Impact of elevated carbon dioxide and temperature on strawberry polyphenols

Himali N. Balasooriya[1*], Kithsiri B. Dassanayake[1,2], Saman Seneweera[1,3], Said Ajlouni[1*]

1. School of Agriculture and Food, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, VIC 3010 Australia.
2. Department of Infrastructure Engineering, Faculty of Engineering, The University of Melbourne, Parkville, VIC 3010 Australia.
3. National Institute of Fundamental Studies, Hantana Road, Kandy, Sri Lanka.

*Corresponding author; Himali N. Balasooriya – phone: +61420620730;

e-mail: hbalasooriya@student.unimelb.edu.au

Abstract

Background: Strawberry cultivars 'Albion' and 'San Andreas' were grown under various combinations of day temperature (25 and 30 °C) and carbon dioxide [CO_2] (400, 650 and 950 µmoL/moL) conditions. The influence of different growth combinations on polyphenol, flavonoid, anthocyanin, antioxidant and individual phenolic compound contents of fresh strawberry fruits were studied. The contents of individual phenolic compounds of fresh strawberry fruits were quantified by using HPLC-UV system.

Results: Elevated [CO_2] and higher temperature caused significant increases in total polyphenol, flavonoid, anthocyanin and antioxidants in both strawberry cultivars when compared with plants grown under ambient growth conditions. Results of HPLC-UV analysis also revealed that individual phenolic compounds of fruits were also increased by increased [CO_2] and temperature. However, the responses were significantly altered by the interaction of elevated [CO_2] and higher temperature. In addition, the individual and interaction effects of [CO_2] and temperature were significantly cultivar dependent. The greatest amounts of flavonoid (482±68 mg/kg FW) and antioxidant (19.0±2.1 µmoL/g FW) were detected in 'Albion' grown under 30 °C and 950 µmoL/moL, and total polyphenol (3350±104 mg

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GAE/kg FW) and anthocyanin (332±16 mg/kg FW) in ‘San Andreas’ grown at 25 °C and 950 μmoL/moL.

Conclusion: Strawberry fruits were rich with polyphenols and antioxidants when they were grown under elevated [CO_2], higher temperature and their interactions. The increase contents of polyphenols and antioxidants of strawberry fruits will be highly beneficial to human health.

Keywords

Carbon dioxide, HPLC, polyphenol, strawberry, temperature

1. Introduction

Horticulture plants are used as the one of the main sources of food, fiber, biofuel, medicine and other products to sustain and enhance human life [1-3]. Strawberry (*Fragaria ananassa* Duch.) continues to acquire higher consumer demand globally due to its excellent nutrition profile including minerals, vitamin C, folates and polyphenols [4]. Additionally, among fruits, strawberry has been consistently appreciated for its health benefits and high dietary fibre content. Phytochemicals in strawberry display an immense biological potential in humans by providing protective and preventing effects against most diseases [4-8]. Ascorbic acid, ellagitannins and polyphenols are the major antioxidant compounds in strawberry which can prevent oxidative stresses and related diseases [8]. Moreover, in addition to the antioxidant property, strawberry polyphenols possess a great interest among other dietary phytochemicals showing, anti-inflammatory, antimicrobial, antiallergy, and antihypertensive properties [6]. More than 40 different compounds of polyphenols have been identified in strawberries [9] having diverse structure and function.

Flavonoids, tannins, phenolic acids, stilbenes and lignans are the major subgroups of polyphenols with the flavonoids being most abundant subgroup in plants [10]. Anthocyanins are quantitatively the most important subclass within flavonoids. They are the naturally occurring water soluble pigments which give strawberry its unique bright red colour, a critical factor in assessing the visual fruit quality of strawberries. The variability of anthocyanins is noteworthy, with more than 25 different anthocyanin compounds discovered in different strawberry cultivars [11]. Pelargonidin and cyanidin derivatives are the main categories in this group and while pelargonidin-3-glucoside (Pel-3-Glu) is the most dominant anthocyanin in strawberry. Cyanidin-3-glucoside (Cy-3-Glu) and pelargonidin-3-rutinoside (Pel-3-Rut) are the other commonly found anthocyanins in smaller amounts compared to Pel-3-Glu. Further, quercetin and kaempferol derivatives which belong to the subclass of flavonols in flavonoids, also function as co-pigments contributing to unique colour of strawberry fruits. Bioavailability, absorption and bioactivity of these compounds in humans are well-known to be relatively high [4] despite the fact that

they are present in strawberry at very low concentrations in comparison to pelargonidin and cyanidin derivatives.

Additionally, a range of other phenolic compounds such as phenolic acids (hydroxycinnamic acids, *p*-coumaroyl, ferulic acid derivatives), stilbenes (resveratrol) and coumarins (*p*-coumaric acid) which are beneficial to human nutrition and health, have also been identified in strawberry fruits [9, 11-13]. However, the strawberry polyphenol profile can widely vary qualitatively and quantitatively as affected by diverse factors, including genotype, environmental factors and their interactions [14-21].

Genetic make-up of strawberries is the most critical contributory factor in the variability of nutritional quality of fruits in terms of total polyphenol content (TPC), total antioxidant content (TAC) and level of individual polyphenol; however, growing conditions or environmental factors also play a significant role in determining the nutritional value of strawberries [14, 16, 18]. Both genotype and environmental factors have been reported to significantly influence the chemical composition of strawberry [12, 14, 22-25]. In general, a range of abiotic (environmental factors including, climate, soil, water stress) and biotic (plant pest and diseases) factors are known to alter strawberry quality and antioxidant profile. However, due to the anticipated climate changes, environmental factors such as, air temperature and CO_2 concentration [CO_2] are important as they are detrimental to the quality and nutritional value of strawberry. Atmospheric [CO_2] may increase from the current levels of 390 μmoL/moL to around 1000 μmoL/moL and temperature by 2.5 to 7.8 ºC at the end of this century [26-29]. These ongoing increases are predicted to cause significant changes in the quantity and quality of agricultural produce [29]. Furthermore, climate change could alter the water availability for crop production and increase the risks of drought and diseases, hence reduce food production and quality [30, 31]. Therefore, these factors, independently or interactively are expected to cause a substantial influence on strawberry phytochemicals.

Among the abiotic factors, growing temperature significantly influences nutritional quality of strawberries [21, 32]. For example, Wang and Zheng[21] demonstrated that increased polyphenol contents and high antioxidant capacity occur with increased day/night growing temperature. Further, Palmieri et

al.[17] reported enhanced concentrations of flavonols and ellagitannins in strawberries when the plants were subjected to higher temperature, ultraviolet irradiation and longer sunshine duration. Higher levels of anthocyanins and phenolic compounds were also reported when strawberries were grown under elevated [CO_2] (e[CO_2]) [33]. For example, resveratrol is an important polyphenol which provides several health benefits, was found in higher contents in strawberries grown either at high temperature or e[CO_2] [34]. Sun et al.[24], on the other hand reported reductions in antioxidant activity and antioxidant compounds occurred in strawberries grown under e[CO_2].

In general, most studies in the past evaluated the effects of temperature and [CO_2] on the nutritional quality of strawberries separately. However, an understanding of the interactive effects of these two environmental factors (high temperature and [CO_2]) on phytochemicals is limited in literature [35]. Thus, it is critical to investigate the interactive effects of e[CO_2] and temperature on the nutritional quality, more importantly the antioxidant contents, especially polyphenols in strawberries.

Polyphenol profile of strawberry shows highly diverse composition and La Barbera et al.[13] have confirmed or tentatively identified over 130 different individual compounds. The diversity of these compounds must be taken into consideration in fruit nutrition since the biological and pharmacological activities are specific to their chemical structure [7]. Therefore, analysing the amounts of individual compounds is important to evaluate the effects of genetic or environmental factors on the nutritional profile of fruits. Previous studies have mostly focused on total polyphenols as a group or a specific subgroup i.e. anthocyanins. However, a broad spectrum of polyphenol compounds would be available in different quantities in strawberries and will be affected in varying degrees by environmental factors. The study examined the effect of e[CO_2] and high temperature and their interactions on various polyphenols in two different strawberry cultivars.

2. Materials and Methods

2.1. Chemicals

HPLC gradient grade methanol, acetonitrile and formic acid were obtained from Thermo Fisher Scientific, Melbourne, Victoria, Australia. Hydrochloric acid, ethanol, Folin-Ciocalteu's reagent (FCR), Gallic acid, Sodium carbonate, 2, 2′-AzinoBis [3-ethylbenzo] Thiazoline-6-Sulfonic acid (ABTS), potassium persulfate, Trolox solution (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), potassium chloride, sodium acetate, potassium persulfate, and formic acid were purchased from Sigma-Aldrich Co, NSW, Australia. For laboratory analysis, polyphenol extractions and HPLC mobile phase, ultrapure water (Milli-Q® water) was used. Milli-Q® water was generated from a Millipore Milli-Q Ultrapure Water Purification System (ZMQP60001), Massachusetts, United States. HPLC grade (≥99.0%) reference standards; catechin, callistephin chloride, cyanidin chloride, *p*-coumaric, 6-O-*p*-Coumaroyl-1,2-digalloylglucose, trans ferulic acid and resveratrol were also purchased from Sigma-Aldrich Co, NSW, Australia. HPLC grade reference standards; pelargonidin-3-rutinoside chloride, kaempferol-3-O-glucoside, kaempferol-3-glucurnoride, quercertin-3,4-di-glucoside and quercertin-3-O-glucuronide were purchased from Extrasynthase, Genay Cedex, France.

2.2. Strawberry Fruits

Strawberries used in this study were produced from strawberry plants grown in controlled environment (CE) chambers (Model: TPG-2400-TH-CO_2, Thermoline Scientific Equipment Pty. Ltd., Wetherill Park, NSW, Australia) at the Parkville Campus of The University of Melbourne, Australia as described by Balasooriya et al.[36]. Briefly, two different day neutral strawberry cultivars, "Albion' and "San Andreas (SA)" were grown under different [CO_2] and temperature combinations inside automated CE chambers. The six different treatment combinations were 400 μmoL/moL×25 ºC, 400 μmoL/moL×30 ºC, 650 μmoL/moL×25 ºC, 650 μmoL/moL×30 ºC, 950 μmoL/moL×25 ºC and 950 μmoL/moL×30 ºC. Each experiment had four replicates and there were three plants per replicate. The treatments were applied during plant growth and development until fruit harvesting. Finally, healthy fruits (including all primary and secondary fruits) with 90% red colour were harvested separately from each chamber for each cultivar and for each replicate.

The antioxidant and polyphenol properties of strawberries were analysed in fresh strawberry fruits. Fruits for fresh analysis were stored in a cold room at 4 ºC until use.

2.3. Extraction of strawberry Polyphenols

Polyphenol compounds in strawberry fruits were extracted using 70% methanol and 0.18 N HCl following the method described by Tow et al. [37] with some modifications. Triplicate samples (5 g each) of fresh strawberry was homogenised with 70% methanol (15 mL) and 0.18 N HCl (5 mL) in 50 mL polyethylene tube (Ultra Turrax homogenizer, Janke and Kunnel, IKA-Labortechnik Ultra-Turrax T25). The homogenate was centrifuged at 8422g (Centrifuge, Thermoline, Scientific Equipment Pvt Ltd) for 15 minutes at room temperature and the resultant supernatant was collected quantitatively. The residue was washed with an additional 5 ml of methanol and the supernatants were combined. Methanol was evaporated from the collected supernatant under vacuum in a rotary evaporator at 60 °C with 8 rpm. The final extract was re-dissolved in Milli-Q water and the final volume was adjusted to 25 mL using a volumetric flask. This final polyphenol extract was then used for analysing the total antioxidants, polyphenols, flavonoid and anthocyanin contents in strawberries. An aliquot (2 mL) of the extract was filtered through 0.45 µm membrane when used for the analysis of individual polyphenol compounds using HPLC.

2.4. Total Polyphenol Content (TPC)

The TPC was measured following the method described by Tow et al. [37]. In this method, a mixture of 20 µL of polyphenol sample extract, 100 µL of 0.2 N Folin–Ciocalteu's reagent (FCR), 200 µL of Milli-Q water and 80 µL of sodium carbonate (7.5% w/v) was incubated at room temperature for one hour in a 96 well microplate. Absorption was then measured at 756 nm using a micro plate reader (Multiskan GO, Thermo Scientific, Australia) and the total polyphenol content was expressed as milligrams of Gallic acid equivalent (GAE) per kilogram of fresh strawberry sample (mg/kg FW). The same procedures were repeated with the solvent blank and the gallic acid standards.

2.5. Total Antioxidant Capacity (TAC)

The TAC in strawberry samples was analysed using ABTS assay by Re et al. [38] with slight modifications. The $ABTS^{\bullet+}$ radicals solution was prepared by reacting the stock solution of ABTS (7 mM) with 2.45 mM potassium per sulfate at the same ratio. The mixture was incubated overnight in the dark to generate the green ABTS• organic free radicals. It was then diluted with methanol to an absorbance of 0.7 at λ=734 nm. Sample extract or standard Trolox solution (25 µL) was mixed with ABTS solution (250 µL) directly in a 96 wells microplate and the absorbance was read at 734 nm after 6 mins. TAC was reported as µmoL Trolox equivalents per gram of strawberry fresh weight (µmoL/g FW).

2.6. Total Monomeric Anthocyanin Content (TMAC)

The TMAC of strawberries was determined using the pH differential method as described by Giusti and Wrolstad [39]. This method is based on the significant reversible changes in absorption of the anthocyanin pigments when the pH changes between 1.0 (highest absorption) and 4.5 (lowest = colourless hemiketal). The strawberry samples were appropriately diluted in 0.025 M potassium chloride (pH 1.0) and 0.4 M sodium acetate (pH 4.5). The mixtures were kept for 15 mins at room temperature before the absorptions were measured at 496 and 700 nm using the microplate reader. TMAC was calculated using molar absorptivity of 27300 L cm/moL and molecular weight of 433.2 g/mol for the anthocyanin Pel-3-glu and expressed as mg/kg FW.

2.7. Total Flavonoid Content (TFC)

A slightly modified colorimetric aluminium chloride method [40] was used in the determination of TFC of samples. In a microplate well, 125 µL of strawberry extract or solvent blank or quercetin standard was mixed with 25 µL of 1 M sodium acetate, 25 µL of 10% aluminium chloride, and 175 µL of Milli-Q water. The mixtures in the microplate were then left at room temperature for 1 h before measuring the absorbance at 415 nm using a micro plate reader. TFC was calculated using quercetin as a standard and expressed as mg of quercetin equivalents per kilogram of strawberry fresh weight (mg/kg FW).

2.8. HPLC-UV analysis for Identification and Quantification of Individual Polyphenols

Separation of polyphenols was performed using a Gemini C18 silica 250 × 4.6 mm, 5 µm column (Phenominex Inc., Lane Cove West, NSW, Australia) connected to a HPLC system equipped with Water 2690 Alliance Separation Module (Waters, Rydalmere NSW, Australia) and coupled with a Waters 2998 Photodiode Array (PDA) Detector. HPLC analysis was performed using a previous method by Kosar et al. [16] with slight modifications. The mobile phase was acidified with 0.2% (v/v) formic acid in Milli-Q water, (A) and acetonitrile (B). The gradient was 5% B for 1 min, 10% B at 10 min, 13% B at 15 min, 20% B at 20 min, 30% B at 25 min, 100% B at 35 min, and returned to 5% B at 40 min. The injection volume was 20 µL and the flow rate was maintained at 1 mL/min. Separation and quantification of polyphenols were performed at room temperature (25 °C). Identification of individual polyphenol peaks was based on both internal and external standards [41]. Identified peaks in strawberry extracts were quantified based on peak area and mass of the external calibration standard that generated the corresponding curve to the peak. During the quantification, average peak area was calculated as the average of two targeted peaks from duplicated HPLC injections. Different polyphenol compounds were detected and quantified at optimum and different wavelengths as required. These included, Pel-3-Glu, Pel-3-Rut and Cyanidin at 510 nm; Kaempferol-3-Glucoside and Kaempferol-3-Glucurnoride at 360 nm; Resveratrol at 320 nm; and Catechin, Ferulic acid, Quercertin-3,4-di Glucoside, Quercertin-3-O-Glucuronide, *p*-coumaric and *p*-Coumaroyl at 280 nm. Results were expressed as milligram or microgram per kilogram of strawberry fresh weight (mg or µg/kg FW) [12].

2.9. Statistical Analysis

Each laboratory experiment was performed in duplicate (2 trial) and each sample was triplicated in each trial. The results were presented as mean ± standard deviation (SD). Data were statistically analysed using Minitab® 17 Statistical Software following Analysis of Variance procedure with a General Linear Model (Minitab 17 Statistical Software (2010). The differences between the means were determined using Tukey's multiple comparison method at 95% confidence level.

3. Results and Discussion

3.1. Effect of high temperature and e[CO_2] on total polyphenols, flavonoid, anthocyanin and antioxidant contents in fresh strawberry

In general, increase in [CO_2] from 400 to 950 μmoL/moL, tended to increase TPC, TFC, TMAC and TAC of strawberry fruits significantly ($P<0.05$) across cultivars. Increase temperature in 5 °C significantly enhanced the TPC, TFC, TMAC and TAC of strawberries irrespective to the cultivar. However, strawberry fruits in cultivar 'Albion' contained significantly higher TAC than 'SA' grown under higher temperature (30 °C). As a whole, considering the effect of strawberry cultivar, fruits of 'San Andreas' had remarkably higher contents of total polyphenols and anthocyanin however, TAC of fruits was significantly higher in cultivar 'Albion'. The interaction of [CO_2] and temperature had significant impacts on TPC, TFC, TMAC and TAC of strawberries and the responses were cultivar dependant.

The effects of temperature, [CO_2] and their interactions on TPC of fruits in strawberry cultivars 'Albion' and 'SA' are shown in Figure I. In general, TPC of strawberry ranged from 970±74 to 2856±134 mg GAE/kg FW for the cultivar 'Albion', and from 904±87 to 3350±104 mg GAE/kg FW for the cultivar 'SA'. Although higher temperature (30 °C) had significant positive impacts on TPC of strawberry grown at ambient (400 μmoL/moL) and moderately high (650 μmoL/moL) [CO_2], the effects turned negative at very high [CO_2], indicating strong temperature and [CO_2] interactions on TPC. The highest TPC were detected at the maximum level of [CO_2] and 25 °C temperature in both 'Albion' and 'SA' while, the lowest TPC were observed under 400 μmoL/moL and 25 °C for both strawberry cultivars (Figure I).

Consequently, it could be concluded that despite the increases in temperature, elevated [CO_2] positively affected on TPC in fruits of both cultivars. Increase in temperature by 5 °C than 25 °C under 400 μmoL/moL [CO_2] enhanced fruit polyphenol contents by 68% and 98% in 'Albion' and 'SA', respectively. At lower [CO_2]; 400 μmoL/moL and 650 μmoL/moL, increased temperature by 5 °C than 25 °C increased the TPC of fruits but, it was opposite at 950 μmoL/moL in both cultivars. However,

both elevated temperature (30 °C) and [CO_2] to 950 μmol mol^{-1} increased polyphenols by 182% in 'Albion' and 206% in 'SA'.

The TFC of fruits in both strawberry cultivars under different temperature, [CO_2] and their interactions are shown in Figure II. The TFC of fruits ranged from 173±48 to 482±68 mg/kg FW for the cultivar 'Albion' and from 155±30 to 405±43 mg/kg FW for the cultivar 'SA'. Increased temperature (30 °C) under 400 μmoL/moL [CO_2] significantly enhanced the contents of strawberry flavonoids by 65% and 113% respectively in 'Albion and 'SA'. The interaction of e[CO_2] and higher temperature also had significant impact on TFC of strawberry and varied across the cultivar. Interactively, 950 μmol mol^{-1} [CO_2] and 30 °C enhanced the TFC by 183% and 173% respectively in 'Albion' and 'SA'. The interaction effect of e[CO_2] and temperature was stronger on TFC of fruits in cultivar 'SA' than 'Albion' (Figure II). Significantly lower contents of total flavonoid were reported at 400 μmol mol^{-1} [CO_2] and 25 °C in both cultivars.

Figure III illustrates the effects of temperature, [CO_2] and their interactions in growth environment on TMAC of strawberry fruits in cultivar 'Albion' and 'SA'. The TMAC ranged from 70±14 to 285±14 mg/kg FW in cultivar 'Albion' and from 67±15 to 331±16 mg/kg FW in 'SA'. Temperature increase in 5 °C under lower and moderately high [CO_2] affected positively on TMAC of fruits in cultivar 'SA' however, altered to a negative at 950 μmoL/moL [CO_2] showing a stronger interaction of [CO_2] and temperature. Although, higher temperature showed significantly positive impacts on TMAC of fruits in cultivar 'Albion' under 400 and 950 μmoL/moL, affected negatively at 650 μmoL/moL. The results also revealed a significant interaction effect of [CO_2], temperature and cultivar on the TMAC of strawberry fruits (Figure III). Moderately high [CO_2] and 30 °C had significantly positive impact on TMAC in cultivar 'SA' however, it was totally opposite for cultivar 'Albion'. Such variation in the responces of the tested culitvars to elevated [CO_2] and temperature could be attributed to the influence of each cultivar genotype on strawberry polyphenols. Increased temperature from 25 to 30 °C at 400 μmoL/moL enhanced strawberry anthocyanins only by 80% and 70% in 'Albion' and 'SA', respectively. The increase in TMAC of strawberries grown under 950 μmoL/moL [CO_2] and 30 °C temperature was

approximately 300% to 350% in comparison to the fruits grown under ambient growth conditions which had the lowest TMAC.

Figure IV shows the effects of [CO_2], temperature and their interactions on the TAC of strawberry fruits. The interaction effect of [CO_2] and temperature on TAC of strawberry was significant and cultivar dependent (Figure IV). Increased temperature to 30 °C positively affected on the content of strawberry antioxidants under all [CO_2] in cultivar 'Albion' however, negatively affected on TAC of fruit in cultivar 'SA' under moderate and highest [CO_2]. Increased temperature by 5 °C than 25 °C under ambient [CO_2] enhanced TAC of strawberry fruits by 46% and 80% respectively in cultivar 'Albion' and 'SA'. However, the highest e[CO_2] and higher temperature increased TAC by 179% and 110%, respectively in 'Albion' and 'SA'. The results explained a stronger interaction of [CO_2], temperature and cultivar on TAC of strawberries. Cultivar 'Albion' had the maximum Trolox equivalents TAC of 19.0±2.1 µmoL/g FW under 950 µmoL/moL and 30 °C and 'SA' contained the maximum of 12.6±1.3 µmoL/g FW under 950 µmoL/moL and 25 °C. Therefore, strawberry would contain greater contents of fruit antioxidants under elevated [CO_2] and high temperature. These findings were in agreement with results reported in section 3.1, regarding the effect of high temperature and elevated CO_2 on total polyphenols contents, and the conclusion of Wang and Lin [42]. Additionally, it has been reported that total antioxidants of fruits would increase significantly in the presence of higher contents of polyphenols [43], flavonoids and anthocyanins [44].

The correlations between TAC and TPC, TFC and TMAC were summarised in Table I. The correlation values between the TAC and TPC, TFC and TMAC were r = 0.94, $p \leq 0.001$, r = 0.79, $p \leq 0.05$ and r = 0.73, $p \leq 0.05$, respectively. Similarly, the results also revealed that TPC significantly correlated with both TFC and TMAC (r = 0.92, $p \leq 0.001$) of strawberries. Further, TFC positively correlated with TMAC (r = 0.85, $p \leq 0.001$) in fresh strawberries.

The positive correlation between increment in antioxidant, phenolic, anthocyanin, and flavonoid compounds was also reported by Wang et al. [33]. It is evident that flavonoids may increase in plants as a

part of general stress response and provide beneficial antioxidant properties [45]. These antioxidant compounds are produced in plants help to reduce oxidative stresses caused by free radicals [46]. The hydroxyl groups attached to the aromatic rings are responsible for the free radical scavenging properties of phenolic compounds. Besides the direct health benefits of polyphenols as antioxidants, strawberry polyphenols indirectly play a fundamental role against chronic metabolic disorders and cancers [7]. The same authors explained that strawberry polyphenols may be involved with cellular signalling, control the gene transcription and expression in cellular metabolism and survival in preventing chronic disorders and cancers.

Previous studies have reported significantly higher contents of polyphenols, flavonoids, anthocyanins and antioxidants under increased growth temperatures [21] or e[CO_2] [33]. In this study, e[CO_2], increased temperature and their interactions enhanced the levels of polyphenols, flavonoids, anthocyanins and antioxidants in strawberry however, these responses were significantly varied across the cultivars. Highest TPC and TMAC were detected in cultivar 'SA'. However, TFC and TAC were significantly ($P<0.05$) higher in cultivar 'Albion'. A previous study by Palmieri et al. [17] demonstrated the effect of genotype and environmental conditions on nutrient contents in nine strawberry cultivars. According to those results, amongst the nine cultivars, 'Albion' was the most sensitive cultivar, which responded to the different environmental conditions. Palmieri et al. [17] attributed the higher contents of plant secondary products under different growth conditions to the acclimatization imposed by environmental stresses. The same authors concluded that genotype was more influential on some strawberry cultivars over all the other parameters. A study by Ariza et al. [47] showed that genotype has a strong influence on strawberry phenolic and anthocyanin contents. Similar to the genotype effect, the current study revealed that e[CO_2] and/or higher temperature could significantly ($P<0.05$) enhance the polyphenol and antioxidant contents of strawberry fruits. Since polyphenols, flavonoids, anthocyanins, and antioxidants are considered as important fruit bioactive compounds, increasing the contents of such fruit nutrients with e[CO_2], higher temperature and their interactions would improve strawberry functional properties.

Consequently, strawberry grown under higher temperature (30 °C) and e[CO_2] (650 and 950 μmoL/moL), could support a better human health.

3.2. Identification and quantitation of individual polyphenols in strawberry gown under high temperature and e[CO_2]

The main polyphenols that have been reported in strawberry belong to chemical classes namely; flavonoids (anthocyanins, flavanols, flavonols), phenolic acids, lignans, stilbens, tannins and coumarins [48]. In this investigation, tweleve different phenolic compounds were identified in strawberry cultivars 'Albion' and 'SA' using external and internal standards at different wavelength (Table II). HPLC chromatograms of polyphenol compounds in strawberries at different wavelengths are shown in Figure V.

Peak 2 detected at 280 nm, 320 nm and 360 nm (Figure V) could not be identified with external or internal standards due to the inaccessibility of the reference standard. According to the literature [11, 49], this peak could be cyanidin-3-glucoside, and was reported to be constant in all strawberry varieties [11]. Most of the identified polyphenol compounds were quantified considering their peak areas at the maximum absorbance. Table III and IV show the concentrations of different polyphenol compounds of fresh fruits of strawberry cultivars 'Albion' and 'SA' and illustrate the effects of different [CO_2], temperature and their interactions on each compound.

3.2.1. Effect of e[CO_2] and high temperature on strawberry anthocyanins

Anthocyanins have an utmost importance in strawberry polyphenol profile, specifically, the major group of pigments accounts for strawberry colour. Specially, the aglycones of pelargonidin and cyanidin are the predominantly found anthocyanins [11] and are highly responsible for the red colour depending on cultivar [22]. Pel-3-Glu revealed the highest contents followed by cyanidin and Pel-3-Rut in both 'Albion' and 'SA'. The impacts of [CO_2], temperature, and their interactions on different anthocyanins compounds of fruits in strawberry cultivars 'Albion' and 'San Andreas' are shown in Table III.

The Pel-3-Glu content ranged from 115±23 to 273±30 mg/kg FW for cultivar 'Albion' and from 114±24 to 348±33 mg/kg FW for cultivar 'SA'. Elevated [CO_2] gradually increased the content of Pel-3-Glu in strawberry fruits in both cultivars. Elevated [CO_2] to 950 μmoL/moL caused 137% and 205% increment in Pel-3-Glu content in 'Albion' and 'SA' respectively in comparison with the growth at 400 μmoL/moL and 25 °C. Higher temperature had positive impact on Pel-3-Glu only at 400 μmoL/moL by increasing the contents of Pel-3-Glu by 15% and 51% in 'Albion' and 'SA' respectively. Under e[CO_2] (650 and 950 μmoL/moL), 30 °C temperature had negative impact on Pel-3-Glu showing the significant interaction effect of [CO_2] and temperature. The interaction of elevated [CO_2] (950 μmoL/moL) and higher temperature (30 °C) enhanced Pel-3-Glu contents by 90% and 103% in 'Albion' and 'SA' respectively, in comparison with plants grown under 400 μmoL/moL and 25 °C. The maximum contents of Pel-3-Glu were detected under maximum e[CO_2] and 25 °C in both cultivars however, cultivar 'SA' contained significantly higher Pel-3-Glu contents than 'Albion'.

Elevated [CO_2] and higher temperature separately showed positive effects on Pel-3-Rut contents in both cultivars (Table III). Highest e[CO_2] and higher temperature interactively enhanced the Pel-3-Rut contents of fruits from 18±3 to 41±4 mg/kg FW for cultivar 'Albion' and from 24±4 to 58±6 mg/kg FW for cultivar 'SA', respectively. The interaction effect of [CO_2] and temperature on Pel-3-Rut contents was significantly cultivar dependant. Higher temperature and 950 μmol mol^{-1} [CO_2] positively affected on Pel-3-Rut contents of strawberries in cultivar 'Albion' however, the effect turned negative in cultivar 'SA' (Table III). Under all growth combinations, 'SA' contained comparatively and significantly higher contents of Pel-3-Rut than 'Albion'.

Cyanidin contents of fruits in strawberry cultivars 'Albion' and 'SA' were increased by e[CO_2] from 400 to 950 μmoL/moL (Table III). However, the interaction of e[CO_2] and temperature was stronger on cyanidin contents of strawberry than their individual impacts. Increased temperature by 5 °C than 25 °C was positively affected on cyanidin contents of strawberry fruits in both cultivars only under 400 and 950 μmoL/moL [CO_2]. Higher temperature had negative impacts on cyanidin contents under 650 μmoL/moL in both cultivars. Elevated [CO_2] of 950 μmoL/moL and 30 °C increased the content of

cyanidin from 27±4 to 175±32 mg/kg FW in cultivar 'Albion' and from 28±3 to 150±22 mg/kg FW in 'SA'. Elevated [CO_2] from 400 to 950 µmoL/moL at 30 °C increased the cyanidin content by 600% and 435% in 'Albion' and 'SA', respectively.

Both, Pel-3-Glu and Pel-3- Rut (pelargonidin derivatives) were detected in larger quantities in cultivar 'SA', while cyanidin was present in large quantities in cultivar 'Albion' when grown under highest e[CO_2] and higher temperature (950 µmoL/moL and 30 °C). These results were in agreement with those reported by Wang and Zheng [21] and Wang et al. [33].

3.2.2. Effect of e[CO_2] and high temperature on strawberry flavonols

Flavonols compounds showed significant variations under increased [CO_2], temperature, and their combination (Table III). Flavonol contents were comparatively lower than the anthocyanins contents in strawberries. Quercetin glucoside (Q-3, 4-di-O-Glu) contents of fruits ranged from 10.1±1.0 to 24.8±2.8 mg/kg of FW and 9.8±1.0 to 25.1±3.0 mg/kg FW in cultivars 'Albion' and 'SA', respectively (Table III). Elevated [CO_2] significantly increased the contents of Q-3, 4-di-O-Glu at 25 °C in both strawberry cultivars. Higher temperature individually enhanced the Q-3, 4-di-O-Glu contents of strawberries however, interactively with 950 µmoL/moL affected negatively on Q-3, 4-di-O-Glu contents in both strawberry cultivars.

Kaempferol glucoside (K-3-Glu'side) and glucuronide (K-3-Glu'nide) were found in comparatively smaller quantities in strawberries compared to other flavonoids (Table III). K-3-Glu'side content varied from 596±81 to 1648±121 µg/kg FW and 603±85 to 1849±33 µg/kg FW in cultivar 'Albion' and 'SA', respectively. Elevated [CO_2], temperature and their interactions significantly increased the kaempferol amounts in strawberries however, the amounts were varied among cultivars. Unsimilar to anthocyanins, which showed the greatest increment at 30 °C and 950 µmoL/moL CO_2, the maximum amounts of K-3-Glu'side were detected under 950 µmoL/moL and 25 °C growth conditions in both cultivars. K-3-Glu'nide content ranged from 512±81 to 1767±121 µg/kg FW and from 591±85 to 1527±28 µg/kg FW in 'Albion' and 'SA', respectively. The lowest amounts were detected under 400 µmoL/moL and 25 °C

and the highest contents under highest e[CO_2] and higher temperature. Similar to the results for anthocyanins, most flavonols contents revealed the greatest amounts under extreme growth condition of 30 °C and 950 µmoL/moL. These findings are in agreement with those reported in the literature [21, 33]. For example, Wang et al. [33] reported higher quercetin glucoside, quercetin glucuronide and kaempferol glucoside concentrations in strawberry fruits under enriched (600 µmoL/moL than ambient) [CO_2] condition. These flavonoids contain hydroxyl or methoxy groups which to contribute their biological and antioxidant properties. Therefore, increased amounts of these flavonoids play pivotal roles in preventing oxidative stresses [50].

3.2.3. Effect of e[CO_2] and high temperature on other phenolic compounds

In addition to flavonols that were discussed before, catechin, ferulic, coumaric, coumaroyl and resveratrol were also detected and quantified in strawberries (Table IV). All these compounds were found to be varied significantly in strawberry cultivars under different [CO_2], temperature and their interactions. Catechin is a monomeric flavanol, generally, reported difficult to be detected [51]. When [CO_2] was elevated from 400 to 950 µmoL/moL in growth environment, catechin content of fruits increased gradually from 9±1.6 to 43±5.8 mg/kg FW and 8±0.8 to 58±4.3 mg/kg FW in 'Albion' and 'SA', respectively. Higher temperature also affected positively on catechin content of fruits in both strawberry cultivars only under elevated [CO_2]. The interaction of e[CO_2], temperature and cultivar was statistically significant ($P<0.05$). Increased temperature by 5 °C than 25 °C interactively with 650 and 950 µmoL/moL [CO_2] enhanced catechin concentrations in strawberry cultivar 'Albion'. However, higher temperature showed negative impact on the catechin content of fruits in cultivar 'SA' at 950 µmoL/moL. Moreover, cultivar 'SA'contained comparatively higher catechin contents than 'Albion' under all e[CO_2] and higher temperature conditions.

Increased temperature significantly influenced on the ferulic acid content of strawberries (Table IV). Ferulic acid content of strawberries showed changes in pattern compared with catechin, with the maximum detected quantities of 63±4 and 67±3 mg/kg FW in cultivar 'Albion' and 'SA' respectively, at

400 μmoL/moL CO_2 and 30 °C. Consequently, increasing the [CO_2] above the 400 μmoL/moL would not have any significant effect on ferulic acid. However, the interaction of [CO_2], temperature and cultivar was significant on ferulic acid contents of fruits. The same results revealed that e[CO_2] at higher temperature caused negative effects on strawberries. Coumaroyl contents ranged from 4.4±0.4 to 14±1.6 mg/kg FW for cultivar 'Albion' and from 4.4±0.3 to 15.4±0.7 mg/kg FW for cultivar 'SA'. The lowest coumaroyl contents were detected under 400 μmoL/moL and 25 °C and the highest under highest e[CO_2] and higher temperature. In previous studies, higher contents of *p*-coumaroyl glucose was reported in strawberry grown under high temperature (30 °C) [21] and enriched [CO_2] [33]. Greater amounts of coumaroyl derivatives were also detected in grapes grown under high growth temperatures [52, 53].

Resveratrol is a stilbene polyphenol and is available only in a few plant sources including strawberries in smaller quantities. As far as resveratrol is concerned, its synthesis can be successfully induced in plants using molecular engineering techniques [54]. From all the polyphenol compounds identified in this study, resveratrol had the lowest quantities in strawberry. The resveratrol content in strawberry varied from 23±1 to 113±5 μg/kg FW and from 23±2 to 82±6 μg/kg FW in 'Albion' and 'SA', respectively. Elevated [CO_2] and high temperature individually and interactively enhanced the resveratrol contents in strawberries. However, 'Albion' revealed the highest resveratrol content under 950 μmoL/moL and 30 °C and 'SA' under 650 μmoL/moL and 30 °C. Similar observation were reported by Wang et al. [34] who demonstrated an increase in resveratrol contents in strawberries under high growth temperatures (25 and 30 °C) or under enriched [CO_2] by 600 μmoL/moL in the growth environment than under ambient growth conditions. In current study, resveratrol content was increased in in strawberries under both e[CO_2] and temperature compared to the strawberries grown under 400 μmoL/moL and 25 °C temperature. Similar to other polyphenols, resveratrol can contribute significantly to the antioxidant contents in strawberry fruits. Resveratrol has also been linked to many health benefits in humans including, preventing and delaying cancers, cardiovascular diseases, heart diseases, pathological inflammations, viral infections, and tumors [54]. Therefore resveratrol produces no harm even when the intake is high [55]. All these phenolic compounds express antagonistic and synergistic activities

interactively with other polyphenols and/or phytochemicals in fruits [40] and could increase the biological potential of strawberry.

The positive impacts of e[CO_2] 650 and 950 μmoL/moL and higher temperature (30 °C) individually and interactively on the contents of individual polyphenols in strawberries have been well established in this investigation. These findings are in agreement with the reported literature, which indicates that under optimal growth conditions, plants promote biomass production and reduce the biosynthesis of secondary metabolites [56]. However, plants stimulate the production of secondary metabolites as a defensive response to the environmental stresses [46]. The biosynthesis of polyphenols starts from phenylalanine to produce phenylpropanoid[57]. *P*-coumaroyl CoA and malonyl CoA are derived from phenylpropanoid and they are the precursors of flavonoid biosynthesis and different enzymes are involved in synthesising major flavonoid classes [45]. These key enzymes usually compete for the same substrate to produce different flavonoid compounds. Generally, the ultimate product of phenylpropanoid pathway is anthocyanin; however, it branches in the middle and, other polyphenols including stilbenes and lignin are synthesised [58]. The synthesis of these compounds was reported to be sensitive to the stresses in the growth environment [57,59].

However, the degree of stress accounts for different secondary metabolites and their varied quantities in plants [58]. In this study, higher temperature (30 °C) in the growth environment individually and interactively with e[CO_2] encouraged the increase of individual polyphenols in different quantities in strawberry. Increasing the temperature in the growth environment would increase the synthesis and accumulation of polyphenolic compounds in response to the increased rate of metabolic processes in plant [58]. However, high temperature could also stop or significantly reduce the metabolic processes in some plants due to the irreversible damages to the plant tissues. These irreversible damages to some plants by heat could be varied in different plant species and cultivars. For example, Downey [58] observed that some phenolic compounds were decreased or stable at high temperature (30 °C) in grapes.

Similarly, high [CO_2] in the growth environment can increase the carbon supply where it promotes higher carbon availability in plants and accumulates more of carbohydrates [60]. These carbohydrates are firstly utilised in plant growth and then the excess amounts are used in the synthesis of carbon based secondary products specially soluble phenols and condensed tannins [61].

In summary, stress growth conditions, e[CO_2] (650 and 950 μmoL/moL) and /or higher temperature (30 °C), can encourage the synthesis and accumulation of phenolic compounds and significantly enhance the corresponding antioxidant properties. Although, a previous study in our lab [36] showed that strawberry fruits had lower yields and physical quality under stress growth conditions, results from this current investigation revealed that strawberry fruits were rich with polyphenols and antioxidants under e[CO_2] (950 μmoL/moL) and higher temperature (30 °C) conditions. The increase contents of total and individual polyphenols and antioxidants of strawberry fruits will be highly beneficial to human health.

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6. Figure Legends

Figure I. Total polyphenolic contents (TPC) of two different strawberry cultivars ('Albion' and 'San Andreas') at different combinations of temperature and [CO_2]. Error bars refer to standard deviation of data (n = 12). Different letters in both cultivars and within each CO_2 concentration are significantly ($p \leq 0.05$) different.

Figure II. Total flavonoid contents (TFC) of two different strawberry cultivars ('Albion' and 'San Andreas') at different combinations of temperature and [CO_2]. Error bars refer to standard deviation of data (n = 12). Different letters in both cultivars and within each CO_2 concentration are significantly ($p \leq 0.05$) different.

Figure III. Total monomeric anthocyanin contents (TMAC) of two different strawberry cultivars ('Albion' and 'San Andreas') at different combinations of temperature and [CO_2]. Error bars refer to standard deviation of data (n = 12). Different letters in both graphs are significantly ($p \leq 0.05$) different**.**

Figure IV. Total antioxidant contents (TAC) (µmoL Trolox equivalents per g of FW) of two different strawberry cultivars ('Albion' and 'San Andreas') at different combinations of temperature and [CO_2]. Error bars refer to standard deviation of data (n = 12). Different letters in both graphs are significantly ($p \leq 0.05$) different.

Figure V. HPLC chromatographs of polyphenols of strawberries obtained at 280, 320, 360 and 510 nm. 1. Catechin, 2. Unknown, 3. Pelargonidin-3-Glucoside (Pel-3-Glu), 4. Pelargonidin-3-Rutinoside (Pel-3-Rut), 5. Cyanidin, 6. Quercetin-3,4-di Glucoside (Q-3,4-diGlu), 7. p-Coumaric, 8. Ferulic acid, 9. Quercetin-3-O-Glucuronide (Q-3-O-Glu'nide), 10. Coumaroyl, 11. Kaempferol-3-Glucoside (K-3-Glu), 12. Kaempferol-3-Glucuronide (K-3-Glu'nide), and 13. Resveratrol

Figure I

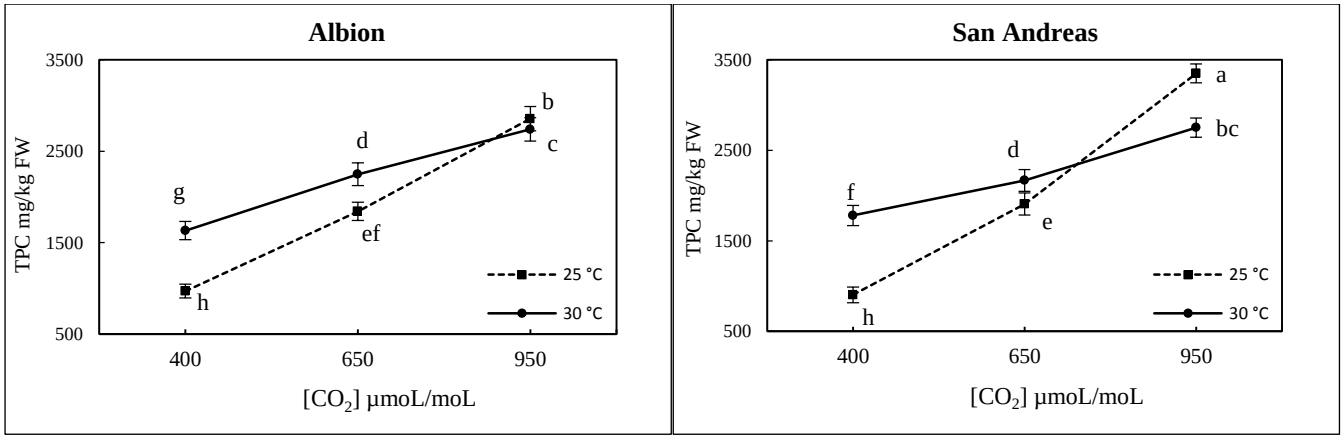


Figure II

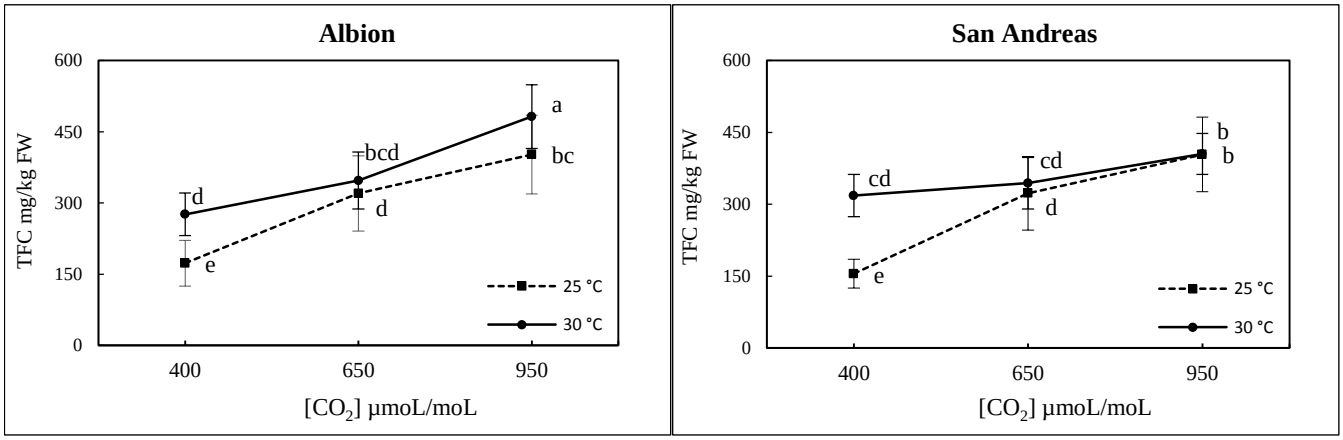
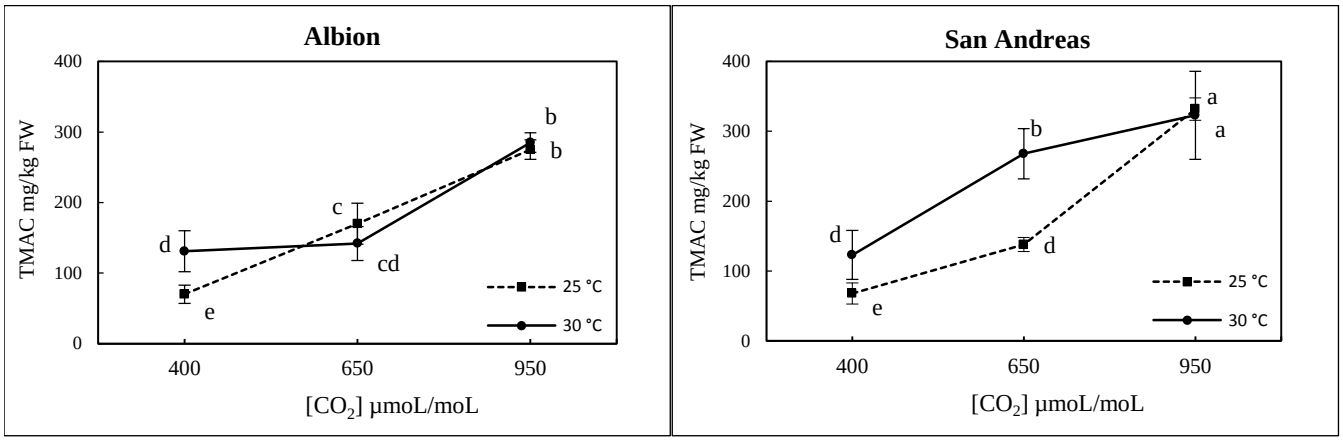


Figure III



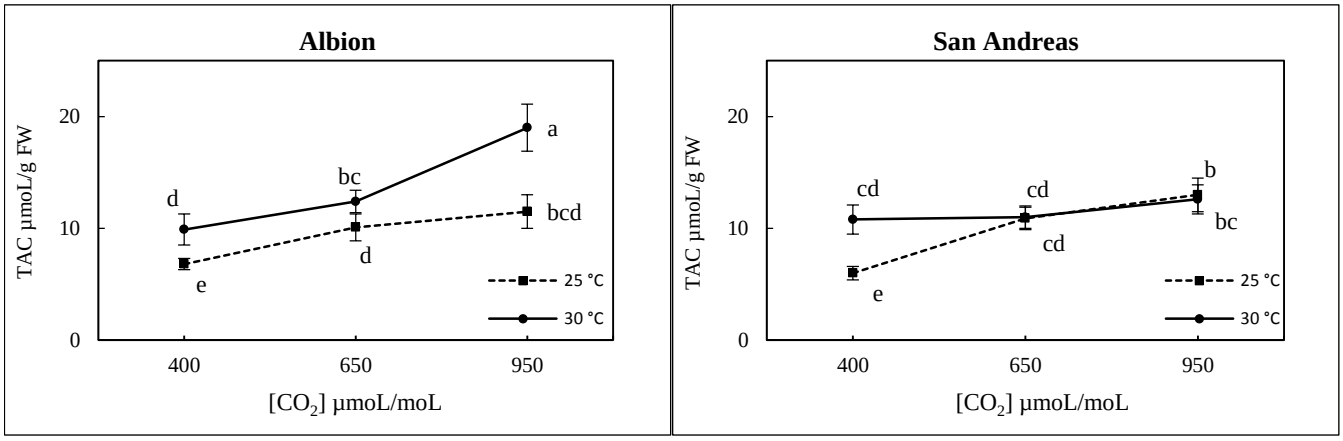


Figure IV

Figure V

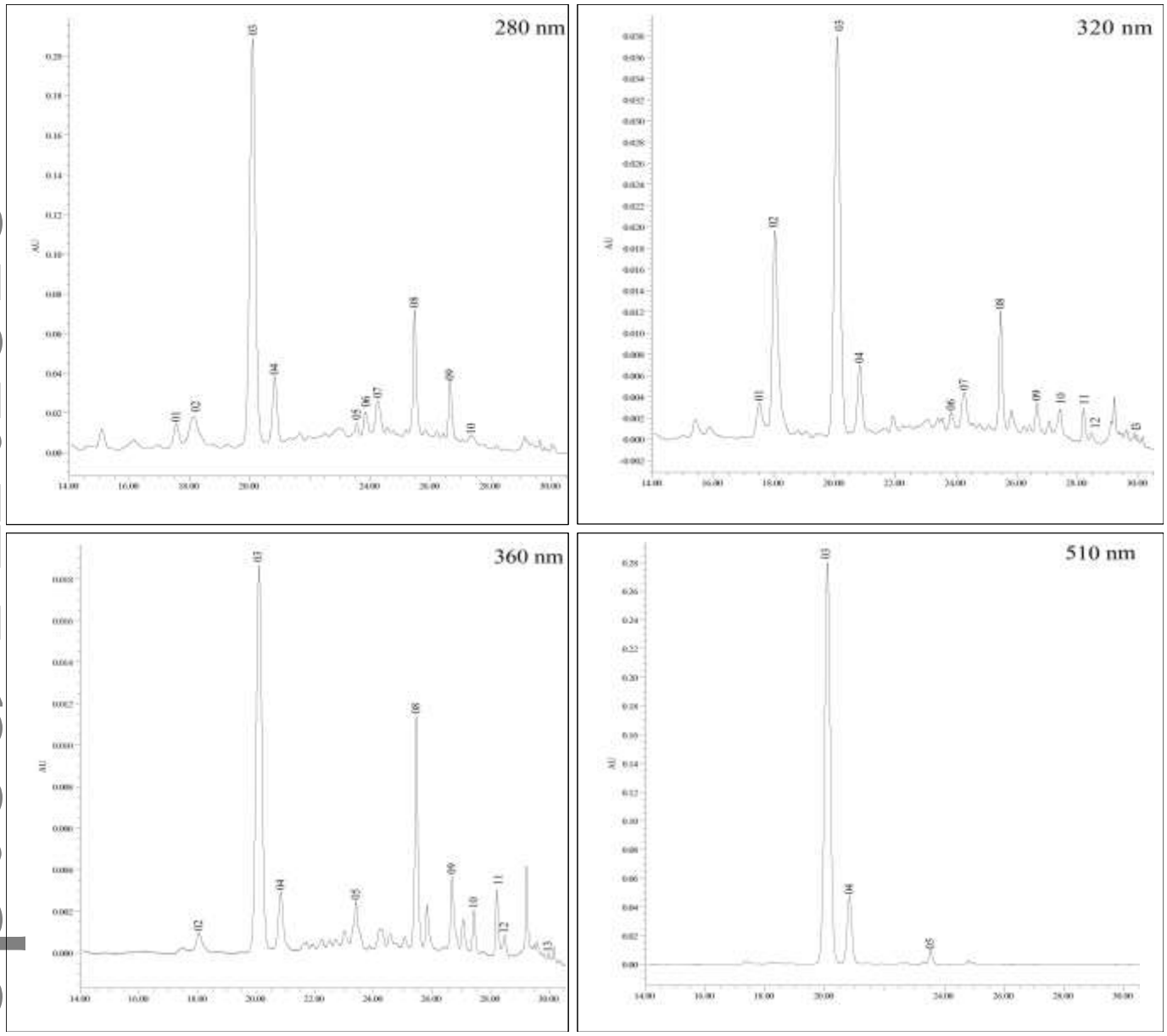


Table I. Pearson's correlation matrix between TPC, TFC and TMAC in strawberry cultivars

Variable	TPC	TFC	TMAC
TFC	0.919**		
TMAC	0.922**	0.851**	
TAC	0.789*	0.937**	0.729*

Values are Pearson correlation coefficients.

* - significant correlation at $p \leq 0.05$, ** - significant correlation at $p \leq 0.001$

Table I

Table II. Identification of strawberry polyphenols

Peak	Polyphenol Compound	RT (Minutes)	λ (nm)
01	Catechin	17.5±0.03	280
02	Unknown	18.2±0.30	280
03	Pelargonidin-3-Glucoside (Pel-3-Glu)	20.0±0.16	510
04	Pelargonidin-3-Rutinoside (Pel-3Rut)	20.8±0.12	510
05	Cyanidin	23.5±0.21	510
06	Quercetin-3,4-di Glucoside (Q-3,4-diGlu)	23.8±0.26	280
07	p-Coumaric	24.2±0.80	280
08	Ferulic acid	25.4±0.21	280
09	Quercetin-3-O-Glucuronide (Q-3-O-Glu'nide)	26.8±0.21	280
10	Coumaroyl	27.4±0.22	280
11	Kaempferol-3-Glucoside (K-3-Glu)	28.3±0.28	360
12	Kaempferol-3-Glucuronide (K-3-Glu'nide)	28.7±0.26	360
13	Resveratrol	29.9±0.31	320

Different polyphenol compounds were identified in a range of 200 – 600 nm wavelength. λ – specific wavelength of compound quantification, RT – retention time (Mean ± SD)

Table II

Table III. Effect of different temperature and [CO_2] on different anthocyanins and flavonols compounds in strawberry cultivars

Cultivar	T	[CO_2]	Anthocyanins			Flavonols			
			Pel-3-Glu [a]	Pel-3-Rut [b]	Cyanidin [c]	Q-3,4-di-O-Glu'side [d]	Q-3-O-Glu'nide [e]	K-3-Glu'side [f]	K-3-Glu'nide [g]
Albion	25 °C	400	115±23 f	18±3 g	27±4 g	10.1±1.0 e	18±2 c	596±81 e	512±81 e
		650	177±29 d	25±3 ef	79±13 d	19.3±2.1 b	6.3±0.8 g	508±58 e	534±58 e
		950	273±30 b	34±4 c	94±9 d	24.6±3.4 a	20±3 c	1432±111 c	733±111 d
	30 °C	400	132±42 ef	29±4 cde	48±6 fg	20.1±1.5 b	39±3 b	619±69 de	624±69 de
		650	165±22 de	29±4 d	61±4 ef	24.8±2.8 a	13±3 fg	760±87 d	572±87 e
		950	219±25 c	41±4 b	175±32 a	16.5±3.3 c	15±2 cde	168±121b	1767±121 a
San Andreas	25 °C	400	114±24 f	24±4 f	28±3 g	9.8±1.1 e	18±3 cd	603±85 e	591±85 e
		650	154±18 de	31±4 de	66±8 ef	20.1±2.6 b	10±2 efg	532±65 e	595±65 e
		950	348±33 a	62±6 a	128±18 c	25.1±3.3 a	52±7 a	1849±33 a	949±33 c
	30 °C	400	172±60 d	34±3 cd	48±5 fg	20.4±2.4 b	45±5 b	469±49 b	1407±49 b
		650	160±28 de	34±4 c	57±4 ef	22.6±3.8 ab	9.9±2 def	626±25 e	590±25 e
		950	231±26 c	58±6 a	150±22 b	13.9±1.7 d	9.4±2 fg	1439±28 c	1527±28 b
Temperature × [CO_2]			*	*	*	*	*	*	*
Temperature × [CO_2] × Cultivar			*	*	*	*	*	*	*

[a,b,c,d,e] expressed as mg/kg FW, [f,g] expressed as µg/kg FW. *significant ($p \leq 0.05$). T – Temperature and [CO_2] = µmoL/moL

Values are mean ± SD (n=12), different letters in each column are significantly ($p \leq 0.05$) different.

Table IV. Effect of different temperature and [CO_2] on other polyphenol compounds in strawberry varieties

Cultivar	Temperature	[CO_2]	Catechin [a]	Ferulic Acid [b]	*p*-Coumaroyl [c]	*p*-Coumaric [d]	Resveratrol [e]
Albion	25 °C	400	9±1.6 f	24±0.4 ef	4.4±0.4 c	1.07±0.03 c	23±1 f
		650	15±2.4 ef	28±1 cd	10.4±0.8 b	1.35±0.03 c	65±8 cd
		950	32±4.8 d	23±1 e	10.7±0.3 b	2.86±0.02 a	59±3 de
	30 °C	400	9±0.7 f	63±4 b	5.5±0.8 c	3.23±0.03 a	66±2 cd
		650	21±0.4 e	30±2 b	9.4±1.9 b	2.98±0.03 a	84±7 b
		950	43±5.8 c	29±1 cd	14.0±1.6 a	2.39±0.03 b	113±5 a
San Andreas	25 °C	400	8±0.8 f	24±0.4 ef	4.4±0.3 c	1.05±0.02 c	23±2 f
		650	20±2.2 e	30±0.9 cd	11.0±0.9 b	2.15±0.02 b	71±6 c
		950	58±4.3 a	25±2 f	13.9±0.9 a	2.79±0.02 a	58±5 e
	30 °C	400	8±0.8 f	67±3 a	5.6±0.8 c	2.89±0.03 a	71±3 c
		650	30±4.4 d	31±2 c	10.2±1.7 b	2.90±0.03 a	82±6 b
		950	50±6.0 b	28±0.4 cd	15.4±0.7 a	2.96±0.08 a	68±2 c
Temperature× [CO_2]			*	*	*	*	*
Temperature× [CO_2] × Cultivar			*	*	*	*	*

[a,b,c d] expressed as mg/kg FW, [e]expressed as µg/kg FW. *significant ($p \leq 0.05$). [CO_2] = µmoL/moL

Values are mean ± SD (n=12), different letters in each column are significantly ($p \leq 0.05$) different.

Table IV