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Physical and Biochemical Evaluation of Blueberries during Long-term Frozen Storage at -20 °C: Implications for Shelf-life and Quality Preservation

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ABSTRACT

Blueberry (*Vaccinium spp.*) is well-known for its high antioxidant capacity and exceptional richness of vitamins, fiber, and polyphenols. Due to its bioactive compounds, flavonoids, tannins, and phenolic acids, blueberry possesses various health benefits, such as anti-diabetic, anti-cardiovascular, and anti-carcinogenic properties. However, its limited production period and short shelf-life generate tremendous post-harvest loss. In this context, post-harvest treatments are necessary to extend the shelf life of blueberries and promote their consumption throughout the year. Thus, this work aimed to evaluate changes in the physical and biochemical quality of blueberries stored at -20 °C for three months. The results showed that one month of frozen storage increased the anthocyanins content in blueberries and preserved all other characteristics. The biochemical fruit quality improved in the second month, while the titratable acidity, pH, total soluble solids, and weight remained preserved. However, at the end of the storage period, there was a continual rise in anthocyanins and flavonoids, whereas polyphenols and antioxidant activity remained stable, surpassing the levels found in fresh blueberries. A notable decline in physical characteristics was observed. In this context, it is recommended to store blueberries at -20 °C for one month at most to maintain fresh consumption quality. Blueberries stored for over two months must be intended for processed blueberry-based products to fully utilize their richness in phenolic compounds and avoid issues related to their sour taste and reduced weight.

Abbreviations: Antioxidant activity (AA), Phenylalanine ammonia-lyase (PAL), Titratable Acidity (TA), Total Anthocyanins Content (TAC), Total Flavonoid content (TFC), Total Polyphenol content (TPC), Total Soluble Solids (TSS)

Introduction

Blueberry (*Vaccinium* spp.) belongs to the Ericaceae family, specifically to the genus Vaccinium, which contains approximately 450

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species (Edger et al., 2022). Since prehistoric times, the blueberry originated from a spontaneous shrub native to the northern regions of Europe and North America. Its distribution expanded in cultivation, regions with mild winters, such as Mediterranean countries (Edger et al., 2022; Pistón et al., 2017). In the last decade, the importance of blueberry species has grown due to their recognized health benefits, including antidiabetic, anti-cardiovascular, and anticancer potential (Silva et al., 2020). These properties are attributable to various bioactive compounds such as flavonoids, tannins, and phenolic acids (Seeram, 2008; Smith et al., 2000). Also, blueberries have a high antioxidant capacity against free radicals and reactive species (Rodriguez-Mateos et al., 2012; Silva et al., 2020; Veberic et al., 2015; Vrhovsek et al., 2012).

However, blueberries are renowned as perishable fruits due to their short shelf life and limited production period, resulting in notable postharvest loss (Duan et al., 2022). Thus, postharvest treatments are necessary to overcome these wastes, including cold storage, heat treatments, irradiation, etc. (Mahajan et al., 2014).

The present work aimed to study the impact of a specific postharvest treatment, specifically the storage of blueberries at -20 °C for three months. The objective was to consider their physical and biochemical characteristics as they changed in storage at the subzero temperature.

Materials and Methods

Plant materials

The plant materials in this study originated from an estate in the Kenitra region in Morocco. The fruits of the "Southern Highbush" cultivar were hand-harvested in March 2017 based on their external appearance: fully colored berries (90%-100%) and absence of visible lesions and rot. Subsequently, the samples were partitioned into three freezer bags, with each containing 42 g of the sample. We stored the samples at -20 °C for three months and took a sample for physicochemical and biochemical analysis at the end of every month.

Phytochemical analysis

Total soluble solids (TSS)

The TSS was measured using a refractometer (ATAGO C.O Ltd; Model PR-1). The results were expressed as °Brix.

pH

Freshly extracted blueberry juice was measured for pH using an electronic pH meter.

Weight

Total weight was counted using a precision electronic scale. The results were expressed in grams.

Titratable acidity (TA)

The titratable acidity was carried out based on a protocol described by Nielsen et al. (2017). Five g of freshly extracted juice from each sample was added to 25 mL of distilled water. The process involved 0.1 N (NaOH) to neutralize the acids present in blueberry juice until the pH reached 8.1.

The results were expressed as citric acid (%) (Nielsen, 2017).

$$\% \text{ TA} = \frac{\text{V(NaOH)} \times \text{Conc (NaOH)} \times \text{K} \times 100}{g \text{ of the sample}}$$

Where V (NaOH) is the required volume of NaOH in mL to reach pH 8.1.

Conc (NaOH) is the NaOH concentration.

K is the specific coefficient of the dominant acid in blueberry juice (citric acid) equal to 0,064 (Adi et al., 2019).

Biochemical analysis

Extract

The extraction involved adding five g of blueberries to 25 mL of methanol and a drop of HCl (0.1% hydrochloric acid). The mixture was stirred using an Ultra Turrax for 15 min and then centrifuged for 15 min at 6000 rpm. Subsequently, methanol was evaporated using a rotary evaporator.

Determination of total polyphenol content (TPC)

Total polyphenols were determined using the Folin-Ciocalteu method (Sánchez-Rangel et al., 2013). The extract was diluted 100 times (D100: 9.9 mL of methanol mixed with 0.1 mL of the sample). Then, 0.25 mL of the sample was added to 0.25 mL of Folin-Ciocalteu reagent, 2 mL of distilled water, and 0.25 mL of sodium carbonate (20%). The mixture was vortexed, and after 30 min of incubation in darkness, the absorbance of the samples was measured using a spectrophotometer at 750 nm. A calibration curve with different concentrations of gallic acid was prepared. The total phenolic content was expressed as milligrams of gallic acid equivalent per gram of fresh sample weight (mg GAE g^{-1} FW).

Determination of antioxidant activity (AA)

The antioxidant activity was evaluated using the DPPH method, based on a procedure described in

previous studies (Brand-Williams et al. 1995; Mensor et al., 2001). A volume of 0.1 mL of the extract was added to 3.9 mL of DPPH solution (6 mg 100 mL^{-1} in 80% methanol) with a dilution factor of 100 (D100). After incubation in darkness for 30 min at room temperature, absorbance readings were taken at 515 nm using a spectrophotometer.
The inhibition percentage was calculated using the following formula:

$$\mathbf{I}\ \% = \frac{(\mathbf{Ac} - \mathbf{As}) \times \mathbf{100}}{\mathbf{Ac}}$$

Where Ac is the absorbance of the control (DPPH).
As is the absorbance of the sample (DPPH with the added test sample).
A calibration curve of Trolox solutions was used and the results were expressed as mg of Trolox equivalent per gram of each sample (mg TE g^{-1}).

Determination of total flavonoid content (TFC)

The total flavonoid content (TFC) was assessed according to a method defined by Lamaison J. and Carnat (1990). A volume of 1 mL of each sample was added to 1 mL of AlCl3 (MeOH). The absorbance was read at 510 nm. Rutin generated a calibration curve, and then the results were expressed as milligrams of rutin equivalent per gram of each sample (mg RE g^{-1}) (Lamaison J. and Carnat, 1990).

Determination of total anthocyanins content (TAC)

A procedure for determining anthocyanin content was based on a protocol described by Giusti and Wrolstad (2001). Two buffers were employed, i.e., a potassium chloride buffer at pH 1.0 and a sodium acetate buffer at pH 4.5. Accordingly, 0.4 mL of the diluted extract was separately mixed with 3.6 mL of each of the two buffers, and then the absorbance was measured by a spectrophotometer at 510 nm and 700 nm. (Giusti and Wrolstad, 2003)
The final absorbance value was obtained by subtracting the measured absorbance at different wavelengths.

$$Abs = (Abs_{510} - Abs_{700})\ pH_1 - (Abs_{510} - Abs_{700})\ pH_{4.5}$$

The results were expressed as micrograms of cyanidin-3-glucoside equivalent per gram of each sample (mg CGE g^{-1}) and were calculated using the following equation:

$$\text{TAC (mg CGE } g^{-1}) = \frac{\text{Abs} \times \text{DF} \times \text{MW} \times 100}{\text{MAC}}$$

Where Abs is absorbance.
DF is dilution factor (100).
MW is the molecular weight of cyanidin-3-glucoside (449,2) (Olivas-Aguirre et al., 2016).
MAC is the molar absorptivity of cyanidin-3-glucoside (26.900) (Young and Abdel-Aal, 2009).

Statistical analysis

All results were statistically conducted using IBM SPSS Statistics 20 software. Both one-way and multi-way analysis of variance (ANOVA) and the Student-Newman-Keuls (SNK) tests were utilized for specific mean comparisons. Statistical significance was considered per treatment group ($p<0.05$). Three replicates were considered for each analysis, and the results were expressed as mean values ± standard deviation (SD).

Results

Biochemical characteristics

Total polyphenol content (TPC)

Average polyphenol content in the fresh blueberries was 2.631 ± 0.168 mg GAE g^{-1}. After one month of freezing storage at -20 °C, TPC remained statistically stable ($p = 0.906$), indicating that no polyphenol loss occurred in blueberries stored at -20 °C for 30 days. After two months of cold storage, we noticed a significant increase compared to fresh fruits. After three months of frozen storage, the polyphenol content continued to rise in a non-significant manner ($p=0.147$), reaching its highest value at 11.366 ± 1.1621 mg GAE g^{-1} (Fig. 1).

Antioxidant activity

The antioxidant activity in blueberries exhibited a similar trend of changes as observed in polyphenol content. The results showed that during the first month of the storage period, the antioxidant activity remained at the same level ($p=0.228$). In the second month, the antioxidant activity increased by 54.57% compared to the berries in their initial state. At the end of freezing storage, a highly significant increase (53.99%) was recorded, compared with the initial fresh berries. The value slightly increased in a non-significant manner compared to the previous month ($p = 0.468$) (Fig. 2).

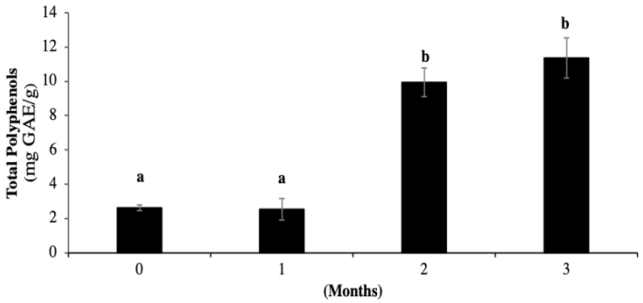

Fig. 1. Comparative analysis of polyphenol levels in three months of frozen storage. a and b are different letters that indicate significant differences ($p<0.05$)

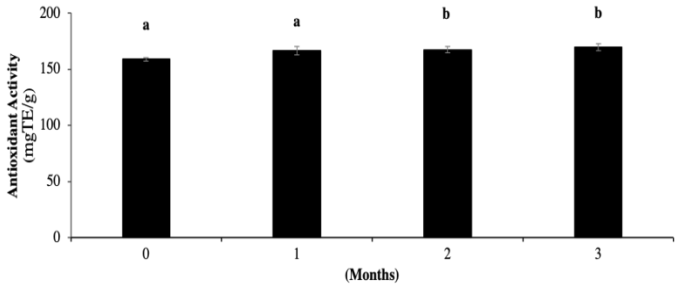

Fig. 2. Changes in antioxidant activity during the three months of frozen storage. a, b: different letters indicate significant differences ($p < 0.05$).

Total flavonoid content (TFC)

Changes in total flavonoid content in blueberries stored at -20 °C showed an interesting trend over time. After one month, blueberries showed a non-significant rise in flavonoids ($p = 0.078$) compared to the fresh samples. However, remarkable changes were detected throughout the latter part of the freezing storage period. A significant increase occurred at the end of the second month. The flavonoid content increased from 2.46 ± 0.221 mg RE g^{-1} to 4.47 ± 0.177 mg RE g^{-1} compared to the previous month. Meanwhile, the flavonoid content of blueberries stored for three months reached a value of 5.172 ± 0.097 mg RE g^{-1}, which denotes a rise of 146% compared to fresh fruits (Fig. 3).

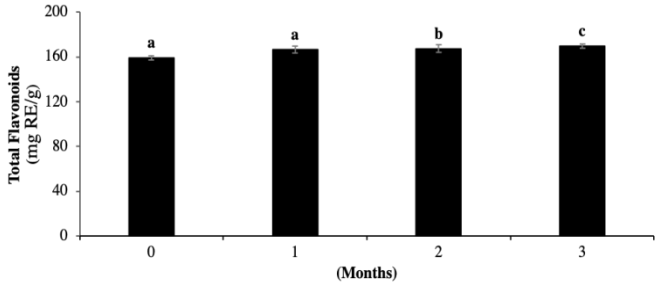

Fig. 3. Variations in total flavonoid content across three months of frozen storage. a, b: different letters indicate significant differences ($p < 0.05$).

Total anthocyanin content (TAC)

The TAC improved significantly by 5% during the first month of frozen storage. However, no significant changes were observed during the second month. By the end of the frozen storage, a significant increase (7%) occurred compared to the fresh berries, reaching a value of 169.744 ± 3.200 mg CGE g^{-1} (Fig. 4).

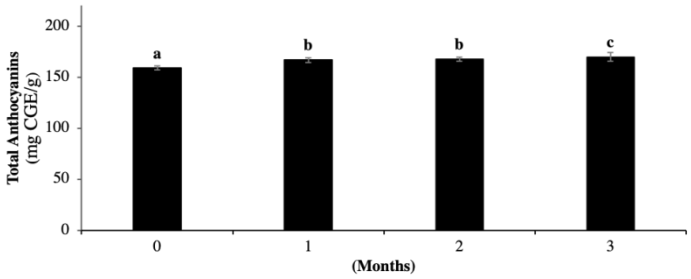

Fig. 4. Changes in anthocyanin levels over three months of frozen storage. a, b: different letters indicate significant differences ($p<0.05$)

Titratable acidity (TA) and pH level

As illustrated in Figure 5, the acidity of frozen blueberries increased through the storage period. However, these changes were not significantly different over the first two months ($p = 0.164$). Conversely, a statistically significant rise was observed at the end of the third month of frozen storage.

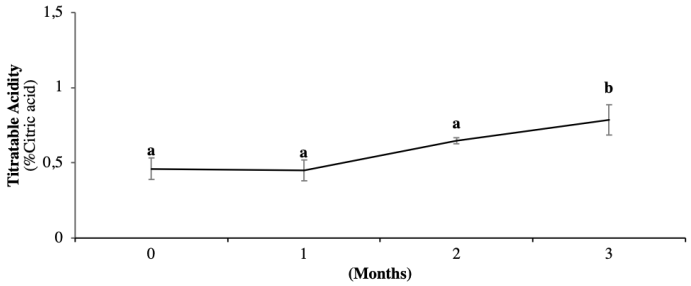

Fig. 5. Changes in titratable acidity over three months of storage at -20 °C. a, b: different letters indicate significant differences ($p<0.05$).

This increase in sour taste was supported by a decrease in pH value. After one month of frozen storage, the pH of blueberries decreased insignificantly from 3.28 ± 0.014 to 2.81 ± 0.035 (Fig. 6). After three months, a significant decrease of 38% was observed compared to the fresh fruits, reaching a minimum value (2.6 ± 0.060).

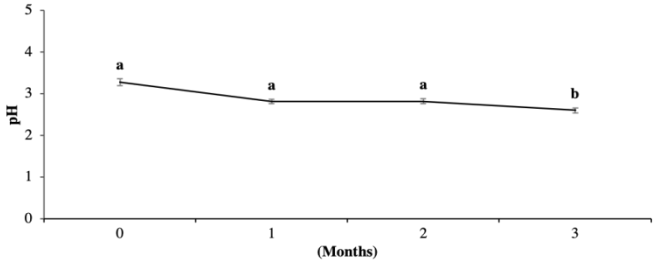

Fig. 6. Changes in pH throughout the three months of frozen storage. a, b: different letters indicate significant differences ($p<0.05$).

Total soluble solids (TSS)

The total soluble solids content in fresh blueberries was 14.5 ± 0.707 °Brix. However, this value decreased by 12% after 30 days of frozen storage (-20 °C) and remained significantly stable throughout the second month. Then, a notable reduction (33%) in soluble solids content was observed, reaching a minimum of 9.75 ± 0.071 °Brix (Fig. 7).

Weight

According to Figure 8, storing blueberries at -20 °C significantly preserved their weight until the end of the second month. At the end of the

third month, a significant decrease (13.26%) was observed compared to the fresh fruits.

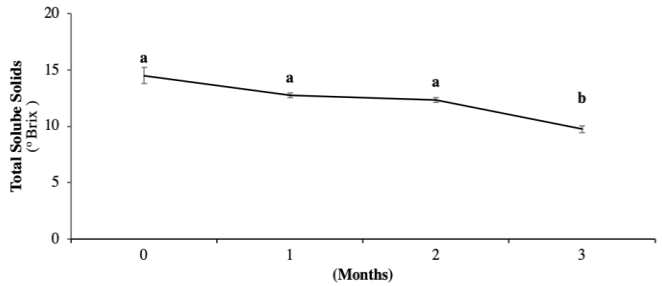

Fig. 7. Changes in total soluble solids (TSS) during the three months of storage (-20 °C). a, b: different letters indicate significant differences ($p<0.05$).

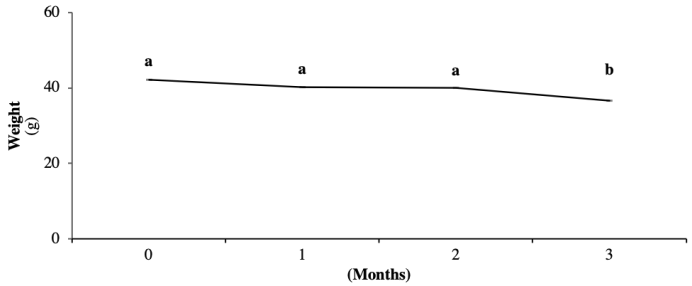

Fig. 8. Changes in weight during the frozen storage of three months. a, b: different letters indicate significant differences ($p<0.05$).

Discussion

Blueberry is a perishable fruit with good health benefits and high economic value. Its beneficial effect emanates from its richness in polyphenolic compounds and antioxidant potential. The total polyphenol content in this research ranged from 2.631 ± 0.168 to 11.366 ± 1.162 mg GAE g^{-1}, which are higher than the values found by Al Hasani et al. (2023). The latter reported an average of 2.09 mg GAE g^{-1} of total polyphenol content (Al Hasani et al., 2023). Indeed, based on the criteria established by Vasco et al. (2008), the researchers classified fruits into three categories having a low (<1 mg GAE g^{-1}), medium (1–5 mg GAE g^{-1}), or high polyphenol content (>5 mg GAE g^{-1}) (Vasco et al., 2008). Accordingly, the berries herein are considered a promising source of polyphenolic compounds. Moreover, the fresh blueberries in this research showed a degree Brix of 14.5 ± 0.707, exceeding the range reported by Matiacevich et al. (2013), which varied between 10.33 and 13.39 depending on the blueberry cultivar (Matiacevich et al., 2013). Nevertheless, it is essential to note that at postharvest, the fruit undergoes a process of senescence and quality deterioration, leading to a rapid loss of its biochemical properties, lower nutrient values, and a shortened shelf life (Tietel et al., 2012). Thus, implementing effective postharvest treatments such as storage conditions could significantly preserve the biochemical properties of blueberries and, consequently, maintain their health benefits (Tietel et al., 2012; Skrovankova et al., 2015).

In the present research, the study of frozen storage on the quality of blueberries illustrated an increase in biochemical properties, including TPC, AA, TAC, and TFC, by the end of the third month of the storage period. As for the physicochemical characteristics, notable changes occurred after the storage duration, with a rise in titratable acidity. This increase in sour taste was simultaneous with a decrease in pH, TSS, and weight.

Our findings align with some previous studies and reports. For instance, Reque et al. (2014) found that the antioxidant activity in blueberries increased significantly during three months of frozen storage at -18 °C, followed by a decline until the end of six months of storage, while maintaining a value greater than that of the initial (Reque et al., 2014). A parallel pattern occurred in research by Bakowska-Barczak and Kolodziejczyk (2008). The authors noticed an increase in the total anthocyanins in three different Saskatoon berry cultivars stored at -20 °C for nine months. The researchers associated this rise with changes in cyanidin-3-arabinoside in the fruits (Bakowska-Barczak and

Kolodziejczyk, 2008).

Indeed, the increase in bioactive compounds (TPC, AA, TFC, TAC) can stem from reactions that continue to occur in the postharvest stage (Piljac-Žegarac and Šamec, 2011). For example, the increase in phenylalanine ammonia-lyase (PAL) was reportedly due to low-temperature stress (King et al., 2022). Based on reports by other authors, this enzyme (PAL) plays a crucial role in the phenylpropanoid pathway. It activates the production of various beneficial compounds, such as anthocyanins and flavonoids in fruits. These bioactive compounds possess antioxidant properties that assist in preserving nutritional quality. Therefore, PAL potentially enhances berry flavor and health benefits (King et al., 2022; Chen et al., 2006). Another possible explanation could be the degradation of cell structures due to thawing the fruit before analysis, resulting in an easy and abundant extraction of these compounds (De Ancos et al., 2000). Some studies suggest that the increase in anthocyanins might result from the co-pigmentation mechanism (Horbowicz et al., 2008). In freezing conditions, transparent co-pigment cooperates with anthocyanins, increasing their stability while intensifying their color. This process highly maintains and amplifies the anthocyanin content in fruits throughout storage. Indeed, 84% of the total antioxidant activity stemmed from anthocyanins, making them the main contributors (Skrovankova et al., 2015). Therefore, increased anthocyanin levels would raise the antioxidant activity and explain the results of this research.

On the contrary, some studies have shown that frozen conditions may either preserve or exert some adverse effects on fruit quality and functional properties. For example, anthocyanin levels remained statistically unchangeable in highbush blueberries (*Vaccinium corymbosum* L.) stored at -18 °C and -35 °C throughout the storage duration of six months (Scibisz and Mitek, 2007). Samec et al. (2015) revealed different fluctuating outcomes of 10 small fruits stored at -20 °C for one year. The authors reported that many fruits maintained the TPC, TFC, and TAC after 12 months of frozen storage, whereas the hawthorn and strawberry fruits showed a decrease in most biochemical components (Šamec and Piljac-Žegarac, 2015).

Moreover, González et al. (2003) evaluated the effect of long-term frozen storage at -24 °C for 12 months on four different raspberry cultivars. During the first three months, the TPC in the 'Heritage' variety showed a significant decrease, while the 'Autumn Bliss' cultivar increased by 11% compared to fresh fruits. The TPC in the 'Zeva' and 'Rubi' varieties remained unchanged (González et al., 2003). Therefore, the diverse fluctuations in bioactive compounds greatly depend on the type of fruits, the cultivar, and the storage conditions, including the temperature and duration (González et al., 2003).

Likewise, other studies highlighted differences in outcomes compared to our results. Chaovanalikit and Wrolstad (2004) illustrated a significant reduction in the antioxidant activity of 'Bing' cherries stored at -23 °C after three months. However, a freezing temperature (-70 °C) enhanced the antioxidant potential by 14% (Chaovanalikit and Wrolstad, 2004). González et al. (2003) found a significant reduction in anthocyanins in four raspberries stored at -24 °C for three months (González et al. 2003). Vollmannová et al. (2009) also showed a decline in anthocyanins in most blueberry cultivars stored for three months at -18 °C (Vollmannová et al., 2009). In addition, Sahari et al. (2004) reported a similar drop in anthocyanins in strawberries stored at different temperatures (-12, -18, and -24 °C) for three months. The same study illustrated an increase in pH values, reaching 3.4 at -12 °C, potentially contributing to the observed decrease in anthocyanin content (Sahari et al., 2004), as suggested by various studies (Sablani, 2015; Wu et al., 2004). Also, Sistrunk et al. (1983) noted that anthocyanins degrade in fruits with a pH above 3.4 (Sistrunk et al., 1983). In contrast, the current research showed a decrease in pH levels by the end of the examined frozen storage, which might explain the increase in anthocyanin content. It could explain how frozen storage affects anthocyanin content in multiple ways, depending on the crop, cultivar, storage duration, temperature, and pH value (Sablani, 2015).

On the other hand, the increased titratable acidity content in our study may arise from the release of additional acids associated with cell wall softening and degradation (Hossain et al., 2014). In contrast, Sahari et al. (2004) showed that the acidity in strawberries varied during storage at different temperatures. At -12 °C, the acidity significantly declined over 90 days, reaching the lowest value (1.08). At -18 °C, the decline reached 1.15 after 60 days before remaining constant. However, a temperature of -24 °C retained the acidity stable after 30 days, and then a gradual decrease in a non-significant manner occurred (Sahari et al., 2004).

However, the decrease in total soluble solids could be due to a loss of water-soluble compounds due to the freezing and thawing process (Pistón et al., 2017). Similar findings by González et al. (2002) revealed a significant drop

of 4% in soluble solids in two different raspberry cultivars, 'Rubi' and 'Zeva,' stored at -24 °C for one year (González et al., 2002). In contrast, the same study revealed the opposite results in other cultivars, 'Heritage' and 'Autumn Bliss,' demonstrating a significant increase of 16% and 4%, respectively (González et al., 2002). Therefore, the total soluble solids strictly depended on the cultivar, environmental circumstances, and storage conditions (González et al., 2003). Similarly, the drop in weight at the end of the storage period can emanate from the loss in fruit water content, thus leading to an increase in the concentration of anthocyanins and phenols in blueberries (Ktenioudaki et al., 2021). The highest phenolic compounds in this research appeared in blueberries stored for three months but with the lowest fruit weight.

Conclusions

Blueberries at freezing temperatures (-20 °C) stored for three months resulted in a noteworthy enhancement of polyphenols, antioxidant activity, flavonoids, and anthocyanins content. This low-temperature storage maintained the titratable acidity, weight, and pH values for 60 days and more. By the end of the third month, a decrease occurred in the weight, pH, and total soluble solids accompanied by an increase in the titratable acidity. Therefore, we recommend using blueberries stored for more than two months in processing methods to fully exploit their richness in phenolic compounds and avoid issues related to their sour taste and reduced weight. In addition, further research can explore specific mechanisms underlying the changes in bioactive compounds during frozen storage, with potential health benefits and applications of frozen blueberries.

Conflict of Interest

The authors indicate no conflict of interest in this work.

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