Non-destructive assessment of highbush blueberry fruit maturity parameters and anthocyanins by using a visible/near infrared (vis/NIR) spectroscopy device: A preliminary approach

A. Ribera-Fonseca^{1,2*}, M. Noferini³, and A.D. Rombolá⁴

¹Departamento de Producción Agropecuaria, Facultad de Ciencias Agropecuarias y Forestales, Universidad de La Frontera, Temuco, Chile. *Corresponding author: alejandra.ribera@ufroterra.cl

²Center of Plant-Soil Interaction and Natural Resources Biotechnology, Scientific and Technological Bioresources Nucleus (BIOREN-UFRO), Universidad de La Frontera, Temuco, Chile.

³FA.MO.SA SRL. (Farm MOnitoring Systems for Agriculture), 40026, Imola (Bo), Italy, email: famosasrl@gmail.com

⁴Dipartimento di Scienze Agrarie (DIPSA), Scuola di Agraria e Medicina Veterinaria, Alma Mater Studiorum, Università di Bologna, 40127 Bologna, Italy, email: adamo.rombola@unibo.it

Abstract

We evaluate the use of a portable and non-invasive technology based on visible and near infrared (vis/NIR) spectroscopy (Cherry-Meter) for monitoring fruit maturity parameters and anthocyanins in two highbush blueberry (*Vaccinium corymbosum* L.) cultivars (Misty and Sharpblue). This device measures an Index of Absorbance Difference (I_{AD}), which showed high correlations with fruit quality parameters in other fruit crops. We found positive but differential correlations between I_{AD} values and fruit anthocyanins, which was higher for Misty ($r = 0.970$, $p \leq 0.01$) than for Sharpblue ($r = 0.714$, $p > 0.05$). Interestingly, Cherry-Meter measurements were also correlated with solid soluble concentrations ($r = 0.685$, $p \leq 0.01$) and fruit firmness ($r = -0.714$, $p \leq 0.01$), but only in Sharpblue. For both cultivars, I_{AD} values were also significantly ($p \leq 0.01$) related with fruit FW (0.447 for Sharpblue and 0.559 for Misty). The High Performance Liquid Chromatography (HPLC) data indicated that I_{AD} variations were associated with significant changes in single berry anthocyanidins levels. These findings are the first approach, highlighting the potentialities of Cherry-Meter for the non-destructive assessment of fruit maturity and anthocyanidin profile in blueberries.

Keywords: Anthocyanins, blueberry, Harvest, non-destructive, vis/NIR spectrometry

1. Introduction

Worldwide highbush blueberry (*Vaccinium corymbosum* L.) production and consumption has significantly augmented in the recent years (Lindberg *et al.*, 2014). The main reason for this success is the remarkable human health benefits of blueberries, including high contents of vitamins and minerals, low calories, and a noticeable antioxidant capacity for anti-aging and degenerative disease prevention (Giongo *et al.*, 2011). The antioxidant properties of blueberries are mostly explained by its great levels of anthocyanins (Ehlenfeldt and Prior, 2001). Several agronomic and physiological factors can influence blueberry quality and phenolic content and composition (Yañez *et al.*, 2015).

Maturity at harvest considerably determine the quality and postharvest-life potential of fruits (Kader, 1999). Fruit maturity heterogeneity difficult the estimation of the optimal harvest start date which is a concern for the fresh fruit supply chain and a crucial aspect to ensure suitable exporting procedures (Infante, 2012). Nowadays, the visual analysis of skin blue color is recognized as one of the most important maturity index used for blueberry fruits (Leiva-Valenzuela *et al.*, 2013), which can result in inadequate homogeneity at harvest. The blue color in blueberries skin is a highly complex attribute, which is mainly function of the accumulation of anthocyanins (Ehlenfeldt and Prior, 2001), the distribution of individual anthocyanins, and the formation of metal complexes of anthocyanins (Harborne, 1976). Nonetheless, the human eye is not capable to sort accurately mature fruit deriving in a high heterogeneity of harvested fruits (Ziosi *et al.*, 2008; Leiva-Valenzuela *et al.*, 2013).

Few years ago, extensive research has been focused on the development of non-destructive techniques for evaluating fruit ripening (Ziosi *et al.*, 2008; Guidetti *et al.* 2009; Infante *et al.*, 2011; Bonora *et al.*,

2013, 2014, 2015). In this way, some technologies operating on visible and near infrared (vis/NIR) spectroscopy can allow checking in real-time the ripening progress of fruits (Bonora *et al.*, 2013), monitoring the physiological changes of a great number of fruits with a high precision. Interestingly, reflectance-spectroscopy-based techniques have been successfully used for the detection of anthocyanins in fruits including grape clusters (Tuccio *et al.*, 2011).

Despite the interesting advances about this topic, the use of vis/NIR spectrophotometry to non-destructively for the *in-situ* estimation of blueberry fruit maturity and quality has been scarcely explored (Guidetti *et al.*, 2009). One of these devices is the Cherry-meter (DA-meter modified), an innovative and more simplified vis/NIR technology developed at the Bologna University (Bologna, Italy). This portable and user-friendly equipment provides an Index of Absorbance Difference (I_{AD}), expressing the ripening stage of fruit, which correlates with some chemical and biochemical parameters (such as chlorophyll content and ethylene emission) to accurately describe fruit ripening (Ziosi *et al.*, 2008; Bonora *et al.*, 2013, 2014; Nagpala *et al.* 2013). The I_{AD} has been recognized as a potential suitable harvest index to *in-situ* monitoring fruit maturation in some cultivars of peaches (Bonora *et al.*, 2013, 2014, 2015), plums (Infante *et al.*, 2011), and apples (Nyasordzi *et al.*, 2013), among others fruit species. Lately, this device has been recently tested for the prediction of cherry maturity through the estimation of fruit skin anthocyanin, with very promising results (Nagpala *et al.*, 2013).

The aim of this work was to study the relationship among I_{AD} measurements and some maturity parameters and anthocyanins in blueberry fruits at harvest, in order to pre-validate the use of this device as a non-invasive tool to predict the fruit harvest date in this fruit species.

2. Materials and Methods

2.1. Experimental procedure and fruit sampling

The study was conducted in a commercial organic farm (Agricultural Society *Masseria Caporelli s.n.c.*, G. Mercatante & C.) located at San Costantino Calabro (VV), Calabria Region, Italy (Latitude: 38° 37' 12" North and 16° 15' 47" East; Altitude: 450 m a.s.l.). Fruits samples (150 berries for each cultivar) of two highbush blueberry (*Vaccinium corymbosum* L.) cultivars (Sharpblue and Misty) were harvested by morning at different maturation stages near to the full maturation stage (from 100% red to 100% blue fruits) estimated visually through the fruit skin color. Berries were collected from six randomly selected bushes (five-year-old plants) per cultivar from cultivated giving a planting density of 3333 bushes per hectare at spacing (3.0 m between rows x 1.0 m within row). Routine horticultural management of the region was applied throughout the season in terms of pruning, irrigation fertilization and pest control. Thus, all bushes were fertilized every year with leonardite (2000 kg ha⁻¹) applied on the soil surface in pre-harvest (March), with organic nitrogen (6 kg ha⁻¹) supplied by fertigation every 2 weeks and canopy-applied nitrogen (4.25 kg ha⁻¹), boron (8 g ha⁻¹) and zinc (27 g ha⁻¹) provided by six treatments. Pest management treatments consisted of copper (0.5 kg ha⁻¹, applied as oxychloride copper) and mineral oil (3.3 kg ha⁻¹) enabling the control of fungal pathogens and cottony cushion, respectively. All fruit samples were transported and stored at 4 °C from orchard to the Vegetal Production Laboratory (*Campus of Food Science*, University of Bologna, Cesena, Italy), where Cherry-Meter measurements and the physical and chemical analyses were performed within the 4 days following sampling. All determinations were made on the same single fruit.

2.2. Non-destructive analysis of fruits: Cherry-Meter data acquisition

In order to calibrate and validate the Index of Absorbance Difference (I_{AD}) as a non-destructive method to estimate the maturity level and quality of blueberries, Cherry-Meter (T.R. Turoni Srl, Forlì, Italy; Figure 1) was used to measure the absorbance of 30 randomly selected fresh intact fruits per cultivar, then obtaining different I_{AD} subclasses based on the I_{AD} value ranges (absorbance values) (Table 1). With this portable instrument, I_{AD} values are obtained from the absorbance differences between two wavelengths associated to the sample molecules (i.e. anthocyanins; 560 and 640 nm) detected in intact fruits, and the reference value at 750 nm (Nagpala *et al.*, 2013). The index calculation is based in the Lambert Beer law ($A = \log_{10} I - I_0$) (Noferini *et al.*, 2009). This instrument consists of a light source composed of six LEDs, positioned around a photodiode: three diode LEDs emitting wavelengths of 560 nm and other three in length of 640 nm. For the I_{AD} calculation, the amount of light remitted from the fruits is captured and measured by the central photodiode, converted by an "Adc conver-ter" ("analog to digital converter") and elaborated by a micro-controller (Costa *et al.*, 2010). The methodology and wavelengths (maximum absorbance of anthocyanins) used here were based on the previous works on cherry fruits, indicating that high I_{AD} values obtained were related to more advanced ripening stage and higher levels of anthocyanins (Nagpala *et al.*, 2013).

In all cases, I_{AD} data were obtained by taking almost two measurements on the center of each fruit and reading the average value on the instrument screen.

Figure 1. The Cherry-Meter device

Table 1. Range of I_{AD} values for the different I_{AD} sub-classes in two cultivars of highbush blueberry fruits

I_{AD} sub-classes	I_{AD} values	
	<i>cv. Sharpblue</i>	<i>cv. Misty</i>
SC 1	≤ 1.5	≤ 2.0
SC 2	$>1.5 - 1.7$	$>2.0 - 2.2$
SC 3	$>1.7 - 1.9$	$>2.2 - 2.4$
SC 4	$>1.9 - 2.1$	$>2.4 - 2.6$
SC 5	$>2.1 - 2.3$	$>2.6 - 2.8$
SC 6	$>2.3 - 2.5$	$>2.8 - 3.0$
SC 7	$>2.5 - 2.7$	$>3.0 - 3.2$
SC 8	$>2.7 - 2.9$	> 3.2
SC 9	> 2.9	-

2.3. Destructive analysis of blueberry fruits

2.3.1. Physical and chemical maturity parameters

As physical quality attributes, the fruit fresh weight (FW) was determined by using an analytical balance. Total soluble solids concentration (SSC) was assessed in juices of whole fruits using a manual thermo-compensated refractometer, standardized with distilled water and expressed as Brix. In addition, Texture Profile Analysis (TPA) was used to evaluate fruit firmness parameters on a 10-berry sample. For this, before the analysis, fruit samples were exposed to room temperature (20 °C) during 2 h, due to commonly fruits decreasing firmness at increasing temperature (Chiabrando *et al.*, 2009). The measurements were carried by TPA using a Texture Analyser (ZWICK ROELL mod. Z2.5TH, UK) equipped with a 5 kg load cell and HDP/90 platform, as previously described Chiabrando *et al.* (2009) for blueberry fruits. Briefly, to evaluate fruit elasticity by using TPA, the samples were deformed to 30% of the original height using a crosshead speed of 0.8 mm s⁻¹ and a 35 mm diameter cylinder stainless flat probe. Each sample was subjected to a two-cycle compression with 10 s between cycles. All these analyses were carried out in the same fruits previously evaluated through Cherry-Meter measurements.

2.3.2. Total anthocyanin spectrophotometric determination

To obtain the anthocyanin extract, samples of 0.1 g of whole fruits, skin or pulp were extracted with 1 mL of acidified ethanol (up to pH 1 with chlorhydric acid) in a mortar, boiled at 95 °C for 1.5 min for spectrophotometric analysis or 60 min for High Performance Liquid Chromatography (HPLC) analyses, incubated at 4 °C in the dark for 24 hours

and centrifuged at 3000 rpm for 10 min, as described earlier by Cheng and Breen (1991). Total anthocyanin (ANTH) concentrations were estimated by the pH differential method as mentioned by Cheng and Breen (1991) with minor modifications Ribera *et al.* (2010). The absorbance of anthocyanin extracts were measured in a spectrophotometer at 530 nm and at 657 nm with a molar extinction coefficient for cyanidin-3-glucoside of 29,600. The concentrations of ANTH were expressed as milligrams of cyanidin-3-glucoside equivalent (c3g) per 100 g of FW. These analyses were carried out in the same fruits previously evaluated through Cherry-Meter measurements. Three different samples of 3 fruits each one were extracted.

2.3.3. Anthocyanidin concentrations

The anthocyanidin concentrations were evaluated by HPLC analysis using the method described by Nyman and Kumpulainen (2001) with minor modifications (Mattivi *et al.*, 2006). Malvidin was used as standard, which was provided by Sigma. This analysis was conducted in a HPLC system (Jasco MD 1510) using a reversed-phase column RP-18, 250×4 mm 5 µm, equipped with a photodiode array detector (DAD, Jasco FP 1520). For anthocyanin extractions, samples of 0.3 g of whole fruits were extracted with 3 mL of acidified ethanol (up to pH 1 with chlorhydric acid) using mortar and pestle. Then, 10 mL of the ethanolic extracts were placed into centrifugation tubes (Falcon type), which were sealed tightly and boiled at 96 °C in a water bath for 60 min hour, incubated at 4 °C in the dark for 24 hours and centrifuged at 3000 rpm for 10 min. Signals were detected in the filtrated supernatants at 530 nm. The mobile phase was performed using acidified water [perchloric acid (HClO₄) 0.3% v/v] (A) and 100% methanol (B). The binary gradient was applied as follows: 0-39.9 min of 90%A-10%B,

40-41.9 min of 60%A-40%B, 42-48.9 min of 20%A-80%B, and 49-62 min of 90%A-10%B.

2.4. Statistical analysis

Results were assessed by one-way ANOVA using Sisvar 3.0 Software. According to the normality and homoscedasticity tests, the multiple comparison tests of means were carried out using Tukey test ($p \leq 0.05$). Statistically different I_{AD} classes were defined based on the significantly diverse anthocyanin total concentration among the I_{AD} sub-classes. Then, with the purpose to confirm the reliability of I_{AD} as a fruit ripening index, the correlation between I_{AD} values with different quality and technological parameters determined destructively for each I_{AD} -classes was evaluated. The software IBM SPSS Statistics 19 was used to test the relationships between two response variables through Pearson correlations.

3. Result and Discussion

Here, we test a portable technique based on vis/NIR spectroscopy, measuring an Index of Absorbance Difference (I_{AD}) (Cherry Meter), to estimate both anthocyanin concentrations and composition, and some quality parameters frequently used to assess fruit maturity and quality in blueberries.

According to our results, the statistical data analysis of the ANTH concentration of blueberries fruits from the different I_{AD} sub-classes, confirmed the identification of three major I_{AD} classes for each blueberry cultivar (Figure 2), which exhibited differential fruit SSC and FW (Table 2). Interestingly, in the present work we found that, inside the I_{AD} sub-classes, spectroscopic data and ANTH concentrations showed significant and positive correlations, which was higher for cv. Misty ($r = 0.970$, $p \leq 0.01$) compared to cv. Sharpblue ($r =$

0.714 , $p \leq 0.05$) (Table 3). Some related works have shown that non-destructive technologies based on vis/NIR spectrometry can be useful to estimate the ANTH concentration in several fruit species, including *V. corymbosum* (Guidetti *et al.*, 2009), *Vitis vinifera* (Tuccio *et al.*, 2011; Giovenzana *et al.*, 2014), and *Prunus avium* (Nagpala *et al.*, 2013). It is noteworthy that blueberry healthy or nutritional properties are generally determined by instrumental techniques (LC-MS; HPLC), which are time consuming, laborious and expensive. Guidetti *et al.* (2009) evaluated the potential of vis/NIR to analyze the nutraceutical properties of blueberry (including total anthocyanins) with interesting results. Additionally, studies reporting suitable relationships among Cherry-Meter measurements (I_{AD} values) and ANTH concentration have been previously reported by Nagpala *et al.* (2013) for cherry fruits. In contrast to other vis/NIR-based portable devices, Cherry-Meter does not require complex models that would need a continuous expensive and laborious up-date. It is important to point out that, for both cultivars, the relationship between I_{AD} data and SSC was even lower than those detected between I_{AD} and ANTH concentration (Table 3). Moreover, inside the I_{AD} sub-classes, blueberries ANTH concentrations and SSC exhibited positive correlations, which was significant ($p \leq 0.05$) for cv. Sharpblue ($r = 0.786$) but not for cv. Misty. In grapevine berries it has been shown that the synthesis of anthocyanins is stimulated by sugars (Hiratsuka *et al.*, 2001), mostly in the initial phase of fruit maturation when ANTH and SSC are strongly correlated. However, in the last ripening phase, the accumulation of anthocyanins and soluble solids in grapes can be uncoupled (Guidoni *et al.*, 2008).

These antecedents can almost partially explain our results showing that for blueberry fruits cv. Misty, blue color intensity and coverage of berry skin was

not highly associated to berry SSC. This outcome could have important implications for blueberry producers and exporters.

Figure 2. Total anthocyanin (ANTH) concentration of different I_{AD} sub-classes in two highbush blueberry cultivars (cv. Sharpblue and cv. Misty). The I_{AD} values of each I_{AD} sub-class are showed in the Table 1. Different letters indicate statistically significant differences between the I_{AD} sub-classes according the Tukey ($p \leq 0.05$) test.

Table 2. Soluble solids concentration (SSC) and fresh weight (FW) of fruits from the different major I_{AD} classes (identified on the basis of total anthocyanin concentration) in two blueberry cultivars. Different letters indicate statistically significant differences between the I_{AD} sub-classes according the Tukey ($p \leq 0.05$) test.

Cultivars	I_{AD} class	I_{AD} values	SSC (Brix)	FW (g)
cv. Sharpblue	C1	≤ 1.5	08.89 c	0.785 b
	C2	1.5 - 2.9	11.16 b	0.972 ab
	C3	> 2.9	12.66 a	1.070 a
			$p = 0.000$	$p = 0.002$
cv. Misty	C1	≤ 2.6	12.09 a	1.467 b
	C2	2.6 - 3.0	12.25 a	1.844 a
	C3	> 3.0	12.16 a	1.920 a
			$p = 0.981$	$p = 0.001$

Table 3. Relationship among I_{AD} values and different fruit maturity parameters in fruits of two cultivars of blueberry. SSC: soluble solid concentration; FW: fresh weight; ANTH: total anthocyanin concentration.

Fruit	Maturity	Blueberry cultivars	
Parameters		cv. Sharpblue	cv. Misty
ANTH		0.714*	0.970**
SSC		0.685**	0.050
FW		0.447**	0.559**
Hardness		-0.713**	-0.350
Elasticity		-0.658**	0.313

Our findings also revealed that I_{AD} values showed significant correlations with fruit FW in both blueberry cultivars (Table 3). In agreement with this observation, Bonora *et al.* (2014) found that I_{AD} data detected by using DA-Meter device and fruit diameter were highly related in nectarines, which allowed accurate predictions of fruit yield.

Jorquera-Fontena (2015) reported that there is a strong correlation between fruit FW and diameter ($R^2 = 0.99$) in highbush blueberry. Therefore, further works are required in order to assess whether Cherry-Meter measurements may be useful to the *in-situ* estimation of fruit yield in highbush blueberry orchards.

In accordance with previous works (Chiabrando *et al.*, 2009), we found high fruit-to-fruit firmness variability in both blueberry cultivars. Furthermore, despite fruit TPA test did not show significant differences among I_{AD} classes, we found that I_{AD} values were negatively correlated with berry hardness ($r = -0.714$, $p \leq 0.01$) and elasticity ($r = -0.658$, $p \leq 0.01$), but only for cv. Sharpblue (Table 3 and Figure 3). Then, our TPA data allowing infer that I_{AD} values from Cherry-Meter measurements could be useful to predict fruit firmness variations in blueberry fruits. Previous works have demonstrated that a series of fruit maturity parameters can be well predicted through vis/NIR spectroscopy-based devices in blueberry. Guidetti *et al.* (2009) found good correlation coefficients (r) between fruit absorbance values (at wavelengths ranging from 450–980 nm) with ripening parameters, including SSC and firmness of fresh blueberries. To the date, related works aimed to evaluate vis/NIR spectroscopy based devices for monitoring the maturity levels of fruits did not provide information about the use of these technologies to determinate the concentration of single anthocyanidin molecules in berries. In the present work, we showed that, independently of the cultivar, the concentrations of single anthocyanidins (analysis

by HPLC) significantly varied among the different I_{AD} classes of blueberry fruits (Figure 4). In agreement with previous works (Ribera *et al.*, 2010), we found that the anthocyanidin profile of blueberry fruits differ among cultivars, with delphinidin as the major molecule. Additionally, our data indicated that the concentration of each single anthocyanidin, including delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin, increased at increasing I_{AD} values, which was more evident in cv. Misty compared to cv. Sharpblue. In both cultivars, the concentration of petunidin and peonidin, but mainly of delphinidin, were even higher compared to the other anthocyanidins (Figure 4). Regardless of the I_{AD} classes, the higher concentration of anthocyanidin molecules was detected in Sharpblue in comparison to Misty. The variation about the relationship between anthocyanidin concentration and I_{AD} values among the single molecules can be due to the differential maximum absorbance of these compounds respect to the emission wavelengths of the Cherry-Meter device. It has been reported that redness and blueness of single anthocyanin molecules are directly related to hydroxylation and methylation pattern of the B ring (He *et al.*, 2010).

Figure 3. Fruit hardness (A) and elasticity (B) as related with I_{AD} values in blueberries cv. Sharpblue. The Pearson correlation was used to test the relationships between the variables. Asterisks denote significance ($**p \leq 0.01$).

Figure 4. Anthocyanidin composition in the different I_{AD} classes of blueberry fresh fruits: (A) cv. Misty and (B) cv. Sharpblue. Delph: delphinidin; Cyan: cyanidin; Pet: petunidin; Pelarg: pelargonidin; Peo: peonidin; Malv: malvidin 3-glucoside. Upper-case letters indicate significant differences among I_{AD} classes for the same molecule, whereas lower-case letter indicate significant differences among different molecules for the same I_{AD} class.

4. Conclusions

Our data shown for the first time that the Cherry-Meter device could be considered as a potential portable instrument used to predict the maturity and health properties of blueberry fruits in a non-destructive way. When used at advanced maturation stages, Cherry-Meter was able to discriminate different maturity levels from apparently similar mature blueberry fruits. Significant correlations between Cherry-Meter measurements and fruit maturity parameters were observed, but not for both cultivars, therefore, we suggest that the calibration of the I_{AD} values as fruit harvest and quality predictors seem to be cultivar-dependent. Interestingly, here we showed that I_{AD} values offers information about the berry anthocyanidin concentration and composition, which is strongly related to the healthy/nutritional properties of this fruit. This aspect could be have a significant impact for the pharmaceutical and cosmetic industry oriented to the development of blueberry-based bio-products as well as for further blueberry breeding programs. Further *in-planta* studies using these and other cultivars should be made in order to confirm and validate the large-scale functionality of this device as a reliable maturity index for blueberries selecting fruits with the biggest anthocyanin concentration, and therefore with both, a better antioxidant performance and longer shelf-life. Our results could contribute to further improve the harvest management and postharvest quality of this esteemed and healthy fruit.

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