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# Genetic and Phytochemical diversity in Bilberry (Vaccinium myrtillus) from a limited Geographical Area

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# Preface

This project exercise was developed by Jens Rohloff, and the work in this exercise has been carried out at Plantebiosenteret at Dragvoll and the Department of Biology at NTNU, campus Gløshaugen in the period autumn 2009 to spring 2011. Responsible supervisor has been Jens Rohloff, who has shared a lot of his time, passion and knowledge. My professional and responsible supervisor on the genetic part, Henrik Jensen has been a great supervisor under the thesis. I want to thank Grete Rakvaag for her time and patience on teaching me the methods used in the phytochemical part and especially the time she spent on helping us in lab. I want also to thank Henrik Berntsen on the time he spent on lab teaching me the procedure for post- and pre-PCR.

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Irene B. Ytterdal

# Abstract

In Norway today no commercial exploitation of the wild growing Norwegian bilberry (*Vaccinium myrtillus*) exists. The market in other regions of Europe, North- and South-America is based on the utilization of cultivated *Vaccinium* species. *V.myrtillus* shows generally a higher content of biochemical compounds with health-beneficial properties. As a consequence of an increasing demand for healthier food, the Nordic Bilberry project started in 2008 with the major goal to find superior clones adapted for different regions with effective production of phytochemicals. In addition, a 4-years Norwegian Bilberry project aiming at cultivation and yield potential aspects, was launched the same year (NFR project no. 184797). The presented master projected was affiliated to this project focusing on phytochemical and genetic diversity in Bymarka.

It is known that life history traits of a plant species influence the clonal diversity and structure within populations. In this thesis different phytochemical methods were used for detection of total phenolics, anthocyanins and antioxidants in berry and plant material from 4 different areas in the geographic restricted area Bymarka, were a total of 80 individuals were collected. Average values detected for total phenols were 490 mg/100 g, 155 mg/ 100 g for anthocyanins and 4 mmol/ 100 g for antioxidants. The results showed little variation among clones in the restricted area. 16 primer pairs for 16 microsatellite loci were tested but only 4 (NA741, NA961, CA421 and CA483) turned out to be of good quality. These microsatellite loci were used to estimate genetic variability within and between populations. Little genetic variation was detected between the different plots, and populations had similar levels of within-population genetic variation. The highest diversity both genetic and phytochemically was found in plot D with berries without any wax layer. This plot was also more genetically different from the other populations than the berries in the same location/area/plot with a wax layer, though no significant differences was found in FST, Heterozygosity observed, Heterozygosity expected or allelic richness ($P<0.05$). The result from this thesis could be used further for improvement of breeding strategies and selection of cultivars with high phenolic contents for production of quality food.

# Content

# 1. Introduction

## 1.1 Ecology

European bilberry, *Vaccinium myrtillus* L., called bilberry is a long-lived low bush that belong to the order *Ericaceae*, subfamily Vacciniaeae. Bilberries grow in temperate woodlands and heaths throughout Europe and produce berries that both animals and humans can eat (Albert et al., 2004) and they have been harvested in centuries by individuals. The color of the berries is deep purple black and it is covered by a wax layer that gives the berries the characteristic blue color (Camp, 1945). Most of the commercial products that can be found in the grocery stores in Norway are imported. Bog whortleberries (*V. uliginosum* L.) is another blueberry species that grows in the wild like *V. myrtillus. V. ulignosum* can be found in Scandinavia, Baltic States, Poland, Belarus, Ukraine, Russia, Germany, Switzerland, France, Italy, Yugoslavia, Bulgaria and Romania. In the early twenties, some of the European countries saw the agronomic value in North American highbush blueberries, and in Germany and Polen cultivars of *V. corymbosum* L. are cultivated. In later years, blueberry cultivation has expanded to non-traditional production countries like Chile, Argentina, Mexico and Spain (Pliszka, 1996; Bañados, 2004; Zadernowski et al., 2005).

The genus *Vaccinium* is believed to origin from South America and spread throughout the world (Camp, 1945). The most common types of sections are *Cyanococcus*, *Oxycoccus*, *Vitis-idaea* and *Myrtillus* (Finn et al., 1991). Bilberries , *Vaccinium myrtillus*, may have derived further from Rocky Mountains of North America (Camp, 1945), and are closely related to other blueberry species such as commercially utilized high bush blueberry species from America (e.g. *V. corymbosum*).

The plant height of bilberry shrubs can be from 10 cm (lowland heaths) to 60 cm (forest), the average height is 25 cm. Bilberries prefer well drained soil with low pH and spreads vegetatively by rhizomes that grow horizontal. The root network from mother to daughter plants can be 5-15 meter in diameter and are important for vegetative reproduction. Bilberries are deciduous, buds are developed in autumn, and in early spring meiosis starts and they produce new leaves and flowers (Ritchie, 1956). The flowers are both insect and self-pollinated, thus self-pollinating has shown to decrease the number of seeds produced compared to cross-pollination (Nuortila et al., 2002). Bilberry are diploid with a chromosome number of 2n=24 (Ritchie 1956). The ripening phase are initiated after seed maturation and

tissue softening, accumulation of flavor compound, aromatic volatiles and pigments occur during ripening phase (Brady, 1987) and development from flower to ripening bilberries, last usually in 8-10 weeks (Sjörs, 1989).

## 1.2 Plant metabolism

Many different definitions of metabolomics have been presented in literature, however the one that provides the best description is: "Metabolomics is a systematic study of the endogenous, small molecule metabolites involved in specific biological processes, providing an assessment of the physiological status of an organism" (Schock et al., 2010). It is an instant picture of the cells metabolites at a given timepoint. The cell's metabolic processes are changing rapidly and can have a reaction time less than 1 second (Dunn and Ellis, 2005).

In plant biology one distinguishes between primary and secondary metabolism. A primary metabolite is an organic substance directly involved in specific biological processes like cell growth, development and reproduction in cells, examples are photosynthesis and respiration and are essential for the plant (Metlen et al., 2009). Compounds like amino and fatty acids, carbohydrates, organic acids and lipids, normally ranging from pmol to mmol levels, can be classified as primary metabolites (Dunn and Ellis, 2005). Uptake and translocation of mineral ions from the soil in plants are essential not just for the plant growth but also for human health including potassium, magnesium, sulfate and nitrogen (Prima-Putra and Botton, 1998). Inorganic nitrogen sources such as nitrate or amides serve as nitrogen sources for protein and amino acid synthesis (Pate,1973), and sulfur is fixed to cystein that is converted to methionine, which is one of the amino acids humans are not capable to synthesize *de novo* (Saito, 2004).

Secondary metabolites are compounds that are not directly involved in essential cellular processes, and they are synthesized by plants and microorganisms. Secondary metabolites include compounds like pigments, aroma, alkaloids and antioxidants (Stafne et al., 2005). The major groups of secondary metabolites in plants are alkaloids, phenolics, terpenes and glycosides. In many cases, secondary metabolites have no vital function for the plant, but have influence on the species development and interaction with the environment. The production of secondary metabolites can be induced by changes in the environment, and give the plants opportunity to respond. Åkerström et al. (2009) showed that light conditions and temperature affect the ripening process, and that there is a context between ripening and

content of secondary metabolites in ripening fruits. There are also shown context between day length, temperature and latitudes, sampling time, nitrogen fertilization and biochemical components in bilberry (Åkerström et al. 2009).

Many secondary metabolites show specific chemical structures and are limited to distinct species (Metlen et al., 2009). If the plant grows in the shadow, this will have an effect on the anthocyanin and antioxidant content in the leaves (Witzell et al., 2003). Many of the secondary metabolites are involved in defense systems against insect herbivores and pathogens, some act as UV-protectants and pollinator attractants (Taiz and Zeiger, 2006). Biological functioning of these compounds has been shown to derive from antioxidative properties and antimicrobial activities.

## 1.3 Nutritional content and phytochemicals

Bilberries are utilized in the food industry, and contain diverse compounds of nutritional value and with health-beneficial properties for humans. The chemicals in the plant show biological activity related to predator and pathogen defense, and are attractants for pollination and seed dispersal (Taiz and Zeiger, 2006). These phytochemicals, or so-called secondary metabolites, comprise phenolics such as flavonoids, anthocyanins, phenolic acids, hydroxycinnamic acids, chlorogenic acid, procyanindins and lignans (Dunn et al., 2005; Kalt et al., 2000; Prior et al., 1998). Other chemicals in bilberries are organic acids (from citrate acid cycle (TCA), sugars, polyols, vitamins and aroma compounds (flavor). The group of flavonoids from bilberry are potent antioxidants, and play an important role to protect against oxidative damage due to their role in scavenging free radicals, so-called reactive oxygen speices (ROS), that can damage biological systems (Dunn et al., 2005).

There are many different types of sugars and acids involved in bilberry primary metabolism having an effect on the berries' taste. Sugars like fructose and glucose are monosaccharides with important functions in cellular respiration. Sucrose is a disaccharide that is derived from glucose and fructose. Glucose is the main product of photosynthesis and start cellular respiration. Sugars are building blocks for the plants. Examples are xylose that is a major component in hemicelluloses, which, together with arabinose and other pentoses, establish the primary cell wall, and bind to pectin forming the middle lamella. Ribose is a sugar important in building RNA molecules, energy carrying molecules such as ATP, NADH and other molecules. Organic acids might also derive from biosynthetic routes other than citric

acid cycle in plants metabolism. Examples on pathways are shikimic acid pathway that is a precursor for the amino acids phenylalanine, tyrosine and tryptophan. Shikimic acid pathway further delivers precursors for phenylpropanoid pathway that produce flavonoids. Citric acid cycle both produce malic acid and citric acid as important intermediates (Campbell and Reece, 2004; Taiz and Zeiger, 2004).

### 1.3.1 Antioxidants

Higher plants that perform aerobic metabolism are all exposed for reactive oxygen species (ROS) and oxygen stress. Oxygen stress arises when antioxidants are not sufficient to neutralize ROS. The plants' defense system is generally activated under cold, drought, pathogen stress, air pollination, UV-radiation in order to scavenge ROS. Such free radicals cause oxidative damage to lipids, proteins and nucleic acids. To protect against the damage of ROS, the cells have developed antioxidant strategies that can neutralize free radicals by transferring electrons to the unstable radicals to stabilize them (Foyer and Noctor, 2005; Prior et al., 1998). Many of the phytochemicals in fruits show antioxidant properties. Researchers have shown that fruits and vegetables contain biological active phenolic compounds, but also other antioxidants such as vitamin A, B, C, E, and carotenoids (Erickson et al., 1996; Kalt et al., 1999b; Wang et al., 1996).

Polyphenols, depending on the substituents of phenolic hydroxyl group, show antioxidant properties and act as radical scavengers of radical breakers, extinguishing strongly oxidative free radicals (Edgecombe et al., 2000).

Flavonoids such as flavones, isoflavones, anthocyanins and catechins posess strong antioxidant capacity increasing proportionally with the number of hydroxyl groups attached to the rings (Blokhina et al., 2001; Cao et al., 1998; Wang et al., 1996).

Halvorsen et al.(Halvorsen et al., 2002) used FRAP (Ferric Reducing/Antioxidant power) and found that the antioxidant content in Norwegian *V. myrtillus* are 8.86 mmol/ 100 g fresh fruit. In Swedish and Polish samples, antioxidant levels were measured to be 8.25 and 8.57 mmol/ 100 g.

### 1.3.2 Flavonoids

Flavonoids are secondary metabolites and one of the largest groups of natural occurring phenolics. Phenolic compounds generally comprise compounds like flavonoids, phenolic acids, hydroxycinnamic acis and lignans. Flavonoids are widely distributed in the plant kingdom and can be found in all plant parts. Approximately 6000 naturally occurring flavonoids are expected to exist in higher plants (Harborne and Williams, 2000). Flavonoids are pigments, but also have other biological functions such as attraction of animals for seed dispersal, and protection against UV-radiation (Jaakola et al., 2004). Flavonoids show antioxidant activity, and can bind metals as well. They are particularly interesting for the food industry due to their antioxidant properties and potential health-beneficial effects. Some of the most common flavonoids are anthocyanidin, cyanidin, delphinidin, peonidin, malvidin, quercetin and catechins (Burdulis et al., 2007a). Environment parameters such as temperature and pH are significant factors affecting flavonoids (Alluis and Dangles, 1999; Stintzing et al., 2002).

Flavonoids are built up of a flavone skeleton built upon C6-C3-C6. One of the aromatic rings derives from phenylalanine, a product from shikimate pathway, and the other from condensation of three malonic acids which is a derivative from malonic acid pathway (Saltveit, 2009). The further synthesis to different flavonoids happens in the phenylpropanoid pathway (Bohm et al., 1998; Marr et al., 1998). The shikimic acid pathway converts carbohydrates derived from glycolysis and the pentose phosphate pathway to the aromatic amino acids. This pathways produces the three aromatic amino acids, phenylalanine, tyrosine and tryptophan. A brief overview over the structures is depicted in Figure 1.1. Since the shikimic acid pathway is not present in humans, the intake of plants in the diet are life essential (Tsao and McCallum, 2009).

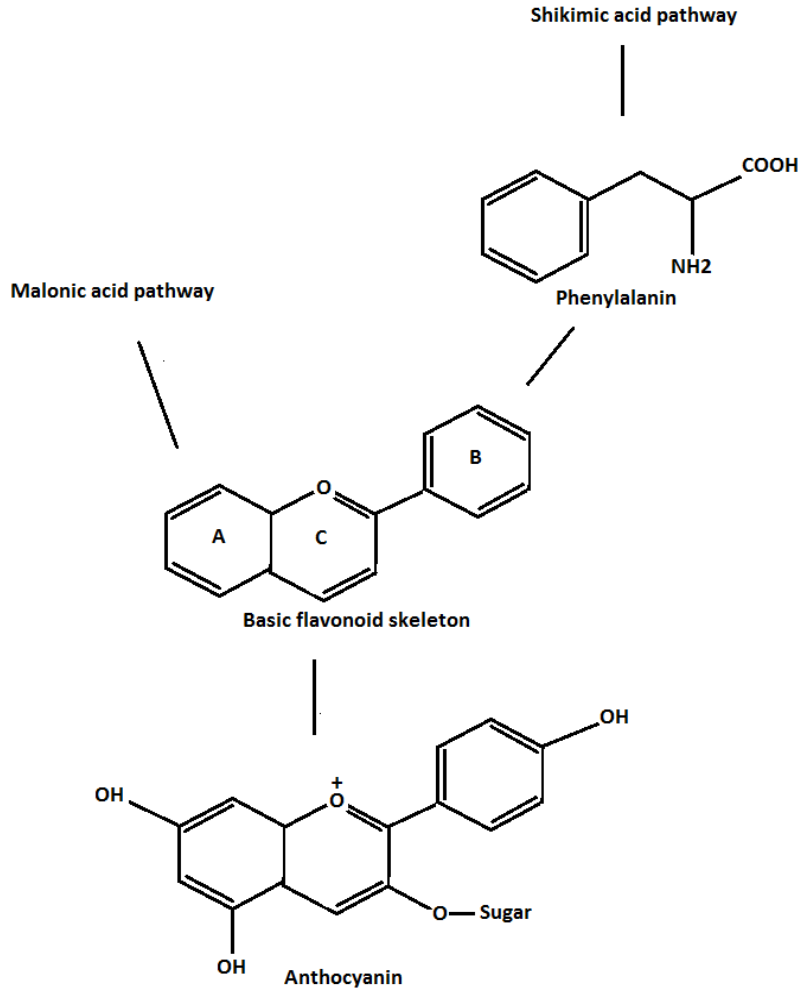


**Figure 1.1: Overview over the structures in anthocyanin biosynthesis. The B ring structure in the basic flavonoid skeleton comes from phenylalanin. The A ring structure is synthesized from three malonic acids from the malonic acid pathway. The C ring is built up with a three carbon bridge. The basic flavonoid skeleton is characteristic for many different flavonoids. Anthocyanins are further synthesized from flavonoid skeleton in several steps.**

The different varieties of flavonoids are specified from the unsaturation, hydrogenation, hydroxylation, methylation and oxidation of the tree-carbon segment in the structure. One or more of the flavonoid hydroxyl groups is bound to a sugar or sugars by an acid-labile hemiacetal bond. Common for flavonoids is that the hydroxyl groups are glycosylated. This makes the flavonoids more water soluble and possible for the plant to store the flavonoids in vacuoles (Blokhina et al., 2001). Four of the major groups of flavonoids are the anthocyanins, the flavones, the flavonols, and the isoflavones (Saltveit,2009; Cook and Samman, 1996). Cinnamic acid is synthesized by elimination of an ammonia molecule phenylalanine. The enzyme phenylalanine ammonia lysase (PAL) catalyzes the reaction. PAL is one important

enzyme for flavonoid synthesized and the enzyme activity increases by stimuli (environmental factors) such as pathogen attack and UV-radioation (Jaakola et al., 2004).

### 1.3.3 Anthocyanins

Anthocyanins are a colored subclass of flavonoids that are pigments with colors ranging from yellow, red, blue and purple. Anthocyanins attract animals for pollination or when fruits are ready to be eaten. The color range from pink to blue are depending on the number and placement of substitutes on one of the aromatic rings and the pH of the cell vacuole where they are stored (Wrolstad et al.,2005).

Anthocyanins are glycosides, which mean that the flavone skeleton contains sugars. Without the sugars these molecules are called anthocyanidins. It is of general interest to measure the anthocyanin content in berries since antioxidant capacities have been directly attributed to their anthocyanin content (Prior et al., 1998). Anthocyanins can also be used as natural food colorants in the food industry (Francis, 1992).

Tests done by Prior et al. (1998) on *V. myrtillus* using the pH-differential method found that the total anthocyanin content in the tested berries was 299.6 ± 12.9 mg/100 g fresh berries. The total phenolics content was measured to be 525 ± 5.0 mg/100 g berries and resembles results by Martinussen and co-workers (2009) found in bilberries in Norway (583.8 mg/100 g (12°C), and 556.8 mg/100 g (18°C). These bilberries were grown at different temperatures showing that total phenolics levels together with sugars (sucrose, fructose and glucose) were higher in plants growing at lower temperatures. Studies carried out by Burdulis et al. (2007b) observed the highest anthocyanin content in berries from northern latitude collected in Russia and Sweden compared to Lithuania. A high content of anthocyanin was also observed in berries collected late in ripening period (August-September) indicating that the anthocyanin level increase as the fruit ripens (Upton, 2001; Suomalainen and Keränen, 1961).

## 1.4 Medicinal properties

Biological functioning of phytochemicals has been shown to derive from antioxidant properties and antimicrobial activities. Antioxidants can neutralize free radicals by transferring electrons to unstable radicals and thus, stabilize them again. Free radicals in the cell can attack important compounds and damage cell structures. Bilberries show high contents of antioxidants (Halvorsen et al., 2002) and a lot of research on the plant's health beneficial properties in humans has been carried out. Both carotenoids and other phytochemicals have been tested on different cancer cell lines, and several anticarcinogenic phytochemicals that can induce detoxification enzymes and prevent initiation of carcenogenesis where found (Azuine et al., 1992; Bagchi et al., 2004; Simic and Jovanovic, 1994). Lutein and zeaxanthin extracts from *V. myrtillus* have been shown to inhibit proliferation on breast cancer cell line MCF-7 and BT-20 (Bomser et al., 1996; Madhavi et al., 1998). Research on the anthocyanins delphinidin and malvidin glycosides *in V. myrtillus* revealed their capability to induce apoptosis in colon cancer cell line HL60, and also inhibition of further growth in colon carcinoma cell line HCT116 (Katsube et al., 2003).

Because of all reported biological activities, and perhaps not yet known beneficial effects attributed to antioxidants in berries, it is of interest to quantitatively determine the levels of antioxidants in berries like *V. myrtillus*. One possibility is to measure the total phenolic content (TPH) with the Folin-Ciocalteu method, a spectrophotometric method. Moyer and co authors (Moyer et al., 2002) studied more than 100 different genotypes of berries (including several *Vaccinium* species*)* to show that TPH is highly correlated with antioxidant capacity.

## 1.5 Nordic bilberry project and market

"Bilberry, toward functional food markets" was founded by the Nordic innovation center (2007-2009)(Häggman et al.,2010). The aim was stimulate companies to cooperate for improvement in wild berry production and utilization and several groups of scientist were set on research the market, quality, biodiversity and traceability of wild berries. 1300 companies dealing with wild berries from Finland, Sweden and Norway were cooperating (Passilta et al., 2009).

Estimates show that bilberry crops in Scandinavia produce approximately 500 million kg per year, and that only 5-10 % are picked and used for food, and about 400 million kg bilberry

are in the nature waiting for the market to be exploited. These resources can be exploited but there are challenges such as logistics (Jaakola et al., 2009) and cultivation techniques.

A major problem in the market today is that products on the market contain a variety of blueberries from different species and the product labeling does not mention this. It is therefore of interest to develop methods to distinguish bilberry from other species. Knowledge about the genome of bilberry is insufficient, and better understanding of the bilberry genome would be beneficial for determining *Vaccinium* species.

## 1.6 *V. myrtillus* genetics

Several studies show that one of the factors influencing chemical content in bilberries beside environmental conditions is genetic variation (Harborne and Williams, 1995; Kalt et al., 2000). For example, qualitative and quantitative variation in phenolic content at different genetic levels have been detected both between and within populations, and in individuals at different physiological and developmental stages (Anttonen and Karjalainen, 2005). The genes underlying chemical content can be activated by environmental signals or stimuli and starts a cascade of reactions that culminate in secondary products. In accordance with this, it has been found in other species such as e.g. spruce trees that the antioxidant and phytochemical concentration are inherent in populations (Lila,2006). Such knowledge can be useful for agronomic purposes.

Genetic diversity of species can mostly be seen between individuals within populations, but there are commonly also often genetic differences among populations of the same species. Individuals within the same species share some percentage of the alleles across all genes. The percentage the individuals of the same species share includes two basic classes of genes. Some are monomorphic within a species and are common to all populations and individuals. Polymorphic loci are the remainders, and vary among individuals in a population and between populations. Differences at polymorphic genes may arise as result of selection due to environmental differences (Husband and Schemske, 1996; Primack and Kang, 1989).

Mutation, migration, natural selection and genetic drift are processes that can lead to changes in allelic frequencies of a population. The allele frequency refers to the frequency of any given allele within a population and is a measure of how common that particular allele is. Genetic drift leads to random changes in allelic frequencies and can produce random genetic changes within populations and thus genetic differentiation among populations. The effect of

genetic drift is largest in small populations. Gene flow occurs when alleles move between populations as a result of dispersal of individuals. Alteration of allelic frequencies of a population can further reduce genetic divergence among populations. Variation in environmental conditions between populations may lead to different optima for different phenotypic characters because these characters' effect on survival and reproduction (fitness) may vary. A relationship between a phenotypic character size or state and fitness is called selection and can also lead to genetic differences among populations when optima differ. This kind of divergence can be especially strong when there is little gene flow between populations. Limited seed dispersal and limited movement of berry eating animals are some examples that can affect the gene flow. Populations that are nearby and are been pollinated by bees may share alleles and have fewer unique alleles in each population, and the populations are more genetically similar. Topography, habitat and physical barriers are also some factors affecting dispersal. Even if variation within a population is low, there may be considerable variability among populations (Russel, 2006). The distribution of genetic variation within and among species is strongly linked to life-history traits, particularly dispersal and reproductive mode (Godt and Hamrick, 1991).

In recent years, developments in molecular biology have resulted in improved methods which may lead to a better understanding of relationships between species, resulting in a more effective and accurate taxonomic classification. This development has also been beneficial for agriculture because it has increased the understanding of importance of genetic diversity in plant breeding. In a study carried out by Albert et al. (2004) on clone diversity and genetic structure in *V.myrtillus* in a geographically limited areas showed high proportion of genetic differences between clones in populations, but low genetic differences between the habitats. Expressed sequence tags-derived polymerase chain reaction (EST-PCR) markers have been shown to be effective in detecting closely related *Vaccinium* species (Boches et al., 2005), and inter simple sequence repeat (ISSR) markers for *V. angustifolium* have recently been developed (Debnath, 2009). Such methods are likely to be very efficient to detect relationships between clones in the same field (Bell et al., 2008).

Polymerase chain reaction (PCR) together with different marker systems have been tested in various species and been shown to be successful to detect genetic differences in different species. A broad variety of molecular markers exists, based on either restriction fragment length polymorphism (RFLPs), random amplification of polymorphic DNAs (RAPDs),

amplified fragment length polymorphism (AFLPs) and microsatellites or simple sequence repeats (SSRs) (Graham and Mcnicol, 1995; Williams et al., 1990).

All methods are beneficial for certain applications, but need to be chosen for the right purpose. However, all are efficient to link phenotypic and genotypic variation in plants. Genetic maps based on RAPD markers have been constructed by Davis and Marquard and their respective co-workers (Davis et al., 1995; Marquard et al., 1997) for diploid blueberries (*Vaccinium* spp.), and been used for fingerprinting and estimating genetic relationships (Bell et al., 2008). The study showed that RAPD markers and AFLP were equally effective in detecting clones in *V. myrtillus* (Albert et al., 2003). Microsatellites, also known as simple sequence repeats (SSR) or short tandem repeats (STR), are short (usually 2-4 bases) repeated sequences flanked with unique DNA sequences and are located in non-coding DNA (Tautz and Renz, 1984). This means that microsatellites usually have high mutation rates. Consequently, they have multiple alleles and are polymorphic (Freeland, 2005). Mutations in the non-coding area of the genes have no influence for the plant's fitness and will therefore be more common than mutations in coding areas.

SSR markers have been used for detecting genetic diversity, fingerprinting, ecological-genetic studies, marker-assisted selection and genetic linkage mapping. They show high information content, co-dominant inheritance, locus specificity, extensive genome coverage and simple detection using labeled primers (Stafne et al., 2005). SSR markers have for example been frequently used for investigation of crop origins. There is need for more reproducible marker systems for blueberries (Powell et al., 1995) as RAPD markers show some limitations because they lack reproducibility between labs due to differences in buffer composition and thermo-cycling conditions. Tag-PCR (EST-PCR) and cleaved amplified polymorphism (CAPS) markers have been developed (Rowland et al., 2003). However, compared to microsatellite or SSR markers, EST-PCR markers are usually not as polymorphic, and there is low throughput in CAPS markers. There have also been designed locus-specific primers to amplify microsatellite loci present in the genes.

Identification of good molecular markers for blueberry genotyping would allow future estimation of inbreeding and mapping of genes underlying phenotypic variation between clones. This will have a significant commercial value. In 2002, the only molecular markers used in lowbush blueberry were RAPD markers (Burgher et al., 2002). EST-PCR markers that were developed for highbush blueberry have been tested in lowbush blueberry for

genetic fingerprinting. Accordingly, Bell et al. (2008) showed that EST-PCR markers developed for highbush blueberry (Rowland et al., 2003), can be used for discerning genetic relationships among species of *Vaccinium*, and be useful for the estimation of intraspesific genetic variability within and among clones of lowbush blueberry.

## 1.7 The aim of the study

The aim of this thesis was to study the genetic and phytochemical diversity in bilberries in a restricted area in Bymarka. The idea for the thesis came from the Nordic bilberry project and the project work done by Jens Rohloff (2009,2010), "Assesment of physiological changes and taste quality of European blueberry (*Vaccinium myrtillus*) using metabolite profiling" (Martinussen et al., 2010), and "Volatile profiles of European Blueberry: Few major players, but complex aroma patterns " (Rohloff et al., 2009). In these projects bilberries from regions of Finland and Norway were harvested from sample fields with high latitudinal differences. Furthermore, there was also interest to study if the phytochemical diversity is coherent with the genetic diversity in bilberries originating from a small region.

Only few reports have focused on microsatellite testing in bilberries, and one part of this study was to examine a set of previously published microsatellite loci to find ones that wouldl be suitable for detecting differences between clones or populations; in this particular case the geographically limited area Bymarka.

# 2 Method and material

## 2.1 Plant collection

Blueberry clones were collected from four different areas in Bymarka: Gråkallen, Trolla, Ramnåsen and Grostadaunet, respectively (locations for the different areas are marked in red in Figure 2.1). Bymarka is a popular hiking area for the residents in Trondheim and area around.

The plant material was harvested in August 2009, by Irene Ytterdal with help from Jens Rohloff. Two plots were selected within each location. Plots covered an area of about 30 $m^2$, and 5 clones were selected in each plot. Mature fruit and leaf material were hand-harvested into tubes and stored in containers with ice elements for transport to the laboratory at NTNU. A description of the different plots is presented in Table 2.1.

**Table 2.1: Description of the location, berry, plant phenotype and day light exposure in the different plots.**

| Plot | Location | Description of location | Berry phenotype | Plant phenotype | Day light exposure |
|---|---|---|---|---|---|
| A | Gråkallen | Near Swamps, power masts and small trees | Small and with wax layer | Small, multiple colors (brown to green) | Almost the whole day |
| B | | Right above a cliff, mostly under pine trees | Medium and with wax layer | High, dark green | Mostly shadow |
| C | Trolla | Plot C and D were in the same place: Above a small lake, in a slope | Medium and with wax layer | Medium size | Mostly in the evening |
| D | | | Medium and without wax layer | Medium size | |
| E | Ramnåsen | In a cliff, right by a ant colony | Big and with wax layer | Medium size | The whole day |
| F | | In a small cliff | Big and with wax layer | Medium size | Moderate shadow |
| G | Grostadaunet | Flat and in a couple of years old logging area | Big and with wax layer | Small, multiple colors (brown to green) | Almost the whole day |
| H | | Same as plot "G", but 20 m more to the south | Big and with wax layer | Small, multiple colors (brown to green) | Almost the whole day |

At arrival in the laboratory the samples were frozen and stored at -30 °C. The selected berries and leaf material was weighed before further processing.


**Figure 2.1: Overview of the four different locations were the 8 different plots were harvested in Bymarka.**

## 2.2 Bilberry Extraction

### 2.2.1 Procedure

1. Approximately 2,000 mg frozen bilberries were crushed with a handblender at high speed and stored on ice in 15 ml microtubes.
2. 180 mg (± 5 %) of the juice were transferred to a microtube (2 ml).
3. 1.8 ml methanol (MeOH) containing ribitol (25 µg/ml) as internal standard for GC-MS, were added to the tube.
4. The mixture was held on ice and shaken for 15 min.
5. Finally, the extract was centrifuged for 5 min (13,000 rpm) at 4 °C and stored in the fridge at -20 °C.

## 2.3 Total phenols

### 2.3.1 Principle

The Folin-Ciocalteu (F-C) method was applied to detect the total content of phenols in the berries . Total content of phenols has been shown to be correlated to antioxidant capacity in plants (Kahkonen et al., 1999; Paixão et al., 2007). Folin and Denis (1912) were the first to develop the method, which was further improved by Singleton and Rossi (1965).

The most common method is to measure the phenolics' ability to react with oxidizing agents. The commercially available Folin-Ciocalteu phenol reagent contains sodium molybdate and sodium tungstate. One aspect to take in consideration is the method's nonspecificity by measuring all phenolic structures in the sample. The colour depends on the hydroxyl groups and their position on the molecules. This reagent produces an intense blue color when reacting with phenols. Julkunen-Tiitto (1985) tested different calibration methods for detection of total phenols, and found that gallic acid was one of the standards to produce the most intense reaction together with the FC-reagent.

The substitution of a hydroxyl group in the position two or three in addition to position four considerably increases the sensitivity of the reaction. When adding an alkali solution, the active compounds are reduced to a compound that produces blue salts which can be measured colorimetrically. Folin and Denis (1912) had earlier shown that the phenol reagent reacts with oxynezol compounds.

### 2.3.2 Procedure

1. 7.5 g waterfree sodium carbonate ($Na_2CO_3$) were diluted in 100 ml distilled water and readily dissolved under stirring.
2. F-C reagens was diluted 1:10 with distilled water. 3 ml F-C reagent was diluted with 27 ml water, and the solution was stored in the dark in a freezer.
3. For the standard curve, different concentrations of gallic acid solutions were prepared. 50 mg gallic acid was mixed with a small amount of EtOH until the powder was dissolved. 100 ml distilled water was then added, and solutions were stored cold. The different standard curve concentrations were mixed as presented in the following table. The different mixtures were all filled up with distilled water, until a total volume of 10 ml was reached.

**Table 2.2: Different standard curve concentrations (GAE: gallic acid equivalent; FW:fresh weight).**

| **Concentration (GAE/g FW)** | 0 | 20 | 40 | 60 | 80 | 100 | 120 |
|---|---|---|---|---|---|---|---|
| **Gallic acid mix. µL** | 0 | 400 | 800 | 1200 | 1600 | 2000 | 2400 |

4. 125 µl F-C-reagent were added on a 96-well microplate, then 25 µL of the different gallic acid concentrations were added, followed by 100 µL of the sodium carbonate. Three different parallels for each concentration and blank were added to the plate.
5. Absorbance was read at 700 nm after 120 min on a spectrophotometer (Labsystems Multiscan MS, type 352). The results were expressed as gallic acid equivalents per gram of fresh weight (GAE/g of FW).
6. The same procedure was carried out with 25 µl berry extract instead of gallic acid.
7. The equation from the gallic acid standard curve was used to find the amount of total phenols in the samples.
8. Weights in mg pr. raw material of berries were divided by the amount of methanol to find the concentration of the berries in the extract. The average for all parallels was calculated, and average values for the blank were subtracted. The last dilution in the well was taken in consideration, and the final values represent total phenols in mg pr. 100 g raw material.

# 2.4 Anthocyanins

## 2.4.1 Principle

Based on change in structural transformation in anthocyanins at different pH (differential method), the total anthocyanin content in the berries can be determined using a colorimetric assay (Moyer et al., 2002). This method was first developed by Sondheimer and Kertesz (1948) who used different pH values for analyses of strawberry jams. Fuleki and Francis (1968a, b) tried this method with buffers using pH 1.0 and 4.5 on cranberries. The anthocyanins produce a colored oxonium form at pH 1.0 and a colorless hemiacetal form at pH 4.5(Wrolstad et al., 2005). The color change is caused by the bisulfate that bind to C-4 position on the anthocyanin chromophore and form a sulfinic acid addition adduct /colorless hemiacetal This conformation change will only happen on monomeric anthocyanins because of the sterical hindrance another phenolic compound make on the polymeric anthocyanins C-4 position (Berke et al., 1998).

To determine the amount of anthocyanins, an absorptive coefficient is required. Several absorptive coefficients exist, including cyanidin-3-glucoside which is one of the most abundant anthocyanins in nature and well applied in similar assays historically (Fuleki and Francis, 1968a). The difference in the absorbance between the two pH are proportional to the anthocyanin content, and the determination is based on Lambert-Beer`s law. The final calculated value is generally expressed as total anthocyanins pr. 100 g raw material.

Content of monomeric anthocyanins were calculated using the coefficient for cyanidin-3-glucoside, and the result were expressed as cyanidin-3-glucoside equivalent per gram of fresh weight (C3-glu equv/ g of FW).

The equation used to calculate the anthocyanin content in the bilberry samples:

$$\text{Absorbance} = (A_{510nm}\ \text{pH 1.0} - A_{700nm}\ \text{pH 1.0}) - (A_{510nm}\ \text{pH 4.5} - A_{700nm}\ \text{pH 4.5})$$

Calculation of the %w/w of total anthocyanins in the sample:

$$\%\ W/W = \frac{A}{\varepsilon L} \times MW \times DF \times \frac{V}{Wt} \times 100\%$$

(Equation 1)

Where:

A = Absorbance
ε = C3-glu molar absorbance (26,900) (Giusti and Wrolstad,2010)
MW = anthocyanin molecular weight (449.2)
DF = dilution factor
V = final volume (ml)
Wt = sample weight (mg)
L = cell path length (usually 1 cm)

### 2.4.2 Procedure

The protocol for buffer mixing handed out by Grete Rakvaag, was adjusted with regarded to sample size:

1. Buffers with different pH were prepared.
    a. pH 1.0: 0.93 g of KCl were mixed and dissolved in a beaker glass with 490 ml distilled water. pH was measured and adjusted with HCl. When correct pH was reached, distilled water was added to the glass to a total volume of 500 ml.
    b. pH 4.5: 27.2 g of $CH_3CO_2Na \cdot 3\ H_20$ were mixed and dissolved with 480 ml disstilled water in a beaker. pH was measured and adjusted with HCl. When correct pH was reached, distilled water was added to the glass until the total volume reached 500 ml.
2. The blueberry extract was diluted further, until an absorbance value below 1 was reached. In order to reach the desired values, all extracts were diluted 1:26.
3. Each sample cuvette was filled with 1 ml of the buffer and 5 µl bilberry sample. Both the buffer with pH 1.0 and pH 4.5 were used, respectively.
4. The absorbance was measured at two different wavelengths in a spectrophotometer (Biorad Smartspec $^{TM}$ plus spectrophotometer, USA) using wavelengths 700 nm and 510 nm
5. Distilled water was used as blank sample.
6. The measured values were used in further calculation to determine the total anthocyanin content pr. 100 g raw material.

## 2.5 Ferric Reducing/Antioxidant Power (FRAP)

### 2.5.1 Principle

FRAP assay measures the reducing capabilities of antioxidants. The method was developed by Benzie and Strain (1996), and was performed with some modifications done by Faria et al. (2005). FRAP assays are inexpensive, simple to prepare and the results are highly reproducible (Xu et al., 2007).

The reduction of ferric ($Fe3^{+}$) to ferrous ($Fe2^{+}$) ion at low pH (3.6) causes the formation of a ferrous tripyridyltriazine ($Fe2^{+}$-TPTZ) complex with a blue color which absorbs at 593 nm (Benzie and Strain, 1996). The absorbance changes are linear when comparing with antioxidant mixtures. Antioxidant content can be read out from the standard curve, and the results are expressed as mmol/l (mM).

### 2.5.2 Procedure

1. Acetatebuffer: 3.1 g $C_2H_3NaO_2 \cdot 3H_2O$/L was mixed with 16 ml $C_2H_4O_2$/l, the buffer was adjusted with HCl until pH 3.6 was reached.
2. 10 mmol/l TPTZ were diluted in 40 mmol/L HCl, and stored cold.
3. A 20 mmol/L $FeCl_3 \cdot 6 H_2O$ solution was prepared and stored cold.
4. The FRAP-reagent was made by mixing 25 ml acetatebuffer, 2.5 ml TPTZ and 2.5 $FeCl_3 \cdot H_2O$
5. 10 µl blueberry extract and 300 µl of the pre-made FRAP-reagent were transferred to a 96-well microplate.
6. The absorbance was measured spectrophotometric (Labsystems Multiscan MS, type 352) after 4 min reaction time at $\lambda$= 595 nm.
7. Blank probes were made by mixing 10 µl water in the well instead of extract.
8. Standard curve was made by mixing aqueous solutions of known $Fe2^{+}$ concentration in the range between 0 to 1500 µmol/l $FeSO4 \cdot 7H_2O$.The results were read out after a reaction time of 4 min and expressed in mmol/l (mM) $Fe2^{+}$.
9. The equation from the standard curve was used to calculate the amount of antioxidants in the samples.
10. Amount of mmol/100 g raw material were found by using the same procedure for total phenols except mg were transformed to mmol/L.

## 2.6 Gas chromatography-mass spectroscopy (GC-MS)

### 2.6.1 Principle

Gas chromatography-mass spectroscopy (GC-MS) is a frequently applied analytical technique in chemistry and biology for identifying compounds in a mixture. It is a combination of gas chromatography which separates the components of a mixture in the gas phase, and mass spectroscopy which characterizes each of the separated components individually (Eiceman et al., 2000). The computer records the compounds' mass spectra by m/z ratios as x-axis and intensity as y-axis. Since a given chemical compound produces a specific mass spectrum, identification of unknown chemicals in the sample mixture can be done by comparing with a spectral library, or by the use of reference compounds. AMDIS (Automated Mass Spectral Deconvolution and Identification System) is a program that helps to deconvolute the GC-MS data files and makes it possible to find and identify all of the separated components. It is further possible to connect AMDIS to libraries by e.g. using the NIST Mass Spectral Database (NIST). This method was applied to identify sugars, acids and secondary metabolites in the berry samples.

### 2.6.2 Procedure

1. Sample material already extracted containing the internal standard ribitol was used (180 mg sample in 1.8 ml methanol 80 % (25 µg ribitol pr. ml MeOH).
2. 0.75 ml of the thawed extract was pipetted into a microtube and vortexed in 10 sec.
3. Sample extracts were dried in a high-capacity SpeedVac concentrator (Savant SC210A, Thermo Electron Corp.) over night without heating, and stored at -80 °C before derivatization.
4. The dried samples were re-dissolved in 80 µl of 20 mg/ml methoxyamine hydrochloride in pyridine, and treated at 30 °C for 90 min in an incubator shaking at 200 rpm.
5. Samples were derivatized with 80 µl of MSTFA (N-Methyl-N(trimethylsilyl) trifluoroacetamide) at 37 °C (shaking incubator, 200 rpm) for 30 min.
6. Samples were then transferred to 1.5 ml autosampler vials with glass inserts and stored at -20 °C prior to GC-MS.
7. GC-MS analyses were performed by Jens Rohloff. A Varian Star 3400 CX, Saturn 3 were used for all analyses. Analysis time was 63 min pr. sample. Sample volume was 1 µL with a split ratio of 25:1. GC separation was carried out on a HP-5MS capillary

column (30 m × 0.25 mm i.d., film thickness 0.25 µm). The injection temperature was set to 230 °C, and the interface at 250 °C. The carrier gas (He) was maintained at a constant flow rate of 1 ml/min. The GC temperature program was held isothermically at 70 °C for 5 min, further ramped from 70 °C to 310 °C at a rate of 5 °C min, and finally held at 310 °C for 7 min. The MS source was adjusted to 230 °C and a mass range of *m/z* 50-550 was recorded. All mass spectra were acquired in EI mode. Detected compounds were quantified based on the internal standard ribitol and expressed as µg/100 g fresh weight.

8. AMDIS software (v.2.64) was used for mass spectra evaluation and peak identification. AMDIS was used in combination with the spectral library NIST05 database and authenticated reference spectra from the *Golm Metabolome Database* (Kopka et al., 2007).
9. MeV 4.6.2 (MultiExperiment Viewer) was used for hierarchical clustering based on a subset of 13 detected metabolites together with values for total phenols, anthocyanins and antioxidants. For this purpose, log2(n) ratios were calculated for each metabolite or metabolite group based on the corresponding median from all samples.

## 2.7 DNA-extraction

Total DNA was extracted from bilberry plant material using QIAGEN DNeasy® Plant mini kit (QIAGEN Inc., Valencia, CA, USA).

## 2.8 Microsatellites

### 2.8.1 Principle

16 primer pairs (forward and reverse) for 16 microsatellite loci were chosen from literature. Since no primers had been developed for *V. myrtillus*, primers that were successfully applied for other *Vaccinium*-species were chosen (Boches et al., 2006; Boches et al., 2005). The primers were preliminarily tested for amplification success, variability and genotype quality (Table 2.3). Only 4 of them were variable and of good quality and thus chosen for further analysis. NA741, NA961, CA421F and CA483F were the 4 microsatellite loci used for final analysis of all samples. The primers were ordered from either Invitrogen or Applied Biosystems.

**Table 2.3: The 16 primers with description of the quality and reference**

| Locus | Amplification | Variability | Quality | Reference |
|---|---|---|---|---|
| CA23F | Yes | Monomorphic | - | Boches et al. 2005 |
| CA112F | No | - | - | Boches et al. 2005 |
| CA169F | Yes | Monomorphic | - | Boches et al. 2005 |
| CA344F | Yes | Monomorphic | - | Boches et al. 2005 |
| CA421F | Yes | 7 Alleles | Good | Boches et al. 2005 |
| CA483F | Yes | 4 Alleles | Good | Boches et al. 2005 |
| CA787F | Yes | Monomorphic | - | Boches et al. 2005 |
| CA794F | Yes | Unspecific amplification | - | Boches et al. 2005 |
| CA94F | Yes | Monomorphic | - | Boches et al. 2005 |
| NA398 | Yes | Monomorphic | - | Boches et al. 2005 |
| NA741 | Yes | 2 Alleles | Good | Boches et al. 2005 |
| NA961 | Yes | 3 Alleles | Good | Boches et al. 2005 |
| NA1040 | Yes | Unspecific amplification | - | Boches et al. 2005 |
| VCC_I2 | Yes | Monomorphic | - | Boches et al. 2005 |
| VCC_I8 | Partly (5/8) | 2 Alleles, but only homozygotes | Null alleles | Boches et al. 2005 |
| VCC_J5 | Partly (5/8) | 3 Alleles? | Can be optimized | Boches et al. 2005 |

### 2.8.2 Procedure

1. All primers were mixed with MilliQ water in Eppendorf tubes to make primer stock solutions of 100 µM concentration. To make working-concentrations (10 µM) for each primer one tube for each of the primers was filled with 18 µl $dH_2O$ and 2 µl of the respective primer stock solution (100 µM).
2. The tubes were vortexed and centrifuged in 10 sec.
3. Each of the microsatellite loci was tested individually.
4. For each microsatellite locus and sample 1 µl of the forward primer and 1 µl of the reverse primer was added to the well of a 96-well plate, together with 3 µl DNA from the sample and 5 µl QIAGEN multiplex solution (QIAGEN, Hilden, Germany).

## 2.9 Polymerase chain reaction (PCR)

The individual alleles at each microsatellite locus were then amplified by PCR. PCR was carried out using GeneAmp PCR system 9700 (Applied Biosystem, Foster City, CA). The following PCR profile was used: initial denaturation at 94 °C for 15 min followed by 12 cycles of 94 °C for 30 s, initially a temperature of 62 °C for 90 s (then decreasing by 1 every cycle), and 72 °C for 60 s. This was followed by 23 cycles of 94 °C for 30 s, 50 °C for 90 s and 72 °C for 60 s. Finally, samples were held at 60 °C for 5 min, and then at an indefinite hold at 4 °C

After PCR:

1. 1.0 µl of the PCR product was transferred to a new 96-well plate (made especially for use in the ABI 3130xl, Applied Biosystems).
2. A mixture with 1000 µl Hi-Di Formamide (provided by ABI-technicians, Applied Biosystem) and 50 µl LIZ (GeneScan 600 LIZ size standard, Applied Biosystem) was made.
3. 10 µl of the Formamide-Liz solution was mixed with PCR-products in each well in the new 96-well plate by pipetting up and down approximately 10 times.
4. The 96-well plate was further centrifuged to avoid bubbles in the mixture prior to fragment analysis (electrophoresis).

# 2.10 Genotyping

1. Electrophoresis of the PCR products was carried out on a 16 capillary 3130xl Genetic Analyser sequencing machine (Applied Biosystems) by a technician.
2. Genotypes were scores using GeneMapper 4.0 (Applied Biosystems) by Henrik Jensen.

# 2.11 Statistical analysis

## 2.11.1 Principle

### 2.11.1.1 Estimates of genetic diversity

Because no data for weather, soil, temperature and other factors influencing on bilberry growth was recorded, and due to the low number of sampled individuals, the factors effect on level of genetic diversity could not be considered. Genetic variation can be partitioned into variation within and among populations.

Genetic variation within populations: By dividing the number of heterozygotes at a particular locus by the number of individuals sampled the observed heterozygosity ($H_o$) is calculated. Expected heterozygosity ($H_{exp}$) is the corresponding frequency of heterozygotes expected when the population is in Hardy-Weinberg equlibium (HWE; see below). Statistical tests based on a comparison of $H_o$ and $H_{exp}$ were used to test whether loci were in Hardy-Weinberg equilibrium or not. For example, if $H_o$ is lower than $H_{exp}$ this suggests the presence of null

alleles at this locus. We used the average $H_{exp}$ across all loci as a single estimate of genetic diversity for each population.

Genetic variation between populations: Wright's inbreeding coefficient ($F_{ST}$) and Nei's coefficient of gene variation ($G_{ST}$) are statistics that quantify the genetic differences among populations. These indices are functions of how heterozygosity is partitioned within and among populations, based on differences in allele frequencies (Wright 1969). Values of $F_{ST}$ and $G_{ST}$ vary from 0 to 1 (Nei 1975; Crow 1986). Low $F_{ST}$ (near 0) means that the majority of variation is within populations, whereas high $F_{ST}$ value (approaching 1) means that individuals within a population are relatively similar but that populations are genetically different. $F_{ST}$-values up to 0.05 suggest little differentiation, between 0.05-0.25 moderate genetic differentiation, and $F_{ST}$ values above 0.25 indicate pronounced levels of genetic differentiation (Freeland, 2005). $F_{ST}$ and $G_{ST}$ are used in similar fashion as indices of genetic differentiation among populations, but the most commonly used is $F_{ST}$ (Crow 1986).

**2.11.1.2. Hardy- Weinberg equilibrium**

The Hardy-Weinberg law states that in large, randomly mating population, free from evolutionary forces, following Mendelian rules for inheritance, the allelic frequencies do not change and the genotypic frequencies stabilize after one generation. The Hardy-Weinberg equilibrium refers to a stable state in such a population, where the genotype and allele frequencies will not change from generation to generation.

Hardy- Weinberg equilibrium (HWE) was checked using exact test based on Markov chain algorithm implemented in the program GENEPOP version 3.4 (Raymond and Rousset, 1995) with Markov chain parameters of 10,000 demomorazation steps followed by 1,000 batches of 10,000 iterations per batch. Bonferroni correction (HE/ number of individuals) was used to evaluate the significance of any deviation from equilibrium.

**2.11.1.3 Analysis of variance (ANOVA)**

ANOVA was used to test the null-hypothesis concerning whether means were equal when several populations are compared. In an ANOVA, the independent variable predicts the value of a dependent variable, and the variables should be normally distributed. The test also estimates explained variation among populations, and unexplained variation, within the populations. T-test or an simple one-way-ANOVA can be used when only two means exist. When no significant differences in the first test are calculated, a post-hoc test is applied in the

next step. A post-hoc test is similar to a series of T-tests but more robust by decreasing the probability of getting significant results by chance. When the *P*-value is lower than 5 % ($P \leq 0.05$), significant differences between the populations exist.

#### 2.11.1.4 Procedure

Genetic diversity within the populations was estimated by: allelic richness (AR) and expected heterozygosity. Allelic richness is a measure of the number of alleles (genetic diversity) that takes into account uneven sample sizes by performing rarefaction, and is hence a statistic that is comparable across samples. Allelic richness and the number of allels (NA) were calculated for each locus using FSTAT 2.9.3.2 (Goudet, 2001) (for AR). Expected and observed heterozygosity was calculated for each population using GENEPOP version 3.4 (Raymond and Rousset, 1995).

The level of genetic differentiation among the populations was assessed as $F_{ST}$ (Wright, 1965). $F_{ST}$ values were estimated using the Hierfstat package in R (Goudet, 2005). $F_{ST}$ was estimated both globally, between all pairs of populations, the different location, and the individuals against plot D. To evaluate the significance of the pair-wise tests, the program used randomizations (1,000 times) for the genotypes.

Mantel's test was used to test for genetic isolation by distance (Slatkin, 1993). The Mantel test tests for independence between $F_{ST}$ estimates and geographical distances by using the regression of a matrix of $F_{ST}/1 - F_{ST}$ values vs. the log of distance between samples. The Mantel test implemented in GENEPOP version 3.4 was used (Raymond and Rousset, 1995).

A one-way ANOVA test was performed together with a post-hoc test on the total phenolics, total anthocyanins, antioxidants and $F_{ST}$ values to investigate significant differences between the plots. Tukey`s least significant difference (LSD) was set to 0.05 (95 % confidence level). *P*-values lower than 0.05 were considered to be significant. ANOVAs were run in Minitab 16.1.0 (Minitab Inc.)

# 3 Results

## 3.1 Chemical analysis

### 3.1.1 Total phenols

Total phenols were determined by spectrophotometric analysis. The results are presented in Figure 3.1 showing the average values and standard deviation for each sample plot (A to H). A relative high variation in phenol content in the different plots was observed. The lowest amount of phenols was found in berries from plot E, followed by plot D, G and H. Plots A, B, C and F showed highest content of total phenols with average levels above 500 mg/100 g. No significant differences could be detected.

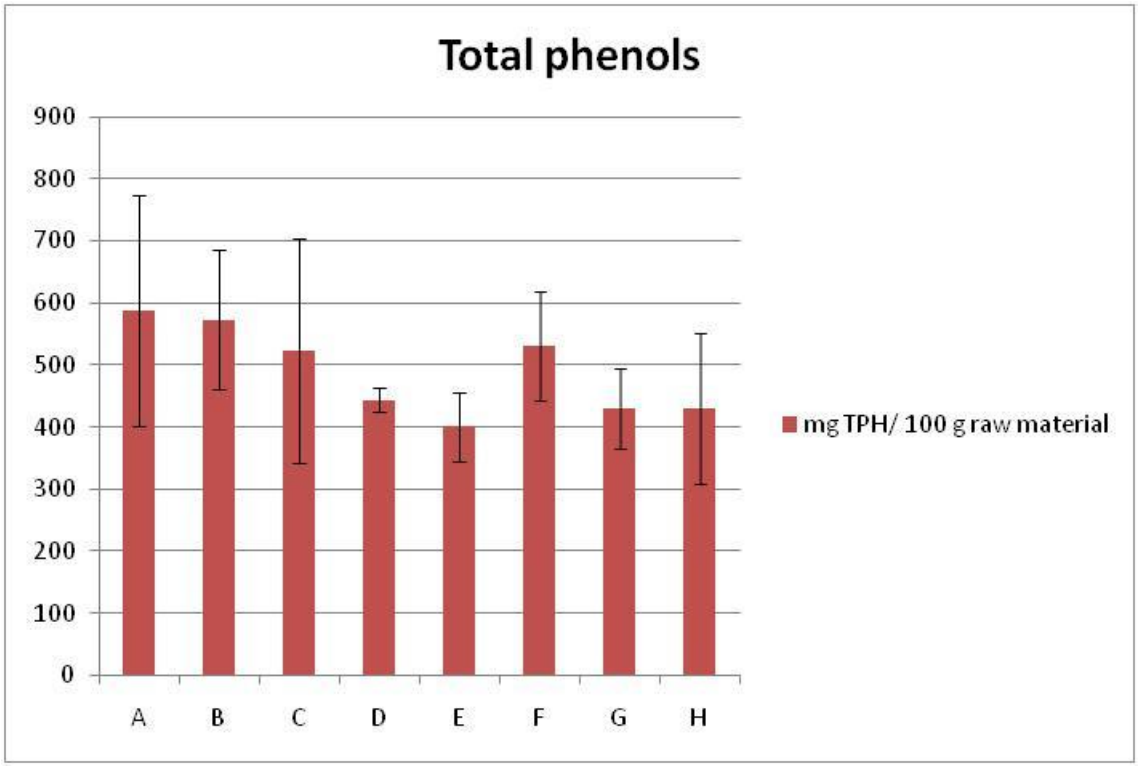


**Figure 3.1: Total phenols (TPH) found with Folin-Ciocalteu method. Bars represent mean values of total phenolic content per 100 g of raw material for each plot and standard deviation (*P*=0.105)**

### 3.1.2 Total anthocyanins

Total anthocyanin content in bilberries was detected using spectrophotometric assays. The average anthocyanin content in the berries from each sample plot was calculated together with standard deviation, and is presented in Figure 3.2. The highest anthocyanin contents were detected in plot D and C (above 150 mg/100 g). All other samples showed similar levels of anthocyanins with relatively high standard deviation. No significant differences were detected.

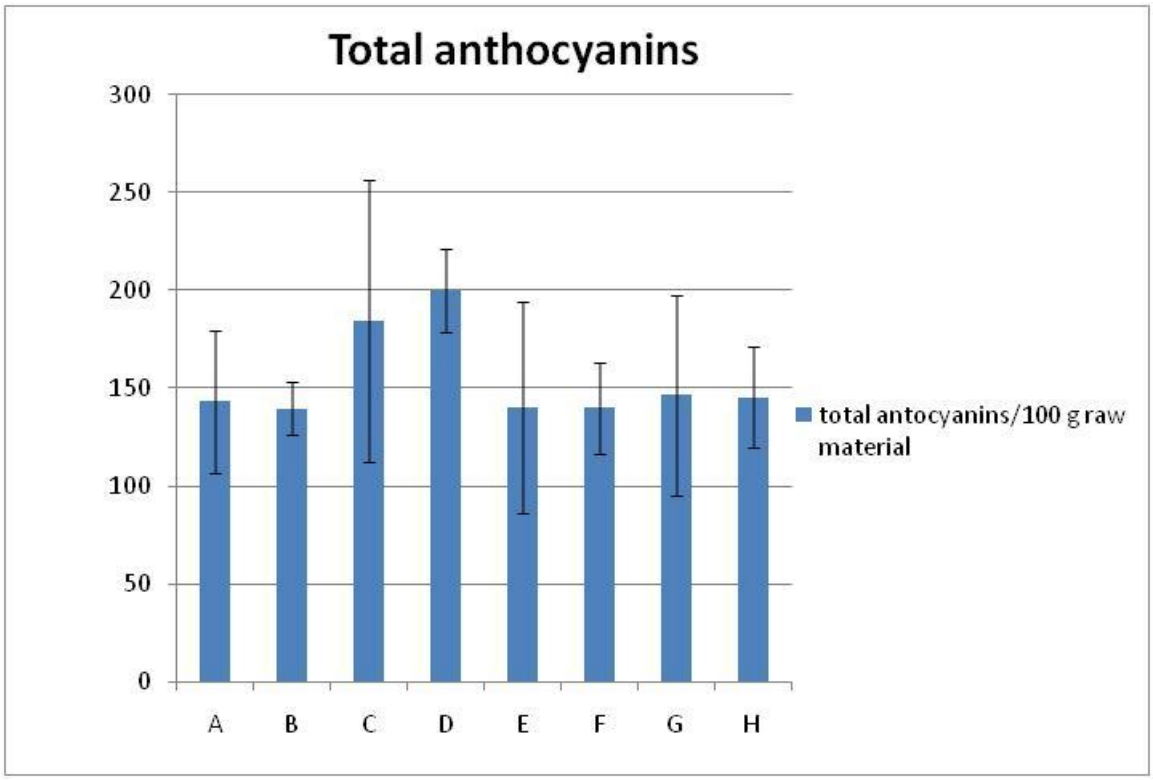


**Figure 3.2: Total anthocyanins found with pH-differential method. Bars represent mean values per 100 g/ raw material for each plot and standard deviation (*P*=0.169).**

### 3.1.3 Antioxidants

Antioxidants were measured spectrophotometrically by detecting the ferric reducing capabilities of the berry juice. The average and standard deviation of total antioxidant content in each of the sample plots is presented in Figure 3.3.

The highest contents of antioxidants were observed in berries from plot A, B, C, and F with levels of 4 mmol/100 g and above. Clearly lowest antioxidant capacity was found in samples from plot E. No significant differences were detected (*P*=0.437).

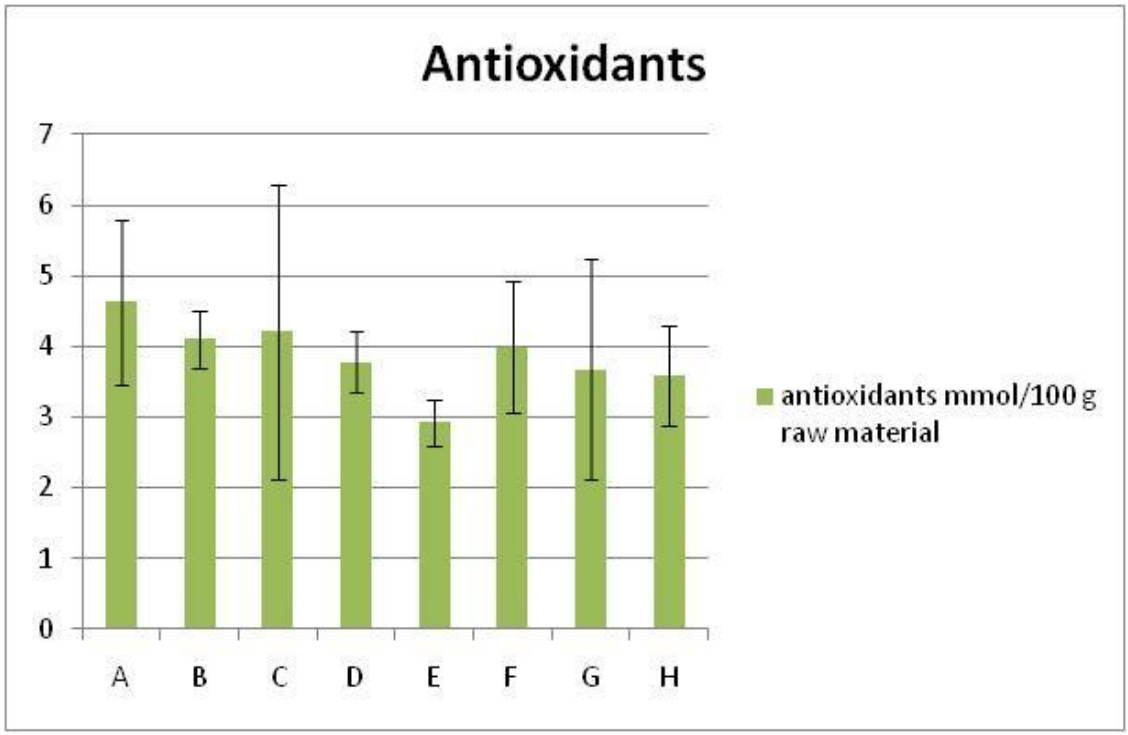


**Figure 3.3: Electron-donating antioxidants were determined by the FRAP assay. Bars represent mean concentration per 100 g raw material in each plot and standard deviation (*P*= 0.437).**

### 3.1.4 GC-MS

The data from GC-MS was analysed using AMDIS in combination with NIST database and *Golm Metabolome Database search*. The compounds that were most abundant and showing highest peaks were chosen. Sugars selected were xylose, arabinose, ribose, fructose, sucrose and glucose. Detected acids were shikimic acid, malic acid, citric acid and quinic acid. Detected polyols comprised myo-inostitol, and chiro-inositol; Chlorogenic acid (3-O-caffeoylquinic acid) was the only phenolic structure included.

The log2(n)-ratios of the detected compounds together with those for antioxidant, total phenols and total anthocyanins were analysed by hierarchical clustering (HCL) using the software tool MultiExperiment Viewer (Figure 3.4). The program clusters the probes that are most common from logarithmic analysis. The results are presented in Figure 3.4.

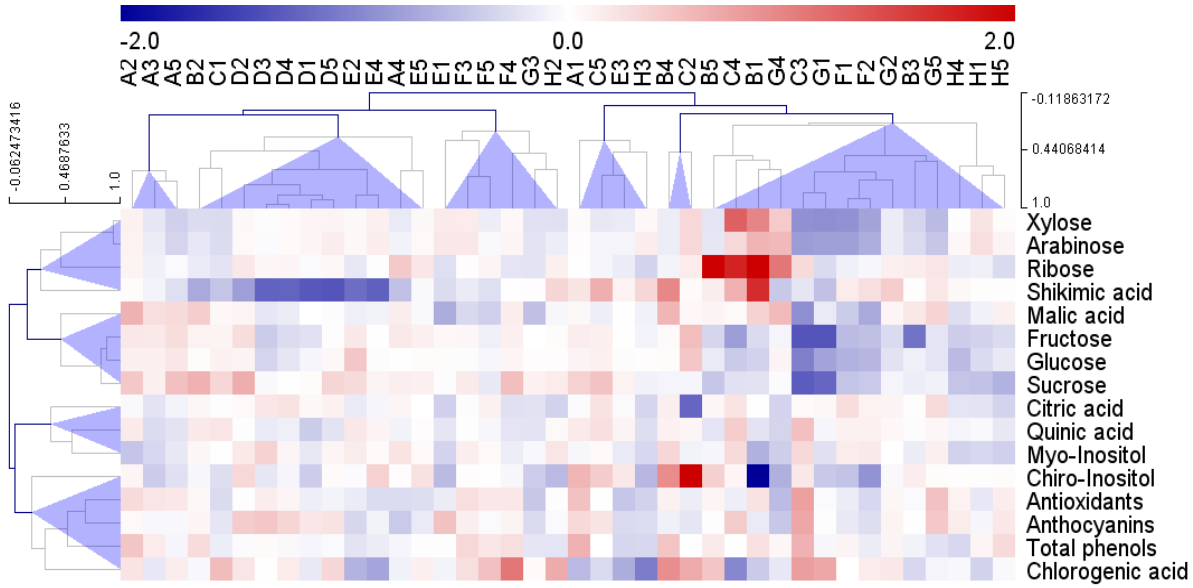


**Figure 3.4: Hierarchical clustering carried out in MeV using a log-value range from -2.0 to 2.0. Dark red color indicate higher and blue indicates lower compound levels.**

Based on statistical analysis when comparing plots significant differences could only be detected for metabolites shikimic acid, quinic acid and malic acid ($P$<0.05).

Based on the chosen settings, the samples clustered into two sub-clusters. The first sub-cluster (left) was divided into three groups: one distinct group clustering three samples from plot A (Gråkallen), the second comprised all samples from plot D (Trolla) together with different other samples, and the third sub-cluster established a group of five samples from the same area (Ramnåsen; E and F). The sample sub-cluster on the right established one big group comprising 14 samples from different locations, among others seven samples from the same area (Grostadaunet; G1, G2, G4, G5, and H1, H4, H5). In addition two minor groups with mixed sample compositions are shown.

The metabolite tree can be subdivided into two cluster groups: one sub-cluster comprising mostly sugars (above), and another sub-cluster including polyols, acids, and the metabolite groups (total phenols, anthocyanins and antioxidants). Hexoses and disaccharides (fructose, glucose, sucrose), together with malic acid from TCA, established one distinct sugar group. Furthermore, all pentoses (xylose, arabinose, and ribose) were grouped together. Pentose-levels (ribose) were higher in samples from individuals B1, B5, C4 and G4. Concentrations of shikimic acid were obviously lower in all samples from plot D (Trolla) compared to the other locations. Levels of the phenol chlorogenic acid were enhanced in several individual samples (Trolla: C1, C2, and C3), and several other samples (B4, B5, F4, F5, G1, and H2).

## 3.2 Genetic analysis

### 3.2.1 Testing loci

Only 4 microsatellite loci were shown to be successful out of the total 16 tested. The microsatellites not chosen had some kind of non-successful PCR amplification and were thus excluded. One tested microsatellite locus did not amplify, 7 of the loci showed no polymorphism, 2 had unspecific amplification, and for 2 there was indication of either null-alleles or duplication of the locus (Table 2.3). The occurrence of null alleles is caused by mutations leading to the loss of a primer binding site, and result in the loss of codominance and presence of some null genotypes making analyses based on such loci unreliable. The 4 successful microsatellites (NA741, NA961, CA421F, and CA483F) varied widely in number of alleles, from 1-6, with a mean value of 2.9 across the 8 sampled populations. As seen in Table 3.1 none of the loci showed significant departure from Hardy-Weinberg equilibrium after Bonferroni correction. A test for Hardy-Weinberg equilibrium was not carried out for NA741 in plot D because in this plot the locus had only 1 allele, giving observed and expected heterozygosity equal to 0 and allelic richness of 1.

### 3.2.2 Genetic variability among and within populations

Allelic richness ranged from 1.99 to 2.69 in the typed microsatellites (Table 3.1), with an overall mean equal to 2.8. Observed heterozygosity ranged from 0.2 to 1.2 with a mean value of 0.6 across all populations (Table 3.1). Expected heterozygosity ranged from 0.08 to 0.12 with mean 0.1 across all populations (Table 3.1).

**Table 3.1: Summary statistics of genetic variability of the 4 microsatellite loci used. Deviance from Hardy-Weinberg equilibrium (H-WE) (Bonferroni-corrected level of significance was *P*=0.00156) Number of alleles per locus (NA), observed and expected heterozygosities (H obs and H exp, respectively), number of individuals typed (N) and Allelic richness (AR).**

| Population | locus | H-WE | NA | H obs | H exp | N | AR |
|---|---|---|---|---|---|---|---|
| A | NA741 | 0.20 | 2 | 0.4 | 0.08 | 5 | 1.98 |
| | NA961 | 0.20 | 2 | 0.4 | 0.08 | 5 | 2.00 |
| | CA421F | 0.01 | 4 | 0.8 | 0.16 | 5 | 4.00 |
| | CA483F | 0.01 | 3 | 0.6 | 0.12 | 5 | 2.96 |
| B | NA741 | 0.20 | 3 | 0.6 | 0.12 | 5 | 3.00 |
| | NA961 | 0.20 | 2 | 0.4 | 0.08 | 5 | 2.00 |
| | CA421F | 0.03 | 6 | 1.2 | 0.24 | 5 | 5.36 |
| | CA483F | 0.20 | 2 | 0.4 | 0.08 | 5 | 2.00 |
| C | NA741 | 0.20 | 3 | 0.6 | 0.12 | 5 | 3.00 |
| | NA961 | 0.20 | 2 | 0.4 | 0.08 | 5 | 2.00 |
| | CA421F | 0.20 | 3 | 0.6 | 0.12 | 5 | 3.00 |
| | CA483F | 0.20 | 3 | 0.6 | 0.12 | 5 | 3.00 |
| D | NA741 | 0.00 | 1 | 0.2 | 0.04 | 5 | 1.00 |
| | NA961 | 0.20 | 2 | 0.4 | 0.08 | 5 | 2.00 |
| | CA421F | 0.12 | 3 | 0.6 | 0.12 | 5 | 2.80 |
| | CA483F | 0.20 | 2 | 0.4 | 0.08 | 5 | 1.98 |
| E | NA741 | 0.06 | 3 | 0.6 | 0.12 | 5 | 2.98 |
| | NA961 | 0.07 | 2 | 0.4 | 0.08 | 5 | 2.00 |
| | CA421F | 0.03 | 6 | 1.2 | 0.24 | 5 | 5.36 |
| | CA483F | 0.20 | 4 | 0.8 | 0.16 | 5 | 3.78 |
| F | NA741 | 0.20 | 2 | 0.4 | 0.08 | 5 | 2.00 |
| | NA961 | 0.20 | 2 | 0.4 | 0.08 | 5 | 1.98 |
| | CA421F | 0.20 | 4 | 0.8 | 0.16 | 5 | 3.60 |
| | CA483F | 0.01 | 2 | 0.4 | 0.08 | 5 | 2.00 |
| G | NA741 | 0.02 | 3 | 0.6 | 0.12 | 5 | 2.60 |
| | NA961 | 0.20 | 2 | 0.4 | 0.08 | 5 | 2.00 |
| | CA421F | 0.04 | 4 | 0.8 | 0.16 | 5 | 3.78 |
| | CA483F | 0.20 | 3 | 0.6 | 0.12 | 5 | 2.98 |
| H | NA741 | 0.12 | 3 | 0.6 | 0.12 | 5 | 2.80 |
| | NA961 | 0.20 | 2 | 0.4 | 0.08 | 5 | 1.98 |
| | CA421F | 0.07 | 5 | 1 | 0.20 | 5 | 4.58 |
| | CA483F | 0.07 | 3 | 0.6 | 0.12 | 5 | 2.80 |

The mean allelic richness within each population ranged from 1.94 in population D to 3.53 in population E (Table 3.2). Observed heterozygosity was lowest in D and highest in E and H (Table 3.2), and expected heterozygosity was lowest in D and highest in E (Table 3.2). There were however no significant differences in allelic richness (ANOVA, F=0.83, df=31, *P* =0.570) or expected heterozygoty (ANOVA, F=1.45, df=31, *P*=0.231) between populations.

**Table 3.2: Over all loci observed and expected heterozygosities (H obs and H exp, respectively), number of individuals typed (N) and Allelic richness (AR).**

| Population | H obs | H exp | AR |
|---|---|---|---|
| A | 0.35 | 0.49 | 1.98 |
| B | 0.45 | 0.54 | 3.09 |
| C | 0.50 | 0.48 | 2.75 |
| D | 0.30 | 0.32 | 1.94 |
| E | 0.55 | 0.71 | 3.53 |
| F | 0.40 | 0.53 | 2.39 |
| G | 0.50 | 0.58 | 2.84 |
| H | 0.55 | 0.61 | 3.04 |

### 3.2.3 Genetic variability among populations

The pairwise $F_{ST}$ estimates between 8 populations of Bilberry in Bymarka ranged from 0 to 0.174 (Table 3.3). The highest level of genetic differentiation was found between C with and D. $F_{ST}$ values from 0 to 0.05 indicate little genetic differention, values between 0.05-0.25 moderate genetic differention and $F_{ST}$ above 0.25 indicate pronounced levels of genetic differention. Consequently, all of the populations have $F_{ST}$ values suggesting low to moderate levels of genetic differention. The low number of loci and individuals sampled from each population means however that the estimates are very uncertain, and hence that conclusions based on these analyses should be made with this in mind.

**Table 3.3: Genetic differentiation ($F_{ST}$) among population pairs of Bilberry from Bymarka. $F_{ST}$ values are given in bold and the respective 95% confidence intervals are given in parentheses. Positive $F_{ST}$ estimates with 95% confidence intervals not including 0 are indicated by a *. As $F_{ST}$ ranges between 0 and 1 negative estimates are a result of very low genetic differences combined with problems with statistical estimation procedures in such cases, and should therefore be interpreted as indicating "no genetic differentiation", i.e. $F_{ST}$ equal to 0.**

| Plot | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| **B** | **0.028** (-0.024/0.076) | | | | | | |
| **C** | **0.103** (-0.042/0.231) | **0.115*** (0.008/0.187) | | | | | |
| **D** | **0.012** (-0.048/0.082) | **0.062** (-0.025/0.119) | **0.174** (-0.026/0.387) | | | | |
| **E** | **0.011** (-0.047/0.67) | **0.002** (-0.043/0.037) | **0.006** (-0.086/0.109) | **0.117*** (0.015/0.217) | | | |
| **F** | **0.018** (-0.94/0.123) | **-0.030** (-0.145/0.039) | **0.007** (-0.046/0.065) | **0.039** (-0.064/0.208) | **-0.006** (-0.069/0.046) | | |
| **G** | **-0.068** (-0.104/-0.045) | **-0.047** (-0.112/-0.013) | **0.037** (-0.032/0.118) | **0.039** (-0.064/0.207) | **-0.019** (-0.037/-0.005) | **-0.026** (-0.075/0.010) | |
| **H** | **0.077** (-0.037/0.185) | **0.021** (-0.097/0.158) | **0.043** (-0.065/0.139) | **0.016*** (0.207/0.450) | **-0.034** (-0.059/-0.011) | **-0.030** (-0.101/0.022) | **0.062** (-0.052/0.204) |

The pairwise level of genetic differentiation between the four locations (Gråkallen, Trolla, Ramnåsen, Grostadaunet) was also very low (Table 3.4). The highest level of pairwise genetic differentiation was found to be between Trolla and Gråkallen, but the estimated level of differentiation between these two populations also suggested a low level of differentiation.

Because of the phenotype difference of berries in plot D (black berries without wax layer) ANOVA-test was performed to test for significant differences. The pairwise genetic differentiation between D and the other populations (mean $F_{ST}$=0.0518) was not significantly different from the genetic differentiation between C and the other populations (mean $F_{ST}$=0.0475) (C vs. D excluded: ANOVA, F=0.031, df=1, P=0.865).

**Table 3.4: Genetic differentiation ($F_{ST}$ estimates) between the four different locations at which the 8 plots were selected. The $F_{ST}$ values are in bold and the confidence interval in parentheses. As $F_{ST}$ ranges between 0 and 1 negative estimates are a result of very low genetic differences combined with problems with statistical estimation procedures in such cases, and should therefore be interpreted as indicating "no genetic differentiation", i.e. $F_{ST}$ equal to 0.**

| Area | Gråkallen | Trolla | Ramnåsen | Grostadaunet |
|---|---|---|---|---|
| Trolla | **0.037** (-0.043/0.133) | | | |
| Ramnåsen | **0.011** (-0.045/0.053) | **0.005** (-0.032/0.051) | | |
| Grostadaunet | **-0.008** (-0.034/0.013) | **0.012**(-0.024/0.050) | **-0.044**(-0.064/-0.022) | |

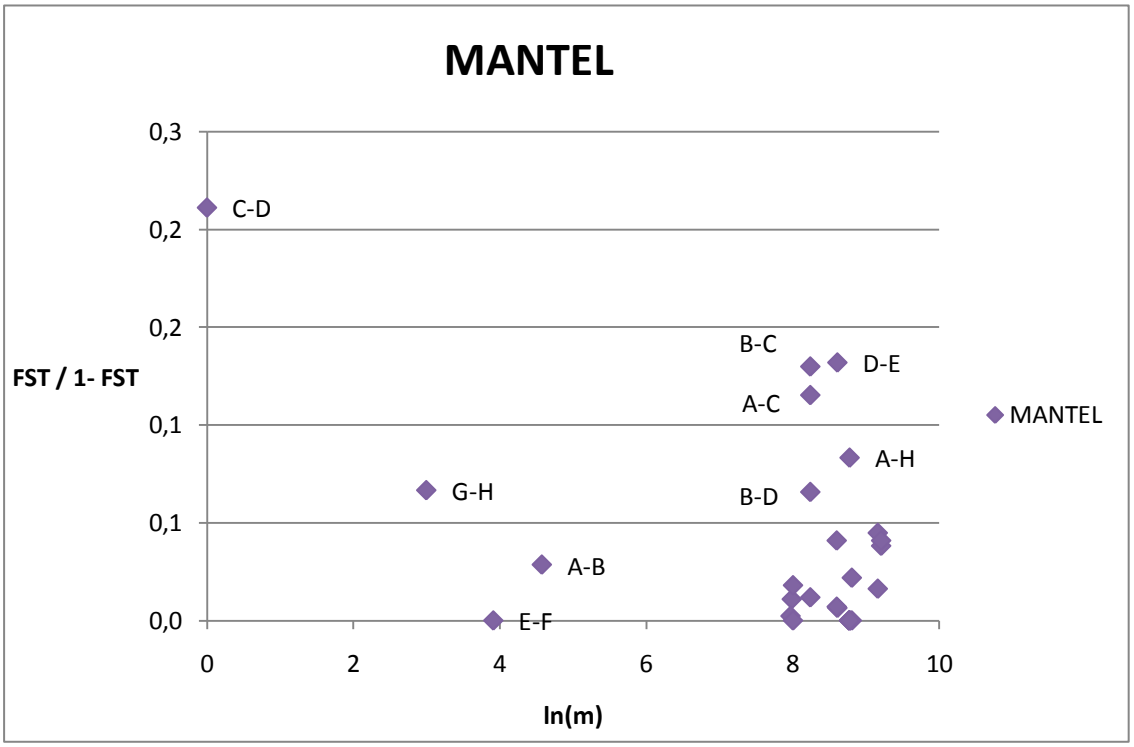


**Figure 3.5: Genetic isolation by distance in Bymarka inferred from multilocus estimates of $F_{ST}$ /1-$F_{ST}$ vs. geographical distance (ln (meter)). Purple dots marked with letters shows the estimates with highest values while the dots clustered together without letters are the rest of the $F_{ST}$ estimated plots.**

There was no significant relationship between pairwise level of genetic differentiation and geographic distance (Figure 3.5), suggesting no isolation by distance (Mantel test: r=0.209, *P*=0.791). It is clear that the level of genetic differentiation between populations C and D is very high, also for the distance between the populations (Figure 3.5).

# 4 Discussion

This thesis was divided into several parts: to quantify groups of phytochemicals such as total phenolics, anthocyanins and antioxidants, estimate levels of selected metabolites, and determine genetic variation in the clones by using microsatellite markers.

In this discussion the methods used for chemical detection and results obtained will first be addressed. The genetic part will be discussed in a separate paragraph, and finally, opportunities for further studies in this subject will be discussed.

## 4.1 Phytochemical variances

The **total phenolic** content was highest in berries from plot A, B, C with an average of 500 mg/100 g raw material. Similar studies done in *V. myrtillus* showed values ranging from 320.4 mg/100 g (Faria et al., 2005) to 525 mg/100 g (Prior et al., 1998). Furthermore, there was little variation between the plots diverging from 401 mg/100 g to 588 mg/100 g. This could be explained due to the fact that total phenolics in the berries show a broad specter. In this thesis the specific phenolic compounds were not detected, but studies done by Baj et al. (1983), Lätti et al. (2008) used techniques such as GC-MS and liquid chromatography-mass spectrometry to specify some of the phenolics in the tested berries. Lätti et al. (2008) revealed higher content of the glycoside delphinidin in northern Finland and, vice versa, higher content of cyanidin in southern locations. A similar relation between berries grown in colder climate was shown by Martinussen et al. (2009). Berries produced at 12 °C in Norway had higher content of phenolics (583.8 mg /100 g) and sugars than berries produced at 18 °C (556.8 mg/100 g).

In this study, no other data was collected expect the observations made of the locations and plant phenotypes in each plot. Environmental growing conditions can impact the levels of phenolics in plants such as growing season, biotic and abiotic influence. Total phenolic content varied 2.4-fold from 2000 to 2001 in studies performed by Cho et al.(2004). Contradictory studies performed by Connor et al. (2002) showed that variation in the total phenolics is more strongly related to genotypic determination rather than effects of growing seasons. Thus, genotyping of plant material is important in order to identify optimal clones and locations for commercial cultivation of *Vaccinium* species.

The F-C-method used to determine the total phenolics showed to be easy and reproducible. The color detected by spectrophotometry is depending on the number and position of hydroxyl groups in the phenolic structures. Since not all phenolics have hydroxyl groups in the detectable position, some structures might be lost. However, this method has been shown be suitable for the determination of total phenolics in vegetables and berries (Moyer et al., 2002; Zadernowski et al., 2005) Moreover, comparative studies clearly clearly showed a strong correlation between total phenolics content and antioxidant capacity (Kahkonen et al., 1999; Paixao et al., 2007).

The highest values of **anthocyanins** were found in plot C and D, but high levels of anthocyanins were also observed in plot A, B and C. The total content of anthocyanins detected ranged from 139 mg/ 100 g raw material to 200 mg/ 100 g. These results are lower than anthocyanins content detected in previously studies. Prior and co-workers (1998) detected 299.6mg/100 g of total anthocyanins in bilberries, while Lätti and co-workers (2008) detected as much as 411 mg /100 g fresh weight. In studies done by Ochmian et al. (2010) on V. corymbosum , fruit quality, phenolic composition and antioxidant activity the highest amount of phenols detected was anthocyanins, then chlorogenic acid and flavonols.

The pH-differential method used to detect anthocyanins is well established, however the method only detects monomeric anthocyanins because the reaction is not able to take place in dimmers where other molecules are attached (Berke et al., 1998).

Anthocyanin levels in bilberries have been shown to be correlated with solar radiation (Jaakola et al., 2004). Interestingly plot D and C were moderately exposed to sun light, B mostly in shadow, and also plot A was less exposed to sun light. In all of these plots it was difficult to harvest enough berries, berries were small and some had already fallen to the ground. Plants appeared with aged leaves, partly with red, purple, and brown spots. Based on the high anthocyanin content, this could indicate that berries were in a late ripening stage. This is confirmed by earlier studies showing that anthocyanin content increases while the fruit ripens (Prior et al., 1998).

**Antioxidant** capacity was highest in plot A, B, and D. The levels of antioxidant content detected ranged from 2.9 to 4.6 mmol/ 100 g. The total amount of antioxidants detected in bilberries varies in previously studies from 0.74 to 1.76 mmol/100 g (Faria et al., 2005) and up to values as high as 8.85 mmol/ 100 g (Halvorsen et al., 2002).

In previously studies on the phytochemical content in *Vaccinium* species a variety of methods has been used. Methods for detecting absorbic acid in Vaccinium berries,(Kalt et al., 1999a; Prior et al., 1998) has been used, and the ORAC (Oxygen radical absorbance capacity) method has been used for detection of antioxidant capacity. Linearity has been shown between antioxidant capacity (ORAC method), anthocyanin, and total phenolic content (Prior et al., 2001). In general, the antioxidant capacity in bilberries is a result of all the chemicals in the berries with antioxidant capacity and thus, also involves the group of phytochemicals.

The method used to detect antioxidants in bilberries, FRAP, might potentially be improved. This method was developed for testing antioxidant capacity in human plasma. In studies on plants the original protocol with heating to 37 °C is still applied. This could lead to misleading results in berries and vegetables since antioxidant capacity might increase with enhanced temperatures (Connor et al., 2002a; Wang and Zheng, 2001). It is also important to remember that this test is not specific, and the method tests only the hydrogen-donating ability leaving out other molecules with antioxidant properties.

The separation of plot D detected in the methods above was also obvious based on data from **GC/MS analysis**. In the clustering done in MeV, Figure 3.4, plot D clearly showed decreased levels of shikimic acid. Shikimic acid is an intermediate in the shikimic acid pathway, leading to the biosynthesis of aromatic amino acids phenylalanine, tyrosine and tryptophan and further to the phenylpropanoid pathway with flavonoids with one of its end products. Together with the high content of phytochemicals (anthocyanins) this could indicate that the intermediate does not accumulate in these berries, i.e. shikimate levels due to high production of flavonoids. The clustering in Figure 3.4 take also in consideration the values found in both GC-MS and phytochemical detection.

Ribose levels were shown to be enhanced in plot B and C. Ribose is one of the components necessary to build RNA and energy carriers. No data is available about ribose accumulation in bilberries.

In general, cluster patterns based on the locations of the plots could be observed. Plot A and D clustered together, D with E, C with B, E with F. Comparing the description of localities in Table 2.1, only plot E and F of the clustered plots showed some similarities in habitat and phenotypes. Plot D and A had little correlation, as plot D was exposed to solar radiation in the evening and plot A almost the whole day. There were also little similarities in the habitat

for plot D and E, as plot E was exposed to day light the whole day. It is important to consider temporal effects when analyzing metabite data since the detected chemical profile in the berries only reflects one timepoint. When doing the same analysis on the same berries some days later may lead to other results. It is therefore important to carry out time course analyses, which was not possible to adress as part of this thesis.

Changes in the phenolic acid content during fruit development in Diospyros lotus has been detected with the use of GC-MS (Ayaz et al., 1997) and a similar study would be interesting to do on *V.myrtillus*. The knowledge of chemical components during ripening would be beneficial to know when the fruits harvesting would give best quality fruit. Factors as winter hardiness, fall-timing, wind and snow cover may also have some effects on the chemical compounds (Fear et al.,1985 ; Gusta et al., 1979).

## 4.2 Genetic analysis

Aroma components, sugar content and total phenolics are found to have a genetic basis and be inherited in some *Vaccinium* species (Cho et al., 2004; Connor et al., 2002b; Connor et al., 2002c; Hirvi and Honkanen, 1983). It is therefore of interest to look closer into the methods developed for genotyping of *V. myrtillus*. Only few studies on this subject could however be found, one exception being that microsatellites which were developed for highbush *Vaccinium* species have been tested in *V. myrtillus* (Boches et al., 2005). As part of the thesis, microsatellite markers were used to examine the level of variation among and within the harvested bilberry populations. It was not expected to find large differences between the plots in the restricted area. Accordingly, previous studies in bilberry have also shown only slight differentiation among populations (Albert et al., 2004).

The DNA extraction was successful as seen in PCR amplification. Though a study done by Al-Saghir (2009) shows that the original Qiagen DNeasy Plant Mini Kit is not suitable for DNA extraction from hard tissues of woody tree crops. In this study plant material from both leafs and woody tree was used. As some loci were successful and gave good results it is however unlikely that this was the reason for the low quality of 12 of the tested microsatellite loci. Secondary metabolites (which *V.myrtillus* has high content of) has also shown to affect the quality in DNA extraction as they react irreversibly with nucleic acids (John, 1992 ; Watanabe and Watanabe, 2000).

16 microsatellite markers of appropriate size ranges and most likely to be successful in bilberry were selected from literature for testing in bilberries from Bymarka (Boches, 2005). Only 4 of the microsatellite loci turned out to be of good quality and were selected to be used for further analysis (NA741, NA961, CA421F, and CA483F). The 4 chosen microsatellite loci in combination with the small number of clones sampled in each plot was not enough to reach sufficient confidence for our genetic estimates. One explanation for the low quality of 12 of the tested microsatellite loci could have been that the primers require specific annealing temperatures. This problem was however avoided in this study because a touch-down PCR profile was used, where a range of annealing temperatures was used.

Tested allelic richness had a mean value of 2.8. Observed heterozygosity and expected heterozygosity had mean values of 0.6 and 0.1 and had no significant differences. There was one loci that showed significant departure from Hardy-Weinberg equilibrium after Bonferroni correction and it was loci NA741 in plot D. Though this is taken in consideration when $F_{ST}$ estimates are done and there is no variation on the locus and will not affect the Mantel-test.

**Differences within and among populations**

The $F_{ST}$ values indicated little genetic population structure among the sampled localities and populations. Furthermore, the Mantel test (isolation-by-distance), showed that there was no effect of geographic distance on the level of genetic differentiation in Bymarka. Interestingly, population D seem to be more genetically differentiated than expected with other populations, in particular with population C which is very close (at the same locality). This result is correlated to recent studies mentioned below, and in the phytochemical analysis plot D also turned out to be exceptional. It is difficult to give a strong conclusion based only on these analyses, but they suggest that the deficiency of the wax layer on the berries in population D may have been due to genetic differences.

A possible explanation for the low level of differentiation between the bilberry populations could be pollination by bees, and seed dispersal in the relatively restricted geographic area by animals. Bymarka is surrounded by the Trondheims Fjord in north, west and south (Figure 2.1), and border to the city Trondheim on the east side. There are therefore limited opportunities for animals to travel from places around, though the birds have free passage in and out of the restricted area which may carry bilberry seeds and thus help to reduce genetic structure and avoid inbreeding in the populations together with knowledge that the main

pollinator in bilberries are bees who flies in long distances (Jaquemart et al.,1994). Regional gene flow could also be explained by humans in the area (Aldrich and Doebley, 1992). Burgher et al. (2002) found no geographical differentiation in his studies using RAPD methods.

Studies shows that *V.corymbosu* that self-fertilize aborts and that this results in a reduction in levels of inbreeding. An aid in this is also that the flowers physical structure helps cross-pollination by insects (Hancock et al., 2008; Krebs and Hancock, 1990; Rowland et al., 2003; Shutak and Kitchin, 1966). Low numbers of SRR alleles per locus has been reported for self-pollinated crops like rice (2.78 alelles/locus for EST-SSR (Cho et al., 2000) in comparison to cross-pollinated crops like maiz (12,6 alleles per locus, (Matsuoka et al.,2002) and 8.02 alleles per locus ( Reif et al.,2004)). In other studies done by Boches et al. (2006) in blueberries 17.7 alleles per locus were found. Observed number of alleles per locus ranged from 1 to 6 in this study and corresponds more to the cross-pollinated crops. Because there is a strong correlation between number of sampled individuals and number of alleles the low number of alleles is however more likely to be a result of the sampling design and the relatively small geographic region which was studied.

As mentioned earlier the life history of the individual clone is important for genetic fingerprint and metabolic pattern (Albert et al.,2004). Another factor that may have an influence on the genetic diversity within a population is the vegetative growth strategy, as phalanx growth, or tightly growing populations are the most common for bilberries. In combination with the result showing that the optimum physical distance between crossing parents are 5-10 m (Nuortila et al., 2002) this suggest that the gene flow will appear at these scales. Studies done by Albert et al. (2004) on *V.myrtillus* driving a Analysis of molecular variance (AMOVA) test, showed that a high proportion of genetic the total variation was within the population (86.19%), and only a low proportion of genetic variation was between populations (13.81%). The distance between populations in the study by Albert et al. (2004) ranged from 1-17 km, which is on about the same scale as in the present study. The same conditions were found in *V.vitis ideae*, where the proportion of total genetic variation was 89.2% within populations (Persson and Gustavsson, 2001) in a geographical region ranging from 6-12 km. Furthermore, in *V.angustifolium* the genetic variation within was estimated to be 92 % and only 8 % was among in a area within a distance on 12.5 to 65 km testing in AMOVA (Bell,2009). In a study done by Ercisli et al. (Ercisli et al., 2008) in Bulgarian

raspberry only 4 microsatellite pairs was found to function. When doing ANOVA test 87.5 % showed to be polymorphic within the populations. In this study the distance ranged from 0.3 to 3.5 km and genetic variation within pop was found to be, between, found using ANOVA. There would also be interesting in driving a AMOVA test on the results, but there was time limitations.

The divergence of plot D is also shown in the chemical analysis and the differences in the genetic part may be realistic. Plot D distinguished a lot from the other plots as all of the clones in this plot had different phenotype and were dark blue, indicating lack of the wax layer. This could be the explanation why plot D showed relatively higher content of all phytochemicals. Only little research on this variant of bilberries has been carried out, and it would therefore be of interest to look further into this phenomenon since higher levels of phytochemicals will be beneficial for the development of clones for commercial purpose.

The results of this study showed low level of genetic differentiation among bilberry populations in a restricted geographical area, Bymarka. However, the small amount of clones tested (80) and a number of 5 individuals in each plot are not enough sufficient to gain conclusive results. The low number of loci used also means that it is not possible to draw reliable conclusions (Raymond and Rousset, 1995). Further analyses are needed where more loci and a larger dataset is used. For estimating average heterozygosity and genetic distance a large number of loci are shown to be more important than large dataset, though number of individual should be over 10 for each population (Nei and Roychoudhury, 1974).

# 5 Conclusion and further studies

This thesis describes methods in detecting total phenols, anthocyanins and antioxidants. All of these methods have been performed with success and some differences in the phytochemical properties have been detected between locations in the geographical restricted area Bymarka. There has also been detected difference both phytochemical and genetic in plot D where all of the berries were black, indicating lack of a wax layer. The higher amount of phytochemicals of the berries in plot D are of interest to study closer as phytochemicals has shown to have a health beneficial effect on humans. There would also be of interest to look closer on the variation due to maturation in the different clones. In this thesis probes from soil from each plot were also collected but due to time and money limitation no further testing was performed on these. Soil acidity and organic matter has shown to have effect in growth in *Vaccinium* species (Finn et al., 1993a ; Finn et al., 1993b ; Hall et al., 1964).

Four of sixteen microsatellite primers developed for other *Vaccinium* species showed to be effective in *V.myrtillus* and give amplifications. The low number of loci detected is not enough to make clear assumptions about the genetic diversity in the clones and further studies using more microsatellite primers are needed.

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# 7 Appendix

## 7.1 Phytochemicals

### 7.1.1 Extraction of phytochemicals

The collected berries were extracted and the amount used was weighed. The measurements are shown in Table 7.1.

**Table 7.1: Weight of berries in extract**

| Population | Weight | *Cont.* | *Cont.* |
|---|---|---|---|
| A1 | 187.4 | E1 | 186.5 |
| A2 | 183.4 | E2 | 181.6 |
| A3 | 180.4 | E3 | 184.0 |
| A4 | 188.4 | E4 | 182.7 |
| A5 | 185.4 | E5 | 188.8 |
| B1 | 184.3 | F1 | 183.7 |
| B2 | 186.6 | F2 | 186.7 |
| B3 | 186.5 | F3 | 187.7 |
| B4 | 184.5 | F4 | 184.7 |
| B5 | 186.5 | F5 | 187.0 |
| C1 | 186.7 | G1 | 180.8 |
| C2 | 185.1 | G2 | 188.4 |
| C3 | 182.5 | G3 | 182.9 |
| C4 | 184.7 | G4 | 181.2 |
| C5 | 186.7 | G5 | 184.3 |
| D1 | 184.3 | H1 | 187.5 |
| D2 | 187.8 | H2 | 182.9 |
| D3 | 184.7 | H3 | 181.3 |
| D4 | 181.6 | H4 | 183.8 |
| D5 | 183.3 | H5 | 184.1 |

### 7.1.2 Total phenols

#### Measurements

The absorbance detected using F-C method is shown in Table 7.2. Pink, green and blue colors indicate the corresponding measurements for blank samples.

**Table 7.2: Measured absorbance using F-C method for total phenols detection.**

| | | | |
|---|---|---|---|
| A1 | 0.467 | 0.53 | 0.493 |
| A2 | 0.43 | 0.455 | 0.377 |
| A3 | 0.406 | 0.272 | 0.496 |
| A4 | 0.274 | 0.334 | 0.275 |
| A5 | 0.402 | 0.412 | 0.373 |
| B1 | 0.393 | 0.37 | 0.447 |
| B2 | 0.369 | 0.37 | 0.375 |
| B3 | 0.372 | 0.342 | 0.331 |
| B4 | 0.39 | 0.384 | 0.373 |
| B5 | 0.365 | 0.373 | 0.392 |
| C1 | 0.298 | 0.311 | 0.294 |
| C2 | 0.321 | 0.328 | 0.327 |
| C3 | 0.586 | 0.599 | 0.6 |
| C4 | 0.315 | 0.313 | 0.322 |
| C5 | 0.375 | 0.357 | 0.317 |
| D1 | 0.32 | 0.306 | 0.322 |
| D2 | 0.36 | 0.355 | 0.345 |
| D3 | 0.383 | 0.401 | 0.359 |
| D4 | 0.375 | 0.332 | 0.376 |
| D5 | 0.304 | 0.34 | 0.328 |

| | | | |
|---|---|---|---|
| E1 | 0.32 | 0.299 | 0.326 |
| E2 | 0.275 | 0.302 | 0.349 |
| E3 | 0.254 | 0.286 | 0.246 |
| E4 | 0.286 | 0.292 | 0.289 |
| E5 | 0.284 | 0.305 | 0.304 |
| F1 | 0.32 | 0.311 | 0.308 |
| F2 | 0.285 | 0.282 | 0.289 |
| F3 | 0.414 | 0.432 | 0.41 |
| F4 | 0.399 | 0.386 | 0.394 |
| F5 | 0.4 | 0.389 | 0.405 |
| G1 | 0.328 | 0.322 | 0.355 |
| G2 | 0.371 | 0.379 | 0.362 |
| G3 | 0.259 | 0.253 | 0.28 |
| G4 | 0.26 | 0.25 | 0.241 |
| G5 | 0.506 | 0.469 | 0.497 |
| H1 | 0.308 | 0.303 | 0.324 |
| H2 | 0.332 | 0.357 | 0.339 |
| H3 | 0.294 | 0.265 | 0.271 |
| H4 | 0.375 | 0.361 | 0.377 |
| H5 | 0.386 | 0.363 | 0.401 |

| | | | |
|---|---|---|---|
| **Blank** | 0.063 | 0.064 | 0.064 |
| **Blank** | 0.053 | 0.054 | 0.054 |
| **Blank** | 0.056 | 0.057 | 0.055 |

**Calculation**

The total content of phenolics was found by using the equation established by the standard curve. Figure 7.1 shows one of the standard curves with equation used for calculating total phenolics in some of the berries. The other equations used were: y = 0.0064x + 0.0458, and y = 0.0063x + 0.0539.

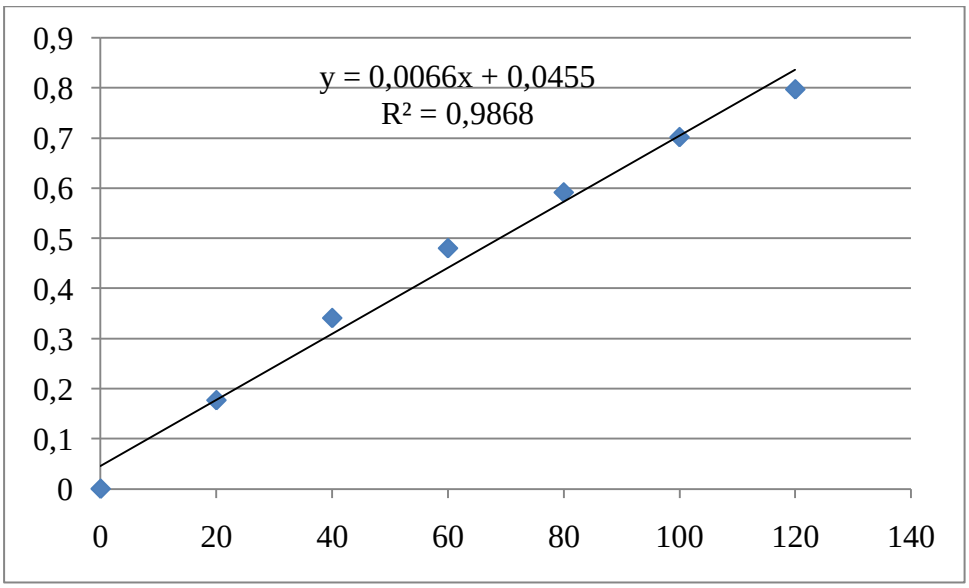


**Figure 7.1: Standard curve used for calculating total phenols, showing known concentrations of gallic acid (Table 2.2) plotted against absorbance.**

Further calculation was done as described.

The equation from the standard curve was used to calculate the total phenol amount in the probes.

The weight of the berries was divided on the amount of methanol added to find the concentration ( X mg berries /1 ml).

In the next step, the average of blanks was subtracted from the average of absorbance (three parallels). This value was further used to find the values for X from the equation from the standard curve of the same day measurements for the berries were carried out. This value is finally multiplied with the final dilution in the wells, 100. The result are presented as mg TPH/ 100 g raw material.

### 7.1.3 Anthocyanins

**Measurements**

Anthocyanins absorbance was both measured in pH 4.5 and pH 1.0. Table 7.3 shows overview of the measured absorbance at different pH and wavelength. The calculation was performed using equation 1, (shown in the description of the method in chapter 2.4.1) using dilution factor 10.

**Table 7.3: Measured absorbance at different pH and wavelengths for the determination of total anthocyanins.**

| | pH 4.5 | | pH 1 | |
|---|---|---|---|---|
| Population | 700 nm | 510 nm | 700 nm | 510 nm |
| A1 | 0 | 0.027 | 0.006 | 0.483 |
| A2 | 0 | 0.019 | 0.009 | 0.378 |
| A3 | -0.002 | 0.017 | 0.001 | 0.336 |
| A4 | -0.011 | 0.005 | -0.005 | 0.229 |
| A5 | -0.012 | 0.005 | -0.012 | 0.32 |
| B1 | -0.002 | 0.012 | -0.013 | 0.284 |
| B2 | -0.017 | 0.005 | -0.02 | 0.363 |
| B3 | -0.009 | 0.012 | -0.004 | 0.353 |
| B4 | -0.011 | 0.011 | -0.009 | 0.31 |
| B5 | -0.006 | 0.016 | -0.009 | 0.342 |
| C1 | -0.009 | 0.01 | -0.017 | 0.275 |
| C2 | -0.009 | 0.007 | -0.004 | 0.284 |
| C3 | -0.012 | 0.034 | -0.012 | 0.7 |
| C4 | 0.008 | 0.013 | -0.007 | 0.5 |
| C5 | 0.001 | 0.022 | -0.011 | 0.421 |
| D1 | -0.007 | 0.021 | -0.006 | 0.427 |
| D2 | -0.002 | 0.02 | -0.008 | 0.513 |
| D3 | -0.009 | 0.026 | -0.008 | 0.545 |
| D4 | -0.01 | 0.019 | -0.006 | 0.487 |
| D5 | -0.006 | 0.017 | -0.013 | 0.429 |
| E1 | -0.009 | 0.013 | -0.005 | 0.559 |
| E2 | -0.009 | 0.009 | -0.009 | 0.251 |
| E3 | -0.008 | 0.008 | -0.001 | 0.262 |
| E4 | -0.015 | 0.004 | 0.001 | 0.322 |
| E5 | -0.005 | 0.014 | -0.007 | 0.295 |
| F1 | -0.008 | 0.009 | -0.005 | 0.294 |
| F2 | -0.012 | -0.001 | -0.007 | 0.262 |
| F3 | -0.007 | 0.018 | -0.003 | 0.403 |
| F4 | -0.012 | 0.018 | -0.005 | 0.392 |
| F5 | -0.009 | 0.017 | -0.003 | 0.345 |
| G1 | -0.002 | -0.003 | -0.009 | 0.329 |
| G2 | -0.007 | 0.013 | -0.004 | 0.382 |
| G3 | -0.001 | 0.002 | -0.006 | 0.248 |
| G4 | -0.01 | 0.001 | -0.005 | 0.221 |
| G5 | -0.006 | 0.015 | 0 | 0.536 |
| H1 | -0.005 | 0.009 | -0.007 | 0.315 |
| H2 | -0.003 | 0.017 | -0.008 | 0.333 |
| H3 | -0.007 | 0.011 | -0.003 | 0.271 |
| H4 | -0.006 | 0.01 | -0.003 | 0.401 |
| H5 | -0.01 | 0.013 | -0.003 | 0.419 |

### 7.1.4 Antioxidant

#### Measurements

Antioxidant properties were found by using the FRAP method. The absorbance measured in this method is presented in Table 7.

**Table 7.4: Absorbance measured for each population. The pink, yellow and blue colour indicate the corresponding blanks.**

| Population | Absorbance | | |
|---|---|---|---|
| A1 | 0.467 | 0.53 | 0.493 |
| A2 | 0.43 | 0.455 | 0.377 |
| A3 | 0.406 | 0.272 | 0.496 |
| A4 | 0.274 | 0.334 | 0.275 |
| A5 | 0.402 | 0.412 | 0.373 |
| B1 | 0.393 | 0.37 | 0.447 |
| B2 | 0.369 | 0.37 | 0.375 |
| B3 | 0.372 | 0.342 | 0.331 |
| B4 | 0.39 | 0.384 | 0.373 |
| B5 | 0.365 | 0.373 | 0.392 |
| C1 | 0.298 | 0.311 | 0.294 |
| C2 | 0.321 | 0.328 | 0.327 |
| C3 | 0.586 | 0.599 | 0.6 |
| C4 | 0.315 | 0.313 | 0.322 |
| C5 | 0.375 | 0.357 | 0.317 |
| D1 | 0.32 | 0.306 | 0.322 |
| D2 | 0.36 | 0.355 | 0.345 |
| D3 | 0.383 | 0.401 | 0.359 |
| D4 | 0.375 | 0.332 | 0.376 |
| D5 | 0.304 | 0.34 | 0.328 |
| E1 | 0.32 | 0.299 | 0.326 |
| E2 | 0.275 | 0.302 | 0.349 |
| E3 | 0.254 | 0.286 | 0.246 |
| E4 | 0.286 | 0.292 | 0.289 |
| E5 | 0.284 | 0.305 | 0.304 |
| F1 | 0.32 | 0.311 | 0.308 |
| F2 | 0.285 | 0.282 | 0.289 |
| F3 | 0.414 | 0.432 | 0.41 |
| F4 | 0.399 | 0.386 | 0.394 |
| F5 | 0.4 | 0.389 | 0.405 |
| G1 | 0.328 | 0.322 | 0.355 |
| G2 | 0.371 | 0.379 | 0.362 |
| G3 | 0.259 | 0.253 | 0.28 |
| G4 | 0.26 | 0.25 | 0.241 |
| G5 | 0.506 | 0.469 | 0.497 |
| H1 | 0.308 | 0.303 | 0.324 |
| H2 | 0.332 | 0.357 | 0.339 |
| H3 | 0.294 | 0.265 | 0.271 |
| H4 | 0.375 | 0.361 | 0.377 |
| H5 | 0.386 | 0.363 | 0.401 |

| | | | |
|---|---|---|---|
| Blank | 0.063 | 0.064 | 0.064 |
| Blank | 0.053 | 0.054 | 0.054 |
| Blank | 0.056 | 0.057 | 0.055 |

## Calculation

For calculating the total content of antioxidants in the berries, the equation from standard curve was used. The standard curve was found by plotting the absorbance against levels of $Fe^{2+}$ in mmol/l (mM). Figure 7.2 shows the standard curve and equation used.

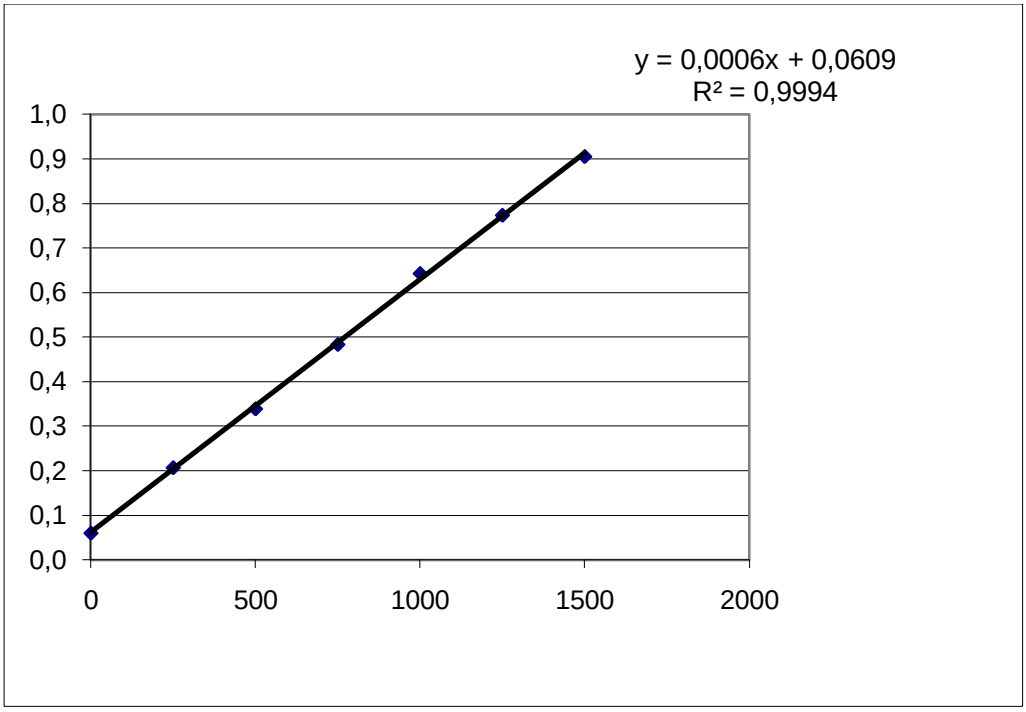


**Figure 7.2: The standard curve with equation used for calculating antioxidant capacity. The figure shows mmol/l (nM) $Fe^{2+}$ plotted against absorbance values.**

Further calculation was done as described. The equation from the standard curve was used to calculate antioxidant amount in the probes. The concentration of the berries (X mg berries /1 ml) extract calculated in finding total phenolic was used. In the next step, the average of blanks was subtracted from the average of absorbance (three parallels). This values was further used to find the values for X from the equation from the standard curve of the same day measurements for the berries were carried out. The values calculated from the standard curve was in mmol/l, this values was converted further to mmol/ 100 g raw material.

### 7.1.5 GC-MS metabolite profiling

The measurement from GC-MS analysis were further used to calculate the concentrations of metabolites detected based on the known concentration of the standard ribitol. Finally, concentrations were used to calculate log2(n) ratios for each metabolite based on the corresponding median from all samples. The results are shown in Table 7.5.

**Table 7.5: Calculated values, ln(cons./median) from the measurements done in GC-MS.**

| ln(cons./median) | A1 | A2 | A3 | A4 | A5 | B1 | B2 | B3 | B4 | B5 | C1 | C2 | C3 | C4 | C5 | D1 | D2 | D3 | D4 | D5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Antioxidants | 0.492 | 0.292 | 0.207 | -0.267 | 0.196 | 0.228 | 0.059 | -0.038 | 0.114 | 0.081 | -0.277 | -0.139 | 0.747 | -0.131 | 0.010 | -0.132 | 0.020 | 0.146 | 0.085 | -0.087 |
| Antocyanins | 0.303 | 0.051 | -0.051 | -0.422 | -0.054 | -0.161 | 0.082 | 0.010 | -0.113 | -0.011 | -0.197 | -0.201 | 0.695 | 0.412 | 0.212 | 0.197 | 0.406 | 0.443 | 0.333 | 0.231 |
| Total phenols | 0.613 | 0.454 | 0.160 | -0.072 | -0.077 | 0.238 | 0.279 | -0.095 | 0.467 | 0.169 | -0.137 | -0.007 | 0.617 | 0.011 | 0.007 | -0.073 | -0.025 | 0.044 | -0.040 | -0.039 |
| Malic acid | 0.059 | 0.610 | 0.254 | 0.142 | 0.286 | 0.348 | 0.399 | 0.185 | 0.575 | 0.090 | 0.065 | 0.067 | -0.885 | 0.053 | -0.128 | 0.004 | 0.083 | -0.194 | -0.146 | -0.018 |
| Xylose | 0.041 | 0.115 | -0.190 | -0.162 | -0.365 | 0.957 | -0.267 | -0.333 | -0.135 | -0.206 | -0.278 | 0.317 | -0.863 | 1.253 | -0.180 | 0.083 | 0.061 | -0.021 | 0.045 | 0.090 |
| Arabinose | 0.027 | 0.105 | -0.149 | -0.100 | -0.336 | 0.590 | -0.184 | -0.278 | -0.135 | -0.194 | -0.269 | 0.316 | -0.772 | 0.306 | -0.080 | 0.081 | 0.049 | -0.028 | 0.042 | 0.144 |
| Ribose | 0.010 | 0.097 | -0.112 | 0.425 | -0.038 | 2.398 | -0.154 | 0.142 | -0.093 | 2.083 | -0.215 | 0.162 | 0.288 | 1.740 | -0.071 | -0.036 | 0.182 | 0.078 | 0.085 | 0.064 |
| Fructose | 0.148 | 0.194 | 0.179 | 0.177 | 0.274 | -0.279 | 0.105 | -1.104 | 0.043 | -0.328 | 0.100 | 0.525 | -1.336 | -0.765 | 0.129 | -0.228 | 0.269 | -0.403 | -0.273 | 0.025 |
| Citric acid | -0.029 | -0.044 | -0.219 | 0.085 | -0.094 | -0.002 | 0.072 | 0.029 | 0.137 | 0.071 | -0.014 | -1.199 | 0.041 | 0.224 | 0.231 | 0.057 | 0.053 | 0.203 | 0.228 | 0.118 |
| Shikimic acid | 0.213 | 0.037 | -0.093 | -0.485 | -0.244 | 1.660 | -0.671 | 0.027 | 0.948 | 0.143 | -0.462 | 0.040 | -0.202 | 0.617 | 0.631 | -1.309 | -0.751 | -1.230 | -1.222 | -1.334 |
| Quinic acid | 0.169 | 0.170 | -0.267 | -0.101 | -0.214 | -0.212 | 0.179 | -0.020 | 0.125 | -0.205 | 0.205 | 0.086 | 0.499 | 0.397 | 0.247 | -0.401 | 0.021 | -0.159 | -0.124 | -0.212 |
| Glucose | 0.094 | 0.126 | 0.128 | 0.015 | 0.201 | -0.346 | 0.037 | -0.112 | 0.036 | -0.162 | 0.115 | 0.443 | -0.999 | -0.413 | 0.124 | -0.075 | 0.087 | -0.336 | -0.197 | 0.067 |
| Chiro-Inositol | 0.588 | 0.083 | -0.396 | 0.025 | -0.243 | -2.485 | -0.059 | 0.145 | 0.906 | 0.374 | -0.252 | 4.502 | 0.185 | 0.085 | 0.402 | -0.392 | 0.315 | -0.192 | -0.073 | 0.047 |
| Myo-Inositol | -0.057 | -0.448 | -0.310 | 0.222 | -0.177 | -0.611 | 0.074 | -0.147 | 0.322 | -0.072 | -0.007 | -0.100 | 0.312 | 0.286 | 0.042 | -0.177 | 0.175 | 0.346 | 0.086 | 0.083 |
| Sucrose | 0.361 | 0.438 | 0.129 | 0.139 | 0.500 | -0.225 | 0.615 | -0.140 | -0.085 | -0.445 | 0.328 | -0.077 | -1.277 | -0.233 | 0.478 | 0.038 | 0.643 | -0.045 | 0.015 | 0.334 |
| Chlorogenic acid | -0.549 | 0.227 | -0.188 | -0.105 | 0.149 | -0.415 | -0.049 | -0.313 | 0.890 | 0.456 | 0.526 | 0.673 | 0.761 | -0.934 | -0.296 | -0.170 | 0.205 | -0.006 | 0.162 | 0.326 |

| *cont.* | E1 | E2 | E3 | E4 | E5 | F1 | F2 | F3 | F4 | F5 | G1 | G2 | G3 | G4 | G5 | H1 | H2 | H3 | H4 | H5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Antioxidants | -0.149 | -0.154 | -0.442 | -0.267 | -0.251 | -0.143 | -0.310 | 0.262 | 0.190 | 0.195 | -0.033 | 0.077 | -0.438 | -0.527 | 0.486 | -0.183 | -0.010 | -0.347 | 0.103 | 0.149 |
| Antocyanins | 0.489 | -0.318 | -0.297 | -0.096 | -0.161 | -0.165 | -0.254 | 0.136 | 0.099 | -0.032 | 0.019 | 0.096 | -0.281 | -0.436 | 0.438 | -0.077 | -0.035 | -0.261 | 0.154 | 0.182 |
| Total phenols | -0.011 | -0.212 | -0.320 | -0.149 | 0.017 | 0.091 | -0.143 | 0.307 | 0.256 | 0.197 | -0.067 | 0.088 | -0.199 | 0.075 | -0.233 | 0.082 | -0.205 | -0.310 | -0.313 | 0.297 |
| Malic acid | -0.650 | -0.125 | -0.004 | -0.097 | -0.047 | -0.407 | -0.668 | -0.300 | 0.147 | -0.375 | -0.159 | 0.080 | -0.491 | 0.535 | 0.341 | -0.102 | -0.058 | -0.109 | -0.251 | 0.127 |
| Xylose | 0.169 | 0.060 | -0.154 | 0.132 | 0.055 | -0.855 | -0.719 | 0.161 | 0.068 | -0.140 | -0.880 | -0.148 | -0.234 | 0.420 | -0.541 | 0.217 | -0.236 | 0.069 | 0.021 | 0.033 |
| Arabinose | 0.187 | 0.062 | -0.086 | 0.146 | 0.049 | -0.741 | -0.632 | 0.181 | 0.082 | -0.043 | -0.739 | -0.148 | -0.216 | 0.549 | -0.443 | 0.255 | -0.139 | 0.146 | 0.064 | 0.094 |
| Ribose | -0.248 | -0.187 | -0.149 | -0.010 | 0.201 | -0.113 | -0.058 | 0.031 | -0.211 | 0.082 | -0.278 | 0.133 | -0.213 | 1.069 | 0.210 | -0.116 | 0.128 | -0.340 | -0.138 | -0.280 |
| Fructose | 0.070 | 0.181 | 0.009 | -0.007 | 0.131 | -0.529 | -0.491 | 0.077 | 0.129 | -0.121 | -1.348 | -0.288 | 0.007 | -0.080 | -0.209 | -0.348 | 0.039 | -0.092 | -0.387 | -0.277 |
| Citric acid | -0.364 | -0.164 | -0.033 | -0.071 | 0.002 | 0.274 | 0.105 | -0.019 | -0.242 | 0.111 | 0.061 | 0.089 | -0.223 | -0.345 | 0.291 | -0.218 | -0.350 | -0.329 | -0.206 | -0.343 |
| Shikimic acid | -0.274 | -1.144 | 0.103 | -1.245 | -0.045 | 0.158 | 0.255 | -0.156 | 0.014 | -0.249 | -0.480 | 0.393 | 0.025 | -0.431 | 0.192 | -0.015 | 0.332 | 0.323 | 0.064 | 0.019 |
| Quinic acid | -0.199 | 0.181 | 0.093 | 0.094 | 0.029 | 0.112 | 0.124 | 0.020 | -0.205 | -0.063 | -0.078 | 0.070 | -0.218 | -0.332 | 0.078 | -0.042 | 0.051 | -0.254 | -0.081 | -0.177 |
| Glucose | 0.034 | 0.441 | -0.017 | -0.021 | 0.036 | -0.603 | -0.570 | -0.016 | 0.086 | -0.060 | -0.718 | -0.225 | 0.029 | 0.092 | -0.206 | -0.288 | 0.031 | 0.029 | -0.537 | -0.167 |
| Chiro-Inositol | -0.537 | -0.026 | 0.188 | 0.362 | 0.243 | -0.464 | -0.845 | 0.026 | -0.056 | 0.015 | -0.430 | -0.040 | -0.392 | -0.612 | 0.039 | -0.015 | -0.546 | -0.559 | 0.031 | 0.035 |
| Myo-Inositol | -0.342 | 0.153 | -0.160 | 0.047 | 0.053 | 0.156 | 0.175 | 0.136 | -0.136 | 0.048 | -0.243 | 0.007 | -0.049 | -0.380 | 0.131 | -0.309 | 0.209 | -0.312 | -0.346 | -0.363 |
| Sucrose | -0.016 | 0.284 | 0.108 | 0.094 | 0.131 | -0.455 | -0.421 | 0.167 | 0.511 | -0.171 | -1.199 | -0.079 | 0.122 | -0.031 | -0.106 | -0.478 | 0.164 | -0.104 | -0.456 | -0.602 |
| Chlorogenic acid | -0.092 | -0.589 | -0.650 | -0.703 | 0.130 | 0.006 | 0.155 | 0.253 | 1.073 | 0.498 | 0.696 | 0.193 | 0.041 | -0.187 | -0.079 | -0.352 | 0.665 | -1.020 | -0.051 | -0.041 |

## 7.2 Genetic analysis

### 7.2.1 Mantel

In the Mantel test, the distance plotted in Figure 3.5 was shown as ln(m). The $F_{ST}$ values plotted were found in the software program R, the plotted $F_{ST}$ formel was $F_{ST}/(1-F_{ST})$. The table for the plotted values for Figure 3.5 is presented in Table 7.6.

**Table 7.6: The calculated values for $F_{ST}/ (1-F_{ST})$ and ln(m) plotted in Figure 3.5.**

| Pop 1 | Pop 2 | $F_{ST}$ | $F_{ST}/ 1-F_{ST}$ | ln (m) |
|---|---|---|---|---|
| A | B | 0.028 | 0.029 | 4.575 |
| A | C | 0.103 | 0.115 | 8.243 |
| A | D | 0.012 | 0.012 | 8.243 |
| A | E | 0.011 | 0.011 | 7.990 |
| A | F | 0.018 | 0.018 | 8.006 |
| A | G | 0.000 | 0.000 | 8.780 |
| A | H | 0.077 | 0.083 | 8.780 |
| B | C | 0.115 | 0.130 | 8.243 |
| B | D | 0.062 | 0.066 | 8.243 |
| B | E | 0.002 | 0.002 | 7.972 |
| B | F | 0.000 | 0.000 | 8.006 |
| B | G | 0.000 | 0.000 | 8.810 |
| B | H | 0.021 | 0.022 | 8.810 |
| C | D | 0.174 | 0.211 | 0.000 |
| C | E | 0.006 | 0.006 | 8.613 |
| C | F | 0.007 | 0.007 | 8.603 |
| C | G | 0.037 | 0.038 | 9.210 |
| C | H | 0.043 | 0.045 | 9.164 |
| D | E | 0.117 | 0.132 | 8.613 |
| D | F | 0.039 | 0.041 | 8.603 |
| D | G | 0.039 | 0.041 | 9.210 |
| D | H | 0.016 | 0.016 | 9.164 |
| E | F | 0.000 | 0.000 | 3.912 |
| E | G | 0.000 | 0.000 | 8.772 |
| E | H | 0.000 | 0.000 | 8.780 |
| F | G | 0.000 | 0.000 | 8.772 |
| F | H | 0.000 | 0.000 | 8.780 |
| G | H | 0.063 | 0.067 | 2.996 |

### 7.2.2 Statistical analysis

Statistical analysis was done in the software Minitab (version 16.1). There was performed a one-way ANOVA test together with Tukey`s post-hoc test.

Source of variation and the p-value, found in the analysis of total phenolics is shown in table 7.7 and Table 7.8.

**Table 7.7: Source of variation for Total phenolics. SS = sum of squares, df = degrees of freedom.**

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 182366.697 | 7 | 26052.385 | 1.886 | 0.105 | 2.313 |
| Within Groups | 442093.725 | 32 | 13815.429 | | | |
| Total | 624460.421 | 39 | | | | |

The grouping information found using Tukey metod in values for Total phenolics are shown in Table 7.8. Means that do not share a letter are significantly different.

**Table 7.8: Grouping information for Total Phenolics using Tukey metod. N= number of individuals.**

| Plot | N | Mean | Grouping |
|---|---|---|---|
| A | 5 | 588,0 | a |
| B | 5 | 572,4 | a |
| F | 5 | 531,5 | a |
| C | 5 | 522,9 | a |
| D | 5 | 444,1 | a |
| G | 5 | 429,9 | a |
| H | 5 | 429,4 | a |
| E | 5 | 401,3 | a |

### 7.2.3 Total anthocyanins

Source of variation and the p-value, found in the analysis done for Total anthocyanins is shown in Table 7.8.

**Table 7.8: Source of variation for Total anthocyanins. SS = sum of squares, df =degrees of freedom.**

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 19513.8 | 7 | 2787.690 | 1.607 | 0.169 | 2.313 |
| Within Groups | 55498.2 | 32 | 1734.319 | | | |
| Total | 75012 | 39 | | | | |

The grouping information found using Tukey metod in values for Anthocyanins is shown in Table 7.9. Means that do not share a letter are significantly different. Plot H are significant different from the other plots.

**Table 7.9: Grouping information for Anthocyanins using Tukey metod. N= number of individuals.**

| Plot | N | Mean | Grouping | |
|---|---|---|---|---|
| H | 5 | 429,41 | a | |
| D | 5 | 200,15 | | b |
| C | 5 | 184,44 | | b |
| G | 5 | 146,40 | | b |
| A | 5 | 143,19 | | b |
| E | 5 | 140,32 | | b |
| F | 5 | 139,80 | | b |
| B | 5 | 139,46 | | b |

### 7.2.4 Antioxidants

Source of variation and the p-value, found in the analysis done for antioxidants is shown in Table 7.10

**Table 7.10: Source of variation for Total phenolics. SS = sum of squares, df = degrees of freedom.**

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 8.895 | 7 | 1.271 | 1.018 | 0.437 | 2.313 |
| Within Groups | 39.934 | 32 | 1.248 | | | |
| Total | 48.830 | 39 | | | | |

The grouping information found using Tukey metod in values forantioxidants is shown in Table 7.11. Means that do not share a letter are significantly different.

**Table 7.11: Grouping information for Anthocyanins using Tukey metod. N= number of individuals.**

| Plot | N | Mean | Grouping |
|---|---|---|---|
| A | 5 | 4,628 | A |
| C | 5 | 4,209 | A |
| B | 5 | 4,103 | A |
| F | 5 | 3,983 | A |
| D | 5 | 3,784 | A |
| G | 5 | 3,673 | A |
| H | 5 | 3,590 | A |
| E | 5 | 2,922 | A |

### 7.2.5 GC-MS

The same tests were performed for concentration of the different volatile compound found in GC-MS.

**Table 7.12: Concentration (µg compound / 100 g fresh material) for each individual in each plot and the p-value.**

| | P-value | Ind. | A | B | C | D | E | F | G | H |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 30487.2 | 19892.6 | 29063.1 | 20924.8 | 28194.3 | 15487.1 | 6831.7 | 18563.9 |
| | | 2 | 31915.1 | 29196.8 | 44431.4 | 34401.5 | 31487.9 | 16093.9 | 19704.9 | 27336.0 |
| | 0.216 | 3 | 31454.7 | 8717.2 | 6912.9 | 17559.9 | 26523.3 | 28402.7 | 26470.2 | 23978.5 |
| | | 4 | 31363.2 | 27449.7 | 12229.1 | 20012.1 | 26104.3 | 29910.7 | 24265.2 | 17857.9 |
| **Fructose** | | 5 | 34586.8 | 18938.7 | 29918.5 | 26943.1 | 29980.3 | 23298.4 | 21328.7 | 19918.8 |
| | | 1 | 2803.6 | 2881.2 | 2845.3 | 3056.5 | 2005.8 | 3797.5 | 3068.8 | 2321.2 |
| | | 2 | 2763.4 | 3101.5 | 870.6 | 3042.3 | 2449.9 | 3205.2 | 3155.2 | 2034.1 |
| | | 3 | 2318.8 | 2972.0 | 3008.1 | 3537.3 | 2792.4 | 2832.7 | 2309.7 | 2077.1 |
| | | 4 | 3142.2 | 3311.5 | 3611.3 | 3625.1 | 2689.9 | 2266.8 | 2043.7 | 2349.5 |
| **Citric acid** | 0.086 | 5 | 2628.6 | 3097.8 | 3636.6 | 3246.8 | 2892.0 | 3226.7 | 3861.3 | 2048.4 |
| | | 1 | 2167.8 | 1206.7 | 2097.3 | 1570.7 | 1488.0 | 959.0 | 455.6 | 937.4 |
| | | 2 | 2342.2 | 2795.1 | 1399.6 | 2874.7 | 2007.4 | 992.6 | 1396.1 | 1781.2 |
| | | 3 | 1719.9 | 1314.1 | 421.6 | 1445.1 | 1683.4 | 1786.9 | 1707.5 | 1362.5 |
| | | 4 | 1737.7 | 1388.0 | 1197.0 | 1535.0 | 1660.4 | 2519.7 | 1464.7 | 957.8 |
| **Sucrose** | 0.199 | 5 | 2492.7 | 969.0 | 2436.7 | 2111.8 | 1723.9 | 1274.5 | 1359.4 | 827.9 |
| | | 1 | 16077.6 | 10356.7 | 16422.4 | 13575.8 | 15144.6 | 8005.1 | 7139.2 | 10972.1 |
| | | 2 | 16602.0 | 15191.4 | 22789.2 | 15967.6 | 22748.1 | 8276.3 | 11689.3 | 15098.2 |
| | | 3 | 16630.7 | 13083.6 | 5391.3 | 10457.1 | 14388.9 | 14404.9 | 15056.4 | 15069.6 |
| | | 4 | 14861.5 | 15171.1 | 9684.9 | 12011.5 | 14332.3 | 15953.1 | 16051.1 | 8551.9 |
| **Glucose** | 0.199 | 5 | 17898.1 | 12448.2 | 16570.5 | 15649.3 | 15172.2 | 13786.8 | 11904.6 | 12378.8 |
| | | 1 | 5340.7 | 3648.0 | 5536.6 | 3021.3 | 3697.7 | 5045.7 | 4173.6 | 4324.8 |
| | | 2 | 5346.4 | 5392.8 | 4914.9 | 4606.6 | 5403.1 | 5105.6 | 4836.4 | 4745.1 |
| | | 3 | 3451.9 | 4420.5 | 7432.0 | 3849.2 | 4951.1 | 4600.6 | 3626.9 | 3498.2 |
| | | 4 | 4077.9 | 5108.9 | 6706.2 | 3983.6 | 4954.5 | 3673.1 | 3234.8 | 4158.8 |
| **Quinic acid** | 0.002 | 5 | 3641.3 | 3673.1 | 5773.9 | 3649.6 | 4641.7 | 4235.1 | 4878.6 | 3777.6 |

*Cont.*

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Malic acid** | 0.003 | 1 | 801.4 | 1070.5 | 806.3 | 759.0 | 394.4 | 503.1 | 644.6 | 682.3 |
| | | 2 | 1390.6 | 1126.8 | 808.2 | 821.1 | 667.1 | 387.6 | 819.0 | 713.3 |
| | | 3 | 974.2 | 909.1 | 311.9 | 622.6 | 752.6 | 560.0 | 462.8 | 677.6 |
| | | 4 | 871.3 | 1343.2 | 797.3 | 652.8 | 685.7 | 875.6 | 1290.0 | 588.0 |
| | | 5 | 1005.9 | 827.1 | 665.2 | 742.2 | 720.9 | 519.4 | 1062.9 | 858.5 |
| **Myo-Inositol** | 0.223 | 1 | 488.8 | 280.8 | 513.5 | 433.3 | 367.5 | 604.7 | 405.7 | 379.8 |
| | | 2 | 330.3 | 556.8 | 468.2 | 616.3 | 602.6 | 616.0 | 521.0 | 637.7 |
| | | 3 | 379.5 | 446.6 | 706.9 | 730.8 | 441.0 | 592.5 | 492.7 | 378.7 |
| | | 4 | 645.7 | 713.4 | 688.5 | 563.6 | 542.0 | 451.5 | 353.7 | 366.1 |
| | | 5 | 433.3 | 481.1 | 539.5 | 562.1 | 545.3 | 542.5 | 589.8 | 359.7 |
| **Chiro-Inositol** | 0.437 | 1 | 83.3 | 3.9 | 36.0 | 31.3 | 27.1 | 29.1 | 30.1 | 45.6 |
| | | 2 | 50.3 | 43.7 | 4176.1 | 63.5 | 45.1 | 19.9 | 44.5 | 26.8 |
| | | 3 | 31.2 | 53.5 | 55.7 | 38.2 | 55.9 | 47.5 | 31.3 | 26.5 |
| | | 4 | 47.5 | 114.5 | 50.4 | 43.0 | 66.5 | 43.8 | 25.1 | 47.8 |
| | | 5 | 36.3 | 67.3 | 69.2 | 48.6 | 59.0 | 47.0 | 48.2 | 48.0 |
| **Xylose** | 0.797 | 1 | 54.1 | 135.3 | 39.4 | 56.5 | 61.5 | 22.1 | 21.5 | 64.5 |
| | | 2 | 58.3 | 39.8 | 71.3 | 55.2 | 55.2 | 25.3 | 44.8 | 41.0 |
| | | 3 | 42.9 | 37.2 | 21.9 | 50.9 | 44.5 | 61.0 | 41.1 | 55.6 |
| | | 4 | 44.2 | 45.4 | 181.8 | 54.3 | 59.3 | 55.6 | 79.1 | 53.0 |
| | | 5 | 36.1 | 42.3 | 43.4 | 56.8 | 54.9 | 45.2 | 30.2 | 53.7 |
| **Arabinose** | 0.877 | 1 | 219.7 | 385.9 | 163.4 | 231.9 | 257.8 | 102.0 | 102.2 | 275.9 |
| | | 2 | 237.4 | 177.8 | 293.2 | 224.6 | 227.5 | 113.6 | 184.5 | 186.1 |
| | | 3 | 184.2 | 162.0 | 98.8 | 208.0 | 196.3 | 256.3 | 172.3 | 247.5 |
| | | 4 | 193.4 | 186.8 | 290.5 | 222.9 | 247.5 | 232.2 | 370.3 | 228.0 |
| | | 5 | 152.8 | 176.1 | 197.4 | 247.0 | 224.6 | 204.8 | 137.3 | 234.9 |
| **Ribose** | 0.086 | 1 | 22.2 | 241.4 | 17.7 | 21.2 | 17.1 | 19.6 | 16.6 | 19.5 |
| | | 2 | 24.2 | 18.8 | 25.8 | 26.3 | 18.2 | 20.7 | 25.1 | 24.9 |
| | | 3 | 19.6 | 25.3 | 29.3 | 23.7 | 18.9 | 22.6 | 17.7 | 15.6 |
| | | 4 | 33.6 | 20.0 | 125.0 | 23.9 | 21.7 | 17.8 | 63.9 | 19.1 |
| | | 5 | 21.1 | 176.1 | 20.4 | 23.4 | 26.8 | 23.8 | 27.1 | 16.6 |
| **Chlorogenic acid** | 0,877 | 1 | 58.7 | 67.1 | 171.9 | 85.7 | 92.7 | 102.2 | 203.9 | 71.5 |
| | | 2 | 127.6 | 96.8 | 199.1 | 124.8 | 56.4 | 118.7 | 123.3 | 197.6 |
| | | 3 | 84.2 | 74.3 | 217.5 | 101.1 | 53.1 | 130.8 | 105.9 | 36.7 |
| | | 4 | 91.5 | 247.5 | 39.9 | 119.5 | 50.3 | 297.1 | 84.3 | 96.6 |
| | | 5 | 118.0 | 160.3 | 75.6 | 140.7 | 115.8 | 167.2 | 93.9 | 97.6 |
| **Shikimic acid** | 0.045 | 1 | 62.4 | 265.2 | 31.8 | 13.6 | 38.3 | 59.1 | 31.2 | 49.7 |
| | | 2 | 52.3 | 25.8 | 52.5 | 23.8 | 16.1 | 65.1 | 74.7 | 70.2 |
| | | 3 | 45.9 | 51.8 | 41.2 | 14.7 | 55.9 | 43.1 | 51.7 | 69.7 |
| | | 4 | 31.0 | 130.1 | 93.4 | 14.9 | 14.5 | 51.1 | 32.8 | 53.7 |
| | | 5 | 39.5 | 58.2 | 94.8 | 13.3 | 48.2 | 39.3 | 61.1 | 51.4 |