Anthocyanin Extraction Method and Sample Preparation Affect Anthocyanin Yield of Strawberries

Toktam Taghavi¹, Hiral Patel¹ and Reza Rafie²

Abstract
Anthocyanins are a group of pigments with antioxidant activities that are present naturally in plants. The role of the pigment in human health and its quantitative analysis has attracted a lot of attention globally. A well-known and accurate method of anthocyanin quantification is based on spectrophotometric methods. However, these methods are subject to interference from impurities and need to be optimized for different plant matrices and extraction conditions. Two experiments were designed to study (1) the effect of plant preparation methods (eg, fresh, frozen, and freeze-dried puree) on anthocyanin yield and (2) the effect of five anthocyanin extraction methods on anthocyanin yield of freeze-dried strawberry puree. Sample preparation methods did not have any effect on anthocyanin yield. Freeze-dried samples were used for their stability (ease of use and flexibility) to compare extraction methods. The anthocyanin yield was affected by the extraction method. Two methods containing chloroform gave the highest anthocyanin yield. One method with methanol:water:HCl gave intermediate results, and the pH differential and the other method with methanol:water:HCl (80:20:1) gave the lowest anthocyanin yields. Processing time (incubation time) was lowest in the pH differential method; however, the haze produced in this method may interfere with the spectrophotometric assessment of anthocyanins.

Keywords
flavonoids, extraction method, organic solvent, methanol extract, chloroform extract, pigments

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Introduction
Strawberries are a rich source of antioxidants,¹ and among them, anthocyanins have attracted a lot of attention.²³ Research on strawberry anthocyanins has been focused on their physiological functions,⁴ and the identification of the best extraction and quantitative methods. A proper quantitative method for anthocyanin extraction has to be identified for different plant matrices.³

The majority of quantitative analytical methods need high-tech and expensive instruments (ie, HPLC) that need to be calibrated with high purity substances of analyzed compounds. The high purity anthocyanins need to be extracted and purified from plant material by very complicated processes and are, therefore, expensive.⁴ However, spectroscopic methods are quantitative analytical methods with an outstanding capability to determine anthocyanin concentration extracted by solvents. Ultrasound-assisted methods have also been used, and according to Li et al.,⁵ the solvent extraction method can preserve the antioxidant properties of blueberry anthocyanins to a greater extent than an ultrasonic-assisted method, and therefore, solvents were selected for this study.

Two main spectroscopic methods are organic solvent-based and pH differential methods, and both are subject to interference from light-absorbing impurities present in the extracts.⁶ The first method uses organic solvents (eg, methanol, ethanol, or acetone) to extract anthocyanins in an acidic environment.² Methanol is commonly used as a solvent for the extraction of anthocyanins⁸ and is most widely used when acidified with HCl.⁹ Solovchenko et al.⁶ claimed that light-absorbing impurities (ie, lipids) should be removed by chloroform for anthocyanin assessment in apple peels. They used the methanol:water-soluble fraction to extract anthocyanins. Neff and Chory¹⁰ also used chloroform: methanol in a different ratio during anthocyanin extraction from Arabidopsis leaf samples.

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The second method for anthocyanin extraction is the pH differential method. This method is based on color changes in anthocyanins due to the reversible structural changes at different pH values. Anthocyanin color is dependent on the pH of the buffer, and the molecule is pigmented at a pH of 1.0 and neutralized and colorless at a pH of 4.5 and higher. Therefore, at a pH of 1.0, the molecule strongly absorbs light between 460 and 550 nm; however, at a pH of 4.5, it is colorless. Thus, the difference in absorbance at 520 nm of the pigment is proportional to its concentration, and accurately and rapidly estimates the total anthocyanins. However, the extracted material is often hazy, and the impurities interfere with the spectroscopic process. A solution to this problem is to avoid haze by extracting anthocyanin first with organic solvents and then use the pH differential method to calculate the concentration using molar absorption coefficient (ϵ) and molecular weight data from previously published works.

However, the application of the solvent increases the process duration, and the solvent properties (eg, polarity, acidity, and concentration of impurities) affects the molar absorption coefficient and maximum wavelength of the absorbance. These reasons make the pH differential method vulnerable.

Due to the complex chemistry of the pigment, the extraction method needs to be optimized for different food matrices. Barnes et al tested a variety of homogenization procedures in blueberries using smashing in a mortar and pestle, grinding in a coffee grinder, and lyophilization in a freeze dryer. Lyophilization did not affect the total anthocyanin content, but increased the reproducibility of the results by eliminating the variability among individual blueberries and supports the need for bulk sample homogenization. However, freeze-dried samples increased the total flavonoid content of blueberries, wheatgrass, and onion, mainly due to the release of the phenolic compounds from the ruptured plant cells during freeze-drying.

Researchers have also used different extraction methods for different fruit crops with unique matrices. The two main methods, pH differential method and acidiﬁcation, have been used for strawberries. However, there is no study to compare their performance for strawberries.

This study had 2 objectives to test the following two hypotheses. The first was to test if different sample preparation and homogenization methods affect strawberry anthocyanin yield using bulk sample homogenization. Bulk homogenization eliminates the inherent sample differences. For the second objective, the freeze-dried sample preparation method was used to test the efﬁcacy of different methanol-based methods and the pH differential method. Freeze-dried samples were used for their stability (ease of use and ﬂexibility) to compare extraction methods. The two main extraction methods were chosen because they are both accurate, simple, and rapid procedures for extracting and measuring monomeric anthocyanin content with high yield.

Materials and Methods

Standard and Blank Preparation

Cyanidin-3-glucoside (cya-3-glu) was used as the internal standard, as it is the most common form of anthocyanin in nature. A stock solution of cyanidin-3-glucoside (cya-3-glu) at 4 mg L−1 in methanol: HCl (0.1%) was prepared and used as an internal standard. One mL was used in all methods to replace the sample as the internal standard. The standard was treated like samples. Distilled water (Mega pure) was used as blank in the spectrophotometer because the buffers have negligible absorbance at 530 nm.

Sample Preparation

About 300 g of strawberry fruits were pureed in a blender (Magic Bullet 600-Watt) to create a bulk representative sample (fresh puree). For objective one, 1 g and for objective two, 5 g of the puree was weighed and stored in small containers with lids and frozen quickly (−30 °C) to reduce oxidative reactions (frozen puree). The frozen samples were then freeze-dried (freeze-dried puree) at −80 °C. The 3 preparation methods tested for objective one were fresh puree, frozen puree, and freeze-dried puree using the Lindoo method, as explained below.

Anthocyanin Extraction Methods

Five anthocyanin extraction methods were compared using freeze-dried strawberry puree. Freeze-dried samples were used for their stability (eg, ease of handling, weighing, and storing) to compare extraction methods. The methods were (1) methanol:water:concentrated HCl (80:20:1) suggested by Lindoo and Caldwell; (2) the methanol:water:HCl:chloroform method suggested by Neff and Chory; (3) the methanol:water:HCl method; and (4) the methanol:HCl:chloroform method, both suggested by Solovechenko et al; and (5) pH differential method suggested by Lee et al. The name of the researchers will follow the methods to distinguish between methods with similarities.

All the methods except the pH differential methods are based on soaking the samples in an organic solvent. The pH differential method is based on color changes of monomeric anthocyanins at 2 different pH values (1.0 and 4.5).

In the first method, (Lindoo and Caldwell), 15 mL of methanol:water:concentrated HCl (80:20:1) was added to strawberry samples and incubated at 4 °C (precision refrigerated incubator, Thermo Fisher Scientiﬁc) in the dark on a shaker (Thermo Fisher Scientiﬁc) for 24 h. The homogenates were centrifuged (Heraeus Multifuge X1R Benchtop Refrigerated Centrifuge, Thermo Fisher Scientiﬁc) at 4 °C at 7000 rpm for 15 min. The supernatant was then removed, and the absorbance was measured using a spectrophotometer (Genesys 150 UV-Vis connected to Visionlite 5 software, Thermo Fisher Scientiﬁc) at 530 and 657 nm.
Neff and Chory\(^{10}\) suggested chloroform and methanol for the extraction of anthocyanins, and therefore, for the second method, 15 mL methanol, 10 mL water, 0.15 mL HCl, and 25 mL chloroform were mixed thoroughly with the samples and incubated at 4 °C for 24 h on a shaker in the dark and then centrifuged at 4 °C, 7000 rpm for 15 min. The supernatant was then removed, and the absorbance was read at 530 and 657 nm.

In the third and fourth methods, (Solovchenko et al\(^{6}\)), anthocyanins were extracted with either 15 mL of methanol: HCl (0.1% HCl) or 10 mL chloroform: methanol (2: 1 v/v; acidified with 0.1% HCl) mixture. Distilled water was added to the amount of 0.2 of the extract volume and incubated on the shaker for 24 h in the dark at 4 °C. The homogenate was centrifuged at 4 °C at 7000 rpm for 15 min, and the absorbance was read at 530 and 657 nm.

Anthocyanin concentrations in the methods mentioned above were determined by formula (1; Tonutare et al\(^{3}\)) and are given as A/g fresh fruit tissue, where TA = total anthocyanin, A = absorbance at 530 and 657 nm, V = volume of extract (ml) and M = mass of the sample (g).

\[
TA = \frac{(A_{530} - 0.3 A_{657}) \ast V}{M}
\]  

In the last method, the pH differential method (Lee et al\(^{13}\)) was used to calculate strawberry fruit anthocyanin content. The freeze-dried strawberry samples were mixed thoroughly with 20 mL of either buffer pH 1.0 (0.025 M potassium chloride) or buffer pH 4.5 (0.4 M sodium acetate buffer) and then incubated for 20 min at room temperature and centrifuged at 4 °C at 7000 rpm for 15 min. The supernatant was then removed, and the absorbance was read at 520 and 700 nm, and anthocyanin was calculated by formula (2).

\[
TA = \frac{A \ast V}{M}
\]

where \(A = (A_{530} - A_{700}, \text{nm}) \) pH 1.0 – \((A_{520} \text{, nm} - A_{700} \text{, nm})\) pH 4.5; V = volume of extract (ml) and M = mass of the sample (g).

The tested methods had different dilution factors (solvent/mass ratios; V/M). The ratio of the solvent to mass was based on the references (20/5, 30.15/5, 20/5, 15.15/5, and 25/5 for methods 1-5, respectively) and was not changed in this experiment (Table 1). The ratio was integrated into the formulas to eliminate the differences in dilution factors to compare the methods. There were four replicates in all the experiments, and the experiments were repeated twice. The average data were used to calculate anthocyanin concentration. The first replicate was used to create the wavelength scanning spectrum, and the dilution factor was not considered in the spectrum of absorbance.

Results

In objective one, three sample preparation methods (eg, fresh, frozen, and freeze-dried) were compared using Lindoo and Caldwell’s\(^{7}\) method (methanol:water:HCl). Sample preparation methods did not have any effect on anthocyanin yield. The average anthocyanin concentration changed from 15.6 to 15.9 \((A/gFW)\) in fresh and frozen sample types, respectively (Figure 1.1 and 1.2). The absorbance spectra of all sample preparations were similar for all bandwidths measured (Figure 1.1–1.3), except at 220 to 280 nm, where fresh samples had lower absorbance (Figure 1.1).

In the second objective, five anthocyanin extraction methods were tested using freeze-dried strawberry fruits. Freeze-dried samples were used for their stability (ease of use) and flexibility (storing capability) compared to either fresh or frozen samples.\(^{16,17}\) All methods used freeze-dried strawberry samples from a homogenized bulk sample. Therefore, differences in anthocyanin concentration are related to the ability of the specific method to soak and extract anthocyanin, not the sample differences. Different methods had different dilution factors (solvent/mass ratio) based on what was suggested in the references. The dilution factors were integrated into the formula to eliminate the differences (Table 1).

Anthocyanins are highly soluble in water. The polyphenolic structure of anthocyanins adds a hydrophobic characteristic to them, and makes them soluble in organic solvents, such as methanol and ethanol. A few anthocyanins, such as pelargonidin, are also soluble in chloroform. The Solovchenko et al\(^{6}\) (with chloroform) and Neff and Chory\(^{10}\) methods both extracted the highest amount of anthocyanins (Table 1), and both used chloroform in their buffers in addition to methanol. The addition of chloroform may have been responsible for the increase in anthocyanin yield, due to increased solubility of specific anthocyanins, such as pelargonidin, compared to other methods that lack chloroform. The addition of both solvents (methanol and chloroform) will increase the likelihood of dissolving a wider range of anthocyanins compared to either solvent alone.\(^{23}\)

The total anthocyanin content in the method of Solovchenko et al\(^{6}\) (methanol:water) was the third-highest among the extraction methods, followed by those of Lee et al\(^{13}\) (pH differential) and Lindoo and Caldwell\(^{7}\) (methanol: water:HCl). The last two extracted almost the same amount of anthocyanins (Table 1).

Cya-3-glu, which was used as an internal standard for anthocyanins, has a maximum absorbance at 530 nm (Figure 2.1). Beside pH differential methods, all other methods are based on organic solvents and have a peak for organic-solvent soluble compounds around 300 nm, which are probably flavonoids and flavor compounds found in high quantities in strawberries.\(^{24-26}\)

When the absorbance spectrum is considered, the Lindoo and Caldwell and Neff and Chory\(^{10}\) methods (Figure 2.2 and 2.3) had a very similar height for the absorbance peak, with
the latter having the maximum absorbance ($\lambda_{\text{max}}$) at 512 nm. The Solovchenko et al$^6$ methanol and chloroform:methanol methods (Figure 2.5) had the highest absorbance ($\lambda_{\text{max}}$) at 510 and 525 nm, respectively, and the pH differential method (Lee et al$^{13}$) the lowest at 500 nm (Figure 2.6). This method is based on the differences between absorption of the colorless structure of anthocyanins at pH 4.5 and the highest absorption for pigmented anthocyanins at pH 1.0. Therefore, the difference reflects monomeric anthocyanins with a peak around 500 nm. The pH differential spectra also lack the peak around 300 nm that exists in other methods.

In the pH differential method, the extract was a little hazy, even after it was centrifuged at 7000 rpm twice. Measuring the absorbance at 700 nm corrects for the haze.$^{13}$ Although the pH differential method gave the lowest anthocyanin concentration, the incubation period is the lowest (20 min) among the other methods (24 h) and is a quick method for the extraction of anthocyanins.

The Solovchenko et al$^6$ chloroform:methanol:HCl method (Figure 2.2 and 2.5) had the highest absorbance ($\lambda_{\text{max}}$) peak at 530 nm. However, this could be due to the lower volume of extraction buffer. Fifteen mL of methanol:water:HCl (80:20:1) was used in the first case and 20 mL chloroform:methanol:HCl (15:5:0.2) in the second. The more concentrated buffer may fall outside the linear range of the spectrum

In the third and fourth methods (adapted from Solovchenko et al$^6$), the addition of chloroform was compared with methanol in removing light-absorbing impurities (ie, chlorophylls, carotenoids, and lipids). The height of the absorption peak for the chloroform-methanol method is higher than the methanol method (Figure 2.2 and 2.5). However, this could be due to the lower volume of extraction buffer. Fifteen mL of methanol:water:HCl (80:20:1) was used in the first case and 20 mL chloroform:methanol:HCl (15:5:0.2) in the second. The more concentrated buffer may fall outside the linear range of the spectrum

### Table 1. Anthocyanin Contents of Freeze-Dried Strawberries Measured by Organic Solvents and pH Differential Methods.

| Method tested       | Anthocyanin concentration A/ gFW | SD* | cya-3-glu* conc. | Formula                        |
|---------------------|----------------------------------|-----|------------------|--------------------------------|
| Lindoo and Caldwell$^7$ Methanol:water:HCl | 8.61 | 0.24 | 1.33 | ($A_{530} - 0.3A_{657} \times 20)/5$ |
| Neff and Chory$^{10}$ Chloroform:methanol:HCl | 14.64 | 0.35 | 2.22 | ($A_{530} - 0.3A_{657} \times 30.15)/5$ |
| Solovchenko et al$^6$ Methanol:water:HCl | 11.52 | 0.16 | 19.25 | ($A_{530} - 0.3A_{657} \times 15.15)/5$ |
| Solovchenko et al$^6$ Chloroform:methanol:HCl | 14.46 | 0.42 | 1.09 | ($A_{520} - A_{700}$ pH 1.0 - ($A_{520} - A_{700}$ pH 4.5 $\times 25/5$) |
| Lee et al$^{13}$ pH differential | 9.30 | 0.09 | 1.09 | ($A_{520} - A_{700}$ pH 1.0 - ($A_{520} - A_{700}$ pH 4.5 $\times 25/5$) |

Notes: *Abbreviations A = absorbance; cya-3-glu conc. = cyanidin 3-glucoside concentration; SD = standard deviation, which is used as the internal standard. The dilution factors are included in each formula to account for differences in solvent to mass ratio for each method.

**Figure 1.** The absorption spectra of strawberry puree in the different forms obtained by the Lindoo and Caldwell method.$^7$ (1) Fresh puree, (2) frozen puree, and (3) freeze-dried puree. All spectra overlap at all wavelengths, except between 220 and 280 nm, which is lower in fresh puree spectrum.
and may not show the accurate concentration of the anthocyanins in the methanol method. Also, chloroform is able to dissolve certain anthocyanins, such as pelargonidin, and its addition to the buffer may increase total anthocyanin content.25

Taghavi et al27 have shown that the methanol:water method was better than the chloroform:methanol method for frozen strawberry samples, which is the opposite of what has been shown here, because water is a better solvent than chloroform for anthocyanins. With freeze-dried samples, there is no water in either the extraction buffer or the samples, the solubility of anthocyanins was limited to methanol, but a few anthocyanins dissolve in chloroform (ie pelargonidin), and therefore, the anthocyanin yield was reduced.

Discussion

In the first objective, sample preparation did not affect anthocyanin yield. Barnes et al16 also expressed that there is no significant difference among a variety of homogenization procedures in blueberries (including lyophilization in a freeze dryer), but an increased reproducibility of the results. In other experiments with blueberries,17 wheaggrass,18 and onion,19 the anthocyanin yield increased in freeze-dried samples. The result emphasizes the importance of method optimization for different food matrixes and extraction methods, as explained by Silva et al.9

Among different factors that affect anthocyanin yield, sample type, and temperature, were considered constant in this experiment. All methods used a 24 h incubation time uniformly, except the pH differential method, which had a 20 min incubation time. The solvent was either methanol or chloroform:methanol, and the solvent/mass ratio was different in the various extraction methods. Samples were dried in freeze dryer. However, water was added to all extraction buffers, except for the chloroform:methanol method (Solovchenko et al).6 The addition of chloroform may affect the anthocyanin yield and determines what extraction method is superior in the presence or lack of it. The solvent/mass ratio (v/m volume/mass) was integrated into the formula to eliminate the solvent/mass ratio differences. The solvent/mass ratio of the previous report was not changed, due to the effect that it may have on the solubility of the anthocyanins and the yield.

The higher amount of anthocyanins in freeze-dried strawberries extracted by Neff and Chory10 and Solovchenko et al6 (chloroform:methanol methods) could be due to the presence of chloroform in their buffers. Solovchenko et al6 claimed that chloroform reduced the light-absorbing impurities, although such an effect was not seen in frozen strawberry.

Figure 2. The absorption spectrum of (1) cyanidin-3-glucoside standard solution used as internal control and measured by Lindoo and Caldwell (1978); freeze-dried strawberry anthocyanin measured by (2) Lindoo and Caldwell (1978), methanol:water:HCl method; (3) Neff and Chory (1998), chloroform:methanol:water:HCl; (4) Solovchenko et al (2001), methanol:water:HCl; (5) Solovchenko et al (2001), chloroform:methanol:HCl; and (6) Lee et al (2005), pH differential method (the differential absorbance of pH 1.0 and pH 4.5 was included in the graph).
The fact that the lack of water in freeze-dried strawberry samples could make a difference is probable. Another reason for higher anthocyanin yield in the presence of chloroform is the solubility of pelargonidin, for which strawberry is a rich source. Therefore, a side-by-side comparison of these two sample types against the different extraction methods with the same dilution factor seems inevitable.

All methods, except the pH differential method (Lee et al13), produced clear supernatants, regardless of whether chloroform was used. Solovchenko et al6 claimed that cuticular lipids affect the turbidity of the samples, but this is not valid for freeze-dried strawberries. The extract was clear, and no turbidity was observed. However, when the samples were dried, and the buffer lacked water, chloroform increased the anthocyanin yield, as was seen in the chloroform:methanol method of Solovchenko et al.6 The highest absorbance spectrum in this method could be due to the higher solvent/mass ratio compared to the Neff and Chory10 method. The more concentrated buffer may fall outside the linear range of the spectrum and does not show the accurate concentration of the anthocyanins in the methanol method.

The Neff and Chory10 method was able to improve the anthocyanin yield compared to the methanol method. The result is opposite to what the authors experienced using frozen strawberry samples.27 The extraction buffer of the Neff and Chory10 method had a higher chloroform to methanol ratio than the chloroform:methanol method (Solovchenko et al6). Neff and Chory10 used it for Arabidopsis leaves, which have a higher chlorophyll content. The chloroform is needed to remove the high chlorophyll content of the leaves, but should not be necessary for strawberry fruits.

Taghavi et al27 have shown that the methanol method (Lindoo and Caldwell7) was the most efficient for anthocyanin extraction of frozen strawberry fruits. However, in this experiment with freeze-dried strawberry fruits, this trend has not been seen, and this method gave the lowest anthocyanin content of all the methods used. The only explanation is that sample type (mainly lack or presence of water or ruptured cells) interacts with the anthocyanin extraction method. A specific method may be ideal for frozen strawberry samples, but not the freeze-dried samples. The results suggest that when water is removed from samples and buffers, the chloroform:methanol method (Solovchenko et al6) extracts the highest amount of anthocyanins.

The low anthocyanin content of the pH differential method was also seen in frozen strawberry samples.27 The other downside of this method is the hazy anthocyanin solution produced that could not be completely clarified by either increasing the centrifugation time or even filtering. This method is extensively used by food scientists and horticulturists to assess the quality of fresh and processed fruits and vegetables. In our opinion, when all these methods are compared, the only benefit of this method is its lower incubation time (20 min) compared to the organic solvent methods (24 h).

The extract absorbance peak at 280 to 320 nm is most probably related to flavonoids and aromatic compounds such as quinones and coumarins, which are abundant in strawberries, as explained by Taniguchi and Lindsey25 and Howard et al.26

Conclusion
A large group of factors such as sample type, temperature, pH, solvent type, and the ratio of its components affect anthocyanin yield. This experiment has shown that the sample type did not affect anthocyanin yield when methanol: water was used as a solvent. Also, there may be an interaction between the sample type and the solvent. When freeze-dried samples were used, the chloroform:methanol method was the best to extract anthocyanin (Solovchenko et al6). Lack of water in this method separates it from the other methods, such as methanol:water (Lindoo and Caldwell7) and chloroform:methanol:water (Neff and Chory10). The pH differential method was not efficient in increasing anthocyanin yield from freeze-dried strawberries. It is recommended to test the sample type and solvent type in a two-factor design to study if there is any interaction between these two parameters. Also, a uniform solvent volume/mass is preferred to compare the data absorption spectrum of the different methods.

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Ethical approval is not applicable for this article.

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Statement of Human and Animal Rights
This article does not contain any studies with human or animal subjects.
Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Trial Registration

Not applicable, because this article does not contain any clinical trials.

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