

Pomegranate's point of harvest through the analysis of bioactive and antioxidant compounds during its development stages

ABSTRACT

Knowing the biochemical and physical-chemical transformations that happen in stage of the fruit development is important to point the maturation rates and to define the point of harvest. In this sense, this work intended to characterize the 'Molar' pomegranate regarding the changes on the bioactive compounds and the antioxidant potential during the phenological stages of its development, as a support to determine its point of harvest. Flowers were marked on the field, in a farm in Várzeas de Sousa, Paraíba, with ribbons of different colors, to count the fruit's age in days after the anthesis (DAA). The harvests to characterize the phenological stages were reaped at 60, 70, 80, 90 and 100 DAA, and those fruits were submitted to the lab, where a randomized experimental design was performed, which treatments were the fruit's ages, counted in days after the anthesis, with four repetitions and five fruit per parcel. 90-day-old fruit display the best quality indicators for the harvest and the commercialization of raw products. At 90 days old, pomegranate cv. 'Molar' reaches the maximum value of soluble solids, SS/TA ratio, total amount of sugars, reducing sugars, ascorbic acid, anthocyanins and antioxidant activity, while it shows a reduction of the titratable acidity, the pH and the phenolic compounds. The ascorbic acid and the anthocyanins were the main bioactive compounds responsible for the pomegranate cv. Molar's antioxidant activity analyzed through the DPPH and ABTS methods.

Keywords: Maturation; antioxidant potential; *Punica granatum* L.

1.INTRODUCTION

The cultivation of the pomegranate tree has a great commercial appeals in the whole world, due to the increase on the demand for raw fruit and for products with functional properties. About 1000 cultivars of *Punica granatum* were identified in the world [30]. The fruit are spherical, with many layered seeds wrapped in pink or carmine pulpy aril [35] They're rich in organic acids, sugars, vitamins, polysaccharides, polyphenols and minerals [1]. Pomegranates are classified as a nonclimacteric fruit, regarding its breathing pattern. Thus, its harvest must be reaped when the fruit reaches full maturity on the plant, when it has the highest quality characteristics [9], since its florescence, maturity and senescence. These changes include physical alterations, and structural, biochemical, and physiological changes, and changes on mineral elements, which reflects on the differences on the fruit's appearance during its maturations, between cultivars [14]. Thus, the harvest time is extremely important, both for its immediate commercialization as a fresh fruit and for its storage, when it's necessary.

The production, commercialization and consumption of pomegranates are growing in the whole world, mainly due to its health-promoting qualities, related to its high concentration of phenolic compounds with antioxidant potential [29, 7].

30 Some of the pomegranate's main phenolic compounds are punicalagins A and B, gallic
31 acid, ellagic acid, flavonoids, vitamin C and anthocyanins that are some of the most
32 important natural antioxidants and are also responsible for the intense red color of the
33 pomegranate juice, is one of the quality parameters that most influence on the sensorial
34 acceptance by the consumers [26, 25, 22].

35 Its juice is considered a source of natural antioxidants, with higher antioxidant potential
36 than other natural juices and drinks, such as green tea and red wine [22, 12].

37 In Brazil, the cultivation of the pomegranate occurs mainly on the states of Paraíba,
38 Pernambuco, Ceará and São Paulo. Semiarid regions favor its cultivation, since it has
39 good aptness in tropical and subtropical climate regions. The spread of the cultivation of
40 pomegranate in Brazil is a fact, but small cultivations, usually made by small producers
41 or by people at home, as an ornamental or medicinal plant, are spread throughout
42 several regions.

43 The characterization of the transformations that occur during the development of the
44 fruits makes it possible to identify the period in which they meet the highest number of
45 characteristics that express the quality, which makes it possible to determine the
46 appropriate collection point for the commercialization in natura or industrial.

47 In this sense, this work aimed at characterizing the 'Molar' pomegranate regarding the
48 changes on the bioactive compounds and the antioxidant potential during the
49 phenological stages of the fruit development, such as the support for the determination
50 of the point of harvest.

51 2.MATERIAL AND METHODS

52 This study was conducted in a commercial orchard of pomegranate cv. Molar that
53 belongs to the Águas de Tamanduá Farm, in Várzeas de Sousa, Paraíba, (longitude
54 38°13'41" and latitude 06°45'33") [21], 30km away from the municipality of Pombal-PB,
55 which production is certified by Associação de Certificação Instituto Biodinâmico (IBD),
56 Law no 10,831 [5]. That area is six years old and has 2,6 ha, established from seedlings
57 produced through seminiferous means. The cultivation area is characterized as a vertisol
58 with clayey texture, which plants were disposed in a triangle-shaped space of 4m x 4m,
59 under an air temperature, a relative humidity (°C) and an average precipitation (mm) of
60 28° C, 68%UR and 250mm, respectively, collected from a meteorological station near
61 the experiment in the city of São Gonçalo-PB [16].

62 At first, healthy, vigorous, adult plants were chosen to mark the flowers using colorful
63 ribbons that resist to high temperatures, insolation, the wind and the rain. By the
64 anthesis time, certified through visual observation, 800 flowers were marked, evenly
65 disposed throughout the orchard. The fruit were reaped and analyzed 60, 70, 80, 90 and
66 100 days after the anthesis (DAA). In each evaluation time, 30 fruit were randomly
67 collected among the marked pomegranates and, immediately after the harvest, the fruit
68 were put in a polystyrene box and disposed in layered, wet paper towels, in order to
69 prevent dehydration, and, soon after, they were transported to the Post-Harvest
70 Technology Lab of the Federal University of Campina Grande (UFCG), Campus Pombal-
71 PB.

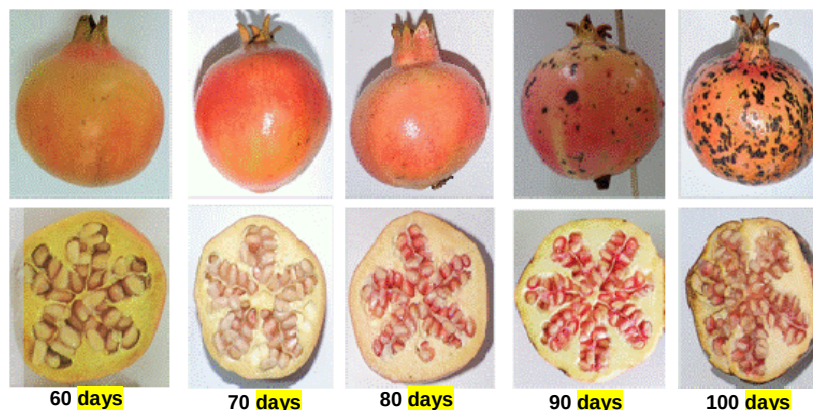


Figure 1. Fruits of the pomegranate (cv. Mollar) at 60, 70, 80, 90 and 100 days, produced at Fazenda Águas de Tamanduá, Várzeas de Sousa-PB, 2015. Source: Own authorship

In the lab, the pomegranates were washed with neutral detergent at 1%, and, after the rinse, they were sanitized with a solution of sodium hypochlorite at 100ppm of free chlorine for fifteen minutes and dried under room temperature. Soon after that, the randomized experimental design was established. Its treatments were the fruit's ages, counted in days after the anthesis, with four repetitions and five fruit per parcel. The arils were separated from the seed through manual pressing in polyethylene plastic bags. After that, the amount of juice extracted from the arils was analyzed.

The following analyses were conducted: Titratable acidity (TA, % of citric acid), conducted through titrimetry using a solution of sodium hydroxide (NaOH) 0,1 N for the sample of 1mL of juice, to which were added 49mL of distilled water and 3 drops of alcoholic phenolphthalein at 1%, standardized with potassium biphthalate [15]; the juice's hydrogen potential (pH) was determined with a pH meter of the brand Tecnozon (mPA model – 210P/Version 7.1), through the direct insertion of the glass membrane electrode in the juice sample [15]; Soluble solids (SS, %): It was determined directly in the homogenized juice, through the measuring in a digital refractometer (PR model – 100, Palette, Atago Co., LTD., Japan), according to the Association of Official Analytical Chemists [3]. SS/TA Ratio: It was conducted by the ratio between the values of soluble solids and titratable acidity; Total sugars (mL 100mL⁻¹), obtained from the extract prepared through the dissolution of 0.2mL of juice dissolved in 100mL of distilled water. Soon after that, an aliquot of 0.2mL of that extract to which 0.8mL of distilled water and 2mL of anthron were added. Those samples were agitated and taken to the double boiler under 100°C for 5 minutes, and the measures were conducted in a spectrophotometer with a wave length of 620nm [35]; Reducing sugars (mL 100mL⁻¹): In order to obtain the initial extract, 0.2mL of the sample that was dissolved in 100mL of distilled water were taken. From that extract, 0.2mL of the sample that was mixed with 1.3mL of water and 1mL of the 3,5-Dinitrosalicylic (DNS), and posteriorly measures in a spectrophotometer at 520nm were conducted [20]; Vitamin C (% of ascorbic acid): it was conducted through the titration with 2,6-Dichlorophenolindofenol (DFI), until the obtainment of a permanent clear pink color, using 1mL of the juice dissolved in 49mL of oxalic acid at 0.5% [3]; Total phenolic compounds (mL 100mL⁻¹): The extracts were prepared from the dissolution of 1mL of juice in 50mL of distilled water and left to rest for 24h. An aliquot of 0.2mL of the juice was transferred to a tube, where 1.925mL of distilled water and 0.125mL of the Folin Ciocalteu reagent were added. That mixture rested for 5 minutes and soon after that, 250µL of sodium carbonate at 20% was added. Then, it was agitated and left to rest in a double boiler at 40°C for 30 minutes. The standard curve was prepared with gallic acid, and the measures were taken in a spectrophotometer at 765nm according to the methodology proposed by Folin and Ciocalteu, described by [33]; Flavonoids and

anthocyanins (mL 100mL⁻¹): 1mL of the pomegranate juice to determine the anthocyanins and 0.5mL for the flavonoids, which were transferred to test tubes enwrapped in aluminum foil. Then, 10mL of the solution for ethanol extraction at 95% with HCl 1,5N at the 85:15 (v/v) ratio, respectively, were added. That sample was homogenized in a Vortex agitator. Then, it was left to rest under refrigeration for six hours, in the absence of light. Posteriorly, the measures were conducted in a spectrophotometer, with a wave length of 535nm, to determine the anthocyanins, calculated through the formula: dissolution factor x absorbance/76.6 [10]; Carotenoids (μL 100mL⁻¹): 1mL of juice, 0.2g of calcium carbonate (CaCO₃) and 5mL of acetone at 80% in a test tube were taken. Then, those samples were centrifuged for 10 minutes at 10°C and 3000rpm. The measures were conducted with lengths of 470, 646 and 663nm in a spectrophotometer, since, in order to determine the concentration of carotenoides, the concentration of chlorophyll a and b in the sample is used [19].

In order to determine the antioxidant potential, two methods were employed: DPPH method (2,2-Dyphenyl-1-picryl-hidrazi) and ABTS method (2,2-Azino-Bis(3-Ethylbenzo-thiazoline-6-sulfonic acid), according with [27] and [28], respectively, with adaptations. The extract was prepared through the dissolution of 1g of pomegranate juice in 49mL of distilled water, left to rest, for, at least, one hour. Then, aliquots of 10 μL, 30 μL and 50 μL were taken from that extract in order to conduct analyses through both methods.

In the DPPH method, the aliquots' volume increased to 100mL with the distilled water, and 3.9mL of the solution of DPPH were add. Posteriorly, the tubes were agitated in order to homogenize them. The measures were conducted in a spectrophotometer at 515nm, and monitored in a span of time ranging from 15 to 20 minutes, which was determined previously according with the treatments. Methanol was used to calibrate the spectrophotometer. All the determinations were made with a third copy and followed by a control (without antioxidant). In order to calculate the values of EC50 (the concentration of the extract needed to reduce 50% of the DPPH radical) of each sample, the antioxidant capacity in different concentrations was calculated, in order to trace a linear curve between the antioxidant capacity of the sample and its concentration. The result was given in g juice g⁻¹ of DPPH.

In the ABTS method, at first, the ABTS radical was formed through the reaction of 7mM of ABTS with 140mM of potassium persulphate, which were incubated under room temperature and in the absence of light, for 16 hours. After then, the solution was diluted in ethanol until the obtainment of a solution with absorbance of 0.70 (± 0.05)nm at 734nm [28]. During the analyses, aliquots of 10μL, 30 μL and 50 μL were taken from the extract and its volume was completed to 100mL with ethyl alcohol and, then, 3mL of the ABTS radical was were added and homogenized in a tube shaker. Posteriorly, after six minutes of waiting, the measuring in the spectrophotometer was conducted at the wave length of 734nm using ethyl alcohol as a solvent. The synthetic antioxidant Trolox was used as a standard solution, at the concentrations of 100; 500; 1000; 1500 and 2,000 μM in ethanol. All the measures was conducted with a third copy, and the results were expressed in μM of Trolox mL⁻¹ of juice. Statistical analysis: The data was submitted for analysis of variance and regression (p<0,05) using the SISVAR software version 5.3 [11].

3.RESULTS AND DISCUSSION

According to the Analysis of Variance, there was a significant effect at the 1% probability level for all the variables studied, except for the antioxidant activity of the juice by the DPPH method, during the development of the fruit of pomegranate cv. Molar that did not differ significantly during the

growth stages of the fruit (Table 1 and 2).

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Table 1. Summary of variance analysis for the variables: titratable acidity (AT), pH, soluble solids (SS), SS / AT ratio, total sugar, Reducing sugars and vitamin C in 'Molar' pomegranates during fruit development.

FV	GL	Quadrado Médio						
		AT	PH	SS	SS/AT	Total sugars	Reducing sugars	Vitamin C
Idade dos								
Frutos	4	0,06728**	0,038832**	4,48583**	54,483632**	15,1890**	0,00070**	18,9810**
Resíduo	15	0,010065	0,004075	0,275425	4,880303	1,4252	0,000002	0,3955
CV (%)		13,83	2,08	4,1	11,94	8,42	13,78	7,66
Média		0,72	3,06	12,81	18,49	14,18	0,0104	8,20

168 **Significativo a 1% de probabilidade, pelo teste de F.

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170

171 **Table 2.** Summary of variance analysis for the variables: Phenolic compounds ,
172 Flavonoids, Anthocyanins, Carotenoids, DPPH and ABTS
173 in 'Molar' pomegranates during fruit development.

FV	GL	Quadrado Médio					
		Phenolic compounds	Flavonoids	Anthocyanins	Carotenoids	DPPH	ABTS
Idade dos							
Frutos	4	38,1648**	68,8261**	4,9612**	0,1630**	3814323,03ns	582076545,62**
Resíduo	15	34,7142	4,0844	0,6527	0,01119	1701292,9	29357570,45
CV (%)		9,52	14,75	19,61	26,15	51,88	22,58
Média		61,86	13,70	4,11	0,40	2514,32	23992.99

174 **Significativo a 1% de probabilidade, pelo teste de F

175

176 Titratable acidity presented a value variation between 0.61% and 0.92% of citric acid, at
177 60 and 80 days, respectively. Posteriorly, a decreased was observed. There were values
178 around 0.66% of citric acid at 100 days old (Figure 2A), in concordance with the behavior
179 observed by [31] and [23]. Those authors indicate the classification of the pomegranate's
180 sweetness as the cultivars' fruit that present less than 1% of acidity.

181 The hydrogen potential (pH) had little variation during the phenological development
182 stages of the fruit, with values ranging from 2.9 to 3.1 (Figure 2B). A similar behavior
183 was observed in the pomegranate's pH by other authors. [8], studying the chemical
184 properties of the pomegranates cultivated in South Africa, observed pH values ranging
185 from 3.32 to 3.64. [21] characterized the quality of the 'Molar' pomegranate during the
186 raw fruit storage under different refrigeration temperatures, and they found out pH values
187 ranging from 3 to 4, throughout the storage.

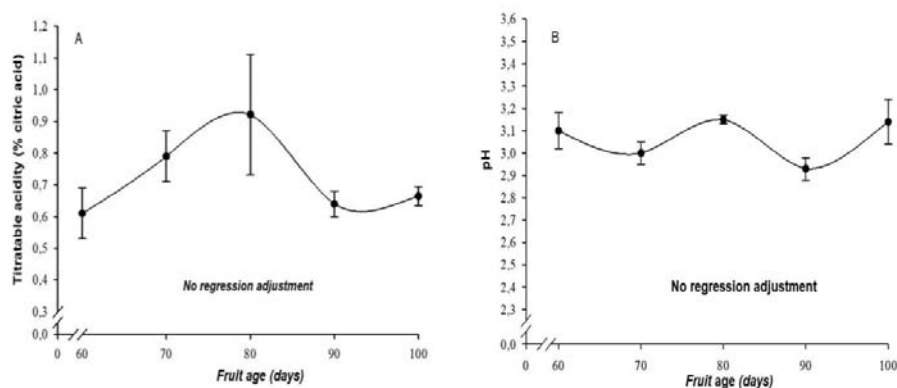


Figure 2. Titratable acidity (A) and pH (B) in the aril of the 'Molar' pomegranate analyzed during the phenological stages of the fruit development.

The soluble solids (SS) had increase between 11% and 14.05, and between 70 and 100 DAA, respectively, corresponding to an increase of 21.7% (Figure 3A).

The soluble solids present in the fruit pulp include important compounds responsible for the taste and the consequent acceptance by the consumers. The most important are sugars and organic acids, and the soluble solids content is the parameter of greater ease of indirect determination of sucrose [34].

[31] report an amount of SS ranging from 12% to 15% in 'Molar' pomegranate tree fruit stored under room conditions. Those values are in the range identified by this work. When [13] evaluated 'Ciparski', 'Konjski zub' and 'Pastun' in Southern Dalmatia, Neretva valley, in Croatia, they described SS values ranging from 13.1% to 15.6%.

The alterations in SS and TA values reflected on the SS/TA ratio (Figure 3B), which varied from 14.42 to 21.30, and the highest SS/TA ratio happened after 90 days, when TA was low and SS was high, which may reveal a sweet fruit to the consumer, agreeing with [31] and [23]. According to [9], the SS/TA ratio is an important indicator about the pomegranate maturation, with values that range from 3.73 to 86.3, depending on the cultivar and the maturation stage.

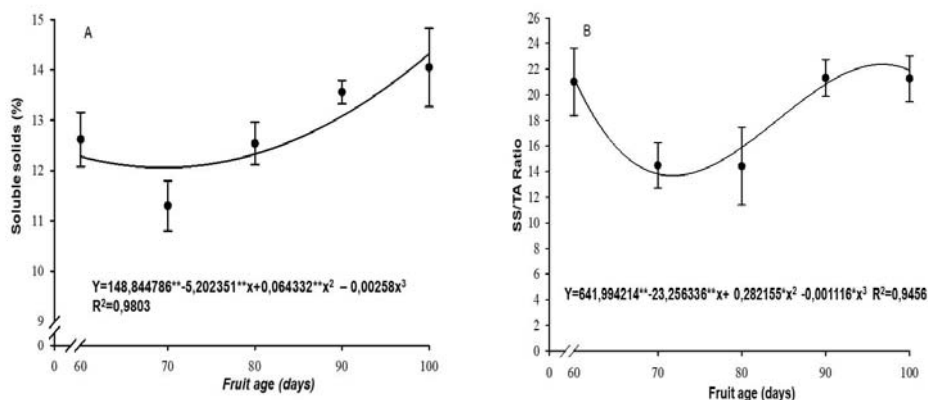


Figure 3. Soluble solids (A) and SS/TA ratio (B) in the 'Molar' pomegranate aril analyzed during the phenological stages of the fruit development.

Total sugars gradually increased to 90 DAA, and even presented a maximum value of $16.70 \text{ mL} \cdot 100 \text{ mL}^{-1}$, with a posterior reduction to 100 DAA (Figure 4A), probably due to the natural senescence of the fruit in the plant, intensifying the consumption of carbohydrates as a source of energy. Reducing sugars presented a similar behavior. They also reached a maximum concentration of $0.016 \text{ mL} \cdot 100 \text{ mL}^{-1}$ at 90 DAA and consequently a reduction at 100 DAA (Figure 4B). Generally speaking, reducing sugars in the pomegranate were very low. Probably, the Molar cultivar has that characteristic, which can indicate a prevalence of non reducing sugars to the detriment of the reducing sugars.

[18] report that differences on the fruit maturation levels causes distinct sugar concentrations. [24] reported an average of 6.4% to 6.8% of fructose and glucose, respectively, in the pomegranate juice in six cultivars ('incekabuk Dikenli', 'Eksi', 'Kan', 'Katirbasi', 'Serife' and 'Tatli') in Turkey. [9] reported an increased on the glucose and fructose concentrations during the fruit maturation, with glucose to fructose ratios ranging between 0.67-0.85 and 0.72-0.86 for the 'Bhágwa' and 'Ruby' cultivars, respectively grown in South Africa.

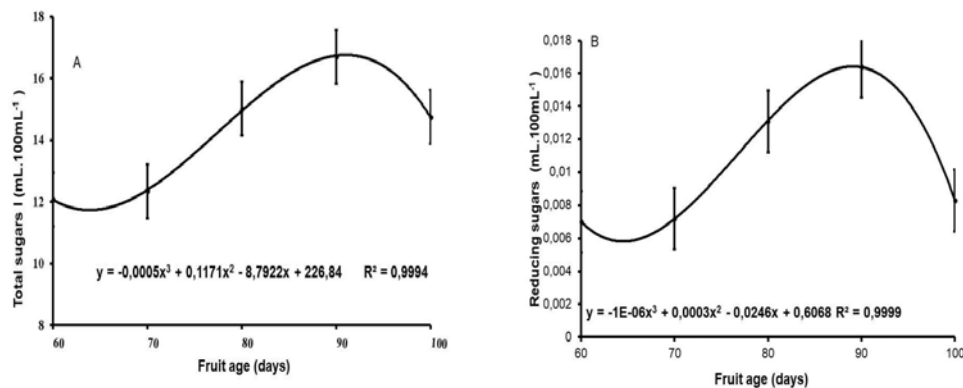


Figure 4. Total sugars (A) and reducing sugars (B) in the 'Molar' pomegranate aril analyzed during the phenological stages of the fruit development.

Vitamin C increased from 60 to 90 DAA, leaping from 4.75% to 10.5% of ascorbic acid, respectively (Figure 5A). From 90 DAA on, as seen with the sugars, there was a reduction, intensifying the possibility of the fruit being in process of senescence at 100 DAA. The phenolic compounds increased from 53.61 to 83.13 $\text{mL} \cdot 100 \text{ mL}^{-1}$, from 60 to 70 DAA, respectively, with a posterior decline to 90 DAA, followed by a small increase to 100 DAA (Figure 5B). This last one was an indicator of the fruit natural senescence. That behavior shows clearly the evolution of the fruit maturation, since in the beginning of the pomegranate development, up to the 80 DAA, agreeing with the phenolic compounds, the highest TA values were observed, but the lowest values for SS, total sugars and reducing sugars. With the evolution of the maturation, the phenolic compounds decreased and reached the lowest values at 90 DAA, when TA decreased, and SS, total sugars, reducing sugars and vitamin C reached the best levels, which might indicate lower astringency and the ideal time for the harvest, regarding the quality characteristics of the pomegranate for it to be consumed raw.

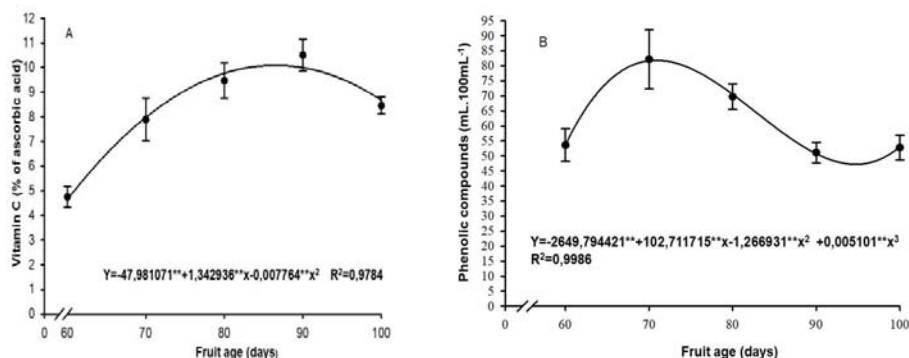


Figure 5. Vitamin C (A) and phenolic compounds (B) in the 'Molar' pomegranate aril analyzed during the phenologic stages of the fruit development.

In concordance with the behavior observed on the phenolic compounds, the flavonoids presented the highest values in the beginning of the pomegranate maturation and decreased linearly with the advance of the of fruit's age (Figure 6A). Anthocyanins increased in between 60 and 90 DAA, like the color transformations on the aril, which are typical for the Molar cultivar, evident at 90 DAA (Figure 6B), the red-pink tonality. Values higher to those found in this study were reported for the 'Wonderful' pomegranate, at the point of harvest, 5.56-30.11mL.100mL⁻¹ [32] and 226.27mL.10mL⁻¹ [2], in Iran. According to [4], the concentration of anthocyanin can be influenced by the climactic conditions during the fruit development and maturation. Thus, the anthocyanins concentration can increase or decreasing according to the climate to which the cultivars are submitted.

Carotenoids also displayed a behavior similar to that displayed by the phenolic compounds and flavonoids. That is, the highest values registered were those by the youngest pomegranates, up to the 80 DAA. Then, the highest concentration of that pigment was registered: 0.58μL.100mL⁻¹ (Figure 5C). However, [6] report that anthocyanins are the main water-soluble pigments responsible for the pomegranate juice's bright red color.

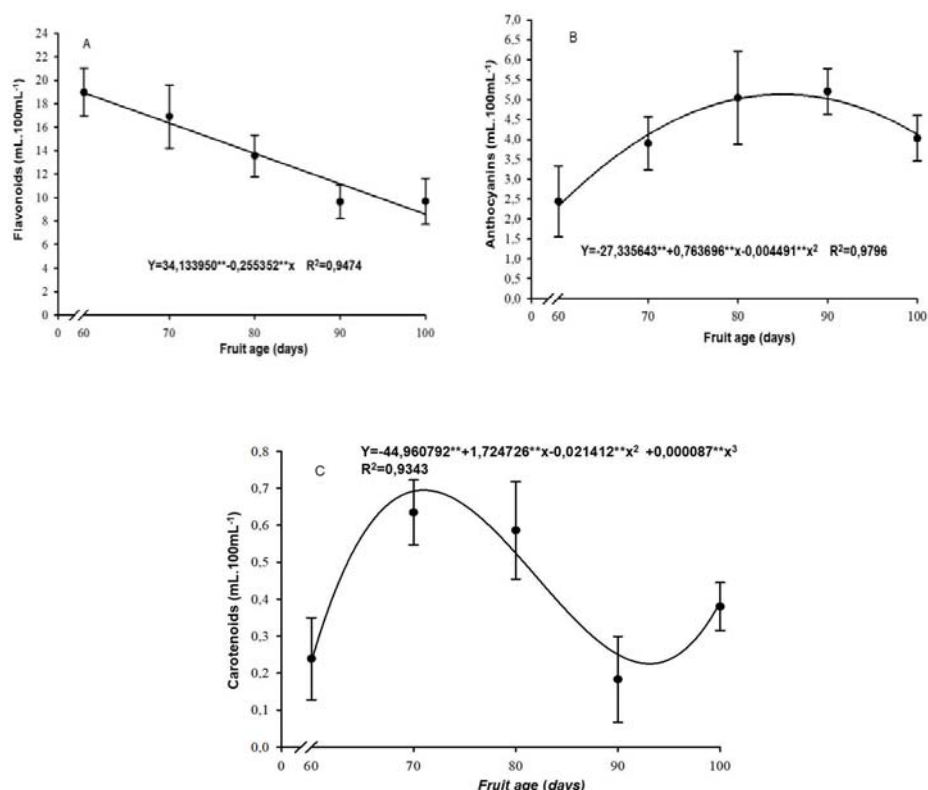


Figure 6. Flavonoids (A), anthocyanins (B) and carotenoids (C) in the 'Molar' pomegranate aril analyzed during the phenologic stages of the fruit development.

According to Figure 7A, an increase in the antioxidant capacity until 90 days old was registered. Fruit reaped at 60 days after the anthesis presented a lower antioxidant activity, with an IC₅₀ value of 1407.17g.juice g⁻¹. DPPH presented the highest antioxidant activity. Regarding the antioxidant capacity of the ABTS method, values of 40,188.13uM Trolox.mL⁻¹ of juice at 60 days and 10,546.52uM Trolox.mL⁻¹ of juice at 90 days, displaying, thus, a higher antioxidant activity at 90 days old (Figure 7B).

The highest antioxidant activity of the fruit at 90 DAA ratifies with the highest values registered at 90 DAA, for ascorbic acid and anthocyanins, respectively. [17], studying the main chemical alterations, and the antioxidant activity and its importance during the pomegranate development also describe a lower antioxidant activity on the pomegranate fruit at 60 days old. Those authors justify that that low activity can be due to a reduced concentration of phenolic compounds and ascorbic acid in the juice.

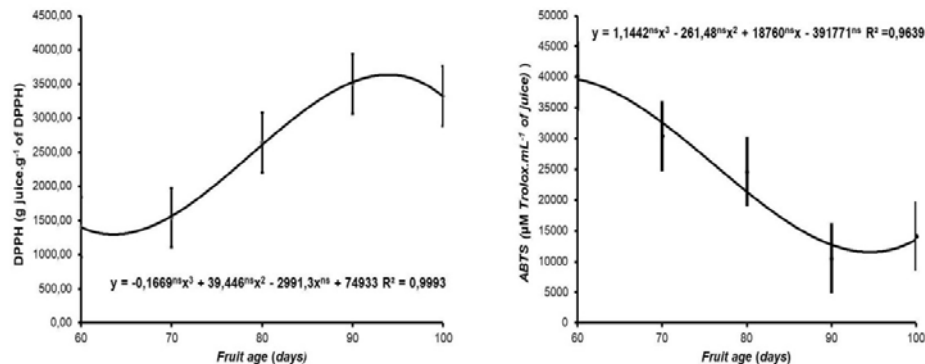


Figure 7: Antioxidant activity, DPPH (A) and ABTS (B) methods for the ‘Molar’ pomegranate aril analyzed during the phenologic stages of the fruit development.

4.CONCLUSIONS

The ‘Molar’ pomegranate produced in Várzeas de Sousa, PB, reaches its best quality indicators for the harvest at 90 DAA. Then, the fruit present the highest rates of soluble solids, total sugars, reducing sugars, vitamin C and anthocyanins, and the highest antioxidant activity.

Ascorbic acid and anthocyanins are the main bioactive compounds responsible for the antioxidant activity in the pomegranate cv. Molar analyzed through the DPPH and ABTS methods.

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