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MICROSCOPIC MEASUREMENT OF APPLE BRUISE

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Abstract

Microstructural differences between unbruised and bruised apple tissues were evaluated. Cell connections appeared to be looser in bruised tissue than in unbruised tissue. Bruised tissue exhibited more empty regions which are not occupied by cells than unbruised tissue. Empty regions in unbruised and bruised tissues were about 0.7 and 2.4 per mm², respectively, comprising 0.7% and 2.7% of the respective total volume. Stereology is a body of mathematical methods relating three-dimensional parameters defining a structure to two-dimensional measurements. Two methods based on a stereological principle were also used to quantify the fraction of total volume occupied by cells. In unbruised tissue, about 99.5% of the volume was occupied by cells compared to only 97.4% in bruised tissue. Both methods successfully quantified microstructural differences between unbruised and bruised apple tissues.

Introduction

Brusing is the damage to plant tissue by external forces which results in physical changes in texture and/or eventual chemical alteration of color and flavor (Mohsenin, 1978; O'Brien et al., 1984). An external force applied to plant tissue initiates bruising which is manifested by a combination of chemical and physical changes (O'Brien et al., 1984).

Goff and Twede (1979) observed that most physical damage to fruits in the retail chain occurred during distribution, which made it difficult to find an unbruised apple in a supermarket. They observed that the incidence of bruising in the retail chain from tree to consumer for 'McIntosh' apples in 3 lb bags ranged from 80 to 100 bruises per bag.

The high incidence of bruise damage in packaged apples and pears was also reported by School and Williams (1972, 1973). They showed that 10-15% and 15-24% of apples were bruised in tray packs and in patterned pack cases, respectively, after a journey of 1600 kilometers and six handling operations.

The most common method to measure bruises on apples, pears and peaches is to measure the diameter, depth, weight or volume of browned tissue (Dedolph and Austin, 1962; Schoorl and Holt, 1974; Mohsenin, 1978; Holt and Schoorl, 1977; Holt et al., 1981; Klein, 1987). The browning reaction in bruised tissue (O'Brien et al., 1984) is an indirect index of mechanical damage. However, direct measurement of bruise size is very subjective due to the difficulty of locating the bruise boundary. There is an intermediate zone where the color changes from white to brown are not clear. A more precise method to evaluate bruise damage is needed.

Electron microscopic techniques have been applied extensively to imaging of animal and plant tissues (Davis et al., 1976a, b; Carroll and Jones, 1979; Chabot, 1979; Davis and Gordon, 1977, 1978, 1980, 1982; Buchheim, 1981, 1982). Many studies with fruits were reported using scanning electron microscopy (SEM) (Gough and Shutak, 1972; Jewell, 1979; Bombein and King, 1982; Glenn et al., 1985; Trakoontivakorn et al., 1988).

Apple tissue bruised by impact became brown and corky during storage not only because of
mechanical damage to cells, but also as a result of enzymatic reactions of polyphenolases and substrates released from ruptured cells (O'Brien et al., 1984). Quantitative differentiation of microstructures between unbruised and bruised apple tissues may be used to follow the progress in the development of the bruise and also can be used to determine the boundary between bruised and unbruised tissues.

'Image analysis' is a numerical method for quantitatively describing geometric or densitometric features of an image (Bradbury, 1979). This method may be applied directly on a specimen or photomicrographs (Underwood, 1969; Bradbury, 1979; Dziezak, 1988). Stereology is a body of mathematical methods relating three-dimensional parameters defining a structure to two-dimensional measurements obtainable on sections of the structure (Weibel, 1980). The method is based on the principle that on a selected phase of a random section through a microstructure, equality exists among volume, area, linear and point ratios (Underwood, 1969).

The objective of this study was to establish an objective method to quantify microstructural differences between unbruised and bruised apple tissues through image analysis.

Materials and Methods

Test Materials

'Rome Beauty' apples were manually harvested from the Northeast Branch of Georgia Experiment Station of the University of Georgia at Blairsville, Georgia on September 29, 1987. They were packed using a shrink wrap machine (Model T14-8, Bestronic, Beseler Corp., Florham Park, NJ) in a gas permeable plastic film (Plastic wrap, D955, CRYOVAC, Simpsonville, SC) and stored at 4°C until evaluated.

Bruise Damage

Five apples were used for this study. Before impact testing, apples were taken from the cooler and unwrapped. Unwrapped apples were impacted at room temperature with a pendulum impactor (Prussia et al., 1987). The apparatus consisted of a 90 mm diameter wooden sphere (135 g) attached to a cord so that the center of the wooden sphere was 1.0 m from the pivot point. The wooden sphere was released from a 45° angle which impacted the apple held at the bottom of the pendulum's swing.

Calculations of Absorbed Energy and Bruise Volume

The amount of energy absorbed by the apples during impact was calculated as the difference between total impact and rebound energy (Prussia et al., 1987).

\[ E_a = m \cdot g \cdot (h_1 - h_2) \]  

where \( E_a \) = energy absorbed (J); \( m \) = mass of the wooden sphere (kg); \( g \) = gravitational constant, 9.8 m/s²; \( h_1 \) = drop height (m); \( h_2 \) = rebound height (m).

After impact, bruises on apples were allowed to develop at room temperature for 24 h. Bruised apples were cut along the stem-calyx axis from the center of the impact point. The radius of apples and the diameter and depth of the browned tissue on the cut surface were measured. Bruise volume was calculated based on the formula described by Holt and Schooler (1977).

Five sections from each apple in the shape of rectangular bars (3 x 3 x 8 mm) were taken starting from the skin and proceeding to the core through the center of the bruised area (Fig. 1). The sections were assigned numbers 1 through 5 from the skin toward core. Sections 1, 2 and 3 were excised from the brown bruised tissue whereas sections 4 and 5 were excised from the unbruised area (Fig. 1).

Sample Preparation for SEM

The excised sections were fixed for 2 h in 2 % glutaraldehyde/0.1 M phosphate buffer (pH 7.2) at 0°C. Fixed sections were washed with 0.1 M phosphate buffer (pH 7.2) for at least 30 min at 0°C and post-fixed in 1% phosphate buffered osmium tetroxide solution for 1 h at 0°C. After another washing step with phosphate buffer, tissues were immediately dehydrated with a series of ethanol concentrations and time as follows: 40% (5 min), 50% (5 min), 60% (5 min), 70% (15 min), 80% (10 min), 90% (15 min), and 100% three times for 10 min each.

The fixed and dehydrated sections were frozen and fractured based on the method described in Humphreys et al. (1974). Fractured specimens were dried using a critical-point drier (Samdri-780A, Tousims Research Corporation, Rockville, MD), and mounted on aluminum stubs with silver paste. Mounted specimens were coated with gold/palladium by a Hummer X Sputter Coater (ANATECH Lim., 5510 Vine St., Alexandria, VA) to a thickness of 40 to 60 nm and stored in a dessicator until examined.

A Scanning Electron Microscope (Model 505, Philips Electronic Instruments, Inc., Mahwah, NJ) with a secondary electron detector operated at an accelerating voltage range between 15 and 20 KeV was employed to examine apple specimens. Specimens were tilted and rotated to obtain uniform scanning and brightness over the viewed specimen surface. Photographs were taken at magnifications to allow viewing a whole specimen under the SEM.

Fig. 1 Excision of specimen from the bruised apple, where shaded area represents a bruise, numbers 1 through 5 represent section 1 to 5.
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Image Analysis

A 20.3 by 25.4 cm print (8 by 10 inch) was developed from the Polaroid (665 P/N Instant Pack Film, ISO 80/20°). Polaroid Corp., Cambridge, MA) negative. Kodak polycontrast RC 11 F print paper was used to reduce the contrast between the bright cell wall area and the dark intracellular area.

Empty regions were defined as regions not occupied or covered by apple cells. They were marked by scratching the areas on the photomicrographs with a sharp needle. The area of each marked region was measured with a digitizer (Hitachi Tablet Digitizer, Model HDG-1118, Hitachi Seiko, Ltd, Japan) connected to a microcomputer (Zenith-200) using a program written in BASIC.

The total number of empty regions (TN) on photomicrographs of each specimen and surface area of the specimen viewed under the SEM were evaluated. Since the viewed surface area varied among specimens, TN was divided by the specimen area and expressed as the number of empty regions per unit area (NPA). Total area of empty regions (TA) was also measured from the photomicrographs and expressed as the total area of empty regions per unit area of specimen (APA).

The volume ratio has been defined as the ratio between volume occupied by cells and total volume and is directly equal to either area or length ratio (Underwood, 1969). For the area or length ratio measurements, test circle was super-imposed at random on the photomicrographs (Fig. 2). Three sizes of circles (diameters of 5.1, 6.4 and 7.6 cm) were tested for consistency in volume ratio measurements.

Volume ratio from linear fraction measurements was calculated as follows:

\[
VRL = \frac{\sum a_i}{L} \times 100
\]

where \( \sum a_i \) is the total distance across intersected cells and \( L \) is the total length on the test line.

VRL was measured from at least ten different locations by randomly super-imposing test circles on the photomicrographs. Consistency of VRL measurements was evaluated by calculating the coefficient of variation (CV) from each size of test circle (SAS, 1985).

The volume ratio is also directly equal to the area ratio (Underwood, 1969). Volume ratio from area fraction (VRA) was calculated from the portion of the area occupied or covered by cells over the total area inside the test line (Fig. 2).

\[
VRA = \frac{100 - \frac{\sum a_i}{A}}{A} \times 100
\]

where, A is the total area inside the test line and \( \sum a_i \) is the area inside the test line which was not occupied by the apple cell. Ten VRA measurements were also obtained from each photomicrograph as described in VRL measurements.

Statistical analysis was performed using ANOVA procedures (SAS, 1985). The effects of apple and section on measured parameters were also analyzed.

Results and Discussion

Measurement of Bruise

Apple diameters and absorbed energies at impact are presented in Table 1. Apple A had a particularly large diameter compared with the other four apples. Apples A and B had slightly higher absorbed energy than the other three apples which was due to the lower angle of rebound during impact. This phenomenon may be due to different textural properties of apples.

Diameter and depth of bruised areas and the calculated bruise volumes for five apples are presented in Table 1. Apple A had particularly large bruise diameter and volume which may be due to the larger contact surface with the wooden sphere. The larger the apple diameter, the greater the contact surface during impact resulting in a larger bruise diameter. Bruise

Table 1. Apple diameter, absorbed impact energy and Bruise measurements of 'Rome Beauty' apples.

<table>
<thead>
<tr>
<th>APPLE</th>
<th>DIAMETER (mm)</th>
<th>ABSORB ED ENERGY (J)</th>
<th>DIAMETER (mm)</th>
<th>DEPTH (mm)</th>
<th>VOLUME (cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>84.0</td>
<td>0.37</td>
<td>27</td>
<td>9</td>
<td>3.6</td>
</tr>
<tr>
<td>B</td>
<td>72.0</td>
<td>0.38</td>
<td>22</td>
<td>8</td>
<td>2.1</td>
</tr>
<tr>
<td>C</td>
<td>68.9</td>
<td>0.36</td>
<td>21</td>
<td>8</td>
<td>1.9</td>
</tr>
<tr>
<td>D</td>
<td>69.6</td>
<td>0.36</td>
<td>20</td>
<td>9</td>
<td>2.0</td>
</tr>
<tr>
<td>E</td>
<td>67.8</td>
<td>0.36</td>
<td>20</td>
<td>8</td>
<td>1.8</td>
</tr>
</tbody>
</table>
Holt and Schoorl (1977) demonstrated that bruise damage expressed as bruise volume was directly proportional to the amount of energy absorbed during impact. The predicted bruise volume for an impact at 0.37 J was reported to be 2.6 cm³ which is close to the measured bruise volume (mean value = 2.3 cm³) from this study even though the apple variety, age and size were different.

Microstructure of Apple Tissues

Typical structural images of five sections are presented in Fig. 3. Cryo-fractured surfaces were even and contained numerous typical open cells. Both unbruised and bruised apple tissues (Fig. 3) exhibited some regions among cells (randomly-shaped black areas on the picture, marked as 'e') that were not occupied by apple cells. Photomicrographs obtained from the first and second sections were from the bruised tissue and contained numerous large empty regions among loosely connected cells which exhibited larger extracellular spaces (Figs. 3A and 3B). The third section (Fig. 3C) was taken from the brown tissue adjacent to the uncolored tissue and had similar numbers of depth was fairly constant and either 8 or 9 mm for five apples.

Fig. 3 Microstructure of apple tissue (A) section 1; (B) section 2; (C) section 3; (D) section 4; (E) section 5. Bar = 1 mm. "e" illustrates empty region.
empty regions as with the first and second sections, but the orderly cell structure remained unaffected. Figs. 3D and 3E were taken from the unbruised tissue and exhibited fewer empty regions and less extracellular spaces than photomicrographs taken from the bruised tissue (Figs. 3A, 3B and 3C). Bruised apple tissue appeared to lose compactness when compared with unbruised apple tissue (Fig. 3).

**Number and Area of Empty Regions**

The number and area of empty regions measured from the photomicrographs of five sections are presented in Table 2. Sections 1, 2, and 3 had 19.7, 18.0, and 14.9 empty regions, respectively; whereas sections 4 and 5 had only 6.3 and 3.7 empty regions, respectively. This indicated that sections 1, 2 and 3 had more damage than sections 4 and 5.

The total number of empty regions per unit area viewed (NPA) obtained from bruised apple tissue had NPA values of 2.41, 2.55 and 2.00 per mm², respectively. Sections 4 and 5 obtained from unbruised apple tissue had NPA values of 0.91 and 0.45 per mm², respectively. Statistical analysis indicated that section 5 exhibited the least NPA among the sections and section 4 exhibited smaller NPA value than sections 1, 2 and 3 (Table 2).

The total areas of empty regions (TA) are presented in Table 2. Bruised tissue exhibited larger TAs than unbruised tissue. Sections 1, 2 and 3 had TA values of 0.24, 0.21, and 0.18 mm², respectively; whereas sections 4 and 5 had TA values of 0.06 and 0.05 mm², respectively. This implies that bruised apple tissue had more areas occupied by empty regions than unbruised tissue.

To account for the variation in the specimen area in view, the percentage of empty area per unit specimen area (APA) was calculated. The greater APA value measured correlated with greater damage in the specimen. Sections 1, 2 and 3 from bruised apple tissue had 2.87, 3.00 and 2.36% of the area occupied by the empty regions, respectively. Section 4 had 0.82% and section 5 had 0.56% of the area occupied by the empty regions in the APA results. The APA results obtained from bruised and unbruised tissues were significant (P < 0.05). However, the difference of APA values within bruised or unbruised tissues was not significant.

Either NPA or APA were found to be useful as an index to distinguish the bruised apple tissues from the unbruised results for each section, however, NPA measurement consumed less time than the APA measurement.

**Size Selection of Test Circle**

Three different sizes of circles were employed to calculate the volume ratio from linear fraction (VRL). Coefficient of variation (CV) is a measurement often used in describing the amount of variation in a population (SAS, 1985). The CV of the VRL for three circles are presented in Table 3. VRL results measured by using a circle with 5.1 cm diameter had the greatest CV whereas VRL results measured by using a circle with 7.6 cm diameter had the smallest CV (Table 3). This implies that the variation among repeated VRL measurements using a 7.6 cm diameter circle was the lowest. Because of the low CV, the 7.6 cm diameter circle was selected for further image analysis on photomicrographs.

For three sizes of circles, VRL results measured from sections 4 and 5 always had lower CV values than those obtained from sections 1, 2 and 3. This implies that the VRL results measured from unbruised apple tissues (fourth and fifth sections) were more consistent than from bruised tissues (first, second and third sections).

**Volume Ratio from Linear (VRL) and Area Fraction (VRA)**

VRL results from five apples measured by using a 7.6 cm diameter were ranged from 98.07 to 99.08% and exhibited no statistical difference (P > 0.05) among apples. The VRA results ranged from 97.67 to 98.36% and also exhibited no statistical difference (P > 0.05).
among apples. This demonstrated that VRL and VRA results obtained from different apples were consistent.

VRL results from different sections are presented in Table 4. Sections 1, 2 and 3 had mean VRL values of 97.88, 97.92 and 97.61%, whereas sections 4 and 5 had 99.66 and 99.62%, respectively. Bruised tissue exhibited an average of 2.2% of the total space not occupied by the apple cells, whereas sections from unbruised tissue exhibited only 0.35%. Standard deviation of VRL from different sections is also presented in Table 4. Sections obtained from the bruised tissue had larger standard deviation than sections obtained from the unbruised tissue.

Table 4. Volume Ratio by Section from Linear and Area Fraction Measurements.

<table>
<thead>
<tr>
<th>SECTION</th>
<th>MEAN VOLUME RATIO FROM LINEAR FRACTION (%)</th>
<th>STANDARD DEVIATION</th>
<th>MEAN VOLUME RATIO FROM AREA FRACTION (%)</th>
<th>STANDARD DEVIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1ST</td>
<td>97.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.83</td>
<td>97.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.91</td>
</tr>
<tr>
<td>2ND</td>
<td>97.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.09</td>
<td>96.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.19</td>
</tr>
<tr>
<td>3RD</td>
<td>97.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.07</td>
<td>96.97&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.99</td>
</tr>
<tr>
<td>4TH</td>
<td>99.66&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.35</td>
<td>99.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.54</td>
</tr>
<tr>
<td>5TH</td>
<td>99.62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.31</td>
<td>99.61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.18</td>
</tr>
</tbody>
</table>

*Mean values of five apples, values in the same column not followed by the same letter are significantly different (P < 0.05).

The mean VRA results and standard deviation obtained from different sections are presented in Table 4. Sections 1, 2 and 3 were obtained from bruised apple tissue and had mean VRA values of 97.22, 96.91 and 96.97%, respectively. Sections 4 and 5 were obtained from unbruised apple tissue and had mean VRA values of 99.25 and 99.61%, respectively. Sections obtained from the bruised tissue exhibited about 3% of the total space not occupied by apple cells (empty regions), whereas sections obtained from the unbruised tissue exhibited only 0.6%. Sections 4 and 5 obtained from unbruised tissue and also had lower standard deviations on VRA results than the bruised tissue (sections 1, 2 and 3). The measured VRL and VRA differences between bruised and unbruised tissues were statistically significant. This demonstrated that the VRL and VRA measurements can be used to distinguish the microstructural difference between bruised and unbruised apple tissues.

From the visual observation of photomicrographs (Fig. 3), specimens obtained from bruised apple tissue exhibited more empty regions than from unbruised apple tissue. Image analysis data support the visual observation and provide numerical results for statistical analysis. Direct measures of empty regions (APA) (Table 2) ranged from 2.36 to 3.00% for bruised tissue and 0.56 to 0.82% for unbruised tissue. Both VRL and VRA results agreed with the APA measurements and can quantify the microstructural difference between bruised and unbruised apple tissues; however, VRA measurements required more time than the VRL measurements.

Major interferences for image analysis on photomicrographs of biological materials are structural complexity and lack of grey level discrimination (Bradbury, 1979; Bolin and Huxoll, 1987). The semi-automatic method (VRL) developed in this study consists of visual observation and computerized data collecting and processing. Structural complexity and different grey levels on photomicrographs were handled manually, but data collection and processing were handled by microcomputer. This method can effectively quantify the microstructural differences between bruised and unbruised apple tissue.

Summary and Conclusions

Apples damaged by a pendulum impactor developed the bruise volumes ranging from 1.8 to 3.6 cm³. Structural images obtained from cryo-facture samples revealed that some regions were not occupied by cells. The bruised tissue exhibited more empty regions than the unbruised tissue based on a visual observation. This criterion was used for image analysis of apple tissues.

There was a clear difference in NPA and APA between bruised and unbruised tissues. NAPAs ranged between 2.00 and 2.55 per mm² for bruised tissues and between 0.45 and 0.91 per mm² for unbruised tissues. APAs ranged between 2.36 and 3.00% for bruised tissues and between 0.56 and 0.82% for unbruised tissues. Statistical analyses of the data showed that both NPA and APA values can be used to quantify microstructural differences between unbruised and bruised apple tissues.

Three different sizes of circles (7.6, 6.4 and 5.1 cm diameter) were used to measure the VRL. The 7.6 cm diameter circle had the smallest coefficient of variation (CV) and is recommended for the volume ratio measurement. There were no significant differences among apples for both VRL and VRA results. This indicates that the measured VRL and VRA among apples were consistent. Both VRL and VRA measured from the unbruised tissue had significantly lower values than the bruised tissue. By using either VRL or VRA measurements, the microstructural difference between bruised and unbruised apple tissues can be quantified and distinguished objectively. However, the VRL method required less measurement time and is recommended.
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Acknowledgment

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References


Discussion with Reviewers

B.G. Swanson: Do you have any evidence to suggest these data will apply to other apple cultivars? Authors: No, we do not believe that degree of structural damage will be the same from one apple cultivar to another; however, we believe that the method developed in this study can be
applied to another cultivar in order to quantify microstructural damage.

B.G. Swanson: Why would you want to differentiate bruised apple tissue by SEM or Image Analysis when we can see discoloration and textural differences?
Authors: Color and texture changes are indirect indicators of bruise damage. However, microscopic observation on bruised apple tissue provides information to support the bruising mechanism that mechanical force ruptures cells to give tissue softness and releases polyphenolases responsible for browning reaction. The microscopic observation on bruised apple tissues in this study provides quantitative data for structural damage and supports the bruising mechanism.

E.A. Davis: Would the authors discuss some of the practical applications in using this technique in evaluating apple damage to the apple industry. Do you think it can implemented in some sort of quality control-decision making capacity? Do you see any reason why this same technique could not be applied to other cellular tissues with similar problems?
Authors: Since SEM sample preparation and semi-automatic image analysis are time-consuming and labor-intensive, the technique developed in this study may not be directly applied to quality control purposes. This study was designed to investigate the bruising mechanism. Similar types of structural damage induced by impact in peaches and pears was reported in the literature. The method developed in this study can be adapted to other cellular tissues with similar problems.

E. Kovacs: In your opinion, which cells are more susceptible to damage? (In Fig 3A, it can be seen, that the damaged cells did not occur systematically)
Authors: Based on our observation on bruised apple tissues, cell damages occurred randomly. It was impossible to notice which cells were more susceptible to mechanical damage.

R.P. Cavalieri: You report that intact cells occupy 2.4% less volume in the bruised tissue. You attribute this to the impact induced bruise. Do you see any evidence of a cell rupture pattern through the tissue from the point of impact or are the ruptured cells more randomly distributed as shown in the figures? If you do see a pattern in some specimens and not in others, can you attribute this to any identifiable difference in structure?
Authors: Cell ruptures occurred randomly without any pattern in all bruised tissues. Based on our observations, distance from the impact point did not affect the degree of structural damage on damaged tissue; in other words, microstructural damages in sections 1, 2 and 3 appeared the same. This was also demonstrated statistically in Table 4.

H.R. Bolin: The bruise, as depicted in Fig. 1, is not a static event, as shown, but is dynamic. How would you envision taking this into account in your "bruised" "not bruised" formula?
Authors: We suppose that the structural damage happens at the time of impact and ruptured cells release enzymes responsible for browning reaction. Released enzymes and substrates play a role for browning reaction. Browning reaction in apple is completed in 12 hours after impact as demonstrated in the literature (Klein, 1987). If our hypothesis is correct, dynamics of bruise is restricted only to color changes which result from enzymatic browning reaction.

E. Kovacs: In your opinion, is bruising dependent on the variety?
Authors: Bruise susceptibility of apple depends on both variety and maturity.