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Physiochemical Behavior Changes During Ripening in the Fruits of *Diospyros Peregrina*

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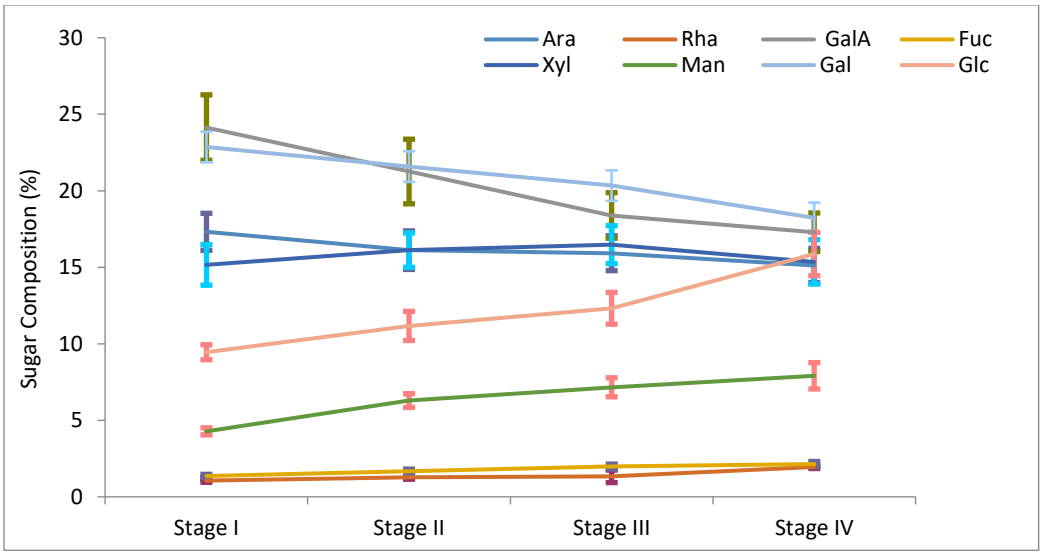
Graphical Abstract



Abstract

Fruit ripening is accompanied by textural changes, which include a loss of fruit hardness and a gradual breakdown of the main cell wall and middle lamella. The crucial stage of ripening is texture change, which results from enzymatic breakdown of the storage polysaccharides and their structure. Pectin and hemicellulose in the cell wall are thought to change as fruit ripens. *Diospyros peregrina* fruit experiments were carried out at different phases of ripening, from the unripe stage to the ripe stage. *Diospyros peregrina* fruits were gathered at various phases of fruit ripening, and changes in physicochemical characteristics, namely pectic, were assessed. *Diospyros peregrina* fruit maturing at various stages had its cell wall structure altered and its tissue stiffness decreased. A decrease in cell wall polysaccharides as acetone insoluble solids, a rise in total soluble sugar with a drop in galacturonic acid, an increase in fruit index, and textural softening were all noted during fruit ripening.

Keywords: Fruit ripening; Cell wall polysaccharides; *Diospyros peregrina*

1. Introduction

Plant cell wall polysaccharides are the most abundant organic compounds; polymers of sugar are the cell wall's main components and form its main structural framework.[1] Polysaccharides are long chains of sugar molecules. The makeup 90% of the plant cell wall can be divided into three polysaccharides: cellulose, hemicellulose, and

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Pectin.[2] Knowledge of polysaccharides' role and behavior during wood and pulp processing is necessary for understanding and controlling these processes.

Studies on the structure and organization of plant cell wall polysaccharides in fruits and vegetables as well as their physiological activities as dietary fibers were made. During ripening, cell wall modifications are crucial for enhancing textural qualities and cell wall-dependent quality traits.[3] However, there are few studies that characterize the neutral sugar content of the main cell wall elements, pectin, hemicellulose, and cellulose, especially for authenticity control. Fruit firmness changes measurement is an excellent way to describe fruit softening. The loss of fruit firmness is a physiological process that occurs during fruit maturation/ripening on the tree.[4]Kader reported that fruit maturity is the most essential term determining storage life and final fruit quality.[5] Immature fruits undergo shriveling and mechanical damage, while ripe fruits not having good flavor quality and were likely to be very soft.

Pectin plays a vital role in the physiological and textural changes during ripening.[6] It has been noted that correlating pectin structure changes with maturity would support elucidating the mechanism of fruit softening at the molecular level.[7]Texture change is the important part of ripening, which occurs due to enzymatic degradation of the structure and storage polysaccharides.[8]All fruit has different specific compositions and nature, so that they have different rates of softening and have varying degrees of sugars.[9] Some fruits like mango, papaya, avocado, and banana undergo drastic and extensive textural softening from the 'stone hard' stage to the 'soft pulpy'stage. The fruit texture changes were affected by several factors, such as primary cell wall and middle lamella, associated with co-extensive polymer networks.[10]

Acid hydrolysis of glycosidic linkages in cell wall polysaccharides has been implicated as the cause of firmness losses after heat treatment of acid fruit and vegetables. According to Doesburg, the process of softening required the hydrolysis of neutral sugar glycosidic bonds.[11] Galacturonans hydrolyze more quickly than neutral sugars because uronic acid was removed from the cell wall. In contrast, in the acidic pH range (pH 2.5-4.5), neutral carbohydrates were discovered in association with pectic compounds.[12]McFeeters *et al*., suggested that pectin hydrolysis at acidic conditions is a possible mechanism involved in softening during ripening.[13]

In ripening fruits, much attention was focused on the loss of neutral sugar side chains from pectin. The cell wall composition of fruits varies from fruit to fruit. Redgwell *et al*., reported that during the ripening of fruits, the cell wall tissue undergoes anet loss of neutral sugars: galactose and arabinose, as shown in several fruits like pear, apple[14], and tomato.[15,16] Even though these results appeared to be familiar to several fruit species, variations in cell-wall compositions could lead to differences in the softening-associated chemical changes for almost every fruit. During ripening, there is a loss of neutral sugar, mainlygalactose, and represented from both the soluble and insoluble pectin fractions of the cell wall.[17]

Pectin are natural, non-toxic polysaccharides and the most complex macromolecule in nature present in the cell wall, i.e., basic building material for plants' cell walls.[18] Pectin is a linear heteropolysaccharide consisting mainly of D-galacturonic acid (GalA) units[19] joined in chains using α (1-4) glycosidic linkage. The carboxyl groups of these uronic acids have partly methanol esterified (1-4)-linked α-D-galacturonic acid with a small fraction of rhamnose, and small side chains might be formed by other neutral sugars such as rhamnose, arabinose, galactose, xylose, and glycose are usually present in 5-10% proportion of the galacturonic acid weight.[20,21] The degree of methoxylation plays a vital role in explaining interactions operating in semi dilute and gelling solutions of these systems.

Pectin revealed an excellent potential for application in the pharmaceutical industry because of its excellent biocompatibility and low immunogenicity, pectin would be rapidly assimilated by the body without being seen as a foreign or intrusive entity. It also serves as a natural, non-toxic diet supplement due to its capacity to absorb water and delay the passage of food through the digestive system. Pectin on adding water and some other substances work as gelling agents, thickener, stabilizers, emulsifiers.[22] Pectin has a wide range of in the cosmetics and food industry because of its gel-forming properties.

Diospyros is a huge genus of trees and shrubs with 500 species that are found throughout the tropics. *Diospyros peregrina* is a member of the Ebenaceae family. Only a few numbers are found in North India; they are usually found in the evergreen forests of the Deccan, Assam, and Bengal. *Diaspyros migrans* (Gaertn.) Gurke, often referred to as Kalatendu, is an ornamental tree grown for its somewhat evergreen shade. It produces velvety fruits and dark green leaves.[23]The most frequent uses of *Diospyros peregrina* (Gaertn.) Gurke (Tinduka) are for the treatment of polyurea and dysentery. It has strong anti-diabetic properties and complements the conventional use of ripe fruits for the management of diabetes.Tree of *D. peregrina* produces about 4000-5000 fruits in a season. The fruits fall to ground from

July onwards under favorable conditions. Fully ripe fruits have a mawkish sweet taste.[24]

Figure 1. Fruits of *Diospyros peregrina* showing different stages of fruit ripening.

2. Material and Methods

2.1 Plant material

The fruits of *Diospyros peregrina* Linn. were freshly harvested from a local Dehradun area in May-June from Dehradun, washed with tap water rinsed with double distilled water, wiped and were stored at -20°C temperature until used. Experiments with *Diospyros peregrina* fruit pulp were conducted at various ripening stages from unripe to the ripe stage. Freshly harvested *Diospyros peregrina* fruits were taken and considered as an unripe stage. In contrast, the subsequent stages of fruits were allowed to ripen on the tree for normal ripening (post-harvesting). The ripening of fruits was post climacteric and ripening as judged by both color development and texture. The four stages of ripe fruits (**Slide 1**) chosen were as follows:

Stage-I. Immature Green

Stage-II. Mature Green

Stage-III. Colour Initiation, Yellowish Green

Stage-IV. Fully Ripe-Reddish Yellow

2.2 Texture measurements

The texture of fruits at all four phases of ripening was measured using a texture analyzer (Llyod Universal Texture Measurement Instrument) to determine the precise variations between the various stages of ripeness. Using a computer-interfaced universal texture analyzer, the three characteristics of penetration, piercing, and compression were used to evaluate the textural qualities. The force (in Newtons) needed by the probe for penetration, piercing, and compression was used to represent the loss in firmness.[25,26,27]

2.2.1 Penetration

The *Diospyros peregrina* fruit's side were penetrated by using a cylindrical 6 mmprobe, which was inserted to a depth of 8 mm with a steady speed of 8 mm/min, and the maximum force required by the probe to penetrate the tissue was recorded (in Newton). One by one, the reading was taken for every six fruits, and the average values were taken to represent the textural value.

2.2.2 Piercing

The sides of the *Diospyros peregrina* fruit were pierced by using a cylindrical 4 mm probe, which was inserted to a depth of 8 mm with a steady speed of 8 mm/min, and the maximum force required by the probe to penetrate the tissue was recorded (in Newton). One by one, the readings were taken for every six fruits, and the average values were taken to represent the textural value.

2.2.3 Compression

The fruits of *Diospyros peregrina* were hand-peeled (control), cut into tissue blocks (10mm×10 mm×10 mm) cubes of fruit were compressed using a 40 mm circular flat plate with a stroke speed of 80 mm/min. For 50% compression, the block, the maximum force required was recorded (in Newton) and expressed the firmness. Forever three fruits, two measurements were taken, and the average values were taken to represent the textural value.

2.3 Preparation of acetone-insoluble solids (AIS)

The extraction of cell wall polysaccharides was carried out by preparing acetone insoluble solids (AIS). The AIS was prepared by fruit pericarp of the fruits *Diospyros peregrina*. Unripe (I) to ripe (IV) fruits were selected for separation and purification of pectic polymers. The fruits were peeled, and 50gm the pericarp was taken. The fruit pericarp was mixed into four volumes (200mL) of acetone (-20ºC) to inactive the endogenous enzymatic hydrolysis and homogenized using a polytronhomogenizer at 15000rpm (PT-MR-2100, Kinematica AG, Switzerland). The resulting slurry was kept at -20°C for 20 minutes for endogenous enzyme inactivity of cell wall material (CWM) and facilitating protein coagulation.[28, 29] The slurry was allowed to be filtered through four layers of micro-cotton cloth. The residue was subsequently washed with 80% 500mL acetone (until the filtrate is sugar-free) and 100% 500mL acetone twice.

2.4 Deactivation of endogenous cell wall degrading enzyme activity

The finally leftover insoluble material above AIS was treated with 10mL PAW/gmtissue (phenol: acetic acid: water in ratio 2:1:1 v/v) at 4°C for 15 minutes with a constant stirrer,[30]4 vol. cold acetone was added, and the final slurry was allowed to filter through GFA paper. The obtained insoluble solids were concentrated and finally washed with 100mL of acetone until a fluffy consistency was obtained. The concentrated extract was dried at 30ºC and stored in a vacuum desiccator for analysis to estimate total soluble sugar anduronic acid contents. The pulp yield of acetone-insoluble solid (AIS) representing cell wall materials (CWM).

2.5 Acid hydrolysis of polysaccharide and preparation of alditol acetate

In cellulose samples and pectin (solid or liquid form), hydrolysis was a very necessary step to prepare alditol acetate. Alditol acetate was prepared by the method as described by Redgwell.[31] For this, 2 mL of conc. sulphuric acid (12*M*) was added to 0.05g (50mg) of the sample and immediately vortexed. Then, the digital water bath was set at 35°C, left the samples on a water bath at 35°C for 1 h and vortexed mixed occasionally, transferred the samples into capped sample bottles (30 mL), and then mixed 22 mL distilled water into capped sample bottles and shake well. Then, set the digital water bath at 100°C, placed the capped sample bottles on the water bath at 100°C for 2 hours to complete the hydrolysis. Started the methodology of preparation of Alditol acetate after hydrolysis; firstly, in the sample, added 3 mL all sugar samples in test tubes and then, mixed 0.5 mL, (1mg/mL) allose (ribose) in 50% saturated benzoic acid, immediately vortex, then, added 0.6 mL ammonium hydroxide (12*M*), vortexed mixture. Added freshly prepared 0.4 mL ammonium hydroxide (3mL) containing 50 mg of sodium borohydride per mL, to the vortexedmixture. Then, set the digital water bath at 40°C, left the samples on a water bath at 40°C for 1 hour and vortexed the mixture occasionally, then added 0.3 mL glacial acetic acid to the vortexed mixture.

In whole reactions, took 0.5 mL samples in another test tubes, then added 0.5 mL 1-methyl imidazole, vortex mixed and added 5 mL acetic-anhydride, vortex mixed. After 10 minutes, added 0.8 mL ethanol and vortexed mixture. After 5 minutes added 5 mL distilled water to the vortexed mixture, and again after 5 minutes added 0.5 mL bromophenol blue indicator (0.04%) and placed in cold water for 10 minutes. Added 5 mL KOH (7.5 M) and mixing by inversion, and after 2 minutes, again added 5 mL KOH (7.5 M), mixing by inversion, there was the formation of brown color layers alditol acetates in the upper part of test tubes.

After that, injected the brown color layers of Alditol acetates with tips and a 100 microlitre micropipette in the 1.5 mL centrifuge tubes. Then, after injection, kept the samples for some time in an open environment or air to concentrate because of the dilution of samples. Alditol acetates were volatile compounds, so they decompose very easily. These were wholly converted alditol acetate compounds.

2.6Determination of galacturonic acid

Take, 0.3 mL of different concentrations (0.01 mg/mL, 0.05 mg/mL, 0.1 mg/mL) of D (+) galacturonic acid

↓

Add, 0.3 mL of a solution containing 2 gm of NaCl and 3 g of boric acid/100 mL in a 50 mL tube and vortexed

↓

Add, 5 mL of conc. $H_2SO_4$and vortexed

↓

Place in a heating block at 70°C, leave for 40 minutes, then cool to room temperature in water

↓

When cool, add, 0.2 mL of di-methyl phenol solution and vortexed immediately

↓

After 10 minutes and within 15 minutes, absorbance was measured by UV-Vis

Spectrophotometer at 400 nm and 450 nm against a water reference

Galacturonic acid was measured using a D (+) galacturonic acid standard that was purchased from Sigma. Galacturonic acid was extracted from the fruit pericarp of *Diospyros peregrina* fruits and measured using AIS following hydrolysis. All the chemicals viz. conc. sulphuric acid, dimethyl phenol, glacial acetic acid, NaCl, boric acid used were of analytical grade reagent.

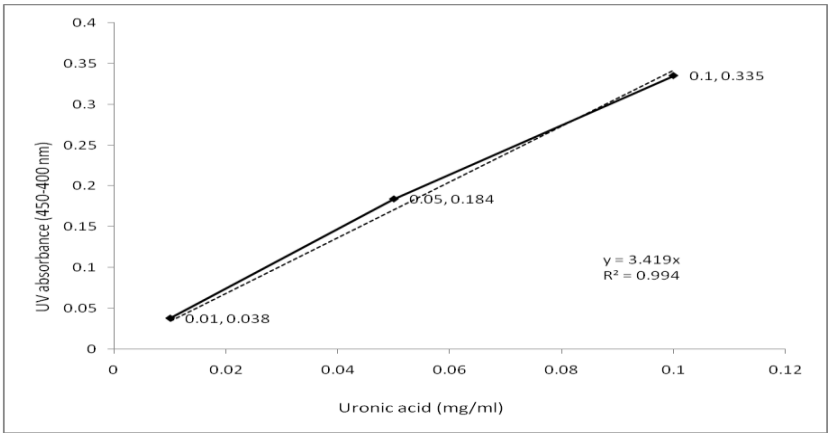

Figure 1. Plot the difference in absorbance between the standards for D (+) galacturonic acid throughout the range in mg/mL. The graph was used to calculate sample concentrations.

Table1. Different quantities of D (+) galacturonic acid standards (0.01 mg/mL, 0.05 mg/mL, and 0.1 mg/mL) were used to create the standard curve at absorbance dilution to ensure that it included no more than 0.10 mg of galacturonic acids.

Sr. No	Conc. (mg/mL)	UV-VIS Absorbance		Optical Density (450nm -400 nm)
		450 nm	400 nm	
1	0.01	0.089nm	0.052 nm	0.036
2	0.05	0.408 nm	0.230 nm	0.185
3	0.10	0.728 nm	0.442 nm	0.338

2.7 Determination of protein content

Protein content was determined by the standard Kjeldahl method (AACC 1990). The conversion factor for protein was 5.95. Protein content was calculated as follows:

$$\text{Protein \%} = \frac{\text{Vol of HCl} \times \text{N of HCl} \times 14.4 \times 5.95}{\text{Weight of Sample}}$$

3. Results and Discussion

3.1 Phenology

The fruits of *Diospyros peregrina* were collected from Dehradun and nearby areas. The plants of *Diospyros peregrina* are unisexual i.e., the male and female plants are separate. The fruits were collected at different stages of fruit ripening Immature Green (Stage I), Mature Green (Stage II), Colour Initiation (Stage III), and Fully Ripe (Stage IV). All the fruit samples were freeze-dried and stored.

3.2 Fruit index

The fruit index (weight of 100 fruits) for the fruits collected at four ripening stages is given below (**Table 2**). The fruit index significantly increased from immature green fruits to mature green fruits, and after that it increased steadily.

Table 2. The fruit index increased from Stage I to II, and after that it increased steadily

Ripening stages	Stage I	Stage II	Stage III	Stage IV
Fruit Index (wt. of 100 fruits)	1470±56g	2243±28 g	2556±65 g	2879±87 g

3.3 Fruit texture

The texture and ripening of fruits of *Diospyros peregrina* were characterized by firmness Fruits' firmness is mostly related to the post-harvesting process of changing turgor pressure (ripening on a tree). Fruit shelf life is affected by the organoleptic properties of texture and firmness during the ripening process. Fruit's firmness is determined by the chemistry of the cell wall. Fruit firmness impacts fruit texture, which in turn affects overall fruit quality and customer acceptance. Research in carbohydrate chemistry, notably on cell wall polysaccharides, particularly pectin and their breakdown, was sparked by the growing interest in manipulating the textural characteristics of fruit.

In the present study, loss of *Diospyros peregrina* fruit firmness was observed during fruit maturation/ripening on the tree. The firmness of fruits is a physiological process during maturation/ripening on the tree. The IInd stage of fruits was considered the phase of rapid change in fruit texture, acquires its maximum at the IIIrd stage of post ripening. The major change in *Diospyros peregrina* fruits during ripening is a loss of firmness. Similar observations are reported in other fruits.[32] Textural properties of fruits were measured by firmness evaluation, which is a key to fruit ripeness. The degree of textural softening and wall alterations during ripening are often correlated with the increasing breakdown and solubilization of pectin at the cell wall. The composition of the fruit's cell walls, in particular, is thought to be responsible for the texture of the fruit. Fruit firmness is most widely determined by simple empirical tests, which involve penetration, piercing, compression, and so on, widely used to determine the fruit's textural properties. Textural softening and changes in cell structure during ripening were reported in many fruits with the help of a texture analyzer.[25,33,34]Textural softening is observed with the distribution of cell wall and middle lamella as reported by the cell wall's swelling and been observed in many fruits such as Kiwifruit. Fruit softening involves swelling the cell wall and is strongly correlated with pectin solubilization.[35]

Diospyros peregrina fruit was subjected to penetration, piercing, and compression during the texture examination (on tissue blocks). At four distinct phases of ripening, it showed a steady reduction in the force needs. Newtons [N] are used to express the firmness data.[36]Between stages III and IV, there was a sharp decline in the force needed to drive a 6 mm probe into the *Diospyros peregrina* fruit, from 200 N at stage I to 50 N at stage IV (150 to 50 N). Compression studies on tissue blocks produced similar results, with the force needed dropping from 250 N to 150 N (from stage I to stage III), and then to 30 N at stage IV. The similar difference in 'piercing' force was observed. As a result, the energy required significantly decreased in stage IV of ripening as opposed to stages I and II, indicating a more pronounced degree of softening at the conclusion of ripening. Fruits like bananas and mangos were discovered to have a similar tissue weakening pattern.[37] Loss of firmness is caused by a change in the organized and compact arrangement of the middle lamella polysaccharides and cell wall.[36]

3.4 Changes in cell wall polysaccharides

Cell-wall polysaccharides include various polysaccharides abundant in fruits and contribute in a substantial way to their texture. Changes in cell wall polysaccharides with special reference to pecticsubstances from the fruits were associated with textural softening, mainly due to cell wall structure and composition changes. Changes in the pectic fraction of the cell wall, significantly increased solubility, depolymerization, de-esterification, and loss of neutral sugar side chains, are reported in many fruits, including tomato,[38,30] Kiwi,[31]apple[39] and bush butter.[40]Cell wall preparations in the form of Acetone Insoluble Solids (AIS) were made, samples were fractionated, and after converting the sugars into volatile derivatives, GLC performed a composition analysis of the monosaccharides in qualitative and quantitative terms. This analysis focused specifically on pectic substances from the fruits of *Diospyros peregrina*.

AIS was prepared from *Diospyros peregrina* unripe and ripe fruit pulp using acetone at -20°C.The % AIS yield, total Carbohydrate content, sugar composition, and protein content is depicted in **Table3**. The deactivation of enzymatic activity was carried out using PAW treatment.

Table 3. Changes in yield and sugar composition of AIS fraction isolated from *Diospyros peregrina* fruit at four ripening stages.

Sample	Yield (a)	Sugar Composition (percentage) (b)								Carbohydrate content (c)	Protein content(c)
		Ara	Rha	GalA	Fuc	Xyl	Man	Gal	Glc		
AIS I	8.9±0.67	17.32±1.21	1.06±0.11	24.13±2.14	1.36±0.11	15.16±1.33	4.28±0.23	22.86±2.21	9.45±0.49	38.38±3.25	14.34±1.24
AIS II	6.8±0.45	16.12±1.26	1.28±0.13	21.26±2.11	1.67±0.14	16.12±1.12	6.29±0.45	21.58±1.78	11.17±.95	33.68±3.14	16.72±1.84
AIS III	4.6±0.53	15.92±1.14	1.34±0.41	18.39±1.49	1.98±0.16	16.48±1.24	7.16±0.62	20.34±2.21	12.32±1.04	27.76±2.28	21.74±2.02
AIS IV	3.8±0.92	15.12±1.12	1.95±0.10	17.29±1.26	2.14±0.17	15.35±1.45	7.91±0.86	18.23±1.65	15.87±1.42	22.63±2.59	23.48±2.32

Ara: Arabinose; Rha: Rhamnose; GalA: Galacturonic acid; Fuc: Fucose; Xyl: Xylose; Man: Mannose; Gal: Galactose; Glc: Glucose.AIS I, II, III and IV are the AIS fractions of *Diospyros peregrina* fruits at different stages of ripening: a Expressed as % of fresh weight pulps; b Expressed as % of total sugar; c Expressed as % AIS (w/w)

The AIS yield decreased from 8.9±0.67% to 3.8±0.92% fresh weight (FW) from unripe to ripe stage, indicating that large acetone-insoluble polymers are degraded to acetone-soluble polymers during ripening and starch to soluble sugars. During ripening, a decrease in AIS and increase in total soluble sugar was reported for many fruits like papaya,[41]strawberry,[42] guava,[43]mango,[44] etc. The carbohydrate content of the AIS was from unripe and ripe fruits were from 38.38±3.25% to 22.63±2.59% (w/w). In addition to carbohydrate content, the AIS fractions contained proteins14.34±1.24% to 23.48±2.32% (w/w) for unripe and ripe fruits. As the fruit ripens, protein content in the AIS of ripe fruit increases due to the generation of more free carboxylic groups (by de-esterification of pectin), which bind more proteins. The remaining part probably contains lignin-like material.[45]

AIS fractions from the fruits of *Diospyros peregrina* at four ripening stages contained arabinose, xylose, galactose, and glucose as the major neutral sugars. Rhamnose, mannose, and fucose were present in relatively small amounts. Arabinose decreased from 17.32±1.21% to 15.12±1.12%; glucose increased from 9.45±0.49% to 15.87±1.42%, galactose decreased from 22.86±2.21% to 18.23±1.65%, mannose increased from 4.28±0.23% to 7.91±0.86%, fucose increased from 1.36±0.11% to 2.14±0.17%, and rhamnose increased from 1.06±0.11% to 1.95±0.10% during ripening. The amount of xylose first increased from 15.16±1.33% (AIS I) to 16.48±1.24% (AIS III) and then decreased to 15.35±1.45% (AIS IV). The amount of galacturonic acid undergoes a consistent decrease from the unripe to the ripe stage from 24.13±2.14% to 17.29±1.26%.

4. Conclusion

Diospyros peregrina fruits were collected at various phases of fruit ripening, and variations in physicochemical characteristics were identified. The textural softening of *Diospyros peregrina* fruit's cell wall carbohydrate polymers is identified in this study as a significant factor. In *Diospyros peregrina*, the fruit index increased as the fruit ripened, softening in texture and losing some of its hardness from immature green fruits to completely ripe fruits. Cell wall polysaccharides as acetone insoluble solids decreased as the fruit ripened, whereas total soluble sugar increased.

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