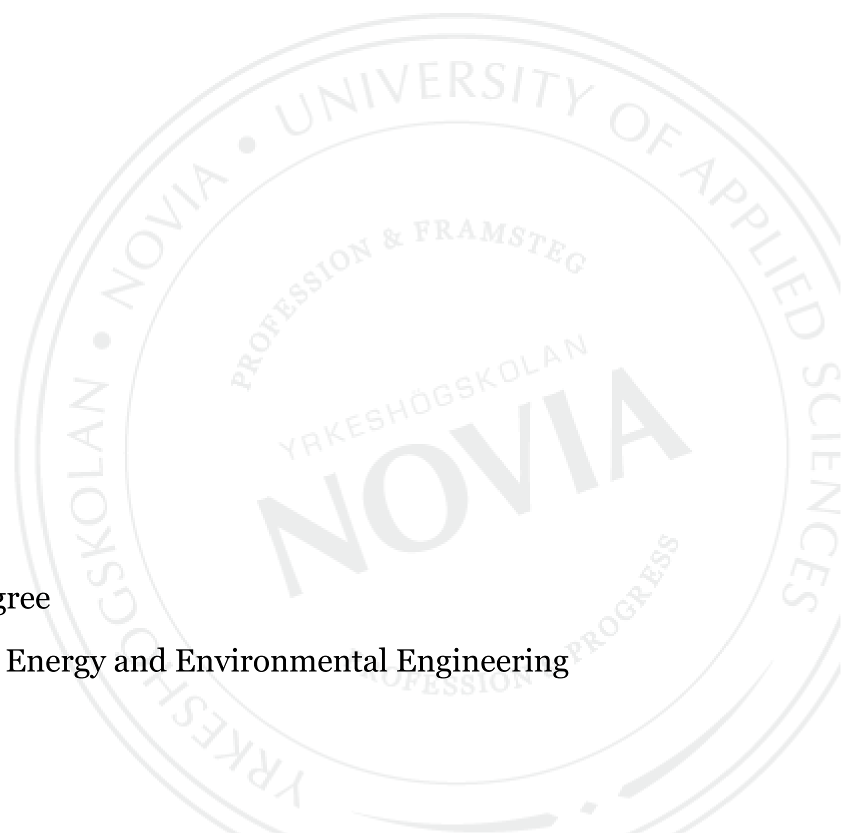
Development of Process for Analyzing Anthocyanin Contents in Bilberries

Sami Lieskoski



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Author: Sami Lieskoski

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Supervisors: Tom Lillhonga

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Abstract

This thesis was conducted as a part of the Industry Nordic project, which aims at increasing the use of non-wood forest products (NWFP) and especially developing the Nordic berry business. The company Marja Bothnia Berries Oy Ltd. obtained a new UV-Vis spectrophotometer for analysis of anthocyanin contents in the bilberries it sells, and wished to have a method developed for this. A method for analyzing anthocyanin contents was developed based on previous literature and on methods used by the customers of the company. The results obtained with the newly obtained UV-Vis spectrophotometer were compared to analysis results from another UV-Vis spectrophotometer and from HPLC analysis conducted by Centria University of Applied Sciences.

This thesis is divided into a theoretical part and a practical part. In the theoretical part, a literature review on UV-Vis spectrophotometry and HPLC was done to better understand these methods for analyzing anthocyanin contents. In the practical part the process and the results are described and discussed. The results show lower anthocyanin contents in bilberries compared to previous literature for analysis conducted using both UV-Vis spectrophotometry and HPLC. Anthocyanin contents measured using UV-Vis spectrophotometry were higher compared to results using HPLC.

Language: English Key words: Anthocyanin, UV-Vis, HPLC, Bilberry, Sampling

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1 Introduction

For sustainable development to occur, resources have to be used in a sustainable manner. Sustainable development is defined as “meeting the needs of the present without compromising the ability of future generations to meet their own needs” [1]. All over the world forests contribute to the economic development and the development of rural areas through timber but also through Non-Wood Forest Products (NWFPs). According to UN’s Food and Agricultural Organization [2], NWFP are “goods of biological origin other than wood derived from forests, other wooded land and trees outside forests”. The importance of forests for economic and rural development is rather significant in Finland where 23 million hectares of forest cover 76% of the area of the country, with forest industry products contributing to 4% of Finnish Gross Domestic Product and 20% of Finland’s exports [3].

NWFPs combined with nature tourism contribute to 30% of the value of forest products in Finland, with the remaining 70% coming from wood. Of this 30% however, nature tourism has the largest share with 88%, while wild berries purchased by companies contribute only with 1%. It is estimated that 95% of berries in the Finnish forests go unharvested [4]. This means that there is untapped potential to expand the market of berries. [3]

This thesis was conducted as a part of the Industry Nordic project, which is a part of the Botnia-Atlantica programme [5]. The programme is a part of European Territorial Cooperation (Interreg), which aims at developing regions through cross-border cooperation. The four priorities of the project are environment, transport, business and innovation [6]. The Botnia-Atlantica programme involves cooperation between regions in Finland, Sweden and Norway. These regions have similar challenges: low population density, long distances and they communicate mainly in the south-north direction, within their countries. The programme’s aim is to improve communications in the east-west direction, strengthen business sectors, increase capacity for innovation and develop natural and cultural heritages. [7]

1.1 The Industry Nordic project

The Industry Nordic project aims at increasing the use of non-wood forest products (NWFP) and especially developing the Nordic berry business. NWFPs include berries, herbs, mushrooms and special NWFPs like sap, pine bark, resin and lichen. These are versatile and pure natural resources acquired from the forest, but they are not yet used to their full potential in Finland and in other Nordic countries. Demographic changes, including a growing and aging population, a consumer base with an increasing level of environmental and health awareness are boosting the demand for NWFPs. Demand is growing for health products such as food and dietary supplements manufactured from Nordic pure natural biomass. It is possible to extract extremely valuable ingredients from these NWFPs which can be used e.g. in the cosmetics or drug industry. This provides new possibilities for entrepreneurship. The estimated size of the NWFP business in Europe is 2-4 billion euros annually. [5]

The production of super foods and refining them from NWFPs requires several steps along the supply chain, from the retrieval of the raw materials to the development of the final product. Currently, NWFP products like bilberries are exported abroad, where they are refined into higher-value products like food supplements. These higher-value products are then imported back to the Nordic markets. Through developing the use of technology in different parts of NWFPs supply chain and by developing new products, from laboratory scale to a pilot run to an industrial scale, the industry’s business can be developed so that more of this high-grade manufacturing takes place locally in the Nordic countries. [5]

1.2 Purpose

The practical work for this thesis was conducted for the company Marja Bothnia Berries Oy Ltd. The company is located in Korsholm, Finland, a neighboring municipality to the city of Vasa. Marja Bothnia Berries coordinates the collection, cleaning, cooling and storage, and the delivery of berries to customers around the world. The company trains their pickers to pick the berries at the ideal time for the content of nutrition and vitamins of the berries. The berries are collected in Finnish forests and are quickly transported using the company's transport system, which is optimized for the preservation of the berries nutrients and vitamins. [8]

Marja Bothnia Berries Oy Ltd and their customers are interested in knowing the amount of anthocyanin in the bilberries sold by the company. Bilberries are divided into different classes: those of low grade might only be good for making juice, while higher class berries are sold to customers who seek high anthocyanin contents. However, Marja Bothnia Berries Oy Ltd and their customers use different methods for analyzing anthocyanin contents in bilberries which can show different results between the methods. Up until now the level of anthocyanin contained by the bilberries has been measured by taking only 5 samples per 100 tons of berries, sent for external analysis in a laboratory using High Performance Liquid Chromatography (HPLC). As there are so few samples taken for a large amount of berries, the reliability and the representability of the results are questionable. Insufficient sampling routines can have a huge impact on the results.

When Marja Bothnia Berries Oy Ltd delivered a load of berries to one customer, the containers with the berries were analyzed and it was found out that the berries found in some containers met the promised contents, but some did not. By taking more samples on their own with a proven method for analysis, the company can ensure that they meet the requirements. Marja Bothnia Berries Oy Ltd can share their method with the customer and then they can communicate about any possible differences in their methods for analysis.

In order to improve sampling and analysis, a new piece of equipment, the Agilent Cary 60 UV-Vis spectrophotometer was obtained for testing. The device gives the opportunity to make several measurements at a lower cost compared to sending samples to an external lab for HPLC analysis. This thesis was conducted in cooperation with Centria University of Applied Sciences, which measured anthocyanin contents in bilberries with High

Performance Liquid Chromatography (HPLC) and with their own UV-Vis spectrophotometer. The goal of this thesis is to find out and test a method to measure anthocyanin contents in bilberries samples accurately and improve the sampling procedure.

1.3 Methodology

The scope of this thesis is separated into two parts. The first part is a theoretical background for which a literature review of UV-Vis spectrophotometry and HPLC has been conducted. Various sources have been consulted in order to get an understanding of these technologies for analyzing anthocyanin contents. The second part consists of actual results from using the Agilent Cary 60 UV-Vis spectrophotometer to measure the anthocyanin content of bilberry samples. Also, different options for sampling procedures are presented, as sampling has a major impact on the anthocyanin content analysis results.

2 Theory

2.1 Bilberries and Non-Wood Forest Products

For sustainable development, harvesting NWFPs from forests can be considered a good alternative for sustainable development to preserve the ecology and biodiversity in forests. Harvesting NWFPs does not destroy habitats unlike e.g. logging and mining. A review of a collection of studies from 2000-2010 on NWFP harvesting in developing countries in the subtropical and tropical areas in Africa, Asia and Latin America showed that the harvesting of NWFPs is overall both economically and environmentally sustainable. A substantial majority of the studies showed that the ecological purenesses of the ecosystems are not under threat by the harvesting of NWFPs at the rates at which they were being harvested at. Furthermore, the harvesting of NWFPs does not pose a threat to the ability of populations and individuals to regenerate. [9]

Some studies do however, show that intensive harvesting can have negative effects on the ecology. The reason for this difference in results could be that there is a wide variety of NWFPs with many differences among them. Some characteristics that are different among NWFPs include the parts that are harvested, the life form of species and life history. The manners in which NWFPs are harvested and end up on the market are also important factors to take into account. It can be more difficult to reach sustainability when the parts of plants that are harvested are crucial for the survival of the plants in the long run, for example stems, roots, or entire plants. Sustainability is easier to achieve when less critical parts of plants are harvested, such as fruits, flowers or seeds. It is the fruit or berries which are harvested from bilberries, so bilberries as a NWFP belong to this more sustainable category. [10]

Achieving sustainability becomes more difficult the more frequently and intensely that products are harvested, and the more the products are sold on the international market instead of locally. The impacts of harvesting NWFPs are usually evaluated over short periods of time, so a system that seems sustainable over a short period might not be sustainable in the long term. When assessing the sustainability of harvesting, the period of time under which one observes the effects on the ecology and economics should be long enough. [10]

Berries are a natural and renewable resource, if harvested in a sustainable manner, which means collecting the berries without damaging the berry plants ability to produce berries in the future. For the picking of berries to be sustainable, it should not harm other life in the forest such as trees, plants and animals. So when berries are harvested in a sustainable manner, it contributes to economic development and well-being in both rural and urban areas. Bilberries (Vaccinium myrtillus L.) are commonly referred to as “blueberries”, but they are actually a different species from blueberries (Vaccinium corymbosum L.) Bilberries are wild berry plants found growing in Finnish forests, while blueberries are larger bushes which are cultivated and do not grow in the wild. Blueberries have lower anthocyanin contents than bilberries. In Finland, about 40 000-50 000 tons of bilberries and lingonberries are harvested each year, with a total economic value of €75-115 million. The berries value after harvesting are about 1500€/ton. [11] [12]

2.2 Anthocyanins and Anthocyanidins

Phenolics are compounds which have at least one aromatic ring and at least one hydroxyl group. The phenolics found in plants include tannins, phenolic acids, flavonoids and the rarer lignans and stilbenes. Bilberries contain large amounts of anthocyanins, which are a big and significant class of flavonoid compounds. Flavonoids are biological pigments without nitrogen [13]. Anthocyanins are found in nature, they are the source of the red, purple, pink orange and blue colors of various fruits, vegetables and flowers. Anthcoyanins may also be found in leaves, seeds, roots and other parts in plants. [14] [15]

The amount of anthocyanins in bilberries depend on several factors, including the region in which they are growing, the conditions of the soil in which the bilberry plants are growing, when the bilberries are harvested and how ripe they are at that moment and how they are stored after harvesting [16]. The red and blue color of bilberries is mostly due to the anthocyanins found in them. Anthocyanins are used as a natural pigment in processed foods. [17]

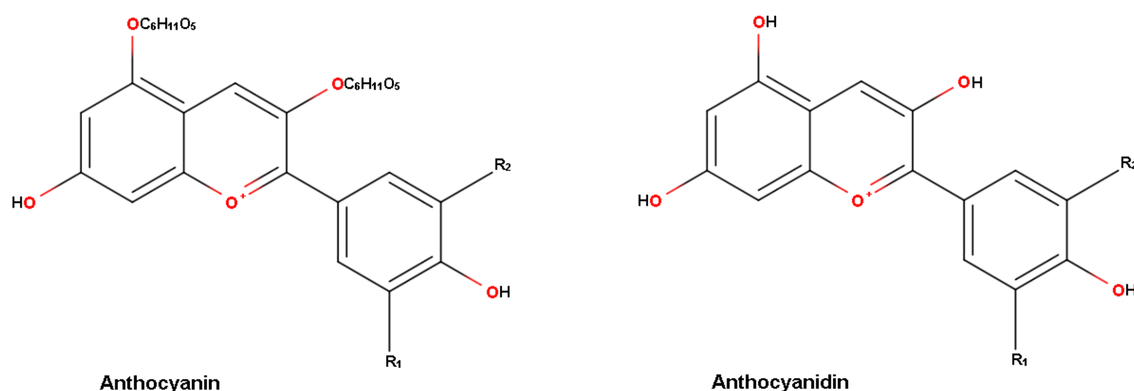

Figure 1. Structure of anthocyanins and anthocyanidins

Anthocyanins are polyphenols, which means that in their chemical structure they contain several phenols. Phenols are aromatic organic compounds consisting of a phenyl group (-C_6H_5) bonded to a hydroxyl group (-OH). The basic structure of anthocyanins are the anthocyanidins. Anthocyanidins are called anthocyanins when they are in their glycoside form. Glycosides are substances occurring in nature in which one part, a carbohydrate, is

attached to a hydroxy compound [18]. So anthocyanidins are like anthocyanins without the sugar molecules attached, as seen in Figure 1. The anthocyanins found in bilberries are glycosides which contain the sugars glucose, arabinose or galactose as the moiety connected to the anthocyanidin. In bilberries, several mixes of anthocyanidins can be found: delphinidin, petunidin, cyanidin, peonidin, pelargonidin and malvidin glycosides. The differences among the anthocyanidins occur in the functional groups R1 and R2. A table showing the different functional groups of the anthocyanidins can be found in Appendix 1 [19]. [20] [17]

Significant characteristics for anthocyanins as pigments are the intensity of the color, its hue and stability. These characteristics are dependent on pH, temperature, light, oxygen, structure and other things. The structures of anthocyanins are subject to change when pH changes, which has a big impact on color. Anthocyanins are challenging to analyze as they are rather unstable. A strong acidic medium is needed to analyze anthocyanins. At a pH of around less than 3 anthocyanins stabilise as red flavylium ions, which is the basic structure anthocyanins are derived from. Anthocyanins are soluble in water and can be extracted with polar, organic compounds such as ethanol and methanol. Anthocyanins dissolved in acidified methanol or acidified ethanol display a characteristic maximum in the visible region of the electromagnetic spectrum (around the range of 510-560 nm). If the extraction of anthocyanins is attempted with only water, the yield of anthocyanin compounds are lower than if organic solvents and acids are used. [21] [16] [22]

Anthocyanins have numerous positive health effects, providing protection against many human diseases. There is a long history of using plants rich in anthocyanin for treating various symptoms and diseases, like improving eyesight. Anthocyanins naturally have an electron deficiency, making them especially reactive against oxygen radicals, in other words they are antioxidants. Studies have shown that it is possible for anthocyanins to have more antioxidative activity than β-carotene, vitamin E and ascorbic acid. Anthocyanins have also been linked to protection from heart attacks, especially from wine and grape juice but also other sources. The reason for this is that these products can decrease inflammation, suppress the formation of platelets which cause blood clots and boost the strength and permeability of capillaries. Studies have also shown that anthocyanins can help in the prevention of diabetes and obesity. Anthocyanin pigments found in purple corn were shown to hinder increases in fat and body weight in studies. [22]

2.3 UV-VIS Spectroscophotometry

Spectrophotometry observes absorption and emission of light through a substance. Light is like waves, and the waves are characterized by their wavelength (λ) and frequency (v) which are related to the speed of light (c), $\lambda v = c$. Molecules in the substance will absorb some of the light transmitted through it. Based on the amount of light transmitted/absorbed it is possible to retrieve information about the composition and structure of the substance being measured. As seen in Figure 2, the electromagnetic spectrum spans from gamma rays (highest energy, smallest wavelength) to radio waves (lowest energy, highest wavelength). In most spectrophotometric applications, mainly wavelengths in the ultraviolet, visible or the infrared area are used. [23]

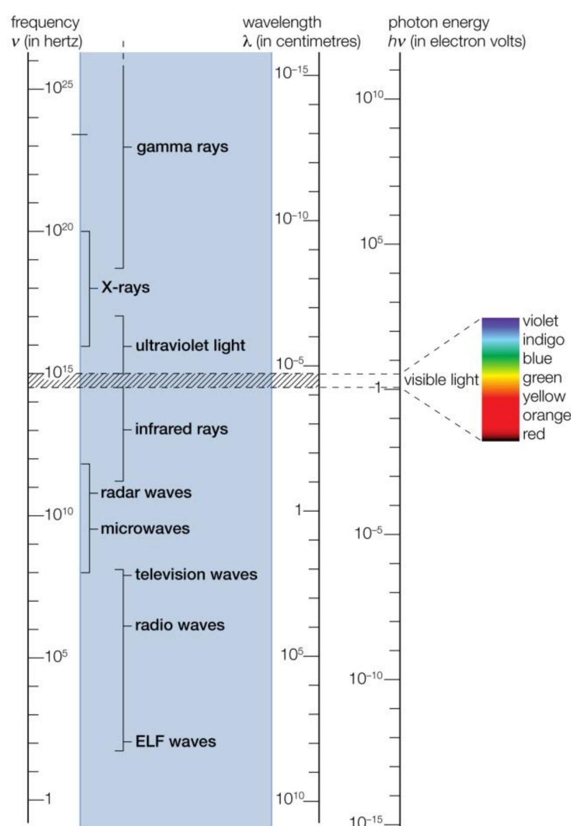

Figure 2. The electromagnetic spectrum [24]

The relationship between different elements and electromagnetic radiation is described by Lambert-Beers law, which expresses proportionality between absorbed radiation and concentration. This physical law is the base which makes spectrophotometry one of the most important disciplines in chemical analysis. UV-Vis spectrophotometry works in the ultraviolet range (UV) 180-400 nm and in the visible range (VISual) 400-800 nm. Many devices can also measure into the near infrared range (NIR), so in reality the technology covers a wider wavelength range of 180-1100 nm. The Agilent Cary 60 UV-Vis can measure in the range of 190-1100 nm [25]. [23]

When a sample that is under measurement absorbs light, the intensity of light passing through the sample decreases. The intensity is called irradiance, which is the energy per unit area of the beam of light. A spectrophotometer works by first letting light pass through a monochromator, which allows one certain wavelength of light to pass through. Monochromatic means "one color", so light of a certain wavelength is called monochromatic light. [26]

The transmittance of light through a sample can be defined with the equation

$$T = \frac{P}{P_0}$$

where T is transmittance, P is the irradiance after the sample and P_0 is the monochromatic light hitting the sample. Due to the nature of this equation, T's range is between 0 and 1 or 0 to 100% when expressed as a percentage.

The absorbance of light can be derived from the transmittance with the following calculation

$$A = \log\left(\frac{P_0}{P}\right) = -\log T$$

If no light is absorbed, then $P = P_0$ and $A = 0$. When 90% of light is absorbed, 10% is transmitted giving $P = 0.10 * P_0$. For this ratio, $A = 1$ when 10% of the light is transmitted, and $A = 2$ when 1% of light is transmitted.

Lambert-Beer's law states that absorbance is directly proportional to the concentration of the substances which absorb light in a sample

$$A = \epsilon bc$$

where c is concentration, b is the pathlength, usually expressed in centimeters, and ε is the molar absorptivity with the units $M^{-1}cm^{-1}$, enabling the product of εbc to be dimensionless. Molar absorptivity tells the amount of light that is absorbed at a specific wavelength by a substance. [26]

Ideally, the absorbance values should be in an intermediate range around A = 0.4-0.9. If the absorbance is too high, meaning that too little light is going through, the intensity of light is difficult to measure and Lambert-Beer's law might not apply. If the absorbance is very low meaning too much light is going through, it is difficult to tell the difference between the sample and the reference. To get the best results, the samples can be diluted so that they fall in this range. [26]

2.4 High Performance Liquid Chromatography (HPLC)

Chromatography are methods to separate and analyze chemical compounds through separating the different components from each other, creating small zones in the mobile phase (liquid or gas) which transition into a stationary phase. The separated components can be isolated, called preparative chromatography or they can be identified and quantified. If the mobile phase contains substances which prefer the stationary phase, these substances will be enriched in the stationary phase until pure solvent passes. The solvent will slowly extract the substances and move them forward in the stationary phase, resulting in a series of extractions. The transport of a substance through the chromatogram will be slower the shorter a compound stays in the mobile phase, or faster the more it is attracted by the mobile phase. Due to this, compounds with different structure pass through the chromatographic system at different speeds. [23]

Liquid chromatography comprises a range of separation techniques which all start with the application of a test mixture on a stationary phase. The separation of the components of samples is done through elution with a liquid; hence HPLC is a form of liquid chromatography. The "high performance" in HPLC is achieved by using a very small and homogenous grain size for the stationary phase, around 3-10 µm. HPLC can be either reversed phase or normal phase. In reversed phase HPLC, the mobile phase is more polar

than the stationary phase, and vice versa for the normal phase. In most applications, reversed phase HPLC is used. [23]

The high pressure pump in the HPLC system is used to make the mobile phase flow at a specified flowrate, most often measured in the scale of milliliters per minute. The sample to be analyzed is injected by the means of an injector and the sample is carried away by the mobile phase into the HPLC column. In the HPLC column, the stationary phase is found. The stationary phase is chromatographic packing material that causes the separation of the different components in the sample. After the separated columns of the sample leave the HPLC column, a detector detects the different compound bands. The mobile phase can be discarded of as waste, or the separated compound bands can be collected to study them further. This type of HPLC is called preparative HPLC. [23]

2.5 Comparison of UV-VIS Spectrophotometry and HPLC

The main difference between HPLC and UV-VIS Spectrophotometry when it comes to analyzing bilberry anthocyanin contents is that HPLC allows for measuring all the different anthocyaninidins individually, while UV-VIS measurements provide an estimate of the total anthocyanin contents. Measuring the individual anthocyanidins requires several different standards as there are many of them. HPLC analysis takes much longer than measuring with UV-Vis spectrophotometry. A UV-Vis spectrophotometer measures almost instantaneously; the lamp just has to be turned on and then the light passing through the sample is read and an absorbance value is obtained. All that is required for measuring is to position the samples for measurement. [27]

In HPLC analysis it takes time for the sample to pass through the column and separate into the different components. A gradient elution HPLC method, in which the composition of the mobile phase is changed during the analysis, was used for analyzing anthocyanin contents in bilberry juices by [20]. The gradient had 10 different compositions of the mobile phase over a time span of 75 minutes, which means that there is 75 minutes of waiting between each injection. In HPLC analysis conducted by [21], the last anthocyanin peak appeared after 93 minutes.

HPLC analysis is more costly than UV-Vis spectrophotometry, the HPLC device itself is more expensive than a UV-Vis spectrophotometer. When analyzing anthocyanin contents of bilberries using HPLC, the contents of six different anthocyanidins are measured. This requires six different expensive anthocyanidin standards to be purchased. HPLC is also more sensitive and requires more maintenance as columns can get clogged, for example. Using HPLC is more challenging than the UV-Vis spectrophotometer, thus a skilled chemist may be required to use the device properly. Analyzing anthocyanin contents with a UV-Vis spectrophotometer is much simpler and the device is more robust.

2.6 Theory of Sampling

The Theory of Sampling (TOS) gives guidelines on all the errors that occur when heterogeneous materials are sampled and how to evaluate the errors, minimize and/or remove them. Bilberries are a heterogeneous material as they are of different size with different properties. Having a good plan for taking samples is an important part in the experimental design of a process. [28]

Sampling is the base for determining the quality of raw materials and products and to identify characteristics like anthocyanin contents. Sample taking is also necessary to determine the homogeneity of a material. There are basically three steps for taking samples: sample taking, sample preparation and analysis. Correctly performing the sample taking is the deciding factor for properly determining quality. Samples are taken from a lot, a lot is defined as all the material that is subject to sampling [28].The value and characteristics of a whole lot is to be determined by a small analyzed sample, weighing as little as a few grams when the whole lot could weigh several tons. Therefore, it is extremely important that the sample taken out fully corresponds to the characteristics of the whole lot. [29]

It is not possible to deduct from a specific sample whether it is a representative sample or not. A “correct sample” i.e. a truly representative sample can only be obtained from a sampling process which meets qualifications. “Incorrect samples”, also called specimens, are all other samples which do not originate from a qualified sampling process. Samples can be taken in increments, this means taking a small partial sample that is later combined

with other partial samples yielding a final sample. This process is called composite sampling. [28]

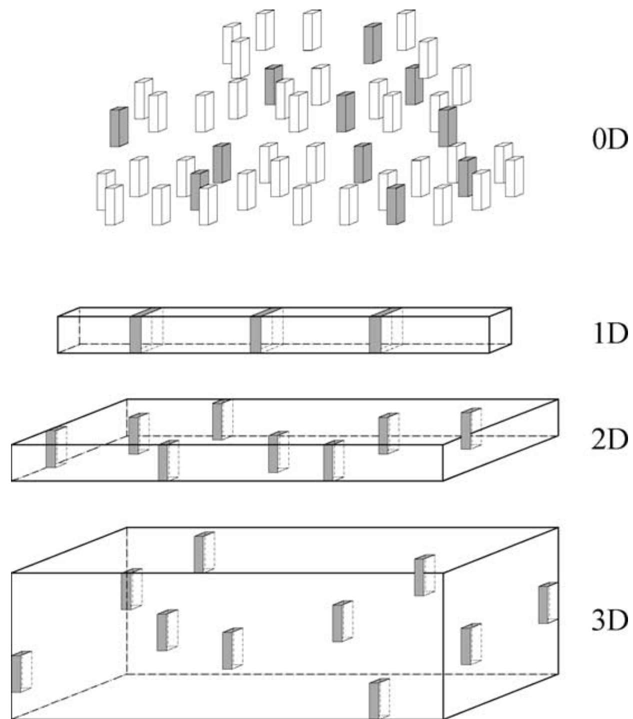

Figure 3. An illustration of sampling from 0D, 1D, 2D and 3D lots **[28]**

The lot that a sample is taken from can be 0D, 1D, 2D or 3D as shown in Figure 3. A 0D lot is a lot for which the material is completely mixed so that there is minimal difference in composition at any point in the lot. A conveyor belt moving materials is an example of a 1D lot. From a 1D lot, a section of the line can be cut out to obtain a sample. A 2D lot can be visualized as a wider 1D lot, where the samples all cover the full height of the lot as in the 1D case, but not the width. A container is an example of a 3D lot. It is always preferable to sample from a 1D lot, so if possible 3D and 2D lots should be turned into 1D. [28]

Figure 4. Grab sampling of material **[28]**

The most commonly used method and the worst method for sampling is grab sampling, shown in Figure 4. Grab sampling is when a spoon or shovel is used to take some material from the top of the lot. This method for sampling should never be used because it leads to great sampling errors as only the topmost material has a chance of being analyzed. [28]

It is stated that 80% of the result of an analysis which gave an error or a discrepancy from the true value is caused by sample taking. Sample preparation makes up 15% of this error, but only 5% is due to the analysis [29]. Sampling, chemical analysis and data analysis do not exist in isolation; they are interdependent. Without taking representative samples it is not possible to estimate the sampling errors that have occurred in the sampling procedure. The results from chemical and data analysis are only reliable if representative samples haves been taken. [28]

3 Materials and methods

3.1 Sampling

The two most important things for taking bilberry samples when analyzing for anthocyanin contents are:

1. When bilberry samples are taken, there must be an equal chance for every berry in the lot to be included in the sample. There should be zero chance of any impurities ending up in the sample, which could prove difficult in reality. The bilberries are cleaned but there is still a chance that there might be e.g. some bilberry leaves in the lot.

2. After the sample is taken, any action which may somehow change the anthocyanin contents must be avoided. [30]

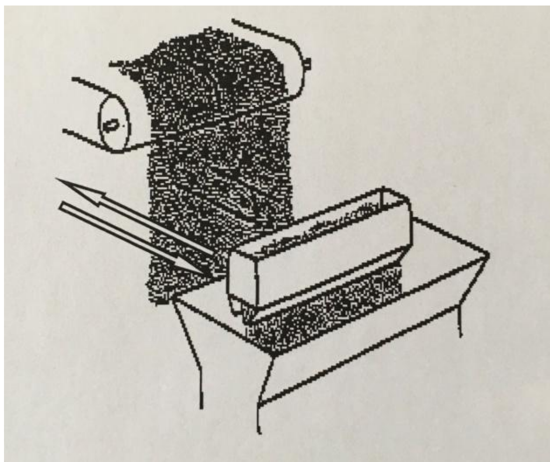

Figure 5. Sampling equipment for a falling stream **[31]**

At Marja Bothnia Berries, there is a falling stream at the place where the berries are processed which would be a good place for sampling. The sampling could be done by means of a mechanical falling stream sampler, which could be automated to take samples at regular time intervals. In ISO standards on sample taking, mechanical sampling is

preferred over manual sampling. The falling stream sampler is like a box which could move under the falling stream from across the stream as shown in Figure 5. [31] [29]

The 12 frozen fresh bilberry samples from Marja Bothnia Berries were stored in plastic bags, thus they were 3D sub-lots. Sub-samples were taken from the plastic bags using a tablespoon. About 5 tablespoons of bilberries were taken from each sample bag while trying to take the bilberries from different spots in the bag. The 5 tablespoons of bilberries were crushed with a stick mixer and yielded enough bilberries to take two parallel 10g samples from each sample bag. Instead of taking sub-samples, it could be better to just take samples that are about 0.5kg in size and then crush all of the berries in the sample. This would homogenize and mix the entire sample.

3.2 Standards and reagent preparation

As anthocyanins dissolve well in organic, acidic compounds, a reagent containing 2% HCl dissolved in methanol was prepared. A previous study by [32] tested reagents containing 20% and 80% of methanol, and the solution which contained 80% methanol led to higher amounts of anthocyanins being extracted. Hence using only methanol and HCl to make the reagent acidic should give the best result. The reagent can be prepared by dissolving 54 ml of 37% HCl in methanol for 1 L of reagent. The standards are also prepared by diluting with the reagent. The standards used for anthocyanin analysis were delphinidin chloride and kuromanin chloride. 5 mL of 20 µg/mL delphinidin chloride standard was prepared from a 400 µg/mL base solution by diluting it by a factor of 20 with 2% HCl-methanol reagent. 5 mL of two more standards, 5 µg/mL and 1.25 µg/mL were prepared by diluting by a factor of 4 and 16, respectively. 5mL of 20 µg/mL kuromanin chloride standard was prepared from a 200 µg/mL base solution by first diluting it by a factor of 2 with 2% HCl-methanol reagent, obtaining a 100 µg/mL standard. The 100 µg/mL standard was diluted by a factor of 5, obtaining a 20 µg/mL standard, and two additional standards were prepared in the same manner as for delphinidin chloride.

3.3 Bilberry sample preparation

The bilberry samples which are frozen fresh bilberries obtained from Marja Bothnia Berries, were taken out from a freezer where they had been stored in -20°C. Exposure to light and high temperatures negatively impacts the anthocyanin contents, therefore it is important that the samples are always stored in a cold and dark place. The samples were crushed with a stick blender; about 10g of berries were weighed with a scale accurate to 0.1 mg for each sample to be analyzed. Two parallel samples were prepared for analysis from every sample. The samples were weighed into 50 ml volumetric flasks, after which 30 ml of 2% HCl-methanol solution was added to rinse the sample into the volumetric flask, and caps were placed on the flasks. After all the samples to be analyzed during one session had been weighed, the volumetric flasks were filled up to the 50 ml mark.

The extractions were conducted at room temperature. The reagent was stored in room temperature, but the berries were frozen so this might have brought down the temperature slightly below room temperature. The anthocyanins from the bilberry samples were extracted using ultrasound. The volumetric flasks containing the samples were placed into an ultrasonic bath, and the cleaner was turned on for five minutes. After that 10 ml plastic syringes and 0.45µm glass microfiber syringe filters were used to filter part of the samples. The filtered samples were then diluted by a factor of 50, using the 2% HCl-methanol solution as the solution to dilute the filtered samples in. After diluting the filtered samples, they were measured against delphinidin chloride standards and kuromanin chloride standards using the Agilent Cary 60 UV-VIS Spectrophotometer, described in the next chapter.

3.4 Measurement of bilberry samples

The Agilent Cary 60 UV-Vis spectrophotometer was used to measure the anthocyanin contents of the bilberry extracts. In UV-Vis spectrophotometry, measurements are usually made at the absorbance maximum. Based on literature and anthocyanin analysis methods by Marja Bothnia Berries customers, a fixed wavelength of 539 nm was decided to ensure

that the analysis processes at Centria and Novia were the same. The device was set to take five replicate measurements for every standard and sample. With the Scan application of the Agilent Cary 60, it is possible to quickly scan the absorbance values over a range of wavelengths. A scan on the delphinidin chloride and kuromanin chloride standards were made over the entire range of the Agilent Cary 60, 1100-190 nm, with a wavelength interval of 1 nm. The measurement results from these scans showed that the absorbance maximum indeed lies close to 539 nm, justifying the use of this fixed wavelength. [21]

Figure 6. Measuring with the Agilent Cary 60 UV-Vis spectrophotometer

Measuring with the Agilent Cary 60 dip probe is very simple: the dip probe is dipped into the volumetric flask containing the standard or sample, the 1 cm measuring gap is checked to ensure that there are no air bubbles, and then the measurement is made. The Agilent Cary 60 UV-Vis Spectrophotometer and some samples are shown in Figure 6. First, the instrument was zeroed by measuring the absorbance of the 2% HCl-Methanol reagent. Then the absorbances of standards were measured to obtain a standard curve for determining the anthocyanin contents of the samples. As earlier stated, the samples were measured twice against two standards, delphinidin chloride and kuromanin chloride. The concentrations of the standards of both of these were 20 µg/mL, 5 µg/mL and 1.25 µg/mL,

and the 2% HCl-Methanol reagent was used as 0 µg/mL, yielding four points on each standard curve.

The standard curves were perfectly linear with correlation coefficients of R = 1.0000, indicating that Lambert-Beers law is obeyed. For kuromanin chloride standards, the absorbance values ranged from 0-1.38. For delphinidin chloride standards, the absorbance values ranged from 0-1.73. Despite these absorbance values not lying in the intermediate range recommended by [26], the standard curves were linear with correlation coefficients of R = 1.0000, so using this range for analysis is acceptable. The absorbance values of the samples were around 1.00-1.72 so the problem of too low absorbance values leading to greater uncertainty is avoided. Between each measurement, the tip of the dip probe was rinsed with distilled water and wiped with a tissue to ensure that no drops from the previously measured standard/sample were carried over to the next standard/sample.

As the standards prepared were in the range of 0-20 µg/mL, the concentrations of the samples also fell in this range, except for a few samples which had higher absorbance values when measured against the standard curve of kuromanin chloride. However, the actual concentration of anthocyanins in bilberries has to be calculated by multiplying the measurement result by 50 to account for the dilution factor. This gives the concentration in µg/mL in the initial 50 mL volumetric flask in which the extraction of anthocyanins was performed. Then to calculate the concentration of anthocyanins per gram of blueberries, this concentration has to be multiplied again by 50 to give the total contents of anthocyanins in the flask in µg, and then be divided by the weight of the sample's bilberries (around 10 grams).

4 Results

There were 12 frozen fresh bilberry samples received from Marja Bothnia Berries which were analyzed for their anthocyanin contents using the Agilent Cary 60 UV-Vis spectrophotometer. For every sample, two parallel samples were analyzed. The results have been compared to measurements made with another UV-Vis spectrophotometer and HPLC by Centria University of Applied Sciences. The results are shown in Table 1.

Table 1. Anthocyanin and anthocyanidin measurement results from bilberry samples

Sample name	UV-Vis Agilent Cary 60		UV-Vis Centria		HPLC Centria
	Delphinidin Chloride (mg/g)	**Kuromanin Chloride (mg/g)**	**Delphinidin Chloride (mg/g)**	**Kuromanin Chloride (mg/g)**	**HPLC (mg/g)**
SWE 15.8_1	4.13	5.16	3.70	4.84	3.02
SWE 15.8_2	3.86	4.88	3.36	4.59	2.86
FIN 15.8_1	5.05	6.34	3.63	4.84	3.01
FIN 15.8_2	4.65	5.84	3.94	5.13	3.38
FIN 10.8_1	3.35	4.19	3.75	4.91	3.24
FIN 10.8_2	3.70	4.63	3.64	4.94	3.31
FIN 20.8_1	3.50	4.41	3.95	5.15	3.39
FIN 20.8_2	4.13	5.16	3.91	5.26	3.34
5102_1	3.65	4.57	3.75	4.83	3.11
5102_2	3.91	4.90	3.73	4.89	3.41
5041_1	3.31	4.24	3.34	4.57	2.98
5041_2	2.91	3.64	3.54	4.73	2.99
5101_1	3.91	4.88	4.01	5.18	3.20
5101_2	3.78	4.73	3.95	5.18	3.07
8.8a_1	3.97	4.96	3.70	4.59	2.93
8.8a_2	4.88	6.11	3.61	4.47	2.74
8.8b_1	4.43	5.53	3.76	4.58	2.92
8.8b_2	4.53	5.67	3.73	4.61	2.86
9.8a_1	3.86	4.83	3.62	4.43	3.04
9.8a_2	3.96	4.75	3.81	4.67	2.95
9.8b_1	3.57	4.48	3.75	4.67	3.04
9.8b_2	3.91	4.91	3.81	4.75	2.62
9.8c_1	3.52	4.39	3.48	4.16	2.74
9.8c_2	4.29	5.35	3.86	4.75	3.02
Minimum	**2.9**	**3.6**	**3.3**	**4.2**	**2.6**
Maximum	**5.1**	**6.3**	**4.0**	**5.3**	**3.4**
Average	**3.9**	**4.9**	**3.7**	**4.8**	**3.0**
Standard Deviation	**0.5**	**0.6**	**0.2**	**0.3**	**0.2**

The anthocyanin contents range when measured with delphinidin chloride standard with the Agilent Cary 60 UV-Vis spectrophotometer is 2.9-5.1 mg/g, with a standard deviation of 0.5 mg/g. This range slightly overlaps with the anthocyanin contents range obtained using HPLC, which is 2.6-3.4 mg/g with a standard deviation of 0.2 mg/g. The range obtained when measuring with delphinidin chloride using Centria's UV-Vis spectrophotometer, 3.3-4.0 mg/g with a standard deviation of 0.2 mg/g, just barely

overlaps with the range of the HPLC measurements. The anthocyanin contents in bilberry samples measured using delphinidin chloride are summarized in Figure 7.

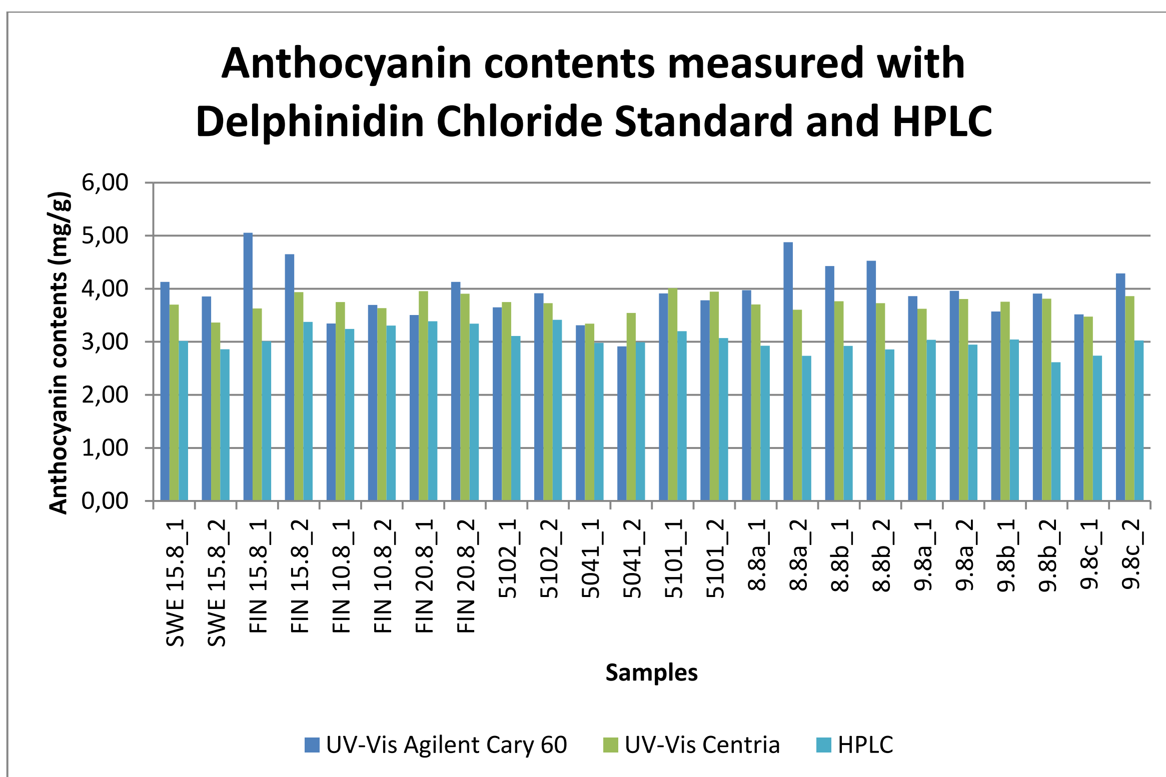

Figure 7. Anthocyanin contents in bilberry samples measured with delphinidin chloride standard

Using kuromanin chloride standards, the range obtained using the Agilent Cary 60 UV-Vis spectrophotometer is 3.6-6.3 mg/g with a standard deviation of 0.6 mg/g, and with Centria's UV-Vis spectrophotometer it is 4.2-5.3 mg/g with a standard deviation of 0.3 mg/g. These ranges do not overlap with the range of the HPLC measurements despite the large ranges. The anthocyanin contents in bilberry samples measured using kuromanin chloride are summarised in Figure 8.

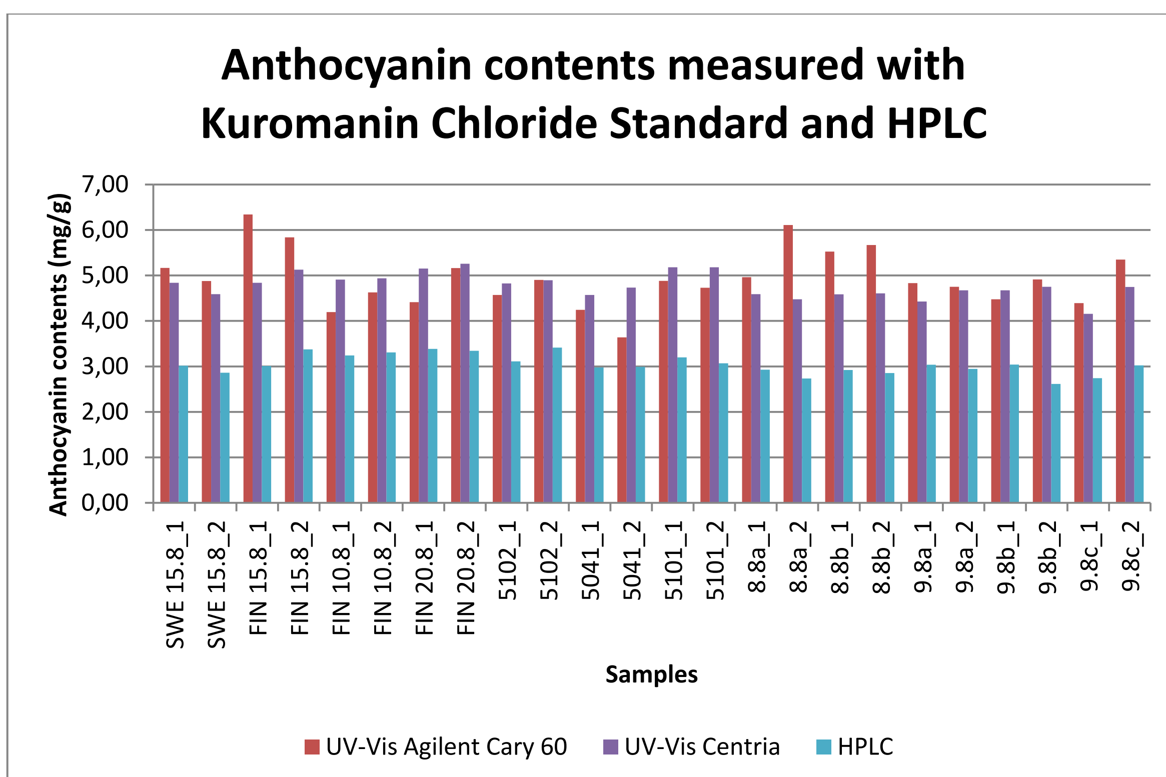

Figure 8. Anthocyanin contents in bilberry samples measured with kuromanin chloride standard

The standard deviations for measurements made with the Agilent Cary 60 UV-Vis spectrophotometer were roughly double that of the standard deviations measured with Centria's UV-Vis and HPLC. This is consistent with the wider range obtained with the Agilent Cary 60 UV-Vis spectrophotometer, and the most likely explanation is sampling errors.

In a study by Heffels et al. [32] in which UHPLC (Ultra High Performance Liquid Chromatography) was used, average total anthocyanin contents in bilberries from Poland, Germany and Ukraine were reported as 6.4 ± 0.8 mg/g, 6.0 ± 0.3 mg/g, and 5.2 ± 0.6 mg/g. For bilberries harvested in Finland, a total anthocyanin content of 6.11 mg/g was reported by [33]. These values from previous studies are much greater than the average concentration of 3.0 mg/g obtained through HPLC analysis in this paper. The measurement results from UV-Vis spectrophotometry lie closer to the reference values from previous papers.

HPLC is the more popular and reliable method for analysis of anthocyanin contents, so it is likely that the results using HPLC are closer to the true values. The wavelength of 539 nm where the anthocyanins show a maximum is far from the maxima of other phenolics.

Phenolics have maxima in the UV range, so there should not be a risk that other phenolics are measured simultaneously with the anthocyanins. Instead the reason for the higher anthocyanin contents obtained by UV-Vis analysis may be due to measuring degradation products polymerized by anthocyanins through browning reactions in addition to the anthocyanins. The discrepancy could also be due to sampling errors and/or the extraction method for anthocyanins. The reagent used in HPLC analysis is different; it contains 10% HCl and 17% water mixed with methanol. In HPLC the samples are subject to hydrolysis to remove the sugar from the anthocyanin, yielding the anthocyanidin. [15]

A table showing the differences between the different UV-Vis spectrophotometers and HPLC is found in Appendix 2. For the Agilent Cary 60 UV-Vis spectrophotometer, the anthocyanin contents were on average 30.33% and 63.06% greater compared to HPLC when measured with delphinidin chloride and kuromanin chloride, respectively. Centria's UV-Vis spectrophotometer had similar results; the anthocyanin contents were on average 22.52% and 57.13% greater when measured with delphinidin chloride and kuromanin chloride, respectively. Measurements made with kuromanin chloride standards yield lower absorbance values despite the concentrations being the same as the delphinidin chloride standards. This resulted in greater anthocyanin contents when measuring with the kuromanin chloride standards. As the results obtained when measuring with delphinidin chloride standards are closer to the results obtained using HPLC, delphinidin chloride seems to be the more reliable standard for analyzing anthocyanin contents.

The average differences between the Agilent Cary 60 UV-Vis spectrophotometer and Centria's UV-Vis spectrophotometer are not large. When measured with delphinidin chloride standard, the average difference between the Agilent Cary 60 compared to Centria's UV-Vis spectrophotometer is 6.17%. With kuromanin chloride standard, the average difference is 3.65%. Even though the results are on average slightly larger with the Agilent Cary 60, there are both negative and positive percentage differences with both standards. This is good as it indicates that there is not a systematic error where one UV-Vis spectrophotometer always yields greater results. The cause of the differences is could be sampling error. As the Agilent Cary 60 UV-Vis spectrophotometer was a new piece of equipment obtained for analyzing anthocyanin contents, it was important to check if it was working. Both UV-Vis spectrophotometers giving similar results is a good indicator that the equipment is working.

5 Discussion

5.1 Possible improvements

Differences in anthocyanin contents greater than 10% were detected in parallel samples using both UV-Vis spectrophotometry and HPLC. The reason for this could be in the sampling of the bilberries, that the samples taken were not an even representation of all the bilberries. Another reason could lie in the sample preparation process, when the bilberries were crushed with the stick blender for extraction. The bilberries were transferred into the volumetric flask using a spoon, and it is possible that in some samples there were more pulp transferred into the flask than peel. Studies have demonstrated that polyphenols and anthocyanins have a tendency to gather in the peel of bilberries. One paper found that anthocyanin content in the peel of bilberries was greater by a factor of 20 compared to the pulp. [34]

As anthocyanins are found in greater concentrations in the peel and dry matter of bilberries, the moisture content is an important factor to take into account. If the moisture contents of the different bilberry samples are determined, it is possible to calculate the anthocyanin contents per gram of dry weight. This can make the results more comparable as higher moisture content ought to lead to lower anthoyanin contents.

5.2 Opportunities for further research

The anthocyanin content analysis through HPLC and UV-Vis gave rather different results and the variance in the results of the UV-Vis measurements are larger than the measurements made with HPLC. In future research, more analysis should be conducted in an attempt to understand why these methods did not give similar results and how they can be made to give similar results. HCl might not be the optimal acid for bringing down the pH value when analyzing anthocyanin contents, because it can cause hydrolysis in some

anthocyanins. Instead organic acids like formic or acetic acid could be used instead. Future research could also investigate how reagents with different levels of acidity of affect the anthocyanin content readings. [22]

The extractions were performed close to room temperature, about 20°C. Temperatures too high cause degradation of anthocyanins as earlier stated. Temperatures above 70°C have been proven to cause rapid degradation of anthocyanins. However, an increase in extraction temperature can also increase the solubility and mass transfer rate of anthocyanins. With increasing temperature, the viscosity and surface tension of the solvents decrease. This facilitates the solvents reaching the matrices of the sample, which also improves the extraction rate. Typically, extraction of anthocyanins is conducted at 20-50°C, so there is still room to try raising the temperature in order to increase the anthocyanin contents. Further research could experiment with higher temperatures. Longer extraction times can also be tested, but there is the same risk of decreasing anthocyanin contents due to oxidation. Having a higher solvent-to-solid ratio might also increase the anthocyanins extracted, so higher solvent-to-solid ratios could be tested. But it costs more to have a higher solvent-to-solid ratio as more solvent is used. [15]

6 Conclusion

A process for analyzing anthocyanin contents in bilberries using UV-Vis spectrophotometry was developed and tested. Values for anthocyanin contents obtained using this process was compared to results from HPLC analysis, which is considered the more reliable method. Frozen fresh bilberry samples were supplied by Marja Bothnia Berries, 12 samples were analyzed and two parallel samples were analyzed for each sample. The bilberries were crushed using a stick blender and anthocyanins were extracted using a 2% HCl-Methanol reagent. Ultrasound was used to extract the anthocyanins using an ultrasonic bath. Anthocyanin contents were measured using two different standards, delphinidin chloride and kuromanin chloride.

Using the Agilent Cary 60 UV-Vis spectrophotometer, the anthocyanin contents in the bilberry samples were between 2.91-5.05 mg/g and 3.64-6.34 mg/g using delphinidin chloride and kuromanin chloride, respectively. The average anthocyanin contents were

3.95 mg/g and 4.94 mg/g for delphinidin chloride and kuromanin chloride, respectively. These averages are lower compared to bilberry anthocyanin contents reported in previous papers, which range from 5.2-6.4 mg/g. The anthocyanin contents measured for the 12 samples using HPLC analysis were between 2.62-3.41 mg/g, even further away from these literature values. The anthocyanin extraction methods for UV-Vis spectrophotometry and HPLC were slightly different. The fact that both HPLC and UV-Vis spectrophotometry yielded lower values could imply that the anthocyanin extraction process and the sampling of bilberries can still be improved. The average percentage difference between the measurements made using the Agilent Cary 60 UV-Vis spectrophotometer and Centria's UV-Vis spectrophotometer were not large, indicating that the new Agilent Cary 60 is working as it should.

Future attempts to improve the anthocyanin analysis process could involve trying a different acid instead of hydrochloric acid, varying the reagent's level of acidity, using longer extraction times, raising the extraction temperature, having a higher solvent-to-solid ratio and using an improved sample taking process.

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Appendix 1

Table 2. Structure of anthocyanidins found in bilberries

Name of Anthocyanidin	**R1 Functional group**	**R2 Functional group**
Pelargonidin (Pg)	H	H
Cyanidin (Cy)	OH	H
Delphinidin (De)	OH	OH
Peonidin (Pn)	OCH_3	H
Petunidin (Pt)	OH	OCH_3
Malvidin (Ma)	OCH_3	OCH_3

Appendix 2

Table 3. Percentage differences between measurement results obtained with the Agilent Cary 60, Centria's UV-Vis spectrophotometer and HPLC

Sample name	UV-Vis Centria (Delphinidin Chloride) and HPLC difference (%)	UV-Vis Centria (Kuromanin Chloride) and HPLC difference (%)	UV-Vis Agilent Cary 60 (Delphinidin Chloride) and HPLC difference (%)	UV-Vis Agilent Cary 60 (Kuromanin Chloride) and HPLC difference (%)	UV-Vis Agilent Cary 60 and UV-Vis Centria (Delphinidin Chloride) difference (%)	UV-Vis Agilent Cary 60 and UV-Vis Centria (Kuromanin Chloride) difference (%)
SWE 15.8_1	22,68	60,35	36,83	71,16	11,53	6,74
SWE 15.8_2	17,55	60,45	34,82	70,56	14,69	6,30
FIN 15.8_1	20,59	60,81	67,98	110,72	39,29	31,03
FIN 15.8_2	16,61	51,84	37,80	72,97	18,16	13,91
FIN 10.8_1	15,61	51,42	3,18	29,37	-10,75	-14,57
FIN 10.8_2	9,95	49,36	11,75	39,95	1,64	-6,30
FIN 20.8_1	16,77	52,15	3,51	30,31	-11,36	-14,35
FIN 20.8_2	16,87	57,23	23,52	54,45	5,69	-1,77
5102_1	20,54	55,14	17,35	46,99	-2,64	-5,25
5102_2	9,17	43,36	14,66	43,55	5,03	0,14
5041_1	12,15	53,41	11,17	42,49	-0,87	-7,12
5041_2	18,43	58,20	-2,66	21,61	-17,81	-23,13
5101_1	25,38	61,93	22,28	52,52	-2,47	-5,81
5101_2	28,53	68,77	23,21	54,04	-4,14	-8,73
8.8a_1	26,51	56,80	35,69	69,54	7,26	8,13
8.8a_2	31,82	63,60	78,27	123,38	35,23	36,55
8.8b_1	28,75	56,84	51,49	89,03	17,66	20,52
8.8b_2	30,53	61,37	58,50	98,53	21,42	23,03
9.8a_1	19,20	45,69	27,11	58,97	6,64	9,11
9.8a_2	29,26	58,62	34,47	61,22	4,03	1,63
9.8b_1	23,39	53,66	17,35	47,16	-4,90	-4,23
9.8b_2	45,69	81,55	49,38	87,67	2,53	3,37
9.8c_1	26,85	51,65	28,39	60,28	1,21	5,69
9.8c_2	27,66	56,95	41,81	76,89	11,09	12,70
Average	22,52	57,13	30,33	63,06	6,17	3,65
Standard deviation	8,16	7,74	20,46	25,51	13,58	14,40

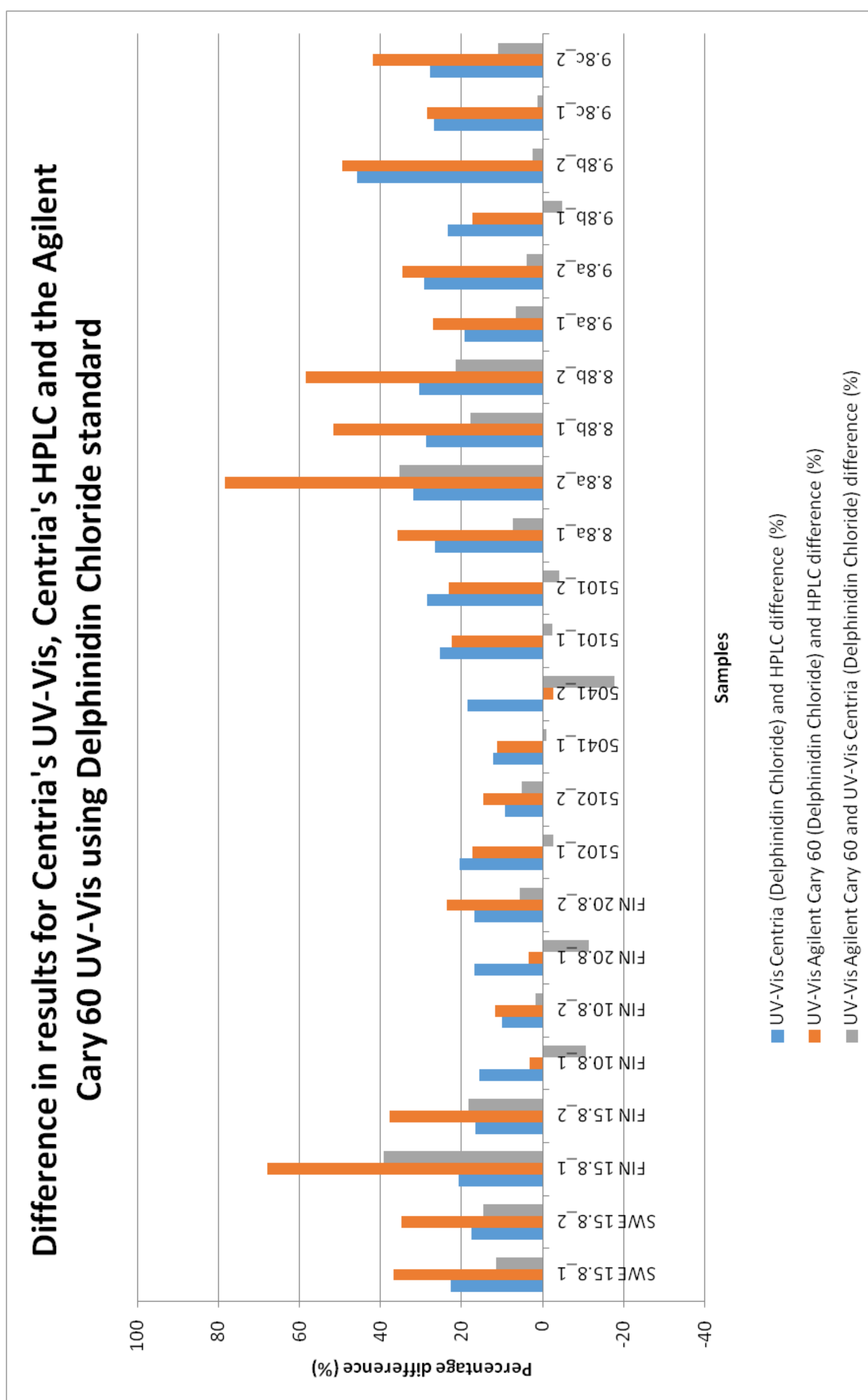

Figure 9. Percentage differences between measurement results obtained with the Agilent Cary 60 and Centria's UV-Vis spectrophotometer using delphinidin chloride, and HPLC

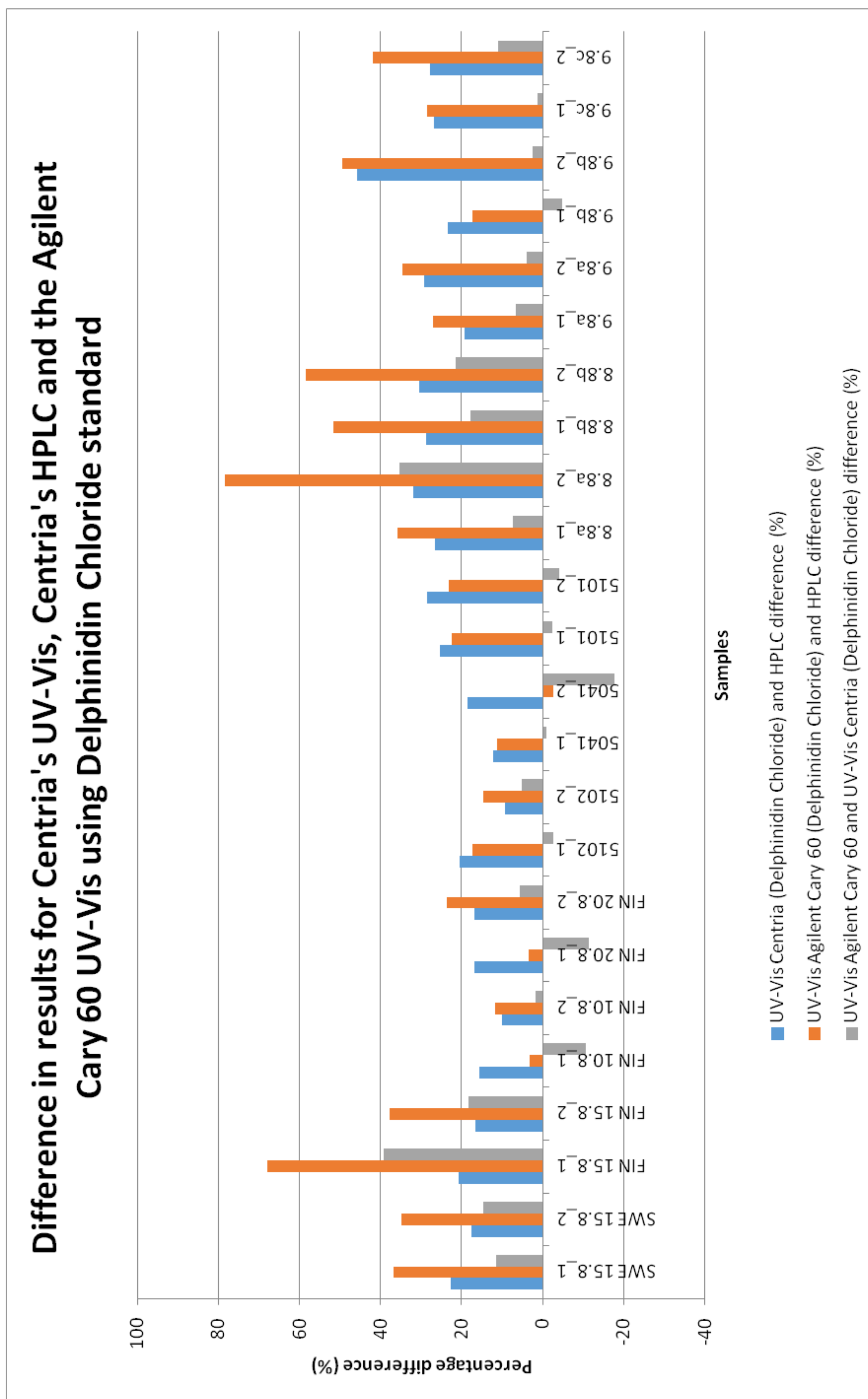

Figure 10. Percentage differences between measurement results obtained with the Agilent Cary 60 and Centria's UV-Vis spectrophotometer using kuromanin chloride, and HPLC