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THE CELLULAR STRUCTURE OF SELECTED APPLE VARIETIES

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Abstract

Apple cultivars (Sauergrauech, Klarapfel, James Grieve, Granny Smith, McIntosh, Rubinette) which had different textures based on sensory and instrumental analysis (particularly in firmness and mealiness) were examined by conventional scanning electron microscopy (SEM), cold-stage SEM (cryoSEM) and confocal scanning laser microscopy (CSLM) using various preparative procedures. Advantages, limitations and artifacts of each technique are discussed.

SEM with glutaraldehyde-fixation and critical-point-drying produced minimal tissue distortion and the fracture pattern and appearance of mealy versus non-mealy tissue was different. Freeze-drying unfixed tissue caused cell collapse and firm versus soft varieties could not be differentiated. Freeze-fracturing and cryoSEM of apple tissue with varying textures revealed the degree of cell adhesion between frozen hydrated cells. CSLM provided more information on the three-dimensional internal structure of intact fresh apple tissue and cell cohesiveness. Details of structural elements were enhanced by staining with ocridine orange.

Key Words: Apple, fruit, texture, structure, scanning electron microscopy, cryo-SEM, firmness, mealiness, confocal microscopy.

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Introduction

The apple (*Malus genus*) is a pome fruit, with the edible cortex developing from tissue of the floral tube (fused bases of the calyx, corolla, and stamens). Cell division is complete early in the growing season and further growth is due to parenchyma cell enlargement and increase in the size of the intercellular spaces (Smith, 1940; Smock and Neubert, 1950). Parenchyma cells and intercellular spaces are loosely arranged in net-like pattern which is inhomogeneous and anisotropic (Khan and Vincent, 1990). Mature cells may be 50-500 μm in diameter with interconnecting air spaces ranging from 210-350 μm across and comprising 20-30% of tissue volume (Reeve, 1953).

The apple cell wall is comprised of cellulose microfibrils loosely woven together and embedded in an amorphous matrix of polysaccharides including pectic substances. Major changes in cell wall composition of senescing apples have been well characterized (Glenn and Poovaiah, 1990; Knee and Bartley, 1981; Melford and Prakash, 1986). Individual parenchyma cells are cemented together by an amorphous layer external to the cell wall called the middle lamella. The cell walls and interlamellar layer constitute 1-3% of the fresh apple weight and impart rigidity to the structure. Chemical changes in the pectic substances and middle lamella are closely related to softening and changes in apple texture (Ilker and Szczesniak, 1990; Mohr, 1989; Stow, 1989).

Texture is an important quality factor of fresh apples (Escher and Lapsley, 1990). Sensory research indicated textural quality was described adequately by the terms crispness, firmness, juiciness and mealiness (Lapsley, 1989). The first three attributes were characterized by mechanical deformative testing. Mealiness, a mouthfeel sensation and a negative texture attribute, could not be recorded instrumentally. Sensory results showed panelists could distinguish between mealy and non-mealy apple and potato varieties whereas an instrumental needle penetration method only differentiated potatoes (Boehler *et al.*, 1987; Lapsley, 1989). Mealiness was described as a condition where the middle lamella has disintegrated to the point that cells separate instead of rupturing when a force is applied (Reeve,

1970). There is little visual evidence of what structural organization in apple tissue correlates with sensorially perceived mealiness, although numerous researchers have used microscopy for direct comparison of physical and structural changes associated with apple softening. This study was undertaken to find a microstructural basis for the relationship between sensory perception of apple texture, mechanical behaviour and apple tissue structure.

Historically the cell structure of apple flesh was examined by light microscopy. Reeve (1953) evaluated the cell size range and percentage intercellular space for five cultivars. There was no consistent correlation between these structural features and the ease of cell separation. Reeve (1953) concluded that differences in metabolism during growth and ripening and the composition of the middle lamella contributed to varied textural qualities.

Transmission electron microscopy (TEM) has provided detailed studies of apple cell wall structure. Nelmes and Preston (1968) followed development of parenchyma cell walls throughout two seasons under conditions which allowed assessment of wall structure as a factor of keeping quality. There were no changes in cell wall thickness or structure during storage and softening. Ben-Arie *et al.* (1979) reported structural alterations in apple and pear cell walls at advanced stages of softening with dissolution of the middle lamella. Mohr (1979) used selective tissue extractions to visualize changes in the cell wall polysaccharides of long storage, firm-type apples versus short storage soft apple types. Once the apples began to soften, there was cell separation, enlargement of intercellular spaces and cell rounding which was linked with mealy texture. Glenn and Poovaiah (1990) used various analytical and microscopic techniques to characterize the influence of calcium on Golden Delicious apple texture. They concluded that disruption of the middle lamella may occur during fixation procedures for TEM.

In recent years scanning electron microscopy (SEM) has provided more information on apple cell structure and orientation since the SEM's depth of field and three dimensional appearance in the micrographs provide details on cell concavity and arrangement (Trakoontivakorn *et al.*, 1988). Diehl and Hamann (1979) examined apple tissue at different stages of ripeness after structural failure. Cellular failure occurred in the cell wall, regardless of whether it was due to torsion, compression or shear testing. Bolin and Huxsoll (1987) observed physical changes in cell structure when cell turgor was modified. Image analysis of glutaraldehyde fixed and critical point dried tissue indicated a 2% moisture loss had a measurable effect on the ratio of cell length to breadth, and on cell roundness index. Configurational changes in the cells were also observed during storage, drying, rehydration and chemical treatment.

Kovacs *et al.* (1988) found low dose irradiation of apple tissue induced softening in the fruit and dissolution of the middle lamella and documented the changes with SEM and glutaraldehyde fixed and critical point dried samples. Cells in the control were opened up by the tissue preparation, with fracture across cells, presumably as a consequence of their firmly attached cell walls and turgor. Cells from irradiated tissue were not cut through, indicating the middle lamella dissolved and the cells separated from each other. TEM observations confirmed that the middle lamella disintegrated, cell walls reticulated and vesicles of different sizes appeared in cell walls, especially in middle lamella areas near intercellular spaces (Kovacs *et al.*, 1988).

Trakoontivakorn *et al.* (1988) compared the cellular structure of immature and mature Granny Smith and Red Delicious apples using SEM with glutaraldehyde osmium tetroxide fixation and critical point drying. Parenchyma cells formed a net-like pattern in sections transverse to the stem-calyx of the apple. Khan and Vincent (1990) used light microscopy and SEM with freeze drying to study anisotropy of apple parenchyma. Morphology varied according to the direction from which tissue was viewed, and cell size increased from the outside to the inside of the cortex.

Problems associated with the effect of preparatory techniques on structural changes in plant tissues for conventional SEM have been discussed by various authors (Boyde and Boyde, 1980; Davis and Gordon, 1980; Falk, 1980). Boyde and Boyde (1980) reported freeze-drying caused less shrinkage than critical point drying for animal tissue, but critical point drying preserved potato tissue better. Flores *et al.* (1987) concluded freeze-drying distorted pimento pepper tissue more than glutaraldehyde fixation with critical point drying. Kalab (1983) outlined techniques to minimize tissue damage during freeze-drying, but no specifics for fruits were presented. Davis and Gordon (1980) compared preparatory techniques for carrots using different combinations of mechanical excision and cryofracture. Cryofracturing tissue after liquid nitrogen immersion, subsequent fixation with glutaraldehyde and osmium tetroxide, and critical point drying after dehydration with acetone resulted in the largest structural differences for different carrot varieties and treatments.

Davis and Gordon (1980) concluded the ideal situation was to leave the tissue hydrated and view the surface directly without any chemical fixation. Cryo-SEM or laser micrographs reveal morphological details about fluid or air filled spaces in plant tissue which are not readily apparent in fixed and dried tissue with conventional SEM (Jeffree *et al.*, 1987). The advantages to viewing frozen hydrated plant tissue directly with cryo-SEM have been reviewed by Crang (1988), Marshall (1987), Robards and Sleytr (1985), and Sargent (1988). Glenn and Poovaiah (1990) studied calcium-mediated postharvest changes in the texture and cell wall structure of Golden Delicious apples. They used conventional and cryo-SEM to evaluate cell-to-cell contact and found that, with cryo-SEM, there was minimal damage to the frozen hydrated tissue.

Confocal scanning laser microscopy (CSLM) has

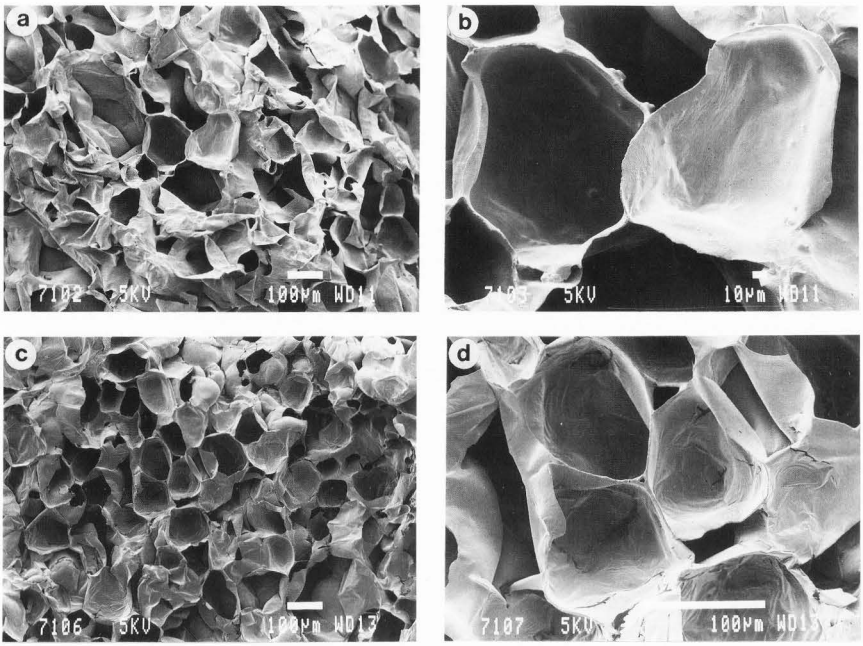


Figure 1. SEM of parenchyma tissue from unfixed, freeze-dried Sauergrauech (**a**, **b**) and Klarapfel (**c**, **d**) apples.

certain advantages over cryo-SEM since tissue is viewed fresh, not subject to artifacts due to freezing, and may be viewed at different levels by focusing (optical sectioning) (Heertje *et al.*, 1987; Brooker, 1991). The basis of the confocal principle is that a point in the tissue is optically illuminated and optically imaged through a detector pinhole which leads to increased resolution and a reduced depth of field (Brakenhoff *et al.*, 1988). To date no CSLM research on fruit tissue has been published.

Materials and Methods

Most apple cultivars were provided by the Swiss Federal Research Station in Waedenswil. The apples were harvested at optimum maturity, sorted, placed in wooden crates (20 kg) and stored at 4 °C and 95% RH until delivery to the Department of Food Science, at Zurich. Some early varieties and domestic Granny Smith apples were purchased locally. Upon receipt in Zurich any apples with defects were rejected and the rest were numbered, sorted by weight and specific gravity, and stored at 4 °C and 90% RH until used (Lapsley, 1989).

Conventional SEM

Cylinders were vertically extracted from individual apples with a 15 mm core borer to provide parenchyma tissue from the equatorial region of the flesh outside the core line. These tissue cylinders were manually broken in half and 3 to 5 mm cubes were cut from fractured surfaces using a scalpel.

Preliminary freeze-drying experiments required development of an apparatus similar to the simple vacuum systems for freeze-drying described by Robards and Sleytr (1985), since no commercial EM freeze-drier was available. A 1.5 kg solid aluminum cylinder was immersed in liquid nitrogen to cool to -150 °C and removed. Six freshly cut apple cubes (3 to 5 mm) were positioned on top of the cylinder, covered by a wire mesh and additional liquid nitrogen was poured over the setup. The cylinder was transferred to a glass chamber

attached to a vacuum pump ($1x10^{-3}$ torr) and the cubes dried for 16 hours. After removal and storage in a desiccator, the cubes were glued to metal stubs with carbon cement and gold or carbon coated before viewing with a JEOL 840 SEM at 5 kV.

Tissue preparation, by critical point drying of 3 to 5 mm cubes, was conducted with and without chemical fixation. Apple cubes were fixed by immersion in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), dehydrated through a graded series of ethanol to absolute, and transferred to a Balzers FL-9496 Critical Point Drier (Balzers Ltd., Liechtenstein). The dehydrated cubes were glued to aluminum stubs using silver cement, sputter coated and viewed in a Hitachi S700 SEM at 20 kV.

Cryo-SEM

The EMscope SP200A non-dedicated system was used (Ashford, England). Cylinders and tissue cubes were cut using the same procedure as for conventional SEM. The 3-5 mm cubes were placed in the EMscope sample holder and frozen in nitrogen slush, transferred to the vacuum chamber of the external cryopreparation unit, freeze-fractured using a cooled scalpel, and carbon coated. Frozen prepared specimens were transferred to the cryostage attached to a Hitachi S-570 operated at 10 kV.

Confocal Scanning Laser Microscopy (CSLM)

CSLM was conducted with a MRC-500 confocal imaging system (Biorad Microscience, Abingdon, England). Cylinders and tissue cubes were extracted with the same procedure as SEM and soaked in a 1% acridine orange solution for 30 minutes. The fluorescent dye was protein specific and excitable in the 450-540 nm wavelength of the laser light source. Each cube was removed from the dye, placed directly on a glass slide and viewed with oil immersion 25x Nikon Neofluor objective (N.A. = 0.8) on a Zeiss inverted microscope. Optical sectioning was possible to a depth of 100 μm. The system was equipped with a flat screen black and white monitor for photography of images using a Nikon F301 35 mm camera.

Results and Discussion

Conventional SEM

The effect of freeze-drying on unfixed tissue for SEM is shown in Figure 1. These preliminary experiments on preparatory techniques were conducted in late summer with early maturity varieties. Sauergrauech is a cider apple and was very firm and juicy whereas Klarapfel does not store well and was very soft and mealy. Parenchyma cell structure is similar in both micrographs and it is not possible to differentiate the varieties or conclude whether tissue shrinkage, cell collapse or distortion is due to the dehydration technique or varying textures. The overall tissue structure is similar to the SEM micrographs of Khan and Vincent (1990) who evaluated anisotropy of apple parenchyma. They acknowledged that freeze-drying rendered the tissue brittle, but felt no surface damage as collapse or cell wall breakage was observed. Glenn and Poovaiah (1990) found less structural preservation was maintained in freeze-dried sections than in chemically fixed apple tissue. Flores *et al.* (1987) found freeze-drying resulted in severe tissue distortion to pimentos, when studying structural changes during lye peeling. For this study it was concluded that freeze-drying was too severe a technique for apple tissue dehydration.

Apple tissue fixation and dehydration with glutaraldehyde and critical-point drying (CPD) improved differentiation of apple tissue with varying textures. Glutaraldehyde fixation renders cell walls more rigid (Robards and Sleytr, 1985). James Grieve, an early variety, was soft and somewhat mealy. As seen in Figure 2b fixed tissue had less shrinkage and minimal connection between cells, which cannot be seen in the collapsed group of cells from unfixed tissue (Figure 2a). This is in agreement with Parsons *et al.* (1974) where CPD, preceded by fixation, of botanical specimens gave good results whereas CPD, without prior fixation, gave poor results. In Figure 3 the microstructure of two varieties with very different textural properties were compared using CPD and fixation. Granny Smith is a storage variety and was very firm, crisp and not mealy. In Figure 3a Granny Smith tissue has more fractures through the cells. Rubinette is a new fall variety (Golden Delicious x Cox Orange) and was very soft and mealy (Kellerhals and Hoehn, 1987). Rubinette tissue had more intact cells with fractures around cells rather than through them (Figure 3b), indicating cell separation rather than fracture of cell walls had taken place upon breaking apart the apple cylinder. This micrograph confirms that mealy Rubinette tissue is an independently compiled group of cells with decreased cell to cell adhesion. Glenn and Poovaiah (1990) found similar differences in cell cohesiveness with calcium treated Golden Delicious apples. SEM of Ca-treated tissue having high tensile strength fractured through cells due to strong cell cohesiveness and the open cellular structure was similar to the Granny Smith tissue. In contrast, untreated fruit having low tensile strength separated between cells due to poor cell cohesion and the closed microstructure was similar to the Rubinette tissue in this study.

Cryo-SEM

Microstructural examination of apple tissue in the frozen hydrated state is preferable due to high water content (90%), thin walls and large fragile cells. Hydrated cells, greater depth of field, and freeze-fracturing the cryo-fixed tissue cube by scalpel in the cryo-stage, present a different overall image of the apple tissue surface and microstructure than conventional SEM. Figure 4 shows three magnifications from firm and crisp Granny Smith tissue and may be contrasted with the conventional SEM image of Granny Smith tissue in Figure 3a. At the lowest magnification (Figure 4a) one intercellular space is visible in the center surrounded by densely

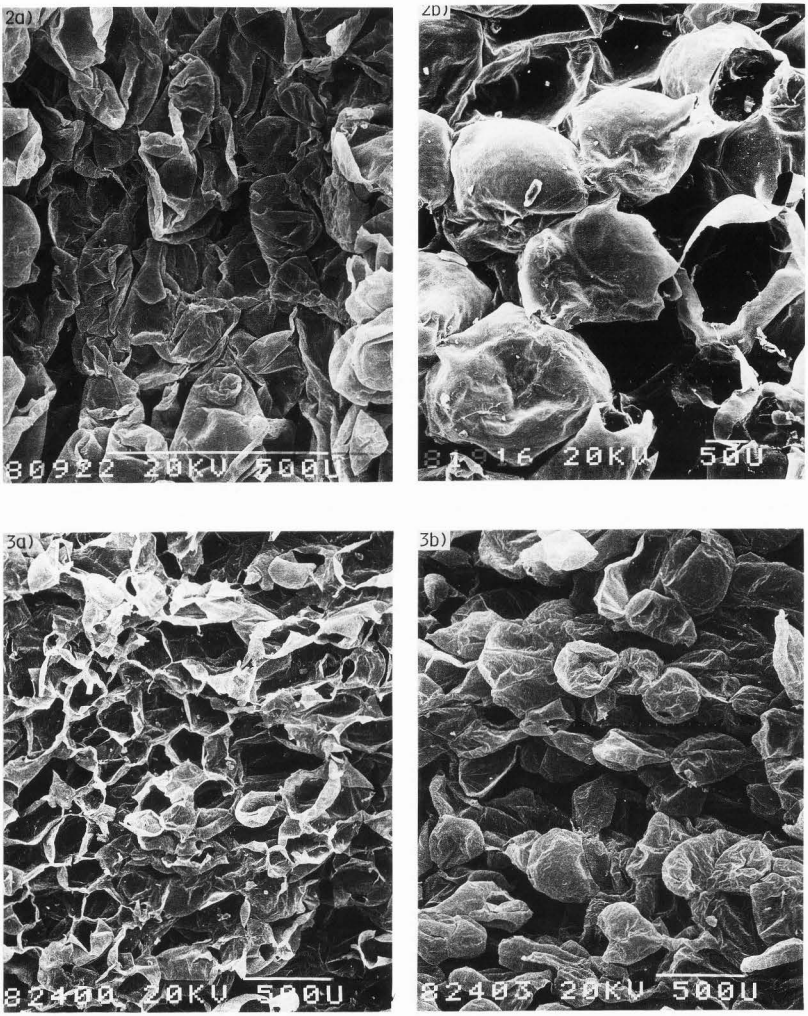
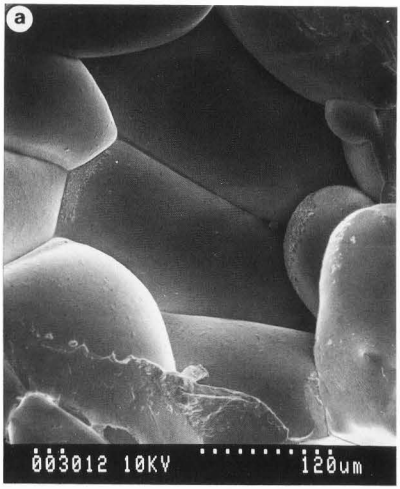
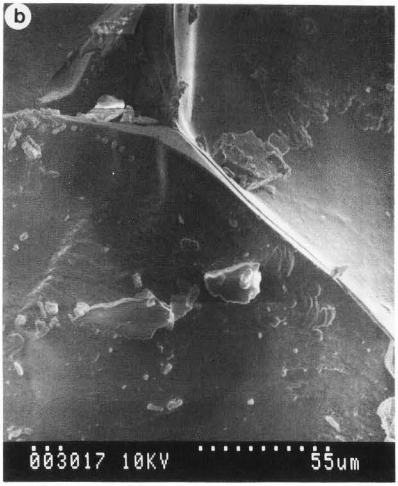


Figure 2. SEM of unfixed (**a**) and fixed (**b**) James Grieve tissue.

Figure 3. SEM of fixed and critical point dried Granny Smith (**a**) and Rubinette (**b**) tissue.





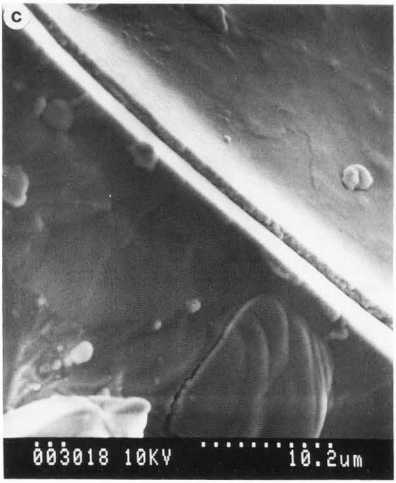


Figure 4. Cryo-SEM of freeze-fractured Granny Smith tissue.

packed hydrated cells. Two higher magnifications of the same tissue (Figures 4b, c) indicate extensive cell to cell contact with cells pressed tightly together. In contrast, Figure 5 is of soft and somewhat mealy McIntosh apple tissue. From two regions of McIntosh tissue (Figures 5a, c) at successive magnifications (Figures 5b, d respectively), weaknesses in the cell walls were observed as spaces between cells, which could result from middle lamella breakdown. Glenn and Poovaiah (1990) used light microscopy, TEM, conventional SEM and cryo-SEM to study the region of cell-to-cell contact in control versus Ca-treated apples after 0 to 6 months cold storage. The cell wall region of Ca-treated apples showed no swelling during storage and cell-to-cell contact was maintained, whereas regions of the middle lamella in untreated tissue stained lightly, appeared distended, and eventually separated. With cryo-SEM untreated hydrated cells appeared intact with cell separation occurring at the middle lamella with minimal tearing. These results indicate that during senescence, and possible development of mealiness, the middle lamella degrades, tissue tensile strength decreases, and cells separate when tissue failure occurs.

Confocal Scanning Laser Microscopy

CSLM of 3 to 5 mm fresh tissue cubes and optical sectioning produced images taken at 10 μm depth from the tissue surface. The fluorochrome used, acridine orange, is protein specific and by staining the cell membrane, the adjacent cell wall was outlined. Fluorescent light images in Figure 6 are from apple tissue of two varieties of similar density but differing textures,

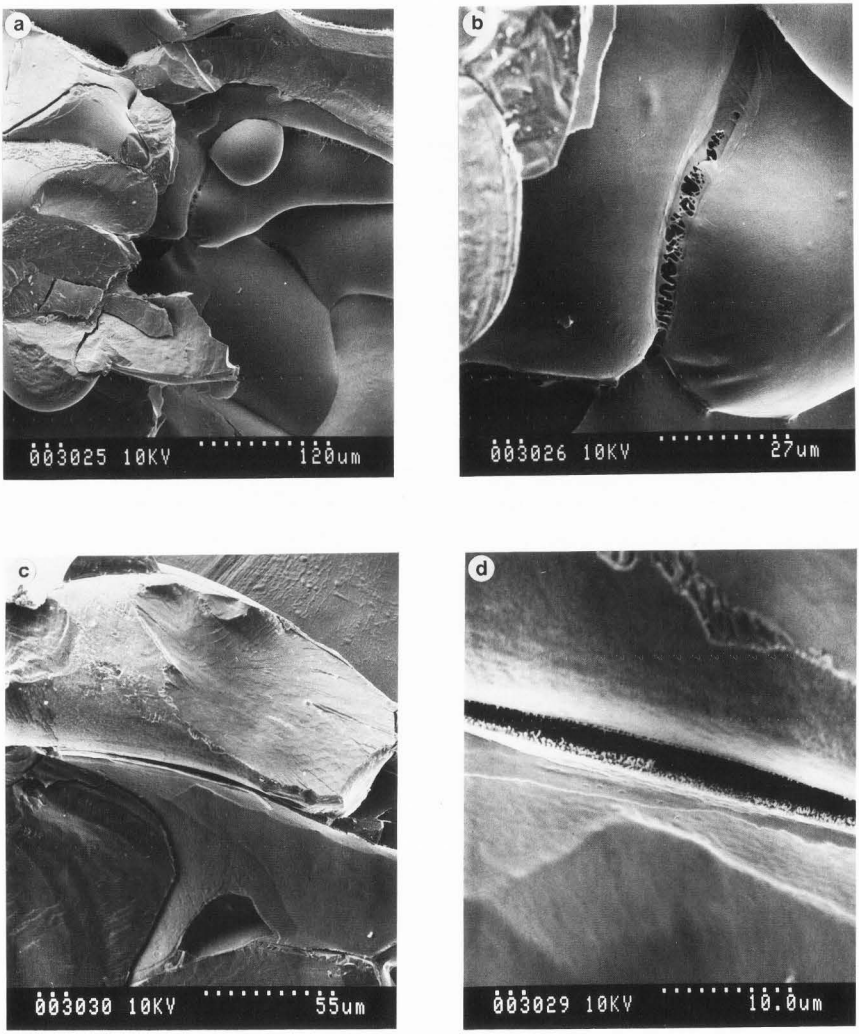


Figure 5. Cryo-SEM of freeze-fractured McIntosh tissue.

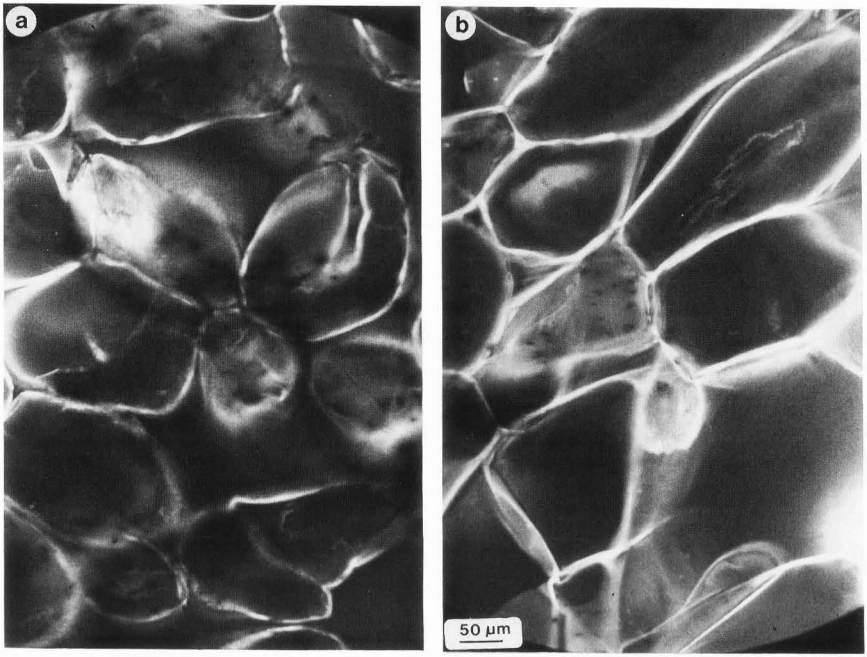


Figure 6. Confocal scanning laser images of Rubinette (**a**) and Granny Smith (**b**) tissue in fluorescence mode.

that were viewed with conventional SEM in Figure 3. Granny Smith were firm, crisp and juicy whereas Rubinette were soft, mealy and dry. In Figure 6a the cells of Rubinette tissue are more rounded with some spaces appearing between cells. Weaknesses at cell junctions may indicate the mealiness perceived sensorially. In contrast, close contact was maintained between cells of the Granny Smith tissue (Figure 6b). The Granny Smith tissue appears more densely packed and cell to cell contact was more extensive, whereas in the Rubinette tissue the cell to cell adhesion was reduced and the individual cells are more separate, as in the conventional SEM micrographs (Figure 3). Although these preliminary results indicated CSLM could differentiate apples of varying textures on the basis of their microstructure, further work should be done to find a fluorochrome in the 450-500 wavelength of the laser light source which is carbohydrate specific, especially for pectins, to better identify cell wall versus middle lamella components. Brooker (1991) has reviewed some of the recent developments in this area.

Conclusions

Use of several tissue preparation and microscopic techniques provided comparative results to evaluate the relationship between textural and structural differences of various apple cultivars but each technique had advantages and disadvantages. Critical-point drying with chemical fixation was the best conventional preparation technique but apple tissue preparation included dehydration which may cause artifacts. Cryo-SEM allowed examination of frozen hydrated tissue but cryofixation may have altered the delicate microstructure. The use of confocal scanning laser microscopy showed the greatest promise since one could visualize to a depth of 100 μm under the surface of a fresh thick specimen with no structure deformation. More research on carbohydrate specific fluorochromes is needed to better identify cell wall versus middle lamella components before this technique could render histochemical results. Microstructural analysis was only one portion of this research. These results indicate that food scientists should have a

better comprehension of the effects of preparatory and microscopy techniques and the number of replicates required before conclusions are made about food structure.

When apple tissue microstructure was examined in conjunction with sensory and instrumental analysis, the characteristics of the fracture plane and the degree of cell to cell contact differed with varying textures. Failure upon mechanical deformation of non-mealy apple parenchyma tissue resulted in rupture through the cells, while in mealy, soft apples, there was disintegration of tissue into individual intact cells or cell agglomerates. Apple senescence researchers have used various terms to explain structural changes they observed. Kovacs *et al.* (1988) called the process architectural weakening. Ilker and Szczesniak (1990) referred to it as intercellular debonding, and Stow (1989) considered the changes in cell to cell cohesion rather than weakening of cell walls. Knee and Bartley (1983) reviewed the cell separation process in senescing apples, disintegration of the middle lamella, and the phenomena of mealiness, but biochemical explanations as to how and why these differences occur in different apple varieties, and in fruits stored under different conditions, are still lacking and need further investigation (Knee, personal communication, 1988).

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Discussion with Reviewers

Reviewer II: Although presenting nice pictures on apple structure, the authors only confirm what was reported by Reeve in 1970. Authors also used different cultivars to study the microstructure of apples prepared by different methods and / or different texture (mealiness). It is difficult to judge whether the texture or combination of texture and cultivar contributed to the observed structural differences!

Authors: Reeve's 1953 paper was a detailed study of cell size and % intercellular spaces for five varieties using light microscopy. Reeve concluded there was no consistent correlation between structural features of cell size or intercellular spaces and the ease of cell separation, and that differences in the metabolism of growth and ripening and the composition of the middle lamella contributed to varying textural qualities (p. 613). The term mealiness was not used. The 1970 paper discussed development of mealiness in some apples in one paragraph (p. 255). He stated: "pectic substances between, as well as within, the matrices of adjacent cell walls form a compound middle lamella. Increases in water-soluble pectins and decreases in the insoluble fractions result in ready cell separation and in the attribute of mealiness. However, many apples and other fruits ripen without becoming mealy". Figure 12 and 13 were light micrographs of apple flesh showing intercellular spaces. There were no micrographs in either publication showing decreased cell to cell adhesion to visually confirm mealiness.

The reviewer's second comment is that we had used different cultivars to study apple microstructure prepared by different methods and/or with different textures and that it is difficult to judge whether the texture or combination of texture and cultivar contributed to the observed differences. The microscopy work was part of a Food Science Ph.D. thesis with the major aim to study the relationship between sensory and instrumental measurement of apple texture. Originally we were of the same opinion as other food scientists that SEM could be used as a tool to visually document apple microstructure. We very quickly learned the importance of the effects of variability and preparative techniques on apple parenchyma tissue and how a microscopist should be involved in initial planning of this type of project.

P. Allan-Wojtas: Why were the apple samples obtained as specified (cylindrical samples, 15 mm in diameter, vertically extracted and manually broken, etc.)? Was the idea to simulate a bite, or to produce a certain type of mechanical failure? Why were the samples not broken by instrumental means (for standardization of procedure) instead? Were samples taken perpendicular to these, and, if so, were they found to behave differently from the samples described in the study?

Authors: An identical, standardized sampling technique was used for instrumental, microscopical and sensory analysis. Previously several researchers had vertically extracted cylinders from individual apples in an attempt to provide as uniform as possible parenchyma tissue from the equatorial region of the flesh, for instrumental testing. Sensory testing was usually done using apple slices. In this study the majority of experimental work was designed so that the instrumental and sensory analysis were conducted simultaneously on the same apple and adjacent cylindrical samples. Panelists were trained to evaluate the four texture parameters with a standard testing procedure for evaluation of each parameter with the same size 15 x 10 mm cylinders as were used for instrumental evaluation. As the accompanying microscopic testing evolved, the microscopists involved suggested standardizing sample extraction as much as possible. Since a variety of microscopes were used, often located in different cities, it was necessary to manually break

the cylinders in two in order to examine fractured tissue surfaces. It was not possible to examine anisotropy of the tissue within the realms of this study.

P. Allan-Wojtas: How many apples (or samples) from each treatment were prepared and/or observed?
Authors: Microscopic testing was always done in duplicate, but not necessarily replicated. Conclusions were not made about a specific technique unless there was a definite trend with several varieties and numerous micrographs. Statistical sampling was done for sensory and instrumental testing only.

P. Allan-Wojtas: How was the degree of cell-to-cell contact measured? Was some equation or calculation used? Were any statistics done?
Authors: Degree of cell-to-cell contact was not measured, only subjectively evaluated after comparing dozens of micrographs. This is an excellent area for further work using image analysis.

E. Kovacs: Do you have any data with using different microscopical methods on the same variety, because it would be very important or do you plan to compare the different methods on the same variety (fresh and stored)?
Authors: It was not within the confines of this study to evaluate the use of different microscopic techniques on the same variety over storage, but it would be an important area to follow up.

E. Kovacs: In this work, did you always use fresh apple?
Authors: Fresh and stored apples only were used in this study.

E. Kovacs: Which preparation method could you recommend for deep-frozen fruits?
Authors: We would recommend cryo-SEM on deep-frozen fruits.

M. Faust: Cells depicted in Figs. 4b, c are totally collapsed and do not show the loose arrangement between the cells. The question is that they are collapsed because the cellulose walls are degraded or because their membrane structure is weakened. Confocal microscopy with acridine orange may give the answer for this.

Fig. 6. The authors describe something that is not readily visible to the reader. I would agree that the cells of "Granny Smith" are longer and appear to have more contact but the difference is slight
Authors: For cryo-SEM the micrographs of Granny Smith and McIntosh have been retained. The authors did not intent to contrast the overall structure of the two varieties but rather the degree of cell to cell contact within the respective tissues. The CSLM images have been used to try to show the degree of cell to cell contact under the surface of fresh intact apple tissue, which is not possible with the other microscopic techniques.