Effects of thermal processing on cell walls of green beans:

a chemical and ultrastructural study

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op het gebied van de Natuurwetenschappen

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LIST OF ABBREVIATIONS

ABTS	:2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
AIR	:alcohol insoluble residue
AUA	:anhydro uronic acid
buffer	:yield of buffer extraction after sterilisation
buf DM	:degree of methylation of buffer fraction after sterilisation
buf DA	:degree of acetylation of buffer fraction after sterilisation
Carb1	:yield of Na_2CO_3 extraction at 4°C after sterilisation
Carb2	:yield of Na_2CO_3 extraction at 20°C after sterilisation
CDTA	:cyclohexane-*trans*-1,2-diamine tetra acetate
CDTA	:yield of CDTA extraction after sterilisation (Chap-7 only)
CDTA DM	:degree of methylation of CDTA fraction after sterilisation
CDTA DA	:degree of methylation of CDTA fraction after sterilisation
CWM	:cell wall material
DM	:degree of methylation
DMSO	:dimethylsulfoxide
DW	:dry weight
FT-IR	:fourier transform infra-red spectroscopy
GalAs	:concentration of galacturonic acid residues in the brine after sterilisation
GalAbrine	:concentration of galacturonic acid residues in the brine after sterilisation
GPC	:Gel permeation chromatography
HEPES	:4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid.
HG	:homogalacturonan
HPLC	:High Performance Liquid Chromatography
HPSEC	:High Performance Size Exclusion Chromatography
M_r	:molecular mass relative to Dextran standards
NIR	:near infra-red spectroscopy
PME	:pectin methylesterase
phAIR	:yield of AIR after preheating
phRha	:rhamnose content of AIR after preheating
phAra	:arabinose content of AIR after preheating
phGal	:galactose content of AIR after preheating
phAUA	:galacturonic acid content of AIR after preheating
phDM	:degree of methylation of AIR after preheating
phDA	:degree of acetylation of AIR after preheating
Res	:yield of residue after sequential extractions of AIR
RG	:rhamnogalacturonan
SEM	:Scanning Electron Microscopy
stAIR	:yield of AIR after sterilisation
stRha	:rhamnose content of AIR after sterilisation
stAra	:arabinose content of AIR after sterilisation
stGal	:galactose content of AIR after sterilisation
stGalA	:galacturonic acid content of AIR after sterilisation
stDM	:degree of methylation of AIR after sterilisation
stDA	:degree of acetylation of AIR after sterilisation
SDS	:sodium dodecyl sulphate
TEM	:Transmission Electron microscopy
TFA	:trifluoroacetic acid
UA	:uronic acid
WIR	:water insoluble residue
WSP	:water soluble polymers

Chapter 1

General introduction

1.1 Vegetable and fruit processing

A large part of the dietary fibre intake in Europe originates from vegetables and fruits[1-3]. Since most fruits and vegetables can be harvested only at selected periods during the season, they are industrially processed to guarantee a year round availability. Food processing is generally understood to encompass all methods by which raw foodstuffs are rendered suitable for storage, cooking or consumption. Techniques for preserving vegetable food from natural deterioration following harvest date to prehistoric times. Among the oldest methods are drying, refrigeration and fermentation[4]. Modern preservation techniques include canning, freezing, the addition of chemicals, such as sucrose, salt, vinegar or benzoate, and to a lesser extend, drying. In addition, packaging has become increasingly important[5]. The principal causes of food spoilage are growth of micro-organisms, endogenous enzyme activity, oxidation and dehydration. The form of spoilage to which food is most susceptible depends on its inherent composition and structure, and exogenous factors, such as the abundance of specific micro-organisms and storage conditions. Activity and growth rates of micro-organisms can be affected by temperature, moisture, oxygen concentration, pH, ionic strength, nutrients available, degree of contamination with spoilage organisms and the presence or absence of growth inhibitors. Control of one or more of these factors suffices to inhibit microbial spoilage.

Heat sterilisation, or thermal processing, essentially consists of subjecting the food in a sealed can or jar to a known temperature long enough to destroy spoilage and pathogenic organisms that might be present in or on the raw food material. Reinfection through exposure to air is prevented by the can's permanent seal. In 1810 Nicholas Appert, a Parisian confectioner, first succeeded in preserving certain foods in sealed glass bottles that had been kept in boiling water. Peter Durand, an Englishman conceived and patented the idea of using tin cans instead of glass bottles and by 1839 tin coated steel containers were widely used. An early obstacle to mass production was the time necessary to process the cans in boiling water. In 1861 it was discovered that the addition of calcium chloride to boiling water raised the temperature of processing from 100 °C to 116 °C or higher. The time necessary to ensure microbial safety was reduced from 4 - 5 hours to 25 - 40 minutes and the average production could be increased from 2,500 to 20,000 cans a day. In 1874 the introduction of closed steam pressure retorts further shortened the time necessary for sterilisation. By 1960 containers of aluminium and plastic material became commercially feasible and also improved glass jars and bottles were in use.

The heat treatments necessary to preserve canned foods are determined by a number of factors, most important being the acidity of the food product in case. Non-acid foods, such as most vegetables, require higher temperatures for adequate processing than acid foods, such as fruits. Operations commonly included in the canning of vegetables, such as green beans, are cleaning, preparatory operations, blanching, filling and exhausting, can sealing, thermal processing, cooling, labelling and storage (Figure 1.1). Food preservation processes are still constantly being (re)designed to optimise microbial safety, nutrient retention, colour, taste, texture and other quality attributes of the end product[6]. Unfortunately one of the negative side effects of heat sterilisation still is extreme softening of the product, resulting in undesirable textural quality.

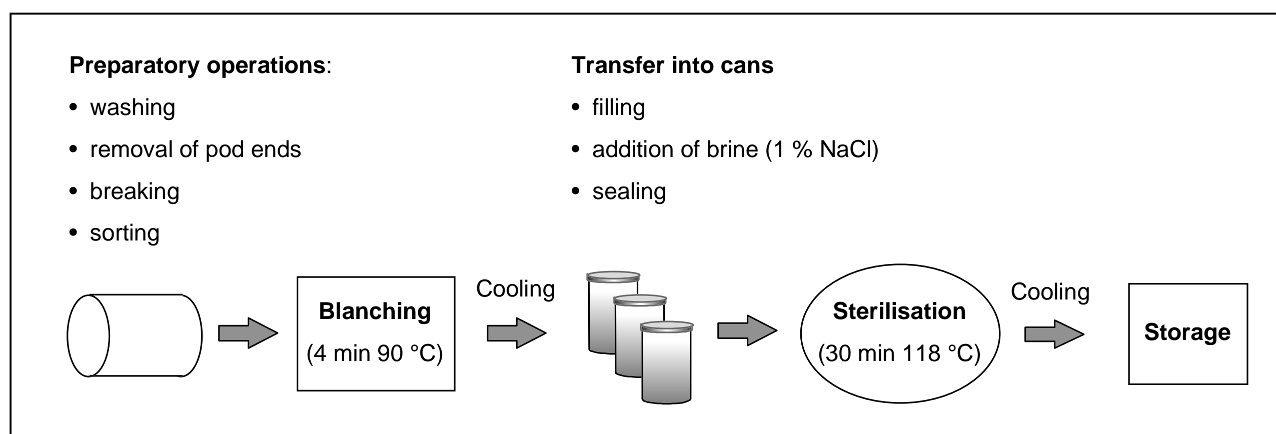

Figure 1.1 *Overview of operations in the standard canning process of green beans.*

1.2 Texture

The texture of processed vegetables is an important quality attribute. Texture is defined as: "all physical characteristics sensed by the feeling of touch, that are related to deformation under an applied force"[7]. Consumer perception of texture is often subconscious, but paradoxically, the pleasure experienced when eating fresh or processed plant foods is largely attributed to their texture. The importance of texture as a quality attribute is accented by the enormous real and economic losses incurred during harvesting and subsequent handling, distribution and storage that ultimately arise from textural deterioration and concomitant physico-mechanical damage.

Texture of vegetables is a sensory sensation and can best be analysed by the use of analytical sensory panels. Examples of typical textural descriptors are 'crisp', 'firm', and 'elastic'. However when processing is investigated, the texture of the raw product as well as the product at several stages during the process has to be quantified, which is not feasible by sensory panels. Therefore, mechanical properties of food, the basic elements of texture, are often measured instrumentally[8].

Texture is a characteristic at the plant organ level, which is dependent on several features of plant tissues, cells, and ultimate basically on structures and interactions of molecules and ions. Plant parts used for food, either stems, leaves, roots, flowers, fruit, seeds, or tubers, contain various tissue types, which play an essential role in determining texture. At first, the relative proportion of fibrous

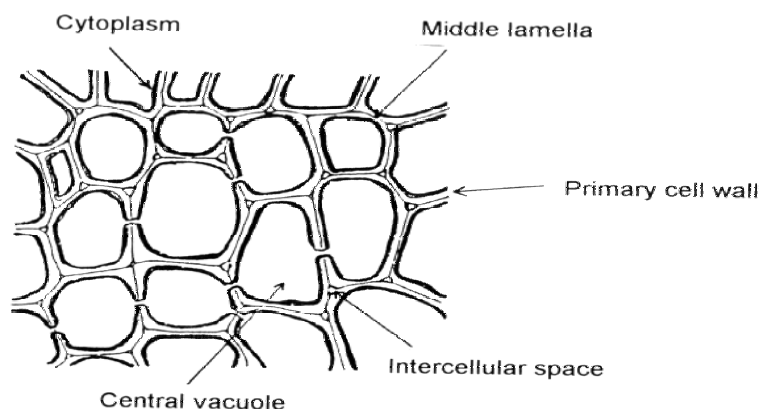

Figure 1.2 Schematic overview of typical parenchyma tissue (from ref[17]).

tissues, such as collenchyma, sclerenchyma and xylem, to the more tender parenchyma tissues should be considered. Most vegetables and fruits consist mainly of parenchyma tissues). Parenchyma cells have a diameter varying from 50 to 500 µm and are polyhedral or spherical in shape. Depending on the spatial arrangement and shapes of the constituent cells, parenchyma tissues can contain significant amounts of air space, which also influence texture[9]. Intracellularly, the cells contain one or more vacuoles, different organelles, a cytoskeleton and inclusion bodies in which important food components, such as starch, proteins and lipids, are stored. These components also effect the texture of the processed products. For example starch is highly associated with the development of a 'waxy' or 'mealy' texture of rice and potatoes[10,11]. The cell is surrounded by the plasma membrane. The texture of fresh products can for a large part be attributed to the turgor pressure generated within cells by osmosis through the plasma membrane. Turgor pressure causes the cells to swell, giving rigidity and crispness to the tissue[7]. However, the contribution of the plasma membrane to texture of processed products is considered to be low, since membrane functions are very quickly lost upon heating[13]. At the cellular level, texture is a manifestation of the ease with which cells can be split open and the ease with which they can be separated[14]. Texture of processed, low-starch vegetables and fruits is therefore mainly determined by the primary cell wall and middle lamella, located exterior to the plasma membrane. The cell wall of a parenchyma cell is in general thin, but strong, thereby limiting expansion and generating turgor pressure[12]. The middle lamella functions as a kind of cement, sticking the cells together and generating tissue strength. For example, analyses of tomato ultrastructure during ripening showed that degradation of the middle lamellae correlated highly with softening[7]. Secondary walls can be deposited internally to the primary wall after cessation of cell growth and are important in some cereal products like wheat bran. Their presence in vegetables is associated with the development of a 'woody' or 'stringy' texture, like for example in asparagus[15,16].

In conclusion, texture of thermally processed vegetables and fruits is mainly determined by (1) the ratio of thin walled parenchymatous tissue to other thick-walled vascular or epidermal tissues, (2) the abundance of specific components such as starch (3) the characteristics of cell walls and middle lamellae of the fresh product and (4) superimposed effects of processing on these characteristics.

1.3 Structural aspects of cell wall and middle lamella constituents

Cell walls contain numerous polymeric compounds that are capable to bear applied stress[18]. This is essential for the main functions of cell walls, which are determining size, shape and strength of plant tissues. The cell wall however also has a role in resistance to pathogens[19], and regulation of various metabolic processes[20-23]. The main cell wall constituents are water, pectin, hemicellulose and cellulose, but also protein and lignin can be present. The middle lamella is merely composed of pectin, and cannot always be discerned from the primary cell wall. Its main function is to cement the cells together.

Older concepts of the cell wall as a static, inert structure[24-26] have given way to new, more dynamic models[27-29]. These newer models differ predominantly from the earliest models in that the individual polymers are not all covalently linked to each other. At present, the cell wall is envisaged as three structurally independent, but interacting domains (figure 1.3). The first domain constitutes the cellulose-hemicellulose network, which is embedded in a second domain, a matrix of pectic substances. The third domain consists of structural proteins, oriented radially within the cell wall matrix. Cell wall polysaccharides are built up of about a dozen main sugars, which can be linked in an enormous variety of ways. The types of linkages, as well as the sugars themselves, dictate the

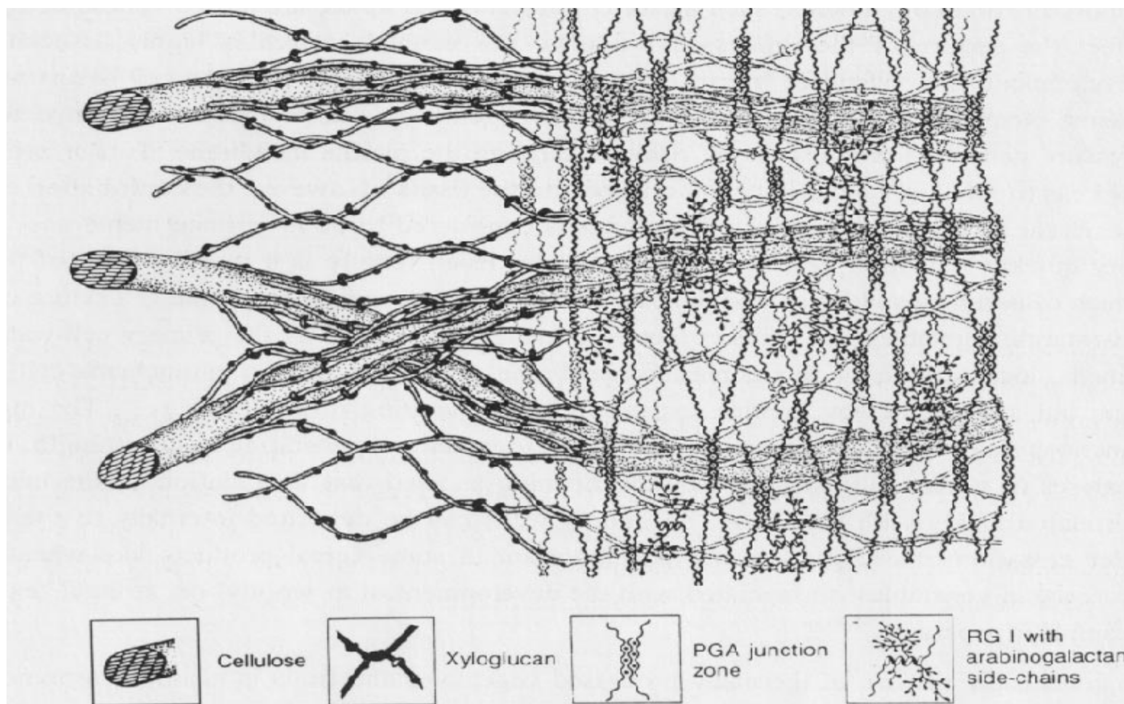

figure 1.3 *A schematic representation of the primary cell wall of most flowering plants except grasses (from ref[28]).*

properties of polysaccharides and thus their possible functions in the plant cell wall and effect on texture[30]. Properties commonly used to characterise the different cell wall polysaccharides are sugar composition, molecular mass distribution, shape, charge, extractability, and binding with other polymers.

1.3.1 Water

The primary cell wall is a highly hydrated structure, with water being one of its most variable features. Water is believed to have four major functions in the cell wall[31]:

- a structural component of the matrix gel
- a wetting agent, hindering direct hydrogen bonding between polymers
- a stabiliser of polymer conformation
- a solvent or transport medium for salts, enzymes and low molecular mass organic compounds.

1.3.2 Pectic substances

Pectic substances are very abundant in fruit and vegetable cell walls. Since they are considered to be very important in determining the texture of processed vegetables, they are discussed here in more detail than the other cell wall constituents. Pectin is not one single polymer, but comprises rather a group of so-called 'pectic substances'. Pectins are defined as hetero-polysaccharides rich in D-galacturonic acid, including side chains of such polysaccharides[30]. The term 'protopectin' is sometimes used to describe *native*, water unextractable, pectic polysaccharides within the cell wall, in recognition of the fact that the methods used to extract them are degradative. Pectins from middle lamella and cell walls are not distinguishable after extraction. There is some evidence that pectins from the middle lamella are less branched than those from the cell wall[32,33]. There are contradictionary data about the degree of methylation of middle lamella pectin, being either low or high[33-36] .

In general, three types of pectic components have been studied: homogalacturonan (HG or 'smooth regions'), and rhamnogalacturonan type I and II (RGI or 'hairy regions' and RGII). Consensus about this nomenclature was reached only recently[37]. While the group of Albersheim already used the nomenclature HG, RGI and RGII since the eighties, based on their work with cell walls from suspension cultured sycamore cells, Voragen and co-workers (De Vries, Renard, Schols) argued that the terms 'smooth' and 'hairy' regions were more appropriate to cover the structure of pectins of all higher plants[26,38-40]. They stated that the precise structure as described for RG I was not universal enough to describe the branched pectins found in all higher plants.

– *Polymer structure*

Pectins are 'block' polymers[41]. They contain linear homogalacturonan blocks ('smooth regions') and elsewhere in the same molecule, branched blocks of rhamnogalacturonan ('hairy regions') with neutral side chains[42] (Figure 1.4).

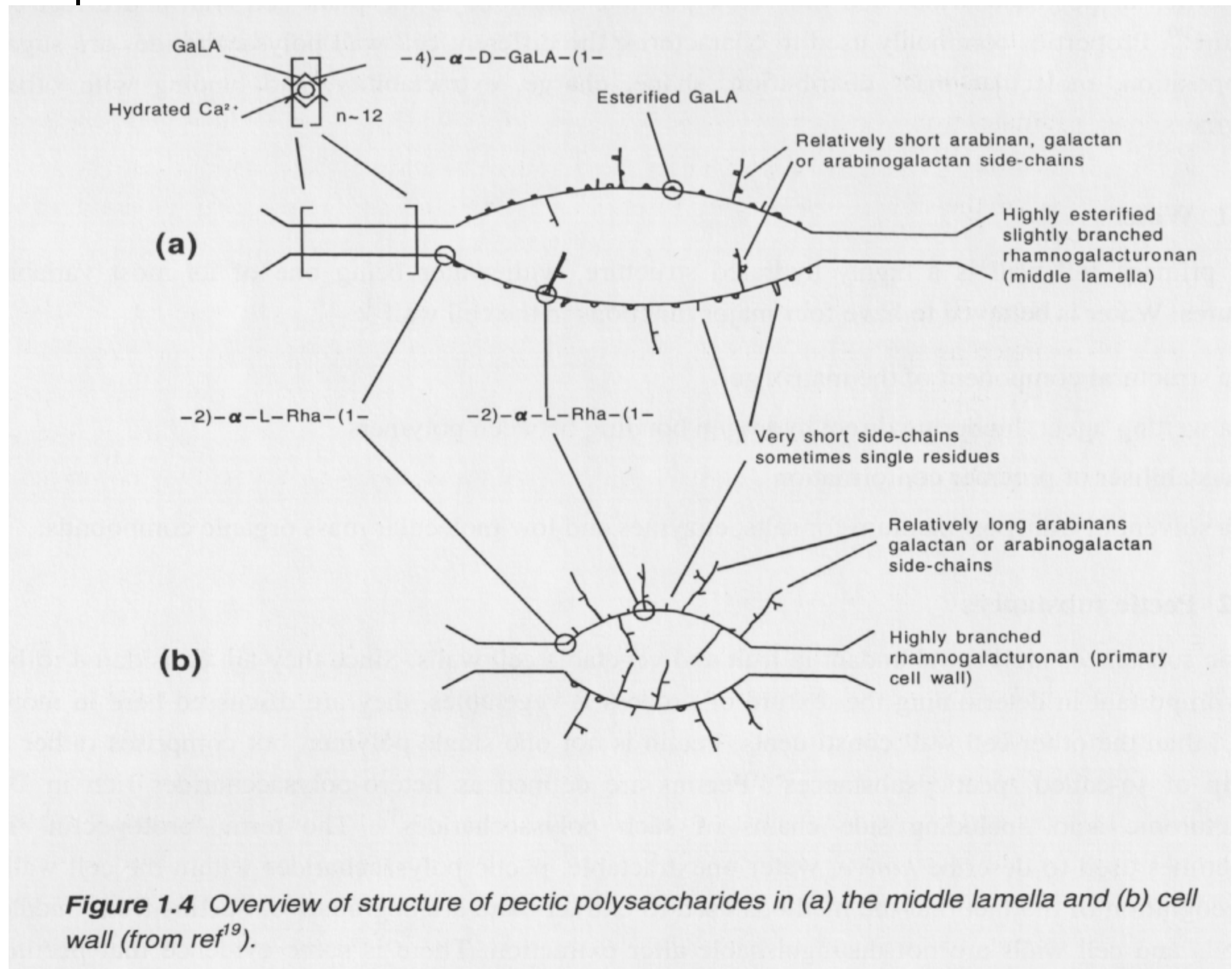

Figure 1.4 *Overview of structure of pectic polysaccharides in (a) the middle lamella and (b) cell wall (from ref[19]).*

Homogalacturonan consists of a backbone of mainly α-(1,4) galacturonic acid which can be interspersed with some α-(1,2) rhamnose residues. The spacing of the rhamnose residues may be regular, but this is not generally established[43]. The galacturonic acid residues can be substituted with methyl and acetyl esters on respectively the carboxylic acids on C-6 and the secondary alcohols on C-2 and C-3[44]. It has been suggested that the rhamnose residues act as molecular 'punctuation marks', with the intervening homogalacturonan blocks being in some cases fully methylesterified and in others not esterified at all[41,45]. The proportion of acetyl groups varies greatly with the plant origin, being for example very high (> 30 %) in sugar beet pectins. The molecular distribution of acetyl groups along the rhamnogalacturonan chain is unknown.

Rhamnogalacturonan consists of a backbone of α-(1,2) rhamnosyl and α-(1,4) galacturonic acid units. The ratio between rhamnose and galacturonic acid is not necessary 1:1, as was proposed in the early RG1 and RGII models of Albersheim and co-workers[46-48] but varies between 0.05 and 1[40]. Side chains consisting essentially of arabinans, galactans, arabinogalactans and single xylose residues are attached to the backbone. The main side for attachment is the O-4 of the rhamnosyl unit. The proportion of

rhamnosyl units with attached side chains varies between 20 to 80 %, depending on the source of the polysaccharide[37]. The amounts and length of the side chains varies with the cell type and physiological state. Talbott and Ray[27] proposed that these neutral polysaccharides also occur as free polymers. It cannot be excluded however, that these free neutral pectic polymers in their experiments originate from side chains split off from the pectic backbone during the cell wall preparation procedure. Feruloyl groups can be ester linked to the non-reducing arabinose and/or galactose termini of the pectic side chains in the 'hairy regions' of for example sugar beet and spinach pectins[49, 50].

Rhamnogalacturonan II is a small and complex pectic polymer[47,51]. The structure is extremely complex with as much as twelve different glycosyl residues, including rare sugars such as apiose, 2-*O*-methyl-L-fucose, aceric acid and common monosaccharides involved in unusual glycosidic linkages such as a fully substituted rhamnose. The main structure seems to be highly conserved during evolution and the complexity of the molecule suggests a role as signal molecule, rather than a structural function.

– *Cross-linking of pectins in the cell wall*

The types of crosslinks in primary cell walls as indicated in Figure 1.5 have been reviewed by Fry[52]. Some pectins can be solubilised with water, which indicates that they are not cross-linked at all. Non-esterified pectic homogalacturonan blocks of sufficient length are able to associate intermolecular in the presence of calcium ions (Figure 1.6)[43,53-55]. This type of association is called 'egg-box'[56]. Liners and co-workers have proposed, using a monoclonal antibody against a calcium induced supramolecular conformation of PGA, that an uninterrupted sequence of at least nine galacturonic acid residues is necessary on each of the two chains to dimerise with five calcium ions[35,57]. Subsequent induced aggregation of these homogalacturonan dimers into tetramers, hexamers etc. can occur[55]. However, at this moment there is some confusion about the existence and importance of 'egg-box' structures *in vivo*[35]. Acidic pectins could also be ionically linked to basic proteins such as extensin[52]. In addition to ionic binding zones in the 'smooth regions', pectins may be covalently cross-linked to other pectins or hemicelluloses by the formation of for example ferulic acid dimers[52,58,59]. There is also some evidence for the existence of other ester linkages between uronic acid residues and neutral sugars from for example cellulose[52].

– *Structure-function relationships of pectins*

As structural polysaccharides, pectins contribute to the strength and flexibility of cell walls from non-lignified plant organs and ripening fruits[41]. McCann and co-workers proposed that pectic compounds, including the neutral side chains, mainly determine the porosity of the cell wall[29,60]. By limiting the porosity below a certain level, pectins inhibit the free diffusion of enzymes through the wall and thereby enzyme-substrate interactions and hence cell wall autolysis.

The cementing function of pectic substances is thought to depend mainly on their non methyl-esterified homogalacturonan regions, which can form 'egg-box' structures with calcium, as is described above. Nevertheless, in suspension cultured carrot cells, the pectin of tightly attached

Figure 1.5 *Overview of the types of crosslinks in the primary wall (from ref[62]) (1. cellulose-cellulose, 2. xyloglucan-cellulose, 3. xylan-cellulose, 4. HG-HG 5. extensin-pectin, 6. extensin-extensin, 7. pectin-pectin, 8. arabinoxylan-arabinoxylan, 9. pectin-cellulose, 10. arabinogalactan-RGI, 11. pectin in extensin, (+)H-bonds, (o) Calcium bridges, (±) other ionic bonds, (:) coupled phenolics, (=) ester bonds, (-)glycosidic bonds, A = arabinose, F = fucose, G = glucose, L = galactose, R = rhamnose, U = galacturonic acid, Û = galacturonic acid methylester, a = amino acid other than tyrosine, y = tyrosine, y:y = isodityrosine, Ø = ferulic acid, Ø:Ø = diferulic acid.*

young callus was found to be composed of high methylesterified galacturonic acid, while that of loosely attached old callus was composed of non-methyl-esterified galacturonic acid[35]. This suggest that the formation of so-called 'egg-box' structures is not associated with intercellular cohesion *in vivo*. Moreover, the ratio of arabinose to galactose was highly correlated (R = 0.94) with the formation of cell clusters and intercellular attachment which, in contrast to the general idea, suggests an important role for these neutral side chains in intercellular adhesion[61]. Very few specific data on the contribution of pectic side chains on textural properties are available[62,63]. Neutral side-chains of pectins are usually considered to hinder gel formation. In fact, they themselves may be capable of weak non-covalent interactions, such as double helices of ß-(1,4)-galactan[64] and partly crystalline aggregates of linear α-(1,5)-arabinans[65]. However, there is no experimental evidence that binding of this type contributes to the strength of plant cell walls. A hint for the importance of side chains in relation to intrinsic texture is presented by Batisse and co-workers[66]. Pectic side chains of crisp cherry fruits were found to be longer and more cross-linked in comparison with pectin from soft

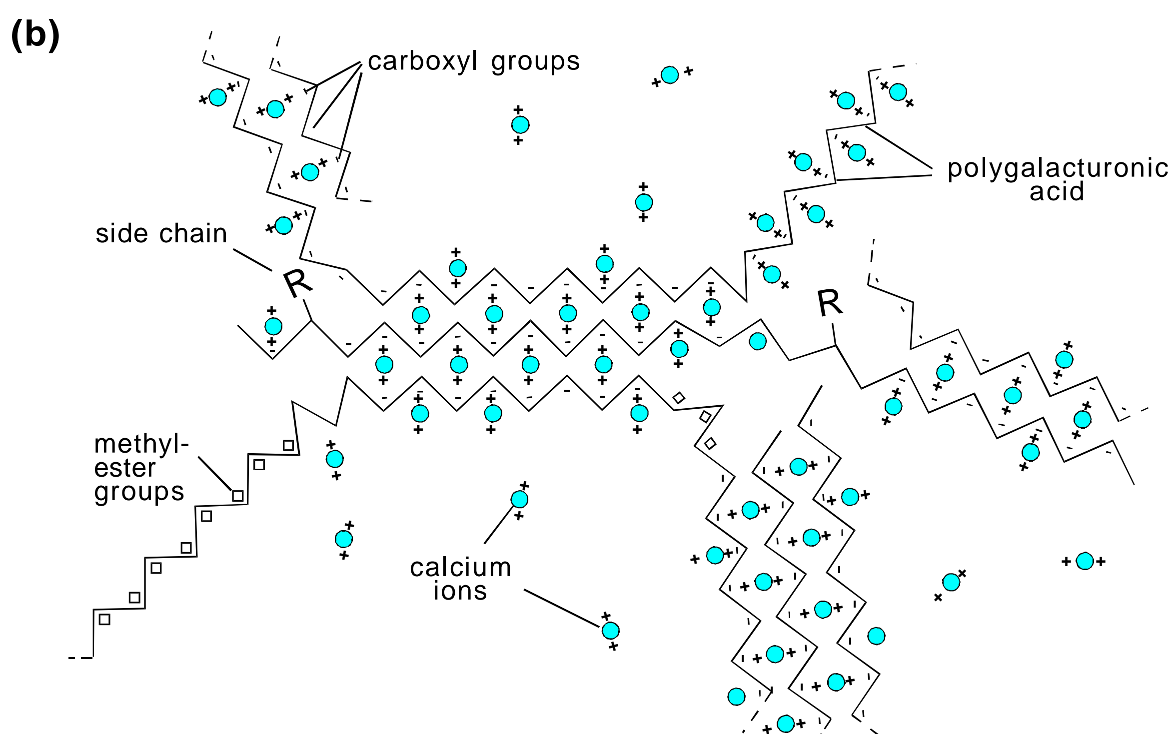

Figure 1.6 *Egg box model of Morris et al.*[55] *showing cross-linking between unsubstituted homogalacturonan and the factors that are likely to influence such cross-linking within the cell wall (from ref*[19]*).*

fruits. For potatoes however, it was reported that either a greater amount, or longer pectic side chains resulted in less firm potatoes and less sensibility to prewarming[67]. (for details on prewarming, see below). A decrease in branching and concomitant higher solubility is described for pectin from many fruits during storage, ripening and concurrent softening. This is the result of either overall degradation of existing pectins, such as described for tomato[68-70], guava[71], mango[72,73], peach,[74], nectarine[75,76] and kiwi[77,78], or a changed metabolic turnover, as was shown for apples[79,80]. In the case of pectin degradation, the softening seemed to be related both with a lower degree of branching and polymerisation. In the case of turnover however, the newly synthesised pectins also had a low degree of branching, but a high degree of polymerisation. This was also accompanied by a higher DM, which was altogether proposed to result in less calcium complexed intercellular cohesion and hence tissue softening[41].

1.3.3 Cellulose

Cellulose is a structural component of all higher plant cell walls. The structure consists of linear chains with ß-(1,4)glucosyl residues. The chains are associated into microfibrils with crystalline and amorphous regions, which make the cellulose structure more divers than the single building units

suggest. The degree of crystallinity depends on the origin of the cellulose. The role of cellulose is generally viewed upon as a skeleton function. The microfibrils are laid down in different parallel layers or lamellae, which must be free to move with respect to each other during elongation[29]

1.3.4 Hemicelluloses

Hemicelluloses are defined as non-cellulosic cell wall polysaccharides other than pectins which can be extracted by aqueous alkali[29]. Hemicelluloses presumably play a role in microfibril spacing, important for wall assembly. The main function of the resulting cellulose-hemicellulose network is to provide shape and strength to the cell wall[29,60]. In the cell walls of dicotyledonous plants xyloglucans and acidic arabinoxylans are the main hemicellulosic polymers found, but also (gluco- or galacto)mannans can be present[28,81,82].

Xyloglucans are structurally related to cellulose in that they possess a ß-(1,4) glucosyl backbone. They differ from cellulose in that approximately 75 % of the glucosyl backbone is substituted at C-6 with a α-xylose residue. Arabinose, galactose and fucose can be attached to the xylose[83]. O'Neill and Selvendran[84] proposed a block type structure for the xyloglucan of *Phaseolus coccineus* (Figure 1.7). Xylans consist of a ß-(1,4) xylose backbone with single arabinose units at the O-3 and single glucuronic acid residues at the O-2 of the xylosyl units[28]. Xyloglucans and unsubstituted xylans can be linked to cellulose through hydrogen bounding[85] (Figure 1.5). In addition there is evidence for covalent linkage of arabinoxylans of grasses to other cell wall polymers through diferuloylesters[52,59].

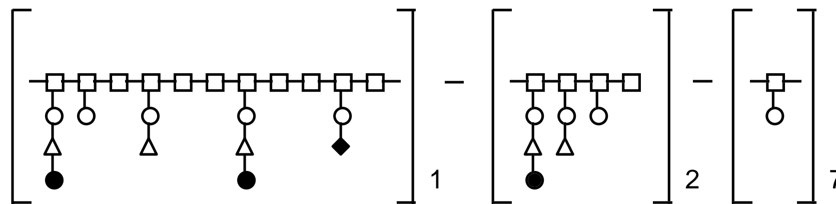

Figure 1.7 *Tentative structure for the xyloglucan from cell walls of Phaseolus coccineus (modified from ref[84]). □, ß(1,4)Glc; O, α(1,6)Xyl; Δ, ß(1,2)Gal; ●, α(1,2) Fuc; ◆, (1,2) Ara.*

1.3.5 Cell wall proteins

Cell walls contain a variety of different proteins, most of which are glycosylated[19]. These include structural proteins as well as enzymes. The most well studied structural proteins are the hydroxyprolin-rich proteins, such as extensin. Extensins are present in the primary cell walls of dicots in varying quantities, making up 1 - 10 % (w/w) of the wall. They are particularly abundant in the cell walls of cultured cells and also in the seed coat of soybeans[30,86]. Extensin is thought to play a role in the skeletal construction of the cell wall, the organisation of the cellulose microfibrils, the restriction of growth and the exclusion of invading pathogens[28,30]. Related proteins are the arabinogalactan proteins

(AGP's), which are slimy, water soluble proteins found in the apoplastic fluid of many tissues. The biological role of AGP's is unclear: suggestions for their function include lubrication and cell/cell recognition[30]. A structural protein which received attention only very recently is expansin, a structural protein involved in the expansion of cells probably by breaking the hydrogen-bonds of hemicellulose and cellulose thereby promoting expansion[87]. The enzymes present in the wall include peroxidase, cellulase, polygalacturonase, pectin methylesterase, and several exo- and endoglycosidases.

1.3.6 Lignin

Certain differentiated cell types contain lignin, a phenolic polymer that is laid down after cell elongation has ceased[19]. The precursors of lignin include the three aromatic alcohols, coumaryl, coniferyl and sinapyl alcohol. These precursors are linked by a wide variety of bounds in the final polymer. Lignin initially is laid down in the middle lamellae and primary walls of certain cells with secondary walls, being mainly vessel elements, tracheids, fibres and sclereids. It later also accumulates in the secondary walls of these cells. Lignin is a filler material, replacing the water and permanently preventing further growth. Its most probable biological function is provision of physical strength[30].

1.4 Effects of thermal processing on cell wall chemistry

Vegetables become softer and lose their desired crispness and firmness during processing mainly due to a loss of cell wall and middle lamella structure and a variety of changes in the cell wall matrix components[88]. Cellulose is a rather inert material. The only change in cellulose during processing is an increase in the degree of hydration[89]. No effect of heating on hemicelluloses has been reported in literature. Pectins are more chemically reactive than other cell wall polymers and can undergo a variety of chemical modifications under conditions similar to those associated with food processing. These modifications are influenced by a number of factors, primarily pH and the concentration and types of salts that are present. Enhanced softening at low pH ($pH < 3$) has been ascribed to hydrolytic cleavage of glycosidic bonds of neutral sugar components of the cell wall[90]. The enhanced softening during heating at neutral and alkaline pH's has likewise been associated with polymer cleavage, in this case through the ß-eliminative degradation of polyuronides[91-94] (Figure 1.8). A higher methylester content results in a larger degradation[92]. In addition Loh and Breene[95] reported thermal protopectin degradation of another kind for potatoes, which seemed more important than ß-elimination. Huang and Bourne[96] studied the kinetics of thermal softening of vegetables and postulated that the rate of softening reflects two simultaneous first order mechanisms. Mechanism 1 was proposed to be due to pectic changes in the middle lamella region and accounted for 85 - 97 % of the original tissue firmness. Its contribution to firmness decreased practically to zero during processing. Mechanism 2, whose nature has not been identified yet, was suggested to be responsible for the residual firmness of vegetables after prolonged heating. The rate constants of both mechanisms could be influenced by preheating[97].

Various ions have been shown to enhance the rate of ß-elimination in native plant tissues[91,98-101] or in a model system of purified pectin[102]. The enhancement effect in a model system with purified carrot

pectin increased in the order $Zn^{+} > Ca^{++} > Cd^{++} \sim Sr^{++} > Mg^{++} \sim Na^{+} \sim K^{+} > NH_4^{+}$. The accelerating effect of ions was more pronounced with low-methoxyl pectins than with high methoxyl pectins. The mechanism behind this effect has not been identified yet, but was not a simple function of the charges, ionic radii or hydration radii of the cations. Calcium has two opposite effects on texture. At one hand it may firm the tissue through complexes with pectic substances, and at the other hand it may soften the tissue by enhancing ß-eliminative degradation. The net result of calcium addition has invariably been to firm the tissue[103,104]. In addition to the effect of salts during the heating process, there is also an effect when salts are added after processing, to the cooked material. Van Buren[105] reported that the firming effect was most prominent with Ca^{++} and Mg^{++}, while monovalent ions had a softening effect.

Another method exploited to reduce tissue softening during thermal processing is preheating of the samples at moderate (50 - 80 °C) temperatures[67,97,106-109]. Bartholome and Hoff[110] suggested that the cell membranes are destroyed, thus allowing salts, such as K^{+}, to activate endogenous pectin methyl esterase (PME). This enzyme can subsequently partially demethylate the pectic compounds. This PME firming effect was hypothesised to involve two separate phenomena: (1) the decrease in susceptibility of the pectin to ß-eliminative degradation during subsequent cooking or sterilisation and (2) increase the formation of pectate-calcium complexes[88,111].

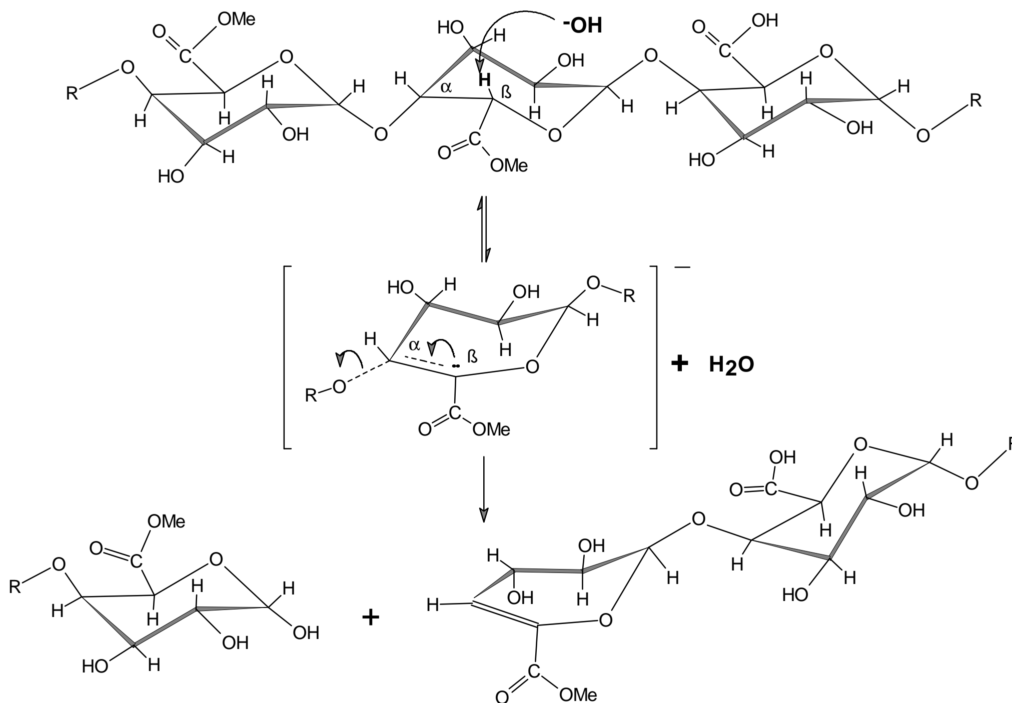

Figure 1.8 *Depolymerisation of a partially esterified pectic galacturonan chain by ß-elimination.*

1.5 Aim and scope of this thesis

Texture is an important quality aspect of fruits and vegetables after industrial processing. The main determinants of textural properties after processing are the plant cell wall and middle lamella. Physical properties of the cell wall and middle lamella are dependent on the chemical composition and

interactions of the constituent polymers. Numerous reports in literature are available about the structure and function of cell wall polymers in relation to plant growth and development. However, there is still a lack of knowledge about the role of the cell wall in determining the texture of plant food materials during storage and/or thermal processing. The aim of the research described in this thesis was (1) to investigate the (bio)chemical and ultrastructural changes in cell walls during processing of green beans and (2) to find the relationship between specific (bio)chemical properties and texture. This research was part of a project funded by the European Community entitled "The biochemistry and archestructure of fruit and vegetable tissue as quality predictors for optimising storage and processing regimes: Basic research leading to applicable models and rules". In this project a range of fruits (peaches, apples and kiwi's) and vegetables (green beans, potatoes and carrots) were compared. To design and to optimise storage and preservation processes in such a way that de desired quality is obtained, it is necessary to know the product behaviour during both storage and processing. In this study green beans were chosen as study objects because they represent a very important vegetable crop in the Netherlands. About 75 % of the produce is industrially processed to either sterilised (> 50%) or frozen products (20%).

First, the effects of blanching and sterilisation on the tissues of green bean pods were studied using scanning (SEM) and transmission electron microscopy (TEM). This is described in Chapter 2. Fracture planes were imaged by SEM to evaluate the contribution of both the outer and inner parenchyma tissue to texture after sterilisation. By using TEM, the ultrastructure of the cell walls and middle lamellae of outer and inner parenchyma tissues was compared during processing. Since the texture and other product properties of vegetables are largely affected by their stage of maturity at harvest[112], the chemical characteristics of cell walls of green beans were subsequently analysed during sequential developmental stages (Chapter 3). The results of this study provided insight in the development of cell wall characteristics, which are important when cell walls of different cultivars are compared to find cultivar specific differences. Next, the identification of cell wall changes during processing is described. Chapter 4 deals with the solubility and composition of the overall cell wall material from fresh, blanched and sterilised beans. Subsequently several pectic and hemicellulosic fractions were isolated from this cell wall material and analysed in more detail (Chapter 5). To study the relation between texture and cell wall characteristics, the effect of a range of blanching conditions on pectic substances was evaluated. The results of the chemical analyses are described in Chapter 6. In Chapter 7 the relation between chemical, texture and spectroscopic data were analysed using multivariate regression techniques. An attempt was made to predict chemical and texture data with a model using near infrared data as input. Chapter 8 concludes this thesis with a summary and discussion of the results obtained.

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Chapter 2

An electron microscopy study to the texture of fresh, blanched and sterilised green bean pods[*]

2.1 Abstract

The influence of blanching and sterilisation on the tissues of green bean pods was studied using scanning (SEM) and transmission electron microscopy (TEM). Fracture planes were examined by SEM showing that the outer and inner parenchyma tissues had a different contribution to texture after heating. After sterilisation, all cells could easily be separated along the middle lamellae, but the adhesion between adjacent cells was more retained in the inner parenchyma. By using TEM, the middle lamellae were shown to be more pronounced in the outer parenchyma tissue. The envisaged causal relationship between middle lamella ultrastructure and fracturing behaviour is discussed. Blanching and sterilisation caused partial degradation of the cell wall and middle lamella as indicated by swelling and loss of contrast. However, differences between the middle lamella of the inner and outer parenchyma tissues remained evident after these heat treatments. Cellulose microfibrils were visualised after extraction of the matrix materials from the cell walls and seemed to be unaffected by the heat treatment.

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2.2 Introduction

Texture is an important quality aspect of vegetable food products. Either control or modification of texture is a major objective in modern food technology. To achieve this there is a need to understand the processes which affect textural parameters. In this respect the histology of the plant tissue, rendering information about the tissue architecture and structure, is of major importance.

The pericarp of green beans can be differentiated into the following tissues: (i) an outer epidermis with a single layer of cells, (ii) a hypodermis consisting of sclerenchyma, (iii) an outer parenchyma, (iv) a second, small celled, fibre layer and (v) an inner parenchyma or seed-cushion[1]. The outer parenchyma shows large intercellular spaces and consists of relatively large cells, with the exception of smaller cells beneath the outer hypodermis and around the vascular bundles. Next to this, they contain thicker cell walls than the cells of the inner parenchyma. In addition they contain large vacuoles and a large number of chloroplasts and starch granules[2]. The cells of the inner parenchyma tend to be more uniform in size and shape, forming a more compact tissue[3,4]. Only cells in the vascular tissues and the hypodermis contain secondary walls at edible maturity.

Pectic substances constitute one of the main matrix components of higher plant cell walls. They are considered to play important roles in cell wall hydration, adhesion of adjacent cells, wall plasticity during growth and recognition reactions between plant cells and bacterial and fungal pathogens[5]. Pectins are based on a backbone of predominantly α-1,4 linked galacturonic acid residues, interspersed at some sites with α-1,2 rhamnose units. Neutral sugar residues, usually arabinose or galactose, can be linked to these rhamnose units as side chains[6]. Homogalacturonans are often partially methylesterified at the carboxyl groups. These methyl groups can be removed by the endogenous enzyme pectin methylesterase. Unesterified, negatively charged, homogalacturonan sequences can associate to form dimers and aggregates by a Ca^{++} mediated co-operative mechanism. The importance of this mechanism to *in situ* cell-cell adherence is however still subject of discussion[7].

When vegetables are thermally processed first turgor is destroyed, leading to loss of crisp succulence. Blanching and sterilisation affect the tissues of vegetables additionally by granular denaturation of cytoplasm, maceration of the tissue and swelling of cell walls[8]. The resulting decrease in firmness is mainly caused by changes in pectins during heating leading to the formation of soluble pectins by ß-eliminative degradation of methylated pectins[9-11].

In this study on the histology of pods of green beans, we applied both scanning and transmission electron microscopy to obtain further information on the ultrastructural alterations in cell walls and middle lamellae, with emphasis on the parenchyma cells, during the course of thermal processing.

2.3 Materials and methods

2.3.1 Plant materials, processing conditions and firmness measurement

Green bean cultivars Masai and Odessa were grown in a greenhouse and harvested at edible maturity (approximately. 20 days after flowering). The beans were subjected to a blanching treatment at either 70 °C or 90 °C during respectively 0, 5, 15 and 60 min. After this blanching treatment samples were cooled by cold running tap water. The samples were stored on ice until either firmness measurements or fixation for microscopy. Portions (410 g) of beans blanched for either 5 min. at 90 °C or 15 min. at 70 °C were subsequently sterilised in cans of 720 mL for 30 min. at 118 °C. The brine consisted of 1 % NaCl.

2.3.2 Firmness measurement

The firmness of fresh, blanched and sterilised bean pods was measured in triplicate using an Instron Universal Testing machine equipped with a Kramer shear cell. 40 g of material was placed in the cell with the length axis of the pods perpendicular to the openings of the shear cell. The maximal force (top value) applied with the shear press to break through the beans was used to quantify the firmness of the beans.

2.3.3 Scanning electron microscopy (SEM)

For SEM, five bean pods of each treatment were broken radially. The fracture planes were cut from the beans and fixed in 1.0 % osmium tetroxide at room temperature. After dehydration in an ethanol series, the samples were critical-point-dried, mounted on specimen holders with carbon cement and coated with gold by sputtering. The specimens were examined in a JEOL JGM T300. Micrographs were made at various magnifications.

2.3.4 Transmission electron microscopy (TEM)

Small pieces of the bean pods were cut, fixed with 3 % glutaraldehyde in 50 mM phosphate buffer pH 6.8 for 2 h, washed in phosphate buffer and finally postfixed in 2 % osmium tetroxide for 1 h. After dehydration in an ethanol series, the samples were embedded in Spurr. Sections (60 - 90 nm) were cut with a diamond knife and poststained in 2 % uranyl acetate for 7 min. followed by 7 min. in Reynolds lead citrate.

Cellulose microfibrils in the cell walls were visualised by embedding small pieces of bean pods in polyethylene glycol. Radial and longitudinal sections, 5 mm thick, were clamped in nickel oyster grids coated with a formvar film. The sections were treated with hydrogen peroxide (30 %) and acetic acid (96 %) 1:1 for 1 h at 90 °C to extract pectin and hemicelulose. After rinsing with water, the grids were transferred to loops covered with a formvar film, subsequently air-dried and Pt and C shadowed at an angle of 45°. The cell wall pattern is not disturbed by this treatment[12]. All preparations were examined

with either a Philips EM400 or a Jeol EM CX 100 II and micrographs were made at various magnifications.

2.4 Results

2.4.1 Firmness after processing

To quantify the texture of fresh, blanched and sterilised beans, the firmness was measured instrumentally (Fig. 2.1). The firmness of both cultivars decreased at 90 °C, but remained more or less the same at 70 °C. After sterilisation at 118 °C the firmness was only 10 % of the original firmness. Blanching at 70 °C before sterilisation resulted in a higher firmness as compared with blanching at 90 °C.

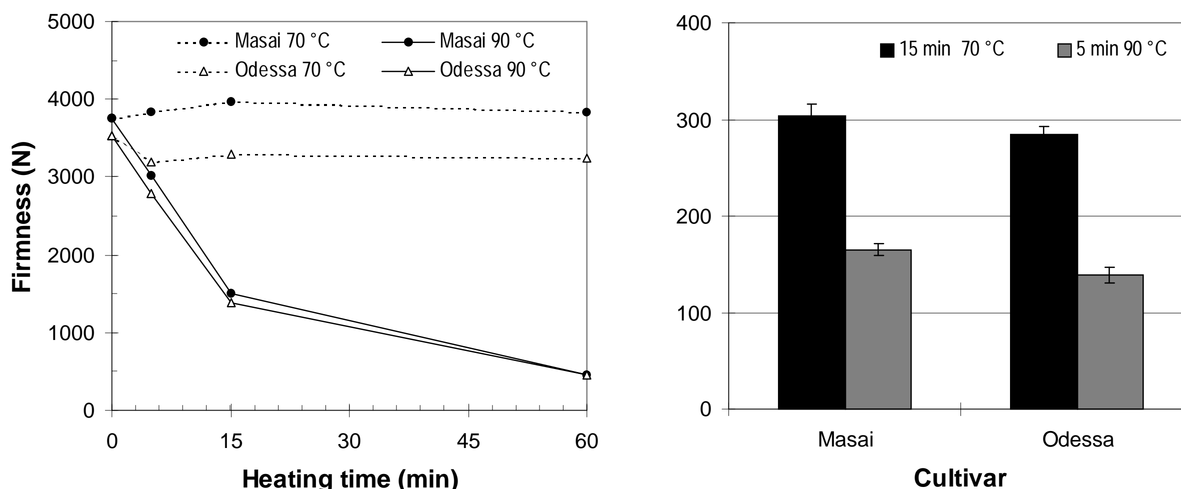


Figure 2.1. *(A) Firmness of green bean cv. Masai and cv. Odessa after incubation at 70 °C and 90 °C and (B) the effect of blanching on the firmness after sterilisation.*

2.4.2 Scanning Electron Microscopy

By using SEM, fracture planes and cutting surfaces of the different samples were examined. No differences in morphology and fracturing behaviour between the two cultivars were observed. In the outer parenchyma large intercellular spaces and, in the fresh tissues, starch granules were visible (Fig. 2.2b). Heat treated cells of the outer parenchyma were filled with gelatinised starch (Fig. 2.3). Secondary wall thickenings were clearly visible in the hypodermis layer. The inner parenchyma cells contained large intercellular adhesion zones and only a few intercellular spaces were present (Fig. 2.2c). For fresh beans and the beans heated for 60 min at 70 °C, fracturing took place through the cells (Fig. 2.2a and 2.3). However, fracturing was different after a heat treatment for 15 min at 90 °C (Fig.

2.4). For the cells of the inner parenchyma fracturing still occurred through the cells (Fig. 2.4c), but for the outer parenchyma tissue fracturing occurred between the cells, along the middle lamellae (Fig. 2.4b). After blanching at 90 °C followed by sterilisation at 118 °C all tissues fractured between the cells. The intercellular adherence however, seemed higher in the inner parenchyma as compared with the outer parenchyma (Fig. 2.5). Between the cells threadlike structures were visible, which were probably remainders of the middle lamella (Fig. 2.5c). No clear differences were observed after sterilisation between the beans blanched at either 70 °C or 90 °C. At some sites it seemed as if the intercellular contact was better preserved in the outer parenchyma when blanched at 70 °C instead of 90 °C (data not shown).

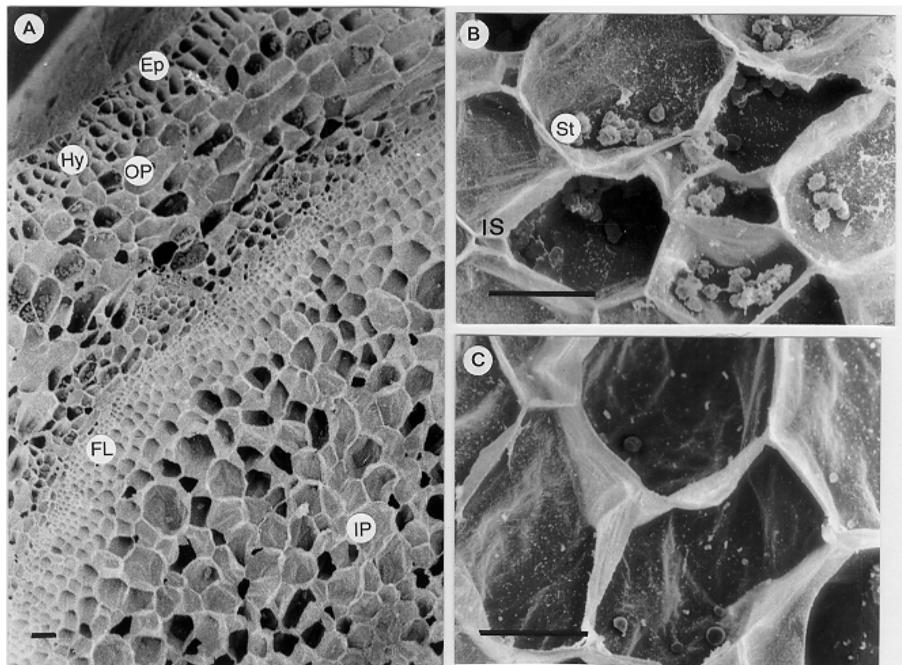
2.4.3 Transmission electron microscopy

The cells of the inner and outer parenchyma of fresh beans possess large central vacuoles and cytoplasmic layers of variable thickness. The cell walls are traversed by many plasmodesmata and at the pit-fields the cell wall is much thinner (Fig. 2.6a). In contrast to the inner parenchyma tissue, the outer parenchyma tissue exhibits predominant, dense middle lamellae, being even more pronounced facing the intercellular spaces (Fig. 2.6a and c). In the inner parenchyma, two adjacent cell walls were visualised as one, homogeneous layer and middle lamellae are only visible at some sites between two adjacent cell walls and at intercellular spaces (Fig. 2.6b and d). At pit-fields the middle lamella appears thicker in both tissues. After heating for 15 min. at 90 °C the cytoplasm is denatured and had a granular appearance. The cell walls appear swollen and have lost much of their electron density (Fig. 2.7a and 7b). A comparable trend was observed for the beans blanched at 70 °C (data not shown). After sterilisation this effect is even more pronounced, since hardly any detail in the cell walls is discernible (Fig. 2.8a and 2.8b). Only in the outer parenchyma and near intercellular spaces the middle lamellae are still visible (Fig. 2.8a). The cells seem to be separated along the middle lamellae in as well the outer as the inner parenchyma tissue. At these areas, dark fibrous material, probably remainders of the middle lamellae, can be seen between adjacent cells (Fig. 2.9). Sometimes larger intercellular spaces are formed. In contrast to the straight cell walls of cv. Masai, heating causes the cell walls of cultivar Odessa to obtain a folded appearance (Fig. 2.10).

After extraction of the sterilised samples with hydrogen peroxide and acetic acid, the cellulose microfibrils in the walls were clearly visible (Fig. 2.11). After this extraction procedure, there seemed to be a greater adherence between the longitudinal sections as compared to the radial sections, which fell very easily apart during rinsing in water. Primary pit-fields of different sizes were present and microfibrils deviated around and across the pit-fields. It seems that more pit-fields were present in the radial walls than in the longitudinal walls of the parenchyma tissues.

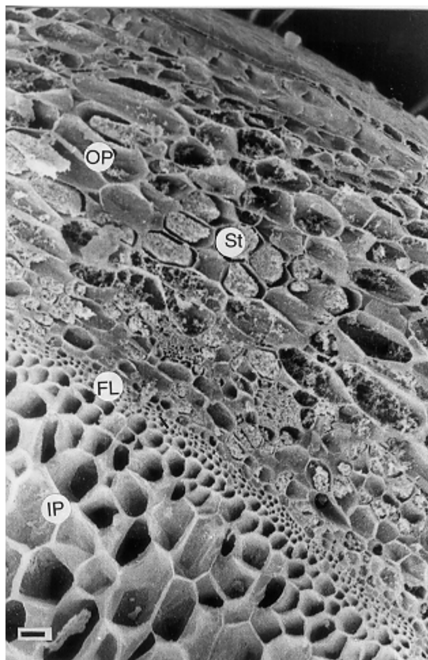
2.5 Discussion

The inner and outer parenchyma tissues of both fresh and processed green bean pods were visualised to be histologically different. Besides a difference in intracellular components like the presence of large amounts of starch and chlorophyll granules, which was already demonstrated by Reeve and



Brown in 1968[3,4], also the structure of cell wall and middle lamella was shown to be different. In the outer parenchyma, the middle lamella was more pronounced than in the inner parenchyma, where the middle lamella could not always be identified by ultrastructural observation (Fig. 2.6). Near intercellular spaces the middle lamellae appeared thicker in both tissues (Fig. 2.6a and b).

Figure 2.2. *SEM micrographs of fracture planes from fresh green beans. (A) Fresh bean, fractured through the cells. (B) Detail of the outer parenchyma showing the interior of the cells with starch granules. Intercellular spaces are visible. (C) Detail of the inner parenchyma showing no starch granules. Abbreviations: Epidermis (Ep), fibrous hypodermis (Hy), outer parenchyma (OP), fiber layer (FL), inner parenchyma (IP), starch (St) and intercellular space (IS) (bar =10 μm).*



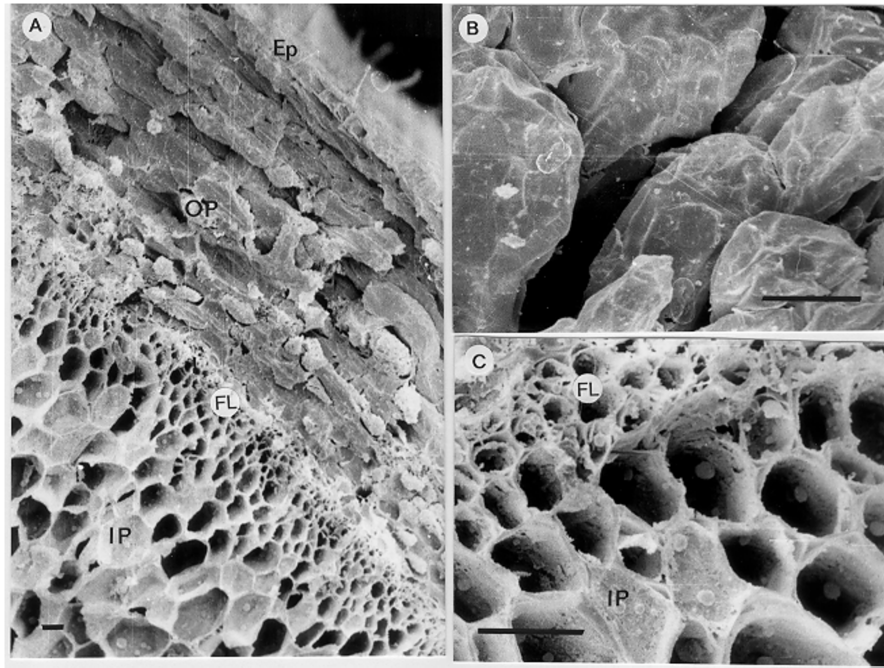
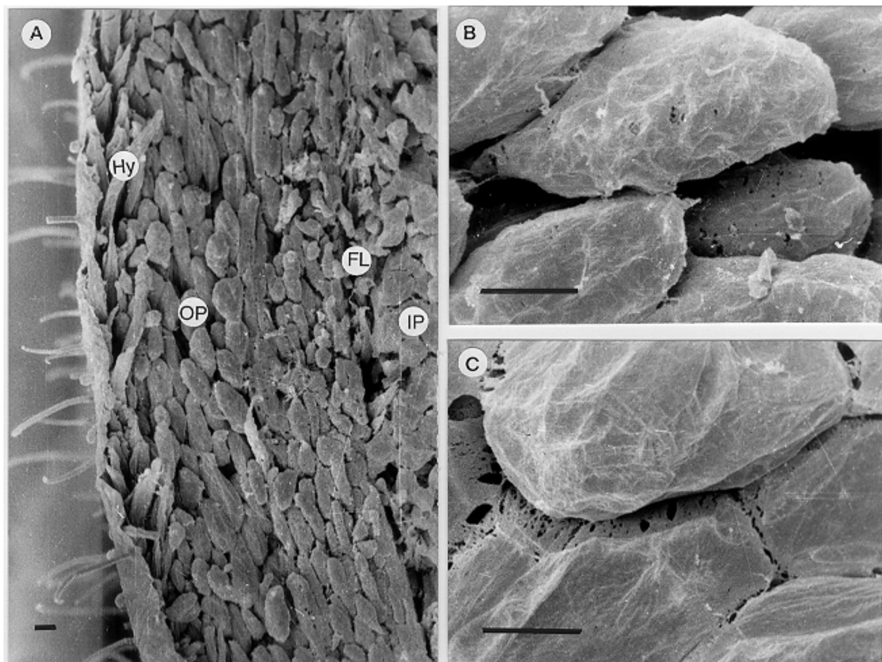


Figure 2.3 *Beans heated for 60 min. at 70 °C, fractured through the cells, showing gelatinised starch in the outer parenchyma tissue (bar =10 µm, abbreviations: see Fig. 2.2).*

Figure 2.4 *SEM micrographs of a fracture plane of green beans, heated for 15 min. at 90 °C.(A) Low magnification of the outer parenchyma fracturing along the middle lamella, leaving the cells intact, and the inner parenchyma fracturing through the cells. (B) Detail of the outer parenchyma*



showing the cell wall surface of intact cells. (C) Detail of the inner parenchyma and fibre layer showing fracturing through the cells. (bar =10 µm, abbreviations: see Fig. 2.2)

Figure 2.5 *SEM micrographs of fracture planes from green beans, blanched at 90 °C and subsequently sterilised for 30 min. at 118 °C. (A) Overview, showing all tissues fracturing along the middle lamellae. (B) Detail of the outer parenchyma. (C) Detail of the inner parenchyma. (bar =10 µm, abbreviations: see Fig. 2.2)*

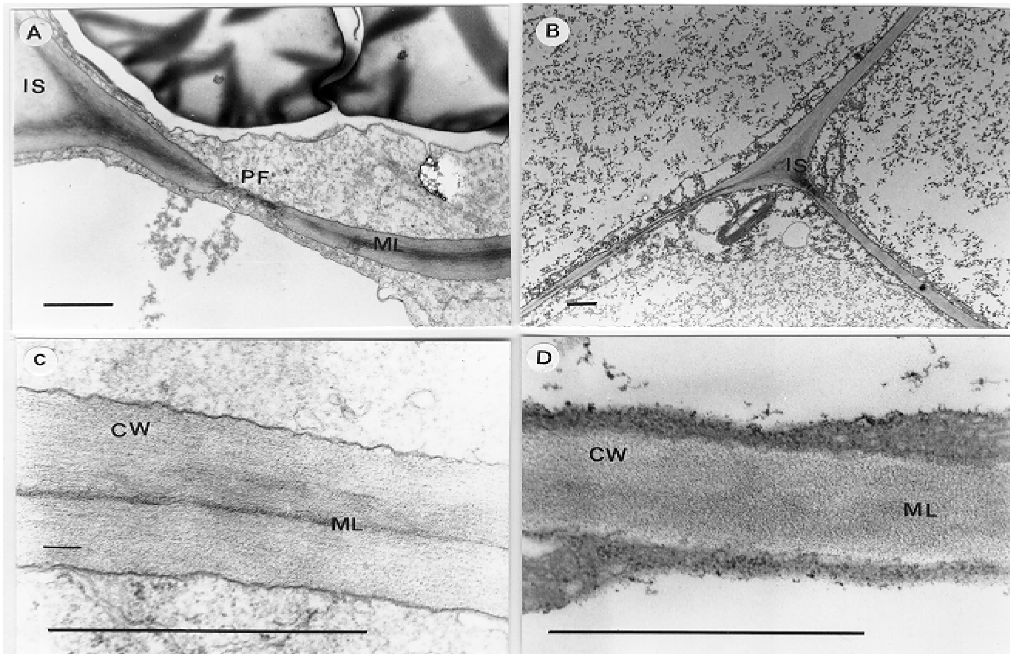


Figure 2.6 *TEM micrograph of fresh beans. (A) Cells of the outer parenchyma with primary pit-field and very thick middle lamellae. (B) Detail inner parenchyma showing middle lamellae only at intercellular spaces and pit-fields. On other sites two adjacent cell walls form a homogeneous layer. (C) Details of cell walls and middle lamellae in outer parenchyma with thick middle lamella. (D) Details of cell walls and middle lamellae in inner parenchyma; two adjacent cell walls form a homogeneous layer. Abbreviations: starch (St), middle lamella (ML), primary pit-field (PF), cell wall (CW) and intercellular space (IS) (bar = 1 µm).*

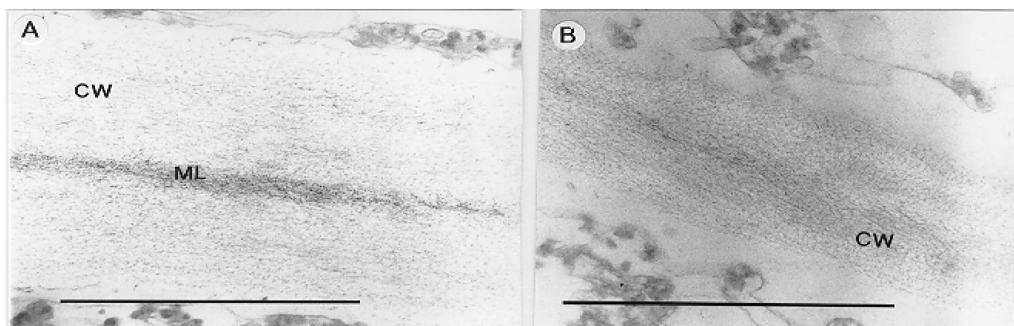


Figure 2.7 *TEM micrograph of beans, heated for 15 min. at 90 °C . (A) Outer parenchyma with thick middle lamella. (B) Inner parenchyma, two adjacent cell walls form a homogeneous layer. (C) (bar = 1 µm, abbreviations: see Fig. 2.6*

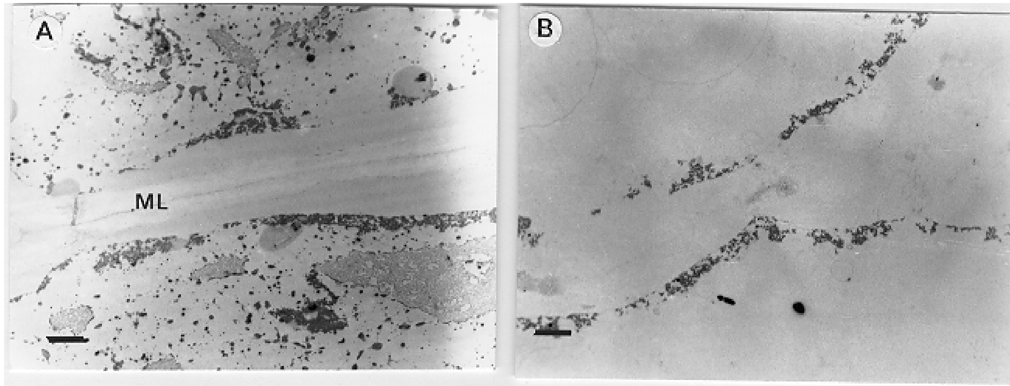


Figure 2.8 *TEM micrograph of beans, blanched at 90 °C and subsequently sterilised for 30 min. at 118 °C. (A) Outer parenchyma with predominant, electron dense middle lamella. (B) Inner parenchyma; two adjacent cell walls form a homogeneous layer, the middle lamella can hardly be identified. (bar = 1 µm, abbreviations: see Fig. 2.6)*

Figure 2.9. *Fibrous structures between cells of beans after blanching and sterilisation (bar = 1 µm, abbreviations: see Fig. 2.6).*

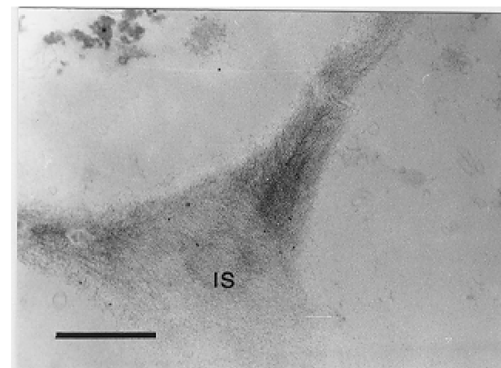
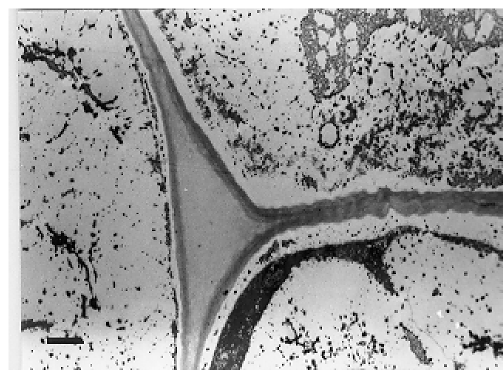


Figure 2.10 *Folded structure of the cell walls from bean cv. Odessa. Similar structures were not observed in cv. Masai (bar = 1 µm).*



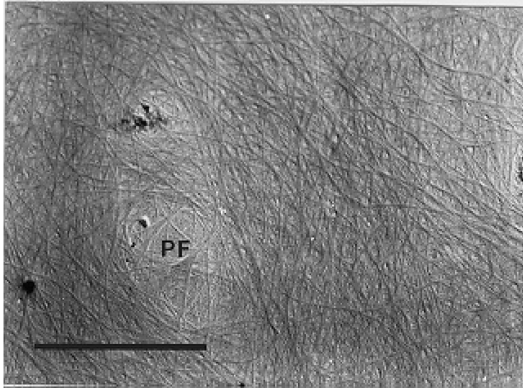


Figure 2.11 *Cell wall texture of a inner parenchyma cell after blanching and sterilisation. The structure of the pith field is retained after sterilisation. (bar = 1 µm, abbreviations: see Fig. 2. 6)*

The heating process caused significant changes in all cell walls and middle lamellae of green beans pods. The middle lamellae and cell walls became loosened, swollen, and less electron dense (Fig. 2.7 and 2.8), most likely caused by breakdown of the matrix material, like pectins. The overall structure of the cellulose framework seemed not to be altered, since the typical pattern of primary walls with pith fields was still clearly visible after sterilisation (Fig. 2.11)

SEM observations showed that the outer and inner parenchyma tissues had a different fracturing behaviour after heating (Fig. 2.4 and 2.5). The cells of the outer tissue separated easier along the middle lamellae, leaving the cells intact, than cells of the inner parenchyma (Fig. 2.4). Even after sterilisation, the intercellular contact seemed higher in the inner parenchyma (Fig. 2.5). The difference in appearance of the middle lamella between the inner and outer parenchyma is preserved throughout the heating process and probably caused the difference in fracturing behaviour of the tissues after heating at 90 °C for 15 min. The main component of the middle lamella of vegetables and fruits is the pectic homogalacturonan[13]. These linear polymers can form aggregates in presence of Ca^{++} ions, but this is hindered if the carboxylic acids are methylesterified or if Ca^{++} is deficient. This implies that the middle lamella polymers can shear along each other and therefore facilitate the fracturing, leaving the cells intact. In addition, Liners and van Cutsem[7] demonstrated that the presence of Ca^{++} associated pectins in the primary walls of senescent suspension-cultured carrot cells did not maintain the cohesion of the walls. In the inner tissue, where the middle lamella was less evident as shown by TEM, it is reasonable to assume that protopectin from the primary wall can extend into the middle lamella zone and contribute to intercellular adhesion. This protopectin, which is highly methylesterified and branched, is secured in the primary wall by acid- and/or alkali labile bonds and can cause the fracturing to proceed through the cells rather than along the middle lamellae. For the inner parenchyma tissues it seemed that, although after heating for 15 min at 90 °C part of the cell wall was degraded, as indicated by the loss of contrast, there was still enough adherence between adjacent cell walls to prevent cell separation upon fracturing.

Blanching beans at 70 °C resulted in a higher firmness than blanching at 90 °C. This difference is generally explained by activity of the enzyme pectin methylesterase, which is relatively heat stabile and has a considerable residual activity after heating for 15 min. at 70 °C[14,15]. The de-esterified pectin is subsequently less susceptible for ß-eliminative degradation and therefore more heat stabile. After processing this results in more insoluble pectin, which might increase the cell-cell adhesion. However since both the 70 °C and the 90 °C blanched beans fractured along the middle lamellae, this was not discernible with ultrastructural observation.

It can be concluded that that the inner and outer parenchyma tissues contribute to a different extend to the texture of thermally processed green beans. This difference seems to be related with a difference in middle lamella ultrastructure.

2.6 References

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Chapter 3

Changes in cell wall (bio)chemistry of green bean pods in relation to development*

3.1 Abstract

The changes in cell wall polysaccharides and cell wall modifying enzymes were studied during the development of green bean pods, from the onset of pod growth, throughout elongation, until senescence and dehydration. An overall increase of cell wall material was observed during pod development. Major changes were detected in the pectic polymers. Very young, exponentially growing cell walls contained large amounts of neutral sugar rich pectic polymers (rhamnogalacturonan), which were water insoluble and relatively tightly connected to the cell wall. During elongation, some more galactose-rich pectic polymers were deposited and cross-linked into the cell wall. Besides this, the level of branched rhamnogalacturonan remained rather constant, while the level of linear homogalacturonan steadily increased. This might suggest that gaps in the cell wall network formed by expansion, are filled in with homogalacturonan, while the structure is being locked by galactose-rich rhamnogalacturonan. During maturation of the pods, these tightly linked galactose-rich pectic polymers were degraded while the accumulation of soluble homogalacturonan continued. During senescence there was an increase in the amount of ionically complexed pectins, mainly at the expense of freely soluble pectins. No significant compositional changes were observed in hemicelluloses during development. The bulk of the hemicellulosic fractions appeared to be mixtures of xyloglucans, xylans and associated pectins. During maturation and senescence there was a relative high amount of mannose containing polymers. The amount of cellulose started to increase during the last growth phase. The most abundant enzyme tested for, was pectin methylesterase. Peroxidase, ß-galactosidase and α-arabinosidase were also detected in appreciable amounts. In general enzyme activities, as expressed on CWM basis, were high during initial stages of pod development, decreased during cessation of elongation and increased again during maturation and senescence. ß-Galactosidase activity reached its maximum during senescence. Polygalacturonase was detected only in very small amounts throughout development. The relation between endogenous enzyme levels and properties of cell wall polymers is discussed with respect to cell wall synthesis and degradation.

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3.2 Introduction

The texture of processed vegetables and fruits is for a large part determined by properties of the cell wall and middle lamella. The cell wall is not a static structure, but is very dynamic in nature. Its composition and structure is being changed continuously during plant development. Plant cell walls consist of cellulose microfibrils, extensively coated by xyloglucans and embedded in a complex matrix of pectic polysaccharides[1,2]. Pectic substances are very abundant in fruit and vegetable cell walls and are considered to be very important in determining texture of processed vegetables. Cell wall pectin consist of two regions, a linear homogalacturonan and a branched rhamnogalacturonan. Neutral side chains, mainly consisting of arabinose and/or galactose, are attached to the rhamnogalacturonan backbone in variable amounts. The carboxyl and hydroxyl groups of the galacturonic acid backbone can be substituted with respectively methyl and acetyl esters. Pectin can be connected to other cell wall components or other pectins by ionic complexes and covalent cross-links[3]. The aim of this study was to analyse the modifications of cell wall composition of green beans during pod growth and senescence, with emphasis on the pectic substances.

Plant development involves a co-ordinated series of biochemical processes that, amongst others, result in the biosynthesis and degradation of cell wall components. During cell expansion non-cellulosic polymers are cleaved enzymatically and internal osmotic pressure pushes the fibrillar components apart. New microfibrils and associated polymers are subsequently deposited on the innermost surface of the wall, forming a highly stratified and cross-linked fabric[1]. The precise role of the pectin matrix in controlling growth is not clear yet. In addition to cell wall polysaccharides, specific structural proteins, called expansins, may be involved in cell expansion by breaking the H-bonds of hemicellulose and cellulose thus allowing shear of the cellulose fibrils. When elongation is complete, the resulting cell wall has to be ‘locked’. This is probably brought about by embedding of other structural proteins or lignin, depending on the type of plant and tissue, in the cell wall matrix[1].

Substantial research has been performed towards the role of pectins in softening of fruits during ripening[4-10]. It has been demonstrated that pectin depolymerisation and high levels of *endo*-polygalacturonase (PG) occur simultaneously in ripening of many fruits. However, the role of PG has been questioned as some fruits apparently lack PG and in spite of this soften normally. Experiments with transgenic tomato fruits have led to proposals that partial breakdown of other structural components, like (hemi)cellulose may be required in addition to the degradation of pectic materials to bring about the extensive softening as observed[8]. Very few data are available concerning cell wall modifications during senescence of plant tissue.

Green beans are a very important vegetable crop in the Netherlands. About 75% of the produce is industrially processed to either sterilised or frozen products. Firmness of green beans after processing is an important quality attribute and is mainly determined by the structural integrity of the cell walls. The properties of the cell walls after processing are for a large part determined by the characteristics of the fresh product. In this study the changes in cell wall polysaccharides and pectin modifying enzymes were studied during the development of green bean pods, from the onset of pod growth, throughout

elongation and maturation, until senescence. The relation between endogenous enzyme levels and structural properties of cell wall polysaccharides will be discussed with respect to cell wall synthesis and degradation.

3.3 Materials and methods

All the analyses, except for the extraction and ion-exchange chromatography were performed in duplicate and the coefficients of variation were in all cases less than 10%.

3.3.1 Plant material.

Green beans (*Phaseolus vulgaris* L.) cvs. Masai and Odessa were grown under standard green house conditions. Green beans were harvested at different sequential developmental stages and classified in days after flowering (daf). Immediately after harvest, the pods were frozen in liquid nitrogen and stored at -50 °C. Next, the seeds were manually removed from the frozen pods if possible, i.e. from stage IIb onwards, and the pods were grounded in liquid nitrogen.

3.3.2 Dry matter determination

The dry matter content of the samples was determined by drying a known weight of homogenised samples overnight at 70 °C, followed by 3 h at 105 °C. After cooling to room temperature, the samples were weighed again. The dry matter and water content were calculated from the weight difference.

3.3.3 Starch content.

To solubilise starch, 5 mL of HCl (8 M) and 20 mL of dimethylsulfoxide were added to 250 mg of a sample and the mixture was placed in a water bath of 60 °C. After an incubation period of 60 min under continuous shaking, 5 mL NaOH (8 M) and citrate-buffer (Titrisol/pH 4, Merck 9884) were added to a final volume of 100 mL. After filtration, 0.1 mL of filtrate was used to quantify the starch content in the sample using test-combination cat. nr. 207748 from Boehringer Mannheim.

3.3.4 Protein content

The nitrogen content of the AIR fractions was measured using a Carlo Erba CHNS-OEA 1108 Elemental analyser. The protein content was estimated by multiplying the nitrogen value by 6.25.

3.3.5 Purification and fractionation of cell walls.

50 g of frozen material was immersed in 180 mL cold (-30 °C) ethanol (96% v/v), homogenised with an ultraturrax by four bursts of 45 sec and collected on a Whatman GF/C filter. The material was suspended in 50 mL cold (-30 °C) aqueous ethanol (80% v/v) and stirred for 1 h at 4 °C. The material was filtered again, washed twice with 50 mL 100% acetone until the filtrate was colourless and dried overnight to yield the Alcohol Insoluble Residue (AIR). The AIR was subsequently grounded in a ball

mill (Retsch MM2). Pectic polymers were extracted using a method from Selvendran et al.[11] with minor modifications (Figure 3.1) To remove starch the AIR (2 g) was suspended in 150 mL 90% DMSO and stirred for 16 h at 20 °C. The suspension was centrifuged (7,000 g for 15 min) and subsequently the pellet was washed twice with 90% DMSO and three times with 80% ethanol. The supernatant, which contained predominantly starch, was discarded. To the pellet, 100 mL of 0.05 M ammonium acetate buffer (pH = 4.7) was added and the suspension was stirred for 16 h at 4 °C. The suspension was centrifuged and the pellet was washed twice with acetate buffer. The supernatants were combined and this "buffer"-fraction, containing free pectins, was dialysed exhaustively against demineralised water at 4 °C. To the pellet, 100 mL 0.05 M CDTA (pH = 6.5) was added and the suspension was stirred for 16 h at 4 °C. The suspension was centrifuged and the pellet was washed once with the CDTA-solution and once with demineralised water. The supernatants, containing calcium complexed pectins, were combined and dialysed at 4 °C for 14 days against demineralised water (CDTA-fraction). Covalently linked pectins were subsequently extracted with respectively 100 mL (O_2-free) of 0.05 M Na_2CO_3 containing 0.01 M $NaBH_4$ at 4 °C, 0.05 M Na_2CO_3 containing 0.01 M $NaBH_4$ at 20 °C, followed by extraction with respectively 0.5 M, 1.0 M, 4.0 M KOH containing 0.01 M $NaBH_4$ and 4.0 M KOH containing 0.65 M H_3BO_3 and 0.01 M $NaBH_4$. All extractions were performed by constant stirring under N_2 for 16 h at 20 °C to leave a residue mainly consisting of cellulose. All Na_2CO_3 and KOH supernatants, containing respectively covalently linked pectins and different hemicelluloses, were filtered, neutralised with acetic acid to pH = 5, dialysed exhaustively against demineralised water and finally lyophilised. During neutralisation of the 0.5 M and 1.0 M KOH supernatants a precipitate formed, which was analysed separately.

3.3.6 Methyl and acetyl substituents.

The amount of methyl and acetyl groups was determined by using a HPLC system under the conditions as described by Voragen et al.[12].

3.3.7 Monosaccharide Composition.

All polysaccharides, including cellulose, from the AIR and Residue were solubilised by dispersing the dried samples in cold 11.5 M H_2SO_4 for 2 h at 20 °C, followed by hydrolysis in 1 M H_2SO_4 for 2 h at 100 °C under continuous stirring (Seaman hydrolysis). The hydrolysates were filtered through a Whatman GF/C glassfibre filter and neutralised with $BaCO_3$. Since the pectic and hemicellulosic fractions extracted from the AIR contained no cellulose, they were hydrolysed by stirring in 2 M TFA for 2 h at 121 °C. Samples were dried under N_2 at 45 °C, washed with NH_4OH, dried under N_2 and dissolved in milli Q water. Samples (10 µL) of the neutralised hydrolysates were analysed for neutral sugars by using a HPLC system (Pharmacia LKB Low pressure mixer, autosampler 2157 and Waters 625 LC pump) equipped with a Carbopack PA1 column (250 x 4 mm, Dionex). Data analysis was performed using Millennium 2010 software (Waters). The eluents, consisting of milli Q water and 150

mM NaOH, were sparged and pressurised with helium. Prior to injection, the system was equilibrated with 30 mM NaOH for 8 minutes at a flowrate of 1.0 mL/min at ambient temperature.

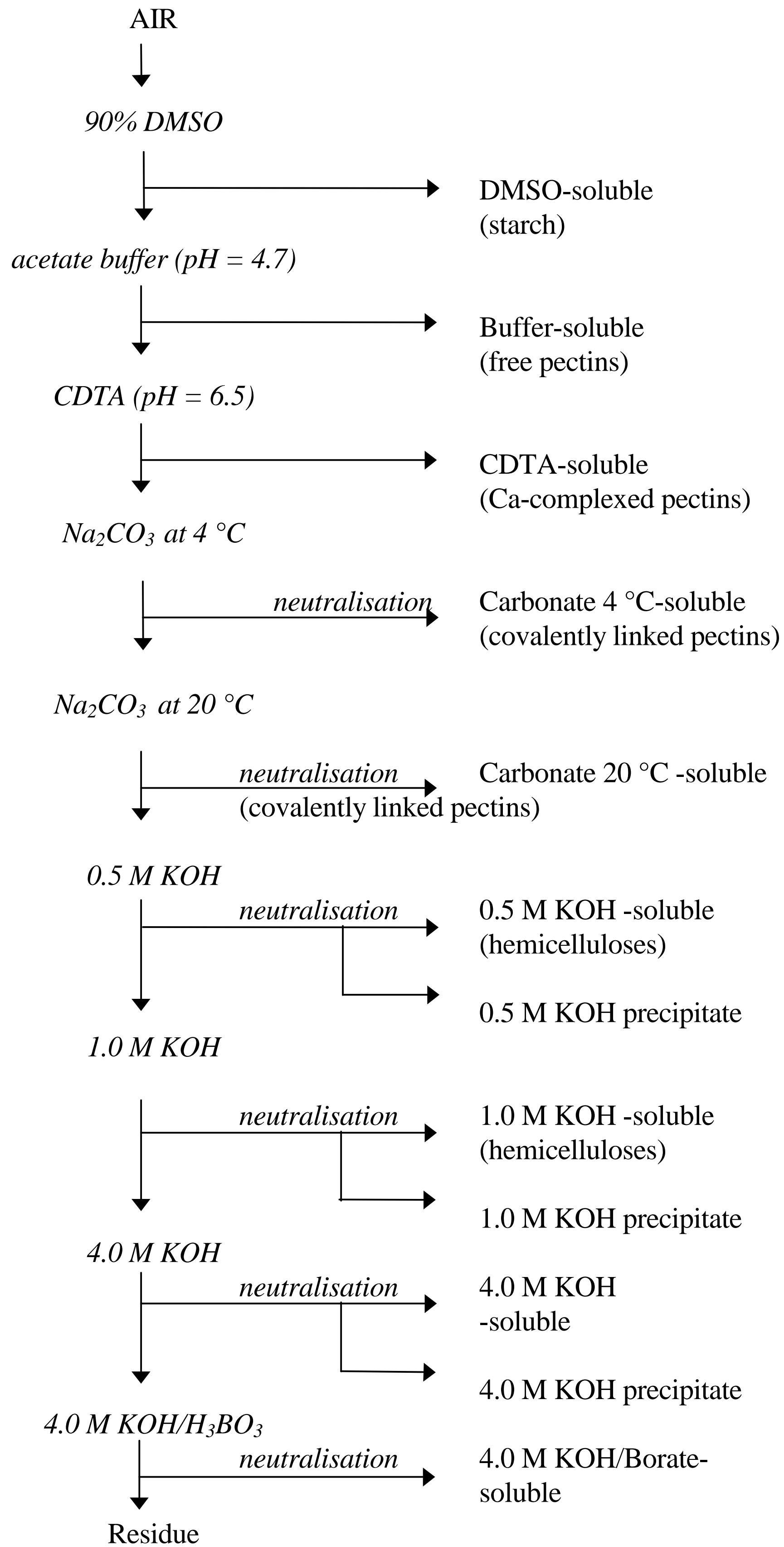


Figure 3.1 *Extraction scheme for pectic and hemicellulosic cell wall fractions.*

The sugars were separated by an initial gradient from 30 - 0 mM NaOH during 3 min and subsequent elution in milli Q water during 30 min. After each run, the column was regenerated with 150 mM NaOH for 15 min. Compounds were detected with a Dionex PED in the pulsed amperometric detection mode fitted with a gold working electrode. The applied potentials were set at E1 = 0.1 V, E2 = 0.6 V and E3 = -0.6 V against a Ag/AgCl reference electrode. Pulse durations for the applied potentials were 500 msec, 100 msec and 50 msec respectively. Trehalose, added after hydrolysis of the samples, was used as an internal standard. Anhydro-uronic acids were determined as described by Ahmed and Labavitch[13].

3.3.8 Size exclusion chromatography

High-Performance Size Exclusion Chromatography (HPSEC) was performed using a HPLC system (Waters UK6 injector, Waters 510 HPLC pump) equipped with a Waters Ultrahydrogel guard column and a Waters Ultrahydrogel 500 (7.8 x 300 mm) and elution with 0.4 M acetic acid/sodium acetate (pH = 3.0) at 0.8 mL/min. For the measurement of enzyme activity the same system, but two columns in series (Ultrahydrogel 2000 and Ultrahydrogel 500; each 7.8 x 300 mm, Waters) were used. The eluate was monitored using a Pharmacia refractive index detector. The system was calibrated using linear pullulans with molecular masses ranging from 6,000 - 1,660,000 Da. Data analysis was performed using Millennium 2010 software (Waters).

3.3.9 Ion exchange chromatography.

The hemicellulosic extracts (20 mg), dissolved in 0.05 M phosphate buffer (pH = 6.5) by stirring for 16 hours at 4 °C, were centrifuged and applied on a DEAE Sepharose fast flow (Pharmacia) column (150 x 10 mm) of a FPLC system (Pharmacia). Elution was performed at a flow rate of 3.0 mL/min using subsequently 30 mL of 0.05 M phosphate buffer (pH = 6.5), 30 mL of buffer containing 0.125 M NaCl, 30 mL of buffer containing 0.250 M NaCl, 30 mL of buffer containing 0.500 M NaCl and 30 mL of buffer containing 1.0 M NaCl. Fractions of 2.0 mL were collected and tested for carbohydrates[14]. Uronic acids in these fractions were monitored by adding 1200 µL of 0.125 M $Na_2B_4O_7$ in conc. H_2SO_4 to 200 µL of the fraction followed by boiling for 5 min. The reaction mixture was cooled in ice-water and 20 µL m-hydroxy-diphenyl (m-HDP, 15% in 1M NaOH) was added. After 10 min incubation at room temperature the extinction at 520 nm was recorded. The fractions eluting at one salt concentration were pooled, dialysed and lyophilised. These pooled fractions were analysed for neutral sugars and uronic acids as is described above.

3.3.10 Enzyme activity assays

All handlings were performed at 4 °C. Ground frozen pods were immersed in 2 M NaCl and homogenised with an ultraturrax by three bursts of 30 sec. After centrifugation, low molecular mass compounds were removed from the extracts by elution over a prepacked Sephadex G-25 column (Pharmacia PM10). Fractions containing proteins were pooled and assayed for enzyme activities and protein. Activities are expressed in katals (1 katal = 1 mole product formed per second).

– *Pectin methylesterase (PME) activity.*

PME activity in the supernatant was determined using a continuous spectrophotometric assay with bromothymol blue as a pH indicator[15].

– *Polygalacturonase (PG) activity.*

PG activity was determined spectrophotometrically following derivatisation of the reaction product with UV-absorbing 2-cyanoacetamide as is described by Gross[16]. In addition the decrease in M_r of polygalacturonic acid caused by PG action was analysed qualitatively using HPSEC.

– *Peroxidase (POD) activity.*

POD activity was determined using a continuous spectrophotometric assay. The reaction mixture (3000 µL) consisted of 0.1 M citric acid buffer (pH = 4.5) containing 0.05 mM ABTS and 0.25 mM H_2O_2. The reaction was started by adding 50 µL sample solution and the decrease in absorbance at 414 nm was monitored using an UVIKON spectrophotometer. POD activities were determined using the molar extinction coefficient of ABTS ($3.6 \times 10^{-4}\ M^{-1}\ cm^{-1}$).

– *Glycosidases.*

The activities of various glycosidases were analysed using the corresponding *p*-nitrophenyl derivatives of α-L-arabino-furanoside, ß-D-galacto-pyranoside (SIGMA) as substrates. The reaction mixture consisted of 1.500 mL of 33 mM acetate buffer of optimum pH for each enzyme (pH 3.5 for galactosidase, pH 4.0 for arabinosidase), 50 mM NaCl and 3 mM of the corresponding PNP-derivative. The reaction mixture was incubated at 30 °C before addition of sample solution. After 20 min incubation at 30 °C the reaction was terminated by the addition of 1.500 mL of 0.2 M Na_2CO_3. The activity was calculated from the amount of PNP formed using the molar extinction coefficient of PNP at 420 nm ($4.8 \times 10^3\ M^{-1}\ cm^{-1}$).

– *Pectinase.*

Enzyme activity of enzyme extracts from stage Ib and IV, using native bean pectin as substrate was tested using a purified green bean pectin (extracted with Na_2CO_3 at 4 °C), containing 48% galacturonic acid, 41% galactose, 10% arabinose and 1 % rhamnose. 1.0 mL of enzyme extract was incubated with 2.0 mL 200 mM acetate buffer and 150 mM NaCl (pH = 4.0) containing 30 mg of pectin for 16 hours at 30 °C. All samples were analysed qualitatively by HPSEC to investigate if molecular mass distributions had shifted upon incubation.

3.4 RESULTS

3.4.1 Development of the pods

Pod length was analysed to monitor the overall development of green beans during the sampling period (Figure 3.2). Previous studies already demonstrated that pods of green beans developed similarly on separate plants and during different seasons[17]. The pattern of growth followed a single

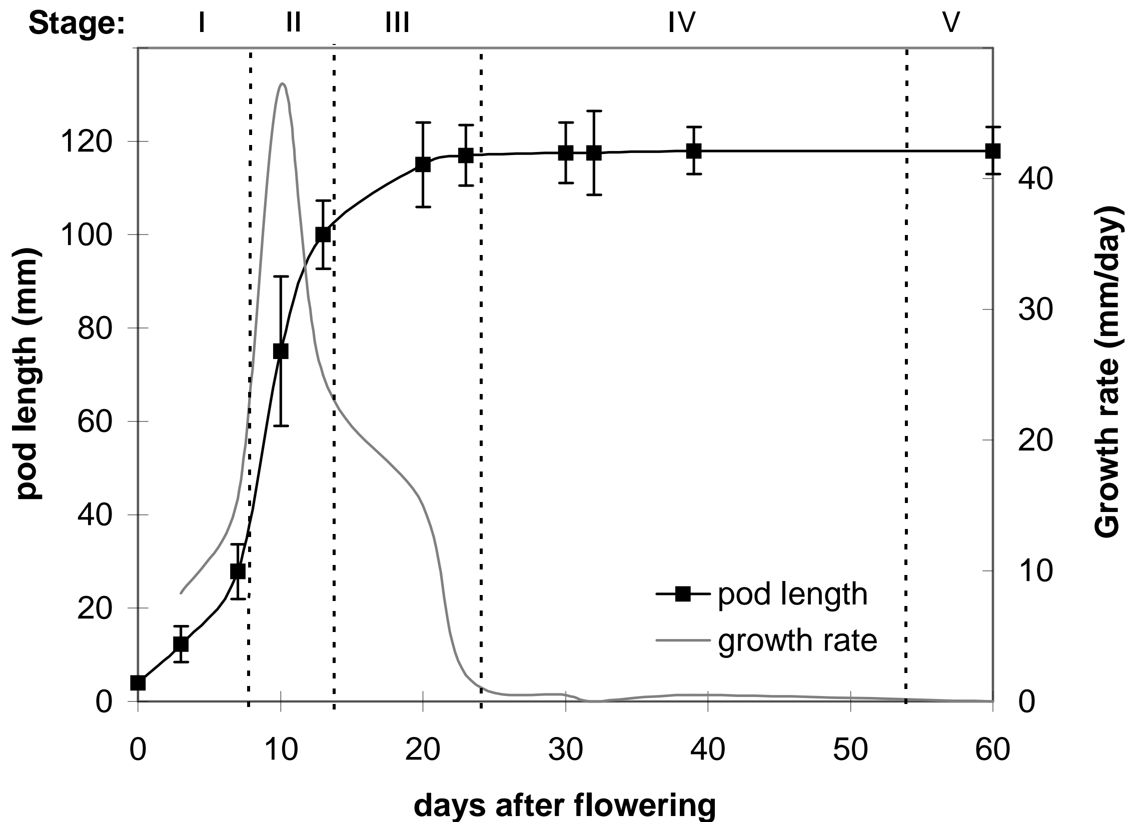


Figure 3.2 *Average length and growth rate of green bean pods during sequential developmental stages.*

sigmoïdal curve. Development of bean pods was preceded by white flowers turning yellow. This yellow flower stage was assigned as 0 days after flowering (daf). Based on growth characteristics, the developing pods were classified into 5 stages (Figure 3.2). Stage I (0-7 daf): initial *exponential* growth phase, the seeds could not be separated from the pods during this stage. This stage was subdivided into two samples: 0-5 daf and 6-7 daf. Stage II (8-13 daf): *linear* growth phase, pods extended approximately 10 mm in length per day This stage was subdivided into three samples: 8-9 daf, 10-11 daf and 12-13 daf. From this stage on, the seeds could be separated from the pods and were discarded. Stage III (14- 23 daf): *cessation* of pod elongation, resulting in pods of on average 117 mm in length. Stage IV (24-55 daf): "*maturation*", further development of seeds, degradation of the inner parenchyma tissue or seed cushion, pods start to turn yellow. Stage V (> 55 daf): "*senescence*", dehydration and browning of the pods, in some cases spontaneous release of the mature seeds.

3.4.2 Overall composition

During the first stages of pod development the water content increased and the AIR content declined (Table 3.1). The AIR contains all high molecular mass components of the beans, including cell wall material (CWM), proteins, and starch. The initial decrease in AIR was mainly due to a strong reduction of protein during these stages. The amount of cell wall material (CWM) increased after stage IIb. In addition, there was an increase of starch content during stage IIb, IIc, III and IV, followed by a

strong reduction during senescence. This all together resulted in a higher yield of AIR. During the last stage of development, i.e. senescence, the pods were dehydrated and contained less than 40% of water. The remaining dry matter contained much AIR, mainly consisting of CWM.

Table 3.1 *Amount of water and contents of AIR, CWM1), protein and starch in green bean pods during sequential developmental stages.*

Developmental stage	days after flowering	Water (mg/g FW)	AIR (mg/g DW)	CWM (mg/g DW)	Protein	Starch
Ia:	0 - 5	867 ± 1	539	243	257	10
Ib:	6 - 7	891 ± 1	529	251	219	9
IIa	8 - 9	911 ± 1	518	272	175	12
IIb:	10 - 11	912 ± 1	463	260	111	33
IIc:	12 - 13	913 ± 1	557	325	101	87
III:	14 - 23	913 ± 1	620	396	101	100
IV:	24 - 55	869 ± 3	665	347	88	143
V:	>55	379 ± 14	857	474	68	20

[1)] The amount of CWM was calculated from the total amount of cell wall carbohydrates after hydrolysis of the AIR.

Table 3.2. *Monosaccharide composition of the AIR from green beans during sequential developmental stages.*

Developmental Stage	days after flowering	Fuc	Rha[1]	Ara	Gal (Mol%)	Glc[2]	Xyl	Man	AUA
I a:	0 - 5	0.8	1.6	17.3	27.2	26.5	7.9	3.6	15.9
I b:	6 - 7	1.0	1.9	13.3	26.5	25.1	6.5	3.1	23.6
II a	8 - 9	1.0	2.0	10.2	25.3	24.0	8.8	4.8	24.9
II b:	10 - 11	1.0	1.6	8.5	23.6	27.8	6.9	2.0	29.5
II c:	12 - 13	0.9	1.7	7.0	20.0	36.5	5.5	3.3	26.0
III:	14 - 23	0.8	1.2	5.7	18.2	40.4	4.5	3.3	26.5
IV:	24 - 55	0.9	1.6	4.6	13.5	40.4	4.8	5.9	29.1
V:	>55	0.8	1.2	4.9	10.3	37.1	5.7	7.1	33.7

[1] Rhamnose was determined after TFA hydrolysis, the other sugars with Seaman hydrolysis. [2] All glucose values have been corrected for contributions made by starch.

3.4.3 Overall cell wall composition and changes during development.

The AIR was used to analyse the general features of the cell wall during development. The molar ratios of cell wall galacturonic acid and neutral sugars in the AIR are shown in Table 3.2. The values

of glucose have been corrected for amounts of glucose originating from starch. Major changes were detected in the pectic sugars arabinose, galactose and galacturonic acid. The percentage of arabinose in the AIR declined during all developmental stages. In the senescent pods only 0.3 times the initial arabinose concentration was left. The percentage of galactose decreased as well, but the decline became significant only after stage IIc and was more rapid in the latter stages to an end value of 0.4 times the initial value. The galacturonic acids increased in two phases: during the exponential growth and during the last two stages. During linear elongation of the pods (Stage II and III) the galacturonic acid percentage of the AIR remained almost constant. In contrast, cell wall glucose percentages increased during the linear elongation phases (stage II and III) to a final concentration of about 1.4 times the initial amount. As the beans aged (stages IV and V) the levels of mannose increased rapidly. The percentages of fucose, rhamnose and xylose remained rather constant throughout development.

For the pectins a decrease in the degree of methylation (DM) was observed at the beginning of development (stage I - IIa) followed by an increase during elongation and senescence of the bean pods (Figure 3.3). The degree of acetylation (DA) was not calculated on galacturonic acid basis because in addition to pectins, other cell wall components like xylans and xyloglucans are also known to be substituted with acetyl groups[1]. The DA was shown to increase rapidly to a level of 8 - 9%, calculated as percentage of total cell wall sugars.

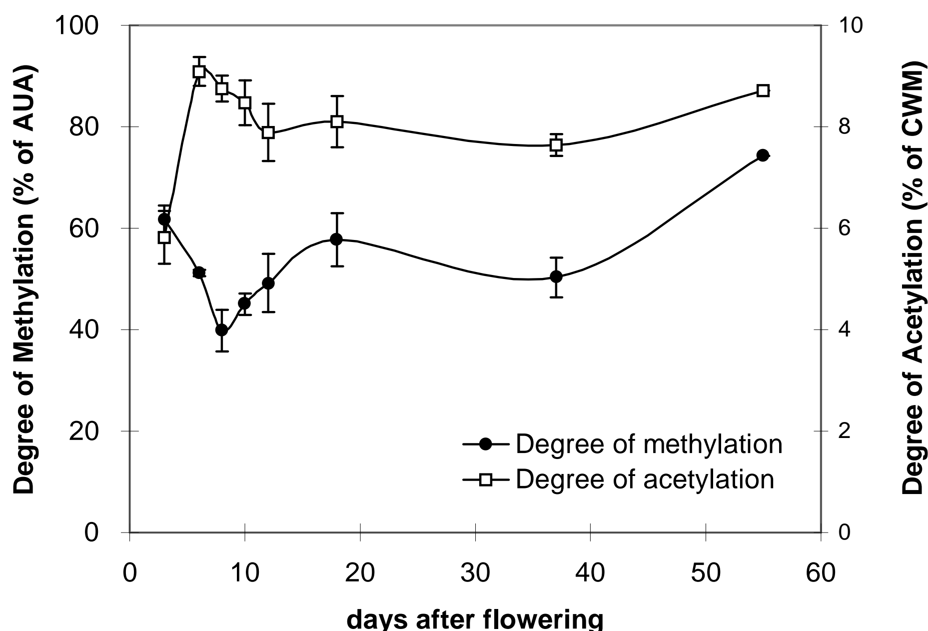


Figure 3.3. *Overall degree of methylation and degree of acetylation of cell walls during sequential developmental stages of green bean pods.*

Table 3.3. *Yields (mg/g DW) of the fractions after extraction of the AIR from green beans during sequential developmental stages.*

Developmental stage	days after flowering	buffer	CDTA	Na_2CO_3 (4 °C)	Na_2CO_3 (20 °C)	0.5 M KOH		1.0 M KOH		4.0 M KOH	KOH/ Borate	Residue
						sol.	prec.	sol.	prec.			
I a:	0 - 5	2	23	32	33	25	93	3	23	19	3	20
I b:	6 - 7	13	26	41	35	20	79	5	33	13	2	37
II a	8 - 9	10	28	40	39	28	54	6	18	16	3	34
II b:	10 - 11	13	23	37	28	14	25	6	10	14	2	32
II c:	12 - 13	18	26	45	33	27	25	4	11	21	3	62
III:	14 - 23	27	31	52	33	40	30	4	2	20	4	63
IV:	24 - 55	68	31	45	4	36	17	12	0	18	1	84
V:	>55	22	113	70	7	39	0	3	1	13	2	94

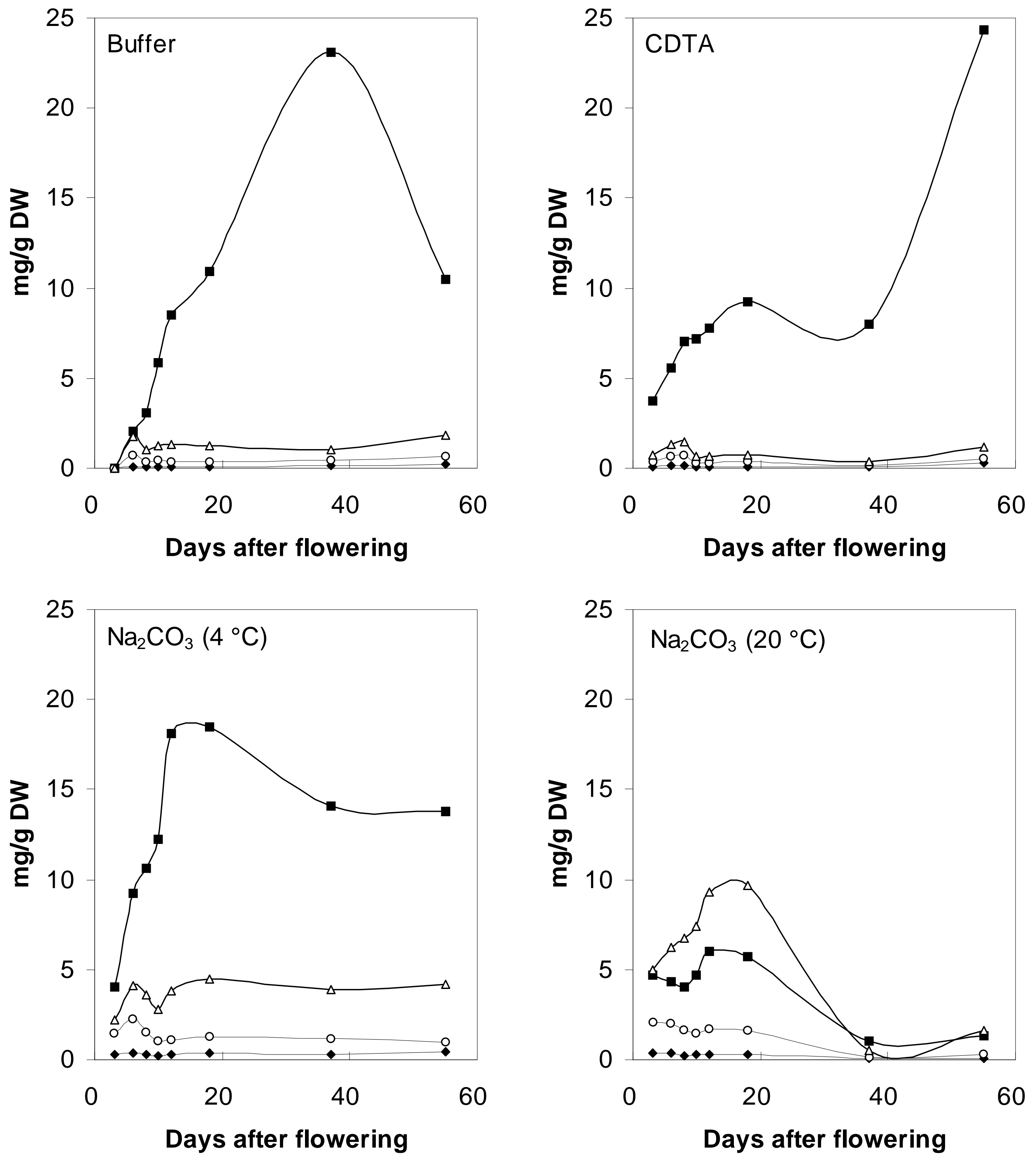


Figure 3.4 *Yield and composition (mg/g pod dry weight) of pectic fractions from green bean pods during sequential developmental stages (AUA: -■-; Rha:-◆-; Ara -Δ-; Gal -○-).*

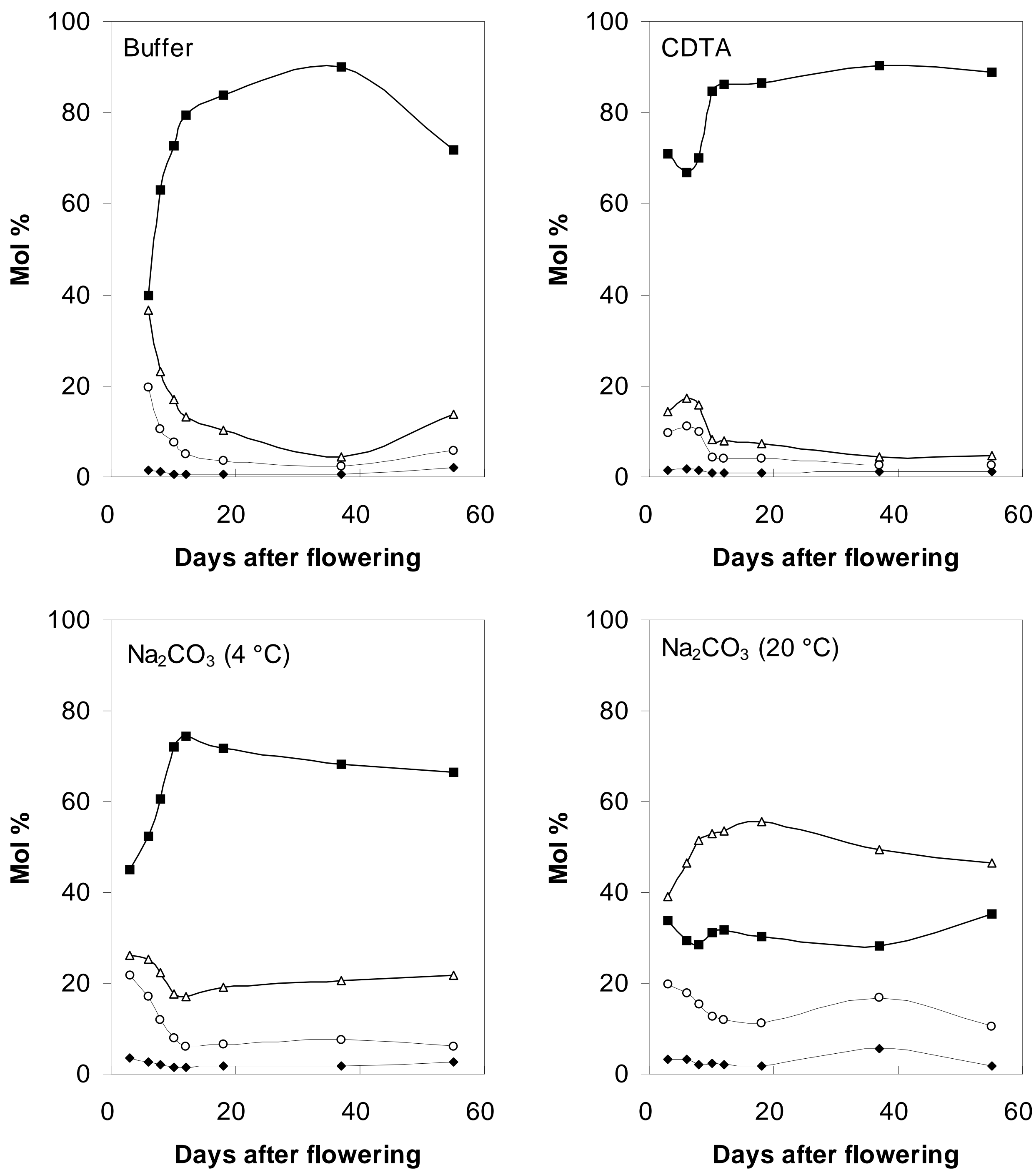


Figure 3.5 *Molar composition of pectic fractions from green bean pods during sequential developmental stages (AU: -■-; Rha:-◆-; Ara -Δ-; Gal -○-).*

3.4.4 Changes in yield and composition of the pectic fractions

The AIR was fractionated into distinct pectic and hemicellulosic fractions by a sequential extraction procedure. The pectic polysaccharides not connected to other cell wall polymers were extracted with the acetate buffer; the ionically linked pectins were most likely solubilised by CDTA. Most of the CDTA-insoluble pectins were subsequently extracted by dilute Na_2CO_3 at 4 °C and 20 °C, presumably by hydrolysis of weak ester crosslinks. This residue was subsequently treated with several KOH solutions to extract hemicelluloses and some residual pectins. During pod development there were significant variations in the amounts of most fractions which indicated changes in connections of the various cell wall polymers to each other (Table 3.3). First, there was an increase in amount of buffer-soluble material, from 0 - 7 % of AIR, with a maximum during maturation (24 - 55 daf). The amount of CDTA-fraction was constant during almost all stages, except for a large increase during senescence. The amount of the 4 °C Na_2CO_3-fraction was approximately constant during pod development at a level of 32 - 48% of pod DW. The amount of the 20 °C Na_2CO_3-fraction decreased and was almost absent during maturation and senescence. Apart from polysaccharide material, all fractions also appeared to contain various amounts of protein and minor amounts of starch (data not shown).

The composition of all pectic fractions was determined by HPLC following TFA hydrolysis. Changes in rhamnose, arabinose, galactose and galacturonic acid content of the pectic fractions during development are shown in Figure 3.4 and 3.5. These sugars represent the major sugar residues in these cell wall fractions. The major compositional changes occurred during the first stages of development, i.e. pod elongation. Galacturonic acid content increased during these stages in all pectic fractions except the 20 °C Na_2CO_3-fraction. The composition of the buffer and CDTA fraction, containing respectively free and calcium complexed pectins, were similar at edible maturity (Figure 3.4). The increase in yield of buffer-fraction up to this stage was mainly caused by an absolute increase of galacturonic acids. The Na_2CO_3 fractions, especially the fraction extracted at 20 °C, contained significantly more neutral sugars from the beginning. These fractions contained most likely covalently linked branched pectins. Pectic sugars were lost, from both Na_2CO_3-fractions during maturation and senescence. At the senescence stage (55 daf) a very large part of the galacturonic acid residues was retrieved in the CDTA-fraction. The net galactose and arabinose levels remained constant in all pectic fractions, with the exception again of the 20 °C Na_2CO_3-fraction, in which galactose increased initially, but decreased during the final developmental stages. In addition to changes in sugar composition, there was also a variation in the DM and DA of the pectins (Figure 3.6). These values were determined only in the buffer and CDTA-fractions since the esters are saponified during the alkaline extraction procedures. The DM and DA of the buffer-soluble pectins were initially very high, respectively 100 and 50%. During the linear growth stage they declined to 70 and 12% respectively, but increased again slightly during maturation and senescence. For the CDTA-soluble pectins a different trend was noticed, the DM and DA being very low throughout development, about 10% each. During initial growth the DM of the CDTA fraction increased only temporarily to a value of 50%. Altogether, on average only 18 % and 7% of the total cell wall methyl~ and acetylesters respectively, were recovered in these fractions.

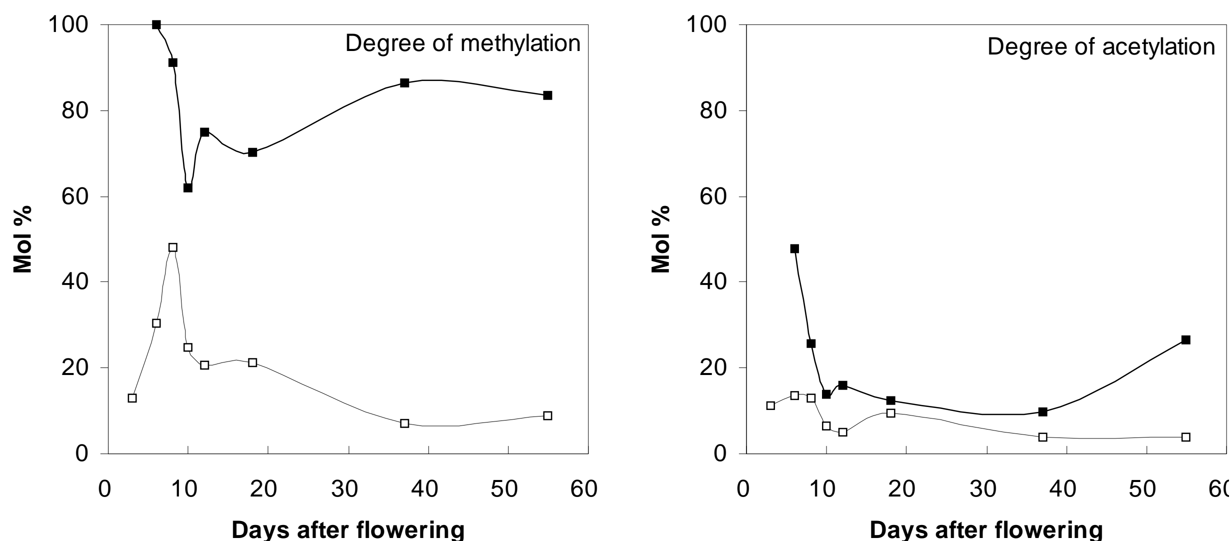


Figure 3.6 *Degree of methylation (left) and degree of acetylation (right) of purified pectic fractions from green bean pods during sequential developmental stages (buffer: -■-; CDTA: -□-)*

3.4.5 Molecular mass distribution of the pectic fractions

The change in M_r distribution of the galacturonic acid rich fractions during development was determined by HPSEC. In Figure 3.7 the elution patterns of the pectins from sequential developmental stages are shown. The peak appearing after 13 min in the CDTA-fraction was caused mainly by residual CDTA present in the sample (Figure 3.7b). The Na_2CO_3-20 °C fractions were very difficult to dissolve and the resulting elution patterns showed no clear peaks and are therefore not shown. All other fractions showed a similar trend. During exponential elongation (stage I), there were large amounts of low and intermediate M_r material. During linear elongation and cessation of growth (stage II III) considerable amounts of high M_r were present, while during maturation (stage IV) the M_r decreased again. This was especially evident in the buffer fraction. At the senescent stage (stage V), all fractions were very heterogeneous in M_r, resulting in a very low, broad peak.

3.4.6 Yield and composition of the hemicellulosic fractions and cellulose residue

Overall yields of the various KOH-(soluble)-fractions showed no clear trend. However, protein content decreased during development from 75 to 30% and 50 to 25% in the 0.5 M and 1.0 M KOH-(soluble)-fractions (not shown). Taking this into account the amount of cell wall material in the 0.5 M(soluble) fraction increased. The protein content of the 4.0 M KOH-fractions and the residue were invariably much lower, respectively 8.9, 7.0 and 2.9%. The yields of the KOH-(precipitate)-fractions, being the major fractions in stage Ia, declined during growth. The protein content of both the 0.5 M and 1.0 M KOH-(precipitate)-fraction was constant and on average 65%. The amount of cellulose residue increased during growth and became one of the major cell wall fractions after pod elongation stopped (stage IV).

Table 3.4 *Carbohydrate composition (mol%) and yield (mg/g Bean DW) of hemicellulose fractions and residue after extraction from AIR from green beans during sequential developmental stages.*

Developmental. stage	days after flowering	Fuc	Rha	Ara	Gal	Glc	Xyl	Man	AUA	total yield
					mol%					mg/g
0.5 M KOH soluble										
I a:	0 - 5	1.2	0.5	13.7	19.8	35.1	8.3	4.8	16.4	4.8
II b:	10 - 11	0.5	1.1	18.1	30.3	36.3	6.5	1.9	5.4	8.7
III:	14 - 23	0.2	0.8	8.6	26.4	49.0	6.0	1.0	8.0	29.9
IV:	24 - 55	0.3	1.2	11.4	33.2	35.1	6.2	1.1	11.4	21.5
V:	>55	0.4	1.2	10.6	27.9	26.5	10.5	1.3	21.7	34.0
0.5 M KOH precipitate										
I a:	0 - 5	2.2	2.9	27.7	33.3	11.8	6.1	6.4	9.5	7.1
II b:	10 - 11	0.9	2.8	25.4	43.2	7.5	4.4	4.2	11.6	2.8
III:	14 - 23	0.7	3.1	21.9	45.1	9.1	3.9	3.8	12.5	4.1
IV:	24 - 55	0.6	3.2	18.8	47.4	2.2	8.6	5.4	13.9	1.5
V:	>55	-	-	-	-	-	-	-	-	0.0
1.0 M KOH soluble										
I a:	0 - 5	3.2	0.7	7.7	18.4	33.5	28.6	4.0	3.9	1.0
II b:	10 - 11	3.3	1.0	10.3	24.9	23.7	28.5	2.5	5.7	2.5
III:	14 - 23	2.2	0.9	9.7	24.9	28.0	24.5	2.2	7.6	1.4
IV:	24 - 55	0.9	0.7	7.2	19.0	46.6	13.0	2.7	10.0	5.5
V:	>55	1.6	1.8	9.8	23.4	14.7	20.0	4.8	23.9	0.8

1.0 M KOH precipitate										
I a:	0 - 5	1.1	1.9	21.6	33.1	17.0	8.6	3.8	12.9	2.0
II b:	10 - 11	0.7	2.1	20.8	40.2	10.8	6.4	3.0	16.1	1.2
III:	14 - 23	0.8	3.1	18.7	42.3	12.5	5.3	1.6	15.8	0.4
IV:	24 - 55	-	-	-	-	-	-	-	-	0.0
V:	>55	-	-	-	-	-	-	-	-	0.0
4.0 M KOH										
I a:	0 - 5	4.2	2.3	9.5	24.4	21.8	17.4	3.3	17.0	13.4
II b:	10 - 11	5.1	1.3	7.0	21.5	26.1	21.8	3.7	13.4	11.2
III:	14 - 23	4.6	1.3	6.5	22.9	28.2	22.2	3.5	10.9	12.8
IV:	24 - 55	3.7	0.7	6.2	22.6	29.6	21.6	5.9	9.6	11.2
V:	>55	3.5	1.1	7.8	17.6	22.9	22.8	10.6	13.8	6.9
4.0 M KOH / Borate										
I a:	0 - 5	0.7	4.0	16.8	39.5	9.6	5.2	4.5	19.7	1.6
II b:	10 - 11	0.9	2.3	15.8	53.0	6.1	4.0	4.3	13.6	1.2
III:	14 - 23	0.8	2.7	13.6	50.6	8.1	4.7	4.0	15.6	2.5
IV:	24 - 55	0.6	2.1	12.2	46.2	8.9	3.4	7.7	18.9	0.6
V:	>55	1.2	4.5	9.3	22.7	15.1	6.2	9.9	31.1	0.3
Residue										
I a:	0 - 5	0.8	0.0	3.2	5.3	74.6	3.3	7.5	5.3	6.4
II b:	10 - 11	0.0	2.4	4.2	7.9	63.9	4.1	9.5	7.9	8.7
III:	14 - 23	0.0	0.0	3.7	6.3	71.3	2.7	10.3	5.7	21.9
IV:	24 - 55	0.0	1.8	2.7	4.4	65.9	2.4	18.1	4.6	28.1
V:	>55	0.0	0.0	3.2	3.5	72.7	3.0	10.7	7.1	24.4

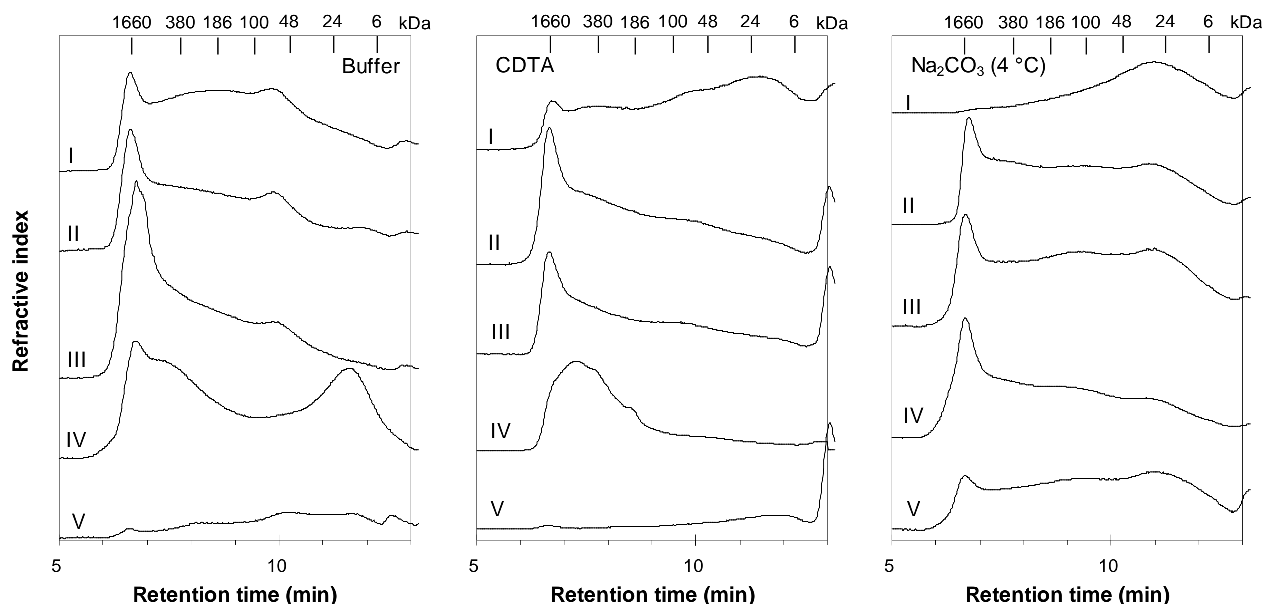


Figure 3.7 *High performance size-exclusion chromatography elution patterns of various pectic fractions from sequential developmental stages (I - V) from green beans.*

The composition of all hemicellulosic fractions was determined by HPLC following TFA hydrolysis. The cellulose residue was analysed after Seaman hydrolysis. With exception of the 0.5 M KOH extraction, which solubilised appreciable amounts of pectin, the sequential KOH extractions solubilised a range of hemicellulosic polymers and small amounts of acidic polymers (Table 3.4). The composition of the fractions was quite stable throughout development and for simplicity reasons only the data from stages Ia, IIb, III IV and V are presented. During neutralisation of the 0.5 M and 1.0 M KOH-fractions a precipitate formed in the early developmental stages, which was analysed separately. These precipitates contained much arabinose, galactose and galacturonic acid. In addition, the levels of protein were very high, being on average 65% of the fraction (not shown). From the molar proportion of the sugars in the 1.0 and 4.0 M KOH fractions the major hemicellulosic component can be inferred to be a xyloglucan[18,19]. In the 1.0 M KOH-fraction the proportion of xylose was relatively high, especially in the final stage. This implied that this fraction most likely also contained some xylans. (1-4) linked xylose residues, typical for xylans, were detected in the cell wall of green beans during earlier studies[20]. The 4.0 M KOH/borate fraction contained mainly arabinose and galactose rich polymers. The residue consisted, as expected, mainly of glucose, but interestingly also contained appreciable amounts of mannose in the final stage.

3.4.7 Anion exchange chromatography of the hemicellulosic fractions

Selected fractions were further resolved by ion-exchange chromatography. After dialysis and lyophilising, the polymers were not completely soluble, the solubility varied between 72- 91% of the extracts. The carbohydrate composition and yields of the major fractions on anion exchange chromatography are listed in Table 3.5. Fractionation of the KOH-(soluble) fractions gave a neutral

fraction, two major acidic fractions, eluting with respectively 0.125 M and 0.250 M NaCl and two very minor acidic fractions eluting by 0.5 M and 1.0 M NaCl.

In the neutral fraction from the 1.0 M and 4.0 M KOH fractions fucose levels were relatively high. Fucose is known to be present in the side chains of xyloglucan from many plant sources, including *Phaseolus coccineus*[19,21]. In addition, the amount of xylose was higher than the amount of glucose, indicating that these fractions contained xylose containing polysaccharides other than xyloglucans. Both the 0.125 and 0.250 M NaCl peak of the 1.0 M and 4.0 M KOH fractions contained high amounts of pectic sugars like galacturonic acid and rhamnose. The 0.125 M peak was also rich in xylose, possibly originating from acidic xylans.

3.4.8 Enzyme activities during development

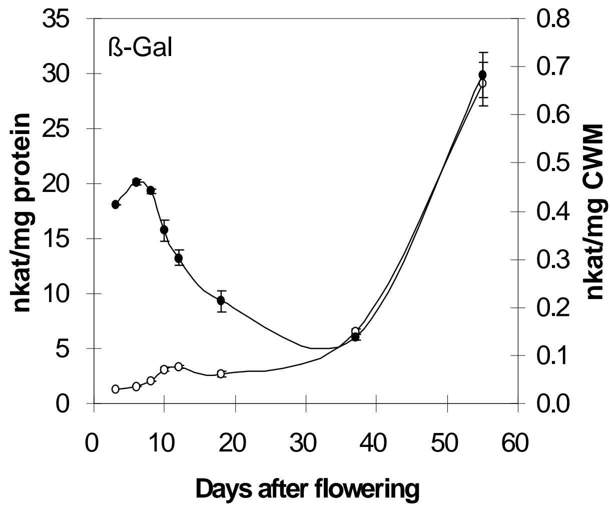
To estimate the potential role of several pectin modifying enzymes as well as peroxidases during development, the activity of crude enzyme extracts were tested *in vitro*. POD was analysed because it can play a role in the cross-linking of pectins and cell wall proteins by catalysing the formation of phenolic couplings, such as dityrosine and diferulic acid. The enzyme activities are plotted as specific activity (nkat/mg protein) as well as activity on basis of cell wall material (CWM), the cell wall being the potential substrate *in vivo* (Figure 3.8).

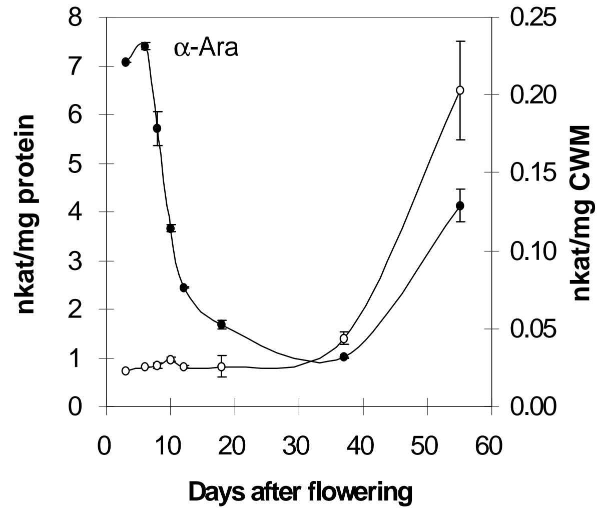
The specific activities were in all cases higher in the older bean pods. In general, the activities on basis of CWM were relatively high in the initial stages of pod development and decreased later on. The highest activity during all developmental stages was found for PME (Figure 3.8). Activity levels increased from 20 to 80 nkat/mg protein during development. PG-activity was detectable only in extremely low amounts (10 - 20 pkat/mg protein) at all developmental stages. Nevertheless, small levels of endo-PG activity might have a large effect on the chain length of pectins. HPSEC analysis of PGA before and after incubation with the enzyme solution however showed no shift in molecular mass distribution, which implied that the observed low activity represents exo-PG activity. ß-Galactosidase as well as α-arabinosidase activity was present throughout pod development (Figure 3.8). The highest activity was found for ß-galactosidase. Interestingly the level of ß-galactosidase was extremely high during the last developmental stage, its specific activity increased over 20-fold. On CWM basis the highest activity for ß-galactosidase was found during senescence. POD activity was also present at all developmental stages, but was very high only during the initial growth phases (Figure 3.9).

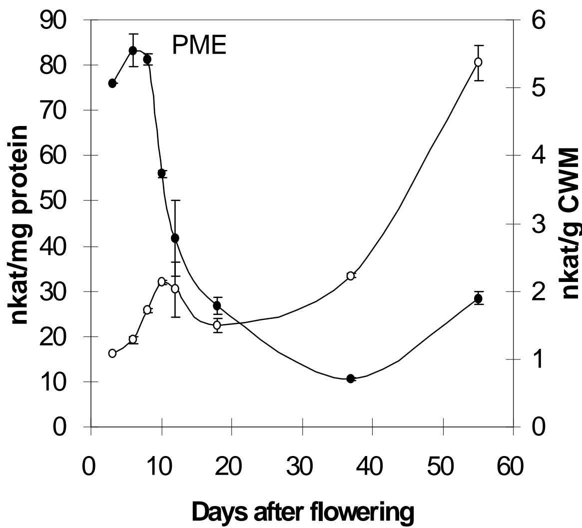
To test for pectin degrading activities, the enzyme extracts from stage Ib (exponential growth) and stage IV (maturation) were tested against a native, highly branched pectic fraction from green beans. HPSEC elution patterns of these enzyme digests are shown in Figure 3.9. The peaks of the digested samples had shifted to a lower M_r. Especially the enzyme preparation from stage Ib contained significant pectin degrading activity. The observed results suggest the presence of rhamnogalacturonase activity, since the shift in retention times can hardly be the result of only side chain degradation and furthermore endo-PG activity was not detected in the beans.

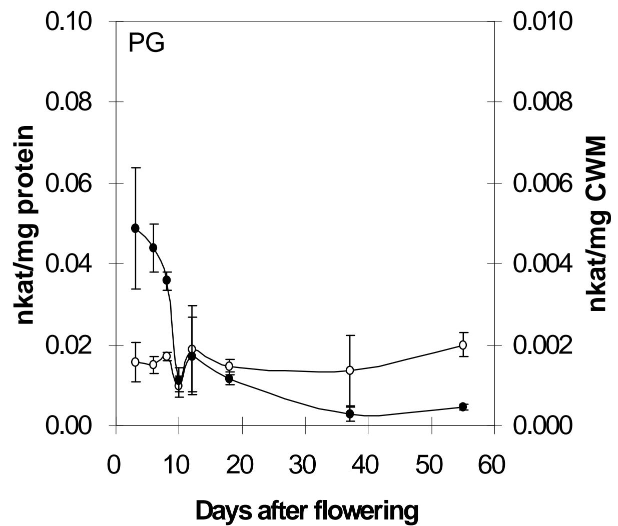
***Table 3.5** Carbohydrate composition (mol%) and yield (mg/g Bean DW) of fractions after anion exchange chromatography of selected hemicellulose fractions.*

Fraction		Fuc	Rha	Ara	Gal	Glc	Xyl	Man	AUA	yield
					mol%					mg/g DW
0.5 M KOH										
buffer	I	1.6	0.3	17.3	19.1	28.1	15.9	4.4	13.4	0.69
	III	0.5	0.7	1.7	1.6	82.3	5.7	1.4	6.1	5.00
	IV	0.4	0.4	3.3	5.8	7.5	5.4	0.8	6.4	6.31
0.125 M NaCl	I	3.0	4.0	20.6	26.1	15.4	7.8	8.9	14.2	0.41
	III	1.8	0.3	8.3	18.3	42.9	6.8	3.5	18.1	0.40
	IV	1.9	0.2	13.5	28.4	27.7	8.8	1.3	16.3	0.61
0.250 M NaCl	I	1.1	0.5	9.9	31.9	12.5	16.9	4.2	23.1	0.43
	III	1.3	5.0	12.1	35.1	16.7	7.3	5.1	17.4	0.66
	IV	0.6	0.5	15.1	49.6	8.8	7.8	1.3	16.3	1.20
1.0 M KOH										
buffer	I	4.2	0.5	3.9	23.3	26.8	31.5	3.8	6.0	0.67
	III	3.5	0.4	5.4	19.3	28.6	32.2	2.9	7.7	0.87
	IV	1.3	0.8	5.4	16.7	39.4	14.2	4.0	18.1	2.69
0.125 M NaCl	I	2.0	3.1	9.6	12.9	12.3	26.4	6.5	27.3	0.09
	III	1.7	3.1	15.7	17.9	14.0	26.2	5.1	16.3	0.11
	IV	1.2	3.4	16.9	28.7	7.8	21.5	3.8	16.8	0.34
0.250 M NaCl	I	0.8	2.1	9.0	15.4	6.6	42.7	3.9	19.6	0.16
	III	0.6	2.1	9.9	14.8	21.2	28.5	3.0	19.9	0.22
	IV	1.0	3.5	12.2	23.6	5.9	22.4	2.7	28.9	0.44
4.0 M KOH										
buffer	I	4.2	0.5	3.8	23.0	26.4	31.1	3.7	7.3	1.92
	III	3.4	0.4	5.2	18.6	27.5	31.1	2.8	11.1	2.94
	IV	1.5	0.9	6.2	19.2	45.3	16.3	4.6	5.9	2.70
0.125 M NaCl	I	2.3	3.5	10.6	14.2	13.6	29.1	7.1	19.6	0.22
	III	1.8	3.2	16.4	18.6	14.6	27.3	5.3	12.7	0.33
	IV	1.2	5.1	9.2	16.5	14.8	13.0	6.6	32.8	0.41
0.250 M NaCl	I	0.9	2.3	9.9	16.9	7.2	46.8	4.3	11.9	0.39
	III	0.4	1.3	6.2	9.2	13.2	17.7	1.8	50.3	1.23
	IV	0.7	2.5	8.8	17.0	4.2	16.1	1.9	48.7	0.74









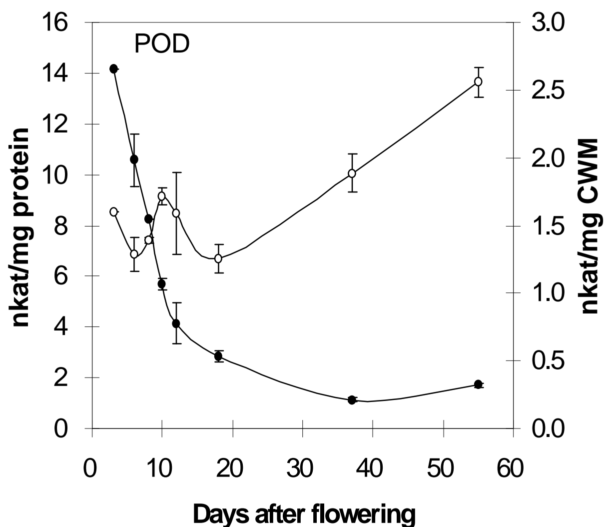


Figure 3.8 *ß-D-galactosidase, α-L-arabinosidase, Pectin methyl-esterase (PME), Poly-galacturonase (PG), Peroxidase (POD) activities in green bean pods during development expressed as nkat/mg protein (-o-) and nkat/mg CWM (-•-).*

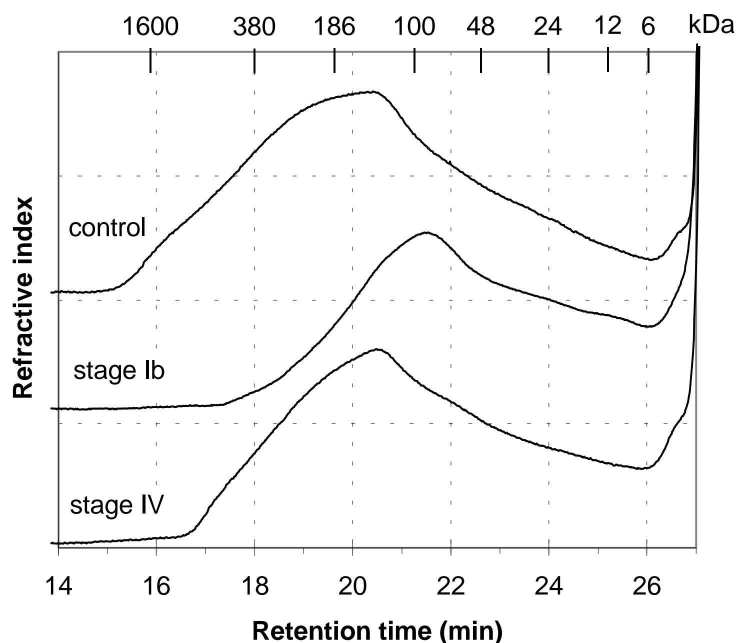


Figure 3.9 *High performance size-exclusion chromatography elution patterns of green bean pectin after digestion with enzymes extracted from exponentially elongating (stage Ib) and maturing (stage IV) green bean pods.*

3.5 DISCUSSION

Growth of green bean pods elongation followed a single sigmoïdal curve (Figure 3.2). Throughout development, there were significant changes in cell wall polysaccharides and enzyme activity levels. Results of cell wall polysaccharide analyses and enzyme activity levels together suggest a high rate of turnover and *de novo* synthesis of cell wall polymers during elongation and a higher rate of autolysis during senescence.

The most significant change in cell wall sugars throughout development, was a relative decrease in neutral pectic sugars and an increase in galacturonic acids (Table 3.2). The overall decrease of arabinose and galactose in the cell wall during development may be due to an enhanced degradation of pectic hairy regions by ß-galactosidase and α-arabinosidase. However, there was no direct relationship between the enzyme levels and the content of arabinose and galactose of the cell wall. This suggests that, in addition to enzymatic degradation, also a reduced incorporation of these sugars into the pectic polymers during synthesis is taking place. This is further supported by the fact that the absolute amounts of arabinose and galactose in the extracted pectic fractions remained more or less constant, while the absolute amount of galacturonic acid increased in all fractions during pod elongation (Figure 3.4). Galactose turnover is suggested to be important in prolonging auxin induced expansion[3]. Furthermore, it has been reported that free arabinogalactan polymers become covalently linked to rhamnogalacturonan within the wall as the cells age[3]. The results obtained in this study suggest that this might also occur in green bean cell walls, since the buffer-soluble pectic fraction of the very small pods contained relatively large amounts of galactose and/or arabinose rich polymers (Figure 3.5). It was not analysed whether these arabinose and galactose polymers occurred as free polymers, or linked to the galacturonan backbone As elongation proceeded more galactose became part of the covalently cross-linked pectic fraction, extractable only with Na_2CO_3 at 20°C. Concurrently, the M_r of all the pectins increased.

During maturation and senescence the larger part of cell wall pectins was easily extractable from the cell wall with buffer and thus probably just held in the wall by physical entanglement. In senescent pods however, there was a decrease in buffer-soluble pectins and most of the pectins were extractable with CDTA and therefore presumably ionically linked. This was accompanied by a decline in M_r of the buffer and CDTA-soluble pectins. This is comparable with results found for many ripening fruits, like kiwi and nectarine[22,23]. Interestingly, the molar sugar composition of the various pectic fractions did not change very much. This is surprising, since the 20 °C Na_2CO_3-soluble pectins consisted for more than 50 mol% of galactose and if they were solubilised one would expect that some of this galactose would end up in the more easily soluble fractions. This galactose was however not recovered in the buffer or CDTA-fraction. This can be the result of exo-galactanase action, which, by hydrolysing side-chains of the branched pectic fractions of the wall, may result in a high rate of degradation of pectic galactan and arabinogalactan side chains[7]. The resulting degradation product, galactose, is lost during preparation of the AIR and dialysis of the cell wall fractions. Solubilisation of galactan was demonstrated to be a general feature of elongating stems and ripening fruit, like tomato, mango, apple and kiwi[6,8,23-25]. For nectarines a decreased degradation of galactan side chains was correlated with the development of mealy fruit[22].

For green beans it seemed that the synthesis of methylated homogalacturonan was still continuing during the maturation phase, while the synthesis of galactose rich polymers and the covalent cross-linking of pectins was already halted. This is supported by the fact that POD activity was low at the final stages of development. POD is postulated to be involved in the oxidative cross-linking of cell wall polymers by catalysing the formation of for example diferulic acid and isodityrosine[3,26]. POD activity was especially high during the elongation phases. Cross-linking of pectins would result in the insolubility of formerly soluble pectins. Cross-linked pectins of this kind may be recovered in the Na_2CO_3-fractions, since the esters between pectin and e.g. diferulic acid may be saponified during the 16 hours incubation. The increase in yields of both Na_2CO_3-fractions ceased already at circa 13 days after flowering. (Figure 3.8). However, peroxidases are enzymes with a very broad spectrum of physiological functions and there are many different isoforms. This makes it difficult to speculate about their function in relation to cell wall modification in the present study, since only total levels were quantified. POD activity was suggested to be a possible parameter in the ripening and senescence of apples and apricots[27]. The increase of CDTA-soluble pectins during senescence might be explained by overall termination of polymer synthesis, but on-going modification of cell wall pectin by PME, which was shown to be still active (Figure 3.8). The demethylated pectins could subsequently become Ca^{++}-complexed to each other and thus extractable with CDTA.

The activity of PME was very high compared to other enzyme levels in the bean pods (Figure 3.8). There was no clear relationship between PME-activity and the average DM of the pectin during development (Figure 3.3 and 3.8). This was also observed for mung bean, tomato and green beans in previous studies[17]. However after comparison of the DM of buffer and CDTA-soluble pectins it is obvious that overall DM values provide very little information about the status of the different pectins in the cell wall *in vivo* (Figure 3.6). The average DM of the total cell wall (Figure 3.3) varied only slightly and was about 50% throughout development. The DM of buffer soluble and CDTA-soluble

pectins however, showed great variation during initial growth and reached extreme values of respectively 80 and 10% after elongation had ceased (Figure 3.6). From these data it is clear that more information is needed about the localisation of the different types of pectin and their DM in order to understand the way in which PME is involved in cell elongation

The most obvious change in the hemicellulosic KOH-fractions during development was a decreased yield of the typical precipitates after neutralisation of the extracts. During maturation and senescence no precipitates were formed anymore. The KOH-precipitates contained most likely highly branched pectins, as could be deduced from the high levels of pectic sugars, and from the high rhamnose /galacturonic acid ratio. In addition to the overall decreased amount, arabinose content declined specifically in these precipitates. Waldron and Selvendran (1990) also noted a maturation related decrease of arabinose in elongating asparagus stems[28]. Since in their case arabinose decreased as the primary wall became more important they presumed that this arabinose was of middle lamella origin. In the present study however, no net loss of arabinose was observed in the buffer, CDTA or 4°C Na_2CO_3-fractions. Therefore it can be concluded that in green bean pods, the overall loss of arabinose, which was already evident in the AIR, originates from highly branched pectins, present in the cell wall in close association with hemicelluloses, which are deeply embedded in the cell wall matrix. This observation seems similar to trends reported for ripening nectarines[22]. The presence of xylose within the pectic KOH-fractions indicates the probable presence of hemicellulose-pectin complexes. Ryden and Selvendran (1990) already stated that the occurrence of such complexes should be taken into account in cell wall models of *Phaseolus* and other leguminous species[21]. The yield and solubility of the precipitates was unfortunately to low to analyse them in more detail. Therefore no conclusions could be obtained whether those precipitates contained just by accident co-precipitated pectic and hemicellulosic polymers or, whether the hemicellulosic sugars glucose, xylose and mannose were in some way or another covalently linked to the pectins. A xylogalacturonan was recently shown to be a structural subunit of the hairy regions of apple pectins[29].

In the present study there was no clear change in xyloglucan and xylan in the bean pods during development (Table 3. 4). Though, we did not examine the M_r distribution of the hemicellulose fractions. Chanda et al.[30] isolated low and high molecular mass xyloglucans from *Phaseolus vulgaris* hypocotyls by extraction with respectively 0.7 and 4.0 M KOH solutions. They recorded a decline in 4 M KOH extracted xyloglucans and concluded that depolymerisation of xyloglucan was involved in cell wall loosening during elongation. The present data do not support their observation, since no significant change in the 4.0 M KOH fraction was detected. Also for ripening tomatoes it was demonstrated that the amounts of cellulose and xyloglucan remained constant while the M_r of xyloglucan decreased markedly during softening[8]. These changes were suggested to result from increased activities of specific ß-glucosidases and xyloglucan endotransglycosylase. However, in contrary to fleshy fruits, pods of green beans do not soften appreciably as they age, the senescent pods become indeed very dry and crisp. Bean pods release their mature seeds by separation of the two pod halves after drying. The cellulose content of the pods increased during the later stages of pod elongation and during maturation senescence (Table 3.3 and 3.4). During maturation and senescence

this was accompanied by an increase in mannose content. Glucomannans occur in moderate amounts in certain secondary cell walls and can crystallise on to cellulose[26].

In conclusion, during development of green bean pods the main change in cell wall composition was a change in the pectic substituents. Very young, exponentially growing cell walls contained large amounts of neutral sugar rich pectic polymers (rhamnogalacturonan), which were water insoluble and relatively tightly connected to other cell wall components. During elongation, additional galactose-rich pectic polymers were deposited and cross-linked into the cell wall. Apart from this, the level of branched rhamnogalacturonan remained rather constant, while the level of linear homogalacturonan increased steadily. This suggests that open gaps in the cell wall network formed by expansion, are filled with homogalacturonan, while the structure is being locked by galactose-rich pectic polymers. During maturation of the pods, these tightly linked galactose-rich pectic polymers were degraded while the accumulation of soluble homogalacturonan continued. During senescence there was an increase in the amount of ionically complexed pectins, mainly at the expense of freely soluble pectins. The results in this study suggest that there is a constant turnover of the cell wall material during pod elongation, with a shift from neutral sugar rich, branched pectins (rhamnogalacturonan) to the synthesis of non-branched, high M_r pectins (homogalacturonan), as elongation proceeds. The synthesis and cross-linking of neutral pectic side-chains seemed to halt as elongation ceased, while the synthesis of highly methylated linear pectins appeared to continue until early senescence. The changes in (hemi)cellulose concerned mainly an increase in cellulose content at the end of the elongation phase and a small shift from xyloglucans to more xylans and mannans during maturation and senescence. Cell walls of senescent pods consisted for the major part of cellulose and linear ionically linked pectins.

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Chapter 4

Cell wall dissolution during industrial processing of green beans[*]

4.1 Abstract

Cell wall material was isolated from fresh and industrially processed green beans using two cultivars and two different isolation methods. The chemical composition of the cell wall fractions was compared and the structure of the water insoluble polymers was investigated by methylation analysis. This study indicated that the major cell wall polysaccharides of green beans pods were cellulose and pectin, but hemicelluloses were also detected. Major cell wall changes occurred during the sterilisation process. Homogalacturonan was partially degraded and on average 68 % of the pectin was solubilised during this process. Sugar analyses indicated that either pectin associated, or hemicellulosic arabinogalactans remained immobilised within the residual cell wall matrix after sterilisation. Processing did not affect the overall degree of acetylation. However, the overall degree of methylation decreased after sterilisation, most likely predominantly caused by ß-eliminative breakdown of highly methylated homogalacturonan regions of the pectin during the heating process.

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[*] Derived from: T. Stolle-Smits, J.G. Beekhuizen, C. van Dijk, A.G.J. Voragen, K. Recourt (1995). J. Agric. Food Chem. 43:2480-2486 .

4.2 Introduction

Plant cell walls are the major determinants of textural properties in fruits and vegetables[1-3]. Since firmness is an important quality attribute of many economically important plant-based food products, there is a need for a better understanding of the relation between the texture of a plant tissue and chemical properties of the cell walls and middle lamellae.

The physical characteristics of the cell wall and middle lamella primarily depend on the composition and the interaction of the constituent polymers and their interactions. Until now, most research on cell walls has been focused on the composition, structure and properties of fresh tissues[4-8]. In most fruits and vegetables the cell wall consists typically of 40 % pectic polymers, 35 % cellulose, 15 % hemicellulosic polymers, 5 % phenolics and 5 % proteins. In this type of cell wall, these constituent polymers are ordered in three structurally independent, but interacting domains[9]. One domain, the fundamental network of cellulose microfibrils and xyloglucans, is embedded in a second domain of matrix pectic polysaccharides. The third domain consists of structural proteins, like extensines.

Pectins have been studied frequently because they are thought to be important cell wall components with respect to growth, ripening and processing of fruits and vegetables. Selvendran[10] supposed that pectins of the middle lamella are highly methylesterified and slightly branched. Cell wall pectin however, consists of two distinguishable regions, a linear homogalacturonan and branched rhamnogalacturonan[11]. There is good evidence for covalent cross-linking of pectins via ester bonds, either connected to other pectic molecules, to hemicellulose, cellulose or protein. Fry[12] suggested that at least some of these are diferulate bridges between adjacent pectin molecules. The additional pectin is connected to the wall only by its ability to form calcium intermediated non-covalent gels[13]. For legumes the overall monosaccharide composition of cell walls of *P. vulgaris* (green beans) was shown to be similar as compared to *P. coccineus* (runner beans)[14-18]. In *P. coccineus* two types of xyloglucan, with different degrees of branching, can be discriminated. In addition complexes of xylans with pectic material have been extracted[17]. Parenchymatous cell walls of *P. coccineus* typically contain a high level of hydroxyproline rich glycoproteins.

Until now, research on the effect of processing on the chemical structure of cell walls has been limited to determinations of total pectin and neutral sugar content[19,20]. Several studies revealed that the major cleavage reaction leading to vegetable softening was a ß-eliminative depolymerisation of intercellular pectin. Increased methylester content resulted in a higher rate of pectin degradation[21]. Some cations and anions were also shown to enhance ß-elimination[22-26]. However, possible modifications of minor but important cell wall constituents remain largely unknown. This study focused on chemical cell wall changes occurring during processing of green beans. We report the sugar composition, acetyl groups, methyl esters and sugar linkages of cell wall polymers isolated from fresh, blanched and sterilised green beans.

4.3 Materials and methods

4.3.1 Plant material and processing conditions.

Green beans (Phaseolus vulgaris L.) cv. Montano (fresh market bean) and cv. Masai (industrial market bean) were grown at the experimental research station PAGV (Lelystad, The Netherlands) and harvested at edible maturity at 68 and 77 days after sowing respectively. The pods were cut into parts of 3 to 4 cm in length and blanched at 90 °C for 4 min. For the canning process portions of 410 g were packed into cans (720 mL) and a 0.25 M NaCl solution (brine) was added. Closed cans were sterilised at 118 °C for 30 min, cooled and stored at 15 °C for 2 weeks. The pH of the brine after sterilisation was 5.5. Quadruplicate samples were taken from fresh, blanched and sterilised beans. Each sample was cryomilled in liquid nitrogen by using a Moulinex food processor. All samples were stored at -20 °C until further analysis.

4.3.2 Firmness measurements.

The firmness of the sterilised bean pods was measured in triplicate using an Instron Universal Testing Machine equipped with a Kramer shear cell. 40 g of material was placed in the cell with the length axis of the pods perpendicular to the openings of the shear cell. The maximal force (top value) applied with the shear press to break through the beans was used to quantify the firmness of the beans.

4.3.3 Isolation of cell wall material.

(a) Alcohol Insoluble Residue (AIR)). 50 g of frozen material was immersed in 100 mL cold (-30 °C) ethanol (96 % v/v), homogenised with an ultraturrax by four bursts of 1 min and collected on a Whatmann GF/C filter. The material was suspended in 50 mL ice-cold aqueous ethanol (70 % v/v) and stirred for 1 h at 2 °C. The material was filtered again, washed twice with 50 mL 100 % acetone until the filtrate was colourless and dried overnight to yield the AIR.

(b) Water Insoluble Residue (WIR) was isolated by a slightly modified method of Selvendran et al. (1985)[27]. 50 g of frozen material was homogenised with an ultraturrax by four bursts of 1 min in 100 mL SDS (1.5 % w/v), 5 mM $Na_2S_2O_5$ and 20 mM HEPES (pH = 7.5). This slurry was centrifuged at 10 °C for 15 min at 2,500 g. The pellet was suspended in 75 mL SDS (0.5 % w/v), 3 mM $Na_2S_2O_5$ and 20 mM HEPES (pH = 7.5) and stirred overnight at 4 °C. Next, this slurry was centrifuged using the conditions as mentioned above. The pellet of this centrifuge step was washed three times with distilled water. After exhaustive dialysis the residue was lyophilised and yielded the water insoluble residue (WIR). The supernatants of the two consecutive centrifugation steps were combined and concentrated by ultrafiltration using an Amicon cell equipped with a PM 10 membrane. Four volumes of ethanol were added to the concentrates to precipitate the polymers overnight at -20 °C. Precipitates were recovered by centrifugation as mentioned above and lyophilised to yield the water soluble polymer (WSP) fraction. All cell wall fractions were milled in a Retsch ball mill prior to further analysis.

4.3.4 Monosaccharide composition.

Each sample was analysed in duplicate. Sugars were released from the samples by dispersing in cold 11.5 M H_2SO_4 for 2 h at 20 °C, followed by hydrolysis in 1 M H_2SO_4 for 2 h at 100 °C (Seaman hydrolysis). By using this method all cell wall polysaccharides were first solubilised, including cellulose. The hydrolysates were filtered through a Whatmann GF/C glassfibre filter and neutralised with $BaCO_3$. Samples (10 µL) of the neutralised hydrolysates were analysed for neutral sugars by using a HPLC system (Pharmacia LKB Low pressure mixer, HPLC pump 2248 and autosampler 2157) equipped with a Carbopack PA1 column (250 x 4 mm, Dionex). The eluents, consisting of milli Q water and 150 mM NaOH, were sparged and pressurised with helium. Prior to injection, the system was equilibrated with 30 mM NaOH for 8 minutes at a flowrate of 1.0 mL/min at ambient temperature. At 0.1 min after injection, the eluent was shifted from 30 mM NaOH to milli Q water. After each run the column was regenerated with 150 mM NaOH for 15 min. Compounds were detected with a Dionex pulsed amperometric detector fitted with a gold working electrode. The applied potentials were set at E1 = 0.1 V, E2 = 0.6 V and E3 = -0.6 V against a Ag/AgCl reference electrode. Pulse durations for the applied potentials were 500 msec, 100 msec and 50 msec respectively. Trehalose, added after hydrolysis of the samples, was used as an internal standard. Anhydro-uronic acids were determined as described by Ahmed and Labavitch[28].

4.3.5 Starch content.

To solubilise starch, 5 mL of HCl (8 M) and 20 mL of dimethylsulfoxide were added to 250 mg of sample. After an incubation period of 60 min under continuous shaking in a water-bath, 5 mL NaOH (8 M) and citrate-buffer (Titrisol/pH 4, Merck 9884) were added to a final volume of 100 mL. After filtration, 0.1 mL of sample was used to quantify the starch content using test-combination cat. nr. 207748 from Boehringer Mannheim.

4.3.6 Protein content.

The nitrogen content of the CWM fractions was measured using a Carlo Erba CHNS-OEA 1108 Elemental analyser. The protein content was estimated by multiplying the nitrogen value by 6.25.

4.3.7 Methyl and acetyl substituents.

The amount of methyl and acetyl groups was determined by using a HPLC system under the conditions as described previously by Voragen et al.[29].

4.3.8 Methylation analysis.

WIR samples were methylated using a modified method of Ciucanu and Kerek[30] . WIR (5 mg) was cryomilled with a SPEX freezer mill and dispersed in anhydrous dimethylsulfoxide (1 mL) by occasional sonification for up to 72 h prior to the methylation procedure. After addition of 20 mg of powdered NaOH and 0.5 mL of CH_3I, the solutions were stirred overnight. The excess of CH_3I was evaporated in a stream of air. The methylated samples were subsequently dialysed using running tap water (16 h), followed by distilled water (6 h). Next, the samples were lyophilised. The lyophilised

powder was methylated again starting with dispersion in anhydrous dimethylsulfoxide. Hydrolysis was performed at 121 °C in TFA (2M). Partially methylated sugars were reduced with $NaBD_4$ and acetylated according to Englyst and Cummings[31]. The partially methylated glycitol acetates were separated and quantified using a fused silica capillary column (30 m x 0.255 mm, wall coated with DB1701, 1.0 μm) in a GC-system (Carlo Erba HRGC 5300 mega series) equipped with a FID. The sample composition was calculated using effective carbon response (ECR) factors[32]. Identification was confirmed by GC-MS (Carlo Erba mega series coupled to a Carlo Erba QMD 1000 MS equipped with a fused silica column coated with CPSIL 5 CB, 25 m x 0.25 mm, 0.12 μm). The 3,4- and 2,3-O-methylated and, separately, the 2- and 4-O-methylated xylitol acetates co-eluted and their relative amounts were calculated from the relative abundance of the ions at m/z 117 and m/z 118 respectively.

4.3.9 Statistical analysis.

Data sets were subjected to analysis of variance (ANOVA, Genstat 5) to determine least significant differences (lsd) among cultivars and processing treatments. All analyses were carried out in duplicate and the entire experiment was repeated four times.

4.4 Results

4.4.1 Firmness of beans.

The firmness, as measured with an Instron, of both green bean cultivars was comparable and decreased during processing (Table 4.1). The strongest decrease occurred during the sterilisation treatment.

Table 4.1 *The firmness[*)] (N) of green bean cultivars Montano and Masai.*

	cultivar	
Treatment	Montano	Masai
Fresh	3851 ± 250[a]	3712 ± 271[a]
Blanched	3132 ± 40[b]	3079 ± 60[b]
sterilised	158 ± 4[c]	155 ± 3[c]

[*)] The mean values in a column are significantly (P<0.05) different for treatment and/or cultivar if followed by a different letter (n = 4, ± SD).

4.4.2 Isolation of cell wall material.

Cell wall material (CWM) was isolated from two green bean cultivars using two different methods. Firstly, AIR was isolated, which contained all CWM in addition to proteins, starch and other high molecular mass components. In addition, the plant material was extracted with SDS-buffers, which

Table 4.2 *Amounts[*)] of dry weight (DW), Alcohol insoluble residue (AIR), Water insoluble residue (WIR), and soluble material (WSP) of green bean cultivars Montano and Masai. Numbers in parenthesis represent the total of cell wall sugars*

treatment	DW	AIR	WIR	WSP
	mg/g fresh	mg/g DW		
Cv. Montano				
Fresh	100 ± 1[a]	582 ± 3[a] (273)	389 ± 82[a,b] (242)	125 ± 13[a] (13)
Blanched	105 ± 4[a]	637 ± 37[a,b] (314)	407 ± 80[a,b] (265)	191 ± 23[b,c] (15)
Sterilised	87 ± 3[b]	691 ± 53[b] (319)	490 ± 45[b] (239)	214 ± 49[c] (61)
Cv. Masai				
Fresh	85 ± 1[b]	584 ± 6[a] (310)	390 ± 58[a,b] (252)	164 ± 13[a,b] (10)
Blanched	85 ± 2[b]	595 ± 49[a] (309)	362 ± 4[b] (245)	185 ± 10[b,c] (22)
Sterilised	71 ± 4[c]	671 ± 39[b] (351)	448 ± 60[a,b] (247)	231 ± 30[c] (81)

[*)] The mean values in a column are significantly ($P<0.05$) different for treatment and/or cultivar if followed by a different letter ($n = 4$, ± SD).

resulted in an immediate separation of water soluble (WSP) and water insoluble cell wall polymers (WIR). The amount of cell wall materials and dry weights of the different fractions are summarised in Table 4.2. During sterilisation the intercellular spaces of the bean pods become filled with brine solution, which resulted in a lower dry weight as compared with the fresh samples. The relative proportion of CWM was higher in the sterilised samples as compared with fresh samples, since small molecules and salts leach out into the brine. Additionally, heat treatment denatures proteins, thereby affecting the overall composition of the WIR. Almost all protein could be extracted from fresh and blanched samples with the SDS-buffer, while after sterilisation most proteins were unextractable and were recovered in the WIR. Protein accounted for 19 - 24 % of the weight of all AIR and WIR samples of sterilised material. WIR of fresh and blanched samples contained approximately 5 - 6 % protein. In contrast, the WSP fractions contained approximately 70 % protein. The AIR and WIR of Montano contained 23 - 30 % of starch whereas the AIR and WIR of Masai contained 13 - 15 % of starch. CWM accounted for 46 - 53 % of the AIR from both cultivars. Processing resulted in an increased solubilization of CWM from both cultivars, reflected by an increase in contents of the WSP-fractions. However, these polymers did not leach out into the brine but remained entangled inside the beans. The amount of polymers in both the blanching water and the brine appeared to be negligible. For this reason no attempts were made to analyse the blanching water and brine in detail.

4.4.3 Analysis of carbohydrate composition.

A comparison of the cell wall fractions during processing revealed that sterilisation drastically altered the overall sugar composition of the cell walls of both cultivars (Figures 4.1 and 4.2). After this treatment the total uronic acid content of the AIR and WIR+WSP was reduced with 34 - 38 % as

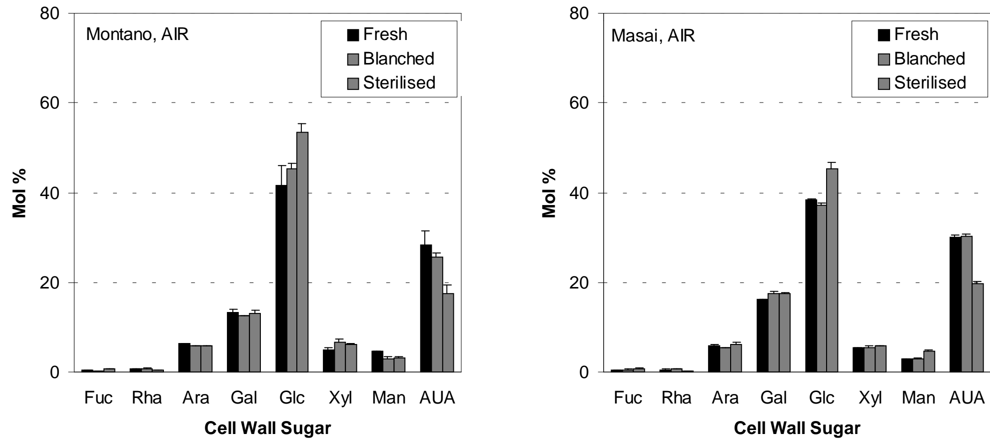

Figure 4.1 *Neutral sugar composition and uronic acid content of the AIR from green bean cultivars Montano and Masai.*

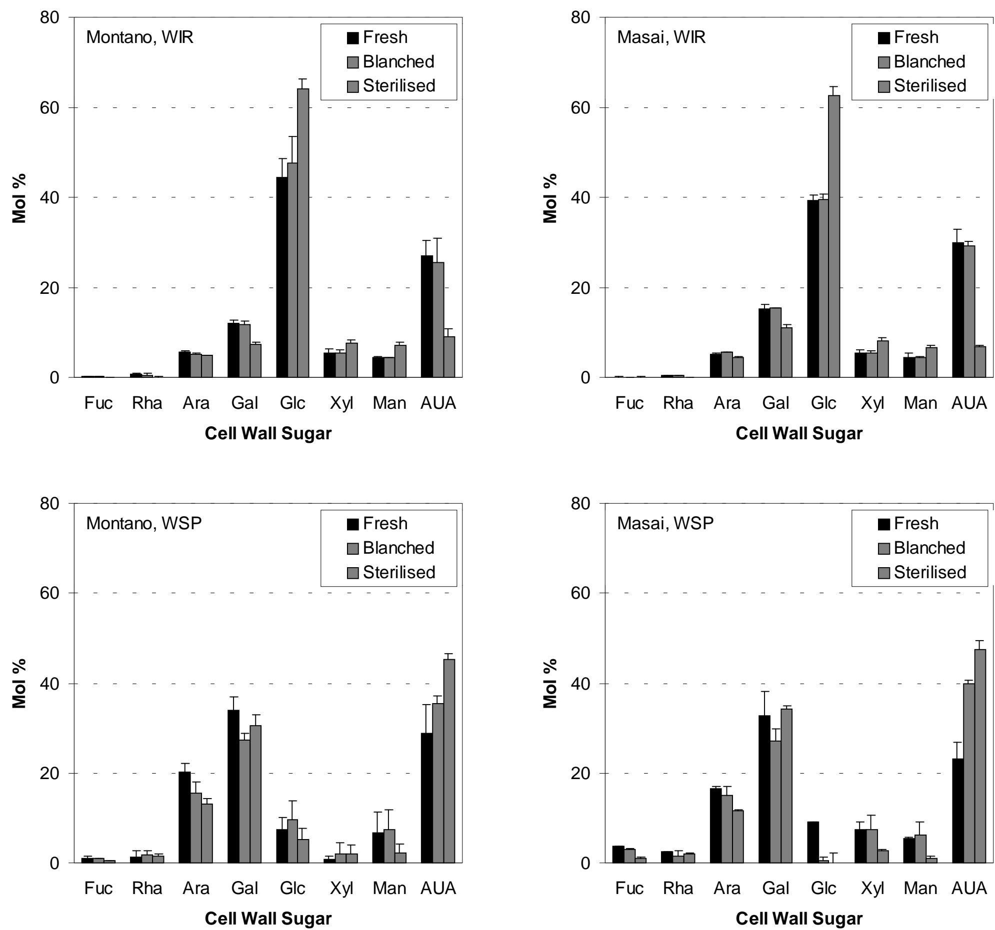

Figure 4.2 *Neutral sugar composition and uronic acid content of the WIR and WSP from green bean cultivars Montano and Masai.*

***Table 4.3** The degree of methylation[*)] (mol % of uronic acid content) of cell wall fractions from green bean cultivars Montano and Masai during processing.*

treatment	AIR	WIR	WSP
Cv. Montano			
Fresh	50.6 ± 1.0[a]	34.0 ± 0.9[a]	55.8 ± 12.8[a,b]
Blanched	49.9 ± 1.0[a]	45.6 ± 3.5[b]	69.5 ± 9.9[a,b]
Sterilised	36.8 ± 2.0[b]	31.1 ± 7.6[a]	46.1 ± 6.7[a]
Cv. Masai			
Fresh	51.7 ± 3.8[a]	33.4 ± 1.5[a]	57.9 ± 13.4[a,b]
Blanched	49.1 ± 4.0[a]	44.4 ± 4.7[b]	49.1 ± 3.9[a]
Sterilised	37.7 ± 0.7[b]	27.3 ± 0.5[a]	40.9 ± 3.8[a]

[*)] The mean values in a column are significantly ($P<0.05$) different for treatment and/or cultivar if followed by a different letter (n = 4, ± SD).

***Table 4.4** Amount of acetyl groups (µmol/g DW) in cell wall fractions from green bean cultivars Montano and Masai during processing.*

treatment	AIR	WIR	WSP
Cv. Montano			
Fresh	124 ± 5[a]	105 ± 9[a,b]	6 ± 3[a]
Blanched	125 ± 1[a]	108 ± 5[a,b,d]	9 ± 3[a]
Sterilised	121 ± 3[a]	83 ± 1[c]	33 ± 6[b]
Cv. Masai			
Fresh	139 ± 1[b]	125 ± 12[d]	9 ± 2[a]
Blanched	144 ± 7[b]	119 ± 10[b,d]	10 ± 1[a]
Sterilised	145 ± 5[b]	91 ± 7[a,c]	50 ± 12[c]

[*)] The mean values in a column are significantly ($P<0.05$) different for treatment and/or cultivar if followed by a different letter (n = 4, ± SD).

can be calculated from the molar sugar composition and the yields of the different fractions (Table 4.2 and Figures 4.1 and 4.2). This reduction was not accompanied by a loss of other pectic sugars like arabinose and galactose. In the WIR there was an additional decrease in pectic sugars, mostly uronic acid, but also substantial amounts of galactose and arabinose. Since the latter pectic sugars were recovered in the WSP fraction, sterilisation apparently solubilised these compounds. The overall cell wall composition of both cultivars was identical, but galactose seemed to be more abundant in the CWM of cv. Masai.

4.4.4 Degree of methylation and acetyl substituents

Similar to the carbohydrate composition, sterilisation affected the degree of methylation (DM) of the cell wall fractions. The DM of the AIR of sterilised beans was on average 14 % lower as compared with the same samples of blanched and fresh beans (Table 4.3). In contrast, the DM of WIR of fresh beans was almost as low as measured for WIR of sterilised samples. The DM of the WSP fractions was higher as compared with the WIR samples. The total amount of acetyl substituents remained constant, 30 % of the acetyl groups was located in the WSP-fractions after processing (Table 4.4).

4.4.5 Methylation analysis

The method of Ciucanu and Kerek[30] for methylation of mono- and disaccharides has been widely adopted to analyse complex carbohydrates. Needs and Selvendran[33] recently published that the undesirable oxidative degradation as noted by York et al.[34] could easily be overcome by addition of sodium hydroxide prior to the addition of methyliodide. The results obtained by these authors indicated almost complete methylation. The methylation analyses performed in this study were not quantitative (Table 4.5). This is based on the low recoveries observed for galactose in comparison with the Seaman hydrolysis without methylation. The bulk of the glucosyl residues were (1-4)-linked, with significant amounts of (1-4,6)- and terminally linked residues. Minor amounts of (1-3,4)- and (1-2,4)-linked residues were also detected. Galactosyl residues were mainly (1-4)-linked, although terminally, and (1-2)-linked residues were also detected. Xylosyl residues were mostly (1-4)- linked and also minor amounts of (1-2)-, (1-2,4)-, (1-3,4)- and terminally linked xylosyl residues were present. Arabinosyl residues were mainly (1-5)-, (1-3,5)- and terminally linked. Rhamnosyl residues were present in small amounts and appeared (1-2)-and (1-2,4)-linked. The detected fucosyl residues were only terminally linked.

4.5 Discussion

4.5.1 Cell wall constituents

Analysis of the AIR and WIR showed that the cell walls of fresh green beans contain cellulose and pectic polysaccharides. Hemicellulosic polymers are also present however, to a lesser extend as can be concluded from the xylose and mannose content (Figures 4.1 and 4.2). This type of cell wall composition is characteristic for parenchyma, which is the major tissue type in pods of green beans[35].

Upon comparing the cell wall composition of both cultivars it was observed that cell walls from cv. Montano contained less galactose than cell walls from cv. Masai. Previously, it has been shown for kiwi and nectarines that levels of galactose decreased during fruit ripening[7,8]. The discrepancy between the two cultivars might therefore be due to harvesting at different developmental stages. On the other hand, it cannot be excluded that the galactose levels are cultivar specific and play a role in determining textural firmness after processing.

Table 4.5 *Abundance of sugar linkages in mol % in WIR samples from fresh, blanched and sterilised green bean cultivars Montano and Masai.*

linkage	Cv. Montano			Cv. Masai		
site	fresh	blanched	sterilised	fresh	blanched	sterilised
fucose						
terminal	0.4	0.4	0.3	0.5	0.6	0.4
rhamnose						
1,2	0.1	0.1	0.1	0.1	0.4	0.1
1,2,4	0.2	0.3	0.1	0.3	0.3	0.2
arabinose						
terminal	1.4	1.3	1.0	1.4	1.7	1.2
1,5	4.7	4.5	2.7	2.8	3.2	3.5
1,3,5	0.8	0.8	1.0	0.9	1.0	1.5
arabinitol	0.9	0.6	0.4	1.4	1.8	2.2
galactose						
terminal	1.2	1.1	0.6	1.5	2.0	1.5
1,2	0.1	0.1	0.1	0.3	0.2	0.3
1,4	3.3	3.3	2.8	4.1	3.9	3.6
1,3,6	0.1	0.0	0.1	0.2	0.2	0.1
1,4,6	0.3	0.3	0.3	0.5	0.8	0.8
1,3,4	0.8	0.7	0.7	0.7	0.7	1.9
1,2,4	0.6	0.6	0.7	0.7	0.5	0.8
galactitol	0.1	0.1	0.1	0.3	0.3	0.7
glucose						
terminal	1.7	0.5	1.6	1.7	1.9	2.1
1,4	67.2	69.4	76.9	63.0	55.8	52.1
1,4,6	3.6	3.9	4.1	4.7	5.3	5.7
1,3,6	0.1	0.1	0.0	0.0	0.5	0.2
glucitol	4.1	1.6	1.0	1.6	2.7	4.3
xylose						
terminal	1.1	1.0	0.9	1.6	1.9	1.7
1,2	0.6	0.9	0.7	1.0	1.1	1.2
1,4	1.6	2.3	1.5	2.6	3.0	3.3
1,2,4	0.8	0.6	0.5	1.3	1.5	1.4
1,2,3	*)	*)	*)	*)	*)	*)
1,3,4	*)	*)	*)	*)	*)	*)
xylitol	0.8	0.9	0.2	1.4	1.7	2.8
mannose						
terminal	0.6	1.1	0.6	0.0	0.1	0.1
1,4	2.3	2.1	1.1	3.4	3.8	3.8
mannitol	0.5	0.4	0.2	0.4	0.6	1.2

*) Not quantified, only detected with GCMS.

For the WIR of fresh samples it was observed that the degree of methylation was very low in comparison to the AIR of fresh samples (Table 4.3). This low value might be explained by pectin

methylesterase (PME) activity during the first homogenisation step. At low temperatures PME might still be active as was previously shown by isolation procedures of tomato fruit cell walls[36]. However, for the WIR of blanched samples the DM was not significantly different as compared with the AIR of blanched samples (Table 4.3). This latter result indicates that no residual enzyme activity was present after blanching. The soluble fraction (WSP), obtained during WIR preparation, contained mostly pectin with a relatively high degree of methylation as compared with the WIR of the same samples. It is not possible to calculate a degree of acetylation for the AIR and WIR samples, because acetyl groups can be linked to pectin as well as to certain hemicelluloses. The WSP fractions however consist mainly of pectin (Figure 4.2), and the degree of acetylation of these soluble pectins can be calculated to be approximately 20 - 30 %.

To obtain insight into the types of polymers contributing to the textural aspects of the tissue a methylation analysis was performed. The bulk of the (1-4)-linked glucose residues arise from cellulose and starch. Also the bulk of the (1,4,6)-linked glucose residues are likely to be derived from starch. Removal of starch with 90 % DMSO was omitted since preliminary experiments had indicated that appreciable amounts of pectins were co-extracted with 90 % DMSO after sterilisation. The bulk of the terminal and (1-5)-linked arabinitol in all fractions most probably originated from side chains of rhamnogalacturonan. The formation of 2,3,5-tri-O-methylarabinose derivatives shows that arabinose is mainly present in the furanoid form. All other cell wall sugars are assumed to be present in the pyranoid form. Most xylose residues are (1,4)-linked. Xylans are the traditional well known origin of (1-4)-linked xylosyl residues and are the main hemicelluloses found in secondary walls of dicotyledonous plants[37]. Secondary walls however, are only abundant in the hypodermal fibre layer of the bean pod[35], consequently xylans most likely are elements of the primary walls of *P. vulgaris*. Ryden and Selvendran[38] however, proposed that, in addition to the common (1,2) linked xylitol residues, some (1,4) linked xylitol residues are an integral part of the xyloglucan of *P. coccineus*, which resembled the overall cell wall composition of *P. vulgaris* very well[14]. Evidence for the occurrence of (1-4)-linked xylosyl residues in isolated xyloglucan was also obtained by Karacsonyi and Kovacik[39] in cell walls from suspension cultured popular cells. In general, methylation of polysaccharides containing high levels of uronic acids is not efficient and may be complicated due to degradation. A low recovery of especially galactose was observed when comparing the results of the methylation analysis with the results of Seaman hydrolysis. This low recovery of galactose might be explained by ß-eliminative breakdown of pectins carrying short side chains of neutral sugars. These segments might be lost during dialysis of the methylated samples[5,38]. However, the analysis rendered sufficient data to outline the general features of the neutral cell wall polymers.

4.5.2 Cell wall changes during processing

In general, cell wall changes occurring during processing can be a result both of enzymatic and chemical reactions. In this study only a high blanching temperature, causing rapid enzyme inactivation, was applied and cell wall changes were expected to be of chemical nature only. Our analyses showed that major alterations occurred during the sterilisation of green beans (Figures 4.1 and 4.2). It was observed that during this treatment approximately 20 % of galacturonite fragments were degraded into

small fragments, which were not retrieved in our cell wall isolations. Since also the overall degree of methylation was lower after sterilisation, de degraded fraction was most likely highly methylated, poorly branched and acetylated, suggesting that this pectin originated from the middle lamellae[38]. Sterilisation presumably results in degradation of the middle lamella pectin thereby reducing the interaction between cells. Recently, a similar observation was made using cryo-SEM of potato cells during steam cooking[40].

Analysis of the WIR and soluble fractions revealed that pectins, as indicated by uronic acid content, were solubilised in the cell wall during the sterilisation process. These pectins did not leach out into the brine, but remained entangled in the cell walls inside the bean pods and were therefore recovered in the WSP-fraction. However, 21 - 28 % of the uronic acids and 70 - 80 % of the galactose and arabinose residues remained insoluble. Arabinose and galactose are characteristic for branched rhamnogalacturonans, but also occur in association with xyloglucan[9,15,16]. Since the methylation analysis (Table 4.5) showed that the arabinosyl and galactosyl residues were typically (1,5)- and (1,4)-linked respectively, these sugars were most likely derived from highly branched pectins with type I arabinogalactan side chains. It can however not be excluded that, after sterilisation, separate arabinan, galactan or arabinogalactan polymers remain embedded within the cell wall matrix. Separate arabinogalactans were isolated from pea cell walls, and were thought to constitute a second matrix layer around the cellulose microfibrils[41].

In conclusion, two major effects of sterilisation on the pectin of cell wall and middle lamella can be discriminated. Firstly, linear homogalacturonan, presumably originating from the middle lamella, is degraded. Secondly, rhamnogalacturonan is partially solubilised probably by breakdown of some covalent linkages. These solubilised polymers however remained entangled within the cell wall matrix, probably due to the branched characteristics. It can be envisaged, that due to both the breakdown as well as the solubilization of the pectins within the cell wall the contribution of these polymers to the firmness of the cell wall is reduced. This results in a less rigid cell wall.

4.6 References

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Chapter **5**

Changes in pectic and hemicellulosic polymers of green beans during industrial processing*

5.1 Abstract

Pectic and hemicellulosic material was extracted from the alcohol-insoluble residues of two fresh and industrially processed green bean varieties. The carbohydrate composition and the relative molecular mass of these fractions were determined. Fresh beans of cv. Odessa contained more pectin and hemicellulose, but less cellulose than cv. Masai. In addition, the pectins from cv. Masai appeared to be more linear in comparison with pectins extracted from cv. Odessa. The major changes occurred during sterilisation and concerned the pectic polymers. In fresh beans the major part of the pectins was most likely covalently linked to other cell wall polymers. Since sterilisation resulted into degradation and solubilisation of pectic polymers from the cell wall and middle lamella. The galacturonic acid backbone was partially degraded. Side chains however, seemed not to be removed from the pectic polymers, resulting in an accumulation of branched pectins of low molecular mass in the buffer soluble fraction. The amount of CDTA-soluble material showed a small increase after blanching but the amount after sterilisation was the same as compared with the fresh material. These results confirm the hypothesis that pectins are solubilised by ß-eliminative degradation of the galacturonic acid backbone during industrial processing. The changes in the pectic polymers during processing are summarised in a scheme. No significant changes were observed in the hemicelluloses and cellulose fractions.

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5.2 Introduction

Texture is a major quality attribute that contributes to the consumer acceptance of fruits and vegetables. Processing of fruit and vegetables is generally accompanied by softening of the plant tissue. Blanching and sterilisation affect plant tissues by destroying turgor, swelling of the cell walls and maceration of the tissue[1,2]. As a result, the texture of processed vegetables can be attributed mainly to the structural integrity of the cell wall and middle lamella[3]. Recent cell wall models envision a cellulose-hemicellulose structural domain embedded in a second domain consisting of pectic substances, while a third domain contains covalently crosslinked proteins[4]. Pectic polymers are key substances determining the mechanical strength of the primary cell wall and adhesion between cells. Therefore, pectins have been the main subject of studies aiming at elucidating the cell wall changes occurring during processing. Unfortunately, most of this work has been limited to quantitative determinations of overall degraded pectins, without more qualitative studies concerning the origin and type of pectins being released[5,6]. Several studies revealed that the major cleavage reaction leading to vegetable softening was a ß-eliminative depolymerisation of pectin. Increased methylester content resulted in a higher rate of pectin degradation[7]. Some cations and anions were also shown to enhance ß-elimination[8-12]. However, possible modifications of minor but important cell wall constituents still remain largely unknown.

In chapter 4 we reported on the overall cell wall changes occurring during processing of green beans[13]. Two major effects of sterilisation on the pectin of cell wall and middle lamella were discriminated. First, linear homogalacturonan, presumably originating from the middle lamella was degraded. Second, branched rhamnogalacturonan was partially solubilised. However, these solubilised polymers remained entangled within the cell wall matrix, probably due to their branched characteristics. We now report in more detail on the changes observed in pectic and hemicellulosic polymers extracted from cell walls during different stages of industrial processing.

5.3 Materials and methods

5.3.1 Plant material and processing conditions.

Green beans (*Phaseolus vulgaris* L.) cvs. Masai and Odessa were grown in a greenhouse and harvested at edible maturity. The pods were cut into parts of 3 to 4 cm in length and blanched at 90 °C for 4 min. For the canning process portions of 410 g were packed into glass jars (720 mL) and a 0.25 M NaCl solution (brine) was added. Closed glass jars were sterilised for 30 min at 118 °C, cooled and stored for 2 weeks at 15 °C. The pH of the brine after sterilisation was 5.5. Triplicate samples were taken from fresh, blanched and sterilised beans. The green beans were split lengthways and the seeds were removed. The pods were cryomilled in liquid nitrogen by using a Moulinex (Masterchief 20) food processor. All samples were stored at -20 °C until further analysis.

5.3.2 Dry matter determination

The dry matter content of the samples was determined by drying a known weight of homogenised samples overnight at 70 °C, followed by 3 h at 105 °C. After cooling to room temperature, the samples were weighed again. The dry matter and water content were calculated from the weight difference.

5.3.3 Firmness measurements.

The firmness of the sterilised bean pods was measured in triplicate using an Instron Universal Testing Machine equipped with a Kramer shear cell. 40 g of material was placed in the cell with the length axis of the pods perpendicular to the openings of the shear cell. The maximal force (top value) applied with the shear press to break through the beans was used to quantify the firmness of the beans.

5.3.4 Isolation and fractionation of Alcohol Insoluble Residues and pectic polymers.

50 g of frozen material was immersed in 180 mL cold (-30 °C) ethanol (96 % v/v), homogenised with an Ultra-Turrax by four bursts of 1 min and collected on a Whatman GF/C glass fibre filter. The material was suspended in 50 mL ice-cold aqueous ethanol (80 % v/v) and stirred for 1 h at 2 °C. The material was filtered again, washed twice with 50 mL 100 % acetone until the filtrate was colourless and dried overnight to yield the Alcohol Insoluble Residue (AIR). Pectic polymers were extracted using a modified method from Selvendran et al.[14] which is summarised in Figure 5.1. To remove starch the AIR (2 g) was suspended in 100 mL 90 % DMSO and stirred for 16 h at 20 °C. The suspension was centrifuged (10,000 g for 15 min) and the pellet was washed twice with 90 % DMSO and three times with 80 % ethanol. To the pellet, 100 mL of 0.05 M ammonium acetate buffer (pH = 4.7) was added and the suspension was stirred for 16 h at 4 °C. The suspension was centrifuged (10,000 g for 15 min) and the pellet was washed once with acetate buffer and once with distilled water. To the pellet, 100 mL of 0.05 M CDTA (pH = 6.5) was added and the suspension was stirred for 16 h at 4 °C. The suspension was centrifuged (10,000 g for 15 min) and the pellet was washed once with the CDTA-solution and once with water. To the pellet, 100 mL of 0.05 M Na_2CO_3 containing 0.01 M $NaBH_4$ was added and the suspension was stirred for 16 h at 4 °C. The suspension was centrifuged (10,000 g for 15 min). To the pellet, 100 mL of 0.05 M Na_2CO_3 containing 0.01 M $NaBH_4$ was added and the suspension was stirred for 16 h at 20 °C. The suspension was centrifuged (10,000 g for 15 min). The depectinated residue was sequentially extracted with 0.5, 1.0 and 4.0 M KOH containing 0.01 M $NaBH_4$ by constant stirring for 16 h at 20 °C to leave a residue essentially consisting of cellulose. All extracts were filtered, neutralised, dialysed exhaustively against demineralised water and finally lyophilised.

5.3.5 Starch and protein content.

The starch and protein content of the samples was analysed as described in chapter 4.

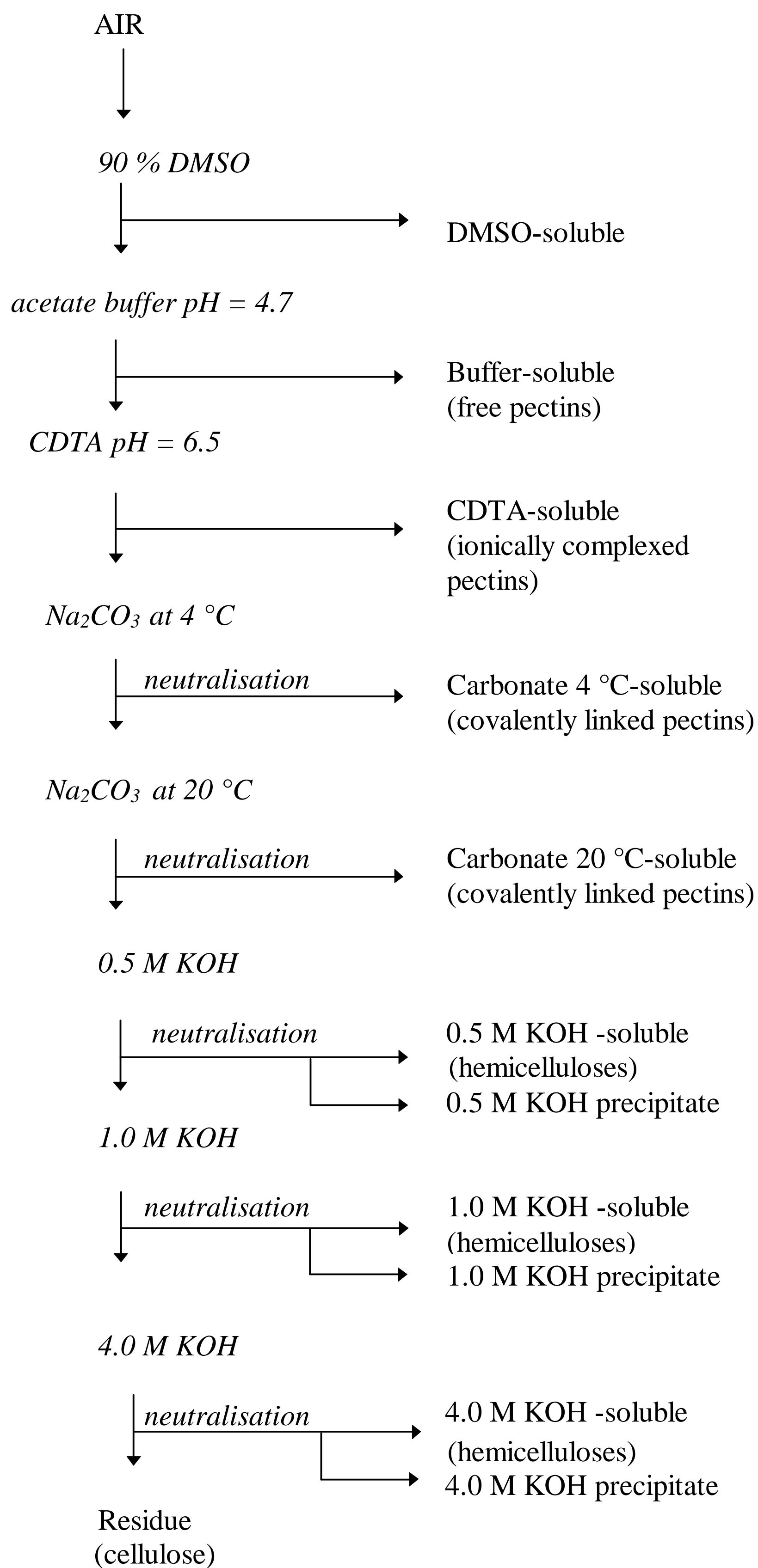

Figure 5.1 *Extraction scheme for pectic and hemicellulosic cell wall fractions.*

5.3.6 Monosaccharide composition

Sugars from the AIR and Residue were hydrolysed by Seaman hydrolysis as described in chapter 4. The pectic and hemicellulosic fractions were hydrolysed by stirring in 2 M TFA for 2 h at 121 °C. Samples were dried under nitrogen at 45 °C, washed with 0.5 mL 1M NH_4OH, dried under nitrogen and dissolved in milli Q water. Samples (10 μL) of the neutralised hydrolysates were analysed for neutral sugars by using a HPLC system as described in chapter 4. Anhydro-uronic acids were determined as described by Ahmed and Labavitch[15].

5.3.7 Methyl and acetyl substituents.

The amount of methyl and acetyl groups was determined by using a HPLC system under the conditions as described previously by Voragen et al.[16]

5.3.8 Molecular mass determinations

Gel Permeation Chromatography (GPC) was performed with a Sepharose CL-4B column (450 x 16mm) (Pharmacia LKB). Desalted cell wall fractions (10 mg in 1.0 mL eluens) were loaded on the column with a sample injector and were eluted with a sodium acetate buffer (0.4 M, pH = 4,5) at 0.5 mL/min. Fractions of 2 mL were collected and assayed for uronic acids as described above. The percentage of uronic acids in each fraction as a percentage of total yield of uronic acids in all fractions was calculated. Dextran standards ranging in average molecular mass from 2,000,000 to 9,300 (Pharmacia) were applied to the column to compare the size of the fractionated polymers with data from the literature. Since the cell wall polymers most likely will have a different conformation than the Dextran standards, thereby affecting the elution behaviour, all M_r values given in this paper should be regarded as “apparent” values.

5.4 Results

5.4.1 Textural measurements

Tissue firmness, as measured with an Instron, of both green bean cultivars decreased approximately 25-fold during processing (Table 5.1). The values for the fresh and blanched beans were comparable for both cultivars, but beans of cv. Masai remained firmer after sterilisation than the beans of cv Odessa.

5.4.2 Isolation and composition of AIR

The yields of AIR were higher in the processed samples as compared with the fresh samples. Besides CWM (28 - 32 %), as estimated from the total amount of cell wall sugars, the AIR also contained starch (28- 31 %) and protein (30 - 39 %) (Table 5.2).

Comparison of the crude cell wall composition of fresh beans revealed that cv. Masai beans contained relatively less arabinose and galactose and more mannose than cv. Odessa beans (Figure

Table 5.1. *Firmness, yield of dry weight (DW) and alcohol-insoluble residue (AIR) of green bean cultivars Masai and Odessa. (n = 4)*

Treatment	Firmness (N)	DW (mg/g of fresh)	AIR (mg/g of DW)
Cv. Masai			
fresh	3754 ± 274	110 ± 3	714 ± 9
blanched	3031 ± 54	94 ± 1	761 ± 11
sterilised	161 ± 3	90 ± 3	775 ± 11
Cv. Odessa			
fresh	3527 ± 106	105 ± 3	617 ± 17
blanched	2793 ± 371	84 ± 4	682 ± 17
sterilised	123 ± 13	89 ± 3	674 ± 38

Table 5.2 *Crude composition of the AIR of green bean cultivars Masai and Odessa (n = 2).*

Treatment	CWM*	Starch (mg/g AIR)	Protein
Cv. Masai			
fresh	310	276	314
blanched	317	283	320
sterilised	291	278	390
Cv. Odessa			
fresh	310	284	300
blanched	324	284	355
sterilised	282	310	308

* CWM is estimated from the total of cell wall sugars in the AIR

5.2). Both the degree of methylation (DM) and the amount of acetyl constituents of both cultivars were comparable (Table 5.3). During sterilisation, the total uronic acid content of the AIR decreased with 12 % and 24 % for cv. Masai and cv. Odessa respectively, as can be calculated from the molar sugar composition and the yields of cell wall material (Table 5.1 and Figure 5.2). This reduction was accompanied by a slight loss of galactose. This resulted in a relative increase of other sugars in the cell walls of both cultivars. Sterilisation also had a significant effect on the DM of the cell walls from

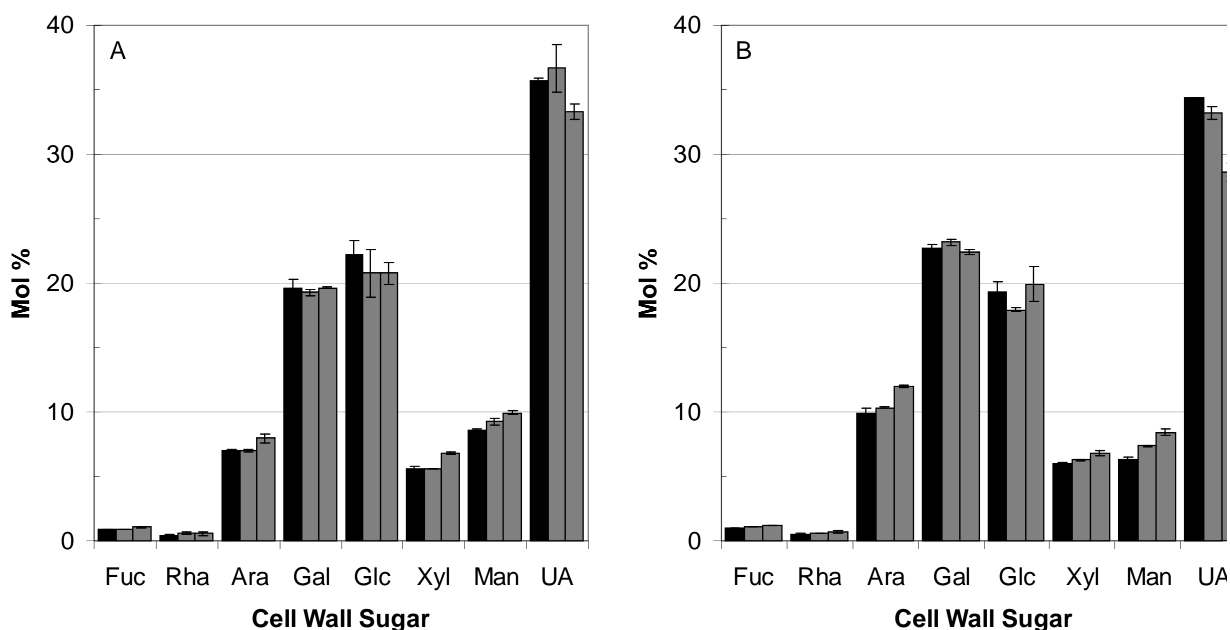

Figure 5.2 *Neutral sugar composition and uronic acid content of AIR from green bean cvs. Masai (A) and Odessa (B) during different stages of industrial processing Solid bars: fresh; striped bars: blanched; dotted bars: sterilised.*

both cultivars (Table 5.3). The DM of the AIR of sterilised beans was on average 17 percentage points lower as compared with the same samples of fresh and blanched beans. The total amount of acetyl substituents remained constant for cv. Masai beans but decreased in cv. Odessa beans (Table 5.3).

5.4.3 Extractability and composition of the cell wall fractions

The pectic polysaccharides not connected to other cell wall polymers were extracted with the acetate buffer; the Ca^{2+} complexed polymers were most likely solubilised by CDTA. Most of the CDTA-insoluble pectins were subsequently extracted by dilute Na_2CO_3. at 4 °C and 20 °C, presumably by hydrolysis of weak ester crosslinks. This residue was subsequently treated with several KOH solutions to extract hemicelluloses and some residual pectins. The cell wall material of the beans generally consisted for about 47 - 50 % of pectins (buffer up to Na_2CO_3 extractions), 21 - 24 % of hemicelluloses (total of KOH extractions) and 16 - 21 % of cellulose (glucose in cellulose residue).

The yield of the various cell wall fractions of processed green beans and changes in cell wall sugar content of the various cell wall fractions are presented in Table 5.4. The DMSO extract also contained a large quantity of starch (data not shown) and some xylose (11 - 22 mol %) and mannose residues (11 - 29 mol %). As expected, the buffer, CDTA and Na_2CO_3 extracts were rich in pectic polysaccharides as can be deduced from their high levels of uronic acid residues. The most abundant neutral sugar in all pectic fractions was galactose, followed by arabinose. The DMSO and

***Table 5.3**. Degree of methylation (DM) and number of acetyl groups in air from green bean cultivars Masai and Odessa during processing.*

Treatment	DM (% of AUA)	Acetyl (µmol/g AIR)
Cv. Masai		
fresh	53.6 ± 5.0	235 ± 10
blanched	45.6 ± 2.5	247 ± 5
sterilised	35.8 ± 2.0	236 ± 6
Cv. Odessa		
fresh	50.3 + 1.0	240 ± 7
blanched	48.5 ± 1.9	256 ± 14
sterilised	34.6 ± 1.8	207 ± 22

carbonate (20 °C) soluble fraction from all samples contained much larger amounts of neutral sugars as compared with the other pectic fractions. The ratios neutral/acid pectic sugars in the buffer, CDTA, Na_2CO_3 (4°C and 20°C) from fresh beans were 0.12, 0.06, 0.13, and 0.60 for cv. Masai and 0.20, 0.15, 0.22 and 1.45 respectively for cv. Odessa.. The pectic extracts derived from cv. Masai contained relatively less neutral sugars and more uronic acids than extracts isolated from cv. Odessa.

Hemicelluloses and additional pectins were subsequently extracted from the depectinated AIR with several KOH solutions of increasing molarity. After neutralisation of the KOH extracts, a precipitate was formed, which was analysed separately (Table 5.5). Polysaccharides in the 0.5 and 1.0 M KOH-precipitates were most likely merely composed of pectins, because they contained large amounts of uronic acids, galactose and arabinose. All soluble KOH extracts were also rich in pectins as can be deduced from the relative high amounts of AUA, Gal and Ara. In addition, the 1.0 and 4.0 M KOH extracts were rich in xylose and glucose containing hemicelluloses. Because the xylose levels were higher than the glucose amounts, the xylose cannot originate from xyloglucans only. These fractions therefore presumably contain many xylans.

The cell wall changes occurring processing were comparable for both cultivars. Most importantly, a change in extractability of the pectic polymers during processing was observed. After blanching more pectins were soluble in CDTA, although the composition of this fraction was not altered. In addition, due to the sterilisation procedure considerably more pectins were extracted with DMSO and buffer. This increase in higher levels of easily extractable material during processing was accompanied by a strong reduction of Na_2CO_3 extractable components. Of course it is also possible that pectins shift from Na_2CO_3 to the CDTA -fractions and pectins from CDTA to the buffer- and DMSO fractions. In addition the amounts recovered in the KOH-precipitates were different after processing. Before processing much precipitate was formed in the 0.5 M KOH extract, while after sterilisation most precipitate was obtained in the 1.0 and 4.0 M KOH fraction. Interestingly sugar analyses

Table 5.4 *Sugar composition and yield (mg/g air) of pectic fractions from green bean cvs. Masai and Odessa during industrial processing.*

Fraction	Fuc		Rha		Ara		Gal		Glc#		Xyl		Man		AUA		Cell Wall Yield mg/g AIR	
	Mol % (of total cell wall sugars)																	
	Ma	*Od*	Ma	*Od*	Ma	*Od*	Ma	*Od*	Ma	*Od*	Ma	*Od*	Ma	*Od*	Ma	*Od*	Ma	*Od*
DMSO																		
fresh	8.3	*6.7*	0.0	*0.0*	8.3	*13.3*	15.0	*16.7*	0.0	*0.0*	21.7	*20.0*	28.3	*20.0*	18.4	*23.2*	47	*34*
blanched	7.2	*5.4*	0.0	*0.0*	8.6	*12.9*	15.8	*19.3*	0.0	*0.0*	18.6	*19.3*	22.9	*22.6*	26.9	*20.5*	43	*52*
sterilised	3.6	*3.0*	0.7	*0.0*	9.4	*10.9*	24.0	*30.2*	0.0	*0.0*	10.9	*10.9*	10.9	*11.5*	40.4	*33.5*	93	*87*
buffer																		
fresh	0.7	*0.3*	0.2	*0.6*	3.4	*5.1*	6.7	*10.6*	0.0	*0.0*	0.0	*0.0*	0.7	*0.6*	88.3	*82.8*	38	*38*
blanched	0.9	*0.3*	0.3	*1.0*	3.7	*6.5*	8.6	*14.7*	0.0	*0.0*	0.0	*0.0*	0.0	*1.4*	86.5	*76.1*	40	*28*
sterilised	0.2	*0.7*	1.6	*1.9*	6.1	*8.7*	24.3	*29.0*	0.0	*0.0*	0.0	*0.0*	0.0	*1.4*	67.8	*58.2*	72	*57*
CDTA																		
fresh	0.0	*0.0*	0.4	*0.5*	2.0	*4.4*	3.5	*8.5*	0.0	*0.0*	0.0	*0.0*	0.0	*0.0*	94.2	*86.7*	39	*30*
blanched	0.0	*0.2*	0.5	*0.5*	1.7	*2.9*	2.9	*5.6*	0.0	*0.0*	0.0	*0.3*	0.0	*0.0*	94.9	*90.4*	57	*43*
sterilised	0.0	*0.3*	1.3	*1.6*	5.8	*8.8*	16.5	*19.5*	0.0	*0.0*	0.0	*0.0*	0.0	*0.0*	76.4	*69.7*	40	*38*
Carbonate 4 °C																		
fresh	0.3	*0.4*	0.6	*0.8*	2.9	*5.2*	7.6	*12.0*	0.0	*0.0*	0.0	*0.0*	0.0	*0.2*	88.6	*81.3*	73	*96*
blanched	0.7	*0.6*	1.0	*1.4*	3.0	*6.0*	7.7	*14.3*	0.0	*0.0*	0.0	*0.0*	0.0	*0.0*	87.6	*77.7*	79	*89*
sterilised	0.9	*0.0*	0.0	*1.0*	10.3	*13.4*	41.0	*37.2*	0.0	*0.0*	0.0	*0.0*	0.0	*0.0*	47.8	*48.3*	40	*45*
Carbonate 20 °C																		
fresh	0.4	*0.3*	1.4	*2.2*	7.8	*12.9*	28.1	*43.5*	0.0	*0.0*	0.0	*0.6*	0.0	*0.2*	62.3	*40.4*	40	*58*
blanched	0.4	*0.3*	2.0	*2.4*	9.3	*13.5*	37.8	*46.1*	0.0	*0.0*	0.4	*0.0*	0.0	*0.3*	50.1	*37.5*	26	*64*
sterilised	2.0	*0.0*	2.0	*2.4*	15.3	*21.8*	48.1	*49.7*	0.0	*0.0*	0.0	*0.0*	0.0	*0.0*	32.5	*26.0*	17	*16*

Values are the mean of duplicate analyses; * Anhydro-values after TFA-hydrolysis;

Glucose values are corrected for non-cell wall glucose derived from starch.

Table 5.5 *Sugar composition (mol %)* * *and yield (mg/g AIR) of hemicellulosic fractions and cellulose residue from green bean cvs. Masai and Odessa during industrial processing.*

Fraction	Fuc		Rha		Ara		Gal		Glc		Xyl		Man		AUA		Yield mg/g AIR	
	Mol % (of total cell wall sugars)																	
	Ma	*Od*	Ma	*Od*	Ma	*Od*	Ma	*Od*	Ma	*Od*	Ma	*Od*	Ma	*Od*	Ma	*Od*	Ma	*Od*
0.5 M KOH																		
soluble																		
fresh	0.7	*0.6*	1.2	*1.4*	16.9	*16.3*	52.8	*49.6*	0.0	*6.7*	11.1	*9.3*	0.0	*1.5*	17.3	*14.6*	18	*29*
blanched	0.0	*0.5*	0.8	*1.6*	12.8	*14.4*	40.8	*42.1*	13.3	*17.4*	12.9	*8.4*	1.3	*0.0*	18.1	*15.6*	19	*30*
sterilised	0.7	*0.5*	1.7	*2.0*	17.2	*19.5*	53.4	*44.7*	5.2	*13.5*	5.2	*4.3*	0.0	*0.0*	16.6	*15.5*	14	*21*
precipitate																		
fresh	0.0	*0.0*	0.0	*1.4*	12.9	*15.5*	48.4	*48.9*	3.8	*2.8*	0.0	*0.9*	0.0	0.0	34.8	*30.5*	24	*31*
blanched	0.0	*0.0*	0.0	*0.0*	14.0	*15.3*	51.8	45.2	2.8	*5.1*	0.0	*0.7*	0.0	0.7	31.5	*33.0*	18	*22*
sterilised	0.0	*0.0*	0.0	*0.0*	0.0	*26.9*	13.9	*9.6*	44.1	*30.7*	0.0	*0.0*	0.0	0.0	41.9	*32.8*	1	*1*
1.0 M KOH																		
soluble																		
fresh	1.6	*1.7*	0.0	*0.0*	7.0	*10.5*	23.7	*39.1*	15.2	*17.7*	39.7	*15.6*	4.5	4.3	8.3	*11.0*	13	*9*
blanched	1.3	*1.7*	0.0	*0.0*	7.3	*10.5*	23.0	39.1	14.2	*25.0*	40.2	*7.8*	6.2	4.7	7.9	*11.1*	10	*23*
sterilised	0.9	*1.0*	0.0	*0.9*	13.2	*17.9*	39.5	*43.7*	10.5	*8.7*	20.7	*13.5*	2.3	0.0	12.3	*14.3*	21	*30*
precipitate																		
fresh	0.0	*0.0*	0.0	*0.0*	18.1	*17.7*	42.0	*40.3*	11.0	*14.7*	2.8	*4.9*	0.0	*0.0*	26.0	*22.3*	11	*14*
blanched	2.6	*0.0*	0.0	*0.0*	16.2	*15.8*	38.7	*33.1*	12.6	*24.4*	2.7	*4.3*	0.0	*0.0*	27.2	*22.4*	14	*22*
sterilised	2.2	*0.0*	0.0	*0.0*	12.8	*15.2*	28.4	*26.7*	11.4	*10.2*	3.8	*2.0*	0.0	*0.0*	41.4	*45.9*	33	*25*

4.0 M KOH																		
soluble																		
fresh	2.8	*3.2*	0.9	*0.7*	9.5	*8.6*	35.1	*29.9*	22.8	*24.1*	18.8	*21.4*	5.2	*5.3*	5.0	*6.8*	36	*37*
blanched	3.0	*2.9*	0.6	*0.7*	7.6	*9.6*	29.2	*29.0*	25.6	*21.5*	20.4	*18.7*	6.5	*6.6*	7.0	*10.9*	30	*41*
sterilised	2.9	*3.5*	0.8	*0.6*	8.7	*8.1*	31.6	*28.3*	24.1	*26.9*	20.2	*21.2*	5.3	*6.5*	6.5	*4.9*	37	*36*
precipitate																		
fresh	0.0	*4.9*	0.0	*0.0*	7.2	*12.1*	18.1	*29.1*	18.1	*17.0*	43.4	*24.3*	0.0	*0.0*	13.3	*12.6*	1	*2*
blanched	1.1	*0.0*	2.3	*3.1*	10.3	*18.5*	24.0	*46.2*	8.0	*10.8*	45.7	*12.9*	0.0	*0.0*	8.6	*8.6*	3	*6*
sterilised	1.4	*1.7*	4.2	*3.4*	15.5	*14.4*	43.7	*39.7*	29.1	*15.2*	46.2	*23.7*	0.0	*0.0*	6.9	*2.0*	15	*21*
Residue																		
fresh	0.0	*0.0*	0.0	*0.0*	6.4	*7.1*	11.0	*13.5*	65.5	*63.0*	0.0	*0.0*	16.5	*15.5*	0.7	*0.9*	162	*130*
blanched	0.0	*0.0*	0.0	*0.0*	5.8	*6.9*	11.0	*13.6*	65.1	*63.0*	0.0	*0.0*	17.3	*15.5*	0.8	*0.9*	205	*131*
sterilised	0.0	*0.0*	0.0	*0.0*	5.3	*3.9*	8.8	*7.3*	68.7	*70.6*	0.0	*3.7*	16.7	*14.3*	0.5	*0.1*	204	*155*

* Anhydro-values after TFA-hydrolysis. Values are the mean of duplicate analyses

of the pectic fractions showed that in addition to the extractability of the pectins, also the composition of the different fractions was modified during the sterilisation process. There was a general loss of galacturonic acids resulting in a relative increase in neutral pectic sugars in all pectic extracts. Galacturonic acid and galacturonic acid oligomers were most likely lost during the cell wall isolation procedure, since they are readily soluble in 80 % ethanol. The ratio neutral/acid pectic sugars in the buffer, CDTA, Na_2CO_3 (4°C and 20°C) after sterilisation was 0.47, 0.31, 1.07, and 2.01 for cv. Masai and 0.68, 0.41, 1.07 and 2.84 respectively for cv. Odessa. Especially the pectins extracted with Na_2CO_3 showed strongly increased levels of galactose and arabinose upon sterilisation. The levels of galactose increased even more than the levels of arabinose. Also in the 1.0 M soluble KOH-fraction and all of the KOH-precipitate fractions the relative amounts of neutral pectic sugars changed after sterilisation.

5.4.4 Molecular mass distribution (M_r) of the pectic fractions.

The M_r distribution of the pectic fractions was determined by GPC (Figures 5.3 - 5.6). Buffer- and CDTA soluble material of the fresh and blanched beans contained considerable amounts of high and intermediate M_r.. The pectic polymers of both carbonate fractions were predominantly of intermediate M_r. For cv. Odessa it seemed that the 4°C carbonate soluble polymers were of slightly lower M_r the cv. Masai polymers (Figure 5.5). For 20 °C carbonate soluble polymers of cv. Odessa a relatively large peak eluted at the void volume, which might be caused by the presence of large molecular aggregates formed during lyophilisation of the samples (Figure 5.6). Sonification or heating of the sample did not result in removal of this high M_r peak. After blanching it seemed that a small part of the polymers eluting after 50 - 60 mL had shifted from the buffer-soluble fraction to the CDTA soluble fraction. This was most obvious for cv. Masai. All pectic fractions of both cultivars showed a marked reduction in M_r after sterilisation (Figures 5.3 - 5.6). The decrease occurred mainly during the sterilisation process. The carbonate fractions from sterilised green beans dissolved very difficult and formed gels. Very little material was recovered which resulted in an erroneous elution pattern when expressed by % of total (Figures 5.5 and 5.6).

5.5 Discussion

Our primary approach to understanding the relation between cell wall composition and vegetable texture after processing was to compare cell walls of fresh and processed green bean cultivars that had different firmness after processing. From experience it is known that cv. Masai generally produced firmer beans than cv. Odessa. The present study confirmed this. In this paper we focused on changes in the chemical composition of cell wall polymers during the conventional industrial conservation process.

5.5.1 Composition of the fresh beans.

The amount of AIR, which reflects all high molecular mass components of the beans, was higher in cv. Masai as compared with cv. Odessa (Table 5.1). The composition of the AIR from fresh beans

however, were comparable for both cultivars. This implies that cv. Masai contains more high molecular components including, CWM, starch and proteins, than cv. Odessa. This is even more pronounced on a fresh weight basis. In addition, the overall composition of the cell walls was slightly different. Cv. Masai contained slightly more cellulose and fewer pectins than cv. Odessa. Also, the overall pectin compositions were different; the amounts of neutral pectic sugars (Rha, Ara and Gal) appeared higher in cv. Odessa, while the amount of uronic acids appeared relatively higher in cv. Masai (Figure 5.2). This suggests that pectin from beans of cv. Masai contained fewer or shorter side chains as compared with the pectin from beans of cv. Odessa. There was no significant difference in the amount of methyl and acetyl substituents of both cultivars. In the present study we found a different overall cell wall composition for green beans when compared with the results of Chapter 4. The beans used in the present study contained much less cell wall glucose than the beans used previously. Since in both cases cv. Masai was used, this variance probably reflects a difference in cellulose content due to growing conditions. The beans used in this study were cultivated in a greenhouse, while the former beans were field-grown. For fresh beans of both cultivars the DMSO, water and CDTA soluble fractions comprised about 3, 23 and 24 % respectively of the total extracted pectic substances on an uronide basis. The major fraction (47 %) of the pectins from fresh beans was represented by the Na_2CO_3- soluble pectins. A small proportion (2 %) of the pectins could additionally be solubilised with hydroxide solutions of increasing molarity. Only 1 % of the total uronide material remained associated with the cellulose residue after extraction. Water soluble pectins contain free, high methoxyl pectic substances, while chelator soluble pectins represent pectin present as low methoxyl pectin or salt chelated molecules (Figure 5.7). Since extraction of intact potato and carrot tissues with chelating agents results in a high tendency for cell separation, it is probable that the bulk of the CDTA extractable pectins originate from the middle lamella[17]. The other fractions, the carbonate and hydroxide soluble cell wall polymers, contain the insoluble and more tightly bound cell wall protopectin[14,18]. Carbonate extractions are harsher treatments than water and chelator extractions and are required to remove pectic substances which are probably linked to other pectins or (hemi)cellulose by weak ester bondings[18]. Because these fractions represent the major pectic fractions of fresh green beans from both cultivars, these green beans appear to contain many of such insoluble, covalently bound pectic substances. For both cultivars the composition of the sequential extracts was clearly different. Especially the 20 °C carbonate soluble pectins appeared more branched than the buffer and CDTA extractable pectins, as can be concluded from the high rhamnose-to-uronic acid ratio. Rhamnose present in the pectic backbone is a major site of side chain attachment. In fresh and blanched beans of cv. Masai there seemed to be relatively more buffer and CDTA extractable pectin, whereas in cv. Odessa there was more carbonate extractable material. Also the composition of the fractions from both cultivars were different. In general the fractions from cv. Masai contained less neutral pectic sugars and more uronic acids. This once more indicates that the pectins from cv. Masai probably contain fewer and/or shorter side chains as compared with the pectin of cv. Odessa. Previously it has been shown for kiwi and nectarines that the levels of galactose decreased during fruit ripening[19,20]. The discrepancy between the two cultivars might therefore be due to harvesting at different developmental stages. On the other hand, it cannot be excluded that the

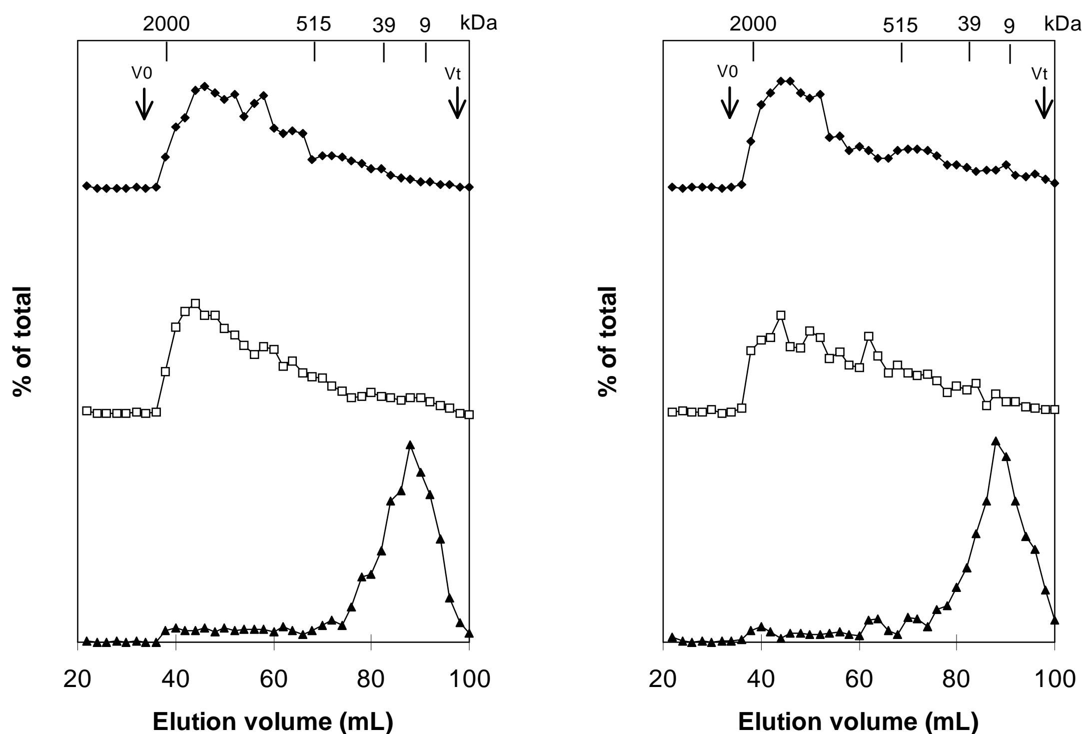

Figure 5.3 *GPC profiles of buffer-soluble fractions from green bean cvs. Masai (left) and Odessa (right) after different stages of industrial processing. Column fractions were assayed for uronic acid. fresh (-◆-), blanched (-□-) and sterilised (-▲-).*

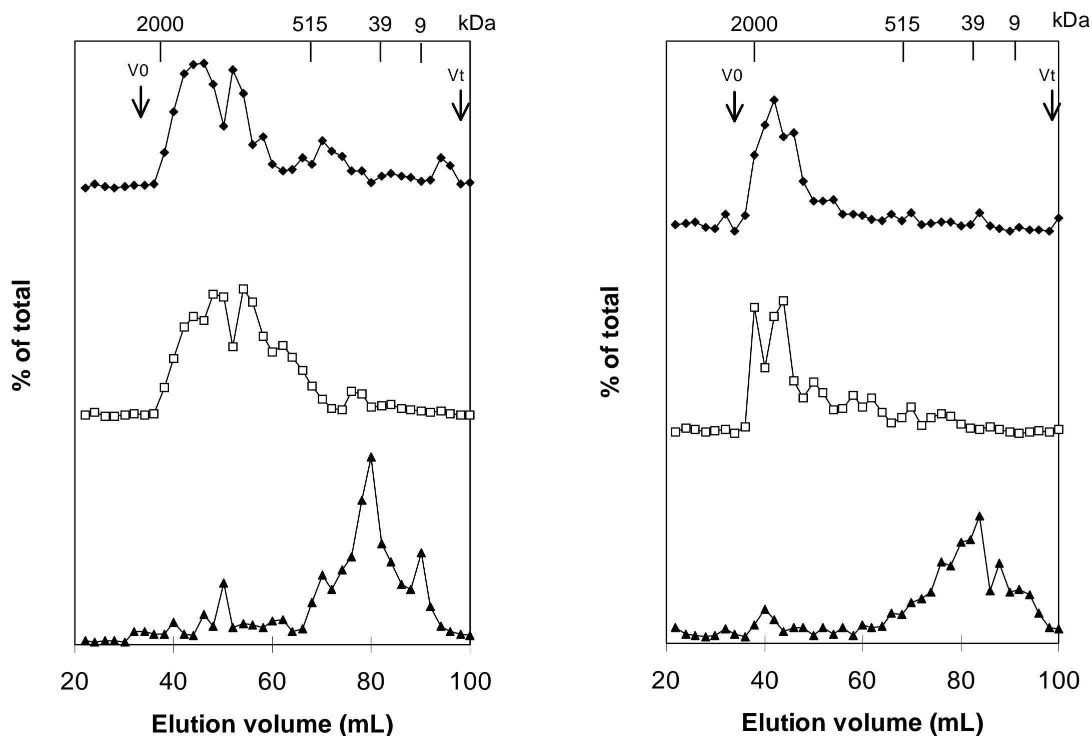

Figure 5.4 *GPC profiles of CDTA-soluble fractions from green bean cvs. Masai (left) and Odessa (right) after different stages of industrial processing. Column fractions were assayed for uronic acid. fresh (-◆-), blanched (-□-) and sterilised (-▲-).*

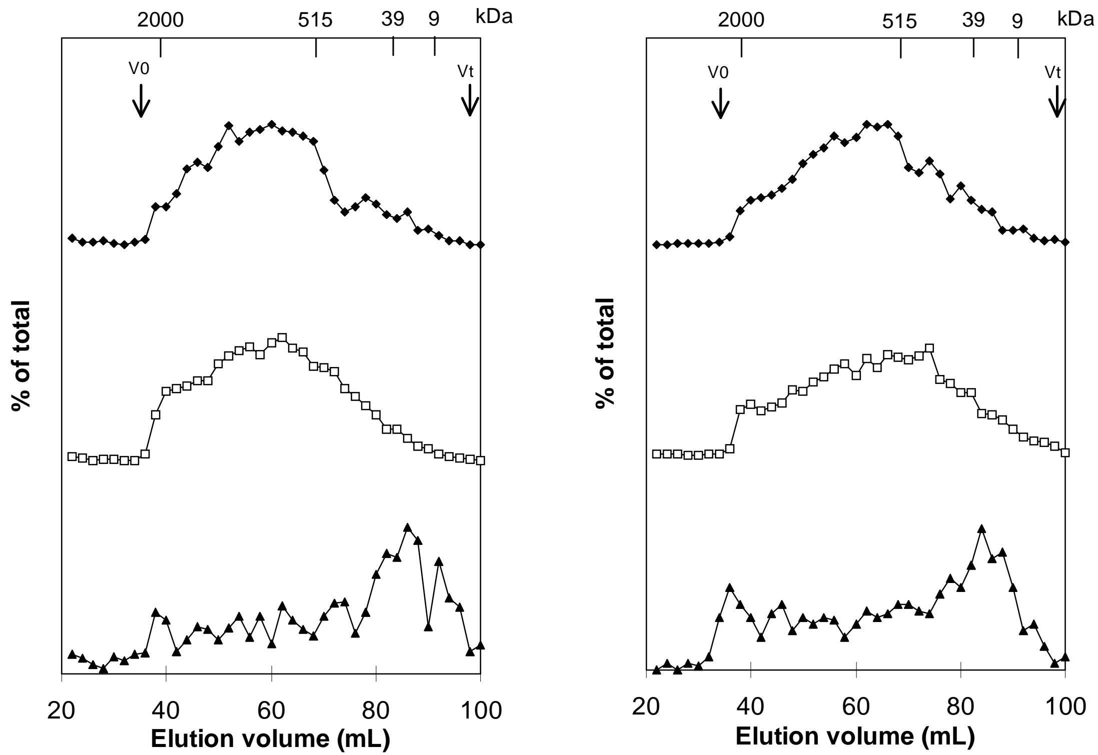

Figure 5.5 *GPC profiles of Carbonate (4 °C)-soluble fractions from cvs. Masai (left) and Odessa (right) after different stages of industrial processing. Column fractions were assayed for uronic acid. fresh (-◆-), blanched (-☐-) and sterilised (-▲-).*

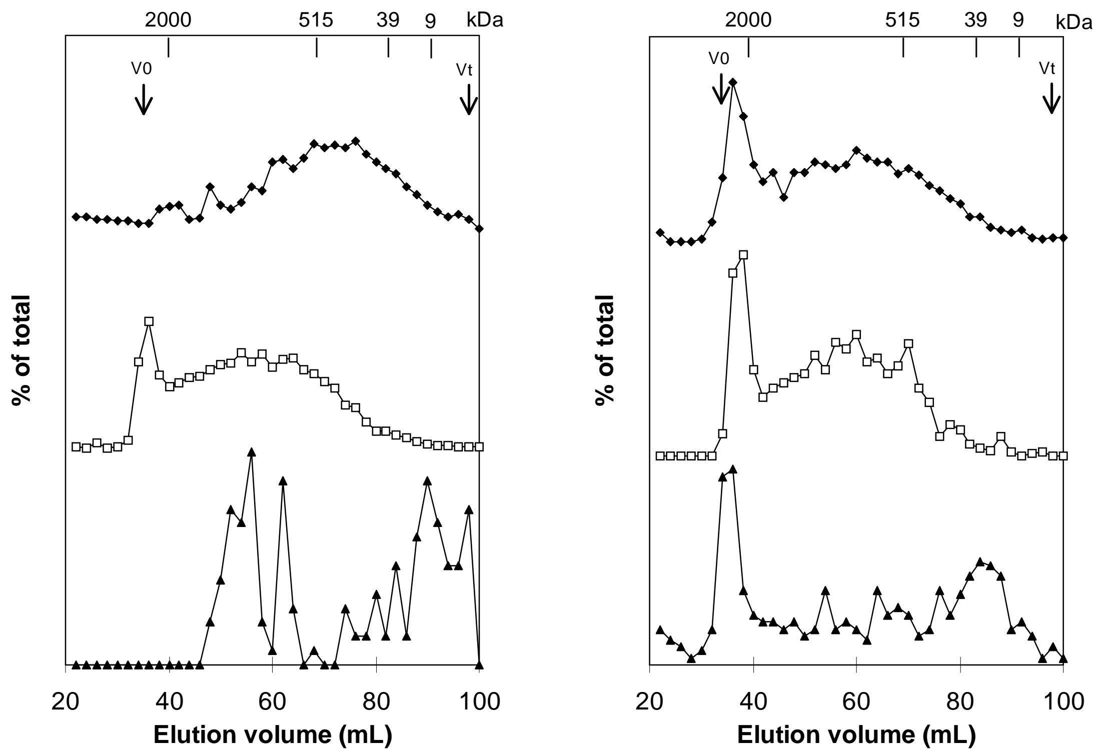

Figure 5.6 *GPC profiles of Carbonate (20 °C)-soluble fractions from cvs. Masai (left) and Odessa (right) after different stages of industrial processing. Column fractions were assayed for uronic acid. fresh (-◆-), blanched (-☐-) and sterilised (-▲-).*

galactose levels are cultivar specific and play a role in determining textural firmness after processing. The presence of side chains in carbohydrate polymers significantly affects a variety of functional properties[21]. However, relative to other functional properties such as retrogradation and gelatinisation of starch, the contribution of side chains to rheological properties has not been studied in depth for carbohydrate polymers. Two contradictory arguments have been issued for the role of side chains of pectins in gelling. Selvendran et al.[14] stated that arabinose and galactose side chains of pectin could contribute to gelling by keeping water molecules within the gel framework. In contrast, BeMiller[22] and Reid[23] stated that the side chains of pectins might tend to limit the extend of interchain association and thus the formation of junction zones required for gelling may be inhibited. Information on the contribution of side chains to the solution properties of pectins is extremely scarce[21]. More systematic research is required to elucidate the role of side chains of pectins in food systems.

KOH solubilised several hemicelluloses and some remaining pectic material. All KOH fractions contained mixtures of pectins and hemicelluloses. From these experiments it is however, not possible to determine whether these different polymers are covalently linked or just co-extracted as separate polymers. A substantial part of the hydroxide soluble pectic material was recovered in precipitates which formed after neutralisation of the fractions. This can be a result of protein-pectin complexes formed at a pH below the iso-electric point of the protein, at which it is positively charged and the pectin is anionic[24]. Preliminary experiments indeed indicated that the precipitates contained appreciable amounts of protein (data not shown). From the molar ratios of the various cell wall sugars it can be deduced that the KOH-soluble hemicellulosic polymers mainly consisted of xyloglucans, which presumably differed in their affinity for cellulose microfibrils. In contrast to cv. Odessa beans, the 1.0 M KOH extract and the 4.0 M KOH-precipitate of cv. Masai contained relatively large amounts of xylose. The xyl/glc ratios for the 1.0 and 4.0 M KOH fractions were respectively 2.61 and 2.14. Using methylation analysis of the water insoluble material from green beans (Chapter 4) it was obvious that a large part of the xylose in green beans was (1-4) linked. Together with the relative amounts of xylose and glucose this suggests that the xylose in the 1.0 M KOH extract and 4.0 M KOH-precipitate are derived from xylans. Another possibility is the presence of xylogalacturonans. In the cell wall material of runner beans it was shown that some galacturonic acid residues were substituted on positions 2 and 3[18] . Ryden and Selvendran[18,25] also stated that they found complexes composed of (1-4) linked xylose containing polymers associated with pectic material in hydroxide fractions from runner beans. They suggested that such complexes may serve as cross-linking polymers within the cell wall matrix of leguminous species. In cell walls of green beans also an appreciable amount of (1-4) linked xylose was found[13]. Probably cell walls of cv. Masai are more heavily cross-linked by this kind of complexes than cell walls of cv. Odessa. In addition, Northcote et al.[26] detected (1-4) linked xylose containing polysaccharides by immunochemistry in the middle lamellae of suspension-cultured bean cells. Waldron and Selvendran[27] suggested that these xylan-pectic polysaccharide complexes might be associated with the concomitant deposition of lignin. Lignification is known to start from the middle lamellae region and involves the deposition of phenolics with xylan. The obtained results therefore might suggest that for cv. Masai the carbohydrate initials for

lignification may be present at a larger amount and that the middle lamellae of this cultivar may even already contain some lignin deposits.

5.5.2 Cell wall changes during processing

During sterilisation pectins were solubilised (Table 5.4). The overall degree of methylation decreased during sterilisation, probably due to complete degradation of highly methylated regions of pectin by ß-elimination (Table 5.3). Analysis of the isolated pectic fractions of the bean pods revealed that substantially more pectin became DMSO and buffer soluble after sterilisation and that the M_r of pectin was reduced. Pectin shifted from the carbonate fractions to the DMSO, buffer and CDTA fraction (Figure 5.7). After blanching, there was a temporary increase in CDTA-extractable material. This might be an effect of some pectin methylesterase (PME) activity during the blanching treatment. Although at 90 °C PME will be inactivated very rapidly, it will take some time before the entire bean pod reaches this temperature. During this warming period PME could have been able to demethylate some of the pectin and made these demethylated pectins available for calcium cross-linking. This idea is supported by a slight decrease of the overall DM of the pectin after blanching. After sterilisation however, the amount of CDTA extractable pectins was equal to the amount extracted from the fresh

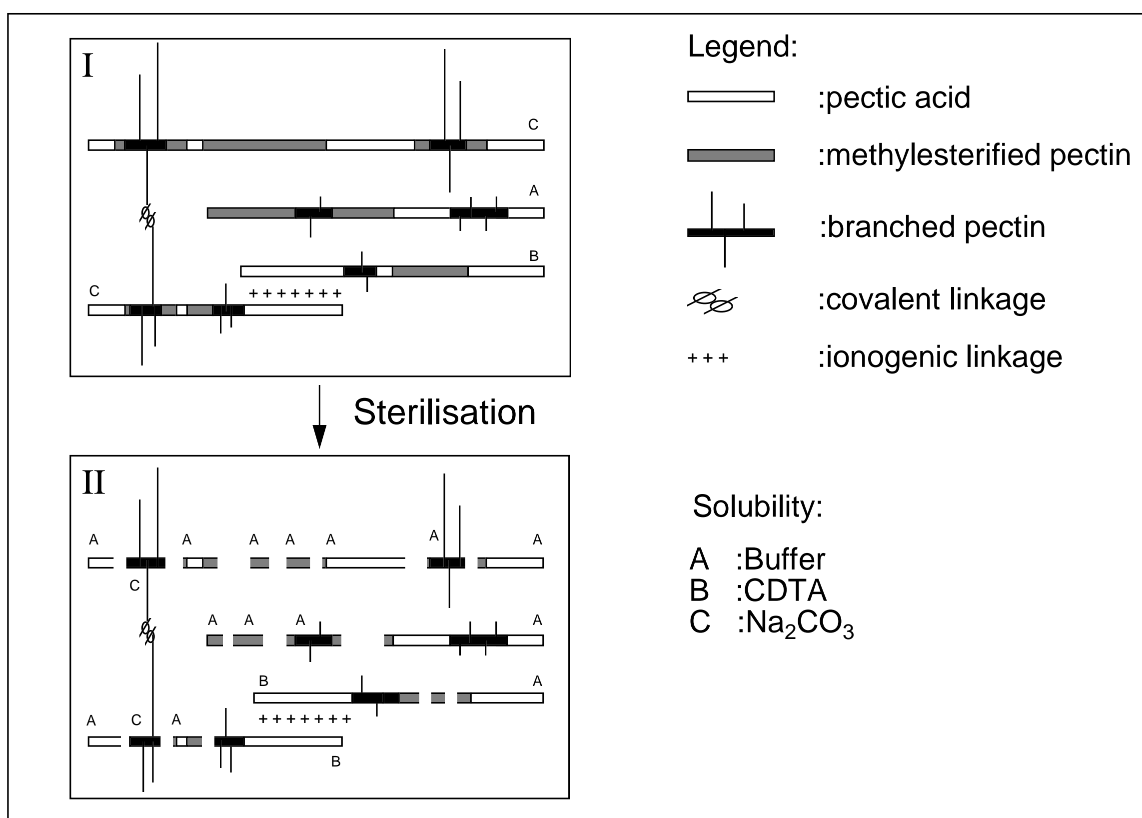

Figure 5.7 Schematic picture of suggested changes in pectic polymers during industrial processing of green beans. In the fresh beans (I) a large part of the pectin appears covalently linked to other cell wall components. During processing the pectin is partly degraded and depolymerised by ß-eliminative degradation of methylated pectic regions. This results in more buffer soluble and less carbonate soluble pectins in the cell wall of sterilised green beans (II).

material due to an overall shift of pectic material. The M_r of all fractions was strongly reduced. After sterilisation there was a relative increase of arabinose and galactose in all the fractions, indicating that all fractions contained relatively highly branched pectins. This is consistent with the general idea that during sterilisation linear, non branched, high methoxyl pectin is degraded into small fragments by ß-elimination. Just a very small amount of the pectins remained extractable only with alkaline solutions or remained associated with the cellulose residue. On the basis of their high arabinose and galactose contents these pectins were most probably very highly branched. No important changes were observed in hemicellulosic and cellulosic polymers. The changes occurring in the hemicellulosic fractions during processing were predominantly caused by pectic polymer modifications. The major changes were changes in the yields of the different fractions, especially the precipitate fractions.

In conclusion, two major effects of blanching and sterilisation on the cell walls of green beans can be discriminated (Figure 5.7): (1) a degradation of linear regions (homogalacturonan) into monomers and small oligomers and (2) a solubilisation of branched regions (rhamnogalacturonan) of the cell wall pectin. Several differences were observed between cell wall material from beans of cultivars Masai and Odessa. It is not possible to determine whether these represent intrinsic differences, since only one batch of each cultivar was analysed. Cultivar Masai beans, which had the highest firmness retention after sterilisation, appeared to have (1) more cell wall material, (2) less branched pectins and (3) more xylans. The present study cannot discriminate between these characteristics with regard to firmness retention because only two cultivars were studied. To determine which of these differences is most important in firmness retention, cell wall characteristics of more cultivars have to be compared including repeats over years.

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Chapter 6

Preheating effects on the textural strength of canned green beans: characterisation of (bio)chemical cell wall features*.

6.1 Abstract

With variable preheating conditions is was possible to modify the firmness of two green bean cultivars after processing. The aim of this study was to elucidate the biochemical basis of this phenomenon. In addition it was attempted to relate differences in pectic properties to different inherent firmness of the two cultivars. Pectic polymers were extracted from the alcohol-insoluble residues from fresh, preheated and sterilised green beans. The carbohydrate composition, degree of esterification and relative molecular mass of the different fractions was analysed. In addition, the residual activities of pectin methylesterase and peroxidase after preheating were determined. The preheating temperature which resulted in the highest retention of firmness after sterilisation corresponded with the optimal temperature for pectin methylesterase activity. After this preheating treatment, there was an overall reduction of the degree of methylation of the cell wall pectin, indicating endogenous PME activity. In addition, the yields of the buffer and CDTA fractions, as well as their average molecular mass were higher after sterilisation. No significant effect on the yield or composition of the carbonate extractable, branched pectins was found. Those results suggest that PME most probably was not able to demethylate pectin flanking these branched regions leaving these pectic regions susceptible for ß-eliminative degradation. With respect to the firmness difference between the two cultivars, the DM and DA of the CDTA-soluble pectins and the total amount of pectins seemed to be important.

Preheating of green beans affects texture after sterilisation most likely by demethylation of pectin by PME thereby (1) decreasing the ß-eliminative degradation of pectin.(2) increasing the capacity of pectin to form Ca^{++} mediated complexes and more important These changes, occurring during preheating and sterilisation, are visualised by a schematic picture.

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6.2 Introduction

Texture is an important quality attribute of fruits and vegetables. Loss of texture during industrial processing of these products is often dramatic and has been the subject of much research. At the practical level, research has been directed towards modifying processing conditions so that more of the products original texture can be retained. At the fundamental level, research has been directed towards understanding which chemical and/or structural parameters of plant tissues contribute to texture and how the preservation process affects these features.

During heating, turgor and membrane integrity are lost rapidly[1]. This initial softening process is enhanced by dissolution of the cell wall and middle lamella[2-4]. Mainly the pectic polymers of the cell wall and middle lamella change during processing. In fresh beans, the major part of the pectins is most likely covalently linked to other cell wall polymers[5]. Heating results in degradation and solubilisation of pectic polymers from the cell wall and middle lamella[2,4-7]. Model experiments with solubilised carrot pectins indicated that ß-elimination was primarily responsible for heat degradation of pectins and that higher methylester content resulted in greater rate of degradation[8]. However, little experimental data on the degradation mechanism of native plant pectin are available. The firmness loss of green beans after sterilisation can be decreased by preheating the beans at moderate temperatures[9,10]. This firming effect is generally attributed to the action of pectin methylesterase (PME) during the preheating period[11-13]. It has been hypothesised that PME forms stretches of consecutive acidic GalA residues, which will bind in a consorted way to a number of Ca^{++} ions[14]. Consequently, strong cross-links can be formed between pectin molecules, probably in a well defined arrangement known as the ‘egg-box’ model[15]. These complexes are thought to have a firming effect on the tissue. At the moment however, there is much discussion in literature whether these ‘eggbox’ structures really exist *in vivo*[16,17]. Another possible firming effect during preheating may be the result of peroxidase (POD) action. A small percentage of the sugars in wall polysaccharides carry ferulic acid and related phenolic groups, which may be cross-linked by the action of POD and H_2O_2[18]. These cross-linked structures form intercellular bridges like diferulate and isodityrosine, thus connecting polymers together in a tight network. In bamboo shoots, sugar beet and Chinese water chestnut these ferulic acid dimers have been associated with thermal stability of texture[19]. These phenolic dimers require a higher pH for their de-esterification than Na_2CO_3-saponifiable uronyl esters[20].

In conclusion, the details of the chemical cell wall changes at moderate preheating temperatures that cause the retention of firmness are not yet fully understood. In this chapter we relate biochemical and chemical aspects of preheating to a reduced loss of firmness during sterilisation of green beans.

6.3 Materials and methods

6.3.1 Plant material

Green beans (*P. vulgaris* L.) cv. Masai and Odessa were grown at the experimental research station PAGV (Lelystad, The Netherlands) and harvested at edible maturity. Both ends of the pods were removed and the middle parts were cut into parts of 3 - 4 cm in length.

6.3.2 Processing conditions

Beans were processed according to Table 6.1. The green bean samples were placed in tap water of various temperatures (30 - 80 °C) and preheated for a certain period of time (10 - 120 min); subsequently, the beans were blanched for 4 min at 90 °C. After the first preheating treatment samples were frozen and lyophilised for enzyme activity measurements and cell wall analyses. For the succeeding canning process, portions (410 g) of beans were packed into glass jars (720 mL) and a 0.25 M NaCl solution (brine) was added. Closed jars were sterilised at 118 °C for 30 min. After sterilisation, the samples were stored at 15 °C until firmness evaluation and cell wall analyses.

Table 6.1. *Overview of processing conditions of green bean cultivars Masai and Odessa.*

Sample	Preheating		Blanching	Sterilisation
	temp (°C)	time (min)	4 min 90 °C	30 min 118 °C
1	30	120	yes	yes
2	40	90	yes	yes
3	50	60	yes	yes
4	60	45	yes	yes
5	70	20	yes	yes
6	80	10	yes	yes
7	90	4	no	yes

6.3.3 Texture assessment

The firmness of the beans was measured using an Instron Universal Testing Machine equipped with a Kramer shear cell. Forty grams of material was placed in the cell with the length axis of the pods perpendicular to the openings of the shear cell. The maximal force (top value) needed to break through the beans was used to quantify the instrumental firmness of the beans.

6.3.4 Isolation of Alcohol Insoluble Residue (AIR) and pectic polymers

AIR was isolated from lyophilised blanched and sterilised bean samples. Sterilised green beans (50 grams) were homogenised in an equal weight volume of water, the lyophilised blanched bean samples were ground in a mill with a sieve of 0.5 mm mesh. AIR was isolated by refluxing for 30 min in 150 mL hot ethanol (80 % v/v), filtering and finally washing the residue with 100 % acetone.

Pectic polymers were extracted using a modified method from Selvendran et al.[21] which is summarised in Figure 6.1. To remove starch, the AIR (2 g) was suspended in 100 mL 90 % DMSO and stirred for 16 h at 20 °C. The suspension was centrifuged (10,000 g for 15 min) and the pellet was washed twice with 90 % DMSO and three times with 80 % ethanol. To the pellet, 100 mL of 0.05 M ammonium acetate buffer (pH = 4.7) was added and the suspension was incubated under constant stirring for 16 h at 4 °C. After centrifugation (10,000 g for 15 min), the pellet was washed once with

acetate buffer and once with distilled water. To the pellet 100 mL of 0.05 M CDTA (pH = 6.5) was added and the suspension incubated with constant stirring for 16 h at 4 °C. The suspension was centrifuged (10,000 g for 15 min) and the pellet was washed once with the CDTA-solution and once with water. To the pellet, 100 mL of 0.05 M Na_2CO_3 containing 0.01 M $NaBH_4$ was added and the suspension incubated with constant stirring for 16 h at 4 °C. The suspension was centrifuged (10,000 g for 15 min). To the pellet 100 mL, of 0.05 M Na_2CO_3 containing 0.01 M $NaBH_4$ was added and the suspension was incubated with constant stirring for 16 h at 20 °C. The suspension was centrifuged (10,000 g for 15 min).

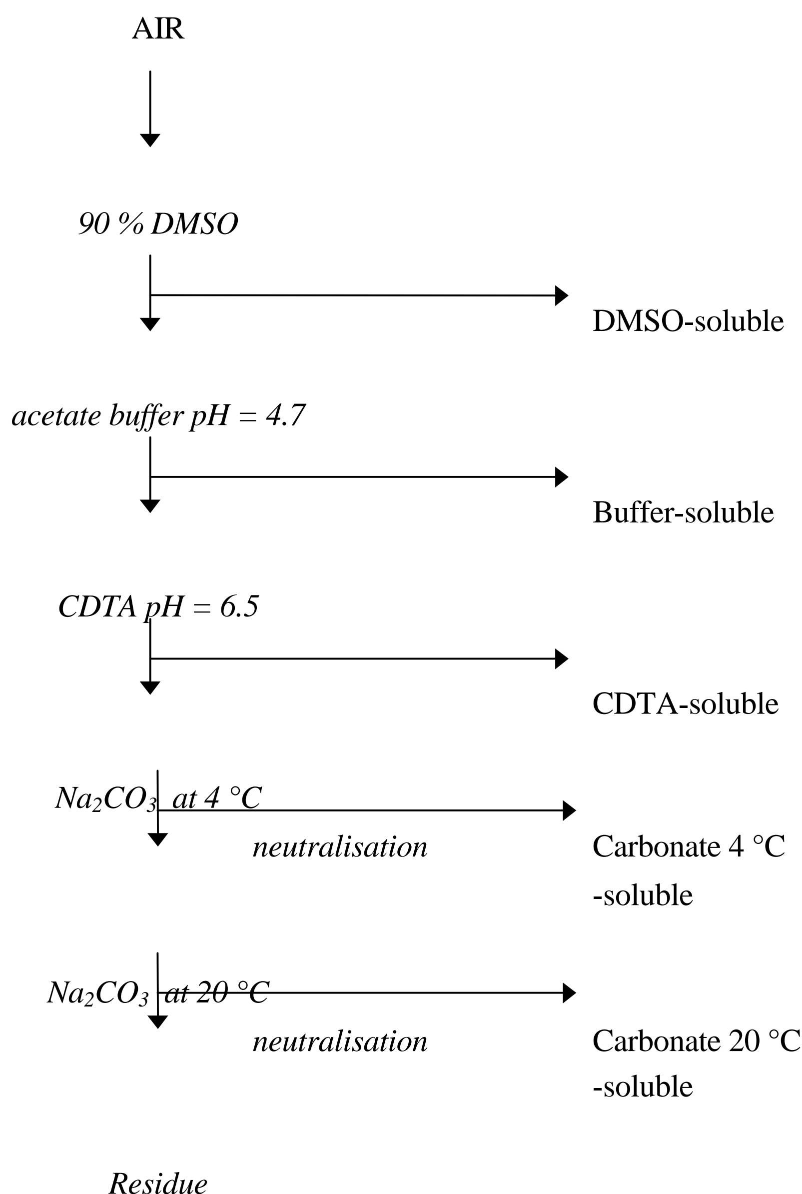

Figure 6.1 *Extraction scheme for the pectic cell wall fractions.*

6.3.5 Dry matter determination

The dry matter content of the processed bean samples was determined as described in Chapter 5.

6.3.6 Monosaccharide composition.

Cell wall sugars in the AIR, Residue and solubilised pectic fractions were released as described in chapter 4 and 5. Samples (10 μL) of the neutralised hydrolysates were analysed for neutral sugars by using a HPLC system (Pharmacia LKB Low pressure mixer, autosampler 2157 and Waters 625 LC pump) equipped with a Carbopack PA1 column (250 x 4 mm, Dionex). Data analysis was performed using Millennium 2010 software (Waters). The eluents, consisting of milli Q water and 150 mM NaOH, were sparged and pressurised with helium. Prior to injection, the system was equilibrated with 30 mM NaOH for 8 minutes at a flowrate of 1.0 mL/min at ambient temperature. The sugars were separated by an initial gradient from 30 - 0 mM NaOH during 3 min and subsequent elution in milli Q during 30 min. After each run, the column was regenerated with 150 mM NaOH for 15 min. Compounds were detected with a Dionex PED in the pulsed amperometric detection mode fitted with a gold working electrode. The applied potentials were set at E1 = 0.1 V, E2 = 0.6 V and E3 = -0.6 V against a Ag/AgCl reference electrode. Pulse durations for the applied potentials were 500 msec, 100 msec and 50 msec respectively. Trehalose, added after hydrolysis of the samples, was used as an internal standard. Anhydro-uronic acids were determined as described by Ahmed and Labavitch[22].

6.3.7 Starch content.

Starch was determined using a test-combination cat. nr. 207748 from Boehringer Mannheim as described previously[4].

6.3.8 Methyl and acetyl substituents.

The amount of methyl and acetyl groups was determined by using a HPLC system under the conditions as described previously[23].

6.3.9 Molecular mass distribution analyses

High Performance Size Exclusion Chromatography (HPSEC) was performed using a HPLC system (Waters UK6 injector, Waters 510 HPLC pump) equipped with two columns (each 7.8 x 300 mm) in series (Ultrahydrogel 500 and Ultrahydrogel 250; Waters) in combination with a Waters Ultrahydrogel guard column and elution with 0.4 M acetic acid/sodium acetate (pH = 3.0). The eluate was monitored using a Pharmacia refractive index detector. The system was calibrated using linear pullulans with molecular masses in the range of 6,000 - 1,660,000 Da. Data analysis was performed using Millennium 2000 software (Waters).

6.3.10 Enzyme activity measurements

-PME extraction and activity assay. Ground, lyophilised bean powder (40 mg) was first washed for 1 h at 4 °C with 1.0 mL demineralised water and subsequently extracted with 1.0 mL 1M NaCl for 1 h at 4 °C. After centrifugation, the total PME activity in the supernatant was determined using a continuous spectrophotometric assay with bromothymol blue as a pH indicator[24]. The reaction mixture (3000 µL) contained 0.14 % citrus pectin (Sigma UK), 0.028 % bromothymol blue, 100 mM NaCl, pH = 7.8. The reaction was started by adding 100 µL sample solution and the decrease in absorbency at 616 nm was monitored using an UVIKON spectrophotometer. PME activities were determined using galacturonic acid as a standard and expressed in katals (1 katal = 1 mole product formed per second).

-POD extraction and activity assay. Ground, lyophilised bean powder (100 mg) was extracted with 1.0 mL 0.1M phosphate buffer pH = 6.0 for 1 h at 4 °C. After centrifugation the activity of soluble POD in the supernatant was determined using a continuous spectrophotometric assay. The reaction mixture (1900 µL) consisted of 0.16 mM dianisidine, 13 mM H_2O_2, pH = 6.0. The reaction was started by adding 300 µL sample solution and the decrease in absorbency at 460 nm was monitored using an UVIKON spectrophotometer. POD activities were determined using the molar extinction coefficient of dianisidine ($11.3 * 10^{-6}$) and expressed in katals (1 katal = 1 mole product formed per second).

6.4 Results

6.4.1 Firmness measurements

Tissue firmness of the sterilised green beans was determined using an Instron Universal Testing machine equipped with a Kramer shear cell. Beans of cv. Masai were firmer than beans of cv. Odessa after any processing condition. Maximal firmness values after sterilisation were obtained for the beans of both cultivars after preheating at 60 °C (Figure 6.2).

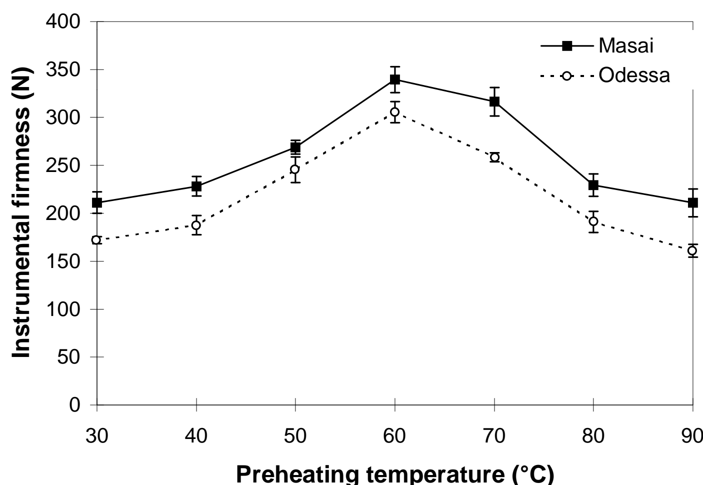

Figure 6.2 *Firmness of sterilised green bean cultivars Masai and Odessa after different preheating treatments.*

6.4.2 Enzyme activity measurements

Bean PME and POD residual activities were compared after different preheating treatments (Figure 6.3). PME and POD activities were higher in cv. Masai as compared with cv. Odessa. The PME activity was not altered by preheating at 30 - 50 °C. Some residual PME activity was still detected after preheating at 60 and 70 °C, but after preheating at 80 or 90 °C, no activity was found. The activity of soluble POD from cv. Masai was strongly increased by preheating at 30 or 40 °C. For cv. Odessa we could not detect a significant increase of POD activity after preheating. The POD activity decreased above heating at 60 °C in comparison with the value observed for no preheating. After blanching at 90 °C no POD activity was detectable anymore.

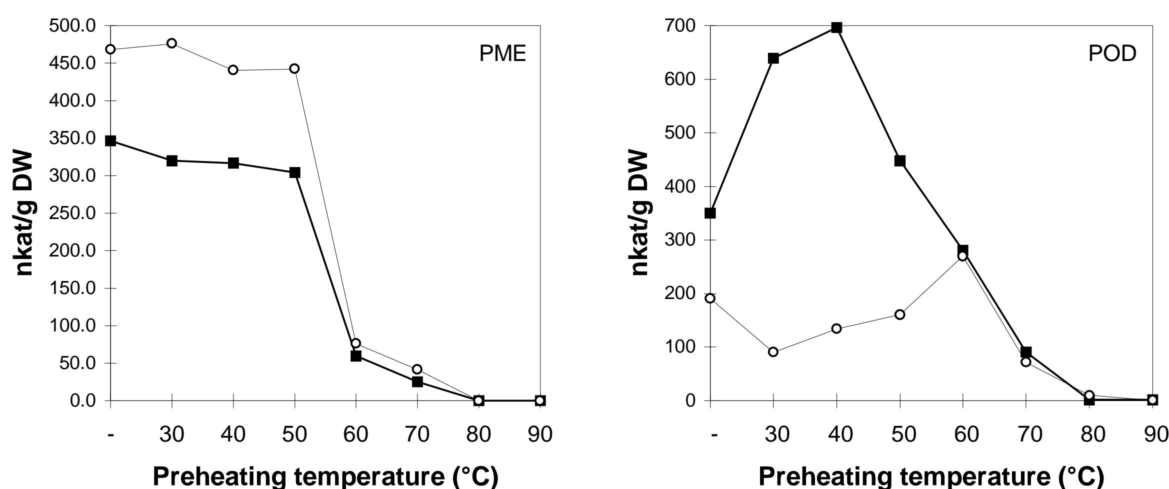

Figure 6.3. *Residual PME and POD activity of green bean cultivars Masai (-●-) and Odessa (-O-) after preheating at different temperatures (for preheating conditions see table 6.1).*

6.4.3 Analysis of alcohol insoluble residues

The pectic sugar composition of all AIR samples was compared (Table 6.2). Interestingly, beans of cv. Masai seemed to contain more pectic material than beans of cv. Odessa. There was no significant difference in the pectin composition of the bean samples of the two cultivars after different preheating treatments. Uronic acid was lost from the AIR during sterilisation. After sterilisation the highest amount of pectic sugars was present in the beans preheated at 60 °C.

Analysis of the degree of methylation (DM) and acetyl esters in the AIR samples from preheated beans revealed that the DM was lowered in beans preheated between 50 - 80 °C in comparison with fresh beans (Table 6.2). The amount of acetyl esters remained constant during all preheating treatments. Analysis of the DM of the sterilised beans showed that for both cultivars the DM was still slightly lower in the 60 - 80 °C preheated samples The difference was however much lower than in the only preheated samples.

***Table 6.2** Yield of AIR and rhamnose, arabinose, galactose and uronic acid content, degree of methylation (DM) and number of acetyl groups in AIR isolated from green beans during processing.*

Sample	preheating temp. (°C)	AIR (mg/g DW)	Pectic sugar composition (µmol/g DW) Rha	Ara	Gal	AUA	DM (mol%)	Acetyl (µmol/g DW)
Cv. Masai								
fresh	-	688	31	127	193	420	65.9	161
preheated	30	727	30	125	175	421	65.1	157
	40	714	30	128	199	444	64.1	165
	50	691	30	124	211	457	58.7	160
	60	692	32	124	219	461	53.9	173
	70	681	31	131	242	446	55.8	172
	80	685	28	128	232	459	58.8	162
	90	736	30	131	206	437	67.6	172
preheated + sterilised	30	745	32	152	249	356	48.5	174
	40	761	29	144	230	357	49.0	172
	50	762	33	152	245	360	44.6	170
	60	767	37	171	284	401	41.8	189
	70	763	34	150	251	420	37.7	177
	80	755	34	143	248	396	45.1	176
	90	742	35	137	218	277	55.9	169
Cv. Odessa								
fresh	-	661	21	98	156	320	68.7	121
preheated	30	698	18	99	164	335	71.2	133
	40	591	17	93	156	322	64.1	123
	50	709	19	98	150	346	65.5	142
	60	726	21	103	157	372	55.5	153
	70	739	21	114	180	377	53.6	144
	80	659	24	103	192	341	63.5	139
	90	650	19	101	204	346	67.3	140
preheated + sterilised	30	716	28	114	191	265	55.5	136
	40	724	27	115	198	258	51.8	134
	50	732	24	130	208	281	48.3	134
	60	730	28	135	229	310	47.9	147
	70	731	31	122	205	297	45.9	137
	80	716	28	122	217	301	45.4	138
	90	730	22	118	182	261	48.9	121

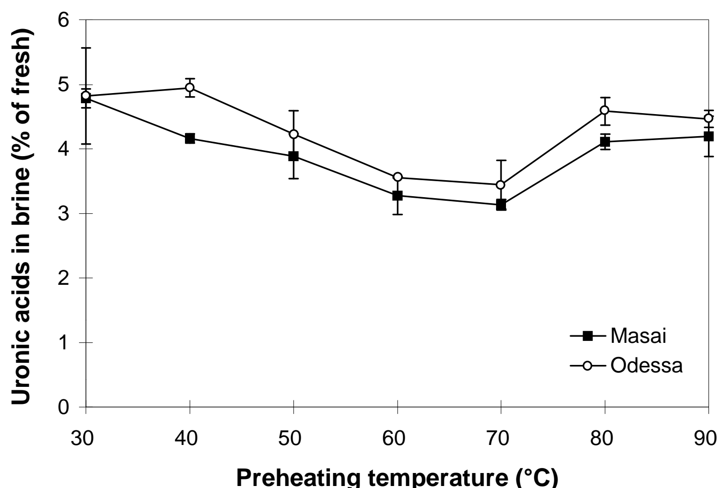

Figure 6.4 *Uronic acids leached out into the brine during sterilisation of two cultivars of green beans after different preheating conditions. Values are expressed as percentage of total uronic acids present in fresh green beans (for preheating conditions see table 6.1) (n=2).*

6.4.4 Solubility and composition of cell wall pectins

The uronic acids solubilised into the brine during sterilisation, were quantified revealing that there was less pectin degradation in the bean samples preheated at 50 - 80 °C (Figure 6.4). Relatively more pectin was degraded in green beans of cv. Odessa than in beans of cv. Masai for all processing conditions.

The AIR from the preheated (40 - 90 °C) samples was fractionated according to Figure 6.1. This extraction procedure was designed to minimise ß-eliminative degradation of pectins during the initial stages of extraction and to solubilise the polymers in as close to their native form as possible (Selvendran, 1985). The pectic polysaccharides not connected to other cell wall polymers were extracted with acetate buffer; the polymers bound in the wall by Ca^{2+} only were solubilised by CDTA. Most of the CDTA-insoluble pectins were subsequently extracted by dilute Na_2CO_3 at 4 °C and 20 °C, presumably by hydrolysis of weak ester cross-links[20]. Cold Na_2CO_3, which would hydrolyse ester bonds, but cause negligible elimination-degradation, solubilised most of the CDTA-insoluble pectins. The sugar composition, degree of methylation, number of acetyl substituents and molecular mass distribution of the resulting pectic fractions and residue was compared. The overall yield of the different fractions is visualised in Figure 6.5. Substantially more pectins could be extracted from beans preheated at 60, or 70 °C. This effect was most obvious in cv. Masai. Especially, the amounts of buffer and CDTA-soluble material increased at these temperatures. The degree of methylation and acetylation of the buffer and CDTA fractions showed no clear trend in relation to the different preheating treatments (Figure 6.6). Both the degree of acetylation and methylation of the CDTA-soluble pectins from cv. Odessa were higher in comparison with the CDTA-soluble pectins from cv. Masai. For all pectic fractions, the pectin yield was higher in the samples preheated at moderate temperatures. In addition, the amount of pectin as expressed per gram dry weight of beans, was higher for cv. Masai as compared with cv. Odessa. However, the sugar composition of all fractions was the same for almost all samples (Figure 6.7). The CDTA-soluble polymers from cv. Odessa contained relatively more neutral pectic sugars than the CDTA

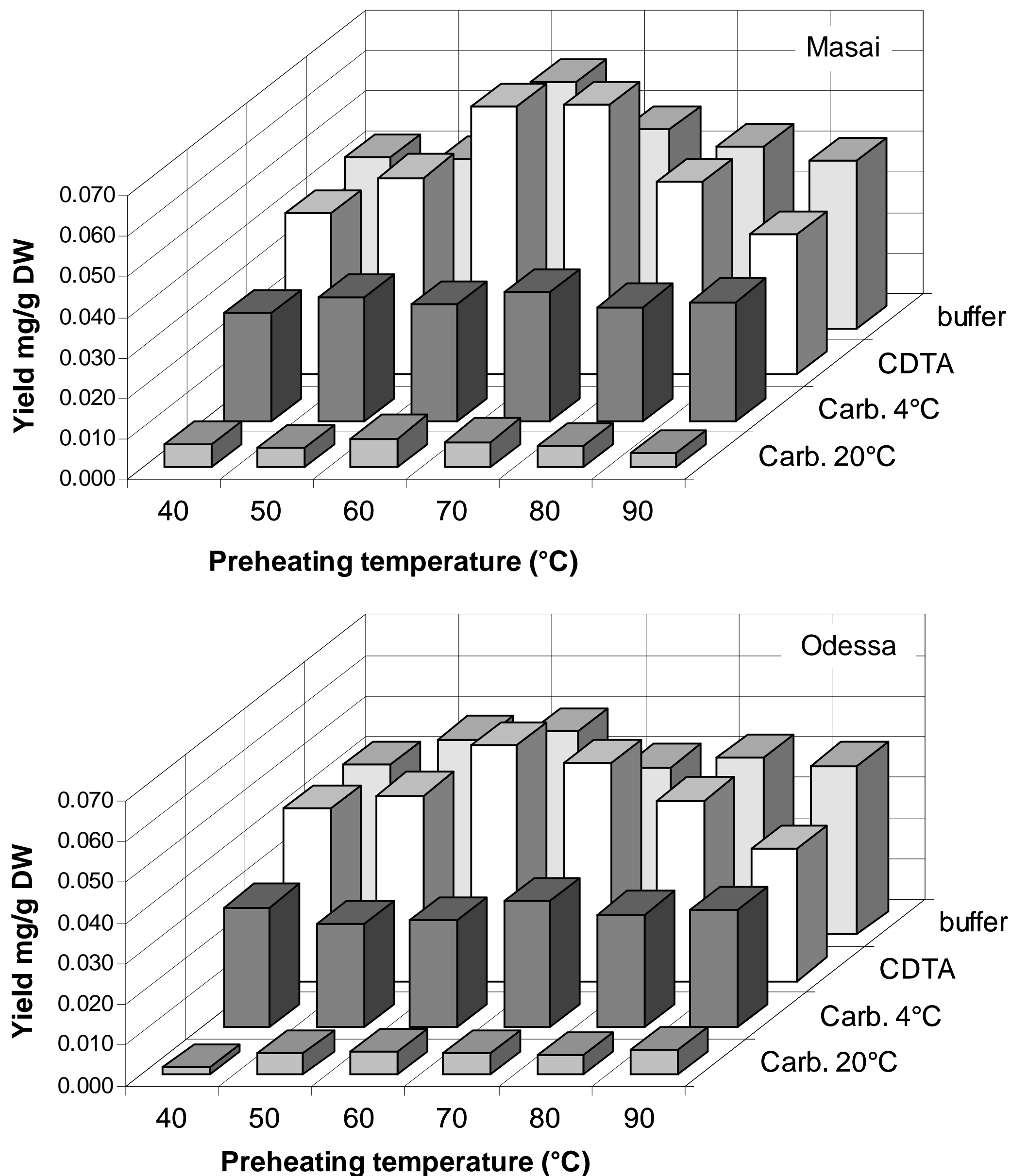

Figure 6.5 *Yields of sequential pectic fractions isolated from green bean cultivars Masai and Odessa after sterilisation and different preheating treatments (for preheating conditions see table 6.1).*

soluble pectins from cv. Masai suggesting that they contain either more or longer sidechains. In the 4 °C carbonate extracts a trend was observed towards a slightly higher relative uronic acid content in the samples preheated at either 60 or 70 °C. There was no effect of preheating on the yield and composition of the Residue of the sterilised beans (Table 6.3). A relative high proportion of the uronic acid containing polymers remained associated with the 'depectinated' residue.

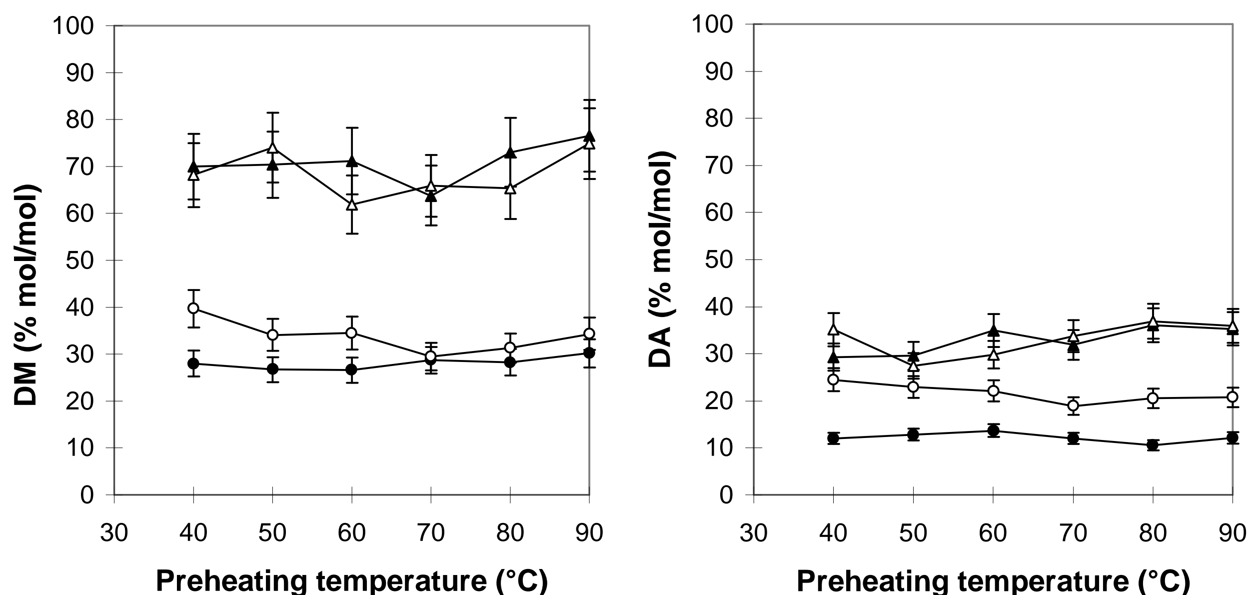

Figure 6.6 *Degree of methylation (DM) and acetylation (DA) of buffer (triangles) and CDTA (circles)-soluble pectic fractions isolated from green bean cultivars Masai (solid markers) and Odessa (open markers) after sterilisation and different preheating treatments. (for preheating conditions see table 6.1).*

Table 6.3 *Yield and composition of residue after extraction of pectins from the AIR of green bean cultivars Masai and Odessa (n=2).*

Preheating temperature	residue yield	cell wall sugars in residue (mol %)							
(°C)	(mg/g DW)	Fuc	Rha	Ara	Gal	Glc	Xyl	Man	AUA
Cv. Masai									
40	0.428	0.3	n.d.[1]	4.9	5.4	59.1	3.9	3.9	22.5
50	0.438	0.4	n.d.	4.7	5.5	60.6	3.9	3.6	21.3
60	0.404	0.5	n.d.	4.6	6.0	59.5	4.0	3.9	21.5
70	0.427	0.5	n.d.	5.0	5.9	58.3	4.2	3.9	22.2
80	0.421	0.5	n.d.	4.9	6.4	61.7	4.1	3.9	18.5
90	0.417	0.5	n.d.	4.8.	5.5	61.6	4.0	3.8	19.8
Cv. Odessa									
40	0.394	0.5	n.d.	4.0	5.3	64.0	3.3	3.2	19.6
50	0.367	0.5	n.d.	4.4	5.5	63.7	3.4	3.3	19.2
60	0.357	0.6	n.d.	4.6	6.2	60.7	3.8	3.8	20.3
70	0.393	0.5	n.d.	4.2	5.4	62.4	3.7	3.4	20.3
80	0.364	0.5	n.d.	4.0	6.0	62.7	3.6	3.4	19.8
90	0.417	0.6	n.d.	3.9	4.5	66.6	2.7	3.0	18.8

[1]*n.d.: not detected*

6.4.5 Molecular mass analyses

The molecular mass (M_r) distribution of all pectic fractions was determined by HPSEC, as shown in Figure 6.8. For each fraction, the differently preheated samples had fairly similar elution patterns, only the relative amounts of the peaks varied. The buffer and carbonate-soluble polymers showed three major peaks, eluting at 13, 14 and 20 min respectively. The molecular mass distribution of the buffer and 4 °C carbonate-soluble fractions of beans preheated at 50 - 70 °C showed a higher peak eluting at 14 - 15 min, in comparison to the conventionally (90 °C) blanched sample. This was most obvious for cv. Odessa. The CDTA-soluble polymers showed a large peak eluting at 17 - 18 min. The CDTA-soluble pectins of beans preheated at 60 or 70 °C were of higher molecular mass than pectins from the other samples. Also, a small peak eluting at 19 - 20 min became visible in the chromatograms. In these fractions only small amounts of high molecular material, eluting at 13 - 14 min, was found. Preheating at 50 - 60 °C resulted in just slight alterations of the elution patterns of the 20 °C Carbonate-soluble polymers.

6.5 Discussion

The firmness of green beans appeared to vary with the conditions of the used preservation process. Sterilised green beans which were preheated at moderate temperatures (50 - 70 °C) during an appropriate period of time before further processing, remained significantly firmer than conventionally processed green beans (Fig. 1). This higher retention of firmness of green beans by preheating was already described by many other authors[9,13,25,26]. A similar effect is found for a diversity of other vegetables including potatoes and carrots[8,27]. The only, very evident, change in the cell walls during preheating was a decrease in DM (Table 6.2). This is consistent with the general idea that the preheating effect can be attributed to demethylation of pectin by endogenous pectin methylesterase (PME) during the preheating period[12,28]. The effect on firmness of this reaction becomes only evident after heating at elevated temperatures, like sterilisation. The optimal temperature for PME extracted from fresh green bean pods was 55 - 60 °C[28]. This was also the temperature of the preheating treatment which resulted in the lowest DM after preheating (Table 6. 2). The residual PME-activity was low after preheating for 45 min at 60 °C, which indicates that a substantial amount of the PME was denatured at this temperature. The overall executed activity during the total preheating period however, may have been very high. Typically, PME is assumed to bind very strongly to pectin electrostatically, and its activity is tightly controlled during plant development[15]. Consequently, when a PME has de-esterified a chain segment, it will remain attached there, and will be released only by heat[29] or perhaps by PG-action. For these reasons, it is indeed very likely that PME is activated during preheating, resulting in removal of methylesters from the pectin. The most important softening process during sterilisation is believed to be ß-elimination[8]. The reaction rate of ß- eliminative degradation depends on temperature, pH and the presence of a methylester at C_6, next to which the chain cleavage occurs. As a consequence, pectin solubilisation will occur to a greater extend upon sterilisation whenever PME could not act optimally either by too low preheating temperature (low activity), or by too high preheating temperature (denaturation).

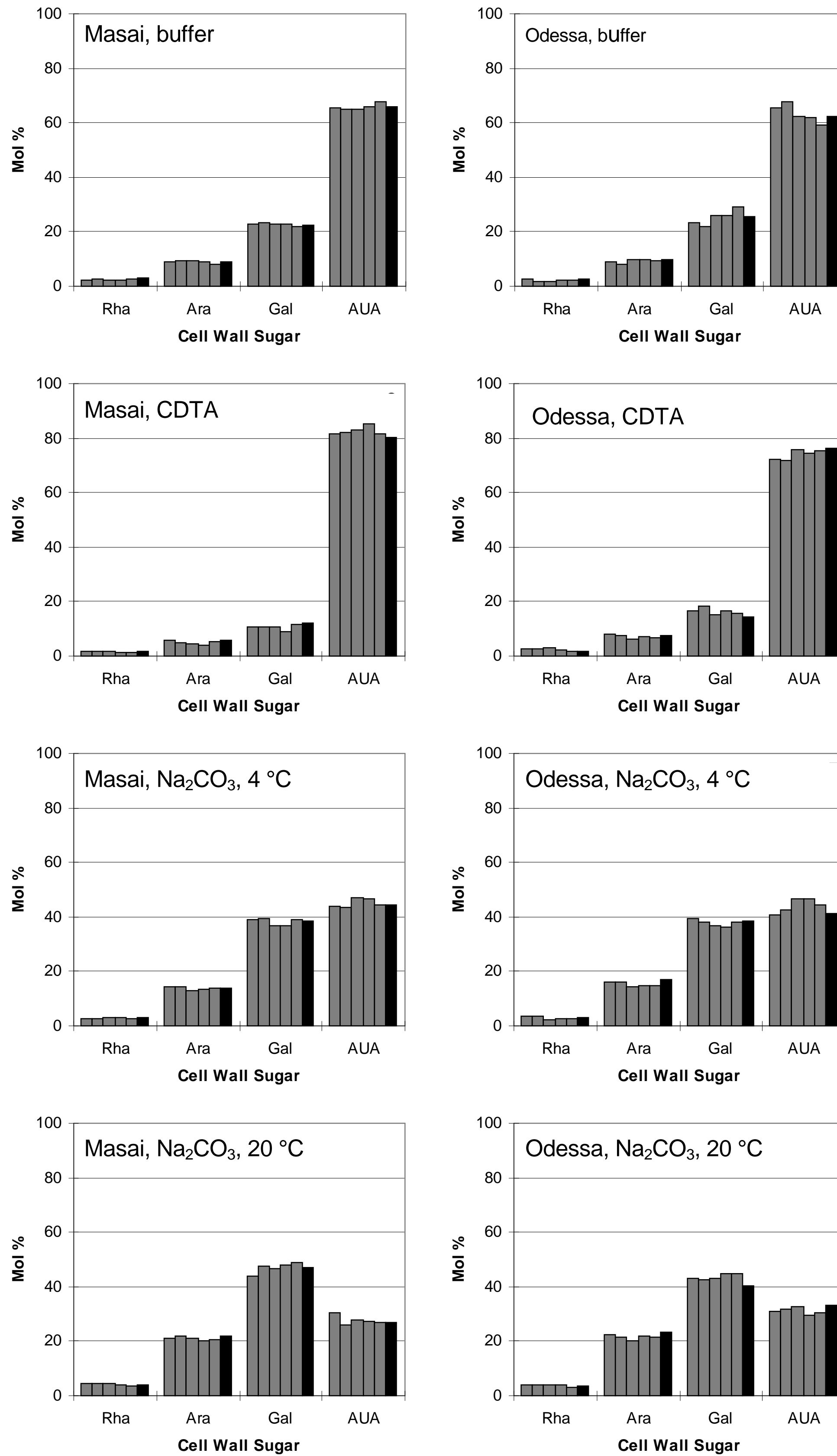

Figure 6.7 *Composition of sequential pectic fractions isolated from green bean cvs. Masai and Odessa after sterilisation with different preheating treatments (: 40 °C, 120 min, :50 °C, 90 min, :60 °C: 45 min, :70 °C: 20 min, :80 °C: 10 min, :90 °C: 4 min) (for preheating conditions see table 6.1).*

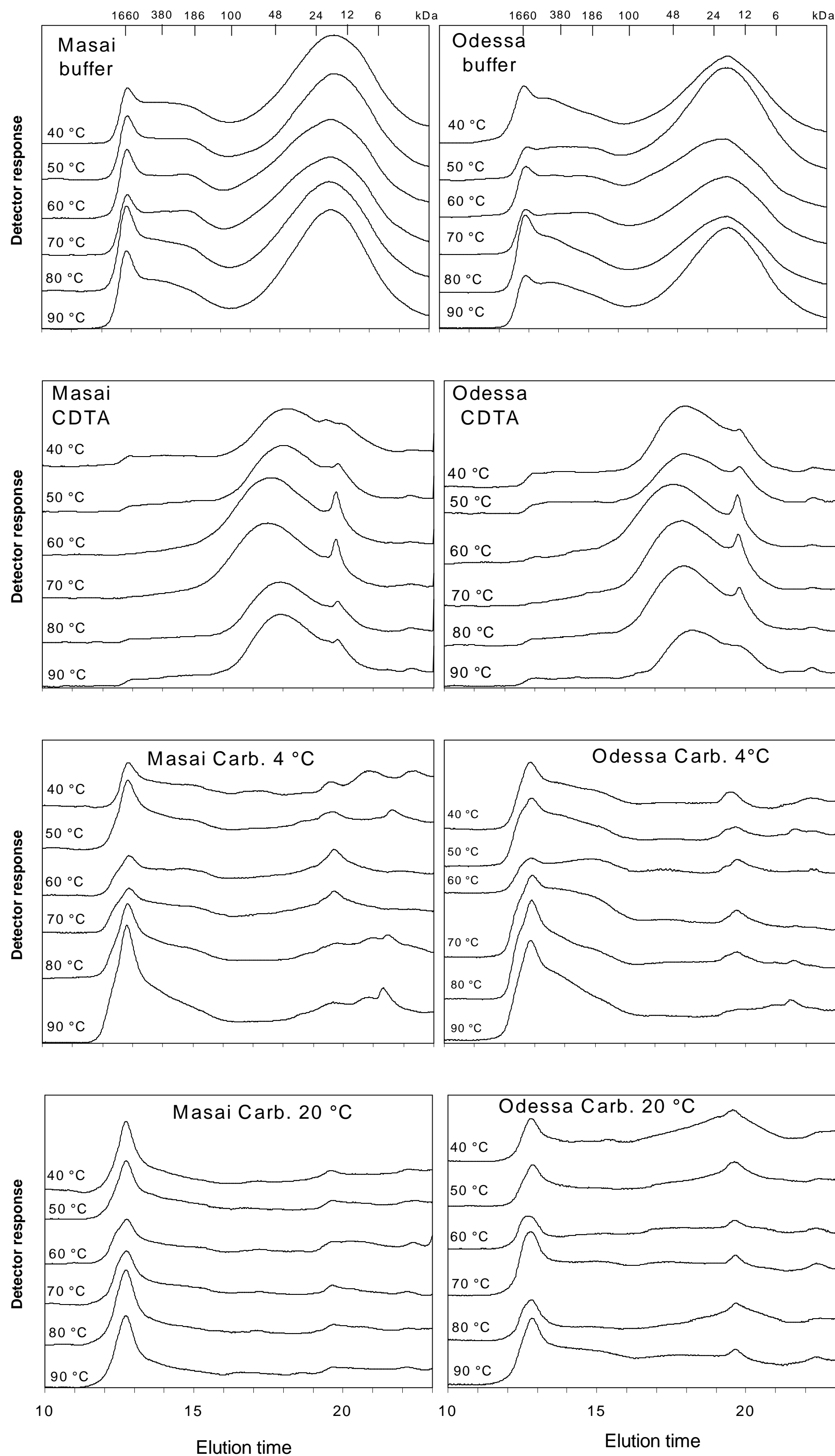

Figure 6.8 *High performance size exclusion chromatography elution patterns of sequential pectic extracts from green bean cvs. Masai and Odessa, after processing with different preheating conditions.*

The increase in M_r of the buffer and CDTA fractions from the beans preheated at 60 °C indicated that less cleavage of the pectic chains has occurred in these samples (Figure 6.8). At the sites were the methylesters are removed the pectin (1) is less heat instable and (2) can form intermolecular complexes with calcium. This is visualised in a diagram in Figure 6.9. Both processes result in a higher firmness retention after sterilisation. Chang et al.[9] also tried to model the texture of green beans in relation to the extractability of cell wall polymers during heat treatments. They as well showed an increased solubility of uronic acid containing polymers during cooking. However, their extraction procedure was highly destructive, since they used both *hot* water and *hot* acid. In addition only little information was provided about the chemical composition of the cell wall polymers. As a result, their model is highly complex, but rather speculative.

The preheating effect was accompanied by an increased yield of buffer and CDTA extracts but had no significant effect on the yield of carbonate extracts. Apparently, the branched pectins, which are recovered predominantly in the carbonate extracts, are flanked by methylated uronic acid residues which are not demethylated by PME during preheating at any of the applied temperatures. This is possibly caused by steric hindrance of PME by the neutral sidechains[30].

A small percentage of the sugars in wall polysaccharides contain ferulic acid and related phenolic side groups[18]. These structures may be cross-linked by either the action of POD and H_2O_2 or polyphenoloxidases to form intercellular bridges like diferulate, thus linking polysaccharides together in a tight network[31]. If there is an enhanced cross-linking of pectins due to POD activity during preheating one could expect that polymers are less easily extracted form the cell wall and consequently that substantially more polymers would be recovered in the carbonate fractions or remain associated with the residue in spite of the buffer or CDTA fraction. More specifically, at least saponification of ester linkages between the phenolic acid derivative and the sugar alcohol is needed to solubilise the cross-linked polymers. Since we measured no significantly increased yields of either carbonate or residue fractions we may deduce that there is very little or zero enhanced pectin cross-linking by POD during preheating. Tyrosine units of extensine, an important structural cell wall protein, however may undergo a similar cross-linking reaction to form isodityrosine cross-links. Extensine is very difficult to extract from the cell wall because isodityrosine cross-links have to be broken. In our study it is most likely associated with the residue in all samples. Therefore we can not exclude that POD has an effect on the texture during preheating by cross-linking of extensines.

Irrespective of the processing conditions, as applied in the present study, cv. Masai appeared firmer as compared with cv. Odessa (Figure 6.2). This was accompanied by a somewhat higher percentage of pectin degradation in beans of cv. Odessa. Possibly firmness is simply related with the overall amount of cell wall material, which is higher for cv. Masai (Table 6.2). A comparable relationship was found for two potato cultivars, which differed with respect to mealiness. Another aspect which might relate to firmness are the properties of the CDTA-soluble pectins. Cv. Masai seems to contain a higher amount of AUA in the CDTA-soluble pectins. Moreover, the DM and especially the DA are much lower in this fraction of cv. Masai. The DM is well known to affect the strength of calcium

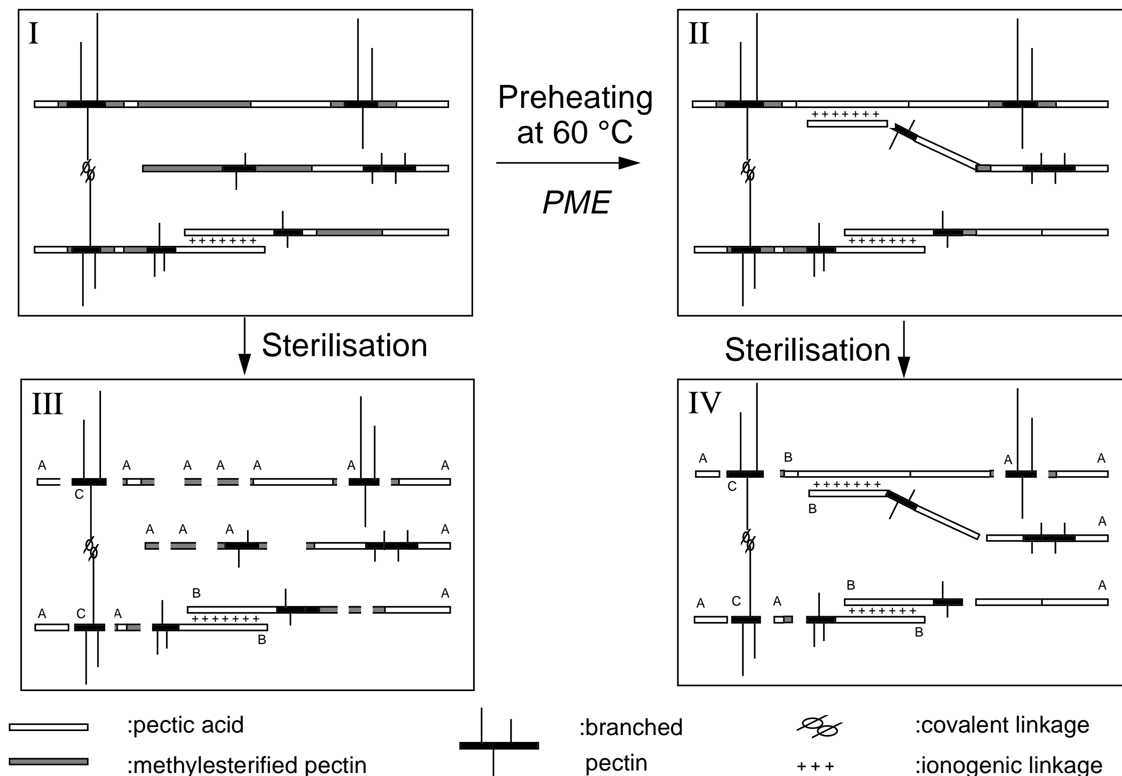

Figure 6.9 *Schematic overview of changes occurring during preheating (II) and sterilisation (III and IV) of fresh green beans (I) in relation to the solubility of pectins. A: buffer-soluble; B: CDTA-soluble; C: Carbonate-soluble/Residue.*

intermediated pectin gels, the normal gel type in cell walls[15]. Methyl groups prevent calcium binding and make the inter-junction segments more flexible. The strength of the calcium gel type depends on the length of the uninterrupted pectate segments that can interact. Acetyl subunits were shown to reduce the binding strength[32]. With this in mind it can be hypothesised that the CDTA-soluble pectins, which reflect more or less the calcium linked pectins, can from stronger, more cohesive, gels in cv. Masai than in cv. Odessa. If CDTA-soluble pectin originates from the middle lamella region, as is often stated, this implies that the cells of cv. Masai may be less easy to separate, resulting in a higher firmness of the tissue.

In conclusion, our results strongly support the general hypothesis that the decrease in softening effect of preheating at moderate temperatures is related with PME. PME is most likely activated during preheating and demethylates specific regions of the pectic polymers, which results in a decreased breakdown of pectin (Figure 6.9).

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Chapter 7

Preheating effects on the textural strength of canned green beans: relating chemical, spectroscopic, and textural properties*.

7.1 Abstract

With variable preheating conditions it was possible to modify the firmness of two green bean cultivars after sterilisation. The products thus obtained were analysed with respect to chemical composition and texture. The texture was measured with sensorial and instrumental analyses. The aim of this study was to define the relations between chemical characteristics of the cell wall material, in particular the amount and structure of cell wall pectins, and the texture of processed green beans. In addition the applicability of spectroscopic techniques was studied. A method based on Fourier Transform Infra-Red spectroscopy (FT-IR) was developed for the chemical characterisation of the pectic fractions. Furthermore, the ability of Near Infra-Red spectroscopy (NIR) to predict the texture and the chemical composition of green beans was investigated. Partial least squares (PLS) regression of chemical and texture data revealed that three significant factors explained 95 % of the variance in texture. From this analysis, it became clear that both instrumental and sensory determined firmness of processed green beans could be related to the cell wall pectins. The DM of buffer and CDTA-extractable pectins and the amount of pectin degradation was negatively correlated with texture. Selected pectic fractions were further investigated by FT-IR. FT-IR proved to be a very promising technique for quantification of the chemical composition of pectines. A FT-IR analysis gave an accurate prediction of the abundance of specific neutral sugars and degree of methylation. NIR spectroscopy proved to be a powerful and rapid technique for assessment and prediction of texture related chemical properties and sensory attributes of green beans.

* Derived from C. Boeriu, T.Stolle-Smits, D. Yuksel and C. van Dijk and C. Boeriu, T.Stolle-Smits and C. Van Dijk, both submitted to J. Agric. Food Chem.

7.2 Introduction

Texture is an important quality attribute of heat processed vegetables like green beans. Texture is a complex parameter, which is affected by a number of factors, including the complexity and dynamics of the plant material and superimposed processing conditions. The sensory perception of texture may be studied by analytical sensory tests, using trained sensory panels and different sensory descriptors to obtain a scaled and quantified analysis of the product[1]. Another approach to study texture is to analyse a physical property related to texture by an objective instrumental measurement, such as the resistance to shear, tensile strength or elasticity[2,3]. The relations between, tissue architecture, objective texture measurements and sensory perceived properties is often poorly characterised or understood. Texture is in fact the external manifestation of micro- and macrostructures resulting from the arrangement of various chemical components by physical forces[4]. To obtain a fundamental understanding of the chemical basis of plant food texture, the complex biological material is characterised by chemical and physical methods. These studies have shown that differences in texture of heat processed plant food materials are caused by variations in tissue architecture and cell wall and middle lamella structure, both (bio)chemical and physical in origin. In some specific cases starch also contributes to textural differences[5-18].

Previous work (Chapter 4, 5) showed that during heat processing of green beans cell wall pectins are degraded due to heat-induced β-elimination[19-27]. Green beans, preheated at moderate temperatures before sterilisation, become less soft than conventionally processed samples[27]. This firming effect of preheating was shown to be related with the demethylation of pectin during preheating, most likely by endogenous pectin methylesterase (PME). This resulted in less pectin degradation due to a decrease in ß-eliminative breakdown of demethylated pectin during further thermal processing (Chapter 6). The aim of this study was to analyse the relations between chemical characteristics of the cell wall material, in particular the amount and structure of cell wall pectins, and the texture of processed green beans. In addition the applicability of spectroscopic techniques was studied. A method based on FT-IR spectroscopy was developed for the chemical characterisation of the pectic fractions. Furthermore, the ability of near infrared spectroscopy (NIR) to predict the texture and the chemical composition of green beans was investigated. The fast and precise analysis method based on NIR finds more and more application for in-line measurement of product properties to optimise process control and as substitute for time consuming laboratory product quality tests[28,29].

7.3 Material and methods

7.3.1 Plant material and processing conditions

Green beans (*P. vulgaris* L.) cvs. Masai and Odessa were grown and processed as is described in Chapter 6. After processing, the samples were stored at 15 °C until analysis.

7.3.2 Texture assessment

– *Sensory evaluation*

The textural characteristics of canned green beans were evaluated by a trained panel of 10 assessors. They rated the firmness of the beans on a line scale anchored on two ends from 'not' to 'very' respectively. The assessors also rated some texture and flavour characteristics on a 3 point ('not', 'detectable', 'present') category scale to check the presence of these characteristics in green beans. These characteristics were 'mealiness', 'fibrousness' and 'graininess' as texture descriptors and 'acidic taste' and 'fishy aroma' as flavour descriptors. At each session all samples of only one cultivar were presented to the assessors in a randomised order. The sessions were repeated 5 times for each cultivar in order to reduce the variance.

– *Instrumental evaluation*

The firmness of the beans was measured using an Instron Universal Testing Machine equipped with a Kramer shear cell as is described in Chapter 6

7.3.3 Cell wall chemistry

The chemical composition of the cell walls of the green beans were analysed as is described in Chapter 6.

7.3.4 Near-infrared reflectance analysis (NIR)

The batches of processed green bean pods were homogenised (Moulinex Masterchef 20) for about 60 seconds prior to NIR analysis. The homogenised samples were packed in standard black cups. Spectra were recorded in the reflectance mode using an InfraAlyzer 500 instrument (Bran and Luebbe), using IDAS software. Measurements were made between at a wavelength range between 1100 to 2500 nm at a 4 nm interval at ambient temperature (20 °C). The detected diffuse reflectances (R) were transformed into apparent absorbencies (log 1/R). The mean spectrum of two repacks for each sample was used for calibration. The spectral data were subjected to multiplicative scatter correction to reduce non-linear scatter effects due to respectively specular reflection, particle size and structure of the sample.

7.3.5 FT-IR spectra

Spectra were obtained on a BIO-RAD Fourier-transformed infrared spectrometer (FTS-60A), equipped with a MTC detector, a DRIFT accessory and a microscope accessory. Spectra were obtained between 4000 and 500 cm^{-1} at a resolution of 4 cm^{-1} in the diffuse reflectance mode, using KBr as reference. 64 intereferograms were co-added for a high signal to noise ratio. Commercially pure pectines, with known degree of methylation (DM), covering the range from 38 % to 76 % DM, were obtained from Sigma. Before measurement, the standard pectins were dissolved in 0.05 M phosphate buffer, pH 7.2, to ionise all the non-esterified carboxyl groups, and subsequently freeze dried.

The spectra were base-line corrected at two points, 1850 and 1500 cm^{-1} . The height and the area of the peaks were determined by peak integration, using the software supplied with the equipment and the

WIN-IR software. The area limits of the peak at 1740 cm^{-1} were set from 1830 cm^{-1} to 1695 cm^{-1} and those of the peak at 1610 cm^{-1} from 1695 to 1570 am^{-1} .

7.3.6 Data analysis

The relationships between (1) sensory variables (Y_1-block) and chemical variables (X_1-block) and (2) texture or chemical variables (Y_2-block) and instrumental (NIR) variables (X_2-block) were studied by a method of Partial Least Squares (PLS) regression on latent variables. PLS regression allows the simultaneous use of strongly interrelated X-variables by focusing the systematic covariances in the X-block into a few latent variables. When only one Y-variable is modelled, the PLS algorithm is non-iterative and is termed PLS1. When several Y-variables are modelled simultaneously, the PLS algorithm is iterative and is called PLS2. The models were validated by using the cross-validation technique, to ensure predictive validity, guarding against over - fitting. The accuracy of the calibrations was expressed by the Standard Error of Prediction (SEP), calculated as the square root of the squared differences between predicted and reference values. The predictive ability of the model is described by:

a) Root-Mean-Square error of Prediction, RMSP, defined for Y variable number i by:

$$RMSP^2 = [\, \sum^{n}_{i=1} (y_i - \bar{y}_i)^2 \,] / n$$

where n is the number of samples used in the validation;

b) Relative Ability of Prediction, RAP:

$$RAP = [\, s_{tot}^{\;2} - RMSP^2 \,] / [\, s_{tot}^{\;2} - s_{ref}^{\;2} \,]$$

were s_{tot}^2 is the total standard deviation of the reference y data, and s_{ref}^2 is the noise level in the reference data themselves.

All the statistical analysis were executed using the chemometric software The Unscrambler version 6.1, CAMO A/S, Trondheim, Norway.

7.4 Results

7.4.1 Texture evaluation

Texture is a sensory sensation and can best be analysed by using analytical sensory panels. However, when consequences of processing are investigated the texture of the raw product as well as the texture at various stages during the process has to be quantified, which is not feasible with sensory panels. For this practical reason we studied the relations between the sensory perceived ‘firmness’ and an instrumentally measured firmness (‘Instron’).

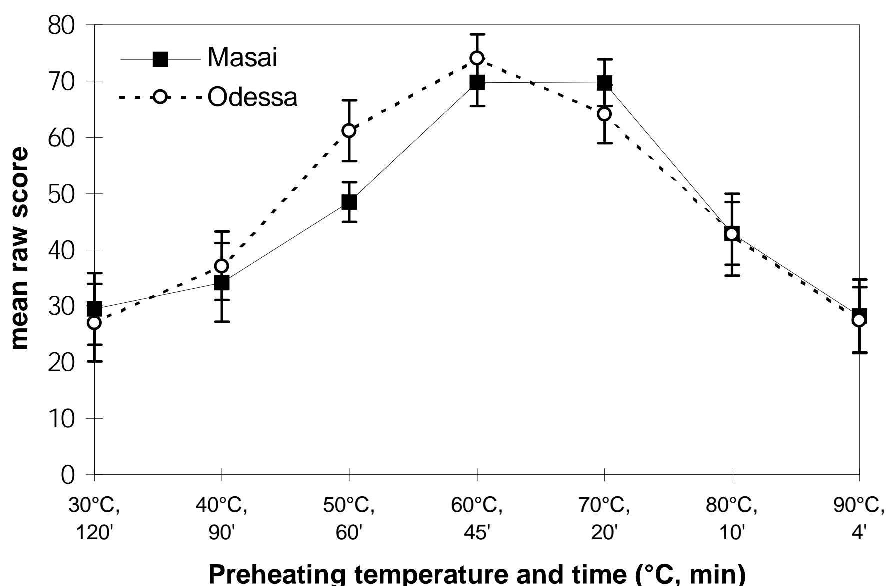


Figure 7.1 *Mean sensory scores for 'firmness' of sterilised green beans of cv. Masai and cv. Odessa.*

– *Sensory evaluation*

Figure 7.1 shows the effect of the preheating conditions on the change in the firmness of sterilised green beans of cv. Masai and cv. Odessa. Green beans blanched at 60 °C for 45 min. had the highest firmness score for both bean cultivars. Lowest firmness scores were obtained at both the low and high temperature end. The beans of cv. Odessa blanched at 50 °C had a clear 'fishy' off-flavour. This off-flavour is likely to be formed by degradation of unsaturated fatty acids by the enzyme lipoxygenase, which is well known to form off-flavours in legumes[30]. Linoleic and linolenic acid are the precursors of volatile short chain aldehydes and of both volatile and non-volatile longer chain aldehydes[30]. Other textural and aroma aspects were not detected in green beans of both cultivars.

– *Instrumental evaluation*

Beans of cv. Masai had a higher instrumental firmness than beans of cv. Odessa after any processing condition as shown in the previous Chapter (Figure 6.2, Chapter 6). Maximal instrumental firmness values after sterilisation were obtained for the beans of both cultivars after preheating at 60 °C. The correlation between the instrumental and sensory evaluation of 'firmness' was very high ($R^2 > 0.95$) for both cvs. as is shown in Figure 7.2. This makes this instrumental method very suitable to quantify the firmness of green beans in a fast and reliable way, with reference to sensory analyses.

The linear regression line obtained for cv. Masai was different than for cv. Odessa. However, sensory analysis showed no significant differences between the two cvs (Figure 7.1). This difference between the two analysis methods may be caused by the fact that the two cvs. were analysed by the

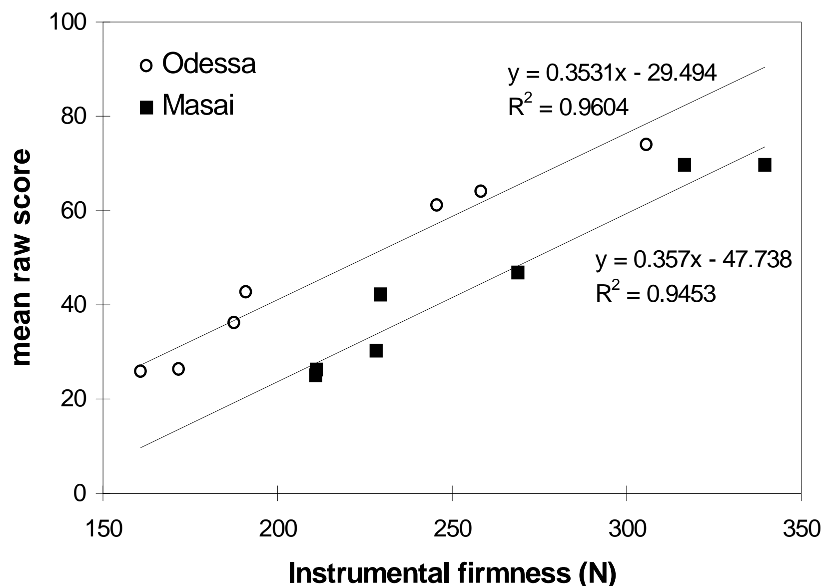


Figure 7.2 *Correlation between sensory (mean raw score) and instrumental evaluation of texture for green bean cvs. Masai and Odessa.*

sensory panel in separate sessions and therefore could not be treated as one data set. This would imply that the sensory method is less objective than the instrumental method. However, it cannot be excluded that the sensory assessed firmness of both cvs. is indeed more comparable than the instrumental measured firmness, since both methods are basically very different and therefore different product properties are probably measured by both methods.

7.4.2 Chemical characterisation of AIR and constituting pectins

The cell wall sugar composition, molecular mass distribution, DM and acetylation and properties of various purified pectic fractions are reported in Chapter 6 of this thesis.

7.4.3 Correlation between the chemical properties of pectins and texture of processed green beans

The relations between perceived and instrumental determined firmness of green beans on one hand and the chemical parameters on the other hand was investigated by a multivariate data analysis technique. The cross-validated PLS2 regression of the two texture Y variables (instrumental firmness, = 'Instron', and sensory 'firmness') versus 26 chemical X variables revealed three significant factors with which 95 % of the total variance in the texture variables was explained (Table 7.1). As can be seen from Table 7.1, less than 4 % of the variance in the Y variables was explained by the three last factors; the main contribution to these factors came from the relations between the rest-variance in the X matrix. From the chemical variables, few (i.e. phAIR, carb1, res, and bufDA) were not well explained by the model. This may be due to either a higher error in the chemical analysis or absence of any relation.

The 'Instron' firmness was most explained by the first PLS factor, while the sensory perceived 'firmness' was most explained by the second factor. The first two factors were most important with respect to reflecting basic variations in the material, due to preheating effects during processing, as already indicated in the input data. These factors will therefore be discussed in more detail. Factor

loadings of the different variables from factor 1 and 2 are plotted against each other in Figure.7.3. Explained variances are shown in Table 7.1. Factor 1 mainly represented the instrumental texture variation. CDTA and buffer soluble pectins contributed most strongly to this factor, followed by carbonate, bufDA and the overall DM after preheating. In the second PLS factor, sensorial 'firmness', the degree of esterification (methylation and acetylation) of both CDTA and buffer fractions, and the total galacturonic acid liberated into the brine due to pectin degradation ('GalAs'), contributed the most. From these results, it was obvious that both instrumental and sensory firmness of processed green beans can be related to the cell wall pectins. The DM of buffer and CDTA-extractable pectins was negatively correlated with texture; this implies that batches characterised by pectins with high DM are relatively less firm, while batches with low DM are relatively firm. In figure 7.4 the distribution of the batches of green beans for the first two factor loadings is shown. Confidence in the ability of the different chemical analysis to predict the sensory determined 'firmness' can be obtained by interpreting each batch in correspondence with the loading plots and checking the results with the raw data. The batches blanched at 60 and 70^{0}C have positive scores for factor 1. This indicates that these samples have a high content CDTA and buffer soluble pectin, with a lower DM and DA than average, reflected in higher firmness. The opposite was observed for samples blanched at extreme temperatures (lower and higher temperatures). Thus, as blanching temperature increased from 30 to 70^{0}C, the firmness of green beans increased, most likely due to demethylation of pectin by PME, which was activated at these temperatures. At temperatures higher than 70^{0}C, this effect is diminished due to fast enzyme inactivation at higher temperatures. Both the sensory as well as the Instron determined 'firmness' are well predicted by the chemical data (Figure 7.5)

Table 7.1 *Results from the PLS regression used to study relationships between the texture (Y block) and chemical (X block) variables. Percent explained variances for the first main factors are given.*

	% Explained variances after factor			
Variables	1	2	3	6
firmness, Instron	82.1	89.2	95.4	99.1
firmness, sensory	42.4	93.7	94.0	96.7
Total explained (Y block)	62.2	91.7	95.4	97.9
Total explained (X block)	40.8	66.3	71.1	84.8

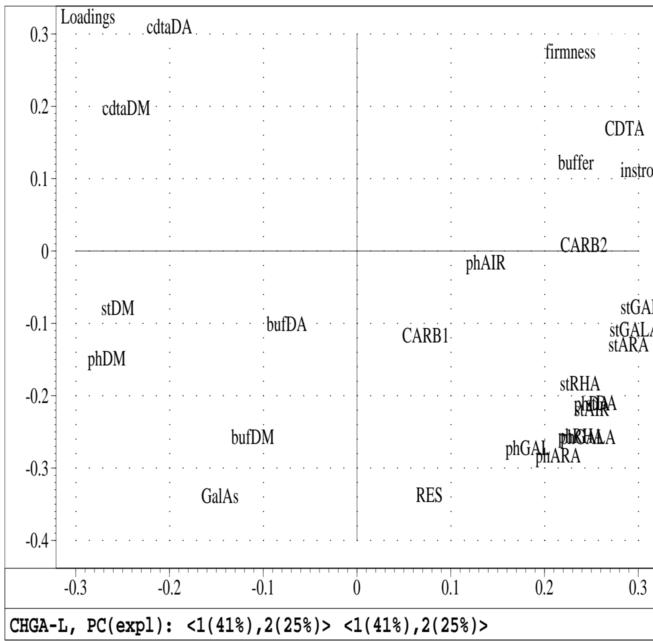


Figure 7.3 *Description of relationships between two texture and 26 chemical variables: PLS loadings for factor 1 and 2.*

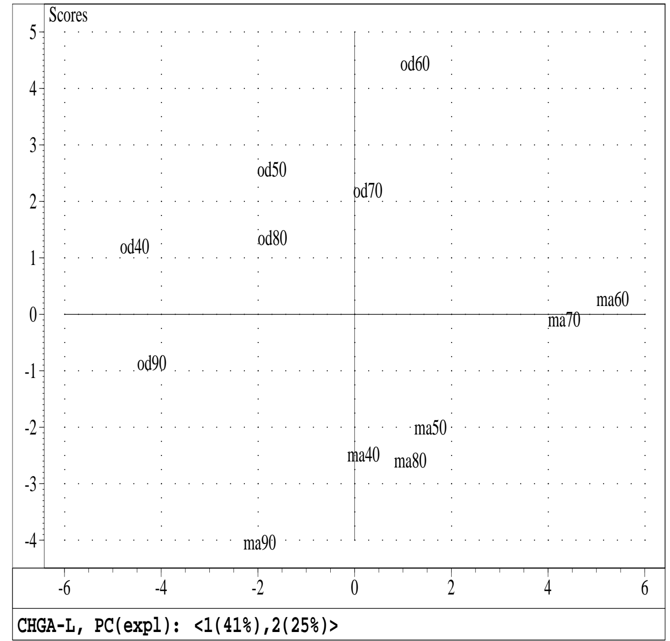
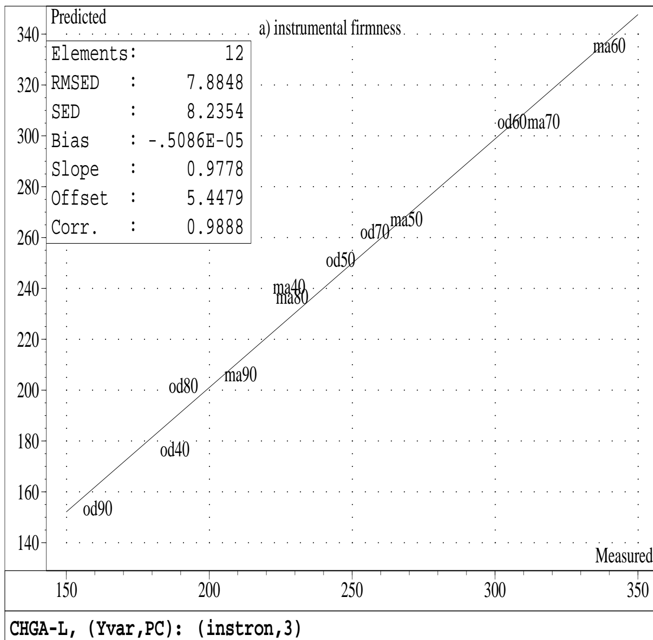


Figure 7.4 *Description of the relative position of the green beans batches to the factors: PLS scores for factors 1 and 2*



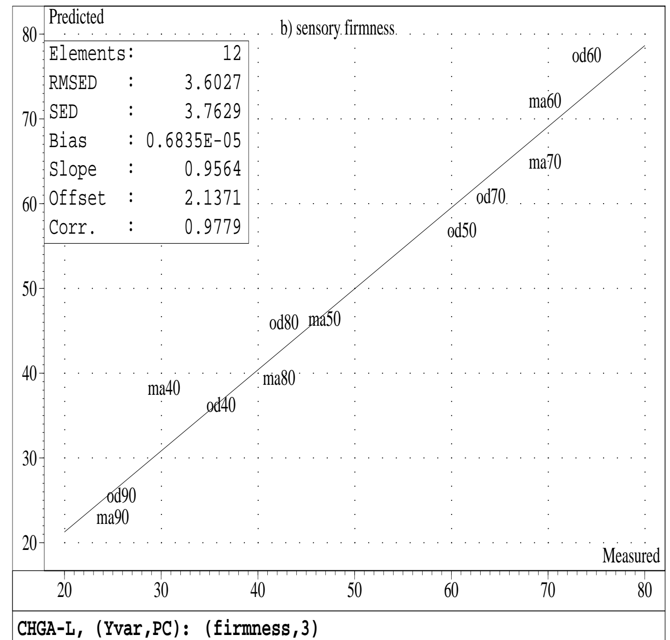


Figure 7.5 *Plot of predicted firmness using chemical data versus instrumentally and sensorial determined firmness of processed green beans.*

7.4.4 Fourier transform infrared spectroscopy (FT-IR)

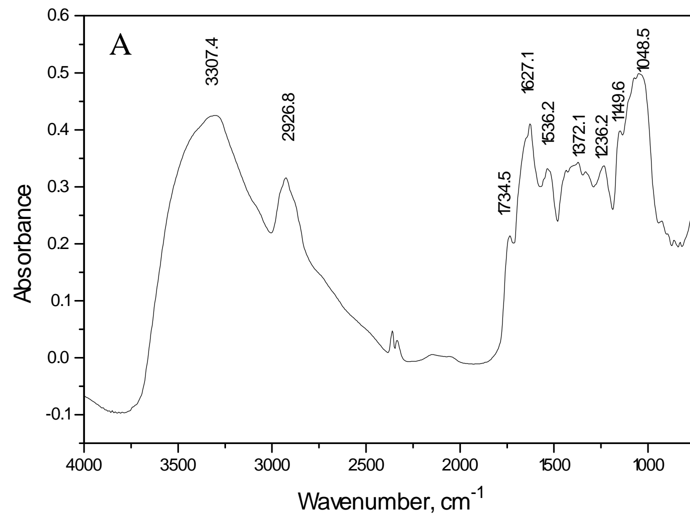
FT-IR spectroscopy has been applied to cell wall material isolated from green beans after different steps of processing (i.e. preheating and sterilisation) and to commercially pectins. The FT-IR spectra provide information in several spectral regions. In the high frequency range, all the spectra have common features, showing bands at 3300-3570 cm^{-1}, corresponding to O-H stretching vibration, and 2900 cm^{-1}, corresponding to C-H stretching vibrations. The most interesting region of the spectrum ranges between 2000 - 900 cm^{-1}. In this region, the following specific absorbencies are present: the C=O stretching in carboxyl and carboxylic ester groups at approximately 1740 cm^{-1}, the absorption of free (1650 cm^{-1}) and associated water (1600 cm^{-1}), the symmetric (1360 - 1440 cm^{-1}) and asymmetric (1550 - 1650 cm^{-1}) stretching vibrations of carboxylate ion, phenolic absorptions between 1600 and 1500 cm^{-1} and absorptions of carbohydrates in the frequency range between 1200 to 900 cm^{-1} [31]. The absorption bands in the ‘fingerprint’ region (1500-900 cm^{-1}) cannot unambiguously be assigned to particular vibration modes due to many complex vibrations that overlap in this region[31]. Therefore, the spectral characteristics of the cell wall pectins will be discussed without identifying every particular vibration that gives rise to the spectrum. The chemical changes of the cell wall pectines were monitored from the FT-IR spectra mainly in relation to the carboxyl ester/carboxylate absorptions.

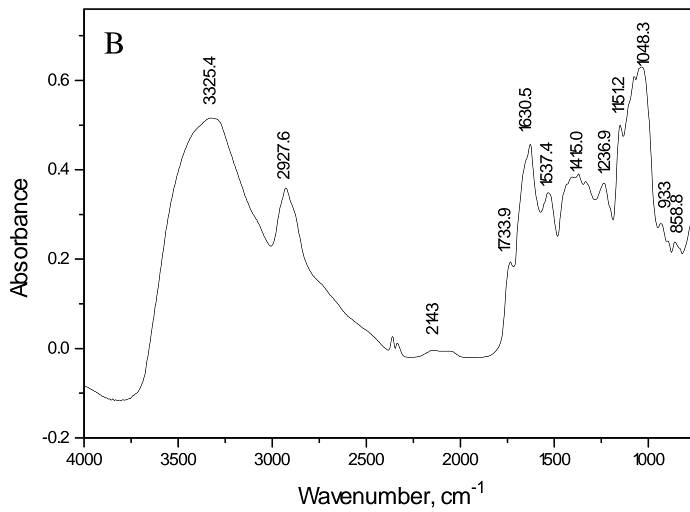
– *Spectra of AIR samples*

FT-IR spectra of the Alcohol Insoluble Residue (AIR) obtained from fresh green beans, from batches of green beans pre-heated at different temperatures according to the scheme presented in table 6.1 and from batches of green beans after sterilisation are presented in Figure 7.6. All the absorptions mentioned above can be detected in the spectra of AIR. In addition, the amide stretching bands of protein at 1650 cm^{-1} is seen in the spectrum. Since AIR is a mixture of polymers, including cell wall pectines, proteins and other alcohol unextractable polysaccharides, such as starch and cellulose, the spectrum obtained represents a summation of all the absorptions of the polymeric constituents. The FT-IR spectra of all AIR samples showed similar characteristic features, regardless the cultivar and the subjected heat treatment.

– *FT-IR of pectic fractions*

FT-IR spectra of extracted pectic polymers (buffer, CDTA, Na_2CO_3 at 4°C and Na_2CO_3 at 20°C) had a number of common features, regardless of the green bean variety. The carbohydrate region, 1200 - 900 cm^{-1}, showed little variation in the different pectic fractions, due to the fact that most of the bonds absorbing in this spectral region (i.e. C-H, O-H, C-C) are common to all sugar molecules (Figure 7.7). The specific absorptions of carbohydrates at 890, 954, 1020, 1050, 1078, 1150 and 1230 cm^{-1} were present in all spectra. The pectin extracted with Na_2CO_3 at 20°C lacks the absorption 954 cm^{-1}. The absorption at 1020 cm^{-1} appeared as a shoulder of the more intense absorption at 1050 cm^{-1}. In the various purified polymers, distinguishable groups are enriched, as seen by the change in the intensity and in the intensity ratios of specific absorptions. However, a positive assignment of these spectral bands to specific groups or sugar molecules is very difficult. The similar shape of IR-spectra of pectic polymers in the carbohydrate region indicates that there are no





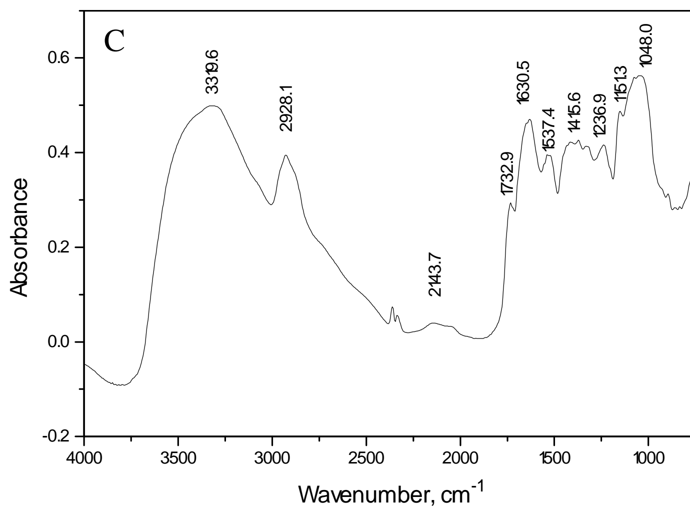


Figure 7.6
FT-IR spectra of AIR from green beans cv. Masai: (a) fresh, (b) blanched at 60°C for 60 minutes, and (c) after sterilisation.

significant differences in the types of sugar present. Differences in ratios of the peaks however indicate that there are differences in the concentrations of specific sugars in different pectic fractions. Comparing the IR spectra of pure carbohydrates (arabinose, galactose, rhamnose, glucose) and pure galacturonic acid and polygalacturonic acid (data not shown) with the spectra of the isolated pectines, it could be seen that the ratio of the intensities of the peaks at 1047-1050 cm^{-1}, characteristic for galactose, and 1015-1020 cm^{-1}, specific for galacturonic acid, varied in different pectin fractions. For example, in the buffer and CDTA extractable pectines, the ratio A_{1050}/A_{1020} was lower than 1, while in the carbonate extracted fractions the ratio A_{1050}/A_{1020} was higher than 1. This indicated that the carbonate extracted pectines contain higher amounts of galactose than the buffer- and CDTA extracted pectines. This is in consensus with the results of the chemical characterisation of pectins presented in Chapter 6, were significant differences in the ratios of sugars in the successive pectic fractions were found (Figure 6.7).

A comparison of the carbohydrate spectral region in spectra of similar pectic fractions from different green bean cultivars (cv. Masai and cv. Odessa) showed a similar trend. The carbohydrate specific absorption bands are all present, but their intensity varied. For example, in the spectra of the CDTA soluble pectins isolated from cell wall material of cvs. Masai and Odessa, the ratio of the intensities of the peaks at 1100 cm^{-1} and 1075 cm^{-1} (A_{1100}/A_{1075}) and those of the peaks at 1050 cm^{-1} and 1020 cm^{-1} (A_{1050}/A_{1020}) were different. This indicates that the ratio in which these sugars are present in the cell wall polymer may vary from one cultivar to another. Clear differences between the spectra of cell wall pectines are observed in the spectral region 1300 - 1800 cm^{-1}. Several absorptions are present in the region 1300 - 1400 cm^{-1}, but with the exception of the peak at 1402 - 1405 cm^{-1}, they cannot be assigned to individual groups. The absorption at 1402 - 1405 cm^{-1}, which is present as a medium intense peak in the spectra of CDTA and carbonate extractable polymers, and as a weak band in the spectrum of buffer soluble pectin, can be identified with the symmetric stretching of carboxylate ion, COO^-. The asymmetric stretching of carboxylate ion is found, in the spectra of buffer and CDTA soluble pectin at 1610 cm^{-1}, while in the spectra of the carbonate extracted pectines, carboxylate absorption is at 1650 cm^{-1}, corresponding to a shift of 40 cm^{-1} to higher frequencies. This shift can be due to an increased hydrophylicity and stronger interaction with water of the completely de-esterified pectin, whose carboxyl groups are totally ionised at the pH of carbonate extraction. It should be mentioned that in this spectral region, also the absorption of free (1650 cm^{-1}) and bound (1600 cm^{-1}) water can be found, and the carboxylate absorption at 1610 cm^{-1} and 1650 cm^{-1} respectively, include also the water contribution. Recently the interaction of water with polymers has attracted much attention[5]. A positive correlation was found between the formation of ionic cross-linking of low methyl pectin and the amount of bound water within the pectic polymers in firm tissue of green bean pods.

A high intensity absorption peak at 1740 cm^{-1} was seen in the spectra of both buffer- and CDTA-extracted pectines. Theoretically, this absorption reflects the contribution of the carbonyl stretching vibration in both the non-ionised (-COOH) and the esterified ($-COOCH_3$) carboxyl group. An additional argument for this assignment was the fact that the peak at 1740 cm^{-1} can be identified in the spectra of polygalacturonic acid ($\nu_{C=O}$, in free -COOH = 1740-1744 cm^{-1}) and of a highly

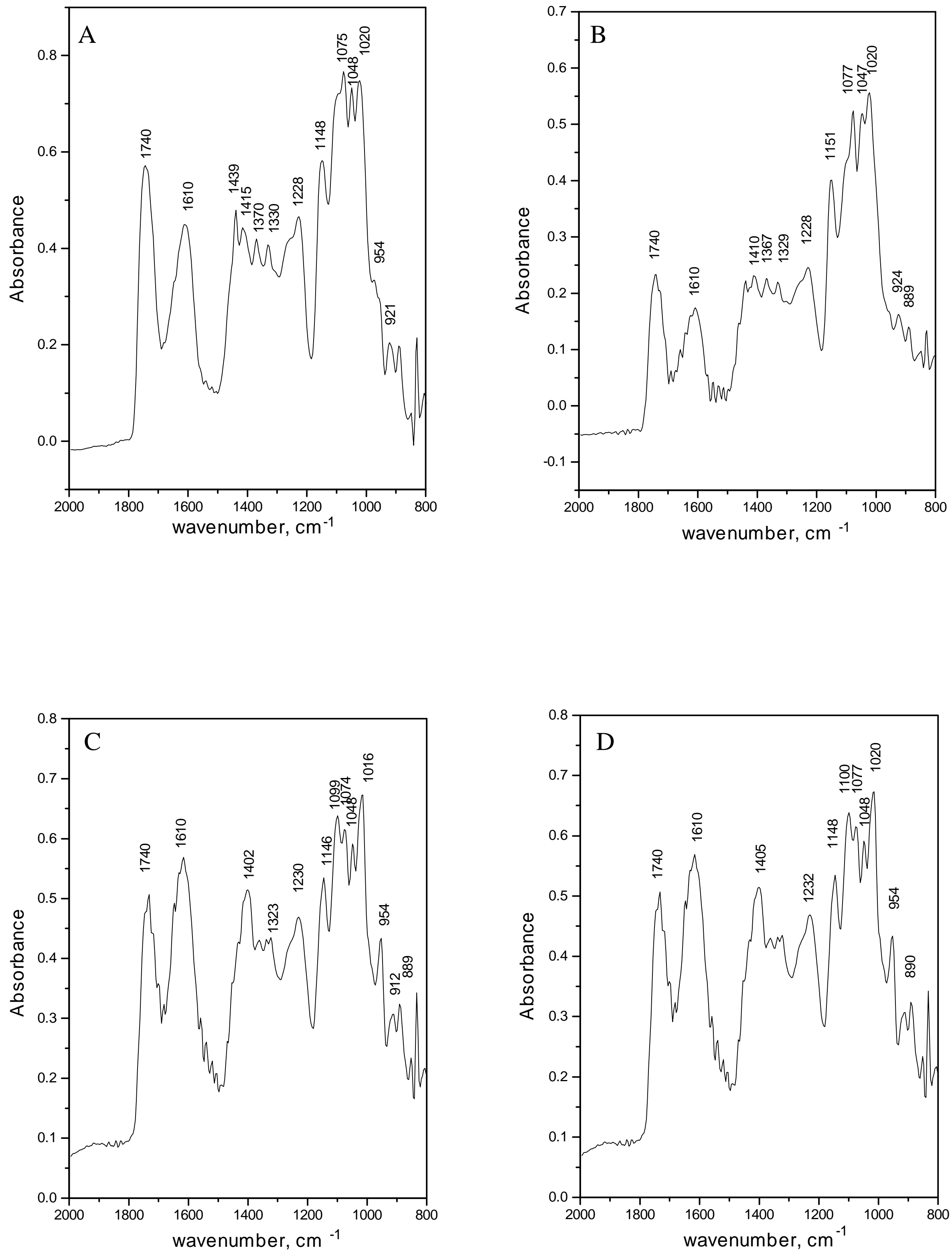
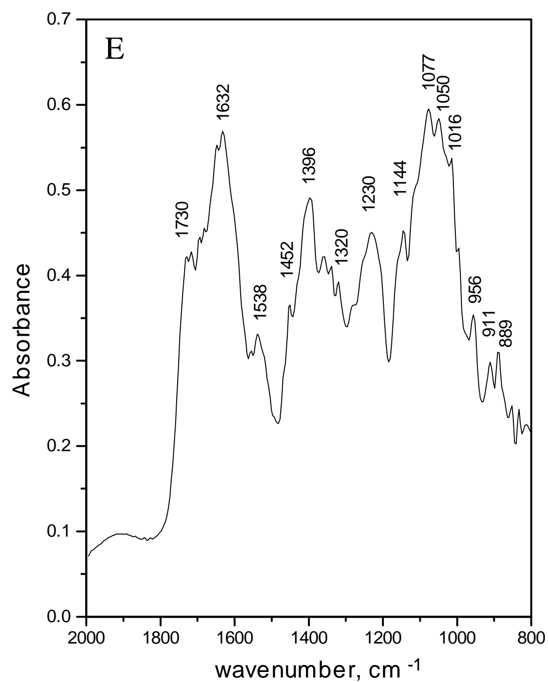
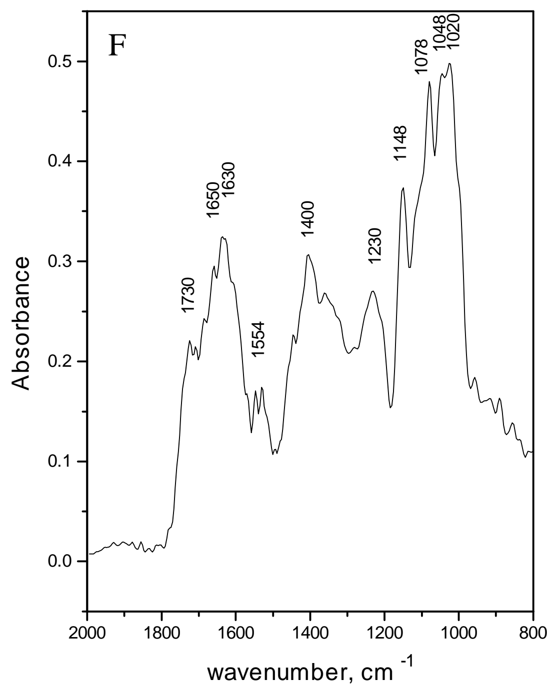
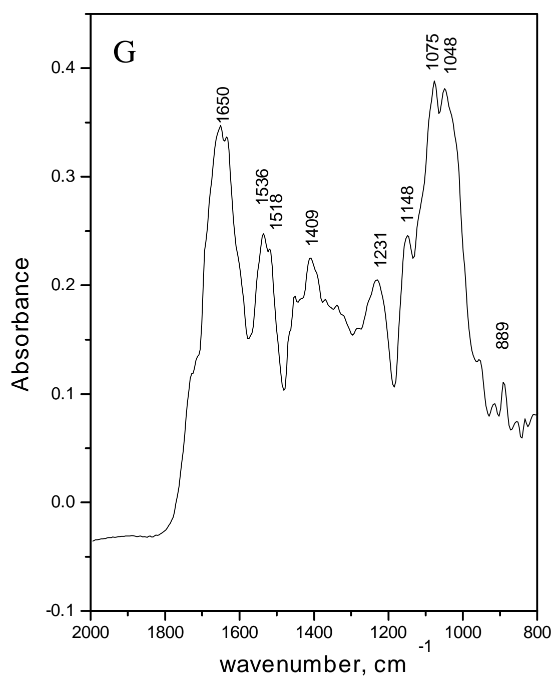
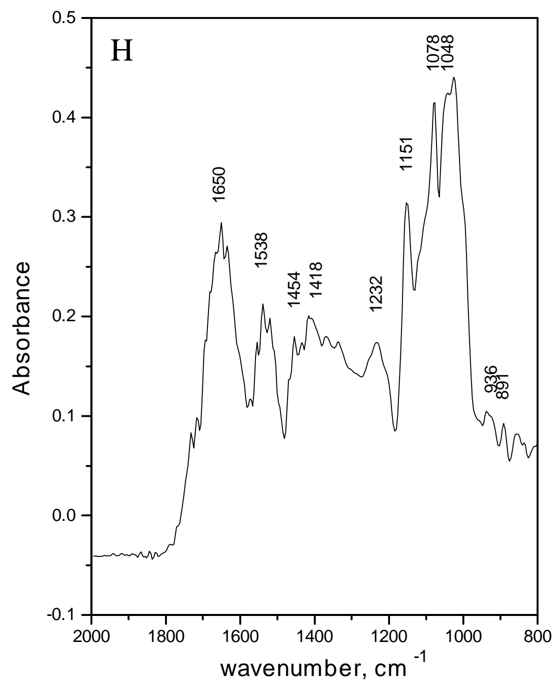


Figure 7.7 *FT-IR spectra of pectic fractions: buffer soluble pectin, cv. Masai (A) and cv. Odessa (B); CDTA soluble pectin, cv. Masai (C) and cv. Odessa (D); pectin extracted with*









Na_2CO_3, at 4° C, cv. Masai (E) and cv. Odessa (F); and pectin extracted with Na_2CO_3, at 20° C, cv. Masai (G) and cv. Odessa (H).

methylated pectin ($\nu_{C=O}$ in -$COOCH_3$ = 1740 cm^{-1}), (data not shown). However, in the case of both buffer and CDTA-extracted pectines, the absorption at 1740 cm^{-1} could unambiguously be attributed to the stretching vibration of the carbonyl in the esterified group, since at the pH of the extraction (pH = 4.7 for the buffer fraction and pH = 6.5 for the CDTA fraction, respectively), all the non-esterified carboxylic groups of the polygalacturonic chain, with a pKa = 3.2[32] will be completely ionised. For a simple and fast comparison between the DM of the buffer and CDTA soluble fractions, the ratio of the absorptions at 1740 cm^{-1} (band A) and 1610 cm^{-1} (band B) which reflects the proportion of esterified vs. non-esterified carboxylate function, can be used. The buffer soluble fraction seemed highly esterified, as could be concluded from the carboxylic ester peak at 1740 cm^{-1} and the ratio A_{1740}/A_{1610} = 1,38 (carboxyl ester/carboxylate). Both the carboxylic ester absorption and the absorption ratio (A_{1740}/A_{1610} = 0.88) were lower in the CDTA soluble pectin, indicating that the CDTA pectin was less esterified and seemed to contain more free carboxylate groups than the buffer extractable pectin. A comparison between the spectra of buffer and CDTA soluble fractions extracted from two different cultivars, showed that the buffer extracted pectins cv. Masai, most likely had a slightly higher DM than the corresponding fraction from cv. Odessa (Table 7.2). There seemed to be no significant difference between the DE of the CDTA soluble pectin from both cultivars. The ester peak at 1740 cm^{-1} decreased significantly in the carbonate extracted (4°C) pectin, showing that during the extraction, the uronic ester groups are hydrolysed. However, a weak absorption at 1720-1730 cm^{-1} could still be seen in the spectrum, as a shoulder of the intense absorption at 1650 cm^{-1}. This absorption could be assigned to the carbonyl stretching vibration in the esterified (acetylated) OH-groups in the pectin, by comparison with the similar vibration found in the spectrum of penta-acetyl glucose (data not shown). In addition ferulic acid esters might be present, linked to the side chains of the pectic polymers. The pectin extracted with carbonate at room temperature was completely de-esterified, as it can be seen by the lack of any absorption in the range 1700-1750 cm^{-1}, characteristic for C=O stretching in esters. This was also reported by McCann and coworkers[31].

Table 7.2 *Ratio of A_{1740} and A_{1610} in FT-IR spectrum of selected buffer and CDTA soluble pectins extracted from pods of cv. Masai and Odessa.*

Sample	A_{1740}/A_{1610}
Buffer soluble pectin, cv. Masai (40 °C)	1.274
Buffer soluble pectin, cv. Odessa (40 °C)	1.388
CDTA soluble pectin, cv. Masai (40 °C)	0.888
CDTA soluble pectin, cv. Odessa (40 °C)	0.880

– *Determination of the DM of pectines based on FT-IR spectra*

The FT-IR spectroscopic data can be used to monitor the structural and chemical changes of pectines during processing. Since the DM of pectin fractions appeared to be a very important parameter in determining the texture of processed green beans, an attempt was made to develop a method based on FT-IR for its quantification.

Since the DM is defined as : [number of esterified carboxyl groups/ total number of carboxyl groups]x100, it follows that the ratio between the area of the peak at 1740 cm^{-1} over the sum of the absorptions at 1740 cm^{-1} and 1610 cm^{-1} should be proportional to the DM of the sample. In order to use these spectroscopic data to determine the DM of a sample, a calibration curve was developed relating the ratio of the areas at the indicated IR frequencies with the DM of pure pectines (Figure 7.8). The equation of the calibration curve, which had a correlation coefficient of 0.97, was used to determine the DM of unknown samples. The standard error in the determination of the DM of a pure pectin using this calibration curve equalled 1. Using this calibration curve, it was possible to determine the DM of pectins of processed green beans. The FT-IR method developed was applied to evaluate the DM of pectins from green beans cv. Masai and Odessa after processing (Figure 7.9). Significant changes occurred in the carboxylic spectral region, from 1850 to 1550 cm^{-1}.

The results for the DM of the buffer fraction generally resemble the results of the chemical determination of the DM (Chapter 6, Figure 6.6) However, the calculated DM for the CDTA fractions was significantly lower using the chemical method, being 25 - 35 %, in comparison with the FT-IR method. It should however kept in mind that these pectic fractions are not very pure and contained also protein and residual CDTA (CDTA fraction), which affect the spectrum in the regions important for DM determination. FT-IR is a very promising technique for quantification of chemical composition of pectines. The results for impure fractions can be improved by using better data analysis methods, such as PLS, and calibration with comparable pectic fractions.

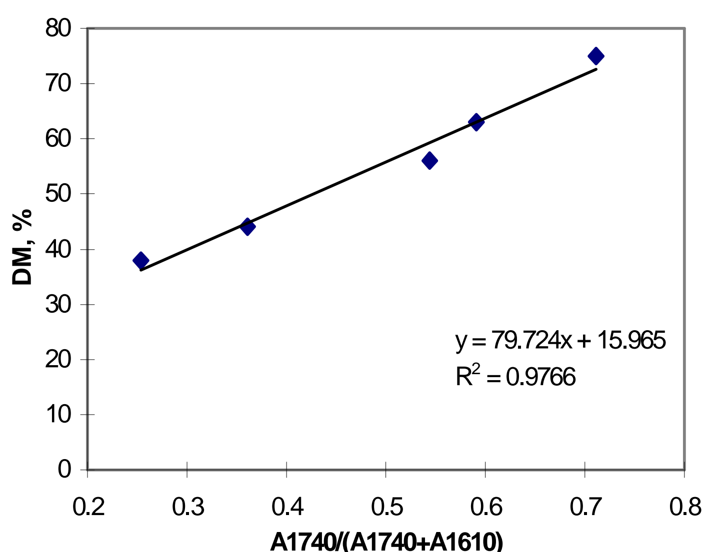


Figure 7.8 *Calibration graph: area ratio A/A+B of the peaks at A (1740 cm^{-1}) and B (1610 cm^{-1}) of a series of standard pectines as a function of their degree of methylation.*

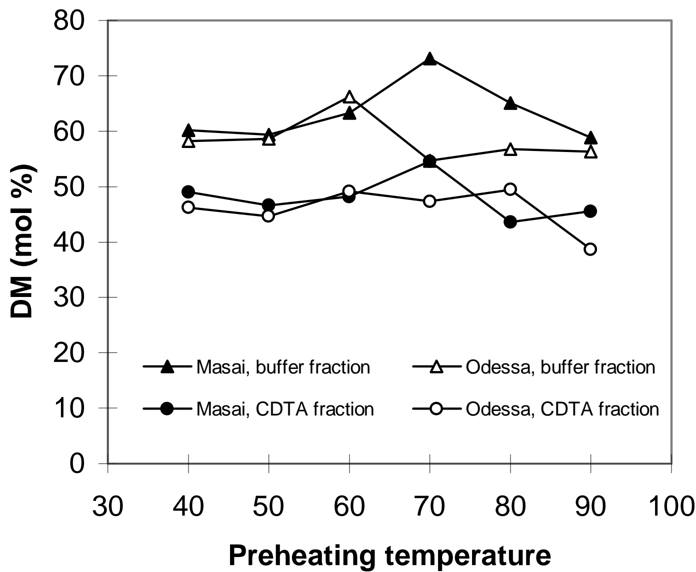


Figure 7.9 *DM of buffer- and CDTA-soluble pectic fractions from green beans after sterilisation as determined with FT-IR.*

7.4.5 Predicting chemical composition and texture of green beans from NIR measurements

The NIR spectrum contains information about the chemical composition of a sample, which determines the amount of near-infrared absorption. The information in a NIR spectrum is however non-selective because of interference of strongly overlapping constituents. The essential relationships between NIR-absorbance spectrum and texture and chemical variables were studied by multivariate regression analysis, as described in the experimental part. In the present study there were only 12 samples, this is very little to perform adequate modelling of the data. The study should only be regarded as a first attempt to study the applicability of NIR spectroscopy in vegetable processing, with emphasis on green beans.

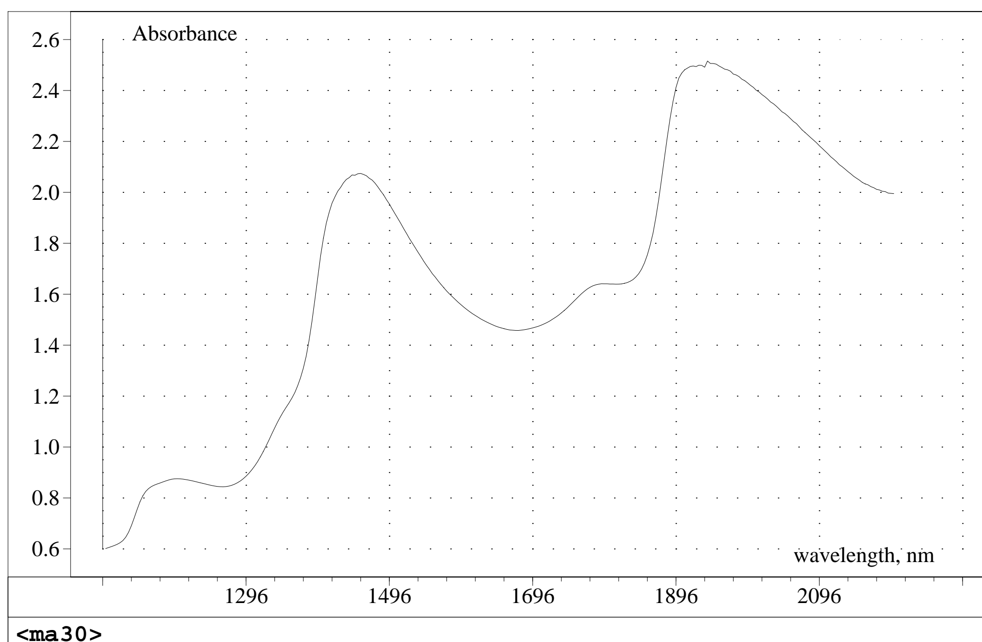


Figure 7.10 *NIR spectrum of sterilised green beans.*

An example of a NIR spectrum for a green bean sample is shown in Figure 7.10. The spectrum showed high absorbency, due to the high levels of water. NIR spectra have peaks at absorption bands for water (1450 nm and 1940 nm). Other absorptions of interest in the spectra may be in the 1520-1570 and 2100-2120 nm areas, in which there are absorption bands for carbohydrates. Other regions of interest, due to absorption of (de)esterified pectins are: 1120-1170 and 1680-1740 nm (C-H stretch in methyl and methylene groups) and 1780-2080nm.

Results from a PLS2 analysis of different chemical variables vs. NIR variables covering the spectral range 1100 nm to 2300 nm are shown in Figure 7.11 in terms of 'relative ability of prediction', RAP. Optimal prediction was obtained after 5 factors. Considering the wide range of variability in the samples, some chemical data seem to be well predicted by NIR. The more texture related compounds, such as CDTA extractable pectins, were found to be well predicted by NIR

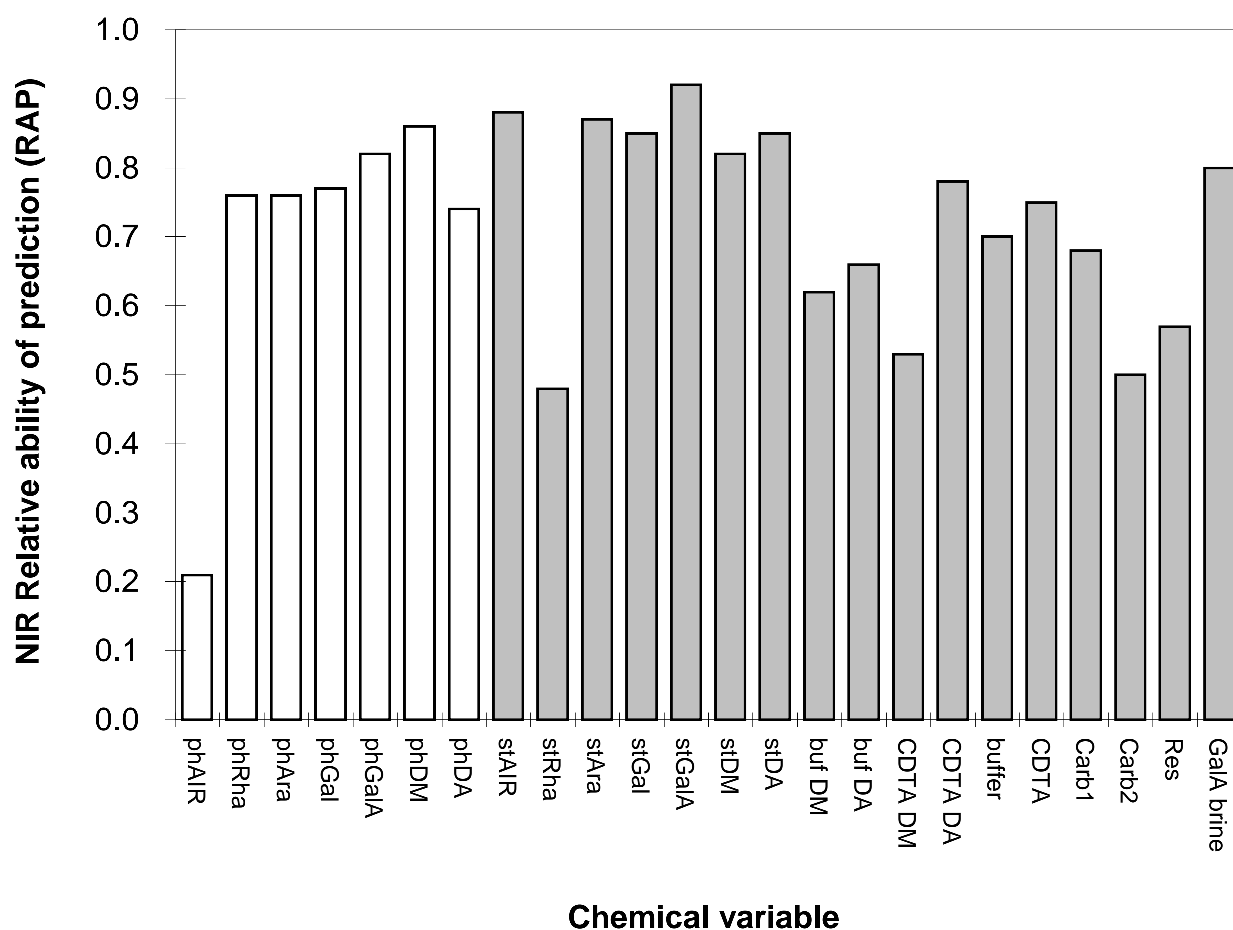


Figure 7.11 *The NIR relative ability of prediction (RAP) of chemical variables (NIR spectrum recorded after sterilisation; white: variables after preheating; grey: variables after sterilisation)*

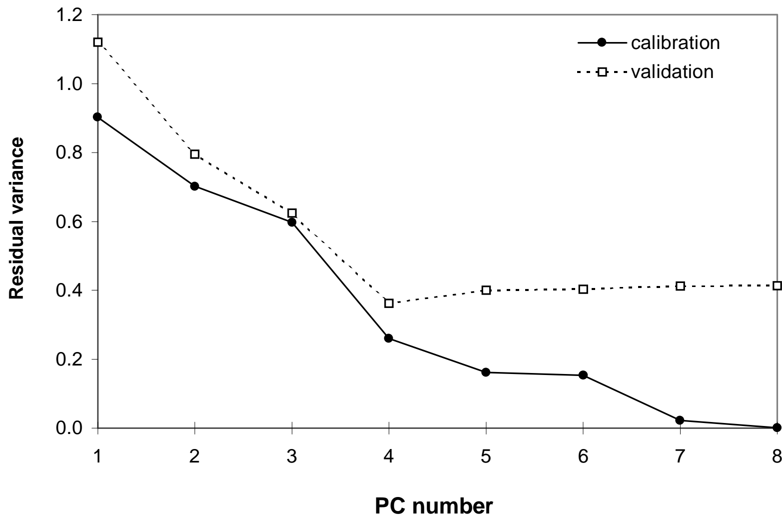


Figure 7.12 Residual variance for calibration and validation in instrumental firmness as a function of the number of PLS regression factors.

Since the chemical variables which highly correlated with firmness of green beans are well described by NIR spectra, an attempt was made to evaluate the ability of NIR to predict the instrumental firmness. Results from a separate PLS1 analysis are shown in Figure 7.12. The residual variance decreases as a function of increasing number of PLS regression factors. After 6 factors a minimum is reached, where after it increases slightly because of statistical over-fitting. The instrumental firmness is well predicted by NIR, as represented by RAP = 0.82. Figure 7.13 shows the relation between the measured and NIR predicted instrumental firmness (r = 0.99, SEP = 3.56). Prediction of the sensory firmness was not studied because the samples of each cv. were analysed in separate sessions and could not be treated as one set of data, resulting in to few data for relevant modelling.

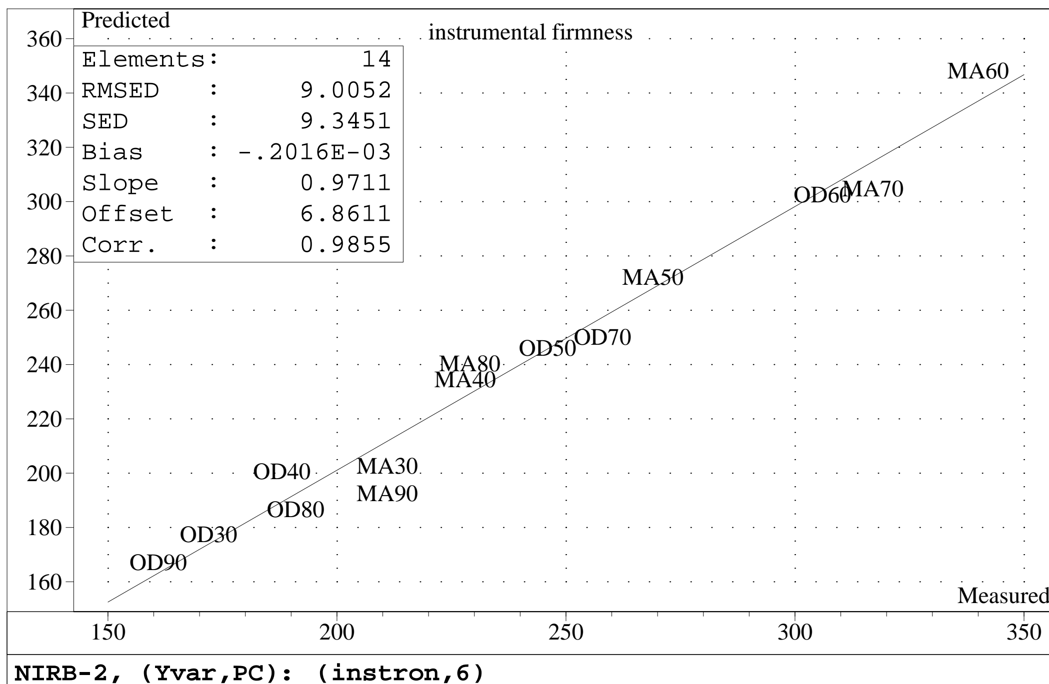


Figure 7.13 *The relation between the measured and NIR predicted instrumental firmness.*

From the results obtained it can be anticipated that IR spectroscopy is a powerful and rapid technique for assessment and prediction of chemical properties and sensory attributes of green beans. The predictive ability of models however is always dependent on the relevance of the data and the quantity and quality of the data. Up to a certain limit the rule is: the more and the better calibration data available, the more accurate prediction is possible[28]. A further calibration and validation of the model with much more samples is necessary before application of NIR for assessment or prediction of chemical and textural green bean properties can be realised in practice.

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Chapter 8

General discussion and conclusions

This thesis deals with cell wall changes in relation to the texture of green beans after heat processing. Although the effects of heat processing on vegetables has received increased attention during the last years[1-10], the number of detailed studies on the (bio)chemical and/or histological events during processing are still rare[11]. In our laboratory a multi-disciplinary approach is being used to study the texture of vegetables, ranging from genetics[12] and (bio)chemistry[13] to processing[14-16], sensory analysis [17] and consumers perception. The work presented here is a combination of a histological and chemical study in order to understand the behaviour of cell walls and middle lamellae during heat processing of vegetables and fruits. Green beans were used as study object, since they are an important crop in Dutch agriculture. The results obtained however, can be transferred to other vegetables as well. In the next sections the results of ultrastructural and chemical cell wall analyses are summarised and discussed in relation to the texture of green beans after heat processing.

8.1 Texture of green beans

Texture is an important quality attribute of green beans after industrial processing. In general, two main approaches are used to investigate texture. One is the separation of texture into more specific properties of the tissue, such as cohesiveness, hardness, elasticity and the classification of the materials in terms of the degree in which they possess these properties. The other, and main approach used in this thesis, is to study the relation between the chemical composition of the product and superimposed processing treatments on a physical parameter related to texture, like the resistance to shear[18].

During industrial heat processing green beans lose their crisp and firm texture and become soft as compared to freshly prepared beans. The most important sensory texture descriptor for green beans was 'firmness' (Chapter 7). Beans preheated at 60 °C before further processing had higher firmness scores in comparison with beans preheated at either lower or higher temperatures. The correlation between the sensory and the instrumental firmness evaluation of green beans was very high ($r^2 > 0.96$). The instrumental method was therefore used as a standard, relatively fast method to quantify the firmness of the beans in all other experiments (Chapters 2 - 6).

8.2 Tissue architecture

Most rheological studies performed on plant tissue disregard the complicated (bio)chemistry and physiology of living tissues and focus on its geometry and mechanical properties. The rheological

properties of plant products is in general considered to be dependent on the interaction and mechanics of tissues and individual cells[19]. Tissue failure is a sequence of individual cell wall ruptures and/or individual intercellular separations. At one end, if the cell wall is stronger than the middle lamella, the tissue will fracture between the cells. At the other end, if the cell wall is weaker than the middle lamella, the cleavage will occur through the cells[20]. Fracture planes of parenchyma tissues of green beans were studied with SEM (Chapter 2). By using this technique it was shown that the inner and outer parenchyma tissues of green beans had different fracture properties after heating. Hence they contribute most likely in a different way to the texture of the bean pods. Even after prolonged heating during sterilisation, the intercellular contacts seemed better preserved in the inner parenchyma as compared to the outer parenchyma tissue. This was accompanied by a different appearance of the cell wall and middle lamella in both tissues. In the outer parenchyma, the middle lamella was much more pronounced, even after heat processing. Intercellular separation is due to mechanical failure of the middle lamella. Possibly intercellular air spaces act as initiation sites for cell separation due to inflation during heating. The middle lamella, which is composed mainly of pectin, is commonly stated to cement adjacent cells together by the formation of calcium complexes[21]. Therefore, it may be assumed that an abundant middle lamella results in optimal intercellular adhesion. However, the present results show that the electron dense middle lamella of the outer parenchyma tissue in fact appears to facilitate the separation of the cells during fracturing (Figure 8.1). This can be explained by the absence of calcium complexes due to methylesterification of the carboxylic acids or of Ca^{++} deficiency. Moreover, Liners and Van Cutsem[22] demonstrated that even the presence of Ca^{++} complexed pectins in the primary walls of senescent suspension-cultured carrot cells did not maintain the cohesion of the walls. The outer parenchyma of the green bean pods is older in origin that the inner parenchyma[23]. During ageing of green beans it was shown that the amount of cross-linking and branching in the cell wall decreased (Chapter 3). If it is assumed that ageing causes comparable cell wall changes in all bean tissues, it can be hypothesised that the pectins of the outer parenchyma tissues of the pods at edible maturity are already less cross-linked and less branched compared to the cells of the inner parenchyma tissue. Both effects most likely facilitate shearing of the polymers alongside each other. In the inner tissue, where the middle lamella was less evident, as shown by TEM, it is reasonable to assume that pectin from the primary wall can extend into the middle lamella zone and contribute to intercellular adhesion. Most pectic molecules are long enough to span the distance between two cells (250 - 400 nm) and pectins may thus covalently link adjacent cells, with primary cell wall pectins on either side[24]. After heat processing, separation of cells was facilitated in all tissues by degradation of the middle lamella, since the constituting pectic polymers are depolymerised (Chapter 5 and 6). Still, differences in fracture planes remained evident between the different parenchyma tissues (Chapter 2). For potatoes, differences in fracture planes after steam cooking were associated with mealiness, more cell separation being found for a mealy cultivar[14]. In comparison to the outer parenchyma of green beans, the mealy potato cultivar also seemed to have a denser cell wall and middle lamella[16]. The mealy potato middle lamella however was also degraded to a larger extend during heating, such in contrast to the middle lamella of the green beans outer parenchyma.

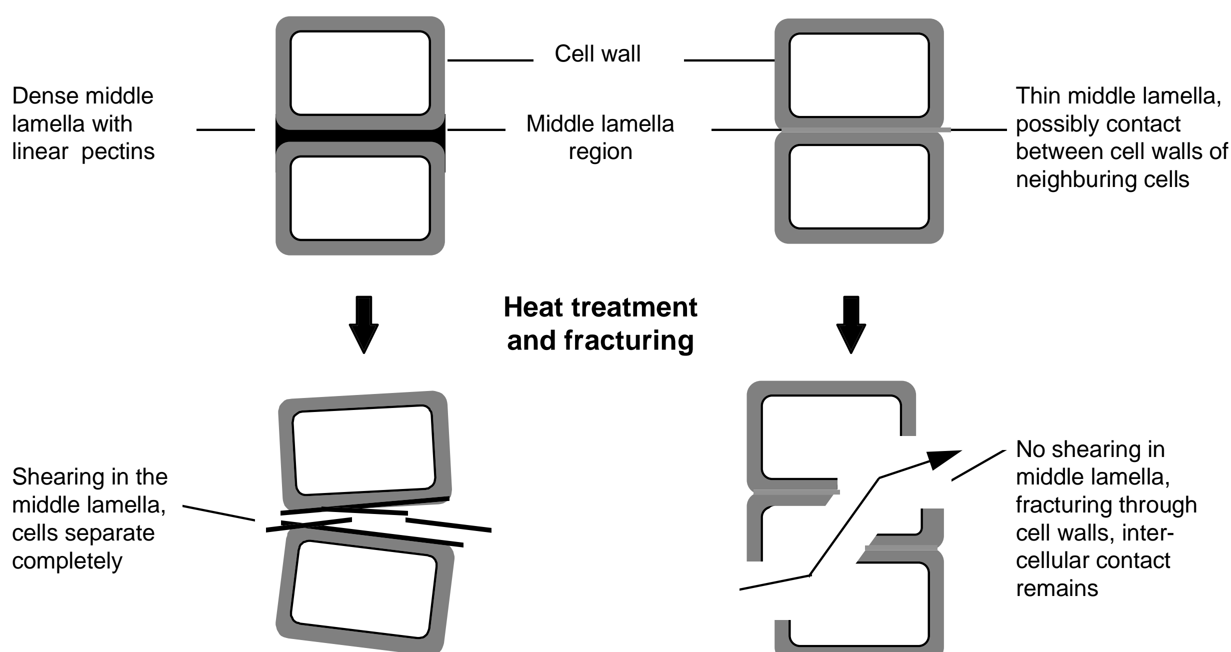

Figure 8.1 *Schematic representation of the fracturing after heat processing of tissues with (left) and without (right) a dense middle lamella.*

8.3 Cell wall characteristics

The cell wall has the most important bearing on the textural properties of heat processed vegetables. The cell walls of green beans are typical for parenchyma cell walls and contain mainly pectin and (hemi)cellulose (Chapter 4). Pectins consist of homogalacturonan and branched rhamnogalacturonan regions, which differ in their connection to each other and other cell wall components (Chapter 5). In fresh beans, one quarter of the pectin was freely soluble, another quarter was ionically complexed and the remaining, more branched pectins, were likely to be covalently linked by ester bonds. From the glycosidic linkage analysis (Chapter 4) it was concluded that the hemicelluloses mainly consisted of xyloglucans and xylans. The exact nature of the xylans could not be established with the methods used. The branching site was mainly at C-2, which occurs for both arabinosyl as well as glucosyluronic acid residues on xylans in this type of cell walls[25].

Cell wall changes during industrial processing are the result of enzymatic and/or chemical reactions, depending on the temperature of the process. At high temperatures, as used in conventional industrial green bean processing, the effects are mainly of a chemical nature, since endogenous enzymes are rapidly inactivated. During heat processing of green beans only the pectic cell wall components were significantly affected (Chapters 4, 5 and 6). Two major effects of sterilisation could be discriminated: (1) linear homogalacturonan degradation into small oligomers and (2) partly solubilisation of branched rhamnogalacturonan, that remained entangled in the wall matrix. Since pectic substances act as a cement, uniting the cells in a tissue, degradation of this super glue must result in a collapse of tissue strength.

8.3.1 Developmental effects

Cell wall composition is among other things dependent on the developmental stage of the cell. To evaluate the degree of cell wall variation in green beans in relation to developmental stage, the composition of cell walls was monitored during growth and maturation (Chapter 3). Major changes were detected in the pectic polymers. Cell walls of very young, exponentially growing pods contained large amounts of neutral sugar rich pectic polymers (rhamnogalacturonan), which were water insoluble and relatively tightly connected to the cell wall. During elongation some more galactose-rich pectic polymers were deposited and cross-linked into the cell wall. Besides this the level of branched rhamnogalacturonan remained rather constant, while the level of linear homogalacturonan steadily increased. This suggests that open spaces in the cell wall, formed by expansion, are filled with homogalacturonan, while the structure is being fixed by galactose-rich pectic polymers. During maturation of the pods these tightly linked galactose-rich pectic polymers were degraded, while the accumulation of soluble homogalacturonan continued. During senescence there was an increase in the amount of ionically complexed pectins, mainly at the expense of freely soluble pectins. The changes in (hemi)cellulose concerned mainly an increase in cellulose content at the end of the elongation phase and a small shift from xyloglucans to more xylans and mannans during maturation and senescence.

For industrial processing, green bean pods are usually harvested at about 20 days post flowering. This corresponds with the cessation of elongation. From the results obtained in Chapter 3 it can be envisaged that harvesting time of the pods will effect the texture of the processed product. In an earlier developmental stage the beans contain relatively less cellulose and high methyl homogalacturonan and more branched rhamnogalacturonan. The implication of those structural differences for the sensory perceived texture are difficult to predict. Methylated homogalacturonan will be largely degraded during heating by ß-eliminative degradation. Thus, when the methylated homogalacturonan is the major type of pectin, as in the older beans, more pectin will be degraded in the processed beans, thereby probably decreasing the pod firmness. In addition the beans are likely to become more tough as the cellulose contents increases.

8.3.2 Cultivar variations

Three cultivars of green beans were used in this thesis, Montano, Odessa and Masai. Cv. Montano is mainly a fresh market bean, while cvs. Odessa and Masai represent industrial market beans. Cvs Odessa and Montano are always softer after heat processing than cv. Masai (Chapter 2, 4, 5 6 and 7). The chemical cell wall analyses were initially performed with cv. Montano and Masai (Chapter 4), but these green beans had a slightly different tissue architecture. The pods of cv. Montano had a somewhat thicker outer parenchyma layer (data not shown). It was therefore decided to use cvs. Masai and Odessa, of which the tissue architecture was more alike, for further comparison of cell wall characteristics in relation to processing characteristics (Chapter 5 and 6).

Small, but consistent differences were found between the cell wall composition of cvs Masai and Odessa, the main difference being the apparently higher abundance of xylans in cv. Masai (Chapter 5). It cannot be excluded that this higher amount of xylans relates with a difference in maturation rate of cv. Masai. In addition, small differences in pectin composition were detected between both cultivars.

Cv. Masai contained pectic compounds with more galacturonic acid residues and less galactose and arabinose at edible maturity (Chapter 5 and 6). However, this difference in pectin composition can possibly also be related with a small difference in maturity at harvest. The beans are judged to be of edible maturity by the breeder and subsequently harvested. Generally, the breeder considers the beans of edible maturity at about 20 days after flowering (see also above). However, the cell wall is still metabolically highly active at this stage and changes every day (Chapter 3). Conclusions about the detected differences in pectin between cultivars can only be made if the development of both cultivars is followed for several years. Since this is beyond the scope of this study, the developmental analyses were focused on one cultivar only. An additional difference between both cultivars was the degree of esterification of the ionically complexed pectins (Chapter 6). For cv. Masai the degree of methylation (DM) as well as the degree of acetylation (DA) were significantly lower than for cv. Odessa. The DM is well know to affect the strength of calcium intermediated pectin gels, the normal type of gel in cell walls. As acetyl substituents have been shown to reduce the binding strength between pectins[26], it may be hypothesised that the calcium complexed pectin gels of cv. Masai are more cohesive than of cv. Odessa. If this type of pectin, originates from the middle lamella, as is often stated[27], than the cells of cv. Masai may be less easy to separate than the cells of cv. Odessa, resulting in a higher firmness of the cv. Masai tissue.

8.3.3 Effects of modified processing procedures

Firmness of vegetables can be better retained by modifying the processing conditions[28-31]. By preheating green beans at moderate temperatures (50 - 70 °C) for an appropriate period of time before further processing, firmness was retained better than with conventional processing conditions (Chapter 6). This firming effect was shown to be related with activation of endogenous pectin methylesterase (PME) activity during preheating. PME demethylates specific regions of the pectic polymers. At the regions where the methylesters are removed the pectin is (1) less heat labile and (2) can form more intermolecular calcium complexes. Both processes result in a higher retention of firmness in processed vegetables.

8.4 Application of infrared spectroscopy

The applicability of spectroscopic techniques for monitoring cell wall changes during processing and quality control of vegetable processing is described in Chapter 7. A method based on Fourier Transform Infra-Red spectroscopy (FT-IR) was developed for the chemical characterisation of the pectic fractions. Furthermore, the ability of Near Infra-Red spectroscopy (NIR) to predict the texture

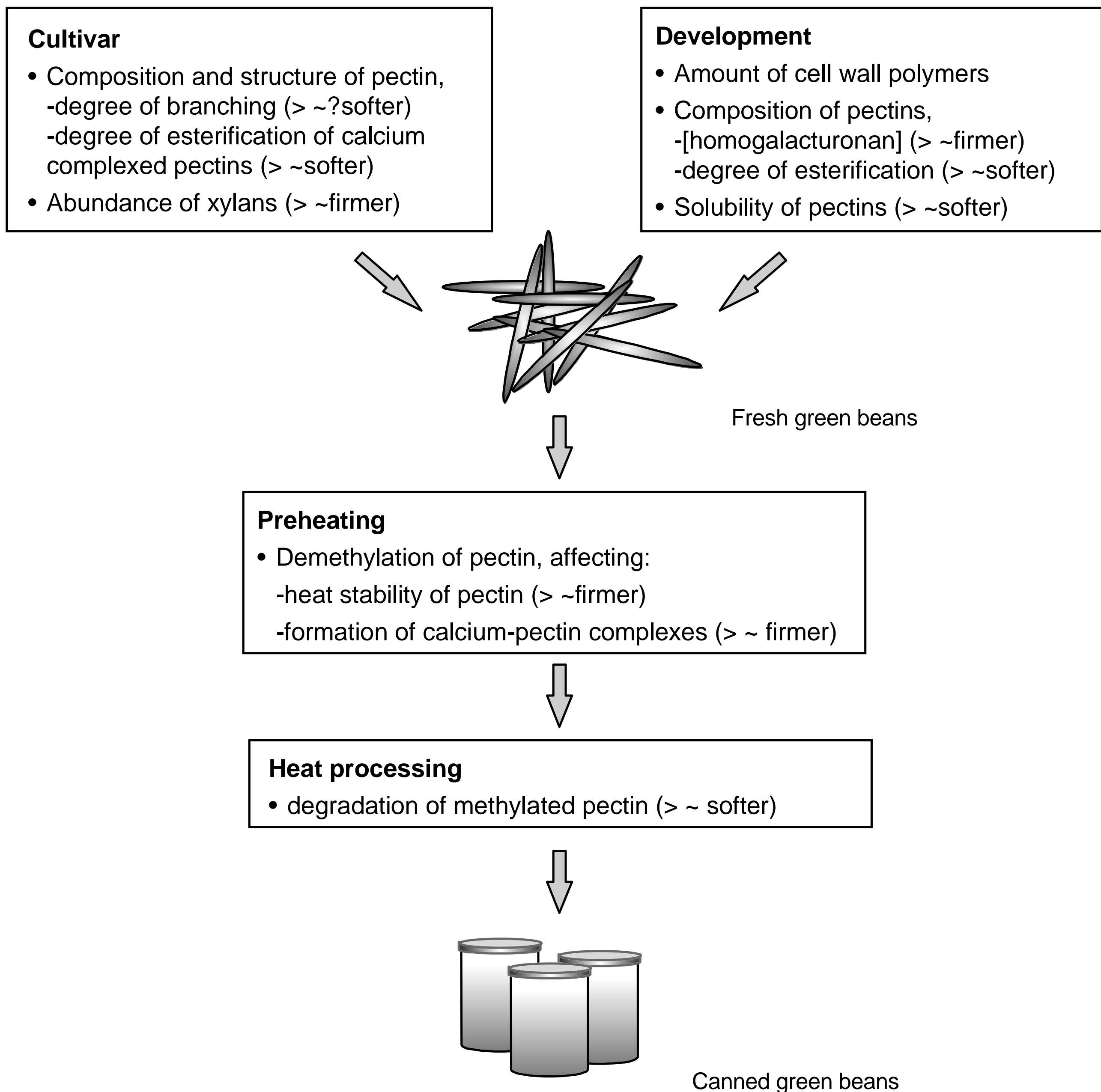

Figure 8.2 *Overview of different factors in the green bean chain influencing cell wall characteristics and their suggested effect on the texture of heat processed green beans.*

and the chemical composition of green beans was investigated. FT-IR showed to be a very promising technique for quantification of chemical composition of pectines. Reliable information can be obtained about the abundance of specific neutral sugars and the DM, when compared with the chemical analyses. However, the pectic fractions contained to much impurities, such as proteins, to allow a highly accurate determination of the DM. The quality of the FT-IR analyses can be improved by using more advanced data analysis methods like partial least square (PLS) regression, and calibration with standardised samples.

NIR spectroscopy is a powerful and rapid technique for assessment and prediction of both chemical properties and the texture of green beans. The instrumental determined firmness and related chemical parameters could very well be predicted with a model based on PLS regression using the NIR spectrum as data input. Unfortunately the number of samples in this study proved insufficient to validate the proposed model. The predictive ability of the developed models remains dependent on both the relevance of the data and the quantity and quality of the data. Up to a certain limit the rule is: the more and the better calibration data available, the more accurate prediction is possible. NIR can be used for in-line measurement of product properties for optimising process control and as substitute for time consuming laboratory product quality tests in the vegetable industry.

8.5 References

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SUMMARY

Texture is an important quality attribute of processed vegetables and fruits. One of the negative side effects of heat sterilisation of vegetables and fruits is the extreme softening of the product, resulting in an undesirable texture. Texture after processing is determined by tissue architecture, the characteristics of cell walls and middle lamellae, the abundance of specific components, such as starch, and superimposed effects of processing on these characteristics. The aim of the research described in this thesis was (1) to study the chemical and ultrastructural changes of cell walls from green beans (*Phaseolus vulgaris* L.) during the course of industrial thermal processing and (2) to gain insight into the key-parameters of fresh (non-starch) vegetables important for firmness after processing.

The influence of blanching and sterilisation on the bean pod tissues was studied using both scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Blanching and sterilisation caused partial degradation of the cell walls and middle lamellae. Cellulose microfibrils were shown to be unaffected by the heat treatment. The outer and inner parenchyma tissues were shown to have a different fracturing behaviour after heating and hence maybe a different contribution to bean texture. The adhesion between cells after processing seemed better preserved in the inner parenchyma tissue. This was accompanied by a different middle lamella ultrastructure, the middle lamella being more abundant in the outer parenchyma tissue. The present observations have led to the following hypothesis: (1) a thick middle lamella may ease cell separation by shearing of its constituting pectic polymers. (2) a thin middle lamella may adhere adjacent cells more firmly by linkage between pectins of the primary wall thus forcing fracturing through, instead of along the cell wall (Chapter 2).

The modification of cell wall composition of green bean pods during growth and senescence was studied with emphasis on its pectic substances (Chapter 3). An overall increase in cell wall material was observed throughout development. Major changes were detected in the pectic polymers. Very young exponentially growing cell walls contained large amounts of rhamnogalacturonan, rich in neutral sugars and tightly connected to the wall. During elongation, some additional galactose-rich rhamnogalacturonan was deposited and the level of homogalacturonan increased steadily. It is suggested that less dense packed regions formed in the cell wall network during expansion are filled up with homogalacturonan, the network itself being conserved by the galactose-rich rhamnogalacturonan. During maturation the tightly linked galactose-rich pectic polymers became degraded. During senescence an increase in ionically complexed pectins occurred, mainly at the expense of the freely soluble pectins. The relation of endogenous enzyme levels and properties of cell wall polymers are discussed with respect to cell wall synthesis and degradation. The harvesting time probably has a large effect on bean texture after processing

The major cell wall polysaccharides of green beans at edible maturity are cellulose and pectin, but hemicelluloses are also detected. Major cell wall changes during industrial processing occurred during the sterilisation procedure (Chapters 4, 5). Only the pectic cell wall components were significantly affected. In fresh beans the major part of the pectins was extractable only after saponification of the tissue and therefore deduced to be covalently linked to other cell wall polymers. However, after sterilisation most pectins were freely soluble. The galacturonic acid backbone was partially degraded,

most likely by ß-eliminative degradation, but side chains seemed not to be split off. In conclusion, two major effects of sterilisation could be discriminated: (1) Linear, methylated homogalacturonan was degraded into small oligomers and monomers and (2) branched rhamnogalacturonan was partially solubilised, but remained entangled in the wall matrix. Since pectic substances act as a kind of cement uniting the cells in a tissue, it is clear that degradation of this super glue results in a collapse of tissue strength.

Firmness of green beans was better retained by preheating at moderate temperatures (50 - 70 °C) for an appropriate period of time before further processing, than with conventional processing conditions (Chapter 6). After this preheating the overall degree of methylation of pectin was reduced, indicating endogenous PME activity. In addition, the yields of the buffer soluble and ionically complexed pectins of the beans preheated at 50 - 70 °C were higher after sterilisation. No significant effect on the yield of the covalently linked, branched pectic fraction was found. This implies that PME was not able to demethylate pectin flanking the branched regions, leaving these pectic regions susceptible for ß-eliminative degradation. In conclusion, preheating affects texture after sterilisation most likely by demethylation of pectin by PME thereby (1) decreasing the heat instability and (2) increase the capacity to form more intermolecular calcium complexes. Both processes result in a higher retention of firmness in processed vegetables. The changes occurring in pectin during preheating and sterilisation are visualised by a schematic picture in Chapter 6.

In Chapter 7 the relation between chemical and texture data was analysed by statistical techniques. In addition, the application of IR-spectroscopy for analysis of selected pectic fractions and for prediction of texture and chemical composition was investigated. Partial least square (PLS) regression analysis of chemical and texture data revealed that three significant factors explained 95 % of the total variation in the texture variables. Both instrumental and sensory determined firmness of the beans could be related to the cell wall pectins. The degree of methylation of buffer soluble and CDTA-extractable pectins and the amount of pectin degradation were negatively correlated with firmness. Since the degree of esterification of pectic fractions is a very important parameter in determining the texture of processed green beans an attempt was made to develop a method based on FT-IR for its quantification. NIR spectroscopy proved to be a rapid technique for assessment and prediction of texture related chemical properties and firmness of green beans.

Finally, all the results regarding cell wall composition and structure in relation to processing are discussed in Chapter 8. Attention is paid to tissue ultrastructure, developmental effects, cultivar differences, influences of the heating process on cell walls and their interrelations.

SAMENVATTING

Textuur is een belangrijk kwaliteitsaspect van industrieel verwerkte groenten en fruit. Een van de nadelen van sterilisatie door een hittebehandeling is het verlies van stevigheid van de produkten. Textuur na verwerking wordt bepaald door de weefselstructuur, de aanwezigheid van specifieke inhoudsstoffen, zoals zetmeel, de kenmerken van celwand en middenlamel en de invloed van het verwerkingsproces hierop. Het doel van de studie beschreven in dit proefschrift was (1) het bestuderen van chemische en histologische veranderingen in celwanden van sperziebonen (*Phaseolus vulgaris*) gedurende industriële verwerking en (2) het verkrijgen van inzicht in sleutelparameters van het verse produkt die van primair belang zijn voor de stevigheid na verwerking.

De invloed van blancheren en steriliseren op de weefsels van sperziebonen werd bestudeerd met behulp van scanning electronen microscopie (SEM) en transmissie electronen microscopie (TEM). De buitenste en binnenste parenchymatische weefsels bleken een ander breukgedrag te vertonen na verhitting en leveren dus waarschijnlijk een andere bijdrage aan de bonentextuur. De verbindingen tussen de cellen bleken na verwerking meer intact in het binnenste parenchym. Dit werd vergezeld door een andere ultrastructuur van de middenlamel, waarbij de middenlamel veel duidelijker te onderscheiden was in de buitenste parenchymlaag. Een mogelijke verklaring hiervoor is dat cellen met een geprononceerde middenlamel gemakkelijker van elkaar te scheiden zijn doordat de pectines in de middenlamel langs elkaar kunnen schuiven. Een dunne middenlamel daarentegen, lijkt gecorreleerd met stevige intercellulaire contacten. Dit wordt mogelijk verklaard door associatie van primaire celwandpectines van naburige cellen, waardoor een breuk gemakkelijker door de celwand gaat, dan via de middenlamel. Blancheren en steriliseren resulteerde in gedeeltelijke afbraak van celwand en middenlamel. Cellulosemicrofibrillen werden niet aangetast door de hittebehandeling.

In Hoofdstuk 3 worden celwandveranderingen tijdens de ontwikkeling van peulen beschreven, met nadruk op pectines. Gedurende de peulontwikkeling was er een algemene toename van celwandmateriaal. De voornaamste veranderingen vonden plaats in de pectines. Zeer kleine, exponentieel groeiende peulen bevatten grote hoeveelheden rhamnogalacturonan, rijk aan neutrale suikers en stevig gebonden aan de rest van de celwand. Gedurende de lineaire lengtegroei nam de hoeveelheid homogalacturonan sterk toe en werd tevens nog wat extra galactose-rijk rhamnogalacturonan gevormd. Dit suggereert dat ruimtes in het celwandnetwerk, gevormd door celstrekking, worden opgevuld met homogalacturonan, terwijl het netwerk wordt verankerd met galactose-rijk rhamnogalacturonan. Gedurende de rijpingsfase worden de sterk gebonden galactose-rijke polymeren afgebroken. Tijdens de verouderingsfase was er een sterke toename van ionogeen gebonden pectines, voornamelijk ten koste van vrije, ongebonden pectines. De relatie tussen endogene enzymniveau's en samenstelling van de celwand worden bediscussieerd in het kader van celwandsynthese en -afbraak. Het oogsttijdstip is waarschijnlijk van grote invloed op de textuur van de peulen na verwerking.

De belangrijkste celwandpolymeren van peulen, rijp voor consumptie, zijn cellulose en pectine, maar ook hemicellulose is aanwezig. De belangrijkste veranderingen tijdens industriele verwerking treden op tijdens de sterilisatie (Hoofdstuk 4 en 5). Alleen pectines veranderden significant. In rauwe peulen was het merendeel van de pectines slechts extraheerbaar na verzeping van het weefsel, en daarom waarschijnlijk covalent gebonden aan andere celwandpolymeren. Echter, na sterilisatie was het merendeel van de pectines vrij oplosbaar. De galacturonzuurrijke hoofdketen was gedeeltelijk afgebroken, waarschijnlijk door ß-eliminatieve afbraak. Zijketens leken verbonden te blijven met de hoofdketen. Twee belangrijke effecten kunnen worden onderscheiden: (1) lineair gemethyleerd homogalacturonan wordt afgebroken tot kleine oligomeren en (2) vertakt rhamnogalacturonan wordt gedeeltelijk oplosbaar, maar blijft verstrikt in de celwandmatrix. Omdat pectines zich gedragen als een soort cement, die de cellen verbinden tot een weefsel, is het logisch dat de afbraak van deze 'superlijm' resulteert in een afname van weefselsterkte.

De stevigheid van verwerkte sperziebonen kan beter worden behouden door de bonen voor te verhitten bij middelhoge temperaturen (50 - 70 °C), alvorens ze verder te verwerken met conventionele verwerkingsmethoden (Hoofdstuk 6). Na deze voorverhitting bleek de gemiddelde methyleringsgraad van het pectine te zijn afgenomen, wat duidt op endogene pectine methylesterase (PME) activiteit. Na steriliseren waren er meer vrij oplosbare en ionogeen gebonden pectines. Er werd geen significant verschil gevonden voor de covalent gebonden, sterk vertakte fractie. Dit doet vermoeden dat PME de hoofdketen in de buurt van zijketens niet kan demethyleren, deze gebieden blijven daardoor gevoelig voor ß-eliminatieve afbraak. Voorverhitting heeft effect op de textuur na sterilisatie door demethylering van het pectine door PME wat (1) de hittestabiliteit verlaagt en (2) de mogelijkheid voor het vormen van intermoleculaire calciumcomplexen vergroot. Beide effecten resulteren in een verhoogde stevigheid van verwerkte produkten. De veranderingen in pectine tijdens voorverhitten en steriliseren zijn samengevat in een schematische tekening.

In Hoofdstuk 7 wordt de relatie tussen chemische en texturele eigenschappen beschreven. Bovendien wordt ingegaan op de toepasbaarheid van infraroodspectroscopie voor de analyse van bepaalde pectine fracties (met FTIR) en voor de voorspelling van chemische samenstelling en textuur (met NIR). PLS regressie analyse van chemische en textuur gegevens resulteerde in drie significante factoren die 95 % van de totale variatie beschreven. Zowel de instrumentele als de sensorische stevigheid van de bonen kon worden gerelateerd aan eigenschappen van celwandpectines. De methyleringsgraad van de vrije ongebonden en ionogeen gebonden pectines, en de pectineafbraak waren sterk gecorreleerd met de stevigheid. Omdat de methyleringsgraad een belangrijke parameter is voor de uiteindelijke stevigheid van het produkt is getracht een kwantitatieve methode te ontwikkelen op basis van FTIR. NIR spectroscopie bleek bovendien een snelle techniek te zijn om textuur gerelateerde chemische eigenschappen en de stevigheid van sperziebonen te voorspellen.

Tot slot worden alle resultaten met betrekking tot celwandsamenstelling en structuur besproken in relatie tot textuur na verwerking in hoofdtuk 8. Hierbij komen aan de orde weefselstructuur, ontwikkelingseffecten, cultivarverschillen, de effecten van het verhittingsproces op de celwanden en hun onderlinge relaties.

NAWOORD

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CURRICULUM VITAE

Trinette Smits werd op 18 september 1966 geboren te Drunen. In 1984 behaalde zij het diploma gymnasium ß (cum laude), aan het Dr. Mollercollege te Waalwijk. In datzelfde jaar werd een start gemaakt met de studie Biologie aan de Katholieke Universiteit Nijmegen. Deze studie werd in augustus 1990 afgerond met microbiologie als hoofdrichting (12 mnd), experimentele plantenoecologie (12 mnd) en moleculaire plantenfysiologie (6 mnd) als nevenrichtingen.

Op 15 september 1990 kwam zij in dienst als wetenschappelijk onderzoeker bij het instituut voor Agrotechnologisch Onderzoek (ATO-DLO) te Wageningen alwaar tot 1 augustus 1991 een analytisch chemisch onderzoek werd uitgevoerd naar de valorisatie van varkensdrijfmest in de hoofdafdeling ‘Bioconversie’. Vervolgens werd in de hoofdafdeling ‘Bio-Chemie en Voedselverwerking’ begonnen met het onderzoek, dat de basis vormde voor dit proefschrift. Na twee en half jaar kreeg ze een vaste aanstelling en werden haar werkzaamheden uitgebreid met het schrijven en begeleiden van andere onderzoeksprojecten. Momenteel is zij werkzaam als sectiehoofd ‘Voedsel Bio-Chemie’ binnen de afdeling ‘Natuurlijke Voedselingrediënten’.

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