using the other two extraction methods. The water extraction method was the mildest of the three methods used, and presumably left the pectin that was naturally solubilised during the softening process in its native state.

5.3.4 Total-NSP and Sugar Composition

Table **5.4** shows the neutral sugar and GalA composition in purified EHF pectin and MHF pectin extracted using the different methods. The value is expressed as % w/w on a dry weight basis. The total neutral and acidic sugar residues present in the purified pectins were approximately 56.37% for EHF pectin and 64.10% for MHF pectin. GalA was the main component of the total-NSP. The percentage of GalA ranged from 28.96 to 48.80% (EHF) and from 51.87 to 58.57% (MHF) based on the total dry weight of the purified pectin.

Table 5.4 Total-NSP and monosaccharide composition (% w/w dry weight) of purified pectins extracted from gold kiwifruit of two different maturities using different extraction methods

Maturity	Extrac. Mtd.	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	Total-NSP
EHF	Acid	0.80[a]	0.48[a]	3.43[a]	2.21[a]	0.63	7.86[a]	4.58[a]	48.80[c]	69.14[a]
	Water	0.86[b]	0.40[a]	2.98[b]	2.03[b]	0.57	7.38[a]	3.67[b]	42.88[d]	60.74[c]
	Enzyme	0.57[c]	0.18[b]	2.29[c]	0.37[d]	0.47	5.27[b]	1.10[d]	28.96[e]	39.21[d]
MHF	Acid	0.91[b]	0.21[b]	1.73[d]	0.49[d]	0.20	3.67[c]	1.21[d]	56.08[a]	64.49[b]
	Water	0.97[b]	0.24[b]	1.78[d]	0.68[c]	0.18	5.57[b]	2.52[c]	51.87[b]	63.77[b]
	Enzyme	0.57[c]	0.19[b]	1.26[d]	0.21[e]	0.16	2.77[d]	0.32[e]	58.57[a]	64.02[b]
SEM[1]		**0.035**	**0.027**	**0.082**	**0.040**	**0.019**	**0.141**	**0.148**	**0.776**	**0.784**
Main Effect										
Fruit Maturity										
EHF		0.74	0.35	2.90	1.54	0.55[a]	6.83	3.12	40.21	56.37
MHF		0.82	0.24	1.59	0.46	0.18[b]	4.00	1.34	55.50	64.10
Extraction Method										
Acid		0.86	0.34	2.58	1.35	0.41[a]	5.76	2.90	52.44	66.82
Water		0.92	0.32	2.38	1.35	0.37[a]	6.47	3.09	47.37	62.26
Enzyme		0.57	0.18	1.78	0.29	0.32[b]	4.01	0.71	43.76	51.62
Probabilities, $P \leq$										
Fruit Maturity		**NS**	***	***	***	***	***	***	***	***
Extraction Mtd.		**	*	**	***	*	***	***	***	***
Fruit Maturity x Extraction Method		*	**	***	***	**NS**	***	***	***	***

[a,b,c]Means in a column with different superscripts differ significantly ($P < 0.05$).
[1]Pooled standard error of mean. NS: not significant; ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$.
Each value represents the mean of two replicates.
Rha: rhamnose; Fuc: fucose; Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; GalA: galacturonic acid.

The occurrence of GalA together with rhamnose, arabinose and galactose in the purified extracts indicated possible branching along the molecular structure of the pectin. Table **5.5** shows an estimation of the degree of branching of the extracted pectins. The degree of branching was calculated by dividing the amount of GalA by the rhamnose concentration, as described by Parkar *et al*. (2010). EHF pectin was more branched, carrying side chains every 47─57 GalA residues, whereas MHF pectin carried side chains every 50─97 GalA residues. EHF pectin also had high proportions of galactose (6.83%) and arabinose (2.90%). A study of green kiwifruit pectin by Parkar *et al.* (2010) showed that the pectin molecules were relatively branched; carrying side chains every 25─51 GalA residues, depending on the extraction methods.

Table 5.5 Degree of branching of purified pectin extracted from gold kiwifruit of two different maturities using different extraction methods

Maturity	Extraction Method	Degree of Branching (GalA/Rha)
Early-harvested Fruits	Acid	57
	Water	47
	Enzyme	47
Main-harvested Fruits	Acid	58
	Water	50
	Enzyme	97

Statistical analysis showed a significant interaction effect between the fruit maturity and the extraction method for the amounts of neutral sugars (except mannose) ($P < 0.05$). Similarly, significant interaction effects between the fruit maturity and the extraction method were observed for the GalA content and total-NSP composition ($P < 0.001$). The highest GalA contents occurred in the purified pectins isolated from MHF by acid treatment (56.08%) and the enzymatic method (58.57%). For EHF, the purified pectin obtained by acid extraction had the highest GalA content (48.80%), followed by water-extracted pectin (42.88%) and then the enzyme-extracted pectin (28.96%). This contradicts the yields of purified pectin (Table **5.2**). This can be explained by: (*i*) cellulase could not extract bound pectin in EHF because, as proposed earlier, the pectin was strongly associated with hemicellulose, cellulose and protein, resulting in lower GalA recovery; (*ii*) pectin hydrolysis by pectinolytic activity of the enzyme, which resulted in a low M_w sugar fraction that could not be precipitated by ethanol and therefore a lower GalA yield; (*iii*) the pectinolytic activity could be higher at pH 3.2. This finding is consistent with the M_w data, which are discussed in section **5.3.5**. The cell wall network of MHF may have been less bound as a result of physiological changes during maturation (*e.g.* cell wall swelling); this may have helped the cellulase to isolate the bound pectin in the cell wall, and therefore more GalA was isolated.

Fucose, xylose, mannose and glucose residues were present in relatively small amounts in all extracts and were regarded as contaminants from the cellulose component and/or the hemicellulosic component. Xylose and glucose could occur as xyloglucan and made up approximately 9.82, 9.38 and 3.75% of the total-NSP in the acid-extracted, water-extracted and enzyme-extracted EHF pectins respectively. However, the xyloglucan contents were estimated to be approximately 2.64, 5.02 and 0.38% in purified MHF pectins obtained by

acid, water and enzymatic extraction methods respectively. Xyloglucan has been reported to be fairly constant at a low level in green kiwifruit during fruit maturation regardless of the extraction method (Li, Sakurai, & Nevins, 2009).

5.3.5 M_w Distribution

Figures **5.3a** and **5.3b** show chromatograms of the light scattering (LS) response at 90º, ultraviolet (UV) and differential refractive index (DRI) signals of purified water-extracted EHF and MHF pectins as a function of the elution volume (mL). Similar chromatograms obtained from acid-extracted and enzyme-extracted pectin samples are given in Appendix **C.2**. The DRI signal is proportional to the concentration of the polymer, whereas the LS signal depends on the size, M_w and concentration of the pectin molecules eluted through the column.

The chromatograms generally showed large LS and UV signals but low DRI signals at the initial elution volume (6 mL). This could indicate the presence of a small amount of protein−pectin aggregates, which were eluted first. At elution volumes of 7–12 mL, the DRI signals of purified MHF pectin showed about four wider peaks, whereas EHF pectin had about five narrower peaks. Figures **5.4a**–**5.4f** compare the DRI chromatograms of EHF and MHF pectins extracted using different methods. The DRI signals for EHF pectin and MHF pectin were distinctly different. In addition, for fruits of both maturities, the DRI signals of the acid- and water-extracted pectins were quite similar but differed somewhat from those of enzyme-extracted pectin. The presence of multiple DRI peaks suggests that different molecular fractions were present in the purified pectins at different concentrations. This is consistent with the sugar composition data.

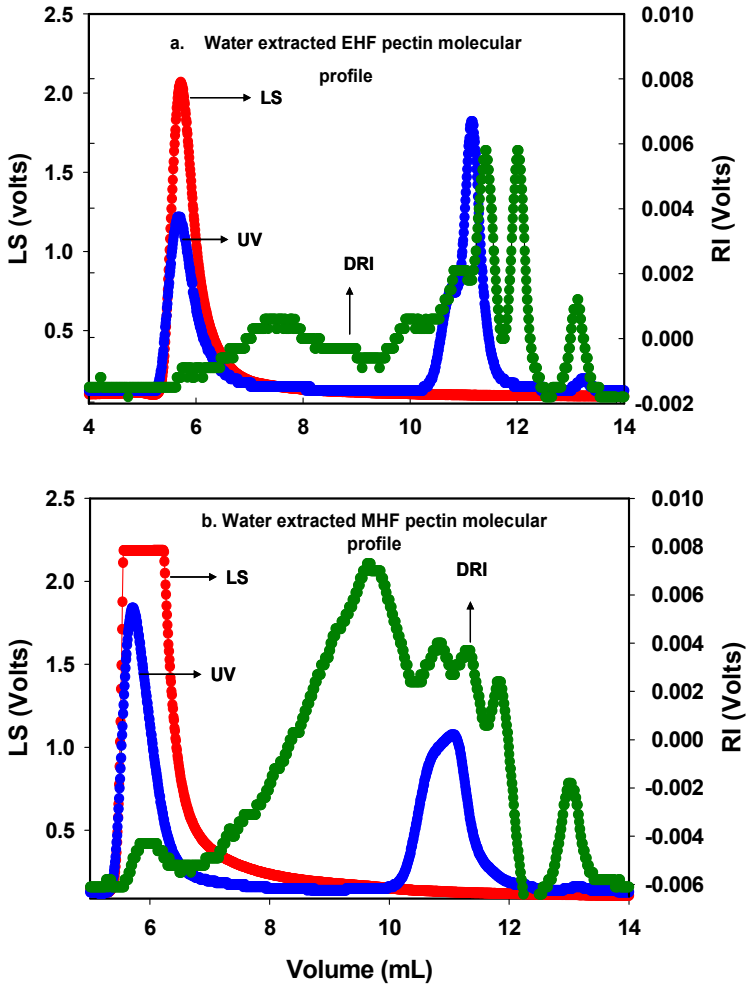


Figure 5.3 LS, UV and DRI profiles of water-extracted (a) EHF pectin and (b) MHF pectin samples prepared, based on 0.5% w/w GalA concentration, in 0.1 M NaCl and 0.2% w/w sodium azide.

The protein fraction, which is regarded as a contaminant, appeared to elute at volumes between 10 and 14 mL, as indicated by the UV signal. A small peak that eluted after 12 mL was thought to be low M_w fractions of sugars, acids and salts. Several studies have indicated that low M_w fractions can be precipitated by ethanol and eluted at the total volume of the column (Yapo, Wathelet, & Paquot, 2007; Emaga, Robert, Ronkart, Wathelet, & Paquot, 2008a). Therefore, the LS data at elution volumes from 7 to 12 mL were used for M_w determination. The results for M_w, polydispersity index and root mean square (RMS) radius are shown in Table **5.6**.

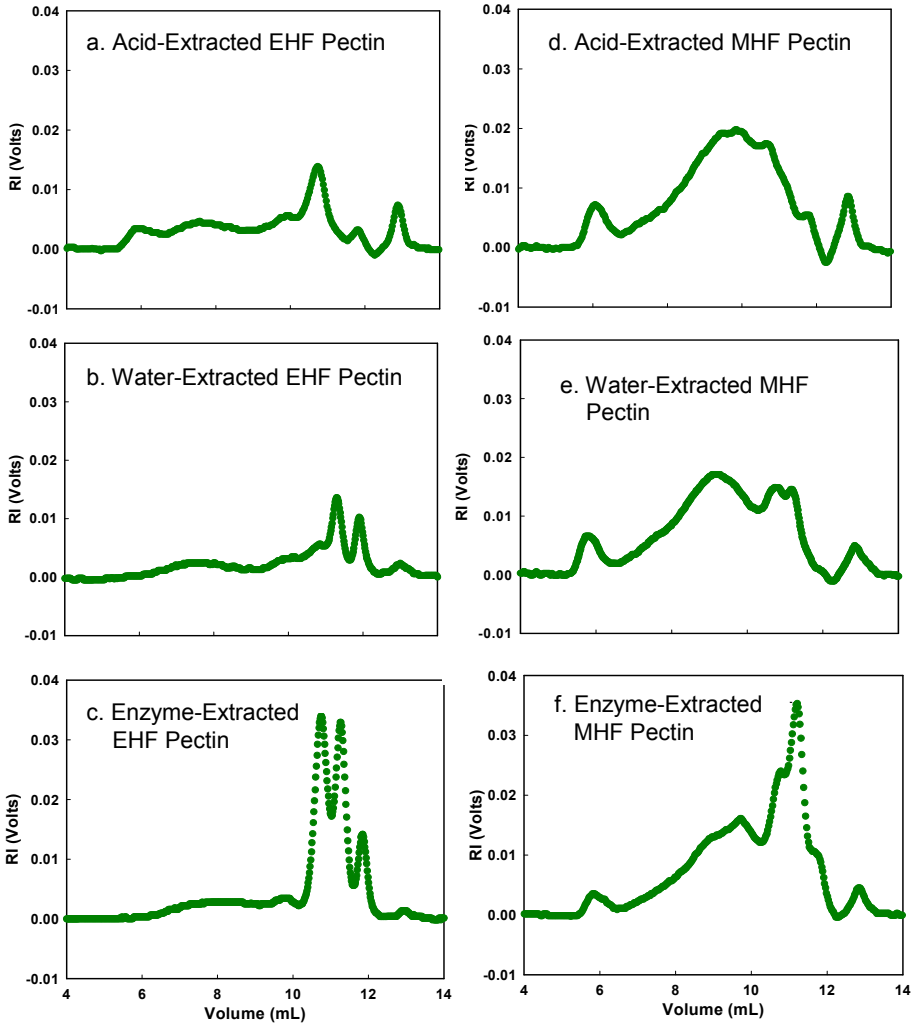


Figure 5.4 DRI profiles of EHF pectin extracted by (a) acid, (b) water and (c) enzyme treatment and MHF pectin extracted by (d) acid, (e) water and (f) enzyme treatment. Based on 0.5% w/w GalA concentration, prepared in 0.1 M NaCl and 0.2% w/w sodium azide.

The *dn*/*dc* value used for the M_w determination was 0.189 mL/g. The M_w ranged from 2.10 x 10^5 to 3.75 x 10^6 g/mol, with the highest for water-extracted MHF pectin and the lowest for enzyme-extracted EHF pectin. In addition enzyme-extracted MHF pectin had lower M_w (1.65 x 10^6 g/mol) than acid-extracted (2.20 x 10^6 g/mol) and water-extracted (3.75 x 10^6 g/mol) MHF pectin.

Table 5.6 Weight-average molecular weight, polydispersity index and RMS radius of purified pectins extracted from gold kiwifruits of two different maturities using different extraction methods

Maturity	Extraction Method	M_w (10^6) (g/mol)	Polydispersity Index	RMS Radius (nm)
Early-harvested Fruits	Acid	1.66[c]	5.00[b]	100.7[d]
	Water	1.03[d]	5.42[b]	114.7[c]
	Enzyme	0.21[e]	12.59[a]	95.8[e]
Main-harvested Fruits	Acid	2.20[b]	1.77[c]	112.6[c]
	Water	3.75[a]	2.43[c]	182.7[a]
	Enzyme	1.65[c]	2.49[c]	162.0[b]
SEM[1]		**0.055**	**0.483**	**0.79**
Main Effect				
Fruit Maturity				
Early-harvested Fruits		0.97	7.67	103.7
Main-harvested Fruits		2.52	2.22	152.4
Extraction Method				
Acid		1.91	3.39	106.7
Water		2.39	3.93	148.7
Enzyme		0.93	7.54	128.9
Probabilities, $P \leq$				
Fruit Maturity		***	***	***
Extraction Method		***	***	***
Fruit Maturity x Extraction Method		***	***	***

[a,b,c,d,e]Means in a column with different superscripts differ significantly ($P < 0.05$).
[1]Pooled standard error of mean. ***: $P < 0.001$.
Each value represents the mean of two replicates.

Both EHF pectin and MHF pectin extracted by enzyme treatment had the lowest M_w, which indicated some hydrolysis of the high M_w fractions of the pectin sample. This finding is in agreement with previous studies (Panouille, *et al*., 2006) on pectin extraction from chicory roots using commercial enzymes (Celluclast 1.5L and Cellulyve TR 400) and from pumpkin using an enzyme from the fungus *Bacillus polymyxa* (Ptitchkina, *et al.*, 2008). These researchers reported that the M_w of pectin isolated by enzyme treatment was relatively lower than that of pectin recovered using acid (0.05 M HCl). The M_ws of pectins obtained from pumpkin and chicory root by enzymatic extraction were approximately 2.0 x 10^5 and 3.0 x 10^5 g/mol respectively. In contrast, the M_ws of pumpkin and chicory root pectins obtained by acid extraction were 7.0 x 10^5 g/mol and 5.0 x 10^5 g/mol respectively.

For EHF, the highest M_w was for pectin extracted using acid, followed by water and then enzyme. Acid extraction could have resulted in solubilisation of the high M_w fractions from cellulose and hemicelluloses, which gave a higher M_w of the pectin sample than that of pectin obtained by the water extraction method.

For MHF, the highest M_w was for pectin extracted using water. Extraction using acid and enzyme gave lower M_ws, possibly as a result of "soluble pectin" (which solubilised during maturation) degradation by acid and enzymes. A similar finding was observed by Oosterveld, Harmsen, Voragen and Schols (2003) for pectin extracted from coffee beans. The acid extraction method resulted in a lower M_w pectin fraction probably because of hydrolysis of the pectin backbone.

Research by Emaga *et al.* (2008a) indicated that the M_ws of banana peel pectins from fruits of different maturities followed the same trend as the M_ws of gold kiwifruit pectin. Their finding could suggest that the M_w of soluble pectin increased with fruit maturity (greenish to yellowish in colour). However, pectin isolated in more mature (yellow with brown spots) banana peels showed lower M_w, because of hydrolysis of the pectin by enzymes such as polygalacturonase (PG) and pectin methyl esterase (PME).

The polydispersity index and RMS radius also varied markedly depending on the fruit maturity and the extraction method. The polydispersity index and the RMS radius ranged from 1.77 to 12.59 and from 95.8 to 182.7 nm respectively. EHF pectin had a higher polydispersity index value (~ 7.67) than MHF pectin (~ 2.22) suggesting that EHF pectin had a wider distribution of polymer fractions with different M_ws. Overall, water-extracted MHF pectin appeared to be the most intact because it had the highest RMS radius (182.7 nm) and M_w (3.75×10^6 g/mol).

EHF pectin extracted by enzyme had the lowest RMS radius and M_w. Significant hydrolysis was observed for EHF pectin, suggesting that pectin from the unripe fruits was susceptible to enzyme treatment.

5.3.6 DE

As the DE of the isolated pectins ranged from 82 to 90% (Table **5.7**), they were classified as high methoxyl pectins (HMPs). There was no significant interaction effect between the extraction method and the fruit maturity. However, the fruit maturity contributed significantly ($P < 0.001$) to the DE, whereas extraction method only has a slight effect on the DE ($P < 0.05$). The DE distributions for pectins from fruit of both maturities are shown in Appendix **C.3**.

Table 5. 7 DE values (%) of gold kiwifruit pectin extracted from fruits of two different maturities using different extraction methods

Maturity	Extraction Method	DE (%)
Early-harvested Fruits	Acid	88
	Water	90
	Enzyme	90
Main-harvested Fruits	Acid	82
	Water	84
	Enzyme	85
SEM [1]		**0.940**
Main Effect		
Fruit Maturity		
Early-harvested Fruits		90[a]
Main-harvested Fruits		84[b]
Extraction Method		
Acid		85[a]
Water		88[b]
Enzyme		88[b]
Probabilities, $P \leq$		
Fruit Maturity		***
Extraction Method		*
Fruit Maturity x Extraction Method		NS

[a,b]Means in a column with different superscripts differ significantly ($P < 0.05$).
[1]Pooled standard error of mean. NS, not significant. ***, $P < 0.001$; *, $P < 0$ .05.
Each value represents the mean of two replicates.

Pectin extracted from less mature fruit (EHF) has higher DE (90%) compared to that of more mature fruit (MHF) (DE 84%). The differences in DE could be related to the following:

(i) The de-esterification of methyl ester groups in pectin during fruit maturation, presumably due to an increase in activity of PME. Therefore, the DE was 90% for

EHF, then as maturation proceeded a decrease in DE for MHF (84%) pectin was observed. A review by Goulao and Oliveira (2008) has indicated a high variation of PME activity during fruit development, maturity and ripening process, for different types of fruit. Additionally, Wegrzyn and MacRae (1992) reported that the activity of PME in ethylene-treated green kiwifruit gradually decreased as the fruit ripens. However this decrease maybe due to the presence of a protein inhibitor of PME (PMEI) (Balestrieri, Castaldo, Giovane, Quagliuolo, & Servillo, 1990). In summary, the role of PME and PMEI on kiwifruit cell wall *in vivo* is still unclear (Schroder & Atkinson, 2006).

(ii) Cell wall of MHF fruit could be more accessible to PME in comparison to EHF cell wall, as result the level of de-esterification of MHF pectin is higher (lower DE) than that of EHF pectin.

The extraction method had a significant effect ($P < 0.05\%$) on the DE. Rombouts and Thibault (1986) have indicated that the DE can be affected by the source of the pectin and its extraction method. Water-extracted and enzyme-extracted pectins from fruits of both maturities had similar DE values (88%), which were higher than those of acid-extracted pectin (85%). This could imply that the methyl ester groups were prone to acid hydrolysis. The DE values obtained for gold kiwifruit pectin were higher than those for pectins from other sources such as fresh sugar beet pulp (63%) (Wang & Chang, 1994) and banana peel (80%) (Emaga, *et al*., 2008a).

5.3.7 Viscosity

The viscosity curves of purified EHF and MHF pectin samples are presented in Figures **5.5a** and **5.5b**. In general, they showed similar trends. The highest viscosity was exhibited by pectin extracted using water, followed by acid and then enzyme. The trends observed for each pectin were in agreement with the M_w data because the viscosity of the pectin solution was influenced by the M_w of the pectin (the higher the M_w, the higher was the viscosity). This has been observed in many polysaccharides (Bourne, 2002). Clearly, the viscosity of

a commercial citrus pectin (Sigma, P9135) is low (~ 25 mPa.s at 53 s^{-1}), probably because of its low M_w (~ 1.66 x 10^5 g/mol) as shown in Figure 5.5.

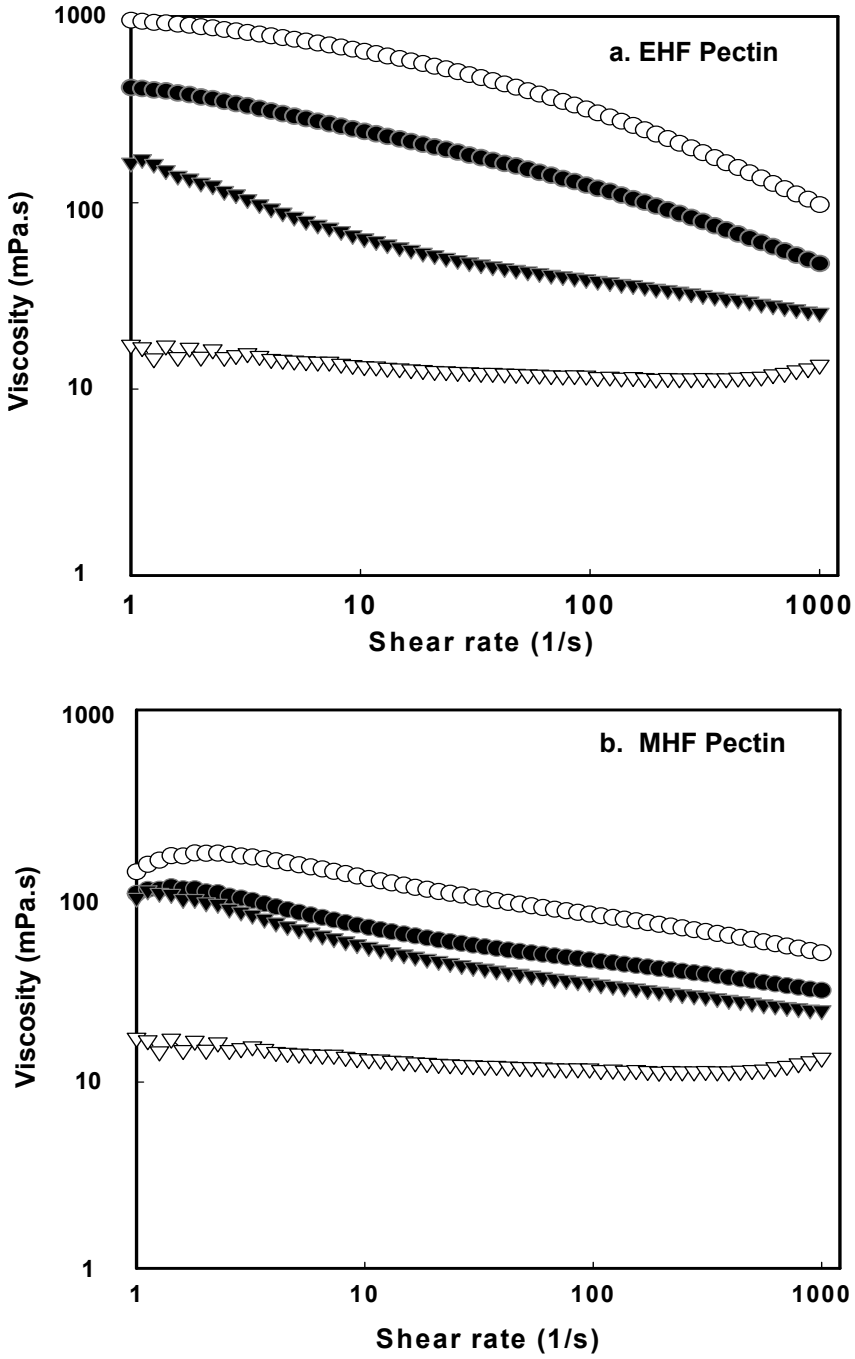


Figure 5.5 Viscosity curves of purified (a) EHF and (b) MHF pectin solutions (in Milli-Q water) extracted using different extraction methods (based on 1.0% w/w GalA concentration at pH 3.50 ± 0.01): (●) acid; (○) water; (▼) enzyme; (▽) commercial citrus pectin (Sigma, P9135).

However, when the viscosity curves of the EHF and MHF pectin solutions were compared, the EHF pectin solution exhibited higher viscosity than the MHF pectin solution for all extraction methods. It is important to note that, despite the lower M_w data obtained for EHF pectin (*i.e.* 1.6 x 10^6 g/mol, water extract) compared to that for MHF pectin (*i.e.* 3.75 x 10^6 g/mol water extract), the viscosity exhibited by EHF pectin was ten times higher than that exhibited by MHF pectin. The relationship between viscosity and M_w appeared to deviate from the expected trend. The proposed explanations for the higher viscosity observed in EHF pectin are as follows.

(i) EHF pectin has a higher DE than MHF pectin, which means that EHF pectin has fewer negatively charged groups along the pectin chain. As a consequence, EHF pectin presents less electrostatic repulsion along the pectin chain (intramolecular repulsion) and between the pectin chains (intermolecular repulsion). A reduction in the intra- and/or intermolecular distance could lead to more chain−chain associations. Consequently, the viscosity of an EHF pectin solution (Figure **5.5a**) could be greater than that of an MHF pectin solution (Figure **5.5b**). Such a relationship between a shorter intermolecular distance and an increased viscosity of a polymer solution has been frequently described (Guimaraes, Coelhe, & Rojas, 2009). A similar explanation has also been given by da Sylva and Rao (2006), who stated that the viscosity of a pectin solution will increase as the DE of the pectin increases. Another study conducted by Femenia *et al.* (2009) has shown that the cell wall materials of half ripe kiwifruits (comparable with our EHF) exhibited improved functional properties such as higher water retention capacity and swelling properties compared with the cell wall materials of the ripe fruits. This could be linked to the DE of the pectin in the fruits.

(ii) EHF pectin has a significantly higher neutral sugar content, as shown in Table **5.4**). The higher galactose and arabinose contents, indicative of a higher degree of branching on the pectin structure, could lead to more chain−chain associations or entanglements, resulting in a higher viscosity (de Man, 1999).

Figure **5.6** illustrates the proposed molecular repulsion in pectin molecules as described above.

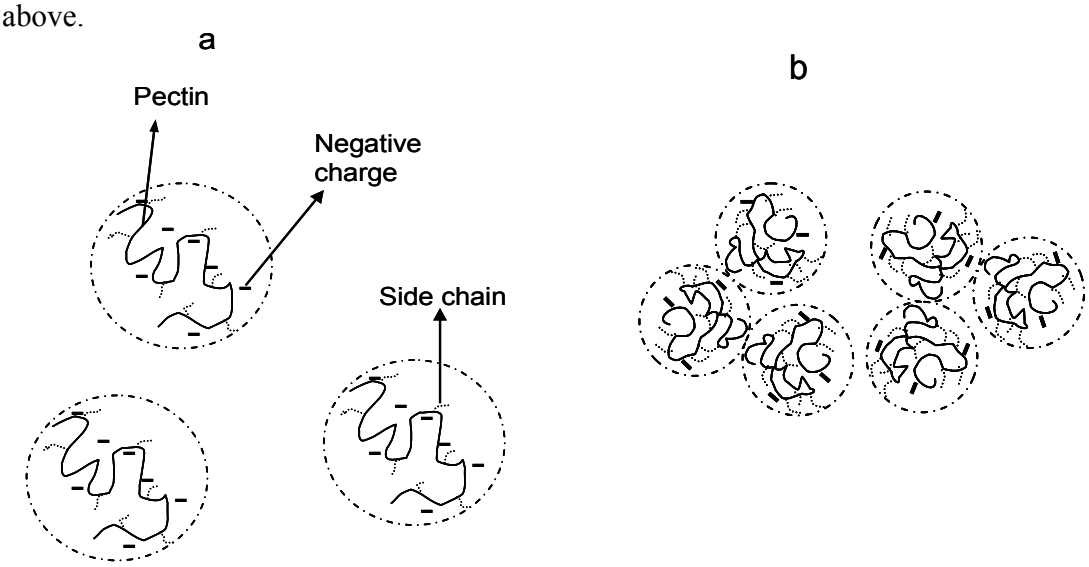


Figure 5. 6 Inter/Intramolecular repulsion in pectin molecules as a result of charge density: (a) MHF − strong repulsion (lower DE, high M_w, lower viscosity); (b) EHF − lower repulsion (higher DE, low M_w, higher viscosity) and more chain−chain interactions.

As shown in Figure **5.7**, gold kiwifruit pectins exhibited a gel-like behaviour; the elastic modulus dominated over the viscous component (*G*") in the whole frequency range tested at 1.0% pectin (GalA) concentration. These frequency sweeps also showed a higher elastic modulus (*G*') for water-extracted EHF pectin than for water-extracted MHF pectin (4.15 and 2.12 Pa for EHF and MHF pectins, respectively at 5.36 Hz). This could imply a higher degree of intermolecular association for EHF pectin than for MHF pectin. The higher network connectivity derived from the pectin associations (higher *G*') probably resulted in the higher viscosity values obtained by rotational measurements.

Additionally, to reinforce the rheological changes when comparing pectin, the viscoelasticity of these two pectins was compared to commercial citrus (DE 61%) and apple pectins (DE 55%) both of similar M_w. The elastic modulus of pectin samples of similar M_w increased when the DE increased (Figure **5.7**). For example, *G*' (at 5.36 Hz) increased from 0.12 Pa for commercial apple pectin (~ DE 55%, M_w 1.70 x 10^5 g/mol) to 0.60 Pa for commercial citrus pectin (~ DE 60.97%, M_w 1.66 x 10^5 g/mol). Apple pectin showed less

chain−chain association than citrus pectin at similar concentration because its *G*" dominated over its *G*' within the frequency range measured.

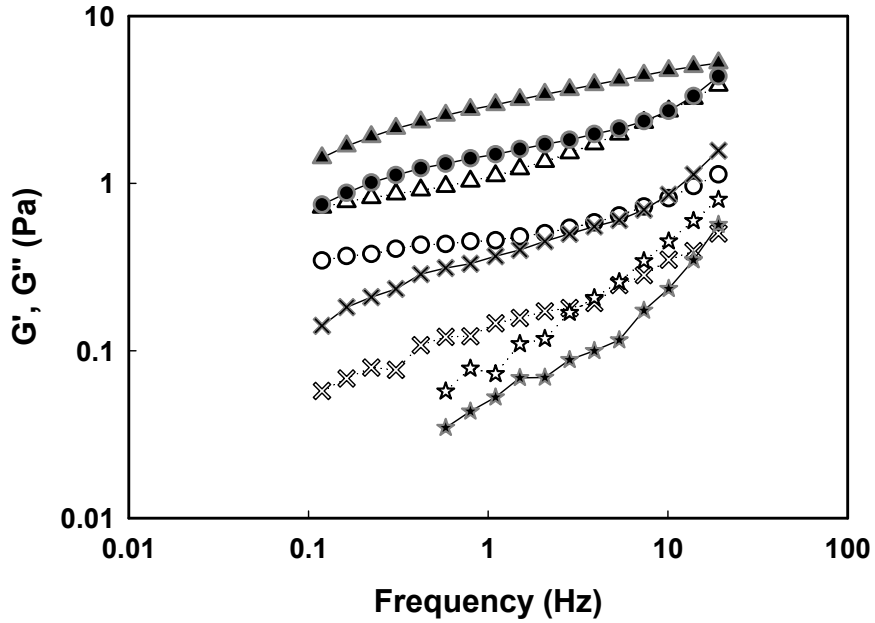


Figure 5. 7 Viscoelastic profiles of gold kiwifruit pectins: (▲) water-extracted EHF pectin; (●) water-extracted MHF pectin; (x) commercial citrus pectin (Sigma, P9135); (★) commercial apple pectin (Herbstreith and Fox KG). Prepared at 1.0% w/w GalA concentration in Milli-Q water, pH 3.50 ± 0.01. *G*', filled symbols; *G*", empty symbols.

5.4 Conclusions

This study has shown that the fruit maturity and the extraction method had significant effects on the yield of pectin, its composition and its rheological behaviour. EHF pectin had a lower yield and GalA content but exhibited higher viscosity than MHF pectin. Water-extracted MHF and EHF pectins had higher M_ws than other extracts, indicating that water extraction did not alter the native form of the pectin in gold kiwifruits. This study demonstrated the importance of the fruit maturity and the extraction method; they could have a significant impact on the physicochemical and functional properties of gold kiwifruit pectin. Pectin was also isolated from gold kiwifruit pomace for comparison purposes (see Appendix **E**). Its physicochemical properties were evaluated and were compared with those of pectin isolated from whole kiwifruit.

CHAPTER 6 Gelation Properties of Gold Kiwifruit Pectin

6.1 Introduction

It is well known that pectin has the functional ability to form a gel. This is one of the main reasons why pectin is used in the food industry, specifically as a gelling agent in fruit-based preserves such as jams, jellies and marmalades, confectionary products such as gum candies, and many yoghurts and dairy desserts (May, 1990; Rolin, 1993).

As previously described in section **1.5.1**, pectin is generally categorised into two types based on the degree of esterification (DE): high methoxyl pectin (HMP) with > 50% DE and low methoxyl pectin (LMP) with ≤ 50% DE. In general, HMP forms a gel in an acidic environment (typically pH ~ 3) and in the presence of low molecular weight co-solutes, such as sucrose, at concentrations of ~ 65% (Evageliou*, et al*., 2000). In contrast, LMP forms a gel in the presence of ionic calcium (Turquois, *et al*., 1999). Whereas HMP cannot form a gel without a high sugar content environment or appropriate acidity, LMP can gel without the addition of sugar, but only in the presence of calcium (Chang & Miyamoto, 1992). This study focused only on the gelation of HMP. The gelation mechanism of HMP has been described briefly in the introduction (section **1.5.2.2.2**).

The presence of a co-solute in the HMP gelation process is very important. It has been shown in many studies (El-Nawawi & Heikel, 1997; Oakenfull & Scott, 1984; Tsoga, Richardson, & Morris, 2004); that a co-solute such as sucrose can stabilise the hydrophobic interactions between the methyl ester groups and that HMP will not gel if the sucrose as co-solute has a concentration less than ~ 65%. The co-solute aids in the formation of the network by competing with pectin for hydrogen bonding to water, by lowering the water activity and by creating conditions under which hydrophobic interactions are likely to occur. Chang (2000) reported that water solutions containing mono- or disaccharides have a lower vapour pressure and, thus, a lower water activity (a_w) than pure water.

It is well established in the literature (Oakenfull & Scott, 1984; Rolin, 1993; Walkinshaw & Arnott, 1981) that the gelation of HMP is the result of the intermolecular formation of junction zones. These junction zones are complex and the molecular structures are collectively held together by the interactions of several chains such as hydrophobic and hydrogen bonds. Ester groups are responsible for the hydrophobic interactions of HMP molecules. The energy contributed by these groups is associated with the contact between these hydrophobic areas and water — the hydrophobic areas tending to aggregate in order to minimise the contacting surface (similar to the coalescence of oil drops in water) (El-Nawawi & Heikel, 1997). The hydrogen bonds that form between adjacent galacturonan chains contribute more (twice as much) free energy to the formation of junction zones than hydrophobic interactions. However, hydrophobic interaction is an essential requirement because hydrogen bonding alone is insufficient to stabilise the junction zones (Oakenfull & Scott, 1984).

The properties of HMP gelation are directly correlated with the properties of the junction zones because these junction zones have the most important role in strengthening and stabilising the gel. They are significantly influenced by factors such as the concentration, DE and molecular weight (M_w) of the pectin as well as the pH of the solution. Other parameters that are generally considered to be important are ionic strength, water activity, sugar type and cooling rate of the gelation process (da Silva & Rao, 2006). Increasing pectin concentration as well as M_w will increase the final gel strength because a larger number of junction zones are formed by galacturonic acid (GalA) units. Rascon-Chu *et al*. (2009) revealed that increasing the concentration of apple pectin from 2% to 3% (w/v) increased the gel hardness to almost double. The DE has a marked impact on gel strength, and on the time and temperature required for gelation to occur (El-Nawawi & Heikel, 1997). Generally, the gel strength increases as the DE decreases, because pectin with a higher number of charges has more active chains to form an intermolecular network. HMP with a DE of 70% or higher will form gels at relatively high temperatures or near boiling, whereas HMP with a DE in the range 50−70% will form gels at lower temperatures.

Pectins generated from different plant sources have different functional properties; in particular, their gelation abilities can vary significantly. Lime, lemon peel and apple pomace are the main sources of pectin and have been reported to contain a particularly large amount of high quality pectin (May, 1990; Rolin, 1993). Gold kiwifruit pectin has been found (Chapter **5**) to possess a high DE in the range 82−90%. To date, there have been no studies on the characterisation of its gelling properties.

The objective of this study was to investigate the gelation properties of gold kiwifruit pectin by varying four different parameters: maturities, pectin concentration, pH of the solution and sucrose concentration. The textural and rheological properties of the gels were studied. Gold kiwifruit pectin gels were compared with gels made from commercial apple and citrus pectins.

6.2 Materials and Methods

6.2.1 Pectin Stock Solution

Three types of pectin were used in this investigation: gold kiwifruit pectin (GKP), apple pectin (AP) and citrus pectin (CP). The DE varied as follows.

- DE 90% pectin was obtained by a water extraction method from early-harvested gold kiwifruit (DE 90% EH-GKP).
- DE 84% pectin was obtained by a water extraction method from main-harvested gold kiwifruit (DE 84% MH-GKP).
- Un-standardised commercial APs were obtained from Herbstreith and Fox KG, Germany, with the following DE values: 55% (DE 55% AP), 65% (DE 65% AP) and 75% (DE 75% AP).
- Un-standardised commercial CP (P9561) was obtained from Sigma Aldrich, UK, with a DE of 90% (DE 90% CP).

The specification of the pectins is shown in Table **6.1**. A pectin stock solution (4% w/w) was prepared by dissolving pectin powder, based on GalA content, in deionised (RO) water under constant stirring at 60ºC for 1 h. The solution was left under agitation at 4ºC overnight to ensure good dispersibility. The final pH of each pectin solution was adjusted to pH 3.0 by the addition of 0.1 M HCl or 0.1 M NaOH.

Table 6. 1 GalA composition of HMPs

Pectin	DE (%)	GalA Concentration (%)
DE 55% AP	55	83
DE 65% AP	65	82
DE 75% AP	75	79
DE 84% MH-GKP	83	77
DE 90% EH-GKP	90	46
DE 90% CP	90	75

6.2.2 Sucrose Solution

Food-grade sucrose was obtained from Chelsea, New Zealand Sugar Ltd. In order to achieve a solution of high sugar content (75% w/w), a mixture of sucrose and glucose (Chelsea) at a ratio of 70% sucrose to 30% glucose on a dry basis was dissolved in RO water at room temperature under constant stirring, as described in section **1.5.2.2.1**. Addition of glucose to sucrose has been reported by Bhandari and Hartel (2002) to aid in the solubility of a high concentration of sucrose by preventing crystallisation.

6.2.3 Pectin Gelation at Various pHs and Fixed Sucrose and Pectin Concentrations

HMP gels were formed based on the method of Rascon-Chu *et al*. (2009) with a slight modification. Table **6.2** shows the gel composition at each pH. The concentrations used in this study are expressed as weight percentages (% w/w). A mixed solution containing fixed concentrations of pectin (1.0%) and sucrose (45%) was used. A certain amount of water was added to obtain 40 g samples and the mixture was stirred for 10 min. In order to achieve the desired pH (1.5, 1.7, 2.0 and 2.5), a certain amount of citric acid (CA) powder (food grade, obtained from Hawkins Watts Limited, New Zealand) was added during

heating (Table **6.2**). The mixed solution was heated at 95ºC in a water bath for approximately 15 min. Continuous stirring during heating was maintained to obtain a homogeneous and consistent gel. The exact amount of CA used at each pH was based on preliminary trials. The relationship between the amount of CA and the final pH of the gel is illustrated in Figure **6.1**. The most extreme pH obtained was pH 1.5, with the addition of 3.1 g of CA. Lubricated cylindrical moulds (1.8 cm diameter, 2.1 cm height) (Figure **6.2**) were filled with the hot mixture and left to set at room temperature. The samples were covered with lubricated foil to minimise evaporation and were left for ~ 18 h at 4ºC before analysis.

Table 6. 2 Pectin gel formulation at various pHs

pH	Pectin solution (g) (4% w/w stock solution, pH 3.0 ± 0.05)	Sucrose solution (g) (75% w/w stock solution)	CA (g)	Water (g)	Final Weight (g)
1.5	10	24	3.1	2.9	40
1.7	10	24	2.4	3.6	40
2.0	10	24	0.6	5.4	40
2.5	10	24	0.2	5.8	40

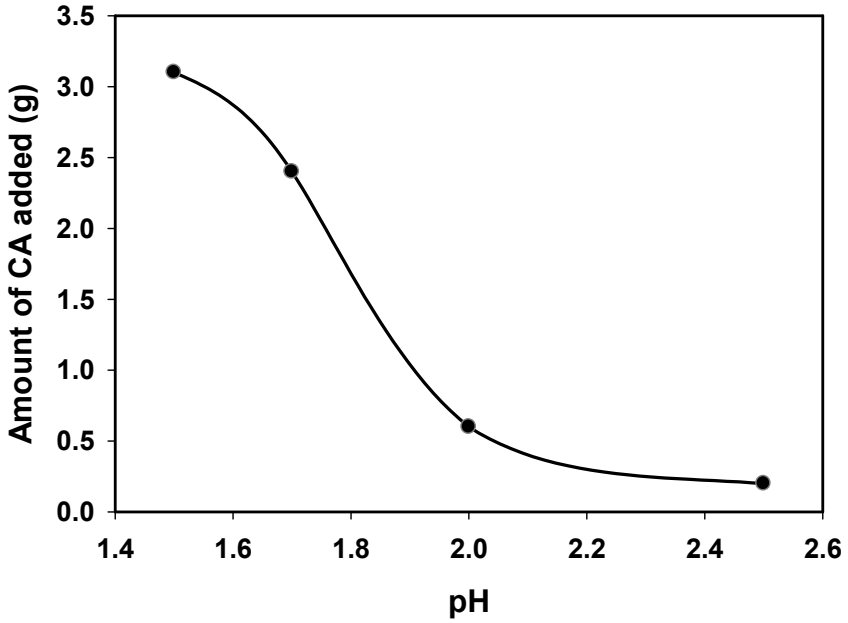


Figure 6. 1 Effect of CA addition (g/40 g pectin + sucrose solution) on the average gel pH (1.0% pectin and 45% sucrose concentration).

6.2.4 Pectin Gelation at Various Pectin Concentrations

Pectin concentrations of 0.5 and 1.0% were used, at a fixed sucrose concentration (45%) and a fixed pH (2.0 ± 0.01). The procedure was similar to that described in section **6.2.3**. The gel formulations are outlined in Table **6.3**.

Table 6.3 Pectin gel formulations at different pectin concentrations

Pectin Concentration (%)	Pectin solution (g) (4% w/w stock solution, pH 3.0 ± 0.05)	Water (g)	Sucrose solution (g) (75% w/w stock solution)	CA (g)	Final Weight (g)
0.5	5	10.6	24	0.4*	40
1.0	10	5.4	24	0.6*	40

*Amount of CA added to obtain pH 2.0.

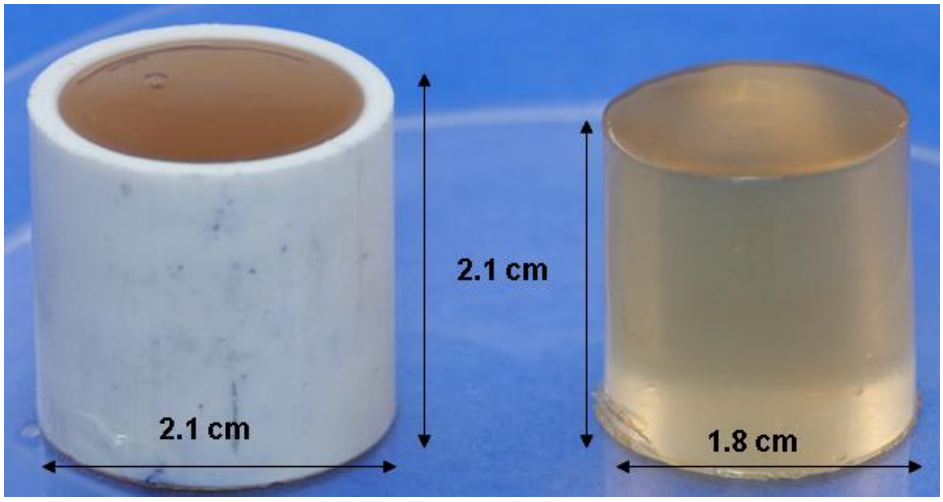


Figure 6. 2 Dimensions of the cylindrical mould and the gel formed.

6.2.5 Pectin Gelation at Various Sucrose Concentrations

The gel formation procedure was similar to that described in section **6.2.3**. The sucrose concentration was varied from 40 to 50% at a constant pH (2.0 ± 0.01) and a fixed pectin concentration (1.0%). The gel formulations are outlined in Table **6.4**.

Table 6.4 Pectin gel formulations at various sucrose concentrations

Sucrose Concentration (%)	Pectin solution (g) (4% w/w stock solution, pH 3.0 ± 0.05)	Water (g)	Sucrose Solution (g) (75% w/w stock solution)	Final Weight (g)
40	10	8.7	21.3	40
45	10	6.0	24.0	40
50	10	3.3	26.7	40

6.2.6 Compression Test

The gel hardness was determined with a two-bite compression test using a TA.XTplus texture analyser (Stable Micro Systems, England) with a 50 kg load cell. This test was carried out after the gels had set (~ 18 h) in 4ºC storage. Prior to measurement, the gels were equilibrated to room temperature. The analysis was conducted at room temperature, using a plastic cylindrical probe of 5 cm diameter. The pre-test and post-test speeds were set at 1 mm/s and 5 mm/s respectively. The samples were compressed to a target deformation of 75% based on the initial height, using a trigger force of 0.04903 N. All measurements were carried out in triplicate. The measurement set-up is illustrated in Figure **6.3**.

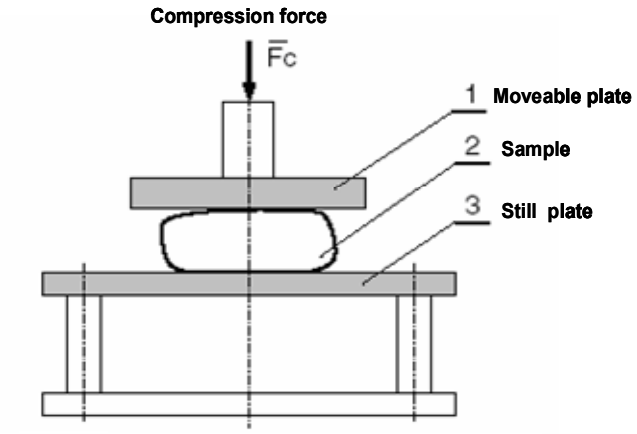


Figure 6. 3 Illustration of the two-bite compression test stand.

6.2.7 Rheological Measurements

The rheological behaviour of the gels was examined using a Paar Physica MCR 301 rheometer (Anton-Paar, GmbH, Germany). Small deformation, oscillatory measurements were performed using a cup and bob measuring system (C-CC27/T200 Cup, B-CC27/Q1 Bob). *G*' and *G*" were monitored during cooling from 96 to 4ºC at a constant frequency of 1 Hz, 0.5% strain (within the linear viscoelastic region) and a cooling rate of 3ºC/min. Samples were loaded at 96ºC and were coated with a thin layer of mineral oil to prevent evaporation during the test. In order to study the gelation behaviour during storage, immediately after cooling, some samples were stored at 4ºC for 13 h under oscillation, also at 0.5% strain and 1 Hz frequency. The large deformation behaviour of all samples was studied using an amplitude sweep test, by applying strains between 0.01 and 1000% at 20ºC and 1 Hz.

6. 3 Results and Discussion

6.3.1 Molecular Characterisation of Pectin and Initial pH

Table **6.5** shows the DE, M_w and initial pH of the pectin solutions used. The pectin M_w varied from 0.04 to 3.75 (10^6 g/mol), with the highest value corresponding to DE 84% MH-GKP and the lowest value corresponding to DE 90% CP. Knowledge of the M_w of pectin is very important in HMP gelation, because the M_w is well correlated with the properties of the junction zones. The longer are the pectin chains, the greater is the number of junction zones formed (da Silva & Rao, 2006). A gel formed by shorter pectin chains is broken more easily than a gel formed by longer pectin chains, because the latter is more flexible and the networks remain cross linked after the breaking test because of the greater number of junction zones (Crandell & Wicker, 1986).

The pH of 4% pectin solutions ranged from approximately 3.0 to 5.30. Commercial AP and CP solutions had similar pHs of 3.0 ± 0.05. In contrast, the pHs of water-extracted GKPs were 4.30 and 5.30 for DE 84% MH-GKP and DE 90% EH-GKP respectively. The pH differences between the commercial pectins and GKP are likely to derive from the

technique used for pectin extraction. For example, GKP solution (1.0% w/w) had a pH value around 3.5 when extracted using CA (Chapter **3**), compared with the higher values obtained using water extraction. Similar results have been observed in the literature; for example, the pHs of pumpkin pectin solution (1.0%) extracted using acid and using enzyme have been reported by Ptitchkina *et al*. (2008) to be 3.2 and 5.2 respectively. In addition, pH differences between GKPs themselves and also compared with commercial pectins may reflect differences in the DE and *pKa* values of the pectins. The term *pKa* is associated with the acid dissociation constant and typically pectin has a *pKa* value of ~ 3.5 (Wang, *et al*., 2002). Rolin, Nielsen and Glahn (1998) and Ralet, Crepeau, Buchholt and Thibault (2003) reported that *pKa* values depend on the negative charge density of the pectin; the *pKa* decreases with increasing DE. The higher pH value obtained for the GKP extracted from early-harvested fruit may correspond to its higher DE value (90%) and probably lower *pKa* compared with the GKP extracted from main-harvested fruit (DE 84%).

Table 6.5 Molecular characterisation and initial pH of pectins

Pectin	DE (%)	M_w (10^6 g/mol)	pH of 4% w/w solution (± 0.05)
DE 55% AP	55	0.17 ± 0.02	2.95
DE 65% AP	65	0.61 ± 0.05	2.90
DE 75% AP	75	0.63 ± 0.05	3.00
DE 84% MH-GKP	84	3.75 ± 0.11	4.30
DE 90% EH-GKP	90	1.03 ± 0.03	5.30
DE 90% CP	90	0.04 ± 0.003	2.98

Mean ± standard error (n = 2)

6.3.2 Effect of pH on Gel Hardness

The gel hardness was expressed in Newton (N), which is the unit of force. The term gel hardness is associated with the texture of the gel, and, in this study, is defined as the force required for the compression of a gel with dimensions 1.8 cm diameter and 2.1 cm height, using a cylindrical probe of 5 cm diameter (Ebrahimi, Alemzadeh, & Seifkordi, 2007). Figure **6.4** shows the effect of pH (1.5−2.5) on the hardness of gels prepared with different pectins. This pH range was tested because, at pH above 2.5 (*i.e.* 3.0, 3.5 and 4.0), either the gels produced were very weak for a reliable compression test or there was no gel formation,

as in the case of DE 90% EH-GKP. In general, maximum gel hardness was obtained at pH 1.7 for APs, with a sharp reduction in hardness observed above and below this pH, regardless of the DE value. However, the DE 84% MH-GKP gel appeared not to be significantly affected by pH variation and formed the weakest gel between pH 1.5 and pH 2.5 compared with the AP gels.

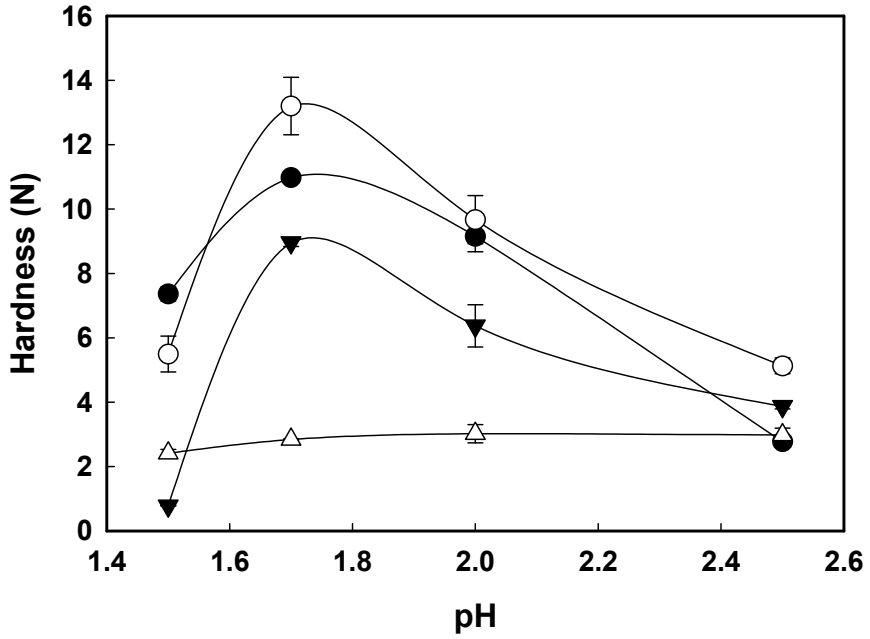


Figure 6. 4 Effect of pH on the hardness of gels prepared with different pectins: (●) DE 55% AP; (○) DE 65% AP; (▼) DE 75% AP; (Δ) DE 84% MH-GKP (1.0% pectin and 45% sucrose concentration).

Pectin gel formation is based on hydrogen bonding and hydrophobic interaction between methyl ester groups (Oakenfull & Scott, 1984). Because of their polyelectrolyte nature, the properties of pectin gels are affected by pH and ionic strength (da Silva & Rao, 2006). Under neutral or less acidic conditions, most free carboxyl groups are negatively charged (COO^-). These charges create repulsive forces that can be strong enough to prevent the formation of a pectin network (Rascon-Chu, *et al*., 2009) via hydrogen and hydrophobic interactions. For example, a reduction in pectin gel (DE 70.3%) viscoelasticity (*G*" and *G*' moduli) was observed by Evageliou *et al*. (2000) as the gel pH was increased above the *pKa* (~ 3.4). As the pH is reduced, the repulsive forces between free carboxyl groups are weaker

because there are fewer charges on the pectin chains (the carboxyl groups are unionised, COOH); therefore more chain–chain interactions are possible, promoting association and gelation of the system (Rascon-Chu, *et al*., 2009). However, further reduction in the pH, in this case below the optimum pH level (pH < 1.7), results in a decrease in gel hardness. A possible reason for this is that, at very acidic pHs, the low repulsion between free carboxyl groups leads to chain-chain interactions occurring too rapidly to achieve a well-arranged polymer network. Therefore, localised aggregations and non-homogeneous gelation are likely to occur, leading to weaker gels (El-Nawawi & Heikel, 1997).

It is important to note that, in the case of DE 84% MH-GKP, variation in the pH had almost no effect on the gel hardness. An almost undetectable maximum was observed at pH 2.0. This phenomenon probably follows the typical behaviour of very low charged pectin networks, which may not be strongly influenced by decreasing or increasing pH. This may also reflect the different *pKa* values of the GKP.

The DE plays the most important role in pectin gelation (da Silva & Rao, 2006). In this study, the influence of the DE on the gel properties was clear; the gel hardness decreased with increasing DE. This observation is in agreement with data obtained by Baker *et al*., (2005), where the gel strength increased with increasing charge density, and will occur progressively only at lower pH (Smit & Bryant, 1968). With the exception of DE 55% pectin, DE 65, 75, and 84% pectins showed this trend, particularly at the optimum pH (1.7). DE 55% pectin formed weaker gels than those prepared using DE 65% pectin. This could have been due to the lower M_w of the pectin. da Silva and Rao (2006) reported that the higher the M_w of the pectin, the stronger is the gel. DE 65% pectin presumably has longer chains (M_w = 0.61 x 10^6 g/mol) than DE 55% pectin (M_w = 0.17 x 10^6 g/mol); as a consequence, DE 65% pectin may generate a greater number of junction zones, leading to stronger gels. However, the pectin DE level seems to be the most important factor in determining the gel strength, given the weaker gels obtained with the high M_w pectins from gold kiwifruit (DE 84% MH-GKP, M_w = 3.75 x 10^6 g/mol).

Overall, the gels prepared from very low charged pectins (DE 90%), such as CP and EH-GKP, tended to form very weak gels that could not be subjected to a compression test. These findings may be explained by the formation of very inhomogeneous gels, with localised aggregation because there is hardly any electrostatic repulsion between the pectin chains.

6.3.3 Effect of Pectin Concentration on Gel Hardness

Because of limited quantities of the extracted GKP, only two levels of pectin were tested to study the influence of pectin concentration on the gel hardness at four different DEs (55, 65, 75 and 84%). GKP and CP with DE 90% were not included in this experiment because these pectins had a tendency to develop very weak gels at these pectin concentrations. The sucrose concentration (45%) and the pH (2.0 ± 0.01) were held fixed. This pH was chosen based on the earlier test in which the gel hardness of GKP was found to be slightly higher at pH 2.0 than at other pHs, and because differences between the other pectins could still be observed. In all cases, the hardness of the pectin gels was enhanced on increasing the pectin concentration from 0.5 to 1.0% (Figure **6.5**). However, the influence of concentration was more marked in the gels formed from low DE pectins (55 and 65%), as shown by the greater slopes.

An increase in the gel hardness with increasing pectin concentration is widely reported in the literature (da Silva & Rao, 2006, Rascon-Chu, *et al*., 2009; Rao, van Buren & Cooley, 1993). When the pH and the sucrose concentration are kept constant, an increase in the number of pectin molecules that are able to interact to form the junction zone network leads to stronger gels. With respect to the impact of the DE of the pectin, a similar trend to that described earlier (section **6.3.1**) was observed; the gel hardness decreased with increasing DE, with the weakest gel formed by DE 84% MH-GKP. The gels formed by DE 90% EH-GKP and DE 90% CP were too weak to perform compression tests.

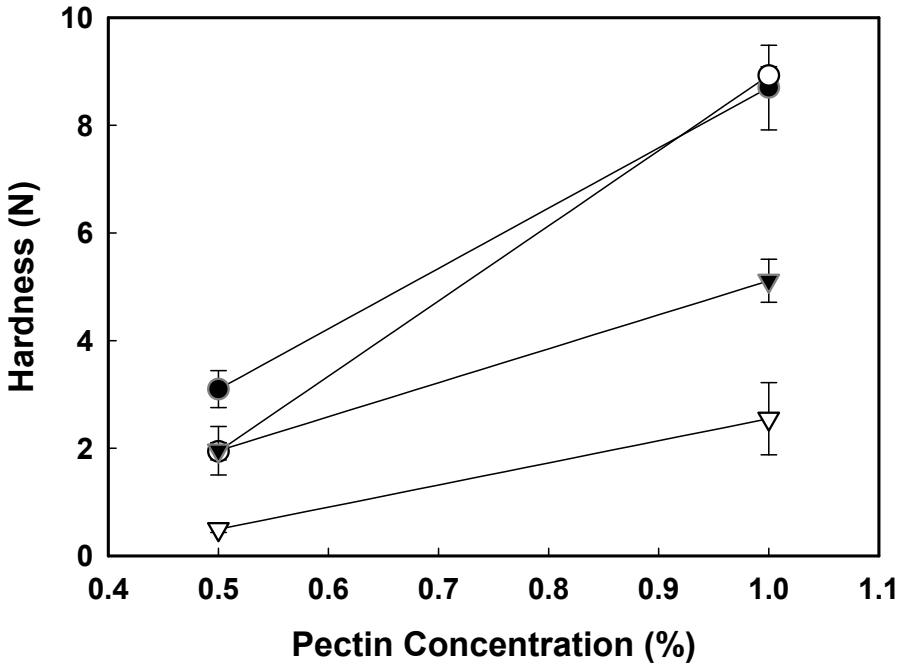


Figure 6. 5 Effect of pectin concentration (w/w) on the hardness of gels prepared with different pectins: (●) DE 55% AP; (○) DE 65% AP; (▼) DE 75% AP; (▽) DE 84% MH-GKP (pH 2.0 ± 0.01, 45% sucrose concentration).

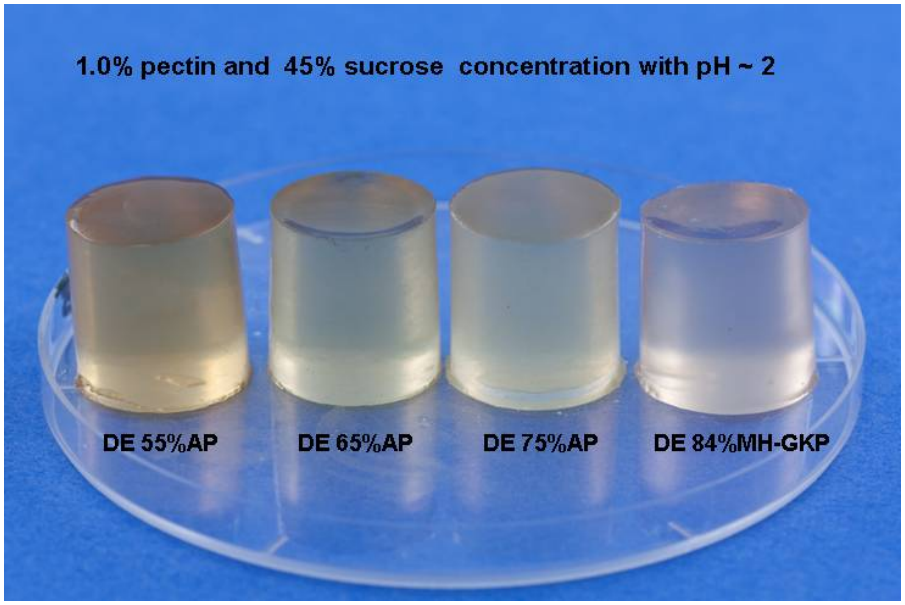


Figure 6. 6 GKP gel (DE 84% MH-GKP) compared with AP gels (DE 55, 65, 75% APs), prepared at pH 2.0 ± 0.01, 1.0% pectin concentration and 45% sucrose concentration.

The pectin concentration had similar effects on the gels prepared from DE 84% MH-GKP and DE 75% AP (slope value). The slope was significantly lower than for the gels prepared from DE 55% AP and DE 65% AP. This may indicate that, when there is a reduction in the negative charge density of the pectins (as for DE 75% and DE 84% pectins), more pectin chains are needed to have an impact on the gel strength. Figure **6.6** shows the relatively similar physical appearances of the "weak" GKP gel and the gels made from APs (1.0% pectin, 45% sucrose and pH 2.0).

6.3.4 Effect of Sucrose Concentration on Gel Hardness

Sucrose was used as a co-solute in this study, because it is commonly found in gel preparations in many food formulations (Rao, *et al*., 1993). Additionally, sucrose normally gives an optimum gel strength compared with other co-solutes (Tsoga, *et al.*, 2004). In preliminary studies, the influence of sucrose concentrations below 40% (30 and 35%) on pectin gelation was investigated. However, no gel was formed for most of the pectins (DE 55% AP, DE 65% AP, DE 84% MH-GKP, DE 90% CP and DE 90% EH-GKP) and a weak gel was observed for DE 75% AP. Therefore, under the conditions of the current study, a minimum of 40% sucrose was required for gel formation. A sucrose concentration range between 40 and 50% was then tested to study its influence on gel hardness. Figure **6.7** shows the hardness of gels made from various DE pectins, at constant pH (2.0 ± 0.01), constant pectin concentration (1.0%) and various sucrose concentrations.

In general, all pectins were significantly affected by sucrose concentration. A maximum gel strength was observed after the addition of 45% sucrose for DE 55% AP and DE 84% MH-GKP. Below and above this concentration, the gels were significantly weaker, whereas DE 65% pectin gels presented a maximum gel strength (9–8.9 N) in the range 45–50% sucrose. Gels prepared using DE 75% AP showed a completely different behaviour within the sucrose range studied. The gel hardness decreased continuously from 10 N at 40% sucrose to ~ 1 N at 50% sucrose. Presumably, maximum hardness corresponded to a sucrose concentration of ≤ 40%. As this is a commercial pectin, and therefore extraction techniques and possible physicochemical modifications are unknown, it is difficult to speculate on the

origin of the different behaviour. Certainly, the sucrose concentration required to yield a strong pectin network was significantly lower, compared with the other pectins.

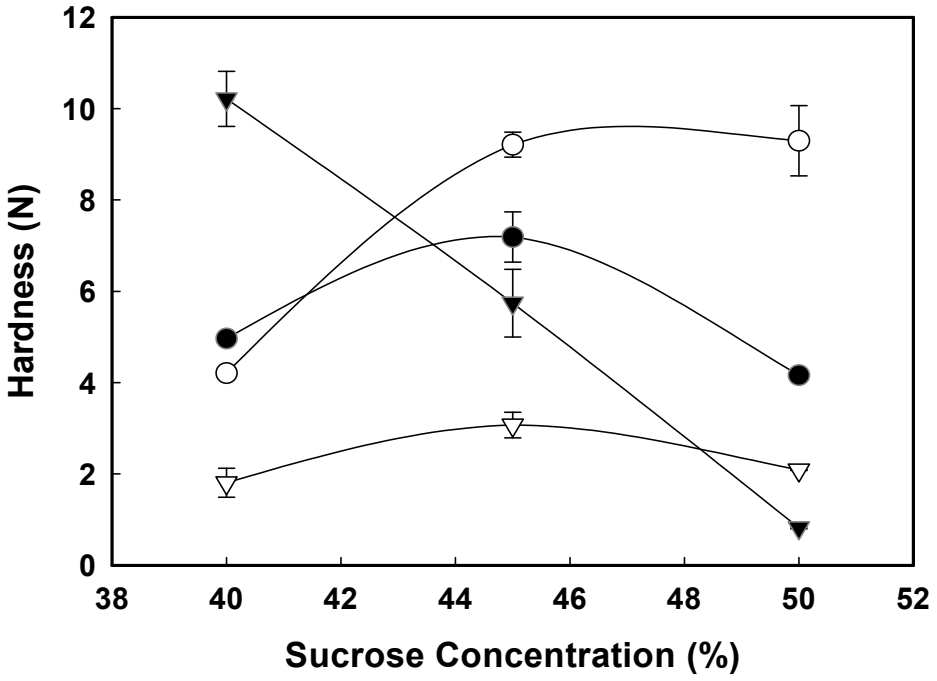


Figure 6. 7 Effect of sucrose concentration (w/w) on the hardness of gels prepared with different pectins: (●) DE 55% AP; (○) DE 65% AP; (▼) DE 75% AP; (∇) DE 84% MH-GKP (pH 2.0 ± 0.01 and 1.0% pectin concentration).

A previous study (da Silva, 1994) on the influence of sucrose concentration on the viscoelasticity of HMP at a fixed pectin concentration and pH demonstrated that an increase in the sucrose concentration from 30 to 55% influenced or significantly increased only the viscous (*G*") character of the final gels. Only high concentrations of sucrose, above 55%, had an impact on the gel strength, as represented by an increase in the elastic modulus (*G*'). However, the fractural properties of these pectin gels were not studied and it is probable that sucrose influences the large deformation properties of the gels, even though the elastic moduli obtained by small deformation oscillatory testing were not significantly affected by a wide range of sucrose concentration.

It is well known that HMP gels are stabilised mainly by hydrogen bonds and hydrophobic interactions (Oakenfull & Scott, 1984). According to Walkinshaw and Arnott (1981), hydrophobic interactions involve mainly the esterified groups and are enhanced by the presence of sucrose in the solution (Bulone, Martorana, Xiao, & San Biagio, 2002). In the presence of sucrose, the formation of the three-dimensional hydrogen-bonded structure in water is more developed because the hydrogen bonding between sucrose hydroxyl groups and water molecules is stronger (Matia-Merino, 2004). The addition of sucrose to HMP gels therefore promotes the formation of hydrophobic interactions between methyl ester groups of pectin, by forming hydrogen bonds with water and thus competing with pectin–water hydrogen bonds and pectin-pectin hydrogen bonds (Tsoga, *et al*., 2004).

In the current study, a sucrose concentration of ≥ 40% was necessary to promote the formation of sufficient stable junction zones to result in gelation. The existence of an optimum concentration of sucrose (*i.e.* 45% for DE 84% MH-GKP) for maximum gel hardness is related to the effects of an excess concentration of sucrose (above 50%): (*i*) strong competition for water between sucrose and pectin may hinder homogeneous network formation, resulting in weaker gels; (*ii*) localised pectin aggregation in the presence of high concentrations of sucrose may again result in the formation of a coarser gel, which requires less force under a compression test. This phenomenon is in agreement with the study conducted by Tsoga *et al*. (2004). These authors revealed that hydrophobic associations formed by heating in the presence of low co-solute concentrations (50 or 55% w/w) resulted in an increase in the pectin (DE 70%) gel strength. However, increasing the concentration further led to a significant decrease in gel strength as a result of excessive pectin aggregation. Overall, the GKP seemed to be the least affected by the variation in sucrose concentration, when compared with the commercial pectins, which was probably due to rapid pectin aggregation as a result of the hydrophobic interactions between the methyl ester groups.

6.3.5 Viscoelasticity Changes during Pectin Gelation: Temperature and Storage Effects

In order to study the rheological behaviour of GKPs, further small deformation oscillatory measurements were carried out to follow the development of the viscoelastic network and to compare GKPs with commercial APs and CP of known DE. This study also aimed to find a correlation between textural properties obtained from the compression test and viscoelastic properties determined by oscillatory measurements in a rheometer. Gels were prepared at pH 2.0 ± 0.01, at a pectin concentration of 1.0% and in the presence of 45% sucrose. Figures **6.8a** and **6.9a** show representative changes in the storage modulus *G*' and the loss modulus *G*" obtained when pectin solutions were cooled from 96 to 4ºC. Figures **6.8b** and **6.9b** illustrate the changes in both viscoelastic values when the gels were held at 4ºC for 13 h.

As described in section **2.2.4.2**, the storage modulus *G*' represents the elastic behaviour of a gel or the solid-like properties whereas the loss modulus *G*" relates to the viscous character of a gel. The presence of a polymer network can be detected when *G*' dominates over *G*", the material behaving more like a solid (elastic deformation). If *G*" is greater than *G*', the liquid-like behaviour dominates, this being typical of a polymer solution that has not set into a gel (Rao, 2007).

As observed in Figure **6.8a** for DE 84% MH-GKP, initially, the viscous character of the system dominated at high temperature (*G*" > *G*'). On cooling, when the gelation temperature (~ 36°C) was reached, the elastic modulus crossed over the loss modulus. However, for DE 90% EH-GKP, there was no cross-over between the storage modulus *G*' and the loss modulus *G*" of the pectin solution on cooling; rather a continuous increase in both moduli with decreasing temperature was observed, with *G*" dominating over the entire temperature range. Figure **6.9a** also illustrates the strong temperature dependence of the cross-over for DE 55% AP, DE 65% AP and DE 75% AP, with gelation temperatures of 25, 28 and 34°C respectively. However, DE 90% CP presented the profile typical of a viscoelastic network (*G*' > *G*") from very high temperatures (96°C), with both elastic and viscous parameters increasing on cooling.

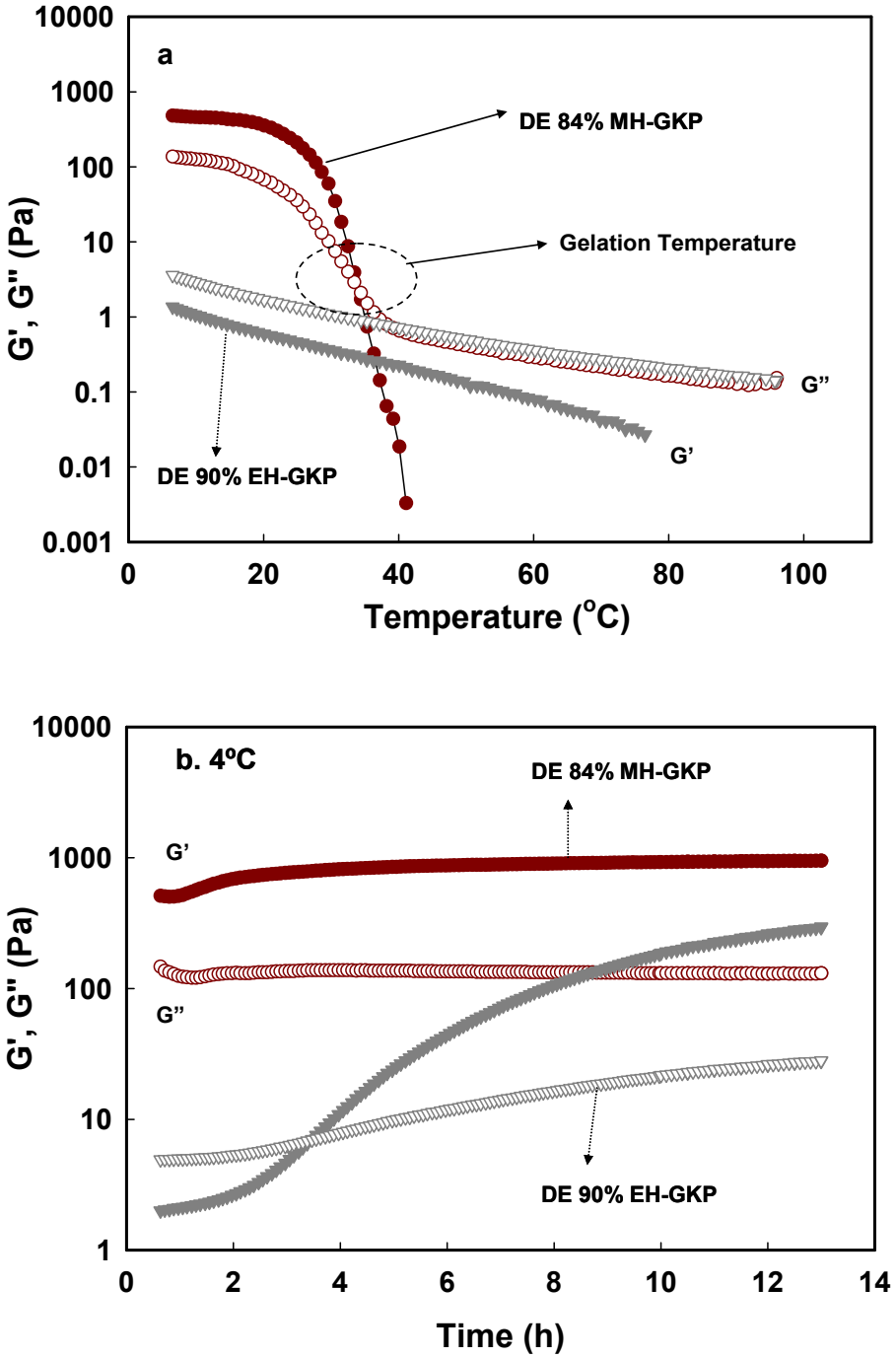


Figure 6. 8 Changes in *G'* (filled) and *G"* (empty) of 1.0% GKP [(●) DE 84% ; (▼) DE 90%]: (a) on cooling from 96 to 4ºC, at 1 Hz, 0.5% strain; (b) during storage for 13 h, at 4ºC, 1 Hz and 0.5% strain (pH 2.0 ± 0.01, 45% sucrose concentration).

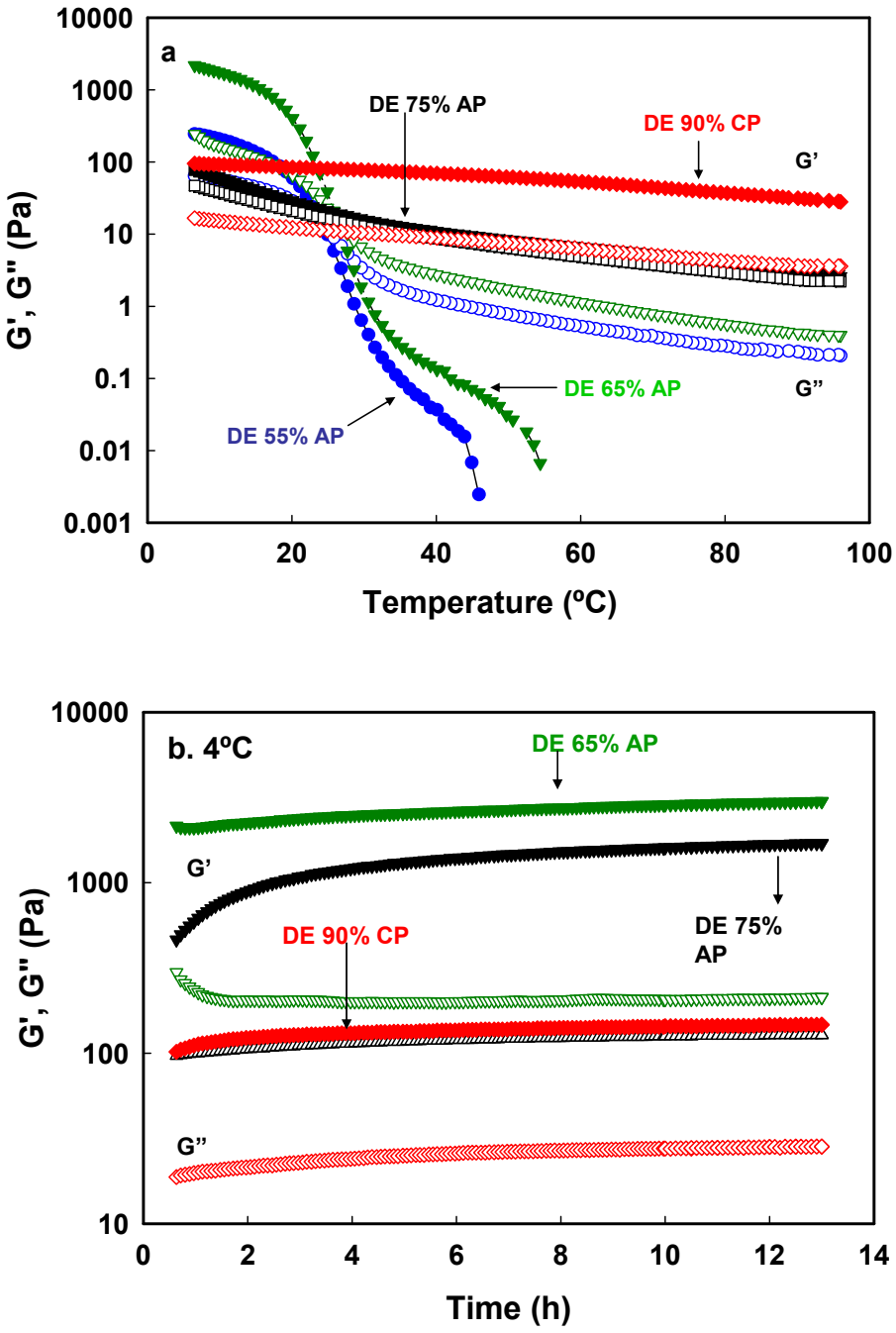


Figure 6. 9 Changes in *G'* (filled) and *G"* (empty) of 1.0% concentration of commercial AP and CP [(●) DE 55 AP; (▼) DE 65% AP; (▲) DE 75% AP; (◊) DE 90% CP]: (a) on cooling from 96 to 4ºC, at 1 Hz, 0.5% strain; (b) during storage for 13 h, at 4ºC, 1 Hz and 0.5% strain (pH 2.0 ± 0.01, 45% sucrose concentration).

According to da Silva, Rao and Fu (1998), both viscoelastic moduli increase during cooling as a consequence of the increasing density of junction zones in a polymer solution. However, *G*' increases more sharply until it crosses over *G*", indicating that the pectin solution has reached the gelation point, at which a sufficient number of cross-links give rise to the formation of a polymer network. The gelation point can be defined as the stage at which a liquid begins to exhibit pseudoplastic properties (Constenla, Ponce, & Lozano, 2002). In the current study, the gelation temperature could be easily identified by the cross-over of *G*' and *G*" and was the criterion followed here. This is one of the classical definitions of gelation time, known as the "cross-over method", which is also signalled by the phase angle crossing 45º (Matia-Merino, 2004). A cross-over on cooling was observed for all pectins studied, with the exception of the very high DE pectins (CP and GKP). Basically, when the mixed solutions of hot pectin and sucrose were cooled, progressive association of GalA units occurred, forming larger associations or clusters until the critical temperature was reached, as described previously by Constenla *et al*. (2002). Beyond this point, the elasticity increased sharply, indicating a significant effect of temperature on the gel network and structure (Angioloni & Collar, 2009). It is believed that, after reaching the gelation point, the *G*' modulus keeps rising gradually as a consequence of a continuous, slower formation and rearrangement of the junction zones, until eventually both moduli *G*' and *G*" reach a pseudo-plateau region (da Silva, *et al*., 1998).

It has been widely reported in the literature (Rolin & de Vries, 1990; Rascon-Chu, *et al.*, 2009) that increasing the DE leads to a faster gelation and therefore to a higher gelling temperature of the pectin. Low charged pectins or low electrostatic repulsive forces between the pectin chains can induce a faster gelling process. The results found here are in agreement with this because the low charged/high DE pectins exhibited more rapid gelation than the high charged/low DE pectins. For example, DE 84% MH-GKP gelled faster (~ 36°C) than DE 75% AP, DE 65% AP and DE 55% AP, with gelling temperatures of 34, 28 and 25°C respectively.

The high DE CP (DE 90%) behaved significantly differently from the other pectins, as the elasticity or *G*' value of the "solution" was greater than the *G*" value (see Figure **6.9a**) from very high temperatures (~ 96ºC). This indicates that this pectin exhibited gel-like properties even before it was cooled. This can be attributed to pectin aggregation effects contributing to the development of viscoelasticity. The aggregation can be attributed to two factors: (*i*) the very low M_w (0.04 x 10^6 g/mol) of this pectin, significantly lower than that of the other pectins, may have influenced the aggregation phenomenon by speeding up the hydrophobic interactions between easily accessible esterified groups on the short chains; (*ii*) the high DE value of the pectin itself may have induced a fast gelling/aggregation process as explained previously, even at very high temperatures. However, the gel formed by this pectin showed severe syneresis under storage (see Figure **6.10b**). Syneresis is defined as the shrinkage of a gel, which occurs concomitantly with expulsion of the continuous phase (Lucey, *et al*., 1998). This suggests that the molecular interactions were not strong enough to maintain the gel network structure. It is very likely that this pectin gel was not very homogeneous and that the large original aggregates (or "particles") signalled by the initial viscoelasticity resulted in the formation of a coarse gel that was prone to syneresis, a phenomenon more commonly observed in traditional particle gel networks. However, no changes in viscoelasticity were observed during storage of the gel at 4ºC for 13 h (see Figure **6.9b**), suggesting that the spontaneous syneresis may have occurred after this period.

In contrast, DE 90% EH-GKP, of similar high DE or charge density to the CP just described, did not develop a viscoelastic network on cooling (see Figure **6.8a**) but set into a gel after ~ 3 h when held at 4ºC (see Figure **6.8b**). This indicated that a slow formation of junction zones between the pectin chains occurred, showing more complex gelation behaviour. This GKP has a higher M_w (1.03 x 10^6 g/mol) than DE 90% CP. Additionally, this type of pectin has a higher concentration of neutral sugars than DE 90% CP (Chapter **5**). It seems reasonable to hypothesise that this could be one of the reasons for the "delay" in gelation—the disruption of junction zone formation because of the presence of neutral sugars, such as rhamnogalacturonan I or xylogalacturonan, in the structure; the junction zones in a gel are normally formed between un-branched pectin chains. The occurrence of hairy regions may affect the chain association, which could lead to size limitation of the

junction zones and, as a result, intermolecular arrangements might take time (BeMiller & Whistler, 1996; Perez, Flores, Marangoni, Gerschenson, & Rojas, 2009). The viscoelasticity of the gel appeared to continue to increase, even after 13 h at 4ºC (Figure **6.8b**). However, after storage, the resulting gel was also weak and unable to support its own weight (Figure **6.10a**), although the syneresis was reduced when compared with the DE 90% CP gel (Figure **6.10b**).

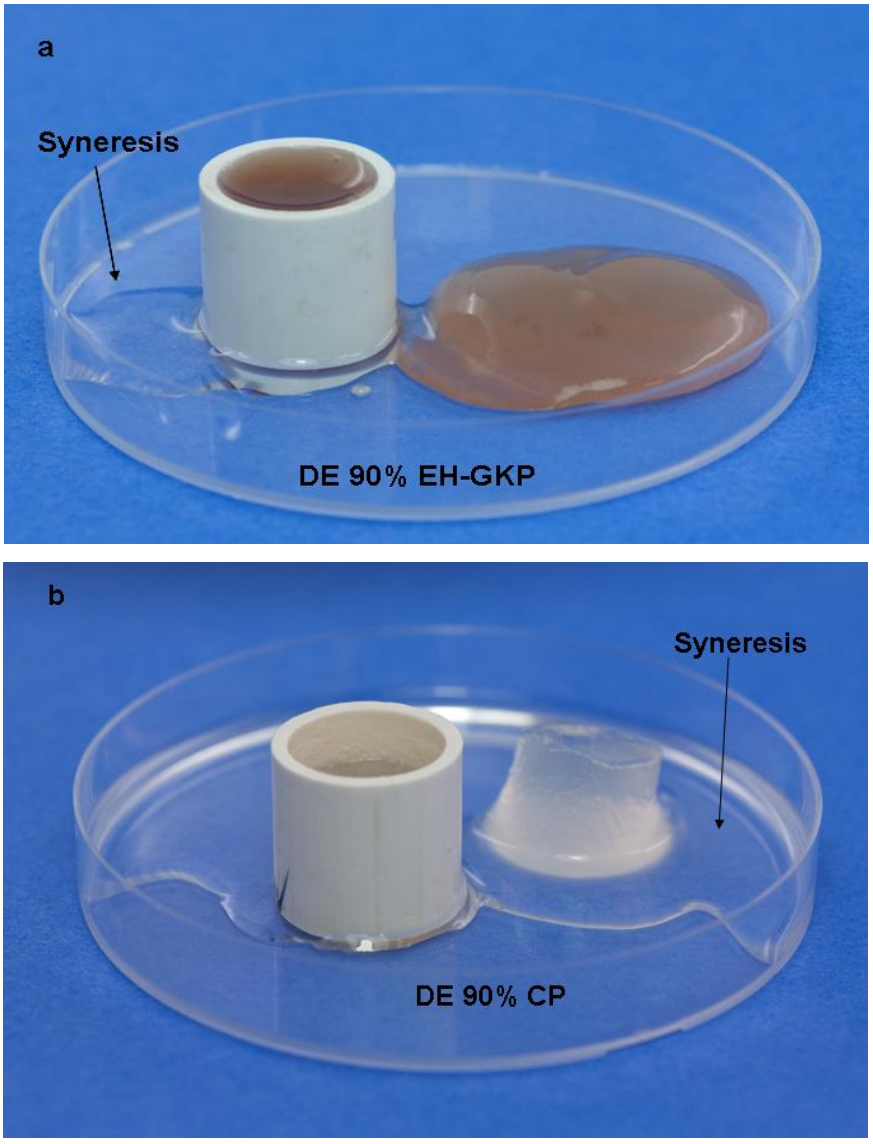


Figure 6. 10 Gel formation of DE 90% (a) EH-GKP; (b) commercial CP. The gel was prepared with 1.0% pectin concentration, pH 2.0 ± 0.01 and in the presence of 45% sucrose, and was stored for ~ 18 h at 4ºC.

For all the pectin gels studied (Figures **6.8b** and **6.9b**), the elastic modulus *G*' almost reached a plateau during storage, with values in the range ~ 100–4000 Pa (except for DE 90% EH-GKP). Initially at 4ºC, the *G*' values of the gels (DE 65% AP, DE 75% AP, DE 84% MH-GKP and DE 90% CP) increased continuously until they reached a plateau (5−6 h), as described previously by da Silva *et al*. (1998). After the first 0.5 h, the *G*" values remained unchanged through the whole storage period at 4ºC. As mentioned previously, a different trend was observed for DE 90% EH-GKP, because gelation occurred only after 3 h of holding the solution at 4ºC.

When comparing the physical properties of the gels, it is appropriate to consider the storage modulus *G*' value because it represents the elasticity or solid-like character of the gels and therefore can be identified with the "gel strength". The higher the value of the storage modulus *G*' at a given frequency, the stronger is the gel formed (da Silva, *et al*., 1998). As shown in both Figure **6.8b** and Figure **6.9b**, the effect of the DE of the pectin on the gel strength was obvious after 13 h of gelation. In terms of gel strength, the DEs are ordered as follows: 65% AP > 75% AP > 84% MH-GKP > 90% EH-GKP > 90% CP. As previously explained, the high charge density (or low DE) pectins have numerous active sites (ionised pectin chains) with higher probability for hydrogen bonding, and sufficient hydrophobic interactions, resulting in more stabilised molecular networks. This leads to a greater "connectivity" of the network and therefore to higher values of elasticity or storage modulus *G'* or gel strength. Differences between DE 90% EH-GKP and DE 90% CP are likely to be due to differences in the M_w and neutral sugars along the chains, as previously discussed.

Interestingly, the *G*' modulus of the DE 84% MH-GKP gel was almost the same as that of the commercial DE 75% AP gel (~ 1000 Pa) after 13 h of gelation. However, the DE 84% MH-GKP gel appeared to have very weak fracture properties compared with the DE 75% AP gel, as previously demonstrated in section **6.3.2**. Additionally, the pectin gels formed by the very high DE 90% EH-GKP and DE 90% CP exhibited syneresis and a very fragile appearance after 18 h of storage, even though they formed networks of *G*' = 100–300 Pa within 13 h of gelation. Based on these facts, it seems likely that network rearrangements affecting the structure and the rheology of the gels take place easily in these pectins with

high DE on storage. What initially appears to be a relatively "strong" network can rearrange itself and undergo bond breakage over time, leading to syneresis. These results confirm that the initial elastic modulus does not necessarily represent the strength of the gels and cannot be directly correlated with the fractural properties of the gels.

6.3.6 Large Deformation Behaviour of the Gels

The large deformation behaviour of the gels was then studied by exploring the change in the complex shear modulus *G** in oscillatory mode, as a function of the shear strain amplitude. The large deformation analysis was carried out to obtain information on the fracture properties of the gels formed in situ, after 13 h of storage. The gel composition has been described previously (1.0% pectin concentration, 45% sucrose concentration, pH 2.0 ± 0.01). In order to better compare the differences between the gels, the data were normalised with respect to the limiting low-strain modulus *Go**. It is well established that deviation from the linear viscoelastic region takes place when the gel is deformed to a strain at which some of the weak gel bonds start to be destroyed (Matia-Merino, 2004).

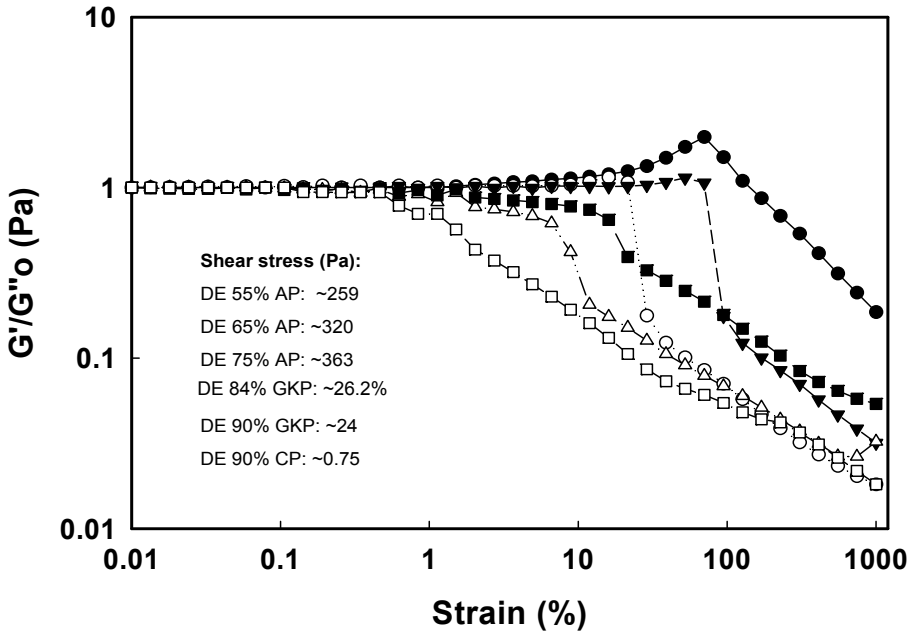


Figure 6. 11 Large deformation behaviour of gels (13 h old) prepared with different pectins (●) DE 55% AP; (○) DE 65% AP; (▼) DE 75% AP; (Δ) DE 84% MH-GKP; (■) DE 90% EH-GKP; (□) DE 90% (CP) at 20°C (1.0% pectin concentration, pH 2.0 ± 0.01, 45% sucrose concentration).

Figure **6.11** illustrates the large deformation rheological behaviour of gels prepared with different DE pectins. The general trend that can be observed is that the gels made from lower charged pectins (DE 90% CP, DE 90% EH-GKP and DE 84% MH-GKP) were typical strain-weakening gels and appeared to have shorter linear regions. These gels exhibited deviation from linear behaviour at strain values in the range 1−2% and required lower shear stress to deform (~ 0.7−26.2 Pa), indicating that these gels were more fragile than the others. In contrast, gels prepared from higher charge density pectins (DE 55% AP, DE 65% AP and DE 75% AP) exhibited longer linear regimes, over 10% strain and high shear stress values (~ 259−363 Pa). This indicates that these gels deformed to a greater extent before they were disrupted. Additionally, they showed more of a strain-hardening behaviour, typical of an elastic-like response from a polymer gel (Zhang, Daubert, & Foegeding, 2007).

Therefore, they could be described as stronger gels. This correlates well with the compression test results in section **6.3.2**, where these gels (DE 55% AP, DE 65% AP and DE 75% AP) were shown to be stronger, requiring higher breaking forces than the gels made from DE 84% MH-GKP.

The number of hydrogen bonds and hydrophobic interactions per cross-section of the strands and the strength of these bonds in the junction zones are some factors commonly considered to influence the large deformation rheology of gels (van Vliet, *et al*., 1991). Consistent with the statement above, it can be speculated that, by increasing the number of active junction zones (or by increasing the charge density of the pectins which corresponds of lower DE pectins), the number of bonds between the polymer chains is increased, producing a stronger connected network that is highly deformable and shows a longer linear viscoelastic region.

The DE 90% CP gel exhibited the shortest linear region, perhaps reflecting the more particulate nature of the gel, as described earlier. It seems possible that the gels obtained from the GKPs also tended to exhibit more of an aggregated/coarse structure that, in the

long term, results in a weaker network (DE 84% MH-GKP) that can show poor water holding capacity (DE 90% EH-GKP).

6.4 Conclusions

In this study, it is clear that the physicochemical properties of pectin influenced their functional properties, *i.e.* their gelation properties. Pectins with dissimilar DEs exhibited different gelation behaviours. Pectins with lower DE were shown to form stronger gels than those made from higher DE pectins. It was also observed in this study that the functional properties of GKP were markedly dependent on fruit maturity. The gel obtained from pectin that was extracted from main-season fruit was considerably firmer than that obtained from pectin extracted from early-season fruit. Consequently, this can affect their applications in food systems. Varying the different parameters of gelation conditions such as pH, and sucrose concentrations have obviously influenced the gelation properties of apple pectins (high charge density). However, these conditions did not have a great affect on that of GKPs (lower charge density). The GKP gels are characterised by 'weak gels'. As result, these pectins could be ideal for applications that require the suspension of fruit particulates, such as marmalades or jams. Further research on their functional properties as acidified milk drink stabilisers is investigated in Chapter 7.

CHAPTER 7 Stabilisation of Acidified Milk Drinks: Addition of Gold Kiwifruit Pectin

7.1 Introduction

In the food industry, there has been increased interest in protein−polysaccharide interactions because of their considerable ability to influence the microstructural, rheological and textural properties of processed food systems. Pectin is added to many milk-based food products mainly for its stabilising ability (Norziah, *et al*., 2000; Matia-Merino, Lau, & Dickinson, 2004). Specifically, pectin is commonly added into acidified milk drinks (AMDs) to prevent protein aggregation. Pectin is also important in controlling the physical properties of the drink, such as texture and mouthfeel (Liu, *et al*., 2006).

The most common defect in AMDs is "wheying off", which is the formation of a clear serum layer at the top of the drink (Sejersen, *et al*., 2007). This occurs because of the decreasing electrostatic and steric repulsion between casein micelles when milk is acidified at pH around 4.6, the isoelectric point (p*I*) of casein (Lucey, *et al*., 1999; Liu, *et al*., 2006). The loss of stability causes aggregation of the casein micelles, resulting in sedimentation and ultimately in the formation of a serum top layer. Pectin prevents the aggregation process by interacting with the casein aggregates, creating a thick steric layer and maintaining the micelles and aggregates in suspension.

The ability of pectin to stabilise acidified milk systems is influenced by its physicochemical properties such as its degree of esterification (DE). High DE pectin or high methoxyl pectin (HMP) is widely used to stabilise AMDs (Liu, *et al*., 2006). The stabilisation of low pH milk protein systems by this pectin involves electrostatic adsorption of pectin chains on to the surface of the casein micelles (below the p*I* of the protein). The interaction occurs only between negatively charged blocks of pectin (at pH > p*Ka*) and positively charged casein segments (at pH < p*I*). There is a block-wise distribution of charges along the pectin chain;

the charged blocks of the chain interact with casein whereas the uncharged blocks extend into the bulk phase of the AMD. This creates entropy-rich loops, which cause a repulsive interaction between micelles, similar to the action of κ-casein at pH 6.7 (Tromp, *et al*., 2004). Pectin with greater charge density or low methoxyl pectin (LMP) or low DE pectin has more electrostatic binding sites available, which may increase its interactions with casein micelles, decreasing the amount of polysaccharide needed for saturation of the casein particles. However, in this case, the steric stability may be restricted because shorter loops are created at the surface of the micelles; therefore sedimentation is likely to occur (Pereyra, *et al*., 1997). Hence, LMP is less able than HMP to achieve milk stabilisation.

The pectin concentration also has a marked influence on the stability of an AMD. The drink will not be stable if the amount of pectin is insufficient to cover the positively charged casein surface. Other physical properties that are considered to be important in stabilising an AMD are the molecular weight and the size of the pectin (Liu, *et al*., 2006). According to these authors, at the low pH of these milk systems, the charge density of pectin is fundamental in driving the adsorption on to the casein micelles; however, the molecular weight and the size of the pectin chains will influence the extent of the steric repulsion between the casein micelles.

This thesis is concerned with low pH AMDs (pH 3.80 ± 0.05). This pH was chosen because it is within the optimum pH range for interaction between casein and pectin (pH 3.6−4.3) in AMDs, as reported by Sejersen *et al*. (2007). According to Tuinier *et al*. (2002), less pectin is needed to achieve stabilisation of the drink at pH ≤ 4.0. A pH of ~ 3.8 seems to be widely used in commercial AMDs to provide maximum efficacy and, therefore, was also the pH of the present study.

In this chapter, stabilisation of AMDs by the addition of pectin derived from gold kiwifruit was investigated and was compared with the stabilisation provided by commercial pectins (apple and citrus). The main aim of this investigation was to study the effect of the concentration and the DE of the pectin on the stability of AMDs. The stability and the rheological properties of the drinks, including the percentage of serum separation, the pectin

concentration in the serum phase, the viscosity (of the AMD and the serum) and the particle size, were determined.

7.2 Materials and Methods

7.2.1 Materials

Low-heat skim milk powder (LHSMP) was obtained from Fonterra Co-operative Group Ltd, New Zealand. Its chemical composition is presented in Table **7.1**. Glucono-δ-lactone (GDL) was obtained from Sigma Aldrich. The various HMPs used in this study were as follows.

- Pectin (P) with DE 90% was extracted using a water (W) extraction method from early-harvested (EH) gold kiwifruit (GK): DE 90% W-EHGKP.
- Pectin with DE 84% was extracted using a water extraction method from main-harvested (MH) gold kiwifruit (DE 84% W-MHGKP).
- Pectin with DE 85% was extracted using an enzymatic (E) extraction method from main-harvested gold kiwifruit (DE 85% E-MHGKP).
- Un-standardised commercial apple pectins (APs) were obtained from Herbstreith and Fox KG, Germany; they had the following DEs: 55% (DE 55% AP), 65% (DE 65% AP) and 75% (DE 75% AP).
- Un-standardised commercial citrus pectin (CP) (P9561) with DE 90% (DE 90% CP) was obtained from Sigma Aldrich, UK.
- Standardised commercial citrus pectin with a minimum DE of ≥ 70%, Grindsted® pectin 780 AMD (GCP), was obtained from Danisco, Copenhagen, Denmark. This is the commercial pectin that is recommended for the production of AMDs.

The DE and the galacturonic acid (GalA) content of each pectin are presented in Table **7.2**.

Table 7.1 Chemical composition of LHSMP

Component	Concentration
Total protein (% w/w)	31.00
Casein (% w/w)	26.15
Whey protein (% w/w)	4.45
Moisture (% w/w)	3.46
Fat (% w/w)	0.66
Lactose (% w/w)	59.50
Ash (% w/w)	7.93
Calcium (mg/kg)	12300
Magnesium (mg/kg)	1090
Sodium (mg/kg)	3790
Phosphorus (mg/kg)	10100

Table 7.2 GalA composition of the HMPs studied

Pectin	DE (%)	GalA Concentration (%)
DE 55% AP	55	83
DE 65% AP	65	82
DE 75% AP	75	79
DE 84% W-MHGKP	84	77
DE 85% E-MHGKP	85	53
DE 90% W-EHGKP	90	46
DE 90% CP	90	75
DE ≥ 70% GCP	≥ 70	83

7.2.2 Methods

7.2.2.1 Preparation of AMDs

In preliminary studies, AMDs were prepared by two methods, *i.e.* the "Pre-mix" method and the "Mixed" method. The "Pre-mix" method involved the addition of pectin powder to the LHSMP before water dispersion. In the "Mixed" method, LHSMP and HMP were dissolved separately before being mixed together. Although both methods led to similar behaviours in terms of stabilisation of the drinks, the "Mixed" method was chosen for further experiments because of its convenience in adjusting the pectin concentration. All percentages in this study are expressed as % w/w, unless otherwise stated.

LHSMP (20%) was dissolved in warm Milli-Q water (Millipore Corp., Bedford, MA, USA) under continuous stirring in a water bath at 50ºC for 60 min. The milk was immediately cooled to room temperature (20ºC) by placing it into a bath of crushed ice. To ensure that mineral equilibrium between the casein micelles and the serum phase was reached, the dispersion was left overnight (~ 18 h) at 4ºC under continuous stirring. The pH of the milk was measured at room temperature. A 2% pectin stock solution (based on the GalA content) of each HMP was prepared by dissolving the pectin in Milli-Q water at 60ºC for 30 min under stirring. Various quantities of pectin solution were added to solutions with a constant protein weight to obtain a constant milk solids non-fat concentration of 10% and final pectin concentrations of 0.1, 0.2, 0.3, 0.5, 0.7 and 1% in the mixed solution (Table **7.3**). The pH of each sample was adjusted to 6.60 ± 0.05 at room temperature by the addition of 0.1 M NaOH, prior to GDL addition, to reach the same final pH (3.80 ± 0.05) after acidification. The milk was acidified by adding 2% GDL at room temperature under stirring for 2 min. As an antimicrobial agent, 0.02% sodium azide was added to each AMD.

Table 7.3 AMD formulations

AMD Formulation	Final Pectin Conc. (%)	2% Pectin Stock (g)	20% LHSMP Stock (g)	Milli-Q Water (g)	Final Solution (g)
1	0.1	2.5	25	22.5	50
2	0.2	5.0	25	20.0	50
3	0.3	7.5	25	17.5	50
4	0.5	12.5	25	12.5	50
5	0.7	17.5	25	7.5	50
6	1.0	25.0	25	0	50

To obtain a homogeneous AMD, the acidification process was carried out with continuous stirring overnight (~ 18 h) at room temperature. Preliminary experiments with and without stirring had been carried out, with a more homogeneous system being obtained when the sample was stirred during the acidification step. All experiments were conducted in duplicate. The procedure is outlined in Figure **7.1**. Because insufficient sample of DE 85% E-MHGKP and DE 90% W-EHGKP was available, measurements at only some pectin concentrations were carried out, as detailed in the following sections. To obtain reproducible data and to minimise the effect of further casein aggregation processes, the measurements were made immediately after the acidification process.

7.2.2.2 Stability of AMDs

In this study, the stability of the AMDs was determined using a centrifugal separation method and by visual observation of serum separation during storage. The results from these two techniques were compared and were related to the stability of the drinks.

7.2.2.2.1 Determination of Physical Stability by Centrifugation

In preliminary studies, to get clear differences between each pectin concentration, various centrifugation speeds (500, 1000, 1500 and 3000 *g*) were tested to determine the percentages of serum obtained under the different forces. An aliquot of 10 mL of AMD was placed in appropriate tubes and centrifuged (Centra, MP4R, rotor 224; International Equipment Company, USA) at 1500 *g* for 15 min at 20ºC (based on the preliminary studies). The separated serum was calculated as a percentage (% v/v), based on the clear serum and the protein layer obtained after centrifugation. No measurements at pectin concentrations of 0.2, 0.5 and 0.7% were carried out for DE 90% W-EHGKP.

7.2.2.2.2 Determination of Physical Stability during Storage

In this method, the stability of the AMDs was observed under static conditions with only gravity as the driving force to evaluate the rate of serum separation over 7 days. AMD aliquots of 10 mL were placed in measurement tubes and were stored statically at room temperature. Serum separation was measured as a function of time for 7 days. The serum percentage was calculated based on the amount of serum produced for each day of observation and at each pectin concentration, considering the initial volume of AMD. In this chapter, the visual appearances of the AMDs after 1 week of storage are shown. No measurements were carried out at pectin concentrations of 0.2, 0.5 and 0.7% for DE 90% W-EHGKP and at a pectin concentration of 0.2% for DE 85% E-MHGKP.

7.2.2.3 Quantification of Pectin in the Serum Phase

AMD aliquots of 20 mL were centrifuged at 15,000 *g* for 30 min at 20ºC. Pectin was considered to be adsorbed on to the casein micelles when it stayed in the protein layer (pellet) after centrifugation ("adsorbed pectin") (Tromp, *et al*., 2004); the "non-adsorbed pectin" remained in the supernatant or serum phase. Both "adsorbed pectin" and "non-adsorbed pectin" were quantified by analysing the pectin concentration in the serum phase and subtracting it from the total amount of pectin added to the AMD. One gram of serum was acid hydrolysed using the Englyst *et al*. (1994) method. The pectin concentration was subsequently analysed calorimetrically by the dimethylphenol method described by Scott (1979) using GalA as a standard.

7.2.2.4 Viscosity Measurements of the AMD and the Serum Phase

Up and down viscosity flow curves were obtained in a controlled-stress rheometer (Paar Physica MCR 301; Anton-Paar, GmbH, Germany) using a cone and plate measuring system (CP 4/40) at a constant temperature of 20 ± 0.1°C and at shear rates between 0.1 and 1000 s^{-1}. Viscosity data at a shear rate of 53 s^{-1} were chosen for comparison purposes as this shear rate is within the range of shear that occurs during mastication in the mouth (~ 50 s^{-1}). Viscosity data at low shear rate (1 s^{-1}) were also chosen for comparison. The viscosities of the AMD and the serum phase were determined. The serum was obtained after centrifuging the AMD at 15,000 *g* for 30 min at 20ºC.

7.2.2.5 Quantification of Average Particle Size in the AMD

The particle size in the AMD was measured as an estimate of the average size of caseins coated with pectin. AMDs with 1.0% added pectin were used for this analysis because the micelle aggregates are fully coated by pectin at this concentration; therefore, bridging flocculation effects are minimised. The AMD was centrifuged at 25,000 *g* for 15 min. The upper part of the pellet was used for analysis. Both the AMD pellet and the pectin were diluted with prepared purified serum (PS) in order to maintain the ionic environment of the casein micelles in the AMD (1:110 dilution times). The PS was obtained by centrifuging an

AMD (10% protein concentration, with no added pectin, pH ~ 3.80) at 25,000 *g* for 2 h at 20ºC. The serum was removed and vacuum filtered twice with membrane filters of sizes 0.45 μm and 0.20 μm to obtain casein-free serum. The determination was carried out by dynamic light scattering using a Zetasizer (Malvern Instruments Ltd, Malvern, Worcestershire, UK). The procedure and all measurements carried out in this study are summarised in Figure **7.1**. The particle size data are reported as the *Z-average* mean diameter in nanometres (nm).

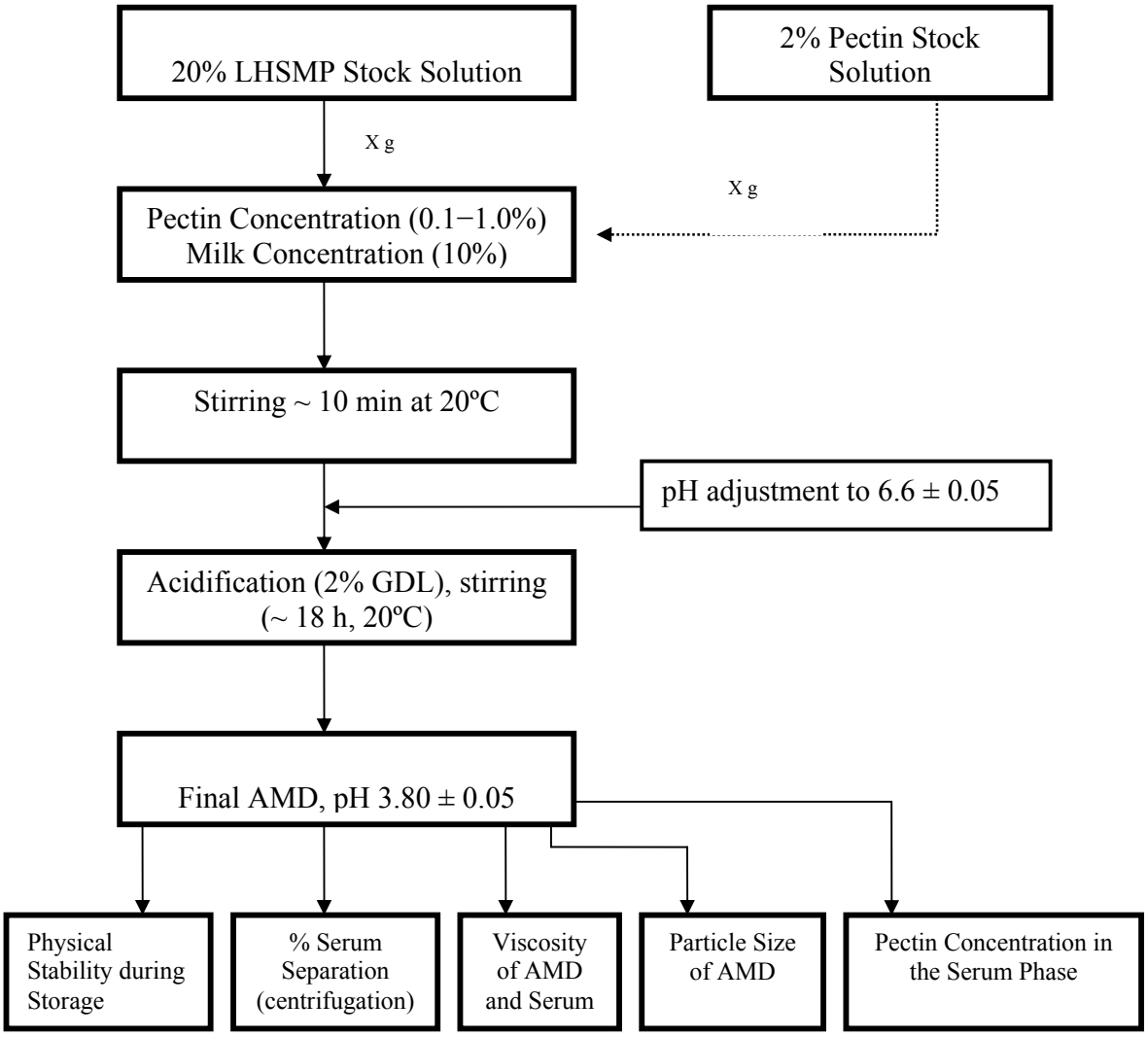


Figure 7.1 Flow chart of AMD preparation.

7.2.2.6. Weight-Average Molecular Weight (M_w) and Root Mean Square (RMS) Radius

The M_w and the RMS radius for commercial pectins (apple pectin and citrus pectin) were determined by SEC-MALLS as previously described in Chapter **4**, section **4.2.4.1**, with sample concentrations of 0.35% for apple pectin and 0.45% for citrus pectin in order to obtain clear peak elutions. The *dn*/*dc* value used for determining the M_w of these commercial pectins was 0.146 mL/g (*dn*/*dc* of commercial citrus pectin) as reported by Fishman *et al*. (2001). The chromatograms of these pectins are shown in Appendix **D.1**.

7.3 Results and Discussion

7.3.1 Physicochemical Properties of HMPs

Table **7.4** shows the size and mass properties of each HMP. The HMPs used in this study varied in M_w, *Z-average* particle size and *Z-average* RMS radius. They ranged from 0.04 to 3.75 x 10^6 g/mol, 108 to 235 nm and 39.9 to 182.7 nm respectively for M_w, *Z-average* particle size and RMS radius. DE 84% W-MHGKP had the highest M_w (~ 3.75 x 10^6 g/mol), *Z-average* value (~ 235 nm) and RMS radius (~ 182.7 nm). In contrast, DE 90% CP had the lowest M_w (~ 0.04 x 10^6 g/mol), *Z-average* value (~ 108 nm) and RMS radius (~ 39.9 nm), probably because of the extraction method and the enzymatic treatment involved when manipulating the DE. Pectin can be modified chemically to give various esterification levels (0−100%) by varying the extraction time, temperature and pH (Baker, *et al*., 2005). Additionally, Ptitchkina *et al*. (2008) have shown that the DE of pumpkin pectin can be manipulated by varying the digestion time. These variations could contribute to different properties of the pectins, leading to differences in their interactions with casein and therefore variation in their stabilisation of AMDs.

Table 7.4 Physicochemical properties of HMPs

Pectin	M_w (10^6 g/mol)	*Z-average* Diameter (nm)	RMS Radius (nm)
DE 55% AP	0.17 ± 0.02	213 ± 0.7	67.6 ± 4.7
DE 65% AP	0.61 ± 0.05	214 ± 27	93.0 ± 1.8
DE 75% AP	0.63 ± 0.05	215 ± 26	108.1 ± 2.5
DE 84% W-MHGKP	3.75 ± 0.11	235 ± 52	182.7 ± 1.1
DE 85% E-MHGKP	1.65 ± 0.04	202 ± 33	162.0 ± 0.60
DE 90% W-EHGKP	1.03 ± 0.03	Not analysed	114.7 ± 0.50
DE 90% CP	0.04 ± 0.003	108 ± 2.3	39.9 ± 4.7

Mean ± standard error (n = 2).

7.3.2 Stability of AMDs

7.3.2.1 AMD Stability by Centrifugation

To establish the effect of the concentration and the DE of the pectins on the stability of AMDs, the pectin concentration was varied (0.1–1.0%) for each DE pectin. Figure **7.2** shows the percentage of serum phase obtained after the centrifugation of each AMD. The amount of serum ranged between 0 and 82%, with the serum quantity decreasing as the pectin concentration increased. When the pectin concentration was ≤ 0.3%, the amount of serum was about greater than 50%; however, when the pectin concentration was increased above 0.5%, the amount of serum dropped below 10%. This trend was observed for all HMPs, except for DE 90% CP where the addition of pectin had no effect on the amount of serum and a constant amount of 80% serum was obtained at all concentrations.

The greater serum separation of the AMDs with ≤ 0.3% pectin implies a poorer efficiency of stabilisation. This could have been due to: (*i*) insufficient steric repulsion created by casein−pectin complexes to span the drink space, probably because of insufficient adsorption of pectin on to the micelles; (*ii*) bridging flocculation between casein micelles sharing the same pectin chains. It is known that an insufficient amount of pectin to cover the positively charged casein micelles can lead to bridging flocculation and subsequent sedimentation of casein micelles (Glahn & Rolin, 1994; Tuinier, *et al*., 2002). Greater amounts of serum were obtained in AMDs prepared from all gold kiwifruit pectins than in those prepared from apple pectin at the same low pectin concentration (0.3%) (*i.e.* ~ 78% serum for DE 84% W-MHGKP versus 58% serum for DE 75% AP). However, when more

pectin was added (*i.e.* 0.5%), this trend was reversed, and the drinks stabilised by gold kiwifruit pectin (DE 84% W-MHGKP and 85% E-MHGKP) showed very little serum separation (0–3%) compared with those stabilised by the other pectins (*i.e.* ~ 20% serum for DE 75% AP). It is likely that the higher DEs of the gold kiwifruit pectins made them less interactive with casein, leading to greater amounts of serum at low pectin concentrations (0.3%).

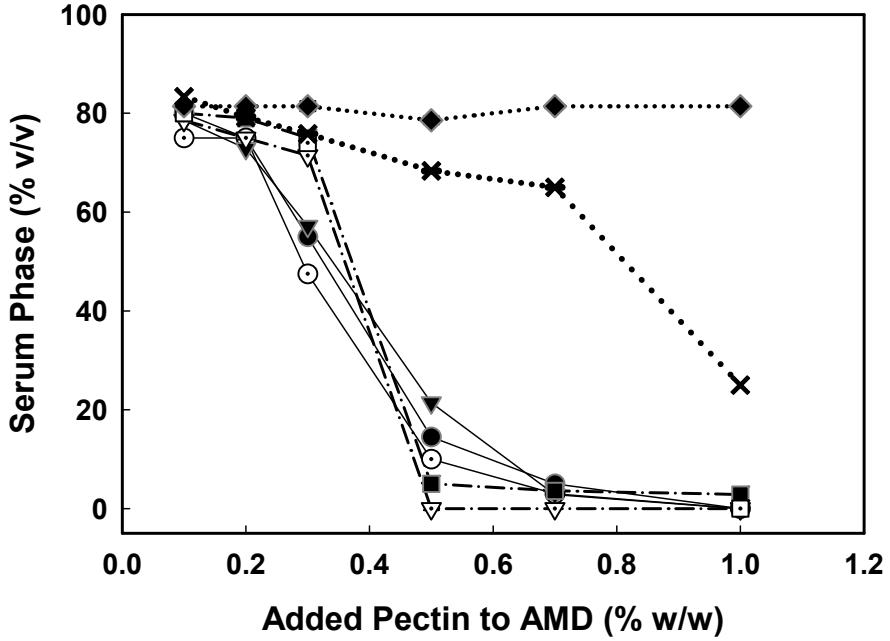


Figure 7.2 Effect of pectin concentration on the serum phase obtained after centrifugation (1500 *g*) of AMDs prepared using pectins with different DEs: (●) DE 55% AP; (○) DE 65% AP; (▼) DE 75% AP; (▽) DE 84% W-MHGKP; (■) DE 85% E-MHGKP; (□) DE 90% W-EHGKP; (♦) DE 90% CP; (x) Grindsted® pectin AMD 780. • – • GKP; •••• CP; ── AP (10% protein concentration, pH 3.80 ± 0.05).

The M_ws of the various HMPs used in this study could have been a key factor in the differences in the stabilities of the AMDs. The serum separation observed at 0.5% pectin concentration decreased from 22% (DE 75% AP) to 0% (DE 84% W-MHGKP) with increasing M_w. DE 84% W-MHGKP, with the greatest M_w (3.75 x 10^6 g/mol), appeared to provide a stable drink (serum ~ 0%) at a lower pectin concentration (0.5%) compared with the other pectins. In contrast, DE 90% CP, with the lowest M_w (0.04 x 10^6 g/mol), showed

strong serum separation at all pectin concentrations tested. The influence of the M_w of the polysaccharide on the stabilisation mechanism of AMDs has been reported by Du *et al*. (2009) when using carboxymethylcellulose (CMC). These authors showed that, the longer the chain, the greater were the extended loops protruding from casein−CMC complexes, resulting in stronger steric repulsion between complexes and therefore providing greater stability. The M_w also has an influence on the viscosity of the aqueous phase. The higher viscosity imparted by non-adsorbed high M_w pectin should theoretically contribute to a greater stability of the system. This is discussed later in this chapter.

For further comparison, an AMD was prepared using the commercial pectin that is recommended for AMD stabilisation (GCP, with DE ≥ 70%). This pectin did not seem to provide greater stability than apple pectin or gold kiwifruit pectin (at the same GalA content). Greater serum separation was obtained at pectin concentrations above 0.3%; in addition, at 1.0% pectin concentration, the drink still showed approximately 20% serum phase whereas those prepared with apple and gold kiwifruit pectins showed nearly no serum separation. This could have been due to the influence of its M_w. Although this was not determined, viscosity analysis of this pectin solution (see Appendix **D.2**) indicated that it provided very low viscosity to the system, with values close to those obtained with DE 90% CP. This suggests that its M_w could be slightly higher than or very close to that of DE 90% CP. It seems that a greater amount of this pectin may be required to achieve stability while still keeping a low viscosity in the drink.

7.3.2.2 AMD Stability during Storage (Static Conditions)

Figure **7.3** illustrates the development of serum separation as a function of the pectin concentration, pectin DE and time. The visual appearances of the AMDs after 1 week of storage are shown in Figure **7.4**. It is obvious that serum separation decreased with increasing pectin concentration, which is in agreement with the findings above (section **7.3.2.1**). All HMPs showed no stability at concentrations ≤ 0.2% (Figures **7.3a**−**7.3b**), with the serum separation increasing considerably after 1 day of storage. These results, as discussed earlier, may indicate chaotic bridging between casein micelle aggregates by insufficient pectin, promoting aggregation and subsequent phase separation as well as

sedimentation of partially pectin-covered, acid-induced aggregated casein micelles over time. At 0.3% pectin concentration (Figure **7.3c**), the AMD prepared from DE 84% W-MHGKP showed almost no phase separation upon storage with only a slight increase on day 4 (~ 4.4%). This gives an indication that stability of the AMD was again reached at a lower concentration of this particular high M_w gold kiwifruit pectin (DE 84% W-MHGKP) compared with other pectins. It was also observed that, at 0.3% pectin concentration, DE 85% E-MHGKP had a similar pattern of serum separation to DE 55% AP. This enzyme-extracted gold kiwifruit pectin of intermediate M_w behaved very similarly to the apple pectins.

At higher concentration, greater AMD stabilisation was found, with the serum phase percentage varying from 0−15% (at 0.3% pectin concentration) to 0−7% (at 0.5% pectin concentration) for DE 55, 65, 84 and 85% pectins over the storage period. Figures **7.3e−7.3f** show that no serum separation was detected in the AMDs with ≥ 0.7% added pectin, indicating possible full coverage of the casein micelles by the pectins (DE 55, 65, 75 and 85%) or sufficient viscosity development in the aqueous phase to avoid protein sedimentation. In agreement with the serum obtained after centrifugation, DE 90% CP did not stabilise the AMDs at similar concentrations to the other pectins.

DE 84% W-MHGKP appeared to successfully stabilise the AMDs at lower concentrations. This pectin (high DE) has fewer negatively charged blocks than DE 55−75% pectins and therefore less interaction with the positive casein groups is expected. However, it has higher M_w. At a pectin concentration of 0.3%, virtually only ~ 4.4% serum separation occurred throughout the storage period, whereas the other HMPs showed ~ 10−35% serum separation. These results indicated that aggregation was almost prevented in the presence of 0.3% DE 84% W-MHGKP for 10% LHSMP, which seems to be better than the stabilisation of AMDs reported by Lucey *et al*. (1999). These authors indicated that AMDs could be stabilised against serum separation under static conditions at low storage temperature (~ 9ºC) by the addition of 0.3% HMP (DE 72%, Genu pectin) at lower milk solids non-fat concentrations (6.5%).

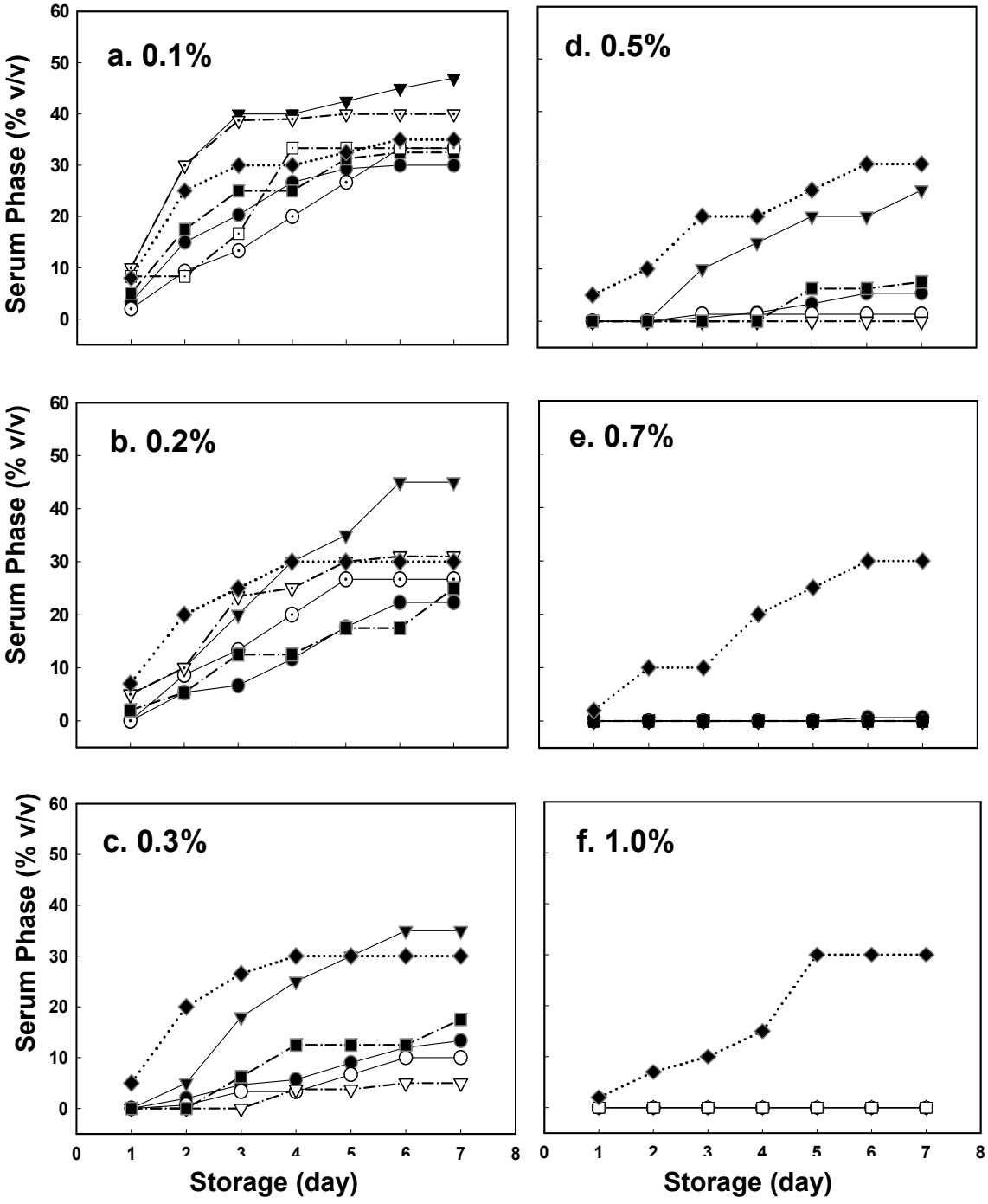


Figure 7.3 Effect of pectin concentration on the stability during storage of AMDs prepared with different pectin types: (●) DE 55% AP; (○) DE 65% AP; (▼) DE 75% AP; (▽) DE 84% W-MHGKP; (■) DE 85% E-MHGKP; (□) DE 90% W-EHGKP; (♦) DE 90% CP. • – • GKP; •••• CP; ── AP. Various pectin concentrations: (a) 0.1%; (b) 0.2%; (c) 0.3%; (d) 0.5%; (e) 0.7%; (f) 1.0%.

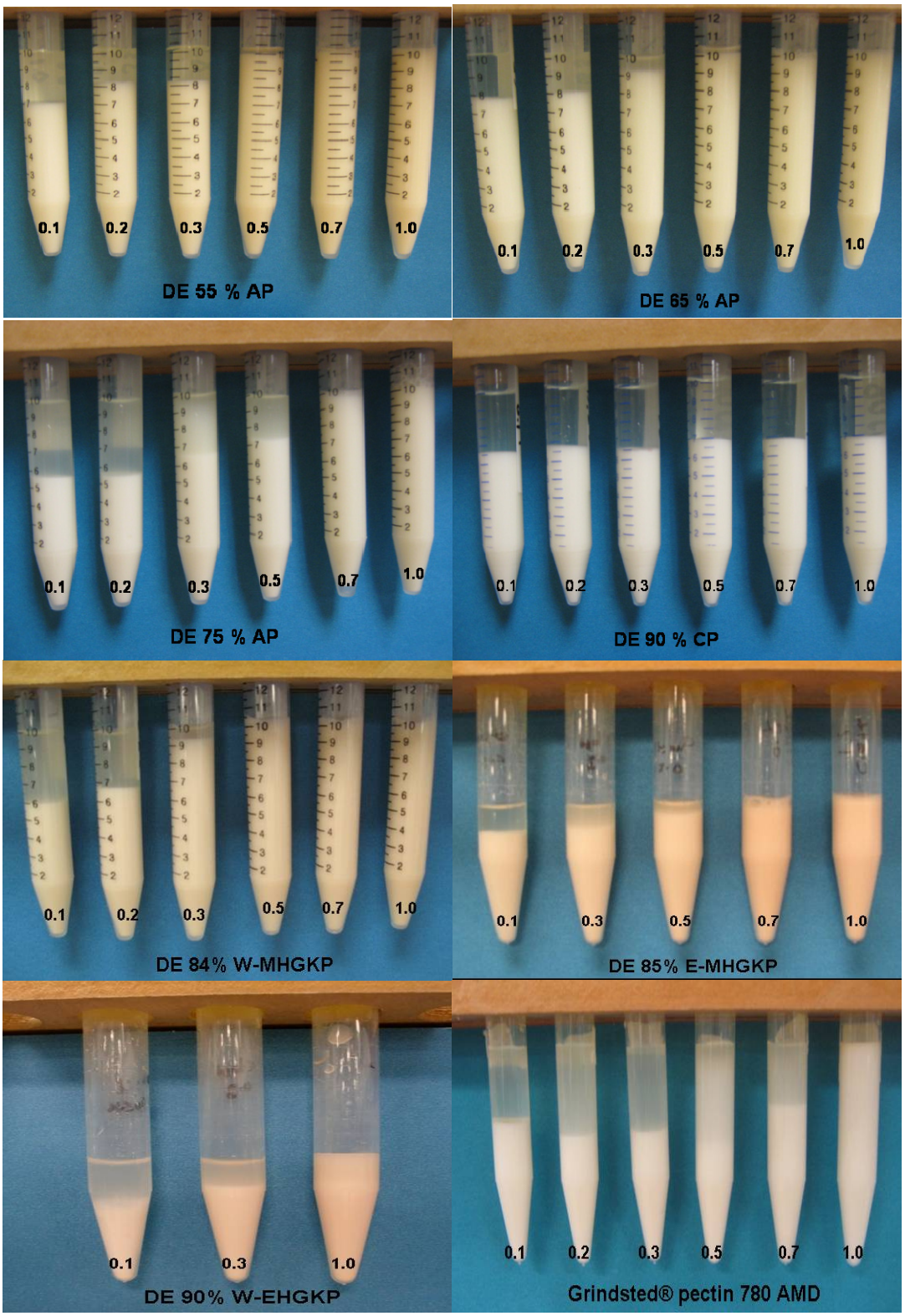


Figure 7.4 Photographs after storage for 7 days (20°C) of AMDs prepared with different pectin concentrations (% w/w) and types.

In general, the centrifugation data agreed well with the static storage results. Both methods showed that DE 84% W-MHGKP (high DE and M_w) provided stability at lower concentration. However, DE 85% E-MHGKP (high DE, intermediate M_w), which showed similar stability to DE 55% AP (Figures **7.3a**−**7.3d**) during storage, behaved like DE 84% W-MHGKP and DE 90% W-EHGKP with centrifugation. Differences between the centrifugation method and the static method may arise if the interaction is weak, as may occur between the low negatively charged pectins (high DE) and the positive charged caseins at low pectin levels. Possible detachment of pectin due to centrifugation may have been the reason for the differences observed.

No clear trend observed for DE 75% AP at concentrations < 0.7% was observed. At 0.5% concentration, this pectin still showed a significant amount of serum phase compared with the other pectins, as also detected after centrifugation of the AMD. This pectin seemed to behave significantly differently from the other pectins. This could have been due to the modification of its structure, leading to differences in charge distribution and therefore variations in its physicochemical properties. Tuinier *et al*. (2002) reported that, because of irregularities of charge density distribution of pectin and casein, it is possible that a certain fraction of pectin could adsorb preferentially at certain patches of the casein surface whereas another pectin fraction may not easily adsorb. The different behaviour of DE 75% AP was also shown by its gelation properties (Chapter **6**).

In contrast to DE 90% W-EHGKP, DE 90% CP (high DE, low M_w) did not stabilise the AMDs at the GalA concentrations studied here. Of the HMPs, DE 90% CP has the lowest charge density (90% of ester groups) and the lowest M_w. AMDs prepared with this pectin showed instability, with high amounts of serum. These results suggested that aggregation of the casein micelles could not be prevented in the presence of this pectin. It is likely that (*i*) not enough electrostatic interaction between casein and pectin occurred because of the high DE, (*ii*) the steric repulsion between pectin-coated caseins was not sufficient to prevent aggregation because the hairy layer provided by this low M_w pectin was not thick enough and (*iii*) the viscosity provided by the non-adsorbed pectin was not high enough to prevent sedimentation.

Figure **7.5** illustrates the turbidity of the serums obtained from the AMDs prepared at 0.1% pectin concentration and after 1 week of storage. Lower amounts of AMD were used in this experiment with DE 85% E-MHGKP and 90% W-EHGKP because the pectin quantity was limited. The serums obtained from the AMDs containing gold kiwifruit pectins (DE 84, 85 and 90%) were cloudier than those obtained from the AMDs containing commercial pectins (DE 55 and 65% AP and DE 90% CP). The tendency to develop a cloudy serum rather than a very clear serum was presumably due to suspension of some of the casein−pectin complexes or small protein aggregates, even at this low pectin concentration. Either viscosity effects or stabilisation of small protein particles at the interface were responsible for the differences observed.

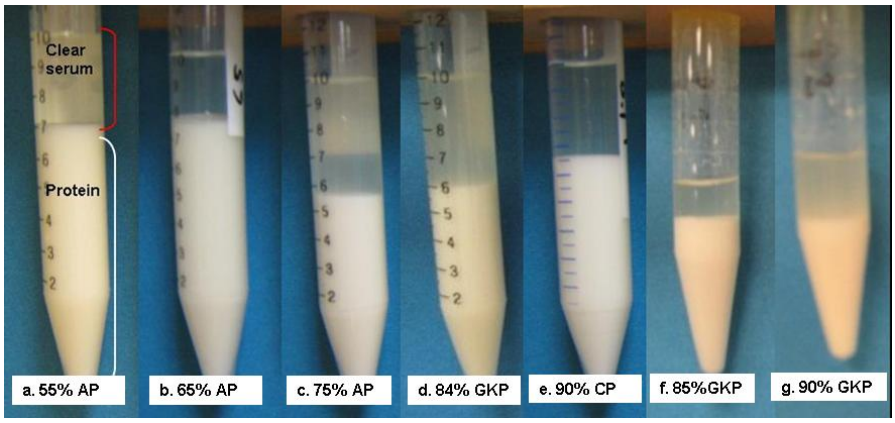


Figure 7.5 Turbidities of AMDs prepared at 0.1% pectin concentration (10% protein, pH 3.80 ± 0.05, day 7 of storage under static conditions): (a) DE 55% AP; (b) DE 65% AP; (c) DE 75% AP; (d) DE 84% W-MHGKP; (e) DE 90% CP; (f) DE 85% E-MHGKP; (g) DE 90% W-EHGKP.

7.3.3 Measurements of Pectin Adsorption

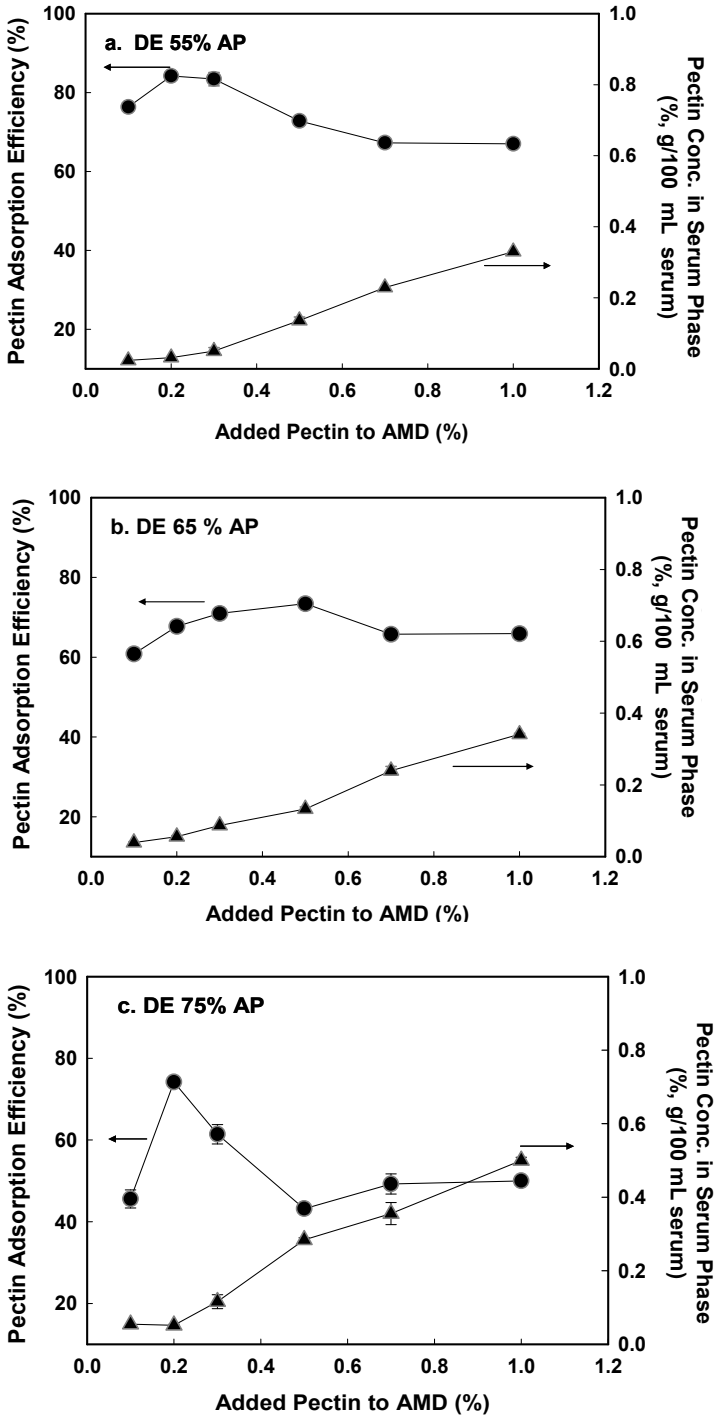
Figure **7.6** illustrates the amount of pectin adsorbed (pectin adsorption efficiency) and the amount of pectin in the serum phase (non-adsorbed) when different pectin concentrations were added to the drinks. The amount of pectin in the serum phase was determined using the dimethylphenol method (Scott, 1979), as described in Chapter **3** (see section **3.2.3.6**). The pectin concentration in the serum phase (non-adsorbed) is expressed as grams of pectin per 100 millilitres of serum. It is clear that not all pectin was adsorbed on to the casein

micelles even when small amounts of pectin were added. For example, the AMDs prepared with apple and gold kiwifruit pectins had approximately 15−55% and 42−58% non-adsorbed pectin respectively at low pectin concentration (0.1−0.3%). It is very likely that pectin was preferentially adsorbed on to casein micelles, driven by differences in the distribution patches of the charges, therefore influencing the pectin−casein electrostatic interaction. Tromp *et al*. (2004) found that 90% of the pectin in an AMD prepared with HMP (DE 72.2%) was non-adsorbed at 0.3% total pectin concentration explained by detachment of pectin during centrifugation at high speed.

In general, the pectin concentration in the serum phase increased gradually with increasing total pectin in the AMD. This indicated that the amount of non-adsorbed pectin increased with increasing stability of the drink (measured by a reduction in the serum phase). A large effect of the DEs of the pectins on the amount of adsorbed pectin was observed, particularly at pectin concentrations ranging from 0.3 up to 1.0%. AMDs prepared with low charged pectins (DE 75% AP, DE 84, 85 and 90% GKPs and DE 90% CP) were demonstrated to have greater amounts of non-adsorbed pectin (45−80%) than AMDs prepared with higher charged pectins (DE 55 and 65% APs) (~ 35%). As a consequence, the pectin adsorption efficiency of low charged pectin was lower than that of higher charged pectins (or low DE pectins such as DE 55% AP). This is in agreement with general knowledge that the electrostatic interaction between pectin and casein is reduced when there are fewer available interactive charges in the pectin chains (high DE pectins) (Liu, *et al*., 2006). Detachment during centrifugation is also more likely to occur.

It was interesting to observe that the gold kiwifruit pectins with almost similar DE (84 and 85%) but different M_w showed different trends in pectin adsorption. Overall, drinks prepared with DE 84% W-MHGKP showed greater pectin adsorption than those prepared with DE 85% E-MHGKP. Enzymatic treatment is likely to have an effect to the adsorption properties of pectin. To emphasise the relationship between non-adsorbed pectin and the stability of AMDs for various DE pectins, Figure **7.7** was plotted at a fixed amount of added pectin (0.7%). Even if the non-adsorbed pectin increased as the charge density of the pectins decreased, the stability (amount of serum separation) was barely affected (except for

84% W-MHGKP). That is, even if less pectin was adsorbed on to the casein micelles or/and aggregates as with the high DE gold kiwifruit pectins, the stability was similar (85% E-MHGKP) or even higher (no serum for 84% W-MHGKP) than for the low charged pectins.



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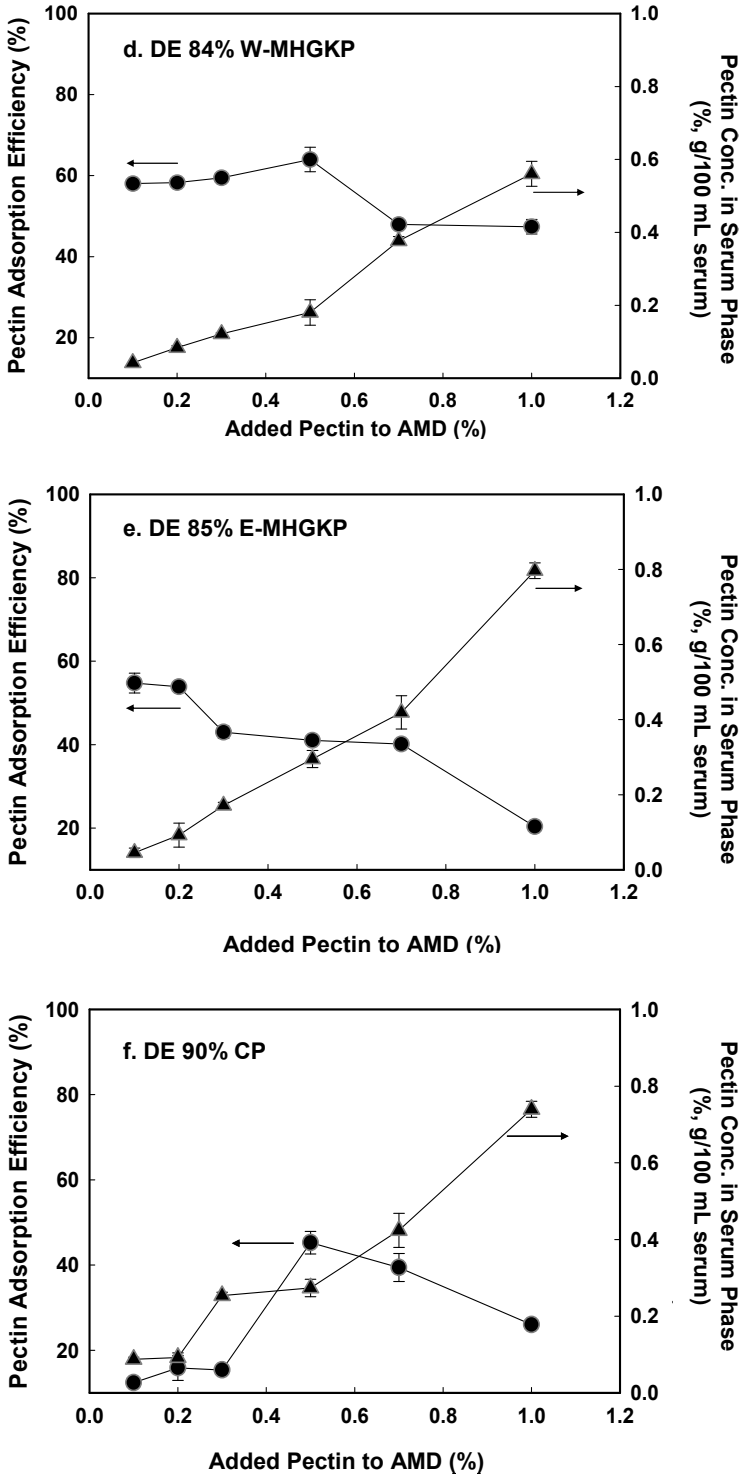

Figure 7.6 Pectin adsorption efficiency (●) and pectin concentration in the serum phase (▲) for AMDs prepared with different pectin concentrations and types: (a) DE 55% AP; (b) DE 65% AP; (c) DE 75% AP; (d) DE 84% W-MHGKP; (e) DE 85% E-MHGKP; (f) DE 90% CP (10% protein concentration, pH 3.80 ± 0.05).

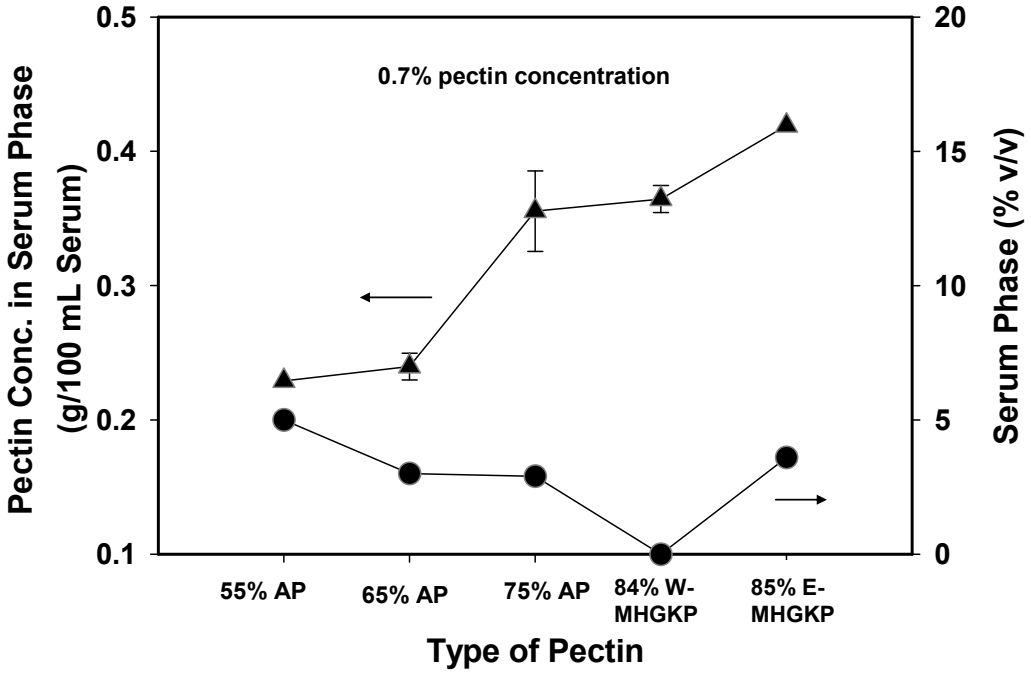

Figure 7.7 Relation between stability (percentage of serum obtained under static conditions) and pectin concentration in the serum phase of AMDs at 0.7% added pectin: (●) serum phase; (▲) pectin concentration in the serum phase (pH 3.80 ± 0.05, 10% protein concentration).

7.3.4 Measurements of Viscosity

In this section, the viscosity behaviour of the AMD as a function of pectin concentration is investigated. Figure **7.8** illustrates the apparent viscosity (up and down curves) of AMDs (**a1**−**a4**) and AMD serum phases (**b1**−**b4**), prepared with different amounts of added pectin and different pectin DEs. Very clearly, AMDs with 0.1% pectin concentration showed shear thinning behaviour for all pectins, except for that prepared with DE 84% W-MHGKP. The thinning behaviour is indicative of the presence of aggregates (pectin-bridged caseins and aggregated caseins) that break apart with increasing shear rate until the behaviour becomes Newtonian (constant viscosity). The incidence of shear thinning behaviour in unstable drinks has been reported by Parker, Boulenguer and Kravtchenko (1994), and Laurent and Boulenguer (2003) and has been explained by bridging flocculation effects in the presence of insufficient pectin. Bridging may occur due to the formation of casein−pectin−casein complexes when a pectin chain interacts with more than one casein

particle, thus "bridging" the proteins together (Tuinier, *et al.*, 2002; Liu, *et al*., 2006). The shear breaks down these aggregated structures. Our results indicate that this process is time independent (similar up and down curves), showing that the process is reversible. In contrast, Newtonian flow through the whole shear rate may indicate that the attraction strength between casein micelles decreases (with increasing pectin concentration), leading to presumably colloidal stability (Parker, *et al*., 1994; Amice-Quemeneur, *et al*., 1995). All AMDs prepared with DE 90% CP were unstable (high amounts of serum phase) at all pectin concentrations and also showed shear thinning behaviour up to 1.0% pectin, where the flow became slightly Newtonian (although still unstable). This type of pectin resulted in a non-homogeneous drink, presumably because the low adsorption level and the low viscosity resulted in a large proportion of aggregated casein, which could lead to sedimentation during the measurement, giving rise to a "fake" Newtonian behaviour.

It is clear that, at 0.1% pectin concentration, the AMD prepared with DE 84% W-MHGKP showed almost a Newtonian type of flow as opposed to the other pectins (shear thinning). It is important to note that the viscosity was determined within 1 h of preparation when all drinks were freshly prepared. In this case, drinks containing DE 84% W-MHGKP were observed to be stable (no separation) over 1 h. However, as the amount of adsorbed pectin was still insufficient to cover the casein micelles and fairly weak electrostatic interaction between pectin and casein (because of the low charge density of the pectin) was expected, the drink started to present serum separation after approximately 2 h of storage.

The shear thinning behaviour disappeared for most pectins by increasing their concentration to 0.2% (except for DE 55% AP, see Appendix **D.3**). At 0.3% pectin concentration, no incidence of shear thinning behaviour was observed in all AMDs (apart from the already mentioned AMD with DE 90% CP). This might indicate that most casein micelle aggregates were very close to full coverage. However, AMD stability was not yet achieved at this concentration because phase separation was still observed over time. This may indicate the importance of the non-adsorbed pectin to provide viscosity and stability.

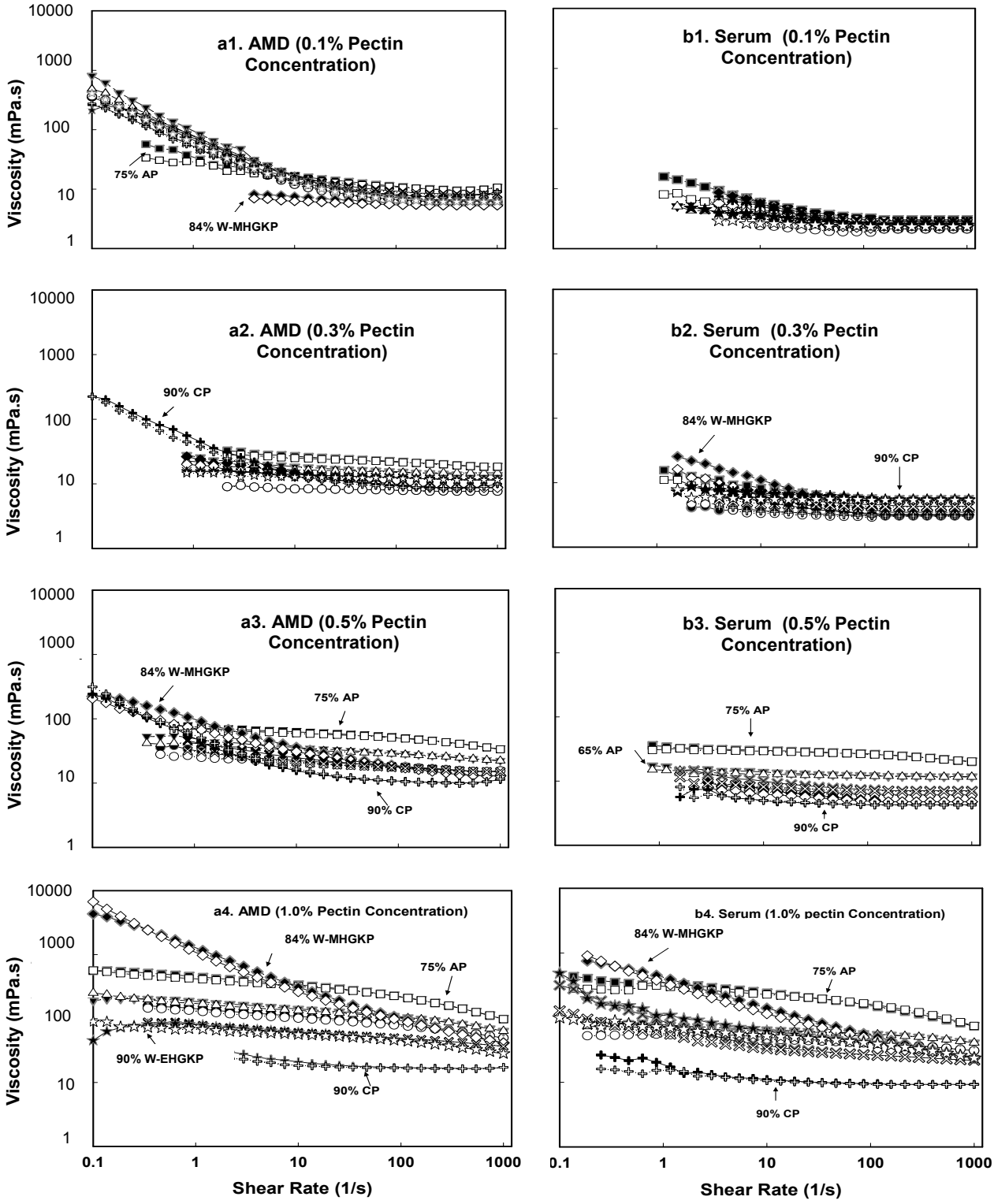

Figure 7. 8 Effect of pectin concentration on up (filled) and down (empty) viscosity curves of AMDs (a) and serum phases (b) prepared with different DE pectins: (●) DE 55% AP, (▼) DE 65% AP, (■) DE 75 AP%, (♦) DE 84% W-MHGKP, (x) DE 85% E-MHGKP, (+) DE 90% CP, (✱) DE 90% W-EHGKP.

In general, the viscosity of the AMD increased with increasing pectin concentration. However, further addition of pectin (> 0.5%) resulted in small shear thinning behaviour effects. This was clear for drinks prepared with DE 84% W-MHGKP at 0.5% pectin concentration (Figure **7.8a3**). This could not have been the result of the rheology of the non-adsorbed pectin in the serum phase, because the viscosity of the serum indicated no occurrence of shear thinning behaviour (Figure **7.8b3**). A possible explanation may be depletion flocculation effects. Depletion flocculation occurs as a result of the segregative interaction between casein micelles and pectin (Maroziene & de Kruif, 2000). In the presence of an excess of non-adsorbed pectin, an attraction force develops between casein micelles, which eventually leads to separation into a protein-rich phase and a pectin-rich phase. Local flocculation of the casein micelles and/or aggregates may occur as the pectin concentration increases (in this case, at 0.5% pectin concentration). More obvious shear thinning was observed at 1.0% pectin concentration for all pectins (except DE 90% CP) and especially for DE 84% W-MHGKP (Figure **7.8a4**). This may have been the result of: (*i*) the rheological properties of the non-adsorbed pectin suspended in the drink (shear thinning at high concentration) (Brejnholt, 2009), because the viscosity of the serum obtained also showed a similar behaviour (Figure **7.8b4**), or/and (*ii*) the occurrence of depletion flocculation forces, as previously described, in the presence of an excess of non-adsorbed pectin. However, because of the high viscosity of the system at high pectin concentration, no phase separation was observed within the time scale of observation.

In summary, the occurrence of shear thinning behaviour was greater for DE 84% W-MHGKP than for the other two gold kiwifruit pectins (DE 85% E-MHGKP and DE 90% W-EHGKP) above 0.5% pectin. This could reflect three facts: (*i*) stronger depletion flocculation forces promoted by an excess of non-adsorbed high M_w/high RMS radius pectin (larger non-adsorbed polymers will cause stronger depletion interactions); (*ii*) higher M_w of the non-adsorbed pectin itself; or (*iii*) the aggregation of non-adsorbed pectin. This resulted in higher viscosity for DE 84% W-MHGKP (high M_w) drinks (116 mPa.s) compared with ~ 78.7 and ~ 78.5 mPa.s respectively at 1.0% pectin for DE 85% E-MHGKP (intermediate M_w) and DE 90% W-EHGKP (intermediate M_w) drinks at 1 s^{-1}. At 1.0% pectin, it is also interesting to note that an AMD prepared with DE 85% E-MHGKP exhibited similar

viscosity to an AMD prepared with DE 90% W-EHGKP (~ 53.1 mPa.s at 53 s^{-1}) even though DE 85% E-MHGKP was slightly higher in M_w (Figure **7.8a4**). It seems that pectin derived using an enzymatic extraction method (DE 85% E-MHGKP) may have different distribution patches of charges influencing the pectin−casein electrostatic interactions, resulting in this pectin behaving similarly to DE 90% W-EHGKP. As reported by Tuinier *et al*. (2002) and described in section **7.3.2.2**, it is possible that certain pectin fractions may be adsorbed preferentially at certain casein patches.

Compared with gold kiwifruit pectins (DE 85 and 90%), AMDs prepared from apple pectins exhibited higher viscosity (Figure **7.8a4**). This could indicate that HMPs with higher negative charge density have more numerous electrostatic binding sites to interact with casein, creating greater layers of adsorbed pectins on the casein particles. The presence of multilayer of pectin as a result of electrostatic sorption has been confirmed by Tuinier *et al*. (2002). This multilayer may be responsible for a higher viscosity (effectively a higher hydrodynamic radius of the protein particles coated by pectin).

Figure **7.9** shows the relation between the stability of an AMD (% serum phase under quiescent conditions) and the viscosity of the serum phase at a low shear rate of ~ 2 s^{-1} (this shear rate was used given the noise observed at a shear rate of 1 s^{-1}) at 0.3 and 1.0% pectin concentration. At 0.3% pectin, all drinks showed serum separation overtime and Newtonian behaviour (measured within 1 h of preparation); drinks prepared with DE 84% W-MHGKP had the lowest serum separation and exhibited the highest serum viscosity (Figure **7.9a**). Drinks prepared with DE 75% AP did exhibit very high serum separation even though the viscosity of the serum was slightly higher than for the other apple pectins, and the M_w was similar to that of DE 65% AP. DE 85% E-MHGKP, with higher M_w than DE 75% AP but similar serum viscosity, led to less serum separation. It seems that there is no straight relationship between pectin M_w, serum viscosity and AMD stability at low pectin concentration.

At 1.0% pectin, there was no serum separation (stable drinks) except for drinks made with DE 90% CP, with the lowest serum viscosity (Figure **7.9b**) and pectin M_w. The viscosity varied among all the other stable drinks. However, DE 84% W-MHGKP exhibited the highest serum viscosity, with DE 75% AP being very close in value.

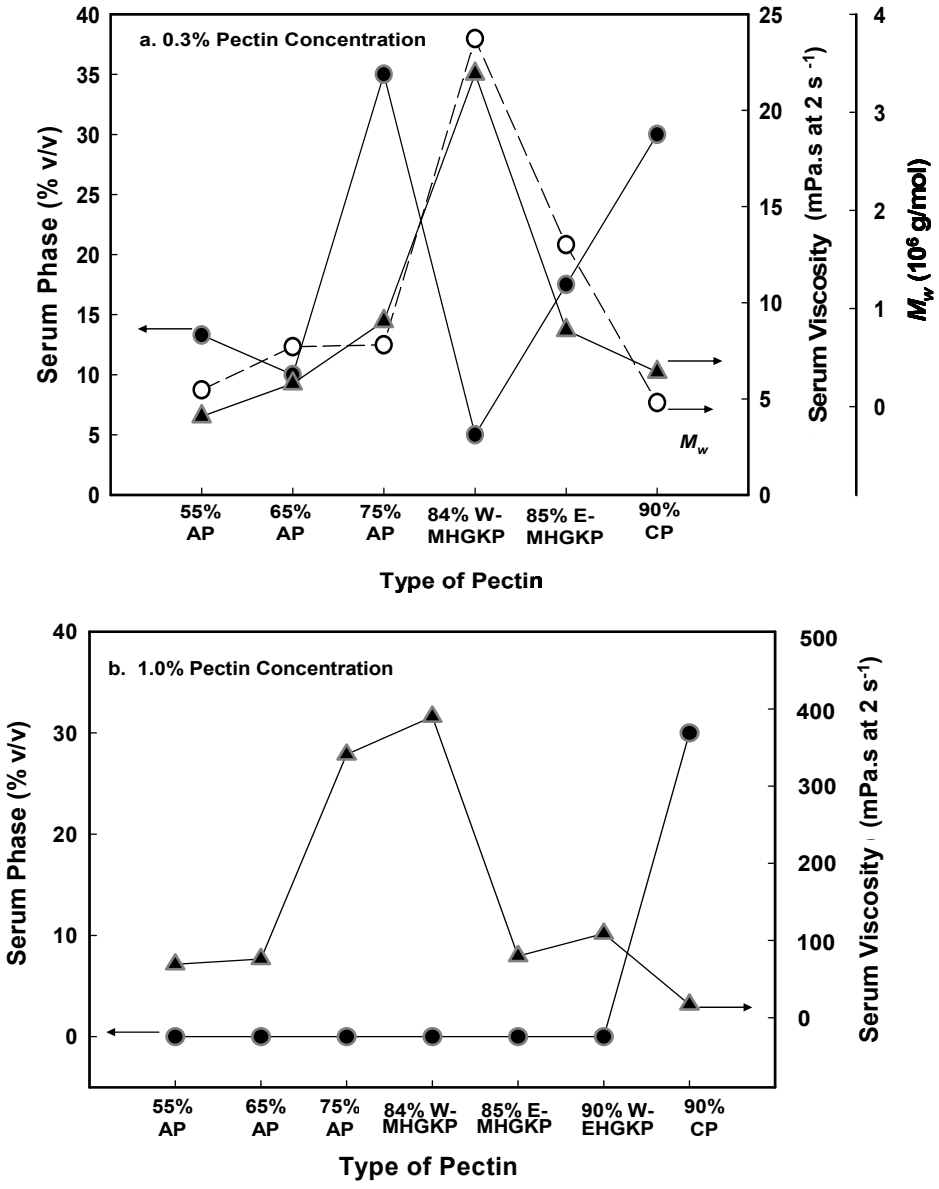

Figure 7.9 Relation between stability (amount of serum after 7 days of storage) (●), serum viscosity (2 s^{-1}) (▲) and pectin M_w (--○--) at: (a) 0.3% and (b) 1.0% pectin concentration, for different types of pectins (DE 55, 65 and 75% APs, DE 84, 85 and 90% GKPs and DE 90% CP) (10% protein concentration, pH 3.80 ± 0.05).

7.3.5 Particle Size Distribution of AMDs

In this section, the particle size of "stable AMD systems" (fully coated pectin–casein micelles and/or aggregates) is investigated and compared with the original size of the caseins (neutral pH) and the pectin polymer (in an acidic environment). The AMD samples were diluted in PS (Pure Serum) obtained from drinks with no added pectin, in order to mimic the environmental conditions of the acidic drinks. The various types of pectin were analysed for the *Z-average* diameter particle size by dissolving the pectin in 0.1 M NaCl (0.5% w/w), to prevent association between pectin molecules, and were further diluted (1:10 w/w) in PS (pH 3.80 ± 0.05). No clear trend for pectin particle size was obtained, as shown in Table **7.4** (108−235 nm); however, DE 84% W-MHGKP had the largest particle size (~ 235 nm) and citrus pectin had the smallest particle size (~ 108 nm). The *Z-average* diameter values obtained in this study were lower than values reported in the literature (Jones, Decker, & McClements, 2009) for sugar beet pectin (~ 500 nm). These authors reported that the large pectin size was influenced by the aggregation of pectin. It is very likely that the wide size distribution obtained here also represented aggregation of pectin. It still gives a very good idea of the trend.

A 10% (w/w) LHSMP dispersion at pH 6.70 ± 0.05 was prepared for casein micelle particle size determination, with further dilution (1:10 times) in imidazole buffer (0.05 M, pH 7.0) to prevent casein micelle dissociation. The *Z-average* diameter of the casein micelles was 200−250 nm, which is in agreement with the widely reported size of micelles (Tuinier, *et al*., 2002). The measurement of the AMD particle size was very challenging given the polydispersity of the system. The following particles may be present in the final drink: (*i*) individual casein micelles–pectin complexes, (*ii*) aggregated casein micelles and sub-micelles coated with pectin, (*iii*) aggregated pectin itself. Therefore, in preliminary studies, different levels of pectin in the AMD (0.5, 0.7 and 1%), various centrifugation speeds (10,000, 15,000 and 25,000 *g*), several type of solvents (0.05 M calcium imidazole buffer, Milli-Q water and PS) and different dilution ranges were tested in order to obtain a valid, reproducible and representative AMD particle average size and distribution. AMD with 1.0% pectin added and centrifuged at 25,000 *g* using pure serum for dilution were the final conditions chosen. Reproducible particle sizes with lower polydispersity (PI < 0.5) were

obtained under these conditions. Based on the results from previous sections, at this level of pectin (1%), the saturation point is likely to have been reached, avoiding bridging of caseins by pectin and maximising the chances of measuring fully covered casein micelles/aggregates (Tuinier, *et al*., 2002). The pellet obtained from centrifuged AMD was dissolved in PS (1:110). At this ratio, the particle size detected was stable and did not change with further dilution. The particle size of casein–pectin complexes of AMD obtained in this study was relatively big (Figure **7.10**) which is likely to reflect the casein–pectin complexes/aggregates. However, the results showed a good trend in relation to their DE as shown in Figure **7.11**.

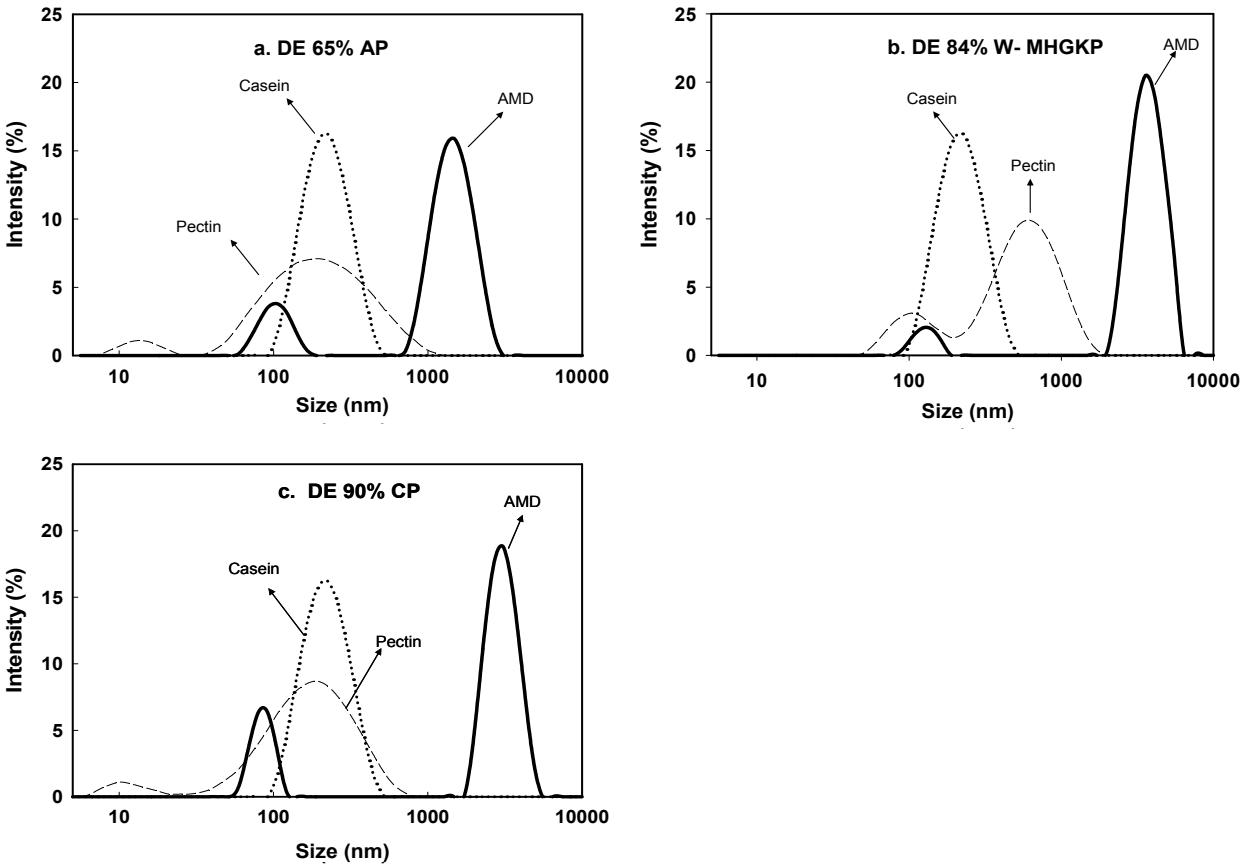

Figure 7.10 Particle size distribution of AMDs (1.0% pectin concentration, in PS 1:110 w/w, pH 3.80 ± 0.05), casein micelles (in calcium imidazole buffer pH 7.0, 0.05 M, 1:10 w/w) and pectins (pH 3.80 ± 0.05 in PS 1:10 w/w): (a) DE 65% AP; (b) DE 84% W-MHGKP; (c) DE 90% CP. (--) pectin; (…) casein; (—) AMD.

Figure **7.10** illustrates the particle size distributions of caseins, pectins and AMDs. These graphs represent stable (no phase separation) (DE 65% AP and DE 84% W-MHGKP) and unstable (DE 90% CP) AMDs. The particle size distributions of casein micelles, pectin and AMD were plotted together to observe the relative sizes for each system. Generally, a bimodal distribution was apparent in all AMDs. Based on size, the first small peak (~ 100 nm) probably corresponded to non-adsorbed pectin (or small protein particles), whereas the biggest size population (> 1 μm) corresponded to pectin-coated casein particles. The disappearance of the sizes at which the majority of the pectin lay indicates that the pectin was electrostatically adsorbed on to the protein. Jensen, Rolin and Ipsen (2010) have recently shown that AMD (pH 4.0) without added pectin presents a monomodal distribution with particle sizes of 5–15 μm. In agreement with our data, they have also shown bimodal distributions in AMDs with added pectin.

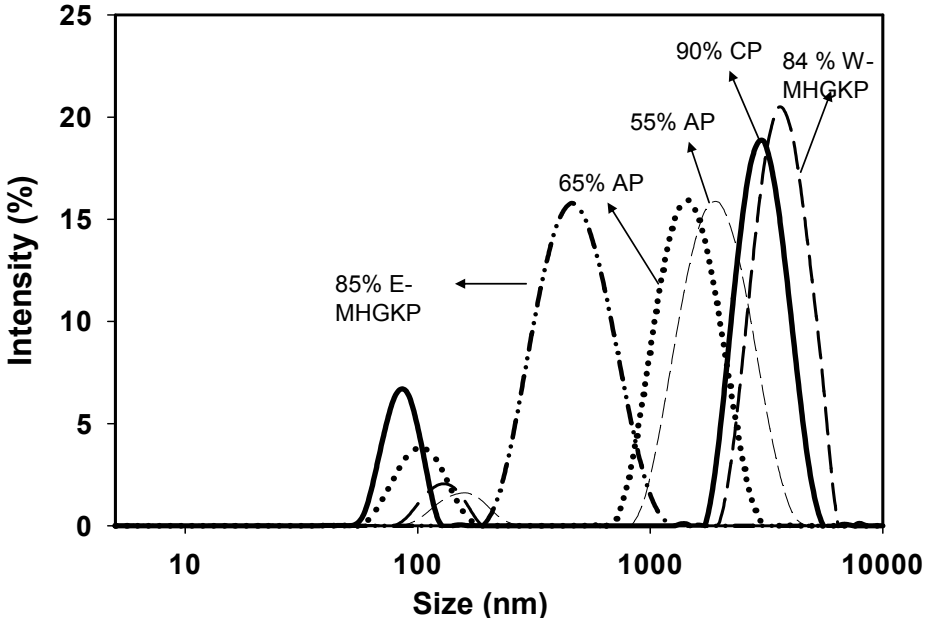

Figure 7.11 Overlay of particle size distributions of AMDs prepared with various HMPs: (---) DE 55% AP; (…) DE 65% AP; (— —) DE 84% W-MHGKP; (-···-) DE 85% E-MHGKP; (▬) DE 90% CP.

Figure **7.11** was plotted to show any pattern in the size distribution of the AMD particles stabilised by the various pectins. Stable drinks prepared from apple pectins and enzyme-extracted gold kiwifruit pectin (85%) showed a reduction in the average particle size as the DE increased (DE 55% > 65% > 75% > 85%), with sizes for the biggest peak ranging from ~ 2000 nm (DE 55%) to ~ 900 nm (DE 85%). The greater particle size in drinks prepared from DE 55% AP could correspond to the thick and more compact pectin layers of casein−pectin complexes developed by higher charged pectins. Liu *et al*. (2006) also reported a greater amount of pectin adsorbed when using high charged pectins to stabilise AMDs at low pH (< 4). At low pH values, caseins increase the number of positive charges, whereas the number of charged carboxyl groups decreases (Tuinier, *et al*., 2002). As a result, the pectin becomes less charged, and fewer sites are available for electrostatic interactions with casein particles; thus, the tendency to adsorb on to the surface of casein particles decreases. Therefore, the larger complexes at DE 55% could represent a greater amount of adsorbed pectin, resulting in more voluminous particles. In contrast, a highly stable drink prepared from gold kiwifruit pectin (DE 84% W-MHGKP) showed a very large average size (~ 5500 nm) of the AMD particles. This may have been due to: (*i*) the M_w and RMS radius values of the pectin being relatively higher (~ 235 nm) than for the other pectins, resulting in more voluminous particles, or (*ii*) large pectin−casein complexes because of the uneven distribution of charged blocks on the pectin (Nakamura, Furuta, Kato, Maeda, & Nagamatsu, 2003). In an unstable drink such as an AMD prepared using DE 90% CP, the particle size (~ 5000 nm) was observed to be similar to that of an AMD prepared with DE 84% W-MHGKP. This may indicate the presence of (*i*) large casein aggregates and (*ii*) protein only partially coated by pectin. In summary, the particle size of AMDs appeared to be largely influenced by the DE, M_w and average size of the pectin and the distribution of carboxyl groups along the pectin chains.

7.4 General Discussion

In this study, it was shown that the ability of pectin to stabilise an AMD was significantly influenced by its concentration, charge and M_w, regardless of the origin of the pectin. The major observation in unstable drinks was serum separation, which could correspond to inadequate steric repulsion created by casein−pectin complexes because of an insufficient amount of pectin, resulting in casein sedimentation. This evidence was consistent with the rheological properties, where this type of drink exhibited shear thinning behaviour as a result of a non-homogeneous drink developed by aggregated casein. This was typical for drinks at low pectin concentrations (≤ 0.3%) for the majority of the systems studied.

In contrast, a stable AMD showed very little serum separation (at higher pectin levels, ≥ 0.5%), which might correspond to: (*i*) adequate steric stabilisation developed by casein−pectin complexes because the amount of pectin was sufficient to cover the casein micelle/aggregate surface; and (*ii*) the viscosity effect of the high M_w non-adsorbed pectin in the serum phase. It is important to note that steric stabilisation is influenced by the structure and the charge density of the pectin. For example, AMD particles prepared from higher charged/short chain pectins (lower M_w) will present a more dense (compact) pectin layer covering the casein micelle/aggregate surface, where more adsorbed pectin is detected. In contrast, the AMD particles prepared from longer chain (high M_w) and less charged pectins will present a lower amount of pectin adsorbed, but with bigger loops, providing stronger steric repulsion depending on the M_w of the pectin.

The evidence of the viscosity effect of non-adsorbed pectin in stabilising the AMD was predominantly observed in drinks prepared with gold kiwifruit pectin of high M_w at 0.3% pectin concentration (DE 84% W-MHGKP) (Figure **7.9a**). The serum of this drink exhibited the highest viscosity compared with the other drink serums; as a result, greater stability was achieved (less serum separation). The drink prepared from the same origin pectin material (DE 85% E-MHGKP with intermediate M_w) showed a different pattern from DE 84% W-MHGKP, because the M_w was lower; more serum separation was observed, probably because of the lower serum viscosity or/and the formation of shorter loops,

coinciding with a greater amount of non-adsorbed pectin detected. The amounts of adsorbed pectin when using these two pectins (DE 84% W-MHGKP and 85% E-MHGKP) were relatively different, even though both had similar DE. It is likely that the difference in pectin adsorption was also driven by the irregularities of pectin distribution charges because of enzymatic modification during isolation of DE 85% E-MHGKP; as a result, this fraction of pectin could adsorb preferentially only at certain patches of the casein surface.

The roles of negatively charged blocks in pectin chains and the M_w of these chains are very important in describing the mechanism of adsorbed and non-adsorbed pectin and for inferring the stability in AMD systems. The charges available are responsible for the electrostatic interaction between casein and pectin, whereas the M_w and the distribution of charges are responsible for creating repulsive interactions between casein−pectin complexes by probably providing larger loops of uncharged chains that protrude into the serum phase of the drink. Liu *et al*. (2006) reported that, in a low pH drink system, the charged blocks of pectin are fundamental in driving the adsorption to casein micelles, the size of the adsorbed pectin chains determining the extent of the steric repulsion between casein−pectin complexes. Based on the results obtained in this study, the following can be inferred and is described schematically in Figure **7.12**.

a. The adsorption efficiency of low negatively charged gold kiwifruit pectins (84 and 85%) decreased as the addition of pectin was increased and was lower than that of DE 55 and 65% APs. For high M_w DE 84% W-MHGKP, the amount of pectin needed to achieve stability was lower than for the other pectins, as described in section **7.3.2**. These results may indicate that, to achieve a stable drink, all positively charged casein micelle sites do not necessarily need to be interacting with pectin; the extended loops protruding from casein−pectin complexes into the serum phase—created by high M_w pectins— may be able to keep the casein−pectin complexes dispersed. A similar mechanism of stabilisation could be postulated for DE 85% E-MHGKP (Figure **7.12a**). However, as the M_w was lower, the protruded loops developed may not have been as big as those in DE 84% W-MHGKP; as a result, the stabilisation effect of this pectin was also lower and more serum separation was observed at low pectin concentration (0.3%).

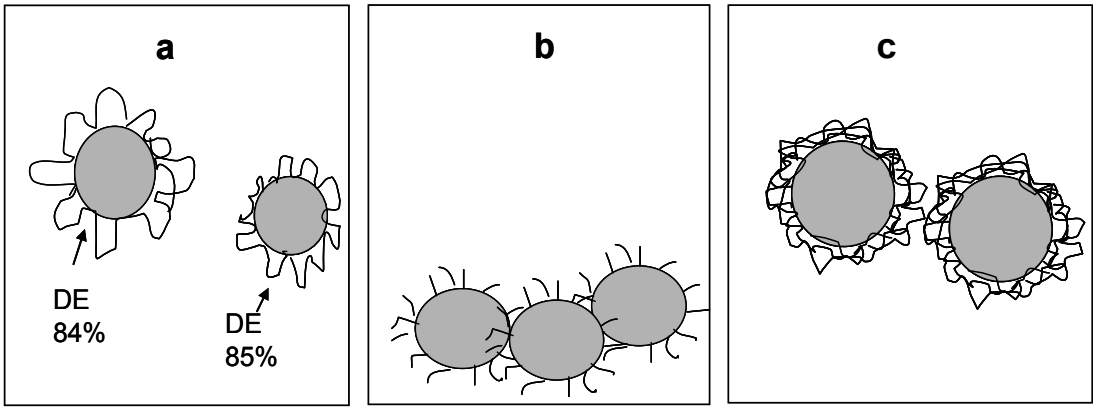

Figure 7.12 Schematic mechanism of casein−pectin interaction: (a) gold kiwifruit pectins (DE 84 and 85% GKP), (b) citrus pectin (DE 90% CP) and (c) commercial apple pectins (DE 55 and 65% AP).

b. Clearly, the large amount of non-adsorbed pectin for a very low charge density pectin (DE 90% CP)—with low casein−pectin interaction—did not lead to stability, probably because of the very low M_w of this high DE pectin. The adsorption efficiency in this case peaked at 0.5% pectin, and in general the adsorption levels were significantly lower than for the other pectins. It was clear that DE 90% CP has no potential in stabilising AMDs at similar GalA concentration to the other pectins. The very short loops/chains protruding from the casein−pectin complexes probably cannot provide enough steric repulsion (**Figure 7.12b**). Therefore, AMDs produced by this pectin had high serum separation and shear thinning viscosity flow. However, addition of gold kiwifruit pectin with a similar charge density or high DE (DE 90% W-EHGKP) and intermediate M_w (1.03×10^6 g/mol) could stabilise the drinks at the concentrations used here. This shows that the magnitude of the M_w and probably the charge distribution are crucial for the stability provided by these high DE pectins.

c. HMPs with greater net negatively charged chains, because of their free carboxylic groups (DE 55 and 65% AP), had greater adsorption efficiencies (Figure **7.12c**). These pectins may adsorb in a more compact layer, leading to greater amounts on the casein micelles compared with the lower charged pectins (DE > 80%). Therefore, the pectin load in these systems was higher and the amount of non-adsorbed pectin was

consistently lower. This is also probably why the drinks containing these pectins had bigger particle sizes.

Evidence of the possible influence of M_w on the stability of AMDs was also shown for the drinks prepared from the commercial pectin GCP (Danisco). This pectin (DE > 70%) is specifically recommended for the stabilisation of AMDs with a recommended dosage of typically 0.35−0.50% for a standard yoghurt drink. However, at this concentration, this pectin—with probably low M_w based on the low viscosity detected—did not stabilise the acid system studied here (~ 3.5% protein), when used as the only stabiliser. The desirable low viscosity in an AMD is probably the reason behind the use of this pectin. The results here suggest that higher pectin concentration is required (probably above 1.0%) to stabilise an AMD under the conditions of this study. DE 84% W-MHGKP, characterised by high DE and M_w, has been shown to stabilise AMDs at only ≥ 0.3% pectin concentration and provides high viscosity, whereas a greater amount of DE 85% E-MHGKP is required but gives a lower viscosity, which is probably more suitable for the preparation of AMDs.

CHAPTER 8 Overall Conclusions and Recommendations

Techniques for isolating pectin from gold kiwifruit, its physicochemical characterisation and its incorporation in an acidified milk drink system have been examined, underlining the importance of its physicochemical and functional properties. Figure **8.1** summarises the pectin isolation techniques, physicochemical characterisations and functional properties investigated.

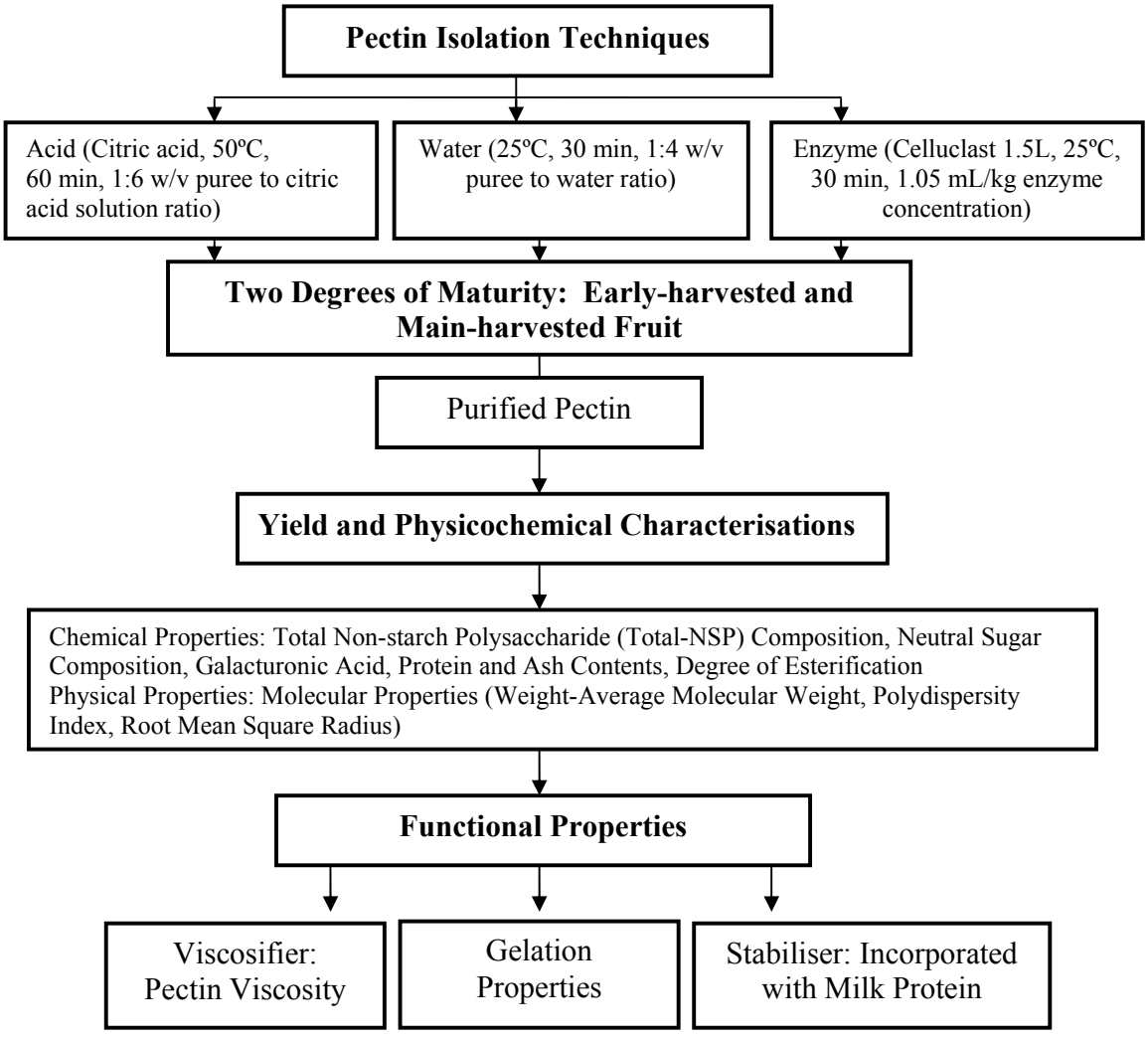

Figure 8.1 General overview of the isolation and characterisation of gold kiwifruit pectin, as investigated in this thesis.

In general, this study set out (*i*) to investigate the effect of techniques (acid, water and enzyme) and conditions (temperature, time and puree to solvent ratio) for extracting pectin from main-season gold kiwifruit (MHF), (*ii*) to study the physicochemical characterisation of the isolated pectin and (*iii*) to relate the physicochemical properties of the pectin to its functional properties in food systems. For comparison purposes, pectin from less mature gold kiwifruit (*i.e*. early-season fruit, EHF) was also isolated using the extraction methods that had been developed for MHF. A large part of this research focused on the pectin isolated from MHF because this more mature fruit forms the major part of the gold kiwifruit harvest. This included evaluation of isolation by treatment with acid, water and enzyme, evaluation of extraction conditions, physicochemical characterisation of the pectin using gas liquid chromatography (sugar composition), capillary electrophoresis (degree of esterification, DE), rheological properties (viscosity) and static light scattering techniques (molecular properties). Pectin isolated from fruit of both maturities was purified and, together with commercial pectin (*i.e.* Sigma pectin P9135), was studied using rheological and light scattering techniques. For comparison purposes, some commercial polysaccharides, such as apple and citrus pectins, were also included in the functional property studies.

8.1 Conclusions

8.1.1 Effect of Extraction Techniques (Acid and Water) and Conditions (Times and Temperatures) on the Physicochemical Properties of Crude Pectin Isolated from MHF

- When extraction techniques and conditions varied, the pectin composition was affected but not the pectin yield. Galacturonic acid (GalA) was observed to be the major component of the water-soluble polysaccharide extracted from gold kiwifruit by acid and water, suggesting that pectin dominated the soluble polymers, as is commonly found in fruits. The GalA content of crude pectin was affected significantly by the extraction method (acid and water) but not by the extraction time (30 and 60 min) or the extraction temperature (25 and 50°C). Crude water-extracted pectin appeared to have a higher GalA content (26.54% w/w) than crude acid-extracted pectin (23.59% w/w), indicating

that pectin hydrolysis occurred in the presence of citric acid. Crude water-extracted pectin was also found to be richer in side chain sugars such as rhamnose, arabinose and galactose (4.45% w/w) than acid-extracted pectin (2.40% w/w), again indicating that more pectin side chain hydrolysis occurred with citric acid treatment than with water treatment.

- The total-NSP content of crude gold kiwifruit pectin was only about 30.33% w/w and, together with protein (~ 6.4% w/w), ash (~ 8.0% w/w) and moisture (~ 12% w/w) (the ash and moisture contents were tested randomly to get an idea of the ash and moisture contents of the crude extracts), comprised about 57% of the crude pectin. The remaining 43% could have been soluble sugars, such as monosaccharides and disaccharides, and organic acids.

- The viscosity of the crude extracts was significantly influenced by (*i*) the polymer concentration in the solution and (*ii*) the technique and temperature of isolation. Crude pectin isolated by acid treatment at 50°C for 60 min exhibited higher viscosity than that isolated by acid treatment under other extraction conditions. Crude pectin isolated by water treatment at 25°C for 30 min had the highest viscosity. Water-extracted pectin had higher GalA content than acid-extracted pectin, showing the strong relationship (R^2 = 0.86) between viscosity and GalA content; the viscosity was lower when the amount of GalA in the solution was also lower.

- Varying the amount of extraction solvent (puree to solvent ratio) was also investigated in preliminary studies. In general, increasing the puree to solvent ratio increased the viscosity of the pectin, as a result of the increasing GalA concentration. The highest viscosities were exhibited by crude pectin extracted by acid treatment with a puree to solvent ratio of 1:6 (w/v), and by crude pectin extracted by water treatment with a puree to solvent ratio of 1:4 (w/v). Therefore, acid extraction at 50°C for 60 min with a puree to solvent ratio of 1:6 and water extraction at 25°C for 30 min with a puree to water ratio of 1:4 were chosen for further investigation.

8.1.2 Effect of Enzymatic Extraction Conditions and Enzyme Concentration on the Physicochemical Properties of Crude Pectin Isolated from MFH

- From this work, Celluclast 1.5L was selected from among several commercial enzyme preparations for the extraction of gold kiwifruit pectin. The effects of extraction time (30 and 60 min) and extraction temperature (25 and 40°C) on the yield, total-NSP composition, sugar composition and viscosity of crude pectin were investigated. The results indicated that the interaction of these parameters had a slight influence on the yield, composition and viscosity of the pectin.

- The highest yield (9.19% w/w) was obtained when the pectin was isolated at 40ºC for 60 min. This was attributed to higher cellulase (but lower polygalacturonase) activity at 40ºC. It is likely that cellulase favoured the release of pectin from the cell wall of the kiwifruit by hydrolysing the cellulose network, therefore increasing the pectin yield.

- As for the crude acid and water extracts (from the studies in Chapter **3**), GalA was a major component of crude enzyme-extracted pectin. A lower GalA content was found at an extraction temperature of 40ºC (39.89% w/w) than at the lower temperature of 25ºC (53.35% w/w) because increasing the extraction temperature significantly promoted pectin hydrolysis. This seemed to contradict the high yield of crude pectin extracted under similar conditions. The presence of contaminants such as protein, ash and other low molecular weight (M_w) sugar fractions, which were isolated during the extraction process, could have contributed to the high yield of pectin. In addition, the extent of hydrolysis was more obvious at the longer extraction time (60 min); increasing the extraction time (at 40ºC) from 30 to 60 min led to a decrease in the GalA content from 44.60 to 35.17% w/w. The neutral sugar content of the pectin side chains was not affected by varying the extraction conditions. The average contents of rhamnose, arabinose and galactose were ~ 0.58, 1.05 and 1.48% w/w respectively.

- The effect of the extraction conditions on the rheological behaviour of pectin was not as clear. However, pectin extracted at 25ºC for 30 min exhibited higher viscosity than pectin extracted under other conditions. Interestingly, pectin extracted at 25ºC for 60

min exhibited lower viscosity even though the GalA contents were similar. At this stage, the M_w of the pectin may have been responsible for the viscosity behaviour.

- Varying the enzyme concentration had a significant effect on the yield, viscosity and molecular properties, but not on the GalA content. The GalA content for all extracts was ~ 50─53% w/w. Determination of the M_w using static light scattering indicated that the high viscosity was related to the high M_w of the pectin. Pectin isolated using a medium enzyme concentration was shown to have higher yield (8.08% w/w), viscosity and M_w (16.50 x 10^5 g/mol) than pectins isolated using a low enzyme concentration (yield, 6.58% w/w; M_w, 3.72 x 10^5 g/mol) and a high enzyme concentration (yield, 7.01% w/w; M_w, 2.44 x 10^5 g/mol). The polydispersity index of all extract was relatively similar (~ 2). The flow properties of enzyme-extracted pectin solutions were consistent with their M_ws.

- The conditions that produced a high pectin yield did not result in a high pectin viscosity. In addition, the amounts of total-NSP and GalA obtained by chemical analysis did not relate directly to the viscosity of the pectin. The viscosity of the pectin depended markedly on its M_w. Celluclast 1.5L at a medium concentration (1.05 mL/kg) and using extraction conditions of 25ºC for 30 min were recommended for the extraction of gold kiwifruit pectin by enzymatic treatment.

8.1.3 Effect of Fruit Maturity and Extraction Technique on Purified Kiwifruit Pectin

- This part of the research involved studies on the physicochemical characterisation of pectin isolated from less mature fruit (EHF). The pectin was obtained using a similar extraction method to that used for MHF. Both extracts were purified to approximately 39−69% w/w of total-NSP using centrifugation and ethanol precipitation. This study showed that the yield of pectin was significantly influenced by both the fruit maturity and the extraction method. The purified yield was higher for MHF (3.64% w/w) than for EHF (1.52% w/w), suggesting that it is more difficult to isolate pectin from less mature fruit than from more mature fruit. In addition, the yield was higher for

enzymatic extraction (3.26% w/w) than for acid extraction and water extraction (~ 2% w/w).

- Interaction of the fruit maturity and the extraction method also influenced the GalA content of the extracts. Pectin from EHF had lower GalA content than pectin from MHF. However, EHF pectin appeared to be more branched (carried side chains every 47−57 GalA residues) than MHF pectin (carried side chains every 50−97 GalA residues). This was also indicated by the high proportion of arabinose (~ 2.90% w/w) and galactose (~ 6.83% w/w). Acid-extracted EHF pectin was higher in GalA content (48.80% w/w) than water-extracted (42.88% w/w) and enzyme-extracted (28.96% w/w) EHF pectin. Acid-extracted and enzyme-extracted MHF pectins had similar GalA contents (56−58% w/w), but enzyme-extracted MHF pectin was less branched. Water-extracted MHF pectin had the lowest GalA content (51.87% w/w), indicating poorer efficiency of its isolation. EHF pectin was higher in protein content (25.94% w/w) than MHF pectin (13.82% w/w), suggesting that it was lower in purity.

- The molecular properties of the purified pectin, such as M_w, polydispersity index and root mean square radius, were influenced by interaction between the fruit maturity and the extraction method. Pectins isolated from fruit of both maturities by enzymatic treatment had the lowest M_w. Water-extracted MHF pectin had the highest M_w (3.75 x 10^6 g/mol), with a polydispersity index of 2.43 and a root mean square radius value of 182.7 nm. In contrast, water-extracted EHF pectin was characterised by lower M_w (1.03 x 10^6 g/mol), with a polydispersity index of 5.42 and a root mean square radius value of 114.7 nm. This study confirmed that pectins extracted from fruits with different degrees of maturity and using different methods have different molecular characteristics.

- The pectin isolated from gold kiwifruit has a high DE and is therefore classified as a high methoxyl pectin (HMP). The maturity of the fruit had a marked influence on the DE. EHF pectin had a higher DE (90%) than MHF pectin (84%), indicating the possibility of pectin de-esterification by pectin methyl esterase (PME) during the maturation process. There was a slight influence of the extraction method on the DE;

acid-extracted pectin had lower DE (85%) than water-extracted and enzyme-extracted pectins (88%).

- The rheological properties of pectins extracted from fruit of two different maturities and using different extraction methods were relatively different. With similar extraction methods, EHF pectin exhibited higher viscosity than MHF pectin. In this study, the contribution of M_w and DE to the viscosity of pectin was obvious. The viscosities of pectins extracted from fruit of similar maturity were dependent on the M_w; water-extracted pectin exhibited the highest viscosity followed by acid-extracted pectin and then enzyme-extracted pectin. However, EHF pectin (with lower M_w) exhibited higher viscosity than MHF pectin (higher M_w). There are two explanations for this finding: (*i*) the low electrostatic repulsion of high DE pectin (EHF) reduced the intra- and/or intermolecular distances, leading to more molecular association; (*ii*) the greater degree of branching in the structure led to more chain−chain associations or entanglements. This finding was confirmed by the viscoelasticities of two water-extracted pectins; EHF pectin exhibited a higher elastic modulus than MHF pectin.

8.1.4 Gelation Properties

- This study investigated the gelation properties of purified water-extracted gold kiwifruit pectins of different DEs (84 and 90%) and extracted from fruit of different maturities (EHF and MHF). Commercial pectins from different sources (apple and citrus) and with different DEs (55, 65, 75 and 90%) were used for comparison purposes. The DE of the pectin had a marked influence on its gelation properties. Pectins with lower DE were shown to form stronger gels than pectins with higher DE. Varying the gelation conditions, such as pH, pectin concentration and sucrose concentration, influenced the gelation properties of apple pectins (high charge density). However, these conditions did not greatly affect the gelation properties of gold kiwifruit pectins (lower charge density). Gold kiwifruit pectin gels were characterised as “weak gels”; these pectins could be ideal for applications that require the suspension of fruit particulates, such as marmalades or jams.

- Fruit maturity had a marked effect on the functional properties of gold kiwifruit pectin. The gel obtained using MHF pectin was considerably firmer than that obtained using EHF pectin. This was attributed to aggregation induced by the DE differences. High charge density (or low DE) pectins have numerous active sites (ionised pectin chains), with higher probability for hydrogen bonding, and sufficient hydrophobic interactions, resulting in more stabilised molecular networks. This leads to a greater "connectivity" of the network and therefore to higher elasticity or storage modulus *G'* or gel strength. High DE pectin gels faster at higher temperature than low DE pectin.

8.1.5 Stabilisation Effect of Gold Kiwifruit Pectins in Acidified Milk Drink Systems

- In this part of the research, gold kiwifruit pectins of different DEs (84, 85 and 90%) and extracted using different methods (water and enzyme) were incorporated into an acidified milk drink (AMD) system as a stabiliser to prevent sedimentation of the casein micelles, which commonly occurs when milk is acidified. The stabilisation effect of gold kiwifruit pectin was compared with that of other commercial pectins (apple and citrus) with different DEs (55, 65, 75 and 90%). Stabilisation of the AMD by gold kiwifruit pectins confirmed that pectin isolated from gold kiwifruit can be used to prevent sedimentation of the casein micelles.

- Regardless of the source of the pectin, stabilisation of an AMD was achieved by pectin with high M_w. In the presence of high M_w, serum separation was reduced at low pectin concentration because, the longer the pectin chain, the greater were the extended loops protruding from the casein−pectin complexes, resulting in stronger steric repulsion between complexes and therefore providing greater stability. In addition, the viscosity effect created by high M_w apparently supports the dispersion of casein–pectin complexes in the drink. For example, water-extracted main-season gold kiwifruit pectin (DE 84% W-MHGKP) provided the greatest stability (lowest serum separation) to an AMD at a pectin concentration of 0.3% compared with other pectins. Furthermore, there was a mutual relationship between M_w and DE, where the charges available were responsible for the electrostatic interaction between casein and pectin, whereas the M_w and the

distribution of charges were responsible for creating repulsive interactions between casein−pectin complexes. In relation to this, pectin with very low M_w and high DE (*e.g.* DE 90% citrus pectin) was not able to provide stability because there was very weak electrostatic interaction between pectin and casein and insufficient steric repulsion. However, gold kiwifruit pectin with similar charge density but high M_w (DE 90% W-EHGKP) was able to stabilise the AMD. It is very likely that the loops protruding (because of high M_w) from the casein−pectin complexes supported the dispersion of the complexes in the drink space or the viscosity effect of the non-adsorbed pectin in the serum phase. Therefore, drink stabilisation was achieved.

- Pectins with higher charge density (*e.g.* DE 55 and 65% apple pectins) had greater adsorption efficiency. These pectins adsorbed in a more compact layer, leading to greater amounts of pectin attached to the casein micelles compared with the lower charge density pectins (*e.g.* DE > 80%). This could imply that the amount of pectin required to achieve a stable system is greater for high charge density pectins than for lower charge density pectins.

- The measurement of AMD particle size is fairly challenging because of (*i*) the polydispersity of the AMD system and (*ii*) the presence of casein–pectin complexes/aggregates or pectin aggregates.

8.1.6 Pomace Pectin

- Pectin could also be extracted from fresh gold kiwifruit pomace, which is a by-product from juice manufacture. Pectin extracted from gold kiwifruit pomace had different physicochemical properties from pectin extracted from the whole fruit even though it was isolated using similar methods. Purified pomace pectin was characterised by a high GalA content (65.7% w/w) and a high neutral sugar content (rhamnose, 1.37% w/w; arabinose, 2.77% w/w; galactose, 5.63% w/w). However, this pectin had inferior rheological properties compared with whole fruit pectin, which could be attributed to its low M_w (7.9×10^5 g/mol).

- There was only a slight effect of the different extraction methods (acid, water and enzyme) on the physicochemical properties of pomace pectin; as a result, the functional properties (viscosities) of the three extracts were also relatively similar.

- Gold kiwifruit juice contained pectin with high M_w (~ 1.36 x 10^6 g/mol) and a GalA content of 48.39% w/w. It is likely that the pectin present in the juice was solubilised from the fruit cell wall during the maturation/ripening process. The solubilisation of pectin during the maturation/ripening process has been well established in some studies on green kiwifruit.

8.2 Recommendations

The recommendations for further research on gold kiwifruit pectin are as follows.

- **Pectin isolation from EHF**. In this study, the method for extracting pectin from EHF is a relatively "weak method"; most of the pectin in EHF is available as insoluble pectin. An investigation of different types of acid used with higher pHs could lead to greater recovery and different functional properties of the pectin.

- **Pectin from fruit of different degrees of maturity**. To determine the relation between the maturity of gold kiwifruit and the physicochemical properties of the pectin, there needs to be a study on subsequent degrees of maturity. This study investigated only two fruit maturities.

- **Biological activity and health**. Pectins isolated from gold kiwifruit have high M_w. Polysaccharides with high M_w have been reported to have high biological activity. This research is in its infancy and merits further investigation.

- **Pomace pectin**. The physicochemical and functional properties of pomace pectin, such as its DE, its gelation properties and its behaviour when incorporated into an AMD

system, need to be investigated further. Gold kiwifruit pomace could be a valuable source of pectin and could offer a new application in food systems.

- **AMD and commercialisation**. Further research on AMD stabilisation by gold kiwifruit pectin needs to be carried out, particularly in relation to the commercialisation part such as its safety, quality of the drink and sensory evaluation.

- **Further research on DE**. Gold kiwifruit pectin was observed to be "a very high" HMP. Investigating the effect of manipulating the DE, by treating the pectin with PME, on the gelation and protein stabilisation properties could be a very challenging future study.

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Appendix A

A.1 Megazyme Assay Kit for Total Starch Determination

Table A.1 Megazyme assay kit information

Sample No	Description	Remarks
1	Thermostable α-Amylase	Activity: 3000 IU/mL
2	Amyloglucosidase	Activity: 3300 IU/mL
3	GOPOD Reagent Buffer	Potassium phosphate buffer (1 M, pH 7.4), *p*-Hydroxybenzoic acid (0.22 M), Sodium azide (0.4% w/w)
4	GOPOD Reagent Enzymes	Glucose oxidase (more than 12,000 IU/g), Peroxidase (more than 650 U/g), 4-Aminoantipyrine (80 mg/g)
5	D-Glucose Standard Solution	1.0 mg D-Glucose/mL in 0.2 % (w/v) Benzoic acid
6	Standardised Regular Maize Starch Control	Starch

A.2 Reagent Preparations for Starch Determination

MOPS Buffer (50 mM, pH 7.0)

11.55 g of MOPS (sodium salt, Sigma cat. no. M-9381) was dissolved into 900 mL of RO water and the pH was adjusted to 7.0 by the addition of 1 M (10% v/v) HCl (approximately 17 mL was required). 0.74 g of calcium chloride dihydrate and 0.2 g of sodium azide were added and the volume was adjusted to 1 L by the addition of RO water.

Sodium Acetate Buffer (200 mM, pH 4.5)

11.8 mL of glacial acetic acid (1.05 g/mL) was dissolved into 900 mL of RO water. The pH was adjusted to 4.5 by the addition of 1 M (4 g/100 mL) sodium hydroxide solution (approximately 60 mL was required). 0.2 g of sodium azide was added and the volume was adjusted to 1 L by the addition of RO water.

Heat-stable α-Amylase Solution

1.0 mL of heat-stable α-amylase was diluted into 30 mL of 50 mM MOPS buffer, pH 7.0. This diluted enzyme was frozen before use and was kept cool (4ºC) during analysis.

A.3 Englyst Fiberzyme Kit Gas Liquid Chromatography (GLC) Information

Contents of Englyst Fiberzyme Kit GLC

1. Heat-stable α-amylase.
2. De-branching enzyme (pullulanase).
3. Pancreatin.
4. GLC stock sugar mixture. This was prepared by dissolving 0.52 g of rhamnose, 0.48 g of fucose, 4.75 g of arabinose, 4.45 g of xylose, 2.82 g of galactose, 9.4 g of glucose and 2.79 g of GalA in RO water followed by the addition of 500 mL of saturated benzoic acid solution (0.5 g/100 mL) and was adjusted to 1 L with RO water.
5. Internal standard. This was prepared by dissolving 500 mg of allose in RO water, followed by the addition of 250 mL of saturated benzoic acid (0.5 g/500 mL) and was made up to 500 mL with RO water. This gave a concentration of 1 mg allose/mL.
6. Reference sample 1 (white flour). This sample was used to check the efficiency of starch hydrolysis.
7. Reference sample 2 (haricot bean). This sample contained all the constituent sugars of NSP and was used to check the efficiency of starch hydrolysis and the washing steps for samples.
8. Reference sample 3 (high-amylose starch resistant to α-amylase). This sample was used through all procedures for total-NSP and was used to check the efficiency of starch hydrolysis and the washing steps
9. Reference sample 4 (cellulose). This sample was used to check the efficiency of the acid hydrolysis step.

A.4 Preparation of Reagents for Total-NSP Composition, Neutral Sugar Composition and GalA Concentration Determination

Dimethylphenol Solution

0.1 g of 3,5-dimethylphenol was dissolved in 100 mL of glacial acetic acid solution.

Sodium Acetate Buffer, 0.1 M, pH 5.2

13.6 g of sodium acetate trihydrate was dissolved and adjusted to 1 L with RO water. The pH was adjusted to 5.2 by the addition of 0.1 M acetic acid. In order to stabilise and activate the enzyme, 4 mL of 1 M NaCl was added to 1 L of buffer.

Sodium Chloride−Boric Acid Solution

2 g of NaCl and 3 g of boric acid were dissolved in 100 mL of RO water.

A.5 Response Factors (RFs) and Chromatogram Reference Sugars from Alditol Acetate Sugars Standard Mixture

Table A.5 Response factors of alditol acetate sugars standard mixture obtained by GLC

Order of Elution	Alditol Acetate	Retention Time (min)	Peak Area	RF Relative to Allitol Hexaacetate
1	Rhamnitol Pentaacetate	2.206	14607	2.245
2	Fucitol Pentaacetate	2.330	17431	0.941
3	Arabinitol Pentaacetate	3.236	161371	1.016
4	Xylitol Pentaacetate	4.207	137425	1.193
5	Allitol Hexaacetate	5.235	98379	1.000
6	Mannitol Hexaacetate	5.609	79452	1.032
7	Galactitol Hexaacetate	6.047	111316	0.884
8	Glucitol Hexaacetate	6.683	346733	0.946

A.6 GLC Chromatograms of Alditol Acetate Derivatives

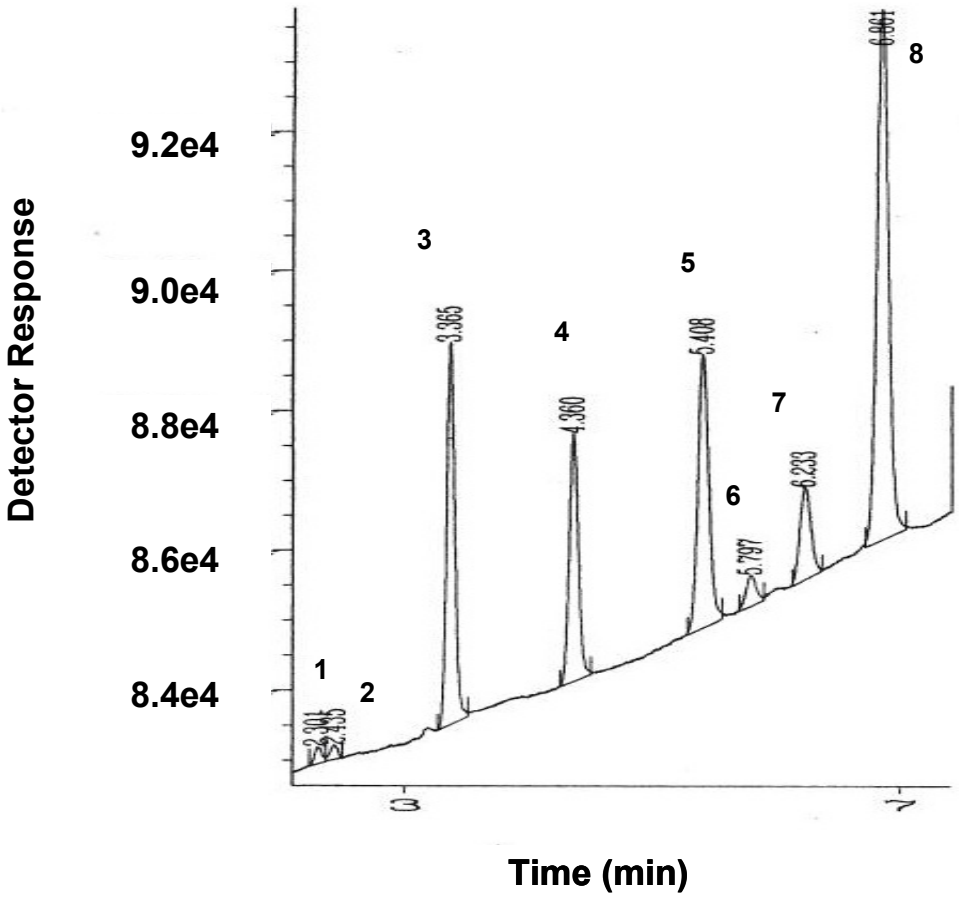


Figure A.6 GLC chromatograms of alditol acetate derivatives. A mixture of sugar standards. 1, rhamnose; 2, fucose; 3, arabinose; 4, xylose; 5, allose; 6, mannose; 7, galactose; 8, glucose.

A.7 Correlation between GalA Concentration and Viscosity

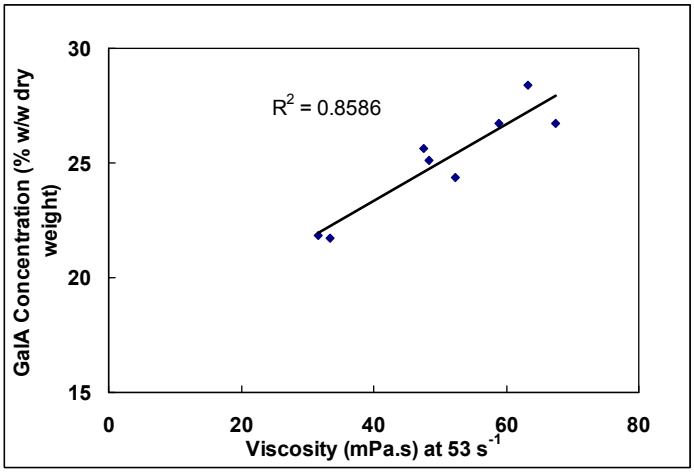


Figure A.7 Correlation between viscosity of acid- and water-extracted pectin at 53 s^{-1} (4% w/w dwb, pH 3.50 ± 0.01) and GalA concentration (% w/w) of pectin extracted at different temperatures and times.

A.8 Effect of Ratio of Gold Kiwifruit Puree to Extraction Solution on Viscosity of Crude Pectin

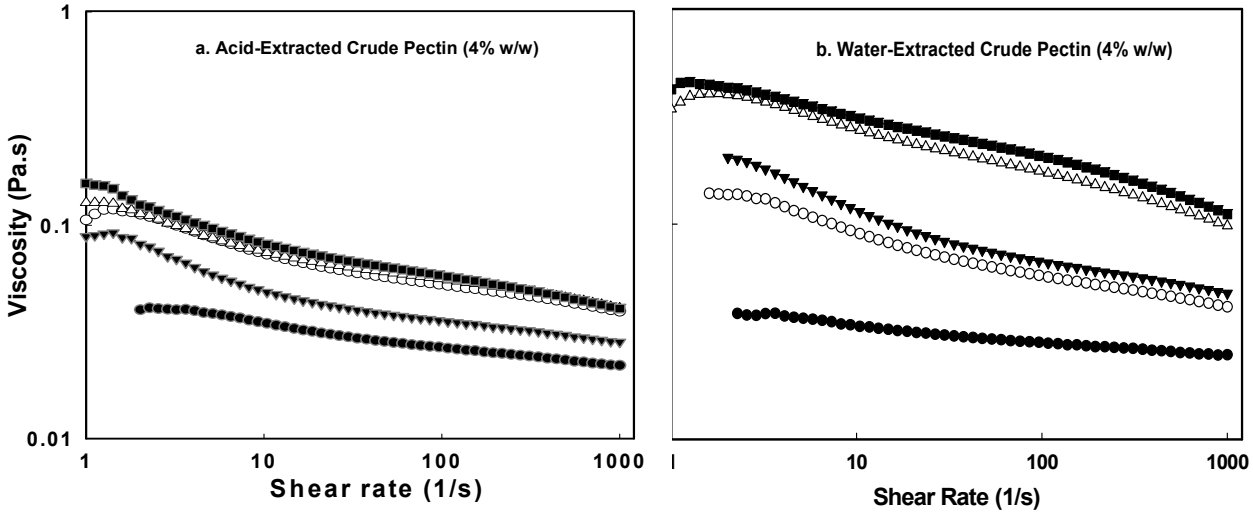


Figure A.8 Viscosity of crude pectin (4% w/w dwb, pH 3.50 ± 0.01) extracted using (a) CA (50°C, 60 min) and (b) water (25°C, 30 min) at different puree to solution ratios (w:v): (●) 1:1; (○) 1:2; (▼) 1:3; (Δ) 1:4; (■) 1:6.

Appendix B

B.1 Commercial Enzyme Specifications

Table B.1 Specifications for commercial enzymes (Adapted from pectin technical information)

Commercial Enzymes*	Optimum Condition	Activity	Source	Company
Celluclast 1.5L FG	pH: 4.5–6.0 Temperature: 50–60ºC	700 IU/mL of Endo-glucanase	*Trichoderma reesei*	Novozymes, Copenhagen, Denmark
Cellulyve TR 400	pH: 4.5–5.5 Temperature: 50ºC	2000 CM IU/g of 1.4-*β*-Endo-D-glucanase, Cellobiase, Cellobiohydrolase	*Trichoderma longibrachiatum*	Lyven, Colombelle, France
Cytolase CL	pH: 3.0–3.5 Temperature: 10–60 °C	613 CM IU/mL of Endo-glucanase, Cellobiase	*Trichoderma longibrachiatum* and *Aspergillus niger*	DSM, The Netherlands

*The commercial enzymes were in liquid form, except Cellulyve TR 400 which was in solid form.
CM: Carboxymethylcellulase; IU: International unit.

B.2 Nelson-Somogyi Method

The Nelson-Somogyi procedure for the determination of reducing sugars (Nelson, 1944) was as follows.

To 0.5 mL of sample aliquot and a sample blank (containing 0.5 mL of RO water), 0.2 mL of RO water and 0.5 mL of solution D were added. The sample and the blank were heated in a boiling water bath for 20 min, then cooled to room temperature (5 min) and stirred for 10 s to release carbon dioxide. A 3.0 mL aliquot of solution E was added to the sample and the blank; vortex mixed for 10 s and allowed to stand for 10 min. The sample and the blank were further mixed to obtain a uniform sample. The absorbance of the sample was measured against that of the blank at 520 nm. A standard curve was prepared by using appropriate amounts of sugars (*e.g.* glucose, GalA and arabinose).

Reagents	Compositions
A	25 g of anhydrous sodium carbonate, 25 g of sodium potassium tartrate and 200 g of sodium sulphate were dissolved in 800 mL of RO water and further diluted to 1 L.
B	30 g of copper sulphate pentahydrate was dissolved in 200 mL of RO water and approximately 0.25 mL of sulphuric acid was added.
C	50 g of ammonium molybdate was dissolved in 900 mL of RO water. 42 mL of concentrated sulphuric acid was added; 6 g of sodium arsenate heptahydrate was dissolved separately in 50 mL of RO water and was added to the above solution. The whole solution was made up to 1 L. This solution was warmed to 55°C to completely dissolve the solute.
D	1 mL of reagent B was added to 25 mL of reagent A.
E	Solution C was diluted using RO water in a ratio of 1:5. This solution should be freshly prepared.

B.3 Standard Curves

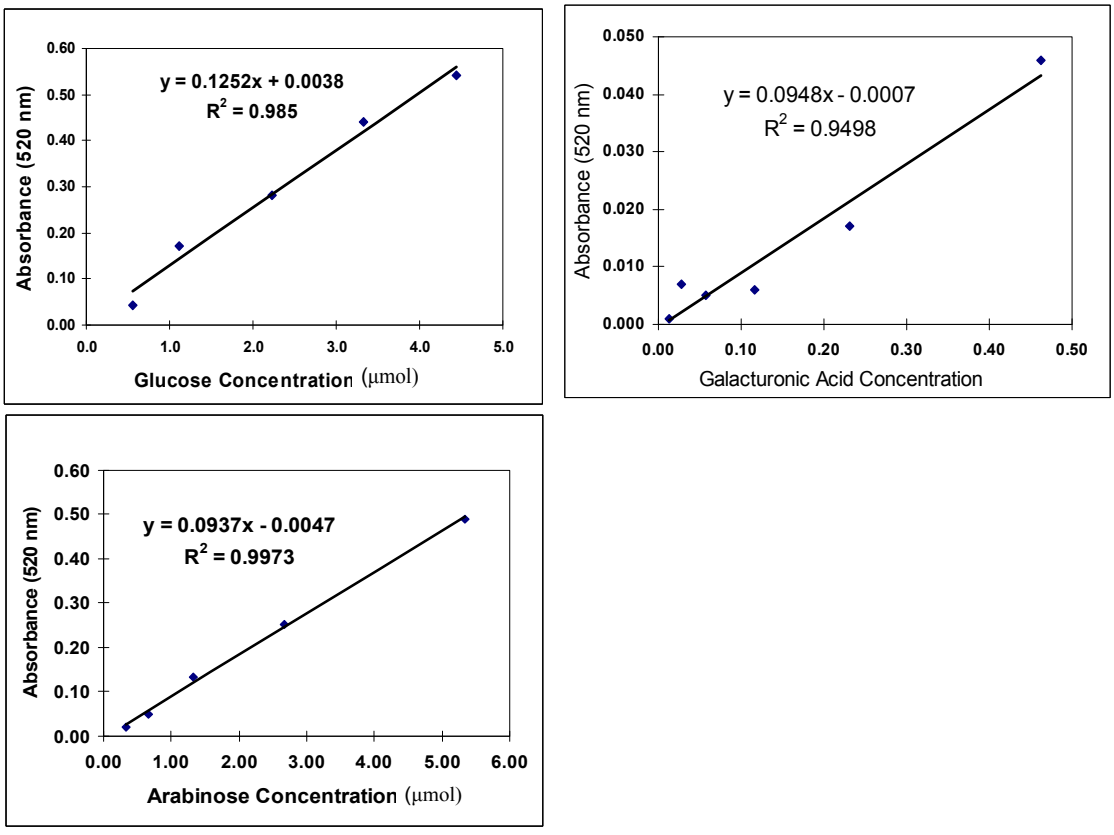


Figure B.3 Standard curves of the individual sugars glucose, GalA and arabinose obtained using the Nelson-Somogyi method.

B.4 Commercial Enzyme Activities

Table B.4.1 Commercial enzyme preparation activities at 25, 40 and 50ºC (*n*katals/mL)

Commercial Enzyme	25ºC			40ºC			50ºC		
	CM	PG	AR	CM	PG	AR	CM	PG	AR
Celluclast 1.5L	51	41	42	63	18	27	29	92	24
Cellulyve TR 400	700	320	322	749	622	431	187	695	690
Cytolase CL	93	192	158	64	98	155	108	65	155
NS 33048	41	101	95	20	96	91	11	196	80

CM: Carboxymethylcellulase; PG: Polygalacturonase; AR: Arabinase; NS 33048: a protease preparation (has a protease activity of 200,000 IU/mL, based on NS 33048 technical information).

Table B.4.2 Commercial enzyme preparation concentrations

Commercial Enzyme	Celluclast 1.5L	Cellulyve TR 400	Cytolase CL
Optimum Range of Concentration*	0.1–2.0 mL/kg	0.05–0.25 g/kg	0.04–0.08 mL/kg
Low	0.1	0.05	0.04
Medium	1.05	0.15	0.06
High	2.0	0.25	0.08

* Optimum ranges of concentrations were obtained from enzyme technical bulletins.

B.5 Viscosity of Enzyme-extracted Crude Pectin I

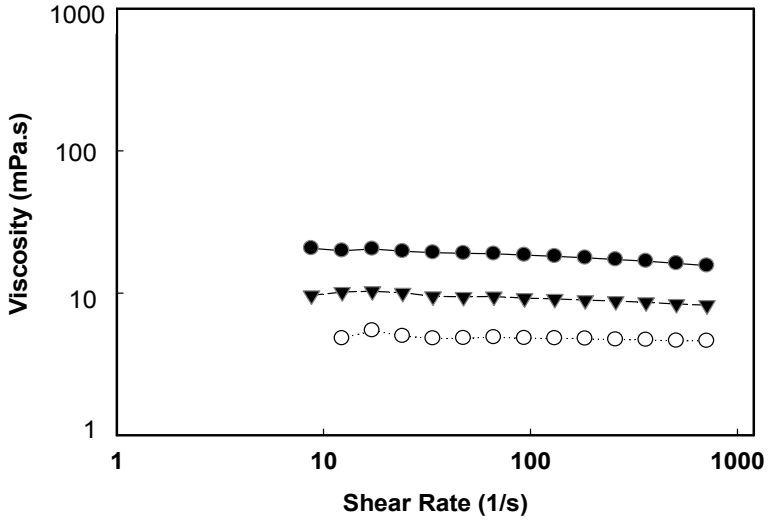


Figure B.5 Viscosity of crude pectin I extracted at 25ºC for 30 min with a medium enzyme concentration. Crude pectin I, 4% w/w dwb in Milli-Q water (pH 3.50 ± 0.01), extracted using: (●) Celluclast 1.5L; (▼) Cellulyve TR 400; (○) Cytolase CL.

B.6 Correlation between M_w and Viscosity of Crude Enzyme-extracted Pectin

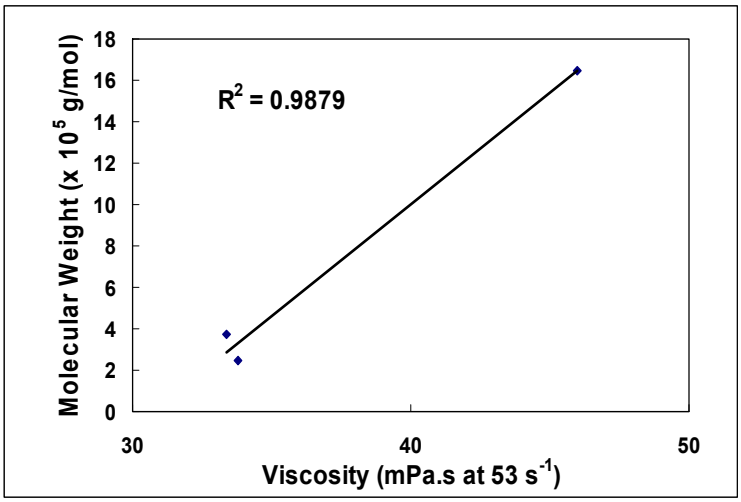


Figure B.6 Correlation between M_w and viscosity at 53 s^{-1} of crude pectin (1.0% GalA concentration (w/w) in Milli-Q water, pH 3.50 ± 0.01) extracted at 25°C, 30 min using different enzyme concentrations.

B.7 Root Mean Square (RMS) Radius of Purified Pectin Extracted Using Different Enzyme Concentrations

Table B.7 Root mean square radius of purified pectin extracted using different Celluclast 1.5L concentrations

Enzyme Concentration	RMS Radius (nm)
Low	87.4 [b]
Medium	162.0[a]
High	84.8 [b]
SEM[1]	0.887
Probability, $P \leq$	***

[a,b]Means in a column with different superscripts differ significantly ($P < 0.05$).
[1]Pooled standard error of mean. ***: $P < 0.001$.
Each value represents the mean of two replicates.

B.8 Extraction of Pectin Using Different Enzyme Preparations (Single Enzymes and Combinations of Enzymes)

Table B.8.1 Total-NSP composition, sugar composition and ash and protein contents (% w/w) of purified gold kiwifruit pectin extracted at 25°C for 30 min using different enzyme preparations

Extraction Method	Ash	Pro-tein	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	Total-NSP
Celluclast 1.5L	7.52[a]	16.91[a]	0.57	0.19	1.26	0.21	0.16[a]	2.77	0.32[a]	58.67[a]	64.03[a]
Cellulyve TR 400	5.25[b]	11.35[b]	1.45	0.13	1.86	0.34	0.96[b]	2.57	0.63[b]	54.36[b]	62.29[a]
Cytolase CL	6.28[c]	10.73[b]	1.41	0.20	1.73	0.34	1.04[b]	3.31	0.41[a]	48.28[c]	56.71[b]
CLN	9.26[d]	5.75[d]	1.00	0.08	1.37	0.25	0.94[b]	2.68	0.62[b]	57.99[a]	64.91[a]
CCN	7.55[a]	5.30[d]	1.19	0.13	1.60	0.45	1.12[b]	2.61	0.50[ab]	49.22[c]	56.80[b]
SEM[1]	**0.05**	**1.37**	**0.32**	**0.03**	**0.20**	**0.07**	**0.15**	**0.22**	**0.05**	**0.69**	**1.15**
Probabilities $P \leq$	***	**	NS	NS	NS	NS	*	NS	*	***	**

[a, b, c,d] Means in a column with different superscripts differ significantly ($P < 0.05$).
[1]Pooled standard error of mean. NS: not significant; ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$.
The total-NSP and sugar compositions were analysed by Englyst Carbohydrate Ltd, UK.
Each value represents the mean of two replicates.
Rha: rhamnose; Fuc: fucose; Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; GalA: galacturonic acid. The value is expressed as % g/100 g dry weight basis.
CLN: Combination of Celluclast 1.5L, Cellulyve TR 400 and NS 33048. CCN: Combination of Celluclast 1.5L, Cytolase CL and NS 33048.

Table B.8.2 Yield, weight-average molecular weight, polydispersity index and RMS radius of purified pectin extracted using different enzyme preparations

Commercial Enzyme	Purified Yield (% w/w)	M_w (x 10^5 g/mol)	Polydispersity Index (M_w/M_n)	RMS Radius (nm)
Celluclast 1.5L	4.39	16.5 ± 0.40	2.49 ± 0.02	162.0 ± 0.85
Cellulyve TR 400	4.82	1.6 ± 0.10	5.85 ± 1.22	164.3 ± 1.48
Cytolase CL	4.00	0.6 ± 0.04	25.08 ± 2.44	59.9 ± 2.01
CLN	5.40	0.13 ± 0.01	9.86 ± 0.66	122.8 ± 1.70
CCN	5.34	0.21 ± 0.03	6.22 ± 3.36	41.2 ± 7.80

CLN: Combination of Celluclast 1.5L, Cellulyve TR 400 and NS 33048. CCN: Combination of Celluclast 1.5L, Cytolase CL and NS 33048.

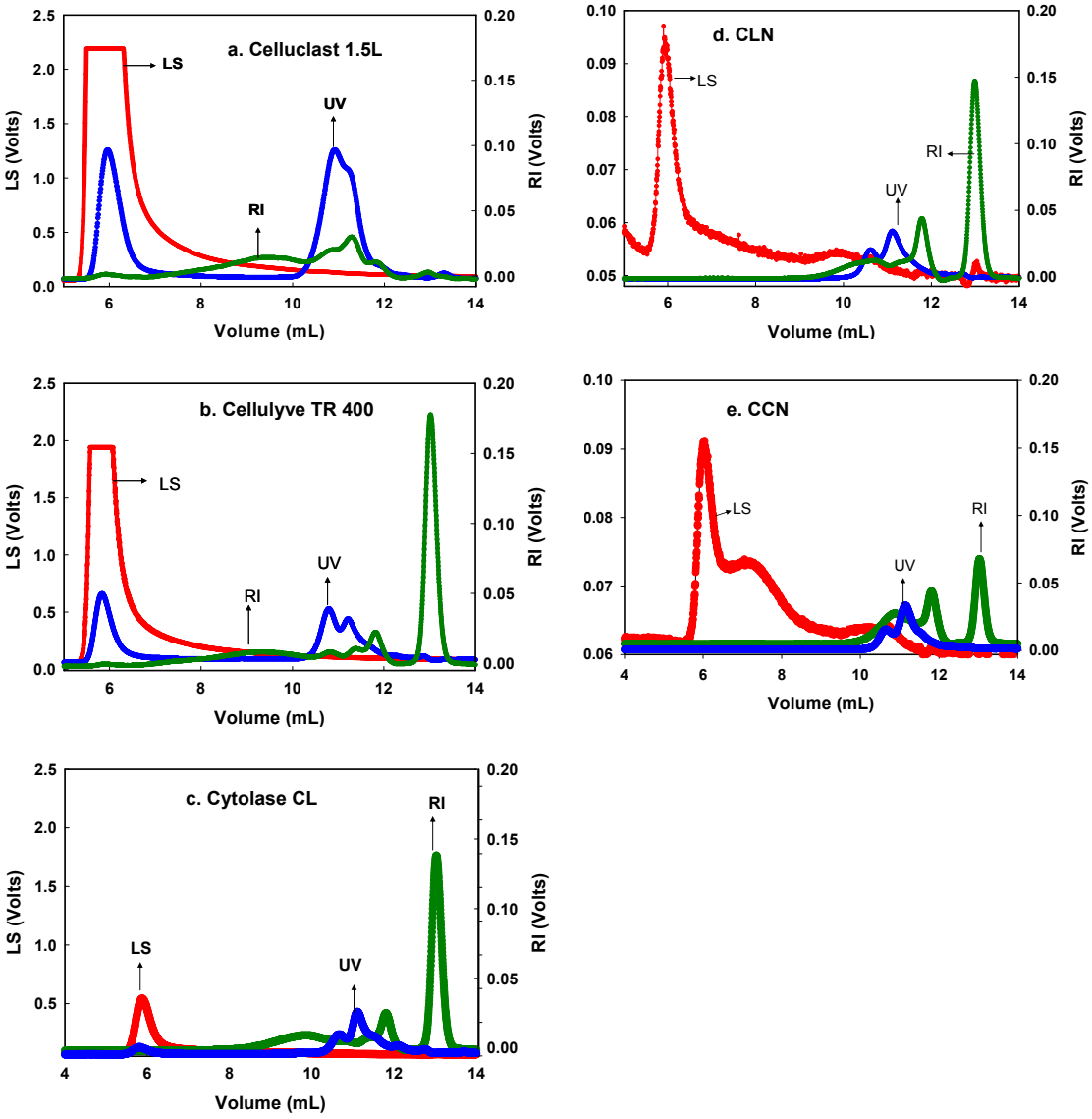
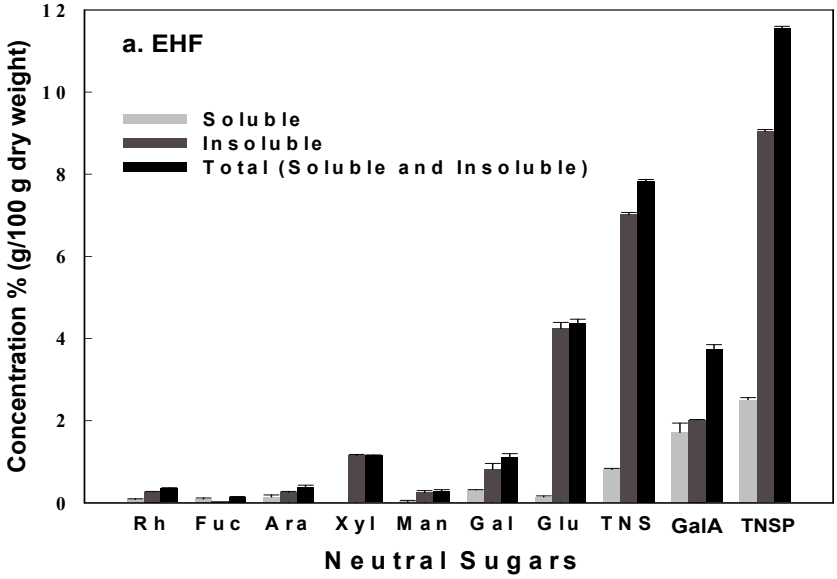


Figure B.8 Molecular property profiles of purified pectin. Light scattering (LS), ultraviolet (UV) and differential refractive index (DRI) signals of purified pectin extracted using single enzymes or a combination of enzymes: (a) Celluclast 1.5L; (b) Cellulyve TR 400; (c) Cytolase CL; (d) CLN (Celluclast 1.5L, Cellulyve TR 400 and NS 33048); (e) CCN (Celluclast 1.5L, Cytolase CL and NS 33048) (based on 0.35% w/w GalA concentration, prepared in 0.1 M NaCl and 0.02% w/w sodium azide).

Appendix C

C.1 Total-NSP Composition, Neutral Sugar Composition and GalA Content of Early-harvested and Main-harvested Fruit



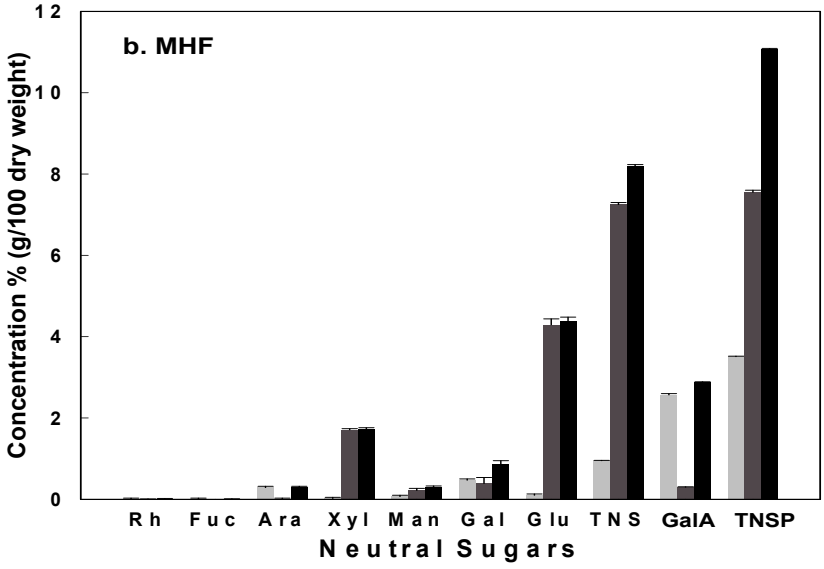


Figure C.1 Total-NSP composition and neutral sugar composition of: (a) early-harvested fruit; (b) main-harvested fruit. Rh, Rhamnose; Fuc, Fucose; Ara, Arabinose; Xyl, Xylose; Man, Mannose; Gal, Galactose; Glu, Glucose; TNS, Total neutral sugars; GalA, Galacturonic acid; TNSP, Total non-starch polysaccharides.

C.2 Molecular Distribution of Early-harvested and Main-harvested Purified Pectin Extracted Using Different Methods

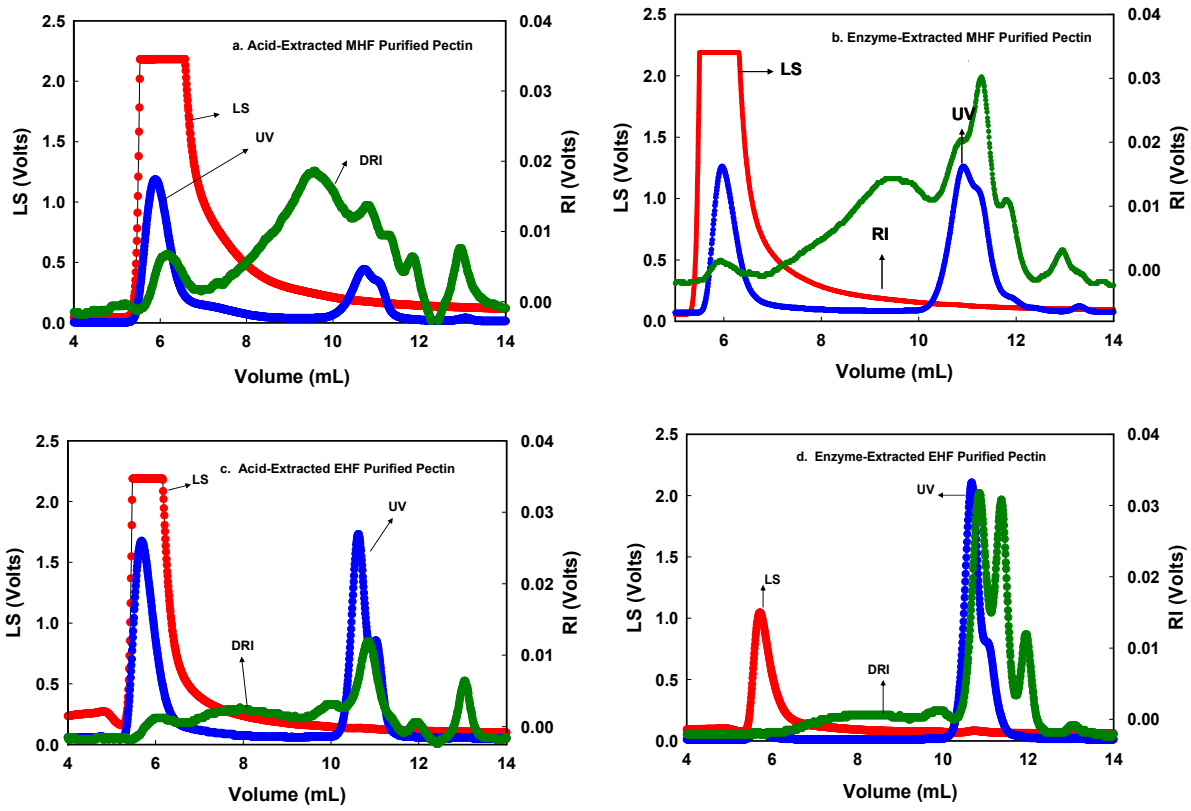


Figure C.2 Molecular property profiles of purified pectin. Light scattering (LS), ultraviolet (UV) and differential refractive index (DRI) signals: (a) acid-extracted MHF pectin; (b) enzyme-extracted MHF pectin; (c) acid-extracted EHF pectin; (d) enzyme-extracted EHF pectin (based on 0.5% w/w GalA concentration, prepared in 0.1 M NaCl and 0.02% w/w sodium azide).

C.3 Degree of Esterification (DE) Distribution

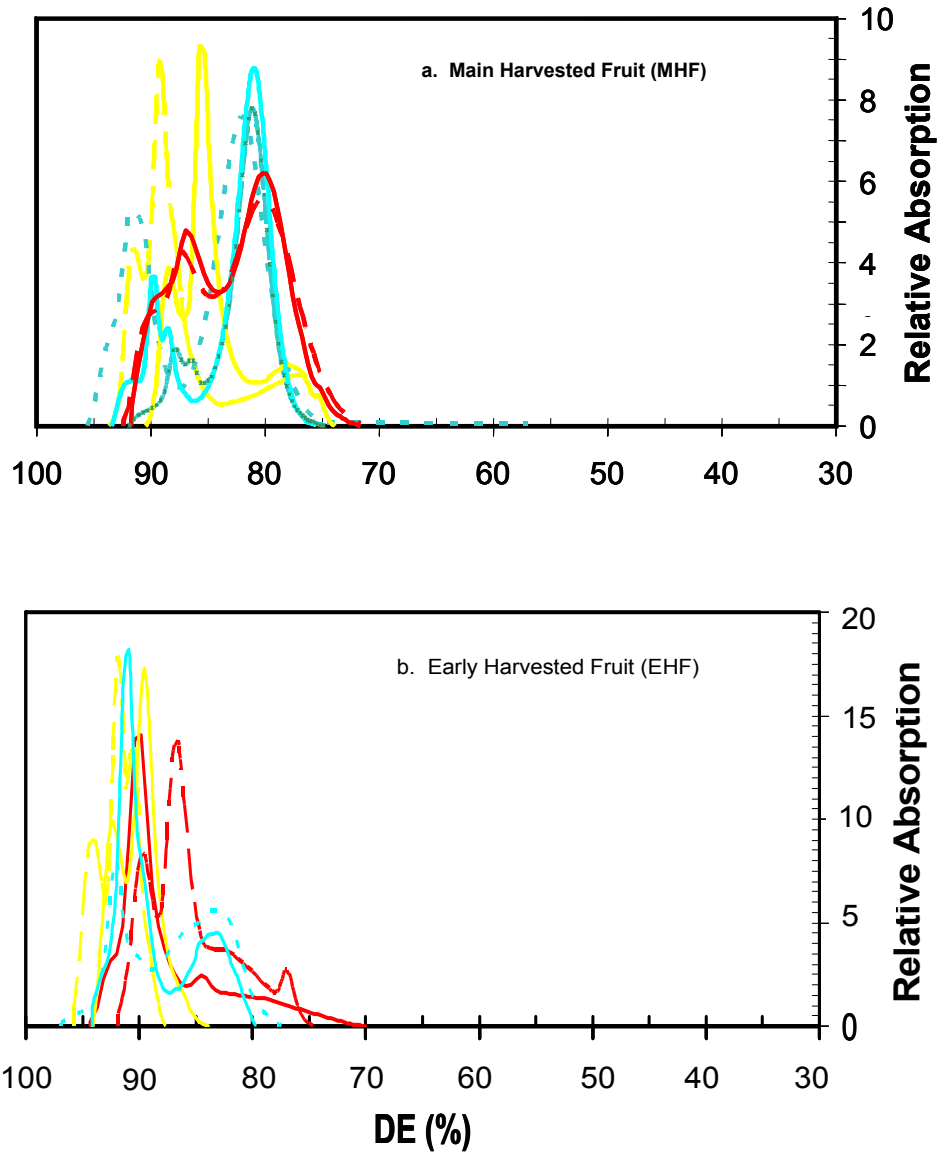


Figure C.3 Degree of esterification (DE) distribution of pectin extracted using different methods from (a) main-harvested and (b) early-harvested gold kiwifruit (acid; water; enzyme). The dotted lines indicate replications.

Appendix D

D.1 Molecular Property Distribution of Commercial Apple and Citrus Pectins

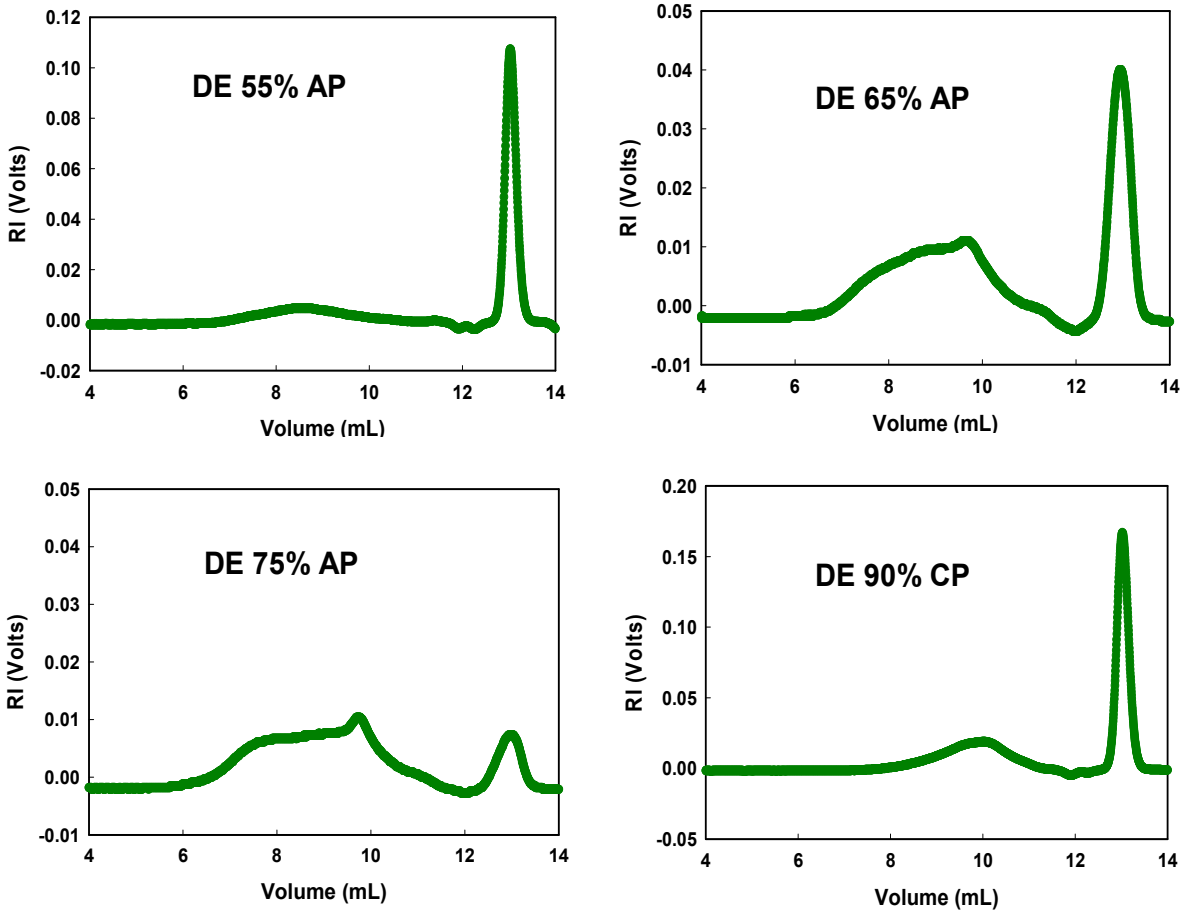


Figure D.1 Differential refractive index profiles of commercial apple (AP) [DE 55, 65 and 75%] and citrus (DE 90% CP) pectins (based on 0.35% and 0.45% w/w GalA respectively for AP and CP prepared in 0.1 M NaCl and 0.02% w/w sodium azide).

D.2 Viscosity of Grindsted® Pectin 780 AMD

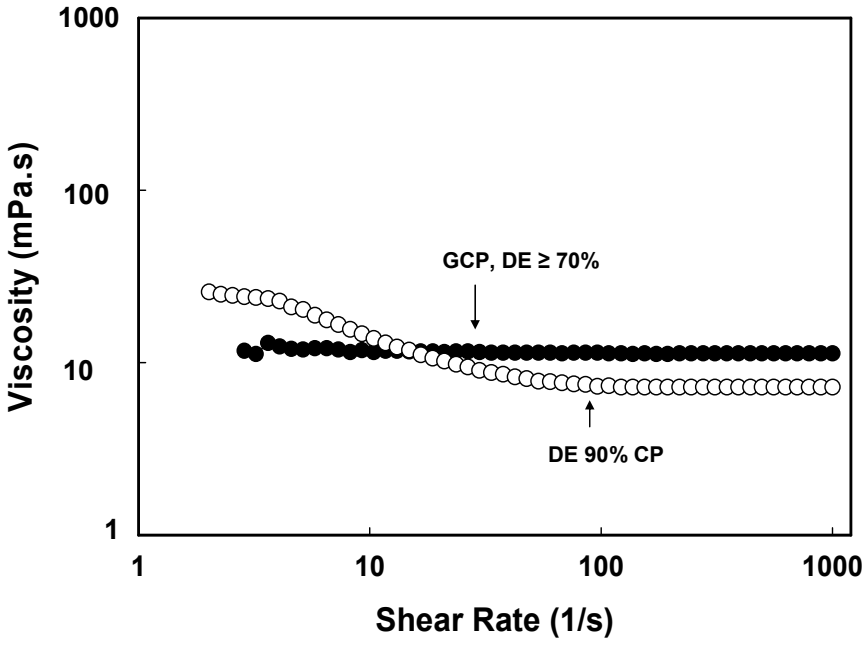


Figure D.2 Viscosity of DE 90% CP and Grindsted® pectin 780 AMD at 1.0% w/w GalA concentration (in Milli-Q water, pH 3.50 ± 0.01).

D.3 Viscosity of AMDs and Serum Phase of AMDs

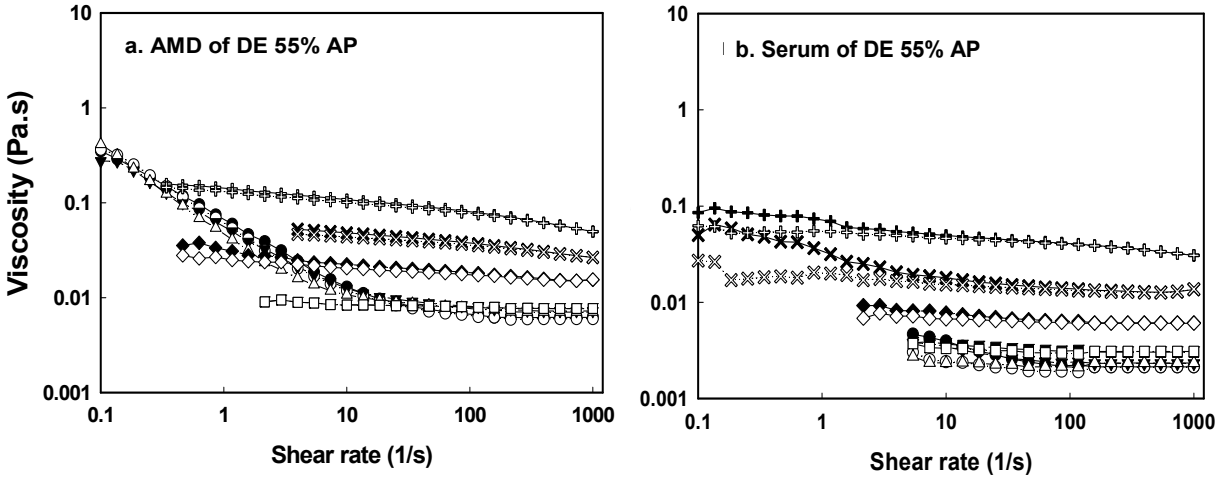


Figure D.3.1 Effect of pectin concentration on up (filled) and down (empty) viscosity of (a) AMD and (b) serum phase of AMD prepared from DE 55% AP: (●) 0.1%; (▼) 0.2%; (■) 0.3%; (♦) 0.5%; (x) 0.7%; (+) 1.0%.

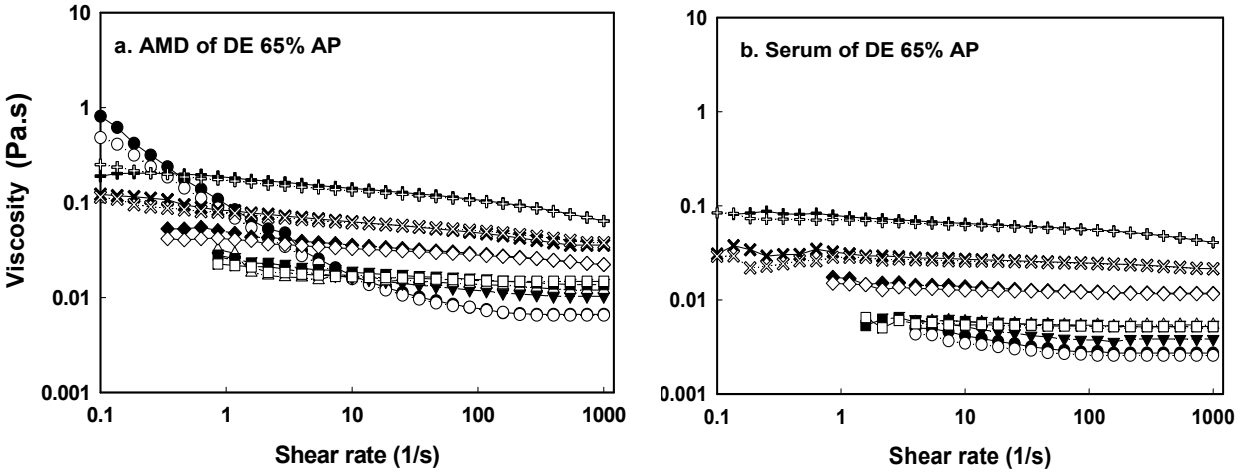


Figure D.3.2 Effect of pectin concentration on up (filled) and down (empty) viscosity of (a) AMD and (b) serum phase of AMD prepared from DE 65% AP: (●) 0.1%; (▼) 0.2%; (■) 0.3%; (♦) 0.5%; (x) 0.7%; (+) 1.0%.

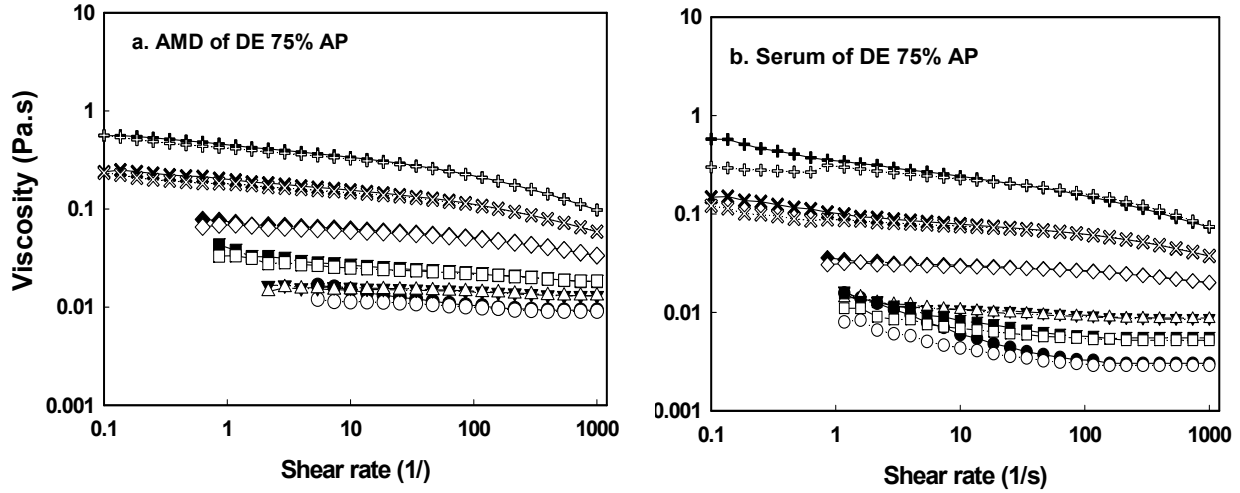


Figure D.3.3 Effect of pectin concentration on up (filled) and down (empty) viscosity of (a) AMD and (b) serum phase of AMD prepared from DE 75% AP: (●) 0.1%; (▼) 0.2%; (■) 0.3%; (♦) 0.5%; (x) 0.7%; (+) 1.0%.

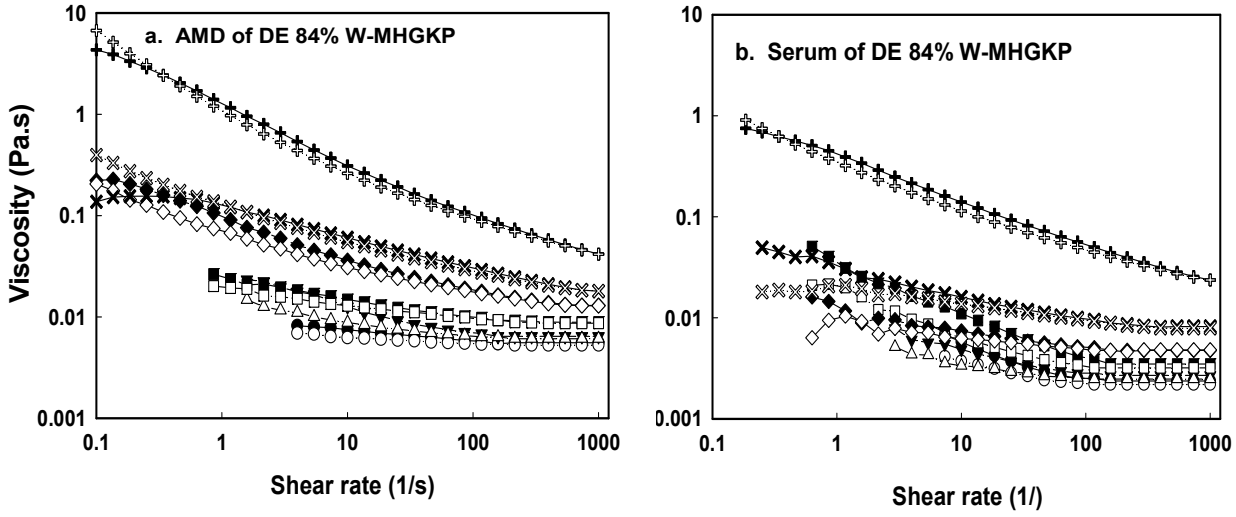


Figure D.3.4 Effect of pectin concentration on up (filled) and down (empty) viscosity of (a) AMD and (b) serum phase of AMD prepared from DE 84% W-MHGKP: (●) 0.1%; (▼) 0.2%; (■) 0.3%; (♦) 0.5%; (x) 0.7%; (+) 1.0%.

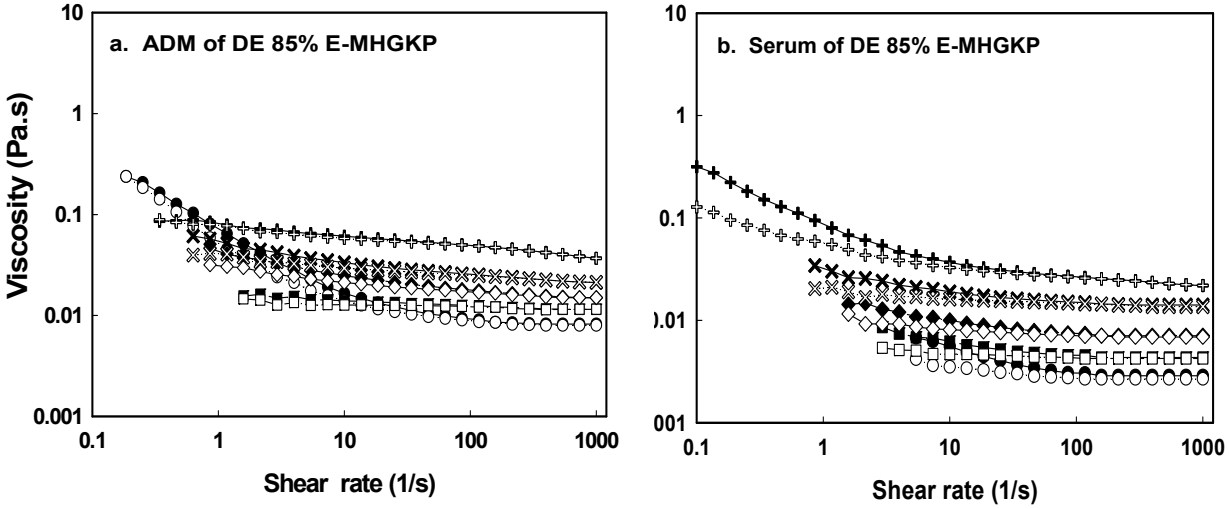


Figure D.3.5 Effect of pectin concentration on up (filled) and down (empty) viscosity of (a) AMD and (b) serum phase of AMD prepared from DE 85% E-MHGKP: (●) 0.1%; (■) 0.3%; (♦) 0.5%; (x) 0.7%; (+) 1.0%.

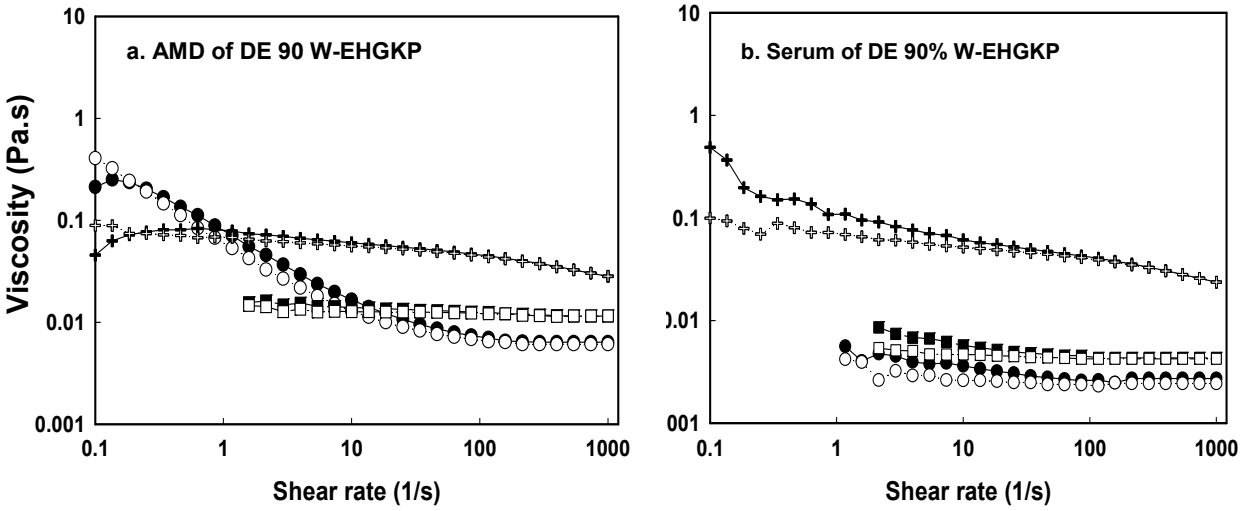


Figure D.3.6 Effect of pectin concentration on up (filled) and down (empty) viscosity of (a) AMD and (b) serum phase of AMD prepared from DE 90% W-EHGKP: (●) 0.1%; (■) 0.3%; (+) 1.0%.

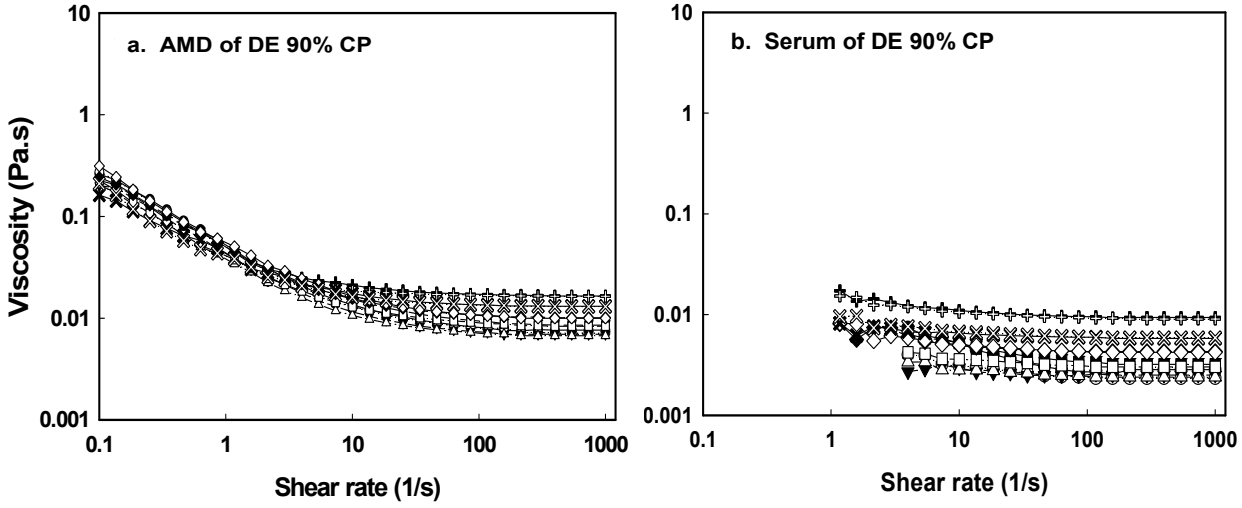


Figure D.3.7 Effect of pectin concentration on up (filled) and down (empty) viscosity of (a) AMD and (b) serum phase of AMD prepared from DE 90% CP: (●) 0.1%; (▼) 0.2%; (■) 0.3%; (♦) 0.5%; (x) 0.7%; (+) 1%.

Appendix E

Characterisation of Pectin from Gold Kiwifruit Pomace

E.1 Introduction

Citrus albedo and apple pomace are by-products of fruit juice manufacture. They are considered to be the main source of the raw material for pectin in many parts of the world (May, 1990). Some other potential raw materials that have been extensively studied include sugar beet pulp (Wang & Chang, 1994), peach (Pagan, *et al*., 2001), mango (Ketsa, Chidtragool, Klein, & Lurie, 1999), banana peel (Emaga, *et al*., 2008b) and chicory roots (Panouille, *et al*., 2006). It has been shown in a recent study that the water-soluble polysaccharides in gold kiwifruit are also a potential source of pectin (Yuliarti, *et al*., 2008).

Kiwifruit is consumed mainly as a fruit; however, it is also processed into kiwifruit juice. In the processing of kiwifruit, a large proportion of the fruit goes to waste and this includes whole fruit, peel, pulp and seeds. Kiwifruit waste has been reported by Wabnitz (2008) to be approximately 18% of the total kiwifruit crop. Gold kiwifruit pomace is produced as a by-product that is left after juice extraction and constitutes about 40−50% of the weight of the fresh fruit. To date, there has been no study on the extraction of pectin from gold kiwifruit pomace. The yield of pomace pectin, its chemical composition and its functional properties may differ from those of the pectin extracted from whole kiwifruit juice and the fruit fraction.

The objectives of this study were (*i*) to explore the possibility of using gold kiwifruit pomace for the isolation of pectin, (*ii*) to examine the composition and the rheological properties of the pectin extracted using different extraction methods (acid, water and enzyme) and to compare them with those of whole kiwifruit pectin (main-harvested fruit; MHF), as studied previously in Chapter **5**, and (*iii*) to investigate the weight-average molecular weight of the soluble pectin extracted from gold kiwifruit pomace.

E.2 Materials and Methods

E.2.1 Preparation of Whole Gold Kiwifruit Pomace

The pomace of main-harvested gold kiwifruit (MHF) used in this study was obtained by squashing whole kiwifruit in a fruit juicer (Avanti, Model 2000 Juicer, FED Australia and New Zealand, ~ 0.5 mm mesh). The fruit was obtained from Zespri International Ltd (Hastings, New Zealand) and belonged to the same batch as that used for the extraction of pectin in the main body of this thesis. As the juice recovered still contained a mixture of very coarse fruit pulp fractions, skin and seeds, further centrifugation (3300 *g*, 20 min and 4°C) was carried out to separate the insoluble fraction from the clear juice. The fruit pulp was hydraulically pressed (Massey University, Palmerston North) to separate more juice. The pellet, which was considered to be pomace, was pooled together with the fruit pomace from the juicer. The skin and seeds of the fruit were included in the pomace preparation. The fresh pomace was analysed immediately for dry matter, ash and protein contents, total-NSP and neutral sugar compositions and galacturonic acid (GalA) content, as described in Chapter **3**, section **3.2.3.** A flow diagram for the preparation of the pomace is summarised in Figure **E.1**. The composition of gold kiwifruit pomace is presented in Table **E.1**.

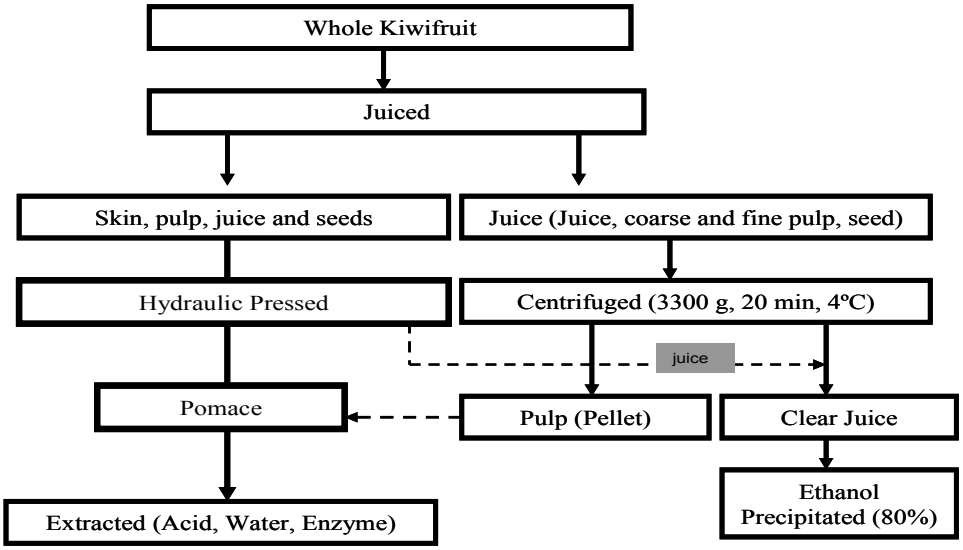


Figure E.1 Schematic flow chart of the preparation of gold kiwifruit pomace.

E.2.2 Pectin Isolation

The isolation of pectin from gold kiwifruit pomace was conducted immediately after juicing to prevent any enzymatic degradation. The extraction methods (acid, water and enzyme) and the conditions for isolating the pectin from the pomace were similar to those described in Chapter **5** (section **5.2.2**). The only exception was the ratio of pomace to solvent used for the extraction (1:3 w/v) as this volume of solvent was sufficient to disperse the pomace properly. The pH of the mixture (pomace and citric acid solution, and pomace and water) was 3.1 ± 0.05 and 3.7 ± 0.05 respectively for 1:3 w/v ratio. For enzymatic extraction, to facilitate a good pomace and enzyme (Celluclast 1.5L) dispersion, RO water was added to the pomace at a ratio of 1:3 (w/v) (pH 3.7 ± 0.05). Figures **E.2** and **E.3** show the pectin obtained by ethanol precipitation from the pomace and the juice.

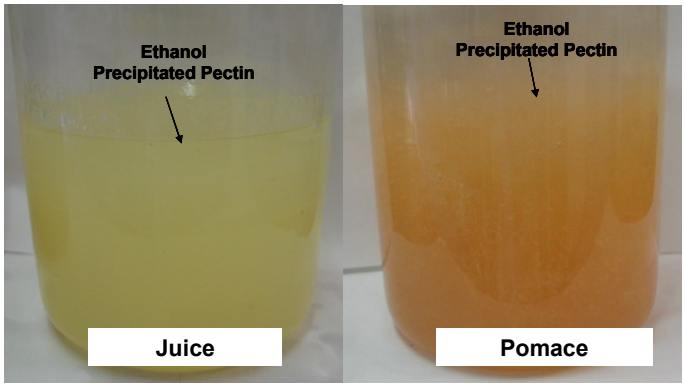


Figure E.2 Precipitation of pectin by 80% ethanol for 4 h at 4°C from an "acid extract" of gold kiwifruit juice and pomace.

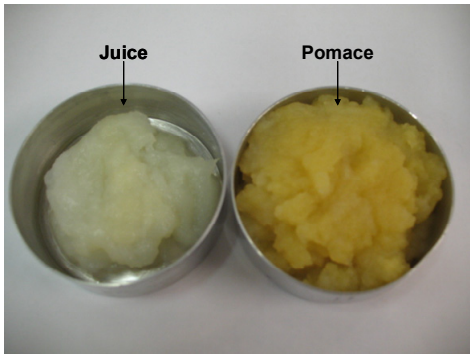


Figure E.3 Ethanol-precipitated gold kiwifruit pectin from juice and pomace (acid extraction method).

E.2.3 Pectin Recovered from Juice and Pomace

As the clear gold kiwifruit juice was assumed to contain the soluble pectin fraction, it was directly precipitated using 80% ethanol. The ethanol-precipitated pectin from the juice was freeze dried as described in Chapter **3**, section **3.2.2.1**, until freeze-dried **crude pectin** was obtained**.**

The freeze-dried crude pectins that were extracted by acid, water and enzyme treatment, and the pectin from the juice, were purified using procedures described in Chapter **5** (section **5.2.2.1**). The yield of purified pectin was calculated as described in Equation **3.1**. The purified pectins were identified as ***purified acid-***, ***water-*** and ***enzyme-extracted pomace pectin*** respectively for the pectins recovered by acid, water and enzyme treatment. The juice pectin was identified as ***purified juice pectin***.

E.2.4 Analytical Methods

The vacuum-dried purified pomace was analysed for total-NSP and sugar compositions (see section **3.2.3.6**), protein content (see section **3.2.3.4**), ash content (see section **3.2.3.3**), weight-average molecular weight (see section **4.2.4.1**) and viscosity (see section **3.2.3.7**). The pectin from the juice was also evaluated for GalA concentration, viscosity and weight-average molecular weight. All analyses were carried out in duplicate.

E.3 Results and Discussion

E.3.1 Pomace Composition

The composition of gold kiwifruit pomace is presented in Table **E.1**. The gold kiwifruit pomace used in this experiment contained approximately 22.12% (w/w) dry matter, 4.51% (w/w) protein (%N x 5.18) and 6.33% w/w ash. It was fairly rich in total GalA content (~ 5%) compared with whole MHF (~ 3%). The insoluble GalA fraction (~ 3%) was greater than the soluble GalA fraction (~ 2%), indicating that a large proportion of the GalA (~ 62% of the total GalA) was insoluble. The pomace was rich in rhamnose (~ 1%), arabinose (~

1%) and galactose (~ 1.5%), suggesting that gold kiwifruit pomace pectin may have branching sites in its structure. The considerable amounts of glucose (~ 10%) and xylose (~ 4%) in the pomace also suggested the presence of cellulose and hemicellulose in significant amounts; the amounts of glucose and xylose were greater than those in whole MHF (~ 5 and 2% respectively).

Table E.1 Composition of fresh gold kiwifruit pomace (% w/w)

Component	Concentration
Dry Matter (%)	22.12 ± 0.47
Moisture (%)	77.80 ± 0.47
Protein (% dwb)	4.51 ± 0.34
Ash (% dwb)	6.33 ± 0.02
Total-NSP (% dwb)	22.97 ± 0.21
Neutral Sugar Composition (% dwb)	
Rhamnose	1.09 ± 0.00
Fucose	0.14 ± 0.03
Arabinose	1.00 ± 0.00
Xylose	3.54 ± 0.07
Mannose	0.95 ± 0.03
Galactose	1.52 ± 0.14
Glucose	10.30 ± 0.18
Galacturonic Acid (% dwb)	5.25 ± 0.04
Soluble GalA	2.05 ± 0.11
Insoluble GalA	3.20 ± 0.14

% dwb: % dry weight basis.
Mean ± standard error (n = 2).

E.3.2 Purified Pectin Yield

Table **E.2** shows the yields of purified pectins that were extracted from gold kiwifruit pomace using different extraction methods (acid, water and enzyme). The pectin yield varied from approximately 3.62 to 4.48% w/w on a dry matter basis. The highest yield was obtained using enzymatic extraction and the lowest yield was obtained using water extraction. These results were relatively similar to the whole MHF pectin yields (3.27–4.39% w/w) reported in Chapter **5**, section **5.3.2**, although the yields from the pomace were slightly higher than those from the whole fruit. In this study, extraction by enzyme (Celluclast 1.5L) was observed to give the highest pectin yields, as also shown in previous

enzymatic extraction studies (Chapters **4** and **5**), indicating that the possible hydrolysis of cellulose led to solubilisation of the pectin from the cell wall.

Table E.2 Purified pectin yield (% w/w dry matter) of gold kiwifruit pomace

Extraction Method	Purified Yield (% w/w)
Acid	3.83 ± 0.07
Water	3.62 ± 0.03
Enzyme	4.48 ± 0.21

Mean ± standard error (n = 2).

E.3.3 Sugar Composition

The compositions of the pectins isolated from gold kiwifruit pomace using different extraction methods are presented in Table **E.3**. The sugar composition was expressed as w/w dry weight. The extract had a total-NSP composition in the range 77–80%. Pomace pectin was richer in GalA (~ 66%) and neutral sugars, particularly rhamnose (~ 1.4%), arabinose (~ 3%) and galactose (~ 6%), than MHF pectin (approximately 55.50, 0.81, 1.6, 4% respectively for GalA, rhamnose, arabinose and galactose). Pomace pectin was observed to be more branching than MHF pectin, indicated by a higher degree of branching in the water extract in particular; pomace pectin carried side chains every ~ 43–46 GalA residues whereas whole MHF pectin carried side chains every ~ 50–97 GalA residues.

Table E.3 Total-NSP and monosaccharide compositions (% w/w dry weight) of purified gold kiwifruit pectin extracted from pomace using different extraction methods

	Extraction Method		
Sugar Composition	**Acid**	**Water**	**Enzyme**
Rhamnose	1.35 ± 0.35	1.40 ± 0.08	1.37 ± 0.27
Fucose	0.39 ± 0.01	0.17 ± 0.02	0.22 ± 0.02
Arabinose	2.40 ± 0.10	2.81 ± 0.14	3.10 ±0.10
Xylose	0.87 ± 0.07	0.86 ± 0.14	0.20 ± 0.00
Mannose	1.07 ± 0.07	0.72 ± 0.15	0.54 ± 0.06
Galactose	4.96 ± 0.04	6.43 ± 0.24	5.50 ± 0.10
Glucose	2.67 ± 0.17	1.42 ± 0.08	1.39 ± 0.01
GalA	63.90 ± 1.69	65.44 ± 1.12	67.80 ± 0.15
Total-NSP	77.59 ± 0.99	79.16 ± 0.27	80.12 ± 0.57
Degree of Branching (GalA/Rha)	50.60 ± 8.80	46.75 ± 2.69	51.42 ± 8.23

Mean ± standard error (n = 2).

The amount of GalA recovered was slightly influenced by the extraction method; the enzymatic method recovered the greatest amount of GalA (~ 67%) followed by the water method (~ 65%) and then the acid method (~ 64%). This occurred because of enzymatic hydrolysis of cellulose in the cell wall network, which in turn caused the release of trapped pectin. This finding was similar to that from a study conducted on chicory roots by Panouille *et al*. (2006). However, a lower amount of GalA was recovered from whole fruit (see section **5.3.4** in Chapter **5**). These discrepancies can be attributed to the composition of the pomace; it had a greater insoluble GalA fraction (3.20%) than the whole fruit (0.3%).

The extraction method had no marked effect on the rhamnose content (1.35–1.40%). This could indicate that all the recovered pectins had similar numbers of rhamnogalacturonan linkages. There were lower amounts of arabinose in the acid-extracted (2.40%) and water-extracted (2.81%) pectins than in the enzyme-extracted pectin (3.10%). The amount of arabinose decreased with increasing acid strength, which could have been due to the hydrolysis of arabinofuranosyl bonds under harsher extraction conditions. Similar findings were reported by Levigne *et al*. (2002) and Garna *et al*. (2007) for extraction from fresh sugar beet pulp and apple pomace.

E.3.4 Ash and Protein Contents

Table **E.4** shows the ash and protein contents of the extracts. There were similar ash (2.59–2.70%) and protein (9.38–10.09%) contents in the extracts obtained using the three extraction methods. Pomace pectin had a higher purity than MHF pectin, which contained approximately 13.82% protein. It was deduced that most of proteins were from the gold kiwifruit juice.

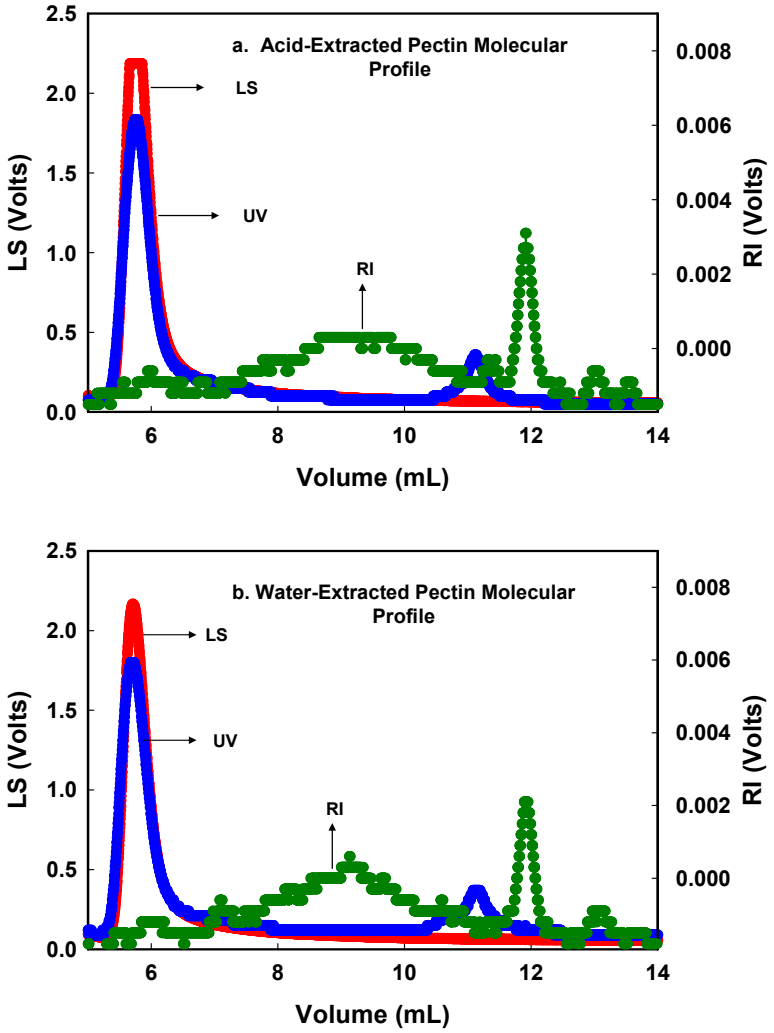
Table E.4 Ash and protein (% w/w dry weight) contents of purified pomace pectin

Extraction Method	Ash	Protein
Acid	2.59 ± 0.01	10.09 ± 0.04
Water	2.65 ± 0.00	9.38 ± 0.02
Enzyme	2.70 ± 0.10	9.82 ± 0.02

Mean ± standard error (n = 2).

E.3.5 Weight-Average Molecular Weight (M_w) of Purified Pectin

Figures **E4a–E4c** show light scattering (LS) at 90º, ultraviolet (UV) and differential refractive index (DRI) signals of pomace pectin samples extracted using the three different methods. In general, the extraction method did not seem to have a great affect on the molecular distribution profile of pomace pectin because all extraction methods resulted in a similar distribution profile. This finding is in agreement with the sugar compositions (GalA in particular); the three extracts had relatively similar compositions.



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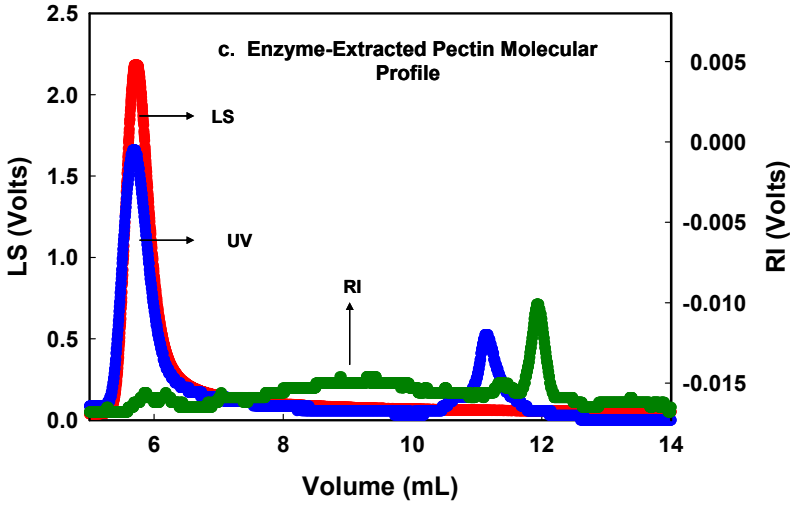

Figure E.4 Light scattering (LS), ultraviolet (UV) and differential refractive index (RI) molecular profiles of gold kiwifruit pomace pectin extracted by (a) acid, (b) water and (c) enzyme treatment.

At the initial elution volume (~ 6 mL), the chromatograms showed high LS but low DRI signals, which could indicate the presence of aggregation (10^7–10^8 g/mol). The subsequent fractions eluted after the first peak (7–12 mL) had higher DRI signals with only a trace in the LS response; they contained lower M_w fractions of approximately 10^5–10^6 g/mol and contained about four peaks. The presence of multiple DRI peaks could suggest different molecular fractions present in the purified pectin at different concentrations. Therefore, the calculation of the M_w of pomace pectin was based on chromatogram slices between elution volumes of 7 and 12 mL.

Table **E.5** compares the M_w, polydispersity index (M_w/M_n) and RMS radius of the pectin extracted from pomace with those of the pectin extracted from whole gold kiwifruit. The *dn/dc* value used for M_w determination was based on the *dn/dc* value of pectin from whole kiwifruit (0.189 ± 0.003 mL/g, Chapter **4**, section **4.2.4.2**). The small variations in the RI signals were in agreement with the M_w (6.7−8.5 x 10^5 g/mol). Enzyme-extracted pectin had a slightly lower M_w (6.7 x 10^5 g/mol) than water-extracted pectin (8.5 x 10^5 g/mol) and acid-extracted pectin (8.4 x 10^5 g/mol). This finding agreed well with previous experiments on whole MHF pectin (Chapter **5**) and with other work on enzymatic extraction by Panouille *et*

al. (2006) and Ptitchkina *et al*. (2008), although the M_w obtained for gold kiwifruit pomace pectin was much higher than those obtained for pectin from chicory roots (3.0 x 10^5 g/mol) and pumpkin (0.45 x 10^5 g/mol).

Table E.5 M_w (x 10^6 g/mol), polydispersity index (M_w/M_n) and root mean square radius of the purified pectin extracted from pomace and whole gold kiwifruit using different extraction methods

Extraction Method	Pomace Pectin			Whole MHF Pectin		
	M_w	M_w/M_n	RMS Radius (nm)	M_w	M_w/M_n	RMS Radius (nm)
Acid	0.84 ± 0.03	9.61 ± 1.15	91.8 ± 2.65	2.20 ± 0.05	1.77 ± 0.06	112.6 ± 1.00
Water	0.85 ± 0.02	11.53 ± 2.24	101.5 ± 0.80	3.75 ± 0.11	2.43 ± 0.09	182.7 ± 1.10
Enzyme	0.67 ± 0.03	10.93 ± 1.80	51.5 ± 1.20	1.65 ± 0.04	2.49 ± 0.05	162.0 ± 0.60

In general, the pomace pectin had a lower M_w (~ 7.9 x 10^5 g/mol), a lower RMS radius (~ 82 nm) but a higher polydispersity index (~ 11) than whole MHF pectin (M_w ~ 2.52 x 10^6 g/mol; RMS radius ~ 152 nm; M_w/M_n ~ 2.22). This applied to the three different extraction methods. It is very likely that the low M_w fraction in all extraction methods was caused by hydrolysis of bound pectin during extraction. This was also indicated by the high polydispersity index of pomace pectin. The results were consistent with the different monosaccharide compositions because pomace pectin contained more neutral sugars and GalA than MHF pectin.

It has been reported that, during the softening on the vine of green kiwifruit, the insoluble pectin present in the cell wall gradually becomes more freely soluble in the apoplast (cell wall) (Redgwell & Percy, 1992). Our results showed that pectin (present in the juice) had a higher M_w than the pectin trapped in the cellular structure of the pomace. Hence, it seemed likely that the high M_w pectin fraction solubilised from the cell wall to the cell. The large polymer molecules, which were capable of holding more moisture, eventually broke away from the cellular structures and became more available in the juice. The above explanation appears to be valid because the pectin from the juice had a higher M_w (~ 1.36 x 10^6 g/mol) than the pectin from the pomace.

Studies on the pectin extracted from sugar beet and sugar beet pomace indicated similar behaviour (Rambouts & Thibault, 1986; Levigne *et al*., 2002), *i.e.* the pectin from sugar beet pomace had a lower M_w than whole sugar beet pectin. Levigne *et al*. (2002) attributed the differences in M_w to the different treatments applied to the materials during processing (such as drying) which may stimulate some artifacts and may modify the solubility and the physicochemical composition of the pomace.

E.3.6 Viscosity of Purified Pomace Pectin

Figure **E.5** illustrates the viscosity curves of pomace and juice pectin solutions prepared in Milli-Q water. The samples were prepared based on the same GalA content (1.0% w/w). The pectins obtained using the three extraction methods exhibited almost similar viscosities (30–34 mPa.s at 53 s^{-1} shear rate), which agreed with the insignificant differences in their M_ws. However, the pectin obtained from the juice exhibited higher viscosity (76.1 mPa.s) than the pomace, close to that of whole MHF pectin (57.3–231.5 mPa.s).

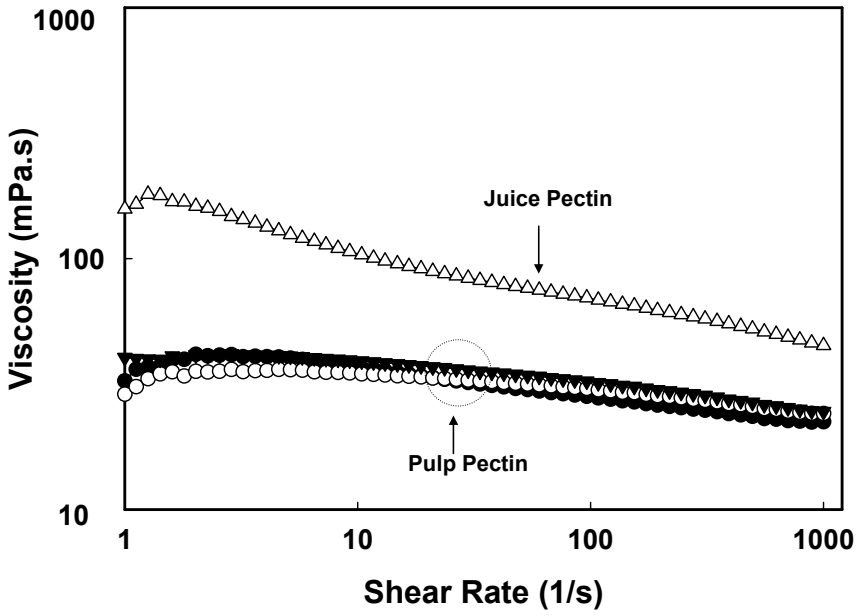

Figure E.5 Viscosity of purified gold kiwifruit pomace pectin extracted using different extraction methods (●, acid; ○, water; ▼, enzyme) and juice pectin (Δ) at 1.0% w/w GalA concentration, pH 3.50 ± 0.01.

E.4 Conclusions

This study showed that pectin accounted for the majority of the water-soluble polysaccharides in gold kiwifruit pomace. The extraction method (acid, water or enzyme) was observed to have a slight effect on the composition, M_w and viscosity of pomace pectin. Based on the sugar composition, this study could imply that there are two distinct pectin fractions in kiwifruit: an insoluble fraction (rich in neutral sugars) and a soluble fraction (lower in neutral sugars). The MHF pectin could be pectin extracted from soluble fraction. The viscosity of pomace pectin was markedly lower than the viscosity of whole MHF pectin, because the latter pectin has higher M_w. However, the viscosity was higher than that of commercial citrus-derived pectin such as Sigma pectin P9135 (~ 20 mPa.s, Chapter **5**, section **5.3.7**). As gold kiwifruit juice pectin was high in M_w, it exhibited higher viscosity in solution than pomace pectin. This study also implied that kiwifruit pomace, which is usually a by-product of juice processing, could be a valuable source of pectin. This pectin fraction may possess useful functional properties in addition to its thickening or gelling properties, for example, in the formation of novel structures resulting from pectin−protein interactions that are yet to be fully understood.