Southern Region Small Fruit Consortium Grant Proposal

Progress Report Research
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Public Abstract:
Strawberry fruit were used to follow the relationship of visual color to anthocyanin type and content. Reflected color was measured on fresh strawberries with a colorimeter and compared to the anthocyanin content of the juice. Additionally, the anthocyanin content of strawberry cultivars from Florida, Alabama, and North Carolina were compared.

Introduction:
Strawberries provided $2.2 billion in the U.S. in 2020 (https://www.agmrc.org/commodities-products/fruits/strawberries) and lead the berry market. Strawberries offer a rich source of vitamin C and are high in phenolic compounds and in the anthocyanin pelargonidin 3-O-glucoside, which has anti-inflammatory properties (Amini et al. 2017; Khoo et al. 2017). The consumer interest for diets high in antioxidant-rich foods has incentivized breeding programs to consider cultivars with higher bioactive compounds (Dzhanfezova et al. 2020). Additionally, consumers vary in their color preference for strawberries. Some equate fully red to dark red fruit with increased ripeness and antioxidant content while others view lighter red or scarlet strawberries as fresher (Dzhanfezova et al. 2020). As the tests used to analyze these compounds can be time-consuming, labor intensive, and expensive, plant breeders desire a rapid yet accurate screening method.
Strawberry fruit can range in color from a white blush to light orange, and from scarlet to nearly black (Figure 1). Most of this visual color is from water-soluble anthocyanins. Wild strawberries were found to have equal amounts of pelargonidin 3-glucoside and cyanidin 3-glucoside, while cultivated octoploid strawberries are dominant in pelargonidin 3-gluocside (Dzhanfezova et al. 2020). Other anthocyanins present in strawberry include pelargonidin 3-rutinoside and pelargonidin 3-malonylglucoside (Voca et al. 2014). A collection of germplasm in Eastern Europe

was screened for anthocyanin pigments by Dzhanfezova et al. (2020), where the darker red cultivars were higher in total anthocyanin and pelargonidin 3-glucoside was 10x higher in concentration than other anthocyanins. The anthocyanin profile of NC germplasm is not known. However, anthocyanin profiles of the strawberry cultivars Camarosa and Chandler compared to those of the NC-developed cultivars Liz, and Rocco showed that the relative percentages of cyanidin 3-glucoside and pelargonidin 3-glucoside differed and that color may be dependent both on total amount of anthocyanin and relative amounts of specific anthocyanins (Perkins-Veazie et al. 2016).

Figure 1. Strawberries from 2022 harvest (Piedmont Research Station, Salisbury NC) showing scarlet, red, and red-black color phenotypes (left to right).

A simple way to quantify strawberry total monomeric anthocyanin content (TMAC) is to extract pigments from an aliquot of puree or juice using the pH differential method and determine spectrophotometric absorbance at 500 to 515 nm (Lee et al. 2002). Despite this, strawberry fruit must be stored or frozen until extracted, and various instruments such as centrifuge, vortexor, microplate reader or spectrophotometer need to be available. The anthocyanin profile can be identified and quantified using chromatography, usually with high performance liquid chromatography (HPLC) or liquid chromatography/mass spectroscopy (LC-MS). Using these machines requires extra preparative steps, trained personnel, and access to the expensive equipment (Heredia et al. 2006).

As fresh market strawberries can lack internal color, using puree or juice may under estimate the external fruit color seen by consumers. Additionally, phenotyping large sets of fresh fruits saves labor in storing or freezing fruit as well as space and the need for additional assays. A relative estimation of total anthocyanin content based on external reflective fruit color (L*a*b*) may provide additional information without the need of additional inputs. Voca et al. (2014) was unable to show consistent correlations of color variables to specific anthocyanin pigments, but authors did not correlate reflective color variables to total anthocyanin content.

We expect to provide information on the variation of anthocyanin type and content in NC germplasm, possible variation in anthocyanin content in fruit of similar cultivars grown in NC and AL, and the feasibility of using a colorimeter test on fresh fruit to predict total anthocyanin content.

This study directly impacts the plant breeding community by providing a means to add a quantitative color value for external strawberry color and as a simplified method for estimating fruit anthocyanin composition in their screening selections and releases.

Objectives:

Our objectives are to 1) determine the anthocyanin profile associated with low and high content of total anthocyanins (TMAC) in strawberries from the North Carolina breeding program; 2) explore the usefulness of using a reflective colorimeter method to correlate color parameters with quantitative values of total anthocyanin content. and 3) determine if that correlation would be a useful parameter in identifying desirable fruit color in the breeding process.

Materials and Methods:

1. Anthocyanin profiles

In 2022, marketable fruit were collected from germplasm trials between April and June at three NCDA&CS research stations in North Carolina. These included the Piedmont Research Station in Salisbury (south central), Central Crops Research Station in Clayton (east), and the Horticultural Crops Research Station in Castle Hayne (southeast). At least three harvests of five fruit per harvest were made per genotype. All fruit were fully red on interior and exterior. Strawberries were weighed and frozen at -20 °C. Frozen fruit were thawed between 1 and 3 months after harvest, juice collected, and a 0.1mL aliquot of juice was mixed with 1.5mL MeOH:water:formic acid solvent and extracted following the method of Perkins-Veazie et al. (2016). The pH differential method (Lee et al., 2002) using sample absorbance at 500 and 700 nm was used to determine total monomeric anthocyanin (TMAC) using a microplate reader Heredia et al. (2006). Absorbance values and dilutions were used to calculate the total monomeric anthocyanin content as follows:

TMAC (mg cyanidin 3-glucoside equivalents/kg) = [($Abs_{510\ at\ pH1}$-$Abs_{700\ at\ pH1}$)-($Abs_{510\ at\ pH4.5}$-$Abs_{700\ at\ pH4.5}$)*dilution factor*26,900(extinction coefficient of C3G)*1000]/449.2(mol wt of C3G).

In 2023, we used these juice extracts to determine the anthocyanin profiles on a subset of 24 genotypes/cultivars (Table 1), separated into 4 groups (6 genotypes per group, 3 samples per genotype) with known TAC values of 100-150 (low), 200-300 (med), 300-400 (high), >400 (very high). An additional sample of a white/blush type such as 'Florida Pearl' was added (expected TAC of <100).

One ml of extract was filtered through PTFE (0.20 uM), and run on a Hitachi LaChrom HPLC using the method of Perkins-Veazie et al. (2016). Anthocyanin concentration was determined using external standards where available (cyanidin 3-glucoside, pelargonidin 3-glucoside, pelargonidin 3-rutinoside, cyanidin 3-rutinoside).

Table 1. Potential cultivars and genotypes for HPLC based on TMAC value.

Classification of total anthocyanin content/concentration			
Low	Medium	High	Very high
10-20	20-30	30-40	>40
	mg cyanidin 3-glucoside equivalents/100 g		
Florida Elyana	Chandler	Camarosa	NC 19-016
NC 19-003	Liz	NC 19-020	NC 19-018
NC 20-047	Albion	NC 20-038	NC 19-020
NC 20-053	NC 20-002	NC 20-085	NC 20-055
NC 20-101	NC 20-008	NC 20-087	NC 21-014
NCK 12-194	Sweet Charlie	NCL 08-067	NCL 05-086

2. Comparison of colorimeter values of strawberries to TAC of fruit grown in NC and AL.

In 2023, 10 genotypes (NC germplasm and other cultivars such as the white/blush 'Florida Pearl') were harvested fully ripe at three harvest dates from PRS. Five fruit per genotype were rated subjectively following the standardized RosBREED 1 to 9 color phenotyping scale (Mathey at al. 2013) Color references were photos showing white/light blush (1), light red/orange (3), scarlet (5), red/red blue (7) and red/red black (9). Colorimeter readings were taken on the darker shoulder of each fruit using a Konica-Minolta CR400 (Figure 1), calibrated with a white standard tile, set at color space D65 and L*a*b* coordinates, and using 8 mm aperture. Lightness, darkness, color intensity and color (hue) were calculated using the methods reported in Ganhao et al. (2019). Bright Red (BR) values were calculated from colorimeter values L* (lightness) and a* (red-green) as sqrt $[(L)^2+(a*)^2]$. Selections and cultivars from the UF breeding program were shipped to NC and used for colorimeter values and UPLC analysis (Table 3).

Figure 1. Minolta-Konica 400 colorimeter showing strawberry shoulder placement for color values.

Each fruit was frozen and thawed and juice used to determine TMAC as described above, and HPLC analysis done on juice from 3 of these fruit per genotype/harvest when possible.
 A comparison of color values, TMAC and specific anthocyanins was done for a select group. Selected cultivars grown in both NC and AL trials (Albion, Camarosa, Victor, Radiance, and Fronteras, 5 fruit per harvest) were collected over three harvest dates to compare location effects on TMAC; due to difficulty in obtaining plant material in AL and stand losses in NC, only 'Camarosa' was in common in both cultivar collections.
Statistical analysis was done using SAS, v. 9.4 and mean separation done where appropriate using Tukey's Honest Significant Difference *t*-Test.

Results
Objective 1. Anthocyanin profiles associated with low and high content of total anthocyanins

Anthocyanin profiles were independent of low or high amounts of total anthocyanins. Profiles appear to be primarily dependent on germplasm (Table 2). The pelargonidin pigments pelargonidin-3-glucoside (P3G) (10-28 mg/100 g juice) and pelargonidin-3-rutinoside dominated in all germplasm (70-90% of total anthocyanin) except 'Florida Pearl', where cyanidin-3-glucoside was in a higher proportion. As juice from 'Florida Pearl' was essentially colorless, peel was scraped from the pinker areas of the fruit to use for anthocyanin extraction to compare pigment profiles, and this may have affected the relative amounts of cyanidin-3-glucoside. The relative amounts of P3G (as percent of total anthocyanin) varied between 70 and 90%, and P3R as 20-30%. Pelargonidin-3-malonylglucoside was relatively high (10%+) for 'Liz', 'Ruby June', NC19-022, NC20-055, NC20-099, Rocco in 2022 NC material, and in 2023 FL 20.27.633 (Table 2).

Objective 2. Comparison of anthocyanin content of cultivars from multiple locations, subjective ratings and colorimeter values of strawberries compared to TMAC of fruit

Colorimeter values could only be obtained for Florida and NC material. Florida results are presented in Figure 3, where L* was highly correlated with TMAC, r=0.93. This high correlation was favored by the presence of two low anthocyanin genotypes. Analysis of data for NC colorimeter and anthocyanin content is ongoing. No obvious relationship with color was found among the types of anthocyanins and reflected color values in this small study. The high correlation found for TMAC and L* indicate the possibility that a colorimeter may be useful in estimation of total anthocyanin. Relative visual color of cultivars and germplasm are shown in Figure 2.

Figure 2. From left to right, 'Florida Pearl', 'Sweet Charlie', 'Felicity', 'NC21-031', 'NC19-020'

Total anthocyanin content and composition of juice obtained from fruit samples from AL, FL, and NC are presented in Table 4. 'Florida Pearl', 'Camarosa', 'Medallion', and 'Felicity' were present in more than one location. Values for TMAC were similar between NC and FL for 'Medallion' and 'Florida Pearl', while 'Felicity' from FL and 'Camarosa' from AL were higher in TMAC than the NC-grown cultivars.

Table 2. Comparison of total anthocyanin and anthocyanin pigments in juice from strawberries harvested in 2022 (NC) and 2023 (FL).

Classification (low to very high anthocyanin)	Cultivar	Total anthocyanin	C3G	PG3G	PG3R	PG3MG	C3G	PG3G	PG3R	PG3MG
			(mg/100g.fw)				(% of total anthocyanin)			
	2022 (NC)									
M	LC146T54	23.22	0.45	18.54	3.91	0.28	1.98	79.79	16.97	1.05
	Stdev	5.09	0.20	4.16	0.74	0.24	0.91	1.46	1.38	0.94
H	Camarosa	33.96	2.66	26.11	5.00	0.18	8.99	75.93	14.59	0.49
	Stdev	10.19	0.89	8.76	1.87	0.13	6.32	6.41	1.55	0.37
H	Chandler	35.18	1.53	30.00	3.52	0.14	4.05	85.41	10.13	0.41
	Stdev	14.46	1.11	12.12	1.34	0.17	1.64	0.53	1.29	0.56
H	Liz	34.42	3.75	22.26	3.47	4.20	11.14	64.64	9.88	12.15
	Stdev	7.47	0.84	4.88	1.17	1.20	2.57	2.79	1.60	1.86
VH	NC19-016	46.43	0.68	38.22	7.39	0.14	1.54	82.41	15.75	0.30
	Stdev	4.93	0.37	3.60	1.62	0.09	1.06	1.33	2.04	0.18
H	NC19-018	37.71	1.54	33.25	2.73	0.19	4.15	88.14	7.21	0.50
	Stdev	4.30	0.32	3.98	0.63	0.17	1.13	1.39	1.35	0.47
VH	NC19-020	41.66	1.95	35.56	4.05	0.11	4.59	85.14	10.06	0.21
	Stdev	13.81	0.90	12.15	1.65	0.13	1.17	2.77	3.88	0.26
M	NC19-022	22.83	0.30	17.17	2.81	2.55	1.21	76.14	12.00	10.64
	Stdev	5.85	0.30	3.86	1.53	1.71	1.01	7.27	4.06	6.92
L	NC20-002	19.91	1.76	17.39	0.77	0.00	8.82	87.33	3.85	0.00
	Stdev	3.63	0.46	3.15	0.18	0.00	1.52	1.41	0.49	0.00
H	NC20-008	32.14	1.59	26.46	3.79	0.30	4.99	82.19	11.84	0.98
	Stdev	8.00	0.48	6.86	0.95	0.15	1.02	1.90	1.50	0.61
M	NC20-018	24.03	1.25	20.87	1.65	0.25	5.27	86.72	6.95	1.06
	Stdev	3.51	0.30	3.38	0.31	0.16	1.19	2.27	1.36	0.69
	NC20-054	30.03	1.21	25.82	2.98	0.02	4.92	85.99	9.09	0.00
	Stdev	7.33	0.60	6.53	1.06	0.00	2.79	1.07	2.69	0.00
H	NC20-055	35.47	0.79	26.55	3.69	4.44	2.26	74.86	10.38	12.50
	Stdev	4.65	0.26	3.50	0.70	0.86	0.76	1.27	1.49	1.49
M	NC20-058	22.46	1.09	19.10	2.09	0.18	4.79	85.07	9.35	0.80
	Stdev	2.58	0.36	2.20	0.23	0.10	1.38	1.21	1.05	0.41
H	NC20-099	32.54	0.73	22.62	3.14	6.05	2.25	69.51	9.64	16.17
	Stdev	12.80	0.49	7.26	1.18	4.24	1.07	9.68	1.06	10.45
H	Rocco	33.92	0.86	24.83	3.48	4.75	2.42	73.13	10.44	14.01
	Stdev	5.27	0.77	4.13	0.45	0.85	1.95	1.98	1.81	1.46
M	Ruby June	21.88	2.28	13.40	1.92	3.80	10.43	61.33	8.73	17.40
	Stdev	2.76	0.38	1.52	0.36	0.43	1.27	2.26	1.02	0.78
	2023 (FL)									
L	Florida Pearl	0.22	0.06	0.13	0.02	0.00	24.18	46.05	7.26	0.00
L	20.12-99	0.89	0.09	0.75	0.05	0.01	11.92	77.39	5.44	0.52
H	20.27-633	35.55	1.19	26.69	3.12	4.54	3.33	75.04	8.70	12.76
M	Medallion	26.77	0.39	21.56	2.96	1.86	1.48	79.99	11.14	7.39
VH	Felicity	40.19	1.21	34.49	4.32	0.17	3.01	85.45	11.00	0.40
VH	Festival	44.22	1.44	37.97	4.68	0.13	3.15	85.92	10.57	0.26

C3G=cyanidin-3-glucoside; PG3G=pelargonidin-3-glucoside; PG3R=pelargonidin-3-rutinoside; PG3MG=pelargonidin-3-malonylglucoside.

Table 3. Comparison of colorimeter values (L*,a*, b*, hue, chroma, BR) to composition and total anthocyanin (TMAC) in fresh strawberries from FL.

	Fruit color	L*	A*	B*	Hue	Chroma	BR (bright redness)	ssc	ph	tacid	TMAC
FL Pearl	white	65.33	11.75	19.88	59.88	23.73	66.60	7.8	3.69	0.55	0.22
20.12-99	light pink	55.56	16.81	18.99	48.75	25.52	58.17	7.7	3.73	0.55	0.89
20.27-633	red	32.03	33.46	16.47	26.08	37.31	46.34	7.2	3.76	0.51	35.55
Medallion	red	34.57	35.31	31.11	23.53	34.09	49.46	8.1	3.57	0.61	26.77
Felicity	red	32.56	37.85	20.52	28.26	43.08	49.95	7.2	3.41	0.79	40.19
FL Festival	red	30.99	32.07	15.95	26.35	35.84	44.60	7.3	3.65	0.56	44.22

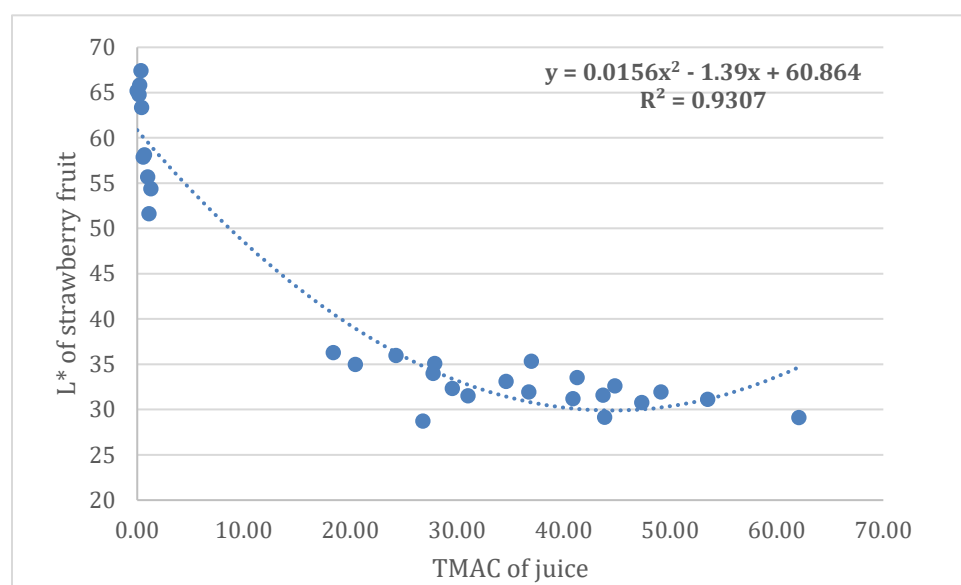$$y = 0.0156x^2 - 1.39x + 60.864$$
$$R^2 = 0.9307$$

Figure 3. Comparison of colorimeter values of whole fruit (peel) to total anthocyanin of juice for FL material.

Table 4. Comparison of fruit composition and total anthocyanin (TMAC) of strawberries obtained from plantings in Wimauma, Florida, Auburn, AL, and Salisbury, NC. Bolded cultivars are those that appear in more than one fruit location. Sorted in order of TMAC values.

Cultivar/ selection	% Soluble solids content	pH	% Titr. acidity (citric acid equiv)	TMAC (mg P3G equiv/100 g juice)
Fruit from Alabama				
Albion	10.2	3.55	0.67	51.68
Camarosa	**9.7**	**3.75**	**0.60**	**59.77**
Fronteras	9.1	3.57	0.52	47.82
Radiance	8.4	3.72	0.50	63.62
RoyalRoyce	9.0	3.60	0.57	37.32
Victor	9.2	3.60	0.55	38.03
Fruit from Florida				
Florida Pearl	**7.8**	**3.69**	**0.55**	**0.22**
20.12-99	7.7	3.73	0.55	0.89
20.27-633	7.2	3.76	0.51	35.55
Medallion	**8.1**	**3.57**	**0.61**	**26.77**
Felicity	**7.2**	**3.41**	**0.79**	**40.19**
Festival	7.3	3.65	0.56	44.22
Fruit from North Carolina (PRS)				
Florida Pearl	**9.1**	**3.89**	**0.62**	**0.81**
Ruby June	9.5	3.66	0.72	17.74
NC21-020	9.3	3.59	0.70	20.14
Sweet Charlie	9.2	3.82	0.63	20.25
NC 19-016	6.3	3.54	0.76	25.15
Medallion	**10.6**	**3.63**	**0.73**	**28.08**
NC20-058	8.0	3.68	0.78	28.90
NC20-055	10.7	3.71	0.76	31.56
Liz	8.5	3.60	0.72	31.88
Felicity	7.7	3.60	0.74	32.29
Rocco	7.4	3.59	0.76	32.43
NC20-008	8.3	3.78	0.60	33.47
Camarosa	**8.9**	**3.65**	**0.75**	**34.45**
NC21-035	9.7	3.71	0.73	37.38
NC21-031	8.6	3.37	0.86	38.90
NC20-012	8.4	3.64	0.70	39.68
Chandler	8.8	3.53	0.79	39.97
NC21-033	9.8	3.60	0.90	40.86
NC 19-020	6.7	3.65	0.75	45.07
HSD (NC fruit)	**3.5**	**0.26**	**0.31**	**17.90**

Impact

Assays of 2023 NC material are not yet finished, due to issues with UPLC and HPLC machines. A wide variation of total anthocyanin values was found in NC germplasm and several of these selections have a pigment profile that is relatively high in pelargonidin-3-malonylglucoside. Colorimeter data from the small set of FL material indicates that color values obtained from fresh strawberry fruit may be useful in estimation of total anthocyanin. Total anthocyanin values can range quite widely within germplasm and with location and indicates a need to adequately sample selections across possible sources that influence values, such as harvest date, location, and year.

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