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Quantification and Distribution of Primary and Secondary Metabolites in the Inner and Outer Parts of Strawberry Fruit

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Abstract: The distribution of primary and secondary metabolites within a fruit can affect its nutritional and organoleptic quality, as fruit can vary in size and shape. This study investigated the differences in the distribution of the primary and secondary metabolites in the fruit of four strawberry cultivars ('Asia', 'Clery', 'Frederica', and 'Sandra') that were collected at one harvest point. The study included an analysis of the individual sugars, organic acids, phenolic compounds, and enzymes responsible for the degradation of phenolics. All of the studied cultivars showed a lower pH, higher total organic acid content, and lower glucose and fructose content in the outer part of the fruit. Differences were also observed in the total phenolic and anthocyanin contents, which were always higher in the outer part. The absolute differences in the total phenolic content ranged from 3723 to 6154 mg kg^{-1} dry weight. Our results provide a basis for understanding the differences in the biosynthesis of these metabolites within this fruit and prove that it is essential to mix samples well before extractions to obtain results that are representative of the whole fruit.

Keywords: phenolics; anthocyanins; sugars; organic acids; enzymes

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1. Introduction

Strawberries are appreciated by consumers for their appearance and characteristic taste, as well as for their health benefits. Strawberries contains various nutrients, including sugars, organic acids, and vitamins [1]. Apart from that, strawberries are also rich in polyphenols, such as anthocyanins, flavanols, flavonols, and others [2]. However, the distribution of these nutrients and phytochemicals can differ within the fruit.

Anthocyanins, as the main pigments, are generally more present in the outer part of the fruit—in peripheral tissues that are exposed to direct light [3]. However, the distribution of anthocyanins in strawberries can differ depending on the type of anthocyanin [4]. In addition, other phenolic compounds show different distribution patterns in strawberry fruit [5,6]. Sugars and organic acids are present in the entire strawberry fruit, but the distribution of the individual sugars can differ [4,7,8]. The distribution of organic acids and sugars can also affect taste perception, since some parts of the fruit can contain fewer sugars and be perceived as sourer [7]. The localisation of metabolites can also differ among different fruit ripening stages [8]. A difference in the distribution of metabolites was also reported for other fruits, such as apples [9,10], blueberries [11], melons [12], and mango fruit [13]. Moreover, the localisation of enzymes such as peroxidase (POD) and polyphenol oxidase (PPO) is not even in the entire fruit [14], and some parts could be more susceptible to colour degradation than others.

Most previous studies on strawberries [4–6,8,15] focused on the spatial distribution of the metabolites, but quantifications of the differences are quite limited. Some metabolites,

such as hexoses (glucose and fructose), cannot be detected separately [16]. Additionally, these studies usually focused on one cultivar, but the genotype is an important factor affecting the content of metabolites [17–21], and wider studies that include different varieties are needed.

This study aims to describe the distribution and to quantify the differences in the contents of metabolites in different parts of the fruit to determine the best way of sampling the fruit and provide a basis for understanding the differences in the biosynthesis of these metabolites. This study analysed the contents of primary metabolites (individual sugars and organic acids) and secondary metabolites (individual anthocyanins and other phenolic compounds) in the inner and outer parts of strawberry fruit of four different cultivars that were collected from a single harvest. Moreover, the difference in the activity of two enzymes responsible for the oxidation of phenolic compounds (peroxidase and polyphenol oxidase) was studied in order to understand if the activity was more notable in a certain part of the fruit and should be considered when sampling the fruit.

2. Materials and Methods

2.1. Plant Material

Strawberry samples were obtained from four economically important short day/June bearing strawberry varieties: 'Sandra', 'Clery', 'Frederica', and 'Asia'. Plants of the selected varieties were planted on 16 August 2020 by using A+ frigo plant material. The strawberries were produced in accordance with the integrated production guidelines. Each individual variety was planted in five blocks, with 10 plants in each block (altogether, 50 plants of each variety). A field trial was conducted in soil that was rich in potassium and nitrogen and low in phosphorus with a drip irrigation system. The soil texture in the orchard was silty loam with a pH value of 6.1 and a mineral composition of 9.5 mg 100 g^{-1} P_2O_5, 27 mg 100 g^{-1} K_2O, and C organic matter stock (2020 soil analysis). The plants were planted on slightly raised beds covered with black polyethylene. They were arranged in a single row, with 0.15 × 0.15 m of spacing between the plants and 1 m of spacing between the rows. The average day and night temperatures in the tunnel ranged between 10.3 and 18.6 °C during the production period. Light levels (0–573.2 W m^{-2}) and humidity (59.2–94.4%) were under ambient spring conditions. The experimental area was covered with a tall polyethylene tunnel from 14 March until the end of harvesting (end of June).

All fruit samples were collected on 21 June 2021 at the research station of the Agricultural Institute of Slovenia located at Brdo pri Lukovici (latitude, 46°10′ N; longitude, 14°41′ E). The fruits were harvested at their optimal ripening stage based on the experience of the collectors and a visual assessment, meaning that they were fully red, including the area around the calyx. The samples were immediately transported to the laboratory in the Department of Agronomy of the Biotechnical Faculty of the University of Ljubljana (Slovenia) for further analysis.

Only strawberry fruits that were free from physical and pathological defects was used for this experiment. The fruits were sorted so that from each cultivar, only fruits of the same size were sampled. The outer and inner parts of the fruit were separated by using a circular sampler with a diameter of 5 mm, as seen in Figure 1. The separated parts were blended with a blender (Multiquick MR 555 MCA, Braun, Germany). The samples were kept at −20 °C for analysis of the organic acid, sugar, and phenolic contents and at −80 °C for enzyme activity measurements.

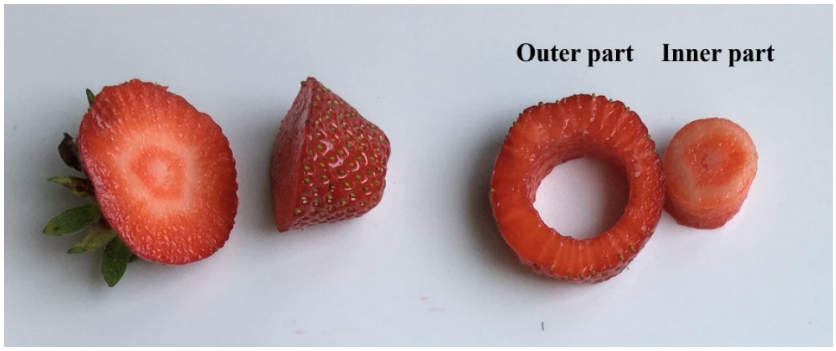

Figure 1. Separation of the inner and outer parts of the strawberry fruit.

2.2. Physical Measurements

The ripening index of the fresh fruit was measured with a DA meter (FRM01F; Sintéleia, Bologna, Italy), which provided data on the chlorophyll content in the fruit based on the absorbance values and was shown to be a good indicator of ripeness in stone fruit, such as nectarines and peaches [22,23]. The measurements were performed on 15 individual fruits for each cultivar.

The outer colour of the fruit and the colour of the blended outer and inner parts were measured with a colourimeter (CR-10 Chroma, Minolta, Osaka, Japan). A total of 15 fruits from each cultivar were measured, and for the outer and inner parts of the strawberry, the colour was measured in 5 replicates. The colour parameters were measured in the CIELAB colour space, where L* corresponds to lightness (0 is black and 100 is white), h° value corresponds to the colour expressed in degrees (0° is red, 90° is yellow, 180° is green, and 270° is blue), C* corresponds to the chroma (a higher value means a more intense colour).

Additionally, the total soluble solids (TSSs) were measured in the blended samples with a refractometer (MA885 Wine Refractometer, Milwaukee, WI, USA), and the pH of these samples was measured with a SevenEasy pH meter with an InLab sensor (Mettler Toledo, Greifensee, Switzerland). The measurements were performed in 5 replicates.

2.3. Dry Matter

For the purpose of the recalculation of the contents per unit of dry weight, the dry weights of the outer and inner parts of the strawberry were measured by placing approximately 5–10 g of the blended sample on a glass dish and then drying the sample for 3 days at 105 °C in an oven.

2.4. Ascorbic Acid Extraction and Determination

The extraction method followed the procedure described by Simkova et al. [24]. Ascorbic acid was extracted from a sample (2.5 g) with 5 mL of 3% metaphosphoric acid, and the sample was shaken for 30 min at room temperature, followed by centrifugation for 10 min at 7000× *g* at 4 °C (Eppendorf Centrifuge 5810 R, Hamburg, Germany). After that, the obtained supernatant was filtered through 0.20 µm cellulose filters (Macherey-Nagel, Düren, Germany). Five repetitions were prepared for each sample. Extracts were stored at −20 °C until an analysis with high-performance liquid chromatography (HPLC).

Ascorbic acid analysis was performed with a Vanquish HPLC (ThermoScientific, Waltham, MA, USA) by using a column Rezex ROA–organic acid H+ 8% (150 mm × 7.8 mm) (Phenomenex, Torans, CA, USA) with 4 mM H_2SO_4 in bi-distilled water as a mobile phase running at a flow rate of 0.6 mL min^{-1} (20 °C) for 15 min. The responses of the samples were measured with a UV detector at 245 nm. The identification and quantification were based on calibration with an external standard (Sigma-Aldrich, Steinheim, Germany).

2.5. Extraction and Determination of Sugars and Organic Acids

The extraction and determination of sugars and organic acids were based on the procedure described by Simkova et al. [24]. Sugars and organic acids were extracted from

the sample (1 g) with 1 mL of bi-distilled water. The samples were placed on a shaker for 30 min at room temperature, followed by centrifugation at 10,000× *g* at 4 °C for 10 min (Eppendorf Centrifuge 5810 R, Hamburg, Germany). The sample supernatant was filtered through 0.20 µm cellulose filters (Macherey-Nagel, Düren, Germany). Extracts were stored at −20 °C until analysis with HPLC.

Organic acid analysis was performed on a Vanquish HPLC (Thermo Scientific, Waltham, MA, USA) under the same conditions as those for ascorbic acid but with 65 °C as the column temperature and while detecting the response at 210 nm. External standards for citric, malic, and fumaric acid from Fluka Chemie (Buchs, Switzerland) and for shikimic acid from Sigma-Aldrich (Steinheim, Germany) were used for identification and quantification.

Individual sugars analysis was performed on Vanquish HPLC (Thermo Scientific, Waltham, MA, USA) by using a column Rezex RCM–monosaccharide Ca+ 2% (300 mm × 7.8 mm) (Phenomenex, Torans, CA, USA) with bi-distilled water as a mobile phase running at a flow rate of 0.6 mL min^{-1} (85 °C) for 15 min. A refractive index (RI) detector was used to measure the responses of the samples. External standards for fructose, glucose, and sucrose (Fluka Chemie, Buchs, Switzerland) were used for identification and quantification.

2.6. Phenolic Extraction and Determination

The phenolic extraction followed the procedure described by Simkova et al. [24]. Phenolic compounds were extracted from a sample (3 g) with 6 mL of 80% methanol acidified with formic acid (3%). The samples were placed in a cooled ultrasonic bath (0 °C) for 1 h, followed by centrifugation for 10 min at 10,000× *g* at 4 °C (Eppendorf Centrifuge 5810 R, Germany). The sample supernatant was then filtered through 0.20 µm polyamide filters (Macherey-Nagel, Düren, Germany). Extracts were stored at −20 °C until analysis with HPLC.

The analysis of the content of phenolic compounds was performed on a Dionex UltiMate 3000 HPLC (Thermo Scientific, Waltham, MA, USA) system. Spectra were measured at 280, 350, and 530 nm. The system ran at a flow rate of 0.6 mL min^{-1} with the following linear gradient: 5% solvent B from 0 to 15 min, 5–20% B from 15 to 20 min, 20–30% B from 20 to 30 min, 30–90% B from 30 to 35 min, 90–100% B from 35 to 45 min, and then 100–5% solvent B from 45 to 50 min. Solvent A was 3% acetonitrile and 0.1% formic acid in bi-distilled water (*v*/*v*/*v*), and solvent B was 3% bi-distilled water and 0.1% formic acid in acetonitrile (*v*/*v*/*v*). The total duration of the analysis was 50 min.

Identification of the phenolic compounds was performed by comparison with the retention times of standards and with an LTQ XL mass spectrometer (Thermo Scientific, Waltham, MA, USA) based on their fragmentation patterns. The sample injection was 10 µL, and the other chromatographic conditions were the same as those described for the HPLC analysis. The mass spectrometer worked in both the negative and positive ion modes with electrospray ionisation (ESI). The capillary temperature was 250 °C, the sheath gas was at 20 units, and the auxiliary gas was at 8 units. The source voltage was 4 kV, with *m/z* scanning from 115 to 1600. The quantification of the phenolic compounds was performed with a calculation according to a corresponding external standard or chemically similar compounds while assuming a response factor equal to 1. The following external standards were used for quantification: cyanidin-3-*O*-glucoside, procyanidin B1, ellagic acid, *p*-coumaric acid, ferulic acid, apigenin-7-glucoside, ellagic acid, caffeic acid, kaempferol-3-glucoside, and quercetin-3 glucoside from Fluka Chemie (Buchs, Switzerland), pelargonidin-3-*O*-glucoside from Sigma-Aldrich (Steinheim, Germany), and isorhamnetin-3-glucoside from Extrasynthese (Genay, France).

2.7. Enzyme Activity Measurements

The enzyme extraction and enzyme activity measurements were performed based on a method previously described by Simkova et al. [24] with minor modifications, as described below.

2.7.1. Extraction of Enzymes

The outer and inner parts of the fruit were ground in a basic IKA A11 grinder (IKA-Werke, Staufen, Germany) at a low temperature by using liquid nitrogen. Extraction from the sample (1.5 g) was performed with 4 mL of extraction buffer (0.01 M TRIS, 0.007 M EDTA, and 0.01 M Borax) and the addition of 0.5 g of Polyclar. The mixture was vortexed for 30 s, followed by centrifugation for 10 min at 10,000× *g* at 4 °C (Eppendorf Centrifuge 5810 R, Hamburg, Germany). After that, the 400 µL of the supernatant was washed through a Sephadex G-25 gel column before measurement.

2.7.2. POD and PPO Assays

Polyphenol oxidase (PPO) activity was assessed by mixing the sample (130 µL) with 300 µL of McIlvaine buffer (0.1 M Na_2 HPO_4 at pH 6.5) and 170 µL of 0.2 M pyrocatechol solution and measuring the absorbance for 20 min at 410 nm.

Peroxidase (POD) activity was assessed by mixing the sample (100 µL) with 1000 µL of H_2 O_2 -Kpi buffer (pH 6.5) and 10 µL of 0.04 M *o*-dianisidine solution in methanol and measuring the absorbance for 20 min at 460 nm.

The absorbance measurements were performed on a Genesys 10S UV–Vis spectrophotometer (Thermo-Scientific, Waltham, MA, USA), and the absorbance values were collected with the VISIONlite software. The enzyme activity was expressed as U (units) per mg protein, where one unit (U) was defined as the change in absorbance in one minute.

The Bradford method was used to determine the protein content, as described by Kruger [25], with minor modifications. The enzyme extract (50 µL) was mixed with Bradford reagent (2.5 mL) and measured at 595 nm after 10 min. The content was calculated as the BSA (bovine serum albumin) equivalent.

2.8. Statistical Analysis

The data were statistically analysed in R Commander x64 4.1.2. The data were expressed as means ± standard error. For significant differences in the physical parameters of the fruit among the cultivars, one-way analysis of variance (ANOVA) was used with Tukey's tests. The significant differences between the inner and outer parts of the strawberry were evaluated by using a *t*-test. Multiway ANOVA was performed to evaluate the interactions of the cultivar and layer (outer or inner part). A significant difference was considered at $p < 0.05$.

3. Results and Discussion

3.1. Physical Parameters

The physical characteristics of the fruits collected from a single harvest are presented in Table 1. The ripening index was the same for the 'Asia', 'Clery', and 'Frederica' fruits, but the ripening index of the 'Sandra' fruit was significantly lower than that in the other cultivars, indicating a lower chlorophyl content. However, the ripening index of the optimal ripening stage for harvest—with fully red fruit that were fit for consumption—could differ depending on the cultivar. Additionally, all of the values were within the same range as that previously reported for ripe strawberries [24]. Regarding colour, the fruit of the 'Sandra' cultivar was lighter but more intense in colour, whereas the 'Asia' fruit was darker but had a lower intensity of colour. In addition, the 'Frederica' cultivar had darker fruit, and the colour hue h° was lower than that for 'Asia', meaning that the colour was redder than for the other cultivars.

The parameters of the blended inner and outer parts of the fruit are presented in Table 2. In general, there was a significant variation in the pH and colour parameters depending on the cultivar and the part of the fruit. The pH was different between the inner and outer parts of the fruit in most of the cultivars, except for 'Sandra'. In the other cultivars, the pH was always higher in the inner part. However, overall, the average pH values were between 3.30 and 3.82, and the absolute differences between the inner and outer parts were relatively small (between 0.24 and 0.46). However, there were no differences in the TSS

between the inner and outer parts for most of the cultivars, except for 'Clery', where the TSS was higher in the outer part of the fruit.

Table 1. Colour parameters and ripening indexes of the fruits in the strawberry cultivars 'Clery', 'Sandra', 'Frederica', and 'Asia'.

Cultivar	Ripening Index	Colour Parameters		
		L*	C*	h°
Asia	0.25 ± 0.01 b	27.5 ± 0.3 a	31.2 ± 0.9 a	31.0 ± 0.7 ab
Clery	0.20 ± 0.02 b	30.5 ± 0.8 b	45.4 ± 0.9 c	32.5 ± 0.7 b
Frederica	0.24 ± 0.02 b	29.2 ± 0.3 ab	36.6 ± 0.9 b	29.5 ± 0.4 a
Sandra	0.12 ± 0.02 a	36.9 ± 0.5 c	48.5 ± 0.9 c	35.7 ± 0.4 c

L*, lightness; C* chroma; h° hue angle. Different lowercase letters indicate statistically significant differences among cultivars (ANOVA, $p < 0.05$).

Table 2. Physical and chemical parameters of the inner and outer parts of the fruit in the 'Clery', 'Sandra', 'Frederica', and 'Asia' strawberry cultivars and the impact of the cultivar and layer on these parameters.

Cultivar	Layer	pH		TSS [°Bx]		Colour Parameters					
						L*		C*		h°	
Asia	Inner	3.76 ± 0.02	***	7.4 ± 0.1	ns	35.4 ± 0.3	***	21.5 ± 0.3	ns	35.4 ± 0.3	*
	Outer	3.30 ± 0.01		7.4 ± 0.1		29.7 ± 0.2		22.2 ± 0.4		29.7 ± 0.1	
Clery	Inner	3.82 ± 0.00	***	9.2 ± 0.0	*	32.2 ± 0.7	**	19.1 ± 0.5	ns	31.4 ± 0.7	ns
	Outer	3.58 ± 0.02		9.7 ± 0.2		28.7 ± 0.2		19.3 ± 0.2		31.0 ± 0.4	
Frederica	Inner	3.62 ± 0.01	***	8.3 ± 0.1	ns	38.9 ± 0.4	***	20.9 ± 0.3	***	33.8 ± 0.1	**
	Outer	3.34 ± 0.01		8.4 ± 0.2		31.4 ± 0.2		25.3 ± 0.2		33.0 ± 0.2	
Sandra	Inner	3.67 ± 0.07	ns	9.6 ± 0.4	ns	35.5 ± 0.7	**	18.9 ± 0.8	***	36.1 ± 0.7	*
	Outer	3.58 ± 0.05		9.4 ± 0.3		31.9 ± 0.1		24.6 ± 0.5		33.7 ± 0.4	
Cultivar		***		***		***		***		***	
Layer		***		ns		***		***		ns	
Cultivar:Layer		***		ns		***		***		**	

ns: not significant; *, ** and ***: significant differences or impact at $p < 0.05$, at $p < 0.01$, and at $p < 0.001$, respectively.

Regarding the colour, for the 'Sandra' and 'Frederica' fruits, there were significant differences in all of the colour parameters between the inner and outer parts. These differences show that the outer part was darker and more intense in colour, and the hue h° was different. In the 'Clery' fruit, there were no significant differences in most of the colour parameters, except for lightness L*, which indicated that the inner part was lighter than the outer part. In addition, in the 'Asia' fruit, the inner part was lighter, and there was also a difference in the hue between the parts of the fruit, but otherwise, there were no significant differences observed for the other parameters.

3.2. Organic Acid Content

Organic acids also serve as flavouring agents, and their content can affect the organoleptic qualities of a fruit. In contrast to other studies [4,7], the organic acid content was higher in the outer part than in the inner part of the fruit (Table 3). The content was around 1.7 times greater in the outer part; the difference was the lowest in the 'Sandra' cultivar (1.5 higher) and highest in 'Asia' (1.8 higher). The total organic acid content was also affected by the interaction of the cultivar and location in the fruit. Similar results for citric acid in strawberries were reported by Crecelius et al. [26], where citric acid was primarily located in the outer part of the fruit in the red development stage, but it did differ among the studied genotypes. These results aligned with the pH values, as no differences were

observed in the pH values of the outer and inner parts of 'Sandra'. This showed that pH can be a good indicator of acidity, but it is not an accurate representation of the organic acid content, which was also shown in a previous study [27], where the strawberry cultivars with the highest titratable acidity and organic acid content did not show the lowest pH values.

Table 3. Total and individual organic acid contents (mg g^{-1} per unit of dry weight) in the inner and outer parts of the fruits in the 'Clery', 'Sandra', 'Frederica', and 'Asia' strawberry cultivars and the impacts of the cultivar and layer on the contents.

Cultivar	Layer	Citric [mg g^{-1}]		Malic [mg g^{-1}]		Shikimic [mg g^{-1}]		Fumaric [mg g^{-1}]		Ascorbic [mg g^{-1}]		Total Organic Acids [mg g^{-1}]	
Clery	Inner	45.30 ± 0.58	***	27.78 ± 1.27	***	0.264 ± 0.009	***	0.082 ± 0.002	***	2.68 ± 0.07	***	76.11 ± 1.72	***
	Outer	88.52 ± 1.04		39.16 ± 0.35		0.323 ± 0.007		0.109 ± 0.003		5.52 ± 0.08		133.63 ± 1.17	
Sandra	Inner	62.24 ± 1.54	***	42.71 ± 1.40	**	0.178 ± 0.009	**	0.076 ± 0.003	***	2.22 ± 0.06	***	107.43 ± 2.49	***
	Outer	104.67 ± 1.87		52.67 ± 1.61		0.231 ± 0.006		0.097 ± 0.002		4.33 ± 0.13		161.99 ± 3.48	
Frederica	Inner	65.12 ± 1.75	***	52.20 ± 2.20	**	0.283 ± 0.004	*	0.173 ± 0.007	ns	1.90 ± 0.06	***	119.67 ± 3.88	***
	Outer	137.24 ± 3.87		63.96 ± 1.83		0.338 ± 0.016		0.194 ± 0.009		3.86 ± 0.06		205.59 ± 5.71	
Asia	Inner	53.71 ± 1.10	***	41.03 ± 1.15	**	0.312 ± 0.025	ns	0.136 ± 0.011	ns	2.03 ± 0.13	***	97.22 ± 2.25	***
	Outer	119.49 ± 0.60		48.40 ± 0.50		0.366 ± 0.007		0.161 ± 0.005		5.24 ± 0.08		173.63 ± 0.93	
Cultivar		***		***		***		***		***		***	
Layer		***		***		***		***		***		***	
Cultivar:Layer		***		ns		ns		ns		***		***	

ns: not significant; *, ** and ***: significant differences or impact at $p < 0.05$, at $p < 0.01$, and at $p < 0.001$, respectively.

Significant differences were also shown in some of the individual organic acid contents that were detected (citric, malic, and ascorbic acids). Additionally, for the citric and ascorbic acid contents, the cultivar influenced the distribution in the outer and inner parts of the fruit. In all of these cases, the contents were higher in the outer part of the fruit. The highest ratio between the inner and outer content was observed for ascorbic acid. The content was up to 2.6 times higher in the outer part in the case of the 'Asia' fruit. Similar results were previously reported in strawberries [28] and mango fruit [13], where a higher vitamin C content was detected in the other part of the fruit. This could be attributed to a difference in the activity of the ascorbate oxidase enzyme, which is responsible for ascorbic acid degradation [29], as differences in ascorbate oxidase and peroxidase activity between the peel and flesh were previously in apples, and higher activity was detected in the flesh of the fruit [30].

In addition, the citric acid content was around two times higher in the outer part of most of the cultivars. The lowest ratio between the outer and inner parts (1.7) was detected in the 'Sandra' fruit. For other organic acids, the differences between the outer and inner parts were relatively small and non-significant in some cases. If a significant difference was detected, the outer/inner ratio was close to 1. The outer/inner ratio for malic acid ranged between 1.2 and 1.4, and for shikimic acid, it ranged between 1.2 and 1.3. A significant difference was detected only in the fruit of 'Clery' and 'Sandra' for fumaric acid content.

3.3. Sugar Content

The sugar content and the sugar/acid ratio are among the most important parameters for the taste perception of fruit [31]. The total sugar content significantly differed between the inner and outer parts for most cultivars, except for 'Sandra' (Table 4), which was in agreement with a previous study [7], where they detected differences in TSS and sugar content within the whole fruit; a similar tendency was also previously reported in melon [12]. The absolute difference between the inner and outer parts of the fruit ranged between 91 and 118 mg g^{-1}, and the content was consistently higher in the inner part of the fruit. However, the ratio of the inner/outer parts was close to 1. The greatest absolute and relative differences were observed in the 'Sandra' cultivar, and the smallest were observed

in 'Frederica'. Additionally, there were differences in the composition of the sugars. For glucose and fructose, the content was always higher in the inner part. In contrast, the sucrose content was higher in the outer part, except for the 'Sandra' cultivar, where no significant differences in sucrose content were detected. The outer/inner ratio for sucrose ranged between 1.3 and 1.8, and for glucose and fructose, it ranged between 0.7 and 0.8. The greatest absolute differences in the contents of all individual sugars were detected in the 'Frederica' cultivar. Differences in the distribution of hexoses (fructose and glucose) and sucrose were previously reported [4], and it was suggested that this trend could be caused by a difference in the activity of the enzymes responsible for sucrose/hexose interchange [32] or by an increase in sucrose imported from photosynthetic tissue. Out of the studied cultivars, the 'Sandra' cultivar showed the most homogenous sugar content and composition in the whole fruit, which consumers could prefer, as homogeneous sugar content is perceived as sweeter [7].

Table 4. Total and individual sugar contents (mg g^{-1} per dry weight) in the inner and outer parts of the fruits in the 'Clery', 'Sandra', 'Frederica', and 'Asia' strawberry cultivars and the impacts of the cultivar and layer on the contents.

Cultivar	Layer	Content [mg g^{-1} of Dry Weight]							
		Sucrose		Glucose		Fructose		Total Sugars	
Clery	Inner	85 ± 2	**	333 ± 7	***	366 ± 7	***	785 ± 16	**
	Outer	111 ± 4		267 ± 2		304 ± 3		681 ± 5	
Sandra	Inner	251 ± 15	ns	283 ± 9	**	292 ± 8	**	827 ± 32	ns
	Outer	221 ± 8		226 ± 10		238 ± 10		686 ± 28	
Frederica	Inner	123 ± 2	***	315 ± 10	***	333 ± 10	***	772 ± 21	*
	Outer	218 ± 6		221 ± 6		242 ± 7		680 ± 19	
Asia	Inner	85 ± 2	***	319 ± 8	***	344 ± 8	***	748 ± 17	**
	Outer	143 ± 4		232 ± 6		256 ± 7		630 ± 16	
Cultivar		***		***		***		***	
Layer		***		***		***		***	
Cultivar:Layer		***		***		***		***	

ns: not significant; *, ** and ***: significant differences or impact at $p < 0.05$, at $p < 0.01$, and at $p < 0.001$, respectively.

3.4. Phenolic Content

Strawberries contain a range of phenolic compounds, such anthocyanins, flavonols, flavanols, and phenolic acids. All of the identified phenolic compounds are listed in Supplementary Table S1, and the results of the phenolic content are presented in Table 5. The total phenolic content was significantly different between the inner and outer parts in all the cultivars that were harvested at the same harvest point. The highest outer/inner ratio was observed in the 'Frederica' cultivar, where the outer part contained 3.4 times more phenolic compounds than the inner part. For the other cultivars, the content of phenolic compounds was more than 1.5 times higher than in the inner part. Additionally, most phenolic compound groups' content showed significant differences between the inner and outer parts in all of the cultivars, and the content was always higher in the outer part. In agreement with a previous study [5,28], flavonols and flavanols were shown to have a higher content in the outer part of the fruit in all cultivars. Flavonols and flavanols follow the same biosynthetic pathway as anthocyanins [33]; in our results, a higher content was detected in the parts with higher anthocyanin content. Additionally, flavonoid accumulation is affected by sunlight [34], which can explain why these metabolites were present in the outer part, which has more exposure to light. In addition, ellagic acid derivatives and other hydroxybenzoic acid derivatives were detected in greater amounts in the outer part of the fruit. This agreed with a previous study [28], where the glycosides of ellagic acid were mainly detected in the outer part of the fruit. The exception among

the phenolic compound groups was the content of hydroxycinnamic acid derivatives and flavanols in the 'Sandra' cultivar, for which no significant differences were detected. In general, the content of hydroxycinnamic acid derivatives did not show a uniform trend among the cultivars. This suggests that the synthesis of these compounds is not related to the part of the fruit but rather depends on the cultivar.

Table 5. Total phenolic content and phenolic content per group (mg kg^{-1} dry weight) in the inner and outer part of the fruit in the 'Clery', 'Sandra', 'Frederica', and 'Asia' strawberry cultivars and the impacts of the cultivar and layer on the contents.

Cultivar	Layer	Content [mg kg^{-1} Dry Weight]									
		Hydroxycinnamic Acid Der.		Flavanols		Hydroxybenzoic Acid Der.		Flavonols		Total Phenolics [1]	
Clery	Inner	1761 ± 81	*	566 ± 53	**	549 ± 7	***	120 ± 12	***	6244 ± 293	***
	Outer	1526 ± 27		885 ± 39		933 ± 29		469 ± 21		10,273 ± 126	
Sandra	Inner	940 ± 53	ns	420 ± 53	ns	518 ± 27	***	74 ± 5	***	3285 ± 27	***
	Outer	769 ± 64		632 ± 82		1064 ± 59		209 ± 7		7008 ± 89	
Frederica	Inner	557 ± 35	***	72 ± 7	**	314 ± 13	***	77 ± 11	***	2597 ± 130	***
	Outer	823 ± 38		227 ± 27		1325 ± 48		347 ± 24		8736 ± 257	
Asia	Inner	1670 ± 56	**	151 ± 13	***	448 ± 17	***	292 ± 28	***	5707 ± 181	***
	Outer	1281 ± 61		409 ± 11		837 ± 40		692 ± 11		11,861 ± 86	
Cultivar		***		***		***		***		***	
Layer		**		***		***		***		***	
Cultivar:Layer		***		ns		***		***		***	

[1] including anthocyanin content; der., derivative; ns, not significant; *, ** and ***: significant differences or impact at $p < 0.05$, at $p < 0.01$, and at $p < 0.001$, respectively.

Anthocyanin Content

Anthocyanins, as the main pigments, can affect the colour of the fruit, which is an important factor that influences consumer preference. There were six different anthocyanins identified in the four studied cultivars (Supplementary Table S1). The total anthocyanin content was significantly higher in the outer part of the fruit than in the inner part in all of the cultivars that were collected at the same harvest point (Table 6). This can be attributed to the fact that anthocyanin synthesis is regulated by light, and they generally accumulate in tissues that are exposed to direct light [3]. However, based on our results, the content of anthocyanins and the differences in the fruits' outer and inner parts also depended on the cultivar. The outer/inner ratio of the total anthocyanin content ranged from 2.1 to 4.8. The highest ratio was detected in the 'Frederica' cultivar, and the lowest was detected in 'Clery'. However, the greatest absolute difference was in the 'Asia' cultivar (5496 mg kg^{-1}), and the smallest was in the 'Sandra' cultivar (3001 mg kg^{-1}). The colour parameters also showed that the cultivar with the lowest average content of anthocyanins in the outer part of the fruit ('Sandra') also had the highest average value of lightness, but the fruit showed the highest value of chroma (C*) compared to the other cultivars, with a higher average content of anthocyanins in the other part. The colour parameters were a good indication of the difference in the anthocyanin contents in the different parts of the fruit, as there were differences detected in all of the colour parameters for the 'Frederica' cultivar, which showed the highest differences.

The colours of different anthocyanin types can be different; therefore, the composition of anthocyanins can also impact the overall colour of the fruit [35]. However, the distribution of the anthocyanins in strawberries differs mainly based on the anthocyanin aglycone, rather than the sugar moiety [4]. Our results showed that the detected cyanidin derivatives, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-(6" malonyl) glucoside, were mainly located in the outer part of the fruit, and the contents detected in the inner part were very low. Especially in the case of cyanidin-3-(6" malonyl) glucoside, the ratio between the inner and outer parts of the fruit was highest out of all the anthocyanins. However, pelargonidin derivatives were also shown to have a higher content in the outer part, the outer/inner

part ratio was not as high as for cyanidin derivatives, and, in the case of pelargonidin-3-(6″ malonyl) glucoside, there was not a significant difference observed between the inner and outer parts in the 'Clery' cultivar. In the 'Asia' fruit, all of the individual anthocyanins had significantly higher contents in the outer part of the fruit. The outer/inner ratio of the individual anthocyanins ranged from 2.2 to 2.8. In the 'Frederica' fruit, the outer/inner ratio for the detected anthocyanins was the highest out of all of the cultivars, which was in line with the colour parameters that were measured as there were significant differences in all of the parameters. Moreover, the interaction results showed that the cultivar affected the differences in contents in the inner and outer parts. These results confirm that pelargonidin species can be found in all parts of the fruit, and cyanidin species are mainly located in the outer part of the fruit, as was previously reported in strawberries [4] and blueberries [11]. These differences can be attributed to differences in the biosynthesis of anthocyanins, since this is a cell-autonomous response controlled at the level of individual cells [3].

Table 6. Total and individual anthocyanin contents (mg kg^{-1} dry weight) in the inner and outer parts of the fruit in the 'Clery', 'Sandra', 'Frederica', and 'Asia' strawberry cultivars and the impacts of the cultivar and layer on the contents.

Cultivar	Layer	Content [mg kg^{-1} Dry Weight]																	
		Cy-3-*O*-glc		Plg-3-*O*-glc		Plg-3-*O*-rut		Cy-3-(6″malonyl) glc		Plg-3-(6″malonyl) glc		Plg-3-*O*-acetylglc		Total Phenolics	
Clery	Inner	18 ± 1	***	2372 ± 22	***	146 ± 7	***	2 ± 1	**	7 ± 2	ns	548 ± 4	***	3093 ± 31	***
	Outer	89 ± 3		4943 ± 45		270 ± 15		18 ± 3		18 ± 5		1140 ± 18		6478 ± 81	
Sandra	Inner	7 ± 0	***	945 ± 54	***	71 ± 1	***	nd		2 ± 0	**	307 ± 16	***	1333 ± 69	***
	Outer	37 ± 1		3100 ± 113		192 ± 3		5 ± 0		6 ± 1		993 ± 55		4334 ± 163	
Frederica	Inner	25 ± 5	***	1502 ± 82	***	nd		nd		15 ± 0	***	35 ± 3	***	1578 ± 86	***
	Outer	173 ± 8		5648 ± 163		nd		nd		49 ± 2		144 ± 6		6013 ± 171	
Asia	Inner	28 ± 2	***	2840 ± 108	***	183 ± 6	***	nd		12 ± 1	***	85 ± 3	***	3147 ± 118	***
	Outer	84 ± 1		7924 ± 45		424 ± 5		nd		26 ± 1		185 ± 8		8642 ± 54	
Cultivar		***		***		***		na		***		***		***	
Layer		***		***		***		na		***		***		***	
Cultivar:Layer		***		***		***		na		***		***		***	

cy: cyanidin; plg: pelargonidin; glc: glucoside; rut: rutinoside; nd: not detected; na: not applicable; ns: not significant; ** and ***: significant differences or impact at $p < 0.01$, and at $p < 0.001$, respectively.

3.5. Enzyme Activity

Enzymatic activity—namely, polyphenol oxidase (PPO) and peroxidase (POD) activity—can cause the oxidation of phenolic compounds and, consequently, colour deterioration [36]. In all studied cultivars, the enzyme activity of peroxidase (POD) and polyphenol oxidase (PPO) was higher in the inner part compared to the outer part of the fruit (Table 7). While the enzyme activity of peroxidase was between 0.54 and 1.05 U mg^{-1} of protein in the outer part, the enzyme activity was between 1.09 and 1.41 U mg^{-1} of protein in the inner part of the fruit. For polyphenol oxidase, the outer part had between 0.33 and 0.98 U mg^{-1} of protein, and there was between 1.17 and 1.37 U mg^{-1} of protein in the inner part. The most significant difference was in the 'Frederica' cultivar, where the inner part had more than two times higher enzyme activity for peroxidase and almost four times higher enzyme activity for polyphenol oxidase. A similar trend was also shown in the protein content obtained during the extraction, where a higher content was obtained in the inner part, and this showed that the differences in absolute activity would be even higher if they were expressed by dry weight. The interaction of the cultivar and inner/outer layer showed that the activity and protein content also depended on the cultivar. López-Serrano and Ros Barceló [14] reported that the PPO was mainly localised in the cortex tissues and that the POD was mainly localised in the vascular bundles in strawberries, which could explain the higher POD activity in the inner part of the fruit. However, in contrast to this study, our results showed a higher PPO activity in the inner part as well, which suggested

that there are other factors that may influence the PPO activity; it has been previously reported that pH and sugar content can affect the PPO activity [36]. Differences in the POD and PPO activity between the inner and outer parts of a fruit can also affect the content of phenolic compounds when the fruit is being cut or mashed and prepared for sampling, as the enzymes from the inner part of the fruit can access the phenolic compounds that are concentrated in the outer part of the fruit and cause their degradation.

Table 7. Peroxidase and polyphenol oxidase activity (U mg^{-1} protein) and protein content (mg BSA per g of dry weight) in the inner and outer parts of the fruit in the 'Clery', 'Sandra', 'Frederica', and 'Asia' strawberry cultivars, as well as the impacts of the cultivar and layer on the activity.

Cultivar	Layer	Activity [U mg^{-1} Protein] POX		PPO		Protein Content [mg g^{-1} Dry Weight]	
Clery	Inner	1.22 ± 0.04	**	1.37 ± 0.09	**	6.28 ± 0.25	***
	Outer	0.95 ± 0.06		0.98 ± 0.06		4.40 ± 0.23	
Sandra	Inner	1.09 ± 0.08	**	1.23 ± 0.06	***	4.59 ± 0.11	***
	Outer	0.60 ± 0.04		0.39 ± 0.03		2.52 ± 0.07	
Frederica	Inner	1.25 ± 0.05	***	1.24 ± 0.02	***	4.84 ± 0.12	***
	Outer	0.54 ± 0.02		0.33 ± 0.04		2.86 ± 0.32	
Asia	Inner	1.41 ± 0.07	**	1.17 ± 0.04	**	10.27 ± 0.14	***
	Outer	1.05 ± 0.05		0.94 ± 0.03		5.08 ± 0.14	
Cultivar		***		***		***	
Layer		***		***		***	
Cultivar:Layer		***		***		***	

, and *: significant differences or impact at $p < 0.01$, and at $p < 0.001$, respectively.

4. Conclusions

Our results showed significant differences in the organic acid and sugar contents, showing that the organic acids accumulated more in the outer part, whereas sugars accumulated more in the inner part of the fruit. All studied cultivars showed higher contents of anthocyanins, flavonols, and hydroxybenzoic acid derivatives in the outer part of the fruit, which showed that the biosynthesis of these compounds was greater in the outer parts of the fruit with exposure to light. Additionally, the activity of peroxidase and polyphenol oxidase showed an uneven distribution. All of these differences should be considered when handling fruit and accounted for when preparing fruit samples for analysis, as they could significantly impact the final results. It is recommended to sample from all parts of a fruit to obtain representative results and to work fast or at lower temperatures to prevent the enzymes from degrading the phenolic compounds once the sample is mixed.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9050605/s1, Table S1: Identification of phenolic compounds and standards with which they are expressed.

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