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An analysis on the impacts of cryogenic freezing on raspberry quality

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Abstract: Counter-season supply of horticultural products is of increasing demand. Consumers are demanding annual supply of raspberries, which has historically been challenging due to their seasonal summer supply and characteristically high metabolism resulting in a short shelf life and limited period of availability. However, the development of freezing technologies for increasing the length of storage of raspberries offers an opportunity for continual supply of premium quality raspberries. We investigated berry quality after freezing whole fresh raspberries, comparing conventional freezing methods with a modern cryogenic freezing method over a period of six months. Significant increases in total soluble solids, titratable acidity, hue and chroma were found when raspberries were frozen compared to fresh raspberries. No overall difference in berry quality was observed between freezing methods for any parameter assessed. When assessed at time intervals, total soluble solids, pH, titratable acidity, chroma and hue were consistent between freezing methods for all durations of time frozen. These findings provide decision support for producers and distributors pursuing a novel counter season supply chain.

1. Introduction

Raspberries (*Rubus ideaus* L.) are a perennial plant that produce a compound fruit composed of many drupelets (Wang *et al.*, 2009). Raspberries grown for fresh production typically have a unique bright red colour with distinctive aroma and taste, as well as health benefits due to their antioxidant properties (Liu *et al.*, 2002). Raspberries are a non-climacteric fruit with a high metabolism, making them perishable with a very short shelf life (Tezotto-Uliana *et al.*, 2014). The ability to maximise fruit quality whilst harvesting and during post-harvest is imperative to commercially viable raspberry production.

The development and adoption of innovative technologies has allowed conventional agricultural systems to pursue increased efficiency, yield, quality, food safety, and sustainability with reduced costs (Sunding and Zilberman, 2001). In the raspberry industry, freezing technologies for post-harvest storage offer producers the ability to extend the market window whilst preserving or increasing fruit quality standards. Additionally, freezing horticultural products allows access to diversified markets and supply chains, creating increased economic feasibility and prosperity (Bora *et al.*, 2021; Safari *et al.*, 2021).

Demand by consumers for out-of-season supply of produce has driven research into the impacts of different storage methods on fruit and berry quality parameters (Boorse et al., 1998; González et al., 2002; Chassagne-Berces et al., 2010; Ceballos et al., 2012; Alhamdan et al., 2018). The perishable characteristics of raspberries caused by high rates of metabolism and limited seasonal production, makes counter season supply problematic for producers (Adobati et al., 2015). Despite extensive literature investigating variances in quality parameters for horticultural products post conventional and cryogenic (liquid nitrogen) freezing, there is currently a gap in research comparing the impacts of the two freezing methods on the quality of whole fresh raspberries (Alhamdan et al., 2018; Otero et al., 2000). Some notable issues with freezing of berry fruit have been identified including ice crystal nucleation and subsequent cell membrane rupture and electrolyte leakage which compromises the eating quality of frozen fruit (Silva et al., 2008). The mouth feel of frozen fruit is related to cell turgor pressure which is driven by each cell having a separate functional semi permeable membrane that splits when ice nucleation occurs, resulting in soft textures in comparison to crisp fresh fruit (Grout et al., 1991). To reduce the damage to cell membranes by crystal nucleation during the freezing of berries, there have been several developments in freezing methodologies. Liquid nitrogen (~-80°C) applied to each berry individually reduces the thermal gradient and creates a snap freeze process that reduces the loss of quality through cell membrane damage caused by slower and higher temperature conventional freezing, resulting in a higher quality product (Ceballos et al., 2012). Studies have demonstrated that the loss of quality, particularly tissue damage and subsequent texture and colour from freezing, is impacted by the rate of freezing. Fruit frozen at lower temperatures (-40°C) have been shown to display better quality parameters than fruits frozen at higher temperatures (-14°C) (Chassagne-Berces et al., 2010).

Whilst the demand for frozen horticultural products has seen considerable investment into studies on the impacts of freezing on vegetables and fruits, there is comparatively limited evidence on the specific effects of freezing on raspberry quality to support industry investment. González *et al.* (2002) found that berry variety, harvest timing and quality at harvest are critical factors influencing the impact freezing has on raspberry fruit quality. De Ancos *et al.* (2000) showed an increase in anthocyanin content during freezing; however, this conclusion was determined to be a result of anthocyanin extraction efficacy after ice nucleation, rather than a biological increase in anthocyanin content. Conversely, Kampuse *et al.* (2001) highlighted a decrease in anthocyanins in frozen raspberries, with varietal differences influencing the degree of anthocyanin depletion.

Cryogenic freezing technology has the potential to improve the quality of frozen berries. This study investigated if the application of freezing technologies can lead to improvements in raspberry fruit quality when frozen. Specifically, using a traditional commercially grown variety and a new propriety variety, we asked - Does freezing method (conventional and cryogenic) and freezing duration (2, 4, and 6 months) influence raspberry quality? The findings of this research are discussed in the context of industry pursuing continuous supply to a market that demands high quality and affordable produce throughout the year.

2. Materials and Methods

Experimental site

The experimental site was located at The Westerway Raspberry Farm (42° 47' 26.34" S, 146° 47' 26.34" E), an established 60-acre raspberry and mixed berry farm at Westerway, in southern Tasmania. The Westerway Raspberry Farm is situated on an alluvial soil deposit adjacent to the Tyenna River. The raspberry canes utilised for the experiment were oriented in a NE SW direction and were trained in a commercial vertical trellis system. Westerway receives an average annual rainfall of 764 mm (BOM, 2019). The traditional and popular 'Willamette' raspberry variety and a newly developed proprietary variety were investigated for the purpose of this experiment. The proprietary variety has been developed for the purpose of machine harvesting, with characteristics of diseases resistance, fruit firmness and low release force from plant.

Sample collection

Harvest coincided with the commercial raspberry season on 27 December 2018. Fruit was hand harvested from randomly selected rows within the block

with berries collected randomly along each row. Consistent with commercial practice, samples were collected into individually labelled commercial plastic 400 g punnets and stored in a commercial cool room (7°C) on site for five hours until transportation.

Treatments and storage conditions

For both raspberry varieties there were two different treatments (post-harvest storage technique and duration frozen) with five replicates for each treatment combination. Treatments were established as a fully factorial trial with two freezing techniques (conventional and cryogenic) and three lengths of storage (2, 4 and 6 months) tested with two varieties of raspberries.

Punnets containing fruit were immediately frozen on site in either a commercial conventional -8°C freezer (Gunter, Germany) or snap frozen in a liquid nitrogen (LN) tunnel at -80°C (Linde Cryoline MT 5-600 quick-freezing (IQF) tunnel, Munich, Germany) depending on treatment. For LN tunnel freezing, the berries were tipped out of the punnets and passed through the LN tunnel individually on the inbuilt conveyor belt and collected into labelled punnets immediately after freezing. All berries from both treatments were then stored in the commercial -8°C freezer for the duration of the storage period. During storage, all samples were stored in the commercial grade plastic punnets with lids and stacked into crates holding 50 punnets per crate, aligned with industry practice. Frozen samples remained in the freezer for three prescribed periods of up to six months.

Laboratory analysis

Samples were transported from the farm freezing facilities back to the laboratory at the Tasmanian Institute of Agriculture (University of Tasmania) for analysis in an ice chilled eski. The samples were then stored in a cool room (4°C) during the laboratory analyses, which were completed within 48 hr after being removed from the freezer.

The pH for every replicate was measured using a diluted sample in an auto-titrator (Metrohm 702 SM Titrino, Herisau, Switzerland). A subsample of 50 berries from each sample was juiced by squeezing berries through a microporous cloth (30-micron mesh filter cloth; Allied Filter Fabrics P/N M0032, Sydney, Australia). A sample of the juice (5 mL) was pipetted (BRANTECH 10 mL Transfer pipette S, San Francisco, America) into a 25 mL beaker and combined with 15 mL of distilled water. The solution was

analysed for initial pH by electrode probes used in the auto-titration. The same solution used for pH was analysed for titratable acidity by the Metrohmautotitrator (702 SM Titrino). The solution was mixed using a magnetic stirrer and combined with 1M sodium hydroxide to determine the titratable acidity (titration to pH = 8.2). Berry titratable acidity was expressed in g/L of citric acid. Total soluble solids (TSS) was determined using a portion of the juice (~3 mL) and a digital refractometer (Atago Pocket Refractometer PAL-1). The sample of juice was analysed immediately after juicing. Using a hand disposable plastic pipette, the sample was placed into the measuring chamber and analysed with the TSS figure (%) recorded manually. The chamber was cleaned between each sample using paper towels and distilled water to remove any residue.

The total anthocyanin content (mg cyanidin-3-glucoside equivalents/g berry fresh weight) was determined using the pH differential method (Lee et al., 2005). Subsamples (30 berries) were homogenised for 30 seconds at 7000 rpm using a Retsch Grindomix GM200 (Haan, Germany). Homogenised subsamples of 10 g were weighed into 50 mL centrifuge tubes and combined with 40 mL of acidified 70 % methanol [700 mL methanol, 300 mL distilled H2O, 0.1 mL concentrated HCl (0.01% v/v)]. The tubes were then placed into an ultrasonic bath (GRANT XUV Digital Ultrasonic Bath (Royston, UK) in darkness for 30 minutes at 10°C. The tubes were then dried and placed into a Hettich Benchtop Centrifuge (Universal 320 R Model Tuttlingen, Germany) and centrifuged for 10 minutes at 1520 g and 4°C. A 0.5 mL sample of the clear supernatant solution was then mixed with each of the buffers; buffer 1 solution being 0.025 M potassium chloride and buffer 2 being 0.4 M sodium acetate. The dilution used was a 1:10 dilution (0.5 mL supernatant to 4.5 mL of buffer). The solutions were then left to equilibrate for 30 minutes. Using a benchtop spectrophotometer (Thermo Scientific Genesys 10S UV-VIS, Waltham, America), the absorption of each solution was measured at 530 nm and 700 nm. This allowed the absorbance of the diluted sample to be calculated using the following equation (Lee et al., 2005):

Absorbance = (A530 - A700) pH1 - (A530 - A700) pH 4.5

where A530 is the spectral wavelength absorption measurement at 530 nm, and A700 is the spectral wavelength absorption at 700 nm.

Using the calculated absorbance value, the monomeric anthocyanin content was calculated using the equation (Lee *et al.*, 2005):

Monomeric Anthocyanin Pigment (mg/L) = (A x MW x DF x 1000)/ (e x 1)

where A = Absorbance, MW = 449.2 g/mol for cyanidin-3-glucoside, DF = dilution factor determined (1 in 10 dilution), 1000 = factor for conversion from g to mg, e = 26,900 molar extinction coefficient, in L X mol-1 X cm-1, for cyd-3-glu and 1 = path length in cm. Max used e = 30200.

The final value from the monomeric anthocyanin pigment (mg/L) was converted to the commonly expressed mg per 100 g fresh weight unit. This conversion was completed by knowing the total amount of anthocyanin in a litre and the initial fresh mass of raspberry sample used.

Anthocyanin (mg per 10 g Fresh weight) = (0.04 x anthocyanin Mg/L value) x 100 g/fresh weigh (g)

Where 0.04 = proportion of 40 mL of a litre, 100 g = final unit, Anthocyanin Mg/L Value = answer from monomeric equation and fresh weight = 10 g weighed initially

Using the homogenised samples, homogenate colour was measured using a colourimeter (Konica Minolta Chroma Meter CR-400, New York, USA) (Edgley *et al.*, 2019). Spectrophotometer tubes were filled with homogenate from each sample and placed in the colourimeter. The values for I*, a* and b* were recorded manually and gave a three-dimensional colour space with each value interpreted as: L* a measure from opaque (0) to completely black (100), a positive a* indicates redness whilst negative a* indicates greenness and a positive b* indicates yellowness whilst negative b* indicates blueness on the hue-circle (Voss, 1992; Gonçalves *et al.*, 2007). Chroma was obtained by the formula:

Chroma = $(a^{*2} + b^{*2})^2$ and Hue from the formula: hue= arctg b^*/a^* (Gonçalves *et al.*, 2007).

Statistical analysis

Analysis of variance and statistical significance of quality parameters (TSS, pH, titratable acidity, anthocyanins, hue and chroma) between treatment interactions and treatment main effects were analysed using the 2019 IBM SPSS Statistics package. Analysis of mean variance was determined using univariate linear models for each differing continuous variable. Post hoc tests were completed using Tukey's range test of statistical significance.

3. Results

There were no three-way interactions between variety, freezing method and length of duration frozen for all quality parameters assessed. However, there were two-way interactions for freezing method and variety, duration frozen and variety, and duration frozen and freezing method for a range of the quality parameters assessed.

Freezing method and variety

Raspberries of both proprietary and 'Willamette' varieties had significantly (P<0.05) increased TSS values after freezing, regardless of method, in comparison to their fresh TSS values (Fig. 1a). Conventionally frozen proprietary berries had significantly (P<0.05) lower pH values in comparison to fresh, however they were not significantly (P>0.05) different to cryogenically frozen proprietary berries (Fig. 1b). Cryogenically frozen 'Willamette' berries and frozen



Fig. 1 - TSS (a), pH (b), Titratable acidity (c), Anthocyanin content (d), Hue (e) and Chroma (f) values for fresh and frozen propriety (grey bars) and 'Willamette' raspberries (black bars). Errors bars denote two times standard error. Letters above bars indicate significant differences from the mean at P<0.05.</p>

proprietary berries had statistically similar pH values in comparison to their respective fresh berries (Fig. 1b.)

Freezing both 'Willamette' and proprietary raspberries significantly increased (P<0.05) titratable acidity (g/L citric acid), whilst the method of freezing made no significant (P>0.05) difference for either variety (Fig. 1c). Anthocyanin content of berries of both varieties were the same whether fresh or frozen, regardless of freezing method (Fig. 1d). Freezing both 'Willamette' and the proprietary variety significantly increased (P<0.05) both the hue and chroma values for berry colour, whilst the method of freezing did not have a significant impact on each variety individually (Fig. 1e and 1f). Proprietary raspberries exposed to cryogenic freezing however had significantly greater (P<0.05) hue and chroma values than 'Willamette' exposed to cryogenic and conventional freezing (Figs. 1e and 1f).

Duration frozen and variety

The TSS (%) for the proprietary raspberry variety was not significantly influenced (P>0.05) within the first two months of freezing, however TSS increased significantly (P<0.05) after four and six months frozen compared to the fresh samples (Fig. 2a). The 'Willamette' variety had significantly increased (P<0.05) TSS two and six months after freezing, however TSS for the four-month frozen sample was not significantly different (P>0.05) from the fresh samples (Fig. 2). Once frozen, there was no significant difference (P>0.05) in TSS for berries frozen for two,



Fig. 2 - Brix (a), pH (b), Titratable acidity (c) and Hue (d) for both the proprietary (black bars) and 'Willamette' (grey bars) variety of raspberry after being frozen for up to six months. Errors bars denote two time standard error. Letters above bars indicate significance differences from the mean at P<0.05 of time frozen values.</p>

four or six months for either variety.

The 'Willamette' variety had no significant (P>0.05) change in pH for the duration frozen. pH of the proprietary variety berries continuously declined at each assessment date but was only significantly lower after being frozen for six months when compared to fresh berries (Fig. 2b).

TA (g/L citric acid) of both the 'Willamette' and proprietary variety significantly (P<0.05) increased once frozen but did not significantly change through the duration of freezing (two, four and six months) (Fig. 2c). The change in titratable acidity to TSS ratio was statistically similar across duration frozen.

The colour (hue units) of both 'Willamette' and the proprietary variety raspberries also significantly (P<0.05) increased when frozen (Fig. 2d). Once frozen, hue of the proprietary variety did not change for the duration of frozen storage, whilst hue for the 'Willamette' berries significantly (P<0.05) reduced at the six-month assessment compared to the second month assessments (Fig. 2d).

Duration frozen and freezing method

Berries that were initially cryogenically frozen had significantly (P<0.05) higher TSS values at four months compared to fresh raspberries, but no significant (P>0.05) difference at six months in comparison to fresh berries (Fig. 3a). For berries that were initially frozen in a conventional freezer, the two- and fourmonth frozen treatments were not significantly different from fresh raspberries in TSS, but at six months there was significantly (P<0.05) greater TSS



Fig. 3 - TSS (%) (a), pH (b), Titratable acidity (c) and Hue (d) for both the conventional (grey bars) and cryogenic (black bars) methods of freezing raspberries. Error bars denote two times standard error. Letters above bars indicate significance differences from the mean at P<0.05.</p>

compared to fresh berries (Fig. 3a).

Cryogenically frozen berries had no significant (P>0.05) differences in pH value for the duration frozen in comparison to fresh berries (Fig. 3b). However, conventionally frozen berries at six months had significantly (P<0.05) lower pH compared to fresh raspberries.

There was a significant increase in TA once frozen, but at each assessment interval (two, four and six months), there was no significant difference in freezing method on the TA concentration (Fig. 3). For both conventional and cryogenic freezing, there was no significant change in TA between two, four and six months frozen (Fig. 3).

For hue, there was no significant difference between freezing method at any single duration measured for the duration of the experiment (Fig. 3). Both freezing methods did exhibit a significant increase in hue colour units initially after freezing, increasing by ~25 % between 0 and two months frozen (Fig. 3).

4. Discussion and Conclusions

Consumer demand for counter season supply of premium horticultural products is driving the need and innovation of storage techniques whilst maintaining premium quality produce (Kuchler and Arnade, 2015; Martindale and Schiebel, 2017). The results of this study found significant differences in quality parameters for whole raspberries after they were frozen compared to fresh berries. Measurements of total soluble solids, titratable acidity, hue and chroma all significantly increased when berries were frozen. This study found no difference however in berry quality once frozen between freezing methods for any parameter assessed. Total soluble solids, pH, titratable acidity and hue were consistent between freezing methods for all durations of time frozen. These results improve the understanding of the impacts of post-harvest storage on raspberry quality and provides critical information and decision support for producers to ensure optimal raspberry quality for consumers.

Raspberries are no exception in the unrelenting demand from consumers for premium quality counter season supply of horticultural products, however, the metabolic characteristics that result in raspberries being highly perishable makes the longterm storage of raspberries comparatively difficult (Gonçalves et al., 2018). For both the 'Willamette' and proprietary varieties, TSS increased once frozen regardless of freezing method. This finding of initial TSS increase after freezing is consistent with González et al. (2002) who showed a similar initial increase in TSS after freezing 'Heritage', 'Autumn Bliss', 'Zeva' and 'Rubi' raspberries. The treatments of two, four- and six-months freezing duration showed differences for TSS between varieties when fresh, with the 'Willamette' variety having greater than 20% higher TSS than the proprietary variety. Varietal difference as shown here is consistent with Shamaila et al. (1993) who found differences for fresh berries for the quality parameters pH, TSS, TA and anthocyanins between 'Chilcotin', 'Chilliwack', 'Meeker', 'Skeena' and 'Tulameen' varieties. This significant difference between varieties persisted for all freezing periods. However, once frozen, TSS values did not significantly change for both varieties for the duration of the trial, and the freezing method, conventional vs cryogenic, also had no impact on TSS. This finding contrasts with the González et al. (2002) study described earlier, which showed that TSS continued to significantly increase during duration frozen. The findings of consistency in TSS levels once frozen for the proprietary and 'Willamette' variety suggest the quality of these varieties once frozen is more stable compared to varieties such as 'Heritage', 'Zeva' and 'Rubi' used by González et al. (2002). This finding is likely a result of differing TSS profiles between varieties having differing stability when frozen, resulting in a variation in TSS level changes.

Titratable acidity contributes to the mouth feel a consumer experiences when stimulated by the multiple acids including citric, ascorbic and phosphoric acid found in raspberries (Haffner et al., 2002; Marsh et al., 2004). Freezing is the most common form of long term storage for fresh produce, with the reduction in metabolism and respiration when frozen allowing raspberries to be stored for long periods of time (Chaves and Zaritzky, 2018). Cryogenic freezing facilitates the rapid transition into the frozen state which reduces membrane damage (and therefore maintains raspberry quality) from slow water crystallization and subsequent membrane damage that occurs during conventional freezing (Cao et al., 2018). Consistent with findings for changes in TSS, there was a significant increase in titratable acidity for frozen berries compared to fresh berries for both varieties, but there was no significant difference resulting from freezing method for either variety.

Titratable acidity significantly increased when initially frozen but did not change with length of duration frozen. These results are consistent with findings of increased total and conjugated acid in frozen raspberries by Mullen *et al.* (2002). However, de Ancos *et al.* (2000) who quantified ellagic acid concentrations, found a significant decrease (14-21%) in ellagic acid content when raspberries were frozen, highlighting that the increase in titratable acidity in this investigation may be due to increase in other acids found in raspberries.

Raspberry quality is derived from multiple parameters with each contributing to the consumer's sensory experience and as such, the ratio of TSS with TA is a major contributor to consumer experience. Despite possible variances of specific acids during freezing, it is the ratio of sugar to acid that influences the consumer's tasting experience (Shamaila et al., 1993). The increase in sugar when frozen is proportional to the increase in acidity and showed no significant difference between freezing methods and duration, and it is this consistency in ratio that is critical to consumer experiences. The consistency in the ratio between TSS and titratable acidity whilst frozen is also broadly consistent with research by González et al. (2002) who used different raspberry varieties and durations frozen. No physiological mechanism or chemical changes induced by freezing/thawing has been described for the increase in titratable acidity post-harvest for raspberries. The measurement of increased titratable acidity in this study may be a physiological response to storage or an increase in extraction efficiency post storage, and until further investigation quantifies the reason for the change, the implications cannot be fully understood. In strawberries, retention of quality parameters including acids, colour and anthocyanin content was associated with the thawing process, where rapid thawing was shown to be more favourable (Holzwarth et al., 2012).

The impact of freezing method and duration on the colour of raspberries is important for influencing consumer demand. A colour that is perceived to be preferable by the consumer increases both the acceptance and perception of other quality parameters such as taste and texture (Clydesdale, 1993). Both varieties had similar hue and chroma values when fresh, however once frozen, colour values increased significantly. When frozen conventionally, both varieties had similar coloured fruit; however, when frozen cryogenically, the proprietary variety had significantly higher hue and chroma unit values than 'Willamette' raspberries (Fig. 1). This was the only quality parameter analysed that showed similarities between varieties for fresh berries but when frozen showed a significant difference between varieties. This highlights a difference in the impacts of freezing methods between the varieties and the subsequent impact on colour. The contrasting influence of freezing between varieties is consistent with findings by other researchers. González *et al.* (2002), for example, showed that for the varieties 'Heritage' and 'Autumn Bliss', chroma and hue was not different when fresh, but after six and nine months of being frozen, hue and chroma values differed between varieties.

The significant change in hue and chroma between fresh and frozen berries was not accompanied by a change in anthocyanin levels. This is inconsistent with findings by Han et al. (2004), who attributed the darker colour of frozen raspberries to anthocyanin synthesis. However, for strawberries, Holcroft and Kader (1999) showed that pH affects the colour expression of anthocyanins, concluding that the red flavylium cation only remains stable in acidic conditions, and strawberries lose their red colour (become pale) when the pH increases. Therefore, if the anthocyanin stability and subsequent redness of raspberries is associated with the amount of acids present (acidity), the increase in titratable acidity observed in this study, may be associated with increasing the amount of stable red flavylium cations, causing the raspberries to be darker when frozen.

This investigation used commercial standards of raspberry quality as a benchmark to compare innovative freezing methods for post-harvest management of raspberry fruit. In this investigation, consistent quantitative results for the quality parameters, benchmarking the commercial standard, provides support for the application of new technology in producing a consistent high-quality product. However, the commercial implications of a non-consistent (significantly different) result are not known, as it may be of better or worse quality in the perception of the consumer. Therefore, results that highlight significant (P<0.05) differences present opportunities for further studies and evaluations of quality as well as potential for changing practices.

The results of this investigation broadly demonstrated consistent quality parameters between conventional freezing and the modern, innovative and comparatively expensive technique of cryogenic freezing. We suggest that based on these findings, there is evidence-based support for the commercial retention of the cheaper conventional freezing over cryogenic freezing for post-harvest storage of raspberries. The study has provided critical decision support for post-harvest management of commercial raspberry production. This is particularly important for the uptake of horticultural technology that aims to access new markets and innovate logistics in supply chains. The findings of this study provide the framework and basis for the uptake and refinement of innovative technologies used in the raspberry industry, and allows freezing method choices to be evidence-based. The changes in titratable acidity during the freezing duration trial is unexplained here nor any published literature to date. Determining whether a physiological increase of titratable acidity occurs or if an increase in extraction efficiency of titratable acidity is the reason behind the results of this study would provide additional insights into the impacts of freezing on raspberries. The quantitative results of the current study also provide impetus for a sensory analysis to investigate consumer perceptions in parallel with the quantitative laboratory evidence provided in this study.

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