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**Post-veraison trimming slow down sugar accumulation without
modifying phenolic ripening in Sangiovese vines**

RUNNING TITLE: Post-veraison trimming of Sangiovese vines

**Gabriele Valentini, Gianluca Allegro, Chiara Pastore, Emilia Colucci, Ilaria
Filippetti***

Dipartimento di Scienze Agrarie, Università di Bologna, viale Fanin 44, 40127,
Bologna, Italy.

***Corresponding author:** ilaria.filippetti@unibo.it; fax +390512096400

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ABSTRACT

BACKGROUND: Vineyard strategies have recently been developed to fight the effects of global warming on grapevine, which is causing fast grape ripening, excessive sugar accumulation in berries and high alcohol levels in wines. Among these, we evaluate the effectiveness of post-veraison trimming to slow down sugar accumulation without modifying phenolic ripening, in Sangiovese vines grown in highly fertile and well-watered soil.

RESULTS: The removal of about two thirds of leaf area by shoot trimming after veraison led to sugar content reduction without effect on yield. Total and extractable anthocyanins, skin and seed tannins showed no significant variation at harvest after the treatment during the three years trial, while the replenishment of carbohydrates in canes at the end of the trial was negatively affected.

CONCLUSIONS: These results indicate that in highly fertile and well-watered soil, post-veraison trimming may represent a powerful tool for decreasing sugar concentration at harvest without affecting yield and total and extractable phenolic compounds but, the starch reserves reduction compared to the control serves as a warning about repeated trimming over the years.

KEYWORDS: anthocyanin, phenolic maturity, source limitation, tannin, technological maturity

INTRODUCTION

In recent years we have been witnessing a variation in climatic conditions affecting mainly a rise in air temperature, a steady carbon dioxide concentration increase in the atmosphere and changes in rainfall distribution throughout the agricultural area and season.^{1,2,3,4} Model output for global wine producing regions predicts an average warming of 2 °C in mean temperature over the next 50 years and a CO₂ concentration range between 541 and 970 ppm.^{5,6}

The climate profoundly influences the production of quality grapes and identifies suitable areas for grapevine growing. In fact, one of the tools that scientists have long used to map areas suitable for the cultivation of specific grape varieties is the definition of bioclimatic indices mainly based on temperature.^{7,8}

Nowadays global warming is affecting grapevine development and phenology, which results in earlier harvest dates,⁹ fast grape ripening, excessive sugar accumulation in berries and a resulting high alcohol wine.^{10,11,3,12,13,14,15} Keller,¹⁶ highlights that in many

cases these phenomena have also been coupled to faster depletion of organic acids, high pH and atypical green flavors in the grape because of the shortening ripening period and shift towards the hotter part of the season.

Focusing on phenolic compounds in black berry varieties it has been clearly verified that the high temperature negatively impacts color intensity^{17,18,19,20} giving rise to poor colored wine. Recent studies focused on the molecular mechanism of lower anthocyanin concentrations under high temperature confirmed the anthocyanin synthesis inhibition and promotion of their degradation.^{21,22} These results supported the occurrence of thermal decoupling of anthocyanin and sugar accumulation already observed in Cabernet Franc and Shiraz varieties exposed to a high temperature regime - 2.3 - 3.2 °C higher than the control ranging from 30° to 40 °C - during the vegetative season.²³ The earlier harvest date caused by these conditions often matched unacceptable phenolic conditions and wines resulting as unbalanced with excessive levels of alcohol, poor color and high astringency.

High temperatures seem to have less influence on the synthesis of tannins during ripening^{24,25,26} compared to anthocyanins, since tannins are the most stable phenolics under diverse growing conditions. These compounds accumulate in the berry skin and seeds from fruit-set to veraison,²⁷ having a rapid increase to a peak followed by a gradual decline after veraison with less or null variation during maturation close to harvest.^{28,29}

Following frequent cases of unbalanced red wines marked by high astringency during the last years, interest in the extractability of flavonoids during ripening is growing. On

Sangiovese berries, a different trend of the main phenolic compounds was noted during ripening, with anthocyanins and skin tannins extractability increasing while seed tannins extractability maintained a stable trend.³⁰

Although in the long term this scenario will result in a latitude shift, certainly influencing the distribution of grapes on Earth with new areas becoming viable on the northern fringes and changes in varietal suitability in the existing growing regions,³¹ in the short term some agronomic techniques can mitigate the negative impact of high temperatures on berry quality.

Different vineyard strategies are now available to slow down sugar accumulation and decelerate an overly quick and unbalanced berry ripening^{32,33,34,35,36} and the most effective of these are based on the induction of source limitations during ripening through post-veraison leaf removal of the upper part of the shoots.^{37,38} The physiological background is that, around veraison, leaves located on the apical part of the canopy are the most functional and are still able to elaborate carbohydrate. In particular, Palliotti et al.³⁸ stated that mechanical post-veraison leaf removal practiced at 16-17 °Brix removing 30-35% of apical leaves reduced sugar accumulation without affecting total anthocyanins and phenols in grape skin.

Previous study on cv. Sangiovese showed that post-veraison trimming could delay sugar accumulation without affecting the evolution of anthocyanins and seed tannins in grape,²⁹ but in our knowledge no research reports the effects of this or similar techniques³⁸ on the evolution of the extractable portion of anthocyanins and skin tannins involved on the phenolic ripening advancement. A recent paper on the same variety

showed that ripening stage had a strong impact on the increase of extractability of anthocyanins and skin tannins independently of the accumulation of their total amount, but not of seed tannins³⁰.

Moreover, since it was evident that when Sangiovese vines were grown on low-vigour conditions, the post veraison trimming over years could induce a yield reduction,²⁹ the aim of our 3-year study was to evaluate whether post-veraison trimming can be applied to slow down sugar accumulation without alteration of the phenolic ripening in highly fertile soil. In order to obtain practical information about the fate of anthocyanins and tannins during ripening, extractions were performed in a model hydroalcoholic solution.

EXPERIMENTAL

Plant material and experimental layout

The trial was conducted over 2012, 2013 and 2014 seasons on an 8-year-old irrigated vineyard of *Vitis vinifera* L. cv. Sangiovese (12 T clone) grafted onto SO4 rootstock, located on flat land close to Bologna, Italy (latitude 44°25'N; longitude 11°28'W). The vines were trained to a vertically shoot positioned (VSP) spur pruned cordon and spaced 1 m intra-row and 2.8 m inter-row, with 12 buds per vine. During the growing season, the number of shoots and bunches were kept uniform by shoot and bunch thinning. All vines were trimmed in June removing only the apical part of the shoots on each vine according to the usual VSP spur-pruned cordon practices.

The experimental design included 32 vines in four randomized blocks, assigned to the following treatments: untrimmed control (C) and shoot trimming applied after full veraison, leaving 8 basal nodes and related laterals (TRIMM).

TRIMM vines were trimmed manually when the total soluble solids (TSS) reached 15-16 °Brix: day of the year (DOY) 221, 231 and 225 in 2012, 2013 and 2014 seasons respectively.

Mean daily air temperature and rainfall data were recorded each year from April to September at a weather station located close to the trial site.

Vegetative and yield data

Each year, after trimming, leaf area was estimated after determining the linear relationship between main and lateral shoots length (cm) and corresponding leaf area (cm²) for 20 shoots collected from C and TRIMM vines. Leaf area was measured with a LI-3000A leaf area meter (Li-Cor Biosciences, Lincoln, NE, USA). Regression curves (data not reported) were used to estimate leaf area on each vine by measuring the length of all main and lateral shoots.

At harvest the collected data included, for both treatments: yield, bunch number and weight, berry mass. Rot incidence was expressed as percentage of infected area per bunch.

Pruning weight per tagged vine was measured every winter.

Berry sampling

Every 10 days, from one week after full veraison to harvest (on October 2 in 2012 and 2013; on September 30 in 2014), a random sample of 80 berries was collected within each block and per treatment (640 berries total) by cutting through the pedicel with scissors. Each sample was then divided into two subsamples that were immediately processed to determine: a) must biochemical parameters (40 berries); b) extractable

anthocyanins and tannins (40 berries). In each year at harvest two extra berry samples (of 20 berries each), collected within each block and per treatment (320 berries total), were frozen and stored at $-80\text{ }^{\circ}\text{C}$ for total anthocyanins and total tannins analyses.

Biochemical analysis of musts

Must parameter subsamples (40 berries each) were analyzed to determine TSS using a temperature-compensating Maselli R50 refractometer (Maselli Misure, Parma, Italy). Must pH and titratable acidity were measured using a Crison Titrator (Crison Instruments, Barcelona, Spain).

Extraction of anthocyanins and tannins using a model hydroalcoholic solution

Whole (not ground) skins and seeds from 40 berries were soaked separately and shaken daily, in tubes containing 80 mL of a hydroalcoholic solution for 15 days at $28\text{ }^{\circ}\text{C}$. The duration and temperature imposed on the extractions were chosen to simulate winemaking conditions and thus determine the concentration of extractable anthocyanins and tannins. The hydroalcoholic solution comprised 6 g/L tartaric acid, 40 mL/L 1 N NaOH, 100 mg/L potassium metabisulphite and a proportion of ethanol that was raised from 0 to 13% in the first 12 days of extraction. This concentration was reached by adding 2 mL of ethanol absolute (12 mL total) every two days to simulate alcoholic fermentation. The extracts were centrifuged (15 minutes, 13000 rpm) and aliquots of the supernatant (400 μL) were dried under vacuum at $20\text{ }^{\circ}\text{C}$. Pellets were stored at $-20\text{ }^{\circ}\text{C}$.

Exhaustive extraction of anthocyanins and tannins

Total anthocyanins were extracted from the skins of 20 berries by soaking the peeled skins in 100 mL methanol for 24 h, then storing the extracts at $-20\text{ }^{\circ}\text{C}$. Total tannins were extracted from the skins and seeds of 20 berries ground separately to a fine powder before extracting 1 mg of the sample in 1 mL 70% (v/v) acetone in water, for 24 hours in dark room.¹⁰ Skin and seed extracts were then centrifuged (15 minutes, 13000 rpm) and two 400 μL aliquots of the supernatant were dried under vacuum at $20\text{ }^{\circ}\text{C}$. Pellets were stored at $-20\text{ }^{\circ}\text{C}$.

Anthocyanin and tannin determinations

Total and extractable anthocyanins were separated by HPLC as described by Mattivi et al.⁴⁰ using a Waters 1525 instrument equipped with a diode array detector (DAD) and a reversed-phase column (RP18 250 x 4 mm, 5 μM) with a pre-column (Phenomenex, Castel Maggiore, BO, Italy). The concentration was determined by measuring absorbance at 520 nm. A calibration curve was established using a malvidin-3-glucoside standard (Sigma-Aldrich, ST. Louis, MO, USA).

Total and extractable tannins were measured by HPLC with the equipment described above. The concentration of tannin in skins and seeds was determined according to the methods previously reported^{41,42} by measuring absorbance at 280 nm. A calibration curve was established with commercial standards of catechin, epicatechin, epicatechin gallate and epigallocatechin (Extrasynthese, Genay, France).

Carbohydrate storage in cane wood

At the end of March 2015, two weeks before budburst, grapevine tissue from basal part of each cane (node 3) was collected during winter pruning and analyzed as reported by

Edward et al.⁴³ to investigate the replenishing of reserves in terms of soluble sugar and starch concentrations.

Statistical analysis

All the data collected were analyzed statistically by the mixed procedure available in SAS v9.0 (SAS Institute, Inc., Cary, NC, USA). Incidence values of rot are subjected to arcsin square root transformation before analysis. Treatment comparisons were analyzed using the Tukey test with a cut-off at $P=0.05$.

RESULTS

A careful analysis of the most important climatic parameters during the three years of the trial showed that 2014 was extraordinarily wet and cool, with total rainfall and average of the mean and maximum air temperature (calculated from April through September) reaching the highest and the lowest values respectively during the period of the trial (Table 1).

Vegetative, productive and berry compositional traits

Starting from uniform vines, trimming reduced leaf area by approximately 67% compared with those of the control and no lateral regrowth was recorded at harvest in any vintage (Table 2).

The bunch number per meter of cordon was uniformed and bunch mass and berry mass were unaffected by trimming. As regards the health of the grapes, no differences between the two treatments were found for rot infections, despite the highest level registered for both in 2014, compared to the previous year (data not reported). TRIMM had a strong effect in reducing leaf-to-fruit ratio and on the soluble solids concentration

(°Brix) that was significantly lower at harvest than that of C berries, while final must titratable acidity and pH content showed no difference among treatments (Table 2).

The levels of both total and extractable anthocyanins and total and extractable tannins from the berry skin and seeds at harvest, were not affected by the trimming (Table 3). As a consequence, the extractability of anthocyanins, skin and seed tannins, calculated as a percentage relative to the total amount at harvest showed no difference between treatments.

Evolution of soluble solids and phenolic compounds during ripening

On a 3-year basis, TRIMM reduced TSS at harvest by 1.4 °Brix as compared with that of C (Table 2, Figure 1). In particular, Figure 1 highlights that starting from ten days after trimming until harvest time, the difference in soluble solids accumulated between TRIMM and C vines remains constant for both 2012 and 2014 (Figure 1A, 1C). The situation was different for 2013 where a significant gap occurs only in the last two weeks before harvest (Figure 1B).

The evolution of extractable anthocyanins (Figure 2) did not vary among treatments and increased until harvest - in the first two years - when the trends appeared very similar with a daily increase of about 1 mg of anthocyanins per kg of grapes (Figure 2A and 2B). In the last year, the concentration of extractable anthocyanins reached maximum values two weeks before harvest and then decreased, especially in the control vines (Figure 2C).

The concentration of both extractable skin (Figure 3) and seed (Figure 4) tannins did not vary among treatments during ripening. In particular, as shown in Figure 3, the level of

extractable skin tannins increased during ripening in 2012 that was hotter and less rainy (Figure 3A, Figure 3B), while, in 2014, the amount of tannins extracted into solution decreased slightly from post-veraison to harvest (Figure 3C).

For extractable seed tannins, it is evident that the highest concentration was found around the time of trimming. Afterwards, the amount decreased strongly during the first ten days of ripening and then stabilized following a plateau.

Pruning weight and carbohydrate storage in cane wood

As expected, TRIMM vines presented a strong reduction (56%) of one-year-old wood for each trial year in comparison to C (Table 4). In March 2015, two weeks before budburst, the sample of basal canes collected to investigate the replenishing of reserves in these parts of the vine showed that TRIMM had accumulated 29% less starch reserves compared to untreated control at the end of the three years trial, while no differences were found in terms of soluble sugars content.

DISCUSSION

As reported by several authors, the level and timing of canopy leaf removal, performed via defoliation or trimming, can alter phenology of berry components during grape ripening and yield.^{44,45} In particular, leaf removal induced a reprogramming of source-sink balance and may mitigate the excess of sugar accumulation due to climate change.⁴⁶

Over three years of study on cv. Sangiovese, the strong reduction of about two thirds of leaf area, corresponding to the distal part of the shoot, imposed after veraison by shoot trimming, led to a decrease of leaf-to-fruit ratio largely below the threshold of 1 m²/kg,

with the expected reduction in sugar content, in agreement with Kliewer and Dokoozlian⁴⁵, but without an effect on yield. Since no lateral re-growth was recorded in any vintages the results are mainly due to well-established leaf age versus net photosynthesis (P_n) relationships^{47,48,49} and not to the competitive effects of new developing laterals.

In a previous research that tested the post-veraison trimming technique for three consecutive years on a low vigor and non-irrigated vineyard of Sangiovese²⁹ a yield constraint of treated vines was found in the last season. The behavior of Sangiovese grown in a severe water stress condition, which tends to sacrifice basal leaves keeping the younger ones much more functional,³⁸ may corroborate this response towards yield reduction. This situation did not occur in our case thanks to the irrigation that limited water stress damage, especially during the hottest season (2012), and to the well-balanced vine conditions at the beginning of the treatments. Furthermore, we can speculate that in present conditions the remaining basal leaves may reveal a photosynthetic compensation mechanism⁵⁰ in order to ensure the production and a sufficient level of reserves for the following year.

Several authors^{50,38,29,36} have previously shown that the limitation of sources immediately after veraison has no influence on the concentration of anthocyanins and tannins in berries and our results at harvest during the three years confirm these findings. A recent work⁴⁶ reported that the strong reduction in leaf-to-fruit ratio (from 1.15 m²/kg to 0.33 m²/kg caused by the removal of 80% of leaf area) at veraison resulted in a decrease of soluble solid accumulations associated with a significant

reduction of secondary metabolites, such as anthocyanins, without substantial differences in their composition. Our results are partially dissimilar to these findings, probably due to the postponing of source limitations - at the end of veraison - and the leaf-to-fruit ratio level, which was set to a higher value, showing a lower reduction of leaf area (about 63%).

Our results, over three years of study, registered no difference between treatments on the concentration of extractable anthocyanins and tannins in wine-like solution during ripening and at harvest, therefore we verify that, despite the drop in soluble solids caused by late trimming, there were no post-veraison trimming effect on concentration of total anthocyanins and tannins in berries as previously reported,^{29,38} but even in grape phenolic maturity⁵¹ traits during ripening, which represent a novel assessment. In particular, the main secondary metabolites involved in phenolic maturity (anthocyanins and skin tannins) increased their extractable portion during ripening in 2012 and 2013 in agreement with other studies,^{52,30} while during 2014, the extractable anthocyanins diminished after reaching a maximum value. The high incidence of rot close to harvest in 2014 could have promoted anthocyanins oxidation and the consequent drop in extractable anthocyanins. After sugar concentration starts to increase, susceptibility to infection by Botrytis cinerea also grows,⁵³ which may be linked to decreases in skin thickness during ripening⁵⁴ or cuticle cracking⁵⁵ that often occurs in wet seasons. Following penetration of the cuticle, Botrytis cinerea facilitates cell host death by induction of oxidative burst culminating in a depletion of phenolic compounds.⁵⁶

On the other hand, skin tannin accumulation showed minor fluctuation from veraison until harvest with no differences between treatments. This result may be caused by a dilution effect due to berry growth⁵⁷ during the third stage of ripening and the ceasing of tannin biosynthesis, which occurs before veraison.⁴¹ Our results are in agreement with those reported for Sangiovese³⁰ and Cabernet Sauvignon⁵⁸ berries extracted in a wine-like solution, where the value of skin tannins – and therefore their extractability – did not increase in the last phase of ripening.

Even though seeds represent a very small part of berry weight, they are a major source of phenolic compounds released in wine. The trend of extractable seed tannins in wine-like solutions in this study is better explained by tannin biosynthesis which is mainly concentrated during the early developmental stages of grape and reduces after veraison.^{59,60,61,62} In addition, in accordance with Allegro et al.,³⁰ the extractable seed tannins dramatically dropped after full veraison. This decline could be linked to solidification of the cells rich in tannins before harvest that could affect the aptitude for extraction of these compounds.^{63,64}

Late trimming in our conditions affected the replenishment of carbohydrates in canes at the end of three years of trial, and it seems that a leaf-to-fruit ratio below 0.6 m²/kg is not sufficient to ensure the yearly recovery of reserves for the following years, as previously suggested by other authors.^{45,65}

CONCLUSIONS

This study on post-veraison shoot trimming above bunch area of Sangiovese cv grown in high fertility conditions clarifies the effects of late source reduction applied over

three consecutive years, showing the vines vegetative, productive and berry compositional responses. In highly fertile and well-watered soil, post-veraison trimming is a powerful tool for decreasing sugar concentration at harvest without affecting yield and total and extractable phenolic compounds. Nevertheless, trimmed vines at the end of the third year had reduced their starch reserves compared to the control, serving as a warning about repeated trimming over the years.

In conclusion, it is important to point out that post-veraison trimming should be applied only when the seasonal climatic conditions would lead to excessive sugar accumulation.

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FIGURE CAPTIONS

Figure 1. Concentration of total soluble solids in berries of control (C) and post-veraison trimmed (TRIMM) Sangiovese vines in 2012 (A), 2013 (B) and 2014 (C). Arrowheads indicate time of trimming. Data are means (n=4) +/- standard error. Asterisks indicate significant differences between treatments within data according to the Tukey test Pd0.05.

Figure 2. Concentration of extractable anthocyanin in berries of control (C) and post-veraison trimmed (TRIMM) Sangiovese vines in 2012 (A), 2013 (B) and 2014 (C). Arrowheads indicate time of trimming. Data are means (n=4) +/- standard error.

Figure 3. Concentration of extractable tannin in berry skins of control (C) and post-veraison trimmed Sangiovese vines (TRIMM) in 2012 (A), 2013 (B) and 2014 (C). Arrowheads indicate time of trimming. Data are means (n=4) +/- standard error. Asterisks indicate significant differences between treatments within data according to the Tukey test Pd0.05.

Figure 4. Concentration of extractable tannin in berry seeds of control (C) and post-veraison trimmed Sangiovese vines (TRIMM) in 2012 (A), 2013 (B) and 2014 (C). Arrowheads indicate time of trimming. Data are means (n=4) +/- standard error. Asterisks indicate significant differences between treatments within data according to the Tukey test Pd0.05.

Table 1. Seasonal trend from April 1st through September 30th in 2012, 2013 and 2014.

	2012	2013	2014
Total rainfall (mm)	288	253	377
Average air temperature (°C)	21.4	20.5	19.9
Minimum air temperature (°C)	14.0	14.1	13.9
Maximum air temperature (°C)	28.7	26.7	22.7

Table 2. Leaf area, yield components, must composition and leaf to fruit ratio recorded at harvest in Sangiovese vines subjected to late trimming applied in post-veraison (TRIMM) and in control vines (C). Data averaged over 2012-2014.

Parameters	C	TRIMM	Year effect
Total LA after trimming (m ² /m)	6.39 a	2.11 b	NS
Bunches (n/m)	14	14	NS
Yield (kg/m)	4.63	4.56	*
Bunch mass (g)	337	341	NS
Berry mass (g)	2.79	2.75	NS
Rot incidence (% of surface area bunch infected)	12.5	9.4	*
Total soluble solids (°Brix)	21.7 a	20.3 b	NS
Titrateable acidity (g/L)	7.40	7.12	NS
pH	3.39	3.40	NS
Leaf-to-fruit ratio (m ² /kg)	1.48 a	0.55 b	NS

Means within rows designated by different letters are significantly different according to Tukey test. Asterisks indicate significance at Pd0.05; NS, not significant. No interaction was found between years and treatments.

Table 3. Total and extractable anthocyanins, skin tannins and seed tannins at harvest in Sangiovese vines subjected to late trimming applied in post veraison (TRIMM) and in control vines (C). Data averaged over 2012-2014.

Parameters	C	TRIMM	Year effect
Total anthocyanins (mg/kg)	627.5	664.9	NS
Extractable anthocyanins (mg/kg)	91.6	89.9	NS
Anthocyanin extractability (%)	13.9	14.6	NS
Total skin tannins (mg/kg)	992.7	906.2	*
Extractable skin tannins (mg/kg)	420.6	404.8	*
Skin tannins extractability (%)	58.3	54.7	NS
Total seed tannins (mg/kg)	1335.2	1332.2	NS
Extractable seed tannins (mg/kg)	629.9	612.6	*
Seed tannins extractability (%)	49.9	48.9	NS

Asterisks indicate significance at Pd0.05; NS = not significant. No interaction was found between years and treatments.

Table 4. Pruning weight and carbohydrates level in cane woods of Sangiovese vines subjected to late trimming applied in post veraison (TRIMM) and in control vines (C). Samples taken in March 2015.

	Pruning weight (kg/m)	Starch (mg/g dry wt)	Soluble sugars (mg/g dry wt)
C	1.10 a	76 a	37
TRIMM	0.49 b	54 b	30

Means within columns designated by different letters are significantly different according to Tukey test (Pd0.05).

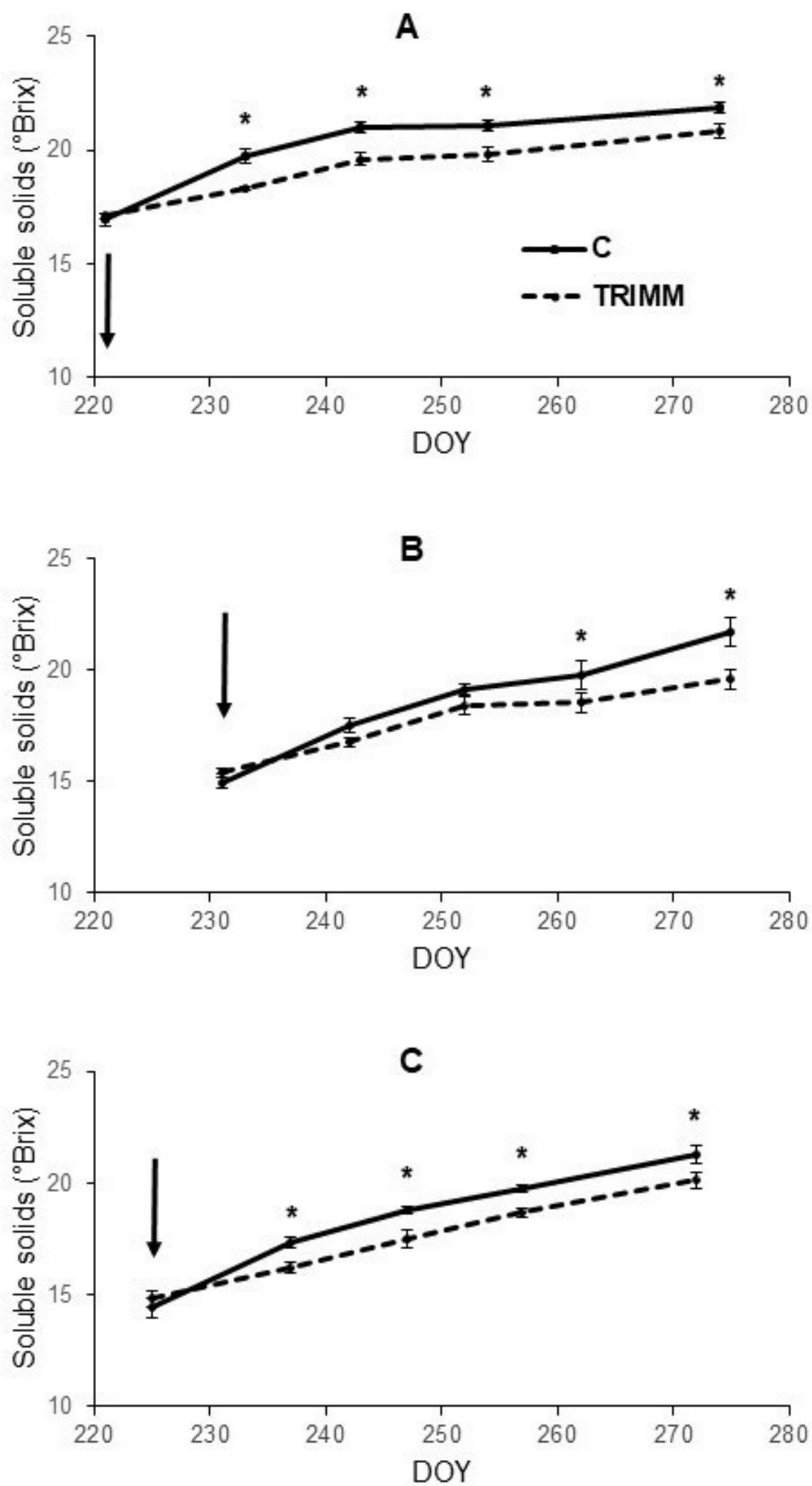


Figure 1.jpg

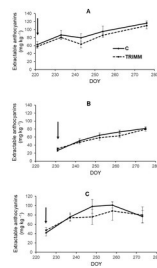


Figure 2.jpg

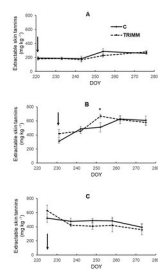


Figure 3.jpg

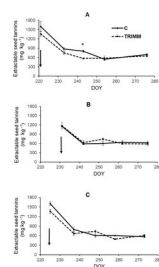


Figure 4.jpg