

STRAWBERRY ANTHOCYANIN DETERMINATION BY pH DIFFERENTIAL SPECTROSCOPIC METHOD – HOW TO GET TRUE RESULTS?

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Abstract. The aim of the research was to analyse the weaknesses of the pH differential method for strawberry anthocyanin determination. The work is based on practical experiments with 12 strawberry cultivars and on analysis of published papers. We used following molar absorption coefficients (ϵ values): 26900 and 29600 $M^{-1} cm^{-1}$ for cyanidin 3 glycoside (C3g) and 15600, 22400, 27300 and 36000 $M^{-1} cm^{-1}$ for pelargonidin 3 glycoside (P3g). In order to show how the calculated value of total anthocyanins depends on the predominant anthocyanin used for the calculations, we compared the results of spectroscopic and chromatographic analysis. Present research demonstrated that different ϵ values may influence the results of total anthocyanins even more than cultivar properties. The most frequently used ϵ values 26900 $M^{-1} cm^{-1}$ and 29600 $M^{-1} cm^{-1}$ gave underestimated values. C3g was present in minor amounts in all cultivars. Conclusively, P3g with the $\epsilon = 15600 M^{-1} cm^{-1}$ should be used for ensuring most precise estimation of total anthocyanin content in strawberries.

Key words: *Fragaria* \times *ananassa*, pelargonidin, molar absorption coefficient

INTRODUCTION

During the past decades fruit anthocyanins have been the topic of numerous scientific investigations [Aaby et al. 2012]. Anthocyanins are considered to have high radical scavenging properties, preventing oxidative stress and helping to maintain physiological functions [Du and Wang 2008]. An important part of the research on anthocyanins has been related to strawberry (*Fragaria* \times *ananassa* Duch.) fruits, because anthocyanins are also responsible for the strawberry antioxidant activity, which is one of the highest among several fruits [Cordenunsi et al. 2005].

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In order to compare the results from different studies and to add new knowledge to the already known, it is extremely important to use proper methods for anthocyanin determination.

During the decades, the pH differential spectroscopic method has been used for the determination of anthocyanin concentration. The advantage of the method is its outstanding possibility to perform quantitative analysis. The majority of quantitative analysis methods need calibration with high purity substances of the analyzed compounds. Since anthocyanins exist in plants as mixtures of several compounds with similar chemical properties and the purification process is complicated, therefore the high purity anthocyanins are expensive [Wrolstad et al. 2005]. The pure anthocyanins are also very unstable and susceptible to degradation [Giusti and Wrolstad 2003]. The lack of commercially available standards in order to determine the acyl derivatives is a problem for several researchers [García-Falcon et al. 2007], especially in the case of very complex mixtures.

The pH differential spectroscopic method gives us the possibility to calculate and report the results using the molar absorption coefficient (ϵ) and molecular weight data from previously published works. However, such simplicity makes the method vulnerable. The wavelength of absorbance maxima and molar absorption coefficient depend on solvent properties (polarity, acidity, concentration of impurities) [Ito et al. 2002]. From published works it appears that several different molar absorption coefficients are used, which may sometimes have two-fold differences. Such differences in ϵ values cause wide fluctuations in reported strawberry total anthocyanins contents and make it problematic to compare results published by different authors. The aim of the current research was to discuss the weaknesses and to analyse step-by-step the bottlenecks of the widely used pH differential method for strawberry anthocyanin determination. The work is based on our own practical experiments and on analysis of previously published results. In our opinion the current work would help to achieve consensus among strawberry scientists on how to determine strawberry total anthocyanin content.

MATERIAL AND METHODS

Plant material. Ripe (fully red) strawberry fruits were harvested in July 2011 from three plantations situated in South Estonia. The longest distance between the experimental sites was 15 km from South to North and 70 km from East to West. All plantations were situated in region representing similar kind of soil (brown pseudopodzolic soil). ‘Senga Sengana’, ‘Chamly’, ‘Induka’, ‘Lucy’, ‘Saljut’ and ‘Dukat’ fruits were harvested from the experimental plantation of Polli Horticultural Research Centre of Estonian University of Life Sciences (58°7’52’’N; 25°32’30’’E). ‘Clery’ and ‘Darselect’ fruits were harvested from Eerika experimental plantation (58°21’55’’N; 26°40’7’’E) of the same university in Tartu. ‘Sonata’, ‘Rumba’ and ‘Polka’ fruits were collected from a commercial strawberry plantation situated 15 km from Tartu (58°7’15’26’’N; 26°35’57’’E). One kg of fruits was transported to the laboratory within two hours and frozen at -30°C. Analyses were performed after four months.

Analytical procedures

Anthocyanin extraction. The frozen fruits (75–120 g) were partly thawed (2 h at room temperature) before homogenizing with a Polytron PE1600 homogenizer. Strawberry puree (4 g) was extracted with 40 ml of solvent ethanol : 0.1 M HCl (85:15%, v:v) and sonicated for 10 minutes. After centrifugation the supernatant was collected and used for anthocyanin determination. Extractions were done in triplicate.

Determination of total anthocyanins by pH differential spectroscopic method. Total anthocyanins were determined according to the pH differential spectroscopic method [Cheng and Breen 1991]. 3 ml of extracts were diluted in 5 ml of two different buffers; 0.025 M potassium chloride pH = 1.0 and 0.4 M sodium acetate pH = 4.5, respectively. After 30 minutes of incubation at room temperature, absorption (A) was measured at $\lambda = 510$ and $\lambda = 700$ nm (Thermo Scientific Helios β , UK). All extracts were analyzed in duplicate.

For calculation of total anthocyanins as C3g, the molar absorptivity coefficient (ϵ) values $26900 \text{ M}^{-1}\text{cm}^{-1}$ [Meyers et al. 2003] and $29600 \text{ M}^{-1}\text{cm}^{-1}$ [Cao et al. 2011] and molecular weight 449 was used. For reporting total anthocyanins as P3g, ϵ values $15600 \text{ M}^{-1}\text{cm}^{-1}$ [Giusti et al. 1999], $22400 \text{ M}^{-1}\text{cm}^{-1}$ [Wicklund et al. 2005] and $27300 \text{ M}^{-1}\text{cm}^{-1}$ [Aaby et al. 2005] and 36000 [Pineli et al. 2011] were used and molecular weight (M) values of 443 and 445 respectively were used. The results were calculated similarly to Giusti and Wrolstad [2001] as follows:

$$A_{sp} = (A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}$$

The content of total anthocyanins (TA) were calculated as follows:

$$TA = (A_{sp} \times M \times DF \times 1000) / (\epsilon \times \lambda \times m),$$

where DF is the dilution factor, λ is the cuvette optical pathlength (1 cm) and m is the weight of the sample (g). The total anthocyanin content was expressed as mg anthocyanin 100 g^{-1} fresh weight.

Determination of anthocyanins by HPLC. Anthocyanins were separated using a Perkin Elmer Series 200 liquid chromatograph (PerkinElmer Inc., Shelton, CT) equipped with a UV-Vis detector and autosampler.

Chromatographic separation was performed on Platinum TM C18 column (250×4.6 i.d., $5 \mu\text{m}$ particle size) from Grace Alltech (W.R. Grace & Co., Maryland, USA). The mobile phase for separation of anthocyanins consisted of 5 ml 85% H_3PO_4 , 25 ml acetonitrile, 470 ml water (A) and acetonitrile (B). The initial mobile phase concentration of 80% A and 20% B was held for 10 minutes, followed a linear gradient to 25% B for 5 min, then a linear increase to 35% B for 2 min and finally linear gradient to 75% for 4 min. Column temperature was ambient.

Calculation of the anthocyanin content was based on the external standard method and P3g and C3g were identified by comparison of their retention times with those of pure standards.

The theoretical absorption values (A_{Cal}) from the results of chromatographic determination of anthocyanins were calculated according the formula:

$$A_{\text{Cal}} = \sum C_i \times \varepsilon \times \lambda = (C_{\text{C3g}} + C_{\text{P3g}}) \times \varepsilon \times \lambda,$$

where C_i is the concentration of individual anthocyanin in the extract, which is calculated as follows:

$$C_i = (C_{\text{iCh}} \times K) / M_i$$

C_{iCh} is chromatographically determined content of individual anthocyanin in strawberry (mg per 100 g), M_i is molecular weight of anthocyanin and all specific factors of experiment are taken into account in the coefficient K (0.000375).

For comparison of calculated chromatographically determinations and spectrometrically measured absorption values (A_{Sp}), the spectrometrically measured values were normalized (A_{N}) for the sample weight 5.0 g:

$$A_{\text{N}} = (A_{\text{Sp}} \times 5.0) / m$$

For anthocyanins limit of detection (LOD) and quantification (LOQ) for anthocyanins were calculated following the IUPAC recommendations. Detection limit was estimated as 3 s and limit of quantification as 10 s, where s was the standard deviation of 10 sample blank measurements. LOD was 8 ng ml⁻¹ and 5 ng ml⁻¹ and LOQ was 26 ng ml⁻¹ and 18 ng ml⁻¹ for C3g and P3g, respectively.

Statistical analysis. All determinations were performed in triplicate and data were expressed as means \pm SD. Statistical analyses were performed with R freeware version 2.13.0 (R Development Core Team).

RESULTS AND DISCUSSION

Major anthocyanins in strawberry fruits – analysis of published works. Despite several factors influencing strawberry fruit total anthocyanin content, it has been proven by several authors that the most common anthocyanin in strawberry fruits is P3g, the content of which ranges from 60 to 95% of total anthocyanins [Aaby et al. 2012, Buendia et al. 2010]. According to Aaby et al. [2012] and Tulipani et al. [2008] the second most abundant anthocyanin in strawberry fruits is pelargonidin 3 malonylglucoside, which may range from 0 to 33.5% of total anthocyanins. Pelargonidin 3 rutinoside (P3r) constitutes from 0.0 to 14.8% of total anthocyanins in strawberry fruits and C3g from 0.9 to 8.9% [Buendia et al. 2010, Aaby et al. 2012]. However, among the published research the total content of strawberry anthocyanins is often expressed as C3g [Wrolstad et al. 2005] and sometimes even as cyanidin 3 galactoside (tab. 1).

Depending on the cultivar and other conditions, the ratio of C3g and P3r may vary significantly; for some cultivars ('Honeoye', 'Korona', 'Sonata', 'Jonsok' and 'Bounty') the C3g had only the fourth position after P3g, P3r and pelargonidin 3 malonylglucoside. In some other cultivars ('Carisma', 'Marlate'), fruits didn't contain P3r at all and C3g was third most abundant [Aaby et al. 2012]. The large variations in single anthocyanin contribution to total anthocyanin content between several studies may be caused by degradation of acylated anthocyanins during the extraction and analysis process [Lopes da Silva et al. 2007].

Table 1. The most commonly used anthocyanins (ACY) and values of molar absorptivity coefficient (ϵ) ($M^{-1}cm^{-1}$) in strawberry total anthocyanin calculations

| Cultivar | ACY | ϵ ($M^{-1}cm^{-1}$) | Wavelength (nm) | Sample analyzed | Extragent | Reference |
|---|-------------------|-----------------------------------|--------------------|---------------------------|---------------------------------|-------------------------|
| n.d. ^a | P3g | 15600 | 496 | fresh fruit | acetone | Giusti et al. 1999 |
| n.d. | P3g | 15600 | | juice concentrate | w.e. ^b | Sadilova et al. 2009 |
| Senga Sengana, Polka, Korona, Honeoye; Inga | P3g | 22400 | 520 | jam | methanol, HCl | Wicklund et al. 2005 |
| Polka | P3g | 22400 | 515 | frozen fruit and juice | methanol, formic acid, water | Klopotek et al. 2005 |
| n.d. | C3g | 25965 | 535 | fresh fruit | acetone, water, acetic acid | Kevers et al. 2007 |
| n.d. | C3g | 26900 | 510 | freeze dried powder | methanol | Heo and Lee 2005 |
| Totem, Puget Reliance | P3g | 27300 | 496 | freeze dried powder | acetone | Aaby et al. 2005 |
| Fengxiang | C3g | 29600 | 510 | fresh fruit | ethanol | Cao et al. 2011 |
| Osogrande, Camino Real | P3g | 36000 | 515 | frozen fruit | methanol | Pineli et al. 2011] |
| Camarosa | P3g | 36000 | | frozen fruit | acetone, HCl | Roussos et al. 2009 |
| Polka | P3g | 36000 | 510 | fresh fruit | ethanol, HCl | Moor et al. 2009 |
| n.d. | C3ga ^c | n.d. | 510 | fresh fruit | acetone, HCl | Erkan et al. 2008 |

^a n.d. – data not obtained

^b w.e. – without extragent, determinations directly from juice

^c C3ga – cyanidin 3 galactoside

The content of C3g and P3g in twelve studied strawberry cultivars. According to the results obtained from chromatographic analysis, where anthocyanins were extracted with acetone, the C3g content in twelve studied cultivars was minimal compared to the content of P3g (tab. 2). The C3g content ranged from 0.5 mg 100 g⁻¹ in ‘Darselect’ to 3.79 mg 100 g⁻¹ in ‘Chamly’. The latter cultivar was the only one in which the sum of C3g and P3g was statistically significantly higher than the P3g content alone. The content of the main strawberry anthocyanin, P3g, was lowest in cultivar ‘Sonata’ (19.3 mg 100 g⁻¹) and highest in ‘Senga Sengana’ (48.5 mg 100 g⁻¹). Our results are in agreement with earlier reported data by Aaby et al. [2012], where P3g content in ‘Sonata’ and ‘Polka’ fruits was 15.3 and 24.9 mg 100 g⁻¹, respectively. The total anthocyanin content calculated from spectrometric data using $\epsilon = 15600$ was 10–36% higher than the sum of chromatographically obtained C3g + P3g. The result is justified by the fact that the content of other anthocyanins (pelargonidin 3 malonylglucoside and pelargonidin 3 rutinoside) were not determined, but may also contribute to the content of total anthocyanins.

Table 2. The content of C3g and P3g and total anthocyanins extracted with acetone in twelve strawberry cultivars C3g, P3g and C3g + P3g content from HPLC analysis for calculation of total anthocyanin content from spectrometrical data a ϵ value of 15600 was used

| Cultivar | Content of anthocyanins, mg 100 g ⁻¹ FW | | | Total anthocyanins |
|---------------|--|-------------|-------------|--------------------|
| | C3g | P3g | C3g + P3g | |
| Senga Sengana | 1.97 ±0.10 | 48.49 ±1.22 | 50.46 ±1.32 | 60.05 ±0.10 |
| Induka | 1.77 ±0.12 | 36.42 ±1.18 | 38.19 ±1.30 | 46.52 ±0.32 |
| Clery | 1.43 ±0.14 | 25.19 ±1.33 | 26.62 ±1.47 | 35.07 ±0.47 |
| Rumba | 1.66 ±0.13 | 25.48 ±0.66 | 27.14 ±0.79 | 30.62 ±0.40 |
| Dukat | 0.62 ±0.04 | 32.93 ±1.28 | 33.55 ±1.32 | 42.23 ±0.08 |
| Polka | 1.89 ±0.02 | 29.30 ±0.76 | 31.18 ±0.78 | 44.05 ±0.44 |
| Delia | 1.26 ±0.06 | 20.30 ±1.00 | 21.56 ±1.06 | 27.83 ±0.03 |
| Sonata | 0.63 ±0.10 | 19.30 ±0.71 | 19.93 ±0.81 | 30.25 ±1.25 |
| Darselect | 0.50 ±0.02 | 23.17 ±1.16 | 23.67 ±1.18 | 27.79 ±0.17 |
| Saljut | 1.73 ±0.09 | 38.14 ±0.67 | 39.87 ±0.76 | 42.68 ±0.28 |
| Lucy | 1.38 ±0.11 | 34.90 ±1.26 | 36.28 ±1.37 | 44.84 ±0.84 |
| Chamly | 3.79 ±0.04 | 44.54 ±1.18 | 48.33 ±1.22 | 55.40 ±0.45 |

Finally, it is possible to conclude that in all twelve strawberry cultivars used in the present study, the C3g had no significant contribution to the total anthocyanin content. If chromatographic analysis is used, the content of total anthocyanins is calculated as a sum of all determined components. Problems arise with the widely used spectroscopic method, where calculations of total anthocyanin content are based on one supposedly predominant anthocyanin. In some cases, if the aim is to compare the amount of total anthocyanins from different fruit species, it could be understandable to use C3g as a reference [Kähkönen et al. 2001, Guerrero et al. 2010], since this compound is a dominant anthocyanin in most of the fruits [Francis and Markakis 1989]. But in papers dealing only with strawberry anthocyanins, it makes no sense to report the content of total strawberry anthocyanins as C3g, which is certainly present in minor amounts compared to the P3g. Since the molar absorption coefficient for P3g is quite different from C3g, we may presume that reported results may be different from the real situation. It is well known that it is not possible to measure the exact content of total anthocyanins by the spectroscopic method, because there is a mixture of several anthocyanins present. In order to obtain results as close as possible to the real situation, it is essential to use the values of major anthocyanin in calculations.

Differences in experimental and calculated optical absorbance. In order to show how the calculated value of total anthocyanins depends on the predominant anthocyanin used for the calculations, we compared the results of spectroscopic and chromatographic analysis in a non-traditional way. We compared the measured value of optical absorbance ($A = (A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}$) in strawberry extracts, which is typical for anthocyanin determinations, with the theoretically calculated values of A, using data from chromatographic measurements.

The theoretical value of A was calculated in a buffer solution pH = 1 using the several different widely used values of ϵ . Since from chromatographic measurements we have data on the content of the two main anthocyanins – P3g and C3g, the calculated value of A should be somewhat lower than the measured value. According to the above mentioned P3r content, our results may differ from real value by 0 to approx 14%, if we presume that ϵ for P3r is close to used the ϵ value in calculations.

Table 3. Comparison of spectrometrically determined optical absorbance (A) value ($\lambda = 510$ nm in glass cuvette with optical pathlength 10 nm) and calculated A value – HPLC data, using different molar absorption coefficients (ϵ) for twelve strawberry extracts

| Cultivar | A value | | | | | | |
|---------------|------------------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
| | spectrometrically determined | calculated | | | | | |
| | | $\epsilon = 15600$ | $\epsilon = 22400$ | $\epsilon = 26900$ | $\epsilon = 27300$ | $\epsilon = 29600$ | $\epsilon = 36000$ |
| Senga Sengana | 0.810 | 0.681 | 0.978 | 1.174 | 1.191 | 1.292 | 1.571 |
| Induka | 0.627 | 0.515 | 0.740 | 0.888 | 0.901 | 0.977 | 1.189 |
| Clery | 0.473 | 0.359 | 0.515 | 0.619 | 0.628 | 0.681 | 0.828 |
| Rumba | 0.413 | 0.366 | 0.525 | 0.631 | 0.640 | 0.694 | 0.844 |
| Dukat | 0.569 | 0.453 | 0.650 | 0.781 | 0.739 | 0.860 | 1.045 |
| Polka | 0.594 | 0.420 | 0.604 | 0.725 | 0.736 | 0.798 | 0.970 |
| Delia | 0.375 | 0.291 | 0.417 | 0.501 | 0.509 | 0.552 | 0.671 |
| Sonata | 0.408 | 0.269 | 0.386 | 0.464 | 0.471 | 0.510 | 0.621 |
| Darselect | 0.376 | 0.320 | 0.459 | 0.551 | 0.559 | 0.606 | 0.737 |
| Saljut | 0.576 | 0.538 | 0.772 | 0.927 | 0.941 | 1.020 | 1.241 |
| Lucy | 0.605 | 0.489 | 0.703 | 0.844 | 0.857 | 0.929 | 1.129 |
| Chamly | 0.747 | 0.651 | 0.935 | 1.123 | 1.139 | 1.235 | 1.503 |

Spectrometrically measured values ranged from A = 0.375 to A = 0.810, which is ideal from the point of view of spectroscopy (tab. 3). The range of calculated values was extended from both sides: the lowest value was 0.269, which was 0.106 units below the lowest actually measured value and the highest calculated value, 1.571, was approx. twice as high as the spectrometrically obtained one. All these values stayed in the experimentally measurable region of A values. It was obvious that the calculated values were mostly higher than the experimentally obtained results, which was in conflict with our expectations. The difference from spectrometrically obtained results originates from different ϵ values used in calculations. If $\epsilon = 22400 \text{ M}^{-1}\text{cm}^{-1}$ is used in the calculations, the results tend to be slightly higher than the experimental values. With increasing ϵ values, the calculated A increases up, reaching the double value of the experimentally measured values. Only the values calculated with the lowest value of ϵ (15600) were lower than the spectroscopically obtained results. As spectrometrically determined content of total anthocyanins also contains other anthocyanins, it is essential that chromatographically determined sum of C3g and P3g value should always be lower.

As an average of 12 studied cultivars, the values of theoretically calculated optical absorbance ranged from 77 to 177% of experimentally obtained values (fig. 1). The closest values to real A were the calculated values with $\epsilon = 15600 \text{ M}^{-1}\text{cm}^{-1}$ and $\epsilon = 22400 \text{ M}^{-1}\text{cm}^{-1}$ (23% lower and 10% higher than the measured values, respectively). According to Aaby et al. [2012], for cultivars ‘Polka’, ‘Senga Sengana’ and ‘Sonata’, the sum of P3g and C3g constitutes 72%, 82% and 70% of total anthocyanins, respectively. Therefore the difference of 23% between measured and calculated A values using $\epsilon = 15600 \text{ M}^{-1}\text{cm}^{-1}$, is in excellent range. The conclusion can be made that commonly used ϵ values $27300 \text{ M}^{-1}\text{cm}^{-1}$ and $36000 \text{ M}^{-1}\text{cm}^{-1}$ for P3g and $26900 \text{ M}^{-1}\text{cm}^{-1}$ and $29600 \text{ M}^{-1}\text{cm}^{-1}$ for C3g give untrue results. Based on what was previously discussed, the best ϵ value for strawberry anthocyanin calculations appears to be 15600.

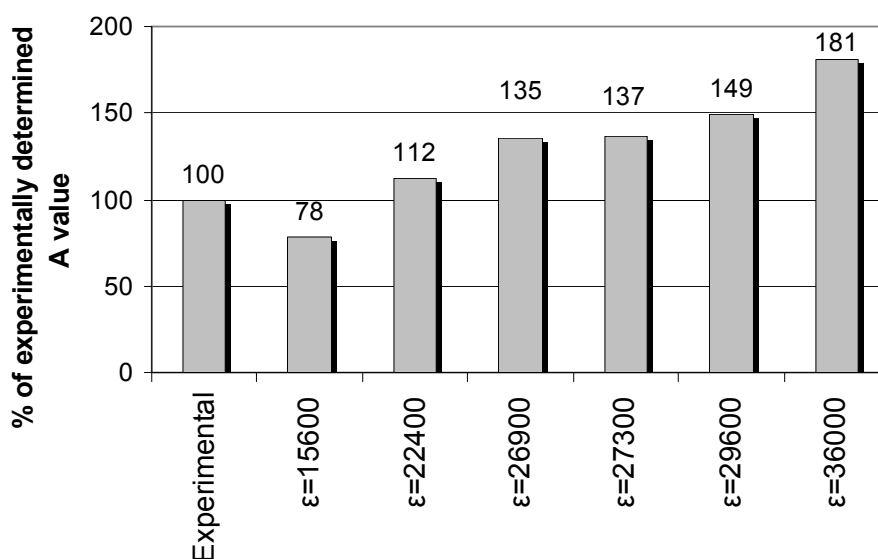


Fig. 1. The relative differences of spectrometrically determined (experimental) optical absorbance (A) value ($\lambda = 510 \text{ nm}$ in glass cuvette with optical pathlength 10 nm) and calculated A value – HPLC data using different molar absorptivity coefficients (ϵ) as an average of twelve strawberry extracts. All A values are normalized to sample fresh weight 5.0 g

Different molar absorption coefficient values in anthocyanin determination – analysis of published papers. The pH differential spectroscopic method is a basic method for determination of total anthocyanin content in fruits, where molar absorptivity coefficient and molecular weight values are used in calculations for determination of total anthocyanin content, used for the calculating the values of anthocyanin molar absorption coefficient and molecular weight values [Giusti and Wrolstad 2001]. According to the Beer-Lambert law, the concentration is inversely proportional to the ϵ value, where C is the concentration of analyte (mol l^{-1}), A is optical absorbance, ϵ is

the molar absorption coefficient ($M^{-1}cm^{-1}$) and l is the optical pathlength of sample (cm). For the calculation of concentration according the Beer-Lambert law the next equation [Parnis and Oldham 2013] is used:

$$C = A / (\epsilon \times l)$$

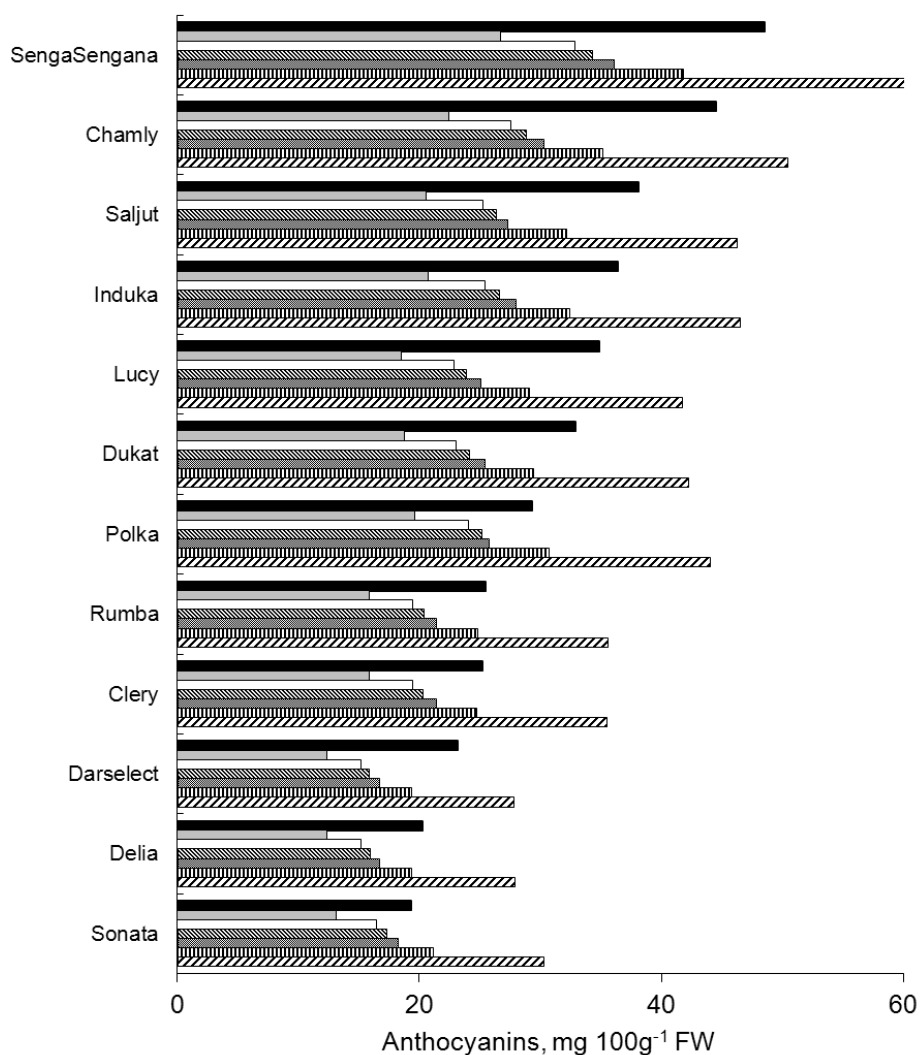


Fig. 2. Content of chromatographically determined sum of C3g and P3g (■) and calculated content of total anthocyanins from spectrometrically determined optical absorbance using ϵ values 36000 (□), 27300 (▨), 22400 (▧), 15600 (▩) $M^{-1}cm^{-1}$ for P3g and 26900 (▦), 29600 (◻) $M^{-1}cm^{-1}$ for C3g based determinations. All results are based on analysis of the same strawberry extracts

In scientific publications several different ϵ values are used for the calculation of anthocyanin content in strawberries (tab. 1). The ϵ value plays a predominant role in the anthocyanin calculations. Based on the Beer-Lambert law, the value of optical absorbance A obtained during an experiment must be divided by the value of the molar absorption coefficient to calculate the molar concentration of the extract for further steps of the calculation. The ϵ values used for strawberry analysis range from $15600 \text{ M}^{-1}\text{cm}^{-1}$ to $36000 \text{ M}^{-1}\text{cm}^{-1}$ (tab. 1). Thus there is more than a two-fold difference in the used ϵ values. For example, if the content of total anthocyanins is expressed as C3g, the ϵ values most frequently used are 26900 and 29600 (tab. 1), meaning that the difference between results is relatively small compared to the results for of P3g, where ϵ values range from 15600 to 36000.

Comparison of total anthocyanin content in twelve strawberry cultivars by using different ϵ values – experimental results. To assess the influence of different ϵ values used in calculations on the total anthocyanin content, we compared the calculated values with chromatographic data (fig. 2). The highest results were obtained from calculations with the lowest ϵ values, 15600. The content of anthocyanins from 12 strawberry cultivars with the mentioned value ranged from $27.8 \text{ mg } 100 \text{ g}^{-1}$ in 'Darselect' and 'Delia' to $60.0 \text{ mg } 100 \text{ g}^{-1}$ in 'Senga Sengana'. For 'Senga Sengana' the calculated total anthocyanin content ranged from $26.0 \text{ mg } 100 \text{ g}^{-1}$ with $\epsilon = 36000 \text{ M}^{-1}\text{cm}^{-1}$ up to $60.0 \text{ mg } 100 \text{ g}^{-1}$ with $\epsilon = 15600 \text{ M}^{-1}\text{cm}^{-1}$. For 'Darselect' the total anthocyanin content ranged from $12.0 \text{ mg } 100 \text{ g}^{-1}$ up to $27.8 \text{ mg } 100 \text{ g}^{-1}$ with $\epsilon = 36000 \text{ M}^{-1}\text{cm}^{-1}$ and $\epsilon = 15600 \text{ M}^{-1}\text{cm}^{-1}$, respectively. Thus, as a result of using different ϵ values, the total anthocyanin content for individual cultivars may differ more than 50%. So we can conclude that different ϵ values used in the calculations may influence the results of total anthocyanins in the same range even more than cultivar, cultural practices or the environmental conditions during the vegetation period.

Comparing the calculated total anthocyanin content with the chromatographic data, it appeared that most of the calculated values were lower compared to the sum of C3g and P3g (fig. 2). When using only $\epsilon = 15600 \text{ M}^{-1}\text{cm}^{-1}$, the results were typically higher than the chromatographic data; this difference ranged from 16 to 34% depending on the cultivar.

The most frequently used ϵ values for strawberry total anthocyanin content reported in literature for C3g are $\epsilon = 26900 \text{ M}^{-1}\text{cm}^{-1}$ and $\epsilon = 29600 \text{ M}^{-1}\text{cm}^{-1}$ [Kähkönen et al. 2001, Wrolstad et al. 2005, Cao et al. 2011]. Our suggestion would be that P3g with the $\epsilon = 15600 \text{ M}^{-1}\text{cm}^{-1}$ would enable more a precise estimation of total anthocyanin content in strawberries.

CONCLUSIONS

It is generally acknowledged that strawberry anthocyanin content depends on cultivar, agroecological conditions during growth, fruit maturity at harvest and postharvest practices, but the importance of methodological aspects in strawberry anthocyanin determinations is often underestimated. In several published studies the total content of strawberry anthocyanins is expressed as C3g. Present study clearly demonstrated that

the C3g content in strawberries was minimal compared to the content of P3g. Furthermore, from published works it appears that several different molar absorption coefficients are used for calculating total anthocyanins. In our research we used all the most frequently used ϵ values for strawberry total anthocyanin content reported in literature. We proved that as a result of using different ϵ values, the total anthocyanin content for individual cultivars may differ more than 50%. Thus, different ϵ values used in the calculations may influence the results of total anthocyanins even more than cultivar differences. Comparing the calculated total anthocyanin content with the chromatographic data, it appeared that the most widely used ϵ values gave underestimated values of total anthocyanins. As a conclusion of present research, in order to achieve the most realistic strawberry total anthocyanin content, the molar absorption coefficient $\epsilon = 15600 \text{ M}^{-1}\text{cm}^{-1}$ for the major strawberry anthocyanin, P3g, should be used.

ACKNOWLEDGEMENTS

Current research was supported by the Estonian Science foundation Grant 7515, the target – financed research project SF0170057s09 and Archimedes Foundation Activity 6 of the ESF DORA programme.

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OKREŚLENIE POZIOMU ANTOCYJANÓW W TRUSKAWKACH METODĄ SPEKTROSKOPII RÓŻNICOWEJ – JAK UZYSKAĆ PRAWDZIWE WYNIKI?

Streszczenie. Celem niniejszego badania była analiza słabości metody różnicowego pH dla ustalenia antocyjanów w truskawkach. Praca powstała na podstawie doświadczeń na 12 odmianach truskawek oraz na podstawie analizy opublikowanych opracowań. Użyto następujących współczynników absorpcji molowej (wartości ϵ): 26900 i 29600 $M^{-1}cm^{-1}$ dla cyjanidyno 3-glukozydu (C3g) oraz 15600, 22400, 27300 i 36000 $M^{-1}cm^{-1}$ dla 3-glukozydu pelargonidyny (P3g). Aby wykazać, w jaki sposób wyliczona całkowita wartość antocyjanów zależy od dominujących antocyjanów, porównano wyniki analizy spektroskopowej i chromatograficznej. W niniejszym badaniu wykazano, że różne wartości ϵ mogą wpływać na wyniki całkowitych antocyjanów nawet bardziej niż cechy odmiany. Najczęściej stosowane wartości ϵ 26900 $M^{-1}cm^{-1}$ oraz 29600 $M^{-1}cm^{-1}$ dały wartości zaniżone. C3g był obecny w niewielkich ilościach we wszystkich odmianach. Podsumowując, można stwierdzić, że P3g o wartości $\epsilon = 15600 M^{-1}cm^{-1}$ należy stosować, aby zapewnić najbardziej precyzyjne oszacowanie całkowitej zawartości antocyjanów w truskawkach.

Słowa kluczowe: *Fragaria × ananassa*, pelargonidyna, współczynnik absorpcji molowej

Accepted for print: 7.01.2014