Southern Region Small Fruit Consortium Grant Progress Report 2025 R-26

Title: Use of Near Infrared Spectroscopy to Rapidly Quantify Anthocyanin of *Vaccinium virgatum* (Rabbiteye Blueberry) Selections

Name, Mailing, and Email Address of Principal Investigator(s):

Principal Investigator	Co-Investigator #1	Co-Investigator #2	Co-Investigator #3
Penelope Perkins-Veazie N.C. State University 600 Laureate Way Kannapolis, NC 28081 penelope_perkins@ncsu.edu	Sushan Ru Auburn Univ. 120 Funchess Hall Auburn, AL 36849 sushan.ru@auburn.edu	Marlee Trandel-Hayse Auburn Univ. 121 Funchess Hall Auburn, AL 36849 mat0141@auburn.edu	Massimo Iorizzo N.C. State University 600 Laureate Way Kannapolis, NC 28081 miorizz@ncsu.edu

Public Abstract:

Rabbiteye blueberries of 15 cultivars from North Carolina and Alabama were used to explore fruit anthocyanin profiles and the feasibility of using Near Infrared Spectroscopy (NIR) to screen germplasm for total anthocyanin content. Mixed results were obtained with the samples. Total anthocyanin was 900-2300 mg/100 g dry weight among blue fruited cultivars. 'Pink Lemonade', a pink-red fruited type, had about 100 mg/100 g dry weight. Anthocyanin content for cultivars grown in both Alabama and North Carolina ('Alapaha', 'Premier', 'Titan', 'Vernon') varied with location. NIR was effective at predicting total anthocyanin content in NC blueberries (R2=90) but was not effective for Alabama blueberries (R2=50). Types of anthocyanins could not be successfully predicted with NIR for either location. Anthocyanin types were primarily malvidin and delphinidin, and were dominated by malvidin-3-galactoside, malvidin-3-arabinoside, delphindin-3-galactoside, delphinidin-3-arabinoside, and petunidin-3-galactoside in blue-fruited rabbiteyes. 'Pink Lemonade' fruit pigments were primarily delphinidin-3-galactoside and delphinidin-3-arabinoside. While use of NIR offers potential for predicting anthocyanin in blueberry fruit, we encountered unexpected results with locations that need to be explored.

Introduction

Blueberries are considered high in total anthocyanins, located in the peel (Ribera et al. 2010). Anthocyanins in blueberry are associated with human health benefits, especially cardiovascular disease (Kalt et al. 2020). Blueberries can have as many as 28 anthocyanins, as five anthocyanidins can be complexed to glucoside, galactoside, and/or arabinoside sugars, and/or acylated with phenolic acids (Grace et al. 2019; Chai et al. 2021). Malvidin and delphinidin anthocyanins appear to dominate in most blueberry germplasm (Stevenson and Scalzo 2012). Additionally, environmental conditions at harvest may also slightly influence anthocyanin profiles (Jung et al. 2021; Timmer et al. 2017; Spinardi et al. 2019). Individual anthocyanins may affect visual color and/or stability of color postharvest and may be important in response to air temperature or light spectra.

Rabbiteye blueberries have better drought tolerance and have wider soil pH adaptation than highbush but are more prone to winter injury (Krewer and NeSmith 2006). Rabbiteye cultivar releases are relatively few compared to northern and southern highbush, although several breeding programs focusing on rabbiteye cultivar improvement are now active. The total anthocyanin content and cyanidin anthocyanins have been reported to be higher in rabbiteye than in highbush blueberries, while acylated anthocyanin content is lower (Chai et al. 2021; Yousef et al. 2013).

The number and complexity of anthocyanins in blueberries makes data collection challenging using LC or LC-MS. Near infrared spectroscopy was found to rapidly estimate total anthocyanin content in two highbush blueberry cultivars, but was not used to identify individual pigments (Sinelli et al. 2008).

In this project, we determined anthocyanins in fruit collected from 9 rabbiteye selections and 12 cultivars in Alabama, and from seven rabbiteye cultivars grown in North Carolina (Table 1). As HPLC analysis is laborious and complex, we tested near infrared spectroscopy as a rapid means of determining relative amounts of individual anthocyanins as well as total anthocyanins.

Table 1. Rabbiteye cultivars from Alabama and North Carolina used for anthocyanin extraction.

Alabama	North Carolina
Alapaha	Alapaha
Brightwell	Montgomery
Climax	Premier
Krewer	Prince
Ochlocknee	Robeson (pentaploid)
Overtime	Titan
Pink Lemonade (50% RE)	Vernon
Powderblue	
Premier	
Tifblue	
Titan	
Vernon	

Methods

Fully ripe rabbiteye blueberry fruit samples (2-5 replicates consisting of 15-20 berries per sample) from 2024 field plots at Auburn University, Alabama and North Carolina were used for anthocyanin determination. The material consisted of 12 cultivars and 9 advanced selections, and 7 cultivars harvested from the Castle Hayne NC. All fruit were frozen at -80 °C, freeze dried, and held at -20 C until analysis. Material from AL was transported on dry ice to the Plants for Human Health Institute (PHHI) in Kannapolis NC. Green to overripe fruit samples, collected in 2024 from NC cultivars, were also used to expand the model for total anthocyanin amounts.

All fruit samples were ground using a Genogrinder (SPEX, New Jersey) for 2 min at 1200 strokes/sec to obtain a uniform small particle size. Vials of powder were scanned three times at room temperature and measured with a resolution of 16 cm^{-1} using a FT-NIR spectrometer (MPA, Bruker Optics, USA) from 3600 to 12500 cm^{-1} spectra in reflectance mode. The instrument is equipped with an integrating sphere to provide diffuse reflectance measurements and a PbS detector and OPUS software version 7.5.18 (Bruker Optics, USA). Raw spectra acquired from the NIR spectrometer was preprocessed before model calibration using preprocessing methods outlined in Perkins-Veazie et al. (2021) Models were constructed using the partial least squares (PLS) regression method by calibrating spectra against total anthocyanin and identified anthocyanin content from HPLC.

Anthocyanin extraction was done using replicates of 20 mg powder mixed with 1.5 ml acidified methanol (formic acid:methanol:deionized water, 1:60:39, v/v/v) in microfuge tubes. Samples were vortexed for 30 sec (Benchmark 1000, Grainger), sonicated for 15 min (Branson Ultrasonicator) and centrifuged for 20 min at 13,000 x g (microcentrifuge Model 5425R, Eppendorf). The supernatant was transferred to a 5 ml tube and the pellet re- extracted with 1.5 ml solvent as described above, which captures 98% of phenolic acids (Kim et al., 2015). Supernatants were combined, and 1 ml aliquots filtered through a 0.20 µM PTFE filter into amber HPLC vials, headspace flushed with nitrogen gas and vials capped with screw top lids.

High performance liquid chromatography (HPLC) was used to identify and quantify anthocyanins, following the method of Kim et al. (2015). A high performance liquid chromatography system (Elite LaChrom, Hitachi Ltd., Tokyo, Japan) equipped with autosampler, diode array detector, and binary solvent delivery manager was used to analyze phenolic compounds. This system includes a reversed phase C18 column (Synergi 4m Hydro-RP 80Å, Phenomenex, Torrance, CA, USA) held at 30 °C. Five percent formic acid (solvent A) and 100% methanol (solvent B) are the mobile phases with a flow rate of 16.7mL/min and a gradient system of 0–5 min, 90% A; 5–15 min, 85% A; 15–20min, 80% A; 20– 25 min, 75% A; 25–45 min, 70% A; 45–47 min, 40% A; 47–60min, 90% A. Ten microliter of sample was injected in duplicate. Anthocyanins were detected at 520 nm. Standard curves of cyanidin-3 glucoside, malvidin-3 galactoside, delphinidin-3 galactoside, delphinidin-3 glucoside, malvidin-3 glucoside, cyanidin-3 galactoside, petunidin-3 glucoside, peonidin-3- glucoside was used to identify and quantify anthocyanins.

Statistical analysis was done using analysis of variance (ANOVA) with SAS v. 9.4. Cultivar means were compared within location and compared using Tukey's honestly significant difference test (HSD) at the 95% significance level.

Results and discussion

As reported by others (Yousef et al., 2013; Timmer et al. 2017), rabbiteye blueberries generally had high amounts of total anthocyanin, ranging from 850-1900 mg/100 g dry weight basis (Fig1). However, some samples from the Auburn locations were lower in total anthocyanin than those from NC (Fig 1A). The majority of pigments in all cultivars were malvidin and delphinidin types (Fig 1B) in fruit from all locations. Of the total anthocyanins, malvidin-3-galactoside represented 17-36%; malvidin-3-arabinoside 7-18%; delphinidin-3-galactoside 12-32%;

delphinidin-3-arabionside 7-10%, and petunidin-3-galactoside 1-13%. The range of differences within specific pigments was more dependent on cultivar than on location. Acylated pigments were also present in small amounts (0.1-1.1% of total anthocyanin) but could not be identified with our HPLC system.

We were able to predict total anthocyanins with NIR with a fit of R2=0.98 for NC and AL-Ru material (Figure 2), but were unable to fit a prediction model for AL-Trandel material (data not shown). We were not successful in fitting NIR models that could predict specific pigments or types of pigments (such as malvidin or delphinidin). We will rerun the AL-Trandel material with NIR, HPLC, and microplate analysis to see if a better NIR model can be developed with this material, and if all three locations can be used to compare the large set with the individual sets of data.

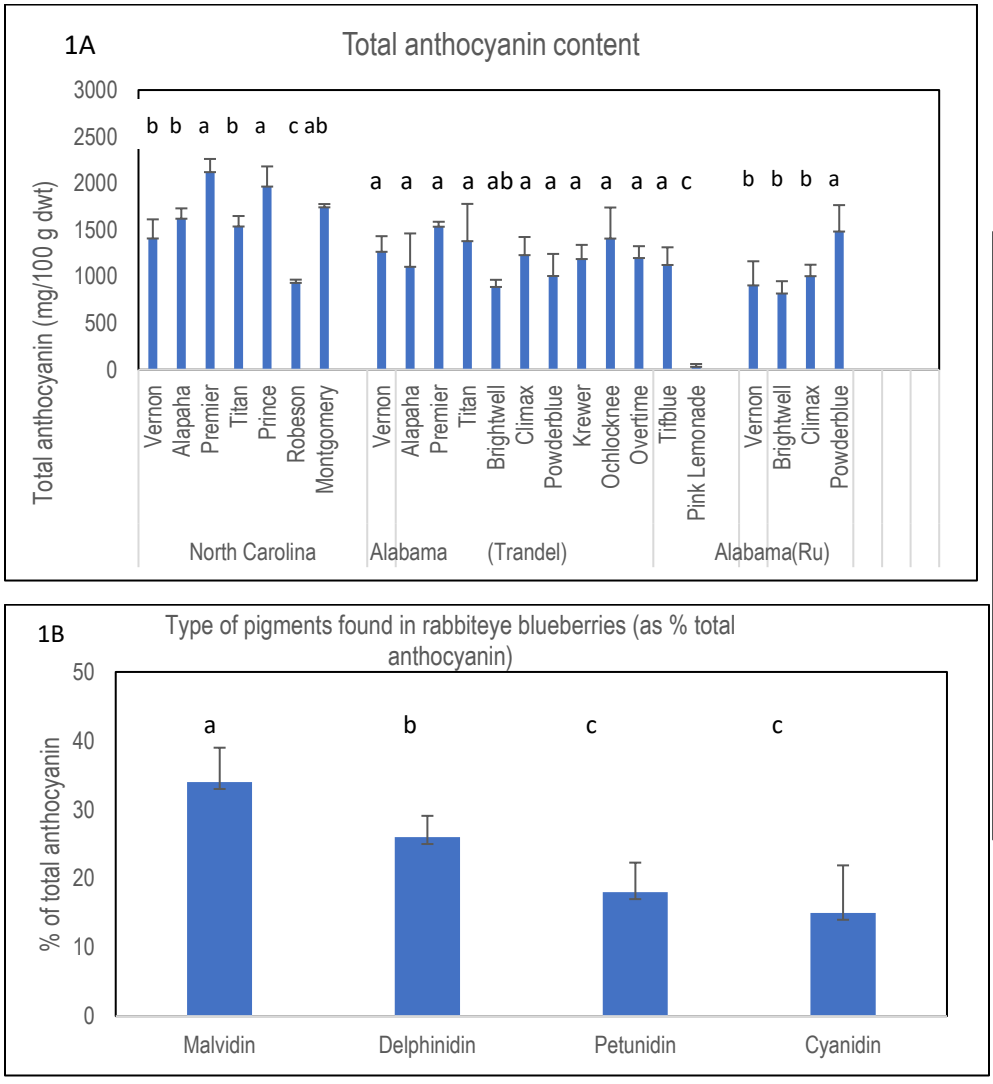

Figure 1. Total anthocyanin content of ripe rabbiteye blueberry fruit from plantings in Alabama and North Carolina (1A) and the relative amounts of anthocyanidins in rabbiteye fruit as a percent of the total anthocyanins (1B). Cultivar means separated within location with HSD, $p<0.01$ and different letters indicate significant differences

.

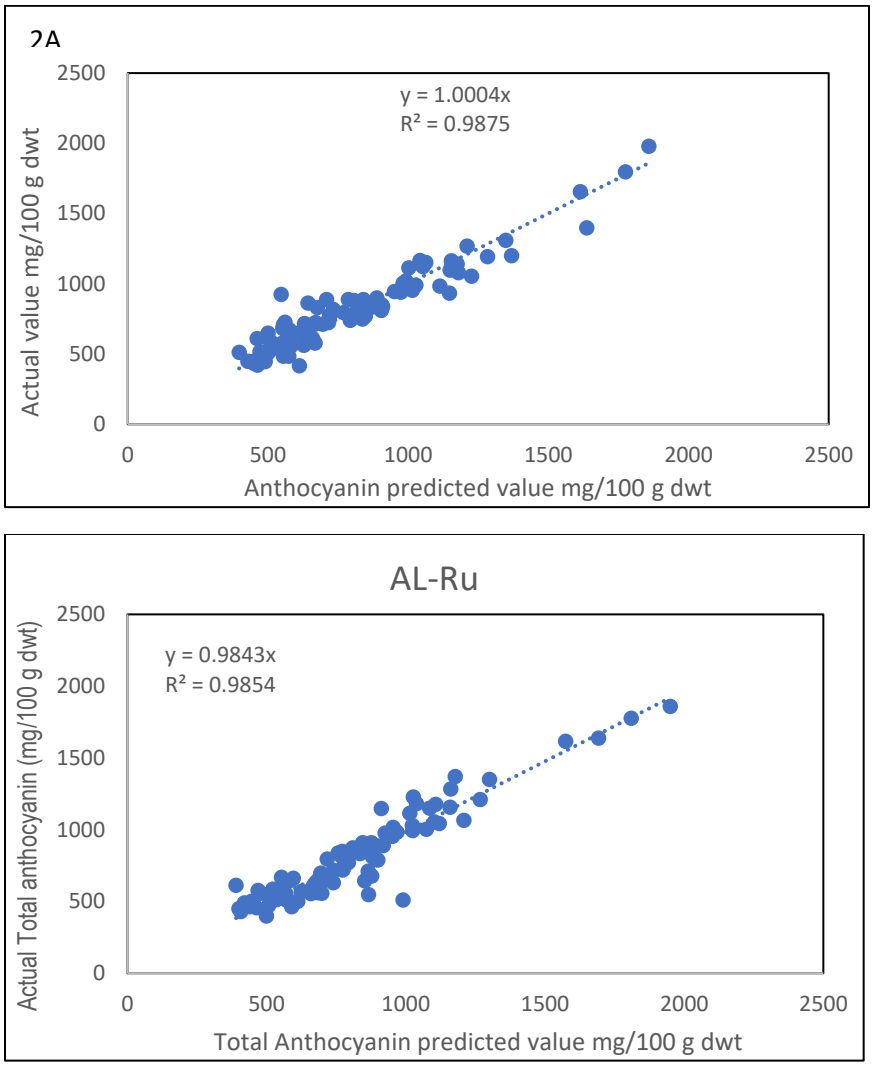

Figure 2. Prediction of total anthocyanin vs actual values for NC blueberries (A) and for AL-Ru blueberries (B).

Table 1. NIR prediction models using freeze dried powders of ripe rabbiteye blueberry fruit.

Location	R2	RMSEP	RPD	Bias	Rank	Preprocessing*	Wavelength (cm-1)
NC	95.91	125	4.94	0.297	4	Min-Max normalization	11602.4-9820.4 6264.1-5369.2
AL-Ru	84.43	122	2.53	2.54	10	1D+Min-Max normalization	10715.3-5369.2
AL-Trandel	53.01	309	1.46	2.86	7	1D+SNV	10715.3-9828.4 6264-5369.1 10715.3-5369.2

* 1D=first derivative; MSC=multiplicative scattering correction; SNV=vector normalization

Literature Cited

Chai, Z., Herrera-Balandrano, D.D., Yu, H., Beta, T., Zeng, Q., Zhang, X., Tian, L., Niu, L. and Huang, W. 2021. A comparative analysis on the anthocyanin composition of 74 blueberry cultivars from China. *Journal of Food Composition and Analysis*, *102*, p.104051.

Grace, M.H., Xiong, J., Esposito, D., Ehlenfeldt, M. and Lila, M.A. 2019. Simultaneous LC-MS quantification of anthocyanins and non-anthocyanin phenolics from blueberries with widely divergent profiles and biological activities. *Food Chemistry*, *277*, pp.336-346.

Jung, Y.S., Kwak, I.A., Lee, S.G., Cho, H.S., Cho, Y.S. and Kim, D.O. 2021. Influence of production systems on phenolic characteristics and antioxidant capacity of highbush blueberry cultivars. *Journal of Food Science*, *86*(7), pp.2949-2961.

Kalt, W., Cassidy, A., Howard, L. R., Krikorian, R., Stull, A. J., Tremblay, F., and Zamora-Ros, R. 2020. Recent research on the health benefits of blueberries and their anthocyanins. *Advances in Nutrition*, *11*(2), 224-236. https://doi.org/10.1093/advances/nmz065

Kim, M.J., Perkins-Veazie, P., Ma, G. and Fernandez, G. 2015. Shelf life and changes in phenolic compounds of organically grown blackberries during refrigerated storge. *Postharvest Biol. Technol*. 110: 257-263.

Krewer and NeSmith 2006. Blueberry Cultivars for Georgia. https://smallfruits.org/files/2019/06/06bbcvproc_Nov0206.pdf

Perkins-Veazie, P., Ma, G., Pottorff, M., Lila, M.A. and Iorizzo, M. 2021, August. New tools for rapid fruit quality analysis in blueberry. In *XII International Vaccinium Symposium 1357*:193-198. 10.17660/ActaHortic.2023.1357.28

Ribera, A. E., Reyes-Diaz, M., Alberdi, M., Zuñiga, G. E., & Mora, M. L. 2010. Antioxidant compounds in skin and pulp of fruits change among genotypes and maturity stages in highbush blueberry (Vaccinium corymbosum L.) grown in southern Chile. *Journal of soil science and plant nutrition*, *10*(4):509-536.

Sinelli, N., Spinardi, A., Di Egidio, V., Mignani, I., and Casiraghi, E. 2008. Evaluation of quality and nutraceutical content of blueberries (Vaccinium corymbosum L.) by near and mid-infrared spectroscopy. *Postharvest Biology and Technology*, *50*(1): 31-36. https://doi.org/10.1016/j.postharvbio.2008.03.013

Spinardi, A., Cola, G., Gardana, C.S. and Mignani, I. 2019. Variation of anthocyanin content and profile throughout fruit development and ripening of highbush blueberry cultivars grown at two different altitudes. *Frontiers in Plant Science*, *10*, p.1045. https://doi.org/10.3389/fpls.2019.01045

Stevenson, D. and Scalzo, J. 2012. Anthocyanin composition and content of blueberries from around the world. *Journal of Berry Research*, *2*(4):179-189.

Timmer, M.A., Grace, M.H., Yousef, G.G. and Lila, M.A. 2017. Inter-and intra-seasonal changes in anthocyanin accumulation and global metabolite profiling of six blueberry genotypes. *Journal of Food Composition and Analysis*, *59:*105-110. https://doi.org/10.1016/j.jfca.2017.02.019

Yousef, G.G, Brown, A., Funakoshi, Y. Mbeunkui, F., Grace, M., Ballington, J.R., Loraine, A. and Lila, M.A. 2013. Efficient quantification of the health-relevant anthocyanin and phenolic acid profiles in commercial cultivars and breeding selections of blueberries (Vaccinium spp*.). J. Agric. Food Chem. 61*(20):4806-4815