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The Application of Polyamines by Pressure or Immersion as a Tool To Maintain Functional Properties in Stored Pomegranate Arils

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Pomegranate fruits were treated with putrescine (Put) or spermidine (Spd) at 1 mM either by pressure infiltration or by immersion and then were stored at 2 °C for 60 days. Samples were taken biweekly and were further stored 3 days at 20 °C for shelf life study. The treatments were effective on maintaining the concentration of ascorbic acid, total phenolic compounds, and total anthocyanins in arils at higher levels than in control samples. In addition, the two ways of polyamine application increased the levels of total antioxidant activity (TAA) during storage, especially when polyamines were applied by pressure infiltration. Moreover, Spd showed the best results on increasing TAA through maintenance of total phenolic compounds.

KEYWORDS: Pomegranate; polyamines; total antioxidant activity; polyphenols; anthocyanins; ascorbic acid

INTRODUCTION

The pomegranate (*Punica granatum* L.) is grown in many countries of the Mediterranean Sea. It is considered one of the oldest known edible fruits with evidence from the Late Bronze age (*1*). Pomegranates are usually consumed as fresh seeds (arils), which contain around 80% of juice and 20% of seed. The juice is rich in several nutrients, namely, sugars, organic acids, vitamins, and minerals (*2*), although great variations depending on cultivar have been reported (*3*). Although pomegranates have been used broadly in the folk medicine of many cultures (*4*), other properties have been claimed recently, such as antioxidant and anticancer activities and protective effects against atherosclerosis, among others (*5*, *6*). Different compounds have been addressed as contributors to the antioxidant capacity of pomegranate. In juices, galloylglucose, ellagic acid, and two anthocyanin glucosides (delphinidin and cyanindin) have been correlated to antioxidant activity (*7*). Another compound with high antioxidant capacity is punicalagin, which can pass to juice from the pith and carpellary membrane during the extraction process (*8*).

It is well-known that during postharvest important quality loss occurs, the main problem being desiccation and browning in both peel and arils, which increases with storage temperature below 5 °C (*9*). However, storage at higher temperature leads to reduction of shelf life by acceleration of the ripening process, desiccation and decay occurrence, which makes necessary the storage at low temperatures. Moreover, losses of anthocyanins and ascorbic acid were reported in both arils and juices (*10*, *11*) that would reduce the potential antioxidant activity. The use of modified atmosphere packaging (MAP) either to husks or arils could delay in part the losses of anthocyanins (*12*, *13*).

The naturally occurring polyamines (putrescine, Put; spermidine, Spd; spermine, Spm) are involved in many developmental processes, and in the case of fruits, the application of exogenous polyamines had led to improve their shelf life (*14*). Thus, exogenous application of Put (usually under pressure infiltration) induced low softening and delayed color changes in lemon, peach, apricot, and plum (*15*−*18*). These effects clearly retarded the ripening process rendering fruits more attractive and palatable, and it seems related to their antisenescence properties. The application of polyamines (1 mM) is considered as nontoxic since concentrations over 2000 mg/kg body weight for Put and 600 for Spd and Spm have been reported as toxic (*19*).

Recently, we have demonstrated that exogenous application of polyamines delayed the maturation process by reducing softening, the increase of the ratio °Brix/acidity and the loss of weight (*20*). In addition, polyamine treatment retarded chilling injury symptoms (skin browning and increased electrolyte

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leakage). Thus, storability and shelf life could be extended in pomegranate stored at low temperatures that would usually develop chilling injury. However, as far as we know, there is not literature available about the effects of polyamines on the chemical composition related to functional properties of the arils. In this sense, the aim of this work was to study the effect of the application of Put or Spd by pressure or immersion on the compounds with functional properties (ascorbic acid, total phenolic compounds, total anthocyanins, and total antioxidant activity) of pomegranate stored at 2 °C followed by 3 days at 20 °C.

MATERIALS AND METHODS

Plant Material and Experimental Design. Pomegranates (*Punica granatum* L. cv. Mollar de Elche) were picked on November 2005 from a commercial orchard in Orihuela (Alicante). This cultivar is late ripening with delicious sweet arils and soft stones. The arils represented 60% of the whole fruit and contained an average moisture of 75%. Fruits were harvested when fully mature according to commercial practice and were immediately transported to the laboratory. Pomegranates with defects (sunburn, crack, bruise, and cut in the husk) were discarded. The remaining fruits were randomized and divided into six lots of 125 fruits for the following treatments in quintuplicate (each replicate contained 25 individual fruits): Half lots were treated by pressure with 1 mM putrescine or 1 mM spermidine or with distilled water, which served as control. Treatments were performed by pressure infiltration adapted from previous experiments in plums (*15*, *18*), with fruit in 20 L of solution, containing Tween-20 (2 g L^{-1}) as nonionic tensioactive, and applying a pressure of 0.05 bar for 4 min at 25 °C. The other half was treated with the same polyamine concentration and control (distilled water) by dips at 25 °C for 4 min. Following treatments, fruits were placed on desiccant Kraft paper and were allowed to dry (room temperature and absence of light) before storage the next day at 2 °C (considered as day 0) in a temperature-controlled chamber, in permanent darkness, and with relative humidity of 90%. After 0, 15, 30, 45, and 60 days, 25 fruits for each treatment (five from each replicate) were sampled and further stored at 20 °C for 3 days (shelf life, SL). Then, each husk was carefully cut at the equatorial zone with sharpened knives, and then arils were manually extracted. The arils of each replicate were combined and frozen in liquid N_2, were milled to obtain homogeneous samples, and were stored at −20 °C until analysis.

Total Antioxidant Activity, Total Phenolic Compounds, and Organic Acids. For each sample, 5 g of arils was homogenized in 10 mL of 50 mM phosphate buffer pH = 7.8 and then was centrifuged at 15 000 rpm for 15 min at 4 °C. The supernatant was used for total antioxidant activity (TAA) and total phenolic compounds quantification in duplicate, as previously described (*21*). Briefly, TAA was determined using the enzymatic system composed of the chromophore 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), the horse radish peroxidase enzyme (HRP), and its oxidant substrate (hydrogen peroxide), in which $ABTS^{\bullet+}$ radicals are generated and monitored at 414 nm. The decrease in absorbance after adding the aril extract was proportional to TAA of the sample. A calibration curve was performed with L-ascorbic acid (0−20 nmol) from Sigma (Poole, Dorset, United Kingdom), and results are expressed as milligrams of L-ascorbic acid equivalent 100 g^{-1}. Total phenolic compounds were quantified using the Folin−Ciocalteu reagent and results were expressed as milligrams gallic acid equivalent 100 g^{-1}. Organic acids were also quantified in duplicate from the same extract by high-performance liquid chromatography (HPLC). The elution system consisted of 0.1% phosphoric acid running isocratically with a flow rate of 0.5 mL min^{-1} through a Supelco column (Supelcogel C-610H, 30 cm × 7.8 mm, Supelco Park, Bellefonte, United States) and detection by absorbance at 210 nm. A standard curve of pure L-ascorbic, citric, malic, oxalic, and succinic acids (Sigma, Poole, Dorset, United Kingdom) was used for quantification and results were expressed as milligrams ascorbic acid 100 g^{-1} and grams 100 g^{-1} for the remaining organic acids. In these chromatographic conditions, the retention times were 8.73, 11.59, 13.57, 14.29, and 17.19 min for oxalic, citric, malic, ascorbic, and succinic acids, respectively.

Total Anthocyanins. The method described by García-Viguera et al. (*22*) was adapted to pomegranate. Five grams of arils was homogenized in 10 mL methanol and was left 1 h at −18 °C. Extracts were centrifuged at 15 000 rpm for 15 min at 4 °C. The supernatant was loaded onto a C18 Sep-Pak cartridge (Waters, Milford, MA), with the following properties: end-capped, surface area of 327 m^2 g^{-1}, and 0.85 mL per filled cartridge. The C18 Sep-Pak was previously conditioned with 5 mL of methanol, 5 mL of pure water, and then with 5 mL of 0.01 N HCl. After sample application, the cartridge was washed with 5 mL of pure water and then was eluted with acidified MeOH (0.01% HCl). Absorbance of the collected fraction was measured at 520 nm. The method gave a recovery percentage of 90%. Total anthocyanin was calculated using cyanidin-3-glucoside (molar absorption coefficient of 23 900 L cm^{-1} mol^{-1} and molecular weight of 449.2 g mol^{-1}) and results were expressed as mg 100 g^{-1} fw and were the mean of determinations made in duplicate in each one of the five samples.

Statistical Analysis. Data for the analytical determinations were subjected to analysis of variance (ANOVA). Sources of variation were storage and treatment. Mean comparisons were performed using HSD the Tukey's test to examine if differences were significant at $p < 0.05$. To know the compounds that contribute to TAA, linear regressions were performed among the functional parameters taking into account data from all sampling dates (from both control and treated). All analyses were performed with SPSS software package v. 12.0 for Windows (*23*).

RESULTS

Total Antioxidant Activity (TAA). In arils, TAA increased during storage, mainly after 15 days at 2 °C plus 3 days at 20 °C compared to value on day 0 plus 3 days at 20 °C (**Figure 1**). This initial TAA increase was higher in Put and Spd treated arils than in control. TAA levels remained higher throughout storage, except for Put treated arils after 60 days in the infiltration method and after 15 and 60 days for Put and Spd treated arils in the immersion method. Comparing the two ways of polyamine application, the pressure infiltration was slightly more effective than immersion on increasing TAA and maintaining it slightly higher until day 30 of storage. In addition, at the end of the storage period, differences between control and treated fruits were significantly higher when treatment was performed by pressure infiltration, especially for Spd treatment with TAA of 69.75 ± 4.51 mg equiv ascorbic acid 100 g^{-1} compared to 53.33 ± 2.33 mg equiv ascorbic acid 100 g^{-1} found in control arils. The final TAA was the same between treated and control fruits when treatments were applied by immersion.

Total Phenolics. The results of total phenolics revealed that they were affected by the way of treatment. The application of polyamines by pressure infiltration led to a significant increase in total phenolics (**Figure 2**), which was detected 1 day after treatment, the Spd treatment being the most effective with values of 139.16 ± 4.12 mg equiv gallic acid 100 g^{-1} compared to Put-treated arils (128.78 ± 2.29 mg equiv gallic acid 100 g^{-1}), and the lowest concentration was found for control fruits (83.34 ± 1.15 mg equiv gallic acid 100 g^{-1}). During storage, the treated arils also showed higher concentration of total phenolics than control, although the final levels were the same in both treated and control arils. When polyamines were applied by immersion, efficacy of the treatment on maintaining differences between control and treated fruits was reduced, reaching similar values at the end of the experiment (≈117 mg equiv gallic acid 100 g^{-1}).

Total Anthocyanins. The levels of total anthocyanins remained significantly higher in treated than in control arils (**Figure 3**), independently of the way of polyamine application. The concentration of total anthocyanins did not change during

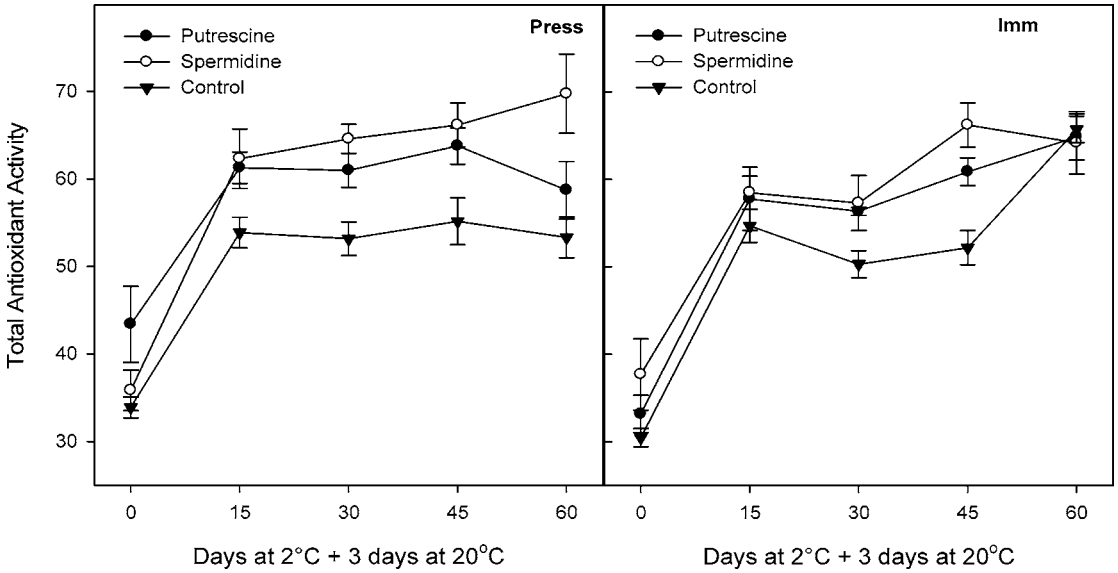

Figure 1. Effect of polyamines by pressure infiltration (Press) or immersion (Imm) treatment on pomegranate total antioxidant activity (mg equiv ascorbic acid 100 g^{-1}) in arils during cold storage (2 °C) + 3 days at 20 °C (SL).

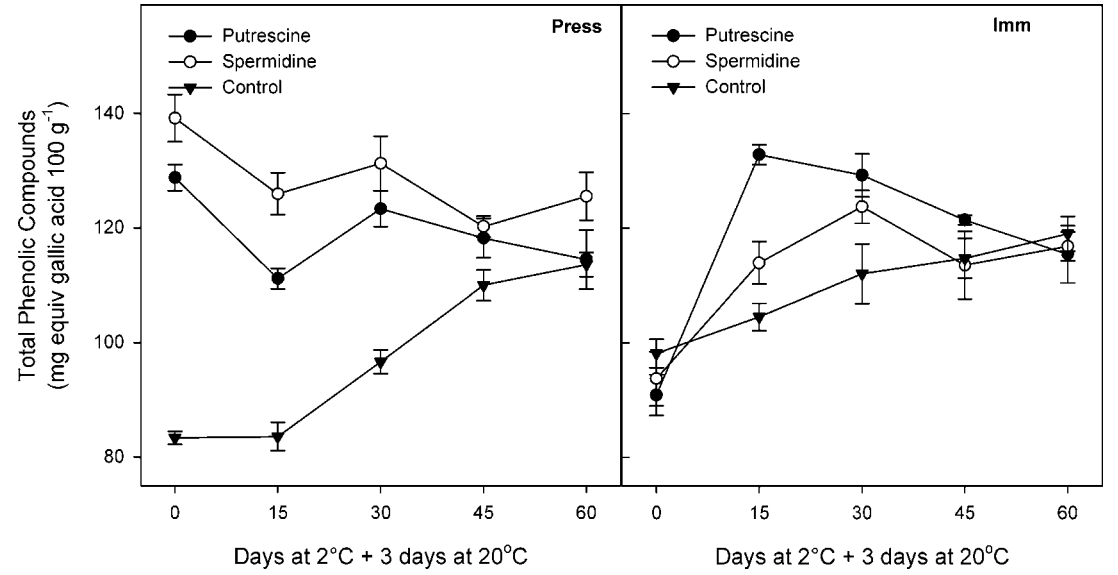

Figure 2. Effect of polyamines by pressure infiltration (Press) or immersion (Imm) treatment on pomegranate total phenolic compounds (mg equiv gallic acid 100 g^{-1}) in arils during cold storage (2 °C) + 3 days at 20 °C (SL).

storage of nontreated fruits, while slight increases were obtained in Spd-infiltrated (from 164.63 ± 7.08 to 229.86 ± 7.47 mg equiv cyanidin-3-glucoside 100 g^{-1}) or Put-immersed (from 160.22 ± 3.07 to 198.57 ± 7.12 mg equiv cyanidin-3-glucoside 100 g^{-1}). The content of total anthocyanin in Put-infiltrated or Spd-immersed remained unchanged during storage.

Organic Acids. The organic acids identified in the arils were oxalic, citric, malic, ascorbic, and succinic acids. The main organic acid was malic acid (0.35−0.40 g 100 g^{-1}) followed by citric acid (0.10−0.20 g 100 g^{-1}), while oxalic and succinic acids were minor (0.02−0.03 g 100 g^{-1}). For these organic acids, no significant differences were attributable either to treatment or storage (data not shown). The effect of polyamine treatments, either by pressure or immersion, was to maintain slight nonsignificant higher levels of ascorbic acid than in control arils (**Figure 4**), which was detectable after 1 day of treatment, with concentration of 115 ± 2 and 112 ± 2 mg 100 g^{-1} ascorbic acid, in pressure-infiltrated Put- and Spd arils, respectively, compared to control fruits (106 ± 2 mg 100 g^{-1} ascorbic acid). During storage, the levels of ascorbic acid remained unchanged for both control and treated fruits.

Correlations. The regressions between TAA and total polyphenols, anthocyanin or ascorbic acid, revealed that the TAA was only correlated to total phenolics in both control and treated fruits ($r^2 > 0.70$) independently of the way of application, while no regression was obtained for ascorbic acid for any fruit. In those treatments which increased total anthocyanins, a high correlation was found with TAA, such as Put immersion ($r^2 = 0.74$) and weaker for Spd under pressure ($r^2 = 0.50$).

DISCUSSION

A large body of evidence from case-control and cohort studies has revealed that fruits and vegetables have a strong protective effect against various types of diseases, and thus the consumption of fruit imparts health-beneficial effects because of occurrence of several chemicals with antioxidant activity, including ascorbic acid, flavonoids, and phenolic compounds such as anthocyanins (*24*). The pomegranate cultivar used in this study

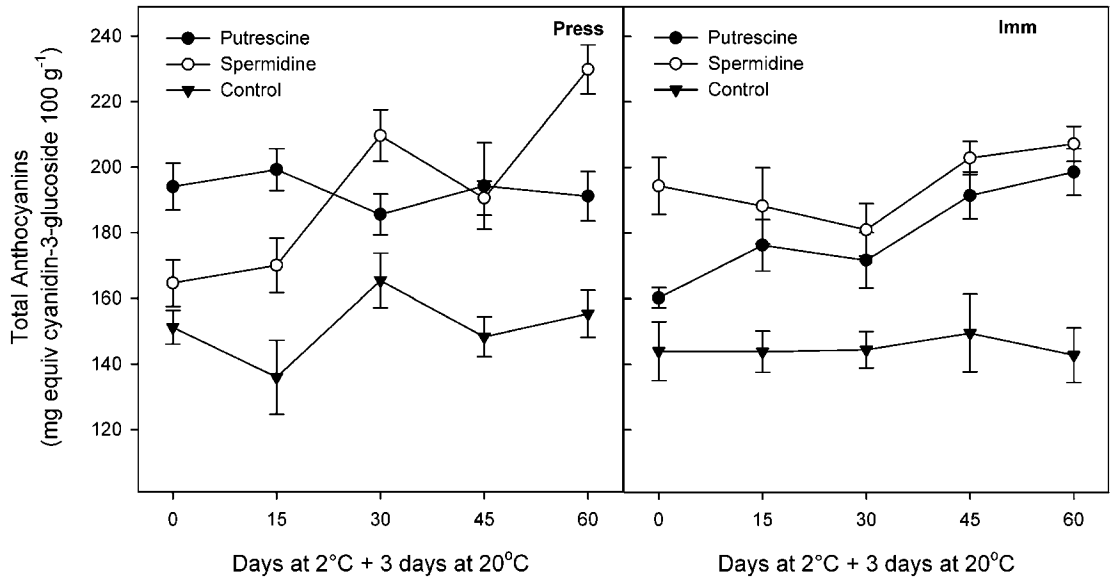

Figure 3. Effect of polyamines by pressure infiltration (Press) or immersion (Imm) treatment on pomegranate total anthocyainins (mg equiv cyanidin-3-glucoside 100 g^{-1}) in arils during cold storage (2 °C) + 3 days at 20 °C (SL).

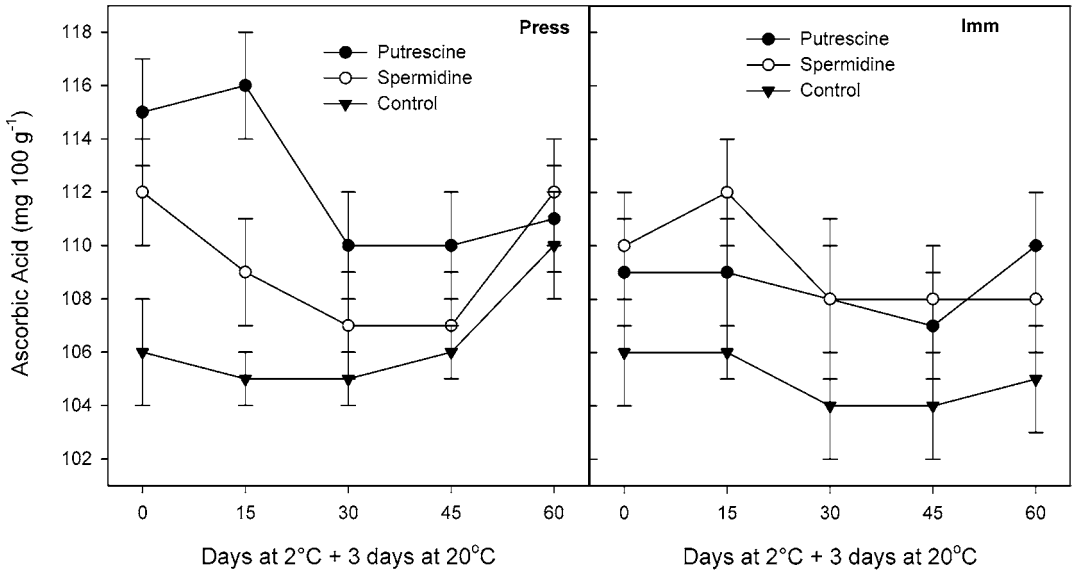

Figure 4. Effect of polyamines by pressure infiltration (Press) or immersion (Imm) treatment on pomegranate ascorbic acid (mg 100 g^{-1}) in arils during cold storage (2 °C) + 3 days at 20 °C (SL).

(Mollar de Elche) is very rich in these compounds compared to data reported for other cultivars, namely, Taifi, Wonderful, and Ganesh, which exhibited lower amounts of ascorbic acid and total phenolics (*2*, *7*, *25*). During pomegranate fruit development and maturation, increases in anthocyanins with concomitant decreases in total phenolics and ascorbic acid have been reported (*26*). In addition, the nonclimacteric pattern reported for pomegranate (*27*) indicates that harvest date marks the degree of maturation together with the maximum levels of functional compounds (*2*). In pomegranate, loss of ascorbic acid (vitamin C) occurred either during cold storage or at ambient temperatures (*25*), while anthocyanins decreased in stored arils (*10*) or juices (*28*). Very few attempts have been carried out to minimize this loss of functional compounds, the use of modified atmosphere packaging (MAP) being slightly effective in the delay of anthocyanins loss in intact pomegranate (*13*) or minimally processed arils (*29*).

In this work, the application of Put or Spd, either by pressure infiltration or immersion, was effective on maintaining the concentration of ascorbic acid, total phenolic compounds, and total anthocyanins higher in treated than in control fruits. Moreover, slight increases or nonsignificant losses of these compounds were observed during storage after polyamine treatments. During storage of control pomegranates, TAA and total phenolics increased, which is in accordance with a previous report (*30*). However, for most of the sampling dates, TAA and total phenolics remained higher in polyamine-treated than in control arils, especially in the pressure-infiltrated pomegranates. As far as we know, this is the first report in which the application of polyamines induced beneficial effects in terms of maintaining or increasing the pomegranate potential antioxidant activity during postharvest storage. The mechanism by which Put or Spd induced these effects is still unknown. However, during the late phases of fruit development and postharvest ripening, a reduction of endogenous Put and Spd has been reported in a wide range of fruits (*14*), which has been associated with the acceleration of the changes related to ripening (color, texture, flavor, and aroma) and the loss of quality. These changes were

greatly delayed or reduced by the postharvest application of Put (*15*−*18*), and the shelf life could be extended. Moreover, the losses of ascorbic acid and the increase in ascorbate oxidase in pepper and tomato development, ripening, and senescence have been associated with decreases in the polyamine content (*31*). Conversely, the engineered polyamine accumulation in tomato enhanced the content of lycopene and the juice quality (*32*). Thus, it is clear that the antisenescence nature reported for polyamines (*14*) would have a role in cell integrity and thus would avoid the contact between substrates and their degrading enzymes. This could explain partially the high TAA in treated arils by lowering the losses of phenolic compounds, since TAA was correlated with total phenolics. In other pomegranate cultivars, anthocyanin, ascorbic acid, and phenolics are responsible for the TAA, alone or in combination (*8*), as has been observed in several fruits (*33*). The major phenolic compound that contributes to TAA of pith and carpellary membrane was punicalagin (*8*), while delphinidin, cyanidin, and pelargonidin were suggested as anthocyanidins participating in the TAA of arils (*34*). On the other hand, the higher TAA found in the arils after the polyamine treatment could be attributed to the polyamine capacity to act as effective scavengers of free radicals, as has been observed in several in vitro systems. Moreover, this capacity was correlated to the number of amino groups (*35*) and could explain the greater effect of Spd (three amino groups) than Put (2 amino groups) in increasing TAA of the pomegranate arils. Recently, it has been proposed that polyamine might function as a protective antioxidant by involving an efficient superoxide dismutase (SOD)/ascorbate-glutathione cycle (*36*).

Although most of the papers dealing with postharvest treatments of polyamines in fruits have been carried out by pressure infiltration (*14*−*18*) to ensure the polyamine intake, we demonstrate in this paper that immersion could also be an effective tool to maintain the functional properties of pomegranate after long storage periods. The immersion method could be considered as lower cost and easier handled than pressure infiltration and could be incorporated as a continuous process at the industry. From the two polyamines assayed, Spd applied by pressure infiltration gave the best results in terms of higher TAA by maintenance of total phenolics. Further research is necessary to get a better knowledge about how polyamines affect the fruit functional properties in general and particularly in pomegranate. Additionally, evaluation of endogenous levels of polyamines in arils after exogenous application of polyamines would confirm the increase in these antisenescence compounds and their putative role on increasing the health-beneficial compounds of pomegranates.

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