# UHPLC Method Development for Studying the Impact of Northern Growth Latitude and Environmental Factors on Tocopherols and Tocotrienols of Sea Buckthorn

Master's Thesis in Technology

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High-performance liquid chromatography (HPLC) and ultra-high-performance liquid chromatography (UHPLC) are both techniques used to separate the components in a mixture. The most significant difference between the methods is the properties of the particle sizes filled into the used columns. When HPLC generally uses a size range from 3 to 5 µm in classical analytics, in UHPLC, the particle size generally stays at ≤ 2 µm. This allows UHPLC to utilize higher pressures, allowing for faster running times, lower solvent consumption, and better analyte separation and detection by the detector.

This thesis studied the effect of northern growth latitude and environmental factors on tocopherol and tocotrienol contents in sea buckthorn berries. The work focused on upgrading the current high-performance liquid chromatography (HPLC) method to ultra-high-performance liquid chromatography (UHPLC) and optimizing the chromatographic separation of tocopherols.

In this work, an HPLC column with a particle size of 3.0 µm was replaced with a UHPLC column with a particle size of 1.9 µm. Also, the flow rate, mobile phase, and injection volumes were optimized. By HPLC, the running time of one sample was 25 minutes, the flow rate was 2.0 ml/min, and the injection volume for the samples was 10 µl. The development of the method made it possible to reduce the running time to eight minutes, reduce the flow rate to 0.4 ml/min, and reduce the samples' injection volume to only 2 µl.

The upgraded UHPLC method was used to study how different environmental conditions affect tocopherol and tocotrienol levels in sea buckthorn berries. As expected, the highest tocopherol concentrations were observed for α- and γ-tocopherols and the lowest for β-tocopherols. Of the tocotrienols, only α-tocotrienol was detected.

The tocochromanol content of the sea buckthorn berries grown in Kittilä was higher than those grown in Turku. The average α-tocopherol concentration for berries grown in Turku was 66.6 ± 20.4 mg/100 g, while for those grown in Kittilä, same result was 91.5 ± 32.9 mg/100 g. Average relative humidity had the most effect on the concentration of tocochromanols compounds. The variety of the berries was not crucial regarding tocochromanol content in seabuckthorn berries.

Keywords: HPLC, liquid chromatography, method development, sea buckthorn, secondary metabolites, tocopherols, tocotrienols, UHPLC

# Content

## Abbreviations

| | |
|---|---|
| α-T | alpha-tocopherol |
| α-T3 | alpha-tocotrienol |
| β-T | beta-tocopherol |
| β-T3 | beta-tocotrienol |
| γ-T | gamma-tocopherol |
| γ-T3 | gamma-tocotrienol |
| δ-T | delta-tocopherol |
| δ-T3 | delta-tocotrienol |
| CLD1 | chlorophyll dephytylase 1 |
| GGDP | geranylgeranyl diphosphate |
| GGDR | geranylgeranyl diphosphate reductase |
| GGPS | geranylgeranyl diphosphate synthase |
| HGA | homogentisic acid |
| HGGT | homogentisate geranylgeranyl transferase |
| HPLC | high-pressure liquid chromatography |
| HPPD | *p*-hydroxyphenylpyruvate dioxygenase |
| HPT | homogentisate phytyltransferase |
| MEP | methylerythritol phosphate |
| MPBQ-MT | MPBQ methyltransferase |
| MT | 2-methyl-6-phytylhydroquinone methyltransferase |
| PAs | proanthocyanidins |
| PCA | principal component analysis |
| PDP | phytyl diphosphate |
| ROS | reactive oxygen species |

| | |
|---|---|
| SMs | secondary metabolites |
| TAT | tyrosine aminotransferase |
| TC | tocopherol cyclase |
| TMT | tocopherol methyltransferase |
| TQ | tocopherol quinone |
| UHPLC | ultra-high-pressure liquid chromatography |

# 1 Introduction

Sea buckthorns are well-known to contain several health-beneficial compounds, including tocopherols and tocotrienols. Tocopherols and tocotrienols, also called tocochromanols, belong to the vitamin E family consisting of α-, β-, γ-, and δ-tocopherols, and four corresponding tocotrienols. They are well-known for their function as antioxidants in membranes and lipoproteins. (Andersson *et al.*, 2008.)

High-pressure liquid chromatography (HPLC) is a prevalent and one of the most common methods for the analysis of tocochromanols and other secondary metabolites. The first separation of tocochromanols took place in the early 1970s (Abidi, 2000). Despite many advantages of HPLC, the technique is quite time-consuming and uses a relatively high amount of organic solvents compared to other analytical methods. Therefore, this thesis aimed to develop a method that would allow the analysis of tocopherols and tocotrienols to be performed by ultra-high performance liquid chromatography (UHPLC) instead of HPLC due to several advantages of UHPLC.

## 1.1 Liquid chromatography

Chromatography was originally discovered as an analytical technique when Russian botanist Mikhail S. Tsvet first used it at the beginning of the 20$^{th}$ century to separate compounds based on color. This is also where the name chromatography is derived from because chroma means color, and graphy refers to writing. (Arnaud, 2016; Barkovich, 2020.)

Tsvet used a chromatographic separation technique to purify individual pigments from various plant pigment mixtures. Tsvet used a combination of calcium carbonate and alumina as the stationary phase, while solvent was used as the mobile phase. The stationary phase was packed into the column, after which the combination of plant pigments and the solvent mixture was poured into it as well. As the plant pigments were eluted at the bottom of the column, Tsvet added solvent until the pigments were divided into bands of pure components and were separated from each other as they moved through the stationary phase. (Barkovich, 2020.)

### 1.1.1 High-performance liquid chromatography (HPLC)

Today's HPLC methods are based on the aforementioned chromatographic separation, although the technology has improved and developed tremendously in the last hundred years. However, the separation of the components is still based on the distribution of the sample between a mobile phase and the column's packing material, allowing qualitative and quantitative analysis of components present in the analyzed sample.

Most lipid oxidants, including tocochromanols, are often analyzed by HPLC, and it is suitable for a wide range of other application areas as well. In general, an HPLC system contains a solvent reservoir, a pump, an injector, a column, a detector unit, and a data processing unit for chromatograms. The components of a basic HPLC system are shown in Figure 1.

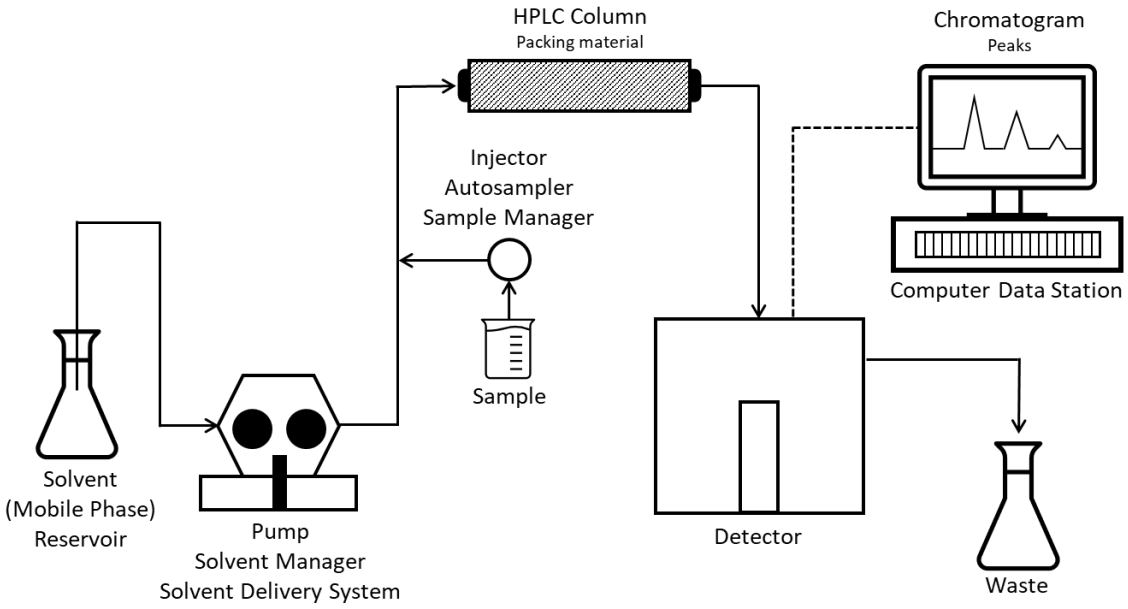


*Figure 1. High-performance liquid chromatography (HPLC) system.*

As Figure 1 implies, the solvent reservoir holds the mobile phase. Typically, the mobile phase consists of two different components: a weak one that helps the analytes stay in the stationary phase and a strong one that helps the analyte elute out of the column. Therefore, the choice of mobile phase depends on the type of interactions required between the

compound, or compounds, under study and the stationary phase. (Science Unfiltered, 2023.)

For normal-phase HPLC, the weak component of the mobile phase must be a non-polar solvent since the attraction of the analyte to the polar stationary phase allows for separation. The non-polar will not draw the analyte molecules, lengthening the analyte's retention time in the stationary phase. A polar organic solvent is then utilized as the active component of the mobile phase when the analytes must be eluted from the column. Normal-phase mobile phase solvents commonly used are hexane, heptane, chloroform, benzene, ethyl acetate dichloromethane, ethanol, and isopropanol. (Science Unfiltered, 2023.)

The pump system generates a specified mobile phase flow rate, typically measured as milliliters per minute (ml/min). Flow rate refers to how fast solvent moves through liquid chromatography equipment. In order to minimize the detector drift and noise, the pump must provide continuous pulse-free flow. (Thermo Fisher Scientific, 2019.)

In the injection stage, the sample is introduced into the continuously flowing mobile phase stream with an injector that carries the sample into the HPLC column containing the stationary phase material to effect the separation. (Waters, 2021; Böttcher *et al.*, 2022.)

Since the working pressure of an HPLC is high, a syringe cannot be inserted directly into the mobile phase. Therefore, an injector that gives a reproducible result without interfering with pressure or the flow rate of the LC system is needed. (Patil, 2021.)

The injector is used so that when the system is balanced, it is filled with the sample solution in its loading position. When the injector is turned to the injection position, it connects to the HPLC system via the loop without disturbing its high pressure or flow rate. Typically, either a manual or an automatic injection system is available. The injection loop is used before the injection begins, whether automatic or manual. (Patil, 2021.)

Different types of HPLC injectors are shown in Figure 2.

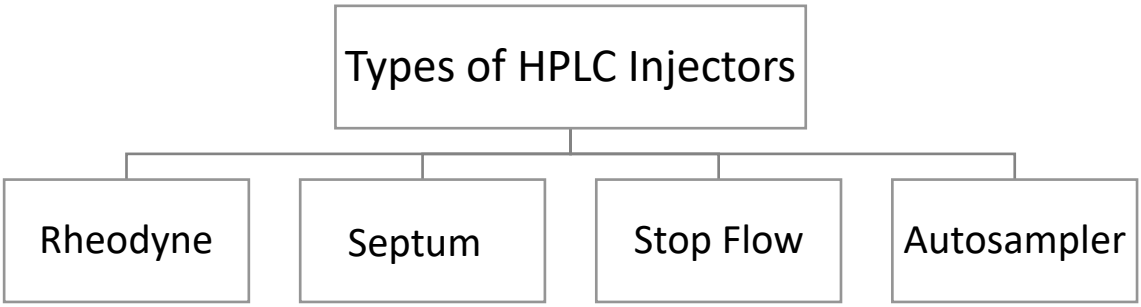


*Figure 2. Different types of HPLC injectors (Patil, 2021).*

It is required that the injection volume must be the same for all samples of one experiment, and the injection must not disturb the pressure or flow rate of the LC system, as stated. Since the volume of the samples is in microliters (usually between 0.1 and 100 µl), it must be accurate. The sample solution must also have no air bubbles or particulate matter. Increasing injection volume also increases peak area and width, so it must be taken into account that if the injection volume is set too large, the peak result may be broadened, which may reduce the resolution between adjacent peaks. (Patil, 2021.)

A detector shows the separated compound bands after the analytes leave the column. In order to generate a chromatogram on display and identify and quantify the concentration of sample components, the detector is connected to a computer data station. (Waters, 2021; Böttcher *et al.*, 2022.)

The actual separation of the components in the samples occurs inside the column, and a detector collects this information. There are several types of detectors, and they should be selected based on the compounds to be analyzed. For example, most natural products have fluorescent absorbance. When suitable wavelengths are used, the analyte atoms are excited to emit a light signal, fluorescence. Then the intensity of emitted light can be monitored to quantify the analyte concentration. A fluorescence detector is often used to analyze tocopherols and tocotrienols due to their native fluorophore properties. It is also the most sensitive means for their detection. (Kallio *et al.*, 2002a; Kallio *et al.*, 2002b; Panfili *et al.*, 2003; Andersson *et al.*, 2008; Huang and Ng, 2011; Górnaś *et al.*, 2015.)

Liquid chromatography can be used in a normal phase (NP) or reversed phase (RP) mode. When analyzing vitamin E, and all eight tocochromanols need to be separated, normal-phase high-performance liquid chromatography (NP-HPLC) is commonly applied (Eccleston *et al.*, 2002; Panfili, Fratianni and Irano, 2003; Huang and Ng, 2011). In NP-HPLC, the stationary phase is polar, while the polarity of the mobile phase is low. In the RP-HPLC version, the polarities are opposite. (Yang, 2017.) To determine tocochromanols, NP-HPLC can be applied, and the elution order is determined by the polarity (number of methyl groups in the chromanol ring) of tocopherols and tocotrienols.

In addition, there are generally two modes of operation depending on the composition of the mobile phase; isocratic elution system or gradient elution system. The mobile phase composition remains constant during the separation process when the HPLC system is defined as an isocratic elution system. If the composition is changed during the run, the system is defined as a gradient elution system. (Böttcher *et al.*, 2022.)

### 1.1.2 Ultra high-performance liquid chromatography (UHPLC)

Like HPLC, ultra-high-performance liquid chromatography (UHPLC) is a liquid chromatography separation technique. The basic principle between HPLC (see Chapter 1.1.1) and UHPLC is the same. However, the needed equipment and used parameters differ from each other.

One of the main differences between HPLC and UHPLC is their dissimilarities in the columns used. Separation efficiency depends on several factors, including the size and type of packing material used in columns, the column's inner diameter, and its length. UHPLC columns are filled with much smaller particles and are shorter and smaller in diameter than HPLC columns. In HPLC, the particle sizes in the columns typically range from 3 to 5 µm, while in UHPLC, the particle size typically stays at 2 µm or less. As with particle sizes, the column dimensions are also reduced compared to HPLC. The length of a typical HPLC column is 150–250 mm with an internal diameter of 3.0 to 4.6 mm. The UHPLC column, on the other hand, has an inner diameter of 2.1 mm or less, and it is much shorter, typically 100 mm. (Thermo Fisher Scientific, 2019; Böttcher *et al.*, 2022.)

Comparatively, there are differences in the possibilities of the flow rate usage of the HPLC and UHPLC methods. UHPLC allows much lower flow rates than HPLC, which simultaneously decreases the time required for analysis. Lower flow rates result in lower solvent consumption, making UHPLC more environmentally friendly and much cheaper

over a longer period when disposal costs associated with solvent usage are reduced. (Thermo Fisher Scientific, 2019; Böttcher *et al.*, 2022.)

Quantitative chromatography aims to separate two or more substances sufficiently to enable accurate measurements. In chromatographic separation, resolution tells how well two elution peaks are differentiated from each other. The resolution is defined as the retention time difference between two peaks, then multiplied by two and divided by the peaks' combined widths by using the following formula (Equation 1) (Henshaw, 2010):

$$R_S = \frac{2\,(t_{R2} - t_{R1})}{W_{b2} + W_{b1}} \qquad (1)$$

In Equation 1, $t_R$ is retention time and $W$ elution peak width, respectively. Numbers 1 and 2 refer to the peaks; 2 with a longer retention time. The resolution is considered partial when the value is 1.0–1.5. The value 1.5–2.0 refers to the baseline resolution, and the value over 2.0 refers to the full resolution approximately. Therefore, resolution increases with increasing peak separation. Peaks can be differentiated successfully with a resolution greater than one, but it is recommended to seek at least a baseline resolution for quantitative analysis. (Guillarme *et al.*, 2008; Henshaw, 2010; Barkovich, 2020.)

In addition, there is also a significant difference between of the system backpressures of UHPLC versus HPLC. Smaller particle sizes in the UHPLC columns causes higher backpressure compared with HPLC. The typical maximum pressure for a HPLC system is between 400 and 600 bar, while UHPLC can have maximum pressure of 1500 bar on some equipment models. Due to small particle size, UHPLC provides better analyte separation, peak capacity, and detection by the detector, allowing higher sensitivity. Overall, UHPLC offers higher throughput and faster run times than HPLC. (Saul, 2018; Thermo Fisher Scientific, 2019.)

## 1.2 Sea buckthorn

Sea buckthorn (genus *Hippophaë,* family *Elaeagnaceae*) includes six species and 12 subspecies, of which *Hippophaë rhamnoides,* also called seaberry or sandthorn, is a unique nitrogen-fixing plant with a natural habitat extending widely to temperate areas in China, Mongolia, Russia, and much of the northern Europe. It is also cultivated in various

other regions, such as Canada. It is a hardy berry-producing plant that can withstand extreme temperatures ranging from –43 °C to +40 °C. It is also considered a very drought-tolerant plant. Sea buckthorn forms a 2–4 m tall shrub or tree, producing berries in late summer. When ripe, the sea buckthorn berries are yellow-orange and usually shiny, varying in size between 3 and 8 mm. The berries are highly berry-rich, containing edible fruit flesh, and enclose one seed. (Li, 2002; Zadernowski *et al.*, 2003; Bal *et al.*, 2011; Michel *et al.*, 2012.)

Due to its strong and complex root system with nitrogen-fixing nodules, and substantial vegetative reproduction, sea buckthorn is considered useful for land reclamation purposes and farmstead protection. Sea buckthorn juice has been characterized and compared with aromas such as strawberry, peach, mango, apricot, papaya, and citrus, but the predominant taste is sour and astringent. Sea buckthorn berries are considered to have broad health potential, and they can be processed for various other foods in addition to juice, such as jams, jellies, syrup, beverages, and seed oils, despite their highly acidic nature and exotic flavor. There has also been a long history of using sea buckthorn berries for medicinal purposes. In addition to many foods and several pharmaceuticals, they are used raw or processed to make cosmetics as well. (Zadernowski *et al.*, 2003; Andersson *et al.*, 2008; Bal *et al.*, 2011.)

Numerous studies show that sea buckthorn berries, leaf extracts, and isolated compounds have pharmacological and therapeutic effects and antioxidant properties, for example, anti-bacterial, anti-radiation, anti-viral, anti-stress, anti-cancer, cardioprotective, and acute, chronic wound healing effects. The juice, pulp, and seeds of sea buckthorn berries are reported to contain more than 190 compounds. (Zadernowski *et al.*, 2003; Andersson *et al.*, 2008; Bal *et al.*, 2011; Michel *et al.*, 2012; Fatima *et al.*, 2015.)

The antioxidant properties of the sea buckthorn are well-known due to its hydrophilic and lipophilic compounds. The berries contain several potentially health-beneficial compounds, such as flavonoids, proanthocyanidins, carotenoids, minerals, and fatty acids, in addition to many chemical elements, such as iron and calcium. Sea buckthorn also accumulates high levels of fat-soluble vitamins A-, E-, and K in addition to vitamin C in its fruit and leaves. The literature has well established that berries also hold high levels of folic acid, phenols, terpenes, and tannins. While the whole sea buckthorn plant offers several benefits in different domains, its most important component is its berries, especially its oils, from which juice is extracted. Due to this, sea buckthorn berries have gained massive popularity as a superfood worldwide over the past years, especially as

consumers' preferences have changed towards more natural products and functional products are becoming more and more common. (Zadernowski *et al.*, 2003; Fatima *et al.*, 2015; Yang, 2017.)

From fat-soluble vitamins, vitamin E is especially abundant in sea buckthorn berries. There are large amounts of vitamin E in the seeds of the berries as well as in the soft parts of the berries, such as the peel and pulp. (Kallio *et al.*, 2002b.)

## 1.3 Tocopherols and tocotrienols

Tocopherols and tocotrienols are important bioactive components and are well known for their function as antioxidants in membranes and lipoproteins, both plants and animals (Fatima *et al.*, 2015; Kallio *et al.*, 2002a).

Tocopherols and tocotrienols, called tocochromanols, belong to the vitamin E family consisting of α-, β-, γ-, and δ-tocopherols, and four corresponding tocotrienols. They all are lipid-soluble and viscous compounds at room temperature, occurring naturally. Tocopherols, especially α-tocopherols, are essential human dietary components, functioning as antioxidants and regulators of cell signaling and gene expression. (Seppänen *et al.*, 2010; Fatima *et al.*, 2015; Górnaś *et al.*, 2015.)

Tocopherols and tocotrienols can be found in whole grains, seeds, nuts, vegetable oils, fruits, green leafy vegetables, meat, fish, milk, and egg products. The tocopherols are mainly present in oilseeds, oils, meats, and green parts of higher plants, whereas the tocotrienols are primarily found in the germ and bran fraction of certain seeds and cereals. (Seppänen *et al.*, 2010.)

α-Tocopherol is considered to be the most active and predominant form of vitamin E, although all tocopherols have some vitamin E activity. Of the tocotrienols, only α-tocotrienol has been claimed to have a significant vitamin E effect. The most abundant natural antioxidants in vegetable oils are the α- and γ-tocopherols. (Andersson *et al.*, 2008; Seppänen *et al.*, 2010.)

The tocochromanol content of sea buckthorn berries varies depending on variety, growth area, or environmental conditions. As an example, in a study by Zadernowski, Naczk, and Amarowicz (2003), the total tocopherol content varied between 101.4 and 128.3 mg/100 g of oil with an average of 110.5 mg/100 g. The study utilized whole sea buckthorn berries, including seeds.

According to the review, Bal *et al.* (2011), the pulp oil is rich in α-tocopherol, concentration being approximately 120–160 mg/100 g. They also stated that the seed oil contains even higher amounts of tocopherols approximately 140 mg/100 ml. Also, Otgonbayar, Matthaus, and Odonmajig (2014) came to the approximately same level result with the total tocopherol content in the seed oil with 94.34 mg/100 g and pulp oil with 90.25 mg/100 g of oil.

As mentioned, unlike other vitamins representing one well-defined chemical structure, natural vitamin E includes two groups, tocopherols and tocotrienols (Azzi and Stocker, 2000). Both groups are the derivatives of a polar 6-chromanol ring and have a similar basic molecular structure (Figure 3).

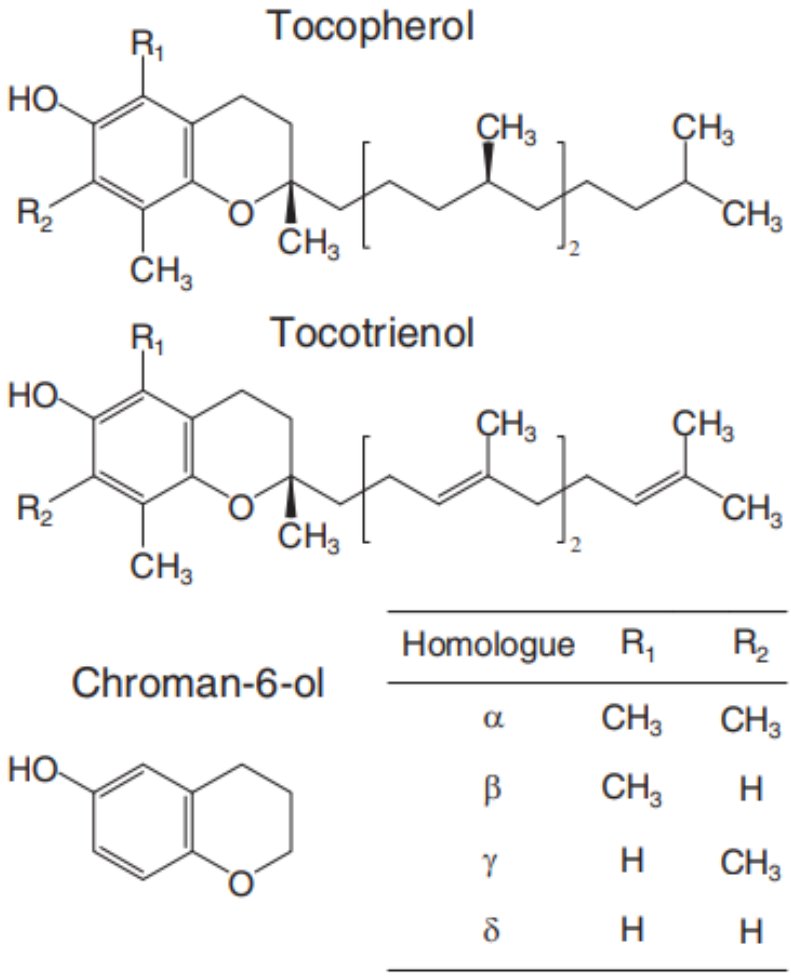


*Figure 3. Tocopherol and tocotrienol chemical structure (Górnaś et al., 2015).*

The difference between the tocopherol and tocotrienol homologs is their side chains' saturation or unsaturation. The tocopherols have a saturated side chain, while the tocotrienols have an unsaturated side chain containing three double bonds. Tocopherol and tocotrienol homologs are named depending on the number and position of the methyl groups in the chromanol ring. The antioxidative nature of the tocochromanols is due to the hydroxy group in the chromanol ring, exposed for oxidation. (Azzi and Stocker, 2000; Górnaś *et al.*, 2015.)

In the middle of the 1980s, radiotrace studies provided insight into the tocopherol biosynthesis pathway in photosynthetic organisms. Mainly the biosynthesis of tocochromanols occurs in higher-growing plant plastids from precursors from two metabolic pathways within the plastid envelope: the shikimate pathway and the methylerythritol phosphate (MEP) pathway. (Lushchak and Semchuk, 2012; Chaalal and Ydjedd, 2021.)

In the shikimate pathway, homogentisic acid (2,5-dihydroxyphenylacetate; HGA) is converted into the chromanol ring via the cytosolic shikimate pathway and used to form the aromatic ring of tocopherols. The MEP pathway produces phytyl diphosphate (PDP) for the tocopherol tail and geranylgeranyl diphosphate (GGDP) for the tocotrienol tail (Figure 4). (Lushchak and Semchuk, 2012; Chaalal and Ydjedd, 2021.)

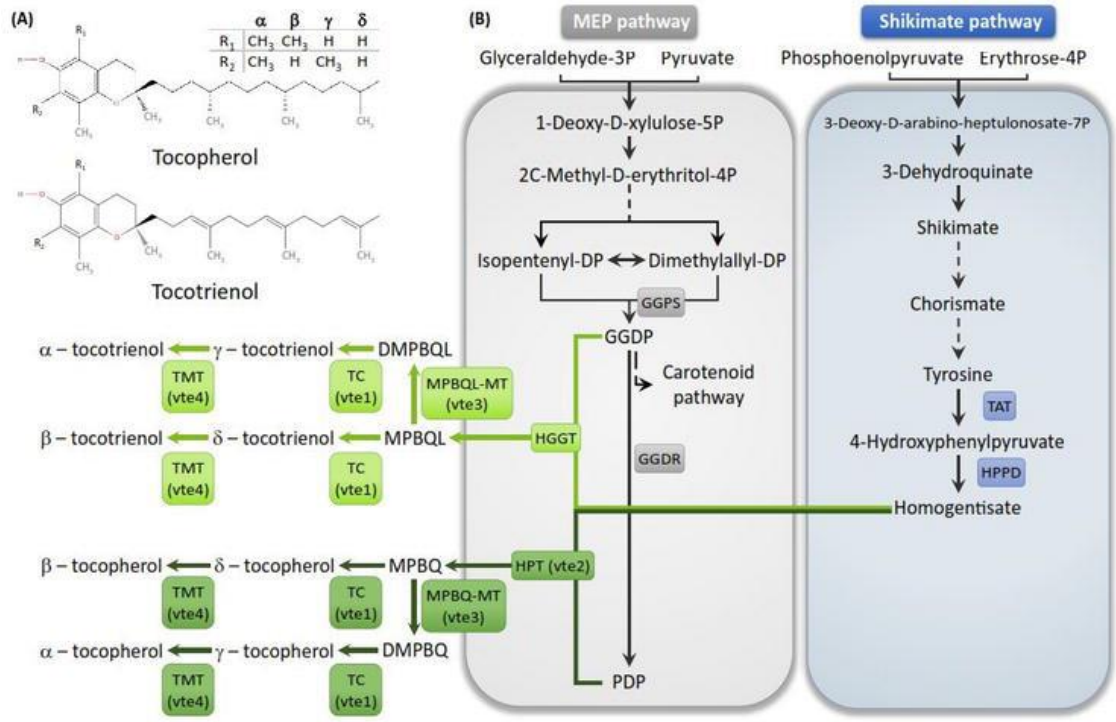


*Figure 4. Chemical composition of tocochromanols and how it is produced in plants. (A) Chemical composition of tocochromanols. (B) Plants' biosynthesis of tocochromanols. Together, the methylerythritol phosphate (MEP) and shikimate routes produce tocopherols and tocotrienols. GGDR, geranylgeranyl diphosphate reductase; GGPS, geranylgeranyl diphosphate synthase; HGGT, homogentisate geranylgeranyl transferase; HPPD, p-hydroxyphenylpyruvate dioxygenase; HPT, homogentisate phytyltransferase; MPBQ-MT, MPBQ methyltransferase; MT, 2-methyl-6-phytylhydroquinone methyltransferase; PDP, phytyl diphosphate; TAT, tyrosine aminotransferase; TC, tocopherol cyclase; TMT, tocopherol methyltransferase. (Chaalal and Ydjedd, 2021.)*

In addition, there is also another pathway for producing PDP by degrading chlorophyll, known as the phytol recycling pathway. While fruits ripen and seeds mature, the hydrolases such as CLD1 may allow phytol to be remobilized. However, it is less clear which enzymes are involved in the dephytylation of chlorophyll (Figure 5). (Chaalal and Ydjedd, 2021.)

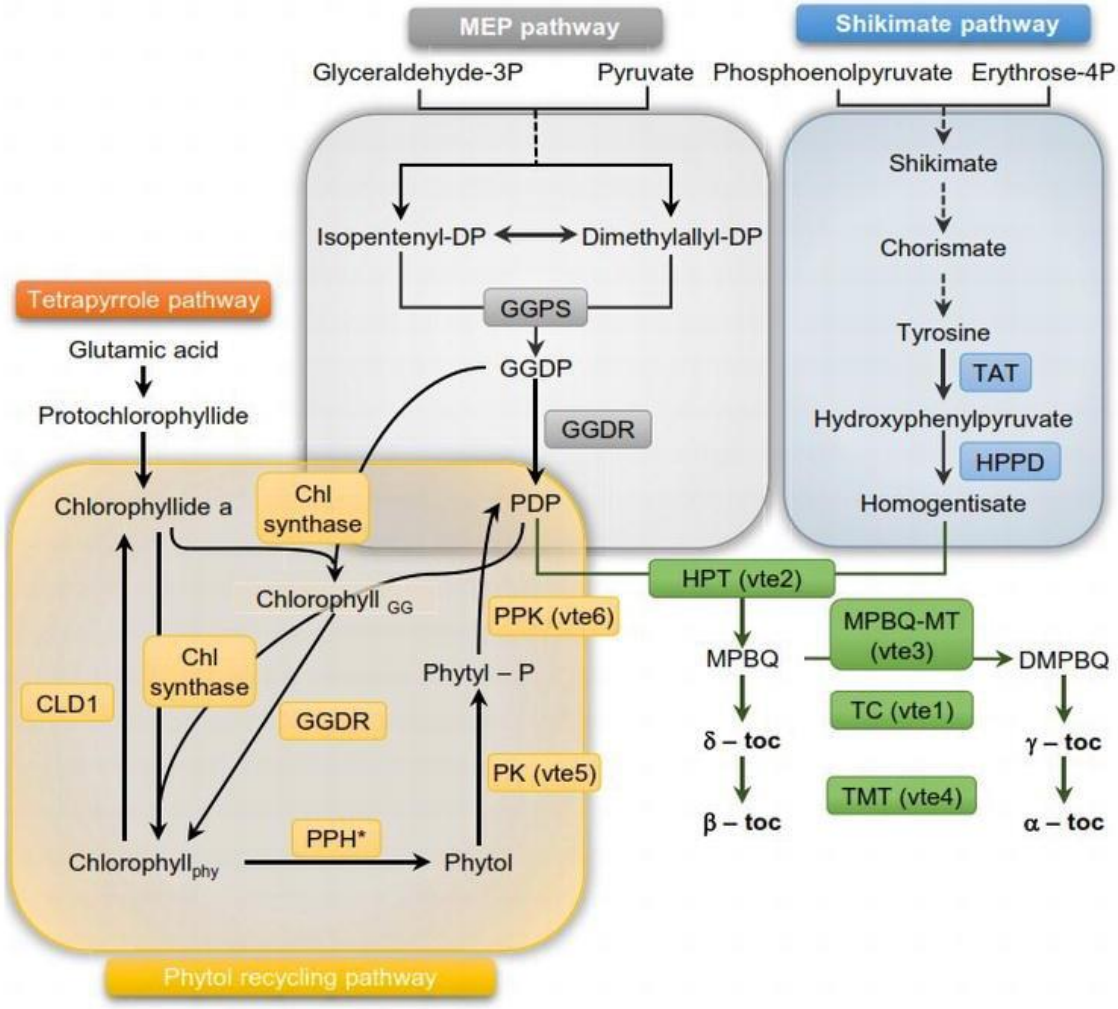


*Figure 5. Tocopherol biosynthesis and chlorophyll degradation in plants. CLD1, chlorophyll dephytylase 1; others: see Figure 4. (Chaalal and Ydjedd, 2021).*

Several enzymes control tocopherol biosynthesis in photosynthetic organisms. According to Lushchak and Semchuk (2012), at least five of these enzymes are believed to be critical in tocopherol biosynthesis. These enzymes include homogentisate phytyltransferase (HPT), which catalyzes the condensation of HGA with either PDP or GGDP, p-hydroxyphenylpyruvate dioxygenase (HPPD) which works as one of the first steps for the biosynthesis of tocopherols as a catalyzer, and tocopherol cyclase (TC) and two methyltransferases.

The antioxidant properties of tocopherols are associated with the production of tocopherol quinone (TQ) and its subsequent recycling or degradation. TQ is the α-tocopherol's oxidation product formed by singlet oxygen and lipid radicals. Thus, it is assumed that

the main functions of tocopherols in photosynthetic organisms are the scavenging of lipid peroxy radicals and the quenching of singlet oxygen. (Lushchak and Semchuk, 2012.)

Biosynthesis changes as plants develop and respond to various stresses such as drought, extreme light, or chilling. (Lushchak and Semchuk, 2012.)

## 1.4 Effects of environment and growth conditions

When plants are exposed to different environmental and growth conditions, the secondary metabolites start to regulate themselves in order for the plant to adapt to the different prevailing stresses (Yang, 2017).

In the Northern Hemisphere, solar irradiance decreases with the increase in latitude. The long days in summer, low solar irradiance, and the short growth period with the Gulf Stream make the agricultural area of Scandinavia unique with the significant climatic difference between the southern and northern parts from 55° to 70°N. Plants have developed strategies to survive and thrive involving stress-based selection, modification of gene expression, and epigenetic changes, resulting in various secondary metabolites that positively impact food quality and biological activities. (Mareri *et al.*, 2022.)

Sea buckthorn is highly adaptable to biotic and abiotic stress, and the effects of geographical origin on sea buckthorns' primary and secondary metabolism have been extensively studied (Yang and Kallio, 2001; Kallio *et al.*, 2002b; Vuorinen *et al.*, 2015; Kortesniemi *et al.*, 2017; Yang, 2017; Yang *et al.*, 2017). Plant secondary metabolites are valuable natural products and integral to the plant's defense system against pathogenic attacks and environmental stresses. Accumulation of the secondary metabolites is strongly dependent on various environmental factors, and plants of the same species grown in different environments or growth conditions may have differences in the concentration of particular secondary metabolites. (Verma and Shukla, 2015; Yang *et al.*, 2018.)

Studies have shown that northern conditions produce berries with higher levels of bioactive compounds that protect against abiotic stressors associated with high latitudes. (Kortesniemi *et al.*, 2017; Yang *et al.*, 2017).

In the far north, latitude significantly influences plants' growth conditions. As growing sites move more and more northward, latitude becomes a more significant factor. At high latitudes, such as Scandinavia, typically, latitude correlates negatively with temperature,

radiation, and length of the growing season, whereas a positive correlation is seen with day length during the growing season. (Yang, 2017.)

As an example, a 2017 study revealed that sea buckthorns (*H. rhamnoides* L. ssp. *rhamnoides*) grown in northern Finland exhibited elevated proanthocyanidins (PA) levels compared to their counterparts grown in southern Finland (Yang *et al.*, 2017).

Each growing site has different environmental profiles due to different locations, latitudes, and altitudes. Variation occurs in growing seasons, temperatures, precipitation, and humidity. Also, light intensity and photoperiod have been reported to influence many secondary metabolites in several plant species' biosynthesis. For instance, plants experience temperature stress when temperatures drop to restore a self-defense mechanism and produce secondary metabolites. It is shown that the attributes of the northern climate, such as long days with cool night temperatures, have mainly a positive impact on the biosynthesis of flavonoids in plants. Variation between species and within individual flavonoid groups has been discovered, however. (Jaakola and Hohtola, 2010; Yang, 2017.)

Several environmental stress factors affect plants and their compounds produced. Due to environmental changes, plants' steady-state levels of reactive oxygen species (ROS), among other things, may increase. In oxidative stress, a temporary or chronic increase in steady-state levels of ROS causes disruption of metabolic processes and signaling in cells, which ultimately leads to cellular oxidation and may cause the death of cells. Antioxidants are tightly associated with the plant's ability to resist stress, especially oxidative stress. (Lushchak and Semchuk, 2012.)

When various stress factors trigger the release of tocopherol, the strong antioxidant protects plants from ROS. The amount of tocopherols in the plant is related to stress intensity, the state of the plant's physiological systems, and the sensitivity of the plant to stress. (Lushchak and Semchuk, 2012.)

For instance, Semchuk *et al.* discovered in 2009 that wild Arabidopsis-plant produced eight- to twelve-fold higher tocopherol amounts of tocopherols than those grown in the laboratory.

## 1.5 Aim of the study

This thesis focused on developing an analyzing method for determining tocopherols and tocotrienols in sea buckthorn berries and optimizing their chromatographic separation.

Previously, the traditional HPLC method has been used, and it was desired to upgrade to the UHPLC method.

The upgraded UHPLC method is intended to be used to study how different environmental conditions affect tocopherol and tocotrienol levels in sea buckthorn berries. The study hypothesizes that the environmental stresses induced by extreme environmental conditions in the far north will positively influence the concentration of the tocochromanols in sea buckthorn berries.

# 2 Materials and Methods

## 2.1 Reagents

For liquid chromatographic eluents, heptane ($C_7H_{16}$) for HPLC ≥ 99% was purchased from Honeywell Riedel-de Haën™ (Germany), 1,4-dioxane ($C_4H_8O_2$) for HPLC ≥ 99.5% and 2-propanol ($(CH_3)_2CHOH$) for LC-MS from Sigma-Aldrich (Darmstadt, Germany). For lipid extraction, chloroform ($CHCl_3$) for HPLC ≥ 99.8% and methanol ($CH_3OH$) for HPLC ≥ 99.9% were purchased from Sigma-Aldrich (Germany). Potassium chloride (KCl) was purchased from VWR Chemicals (Leuven, Belgium), and a 0.88% solution was prepared in Milli-Q water. For the preparation of standard solutions, ethanol ($C_2H_5OH$, min. 99.5 p-%) was purchased from Altia Oyj (Rajamäki, Finland). α-, γ-, and δ-tocopherol standard solutions were ready-prepared as stock solutions in the freezer at the University of Turku. The β-tocopherol standard was purchased from Sigma-Aldrich (Darmstadt, Germany).

## 2.2 Standards

Each tocopherol standard solution was prepared at approximately 500 µg/ml concentration, diluting in ethanol. To measure the exact concentration of all stock solutions, they were diluted further 1:9 in ethanol using 10 ml volumetric flasks and measuring absorbance with a spectrophotometer (Evolution 300 UV-VIS, Thermo Scientific™, Madison, Wisconsin, U.S.A) at an appropriate wavelength (Table 1). To calculate the exact concentration of the standard solutions, the Beer-Lambert Law was used in the calculations (Equation 2), where $A =$ absorbance, $\varepsilon =$ molar absorption coefficient ($M^{-1}$ $cm^{-1}$), $b =$ molecular weight/optical length (cm), and $c =$ concentration (mol/l):

$$A = \varepsilon\, b\, c \quad \rightarrow \quad c = \frac{A}{\varepsilon\, b} \qquad (2)$$

Physiochemical data of tocopherols are shown in Table 1.

*Table 1. Physiochemical data of tocopherols.*

| Substance | Molecular weight | $\lambda_{max}$ (nm) | $\varepsilon$ ($M^{-1}$ $cm^{-1}$) |
|---|---|---|---|
| α-Tocopherol | 430.7 | 292 | 3270 |
| β-Tocopherol | 416.7 | 296 | 3730 |
| γ-Tocopherol | 416.7 | 298 | 3810 |
| δ-Tocopherol | 402.7 | 298 | 3520 |

Stock solutions were stored in glass bottles at –20 °C and protected from light for further handling.

Working solutions with 0.2, 2.0, 10, and 20 µg/ml concentrations were prepared in heptane for the analysis. Before dilution, ethanol evaporated away with gentle nitrogen flow. Suitable combinations (1, 4, 10, 20, 30, 40, and 50 ng) were made to give appropriate levels corresponding to sample tocopherol amounts. A standard solution was prepared to contain all four tocopherols (α-T, β-T, γ-T, and δ-T) together so that all curves could be determined simultaneously. Working solutions stay stable for about a month. Concentrations were checked at the time by spectrophotometric measurements, and new solutions were made if necessary. Calibration curves were made for each run.

## 2.3 Samples

Sea buckthorn berries (*Hippophaë rhamnoides* L. ssp. *rhamnoides*) were obtained from the University of Turku. Two varieties of sea buckthorn berries, 'Tytti' and 'Terhi', were collected from two different growth areas, Kittilä and Turku, Finland, between September to November in the years 2016 and 2017. Growth areas range between longitudes 60°N and 68°N. The berries were collected from several different bushes at different time points. Berries collected from different bushes at the same time points were pooled together for analysis. Prior to analysis, the berries were pretreated with lipid extraction. Sea buckthorn samples of both varieties with growth areas, dates of collection, and bushes are listed in Tables 2 and 3.

*Table 2. Samples of the sea buckthorn variety Terhi.*

| Time point | Growth area | Date of collection | Bushes |
|---|---|---|---|
| 1 | Kittilä | 11.9.2016 | D08, D14, E17 |
| 2 | Kittilä | 16.9.2016 | D08, D14, E17 |
| 3 | Kittilä | 21.9.2016 | D08, D14, E17 |
| 4 | Kittilä | 26.9.2016 | D08, D14, E17 |
| 5 | Kittilä | 1.10.2016 | D08, D14, E17 |
| 6 | Kittilä | 6.10.2016 | D08, D14, E17 |
| 7 | Turku | 30.8.2017 | 1, 3, 5, 7 |
| 8 | Turku | 5.9.2017 | 1, 3, 5, 7 |
| 9 | Turku | 13.9.2017 | 1, 3, 5, 7 |
| 10 | Turku | 22.9.2017 | 1, 3, 5, 7 |
| 11 | Turku | 29.9.2017 | 1, 3, 5, 7 |
| 12 | Turku | 13.10.2017 | 1, 3, 5, 7 |
| 13 | Turku | 20.10.2017 | 1, 3, 5, 7 |
| 14 | Turku | 27.10.2017 | 1, 3, 5, 7 |
| 15 | Turku | 3.11.2017 | 1, 3, 5, 7 |
| 16 | Kittilä | 15.9.2017 | B15, D14, E17 |
| 17 | Kittilä | 23.9.2017 | B15, D14, E17 |
| 18 | Kittilä | 28.9.2017 | B15, D14, E17 |
| 19 | Kittilä | 6.10.2017 | B15, D14, E17 |
| 20 | Kittilä | 13.10.2017 | B15, D14, E17 |
| 21 | Kittilä | 20.10.2017 | B15, D14, E17 |
| 22 | Kittilä | 27.10.2017 | B15, D14, E17 |
| 23 | Kittilä | 3.11.2017 | B15, D14, E17 |
| 24 | Kittilä | 10.11.2017 | B15, D14, E17 |
| 25 | Kittilä | 17.11.2017 | B15, D14, E17 |

*Table 3. Samples of the sea buckthorn variety Tytti.*

| Time point | Growth area | Date of collection | Bushes |
|---|---|---|---|
| 1 | Kittilä | 11.9.2016 | B08, C10, C13, E16, E19 |
| 2 | Kittilä | 16.9.2016 | B08, C10, C13, E16, E19 |
| 3 | Kittilä | 21.9.2016 | B08, C10, C13, E16, E19 |
| 4 | Kittilä | 26.9.2016 | B08, C10, C13, E16, E19 |
| 5 | Kittilä | 1.10.2016 | B08, C10, C13, E16, E19 |
| 6 | Kittilä | 6.10.2016 | B08, C10, C13, E16, E19 |
| 7 | Kittilä | 16.10.2016 | B08, C10, C13, E16, E19 |
| 8 | Kittilä | 21.10.2016 | B08, C10, C13, E16, E19 |
| 9 | Turku | 30.8.2017 | 2, 4, 8 |
| 10 | Turku | 5.9.2017 | 2, 4, 8 |
| 11 | Turku | 13.9.2017 | 2, 4, 8 |
| 12 | Turku | 22.9.2017 | 2, 4, 8 |
| 13 | Turku | 29.9.2017 | 2, 4, 8 |
| 14 | Turku | 13.10.2017 | 2, 4, 8 |
| 15 | Turku | 20.10.2017 | 2, 4, 8 |
| 16 | Turku | 27.10.2017 | 2, 4, 8 |
| 17 | Turku | 3.11.2017 | 2, 4, 8 |
| 18 | Kittilä | 15.9.2017 | B08, C10, C13 |
| 19 | Kittilä | 23.9.2017 | B08, C10, C13 |
| 20 | Kittilä | 28.9.2017 | B08, C10, C13 |
| 21 | Kittilä | 6.10.2017 | B08, C10, C13 |
| 22 | Kittilä | 13.10.2017 | B08, C10, C13 |
| 23 | Kittilä | 20.10.2017 | B08, C10, C13 |
| 24 | Kittilä | 27.10.2017 | B08, C10, C13 |
| 25 | Kittilä | 3.11.2017 | B08, C10, C13 |
| 26 | Kittilä | 10.11.2017 | B08, C10, C13 |
| 27 | Kittilä | 17.11.2017 | B08, C10, C13 |

Both berry varieties were collected at the same time points. However, Tables 2 and 3 show that Terhi has 25 time points and Tytti has 27. There were not enough berries for analysis at two time points for variety Terhi, so they were excluded from the study.

*Sample pre-treatment*

Since tocochromanols are easily oxidized, avoiding conditions that would promote oxidation during sample preparation or storage was essential. Samples were analyzed as fresh as possible, stored at low temperatures in the dark, and worked under subdued light. Only the soft parts of the berries were included in the analysis. No seeds were included.

The whole sea buckthorn berries were crushed in a mortar in liquid nitrogen to break the surface of the berries. Crushed berries were freeze-dried (Lyovapor™ L-200, Büchi, Flawil, Switzerland) for approximately 24 hours, after which they were frozen for further preparation at approximately ~80 °C. The lipid contents were isolated from the berries using methanol–chloroform extraction. When homogenate is diluted with chloroform and methanol, it separates homogenate into two layers. The lipids move into the chloroform layer, and the non-lipids into the methanolic layer. By isolating the chloroform layer, a purified lipid extract is obtained.

Freeze-dried berries without the seeds (100 mg) were allowed to stand in test tubes in 900 µl of chloroform and 1.5 ml methanol for one hour, protected from light. Afterward, berries were crushed in this solvent mixture with Ultra Turrax (IKA® T25 Digital, IKA-Werke GmbH & Co. KG) for approximately one minute. 2 ml of chloroform was added to the berry crush, after which the berries were sonicated for 30 minutes.

Into the sonicated samples, 1.2 mL of 0.88% KCl was added, vortexed for 5 seconds, and centrifuged at 3500 rpm for 3 minutes. The lower phase (lipid extract) was salvaged into another tube. 1.5 ml of chloroform was added to the upper phase, vortexed for 30 seconds, and recentrifuged at 3500 rpm for 3 minutes. The lower phase was salvaged again. 1.5 ml of chloroform was added to the upper phase once more, centrifuged with the same settings, and the lower phase was salvaged. After collecting all the lipid extract, chloroform was evaporated to dryness under a stream of nitrogen at 50 C for further analysis. The obtained oil contents from the fresh berries stayed between 0.39 and 0.47% of weighed berries, as shown in Table 4.

*Table 4. The obtained oil content of sea buckthorn berry pulps of both varieties. Values are averages from averages of pooled time points from three replicates.*

| Cultivar | Oil content average (mg) | % of fresh berries | % of freeze-dried berries |
|---|---|---|---|
| Terhi | 31.16 ± 15.01 | 0.39 ± 0.08 | 2.57 ± 0.52 |
| Tytti | 34.37 ± 17.02 | 0.47 ± 0.11 | 2.83 ± 0.62 |

The extracted lipids were dissolved in 3 ml of heptane, and the solution was filtered through 0.45 µm PTFE filters before injecting it into the HPLC system. Each sample time point was prepared in triplicate.

## 2.4 NP-UHPLC-FLD

Tocopherols and tocotrienols of the lipid extracts of the sea buckthorn berries shown in Tables 2 and 3 were analyzed with normal phase ultra-high-performance liquid chromatography equipped with an RF-20A prominence fluorescence detector (NP-UHPLC-FLD), LC-20AD XR pump, SIL-20AC autosampler, and CTO-20AC prominence column oven (Shimadzu Corporation, Kyoto, Japan). As a column, Restek Pinnacle DB Silica UHPLC column 1.9 µm, 100 × 2.1 mm (Bellefonte, Pennsylvania, USA) with a temperature of 30 °C was used. Tocopherols were separated isocratically within 8 min by using a mobile phase containing 98% heptane and 2% 1,4-dioxane (v/v) with a flow rate of 0.4 ml/min. The injection volume for the samples was 10 µl. Chromatograms were recorded at 292 nm and 325 nm. When needed, the UHPLC instrument was washed with 2-propanol between runs.

The concentrations of tocopherols were determined by an external standard method using α-, β-, γ-, and δ-tocopherol. Tocotrienols exhibit similar fluorescent responses to their corresponding tocopherols, so tocotrienols could be quantified using tocopherol standards (Lampi, 2021). Chromatograms were processed with LabSolutions version 5.106 software (Shimadzu Corporation, Kyoto, Japan).

## 2.5 Environmental conditions

The study compared the variability of tocochromanol concentrations in sea buckthorn berries concerning environmental conditions, including precipitation, effective

temperature sum, and average relative humidity. Latitudes between the southern and northern parts, from 60° to 68°N, were also included. Weather data was provided by the Finnish Meteorological Institute (Helsinki). Weather data were recorded at weather stations in Kittilä (Pokka, 68°02′N, 24°37′E, 210 m) in the years 2016–2017 and Turku (Artukainen, 60°23′N, 22°09′E, 1 m) in the years 2016–2017.

The Kittilä weather station is in a small clearing area in a mixed forest. The surrounding terrain is flat but descends 200 m to the south to the streams of Kitinen and Takahaara, 267 m above sea level. The weather station is surrounded from the north and east by a string fen, approximately 270 m above sea level at a distance of 130–200 m. The environment is moraine land with pines and spruce growing, mainly in places of sandy pine fabrics 260–300 m above sea level, especially on the east side of the wide bay about 270 m above sea level. The highest summits are 350–400 m above sea level. (Finnish Meteorological Institute, 2023.)

Turku station is located in the suburbs of Turku, approximately 5 km from the city center. The environment of the land is half clay soil land about 0-10 m above sea level and half forested rocky ridge 20-40 m above sea level. The station itself is located on clay soil land (Raisionjokilaakso). The average coastline of the archipelago sea is about 6 km to the southwest, and the nearest (North Strait) is about 1 km south. (Finnish Meteorological Institute, 2023.)

## 2.6 Multivariate data analysis and statistics

A multivariate analysis was conducted using values of environmental conditions calculated from the start of the growing season until harvest.

Principal component analysis (PCA) was used to compare tocopherol and tocotrienol concentrations and latitude between 60°N and 68°N with different growth conditions, including precipitation summary (mm), the effective thermal summary (°Cd with the baseline of 5 °C), and average relative humidity (%), to examine how these variables affected tocochromanol concentrations in the sea buckthorn samples.

The automatic unit variance (UV) scaling technique was used to scale the data. The automatic scaling method compares samples based on correlations, while the mean scaling method focuses only on the differences. With the automatic scaling method, all samples can be compared and viewed without excluding the examination of similarities

if it is assumed that there could be ones between the metabolites. (van den Berg *et al.*, 2006.)

The SIMCA® 16.0.1 software (Sartorius Stedim Biotech, Göttingen, Germany) was used for the multivariate data analyses.

# 3 Results and discussion

The method development started by checking that the standard solutions were prepared correctly. A calibration curve was run using the old traditional HPLC method, and the chromatogram was checked to be appropriate. The test run of standard solutions analysis was run isocratically using the equipment described in Chapter 2.4 with the following analytical conditions: flow rate of 2.0 ml/min, analysis time of 25 min, and solvent composition of 3% 1,4-dioxane and 97% heptane. Phenomenex Luna® Silica, 3.0 µm, 250 × 4.6 mm, 100 Å (Torrance, California, USA) with a temperature of 30 °C was used. In addition, a test run was also carried out with extracted berry samples to verify that the pre-treatment of the berries would be suitable.

Optimization for the new method was performed with a standard mixture consisting known of all four (α-, β-, γ-, and δ) tocopherols with a concentration of 20 µg/ml. The method was developed by trying different analysis conditions and comparing the results to find the best settings. Flow rate, solvent composition ratios, a few different columns, different injection volumes, and the impact of column oven temperature were tested.

## 3.1 NP-UHPLC-FLD

### 3.1.1 Column

During the study, two different columns were tested. Columns are listed in Table 5. In the traditional HPLC method, Phenomenex Luna® Silica HPLC column (3.0 µm, 250 × 4.6 mm, 100 Å Torrance, California, USA) was used.

*Table 5. Tested columns during method development.*

| | |
|---|---|
| **Column 1** | Restek Pinnacle DB Silica UHPLC column 1.9 µm, 100 × 2.1 mm, 140 Å (Bellefonte, Pennsylvania, USA) |
| **Column 2** | Phenomenex Kinetex® Hilic UHPLC column 2.6 µm, 100 × 2.1 mm, 100 Å (Torrance, California, USA) |

The HPLC column was included in the test runs so that the performance of the UHPLC columns could be compared with the operation of the column of the traditional method.

Columns were tested with the standard solution using a mobile phase used in the traditional HPLC method (composition ratio of 3% 1,4-dioxane and 97% heptane). Column's performances were estimated and measured with a resolution value between β- and γ-tocopherol. More about the resolution and its meaning in this study is discussed in Chapter 3.1.2.

As can be seen in Figure 6, the best result was obtained with the traditional HPLC column. However, it was decided to change the column to the Restek Pinnacle DB Silica UHPLC column. The test results of column 2 were inadequate due to the column's low resolution. Hence, column 2 was excluded from the study after the first test runs.

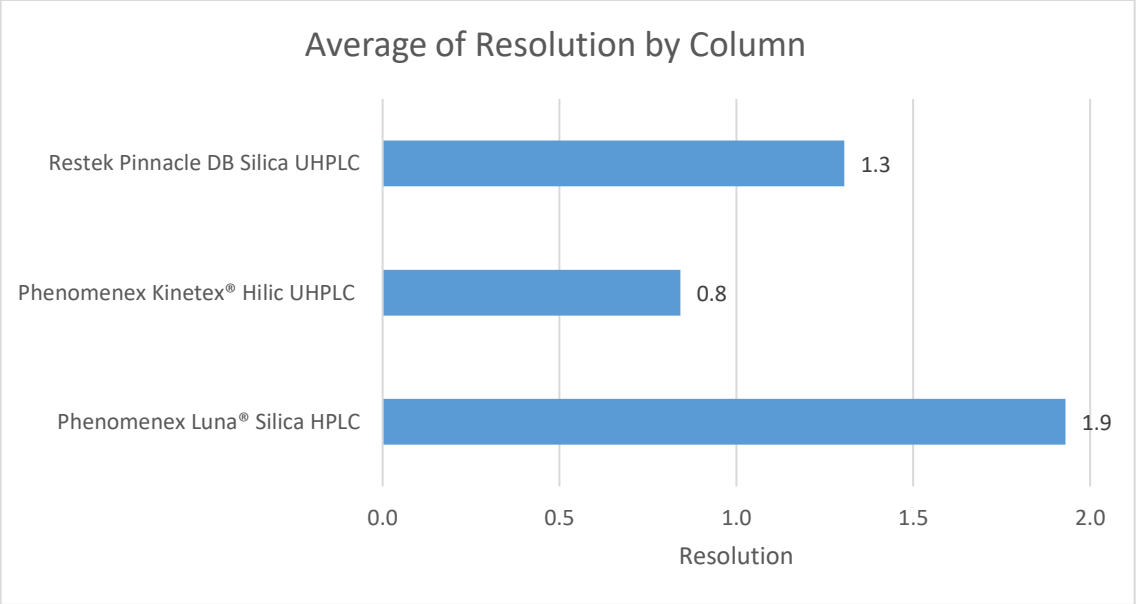


*Figure 6. Averages of resolutions by columns with the standard solution between the β- and γ-tocopherol. A traditional HPLC mobile phase was used with a composition ratio of 3% 1,4-dioxane and 97% heptane.*

Although the resolution was better with the traditional HPLC column than with the Restek column, peak shifting brought challenges to the usage of the HPLC column. Figure 7 demonstrates how peaks are shifting in the chromatogram while using the traditional HPLC column. Regardless, peaks of the same compound should always settle on top of one another.

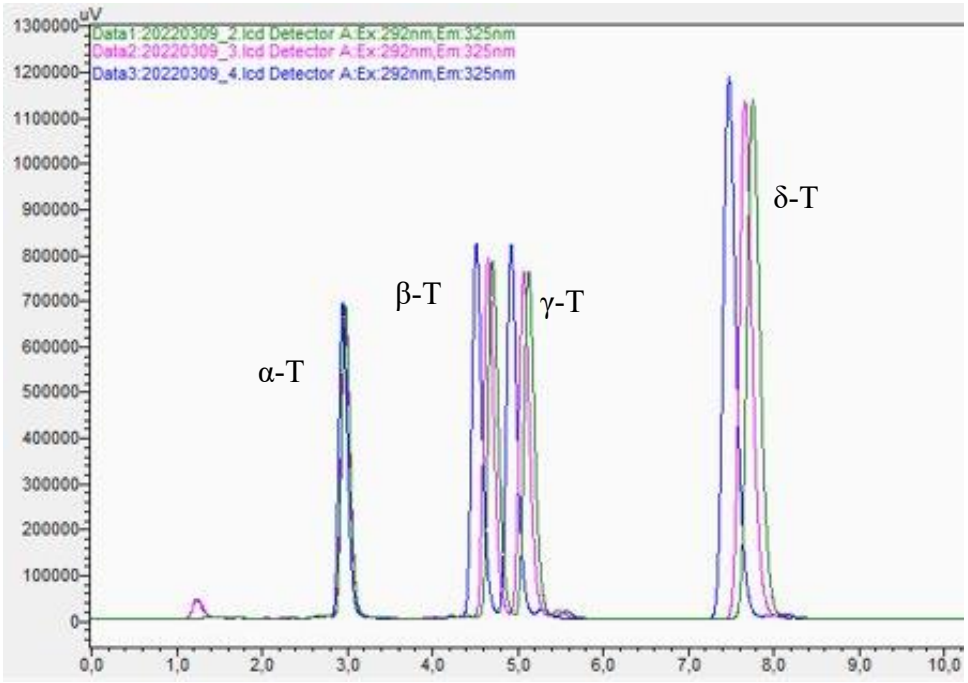


*Figure 7. Chromatogram and peak shifting of standard mixture with the traditional HPLC column.*

Figure 8 shows how peak shifting was controlled by replacing the new HPLC column.

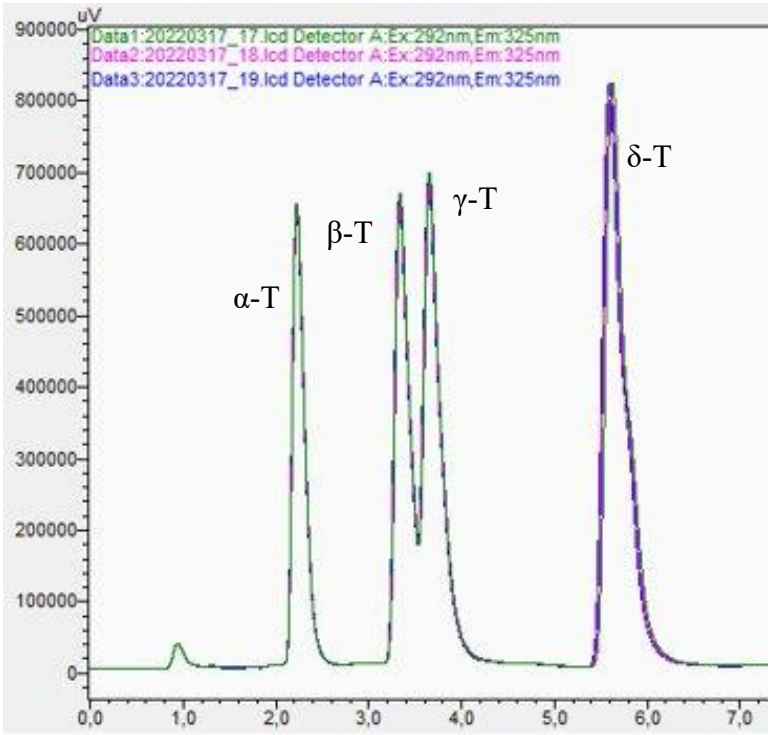


*Figure 8. Chromatogram and peak shifting of standard mixture with the Restek Pinnacle DB Silica UHPLC column.*

Although the resolution was better with the traditional HPLC column, peak shifting was one of the turning points for changing the HPLC column to a new UHPLC column, along with a shorter analysis time and saving solvent consumption.

All columns were used at an oven temperature of 30 °C. In addition, a test run was carried out at a slightly higher temperature when the final column was chosen. The oven temperature was increased to 40 °C to be observed if any benefits occurred. However, increasing the oven temperature did not add any value to the method or bring advantages compared to the lower temperature. Therefore, it was decided to keep the temperature at the same 30 °C as the traditional HPLC method.

### 3.1.2 Resolution

As can be seen visually from Figure 9, the tocopherols are very well separated from each other with the traditional HPLC. Even when β-T and γ-T elute closely in succession, they have an excellent resolution value of 1.93.

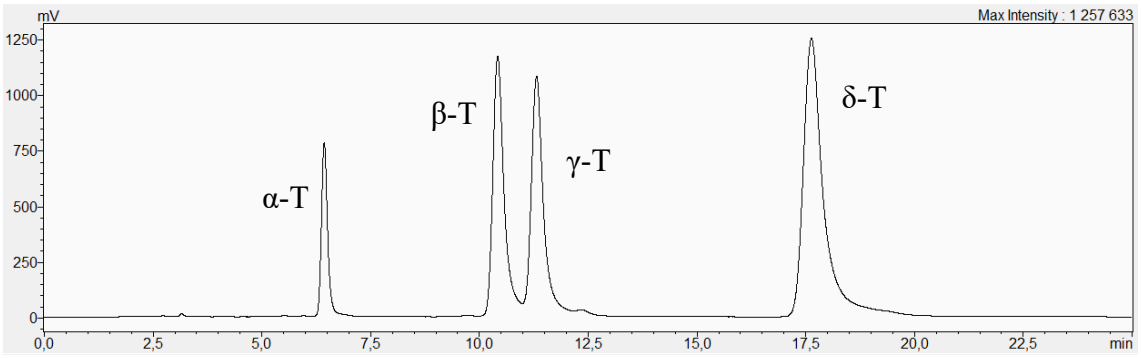


*Figure 9. Chromatogram of tocopherol standards with HPLC; α-tocopherol (α-T), β-tocopherol (β-T), γ-tocopherol (γ-T), and δ-tocopherol (δ-T).*

During method development, it was noticed that the new method did not achieve as good a resolution between β- and γ-T as the traditional one, as discussed. When comparing the separation of β- and γ-T in Figure 9 (HPLC) to Figure 10 (UHPLC), the separation is now partial instead of baseline resolution, with a resolution value 1.32.

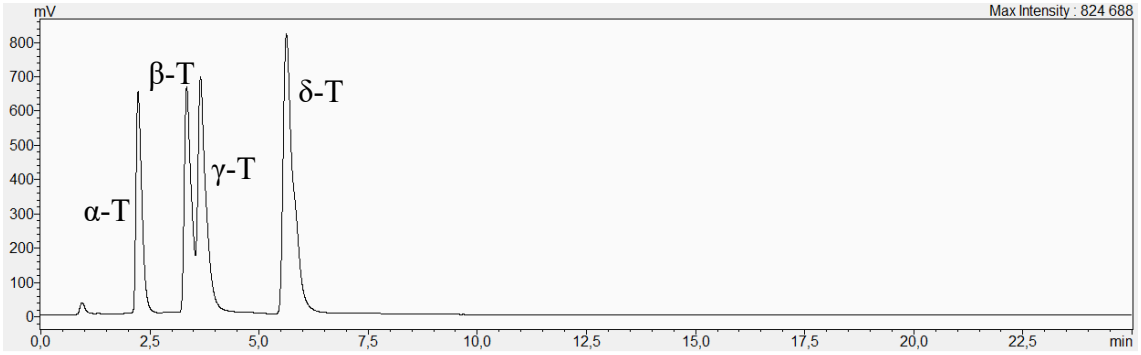


*Figure 10. Chromatogram of tocopherol standards with UHPLC; α-tocopherol (α-T), β-tocopherol (β-T), γ-tocopherol (γ-T), and δ-tocopherol (δ-T).*

Although the resolution between these two compounds decreased with the new method, the resolution value is still good (> 1), and the quantification can be done successfully.

As an addendum, most plants lack β-tocopherol entirely or are only in minor concentrations (Górnaś *et al.*, 2016). Hence, considering the overall tocopherol content of sea buckthorn berries, this is not a critical challenge for method development for UHPLC. Also, when tocochromanols are analyzed, the β-T peak in the sample chromatogram will not be as dominant as in Figure 10, where the standard solution was used.

### 3.1.3 Flow rate

The used flow rate with traditional HPLC was 2.0 ml/min. The aim was to lower it approximately to 0.8 ml/min by upgrading the method to UHPLC. Flow rates were tested between 0.1 and 0.65 ml/min. Flow rate suitability was tested by comparing resolution changes between β- and γ-tocopherols. Figure 11 shows how the resolution decreases when the flow rate increases too much.


*Figure 11. Flow rate (ml/min) compared to resolution.*

The best results were achieved with a 0.4 ml/min flow rate without negatively affecting other parameters while sticking to the goal of the method development to shorten the run time. Therefore, 0.4 ml/min was selected as the final flow rate.

### 3.1.4 Injection

No manual injectors were used in this study, and a SIL-20AC autosampler was selected. 50% heptane and 50% 2-propanol (v/v) solution was used to wash the injectors during runs.

For the samples in the traditional HPLC, the injection volume was 10 µl. UHPLC method allowed for the reduction of the injection volume to 2 µl. The injection volumes for the standard mixture were adjusted proportionately to the variable sample volume. Volumes for the standard mixture used with the traditional HPLC method and new volumes with the UHPLC method are presented in Table 6.

*Table 6. Injection volumes (µl) used in HPLC versus UHPLC methods.*

| VIAL | HPLC (µl) | UHPLC (µl) |
|---|---|---|
| ***1 (blank)*** | 5 | 5 |
| ***2*** | 25 | 5 |
| ***3*** | 10 | 2 |
| ***3*** | 25 | 5 |
| ***4*** | 10 | 2 |
| ***5*** | 15 | 3 |
| ***4*** | 20 | 4 |
| ***5*** | 25 | 5 |

The standard solution was prepared as a mixture of traditional HPCL and new UHPLC methods. Hence the same vials could be used, and the concentrations could adjust with the injection volumes.

### 3.1.5 Mobile phase

No problems or particular challenges have been found in the solvent composition ratios in traditional HPLC, which would make it necessary to change them. However, in terms of the development of the method, different composition ratios were tested.

As a mobile phase, 3% 1,4-dioxane and 97% heptane have been used in the traditional HPLC method, where 1,4-dioxane is used as a stabilizer. Two different solvent compositions were tested in addition to the traditional one. Composition mixtures of 5% 1,4-dioxane and 95%, and 3% 1,4-dioxane and 97% heptane were tested in addition to the mixture used with traditional HPLC.

The suitability of different solvent composition mixtures was compared to obtained resolution values. Results are shown in Figure 12.

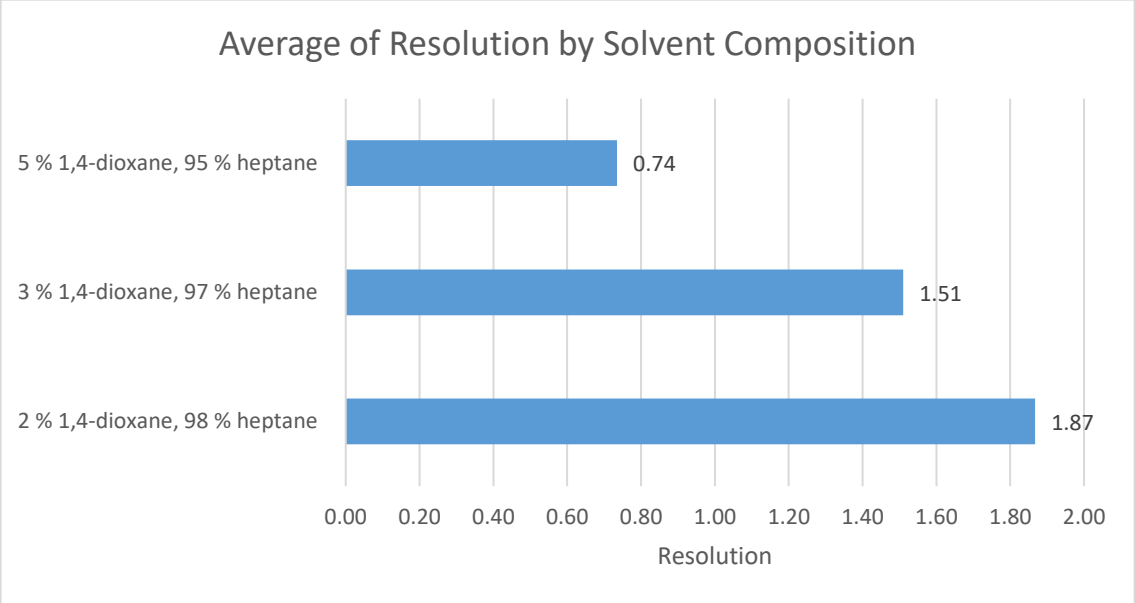


*Figure 12. Solvent compositions compared with the averages of achieved resolutions.*

Ultimately, it was decided to change the solvent composition of the 2% 1,4-dioxane and 98% heptane for the new UHPLC method since a better resolution was achieved.

## 3.2 Tocopherol and tocotrienol concentrations in sea buckthorn berries

Developed normal phase ultra-high-performance liquid chromatography equipped with fluorescence detectors (NP-UHPLC-FLD) was used to determine tocopherols and tocotrienols from freeze-dried sea buckthorn berries. Extracted lipid content was used as a sample. The samples were analyzed in 11 batches on different days. Calibration curves were made for each run and were prepared to contain all four tocopherols together to determine them simultaneously. Figures 13–16 show exemplary calibration curves for each tocopherol.

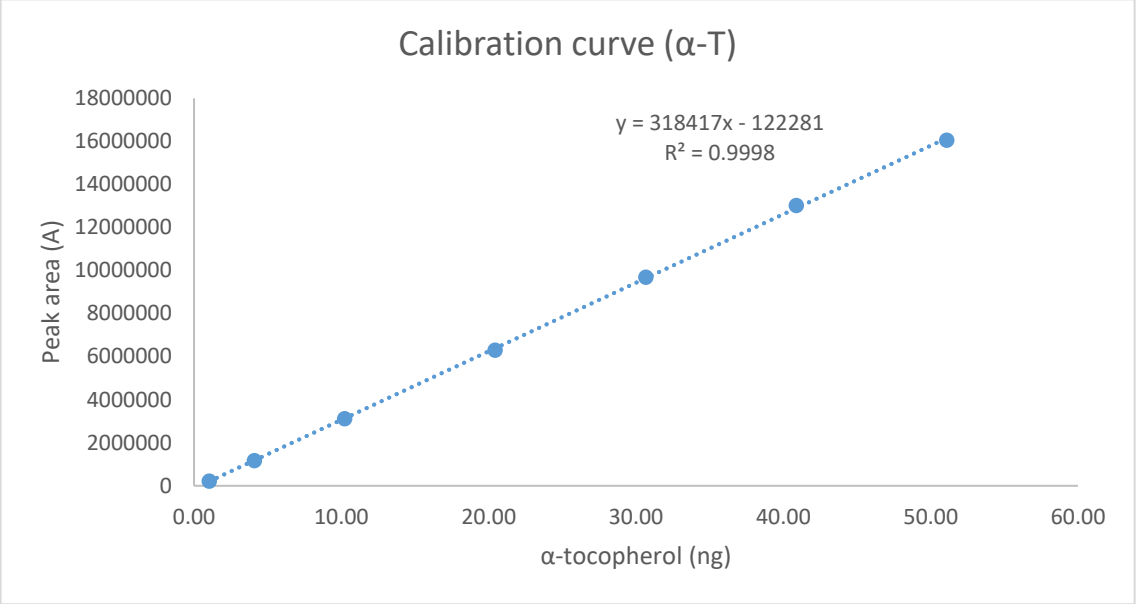


*Figure 13. An exemplary calibration curve of α-tocopherol. y=318417x-122281. $R^2$=0.9998.*

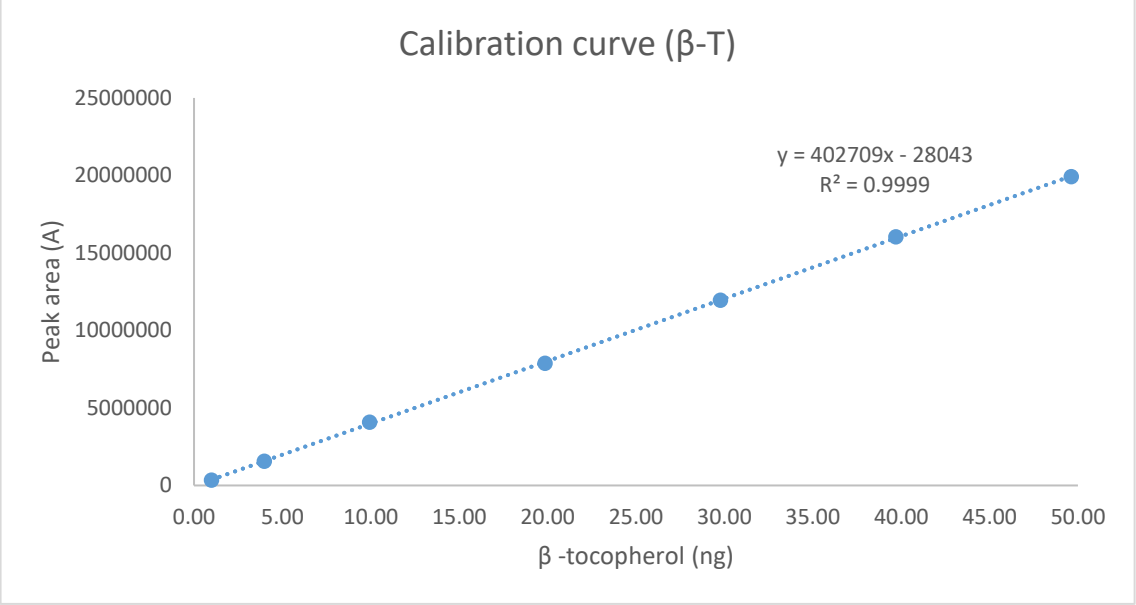


*Figure 14 An exemplary calibration curve of β-tocopherol. y=402709x-28043. $R^2$=0.9999.*

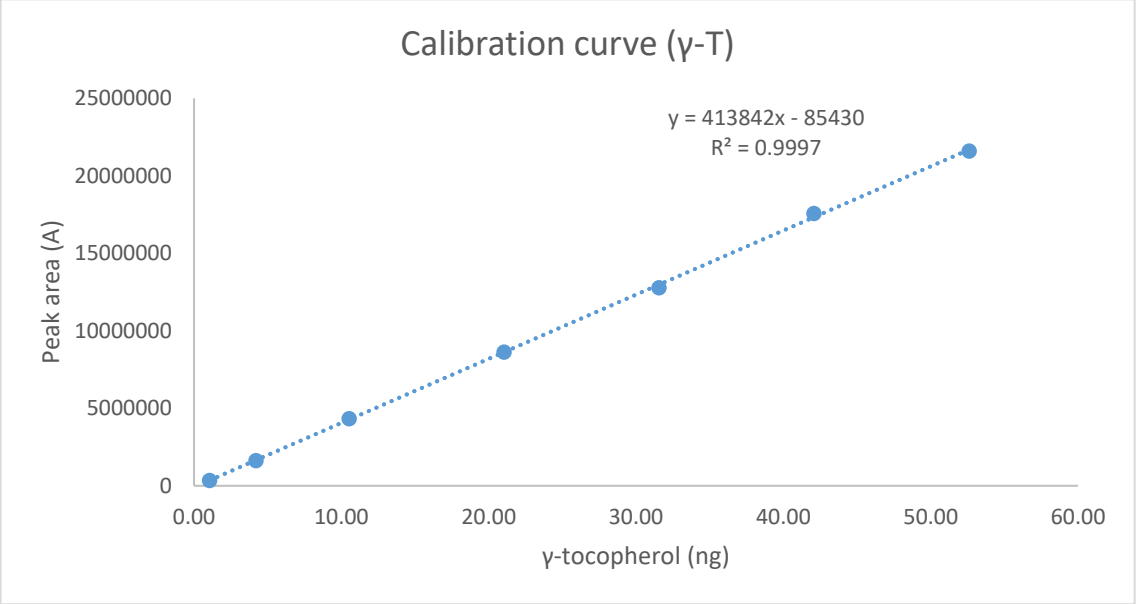


*Figure 15. An exemplary calibration curve of γ-tocopherol. y=413842x-85430. $R^2$=0.9997.*

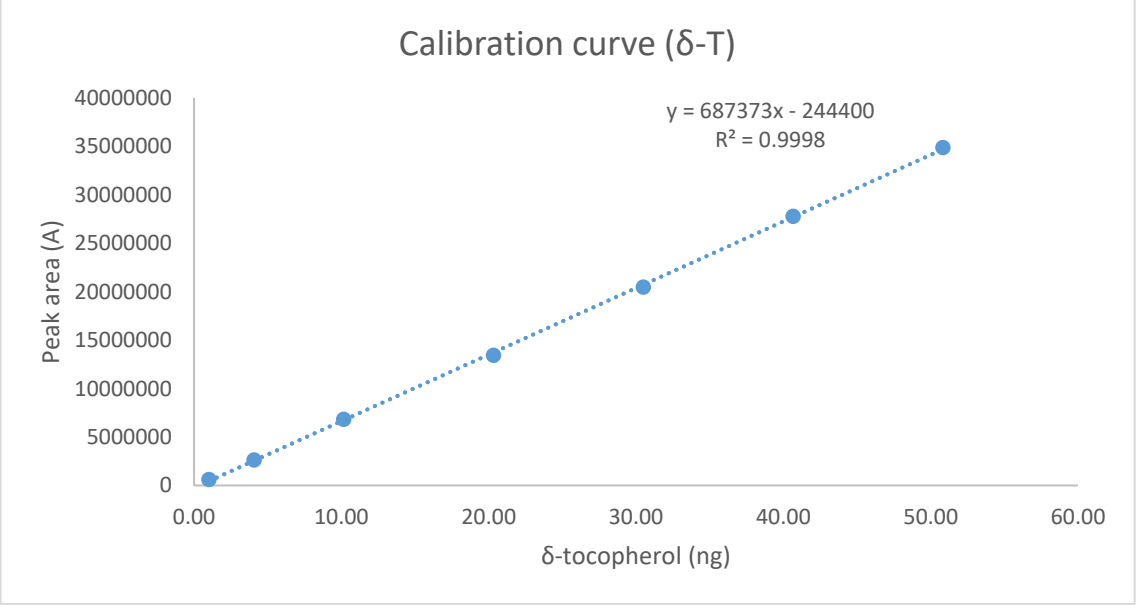


*Figure 16. An exemplary calibration curve of δ-tocopherol. y=687373x-244400. $R^2$=0.9998.*

As a result, all four tocopherols (α-, β-, γ-, and δ-T) were identified from the sea buckthorn berries, with α-T and γ-T having the highest concentrations. The lowest concentrations were observed for β-T, as expected. Of the tocotrienols, only α-tocotrienols (α-T3) were detected in some of the samples.

Example chromatogram from variety Terhi (time point 5, first parallel) is shown in Figure 17. For this sample, all tocopherols and α-T3 were identified.

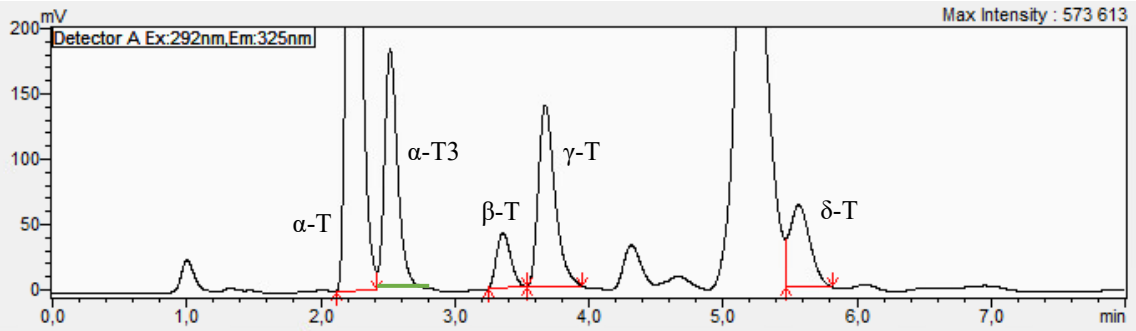


*Figure 17. Example chromatogram of variety Terhi (time point 5, first parallel) with concentrations (mg/100g) α-T: 80.8, β-T: 5.3, γ-T: 20.5, δ-T: 8.4 and α-T3: 27.1.*

Tables 7 and 8 lists the observed concentrations of tocopherols and tocotrienols in the sea buckthorn samples. The given values are the averages of three replicates, expressed in mg/100 g of freeze-dried berries.

Identified tocochromanol concentrations and standard deviations for the variety of Terhi are shown in Table 7.

*Table 7. Concentrations (mg/100 g) of identified tocochromanols in variety Terhi.*

| Time point | Growth area | α-T | β-T | γ-T | δ-T | α-T3 |
|---|---|---|---|---|---|---|
| 1 | Kittilä | 119.2 ± 6.5 | 4.0 ± 3.4 | 18.4 ± 0.6 | 0.0 ± 0.0 | 9.3 ± 16.1 |
| 2 | Kittilä | 88.3 ± 11.9 | 5.1 ± 0.8 | 17.9 ± 2.3 | 1.9 ± 3.3 | 11.8 ± 8.5 |
| 3 | Kittilä | 53.8 ± 4.0 | 3.3 ± 0.3 | 10.7 ± 0.7 | 0.0 ± 0.0 | 3.8 ± 3.5 |
| 4 | Kittilä | 37.3 ± 9.4 | 2.5 ± 0.7 | 8.5 ± 1.8 | 0.7 ± 1.3 | 9.8 ± 10.2 |
| 5 | Kittilä | 67.8 ± 11.8 | 4.5 ± 0.7 | 16.8 ± 3.5 | 6.5 ± 1.7 | 30.9 ± 11.6 |
| 6 | Kittilä | 85.0 ± 23.3 | 0.0 ± 0.0 | 19.3 ± 5.1 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 7 | Turku | 59.1 ± 1.7 | 2.8 ± 0.2 | 3.3 ± 0.5 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 8 | Turku | 66.7 ± 8.6 | 3.5 ± 0.5 | 3.1 ± 0.8 | 0.0 ± 0.0 | 2.5 ± 4.3 |
| 9 | Turku | 62.3 ± 19.0 | 3.7 ± 0.9 | 2.5 ± 0.6 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 10 | Turku | 48.0 ± 19.1 | 3.3 ± 1.3 | 1.6 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 11 | Turku | 73.8 ± 21.3 | 4.8 ± 1.3 | 0.0 ± 0.0 | 0.0 ± 0.0 | 2.4 ± 2.6 |
| 12 | Turku | 53.7 ± 21.1 | 3.8 ± 1.5 | 0.0 ± 0.0 | 0.0 ± 0.0 | 2.3 ± 0.6 |
| 13 | Turku | 59.6 ± 13.9 | 4.2 ± 0.9 | 0.0 ± 0.0 | 0.0 ± 0.0 | 1.0 ± 1.6 |
| 14 | Turku | 65.4 ± 26.2 | 4.7 ± 2.1 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 15 | Turku | 91.5 ± 0.7 | 7.5 ± 1.2 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 16 | Kittilä | 125.8 ± 9.9 | 0.0 ± 0.0 | 12.8 ± 1.6 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 17 | Kittilä | 127.0 ± 3.1 | 0.0 ± 0.0 | 15.0 ± 0.6 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 18 | Kittilä | 102.6 ± 25.5 | 0.0 ± 0.0 | 16.4 ± 4.2 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 19 | Kittilä | 99.4 ± 13.3 | 6.6 ± 0.9 | 21.4 ± 2.1 | 4.8 ± 4.2 | 23.6 ± 10.2 |
| 20 | Kittilä | 112.0 ± 3.4 | 0.0 ± 0.0 | 26.4 ± 1.0 | 0.0 ± 0.0 | 2.9 ± 2.9 |
| 21 | Kittilä | 123.4 ± 3.6 | 0.0 ± 0.0 | 31.2 ± 0.9 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 22 | Kittilä | 78.2 ± 32.7 | 0.0 ± 0.0 | 20.5 ± 8.4 | 0.0 ± 0.0 | 3.9 ± 5.5 |
| 23 | Kittilä | 85.1 ± 4.7 | 0.0 ± 0.0 | 24.5 ± 0.7 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 24 | Kittilä | 71.6 ± 6.0 | 0.0 ± 0.0 | 17.3 ± 2.2 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 25 | Kittilä | 70.1 ± 12.5 | 0.0 ± 0.0 | 16.7 ± 1.5 | 0.0 ± 0.0 | 0.0 ± 0.0 |

The concentrations of identified tocochromanols for the variety Terhi are demonstrated in Figure 18.

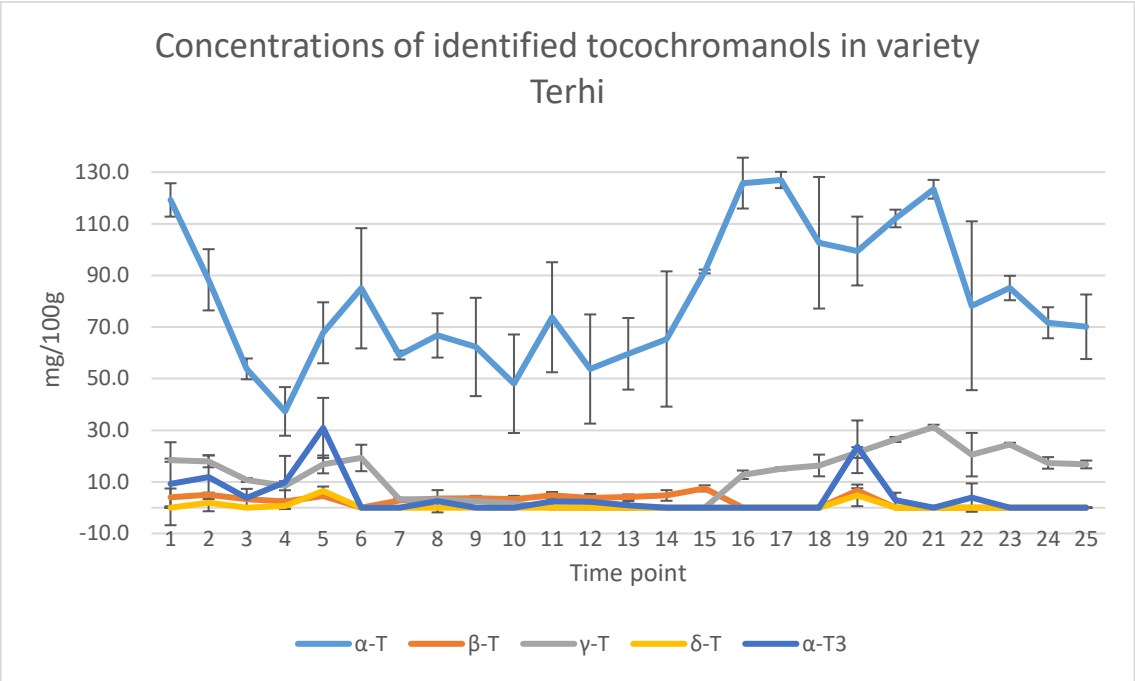


*Figure 18. The concentration of identified tocochromanols and their standard deviations for various Terhi.*

Table 8 shows identified concentrations and standard deviations for the variety Tytti.

*Table 8. Concentrations (mg/100 g) of identified tocochromanols in variety Tytti.*

| Time point | Growth area | α-T | β-T | γ-T | δ-T | α-T3 |
|---|---|---|---|---|---|---|
| 1 | Kittilä | 121.1 ± 16.5 | 7.1 ± 1.0 | 25.0 ± 3.3 | 8.4 ± 1.3 | 0.0 ± 0.0 |
| 2 | Kittilä | 94.8 ± 15.4 | 6.2 ± 0.7 | 20.2 ± 2.6 | 4.4 ± 3.8 | 2.9 ± 5.0 |
| 3 | Kittilä | 49.1 ± 2.4 | 3.6 ± 0.1 | 10.0 ± 1.0 | 3.8 ± 0.3 | 0.0 ± 0.0 |
| 4 | Kittilä | 44.7 ± 7.1 | 3.7 ± 0.8 | 8.6 ± 1.5 | 1.3 ± 2.3 | 2.3 ± 0.2 |
| 5 | Kittilä | 63.5 ± 19.6 | 4.9 ± 1.4 | 10.4 ± 3.3 | 1.5 ± 2.6 | 17.7 ± 2.5 |
| 6 | Kittilä | 75.2 ± 22.1 | 1.3 ± 2.2 | 20.6 ± 6.4 | 1.8 ± 3.1 | 11.0 ± 12.4 |
| 7 | Kittilä | 84.9 ± 23.9 | 8.0 ± 2.6 | 20.2 ± 6.4 | 4.7 ± 4.2 | 22.3 ± 15.6 |
| 8 | Kittilä | 86.5 ± 16.8 | 7.6 ± 2.0 | 21.5 ± 4.6 | 9.3 ± 2.3 | 14.2 ± 1.7 |
| 9 | Turku | 64.5 ± 10.1 | 3.8 ± 0.5 | 3.1 ± 0.3 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 10 | Turku | 55.4 ± 2.9 | 3.7 ± 0.1 | 1.1 ± 0.9 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 11 | Turku | 35.0 ± 5.5 | 2.7 ± 0.3 | 1.1 ± 0.2 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 12 | Turku | 66.9 ± 11.3 | 5.3 ± 0.9 | 0.6 ± 1.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 13 | Turku | 82.5 ± 22.5 | 7.0 ± 1.8 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 14 | Turku | 51.6 ± 8.9 | 4.3 ± 0.8 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.7 ± 1.2 |
| 15 | Turku | 95.3 ± 18.7 | 8.4 ± 1.7 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 16 | Turku | 68.8 ± 18.3 | 6.0 ± 1.6 | 0.0 ± 0.0 | 1.5 ± 2.6 | 0.0 ± 0.0 |
| 17 | Turku | 100.0 ± 6.7 | 8.2 ± 1.2 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 18 | Kittilä | 147.6 ± 8.5 | 0.0 ± 0.0 | 17.0 ± 1.1 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 19 | Kittilä | 143.9 ± 1.8 | 0.0 ± 0.0 | 22.7 ± 1.6 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 20 | Kittilä | 135.5 ± 2.2 | 0.0 ± 0.0 | 28.1 ± 1.7 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 21 | Kittilä | 98.5 ± 34.3 | 7.5 ± 2.5 | 17.2 ± 6.3 | 6.6 ± 2.3 | 9.4 ± 5.4 |
| 22 | Kittilä | 96.5 ± 16.3 | 0.0 ± 0.0 | 28.8 ± 4.5 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 23 | Kittilä | 135.9 ± 11.9 | 0.0 ± 0.0 | 44.4 ± 3.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 24 | Kittilä | 83.6 ± 45.0 | 0.0 ± 0.0 | 27.9 ± 15.1 | 0.0 ± 0.0 | 2.0 ± 2.9 |
| 25 | Kittilä | 71.9 ± 19.2 | 2.9 ± 0.8 | 21.8 ± 7.2 | 4.4 ± 1.1 | 0.0 ± 0.0 |
| 26 | Kittilä | 74.6 ± 47.8 | 2.8 ± 2.5 | 4.6 ± 0.8 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 27 | Kittilä | 55.1 ± 10.3 | 2.5 ± 0.5 | 14.9 ± 2.8 | 0.0 ± 0.0 | 0.0 ± 0.0 |

Demonstration of the results of tocochromanols concentrations of variety Tytti are shown in Figure 19.

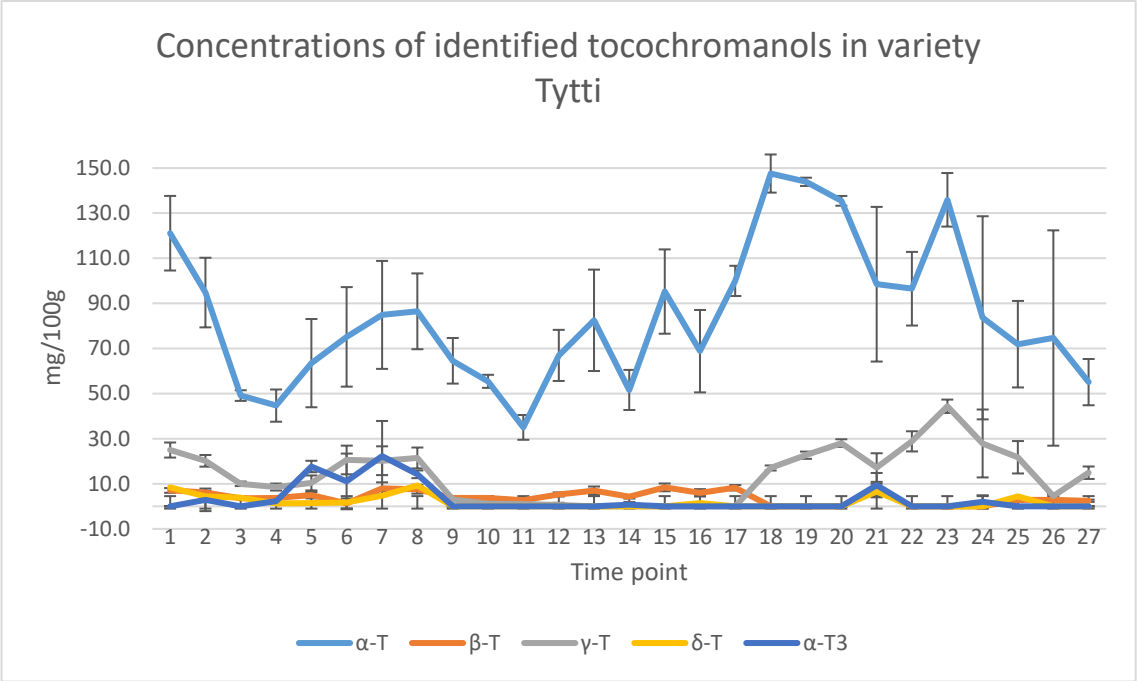


*Figure 19. The concentration of identified tocochromanols and their standard deviations for various Tytti.*

As both Tables 7 and 8 and Figures 18 and 19 indicate, some samples have much of a variation in the standard deviations.

## 3.3 Effects of environmental conditions on tocochromanols

The concentration of tocochromanols was compared to environmental conditions such as precipitation, effective temperature sum, and average relative humidity. Also, latitudes between the southern and northern parts, from 60° to 68°N, were compared. The weather conditions were calculated considering the values from the start of the growing season to the collection date.

The effective temperature sum consists of the sums of the average temperatures of the days of the growing season, which take into account the part of the average temperatures exceeding five degrees. The temperature sum is calculated for those days of the year when the temperature exceeds +5 ° C.

Figure 20 compares tocochromanols concentrations in different growth conditions mentioned above and is colored based on the growth areas. It is clearly seen that the sea

buckthorn berries grown in Turku differ significantly from those grown in Kittilä. Berries grown in Turku are clearly separated from those grown in Kittilä, and there is no overlapping between these two. Berries grown in Turku also appear to have many similarities in their tocochromanol composition, and they correlate with each other quite well. Also, the sea buckthorn berries grown in Kittilä seem more varied in their tocochromanol compositions and are explicitly more dispersed.

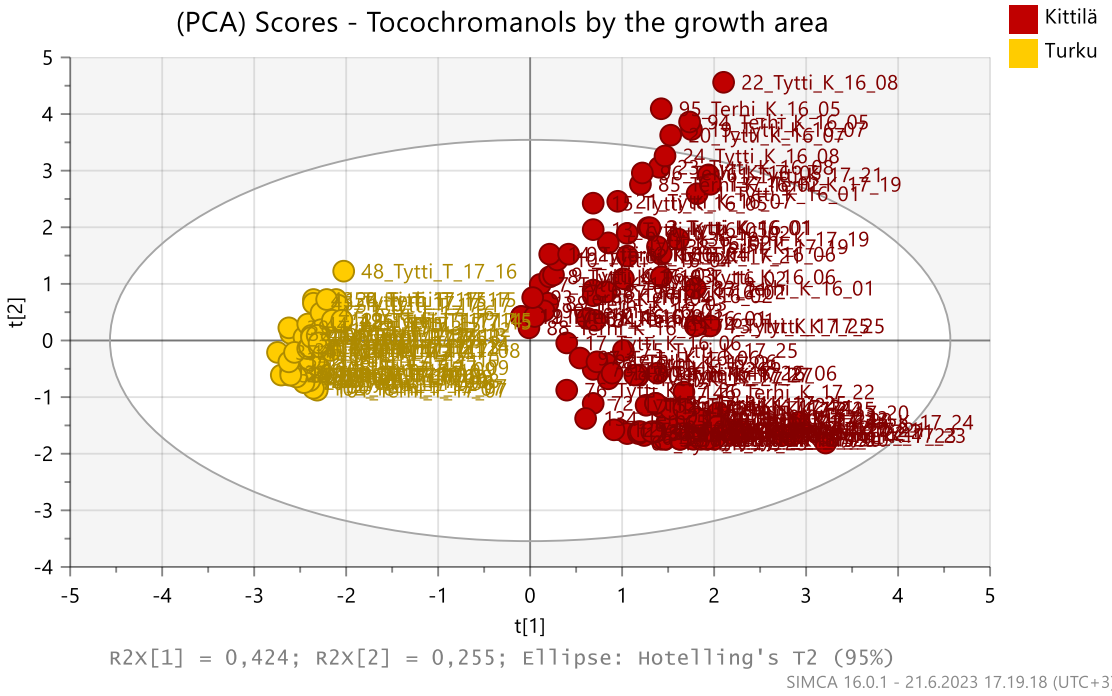


*Figure 20. Comparison of tocochromanol concentrations with growth conditions. Coloring is based on growth areas. PCA model of 156 objects and 8 variables (UV-scaled; $R^2X[1] = 0.424$, $R^2X[1]_{(cum)} = 0.424$, $Q^2[1] = 0.285$, $Q^2[1]_{(cum)} = 0.285$; $R^2X[2] = 0.255$, $R^2X[2]_{(cum)} = 0.679$, $Q2[2] = 0.179$, $Q^2[2]_{(cum)} = 0.413$).*

A clear distribution of the samples was also seen when the PCA model was made with an emphasis on classification according to the year of berries collection, which is shown in Figure 21. Berries collected in 2016 seem more scattered than those collected in 2017.

On average, berries collected in 2017 had slightly higher tocochromanols concentrations than those collected in 2016, but this may be a result of the 2017 berries being somewhat fresher.

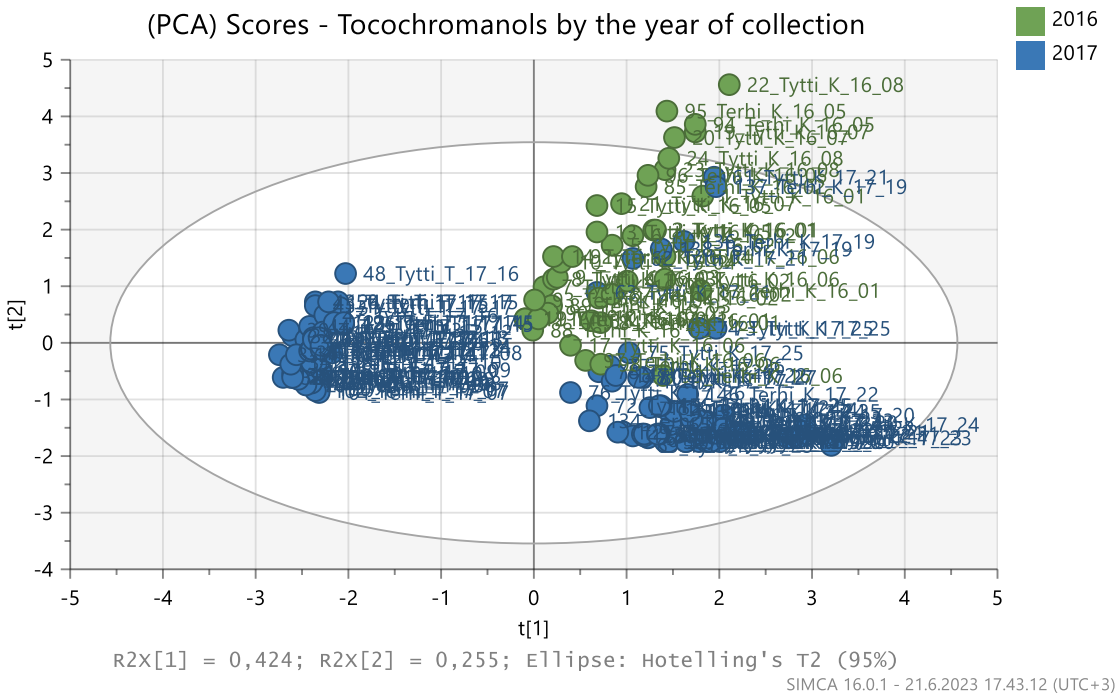


*Figure 21. Comparison of tocochromanol concentrations with growth conditions. Coloring is based on collection years. PCA model of 156 objects and 8 variables (UV-scaled; $R^2X[1] = 0.424$, $R^2X[1]_{(cum)} = 0.424$, $Q^2[1] = 0.285$, $Q^2[1]_{(cum)} = 0.285$; $R^2X[2] = 0.255$, $R^2X[2]_{(cum)} = 0.679$, $Q^2[2] = 0.179$, $Q^2[2]_{(cum)} = 0.413$).*

When the PCA was examined based on the varieties, it was noticed that the variety of the berries does not seem to matter in terms of tocochromanol concentrations. Results can be observed in Figure 22, where the PCA model is colored based on the berry varieties.

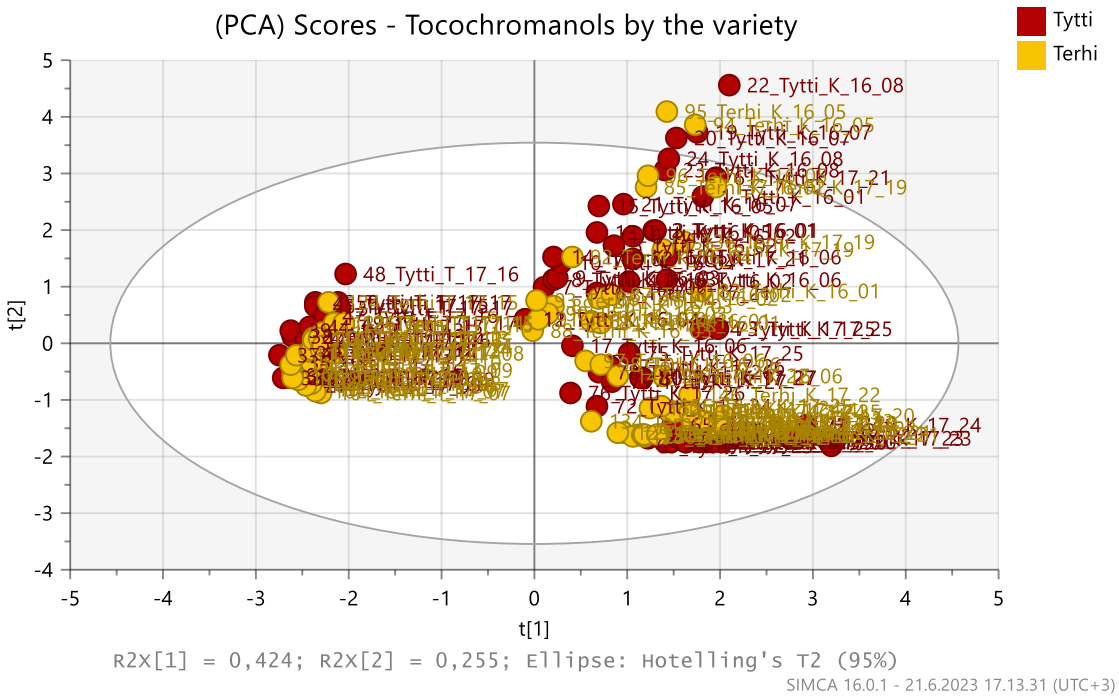


*Figure 22. Comparison of tocochromanol concentrations with growth conditions. Coloring is based on berries varieties. PCA model of 156 objects and 8 variables (UV-scaled; $R^2X[1] = 0.424$, $R^2X[1]_{(cum)} = 0.424$, $Q^2[1] = 0.285$, $Q^2[1]_{(cum)} = 0.285$; $R^2X[2] = 0.255$, $R^2X[2]_{(cum)} = 0.679$, $Q^2[2] = 0.179$, $Q^2[2]_{(cum)} = 0.413$).*

PCA loadings are represented in Figure 23.

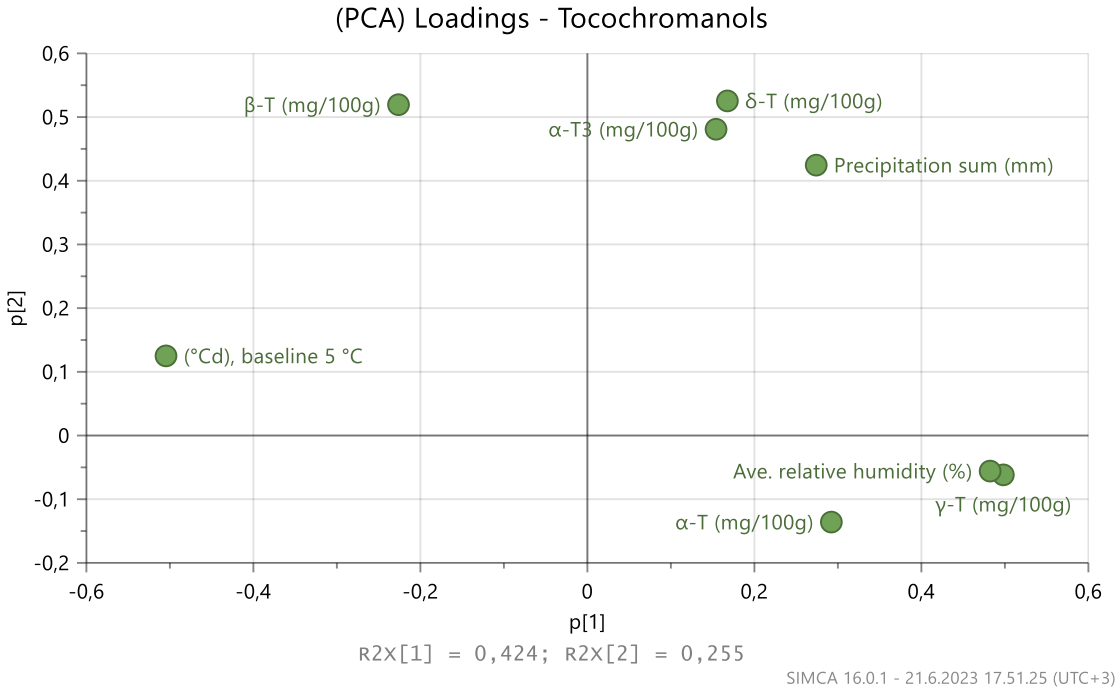


*Figure 23. PCA loadings plot p[2] vs. p[1].*

Since PCA scores did not separate the samples well regarding the different varieties, the PLS-DA model was also tested (data not shown). However, this model did not give any more precise results either. Eventually, the PCA biplot was tried. All the growth conditions were removed from the variables, and the data were scaled with the Pareto scaling technique. The PCA Biplot is presented in Figure 24.

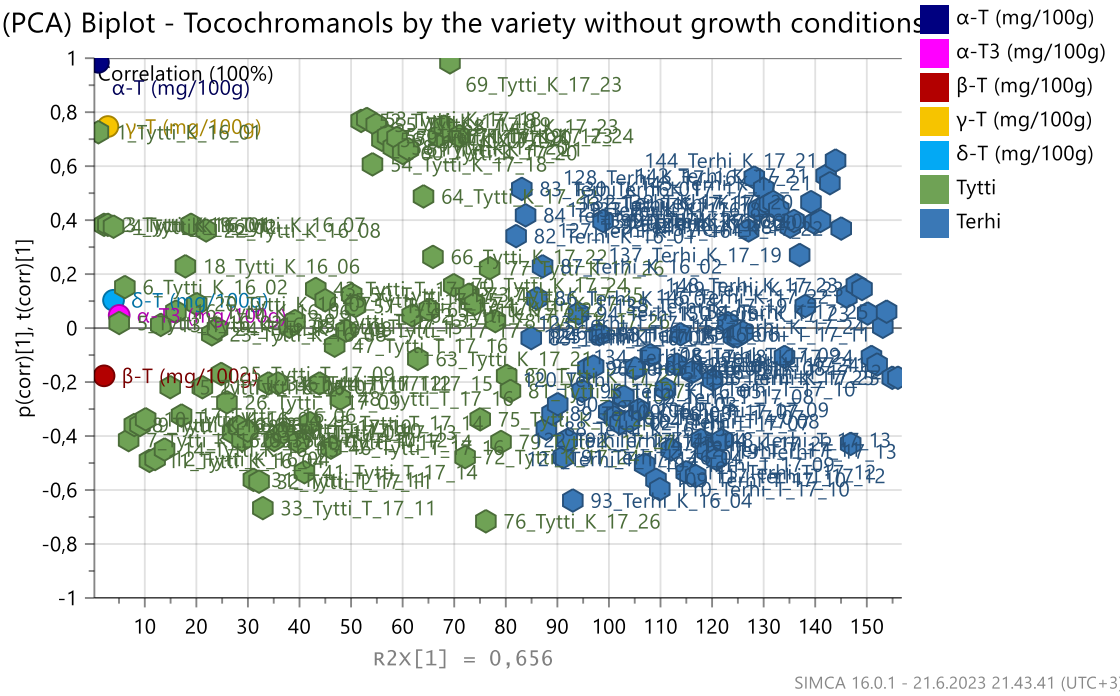


*Figure 24. Comparison of tocochromanol concentrations by variety without growth conditions. PCA biplot model of 156 objects and 5 variables (Pareto-scaled; $R^2X = 0.656$, $R^2X_{(cum)} = 0.656$, $Q^2 = 0.225$, $Q^2_{(cum)} = 0.225$).*

As Figure 24 implies, the tocochromanol concentrations of both varieties were moderately scattered. Repercussion might be due to the samples' high variation of the standard deviations, as stated in Chapter 3.2 Result indicates that the chosen principal component do not sufficiently capture the data variability and do not distinguish between them effectively. Nevertheless, it can be stated that the variety of berries has hardly any significance in forming tocochromanol concentrations in sea buckthorn berries.

As stated in Chapter 3.2, the highest concentrations were detected with α- and γ-tocopherols. PCA loadings (Figure 23) show that these compounds are most affected by average relative humidity.

# 4 Conclusions

A new chromatographic method was successfully developed in such a way that it can be performed with UHPLC in the future, bringing many advantages for analyzing tocopherols and tocotrienols in sea buckthorn berries.

The HPLC column was changed from Phenomenex Luna® Silica column 3.0 µm, 250 × 4.6 mm, 100 Å (Torrance, California, USA) to UHPLC Restek Pinnacle DB Silica column 1.9 µm, 100 × 2.1 mm, 140 Å (Bellefonte, Pennsylvania, USA), in order to control peak shifting and achieve optimum resolution. The oven temperature was not necessary to change. The run characteristics of both traditional HPLC and new UHPLC methods are listed in Table 9.

*Table 9. Run characteristics of HPLC and UHPLC methods.*

| | **HPLC** | **UHPLC** |
|---|---|---|
| *Flow rate* | 2.0 ml/min | 0.4 ml/min |
| *Analysis time* | 25 min | 8 min |
| *Oven temp.* | 30 °C | 30 °C |
| *Solvent comp.* | 3% 1,4-dioxane, 97% heptane | 2% 1,4-dioxane, 98% heptane |
| *Column* | Phenomenex Luna® Silica 3.0 µm, 250 × 4.6 mm, 100 Å | Restek Pinnacle DB Silica 1.9 µm, 100 × 2.1 mm, 140 Å |

Due to method development, the analysis time could be reduced to eight minutes instead of the previous 25 minutes. The solvent composition ratio was also changed from 3% 1,4-dioxane and 97% heptane to 2% 1,4-dioxane and 98% heptane due to better results in resolution values.

With the traditional HPLC method, the solvent consumption was 2.0 ml/min of flow rate, while with the new UHPLC method, consumption was able to decrease even to 0.4 ml/min of flow rate reducing solvent composition by 92%. Lower reagent consumption will also positively affect the environment's point of view when the amount of used solvents is much less. Therefore, developing the method from HPLC to UHPLC was also an environmental act in addition to the fact that it will be helpful considering the lipid analytics of sea buckthorn berries (Cielecka-Piontek *et al.*, 2013).

The upgraded UHPLC method was used to study how different environmental conditions affect tocopherol and tocotrienol levels in sea buckthorn berries. It was hypothesized that the environmental stresses induced by extreme environmental conditions in the far north would positively influence the concentration of the tocochromanol content in the sea buckthorn berries.

Verma and Shukla (2015) and Yang *et al.* (2018) discovered that the same species grown in different environments or growth conditions may have differences in the concentration of particular secondary metabolites. The results of this study supported this claim and aligned with the research hypothesis.

The highest concentrations were observed for α- and γ-tocopherols and the lowest for β-tocopherols. The average tocochromanol content of the berries grown in Kittilä is higher than those grown in Turku. The average α-tocopherol concentration for berries grown in Turku was 66.6 ± 20.4 mg/100 g, while for those grown in Kittilä same result was 91.5 ± 32.9 mg/100 g. However, as can be deduced from the standard deviations, there was much variation in the results, even between the berries grown in the same area. Nevertheless, it can be stated that the tocochromanol contents of the sea buckthorn berries grown in Kittilä were higher than those grown in Turku.

Of the environmental conditions, average relative humidity had the most significant effect on the concentration of compounds. The variety of the berries was not crucial regarding tocochromanol content in seabuckthorn berries.

Although the method development was carried out with the properties of sea buckthorn berries in mind, it can be used to determine the tocochromanols of any oils or extracted lipids in the future.

For further development, it is possible to refine the method. As perceived, a lot of standard deviation variations were observed between the results. This could be the result of non-homogeneous samples or inaccuracy in the sample pre-treatment step. One possibility to improve the accuracy of the results could be a more critical examination of the sample pre-treatment method and possible changes to the work steps, so the accuracy of the results could be further improved in the future.

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