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Market driven authentic non-timber forest products from the Baltic Sea region

WP5 Literature survey on quality characterization methods

O5.1 Quality characterization methods for selected species and non-wood forest products

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Abstract

Plants are used as a part of diets, medicines and cosmetics, and therefore it is important to show proof of the quality of plants and plant-based products. There are several approaches to prove quality, but the focus of the review is on the polyphenolic composition of plants. Polyphenols are a group of over 8000 aromatic compounds with a phenol ring(s) in their structure. Different plant species contain several polyphenols in varying concentrations. The concentration of polyphenols is affected by specie, growing conditions (soil, weather), growth stage (ripeness) and geographical location. In order to prove quality, it is important to know how polyphenols are extracted from the material and which polyphenols are the most important for quality. Total polyphenol content and antioxidant capacity can be used as quality indicators for all plants and plant-based products. However, to show plant uniqueness, individual polyphenols such as bilberry anthocyanins, lingonberry resveratrol, or chaga styrylpyrones must also be analyzed.

Keywords

Bilberry | Lingonberry | Chaga | Extraction | Polyphenols | Quality characterization

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Content

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1. Introduction

The World Health Organization (WHO) has estimated that two thirds of premature deaths in the Europe Region are caused by four major non-communicable diseases (NCDs): cardiovascular disease, diabetes, cancers and chronic respiratory disease. At least 80% of all heart disease, stroke and diabetes and 40% of cancer could be prevented by tackling major risk factors, such as tobacco, alcohol use, unhealthy diets, physical inactivity, hypertension, obesity and environmental factors. As one of the recommendations, WHO urges to increase the intake of fruits, vegetables and fibers in a diet (World Health Organization, 2016).

Plants contain nutrients, vitamins and minerals, which are important to our biological activity. However, they also contain other valuable compounds, such as polyphenols, a class of phytochemicals, that could have several health benefits related to non-communicable diseases. In clinical trials, some of the polyphenols have showed promising results in certain NCDs (Fraga, et al., 2019). Polyphenols also have antioxidative properties, which are often linked to suspected health effects of polyphenols (Manach, et al., 2004). In human diet, polyphenols are the most abundant source of antioxidants, and their intake is 10 times higher than the intake of vitamin C and 100 times higher than intake of vitamin E (Scalbert, et al., 2005).

Main sources of polyphenols are fruits, leaves and roots, and their derived products like powders, jams, juices, teas, wine and coffee (Fraga, et al., 2019; Scalbert, et al., 2005; Ignat, et al., 2011). Nevertheless, polyphenols are rarely discussed as a part of a healthy diet nor their content displayed on food packings even though the consumers are getting more aware of the health benefits associated to high consumption of fruits and vegetables.

There is a lot of research trying to measure the content of different polyphenols and demonstrate their beneficial role in human diet. However, a large structure variety of polyphenols, complex plant and food matrices and complicated pathways of human metabolic hinders the research (Bresciani, et al., 2017). In chemical research, the lack of standards and standardized methods also causes obstacles in addition to the variations in polyphenol content. The polyphenol content varies depending on the geographical location, growing conditions and plant processing (Manach, et al., 2004).

This literature survey presents polyphenolic compounds and their classification and occurrence in three selected plant species. However, the main focus of this review is placed on extraction and analysis of polyphenols in order to prove quality of the selected species and their derived products. Practical examples are given on extraction and analysis of polyphenolic compounds as well as method recommendations for each selected plant species.

2. Polyphenols

Polyphenols are secondary metabolites of plants, that are generally involved in defense against ultraviolet radiation or aggression of pathogens (Pandey & Rizvi, 2009). They are considered to be anti-inflammatory, antiviral, antimicrobial and antioxidative agents and therefore they are suspected to decrease the risk of cancers, cardiovascular diseases, neurodegenerative diseases and osteoporosis (Pandey & Rizvi, 2009; Chandra, et al., 2014). Besides their believed health benefits, polyphenols impact flavor, color, scent and astringency of plants and derived products (Pandey & Rizvi, 2009).

Over 8000 polyphenolic compounds have been identified in plants, but not all of them are found in edible plants (Manach, et al., 2004; Pandey & Rizvi, 2009). Polyphenols can be subdivided into different groups based on the number of phenol rings and structural elements between the rings binding them together. Four main subgroups of polyphenols (Fig. 1) are phenolic acids, flavonoids, stilbenes and lignans (Pandey & Rizvi, 2009).

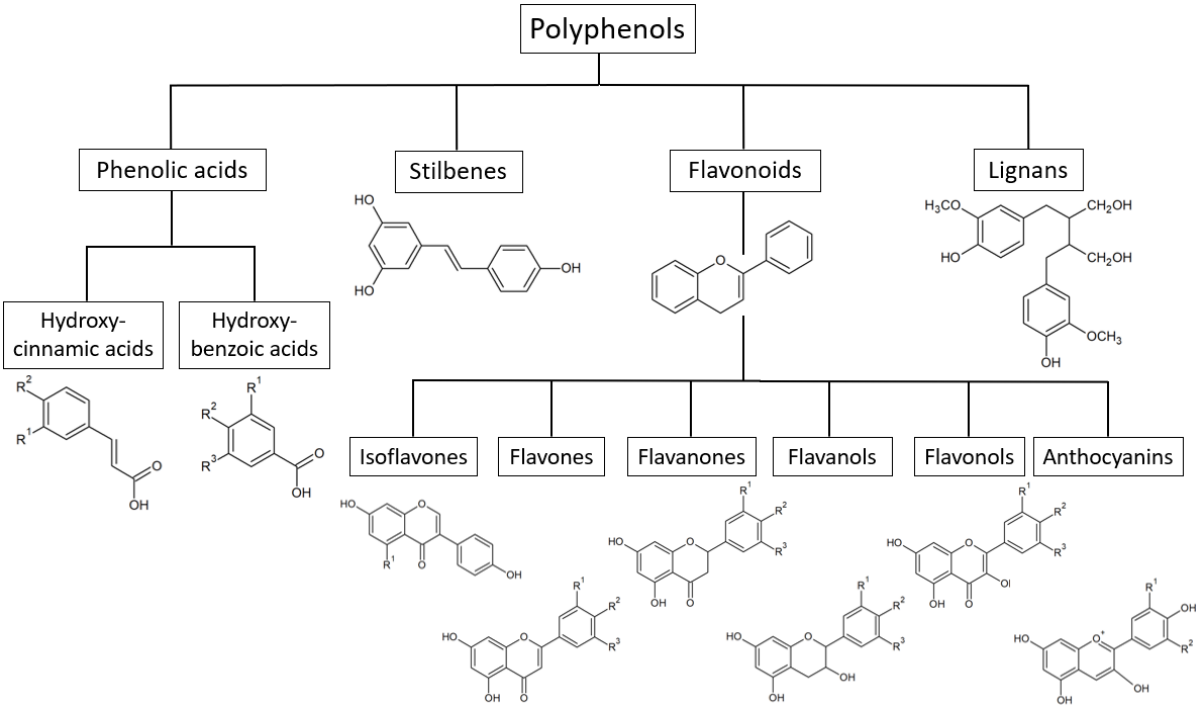

Figure 1. Polyphenols and the main subgroups (Manach, et al., 2004; Pandey & Rizvi, 2009).

Flavonoids are probably the most studied group of polyphenols as there are over 4000 compounds in this particular subgroup (Ignat, et al., 2011; Pandey & Rizvi, 2009). Flavonoids can be recognized from two aromatic rings bound together by oxygenated heterocycle, which is formed by three carbon atoms (Manach, et al., 2004; Ignat, et al., 2011). Flavonoids can be further subdivided to isoflavones, flavones, flavanones, flavanols (catechin and proanthocyanidins), flavonols and anthocyanins.

(Manach, et al., 2004; Ignat, et al., 2011; Pandey & Rizvi, 2009). Many of the flavonoids functions as pigments in fruits, leaves and flower petals (Pandey & Rizvi, 2009).

In food, flavonols are the most represent group of flavonoids (Manach, et al., 2004). Common flavonols in plants are quercetin, kaempferol, myricetin and isorhamnetin (Manach, et al., 2004; Riihinen, et al., 2008). Flavonols exists usually in glycosylated form, meaning they are attached to sugars, often to glucose or rhamnose. Typically, fruits contain 5 to 10 glycosylated flavonols. Flavonol concentration can vary a lot in plants, even between the fruits of the same tree or sides of the same fruit. This is due to the biosynthesis of flavonoids stimulated by light, which also causes flavonols to concentrate on the leaves and peels of the fruit (Manach, et al., 2004).

Flavones, flavanones and isoflavones are less common compared to flavonols. Flavones constitute mostly glycosides of luteolin and apigenin and are rarely present in edible plants (Manach, et al., 2004). Flavanones instead can be found at high concentrations in citrus fruits and at lower concentrations in tomatoes and aromatic plants like mint. Some examples of flavanones are naringenin in grapefruits and hesperetin in oranges (Manach, et al., 2004; Ignat, et al., 2011). Isoflavones are almost solely found in leguminous plants like soya, which contains genistein, daidzein and glycitein in glycosylated forms. Isoflavones are heat sensitive and are usually hydrolyzed to glycosides if the plant is processed (Manach, et al., 2004).

As other flavonoids are usually in glycosylated form, flavanols are an exception. There are two types of flavanols, monomers (catechins) and polymers (proanthocyanidins). Catechins are found in many fruits and their richest source is apricots. Common flavanol monomers found in fruits are catechin and epicatechin. Other catechins, such as gallocatechin, epigallocatechin and epigallocatechin gallate, are more abundant in grapes, tea and seeds of leguminous plants (Manach, et al., 2004).

Proanthocyanidins are also known as condensed tannins. They are dimers, oligomers and polymers composed of monomeric flavanols, such as catechin and epicatechin, linked together by aromatic rings (Manach, et al., 2004; Ignat, et al., 2011). The degree of polymerization of proanthocyanidins (number of monomeric units in a polymer) is not often determined due to the large variability, which hampers the estimation of proanthocyanidin content. For example, in some apples, the degree of polymerization ranges between 4 and 11. In fruits, proanthocyanidins have an effect on astringency, which decreases during the maturation of the fruit (Manach, et al., 2004).

Anthocyanins are known as plant pigments as they give pink, red, purple and blue color to plants depending on the compound and pH. They are abundant in flower petals and fruits (especially in the fruit peels), and often dark berries are highly rich in anthocyanins (Manach, et al., 2004). Anthocyanins can be found in glycosylated form or in sugar-free form (aglycone), referred to as anthocyanidins (Manach, et al., 2004; Ignat, et al., 2011). Anthocyanins can also be found in esterified form or complex form. Esterified form is created in the reaction between anthocyanins and phenolic acids or organic acids (e.g. citric acid and malic acid) and the complex form in the reaction between anthocyanins and

other flavonoids (Manach, et al., 2004). Typical anthocyanins in plants are cyanidin, delphinidin, malvidin, peonidin, petunidin, and pelargonidin aglycones attached to sugar units, such as galactose, glucose or arabinose (Ignat, et al., 2011; Primetta, et al., 2013).

Phenolic acids are derivatives of benzoic acid (hydroxybenzoic acids) or cinnamic acid (hydroxycinnamic acids). They can be found in the nature in free molecular form or in bound form linked to other components by ester, ether or acetal bonds (Manach, et al., 2004; Ignat, et al., 2011). Content of hydroxybenzoic acids is usually quite low in edible plants (Manach, et al., 2004). However, some common hydroxybenzoic acids are gallic, ellagic, vanillic, syringic, protocatechuic, and *p*-hydroxybenzoic acid (Ignat, et al., 2011; Häkkinen, et al., 1999). Hydroxybenzoic acids are also components of polymers (hydrolysable tannins), such as gallotannins and ellagitannins. Gallotannins can be found in mangoes and ellagitannins in some of the red fruits like strawberries, raspberries and blackberries (Manach, et al., 2004).

Hydroxycinnamic acids are more common in edible plants than hydroxybenzoic acids (Manach, et al., 2004). Some quite common hydroxycinnamic acids are *p*-coumaric, caffeic, ferulic, and sinapic acid (Manach, et al., 2004; Ignat, et al., 2011). Caffeic acid, in free and bound form, is the most common hydroxycinnamic acids as it represents 75 – 100 % of the total hydroxycinnamic acid content in most fruits. Hydroxycinnamic acids are found in all parts of the fruit, but the highest concentrations occur in the outer parts of a ripe fruit. Generally, the concentration of hydroxycinnamic acids locally decrease during the ripening, but the total concentration increases as the size of the fruit increases (Manach, et al., 2004).

Stilbenes are present in only few edible plants (Manach, et al., 2004; Ignat, et al., 2011; Pandey & Rizvi, 2009). Their structure contains two phenyl rings linked to each other by a methylene bridge formed by two carbon atoms (Pandey & Rizvi, 2009). Resveratrol is one of the most studied stilbenes and it is suspected to have many health effects (Ignat, et al., 2011; Pandey & Rizvi, 2009). It is present mainly in glycosylated, either cis- or trans isomeric form. Resveratrol is found, for example, in lingonberries, cranberries and grapes, but only in low concentrations (Petrovski, et al., 2011).

Lignans are diphenolic compounds that have 2,3-dibenzylbutane structure formed by dimerization of two cinnamic acid residues (Pandey & Rizvi, 2009). The most important lignans in plants are secoisolariciresinol and matairesinol. They are mainly found in oilseeds and flaxseeds, of which flaxseeds are richest source of lignans (Manach, et al., 2004; Pandey & Rizvi, 2009). Also, some grains, leguminous plants, vegetables and fruits (pears and prunes) contain lignans, but in a much lesser extent (Manach, et al., 2004).

3. Polyphenols in selected species

Three Baltic Sea region plant species, bilberry, lingonberry and chaga, were selected for a closer study. In the following chapters, their appearance, polyphenol content and extraction methods will be discussed.

3.1 Bilberry (*Vaccinium myrtillus* L.)

Bilberry, also known as European blueberry, grows wild in Northern Europe, Asia and Western North America. Both fruit and leaves of bilberry can be utilized. The fruit itself has dark bluish peal, flesh and juice, which has a distinct and pleasant flavor (Fig. 2). Growing condition, such as day length, temperature and light intensity, have a strong impact on polyphenol composition of the fruit. Several studies have shown, that bilberries grown northern, have higher polyphenol concentrations than bilberries grown southern. This is believed to result from a longer day length and colder climate during the growing season (Uleberg, et al., 2012).

Figure 2. Ripe *Vaccinium myrtillus* L. berries in nature (from Pixabay, Kjerstin Michaela Haraldsen).

3.1.1 Valuable compounds

The major polyphenols in bilberries are anthocyanins, proanthocyanidins, flavonols and hydroxycinnamic acids. Polyphenols are not evenly distributed in berries, typically 10 % of polyphenols are located in berry pulp, 28 – 35 % in peel and the remaining 60 – 70 % in seeds (Häkkinen, et al., 1998). Generally, the quality measurement of bilberries focuses on anthocyanins, because bilberries are considered one of the best sources of anthocyanins. Anthocyanins are found mainly in the peel of the fruit and to a lesser extent in the pulp (Riihinen, et al., 2008).

There are 15 different anthocyanins in bilberries, delphinidin-3-*O*-galactoside, delphinidin-3-*O*-glucoside, cyanidin-3-*O*-galactoside, delphinidin-3-*O*-arabinoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-galactoside, cyanidin-3-*O*-arabinoside, petunidin-3-*O*-glucoside, peonidin-3-*O*-galactoside, petunidin-3-*O*-arabinoside, peonidin-3-*O*-glucoside, malvidin-3-*O*-galactoside, peonidin-3-*O*-

arabinoside, malvidin-3-*O*-glucoside and malvidin-3-*O*-arabinoside (Jaakola, et al., 2002). From aforesaid, glycosylated forms of cyanidin and delphinidin constitute the most of anthocyanins in bilberry (Primetta, et al., 2013). Bilberries also contain large amounts of proanthocyanidins, which are located in the pulp and especially in the leaves. Red and green leaves contain relatively high levels of proanthocyanidins, up to 7 % of the dry weight. Therefore, bilberry leaves are also utilized, for example, in plant-based cosmetics (Riihinen, et al., 2008).

Bilberry leaves also contain flavonols up to 1 % of the fresh weight. The main flavonols in the leaves are quercetin and kaempferol, which are believed to contribute to the photo-protection of plants. This explains, why red leaves contain more flavonols than the green ones. The peel and pulp of the fruit also contains quercetin, making quercetin the most dominant flavonol in the plant. Moreover, the peel of the fruit contains substantial amount of myricetin and some isorhamnetin (Riihinen, et al., 2008).

Almost all plants, regardless of origin, contain at least one of the most common hydroxycinnamic acids: *p*-coumaric, caffeic, ferulic, or sinapic acid (Riihinen, et al., 2008). Of these, *p*-coumaric acid, caffeic, and ferulic acid are present in bilberry peel and pulp and in high concentrations in leaves (Riihinen, et al., 2008; Häkkinen, et al., 1999). Bilberry also contains some hydroxybenzoic acids, such as *p*-hydroxybenzoic, and ellagic acid, but only in very low concentrations (Häkkinen, et al., 1999).

3.1.2 Extraction

There are many variables in the extraction: handling of the material (grinding, sieving, drying, freezing), selection of the extraction method (conventional, pressurized liquid, supercritical fluid, pulsed electric field, ultrasonic-assisted, microwave-assisted or enzyme-assisted extraction) and selection of the extraction parameters (temperature, solvent, solid-solvent ratio, extraction time, re-extractions) (Aaby, et al., 2013; Azmir, et al., 2013). Often the extraction method and parameters are selected based on the target compounds to be extracted. For example, more polar methanol extracts anthocyanins slightly better than less polar ethanol. In general, environmentally friendly solvents are still preferred when extracting plant-based materials, although more hazardous or toxic solvents could provide better yields (Azmir, et al., 2013).

Sometimes maximizing the yield of some individual compounds can lead to a decrease in the total yield of extracted compounds. For example, maximizing anthocyanin yield can lead to a decrease in the total polyphenol yield. Therefore, it is important to compromise or choose which compounds to extract (Klavins, et al., 2018; Paes, et al., 2014). The choice of extraction parameters also depends on the form of the material. For example, whole frozen bilberries favor different extraction parameters than bilberry press cakes (residue from juice press) (Klavins, et al., 2018). Therefore, before selecting the extraction method and parameters, it is necessary to determine, what is the target of extraction and what is the part or form of the plant in the extraction. All polyphenols can never be extracted from the material, but the highest possible yield should be sought.

Bilberries are often extracted using conventional or ultrasonic-assisted extraction and aqueous methanol or ethanol as a solvent. Typically, the content of methanol or ethanol in aqueous solutions varies between 50 - 100 %. With small additions of acid (≤ 1 % v/v), for example, acetic acid, citric acid, trifluoroacetic acid or hydrochloric acid, polyphenol yield is often improved because the acid stabilizes some polyphenolic compounds, such as anthocyanins (Azmir, et al., 2013; Klavins, et al., 2018; Denev, et al., 2010; Klavins, et al., 2015). Organic acids are typically preferred over hydrochloric acid because hydrochloric acid can degrade esterified anthocyanins (Denev, et al., 2010). In the next chapters, some extraction methods for bilberries and their derived products are described.

Klavins et al. studied the effect of different solvents on polyphenol content in ultrasonic-assisted extraction. 0.1 g of dried bilberry powder was extracted in 50 ml of aqueous ethanol (0 - 96 %), methanol (20 - 100 %), acetone (20 - 100 %) and dimethyl sulfoxide (20 - 100 %). The sample was treated in an ultrasound bath for 40 minutes and again for 20 minutes. The temperature maintained at 40 °C. After the ultrasonic treatment, the samples were shaken for 24 hours at 140 rpm and treated again in the ultrasound bath for 40 minutes and 20 minutes. Finally, the samples were filtered. The highest polyphenol content was reached using 96 % ethanol, followed by 100 % acetone and 80 % methanol. The highest flavonoid content was obtained using 100 % methanol, followed by 60 % ethanol and 80 % acetone (Klavins, et al., 2015).

Aaby et al. optimized the extraction time and temperature in a conventional extraction method. 5 g of bilberry press cake was mixed with 15 ml of water and homogenized for 20 seconds. The mixture was then extracted using different extraction times (4, 15, 30 and 45 min) and temperatures (22, 40, 60, 80 and 100 °C). The supernatant (aqueous extract) was separated from the press cake residue by centrifuging 10 minutes at 4 °C, 1300 g. Finally, the volume was made up to 25 ml with water (Aaby, et al., 2013).

Aaby et al. reported that the extraction temperature had a greater effect on total content of polyphenols and anthocyanins than the extraction time. The highest polyphenol yield was obtained at high temperature and long extraction time, and the highest anthocyanin yield was obtained at high temperature and short extraction time. However, the effect of temperature was greater on anthocyanin yield than on polyphenol yield, and when combined with the long extraction time, high temperature had a negative effect on the anthocyanin yield. Therefore, the best extraction yield for both, polyphenols and anthocyanins, is obtained when the extraction temperature is high (80 - 100 °C) and the extraction time is short (4 - 15 min) (Aaby, et al., 2013).

In the same study, Aaby et al. also tested ultrasonic-assisted acetone extraction. They homogenized 5 g of bilberry press cake with 15 ml of 100 % acetone. The solution was extracted in an ultrasound bath at 45 kHz for 10 minutes and centrifuged for 10 minutes at 4 °C, 1300 g to separate the bilberry press cake and supernatant. The supernatant was collected and the press cake was re-extracted three times with 10 ml of 70 % aqueous acetone under the same conditions in an ultrasound bath. Finally, the

supernatants were combined and the acetone was removed by a nitrogen flow at room temperature. The volume of the remaining extract was made up to 25 ml with water (Aaby, et al., 2013).

The acetone extracted bilberry press cake had a polyphenol content of 1447 mg GAE/100 g fw (fresh weight) and an anthocyanin content of 458 mg/100 g fw. In the optimized water extraction, bilberry press cake had a polyphenol content of 528 mg GAE/100 g fw and anthocyanin content of 305 mg/100 g fw. Thus, ultrasonic-assisted acetone extraction provided better yields for both polyphenols and anthocyanins than conventional water extraction (Aaby, et al., 2013).

Dinkova et al. used enzyme-assisted extraction for bilberries. One gram of ground bilberry peel was mixed with 50 ml of water, which was acidified to pH 3 with 1 M hydrochloric acid. The mixture was treated in a 50 ℃ water bath for 15 minutes. Then, 5 ml of an acidified aqueous solution (1 % v/v) of the enzyme preparation was added to the mixture. The mixture was incubated for 2 hours at 50 ℃ and then placed in a boiling water bath for 5 minutes to inactivate the enzymes. The extract was then immediately cooled in an ice bath and centrifuged at 4730 g for 15 minutes. Finally, the supernatant was separated from the residue and collected (Dinkova, et al., 2014).

Dinkova et al. reported that enzyme-assisted extraction provided 1.2 – 4.7-fold higher concentrations of individual anthocyanins (excluding galactosides of cyanidin and peonidin) compared to a control extract made without enzyme addition. This is likely to mean that the enzymes help to break down bilberry peel matrix and increase the extraction yield. However, enzyme-assisted extraction altered the proportions of extracted anthocyanins as the concentration of delphinidin glucoside increased and the concentration of cyanidin glucoside decreased compared to the control extract (Dinkova, et al., 2014).

Dinkova et al. also reported that the enzyme addition improved the shelf life of anthocyanins in chilled storage indicating that enzymes may provide higher pigment stability. Also, in bilberry juice production, enzymatic treatment (maceration) increased the yield of anthocyanins. However, enzymatic treatment did not provide better chilled storage of juices. Of the three enzymes tested, Panzym BE XXL, Panzym Pro Color and Pectinex Ultra Color, Pectinex Ultra Color gave the highest extraction yield (811.1 $\pm$ 36.5 mg MGE/l) and the best chilled storage stability for bilberry peel anthocyanins (Dinkova, et al., 2014).

Kerbstadt et al. and Eliasson et al. experimented different drying methods and tested their effect on anthocyanin yield (Kerbstadt, et al., 2015; Eliasson, et al., 2017). Kerbstadt et al. used infrared drying, infrared impingement drying, microwave-assisted hot air drying and freeze drying. They noticed that total anthocyanin content of bilberries ranged from 14 to 44 mg/g dw (dry weight) depending on the drying method, temperature and the end moisture content. The highest total anthocyanin content was obtained when the bilberry press cake was dried by infrared impingement drying at 70 °C to 20 w-% end moisture content (Kerbstadt, et al., 2015).

Eliasson et al. used hot air drying, microwave-assisted hot-air drying and freeze drying. In their experiment, the total anthocyanin content of bilberries varied from 60 to 87 mg/g dw depending on the drying method, temperature and particle size. The highest anthocyanin content was obtained by freeze drying and using sieved particles below 710 µm (Eliasson, et al., 2017). As an extraction method, Eliasson et al. used pressurized liquid (CO_2) extraction and Kerbstadt et al. used supercritical fluid (CO_2) extraction (Kerbstadt, et al., 2015; Eliasson, et al., 2017).

Eliasson et al. placed 5 g of ground bilberry press cake in a 100 ml extraction basket in a pressurized carbon dioxide extractor. The remaining space was filled with glass wool. Extraction conditions of 350 bar and 50 °C were used. 80 % aqueous ethanol was used as a solvent and the purity of the carbon dioxide was 2.8. The extraction solvent and carbon dioxide were added to the extraction basket at a flow rate of 3 g/min for 60 minutes. Finally, the extract was separated from the carbon dioxide in a 500 ml cyclone at 25 °C and 10 bars and recovered (Eliasson, et al., 2017).

Eliasson et al. compared pressurized liquid (CO_2) extraction with conventional extraction using acidified methanol (1 % v/v HCl) as a solvent. In almost every scenario, with different drying methods and particle sizes, conventional methanol extraction (60 – 87 g/kg dw) gave higher anthocyanin yield than pressurized liquid (CO_2) extraction (50 – 85 g/kg dw) (Eliasson, et al., 2017).

3.2 Lingonberry (*Vaccinium vitis-idaea*)

Lingonberries grow in the same regions as bilberries, in Northern Europe, Asia and North America. Lingonberry fruits have a bright or dark red peel, light red pulp and strong acidic flavor (Fig. 3) (Penhallegon, 2006). Like bilberries, lingonberry leaves can be utilized, for example, in food industry, traditional medicine or cosmetics (Bujor, et al., 2018).

Figure 3. *Vaccinium vitis-idaea* berries growing on top of moss (from Pixabay, Alisa Nykänen).

3.2.1 Valuable compounds

Lingonberry fruits constitute mostly flavonols, hydroxycinnamic acids, proanthocyanidins and some anthocyanins (Häkkinen, et al., 1999; Bujor, et al., 2018). Common flavonols are quercetin, myricetin and kaempferol, of which quercetin is the major singular polyphenol in fruits (Häkkinen, et al., 1999; Ek, et al., 2006). Lingonberry fruit also contain some catechins, which are also present in leaves. Arbutin is the major singular polyphenol in leaves as it represents 31 - 50 % of all the polyphenols in them (Bujor, et al., 2018).

Proanthocyanidins appear to be the largest group of polyphenols in lingonberry fruits and leaves (Bujor, et al., 2018). However, due to the different levels of polymerization, they are difficult to analyse and quantify. Anthocyanins are easier to analyse, but they are present in fruit only at low concentrations. This can be also be observed from the color of the fruit as the anthocyanin concentration is highest in dark bluish and blackish berries (Manach, et al., 2004). The three anthocyanins identified in lingonberry are cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, and cyanidin-3-*O*-arabinoside, of which cyanidin-3-*O*-galactoside is the most abundant one (Bujor, et al., 2018; Ek, et al., 2006).

Lingonberries also contain small amounts of resveratrol. Resveratrol occurs only in few edible berries and flower petals, but it is difficult to detect due to small quantities (Petrovski, et al., 2011). In addition, lingonberries contain a lot of hydroxycinnamic acids and some hydroxybenzoic acids such as *p*-hydroxybenzoic acid and ellagic acid. Of the hydroxycinnamic acids, lingonberry fruit contains *p*-coumaric acid, caffeic acid and some small amounts of ferulic acid (Häkkinen, et al., 1999; Bujor, et al., 2018). The leaves contain *p*-coumaric acid, caffeic acid and some small amounts of sinapic acid. Of these, *p*-coumaric acid is the most abundant hydroxycinnamic acid in lingonberries. Overall the fruits contain hydroxycinnamic acids in amounts of 2 - 3 w-% of dried extract and the leaves contain 6 - 14 w-% of dried extract (Bujor, et al., 2018).

3.2.2 Extraction

Bilberry and lingonberry are both *Vaccinium* genus berries and they have many similarities in their shape, structure and polyphenol constitution. Therefore, similar extraction methods and parameters are often used to extract both bilberries and lingonberries (Klavins, et al., 2018; Tian, et al., 2017; Kähkönen, et al., 1999). Hence, only a few examples are given on the extraction of lingonberries.

Many have used ultrasonic-assisted extraction or conventional extraction method for lingonberries (Klavins, et al., 2018; Tian, et al., 2017; Kähkönen, et al., 1999). For example, Tian et al. used ultrasonic-assisted extraction to extract polyphenolic compounds. They ground fresh lingonberries with liquid nitrogen to a fine powder. 4 g of lingonberry powder was mixed with 40 ml of 70 % aqueous ethanol acidified with acetic acid (70:30:1 v/v/v). The mixture was treated in ultrasonication for 30 minutes and shaken for 20 minutes at room temperature. The extract was then centrifuged at 4420 g for 10

minutes and the supernatant was collected. The same method was also used to extract polyphenols from lingonberry leaves (Tian, et al., 2017).

Tian et al. chose aqueous ethanol as the extraction solvent because it can be easily up-scaled and applied in the food industry. By using simple ethanol extraction, they were able to identify 20 individual polyphenols in lingonberry fruits and 18 polyphenols in lingonberry leaves. The identified compounds were mainly flavanols, proanthocyanidins, ellagitannins, anthocyanins, phenolic acids, flavonols, flavones and flavanones. They reported total polyphenol content of 440 mg/100 g fw in fruits and 6138 mg/100 g fw in leaves (Tian, et al., 2017).

Kähkönen et al. used conventional extraction method to treat lingonberries. They mixed 0.5 g of air-dried lingonberries with 10 ml of 70 % aqueous acetone and homogenized for one minute. The extract was then centrifuged at 3000 g for 15 minutes and the supernatant was collected. The extraction was repeated with another 10 ml of the same solvent, centrifuged and the supernatant collected and combined with the first. The supernatants were evaporated to dryness under vacuum and dissolved in methanol. Sugars were removed from the extract in a solid-phase extraction in order to avoid interference in measurements. Total polyphenol content measured from the lingonberries was 24.9 $\pm$ 0.4 mg GAE/g dw (Kähkönen, et al., 1999).

The last example of extraction is the microwave-assisted extraction method. Bujor et al. placed 1 g of lingonberry powder in a vial with a condenser. They added 30 ml of 1 % aqueous citric acid to the vial and treated the mixture in a microwave oven (300 W) for 7 minutes at 40 °C. They extracted lingonberries under the same conditions, also using 55 % aqueous ethanol as a solvent. After the extractions, the extracts were filtered and the volume was adjusted to 30 ml (Bujor, et al., 2018; Bujor, et al., 2016).

Bujor et al. reported small differences in polyphenol contents between extraction solvents. When extracted with aqueous citric acid, the polyphenol content was 13.3 $\pm$ 0.6 mg GAE/g dw and when extracted with 55 % ethanol, the polyphenol content was 15.4 $\pm$ 0.3 mg GAE/g dw. Anthocyanin content, on the other hand, was almost the same between the extraction solvents, 3.44 $\pm$ 0.75 mg/g de (dry extract) with aqueous citric acid and 3.40 $\pm$ 0.49 mg/g de with 55 % ethanol (Bujor, et al., 2018).

Bujor et al. also reported variations in the polyphenol content of lingonberry fruits, leaves and stems harvested in different years. In September 2013, lingonberry leaves contained polyphenols 114.6 $\pm$ 21.1 mg GAE/g dw and in September 2014 99.8 $\pm$ 3.7 mg GAE/g dw. Lingonberry stems contained polyphenols 82.3 $\pm$ 11.8 mg/g GAE dw in 2013 and 77.6 $\pm$ 1.3 mg/g GAE dw in 2014. However, the polyphenol content of the fruits was similar in 2013 and 2014, 13.5 $\pm$ 0.9 mg GAE/g dw and 13.3 $\pm$ 0.6 mg/g GAE dw, although higher concentrations were observed in lingonberry leaves and stems harvested in 2013. (Bujor, et al., 2018).

3.3 Chaga mushroom (*Inonotus obliquus*)

Chaga "mushroom" is a white-rot fungus, which belongs to the *Hymenochaetaceae* genus (Ma, et al., 2013). Chaga is found in Europe, Asia and northern parts of America (Ma, et al., 2013; Glamočlija, et al., 2015; Nakajima, et al., 2007). It lives as a parasite in birch trunks in cold climates and appears as an overgrown dark brown chunk consisting of wood and mycelium (Fig. 4) (Ma, et al., 2013; Nakajima, et al., 2007).

Figure 4. Pieces of chopped chaga (from Pixabay, Melanie Tickell).

3.3.1 Valuable compounds

Compared to bilberry and lingonberry, chaga constitutes from quite different compounds. The main polyphenols in chaga are styrylpyrones, which are not present in berries. Styrylpyrones do not belong to the polyphenolic subgroups presented in Chapter 2, but like other polyphenols, styrylpyrones have large aromatic structures containing one or more aromatic rings (Fig. 5) (Lee & Yun, 2011).

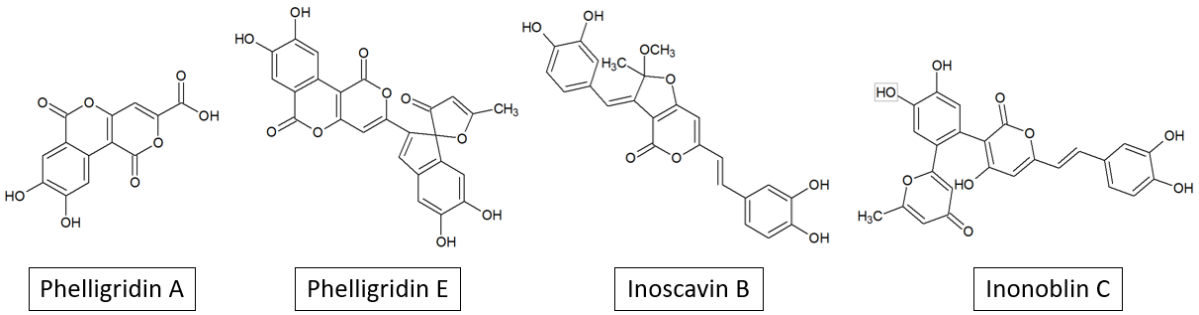

Figure 5. Examples of styrylpyrones found in *Inonotus obliquus* (Lee & Yun, 2011).

The major styrylpyrones in chaga are hispidin, inoscavins, inonoblins and phelligridins (Lee & Yun, 2011). Chaga also contains small amounts of familiar hydroxybenzoic acids, such as vanillic, syringic, protocatechuic, *p*-hydroxybenzoic, and 2,5-dihydroxyterephthalic acid (Glamočlija, et al., 2015; Ju, et al., 2010; Barros, et al., 2009). A few flavonoids, such as naringenin, kaempferol, epigallocatechin, narirutin (flavanone) and fortuneletin (flavone), are also present at low concentrations (Zheng, et al., 2008).

3.3.2 Extraction

The structure of chaga is completely different from that of berries. It consists mainly of two evident parts, black (exterior of mushroom) and brown (interior of mushroom) (Nakajima, et al., 2007). It has a hard wood-like structure, which is why chaga is often ground and extracted at high temperatures (Glamočlija, et al., 2015; Ju, et al., 2010; Hwang, et al., 2019). Sometimes the interior, and exterior part of chaga is extracted separately or only the interior part is extracted (Nakajima, et al., 2007; Hwang, et al., 2019). Conventional extraction methods are commonly used to extract polyphenols from chaga (Glamočlija, et al., 2015; Nakajima, et al., 2007; Ju, et al., 2010; Hwang, et al., 2019; Cui, et al., 2005).

Hwang et al. tested the effect of four different extraction methods on the polyphenol content of chaga. In each extraction, they treated 10 g of interior part of chaga with 400 ml of water. Hot water extract was obtained by treating chaga in a water bath at 100 ℃ for 2 hours. High temperature and pressure extract was obtained by treating chaga in an autoclave at 121 ℃ and 1.1 – 1.2 kg/cm^2 for 2 hours. Enzyme extract was obtained by dispersing chaga powder in water and adjusting the pH to 4.5 with 1 M hydrochloric acid. Viscozyme L. was added 5 % (v/w) to the solution. The mixture was stirred and heated in a water bath at 200 rpm, 50 ℃ for 2 hours. After the extraction, the enzyme was inactivated by heating the mixture at 90 ℃ for 10 minutes. Ultrasonic-assisted extraction was performed by treating chaga powder in an ultrasound bath (42 kHz) at 50 ℃ for 2 hours. Each extract was filtered through Whatman No. 4 filter paper and concentrated with a rotary evaporator at 50 ℃ to a volume of 45 ml. Finally, the extracts were frozen and freeze dried (Hwang, et al., 2019).

Hwang et al. obtained the highest total polyphenol content with high temperature and pressure extract, followed by hot water extract, ultrasonic extract and enzyme extract. The high polyphenol content is believed to result from high extraction temperature. However, when Hwang et al. measured the concentrations of five individual polyphenols (gallic, protocatechuic, 3,4-dihydroxybenzaldehyde, caffeic, and syringic acid), lower extraction temperatures gave better results for some individual compounds than higher temperatures. The highest concentrations of gallic acid, protocatechuic acid and caffeic acid were obtained from the ultrasonic extract with the extraction temperature of 50 ℃ . The highest concentrations of 3,4-dihydroxybenzaldehyde and syringic acid were obtained in the high temperature and pressure extract with the extraction temperature of 121 ℃. Hwang et al. concluded that some individual polyphenols, such as gallic acid and caffeic acid, are more sensitive to high temperatures and can be destroyed when heated (Hwang, et al., 2019).

Glamočlija et al. used conventional extraction method to study the polyphenols Finnish and Russian chaga. 25 g of chaga powder was mixed with 0.5 l of water and heated at 80 °C for 2 hours. The aqueous extract was centrifuged at 3000 rpm for 15 minutes and filtered with Whatman no. 4 filter paper. Finally, the filtrated extract was concentrated to dryness with a rotary evaporator at 40 °C. The residue from the aqueous extraction was further mixed with 70 % ethanol and extracted at 70 °C overnight. The ethanol extract was centrifuged, filtered and evaporated to dryness in the same manner as the water extract (Glamočlija, et al., 2015).

When Glamočlija et al. analysed the total polyphenol content of the extracts, it was found that the 70 % ethanol extract contained 26 - 39 % of all the polyphenolic compounds in Finnish and Russian chaga – although the ethanol extraction was made from the residue of aqueous extract. In the case of antioxidant capacity, the 70 % ethanol extract contained 87 - 95 % of the total antioxidant capacity. This shows the importance of the extraction solvents. If Glamočlija et al. had used only 70 % ethanol as a solvent, they would have obtained a much lower polyphenol content. However, if they had used only water as a solvent, they would have obtained significantly lower antioxidant capacity (Glamočlija, et al., 2015).

Cui et al. also extracted chaga conventionally. They used several solvents in order to separate effective compounds of chaga into different extracts. 100 g of ground chaga was mixed with 80 % ethanol and extracted at room temperature overnight. The supernatant was separated from the residue and freeze-dried (Fa extract). The residue from the 80 % ethanol extraction was mixed with water and treated in a boiling water bath for 4 hours. The aqueous phase was separated from the residue, evaporated to half its volume and mixed with 95 % ethanol in a ratio of 1:4 (v/v) to obtain a precipitate. The mixture was centrifuged and the precipitated fraction was freeze-dried (Fb extract). The resulting supernatant was evaporated to remove ethanol. The remaining aqueous supernatant was mixed with ethyl acetate in a ratio of 2:1 (v/v). The upper ethyl acetate phase (Fc extract) and the lower aqueous phase (Fd extract) were separated, evaporated and freeze-dried. Finally, all extracts were reconstituted with 80 % ethanol (Cui, et al., 2005).

The dried ethanol extract (Fa) contained mostly triterpenoids and steroids, the precipitated and dried extract (Fb) contained mostly soluble polysaccharides, the dried ethyl acetate extract (Fc) polyphenolic compounds and the dried aqueous extract (Fd) remnant polyphenols and low molecular weight polysaccharides. This evidently demonstrates the importance of the solvent in selecting the target compounds for extraction. When the antioxidant capacity of the extracts was measured with three different methods, the Fc extract had the highest value in all cases due to the highest polyphenol content. Also, Fa extract had a high antioxidant capacity due to the presence of triterpenoids and steroids such as lanosterol, inotodiol, trametenolic acid and ergosterol peroxide. However, the Fb and Fd extracts were almost inactive as they contained mainly polysaccharides. As a conclusion, Cui et al. stated that polyphenols have high antioxidant capacity and can protect cells from oxidative stress (Cui, et al., 2005).

4. Verification of quality

In the coming chapters, methods and assays for the measurement of polyphenolic compounds in bilberry, lingonberry, and chaga extracts are presented.

4.1 Total polyphenol content

Total polyphenol content is a common analysis for all plant-based materials. It gives information on the entire concentration of polyphenols. The assay is fast and easy to perform, which makes analysis quite popular in the research of plants and their derived products. Although, the analysis provides good general information, it does not give information on individual polyphenols. In other words, it does not tell what polyphenols the material contains and in what quantities (Waterhouse, 2002; Ainsworth & Gillespie, 2007).

Total polyphenol content is usually determined by Folin-Ciocalteu (F-C) assay. The assay is based on the fact, that in alkaline solutions polyphenols donate electrons to colorimetric reagents, such as the Folin-Ciocalteu reagent. This leads in the oxidation of polyphenols and the reduction of F-C reagent being reduced. When F-C reagent, composed of phosphomolybdic/phosphotungstic acid complexes, is reduced, it turns blue, making complexes possible to be detected in the visible portion of the spectrum. There are many variations on the F-C assay, but the basics (reagents, quantifying method) remain unchanged (Waterhouse, 2002; Ainsworth & Gillespie, 2007). Below, an example of the Folin-Ciocalteu assay is described.

100 µl of extract is mixed with 200 µl of 10 % (v/v) Folin-Ciocalteu reagent. 800 µl of 700 mM sodium carbonate solution is added to the mixture. The solution is incubated at room temperature for 2 hours, and the absorbance is measured with UV-Vis spectrophotometer at 765 nm. Gallic acid (5 – 25 nM) is used as a calibration standard and the results are expressed in gallic acid equivalents per gram of dry weight (mg GAE/g dw) (Ainsworth & Gillespie, 2007).

The F-C assay is not specific for polyphenolic compounds alone. If the extract contains non-polyphenolic compounds that can be oxidized or reduced, this will have increasing or decreasing effect on total polyphenol content (Waterhouse, 2002; Ainsworth & Gillespie, 2007). For example, aromatic amines, high sugar levels, and ascorbic acid can increase the measured total polyphenol content. Therefore, the F-C assay is said to be a measurement of polyphenols and other oxidizing compounds (Ainsworth & Gillespie, 2007). However, the F-C assay is an easy and straightforward assay that gives repeatable results, and is therefore widely used to determine total polyphenol content (Waterhouse, 2002; Ainsworth & Gillespie, 2007).

4.2 Antioxidant capacity

Many polyphenolic compounds, such as flavonoids and phenolic acids, act as antioxidants. Antioxidants inhibit the oxidation of other compounds, leading themselves being oxidized. In animal cells, antioxidants prevent free radicals, such as nitrogen, and oxygen radicals, from damaging

proteins, lipids, and carbohydrates, which are valuable building blocks of cellular structures. They also prevent free radicals from damaging DNA, which can prevent the formation of cancer and other diseases. Thus, antioxidants can improve human health (Uttara, et al., 2009).

The concentration of antioxidants is expressed as antioxidant capacity, reflecting the ability of antioxidants to inhibit oxidation. There are several antioxidant capacity assays, but all are based on oxidation of antioxidants and reduction of radicals. Reduction causes radicals to change color, decolorate or emit light, that can be easily detected even in the visible part of the spectrum (Prior, et al., 2005; Huang, et al., 2005).

In addition to the concentration of antioxidants, the rate of oxidation also affects the antioxidant capacity. Plants contain fast and slow antioxidants with have different reaction rates. Fast antioxidants typically oxidize in 4 minutes in the presence of radicals, but slow antioxidants take longer to oxidize (Prior, et al., 2005). The reaction time required to obtain the overall result can vary considerably depending on the assay (conditions and reagents). Thus, the time spent on redox reactions affects the final result (Prior, et al., 2005; Huang, et al., 2005).

Antioxidant capacity assays are typically divided into two categories based on the oxidation reaction mechanism. These mechanisms are 1) single electron transfer (SET) and 2) hydrogen atom transfer (HAT). The SET method is based on the ability of an antioxidant to donate a single electron and HAT method is based on the ability of an antioxidant to donate a hydrogen atom to reduce free radicals (Prior, et al., 2005; Huang, et al., 2005). The HAT reaction mechanism is typically rapid and solvent and pH-independent. The presence of reducing agents, such as metals, can incorrectly increase the antioxidant capacity of the HAT method (Prior, et al., 2005).

SET and HAT reactions almost always occur together in a balance affected by pH and the structure of antioxidants. Unlike HAT, the SET reaction mechanism is pH-dependent and relatively slow. Due to the slow reaction rate, the antioxidant capacity in SET method is often calculated as a percentage decrease. If long reaction times are used, secondary reactions may interfere with the SET reaction mechanism. Some trace elements, especially metals, can also interfere and cause high variability and poor consistency of results (Prior, et al., 2005).

Common SET-based assays include DPPH (2,2-diphenyl-1-picrylhydrazyl assay), FRAP (ferric ion reducing antioxidant parameter), CUPRAC (copper reduction assay) and ABTS (2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) assay). Common HAT-based assays include ORAC (oxygen radical absorbance capacity), TRAP (total radical trapping antioxidant parameter) and TOSC (total oxidant scavenging capacity) (Prior, et al., 2005; Huang, et al., 2005). In many research articles, SET-based antioxidant assays have been used to determine antioxidant capacity. Below are examples of SET-based ABTS and DPPH assays.

The ABTS assay is based on decolorization of the solution in the presence of antioxidants. ABTS-reagent is dissolved in water to give a concentration of 7 mM. The $ABTS^{\bullet+}$ radical is formed when the reagent solution is further mixed with 2.45 mM potassium persulfate solution. The mixture is allowed to stand at room temperature in the dark for 12 - 16 hours before usage to obtain maximum and stable absorbance. The mixture is then diluted with ethanol to adjust the absorbance to 0.70 ± 0.020 at 734 nm. Samples are prepared by mixing 10 µl of the extract with 1.0 ml of the reagent mixture. After stirring for 6 minutes, the percentage inhibition of the absorbance at 734 nm is measured. Trolox (0 - 15 µM) is used as a calibration standard. Results are expressed in Trolox equivalents per gram of dry weight (mmol TE/kg dw) (Re, et al., 1999).

Like ABTS, DPPH assay is also based on decolorization of the solution in the presence of antioxidants. The $DPPH^{\bullet}$ reagent is dissolved in 80 % aqueous methanol to give a concentration of 1 mM. The solution is further stirred for 40 minutes. The absorbance of the $DPPH^{\bullet}$ solution is adjusted to 0.650 ± 0.020 at 517 nm with fresh 80 % aqueous methanol. The sample is prepared by mixing 50 µl of the extract with 2.95 ml of the reagent solution and incubating for 30 minutes in the dark. Immediately after incubation, the percentage inhibition of absorbance is measured at 517 nm. Ascorbic acid (10 - 100 mg/l) is used as a calibration standard, and the results are expressed in ascorbic acid (vitamin C) equivalents per gram of fresh weight (mg VCE/100 g fw) (Floegel, et al., 2011).

In addition to being based on either the HAT or SET mechanism, antioxidant assays may also be applicable to either lipophilic (fat-soluble) or hydrophilic (water-soluble) antioxidants. Only two assays, ABTS and ORAC, are considered suitable for both lipophilic and hydrophilic antioxidants (Prior, et al., 2005). Due to the different nature of antioxidants (slow, fast, fat-soluble, water-soluble), two reaction mechanisms, and the lack of standardized methods, selection of the assay and measurement of antioxidant capacity can be challenging (Prior, et al., 2005; Huang, et al., 2005). Fortunately, antioxidant capacity is often expressed in Trolox equivalents (TE) or vitamin C equivalents (VCE), which eases the comparison of the assays (Huang, et al., 2005; Floegel, et al., 2011). Admittedly, the results of the same sample can be very different when measured by different assays, even if the results are expressed with the same standard.

4.3 Determination of anthocyanins

There are several ways to determine anthocyanins in plant-based materials, including: 1) total anthocyanin content, 2) individual anthocyanins (anthocyanin profile) and 3) individual anthocyanidins (Lee, et al., 2008). Typically, high-pressure liquid chromatography (HPLC) or ultra-performance liquid chromatography (UPLC) is used in anthocyanin analyses (methods 1 - 3), but also UV-Vis-spectrophotometer can be used in total anthocyanin content (method 1) (Lee, et al., 2008; Grunovaitė, et al., 2016). The advantages and disadvantages of anthocyanin methods are discussed in the following chapters.

Anthocyanin profile provides qualitative and quantitative information on the anthocyanins in the sample. The samples are injected onto an HPLC column, which separates the anthocyanins based on their polarity and interactions with the column material. This causes the anthocyanins to elute to the detector at different times. Each anthocyanin produces a signal on the detector and these signals are constructed into a spectrum. The concentration of individual anthocyanins is determined from the area of the signals in the spectrum (Lee, et al., 2008). Due to the high price and lack of anthocyanin standards, the concentration of individual anthocyanins is often expressed as a percentage of the total anthocyanin yield (Zhang, et al., 2004). Below, an example of the anthocyanin profile determination is described.

The extract is filtered through a 0.45 µm PTFE filter and transferred to a vial. The sample is analysed by HPLC-DAD (diode array detection) using a Beckman Ultrasphere ODS column (5 µm, 4.6 mm × 250 mm) to separate anthocyanins. The mobile phases, A: 0.4 % TFA in water and B: 0.4 % TFA in acetonitrile, are used to pump the sample through the column. The gradient conditions are 0-6 min 15% B; 6-20 min 15-22% B and 20-35 min 22-30% B. Other parameters are 1 ml/min flow rate, 35 °C column temperature, 20 µL sample injection and 5 min post run time. Detection of anthocyanins occurs at a wavelength of 525 nm. The standard mixture is prepared from commercially available standards, cyanidin-3-glucoside chloride, pelargonidin-3-glucoside chloride, peonidin-3-glucoside chloride and malvidin-3-glucoside chloride, by mixing them in 50 % aqueous methanol solution containing 2 % of concentrated HCl (37 %). The concentration of the individual standards in the mixture varies between 0.040 - 0.130 mg/ml. The standard mixture is used to identify anthocyanin peaks, and the results are expresses as a percentage of the total anthocyanin yield (Zhang, et al., 2004).

The anthocyanin profile provides useful information for identifying materials, controlling product quality, and examining the consistency of raw materials. But the unavailability of anthocyanin standards is a major disadvantage as plants have more than 250 anthocyanins and only a few have a commercial standard required to determine concentration of an individual anthocyanin (Zhang, et al., 2004). A more straightforward way to measure the content of anthocyanins is to hydrolyze them to anthocyanidins. This often reduces the number of analyzable compounds to six most common anthocyanidins: cyanidin, delphinidin, malvidin, petunidin, peonidin and pelargonidin, all of which have available standards (Lee, et al., 2008; Zhang, et al., 2004).

Bilberry contains fifteen anthocyanins, and if hydrolysed to anthocyanidins, the number of detectable compounds decreases to five as the sugars are seperated from the aglycones (Primetta, et al., 2013; Zhang, et al., 2004). Lingonberry contains three anthocyanins, and if hydrolysed to anthocyanidins, the number of detectable compounds decreases to one (Bujor, et al., 2018). A smaller number of compounds not only help in the availability of standards, but also eases analytical determination. When a sample consists of several anthocyanins, it is difficult to separate them in a single HPLC run. This can lead to overlapping of signals leaving anthocyanins undetected. On the other hand, if

anthocyanins are hydrolysed to anthocyanidins, information on anthocyanins and their sugar units is lost (Zhang, et al., 2004). An example of hydrolysis is shown below.

Prior to hydrolysis, extraction often occurs using more acidic solvents, such as 50 % aqueous methanol containing 17 % of concentrated HCl (37 %). The acidic extract is filtered and transferred into a vial, which is placed in a preheated dry bath. The sample is hydrolysed in a dry bath at 100 $\pm$ 2 ℃ for 60 minutes, after which it is immediately cooled to room temperature. Anthocyanidins are also analysed with HPLC using similar parameters as in anthocyanin analysis. For example, Zhang et. al analysed both anthocyanins (method described above) and anthocyanidins by HPLC-DAD using only different wavelengths (530 nm) and gradient conditions (isocraticly 18 % B) for the determination of anthocyanidins. A separate standard mixture was also used to quantify anthocyanidins (Zhang, et al., 2004).

Both, the anthocyanin profile and the total anthocyanin content can be determined by HPLC in the same run. To obtain a more accurate total anthocyanin content, each anthocyanin should be calibrated with a corresponding standard and the total content calculated from the sum of the individual anthocyanin concentrations (Lee, et al., 2008). However, this is not practical due to the lack of standards (Zhang, et al., 2004). Therefore, total content is calculated using an external standard, which should be the most abundant anthocyanin of the material studied. For bilberries, this is cyanidin-3-O-glucoside and for lingonberries cyanidin-3-*O*-galactoside (Primetta, et al., 2013; Bujor, et al., 2018; Lee, et al., 2008).

The total anthocyanin content provides quantitative information on anthocyanins as it expresses the concentration of all the anthocyanins in the material. In addition to HPLC, total anthocyanin content can also be measured by UV-Vis-spectrophotometer (Lee, et al., 2008; Lee, et al., 2005). The UV-Vis method is based on monomeric anthocyanins going through reversible structural transformation when pH changes. At pH 1.0, anthocyanins are in colored oxonium form and at pH 4.5, anthocyanins are in colorless hemiketal form. When the absorbance is measured at 520 nm and 700 nm, the difference in absorbance between pH is proportional to the concentration of monomeric anthocyanins. Degraded anthocyanins (in polymeric form) are pH resistant and do not change color, and therefore cannot be detected with UV-Vis-spectrophotometer. As a result, the pH differential assay typically gives too low total anthocyanin content (Lee, et al., 2005).

If the total anthocyanin content determined by HPLC is compared with a content determined by UV-Vis-spectrophotometer, much more accurate and higher values are obtained with HPLC. For example, Lee et al. 2008 found that HPLC (14.09 mg C_3Gl/100 ml) gave almost four times greater result for total anthocyanin content in bilberry juice than UV-Vis-spectrophotometer (3.56 mg C_3Gl/100 ml) (Lee, et al., 2008). Thus, HPLC is a more reliable method for determining the total anthocyanin content, but UV-Vis is still faster, cheaper and easier to use.

4.4 Total proanthocyanidin content

Proanthocyanidins (PACs) are dimers, oligomers and polymers with a wide variety of complex structures. Therefore, the determination of total proanthocyanidin content is difficult and very reliable nor accurate methods are not available (Manach, et al., 2004). However, the proanthocyanidin content is often determined using vanillin, butanol-HCl, or DMAC (4-(dimethylamino)cinnamaldehyde) assay. Proanthocyanidins can be analysed either as proanthocyanidins (vanillin and DMAC assay) or can be depolymerized to anthocyanins by butanol-HCl assay (Krueger, et al., 2016; Feliciano, et al., 2012; Nakamura, et al., 2003; Wangensteen, et al., 2014). The following chapters focus on the DMAC assay in which proanthocyanidins are analysed as proanthocyanidins.

DMAC is a commonly used assay to quantify proanthocyanidins (Krueger, et al., 2016; Feliciano, et al., 2012). The assay is specific for phenolic compounds with meta-oriented di- or trihydroxyphenols, including both proanthocyanidins and flavanols. Thus, the assay is not selective only for proanthocyanidins. However, UV-Vis spectrophotometer at 640 nm is used in the assay, which excludes the spectra of anthocyanins preventing further interference (Feliciano, et al., 2012). Many other assays use lower wavelengths are that allow anthocyanin interference. However, it is common in all proanthocyanin assays that the proanthocyanidin content is underestimated because the assays do not consider different degrees of polymerization (Krueger, et al., 2016; Feliciano, et al., 2012; Nakamura, et al., 2003; Wangensteen, et al., 2014). Below, an example of DMAC assay is described.

DMAC reagent is prepared by dissolving 0.05 g of DMAC reagent in 50 ml of buffer solution (75 % aqueous ethanol containing 12.5 % concentrated HCl). 70 µl of extract is mixed with 210 µl of DMAC reagent in a microplate well. The plate is placed in a UV-Vis microplate reader and mixed for 10 seconds at 600 shakes/min. The absorbance is measured at 640 nm after 30 minutes. Procyanidin A2 dimer (0.005 - 0.03 mg/ml) is used as a calibration standard and the results are expressed in procyanidin A2 equivalents per gram (mg/g) or milliliter (mg/ml) (Krueger, et al., 2016). Also, procyanidin B2 or more affordable catechin and epicatechin can be used as a standard (Payne, et al., 2010).

4.5 Total flavonoid content

Total flavonoid content gives an estimate of all flavonoids in the sample (see Chapter 2). It is typically measured by colorimetric aluminum chloride assay or 2,4-dinitrophenylhydrazine assay (Chang, et al., 2002; Do, et al., 2014). The aluminum chloride assay is specific mainly for flavones and flavonols because they form stable complexes with aluminum chloride. In contrast, the 2,4-dinitrophenylhydrazine assay is more specific for flavanones. Thus, the total flavonoid content does not really represent the total content of all flavonoids. For the most accurate result, it is recommended to use both the aluminum chloride and 2,4-dinitrophenylhydrazine assays and to sum the results (Chang, et al., 2002). Nevertheless, only the aluminum chloride assay is often used to determine the total flavonoid content (Do, et al., 2014). Examples of these two assays are shown below.

In the aluminum chloride assay, 0.5 ml of extract is mixed with 1.5 ml of 95 % ethanol, 0.1 ml of 10 % aluminum chloride solution, 0.1 ml of 1 M potassium acetate solution and 2.8 ml of water. The mixture is incubated at room temperature for 30 minutes. The absorbance of the mixture is measured at 415 nm with UV-Vis spectrophotometer. Quercetin is used as a calibration standard (25 - 100 µg/ml) and the results are expressed in quercetin equivalents by mass of the sample (%) (Chang, et al., 2002).

In 2,4-dinitrophenylhydrazine assay, 1 ml of extract is reacted with 2 ml of 1 % 2,4-dinitrophenyl-hydrazine reagent and 2 ml of methanol at 50 °C for 50 minutes. The sample is then cooled to room temperature and mixed with 5 ml of 1 % KOH in 70 % methanol. The mixture is incubated at room temperature for 2 minutes. 1 ml is taken of the mixture, which is further mixed with 5 ml of methanol and centrifuged at 1000 g for 10 minutes to separate the formed precipitate. The supernatant is then collected and the volume adjusted to 25 ml. The absorbance of the supernatant is measured at 495 nm. (±)-naringenin is used as a calibration standard (0 – 2000 µg/mL) and the results are expressed in (±)-naringenin equivalent by mass of the sample (%) (Chang, et al., 2002).

4.6 Individual polyphenols

Bilberry, lingonberry and chaga contain various polyphenolic compounds that can be identified and the concentration determined. However, in the terms of quality, the focus is typically on polyphenols, which are present in larger amounts. In bilberries, such polyphenols include, for example, quercetin, *p*-coumaric acid and ferulic acid, and in lingonberries, quercetin and *p*-coumaric acid. Both berries are also rich in individual anthocyanins and proanthocyanidins (Riihinen, et al., 2008; Häkkinen, et al., 1999). HPLC or UPLC is typically used to determine individual polyphenols due to resolution and gentle sample handling (Riihinen, et al., 2008; Grunovaitė, et al., 2016). Below, an example of the analysis of individual polyphenols is described. However, the analytical parameters vary greatly depending on the compound to be analysed.

Bioactive compounds are analysed by UPLC-DAD using an Acquity BEH C_{18} column (1.7 µm, 2.1 mm x 100 mm, 1.7 µm) at 40 °C. Mobile phases, A: 1 % formic acid in water and B: 100 % acetonitrile are used to pump the sample through the column. The gradient condition is 0 min 1 % B; 4.5 min 10 % B; 7.5 min 26 % B; 13 min 100 % B; 15 min 80 % B; 16 min 50 % B and 17 min 99 % B. Other parameters are 0.4 ml/min flow rate and 20 µL sample injection. The detection of active compounds occurs at wavelengths of 220 - 500 nm. The UPLC effluent (waste) is introduced to a quadrupole time of flight mass spectrometer (QTOF) equipped with an electrospray ionization probe. The ionization is performed at + 4000 V. Nitrogen is used as a nebulizer gas in 2.0 bar and as a drying gas at flow rate of 10 l/min. MS data is also recorded in ESI negative ionization mode and spectra is acquired by a full scan acquisition covering m/z of 100 - 1500. From the UPLC-QTOF spectrum, polyphenols such as hyperoside, rutin and chlorogenic acid, are quantified using corresponding standards (4.0 - 150 µg/ml) (Grunovaitė, et al., 2016).

At the moment, chaga is still a relatively unexplored and mysterious material. Several research articles on chaga provide qualitative rather than quantitative information on phenolic compounds (Glamočlija, et al., 2015; Lee & Yun, 2011; Zheng, et al., 2008). Some individual polyphenols quantified from chaga are *p*-hydroxybenzoic acid, cinnamic acid, kaempferol, naringenin and narirutin (Glamočlija, et al., 2015; Zheng, et al., 2008). An example of chaga's phenolic acid analysis is described below.

Phenolic acids are determined by HPLC-DAD using a Spherisorb reverse phase C_{18} column (3 µm, 150 mm x 4.6 mm) at 25 °C. Mobile phases are A: 2.5 % aqueous acetic acid, B: 2.5 % acetic acid/acetonitrile (90:10 v/v) and C: 100 % acetonitrile. The gradient condition is 10 min isocratic 100 % A; 10 min 50% A and 50% B; 15 min isocratic 100% B; 10 min 90% B and 10% C; 10 min 70% B and 30% C; 5 min 50% B and 50% C; 5 min 20% B and 80% C and 5 min 100% A, and the flow rate is 0.5 ml/min. Phenolic acids are characterized by DAD at 280 nm, and identified by a mass spectrometer (MS) connected to the HPLC system via DAD cell outlet. MS is equipped with electrospray ionization (ESI) interface. Nitrogen is used as sheath, and auxiliary gas at flow rates of 1.2 and 6 l/min. Capillary and source voltages are 10 V and 3.5 kV and the capillary temperature is 175 °C. Spectra is recorded in negative ion mode covering m/z of 80 - 620. Protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid and cinnamic acid are used as standards for quantification of phenolic acids and the results are expressed as mg/kg of dry weight (Barros, et al., 2009).

5. Conclusions

Nature is filled with plants that are utilized in food, cosmetics, pharmacy and supplements. A group of compounds of particular interest is polyphenols. However, the composition of polyphenols varies between species and within a specie depending on genetic variation, geographical location, growing conditions and growth stage. Also, processing such as drying, chopping, grinding, boiling, pressing, extracting, fermenting and pasteurizing, can change the polyphenol content because it requires mechanical breakage of tissues, heating, pressurization or addition of enzymes and bacteria. Therefore, it is important to show proof that the quality of the plants is maintained even if converted to products. In the worst case, processing can reduce the concentration of valuable compounds or even destroy them.

When the quality of the plant or product is verified, simple and fast methods are often preferred over complicated and expensive ones. This enables enterprises of all sizes to show the proof of their product's quality. However, there are many challenges when estimating the quality of materials and products. The critical step is the selection of appropriate extraction method and parameters because different materials favor different conditions. Therefore, extraction should always be examined and optimized for each specie and product prior the analyses.

As plants consist of hundreds of compounds, it is important to determine, which compounds the plant contains and which compounds are the target of measure. In terms of quality, it is often sufficient to focus on total contents or on individual compounds that are either species-specific or present in high concentrations. Of the analyses presented in the review, total polyphenol content and antioxidant capacity are suitable and recommended analyses for all plants and plant-based products.

Total anthocyanins, total proanthocyanidins and total flavonoids are often measured in berries and derived products. Anthocyanins are specific compound in bilberries and are also present in high concentrations. Therefore, anthocyanins are often analysed from bilberries and derived products. Lingonberry contains a lot of proanthocyanidins and hydroxycinnamic acids. However, due to the complexity of proanthocyanidins, only the total content of proanthocyanidins is often analysed. Lingonberry also contains resveratrol, but it is present in such low concentrations that detection is challenging.

Anthocyanins, proanthocyanidins or flavonoids are not relevant compounds in chaga. The main polyphenols in chaga are styrylpyrones, which can be analysed as individual compounds. However, quantitative analyses of styrylpyrones are challenging and still under development. Meanwhile, total polyphenols, antioxidant capacity, and some phenolic acids can be quantified from chaga.

The purpose of analysing plants and plant-based products is to show proof of quality. The results of total contents and individual compounds can be utilized in product development and commercialization. Showing quality also helps consumers compare products. And nowadays, quality is valued over quantity.

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