STUDIES ON THE STRUCTURE OF INDUSTRIAL HIGH METHOXYL PECTINS

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THESE

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THEOREMS (STELLINGEN)

1. Because of its widespread occurence in the plant kingdom and because of its varied properties, pectin plays an essential role in food technology.
(This thesis, chapter 1)

2. Medical and nutritional studies have provided evidence for the old adage "an apple a day keeps the doctor away".
(This thesis, chapter 1)

3. There is no evident chemical test to differentiate between pectins from apple pomace and those from citrus peels.
(This thesis, chapters 2, 3, 4 and 6)

4. The excess of scattered light that is often observed from pectin solutions is probably due to some molecular aggregation phenomena.
(This thesis, chapter 3)

5. EndoPG should not only act on a sequence of fully de-esterified galacturonic acid residues, but also on a sequence of strictly alternating esterified and non esterified galacturonic acid units.
(Rexova-Benkova & Markovic, 1976)

6. It is probable that the quality of pectins extracted from apple and citrus fruits depends largely on the processing of pomaces and peels after the juice extraction.
(This thesis, chapter 8)

7. In the cell-wall model of Keegstra *et al.* (1973), pectin molecules are linked to xyloglucans via their arabinogalactan side-chains. Several evidences suggest that pectic substances may be directly associated to other cell-wall constituants.
(This thesis, chapter 2)

8. The distribution of rhamnose units in pectin molecules has not yet been elucidated. However, it may represent an important structural feature in the explanation of the gelling ability of pectins.

9. Ideas are intangible. However, they do not exist without tangible support. When an idea is written down on paper, the eyes see first ink but then the brain starts working and the idea exists. Organizing ink on paper brings our ideas to life.

10. The meaning of "quality" depends on the point of view. Lack of a common definition may lead to profound differences between the intentions of food producers and the perceptions of food consumers.

11. Progress in Food Technology brings both safety and profitability but contradicts with the pleasure that consumers expect from food consomption.

Je dédie cette thèse à ma compagne Martine
et à ma fille Manon
qui m'ont procuré la force d'accomplir ce travail

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ABSTRACT

T.P. Kravtchenko, 1992, Studies on the Structure of Industrial High Methoxyl Pectins. Ph.D. Thesis, Agricultural University of Wageningen, The Netherlands.

The chemical structure of three industrial high methoxyl pectins (one extracted from apple pomace and two from lemon peels) has been extensively investigated. The apple pectin differs from the lemon ones by having a higher apparent molecular size, a higher neutral-sugar content, present either as covalently-linked side chains or as free neutral polysaccharides, a higher acetyl content and a higher phenolic content but a lower protein content. The lemon pectins appear to be very similar to each other except for their calcium cation content. Preparative size exclusion chromatography and ion exchange chromatography show that pectin samples are not homogeneous and that within one pectin preparation, the composition of pectin molecules varies with their molecular size or ionic charge. The lemon pectin sample richer in calcium cations contains a higher proportion of molecules with low degree of esterification, probably explaining its higher tendancy to form aggregates in the presence of calcium. Enzymic degradation of the pectin samples and subsequent analysis of the resulting fragments showed that at least part of the non-esterified carboxylic acids are grouped in blocks. However, such blocks are more numerous in the lemon pectin that is richer in calcium cations than in the apple or the other lemon pectin. Their presence explains the higher calcium-sensitivity of certain pectins and thus strongly influences their physical behaviour on application.

LIST OF ABBREVIATIONS

AAS:	Atomic absorption spectroscopy
AES:	Atomic emission spectroscopy
AUA:	Anhydro uronic acid
CyDTA:	Cyclo hexane diamino tetra acetic acid (Titriplex IV)
DAc:	Degree of acetylation (%)
DM:	Degree of methoxylation (%)
DP:	Degree of polymerization
EDTA:	Ethylene diamine tetra acetic acid (Titriplex III)
endoPG:	Endopolygalacturonase
GalA:	Galacturonic acid
GLC:	Gas-liquid chromatography
HM:	High-methoxyl
HPIEC:	High performance ion exchange chromatography
HPLC:	High performance liquid chromatography
HPSEC:	High performance size exclusion chromatography
IEC:	Ion exchange chromatography
K:	Stability constant of calcium-pectinate
K_{av}:	Partition coefficient
LM:	Low-methoxyl
M_n:	Number average molecular weight
M_w:	Weight average molecular weight
MW:	Molecular weight (daltons), *i.e.* molar mass (g/mole)
NS:	Neutral sugar(s)
η_{sp}:	Specific viscosity
$[\eta]$:	Intrinsic viscosity (l/mol)
PME:	Pectin methyl esterase
Rha:	Rhamnose
RI:	Refractive index
RT:	Retention time (min)
°SAG:	Degree SAG
SEC:	Size exclusion chromatography
TFA:	Trifluoro acetic acid
UV:	Ultra violet

CONTENTS

Chapter 1:

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

OCCURENCE OF PECTIC SUBSTANCES

Pectic substances are carbohydrate macromolecules present in virtually all higher plants (Ovodov, 1975), *Zosteraceae* seeweads (Ovodova *et al.*, 1968) and certain fresh water algae (Anderson & King, 1961). They are major components of the primary cell-wall and the middle lamella of young growing plant tissues but are absent from the secondary walls of more mature tissues (Thornber & Northcote, 1961). They are mainly deposited in the primary cell-wall during the early stages of growth when the area of the wall is increasing (Neukom, 1967; Northcote, 1986). The location of pectin in plant cell-walls has been reviewed by Joslyn (1962), Doesburg (1965), Lamport (1970), Northcote (1972), McNeil *et al.* (1979) and Dey & Brinson (1984). Several models of insertion of pectic substances in the cell-wall have been proposed (Keegstra *et al.*, 1973; Monro *et al.*, 1976; Lamport & Epstein, 1983; Selvendran, 1985; Fry, 1986; Renard, 1989). Besides in cell-walls, pectic substances may be present in the vacuoles of some plants such as coloured flowers of *Boraginaceae* species (Bayer *et al.*, 1966).

NOMENCLATURE

The term pectic substances is generally used to describe the group of complex plant polysaccharides in which D-galacturonic acid esterified to various extents with methanol is the main component. The great diversity in composition and forms of occurence of pectic substances in plants led to the development of several more restrictive definitions. The nomenclature of pectic substances is essentially based on the degree of methoxylation (DM) of the carboxyl groups of the polygalacturonan chain. DM is defined as the proportion of galacturonic-acid units esterified with methanol and is expressed in %. After several attempts, a nomenclature has been accepted by a committee of the American Chemical Society (Kertesz *et al.*, 1944). This terminology, with slight modifications is now generally accepted.

The term pectic acid applies to pectic substances mostly free of methyl ester groups (DM less than 5%). The salts of pectic acid are called pectates.

The term pectinic acid is used to designate the pectic substances mostly composed of polygalacturonic acids carrying more than a negligible proportion of

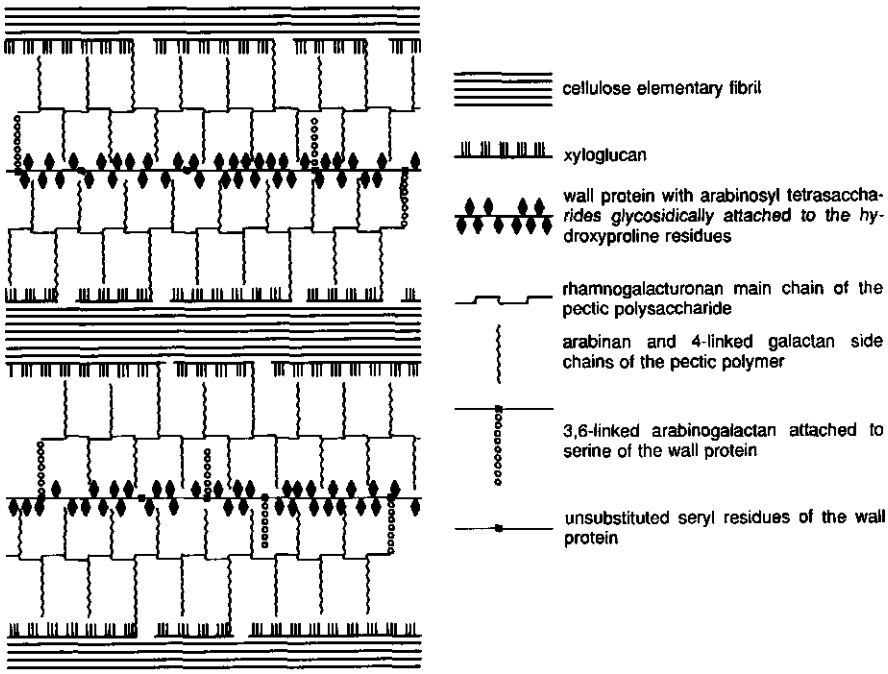

Figure 1: model of insertion of the pectic substances in primary cell-wall of suspension-cultured sycamore cells (Keegstra *et al., 1973).*

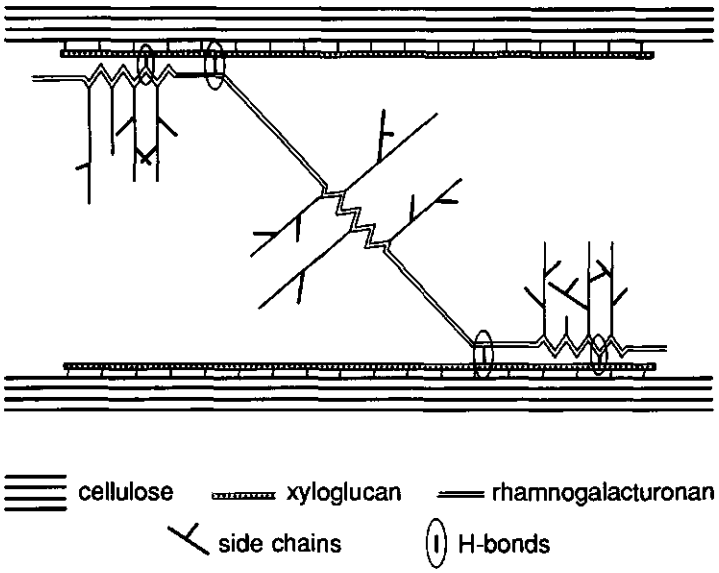

Figure 2: model of insertion of the pectic substances in apple cell-walls (Renard, 1989).

methyl ester groups. The salts of pectinic acid are called pectinates.

The name pectin is derived from the greek *pectos* which means coagulum and is mainly used to designate those water soluble pectic substances which are capable of forming gels under suitable conditions.

The term protopectin applies to the water insoluble pectic substances in plants. It is considered as the parent pectic substance which can, upon restricted hydrolysis, yield pectin.

Highly esterified pectic substances are industrially extracted and subsequently de-esterified to various DMs. The commercial use of these pectins as gelling agents led to the development of more specific definitions. High-methoxyl (HM) or high ester pectins have a DM over 50% and are capable of forming jellies at low water activity and low pH. HM pectins are further classified according to their "setting time" (table 1). Commercial low-methoxyl (LM) or low-ester pectins have a DM ranging from 30 to 50% and are capable of forming gels in the presence of polyvalent cations.

Table 1: Example of denomination of commercial HM pectins.

	Degree of methoxylation (%)	Setting time (min)
Ultra Rapid Set	74-77	<3
Rapid Set	71-74	4-8
Medium Rapid Set	66-70	15-25
Slow Set	53-65	50-65

The development of the knowledge on pectic substances have raised some lack or inconsistencies in these definitions. It is interesting to note that this nomenclature does not mention all the other structural constituents of pectic substances such as neutral sugars or acetyl esters (see below). Since pectin extracts very often contain accompanying substances of structures very similar to that of the pectin side-chains, some authors (Henglein, 1947; Aspinall, 1970) have included under the term pectic substances araban, galactan and arabino-galactan polymers. Indeed, all these molecules are thought to be covalently associated in protopectin. Also, some polygalacturonates, *e.g.* from sugar-beet are designated as pectins

although they have no natural gelling power. McCready & Gee (1960) pointed out that the extreme heterogeneity of the pectic substances contributes to the uncertainty of their definition.

ROLE AND FUNCTIONS OF PECTIC SUBSTANCES *IN SITU*

Since they were discovered by Braconnot in 1824, pectic substances have been studied in many various fields of science because of their wide distribution over the plant kingdom as well as their multiple properties.

Pectic substances play a function in the cell-wall of plants as a "lubricating" or "cementing" agent (Rees & Wight, 1969) and therefore participate in the maintenance of tissue cohesion by acting as an intercellular adhesive substance (Pilnik & Voragen, 1970). The restriction of cell enlargement to cells lacking a secondary wall has led to theories of cell-wall extension involving an important rôle for pectic substances (Northcote, 1963). The ion exchange capacity of pectic substances is used by the roots of plants to absorb calcium ions from the soil (Ramamoorthy & Leppard, 1977; Oades, 1978). More recently it was discovered that oligogalacturonides of specific size can play an extremely important rôle in plant development and can regulate plant morphogenesis (Albersheim & Darvill, 1989): acidic fragments (presumably pectin) of plant cell-walls inhibit flowering in *lemna* (Gollin *et al.*, 1984); cell-wall pectic fragments from suspension-cultured sycamore cells can inhibit the formation or alter the position of roots, cause marked tissue enlargement or induce flower formation on tobacco thin-layer explants (Eberhard *et al.*, 1989).

Pectic substances act as a barrier to pathogens. According to Weintraub & Ragetli (1961) pest infections induce the enzymic de-esterification of the pectin and the formation of insoluble calcium-pectate which prevents further invasion. Oligogalacturonides were also shown to activate more specific plant defense responses: they elicit the accumulation of phytoalexin (Hahn *et al.*, 1981; Nothnagel *et al.*, 1983; Walker-Simmons *et al.*, 1983; Jin & West, 1984; Davis *et al.*, 1986; Komae *et al.*, 1990) which has a wide spectrum of antimicrobial activity, they induce the lignification (Robertsen, 1986) and the accumulation of protease inhibitors (Bishop *et al.*, 1984) in plant tissues. Such pectic fragments may originate from the action of the pectolytic enzymes of the invading organisms.

Since fresh and processed plant products constitute a large part of human food supply, pectic substances have a nutritional function as natural food fiber (Bock & Krause, 1978; Cummings *et al.*, 1979; Jenkins, 1980). Pectin lowers the

cholesterol level of serum and/or liver (Lin *et al.*, 1957; Mokady, 1973; Fisher *et al.*, 1974; Kay & Truswell, 1977; Jenkins *et al.*, 1979) and decreases the amount of glucose in the serum of diabetic or obese subjects (Jenkins *et al.*, 1976; Monnier *et al.*, 1978; Williams *et al.*, 1980; Vaaler *et al.*, 1980; Poynard *et al.*, 1980; Kanter *et al.*, 1980). Moreover, pectin has been reported to increase the frequency of defecation (Kay & Truswell, 1977; Cummings *et al.*, 1979).

Softening in fruit occurs by enzymic breakdown of the cell-wall components (Pressey, 1977; Knee & Bartley, 1981; Labavitch, 1981; Huber, 1983). The amount and the nature of pectic substances play therefore an important rôle in regulating the firmness of fruits and vegetables on growing, ripening and storage (Pilnik & Voragen, 1970; Knee, 1978a, 1978b; de Vries *et al.*, 1984; Reid *et al.*, 1986). Fruits and vegetables are subject to spoilage by pectolytic micro-organisms (Vaughn *et al.*, 1969, 1972; Barash & Eyal, 1970; Lund, 1971).

During the processing of fruits and vegetables pectic substances are subjected to both desirable and undesirable modifications (Doesburg, 1965; Pilnik & Voragen, 1970; Rombouts & Pilnik, 1978; van Buren, 1986; McFeeters, 1986). It is recognized that eliminative breakdown of pectin is responsible for the softening of vegetables during heat treatment (Keijbets & Pilnik, 1976). Pectin is also greatly involved in the problems of cloud stability in fruit juices (Krop & Pilnik, 1974a; Rombouts & Pilnik, 1978; Siliha & Pilnik, 1985). In the fruit juice industry, pectin changes are frequently introduced on purpose by the addition of commercial pectolytic enzymes for the extraction (Pilnik, 1982), the clarification (Endo, 1965; Baron & Drilleau, 1982) or the stabilization of cloud (Krop & Pilnik, 1974b; Siliha & Pilnik, 1985).

Pectic substances also proved their importance for other technological operations. It has been recognized very early (Beyerinck & van Delden, 1904) that the degradation of pectic substances by certain *Clostridium* species is responsible for the loosening of cellulose fibres of flax. The addition of calcium to fresh sugar-beet cossettes (Randall *et al.*, 1982) or citrus peels (Rebeck & Cook, 1977) favours the de-esterification of the pectin and, at the same time, insoluble calcium-pectate is formed. This results in firmer pulps which are easier to dewater mechanically.

USES OF EXTRACTED PECTINS

Pectic substances are industrially extracted, mainly from lemon peels and apple pomaces (Neukom, 1967; Nelson *et al.*, 1977; Thom *et al.*, 1982; May,

1990; Rolin & de Vries, 1990).

The most important field of application of extracted pectins still remains the food industry. Pectins can form gels under certain circumstances and are traditionally used as gelling agents in jams, jellies and marmelades to compensate for the lack of pectin (the natural gelling agent) in fruits (Nelson *et al.*, 1977; May, 1990; Rolin & de Vries, 1990). They are also used as gelling agents in confectionery, bakery fillings and milk puddings. LM pectins have found very interesting applications with the development of low-calorie gels. Pectins are used as thickening or stabilizing agent in dairy products, fruit drinks, fruit and tomato pastes. Mitchell (1980) showed that pectates may be used in canned products. Very recently, pectin-based preparations have been introduced in food technology as fat replacer. For all these purposes, pectin manufacturers have developed a complete set of more or less modified pectins.

Pectin possesses many interesting pharmacological activities and is therefore also used in medical preparations. Pectin may be incorporated in human food to correct diets lacking fibers (see above). Pectin has proved its value as a prophylactic in poisoning with heavy metals (Kohn *et al.*, 1968; Malovikova & Kohn, 1979; Walzel *et al.*, 1987) and radioactive elements (Waldron-Edward *et al.*, 1965). This has led to the development by the Moscovian Experimental Preserve Factory of a so-called "prophylactic apple-pectin juice" containing *ca.* 10% of sugar-beet pectin (Obodovkaja *et al.*, 1978). Pectin has a haemostatic and anti fibrinolytic effect (Bock et al., 1964). It has been used as blood plasma extender (Schultz *et al.*, 1952) and for the treatment of gastric deseases (Gorin *et al.*, 1964; Neukom, 1967).

Pectic acid added to wine reduces heavy metal content and can be filtered off quantitatively (Wucherpfennig, 1984). Rexova-Benkova & Tibensky (1972) showed that cross-linked pectic acid may be used for the affinity chromatography of pectic enzymes for a selective purification of endo-polygalacturonase. Pectin can be used to remove traces of metallic cations (Jellinek & Sangal, 1972) or radioactive contaminants (Langehorst *et al.*, 1961) from waste water.

From the great variety of their rôles, functions and applications, it is clear that research on pectic substances has captured the attention of plant physiologists, phytopathologists, nutritionists, pharmacologists, food technologists and pectin manufacturers. In order to better understand their physico-chemical properties, the structure of pectic substances has been particularly well investigated. Moreover, since they contain some of the most complex structures which have been found in

polysaccharides (McNeil *et al.*, 1979; Lau *et al.*, 1985), pectins have also attracted many research teams in search of a challenge. All this causes the literature on the subject to be so abundant and dispersed that an exhaustive review becomes now almost impossible. Indeed, in the years 1967-1990, Chemical Abstracts recorded more than 7000 references dealing with pectic substances, with more than 400 just for the year 1990. In this thesis, relevant literature about the structure and the analysis of the pectic substances has been reviewed and discussed in chapters 2 to 7.

AIMS OF THE THESIS

Despite the tremendous efforts accomplished for several decades, the complete elucidation of the structure of pectic substances is not yet achieved. The fine chemical structure of pectic substances extracted in mild conditions from various plants and fruits has been particularly well studied. In contrast, informations about the structural features of industrial pectins are very scarce. Indeed, much more effort has been devoted to the rheological characterization of industrial pectins. The main problem of pectin manufacturers and users is the prediction of the behaviour of commercial pectin preparations on application in different food systems. Pectin preparations with very similar global chemical characteristics can behave very differently on application.

The aim of this thesis was to investigate some methods of characterization of the chemical structure of industrial pectins in order to identify the structural feature(s) which may explain differences of behaviour of high methoxyl pectins. A better knowledge of pectin structure hopefully will lead to a better understanding of the rôles of pectic substances in fruit and vegetables and of the rheological performance of extracted pectins.

This study was performed with three pectins extracted under industrial conditions from lemon peels and apple pomace. These three samples have been chosen because of their very different behaviour on application, especially in the presence of calcium cations. The chapters 2 to 8 represent the experimental part of this thesis. Chapter 2 describes a set of modern techniques for the chemical analysis of pectic substances and these methods have been applied for the characterization of three industrial pectin samples. Chapter 3 describes a method of calculation of the intrinsic viscosity of aqueous pectin solutions. In chapters 4

and 5 the same pectin samples have been fractionated by preparative size exclusion chromatography and ion exchange chromatography, respectively. The resulting fractions have been investigated by means of the methods described in chapter 2 in order to establish the inter-molecular distribution of industrial pectins. Chapter 6 describes the improvement of the selective depolymerisation of pectic substances by chemical ß-elimination in aqueous solution. In chapter 7, the intra-molecular distribution of the pectin structural features has been investigated by means of several chemical and enzymatic methods of degradation and the subsequent analysis of the resulting fragments. Chapter 8 describes some differences in physical behaviour of the three industrial pectin samples characterized by chemical analysis in chapters 2 to 7. Chapter 9 discusses the relationship between physical properties and structural features of industrial pectins on the basis of collected and published data.

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Chapter 2:

ANALYTICAL COMPARISON OF THREE INDUSTRIAL PECTIN SAMPLES

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2. ANALYTICAL COMPARISON OF THREE INDUSTRIAL PECTIN PREPARATIONS

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ABSTRACT

The chemical composition of three industrial "rapid set" pectin samples, one from apple and two from lemon has been extensively investigated. The so-called "ballast" that has been removed by copper precipitation appears to be mainly constituted of neutral polysaccharides and, to a less extent, of proteins and phenolic compounds. Purified pectin molecules are composed of galacturonic acid and neutral-sugar residues and also carry some phenolic and proteinaceous material. Pectin molecules of industrial apple and citrus preparations are very similar to those extracted under mild conditions from similar sources but carry fewer neutral sugars. All three pectins have been found to be slightly acetylated (DAc 1.5 and 5.0% for lemon and apple pectins, respectively). The pectin extracted from apple contains more neutral sugars and more phenolics but fewer proteins than the two lemon pectins, which are very close to each other. However, one of the lemon pectins contains a pectin fraction that appears to be less esterified. The same lemon pectin also differs from the other pectin samples by its higher calcium ion content. Both structural and analytical consequences are discussed.

INTRODUCTION

Pectic substances are glycosidic macromolecules that occur exclusively in plants. They are industrially extracted to be used as food additive (Nelson *et al.*, 1977; Thom *et al.*, 1982; May, 1990). One of the greatest difficulties in their optimal utilization is that some industrial pectin preparations that appear to be very similar on gross analysis exhibit different physical behaviour. A better knowledge of the chemical structure of industrial pectins is thus of major importance to understanding and improving their technological applications.

The chemical structure of pectic substances has been intensively studied for more than 60 years because of their great importance in many fields. They act as "lubricating" or "cementing" agents in the cell-walls of plants (Rees & Wight, 1969). Pectic substances are involved in the interactions between plant hosts and their pathogens (Talmadge *et al.*, 1973; Albersheim *et al.*, 1981). The amount and nature of pectin strongly influence the texture of fruits and vegetables on growing, ripening and storage (Voragen & Pilnik, 1970; Knee, 1978a, 1978b), and also affect the processing of fruits and vegetables (Rombouts & Pilnik, 1978). Pectin is a nutritional fibre (Cummings *et al.*, 1979) and possesses many interesting medical properties (Deuel & Stutz, 1958; Verstraete, 1979). Curiously, relatively little work dealing with the chemical composition and structure of industrial pectins has been published.

Pectin is primarily a linear polymer of D-galacturonic acid units joined by α(1-4)glycosidic linkages. The pyranose ring of D-galacturonic acid occurs in the chair form $^4C_1$ corresponding to the most stable conformation (Deuel & Stutz, 1958; Rees & Wight, 1971; Morris *et al.*, 1975). The glycosidic bonds at C-1 and C-4 are therefore in axial-axial position.

Only few examples of the isolation of pure homogalacturonan fractions are reported (Bishop, 1955; Zitko & Bishop, 1965; Aspinall & Canas-Rodriguez, 1958; Bhattarcharjee & Timell, 1965; Chambat & Joseleau, 1980). However, these fractions were obtained by severe treatments probably generating artifacts (Aspinall, 1980; de Vries *et al.*, 1982). From many studies it has become clear that pectin is not a homopolysaccharide but that neutral sugars are part of the pectin molecules.

L-rhamnosyl residues are present in the galacturonan backbone (Aspinall & Fanshave, 1961; Barrett & Northcote, 1965; Aspinall *et al.*, 1967a, 1967b, 1968a, 1968b, 1969, 1970; Stoddart *et al.*, 1967; Foglietti & Percheron, 1968; Talmadge *et al.*, 1973; Kato & Noguchi, 1976; Toman *et al.*, 1976; Simson & Timell, 1978; McNeil *et al.*, 1980; Eda & Kato, 1980; Lau *et al.*, 1985; Sun *et al.*, 1987, Schols *et al.*, 1990), inserted in the following manner: O-α-D-GalA-(1-2)-O-L-Rha-(1-4)-O-α-D-GalA. Recent NMR studies on apple pectin fragments have proved that the configuration of the L-rhamnosyl linkage is α (Colquhoun *et al.*, 1990).

Other neutral sugars, mainly L-arabinose, D-galactose, D-xylose and D-glucose occur as side-chains (Aspinall & Canas-Rodriguez, 1958; Neukom *et al.*, 1960; McCready & Gee, 1960; Aspinall & Fanshave, 1961; Barrett & Northcote, 1965; Zitko & Bishop, 1965, Hatanaka & Ozawa, 1966; Jacin *et al.*, 1967; Aspinall *et al.*, 1968b; Foglietti & Percheron, 1968; de Vries *et al.*, 1982;

Schols *et al.*, 1990). The neutral side chains are linked to the main chain via the C-4 of rhamnose units (Aspinall *et al.*, 1967a; Talmadge *et al.*, 1973; Eda & Kato, 1980; McNeil *et al.*, 1980; Lau *et al.*, 1985; Sun *et al.*, 1987; Schols *et al.*, 1990). However, galactose, arabinose or xylose have often been found covalently linked to galacturonic acid (Bouveng, 1965; Barrett & Northcote, 1965; Aspinall *et al.*, 1967a, 1967b, 1968a; Stoddart *et al.*, 1967; Foglietti & Percheron, 1968; Kikuchi & Sugimito, 1976; Ishii, 1981; Schols *et al.*, 1990).

Some of the carboxyl groups of the rhamnogalacturonan backbone are esterified with methanol and some hydroxyl groups at C-2 and C-3 of the galacturonic acid units are esterified with acetic acid (McCready, 1970; Kim *et al.*, 1978; Rombouts & Thibault, 1986a). The free-acid groups may be partly or fully neutralized with sodium, potassium, calcium or magnesium (Kawabata, 1977).

It has recently been shown that pectin molecules from spinach (Fry, 1983) and sugar beet (Rombouts & Thibault, 1986a) also carry some feruloyl groups. They are located mainly in the side chains (Rombouts & Thibault, 1986b) and may be covalently bound to the non-reducing termini of arabinose and/or galactose chains (Fry, 1983).

L-fucose (Aspinall *et al.*, 1967b, 1968a, 1968b; Lau *et al.*, 1985), apiose (Darvill *et al.*, 1978) and some other rare sugars (Aspinall *et al.*, 1958, 1961; Barrett & Northcote, 1965; Foglietti & Percheron, 1968; Darvill *et al.*, 1978) were found as trace constituents in certain pectic substances but there is no information available about their structural location.

In this work, the chemical composition of three industrial pectins was determined by using modern analytical methods. This includes minerals, acetyl esters, protein and amino acids which are often neglected in the characterization of pectin preparations.

EXPERIMENTAL

Material

Three unstandardized industrial pectins classified as "rapid set" were obtained from Sanofi Bio Industries (France): two from lemon peels (A and B) and one from apple pomace. All three were industrially extracted by the same classical hot-acid procedure (May, 1990).

Copper purification

Commercial samples (0.5% solutions, 1000 ml) were purified by precipitation with a 7% aqueous copper acetate solution (75 ml). The precipitate that formed was collected on a G3 fritted-glass filter and abundantly washed with distilled water. The precipitate was then redissolved in 20 mM Na-CyDTA (titriplex IV, Merck) at pH 5, dialysed against distilled water and freeze-dried.

Analytical methods

The anhydro-uronic acid content (MW=176) was determined by the automated *meta*-hydroxydiphenyl assay (*m*hdp, Thibault, 1979).

Underivatized uronic acid units were analysed by HPLC after hydrolysis of the pectin samples in 2N H_2SO_4 at 100°C for 3 hours. HPLC analysis was performed with a Dionex system (Sunnyvale, CA, USA) equipped with a CarboPac PA1 column (9x250 mm) and a pulsed amperometric detector. Conditions were very similar to those used by Martens & Frankenberger (1990).

Neutral sugars were determined as their alditol acetates by GLC. 2 mg samples were hydrolysed for 1 hour at 121°C with 2N TFA (Albersheim *et al.*, 1967). Hydrolysates were dried under a stream of air at 40°C, reduced with $NaBH_4$ (10 mg) in 1.5N ammonia (0.2 ml) and acetylated with acetic anhydride (3 ml, 30 min, room temperature) in the presence of 1-methyl imidazole (0.45 ml) as catalyst (Blakeney *et al.*, 1983). The alditol acetate derivatives of the sugars were then separated from the aqueous phase by extraction with dichloromethan (2 x 3 ml) and determined by GLC equipped with an OV 275 packed column.

The methoxyl and the acetyl contents were determined by HPLC analysis of the methanol and the acetic acid released on alkaline deesterification (1 hour, 5°C, 0.5M KOH; Voragen *et al.*, 1986). The degree of methoxylation (DM) and the degree of acetylation (DAc) were calculated using the galacturonic acid content measured by the *m*hdp assay.

The total nitrogen content was determined by a semi automated micro-Kjeldhal method. Protein content was estimated by multiplying the N content by 6.25.

Amino acid compositions were determined with a Biotronic LC 600 E automatic analyser. Samples were hydrolysed in 6M HCl for 21 hours at 110°C under nitrogen.

Starch content was determined enzymatically by using a test kit (Boehringer, Mannheim, FRG)

Total phenols were determined with the Folin Ciocalteu reagent without copper treatment and with ferulic acid used as standard (Swain & Hillis, 1959). 0.25M Folin-Ciocalteu reagent (0.4 ml) being added to the sample solution (0.5%, 0.4 ml), followed after 5 min by 1M Na_2CO_3 (0.4 ml). The absorbance at 750 nm was read after 1 hour.

Phenolic acids were assayed by HPLC. Pectin samples (100 mg) were treated with 0.5M KOH (5ml) in a screw-cap tube under N_2 at room temperature for 24 hours with *p*-hydroxybenzoic acid as internal standard, after which 6M HCl (0.75 ml) was added to the mixture, and the phenolic components were recovered from the acidified solution by extraction with ethyl acetate (2x4 ml). The combined ethyl acetate extracts were dried under vacuum at 40°C, the residues were dissolved in methanol (1 ml) and aliquots (20 μl) were injected on a reverse-phase Spherisorb 10 ODS column (Chrompack, 250x4.6 mm). The column was eluted with a linear gradient mixture of 4% (v/v) AcOH in MeOH/4% (v/v) AcOH in H_2O (10-50%) at a flow rate of 1.5 ml/min. The eluate was monitored in UV at 280 nm.

Sodium, potassium and calcium were simultaneously determined with an ELEX 6361 flame AES (Eppendorf), magnesium with an IL 357 flame AAS (Instrumental Laboratory) and phosphorus, colorimetrically with molybdene blue (ISO 3946-1982, UDC 664-2:543.847).

Molecular size distributions were determined by high performance size exclusion chromatography (HPSEC), a series of Biogel TSK columns 60XL, 40XL and 30XL (300x7.5 mm) being used in combination with a Biogel TSK guard column (75x7.5 mm). Columns were eluted with 0.4M Na-acetate buffer at pH 3.0 with a flow rate of 0.8 ml/min at 30°C and detected with a Shodex SE 61 RI detector at 40°C.

Charge distributions were determined by high performance ion exchange chromatography (HPIEC) as described by Schols *et al.* (1989). A Biorad MA7P column (50x7.8 mm) was eluted with a linear gradient of 15-270mM Na-phosphate buffer at pH 6.0 at a flow rate of 1.5 ml/min. Detection was done by reading the absorbance in UV at 215 nm. The increase in baseline signal was corrected by substracting the chromatogram obtained for a blank run from those of sample runs.

RESULTS AND DISCUSSION

Three unstandardized industrial pectin samples, one from apple pomace and two from lemon peels, empirically known for their different solubility and gel behaviour, especially in the presence of calcium, have been extensively investigated for their chemical composition.

Table 1: Composition (as percentage weight of dry matter) of the unstandardized industrial pectin samples.

	Lemon A	Lemon B	Apple
Galacturonic acid[a]	76.4	77.1	60.8
Methoxyl groups[b]	4.4(71.5)	4.4(72.1)	3.6(74.3)
Acetyl groups[b]	0.26(1.4)	0.30(1.6)	0.72(5.0)
Total neutral sugars[a]	8.5	9.2	27.0
Proteins (N x 6.25)	3.0	3.3	1.6
Total phenols	0.18	0.15	0.59
Ash	2.38	1.96	1.89
Total	95.1	96.3	95.9

[a] Values recorded as "anhydro" residues.

[b] Values in parentheses are degree of methoxylation (DM) or degree of acetylation (DAc).

Uronide residues

All three industrial samples contain a high amount of uronide (table 1) as evidenced by the *m*hdp assay. Moreover, HPLC analysis of pectin hydrolysates did not reveal the presence of any uronic acid other than galacturonic acid. This does not confirm the identification of some glucuronic acid in pectins by Aspinall *et al.* (1967b, 1968a, 1968b) and McNeil *et al.* (1980). Since glucuronic acid units are probably accomodated in side chains (McNeil *et al.*, 1980), they may have been

removed from the native pectin during the industrial-extraction process. However, complete hydrolysis of the galacturonan backbone was not achieved since some oligomeric fragments were detectable. It is thus possible that glucuronic acid residues remained attached to oligomers that could not be resolved by the HPLC analysis.

The apple-pectin sample contains less galacturonic acid (60.8%) than the two lemon pectins (76.4 and 77.1% for lemon A and lemon B, respectively). This is mainly due to the difference in neutral-sugar contents (table 1).

Neutral sugars

The industrial apple-pectin preparation contains about 25 times as many glucose, 12 times as many xylose, 4 times as many arabinose and 1.5 times as many rhamnose residues per 100 galacturonide residues as the lemon pectin samples (table 2). However, purification with copper ions shows that these neutral sugars are not all covalently bound to the pectin molecules.

Table 2: Neutral-sugar composition (as mol/100 mol gal A) of the unstandardized industrial and corresponding copper-purified pectin samples.

	Industrial samples			Cu-purified samples		
	Lemon A	Lemon B	Apple	Lemon A	Lemon B	Apple
Rhamnose	2.1	1.6	2.9	1.5	1.3	2.1
Arabinose	3.3	3.1	12.8	2.1	2.3	3.5
Xylose	0.2	0.2	2.1	0.1	0.1	1.9
Mannose	0.2	0.2	0.2	t	t	t
Galactose	6.8	7.7	8.9	3.8	4.8	3.8
Glucose	0.6	1.0	23.3	0.3	0.5	8.5
Total	13.2	13.8	60.2	7.8	9.0	19.8

Copper precipitation removed more than 60% of the glucose units present in the industrial apple pectin. Most of it is of starch origin which represents 10.5% (dry weight) of the industrial apple pectin. This justifies the enzymatic removal of starch molecules, performed in some factories in order to avoid too large dilution of industrial apple pectins and potential problems of precipitation on application (May, 1990). Commercial lemon pectins also contain some starch (0.16 and 0.51% for lemon A and lemon B pectins, respectively) but in much lower quantity than the apple pectin. In the apple pectin, starch accounts for more glucose than that which has been removed by copper precipitation (7.6% dry weight), indicating that, despite intensive washing, some starch could not been removed from the copper pectinate precipitate.

Copper precipitation also removed almost all mannose and parts of arabinose, galactose and xylose units. The proportion of arabinose removed is higher than that of galactose, especially in the apple pectin. The removal of neutral sugar residues by copper-purification is due to the occurence of free neutral polysaccharides such as arabans, arabino-galactans, xyloglucans or mannans that have been co-extracted with the pectin fraction (Aspinall, 1980).

Some rhamnose units did not precipitate with copper ions from the three industrial pectins. The literature on plant cell-walls does not relate the occurence of rhamnose units with any other polysaccharide than pectin. Moreover, some galacturonic acid did not precipitate either during the purification treatment (0.40, 0.34 and 1.45% from lemon A, lemon B and apple pectins, respectively). This would mean that copper ions do not precipitate some rhamnose-rich pectin molecules, molecules which are known to carry many other neutral sugar residues (McNeil *et al.*, 1980; de Vries *et al.*, 1981; Schols *et al.*, 1990). McCready & Gee (1960) and Michel *et al.* (1981) observed a similar loss of rhamnose and galacturonic acid during copper purification but with a much lower rhamnose/galacturonic acid ratio.

After purification, the sugar composition of the lemon B pectin is very close to that of the lemon A pectin, both qualitatively and quantitatively, except for a slightly higher content of galactose. On the other hand, the apple pectin differs from the lemon pectins by its higher content of neutral sugars, especially glucose, xylose, arabinose and rhamnose. This confirms that the neutral-sugar content appears to be determined by the plant source (Nelson, 1977, de Vries *et al.*, 1984b). These differences in neutral sugar composition may explain some differences in molecular conformation and thus physical properties: rhamnose is thought to disturb the regularity of the galacturonan backbone (Rees & Wight, 1971; Talmadge *et al.*, 1973) and may play an important rôle in the formation of

junction zones (Thom *et al.*, 1982), other neutral sugars constitute side chains which may limit inter-chain associations. It is interesting to note that all three pectins still contain some mannose and xylose residues and significant amounts of glucose. Some starch molecules are still present in the purified apple pectin (see above). However, in all three pectins, the starch content of the industrial samples does not account for all the glucose present, indicating that some non-starchy glucose units may be bound to pectin molecules. Xylose and glucose are generally found in apple pectins (Zitko & Bishop, 1965; Knee, 1978a; de Vries *et al.*, 1981; Aspinall & Fanous, 1984). In lemon pectins, xylose is also present but in much lower quantity than in apple pectins (Aspinall *et al.*, 1968a; de Vries *et al.*, 1984b; Thibault *et al.*, 1988; Axelos *et al.*, 1989). Simultaneous occurence of xylose and glucose in purified pectins has been ascribed to the presence of some xyloglucan fragments attached to the pectin side chains (Talmadge *et al.*, 1973; de Vries *et al.*, 1981). However, several observations (Aspinall *et al.*, 1968a; de Vries *et al.*, 1982; Schols *et al.*, 1990) indicate that some xylogalacturonan regions exist. Contrarily to de Vries *et al.* (1981) but in agreement with Knee (1978a), Stevens & Selvendran (1984a) and Thibault *et al.* (1988), we have been able to detect some traces of mannose in all three purified pectin samples analysed. According to Leigh & Krzeminski (1966) mannose may arise from epimerization of glucose during the hydrolysis step of the neutral-sugar analysis. However, in the conditions we used no mannose appeared during the analysis of a glucose control.

Compared to pectins of similar sources extracted in mild conditions (Knee, 1978a; de Vries *et al.*, 1981, 1984b; Stevens & Selvendran, 1984a; Aspinall & Fanous, 1984; Thibault *et al.*, 1988), these industrial samples contain relatively few neutral sugars. This seems to be a general feature of industrial pectins (Kawabata, 1977; Michel *et al.*, 1981; Axelos *et al.*, 1989), and it may be explained by the rather severe conditions applied to the pectin during its industrial extraction. Indeed, in hot-acid conditions, acid-labile bonds, especially arabinosidic linkages, undergo degradation, resulting in a "trimming" phenomenon of the pectin side chains.

Methoxyl/Acetyl esters

Table 1 shows the methoxyl and acetyl contents of the three industrial pectins. In order to maintain the mass balance, they were calculated by using masses of 14 and 43g for methoxyl and acetyl respectively. In fact, the

polygalacturonic acid content was calculated by using a mass of 176 (see above) irrespective of its degree of methoxyl and/or acetylation.

All three industrial pectins are highly methoxylated. The observed degrees of methoxylation (DM) are in agreement with those generally observed for both "rapid set" industrial pectins (Kawabata, 1977; Axelos *et al.*, 1989) and pectins from similar sources extracted in mild conditions (Aspinall *et al.*, 1968a; Knee, 1978a; de Vries *et al.*, 1984b; Stevens & Selvendran, 1984a), indicating that the industrial procedure does not affect the methoxyl esters very much. Since the two lemon pectins are very close to each other, the apple pectin differs from them by reason of its higher DM. That confirms the generally accepted fact (May, 1990) that industrial apple pectins have a higher DM than industrial citrus pectins.

The three industrial pectins also contain some acetyl esters. The amounts measured are higher than those described in the literature, especially for the apple pectin (McComb & McCready, 1957). However, Thibault *et al.* (1988) and Voragen *et al.* (1986) analysed citrus pectins with DAc values in the order of two. Although DAc values lower than 12.5% do not hinder gelation (BeMiller, 1986), it is likely that acetylation influences gel properties. This has been neglected in rheological studies on apple and citrus pectins. Moreover, a relatively high acetyl content may explain the differences generally observed between DM obtained by titration or by methanol determination. Indeed, with the titration procedure, acetyl residues released on saponification add up to esterified galacturonic acid units. For instance, a 70% methoxylated and 4% acetylated pectin would appear to be 71.2% methoxyl-esterified.

Proteins/Amino acids

The protein content of the three industrial pectins is given in table 1 and the amino acid composition is given in table 3.

Lemon A and lemon B pectin samples contain very similar amounts of protein and their amino-acid compositions are very close to each other. However, lemon B pectin exhibits a lower content of asparagine and hydroxyproline but a higher amount of glutamine and leucine. On the other hand, apple pectin contains much less protein than the lemon pectins and its amino-acid composition is clearly different. Apple pectin contains less tyrosine, phenylalanine, lysine, histidine and proline but more asparagine, glutamine and alanine. Anderson *et al.* (1987) found very similar differences between industrial pectins from apple and lemon.

Table 3: Amino-acid composition (as mole %) of the unstandardized industrial pectin samples.

	Lemon A	Lemon B	Apple
Asparagine	13.2	0.5	16.9
Threonine	6.2	6.7	7.2
Serine	5.4	5.3	6.4
Glutamine	10.3	12.7	15.2
Glycine	9.8	9.5	9.9
Alanine	7.1	7.3	8.9
Valine	5.7	5.7	4.5
Cystine	t	t	t
Methionine	t	t	t
Isoleucine	3.6	3.7	3.1
Leucine	6.2	7.0	6.1
Tyrosine	3.1	3.4	1.9
Phenylalanine	3.4	4.0	2.4
Lysine	6.7	6.6	4.1
Histidine	2.7	2.4	1.8
Arginine	3.9	3.8	3.4
Hydroxyproline	5.8	4.7	4.4
Proline	6.8	6.7	3.8

Copper precipitation removed about 35% of the proteinaceous compounds from lemon A pectin and 30% from lemon B pectin but none from apple pectin. Even after this purification, the protein contents of the lemon pectins remain significantly higher than that of the apple pectin.

The amino-acid composition of copper-purified pectins has not been investigated. It is thus impossible to draw any qualitative conclusion about the nature of the proteinaceous compounds that are attached to the pectin molecules. Moreover, although covalent bonds have already been identified between amino acids and neutral-sugar residues (Lamport, 1969; Lamport *et al.*, 1973), the mode of attachment of these proteins to the pectin molecules remains unknown.

The high content of hydroxyproline, proline, serine, threonine, valine, tyrosine and lysine suggests that the proteinaceous compounds present in the

industrial samples may be fragments of one of the various structural proteins that occur in plant cell-walls (Cassab & Varner, 1988).

The presence of asparagine and glutamine may explain the detection of some naturally-occuring amidated uronide units in sunflower pectins (Lin *et al.*, 1976). Indeed, the ammonia released on alkaline treatment (National Research Council, 1972) may come from amidated amino acids. However, even the industrial lemon pectin A, which contains about 0.9 mg of asparagine and 0.7 mg of glutamine (if complete amidation is assumed) would appear to be less than 0.25% amidated with the procedure of the National Research Council (1972). Moreover, since the association of proteins with pectic substances appears to be a general phenomenon (McNeil *et al.*, 1982; Stevens & Selvendran, 1984a), the Kjeldhal procedure does not fit with the accurate determination of the degree of amidation of industrial pectins. For instance, the lemon A industrial pectin which contains 0.48% (w/w) of total nitrogen, would appear to be almost 8% amidated with the Kjeldhal procedure.

Phenolics

The total phenol contents of the industrial pectin samples are given in table 1. The apple pectin contains three times as many phenolic compounds as the lemon pectins. This may explain the brownish colour of apple pectins solutions.

The composition of these phenolics has not been investigated. However, HPLC analysis did not reveal the presence of any phenolic acids, such as ferulic acid (Rombouts & Thibault, 1986a) or *p*-coumaric acid (Guillon & Thibault, 1988) which are known to occur in sugar-beet pectin.

Only part of the total phenolics could be removed by copper purification (39, 13 and 27%, for lemon A, lemon B and apple pectins, respectively) which suggested that at least some phenolic compounds other than phenolic acids might be bound to the pectin molecules. Moreover, treatment with polyclar AT, which is often used to remove polyphenolic residues did not remove any phenolic compounds from the industrial pectin samples. Since polyclar treatment also did not remove any glycoside, it seems that polyphenolic material present in industrial preparations is entirely complexed with pectic substances as suggested by Stevens & Selvendran (1984b) or with neutral polysaccharides.

Minerals

Table 4 shows the mineral composition of the three industrial pectin samples. The apple pectin differs from the lemon samples by reason of its higher content of potassium, magnesium, phosphorus and a lower content of sodium. The difference in magnesium content between apple and lemon pectins is not as marked as that observed by Kawabata (1977). The origin of phosphorus in these pectins has not been established but, Henglein *et al.* (1949) verified the occurence of phosphoric acid associated with pectin via ester or ionic linkages. Whereas almost all phosphorus could be removed by percolation through a column of Amberlite IR 45 anion exchanger, the hypothesis of ionic linkage seems to be more reliable.

Table 4: Mineral composition (as percentage weight of dry matter) of the three industrial pectin samples.

	Lemon A	Lemon B	Apple
Na^+	0.17	0.15	0.04
K^+	0.27	0.20	0.62
Ca^{2+}	0.74	0.36	0.31
Mg^{2+}	0.05	0.05	0.06
PO_4^{2-}	0.04	0.04	0.15

Among the three industrial pectin samples, irrespective of the plant source, the lemon A pectin is characterized by a much higher calcium content. That seems to indicate the presence of some regions that strongly retain calcium ions along with the pectin molecules of the lemon A sample, *e.g.* blocks of de-esterified galacturonide units that may have been created by the attack of native pectin esterase (Kohn *et al.*, 1968).

It appears that non-esterified galacturonic acids are only neutralized up to 44.4, 27.9 and 43.8% for the lemon A, lemon B an apple pectins respectively, with the cations that have been determined (see table 4). Since all three industrial samples have been extracted under the same conditions, no explanation can at present be given for this observation.

Molecular size distribution

HPSEC was used for the rapid characterization of the pectin molecular size. Figure 1 shows the elution pattern of the three industrial pectin samples. The system has not been calibrated for molecular-mass determination on purpose. Indeed, SEC separates molecules according to their molecular size (Laurent & Killander, 1964). Calibration and subsequent determination of molecular mass are only possible for series of compounds of similar molecular shape and density. Chemical analysis has shown that apple pectin contains more neutral sugar residues than lemon pectins. Side chains as well as rhamnose units, which increase the main chain flexibility may render apple-pectin molecules more compact and more dense than those of lemon and, at similar molecular mass, they should elute later because of their relative smaller molecular size. Without using specific detection such as light scattering or on-line viscosity, it thus appears impossible to derive the molecular mass of pectins from HPSEC when the system is calibrated with pectins of different origin.

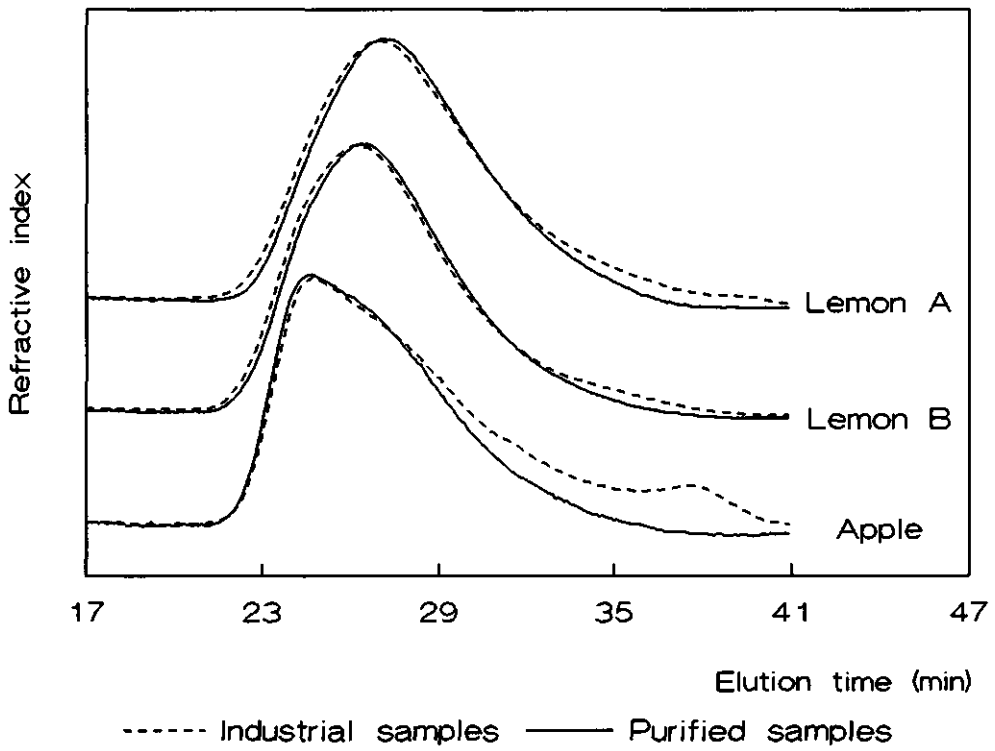

Figure 1: HPSEC of the industrial and corresponding Cu-purified samples

Lemon pectins were found to elute within one single peak. The apple pectin however, exhibits a second peak of smaller size which elutes in the tail of the main peak. Differences in elution time indicate that the lemon B pectin sample has a larger hydrodynamic volume than the lemon A sample and that both lemon pectins have a smaller hydrodynamic volume than the apple pectin. This latter observation suggests a very high average molecular mass for the apple pectin, all the more as it is expected to have a relatively small molecular size compared to its molecular mass (see above). The broadness of the peaks also indicates that within one pectin sample, the size of the molecules is not homogeneous.

Figure 1 also shows that copper purification mainly removed molecules that elute in the tail of the peak. This indicates that free neutral polysaccharides have a relatively low hydrodynamic volume. These results confirm those obtained by Michel *et al.* (1981) and Brigand *et al.* (1990).

Intermolecular charge distribution

The determination of degrees of methoxylation (DM) and acetylation (DAc) as described above provides only average values. Figure 2 shows the chromatograms obtained for the three industrial samples by high performance ion exchange chromatography on a MA7P column. As expected for highly methoxylated pectins (van Deventer-Schriemer & Pilnik, 1976; Schols *et al.*, 1989), the main bulk of the three samples elutes at a rather low ionic strength. However, some other peaks appear at a higher ionic strength. All three chromatograms exhibit a well-defined peak, eluting always at the same elution time (8.96 min). Schols *et al.* (1979) suggested that this peak is due to the elution of some pectin molecules under conditions which are not yet elucidated. We have isolated this peak by collecting the eluate from repeated runs and have found that it reacts positively with the *m*hdp test. However, since this peak also occurs during blank elutions, it could also be due to the elution of some salt impurities bound during the column regeneration at low ionic strength. Their elution at a given concentration of phosphate buffer may suddenly increase the ionic strength of the eluent and provoke the elution of some pectin molecules that should elute later. A third broader peak elutes after 9 minutes, *i.e.* high ionic strength. This peak is due to the elution of pectin molecules of low DM (Schols *et al.*, 1989).

The apple pectin that has the highest DM (table 1) elutes first. Surprisingly, the lemon B pectin which is slightly but significantly more methoxylated than the lemon A elutes somewhat later. This may be explained by

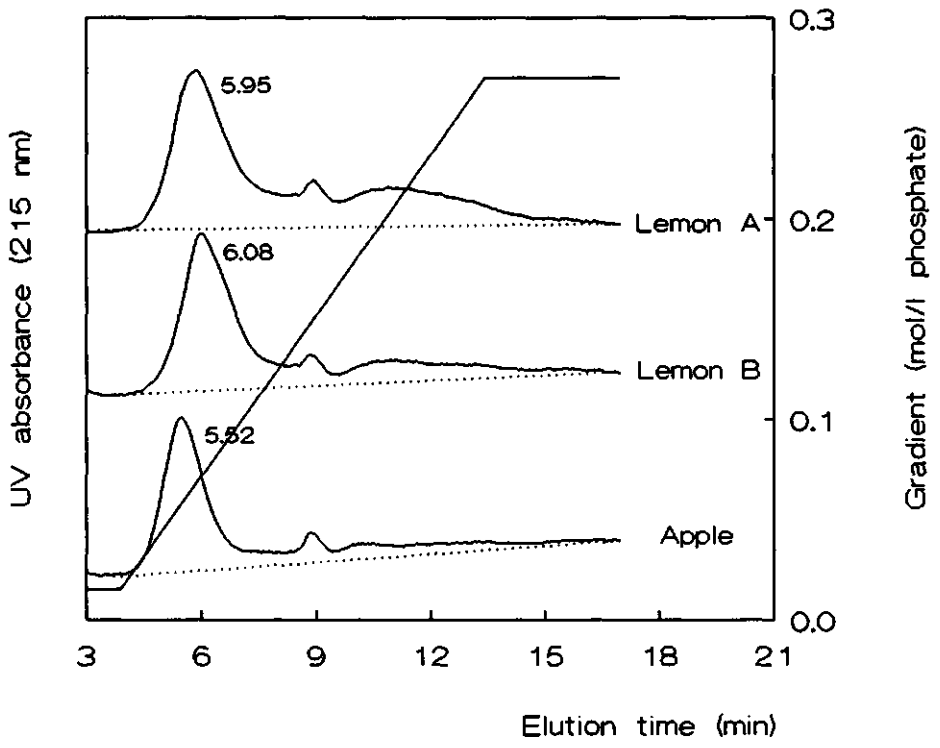

Figure 2: HPIEC of the industrial samples on MA7P column.

the relative importance of the third peak in the lemon A pectin, indicating the presence of numerous low-esterified molecules that lower the average DM of the whole sample. Such molecules also occur in the lemon B pectin, but in a much lower proportion. They are almost absent in the apple pectin. The determination of the average DM of industrial pectins by ion-exchange chromatography (van Deventer-Schriemer & Pilnik, 1976; Schols *et al.*, 1989) is thus very inaccurate as soon as the distribution of DMs is not homogeneous. Low methoxylated pectin molecules exhibit a high affinity for calcium ions (Kohn, 1975) and their presence in larger proportion in the lemon A pectin sample may explain its higher calcium content as well as some gelling properties.

Copper purification did not change the elution pattern of the pectin samples on HPIEC, indicating that no significant de-esterification occured during the treatment. This confirms the findings of Michel *et al.* (1981).

CONCLUSION

Apple and lemon industrial pectin preparations differ from each other in their chemical composition, both quantitatively and qualitatively. Apple pectin appears to contain more neutral sugars and more phenolics but less proteins than lemon pectins. HPSEC indicates that apple pectin also has a much larger molecular size than lemon pectins.

Copper purification proved that only part of these compounds is associated with pectin molecules. All three industrial pectin preparations contain free neutral polysaccharides (especially starch in the apple pectin), free phenolic compounds and free proteins which constitute the so-called "ballast" (Michel *et al.*, 1981; Brigand *et al.*, 1990). The presence of such impurities result from the incomplete purification during the industrial extraction, probably because of the low specificity of the alcohol precipitation. Their effects on the physical properties of pectins remain unknown.

The presence of neutral sugars that could not be separated from the galacturonide fraction by copper purification indicates that industrial pectins may carry neutral side chains as has often been demonstrated for laboratory-extracted pectins. However, the degradative hot-acid conditions of the industrial extraction lead to a "trimming" of the side chains. Side chains are thus shorter and/or less abundant in industrial pectins than in pectins from similar sources extracted in mild conditions.

It is interesting to note that purification tends to reduce compositional differences between apple and lemon pectins. Analytical differences between apple and lemon industrial pectins appear to be due more to the presence of accompanying molecules than intrinsic differences of the pectin molecules. However, the purified apple pectin clearly differs from the purified lemon pectins. Although differences may arise from the physiological state (Gould *et al.*, 1965; Knee, 1973, 1978a; de Vries *et al.*, 1984a) and extraction conditions (Joslyn & Deuel, 1963; de Vries *et al.*, 1981) our results seem to confirm that pectin composition depends very much on plant origin (Zitko & Bishop, 1965; Kawabata, 1977; de Vries *et al.*, 1984b). Apple pectin molecules contain more neutral sugars and probably more phenolic compounds but fewer proteins than lemon pectin molecules.

Of the two lemon pectins studied, lemon A has been found to retain many more calcium ions than the other one.

Some of these analytical differences may explain some empirically known differences in physical behaviour that are of great technological importance. However, because of the many varying parameters, the establishment of the composition-properties relationship may require a statistical investigation carried out on a large number of industrial pectin samples. Moreover, the analysis of the whole molecules does not provide any information about differences between molecules (inter-molecular structure) or on the sequence of the different constituents (intra-molecular structure). These industrial samples have also been preparatively fractionated by size exclusion and ion exchange chromatographies, and have been specifically depolymerized by using both chemical and enzymic methods. These data will be the object of further papers in this series.

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Chapter 3:

A SIMPLIFIED METHOD FOR THE DETERMINATION OF THE INTRINSIC VISCOSITY OF PECTIN SOLUTIONS BY CLASSICAL VISCOSIMETRY

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3. A SIMPLIFIED METHOD FOR THE DETERMINATION OF THE INTRINSIC VISCOSITY OF PECTIN SOLUTIONS BY CLASSICAL VISCOSIMETRY

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ABSTRACT

Viscosimetric measurements were performed on various commercial pectin solutions with an Ubbelohde viscosimeter. Intrinsic viscosities were determined by extrapolating to zero concentration:

- the reduced viscosity: η_{sp}/C
- the logarithm of the reduced viscosity: $\ln(\eta_{sp}/C)$
- the inherent viscosity: $(\ln\eta_{rel})/C$
- the combined relation: $\frac{1}{C}\left[2\left(\frac{\eta}{\eta_0} - 1 - \ln\frac{\eta}{\eta_0}\right)\right]^{1/2}$

The classical η_{sp}/C *vs.* C relation exhibits a non linear behaviour. Thus, the determination of intrinsic viscosity by linear extrapolation to zero concentration of η_{sp}/C leads to a 5-10% under-evaluation. The empirical $\ln(\eta_{sp}/C)$-C relation provides intrinsic viscosity values very close to those given by the inherent viscosity and combined relation. Moreover, the values provided by the combined relation are fairly constant with concentration. This behaviour allows to determine the intrinsic viscosity from one single-concentration measurement without loss of accuracy.

INTRODUCTION

Many practical applications of pectins such as formation of gels are directly related to their molar mass. Thus, the determination of viscosity of pectin solutions has often been used to calculate their molar mass according to the principles initiated by Staudinger in the 30s.

Solution viscosity is basically a measure of the size or extension in space

of polymer molecules (Billmeyer Jr., 1971). It is therefore empirically related to the molar mass. Mark (1938) and Houwink (1940) proposed the relation:

$$[\eta] = k M^{\alpha}$$

where $[\eta]$ is the limiting viscosity number, more commonly called intrinsic viscosity, and k and α are two constants dependent on the couple molecular shape-solvent. The simplicity of measurement and the usefulness of the viscosity-molar mass relation make of viscosity measurement an extremely valuable tool for the molecular characterization of pectins.

Part of the problem is then the accurate determination of the intrinsic viscosity. Several methods were proposed to extrapolate the intrinsic viscosity from viscosity measurements performed at different concentrations. Some of the available methods for the intrinsic viscosity determination of polymers were compared in order to improve the accuracy as well as the ease of the current intrinsic viscosity determination of pectins.

THEORY

The concentration dependence of the specific viscosity in the very dilute region may usually be expressed in a power series in the concentration (Eisenberg, 1976):

$$\eta_{sp} = [\eta]C + k[\eta]^2C^2 + k'[\eta]^3C^3 + \ldots \qquad (1)$$

where k is a dimensionless constant known as the Huggins factor (Huggins, 1942).

From this expression one can easily recognize the relations currently used to determine the intrinsic viscosity:

$$[\eta] = \lim_{C \to 0} \frac{\eta_{sp}}{C} \qquad (2)$$

η_{sp}/C being called the reduced viscosity

$$[\eta] = \lim_{C \to 0} \frac{1}{C} \ln \frac{\eta}{\eta_0} \qquad (3)$$

$\ln(\eta/\eta_0)/C$ being called the inherent viscosity

In order to correct the imperfect linearity of the η_{sp}/C-C relation of polystyrene solutions, Staudinger and Heuer (1934) established the empirical relation:

$$[\eta] = \lim_{C \to 0} \ln \frac{\eta_{sp}}{C} \qquad (4)$$

In their study on pectin viscosity, Owens *et al.* (1946) used the relation 4 by plotting η_{sp}/C on a semi-logarithmic scale.

Moreover, combination of the former relations using the reduced viscosity (relation 2) and the inherent viscosity (relation 3) provides the following combined relation that has already been reported (Lecacheux, 1982; Morris, 1984):

$$[\eta] = \lim_{C \to 0} \frac{1}{C} \left[2 \left(\frac{\eta}{\eta_0} - 1 - \ln \frac{\eta}{\eta_0} \right) \right]^{1/2} \qquad (5)$$

MATERIAL AND METHODS

Various unstandardized commercial pectins were taken in our own collection. Their brief analytical characteristics are given in table 1.

Viscosities were measured in an Ubbelohde capillary viscosimeter as described by van Deventer-Schriemer & Pilnik (1987). Pectins were dissolved in 0.1M tris-succinate buffer pH 6.0 (molarity refers to succinic acid) with 0.01 % thiomersal. Temperature was set at 30°C. Flow times were recorded with a stopwatch with a precision of 0.1 second. All concentrations are expressed in galacturonic acid content.

RESULTS AND DISCUSSION

Flow times through a capillary viscosimeter were recorded for 11 different commercial pectin samples at different concentrations in order to determine the intrinsic viscosity by means of various available procedures. Table 2 gives the intrinsic viscosities calculated with the use of relations given in the theoritical part of this paper.

Table 1: Analytical characteristics of the pectin samples as determined by the titration procedure.

	Galacturonan content (% raw pectin)	Degree of Methoxylation (%)
Apple A	68.8	50.4
Apple B	72.3	74.3
Apple C	69.1	60.3
Apple D	63.7	62.1
Apple E	60.9	73.6
Lemon A	75.1	71.8
Lemon B	74.4	72.8
Lemon C	69.5	61.1
Lemon D	70.1	71.5
Lime A	71.3	62.6
Lime B	70.2	69.5

Table 2: Intrinsic viscosities of pectin samples determined by extrapolation to zero concentration by using 4 different procedures.

	Reduced viscosity	ln of red.visc.	Inherent viscosity	Combined relation
Apple A	5.48	5.70	5.74	5.75
Apple B	6.88	7.80	7.78	7.75
Apple C	6.47	7.10	6.96	6.96
Apple D	4.72	4.76	4.78	4.78
Apple E	5.83	5.93	5.94	5.92
Lemon A	4.30	4.48	4.47	4.48
Lemon B	5.23	5.64	5.63	5.62
Lemon C	4.72	4.76	4.73	4.73
Lemon D	6.20	6.36	6.34	6.34
Lime A	5.87	5.99	5.95	5.99
Lime B	6.26	6.49	6.52	6.50

The values obtained by different procedures are very close to each other (SD < 10^{-2}) except for the reduced viscosity method that provides values 5-10% lower. While using the relation given by Owens *et al.* (1946), this difference leads to an under-evaluation of the weight average molar mass of 2-10%.

This difference may be explained by the non linear behaviour of the η_{sp}/C-C relation as it can be seen from figure 1. Logarithmic regression of the reduced viscosity against C provides a better extrapolation to zero concentration of the intrinsic viscosity than the linear regression.

Inherent viscosity and combined relation also allow a very good extrapolation of the intrinsic viscosity. This may be explained by the fact that both relations are better mathematical simplifications of the general concentration dependence of the reduced viscosity (relation 1) than the reduced viscosity.

In order to improve the accuracy of the intrinsic viscosity determination of pectin solutions we must therefore recommend the use of either the logarithmic

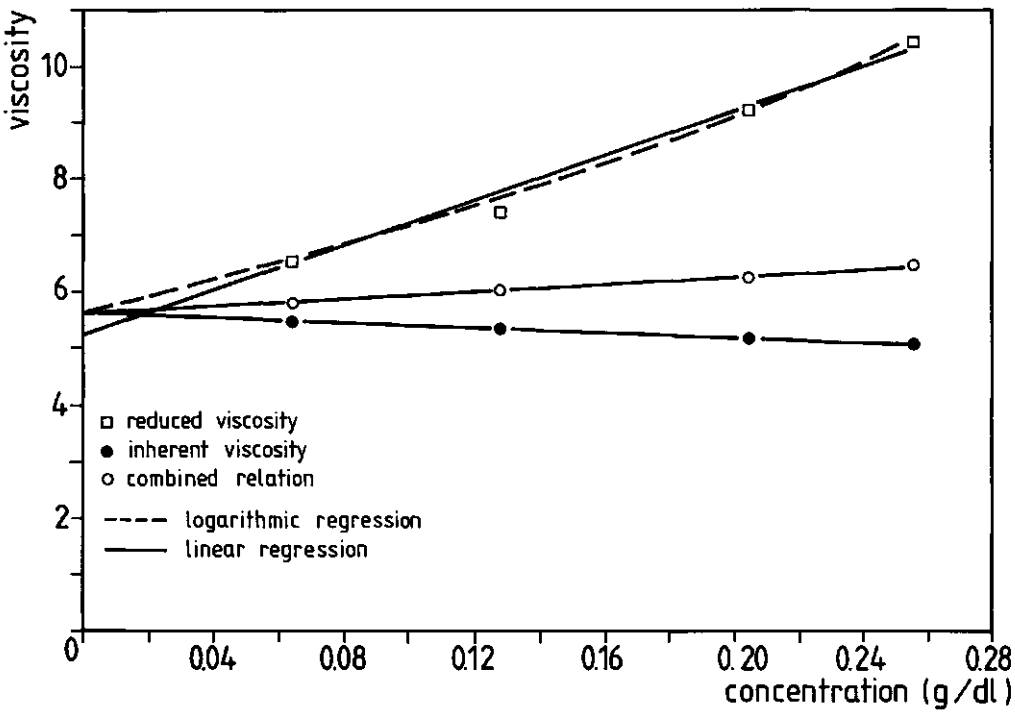

Figure 1: Intrinsic viscosity determination of the lemon pectin sample B by extrapolation to zero concentration by using 4 different relations.

extrapolation of the reduced viscosity, the extrapolation of the inherent viscosity or the extrapolation of the combined relation instead of the currently used linear reduced viscosity extrapolation.

As it can be seen from figure 1, the slope of the combined relation is very small. This suggests that a single measurement at low concentration allows to estimate the intrinsic viscosity.

Table 3 compares the values of intrinsic viscosity obtained by either multi-point or single-point (0.03-0.06 g/dl concentration) extrapolation to zero concentration of the combined relation (relation 5). The latest method provides an estimation of the intrinsic viscosity with an error that is not higher than 5%. It is still possible to reduce the concentration of the solution to be measured until 0.01 g/dl. Moreover, accuracy may be improved by using an automated system for flow time measurements. In such conditions, the intrinsic viscosity of pectin solutions can be accurately estimated (error $< 1\%$) by a single measurement at low concentration by using the combined relation. This makes the viscosimetric procedure much faster without loss of accuracy.

Table 3: Comparison of multi-point and single-point extrapolation for the determination of the intrinsic viscosity of pectins using the combined relation (relation 5).

	Multi-point Extrapolation	Single-point Extrapolation	
Apple A	5.75	5.89	(0.055)*
Apple B	7.75	8.13	(0.057)
Apple C	6.95	7.24	(0.057)
Apple D	4.78	4.86	(0.032)
Apple E	5.92	6.05	(0.031)
Lemon A	4.48	4.60	(0.065)
Lemon B	5.62	5.82	(0.064)
Lemon C	4.73	4.80	(0.035)
Lemon D	6.34	6.49	(0.035)
Lime A	5.95	6.08	(0.036)
Lime B	6.50	6.67	(0.035)

* pectin concentration (g/dl)

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Chapitre 4:

STUDIES ON THE INTERMOLECULAR DISTRIBUTION OF INDUSTRIAL PECTINS BY MEANS OF PREPARATIVE SIZE EXCLUSION CHROMATOGRAPHY

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4. STUDIES ON THE INTERMOLECULAR DISTRIBUTION OF INDUSTRIAL PECTINS BY MEANS OF PREPARATIVE SIZE EXCLUSION CHROMATOGRAPHY

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ABSTRACT

Three industrial high methoxyl pectins have been fractionated by SEC on a preparative scale and the chemical composition, viscosity and light scattering behaviour of the fractions have been investigated. Chemical analysis revealed that the composition varies greatly from one SEC fraction to another. In all three pectin samples, the fractions of low molecular size contain most of the free neutral polysaccharides as well as some free pectin "hairy regions". In addition, the lemon pectin samples contain some pectin molecules of large size that are rich in neutral sugars. Phenolic and proteinaceous compounds coelute with neutral sugar-rich fractions. However, in the apple pectin, phenolics and proteins occur predominantly in the fractions of low molecular size. Lemon pectin molecules, especially that of the lemon A sample, are prone to aggregation in the presence of calcium cations. The aggregate fraction can be disrupted by shear forces, heating or the presence of a chelating agent. The formation of such calcium-pectate aggregates seems to be due to the presence of some molecules with low degrees of methoxylation. Light scattering measurements also suggest that even very narrow SEC fractions remain highly heterogeneous on the basis of their molecular weight, thus indicating large differences in molecular conformation.

INTRODUCTION

In a previous paper (Kravtchenko *et al.*, 1992; chapter 2), the chemical composition of three industrial high methoxyl pectins from apple and lemon have been extensively investigated. Although they are known to behave very differently on application, especially in the presence of calcium ions, the two lemon pectins have been shown to be chemically very close to each other. In contrast, the apple

pectin is richer in neutral sugars, which are present either as neutral side-chains or as free neutral polysaccharides, but does not behave very differently from the lemon B pectin sample on application. Extensive analysis of whole pectin samples only provided average values and was thus not sufficient to reveal the structural differences that could explain the behaviour on application.

With the many possible variations in methoxylation, acetylation, content and type of binding of neutral sugars, content of non-glycosidic residues and molecular size, it is very unlikely that in a given preparation, one pectin molecule is identical to another. High performance size exclusion chromatography (HPSEC) indicated some heterogeneity in the molecular size of pectin molecules within the three pectin preparations investigated (Kravtchenko *et al.*, 1992; chapter 2), but data about the inter-molecular distribution of the chemical composition of pectins can only be obtained by extensive fractionation.

Size exclusion chromatography (SEC) is a well known method of separating substances differing in molecular size. In SEC, the elution volume of a molecule of given size depends on its hydrodynamic volume (Yau *et al.*, 1979; Kato *et al.*, 1983). Since the molecular size distribution of a polymer can be important in understanding its functional behaviour (Mitchell, 1976), SEC has often been used to fractionate pectin preparations (Jordan & Brant, 1978; Davis *et al.*, 1980; Anger & Berth, 1985, 1986; Rombouts & Thibault, 1986; Lecacheux *et al.*, 1987; Hourdet & Muller, 1987).

In this paper we describe the inter-molecular distribution of sugar units, methoxyl and acetyl groups, phenolics, proteinaceous compounds and molecular parameters obtained by viscosity and light scattering measurements of three industrial pectins by means of preparative SEC.

MATERIAL AND METHODS

Pectin samples

Three unstandardized industrial pectins classified as "rapid set" were obtained from Sanofi Bio Industries (France): two extracted from lemon peels (lemon A and B) and one from apple pomace. Their chemical composition has been extensively described in a previous paper (Kravtchenko *et al.*, 1992; chapter 2).

Fractionation on Sepharose CL-2B/Sepharose CL-4B

Pectin samples were fractionated on two coupled columns (2.5x40 cm), one packed with Sepharose CL-2B and the other packed with Sepharose CL-4B (Pharmacia, Sweden). The fractionation range as determined with dextrans is $3x10^4$-$5x10^6$ daltons for the CL-4B gel and 10^5-$2x10^7$ daltons for the CL-2B gel. The columns were eluted with 0.037M phosphate buffer pH 6.5 containing 0.001M Na_2-EDTA and the polysaccharide concentration was recorded continuously with a differential refractometer (Knauer, Germany). For each SEC run, 30 mg pectin were injected. 10 ml fractions were collected for subsequent viscosity and light scattering measurements.

Fractionation on Fractogel TSK 55(S)/75(S)

Pectin samples were also fractionated on a larger scale on a column (5x90 cm) packed with a mixed bed of Fractogel TSK HW 55(S)/75(S) (1:1) (Merck, Germany). The fractionation range as determined with dextrans is 500-$2x10^5$ daltons for the 55(S) gel and 10^5-10^7 daltons for the 75(S) gel. The column was eluted with 0.1M Na-succinate buffer pH 4.8 at a flow rate of 120 ml/h. For each run, 150 mg pectin were injected. 20 ml fractions were collected, assayed for their uronide and total neutral sugar contents and combined into 9 pools. Each pool was ultrafiltered on PM10 membrane (Amicon, USA) and freeze-dried before further chemical analysis. Corresponding pools from several injections were combined in order to obtain enough material.

Chemical analysis

The anhydrouronic acid (MW=176) content was determined by the automated *meta*-hydroxydiphenyl assay (*m*hdp, Thibault, 1979). Total neutral sugars were estimated with the automated orcinol assay (Tollier & Robin, 1979), using anhydroarabinose (MW=132) as standard.

Neutral sugars were determined by GLC as their alditol acetates (Kravtchenko *et al.*, 1992; chapter 2).

The methoxyl and acetyl contents were determined by HPLC analysis of the methanol and the acetic acid released on alkaline de-esterification (Voragen *et al.*, 1986). About 5 mg pectin was saponified with 250 µl of a 0.8M NaOH/

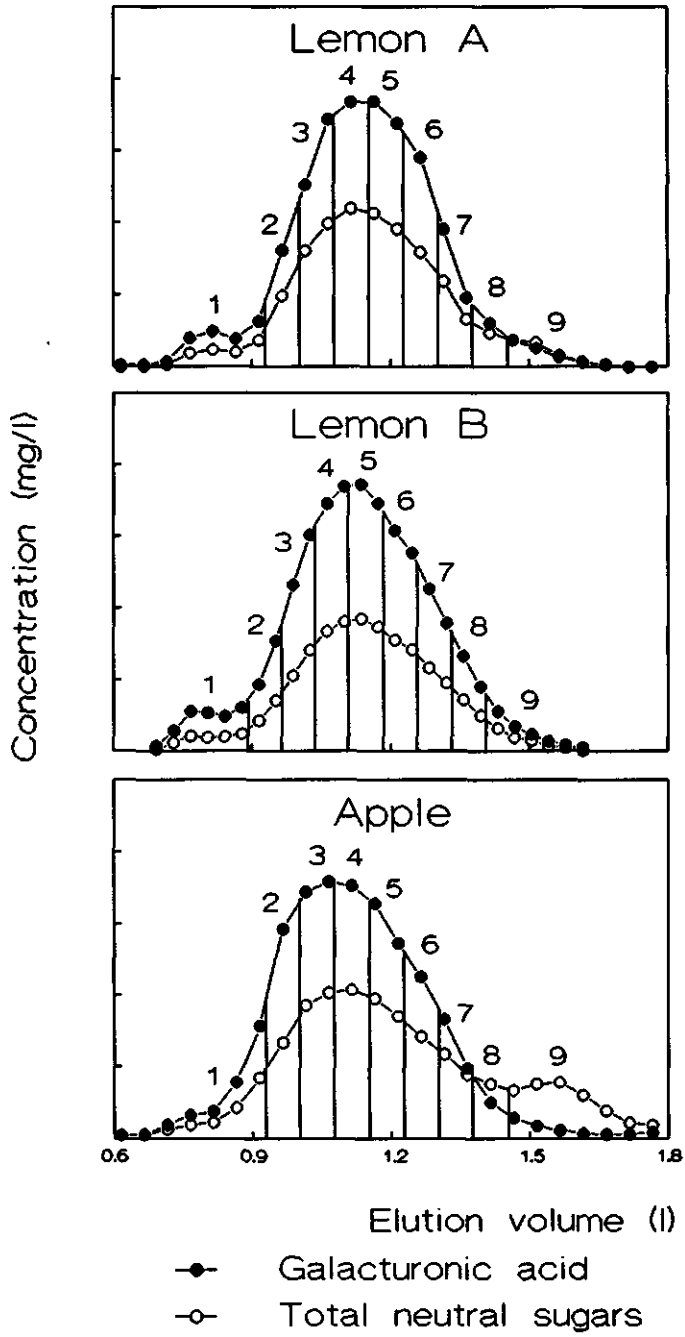

Figure 1: Elution patterns of the three industrial pectins on Fractogel TSK HW 55(S)/75(S) eluted with 0.1M Na-succinate buffer at pH 4.8 containing 29 mg/l of calcium cations.

isopropanol (1:1) mixture. After centrifugation 20 μl of the supernatant was injected on an Aminex HPX87H column for methanol and acetic acid determinations. The pectic acid precipitate was redissolved in 25 ml of 0.05 M NH_3-oxalate and the galacturonide content was determined with the *m*hdp assay.

Protein content was evaluated by the Sedmak & Grossberg assay (1977) using micro-titer plates as described by Rylatt & Parish (1982).

Total phenols were estimated with the Folin-Ciocalteu reagent without copper treatment and with ferulic acid used as standard (Swain & Hillis, 1959), 0.2 ml of Folin-Ciocalteu reagent (Merck, Germany) being added to the sample solution (0.4 ml, 0.5%), followed after 5 min by 0.2 ml of saturated Na-carbonate solution. The absorbance at 750 nm was read after 1 hour.

High performance size exclusion chromatography

High performance size exclusion chromatography (HPSEC) was performed with a series of Biogel TSK columns (Biorad, USA) 60XL, 40XL and 30XL (300x7.5 mm) being used in combination with a Biogel TSK guard column (75x7.5 mm). Columns were eluted with 0.4M Na-acetate buffer pH 3.0 at a flow rate of 0.8 ml/min and at 30°C. Detection was performed with a Shodex SE 61 refractive index detector at 40°C.

High performance ion exchange chromatography

High performance ion exchange chromatography (HPIEC) was performed as described by Schols *et al.* (1989). A Biorad MA7P column (50x7.8 mm) was eluted with a linear gradient of 15-270mM Na-phosphate buffer at pH 6.0 with a flow rate of 1.5 ml/min. Detection was carried out by reading the absorbance in the UV at 215 nm. The increase in baseline signal was corrected by substracting the chromatogram obtained for a blank run from those of sample runs.

Physical measurements

Relative viscosities were recorded with a capillary viscometer (Viscomatic, Fica, France). Intrinsic viscosities were determined from one single point (Kravtchenko & Pilnik, 1990; chapter 3).

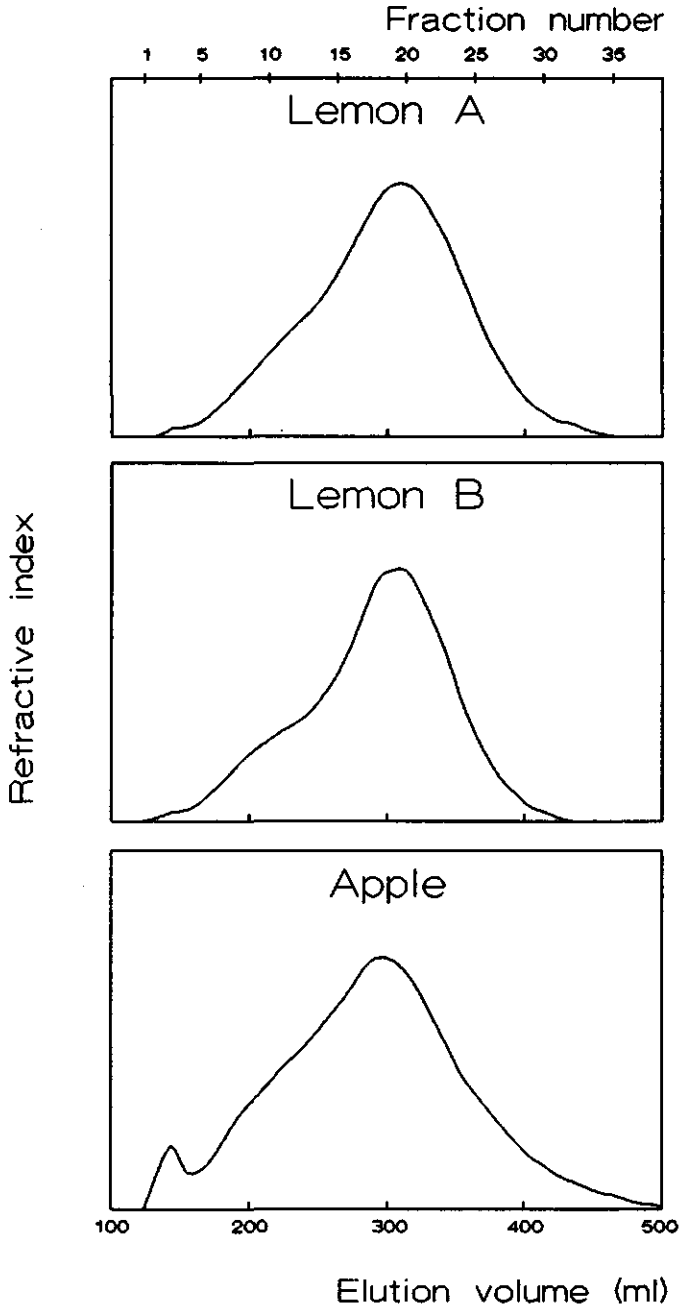

Figure 2: Elution patterns of the three industrial pectins on Sepharose CL-2B/Separose CL-4B eluted with 0.037M phosphate buffer at pH 6.5 with 0.001M Na_2-EDTA.

Light scattering measurements were performed with a Sofica light-scattering photometer (Fica, France) equipped with a helium-neon laser (Zeiss, Germany) of wavelength 632 nm. Details are given elsewhere (Berth *et al.*, 1990).

RESULTS AND DISCUSSION

Fractionation by preparative SEC

Industrial samples have been fractionated by preparative SEC without any previous purification. Prior to injection, the samples were dissolved in the appropriate buffer by shaking overnight at room temperature. Figures 1 and 2 show the elution patterns obtained by chromatography on Fractogel HW 55(S)/75(S) and Sepharose CL-2B/sepharose CL-4B, respectively. Recoveries were not significantly different from 100%. Rechromatography on HPSEC as shown in figure 3 demonstrates the efficiency of the fractionation on the Fractogel TSK column.

Since pectin molecules from different preparations with the same molecular weight can have different hydrodynamic volumes by reason of differences in degree of methoxylation (Smidsrod & Haug, 1971; Michel *et al.*, 1982; Fishman *et al.*, 1984) or degree of branching with neutral-sugar side-chains (Berth, 1988; Kravtchenko *et al.*, 1992; chapter 2), SEC is not suitable for the direct determination of the molecular weight of pectins (Masuda *et al.*, 1979; Anger & Berth, 1985, 1986; Berth, 1988; Kravtchenko *et al.*, 1992; chapter 2). However, simple qualitative inspection of chromatograms may reveal important information about the molecular size distribution of pectin preparations.

Figures 1 and 2 show that the three industrial pectins have large hydrodynamic volumes and wide size distribution. The apple pectin which elutes somewhat earlier, appears to have a higher average molecular size than the two lemon pectins. However, the apple pectin also contains a higher proportion of smaller molecules that elute in the tail of the main peak, thus resulting in a broader size distribution. These results confirm those obtained previously by low pressure SEC (de Vries *et al.*, 1984; Brigand *et al.*, 1990) and HPSEC (Brigand *et al.*, 1990; Kravtchenko *et al.*, 1992; chapter 2).

Compared with fractionation on the Fractogel TSK column (figure 1) or HPSEC (Kravtchenko *et al.*, 1992; chapter 2), the chromatograms obtained with

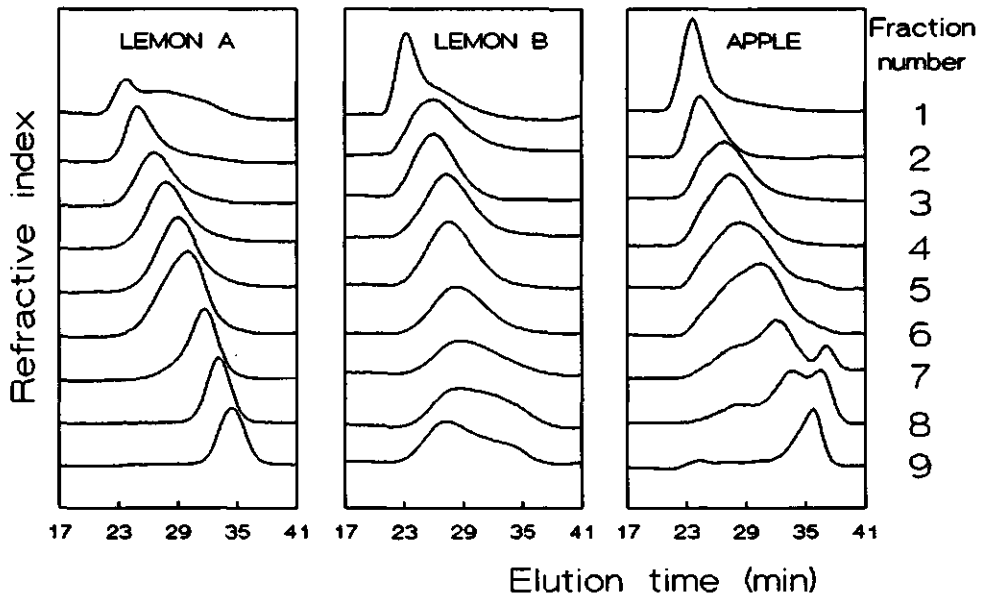

Figure 3: HPSEC elution patterns of the fractions obtained by preparative SEC on Fractogel TSK HW 55(S)/75(S) for the three industrial pectins as shown in figure 1.

the Sepharose columns (figure 2) exhibit a wide distribution for the largest molecules. This may be due to the wide overlap of the fractionation range for the Sepharose CL-4B and Sepharose CL-2B gels, leading to an increased resolution in that region.

By fractionation on the Fractogel TSK column (figure 1) both lemon pectins give a separate peak eluting at the void volume of the column. This early peak disappears when samples are heated at 100°C for about 10 min just before the injection, although the rest of the chromatogram remains unchanged. Moreover, when heated samples are kept at room temperature for a few hours before injection, the peak reappears with the same size. Such a peak does not occur by chromatography on HPSEC (Kravtchenko *et al.*, 1992; chapter 2). On rechromatography on HPSEC (figure 3), fractions 1, especially that of the lemon A sample, exhibit an unexpected wide molecular size distribution compared to the other fractions. Indeed the elution pattern of the void fraction resembles that of the corresponding unfractionated pectin sample (Kravtchenko *et al.*, 1992; chapter 2). Discrepancy in elution behaviour may be explained by molecular disruption caused

by high shear forces that occur in the HPSEC system. These results are consistant with the hypothesis of the presence of a microgel component in pectin solutions (Sorochan *et al.*, 1971; Smith, 1976; Kawabata & Sawayama, 1977; Berth *et al.*, 1977, 1982; Jordan & Brant, 1978; Davis *et al.*, 1980; Plashchina *et al.*, 1985) that can be disrupted by heat (Sorochan *et al.*, 1971; Smith, 1976; Fishman, 1986) or shear forces (Fishman, 1986). For large-scale fractionation on the Fractogel column, the eluant was prepared with tap-water containing 29 mg/l of calcium. When the buffer was prepared with distilled water, the same voiding peak did not occur. This may indicate the involvement of calcium cations in the phenomenon of aggregation that we have observed.

Comparing chromatograms obtained for samples heated and unheated, this aggregate fraction can be estimated to represent 4 and 2 % for lemon A and lemon B, respectively. However, even operated at a relatively low flow rate, the SEC column may still generate shear forces able to disrupt some aggregates. Thus, SEC cannot be considered as a reliable technique to quantify the phenomenon of aggregation.

Similar void peak occurs in the apple pectin sample too, but it has proved to remain stable when the sample was pre-heated prior to injection onto SEC. Moreover, this fraction remains homogeneous on HPSEC (figure 3). This indicates that the high molecular size fraction of apple pectin is of a different nature to that of the lemon pectins. It may consist of individual molecules of very large size.

On fractionation on Sepharose columns, both lemon pectins do not show this high molecular size fraction. This may be due to the presence of EDTA in the eluent and may be a further indication of the involvement of divalent cations in the aggregation observed previously on the Fractogel column. On the other hand, the apple pectin sample still exhibits a well separated void peak on Sepharose columns. This seems to confirm our previous interpretation (see above).

Industrial pectin samples have been fractionated on the Fractogel TSK column in order to investigate their inter-molecular chemical differences. Since the final goal is a better understanding of the physical behaviour of these pectins we have decided to use buffer prepared with tap-water and to avoid preheating of samples in order to isolate and further investigate the high molecular size fractions that occur in the lemon pectin samples.

Table 1: Chemical composition of the fractions obtained by SEC on the TSK HW 55(S)/75(S) column (expressed as percentage weight of AUA).

Lemon A									
Fraction	1	2	3	4	5	6	7	8	9
AUA (%)[a]	5.9	8.4	16.0	19.6	19.5	15.5	8.2	3.6	2.7
Rha	1.9	2.0	1.7	1.3	1.0	1.3	2.2	2.5	4.3
Fuc	0.4	0.3	0.3	0.3	0.3	0.4	0.4	0.6	1.0
Ara	1.8	2.0	1.5	1.3	1.4	1.7	2.7	4.8	14.7
Xyl	0.3	0.3	0.3	0.2	0.2	0.1	0.2	0.3	1.5
Man	0.4	0.1	0.3	0.3	0.2	0.1	0.2	0.9	4.9
Gal	5.5	5.3	3.7	3.5	3.5	4.6	6.2	17.0	44.4
Glc	0.5	0.3	0.4	0.3	0.3	0.4	0.8	3.0	4.9
Total NS	10.8	10.4	8.1	7.3	6.8	8.6	12.6	28.9	75.6
DM (%)[b]	62	70	71	73	72	72	65	66	n.d.
DAc (%)[b]	1.8	1.9	1.8	1.4	1.4	1.3	1.5	1.9	n.d.
Phenolics	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.6	2.8
Proteins	1.2	0.8	0.7	0.7	0.5	0.5	0.5	0.7	3.8

Lemon B									
Fraction	1	2	3	4	5	6	7	8	9
AUA(%)[a]	5.7	7.5	13.0	17.4	17.9	15.4	12.0	7.1	4.0
Rha	1.1	1.9	1.5	1.3	1.2	1.2	1.3	1.1	1.5
Fuc	0.2	0.3	0.4	0.2	0.3	0.3	0.3	0.4	0.4
Ara	1.5	3.3	2.0	1.4	1.5	1.7	2.3	2.3	3.0
Xyl	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Man	0.2	0.8	0.2	0.1	0.1	0.2	0.7	0.6	0.4
Gal	4.2	5.3	5.4	4.3	4.4	5.4	8.1	8.7	9.4
Glc	0.9	0.5	0.3	0.3	0.5	0.6	1.0	1.2	1.7
Total NS	8.3	12.3	9.9	7.7	8.1	9.6	13.8	14.3	16.6
DM (%)[b]	69	75	77	78	73	73	66	67	80
DAc (%)[b]	1.4	1.9	1.5	1.7	1.3	1.5	1.7	2.3	1.7
Phenolics	0.3	0.4	0.2	0.1	0.1	0.2	0.5	0.6	0.2
Proteins	2.3	1.2	0.7	0.5	0.4	0.6	0.8	0.9	0.5

Apple									
Fraction	1	2	3	4	5	6	7	8	9
AUA(%)[a]	9.8	14.3	18.3	18.1	15.5	11.5	6.7	2.9	2.0
Rha	1.4	1.7	1.6	1.6	1.4	1.9	2.4	2.2	7.1
Fuc	0.2	0.3	0.2	0.2	0.2	0.3	0.6	0.7	0.4
Ara	2.1	2.1	2.3	2.5	2.8	3.7	7.3	4.5	44.2
Xyl	1.2	1.3	1.3	1.3	1.2	1.3	1.4	1.8	3.0
Man	0.1	0.2	0.1	0.1	0.2	0.3	0.7	1.6	1.6
Gal	3.3	2.9	3.7	3.9	4.4	6.4	11.0	22.5	27.0
Glc	3.4	3.6	7.2	10.8	14.1	16.9	24.5	30.7	52.9
Total NS	11.6	12.2	16.4	20.5	24.2	30.8	48.0	84.0	136.1
DM (%)[b]	76	76	79	76	74	68	56	43	157
DAc (%)[b]	3.6	3.4	4.2	5.2	6.5	5.7	6.8	24.1	n.d.
Phenolics	0.8	0.2	0.2	0.1	0.2	0.2	0.6	2.2	2.4
Proteins	1.0	0.6	0.4	0.3	0.5	0.5	1.0	2.2	3.0

[a] fraction % of the whole sample
[b] expressed as mole % of AUA

Distribution of Neutral Sugars

The orcinol assay indicates that the neutral-sugar (NS) content is not constant over the pectin molecules fractionated on the Fractogel TSK column (figure 1). Results of the orcinol assay have not been corrected for interference with galacturonide. Indeed, we have found (not published) that the response of galacturonic acid with orcinol depends on the degree of polymerization: polygalacturonic acid gives less colour than monogalacturonic acid, probably because of incomplete hydrolysis during performance of the test. A calculation based on the colour generated by monogalacturonic acid (used as standard) thus under-estimates the total NS content. Moreover, the orcinol reagent does not react with the same intensity for different sugar species and differences in quantity are partly due to differences in sugar composition.

In addition, NS were also individually determined by GLC in the pools obtained by fractionation on Fractogel TSK. Table 1 gives the NS composition of the fractions as indicated in figure 1, expressed as percentage weight of NS per galacturonic acid.

In lemon pectins, a minimum NS content was found in fractions of intermediate size which represent the main bulk of the samples. Except for fraction 1, the fractions of high molecular size exhibit a higher amount of NS (about twice that of the molecules of intermediate size) but the most spectacular difference occurs for the smallest molecules. In lemon A pectin, fraction 9 contains 4 times as many rhamnose, 7 times as many xylose, 10 times as many arabinose and 13 times as many galactose residues as fraction 5. This high NS content is partly due to the presence of free neutral polysaccharides which have proved to elute very late on SEC (Michel *et al.*, 1981; Le Quéré *et al.*, 1981; Brigand *et al.*, 1990; Kravtchenko *et al.*, 1992; chapter 2). However, the high content of rhamnose indicates the presence of "hairy regions" (de Vries *et al.*, 1982).

The high NS content of the largest pectin molecules cannot be explained by the presence of free "hairy regions" that are parts of the whole molecules. Moreover, since free neutral polysaccharides elute later, it seems that some highly branched pectin molecules of large size occur in lemon pectins. Lecacheux *et al.* (1987), Berth *et al.* (1990) and Berth & Lexow (1991) observed a similar enrichment of NS at the beginning and at the end of the elution pattern of other industrial pectins.

Fraction 1 of the lemon pectin samples exhibit a sugar composition very similar to that of the unfractionated samples, further suggesting that it may be constituted of aggregated molecules of intermediate size.

In the apple pectin, NS content also increases with decreasing molecular size but the NS content of the largest molecules is not significantly higher than that of the unfractionated sample. The NS content increases regularly from fraction 5 to fraction 9.

For all three pectins, glucose units, which mainly belong to starch molecules (Kravtchenko *et al.*, 1992; chapter 2) are located in the latest fractions, especially in the apple sample. However, a small but significant amount of glucose is present in all fractions, possibly attached to pectin molecules.

Degree of esterification and ester distribution

Table 1 shows the degree of methoxylation (DM) of the fractions obtained by SEC on the Fractogel column from the three industrial pectin samples. DM values measured on very small samples suffered from rather high standard deviations (about 5%). However, we can observe a trend of decreasing DM with decreasing the molecular size. Such a variation has already been reported by Brigand *et al.* (1990). In the case of the lemon pectins, especially for lemon A, fraction 1 exhibits a lower DM than the following fractions. This may explain the tendency of these fractions to aggregate in the presence of calcium cations. On the other hand, fraction 9, seems to be very highly methoxylated. This is explained by the presence in these fractions of free pectin "hairy regions" which have been shown to be almost completely methoxylated (De Vries *et al.*, 1982).

Figure 4 shows the elution patterns on high performance ion exchange chromatography (HPIEC) of the SEC fractions from the three pectin samples. Pectin fractions clearly elute earlier and earlier with increasing fraction number, *i.e.* decreasing hydrodynamic volume. As elution volume on IEC is thought to increase with decreasing DM (van Deventer-Schriemer & Pilnik, 1976; Schols *et al.*, 1989) these results seems to contradict those presented above. However, mechanically-degraded (Anger *et al.*, 1977) and enzyme-degraded (van Deventer-Schriemer & Pilnik, 1987) pectins have been shown to elute at lower ionic strength than undegraded pectins on IEC, making them appear to be more esterified. Differences in elution time on HPIEC may thus be due to some molecular size effect rather than any real difference in DM.

It has been shown (Kravtchenko *et al.*, 1992; chapter 2) that the lemon A pectin sample contains a high proportion of pectin molecules eluting at high ionic strength on HPIEC. Figure 4 shows that almost all these low DM molecules are concentrated in the fractions of high molecular size (fractions 1 to 3). Since they

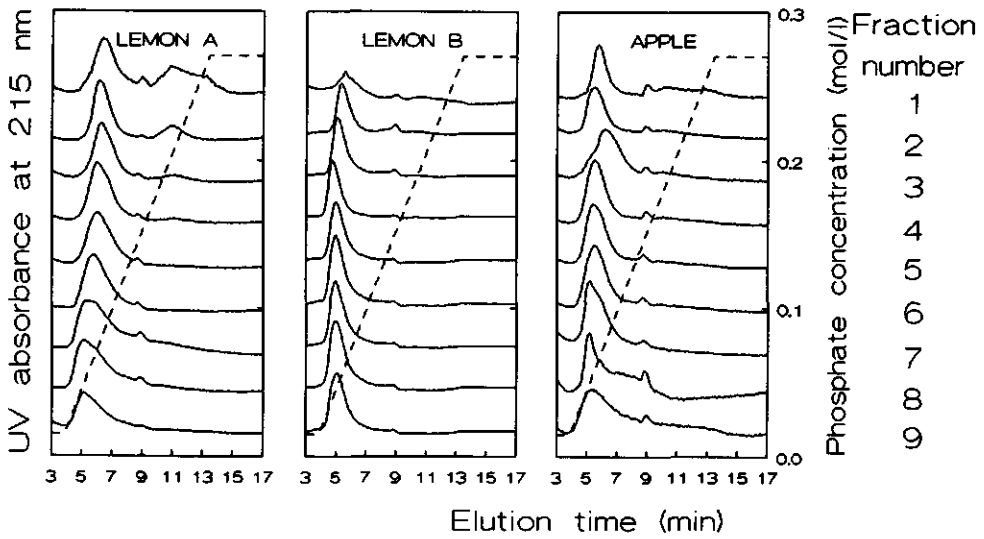

Figure 4: HPIEC elution patterns of the fractions obtained by preparative SEC on Fractogel TSK HW 55(S)/75(S) for the three industrial pectins as shown in figure 1.

do not contain so much of these low DM molecules, lemon B and apple pectin samples do not exhibit such a phenomenon so clearly. The presence of these low DM pectin molecules in the lemon A sample explains the lower average DM found for fraction 1 and may explain its strong tendency to form aggregates in the presence of calcium (Fishman *et al.*, 1984; Paoletti *et al.*, 1986).

Degree of acetylation (DAc) has also been determined on the fractions obtained by preparative SEC (table 1). Again, with so small samples, the HPLC technique was not accurate enough to provide very reliable results (standard deviations of about 5%). However, it can be seen from table 1 that the neutral sugar-rich fractions are the most acetylated. This is in agreement with Schols *et al.* (1990) who found a DAc of 60% in neutral sugar-rich pectin fragments from apple. Moreover, it has been shown that acetyl residues are mainly linked to the galacturonosyl residues instead of neutral-sugar side-chains (Schols *et al.*, 1990). All fractions from the apple pectin sample are more acetylated than those from the lemon samples. This is in agreement with the fact that apple pectin is more acetylated than lemon pectins (Kravtchenko *et al.*, 1992; chapter 2).

Distribution of phenolic and proteinaceous compounds

Table 1 shows the distribution of total phenolics and proteins over the fractions obtained by fractionation on the TSK column. Phenolics and proteins are present in all fractions from the three pectin samples but in varying amounts. The content of phenolic compounds is the highest in the fractions rich in neutral sugars. This also indicates that they may be associated to pectin molecules via the neutral-sugar side-chains (Kravtchenko *et al.*, 1992; chapter 2), as is the case for phenolic acids in sugar-beet pectin (Rombouts & Thibault, 1986). However, the high molecular size fraction of the apple pectin also contains a high amount of phenolics although the neutral-sugar content is low compared to the fractions of lower molecular size. This suggests the presence of some free polyphenols of very large size. Proteins also seem to coelute with the neutral sugar-rich pectin fractions, *i.e.* fractions of low molecular size. However, the ratio of protein to total neutral sugars decreases regularly with increasing elution volume, suggesting that proteins occur preferentially with the largest pectin molecules. We did not investigate the possible linkage of proteins and phenolics with pectin molecules. Their order of elution on SEC may thus be either due to their own molecular size or to the size of the pectin molecule to which they are linked.

Viscosity measurements

Relative viscosities of the fractions obtained by fractionation on Sepharose columns have been recorded. Figure 5 shows the intrinsic viscosities plotted on a logarithmic scale against the elution volume. Intrinsic viscosities were calculated on the basis of the concentrations measured by refractometry. As observed repeatedly for other pectin samples (Anger & Berth, 1985, 1986; Berth *et al.*, 1990; Berth & Lexow, 1991) intrinsic viscosity does not increase regularly with hydrodynamic volume. This may be ascribed to the presence of molecules that are built up in different ways. Above an elution volume of about 180 ml, intrinsic viscosity decreases with increasing hydrodynamic volume. However, the relation is not linear as expected for homologous polymers. The intrinsic viscosity is markedly lowered for both the largest and smallest molecules which were shown to be richer in NS and therefore probably more branched. Surprisingly, the intrinsic viscosity drops rapidly with decreasing elution volume, *i.e.* increasing hydrodynamic volume, for the fractions eluting before 180 ml. This indicates the presence of highly dense and spherical particles which may be either branched

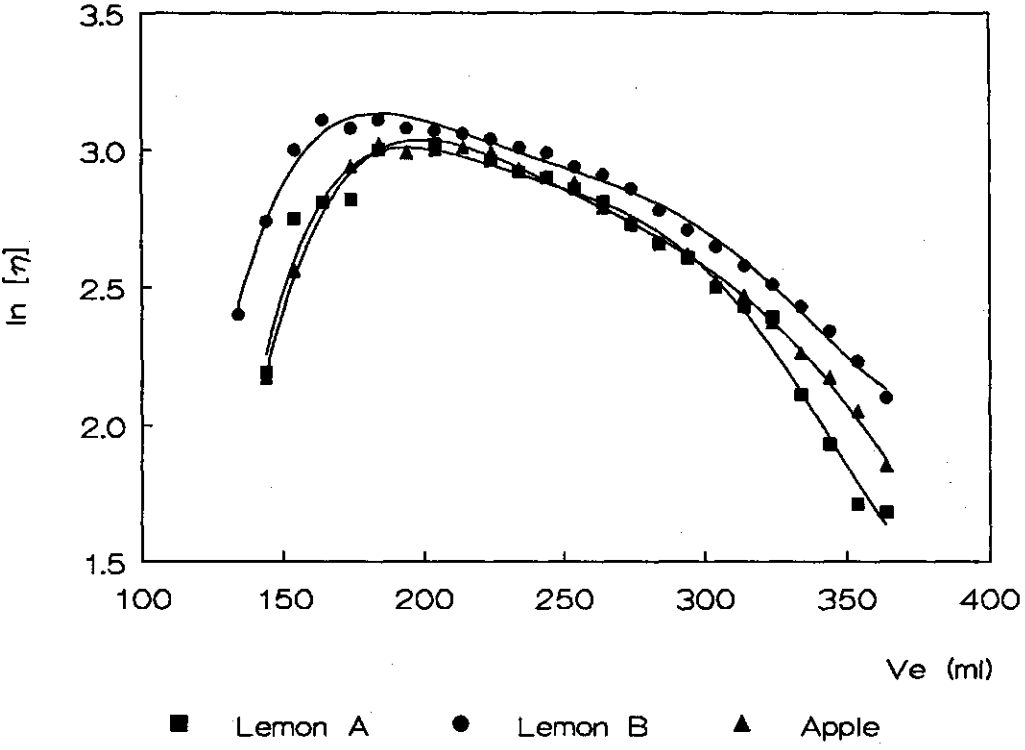

Figure 5: Change in intrinsic viscosities of the fractions obtained by preparative SEC on Sepharose CL 2B/Sepharose CL 6B as shown in figure 2.

pectin molecules or relatively stable aggregates.

For all fractions, at a given elution volume, lemon B exhibits higher intrinsic viscosity than lemon A and apple pectins. In the light of all the previous observations, the lower intrinsic viscosity of the apple pectin fractions may be due to a higher degree of branching with side chains than the lemon pectins. For the lemon A pectin, the difference may be explained by the presence of some dense particles in all the fractions which do not contribute substantially to the viscosity.

Light scattering measurements

The SEC fractions obtained with the Sepharose columns for the three industrial pectins (figure 2) have been investigated by light scattering between 30 and 150°. Measurements were carried out at one single concentration on the solutions resulting from SEC. Since pectin solutions were injected

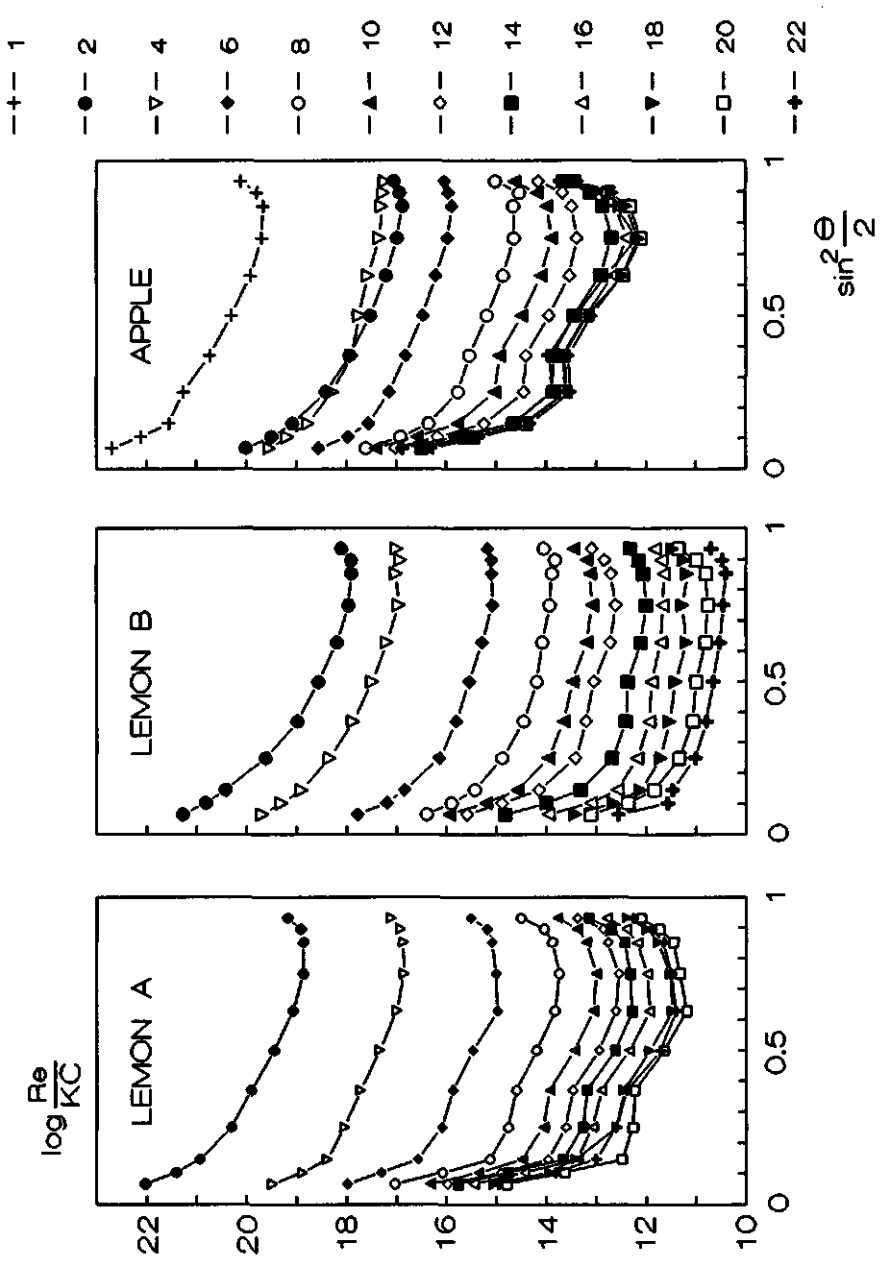

Figure 6: Guinier plot of the fractions obtained by preparative SEC on Sepharose CL 2B/Sepharose CL 6B as shown in figure 2. Fractions were filtered through 0.8 μm pore size filter prior to light scattering measurement.

without any preliminary purification and collected in a non dust-free environnement, SEC fractions were filtered prior to light scattering measurements. Results obtained using membrane filters of pore size 0.8 and 0.45 μm are shown as Guinier plots (Kerker, 1969; Berth *et al.*, 1990) in figure 6 and 7, respectively. It should be noted that these data have not been obtained by the analysis of the same fractions after successive filtrations through membrane of diminished pore size, but by the analysis of the fractions obtained from two independant SEC runs. However, SEC fractionation showed excellent reproducibility.

As expected, the scattering intensity decreases with increasing SEC elution volume, indicating decreasing average molecular weigths. Only in the case of apple pectin we observe a slight increase of the level for the last eluting fractions. That may be explained by the occurence of compact molecules such as pectin hairy regions and/or free neutral polysaccharides in the low molecular size fractions (see above).

After filtration through 0.8 μm pore size filters, all the curves exhibit very similar shapes. The strong curvation at angles below 50° followed by a steady flattening above 50° indicate the presence of particles of very high molecular weight (Huglin, 1972; Berth *et al.*, 1990). Strongly curved scattering functions indicate an extremely broad mass distribution or bimodal system (Dautzenberg & Rother, 1988). Such an excess of light scattering along the chromatogram has already been reported by Brigand *et al.* (1990). Berth (1988) and Berth *et al.* (1990) also reported the presence of high molecular weight components in SEC fractions of several pectins and pointed out that the very similar curvature within the low angle range suggests a similar particle component of the same size level in all fractions. The upward trends at angles above 135° are probably due to reflexions within the measurement system and should be ignored.

The use of 0.45 μm pore size filters instead of 0.8 μm ones reduces considerably the scattering level, although any measurable loss of polymer cannot be detected. Also, scattering curves become more flat and exhibit a more constant angular dependence of the scattered light, indicating the removal of at least some large sphere-like particles. Although dissolved macromolecules can usually pass unrestrictedly through membranes of the pore sizes applied here, it is clear that 0.45 μm pore size membrane causes some fractionation of the industrial pectins. However, the fraction removed by the membrane represents only a very low (negligible) mass contribution of the total concentration. For the apple pectin sample, filtration through 0.45 μm pore size filter removes the large particles which caused the increase of the light scattering intensity with increasing SEC elution volume for the high elution volume fractions. Whereas filtration of the

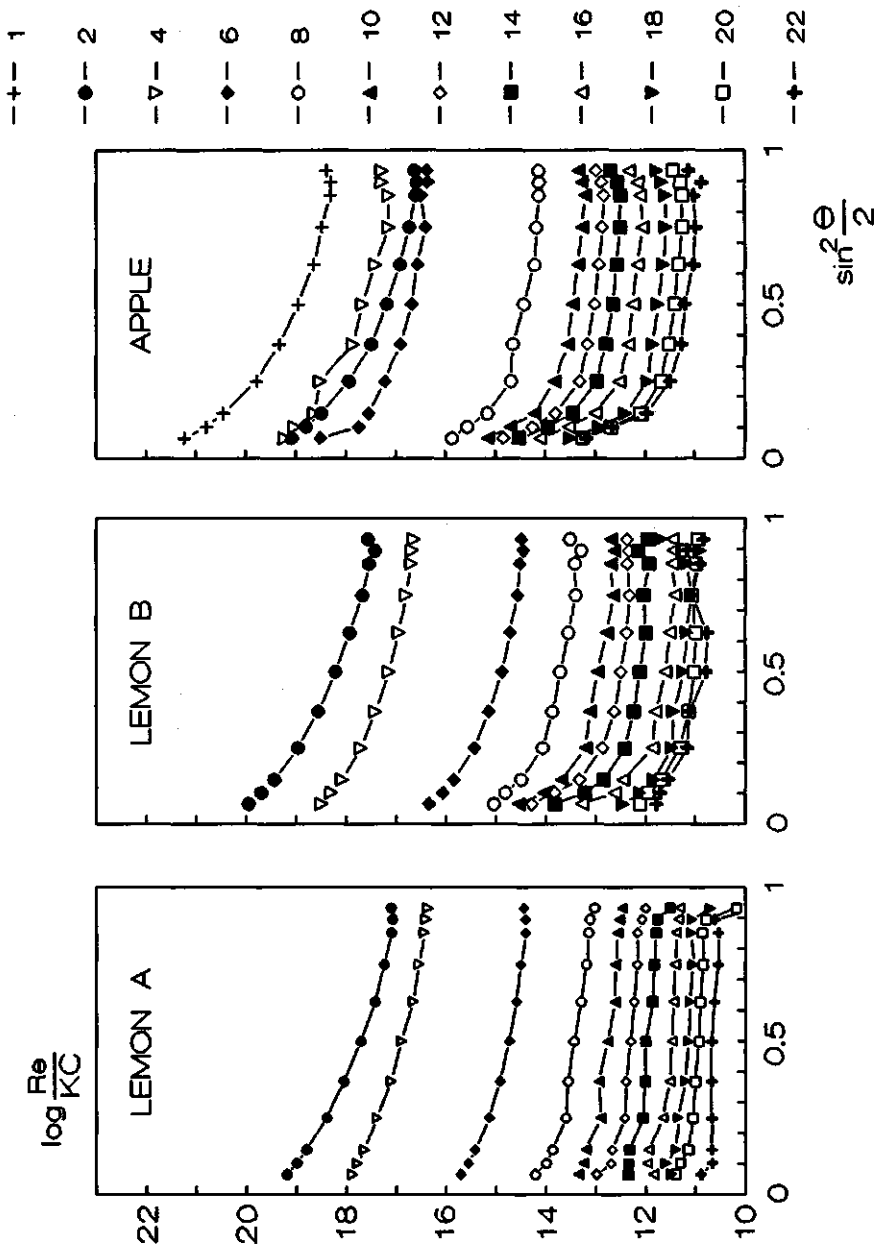

Figure 7: Guinier plot of the fractions obtained by preparative SEC on Sepharose CL 2B/Sepharose CL 6B as shown in figure 2. Fractions were filtered through 0.45 μm pore size filter prior to light scattering measurement.

lemon A pectin sample through 0.45 μm pore-size filter gives almost ideal scattering curves for homogeneous systems, scattering curves remains strongly curved for the lemon B and apple samples. Moreover, unlike previous results obtained on another citrus pectin (Berth *et al.*, 1990), the initial slope of the scattering curves increases with the elution volume indicating an increased average radius of gyration.

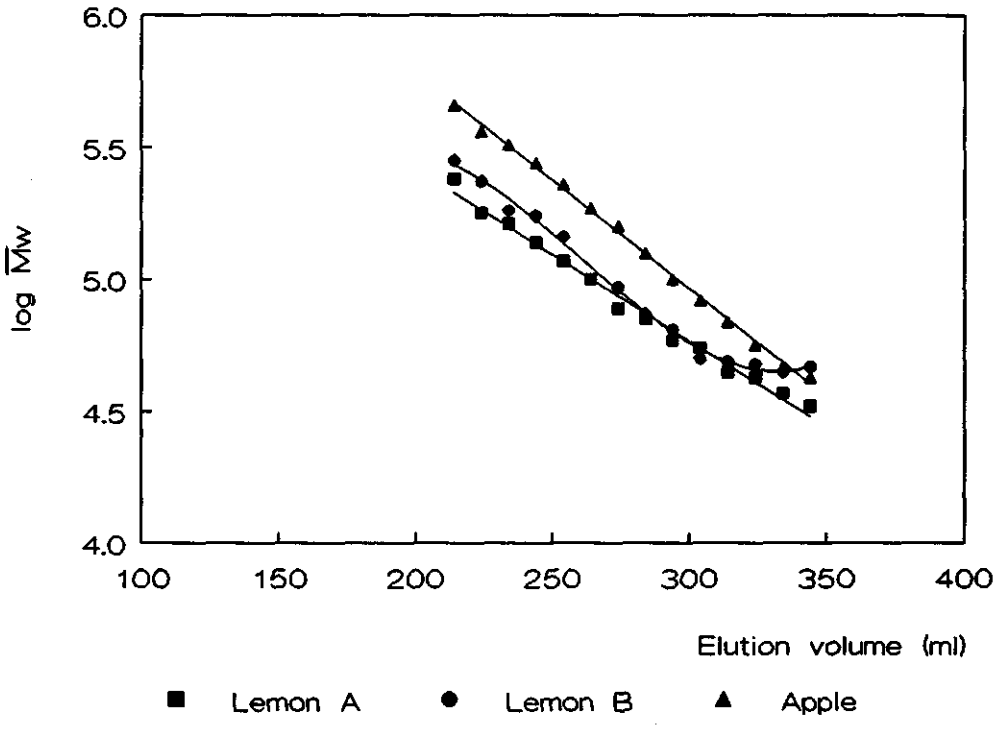

Figure 8: Change in M_w of the fractions obtained by preparative SEC on sepharose CL 2B/Sepharose CL 6B as shown in figure 2.

M_w were calculated from light scattering curves obtained after filtration of SEC fractions through 0.45 μm pore size filters. The two component interpretation (Berth *et al.*, 1990) was applied with the computerized algorithm described by Berth & Lexow (1991), taking fraction 2 as the model curve for the pure particle component in order to eliminate the particle contribution in favour of the molecularly dispersed fraction. Although in principle the same considerations with respect to the heterogeneity of the fractions may hold, calculations failed without

neglecting the angular region below 45°. Plotting the logarithm of MW against the elution volume (figure 8) clearly shows that pectin molecules of identical MW but from different sources elute on SEC at different elution volumes. This definitively demonstrates that SEC is not suitable for the direct determination of the MW of pectins from different sources by the use of a single calibration.

At equivalent elution volumes on SEC, the apple pectin always exhibits higher molecular weights than the two lemon pectins. This is due to two factors: 1- the fractions from the apple pectin contain more NS units than those from the lemon pectins; 2- all fractions from the apple pectin contain large amounts of glucose, possibly originating from starch. This supports the importance of neutral sugars emphasized by Berth (1988) and Berth *et al.* (1990).

Figures 6 and 7 demonstrate that preparation of the sample solutions and scattering functions must be carefully considered to get reliable information from light scattering measurements. Indeed, as systems containing traces of particulate matter, pectin solutions are extremely sensitive to any manipulation. For this reason it appears advisable to perform MW determinations with other absolute techniques such as membrane osmometry and/or sedimentation equilibrium analysis which are less sensitive to the presence of minor amounts of very large particles.

CONCLUSION

Chemical analysis of SEC fractions showed that within one pectin preparation, molecules differing in hydrodynamic volume vary in composition. In particular, the largest and the smallest molecules are richer in neutral sugars than those of intermediate size. However, the molecules only differ by the proportion of the same building units. They therefore belong to the same polysaccharide species. Aspinall (1970) described pectins as "chemically homogeneous polydisperse systems consisting of structurally related molecular species with continuously variable proportion of neutral sugar residues" and Anderson & Stoddart (1966) proposed the term "heteropolymolecular" to describe such systems. Anyway, industrial pectin preparations are undoubtedly complex mixtures of molecules differing not only in molecular size. They can therefore not be considered as series of homologue polymers.

Light scattering measurements clearly indicate that within one pectin sample, fractions of a given hydrodynamic volume remain highly heterogeneous on the basis of their molecular weight. This clearly indicates the coexistence in industrial pectins of molecules of very different shapes. All SEC fractions contain

various amounts of a very high molecular weight component. Since this high molecular weight fraction has a dominant effect on light scattering measurements, most investigators have tried to remove it by ultracentrifugation (Berth *et al.*, 1977; Smith, 1976; Kawabata & Sawayama, 1977; Jordan & Brant, 1978; Plashchina *et al.*, 1985; Berth, 1988; Sawayama *et al.*, 1988), filtration (Smith, 1976; Jordan & Brant, 1978; Axelos *et al.*, 1987; Hourdet & Muller, 1987) or heating (Smith, 1976, Sawayama *et al.*, 1988) but none of these techniques has proven to be completely successful. However, it is evident that the use of filters or any other "purification" procedure is quite likely to affect pectin solutions so that they are no longer representative of the material originally dissolved. Rejecting any amount of sample raises the question as to wether the purified solution can still be related to the properties of the original material. It appears that pectins which are polydisperse with respect to molecular weight are also polydisperse with respect to the conformation and/or reactivity of molecules. It is now a question of how these particular features affect the physical behaviour of industrial pectins.

The nature of this high molecular weight component is not yet definitively elucidated. Since the amount of particle component appears to increase with increasing quantity of total neutral sugars in the early SEC fractions, Berth (1988) and Berth *et al.* (1990) suggested that these particle components may be molecules rich in neutral sugars (*i.e.* highly branched). This is in agreement with the view that molecules with very different molecular weight but of similar hydrodynamic volume may coelute on SEC. Brigand *et al.* (1990) further suggested that starch is probably the main reason for the excess of light scattering. However, free neutral polysaccharides including starch have been shown to elute only in the tail of the SEC elution pattern (Kravtchenko *et al.*, 1992; chapter 2) and they therefore may not explain the presence of high molecular weight particles in the high molecular size fractions. The fact that these large particles are at least partly retained by membranes with size as large as 0.45 μm, strongly suggests that they may be extremely large aggregates instead of molecularly dispersed molecules. It is thus very surprising to find such large particles in almost all SEC fractions. Berth & Lexow (1991) suggested that a mechanism other than the simple size exclusion may govern the elution pattern of these high molecular species. In addition, it may also be possible that some of these high molecular weight particles are aggregates that could have been formed after SEC fractionation. Indeed, SEC experiments showed that pectin molecules are prone to aggregation and that aggregates can be disrupted by shear forces. These aggregates may thus have been disrupted during SEC fractionation, their constituent molecules fractionated according to classical SEC theory and reforming on resting before further

characterization. This hypothesis is supported by the experiments of Smith (1976) who could still modify the light scattering behaviour of pectin solutions even after 20 filtrations through fresh membranes of the same pore size. Each filtration step may have removed only a part of the aggregates, the other being disrupted by the physical treatment. Indeed, all the purification procedures described above failed, probably because they only temporarily disrupt aggregates. Only ultracentrifugation that removes dense particles without shear forces and ion exchange chromatography which may remove the molecules responsible for aggregation may be expected to be efficient. We have obtained strong indications that the tendency for aggregation of certain pectin preparations may be due to the presence of some molecules of low DM (inter-molecular distribution). However, other mechanisms of aggregation such as hydrogen bonding or hydrophobic interactions (Davis *et al.*, 1980) may also occur in the absence of calcium ions. The simultaneous presence of proteins and phenolic compounds in all SEC fractions may also explain aggregation.

It seems that the application of more and more advanced techniques reveals an increasing complexity in the inter-molecular distribution of pectin molecules within industrial preparations. However the importance of such an heterogeneity in determining the physical behaviour still remains largely unexplained.

Since the inter-molecular distribution of methyl esters appears to be of great importance to understand the reactivity and probably the physical behaviour of industrial pectins, the same samples have been fractionated by preparative ion exchange chromatography. The resulting fractions have been extensively analysed in order to establish the distribution of the structural constituents among pectin molecules differing in charge. The results of these investigations are the object of a following paper.

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Chapter 5:

STUDIES ON THE INTERMOLECULAR DISTRIBUTION OF INDUSTRIAL PECTINS BY MEANS OF PREPARATIVE ION EXCHANGE CHROMATOGRAPHY

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5. STUDIES ON THE INTERMOLECULAR DISTRIBUTION OF INDUSTRIAL PECTINS BY MEANS OF PREPARATIVE ION EXCHANGE CHROMATOGRAPHY

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ABSTRACT

Three industrial high methoxyl pectins have been fractionated by ion exchange chromatography on a preparative scale. Chemical analysis of the resulting fractions revealed that pectin molecules varying in charge, also differ in composition. Neutral sugars, phenolic and proteinaceous compounds were found to coelute with pectin molecules. The non-uronide material appeared to be associated with all pectin molecules but mainly attached to those requiring a high ionic strength to be released from the ion exchanger. Moreover, despite large quantitative differences, the distribution of individual neutral sugars among the molecules was found to be very similar for pectins from lemon and apple, indicating a great similarity in structural features. Separation of pectin molecules by ion exchange chromatography was found to depend on many different chemical parameters such as degree of methoxylation but probably also on the intra-molecular distribution of free carboxyl groups and the presence of phenolics. This renders the interpretation of ion exchange elution profiles difficult and uncertain.

INTRODUCTION

Fractionation by size exclusion chromatography has repeatedly shown that industrial pectins are not chemically homogeneous (Brigand *et al.*, 1990; Kravtchenko *et al.*, 1992b; chapter 4); molecules varying in molecular size also differ in chemical composition. High performance ion exchange chromatography (HPIEC) showed that industrial pectins are also heterogeneous with respect to their charge (Kravtchenko *et al.*, 1992a, chapter 2). However, HPIEC analysis did not allow a deeper insight into the chemical variations which may accompany differences in molecular charge.

Since the introduction of DEAE-cellulose columns in pectin research by the

team of Deuel (Neukom *et al.*, 1960; Heri *et al.*, 1961a, 1961b), ion exchange chromatography (IEC) has been used by almost all investigators of pectin structure. The basic principle is that separation is achieved according to the charges of pectin molecules, which depend mainly on the number of dissociated carboxyl groups present on the individual molecules. Basically, pectins can thus be fractionated by IEC according to their degree of methoxylation (Heri *et al.*, 1961a; Hatanaka & Ozawa, 1964; van Deventer-Schriemer & Pilnik, 1976; Anger *et al.*, 1977) and their covalently-linked neutral-sugar content (Heri *et al.*, 1961a; Hatanaka *et al.*, 1966; Anger *et al.*, 1977; de Vries *et al.*, 1981). However, it is likely that the size of the pectin molecules also affects the elution pattern (Heri *et al.*, 1961a). Anger *et al.* (1977) observed that mechanically degraded pectins of similar DM elute earlier and earlier with decreasing molecular size. This contradicts the asumption of Walker & Saunders (1970), who ascribed the influence of the molecular size on the desorption of pectin to a superimposed size exclusion effect. Interactions other than ionic ones, independant of the degree of methoxylation but dependant on the size of the molecules might also be involved (Anger *et al.*, 1977).

IEC has therefore widely been used to fractionate pectin molecules in relation to their degree of methoxylation (van Deventer-Schriemer & Pilnik, 1976; Schols *et al.*, 1989). Since free neutral-polysaccharides are not retained by the exchanger at low ionic strength, IEC has also been used to isolate pectic substances from mixtures with other neutral polysaccharides (Aspinall *et al.*, 1968; Ishii, 1981, 1982; Barbier & Thibault, 1982; Thibault, 1983; Rombouts & Thibault, 1986; Berth, 1988). More generally, IEC has been used to characterize the inter-molecular distribution of pectin samples, the elution profile being considered as a "fingerprint" (Smit & Bryant, 1967; Brigand *et al.*, 1990). IEC provided a meaningful indication of changes occuring in stored fruits (Knee, 1970) or of differences between various cell-wall extracts (Rombouts & Thibault, 1986; Thibault, 1988; Renard *et al.*, 1990, 1991). IEC also allows the differenciation of pectins de-esterified by different ways; pectins saponified by acid or alkali appear to be homogeneous on IEC whereas pectins saponified by plant pectin-esterase elute in large fractions of various degrees of methoxylation (Heri *et al.*, 1961b; Schols *et al.*, 1989).

Diethyl-amino-ethyl (DEAE) linked to various matrices has been the most widely used anion exchanger for the fractionation of pectins. Deuel and coworkers (Neukom *et al.*, 1960; Heri *et al.*, 1961a, 1961b) as well as many other investigators (*e.g.* Aspinall & Fanshawe, 1961; Rosik *et al.*, 1962; Smit & Bryant, 1967; Hatanaka & Ozawa, 1966; Knee, 1970; van Deventer-Schriemer & Pilnik,

1976; Anger *et al.*, 1977; Ishii, 1978; de Vries *et al.*, 1981) used DEAE-cellulose columns. Because of the low capacity and the poor flow capacity of cellulose ion exchangers, DEAE linked to other matrices such as microcristalline cellulose (Stevens & Selvendran, 1984a; Anger & Dongowski, 1984; Saulnier & Thibault, 1987; Racape *et al.*, 1987; Thibault, 1988), cross-linked dextrans (Aspinall *et al.*, 1968; de Vries *et al.*, 1981; Ishii, 1981; Stevens & Selvendran, 1984b) or cross-linked agarose (Michel *et al.*, 1981; Barbier & Thibault, 1982; Stevens & Selvendran 1984b; Rombouts & Thibault, 1986; Axelos *et al.*, 1989; Renard *et al.*, 1990) have been favourably used. All these commercially available column materials differ from each other by their flow properties, their ionic capacity and their size exclusion limit which may be very important for the proper binding of high molecular weight molecules such as pectins. Antal & Toman (1976) introduced diethyl-amino-hydroxypropyl and Sun *et al.* (1987) used quaternary amino-ethyl (QAE) as alternative exchangers.

After sample application and adsorption on the ion exchanger, the column is washed with low ionic strength buffer in order to remove the unbound substances. Then, the separation is obtained by varying conditions of elution. Some of the earlier investigators (Neukom *et al.*, 1960; Aspinall & Fanshawe, 1961; Hatanaka & Ozawa, 1964, 1966) fractionated pectins by increasing the pH. This procedure can be criticized since at high pH values, methyl-esterified pectins undergo de-esterification and ß-eliminative depolymerization (Albersheim *et al.*, 1960). Most investigators preferred to elute pectic substances by increasing the concentration of the buffer used as mobile phase at constant pH. Thus, buffers such as phosphate (Heri *et al.*, 1961b; Rosik *et al.*, 1962; Smit & Bryant, 1967; Knee, 1973b; van Deventer-Schriemer & Pilnik, 1976; Anger *et al.*, 1977; de Vries *et al.*, 1981; Stevens & Selvendran, 1984a), acetate (Hatanaka & Ozawa, 1969; Ishii, 1978; Barbier & Thibault, 1982; Stevens & Selvendran 1984a; Rombouts & Thibault, 1986), carbonate (Hatanaka & Ozawa, 1964), formate (Aspinall *et al.*, 1969), borate (Neukom *et al.*, 1960; Knee, 1973a) and succinate (Renard *et al.*, 1990, 1991) have been used in the pH range 4-6.5. Stevens and Selvendran (1984b) increased the ionic strength of the mobile phase with a gradient of NaCl concentration in Na-borate buffer and, Racape *et al.* (1987) used a non-buffered gradient of NaCl. The choice of the buffer is mainly directed by the conditions of stability of pectin molecules. However, some other criteria such as compatibility with further chemical analysis must also be taken into account. Knee (1970) claimed to improve the desorption by adding some EDTA to the mobile phase in order to avoid binding of polyuronides to the matrix by divalent metal ions.

Recently, high performance chromatographic procedures have been

developed making the IEC analysis much faster. Schols *et al.* (1989) used a MA7P column (Biorad, USA) coupled to a UV detector reading the absorbance at 215 nm. Brigand *et al.* (1990) used a monoQ column (Pharmacia, Sweden) coupled with an on-line analyzer determining the carbohydrate content of the eluate. Unfortunatly, by these HPLC techniques, it is practically difficult to collect large fractions for further investigation.

This report deals with the preparative fractionation of industrial pectin samples by IEC and the subsequent chemical analysis of the resulting fractions.

MATERIAL AND METHODS

Pectin samples

Three unstandardized industrial pectins have been obtained from Sanofi Bio Industries (France): two from lemon peels (lemon A and B) and one from apple pomace. Their chemical composition has been extensively described in a previous paper (Kravtchenko *et al.*, 1992a; chapter 2).

For the calibration of the IEC column, highly methoxylated pectins were prepared by trans-esterification (van Deventer-Schriemer & Pilnik, 1976) of lemon B pectin, pectic acid was obtained by alkaline saponification (van Deventer-Schriemer & Pilnik, 1976) of the apple pectin sample and other pectin standards were commercial apple pectins obtained by industrial acid de-esterification (Nelson *et al.*, 1977).

Preparative ion exchange chromatography

500 mg of pectin were applied onto a column (23x5 cm) of DEAE-sepharose CL6B (Pharmacia, Sweden) equilibrated with 0.005M Na-succinate buffer at pH 4.8. After loading, the column was washed with 0.005M Na-succinate buffer (250 ml) and eluted with a linear gradient from 0.005 to 0.5M of Na-succinate buffer (2000 ml). The gradient was generated by two peristaltic pumps governed by an LCC500 pump controller (Pharmacia, Sweden). Elution was continued with 1000 ml of 0.5M Na-succinate buffer and 250 ml of 0.5M NaOH. 20 ml fractions were collected and assayed for uronic acid and total neutral-sugar contents. Fractions were grouped to constitute 12 pools. Each pool was ultrafiltered

through PM10 membranes (nominal cut-off of 10,000 daltons; Amicon, USA) and freeze-dried prior to further characterization.

High performance size exclusion chromatography

High performance size exclusion chromatography (HPSEC) was performed as described by Kravtchenko *et al.* (1992a; chapter 2).

Chemical analysis

The anhydrouronic acid (AUA, MW = 176) content was determined by the automated *m*-hydroxydiphenyl assay (*m*hdp, Thibault, 1979). Total neutral-sugar content was evaluated with the automated orcinol assay (Tollier & Robin, 1979), using anhydroarabinose (MW = 132) as standard.

Neutral sugars were determined by GLC as their alditol acetates (Kravtchenko *et al.*, 1992a; chapter 2).

The methoxyl and acetyl contents were determined by HPLC analysis of the methanol and the acetic acid released on alkaline de-esterification (Voragen *et al.*, 1986). The method was modified as described previously (Kravtchenko *et al.*, 1992b; chapter 4).

Protein content was evaluated by the Sedmak & Grossberg assay (1977), using micro-titer plates as described by Rylatt & Parish (1982).

Total phenols were estimated with the Folin Ciocalteu reagent without copper treatment and with ferulic acid used as standard (Swain & Hillis, 1959). 0.2 ml of Folin Ciocalteu reagent (Merck, Germany) was added to the sample solution (*ca.* 0.5%), followed after 5 min by 0.4 ml of saturated Na-carbonate solution. The absorbance at 750 nm was read after 1 hour.

RESULTS

Preparative IEC

Large scale ion exchange chromatography (IEC) was used in order to fractionate industrial pectin samples according to the charge of their constituting molecules. DEAE Sepharose CL6B (cross-linked agarose) was chosen because of

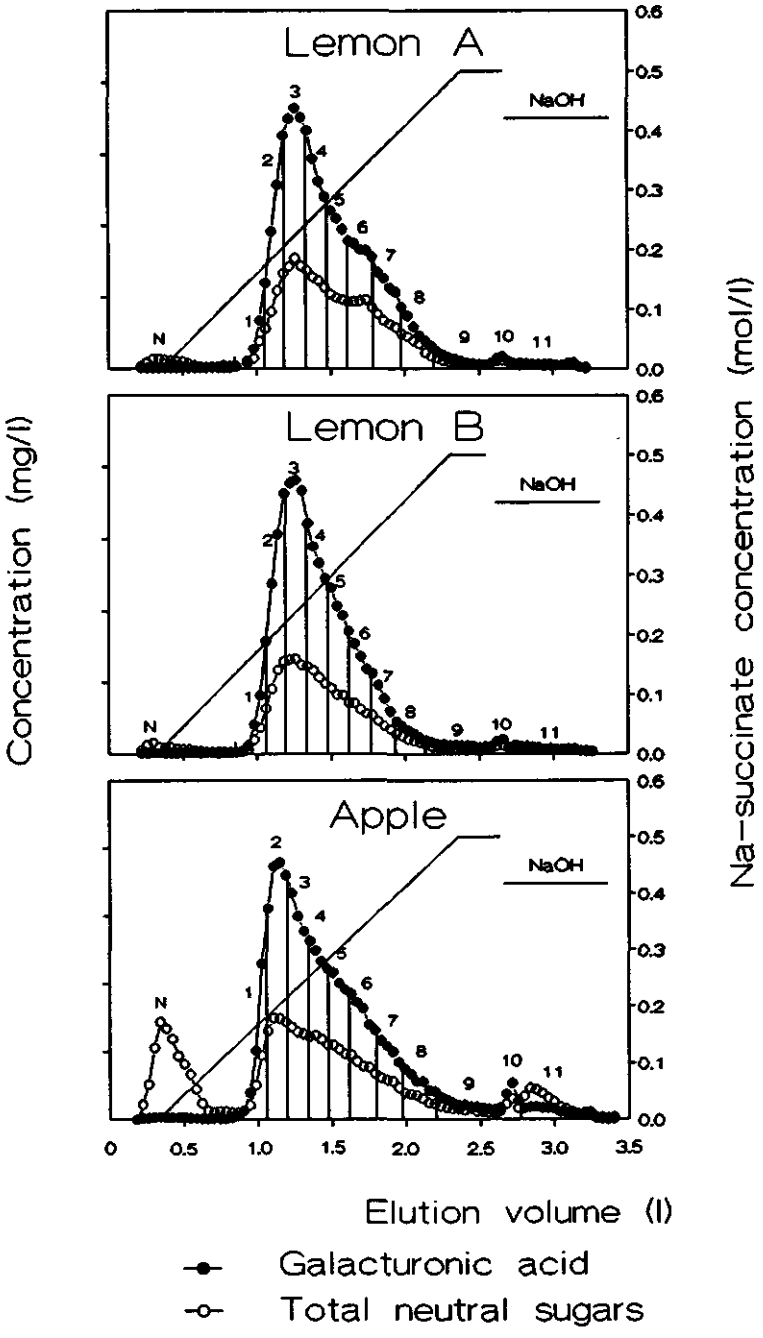

Figure 1: IEC elution pattern of the three industrial pectin samples on DEAE-Sepharose CL 6B.

its excellent flow properties allowing the use of high flow rates, its high exclusion limit ($4x10^6$ daltons) and its high total charge capacity, all resulting in high loading capacity. Na-succinate buffer at pH 4.8 was chosen because the pH of maximum stability for pectin molecules is around 4. Moreover, unlike acetate, succinate buffer disturbs neither the determination of galacturonic acid with the *m*hdp assay nor the determination of the degree of acetylation.

Figure 1 shows the elution patterns obtained by IEC of the three industrial pectin samples. For the reasons invoked elsewhere (Kravtchenko *et al.*, 1992b; chapter 4), the results obtained by colorimetry with orcinol and *m*hdp have not been corrected for mutual interference. The amount of neutral sugars thus appears largely over-estimated.

AUA recoveries were measured by determining the AUA content in the eluate of 5 ml columns of DEAE-Sepharose CL6B, eluted with 0.5M Na-succinate buffer and 0.5M NaOH after injection of a known amount of pectin. Recoveries were 95, 99 and 89% for the lemon A, lemon B and apple pectins, respectively. Even after washing with NaOH, the top of the column exhibited a brownish coloration which indicates that some phenolic compounds bound irreversibly to the column material. This was especially clear for the apple pectin sample which was found to contain a higher amount of phenolics than the lemon ones (Kravtchenko *et al.*, 1992a; chapter 2). Since polyphenols may be attached to some pectin molecules, non-ionic binding via phenolic compounds may explain the incomplete recovery of the apple pectin from the IEC column. Indeed, at alkaline pH, the ion exchanger is completely uncharged and ionic interaction cannot be taken responsible for the irreversible binding of some uronide material. Polyphenols could only be removed from the IEC column by washing with 0.1M $NaClO_2$.

A fraction which is mainly constituted of neutral sugars (fractions N) is not retained by the column. This fraction represents the "ballast" (Michel *et al.*, 1981; Brigand *et al.*, 1990), neutral polysaccharides which are not covalently bound to the pectin molecules. The free neutral polysaccharides represent only a small fraction of the lemon pectin samples but they are very abundant in the apple pectin sample. This is in agreement with the results derived from copper purification experiments of these same pectin samples (Kravtchenko *et al.*, 1992a; chapter 2).

All the galacturonic acid containing material bind to the column at low ionic strength and elutes by raising the buffer concentration. Only a small percentage (fraction 11) of it, requires NaOH to be released. As already observed by HPIEC (Kravtchenko *et al.*, 1992a; chapter 4), the broadness of peaks indicates that pectin molecules are distributed over a wide range of degrees of methoxylation (DM). Axelos *et al.* (1989) found a similar elution pattern for a commercial high

methoxyl pectin sample. The shoulder which occurs on the tail of the main peak (fractions 5-7) can be interpreted as the second peak found, with the same technique, with other pectins extracted from apple (Knee, 1970) and sugar-beet (Le Quéré *et al*, 1981).

Coloration with orcinol shows that some neutral sugars coelute with the galacturonide material, confirming that neutral sugars are not only present as co-extract but are also covalently linked to the galacturonan backbone (Neukom *et al.*, 1960; Ishii, 1978; Dea & Madden, 1986; Kravtchenko *et al.*, 1992a; chapter 2).

It is interesting to note that after ultrafiltration and freeze-drying, fractions 6 and 7 from the lemon pectin samples could only be redissolved in water or alkali in the presence of CyDTA. This indicates that pectin molecules of these fractions were associated via polyvalent cations (Rees *et al.*, 1973) that may originate from the buffer solution or the pectin itself.

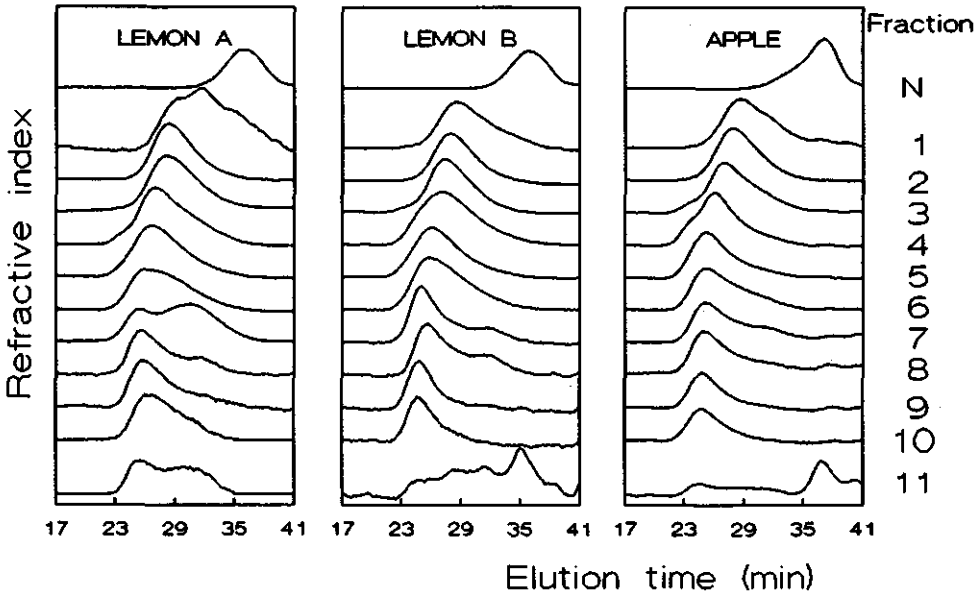

Figure 2: HPSEC elution pattern of the fractions obtained by preparative IEC of the three industrial pectin samples as indicated in figure 1.

Molecular size distribution

The fractions obtained by preparative IEC have been rechromatographed on HPSEC. The elution patterns obtained are shown in figure 2.

The unbound fractions from the three industrial pectin samples (fractions N) elute relatively late on HPSEC, confirming that free neutral polysaccharides have a much smaller hydrodynamic volume than pectin molecules (Michel *et al.*, 1981; Le Quéré *et al.*, 1981; Brigand *et al.*, 1990; Kravtchenko *et al.*, 1992a; chapter 2).

The subsequent acidic fractions elute very early on HPSEC, indicating that they are constituted of molecules of large hydrodynamic volume. However, fractions 1, 7 and 11 appear to be not homogeneous on SEC. Fraction 1, especially that from the lemon A pectin appears to be constituted of three partly resolved peaks. Fraction 7 is characterized by the presence of a second peak which elute after the main peak. Fraction 11 is mainly constituted of small molecules which elute at retention time very similar to that of the neutral fraction (fraction N).

These data do not support those presented by Smit & Bryant (1967). These authors did not find any significant change of viscosity in IEC fractions obtained from an industrial citrus pectin and thus concluded that the molecular weight is nearly identical for all pectin molecules, irrespective of their charge. From rough IEC fractionation, Sun *et al.* (1987) also, could not detect any change in the SEC elution pattern of the IEC fractions.

Neutral-sugar distribution

Table 1 shows the sugar composition of the fractions from the three industrial pectin samples which do not bind to the IEC column at low ionic strength (fractions N). All three neutral fractions contain arabinose, galactose, glucose, xylose, fucose and mannose. These neutral polysaccharides may come from the partial solubilisation of other cell-wall polysaccharides or may have been separated from the pectin molecules by the "trimming" reaction which occur during the industrial hot-acid treatment (Kravtchenko *et al.*, 1992a; chapter 2). The neutral polysaccharides isolated from the two lemon pectin samples are remarkably close to each other, both in quantity and composition. The free neutral-polysaccharides from lemon pectins are mainly composed of galactose and arabinose in the ratio 4.8:1. Glucose represents only about 10% of the neutral-sugar units, confirming the low starch content of lemon pectins (Kravtchenko *et al.*, 1992a; chapter 2).

Table 2: Chemical composition of the fractions obtained by preparative IEC (expressed as percentage weight of AUA).

Lemon A											
Fraction	1	2	3	4	5	6	7	8	9	10	11
AUA (%)[a]	2.5	13.8	21.7	17.4	12.9	13.3	9.5	5.3	2.1	0.7	0.8
Rha	0.9	0.2	0.3	1.1	1.6	2.6	3.0	4.7	3.6	3.4	5.0
Fuc	0.6	0.1	0.1	0.2	0.2	0.3	0.4	0.2	0.2	0.3	0.7
Ara	9.0	0.8	0.8	1.8	2.5	2.6	1.9	3.3	2.3	2.6	11.2
Xyl	0.4	0.1	0.1	0.2	0.2	0.2	0.2	0.4	0.3	0.8	0.8
Man	1.0	t	t	t	t	t	0.1	0.7	0.7	2.8	2.7
Gal	21.8	2.7	3.0	5.3	5.0	5.3	4.2	6.2	5.7	6.6	24.6
Glc	1.6	0.1	0.1	0.2	0.2	0.2	0.2	0.8	0.8	3.1	4.9
Total NS	34.7	3.9	4.3	8.5	9.5	11.0	9.6	15.9	13.5	19.2	49.0
DM (%)[b]	83	95	78	79	70	66	52	37	60	59	n.d.
DAc (%)[b]	1.0	0.9	0.9	1.6	1.9	1.9	1.7	1.7	2.1	3.0	n.d.
Phenolics	0.1	0.1	0.1	0.1	0.1	n.d.	n.d.	1.4	1.3	7.0	13.3
Proteins	0.6	t	t	t	0.1	0.2	0.3	1.8	1.2	1.7	13.5

Lemon B											
Fraction	1	2	3	4	5	6	7	8	9	10	11
AUA (%)[a]	3.3	17.4	24.2	18.2	13.7	10.4	6.0	2.8	1.9	1.1	1.0
Rha	0.4	0.3	0.7	1.2	2.3	2.8	4.4	3.1	3.3	1.6	11.1
Fuc	0.3	0.3	0.4	0.5	0.4	0.4	0.5	0.4	0.2	0.1	1.2
Ara	2.7	0.8	1.2	1.9	2.9	2.8	2.9	2.5	2.5	2.0	12.8
Xyl	0.3	0.1	0.1	0.2	0.2	0.2	0.4	0.4	0.4	0.3	1.0
Man	0.5	0.1	0.1	0.1	0.1	0.1	0.4	0.6	1.O	1.3	2.7
Gal	6.7	3.2	4.7	6.2	6.8	5.3	6.3	7.1	6.8	6.8	53.7
Glc	1.3	0.2	0.2	0.2	0.3	0.3	0.8	0.8	1.5	4.2	9.3
Total NS	14.0	4.7	6.9	9.8	2.7	1.5	15.1	14.5	15.5	16.2	90.6
DM (%)[b]	77	95	75	76	64	66	54	35	63	66	n.d.
DAc (%)[b]	1.3	1.0	1.0	2.1	2.3	2.1	1.6	1.4	3.7	3.7	n.d.
Phenolics	1.0	0.2	0.1	0.7	0.1	0.1	n.d.	n.d.	1.6	1.9	10.1
Proteins	0.7	t	t	0.4	0.1	0.2	0.7	1.7	0.6	3.8	9.5

Apple											
Fraction	1	2	3	4	5	6	7	8	9	10	11
AUA(%)[a]	7.3	20.2	17.1	13.5	11.4	11.3	7.2	4.7	3.2	1.9	2.1
Rha	0.4	0.4	0.7	2.0	3.7	4.0	3.9	4.1	3.1	2.2	5.4
Fuc	0.3	0.1	0.3	0.3	0.3	0.3	0.3	0.7	0.3	0.3	0.4
Ara	2.6	1.1	1.6	3.6	5.2	5.0	3.8	4.0	3.8	2.9	11.7
Xyl	1.2	0.7	0.8	1.5	1.9	1.9	2.3	2.7	2.0	1.9	3.8
Man	0.4	0.2	0.2	0.3	0.3	0.3	0.3	1.4	1.1	0.6	3.0
Gal	4.4	2.6	4.1	6.4	7.4	6.0	5.2	6.3	6.3	4.6	14.2
Glc	7.6	2.0	3.9	4.0	5.1	4.7	4.5	7.9	9.6	5.6	112.6
Total NS	16.5	7.0	11.3	17.8	23.5	21.9	20.0	26.5	25.8	17.8	150.6
DM (%)[b]	76	94	72	69	60	58	50	41	58	55	n.d.
DAc (%)[b]	2.0	2.4	3.1	5.0	5.6	5.4	4.2	3.7	6.1	4.3	n.d.
Phenolics	0.4	0.2	0.2	0.3	0.4	0.6	2.3	5.2	1.6	1.2	9.1
Proteins	t	t	t	0.1	t	0.1	0.3	1.6	1.6	1.2	4.8

[a] fraction % of the whole sample.
[b] expressed as mol % of AUA.
n.d. not determined

Table 1: Sugar composition of the unbound fractions from the three industrial pectin samples (expressed as percentage weight of total sugars)

	Lemon A	Lemon B	Apple
Galacturonic acid	1.3	0.8	1.0
Rhamnose	0.5	0.5	0.1
Fucose	1.2	1.2	0.3
Arabinose	14.3	13.9	40.2
Xylose	0.6	0.4	0.4
Mannose	5.5	5.5	0.8
Galactose	69.0	66.4	16.1
Glucose	7.5	11.3	41.1

In contrast, the neutral fraction from the apple pectin contains a higher amount of neutral polysaccharides of different composition. The proportion of arabinose is higher whereas that of galactose is much lower (ratio gal/ara 1:2.5). This difference may be due to the presence of arabinogalactans of type I (1,4-linked galactose units) and type II (1,3/1,6-linked galactose units) (Clarke *et al.*, 1979) in different proportions in the free neutral-polysaccharides from apple and lemon. In addition, the neutral fraction from the apple sample contains a larger proportion of glucose but relatively less fucose and mannose than those of the lemon samples. The three pectin samples also contain some rhamnose in their free neutral-polysaccharide fraction although only traces of galacturonic acid could be detected. The rhamnose to galacturonic acid ratio is very high (0.1-0.5). These rhamnose-rich pectin molecules of small size (see above) may be free "hairy regions" which do not bind to the ion exchanger because of their high neutral-sugar content and/or their high DM (de Vries *et al.*, 1982; Saulnier & Thibault, 1987). Indeed, de Vries *et al.* (1982) mentioned that about 10% of the pectin obtained by mild extraction from apple only bind to the IEC column after partial saponification. Molecules with a very high rhamnose/galacturonic acid ratio have also been found in the fraction from the same pectin samples which do not precipitate with copper ions (Kravtchenko *et al.*, 1992a; chapter 2).

The following fractions (1 to 11) that elute by increasing the ionic strength contain both galacturonic acid and neutral-sugar units, but in varying proportion. According to their total neutral-sugar content as shown in table 1, pectin molecules can be divided into five distinct groups. Fractions 1, especially that of the lemon A sample are characterized by a very high neutral-sugar content. Then, the total neutral-sugar content reachs a minimum in the fractions 2 to 4 which represent the main bulk of the galacturonic acid of the whole samples. The shoulder which

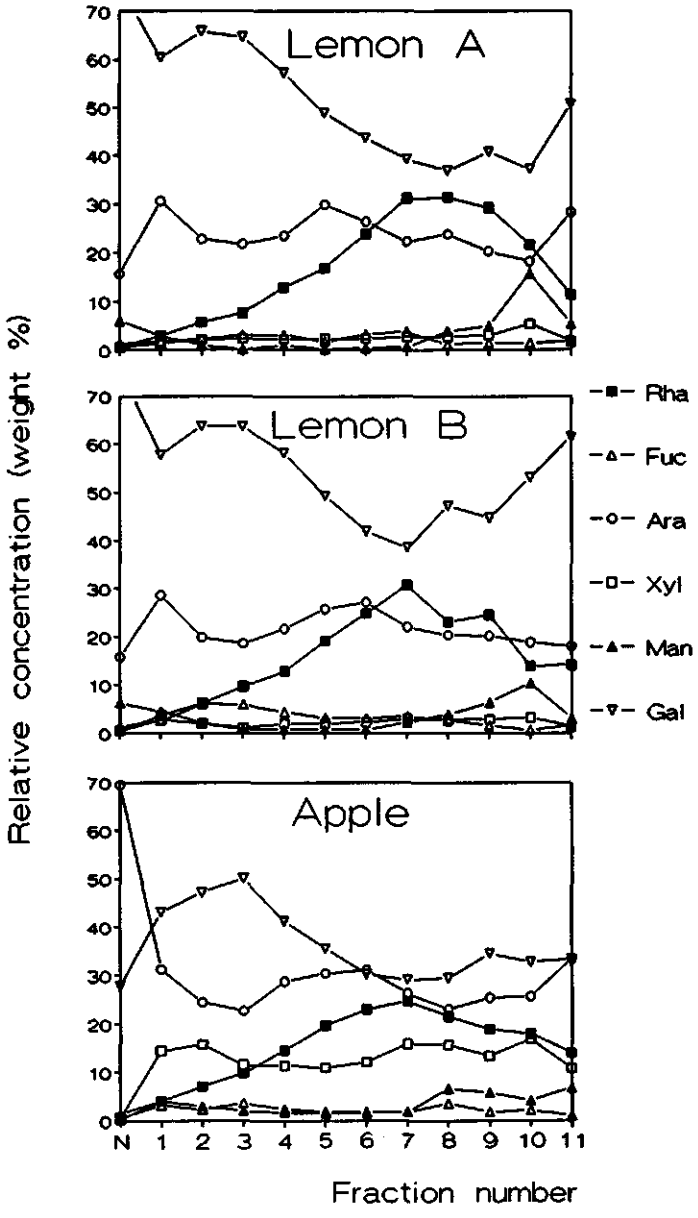

Figure 3: Relative composition of the neutral sugars other than glucose in the fractions obtained by preparative IEC of the three industrial pectin samples as indicated in figure 1.

occurs on the tail of the main peak (fractions 5-7) is characterized by a relatively high total neutral-sugar content. The total neutral-sugar content decreases again in fractions 8 to 10 before increasing dramatically in the fraction eluted by NaOH (fraction 11). This neutral-sugar distribution, particularly clear for the lemon pectins is masked in the apple sample by a rapid increase of the glucose content with increasing ionic strength. Such a neutral-sugar distribution in the IEC chromatograms is very surprising since the charge of the molecules could be expected to decrease with increasing their neutral-sugar content. The high amount of glucose in fraction 11 may be due to the presence of retrograded starch molecules which remained on the top of the column until the pH was high enough to allow their solubilization and their subsequent elution. The high neutral-sugar content of the pectin fractions eluting at low ionic strength (fractions 1) has already been observed for pectins from tobacco (Sun *et al.*, 1987) and cherry fruits (Barbier & Thibault, 1982). It is possible that these fractions contain some free neutral polysaccharides that can bind to the ion exchanger and require some increase of the ionic strength to be released. Indeed, Neukom *et al.* (1960) have been able to fractionate arabans on DEAE-cellulose with phosphate buffer in the range 0.025-0.25M. This indicates that complete removal of neutral polysaccharides cannot be reached by ion exchange chromatography. Copper precipitation, which is more specific for the presence of uronide, should be preferred. It is interesting to note that despite differences of origin and procedure of extraction, we found that in the three industrial pectins the distribution of total neutral sugars was very similar to that reported for other pectin samples (Knee, 1970; Le Quéré *et al.*, 1981; de Vries *et al.*, 1982). Minor discrepancies are probably due to differences in IEC procedures and/or sharpness of the fractions analysed.

A closer examination of the neutral-sugar composition reveals that the relative proportion of the neutral-sugar units also varies in large proportions. Figure 3 shows the relative composition of the neutral sugars other than glucose within the IEC fractions from the three industrial pectins. Without taking into account glucose units, all three pectin samples exhibit very similar distribution of the neutral-sugar units. The proportion of galactose which is the major neutral sugar is maximum in fractions 2 and 3, decreases to a minimum in fractions 7 and 8 and then increases again up to fraction 11. The proportion of arabinose is just opposite to that of galactose and shows maxima in fractions 1 and 5-6. However, it also increases in fraction 11. The proportion of rhamnose units is clearly maximal in the fractions 7 to 9 for the lemon pectins and 6 to 8 for the apple

pectin. The proportion of xylose and fucose seems to be nearly constant except for a sligth minimum in fractions 3 to 6. The proportion of mannose units is clearly higher in the last four fractions with a clear maximum in fraction 10 for the lemon pectins. These data suggest a discontinuous rather than a continuous distribution of neutral-sugar units in industrial pectins.

Distribution of proteinaceous and phenolic compounds

Table 2 shows the distribution of phenolic and proteinaceous compounds among the fractions obtained by fractionation of the three industrial pectins by IEC. Phenolics could not be determined in certain lemon pectin fractions because of the presence of CyDTA (see above) which reacts positively with the Folin-Ciocalteu reagent.

Except for lemon B pectin where a high phenolic content is also found in the first fraction, phenolic compounds appear to coelute mainly with pectin molecules that require a high ionic strength to be released from the IEC column. The apple pectin differs from the lemon pectin samples by a higher phenolic content in fractions 2 to 6. However, in the apple, the amount of phenolic compounds present in the IEC fractions is lower than expected from the total phenol content of the unfractionated sample (Kravtchenko *et al.*, 1992a; chapter 2). Indeed, phenolic compounds seem to bind very strongly to the column (see above) and some of them may not be recovered from the column.

Proteins exhibit a distribution very similar to that of phenolics. Coelution of proteins and phenolics has already been observed in fractions obtained by SEC of the same industrial samples (Kravtchenko *et al.*, 1992b; chapter 4). All the fractions obtained from the apple pectin contain less proteins than the corresponding ones from the lemon pectins. Although the method of estimation is different, these results are in excellent agreement with total protein contents found previously (Kravtchenko *et al.*, 1992a; chapter 2).

Distribution of methoxyl and acetyl esters

Table 2 shows the degree of methoxylation (DM) and the degree of acetylation (DAc) of the fractions obtained by preparative IEC of the three industrial pectins. Since they were eluted by NaOH and therefore at least partly saponified, degrees of esterification of fractions 11 were not determined.

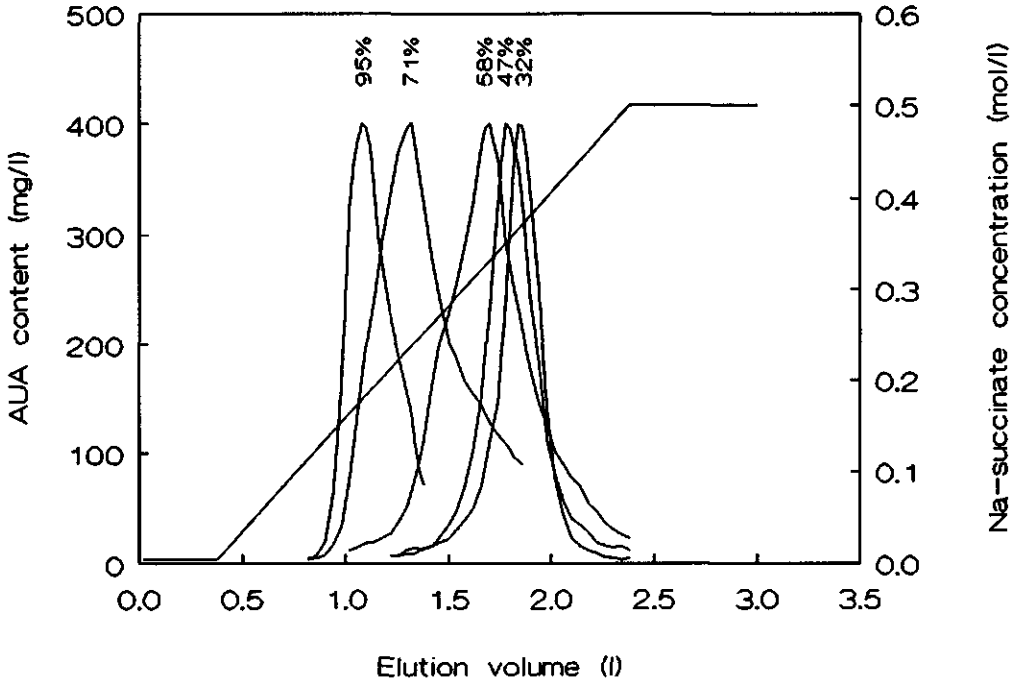

Figure 4: Calibration of the preparative IEC column with pectin samples of different DMs as indicated in percentages.

For comparison, the preparative IEC column has been calibrated using a series of pectins varying in average DM. Since they were produced by acid de-esterification in aqueous solution, the distribution of their free carboxylic groups may be considered to be random (Speiser *et al.*, 1947). Figure 4 clearly shows that standard pectins elute in a rather narrow peak, their elution time increasing with decreasing DM.

As expected, DM of the fractions obtained by IEC of the three industrial samples decreases regularly from fractions 2 to 8. However, except for fractions 2 to 4, DM appears to be lower than expected from the elution volume of the fraction. This indicates that structural features other than the average DM govern the strength of binding to the ion exchanger. Since all these fractions have similar hydrodynamic volumes, some molecular size effect cannot be taken responsible for their elution delay. Anger & Dongowski (1984) suggested that this is due to differences in the distribution of the free carboxyl groups along the pectin

backbone. A blockwise distribution will result in zones of higher charge density which bind strongly to the ion exchanger. Moreover, fractions with a DM very different from that expected were found to have a higher content of phenolics. This is especially true for the two last fractions from the three pectin samples. Binding by a non-ionic mechanism via phenolics may thus explain that some pectin molecules elute later than expected from their average DM. This hypothesis is reinforced by the fact that the fractions from the apple pectin sample which contain more phenolics than those of the lemon pectin fractions have a lower DM. Another possible cause for the elution delay of the pectin fractions has been evidenced during the column calibration. After injection, the pectic acid standard (DM 0%) could not be released from the column even by elution with NaOH. Elution could only be obtained with 0.05M NH_3-oxalate in 0.5M NaOH, indicating that the pectic acid was insolubilized in the column by reaction with polyvalent cations which occur as impurity in buffer solutions. However, since recoveries were close to 100%, this phenomenon is probably negligible for the IEC fractionation of high methoxyl pectin samples. Average DMs have been recalculated for the three pectin samples from the contribution of each of their constituting fractions. For the lemon pectins, recalculated DMs were found to be in good agreement with average DMs measured on the whole samples (Kravtchenko *et al.*, 1992a; chapter 2). For the apple pectin, recalculated DM was found to be 68% although the average DM was 74%. This indicates that the fraction which remained bound to the IEC column is highly esterified.

On the other hand, all the fractions 1 are less methyl-esterified than expected from their order of elution. This is probably due to their high neutral-sugar content (table 2) and/or their small molecular size (figure 3).

For the three pectin samples, DAc increases with increasing elution volume on IEC. The variation is not continuous but seems to progress by steps of increasing DAc: fractions 1-3, 4-9 and 10-11. This particular distribution remains completely unexplained. As expected from average DAc of the whole samples (Kravtchenko *et al.*, 1992a; chapter 2), the fractions from the apple pectin sample are always more acetylated than those of the lemon pectin samples.

CONCLUSION

Large scale preparative IEC revealed that pectic substances from industrial samples varying in charge are heterogeneous with respect to their content of neutral sugars. Such a heterogeneity has already been observed earlier, for instance in

pectins from apple (Neukom *et al.*, 1980; de Vries *et al.*, 1981), sugar-beet (Le Quéré *et al.*, 1981), cherry fruit (Barbier & Thibault, 1982) or tobacco (Sun *et al.*, 1987). Both the total content and the relative composition of the neutral sugars vary discontinuously among pectin fractions varying in charge. It is very interesting to note however that, despite large differences in total neutral-sugar content, the relative proportion of the different neutral-sugar units other than glucose exhibits very similar distributions for the three industrial pectins, irrespective of their botanical origin. Moreover, pectin samples are also heterogeneous with respect to their content of phenolic and proteinaceous compounds. Proteinaceous and phenolic compounds coelute mainly with pectin fractions requiring a high ionic strength to be released from the IEC column.

Surprisingly, the last eluting fractions exhibit a high DM as well as a high neutral-sugar content and are therefore only weakly charged. In fact, the mechanism of ion exchange chromatography of pectic substances appears to be much more complex than generally thought and is still not completely understood. The strength of binding to the column depends on many different parameters such as average DM and distribution of free carboxyl groups, neutral-sugar content, molecular size or phenolic content. Interpretation of the IEC elution patterns is rendered extremely difficult by the complexity of the pectin composition and structure. The problem is also accentuated by the fact that some low methoxylated pectin molecules can precipitate inside the column if calcium ions are present. Non-ionic adsorption via phenolic compounds and/or precipitation with multivalent cations may be responsible for the incomplete recovery of galacturonic acid observed by many investigators (Thibault, 1983; Saulnier & Thibault, 1987; Renard *et al.*, 1991). Release of calcium-pectinate can be achieved by adding a calcium-complexant agent such as oxalic acid or EDTA. Phenolic rich pectin molecules can be released from the ion exchanger by washing the column with $NaClO_2$.

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Chapter 6:

IMPROVEMENT OF THE SELECTIVE DEPOLYMERIZATION OF PECTIC SUBSTANCES BY CHEMICAL β-ELIMINATION IN AQUEOUS SOLUTION

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6. IMPROVEMENT OF THE SELECTIVE DEPOLYMERIZATION OF PECTIC SUBSTANCES BY CHEMICAL β-ELIMINATION IN AQUEOUS SOLUTION

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ABSTRACT

The reaction of β-elimination which splits specifically the glycosidic linkages next to methoxylated galacturonic acid units without steric limitation may be used for the determination of the sequence of methyl esters along pectin molecules. However, because of competitive de-esterification, completeness of the reaction has never been achieved. In order to improve the extent of degradation, the influence of temperature, pH and buffer concentration on the β-elimination reaction in aqueous solutions has been investigated. Examination of kinetics revealed that any increase of temperature increases the rate of the β-elimination reaction more than that of the de-esterification. On the other hand, any decrease of pH decreases the rate of β-elimination but, much less than the rate of de-esterification. Buffer concentration plays an important role by controlling the pH through its buffering capacity as well as the availability of hydroxyl ions. Increasing the temperature up to 115°C and decreasing the pH down to 5.0 improved the splitting of the glycosidic bonds of a high methoxyl pectin backbone from 5.5 to 38% (53% of the glycosidic bonds contiguous to a methoxylated galacturonide unit). In spite of the appearance of various secondary reaction products, the absorbance at 235 nm can be used to estimate the extent of degradation.

INTRODUCTION

Pectin is mainly constituted of linear chains of 1,4-linked α-D-galacturonic acid units. The carboxyl groups of the galacturonic acid units are partially esterified with methanol. Although the average degree of methoxylation can be measured easily (Doner, 1986), the distribution of the methoxyl esters along the polygalactunic acid chain is still largely unknown (Taylor, 1982). The development

of a reaction, splitting specifically and completely the glycosidic bonds adjacent to methoxylated or non-methoxylated galacturonic acid units would provide a unique tool to investigate the sequence of the methyl ester groups along the pectin chains.

Vollmert (1950) stated that glycosidic linkages between galacturonic acid units are broken during the alkaline de-esterification of pectins at room temperature. Kenner (1955), Neukom & Deuel (1958) and Whistler & BeMiller (1958) suggested that the splitting of pectin chains in alkaline solutions results from a base-catalyzed β-elimination reaction (also called "β-dealkoxylation"). Albersheim (1959) observed a similar degradation in hot neutral or weakly acidic conditions, which is an unusual phenomenon for most polysaccharides.

The β-elimination reaction proceeds on uronic acids that possess a glycosidic linkage on C-4 in the β-position of the carboxyl group at C-5. According to this mechanism, the activated hydrogen atom on C-5 is removed by suitable proton-acceptors, leading to unstable, intermediary anions which are stabilized by losing the C-O linkage in the β position (Kiss, 1974). A double bond appears between C-4 and C-5 at the non-reducing end. Evidence from model compound studies (BeMiller & Kumari, 1972) suggests that the elimination does not take place by a concerted E2 process but by an E1cB anionic mechanism. In pectin, since the D-galacturonate residues are in the $^4C_1$ conformation (Deuel & Stutz, 1958; Rees & Wight, 1971), both substituents directly involved in the β-elimination are in the axial position. Therefore, β-elimination of pectin is a diaxial procedure (Kiss, 1974), *i.e.* "trans-elimination". This particular conformation makes the β-elimination reaction of pectin much easier and much faster than the cis (e,a) elimination of other natural polyuronates such as alginate (Albersheim *et al.*, 1960; Haug *et al.*, 1963; BeMiller & Kumari, 1972).

Albersheim (1959) suggested that the susceptibility of pectin to depolymerization by β-elimination is determined by the presence of its esters. Indeed, pectic acid was shown to be quite stable when similarly treated (Albersheim, 1959). Moreover, the β-elimination limit is closely related to the degree of methyl esterification of the pectin (Albersheim *et al.*, 1960, Rombouts & Thibault, 1986). The β-elimination reaction which depends on the presence of an ester group on galacturonic acid residues may thus be used for the specific depolymerization of the pectin backbone leading to the determination of the distribution of the acid groups (Henfrey, 1973).

This reaction performed in 0.1M Na-phosphate buffer at pH 6-6.8 has been used by several investigators (Barrett & Northcote, 1965; Stoddart *et al.*, 1967; de Vries *et al.*, 1983; Rouau & Thibault, 1984; Rombouts & Thibault, 1986). In such conditions, β-elimination is never complete because of a competitive reaction of de-

esterification (Albersheim *et al.*, 1960; Rouau & Thibault, 1984). This partial depolymerization provided valuable information about the distribution of the side chains in pectins and led to the identification of "hairy" and "smooth" regions (Barrett & Northcote, 1965; Stoddart *et al.*, 1967) as well as valuable information about structural feature of the neutral side-chains of pectins (de Vries *et al.*, 1983; Kiyohara *et al.*, 1989) but was not sufficient to draw any conclusion about the distribution of methyl esters along the galacturonic acid chains.

β-elimination can also be catalyzed without de-esterification by enzymes called "trans eliminases" or "lyases" (Pilnik & Rombouts, 1981), but complications due to steric limitation occur: *e.g.* neither pectin lyase nor pectate lyase can degrade pectin "hairy regions" (de Vries *et al.*, 1982). Complete degradation is further limited by the fact that pectolytic enzymes require a complex binding subsite to recognize their catalytic site (Rexova-Benkova & Markovic, 1976): *e.g.* pectin lyase does not degrade esters of oligogalacturonides smaller than 4 units nor the two glycosidic bonds nearest the reducing end (Edstrom & Phaff, 1964), exopectate lyase from *Clostridium multifermentans* attacks the reducing end of the molecule where two units are cleaved at a time until any esterified group is encountered, when reaction stops (McMillan *et al.*, 1964; Voragen, 1972)... Moreover, pure enzymes are not always available. The chemical method which is thought to be not limited by steric hindrance (Rouau & Thibault, 1984) thus constitutes a prime method of obtaining the selective depolymerization of the pectin backbone.

Some factors of the chemical β-elimination reaction have already been studied. At pH 6.8, Albersheim *et al.* (1960) determined an approximative Q_{10} of 3.5 between 50 and 95°C, demonstrating the strong temperature dependence of β-elimination. Keijbets & Pilnik (1974) stated that the cations Ca, Mg and K and the anions citrate, malate, phytate and chloride stimulate β-elimination at pH 6.1 and 100°C. The rate of β-elimination increases with increasing the pH because hydroxyl ions initiate the reaction (Neukom & Deuel, 1958) but the rate of de-esterification is also greatly increased. On the other hand, Doesburg & Grevers (1960) observed that, at pH 4.5-5.5 a marked decrease in the gelling power (*i.e.* degradation) of pectinic acid can be achieved without any appreciable decrease in the degree of esterification.

The β-elimination reaction can be followed by various methods. Since β-elimination results in a depolymerization, Albersheim *et al.* (1960) measured the drop of viscosity of pectin solutions. The decrease in molecular size can also be measured by HPSEC (Deckers *et al.*, 1986). However, as the depolymerization progresses, any new splitting of the polymer chain produces only a slight decrease

in molecular size. It is thus difficult to quantify accuratly the extent of degradation of highly degraded samples by evaluating the degree of polymerization. The periodate-thiobarbituric acid (TBA) test has been used (Albersheim *et al.*, 1960; Rombouts, 1972; Keijbets *et al.*, 1976) to detect unsaturated uronides produced by the β-elimination reaction. This test is very sensible and specific but, the molar extinction coefficient varies with the chain length (Voragen, 1972). Conjugation of the double bond with the carboxyl group on C-5 in the reaction products produces a strong absorption in UV at 235 nm.

In this paper, the effects of pH, temperature and buffer concentration have been investigated in order to reduce the unexpected de-esterification and thus increase the extent of degradation by chemical β-elimination.

EXPERIMENTAL

Pectin

An industrial "rapid set" pectin from lemon peels (lemon A, Sanofi Bio Industries, France) with an uronide content of 84.2%, and a degree of methoxylation of 71.8% was used as substrate.

β-elimination

A fresh aqueous pectin solution (10 mg/ml) was mixed with an equal volume of the appropriate buffer solution. When necessary, the pH was corrected by addition of 0.1M NaOH. Solutions were immediately divided into a series of sealed tubes and heated in a thermostated oil-bath for various times. Anhydrogalacturonic acid (AUA, MW = 176) content was determined in each final pectin solution by using the automated *meta*-hydroxydiphenyl reaction (*m*hdp, Thibault, 1979).

Extent of degradation

Appearance of unsaturated uronide residues was determined by measuring the absorbance at 235 nm after appropriate dilution (20-60 fold). Extent of

degradation (ED) is defined as the fraction of degradable glycosidic bonds that have been broken, degradable bonds being those contiguous to a methoxylated galacturonide unit. It was calculated using the following relation:

$$ED\ (\%) = \frac{A_{235} \times d / k}{AUA \times DM / 100} \times 100$$

where A_{235} is the absorbance at 235 nm, d the dilution factor, k the molar extinction coefficient ($l.mol^{-1}.cm^{-1}$), AUA the anhydro galacturonic acid (MW=176) content ($mol.l^{-1}$) and DM the degree of methoxylation (%).

High Performance Size Exclusion Chromatography

HPSEC was performed with a series of Biogel TSK columns 40XL, 30XL and 20XL (300x7.5 mm) being used in combination with a Biogel TSK guard column (75x7.5 mm). Columns were eluted with 0.4M Na-acetate buffer pH 3.0 at a flow rate of 0.8 ml/min and at 30°C. Detection was performed with a Shodex SE 61 refractive index detector at 40°C.

Enzymic β-elimination

Enzymic β-elimination was performed with an endopectin lyase (EC 4.2.2.10, type 2, van Houdenhoven, 1975) purified from a commercial enzyme preparation (Ultrazym, Gist Brocades, NL). 5 mg/ml pectin solution was incubated with pectin lyase (5 U/g pectin) in 0.1M Na-acetate buffer pH 4.2 at 30°C for 24 hours.

RESULTS AND DISCUSSION

The effects of temperature, pH, buffer concentration and time of treatment on β-elimination have been investigated in order to optimize the reaction of β-elimination. In the light of published data (see introduction) it was decided to explore the following conditions:

- temperature: 95, 105 and 115°C,
- pH: 5.0, 5.4, 5.7 and 6.0,

Na-citrate was chosen as buffering agent from those tested by Keijbets &

Pilnik (1974) because of its stimulating effect on β-elimination. Moreover, this buffer solution has proved to be perfectly stable (pH, color and UV spectrum) on heating at 115°C for 3 days. As the stability of the pH during the reaction depends on the strength of the buffer, kinetic studies would have required the use of highly concentrated citrate solutions. However, high salt concentration would cause problems for further characterization of the degradation products and we have tried to reduce it as much as possible. We have investigated the concentrations 0.01, 0.05, 0.1 and 0.5 mol/l.

All combinations of these parameters have been treated to reveal possible interactions. Thus, 48 different conditions were tested. Samples treated in 0.5M buffer could not be analyzed because pectin precipitated very soon on heating.

Double bonds produced by the reaction of β-elimination were quantified by reading the absorbance at 235 nm (Albersheim *et al.*, 1960). Edstrom & Phaff (1964) found a molar extinction coefficient of 5,500 $l.mol^{-1}.cm^{-1}$ for unsaturated methyl galacturonides but slightly different values were found for non-esterified unsaturated oligo-uronic acids (Voragen, 1972; Nagel & Wilson, 1969). We have measured the molar extinction coefficient of non-methoxylated unsaturated digalacturonic acid and we have found a value of 5,610 $l.mol^{-1}.cm^{-1}$ which is not significantly different from that obtained by Edstrom and Phaff (1964). Thus, a molar extinction coefficient of 5,500 $l.mol^{-1}.cm^{-1}$ has been used for all the calculations.

As first trials, degradations in the various conditions described above were performed during 24 hours.

Extent of degradation after 24 hours of treatment

The ED after 24 hours (ED_{24}) increases clearly with the temperature (fig. 1), confirming the findings of Albersheim *et al.* (1960) at lower temperatures. The best results of depolymerization were thus obtained at the highest temperature tested (*i.e.* 115°C). On average, ED_{24} increased by a factor 3 between 95 and 105°C and by a factor 1.8 between 105 and 115°C. At high buffer concentration, the positive effect of raising the temperature decreases with increasing pH. For instance, at buffer concentration 0.05M, ED_{24} increases by a factor 5.8, 3.3, 2.5 and 2.1 between 95 and 115°C at pH 5.0, 5.4, 5.7 and 6.0, respectively. In contrast, at lower buffer concentration (0.01M), ED_{24} is seen to increase by increasing pH (fig. 1)

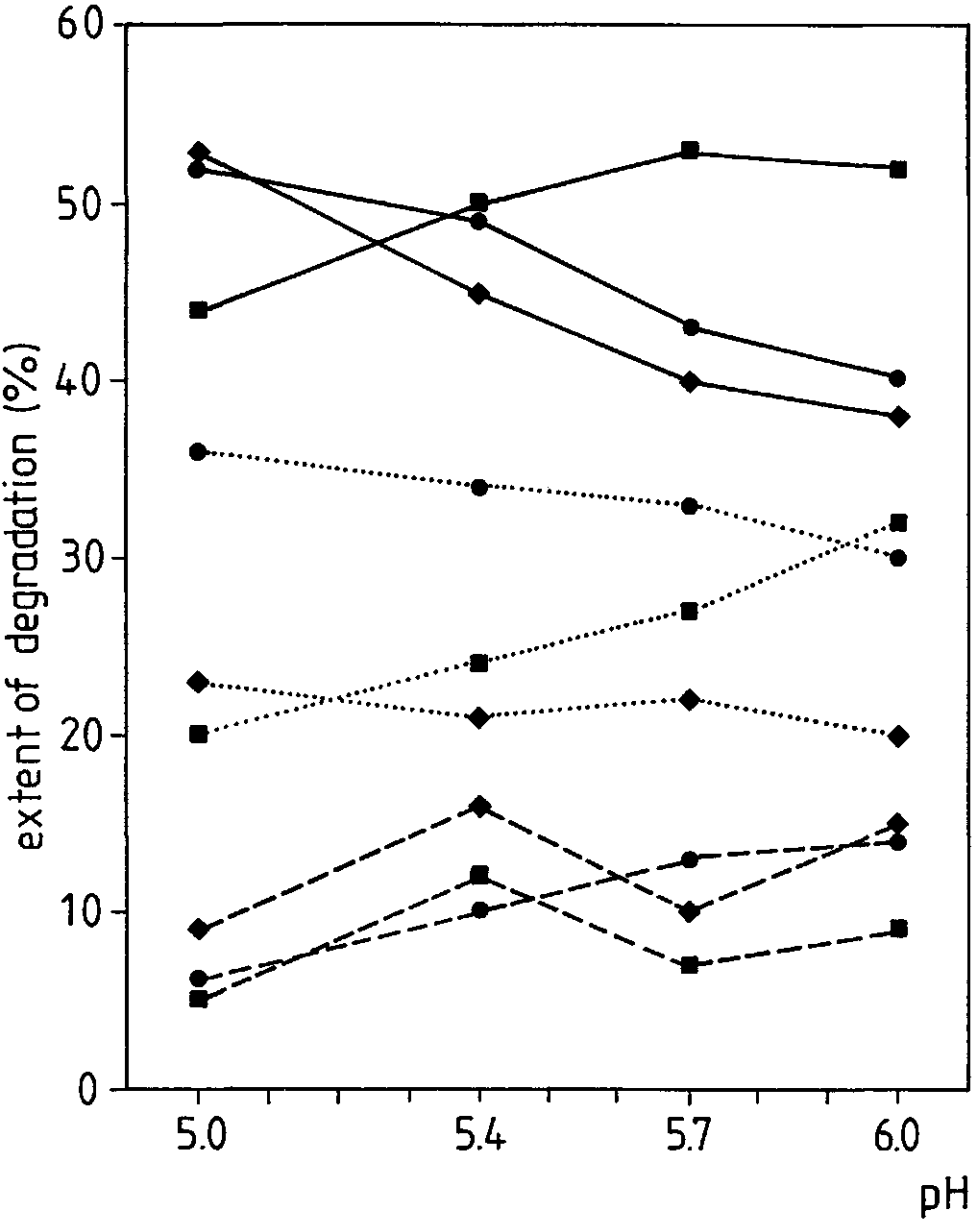

Figure 1: Extent of degradation after 24 hours of treatment in various conditions of pH, buffer concentration (■, 0.01M; ●, 0.05M; ◆, 0.1M) and temperature (– –, 95°C; ···· , 105°C; — , 115°C). Degradation is expressed as the percentage of split glycosiduronic linkages of all glycosiduronic acid linkages situated next to an esterified galacturonic acid unit.

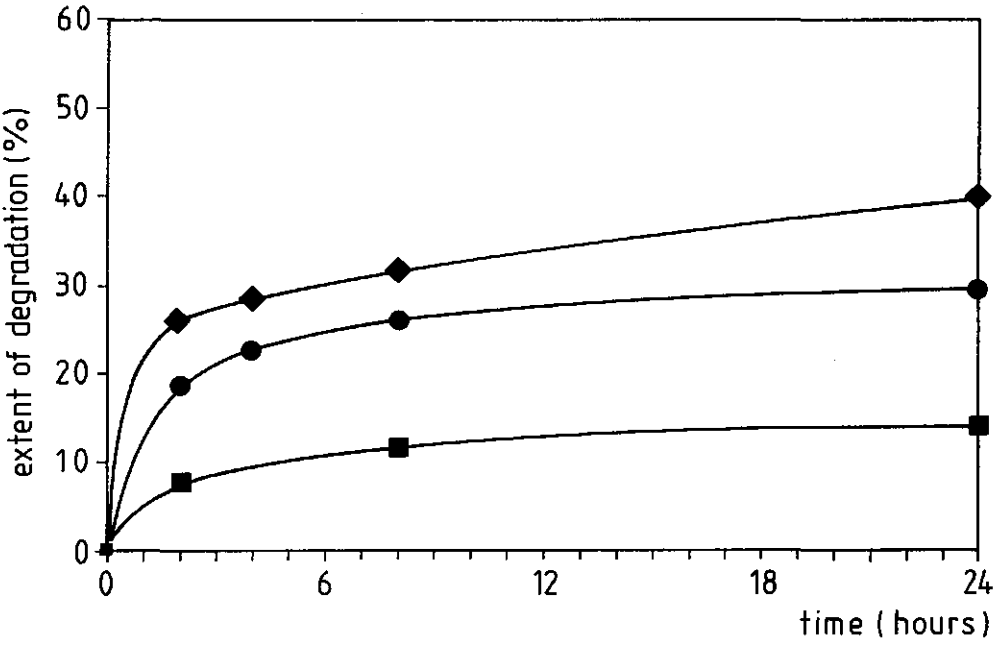

Figure 2: Influence of the temperature on the rate of depolymerisation by β-elimination in 0.05M Na-citrate buffer at pH 6.0:■, 95C°;●, 105°C;◆, 115°C.

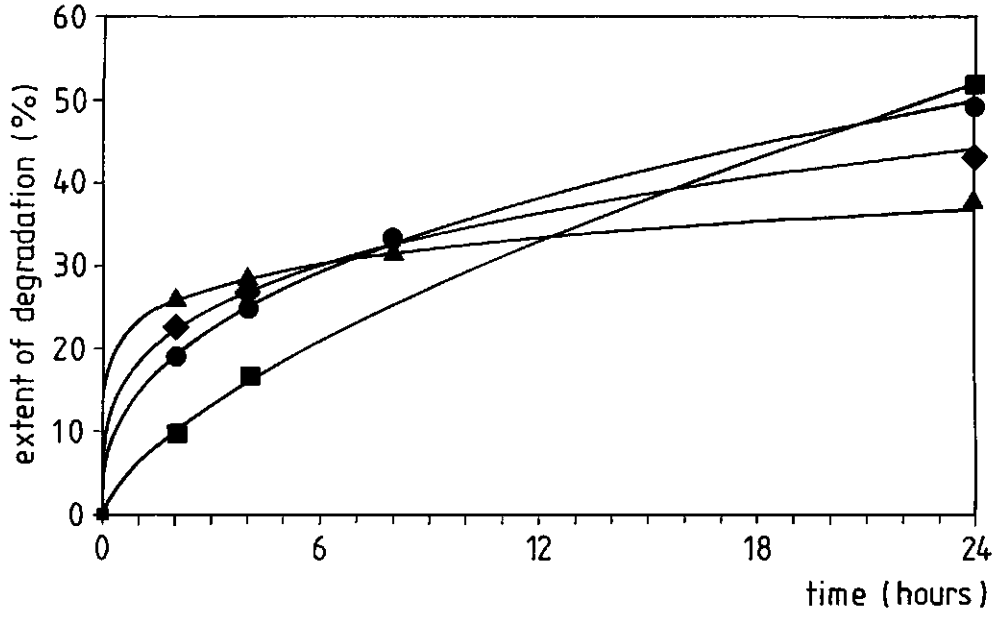

Figure 3: Influence of the pH on the rate of depolymerisation by β-elimination in 0.05M Na-citrate buffer at 115°C: ■, pH 5.0; ●, pH 5.4; ◆, pH 5.7; ▲, pH 6.0.

The effect of the buffer concentration on ED_{24} is much more complex. Indeed, it is very dependent on the level of the other factors. Especially, we already pointed out the very clear interaction that occurs between the pH and the buffer concentration.

By raising the temperature up to 115°C, and decreasing the pH below 6, we obtained EDs higher than 50% after 24 hours of treatment. For comparison, the same pectin sample has also been treated in 0.1M Na-phosphate buffer pH 6.8 at 80°C for 24 hours, and by pectin lyase (5 U/g, 24 hours). EDs were 8 and 27% for chemical and enzymic β-elimination, respectively.

The effect of the various factors tested appeared to be very complex, and we have therefore examined the rates of depolymerization.

Rates of depolymerization

Rates of reaction for the various sets of conditions have been observed up to 24 hours. All reaction rate curves display a similar general shape. They all tend to a plateau at which the reaction of β-elimination stops. It is likely that this limitation is due to the competitive reaction of de-esterification (Albersheim *et al.*, 1960). The level of the plateau can be interpreted as the ratio of the rate of de-esterification to the rate of β-elimination. This level decreases with increasing the ratio de-esterification/β-elimination. The kinetic curves also differ in their slope at the origin. At the beginning of the reaction, de-esterification has not removed enough methyl esters to limit the reaction of β-elimination. Therefore, it can be assumed that the slope at the origin is directly related to the rate of β-elimination.

When the temperature is raised from 95 to 115°C, the β-elimination reaction is accelerated (fig. 2). Although the rate of de-esterification is also probably increased, the higher the temperature, the closer the plateau comes to 100%. Thus, any increase of temperature favoures β-elimination more than de-esterification.

When the pH is raised from 5 to 6, the reaction starts much faster (fig. 3), confirming that β-elimination is favoured by an increase of pH (Neukom & Deuel, 1958) but, the reaction also stops much earlier. Indeed, de-esterification is much more favoured by an increase of pH between 5 and 6 than β-elimination.

The buffer concentration does not exhibit such clear effects. It seems that the depolymerization occuring at high pH and low buffer concentration behaves more like those occuring at low pH and high buffer concentration (fig. 4).

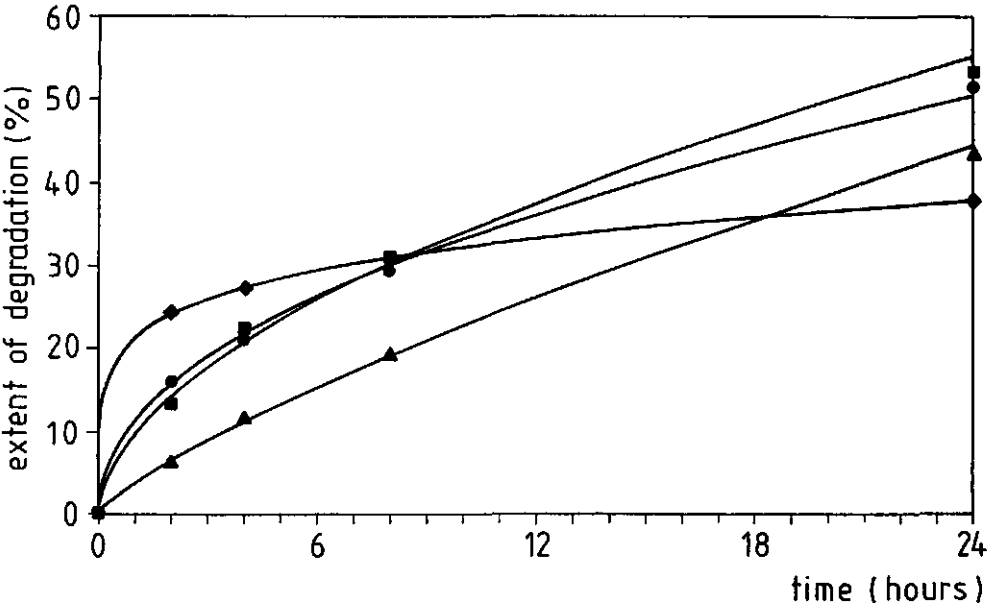

Figure 4: Influence of the pH and the buffer concentration on the rate of depolymerisation by β-elimination at 115°C: ■, 0.1M, pH 5.0; ●, 0.01M, pH 5.0; ◆, 0.1M, pH 6.0; ▲, 0.01M, pH 6.0.

During the reaction, the pH of the solutions has been measured after cooling: after 24 hours of treatment in 0.01M buffer, the pH dropped to 4.4 when starting from 5.0 and to 4.8 when starting from 6.0, whereas it remained almost constant in 0.1M buffer. Consequently, when the initial pH is high and the buffer concentration low, the pH-drop decreases the rate of de-esterification and the extent of degradation is much higher. On the other hand, when both the initial pH and the buffer concentration are low, the pH-drop induces slowing down of the β-elimination reaction. The drop of pH may be due to de-esterification of pectin which release free carboxylic acids. Besides, the drop of pH is smaller at low pH values that are less favourable to de-esterification.

Since salt concentration probably affects the rate of ß-elimination (Keijbets & Pilnik, 1974) only slightly, it seems that buffer concentration only influences the extent of degradation by controlling the pH via the buffer capacity. However, the buffer concentration also influences the speed of de-esterification because neutral salts decrease the electrical potential of the molecules (Deuel & Stutz, 1958).

Examination of reaction rate curves shows that at low pH and/or low buffer

concentration, even at high temperature, the trans-elimination reaction is still proceeding after 24 hours, suggesting that there are still suitable reaction sites available, *i.e.* esterified galacturonide residues.

Reliability of the calculated extent of degradation

Upon continued heating, the pectin solution received a dark colour, possibly due to unsaturated compounds of increased conjugation (Albersheim *et al.*,

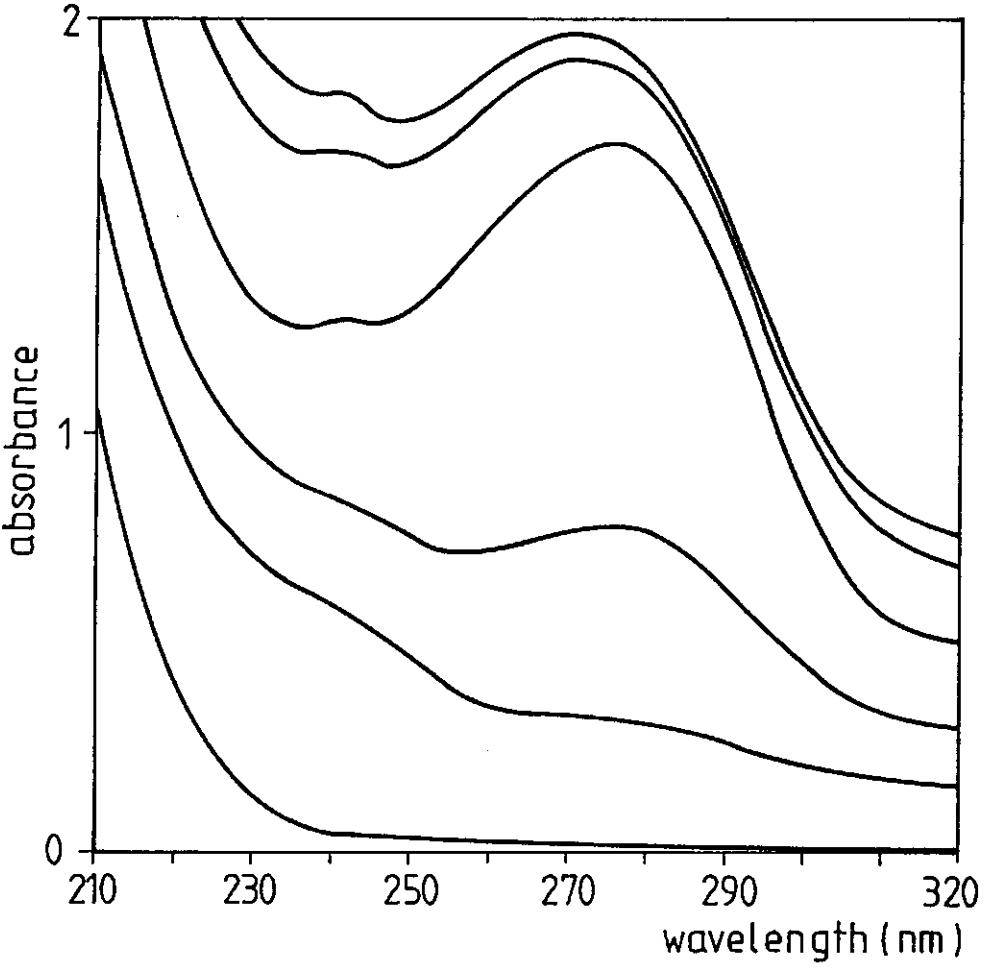

Figure 5: Changes in the UV spectrum of the reaction products of chemical β-elimination after 0, 4, 8, 24, 48 and 72 hours of treatment at pH 5 and 115°C.

1960). Moreover, unsaturated oligogalacturonides produced by the β-elimination reaction are very unstable. Depending on the pH, their degradation produces 2-furoic acid, 5-formyl-2-furoic acid and a number of non-identified reaction products (Voragen *et al.*, 1988). Indeed, on prolongated heating, a second absorbance peak, probably due to these secondary reaction products appears at 265-270 nm (fig. 5). Henfrey (1973) suggested that the increase in absorbance at 235 nm may partly be due to an overlap of this second peak. Pectin depolymerisation in certain conditions has also been monitored by HPSEC. It appears (fig. 6) that samples treated with pectin lyase, *i.e.* in very mild conditions of temperature and pH elute much later on HPSEC than samples of similar ED degraded by chemical β-elimination. Even if a difference in elution time at the top of the peak does not imply a large difference in degree of polymerization, it seems that the appearance of secondary reaction products leads to an over-estimation of the true extent of degradation. On the other hand, Voragen *et al.* (1988) showed that the unsaturated trimer and dimer are degraded to the corresponding saturated

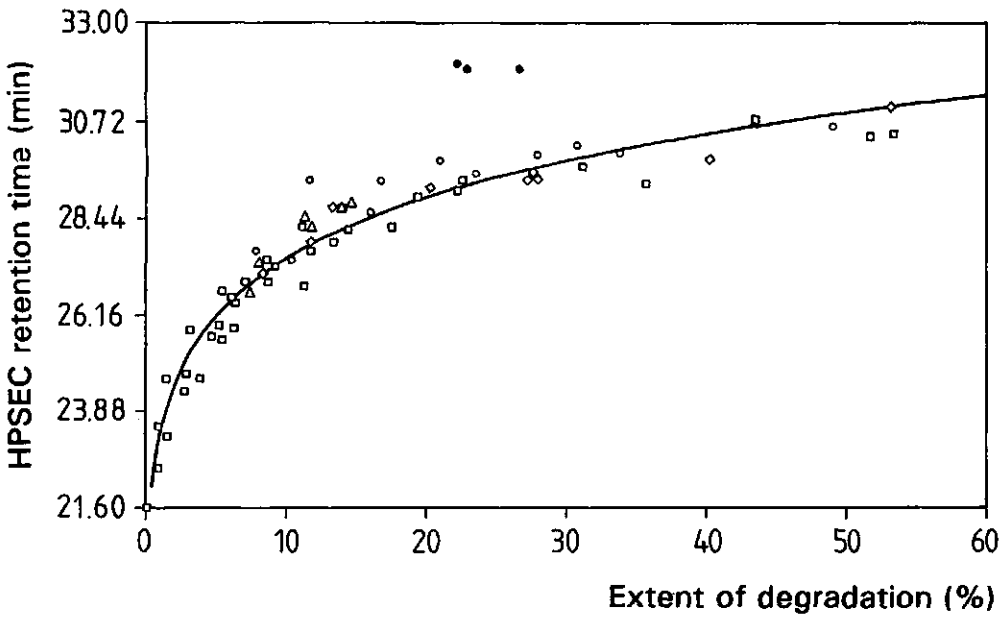

Figure 6: Relationship between the extent of degradation calculated on the basis of the absorbance at 235 nm and the HPSEC elution time at the top of the peak for samples treated in various conditions of temperature, buffer concentration and pH: □, pH 5.0; o, pH 5.4; ◇, pH 5.7; △, pH 6.0; ●, sample treated with pectin lyase.

dimer and monomer, next to the other reaction products. We observed that the absorbance at 235 nm of a solution of unsaturated digalacturonide decreases on heating at 115°C and pH 5. Disappearance of 4,5-unsaturated units leads to an under-estimation of the true extent of degradation. Thus, it is very unlikely that the ED calculated on the basis of the absorbance at 235 nm measures accuratly the real extent of degradation. Nevertheless, calculated ED can be accepted as a good indicator of the extent of reaction, since both secondary reactions which may alter its validity are closely related to the amount of unsaturated galacturonide units. This assessment is demonstrated by the clear correlation existing between ED and HPSEC elution time of chemically treated samples (fig. 6).

CONCLUSION

Systematic study of the rates of reaction between 95 and 115°C and from pH 5.0 to 6.0 in buffers of various concentrations showed that both β-elimination and competitive de-esterification are favoured by increasing the temperature and increasing the pH. However, any increase of temperature favoures β-elimination more than de-esterification and any increase of pH favours de-esterification more than β-elimination. Thus, to increase the extent of degradation, it is advantageous to increase the temperature and decrease the pH. The buffer concentration must be high enough to avoid any change of the pH during the reaction.

Within the conditions that we have tested, it can be recommended to perform the β-elimination reaction in 0.1M Na-citrate buffer pH 5.0 at 115°C. In such conditions we could split 38% of the glycosidic bonds of a high methoxyl pectin galacturonan chain after 24 hours of treatment. For comparison, the same pectin sample treated in the usual conditions (80°C, pH 6.8, 24 hours) could only be degraded up to 5.5%.

The rate of reaction in such conditions showed that the reaction can still be improved by increasing the reaction time. Moreover, it seems that high ionic strength stimulates the reaction of de-esterification (Deuel & Stutz, 1958). The extent of degradation may thus still be improved by the use of lower concentration of a buffer of higher capacity at pH 5.0.

This technique has been applied for the specific depolymerization of three industrial high methoxyl pectins differing in physical behaviour. The results obtained will be presented in a following paper.

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Chapter 7:

ENZYMIC AND CHEMICAL DEGRADATION OF SOME INDUSTRIAL PECTINS

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7. ENZYMIC AND CHEMICAL DEGRADATION OF SOME INDUSTRIAL PECTINS

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ABSTRACT

Three industrial high methoxyl pectins purified by copper precipitation have been degraded by acid, chemical β-elimination and endopolygalacturonase. Degraded pectins from β-elimination and enzyme treatments have been fractionated by preparative SEC and the resulting fractions have been analysed for their neutral-sugar, protein and phenolic contents. Distribution of neutral sugars indicates that the model of alternating "hairy" and "smooth" regions is also valid for industrial pectins. Most of the phenolics present in the copper purified samples are probably not bound to the pectin molecules except for a small proportion which was found to be associated with the segments rich in neutral sugars. Proteins are associated with the hairy regions. Relative proportions of the oligomers with DP up to 17 produced by the action of endoPG and analysed by high performance ion exchange chromatography were compared with a theoretical distribution model. The abundance of mono-, di- and trigalacturonides suggests that, in the smooth regions of industrial pectins, free carboxyl groups are present as blocks of non-esterified galacturonide units. HPSEC elution patterns of acid-degraded samples suggest that homogalacturonan blocks of average DP 40-60 are interspaced by rhamnose units. Degradations by β-elimination and endoPG indicate that most of the rhamnose units are concentrated in the regions carrying the side chains. This would mean that the pectin backbone is constituted of rhamnogalacturonan regions alternated with homogalacturonans regions, the homogalacturonan regions being interrupted at regular intervals by rhamnose units.

INTRODUCTION

In order to explain differences in the physical behaviour of three industrial high methoxyl pectins, the nature of the structural constituting units (Kravtchenko *et al.*, 1992a; chapter 2) as well as their distribution among the pectin molecules

(inter-molecular distribution, Kravtchenko *et al.*, 1992c; chapter 4; Kravtchenko *et al.*, 1992d; chapter 5) have been investigated. For instance, the inter-molecular distribution of the methyl esters was shown to be important in determining the reactivity of pectin molecules towards calcium ions (Kravtchenko *et al.*, 1992c; chapter 4). However, differences in physical behaviour may also depend on the sequence in which the various structural units are arranged (intra-molecular distribution). The specific splitting of glycosidic bonds and the subsequent analysis of the reaction products is still one of the most efficient means to provide information for the sequences in which pectin constituents are linked (Aspinall, 1977).

Acid hydrolysis has been used for the selective degradation of pectic substances (Aspinall *et al.*, 1967a, 1967b; Morris *et al.*, 1980; Powell *et al.*, 1982; Durand *et al.*, 1990), using the fact that the different glycosidic linkages are hydrolysed at different rates. Glycosidic linkages at C-1 of neutral sugars, especially those of furanosides are hydrolysed very readily whereas glycosiduronic acid linkages are highly resistant to acidic conditions (Aspinall, 1970; Fry, 1988). Under appropriate conditions of hydrolysis it is therefore expected to degrade totally the neutral-sugar side-chains as well as the pectin backbone at all insertions of a rhamnose unit without affecting the homopolygalacturonan sequences.

Neukom & Deuel (1958) discovered that linkages between galacturonic acid units are subjected to depolymerization in hot alkaline or weakly acidic conditions following a reaction of β-elimination. In natural pectins, this reaction has been shown to depend on the presence of an ester group on the C-6 next to the glycosidic bond (Albersheim, 1959) and may therefore be used for the specific depolymerization of the galacturonan backbone according to the distribution of their methyl ester groups.

Specific depolymerization of the polygalacturonic acid chain can also be achieved by using purified pectolytic enzymes. Polygalacturonase hydrolyses glycosidic linkages of low methoxylated pectins. Pectin lyase and pectate lyase split the glycosidic bonds of high methoxyl and low methoxyl pectins, respectively, by a mechanism of β-elimination. Properties as well as purification procedures of pectolytic enzymes have been particularly well reviewed (Rexova-Benkova & Markovic, 1976; Pilnik & Rombouts, 1979, 1981). Endopolygalacturonase (endoPG) has been used to extract defined pectic fragments from cell materials (Talmadge *et al.*, 1973; Knee *et al.*, 1975; Ishii, 1976; Renard *et al.*, 1991) and allowed Darvill *et al.* (1978) to isolate rhamnogalacturonan II, a very complicated pectic polysaccharide from suspension-cultured sycamore cell-wall. EndoPG has also been used to degrade isolated pectins (Hatanaka & Ozawa, 1969; Kikuchi &

Sugimoto, 1976; Thibault, 1983; Saulnier & Thibault, 1987; Rombouts & Thibault, 1986). The problem is that conclusions about the distribution of the bonds broken can only be drawn when the knowledge of the mode of action of the enzyme used is sufficient. In fact, the activity of pectolytic enzymes does not depend only on the presence or the absence of methoxyl esters on the galacturonan backbone. However, the information we get from the study of enzymic degradation is not limited to that obtained from the characterization of the low molecular weight cleavage products. Significance must also be attached to the resistance of certain fragments (Aspinall, 1970).

This report deals with the characterization of the degradation products obtained from the specific depolymerization of three purified industrial pectins by acid hydrolysis, chemical β-elimination and enzymic degradation with endopolygalacturonase.

MATERIAL AND METHODS

Pectin Samples

Three unstandardized industrial pectins were obtained from Sanofi Bio Industries (France): two from lemon peels (lemon A and B) and one from apple pomace extracted by the same hot acid industrial process, in the same factory. They have been purified by copper precipitation as described previously (Kravtchenko *et al.*, 1992a; chapter 2). Their chemical composition has been extensively described in a previous paper (Kravtchenko *et al.*, 1992a; chapter 2).

Polygalacturonic acid was obtained from Fluka AG (Switzerland).

Enzymic degradation

Highly purified endopolygalacturonase (endoPG, E.C. 3.2.1.15) was isolated from a preparation of *Kluyveromyces fragilis* as described by Versteeg (1979). Enzyme activity is expressed in units, one unit being the amount of enzyme which splits one μmol of glycosidic bond per min under defined conditions. Enzyme activity was assayed by the formation of reducing groups under the conditions used for the pectin degradation (see below). The enzyme preparation was devoid of activity of pectin lyase, pectate lyase and pectinesterase.

2 mg/ml pectin solutions in 0.05M NH_3-acetate buffer at pH 4.2 were treated with 0.03 U/ml of endoPG at 30°C for 24 hours. Extent of hydrolysis was evaluated by measuring the appearance of reducing end groups.

β-elimination

β-elimination was achieved by heating pectin solutions (5 mg/ml) in 0.2M NH_3-carbonate/HCl buffer pH 6.8 at 80°C for 8 hours.

Resulting 4,5-unsaturated galacturonide residues were determined spectrophotometrically at 235 nm, assuming a molar extinction coefficient of 5,500 $mol^{-1}cm^{-1}$ for the unsaturated products (Kravtchenko *et al.*, 1992b; chapter 6).

Acid hydrolysis

Pectins were heated in 0.5M TFA at 100°C for various times. After the treatment, TFA was evaporated under a stream of air and degradation products were redissolved in distilled water before HPSEC analysis.

Preparative Size Exclusion Chromatography

Pectin degradation products (200 mg) were fractionated by chromatography on a column (100x2.5 cm) of Sephacryl S200 gel (Pharmacia, Sweden). The column was eluted at 40 ml/h with 0.2M NH_3-acetate buffer pH 4.0. Retentions were expressed as a function of the partition coefficient K_{av}. The void (V_0) and the total (V_t) volumes were determined using undegraded lemon B pectin and galacturonic acid, respectively. 7.5 ml fractions were collected, assayed for their contents of galacturonic acid and total neutral sugars, and pooled. Pools were directly freeze-dried prior to further analysis.

Volatile buffers were used for pectin degradation and preparative fractionation in order to avoid dialysis.

High Performance Size Exclusion Chromatography (HPSEC)

Degraded pectins or fractions from the Sephacryl S200 column were injected on a series of Biogel columns TSK 40 XL, 30 XL and 20 XL (300x7.5

mm) being used in combination with a Biogel TSK XL guard column (75x7.5 mm). Elution was performed with 0.4M Na-acetate buffer pH 3.0 at a flow rate of 0.8 ml/min at 30°C. Elution products were detected with a Shodex SE 61 refractive index detector at 40°C.

High Performance Ion Exchange Chromatography (HPIEC)

High performance ion exchange chromatography of the degradation products was performed with a Dionex BioLC system (Dionex, Sunnyvale, CA) equipped with a Dionex Carbopac PA-1 column (250x4 mm). After sample injection, the column, pre-equilibrated with 0.1M NaOH, was eluted with two successive linear gradients of NaAc in 0.1M NaOH (0.35-0.7M, 35 min and 0.7-1M, 5 min), washed for 5 min with 1M NaAc in 0.1M NaOH and then re-equilibrated for 15 min with 0.1M NaOH. The flow rate was 1 ml/min. Detection was made with a PAD II pulsed-amperometric detector (Dionex, Sunnyvale, CA) equipped with a gold working-electrode and an Ag/AgCl reference electrode. The following pulse potentials and durations were used: $E_1=0.1V$, $t_1=500ms$; $E_2=0.6V$, $t_2=100ms$; $E_3=-0.6V$, $t_3=100ms$.

Analytical methods

Galacturonic acid and total neutral sugars (expressed as arabinose) were determined by the automated *m*-hydroxydiphenyl (*m*hdp, Thibault, 1979) and orcinol (Tollier & Robin, 1979) methods, respectively. Neutral-sugar content was corrected for interference of galacturonic acid with the orcinol assay.

Reducing-end groups were determined by the Nelson-Somogyi method (Spiro, 1966) with galacturonic acid as standard.

Neutral sugars were determined by GLC as described previously (Kravtchenko *et al.*, 1992a; chapter 2).

Degree of methoxylation (DM) was determined by HPLC with the method of Voragen *et al.* (1986) modified by Kravtchenko *et al.*, (1992c; chapter 4).

Total phenols were estimated with the Folin-Ciocalteu reagent as described elsewhere (Kravtchenko *et al.*, 1992c; chapter 4).

Protein content was evaluated by the Sedmak & Grossberg assay (1977) using micro-titer plates as described by Rylatt & Parish (1982) and bovine serum albumin as standard.

RESULTS AND DISCUSSION

Acid hydrolysis

Acid hydrolysis of pectins was performed in 0.5M TFA at 100°C in order to determine the distribution pattern of rhamnose units in the main chain. Under these conditions, the polyuronate chain sequences would be expected to remain intact whereas Rha-GalA linkages should be completely hydrolyzed (Fry, 1988).

HPSEC analysis of the degradation products after different times of acid treatment (figure 1) shows the appearance of a peak with narrow distribution at a retention time of *ca.* 27.5 min which is particularly visible after 5-16 hours of treatment. This peak can be ascribed to homopolygalacturonic acid sequences. Its narrowness suggests that rhamnose residues interrupt regularly polygalacturonic acid chains of nearly constant length. This peak elutes at the same retention time and with the same distribution pattern as the polygalacturonic acid obtained from

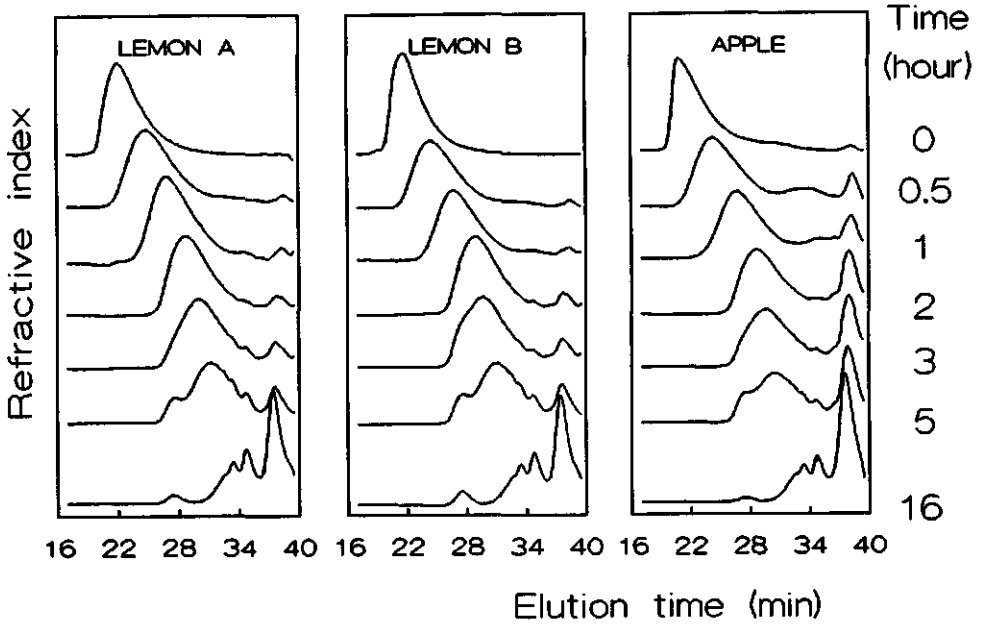

Figure 1: Change in the HPSEC elution pattern of the three purified pectin samples under various times of heating at 100°C in 0.5M TFA.

Fluka. Dortland (1972) used the method of Rombouts *et al.* (1970) to determine the number average DP of several commercial polygalacturonic acid preparations. Since they are probably all prepared by the same way (*i.e.* acid hydrolysis of pectin), they were found to have an average DP in the range 40-60. We therefore assumed that the polygalacturonic acid that we used has an average DP of about 40-60. From similar acid degradations of other pectin samples, galacturonic acids were found to be arranged in sequences of 25 (Powell *et al.*, 1982) to 50 monomers (Durand *et al*, 1990).

The peak, presumed to consist of homogalacturonate segments, was slowly degraded into oligomeric fragments of very low molecular weight without formation of fragments of intermediate size. A similar phenomenon was observed during the treatment of polygalacturonic acid (not shown). This suggests that homogalacturonan segments are slowly degraded in acid conditions by a reaction affecting only chain extremities.

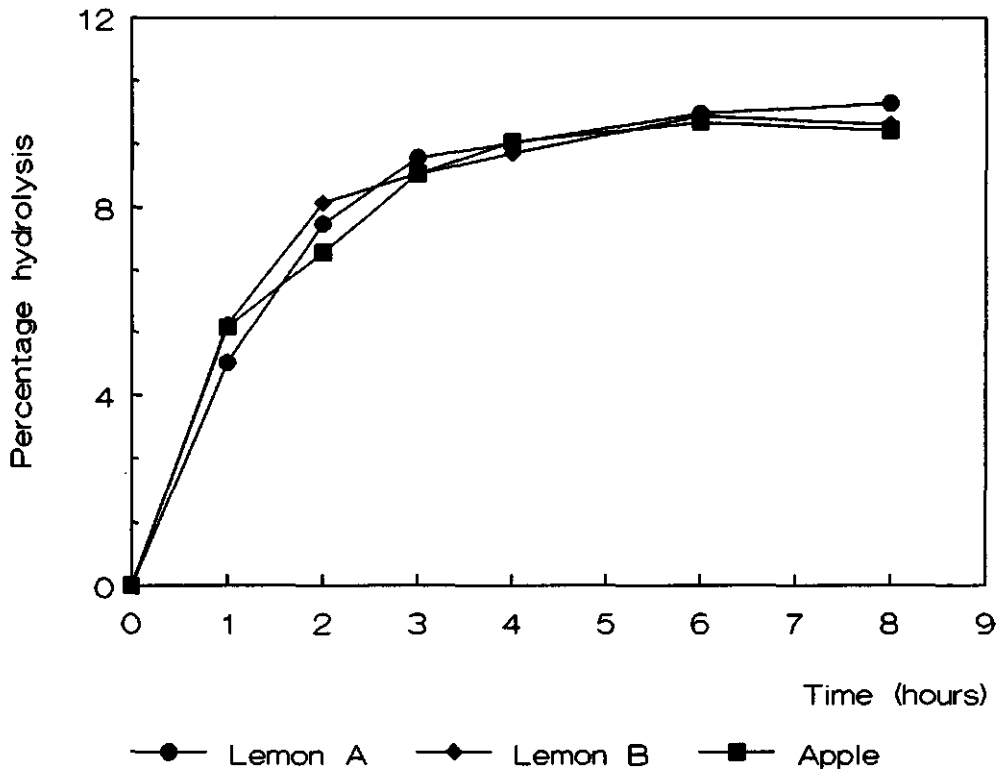

Figure 2: Rate of degradation of the three purified pectin samples by chemical β-elimination.

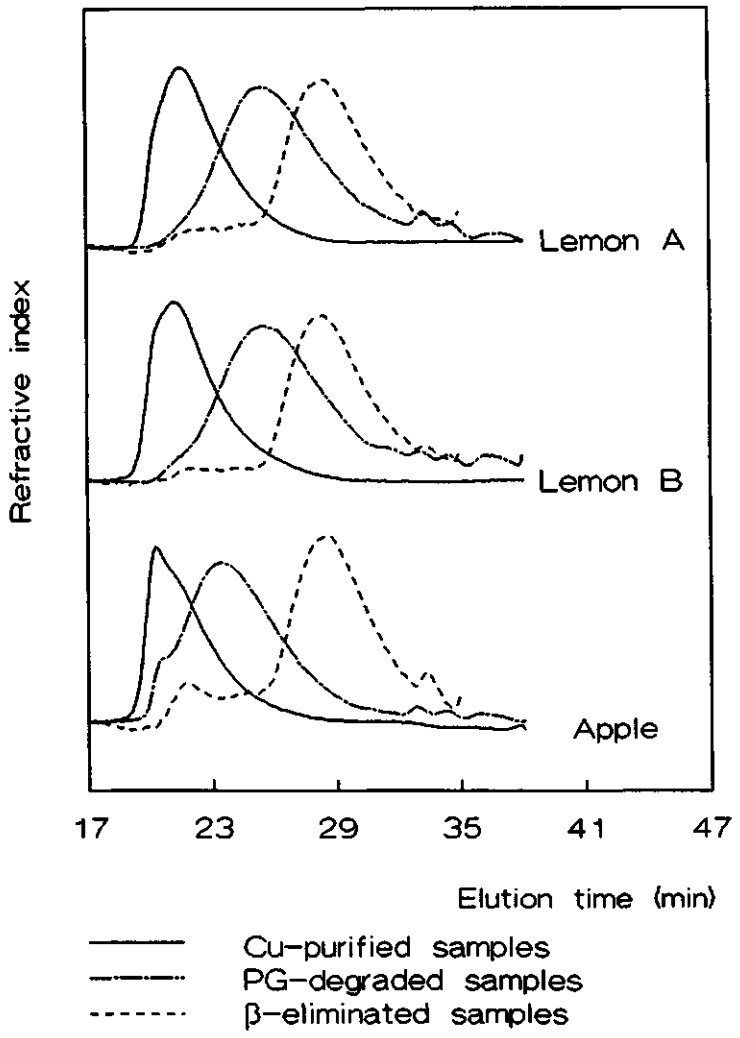

Figure 3: HPSEC elution pattern of the three purified pectin sample degraded by chemical β-elimination and endoPG.

Chemical β-elimination

Cu-purified pectin samples were degraded by chemical β-elimination at pH 6.8 and 80°C in order to split the glycosiduronic bonds next to methoxylated galacturonic acid units. Figure 2 shows the rate of degradation of the three purified

industrial pectins. The depolymerization was very fast in the first stage of the reaction. Then, the speed of the reaction decreased rapidly to stop completely after 6 hours. After 8 hours, the percentage of glycosiduronic bonds broken was only 10.0, 9.8 and 9.9% for lemon A, lemon B and apple pectins, respectively. Indeed, the reaction stops because of the removal of methoxyl groups by saponification (Albersheim *et al.*, 1960). Similar degrees of degradation have been obtained by other investigators for pectins of similar DM under similar conditions (Thibault, 1983; Rombouts & Thibault, 1986). Since, apple and lemon pectins differ greatly in neutral-sugar and acetyl-ester contents (Kravtchenko *et al.*, 1992a; chapter 2), these results confirm that chemical β-elimination is not affected by other structural features than the presence of a methyl ester next to the glycosidic bond to be split (Rouau & Thibault, 1984).

HPSEC analysis of the degradation products (figure 3) indicates that chemical β-elimination led to a reduction in molecular size of almost all the pectin molecules. Since hydroxide ions which induce the reaction are not limited by the presence of side chains or other substituents, and assuming a random distribution of methyl esters, the galacturonan backbone should be degraded in fragments with an average size of *ca.* 10 units. However, especially in the case of the apple pectin sample, some molecules elute very early on HPSEC and thus appear to have a much larger molecular size. These high molecular weight fragments elute in two peaks at *ca.* 22 and 24 min, respectively.

Degradation products were separated on Sephacryl S200 as shown in figure 4. The elution patterns obtained show two quite different polysaccharide populations. The peak excluded from the gel contains only a small fraction of the galacturonide but most of the neutral-sugar units whereas the peak eluting in the fractionation range of the column is composed mainly of galacturonic acid units. In addition, lemon pectins exhibit an incompletely resolved peak at K_{av} 0.5 that corresponds to undegraded fragments of intermediate size. These fragments may correspond to blocks of non-esterified galacturonic acids. This peak appears to be more important in the degradation products from the lemon A pectin sample.

SEC fractions were grouped in 7 pools as shown in figure 4. Neutral sugars were determined in each pool as their alditol acetates. Table 1 clearly confirms that most of the neutral-sugar units are concentrated on large fragments which elute close to the void volume of the column. More than 70% of the neutral-sugar units are concentrated in fractions 1 and 2 which represent only about 7% of the galacturonic acid residues. This confirms that pectin molecules are made of a succession of branched and unbranched galacturonan segments (Barrett & Northcote, 1965; Kikuchi & Sugimoto, 1976; de Vries *et al.*, 1982; Thibault,

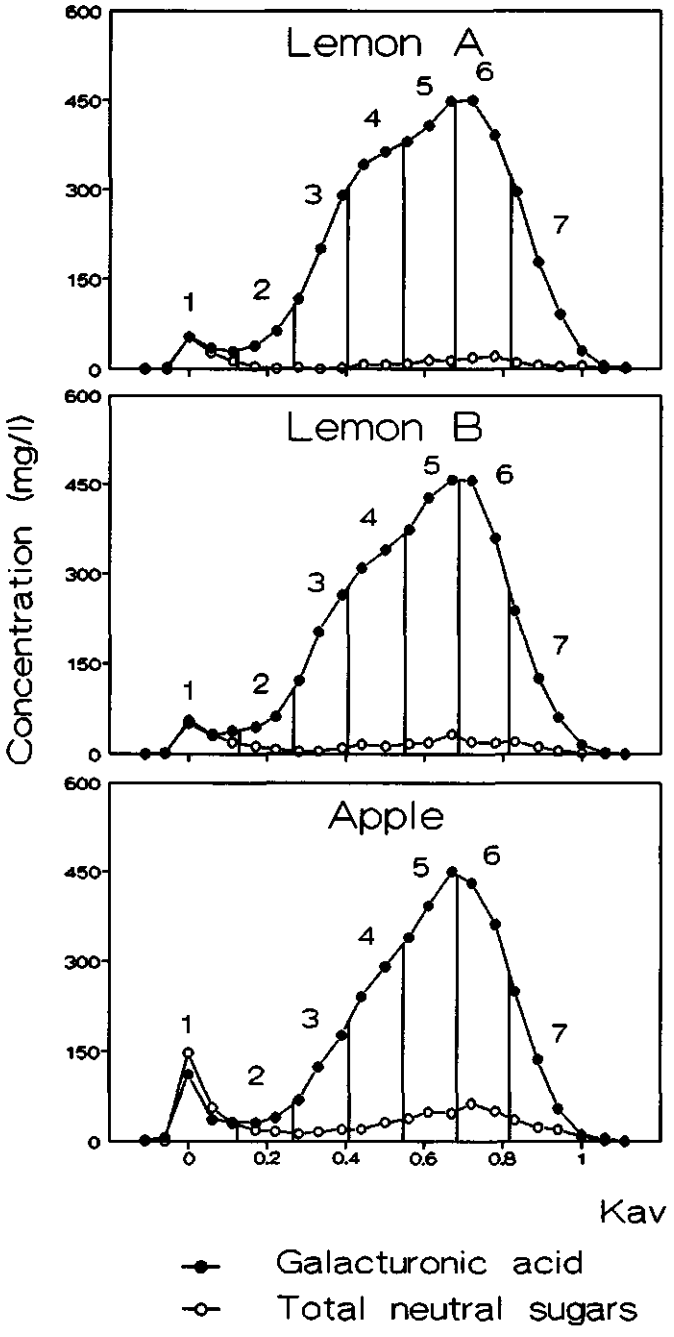

Figure 4: SEC elution pattern on Sephacryl S200 of the three purified pectin samples degraded by chemical β-elimination.

Table 1: Chemical composition of the SEC fractions obtained by β-elimination (expressed as percentage weight of AUA).

Lemon A							
Fraction	1	2	3	4	5	6	7
AUA (%)[a]	1.9	3.4	12.1	20.8	24.5	24.5	12.7
Rhamnose	75.5	34.9	2.0	0.6	O.4	0.3	0.4
Arabinose	50.2	25.5	1.3	0.5	0.6	0.6	1.1
Xylose	6.3	1.6	0.2	0.2	0.1	0.2	0.5
Mannose	t	0.2	t	t	t	t	0.2
Galactose	133.0	32.2	1.9	1.1	1.1	1.0	1.6
Glucose	4.5	3.7	0.5	0.2	0.2	0.2	1.5
Total NS	269.5	98.1	5.9	2.6	2.4	2.3	5.3
Phenolics	0.4	0.2	0.2	0.1	0.2	0.2	1.2
Proteins	0.9	0.8	0.2	0.2	0.1	0.1	0.1
Lemon B							
Fraction	1	2	3	4	5	6	7
AUA (%)[a]	2.0	3.7	12.3	20.2	26.3	24.8	9.5
Rhamnose	70.1	44.8	2.1	0.7	0.4	0.2	0.3
Arabinose	50.2	37.4	2.0	0.9	0.6	0.5	0.9
Xylose	4.9	2.2	0.3	0.2	0.1	0.1	0.4
Mannose	t	0.4	t	0.1	0.1	0.1	0.3
Galactose	151.1	49.8	4.5	2.5	1.3	0.8	0.8
Glucose	4.1	8.9	1.1	0.7	0.4	0.2	1.9
Total NS	280.4	143.5	10.0	5.1	2.9	1.9	4.6
Phenolics	0.2	0.1	0.1	0.1	0.1	0.3	1.7
Proteins	0.9	0.5	0.2	0.2	0.1	0.1	0.3
Apple							
Fraction	1	2	3	4	5	6	7
AUA (%)[a]	4.8	2.7	8.5	19.1	27.2	26.8	10.9
Rhamnose	71.2	45.6	3.4	0.8	0.5	0.2	0.2
Arabinose	68.0	42.8	3.2	1.4	1.3	0.7	0.4
Xylose	38.4	22.0	3.6	0.9	0.4	0.2	0.2
Mannose	0.8	0.8	0.3	0.1	0.1	0.1	0.1
Galactose	112.0	44.0	4.2	1.7	1.3	0.6	0.4
Glucose	214.8	80.8	11.9	3.7	1.2	0.5	2.4
Total NS	505.2	236.0	26.6	8.6	4.8	2.3	3.7
Phenolics	0.7	1.0	1.1	0.1	0.2	0.2	4.1
Proteins	0.9	0.3	0.2	0.2	0.1	0.1	0.1

[a] fraction % of the whole sample

1983; Rombouts & Thibault, 1986) that de Vries *et al.* (1982) called "hairy" and "smooth" regions, respectively.

Since the galacturonan backbone can be expected to have been broken in fragments of equivalent sizes (see above), the large molecular size of the fragments rich in neutral sugars indicates that neutral-sugar units are arranged as long side chains. Talmadge *et al.* (1973) suggested the presence of long homo-arabinans in pectic substances from sycamore while galactans of high degree of polymerization (DP) have also been obtained (Aspinall & Jiang, 1974; Eda & Kato, 1980; Darvill *et al.*, 1980).

Rechromatography on HPSEC (not shown) indicated that the fraction eluting at the void volume of the Sephacryl S200 column contains the two high molecular weight fractions which were also observed by HPSEC analysis of the unfractionated degradation products (figure 3). These two separated peaks as well as fraction 2 from the preparative column may represent different fragment populations of fragments which probably differ in neutral-sugar content and therefore in size of their side chains. The fractions eluting on HPSEC at RT 22 min probably correspond to fraction "a" isolated from apple pectin by de Vries *et al.* (1982) which contained 90% of the neutral sugars and only 5% of the galacturonide of the sample. This is in agreement with Knee *et al.* (1975) and Thibault (1983) who observed that pectin fragments from apple contain two types of side chains distinguishable by their length.

In addition, the amount of neutral sugars is higher in fragments of low size from the apple pectin, indicating that some short side chains or single neutral-sugar units may also be present on the smooth regions of apple pectin molecules.

The molar ratios of rhamnose residues to galacturonic acids were found to be *ca.* 0.9 in fractions 1, irrespective of the plant origin. This means that there is about one rhamnose residue for every galacturonic acid residue in hairy regions. In contrast, smooth regions (fractions 3 to 7) contain only about 1 rhamnose unit per 200 galacturonic acid units.

Table 1 also shows the distribution of phenolic and proteinaceous compounds over the SEC fractions obtained from the three β-eliminated samples. Most of the phenolic compounds elute with the low molecular weight fragments (fractions 6 and 7). However, some phenolic compounds coelute with the high molecular weight fragments, and therefore seem to be associated with hairy regions. In contrast, proteins appear to be mainly present in the fractions rich in neutral sugars, probably associated with neutral side-chains (Lamport 1969; Lamport *et al.*, 1973).

Enzymic degradation

The three Cu-purified pectins were submitted to the action of a purified endoPG from *Kluyveromyces fragilis*. After maximum degradation with excess enzyme (addition of enzyme did not lead to any further degradation), the percentage hydrolysis of the glycosiduronic linkages were 4.1, 3.8 and 1.4% for lemon A, lemon B and apple pectin samples, respectively. Assuming the complete degradation of all glycosidic linkages next to free carboxylic acid, the extent of degradation of such pectins should be over 25%. However, to act on the pectin backbone, endoPG requires not only a single non-methoxylated galacturonic acid unit, but a sequence of non-methoxylated galacturonides (Rexova-Benkova & Markovic, 1976). The endoPG that we used has been incubated with polygalacturonic acid and the degradation products were chromatographed on the dionex PA-1 column (not shown). At the maximum hydrolysis, mono-, di- and trigalacturonic acids remained as end products indicating that endoPG is not able to degrade di- and trigalacturonic acids. The probability of finding sequences of non-esterified galacturonides of a given length and therefore the potential extent of degradation by endoPG decreases very quickly with increasing DM (Pilnik *et al.*, 1973). Koller & Neukom (1969) found that the hydrolysis of pectin decreases with increasing DM: pectins with a DM above 75% were not hydrolyzed. This may explain why the extent of degradation of the apple pectin is so low compared to that of the lemon pectins although its average DM is only slightly higher (Kravtchenko *et al.*, 1992a). However, other structural features may also explain the difference of extent of degradation between apple and lemon pectins. The action of endoPG is limited by the presence of neutral side chains by steric hindrance (de Vries *et al.*, 1982; Thibault, 1983; Pasculli *et al.*, 1991) or the presence of acetyl esters on C-2 or C-3 of the galacturonic acid units (Solms & Deuel, 1951; Rexova-Benkova *et al.*, 1977; Pasculli *et al.*, 1991). A blockwise distribution of the free carboxyl groups in the lemon pectins would increase the frequency of sequences of non-esterified galacturonides with the required length for endoPG attack. The low degradation limits observed in this study are in agreement with those obtained by other investigators with high methoxyl pectins (Thibault & Mercier, 1978; Rombouts & Thibault, 1986).

Figure 3 shows the change in HPSEC elution pattern of the three pectin samples on treatment with endoPG. Despite low extents of degradation there is a clear shift towards fragments of lower molecular size. These patterns are as expected for an endoenzyme. The lesser change in molecular size of the apple pectin confirms its lower extent of degradation compared to lemon pectins.

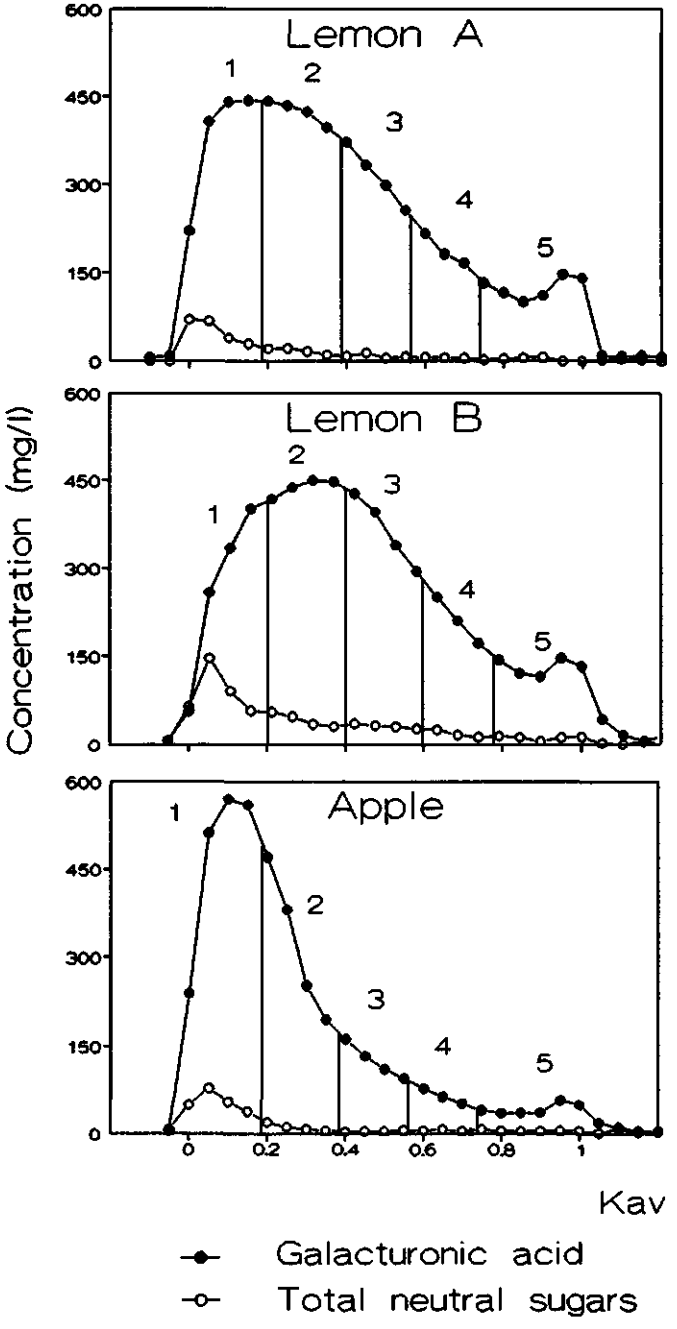

Figure 5: SEC elution pattern on Sephacryl S200 of the three purified pectin samples degraded by endoPG.

Table 2: Chemical composition of the SEC fractions obtained by degradation with endoPG (expressed as percentage weight of AUA)

Lemon A					
Fraction	1	2	3	4	5
AUA (%)[a]	28.2	29.2	19.1	11.3	12.2
Rhamnose	9.3	1.5	0.4	0.3	0.6
Arabinose	5.9	1.4	0.7	0.7	1.5
Xylose	0.7	0.1	t	t	0.3
Mannose	0.1	0.1	0.1	0.1	0.2
Galactose	13.2	1.7	1.4	1.5	1.3
Glucose	1.2	1.0	1.1	1.6	3.8
Total NS	30.4	5.8	3.7	4.2	7.7
Phenolics	0.1	0.1	0.2	0.3	1.9
Lemon B					
Fraction	1	2	3	4	5
AUA (%)[a]	20.7	31.5	22.9	13.3	11.6
Rhamnose	5.4	0.6	0.1	0.1	0.2
Arabinose	4.2	0.7	0.4	0.3	0.8
Xylose	0.4	0.1	t	t	0.1
Mannose	0.1	t	t	0.1	t
Galactose	13.8	3.7	3.0	2.8	1.8
Glucose	0.8	0.4	0.4	0.5	1.0
Total NS	24.7	5.5	3.9	3.8	3.9
Phenolics	0.1	t	0.1	0.1	4.5
Apple					
Fraction	1	2	3	4	5
AUA (%)[a]	46.0	32.2	10.1	5.2	6.5
Rhamnose	8.6	2.4	0.7	0.9	1.5
Arabinose	8.3	2.7	1.8	2.5	5.2
Xylose	5.2	1.2	0.7	0.6	0.5
Mannose	0.1	t	0.1	0.2	0.2
Galactose	10.7	3.2	3.9	5.2	4.4
Glucose	11.8	3.9	4.5	7.3	7.1
Total NS	44.7	13.4	12.6	16.7	18.9
Phenolics	0.4	0.2	0.4	0.7	3.0

[a] fraction % of the whole sample

After heat inactivation of the enzyme, the reaction products were directly freeze-dried. Pectin fragments were separated by preparative SEC on Sephacryl S200 (figure 5). Elution patterns are similar to those obtained by de Vries *et al.* (1982) with apple pectins degraded by pectin and pectate lyases. However, the SEC column that we used had a more restricted fractionation range and did not allow the separation of the fraction "a" isolated by de Vries *et al.* (1982).

Because of the lower extent of degradation, separation between smooth and hairy regions is not as clear as in the case of β-elimination. However, it is clear that most of the neutral-sugar units are located in the fractions of large molecular size. Table 2 shows the neutral-sugar composition of the S200 fractions. The distribution of neutral sugars over the degradation products obtained by treatment with endoPG is similar to that of the fragments obtained by treatment with chemical β-elimination. However, the neutral-sugar/galacturonic acid ratio is lower in the high molecular weight range because the same neutral-sugar side-chains are carried by longer segments of galacturonic acid. On the other hand, it seems that treatment with endoPG has led to the formation of a larger proportion of fragments of much lower size than chemical β-elimination.

Apple pectin differs from the lemon pectins by the presence of large amounts of glucose and xylose units. This may indicate the presence of separate xyloglucan and/or single unit xylose side chains. Indeed, covalent bonds between xylose and galacturonic acid have often been found (Barrett & Northcote, 1965; Aspinall *et al.*, 1967a, 1967c; Schols *et al.*, 1990).

The distribution of phenolic compounds over the endoPG degradation products (table 2) is similar to that observed on pectins degraded by β-elimination.

Distribution of the oligomers

After freeze-drying, an aliquot of the degradation products obtained by endoPG was chromatographed on a Dionex PA-1 column. Figure 6 shows the chromatograms obtained by degradation of the three pectin samples. Oligogalacturonides of DP 1 to 17 elute as perfectly well separated peaks. Since the separation was performed at very high pH, all methoxyl and acetyl esters were probably hydrolyzed very rapidly and thus did not affect the separation. The proportion of galacturonic acid present among the oligomers of DP up to 17 was calculated assuming that the response of the pulsed amperometric detector is proportionnal to the concentration of HCOH groups (Koizumi *et al.*, 1989).

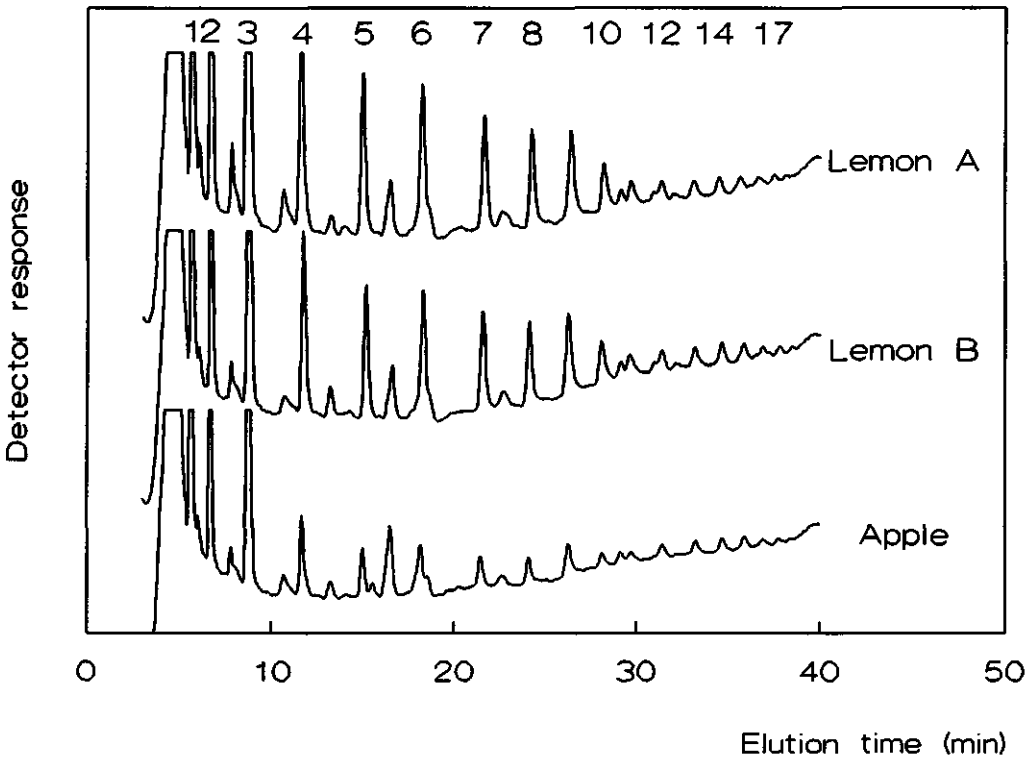

Figure 6: High performance ion exchange chromatography of the oligogalacturonides produced by the action of endoPG on the three pectin samples. Figures indicate the oligomer size.

With a computerized mathematical model, galacturonan chains with the DM of the industrial samples but with a random distribution of methyl esters were generated. From these model molecules, the theoretical distribution of the galacturonic acid units among segments of contiguous esterified or non-esterified residues with a given DP was established. Assuming the place of splitting for each method of degradation used, we calculated the theoretical distribution of the oligomers formed. These theoretical results were compared with the experimental ones obtained by the treatment of the pectin samples with endoPG (figure 7).

The proportion of mono-, di- and trimers produced by treatment with endoPG is much higher than expected from the theoretical distribution of the methoxyl groups. A large proportion of oligomers of DP up to 3 indicates that large galacturonan regions, *i.e.* non-esterified regions, have been readily

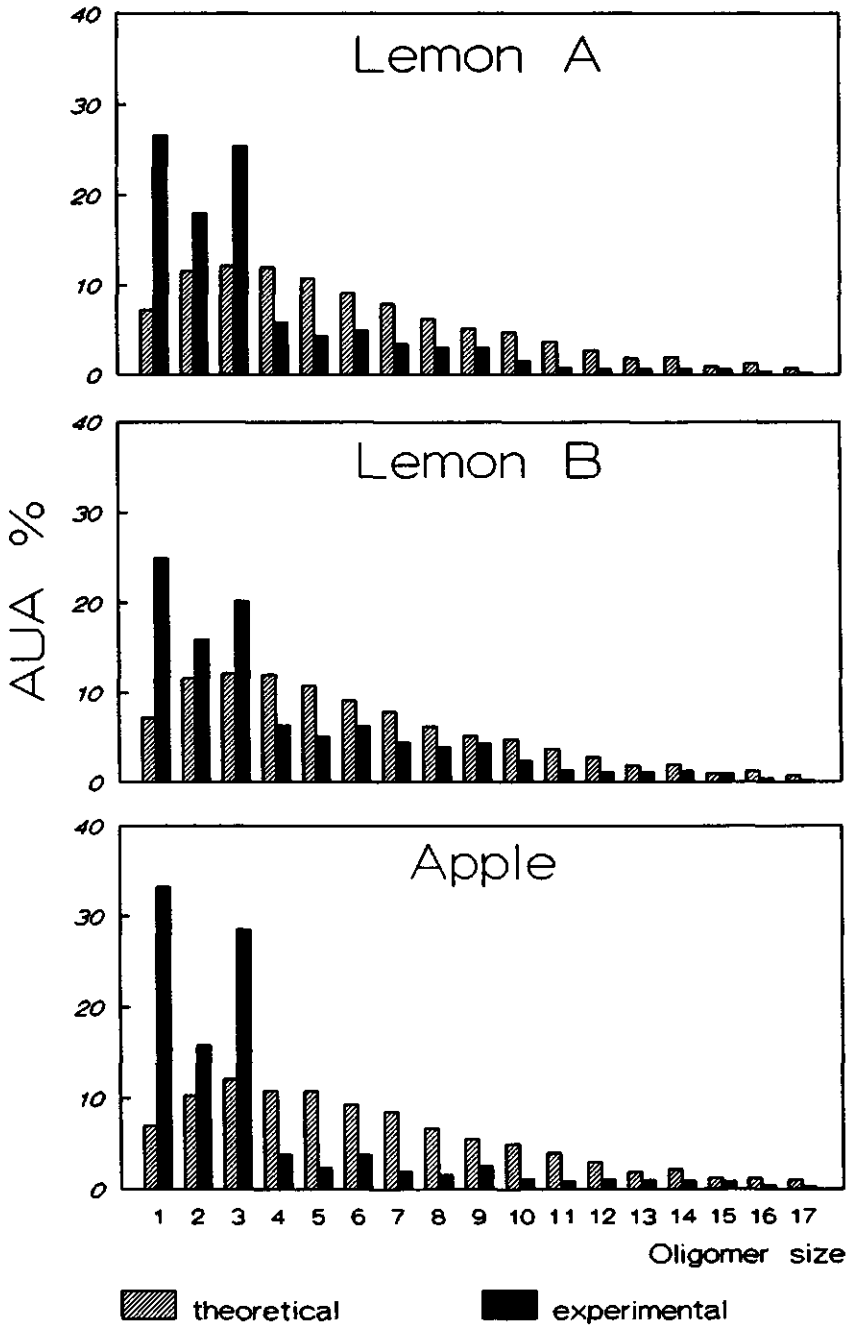

Figure 7: Experimental versus theoretical random distribution of the oligomers produced by the action of endoPG on the three industrial pectin samples.

degraded by endoPG. This suggests that in these industrial pectins, free carboxylic acids are unevenly distributed.

EndoPG is known to require a certain sequence of unesterified galacturonic units. Partly methoxylated galacturonic acid chains, especially those with a high DM would therefore be less degraded and the release of oligomers of small size would be lower than with the simple mode of degradation that we have assumed in our model. Thus, taking into account a more realistic mode of action for endoPG supports the view that free carboxyl groups appear in blocks.

It must be kept in mind that endoPG can be expected to degrade the smooth regions more readily than the hairy regions. de Vries *et al.* (1982) found that the DM is much higher in hairy than in smooth regions. This means that homogalacturonan regions would be better substrates for endoPG than expected from the average DM of the whole samples. DMs have been measured in the high molecular weight fragments (fractions 1) produced by the action of endoPG on the three purified pectins. Values of 58, 57 and 24% were found for lemon A, lemon B and apple, respectively, indicating that in industrial pectins, smooth regions are more methoxylated than hairy regions. Assuming a random distribution of the free carboxyl groups, their degradation with endoPG should therefore produce less low molecular weight oligomers than expected from their average DM. Again, the high proportion of low molecular weight oligomers suggests a blockwise distribution of free carboxyl groups.

An argument against our conclusions may be found in the fact that some oligogalacturonides are able to aggregate with high molecular weight fragments (Rombouts & Thibault, 1986). Such a phenomenon may hinder the chromatographic separation of the oligomers and therefore modify the observed distribution. However, no evidence was provided to assume that oligomers of DP higher than 3 are more prone to such an aggregation than lower oligomers.

Among the oligomers of DP lower than 17, produced by the action of endoPG on non-methoxylated regions of the molecules, compared to lemon pectins, the apple pectin contains a higher proportion of mono-, di- and trimers, indicating that acid blocks are probably larger than in the lemon pectins. However, figure 5 clearly shows that compared to the total pectin content, the production of low molecular weight fragments which elute close to the total volume of the column is much lower for the apple than for the lemon pectins. This indicates that in the apple, there are less suitable sites for the action of endoPG, *i.e.* less blocks of free carboxyl groups.

CONCLUSION

Each of the degradation methods applied to the three industrial pectin samples had a different specificity. Chemical β-elimination occurred all along the galacturonan backbone without hindrance by side chains or other substituents. EndoPG requires a certain sequence of non-methoxylated galacturonate residues and is hindered or blocked by side chains, rhamnosyl residues or acetyl groups at C-2 and/or C-3 of galacturonic acid units. Both methods showed that neutral sugars are unevenly distributed along the pectin molecules. It may thus be concluded that industrial pectins are also made of alternating smooth galacturonan and hairy rhamnogalacturonan regions. However, hairy regions of industrial pectins carry less neutral sugars than pectins extracted in mild conditions (de Vries *et al.*, 1982; Thibault, 1983; Rouau & Thibault, 1984; Rombouts & Thibault, 1986) because of the trimming reactions which occur during the industrial process of extraction (Kravtchenko *et al.*, 1992a). In addition to arabinogalactan side chains, apple pectin hairy regions may carry separate xylan and/or xyloglucan side chains.

Degradation with chemical β-elimination and endoPG showed that, rhamnose units are concentrated in the segments of the galacturonan backbone rich in neutral side chains, leaving more than 90% of the galacturonan chain almost devoid of rhamnose insertions. This is in agreement with the fact that most of side chains are linked to C-4 of the rhamnose units (Aspinall *et al.*, 1967c; Talmadge *et al.*, 1973; Eda & Kato, 1980; McNeil *et al.*, 1980; Sun *et al.*, 1985; Schols *et al.*, 1990). Acid hydrolysis suggests that at least one rhamnose unit is present between homogalacturonan regions of about 40-60 units. Smooth regions however, were found to contain only 1 rhamnose unit per 200 galacturonic acid units. This would mean that smooth regions are almost completely devoid of rhamnose. This discrepancy may be explained by the fact that with the usual hydrolysis conditions, because of the high stability of glycosiduronic linkages, most of rhamnose units remain attached to galacturonic acid units as aldobiuronic acids (Aspinall, 1970), whereas stronger acid conditions lead to a substantial destruction of rhamnose units. Therefore, rhamnose content may be considerably under-estimated. An alternative method would be to reduce galacturonic acids to galactose residues so that acid-stable glycosiduronic bonds are converted into normal glycosidic linkages. Thus, with a higher rhamnose content than measured, rhamnose units may very well be present in alternance with galacturonic acid units in hairy regions (Schols *et al.*, 1990) as well as at regular intervals with homogalacturonan chains in smooth regions (Powell *et al.*, 1982; Durand *et al.*, 1990).

After degradation by β-elimination or endoPG, most of phenolic compounds co-eluted on SEC with the smallest pectin fragments. Rombouts & Thibault (1986) showed that most of the polyphenols from sugar-beet which also eluted with the smallest enzyme degradion products are not covalently linked to the pectin molecules. These are probably the same phenolic compounds which eluted with molecules of small size on preparative SEC of the undegraded pectin samples (Kravtchenko *et al.*, 1992c; chapter 4). The reason why these phenols remain associated with pectin during purification and only separate upon extensive degradation is not known. However, another part of the phenols, especially in the case of the apple pectin, co-elutes with the fragments rich in neutral sugars. Ferulic acids were found to be covalently associated with pectin side-chains of spinach (Fry, 1983) and sugar-beet (Rombouts & Thibault, 1986). Industrial pectins from apple and lemon do not contain any phenolic acids (Kravtchenko *et al.*, 1992a; chapter 2) but some other phenolic compounds are probably bound to their neutral side-chains.

DM was found lower for the fractions rich in neutral sugars than for the whole undegraded samples. This means that DM is lower in hairy regions than in smooth regions. This contradicts the findings of de Vries *et al.* (1982) but, is in agreement with Saulnier & Thibault (1987) who found that hairy regions of acid-extracted pectins require a high ionic strength to elute from IEC column. In apple hairy regions, Schols *et al.* (1990) also found an average DM of 40%. DM of hairy regions from the industrial apple pectin was found lower than that of the two lemon pectins. This explains the much lower neutral-sugar/galacturonic acid ratio of the fragments rich in neutral sugars from the β-eliminated apple compared to that of the lemon pectins: apple hairy regions were probably less degraded by chemical β-elimination than those from lemon and, rhamnogalacturonan fragments carrying the side chains were therefore longer. Analysis of the oligomers produced by the action of endoPG suggests that in smooth regions, free carboxyl groups are arranged as blocks. This contradicts previous studies (Fielding, 1975; Kohn, 1975; de Vries *et al.*, 1983, 1986; Westerlund *et al*, 1991) which indicated that in HM pectins free carboxyl groups are distributed at random. This is probably true on average, although the presence of a few blocks of free carboxyl groups would have been difficult to detect. Blocks of free carboxyl groups probably result from the action of pectin methyl esterase (Taylor, 1982) on HM pectins, *i.e.* with DM in the range 70-75%. However, even after some action of the enzyme, the average DM remains higher than 70%, indicating that blocks only involve a very small proportion of the free carboxyl groups, the others being distributed at random. These blocks which are of very high technological importance are therefore very

difficult to detect and quantify. EndoPG degrades specifically acid blocks and ignores isolated carboxylic acids randomly spread in between methoxyl esters. The extent of degradation is indicative of the amount of galacturonic acid units involved in blocks and the size distribution of the resulting oligomers indicates the size of the blocks. It should be noted that the degradation of pectin molecules and the appearance of small oligomers may also result from the action of endoPG on pectin molecules of low DM.

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Chapter 8:

SOME PHYSICAL CHARACTERISTICS OF THREE INDUSTRIAL HIGH METHOXYL PECTINS

8. SOME PHYSICAL CHARACTERISTICS OF THREE INDUSTRIAL HIGH METHOXYL PECTINS

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ABSTRACT

Three industrial HM pectins, two from lemon and one from apple have been compared on the basis of their physical behaviour. Gel strength appears to depend mainly on the content of galacturonic acid and is only slightly affected by the amount of side chains and/or free neutral polysaccharides. On the other hand, one of the two lemon pectins exhibited a lower gel strength as well as a higher stability constant of calcium-pectinate. A simple, sensitive and reproducible test has been developed to characterize the calcium sensitivity of HM pectins. Higher affinity for calcium may explain higher calcium sensitivity, lower solubility and consequently lower gelling performance in the presence of calcium ions. Such affinity for calcium ions may be due to the action of pectin methyl esterase in lemon peels before the pectin extraction.

INTRODUCTION

Pectic substances are extracted industrially and are widely used in the food industry, mainly as gelling agents (Crandall & Wicker, 1986). Food technologists are therefore primarily interested in their physical behaviour. Together with rheological measurements, the fine structure of extracted pectins has been intensively investigated during the last decades. These studies clearly show that the strength of pectin gels is affected by numerous structural features such as molecular size and degree of methoxylation. However, the complexity of pectin preparations has not yet allowed to reach the ultimate stage which would be the prediction of the physical behaviour from the chemical analysis.

The gel formation mechanism of pectin gels is similar to that of other gelling polysaccharides (Rees, 1969): some regions of the polymer molecules associate in junction zones to form a three dimentional network which traps the solvent. Gelling ability of pectin molecules depends primarily on their molecular

size. Elasticity is mainly influenced by short stiff chains and, above a minimum, is independent on MW whereas, breaking strength is influenced by longer, more flexible chains which are largely MW-dependent and remain cross linked after the bonds between the short stiff chains have been ruptured (Crandall & Wicker, 1986). This assumption is based on the observations of Swenson *et al.* (1953) and Christensen (1954) who showed that breaking strength is more influenced by MW than gel elasticity. In high methoxyl pectins (HM pectins, degree of methoxylation higher than 50%), the binding forces between the chains are hydrogen bridges and hydrophobic forces between methoxyl groups (Oakenfull & Scott, 1985), which are both promoted by low water activity and low pH values. Indeed, degree of methoxylation (DM) affects the thermodynamic affinity of the pectin molecules for the solvent which is believed to be the key of the gelation of HM pectins (Owens *et al.*, 1954) and therefore influences the gel breaking strength. Jelly grade can be increased by decreasing the DM value (Deuel *et al.*, 1950; Doesburg & Greevers, 1960) although gel strength was shown to increase with increasing methoxyl ester content up to a level of around 70% (Morris *et al.*, 1980). The influence of DM on the setting time has been particularly well established: the setting time (or setting temperature at constant cooling rate) increases very regularly with decreasing DM (Smit & Bryant, 1968). This property is the base for the classification of high methoxyl pectins and, determination of the DM is probably the only chemical determination which is systematically performed on commercial pectins. However, the distribution of methoxyl groups among and/or along pectin molecules may also play a very important role. Walter & Sherman (1984) found that at a specific DM value, the physical properties of HM pectins are influenced by the dispersion and location of free carboxyls. The DM also interacts with gel pH to determine the maximum gel strength: the optimum firmness occurs at higher pH values for higher DM pectins (Ehrlich, 1968). To be able to associate, the pectin chains must have a regular conformational structure but also need structural elements causing the termination of junction zones and prevent the formation of insoluble micelles (Pilnik, 1989). Terminating elements can be supposed to be rhamnose insertions, acetyl esters or neutral side-chains (Rees & Wight, 1971). Pippen *et al.* (1950) showed that the introduction of one acetyl group for every eight galacturonide units prevents jelly formation from a pectin-sugar-acid-water system. Since side chains have proved to be concentrated in hairy regions (de Vries *et al.*, 1982; Kravtchenko *et al.*, 1992c; chapter 7), it is obvious that junction zones must be formed within the smooth regions. Although homogalacturonan segments are very stiff (Rees & Wight, 1971; Hallman & Whittington, 1973), insertion of α-linked L-rhamnose units (Colquhoun *et al.*, 1990) confers some

flexibility to the pectin backbone (Rees & Wight, 1971; Hallman & Whittington, 1973). Pectin molecules can thus be described as jointed chains of rod-like segments. All these structural features determine the relative proportion of the pectin chains which are involved in junction zones and are thus important factors in determining the final gel properties.

Despite intensive research efforts, the relationship between chemical structure and physical properties is still not completely elucidated. The problem is that pectins with similar analytical characteristics may behave very differently on application. This report deals with the comparison of three well-characterized industrial pectins, all classified as "rapid set" which, despite large analytical similarities exhibit different physical behaviour.

MATERIAL AND METHODS

Material

Three unstandardized industrial high methoxyl pectins were obtained from Sanofi Bio Industries (France): two from lemon peels (lemons A and B) and one from apple pomace. Their chemical composition has been extensively described in previous papers (Kravtchenko *et al.*, 1992a, 1992b, 1992c; chapters 2, 4 and 5).

Intrinsic viscosity

Intrinsic viscosities were determined from one single point measurement with an Ubbelohde viscosimeter in 0.1M Na-succinate buffer pH 4.8 at 30°C (Kravtchenko & Pilnik, 1990; chapter 3).

Molecular weight

Number average molecular weights ($\overline{M}_n$) were determined with an osmometer (Knauer, Germany) equipped with a SM 115 39 membrane (Sartorius, Germany). Measurements were carried out in 0.037M phosphate buffer pH 6.5 with 0.001M Na_2EDTA and 0.02% NaN_3 at 37°C in three or more pectin concentrations. $\overline{M}_n$ were calculated in the usual manner (Stuart, 1953).

Weight average molecular weights ($\overline{M}_w$) were determined by light scattering with a Sofica apparatus (Fica, France) equipped with a helium-neon laser of wavelength 632 nm. Pectins were dissolved in 0.037M phosphate buffer at pH 6.5 and solutions were filtered through 0.5μm pore size filter prior to measurement. $\overline{M}_w$ were calculated according to Zimm (1948). Further details are given elsewhere (Berth *et al.*, 1990).

Gel characteristics

The "gelly grade" was determined by the IFT-SAG method (IFT, 1959). SAG values were corrected for the jelly soluble solid content as determined by refractometry. "SAG jelly grades" were obtained from the Cox & Highby curve (Cox & Highby, 1944).

Breaking strength was determined with a Stevens apparatus equipped with a ϕ 0.5 inch HF plugger and operated at 1 mm/s. Gellies were prepared as described for SAG determinations but in petri dishes (inside ϕ 60 mm, height 9 mm).

Calcium activity

Stability constants of calcium-pectinate (K) were determined according to Kohn & Furda (1967a, 1967b), using tetramethylmurexide (TTM) as auxiliary ligand. Solutions contained 0.6 meq of pectin free carboxyl groups, 0.6 meq of Ca^{++} and 4.10^{-5} mol/l of TTM. Measurements were carried out at 20°C in 3.10^{-3}M LiNO3/LiOH at pH 7. The stability constant K was calculated for the complex of calcium with pectin, where the ligand was assumed to be constituted of two free carboxyl groups binding one Ca^{++}.

Calcium sensitivity

0.40 g pectin (dry weight) was stirred for 30 min at room temperature in 50 ml of distilled water. To insure complete dissolution, stock pectin solutions were heated at 100°C for 10 min and cooled rapidly at room temperature. 3 ml aliquots were diluted with 4 volumes of 0.1M Na-succinate buffer pH 4.8 containing the appropriate amount of $CaCl_2$. The difference of ionic strength were

compensated by the addition of NaCl (Michel, 1982).

The turbidity of pectin solutions was measured with a Hach ratio turbidimeter model 18900 at a wavelength of 550 nm, in a 23 mm cell and at 30°C. The solutions were measured after complete stability of the signal (*ca.* 10 min). The intensity of light scattered at the nephelometric angle (90°) was ratioed to the sum of transmitted (180°) and forward scattered (165°) light intensities. Turbidity was thus expressed as Nephelometric Turbidity Units (NTU).

Analytical methods

The anhydrouronic acid (AUA, MW=176) content was determined by the automated *m*-hydroxydiphenyl assay (*m*hdp, Thibault, 1979).

Calcium content was determined with an ELEX 6361 flame AES (Eppendorf, Germany).

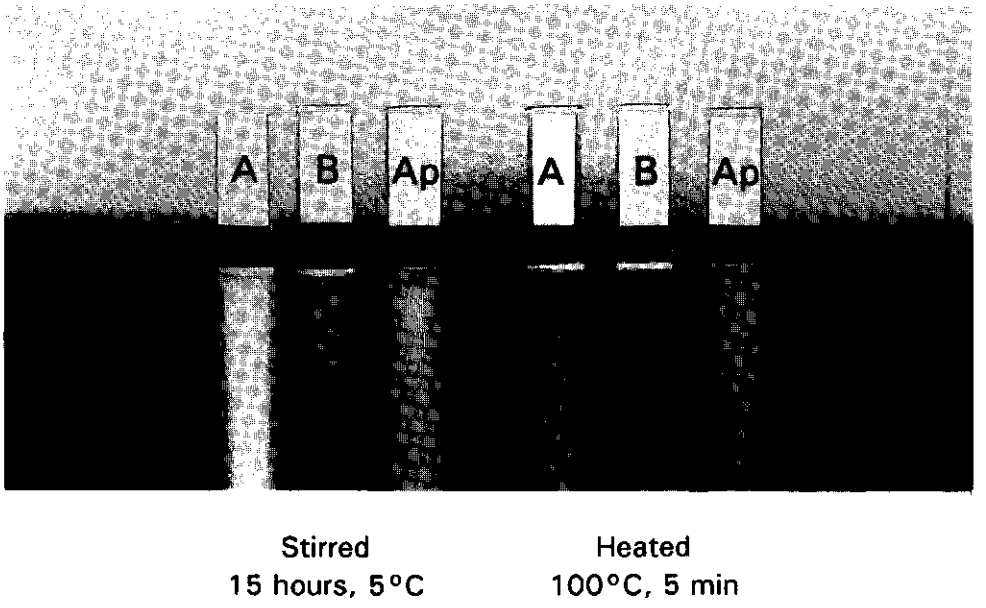

Figure 1: Comparison of the turbidity of the solutions (10 mg/ml) from the three pectin samples with and without heating. A: lemon pectin A; B: lemon pectin B and Ap: apple pectin.

RESULTS AND DISCUSSION

As first visual observation, it can be seen (figure 1) that even after several hours stirring at room temperature, solutions of lemon A and apple pectins remain very turbid although those of lemon B became clear. Lemon A pectin was found to be more difficult to dissolve, *i.e.* complete disappearance of solid particles took more time than lemon B and apple pectins. Also, gels prepared from lemon A pectin were slightly grainy although those from lemon B and apple pectins were perfectly smooth. However, these observations are only qualitative and do not give direct indication of the pectin behaviour in the final food products. Through some physical measurements, we have quantified and discussed these physical differences.

Molecular weight

Table 1 shows the molecular weight averages of the three industrial pectin samples. Weight average molecular weight ($\overline{M}_w$) values are consistent with qualitative data obtained by high performance size exclusion chromatography (Kravthenko *et al.*, 1992a; chapter 2): lemon B pectin has a slight larger molecular size than lemon A pectin whereas apple pectin has a much higher molecular size than lemon pectins. Number average molecular weights ($\overline{M}_n$) are much lower than $\overline{M}_w$. This indicates that these three industrial pectin samples are highly heterogeneous on the basis of their molecular size and confirms the large distribution patterns previously observed by size exclusion chromatography. The apple pectin exhibits a particularly low $\overline{M}_n$ compared to $\overline{M}_w$, probably because of the presence in large amount of free neutral polysaccharides of small size (Kravtchenko *et al.*, 1992a; chapter 2; Kravtchenko *et al.*, 1992b; chapter 4). In contrast, the lemon A pectin exhibits a relatively lower $\overline{M}_w/\overline{M}_n$ ratio than lemon B pectin although size exclusion elution patterns were found rather similar. This may be due to the association of some pectin molecules, resulting in an apparent higher $\overline{M}_n$ value. Indeed, lemon A pectin molecules have proved to be prone to aggregation in the presence of calcium ions (Kravtchenko *et al.*, 1992b; chapter 4) and was also shown to contain more calcium than molecules from the two other pectins (Kravtchenko *et al.*, 1992a; chapter 2).

$\overline{M}_w$ values obtained by light scattering are much higher than those obtained by other methods for other industrial high methoxyl pectins. Although they clearly indicate differences in molecular size, light scattering data must not be taken as an

Table 1: Molecular parameters, viscosity and gelling strength of the three industrial pectins.

	Lemon A	Lemon B	Apple
$\overline{M}_w$	44,670,000	50,120,000	223,870,000
$\overline{M}_n$	77,300	55,350	72,600
[η]	3.5	4.5	4.9
°SAG	214	244	188
Ca content (w %)	0.74	0.36	0.31
log K	3.15	2.68	2.70

absolute truth. Indeed, light scattering gives too much emphasis to extremely large pectin molecules, even if they represent only a minor proportion of the total pectin material. Other methods of determination of the $\overline{M}_w$ such as sedimentation equilibrium should be preferred.

Intrinsic viscosity

Since the work of Owens *et al.* (1946) viscosimetry has been extensively used for the determination of pectin molecular weight. In addition, a close relationship between viscosity and jelly strength has been established (Swenson *et al.*, 1953; Christensen, 1954). Table 1 shows that apple and lemon B pectins have very similar intrinsic viscosities whereas lemon A pectin appears to be much less viscous. Following the Mark-Houwing relationships (Mark, 1938; Houwink, 1940) this would mean that lemon A pectin has a lower $\overline{M}_w$ than lemon B and apple pectins. This contradicts the data obtained by light scattering. When recalculated on the basis of the concentration of galacturonic acid, intrinsic viscosities become 4.6, 5.8 and 8.1 for lemon A, lemon B and apple pectins respectively, which are in better agreement with light scattering $\overline{M}_w$ as well as HPSEC elution patterns (Kravtchenko *et al.*, 1992a; chapter 2). This is mainly explained by differences in neutral sugar content (Kravtchenko *et al.*, 1992a; chapter 2): apple pectin contains more free neutral polysaccharides than lemon pectins. These free molecules have also been shown to have a low hydrodynamic volume (Kravtchenko *et al.*, 1992a; chapter 2; Kravtchenko *et al.*, 1992b; chapter 4) and therefore do not contribute

significantly to the viscosity of the whole sample. Apple pectin molecules also carry more bound neutral sugars, mainly as side chains than lemon pectins. However, these side chains are relatively short compared to the length of the main chain and it is likely that they do not modify significantly the hydrodynamic volume of the whole molecules. Indeed, McCleary & Neukom (1982) showed that single-unit galactose side chains do not participate to the viscosity of galactomannan macromolecules (figure 2). It may therefore be assumed that in pectins, intrinsic viscosity depends more on the length of the main chain, *i.e.* degree of polymerization of the rhamnogalacturonan backbone, than the true molecular weight. Discrepancies on application of the Mark-Houwink relation to pectin are probably due to differences in neutral sugar content. Viscosimetry, which only allows the determination of molecular weight of homologous polymer series, is thus not suitable for the molecular weight determination of pectins from different sources, which may contain various amounts of neutral side chains.

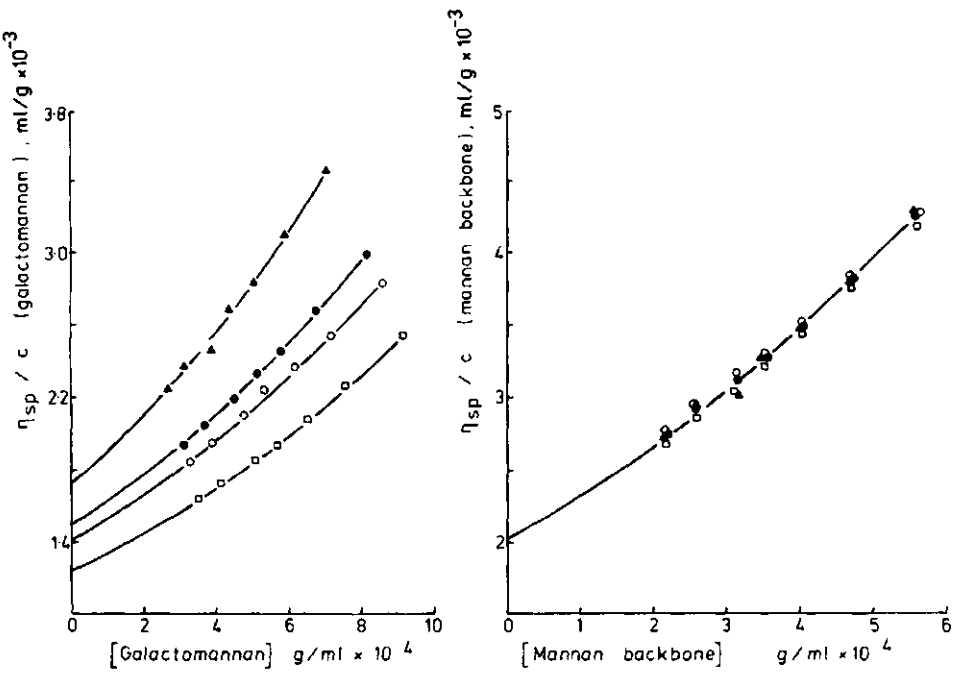

Figure 2: Effect of galactose removal from guar galactomannan on limiting viscosity number. The galactose/mannose ratios of the polysaccharides are 38:62 (□), 32/68 (○), 27/73 (●) and 15/85 (▲). (McCleary & Neukom, 1982)

Gelling grade

The "grade" is defined as the sugar carrying-power, that is the parts (by weight) of sugar which will form, under standard conditions a jelly of satisfactory properties with one part of pectin. Thus, 1 g of 150 grade pectin will form the proper jelly with 150 g of sugar. Gel strength of HM pectins is generally given by the so-called SAG grade, calculated from the sagging of a standard gel under its own weight (IFT, 1959).

Table 1 shows the grade values obtained for the three industrial pectins. SAG values of the lemon pectins are consistent with molecular weight and intrinsic viscosity values: lemon B which has a larger molecular size exhibits a higher gelling ability. In contrast, the apple pectin which has a much higher molecular size exhibits a much lower grade value. When recalculated on the basis of the galacturonic acid content, grade values come much closer to each other and become 280, 316 and 309 for lemon A, lemon B and apple respectively. This may be explained by the higher neutral-sugar content of the apple pectin sample (Kravtchenko *et al.*, 1992a; chapter 2). Indeed, since side chains are mainly located in "hairy regions" (de vries *et al.*, 1982; Kravtchenko *et al.*, 1992c; chapter 7) and junction zones can only be formed in the "smooth regions", it is likely that the size of neutral side-chains does not affect the gel formation. Anyway, the SAG grade of the apple pectin still remains lower than expected from the relations of Swenson *et al.* (1953) and Christensen (1954) as compared with the lemon pectins. This may be explained by the fact that the apple pectin also carries some short side chains in the smooth regions (Kravtchenko *et al.*, 1992c; chapter 7) as well as acetyl groups which act as termination points and thus limit the formation of junction zones (Rees & Wight, 1971). In order to evaluate more specifically the influence of the free neutral polysaccharides, two gels were compared; one from lemon B pectin diluted with 10% of starch and the other from the same pectin diluted with 10% of saccharose. SAG grades were found identical for both preparations (203 °SAG) indicating that free neutral polysaccharides do not affect directly the gel elasticity. In contrast, the breaking strength, which is a measure of the elastic limit (Christensen, 1954) was found higher for the gel prepared with native starch (104 ± 2 N) than for the gel prepared only with saccharose (92 ± 2 N). This means that starch, which constitutes the largest part of the free neutral polysaccharides in apple, reduces the brittleness of pectin gels. It must be supposed that destarching performed by certain pectin manufacturers (May, 1990) has a larger effect on the pectin behaviour than usually thought. In addition, the gel prepared in the presence of additional starch was less bright and limpid.

Calcium activity

Although they were extracted in the same factory following the same process, these three industrial pectin samples were found to differ in calcium content (Kravtchenko *et al.*, 1992a; chapter 2). This was thus ascribed to differences in affinity for calcium ions.

Table 1 shows the stability constant K of calcium-pectinate for the three pectin samples. Lemon A differs from lemon B and apple pectins by a higher stability constant. The stability constant K depends on the density of the electric charge on the pectin molecule (Kohn & Furda, 1967b) and rises rapidly with increasing charge density. Differences in K value for pectins of similar DM were ascribed to differences in the distribution pattern of free carboxyl groups along the pectin chain (Kohn *et al.*, 1968).

Calcium sensitivity

The term "calcium sensitivity" is often used to describe abnormal behaviour which occurs with certain pectins in the presence of calcium ions. It expresses itself in difficulties dissolving the pectin powder or preparing the gels. Despite the practical importance of this parameter, there is no simple test which allows to quantify the calcium sensitivity of different pectins. The addition of calcium ions to pectin solutions lead to an increase of the turbidity. Since the intensity of the turbidity depends on the size of particles in suspension, nephelometry was thought to be a simple and accurate indicator of the aggregation process which accompany the addition of calcium.

Preliminary experiments showed the importance of the procedure of adding calcium to pectin solutions. Indeed, the addition of small volumes of concentrated calcium solutions led to the formation of local microgel particles which affected very much the reproducibility of the test. It was thus prefered to add concentrated pectin solutions to diluted buffered calcium solutions under vigorous stirring.

Figure 3 shows that the turbidity of the three HM pectins increases with increasing the calcium content of the solutions. This indicates they are all calcium sensitive. However, the lemon A pectin seems to be much more sensitive to calcium ions than lemon B and apple pectins. The increase in turbidity indicates that lemon A is more prone to aggregation in the presence of calcium ions than the two other pectins.

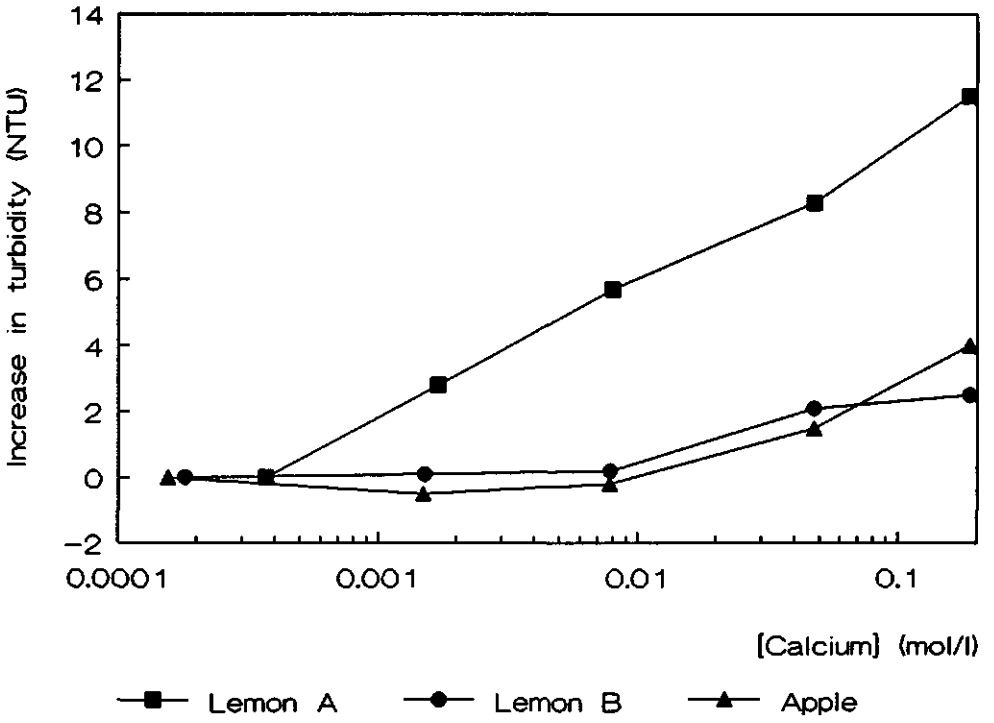

Figure 3: Increase of the turbidity of pectin solutions on addition of calcium cations.

This simple and inexpensive test would be very useful in the pectin industry for predicting the calcium sensitivity and orientate industrial pectins towards applications where calcium will not cause depreciation.

DISCUSSION

Earlier workers believed that pectin was only constituted of long homopolygalacturonan chains. The presence of neutral sugars in pectin preparations was therefore ascribed to the presence of co-extracted free neutral polysaccharides which were embodied under the term "ballast". Despite the fact that pectins have proved to contain covalently bound neutral sugars, our experiments showed that viscosity and gelly grade depend mainly on the length of the pectin galacturonan backbone, confirming the early view that neutral sugars act mainly as diluent.

However, although free neutral sugars only play a minor role in the determination of the SAG jelly-grade, they modify significantly the gel properties. For instance, the presence of starch confer elasticity to the gel. Also, although the length of the side chains probably does not affect the mechanism of gelification, it is likely that their distribution along the pectin molecules will determine the size of the junction zones and therefore the properties of the gel.

Pectin molecular size was not found to be very indicative of the SAG jelly-grade. In our study, MWs were estimated by methods providing the true molecular weight which take into account the neutral sugars. The high neutral sugar content of the apple pectin may thus explain its relative low jelly grade compared to its high MW. Gel strength seems to be more closely related to the length of the pectin chains. The opinion widely accepted that the increase in MW results in an increase in gel strength should be revised in favour of the degree of polymerization of the pectin main chain. Therefore, viscosity or other methods of estimation of the degree of polymerization of the polygalacturonan chain would be better estimations of the gelly grade than true molecular weights investigated by means of more sophisticated techniques. However, the neutral-sugar content does not account alone for the large differences in MW that we observed. The small differences in jelly grade can also be explained by the fact that above a certain limit, gel elasticity is not very much influenced by molecular size (Swenson *et al.*, 1953; Christensen, 1954). It is mainly for this later reason that some pectin producers, especially those producing high molecular weight pectins would prefer to grade pectins by means of breaking strength (Beach *et al.*, 1986).

Since pectins are charged polymers, all three pectin samples exhibit some affinity for calcium ions. Despite extraction in acidic conditions and subsequent extensive washing, they all still contain some calcium ions. Stability constant of calcium-pectinate and turbidimetric measurements showed that the three pectins are sensitive to calcium ions. Indeed, even HM pectins are calcium sensitive if the proper blocks of free carboxyl groups occur (Doesburg, 1965). However, the lemon A pectin differs from the two other samples by a higher native calcium content, a higher stability constant of calcium pectinate and a greater susceptibility for aggregation in the presence of calcium ions. This difference may be ascribed to a difference in the distribution of free carboxyl groups. Statistical calculations (Powell *et al.*, 1982) and Ca-activity measurements (Kohn, 1975; Kohn & Luknar, 1977) showed that Ca-pectinate junction zones occur when at least 14 free consecutive carboxyl groups are present. The presence of blocks of deesterified galacturonic acid units thus increases the affinity for calcium ions (Kohn *et al.*, 1968). Previous studies (Fielding, 1975; Kohn, 1975; de Vries *et al.*, 1983, 1986;

Westerlund *et al.*, 1991) showed that in HM pectins the distribution of free carboxyl groups cannot be distinguished from random. According to Cook & Stoddart (1973), at an early stage in the biosynthesis pathway, pectin exists in an essentially fully esterified form. The presence in plant tissue of pectin which can be extracted only by the use of calcium sequestrants (Gould *et al.*, 1965; Rees & Wight, 1969; Cook & Stoddart, 1973) however indicates that some chains have undergone substantial de-esterification which is believed to occur at a later stage of biosynthesis by the action of pectin methyl esterase (PME) and would give rise to a blockwise arrangement of free carboxyl groups. Such blocks may also be due to the action of PME during the storage or during the juice extraction of the lemon fruits. Indeed, citrus fruits contain enough PME for fully de-esterify all the pectin in less than 15 min at the pH of the juice (Versteeg, 1979). Apple fruits which are known to contain less PME, are less affected by the problem of deesterification. The difference between lemon A and lemon B pectins may be due to botanical differences but also to the processing of the fruits before the pectin factory. In fruits, pectin is hardly accessible by PME because it is located in different cell parts. The process of juice extraction partly destroys the cell structures and allow PME to reach the pectin. A delay between juice extraction and peel drying allows PME to act on the pectin. Inactivation of the enzyme must thus be done as soon as possible, more especially as juice factories are mostly situated under tropical climates where high temperature favours the action of the enzyme. Plant PME are known to proceed along the pectin molecules giving rise to blocks of free carboxyl groups. Even a slight decrease of the average DM may produce a few blocks of carboxyl groups which deeply modify the behaviour of the pectin.

Turbidity measurements indicate that calcium sensitivity expresses itself as an increasing tendancy for aggregation and thus decreases the pectin solubility. Relative lower strength of the gels made of the lemon A pectin may be due to the association of the molecules via calcium ions, making them less available for participating in the gel network.

It is evident that gelling properties of HM pectins are not governed by one single parameter such as the MW but by a combination of many structural features. The determination of the influence of each individual parameter is greatly limited by the lack of techniques which allow the specific modification of one single parameter without affecting the others. One hope would be the synthesis of simplified pectin molecules. For real complex pectins, an alternative would be to derive multifactor relationships from the analysis of a large number of pectin samples. This is nowadays possible owing to the development of computerized multivariate analysis such as principal component analysis (PCA). However, this

requires that complex structural features such as the distribution of neutral side chains or methoxyl esters can be determined and accuratly quantified. Obviously it is in this direction that our future efforts must be directed.

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SUMMARY

The physical behaviour of industrial pectins is not yet completely understood. Pectin preparations which appear to be very similar on routine analysis may exhibit very different behaviour on applications such as jam making or protein stabilisation, especially in the presence of calcium ions. Despite intensive research efforts, there is still a lack of knowledge over specific structural features of industrial high methoxyl (HM) pectins. The aim of this work was to develop methods for the characterization of the chemical structure of three industrial HM pectins, chosen for their differences of behaviour on application.

Literature data relative to the nomenclature, role and applications of pectic substances are reviewed in chapter 1. Relevant literature about the structure and the analysis of pectins has been reviewed and discussed in chapters 2 to 7.

Chapter 2 describes the chemical composition of the three industrial HM pectins to be compared. The fraction which is not covalently bound to polygalacturonic acid, the so-called "ballast", has been removed by copper treatment. It contains neutral polysaccharides and, to a less extent, proteins and phenolic compounds. Purified pectin molecules are mainly composed of partly methoxylated polygalacturonic acid and neutral sugars but, also carry some phenolic and proteinaceous material. Pectin molecules of industrial apple and citrus preparations are very similar to those extracted under mild conditions from similar sources but carry fewer neutral sugars. This is probably due to the "trimming" of the side chains which occurs during the industrial hot-acid extraction. All three pectins have been found to be slightly acetylated (DAc 1.5 and 5.0 for lemon and apple pectins, respectively). The pectin extracted from apple contains more neutral sugars and more phenolics but fewer proteins than the two lemon pectins, which are very close to each other. However, one of the two lemon pectins (lemon A) contains a fraction that appears to be less esterified. The same lemon pectin also differs from the other pectin samples by its higher calcium ion content. Analytical methods have been discussed and improved in view of analysing small-quantity samples.

In chapter 3, a method has been developped for the determination of the intrinsic viscosity of pectin solutions by classical viscosimetry. A relation obtained by combining classical viscosity expressions allows the determination of the intrinsic viscosity from one single-concentration measurement without loss of accuracy.

The analysis of the whole pectin samples does not provide any information about differences between molecules (inter-molecular distribution). To get deeper insight into the sample heterogeneity, the three industrial pectins have been fractionated by means of preparative chromatographies.

Chapter 4 describes the size distribution of industrial pectins. The three industrial pectin samples have been fractionated on a preparative scale by size exclusion chromatography (SEC) and the chemical composition, viscosity and light scattering of the fractions have been investigated. Chemical analysis revealed that the composition varies greatly from one SEC fraction to another. In all three pectin samples, the fractions of low molecular size contain most of the free neutral polysaccharides as well as small pectin molecules rich in neutral sugars, probably originating from the degradation of some pectin molecules during the extraction process. In addition, the lemon pectin samples contain some pectin molecules of large size which are rich in neutral sugars. Phenolic and proteinaceous compounds co-elute with neutral sugar rich fractions. In the apple pectin, phenolics and proteins occur predominantly in the fractions of low molecular size. Lemon pectin molecules, especially that of the lemon A sample are prone to aggregation in the presence of calcium cations. The aggregate fraction can be disrupted by shear forces, heating or the addition of a chelating agent. The formation of such calcium-pectinate aggregates seems to be due to the presence of some molecules of low degree of methoxylation. Light scattering measurements suggest that even very narrow SEC fractions remain highly heterogeneous on the basis of their molecular weight, thus indicating large differences in molecular conformation.

Chapter 5 describes the intermolecular distribution of electrical charges in industrial pectins. The three industrial pectin samples have been fractionated on a preparative scale by ion exchange chromatography (IEC) and chemical analysis of the resulting fractions revealed that pectin molecules varying in charge also differ in composition. Neutral sugars, phenolic and proteinaceous compounds were found to coelute with pectin molecules. The non-uronide material appeared to be associated with all pectin molecules, but mainly attached to those requiring a high ionic strength to be released from the ion exchanger. Despite large quantitative differences, the distribution of individual neutral sugars among the molecules was found very similar for pectins from lemon and apple, indicating a great similarity in structural features. Separation of pectin molecules by IEC was found to depend on many different chemical parameters such as degree of methoxylation but probably also the intramolecular distribution of free carboxyl groups and the presence of phenolics. This makes the interpretation of ion exchange elution profiles difficult and uncertain.

The inter-molecular distribution of the structural features, especially that of methoxyl esters has proved to be important in explaining the reactivity of pectin molecules towards calcium ions. In addition, differences in physical behaviour may also depend on the sequence in which the various structural units are arranged (intra-molecular distribution).

The β-elimination reaction which splits specifically the glycosidic linkages next to methoxylated galacturonic acid units without steric limitation may be used for the determination of the sequence of methoxyl esters along pectin molecules. However, because of competitive de-esterification, completeness of the reaction has never been achieved. In chapter 6, the influence of temperature, pH and buffer concentration on the β-elimination reaction in aqueous solution has been investigated in order to improve the extent of degradation. Examination of the rates of reaction revealed that any increase of temperature increases the speed of the β-elimination reaction more than that of the de-esterification. On the other hand, any decrease of pH decreases the speed of β-elimination but, much less than that of de-esterification. Buffer concentration plays an important rôle by controling the pH through its buffering capacity as well as the availability of hydroxyl ions. Increasing the temperature up to 115°C and decreasing the pH down to 5.0 improved the splitting of the glycosidic bonds of a high methoxyl pectin backbone from 5.5 to 38% (53% of the glycosidic bonds contiguous to a methoxylated galacturonide unit). In spite of the appearance of various secondary reaction products, the absorbance at 235 nm can be used to estimate the extent of degradation.

In chapter 7, the three industrial HM pectins purified by copper precipitation have been degraded by acid, chemical β-elimination and endopolygalacturonase. Degraded pectins from β-elimination and enzyme treatment have been fractionated by preparative SEC and the resulting fractions have been analysed for their neutral sugar, protein and phenolic contents. Distribution of neutral sugars indicates that the model of alternating "hairy" and "smooth" regions is also valid for industrial pectins. Only some of the phenolics present in the copper purified samples were found to be bound to the segments rich in neutral sugars. Proteins are associated with the hairy regions. Oligomers with DP up to 17 produced by the action of endoPG were analysed by high performance ion exchange chromatography and their relative proportions were compared with a theoritical distribution model. The abundance of mono-, di- and trigalacturonides suggests that, in the smooth regions of industrial pectins, free carboxyl groups are present as blocks of unesterified galacturonide units. SEC elution patterns of acid-degraded samples suggest that homogalacturonan blocks of average DP 40-60

are interrupted by rhamnose units whereas degradations by β-elimination and endoPG indicate that most of the rhamnose units are concentrated in the regions carrying the side chains. This would mean that the pectin backbone is constituted of rhamnogalacturonan regions alternating with homogalacturonans which are interrupted at regular intervals by rhamnose units.

In chapter 8, the three industrial HM pectins have been compared on the basis of their physical behaviour. In aggreement with published data, gel strength appeared to depend mainly on the degree of polymerization and the content of galacturonic acid, and is only slightly affected by the amount of side chains and/or free neutral polysaccharides. Furthermore, one of the two lemon pectins exhibited a lower gel strength as well as a higher stability constant of calcium. A simple, sensitive and reproducible test has been developed to characterize the calcium sensitivity of HM pectins. Higher affinity for calcium may explain higher calcium sensitivity, lower solubility and consequently lower gelling performance in the presence of calcium ions. Such affinity for calcium ions can be ascribed to the action of pectin methyl esterase in lemon peels before the pectin extraction.

The data from only three pectin samples do not allow to draw unquestionable conclusions about the relation structure-physical behaviour of industrial HM pectins. However, it seems that several properties difficult to understand such as solubility or gel strength, are strongly related to the inter- and/or intra-molecular distribution of methoxyl esters in the pectin samples. A blockwise distribution of even a minor proportion of the free carboxyl acids leads to a higher sensitivity towards calcium ions and may decrease the gelling strength. From the data collected in this thesis, it appears that it is extremely difficult to analyse blocks of non-methoxylated galacturonosyl residues when they only represent a minor proportion of the total non-methoxylated galacturonosyl residues (the others being probably randomly distributed along the pectin backbone). However, the effect of their presence can be easily quantified by measuring the calcium-sensitivity.

SAMENVATTING

Voor het fysisch-chemische gedrag van industriële pektines bestaat nog geen eenduidige verklaring. Pektinepreparaten die op basis van gangbare analytische parameters grote overeenkomsten vertonen kunnen onderling zeer verschillen in hun gedrag bij toepassingen zoals jambereiding of eiwitstabilisatie. Dit geldt met name wanneer calciumionen aanwezig zijn. Ondanks intensieve onderzoeksinspanningen ontbreekt nog steeds de nodige kennis van de specifieke structuureigenschappen van industriële pektines. Het doel van het voorliggende onderzoek was het ontwikkelen van methodes voor de karakterisering van de chemische structuur van drie industriële hoogveresterde pektines (high methoxyl = HM) die gekozen werden vanwege hun onderling verschillend gedrag bij applicatie.

Hoofdstuk 1 bevat een overzicht van literatuurgegevens met betrekking tot de nomenclatuur, rol en toepassingen van pektines. Relevante literatuur met betrekking tot de structuur en analyse van pektines wordt gegeven en besproken in de hoofdstukken 2 tot en met 7.

In hoofdstuk 2 wordt de chemische samenstelling van de drie te vergelijken industriële HM pektines gegeven. De pektines werden allereerst gezuiverd door de fraktie die niet covalent gebonden is aan het polygalacturonzuur, de zogenaamde "ballast", te verwijderen door behandeling met koper. Deze fraktie bestaat uit neutrale polysacchariden en in mindere mate uit eiwitten en fenolen. Gezuiverde pektinemolekulen zijn grotendeels opgebouwd uit gedeeltelijk veresterde galacturonzuurbouwstenen en neutrale suikers maar bevatten ook wat fenolen en eiwitten. De pektinemolekulen van industriële appel- en citruspreparaten vertonen veel overeenkomsten met pektines die onder milde omstandigheden geëxtraheerd worden uit dezelfde bronnen, ze bevatten echter minder neutrale suikers. Dit is waarschijnlijk te wijten aan het afsplitsen van pektine-zijketens tijdens de in de industrie gangbare extractie bij temperaturen tussen 60 en 100 °C. Alle drie pektinepreparaten bleken in lichte mate geacetyleerd te zijn (acetyleringsgraad 1,5 en 5,0 voor citrus- en appelpektine respectievelijk). Het uit appel geëxtraheerde pektine bevat meer neutrale suikers en meer fenolen maar minder eiwit dan de twee citruspektines, die sterk op elkaar lijken. Eén van de twee citruspektines echter bevat een fraktie die minder veresterd blijkt te zijn. Dit citruspektine verschilt ook van de andere pektinemonsters door een hoger gehalte aan calciumionen. Analysemethoden zijn besproken en verbeterd met het oog op analyse van monsters op kleine schaal.

In hoofdstuk 3 is de ontwikkeling beschreven van een methode om de intrinsieke viscositeit van pektine-oplossingen te meten met behulp van klassieke viscosimetrie. Een formule die verkregen werd door klassieke viscositeitsvergelijkingen te combineren maakt het mogelijk de intrinsieke viscositeit te bepalen door meting bij één enkele concentratie zonder dat dit leidt tot verlies van nauwkeurigheid.

De analyse van de pektinemonsters als geheel verschaft geen informatie over de verschillen tussen molekulen onderling (intermolekulaire verdeling). Om een beter inzicht te verkrijgen in de heterogeniteit van de monsters zijn de drie industriële pektines gefraktioneerd met behulp van preparatieve chromatografische technieken.

Hoofdstuk 4 beschrijft de molekuulgewichtsverdeling van de industriële pektines. De drie pektines zijn gefraktioneerd op preparatieve schaal met behulp van gelpermeatiechromatografie en de chemische samenstelling, viscositeit en lichtverstrooiing van de frakties is bepaald. Uit chemische analyse bleek dat de samenstelling sterk varieerde tussen de verschillende gelpermeatiefrakties. Voor alle drie de monsters geldt dat de fraktie met het laagste molekuulgewicht de meeste neutrale suikers bevat. Alle drie pektines bevatten ook kleinere pektinemolekulen die rijk zijn aan neutrale suikers en waarschijnlijk ontstaan zijn door afbraak van pektinemolekulen tijdens het extractieproces. Bovendien bevatten de citruspektines grote pektinemolekulen die rijk zijn aan neutrale suikers. In het appelpektine komen fenolen en eiwitten vooral voor in de frakties met het laagste molekuulgewicht. Citruspektine-molekulen, met name die van citrus A, zijn gevoelig voor aggregatie in de aanwezigheid van calciumionen. De aggregaten kunnen verbroken worden door afschuifkrachten, verhitten of toevoegen van een chelator. De vorming van zulke calciumpektinaat-aggregaten is waarschijnlijk te wijten aan de aanwezigheid van molekulen met een lage veresteringsgraad. Uit lichtverstrooiingsmetingen volgt dat zelfs gelpermeatiefrakties met een smalle molekuulgewichtsverdeling eveneens in hoge mate heterogeen zijn in molekuulgewicht, hetgeen duidt op grote verschillen in molekulaire konformatie.

Hoofdstuk 5 beschrijft de intermolekulaire verdeling van ongemethoxyleerde galacturonzuurbouwstenen in industriële pektines. Daartoe zijn de drie pektinemonsters op preparatieve schaal gefraktioneerd met behulp van ionenwisselingschromatografie. Chemische analyse van de resulterende frakties laat zien dat de pektinemolekulen die in lading verschillen ook verschillen in samenstelling. Neutrale suikers, fenolen en eiwitten bleken te coëlueren met pektinemolekulen. Het niet-uronide materiaal bleek voor te komen in alle pektinefrakties, maar vooral in die frakties die pas bij hoge ionsterkte van de ionenwisselaar vrijkwamen.

Ondanks grote kwantitatieve verschillen was de verdeling van de afzonderlijke neutrale suikers over de molekulen grotendeels hetzelfde voor citrus- en appelpektine, hetgeen wijst op grote overeenkomsten in hun fijnstructuur. Scheiding van pektinemolekulen met ionenwisselingschromatografie bleek afhankelijk te zijn van meerdere chemische parameters zoals methoxyleringsgraad maar waarschijnlijk ook de intramolekulaire verdeling van vrije carboxylgroepen en de aanwezigheid van fenolen. Dit maakt de interpretatie van ionenwisselings-elutieprofielen moeilijk en onzeker.

De intermolekulaire verdeling van met name de methoxylesters blijkt erg belangrijk te zijn bij het verklaren van de reaktiviteit van pektinemolekulen met betrekking tot calciumionen. Bovendien kunnen verschillen in fysisch-chemisch gedrag ook afhangen van de volgorde waarin de verschillende structuureenheden gerangschikt zijn (intramolekulaire verdeling).

De ß-eliminatiereaktie, die specifiek die glycosidische bindingen verbreekt die grenzen aan gemethoxyleerde galacturonzuureenheden zonder daarbij hinder te ondervinden van vertakkingen, kan gebruikt worden voor de bepaling van de verdeling van methylesters over pektinemolekulen. Vanwege competitieve deësterificatie heeft men echter nog nooit een volledige afbraak kunnen bewerkstelligen. In hoofdstuk 6 is de invloed van temperatuur, pH en bufferconcentratie op de ß-eliminatiereaktie in waterige oplossing onderzocht om de mate van afbraak te verhogen. Uit kinetische studies bleek dat elke temperatuurtoename de snelheid van ß-eliminatie sterker deed toenemen dan de snelheid van deësterificatie. Aan de andere kant zorgde pH-daling voor een daling van de ß-eliminatiesnelheid, echter in veel mindere mate dan de snelheid van deësterificatie. De bufferconcentratie speelt een belangrijke rol in verband met zowel het constant houden van de pH door middel van de buffercapaciteit als de beschikbaarheid van hydroxylionen. Verhoging van de temperatuur naar 115 °C en verlaging van de pH naar 5,0 betekende een verhoging van het percentage gesplitste glycosidische bindingen van een hoogveresterde pektinebackbone van 5,5 naar 38% (53% van de glycosidische bindingen grenzend aan een veresterde galacturonzuurbouwsteen). Ondanks het ontstaan van verschillende secondaire reaktieprodukten kan de extinctie bij 235 nm gebruikt worden om de mate van degradatie te schatten.

In hoofdstuk 7 is de afbraak van de drie door middel van koperprecipitatie gezuiverde pektines met zuur, chemische ß-eliminatie en endopolygalacturonase besproken. De door ß-eliminatie en enzymbehandeling afgebroken pektines zijn gefraktioneerd met behulp van preparatieve gelpermeatiechromatografie (size exclusion chromatography = SEC) en de resulterende frakties zijn geanalyseerd op

neutrale suikers, eiwit en fenolen. De verdeling van de neutrale suikers geeft aan dat het model van afwisselend voorkomende "hairy" en "smooth regions" ook geldt voor industriële pektines. Slechts een gedeelte van de fenolen aanwezig in de met koper gezuiverde monsters bleek gebonden aan de neutrale suiker-rijke frakties. Eiwitten zijn gekoppeld aan de hairy regions. Oligomeren met een polymerisatiegraad tot en met 7, verkregen door endoPG inwerking, werden geanalyseerd door middel van HPIEC (high performance ion exchange chromatography). Hun onderlinge verhoudingen werden vergeleken met een theoretisch verdelingsmodel. Het overvloedig voorkomen van mono-, di- en trigalacturoniden geeft aan dat in de smooth regions van industriële pektines vrije carboxylgroepen voorkomen als blokken van onveresterde galacturonide-eenheden. SEC elutiepatronen van door zuur afgebroken monsters maken aannemelijk dat homogalacturonaanblokken met een gemiddelde polymerisatiegraad van 40 tot 60 onderbroken worden door rhamnose-eenheden terwijl afbraak met ß-eliminatie en endoPG aangeeft dat de meeste rhamnose-eenheden geconcentreerd voorkomen in die gebieden die zijketens bevatten. Dit zou betekenen dat de pektinehoofdketen is opgebouwd uit afwisselend rhamnogalacturonanen en homogalacturonanen, welke laatste met regelmatige intervallen onderbroken worden door rhamnose-eenheden.

In hoofdstuk 8 zijn de drie industriële HM pektines vergeleken op basis van hun fysisch-chemische gedrag. In overeenstemming met gepubliceerde gegevens bleek de gelsterkte voornamelijk af te hangen van de polymerisatiegraad en het galacturonzuurgehalte en wordt zij slechts in lichte mate beïnvloed door de hoeveelheid zijketens en/of vrije neutrale polysacchariden. Verder vertoonde één van de twee citruspektines zowel een lagere gelsterkte als een hogere stabiliteitsconstante voor calcium. Een simpele, gevoelige en reproduceerbare test is ontwikkeld om de calciumgevoeligheid van HM pektines te karakteriseren. Een grotere affiniteit voor calcium kan een hogere calciumgevoeligheid verklaren alsmede een lagere oplosbaarheid en dientengevolge slechtere geleringseigenschappen in de aanwezigheid van calciumionen. Deze affiniteit voor calciumionen kan toegeschreven worden aan de werking van pektinemethylesterase in de citrusschil voorafgaand aan de extractie.

De verzamelde gegevens van slechts drie pektinemonsters laten geen harde conclusies toe omtrent de relatie tussen structuur en fysisch-chemisch gedrag van industriële HM pectines. Het lijkt er echter op dat een aantal moeilijk te begrijpen eigenschappen zoals oplosbaarheid of gelsterkte sterk gerelateerd zijn aan de inter- en/of intramolekulaire verdeling van methoxylesters in de pektinemonsters. Een bloksgewijze verdeling van zelfs een klein gedeelte van de vrije carboxylzuren leidt al tot een hogere gevoeligheid ten opzichte van calciumionen en kan de gelsterkte

al verlagen. Uit de verzamelde gegevens blijkt dat het uitzonderlijk moeilijk is om blokken van ongemethoxyleerde galacturonzuureenheden te analyseren die slechts een klein gedeelte van het totaal aan ongemethyleerde galacturonzuurresiduen vertegenwoordigen (de rest is waarschijnlijk at random verdeeld over de pektinebackbone). Het effect van de aanwezigheid van deze blokken echter kan gemakkelijk gekwantificeerd worden door de calciumgevoeligheid te meten.

RESUME

Le comportement physique des pectines industrielles n'est encore pas complètement élucidé. Des préparations de pectine qui apparaissent comme étant très proches l'une de l'autre en analyse de routine peuvent se comporter de façon très différente dans des applications telles que la confection de confitures ou la stabilisation des protéines, particulièrement en présence d'ions calcium. Malgré des efforts de recherche très intenses, notre connaissance des éléments structuraux constituant les pectines industrielles hautement méthylées (pectines HM) présente encore de larges lacunes. Le but de ce travail était de développer des méthodes de caractérisation de la structure chimique de trois pectines HM industrielles, choisies pour leurs différences de comportement en application.

Les données bibliographiques relatives à la nomenclature, au rôle et aux applications des substances pectiques ont été passées en revue dans le chapitre 1. La bibliographie concernant la structure et l'analyse des pectines a été décrite et discutée dans les chapitres 2 à 7.

Le chapitre 2 décrit la composition chimique des trois pectines HM industrielles qui doivent être comparées. La fraction qui n'est pas liée à l'acide polygalacturonique par des liaisons covalentes, appelée "ballast", a été éliminée par traitement au cuivre. Elle contient des polysaccharides neutres et, dans en moindre quantité, des protéines et des composés phénoliques. Les molécules de pectine purifiées sont principalement constituées d'acide polygalacturonique partiellement méthoxylé et de sucres neutres, mais elles portent aussi quelques matières phénoliques et protéiques. Les molécules de pectine des préparations industrielles extraites de citron et de pomme sont très similaires à celles extraites des mêmes sources par des procédures douces, mais portent moins de sucres neutres. Ceci est probablement dû au phénomène de "rognage" des chaines latérales qui se produit pendant l'extraction industrielle, en milieu acide et chaud. Les trois pectines ont été trouvées légèrement acétylées (DAc 1,5 et 5,0 pour les pectines de citron et de pomme, respectivement). La pectine extraite de pomme contient plus de sucres neutres et plus de composés phénoliques mais moins de protéines que les deux pectines de citron qui sont très proches l'une de l'autre. Néanmoins, l'une des deux pectines de citron (citron A) contient une fraction qui semble être moins estérifiée. Cette même pectine de citron se différencie aussi des deux autres échantillons par sa plus forte teneur en calcium. Les méthodes analytiques ont été examinées de façon critique et améliorées dans le but d'analyser des micro-échantillons.

Dans le chapitre 3, une méthode de détermination de la viscosité intrinsèque des solutions de pectine par viscosimetrie classique a été développée. Une relation obtenue par la combinaison de plusieurs expressions classiques de la viscosité permet la détermination de la viscosité intrinsèque à partir d'un seul point expérimental sans perte de précision.

L'analyse globale des échantillons de pectine ne fournit pas d'information sur les differences existant entre les molécules (distribution intermoléculaire). Afin de mieux comprendre l'hétérogénéité des échantillons, les pectines industrielles ont été fractionnées par chromatographie préparative.

Le chapitre 4 décrit la distribution de taille des pectines industrielles. Les trois échantillons de pectine industrielle ont été fractionnés à l'échelle préparative par chromatographie d'exclusion stérique (SEC), et la composition chimique, la viscosité et la diffusion de la lumière de chaque fraction ont été étudiées. L'analyse chimique révèle que la composition varie de façon importante d'une fraction à l'autre. Dans chacun des trois échantillons, la fraction des petites masses moléculaires contient la plupart des polysaccharides neutres ainsi que quelques petites molécules de pectine riches en sucres neutres, provenant sans doûte de la dégradation de quelques molécules de pectine pendant la procédure d'extraction. De plus, les échantillons de pectine de citron contiennent quelques molécules de grande taille qui sont riches en sucres neutres. Les composés phénoliques et les protéines co-éluent avec les fractions riches en sucres neutres. Dans la pectine de pomme, les composés phénoliques et les protéines sont principalement présents dans les fractions de faible masse moléculaire. Les molécules des pectines de citron, particulièrement celles de l'échantillon A ont tendance à s'aggréger en présence d'ions calcium. Les aggrégats peuvent être brisés par des forces de cisaillement, un chauffage ou l'addition d'agents chélatants. La formation de tels aggrégats de pectinate de calcium semble être dûe à la présence de quelques molécules de faible degré de methoxylation. Les mesures de diffusion de la lumière suggèrent que même des fractions de SEC très étroites sont encore très hétérogènes sur la base de leur masse moléculaire, ce qui indique de larges différences de conformation moléculaire.

Le chapitre 5 décrit la distribution intermoléculaire des charges électriques dans les pectines industrielles. Les trois échantillons de pectine ont été fractionnés à l'échelle préparative par chromatographie d'échange d'ions (IEC) et l'analyse chimique des fractions a révelé que les molécules de pectine qui diffèrent en charge se différencient aussi par leur composition. Les sucres neutres, les composés phénoliques et les protéines co-éluent avec les molécules de pectine. Les constituants autres que l'acide galacturonique semblent être associés avec toutes les

molécules de pectine, mais principalement avec celles qui nécessitent une haute force ionique pour éluer de la colonne d'échange d'ions. Malgré de larges différences quantitatives, la distribution des divers sucres neutres parmi les molécules de pectine est apparue très similaire pour les pectines de citron et de pomme, indicant de grandes similarités de structure. La séparation des molécules de pectine par échange d'ion s'est révélée être dépendante de plusieurs paramètres chimiques tels que le degré de méthoxylation mais probablement aussi de la distribution intramoléculaire des acides carboxyliques libres et de la présence de composés phénoliques. Cela rend l'interprétation des profils d'élution d'échange d'ion difficile et incertaine.

La distribution intermoléculaire des éléments structuraux, particulièrement celle des esters methyliques est apparue comme très importante pour expliquer la réactivité des molécules de pectine envers les ions calcium. Par ailleurs, des différences de comportement physique peuvent aussi dépendre de la séquence dans laquelle les différents éléments structuraux sont arrangés (distribution intramoléculaire).

Il aété proposé que la réaction de ß-élimination qui rompt spécifiquement les liaisons glycosidiques contigües à un résidu acide galacturonique méthoxylé, sans limitation stérique, soit utilisée pour la détermination de la distribution des esters méthyliques lelong des molécules de pectine. Néanmoins, à cause de la compétition avec une réaction de désestérification, la réaction complète n'a jamais pu être obtenue. Dans le chapitre 6, l'influence de la température, du pH et de la concentration de la solution tampon sur la réaction de ß-élimination en milieu acqueux a été étudiée dans le but d'améliorer le rendement de la réaction. L'étude des vitesses de réaction a montré que toute augmentation de température augmente le rendement de la réaction plus que celui de la réaction de désestérification. D'autre part, toute diminution de pH diminue la vitesse de ß-élimination mais bien moins que la vitesse de désestérification. La concentration de la solution joue un rôle très important en controlant le pH par l'intermédiaire du pouvoir tampon, ainsi qu'en déterminant la disponibilité des ions hydroxyle. En augmentant la température jusqu'à 115°C et en abaissant le pH jusqu'à 5,0, le pourcentage de liaisons glycosidiques rompues a été amélioré de 5,5 à 38% (53% des liaisons glycosidiques contigües à un acide galacturonique méthoxylé). Malgré l'apparition de nombreux produits de réaction secondaires, l'absorbance à 235 nm peut être utilisée pour estimer l'état d'avancement de la réaction de ß-élimination.

Dans le chapitre 7, les trois échantillons de pectine industrielle purifiées par précipitation au cuivre ont été dégradées par hydrolyse acide, ß-élimination chimique et l'enzyme endopolygalacturonase. Les produits de dégradation obtenus

par ß-élimination et traitement enzymatique ont été fractionés par SEC préparative, et les fractions obtenues ont été analysées pour leur composition en sucres, en protéines et en composés phénoliques. La distribution des sucres neutres indique que le modèle des zones chevelues alternant avec des zones lisses est aussi valable pour les pectines industrielles. Seule une petite partie des composés phénoliques présent dans les pectines purifiées se trouve associée aux segments riches en sucres neutres. Les protéines sont associées aux zones chevelues. Les oligomères de taille inférieure ou égale à 17 produits par l'action de l'endopolygalacturonase ont été analysés par chromatographie d'échange d'ions à haute performance, et leur proportions relatives ont été comparées à une distribution théorique modèle. L'abondance des mono-, di- et trimères d'acide galacturonique suggère que, dans les zones lisses des pectines industrielles, les acides carboxyliques libres sont regroupés en blocs de résidus acides carboxyliques non estérifiés. Les profils d'élution SEC des pectines dégradées par hydrolyse acide suggèrent que des séquences homogènes d'acide galacturonique de 40 à 60 résidus alternent avec des unités de rhamnose, alors que les dégradations par ß-élimination et endopolygalacturonase indiquent que la plupart des unités rhamnose sont concentrées dans les régions portant les chaines latérales de sucres neutres. Cela pourrait vouloir dire que la chaine principale de la pectine est en fait constituée de régions rhamno-galacturoniques alternant avec des zones homogènes d'acide galacturonique, ces dernières étant interrompues à intervalle régulier par des unités rhamnose isolées.

Dans le chapitre 8, les trois pectines HM industrielles ont été comparées sur la base de leur comportement physique. En accord avec les données publiées, la force de gel est apparue dépendre principalement du degré de polymérisation et de la teneur en acide galacturonique, et n'est que faiblement affectée par la quantité de chaines latérales ou de polysaccharides neutres libres. De plus, une des deux pectines de citron a montré une plus faible force de gel ainsi qu'une forte constante de stabilité avec le calcium. Un test simple, sensible et reproductible a été développé pour caractériser la sensibilité au calcium des pectines HM. Une plus forte affinité pour les ions calcium pourrait expliquer une moindre solubilité et donc de moins bonne performances en gélification en présence d'ions calcium. Une telle affinité pour les ions calciums peut être attribuée à l'action de pectin méthyl estérase dans les peaux de citron avant l'extraction de la pectine.

Les données collectées à partir de trois échantillons de pectine seulement ne permettent pas de dresser des conclusions définitives quant à la relation structure chimique-comportement physique des pectines HM industrielles. Néanmoins, il semble que plusieurs propriétés difficiles à comprendre telles que la solubilité ou

la force de gel sont fortement liées à la distribution inter et/ou intramoléculaire des esters méthyliques dans les échantillons de pectine. Une distribution en bloc, même d'une très faible partie des acides carboxyliques libres conduit à une une plus forte sensibilité aux ions calcium et pourrait réduire le pouvoir gélifiant. Des données accumulées dans cette thèse, il apparait qu'il est extrêmement difficile d'analyser des blocs de résidus d'acide galacturonique non estérifiés quand ils ne représentent qu'une faible proportion des résidus d'acide galacturonique totaux (les autres étant probablement répartis de façon aléatoire lelong de la chaine pectique). Néanmoins, les effets de leur présence peuvent facilement être quantifiés par la mesure de la sensibilité au calcium.

CURRICULUM VITAE

Thierry Kravtchenko was born on the 9th of august, 1962 in Paris, France. After obtaining his Baccalaureat diploma in 1980, he graduated in 1986 from the Ecole Nationale d'Ingénieurs des Travaux Agricoles (ENITA, Dijon, France), with special emphasis on the characterization and analysis of agricultural products. In 1985-86 he was a research fellow at the department of Oenology of the University of Dijon (France) and obtained the first part of the National Diploma of Oenology. In 1986-87, he was a research fellow at the Department of Flavour Research of the National Institute for Agronomic Research (INRA, Dijon, France) and consequently received his M.Sc. degree from the Ecole Nationale Supérieure de Biologie Appliquée à la Nutrition et à l'Alimentation (ENSBANA, Dijon, France). Since december 1987 he has been working in the Laboratory of Food Chemistry, Department of Food Science at the Agricultural University of Wageningen (The Netherlands), conducting research on the structural features of industrial pectins. This work was supported by Sanofi Bio-Industries (Paris, France).
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