COMPOSITIONAL, STRUCTURAL AND FUNCTIONAL PROPERTIES OF KRUEO MA NOY EXTRACTS

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Technology

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สมบัติทางองค์ประกอบ โครงสร้าง และเชิงหน้าที่ของสารสกัดจากเครือหมาน้อย

นางจิตรา สิงห์ทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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สารสกัดประเภทโพลีแซคคาไรด์ที่ได้จากใบเครือหมาน้อย (*Cissampelos pareira*) ซึ่งเป็นพืชพื้นบ้านของประเทศไทย คือ เพคติน ซึ่งมีองค์ประกอบของกรดกาแลคทูโรนิคอยู่สูงถึงร้อยละ 70 และมีปริมาณโมโนแซคคาไรด์เพียงเล็กน้อย โครงสร้างหลักคือ กรดกาแลคทูโรนิค ที่ต่อกันด้วยพันธะ α-(1,4) ซึ่งได้จากการลดปริมาณกรดคาร์บอซิลิคและวิเคราะห์ด้วยวิธีเมธิลเลชั่น นอกจากนั้นยังวิเคราะห์ด้วย ฟูริเออร์ทรานฟอร์ม อินฟราเรดสเปกโทรสโกปี (FT-IR spectroscopy) และ นิวเคลียร์แมกเนติกเรโซแนนซ์สเปกโทรสโกปี (NMR spectroscopy) เพื่อเป็นการยืนยันผลที่ได้ สำหรับระดับเอสเทอริฟิเคชั่น (degree of esterification) ของเพคตินจากเครือหมาน้อยคิดเป็นร้อยละ 41.7 สำหรับสารสกัดหยาบ (crude extract) และร้อยละ 33.7 สำหรับสารสกัดที่ผ่านการแยกด้วยเยื่อบาง (dialyzed extract) เพคตินจากเครือหมาน้อยมีน้ำหนักโมเลกุลเฉลี่ย (average molecular weight : Mw) 55 กิโลดาลตัน เส้นผ่านศูนย์กลางอนุภาค (radius of gyration : Rg) 15.2 นาโนเมตร และค่าความหนืด (intrinsic viscosity : [η]) 2.3 เดซิลิตร/กรัม สารละลายเพคตินจากเครือหมาน้อยแสดงพฤติกรรมการไหลแบบนิวโตเนียน (Newtonian behavior) ที่ความเข้มข้นน้อยกว่าร้อยละ 0.5 และแสดงพฤติกรรมการไหลแบบนอนนิวโตเนียนชนิดเชียร์ธินนิ่ง (Shear-thinning behavior) ที่ความเข้มข้นมากกว่าหรือเท่ากับร้อยละ 0.5 สารละลายเพคตินจากเครือหมาน้อยเกิดเป็นเจลที่ความเข้มข้นร้อยละ 1.0 สารละลายเพคตินที่ความเข้มข้นร้อยละ 0.5 เกิดเจลที่สภาพความเป็นกรด (pH 2-4) การเติมเกลือโซเดียมคลอไรด์ที่ความเข้มข้นต่ำกว่าร้อยละ 0.4 โมล ทำให้ความแข็งของเจลเพิ่มขึ้น เพคตินจากเครือหมาน้อยไวต่อปริมาณแคลเซียม การเติมแคลเซียมคลอไรด์ 1 มิลลิโมล ทำให้ความแข็งของเจลเพิ่มขึ้นอย่างมีนัยสำคัญ อย่างไรก็ตามเมื่อเพิ่มความเข้มข้นของแคลเซียมคลอไรด์จนถึง 3 มิลลิโมล จะเกิดการตกตะกอน ยิ่งไปกว่านั้นการเติมน้ำตาลทราย ช่วยทำให้ความแข็งของเจลเพิ่มขึ้น เมื่อความเข้มข้นของเพคตินเพิ่มขึ้น ความแข็งของเจล จุดหลอมเหลว และพลังงานที่ใช้ในการหลอมเจลจะเพิ่มขึ้น

การใช้วิธีการแสดงผลตอบสนองแบบโครงร่างพื้นผิว (response surface methodology : RSM) สำหรับประเมินผลความเข้มข้นของเพคตินจากเครือหมาน้อย (ร้อยละ 0-2), น้ำตาลทราย (ร้อยละ 0-60), แคลเซียมคลอไรด์ (0-4 มิลลิโมล) และสภาพความเป็นกรด-ด่าง (pH 2-8) ที่มี

ต่อสมบัติการเป็นเจล โดยวัดในรูปของพลังงานสะสม (Storage modulus : G') พบว่าทุกปัจจัยมีผลอย่างมีนัยสำคัญทางสถิติต่อความแข็งของเจล ความแข็งของเจลเพิ่มขึ้นเมื่อเพิ่มความเข้มข้นของเพคตินจากเครือหมาน้อย นอกจากนั้นปริมาณแคลเซียมคลอไรด์ที่เพิ่มขึ้นยังมีผลทำให้ความแข็งของเจลเพิ่มขึ้นจนปริมาณแคลเซียมคลอไรด์ถึง 2 มิลลิโมล เมื่อเพิ่มปริมาณแคลเซียมคลอไรด์ต่อไป ความแข็งของเจลจะลดลง เมื่อให้ความเข้มข้นของเพคติน และสภาพความเป็นกรด-ด่างคงที่ ความแข็งของเจลจะสูงสุดที่ปริมาณแคลเซียมคลอไรด์ 2 มิลลิโมล และน้ำตาลทรายร้อยละ 50 เช่นเดียวกันกับการลดสภาพความเป็นกรด-ด่าง ทำให้ความแข็งของเจลเพิ่มขึ้น

สาขาวิชาเทคโนโลยีอาหาร
ปีการศึกษา 2547

ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

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A polysaccharide was extracted from the leaves of Krueo Ma Noy (*Cissampelos pareira*), a woody climbing plant from Thailand. It was found that Krueo Ma Noy polysaccharide is a pectin consisting mainly of a high level of galacturonic acid (~70%) and a small amount of monosaccharides. The dominant structure was established as a α-1,4-link-galacturonan with a combination of carboxyl reduction and methylation analysis and this structure was confirmed by FT-IR and NMR spectroscopy. The degree of esterification of crude and dialyzed Krueo Ma Noy pectins was found to be 41.7% and 33.7%, respectively. Krueo Ma Noy pectin had an average molecular weight of 55 KDa, a radius of gyration of 15.2 nm and an intrinsic viscosity of 2.3 dL/g. In diluted solutions, Krueo Ma Noy pectin exhibited Newtonian flow behavior. Shear-thinning behavior was observed at a concentration of ≥ 0.5% (w/v). Both crude and purified extracts gelled in aqueous solutions at 1.0% (w/v). Krueo Ma Noy pectin (0.5%,w/v) also formed gel under acidic conditions (pH 2-4). The addition of NaCl significantly increased the gel strength when salt concentration was below 0.4 M. Krueo Ma Noy pectin was very sensitive to the presence of $CaCl_2$. The addition of 1 mM $CaCl_2$ significantly increases gel strength. However, when $CaCl_2$ concentration was greater than 3 mM, precipitation occurred. In addition, the presence of sugar increased G′ with increasing sugar concentration. Gel strength,

melting point and melting enthalpy of Krueo Ma Noy pectin increased with increases in polymer concentration.

The response surface methodology was employed to evaluate the concentration effect of pectin (0-2%, w/v), sucrose (0-60%, w/v), $CaCl_2$ (0-4 mM) and pH (2-8) on gelling properties. Gel strength was evaluated from the storage modulus G′. It was found that all the factors examined had significant effects on the strength of Krueo Ma Noy pectin gel. The gel strength increased with increases in Krueo Ma Noy pectin concentration. Increases in $CaCl_2$ concentration resulted in increases in gel strength until $CaCl_2$ concentration reached 2 mM. Further increases in $CaCl_2$ concentration decreased gel strength. When pH and pectin concentrations were fixed, the maximum gel strength was at 2 mM $CaCl_2$ and ~50% sucrose. Also, decreases in pH strengthened Krueo Ma Noy pectin gel.

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Jittra Singthong

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List of Abbreviations

Abbreviations

D_2O = deuterium oxide

DMSO = dimethylsulfoxide

$NaBD_4$ = sodium borodeuteride

TFA = trifluoroacetic acid

PMAA = partially methylated alditol acetates

GC = gas chromatography

MS = mass spectrometry

GC-MS = gas chromatography- mass spectrometry

EI = electron impact ionization

m/z = mass/charge ratio

HPLC = high performance liquid chromatography

DP = degree of polymerization

RI = refractive index

UV = ultraviolet

HPAEC = high performance anion exchange chromatography

PAD = pulsed amperometric detection

NMR = nuclear magnetic resonance

^{1}H NMR = proton nuclear magnetic resonance

^{13}C NMR = carbon 13 nuclear magnetic resonance

FT-IR = fourier transform infrared spectroscopy

List of Abbreviations (Continued)

DE = degree of esterification

HPSEC = high performance size exclusion chromatography

LS = light scattering

dn/dc = refractive index increment

MALLS = multi-angle laser light scattering

RALLS = right-angle laser light scattering

SEC-MALLS = size exclusion chromatography couple to multi-angle laser light scattering

LBG = locust bean gum

LMP = low methoxyl pectin

HMP = high methoxyl pectin

DSC = differential scanning calorimetry

Symbols

$[\eta]$ = intrinsic viscosity

η_{rel} = relative viscosity

η_{sp} = specific viscosity

c = concentration

c* = critical concentration

K' = Huggin's coefficient

K" = Kraemer's coefficient

α = Mark-Houwink exponent

List of Abbreviations (Continued)

Mr = average molecular weight for the Mark-Houwink-Sakurada equation

Rg = radius of gyration

Mw = molecular weight

τ = shear stress

η = apparent viscosity

γ = shear rate

k = consistency index

n = parameter which varies with the type of flow

Pa = Pascal

ω = frequency

G' = storage modulus

G" = loss modulus

σ_0 = shear stress amplitude

γ_0 = strain amplitude

δ = phase angle

G* = complex modulus

η* = complex viscosity

tan (δ) = tangent of the phase angle (tan delta)

A = area

ΔH = melting enthalpy

Tm = melting temperature

Chapter I

Introduction

Hydrocolloids are used as food ingredients in the food industry because of their ability to modify/control the functional properties of food systems. The food hydrocolloid industry represents a market of over US$ 3.0 billion (Seisum, 2002). Most important properties of hydrocolloids are their viscosity (including thickening and gelling) and water binding. Other significant functions include emulsion, stabilization, prevention of ice recrystallization and organoleptic properties. Food stuffs are very complex systems, therefore an application of multifunctional hydrocolloids would result in food property modification.

The scientific classification of food hydrocolloids is based on their functional properties or the origin of raw materials, for example, gelling agents, thickening agents, seaweed extracts, seed gums, plant exudates, fermentation polymers (Seisum, 2002, Norton and Foster, 2002). The commercially important hydrocolloids, their origin and their main functional properties are given in Table 1.1 (Phillips and Williams, 2000) and Table 1.2 (Norton and Foster, 2002), respectively. Hydrocolloids extracted from plant are interesting additives for several industries, in particular for the food industry. These polysaccharides have the advantage of being regarded as totally natural products for many consumers.

Table 1.1 Sources of commercially important hydrocolloids.

Botanical	
	trees
	cellulose
	tree gum exudates
	gum arabic, gum karaya, gum ghatti, gum tragacanth
	plants
	starch, pectin, cellulose
	seeds
	guar gum, locust bean gum, tara gum, tamarind gum
	tubers
	konjac mannan
Algal	
	red seaweeds
	agar, carrageenen
	brown seaweeds
	alginate
Microbial	
	xanthan gum, curdlan, dextran, gellan gum, cellulos
Animal	
	gelatin, caseinate, whey protein, chitosan

Source : Phillips and Williams (2000).

Table 1.2 Food hydrocolloids and their functional properties.

Gelling	Thickening	Emulsification
Pectin	Pectin	Gum Arabic
Alginate	Alginate	Gelatin
starch	Starch	Milk proteins
Agar	Locust bean gum	
Carrageenan	Guar gum	
Gellan gum	Xanthan gum	
Gelatin	Gum Arabic	
Milk proteins		

Source : Norton and Foster (2002).

Krueo Ma Noy (*Cissampelos p*areira) is a local northeastern plant in the family of *Menispermaceae*. Krueo Ma Noy leaf extract can form gel in a short period of time after water extraction. This gel from leaf extract is consumed as a dessert by northeastern Thais and used by local people as cool medicine for treating fever. This plant is found throughout warm parts of Asia, East Africa and America (Smitinand and Larson, 1991). It is widespread in the northeast of Thailand. The gelling components in Krueo Ma Noy could be another source of natural polysaccharide. However, there is a little information available in the literature on the chemical composition and functional properties of this plant extract.

Therefore, the purpose of this research project is to systematically investigate the chemical and functional components of Krueo Ma Noy extract, especially its gelling property in order to understand its useful functionality including structural characterization. Furthermore, the results may lead to the development of a food ingredient for industrial uses.

1.1 Research objectives

1. To optimize the extraction process of Krueo Ma Noy.
2. To identify chemical and functional properties of its leaf extracts.
3. To elucidate the fine structure and determine its molecular properties.
4. To study the relationship between its structure and its functional properties.

1.2 Research hypothesis

1. The active component causing gelation in the leaf extract is hydrocolloid/gum.
2. The active component can be used as a food ingredient.

1.3 Scope and limitation of the study

Krueo Ma Noy's leaf extract will be analyzed in order to find the active component that causes gelation. The active component will be analyzed using various techniques in order to determine its chemical structure. The chemical and functional properties of the active component will then be studied.

1.4 Expected results

1. To understand the chemical, structural and functional properties of the Krueo Ma Noy extract.
2. To produce active components to be used as a food ingredient.

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Norton, I.T. and Foster, T.J. (2002). Hydrocolloids in real food systems. In Williams, P.A. and Phillips, G.O., **Gums and Stabilizers for the Food Industry 11** (pp. 4-9). UK : The Royal Society of Chemistry.

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Chapter II

Literature review

2.1 *Cissampelos pareira*

Cissampelos pareira is a local plant in the family of *Menispermaceae*, shown in Figure 2.1. Its local name is Krueo Ma Noy, Khong Khamao, Kon Pit, Krung Khamao and Sifan (Smitinand and Larsen, 1991).

Figure 2.1 *Cissampelos pareira*

C. pareira is a scandent shrub with leafy slender stems, which is densely to sparsely pubescent. Its leaves are broadly ovate 4.5-12 cm, rounded at the base, truncate or cordate with the apex mostly obtusely to acutely acuminate with a pubescent lower surface and an upper surface which is sparsely pubescent. The composition of the male inflorescences is having flowers in subcorymbose, peduncled cymes, 2-4 cm long, solitary or a few rising into a fascicle. Male flowers are green to yellowish on pedicles of 1-2 mm sepals, with the ovary 1.25-1.5 mm long and pilose outside. Corolla cupuliform is ca 0.5 mm long and pubescent outside and the synandrium is ca 0.75 mm long. The female inflorescence is composed of a pseudoraceme of fascicles which have a suborbicular bract in the axial of the accrescent. Female flowers are 1-1.5 mm on pedicles (Smitinand and Larsen, 1991). Figure 2.2 shows the characteristics of the *C. pareira*.

This plant is found throughout the warm parts of Asia, East Africa and America. It is widespread in the northeast of Thailand. The extract from its leaves can be used to make gel. The dark green gel is used as medicine for treating fever in local people. Local people use this plant as a diuretic and for the treatment of a variety of ailments, including asthma and for traumas (Mukerji and Bhandari, 1959).

C. pareira is rich in alkaloids which western scientists have shown an interest in even as far back as 1965 to 1968. Saponins and sterols are common; occasional triterpenes, ethereal oils, polyterpenes, and polyphenols are also present. This plant contains tetrandrine, which is analgesic, anti-inflammatory, and febrifuge and has recently been shown to have anti-tumor and anti-leukemic properties as well. In reviews of the alkaloids in general, the aporphine alkaloids, and their role in the preparation of curares have been published. In addition to tetrandrine, *C. pareira*

contains cycleanine, hayatine and other berbine derivatives. In more recent research, the bisbenzylisoquinoline alkaloids have been found to be anti-inflammatory constituents of this plant. In clinical experiments, the alkaloids suppressed the production of nitric oxide, a critical mediator in inflammation, which explains some aspects of the anti-inflammatory mechanisms present in the alkaloids of this plant (Dwuma-badu, *et al*., 1975). Moreover, *C. pareira* contains Cissampareine which can inhibit tumors (Kupchan, Patel, and Fujita, 1965). This plant can be used as a diuretic and as a muscle relaxant (Caceres, Giron, and Martinez, 1987, Tang and Eisenbrand, 1992). Additionally, *C. pareira* contains tropoloisoquinoline alkaloids, Pareirubrines A and B, which have been isolated as alkaloids with anti-leukemic properties (Itokawa, Morita, Matsumoto, and Takeya, 1993).

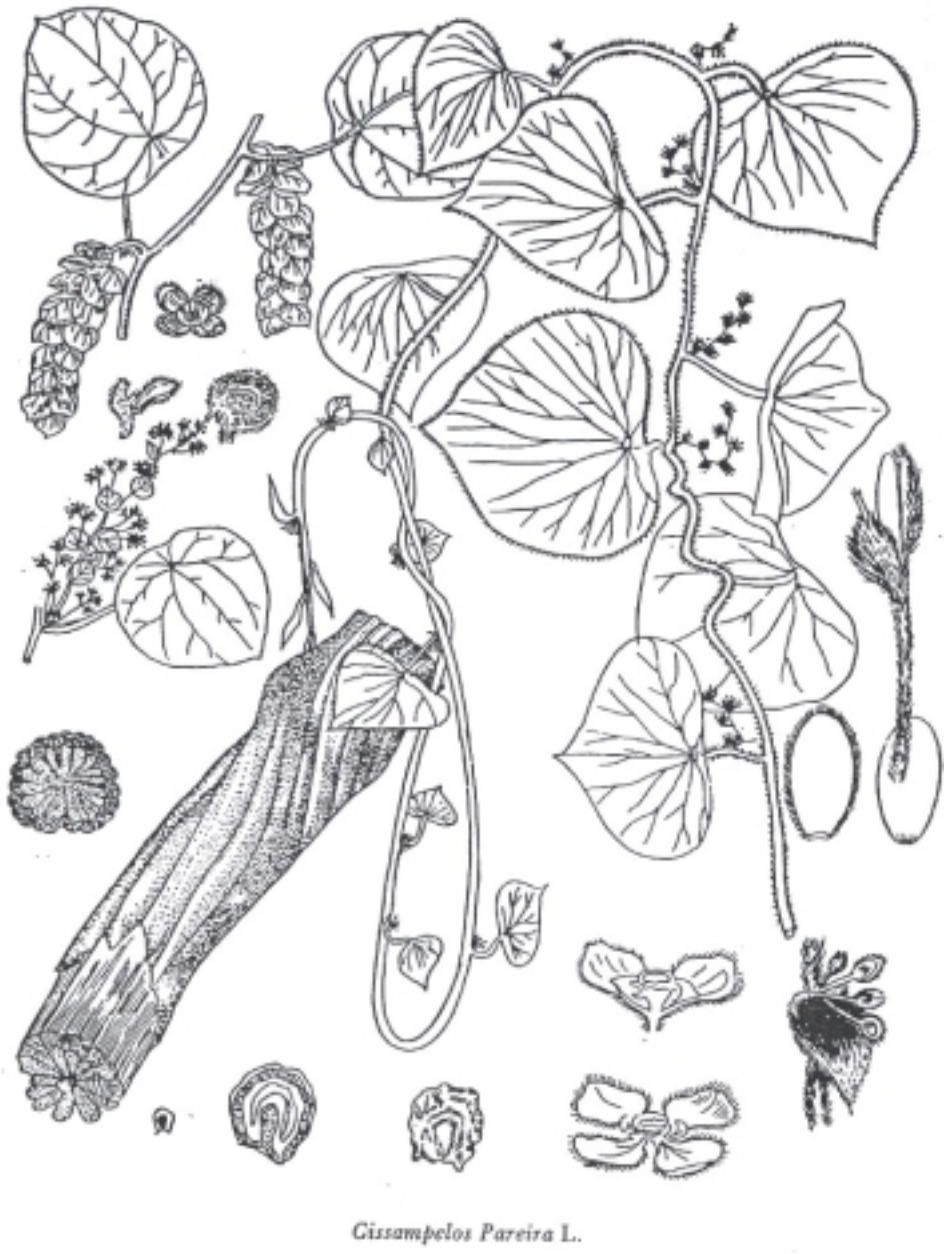

Figure 2.2 Characteristics of *C. pareira*

(Source: Mukerji and Bhandari, 1959)

2.2 Structural Characterization of Polysaccharides

2.2.1 Introduction

Polysaccharides have well-defined structures that vary with the functional properties. Polysaccharides may contain a number of different monosaccharide units. The number and distribution of monosaccharides along the polymer chain constitute the primary structure. These monosaccharides can be linked in different ways that restrict their freedom of motion with respect to each other and impose secondary structure on the polymer chain. Where they are regularities in primary structure, polysaccharides can adopt sterically regular secondary conformations. These ordered structures can sometimes further aggregate to form tertiary, crystal-like structures. Association of ordered tertiary structures may lead to quaternary structures, as occur when polysaccharides form gels (Oakenfull, 1998).

Polysaccharide structures are fundamental to understand about chemical structures (chemical composition, linkage patterns) and molecular structures (chain conformation, molecular weight) to the point that these determinations are fairly routine, and conformational analysis of polysaccharides by molecular modeling is a rapidly advancing field (BeMiller, 1996). In this research project, many methods may be used to elucidate chemical structure and molecular structure of a polysaccharide. The analysis of Krueo Ma Noy extracts will be covered in the following sections.

2.2.2 Methylation analysis

Methylation analysis has been an important method in the structural analysis of polysaccharides for many years (Chaplin and Kennedy, 1994). Elucidation of the linkage position is achieved by permethylation of the polysaccharides. Acidic hydrolysis of the resulting poly-methylethers cleaves only the interglycosidic linkages and leaves with the methylether bonds intact. Reduction and acetylation then yields partially methylated alditols, which are acetylated in the former linkage positions. The products of this so-called standard methylation analysis are then characterized by gas chromatography and mass spectrometry (Lindhorst, 2000).

For the first step in the methylation analysis, it is essential to carry out a complete methylation of all of the hydroxyl groups, allowing their methylation via a suitable methylating agent. The choice of reagents for this step is crucial, as incomplete methylation will yield inaccurate results. In early studies of polysaccharide structure, the methylations were performed with dimethyl sulfate in sodium hydroxide or with methyl iodide and a silver oxide catalyst and such reagents quite often give incomplete methylation. Prolonged reaction times and corresponding delays in completion of the analysis are encountered. Further, large samples of polysaccharides were needed for these methylations in order to obtain sufficient derivatives for analysis by fractional distillation methods (Chaplin and Kennedy, 1994). A method superior to the older ones for methylating polysaccharides has been developed using a strong base, methylsulfinyl methyl sodium, to ionize free hydroxyl groups of the polymer and using methyl iodide for methylating these groups. In this reaction sequence, alkoxide ions are generated readily from free hydroxyl groups by the strong base and methylation of these ions which occur rapidly (Phillips and Fraser,

1981). More recent methods have obtained greater yields in shorter reaction times using dimethyl sulfoxide as a solvent and methyl iodide and sodium or potassium hydroxide as the methylating agent and base, respectively (Ciucanu and Kerek, 1984).

Polysaccharides containing uronic acid or hexosamine residues are more difficult to be methylated and may yield secondary products. As a result, the determination of the primary sequences of polysaccharides containing uronic acid is often complicated by an unusual stability to acid hydrolysis of glycosidic bonds formed by these residues (Taylor and Conrad, 1972). Such derivatives may require special analytical techniques for identification (Pazur, 1986). The method of O'Neill, Darvill and Albersheim (1990) used lithium triethylborodueteride in tetrahydrofuran as a solvent to reduce the carboxyl group of the methylated polysaccharides. Furthermore, Voragen (1996), Strasser and Amado (2001) and Strasser and Amado (2002) studied the structure of pectic polysaccharides from sugar beet. Linkage analysis of polysaccharides was performed by methylation analysis after carbodiimide-activated reduction with sodium borodeuteride ($NaBD_4$). In addition, linkage analysis of flaxseed gum and water soluble yellow mustard polysaccharide was studied by Cui, Mazza and Biliaderis (1994) and Cui, Eskin and Biliaderis (1994) respectively, using methylation analysis that was carried out according to the method of Ciucanu and Kerek (1984) where as the reduction of carboxyl group after methylation was conducted following a procedure of O'Neill *et al*. (1990).

When the methylation is complete, the polysaccharide is hydrolysed in acid to constituent monosaccharide units which are methylated in specific positions. The hydrolysis products are reduced using sodium borodeuteride and acetylated using acetic anhydride to yield partially methylated alditol acetates (PMAA). The structure

of PMAA must be identified and quantified using gas chromatography (GC) to separate the PMAA and mass spectrometry (MS) to identify them (Biermann and McGinnis, 1989).

PMAA are broken into fragments when attacked to form a stream of high energy electrons at a raised temperature. The cleavage fragments, with their characteristic m/z values, can be identified and their fragmentation patterns will also indicate their substitution pattern (Biermann and McGinnis, 1989).

2.2.3 High performance liquid chromatography (HPLC)

HPLC is a powerful method of separation and identification of carbohydrates, of low to medium molecular weight (DP 1-30). The analysis of carbohydrates can be applied using both normal and reverse phase chromatography. There are extremely wide selection of column packing, elution phase and detection system (Harris, Morrison and Dacombe, 1995, BeMiller, 1996). Methods for analysis of carbohydrates have often employed silica-based amino-bounded or polymer-based metal-bounded cation exchange columns, with a refractive index (RI) or a low wavelength ultraviolet (UV) detector. These analytical methods require attention to sample solubility, sample concentration and, in the case of the metal-bounded cation exchange columns, also require column heating. In addition, RI and low wavelength UV detection methods are sensitive to eluent and sample matrix components. This usually obstructs the use of gradients and often requires stringent sample clean-up prior to injection (Technical note 20 in www.dionex.com).

An improved HPLC technique is known as high performance anion exchange chromatography (HPAEC) which carbohydrates are ionized by elution in a

strongly basic (pH 11-13) mobile phase. Carbohydrates in such an environment are ionized, permitting their separation on an anion exchange column. An electrochemical detector is used to determine separation between sample molecules. There are several types of electrochemical detection, which pulsed amperometric detection (PAD) is used for carbohydrates (Harris *et al*., 1995). Pulsed amperometry measures the current that results from the oxidation or reduction of sample molecules on an electrode (the actual exchange of electrons) which, unlike conduction, measures the difference in current flowing between two cells when a potential or voltage is applied across them based on the presence of ion in solution.

2.2.4 Nuclear magnetic resonance (NMR)

NMR has not been used for quantitative analysis but ^{13}C- and ^{1}H-NMR spectra have been published for many hydrocolloids. NMR analysis was used for determination of the fine structure of the polysaccharides. NMR spectra provide an accurate fingerprint of structure to identify polysaccharide mixtures and closely related polysaccharides (Whistler and BeMiller, 1993). NMR spectroscopy is the most important technique for structural determination of polysaccharides of biological or synthetic origin (Boong, 1998). It allows the elucidation of not only the static but also the dynamic structure of polysaccharides. NMR relies on the interaction between magnetically sensitive nuclei which are exposed to both a strong magnetic field and a pulsed or continuous wave radio frequency. Almost all elements have a magnetically active isotope which can be observed by NMR spectroscopy. The most frequently encountered atoms in organic molecules were proton (^{1}H) and carbon (^{13}C). Most NMR studies of carbohydrates have therefore been concerned with protons and

carbons. Other less sensitive and less commonly used nuclei include ^{31}P, ^{15}N and ^{17}O (Birch, 1985).

NMR spectra are often recorded as one-dimensional spectra for rapid identification of a compound or to check the purity of a substance. With the advent of two- and three-dimensional NMR experiments are available for the complete assignment of molecules and these NMR techniques are of paramount importance in structural and dynamic studies of carbohydrates ranging from monosaccharides to polysaccharides (Boong, 1998).

The NMR spectra of polysaccharides are most often obtained in solution, that is in D_2O or d_6-DMSO for unsubstituted products. All chemical shifts (δ) in the ^1H-NMR spectra derived from polysaccharides are in the range of 1 to 6 ppm from internal reference. The anomeric protons from each type of monosaccharide give recognizable signals depending on their α or β configurations. ^{13}C-NMR has a weaker signal, it has significant advantages over ^1H-NMR spectroscopy in the structure analysis of polysaccharides, because the chemical shifts in ^{13}C-NMR spectra are spread out over a very broad range. The broad distribution of the signal helps to solve the overlapping problems associated with the proton spectrum. The signal from ^1H of α-anomers is in a lower field than that from the corresponding β-anomers, and that ^{13}C (β) is in a lower field than ^{13}C (α) (Atkins, 1985). The signals of ^{13}C-NMR spectra from the anomeric carbons appear in the range of 90-110 ppm while the non-anomeric carbons show in the range of 60-85 ppm. For polysaccharides containing uronic acids, signals from the carboxyl carbons will appear in a lower field around 170-180 ppm. The signals of carbon atoms with primary hydroxyl groups

(C-6 in pyranoses and C-5 in furanoses) will appear in the higher field around 60-64 ppm while the signals of carbon atoms with secondary hydroxyl groups (C-2,3,4 in pyranoses and C-2,3 in furanoses) will appear in the range of 65-85 ppm (Atkins, 1985).

2.2.5 Fourier transform infrared spectroscopy (FT-IR)

The vibrational spectra are important in the analysis and identification of polysaccharides in food polysaccharides. When infrared (IR) radiation passes through a sample, molecules can absorb the infrared light of frequencies that match the energy of changes in molecular vibrations. Fourier transform infrared (FT-IR) provides infrared spectra by first collecting an interferogram of signals using an interferometer which measures all of the infrared frequencies simultaneously. Once an interferogram is collected, it is translated into a spectrum (emission, absorption, transmission, etc.) through the fast Fourier transform algorithm. The FT-IR spectroscopy can provide a spectrum in seconds compared to a few minutes when using a dispersive IR spectrometer, in which wavenumbers are observed sequentially as the grating is scanned. (Ćerná *et al*., 2003, Kačuráková and Wilson, 2001, Smith, 1996).

FT-IR spectroscopy is a rapid, versatile and sensitive tool for elucidating the structure, physical properties and interactions of carbohydrates, for studying pectic polysaccharides and hemicelluloses extracted from plants and for detecting structural and compositional changes occurred in the cell walls (Ćerná *et al*., 2003, Kačuráková, Capek, Sasinková, Wellner and Ebringerová, 2000). Carbohydrates show high absorbencies in the region of 1200-850 cm^{-1}, that is within the so-called fingerprint

region, where the position and intensity of identification are employed (Filippov, 1992).

Particular polysaccharides were identified using infrared data of model compounds representing individual polysaccharide type and its mixtures, based on major sugar components. The role of COOH side groups in ring vibrations was verified with monosaccharide models measured in aqueous solutions, which represent structural moieties of the polysaccharides. FT-IR spectra in the 1200-850 cm^{-1} region provided information about the main polysaccharides appearing in the complicated system of polysaccharide mixtures. The overall shape of a polysaccharide spectrum was determined by the backbone polysaccharide composition but it could also be strongly influenced by the side chain constituents. At least one very intense band was identified for each particular polysaccharide structural moiety. The IR band of β-(1→6) or β-(1→3)-linked galactan showed an intense band at about 1078-1072 cm^{-1}. The β-(1→4)-mannan was found at 1066-1064 cm^{-1}. The main chain forming arabinan was at 1039 cm^{-1} while the arabinans side chain was at about 1044 cm^{-1}. Xyloglucan and β–glucan showed bands at 1041 cm^{-1} while α–glucan band showed an additional band at 1026 cm^{-1}. Rhamnose in side chains showed a band at 1043 cm^{-1}. These characteristic band maxima were due to the influence of the constituent monosaccharides of the studied pectic and hemicellulosic polysaccharides. Galactose showed the strongest IR band at 1078 cm^{-1}, mannose at 1070 cm^{-1} and glucose at 1035 cm^{-1}. The relative position of axial and equatorial (OH) side groups influenced the main band frequency positions at 1100-1000 cm^{-1} and the maxima assigned to ring and side group vibrations were related to the polysaccharide spectra. Therefore, the

distinctive band positions allowed the identification of polysaccharide structures and their composition (Kačuráková *et al*., 2000).

Coimbra, Barros, Barros, Rutledge and Delgadillo (1998) studied multivariate analysis of uronic acid and neutral sugars in whole pectin samples of olive and orange pulps by FT-IR spectroscopy. The FT-IR spectrum of a pectic polysaccharide sample showed characteristic absorbances in the region between 1200 and 850 cm^{-1} ; a sample rich in uronic acid showed two intense peaks at 1110 and 1020 cm^{-1}.

Pectin is a polymer of D-galacturonic acid that has a carboxyl group on C5, some of which are esterified to form methyl esters. Most of the functional uses of pectin are directly or indirectly related to the extent to which the carboxyl group are esterified (Gnanasambandam and Proctor, 2000, Walter, 1991). Stronger bands occurring between 1760-1730 cm^{-1}, and between 1630-1600 cm^{-1} indicated the ester carbonyl (C=O) groups and carboxylate ion stretching band (COO-), respectively (Manrique and Lajolo, 2002, Chatjigakis, Pappas, Proxenia, Kalantzi, Rodis and Polissiou, 1998). It was observed that the ester carbonyl groups increased in their intensity and band area as the degree of esterification increased, while the intensity of the carboxylate stretching band decreased.

Measurement of the degree of esterification (DE) is a routine analytical procedure in pectin analysis. The DE may be expressed as the percent of the total number of carboxyl groups esterified or as the percentage of methoxyl contents of the total pectins (Walter, 1991). Several methods for measuring the degree of esterification were reported in the literature. A titrimetric method proposed by Food Chemical Codex (FCC, 1981) and USP26 NF21, 2003 is commonly used for the

determination of DE. The DE of pectins can also be determined by means of instrumental methods, such as HPLC (Plöger, 1992, Levigne, Thomas, Ralet, Quemener and Thibault, 2002) and ^{1}H-NMR spectroscopy (Grasdalen, BakØy and Larsen, 1988). Compared with chemical methods, these instrumental techniques have an advantage in being faster with no hydrolysis, even though they also require isolation of pectin samples. FT-IR spectroscopy has become useful as a simple, quick and non-destructive method for determination of DE in the pectic polysaccharides of the cell wall material extracts. The estimation of DE is based on a calibration curve using standard pectins with known DE and spectra bands at around 1749 and 1630 cm^{-1}. These absorptions are attributes of the esterified and non-esterified carboxyl groups of pectin molecules, respectively (Chatjigakis *et al.*, 1998).

The FT-IR spectroscopy method was developed to measure the DE of commercial pectin samples (Gnanasambandam and Proctor, 2000). The mean values of the DE of pectin samples obtained from FT-IR spectroscopy and the titrimetric method were similar. The standard deviation values for the FT-IR method were smaller compared to those obtained from the titrimetric method. FT-IR spectroscopy can be a rapid, alternative method to titrimetric analysis of DE of pectin.

FT-IR spectroscopy is a method suitable for monitoring chemical changes in cell walls and more specifically changes in the DE. Chatjigakis and co-workers (1998) applied FT-IR spectroscopy to the study of peach cell walls and revealed the existence of two peaks absorbing at 1749 and 1630 cm^{-1}, which are ascribed to the absorption of the esterified and non-esterified carboxyl groups of pectin molecules, respectively. A linear relationship between the DE and the ratio of the area underneath the peak at 1749 cm^{-1}over the sum of the areas underneath the two peaks

at 1749 and 1630 $cm^{-1}[A_{1749}/(A_{1749}+A_{1630})]$, was established using the FT-IR spectra of standard compounds. During storage at 0ºC, the DE remained practically constant up to 35 days. Storage at higher temperatures resulted in the decrease of DE. The changes in the DE during storage correlated well to fruit firmness.

Manrique and Lajolo (2002) studied the DE in pectins isolated from ripening papaya fruit using FT-IR spectroscopy. The method was used for measuring the methylation level of different pectin fractions isolated from papaya (*Carica papaya*) fruit in three stages of ripening (green, intermediate and ripened). DE values were calculated from absorbance spectra of the samples, using a relationship involving absorbance intensities for 1630 and 1745 cm^{-1} bands. Under present experimental conditions these bands could be assigned to stretching frequencies for the carbonyl groups of galacturonic acid and its methyl ester, respectively. DE values were compared with those calculated from independent measurements of galacturonic acid and methanol. There were no significant differences between the DE results obtained by the two methods for each sample, indicating the good reliability of the FT-IR technique. Firmness values for green, intermediate and ripened papaya were 95.42, 50.74 and 9.61 N, respectively (with a mean SD less than 2%, each case). There seemed to be an inverse relationship between firmness and DE of pectins associated to middle lamella and primary cell wall during fruit ripening.

2.2.6 High performance size exclusion chromatography (HPSEC)

Polysaccharides are used increasingly in the food industry to improve the functionality of processed foods. Their structure-function relationships need to be known in detail to understand better the use of these polysaccharides in food systems.

The effect of molecular weight on functionality has been studied (Corredig, Kerr and Wicker, 2000).

HPSEC has been widely used in the food industry for the determination of molecular weight and quality control of polysaccharides. This separation technique has been used in combination with component analysis of the elution chromatography to characterize size and viscosity distribution of polysaccharides (Beri, Walker, Reese and Rollings, 1993, Fishman, Cooke, Hotchkiss and Damert, 1993). Because intrinsic viscosity is related to the hydrodynamic volume of the polysaccharides, differential refractometric and viscometric detectors have been used in series for studying changes in molecular weight distribution of polysaccharides eluted from size exclusion columns (Fishman, Pfeffer, Barford and Doner, 1984, Fishman, Chau, Kolpak and Brady, 2001).

Application of complex polysaccharides obtained from plants and microbes are found to increase in the food industry to improve the functional properties of processed foods. HPSEC with concentration-viscosity detector, coupled with Gaussian curve fitting of concentration and viscosity chromatograms has been applied to pectin, tragacanthin, logust bean gum, carboxyl methyl cellulose, alginates and gum arabic. Weight average intrinsic viscosity was determined directly from areas under the concentration and specific viscosity curve. In addition, the radius of gyration (Rg) and molecular weight (Mw) were determined from both size and the universal calibration of columns with pullulans. This information can be useful for detection of adulteration, for quality control, for monitoring polymer processing and for fundamental solution behavior of aggregation water-soluble polysaccharides (Hoagland, Fishman, Konja and Clauss, 1993).

The development of a light scattering (LS) detector has simplified the absolute determination of Mw and Rg, eliminating the need for column calibration with molecular weight standards and the need for principal component analysis of chromatograms. If the refractive index increment (dn/dc) of the eluting solute is known, it is possible to obtain absolute values of Mw by coupling LS measurements with data derived from a different refractometer. In addition, when using a multi-angle light scattering detector (MALLS), the scattered intensity generated from a sample is simultaneously measured by a series of fixed angle detectors (Burchard, 1994, Corredig *et al.*, 2000). The average values of Mw and Rg, including Mw and Rg distributions, were calculated using a Zimm or Debye plot.

Morris, Foster and Harding (2000) studied the effect of the degree of esterification on the hydrodynamic properties of citrus pectin. Five citrus pectins with an average degree of esterification of 77.8, 65.0, 53.9, 37.8 and 27.9% were studied using capillary viscometry, sedimentation velocity, sedimentation equilibrium and size exclusion chromatography coupled with multi-angle laser light scattering (SEC-MALLS). Molecular weight for all five pectin samples were within the range 190,000±30,000 g/mol. The results indicated increasing chain stiffness with decreasing DE. Molecular characterization of four commercial pectins with different DE was analyzed using HPSEC in-line with MALLS and refractive index detectors. Molecular weight distributions and the root mean square radius were calculated after elution under different conditions such as mobile phase, flow rates and amounts of pectin injected. Pectins were polydispersed with respect to Mw. The molecular weight distribution ranged from $8x10^3$ to 10^6, depending on the source and was not

affected by buffer composition or flow rates of separation. The four pectins had similar values for Rg of 50 nm (Corredig *et al*., 2000).

Orange albedo pectin extracted using microwave heating under pressure, was characterized for Mw, Rg and intrinsic viscosity [η] by HPSEC with online light scattering and viscosity detectors. Mw, Rg and [η] all decreased with increasing extraction time from 2.5 to 8 min. At heating time of 2.5 min, Mw $3.6x10^5$, Rg 38 nm and [η] 10.8 dL/g, were significantly higher than a commercial citrus pectin. The results revealed that solubilized pectin distributions were bimodal in nature and that the low-molar-mass fraction increased at the expense of the high-molar-mass fraction with increasing extraction time. Possibly these results indicated that at short extraction times, pectin was solubilized in compact aggregated network structures that were broken down to their more asymmetric components with increased heating times (Fishman, Chau, Hoagland and Ayyad, 2000). Furthermore, Corredig and Wicker (2001) studied changes in the molecular weight distribution of three commercial pectins after valve homogenization using HPSEC coupled with MALLS detector. Pectin sampled after homogenization at 124 MPa showed a significant effect on the molecular weight distribution and the molecular weight averages of high methoxyl pectin samples. On the other hand, no changes occurred in the apparent viscosity of the diluted pectin solution after homogenization at 17 MPa, and only one of the pectins analyzed showed an effect of high pressure (124 MPa) on viscosity. While the apparent viscosity of pectin with a wide molecular size distribution (large polydispersity) did not change upon pressure treatment, one of the pectins studied showed increased polydispersity and a significant change in flow behavior after treatment at 124 MPa. The results showed that molecular changes may occur upon

valve homogenization, and that the polydispersity of the molecular weight distribution may hinder an accurate estimation of such changes.

2.3 Rheological Properties

2.3.1 Introduction

Functional properties of polysaccharides (gums, hydrocolloids) are generally considered with regard to uses in fabricated foods mainly because of their thickening and gelling properties and to modify and/or control the flow and texture properties in the food industry (BeMiller and Wistler, 1996, Eliasson, 1996). Functional properties of hydrocolloids are mostly related to the physicochemical mechanisms underlying their behavior in an aqueous medium. All these mechanisms are caused by the thermodynamics of the system. Water solubility is related to solvent quality, the strength of interactions between the polysaccharide and water. The hydrodynamic volume and the thickening properties are related to water-polysaccharide interactions. On the contrary, gelling properties take place as a result of an equilibrium between polymer-polymer and polymer-solvent interactions (Eliasson, 1996). Thus with the use of polysaccharides, individually or in combination with others, it is possible to fabricate products with rheological properties ranging from viscous to elastic. Rheological properties, involving the flow and deformation characteristics of materials under stress, are important in understanding these structures and in the handling, processing, mastication and utilization of foods. Therefore, rheological methods are the appropriate tools for studying functional properties of hydrocolloids (Walter, 1998, Blanshard and Mitchell, 1978). Applying rheological measurements and concepts to polysaccharide

solutions facilitates the acquisition of information about molecular size, polymer structure, molecular shape, solvent-polysaccharide interactions and intermolecular networks (BeMiller and Whistler, 1996, Walter, 1998). The information obtained depends on the analysis performed and the solution concentration.

The behavior of dilute and concentrated polysaccharide solutions will yield different types of information. So it is important to understand what the terms "dilute" and "concentrated" mean with respect to polysaccharide in solution. When the concentration dependence of the zero-shear specific viscosity (η_{sp}) of a very dilute polysaccharide solution is plotted versus the coil overlap parameter, $c[\eta]$ (where $[\eta]$ is intrinsic viscosity, a measure of the space occupancy of a molecule), the resulting curve will increase linearly with concentration up until a critical concentration (c*), after which the viscosity-concentration curve deviates from linearity (Figure 2.3). Below the critical concentration (c*), the polysaccharide solution is in the dilute region, where each molecule occupies a discrete amount of space. The critical concentration represents the concentration where polysaccharides in solution begin to overlap and entangle with one another. The entanglements between polymers in solution create friction and cause a sharp increase in viscosity (BeMiller and Whistler, 1996, Dickinson, 1992).

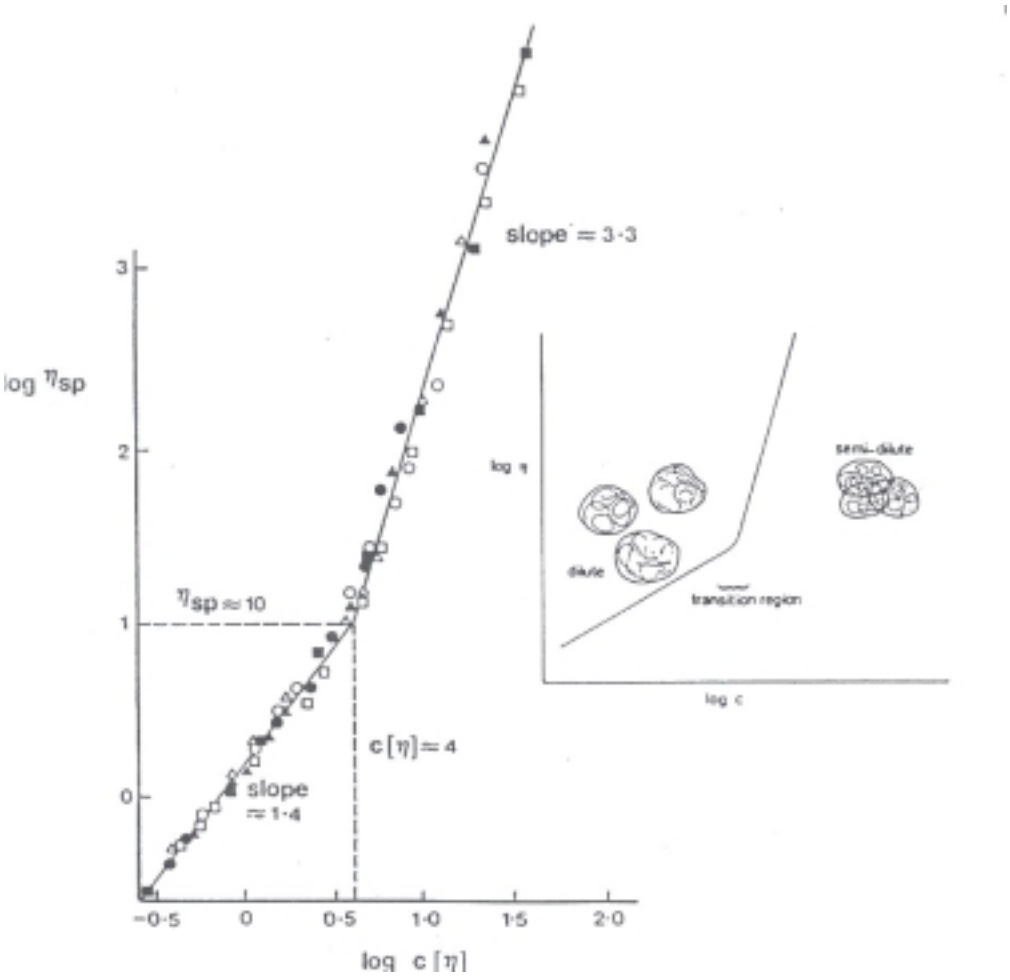

Figure 2.3 Generalized concentration dependence of viscosity for conformationally disordered (random coil) polysaccharides.
(Source: Whistler and BeMiller, 1993; Ross-Murphy, 1994)

In the real of polymer solution, the term dilute solution refers to a solution where each polymer occupies a discreet amount of space (Figure 2.4a). As the amount of polymer in the solution increases, a polymer concentration is reached (c=c*) at which the polymer coils begin to overlap as in Figure 2.4b, the term "semi-dilute solution" refers to a solution of macromolcules above the overlap concentration

(c>c*) (Figure 2.4c), and the term "concentrated" is used to denote solutions with a very high chain density, approaching a polymer melt (Dickinson, 1992, Ross-Murphy, 1994). So, in solution concentration below and above the coil overlap parameter will be termed dilute and concentrated, respectively. An analysis of dilute polysaccharide solutions offers information about hydrodynamic volume and polymer chain conformation while concentrated solution measurements are related to the magnitude of viscosity, shear rate dependence of viscosity and intermolecular networks. This information can be obtained by analyzing the results of small deformation, steady shear and oscillatory measurements.

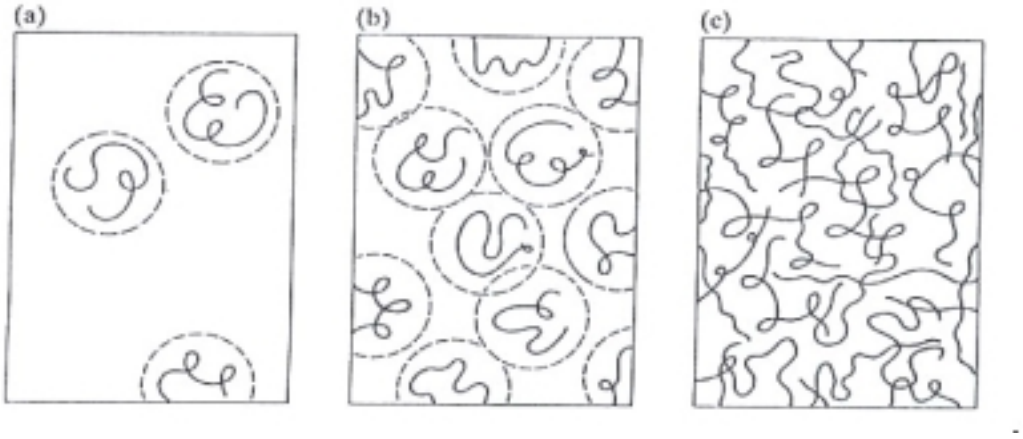

Figure 2.4 Illustration of the effect of polymer concentration c on the structure of a macromolecule solution: (a) dilute solution (c<c*); (b) onset of coil overlap (c≅c*); and (c) semi-dilute solution (c>c*).

(Source: Dickinson, 1992)

2.3.2 Dilute solutions : Intrinsic viscosity

With the solubilization of polysaccharides, it is an important to develop the functional properties, viscosity measurement in dilute condition in order to provide an easy and interesting means to characterize its behavior in aqueous solution (Eliasson, 1996).

Intrinsic viscosity, [η], represents the volume occupancy of the mass unit of the polymer molecule. It is not actually a viscosity. This parameter is generally measured in order to obtain information about the molecular weight and conformation of a polymer (Hill, Ledward and Mitchell, 1998, Blanshard and Mitchell, 1978). Intrinsic viscosity is determined experimentally from measurements of the viscosity of solutions of very low concentration ($c<c^*$). Denoting solution and solvent viscosity as, η and η_s, respectively, is defined formally by the following standard relationships:

Relative viscosity: $\eta_{rel} = \eta / \eta_s$

Specific viscosity: $\eta_{sp} = (\eta-\eta_s) / \eta_s = \eta_{rel} - 1$

Intrinsic viscosity: $[\eta] = \lim_{c\to 0} \eta_{sp} / c$

Intrinsic viscosity may be taken as the intercept of a combined Huggins-Kraemer plot extrapolated to infinite dilution where

$$\eta_{sp} / c = [\eta] + K' [\eta]^2 c$$

and

$$(\ln \eta_{sp})/c = [\eta] + K'' [\eta]^2 c$$

are the Huggins and Kraemer equations respectively, K′ is the Huggins coefficient, K″ is the Kraemer coefficient, and [η] is the intrinsic viscosity of dL/g or in mL/g. Assuming that in dilute solutions isolated polysaccharide coils interfere with the flow of solvent and increase viscosity, the magnitude of viscosity increase should be proportional to molecular volume (Walter, 1998, Ross-Murphy, 1994, Eliasson, 1996). Therefore, intrinsic viscosity values can provide valuable information about chain branching, stiffness and polymer size and shape. For every polymer-solvent system, the intrinsic viscosity is directly related to the molecular weight according to the Mark-Houwink-Sakurada equation:

$$[\eta] = K\, Mw^{\propto}$$

where $\propto$ is the Mark-Houwink exponent and Mw is the average molecular weight. K and $\propto$ are related to the degree of molecular expansion and hence depend upon the local stiffness of the polymer backbone and polymer-solvent interaction: $\propto = 0$ for a sphere, 1.8 for a rod and ideally 0.5-0.8 for flexible polymer in marginal and good solvents.

The intrinsic viscosity of polysaccharide varied with molecular weight, solvent quality and molecular conformation. Iglesias and Lozano (2004) found that an increase in the intrinsic viscosity with increasing molecular weight for sunflower pectin occured when all other pertinent parameters (solvent, temperature) were constant. In a similar manner, guar galactomannan increased in molecular weight by increasing the intrinsic viscosity between 4.5 and 12.5 dL/g (Robinson, Ross-Murphy and Morris, 1982).

Differences in intrinsic viscosity based on solvent type have also been observed. These differences arise either from solvent quality leading to an expansion

or compaction of polymer structure and/or solvent system acting in a manner such that polymer-polymer interaction, which can artificially increase intrinsic viscosity measurements, are inhibited. Richardson, Willmer and Foster (1998) found the intrinsic viscosity of locus bean gum (LBG) and guar gum decreased for every level of sucrose addition except 20% sucrose, which resulted in the highest intrinsic viscosity. Intrinsic viscosity values obtained for galactomannans are affected by the degree of galactose substitution. It has been suggested that the intrinsic viscosity of LBG is artificially high due to a contribution from polymer-polymer association between minimally substituted regions of the mannan backbone. This assertion is supported by Goycoolea, Morris and Gidley (1995) showing that when polymer-polymer interactions were inhibited by salvation in an alkaline solution, the intrinsic viscosity of LBG decreased from 12.1 dL/g at neutral pH to 5.2 dL/g in alkali causing same charge repulsion between polymers. Consistent with a much greater galactose substitution for guar gum, reduction in intrinsic viscosity also occurred from 12.5 dL/g in a neutral solution to 11.49 dL/g in alkali solution.

2.3.3 Semi-dilute solutions

A. Steady shear viscosity

For a liquid, the stress (shear) depends on the rate of deformation (ability to resist motion). The apparent viscosity is defined by

$$\eta = \tau / \dot{\gamma}$$

where η is viscosity, τ is shear stress and $\dot{\gamma}$ is shear rate. Shear stress is the force (Pa) applied to a sample and shear rate (sec^{-1}) is the velocity gradient generated in a sample as a result of the applied stress (Dickinson, 1992).

A Newtonian flow is a behavior in which shear stress is a strictly linear function of the shear rate. If shear stress does not have a linear relationship with the shear rate, its behavior is said to be non-Newtonian. Flow curves are shown in Figure 2.5 and the gradient $d\tau/d\dot{\gamma}$ at a point on a graph of shear stress versus shear rate is known as the apparent viscosity (η) of the material at that particular shear rate. Most food systems are non-Newtonian behavior and shear-thinning behavior (Pseudoplastic) is the most common type of non-Newtonian flow in food systems in which the viscosity decreases with increasing shear rate (Figure 2.5). The converse phenomenon is shear-thickening behavior (Dilatant) which shows an increase in viscosity which corresponds to an increase in the shear rate.

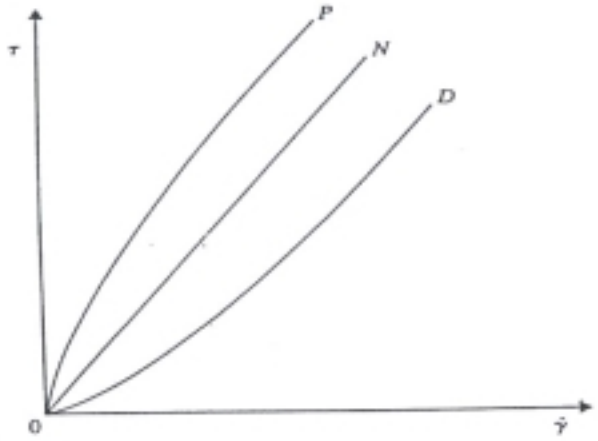

Figure 2.5 Flow behavior, the shear stress τ is plotted against the shear rate $\dot{\gamma}$: P, Pseudoplastic (shear-thinning); D, Dilatant (shear-thickening); N, Newtonian flow.
(Source**:** Dickinson, 1992)

Steady shear viscosity of polysaccharide solutions is a function of the size, shape and the conformations of a polysaccharide (BeMiller and Whistler, 1996). Linear molecules in solution will gyrate and flex, sweeping out a large space and colloiding with each other that creates friction and produces high viscosity. Therefore, a linear molecule in solution at a specified concentration will increase viscosity more than a highly branched molecule of similar molecular weight at the same concentration. Moreover, the linear chains contain charged groups (always a negative charge) which also form stable solutions causing extended configuration due to charge repulsion, increasing the end-to-end chain length and the volume swept out by the polymer which provides high viscosity.

The flow curve obtained for concentrated solutions has often been described by the power law model, for which the constitutive equation is

$$\tau = k\dot{\gamma}^{n}$$

where k is the consistency index and n is a parameter which varies with the type of flow: $n<1$ for shear-thinning, $n=1$ for Newtonian and $n>1$ for shear-thickening behavior.

Medina-Torres, Brito-DeLa Fuente, Torrestiana-Sanchez and Katthain (2000) studied the rheological properties of aqueous solution of mucilage isolated from *Opuntia ficus idica*. Steady shear viscosity measurements showed a non-Newtonian shear thinning behavior, which could be correlated to the mucilage concentration, in the range from 1 to 10% (w/w). Mucilage viscous solutions were found to be slightly temperature dependent and this behavior is almost independent of mucilage concentration. On the other hand, the steady-shear viscosity was dependent on ionic strength, i.e. increased ionic strength with decreased viscosity. The dependence of

viscosity on pH was also observed. As pH was increased from acid to alkaline conditions, viscosity increased.

B. Oscillatory measurements

An oscillatory rheological method is the best technique to separate out the solid-like and liquid-like characteristics of a food system. Results are very sensitive to chemical composition and physical structure thus they are useful in a variety of applications including gel strength evaluation, monitoring starch gelatinization, studying the glass transition phenomenon, observing protein coagulation or denaturation, evaluating curd formation in dairy products, cheese melting, texture development in bakery and meat products, shelf-life testing and the correlation of rheological properties to human sensory perception (Steffe, 1996, Dickinson, 1992). The viscoelastic parameters of the material are determined by comparing the strain with the resulting oscillating stress. The stress is directly in phase with the strain when a sinusoidal strain wave is applied to a perfectly elastic solid. In contrast, for an ideal viscous liquid, the stress is exactly 90° out of phase with strain.

Figure 2.6 illustrates the principle of sinusoidal oscillation. A sinusoidal oscillation of maximum strain γ_m with frequency ω is applied to a sample in a parallel plate geometry. The rate of change of the sinusoidal oscillation is maximum when the strain is zero, the resultant stress wave will be exactly 90° out of phase with the imposed deformation (Ross-Murphy, 1994).

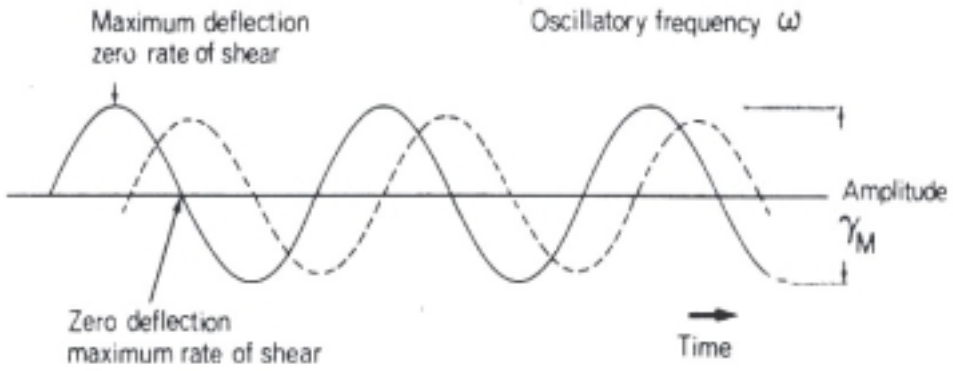

Figure 2.6 Sinusoidal oscillatory shear. At the extremes of the oscillatory cycle the shear strain (—) is maximum, but the shear rate (-----) is zero, while the converse is the case at the zero strain position.

(Source: Ross-Murphy, 1994)

The behavior of most polysaccharide solutions can be described as viscoelastic, following somewhere between the two extremes of behavior, in phase and out of phase, ($0<\delta<90$). The value of solid-like and liquid-like behavior in polysaccharide systems depends on the relative amounts of elastic and viscous behavior. The ratio of in phase stress to applied strain is the elastic modulus, G′, defined by

$$G' = (\sigma_0 / \gamma_0) \cos (\delta)$$

And the ratio of out of phase stress for an applied strain is the viscous modulus, G″, defined by

$$G'' = (\sigma_0 / \gamma_0) \sin (\delta)$$

Where σ_0 is the shear stress amplitude, γ_0 is the strain amplitude and δ is the phase shift or phase angle relative to the strain.

The energy used in the deformation of an elastic solid is recovered as the sample springs back to its original state (stored), while for a perfect liquid there is no such recovery and the energy is lost. Hence G′ and G″ are known as the storage and loss moduli, respectively (Walter, 1998, Steffe, 1996).

The overall response of the sample may be characterized by the complex modulus, G*:

$$G^* = (G'^2 + G''^2)^{1/2}$$

A closely related parameter is complex viscosity (η*), which is the ratio of total stress to frequency of oscillation (ω). It is defined as:

$$\eta^* = G^*/ \omega = (G'^2 + G''^2)^{1/2}/ \omega$$

Another popular material function used to describe viscoelastic behavior is the tangent of the phase shift or phase angle (called tan delta) which is also a function of frequency:

$$\tan(\delta) = G''/ G'$$

The amplitudes of the stress and strain waves are usually adjusted to sufficiently low values that stress is proportional to strain and this is known as the region of linear viscoelasticity (Figure 2.7). The main advantage of the oscillatory measurement is that it leads to minimal disruption in the structure of the material being investigated because it is carried out without significantly perturbing the process such as low stress and strain amplitudes (Walter, 1998, Dickinson, 1992).

For dilute and concentrated solutions at low frequency where there is sufficient time for network re-entanglement compared to impose movement, complex viscosity remains constant. As frequency increases, a small number of molecular chains have time to re-entangle within the oscillatory period and the complex

viscosity drops (Aspinall, 1982). The frequency dependence of complex viscosity is nearly equal to the steady shear viscosity when the shear rate and frequency are equal. This relationship referred to as the "Cox-Merz rule" may be useful for materials that are more easily tested under oscillatory instead of steady shear conditions (Aspinall, 1982, Steffe, 1996). The most common mode of oscillatory testing is the frequency sweep because it shows how the viscous and elastic behavior of the material changes with the rate of application of strain or stress. For polysaccharide solutions, G′ and G″ will vary based on polysaccharide type, solution concentration and frequency of oscillation. Analysis of G′ and G″ provides information about the degree of solid-like and liquid-like behavior of a solution, which can be related to molecular structure and the presence of intermolecular networks.

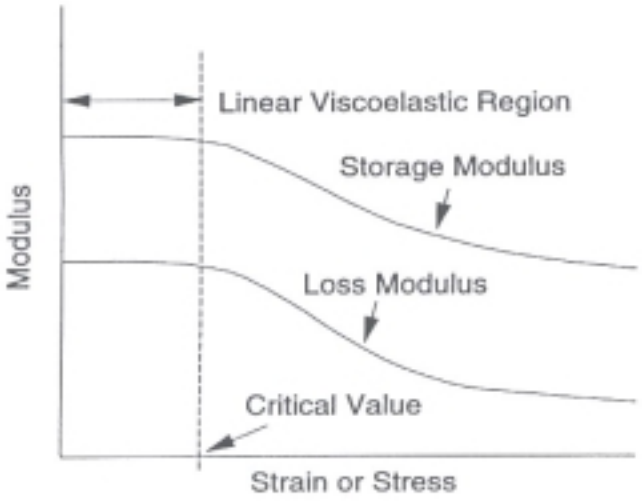

Figure 2.7 Typical response to a strain or stress sweep showing the linear viscoelastic region defined by the critical value of the sweep parameter.

(Source: Steffe, 1996)

Figure 2.8 presents the mechanical spectra of polysaccharide systems. For dilute solution (Figure 2.8c), G″ is higher than G′ over the entire frequency range but they approach each other at higher frequencies. At high frequencies of oscillation concentrated solution of disordered polymers behave similarly to gels, whereas at lower frequencies G″ becomes predominant, as shown in Figure 2.8b. The crossover frequency is sometimes a useful criteria for product evaluation. G′ is significantly higher than G″ throughout the frequency range for the gel (Figure 2.8a). It is meaningful to observe that moduli are a strong function of frequency in the dilute and concentrated solutions but are practically constant with the gel (Aspinall, 1982, Ross-Murphy, 1994, Steffe, 1996, Walter, 1998).

Figure 2.8 also displays complex viscosity (η*) curves for polysaccharide systems. Complex viscosity is frequency independent at low concentrations because there are insufficient molecules in solution to contribute to the development of a network. In concentrated solutions, η* is constant at low frequency because there is sufficient time for network rearrangement relative to movement imposed on the solution. As frequency increases, more and more molecular chains do not have time to rearrange themselves within the period of oscillatory movement and η* decreases.

2.3.4 Gelling system

Gels are generally defined as a class of systems which show solid-like properties in the presence of excess solvent (Lapasin and Pricl, 1995, Doublier and Cuvelier, 1996, BeMiller and Whistler, 1996). Gelation can be divided into two main classes of mechanisms, chemical and physical gels (Lapsin and Pricl, 1995, Ross-Murphy, 1995).

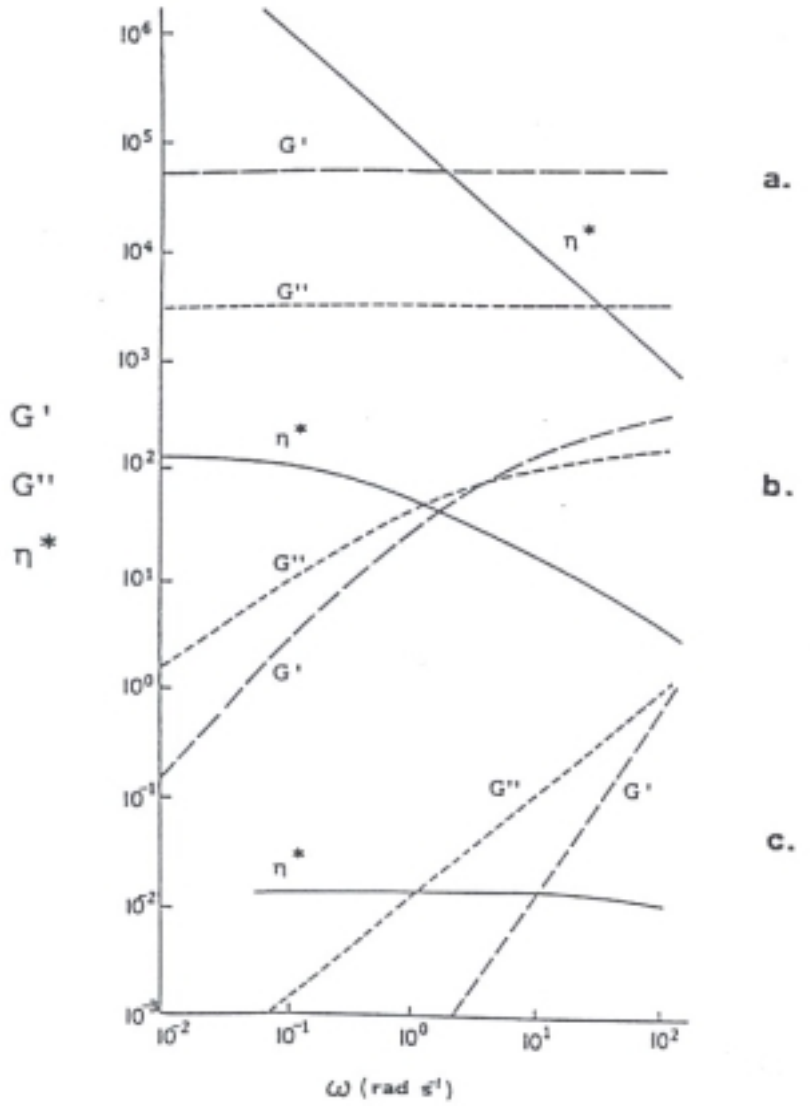

Figure 2.8 Typical mechanical spectra of polysaccharides systems. (a) strong gel; (b) concentrated solution (c>c*); (c) dilute solution (c<c*), where c* denotes polymer concentration at the onset of coil overlap and entanglement.

(Source: Walter, 1998)

Chemical gels are characterized by a permanent stability of the network causing the strength of chemical bonds, this type of gel is mainly the domain of synthetic polymers. In physical gels, the point cross-links are replaced by weaker and potentially more reversible forms of chain-chain interactions from london forces to hydrogen bonding. Gelation of polysaccharides arises from physical cross-linking through polymer-polymer interactions. Typically, polysaccharide gels contain more than 90% by weight of water or aqueous electrolyte. Two major classes of networks are often distinguished, referred to as the strong and weak gels (Lapasin and Pricl, 1995, Ross-Murphy, 1995, Burchard and Ross-Murphy, 1990).

Evaluation of G′ and G″ as a function of frequency, time and temperature provides useful information on the phenomenon of gelation (Walter, 1998). Strong gels show the characteristic of true gels under small deformation conditions, they manifest the typical behavior of viscoelastic solids. They rupture rather than flow above a critical deformation value. The behavior is intermediate in properties between the entanglement networks and true gels for which the term weak gels is used. The difference between the strong gels, weak gels and entangled solutions can be clearly evidenced by means of oscillatory flow measurement. However, a reliable discrimination necessarily requires an extended experimental frequency range, particularly in the region of low frequencies. Strong and weak gels can be unambiguously classified on the basis of their mechanical spectra. A typical strong gel spectrum, over the frequency range 10^{-2} to 10^{2} Hz, consists of two nearly horizontal straight lines. G′ is typically 1-2 orders of magnitude greater than G″, and both may show slight increases at higher frequencies (Lapasin and Pricl, 1995).

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Chapter III

Extraction, Purification and Physicochemical Characterization of Krueo Ma Noy Extracts

Abstract

A polysaccharide was extracted from the leaves of Krueo Ma Noy (*Cissampelos pareira*), a woody climbing plant from Thailand. The extraction conditions were 2% solids in distilled water at 25-28°C and natural pH (3.8-4.0). Alcohol precipitation and drying produced a crude extract and further dialysis and lyophilization produced a dialyzed extract. It has been found that Krueo Ma Noy polysaccharide was a pectin consisting mainly of high levels of galacturonic acid (~70%) and small amounts of monosaccharides. In diluted solutions, Krueo Ma Noy pectin exhibited Newtonian flow behavior. Shear-thinning behavior was observed at a concentration of ≥ 0.5% (w/v). Both crude and dialyzed extracts gelled in aqueous solutions at 1.0% (w/v). Krueo Ma Noy pectin (0.5%,w/v) also formed gel under acidic conditions (pH 2-4). The addition of NaCl significantly increased gel strength when salt concentration was below 0.4 M. Krueo Ma Noy pectin was very sensitive to the presence of $CaCl_2$. The addition of 1 mM $CaCl_2$ significantly increased gel strength. However, precipitation occurred when $CaCl_2$ concentration was greater than 3 mM. The divalent cation (Ca^{2+}) showed more effect on increasing viscoelastic properties of Krueo Ma Noy pectin than monovalent cation (Na^{+}) did, which indicated

a typical property of low methoxy pectins. In addition, the presence of sucrose increased G′ with an increase in sucrose concentration.

Keywords: Krueo Ma Noy; *Cissampelos pareira*; pectin; galacturonic acid; FT-IR spectroscopy; rheological properties

3.1 Introduction

Krueo Ma Noy, *Cissampelos pareira* , is in the family of *Menispermaceae*. It is a woody climbing vine with leaves of up to 30 cm in length. The plant is found throughout warm parts of Asia, East Africa and America (Smitinand and Larsen, 1991). It is widespread in the northeast of Thailand.

Krueo Ma Noy is commonly referred to as a medicinal herb by indigenous people and is used for treating a variety of ailments such as asthma, dysentery, diuretic and traumatic pain (Mukerji and Bhandari, 1959). The extracts from the root of this plant contain a number of alkaloids, especially bisbenzylisoquinoline alkaloids (Dwuma-badu et al., 1975, Manske and Holmes, 1954). The root extracts exhibit four biological functions: antitumor (Kupchan, Patel and Fujita, 1965), antileukemia (Itokawa, Morita, Matsumoto and Takeya, 1993), diuretic (Caceres, Giron and Martinez, 1987) and a muscle relaxant (Tang and Eisenbrand, 1992). Water extracts from leaves can form gel in a short period of time but it is not the same for the extracts from the stem or root. The gel from the leaf extract is consumed as a dessert by northeastern Thais.

Polysaccharides obtained from different sources are widely used in food systems for various purposes, such as thickeners, stabilizers, gelling agents and

texture modifiers. The gelling component in Krueo Ma Noy could be another source of natural polysaccharides. However, there is little information available in the literature on the chemical composition and rheological properties of this material. Therefore, the objectives of this study were to (i) develop process of extracting the gelling material; (ii) characterize the physicochemical properties of the gelling component extracted from Krueo Ma Noy leaves.

3.2 Materials and methods

3.2.1 Preparation of plant materials

Krueo Ma Noy leaves, procured from the farmers' market in the northeast of Thailand, were cleaned with water to remove dirts and infected leaves before being dried at 60°C for 3 hours. The dried leaves were ground and stored at room temperature in a vacuum packed container until use.

Dried Krueo Ma Noy leaves were analyzed for moisture, ash, protein, lipid and dietary fiber contents according to the methods of AOAC (1997).

3.2.2 Extraction and purification

Dried Krueo Ma Noy leaves were extracted with distilled water at a solid:water ratio of 1:50. The extraction was carried out under various conditions on a high temperature range as shown in Table 3.1 using a partial factorial design to determine the effect of the three extraction variables (pH, temperature and time). The coefficients were tested for significance using SAS version 6.0 at a significance level of $p \leq 0.05$. The mixture was filtrated through a vacuum filter and centrifuged at

10,000 rpm for 15 min. The supernatant was concentrated to about half of its volume in a rotary evaporator before being precipitated with ethanol 95%(w/v) at the concentrate to ethanol ratio of 1:3 (final ethanol concentration was~ 70%). The precipitate was dried in a vacuum oven and ground to obtain a crude extract.

Yield, apparent viscosity and protein content were used as criteria for the selection of the extraction conditions offering high yield, low protein and high apparent viscosity. Apparent viscosity was measured using Viscometer models RVT at a concentration of 0.5% (w/v). Protein content was determined from nitrogen content (Nx6.25) using a CNS-2000 Elemental Analyzer (Leco corporation, St. Joseph, Michigan, USA).

The crude extract obtained under optimum conditions was solubilized in distilled water (0.1% w/v) and heated to 85°C for 1 hour, centrifuged at 10,000 rpm for 10 min at room temperature and the supernatant was dialyzed against distilled water for 3 days (15,000 molecular weight cutoff) at room temperature. The dialyzed sample was lyophilized to obtain a purified or dialyzed extract.

Table 3.1 Partial factorial experiment design for extraction.

Treatments	Study factors		
	Temperature (°C)	Time (min.)	pH
1	- (75)	- (20)	- (3)
2	+ (95)	- (20)	- (3)
3	- (75)	+ (60)	- (3)
4	+ (95)	+ (60)	- (3)
5	- (75)	- (20)	+ (5)
6	+ (95)	- (20)	+ (5)
7	- (75)	+ (60)	+ (5)
8	+ (95)	+ (60)	+ (5)
9	0 (85)	0 (40)	0 (4)
10	0 (85)	0 (40)	0 (4)
11	0 (85)	0 (40)	0 (4)
12	0 (85)	0 (40)	0 (4)
Control	Room temp. (25-28)	20	3.8-4.0

+= upper level, -= lower level and 0= center points

3.2.3 Chemical compositions

Moisture, ash, and mineral contents were determined according to the AOAC method (AOAC, 1997). Protein content was determined from nitrogen content (Nx6.25) using an Automatic Elemental Analyzer (Strada Rivoltana, Milan, Italy).

Total sugar content was determined using the method of Dubois, Gilles, Hamilton, Rebers and Smith (1956). Two millilitres of sugar solution containing between 10 and 70 μg of sugar is pipetted into a colorimetric tube, and 0.05 mL of 80% phenol is added. Then 5 mL of concentrated sulfuric acid is added rapidly. The tubes are allowed to stand for 10 minutes, then they are shaken and placed for 10 to 20 minutes in a water bath at 25 °C to 30 °C before readings are taken. A Varian Cary 3C UV-Visible spectrophotometer was used to measure the absorbance at 490 nm for hexoses.

The uronic acid content was determined using the method of Blumenkrantz and Asboe-Hansen (1973). To 0.2 mL of the sample solution containing 0.5 to 20 μg uronic acid, 1.2 mL of sulfuric acid/tetraborate was added. The tubes were refrigerated in crushed ice. The mixture was shaken in a Vortex mixture and the tubes heated in a water bath at 100°C for 5 minutes. After cooling in a water-ice bath, 20 μL of the meta hydroxybiphenyl reagent was added. The tubes were shaken and left for 5-10 minutes, absorbance measurements were taken at 520 nm in a Varian Cary 3C UV-Visible spectrophotometer.

Monosaccharide composition was analyzed following the procedure of Wood, Weisz and Blackwell (1994). Monosaccharide content was determined using a DIONEX HPLC system with pulsed amperometric detection (PAD). Samples were hydrolyzed in 12 M H_2SO_4 for 30 minutes at 30 °C, diluted with water, hydrolyzed

for 3 hours at 100°C, cooled, filtered through a 0.45 μm filter and injected onto the HPLC column and quantitatively analyzed with comparison to standards.

3.2.4 FT-IR Spectroscopy

All samples were dried and desiccated in a vacuum jar containing blue silica gel prior to FT-IR analysis. FT-IR spectra of samples were obtained using a Golden-gate Diamond single reflectance ATR on a FTS 7000 FT-IR spectrophotometer with a DTGS detector (DIGILAB, Randolph, MA). The spectra were recorded at the absorbance mode from 4000 to 400 cm^{-1} (mid infrared region) at a resolution of 4 cm^{-1} and 128 interferograms were collected to obtain a high signal to noise ratio. At least triplicate spectra readings for each sample were performed.

3.2.5 Rheological properties

All rheological properties were determined on a Bohlin CVO Rheometer (Bohlin Instruments, East Brunswick, NJ). A parallell plate geometry (40 mm diameter, 1.0 mm gap) was used for both steady shear and oscillatory measurements. Steady shear viscosity was determined at various concentrations and temperatures. The viscoelastic properties, i.e. storage modulus (G′) and loss modulus (G″), were determined through small amplitude oscillatory tests at frequencies from 0.1 to 10 Hz. Prior to any dynamic experiments, a strain sweep test at a constant frequency of 0.1 Hz was conducted to set the upper limit of the linear viscoelastic zone. All oscillatory tests were performed at a strain value of 0.02 (2%), which was within the linear viscoelastic region. A thin layer of low viscosity mineral oil was added to cover the sample in order to prevent solvent evaporation during measurements.

All crude and dialyzed extracts were solubilized in distilled water at 85°C for 1 hour to obtain solutions. The curing time experiment of 0.5 and 1% (w/v) extracts was performed at 5°C and the change in storage modulus was recorded at 2% strain and a frequency of 0.1 Hz. The hot sample solution was loaded onto the pre-heated plate of the rheometer (70°C) immediately after preparation, and the temperature was rapidly lowered to 5°C.

Changes in viscoelastic properties of 0.5% (w/v) solution of crude and dialyzed extracts due to pH variation, in the range of 2-8 (adjusted with HCl or NaOH), NaCl, $CaCl_2$ and sucrose were examined.

3.3 Results and discussion

3.3.1 Extraction, purification and chemical compositions

Dried Krueo Ma Noy leaves contained, on average, 6.43% moisture, 11.18% ash, 6.78% protein, 2.22% lipid and 65.98% total dietary fiber. Extraction at a high temperature range in Table 3.2 showed that high yield but low viscosity were obtained with various protein contents of no significant difference among each other, whereas extraction at control conditions of room temperature, natural pH (3.8-4.0) and short extraction time (20 min) gave a significantly higher viscosity. Then, the extraction procedure was varied in extraction temperature at natural pH and lower extraction time. The results (Table 3.3) showed that lower extraction temperatures gave higher viscosity and greater gel strength. Moreover, the extraction procedure was varied in extraction time at natural pH and a lower extraction temperature (room temperature). The results were shown in Table 3.4.

It showed that a high extraction time (60 min) gave apropos to acquire high viscosity and gel strength. Based on functional properties, extraction at room temperature of natural pH for 60 min would be preferably considered as a suitable method of extraction. Therefore, the crude extract obtained from room temperature extraction was used for preparation of the dialyzed extract.

The chemical and sugar compositions of crude extract as well as the dialyzed extract of Krueo Ma Noy leaves were presented in Table 3.5. The yield of the dialyzed extract was about 85% of the crude one. The protein content of the crude extract was significantly reduced after dialysis. The dialysis process appeared not to change the monosaccharide profile, however, the total uronic acid content was increased from 70.56% in the crude extract to 75.93% in the dialyzed extract. The dialysis process also significantly reduced the level of potassium. In contrast, the levels of calcium and sodium were increased after dialysis, indicating strong binding properties of the two cations with the acidic polysaccharides. The preliminary composition analysis indicated that the polysaccharides extracted from Krueo Ma Noy leaves was a pectin.

Table 3.2 Effects of extraction conditions on yield, viscosity and protein of Krueo Ma Noy extract.

Treatment	Studied factor			Results		
	Temp(°C)	Time(min)	pH	Yield(%)	Viscosity(centipoise)*	Protein(%)**
1	75	20	3	27.16±1.00^{ab}	279.47±0.92^{b}	1.96±0.52^{b}
2	95	20	3	28.92±3.22^{a}	48.17±0.29^{c}	2.45±0.57^{ab}
3	75	60	3	26.52±0.49^{ab}	116.80±5.54^{d}	1.80±0.42^{ab}
4	95	60	3	26.65±0.51^{ab}	29.33±0.92^{c}	2.05±0.39^{b}
5	75	20	5	25.94±0.97^{ab}	278.67±2.31^{b}	1.89±0.36^{b}
6	95	20	5	26.18±0.80^{ab}	48.33±0.58^{c}	2.37±0.49^{ab}
7	75	60	5	24.50±1.36^{ab}	113.60±5.54^{d}	2.49±0.47^{ab}
8	95	60	5	27.11±1.71^{ab}	27.20±1.39^{c}	2.48±0.42^{ab}
9	85	40	4	25.52±2.28^{ab}	93.33±0.92^{d}	1.79±0.31^{b}
10	85	40	4	26.15±4.02^{ab}	92.93±2.54^{d}	1.84±0.19^{b}
11	85	40	4	25.78±2.07^{ab}	93.87±0.92^{d}	1.74±0.20^{b}
12	85	40	4	25.84±3.18^{ab}	93.33±0.92^{d}	1.74±0.43^{b}
control	RT	20	3.8-4.0	21.54±1.87^{b}	681.07±3.37^{a}	3.17±0.26^{a}

* 0.5% sample (w/v) using spindle number 2 speed 50 rpm.
The viscosities of samples were measured by viscometer model RVT.

** Protein content was determined from nitrogen content (Nx6.25) using a CNS-2000 Elemental Analyzer (Leco corporation, St. Joseph, Michigan, USA).

^{a,b,c,d} Values are means of triplicate measurements.
Values with different letters in each column are significantly ($p \leq 0.05$) different from each other.

Table 3.3 Effects of extraction temperature at pH 3.8 and 20 min of extraction time on yield, viscosity and gel strength of Krueo Ma Noy extract.

Treatment	Studied factor			Results		
	pH	Time(min)	Temp(°C)	Yield(%)	Viscosity(centipoise)*	Max. force (g)**
1	3.8	20	28	21.24±0.59^{a}	686.67±30.55^{a}	297.80±6.94^{a}
2	3.8	20	35	21.25±1.00^{a}	667.33±11.02^{a}	282.97±8.73^{b}
3	3.8	20	45	22.29±0.88^{ab}	413.33±4.62^{b}	116.43±3.06^{c}
4	3.8	20	55	22.46±1.39^{ab}	306.67±4.62^{c}	38.07±2.15^{d}
5	3.8	20	65	23.08±2.21^{ab}	269.33±4.62^{cd}	27.17±2.60^{de}
6	3.8	20	75	23.85±2.51^{b}	245.33±4.62^{d}	18.40±2.30^{e}

* 0.5% sample (w/v) using spindle number 2 speed 50 rpm.

The viscosities of samples were measured by viscometer model RVT.

** 1% sample (w/v) and 30% sugar (w/v)

The gel strength (maximum force) of the gels were measured by texture analyzer model TA.XT2i. (using cylinder probe: ∅ 1/2" ∅ delrin AOAC for gelatin. Code: P/0.5 and speed 0.5 mm/sec).

^{a,b,c,d,e} Values are means of triplicate measurements.

Values with different letters in each column are significantly ($p \leq 0.05$) different from each other.

Table 3.4 Effects of extraction time at pH 3.8 and extraction temperature 28°C on yield, viscosity and gel strength of Krueo Ma Noy extract.

Treatment	Studied factor			Results		
	pH	Temp(°C)	Time(min)	Yield(%)	Viscosity(centipoise)*	Max. force(g)**
1	3.8	28	20	21.09±0.24[b]	704.00±16.00[b]	286.23±7.67[a]
2	3.8	28	40	23.43±0.40[b]	1043.33 ±35.12[a]	296.23±6.45[a]
3	3.8	28	60	26.77±1.78[a]	1066.67 ±30.55[a]	305.17±5.42[a]

* 0.5% sample (w/v) using spindle number 2 speed 50 rpm for treatment 1.

0.5% sample (w/v) using spindle number 3 speed 50 rpm for treatment 2 and 3.

The viscosities of samples were measured by viscometer model RVT.

** 1% sample (w/v) and 30% sugar (w/v)

The gel strength (maximum force) of the gels were measured by texture analyzer model TA.XT2i. (using cylinder probe: ∅ 1/2" ∅ delrin AOAC for gelatin. Code: P/0.5 and speed 0.5 mm/sec).

[a,b,c,d] Values are means of triplicate measurements.

Values with different letters in each column are significantly (P≤0.05) different from each other.

Table 3.5 Chemical composition of crude and dialyzed extracts.

Composition (%dry basis)	Crude extract	Dialyzed extract
Moisture	10.57±0.54	8.53±0.39
Ash	9.86±0.01	8.40±0.69
Protein	3.19±0.10	0.29±0.07
Total sugar (as glucose)	9.06±0.02	8.56±0.01
Uronic acid (as galacturonic acid)	70.56±0.10	75.93±0.83
Monosaccharide		
Rhamnose	1.07±0.00	1.07±0.00
Arabinose	0.60±0.01	0.51±0.00
Galactose	0.78±0.01	0.64±0.01
Glucose	0.61±0.03	0.32±0.01
Xylose	0.28±0.00	tr
Mannose	0.58±0.02	0.56±0.00
Minerals		
Na	0.16±0.00	0.26±0.00
K	3.90±0.00	1.60±0.00
Ca	1.40±0.00	2.70±0.00
Mg	0.90±0.00	0.71±0.00
Fe (mg/g)	0.03±0.00	0.03±0.00
Zn (mg/g)	0.12±0.00	0.14±0.00

tr = trace amount

3.3.2 FT-IR spectroscopy

FT-IR spectra in the wave number between 950-1200 cm^{-1} is considered as the "finger print" region for carbohydrates which allows the identification of major chemical groups in polysaccharides: the position and intensity of the bands are specific for every polysaccharide (Cěrná *et al*., 2003, Kalapathy and Proctor, 2001). In order to confirm the identity of Krueo Ma Noy extracts, both crude and dialyzed extracts samples were analyzed by FT-IR and their spectra were compared with three commercial pectin standards (Figure 3.1). It was found that the FT-IR spectra of both crude and dialyzed extracts exhibited similarities to that of the commercial pectin standard, confirming the conclusion from the chemical composition analysis that the extract from Krueo Ma Noy leaves is a pectin.

In addition to the broader band of absorption between 3600 and 2500 cm^{-1} due to O-H stretching, strong absorbance were observed at 1730-1760 cm^{-1} and 1600-1630 cm^{-1}, which were caused by the ester carbonyl (C=O) groups and carboxyl ion stretching band (COO^-), respectively (Kamnev, Colina, Rodriguez, Ptitchkina and Ignatov, 1998, Silverstein, Bassler and Morril, 1991). Carboxylate groups show two bands, an asymmetrical stretching band near 1550-1650 cm^{-1}, and a weaker symmetric stretching band near 1400 cm^{-1}. In pectin samples, the weaker symmetric COO^- stretching is followed by moderately intense absorption patterns between 800-1300 cm^{-1}, collectively referred to as the finger print region that is unique to a compound. These bands are usually difficult to interpret. Other bands of lesser importance in pectin samples are C-H bending, occurring at 1380 cm^{-1} and C=O stretching occurring at 1000-1300 cm^{-1}. In order to determine if Krueo Ma Noy pectin is a low methoxy pectin or a high methoxy pectin, the FT-IR spectra of both

crude and dialyzed extracts were compared against three commercial citrus pectins at various degree of esterification (DE) (P9311:26%DE, P9436:59%DE and P9561:94%DE, Sigma Chemical Co., Steinhem, Germany). The intensity of the absorbance of the ester carbonyl groups (1730-1760 cm^{-1}) increased with an increase of the degree of esterification (Figure 3.2), corresponding to the increase of ester carbonyl absorbance, the intensity of the carboxyl stretching band decreased (Chatjigakis, Pappas, Proxenia, Kalantzi, Rodis and Polissiou, 1998, Filippov, 1992, Manrique and Lajolo, 2002). It can be concluded that the pectin extracted from Krueo Ma Noy leaves is a low methoxy pectin and the degree of esterification of the dialyzed extract is lower than the crude extract. The observed results also suggest that the dialysis process removed some higher methoxy pectin moieties with smaller molecular weight.

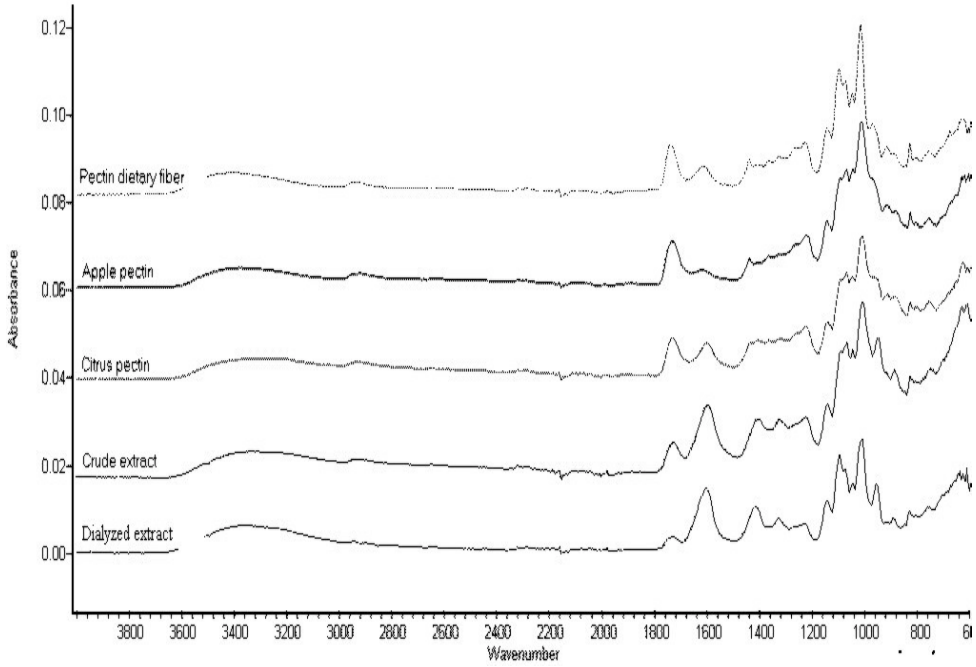


Figure 3.1 Fourier transform infrared spectra of crude and dialyzed extracts from Krueo Ma Noy leaves and pectins (apple pectin, citrus pectin and pectin dietary fiber control).

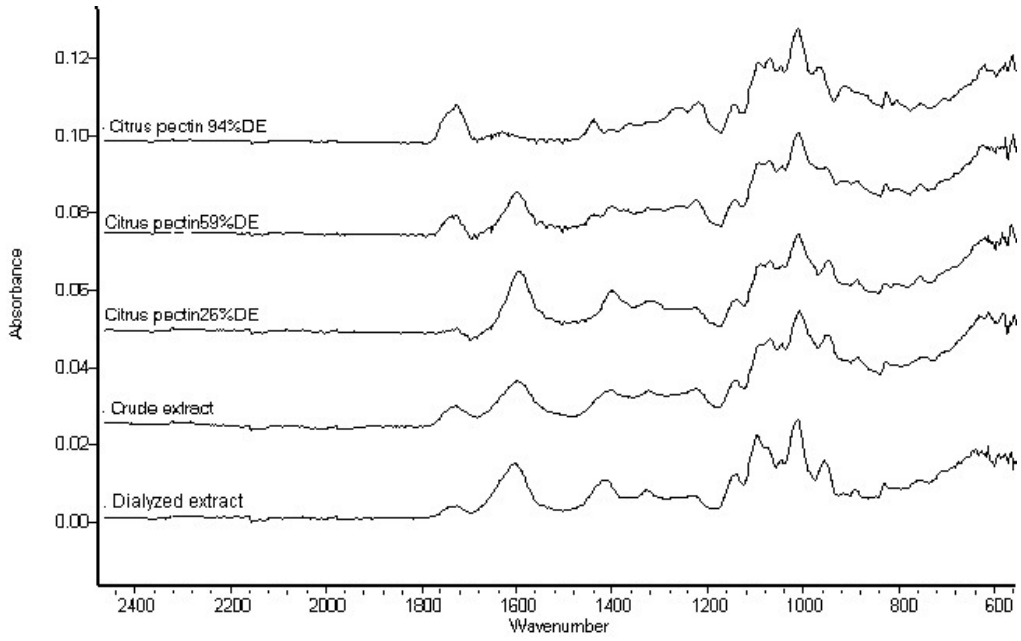


Figure 3.2 Fourier transform infrared spectra of commercial pectin standards and Krueo Ma Noy pectins (crude and dialyzed extracts).

3.3.3 Rheological properties

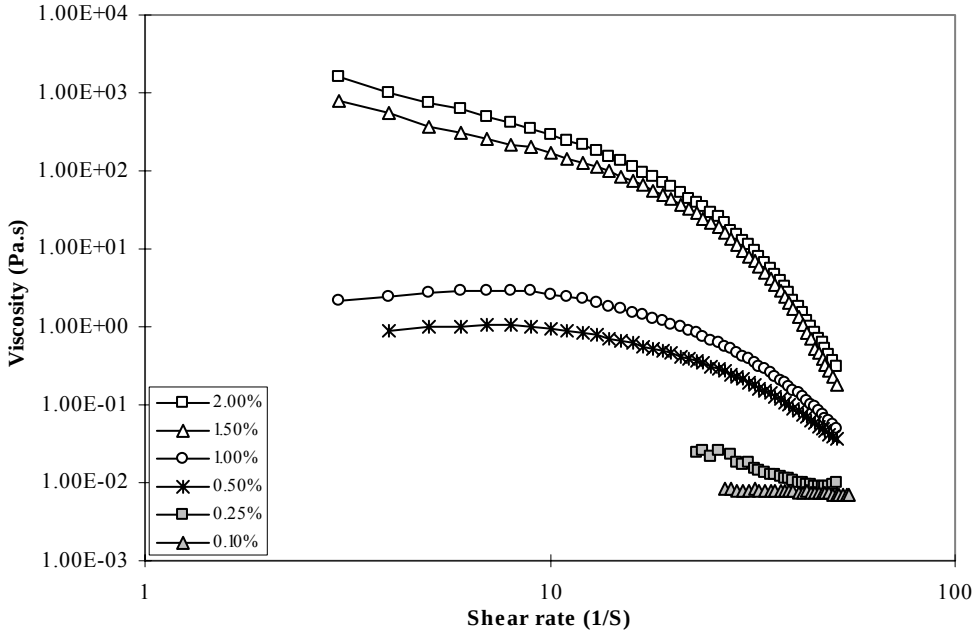
3.3.3.1 Flow behavior

Effects of concentration:

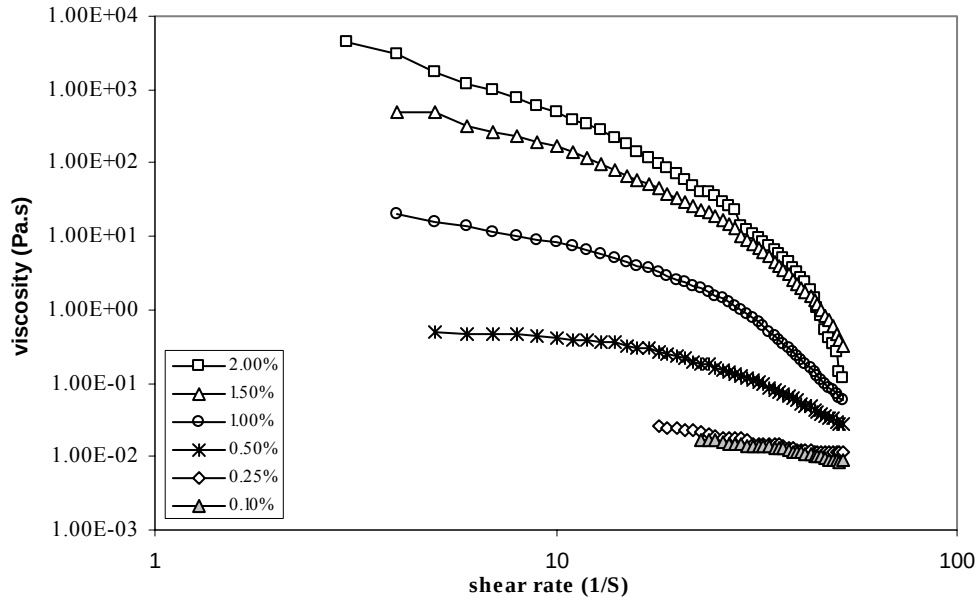
The flow curves of crude and dialyzed extracts obtained at concentrations of 0.1-2% (w/v) at 25°C showed a Newtonian flow behavior at concentrations below 0.5% (w/v) and shear thinning (pseudoplastic) flow behavior at higher concentrations (Figure 3.3a and b). The extent of shear thinning increased with pectin concentration and shear rate.

Effects of temperature:

The crude extract showed a Newtonian flow behavior at 65°C and shear thinning flow behavior at a lower temperature (Figure 3.4a). On the other hand, the dialyzed extract showed shear thinning behavior at all temperatures (Figure 3.4b), which might reflect the effective concentration of pectin in the sample. The dialyzed extract contained a higher amount of pectin than the crude extract when they were prepared with the same concentration.



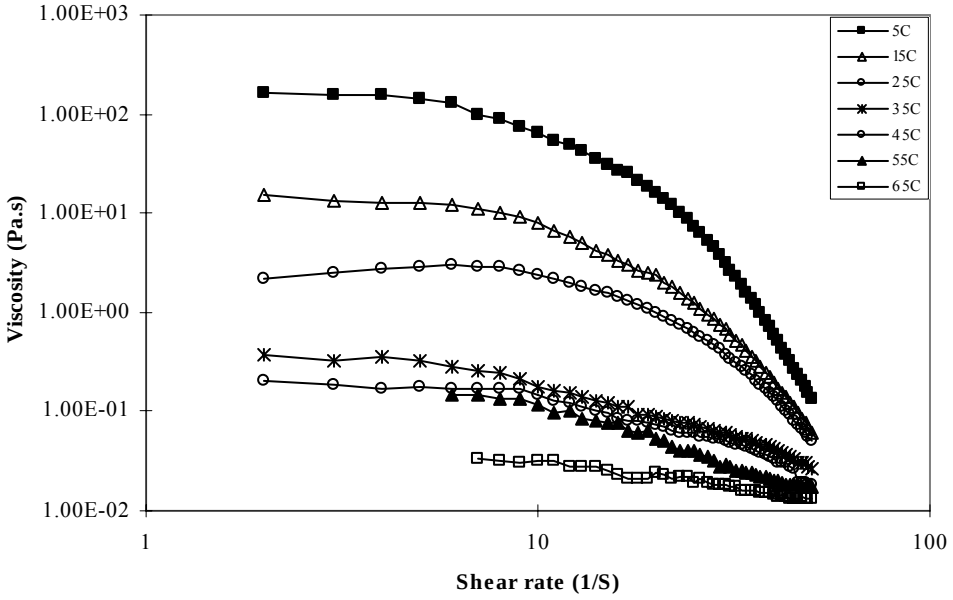
(a)



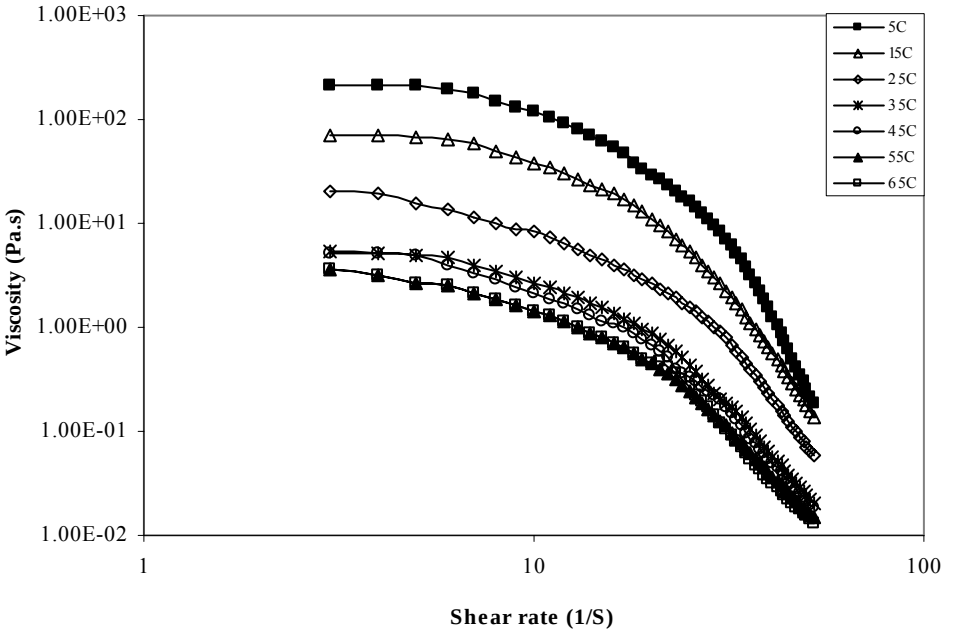
(b)

Figure 3.3 Steady shear flow for different concentrations at 25°C.

(a) Crude extract (b) Dialyzed extract



(a)



(b)

Figure 3.4 Steady shear flow for different temperatures at a concentration of 0.5%(w/v).

(a) Crude extract (b) Dialyzed extract

3.3.3.2 Viscoelastic behavior

Effects of concentration:

The viscoelastic properties of Krueo Ma Noy pectin were examined by oscillatory experimental measurements (Figure 3.5). The storage modulus increased with increasing pectin concentration and decreasing temperature. When polymer concentrations were increased to 0.5%(w/v), the mechanical spectrum at 25°C was typical of a semi-dilute to concentrated solution where the loss modulus was higher than the storage modulus at low frequencies and the reverse was observed at higher frequencies (Figure3.6a and b). It was worth noting that at the same polymer concentration, the moduli of the dialyzed extract was much higher than that of the crude extract, although the nature of the mechanical spectrum remained the same. However, the mechanical spectra of both dialyzed and crude extracts samples changed significantly when they were measured at 5°C, both mechanical spectra behaved like a gel. In order to evaluate the gelling properties of Krueo Ma Noy pectin, both the crude and dialyzed extracts were monitored on the rheometer at a constant temperature of 5°C (Figure 3.7). During the aging process, G′ continued to increase with increasing time, and then started to plateau at~50 min for the 1%(w/v) crude extract and ~40 min for the 1%(w/v) dialyzed extract. For the 0.5%(w/v) Krueo Ma Noy pectin, both the crude and dialyzed extracts, G′ increased with increasing time, and then started to plateau around 90 min. Further, magnitudes of G′ were strongly dependent on pectin concentrations, they increased with increasing pectin concentration, presumably due to an increase in the number of junction zones. The mechanical properties of the Krueo Ma Noy gels were examined at two temperatures after gel formation at 4°C for at least 12 hours (Figure 3.8). At both 5°C and 25°C,

the crude extract exhibited a strong gel structure when its storage modulus G′ was much greater than the corresponding loss modulus G″ and the two moduli were independent of frequency. The storage modulus increased with increasing pectin concentration and decreasing temperature. The dialyzed Krueo Ma Noy pectin exhibited similar rheological behavior, but the gel strength was much stronger at 2% (w/v). The stronger gel strength of the dialyzed pectin is consistent with the previous observation of steady shear measurement suggesting a higher effective polymer concentration and/or higher polymer molecular weight.

Thus by the normal criteria of biopolymer network rheology the mechanical response obtained from Krueo Ma Noy extracts at room temperature showed a smooth progression from a solution-like response at a concentration of 0.1%(w/v) to typical gel-like properties at a concentration of 1.0%(w/v) and above. At 0.5% polymer solutions, a viscoelastic flow behavior was observed at 25°C and a gel structure obtained at 5°C.

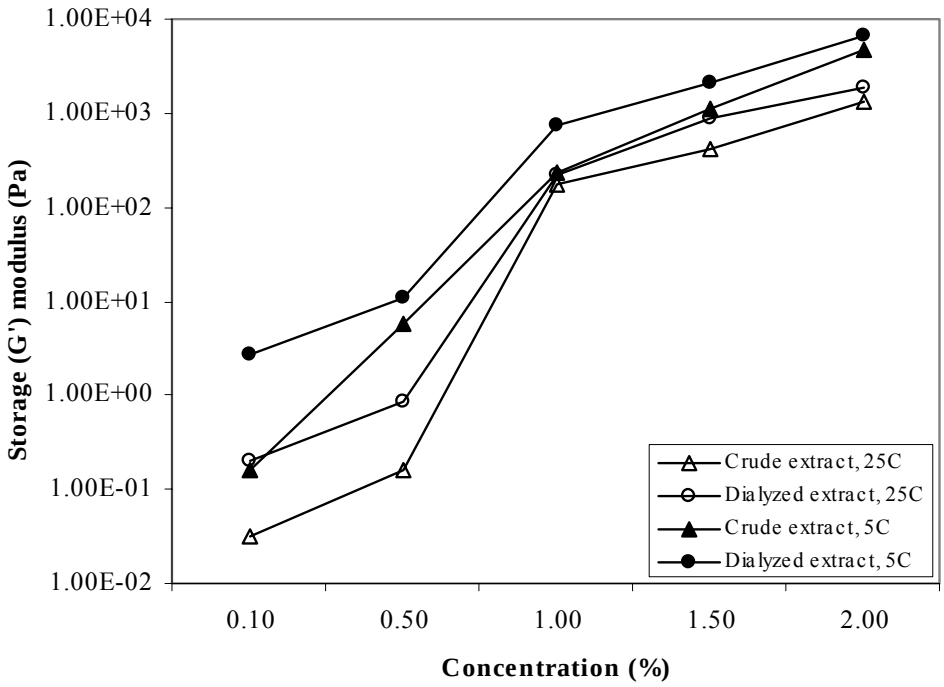
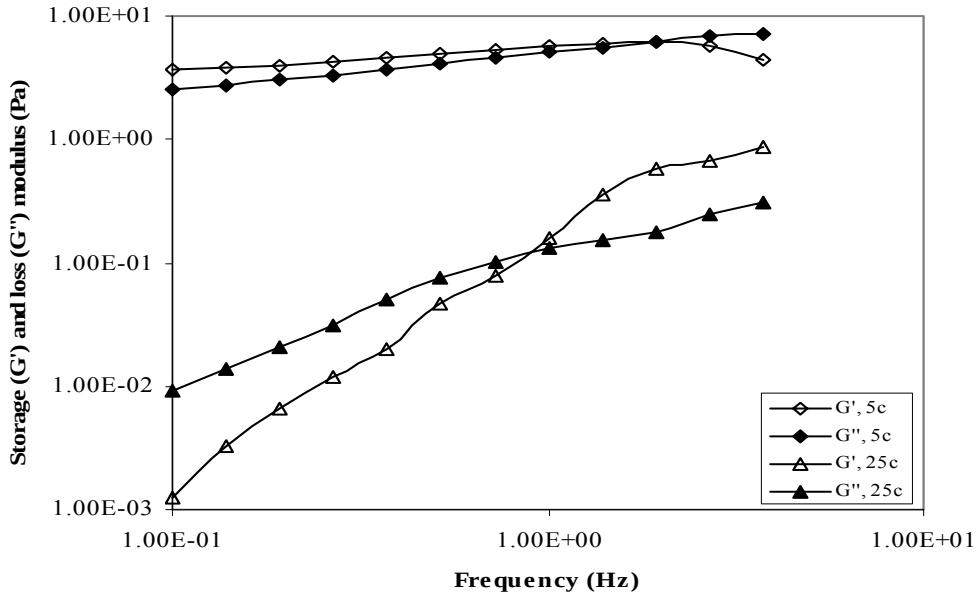
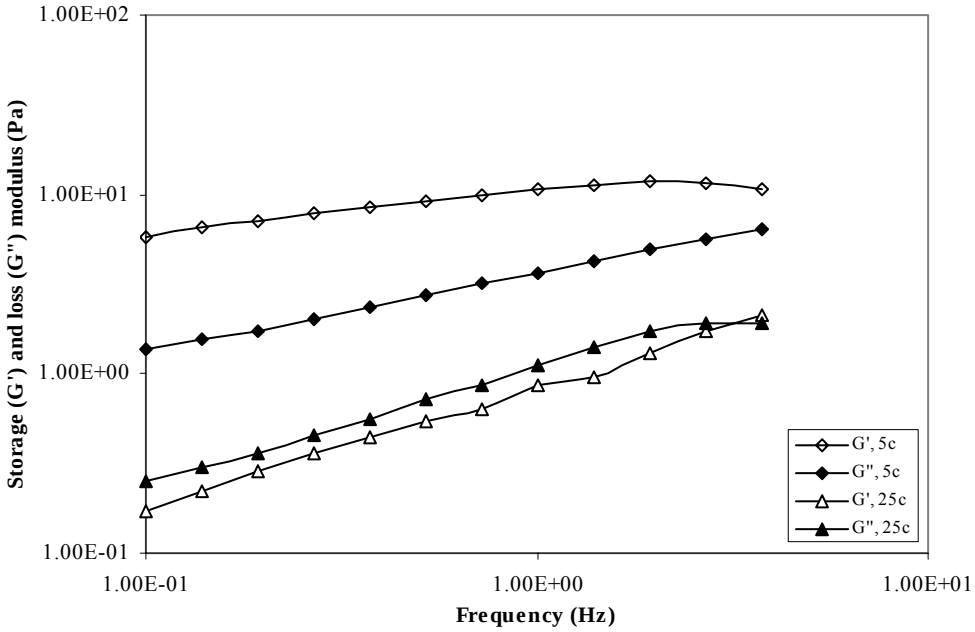


Figure 3.5 Storage modulus (G′) at 1 Hz for different concentrations of crude and dialyzed extracts at 25°C and 5°C.



(a)



(b)

Figure 3.6 Frequency dependence of storage (G′) and loss (G″) modulus at 25°C and 5°C. (a) 0.5% Crude extract (b) 0.5% Dialyzed extract

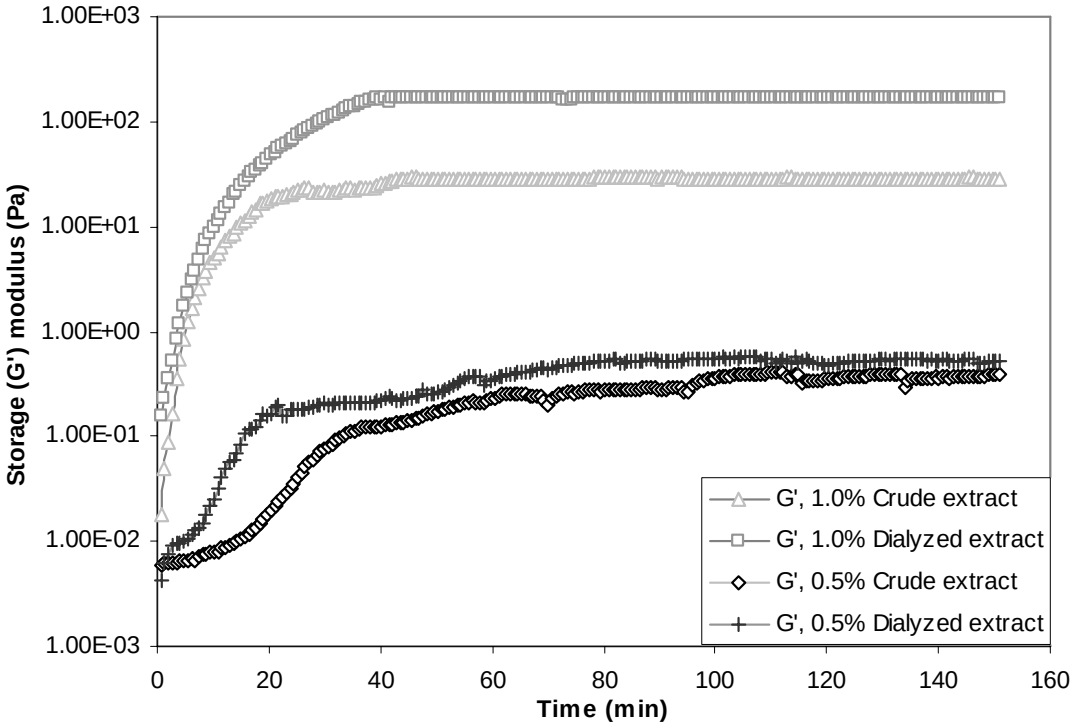
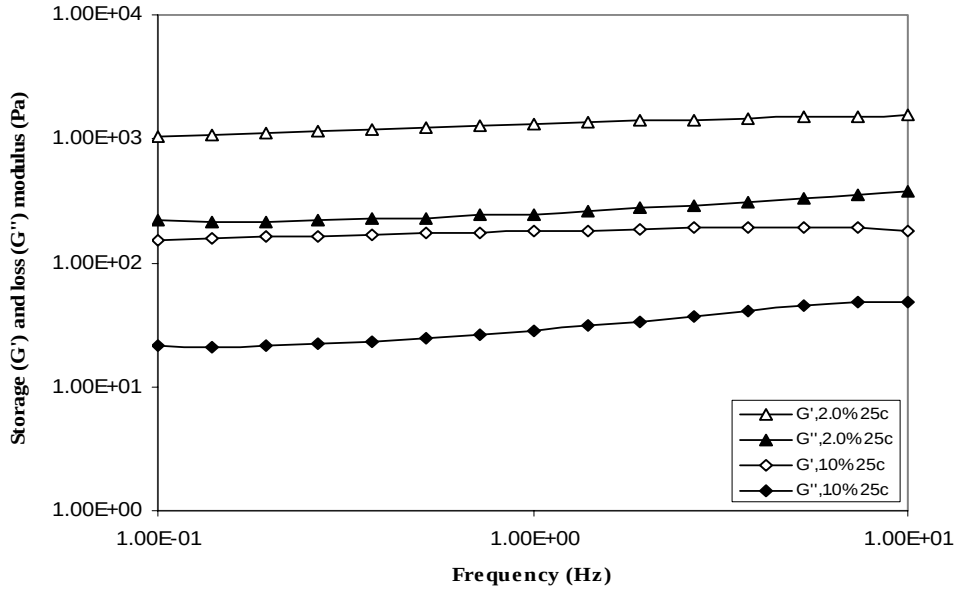
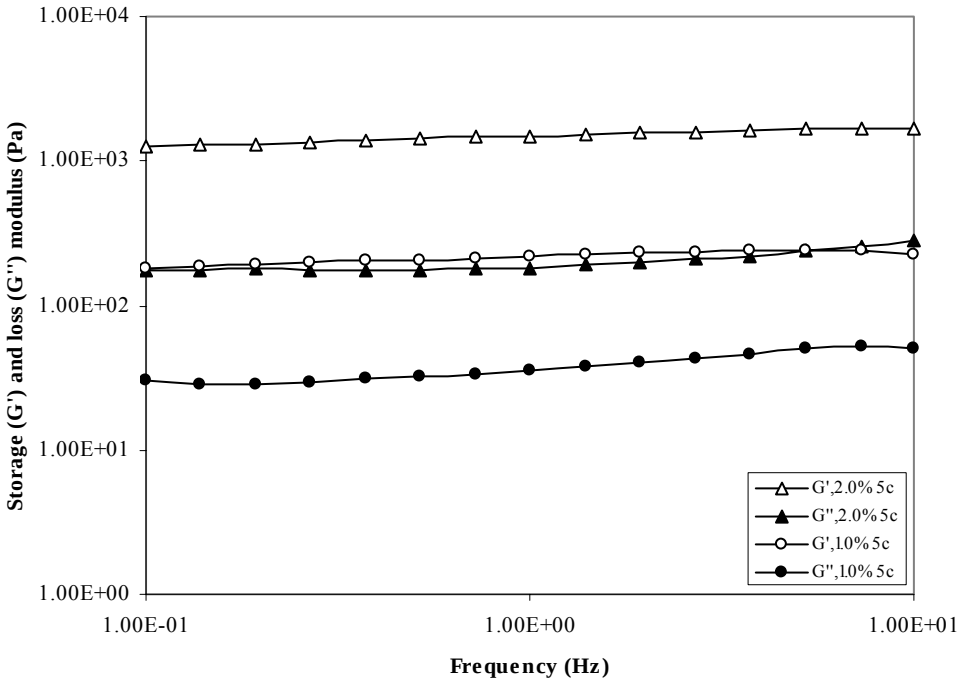


Figure 3.7 Evolution of storage modulus (G′) with time of crude and dialyzed extracts at 5°C



(a)



(b)

Figure 3.8 Frequency dependence of storage (G′) and loss (G″) modulus of 1% and 2 % (w/v) of the crude extract. (a) 25°C (b) 5°C

Effects of pH:

Decreasing the pH causes an increase in the G′ values (Figure 3.9). In the acidic region (pH 2-4), the storage modulus showed a tendency to a constant value to form gel. A similar argument was given by Gilsenan, Richardson and Morris (2000) to explain the acid-induced gelation of low methoxy pectin. The effect of further reduction of the pH was to reduce the charge density of the pectin chain thereby reducing electrostatic repulsion. On the other hand, at the pH range of 5-8 at a polymer concentration of 0.5% (w/v), the mechanical responses were predominantly liquid-like behavior.

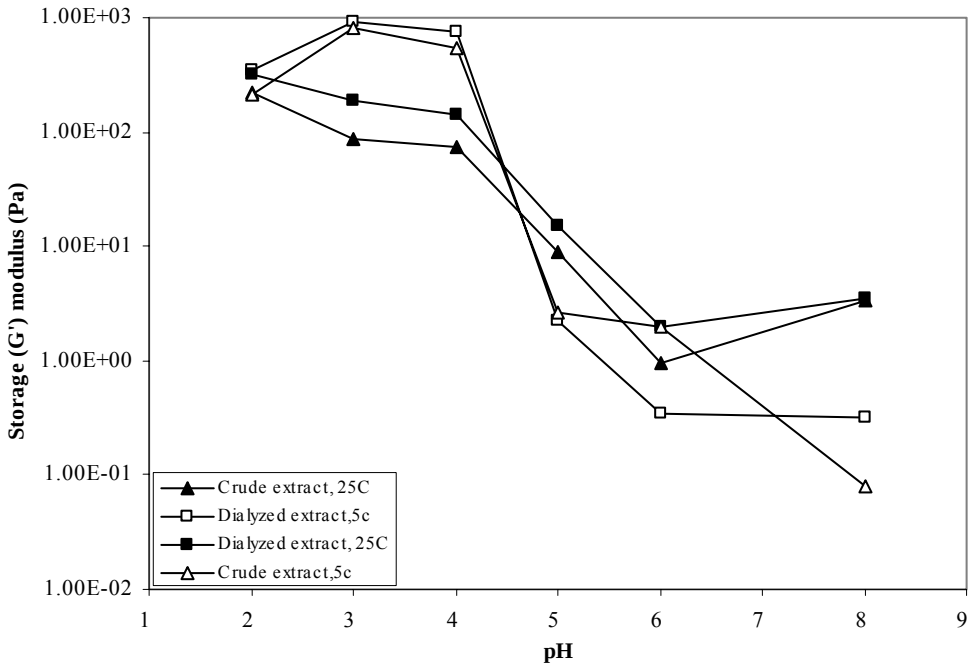
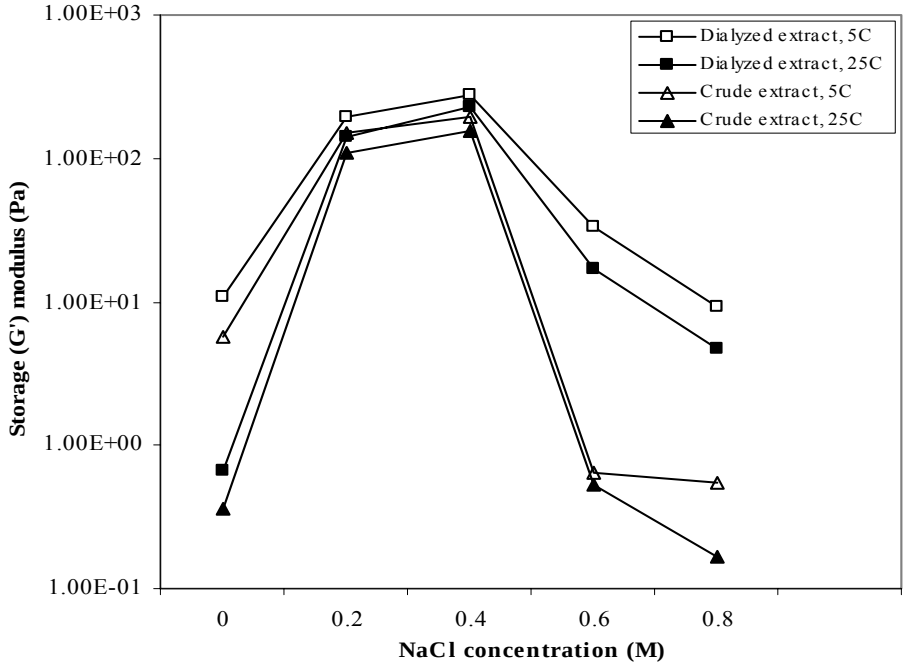


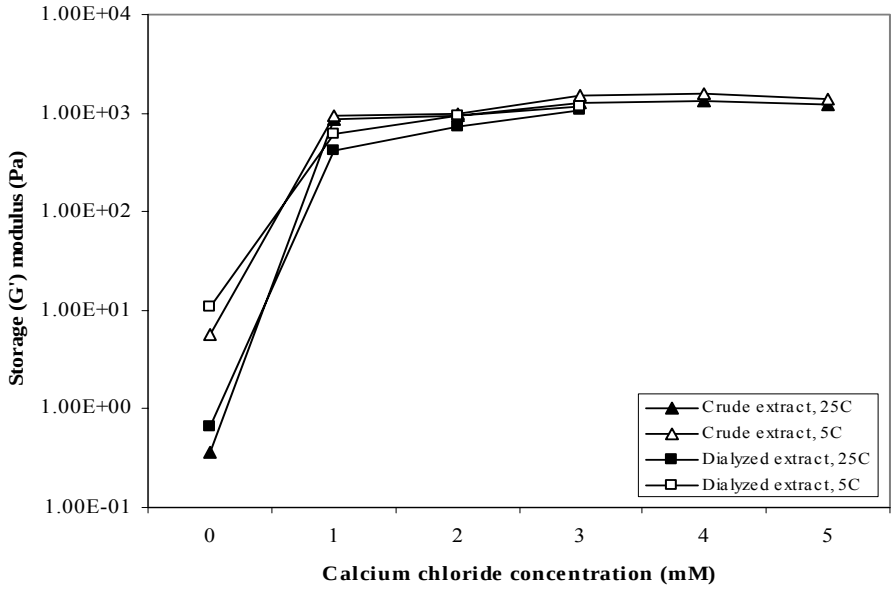
Figure 3.9 Effects of pH on the storage modulus (G′) at 1 Hz of 0.5%(w/v) crude and dialyzed extracts.

Effects of co-solutes:

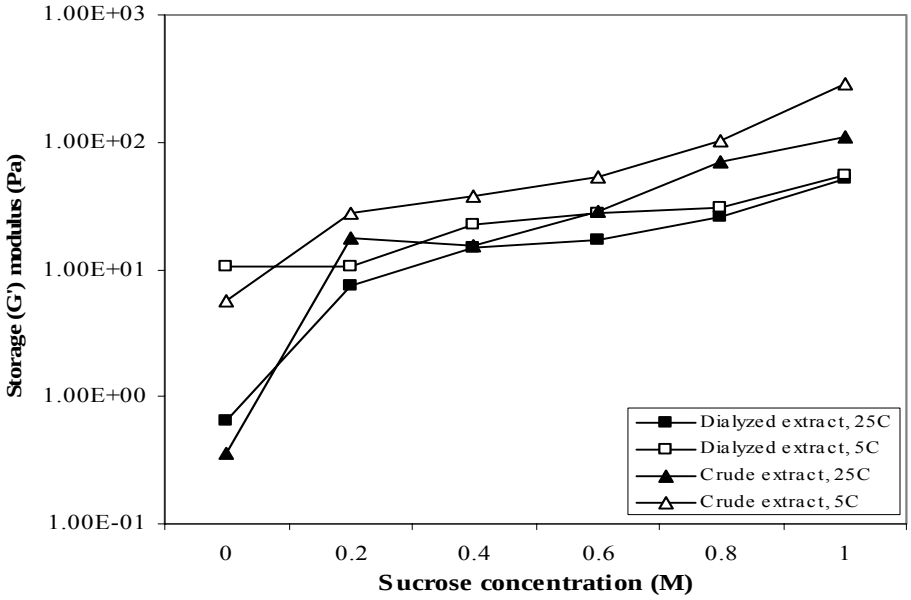
The effect of ionic strength and ion types on viscoelastic behavior is important not only to determine whether the sample behaves as a polyelectrolyte but also to estimate its functional rheological properties (Medina-Torres, Brito-De La Fuente, Torrestiana-Sanchez and Katthain, 2000). The solutions formed gel at NaCl concentrations between 0.2 and 0.4 M whereas the others did not gel (Figure 3.10a). The effect of $CaCl_2$ concentration (divalent cation) on the storage modulus of the crude extract solution showed gel formation between 1 and 5 mM whereas the dialyzed extract solution showed gel formation between 1 and 3 mM (Figure 3.10b). It should be noted that at concentrations higher than 3 mM $CaCl_2$, the dialyzed extract solution formed an aggregate resulting in precipitation. Thus, the dialyzed extract was very sensitve to Ca^{2+}. Ca^{2+}-pectin gels can be formed only in a narrow range of Ca^{2+} concentrations between 1 and 3 mM. The Ca^{2+} content in the dialyzed extract was about twice of that in the crude extract. The viscoelastic behavior was more dependent on Ca^{2+} ion than on Na^{+} ion (Figure 3.10a and b). The divalent cation showed a more pronounced effect on the viscoelastic properties of the extracts than the monovalent cation. The storage modulus increased with increasing sucrose content of both the crude and dialyzed extracts (Figure 3.10c).


1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.00E-01
Storage (G') modulus (Pa)
0
0.2
0.4
0.6
0.8
NaCl concentration (M)
Dialyzed extract, 5C
Dialyzed extract, 25C
Crude extract, 5C
Crude extract, 25C

(a)


1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.00E-01
Storage (G') modulus (Pa)
0
1
2
3
4
5
Calcium chloride concentration (mM)
Crude extract, 25C
Crude extract, 5C
Dialyzed extract, 25C
Dialyzed extract, 5C

(b)



(c)

Figure 3.10 Effects of co-solutes on the storage modulus (G′) at 1 Hz of 0.5%(w/v) crude and dialyzed extracts:

(a) sodium chloride (b) calcium chloride

(c) sucrose

3.4 Conclusion

The crude and dialyzed extracts of Krueo Ma Noy leaves were found to consist mainly of pectic-like polysaccharides containing high galacturonic acid. The FT-IR spectra in the region 1200-950 cm^{-1} allowed the prediction of neutral sugars and uronic acid in the pectic polysaccharides. The carbonyl absorption bands at 1600-1630 and 1730-1760 cm^{-1} were from free and esterified carboxyl groups of pectin, respectively. The crude and dialyzed extracts exhibited gelling properties in aqueous solution at 1.0% (w/v). Diluted solutions of Krueo Ma Noy pectin exhibited Newtonian behavior whereas Shear-thinning behavior occurred at a concentration of ≥ 0.5% (w/v). The extracts at a concentration of 0.5% (w/v) showed gelation in the acidic region (pH 2-4). Lowering the pH of Krueo Ma Noy pectin gels below pH 4 led to a small increase of the modulus. On the other hand, the mechanical responses displayed liquid-like behavior in the pH range of 5-8. The effect of NaCl concentration on the viscoelastic behavior of the extracts (0.5%,w/v) showed gel formation between 0.2 and 0.4 M. The crude extract formed gel at $CaCl_2$ concentrations between 1 and 5 mM whereas the dialyzed extract showed gel formation between 1 and 3 mM, higher Ca^{2+} concentration resulted in precipitation. The divalent cation (Ca^{2+}) showed a more pronounced effect on viscoelastic behavior than monovalent cation (Na^{+}) did. The storage modulus increased with increasing sucrose content of both the crude and dialyzed extracts.

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Chapter IV

Structural Characterization and Determination of Degree of Esterification of Krueo Ma Noy Pectin

Abstract

Pectins extracted from Krueo Ma Noy (*Cissampelos pareira*) leaves consisted mainly of galacturonic acid with trace amounts of neutral sugars. The dominant structure of Krueo Ma Noy pectin was established as a α-1,4-link-galacturonan by a combination of carboxyl reduction and methylation analysis and this structure was confirmed by FT-IR and NMR spectroscopy. The degree of esterification of Krueo Ma Noy pectins was 41.65% and 33.69% for crude and dialyzed pectins, respectively. Krueo Ma Noy pectin had an average molecular weight of 55 KDa, a radius of gyration of 15.2 nm and an intrinsic viscosity of 2.3 dL/g.

Keywords: Krueo Ma Noy pectin; *Cissampelos pareira*; FT-IR spectroscopy; degree of esterification; HPSEC

4.1 Introduction

Pectin is a complex heterogeneous polysaccharide found in the primary cell walls of most plants, in which it provides mechanical strength and flexibility due to its interaction with other cell wall components. The dominant structural feature of pectin is a linear chain of poly-α-(1-4)-D-galacturonic acid with varying degrees of esterification of the carboxylic groups. However the fine structures of pectins vary substantially on the amount of α-(1-2)-linked-L-rhamnosyl residues in the backbone chain and the presence of branches consisting of arabinan and galactan. In the food industry, pectin is an important gelling agent and thickener. Gelling properties and mechanisms of pectins are primarily dependent on degree of esterification and molecular weight of the polymer (Manrique and Lajolo, 2002, Barros *et al*., 2002).

Krueo Ma Noy, *Cissampelos pareira,* is a woody climbing vine from the family of *Menispermaceae*. It is found throughout the warm regions of Asia, East Africa and America (Smitinand and Larsen, 1991). It is widespread in the northeast of Thailand. Krueo Ma Noy leaves (up to 30 cm in length) are commonly used as a herb by the indigenous people due to their analgesic properties and they have been used for many years for ailments of women. The dark green gel formed after cold extraction of the leaves with water has been used by the indigenous people as a cooling medicine for treating fever. Local medicine also uses this plant for a number of ailments such as asthma, dysentery, diuretic and treatment of traumatic pain (Mukerji and Bhandari, 1959). It is interesting to note that the formation of the gel occurs in a very short period of time after water extraction from the leaf. Chapter 3

has recently shown that the polysaccharide responsibility for the gelation of Krueo Ma Noy extract is a pectin but the structure of this pectin has not been determined.

Determination of pectin structures is the first step toward understanding of its functionality. The monosaccharide composition and linkage patterns are primary information of the structure. Enzymatic hydrolysis, NMR and FT-IR spectroscopy have proven to be effective methods for determining the structure of polysaccharides, including pectins (Voragen, Pilnik, Thibault, Axelos and Renard, 1995). The degree of esterification, the percentage of the total number of carboxyl groups esterified, has a significant effect on the gel strength and gelling mechanisms of pectins (Walter, 1991). Several methods have been reported in the literature to determine the degree of esterification, e.g. a titrimetric method adopted by Food Chemical Codex (FCC, 1981) and USP26 NF21 (2003). The degree of esterification of pectins can also be determined by means of instrumental methods, such as HPLC (Plöger, 1992, Levigne, Thomas, Ralet, Quemener and Thibault, 2002) and ^{1}H-NMR spectroscopy (Grasdalen, Bakϕy and Larsen, 1988). Recently, Fourier transform infrared spectroscopy has become a preferred technique for determining the degree of esterification due to its ease of use and non-destructive mechanism (Filippov, 1992, Manrique and Lajolo, 2002). In addition, FT-IR spectroscopy has become a powerful research tool to provide structural information about plant cell walls. The objectives of this chapter are to determine the structural features, degree of esterification and molecular characteristics of Krueo Ma Noy pectin.

4.2 Materials and methods

4.2.1 Preparation of standard samples

Pectin standards with a known degree of esterification, 26%, 59% and 94%, were obtained from SIGMA (Steinheim, Germany). Subsequent pectin standards with a known degree of esterification, such as 42.5% and 76.5%, were prepared from these three commercial standards.

4.2.2 Preparation of pectin from Krueo Ma Noy

Krueo Ma Noy leaves, procured from markets in the northeast of Thailand, were cleaned with water to remove dirts and infected leaves then dried at 60°C for 3 hours. The dried leaves were ground and stored at room temperature in a vacuum-packed PP bag. Krueo Ma Noy pectins were prepared according to the method described previously in Chapter III. The extraction conditions were 2% solids in distilled water at 25°-28°C and natural pH (3.8-4.0). Alcohol precipitation and drying produced a crude extract and further dialysis and lyophilization produced a purified or dialyzed extract.

4.2.3 Enzyme assay for identification of pectin

The identification to confirm of pectins from Krueo Ma Noy leaves extract was carried out using an assay available in a kit from Megazyme (Megazyme International Ireland Ltd., Ireland). Sample was dissolved in deionized water, and adjusted to pH 12 to catalyze the demethylation of the esterified galacturonic acid. The pectate was then incubated with pectate lyase which cleaved the polygalacturonic

acid and released unsaturated oligosaccharides to be observed at the absorbance of 235 nm.

The amount of unsaturated product produced was calculated as:

Blank Absorbance = Enzyme Blank + Sample Blank

ΔAbsorbance = Reaction Absorbance – Blank Absorbance

Unsaturated product = ΔAbs x 1/L x 1/ε

where ΔAbs is the change of reaction absorbance minus blank absorbance measured after 30 min, L is the cuvette path length (=1 cm.) and ε is the molar extinction coefficient of the reaction product (4600 $M^{-1}cm^{-1}$). A value of unsaturated product more than $0.5x10^{-5}$ Molar indicates the presence of pectin and conversely an unsaturated product concentration less than $0.5x10^{-5}$ Molar indicates the absence of pectin (Hansen, Thuesen and Soderberg, 2001).

4.2.4 Methylation and GC-MS of Partially Methylated Alditol Acetate (PMAA)

Methylation analysis of pectic substances has been a difficult task due to the present of large quantities of uronic acids. In this study, the uronic acid was reduced into neutral sugars then normal methylation analysis was carried out for neutral sugars. The two steps were described in the following subsections.

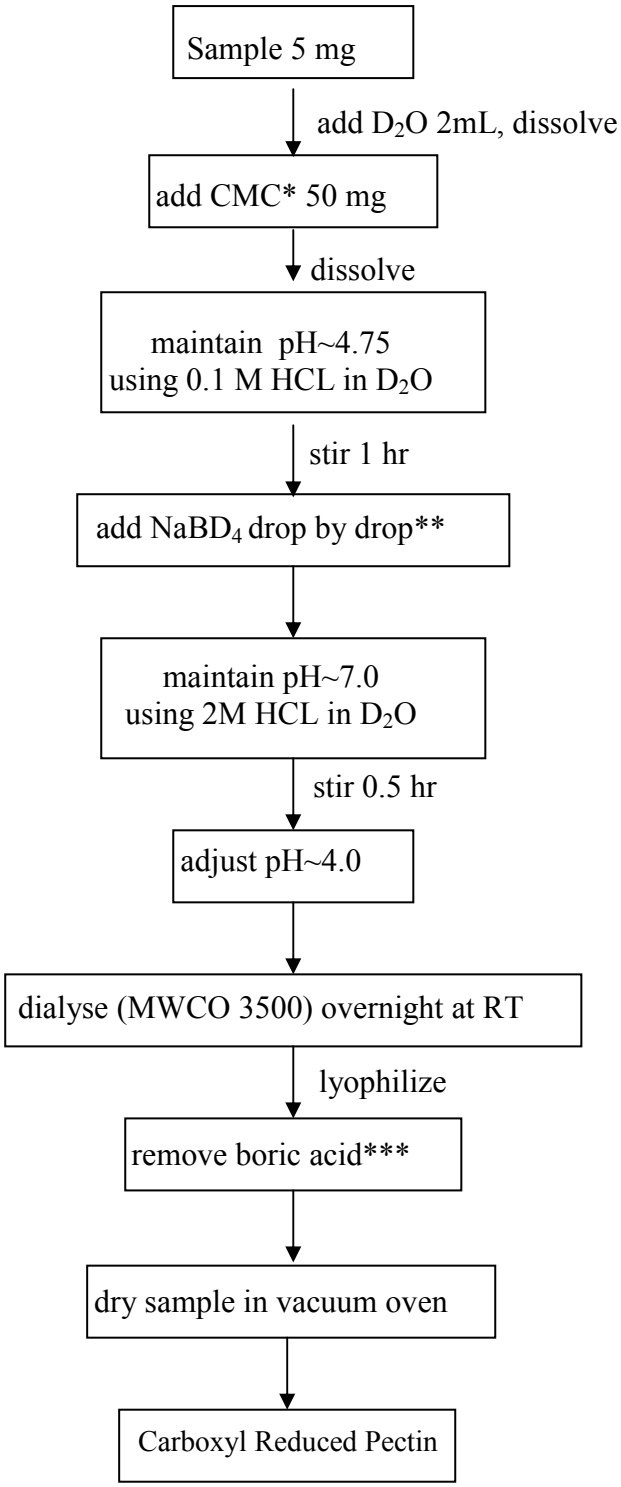
4.2.4.1. Reduction of Uronic Acids

The reduction of the uronic acid was conducted following a procedure described by Taylor and Conrad (1972) and York, Darvill, McNeil, Stevenson and Albersheim (1986) with slight modification (Figure 4.1). A sample of 5 mg was

dissolved in deuterium oxide (2 mL) and 50 mg of 1-cyclohexyl-3-(2-carbodiimide metho-p-toluenesulfonate (CMC, Sigma) was added. The pH was adjusted and maintained at 4.75 using 0.1 M HCl in deuterium oxide. After 1 hour, 800 mg of sodium borodeuteride dissolved in 5 mL of deuterium oxide was added over a period of 0.5 hour and maintained at pH 7.0 using 2.0 M HCl in deuterium oxide during the subsequent reduction reaction.

The reaction was allowed to continue for 0.5 hour at pH 7.0 after the addition of sodium borodeuteride with constant stirring. After titration of the solution to pH 4.0, the reduced polysaccharide was dialysed against distilled water overnight at room temperature (3,500 molecular weight cutoff) to separated salts and then lyophilized. The polysaccharide was dissolved in distilled water, then methanol with 10% acetic acid was added. The mixture was dried with a stream of nitrogen to remove boric acid. This process was repeated 3-4 times to ensure that most of the boric acid was removed. Finally, a few drops of methanol were added and evaporated (twice) to remove any boric acid left. A complete removal of boric acid from the sample is important.

Samples with reduced uronic acid (2-3 mg) were dried at 80°C for 4-5 hours and stored over night in phosphorus pentoxide (P_2O_5) in desiccators under vacuum.



* 1-cyclohexyl-3-(2-morpholino-ethyl)carbodiimide metho-*p*-toluenesulfonate (CMC, Sigma)

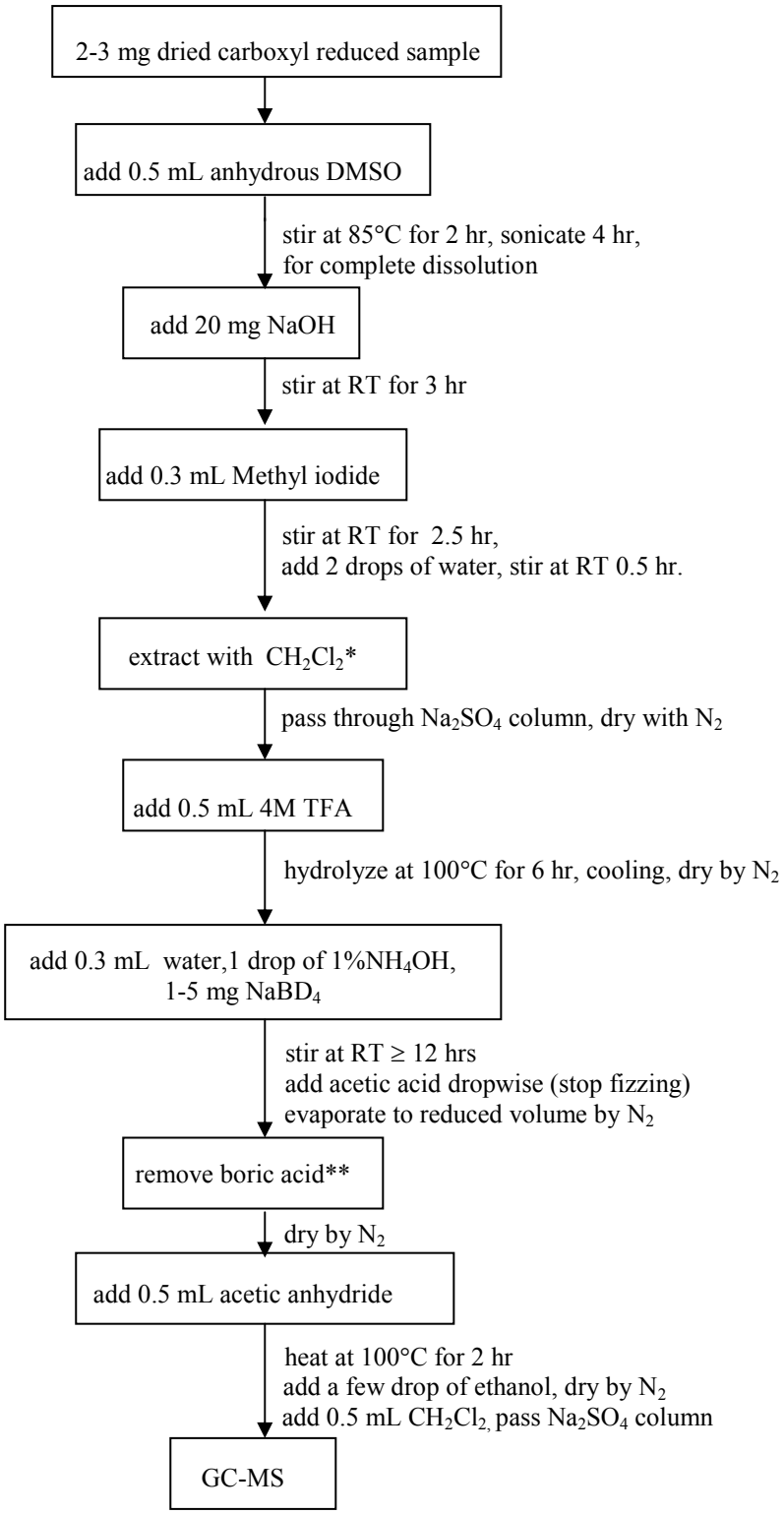
** 800 mg of sodium borodeuteride dissolve in 5 mL of D_2O is added over 30 min. $NaBD_4$ can be replaced with $NaBH_4$

*** add 0.5 mL distilled water and 0.5 mL 10% acetic acid in methanol, evaporated, repeat 3 times, finally add 0.5 mL methanol, evaporated (2 times).

Figure 4.1 Flow chart of the carboxyl reduction of uronic acids.

4.2.4.2. Methylation Analysis

The methylation analysis of the pectin samples after reduction of uronic acid was carried out according to the method of Ciucanu and Kerek (1984) with slight modification (Figure 4.2). The dried samples were dissolved in anhydrous DMSO at 85°C for 2 hours with constant stirring and sonicated for 4 hours to ensure complete dissolution of the samples. Dry powder of sodium hydroxide (20 mg) was added and the mixture was stirred at room temperature for 3 hours. The mixture was stirred for an additional 2.5 hours after adding methyl iodide (0.3 mL). The methylated polysaccharide was extracted with methylene chloride. The methylene chloride extract was passed through a sodium sulphate column to remove water and the solvent was evaporated by a stream of nitrogen. The dried methylated polysaccharide was hydrolyzed in 0.5 ml of 4.0 M trifluoroacetic acid (TFA) at 100°C for 6 hours and the TFA was removed by evaporation under a stream of nitrogen. The acid hydrolysate was subsequently reduced using sodium borodeuteride (1-5 mg) and acetylated with acetic anhydride (0.5 mL). Aliquots of the resultant of partially methylated alditol acetates (PMAA) were injected into GC-MS system (ThermoQuest Finnigan, San Diego, CA) fitted with a SP-2330 (Supelco, Bellefonte, Pa) column (30 m x0.25 mm, 0.2 μm film thickness, 160°C to 210°C at 2°C /min, then 210°C to 240°C at 5°C /min) equipped with an ion trap MS detector.



* transfer the mixture to a flat bottom test tube using a glass pipette, wash reaction vial with 1 mL CH_2Cl_2 3 times, wash test tube with deionized water 3-5 mL 3 times

**add 0.5 mL 5% acetic acid in methanol, evaporated, add 0.5 mL methanol, evaporated (many times).

Figure 4.2 Flow chart of methylation analysis.

4.2.5 Nuclear magnetic resonance (NMR) spectroscopy

The proton and ^{13}C NMR spectra were recorded on a Bruker AMX 500 FT spectrometer following the procedure of Cui, Mazza and Biliaderis (1994). All samples were dissolved in deuterium oxide (D_2O) at 90°C for 3 hours.

4.2.6 Determination of the Degree of Esterification

4.2.6.1. Titrimetric method

The degree of esterification of Krueo Ma Noy pectin was determined using the titrimetric method of Food Chemical Codex (FCC, 1981) and USP 26 NF 21 (2003) with slight modifications. The dried sample of 500 mg was transferred to a 250 mL flask, moistened with 2 mL of ethanol and dissolved in 100 mL of carbon dioxide-free water. After the sample was completely dissolved, 5 drops of phenolphthalein were added, the sample was titrated with 0.5 N sodium hydroxide and the result was recorded as the initial titer. Then, 10 mL of 0.5 N sodium hydroxide were added and shook vigorously before allowing stand for 15 min. 10 mL of 0.5 N hydrochloric acid were added and shook until the pink color disappeared. 5 drops of phenolphthalein were added before titration with 0.5 N sodium hydroxide to give a faint pink color that persisted after vigorous shaking and this value was recorded as the saponification titer (the final titer).

The degree of esterification (DE) was calculated from the following formula:

% DE = the final titer / (the initial titer + the final titer) x 100

4.2.6.2. FT-IR spectroscopic method

Pectin standards and Krueo Ma Noy pectin were dried and desiccated in a vacuum jar prior to FT-IR analysis. FT-IR spectra of pectins were obtained using a Golden-gate Diamond single reflectance ATR in a FTS 7000 FT-IR spectrometer equipped with a DTGS detector (DIGILAB, Randolph, MA). The spectra were recorded at the absorbance mode from 4000 to 400 cm^{-1}(mid infrared region) at the resolution of 4 cm^{-1} with 128 co-added scans. At least triplicate spectra readings were performed for each sample. Because the degree of esterification is defined as the (number of esterified carboxylic groups/number of total carboxylic groups) x 100, it is inferred that the ratio of the area of the band at 1730 cm^{-1} (corresponding to the number of esterified carboxylic groups) over the sum of the areas of the bands at 1730 and 1600 cm^{-1} (corresponding to the number of total carboxylic groups) should be proportional to the degree of esterification, i.e. $DE=A_{1730}/(A_{1730} + A_{1600})$ (Chatjigakis, Pappas, Proxenia, Kalantzi, Rodis and Polissiou, 1998, Manrique and Lajolo, 2002).

4.2.7 Molecular characterization

Molecular weight, molecular weight distribution, a radius of gyration and an intrinsic viscosity of Krueo Ma Noy pectin were determined using high performance size exclusion chromatography according to a method described by Wang, Wood, Huang and Cui (2003). HPSEC system used was a Shimadzu SCL-10Avp unit (Shimadzu Scientific Instruments Inc., Columbia, Maryland, USA). The column set consisted of two columns in series, a Shodex OhPak KB-806M (Showa Denko K.K., Tokyo, Japan) and an Ultrahydrogel linear (Waters, Milford, CT, USA) were

maintained at 40°C during measurements. The mobile phase was 50 mM $NaNO_3$ (pH 5.8) with 0.03% (w/w) NaN_3 with a flow rate of 0.6 mL/min. Triple detectors, a right angle laser light detector, a refractive index detector and a viscosity detector, were used for characterizing the molecular weight and molecular weight distribution. Pullulan standards (Showa Denko K.K., Tokyo, Japan) of known molecular weight and intrinsic viscosity and a dn/dc of 0.146 mL/g were used.

4.3 Results and discussion

4.3.1 Pectin identification assay

Preliminary tests indicated the extract from Krueo Ma Noy leaves was acidic polysaccharides, and possibly a pectin. A pectin identification assay was used to confirm the identity of the polysaccharide as described in the experimental section. When pectin was demethylated and treated with pectate lyase, the glycosidic bonds of the galacturonide chain would be split by β-elimination of hydrogens from the 4 and 5 carbon positions of a galacturonosyl moiety. This reaction resulted in a double bond, which gave an increase in absorbance at 235 nm. The contents of unsaturated oligosaccharides of the dialyzed extract was much greater than $0.5x10^{-5}$ Molar (Table 4.1), suggesting the presence of pectin (Hansen *et al.*, 2001). For comparison purposes, commercial pectin standards and non-pectin samples were also examined (Table 4.1).

Table 4.1 Determination of content of unsaturated oligosaccharides in pectin and non- pectin polysaccharides.

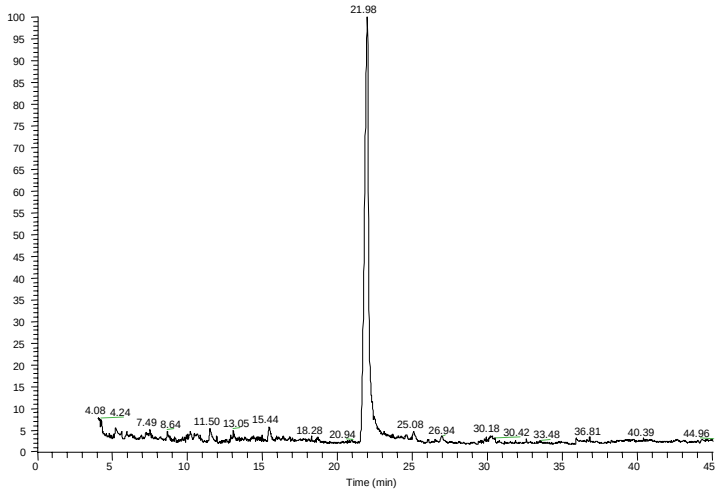
Polysaccharide Type	Absorbance Value*				Unsaturated product x 10^{-4}**
	Enzyme Blank	Sample Blank	Reaction	ΔAbs	
Carrageenan	0.009	0.037	0.050	0.004	0.01
Amidated low ester pectin	0.044	0.037	0.845	0.763	1.66
Low ester pectin	0.024	0.037	1.320	1.259	2.74
Sugar beet pectin	0.140	0.037	0.604	0.427	0.93
High ester pectin	0.071	0.037	0.808	0.700	1.52
Dialyzed extract	0.052	0.037	1.176	1.087	2.36

* All samples were analyzed as double determinations.

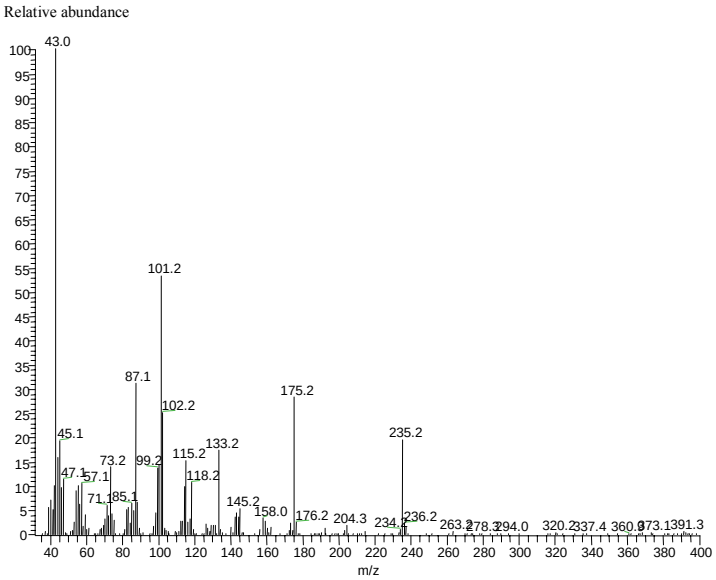
** Concentration of unsaturated product (Molar or mole/liter).

4.3.2 Methylation Analysis

Carbodiimide-activated reduction of the carboxyl groups of glycosyluronic acids with sodium borodeuteride ($NaBD_4$) resulted in an easily identified sugar (deuterized). There was only one major peak detected from the GC-MS analysis of the partially methylated alditol acetate (PMAA) derived from the carboxyl reduced Krueo Ma Noy pectin (Figure 4.3a), and its corresponding mass spectrum is showed in Figure 4.3b. The combination of the fragmentation pattern and retention time of the PMAA suggested that the polysaccharide was made of 1,4-linkaged D-galactosyl residues. The diagnostic fragment m/z 235 which was shifted two mass units higher than the m/z 233 expected from a 4-linked hexopyranosyl unit (Figure 4.4) (Biermann and McGinnis, 1989). Because there was no GC peaks detected from the non-reduced Krueo Ma Noy extract there was no 1,4-D-galactosyl residues in the polymer. The major peak in Figure 4.3a was only from 1,4-D-galacturonic acid. This result suggested that the Krueo Ma Noy extract was a pectin which had a linear backbone chain of α-1,4-D-galacturonic acid (Walter, 1991).



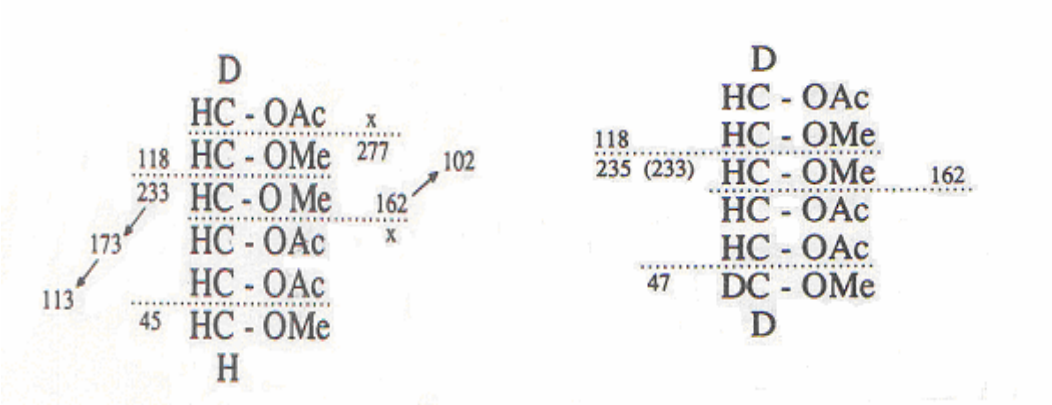
(a)



(b)

Figure 4.3 GC Chromatogram and mass spectrum of dialyzed extract.

(a) GC Chromatogram (b) Mass spectrum



(a) (b)

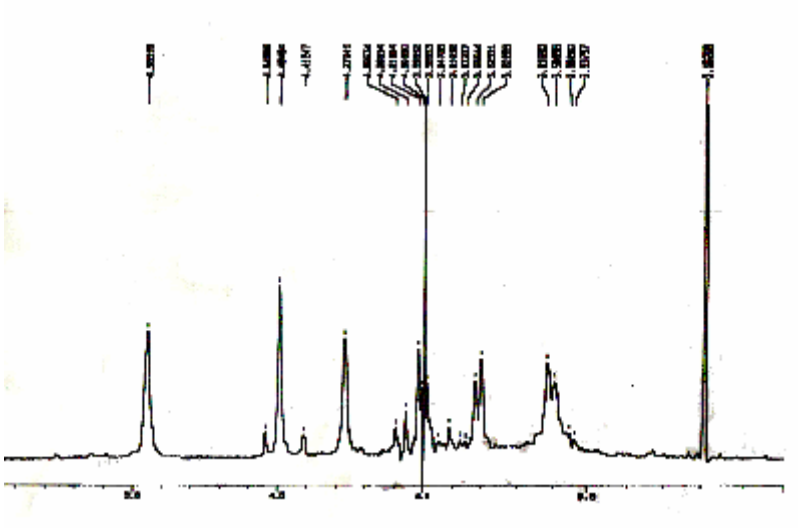
Fig. 4.4 The fragmentation of 1,4-inkaged hexitol.

(a)1,4,5-tri-O-acetyl-(1-deuterio)-2,3,6-tri-O-methyl hexitol

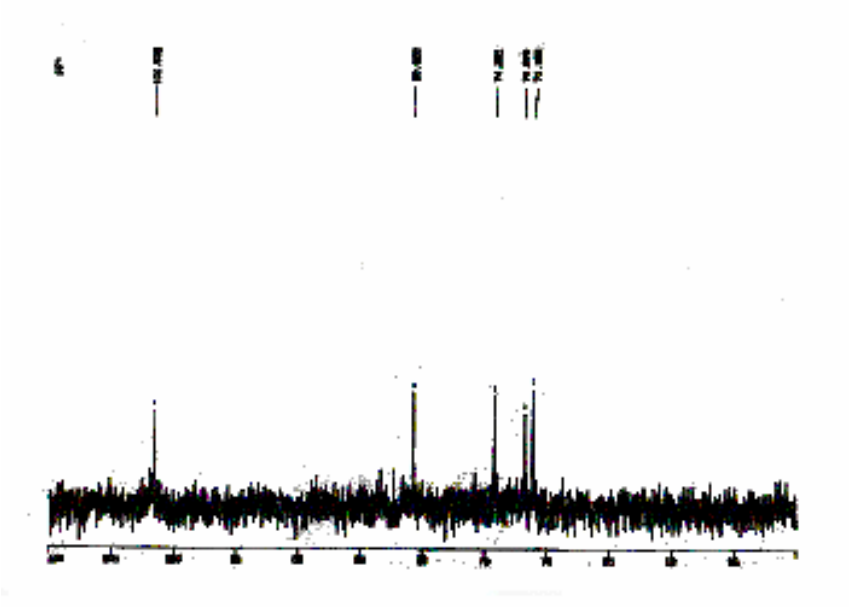
(b)1,4,5,-tri-O-acetyl-(1,6,6-trideuterio)-2,3,6-tri-O-methyl hexitol

4.3.3 Nuclear magnetic resonance (NMR) spectroscopy

The chemical structure of components from dialyzed extracts was studied by ^{13}C- and ^{1}H- NMR spectroscopy analysis (Figure 4.5). The signal at 175 ppm is from C-6 of uronic acid and that at 101.6 ppm corresponds to C-1 of uronic acid. The signals between ^{13}C- and ^{1}H- are shown in Table 4.2. The evidence for citrus pectin was the characteristic signals in its ^{13}C- and ^{1}H-spectrum (Table 8) at 102.6/4.9 ppm (C-1/H-1), 63.5/3.6 ppm (C-2/H-2), 70.8/3.8 ppm (C-3/H-3), 80.9/4.9 ppm (C-4/H-4), 74.3/4.3 ppm (C-5/H-5) and 173.1 ppm (C-6). The results for polygalacturonic acid were similar to citrus pectin and the dialyzed extract.



(a)



(b)

Fig. 4.5 ^{1}H -and ^{13}C-NMR spectrum of the dialyzed extract.

(a) ^{1}H –NMR spectrum (b) ^{13}C-NMR spectrum

Table 4.2 ^{1}H and ^{13}C of dialyzed extract, citrus pectin and polygalacturonic acid.

	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5	C-6/H-6
Polygalacturonic acid	102.4/4.9	70.7/3.6	71.0/3.8	80.7/4.8	72.9/4.3	174.5/-
Citrus pectin	102.6/5.2	63.5/3.6	70.8/3.8	80.9/4.9	74.3/4.3	173.1/-
Dialyzed extract	101.6/4.9	71.2/3.6	71.9/3.9	80.8/4.5	74.3/4.2	175.0/-

4.3.4 FT-IR spectra and the degree of esterification of pectins

The FT-IR spectra of Krueo Ma Noy pectin and commercial pectin standards were presented in Figure 4.6. The functional groups of pectins and their corresponding frequencies and nature of the bands were presented in Table 4.3. The broad, strong areas of absorption between 3600 and 2500 cm^{-1} referred to O-H stretching absorption due to inter- and intra-molecular hydrogen bonds. The O-H stretching vibrations occurred within a broad range of frequencies and indicated several features of a compound, including free hydroxyl groups stretching bonds that occurred in samples in vapor phase and bonded O-H bands of carboxylic acid (Silverstein, Bassler and Morril, 1991). In the case of pectin samples, absorption in the O-H region was due to the inter- and intra-molecular hydrogen bonding of the galacturonic acid backbone. Bands around 2950 cm^{-1}(3000-2800 cm^{-1}) referred to C-H absorption, these included CH, CH_2 and CH_3 stretching and bending vibrations. Typically, two moderately intense bands were observed in the C-H region of aliphatic compounds. In the pectin samples, the C-H stretching and bending vibrations usually

responded as a band superimposed upon the broader O-H band ranging from 2500 to 3600 cm^{-1} (Filippov, 1992, Kačuráková, Kapek, Sasinková, Wellner and Ebringerová, 2000).

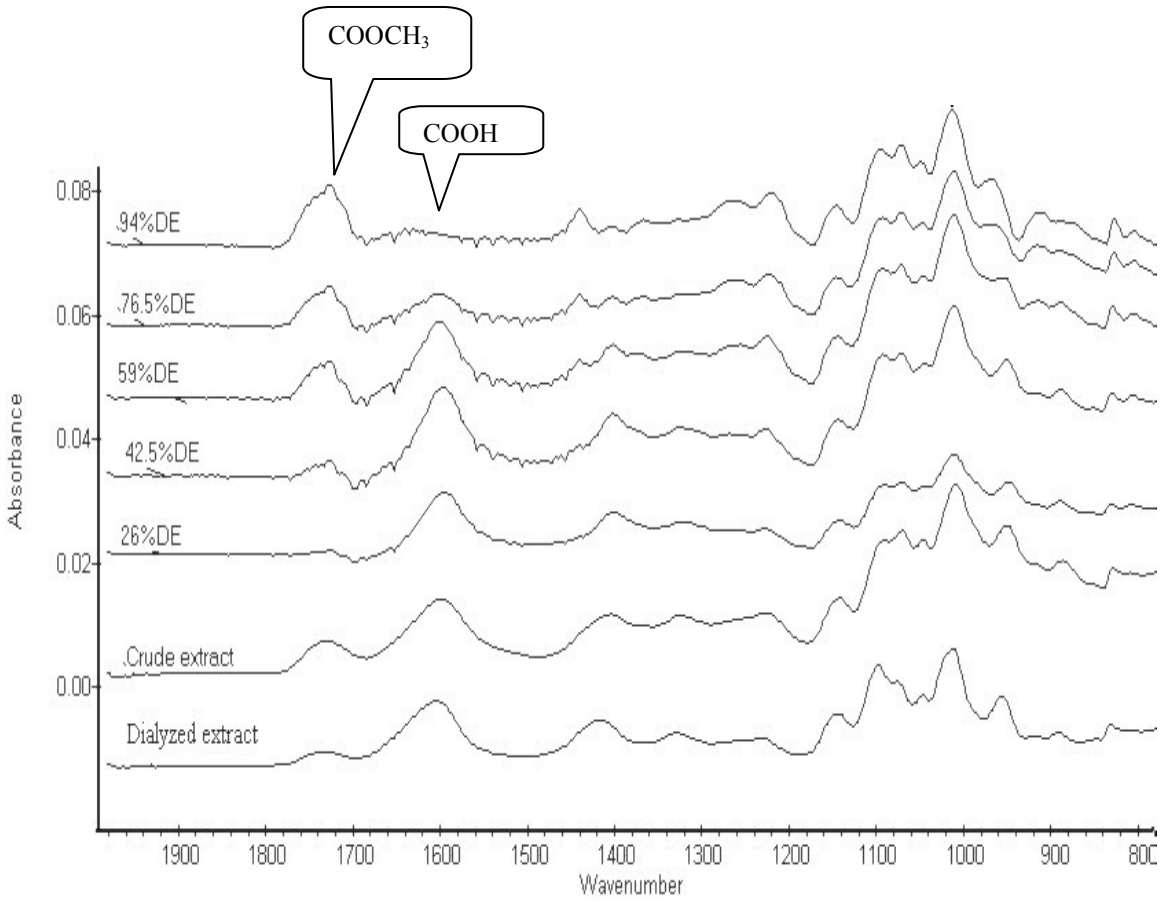


Figure 4.6 Fourier transform infrared spectra of commercial pectin standards and Krueo Ma Noy pectins.

Table 4.3 Wave numbers and intensities of functional groups present in commercial pectin samples analyzed by FT-IR spectroscopy.

Wave number(cm^{-1})	Functional groups	Intensity
3600-2500	O-H stretching	Broad, strong
3000-2800	C-H stretching, symmetric, asymmetric	Sharp, occasionally double overlapping with O-H
1760-1730	C=O, esterified	Strong
1630-1600	COO-asymmetric stretching	Strong
1400	COO-symmetric stretching	Weak
1380	C-H bending	Weak
1300-1000	C=O stretching	Weak

These absorbencies were observed with all pectin standards studied. In the case of esterified pectins, an O-CH_3 stretching band would be expected between 2950 and 2750 cm^{-1} due to methyl esters of galacturonic acid. However, due to a large O-H stretching response occurring in a broad region (3600-2500 cm^{-1}), the O-CH_3 activity was masked and therefore was not a reliable indicator of methoxylation. Stronger bands occurring between 1760-1730 cm^{-1}, and between 1630-1600 cm^{-1} were derived from the ester carbonyl (C=O) groups and carboxylate ion stretching band (COO-), respectively (Manrique and Lajolo, 2002; Chatjigakis *et al*., 1998). It was observed that the intensity of the absorbance or band area of the ester carbonyl groups increased with the increase in degree of esterification, in contrast, the absorbance intensity or the band area of the carboxylate stretching band decreased at 1730-1760 cm^{-1} (Fig.

4.6). In a similar manner, the intensity of the absorbance or band area of the free carboxylate groups (1630-1600 cm^{-1}) increased with the decrease in the degree of esterification. These observations established the basis for quantitative analysis of the degree of esterification of pectins from FT-IR, the 1760-1730 cm^{-1} bands representing ester carbonyl groups and the 1630-1600 cm^{-1} band representing the free carboxylate groups.

It would be worth noting that the carboxylate groups showed two bands, an asymmetrical stretching band near 1650-1550 cm^{-1}, and a weaker symmetric stretching band near 1400 cm^{-1}. In the pectin samples, the weaker symmetric COO- stretching was followed by moderately intensed absorption patterns between 1300-800 cm^{-1}; these collectively referred to as the finger print region for pectins. Other bands of lesser importance in the pectin samples were C-H bending, occurring at 1380 cm^{-1}, and C=O stretching occurring at 1300-1000 cm^{-1} (Coimbra, Barros, Barros, Rutledge and Delgadillo, 1998, Gnanasambandam and Proctor, 2000).

In order to quantify the degree of esterification of pectins, a calibration curve was constructed based on the pectin standards of known degrees of esterification. The calibration curve was established from the ratio of $A_{1730}/(A_{1730} + A_{1600})$, as presented in Figure 4.7. For every triplet of measurements of pectin standards, the coefficient of variation of the ratios was less than 2%, indicating an excellent reproducibility. The high value of the square of the linear correlation coefficient (r^2=0.98) indicated a highly linear relationship between the degree of esterification and absorbent area at 1730 cm^{-1} and 1600 cm^{-1}. Using this calibration curve, the degree of esterification of the pectin from Krueo Ma Noy extracts was calculated and the results were presented in Table 4.4. The degree of esterification of

the Krueo Ma Noy pectins was 41.65% and 33.69% for crude and dialyzed pectin samples, respectively. The degree of esterification of the dialyzed extract was lower than that of the crude extract, suggesting that some small molecules of pectin with higher degree of esterification was removed during dialysis process.

To evaluate the validation of the FT-IR method, the degree of esterification values obtained by FT-IR method were compared with those obtained from the titrimetric method as shown in Table 4.4. The results confirmed that FT-IR was a reliable method for determining the degree of esterification of pectins since there was no significant difference observed between the degree of esterification from the two methods for both samples.

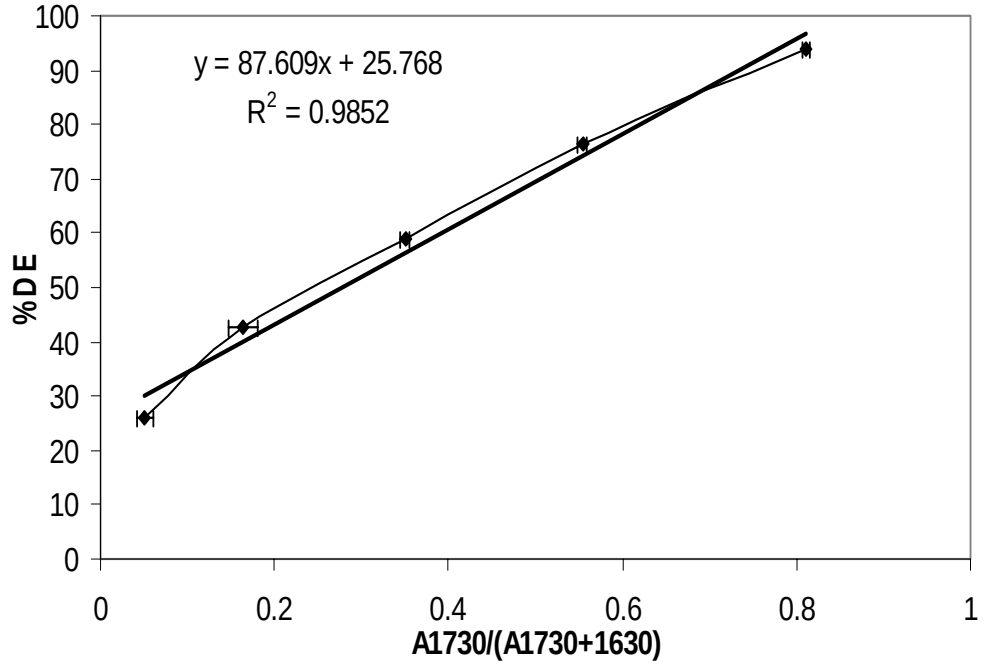


Figure 4.7 Calibration curve of the FT-IR spectra of pectin standards : ratio of the peak area at 1730 cm^{-1} over the sum of the peak areas at 1730 and 1600 cm^{-1} versus the degree of esterification of pectins (%).

Table 4.4 Degree of esterification of pectins from the crude and dialyzed extracts of Krueo Ma Noy obtained by different methods.

Method	Degree of esterification (%)	
	Crude extract	Dialyzed extract
FT-IR spectroscopy	41.65±0.04[a]	33.69±0.17[b]
Titrimetric	43.20±1.00[a]	36.20±2.97[b]

[a,b] Values are means of triplicate measurements.

Values with different letters in each column are significantly ($p \leq 0.05$) different from each other.

4.3.5 Molecular characterization

Molecular weight, molecular weight distribution, radius of gyration and intrinsic viscosity of Krueo Ma Noy pectin were determined using high performance size exclusion chromatography (HPSEC) according to a method described by Wang *et al.* (2003), (Figure 4.8 and Table 4.5). The average molecular weight (Mw) of the dialyzed Krueo Ma Noy pectin was 55 KDa. This value was significantly lower than the values from 85 KDa to 103 KDa reported for citrus pectin but in the general range of 10 KDa to 100 KDa for various fruit sources (Corredig, Kerr and Wicker, 2000). The polydispersity parameter of 1.9 indicated a broad molecular weight distribution of the dialyzed Krueo Ma Noy pectin. The HPSEC method also provided information of radius of gyration (Rg) and intrinsic viscosity [η] of the dialyzed Krueo Ma Noy pectin (15.2 nm and 2.3 dL/g, respectively), reflecting the conformation of the polymer in the solvent system. The Rg (15.2 nm) value of the dialyzed Krueo Ma Noy pectin was much lower than that of the four commercial pectins (50 -59 nm) with molecular weights ranging from 90 KDa to 200 KDa (Corredig *et al.*, 2000). Although the flow rate and solvent had some influence on the values of Rg, experimental data from different laboratories were still comparable.

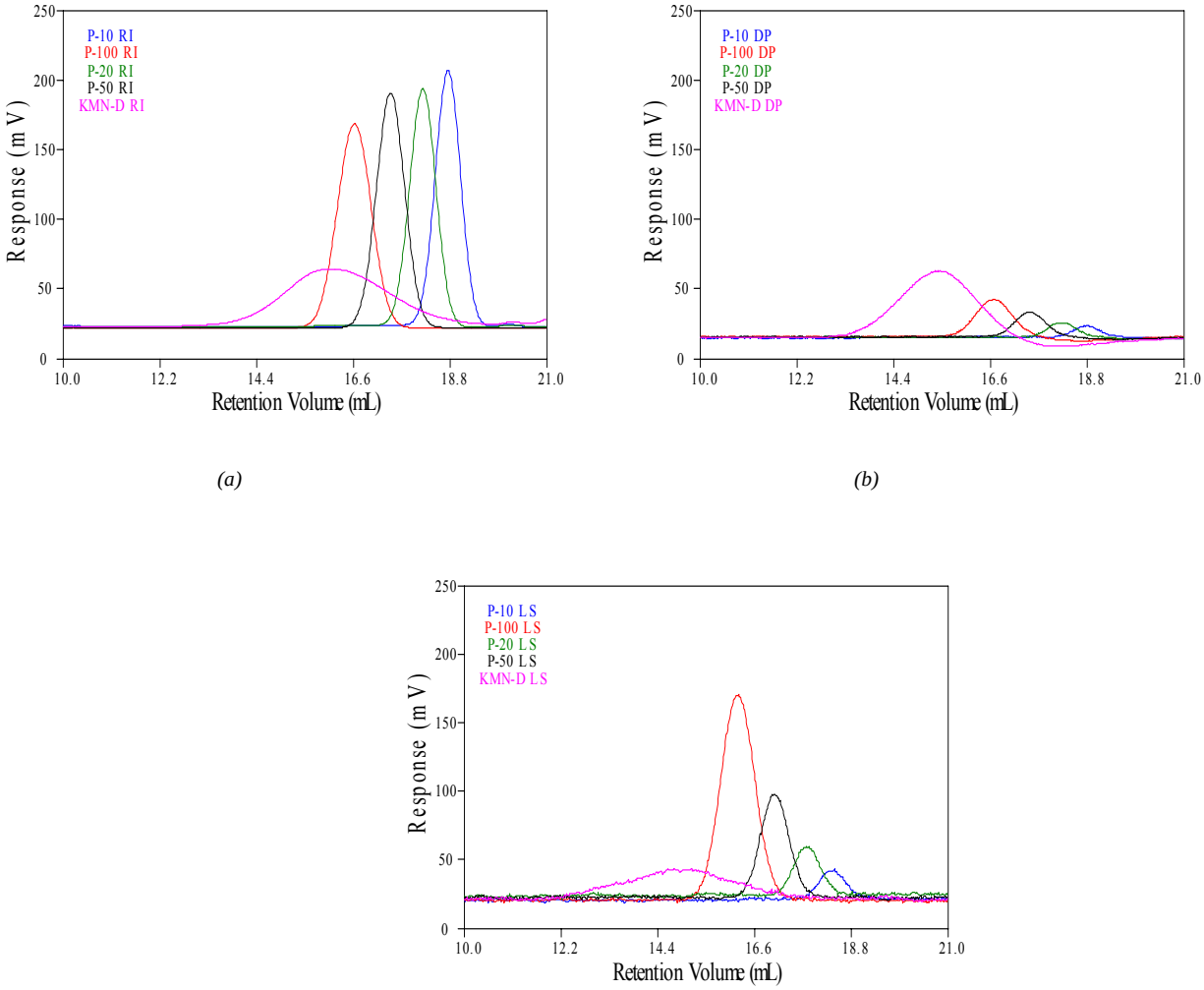


Figure 4.8 HPSEC elution profile of dialyzed extracts and pullulan standards detected by RI (a), DP (b), and LS (c).

KMN-D = dialyzed extract, P-100 = pullulan 100, P-50 = pullulan 50, P-20 = pullulan 20, P-10 = pullulan 10

Table 4.5 Means number average (Mn), weight average (Mw) and z-average (Mz)) of molecular weight, radius of gyration (Rg), intrinsic viscosity ([η]) and polydisperse (Pd) of dialyzed extract.

Mn (x 10^3 Da)	29.08 ± 2.45
Mw (x 10^3 Da)	55.08 ± 2.62
Mz (x 10^3 Da)	99.00 ± 7.55
Rg (nm)	15.21 ± 0.28
[η] (dL/g)	2.30 ± 0.04
Pd (Mw/Mn)	1.90 ± 0.11

4.4 Conclusions

The polysaccharide extracted from Krueo Ma Noy (Cissampelos pareira) leaves consisted mainly of pectin. The dominant structure of dialyzed pectin was 1,4-D-galacturonic acid. The FT-IR spectroscopy confirmed the pectin structure. The two characteristic peaks, 1730 and 1600 cm^{-1} for absorption of the esterified and non-esterified carboxyl groups of pectin, respectively, were used to quantify the degree of esterification of Krueo Ma Noy pectin using standards of known degree of esterification to construct the calibration curve. The degree of esterification of Krueo Ma Noy pectins was estimated to be 41.65 and 33.69 % for the crude extract and the dialyzed pectin samples, respectively. These results were confirmed by a titrimetric method. The average molecular weight, a radius of gyration and an intrinsic viscosity of the dialyzed extract were 55 KDa, 15.2 nm and 2.3 dL/g, respectively.

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Chapter V

Gelling Properties of Krueo Ma Noy Pectin

Abstract

Krueo Ma Noy pectin (KMNP) was extracted from Krueo Ma Noy leaves (*Cissampelos pareira*). Its structure was typical of a pectin, consisting of 1,4-linked α-D-galacturonic acid with trace amounts of neutral sugars. Krueo Ma Noy pectin exhibited gelling properties in aqueous solutions at 1.0% (w/v) and above room temperature. The gel strength, melting point and melting enthalpy of Krueo Ma Noy pectin increased with polymer concentration. The gelling characteristics were affected by a number of factors, such as pectin concentration, pH and the presence of co-solute and salt. A response surface methodology was employed to evaluate the concentration effect of pectin (0-2%, w/v), sucrose (0-60%, w/v), calcium chloride (0-4 mM) and pH (2-8) on the gelling properties. The gel strength was evaluated by the storage modulus G′. It was found that all the factors examined had significant effects on the strength of KMNP gel. The gel strength increased with an increase in KMNP concentration. Increases of calcium chloride concentration resulted in increased gel strength until calcium chloride concentration reached 2 mM. Further increases in calcium chloride concentration decreased gel strength. When pH and pectin concentration were fixed, maximum gel strength was at 2 mM calcium chloride and ~50% sucrose. Also, the decreases in pH strengthened KMNP gel.

Keywords: Krueo Ma Noy pectin; gelling property, response surface methodology, rheology; DSC

5.1 Introduction

Gelation of polysaccharides is of special importance when they are used to modify texture in many foods. Pectins have been widely used as gelling agents, stabilizers, thickeners and emulsifiers in many food products. They are anionic polysaccharides consisting of a linear backbone of α–link D-galacturonic acid residues partially esterified with methanol (Walter, 1991). Pectin with a degree of esterification < 50% (the degree of substitution of methyl esters) are considered to be low methoxyl pectins (LMP) while those with a degree of esterification greater than 50% are considered to be high methoxyl pectins (HMP). The degree of esterification determines the mechanism of the formation of pectin gels, their conformation and rheological properties (Fishman, Pfeffer, Barford and Doner, 1984, Grusso and Rao, 1998, Fu and Rao, 2001).

Pectin extracted from Krueo Ma Noy leaves mainly consisted of galacturonic acid with trace amounts of neutral sugars. The degree of esterification of Krueo Ma Noy pectin was less than 50% (LMP), as shown in the previous chapters. The study of structure formation in polysaccharide systems used as gelling agents in food formulations could be useful in understanding the kinetic and thermal behavior of the food products in which they are associated as well as providing additional insights into the nature of the gelation process of these polymers (Norziah, Kong, Karim and Seow, 2001).

This study was designed to examine the effects of Krueo Ma Noy pectin, sucrose, calcium and pH on rheological properties including gelling properties of Krueo Ma Noy pectin.

5.2 Materials and methods

5.2.1 Preparation of pectin from Krueo Ma Noy

Krueo Ma Noy leaves, procured from the markets in the northeast of Thailand, were cleaned with water to remove dirts and infected leaves were also removed before being dried at 60°C for 3 hours. The dried leaves were ground and stored at room temperature in a vacuum-packed PP bag. The Krueo Ma Noy pectins were prepared according to a method described in Chapter III. The extraction was conducted at 2% solid in distilled water at 25°-28°C and natural pH (3.8-4.0). Alcohol precipitation and drying provided a crude extract and further dialysis and lyophilization produced a purified or dialyzed extract.

5.2.2 Rheological properties

All rheological properties were determined on a Bohlin CVO Rheometer (Bohlin Instruments, East Brunswick, NJ). A parallel plate geometry (40 mm diameter, 1.0 mm gap) was used for oscillatory measurements. The viscoelastic properties, such as the storage modulus (G′) and the loss modulus (G″), were determined through small amplitude oscillatory tests at frequencies from 0.1 to 10 Hz. Prior to any dynamic experiments, a strain sweep test at a constant frequency of 0.1

Hz was used to determine the linear viscoelastic region. All oscillatory tests were performed at a strain value of 0.02 (2%, within the linear viscoelastic region). A thin layer of low viscosity mineral oil was used to cover the sample in order to prevent solvent evaporation during measurements. Temperature sweeps were performed between 5° and 80°C. Samples were loaded onto the rheometer in a gel state at 5°C and the heating rate was set at 1°C/min.

A response surface methodology with 5 level composite design was used for dispersion preparation. The variables (KMNP, sucrose as a co-solute, calcium, and pH) were coded as -2, -1, 0, +1 and +2 corresponding with the concentration of KMNP (0-2%w/v), sucrose (0-60%w/v), calcium chloride (0-4 mM) and pH (2-8), as shown in Table 5.1. The design resulted in 30 different treatments. Each dispersion of KMNP was prepared in distilled water and heated to 85ºC using mechanical stirring. The solution was then adjusted with NaOH or HCl to the desired pH. The required amount of sucrose and calcium chloride was added slowly to the solution with continuous stirring at elevated temperatures to complete dissolution. Multiple regression was applied in data analysis using SAS version 6.0. Response surface plots were presented to visualize responses of the interaction between independent variables.

Table 5.1 Central composite design 2^4 for Krueo Ma Noy pectin interaction.

Treatments	Pectin	Sucrose	Calcium	pH
1	+1	+1	+1	+1
2	+1	+1	+1	-1
3	+1	+1	-1	+1
4	+1	+1	-1	-1
5	+1	-1	+1	+1
6	+1	-1	+1	-1
7	+1	-1	-1	+1
8	+1	-1	-1	-1
9	-1	+1	+1	+1
10	-1	+1	+1	-1
11	-1	+1	-1	+1
12	-1	+1	-1	-1
13	-1	-1	+1	+1
14	-1	-1	+1	-1
15	-1	-1	-1	+1
16	-1	-1	-1	-1
17	-2	0	0	0
18	+2	0	0	0
19	0	-2	0	0
20	0	+2	0	0
21	0	0	-2	0
22	0	0	+2	0
23	0	0	0	-2
24	0	0	0	+2
25	0	0	0	0
26	0	0	0	0
27	0	0	0	0
28	0	0	0	0
29	0	0	0	0
30	0	0	0	0

Concentrations are based on coded levels of design factors : -2, -1, 0, +1, +2

Krueo Ma Noy pectin 0-2% (w/v) : 0, 0.5, 1.0, 1.5, 2

Sucrose 0-60% (w/v) : 0, 15, 30, 45, 60

$CaCl_2$: 0-4 mM : 0,1, 2, 3, 4

pH 2-8 : 2.0, 3.5, 5.0, 6.5, 8.0

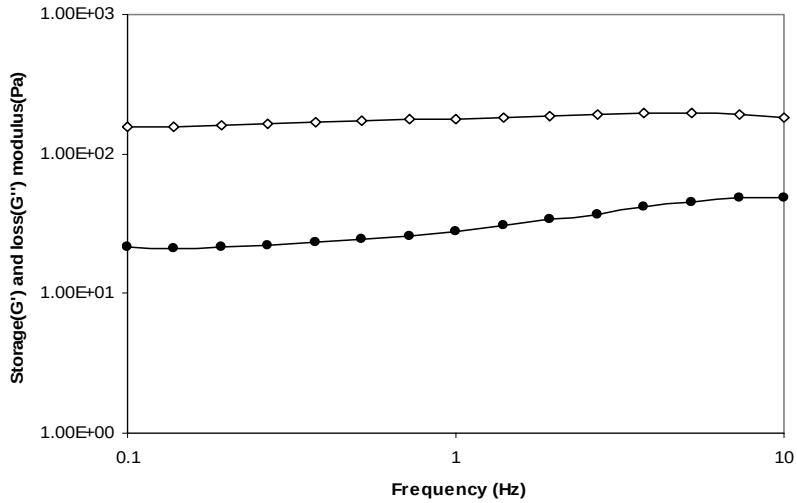
5.2.3 Differential scanning calorimetry (DSC)

Thermal analyses were performed using a Differential Scanning Calorimeter (2920 modulated DSC; TA Instruments, New Castle, DE, USA). The samples of 80-90 mg were weighed into high-volume pans (Part number: 900825-902; TA Instruments, New Castle, DE, USA). The measurements were carried out at a heating rate of 5°C /min from 5 to 140°C. The reported values are the result of means of duplicate measurements. The instrument was calibrated using indium and an empty pan as reference. The enthalpy (ΔH) of endotherm was measured from DSC thermograms using provided software (Universal Analysis, version 2.6D, TA Instruments).

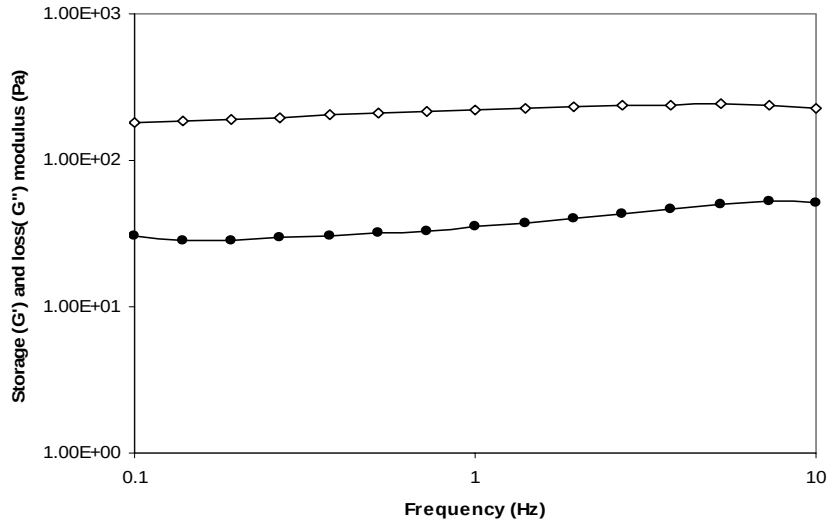
5.3 Results and discussion

5.3.1 Frequency sweep

The viscoelastic properties of Krueo Ma Noy pectin were examined by oscillatory experimental measurements and the results were shown in Figure 5.1. The crude extract exhibited a strong gel structure at 1.0% (w/v) as its storage modulus G′ was much greater than the corresponding loss modulus G″ and the two moduli were independent of frequency (Figure 5.1a). The dialyzed Krueo Ma Noy pectin exhibited similar rheological behaviour, but the gel strength was stronger than that of the crude extract, as shown in Figure 5.1b. The stronger gel strength of the dialyzed pectin may be due to a higher effective polymer concentration. The gel strength was increased with the increase of sample concentration (Figure 5.2).



(a)



(b)

Figure 5.1 Frequency dependence of storage (G′,—o—) and loss (G″,—●—) modulus of 1%(w/v) Krueo Ma Noy pectin at 25°C:

(a) Crude extract (b) Dialyzed extract

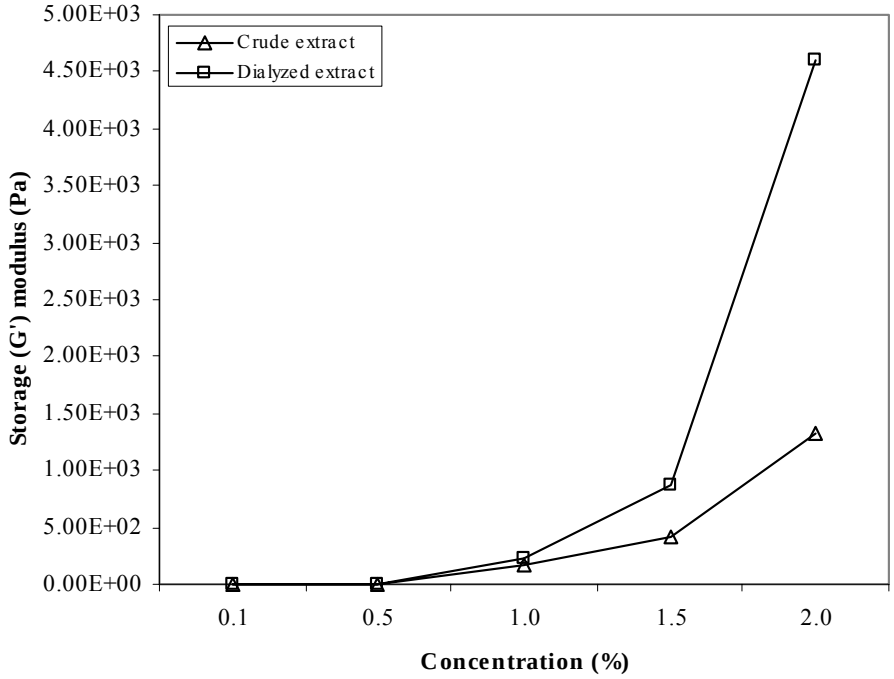


Figure 5.2 Storage (G′) modulus at 1 Hz for different concentration of crude and dialyzed extracts at 25°C.

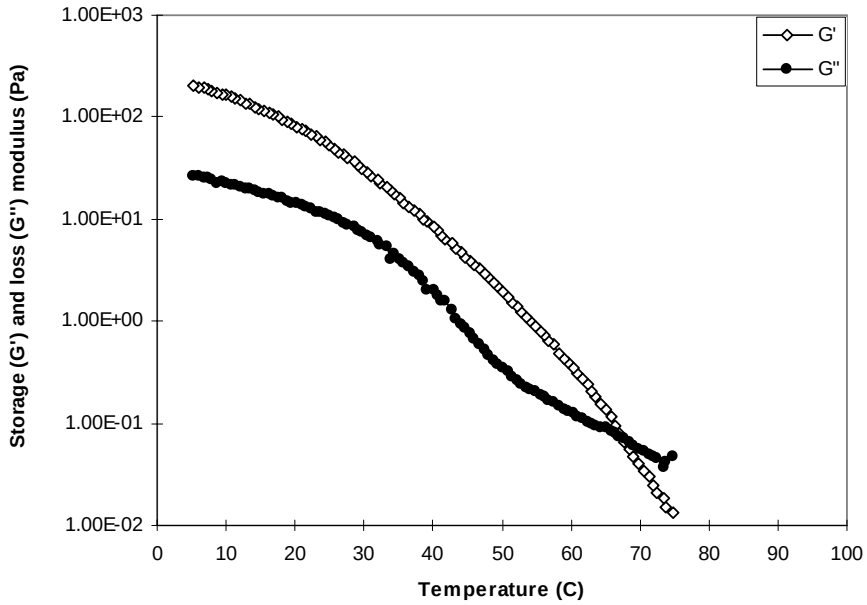
5.3.2 Temperature sweep

Krueo Ma Noy pectin did not show a clear melting point when the gel was heated at 1°C/min at a constant frequency (0.1 Hz) (Figure 5.3). At the beginning of the heating, the storage modulus G′ decreased with the increase of temperature. The storage modulus G′ and loss modulus G″ showed a crossover around 67ºC as shown in Figure 5.3a which indicates a melting point for the crude extract. The rate of decrease of the storage modulus G′ was much faster after the temperature exceeded 40°C; this was especially the same for the dialyzed extract, as shown in Figure 5.3b. The melting point of Krueo Ma Noy pectin gels was difficult to determine due to the sensitivity and operational limitations of the rheometer. An attempt was made to

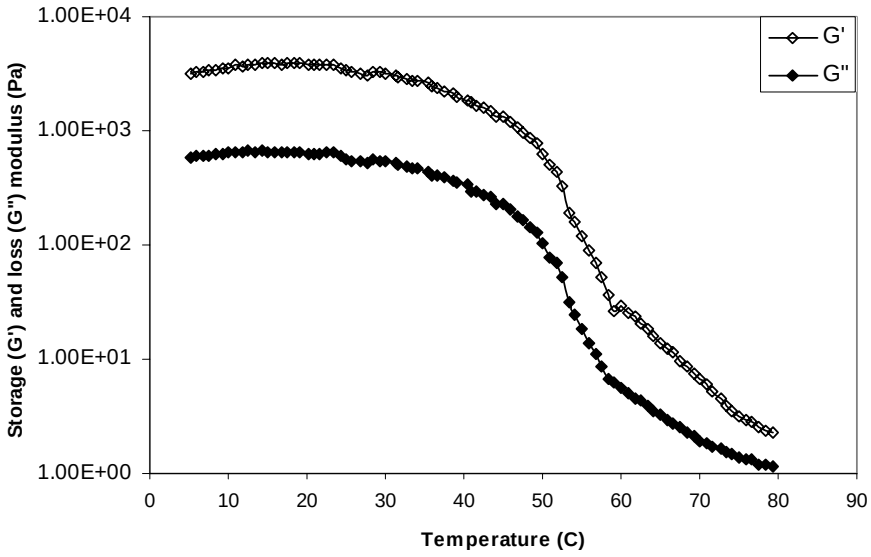
determine the melting temperature of Krueo Ma Noy pectin samples by the differential scanning calorimetry method, as described in the following section.

5.3.3 Differential scanning calorimetry

The effect of Krueo Ma Noy pectin concentration on the melting temperature and melting enthalpy involved was determined by DSC, and the results are presented in Figure 5.4 and Table 5.2. The increase of polymer concentration had a positive influence on the melting enthalpy (ΔH) and melting temperature(Tm). For the crude extract, the ΔH values increased from 0.11 to 10.84 J/g and T_m increased from 75.15 to 113.49 °C when the polymer concentration was increased from 2 to 4%. The ΔH and T_m values for the dialyzed extract increased from 0.56 to 5.85 J/g and 76.10 to 104.21 °C, respectively when the polymer concentration was increased from1% to 3% (Table 5.2). At the same polymer concentration, the ΔH values of the dialyzed extract were higher than that of the crude extract. This might be due to the higher effective concentration of the dialyzed pectin sample.



(a)



(b)

Figure 5.3 Temperature dependence of G′ and G″ during heating from 5ºC to 80ºC at the rate of 1ºC/min for 1%(w/v) of Krueo Ma Noy pectin solution: (a) Crude extract (b) Dialyzed extract

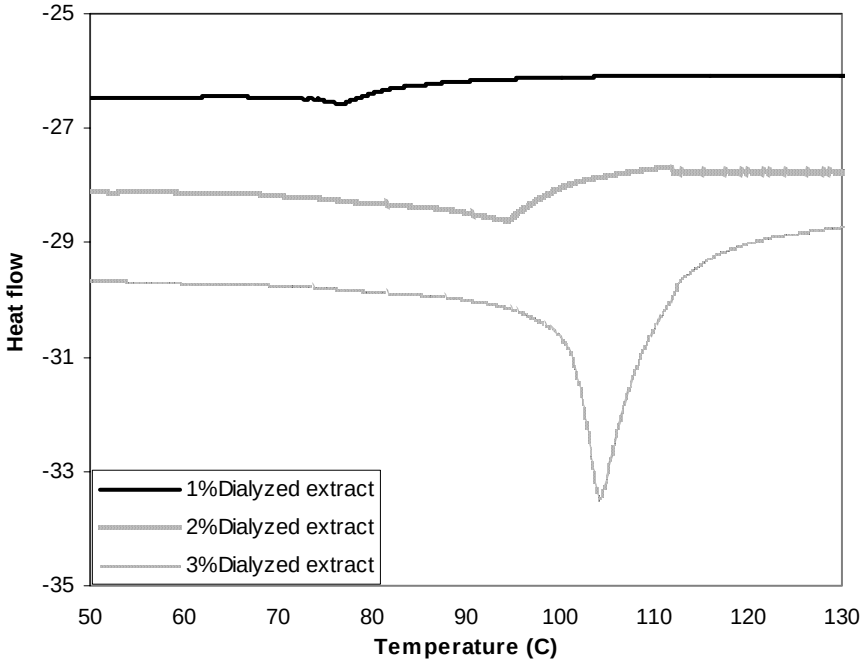


Figure 5.4 DSC thermograms of dialyzed Krueo Ma Noy pectin.

Table 5.2 Thermal properties of crude and dialyzed extracts determined by DSC**.**

Krueo Ma Noy pectin	Tm (°C)*	ΔH (J/g)*
2% Crude extract	75.15±0.47	0.11±0.02
3% Crude extract	105.06±0.85	2.48±0.13
4% Crude extract	113.49±1.58	10.84±1.37
1% Dialyzed extract	76.10±0.76	0.56±0.15
2% Dialyzed extract	92.65±2.43	0.65±0.05
3% Dialyzed extract	104.21±0.45	5.86±1.77

Tm: peak temperature of melting (°C)

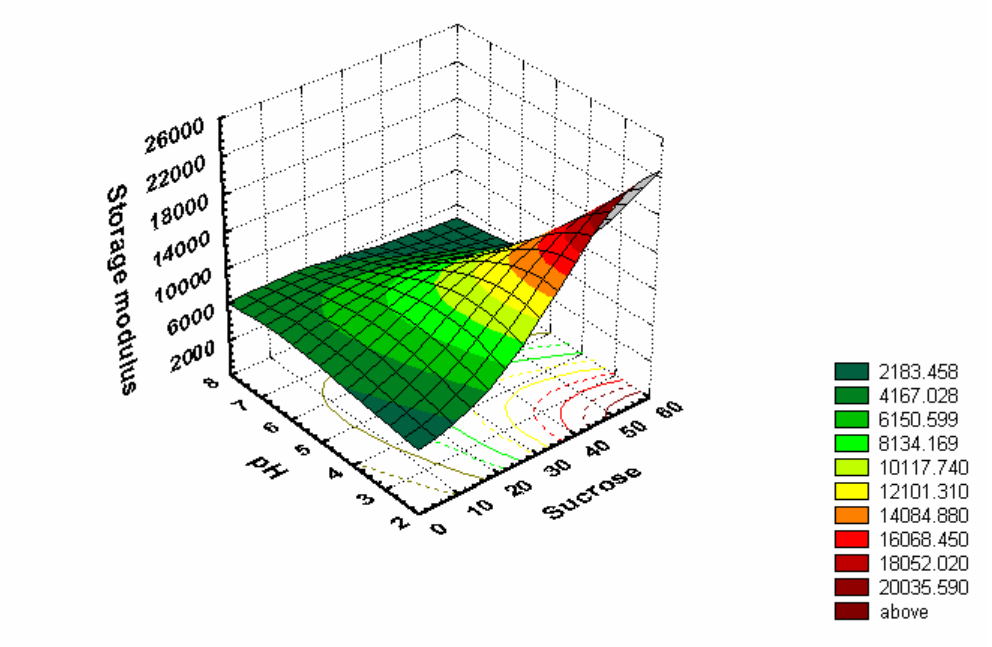
ΔH: enthalpy (J/g)

*Each result represents the average of duplicate values ± SD.

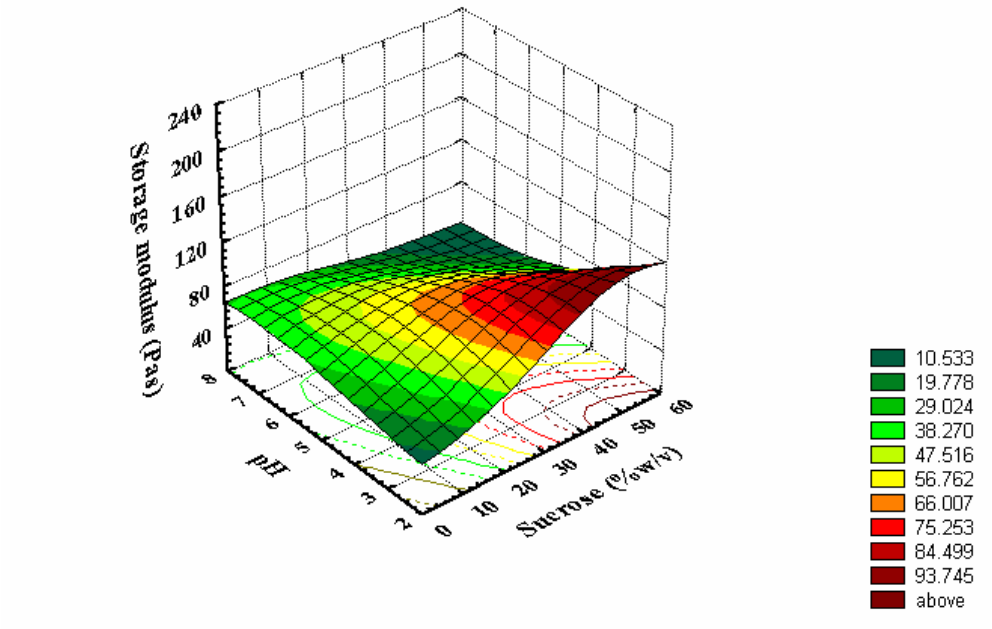
5.3.4 Krueo Ma Noy pectin-sucrose-calcium-pH interaction

The effects of dialyzed Krueo Ma Noy pectin (KMNP), sucrose, calcium and pH on the storage modulus (G′) at 1.0 Hz are shown in Figure 5.5, 5.6 and 5.7. The interaction of variable factors was statistically significant with R^2 value of 0.937 (equation: G' = Exp [-3.95 + 12.07KMNP + 0.14sucrose + 0.23$CaCl_2$ + 0.63pH - 4.24$KMNP^2$ + 0.01KMNP*sucrose - 0.001$sucrose^2$ + 0.65KMNP*$CaCl_2$ - 0.01sucrose* $CaCl_2$ – 0.47 $CaCl_2^2$ – 0.06KMNP*pH – 0.02sucrose*pH + 0.18 $CaCl_2$*pH – 0.07pH^2]). A concentration of Krueo Ma Noy pectin was the major factor effecting the storage modulus values of the system. The highest G′ was obtained at the concentration of Krueo Ma Noy pectin 1.5% (w/v), sucrose 45% (w/v), calcium chloride 2 mM and pH 3.5. Figure 5.5a and 5.5b showed the response surface plots illustrating the effect of sucrose concentrations and pH on G′ of KMNP solutions at the constant calcium chloride concentration of 2 mM. At both high and low KMNP concentrations, the interaction between sucrose and pH gave a rise to the maximum G′ at low pH and high sucrose concentration. This indicated that low pH promoted a conformational transition of pectin to be more compact three-dimensional structure.

Figure 5.6a and 5.6b displayed response surface plots of the effect of sucrose and calcium chloride concentrations on G′ of KMNP solutions at the constant pH of 3.5. At low KMNP concentration, the maximum G′ was at high sugar concentration and about 1 mM calcium chloride whereas at high KMNP concentration, calcium chloride concentration was shifted to about 2 mM at the maximum G′ as well as a little bit shifting of sucrose to higher concentration. This indicated that more cations were needed to form a complex gel conformation with higher KMNP concentration to obtain a strong gel system.



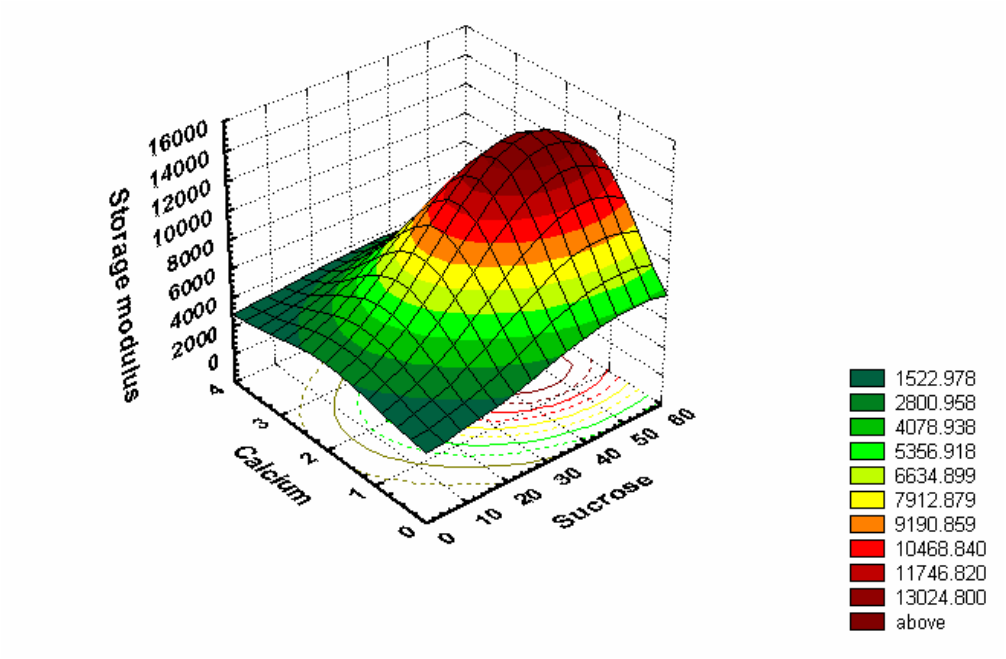
(a)



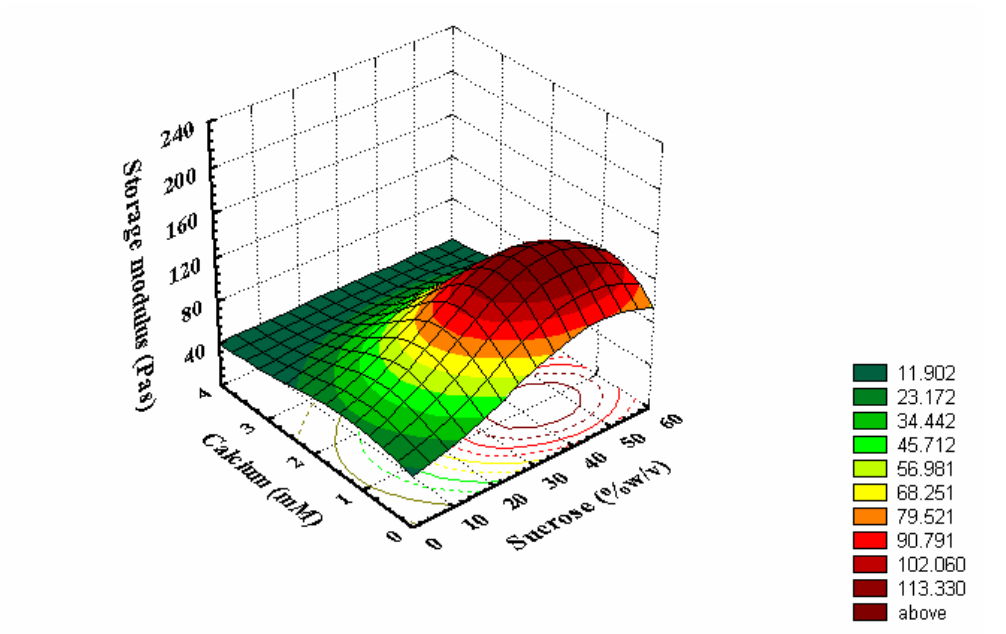
(b)

Figure 5.5 Response surface plots of the effects of sucrose and pH on G′ at 2mM $CaCl_2$:

(a) KMNP 1.5% (w/v) (b) KMNP 0.5% (w/v)



(a)



(b)

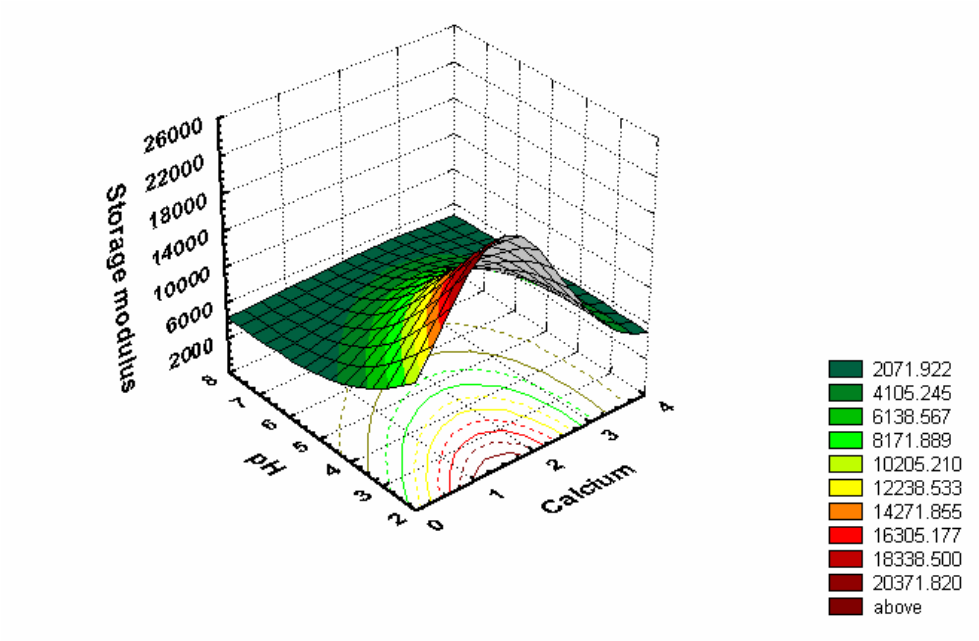
Figure 5.6 Response surface plots of the effects of sucrose and $CaCl_2$ on G′ at pH 3.5 :

(a) KMNP 1.5% (w/v) (b) KMNP 0.5% (w/v)

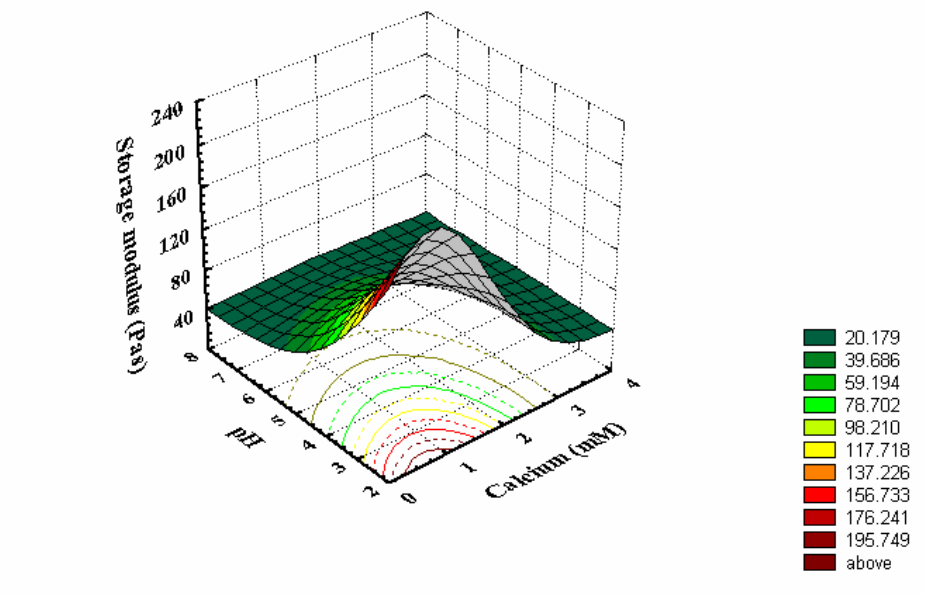
Figure 5.7a and 5.7b revealed the effect of calcium chloride concentration and pH on the maximum G′ of KMNP solutions. The interaction between calcium chloride and pH gave a rise to in the maximum G′ at low pH and about 1.3 mM calcium chloride for high KMNP concentration. At low KMNP concentration, similar shifting in calcium chloride concentration was observed as the required calcium chloride for the maximum G′ was reduced to around 0.8 mM.

5.4 Conclusion

The crude and dialyzed extracts from Krueo Ma Noy leaves exhibited gelling property in solution at 1% (w/v). The gel strength was increased with increasing polymer concentration. In addition, the melting point and melting enthalpy of Krueo Ma Noy pectin gel also increased with polymer concentration. Gelling characteristics were affected by a number of factors, such as KMNP concentration, pH and the present of co-solute and salt. A response surface methodology using 5 level central composite design was employed to evaluate, from the storage modulus G′, the effect of KMNP concentration (0-2%, w/v), sucrose (0-60%, w/v), calcium chloride (0-4 mM) and pH (2-8) on gelling properties. It was found that all the factors examined had significant effects on the strength of KMNP gel. The gel strength increased with increased KMNP concentration. The best interaction of sucrose, calcium chloride, and pH giving the maximum G′ under studied ranges was found at 45% (w/v) sucrose, 2 mM calcium chloride, and pH of 3.5 at KMNP 1.5% (w/v)



(a)



(b)

Figure 5.7 Response surface plots of the effects of $CaCl_2$ and pH on G′ at 45% (w/v) sucrose :

(a) KMNP 1.5% (w/v) (b) KMNP 0.5% (w/v)

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Chapter VI

SUMMARY AND RECOMMENDATIONS

A polysaccharide was extracted from the leaves of Krueo Ma Noy (*Cissampelos pareira*), a woody climbing plant from Thailand. The extraction conditions were 2% solids in distilled water at 25-28°C and natural pH (3.8-4.0). Alcohol precipitation and drying provided a crude extract and further dialysis and lyophilization provided a dialyzed extract. It was found that Krueo Ma Noy polysaccharide is a pectin that consisted mainly of high levels of galacturonic acid (~70%) and small amounts of monosaccharides. The dominant structure of Krueo Ma Noy pectin was established as a α-1,4-link-galacturonan by a combination of carboxyl reduction and methylation analysis and this structure was confirmed by FT-IR and NMR spectroscopy. The degree of esterification of Krueo Ma Noy pectins was 41.65% and 33.69% for crude and dialyzed pectins, respectively. Krueo Ma Noy pectin had an average molecular weight of 55 KDa, a radius of gyration of 15.2 nm and an intrinsic viscosity of 2.3 dL/g.

In diluted solutions, Krueo Ma Noy pectin exhibited Newtonian flow behavior while shear-thinning behavior was observed at a concentration of ≥ 0.5% (w/v). Both crude and dialyzed extracts gelled in aqueous solutions at 1.0% (w/v) and above at room temperature (25°). Krueo Ma Noy pectin (0.5%,w/v) also formed gel under acidic conditions (pH 2-4). The addition of NaCl significantly increased the gel strength when salt concentration was below 0.4 M. Krueo Ma Noy pectin was very

sensitive to the presence of $CaCl_2$: the addition of 1mM calcium chloride significantly increased gel strength. However, when $CaCl_2$ concentration was greater than 3 mM, precipitation occurred. The divalent cation (Ca^{2+}) showed greater effect on increasing the viscoelastic properties of Krueo Ma Noy pectin than monovalent cation (Na^{+}), exhibiting a typical property of low methoxy pectins. In addition, the presence of sugar increased G′ with an increase in sugar concentration. The gel strength, melting point and melting enthalpy of Krueo Ma Noy pectin increased with increasing polymer concentration.

The gelling characteristics were affected by a number of factors, such as pectin concentration, pH and the presence of co-solute and salt. A response surface methodology was employed to evaluate the concentration effect of pectin (0-2%, w/v), sucrose (0-60%, w/v), $CaCl_2$ (0-4 mM) and pH (2-8) on the gelling properties. The gel strength was evaluated from the storage modulus G′. It was found that all the factors examined had significant effects on the strength of Krueo Ma Noy pectin gel. The gel strength increased with an increase in Krueo Ma Noy pectin concentration. Increases of $CaCl_2$ concentration resulted in increases of gel strength until $CaCl_2$ concentration reached 2 mM. Further increases in $CaCl_2$ concentration decreased gel strength. When pH and pectin concentration were fixed, maximum gel strength was at 2 mM $CaCl_2$ and ~50% sucrose. Also, decreases in pH strengthened Krueo Ma Noy pectin gel.

On the basis of these conclusions, it is possible to make suggestions with respect to the future direction of research concerning Krueo Ma Noy pectin. Firstly, the complete study of the molecular structure and conformational analysis of Krueo Ma Noy pectin is needed. Secondly, comparison of functional properties between

Krueo Ma Noy pectin and commercial pectins should be carried out for potential applications of Krueo Ma Noy pectin. Thirdly, the effects of Krueo Ma Noy pectin and other hydrocolloids and/or protein interactions on its functional properties and microstructure should be studied. In addition, it would be useful to extract the Krueo Ma Noy pectin using several different methods to see how each method affects the galacturonic acid and sugar distribution, including the degree of esterification and its functional properties.

Biography

Jittra Singthong was born and brought up in Nakhon Ratchasima, Thailand. She attended Khon Kaen University, Thailand, and received her Bachelor's degree in Food Technology (1993). She worked at Thai President Food Co., Ltd. in the research and development in biscuit unit for one year. In 1997 she received a Master's degree in Food Science and Technology from Chiang Mai University, Thailand, and worked at Ubon Rajathanee University as a lecturer in the Department of Agro-Industry, Faculty of Agriculture. From 2000-2003 she obtained a scholarship from the Office of the Higher Education Commission, Ministry of Education (then Ministry of University Affairs) under the Consortium Sandwich Ph. D. Program, to pursue her Ph. D. study at the School of Food Technology, Suranaree University of Technology, Thailand and the Department of Food Science, University of Guelph, Canada together with the collaborative research at Food Research Program, Agriculture and Agri-Food Canada.