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Investigating the influence of biotic and abiotic factors on the phytochemical variation of lingonberries (*Vaccinium Vitis-idaea* L.) in Norway

Mathias Rudolf Amundsen

A dissertation for the degree of Philosophiae Doctor – October 2023

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1 Acknowledgements

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Writing a doctoral thesis is not just blueberries, but if this thesis inspires at least one more person to go out to pick wild berries, I consider it a success.

2 Abstract

Lingonberry is an evergreen dwarf shrub abundant in the area of Nordic countries and a food traditionally regarded as a staple of Nordic diets. There is however limited commercial harvest of these readily available berries. The objective of this doctoral thesis is to investigate how the composition Norwegian lingonberries vary and is affected by abiotic and biotic growth conditions. The thesis consists of four papers of which three studies were conducted in controlled conditions investigating the effects of ripening, light conditions, and temperature during ripening on the composition of the targeted compounds in lingonberries. The fourth paper of the thesis investigates the variation in composition of wild Norwegian lingonberries and how different environmental factors influence this composition. In lingonberries, in total 29 phenolic compounds, sucrose, glucose and fructose as well as 4 organic acids and 77 volatile organic compounds were detected.. In the controlled studies time of harvest significantly influenced the quality of the lingonberries. Spectral light composition with supplemental blue wavelengths increased the content of anthocyanins, and slightly influenced the ratio of sugars to organic acids. There was only a limited effect of light intensity on the content of anthocyanins in lingonberries. Berries grown at lower temperatures had a higher content of anthocyanins and organic acids, whereas the other phenolic compounds were not significantly influenced. Latitude and temperature had the most significant effect on the content of anthocyanins in lingonberries. While light conditions only slightly influence berry quality, factors such as amount of precipitation during ripening, the density of deciduous trees, and altitude also significantly influenced berry quality. Precipitation influenced the content of organic acids in the field study. The result from this study further strengthens the evidence that lingonberries are a rich source for dietary polyphenols, and that berry quality increases with later harvest times. High quality lingonberries can be found across the country with large variation within local areas. The combination of field experiments and controlled experiments showed that weather conditions during ripening, latitude and density of deciduous trees all influence berry quality.

3 List of papers

The thesis is based on four papers. Papers I and III are reprinted from the respective journal publishers, under creative commons attributions license CC-BY. The papers are referred throughout the text by their Roman numerals.

Paper I – Composition of sugars, organic acids, phenolic compounds and volatile organic compounds in lingonberries (*Vaccinium Vitis-idaea* L.) at five ripening stages

M. Amundsen, A L. Hykkerud, N. Kelanne, S. Tuominen, G. Schmidt , O. Laaksonen, B. Yang, I. Martinussen, L. Jaakola, and K. Aaby¨

Published in Foods https://doi.org/10.3390/foods12112154.

Paper II - Effect of light conditions on the chemical composition (*Vaccinium vitis-idaea* L.)

A L. Hykkerud, **M. Amundsen**, I. Martinussen, K. Aaby and L. Jaakola

Manuscript prepared for submission.

Paper III - Effect of temperature on the chemical composition of northern and southern ecotypes of lingonberries (*Vaccinium vitis-idaea* L.)

M. Amundsen, L. Jaakola, K. Aaby, I. Martinussen, N. Kelanne, S. Tuominen, O. Laaksonen, B. Yang and A L. Hykkerud

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Paper IV- Analysis of variation in content of anthocyanins, sugars, and organic acids in wild populations of lingonberry (*Vaccinium vitis-idaea* L.) in Norway

M. Amundsen, I. Martinussen, K. Aaby, A. Granhus, M. Hauglin, L. Jaakola and A L. Hykkerud,
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4 Abbreviations

CAD – Cinnamic acid derivative

DMAC– Dimethylaminocinnamaldehyde

DW– Dry weight

FW– Fresh weight

GC – Gas chromatography

HPLC – High performance liquid chromatography

HS – Head space

MS– Mass spectrometry

PA – Proanthocyanins

PAR – Photosynthetically active radiation

SPME – Solid phase micro extraction

UTI – Urinary tract infection

UV – Ultra violet

VOC – Volatile organic compounds

5 Introduction

5.1 Background

It has been estimated that Norwegian consumption of fruits and berries has nearly doubled in the last 70 years [1]. In 1979, 29 % of the Norwegian fruit and berry supply was harvested from Norwegian sources. This has drastically been reduced with 92.5 % of fruits and berries being imported in 2021 [2,3]. Traditionally, wild berries have been an important source for nutrition in the Nordic region. However, these resources remain largely untapped, with an estimated annual yield of over a million tons of wild berries in Nordic forests [4]. Harvest of berries, mushrooms, and plant parts from outfield areas is permitted in Norway as long as it is done with consciousness and care for nature [5]. Such harvesting cause minimal damage to the foraging areas while providing a substantial annual berry yield without significant encroachment on nature [6]. There are a number of berries growing in Norwegian forests that are used as food including; bog bilberries (*Vaccinium uglinosum*), lingonberries (V. *vitis-idaea* L.), raspberries (*Rubus idaeus*), wild strawberries (*Fragaria vesca*), cloudberries (*R. chamaemorus*), European blueberry/bilberry (*V. myrtillus*), arctic bramble (*R. arcticus*), crowberries (*Empetrum nigrum*), blackberries (*R. ursinus*), cranberries (*V. oxycoccos and V. microcarpum*), rowanberries (*Sorbus aucuparia* L.), hawthorns (*Hippophae rhamnosides*), elderberries (*Sambucus*) and junipers (*Juniperus communis*). Finland and Sweden have the most developed wild berry industries in the Nordic region [7]. The value of the wild berry industry has been reported to be worth between 8.7 to 25.3 mill. €/year in the timespan between 2008–17 in Finland [4]. Although there is growing interest in the wild berry processing in Norway, it is still a relatively small industry. However, considering the historical context of commercial berry harvest in Norway, there is a potential for increase in the size of the berry industry. The first reference of export of Norwegian lingonberries is dated 1835 and it reached its height at the turn of the 20th century with the development of the railways [7]. It has been estimated that it in 1896 it was exported as much as 170000 kg berries annually from Norway [8], and that as much as 20 million kg of Swedish lingonberries was exported annually at the turn of the century [9]. These figures show a substantial potential for market increase of wild Norwegian berries. These berries could play an important part to achieve the political targets to increase the degree of self-sufficiency in Norway back up to at least 50 %, with a 50 %

increase in the proportion of Norwegian fruits and vegetables [2]. Furthermore, increased utilization of wild berries aligns with these targets while preserving traditional food traditions and promoting public health.

5.2 Lingonberries

Lingonberries (*Vaccinium vitis-idaea* L.) are a species of heath plant in the Ericaceae family (Figure 1). The *Vaccinium* genus contains about 450 species that produce small to medium-sized fleshy berries. In addition to lingonberries, four wildly edible species are found in the Nordic countries, European blueberry (bilberry), bog bilberry, two cranberry species [12]. There is evidence that lingonberries have been used in Scandinavia since the bronze age, but the first scientific description of lingonberries was made by the Italian diplomat Lorenzo Magalotti in 1674 [9]. Several other scientists including Carl von Linné (1748) and Anders Jahan Retzius (1806) have described the botany of lingonberries and the Swedish botanists Linné gave lingonberries the Latin name *Vaccinium vitis-idaea* in his classification of species [9]. Lingonberries are found in the circumpolar areas of the world, in two subspecies that are geographically separated: *V. vitis-idaea* var. *vitis-idaea* and L *V. vitis-idaea var. minus* Lodd.

Figure 1 Illustration of blooming branch (1), berries (2), flowers cut in half (3) and stamen (4) of lingonberries [10].

5.2.1 Characteristics and growth

Lingonberries are evergreen dwarf shrubs that have an extensive subterranean rhizome system with aerial systems typically reaching between 10 to 30 cm above ground [11]. On the aerial shoots the leaves typically form 2-3 ranked leaves, that are between 1-3 cm and have characteristic oval glossy, dark green leaves with a leathery texture [11]. The Eurasian subspecies (*V. vitis-idaea* var. *vitis-idaea)* have slightly larger leaves than the North American subspecies. Lingonberry flowers are bell-shaped flowers that typically are pink or pale red and hang in clusters. Flowering of lingonberries typically occurs in late spring. Pollination can be through self-pollination, but they are commonly insect-pollinated [11]. Long-term follow-up of Finnish lingonberry sites have shown a pollination rate at about 60 % but with up to 90 % in good years giving abundant yields found in forests around the northern hemisphere [9]. The green berries form during summer and ripen in late summer/early autumn. Lingonberries are scarlet red, and mostly globous, but there have been found berries of several different shapes [9]. Berries from the Wildberries project have varied from round to oval and pear shaped (Figure 2). The lingonberries have on average 7 seeds [11]. In Finland the estimated annual yield has been on average 257.2 million kg with variation from 182.6 to 385.7 million kg, making them the most abundant berry species in the country [4]. In 1991 it was estimated that the total yield of lingonberry production in Norwegian forests varied between 44 and 115 million kg per annum [9]. It has been estimated that approximately 80 % of Nordic wild berries are harvestable, whereas only 2-12 % with an average of about 7.6 % has been harvested between 1997 and 2008 in Finland [4]

Figure 2 Shapes lingonberries picked in Ås during the 2020 growth season (cropped picture by Mathias Amundsen).

5.2.2 Environmental adaptation

Found across the northern hemisphere the forests in which lingonberries grow are largely varying. Lingonberries prefer to grow in in leached soils with low pH, low base saturation, and low calcium content [11]. They thrive in areas with little light, as the canopy closes, they cover the floor at the expense of more light-dependent species [9]. Lingonberries have a generally strong ability to adapt to variation in growth conditions. In 1955 Ritchie [11] described how lingonberries adapt quickly from extreme events like wildfires compared to many of its heath concomitants. He also noticed how lingonberry's ability to reproduce was closely linked to temperature [11]. Other plants can generally grow in temperature ranges from of -10 to +60°C, defined by the freezing point of intracellular water and the temperature of substantial protein denaturation. However, lingonberries have made adaptational changes and buds are able to withstand minimum temperatures as low as -25° and -32°C in January. Lingonberries can tolerate temperatures down to at least -40°C but grow poorly in areas with warm summers [12]. It is thought that lingonberries have the thick leathery leaves to better withstand the cold temperature stress, as it has been shown that the evolutionary process has influenced the resistance of plants to the temperatures in which they grow [13]. In Norway heaths have been found at altitudes up to 1800 m, though they are known to grow at higher altitudes further south on the continent [9,14]. Despite poorly withstanding higher temperatures, lingonberries have a great ability to survive in dry environments [9]. Lingonberries prefer acidic and nutrient poor environments, but they can be found on a variety of forest floors [11]. Weather conditions during the growth season are very important for the yield of lingonberries, as frost, severe drought, or abundant rain may lead to higher losses of buds, flowers, and unripe berries, and maximum growth was reached at low photosynthetic light flux [9]. Both thinning of forests and clear cutting of forests reduced the biomass of lingonberries, but as it was regained quickly it gives the berries a competitive advantage. The highest number of berries are typically found in clear cut forest a few years after recovery [9]. Commercial cultivation has been attempted extensively for the last 100 years but has not reached major commercial success [9].

5.2.3 Uses as food and medicine

Lingonberries have traditionally been used as food, medicine, as an ornamental plants and a food storage agent [9]. Typically, they have been eaten mostly frozen, or produced into jams and they are essential to many traditional dishes in the Nordic countries [9]. The berries, have a particular complex flavour profile, with the sour and bitter flavours being predominant, and with astringent properties that some find challenging [15]. Lingonberries have been part of the traditional medicine in the Nordics, Russia, China, Canada and the US [16-18]. Traditionally lingonberries and their leaves have been used for treatment of a variety of different diseases and health issues, including issues related to the blood, which was attributed to their scarlet red colour [19]. In the recently published Nordic nutrition recommendations increased consumption of fruits and vegetables and berries was considered a key part of changes considered beneficial for the general population [20]. There is also promising research that has shown how inclusion of lingonberries to our diets can help prevent several chronic and acute diseases [17,21]. There has long been evidence supporting that lingonberries can help in reduction of risk for urinary tract infections (UTI). In clinical trials evidence has been found supporting that lingonberry and cranberry phenolic compounds can reduce adhesion of bacteria to the urinary tract wall [22-24]. Additionally, lingonberry consumption has been found to reduce the prevalence and risk factors of several of modern lifestyle diets. Recent studies have shown reduction of cholesterol postprandial glucose levels, in the weight and visceral fat gain, and a potential for management of type 2 diabetes [25-29]. Due to the increasing amount of evidence for benefits of lingonberry consumption, many consider lingonberries to be a high value nutrient source [17,21]. By increasing the proportion of local fruits and berries in the diet, many consider that both public health and the sustainability of our diet would improve [30].

5.3 Quality and health related compounds in lingonberries

Compared to earlier times there are today a large array of fruits and vegetables for curious consumers conscious about quality. Studies have shown that nutritional value and the nature of health-promoting constituents are important when consumers choose what products to pick. Beliefs, attitudes, perceptions, and preferences all affect consumers in in their choice of fruits in the stores [31]. In studies of berries, it has been found that people tend to prefer sweeter

and less sour and bitter berries and prefer berries they are familiar with [15]. This has been found as a challenge to lingonberries as they are often referred to as bitter, sour, and astringent. The most abundant compound in lingonberries is as in most fruits and berries is water. Water content of lingonberries is approximately is approximately 83 % of the berry [32]. Per 100 g fresh weight (fw) lingonberries also contain 7-8 g sugar, 2.2 g dietary fibers, 2.2 g organic acids, 0.7g protein and 0.5 g lipids [32,33]. This gives an average energy content of in around 44- 56 kcal/100 g in fresh berries [34,35]. These macronutrients contribute to both the flavour and energy content of the berries which is between 44 and 56 kcal/100 g in fresh berries [34,35]. Micronutrients in lingonberries include a vitamin content of 1,5mg alfa-tocopherol equivalents, approximately 8 mg vitamin C, 0,67 retinol equivalents of vitamin A, and small amounts of the vitamin B's per 100g. Eating 100g of lingonberries, would thus give approximately 10 % of the average daily requirement for adults of Vitamins C and E [36,37]. Lingonberries are also rich sources of several health promoting compounds including high levels of phenolics like flavan-3-ols, cinnamic acid derivatives and flavonols, they are increasing in popularity [38]. These compounds in berries are recognised as potential agents reducing the risks of several lifestyle related diseases [17].

5.3.1 Phenolic compounds

Phenolic compounds are a diverse and important group of phytochemicals found in most plants. Common characteristics of the compounds are a carbon structure with one or more aromatic ring(s) with at least one hydroxy substituent [39]. They are among the most abundant plant specialized metabolites and prevalent bioactive compounds in fruits and berries, with more than 10000 phenolic compounds identified to date [40]. Though tannins previously were considered antinutrients due to their binding to iron and certain proteins inhibiting their uptake in the body [40]. Phenolic compounds also give olfactory sensations with colour flavour and astringency [41,42]. In plants, polyphenolic compounds have several different functions as attractants to pollinators, as protective agents against attackers and outside stressors as well as being structural materials in cells [39]. Among the numerous compounds there is a large variety in complexity, and subclassification is most commonly done based on their chemical structure. The five most common subclasses are flavonoids, phenolic acids, stilbenes, lignans, and tannins[40]. Flavonoids are both the most numerous and abundant among the naturally

occurring phenolic compounds. All the flavonoids have a basic structure with of two aromatic rings connected by a three-carbon chain in a C6-C3-C6 backbone (Figure 3). Flavonoids are divided into six subclasses with various oxidation and saturation of the C ring; anthocyanins, flavonols, flavones, isoflavones, flavanols and flavonones, that give them different properties. Anthocyanins are the main pigments in flowers and fruits responsible for most red, blue and purple colours found in the nature. In different pH anthocyanins change colour, in low pH conditions appear as red but turn bluer with increases of ph. There are six main classes of anthocyanidins; cyanidin, delphinidin, pelargonidin, peonidin, petunidin and malvidin, that in their glycosylated forms are known as anthocyanins. Cyanidin glycosides are most abundantly found anthocyanins in nature. As much as 82% of anthocyanins identified in fruits and berries have been cyanidin glycosides [43]. Flavonols are also important antioxidants and UV-protectants that a double bond between the C2 and the C3 position and a hydroxy group at the C3 position. In plants, flavonols are represented mainly by quercetins, kaempferols, and myricetins. Differing in a hydroxyl group at C3-position on the C-ring compared to the flavanols, the flavonols is the most abundant group of the flavonoids found in nature. The simplest structures among the proanthocyanidins are catechin and epicatechin, which can bind to each other in cells to create large complex molecules. The most known of these are the polymeric compounds more commonly known as tannins that amongst others give wines their astringent properties [40].

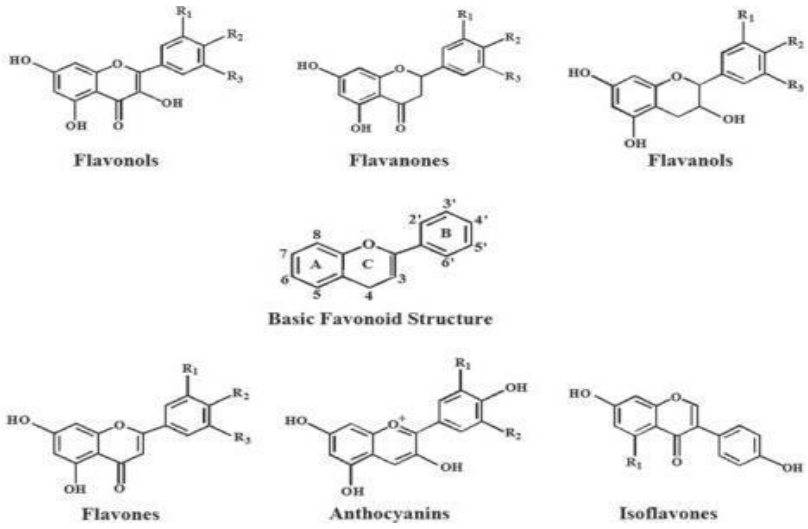

Figure 3 Basic flavonoid structures, including flavonols, flavanones, flavanols, flavones, anthocyanins and isoflavones.

Non-flavonoid compounds typically have simpler structures generally with an aromatic ring with a C-1 or C3 bound, which again are further sub-categorized onto simple phenols, phenolic acids, benzoic aldehydes, hydrolysable tannins, acetophenones and phenylacetic acids, hydroxycinnamic acids, coumarins, benzophenones, xanthones, stilbenes, lignans and secoiridoids. However, also other phenolic acids, lignans, stilbenes, coumarins and tannins belong to the group of phenolics [40]. Phenolic acids in plants are involved in various functions, including photosynthesis, nutrient uptake, protein synthesis, and in enzyme activity. Hydroxycinnamic acids are aromatic acids with a C6–C3 skeleton typically found in foods as simple esters bound to quinic acid or glucose. The most abundant cinnamic acid derivatives (CADs) are p-coumaric, caffeic, ferulic and sinapic acids. Most hydroxybenzoic acids have a C6-C1 backbone derived from benzoic acid. Benzoic acids are well known preservatives in foods inhibiting the growth of fungi. Biosynthesis of flavonoids has been extensively studied and has been characterized in various plant species. Most plants' phenolic compounds are synthesized through the phenylpropanoid pathway. The precursor of all phenolic compounds is L-phenylalanine (or L-tyrosine), which is synthetised through the shikimate pathway, then converted to cinnamic acid and 4-coumaroyl- CoA. 4-coumaroyl- CoA is the precursor for the phenylpropanoid pathway, which takes place in the plant cytosol and is the pathway leading to the synthesis of numerous flavonoids (Figure 4) [40,44]. After synthesis the compounds are mostly conjugated to sugars such as glucose, rutinose and rhamnose and accumulated in the plant vacuoles and various other subcellular locations as glycosides. Biosynthesis of most phenolic compounds in lingonberries begins already at fruit set, with highest content of phenolic compounds observed in immature fruit [45-47]. However, changes in the composition of phenolics and terpenoids occur during the fruit development and ripening [46,48,49]. The initiation of synthesis of anthocyanins during the later stages of ripening is the most visible change occurring in this process [49].

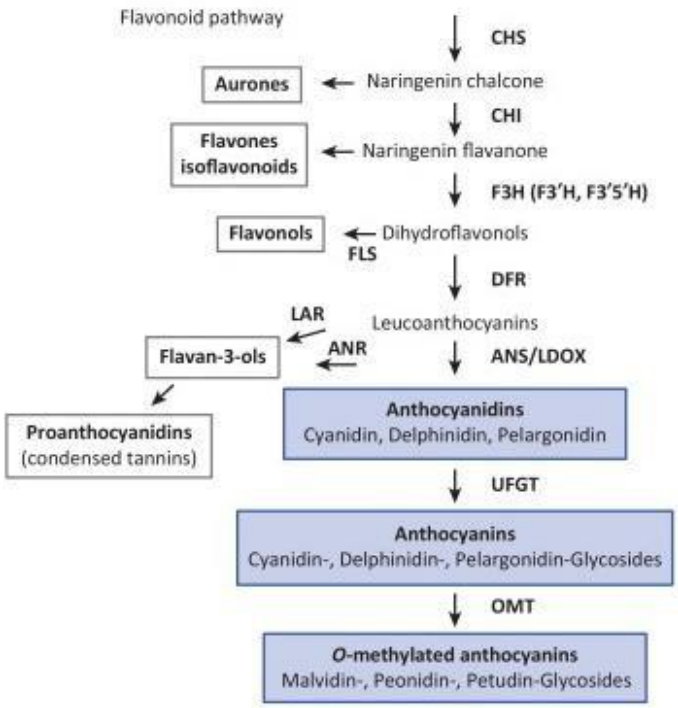

Figure 4 - General overview of the flavonoid biosynthesis pathway from Jaakola [43].

Several studies have previously investigated the phenolic profile of lingonberries and lingonberry leaves. The highest number of phenolic compounds identified in one study, was 56 phenolic compounds in lingonberry fruit extracts and 127 across all parts of the plant [50]. The most abundant of the phenolic compounds in lingonberries proanthocyanins, but also several anthocyanins, hydroxycinnamic acids, hydroxybenzoic acids and flavanols have been identified. Between 63 and 71 % of the total content of phenolic content in lingonberries have been reported to be proanthocyanins [51]. Cyanidin-galactoside, -glucoside and -arabinoside are the three main anthocyanin compounds in lingonberries, but also small amounts of other anthocyanins have been identified [48,52].

5.3.2 Soluble sugars

The commonly used term sugars, refers to the simplest form of carbohydrates namely the mono- or disaccharides. Monosaccharides can be found as three to seven carbon molecules of which the five or six carbon saccharides are being most common. These compounds are sweet tasting caloric compounds found abundantly in nature. Carbohydrates are the main source of fixed carbon for all life and source of energy in the human diet providing approximately 4 kcal/g of

energy per gram eaten. It is recommended that between 40-60 % of our energy in the diet is through carbohydrate with a maximum of 10 % of these coming from the simple sugars. The soluble sugars have a sweet flavour favourable to many consumers, which varies between different sugars. The sweetest of the naturally occurring sugars found in lingonberries is fructose with almost double the sweetening effect compared to sucrose [53]. In plants and in all living cells sugars play important roles. They act as a key source of energy in plants and can also act as osmolytes in regulation of the osmotic pressure in response to stress in cells. Among the most common monosaccharides are glucose, fructose and galactose varying in the structural orientation around several of the chiral centres found in the compounds. Dimers of the monosaccharides are known as disaccharides, and carbohydrates are generally characterised dependent on the number of monosaccharide units into oligo- or polysaccharides and their derivatives. Biosynthesis of the simple sugars occurs through photosynthesis in the photosynthetic tissues has been extensively described. The synthetised sugars are transported to fruits through sieve elements of the phloem in the plant. In addition to simple sugars, other complex carbohydrates such as starch are produced through photosynthesis, and used for storage, and cleaved for utilization when needed by the plant to other tissues [54]. Previous findings have shown that the sugars in lingonberries consist of fructose, glucose in approximately equal parts and sucrose in lower concentrations [55].

5.3.3 Organic aids

Organic acids are organic compounds characterized by one or more carboxylic acid groups with weak acidic properties and they do not dissociate completely in water. In plants, organic acids provide redox equilibrium and regulate pH and osmotic pressure. Organic acids typically have a tart, sour, or acidic flavour and can give astringent characters to a food [38]. However, organic acids also regulate the sweetness in flavour of berries, as the ratio of sugars to organic acids is determinant of how sweet flavour is perceived [15,56]. Among Nordic berries that are generally characterised by having a high organic acid content, lingonberries are among the ones with the highest [35,38,57]. Fumaric, malic, succinic and citric acids are intermediates of the oxidative tricarboxylic acid cycle, whereas compounds like quinic and shikimic acids are intermediates in synthesis of aromatic compounds (Figure 5) [58]. The studies of lingonberry juices on the content of organic acids have reported levels of 2.0-3.2 g/100 ml [35,57,59,60]. Lingonberries

have previously been shown to contain several organic acids, such as shikimic-, fumaric-, citric-, tartaric- malic acid, though not all of these have been found in different studies [34,35,57].

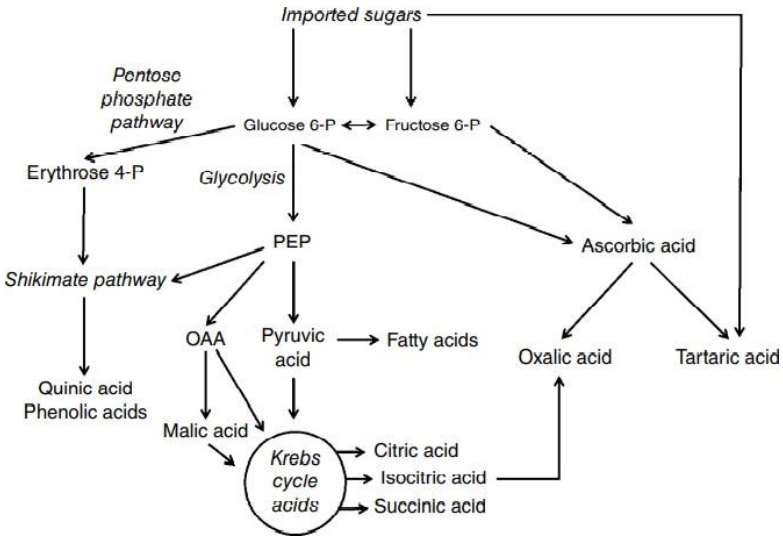

Figure 5 General overview of the synthesis pathways for several organic acids adapted from Walker and Famiani [58].

5.3.4 Volatile organic compounds

Volatile organic compounds (VOCs) are a large group of heterogenous small molecules that have a low solubility in water and a high vapor pressure. More than 1700 compounds have been identified from 90 plant families [61] VOCs volatility makes them act as compounds that alone or together gives aroma to foods and can contribute to the perception of sweetness [61]. In all fruits and berries and VOCs are present in large numbers. While there are often hundreds of such VOCs in a food,s the characteristic odours are often determined by the relative ratios of a relatively low number of compounds [62]. The ability of a compound to give an olfactory sensation is dependent on the concentration of the compound, or compounds in a matrix and the interaction between compounds [63]. Some volatile compounds form bonds to other compounds and structures in the plants which influences their volatility. Both the matrix and composition of a berry or berry product therefore strongly influences the results of volatile analysis across different studies [64]. Aromas of different compounds are however not always dose dependent. Each compound has a threshold aroma, and compounds varying 100 or even 1000-fold can contribute to aroma profiles in similar or different ways dependent on their individual or relative proportions [62]. In plants, VOCs have a variety of

roles including roles in plant communication and defence mechanisms. Due to the diversity of the VOCs and number of classes, there is also a larger complexity in the biosynthesis pathways compared to many other group of compounds (Figure 6). These pathways include the metabolism of amino acids, sugars, and lipids where the volatile compounds are often intermediates in complex parts of these biosynthesis processes [65]. The main classes of VOCs have been identified as being alcohols, aldehydes, amines, esters, ketones, lactones and terpenes and within each class there can be thousands of compounds. Some of the most odoriferous are monoterpene alcohols. In the studies analysing lingonberry aroma there has been to date identified more than 130 individual compounds, that belong to many of the groups mentioned. Prior to this thesis, only one study analysing VOCs of unprocessed lingonberries was found, which identified 38 distinct volatile aroma compounds [56]. However, also three other studies have been conducted on VOCs of lingonberry juice or juice biproduct [59,66,67]. A key compound, making up a large proportion of the total lingonberry volatiles in the studies was 2-methylbutanoic acid [66,67]. Using GC-olfactometry some of the key volatiles in lingonberries for aroma 2-methylbutanoic acid, eucalyptol, hexanal, linalool, methyl benzoate Diacetyl and 2-methylpropanoate were identified [59].

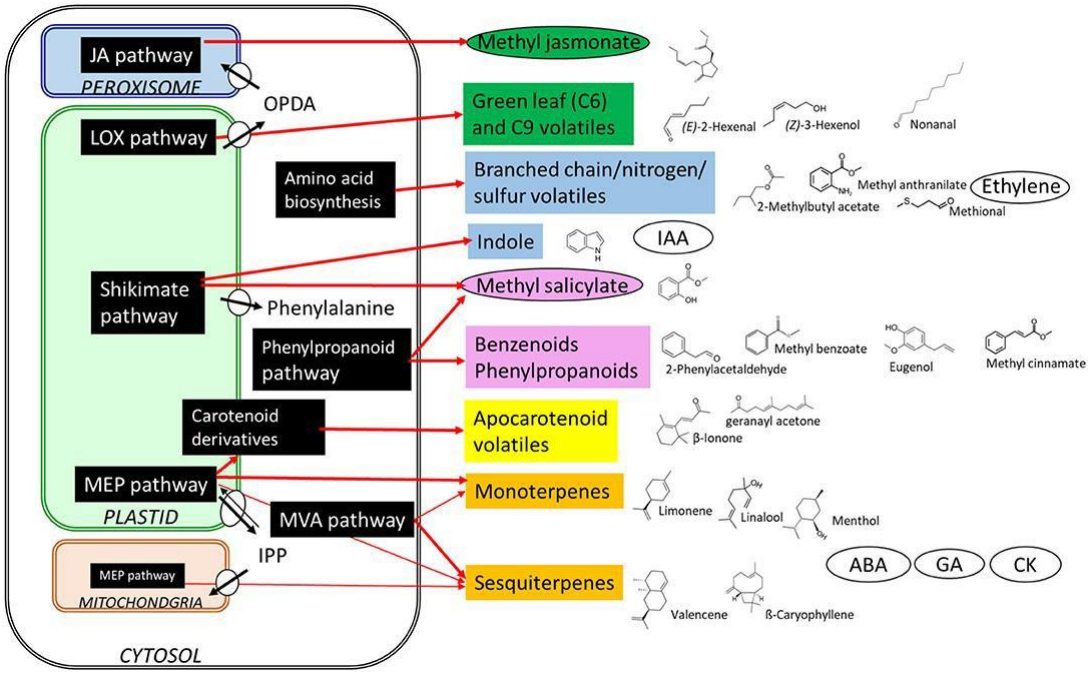

Figure 6 General overview of synthesis of volatile organic compounds in plants adapted from Bai, Jordán and Li [61].

5.4 Environmental factors and their effect on berry quality

Norway stretches from the latitude of 57°N in the south to 71°N in the north, and from 4°E in the west to 31°E in the east and has a very diverse landscape. Altitudes range from sea level to 2469 m and there are both coastal and inland regions. The Norwegian Ocean Current creates a special climate for such high latitudes, and there is a large variation in the climate in Norway with everything from arctic to sub-European climate [68]. Norway has abundance of water and large areas of outlands and therefore the Nordic region presents unique ecosystems for plant growth and there is large variation in the local flora, as 95% of the land area is wilderness [69]. Along with ongoing Climate warming, substantial changes are predicted, which will be most substantial in the arctic regions. These kind of changes inabiotic conditions have been shown to influence the expression of metabolites in fruits and berries [70]. These will naturally also influence the local flora [71], which together with the local fauna are the biotic factors that can influence the metabolism of the fruits and berries. Adaptations of plants to variations in the abiotic and biotic differences are species dependent [70,72]. These changes influence rates of processes and availability of energy or nutrients to perform essential functions, such as photosynthesis, respiration rate, nutrient uptake, osmotic balance and enzymatic activities (Figure 7). To understand berry quality, it is key to understand the adaptations in the plant metabolism to environmental changes [71].

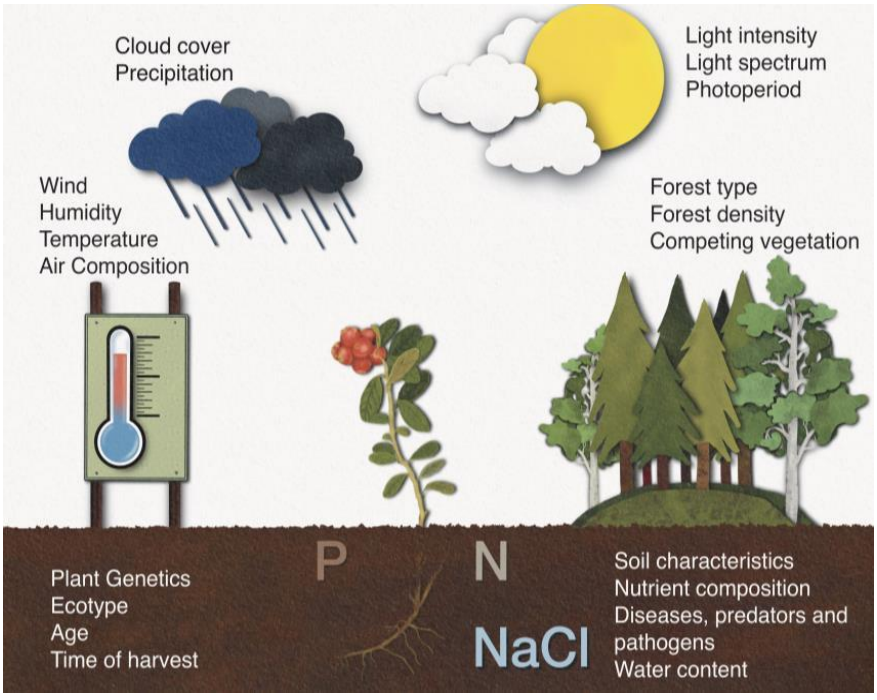

Figure 7 Illustration of selection of factors considered to influence the composition of lingonberries illustration by Magnus Halsnes.

5.4.1 Light

Light is considered the most important factor influencing plant growth, biomass production and crop quality, being their main source of energy [44,73]. Light possesses several qualities, including photoperiod, spectrum, and intensity, that can vary with seasons and location on the planet. Earth has an angle of approximately 23.45° compared to the sun which contributes to many of the differences mentioned (Figure 8). Photoperiod is closely related to the solar tilt, and during the summer months above the Arctic Circle there is light available 24-hours a day, and oppositely there is no direct sunlight during the winter months. As lingonberries flower around may to June there can be large variation in the photoperiod during flowering and fruit development. However, ripening of the berries in early autumn is closer to equinox where day and night are equally long and thus there is less difference in photoperiod during ripening of the berries at different latitudes, though there are some differences.

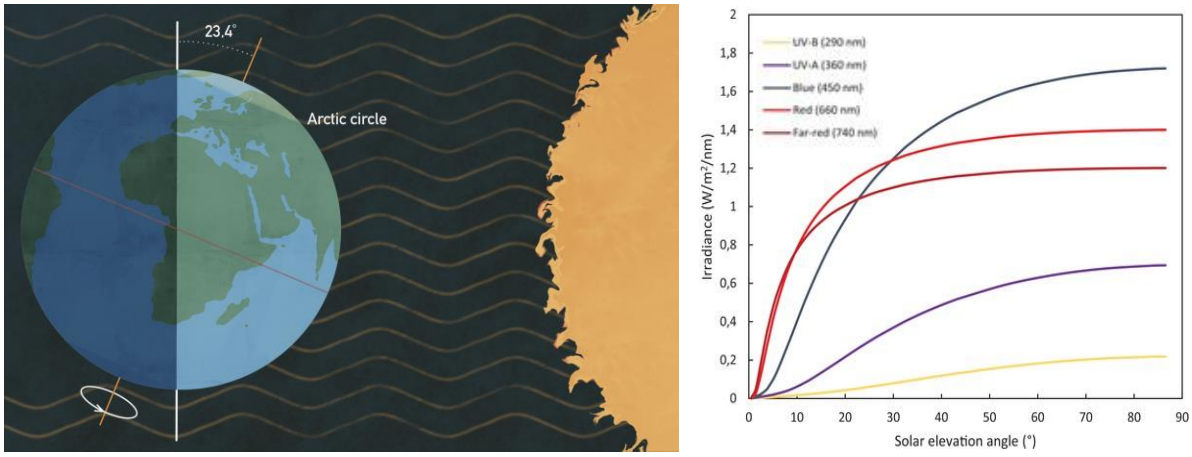

Figure 8 Illustration of the solar tilt compared to the sun by Magnus Halsnes, and figure showing the irradiance of light of different wavelengths with solar elevation angle adapted from Mølmann et al [68].

During the 24-hour photoperiod in Arctic in summer the sun is lower above the horizoncompared to southern latitudes, which influences the radiation properties of the light. The light scattering properties of the ozone layer is dependent on the angle at which the ray enters (Figure 8) and changes the length of path through the atmosphere. As the solar angle in the Arctic is narrower during the summer months, the light spectrum has a lower proportion of blue light and higher proportion of far red light influencing amongst others the red to far red rate which is known to influence plant growth [68]. In the field studies comparing northern Norway and the Italian alps, a clear difference in the light radiation between the two regions

was shown [74]. Plants have a range of photoreceptors making them able to utilise and detect different wavelengths of light from ultraviolet UV-B to far-red (280-700 nm) for photosynthesis [75]. The conversion of light energy to metabolites in plants is not very efficient with rates ranging from 4-6 % [76]. These efficiency differences are in part due to varying efficacy between receptors, but also due to the variation of light intensity due to a number of factors. The sum of receptor efficiency and amount of radiation has previously been shown to be the primary determinant of the crop yield in some species [75]. It has been shown that variation in the light spectrum influences the content and composition of flavonoids in many fruits and berries [70,77,78]. Light spectral characteristics have been shown to influence signal transduction and transport mechanisms in anthocyanin biosynthesis [54]. Light scattering in the atmosphere also decreases the energy of the light available at the earth's surface, with a lower energy content on a horizontal surface received in the regions the furthest regions [79]. The energy received on the earth surface is however not clearly linear but is influenced by several factors like differences in weather conditions in these regions, and with cloud cover scattering the light [68]. There are several factors determining the light reflected to a plant including geographic location, time of day, time of year, landscape, and the forest and weather conditions [80]. In several berries there has been found correlation between amount of radiation the plants receive and the profile and content of secondary metabolites [70].

5.4.2 Forest and soil characteristics

Mainland Norway covers an area of 323 810 km^2 of which 38% is covered by woods (127 604 m^2) [81]. Characterization of forests is in Norway typically done based on their age, bonity and forest density [69]. Since the beginning of measurement of Norwegian forests around 1920, there has been a continuous increase in the volume of all the forest species. On average the dominant species of the forests are 28 % spruce, 29 % pine, 42 % broadleaved and < 1 % unstocked forest [69]. There are, large differences in the cover of trees and predominant species in different regions of Norway, and large variations from region to region in both forest types and abundances. Occurrence of lingonberry shrubs in forests has been shown not to vary with forest density, with an average of about 5-7 % coverage being slightly more abundant in Scots pine forests compared to Norwegian spruce forests [82]. This is different compared to bilberries where the coverage is dependent not only on stand age but also irradiance and tree species composition [82,83]. Variation in branching of trees due to forest type, and the density of the tree stands can strongly influence the light available at the ground. The average stand age classification is species dependent, but with older forests of pine dominant forests, however the trend the last 40 years has been that the average age of the forest is increasing [69]. As light intensity has been shown to significantly influence the metabolism of other plants, it is considered that the increased age and density of Norwegian forests can influence the lingonberry metabolism. Forest density and type can also influence the light spectra due to absorption of radiation from tree leaves. Soil characteristics and forest types are often connected. Pine trees typically have an extensive and deep root system and thus they can thrive in dry and nutrient poor areas. Spruce on the other hand has a shallower root system and is thus found in the areas with the deepest nutrient rich soils. Soil can vary in both organic and non-organic composition, density, water content, mechanical composition and geological variability [84]. There is a large variation in the soil characteristics of Norway, but the most abundant type of soil is podzol [84,85]. Variation in soil nutrient composition plays an essential part in the plant metabolism. In addition, different factors associated to the lice insect pests, fungi, competing vegetation, salinity, drought, heavy metals, and pesticides all can influence the metabolites in plants [72]. Factors in the soil that have been shown to influence the metabolism of lingonberries are acidity, content of essential elements such as nitrogen, potassium and phosphorus and conductivity [48]. On the British Isles it has been found that

lingonberries are found on mainly three types of vegetation, on shallow poorly developed and mountainous heaths, on well-drained podzols of upland heaths, and on humus and drained pea of edge scree and eroded bog communities [11].

5.4.3 Temperature and precipitation

The Norwegian Ocean Current makes the northern regions of Norway, Sweden, Finland and parts of western Russia fertile and inhibits permafrost seen in other arctic regions worldwide [68]. However, with the ocean current a unique environment sustaining more high life is created also in these regions. In Norway the mean annual temperatures in the normal period between 1971 and 2000 was +1.3°C and the mean annual precipitation of 1600 mm [86]. In addition to variation in the energy received from the sun, the reasons for variation in temperature include latitude and altitude. Thus, there is a large variation between different growth locations and from season to season in temperature and precipitation (Figure 9). The length of the growth season, the number of days with mean temperature above 5°C, in Norway ranges from 210-240 days along the Western coast of Norway, to less than 60 days in the mountainous areas. There is approximately 37,000 km2 that has a growing season lasting more than six months [87]. There has been an increase in temperature and precipitation across most of Norway from the periods of 1961–1990 to 1991–2020. According to the climate report in Norway, it has been estimated that annual temperature will increase by ca. 4.5 ºC (interval: 3.3 to 6.4 ºC) by 2100, and annual precipitation will increase by ca. 18 % (interval: 7 to 23 %), snow cover becoming negligible or non-existent, while snow volumes may increase in some areas in the high mountains. Towards 2100, the thermal growing season will become longer, especially along the coast [87], and the onset of heavy rainfalls is also likely to increase in this period [88].

The growth temperature of the plant influences the rates of the naturally occurring processes and accumulation of different compounds in the fruit tissues. Several of the biosynthesis pathways in plants have been linked to temperature responses. Amongst others changes in temperatures influence the ability to synthesise compounds that increase the osmotic pressure and make the plants available to attract more temperature from the soil [13]. Soluble sugars are the key-compounds affecting the osmotic pressure as they also store the excess energy in the plants and increase the carbon available to the plants, without causing much harm. However, some studies have shown that some plants increase the synthesis of sugars in response to

chilling. In *Vaccinium* berries it has been shown a reduction in the rate of photosynthesis at lower temperatures, with increased capturing of carbon by enhancement in the synthesis of phenolic compounds [89]. Lower temperatures have been shown to increase the synthesis of phenolic compounds including anthocyanins, but low temperatures in the absence of light can decrease the content of anthocyanins [89]. Changes in temperature are recognised by the plant as a direct influence on the change in the plant metabolism and development to achieve homeostasis [13].

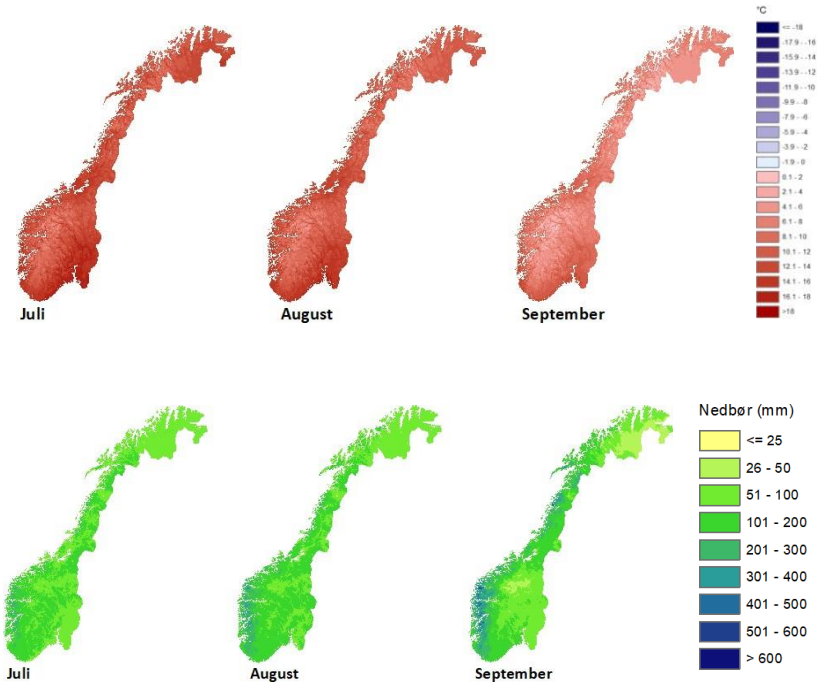

Figure 9 Mean temperature and percipitation from July to september in the 1991-2020 climate normal. Figure adapted from MET Norway and used under CC-BY 4.0.

6 Aim of the study

The objective of this doctoral thesis was to study how the composition of sugars, organic acids, phenolic compounds, and volatile organic compounds varies in Norwegian lingonberries. The thesis aims to study how different growth conditions and changes in these (e.g. forest type, light and temperature) affect the production of sugars, organic acids and phenolic compounds during lingonberry ripening.

The following research work was carried out to reach the objectives of the study:

1. *To study the effect of ripening stage on the composition of lingonberries (Paper I)*
2. *To study the effect of changes in light conditions and temperature on the quality of lingonberries under controlled conditions (Papers II and III)*
3. *To study the in-field effects of the variation in forest density and type, temperature, precipitation, and latitude on a large-scale field trial on the quality of lingonberries grown across Norway (Paper IV)*

7 Materials and methods

In the **paper I** and **IV**, berry samples were collected from defined wild stands of 250 m^2 based on predefined criterion (Table 1). In **paper II** and **III** however, whole plants after pollination with green developing berries were transferred to growing pots and were grown under controlled treatments in the experiments. From these berries the ripe berries were harvested and analysed. Across the four studies there were four analytical methods used to analyse the content of sugars and organic acids (HPLC-RI-UV), phenolic compounds (HPLC-UV-ESI-IonTrap-MS), total proanthocyanins (DMAC method) and VOCs (HS-SPME-GC-MS) (Table 1). Methods were chosen based on the availability of equipment, accuracy of the measurements and because they were established in the group for analyses of the individual compounds. Analyses of volatile compounds were chosen based on the availability of instrumentation and to comprehensively explore the volatiles in lingonberries, which has so far been relatively little studied.

Table 1 Overview of sample material, analytical and statistical methods

PAPER	MATERIAL	ANALYTICAL METHOD	STATISTICAL METHOD
I	- Berries from 3 stands - Collected at 5 timepoints.	HPLC-RI-UV[a] HPLC-U-ESI-IonTrap-MS[b] HS-SPME-GC -MS [c] DMAC method [d]	Anova Tukey's HSD test PCA
II	- Plants from 3 stands - 5 light treatments. - Collected at 4 timepoints	HPLC-RI-UV HPLC-U-ESI-IonTrap-MS DMAC method	Anova
III	- Plants from 3 stands at 2 locations - 2 temperature treatments - Collected at 1 timepoint - Ripe berries from paper I	HPLC-RI-UV HPLC-U-ESI-IonTrap-MS DMAC method HS-SPME-GC -MS	Anova Tukey's HSD test PCA
IV	- Berries from 7 locations - 8 stands at each location - Collected in triplicate annually for 3 years	HPLC-RI-UV HPLC-U-ESI-IonTrap-MS	Anova Tukey's HSD test Pearson's correlation PCA Linear regression

[a] Analysis of sugars and organic acids [b] Analysis of phenolic compounds, [c] Analysis of volatile organic compounds, [d] Analysis of total proanthocyanin content with dimethylacetamide method.

8 Summary of papers

8.1 Paper I

Composition of sugars, organic acids, phenolic compounds and volatile organic compounds in lingonberries (*Vaccinium Vitis-idaea* L.) at five ripening stages

Mathias Amundsen, Anne Linn Hykkerud , Niina Kelanne, Sanni Tuominen, Gesine Schmidt, Oskar Laaksonen, Baoru Yang, Inger Martinussen, Laura Jaakola, and Kjersti Aaby

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The differences in composition between lingonberries harvested at five ripeness stages was studied in the first paper of the thesis. The highest content of phenolic compounds and organic acids was found in berries harvested first. In these berries, particularly the content of proanthocyanins, flavonols and cinnamic acid derivatives was the highest. The results show that lingonberries follow a pattern of ripening often seen in non-climacteric berries. As the berries ripened, the content of organic acids, proanthocyanins, flavonols and cinnamic acid decreased while the content of anthocyanins and sugars increased. The proanthocyanin content decreased as the berries ripened, and it was found that the proportion of procyanidin A in unripe berries was higher than that detected in ripe berries. The study was the first to investigate the content of volatiles during ripening in lingonberries. Volatile acids increased continuously as the berries ripened, whereas several other volatile compounds showed the highest levels just prior to ripeness and in late season berries. In addition to the changes occurring due to ripening, variation was observed in the profile of both phenolic compounds and volatiles, depending on the growth location of the berries. Particularly, a distinct increase in the content of terpenoids was detected in berries from one location. There were differences in the responses between the three stands studied.

8.2 Paper II

Effect of light conditions on the chemical composition of lingonberries (*Vaccinium vitis-idaea* L.)

Anne Linn Hykkerud , Mathias Amundsen, Inger Martinussen, Kjersti Aaby, and Laura Jaakola

Manuscript prepared for submission.

In the second paper of the thesis, the effect of light quality and intensity on the composition of lingonberries was studied. As latitude influences the light conditions, and there can be large variation in the amount of sunlight received by plants under the the forest canopy, it is important to develop more knowledge on the effect of light conditions to berry quality. It was found out that light quality with supplemental radiation of red, far red, blue, and white light significantly influenced both the content of sugars and anthocyanins in lingonberries, even if the effect were proportionally minor. Blue light treatment increased the major anthocyanins cyanidin-3-O-glucoside, cyanidin-3-O-arabinoside and cyanidin-3-O- galactoside in ripe lingonberries. Contrarily, the level of procyanidin B2 was affected mostly by far-red treatment. The total content of sugars as well as the levels of glucose and fructose was highest in berries treated with white light, and lowest in berries treated with far red light. Light intensity also influenced several compounds in lingonberries, in particularly two of the quercetins, cyanidin-galactoside, ferulic acid hexoside 1 and procyanidin B2. Interestingly, a higher content of benzoic acid, kaempferol-3-O-(3-HMG)-rhamnoside and quercetin-3-O-pentoside was detected under low light intensity. Sugars, however, were not affected by light intensity. The berries ripening for a longer period of time under light treatments showed much more significant changes in composition. The harvest time clearly influenced the berry chemical composition and from the first stage of ripe berries, a fivefold increase in benzoic acid content and an increase in the total phenolic content was detected.

8.3 Paper III

Effect of temperature on the chemical composition of northern and southern ecotypes of lingonberries (*Vaccinium vitis-idaea* L.)

Mathias Amundsen, Laura Jaakola, Kjersti Aaby, Niina Kelanne, Sanni Tuominen, Oskar Laaksonen, Baoru Yang, Inger Martinussen, and Anne Linn Hykkerud

Published in Food Research International, 167(2023), 112738. doi:10.1016/j.foodres.2023.112738

In the third paper of the thesis the effects of ripening temperature were studied. As temperature has previously been associated with large changes in lingonberry quality in field studies, controlled studies were performed to get deeper understanding on the effect of temperature on the lingonberry composition. Lingonberries of both southern and northern ecotypes ripened under controlled growth conditions in phytotronto study the responses and whether there were different adaptations between the growth locations. The study clearly showed that the low temperature treatment, where the plants were subjected to 9 °C during ripening, gave higher contents of both anthocyanins and cinnamic acid derivatives. The lingonberries originating from the northern location had a higher proportion of cyanidin-3-O-glucoside and -arabinoside compared to the southern berries. Also, the content of organic acids was significantly influenced by latitude, with higher content in berries from the northern origin, whereas there was no effect of neither origin nor temperature on the content of sugars in lingonberries. Regarding the volatiles,, 40 of the 77 VOCs identified in this study, had not previously been reported in lingonberries. The profile consisted of a large variety of different groups of VOCs. The volatile composition appeared not to be significantly influenced by temperature. However, the profile of VOCs was mostly significantly influenced by geographical origin, and there also appeared to be clear differences within berry ecotypes from the same region of the country.

8.4 Paper IV

Analysis of variation in content of anthocyanins, sugars, and organic acids in wild populations of lingonberry (*Vaccinium vitis-idaea* L.) in Norway

Mathias Amundsen, Inger Martinussen, Kjersti Aaby, Aksel Granhus, Marius Hauglin, Laura Jaakola and Anne Linn Hykkerud

Manuscript prepared for submission.

In the fourth and most comprehensive paper of the thesis the quality of Norwegian lingonberries and factors influencing the quality was studied. In previous field studies it has been found out that growth conditions of wild berries affect their composition. The results show that the content of anthocyanins, sugar, and organic acids varies significantly in wild populations of Norwegian lingonberries. The mean content of anthocyanins in wild Norwegian lingonberries was 100 mg/100 g fw, of sugars 6.9 g/100 g fw, and of organic acids was 2.8 g/100 g fw. There was a large variation in the quality of Norwegian lingonberries harvested from the different stands and locations, even with some variation within different sectors of the same stand and over the three-year period. Temperature and latitude had the most significant influence on the lingonberry quality, but there were also effects of both altitude, forest type and density on the anthocyanin composition of lingonberries. Higher latitudes and increased temperatures both increased the content of anthocyanins in lingonberries. A strong correlation between temperature and latitude was detected in this experiment. Higher latitude also increased the content of sugars in lingonberries, whereas higher temperature had the opposite effect. Organic acids were most significantly influenced by temperature with increased content of both citric and quinic acid with more precipitation. Cyanidin-3-O-galactoside, -arabinoside, -pentoside and -acetyl hexoside all were found in higher content in lingonberries grown at lower temperatures in the north, whereas lingonberries at the southern location had higher content of cyanidin-3-*O*-glucoside. Precipitation as measured in the amount of mm during ripening did not influence the composition of anthocyanins in lingonberries. Increased density of deciduous trees within the sample plots significantly but with small effect increased the content of both anthocyanins and organic acids in lingonberries.

9 Discussion

9.1 Composition and quality of Norwegian lingonberries

Across the four papers of the thesis total of 113 individual compounds were detected, of which 77 were volatile organic compounds, 29 were phenolic compounds, three were sugars and four organic acids. In all **papers I-IV** the content of anthocyanins, sugars, and organic acids were analysed as they are considered the most important compounds for quality of the lingonberries. However, additional analyses of volatiles, other phenolic compounds and total proanthocyanins were also performed in **papers I-III**. The selection of compounds for **paper IV** was done based on considereding the most important contributors to quality, and their ease of analysis, and will be the focus of the discussion. In apples or other fruit, quality is by often determined by attributes such as appearance, firmness and flavour, as well as by the absence of physiological and pathological disorders [31]. While exploring other quality aspects like size and firmness of lingonberries or investigating additional chemical compositions could have provided valuable insights, however, this study primarily focuses on phenolics, sugars, and organic acids being the key-compounds affecting quality in lingonberries [15,17]. A higher content of phenolic compounds in lingonberries is considered beneficial due to the association with health benefits, attractive colour, and taste [17,21]. Considering the general preference for sweeter berries , this study also examines the sugars and the organic acid contents [15]. Therefore, high-quality lingonberries are characterized by a higher phenolic and sugar content as well as lower organic acid content. The profile of volatile compounds was studied as they significantly influence the berries' aroma [63]. To date, there has been less emphasis on the profile of volatiles and aroma of berries, disregarding their essential role in the organoleptic properties [63]. The compounds analysed do not give a complete picture of all compounds present in lingonberries, but rather targeted groups of compounds due to their known influence on the key properties in lingonberries, and thus the berry quality. Various other compounds such as vitamins, lipids, proteins, carbohydrates, fibre, and minerals also affect the composition of berries. Some concerns have been raised regarding Nordic wild berries as a potential source of radioactivity in food following the 1989 Chernobyl accident. However, studies have shown that that the berries do not accumulate large amounts of radioactivity, and the latest reports of the Norwegian scientific committee of food

showed that berries do not contribute to the radioactivity in the Norwegian diets [91,92]. While studying a broader range of compounds could enhance the understanding of metabolic processes within the berries, this study focuses on the primary compounds that influence lingonberry quality.

9.1.1 Phenolic compounds, anthocyanins, and health effects

In **papers I-III** the content of phenolic compounds in ripe lingonberries was found to be approximately 600 mg/100 g, among the analysis of total PAs and 29 compounds identified in lingonberries of which five were anthocyanins, 11 flavonols, 9 phenolic acids, and four flavan-3-ols analysed. The quantity of phenolic compounds in lingonberries can be considered relatively high, even if some berries like black elderberry and chokeberry have even higher contents of phenolic compounds reported (>1700 mg/100g), [93]. The content of phenolic compounds in unripe berries was even higher, and unripe berries were found to have the highest total content of phenolics, flavonols, cinnamic acids, and proanthocyanins, including a higher proportion of the less common A-type procyanidins. In unripe berries, the total PA content alone was above 600 mg/100 g. These patterns were in line with findings previously shown inripening lingonberries [48], and follow trends also seen in other berries [70]. The content of anthocyanins however, increased with time until harvest. Anthocyanins are among the most extensively studied phenolic compounds. In lingonberries all the anthocyanins identified across the four papers were cyanidin-glycosides. These cyanidin-glycosides have similar spectral characteristics and give lingonberries their scarlet red colour. Low contents of other non-cyanidin anthocyanins such as delphinidin, petunidin, peonidin, and malvidin have also been reported in other studies in very low concentrations [48,52]. The HPLC-DAD-MS method used for analyses of phenolic compounds in lingonberries has been used in different varieties for decades and gives an accurate quantification of most phenolic compounds with certainty of identification as both UV-spectral and MS are used to determine the identity of the compounds [94-96]. While it is an accurate and reliable method the number of phenolic compounds identified in the thesis is, however, much lower than what has been published by others [48,50]. It is recognised that identification of a higher number of phenolic compounds could have been achieved by using higher resolution mass spectrometry. Another challenge with the method is analysing PAs as separation of peaks due to co-elution with other phenolic

compounds and low extinction coefficients of the compounds reduces the reliability. Therefore, the total content of the PAs was also determined through the DMAC method in **papers I-III.** To improve the understanding of the variation in the PA content and polymerisation of the PAs a study using selected samples from the samples in this thesis is currently conducted. Differences in anthocyanin structures gives variation in chemical properties and variation in uptake in the body, even within similar structures such as those found in lingonberries [97]. Compared to a larger group of fruits and vegetables, lingonberries have been found to have a moderate level of anthocyanins [98]. A report on anthocyanin content in berries however, ranked lingonberries as one of the least abundant sources [93]. Notably, the content of anthocyanins observed in our study, ranging from 60 mg/100 g to 160 mg/100 g, is higher than the lower levels reported in the mentioned study [93]. Using the upper level of anthocyanin content detected in our study would classify lingonberries at an intermediate level of anthocyanin source, comparable to lowbush blueberry, highbush blueberry, and blackberry [93]. Nevertheless, these levels are significantly lower than those found in darker berries such as black elderberry, black chokeberry, blackcurrant, black raspberry, evergreen huckleberry, and European blueberry, which have content exceeding 300 mg/100g [93].

Though the use of unripe berries on large in home or industry use is unlikely, separation of berries based on surface colour could be interesting on a large scale to improve product quality. From **paper I-II** it could be found that berries that are partially red have a higher content of flavonols or PAs, which areas in relatively high contents in lingonberries [43,51,70]. Health effects of anthocyanins and other phenolic compounds is dependent on dose, and therefore the variation in content is of high importance. Findings from **paper I** may give reason for interest in the harvest of unripe berries for certain nutraceutical applications, where high phenolic and proanthocyanin content is sought after due to the numerous health benefits. Whereas in **paper I** and **II** it was showed that anthocyanin content in the berries continued to increase as long as the berries ripened and along the season, which has also previously reported in lingonberries [48]. Higher contents of anthocyanins have been associated with several health benefits of lingonberries [17,21]. This is a challenging market, but a market with large potential as more than 80 % of Europeans responded that they had taken food supplements in the last 12

months according to a study commissioned by Food supplements Europe [99]. This research has shown potential use of both ripe and unripe berries as a potential resource, as plant extracts had more than 9 % of this market share [99]. Using diverging evidence to substantiate the use of food supplements should however be limited. Though there is much attention to the effects of phenolic compounds in fruit and berry research, like the effects against UTI and metabolic adverse outcomes, using health claims in sales of lingonberries is currently not permitted in the EU. According to Regulation (EU) No 432/2012 it is currently only two health claims of phenolic compounds that have been evaluated and been permitted. These two are: Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress [100] and Cocoa flavanols help maintain the elasticity of blood vessels, which contributes to normal blood flow [101]. The results of health effects of lingonberry consumption are very promising, and there is potential for such claims to be allowed in the future. Having a clearer understanding of the composition of lingonberries and variability in the constituents is an essential part of this process. Therefore, the results from this thesis can contribute to the body of evidence to this. Inclusion of lingonberries to any diet to increase the proportion of plant-based foods will however still be regarded as beneficial [102].

9.1.2 Sugars and organic acids and sweetness

Lingonberries flavour is a result of the combination of all compounds they contain but is particularly influenced by the content of sugars and organic acids [103]. The high content of organic acids and phenolic compounds in lingonberries contributes to their astringent and bitter characteristics. The sweetness of a berry is commonly associated with its sugar content, and Norwegian lingonberries were in **paper IV** found to contain sugars ranging from 4.4 to 11.4 g/100 g fw. Most of these sugars were fructose and glucose in equal proportions, with only a small amount of sucrose. However, the perceived sweetness of a berry is not solely determined by its sugar content but also by the ratio of sugars to organic acids [15,34]. The content of organic acids was between 1.8 and 4.8 g/100 g fw in wild Norwegian lingonberries. The two predominant organic acids, citric and quinic acid, varied with different growth conditions and during berry ripening. The content of sugars and organic acids observed in this study was

consistent with previous literature, although different studies have reported slight variations in organic acid content, with some describing lingonberries as having medium content compared to other berries [35] whereas another study found lingonberries to have a high content compared to others [38]. The average ratio of sugars to organic acids in Norwegian lingonberries was 2.48, but there was considerable variation observed, ranging from 0.97 to 3.8. These values align with previously reported ratios for lingonberries (2.27 and 2.72) [35,38]. Previous studies have revealed that many consumers prefer berries with a sweeter taste and less bitterness [15]. As lingonberries ripen, they undergo changes in taste and texture. Sour and tart berries with a firm structure and small size become slightly less acidic, more palatable, and grow in size as they ripen [104]. In recent research there has been emphasis on trying to mask or influence the taste of lingonberries in different ways [56,60,105-107]. The most used technique is addition of sugars, however adding more sugars will not only make the products sweeter, but also add to its caloric content. There are several other approaches to such masking, including addition of non-caloric sweeteners, addition of other compounds, using bioprocessing or change of texture. However, I believe that, rather than masking these properties, highlighting the unique flavour of lingonberries, and finding the right application and proportions in different products could lead to their success in the long term. By identifying locations with the sweetest tasting berries of highest quality there may however be possibilities to achieve products of higher quality where added sweeteners could be avoided. To achieve the harvesting lingonberries at the right time is important, as berries harvested later in the season had higher content of sugars and lower content of organic acids. In **paper I** and **paper III** it was shown that ripe lingonberries get a sweeter taste due to changes in sugar to acid rate until they were picked off the bush. This was also supported by the work in a Lithuanian field study of lingonberries were substantial variation in berry quality depending on the time of harvest was observed [48].

The choice to use an ultrasound assisted extraction with 70 % methanol as a solvent for extraction of sugars, organic acids and phenolics was based on a test of these different extraction solvents. Three different solvent combinations and effects of using frozen or freeze-dried berries published in the conference proceedings of the 12th international *Vaccinium* symposium [108]. Sugars and acids are often analysed from diluted berry juice [34,57,109],

whereas the extraction of phenolic compounds is typically done using methanol and acetone or aqueous methanol or acetone solutions [110]. Different additions to the solvent solutions or procedures during the treatments can be used to improve efficiency and stability of the end solutions [111]. It was considered that extracting freeze-dried berries with 70% methanol gave the most accurate and time efficient method for extractions of the target compounds. This simultaneous extraction of both sugars and phenolic compounds was essential to achieve the analyses of the large number of samples presented in this thesis. The use of freeze-dried samples was thought to give the most homogenous, stable, and easy to handle samples. However, in **paper II** it was considered that the sample size was too small to have a homogenous freeze-dried sample without losses. A deviation from this approach was done in **paper III,** where 5 berries of each stand, treatment and timepoint were analysed rather than a freeze-dried homogenous sample. This was done due to the limited amount of sample material. The results of sugars, organic acids and phenolic compounds in all of the papers are expressed as a per 100g fresh weight (fw) rather than dry weight (dw) basis. While it could be argued that to study the concentrations per unit dw is more biologically relevant in several instances, we decided to show in the initial publication of the field paper the results as per 100 g dw [112]. However, as the focus of the thesis is to discuss the effects of the changes in the relevance of lingonberries as a food it was considered that expressed as a per 100g fw would make it easier to compare to other nutrients and studies. As a standard in food composition databases, the content of nutrients is generally reported on a per 100 g fw basis throughout the European region.

9.1.3 Volatile organic compounds and aroma

With more than 70 compounds tentatively identified in ripe lingonberries in **papers I and III**, the VOCs were the group with the highest number of compounds identified. The number of VOCs that have been identified in **papers I and III** is high compared to other species in the *Vaccinium* genus [63]. However, in both earlier papers published in the 1960's the content of volatiles measured was significantly higher [66,67]. A part of the differences can be attributed to the differences in the analysed materials as well as analytical techniques. Release of aroma in the berries is dependent on a number of factors including the food matrix and processing and other extrinsic parameters [64]. In this study, it was chosen to analyse VOCs using HS-

SPME extraction from frozen slightly crushed berries as this mimices the most commonly use of lingonberries as food perceived. There have been identified approximately 100 volatiles in aspecies of *Vaccinium* berries, whereas more 300 compounds identified in apples [113], and in grapes several hundreds have also been identified [114]. Among the compounds in this study hexanal (green, grass), eucalyptol (mint), linalool (fruity, flower), and methyl benzoate (aromatic, fruit) had previously been identified as odorants [59]. Diacetyl (2,3-butanedione; butter, cream), 2-methylpropanoate (fruity) which had also previously been found to give odour however, could not be identified in the current study. In the two papers studying aroma content it was found that aroma was influenced both by ripening and the origin of the berries. In **paper III** there was clear evidence that while the growth temperature did not influence the composition of volatiles, there was a larger effect of growth location. In wine, it has been found that the varietal aroma of the grapes, that varies between locations and develops during ripening can give the local products their characteristics. It has been suggested in wine that the aroma distinct from the grape raw material influences the wine aroma even though substantial changes occur during fermentation and storage [114]. Optimal aroma of a product is dependent on personal preferences, and increased awareness of the contributing factors can help understanding how berries and berry products are considered. Though some analyses of the volatiles of lingonberries have been done for more than 60 years, there is still limited understanding on what compounds are of importance for the flavour, and there is no clear understanding of the volatile profile. In general, there is still too limited understanding of the volatile profile of berries within the *Vaccinium* genus and the relationship between these aroma compounds and perceived aroma [63]. As seen in the other species the heterogenicity between compounds identified in different studies shows the need for improved understanding of the volatile compounds and aroma. As in these studies there was limited information available to identify how abiotic and biotic growth factors influence the content of VOCs, studies to identify these would also be interesting.

9.2 Environmental influence on quality of lingonberries

Growing across Norway, wild lingonberries are ripening in condition that can vary significantly. These growth conditions can often covary making interpretation of results from the field challenging [12,70]. Therefore, the approach taken in this thesis, was a combination of studies where certain factors were controlled in **papers I-III** and a multi-year field study of several growth locations and stands in **paper IV**. This was taken to increase the understanding of the influence of the specific factors and the aim was to utilise the results and knowledge from these three first papers when interpreting the results from **paper IV.** The results from the three papers investigating the controlled conditions however also stand well on their own. This type of approach on a combination of controlled studies with field studies has previously been suggested critical for understanding the variation in composition of wild berries [12].

9.2.1 Influence of temperature, light and latitude

A ew field earlier studies of wild lingonberry populations were identified in this thesis, from Bulgaria, Canada, Finland, Lithuania and Poland [14,48,55,115-117]. These studies however, varied in complexity and sample sizes. Most of the studies investigated the content of anthocyanins and antioxidant activity, whereas only one investigated the sugar content. No studies were identified investigating the content of organic acids or volatile organic compounds in larger populations of lingonberries. Latitude has been identified in several as a key factor influencing lingonberry quality[48,55,115]. In field studies of bilberries, bog blueberries, buckthorn and currants, latitude has also been found to influence the quality of the berries greatly [118-123]. Latitude is among the often-studied factors that covary with other factors. There have been several different hypotheses on the characteristics of latitude that influence quality the most, light and temperature conditions receiving the most attention [44,68].

Light quality is a key factor in influencing the development of several of the compounds related to quality in lingonberries [70]. Treatments with red and blue light have previously shown to increase the synthesis of both primary and secondary metabolites in bilberries, other berries, fruits, and vegetables [54,74]. In **paper II** cyanidin-3-O-galactoside, cyanidin-3-O-arabinoside and cyanidin-3-O-pentoside all were upregulated when the lingonberries were

grown under supplemental blue light, whereas no effect was observed for the content of phenolic acids or flavonols. Thus, an increase in the total anthocyanin content was found in the lingonberries treated with supplemental blue light. Blue light which has previously been shown to increase synthesis of flavonoids. The phenolic acids have previously been shown being the main protector against UV radiation in the lipophilic fraction of lingonberries [124], but both phenolic acids and flavonols are known as protectors against UV- radiation in plants. UV radiation treatments have also shown in other studies to increase the content of flavonoids and phenolic acids in berries [70]. The effect on anthocyanins was different and lower than what has been previously seen in other *Vaccinium* berries [70]. In bilberries it has been found that the delphinidins were strongly related to changes in light conditions, with little effect on cyanidins [54]. A higher total anthocyanin content has also been found in the northern bilberry clones [118,125]. Also in cranberries the highest accumulation of anthocyanins has been found in berries grown under supplemental red light wavelengths [70]. Also, the content of sugars and organic acids were influenced by the spectral light treatments. The effects on organic acids were minor, but it was notable that the sugar content of lingonberries ripened under far-red light was lower compared to those treated with blue, red and white light. Similar effects of red and blue light supplementation have also been shown in several vegetables, but this is contrasting to the results observed in other berries [103]. Light differences related to latitude have previously been associated with changes in the sugar content of lingonberries and bilberries [55]. In locations further north, the ratio of red to far-red light is reduced over a whole day [68]. Whereas irradiance of blue light is higher at low latitudes, with some effects of twilight [126]. The results from **paper II** would therefore indicate that locations further south would increase synthesis of phenolic compounds based on their response to the light spectra.

Temperature has also been found to significantly influence the secondary metabolism of *Vaccinium* berries [70]. The difference of 6 degrees and the two temperatures (9 °C and 15 °C) in **paper III**, were chosen as they represented the temperatures at the two collection site locations in the ripening period the year prior. Results from the papers show that the lower temperature of 9 °C gave a clearly higher content of anthocyanins and cinnamic acids in lingonberries, and there was also an effect on the glycosylation of the anthocyanins. There

was however no effect of temperature on the other groups of compounds in lingonberries. Compared to the direct effects of changes in light, the effects by temperature are more indirect. Changes in temperature during ripening influences the rates of the chemical processes occurring in the plants, and the responses vary greatly between species [103]. Also, the relative ratios of other phenolic compounds varied between the two temperatures. Variation in glycosylation together with hydroxylation and methylation pattern has previously been suggested to be a part of a plant's response to outside stressors [127]. Additionally, there is variation in the uptake of different anthocyanins [97], which can influence the health effects of these compounds. Combining the results from **paper II** and **paper III** would suggest that anthocyanin content would increase with lower temperatures and stands further south. As expected, **Papers I-III** showed that while the studied factors influenced the composition of the lingonberries, there was variation occurring due to factors that could not be explained. **Papers II** and **III** showed that while lower temperature significantly increased the content of phenolic acids in lingonberries, there was a generally low effect of environmental influence on the synthesis on non-anthocyanin phenolic compounds.

In the field study in **paper IV**, lingonberry stands representing a large variation of the Norwegian latitudes from 59 to 69°N were included. In total 56 field stands from 7 locations were included in the study and followed for three years. Studying the metadata, as expected, a strong correlation between latitude (°N), and temperature during the ripening period (°C) was found. Most of the growth factors studied in **paper IV** influenced lingonberry quality, but the most prominent effect on quality was from the latitude and temperature. The effect of latitude and temperature on anthocyanins and sugars was significant, but these did not influence the content of organic acids. The effects of latitude and temperature were also larger on the content of anthocyanins than on the content of sugars. There was no correlation between anthocyanins, sugars and organic acids found in **paper IV.** However, in the regression analyses the sugar to acid ratio appeared to have the opposite direction in the statistical tests for most compounds. Though the exact light conditions at each stand was not measured in **paper IV,** it is considered that stands further north have a higher proportion of red and far-red light, as the low solar angle during midday in the north scatters the light to reduce the amount of blue light in the spectrum [68]. The direct effects in the regression model showed that

stands further north and at higher temperatures gave increased content of anthocyanins. The interaction coefficient between the two was however negative. Of the findings in the field studies of lingonberries, the temperature showed a correlation to the total content of sugars [55] and the total content of phenolics flavan-3-ols an Proanthocyanidins [48] but correlated negatively to phenolic acids [48]. There have been slightly varying effects of temperature detected in lingonberries, as one study showed no effect of temperature [48], whereas the antioxidant compounds in leaves increased with reduced temperature [115]. To facilitate interpretation in light of **papers II** and **III** it was chosen to use mean temperature and precipitation during the final 30 days prior to harvest. It is recognised that precipitation and temperature prior to this may also influence the composition, but it is considered that this is a key period in the ripening process. Interpreting the results considering these studies it could be considered that when held at a same temperature as in **paper II,** stands further north would have lower content of anthocyanins. Additionally, in the light of the results from **paper III** it would be understood that berries grown at one location would accumulate an increased content of anthocyanins when the temperature is lower. The higher content of organic acids that was found at lower temperatures in **paper III**, was not found in the field results of **paper IV**, where no influence by neither temperature nor latitude was found. The content of quinic acid however, was following a similar trend to that seen for the anthocyanins, as quinic acids is related to the synthesis of anthocyanins [58]. The IPCC has projected that temperatures will increase in the arctic regions by as much as 6 degrees in the coming 50 years [71]. The same difference in temperature as that studied in **paper III**, showed a clear effect of this temperature change**.** The obvious effects for quality will naturally be influenced by the location of the high-quality berry plots,and will likely also influence high yield locations. The effects were however not linear to temperatures and light, and some variation in chemical composition was observed even between different sectors within the same stand.

9.2.2 Influence of light intensity, forest density and precipitation

Light intensity available for lingonberry plants varies with a number of factors like latitude, cloud cover, forest density, and there is a lower energy content on a horizontal surface received in the regions the furthest north of Norway [79]. It has been found in studies with many species that increase in light intencity increases sugar content [103], however, it has previously been

hypothesized that it is less important for species in the *Vaccinium* genus. Increases in light intensity in berries of the *Vaccinium* genus only fine-tunes the flavonoid biosynthesis, as the species are often adapted to grow in shaded habitats [70]. Results from **paper II**, supported this hypothesis with findings indicating that there was a generally a low effect of supplemental light irradiation on the synthesis of most phenolics, sugars, and organic acids. Though there was a significant increase in the content of anthocyanins when subject to the higher light intensity. The two light intensities used in **paper II** were representing open and covered growth locations in the forests. In the **paper IV** the forest volume was measured according to the Norwegian standard procedure [69]. While the trees represent biotic variation, the focus of the thesis has been on the abiotic effects of changes in tree volumes. Only the volume of deciduous trees, and not pine and spruces significantly influenced the quality of lingonberries. Increased volume of deciduous trees increased the content of anthocyanins and organic acids, with no effect on sugars. As light intensity in **paper II** was associated with higher anthocyanin content, it was expected that the denser forests of pine and spruce would give a lower content of anthocyanins. However, it can be expected that the increase of forest density that has occurred over the last 100 years [69] has had limited effect on the lingonberry quality. The higher content of anthocyanins and organic acids in deciduous forests could also be linked to the soil characteristics in these locations as soil characteristics have previously been found to influence quality of lingonberries [48]. Precipitation was the factor that significantly had the highest effect on the content of the organic acids in **paper IV.** The effect of the changes in the available water is however largely different due to the variation in needs for water by plant species [128]. In a previous lingonberry study, it was found that higher amount of annual precipitation gave higher levels of antioxidant compounds in lingonberries, but no effect on total content of phenolic compounds and anthocyanins [115]. Both scarcity of water and water surplus have the potential to act as stressors to plants influencing their metabolism. Though the availability of water for a plant in the case of drought can influence the composition of phenolic compounds [72], it appears that the range of tolerance for water in lingonberries is wide. In some plants low availability of water can cause partial closure of stomata that gives a decrease in the level of intracellular CO_2 which can cause increases in phenolic synthesis to protect the photosynthetic apparatus. The availability of water and thus the water conditions have to be more extreme to exhibit an effect on the quality of lingonberries.

9.2.3 Influence of harvest time and ecotypes

Variation in quality depending on harvest time has been shown in several other berries in the *Vaccinium* genus [70]. Though all the berries in **paper IV** study were harvested ripe, lingonberry maturity occurs late in the growth season, and the timing of ripening varies across different regions. With berries in **Paper IV** being harvested from regions across Norway, there is naturally also variation in the expected harvest times, and during the three years of the study the harvest dates ranged from 25.08 to 28.09. However, considering the large effects of harvest time seen in both **paper I** and **III,** with the content of anthocyanins, sugars and organic acids all increasing with time. It can be expected that harvest time has also influenced lingonberry quality in **paper IV** in some way though the size of this effect is unclear. There seems to be a slight trade of between sweetness to anthocyanin content in lingonberries. Waiting until the berries are fully ripe and to a point later in the season can give berries that contain a higher level of anthocyanins and that have a sweeter taste. However, berries from early in the season also have a high quality and can be of better quality for many applications. As there is not a single optimal growth environment for all quality aspects in lingonberries, it is considered that the optimal place for harvest can be determined based on the intended and sought-after quality characteristics. Norwegian lingonberries have a generally high quality, but in **paper IV** it was found that there was a large variation in the quality between stands. The quality of these berries was influenced by a number of factors but across all the four papers of the thesis, it was found that the local characteristics even within a stand could influence the quality of the berries. While the thesis comprehensively studied a variety of important factors, there are probably several more factors that also could affect the quality of berries. The models applied in **paper IV** had a fit (R^2) of < 0.4 which indicate that while they explain a part of the variation in the composition, there are still a number of factors and interactions that need to be included to fully understand the synthesis. Among the most important the results could be linked to genetic variations between different stands, soil composition, and water content. There has been shown differences in the rate between different genotypes of the same species of bilberries [89]. Expression of anthocyanins are also clearly different in different genotypes, as there have been identified several wild cultivars of white lingonberries not synthesising

anthocyanins [9]. There have in general only been a few studies addressing the genetic diversity of cultivars and wild populations of lingonberries [129- 131]. The composition of different lingonberry cultivars or lingonberries collected at different locations and later grown under the same growth conditions had a variation in their chemical composition of phenolics and triterpenoids [132]. This shows that high quality lingonberries deserve also future research endeavours.

10 Main conclusions

In total 113 individual compounds were identified in lingonberries in the thesis, of which 77 were volatile organic compounds, 29 phenolic compounds, three sugars and four organic acids. Lingonberries have in this thesis been shown to be a rich source for dietary polyphenols, and interesting food source due to unique taste, aroma, health benefits and colour. With the combination of field and controlled condition experiments, this thesis has given a valuable insight into both primary and secondary metabolism in lingonberries. Therefore, the thesis gives a new insight into variation in quality of lingonberries across Norway.

Previously, there were 40 volatile compounds described in lingonberries identified in the earlier papers presented in this thesis. In the present study, the volatile composition varied greatly between different lingonberry stands, but no clear conclusions of environmental variation on their composition could be found. The ripening process in lingonberries followed a similar pattern for the development of phenolic compounds, sugars and organic acids as have previously been described in many other fruits and berries. The work, however, gave a clearer understanding of the importance of harvest time on the quality of lingonberries, with substantial quality increase along with time. As unripe berries had the highest total content of phenolic compounds and proanthocyanidins, they also can be interesting in certain applications.

Weather conditions were identified as a key-factors influencing lingonberry quality. Temperatures generally seen in Northern regions of Norway during ripening were associated with higher content of anthocyanins and phenolic acids. Whereas increased amounts of precipitation during ripening gave berries with higher content of organic acids, that will most likely be perceived as sourer taste. Light conditions and intensity influenced lingonberry quality less than expected, though supplemental blue light gave higher content of anthocyanins compared to supplemental red or far-red radiation. These light conditions also appear to influence the sugar to acid ratio which was lower at far-red treated berries. Increased light intensity increased the synthesis of some phenolics. Interestingly the increased volume of deciduous trees within a stand improved berry quality, while there was no influence of spruce and pine forests. Results from the field study further showed that quality of wild

lingonberries varied substantially between stands in Norway. While much of the variation could be explained by the studied variables, there is yet much to learn about their metabolism.

The high-quality Norwegian berries possess a large potential for increased commercial use, and give incentive for increased private harvest. As both an increased consumption and industry use should be a target for Norway, Increased use of Norwegian lingonberries could replace the importation of large amounts of fruits and berries and in a sustainable way improve the diets of Norwegians. Going out to the forest to harvest food is also good for both physical and mental health. Use of lingonberries and other wild fruits and berries not only gives a larger variety in the tastes, but will give more local flavours based on the local flora. Norway is also rich with other species than not only berries but also other edible plants, and future research could focus on better sustainable utilisation of these high-quality raw materials for foods.

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Composition of Sugars, Organic Acids, Phenolic Compounds, and Volatile Organic Compounds in Lingonberries (*Vaccinium vitis-idaea* L.) at Five Ripening Stages

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Abstract: Wild lingonberries are a traditional source of food in the Nordic countries and an important contributor to economic activity of non-wood forest products in the region. Lingonberries are a rich source of bioactive compounds and can be a valuable contributor to a healthy diet. However, there are few studies available on how the bioactive compounds in lingonberries develop as they ripen. In this investigation, we examined the content of 27 phenolic compounds, three sugars, four organic acids, and 71 volatile organic compounds at five ripening stages. The study showed that, while the highest content of phenolic compounds was found early in the development, the organoleptic quality of the fruits improved as they ripened. From the first to the last stage of development, anthocyanins went from being nearly absent to 100 mg/100 g fw, and there was an increased content of sugars from 2.7 to 7.2 g/100 g fw, whereas the content of organic acids decreased from 4.9 to 2.7 g/100 g fw, and there were several changes in the profile of volatiles. The contents of flavonols, cinnamic acid derivatives, flavan-3-ols, and the total concentration of phenolic compounds were significantly lower in the fully ripe berries compared to berries in the early green stage. In addition to the changes occurring due to ripening, there was observed variation in the profile of both phenolic compounds and volatiles, depending on the growth location of the berries. The present data are useful for the assessment of harvest time to obtain the desired quality of lingonberries.

Keywords: wild berries; cowberry; fruit quality; health; taste; aroma

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1. Introduction

Lingonberry (*Vaccinium vitis-idaea* L.), belonging to the family of the *Ericaceae* and the genus *Vaccinium*, is a perennial dwarf shrub with scarlet red fruits [1]. The *Vaccinium* genus also contains blueberries (*V. corymbosum*), cranberries (*V. macrocarpon*), and bilberries (*V. myrtillus*), which are increasing in popularity worldwide. Lingonberries grow wildly and abundantly in forests across the Nordic countries and are an important source of income for the Nordic rural communities. Despite an increasing interest in wild berries, only an average of 7.6% of the total yield of lingonberries is harvested annually [2,3]. Lingonberries are mainly sold as fresh or frozen, or produced into jams. These berries are known to have a sour and astringent taste found challenging for many consumers, and appraised the reason for the relatively low consumption of lingonberries [4]. The flavor profile of lingonberries is mostly attributed to both the low content of sugars and the high content of phenolic compounds and organic acids in the berries [5–8]. Flavour is also

affected by volatile organic compounds (VOCs), but little is known about the aroma profile of lingonberries [9]. Lingonberries have a super-food potential, as several studies have found health beneficial effects of including them in the diet [10–14].

The ripening of berries in the *Vaccinium* genus is non-climacteric, with a continuous respiration rate throughout the process. After berry formation, several processes occur simultaneously, causing changes in the composition of the berries. In various *Vaccinium* berries, large changes in the composition of phenolics and terpenoids during the season have been observed [15–20]. The synthesis of most phenolic compounds starts at fruit onset, with the highest content of total phenolic compounds observed in immature fruits [16,19,21]. In immature fruits, the content of sugars is low and organic acids is high, and, as the berries ripen, the sugar content increases and organic acid content decreases [22]. As reduced astringency in the berries is considered an important factor for increased use of lingonberries, it is important to study the effects of ripening on composition. The development of volatile compounds has not been extensively studied in lingonberries [9]. In grapes, however, ripening has generally been found to be characterized by accumulation of alcohols, fruity esters, and terpenes during the later stages of development [23]. It is also known that the ripening process is influenced by growth conditions [15,18,24].

The process of berry ripening is visually expressed with an increase in size and accumulation of color in their skin [17,20]. Due to the sour taste and astringency of the berries, it is key to understand how the targeted compounds, influencing the flavor, develop during ripening. Ripening also influences the health properties of lingonberries. The objective of this study was to investigate how sugars, organic acids, and phenolic and volatile organic compounds vary in lingonberries as they ripen. Changes in the metabolic profile during maturation are considered to influence the flavor and antioxidant and antimicrobial potential of the berries. Improved understanding of the ripening processes is essential to increase the use of lingonberries and can be used to optimize harvest time and quality control.

2. Materials and Methods

2.1. Lingonberry Sample Material

2.1.1. Harvest of Wild Berry Samples

Wild lingonberries were sampled between July and September 2020, from three natural stands (250 m^2) in southern Norway within a 3 km radius (Supplementary Material Figure S1). Whole undamaged lingonberries (100 g) were harvested by hand at five stages of ripening based on predetermined visual criteria of the berry skin surface color, ranging from large unripe green fruit in July to fully ripe fruit in September (Table 1). After harvest, the berries were frozen within three hours and stored at −40 °C until further analysis.

2.1.2. Chemicals

Water used in the experiments was purified by a Milli-Q purification system (Millipore Sigma, Burlington, MA, USA), and solvents used were of HPLC-isocratic grade or higher. The chemicals used as standards in the experiments were purchased from several different vendors. From Acros Organics (Antwerp, Belgium), we purchased 2-hexenal. From Chem Service Inc. (West Chester, PA, USA), we purchased acetophenone, fructose, glucose, and sucrose. From Fluka (Steinheim, Switzerland), we purchased chlorogenic acid and eucalyptol. From Merck (Darmstadt, Germany), we purchased quinic acid. From Polyphenols AS (Sandnes, Norway), we purchased cyanidin-3-O-galactoside. From Sigma-Aldrich (St. Louis, MO, USA), we purchased benzaldehyde, catechin hydrate, citric acid, o-cymene, *p*-cymene, 2-ethyl furan, ethyl octanoate, (*E*,*E*)-2,4-heptadienal, (*E*,*E*)-2,4-hexadienal, hexanal, hexanoic acid, 1-hexanol, hexyl acetate, linalool, D-limonene, malic acid, methylbutanal, 3-methyl-1-butanol acetate, 6-methyl-5-hepten-2-one, 4-Methyl-2-pentanol, n-alkane mixture (C7–C30), neryl acetate, 1-octen-3-ol, 1-pentanol, quercetin-3-O-rutinoside, 2-α-pinene, shikimic acid, and γ-terpinene.

Table 1. Characterisation of the lingonberry samples [a] and weather characteristics at the sampling day [b].

Harvest Date	Fruit Color		Weight (g/berry)	Dry Weight (%)	Temp Mean (°C)	Max (°C)	Min (°C)	Rain (mm)
23 July 2020	Unripe green		0.21 ± 0.05	15.5 ± 0.2	13.2	19.4	5.0	0
24 July 2020	Half red		0.23 ± 0.08	15.3 ± 1.1	14.2	20.4	7.8	0
8 August 2020	Ripening fully red		0.28 ± 0.10	15.6 ± 1.2	19.0	25.2	12.4	0.1
27 August 2020	Fully ripe scarlet red		0.28 ± 0.05	15.0 ± 1.5	11.8	19.9	4.2	0
28 September 2020	Late season scarlet red		0.27 ± 0.02	15.1 ± 1.2	9.7	15.4	2.5	0.1

[a] Weight and dry weights are mean values ± standard deviation of three samples. [b] Weather data were obtained from the Norwegian Meteorological Institutes Ås weather station and represent mean, minimum, and maximum daily temperature and precipitation during the day of harvest [25].

2.1.3. Methanolic Extraction of Lingonberries

The non-volatile metabolites in lingonberries were extracted in duplicate at ambient temperature (20–22 °C) following the methanolic extraction method described previously [26,27]. Frozen lingonberries (~50 g) were milled before lyophilization for 72 h at a pressure of 0.633 mbar (Gamma 1–16, Christ GmbH, Osterode am Harz, Germany). Dry samples (400 ± 10 mg) were mixed with 5 mL 70% methanol in water (*v*/*v*) in a vortex mixer for 15 s before sonication for 10 min (Ultrasonic Cleaner, VWR International, Radnor, PA, USA) and centrifugation for 10 min at 39,200× *g* (Avanti J-26 XP Centrifuge, Beckman Coulter, Brea, CA, USA). After collection of the supernatant, a re-extraction of the insoluble material was performed. Supernatants were pooled, and the volume was made up to 20 mL with the extraction solvent (70% methanol in water). The extracts were filtered through Millex HA 0.45 µm filters (Millipore Corp., Burlington, MA, USA) before transfer to HPLC vials and stored at −80 °C until analysis.

2.2. Analysis of Sugars and Organic Acids

Sugars and organic acid content were determined using an Agilent 1100 series HPLC system, equipped with a diode array detector (DAD) and a refractometer index (RI) detector (Model 132; Gilson, Villiers-le-Bel, France), as previously described by Woznicki et al. [28]. The methanolic extracts (20 µL) were injected in a randomized order, and separation was performed on a Rezex ROA-Organic acid H+ (8%) column (300 × 7.8 mm; Phenomenex, Torrance, CA, USA) at 45 °C with mobile phase 7.2 mmol/L H_2SO_4 run at a flow rate of 0.5 mL/min. The detection of the sugars was performed with a RI detector and the organic acid detection was performed with DAD at 210 nm. Identification and quantification was performed according to the method described by Amundsen, et al. [24], and results were presented on a equivalent g/100 g fresh weight (fw) basis.

2.3. Analysis of Anthocyanins, Flavonols, Cinnamic Acid Derivatives and Procyanidins

Targeted analysis of phenolic compounds was performed using an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler cooled to 4 °C, a DAD, and a MSD XCT ion trap mass spectrometer was fitted with an electrospray ionization interface with the method described by Aaby, et al. [29]. In a

randomized order, 10 µL of the extracts were injected, and separation was performed on a Synergi 4 µm MAX RP C12 column (250 mm × 2.0 mm i.d.), to which a 5 µm C12 guard column (4.0 mm × 2.0 mm i.d.) was equipped. Both columns were produced by Phenomenex (Torrance, CA, USA). The mobile phases used were set up as a binary solvent system, which consisted of: (A) formic acid/water (2/98, *v/v*) and (B) acetonitrile. The two solvents were used in a gradient of: 0–10 min 5–10% B, 10–22 min 10–12.4% B, 22–42 min 12.4–28% B, 42–50 min 28–60% B, 50–55 min 60% B, and 55–58 min 60–5% B. Elution was performed with a flow rate of 0.25 mL/min at 40 °C, with a total run time of 60 min. The mass spectrometer (MS) was operated in positive and negative ion modes according to a previously described method by Aaby et al. [30]. Identification and quantification were performed according to the method described by [24], and results were presented on a equivalent mg/100 g fresh weight (fw) basis.

2.4. Analysis of Volatile Organic Compounds

The analysis on the composition of volatile organic compounds (VOCs) was performed according to the method previously described by Marsol-Vall et al. [31]. Determination of the VOC composition was performed with a Trace 1310 gas chromatograph coupled with a TSQ 7000 EVO mass spectrometer (Thermo Scientific, Reinach, Switzerland). To extract volatile compounds, a TriPlus RSH multipurpose autosampler (Thermo Scientific, Reinach, Switzerland) equipped with a HS-SPME with a 2 cm DVB/CAR/PDMS 50/30 µm fiber (Supelco, Bellefonte, PA, USA) was used. Two grams of a lingonberry sample, which was partially thawed, was weighed to a 20 mL headspace vial and spiked with 10 µL of the internal standard mix (4-methyl-2-pentanol at 100 µg/mL and neryl acetate at 113 µg/L in methanol). Then, the berries were crushed. Equilibration of the sample was performed for 10 min at 45 °C, followed by exposure of the fiber to the headspace of the sample vial for 30 min at 45 °C. Separation was performed using a polar capillary column (DB-WAX, 60 m × 0.25 mm × 0.25 µm: J&W Scientific, Folsom, CA, USA). The carrier gas helium was used with a constant flow of 1.6 mL/min. Identification and quantification were performed according to the method described by [24] and expressed as normalized peak areas (compound area/ISTD area). Each lingonberry sample was analyzed in quadruplicate.

2.5. Statistical Analysis

To assess the effect of ripening on groups of compounds in lingonberry, a two-way analysis of variance (ANOVA) and a Tukey's Honestly Significant Difference (HSD) test were performed, reporting significant differences at level $p < 0.05$. To illustrate the variation in the composition of volatile compounds in the samples, a principal component analysis (PCA) was performed with the Unscrambler Software (The Unscrambler®X version 10.4.1, CAMO Software AS, Oslo, Norway). Pareto scaling (weighed by 1/square root of the standard deviation) was applied before the multivariate data analysis.

3. Results and Discussion

3.1. Sugars and Organic Acids

There were three sugars and four organic acids identified in lingonberries in this study: sucrose, glucose and fructose, and quinic, citric, malic, and shikimic acid, respectively. During ripening, the total content of sugars increased from 2.7 g/100 g fw in the early green berries to 7.2 g/100 g fw in the late season ripe berries (Table 2, Supplementary Material Table S1), whereas the content of organic acids decreased from 4.9 g/100 g fw to 2.7 g/100 g fw during the same period. There were no major changes in the profile of sugars during ripening, though the proportion of sucrose (8.8–4.3%) and glucose (50–47%) slightly decreased, whereas the proportion of fructose (42–49%) increased during ripening. Although both glucose and fructose increased in absolute amounts, the sucrose content remained relatively stable throughout ripening. During ripening, particularly the concentrations of quinic and malic acid decreased, whereas only insignificant changes were

observed in the concentration of citric acid. The proportion of citric acid thus increased (42–64%), whereas the proportion of quinic (55–34%) and malic acid (3.2–1.8%) decreased. Shikimic acid contributed only to >1% of the total content of lingonberry organic acids. Shikimic acid has been shown to play a role in the synthesis of phenolic compounds [32]. However, no major changes were detected in its content in the course of lingonberry ripening. In a previous study of blueberries, no changes in the proportions of sugars were detected [22], whereas there was a slightly higher content of fructose than glucose, as well as lower content of sucrose in the berries throughout the ripening period in bilberries [33]. In cranberries, the content of quinic acid decreased, and malic acid increased, whereas there was no change in the citric acid content [34]. The total concentrations of sugars and organic acids in ripe berries were comparable to previous findings in lingonberries [5,35,36]. The simultaneous increase in sugar content with a decrease in organic acids is among the most important and characteristic features of fruit and berry ripening and was thus expected [22]. Additionally, earlier studies on lingonberries and bilberries have indicated increasing content of sugars towards the end of ripening [33,37]. The increase in sugar concentration and decrease in the organic acid concentration improve the palatability of the berries. The ratio of sugar to organic acids, which increased from 0.6 to 2.7 during ripening, influences the perceived sweetness of berries and is likely to influence the liking of the lingonberries [4,8]. As lingonberries are generally considered to have a low degree of sweetness, and addition of sugars is often seen as a necessity in products, and berries with a high ratio of sugars to organic acids would be preferred. Harvest of berries later in the season could thus improve quality as the ratio of sugars to organic acids is significantly higher than earlier in the season.

Table 2. Concentration of sugars and organic acids in lingonberries picked at five ripening stages [a].

	Green	Half Red	Red	Fully Ripe	Late Season
Sucrose	0.23 ± 0.01	0.37 ± 0.07	0.38 ± 0.07	0.34 ± 0.06	0.31 ± 0.02
Fructose	1.1 ± 0.3 d	1.9 ± 0.3 c	2.7 ± 0.5 b	3.2 ± 0.1 ab	3.5 ± 0.4 a
Glucose	1.3 ± 0.2 b	2.2 ± 0.2 b	3.0 ± 0.5 a	3.3 ± 0.4 a	3.4 ± 0.1 a
Total sugars	2.7 ± 0.5 c	4.4 ± 0.4 b	6.2 ± 1.0 a	6.9 ± 0.4 a	7.2 ± 0.4 a
Citric acids	2.1 ± 0.4	2.2 ± 0.3	2.1 ± 0.3	2.1 ± 0.6	1.7 ± 0.4
Quinic acid	2.7 ± 0.5 a	1.9 ± 0.1 b	1.5 ± 0.2 bc	1.1 ± 0.2 cd	0.9 ± 0.1 d
Malic acid	155 ± 39 a	114 ± 54 ab	93 ± 24 abc	79 ± 25 bc	48 ± 17 c
Shikimic acid	3.0 ± 0.7	2.4 ± 0.3	2.3 ± 0.5	2.3 ± 1.0	2.3 ± 0.9
Total organic acids	4.9 ± 0.4 a	4.2 ± 40.3 ab	3.8 ± 0.1 bc	3.2 ± 0.7 cd	2.7 ± 0.5 d
Sugar: acid ratio	0.6 ± 0.2 b	1.0 ± 0.1 b	1.6 ± 0.3 b	2.2 ± 0.4 ab	2.7 ± 0.3 a

[a] All concentrations are mean values ± standard deviation of three samples analyzed in duplicate presented as concentration in g/100 g fw, except shikimic and malic acid in mg/100 g fw. Different letters (a–d) indicate significant differences ($p < 0.05$) between the samples, as determined by Tukey's HSD test.

3.2. Phenolic Compounds

There were in total 27 phenolic compounds tentatively identified (Supplementary Material Table S2) and quantified in lingonberries during the five stages of ripening (Table 3). Five anthocyanins, eleven flavonols, eight cinnamic acid derivatives, and three flavan-3-ols were identified. The anthocyanins identified were all glycosides of cyanidin. Flavonols tentatively identified were mostly glycosides of quercetin, with small amounts of kaempferol glycosides. Cinnamic acid derivatives (CADs) identified were hexosides of ferulic, caffeic, sinapic and *p*-coumaric acid, a coumaroyl iridoid and chlorogenic acid. Exact quantification of all flavan-3-ols was not possible due to coelution and low molar absorptivity. However, catechin, a B-type dimer, and an A-type dimer could be quantified. Chemical composition of ripe lingonberries has been investigated in several publications, and the composition of anthocyanins, flavonols, and flavan-3-ols were in line to what has been previously reported [7,15,36,38–42].

Table 3. Content of phenolic compounds in lingonberries picked at five ripening stages [a].

	Green	Half Red	Red	Fully Ripe	Late Season
Cyanidin-3-O-galactoside	0.4 ± 0.1 b	17.8 ± 1.3 b	57.9 ± 4.0 a	65.4 ± 11.0 a	79.3 ± 17.5 a
Cyanidin-3-O-glucoside	0.0 ± 0.0 c	0.6 ± 0.1 c	3.1 ± 0.5 b	4.5 ± 1.0 ab	5.6 ± 1.5 a
Cyanidin-3-O-arabinoside	0.0 ± 0.0 c	0.9 ± 0.0 c	6.6 ± 0.2 b	10.0 ± 0.7 ab	13.0 ± 3.4 a
Cyanidin-3-O-pentoside	0.0 ± 0.0 c	0.1 ± 0.0 c	0.6 ± 0.1 b	0.9 ± 0.1 ab	1.2 ± 0.4 a
Cyanidin-3-O-(acetyl)glucoside	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
Total anthocyanins	0.4 ± 0.1 c	19.4 ± 0.7 c	68.3 ± 2.3 b	80.9 ± 7.0 ab	98.5 ± 12.6 a
Quercetin-3-*O*-galactoside	10.4 ± 2.3 a	10.5 ± 4.1 a	7.6 ± 2.8 ab	5.5 ± 0.6 b	4.6 ± 2.0 b
Quercetin-3-*O*-glucoside	2.4 ± 0.2 a	2.3 ± 0.4 a	1.6 ± 0.3 ab	1.2 ± 0.1 b	1.2 ± 0.3 b
Quercetin-3-*O*-xyloside	1.7 ± 0.2	1.6 ± 0.3	1.4 ± 0.1	1.3 ± 0.2	1.2 ± 0.2
Quercetin-3-*O*-arabinoside	10.4 ± 0.8 a	10.8 ± 0.6 a	9.5 ± 1.3 ab	7.8 ± 0.5 bc	6.6 ± 0.8 c
Quercetin-3-*O*-arabinofuranoside	1.0 ± 0.1 a	0.8 ± 0.1 b	0.6 ± 0.1 c	0.5 ± 0.1 d	0.4 ± 0.1 d
Quercetin-3-*O*-rhamnoside	6.3 ± 4.5	6.3 ± 4.8	5.2 ± 3.6	3.8 ± 2.6	2.4 ± 1.7
Quercetin-(HMG)-pentoside	0.4 ± 0.1 a	0.4 ± 0.1 ab	0.3 ± 0.0 abc	0.3 ± 0.0 bc	0.2 ± 0.0 c
Quercetin-3-*O*-(HMG)-pentoside 2 [b]	0.0 ± 0.0 b	0.1 ± 0.1 ab	0.2 ± 0.1 a	0.0 ± 0.1 b	0.0 ± 0.0 b
Kaempferol-3-O-rhamnoside	0.4 ± 0.2 a	0.3 ± 0.2 ab	0.3 ± 0.1 ab	0.2 ± 0.1 ab	0.2 ± 0.0 b
Quercetin-3-*O*-(HMG)-rhamnoside [b]	8.2 ± 7.8	7.6 ± 5.8	5.7 ± 4.9	4.6 ± 4.3	2.8 ± 2.6
Kaempferol-3-*O*-(HMG)-rhamnoside [b]	0.0 ± 0.0 b	0.1 ± 0.0 b	0.4 ± 0.1 ab	0.7 ± 0.2 b	0.5 ± 0.1 b
Total flavonols	41.2 ± 7.7 a	40.8 ± 7.3 a	32.8 ± 5.3 ab	25.8 ± 4.2 b	20.1 ± 1.2 b
Ferulic acid-hexoside 1	1.2 ± 0.8 c	2.4 ± 0.4 ab	2.8 ± 0.6 a	1.5 ± 0.2 bc	1.3 ± 0.2 c
Ferulic acid-hexoside 2	31.4 ± 11.2 a	30 ± 19.2 a	3.6 ± 1.5 b	0.8 ± 0.1 b	0.7 ± 0.1 b
Coumaroyl iridoid	5.7 ± 2.2 a	2.9 ± 1.4 ab	1.6 ± 0.8 b	1.1 ± 0.8 b	1.0 ± 0.8 b
Caffeic acid hexoside 1	2.1 ± 0.3 a	1.9 ± 0.5 ab	1.1 ± 0.1 bc	0.8 ± 0.2 c	0.7 ± 0.1 c
Caffeic acid hexoside 2	3.1 ± 0.3 a	2.9 ± 1.6 a	1.0 ± 0.3 b	0.5 ± 0.1 b	0.3 ± 0.0 b
p-Coumaric acid hexoside	5.7 ± 0.5	4.7 ± 1.1	5.3 ± 0.1	4.2 ± 0.3	5.7 ± 1.6
Chlorogenic acid	2.6 ± 0.6	3.8 ± 4.4	1.8 ± 1.0	1.3 ± 0.1	1.7 ± 0.7
Sinapic acid hexoside	0.1 ± 0.0 a	0.1 ± 0.0 ab	0.1 ± 0.0 ab	0.1 ± 0.0 b	0.1 ± 0.0 b
Total cinnamic acid derivatives	51.9 ± 6.4 a	48.8 ± 14.1 a	17.4 ± 1.7 b	10.3 ± 0.7 b	11.3 ± 1.1 b
Proanthocyanidin dimer A	103.8 ± 41.7 a	78.8 ± 34.6 ab	46.8 ± 28.3 b	34.1 ± 13.6 b	27.2 ± 4.3 b
Proanthocyanidin dimer B	30.8 ± 7.4 a	26.7 ± 8.3 ab	17.8 ± 3.5 bc	14.1 ± 3.5 c	14.2 ± 4.7 c
Catechin	71.4 ± 24.6 a	54.4 ± 20.3 ab	36.7 ± 10.7 bc	26.1 ± 7.9 c	31.4 ± 11.4 c
Total Flavan-3-ols	206.0 ± 67.8 a	159.8 ± 61 ab	101.4 ± 33.4 bc	74.3 ± 10.7 c	72.8 ± 14.6 c

[a] Concentrations are mean values ± standard deviation of three samples analyzed in duplicate. Anthocyanins were quantified as mg/100 g fresh weight (fw) equivalents of cyanidin-3-galactoside at 520 nm, flavonol glycosides as quercetin-3-rutinoside at 360 nm, cinnamic acid derivatives as chlorogenic acid at 320 nm, and flavan-3-ols as catechin at 280 nm. Different letters (a–d) indicate significant differences ($p < 0.05$) between the samples, as determined by Tukey's HSD test. [b] HMG = Hydroxy-3-methylglutaroyl.

In the green lingonberries, the accumulation of anthocyanins had just started with 0.4 mg/100 g fw measured. As the berries ripened, accumulation increased, and the content of anthocyanins was 80 mg/100 g fw in ripe berries and increased to 99 mg/100 g fw late in the season (Table 3, Figure 1). All anthocyanins increased with ripening, but the proportion of cyanidin-3-O-galactoside decreased from 92% to 80% in the ripe berries. This was mostly due to a strong increase in the proportions of cyanidin-3-O-arabinoside and cyanidin-3-O-glucoside that were absent in green berries and had a proportion of 13.1% and 5.7%, respectively, in late season berries. In a previous study, the proportion of cyanidin-3-O-galactoside also decreased from 94% to 81% between early berries on 29th of July and late season berries on the 4th of October. In the same study, increases in both the proportion of cyanidin-3-O-arabinoside from 1% to 11% and cyandin-3-O-glucoside from 5% to 6% were detected [15]. The content of the individual compounds is of importance, as studies have shown that the glycosylation influences the absorption of the lingonberry anthocyanins in humans [43]. The anthocyanins of lingonberries accumulate mostly in the skin with little coloration in the fruit flesh [18]. The increase in the anthocyanin content in the fruit skin is the visual expression of ripening [18]. It is thought that plants accumulate anthocyanins to attract seed dispersers, indicating a ripe berry, but they also protect the berry against outside stressors, such as radiation [16–20]. It has been found that birds prefer lingonberries with higher anthocyanin content [1]. Though the process was gradual, most anthocyanin

accumulation had taken place as the berry had turned fully red. However, a significantly higher content was detected in the fully ripe late season berries.

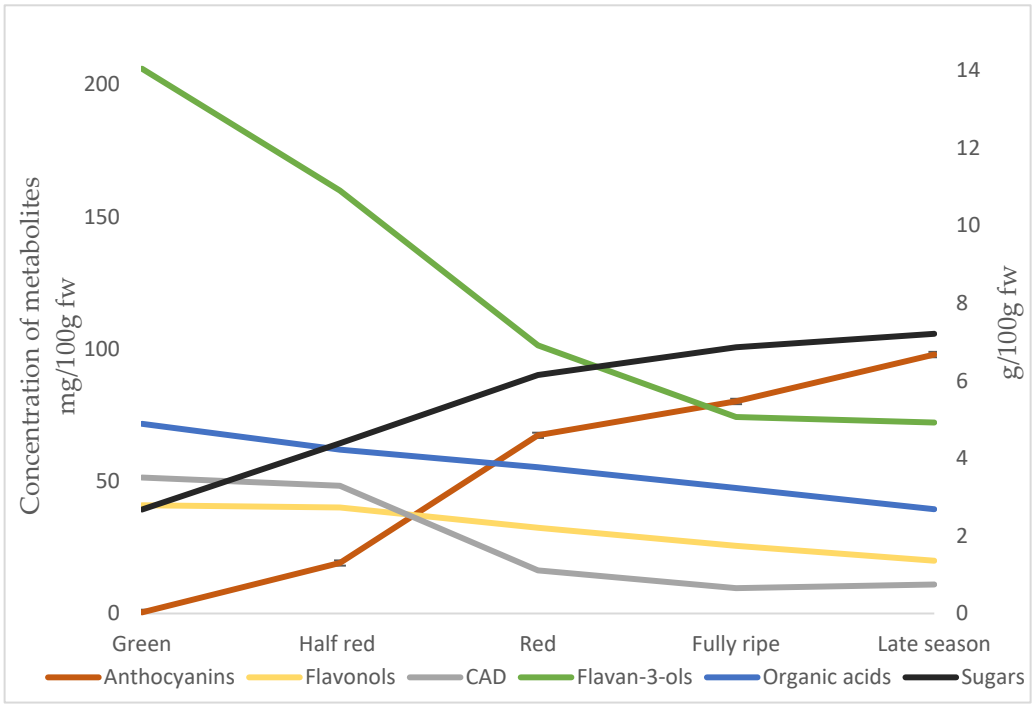


Figure 1. Concentrations of groups of phenolic compounds (mg/100 g fw), sugars, and organic acids (g/100 g fw) in lingonberries at the five different stages of ripening. CAD—cinnamic acid derivatives.

While the anthocyanins accumulate throughout the season from 0.4 to 98.5 mg/100 g fw, the content of flavan-3-ols and CADs decreased from 206.0 to 72.6 mg/100 g fw and from 51.9 to 11.3 mg/100 g fw (Table 3). Additionally, the flavonols decreased from 41.2 and 20.1 mg/100 g fw, but their decrease was more gradual throughout the season. The highest total content of phenolic compounds, consisting of flavonols, flavan-3-ols, and CADs, were found in early green berries (Figure 1). Similar results have been found in many fruits and berries, and it is thought that they are a defense mechanism of the plants to protect the immature seeds against predators and herbivores early in the season [18]. In cranberries, the content of flavonols and proanthocyanidins approximately halved as they ripened [44]. In the present study, the profile of flavonols changed during ripening. The content of all flavonols besides kaempferol-3-O-(HMG)-rhamnoside decreased or remained stable during ripening. A previous study reported a decrease in the total concentration of flavonols in lingonberries during ripening, with a particularly large decrease in the content of glucosides and rhamnosides [15]. In the present study, in the early green and half red lingonberries, the proportion of ferulic acid among the CADs was between 55–60%. As the berries ripened, the large decrease in CAD content was mainly due to the decrease in the concentration of a ferulic acid, hexoside. In ripe berries, the proportion of ferulic acid was 8.5%. The high content of ferulic acid in green berries could be linked to the role of ferulic acid in the crosslinks of structural carbohydrates prior to the softening of cell tissues. The content of a coumaric acid, hexoside, remained high throughout ripening. This is probably due to coumaric acid hexoside being a precursor in the cyanidin synthesis [28]. The two dimeric procyanidins measured (A- and B-type procyanidins) and catechin all decreased during ripening. However, there was a stronger decrease in the concentration of the A-type (74%) than the B-type (54%) procyanidin during ripening. Similarly, in a previous study of lingonberries harvested at several timepoints during the season, a relative decrease in the A-type and increase in the B-type proanthocyanidins was observed [15]. Proanthocyanins are among the most interesting health-promoting compounds in lingonberries [10]. The

A-type proanthocyanins found in the highest content in the early fruit are less common and have been shown to have the strongest anti-lipid peroxidation activity [45]. This shows that in the early stage, green berries also contain a high content of interesting compounds that could be utilized.

3.3. Volatile Compounds

A total of 71 volatile organic compounds (VOCs) were tentatively identified in lingonberries during ripening (Supplementary Material Table S3). The VOCs consisted of twenty aldehydes, nine esters, of which six were acetates, six ketones, eleven alcohols, eight acids, sixteen terpenoids, and one furan. Previously, five studies have been performed analyzing volatile organic compounds in lingonberries [6,24,31,46,47]. Among the studies, only one has analyzed whole crushed berries [24], three have analyzed lingonberry juices [6,31,46], and one analyzed the press residue from juice production [47]. Aldehydes were the most abundant group of VOCs found in the present study (Figure 2). Among aldehydes, many compounds are known to give various aromas, and they most often have green, fatty, or tallow aromas [48]. In previous research of lingonberries, several compounds that were also identified in this study were highlighted as contributors to aroma using GC-olfactometry, such as 2-methylbutanoic acid [46,47], eucalyptol, hexanal, linalool, and methyl benzoate [31]. Diacetyl and 2-methylpropanoate were also identified, but they could not be quantified in this study [31]. Additionally, in cranberries, large variation in the profile of VOC compounds has been found [9]. Most studies of cranberries report the presence of benzyl compounds, including benzyl alcohol, benzoic acid, and benzaldehyde. Additionally, a major part of the studies also have found the terpene α-terpineol in relatively high levels. These compounds were also identified in lingonberries in this study.

During ripening, several changes occurred in the profile of VOCs in lingonberries (Figure 2). During ripening, also, different numbers of compounds were measured: 57 at the first stage of ripening, 63 at the second stage, 67 at the third, 63 at fourth, and 65 at the fifth (Supplementary Material Table S3). The highest total content of VOCs was found in the late season berries closely followed by the red berries. This is in contrast to what has been shown in grapes, in which a higher number of volatiles was found before initiation of ripening (pre- compared to post-veraison fruit) [49]. Previous research has shown that VOCs, amongst others, act as signaling molecules to attract pollinators and seed dispersers, and it is, therefore, natural that a high content is found just prior to ripening. This has been shown in highbush blueberries (*V. corymbosum* L.), in which the highest content of VOCs was found just prior to ripening [50]. In lingonberries, an increase in the content of volatile acids and a tendency for increase in the content of ketones, terpenes, and furan during ripening was detected (Figure 2). Synthesis of volatile compounds is part of other metabolic processes occurring in the berries during ripening. The continuous increase in volatile, short chained fatty acids (C5–C9) during ripening could be due to an increase in the fatty acid fraction in the wax layer of the lingonberry surface during berry ripening [51]. The content of esters was highest in green fruits. When the coloration of the skin had begun, there was little variation in their contents. This is contrary to their role in fully mature blueberries, where esters previously have been proposed to be responsible for fruity aroma characteristics [9]. In lingonberries, a high content of alcohols in the green berries was detected, as well as a tendency for an increase in the late season berries. Two studies of *V. padifolium* showed an increase in the content of alcohols in the ripe berries compared to earlier in the season [19]. Additionally in red grapes, increased alcohol concentrations occur as the berries ripen, whereas the concentration of aldehydes decreased [23]. The content of aldehydes in the present study, however, did not decrease as the berries ripen, as the content was highest in red and late season berries (Figure 2). The high temperature detected when collecting the red berries (Table 1) may have influenced the release of aldehydes and terpenes at this stage. Grapes harvested at higher temperatures have previously been shown to contain a higher content of terpenes [52]. The content of benzyl acetate, benzyl

alcohol, and methyl benzoate increased in lingonberries as the berries ripened (Supplementary Material Table S3). This is in line with previous findings showing that the content of benzoic acids increases as the berries ripen [34].

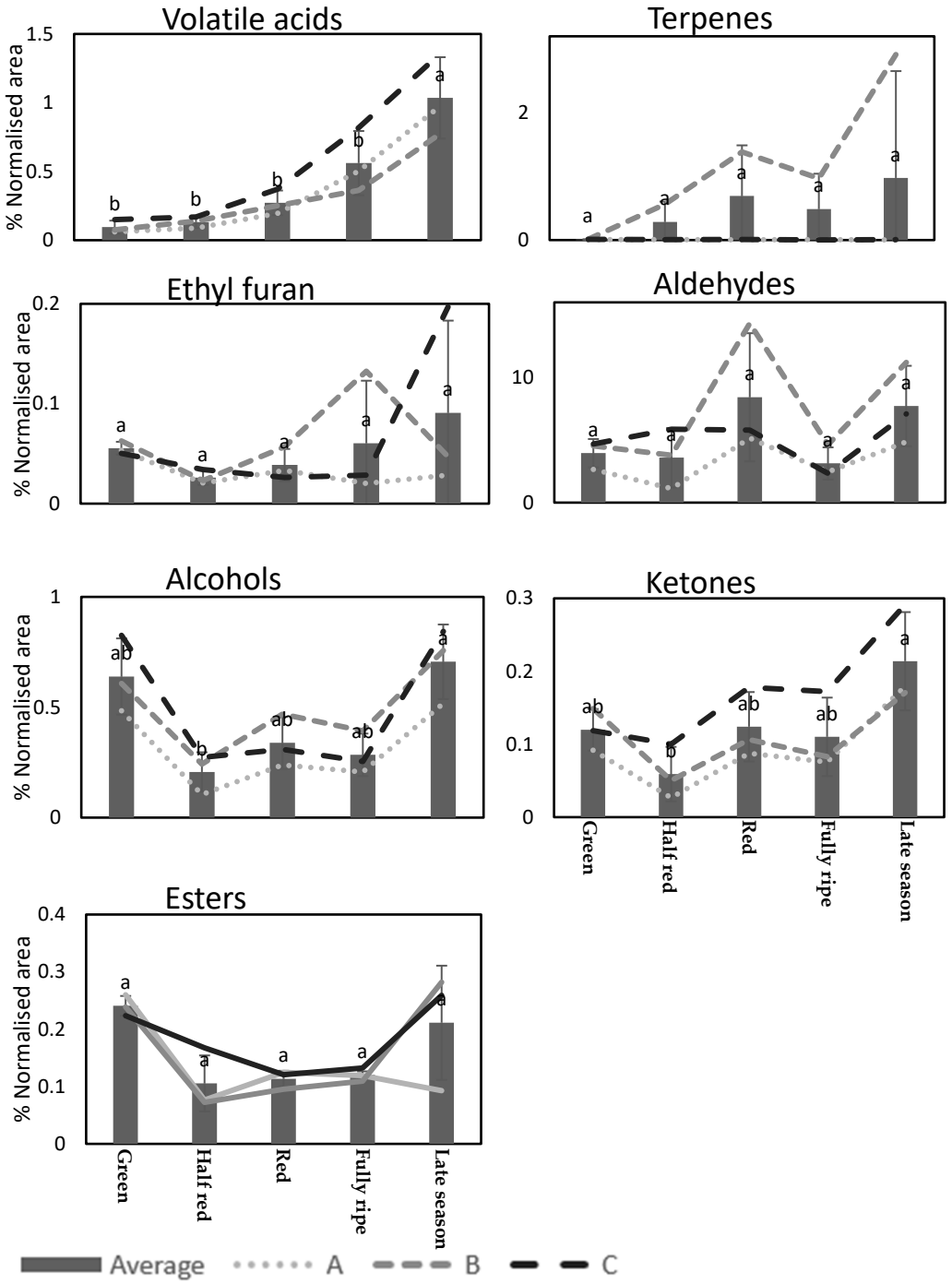


Figure 2. Changes in the mean content of aldehydes, volatile acids, alcohols, ketones, esters, terpenes, and ethyl furan in % normalized area of the internal standard in lingonberries at the three stands (A–C) at the five stages of ripening. Different letters (a–b) indicate significant differences ($p < 0.05$) between the samples, as determined by Tukey's HSD test.

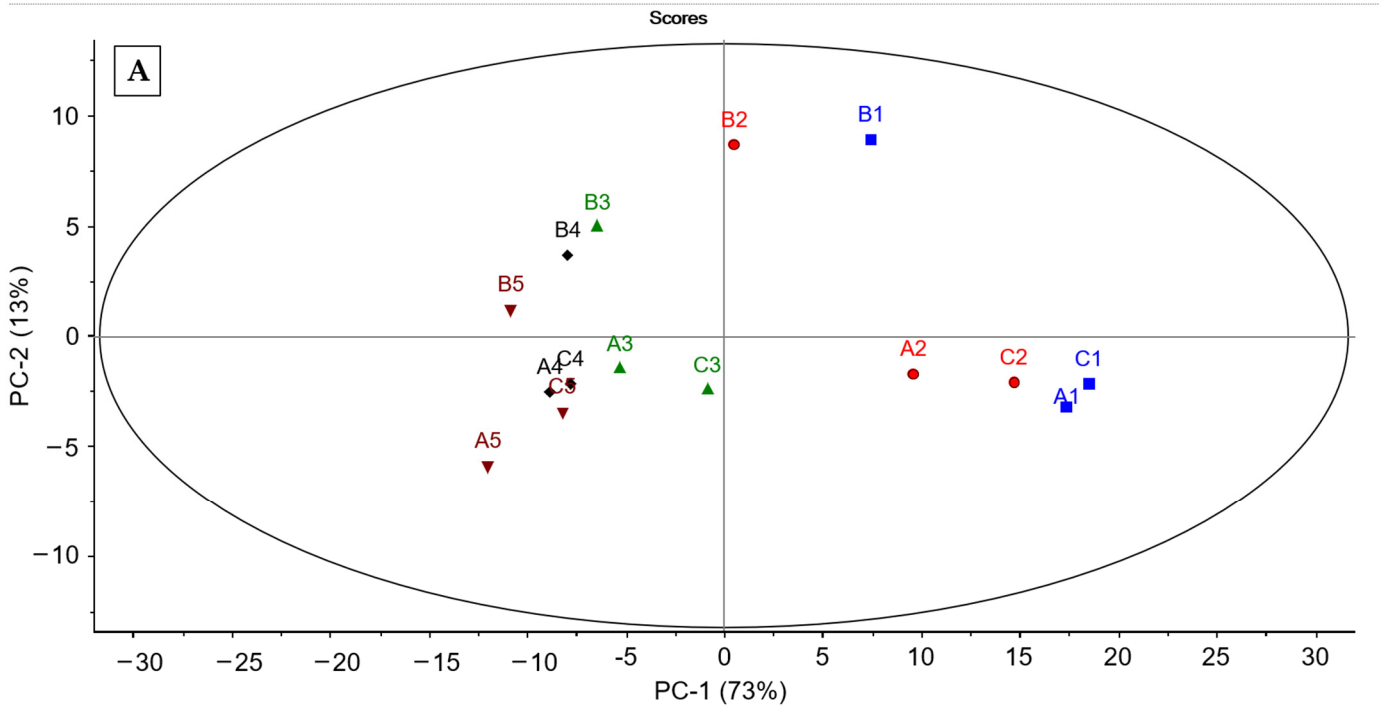
Clear differences in the profiles and responses between the three stands were found, particularly, in the profile of aldehydes and terpenes (Figure 2). Berries from stands A and C were picked from pine forest with medium forest density, whereas the berries

from stand B were picked from a clear-cut area. As berries in a clearcut forest are more exposed to radiation than berries grown in a denser forest, this may affect the synthesis of metabolites, such as terpenes. However, lingonberry genetics, growth environment, and their interaction can all influence the synthesis of volatile compounds in a complex manner [18]. In grapes, large variation in aroma compounds has been between different cultivars independent of grape color [23]. Similarly to the results from our study, it has previously been shown in grapes that the terpenes were influenced significantly by UV-B radiation during ripening [52]. This could also explain why the distance from forests has been shown to influence the composition of grapes, which could be due to shading effect [49]. Additionally, genetic background affects the VOC composition, for example, in both highbush and rabbiteye blueberries, large variations have been found between different cultivars during ripening [9]. With the changes observed in the number of VOCs, and their relative quantities, this study indicates that the ripeness of lingonberries affects the profile of VOCs in the berries, which is likely to influence the perceived aroma.

3.4. General Discussion

In the PCA of all the measured compounds from the three locations (A, B, C) at the five ripening stages (1–5), the first two components explained 86% of the variation in the data set (Figure 3A). There was a clear clustering of both ripening stage and location. The five ripeness stages spread in the first principal component (73%), showing that the ripeness most significantly influenced the chemical composition of lingonberries. The difference between stands spread in the second principal component (13%) with berries from stand B being separated from berries from stands A and C. Though there was a clear horizontal separation between each ripening stage, it was an evident clustering of berries before (1–2) and after full coloration of the skin (3–5). The cluster of the most ripe berries was characterized by the highest content of anthocyanins, sugars, volatile acids, benzyl alcohol, and acetophenone (Figure 3B). In contrast, the berries prior to ripening were characterized by high content of several of the CADs and flavonols, the flavan-3-ols, malic and quinic acid, methyl acetate, 1-octen-3-ol, 5-methyl-hepthen-one, caryophyllene, and 2,4-heptadienal. Early in the season, lingonberries from a single cluster can vary in ripeness depending on their position. Berries less exposed to sunlight appear to ripen at a slower rate. These differences in ripening on a bush can influence the overall quality of the product. Therefore, efforts should be placed on separation of berries based on color if the berries are harvested early in the season. This study gives clear evidence for a variation in the profile of volatiles in lingonberries with ripening. However, there is still a large gap within the understanding of the influence of changes in composition and their relation to fruit flavor itself. This is in part due to the complex nature of the interactions between the compounds in aroma. Additionally, while lingonberries generally have a high content of phenolic compounds, there is not enough evidence available to determine how variation in these compounds during ripening will influence the berry aroma.

The samples from each of the sampling stands were most clustered in the second and third dimension. Particularly, stand B stood out, but there were differences observed between the five stages of ripening. Though the lingonberries were collected from three locations within a 3 km radius from one another, there was a large variation in the chemical composition particularly in the composition of terpenes and aldehydes between the three locations. This could be due to different forest density, thus different solar radiation, at the three stands, as previously discussed (Section 3.3). The variation in the content of flavonoids is also likely influenced by differences in the growth environment as both radiation and temperature have previously been shown to influence their composition [24,37,53]. However, a recently published paper shows that the composition of both phenolic compounds and triterpenoids in lingonberries grown under the same conditions is strongly influenced by genetic variation of the plants [54]. Plants with different genetic backgrounds may also react differently to different environmental conditions [15,18,24,37,55].



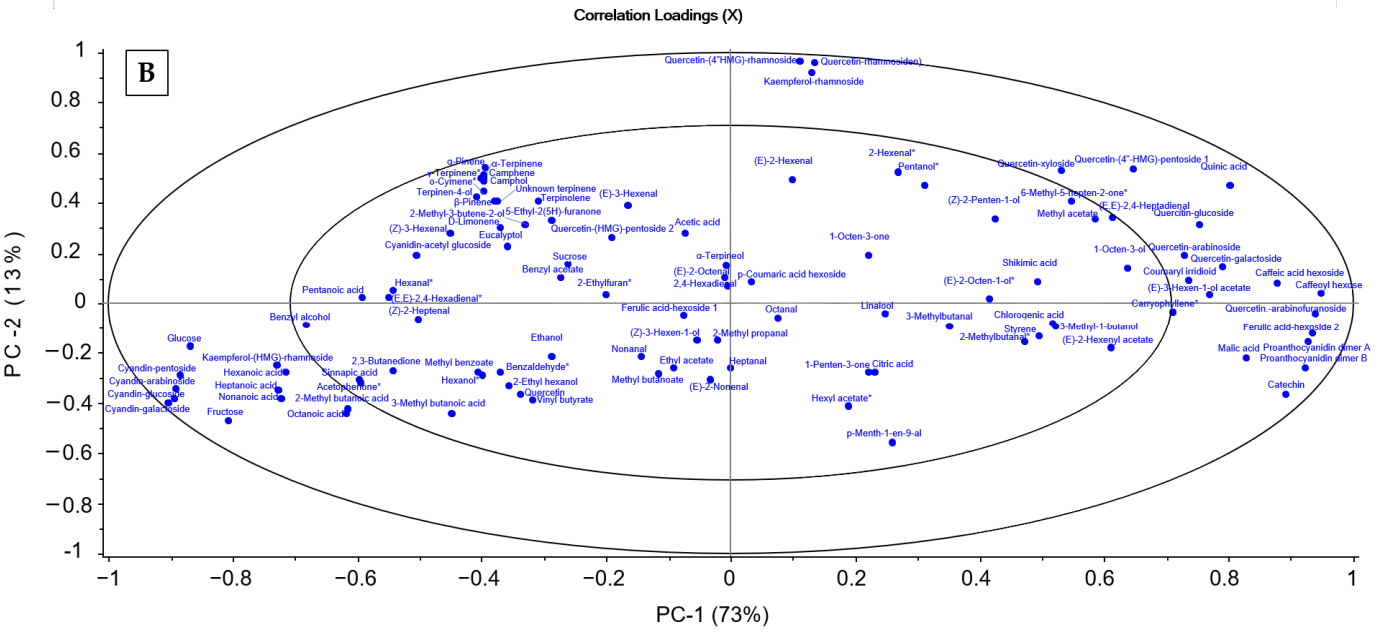


Figure 3. Plots after principal component analysis (PCA) of sugars, organic acids, phenolic compounds, and volatile organic compounds in the lingonberry samples. (**A**) Score plot of berries harvested from three locations (A–C) at five ripening stages; green berries (1; blue squares), half red berries (2; red circles), red but not fully ripe (3; green triangles), ripe berries (4; black diamonds); and late season berries (5; inverted brown triangles). (**B**) Loading plot showing the contribution of each compound in the experiment to the differences between the samples.

4. Conclusions

The chemical composition of lingonberries changed markedly during fruit ripening. The highest contents of phenolic compounds, especially proanthocyanidins, were found in the early stages of berry development, whereas the highest content of anthocyanins

and a richer aroma profile was detected in the late harvested lingonberries. Therefore, for the traditional food uses, highest quality berries are achieved by waiting until late in the season with harvest. Potentially, the green berries often harvested together with the first ripe berries in the early season, could be separated and utilized for other purposes. The results reported are of interest for both consumers and producers, as they highlight the influence of ripening and harvest time on the quality of the berries. Further studies are needed for better understanding the relation between volatile compounds and aroma profiles of lingonberries, and the impact of genetic and environmental factors on variation of the aroma profiles and flavor of the berries.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods12112154/s1, Figure S1: Coordinates of stands in the experiment. Table S1: Content of all compounds analyzed in lingonberries from the three stands in Ås Norway, at five ripening stages; Table S2: Characterization of phenolic compounds in lingonberries using HPLC-DAD-MS; Table S3: Characterization of volatile organic compounds in lingonberries using HS-SPME-GC-MS.

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PAPER II

Effect of light spectrum, light intensity, and treatment time on chemical composition in lingonberries (*Vaccinium vitis-idaea* L.)

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Abstract

Lingonberries (*Vaccinium vitis-idaea* L.) are renowned for their unique flavor profile and potential health benefits due to their rich content of bioactive compound. Understanding how environmental factors impact synthesis of bioactive components of fruits and berries has garnered significant interest in recent years. These light conditions have been identified as key factors influencing plant metabolism. The aim of this study was to investigate how light intensity and quality on the accumulation of phenolic compounds, sugars, and organic acids in lingonberries (*Vaccinium vitis-idaea* L.). Lingonberries were subjected to supplemental light treatments of blue, red, and far-red light in addition to high and low light intensities in a carefully designed growth chamber setup. Ripe berries from each light treatments were harvested over a period of four weeks and the berries were harvested and analyzed for changes in their content of phenolic compounds, sugar composition, and organic acid levels. High-performance liquid chromatography (HPLC) methods were employed to quantify the specific changes in the metabolite profiles. The findings from this study revealed that both light spectral treatments and light intensity treatments influenced the composition of lingonberries. Blue light and high light intensity conditions elevated the levels of anthocyanins in lingonberries. There were also slight effects of the treatments on the content of sugars and organic acids. Harvest time was very important influencing quality of the berries. Later stages of harvest generally led to an accumulation of sugars and anthocyanins, while the levels of CADs decreased. The findings from this study indicate that that variation in light conditions and the time of harvest can significantly impact the quality of lingonberries. This investigation provides valuable insights into the intricate relationship between light conditions and the composition of bioactive compounds in lingonberries.

Introduction

Berries are a well-acknowledged source of compounds that may contribute to reducing cholesterol, blood pressure and levels of homocysteine (Olas 2016, Erlund et al., 2008). Additionally, they improve the endothelial function through the presence of high amounts of polyphenolic compounds (Ghosh and Scheepens 2009, Alotaibi et al., 2021). The *Vaccinium* genus contains many commercially important berry species, among them the wild growing lingonberry (*Vaccinium vitis-idaea*). Lingonberry is a small evergreen shrub native to the circumpolar region. The berries, predominantly utilized in Northern Europe, contain a high content of health promoting compounds including phenolics such as

anthocyanins, flavan-3-ols, cinnamic acid derivatives and flavonols (Nestby et al., 2018). There has been an increasing interest in the research of phenolic compounds in the last decades. Several studies have demonstrated marked variations in phenolic content (Anttonen and Karjalainen 2005, Teixeiro et al., 2013, Karppinen et al., 2016) as well as sugars and acids (Akagić et al., 2020) in wild berries, as responseto both biotic and abiotic factors. The wild growing lingonberries are distributed over large areas exposed to varying environmental conditions (Nestby et al., 2018). Therefore, it is of interest to have a better understanding on how the concentration and composition of phenolic compounds are affected by external in addition to internal factors.

Our previous study demonstrated that the content of phenolic compounds in lingonberries is influenced by their origin and the temperature associated with the growth location (Amundsen et al., 2023). Other important environmental factors affecting the composition of metabolites include light conditions during berry development and ripening as well as the time of harvest. Together, these factors impact the production of metabolites associated with light protection, such as phenolic compounds, as well as compounds associated with taste like sugars and acids (Briggs and Olney 2001, Zoratti, et al., 2014, Ouzounis et al., 2015, Samkumar et al., 2022a). The level of irradiance and spectral distribution of solar radiation vary based on latitudes, altitudes, time of day and season. Decreasing solar elevation due to increased latitudes causes changes in red:far-red ratio during the twilight hours (Mølmann et al., 2021). Plants' photoreceptors perceive alterations in irradiance level and wavelength composition, enabling them to sense light signals within the solar spectrum (280-750 nm where 450-495 nm is blue, 600-700 nm is red, and 750-800 nm is far-red). These signals induce developmental and metabolic changes throughout plants' growth cycle, including fruit development and ripening (Kang et al., 2020).

In bilberries (*Vaccinium myrtillus*), light conditions are found to play a significant role in both the content and composition of anthocyanins (Zoratti et al., 2014; Samkumar et al., 2021b, 2022a). Even during the early development of bilberry fruit, short exposures to specific light spectrums are found to impact the final flavonoid profile in ripe berries (Zoratti et al., 2014). Exposure to blue wavelengths has been shown to increase the accumulation of the hydroxylated anthocyanins; delphinidins, petunidins and malvidins, in ripening bilberries (Mikulic-Petkovsek et al., 2015). Bilberries grown in sites with higher photosynthetic active radiation had higher levels of anthocyanins, flavonols, hydroxycinnamic acids, and total phenolics. A study on the molecular mechanisms regulating these specific light spectrum effects in bilberry suggests different signal transduction and transport mechanisms between red and blue light responses in abscisic acid (ABA)-regulated anthocyanin, especially delphinidin branch biosynthesis, during bilberry fruit ripening (Samkumar et al., 2021b). In cranberries (*V. macrocarpon*), a short exposure to red wavelengths increased anthocyanin accumulation compared to berries treated with white light or kept in darkness (Zhou and Singh, 2002). Another study on cranberries, light treatments (natural light, red light, and far-red light) during postharvest caused increased accumulation of anthocyanins (Zhou and Singh, 2004), affecting the individual anthocyanins differently between

treatments. These findings indicate that the light environment influences the regulation of anthocyanin biosynthesis.

The intensity of light has been found to impact the composition and concentration of compounds associated with fruit quality (Jovancevic et al., 2011; Mikulic-Petkovsek et al., 2015). Light intensity is influenced by factors such as season, cloudiness, latitude, and forest density. High light intensities have been associated with higher concentrations of anthocyanins and other phenolic compounds in bilberries (Jovancevic et al., 2011; Mikulic-Petkovsek et al., 2015), raspberries (*Rubus idaeus*) (Wang et al., 2009), *Berberis microphylla* berries (Arena et al., 2018), and grapes (*Vitis vinifera*) (Downey et al., 2004, Jamshidian at al., 2010, Šebela et al., 2017). However, results from studies on *Vaccinium* spp. indicate that the demand for high light intensities may differ between species (Zoratti et al., 2015b).

The lingonberries mature over a course of 3-4 weeks in the late season, typically from late August to September. During this period the light conditions vary and can significantly impact the berry phenolic composition. A previous study indicates that the ripening time of lingonberries does not appear to be affected by temperature (Amundsen et al., 2023a). Furthermore, the maturity stage plays a substantial role in determining the levels of metabolites in berry fruits (Prior et al.,1998; Amundsen et al.,2023b). The effect of fruit maturation and ripening on development and changes of phenolic compounds in *Vaccinium* spp. has been documented (Celik et al.,2008). Łata et al. (2005) observed a significant impact of harvest dates within the season on the levels of selected phenolics in blueberries (*V. corymbosum*). However, there is limited knowledge regarding seasonal variation in ripe berry fruits (Vilkickyte and Raudone 2021; Amundsen at al 2023b). Since both the light spectrum and light intensities undergo marked changes during the harvesting period, exposure time and harvest time may affect the levels of phenolic compounds in berries.

Previous findings indicate that plant responses to different light treatments can vary significantly, even among closely related species (Zoratti et al., 2015b). The impact of light on the accumulation of secondary metabolites in *Vaccinium* berries appears to be species-specific. So far, little is known on the effect of light conditions on the lingonberry fruit ripening. Therefore, this study aims to investigate the effects of light intensity and specific light spectra on the concentrations of phenolic compounds, sugars, and organic acids in lingonberries during berry maturation under controlled conditions.

MATERIALS AND METHODS

Light treatments and berry samples. Wild lingonberry shrubs with intact root systems and forest soil were collected from three different open vegetation covers in Målselv, Norway (69°11´N, 18°25´E) during late summer (August 2. 2021). The shrubs were collected after pollination and development of green berries and placed in large boxes (50 cm × 70 cm). The plants were grown in chambers covered

by photo reflective sheets with ambient light provided from the top under controlled conditions in a phytotron in Tromsø, Norway (69°42′N, 18°56′E). The temperature was maintained at 12 °C throughout the ripening period. The light treatments consisted of additional irradiation given to the plants inside the chambers by Heliospectra RX30 lamps (Heliopsectra AB., Gothenburg, Sweden), with blue (460 nm), red (660 nm) or far-red (735 nm) light wavelengths as well as white light with high (250 µmol/m^2) and low light intensity (80 µmol/m^2) (Figure 1). The treatments were compared to control plants which were grown in the chambers without additional irradiation but otherwise underwent the same treatments. The irradiation energy flux (µW/cm^2) and the distance from light source to plants from all the light treatments were measured using JAZ Spectrometer (Ocean Optics Inc., Orlando, FL, USA). Lingonberry plants were grown for two weeks before the first harvesting timepoint (23rd of August), thereafter harvested every week until 14th of September. All treatments were performed as triplicates. Berries were harvested at full redness with a minimum of 10 berries at each timepoint. After harvest all berries were rapidly frozen to -20 °C and extraction performed within two months.

Extraction procedure. For extraction of phenolic compounds, sugars, and organic acids, a modified version of the method described by Davik et al., (2020) was used. Five randomly selected berries (0.95-1.48 g) were homogenized with 70% methanol in water (v/v) (5 mL) in a Polytron PT3100 homogenizer (Kinematica AG, Littau, Switzerland) for 15 seconds. This mixture was sonicated for 10 min (Ultrasonic Cleaner, VWR International, Pennsylvania, USA) and centrifuged for 10 min at 39200 × g (Avanti J-26 XP Centrifuge, Beckman Coulter, California, USA). After collection of the supernatant, the insoluble plant material was re-extracted with the extraction solvent (5 mL). Supernatants were pooled, and the volume was brought up to 20 mL with 70% methanol in water. The extractions were performed at ambient temperature (20-22 °C).

Chemicals. Standards for HPLC analyses of phenolic compounds were cyanidin-3-O-galactoside (Polyphenols AS, Sandnes, Norway), catechin hydrate and quercetin-3-O-rutinoside (Sigma–Aldrich, MO, USA), and chlorogenic acid (Fluka, St. Gallen, Switzerland). The standards for analysis of sugars and organic acids were citric, malic, and shikimic acids (Sigma–Aldrich, MO, USA) and quinic acid (Merck, Darmstadt, Germany) and glucose, sucrose, and fructose (Chem Service Inc., West Chester, PA, USA). All chemicals and solvents used were of HPLC-isocratic grade or higher and water used was purified using a Milli-Q purification system (Millipore Sigma, MA, USA). For total proanthocyanidin analysis dimethylaminocinnamaldehyde (DMAC) (Sigma–Aldrich, MO, USA) and procyanidin A2 (Extrasynthese, Genay, France) were used.

Analysis of phenolic compounds. The analysis of phenolic compounds in berries has previously been described by Aaby et al., (2013) and Amundsen et al. (2023a, b). The instrumentation used consisted of an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler cooled to 4 °C, a diode array detector (DAD), and an MSD XCT ion trap mass spectrometer fitted with an electrospray ionization interface. Samples were analyzed in randomized

order. Separation of the compounds was performed by injecting 10 µL of the methanolic extracts on a Synergi 4 µm MAX RP C12 column (250 mm × 3.0 mm i.d.) equipped with a 5 µm C12 guard column (4.0 mm × 2.0 mm i.d.), both from Phenomenex (Torrance, California, USA). The mobile phase was a binary solvent system consisting of (A) formic acid/water (2/98, v/v) and (B) acetonitrile. The following gradient: 0–10 min 5-10% B, 10-22 min 10-12.4% B, 22-42 min 12.4-28% B, 42-50 min 28-60% B, 50-55 min 60% B, and 55-58 min 60–5% B, was eluted at a flow rate of 0.5 mL/min at 40 °C with a total run time of 60 min. The DAD recorded signals between 220-600 nm. The ESI-MS was operated at positive and negative modes and had a nebulizer pressure of 40 psi; dry gas flow of 10 L/min, drying temperature of 350 °C; and capillary voltage of 3.5 kV. For identification of the compounds, the retention times and UV–vis and mass spectra were compared to previous reports of phenolic compounds in lingonberries (Bujor et al., 2018; Ek et al., 2006, Hokkanen et al., 2009; Marsol-Vall et al., 2020, Amundsen et al., 2023a,b). For quantification external standard curves were used and content of anthocyanins were expressed as equivalents of cyanidin-3-O-galactoside at 520 nm, flavonol glycosides as quercetin-3-O-rutinoside at 360 nm, cinnamic acid derivatives as chlorogenic acid at 320 nm, flavan-3-ols as catechin at 280 nm and benzoic acid as benzoic acid at 280 nm and expressed as mg/100 g fresh weight (fw).

Total proanthocyanidins. The total proanthocyanidin content was quantified according to a method described by Sintara et al. (2018). The methanolic sample extracts were diluted with methanol 1/24 (v/v). The diluted sample (20 µL) was pipetted into a 96-well plate (Thermo Fisher, Massachusetts, USA), and 100 µL of 1 mg/mL dimethylaminocinnamaldehyde (DMAC) in acidic methanol (0.4 N H_2SO_4) was added. Immediately after the addition of the DMAC solution, the sample absorption was measured at 640 nm on a spectrophotometer (SpektrostarNano, BMG Labtech, Baden-Wuerttemberg, Germany), with subsequent readings every minute for 10 min. Quantification was performed based on calibration curves of an external standard of procyanidin A2 using the average of the three last readings of the samples.

Analysis of sugars and organic acids. The analysis of sugars and organic acids in lingonberries has previously been described by Amundsen et al., (2023a). The instrumentation used consisted of an Agilent 1100 series HPLC system equipped with a DAD and a refractometer index (RI) detector (Model 132; Gilson, Villiers-le-Bel, France). Separation of the compounds was performed by injecting 20 µL of the methanolic extracts on a Rezex ROA-Organic acid H+ (8 %) column (300 × 7.8 mm; Phenomenex, California, USA) at 45 °C with a mobile phase of 7.2 mmol/L H_2SO_4 and a flow rate of 0.5 mL/min. The detection of sugars was done on the RI detector, and the organic acids on the DAD detector at 210 nm. Quantification was performed based on external calibration curves of glucose, sucrose, fructose, and citric, malic, shikimic and quinic acids and expressed as g/100 g fw.

Statistical analysis. The influence of light spectrum and light intensity and time of harvest and the interaction of the treatments and time were assessed by ANOVA ($\rho < 0.05$). The correlation between

compounds measured were analyzed with a Pearson's correlation test using Minitab (Minitab LLC., State College, PA, USA).

RESULTS

In total 27 phenolic compounds, three sugars and three organic acids were detected in lingonberries (Table 1-7). The identification and contents of the major anthocyanins (Table 1), flavonols (Table 2), cinnamic acid derivatives (CADs) and a benzoic acid (Table 3), flavan-3-ols (Table 4), sugars (Table 5) and organic acids (Table 6) were similar to previous studies on lingonberries (Andersen, 1985; Ek et al., 2006; Hokkanen et al., 2009; Kelanne et al., 2019; Lee and Finn, 2012 Amundsen et al 2023ab).

Anthocyanins. The total anthocyanin content ranged from 57-95 mg/100 g fw (Table 1). The anthocyanins detected were all glycosides of cyanidin. The dominant compound was cyanidin-3-O-galactoside followed by cyanidin-3-O-arabinoside, cyanidin-3-O-glucoside, cyanidin-3-O-(acetyl)glucoside and a cyanidin-3-O-pentoside. Cyanidin-3-O-galactoside, cyanidin-3-O-arabinoside and cyanidin-3-O-pentoside responded to the light spectrum treatments, all having higher levels when grown under blue light compared to red and far-red light (Table 1 and 7). The levels of cyanidin-3-O-arabinoside and cyanidin-3-O-pentoside were also affected by the light intensity treatments with increased concentration at high light intensity. There was little interaction between the harvesting time and light treatments. While there was a clear effect of harvesting time for cyanidin-3-O-arabinoside, cyanidin-3-O-pentoside and cyanidin-3-O-glucoside all were significantly lowest at the earliest harvesting points. The harvesting time also caused alterations in the composition of cyanidins. There was a significant difference in the composition of cyanidin-3-O-arabinoside and cyanidin-3-O-galactoside at different harvest times. The proportion of cyanidin-3-O-galactoside increased from 68% at the earliest harvesting date to 81% at the later date, while levels of cyanidin-3-O-arabinoside was reduced from 22% to 14% at the same time.

Flavonols. Two kaempferol and nine quercetin glycosides were detected (Table 7), quercetin-3-O-arabinoside and quercetin-3-O-rhamnoside being the dominating (Table 2). The flavonol levels were relatively unaffected by light treatments. Quercetin-3-O-pentoside had higher levels at high light intensity (Table 7).

Phenolic acids. In total six cinnamic acid derivatives (CADs) were detected and quantified, a p-Coumaric acid hexoside and a ferulic acid hexoside being the dominating (Table 3). In addition, coumaryl iridoid, another ferulic acid hexoside, and two caffeic acid hexosides and one benzoic acid derivative were detected. CADs were not affected by the light treatment (Table 7). The level of total CADs decreased with increased time of harvest (in both light treatments). The content of benzoic acid no effect of light treatment but a strong significant effect of harvesting time. The highest levels were

detected under blue light and the latest harvesting dates. The proportion of BAS was negatively correlated to CADs (-0.840), flavonols (-0.842) and anthocyanins (-0.740) (not reported).

Flavan-3-ols. The content of four flavan-3-ols were quantified with the applied HPLC-DAD method: procyanidin b2, procyanidin a2, catechin and epicatechin (Table 4). The total concentration of flavan-3-ols and their oligomers and polymers, that is, proanthocyanidins (PAs), determined by a spectrophotometric method, varied from 244 to 765 mg/100 g fw (Table 4), and confirms previous results as the most abundant phenolic group in lingonberries (Dudonne et al., 2015; Hellström et al., 2009; Kylli et al., 2011). Regression analysis revealed that the level of procyanidin b2 was affected by the light spectrum treatments (Table 7) and the level was highest at far-red treatment (Table 4). Catechin was affected by harvesting time, being highest at the first weeks of harvest.

Sugars and organic acids. Three sugars were detected and quantified in lingonberries (Table 5). The total content of sugars in lingonberries varied from 4.7 to 7.8 g/100 g fw. Fructose, glucose, and total level of sugars were affected by the light spectrum treatments, with highest content in the berries treated with white light, and lowest in the berries ripened under far red light. Three organic acids were detected and quantified in lingonberries in concentration from 3.2 to 5.4 g/100 g fw (Table 6). The content of citric and malic acids and total organic acids were significantly affected by the spectrum treatments with lowest contents in the lingonberries ripened under red light. The content of organic acids correlated to total CADs (0.459) and total proanthocyanidins (0.639). There were clear effects of treatment time on the composition of both sugars and organic acids in lingonberries particularly in the light intensity experiment.

DISCUSSION

LIGHT SPECTRUM

Several studies have demonstrated that specific spectral light wavelengths given as supplemental irradiation during ripening can stimulate the biosynthesis of flavonoids in fruit crops, including species from the *Vaccinium* genus (Karppinen et al., 2016, Zoratti et al., 2014a, Samkumar et al., 2021b, 2022a). In the present study, the total anthocyanin level was elevated in berries ripening under blue light. This finding is consistent with previous studies that have reported a positive effect of blue light on anthocyanin levels in different fruit and berry species, such as bilberry (Samkumar et al., 2021a), strawberry (*Fragaria* x *ananassa*) (Kadomura-Ishikawa et al., 2013), sweet cherries (*Prunus avium*) (Kokalj et al., 2019), grape (*Vitis vinifera*) (Zhang, 2021) and Asian pear (*Pyrus pyrifolia*) (Tao et al., 2018). In contrast to our result on lingonberries, other studies have also found effects of red light on increasing anthocyanin content (Zhang et al., 2018, Zhang et al., 2021). In cranberries, both red and far-red light have been shown to increase anthocyanin concentrations (Zhou and Singh 2004), while red light irradiation had a negative or no effect on anthocyanin accumulation in strawberries (Kadomura-Ishikawa et al. 2013). Cyanidin glycosides are the only group of anthocyanins detected in lingonberries.

Previous studies on monochromatic light treatments in bilberries found a more significant impact of light spectrum treatment on the accumulation of total anthocyanins compared to our study (Samkumar et al. 2021b; Zoratti et al. 2014b). However, in bilberries, the impact was more prominent on delphinidins, which are not present in lingonberries, while cyanidins were not significantly affected (Zoratti et al. 2014b). Samkumar et al. (2021) also investigated the effects of supplemental red and blue light on anthocyanins in bilberries and found that delphinidins showed the largest enhancement under both red and blue light, while cyanidin levels exhibited smaller alterations. The same study detected smaller alteration also in the cyanidin levels. These results suggest that treatments with specific light spectra can influence the levels of anthocyanins in lingonberries; however, the response appears to be less pronounced compared to closely related *Vaccinium* berries with a different anthocyanin profile.

In the present study the level of procyanidin b2 was elevated under far-red light. Zhang et al. (2018) investigated the effect of different light spectra on strawberries and found that proanthocyanidins responded to red-blue light treatments. Among the flavonols, quercetin-(4"-HMG)-pentoside and quercetin-3-O-pentoside were the only compounds affected by the light spectrum treatments in lingonberries. In bilberries, both increases and decreases in the content of individual flavonols and flavan-3-ols were observed with supplemental light treatments (Zoratti et al., 2014), while no significant differences were observed in strawberries (Nadalini et al., 2017). These results suggest that, although flavonols have previously been shown to act as photo protectors and UV-protectants, their response to blue, red, and far-red light treatments in lingonberries is limited. Previous findings have indicated that the effects of UV treatments are stronger than those of visible light on flavonol compounds (Bian et al., 2014).

In the present study of lingonberries, lowest content of sugars was found in lingonberries ripened under far-red light. There was a minor effect observed on the content of organic acids. In previous studies of bilberries (Samkumar et al., 2022b), strawberries (Nadalini et al., 2017), and grapes (Zhang et al., 2021), where sugar content was higher and organic acid content was lower in berries grown with red and blue light supplementation. Similar effects of red and blue light supplementation on sugar levels have also been shown in several vegetables (Bian et al., 2014). In bilberries, it was observed that the genes associated with the metabolism and transport of sugars from leaves, which are the photosynthetic source tissues, to the berries, were upregulated by supplemental light, particularly red-light treatment (Samkumar et al., 2022b). Light differences related to latitude have previously been associated with changes in the sugar content of lingonberries and bilberries (Vilkickyte et al., 2019). In locations further north, the ratio of red to far-red light is reduced (Mølmann et al., 2021). Therefore, the results from our study suggest that sugar synthesis at higher latitudes could be reduced due to a higher proportion of far-red radiation.

LIGHT INTENSITY

The light intensity is directly affecting the photosynthesis as well as levels of compounds influenced by abiotic stress. Low light intensities are closely connected to high forest density. However, lingonberry is often found growing in shaded forest habitats and therefore high light intensities are not required for the induction of anthocyanin biosynthesis (Karppinen et al., 2016). Previous studies have shown that low light intensity in forests can be associated with lower levels of phenolic compounds in bilberry leaves (Martz et al., 2010).

In the present study, the lingonberries ripening under the two light intensity conditions reflecting open and covered growth locations, appear to have less effects on flavonoid biosynthesis compared to other studies. However, increased light intensity did lead to higher levels of anthocyanins, catechins, and procyanidin b2. For the other phenolic compounds, there were minor effects of light intensity. In bilberry leaves, the content of both anthocyanins, catechins, flavonols and hydroxycinnamic acids responded stronger to high light intensity conditions compared to our results (Jaakola et al., 2004). Similar trends have been reported in bilberries grown in locations with higher photosynthetic active radiation, where higher levels of anthocyanins, flavonols, hydroxycinnamic acids, and total phenolics have been detected (Jovancevic et al., 2011; Mikulic-Petkovsek et al., 2015). Studies on grape berries have also demonstrated the impact of light intensity and shading. Increased light intensity through reflected film was found to increase the levels of quercetin and catechin in grapes (Jamshidian et al., 2010), while low light conditions resulted in decreased flavonol synthesis (Downey et al., 2004). In raspberries, both light intensity and duration of light treatment increased the levels of total anthocyanins and total phenolics (Wang et al., 2009). Shading had a moderate effect on total anthocyanins in strawberries, with higher levels observed under high light exposure (Anttonen et al., 2006). However, it has been shown that the requirement for higher light exposure varies markedly between *Vaccinium* spp. (Zoratti et al., 2015b). In the present study, no influence of light intensity on the accumulation of sugars or organic acids was found in lingonberries.

This contrasts with previous results on lingonberries where higher sugar content has been found in medium to low density forests (Vilkickyte et al., 2019). Similar result has also been seen in bilberries and grapes (Mikulic-Petkovsek et al., 2015a; Teixeira et al., 2015). Generally, it has been observed that fruits and berries tend to accumulate higher sugar content and lower organic acid content under higher irradiation rates (Zheng et al., 2018). However, in raspberries grown under controlled conditions, no effect of light intensity was observed on the content and composition of sugars and organic acids (Wang et al., 2009). Similar results were found in studies on bilberries, black currants, and white currants, where elevated radiation did not affect sugar levels (Zheng et al., 2018). As many *Vaccinium* species are shade adapted, the low effect of irradiance on the sugar and organic acid content in lingonberries, could be due to a lower photosynthetic saturation point, to limit the influence of forest densities on the

plants. Though the saturation point of photosynthesis is rarely reached in whole plants (Zheng et al., 2018).

TREATMENT TIME

The results of the study indicate that treatment time, independent of light conditions, had the greatest impact on the concentration and composition of polyphenols, sugars, and organic acids in lingonberries. Phenolic acids decreased over time, while anthocyanins increased. The influence of time from the first to latest harvest of ripe berries on the composition of lingonberries has been previously demonstrated (Vilkickytė and Raudonne, 2021; Amundsen et al., 2023b). It has been observed that the content of flavan-3-ols and condensed tannins is highest in the early stages of development and decreases throughout the season in various fruits and berries (Karppinen et al., 2016). In lingonberries, anthocyanins are primarily located in the fruit skin, and their biosynthesis is influenced by environmental factors such as light (Zoratti et al., 2014b). The effect of harvest time on flavonoid composition has been observed in other berry species as well (Anttonen et al., 2006; Zorenc et al., 2016; Wang et al., 2017; King et al., 2021). In cranberries, both the concentration and the composition of flavonols were not significantly affected during fruit development (Wang et al., 2017), while the harvest time caused a 1.5-2.0-fold difference in the content of phenolics in strawberries (Anttonen et al., 2006). Similarly, in *V. corymbosum*, berries harvested later in the season generally contained more total anthocyanins and sugars, and less flavonols, hydroxycinnamic acids, and organic acids (Zorenc et al., 2016). In *Aronia mitschurinii* the levels of anthocyanins and ◦brix concentration also increased during the harvesting season (King et al., 2021). In raspberries the content of anthocyanins and sugars increased and the content of flavonols and organic acids decreased comparing earlier and latter harvesting times (Wang et al., 2009). The interaction between the light treatments and treatment time showed that there was some influence of the duration of this treatment and the total content of anthocyanins, though this was not observed for the compounds individually. For all other compounds, however, no significant interactions were observed.

CONCLUSIONS

The study demonstrated that the levels of quality-related compounds in lingonberries were influenced by both light spectral treatments, light intensity treatments, and by the time of ripening. Cyanidin glycosides, the primary anthocyanins in lingonberries, were particularly sensitive to the light treatments, with elevated levels observed under blue light and high light intensity conditions. However, the duration of the specific light treatments had minimal effect on the levels of these compounds. On the other hand, sugar and acid levels, as well as cinnamic

acid derivatives (CADs) and anthocyanins, responded to the time of ripening. Later stages of harvest season generally led to an accumulation of sugars and anthocyanins, while the levels of CADs decreased. These findings suggest that the time of harvest can significantly impact the quality of lingonberries. The results of the study suggest that differences in the quality of lingonberries between different growth locations may be attributed to variations in light conditions as well as the timing of harvest.

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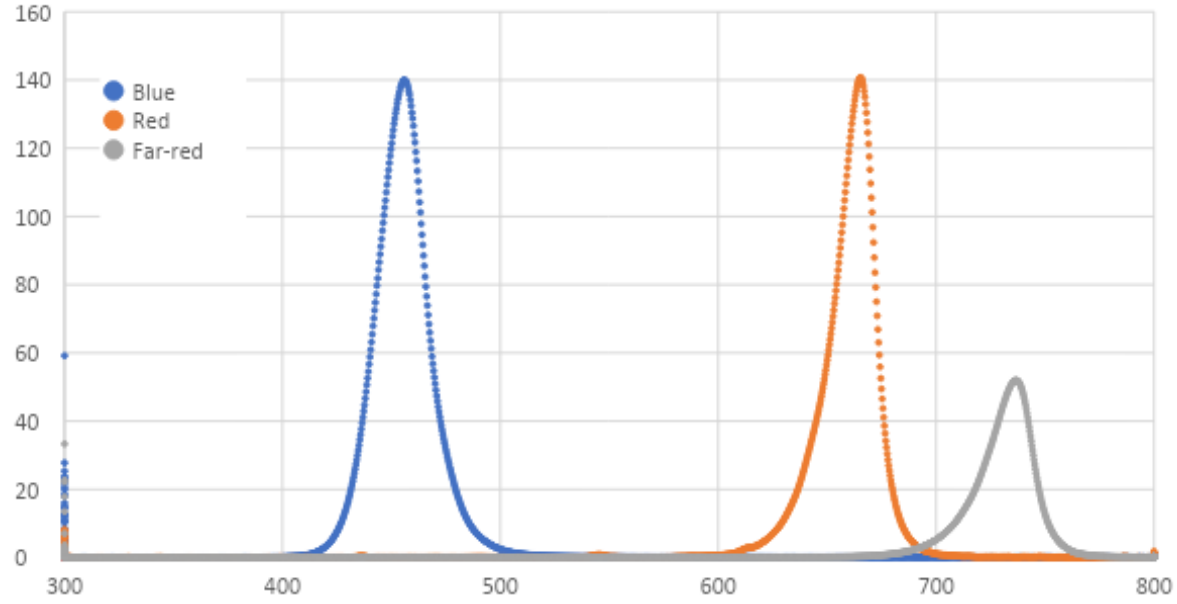

Figure 1 Spectra and intensities of the supplemental lights used in the experiments.

Table 1 Concentrations (mg/100 g) of five anthocyanins found in lingonberries ripened under four different light spectrum light treatments (red, far red, blue, and white) and two different light intensities (high and low) after four harvesting dates (weeks). The values represent means ± SE of three biological replicates.

Light	Time (weeks)	Cyanidin-3-O-galactoside	Cyanidin-3-O-arabinoside	Cyanidin-3-O-glucoside	Cyanidin-3-O-pentoside	Cyanidin-3-O-(acetyl) glucoside	Total anthocyanins
White	1	53.4 ± 5.2	9.5 ± 0.9	2.03 ± 0.09	0.63 ± 0.1	0.10 ± 0.10	65.6 ± 5.7
High	2	53.0 ± 8.1	16.7 ± 1.3	4.80 ± 0.56	0.93 ± 0.1	1.27 ± 0.41	76.7 ± 7.4
	3	56.4 ± 3.0	15.4 ± 0.6	4.13 ± 0.62	0.90 ± 0.0	0.33 ± 0.17	77.2 ± 3.0
	4	68.9 ± 3.0	19.3 ± 0.9	5.17 ± 1.07	1.13 ± 0.0	0.10 ± 0.06	94,6 ± 3.0
White	1	44.6 ± 4.6	10.0 ± 1.0	2.63 ± 0.18	0.60 ± 0.1	0.60 ± 0.32	58.4 ± 4.8
Low	2	45.8 ± 5.1	12.0 ± 0.3	3.30 ± 0.15	0.70 ± 0.0	0.20 ± 0.15	61.9 ± 5.1
	3	35.8 ± 2.6	12.1 ± 0.9	4.27 ± 0.41	0.73 ± 0.0	1.10 ± 0.27	54.0 ± 3.6
	4	54.4 ± 6.9	16.6 ± 0.5	5.03 ± 0.58	0.93 ± 0.1	0.60 ± 0.55	77.5 ± 5.7
Blue	1	46.6 ± 7.9	9.6 ± 2.0	2.37 ± 0.19	0.70 ± 0.12	0.63 ± 0.07	59.9 ± 9.8
	2	51.8 ± 3.6	13.5 ± 0.4	3.73 ± 0.50	0.90 ± 0.06	0.53 ± 0.09	70.4 ± 2.8
	3	59.7 ± 6.5	20.3 ± 3.0	5.00 ± 1.40	1.20 ± 0.17	0.77 ± 0.38	86.9 ± 8.7
	4	58.0 ± 4.5	17.0 ± 3.3	4.77 ± 1.39	0.97 ± 0.18	0.57 ± 0.29	81.3 ± 9.5
Red	1	45.2 ± 1.5	10.1 ± 0.4	2.70 ± 0.12	0.70 ± 0.00	1.17 ± 0.13	59.9 ± 1.0
	2	48.0 ± 2.4	14.1 ± 1.6	3.83 ± 0.44	0.80 ± 0.10	0.77 ± 0.38	67.5 ± 4.9
	3	42.2 ± 5.5	11.2 ± 0.8	3.33 ± 0.19	0.70 ± 0.06	0.33 ± 0.07	57.8 ± 6.4
	4	52.4 ± 5.0	12.3 ± 1.0	4.17 ± 0.64	0.83 ± 0.03	0.00 ± 0.00	69.6 ± 5.3
Far red	1	43.4 ± 4.1	9.5 ± 1.0	2.33 ± 0.12	0.67 ± 0.09	0.83 ± 0.15	56.7 ± 5.2
	2	45.8 ± 1.2	15.6 ± 1.3	4.33 ± 0.48	0.97 ± 0.09	1.10 ± 0.31	67.8 ± 0.9
	3	49.8 ± 1.2	14.5 ± 2.4	4.30 ± 0.59	0.87 ± 0.12	0.60 ± 0.21	70.1 ± 2.6
	4	50.0 ± 3.7	15.2 ± 1.3	4.87 ± 0.30	0.93 ± 0.07	0.13 ± 0.07	71.1 ± 5.3

Table 2 Concentrations (mg/100 g) of the five dominating flavonols found in lingonberries ripened under four different light spectrum light treatments (red, far red, blue, and white) and two different white light intensities (high and low) after four harvesting dates (weeks). The values represent means ± SE of three biological replicates.

Light	Time (weeks)	Quercetin -3-O-rhamnoside	Quercetin -3-O-arabinoside	Quercetin -3-O-galactoside	Quercetin -3-O-xyloside	Quercetin -3-O- (3-HMG) - rhamnoside	Total flavonols
White	1	11.97 ± 0.44	9.70 ± 0.95	7.97 ± 1.07	1.23 ± 0.03	3.83 ± 0.29	35.90 ± 2.50
High	2	9.13 ± 2.21	7.53 ± 1.30	6.40 ± 1.21	1.07 ± 0.18	4.77 ± 0.23	32.20 ± 5.10
	3	11.13 ± 1.98	7.53 ± 0.84	6.17 ± 1.12	1.03 ± 0.13	4.03 ± 0.24	33.00 ± 4.20
	4	9.40 ± 2.44	6.67 ± 1.66	4.73 ± 1.19	1.00 ± 0.21	3.47 ± 0.48	27.80 ± 6.40
White	1	10.53 ± 1.98	8.57 ± 0.96	6.77 ± 0.91	1.20 ± 0.15	4.97 ± 0.29	35.50 ± 4.00
Low	2	7.43 ± 1.39	6.50 ± 0.79	4.80 ± 0.66	0.87 ± 0.09	3.47 ± 0.29	25.60 ± 3.30
	3	5.00 ± 2.52	6.27 ± 0.44	4.93 ± 0.52	0.87 ± 0.09	5.97 ± 0.48	28.20 ± 1.90
	4	7.73 ± 1.58	6.13 ± 0.69	4.37 ± 0.32	0.87 ± 0.09	4.47 ± 0.15	26.40 ± 2.80
Blue	1	11.10 ± 2.21	7.83 ± 1.31	6.50 ± 1.21	1.13 ± 0.19	5.63 ± 0.50	35.40 ± 5.90
	2	6.60 ± 1.61	7.03 ± 0.62	4.87 ± 0.71	0.90 ± 0.12	4.23 ± 0.54	26.30 ± 3.50
	3	7.27 ± 1.20	6.43 ± 1.03	4.03 ± 0.67	0.83 ± 0.12	4.47 ± 0.22	25.80 ± 3.10
	4	7.00 ± 1.30	5.60 ± 0.80	3.73 ± 0.83	0.73 ± 0.13	3.37 ± 0.23	22.70 ± 3.10
Red	1	7.10 ± 0.93	7.27 ± 1.42	5.17 ± 1.47	0.97 ± 0.22	5.43 ± 0.58	28.80 ± 5.20
	2	6.77 ± 1.38	5.67 ± 0.79	4.20 ± 0.67	0.73 ± 0.07	4.60 ± 0.78	25.10 ± 2.90
	3	5.30 ± 0.53	4.43 ± 0.41	3.27 ± 0.35	0.60 ± 0.06	4.03 ± 0.22	19.50 ± 1.70
	4	5.10 ± 0.67	4.37 ± 0.44	2.67 ± 0.37	0.60 ± 0.06	3.13 ± 0.61	21.40 ± 4.20
Far red	1	10.53 ± 1.59	9.03 ± 1.16	6.50 ± 1.19	1.13 ± 0.15	5.33 ± 0.84	35.90 ± 5.30
	2	7.20 ± 0.30	7.00 ± 0.60	4.87 ± 0.45	0.93 ± 0.09	4.97 ± 0.73	27.60 ± 2.20
	3	7.83 ± 1.17	6.40 ± 0.21	4.57 ± 0.48	0.83 ± 0.03	4.40 ± 0.29	26.60 ± 2.20
	4	7.20 ± 1.18	5.53 ± 0.26	3.77 ± 0.29	0.77 ± 0.07	4.23 ± 0.74	23.70 ± 2.70

Table 3. Concentrations (mg/100 g) of eight cinnamic acid derivatives (CADs) and benzoic acid *detected in lingonberries ripened under four different light spectrum light treatments (red, far red, blue, and white) and two different white light intensities (high and low) after four harvesting dates (weeks). The values represent means ± SE of three biological replicates.*

Light	Time (weeks)	Ferulic acid -hexoside 1	Ferulic acid -hexoside 2	Coumaroyl iridioid	p-Coumaric acid - hexoside	Caffeic acid - hexoside 1	Caffeic acid - hexoside 2	Total CADs	Benzoic acid
White	1	3.10 ± 0.87	0.20 ± 0.10	0.43 ± 0.09	3.87 ± 0.61	0.43 ± 0.13	0.57 ± 0.07	15.80 ± 1.40	14.70 ± 4.10
High	2	2.60 ± 0.25	0.17 ± 0.03	0.33 ± 0.12	4.70 ± 0.38	0.40 ± 0.06	0.57 ± 0.09	9.20 ± 1.70	40.30 ± 9.30
	2	2.00 ± 0.20	0.10 ± 0.00	0.43 ± 0.09	5.20 ± 0.68	0.43 ± 0.03	0.47 ± 0.07	9.30 ± 0.40	58.10 ± 7.50
	4	5.40 ± 0.44	0.47 ± 0.17	0.57 ± 0.09	6.33 ± 1.03	0.77 ± 0.03	0.83 ± 0.09	9.10 ± 1.00	94.60 ± 15.80
White	1	2.87 ± 0.41	0.10 ± 0.00	0.50 ± 0.12	5.33 ± 0.48	0.53 ± 0.03	0.53 ± 0.03	13.20 ± 1.90	22.80 ± 2.80
Low	2	2.43 ± 0.22	0.33 ± 0.12	0.60 ± 0.21	3.00 ± 0.17	0.37 ± 0.03	0.63 ± 0.07	10.40 ± 0.80	21.70 ± 0.90
	2	2.70 ± 1.01	0.13 ± 0.03	0.33 ± 0.03	3.67 ± 0.45	0.47 ± 0.07	0.60 ± 0.10	8.00 ± 0.30	62.10 ± 6.60
	4	4.57 ± 0.71	0.20 ± 0.06	0.50 ± 0.15	5.27 ± 1.08	0.67 ± 0.15	1.07 ± 0.09	8.40 ± 1.50	88.40 ± 22.60
Blue	1	4.40 ± 0.62	0.60 ± 0.15	0.70 ± 0.21	4.33 ± 0.90	0.63 ± 0.09	1.17 ± 0.27	12.80 ± 1.70	15.70 ± 2.20
	2	4.33 ± 0.82	0.17 ± 0.03	0.53 ± 0.15	5.30 ± 0.76	0.50 ± 0.06	1.00 ± 0.15	12.50 ± 1.50	28.20 ± 1.90
	2	2.23 ± 0.22	0.23 ± 0.09	0.77 ± 0.15	3.73 ± 0.88	0.47 ± 0.09	0.50 ± 0.06	9.30 ± 1.10	70.10 ± 12.10
	4	1.83 ± 0.38	0.10 ± 0.00	0.40 ± 0.12	3.57 ± 0.73	0.47 ± 0.09	0.37 ± 0.07	7.00 ± 1.10	129.80 ± 25.40
Red	1	3.23 ± 0.18	0.80 ± 0.40	1.23 ± 0.12	2.87 ± 0.34	0.30 ± 0.10	1.23 ± 0.03	10.60 ± 0.10	17.90 ± 2.20
	2	2.97 ± 0.32	0.30 ± 0.10	0.73 ± 0.22	3.20 ± 0.49	0.47 ± 0.03	0.77 ± 0.18	9.70 ± 0.60	29.10 ± 8.60
	2	2.80 ± 0.47	0.37 ± 0.12	0.50 ± 0.00	2.80 ± 0.15	0.37 ± 0.03	0.80 ± 0.35	8.20 ± 0.70	33.80 ± 7.40
	4	3.70 ± 0.65	0.20 ± 0.06	0.27 ± 0.03	4.03 ± 0.41	0.43 ± 0.03	0.57 ± 0.17	8.40 ± 0.40	68.60 ± 11.50
Far red	1	5.17 ± 1.17	0.50 ± 0.06	1.57 ± 0.45	4.77 ± 0.75	0.63 ± 0.09	1.57 ± 0.15	15.30 ± 2.00	10.40 ± 1.50
	2	3.27 ± 0.26	0.33 ± 0.03	0.80 ± 0.12	2.80 ± 0.25	0.53 ± 0.07	0.67 ± 0.09	9.00 ± 0.70	35.10 ± 4.40
	2	2.43 ± 0.18	0.17 ± 0.07	0.60 ± 0.15	4.03 ± 0.52	0.47 ± 0.03	0.50 ± 0.06	8.70 ± 0.60	53.40 ± 6.90
	4	2.80 ± 0.06	0.20 ± 0.06	0.53 ± 0.03	3.43 ± 0.33	0.40 ± 0.06	0.50 ± 0.06	8.40 ± 0.40	75.80 ± 7.60

Table 4 Concentrations (mg/100 g) of four flavan-3-ols and total proanthocyanidins[a] found in lingonberries ripened under four different light spectrum light treatments (red, far red, blue, and white) and two different white light intensities (high and low) after four harvesting dates (weeks). The values represent means ± SE of three biological replicates.

Light	Time (weeks)	Procyanidin b2	Procyanidin a2	Catechin	Epicatechin	Total proanthocyanidins [a]
White High	1	21.6 ± 0.4	13.0 ± 3.7	50.6 ± 5.9	2.6 ± 0.92	735 ± 61
	2	23.9 ± 1.2	13.2 ± 3.2	36.7 ± 5.8	2.9 ± 0.82	653 ± 78
	3	18.8 ± 1.3	15.0 ± 1.6	34.8 ± 3.6	3.9 ± 0.71	408 ± 21
	4	16.8 ± 0.8	21.0 ± 2.1	44.4 ± 3.6	3.7 ± 0.15	352 ± 99
White Low	1	21.0 ± 1.0	16.8 ± 2.1	42.5 ±[a] 2.2	4.2 ± 0.18	746 ± 119
	2	18.1 ± 1.0	16.7 ± 3.3	38.3 ± 3.6	4.5 ± 0.93	616 ± 51
	3	15.9 ± 1.3	11.0 ± 0.3	30.3 ± 0.2	7.5 ± 2.61	378 ± 54
	4	18.6 ± 1.7	15.1 ± 3.5	35.3 ± 5.4	6.5 ± 3.63	336 ± 98
Blue	1	18.6 ± 1.8	19.7 ± 0.9	50.9 ± 3.5	5.4 ± 0.21	766 ± 120
	2	17.3 ± 0.7	13.3 ± 3.3	34.5 ± 4.7	10.6 ± 3.23	614 ± 107
	3	15.2 ± 1.0	18.1 ± 3.6	40.6 ± 5.2	2.4 ± 0.96	639 ± 52
	4	19.0 ± 0.7	18.9 ± 1.3	39.5 ± 1.1	3.9 ± 0.32	319 ± 53
Red	1	17.1 ± 0.4	14.0 ± 1.8	42.4 ± 3.0	6.0 ± 0.93	428 ± 139
	2	18.3 ± 5.1	15.0 ± 2.9	38.1 ± 4.0	4.8 ± 0.80	472 ± 132
	3	15.3 ± 3.3	10.8 ± 4.0	29.4 ± 5.3	10.6 ± 3.72	244 ± 64
	4	17.8 ± 1.3	14.6 ± 4.2	29.8 ± 6.2	13.6 ± 4.38	446 ± 73
Far red	1	21.5 ± 2.0	19.3 ± 3.0	53.4 ± 6.3	6.8 ± 1.39	681 ± 209
	2	21.0 ± 3.1	14.5 ± 0.5	39.8 ± 0.9	4.4 ± 0.18	461 ± 70
	3	24.0 ± 2.6	18.5 ± 2.6	39.0 ± 4.2	5.2 ± 1.39	388 ± 92
	4	24.7 ± 1.4	14.5 ± 0.8	32.4 ± 1.7	10.4 ± 0.41	367 ± 32

[a] Determined with the DMAC method

Table 5 Concentrations (g/100 g) of sugars in lingonberries found in lingonberries ripened under four different light spectrum light treatments (red, far red, blue, and white) and two different white light intensities (high and low) after four harvesting dates (weeks). The values represent means ± SE of three biological replicates.

Light	time (weeks)	Sucrose	Glucose	Fructose	Total sugars
White	1	0.53 ± 0.03	3.09 ± 0.09	2.21 ± 0.14	5.84 ± 0.25
High	2	0.60 ± 0.01	3.51 ± 0.15	2.50 ± 0.05	6.61 ± 0.14
	3	0.68 ± 0.07	3.82 ± 0.21	2.86 ± 0.23	7.37 ± 0.51
	4	0.60 ± 0.12	4.03 ± 0.39	2.76 ± 0.30	7.40 ± 0.80
White	1	0.54 ± 0.11	3.20 ± 0.25	2.25 ± 0.34	5.99 ± 0.70
Low	2	0.57 ± 0.11	3.61 ± 0.43	2.43 ± 0.42	6.61 ± 0.96
	3	0.55 ± 0.01	4.22 ± 0.21	2.97 ± 0.36	7.75 ± 0.58
	4	0.47 ± 0.12	4.10 ± 0.37	2.84 ± 0.43	7.43 ± 0.91
Blue	1	0.54 ± 0.09	2.78 ± 0.18	2.03 ± 0.26	5.36 ± 0.51
	2	0.68 ± 0.13	3.48 ± 0.33	2.62 ± 0.25	6.79 ± 0.71
	3	0.47 ± 0.05	3.06 ± 0.09	2.04 ± 0.26	5.57 ± 0.40
	4	0.40 ± 0.04	3.82 ± 0.02	2.78 ± 0.05	7.02 ± 0.02
Red	1	0.52 ± 0.02	2.5 ± 0.29	2.04 ± 0.24	5.07 ± 0.55
	2	0.64 ± 0.09	3.37 ± 0.33	2.75 ± 0.18	6.77 ± 0.61
	3	0.38 ± 0.03	2.94 ± 0.13	2.20 ± 0.24	5.52 ± 0.40
	4	0.46 ± 0.11	4.15 ± 0.40	2.72 ± 0.26	7.33 ± 0.77
Far red	1	0.48 ± 0.06	2.44 ± 0.12	1.78 ± 0.11	4.71 ± 0.27
	2	0.45 ± 0.00	2.83 ± 0.12	2.08 ± 0.06	5.36 ± 0.18
	3	0.37 ± 0.03	2.63 ± 0.17	1.71 ± 0.12	4.72 ± 0.32
	4	0.40 ± 0.06	3.42 ± 0.43	2.15 ± 0.31	5.98 ± 0.81

Table 6 Concentration (g/100 g) of organic acids found in lingonberries ripened under four different light spectrum light treatments (red, far red, blue, and white) and two different white light intensities (high and low) after four harvesting dates (weeks). The values represent means ± SE of three biological replicates.

Light	Time	Citic acid	Quinic acid	Malic acid	Total acids
White	1	2.46 ± 0.08	2.38 ± 0.10	0.17 ± 0.05	5.03 ± 0.23
High	2	2.34 ± 0.03	2.67 ± 0.56	0.37 ± 0.11	5.39 ± 0.43
	3	2.25 ± 0.05	1.68 ± 0.20	0.32 ± 0.01	4.26 ± 0.24
	4	1.85 ± 0.04	1.80 ± 0.28	0.67 ± 0.02	4.34 ± 0.25
White	1	2.54 ± 0.13	2.33 ± 0.48	0.17 ± 0.04	5.06 ± 0.40
Low	2	2.50 ± 0.13	1.77 ± 0.29	0.54 ± 0.05	4.83 ± 0.35
	3	2.17 ± 0.17	1.31 ± 0.04	0.55 ± 0.08	4.04 ± 0.30
	4	2.05 ± 0.14	1.42 ± 0.23	0.42 ± 0.05	3.91 ± 0.06
Blue	1	2.12 ± 0.08	2.45 ± 0.58	0.10 ± 0.03	4.69 ± 0.64
	2	2.17 ± 0.20	1.74 ± 0.20	0.15 ± 0.02	4.08 ± 0.11
	3	2.15 ± 0.08	1.62 ± 0.25	0.27 ± 0.02	4.07 ± 0.20
	4	1.74 ± 0.03	1.51 ± 0.32	0.34 ± 0.10	3.61 ± 0.31
Far red	1	2.36 ± 0.16	2.31 ± 0.47	0.09 ± 0.01	4.77 ± 0.33
	2	2.17 ± 0.08	1.58 ± 0.03	0.21 ± 0.05	3.98 ± 0.00
	3	1.97 ± 0.08	1.84 ± 0.36	0.25 ± 0.01	4.08 ± 0.43
	4	2.10 ± 0.22	1.62 ± 0.08	0.33 ± 0.00	4.07 ± 0.30
Red	1	1.86 ± 0.24	1.60 ± 0.17	0.03 ± 0.02	3.51 ± 0.39
	2	2.12 ± 0.12	1.36 ± 0.03	0.06 ± 0.02	3.57 ± 0.11
	3	1.74 ± 0.08	1.28 ± 0.17	0.12 ± 0.03	3.16 ± 0.28
	4	1.90 ± 0.05	2.12 ± 0.08	0.29 ± 0.06	4.33 ± 0.18

Table 7. Significance levels[a] of ANOVA accounted by light spectrum (LS), light intensity (LI), harvest time (T) and light × harvest time on content of phenolic compounds, sugars, and organic acids in lingonberries. The plants were grown at five different light treatments (red, blue, far-red, white high and low light intensity LED) and four different harvesting times.

	Light spectrum (LS)	Time (T)	LS x T	Light intensity (LI)	Time (T)	LI x T
Cyanidin-3-O-galactoside	*	ns	ns	ns	ns	ns
Cyanidin-3-O-arabinoside	***	***	ns	*	***	ns
Cyanidin-3-O-pentoside	**	*	ns	*	***	
Cyanidin-3-O-glucoside	ns	*	ns	ns	***	ns
Cyanidin-3-O-(acetyl)glucoside	ns	ns	ns	ns	ns	ns
Total anthocyanins	***	**	ns	**	**	ns
Kaempferol-3-O-(3-HMG)-rhamnoside	ns	ns	ns	ns	ns	ns
Kaempferol-3-O-rhamnoside	ns	ns	ns	ns	ns	ns
Quercetin-3-O-(3-HMG)-rhamnoside	ns	*	ns	ns	ns	ns
Quercetin-(4»-HMG)-pentoside	ns	ns	ns	ns	ns	ns
Quercetin-3-O-rhamnoside	ns	ns	ns	ns	ns	ns
Quercetin-3-O-pentoside	ns	ns	ns	*	ns	ns
Quercetin-3-O-arabinofuranoside	ns	ns	ns	ns	ns	ns
Quercetin-3-O-arabinoside	ns	ns	ns	ns	*	ns
Quercetin-3-O-xyloside	ns	ns	ns	ns	ns	ns
Quercetin-3-O-glucoside	ns	ns	ns	ns	ns	ns
Quercetin-3-O-galactoside	ns	ns	ns	ns	ns	ns
Total flavonols	ns	ns	ns	ns	ns	ns
Coumaryl iridioid	ns	ns	ns	ns	ns	ns
Ferulic acid hexoside 1	ns	**	ns	ns	***	ns
Ferulic acid hexoside 2	ns	*	ns	ns	*	ns
p-Coumaric acid – hexoside	ns	ns	ns	ns	*	ns
Caffeic acid hexoside 1	ns	***	ns	ns	*	ns
Caffeic acid hexoside 2	ns	ns	ns	ns	*	ns
Total cinnamic acid derivatives (CADs)	ns	**	ns	ns	***	ns
Benzoic acid	ns	***	ns	ns	***	ns
Procyanidin b2	*	ns	ns	ns	ns	ns
Procyanidin a2	ns	ns	ns	ns	ns	ns
Catechin	ns	*	ns	ns	*	ns
Epicatechin	ns	ns	ns	ns	ns	ns
Total proanthocyanidins	ns	*	ns	ns	ns	ns
Sucrose	ns	ns	ns	ns	*	ns
Fructose	*	ns	ns	ns	*	ns
Glucose	*	***	ns	ns	**	Ns
Total sugars	*	*	ns	ns	*	ns
Citric acid	ns	*	ns	ns	***	ns
Quinic acid	ns	ns	ns	ns	*	ns
Malic acid	**	***	ns	ns	***	ns
Total organic acids	*	ns	*	ns	**	ns

[a] *** P < 0.001; ** P < 0.01; *P < 0.05;

PAPER III

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Effect of ripening temperature on the chemical composition of lingonberries (*Vaccinium vitis-idaea L.*) of northern and southern origin

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ABSTRACT

Lingonberries (*Vaccinium vitis-idaea* L.) from two locations, northern (69°N, 18°E) and southern (59°N, 10°E) Norway, were grown under controlled conditions in a phytotron at two temperatures (9 and 15 °C) to study the effects of the ripening temperature and origin on the chemical composition of the berries. The concentrations of phenolic compounds, sugars, and organic acids as well as the profile of volatile organic compounds (VOCs) were determined using chromatographic and mass spectrometric methods. Five anthocyanins, eleven flavonols, eight cinnamic acid derivatives, three flavan-3-ols, three sugars, three organic acids, and 77 VOCs were identified, of which 40 VOCs had not previously been reported in lingonberries. Berries from both locations, were found to have higher contents of anthocyanins and cinnamic acid derivatives when ripened at lower temperature (9 °C), compared to the higher temperature (15 °C). Lingonberries of northern origin had a different VOC profile and higher contents of anthocyanins and organic acids than berries originating from the south. Lingonberries from the northern location also had higher proportions of cyanidin-3-*O*-glucoside and cyanidin-3-*O*-arabinoside than lingonberries from the southern location. The results show that the composition of lingonberries is influenced by both the environment and the origin of the plants, with phenolic compounds mainly influenced by the growth temperature and VOCs mainly influenced by plant origin.

1. Introduction

Lingonberry (*Vaccinium vitis-idaea* L.) is an evergreen dwarf shrub native to the northern hemisphere that bears bright scarlet red berries in autumn. It has been estimated that between 130 and 390 million kilograms of edible lingonberries are ripening in Finnish forests alone, of which only around 7 % is harvested annually (Salo, 2015). Lingonberries are a traditional part of the Nordic diet, primarily consumed as jam, juices and in desserts. Lingonberries are also an important source of income for rural communities and a commodity for export (Hjalmarsson and Ortiz, 2001; Salo, 2015). Berries from the *Vaccinium* genus, have increased in popularity over the last years, attributed to their high content of a variety of bioactive compounds, dietary fibres, and micronutrients (Kowalska, 2021), making them a valuable part of a healthy diet.

Lingonberry is generally considered as a stress-tolerant species, which can grow in areas with varying temperatures, although it prefers areas with relatively low temperatures (Hjalmarsson and Ortiz, 2001). The growth environment influences the biosynthesis of specialized metabolites in plants and affects the adaptation of plants to different growth locations by changing the accumulation of metabolites (Kissen et al., 2016; Zoratti et al., 2014). Phenolic compounds are the most widely studied secondary metabolites in lingonberries (Andersen, 1985; Bujor et al., 2018; Ek et al., 2006; Viljanen et al., 2014). Lingonberries are among the *Vaccinium* species with the highest number of volatile organic compounds (VOCs) (Sater et al., 2020). VOCs are important for

Abbreviations: Dw, Dry weight; DAD, Diode array detector; Fw, fresh weight; GC, Gas chromatography; HPLC, High-performance liquid chromatography; HS-SPME, Head space solid phase micro extraction; MS, Mass spectrometry; PA, Proanthocyanidin; PCA, Principal component analysis; RI, Refraction index; VOC, Volatile Organic compounds; DVB/CAR/PDMS, Divinylbenzene-carboxen-polydimethysiloxane.

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the aroma and flavour of foods. However, only a few studies have assessed the contents of these compounds in lingonberries (Sater et al., 2020). It is challenging to understand the drivers of the quality traits of wild species such as lingonberries. The contents of both primary and secondary plant metabolites associated with quality are influenced by factors such as the ripening stage, genotype, and preharvest biotic and abiotic conditions (Karppinen et al., 2016; Farneti et al., 2017). The contents of phenolic compounds have been shown to vary markedly from northern to southern latitudes in other wild *Vaccinium* species (Lätti et al. 2008, Lätti et al. 2010, Åkerström et al. 2010). Earlier field studies have indicated association between temperature and the accumulation of different groups of phenolic compounds and total phenolic content in wild stands of lingonberries (Alam et al., 2016; Vyas et al., 2015; Bujor et al., 2018; Vilkickyte and Raudone, 2021b). The sugar content in lingonberries has been found to be inversely correlated with latitude (Vilkickyte and Raudone, 2021b). Even though field studies provide valuable information on the effects of environmental factors on the quality of berries, the fluctuation of biotic and abiotic conditions and their covariation makes the estimation of the role of specific environmental factors difficult. The knowledge of temperature effects will become increasingly important due to predicted increases in mean temperatures across the globe, with the largest effects seen in Arctic and Antarctic areas(IPCC, 2021). Furthermore, no previous studies have investigated the association between the growth conditions and VOCs and organic acids in lingonberries. As phenolic compounds, VOCs, sugars, and organic acids are known to influence the taste and flavour of lingonberries, more studies are needed to highlight the key factors affecting the accumulation of these compounds.

Therefore, the aim of this study was to investigate the influence of the ripening temperature and latitudinal origin on the metabolomic profile and chemical composition of lingonberries grown under controlled conditions. Lingonberries originating from northern and southern Norway ripened at 9 and 15 °C in a phytotron to assess the effects of origin and the response to temperature on phenolic compounds, VOCs, sugars, and organic acids. This study is the first comprehensive report on the chemical quality of lingonberries in

Table 1
Concentrations of phenolic compounds (mg/100 g fw), sugars (g/100 g fw) and organic acids (g/100 g fw) in lingonberries from two locations in Norway (north and south) ripened in a phytotrone at 9 and 15 °C and harvested from the wild[a]. Results from ANOVA (p values) of significant differences between samples from the phytotron experiment[b].

	Significance (P-values) [b]			North	North	South	South	North	South
	T	O	T × O	9 °C	15 °C	9 °C	15 °C	Wild stands	Wild stands
Cyanidin-3-*O*-galactoside	0.000	0.038	0.006	61.1 ± 1.8 bc	44.2 ± 1.4c	71.3 ± 3.3b	42.4 ± 3.8c	101.6 ± 11.1 a	65.4 ± 11.0b
Cyanidin-3-*O*-glucoside	0.018	0.000	0.274	4.1 ± 0.2 abc	5.0 ± 0.4 a	2.8 ± 0.3c	3.2 ± 0.4 bc	3.1 ± 0.3c	4.5 ± 1.0 ab
Cyanidin-3-*O*-arabinoside	0.004	0.000	0.179	18.4 ± 1.1 a	13.7 ± 1.3 bc	9.0 ± 1.1 d	6.8 ± 2.2 d	14.3 ± 2.4 ab	10.0 ± 0.7 cd
Cyanidin-3-*O*-pentoside	0.000	0.002	0.271	1.4 ± 0.1 a	0.9 ± 0.1 bc	1.0 ± 0.1b	0.6 ± 0.2c	1.4 ± 0.1 a	0.9 ± 0.1 bc
Cyanidin-3-*O*- (acetyl)glucoside	0.041	0.044	0.506	0.8 ± 0.0	0.3 ± 0.0	0.3 ± 0.5	0.1 ± 0.1	0.5 ± 1.0	0.1 ± 0.1
Total anthocyanins	**0.000**	**0.016**	**0.067**	**85.9 ± 2.6b**	**64.2 ± 2.9 bc**	**84.5 ± 2.0b**	**53.1 ± 5.8c**	**120.9 ± 12.8 a**	**80.9 ± 12.0b**
Quercetin-3-*O*-galactoside	0.090	0.065	0.128	5.1 ± 0.4b	5.0 ± 0.3b	6.9 ± 0.3b	5.2 ± 1.5b	17.4 ± 5.2 a	5.5 ± 0.6b
Quercetin-3-*O*-glucoside	0.216	0.363	0.982	0.8 ± 0.1b	0.9 ± 0.1b	1.4 ± 0.0b	1.2 ± 0.3b	3.0 ± 1.0 a	1.2 ± 0.3b
Quercetin-3-*O*-xyloside	0.839	0.392	0.886	1.0 ± 0.1	1.0 ± 0.1	1.3 ± 0.1	1.2 ± 0.2	1.3 ± 0.1	1.3 ± 0.2
Quercetin-3-*O*-arabinoside	0.000	0.000	0.028	6.8 ± 0.3b	6.4 ± 0.2b	8.5 ± 0.5b	6.4 ± 1.7b	12.8 ± 2.2 a	7.8 ± 0.8b
Quercetin-3-*O*-arabinofuranoside	0.398	0.003	0.165	0.4 ± 0.0b	0.4 ± 0.0b	0.7 ± 0.2 a	0.4 ± 0.0b	0.6 ± 0.0 ab	0.5 ± 0.1b
Quercetin-3-*O*-rhamnoside	0.399	0.009	0.419	7.2 ± 0.1 ab	6.7 ± 0.6 ab	10.6 ± 1.2a	10.5 ± 0.5 a	7.4 ± 2.0 ab	3.8 ± 2.6b
Quercetin-3-*O* -(HMG)-pentoside 1[c]	0.039	0.161	0.148	0.6 ± 0.1 a	0.5 ± 0.1 a	0.3 ± 0.0b	0.2 ± 0.1b	0.3 ± 0.1b	0.3 ± 0.0b
Quercetin-3-*O*-(HMG)-pentoside 2[c]	0.006	0.006	0.058	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.1	0.4 ± 0.0	0.3 ± 0.1	0.2 ± 0.1
Kaempferol-3-O-rhamnoside	0.458	0.000	0.567	0.2 ± 0.0b	0.3 ± 0.0b	0.1 ± 0.0b	0.2 ± 0.0b	0.3 ± 0.0b	0.7 ± 0.3 a
Quercetin-3-*O*-(HMG)-rhamnoside [c]	0.347	0.001	0.694	7.5 ± 0.6	6.9 ± 0.7	5.6 ± 3.3	5.5 ± 5.3	5.9 ± 3.1	4.6 ± 4.3
Kaempferol-3-*O*-(HMG)-rhamnoside [c]	0.132	0.000	0.515	0.3 ± 0.0 ab	0.4 ± 0.0 a	0.2 ± 0.1 bc	0.2 ± 0.0 bc	0.0 ± 0.0c	0.0 ± 0.0c
Total flavonols	**0.298**	**0.193**	**0.617**	**30.4 ± 1.7b**	**28.7 ± 0.8b**	**36.1 ± 9.0b**	**31.3 ± 3.9b**	**49.2 ± 3.9 a**	**25.8 ± 7.3b**
Ferulic acid-hexoside 1	0.273	0.619	0.699	4.8 ± 0.1	3.9 ± 0.2	4.3 ± 1.2	3.9 ± 0.9	3.5 ± 0.2	1.5 ± 0.2
Ferulic acid-hexoside 2	0.000	0.729	0.752	8.3 ± 0.7 a	3.4 ± 0.3 bc	8.4 ± 2.5 a	3.9 ± 0.9b	4.6 ± 0.7b	0.8 ± 0.1c
Coumaroyl iridoid	0.323	0.949	0.253	1.2 ± 0.2	1.3 ± 0.2	1.6 ± 0.9	0.9 ± 0.4	0.8 ± 0.9	1.1 ± 0.8
Caffeic acid hexoside 1	0.047	0.034	0.355	2.0 ± 0.1a	1.5 ± 0.1b	1.7 ± 0.2 ab	1.4 ± 0.3b	0.9 ± 0.1c	0.8 ± 0.2c
Caffeic acid hexoside 2	0.001	0.003	0.081	2.1 ± 0.0 a	1.3 ± 0.1b	1.4 ± 0.4b	1.0 ± 0.1 bc	0.7 ± 0.1 cd	0.5 ± 0.1 d
Coumaric acid – hexoside	0.527	0.021	0.708	3.7 ± 0.1	4.4 ± 0.2	6.2 ± 2.1	6.3 ± 1.6	5.7 ± 1.1	4.2 ± 0.3
Caffeoylquinic acid (chlorogenic acid)	0.919	0.011	0.767	0.7 ± 0.2b	0.8 ± 0.3b	2.0 ± 1.0 ab	1.8 ± 0.5b	6.0 ± 3.1 a	1.3 ± 0.1b
Sinapic acid hexoside	0.978	0.052	0.029	0.1 ± 0.0 ab	0.1 ± 0.0 ab	0.2 ± 0.0 a	0.1 ± 0.0 a	0.1 ± 0.0 ab	0.1 ± 0.0b
Total cinnamic acid derivatives	**0.050**	**0.361**	**0.951**	**23.1 ± 0.8 a**	**17.0 ± 0.9 ab**	**25.8 ± 8.0 a**	**19.4 ± 4.4 ab**	**22.4 ± 4.0 a**	**10.4 ± 1.1b**
Catechin	0.633	0.102	0.894	48.2 ± 0.9	44.5 ± 2.0	58.5 ± 25.6	33.9 ± 16.5	45.5 ± 15.6	26.1 ± 7.9
Procyanidin a2	0.004	0.333	0.011	41.4 ± 4.3 ab	38.2 ± 7.5 ab	50.0 ± 12.1ab	40.7 ± 8.6 a	57.1 ± 2.3 a	34.1 ± 13.6b
Procyanidin b2	0.919	0.584	0.654	25.2 ± 0.9	21.5 ± 0.2	24.3 ± 6.9	18.5 ± 6.6	19.2 ± 5.6	14.1 ± 3.4
Total proanthocyanidins	**0.269**	**0.202**	**0.419**	**497 ± 17 ab**	**473 ± 7 ab**	**651 ± 116 ab**	**510 ± 73 ab**	**401 ± 97 ab**	**269 ± 47b**
Sucrose	0.021	0.001	0.748	0.6 ± 0.0 a	0.5 ± 0.0 ab	0.5 ± 0.1 abc	0.4 ± 0.1 bcd	0.3 ± 0.1 d	0.3 ± 0.1 cd
Glucose	0.055	0.567	0.314	2.8 ± 0.1	3.0 ± 0.0	2.8 ± 0.2	3.2 ± 0.4	3.0 ± 0.3	3.3 ± 0.4
Fructose	0.011	0.000	0.026	2.4 ± 0.1c	2.4 ± 0.1c	2.6 ± 0.2 bc	3.1 ± 0.1 ab	2.6 ± 0.3c	3.2 ± 0.1 a
Total sugars	**0.073**	**0.143**	**0.169**	**5.9 ± 0.3**	**6.0 ± 0.1**	**5.9 ± 0.4**	**6.7 ± 0.5**	**5.9 ± 0.7**	**6.9 ± 0.4**
Citric acid	0.169	0.000	0.038	2.5 ± 0.1 a	2.4 ± 0.0 a	1.8 ± 0.0 ab	2.0 ± 0.1 ab	1.4 ± 0.3b	2.1 ± 0.6 ab
Malic acid	0.676	0.001	0.003	0.1 ± 0.0 ab	0.1 ± 0.0 a	0.1 ± 0.0 ab	0.0 ± 0.0b	0.0 ± 0.0 ab	0.1 ± 0.0 a
Quinic acid	0.016	0.062	0.391	1.9 ± 0.1 a	1.6 ± 0.1 ab	1.7 ± 0.2 ab	1.5 ± 0.1b	1.5 ± 0.1b	1.1 ± 0.2c
Total organic acids	**0.234**	**0.000**	**0.120**	**4.5 ± 0.2 a**	**4.1 ± 0.1 ab**	**3.5 ± 0.2 bc**	**3.6 ± 0.2 bc**	**3.0 ± 0.2c**	**3.2 ± 0.7c**

[a] All concentrations are mean values ± standard deviation of three samples, except in the northern wild stand with four samples. Anthocyanins were quantified as mg/100 g fresh weight (fw) equivalents of cyanidin-3-*O*-galactoside at 520 nm, flavonol glycosides as quercetin-3-*O*-rutinoside at 360 nm, cinnamic acid derivatives as chlorogenic acid at 320 nm, and individual flavan-3-ols as catechin at 280 nm. Total proanthocyanidins was determined spectrophotometrically by the DMAC method. For the other compound groups, total content is the sum of individual compounds. Different letters (a-c) indicate significant differences ($p < 0.05$) between the samples as determined by the Tukey's HSD test.

[b] ANOVA with the factors temperature (T) and origin (O) and their interaction (T × O).

[c] HMG = Hydroxy-3-methylglutaroyl.

relation to temperature and place of origin.

2. Results and discussion

2.1. Phenolic compounds

2.1.1. Phenolic composition

A total of 27 phenolic compounds were tentatively identified and quantified in lingonberries by comparison of UV and MS spectra to previously published reports of lingonberries (Table 1 and Table s1, Supporting information). The identification and contents of the major anthocyanins, flavonols and cinnamic acid derivatives (CADs) were comparable to previous analyses in lingonberries (Andersen, 1985; Ek, Kartimo, Mattila, & Tolonen, 2006; Hokkanen, Mattila, Jaakola, Pirttilä, & Tolonen, 2009; Kelanne et al., 2019; Lee & Finn, 2012; Vollmannova et al., 2009). However, earlier as many as 63 phenolic compounds have been identified in lingonberries, although several only in small quantities (Bujor et al., 2018). The five anthocyanins identified in the present study were all glycosides of cyanidin, and the flavonols were largely glycosides of quercetin. Eight CADs were quantified in the lingonberries. The total concentrations of anthocyanins, flavonols, and CADs were 53–121, 26–49, and 10–26 mg/100 g fresh weight (fw), respectively (Table 1). The contents of only three flavan-3-ols were accurately quantified with the applied HPLC-DAD method due to low molar absorptivity and coelution with other phenolic compounds. The total concentration of flavan-3-ols and their oligomers and polymers, that is, proanthocyanidins (PAs), determined by a spectrophotometric method, varied from 269 to 651 mg/100 g fw, confirming earlier research identifying PAs as the most abundant group of phenolic compounds in lingonberries (Dudonne et al., 2015; Hellström et al., 2009; Kylli et al., 2011).

2.1.2. Influence of temperature and origin on the phenolic profile

Lingonberries grown at 9 °C had significantly higher contents of anthocyanins and cinnamic acid derivatives than berries grown at 15 °C (Table 1). Previous field studies of wild lingonberries have indicated that both the leaves and berries of lingonberries accumulate higher amounts of anthocyanins, phenolic acids and flavonols and lower contents of flavan-3-ols and proanthocyanidins at lower growth temperatures (Vilkickyte and Raudone, 2021b; Vyas et al., 2015). However, these results were limited by the temperature in the local settings as the study of Vyas et al. (2015) was performed under lower temperatures and in a narrow temperature range (3.9 – 6.4 °C), while the temperature in the study by Vilkickyte and Raudone (2021b) was higher (~18 °C) during the growth season. An increase in the synthesis of phenolic compounds at low temperatures has been suggested as a part of the specialized protective action in berries, as they can maintain high photosynthetic rates and thereby fixate carbon (Jaakola and Hohtola, 2010). CADs are associated to the upstream synthesis of anthocyanins (Jaakola and Hohtola, 2010), and a simultaneous increase in the CADs and anthocyanins could be due to the upregulation of anthocyanin biosynthesis genes. In controlled temperature studies of bilberries (*Vaccinium myrtillus*) (grown at 12 and 18 °C), black currants (grown at 12, 18 and 24 °C) and raspberries (*Rubus idaeus*) (grown at 12, 18 and 24 °C), the optimal temperature for anthocyanin synthesis was found to be 18 °C (Remberg et al., 2010; Uleberg et al., 2012; Woznicki et al., 2016). A controlled study of cloudberries (*Rubus chamaemorus*) grown at 9, 12, 15 and 18 °C showed the highest anthocyanin content at lower growth temperatures (Martinussen et al., 2010; McDougall et al., 2011). In both lingonberries and black currants (*Ribes nigrum*), also the contents of most phenolic acids increased with decreasing temperature (Vilkickyte and Raudone, 2021b; Woznicki et al., 2016). Similarly, increases in the hydroxycinnamic acid contents in bilberries were observed at lower growth temperatures (Uleberg et al., 2012). As lingonberries ripen late in the growth season and cloudberries thrive in low-temperature areas, the increase in the synthesis of phenolic compounds could therefore be an adaptation strategy to low temperatures. In addition to being beneficial to the plant, it has been shown that a higher content of PAs and other phenolic compounds in lingonberries give the berries a bitter taste that can be challenging by consumers (Laaksonen et al., 2016) but beneficial in relation to several health effects of the berries (Kowalska, 2021).

Among the anthocyanins, the concentration of cyanidin-3-*O*-glucoside was higher at 15 °C, which was the inverse of the other cyanidin-glycosides (Table 1). The profile of CADs was also affected by temperature, as the contents of ferulic acid and caffeic acid hexosides were significantly higher in lingonberries ripened at 9 °C than at 15 °C. Total flavonols were not affected by temperature and only minor effects were observed for the individual flavonols. Environmental stressors have been shown to influence the patterns of hydroxylation, methylation, and glycosylation in other plants (Alseekh et al., 2020). The hydroxylation of flavonoids has been considered a genetic trait in fruits with different species seemingly responding differently to stressors (Karppinen et al., 2016; Zoratti et al., 2014). In bilberries, decreases in both cyanidin-3-*O*-galactoside and cyanidin-3-*O*-glucoside were observed at lower growth temperatures with simultaneous increases in the concentrations of delphinidin glycosides (Uleberg et al., 2012).

Origin significantly influenced the total anthocyanin concentration in lingonberries, with the highest content found in berries of northern origin (Table 1). In lingonberries, it has previously been shown that the contents of anthocyanins, proanthocyanidins, phenolic acids and total antioxidant activity positively correlated with latitude, while total phenolics and the total flavonoid content showed less correlation (Vilkickyte and Raudone, 2021a,b; Vyas et al., 2015). The effect of origin on the anthocyanins was less significant than the variation caused by temperature. The glycosylation of the cyanidins was also affected by origin, with higher proportions of cyanidin-3-*O*-glucoside and cyanidin-3-*O*-arabinoside in berries of northern origin than in berries originating further south. In other *Vaccinium* species, such as bilberries (Lätti et al., 2008; Uleberg et al., 2012; Martz et al. 2010), and bog bilberries (*V. uliginosum*) (Lätti et al., 2010) it has also been found that northern clones produced more anthocyanins than southern clones. Latitude affects the synthesis of anthocyanins differently in different berry species; for instance, in bilberries, content of all anthocyanins increased and the proportion of cyanidin-3-*O*-galactoside was higher in the northern ecoregion (Uleberg et al., 2012), whereas in currants a higher proportion of several of the cyanidin-glycosides but a lower proportion of cyanidin-3-*O*-rutinoside in berries of the northern latitude was found (Yang et al., 2013). There was no effect of origin on the total flavonol content but significant effects were observed for many of the individual compounds including quercetin-xyloside, -arabinofuranoside -rhamnoside, -(HMG)-rhamnoside and –(HMG)-pentoside), with most compounds being more prevalent in berries from the southern origin (Table 1). In bog bilberries, the flavonol content was higher at higher latitudes compared to more southern growth places (Lätti et al., 2010). There was no effect on origin of total CADs. However, there were higher concentrations of *p*-coumaric acid hexoside and chlorogenic acid detected in lingonberries originating from the south, while the caffeic acid hexosides were more abundant in berries of northern origin. The profiles of certain phenolic acids in wild berries have also previously been shown to be affected by latitude (Vilkickyte and Raudone, 2021b). In bilberries from different origins grown under controlled conditions, the concentration of hydroxycinnamic acid derivatives was higher in bilberries from southern clones (Uleberg et al., 2012).

At the field stands, all measured phenolic groups were detected in higher concentrations in berries from the northern location compared to berries from the southern location (Table 1). There was a lower total anthocyanin content in lingonberries from the southern field stands with a higher proportion of cyanidin-3-*O*-glucoside. This trend could be due to both the effects of temperature and origin and their interaction, as observed in the phytotron experiment. There were also higher concentrations of total flavonols and chlorogenic acid detected in lingonberries

from the northern field stands. UV-radiation is known to influence the flavonol content as well as the content of anthocyanins in berries (Jaakola and Hohtola, 2010). Lingonberries collected from the field stands were influenced by larger fluctuations in environmental factors, including UV-radiation, temperature, and precipitation compared to berries ripened in the phytotron (Jaakola and Hohtola, 2010; Yang et al., 2013). In the northern field stands elevated levels of quercetin-3-*O*-galactoside, -glucoside and -arabinoside in addition to the total increase in the anthocyanins were found compared with berries ripened in the phytotron, likely due to increased UV-radiation under the open field conditions. In summary, the results indicate that both temperature and place of origin influenced the composition of phenolic compounds in lingonberries, and that temperature was the strongest contributor.

2.2. Sugars and organic acids

2.2.1. Composition of sugars and organic acids

The total sugar content in the berries varied between 5.9 and 6.9 g/100 g fw, and the content of organic acids varied between 3.0 and 4.5 g/100 g fw (Table 1). The contents of the three sugars, sucrose, glucose and fructose, were in accordance with previous reports of lingonberries, whereas the content of organic acids was higher than previously reported (Jensen et al., 2002; Kelanne et al., 2019; Mikulic-Petkovsek et al., 2012; Viljakainen et al., 2002). Some studies have reported the presence of malic or tartaric acid in lingonberries, whereas in our study, quinic acid was identified, similarly to what has been reported in Swedish lingonberries (Jensen et al., 2002).

2.2.2. Influence of temperature and origin on sugars and organic acids

Neither the total concentration of sugars nor organic acids in lingonberries ripened in the phytotron were significantly influenced by temperature (Table 1). However, higher fructose and lower sucrose contents were found in lingonberries ripened at higher temperature, particularly in berries from the south. A positive correlation between the growth temperature and total sugar concentration has been found in a field study of wild lingonberries (Vilkickyte et al., 2019). Similar results were observed in bilberries, where higher sugar concentrations were detected at 18 °C than at 12 °C (Uleberg et al., 2012). Organic acids were not significantly affected by temperature, except quinic acid, which was found at higher concentrations at 9 °C (Table 1). In accordance with our results, the temperature did not affect the total concentration of organic acids in bilberries, but a lower content of quinic acid was detected at higher growth temperatures (Uleberg et al., 2012).

The origin of lingonberries did not significantly affect the total sugar concentration in the present study, but the concentration of fructose was higher in berries of southern origin, and sucrose was slightly higher in berries of northern origin (Table 1). Similar results were found in bilberries grown under controlled conditions, where the origin of the plants did not influence the total content of sugars, apart from sucrose, which was higher in the northern clones (Uleberg et al., 2012). Lingonberries from the north had significantly higher concentrations of all three quantified acids, and there was a significant interaction between temperature and origin for citric acid and malic acid (Table 1). Uleberg et al. (2012) also reported a higher concentration of malic acid but a lower content of quinic acid in bilberries from northern clones, whereas no clear effect of clones on the total organic acid concentration was observed. Although sugars typically provide a sweet taste, the high concentration of organic acids in lingonberries may mask sweetness, and therefore, the combined content or ratio between sugars and organic acids is important for their flavour profile (Viljanen et al., 2014). This ratio is also known to influence the perceived sourness and astringency in berries (Laaksonen et al., 2016). Thus, with a higher concentration of organic acids and the same sugar concentration lingonberries from the northern location are likely to be perceived as sourer and less sweet than berries from the southern location.

No significant differences in total sugar or acid concentrations were found in lingonberries from the wild stands in the present study compared to berries ripened in the phytotron (Table 1). There was a significantly higher content of quinic acid in lingonberries of northern origin than of southern origin in berries harvested from the field stand. As the higher total content of organic acids in berries from the northern location observed in the phytotron was not observed in berries harvested from wild stands, it indicates that other factors than temperature also affected the content of the sugars and organic acids in lingonberries.

2.3. Volatile organic compounds

2.3.1. Composition of VOCs

A total of 77 VOCs were tentatively identified in lingonberries (Table 2). Among these compounds there were 21 aldehydes, twelve esters of which nine were acetates, seven ketones, ten alcohols, eight volatile acids, 18 terpenes, of which two were unidentified, and one furan. Among the 77 compounds, 65 were found in the phytotron samples, and 12 solely found in the samples collected from the wild stands. In an earlier study, using olfactory GC–MS, 2-methylbutanoic acid, 2-methylpropanoate, hexanal, linalool, eucalyptol, diacetyl and methyl benzoate were identified as odorants in lingonberries (Marsol-Vall et al. 2020). Of these compounds, diacetyl and 2-methylpropanoate were not identified in the present study. Anjou and von Sydow, (1967, 1969) identified 2-methylbutanoic acid as the most abundant VOC and thus considered it a key compound in lingonberries, indicating that 2-methylbutanoic acid may be important for the lingonberry aroma. However, no studies have to date determined the contribution of the volatile compounds to the aroma profile of lingonberries. In cranberries, however, the compounds with the highest odour activities and thus contributing to aroma, were aldehydes and esters (Cosme et al., 2022; Zhu et al., 2016). Since lingonberries and cranberries have similar flavour characteristics, it is likely that the same groups of compounds contribute to the aroma profile of both of these berries. The aldehydes, shown to contribute to the aroma of cranberries, were also found in high abundances in the present study. However, aroma thresholds vary for the different compounds, and high concentration does not automatically mean high contribution to aroma (Maffei, 2010). The twelve esters detected in lingonberries are thought to be among important flavour and fragrance components as esters have been shown to contribute significantly to aroma of other fruits and berries. For example, esters have been among the proposed compounds responsible for the fruity flavours of blueberries at full maturity (Sater et al., 2020).

Among the 77 VOCs identified in lingonberries, 40 had not previously been identified (Table 2) (Anjou and von Sydow, 1967, 1969; Marsol-Vall et al., 2020; Viljanen et al., 2014). Also, among studies of several other *Vaccinium* species and among studies of grapes, there has been a large variation in the VOC profiles (Sater et al., 2020; González-Barreiro et al., 2015). These variations are likely to be influenced by genetic differences, or biotic and abiotic conditions (Karppinen et al., 2016). In studies of cranberries there has been some variation of compounds identified, but high contents of benzyl compounds and terpineol are generally found (Sater et al., 2020). These compounds were also detected in lingonberries in the current study (Table 2), and the benzyl compounds have been reported in all previous studies of lingonberries (Anjou and von Sydow, 1967, 1969; Marsol-Vall et al., 2020; Viljanen et al., 2014). It is recognised that the analytical technique used to determine VOCs can influence the composition of compounds (Sater et al., 2020). The studies analysing concentrated essential oils of lingonberries (Anjou and von Sydow, 1967, 1969) obtained higher number of volatile compounds and a larger variation in the compound profile than studies using HS-SPME-GC–MS (Marsol-Vall et al., 2020; Viljanen et al., 2014). Our study using HS-SPME-GC–MS gave a larger number of VOCs than the previous studies using this technique. The present study was, however, the first investigation of whole or crushed berries. It is recognized that the sample pre-treatment with freezing, partial thawing and mashing used in the present study will influence the VOC profile of

Table 2
Volatile compounds (% normalized peak area) in lingonberries from two locations in Norway (north and south) ripened in a phytotron at 9 and 15 °C and harvested from the wild[a]. Results from ANOVA (p values) of significant differences between samples from the phytotron experiment[b].

				Significance (P-values)[b]			North		South		North	South
	RI Measured [d]	RI literature	Ref [e]	T	O	T*O	9 °C	15 °C	9 °C	15 °C	Wild stands	Wild stands
Aldehydes												
Acetaldehyde	716	690	1, 2	0.004	0.000	0.010	0.004 ± 0.001b	0.005 ± 0.001b	0.014 ± 0.001ab	0.024 ± 0.008ab	–	0.041 ± 0.022a
2-Methyl propanal	811	818					–	–	–	–	0.006	–
2-Methylbutanal [c]	921	910		0.365	0.000	0.365	0.003 ± 0.000b	0.003 ± 0.001b	–	–	0.019a	0.006 ± 0.006b
3-Methylbutanal	924	914		0.340	0.424	0.563	0.001 ± 0.000	0.002 ± 0.001	0.002 ± 0.001	0.003 ± 0.005	0.01	0.006 ± 0.007
Hexanal [c]	1086	1084	1,2,3	0.361	0.000	0.075	0.147 ± 0.041bc	0.255 ± 0.071c	1.528 ± 0.517a	1.199 ± 0.656abc	0.324abc	1.31 ± 0.301ab
(Z)-3-Hexenal	1142	1139		0.065	0.000	0.061	0.030 ± 0.015	0.032 ± 0.008	0.633 ± 0.342	0.383 ± 0.326	0.046	0.156 ± 0.099
(E)-3-Hexenal	1147	1131		0.213	0.000	0.189	0.092 ± 0.034	0.109 ± 0.01	2.242 ± 1.133	1.646 ± 1.288	0.132	1.104 ± 0.594
Heptanal	1187	1186	3	0.011	0.000	0.011	0.005 ± 0.003	0.009 ± 0.001	–	–	–	0.011 ± 0.019
(E)-2-Hexenal	1205	1216		0.335	0.000	0.257	0.005 ± 0.002	0.007 ± 0.001	0.086 ± 0.042	0.064 ± 0.061	–	0.018 ± 0.025
(Z)-2-Hexenal [c]	1224	1226		0.642	0.000	0.506	0.236 ± 0.081	0.265 ± 0.051	0.964 ± 0.725	0.8 ± 0.758	–	–
Octanal	1290	1291	1,2,3	0.402	0.000	0.263	0.004 ± 0.000	0.005 ± 0.000	0.003 ± 0.000	0.003 ± 0.002	–	0.005 ± 0.008
(Z)-2-Heptenal	1328	1318	1	0.052	0.001	0.254	0.008 ± 0.001	0.014 ± 0.003	0.016 ± 0.007	0.018 ± 0.01	0.007	–
Nonanal	1397	1397	1,3	0.670	0.000	0.111	0.013 ± 0.000c	0.015 ± 0.002c	0.008 ± 0.002c	0.007 ± 0.004c	0.298a	0.083 ± 0.013b
(E,E)-2,4-Hexadienal [c]	1405	1407	3	0.468	0.000	0.468	–	–	0.015 ± 0.007ab	0.012 ± 0.009ab	0.029a	0.009 ± 0.002ab
2,4-Hexadienal	1412	1406		0.749	0.000	0.995	0.094 ± 0.017a	0.097 ± 0.023a	0.029 ± 0.019b	0.032 ± 0.019b	–	0.008 ± 0.003b
(E)-2-Octenal	1441	1430	3	0.466	0.181	0.154	0.005 ± 0.001b	0.007 ± 0.001b	0.013 ± 0.009b	0.007 ± 0.003b	0.035a	0.015 ± 0.005b
2-Furaldehyde	1475	1467	1, 3	0.602	0.000	0.602	0.003 ± 0.001	0.003 ± 0.002	–	–	–	–
(E,E)-2,4-Heptadienal [c]	1477	1508	3	0.395	0.000	0.023	–	0.001 ± 0.001b	0.004 ± 0.002b	0.004 ± 0.002b	0.021a	0.014 ± 0.004a
Benzaldehyde [c]	1540	1529	1,2,3	0.238	0.594	0.508	0.006 ± 0.001	0.01 ± 0.002	0.005 ± 0.000	0.018 ± 0.023	0.006	0.006 ± 0.002
(E)-2-Nonenal	1549	1542					–	–	–	–	0.016a	0.009 ± 0.002b
p-Menth-1-en-9-al	1636	1629					–	–	–	–	–	0.001 ± 0.001
Acetate esters												
Methyl acetate	832	827		0.481	0.603	0.309	0.001 ± 0.000	0.004 ± 0.005	0.002 ± 0.000	0.002 ± 0.001	0.008	0.004 ± 0.001
Ethyl acetate	893	885	1,2,3	0.521	0.937	0.052	0.012 ± 0.000b	0.011 ± 0.002b	0.01 ± 0.002b	0.012 ± 0.001b	0.032a	0.027 ± 0.007a
Hexyl acetate [c]	1274	1271		0.132	0.000	0.026	0.045 ± 0.007a	0.069 ± 0.021ab	0.009 ± 0.005b	0.004 ± 0.002b	–	0.022 ± 0.039ab
(E)-3-Hexen-1-yl acetate	1300	1306		0.007	0.000	0.007	–	0.001 ± 0.000	–	–	–	–
(E)-3-Hexen-1-ol acetate	1317	1320					–	–	–	–	0.003	0.004 ± 0.002
(Z)-3-Hexen-1-yl acetate	1310	1316		0.661	0.699	0.029	0.034 ± 0.015	0.08 ± 0.043	0.066 ± 0.064	0.035 ± 0.026	–	–
(E)-2-Hexenyl acetate	1355	1340					–	–	–	–	0.009b	0.026 ± 0.005a
(E)-2-Hexen-1-yl acetate	1328	1333		0.614	0.000	0.629	0.08 ± 0.014a	0.073 ± 0.005a	0.001 ± 0.002b	0.001 ± 0.001b	–	–
Benzyl acetate [c]	1733	1726		0.205	0.000	0.205	–	–	0.004 ± 0.003	0.002 ± 0.002	–	0.005 ± 0.008
Other esters												
Methyl butanoate	993	984	2	0.893	0.004	0.983	0.003 ± 0.000b	0.003 ± 0.000b	0.003 ± 0.001b	0.003 ± 0.000b	0.016a	0.013 ± 0.003a
Vinyl butanoate	1046	1045					–	–	–	–	–	0.002 ± 0.004
Methyl benzoate	1642	1641	1,2,3	0.025	0.000	0.165	0.007 ± 0.001bc	0.006 ± 0.001ab	0.011 ± 0.001a	0.008 ± 0.002ab	–	0.002 ± 0.003c
Ketones												

(continued on next page)

Table 2 (*continued*)

				Significance (P-values)[b]			North		South		North	South
	RI Measured [d]	RI literature	Ref [e]	T	O	T*O	9 °C	15 °C	9 °C	15 °C	Wild stands	Wild stands
2,3-Butanedione	979	978	1,2,3	0.000	0.000	0.044	0.017 ± 0.001	0.04 ± 0.01	0.005 ± 0.009	0.012 ± 0.021	0.055	0.018 ± 0.017
1-Penten-3-one	1029	1022		0.604	0.000	0.663	0.005 ± 0.002	0.001 ± 0.002	0.034 ± 0.018	0.033 ± 0.021	0.015	0.024 ± 0.021
Acetoin	1293	1287		0.001	0.009	0.989	0.003 ± 0.000	0.006 ± 0.001	0.001 ± 0.001	0.004 ± 0.007	–	–
1-Octen-3-one	1300	1304		0.006	0.002	0.016	0.001 ± 0.002	0.004 ± 0.001	0.004 ± 0.001	0.004 ± 0.002	0.008	0.004 ± 0.004
6-Methyl-5-hepten-2-one [c]	1341	1332	1,2,3	0.961	0.258	0.724	0.005 ± 0.002b	0.005 ± 0.000b	0.005 ± 0.001b	0.005 ± 0.002b	0.015a	–
Acetophenone [c]	1672	1679	1, 3	0.588	0.000	0.033	0.018 ± 0.000bc	0.017 ± 0.002b	0.009 ± 0.002c	0.012 ± 0.004bc	0.021ab	0.030 ± 0.002a
5-Ethyl-2(5H)-furanone	1777	1754		0.428	0.000	0.428	–	–	0.018 ± 0.011	0.015 ± 0.013	0.019	0.019 ± 0.004
Alcohols												
Ethanol	943	937		0.057	0.677	0.003	0.008 ± 0.002b	0.006 ± 0.001b	0.004 ± 0.000b	0.011 ± 0.009b	0.030a	0.011 ± 0.004b
2-Methyl-3-butene-2-ol	1047	1036	3	0.000	0.000	0.832	0.005 ± 0.001	0.008 ± 0.002	–	0.003 ± 0.004	–	0.019 ± 0.033
3-Methyl-1-butanol	1215	1236	1	0.391	0.009	0.631	0.001 ± 0.001	0.001 ± 0.001	0.005 ± 0.005	0.003 ± 0.006	–	0.104 ± 0.18
1-Pentanol [c]	1256	1259	3	0.579	0.000	0.824	0.011 ± 0.002	0.012 ± 0.001	0.016 ± 0.001	0.017 ± 0.006	–	0.023 ± 0.04
(Z)-2-Penten-1-ol	1322	1322		0.591	0.000	0.079	0.002 ± 0.001c	0.004 ± 0.001c	0.008 ± 0.005c	0.007 ± 0.005c	0.146a	0.036 ± 0.003b
1-Hexanol [c]	1357	1345	3	0.193	0.000	0.086	0.096 ± 0.022a	0.133 ± 0.027ab	0.068 ± 0.005b	0.063 ± 0.031bc	0.005c	0.006 ± 0.002c
(Z)-3-Hexen-1-ol	1390	1390	1, 3	0.628	0.955	0.127	0.080 ± 0.046	0.132 ± 0.077	0.121 ± 0.066	0.094 ± 0.062	0.09	0.052 ± 0.003
1-Octen-3-ol [c]	1458	1454	3	0.996	0.542	0.830	0.007 ± 0.000b	0.007 ± 0.001b	0.007 ± 0.000b	0.007 ± 0.001b	0.011a	0.012 ± 0.000a
Ethyl hexanol	1500	1499	1	0.271	0.051	0.409	0.004 ± 0.000	0.004 ± 0.000	0.004 ± 0.001	0.005 ± 0.001	–	–
Benzyl alcohol	1896	1879	1, 3	0.107	0.437	0.944	0.093 ± 0.01	0.16 ± 0.054	0.056 ± 0.011	0.129 ± 0.122	0.082	0.102 ± 0.005
Volatile acids												
Acetic acid	1462	1448		0.131	0.012	0.589	0.008 ± 0.002ab	0.01 ± 0.001b	0.007 ± 0.002b	0.008 ± 0.001b	0.005b	0.015 ± 0.003a
3-Methylbutanoic acid	1681	1676	1	0.284	0.081	0.284	–	–	0.002 ± 0.003ab	0.008 ± 0.014ab	0.049a	0.048 ± 0.032a
2-Methylbutanoic acid	1684	1670	1	0.000	0.000	0.000	0.243 ± 0.026b	0.750 ± 0.124a	0.025 ± 0.011c	0.019 ± 0.014c	–	0.037 ± 0.065c
Pentanoic acid	1737	1743		0.098	0.001	0.877	0.007 ± 0.001ab	0.009 ± 0.002ab	0.012 ± 0.003a	0.014 ± 0.003a	0.007ab	0.002 ± 0.003b
Hexanoic acid [c]	1858	1852		0.126	0.000	0.335	0.037 ± 0.009ab	0.048 ± 0.01bc	0.067 ± 0.015a	0.07 ± 0.003a	–	0.011 ± 0.006c
Heptanoic acid	1967	1956					–	–	–	–	0.14	0.075 ± 0.045
Octanoic acid	2067	2072					–	–	–	–	0.011	0.015 ± 0.004
Nonanoic acid	2169	2165		0.590	0.000	0.024	0.132 ± 0.012a	0.119 ± 0.014a	0.072 ± 0.012b	0.093 ± 0.023ab	0.012c	0.014 ± 0.007c
Terpenes												
α-Pinene [c]	1028	1027	1,2,3	0.232	0.004	0.232	0.025 ± 0.022	0.059 ± 0.040	–	–	–	0.142 ± 0.246
Camphene	1070	1063	3	0.065	0.007	0.065	0.001 ± 0.001	0.003 ± 0.002	–	–	–	0.008 ± 0.014
β-Pinene	1105	1112	3	0.056	0.015	0.056	0.001 ± 0.001	0.007 ± 0.003	–	–	–	0.013 ± 0.023
β-Thujene	1116	1124		0.221	0.013	0.221	0.002 ± 0.003	0.004 ± 0.003	–	–	–	–
Unknown terpinene	1119						–	–	–	–	–	0.005 ± 0.009a
α-Terpinene	1180	1187					–	–	–	–	1.000	1.000 ± 0.000
D-Limonene [c]	1196	1202	1, 3	0.780	0.000	0.780	0.009 ± 0.005a	0.011 ± 0.004ab	–	–	–	0.005 ± 0.005ab
Eucalyptol [c]	1215	1204	1,2,3	0.199	0.000	0.409	0.035 ± 0.030ab	0.057 ± 0.008a	–	0.005 ± 0.008b	0.007ab	0.028 ± 0.02ab
γ-Terpinene [c]	1247	1251	3	0.330	0.001	0.330	0.006 ± 0.006b	0.011 ± 0.006b	–	–	0.166ab	0.391 ± 0.283a

(*continued on next page*)

Table 2 (*continued*)

				Significance (P-values)[b]			North		South		North	South
	RI Measured [d]	RI literature	Ref [e]	T	O	T*O	9 °C	15 °C	9 °C	15 °C	Wild stands	Wild stands
Styrene	1262	1255	1				–	–	–	–	0.012b	0.023 ± 0.003a
o-Cymene [c]	1272	1265		0.303	0.001	0.303	0.005 ± 0.005	0.01 ± 0.006	–	–	–	–
Terpinolene	1283	1280		0.236	0.002	0.236	0.001 ± 0.002b	0.003 ± 0.002b	–	–	0.023a	0.005 ± 0.005b
trans-Linalool oxide (furanoid)	1487	1471		0.000	0.000	0.131	0.044 ± 0.017a	0.025 ± 0.006ab	0.011 ± 0.001bc	0.001 ± 0.001c	–	–
Linalool [c]	1555	1550	1,2,3	0.001	0.055	0.242	0.059 ± 0.013a	0.048 ± 0.011a	0.055 ± 0.01a	0.029 ± 0.006ab	0.005ab	0.002 ± 0.002b
Terpinen-4-ol	1616	1612	3	0.122	0.000	0.122	0.034 ± 0.029	0.077 ± 0.035	0.020 ± 0.034	–	–	–
α-Terpienol	1700	1696	3	0.103	0.002	0.871	0.008 ± 0.001	0.007 ± 0.001	0.007 ± 0.001	0.006 ± 0.001	–	–
Unknown terpine 2	1775			0.000	0.000	0.000	0.012 ± 0.004a	0.006 ± 0.001b	0.001 ± 0.003c	–	–	–
Camphol	1700	1698					–	–	–	–	0.008	0.010 ± 0.003
Furans												
2-Ethyl furan [c]	958	944		0.716	0.000	0.268	0.007 ± 0.002	0.009 ± 0.001	0.015 ± 0.005	0.021 ± 0.016	0.023	0.06 ± 0.063
Internal standards												
4-Methyl-2-pentanol	1172											
Neryl acetate	1728											

[a] All concentrations are mean values ± standard deviation of three samples at each temperature and from each origin in berries grown in the phytotron, one sample from berries grown in the wild in berries from the northern origin and three samples from berries grown in the wild from the southern origin. Different letters (a-c) indicate significant differences ($p < 0.05$) between the samples as determined by the Tukey's HSD test.
[b] ANOVA with the factors temperature (T) and origin (O) and their interaction (T × O).
[c] Identified with authentic standard compound.
[d] Kovats retention index for 60 m DB-WAX column (from literature).
[e] References; 1, Viljanen et al. (2014); 2, Marsol-Vall et al. (2021); 3, Anjou and von Sydow (1967).

the berries. The pre-treatment was chosen to secure a consistent treatment of the berries prior to analysis and to mimic the consumer perception of aroma of lingonberries when eaten.

2.3.2. *Influence of temperature and origin on the profile of VOCs*

There was generally little effect of temperature on the composition of VOCs (Table 2). Temperature significantly influencing the contents of only 13 of the 65 compounds found in lingonberries ripened in the phytotron. There were significant effects of three of seven ketones and three of eleven terpenes, and the seven other belonged to the other groups. While there was no clear trend for the ketones, the three terpenes were higher at the lower growth temperature. In blackcurrants and grapes, a lower growth temperature during the last month of ripening increased the total concentration of VOCs (Marsol-Vall et al., 2018; Xie et al., 2019). The effect of temperature in these studies, however, were largely due to differences in temperature between the growth stands and could therefore also be influenced by other environmental factors than temperature (Marsol-Vall et al., 2018). In a PCA of the volatile compounds in the lingonberry samples the two first components explained 74% of the variation in the dataset (Fig. 1). There was a slight clustering and separation of the samples based on growth temperature, but the separation based on origin and samples from the wild compared to those ripened in the phytotron explained substantially more of the variation in the dataset than the temperature. Previous investigations of other species of the Vaccinium genus have neither managed to identify which growth factors induce changes in VOCs profile (Sater et al., 2020).

The origin of the lingonberries significantly influenced the contents of 55 of the 65 VOCs in lingonberries ripened in the phytotron, indicating a larger effect of the origin than of the temperature. Origin also affected the composition of VOCs in bilberries (Rohloff et al., 2009), highbush blueberries (*V. corymbosum*) (Du et al., 2011), blackcurrants (Marsol-Vall et al., 2018), rabbiteye blueberries (*V. ashei*) and cranberries (*V. macrocarpon*) (Sater et al., 2020). In both blackcurrants and highbush blueberries, the same compounds were found at both northern and southern locations but with higher total concentrations in berries at the northern locations (Marsol-Vall et al., 2018, Du et al., 2011). In the PCA (Fig. 1), berries from different origin spread across PC1, which explained 51 % of the variation in the dataset, but with a large variation between the three stands in the southern origin. The variations in VOCs in berries from different places of origin may be linked to adaptations caused by genetic and environmental associations as known in several species (Karppinen et al., 2016). In blackcurrants and bilberries there was, however, a large variation between different cultivars of the same species (Marsol-Vall et al., 2018, Du et al., 2011). Among the large contributors to the variation across PC1 were the aldehydes (Fig. 1B). The aldehydes have previously been identified as key aroma compounds that could influence the flavour of *Vaccinium* berries (Sater et al., 2020). Aldehydes are mostly formed from the breakdown of lipids with C6 and C9 compounds formed through the lipoxygenase and hydroperoxide lyase pathways. In red grapes, the volatile profile is characterised by esters during early berry development, aldehydes in the middle, and alcohols being most predominant during late development (González-Barreiro et al., 2015). In addition to the higher content of the aldehydes, the berries from the southern origin had a higher content of 5-ethyl-2 (5H)-furanone. There was a large variation in profile and concentration of all terpenes between samples from the two origins with higher content in berries from the northern origin (Table 2). The terpenoids have a large variety of functions, including being important signalling molecules, often with pleasant floral and fruity scents. Previously, it has been shown that genetic differences can contribute to variation in the content of triterpenoid in lingonberries (Vilkickyte et al., 2022). There were significant interactions between temperature and origin in twelve of the VOCs, but as these were individual compounds from all groups, no

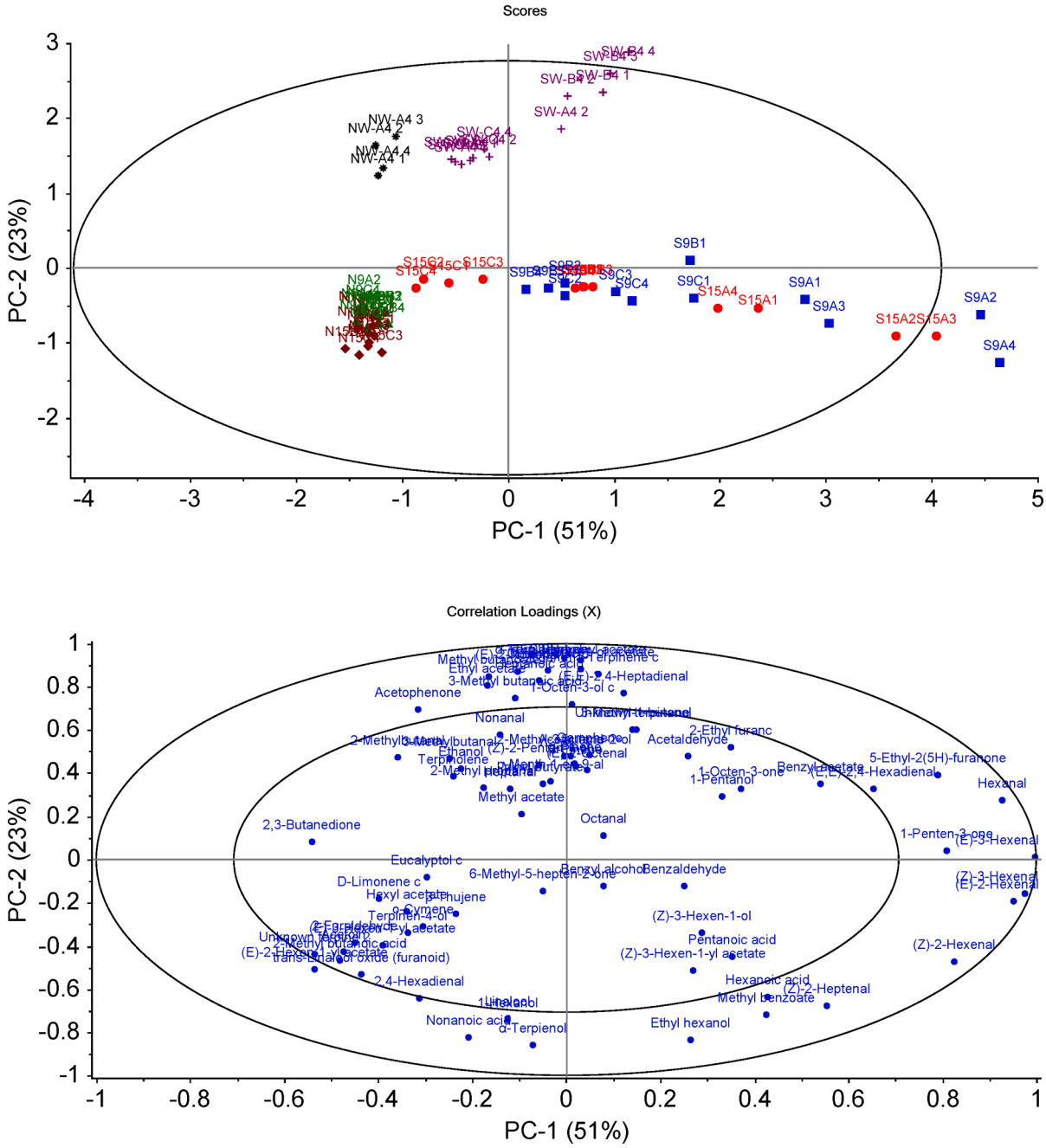

Fig. 1. Plots after principal component analysis (PCA) of volatile organic compounds in the lingonberry samples ripened in the phytotron and harvested from the wild. A: Score plot of berries of southern origin (S) at 15 °C (red circles) and 9 °C (blue squares) and of northern origin (N) at 15 °C (brown diamonds) and 9 °C (green triangles). A, B and C are the three sample parallels from each location and growth condition. There were berries analysed from four locations in the wild (W) in the southern origin (purple cross) and one in the northern origin (black star). B: Loading plot showing the contribution of each compound in the experiment to the differences between the samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

clear trends were seen (Table 2). The variation in both the quantitative and qualitative content of volatiles in lingonberries, in particular the terpenoids and aldehydes, gives indication that the aroma profile of berries from different locations may differ. As aldehydes have low aroma threshold and have been associated to the odour of cranberries, they may be particularly important to distinguish different origins. Variation among the terpenoids and aldehydes could be due to the variation and adaptation to growth conditions at each location, and the complexity of VOC biosynthesis which is comprised of several different regulatory pathways and environmental and genetic interactions (Maffei, 2010). Mechanisms influencing the synthesis of VOCs have been linked to each other, but the exact regulation of biosynthesis is not fully understood (Karppinen et al., 2016).

There was no major difference in the number of VOCs from lingonberries collected from the wild compared to the berries ripened in the phytotron (Table 2). There was, however, a clear difference in the compounds identified, and a separation between lingonberries harvested from wild stands compared to berries ripened in the phytotron in the PCA analysis. Berries from the wild stands clustered with a spread in the direction of PC2 compared to berries ripened in the phytotron, with PC2 explaining 23% of the variation of the dataset (Fig. 1A). Samples collected from the north and the south, however, were clustered and separated in the PC1 in a similar manner as seen for berries ripened in the phytotron. Differences between lingonberries from the northern and

the southern wild stands include a higher number of quantifiable volatiles observed in berries from the southern location.. It has previously been shown that cultivated cranberries (*V. macrocarpon*.) had a smaller number of VOCs than the wild European relative (*V. oxycoccos*) (Sater et al. 2020). The clear differences between lingonberries collected from the wild compared to the berries ripened in the phytotron, indicate that some biotic or abiotic conditions during ripening influenced the profile of VOCs in lingonberries. Different growth conditions like light, weather, and soil composition have comparatively previously been shown to influence the volatile composition in wine grapes (González-Barreiro 2015), and are thus also likely to influence the composition of volatiles in lingonberries. The results from this study show that while there were differences in chemical composition of lingonberries from individual stands, and between berries grown in the wild or ripened in the phytotron, there also were clear differences in the VOC profile between berries from the north and south. These differences are likely to influence the perceived aroma which could give lingonberry products local flavour characteristics.

3. Conclusions

The current investigation of lingonberries showed clear effects of both the ripening temperature and the origin of the lingonberries on their metabolomic profile. Findings from the present study provide insight into how lingonberries accumulate different compounds in relation to external stressors and help us to understand the complexity of the regulation of the metabolites in these berries. Although the effects of both the temperature and place of origin were observed in many compounds, the phenolic compounds were mostly affected by temperature, and the VOCs were largely affected by origin of the berries. Altogether 40 previously undescribed VOCs were identified in lingonberries in this study. The content and composition of metabolites are important to consider, as they are crucial in plant defence against outside stressors and influence the nutritional and sensorial properties of berries. Difference in perceived aroma between lingonberries from different locations could give lingonberry products local flavour characteristics. These types of local flavoured products would be like in wine "terroir" products that are recognized to have local characteristic (González-Barreiro 2015). However, further investigation of the VOCs in relation to sensory perception are needed to assess these differences. As the biosynthesis of volatiles are complex interactional systems, further studies on both the compositional and genetic effects of different environmental conditions are needed to fully understand these interactions in lingonberries. Consequently, ongoing climate change may differentially affect the chemical profile of lingonberries in different growth locations.

4. Experimental

4.1. Chemicals

The chemicals and solvents used in this study were of HPLC-isocratic grade or higher. The water used was purified using a Milli-Q purification system (Millipore Sigma, MA, USA). Standards for HPLC and GC analyses are listed in supplementary material table s3 and included cyanidin-3-*O*-galactoside (Polyphenols AS, Sandnes, Norway), catechin hydrate, quercetin-3-*O*-rutinoside (Sigma–Aldrich, MO, USA), and chlorogenic acid (Fluka, St. Gallen, Switzerland) for analysis of phenolic compounds. Citric, malic and shikimic acids (Sigma–Aldrich, MO, USA), glucose, sucrose, and fructose (Chem Service Inc., West Chester, PA, USA), and quinic acid (Merck, Darmstadt, Germany) for analysis of sugars and organic acids. 4-Methyl-2-pentanol and neryl acetate (Sigma–Aldrich, St. Louis, MO, USA) were used as internal standards in GC–MS analysis. Hexanal, methylbutanal, (*E,E*)-2,4-hexadienal, (*E,E*)-2,4-heptadienal, benzaldehyde, 3-methyl-1-butanol acetate, hexyl acetate, ethyl octanoate, 6-methyl-5-hepten-2-one, 1-pentanol, 1-hexanol, 1-octen-3-ol, hexanoic acid, γ-terpinene, *p*-cymene, linalool, o-cymene, 2-α-pinene, D-limonene 2-ethyl furan, and an n-alkane mixture (C7–C30) were purchased from Sigma–Aldrich (St. Louis, MO, USA), 2-hexenal was purchased from Acros Organics (Antwerp, Belgium), eucalyptol, was purchased from Fluka (Steinheim, Switzerland), and acetophenone was purchased from (Chem Service Inc., West Chester, PA, USA) and used as standards in analysis of volatile organic compounds. Dimethylaminocinnamaldehyde (DMAC) (Sigma–Aldrich, MO, USA) and procyanidin A2 (Extrasynthese, Genay, France) were used for total proanthocyanidin analysis.

4.2. Plant material and experimental design

After the development of early green-stage fruits (Fig. 2), lingonberry plants were collected with intact root systems along with native soil from three wild stands (replicates) in southern (59°4N, 10°5 E, Ås) and northern (69°1N, 18°6 E, Tromsø) Norway in the 2020 growth season. The three wild stands chosen at each location were areas with many plants with fruits set within one location but with some geographical spread (Table s2, Supporting information). The locations in northern and southern Norway were chosen due to a large variation in temperature during the growth season to evaluate whether the lingonberries had made a natural adaptation to temperature. The mean temperatures during the 2020 growth season in the areas chosen for testing were 9.3 °C in the north (August-September) and 17.0 °C in the south (July-August), with 3.2 mm and 4.2 mm of average daily precipitation, respectively (Lussana, 2021). The plants were transported to identical phytotron facilities in Ås (Centre for Climatic Research at the Norwegian University of Life Sciences) and Tromsø (The Arctic University of Norway and NIBIO Climate Laboratory) in their native soil. At the phytotrons, plants were placed in large growth containers and randomly assigned to one of two temperature treatments of 9 °C and 15 °C. The berries were grown under natural light with no artificial illumination and the relative humidity kept at 100 %. The day length at the period of ripening were similar at the different location, with an approximate

Fig. 2. Lingonberry plant before the collection and transport to the phytotron in Ås.

15–13 h light. The plants were watered every other day. The temperature treatments lasted for approximately 4 weeks, until most of the berries were classified as ripe, with the classification of ripeness based on surface colour being completely red. After the first harvest, berries were left to ripen for up to two weeks and picked when they were considered ripe. Within 2 h after harvest, the berries were frozen and kept frozen until analysis to maintain a coherent treatment between the samples from the two testing locations.

4.3. Methanolic extraction

Extraction of phenolic compounds, sugars and organic acids was performed following a modified version of the method described by Davik et al. (2020). Frozen lingonberries (~50 g) were milled for 15 s in a small blade mill and lyophilised for 72 h (Gamma 1–16, Christ GmbH, Osterode am Harz, Germany). The average dry matter in the lyophilised lingonberries was 16.8 ± 0.8 % (Supplementary material, Table s2). A dry lingonberry sample (400 ± 10 mg) was mixed with 70 % methanol in water (v/v) (5 mL) in a vortex mixer for 15 s. This mixture was sonicated for 10 min (Ultrasonic Cleaner, VWR International, Pennsylvania, USA) and centrifuged for 10 min at 39200 × *g* (Avanti J-26 XP Centrifuge, Beckman Coulter, California, USA). After collection of the supernatant, the insoluble plant material was re-extracted with the extraction solvent. The extractions were performed at ambient temperature (20–22 °C). Supernatants were pooled, and the volume was brought up to 20 mL with 70 % methanol in water. Extractions were performed in duplicate. The extracts were filtered through Millex HA 0.45 μm filters (Millipore Corp., Massachusetts, USA) before being transferred to HPLC vials and stored at −80 °C until analysis.

4.4. Analysis of phenolic compounds with HPLC-DAD-ESI-MS

Phenolic compounds were determined using an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler cooled to 4 °C, a diode array detector (DAD), and an MSD XCT ion trap mass spectrometer fitted with an electrospray ionization interface. The method has been described by Aaby et al. (2013). For analysis, 10 μL of methanolic extract was injected with separation on a Synergi 4 μm MAX RP C12 column (250 mm × 2.0 mm i.d.) equipped with a 5 μm C12 guard column (4.0 mm × 2.0 mm i.d.), both from Phenomenex (Torrance, California, USA). The mobile phase was a binary solvent system consisting of (A) formic acid/water (2/98, v/v) and (B) acetonitrile. The gradient (0–60 min with 5–60 % of B in gradual steps) eluted at a flow rate of 0.25 mL/min at 40 °C. The mass spectrometer was operated in positive and negative ion modes. Identification of phenolic compounds was performed based on their UV–vis (220–600 nm) and mass spectra and retention times relative to external standards, which were compared to previous reports of phenolic compounds in lingonberries (Bujor et al., 2018; Ek et al., 2006; Hokkanen et al., 2009; Marsol-Vall et al., 2020). Quantification was performed based on calibration curves of external standards. Anthocyanins were quantified as mg/100 g fresh weight (fw) equivalents of cyanidin-3-*O*-galactoside at 520 nm, flavonol glycosides as quercetin-3-*O*-rutinoside at 360 nm, cinnamic acid derivatives as chlorogenic acid at 320 nm, and flavan-3-ols as catechin at 280 nm.

4.5. Spectrophotometric analysis of total proanthocyanidins

The total proanthocyanidin content was quantified according to a method described by Sintara et al. (2018). The methanolic sample extracts were diluted with methanol 1/24 (v/v). The diluted sample (20 μL) was pipetted into a 96-well plate (Thermo Fisher, Massachusetts, USA), and 100 μL of 1 mg/mL dimethylaminocinnamaldehyde (DMAC) in acidic methanol (0.4 N H_2SO_4) was added. Immediately after the addition of the DMAC solution, the sample absorption was measured at 640 nm on a spectrophotometer (SpektrostarNano, BMG Labtech, Baden-Wuerttemberg, Germany), with subsequent readings every minute for 10 min. Quantification was performed based on calibration curves of an external standard of procyanidin A2 using the average of the three last readings of the samples.

4.6. Analysis of volatile organic compounds by HS-SPME-GC-MS

VOCs were determined with a Trace 1310 gas chromatograph coupled with a TSQ 8000 EVO mass spectrometer (Thermo Scientific, Reinach, Switzerland). Extraction of volatiles was performed in a TriPlus RSH multipurpose autosampler (Thermo Scientific, Reinach, Switzerland) by using HS-SPME with a 2 cm DVB/CAR/PDMS 50/30 μm fibre (Supelco, CA, USA). Two grams of partially thawed lingonberry samples were weighed into a 20 mL HS vial. Samples were spiked with 10 μL of the internal standard mix (4-methyl-2-pentanol at 100 μg/mL and neryl acetate at 113 μg/L in methanol), and then the berries were crushed. Initially, the samples were equilibrated for 10 min at 45 °C, and the fibre was exposed to the headspace of the sample vial for 30 min at 45 °C. Volatiles were thermally desorbed in the injection port at 220 °C, and spitless injection was applied. The separation of compounds was performed with a polar capillary column (DB-WAX, 60 m × 0.25 mm × 0.25 μm: J&W Scientific, Folsom, CA, USA). Helium was used as a carrier gas with a constant flow of 1.6 mL/min. The oven was temperature-programmed from 50 °C (hold = 3 min) to 200 °C with a constant ramp of 5 °C/min (hold = 14 min). Mass selective detection was performed in the scan mode (33–300 *m/z*; EI (70 eV)). The interface temperature was set to 200 °C, and the ion source was set to 220 °C. Identification of the VOCs was performed by probability-based matching of the obtained mass spectra with the mass spectra from the National Institute of Standards and Technology database (NIST20) with authentic standard compounds when available. As a second criterion for the identification, Kovats retention indices (RIs) were calculated using an n-alkane mixture (C7–C30). Chromeleon (7.2.10, Thermo Scientific, Reinach, Switzerland) was used to perform peak detection, base ion detection, and peak area integration. Peak areas were normalized to the internal standards (compound area/ISTD area) previously added to each sample. Samples were analysed in quadruplicate.

4.7. Analysis of sugars and organic acids by HPLC-DAD-RI

Sugars and organic acids were determined using an Agilent 1100 series HPLC system equipped with a DAD and a refractometer index (RI) detector (Model 132; Gilson, Villiers-le-Bel, France) as described (Woznicki et al., 2017). Methanolic extracts (20 μL) were injected, and separation was performed on a Rezex ROA-Organic acid H+ (8 %) column (300 × 7.8 mm; Phenomenex, California, USA) at 45 °C with a mobile phase of 7.2 mmol/L H_2SO_4 and a flow rate of 0.5 mL/min. External standards of glucose, sucrose, fructose, and citric, malic, shikimic and quinic acids were used for quantification. The sugars were detected with an RI detector, and the organic acids were detected with DAD at 210 nm.

4.8. Statistical analyses

To assess the effect of origin and growth temperature on lingonberries grown in the phytotron, two-way analysis of variance (ANOVA) was performed. Tukey's honestly significant difference (HSD) test was performed to determine significant differences ($p < 0.05$) between all the samples. These tests were performed using the R-core package (R Core Team, 2020). To illustrate the variation among the samples in the profile of volatile organic compounds, principal component analysis (PCA) was performed with Unscrambler Software (Unscrambler®X version 10.4.1, CAMO Software AS, Oslo, Norway). All variables were weighed by 1/square root of the standard deviation before analysis.

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CRediT authorship contribution statement

M. Amundsen: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft. **L. Jaakola:** Conceptualization, Methodology, Resources, Writing – review & editing. **K. Aaby:** Resources, Methodology, Writing – review & editing, Funding acquisition. **I. Martinussen:** Conceptualization, Project administration, Funding acquisition. **N. Kelanne:** Investigation, Data curation, Methodology, Writing – review & editing. **S. Tuominen:** Investigation, Data curation, Formal analysis. **O. Laaksonen:** Methodology, Writing – review & editing. **B. Yang:** Methodology, Writing – review & editing, Funding acquisition. **AL. Hykkerud:** Conceptualization, Methodology, Investigation, Writing – review & editing, Methodology, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.112738.

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PAPER IV

Influence of growth conditions on the content of anthocyanins, sugars, and organic acids in wild populations of lingonberries (*Vaccinium vitis-idaea* L.) across Norway

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Abstract: Lingonberry (*Vaccinium vitis-idaea* L.) is a wildly growing dwarf shrub that gives a scarlet red berry, rich in several health promoting compounds. Lingonberries from 56 wild stands at seven locations across Norway from 59°N, 10°E to 69°N, 18°E were harvested in triplicate samples for three consecutive years to study the effects of latitude, altitude, climatic and forest characteristics on the composition of sugars, organic acids, and anthocyanins. Five anthocyanins all cyanidin-glycosides, three sugars and four organic acids were identified. The mean contents of anthocyanins, organic acids, and sugars were 100 mg/100 g fw, 2.8 g/100 g fw, and 6.9 g/100 g fw, respectively. Temperature during the last 30 days of ripening and latitude were the stand characteristics that influenced the concentration of anthocyanins, organic acids, and sugars most significantly. There was also a slight effect of both precipitation during the last 30 days of ripening and tree volume on these compounds. The volume of pine trees in the stand did not influence the contents of anthocyanins, organic acids, or sugars in lingonberries. On the contrary a volume of spruce decreased the total content of anthocyanins, whereas of deciduous trees density slightly increased the content of anthocyanins and organic acids. The precipitation level negatively correlated with the organic acid content in lingonberries. This study is the first to comprehensively investigate the effects of forestall conditions on the chemical composition in lingonberries and show that it is influenced by both the environment and the geographical origin of the plants. The study will help unravel how different growth conditions influence the nutritional quality and sweetness of wild lingonberries.

Keywords: Wild berries, Fruit quality, Phenolic compounds

1. Introduction

Lingonberries (*Vaccinium vitis-idaea* L.) is a dwarf shrub native to the circumpolar region. The scarlet red berries are harvested every autumn from wild stands, and they are a staple of the Nordic cuisine and diets. The lingonberries are known for their characteristic flavour often described as sour and quite tart, bitter, and astringent [1-2]. Lingonberries have been used in traditional Nordic, North American, Russian and Chinese medicine against a number of diseases[3]. Recently, there has been a growing interest in lingonberries due to their beneficial health effects against many of the modern lifestyle diseases[4]. Berries contain high levels of phenolic compounds and are considered beneficial as they have been identified to reduce prevalence of several cardiovascular diseases [5-6]. Increased consumption of fruits, berries and vegetables is recommended to improve human health and improve sustainability of our diets [7-8]. Therefore, there is an aim to increase the proportion of fruits and berries in nutrition recommendations of the Nordic countries[9]. Anthocyanins are among the important bioactive compounds responsible for the health benefits of berries. Anthocyanins are also important water-soluble pigments responsible for much of the red, blue and purple colours in nature[10]. Contents of sugars and organic acids, and their relative abundance is the most important contributor of the flavour of fruits and berries[11] It has been identified that berries with higher proportion of sugars to organic acids are generally preferred [2].

Among the berries found in Nordic forests, lingonberries are the most prosperous[12]. *Vaccinium vitis-idaea* L is an adaptable species, that thrives in leached soils with low pH, low base saturation, and low calcium content[13]. Lingonberries can, however, grow on a variety of different soils and nutrient contents, as well as forests of different types. The

reproductive performance of lingonberries is linked to changes in temperature [14]. In Scandinavia their altitude limit is largely determined by snow cover and exposure to extreme cold temperatures [13]. In Norway, lingonberries are found across the country from the very south (57°N) to the north (71°N) with heathers reported at altitudes as high as 1800 m above sea level [15]. Field studies of various berries have shown that their chemical composition can vary greatly based on the year of harvest and from where the berries are harvested[16-21].Environmental conditions influence the ability to synthesize secondary metabolites in berries. It is important to improve the understanding of how the concentration and composition of phenolic compounds, sugars and organic acids are affected by external as well as internal factors to identify high quality berries. Though the biosynthesis of anthocyanins, sugars and organic acids is well understood, the influence of genetic and environmental factors on these processes' is an ongoing field of research[11, 22-23]. Both abiotic and biotic factors can influence the composition of these compounds through different ways of regulation. Light is among the most important factors regulating synthesis of metabolites in plants. Solar radiation on the forest floor varies with latitudes, altitudes, time of day and season in addition to forest characteristics. Besides the day length, difference in the ratio between red and far-red radiation is the major factor affecting the variation in light conditions between latitudes[24]. Additionally, the amount of radiation that reaches the plants has been shown to influence the composition of various compounds in the berries [11]. Temperature is another key factor in the regulation of metabolites in plants, in addition to the growth medium composition[11]. Previous field studies of lingonberries have indicated that higher latitudes, and lower temperatures are preferred for synthesis of anthocyanins. In a controlled condition phytotron study it was found higher concentrations of anthocyanins and organic acids in lingonberries ripened under lower temperatures. In the same study no effect was found on the content of sugars[25]. No earlier study was identified analysing the variation in content of organic acids of wild lingonberries. This study aims at providing a comprehensive analysis of how growth conditions affect the composition of anthocyanins, sugars, and organic acids of wild lingonberry populations across Norway. This study is the first multi-year screening of quality in wild stands of lingonberries in Norway.

2. Materials and Methods

2.1. Berry samples and sample pretreatment

Lingonberries were collected from seven locations across Norway from 58°N, 10°E to 69°N, 12°E (Figure 1), with eight stands at each location representing the local forest variation. Berry samples were collected three consecutive growth seasons (2019-2021). The stands were circular plots of 250 m^2, divided in three sectors being replicates representing local biological variation. Data on the forest characteristics of all field stands was recorded during the 2020 growth season according to the standard procedure of the Norwegian forest inventory[26], and climatic data was collected from the Meteorological Institute of Norway[27] (Figure 1). Mean daily temperature and precipitation was collected during the last thirty days prior to harvest and ranged from 6.2 – 15.9 °C and 0.44 – 6-7 mm/day in the different stands respectively[27]. From each of the sectors, up to 250 ml of undamaged lingonberries were harvested by hand. After harvest, the berries were frozen as rapidly as possible and within 8 hours, and shipped frozen to the testing facilities, where they were kept frozen at -40 °C until analysis. Samples that did not adhere to the quality criterion due to incomplete ripeness were excluded from the analysis. In total 438 lingonberry samples were analysed in duplicate over the study period. Frozen lingonberries were milled before were freeze-drying for 72 h with a pressure limit of 0.633 mbar (Gamma 1-16, Christ GmbH, Osterode am Harz, Germany)with an average water loss of 83.6 % (± 1.3 %). Extraction of the targeted compounds from the freeze-dried lingonberries was performed was performed at ambient temperature (20–22 ◦C) in duplicate using 70% methanol (VWR, Radnor, Pennsylvania, USA) in Milli-Q water (Millipore Sigma, Massachusetts, USA) according to a previously described method[28-29]. Dry lingonberry samples (400 ± 10 mg) were mixed with 5 mL of the 70 % methanol in water (v/v) in a vortex mixer for 15 s. The mixture was sonicated for 10 min (Ultrasonic Cleaner, VWR International, Pennsylvania, USA) and centrifuged for 10 min at 39200 × g (Avanti J-26 XP Centrifuge, Beckman Coulter, California, USA). After collection of the supernatant, the insoluble plant material was re-extracted with the extraction solvent. Supernatants were pooled, and the volume was brought up to 20 mL with the 70 % methanol in water. Results of the analyses are expressed on a 100 g of fresh weight (fw) of berries recalculated from the dry matter using the individual sample water loss during drying.

Location	Latitude (°N)	Temperature (°C/day)	Precipitation (mm/day)	Altitude (m)	Tree Volume (m^3/stand)
AAS	59.6	15.0	2.9	107	65
KON	60.2	13.9	1.2	268	139
REN	61.6	10.4	4.5	240	23
MID	63.9	12.5	1.8	345	78
TR1	69.0	10.0	2.6	578	109
TR2	69.0	9.3	2.7	143	105
PAS	69.3	9.0	1.1	83	66

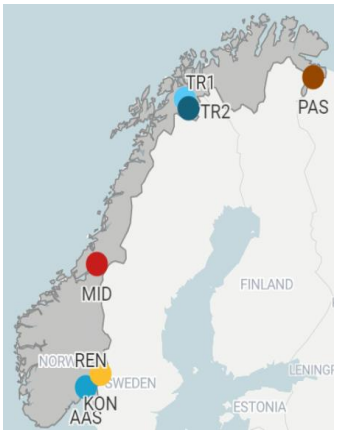


Figure 1 Locations for collection of lingonberry samples in Norway, and mean characteristics of the stands. At each location 8 stands were set up representing local variation in forest conditions (Created using datawrapper.de)

2.2. Analysis of anthocyanins

The contents of the anthocyanins were determined using an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler cooled to 4 °C, a diode array detector (DAD), and an MSD XCT ion trap mass spectrometer fitted with an electrospray ionization interface. The method followed a previously described protocol[29]. For analysis, 10 µL of methanolic extract was injected with separation on a Synergi 4 µm MAX RP C12 column (250 mm × 2.0 mm i.d.) equipped with a 5 µm C12 guard column (4.0 mm × 2.0 mm i.d.), both from Phenomenex (Torrance, California, USA). The mobile phase was a binary solvent system consisting of (A) formic acid/water (2/98, v/v), and (B) acetonitrile. The gradient (0-60 min with 5-60% of B in gradual steps) eluted at a flow rate of 0.25 mL/min at 40 °C. The mass spectrometer was operated in positive and negative ion modes. The nebulizer pressure was 40 psi; dry gas flow, 10 L/min; dry temperature, 350 °C; and capillary voltage, 3.5 kV. Identification of the anthocyanins was done based on their UV-vis (220–600 nm) and mass spectra and retention times, that were compared to authentic standards and previous findings [30-32]. Quantification of all anthocyanins was performed based on an external standard calibration curve of cyanidin-3-*O*-galactoside (Polyphenols AS, Sandnes, Norway). The results are reported as equivalents of cyanidin-3-galactoside at 520 nm and expressed as mg per 100 g of fresh weight (fw) of berries.

2.3. Analysis of sugars and organic acids

For determination of sugars and organic acids, 20 µl of methanolic sample extract was injected to an Agilent 1100 series HPLC system, equipped with a DAD and a refractometer index (RI) detector (Model 132; Gilson, Villiers-le-Bel, France) as previously described [33]. Separation was performed on a Rezex ROA-Organic acid H+ (8 %) column (300 × 7.8 mm; Phenomenex, California, USA) at 45 °C with mobile phase 7.2 mmol/L H_2SO_4 and flow rate 0.5 mL/min. External standards of glucose, sucrose, and fructose (Chem Service Inc., West Chester, PA, USA), and citric, shikimic, malic (Sigma–Aldrich, MO, USA), and quinic acid (Merck, Darmstadt, Germany) were used for identification and quantification. The sugars were detected with RI detector and the organic acids with DAD at 210 nm.

2.4. Statistical analyses

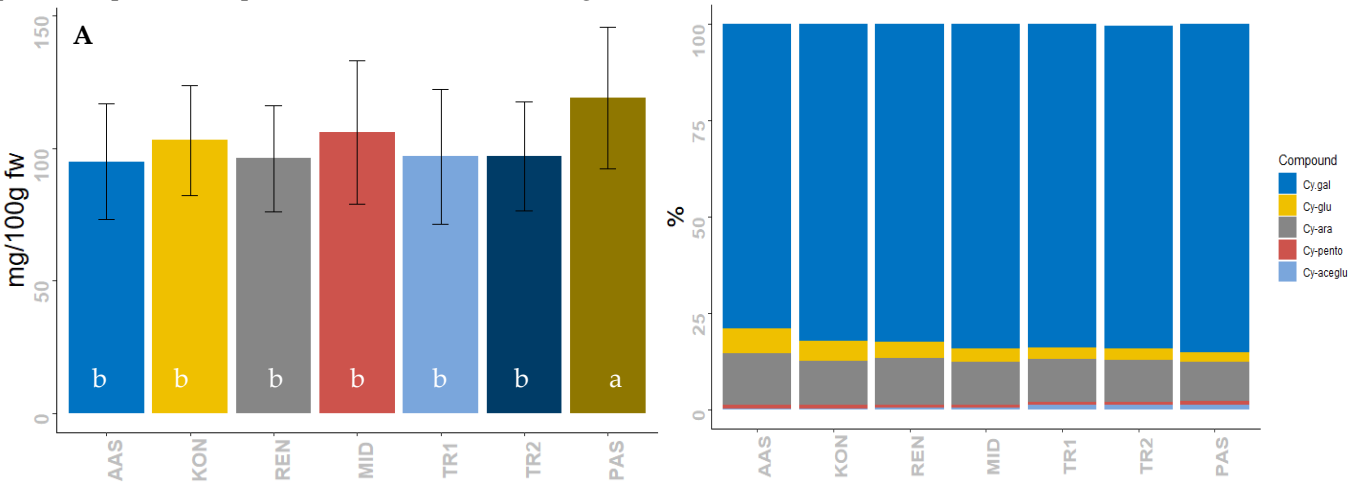
To study the significant differences ($p < 0.05$) in the content of sugars, organic acids, and anthocyanins between the seven locations, an analysis of variance (ANOVA) with a Tukey's honestly significant difference (HSD) test was performed. For assessment of relationships and co-variability between the content of the different compounds, a Pearson's-correlation test was performed. Linear regression models with latitude, altitude, temperature, precipitation, stand volume of spruce, pine, and deciduous trees and interactions between latitude, temperature and altitude as independent variables, and the composition of each of the compounds measured as dependent variables. The statistical analyses were performed using R with the core and ggcorr packages [34]. The interactions in the regression models were chosen due to the strong correlation between temperature and latitude (Pearson's correlation factor ~0.8) and the known relationship between temperature and altitude. To illustrate the variation among the samples, principal component analysis (PCA) with the individual compounds as variables was performed with the Unscrambler Software (The Unscrambler®X version 10.4.1, CAMO Software AS, Oslo, Norway). In the PCA, all variables were weighed by 1/standard deviation before analyses.

3. Results and Discussion

3.1. Qulity of wild Norwegian lingonberries

3.1.1. Anthocyanins

The mean anthocyanin content in Norwegian lingonberries was 100 mg/100 g fw (Figure 2). There were differences between locations and stands, with the stand with the highest content of anthocyanins (160 mg/100 g FW), having 2.8 times higher content of anthocyanins than the stand with the lowest content (56 mg/100 g FW). Lingonberries collected from the location the furthest north on average had a significantly higher content of anthocyanins than the other stands, which were not significantly different from each other. Previous studies have found the content of anthocyanins to vary greatly, ranging from 30-200 mg/100 g FW[16, 35-36]. In a study of wild Lithuanian lingonberries it has been reported a large difference in difference between the stands with the highest and lowest anthocyanin content[16]. Also in lingonberries of different cultivars grown under the same growth environment large differences in the anthocyanin content was found[37]. In wild bilberry populations in Finland 4.5 difference between different plots has been detected [20], with large variation also found in sea buckthorn and blackcurrant between different plots and years[38-39]. The anthocyanin content of lingonberries is significantly lower than in darker berries like bog bilberries (*V. uglinosum* L.) (194 mg/100 g FW) and bilberries (*V. myrtillus* L.) (411 mg/100 g FW), but comparable to the content found in cranberries (140 mg/100 g FW), strawberries (21.2 - 41.7 mg/100 g FW), raspberries (92.1 mg/100 g FW) and sweet cherries (122 mg/100 g FW)[40]. The UV and mass spectra revealed that only cyanidin glycosides were present in lingonberries, with a fragment of m/z 285 in negative mode and m/z 287 in positive mode. The neutral loss revealed the presence of two hexosides (galactose and glucose) and two pentosides (arabinose and unknown) and a compound with neutral loss of 204 amu, which was identified as acetyl glucoside, as this compound has been previously reported in lingonberries. The most abundant anthocyanin was cyanidin-3-O-galactoside with a mean of 83.2 % (69-90 %), followed by 11.5 % (6.1-22.3 %) of cyanidin-3-O-arabinoside, 3.7 % (1.5-9.3 %) of cyanidin-3-O-glucoside, and 0.8 % of both cyanidin-3-O-pentoside (0-1.7 %) and -acetyl hexoside (0-3.8 %) (Figure 2). The three most abundant anthocyanins, cyanidin-3-O-galacoside, -glucoside and arabinoside, have also been the most widely studied and typically contribute to more than 98 % of the total anthocyanin content in lingonberries [32, 41-42]. In line with our results, also previous reports of lingonberry anthocyanins showed a large variation in the relative proportions of the main anthocyanins with 65-88 % cyanidin-3-O-galactoside, 10-20 % cyanidin-3-O-arabinoside and 1.4-12.3 % cyanidin-3-O-glucoside[18, 41-43]. One study was identified that reported traces of delphinidin, petunidin, peonidin and malvidin in lingonberries [16].



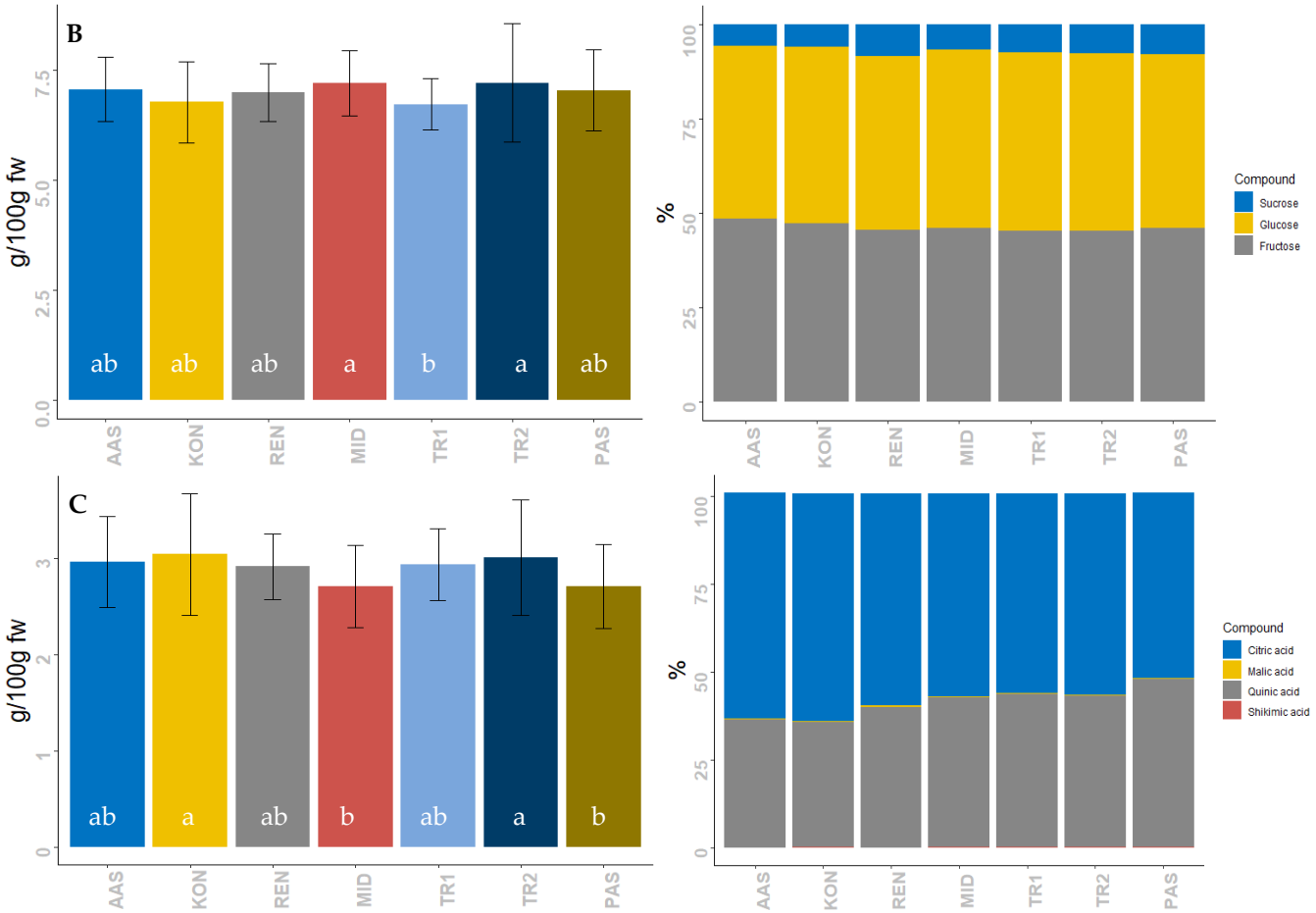


Figure 2 Mean content ± standard deviation of anthocyanins (mg/100 g fw) (A), sugars (g/100 g fw) (B) and organic acids (g/100 g fw) (C) in wild Norwegian lingonberries. Different letters (a-c) specify significant differences ($p < 0.05$) between the samples as determined by the Tukey's HSD test. Proportion of the individual anthocyanins, sugars and organic acids in wild Norwegian berries as expressed by % share of content.

3.1.2. Sugars and organic acids

The mean content of sugars in wild stands of lingonberries was 6.9 g/100 g fw (4.4 – 11.4 g/100 g fw) (Figure 2). The content of sugars measured was comparable to that previously reported in the Norwegian food composition table (7.0 g/100 g fw). In berries, mean content from 8.2 g/100 g fw to 9.4 g/100g fw (553 mg/g dry weight), and in juices mean sugar content from 7.3 to 9.0 g/100 ml were reported [32, 41, 44-45]. Compared to other berries, lingonberries are neither in the high- or low-end considering content of sugars [1, 46]. The saccharides identified in wild lingonberries in this study were fructose (40 – 51 %), glucose (42 – 52 %) and sucrose (0 – 12 %). Some variation in the proportions of the sugars observed between studies[46-48], with the two that have analysed juices of lingonberries showing little to no content of sucrose[1, 41]. This could be due to an enzymatic degradation of sucrose to fructose and sucrose during juice processing. As expected, there was a close association between glucose and fructose, but only a weak correlation between the two and content of sucrose (Figure 3). Though there was not a huge difference in content of sugars between the seven growth locations, both content and proportions of sugars was significantly influenced by growth locations.

The mean content of organic acids was 2.8 g/100 g fw (1.8 – 4.8 g/100 g fw) (Figure 2). Lingonberries are among the Nordic berries with the highest content of organic acids. In lingonberry juices, concentrations from 2.0 - 3.2 g/100 ml are reported. This is also comparable to what has been reported in wild Finnish, Swedish and Slovenian lingonberries[1, 41, 46, 49].Despite their importance in flavour there has been relatively little research on the organic acid content in lingonberries compared to the research on sugars and phenolic content. The acids measured were mostly citric (35 – 64 %) and quinic (25 - 79 % acid), with a low proportion of malic and shikimic acid (< 1 %). Organic acid content is also comparable to previously reported results [1, 32, 41, 46, 49-50]. The ratio of sugars to acids in the present study was on average 2.48, with a large variation around the mean (0.97-3.8). The sugar to acid ratio was in the medium range of what has been reported previously in lingonberry juices (2.27and 2.72) [41, 46]. The sugar to acid ratio is important in determining the

taste perception of sweetness in berries and fruits. Strawberries that are perceived as a sweeter berry, and have according to the Finnish National Institute for Health and Welfare typically have the same sugar content as lingonberries (7.0 g/100 g fw) but a lower content of organic acids (1.6 g/100 g fw) [51] making this ratio higher and the reason why strawberries are considered sweeter, whereas sea-buckthorn which similarly to lingonberries has a very acidic and bitter taste, has a sugar content of 2.1 g/100 g fw and an acid content of 1.9 g/100 g fw, the ratio being close to 1 [52]. In a study comparing this ratio between different species lingonberries was among the least sweet tasting together with berries in the *Grossulariaceae* family[46]. Though the overall content of the sugars is considered to play the most significant role in the taste, quality, and perceived sweetness and flavour, it is well known that there is variation between different sugars in sweetness.

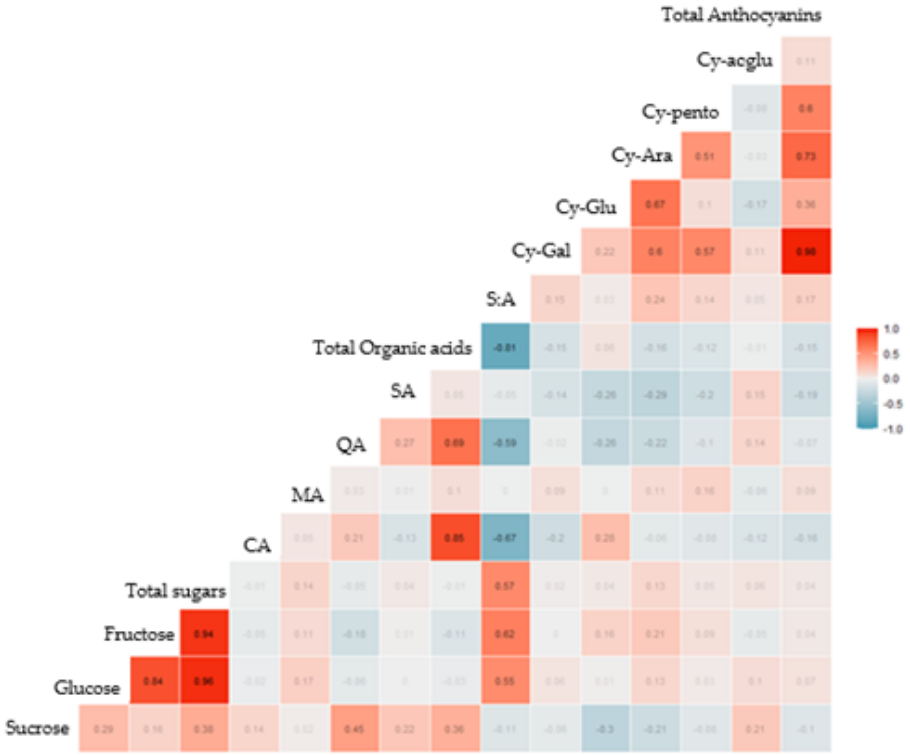


Figure 3 Pearson's correlation coefficients of relationships between different compounds found in lingonberries. Abbreviations: Cy-cyanidin. Gal-galactoside, Glu-glucoside, Ara-arabinoside, pento-pentoside, acglu-acetyl glucoside, CA-citric acid, QA-quinic acid, MA-malic acid, SA-shikimic acid, S:A - sugar to organic acid ratio.

3.1.3. Variation between stands and locations

There were slight significant differences in content of anthocyanins, sugars and organic acids between the seven locations (Figure 2), with significant differences mostly seen between one or two of the stands.

The average variation between the three biological replicates collected from the same stand had a higher variation with an average anthocyanin standard deviation of 11.1 mg/100 g fw, 0.43 g/100g fw for sugars and 0.24 g/100 g fw of organic acids, while the mean deviation between replicate analysis was 3.5 mg/100g fw for anthocyanins, 0.16 g/100g fw for sugars and 0.07 g/100g fw for organic acids and. This shows that even within a single stand there can be an approximately 10 % variation in quality. Previous studies on ripeness of lingonberries and cranberries have shown that during the early stages of berry ripening there is high content of organic acids, but as the berries ripen, they accumulate more sugars and anthocyanins and organic acid content decreases [16, 53-55]. Time from ripeness to harvest has been shown to influence the composition of the berries[55]. Therefore, it is recognized that this could also have influenced the results of this study, though there was no correlation between the total content of anthocyanins or any of the individual compounds and the content of sugars or organic acids (Figure 3). The differences within a single stand (250 m^2) could also be due to variation in clones of lingonberries or differences in the shading or soil quality parameters. Soil quality has previously been shown to influence the composition of lingonberries [16]. Recent studies have also shown that lingonberry quality can be associated with the genetic background of the plant[37]. The genetic background is likely to interact with the changes in growth environment to influence the composition of the berries [22]

3.2. *Effects of growth conditions on chemical composition of lingonberries*

The two first components of the PCA explained 89 % of the variation in the dataset (Figure 4A). The PCA showed that variation in anthocyanin contents explained more of the variation between the samples than the variation in contents of sugars and acids in the lingonberries in this study (Figure 4B). The largest variation was seen in the content of cyanidin-3-*O*-galactoside. As there were minor significant differences between berries from the seven stands (Figure 2) and low correlation coefficients between the groups of compounds indicate that different growth conditions affected the various compounds (anthocyanins, sugars, and acids) differently.

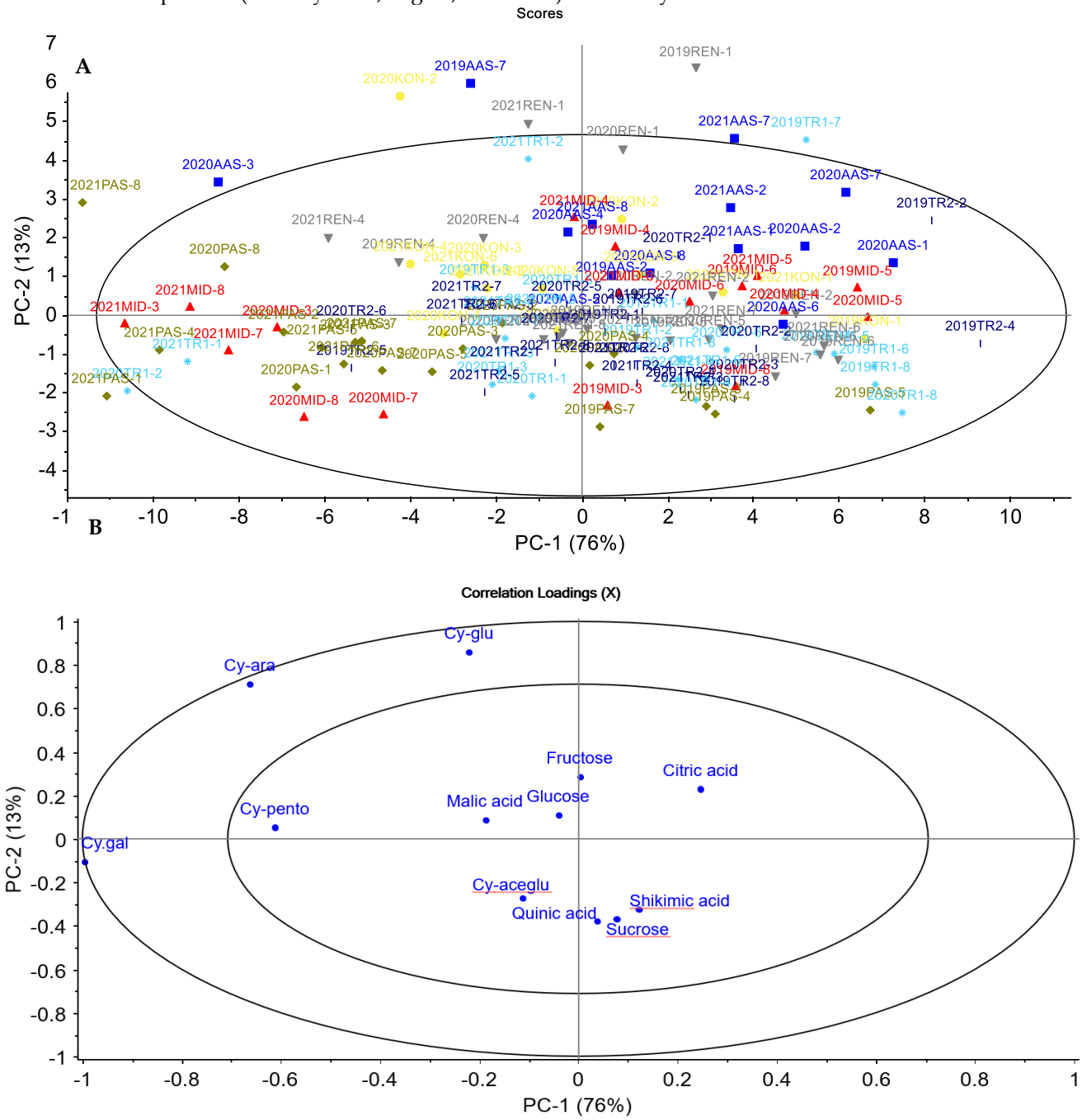


Figure 4 Principal component analysis (PCA), A: Score plot of lingonberries harvested from seven locations and up to eight stands from each location (1-8) over three years. The colour and symbols in locations from south to north; AAS = blue box, KON = yellow dot, REN = grey inverted triangle, MID = red triangle, TR1 = light blue star, TR2 = dark blue line and PAS = green diamond. B: Loading plot showing the contribution of each compound in the experiment to the differences between the samples. Abbreviations: Cy-Cyanidin, gal-galactoside, glu-glucoside, ara-arabinoside, pento-pentoside, aceglu-acetyl glucoside.

The overall linear regression for sugars and anthocyanins were significant, whereas the total model for organic acids was not (Table 1). The regression models for content of anthocyanins, organic acids, and sugars in lingonberries showed that latitude, temperature, altitude precipitation and forest stand density all significantly explained part of the variation in the composition of these compounds. The two clearly most dominant factors influencing the quality of sugars and anthocyanins in lingonberries were latitude and temperature. For organic acids however, precipitation significantly explained most of the variation in the composition. Latitudes do not inherently influence quality but they do influence the composition due to natural differences in growth temperatures, and several aspects of light. At higher latitudes there are changes in the light conditions due to earths angle compared to the sun[24]. A key aspect in these changes in the daily irradiance distribution with a prolonged period with shift toward red and far-red light, due to light scattering in the atmosphere [24]. The ratio of red to far red light is particularly important in the metabolism of plants. It is of particular importance as light quality has been shown to influence the metabolism of several fruits and berries [22, 24]. Also, in other *Vaccinium* species it has been shown that supplemental radiation of both blue and red light influences the synthesis of anthocyanins[56] and sugar [57]. Regression models for the quality compounds in lingonberries had model fits (R^2) between 0.069 (Sucrose) and 0.371 (Cyanidin-glucoside) (Table 1). It is generally challenging to interpret results from wild stands of berries, due to strong correlations between several of the factors that influence metabolisms in lingonberries. The regulation through influence of different growth factors can be challenging to interpret, and is also influenced by genetic factors [22]. Improved understanding of the mechanistic influences is needed to make interpretations of how these factors are coregulated. While a substantial amount of the variation in anthocyanins can be explained by the model, there is still much to discover surrounding environmental and genetic influence on the quality of lingonberries. The content of sugars as primary metabolites was more poorly described by the model, which could be due to the complexity of the metabolism, compared to the secondary metabolites like anthocyanins. This is a common problem when studying fruits and berries in the wild, since latitude and temperature often co-varies, with lower temperatures at higher latitudes[14].

Table 1 General linear regression model coefficients (R and model constant/intercept), beta values[a] and significance levels[b] of latitude, altitude, forest characteristics, and weather conditions on the composition of sugars and organic acids (g/100g fw) and anthocyanins (mg/100g fw) in lingonberries.

	Cy-Gal[c]	Cy-Glu	Cy-Ara	Cy-Pento	Cy-Acglu	ACN	Suc	Glu	Fru	Sugars	CA	QA	MA	SA	Acids	S:A
R^2	0.232	0.371	0.122	0.290	0.290	0.195	0.188	0.069	0.125	0.077	0.312	0.236	0.079	0.144	0.221	0.166
Intercept	-1345***	32.36	47.2	-32.45***	7.11	-1295***	0.23	18.4**	29.09***	47.72***	3.02	-11.8***	-0.16	-0.02	-8.9	25.61** *
Latitude (°N)	21.86***	-0.44	-0.41	0.51***	-0.09	21.49***	0	-0.23*	-0.38***	-0.6***	-0.03	0.19***	0	0	0.16	-0.33***
Temperature (°C/30 days)	111.01***	-0.85	0.19	2.93***	-1.23	112.19***	-0.02	-1.01*	-1.44***	-2.46*	-0.02	0.78***	0.01	0	0.76	-1.41*
Altitude (m)	7.1***	-0.13	0.37	0.19***	-0.07	7.44***	0.01	-0.05	-0.06*	-0.1	-0.08***	0	0	0	-0.08**	0.03
Precipitation (mm/30days)	2.47	-0.16	-1.16	0.48***	-0.68***	1.12	-0.01	0.06	-0.13	-0.08	0.46***	0.15***	0	0	0.62***	-0.54***
VMPRHAL (m^3/stand)	0.73***	0.02*	0.12***	0.01*	0.01	0.89***	0***	0	0	0	0.01*	0*	0	4.3E-5***	0.01***	-0.01
VMPRHAF (m^3/stand)	0.02	0	0	0	0	0.02	0	0	0	0	0.001***	0	0	3.7E-6*	0	0
VMPRHAG (m^3/stand)	0.04	-0.03***	-0.08***	0	0	-0.07	0	0	-0.01***	-0.01	0	0	0	0	0	0
Lat x Temp	-1.73***	0.01	-0.01	-0.04***	0.02	-1.76***	0	0.02*	0.02***	0.04*	0	-0.01***	0	0	-0.01	0.02*
Lat x Alt	-0.11***	0	-0.01	-0.002***	0	-0.11***	0	0	0*	0	-1.9E-5***	0	0	0	0.001**	0
Temp x Alt	-0.54***	0.02*	-0.02	-0.01***	0.01	-0.55***	0	0	0	0	0.01***	0	0	0	0.01*	0
Temp x Perc	-0.11	0.02	0.1	-0.05***	0.06***	0	0	0	0.01	0.01	-0.04***	-0.01*	0	0	-0.04 ***	0.04***
Lat x Temp x Alt	0.01***	-0.0003*	0	0.0002***	0	0.01***	0*	0	0	0	-9.4E-5***	0	0	0	-8.3E-5*	0

[a]Slope coefficient (b-values) and significance levels (p) ***<0.05 **<0.01, *<0.05. [b]VMPRHA, stem volume per hectare; G, spruce, L-deciduous, F- pine. [c]Abbreviations: Cy, cyanidin; Gal, galactoside; Glu-glucoside, Ara-arabinoside, Pento-pentoside, Acglu-acetyl glucoside, Suc-sucrose, Glu-glucose, Fru-fructose, CA-citric acid, Qa-quinic acid, Ma-malic acid, Sa-shikimic acid, S:A - sugar to organic acid ratio.

3.2.1. Anthocyanins

Latitude and mean temperature during the last 30 days gave the most significant influence on the content of anthocyanins in lingonberries (Table 1). There was also an effect of the interaction of temperature and latitude, and temperature and altitude. Previous studies conducted in field conditions have also identified latitude and temperature as key factors that affect anthocyanin content [16-17, 58]. A study on Lithuanian lingonberries found no impact of latitude or temperature on anthocyanin content, but there was little geographical variation among the samples[58]. Conversely, a study on lingonberries from Newfoundland demonstrated that lower temperatures and higher latitudes increase the anthocyanin content [17]. In this study higher latitudes and temperatures both increased content of anthocyanins in lingonberries. This finding aligns with a study conducted on lingonberries grown under controlled conditions at two latitudes, which showed that lower temperatures increased anthocyanin content in both northern and southern clones, with slightly higher content at higher latitudes[25]. Additionally, another separate study indicated that light conditions could influence anthocyanin synthesis in lingonberries, as those grown under controlled conditions with supplementary blue light exhibited slightly higher concentrations of anthocyanins compared to red and far-red light (Unpublished results). The controlled condition studies can explain the effect of the interaction coefficient between the temperature and latitude. As temperatures rise at a specific latitude, the anthocyanin content decreases, whereas berries grown at the same temperature, will have lower content of anthocyanins at higher latitudes. Also, in previous field studies of other bilberries, bog blueberries and currants, latitude has been identified as key factors influencing the content of anthocyanins[19-20, 59-60]. In currants increased temperature has also been associated with lower content of anthocyanins[59-60]. Lower temperatures are typically associated with carbon storage in more challenging environments [61]. It has previously been established that cooler temperatures favour the synthesis of bioactive compounds like phenolics and vitamin C, though the optimal temperature is species dependent[22]. The contents and proportions of the anthocyanidin glycosides were significantly different between the 7 locations (Figure 2).The correlation between total anthocyanin content and cyanidin-glucoside and -acetyl glucoside was weak. This is in line with a previous study, where an opposite relation between temperature and the glucoside of cyanidin compared to the galactoside and arabinoside was found[25]. Though similar effects are seen in both bilberry and currants, different glycosides have been affected by the changes in temperature [62-63]. Cyanidin-3-*O*-glucoside, -arabinoside and -acetyl-glucoside were not significantly affected by temperature but were weakly influenced by the volume of spruce and the interaction of temperature and altitude.

There was a significant negative effect of the volume of spruce but effect of volume of deciduous trees in lingonberries (Table 1). Differences in stand volume of spruce and volume of deciduous trees could cause differences in available light for the shrubs on the forest floor. It is well known that while lingonberries are adaptable, they produce the highest volume of berries in areas with high availability of light, and in pine forests[14-15]. However, we found no effect of light intensities reflecting open and dense forests on the content of anthocyanins in lingonberries (unpublished results). One earlier study found that there was a slight effect of soil type on the composition of wild lingonberries[16]. Also, cloud cover and rainfall during the study period can influence the light conditions, but only a slight effect of precipitation on the content of the cyanidin-pentoside and -acetyl glucoside was found in this study (Table 2). This is in line with previous findings that have shown no correlation between amount of precipitation and total anthocyanin content during the lingonberry growth period [17, 64].

3.2.2. Sugars and organic acids

The regression models used explained little of the variation in sugar content of lingonberries (Table 1). There was nevertheless a slight negative effect of both higher latitudes and temperature on both the content of glucose and fructose. The interaction coefficient of latitude and temperature was however positive. In a study of Lithuanian lingonberries, it was found that lingonberries have a higher sugar content at locations further south, and a higher sugar content at higher temperatures[47]. In our separate study supplemental blue and red light gave higher sugar content than far red light, whereas temperature was found not to influence the content of sugars in lingonberries when ripened in a phytotron[25]. There have been conflicting evidence on the effects of light on sugar content in berries, while it in buckthorn was found that higher latitudes were negatively correlated with sugar content[39], in currants the effects were dependent on the cultivar[59]. In strawberries a negative relationship between temperature and sugar content was detected [29], whereas higher sugar content was found in bilberries grown at higher temperatures[62]. This shows the complexity of the sugar metabolism, and the complexity and variability among species. The content of fructose was slightly significantly affected by the volume of spruce and the content of sucrose of the presence of deciduous trees. There was no significant effect of precipitation on the content of sugars in lingonberries. This is in contrast to a previous study which reported that lingonberries from sites with medium humidity have the highest content of sugars[16].

The total content of organic acids was most significantly influenced by altitude, precipitation, volume of deciduous trees and the interaction between temperature and precipitation. Quinic acid was significantly influenced both by latitude, temperature, and precipitation, whereas citric acid was most significantly influenced by altitude and precipitation. In the stands furthest north there was a higher proportion of quinic acid and lower proportion of citric acid (Figure 1). As quinic acid is associated to the synthesis pathway of anthocyanins, the increased content at higher latitudes was expected as the content of these increased with latitude. There has however been conflicting reports in the previous literature, with both higher and lower content of these acids at varying latitudes [49]. In lingonberries, content of organic acids was influenced by both temperature[25] and light conditions (unpublished results). Also in other fruits and berries, both abiotic and biotic stressors have been found to influence the content of organic acids [11]. There was a large variation in the mean daily precipitation during ripening in the lingonberry stands from 0.22 to 10.83 mm. Increased precipitation appeared to increase both the total content of organic acids and individual organic acids (Table 1). The precipitation is an important factor together with the soil characteristics and slope determining runoff and consequently the water balance in the plant, which again impacts plant metabolism[22]. The content of organic acids was slightly influenced by increased volume of spruce, and deciduous trees. The ratio of sugars to organic acids were mostly influenced by latitude and precipitation, whereas anthocyanins were affected by temperature, latitude, and altitude. The results show that the quality aspects studied were affected in different ways, meaning that selecting harvest locations for specific quality purposes can be possible. The environment in which the plants grow is also predicted to change and has changed over the last 100 years. Temperature is predicted to increase more than 6 °C in the coming 30 years [65], while the total tree volume and the volume of all tree species in Norwegian forests has been increasing over the last 100 years making the forests denser [26]. Results from this study shows that temperature changes and variation in forest density likely will influence the quality of lingonberries. There may also be differences compared to current high quality habitats can be found. One study predicted that the volume of lingonberries and other berries within *Vaccinium* species will increase over the next 30 years[66].

4. Conclusion

A large variation in the content and composition of anthocyanins, sugars, and organic acids in wild Norwegian lingonberries was detected. Norwegian lingonberries appeared to have high contents of anthocyanins and have a ratio between sugars and organic acid similar to what has been previously reported, making them high quality berries. The study gives indication to some of the complexity surrounding analysis of wild berries. All the studied factors including, latitude, temperature, precipitation, tree volume, and type influenced the quality of wild Norwegian lingonberries. It was found that at higher latitudes and temperatures berries accumulated higher contents of anthocyanins and lower content of sugars, but the interaction coefficient of temperature and latitude was opposite. Whereas the content on organic acids was most influenced by precipitation and tree volume in the stand. The study gives valuable new insight into the complex metabolic processes occurring in berries and the need to understand the complex interactions between growth environment and the composition of lingonberries. This can showcase regional differences in the berry quality or give information on the terroir of the berries for the consumers. However, the research emphasizes the need for further studies also assessing the influence of genetic adaptations.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, AH, IM, LJ, AG and MH.; methodology, MA, KA, AG, LJ, IM and ALH; software, MA, MH; formal analysis, MA.; investigation, MA; resources, KA, IM, AG.; data curation, MA and MH.; writing—original draft preparation, MA.; writing—review and editing, KA, AG, MH, LJ, IM and ALH.; visualization, MA.; supervision, KA and LJ.; project administration, IM.; funding acquisition, IM, LJ and KA. All authors have read and agreed to the published version of the manuscript." Please turn to the CRediT taxonomy for the term explanation. Authorship must be limited to those who have contributed substantially to the work reported.

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