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Characterization of Commercial Pectin Preparations by Spectroscopic and Chromatographic Techniques.

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Characterization of Commercial Pectin Preparations by Spectroscopic and Chromatographic Techniques

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by

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ABSTRACT

Characterization of Commercial Pectin Preparations by

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Pectin has a long history as a food additive. However, elucidation of its fine structural and property relationships remains elusive. Recent research has focused on pectin's ability to complex with divalent heavy metals to aid in characterizing it. Commercial pectins of unknown composition were obtained from local grocers. Purified pectin samples from orange peel, lemon peel, and apple pomace, each of low and high levels of methyl esterification and of unknown distribution pattern were also purchased. Instead of metal complexation, several highly absorbing dyes such as Ruthenium Red, Nile Blue, and Acridine Orange were used to complex with the pectins and their resulting UV-Vis spectral patterns were employed to determine if one can characterize the different pectins. Chemometric methods are also included to aid in distinguishing them apart.

DEDICATION

The author wishes to dedicate this work to the memory of his grandparents, Glenn Alvin and Gladys Eleree Dalton. Their unconditional love, humility, and appreciation for the small things were an example to all.

ACKNOWLEDGEMENTS

The author wishes to express sincere appreciation to Professors Ho, Davidson, Jiang, and Wardeska for their assistance in the research, preparation of this manuscript, and presiding over the graduate committee. In addition, special thanks to Danessa Dixon whose love and support was my cornerstone during all phases of this undertaking. Thanks also to all faculty members of the Department of Chemistry for their valuable input into my education.

CONTENTS

Page

Chapter Page

Chapter Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Figure Page

LIST OF ABBREVIATIONS

Abbreviation or Symbol	**Term**
AO	Acridine Orange
AUA	Anhydrouronic Acid
DA	Degree of Amidation
DE	Degree of Esterification
DM	Degree of Methylation
ED	Euclidean Distance
GalA	Galacturonic Acid
GPC	Gel Permeation Chromatography
IP	Inner Products
MOPS	3-Morpholinopropanesulfonic acid
MW	Molecular Weight
NB	Nile Blue
PGA	Polygalacturonic Acid
R^2	Correlation Coefficient (%)
r	Correlation
RI	Refractive Index
RR	Ruthenium Red
UV-Vis	Ultraviolet-Visible

GLOSSARY

Degree of Amidation. Percentage of the C-6 carboxylate groups that are amide groups.

Degree of Esterification. Amount of esterification along the polymer backbone.

Degree of Methylation. 1. Moles of methanol present per 100 moles of galacturonic acid. 2. Percentage of the C-6 carboxylate groups that are esterified with methanol.

High Methoxyl Pectin. Pectin containing > 50% (of total ester groups) amount of methyl esterification (> 50 DM) along its backbone.

Low Methoxyl Pectin. Pectin containing < 50% (of total ester groups) amount of methyl esterification (< 50 DM) along its backbone.

Protopectin. Water insoluble parent pectic substance, which upon hydrolysis, yields pectinic acids.

Pectic Substance. Polyuronide composed mostly of anhydrogalacturonic acid residues.

Pectinic Acids. Colloidal polygalacturonic acid with very low methyl ester content.

Pectic Acids. Colloidal polygalacturonic acid devoid of methyl esterification.

Pectates. Mg, Ca, Na, or other Pectic acid salts.

Pectin. 1. A large, naturally occurring polymer contained within the middle lamella of plants. 2. Water soluble pectinic acid of varying methyl esterification- capable of forming gels under suitable conditions.

Pectinates. Mg, Ca, Na, or other Pectin salts.

CHAPTER 1

INTRODUCTION

History of Pectin

Pectin has a very long chemical history. As early as 1750, apple, currant, and quince jelly recipes were published in the "London Housewife's Family Companion"[1]. In 1790, Vauquelin reported[2] pectin as a soluble substance in fruit juice. Scientific study in 1825 by Henri Braconnot[3] led to a detailed description of "Pectin-Acid", derived from Greek "*pectos*" which means to solidify, congeal, or curdle. The commercial production of liquefied extract of pectin began in 1908 Germany and was quickly patented in the United States[4].

Sources of Pectin

General acceptance states that pectin comprises up to 35%[5] of the cell wall of most terrestrial plants where alginates and carrageenans play a similar role for their marine counterparts. All green land plants contain pectin to a certain degree. Pectin content in dicotyledonous (flowering) plants far outweighs that contained in monocotyledonous (seed-bearing) plants and grasses. See Table 1 for a representation of the content of pectin in monocots versus dicots.

Table 1. Classes of pectin

Components	Monocots (%)	Dicots (%)
Cellulose	30	30
Pectin	5	35
Arabinoxylan	30	5
Xyloglucan	4	25
β-(1,3),(1,4)-Glucans	30	0
Glycoprotiens	1	5

Classes of Pectin

Two main classes and several subclasses of pectin exist[6]. High Methyl Ester (HM-) pectin is the first general class of pectin. In this type of pectin, a high portion (>50%) of carboxyl groups exists as a methyl ester. The remaining carboxyl groups exist as the free acid, ammonium, sodium, calcium, or other rarer salts. Low Methyl Ester (LM-) pectin is the second general type. For this type of pectin, less than 50% of the carboxyl groups exist as the methyl ester variant. These are usually obtained from mild alkali or acidic treatment of HM-Pectin. Small quantities of acetylated pectin (small amount of acetyl esterification) can be found in all plant sources. Amidated pectin[7] is obtained from HM-Pectin when ammonia is used in the alkaline deesterification process. During this process, some of the carboxyl groups are converted to the acid amide. Small quantities of amidated pectin can be found naturally in sugar beets and certain other sources.

Chemistry of Pectin

Pectins are a family of complex, anionic polysaccharides found in the primary cell wall and intercellular regions of higher plants[8]. Pectins, as a compound, are linear polysaccharides composed primarily of D-galactopyranosyluronic acids joined via $\alpha(1\rightarrow4)$ glycosidic linkages. This regular structure is intradispersed with L-rhamnopyranosyl units, or “hairy regions”, methyl ester groups and rarely, neutral-sugar side-chains. The galacturonic acid units contained within pectins can be either partially methyl-esterified, acetylated or both. Figures 1-3 are typical representations. Pectins, as naturally found, generally have an average molecular weight (MW)

of approximately 200 kDa (with 300 kDa being a normal occurrence) and form strong gels in the presence of cations (divalent or monovalent) or small sugars.

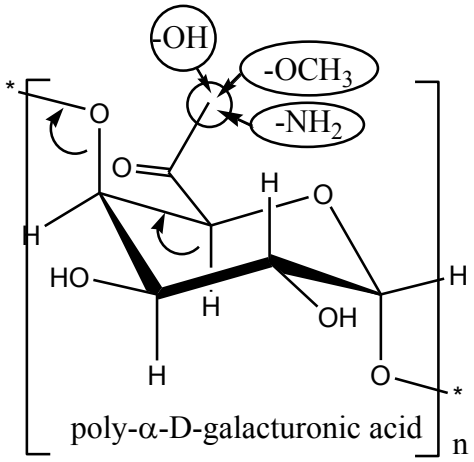


Figure 1. Naturally occurring forms of D-galacturonic acid residues where arrows indicate possible β-elimination in the ester form

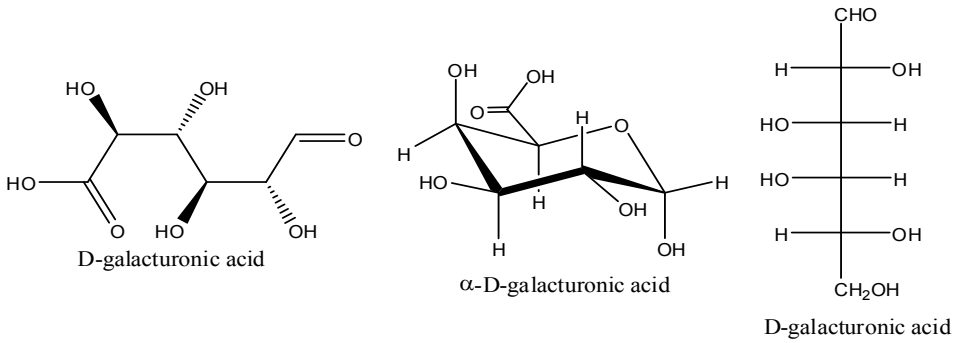


Figure 2. Alternative representations of D-Galacturonic Acid

Pectin nomenclature includes many terms as defined by the Committee for the Revision of the Nomenclature of Pectic Substances[9] and has seen many changes over the years. A current definition for these molecules is lacking. Effort will be made to maintain current commercial nomenclature within this text. Pectins are employed in several different forms

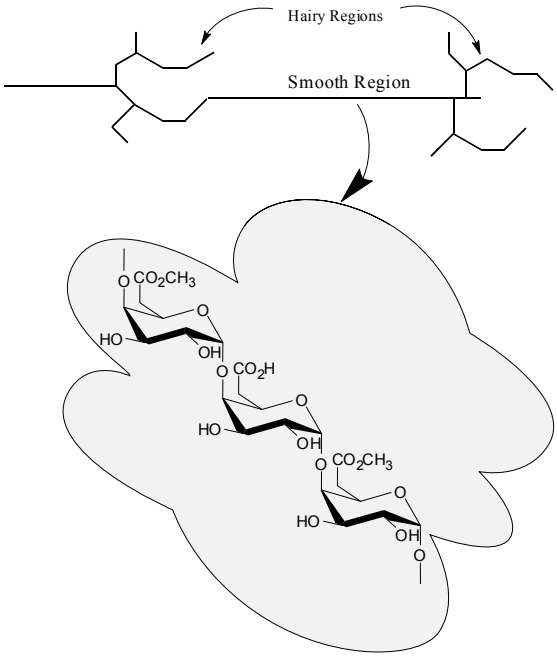


Figure 3. Overview of the pectin structure. Homogalaturonan with partially methyl-esterified α-(1→4)-linked D-Galacturonic acids comprises the backbone in the smooth region

such as pectinic acids, pectinates, pectic acids, and pectates, where pectin, as an over-arching term, encompasses all listed forms.

Pectic acids, with various degrees of neutralization, are poly(α-D-galacturonopyranosyluronic) acids (poly-D-galacturonic acids for short) with no or very small amounts of methyl esterification. Pectinic acids, on the other hand, contain appreciable levels of methyl esterification along its backbone. Pectates and pectinates are salts of these molecules, respectively. Pectins are generally classified based on their degree of esterification (DE) or degree of methylation (DM) of contained carboxyl groups with methanol. High-methoxyl, or HM-pectins, have a DE of 50% or greater while low-methoxyl (LM-) pectins have a DE of less than 50%.

Commercially, about 17000 metric tons of combined HM- and LM-pectin were produced in 1998[10]. The most common industrial sources of pectins are apple pomace and citrus peel. However, other sources have been used, including sunflower heads, sugar beet pulp, and potato pulp, all depending on a given region's agricultural base. The commercial process for pectin isolation was first described by May in 1990[6] and followed in more detail by Voragen et al. in 1995[11]. The predominant commercial class of pectins includes fast-gelling pectins (DE > 70%) or slow- to medium-set pectins (DE 60-70%). Conversely, LM-pectins do not have a grading system because of the number of variables that affect gelation. Pectins of all forms are typically sold as powders. The major usage of pectins is in food applications. Their main function in this role is as a thickening or gelling agent for jams and jellies, bakery glazing and fillings, fruit preparations for dairy products, confectionary, or as a stabilizer in milk or fruit beverages[12]. An added characteristic of pectins is that they function as water-soluble dietary fibers. In this utility, pectins are resistant to hydrolysis by human digestive enzymes and only partially broken down by intestinal bacteria to short-chain fatty acids, methane, carbon dioxide, and water. In this light, new pectins have been developed as fat-substitutes[13, 14].

Generally, pectins are soluble in water thereby making them insoluble in most organic solvents. Further, pectic acids are only soluble when complexed with monovalent salts. Divalent salt complexes of pectic acids are of limited solubility while trivalent salts are insoluble. The typical solubility trend is observed as decreasing with increasing ionic strength and MW and decreasing DE. If gelling conditions exist, pectins will not dissolve. A gel is simply a system within which the contained polymer is in a state of flux between being fully dissolved and being

precipitated. Flory[15] describes gels as polymer molecules cross-linked to form an interconnected three-dimensional network immersed in a liquid medium. In Pectins, as with most other food gels, cross-linking occurs as additive, weak, intermolecular interactions with no to very low kinetic activity, atypical to the covalent-linkages usually seen in synthetic polymer gels. Therefore, pectin gels suffer from a temperature threshold above which no gelation can occur. Generally, in polysaccharide gels changing either the temperature or counterionic pH variations induce cross-linking[16]. In typical use, pectins form two types of gels: pH and sugar dependant and cation dependant. In the first case, high DM pectin is required with an acidic pH (< 3.6) and a high concentration of simple sugar (> 55% w/w)[17]. This particular gel system is widely seen in the manufacture of jams and jellies. The other type of gel involves the use of lower DM pectin and the presence of a divalent cation (e.g. calcium). This system has the advantage of gelation over a wide pH range without the presence of a sugar.

High-methoxyl pectins, as mentioned above, form gels with the presence of a high concentration of co-dissolved sugar and at an acidic pH. The presence of the sugar as a co-solute decreases water activity and effectively dehydrates the pectin (by robbing the pectin of needed solvent to induce precipitation) while the low pH hinders ionization of the carboxyl groups thereby decreasing electrostatic, intramolecular repulsion within the pectin chains. Pectin is inherently a complex, polyprotic, weak carboxylic acid with a pKa of approximately 3.5 to 4.5. By lowering the pH below this pKa, the molecule becomes less hydrophilic and thereby increasing the tendency to form gels. These effects increase chain-to-chain interaction and lead to partial precipitation, or gelation. Hydrogen bonding[18] and hydrophobic interactions of the

ester groups[19] stabilize the structure. As previously mentioned, though these interactions are weak, their cumulative effects are sufficient to provide thermodynamic stability to the three-dimensional network as shown in Figure 4 where interaction zones for High-methoxyl pectin is a typical representation.

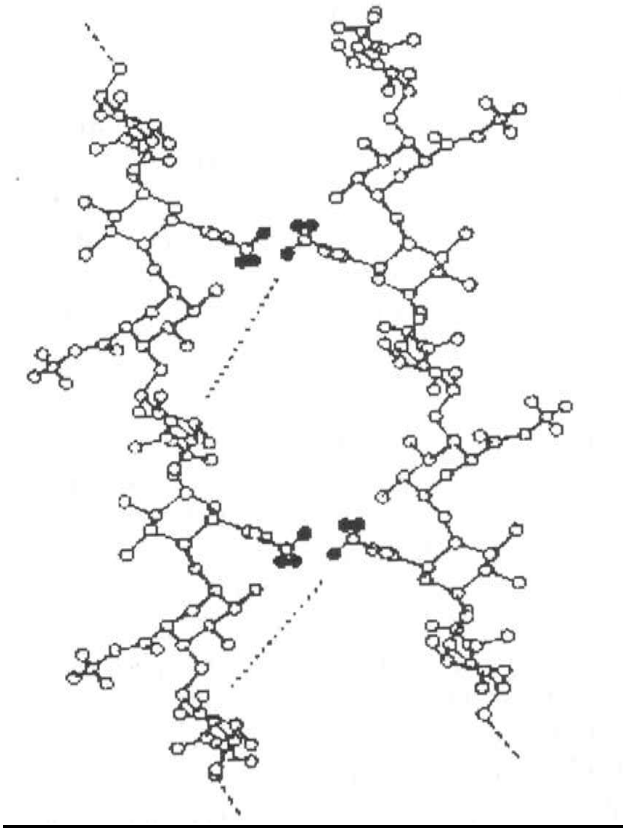


Figure 4. Interaction zones for High-methoxyl Pectin.[20] *Source*: Walkinshaw, M. D.; Arnott, S., Conformations and interactions of pectins. II. Models for junction zones in pectinic acid and calcium pectate gels. *J. Mol. Biol.* **1981,** 153, 1075-1085.

Low-methoxyl pectins, as previously mentioned, form gels in the presence of a counter cation (most commonly calcium) with little or no co-solute sugar. This occurs within a wide pH range and with higher temperature stability. As is commonly known[21], the lower the pectin DE the greater the tendency to precipitate in the presence of a cation, generally calcium. Calcium

and *d*-orbital, divalent, transition metals (e.g. Cu^{2+}, Co^{2+}, Pb^{2+}, etc.), use unfilled orbitals to form coordination complexes with neutral and acidic carbohydrates. It is suggested that calcium's radius (0.1 nm) is large enough to coordinate with the spatial arrangement of oxygen atoms in many sugars and can have extreme flexibility in the direction of its own coordinate bonds[22]. A proposed structure for calcium-induced gelation is shown in Figure 5. By cooperatively and

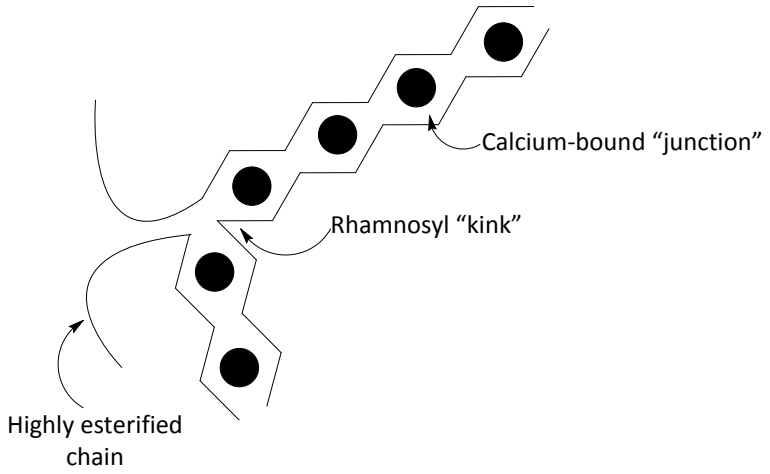


Figure 5. Low-methoxyl calcium pectate gel.

consecutively "cross-linking" 7-14 oxygens of two adjacent chains a high degree of gel stability can be achieved[23]. Clearly, in this case a blockwise distribution of carboxyl groups can be very sensitive to low calcium levels[24]. As with high-methoxyl pectins, several factors influence gelation of low-methoxyl pectins. As previously alluded to, the presence of methoxyl groups hinder gelation; therefore, low DM is desirable. Also, the lower the DM the less calcium required to induce precipitation. Conversely, the lower the MW of respective pectin chains the more calcium is required to induce gelation[20]. Calcium typically will not precipitate pectins with a DE greater than 60%. As with methoxyl groups, anything that disrupts the blockwise

distribution of carboxyl groups will adversely affect gelation (e.g. acetyl groups, rhamnose side chains, etc.) other than when the disruption promotes chain association as occurs with amide groups. Figure 6 shows a plot of calcium-concentration effects and the optimal calcium level for a given pectin as related by gel strength.

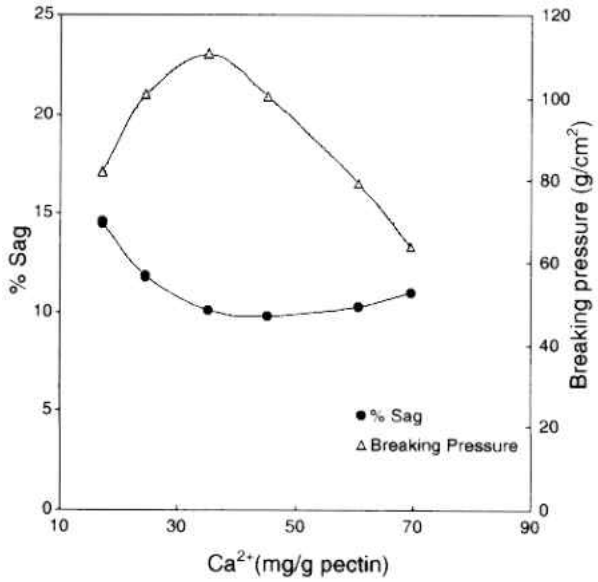


Figure 6. Calcium concentration effects on LM-pectin gels. (pH=6, 30% sugar, 1% pectin)[25]
Source: El-Nawawi, S. A.; Heikel, Y. A., Factors affecting the production of low-ester pectin gels. *Carbohyd. Polym.* **1995,** 26, (3), 189-193.

This optimum will shift as determined by interaction of previously listed variables. Although LM-pectin can form gels over a wide pH range, optimal pH occurs above the pKa (~ 3.5). From 3.5 to 7.0 pH, carboxyl groups become ionized and consequently increase chain repulsion and have a greater tendency to interact with a cation. Co-solute sugars are not necessary to induce gelation as with HM-pectins, but at levels up to 30% (w/w) gelation of LM-pectins is enhanced[26].

At a pH higher than neutral (about pH 8) pectins become unstable, being subjected to β-elimination at the C-4 position when the C-6 carboxylate group is esterified. The arrows shown in Figure 1 indicate this process. Therefore, HM-pectin is very vulnerable to degradation even at room temperature and pH 5[27]. Pectins, HM and LM, are subject to limited hydrolysis at lower pH values. However, this is miniscule when compared to depolymerization though β-elimination at high pH ranges. Chain stability at low pH remains high. This feature is a very desirable pectin trait as food preparations are typically done at acidic pH. In addition, pectins as a solid lose less than 5% of their grading level per year when stored at room temperature and low humidity, making them stable during long-term storage.

Jams and jellies remain the largest commercial outlet for pectins. Prior to the commercial availability of pectins, jam (and jelly) manufacturing had to rely on the inherent pectin content of the given fruit and very high temperatures. Under these harsh conditions, traditional jams were very limited on fruit selection. In addition, jam color, aroma, and vitamin content were lacking. Other commercial applications of pectins are listed *vide infra*. The use of pectins in food is a rapidly expanding market- not only from a gelling perspective but as a thickening agent also. Some of these new applications include reduced-sugar jams, heat-resistant jam for baked goods, heat-reversible glazing, yogurt preparations, fruit jellied candies, etc. In addition to these food preparations, pectins are becoming widely used for stabilization of oil-in-water emulsions[28], fat-replacement food components[29] and limited pharmaceutical uses (e.g. wound dressings[30], gastric ulcer treatment[31], acid reflux prevention[32], etc.).

Pectins are becoming a very important product derived from renewable resources. The "Green Initiative" in many countries is pushing this type of research to the forefront. Pectins have a variety of very important uses in more than just the food industry. Unfortunately, as a class of compounds pectins are very complex polymers, difficult to analyze and ungainly to functionalize on a manufacturing scale. Thankfully, interest in renewable chemistry is driving research in this area to new discoveries on a daily basis. As a cheap and versatile raw material, pectin is poised for many great applications.

Industrial Production of Pectins

As an abundant raw material, apple pomace and citrus peels are the sources from which most industrial pectins are derived. However, raw materials are highly dependent upon local crop sources. In some parts of the world, sugar beet pulp, sunflower heads, or potato pulp are used. May[6] and Voragen[11] previously described, as summarized in Figure 7, the industrial process for pectin extraction in detail.

The source materials are refluxed with dilute mineral acid (~ pH 2) at 60-100°C for 1-10 hours. The hot pectin extract is separated from the solid residue and pectinase-free α-amylase is added to hydrolyze starch if the source is apple or peach pomace. The clarified extract is concentrated under vacuum to ~ 4% pectin content and precipitated with 2-propanol. The precipitate is washed, dried, and ground to a powder form. Desired yield and DE determine extraction temperature and time (de-esterification proceeds faster than de-polymerization at lower temperatures). Low DE types of pectin are produced via acidic treatments at various stages during the extraction process. When ammonia is used for this purpose, amidated

pectins are obtained as a final product. To get a product that is consistent over a range of properties, blending batches and dilution with sugars is customary[6].

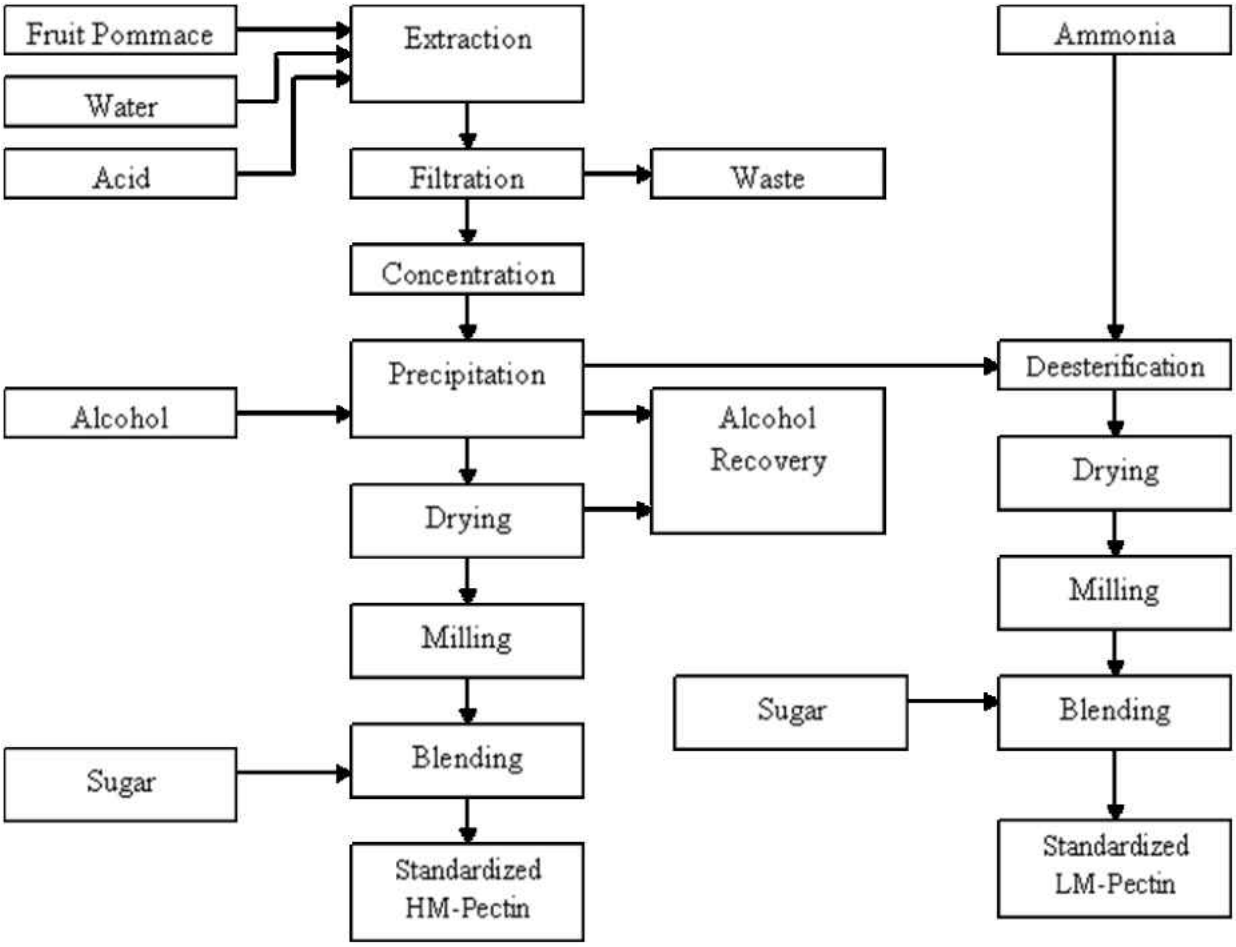


Figure 7. Industrial production of pectins

Important Uses

Historically, Pectins have found use as general texture modifiers and gelling agents. Among the more common applications, pectin is used extensively in jams, jellies,

confectionaries, deserts, yogurts, and anti-diarrheal agents. Some of the more important modern uses include: 1) Ca^{2+} sequestering agent in detergents, 2) fillers in low calorie food products, 3) edible acidifying agents, 4) rheology modification, 5) biodegradable surfactants and emulsifiers, 6) edible packaging, 7) dairy stabilizers, and 8) dietary fat replacements (e.g. Slendid™, GENU™, and Olestra™)[33, 34].

Recently, new applications of pectin have become very important. Most prominent is the treatment of wastewater effluents where pectin has found extensive use in treatment regimens involving contamination with heavy metals. Pectins of most configurations show affinity for complexation with metal ions in aqueous solutions. Also, as an excipient, pectin efficiently encapsulates many pharmaceutical actives that are expatriated in the human large intestine and colon thereby greatly increasing drug efficacy.

Commercially Important Measurables

Years of pectin research have produced several important commercial analytical measures. While not all-inclusive, discussions of seven methods are included below.

Absolute, Relative, or Weight Average Molecular Weight

MW is a very important physical property of pectin. It is the most important characteristic in determining the functional behavior of pectin. Gelling abilities of individual pectins are tied very closely with MW.

Total % Galacturonic Acid (%GalA)

GalA content, as with MW measurement, is important to the gelling capabilities of given pectin. For this measurement the polymer is degraded to monosaccharide via one of numerous chemical or enzymatic methods and subsequently analyzed.

Degree of Esterification (DE, %DE)

DE is an important molecular index for pectin classification that describes the extent to which carboxyl groups in pectin molecules exist as the methyl ester. Depending upon method of analysis, %DE can be expressed as either the ratio of esterified carboxyl groups to total carboxyl groups (100% theoretical maximum) or as percentage ester content (~ 16.3% theoretical maximum). DE is measured through various techniques, but titrimetry is a long-standing, classical method of DE determination[35]. For the titrimetric method the free carboxyl groups are protonated via washing the pectin with acidic alcohol and then drying. Subsequent dissolution in water and titration with a standard base, DE of the pectin sample is determined. Acetyl content of the pectin can lead to overestimation of DE content. %DE can be calculated as shown in Equation 1.

$$\%DE = 176 \times \frac{176 \times CH_3O\%}{31 \times AUA\%} \times 100 \qquad \text{Equation 1}$$

where AUA (GalA) is anhydrouronic acid content. In addition, 176 and 31 are MWs of AUA and MeO respectively.

Degree of Methylation (%DM)

%DM is an important molecular index for pectin classification that describes the extent to which carboxyl groups in pectin molecules exist as the methyl ester in ratio to all esterified

groups. Methoxyl content can be determined through enzymatic or alkaline demethylation. Analysis of methanol is achieved through various techniques. Most notable of these is a spectrophotometric technique involving reaction with potassium permanganate or alcohol oxidase and subsequent condensation with pentane-2,4-dione to yield a colored product. A common HPLC method[36] provides simultaneous measure of methyl and acetyl content.

Degree of Amidation (%DA)

%DA is another important molecular index for pectin classification that describes the extent to which carboxyl groups in pectin molecules exist as the amidated ester in ratio to all esterified groups. This measure can be a fingerprint for pectin characterization.

Neutral Sugars

Total acid hydrolysis of a pectin sample is typically used to determine total neutral monosaccharide content. This modern standard of analysis involves methanolysis with 2M HCl prior to trifluoroacetic acid hydrolysis. Subsequent monosaccharides are converted to alditol acetates and analyzed via GLC or HPLC with refractive index detection.

Random or Blockwise Carboxyl Distribution

Distribution pattern of esterification greatly influences gelling power or complexing ability of a pectin polymer. This is a very important property for the emerging pharmaceutical applications of pectin. Several methods exist for this determination including measurement via UV-Vis with Ruthenium Red (RR), carbazole, or hydroxamic acid (hydroxylamine).

CHAPTER 2

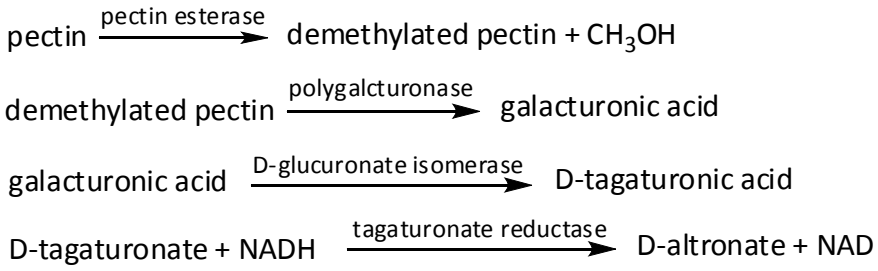
TECHNIQUES FOR STRUCTURAL ANALYSIS OF PECTINS

Current research includes numerous techniques for structural analysis of pectins. No one current method is all-inclusive. Therefore, analyses of pectins are a combination of many techniques. Discussions of only the more common techniques are included in this chapter.

Enzymatic Analysis

Enzymatic analysis is an old technique having been used in the 1880s for starch and sucrose in carbohydrates[37]. Availability of spectrophotometers after World War II allowed widespread enzyme use in the analytical laboratory. Enzymes are highly specialized proteins that exhibit catalytic activity towards specific substrates. Enzymes produced in higher plants or microorganisms easily degrade pectic substances. This degradation produces textural changes in fruits and vegetables during ripening, storage, and processing. Pectic enzymes comprise two classes: esterases and depolymerases. Pectinesterases include many forms, such as methyl esterase and acetyl esterase, which split off methoxyl and acetyl groups, respectively. Pectin depolymerases, such as polygalacturonase and lyase, degrade the polymeric backbone by cleaving α-D-(1→4) linkages. Another group of enzymes that achieve this same degradation via β-elimination are characterized as transeleminases[38].

Enzymatic degradation precedes several analytical measures for pectin characterization. The enzymatic route of pectin analysis involves multiple steps and analyses of reactions or products. This is conducive to real time, online analysis. This route is shown below:



For pectins, GC and HPLC analysis can be used to analyze the methanol released during the reaction or the reduction of NADH can be spectrophotometrically measured at 340 nm.

Enzymes offer excellent specificity, high sensitivity, rapid sample preparation for analysis, low-cost equipment, and ease of automation. Unfortunately, several enzymes used for pectin analysis can often be quite expensive due to supply and demand. In addition, they are prone to degradation after prolonged reaction times under conditions necessary for some pectin analyses. Another disadvantage is factors affecting enzymatic determination must be carefully controlled. The most important factor is temperature as higher temperatures increase enzymatic activity but can quickly denature the enzyme. Temperature control is typically within the range of +/- 0.1 °C[37].

Most enzymes have a narrow pH range for optimal activity. Outside their pH range, enzymes can be denatured and rendered ineffective. Therefore, buffer solutions are necessary to maintain desirable conditions. Unfortunately, some characteristics of pectins preclude the use of many common biological buffers. In addition, due to pectin's high affinity for heavy metals, it is common for some metal contamination to be present in a pectin preparation. This cationic impurity can act as an inhibitor to the enzymatic catalysis and, thus, slow the desired modification of the pectin.

Various techniques are used to measure the products of an enzymatic modification. As hinted previously, spectrophotometric analysis is a common and preferred method. For products that absorb appreciably in the ultraviolet, visible, or infrared region of the electromagnetic spectrum, concentrations can be directly measured. Indicators are added to produce colored complexes when native absorbances of the analytes are low. Pectins can interact with added indicators through numerous mechanisms and produce desirable or undesirable results.

Enzyme electrodes have also been commonly used to analyze specific carbohydrates. The choice enzyme is immobilized upon a membrane or film coated electrode. For this system, amperometric detection is commonly employed. Electrodes are available for the determination of glucose, maltose, sucrose, lactose, and most importantly (for pectin analysis) galactose. However, for the determination of galactose, galactose oxidase electrodes lack specificity. Stoecker and Yacynyeh immobilized galactose oxidase on a solid resin support to produce a column that catalyzes the oxidation of galactose to an aldehyde[39]. Hydrogen peroxide produced in the reaction could be measured via amperometry or chemiluminescence.

Ultraviolet-Visible Spectrophotometry

Spectrophotometry is the quantitative study of electromagnetic spectra. A spectrophotometer measures light intensity as a function of the wavelength of light. UV-Vis spectroscopy optimally deals with the spectrum between 190-750 nm. The Beer-Lambert law states that the absorbance of a solution is directly proportional to its concentration. Thus, UV-

Vis spectroscopy can be used to determine the concentration of a solution. The law is expressed mathematically in Equation 2:

$$A = -\log_{10}(\frac{I}{I_0}) = \mathcal{E} \times c \times L \qquad \text{Equation 2}$$

where A is measured absorbance, I_0 is intensity of incident light at measured wavelength, I is transmitted intensity through the sample, L is the pathlength through the sample, c the molar concentration of the absorbing species, and $\mathcal{E}$ the molar absorptivity given in mol/dm^3. Molar absorptivity is a constant, fundamental molecular property in a given molecule at a given temperature and can easily be calculated using a solution of the compound with known concentration. The Beer-Lambert Law is useful for characterizing many compounds but is not universal. A second order polynomial relationship between absorption and concentration is sometimes encountered for very large, complex molecules such as organic dyes (e.g. Acridine Orange and Ruthenium Red). Figure 8 highlights the basic components of a spectrophotometer. When the spectrophotometer uses a photodiode array detector the monochromator is placed after the sample in order to disperse the light onto the detector.

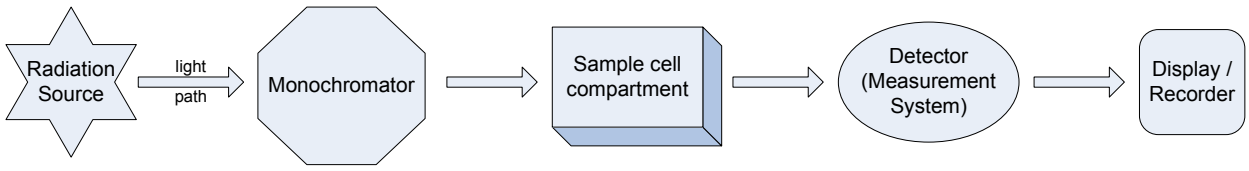


Figure 8. Basic construction of a spectrophotometer

Older spectrophotometers used phototubes and photovoltaic cells to provide an electrical signal proportional to the intensity of impinging radiation upon itself. Many modern

instruments are constructed as multichannel systems. This capability stems from the usage of a silicon photodiode array as the detector. The diode arrays are an assembly of several hundred silicon diodes detectors in a linear pattern on a silicon chip. These chips average 1 to 6 cm in length with the width of individual diodes being 0.015 to 0.050 mm. The chip contains a switch and capacitor for each diode element. A shift register closes each switch for a fraction of a second in order to charge its companion capacitor to -5V. Impinging radiation on a diode surface causes partial discharge of its matched capacitor, proportional to the incident light flux. This lost charge is replaced during the next switching cycle. The amount of current needed for this recharge is digitized into a useable signal. With one or more diode arrays placed along the focal plane of a grating monochromator, the entire spectral wavelength can be measured simultaneously within a fraction of a second.

Due to advances in instrument design, numerous accessories are available to accommodate specialized functions. Some of the more important add-ons are special cell holders, peristaltic pumps for continuous flow sample analysis, auto samplers, multi-cell transporters, Peltier temperature controllers for thermostating, and advanced software for time-series analysis.

Colorimetric Spectrophotometry

Colorimetric analysis (colorimetry) is an old and proven analytical technique. It is traditionally a subset of visible spectroscopy. Colorimetry has the advantages of rapid analysis, small sample size, versatility, inexpensive equipment, and good sensitivity. For pectins, total AUA content is a very important measure[40]. While several analytical techniques[40-43] currently

exist for this measure, due to the aforementioned traits (e.g. size of polymer and heterogeneous nature), colorimetry is the most common[44]. In addition, industrial pectin preparations appear to be similar on gross analysis but exhibit differing physical properties that can be modeled, in part, through UV-Vis analysis. Through colorimetric analysis, the original pectin polymer can be analyzed with minimal chemical modification[45]. Several of the more common colorimetric methods follow.

First described by Bergman[46], hydroxylamine has been used for many years as a colorimetric reagent in the determination of amides. For this reaction, a concentrated aqueous solution of hydroxylamine hydrochloride converts esters to hydroxamic acids. Red complex formation of hydroxamic acid is measured at 600 nm. It is important to note PGA gives no reaction with hydroxylamine.

Dische developed a method for quantitating uronic acids using carbazole in sulfuric acid[47]. This method was inadequate in distinguishing between uronic acid monomers; therefore, Dische further refined the procedure[48]. This newer method was able to distinguish between GalA and other polyuronides. Exact timing of the reaction was critical to the determination. In efforts to simplify the assay, numerous researchers have refined this method but all suffered from lack of specificity[49-53]. An extensively modified version[54] of this method involves purification and solubilization of pectin with EDTA, deesterification with sodium hydroxide and subsequent reaction with a pectinase. Further treatment with carbazole and measurement at 530 nm determines AUA content. This reaction has limited interference from organic acid, hydroxyl acids, and common sugars. Unfortunately, it does not discriminate

between uronic acids and hexoses. In addition, an amount of cross-reactivity occurs with aldose sugars.

Orcinol has been extensively used to determine total uronic acid content of polysaccharides[55]. For this method, a polyuronide is reacted with a modified Tollens'/Bial reagent and the extinction coefficient measured at 650 nm. This method is very labor intensive, lacks accuracy of other methods, and is highly sensitive to interferences such as pentoses and furfurals.

A common reagent, 3,5-dimethylphenol is used to determine total uronic acid content in polysaccharides[56, 57]. When pectin is hydrolyzed and the reagent added, a colored complex is produced suitable for spectroscopic analysis. Absorbance is measured at 400 and 450 nm. The calculated difference between these absorbances indicates amount of uronic acid in the sample.

Developed by Thibault[58], m-Hydroxydiphenyl assay has been extensively used to determine total AUA content in pectin. When uronic acid is heated in concentrated sulfuric acid/tetraborate and further treated with meta-hydroxydiphenyl, a chromogen develops. This chromogen greatly reduces interferences of neutral sugars prevalent in other colorimetric methods. In addition, specificity and increased sensitivity are enhanced versus other listed colorimetric methods.

Metachromasy

Since the discovery of metachromasy in 1875, aggregation of cationic dyes with acid polysaccharides in aqueous solutions has been extensively studied[59-63]. Higher MW

polysaccharides have been shown to produce metachromic shifts among popular cationic dyes[62-67]. Metachromasy can occur through superposition of neighboring dye molecules caused by coiling of the polymer chain. Also, Stone[63] deduced that dye-dye interactions played a role in metachromasy. Stoddart[68] explained both a spectral shift and hyperchromic effect caused by interaction of RR and PGA. Stoddart's study postulated RR would continue to interact with pectin even after full protonation of the pectin carboxyl groups.

Complexometric Analysis

Recent *in vitro* studies have shown varying levels of pectin complexation with divalent and trivalent metal cations[69, 70]. These studies indicated different classes of pectin show high selectivity toward metal ions. Thus, differentiation of unknown aqueous pectins can be achieved by targeted complexation and precipitation by select metal cations. Binding affinity[69, 70] is generally in the order of $Cu^{2+} \approx Pb^{2+} >> Co^{2+} \approx V^{2+} \approx Zn^{2+} > Cd^{2+} \approx Ni^{2+} > Ca^{2+}$ with several other metal cations known to form complexes with pectin. Researchers have shown different classes of pectin (e.g. citrus, apple, sugar beet, etc.) to be highly selective within each of these categories.

Molecular Weight Determination

'Molecular weight' (MW) or 'relative molecular mass' is a fundamental parameter in characterizing a macromolecule. Despite this fundamental nature, polysaccharides have proven very difficult to analyze. Theory and practice suggest numerous reasons for this but can be summarized within four basic categories that are true for all polysaccharides. First is polydispersity where the polymer consists of chains of multiple and different MW species.

Next, the polymer can be thermodynamically non-ideal. This results when high thermodynamic exclusion volumes result from high solvent affinity or asymmetry. A further complication here is the polymer can exhibit polyelectrolyte behavior. Analytical techniques whose results rely upon certain assumptions about chain conformation can be seriously affected by the polymer in solution (e.g. MW). Finally, numerous polysaccharides aggregate in aqueous solutions giving rise to false MW determinations. This is partly indicative of the large diversity of data reported in literature.

The performance of pectin as a gelling or thickening agent is diminished by the presence of low MW components in the MW distribution. As mentioned, measuring MW of pectins and other naturally occurring polysaccharides is difficult due to their polydisperse nature and the tendency to form aggregates in aqueous solutions. This lends to the diverse data recorded in literature for pectins. All MW methods fit into one of three categories: absolute, relative, and a combination of both. Absolute techniques include light scattering[71, 72], membrane osmometry[73], and sedimentation equilibrium[74]. Being absolute, these methods do not require comparison to a known standard. Light scattering is the most common technique for pectin analysis. However, light scattering is very susceptible to error caused by chain aggregation.

Relative techniques include gel permeation chromatography (GPC)[75, 76], viscometry[76], and sedimentation velocity[77]. These methods require standardization against a known MW compound. For gel permeation, neutral, particulate gel media has been shown to give separations based upon size of components in a mixture. It is important to note this method is both nondestructive and occurs under mild conditions. In addition, for most separations,

composition of the mobile phase does not play a large role. This gives the advantage of being able to choose a suitable mobile-phase based upon particular species of interest. However, because of this phenomenon, gradient elutions are not available for this type of analysis.

As mentioned, particulate gels comprise the stationary phase within which the solute can penetrate through the entire volume of the compacted particles. Adsorption is an undesirable trait for this type of analysis. Therefore, conditions are chosen so that elution volumes depend only on the sizes of solutes. Large solutes elute first due to lack of penetration into the particulate bed. GPC, like other chromatographic techniques, are governed by the van Deemter equation.

Recent years have seen the introduction of new methods relying on a combination of absolute and relative measurements to provide a more complete MW profile of a complex carbohydrate[78].

CHAPTER 3

METHODOLOGY

The following sections describe historical techniques and proposed research and provide a cursory examination of the techniques and methods used to develop this study.

Spectrophotometry

A Hewlet Packard 8452A instrument was used for experimentation. The HP 8452A Diode-Array Spectrophotometer is a single-beam, microprocessor-controlled spectrophotometer.

The 8452A spectrophotometer uses a photodiode array consisting of 316 elements each with a dimension of 18 x 0.5 mm. The dispersion of the grating and the size of the diode elements are such that a resolution of 2 nm is realized throughout the UV-Visible range of 190 to 820 nm. With this installed photodiode array, few optical components are needed thus resulting in a radiation throughput much higher than that of traditional spectrophotometers. This throughput allows a single, low-noise deuterium lamp to be used as the source for not only the ultraviolet but for the visible region of the spectrum as well. Additionally, sample photodecomposition is minimal, in comparison to traditional instruments, due to very short exposure times.

For experimentation, precisely matched, fused quartz, 1 cm path-length, low-volume cuvettes are used. During analysis, the source polychromatic light is collimated and passes through the sample onto the monochromator entrance slit. From there, the beam passes onto a fixed, aluminum-coated holographic grating to disperse onto the photodiode array. Because

the system employs no moving parts, wavelength reproducibility from scan to scan is extremely high (± 0.05 nm) and a single scan only requiring 0.1 s. To improve measurement precision, the instrument scans the spectra over a 3 s time interval and records the spectral mean and standard deviation at each wavelength. Extreme stability of the source and electronic system is such that the signal of the blank only need be analyzed every 5 to 10 minutes.

Gel-Permeation Chromatography

The origins of chromatography dates to 1906 when discovered by Russian botanist, Mikhail Tswett, while analyzing and separating vegetable pigments[79]. Tswett named the process chromatography from the Greek words *chroma* and *graphy* translated as "color writing". For chromatography, the analyte must be dissolved into a liquid that is then passed into the chromatographic device containing fixed chromatographic particles. Liquid Chromatography is a type of chromatography employing a liquid mobile phase and a finely divided, immobilized stationary phase. Although many classical chromatographic techniques exist, modern HPLC did not come into existence until 1967 with the development of technologies allowing its advancement[80]. The name *High Performance Liquid Chromatography* was coined to distinguish these newer techniques from their classical counterparts. Several types of HPLC are commonly employed. Gel-Permeation Chromatography (GPC) is a modern variation of High Performance Liquid Chromatography (HPLC) relying on size-exclusion principles for non-polar species. Because of its wide versatility and applicability, HPLC is one of the most commonly used separation techniques today.

Liquid-chromatographic columns are mostly constructed from stainless steel but heavy-walled glass tubing is sometimes employed for specific analyses. A typical column length is 10 to 30 cm with an inner diameter of 4 to 10 mm. Column packings are as diverse as the types of analyses performed but typically have particle sizes of 5 to 10 µm. Sepharose™ is a common stationary phase used for polysaccharide analysis as it provides good separation for a wide range of these polymers. Sepharose is a cross-linked, beaded form of agarose providing gel filtration in a broad fractionation range. Agarose contents are available in 2%, 4%, and 6% and marketed as 2B, 4B, and 6B respectively.

No highly sensitive, universal detector system exists for GPC. However, refractive index (RI) is used in this project as it has found considerable use in modern GPC and HPLC analysis in general. Measurement is based upon changes in the refractive index of the solvent that is caused by analyte molecules. Refractive index is a general rather than selective method as it reacts to the presence of all solutes in a solvent. RI detectors are sensitive to temperature change, pressure, and solvent composition. All solvents used for the mobile phase must be carefully degassed and the detector thermostated. This detector suffers from limited sensitivity with a LOD of 100 ng to 1 µg. However, refractive index has an order of magnitude higher sensitivity than light scattering detection.

Colorimetry

Carboxyl substitution pattern and MW play a vital role in pectin's ability to form complexes especially with heavy metals[69, 70, 81]. Ruthenium Red (RR) has been shown as an

acceptable indicator for measuring distribution patterns under controlled conditions[45] (< 60% DE); however, numerous interferences occur with the measurement.

Acridine Orange (AO), Nile Blue (NB), and several other cationic dyes have traditionally been used as biological stains for polyanions in uncontrolled conditions[62, 63, 82, 83]. It is unknown if these dyes will interact reproducibly with pectin of up to and beyond 60% DE nor suffer from the same interferences as RR.

Hydroxylamine

Hydroxylamine has a long history of use in colorimetric analysis[46]. The predominate method involves mixing 0.5 mL 2 N hydroxylamine hydrochloride (in 60% ethanol) with 0.5 mL of 3.5 N sodium hydroxide. This solution is added to 2 mL of a 1% (w/v) solution of pectin and mixed gently at 25°C for 2 hours. After reaction, add 1 mL of 3.5 N hydrochloric acid with mixing and follow with 1 mL of 0.74 M ferric chloride in 0.1 N hydrochloric acid. Red complex formation is measured at 600 nm.

Carbazole

Carbazole colorimetric methods continue to be a popular method for carbohydrate analysis. The method as pertains to pectins is as follows. Moisten 1.0 g of 70% ethanol-extracted pectin in a 250 mL beaker with 95% ethanol. To the beaker, add 200 mL of 0.5% EDTA in order to complex the divalent pectic cations and thereby dissolve. Adjust to pH 11.5 with 1 N sodium hydroxide and hold at 25°C for 30 minutes. Adjust to pH 5.0 with acetic acid and add 0.1 g of pectinase with stirring. Mix for 1 hour and dilute to 250 mL. Discard initial small amount of filtrate but retain remainder for carbazole analysis. For AUA analysis, cool 12

mL of conc. hydrochloric acid in a test tube to 3°C. To this, add 2 mL aliquot of galacturonide solution and mix thoroughly at low temperature. Heat for 10 min in boiling water, cool to 20°C, and add 1.0 mL 0.15% carbazole solution. Let stand at room temperature for 25 min. and measure AUA at 520 nm using a standard curve.

Proposed Research

The analysis of complex polysaccharides presents a challenge. As previously mentioned, RR can be used as a colorimetric technique to characterize directly or indirectly several properties of Pectin. However, in colorimetric analysis of carbohydrates, RR has been shown to lack specificity and suffer numerous interferences. Researchers are beginning to explore relationships of complex biopolymers and carbohydrates as they relate to alternative cationic dyes[84]. Cationic dye research, as it relates to the field of food science, is still yet relatively unexplored due to the numerous problems previously discussed. While numerous analytical methods exist for food polysaccharide analysis[11, 27, 35, 78], many are difficult, labor intensive, time consuming, suffer from interferences, or expensive. Therefore, the primary focus of this research is to determine if several classes of laboratory grade pectins of known composition will show reproducible spectrophotometric performance, similar to RR, with a selection of cationic dyes. Additionally, the analysis should be relatively quick, inexpensive, show good selectivity, and require little chemical modification of the pectin polymer. The proposed objectives of this project are described below.

1. To investigate if any of the following metachromic, cationic dyes provide qualitative or quantitative interaction with pectin: Aniline Blue, Toluidine Blue, Congo Red, Coriphosphine O, Alcian Blue, Nile Blue, and Acridine Orange.

2. To investigate if any of the listed dyes can be used alone or in conjunction with other dyes to differentiate spectrophotometrically between pectin of known and unknown composition including DE above ca. 70% (upper practical limit for RR).

3. To investigate if lab grade or consumer grade pectins cause metachromic aberrations in the cationic dyes such that these provide a "fingerprint" for a particular pectin type at any concentration

4. To investigate if MW (by GPC) has an effect on the above characterization techniques

CHAPTER 4

EXPERIMENTAL PROCEDURES, RESULTS, AND DISCUSSION

The following sections describe the experimental procedures performed in order to develop a scientific understanding of pectin/cationic dye relationships.

Reagents

The list of reagents used for this project, their grades, suppliers, information, and chemical structure (where applicable) is given below.

Deionized, ultrapure, filtered water was obtained from a Millipore Q™ distillation apparatus provided by Continental Water System (Millipore, Bedford, MA).

Copper II Sulfate, 100% purity was obtained from J.T. Baker (Phillipsburg, NJ).

Lead Acetate, 100% purity was obtained from EMD Chemicals Inc. (Gibbstown, NJ).

Ethanol, 95% purity, and Glacial Acetic acid, 99.5% purity was provided by Eastman Chemical Company (Kingsport, TN) and diluted to 50% (v/v) with Millipore Q distilled water.

Polygalacturonic acid (from Orange), 95% purity, lot 407261, was obtained from Fluka Chemical Corporation (Milwaukee, WI).

Consumer canning pectin SureGel #1: “Low Sugar” Lot #: 08 May 2005 D4 10:52, SureGel #2: “Normal Sugar” Lot #: 25 Nov 2005 D4 02:48Supplier: Kraft Foods Global, Inc. (Northfield, IL).

Coriphosphine O or 3-amino-6-(dimethylamino)-2-methylacridine monohydrochloride (Figure 9), 100% purity, was obtained from Trust Chemicals Industry (Port Said, Egypt).

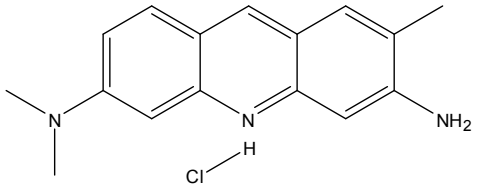


Figure 9. Chemical structure of Coriphosphine O

3-Morpholinopropanesulfonic acid (MOPS) (Figure 10), 100% purity, was obtained from Lancaster Synthesis, Inc. (Pelham, NH).

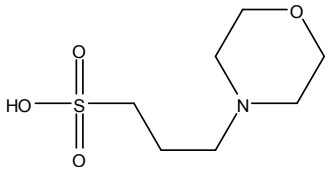


Figure 10. Chemical structure of MOPS

The following compounds were obtained from Sigma-Aldrich (St. Louis, MO): 2-propanol, 99% purity, Pectinesterase enzyme, from orange peel, ammonium sulfate suspension, 50-350 units/mg protein, Trichloroacetic acid, 99% purity, Apple Pectin, 9.5% methyl esterification, 88% galacturonic acid content, 75% DE, lot 67H16351, Citrus Pectin, 12% methyl esterification, 82% galacturonic acid content, 92% DE, lot 116H0569, Ruthenium Red or ammoniated ruthenium oxychloride ($[(NH_3)_5RuORu(NH_3)_4ORu(NH_3)_5]Cl_6$) (Figure 11), 100% purity, Sodium Titriplex™ Salt or trans-1,2-Diaminocyclohexane-N,N,N',N'-tetraacetic acid, monohydrate sodium salt (Na-CyDTA) (Figure 12), 100% purity, Aniline Blue, a mixture of Methyl Blue and Water Blue (Figure 13), 100% purity, Toluidine Blue or Tolonium Chloride (Figure 14), 87% purity, Congo Red or benzidinediazo-bis-1-naphtylamine-4-sulfonic acid

sodium salt (Figure 15), 0.1 wt% solution, Alcian Blue, a copper phthalocyanine dye (Figure 16), 52% purity, Nile Blue (Figure 17), 75% purity, Acridine Orange (Figure 18), 90% purity.

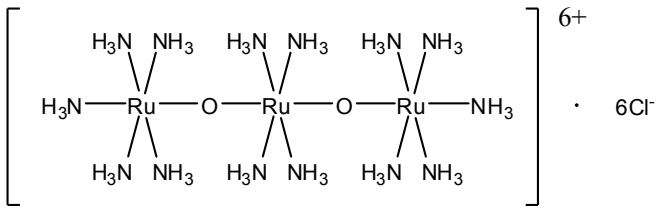


Figure 11. Chemical structure of Ruthenium Red

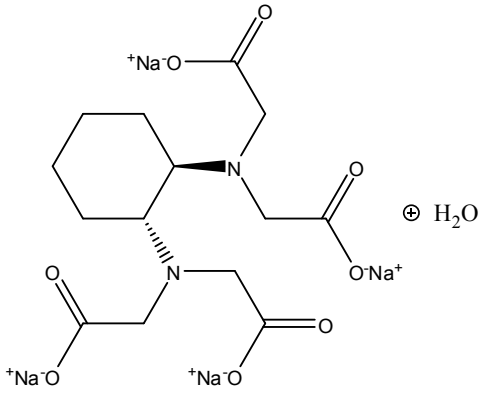


Figure 12. Chemical structure of Na-CyDTA

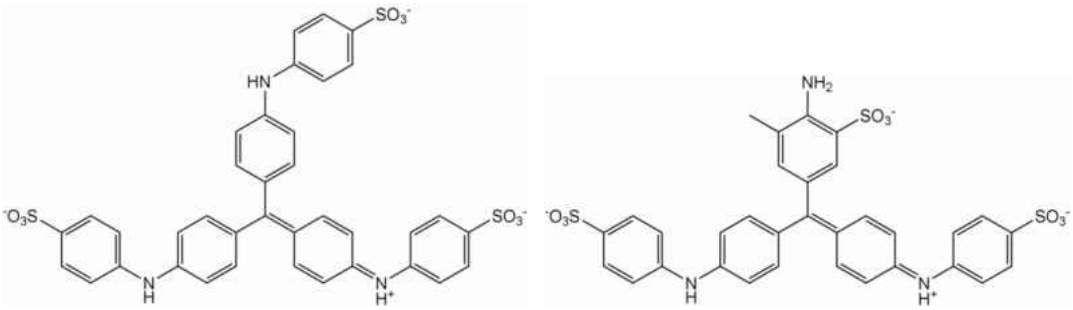


Figure 13. Chemical structure of Methyl Blue and Water Blue

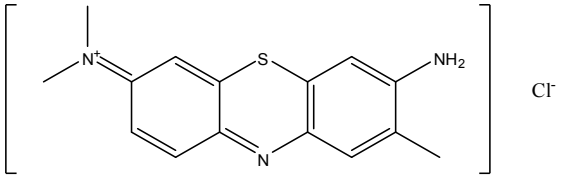


Figure 14. Chemical structure of Toluidine Blue

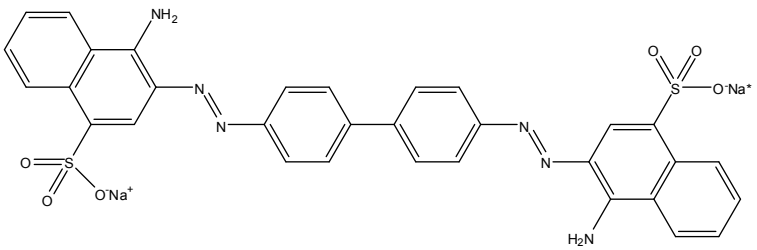


Figure 15. Chemical structure of Congo Red

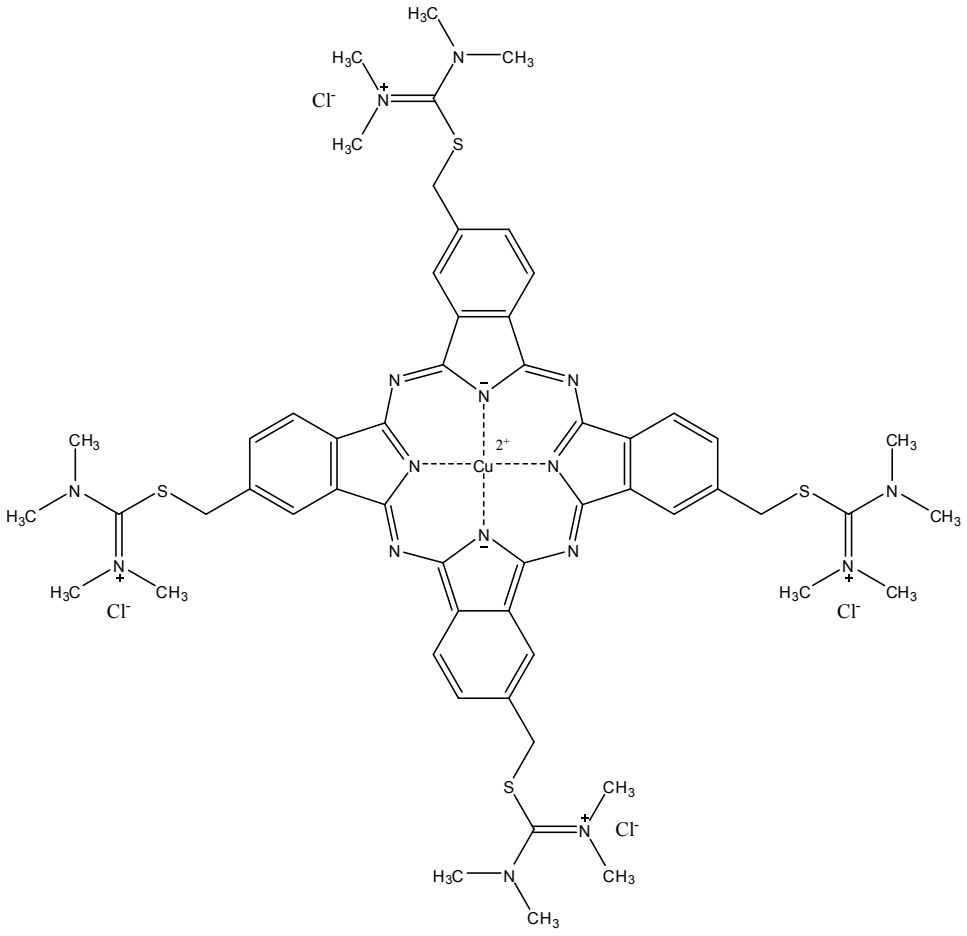


Figure 16. Chemical structure of Alcian Blue

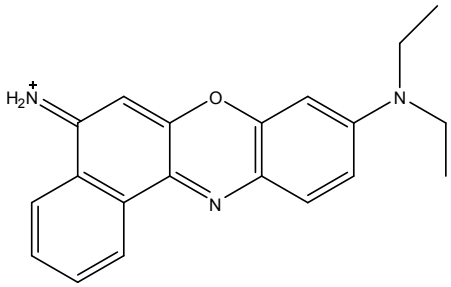


Figure 17. Chemical structure of Nile Blue

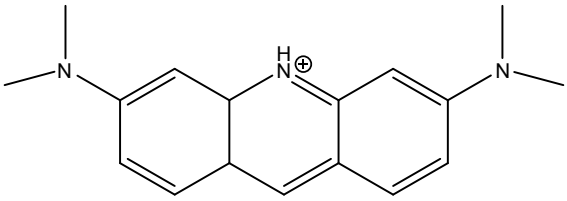


Figure 18. Chemical structure of Acridine Orange

Instrumentation

For UV-Vis determination, a Hewlett Packard 8452A spectrophotometer (described earlier) with an installed photodiode array detector was the instrument of choice. The installed Peltier temperature controller was set for 30°C.

For GPC measurements, the system used consisted of a Waters 510 LC pump, a Perkin Elmer ISS 200 autosampler, and a Perkin Elmer 200 series Refractive Index detector. A Sepharose 2B column of 2.5 cm x 60 cm with a MW fractionation capability ranging from 70 to 40,000 kDa was installed in the system. The system had an injection volume of 50 microliters. Due to the nature of the analytes in this study, two mobile phases were employed. The mobile phase for laboratory pectin standards consisted of 3 mM Na_2CO_3. For unpurified, consumer grade materials it was necessary to employ 3 mM NaOH as the eluent. After properly degassing the solvents, the RI detector, the column, and the solvents were thermostated to 40°C.

The following miscellaneous equipment was also employed: Parafilm® (Pechiney Plastic Packaging, Chicago, IL), High Shear Omni Mixer homogenizer (Omni International, Marietta, GA), Eppendorf adjustable pipettes with removable tips and an Eppendorf micro-centrifuge (Westbury, NY), polypropylene, factory sterilized, snap-cap micro-centrifuge tubes (Bio Plas Inc, San Rafael, CA), and a Vortex-Genie® 2 (Scientific Instruments Inc., Bohemia, NY).

Preparation of Reagents and Stock Solutions

The following reagents were prepared as described below.

1. Sodium Hydroxide (1 M): In 50-mL volumetric flask, dissolved 2 g of solid sodium hydroxide in Millipore Q water.
2. MOPS Buffer (0.10 M): In 1-L volumetric flask dissolved 20.92 g of 3-morpholinopropanesulfonic acid in Millipore Q water and adjusted to pH 6.5 with sodium hydroxide from reagent 1 above.
3. Copper II Sulfate (0.010 M): In 50-mL volumetric flask, dissolved 0.10 g of copper II sulfate and 0.75 mg of 2-propanol in MOPS buffer from reagent 2 above.
4. Lead Acetate (0.60 M): In 50-mL volumetric flask, dissolved 11.38 g of lead acetate in MOPS buffer from reagent 2 above and refrigerated. Solution has shelf life of 2 days.
5. CyDTA (0.020 M): In 500-mL volumetric flask, dissolved 45.28 g of sodium titriplex with MOPS buffer from reagent 2 above.

Preparation of Standard Solutions

Stock standard solutions were prepared for polygalacturonic acid (PGA), apple pectin, citrus pectin, ruthenium red, aniline blue, toluidine blue, congo red, coriphosphine o, alcian blue, nile blue, and acridine orange.

Dye standards were prepared as described below. When not in use, these solutions were sealed with Parafilm and stored refrigerated.

1. Ruthenium Red (0.25 mM): In 10-mL volumetric flask, dissolved 2 mg of ruthenium red in MOPS buffer.
2. Aniline Blue (0.125 mM): In 10-mL volumetric flask, dissolved 1 mg of aniline blue in MOPS buffer.
3. Toluidine Blue (0.0625 mM): In 10-mL volumetric flask, dissolved 0.57 mg of toluidine blue in MOPS buffer.
4. Congo Red (0.125 mM): In 10-mL volumetric flask, diluted 1 mL of congo red in MOPS buffer.
5. Coriphosphine O (0.25 mM): In 10-mL volumetric flask, dissolved 2 mg of coriphosphine o in MOPS buffer.
6. Alcian Blue (0.25 mM): In 10-mL volumetric flask, dissolved 1.52 mg of alcian blue dye in MOPS buffer.
7. Nile Blue (0.125 mM): In 10-mL volumetric flask, dissolved 1.25 mg of NB in MOPS buffer.

8. Acridine Orange (0.125 mM): In 10-mL volumetric flask, dissolved 1.1 mg of AO in MOPS buffer.

Pectin standards sample were prepared as described below. When not in use, these solutions were sealed with Parafilm and refrigerated.

1. The pectin source was dried in a standard laboratory vacuum oven *in vacuo* at 45°C for 24 hours.
2. Dried pectin obtained in Step 1 was used to prepare a 2 wt% solution by slow addition of pectin powder, 2 g, to 100 mL room temperature Millipore Q water. To prevent clumping, this addition was accompanied with homogenation.
3. The solution in Step 2 was gently mixed for 1 hour to allow for complete dissolution of any impurities present in the pectin sample.
4. From the solution in Step 3, pectin was precipitated by adding 7.5 mL of 10 mM copper II reagent.
5. The precipitate in Step 4 was filtered onto a medium fritted funnel and washed with four 500 mL volumes of 5% ethanol.
6. The solid in Step 5 was re-dissolved in 100 mL of 20 mM sodium titriplex (Na-CyDTA) reagent.
7. The solution obtained in Step 6 was acidified to pH 5 with 50% acetic acid reagent.
8. The acidified solution in Step 7 was placed into a 5000 MW-cutoff cellulosic membrane dialysis tube and dialyzed against a countercurrent flow of distilled water for 14 hours.

9. The purified pectin solution from Step 8 was slowly added to 300 mL of 95% ethanol to precipitate solid pectin.
10. The precipitate from Step 9 was filtered on a medium fritted funnel and dried in a standard laboratory vacuum oven at 45°C *in vacuo* for 24 hours.
11. Dried solid from Step 10 was used to prepare a 0.2 wt% solution by slow addition of 0.02 g powder to 0.1M MOPS buffer reagent, with mixing, to a 10-mL volumetric flask. This standard solution was sealed with Parafilm and refrigerated when not in use.

Preparation of Commercial Samples

Consumer grade commercial samples in powder form were purchased from local retailers. As discussed previously, consumer pectins are sold in two varieties: regular and low sugar. Manufacturer information does not include pectin source or content of these materials. Only the moniker of "Low Sugar" infers a pectin of low %DM and high calcium content. The "normal sugar" pectins are assumed high %DM material with low MW sugar content > 55% (w/w) based upon accepted prior scientific knowledge. Each sample was prepared as described below without further purification.

1. The source sample was dried in a standard laboratory vacuum oven *in vacuo* at 45°C for 24 hours.
2. Dried solid from Step 1 was used to prepare a 0.2 wt% solution by slow addition of 0.04 g powder (assumed 50% by weight to be low MW sugars) to 0.1 M MOPS buffer reagent, with mixing, to a 10-mL volumetric flask.

3. The solution in Step 2 was sealed with Parafilm and refrigerated when not in use.

Typical and accepted specifications of various agencies for consumer grade pectins are summarized in Table 2.

Table 2. Commercial pectin specifications

Reference	FAO	FCC	EEC
Loss on Drying	max. 12%	max. 12%	max. 12%
Acid-insoluble ash	max. 1%	max. 1%	max. 1%
Sulfur dioxide	max. 50 mg/kg	max. 50 mg/kg	max. 50 mg/kg
Sodium methyl sulfate		max. 1%	
Methyl-, ethyl-, 2-propanol	max. 1%	max. 1%	max. 1%
Nitrogen content, amidated pectin			max. 2.5%
Nitrogen content, pectins	max. 2.5%		max. 0.5%
Galacturonic acid	min. 65%		min. 65%
Total anhydrogalacturonides		min. 65%	
Degree of Amidation	max. 25%	max. 25%	max. 25%
Arsenic, ppm	max. 3	max. 3	max. 3
Lead, ppm	max. 10	max. 5	max. 10
Copper, ppm	max. 50		max. 50
Zinc, ppm			max. 50
Other heavy metals, ppm		max. 20	

Preparation of Samples for Analysis

Prior to spectrophotometric analysis, the spectrophotometer was allowed to warm up. In addition, the quartz cuvettes were cleaned and prepared for use. A series of five solutions were prepared for each pectin sample as described below in Table 3.

Table 3. Reagent volumes for preparation of spectrophotometric experiments

Sample ID	Pectin (µL)	Pb^{2+} (µL)	Dye (µL)	MOPS (µL)
Conc1	50.0	50.0	5.0	1395.0
Conc2	50.0	50.0	10.0	1390.0
Conc3	50.0	50.0	15.0	1385.0
Conc4	50.0	50.0	20.0	1380.0
Conc5	50.0	50.0	25.0	1375.0

Using the appropriate Eppendorf pipette, 0.05 mL of pectin solution was pipetted into a 1.8-mL micro-centrifuge tube. To this was added the listed amount of dye solution as shown in Table 3. The tube was capped and vortexed for 15 s. The tube was then uncapped and 0.05 mL of Pb^{2+} reagent was added along with the specified amount of MOPS reagent. Again, the tube was capped and shaken vigorously on the vortex for 30 s. The sample was allowed to stand for 30 minutes. After standing, the sample was placed into the micro-centrifuge and spun at 7000 RPMs for 10 min. A transfer pipette was used to transfer the supernatant from the centrifuge tube to the quartz cuvette for spectroscopic analysis.

UV-Vis Conditions

For UV-Vis analysis, the HP8452A diode array spectrophotometer was allowed to warm up for 1 hr prior to analysis. The spectrophotometer was equipped with a peltier temperature controller thermostated at 30°C. Once placed into the instrument sample holder, each sample was allowed to equilibrate for 5 minutes prior to analysis. Two matched, quartz, low volume (2 mL) cuvettes were used for analysis. The cuvettes were cleansed with tepid Millipore Q water, rinsed with spectroscopic grade acetone, and dried with a thin stream of nitrogen between analyses.

GPC Conditions

For GPC analysis, the GPC system was primed and allowed to purge for 1 hour when powering up from a cold state or changing solvent composition. When starting from a low-flow, inactive state, the system was allowed to purge for 30 minutes. The system was further allowed to purge for 5 minutes between successive sample measurements. The refractive

index detector, column, and solvents were thermostated to 40°C and allowed to equilibrate during instrument purging.

Data Analysis

For spectrophotometric methods, data were collected directly from the HP8452A spectrophotometer system with HP UV-Vis ChemStation software and later exported to Microsoft Excel with Microsoft Windows XP. Data analysis and charting for this project were done with a combination of Microsoft Excel 2007, MathWorks MATLAB R2007a, and Minitab Release 14.12. Chemical structures were drawn with CambridgeSoft BioDraw Ultra 10.0 and Bio3D 10.0. After plotting, the raw data were processed using a Savitsky-Golay smoothing filter algorithm. After that process, baseline correction was performed. From this baseline spectral data, the corresponding blank was subtracted. Finally, after correcting for the blank, the spectral absorbance data were autoscaled from 0 to 1. Further analysis involved calculating Euclidean distance (ED) and correlation coefficients percentages (R^2) between pectin standards and consumer unknowns for each of the above datasets. Additionally, eigenanalysis was performed for each of the datasets by calculating eigenvalues and eigenvectors. Finally, inner products (IP) were calculated between eigenvectors for pectin standards and consumer unknowns. For GPC analysis, data were collected directly from the LC instrument with Turbochrom and exported to Microsoft Excel as resizable images.

The data collected during the described experiments are discussed in the sections to follow. In order to establish reproducibility, data were collected and analyzed in triplicates. The triplicate data indicates very stable and reproducible measurements.

Verification of Current Ruthenium Red Substitution Pattern Technique

The citrus standard of this study was subjected to a previously devised experiment by Hou et al.[45] in order to verify RR does interact with the pectin. The 92% DE citrus pectin standard was used to prepare two distinct carboxyl distribution patterns by the following methods. The method of Thibault and Rinaudo[85] was used to produce a random esterification pattern along the polymer backbone. A 0.5 wt% solution of pectin was prepared in 0.5 M sodium hydroxide and stirred gently at 5°C. Aliquots were taken and neutralized with 1 N hydrochloric acid. The pectin was precipitated with three volumes of 2-propanol (isopropyl alcohol), filtered, washed with acetone, and dried overnight at 40°C *in vacuo*. The method of Powell et al.[86] was used to prepare a blockwise carboxyl distributed pectin. A solution of 1.5 g citrus pectin in 0.1 M sodium chloride, at pH 6.8, was mixed with 40 mg/100 mL pectinesterase and digested at 25°C. Aliquots were taken over the course of the reaction and quenched with 0.1 wt% trichloroacetic acid, precipitated with three volumes of isopropyl alcohol, filtered, washed with acetone, and dried overnight at 40°C *in vacuo*.

For spectrophotometric analysis of each of the above samples, solutions of 0.1% (w/v) pectins were prepared in 100 mM MOPS buffer as previously described. Each sample was doped with 0.5 mL of 0.02% RR, mixed, and allowed to stand for 5 minutes. Upon standing, 0.5 mL of 0.6 M lead acetate was added to each in order to precipitate pectin. The mixtures were centrifuged at 1,400 *g* for 15 minutes. The absorbances were measured at 534 nm and recorded. A blank was prepared without pectin and subtracted from each of the measured absorbances with the results shown in Figure 19.

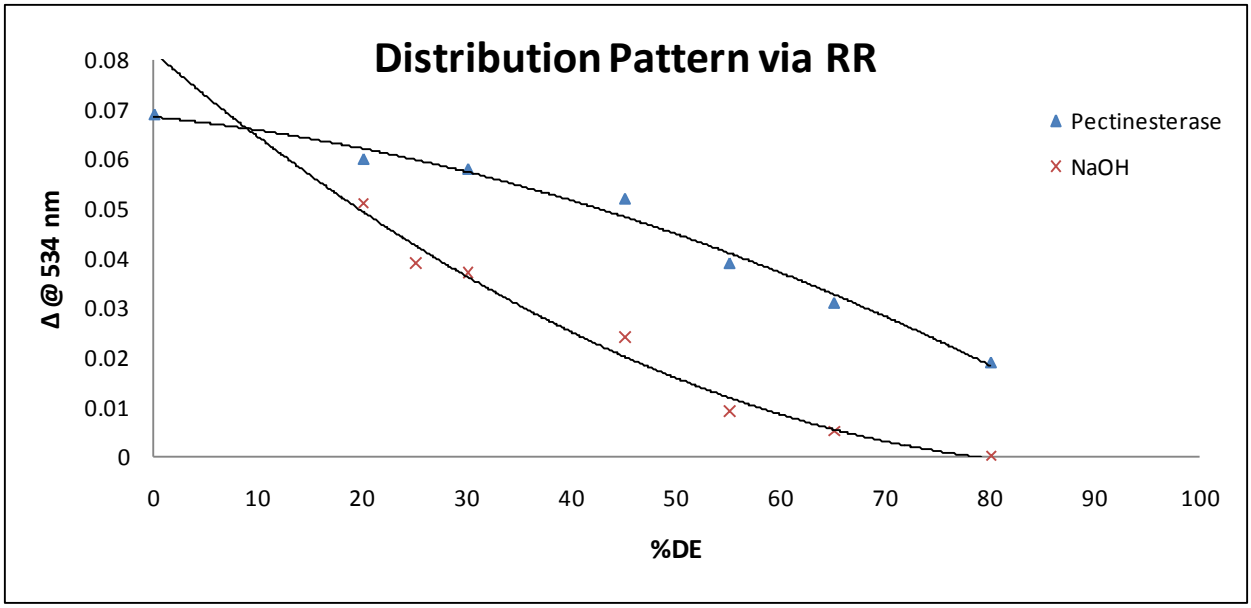


Figure 19. Absorbance difference at 534 nm, against blank, of RR with pectin of known %DM

The results show conclusively that RR does complex quantitatively with the citrus pectin used in this study up to a %DE of approximately 79 when the polymer is randomly substituted (NaOH). As shown, RR complexes quantitatively with a blockwise substituted (pectinesterase) pectin beyond 80 %DE up to about a 92 %DE when the fit is forecasted to x-axis intercept. It is important to notice as the %DE goes up, the ability of pectin to complex RR drops as there are less reactive sites along the polymer backbone available.

Results and Discussions

Multiple cationic dyes were explored for this study. Prior knowledge shows pectin can interact quantitatively with RR. Experimentation shows limited interaction of NB and AO with pectin. These are discussed below. Alcian Blue and Coriphosphine O was dropped from this study as they exhibited solubility problems and gave erratic results. Toluidine Blue was also discarded from the study as it exhibited large and inconsistent spectral shifts during sample

analysis. Aniline Blue and Congo Red were also dropped from the study as they precipitated out of solution upon addition of other necessary reagents. Numerous trials with RR, AO, and NB were attempted until the optimal concentrations for this study were determined.

Optimization of Spectral Detail, Manipulation, and Analysis

The microprocessor evolution has enabled the chemist to collect vast amounts of information quickly. The bottleneck has rapidly become data interpretation and processing. With the rise of computerized instrumentation, sequential digitized signals have become commonplace in analytical chemistry. UV-Vis spectra are a perfect example of data series that are sequential in frequency. Raw spectral information such as peak shifts, positions, and integrals is most often dependent upon how the information on the computer is first processed. Several techniques for handling sequential series spectra are emphasized below.

In digital signal processing, a very important first step involves noise filtering. For low concentrations, the analyte signal can be difficult to distinguish from background. Measurement noise is typically broken into two classes[87]: stationary and correlated noise. Stationary noise is applicable to spectroscopic analysis and can be further broken down into homoscedastic and heteroscedastic noise. For homoscedastic noise, the noise mean and standard deviation typically remain constant across the entire signal. In absence of detailed knowledge of a system, filters for homoscedastic noise provide good approximations. Heteroscedastic noise is dependent upon and is often proportional to signal intensity. In spectroscopy, heteroscedastic noise typically arises when data are transformed prior to

processing in the case of converting transmittance to absorbance. The transformed information distorts the true noise distribution in the raw data.

Due to the multitude of unknowns, it is not possible to create an all-inclusive noise filter for use in spectrophotometric analysis. However, it is possible to create general smoothing functions that model spectrographic data well without causing much loss in spectral detail[88]. Moving average filters are popular for many types of digital signals but have the disadvantage of using linear approximations for the data. Spectral data are better approximated by polynomial curves[89], especially at the center of the peak where signal intensity and integrity are important. Linear models will always underestimate peak intensity. Prior to high-speed computers and sophisticated software packages[90], cubic, quartic, and quintic polynomial regressions[91] of full spectra would be prohibitively time consuming and computationally intensive[92]. To address this problem, Savitsky and Golay[93] developed a simplified method of calculating filtered datapoints along the frequency series of the spectrum. The filter is moved along the frequency series and each datapoint is replaced successively by the corresponding filtered datapoint. Performance of the filter is tied to the number of data points used in each successive calculation and is known as the 'window'. Savitsky-Golay filters are often represented in tabular form providing coefficients for window size, polynomial order, and normalization constants. The coefficients obtained after selecting polynomial order and window size are used to multiply the raw data and summed to obtain the smoothed value. Figure 20 shows an example of Savitsky-Golay filtering on low concentration RR data. This smoothing is especially important at low concentrations where the analyte signal can be

obscured by noise. At high concentrations, the analyte signal is much greater than background noise; therefore, little is gained from smoothing. Figure 20 and Figure 21 contrast the raw signal and smoothed spectra at the two concentration extremes.

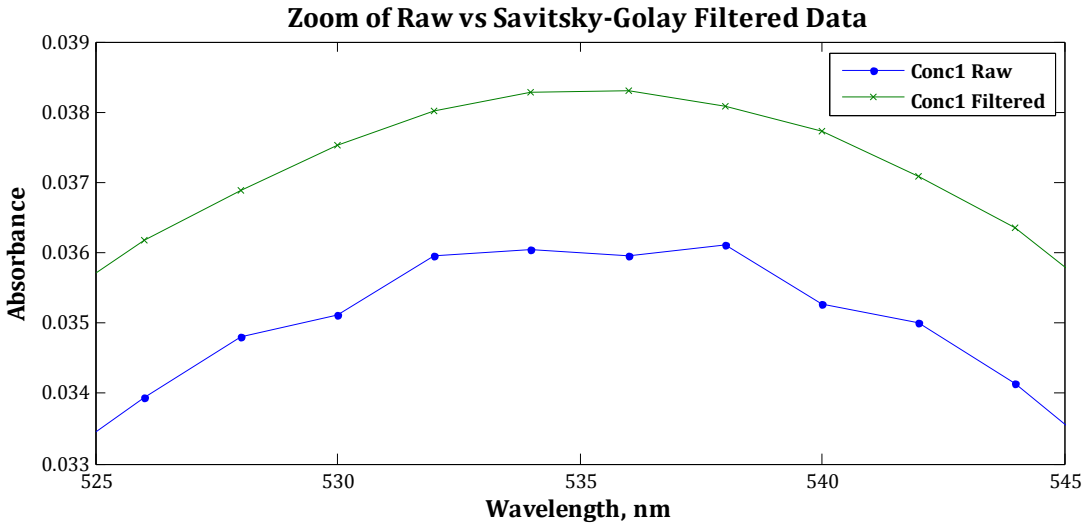


Figure 20. Raw vs. Savitsky-Golay filtered data for Citrus at low concentration with RR

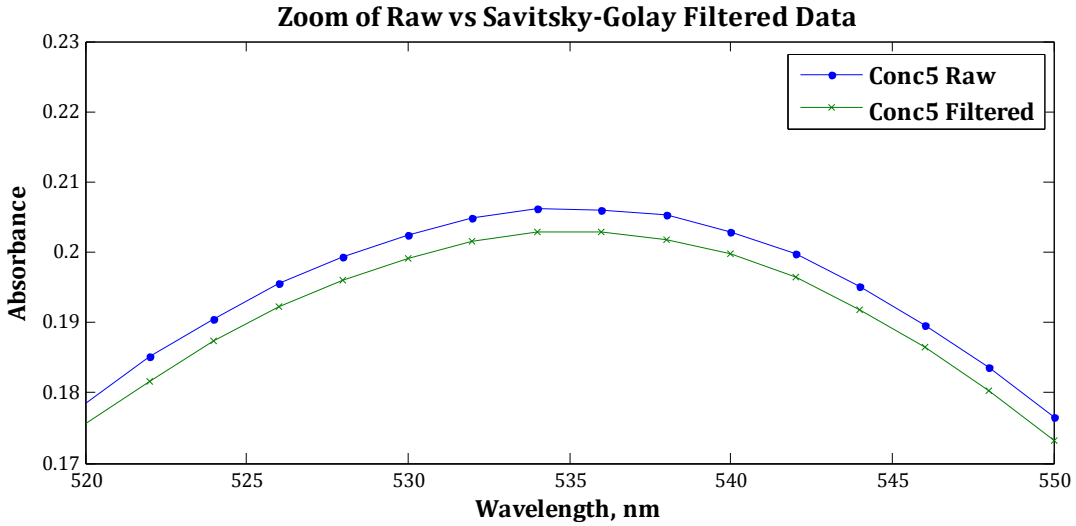


Figure 21. Raw vs. Savitsky-Golay filtered data for Citrus at high concentration with RR

To maintain consistency and data integrity, all data for this study were filtered using the Savitsky-Golay algorithm provided in MATLAB with 3rd order polynomial and 5-point window parameters.

Due to measurement error, noise, and a multitude of other possible causes, it is often necessary to adjust data for baseline shift and zero-level axis translation. For these data manipulation techniques to be applied successfully, it is necessary to identify a region of the spectra where no chemical species absorbs. This is known as the *zero-component region*. By definition, these regions have a chemical rank of zero and spectral manipulation calculations are anchored on these points. Any spectral shifts in these regions can be attributed to instrument and measurement noise that can be subsequently corrected. As previously alluded to, the zero-component regions can also be used to calculate systematic background and baseline offsets. For this project, spectral data were first translated such that the minimum 'zero' absorbance fell at zero on the y-axis, filtered with a Savitsky-Golay function as previously discussed, and finally subjected to baseline correction. In axis translation, the absorbance minimum is simply subtracted from each data point to yield a new, zero-corrected data vector. Next, data smoothing was performed as previously discussed. Finally, baseline correction involved identifying two 'zero' points at opposite ends of the spectra and calculating the slope and intercept of a line connecting them. This resulting linear equation was then used point-by-point to recalculate old spectral data into 'baseline' corrected data. While not necessary for this project, it is possible to calculate baseline corrections for multiple peaks realizing these multiple data sets must then be treated independently.

Chemometric Techniques

When *a priori* data are not available for a sample, numerous techniques can be employed to identify the compound. In spectroscopic analysis, it is possible to use chemometric techniques to aid in identifying these compounds. The first step in any chemometric analysis is to determine similarities with known compounds. This process is known collectively as pattern recognition or pattern matching. Many types of pattern recognition and pattern matching exist in chemometric analysis. For online spectral searches of known compounds, pattern matching is an important tool for the spectroscopist in identifying unknowns under standard conditions. Additionally, exploratory data analysis allows the extraction of useful data from samples. Unfortunately, the 1-dimensional, nonlinear signal in spectral analysis limits the available techniques. Of the available techniques, the more useful include Euclidian distance, Mahalanobis distance, simple correlation (r), and eigenanalysis. In order to use these techniques, further preprocessing of the spectral data is necessary. These preprocessing techniques include axes translation (previously discussed), vector normalization, scaling, range scaling, autoscaling, feature weighting, and rotation among others. Good pattern recognition techniques are typically based upon normalized data set comparisons. Normalization sets the length of all the data vectors in the data set to equivalent lengths. Normalization removes the variance in the data set due to differences in the magnitude of measurements. However, this effect on the data can be undesirable if variance in the data set is meaningful. For this project, minute variance is important to data analysis. Fortunately, range scaling can be used as a preprocessing technique as it does not obscure variance in the

data set and removes baseline-offset effects. The calculation for range scaling is done by using Equation 3.

$$x_j^* = \frac{(x_j - Min_x)}{(Max_x - Min_x)} \qquad \text{Equation 3}$$

where x_j^* is the absorbance of the normalized spectrum x^* and x_j the absorbance of spectrum x at wavelength j. Max_x and Min_x are the maximum and minimum absorbances, respectively, of spectrum x. Unfortunately, range scaling is sensitive to the presence of outliers. These methods are the only preprocessing steps relevant to the current work.

Three categories of chemometric pattern-matching techniques were employed to characterize data for this study and further combined via feature weighting calculation. These techniques included ED, r, and eigenanalysis (with eigenvalues and eigenvector IP calculations). After preprocessing was completed on the spectral data, MATLAB was used to calculate EDs between each of the standards and the unknowns. ED uses simple geometry to evaluate the 'distance' between two spectra. A shorter calculated distance indicates a closer match between the spectra. The distance between two samples k and l is defined by Equation 4:

$$d_{kl} = \sqrt{\sum_{j=1}^{J} (x_{kj} - x_{lj})^2} \qquad \text{Equation 4}$$

where there are j measurements and x_{ij} is the j*th* measurement on sample i. The smaller this value, the more similar are the samples. ED can also be represented in vector matrix format as

in Equation 5:

$$d_{kl} = \sqrt{(\vec{x}_k - \vec{x}_l) \times (\vec{x}_k - \vec{x}_l)'} \qquad \text{Equation 5}$$

where the objects $\vec{x}_k$ and $\vec{x}_l$ are row vectors and $(\vec{x}_k - \vec{x}_l)'$ is the transpose of $(\vec{x}_k - \vec{x}_l)$ to provide a square matrix. After calculating, the results were tabulated in distance matrices and used for comparison purposes.

Next, Microsoft Excel was used to calculate correlations between the data vectors of the standards and the unknowns. If the spectrum of an unknown is treated as a set of dependent variables, the standard spectrum can be used as a set of independent variables to perform a linear regression of the two. The correlation coefficient, R^2, is a measure of the spectral similarity in terms of peak positions, shapes, and intensities. Further, the slope, m, of the regression line is a measure of the match of the baseline corrected absorption intensities. The intercept, c, is a measure of baseline offsets between the spectra. As spectral data have been baseline corrected, only the R^2 values were tabulated in an abbreviated correlation percentage matrix and used for comparison purposes. It is important to note the closer the R^2 value to 1, the more similar are the objects.

Finally, MATLAB was used to calculate the eigenvalues and their associated eigenvectors for the standards and the unknowns. Once the eigenvectors were obtained, IPs were calculated between the standard and unknown eigenvectors.

ED and correlation calculations were performed on one data vector. It is possible for the samples in this study to contain more than one component. The acquired spectra can be factor analyzed to determine how many potential components are contained. Factor and

principal component analyses are powerful multivariate techniques. The goal of factor analysis is to provide a spatial dimensional representation of the data vectors. It expresses the original data matrix in terms of linear combinations of orthogonal vectors and contains the same information as the original data. Reducing the measurement dimensionality to the smallest possible intrinsic dimensionality allows for efficient manipulation of the data and better understanding of the data under study. This can be achieved by observing correlation between pairs of vectors. Normally, the correlation between two unit-length vectors is the cosine of the angle between them. For this study, it is more useful to determine vector correlation through calculation of IPs of two eigenvectors. If an $m \times n$ data matrix is normalized (or range scaled in this case) and factor analyzed, n orthogonal vectors are obtained. These are referred to as eigenvectors. Eigenvectors are a set of vectors associated with a matrix equation that are also known as characteristic vectors[94]. Associated with each eigenvector is a descriptor of its importance referred to as the eigenvalue. Eigenvalues are a set of scalars associated with a matrix equation that are sometimes known as characteristics roots[95]. If the eigenvalue is multiplied with its associated eigenvector, the original data magnitude is obtained. The determination of the eigenvalues and eigenvectors of a system is known as matrix diagonalization. Matrix diagonalization is the process of taking a square matrix and converting it into a diagonal matrix. The diagonal matrix will share the same fundamental properties of the underlying matrix and is equivalent to transforming the underlying system into a set of coordinate axes in which the matrix takes a canonical form. The entries of the diagonalized matrix represent the eigenvalues of the original matrix. Similarly, the new set of axes

corresponding to the diagonal matrix is the eigenvectors of the original matrix. This eigen decomposition of a square matrix A is shown in Equation 6:

$$A = PDP^{-1} \qquad \text{Equation 6}$$

where P is a matrix composed of the eigenvectors of A, D is the diagonal matrix constructed from the corresponding eigenvalues, and P^{-1} is the matrix inverse of P. The eigenvectors that account for the largest variance in the dataset are represented by large eigenvalues. Because of experimental noise, the largest eigenvectors usually do not account for 100% of the data variance. Therefore, smaller values are normally found. An example of a spectral eigenvalue matrix is shown in Figure 22 and its associated eigenvectors are shown in Figure 23. It can be seen that the first eigenvalue is much larger than the rest indicating the presence of only one spectral pattern in the data.

$$\begin{bmatrix} 28.14 & 0 & 0 & 0 & 0 & 0 \\ 0 & 5.08x10^{-15} & 0 & 0 & 0 & 0 \\ 0 & 0 & 1.01x10^{-15} & 0 & 0 & 0 \\ 0 & 0 & 0 & 9.85x10^{-16} & 0 & 0 \\ 0 & 0 & 0 & 0 & -1.09x10^{-15} & 0 \\ 0 & 0 & 0 & 0 & 0 & -6.32x10^{-15} \end{bmatrix}$$

Figure 22. Eigenvalue matrix of RR Apple range scaled concentration 3 spectra

This matrix and graph indicates only 1 component accounts for the total variance in the data set. The other eigenvectors have no discernable spectral characteristics and are mostly noise. Factor and multicomponent analyses of the unknowns used in this study indicate the presence of more than 1 compound contained in each sample.

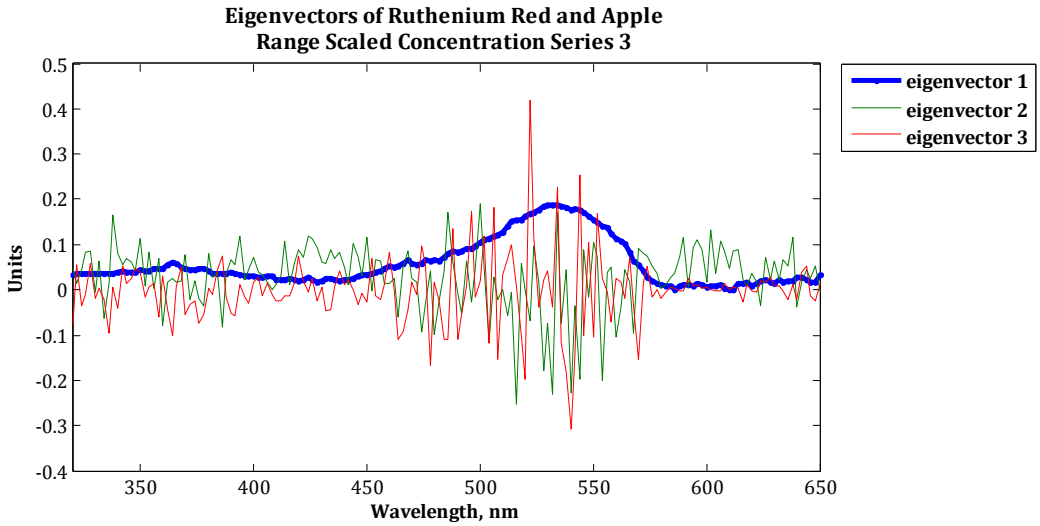


Figure 23. Eigenvectors of RR apple range scaled concentration series 3 data

The command in Equation 7 is used to calculate eigenvalues and eigenvectors within MATLAB.

$$[V, D] = eigs(A * A^{-1}) \qquad \text{Equation 7}$$

Equation 7 determines the six largest eigenvalues and eigenvectors of matrix A and assigns the resulting eigenvalue matrix to D and eigenvector matrix to V. For this calculation, matrix A must be a square matrix (multiplying by the matrix transpose provides a square matrix). The IPs of the eigenvectors were calculated in Excel using Equation 8.

$$= MMULT(MINVERSE\big(MMULT(TRANSPOSE(eVect1), eVect1)\big), MMULT(TRANSPOSE(eVect1), eVect2)) \qquad \text{Equation 8}$$

where $MMULT$ returns the matrix products of two arrays, $MINVERSE$ returns the inverse matrix of the array, $TRANSPOSE$ shifts the vertical and horizontal orientation of the array, $eVect1$ is eigenvector 1, and $eVect2$ is eigenvector 2. An IP value closer to 1 indicates a better match.

Examples of Single Dye Spectra Used for Calculation

Due to the large amounts of data, selected spectra that are informative are included to demonstrate the important features found. These and similar spectra were used for subsequent data analysis. Shown in Figure 24, Figure 25, and Figure 26 are baseline corrected data for the three standards, PGA, apple pectin, citrus pectin, the two unknowns, and an overlay of the complexation spectra of all three standards and the two unknowns at concentration level 4 of respectively, ruthenium red, nile blue, and acridine orange. As can be seen in Figure 25 and particularly Figure 26, the spectra of NB and AO for all three standards and two unknowns are closely overlapped. This portends that it would be difficult to distinguish them using NB and AO as complexing dyes. In Figure 24 the spectra of the three standards are quite well separated and the two unknowns are somewhat discernable. Therefore, it would indicate that RR would work the best out of the three colorimetric reagents.

Validation of Chemometric Techniques Employed and Standard

An integral part of any pattern matching technique is to determine the resolving power of the standards in the data set. For this purpose, the unknowns are subjected to the same mathematical treatments of the chemometric techniques as the standards. These data indicate how well the calculated metric can distinguish between known and unknown sample spectra. If the metric cannot reliably distinguish between its own standard spectra then it will not be able to provide unambiguous results when the techniques are used to determine unknowns. For all subsequent calculations, the spectral wavelength ranges were:

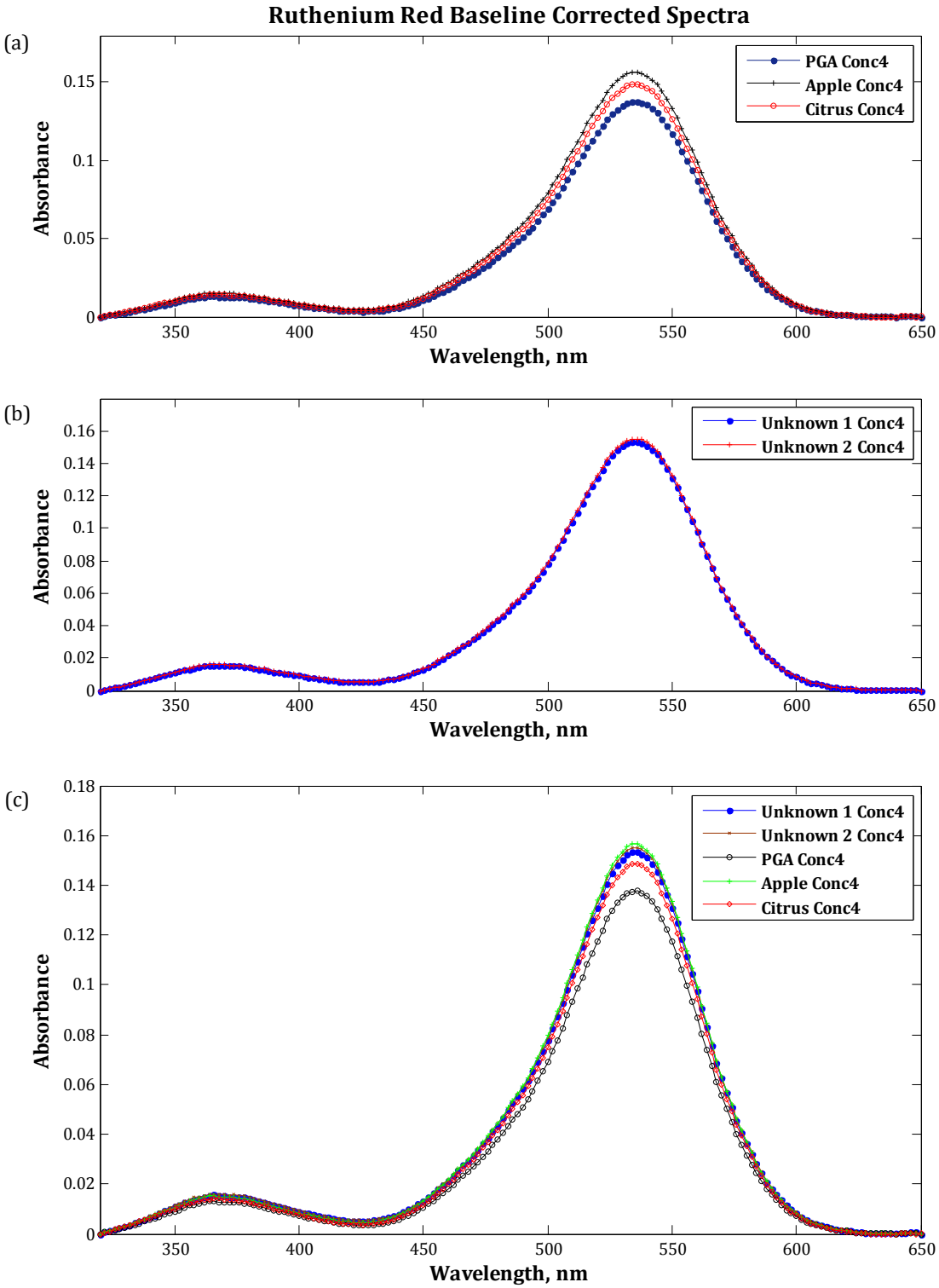


Figure 24. Baseline corrected spectra of RR complex of standards (a), unknowns (b), and (c) overlay of all at concentration level 4

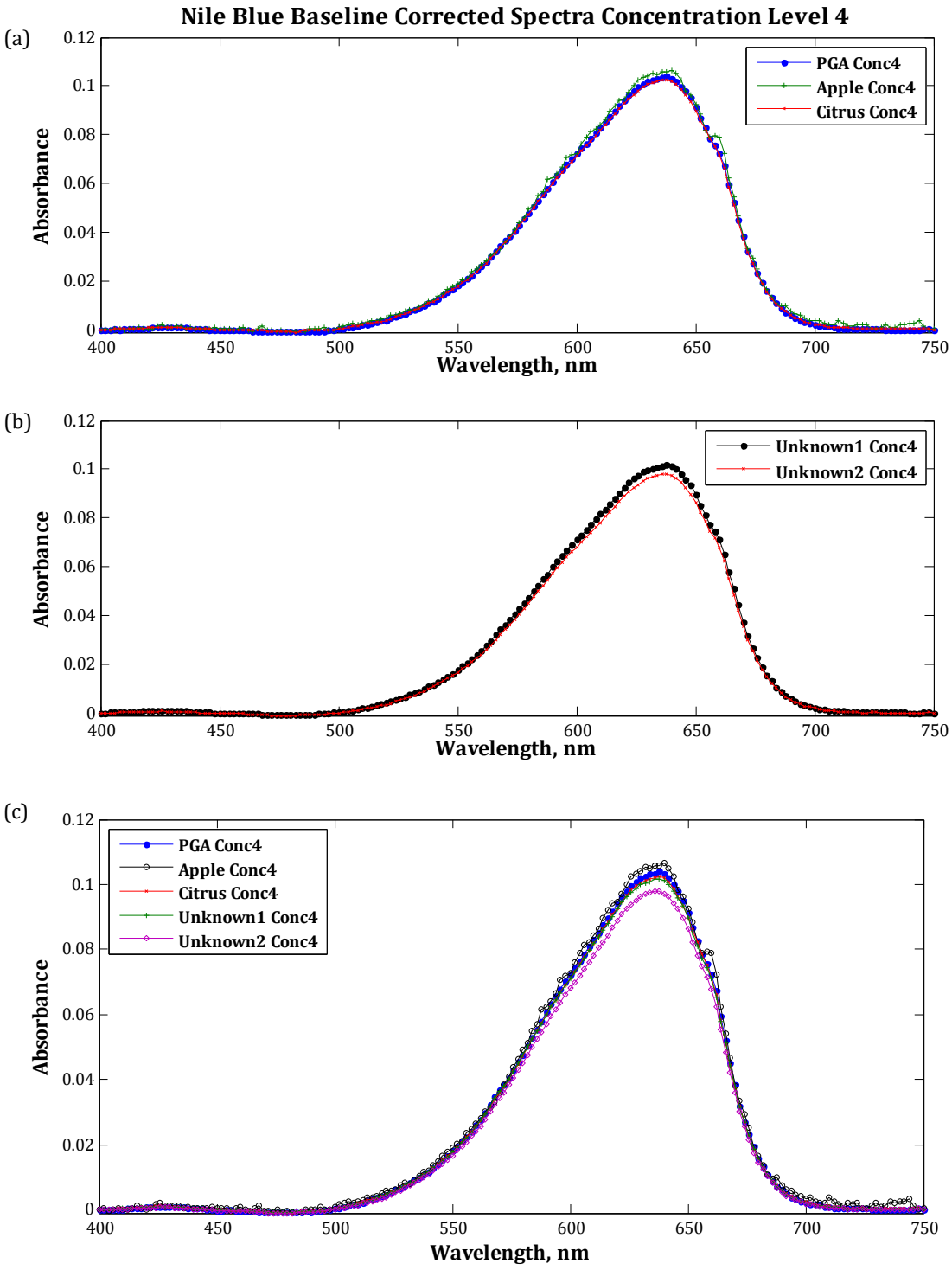


Figure 25. Baseline corrected spectra of NB complexation of standards (a), unknowns (b), and (c) overlay of all at concentration level 4

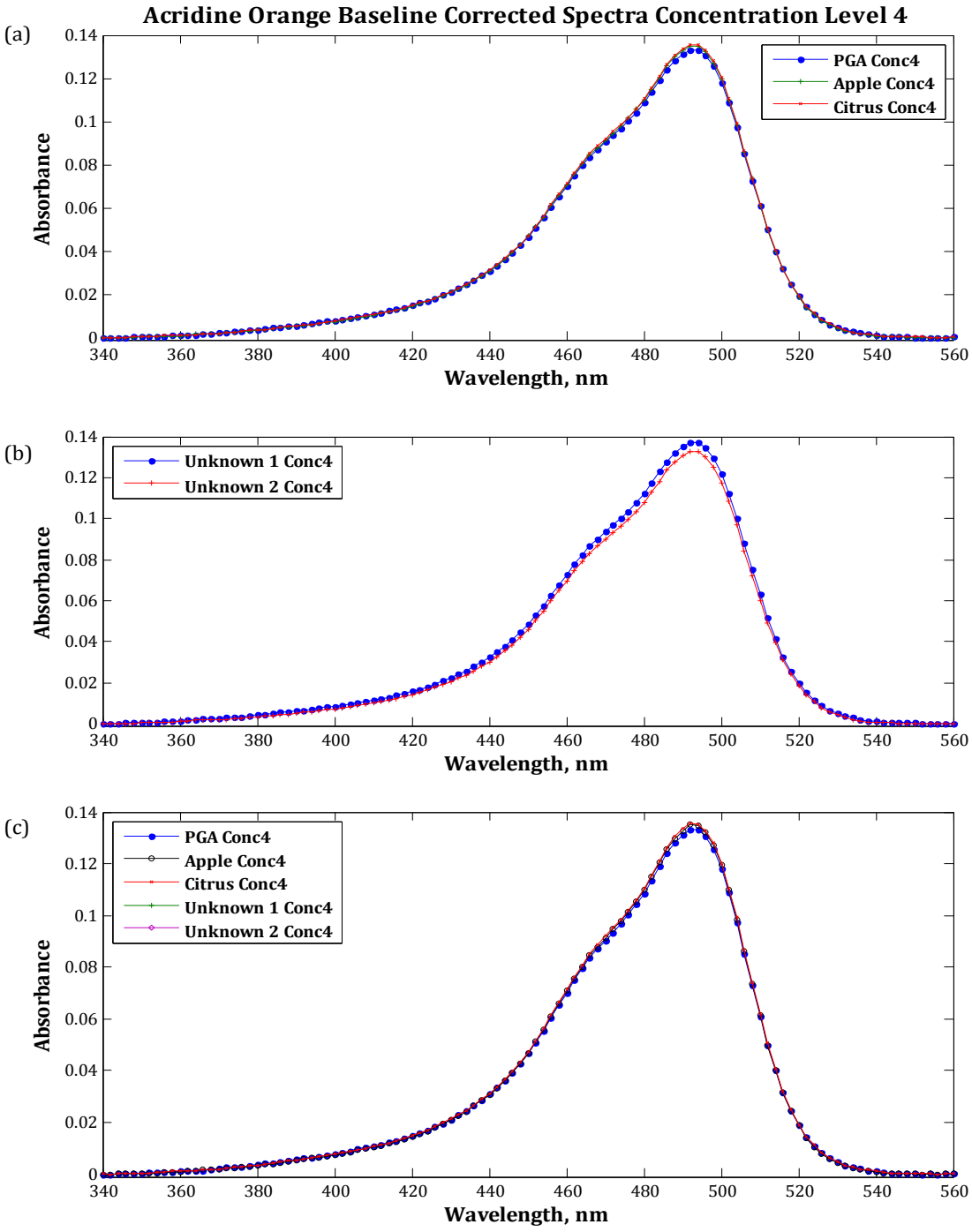


Figure 26. Baseline corrected spectra of AO complex of standards (a), unknowns (b), and (c) overlay of all at concentration level 4

RR 320 nm to 650 nm, NB 400 nm to 750 nm, and AO 340 nm to 560 nm where the major spectral features are present.

First, Euclidean Distance values between different pectin standards were calculated. This was done for all concentrations of the colorimetric reagents used. The ED values between any two datasets indicate how similar they are. Smaller values equate to greater similarity between the two sets of data. The interstandard Euclidean Distances of range scaled data among the pectin standards using the three colorimetric reagents RR, NB, and AO are tabulated in Table 4.

For all reagents, the ED values calculated for identical datasets did give a value of zero indicating they were the same or no "distance" apart. The ED values for all reagents at the lowest and highest concentrations tend to show little discrimination, while the best discrimination was observed for concentration levels 3 or 4. There was a distinct reagent concentration dependence observed. At too low a concentration, there were still many complexation sites available while at too high a concentration, saturation and excess reagent

Table 4. Interstandard ED of each dye at all concentrations

Euclidean Distances									
	PGA to Apple			**PGA to Citrus**			**Apple to Citrus**		
Concentration	**RR**	**NB**	**AO**	**RR**	**NB**	**AO**	**RR**	**NB**	**AO**
1	1.583	0.279	0.224	1.485	0.213	0.219	0.945	0.264	0.267
2	0.358	0.527	0.254	0.463	0.656	0.192	0.209	0.869	0.312
3	1.867	0.374	0.163	1.755	0.269	0.065	0.499	0.372	0.134
4	3.325	0.759	0.244	3.434	0.161	0.130	0.572	0.655	0.144
5	0.738	0.910	0.098	1.487	0.282	0.078	2.032	0.679	0.091
	PGA to PGA			**Apple to Apple**			**Citrus to Citrus**		
Concentration	**RR**	**NB**	**AO**	**RR**	**NB**	**AO**	**RR**	**NB**	**AO**
All	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

"smear" the spectral features and obscure whatever small differences that might be present. For RR, one observes that the ED values were large between PGA and apple (3.325) and between PGA and citrus (3.434) at concentration level 4 and reasonably large at concentration level 3. These indicate that RR seems to be able, at least from our knowledge of the pectins structures, to tell apart pectin structures that are distinctly different. However, the NB and the AO spectral data gave ED values that are not as useful. For instance, both dyes do not give ED values that clearly discriminate PGA pectin from the two other pectins as RR ED values can. Thus, it seems that NB and AO are not going to be reliable in the analysis of the unknown pectins. The difference between apple and citrus pectin standards, however, are not as large as one would hope. This may present difficulty in further analysis. These results indicate the standards library should be adequate for discrimination of unknowns based upon ED.

The interstandard R^2 values of range scaled data among the pectin standards using the three colorimetric reagents RR, NB, and AO are tabulated in Table 5.

Table 5. Interstandard R^2 values of each dye at all concentrations

R^2									
	PGA to Apple			**PGA to Citrus**			**Apple to Citrus**		
Concentration	**RR**	**NB**	**AO**	**RR**	**NB**	**AO**	**RR**	**NB**	**AO**
1	0.913	0.998	0.998	0.928	0.999	0.998	0.973	0.998	0.998
2	0.996	0.994	0.997	0.993	0.995	0.999	0.999	0.983	0.995
3	0.872	0.997	0.999	0.898	0.999	1.000	0.996	0.998	1.000
4	0.530	0.993	0.999	0.441	1.000	1.000	0.993	0.994	1.000
5	0.980	0.988	1.000	0.927	0.999	1.000	1.000	1.000	1.000
	PGA to PGA			**Apple to Apple**			**Citrus to Citrus**		
Concentration	**RR**	**NB**	**AO**	**RR**	**NB**	**AO**	**RR**	**NB**	**AO**
All	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

As shown previously in the calculation of ED values for RR, correlation data again indicated good discriminating power. In particular, at concentration level 4 the correlation coefficients between PGA and apple (0.530) and between PGA and citrus (0.441) were sufficiently low to allow one to conclude that the pectins were quite different. However, the same difficulty was evident in distinguishing apple from citrus. The correlation coefficient indicated they were identical. Unlike ED values, correlation date for NB and AO showed little to no discriminating power. This correlation dataset by itself would not be adequate for determinations of unknowns of natural sources.

The interstandard inner products (IPs) for eigenvectors of range scaled data among the pectin standards using the three colorimetric reagents RR, NB, and AO are tabulated in Table 6.

Table 6. Interstandard eigenvector IPs of each dye at all concentrations

Inner Products									
	PGA to Apple			**PGA to Citrus**			**Apple to Citrus**		
Concentration	**RR**	**NB**	**AO**	**RR**	**NB**	**AO**	**RR**	**NB**	**AO**
1	0.953	0.999	0.999	0.961	0.999	0.999	0.985	0.999	0.998
2	0.998	0.996	0.998	0.996	0.993	0.999	0.999	0.989	0.997
3	0.942	0.998	0.999	0.950	0.999	1.000	0.996	0.998	1.000
4	0.797	0.991	0.999	0.790	1.000	1.000	0.995	0.993	1.000
5	0.992	0.987	1.000	0.965	0.999	1.000	0.935	0.993	1.000
	PGA to PGA			**Apple to Apple**			**Citrus to Citrus**		
Concentration	**RR**	**NB**	**AO**	**RR**	**NB**	**AO**	**RR**	**NB**	**AO**
All	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

The inner product calculations for the standards library indicate poor resolving power among all the dyes. RR, as with the previous mathematical treatments, shows the best discriminating power at concentration level 4 of pectins known to be very different. The IP

values using RR spectral data are between PGA and apple (0.797) and between PGA and citrus (0.790), distinctly smaller than those between the other pectins. While spectral data of NB and AO do not, again, show such discrimination as RR even for well-known, distinctly different pectins.

Overall, the IP values are similar to R^2 values in their poor discriminating power between the different pectins that are not very different. These values do not offer any discrimination for all concentrations of NB and AO spectral date. Therefore, the IPs of NB and AO show these dyes are not suitable for unknowns. The PGA is partially distinguished from the apple and citrus as the IP values with them are 0.797 and 0.790 respectively. However, it is still difficult to distinguish between the apple and citrus pectin.

Determination of Unknowns Using Single Dye System

The ED, R^2, and IP data obtained from the use of RR, NB, and AO as "complexing" agents with pectin and unknowns 1 and 2 are tabulated in Table 7, Table 8, and Table 9 respectively. Pectin unknown prediction results based on the three factors listed in these tables are tabulated in Table 10.

From the data in Table 7, it can be again observed that at concentration levels of 1, 2, and 5, the results were ambiguous, inconsistent, and unsatisfactory. The reasons being given previously that the degree of complexation of the dyes with the available sites on the pectin were either incomplete or saturated. Thus, the discussion will focus on the results from dye concentration levels 3 and 4.

For unknowns 1 and 2, at level 3, the R^2 and IP values were distinctly the smallest with PGA, thus quite correctly hinting the absence of PGA structure in them for RR spectral data. However, this was not the case for dyes NB and AO spectral data. The R^2 and IP values were all close to one with PGA, apple, or citrus. Even at concentration level 4, the R^2 and IP values using these two dyes were all close to one. Thus, no discrimination was possible using NB or AO. In the case of RR, the results were more distinct at concentration level 4. The R^2 and IP values were clearly low for PGA, 0.493 and 0.799, and 0.504 and 0.796, respectively in unknown 1 and unknown 2, indicating the absence of PGA in both unknowns. However, R^2 and IP values were not able to allow one to discriminate the presence of apple or citrus features in both unknowns as their values were all very close to unity.

Table 7. Tabulated results of RR comparison calculations

Comparison Metric for RR					
	Concentration	**Comparison**	**w/ PGA**	**w/ Apple**	**w/ Citrus**
Unknown 1	**1**	**ED**	7.386	6.965	6.557
		R^2	0.301	0.041	0.052
		IP	0.584	0.651	0.703
	2	**ED**	0.414	0.265	0.190
		R^2	0.996	0.999	0.999
		IP	0.997	0.999	0.999
	3	**ED**	1.762	0.329	0.608
		R^2	0.886	0.996	0.995
		IP	0.949	0.998	0.994
	4	**ED**	3.352	0.383	0.414
		R^2	0.493	0.997	0.994
		IP	0.799	0.998	0.997
	5	**ED**	2.991	2.489	3.887
		R^2	0.624	0.753	0.362
		IP	0.862	0.906	0.755
Unknown 2	**1**	**ED**	2.010	1.489	0.723
		R^2	0.877	0.940	0.987
		IP	0.932	0.965	0.992
	2	**ED**	0.476	0.201	0.289
		R^2	0.993	0.999	0.998
		IP	0.996	0.999	1.000
	3	**ED**	2.485	1.299	1.752
		R^2	0.790	0.970	0.949
		IP	0.909	0.980	0.960
	4	**ED**	3.349	0.312	0.494
		R^2	0.504	0.997	0.993
		IP	0.796	0.998	0.996
	5	**ED**	0.942	0.680	1.927
		R^2	0.967	0.984	0.871
		IP	0.986	0.993	0.940

Table 8. Tabulated results of NB comparison calculations

Comparison Metric for NB					
	Concentration	**Comparison**	**w/ PGA**	**w/ Apple**	**w/ Citrus**
Unknown 1	**1**	**ED**	0.174	0.302	0.228
		R^2	0.999	0.998	0.999
		IP	0.999	0.998	0.999
	2	**ED**	1.040	1.128	0.666
		R^2	0.986	0.974	0.989
		IP	0.982	0.979	0.993
	3	**ED**	0.249	0.414	0.130
		R^2	0.999	0.997	1.000
		IP	0.999	0.997	1.000
	4	**ED**	0.148	0.742	0.158
		R^2	1.000	0.992	1.000
		IP	1.000	0.991	1.000
	5	**ED**	0.196	0.904	0.283
		R^2	0.999	0.986	0.998
		IP	0.999	0.987	0.999
Unknown 2	**1**	**ED**	0.217	0.295	0.271
		R^2	0.999	0.999	0.999
		IP	0.999	0.999	0.999
	2	**ED**	0.491	0.703	0.392
		R^2	0.997	0.990	0.996
		IP	0.996	0.993	0.997
	3	**ED**	0.279	0.407	0.124
		R^2	0.999	0.998	1.000
		IP	0.999	0.997	1.000
	4	**ED**	0.169	0.714	0.144
		R^2	0.999	0.993	1.000
		IP	1.000	0.992	1.000
	5	**ED**	0.146	0.845	0.248
		R^2	1.000	0.989	0.999
		IP	1.000	0.988	0.999

Table 9. Tabulated results of AO comparison calculations

Comparison Metric for AO					
	Concentration	**Comparison**	**w/ PGA**	**w/ Apple**	**w/ Citrus**
Unknown 1	**1**	**ED**	0.268	0.171	0.283
		R^2	0.997	0.999	0.997
		IP	0.998	0.999	0.998
	2	**ED**	0.151	0.268	0.254
		R^2	0.999	0.998	0.998
		IP	0.999	0.998	0.998
	3	**ED**	0.143	0.103	0.117
		R^2	0.999	1.000	0.999
		IP	0.999	1.000	1.000
	4	**ED**	0.122	0.200	0.139
		R^2	1.000	0.999	0.999
		IP	1.000	0.999	0.999
	5	**ED**	0.095	0.086	0.084
		R^2	1.000	1.000	1.000
		IP	1.000	1.000	1.000
Unknown 2	**1**	**ED**	0.243	0.306	0.203
		R^2	0.998	0.998	0.998
		IP	0.998	0.997	0.999
	2	**ED**	0.225	0.391	0.270
		R^2	0.999	0.995	1.000
		IP	0.999	0.996	0.998
	3	**ED**	0.083	0.161	0.085
		R^2	1.000	1.000	1.000
		IP	1.000	0.999	1.000
	4	**ED**	0.263	0.066	0.158
		R^2	0.999	1.000	1.000
		IP	0.998	1.000	0.999
	5	**ED**	0.101	0.130	0.106
		R^2	1.000	0.999	0.999
		IP	1.000	1.000	1.000

For the pectin spectral data with NB and AO, and at concentration levels of 3 and 4, the ED values obtained for both unknowns were nondiscriminating and inconsistent from one concentration to another. For instance, at concentration level 3, NB ED results indicated citrus features well in unknown 1, but at concentration 4 PGA was the choice. From our knowledge of the pectins, PGA was quite surely not a component of the natural pectins. For unknown 2, the ED values from NB data did indicate at both concentration levels 3 and 4 that the citrus feature seems to be the closest one, but the distinction was not as convincing. The data for AO were more inconsistent and confusing. However, the ED values from RR data were much more consistent. At both concentration levels 3 and 4, for both unknowns, the apple features was closest with citrus features not that far apart. The ED values were 0.329 and 0.383 for apple versus 0.608 and 0.414 for citrus, and 1.299 and 1.752 versus 0.312 and 0.494 respectively, in unknown 1 and unknown 2. Therefore, the ED values of RR data seemed to indicate that both unknowns might have both apple and citrus features with the apple feature somewhat more prominent.

The predictions of these data were summarized in Table 10. One can quite clearly see the conclusions that were arrived at from the above discussions. The prediction using the RR spectral data consistently point to the apple feature being the dominant structure in pectin unknown 1 and 2 at concentration level 4.

Table 10. Summarized predictions for all dyes and data

Prediction Matrix for Data of all Dyes					
	Concentration	**Dye**	**ED**	R^2	**IP**
Unknown 1	**1**	**RR**	citrus	pga	citrus
		NB	pga	pga≈citrus	pga≈citrus
		AO	apple	apple	apple
	2	**RR**	citrus	citrus	citrus
		NB	citrus	citrus	citrus
		AO	pga	pga	pga
	3	**RR**	apple	apple	apple
		NB	citrus	citrus	citrus
		AO	apple	apple	apple≈citrus
	4	**RR**	apple	apple	apple
		NB	pga	pga≈citrus	pga≈citrus
		AO	pga	pga	pga
	5	**RR**	apple	apple	apple
		NB	pga	pga	pga
		AO	citrus	???	???
Unknown 2	**1**	**RR**	citrus	citrus	citrus
		NB	pga	???	???
		AO	citrus	???	citrus
	2	**RR**	apple	apple	citrus
		NB	citrus	pga	citrus
		AO	pga	citrus	pga
	3	**RR**	apple	apple	apple
		NB	citrus	citrus	citrus
		AO	pga	???	pga≈citrus
	4	**RR**	apple	apple	apple
		NB	citrus	citrus	citrus
		AO	apple	apple≈citrus	apple
	5	**RR**	apple	apple	apple
		NB	pga	pga	pga
		AO	pga	pga	???

Combined Factors as Predictors of Features in Unknowns

In an attempt to improve the predictive power of the obtained data, the three factors of ED, R^2, and IP were combined into one quantitative quotient, Q, as given in Equation 9.

$$Q = \frac{R^2 \times IP}{ED^2} \qquad \text{Equation 9}$$

The Q value attempts to expand the differences between the pectin data through adding weighting to ED. The larger the Q value, the more specific standards feature is present in the unknown data. The resulting values were tabulated in Table 11. To highlight differences, the Q values from Table 11 were scaled by dividing each row data by the maximum value for that row. The results were tabulated in Table 12 where 1.000 indicates the strongest match. The predictions were summarized in Table 13. Again, the discussions will focus only on data for these reagents at concentration levels 3 and 4. Again, because the RR pectin spectral data at concentrations 3 and 4 have been shown previously to provide the best and most robust results, the discussion will focus on these sets of data. The Q values at concentration 3 for RR spectral data gave a 1.000 for the apple standard in unknown 1 compared to 0.317 with citrus standard while at concentration 4, the values are 1.000 for the apple standard and 0.924 for citrus standard. Thus, the Q value showed a greater distinction between the 2 standard features in the unknown. For unknown 2, similar improvement is seen. At concentration level 3, the apple standard value of 1.000 is contrasted at 0.581 for the citrus standard. At concentration 4, the values are now 1.000 compared to 0.430. Again, the Q value seems to improve the predictive value and allow a clearer distinction to be observed.

Table 11. Combined Q table of ED, R^2, and IP values for all dyes

Combined Comparison Matrix					
	Concentration	**Comparison**	**w/ PGA**	**w/ Apple**	**w/ Citrus**
Unknown 1	**1**	**RR**	0.003	0.001	0.001
		NB	32.897	10.929	19.220
		AO	13.815	34.103	12.455
	2	**RR**	3.389	9.253	19.397
		NB	0.911	0.764	2.227
		AO	43.863	13.894	15.398
	3	**RR**	0.167	5.978	1.893
		NB	16.112	5.799	58.956
		AO	48.478	93.866	72.278
	4	**RR**	0.026	4.413	4.076
		NB	45.622	1.798	39.944
		AO	66.614	24.975	51.344
	5	**RR**	0.041	0.079	0.017
		NB	25.984	1.205	12.493
		AO	109.647	134.890	142.685
Unknown 2	**1**	**RR**	0.127	0.276	1.328
		NB	21.183	11.466	13.568
		AO	16.821	10.653	24.289
	2	**RR**	2.559	16.148	8.387
		NB	4.133	1.998	6.468
		AO	19.620	6.522	13.652
	3	**RR**	0.075	0.374	0.217
		NB	12.810	6.002	64.650
		AO	145.826	38.758	139.516
	4	**RR**	0.026	6.647	2.859
		NB	35.152	1.944	48.288
		AO	14.415	230.723	39.815
	5	**RR**	0.636	1.385	0.165
		NB	46.984	1.383	16.258
		AO	98.173	59.077	88.068

Table 12. Prediction Matrix using scaled Q-table values

Combined Comparison Matrix					
	Concentration	**Comparison**	**w/ PGA**	**w/ Apple**	**w/ Citrus**
Unknown 1	**1**	**RR**	1.000	0.170	0.266
		NB	1.000	0.332	0.584
		AO	0.405	1.000	0.365
	2	**RR**	0.175	0.477	1.000
		NB	0.409	0.343	1.000
		AO	1.000	0.317	0.351
	3	**RR**	0.028	1.000	0.317
		NB	0.273	0.098	1.000
		AO	0.516	1.000	0.770
	4	**RR**	0.006	1.000	0.924
		NB	1.000	0.039	0.876
		AO	1.000	0.375	0.771
	5	**RR**	0.514	1.000	0.213
		NB	1.000	0.046	0.481
		AO	0.768	0.945	1.000
Unknown 2	**1**	**RR**	0.095	0.208	1.000
		NB	1.000	0.541	0.641
		AO	0.693	0.439	1.000
	2	**RR**	0.158	1.000	0.519
		NB	0.639	0.309	1.000
		AO	1.000	0.332	0.696
	3	**RR**	0.200	1.000	0.581
		NB	0.198	0.093	1.000
		AO	1.000	0.266	0.957
	4	**RR**	0.004	1.000	0.430
		NB	0.728	0.040	1.000
		AO	0.062	1.000	0.173
	5	**RR**	0.459	1.000	0.119
		NB	1.000	0.029	0.346
		AO	1.000	0.602	0.897

Table 13. Predicted results from combined datasets

	Concentration	RR	NB	AO
Unknown 1	**1**	pga	pga	apple
	2	citrus	citrus	pga
	3	apple	citrus	apple
	4	apple	pga	pga
	5	apple	pga	citrus
Unknown 2	**1**	citrus	pga	citrus
	2	apple	citrus	pga
	3	apple	citrus	pga
	4	apple	citrus	apple
	5	apple	pga	pga

Selection of Samples Based Upon Molecular Weight

To further aid in characterizing pectins, commercial samples were purchased and subjected to GPC analysis. The unknown samples used in the study were selected from this list based upon proximity to one of the pectin standards used earlier. A tabulated list of GPC results is included in Table 14 for commercial samples and Table 15 for samples used in this study.

Table 14. MW results of GPC studies on diverse samples

Manufacturer	**M_w**	**M_n**	$\overline{x}_{M_w}$	**As Branded**
SureGel	931803	40124	N/A	Low Sugar
SureGel 1A	902542	49521	884607	Low Sugar
SureGel 1B	866672	57450		Low Sugar
SureGel	1269073	43785	N/A	High Sugar
SureGel	915064	19616	N/A	High Sugar
SureGel	829814	35822	N/A	High Sugar
SureGel 2A	785962	32957	742409	High Sugar
SureGel 2B	677902	33174		High Sugar
SureGel 2C	763364	45010		High Sugar
Mrs. Wages	400355	2539	395984	Low Sugar
Mrs. Wages	391614	2707		Low Sugar
Ball 1A	1092706	121837	1057638	High Sugar
Ball 1B	1022570	102155		High Sugar
Ball	970401	66216	N/A	High Sugar
Ball 2A	2023990	783468	1990572	Low Sugar
Ball 2B	1957155	699325		Low Sugar
Ball	1740333	760892	N/A	Low Sugar
Kroger 1A	881469	28269	877470	High Sugar
Kroger 1B	880487	27529		High Sugar
Kroger 1C	870455	55952		High Sugar
Aldrich	1578538	906765	N/A	HM-Citrus
Aldrich	305651	82102	N/A	Citrus
Aldrich	146601	59834	N/A	95% PGA

Table 15. Chromatographic GPC results of samples used in this study

Sample	M_w	M_n	$\bar{x}_{M_w}$
Unknown #1	845328	62143	864568
	883808	64832	
	720050	43340	outlier
Unknown #2	494380	36103	492712
	491044	32216	
	361973	33554	outlier
PGA Standard	383546	99885	383932
	384318	95499	
	392133	102470	
Apple Standard	708248	51437	702726
	700991	57336	
	698939	61700	
Citrus Standard	380486	85946	382765
	389263	78302	
	378546	73533	

The MW data show that apple pectin standard, on average, have lower MW compared to that of citrus pectin standard. PGA pectin standard has the highest MW. Thus, PGA is structurally much different from the others and thus quite easily distinguished. Our data clearly bear this out. Unknown 2 has lower MW than apple pectin standard and has the lowest MW compared to all pectin standards. So unknown 2 should have structure closer to apple pectin more so than the other two standards. For unknown 1, the average MW is quite similar to that of the apple pectin especially in M_n so this unknown should have structure very close to that of the apple pectin. Although one of the three MW measurements seemed to be an outlier, if one omitted it from the mean calculation, the MW of unknown 1 is substantially higher than the MW of apple pectin but still quite distant from that of citrus pectin. Therefore, it seems that our analysis using the Q value from RR spectral data is in line with the GPC MW results.

As mentioned previously, euclidean distance, correlation coefficients, and eigenanalysis data failed to predict consistently with NB and AO. When looking at MW data, it is readily apparent polymer size greatly influences spectral results as with %DE values. MW of citrus and PGA standards are relatively small when compared to apple. Pectin Unknown 2 was closer to PGA and citrus standards in MW. Pectin Unknown 1 MW was closer to that of apple. Further, %DE of the pectins

CHAPTER 5

CONCLUSION

Pectins are a family of abundant, naturally occurring, complex, anionic polysaccharides comprising up to 35% of the cell wall of most terrestrial plants. Pectin has had a long and varied history in the food and more recently the pharmaceutical industry. Incredibly, with this history, not much was known of pectin's structural and chemical properties until recent years. Two main classes of pectin exist, low methoxyl and high methoxyl pectin. These play very important roles in pectin's chemical and physical properties and provide for a diversity of uses. The two main classes can be further subdivided into slow, medium, and rapid set gelation types. The speed of gelation is directly proportional to the %DE of the molecule. Pectin contains a very high percentage of galactouronides but can contain substantial amounts of other uronide impurities that further differentiate its properties.

Numerous techniques exist for the analysis of pectin. However, due to the complexity and heterogeneous nature of the pectin polymer, distinctive analyses are very difficult. Additionally, pectins are insoluble in most organic solvents and only soluble in water when complexed with a monovalent salt. This adds to the complexity of analysis. Two techniques often used for characterization of pectin are molecular weight determination through gel permeation chromatography and UV-Vis analysis with reactions producing colored complexes. Molecular weight (MW) has the second largest influence on pectin chemical and physical properties with %DE being the most important. The MW of the polymer is a direct indication of the number of reactive sites available for complexation. Larger sizes also increase polymer self-

aggregation where the pectin will fold and crosslink inter- and intra-molecularly. The size can also influence solution dynamics and viscosity (e.g. gelation). For this reason, GPC analysis can be greatly influenced by how the sample matrix is prepared. Only meticulous preparation and measurement conditions will provide accurate MW results.

Spectrophotometric measurement techniques generally suffer from a lack of specificity in complex carbohydrate research. Because pectin is not homogeneous, the reagents used to produce color in reactions with decomposed pectin suffer numerous interferences. Even reagents targeted specifically to the galactouronides will show limited reactivity to other uronides in the system. Due to these deficiencies, researchers have recently intensified research on complexation reactions rather than chemical modification to analyze pectins. In complexation reactions, pectin has a very high affinity for divalent and trivalent ionic compounds. Divalent salts of pectin have limited solubility while trivalent salts are insoluble. Pectin complexation with colored metal complexes or other ionic compounds that produce color in the UV-Vis region of the electromagnetic spectrum allow for low cost, rapid analysis through UV-Vis measurements. Ruthenium Red is a metachromic, ionic dye that has shown quantitative interaction with pectin at lower %DE values. Lower %DE pectin polymers provide numerous sites for complexing with RR. MW and polymer solution dynamics will greatly influence pectin's ability to form complexes. Therefore, the best complexation will occur at moderate MW and in specifically controlled conditions.

The first goal of this work was to define the best complexing conditions for the pectin under study. Experimentation was conducted on pectins with a moderate range of molecular

weights in a slightly acidic biological buffer. Most organic and inorganic buffers show varying levels of complexation with pectin and cause precipitation and experimental error. Thus, it was necessary to choose the buffer carefully for this work. The versatile biological buffer, 3-morpholinopropanesulfonic acid, was chosen as it provides the needed pH buffering capacity and aids in the ionization of the pectin without complexation. Low pH hinders ionization of carboxyl groups to decrease electrostatic intramolecular repulsion within the chains thus allowing gelation. We want to keep pH higher to keep chains available for reactions and to minimize chain-chain interactions.

As previously discussed, RR complexes quantitatively with pectin up to roughly 80 %DE. The intent of this research is to devise a method whereby pectins of any range %DE can be characterized with RR or other metachromic dyes. Additionally, the method should also characterize off-the-shelf pectin with minimal purification. The standards used in this study fall within a high range of %DE. PGA (pectin with very low %DE) was also employed as a standard to cover the full range of %DE. Prior knowledge shows calcium is a good precipitating agent for lower %DE values but poor for higher levels of %DE. Based upon *a priori* knowledge, Pb^{2+} was chosen as the best candidate for precipitating pectin under a variety of conditions and physical properties. To develop the method, reagent grade pectins of known composition were acquired for study. The standards were purified to remove as much interference as possible. Experimentation was done to determine optimal concentrations of buffer, dye, Pb^{2+} precipitating agent, and pectin to use for the study. RR complexation with the standards was verified by replicating previous experimentation by other researchers. The pectin was dissolved

in a dilute aqueous buffer solution, the dye of known concentration was added, allowed time to interact, the pectin-dye complex precipitated out of solution and the supernatant with residual dye analyze via UV-Vis. Because of the minute concentrations used, the deltas in the absorbances were very small. Differences in the spectra could be seen visually but in order to fully explain the results and validate the data, it was necessary to employ chemometric techniques. As expected, RR did show quantifiable interactions (using euclidean distance, correlation coefficients, and eigenvector inner products) with the various pectin standards. Numerous other cationic dyes were tested but most showed no discernable results. Nile Blue and Acridine Orange did, however, show some promise. Although showing some interaction, when compared singularly with RR, these dyes failed to provide data of high confidence. To increase predictive resolution, the chemometric factors were combined into a single quotient, Q, given by $Q = (R^2 \times IP)/(ED)^2$.

The range of concentrations used for this study was important. The spectral features did show at too low of dye concentration, signal noise and lack of pectin complex interaction with the dyes gave results of poor precision. Conversely, high dye concentrations saturated the pectin sites and excess amount in the solution led to the loss of resolution. Mid-range dye concentration showed the most reproducible results.

Commercial samples of both “low sugar” (low %DE) and “normal” (high %DE) were obtained from local grocers and classified as unknown 1 and unknown 2, respectively. These samples were not further purified and analyzed under the same conditions as the standards. Using the same chemometric techniques, the unknowns were compared to the standards and

these data were used to predict which standard the unknown was closest to at the chosen concentration range.

The results obtained showed that the pectins treated with NB and AO did not give the analysts any useful information or predictive value at all concentrations and employing any of the three chemometric factors chosen. Even at the optimal concentration level 3 or 4, these dye data did not afford any robust consistent predictive values.

Ruthenium red seemed to be able to provide discrimination when used as the colorimetric reagent for the different pectins. Among the concentration range attempted, concentrations level 3 and 4, particularly the later, gave good results. With the three chemometric treatments, the Euclidean Distance (ED) values were the greatest predictive factor. The correlation coefficients (R^2) and the inner product (IP) were useful in ruling out the feature, i.e. the feature not present, in the unknown rather than distinguishing among the features that might be present or similar to one another. Thus, the quotient, Q, was conceived to include all these factors while amplifying the most predictively useful factor. So, $Q = (R^2 \times IP)/(ED)^2$ was used and it indeed was able to separate the features apart consistently. Furthermore, the predictions of the Q values were corroborated by the gel permeation MW results.

Thus, from all the results obtained and the discussions therein, the objectives of this research have been mostly achieved. We have shown that, among several, the pectin spectral data of a colorimetric reagent, ruthenium red, coupled with the three chosen chemometric

factors, ED, R^2, and IP, allowed one to distinguish the features in the unknown pectins. These results were also further corroborated by GPC MW data.

For further investigations, the predictive value of the dyes may be enhanced by combining 2 or 3 of them simultaneously in a single pectin. Because of the structural features of the pectins and that of the colorimetric reagents, there will be a competition among the dyes for the sites in a given pectin. Some dyes may be more competitive for a certain feature. This competition can be used to enhance the distinction among the pectins studied.

BIBLIOGRAPHY

1. International Pectin Producers Association, Pectin: Discovery & History. In Internation Pectin Producers Association: 2001.
2. Vauquelin, M. L.-N., Analyse due Tamarin. In *Ann. Chim.*, Paris, 1790; pp 92-106.
3. Braconnot, H., Recherches sur un novel acide univerellement rependu dans tous les vegetaux. In *Ann. Chim. Phys. Ser. II*, Paris, 1825; pp 173-178.
4. Douglas, R. Pectin Food Product. U.S. Patent 1082682, 1913.
5. Tombs, M. P.; Harding, S. E., *An Introduction to Polysaccharide Biotechnology*. Taylor & Francis: Pennsylvania, 1998.
6. May, C. D., Industrial pectins: Sources, production and applications *Carbohydrate Polymers* **1990,** 12, (1), 79-99.
7. Pilnik, W.; Reitsma, J. C. E., Analysis of mixtures of pectins and amidated pectins. *Carbohydrate Polymers* **1989,** 10, (2), 315-319.
8. Visser, J.; Voragen, A. G. J., *Pectins and Pectinases*. Elsevier Science: Amsterdam, 1996.
9. Kertesz, Z. I.; Baker, G. I.; Joseph, G. H.; Mottern, H. H.; Olsen, A. G., *Chem. Eng. News* **1944,** 22, 105.
10. Korner, R.; Limberg, G.; Christensen, T. M. I. E.; Mikkelsen, J. D.; Roepstorff, P., Sequencing of Partially Methyl-Esterified Oligogalacturonates by Tandem Mass Spectrometry and Its Use To Determine Pectinase Specificities. *Anal. Chem.* **1999,** 71, (7), 1421-1427.

11. Voragen, A. G. J.; Pilnik, W.; Thibault, J. F.; Axelos, M. A. V.; Renard, C. M. G. C., *Food Polysaccharides and Their Applications*. Marcel Dekker, Inc.: New York, 1995; p 287-389.

12. Rolin, C.; Nielsen, B. U.; Glahn, P.-E., *Polysaccharides: Structural Diversity and Functional Versatility*. Marcel Dekker, Inc.: New York, 1998; p 377-431.

13. Bishay, I. E.; Clark, D. R. Carbohydrate protein cream substitutes. U.S. Patent 5536514, 1996.

14. Speirs, C.; White, K. E. Gelling system. U.S. Patent 5356654, 1994.

15. Flory, P. J., *Priciples of Polymer Chemistry*. Cornell University Press: Ithaca, New York, 1953.

16. Lapasin, R.; Pricl, S., *Rheology of Industrial Polysaccharides: Theory and Applications*. Blackie Academic & Professional: London, 1995.

17. Wang, Q.; Pagan, J.; Shi, J., Pectin From Fruits. In *Functional Foods: Biochemical and Processing Aspects*, Shi, J.; Mazza, G.; Le Maguer, M., Eds. CRC Press LLC: Washington, 2002; Vol. 2.

18. Jenchs, W. P., *Catalysis in Chemistry and Enzymology*. McGraw-Hill: New York, 1969.

19. Chen, T. S.; Joslyn, M. A., The effect of sugars on viscosity of pectin solutions. II. Comparison of dextrose, maltose, and dextrins. *J. Colloid Interface Sci.* **1967,** 25, 246-352.

20. Walkinshaw, M. D.; Arnott, S., Conformations and interactions of pectins. II. Models for junction zones in pectinic acid and calcium pectate gels. *J. Mol. Biol.* **1981,** 153, 1075-1085.

21. Anyas-Weisz, L.; Deuel, H., Über das Verhalten löslicher Polyelektrolyte gegenüber Ionenaustauschern. *Helv. Chem. Acta* **1950,** 33, 559-562.

22. Van Buren, J. P., Function of pectin in plant tissue structure and firmness. In *The Chemistry and Technology of Pectin*, Walter, R. H., Ed. Academic Press, Inc.: New York, 1991; pp 1-22.

23. Kohn, R.; Luknar, O., Intermolecular calcium ion binding on polyuronates – polygalacturonate and polyguluronate. *Coll. Czech. Commun.* **1977,** 42, 731-744.

24. Thibault, J. F.; Rinaudo, M., Chain association of pectic molecules during calcium-induced gelation. *Biopolymers* **1986,** 25, (3), 455-468.

25. El-Nawawi, S. A.; Heikel, Y. A., Factors affecting the production of low-ester pectin gels. *Carbohyd. Polym.* **1995,** 26, (3), 189-193.

26. Grosso, C. R. F.; Rao, M. A., Dynamic rheology of structure development in low-methoxyl pectin+Ca^{2+}+sugar gels. *Food Hydrocolloids* **1998,** 12, (3), 357-363.

27. Rolin, C., Commercial Pectin Preparations. In *Pectins and their Manipulation*, Seymour, G. B.; Knox, J. P., Eds. CRC Press, LLC: Boca Raton, Florida, 2000.

28. Dea, I. C. M.; Madden, J. K., *Food Hydrocolloids* **1986,** 1, 71.

29. Glahn, P.-E. Pectin Process and Composition. U.S. Patent 6207194, 2001.

30. Hetland, G.; Samuelsen, A. B., Protective Effect of *Plantago major* L. Pectin Polysaccharide against Systemic *Streptococcus pneumoniae* Infection in Mice. *Scand. J. Immunol.* **2000,** 52, (4), 348-255.

31. Sun, X.-B., Matsumoto, T.; Yamada, H., Anti-ulcer activity and mode of action of the polysaccharide fraction from the leaves of Panax ginseng. *Planta Med.* **1992**, 58, 432-435.

32. Waterhouse, E. T., Washington, C., Washington, N., An investigation into the efficacy of the pectin based anti-reflux formulation-Aflurax. *Int. J. Pharm.* **2000,** 209, 79-85.

33. Thayer, A. M., Food Additives. *Chem. Eng. News* **1992**, 70, pp 26-44.

34. Rhodes, K. H. Process for making whey-derived fat substitute product and products thereof. U.S. Patent 5413804, 1992.

35. National Research Council, Pectins. In *Food Chemical Codex*, 2 ed.; National Academy of Sciences: Washington, D.C., 1972; pp 577-581.

36. Voragen, A. G. J.; Schols, H. A.; Pilnik, W., Determination of the degree of methylation and acetylation of pectins by HPLC. *Food Hydrocolloids* **1986,** 1, (1), 65-70.

37. Kearsley, M. W.; Verwaerde, F., Enzymatic determination of carbohydrates. In *Advances in Carbohydrate Analysis*, White, C., Ed. Elsevier Science & Technology: London, 1991; Vol. 1.

38. Albersheim, P.; Neukom, H.; Deuel, H., Über die Bildung von Ungesättigen Abbauprodukten durch ein Pektinabbauendes Enzym. *Helv. Chim. Acta* **1960,** 43, 1422-1426.

39. Stoecker, P. W.; Yacynyeh, A. M., Chemically modified electrodes as biosensors. *Sel. Elec. Rev.* **1990**, (12), 137-160.

40. Moraru, C.; Logendra, L.; Lee, T.-C.; Janes, H., Characteristics of 10 processing tomato cultivars grown hydroponically for the NASA Advanced Life Support (ALS) Program. *Journal of Food Composition and Analysis* **2004,** 17, (2), 141-154.

41. McCready, R. M.; McComb, E. A., Pectic constituents in ripe and unripe fruit. *Journal of Food Science* **1954,** 19, (1-6), 530-535.

42. Smith, J. E.; Stainsby, G., Studies on pectins I. Light-scattering and weight average molecular weights. *British Polymer Journal* **2006,** 9, (4), 284-289.

43. Hobson, G. E., Pectinesterase in normal and abnormal tomato fruit. *Biochem. J.* **1963,** 86, 358-365.

44. Robertson, G. L., The determination of pectic substances in citrus juice and grapes by two spectrophotometric methods. *Journal of Food Biochemistry* **1981,** 5, (2), 139-143.

45. Hou, W.-C.; Chang, W.-H.; Jiang, C.-M., Qualitative distinction of carboxyl group distributions in pectins with ruthenium red. *Bot. Bull. Acad.Sin.* **1999,** 40, 115-119.

46. Bergman, F., Colorimetric determination of amides as hydroxamic acids. *Anal. Chem.* **1952,** 24, 1367-1369.

47. Dische, Z., A new specific color reaction of hexuronic acids. *J. Biol. Chem.* **1946,** 167, 189-198.

48. Dische, Z., A modification of the carbazole reaction of hexuronic acids for the study of polyuronides. *J. Biol. Chem.* **1950,** 183, 489-494.

49. Taylor, K. A.; Buchanan-Smith, J. G., A colorimetric method for the quantitation of uronic acids and a specific assay for galacturonic acid. *Anal. Biochem.* **1992,** 201, 190-196.

50. Dekker, R. F. H.; Richards, G. N., Determination of pectic substances in plant material. *J. Sci. Food Agric.* **1972,** 23, 475-483.

51. McComb, E. A.; McCready, R. M., Extraction and determination of total pectic materials in fruits. *Anal. Chem.* **1952,** 24, 1630-1632.

52. Bitter, T.; Muir, H. M., A modified uronic acid carbazole reaction. *Anal. Biochem.* **1962,** 4, 330-334.

53. Rouse, A. H.; Atkins, C. D., Pectinesterase and pectin in commercial citrus juices as determined by methods used at the citrus experiment station. *Fla. Agric. Exp. Stn. Bull.* **1955,** 570.

54. McCready, R. M., In *Methods in Food Analysis*, Joslyn, M. A., Ed. Academic Press: New York, 1970; pp 565-599.

55. Brown, A. H., Determinations of pentose in the presence of large quantities of glucose. *Arch. Biochem.* **1946,** 11, 269.

56. Blumenkrantz, N.; Asboe-Hansen, G., New method for quantitative determination of uronic acids. *Anal. Biochem.* **1973,** 42, (2), 484-489.

57. Scott, R. W., Colorimetric determination of hexuronic acids in plant materials. *Anal. Chem.* **1979,** 51, 936-941.

58. Thibault, J. F., Automatisation du dosage des substances pectiques. par la méthode au méta-hydroxydiphényl. *Lebensm. Wiss. u.-Technol.* **1979,** 12, 247.

59. Mitra, A.; Nath, R. K.; Chakraborty, A. K., Interaction of bacterial acidic polysaccharides with cationic dyes. *Colloid & Polymer Science* **1993,** 271, (11), 1042-1048.

60. Mandal, N. C.; Biswas, B. B.; Pal, M. K., Metachromasia induced by inositol-hexa-phosphate with toluidene blue. *Histochemistry and Cell Biology* **1969,** 18, (3), 202-209.

61. Stone, A. L., Anomalous rotary dispersion of metachromatic mucopolysaccharide-dye complexes. I. Heparin. *Biopolymers* **2004,** 2, (4), 315-325.

62. Stone, A. L.; Bradley, D. F., Aggregation of cationic dyes on acid polysaccharides: I. Spectrophotometric titration with acridine orange and other metachromic dyes. *Biochim. Biophys. Acta* **1967,** 148, 172-192.

63. Stone, A. L., Aggregation of cationic dyes on acid polysaccharides. II. Quantitative parameters of metachromacy. *Biochim. Biophys. Acta* **1967,** 148, 193-206.

64. Seely, G. R.; Knotts, R. R., Binding of methylene blue to alginate: Effect of acid treatment on metachromasy. *Carbohydrate Polymers* **1983,** 3, (2), 109-127.

65. Chakrabarti, A.; Nath, R. K.; Chakraborty, A. K., Induction of metachromasy in cationic dye by bacterial cell surface polysaccharide from *Klebsiella* K10. *Spectrochimica Acta Part A: Molecular Spectroscopy* **1989,** 45, (9), 981.

66. Jana-Sur, P.; Chakraborty, A. K., Studies on the interaction of bacterial capsular polysaccharide-*Klebsiella* K16 with cationic dyes. *Journal of Photochemistry and Photobiology A: Chemistry* **2005,** 173, (1), 64-69.

67. Dasgupta, S.; Nath, R. K.; Biswas, S.; Hossain, J.; Mitra, A.; Panda, A. K., Spectral studies on the interaction of cationic dye/surfactants with *Klebsiella* K28 capsular polysaccharide. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **2007,** 302, (1-3), 17-23.

68. Stoddart, R. W.; Spires, I. P. C.; Tipton, K. F., Solution properties of polygalacturonic acid. *Biochem. J.* **1969,** 114, 863-870.

69. Kartel, M. T.; Kupchik, L. A.; Veisov, B. K., Evaluation of pectin binding of heavy metal ions in aqueous solutions. *Chemosphere* **1999,** 38, (11), 2591-2596.

70. Lakatos, B.; Meisel, J.; Rockenbauer, A.; Simon, P., EPR and Mössbauer spectroscopic studies on metal complexes of pectic acids and their derivatives. *Inorgan. Chim. Acta* **1983,** 79, 269-270.

71. Dzizenko, A. K.; Sorochan, V. D.; Prudnikova, T. I.; Ovodov, Y. S., Determination of the molecular weight of polysaccharides by the light-scattering method. *Russian Chemical Bulletin* **1969,** 18, (12), 2642-2643.

72. Turquois, T.; Gloria, H., Determination of the absolute molecular weight averages and molecular weight distributions of alginates used as ice cream stabilizers by using multiangle laser light scattering measurements. *J. Agric. Food Chem.* **2000,** 48, (11), 5455-5458.

73. Selin, M. M.; Donetskii, I. A.; Volodin, A. A.; Kochergin, P. M., Determination of the molecular weight of dextrans using an anisotropic semipermeable membrane. *Pharmaceutical Chemistry Journal* **1977,** 12, (3), 412-414.

74. Hexner, P. E.; Radford, L. E.; Beams, J. W., Achievement of Sedimentation Equilibrium. *Proceedings of the National Academy of Sciences of the United States of America* **1961,** 47, (11), 1848-1852.

75. Yau, W. W.; Kirkland, J. J.; Bly, D. D., *Modern Size-Exclusion Liquid Chromatography: Practice of Gel Permeation and Gel Filtration Chromatography*. 1 ed.; Wiley-Interscience: New York, 1979.

76. Spatorico, A. L.; Coulter, B., Molecular weight determinations by gel-permeation chromatography and viscometry. *Journal of Polymer Science: Polymer Physics Edition* **1973,** 11, (6), 1139-1150.

77. Hubbard, A. T., *Encyclopedia of Surface and Colloid Science*. CRC Press: New York, 2002.

78. Cho, S.; Prosky, L.; Dreher, M. L., *Complex Carbohydrates in Foods*. CRC Press: New York, 1999; p 700.

79. Tswett, M., On a new category of adsorption phenomena and their application to biochemical analysis. *Ber. Deut. Botan. Ges.* **1906,** 24, 316, 384.

80. Ettre, L. S.; Zlatkis, A., *75 Years of Chromatography - A Historical Dialogue*. Elsevier: New York, 1979.

81. Dronnet, V. M.; Renard, C. M. G. C.; Axelos, M. A. V.; Thibault, J.-F., Binding of divalent metal cations by sugar-beet pulp. *Carbohyd. Polym.* **1997,** 34, 73-82.

82. Wood, P. J., Factors affecting precipitation and spectral changes associated with complex-formation between dyes and β–D-glucans. *Carbohyd. Res.* **1982,** 102, 283-293.

83. Wood, P. J., Specificity in the interaction of direct dyes with polysaccharides. *Carbohyd. Res.* **1980,** 85, 271-287.

84. Zacharias, D., New Color Reactions for Determination of Sugars in Polysaccharides. In *Methods of Biochemical Analysis*, David, G., Ed. 2006; pp 313-358.

85. Thibault, J. F.; Renaudo, M., Interactions of Mono- and divalent counterions with alkali- and enzyme-deesterified pectins in salt-free solutions. *Biopolymers* **1985,** 24, 2131-2138.

86. Powell, D. A.; Morris, E. R.; Gidley, M. J.; Rees, D. A., Conformations and interactions of pectins. II. Influence of residue sequence on chain association in calcium pectate gels. *J. Mol. Biol.* **1982,** 155, 517-525.

87. Brereton, R. G., *Data Analysis for the Laboratory and Chemical Plant*. John Wiley & Sons Ltd: West Sussex, UK, 2003.

88. Rowell, R. L., Signal Averagers. *J. Chem. Ed.* **1974,** 51, A71.

89. Wallace, R. M., Analysis of absorption spectra of multicomponent systems. *J. Phys. Chem.* **1960,** 64, 899.

90. LaFara, R. L., *Computer Methods for Science and Engineering*. Hayden: Rochelle Park, NJ, 1978.

91. Enke, C. G.; Nieman, T. A., Signal-to-Noise Ratio Enhancement by Least-Squares Polynomial Smoothing. *Anal. Chem.* **1976,** 48, 705A.

92. Malinowski, E. R., *Factory Analysis in Chemistry*. 2 ed.; Wiley: New York, 1991.

93. Savitsky, A.; Golay, M. J. E., Smoothing and Differentiation of Data by Simplified Least Squares Procedures. *Anal. Chem.* **1964,** 36, 1627-1639.

94. Marcus, M.; Minc, H., *Introduction to Linear Algebra*. Dover: New York, 1988.

95. Hoffman, K.; Kunze, R., *Characteristic Values*. Prentice-Hall: Englewood Cliffs, NJ, 1971.

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