Turun yliopisto
University of Turku

Effects of Wine Yeast to Chemical Composition of Black Currant Wine – Anthocyanins and Alcohols

Master thesis in Technology
University of Turku
Department of Biochemistry
Master's Degree Programme in Food Development
August 2016

Niina Kelanne

The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service

University of Turku
Biochemistry
Kelanne, Niina: Effects of Wine Yeast to Chemical Composition of Black Currant Wine – Anthocyanins and Alcohols
Master Thesis of Science, 45 p, 8 appendix p.
Food Development
August 2016

Wine has been made over 7000 years. Usually wine is made from fruits of *Vitis vinifera*, but other raw material can be used and then wine is called fruit wine. Grape vines do not grow well in Finland. That is why the most of the wines produced in Finland are produced from other raw materials than grapes. Even 200 000 liters of fruit wines are produced yearly. The most used wine yeasts species are different *Saccharomyces cerevisiae* varieties, because they can use many different sugars as a source of energy and they tolerate the high concentrations of ethanol. Use of non-*Saccharomyces* species have increased, because they have positive effects on sensory properties of the wine. The yeast species effects on fermentation time, concentrations of ethanol and other alcohols, stability of anthocyanins and sensory properties. It is important to know differences between yeast species to get best result in fermentation and aromatic compounds.

In this study 11 wines were made by using two black currant varieties, Mikael (LUKE's variety 17) and LUKE's variety 15, and four commercially available yeast products, two *Saccharomyces cerevisiae*, one *S. bayanus* and one *Torulaspora delbrueckii* species. The wines were made by applying commercially available home wine methods. Fermentation was monitored with pH, °Brix and specific gravity assays. Alcohols were analyzed with GC-FID instrument and anthocyanins were analyzed and identified first with HPLC-DAD instrument and final identification was made with UHPLC-DAD-MS instrument.

Yeasts were noticed to have different lag phases and fermentation time. Ethanol and three other alcohols, methanol, isoamylalcohol and isobutanol, were identified from wines. The concentration of ethanol was the highest in the *S. cerevisiae* wines and lowest in the *S. bayanus* wines. The amount of the higher alcohols were the highest in wines fermented with *T. delbrueckii*. Four main anthocyanins, characteristic in black currant, and six other anthocyanins were identified from the wines. Two of anthocyanins were acylated anthocyanins. The yeasts had significant effect on final anthocyanin concentration than black currant species. The final concentrations of the anthocyanins were higher in wines made from *S. cerevisiae* than in *S. bayanus* wines.

Key words: alcohol, anthocyanin, black currant, chromatography, fermentation, fruit wine, yeast

Turun Yliopisto
Biokemia
Kelanne, Niina: Viinihiivan vaikutus mustaherukkaviinin kemialliseen koostumukseen – antosyaanit ja alkoholit
Diplomityö, 45 s, 8 liites.
Elintarvikekehitys
Elokuu 2016

Viiniä on valmistettu suunnitelmallisesti viinirypäleistä yli 7000 vuotta. Viiniä voidaan valmistaa myös muista raaka-aineista kuin viinirypäleistä, jolloin niitä kutsutaan hedelmäviineiksi. Suomessa viinirypäleiden kasvattaminen on haasteellista kylmän ilmaston takia. Muiden marjojen, kuten mustaherukan, kasvattamiseen Suomen ilmasto on sopiva. Ammattimaisesti hedelmäviinejä valmistetaan Suomessa noin 200 000 litraa vuodessa. *Saccharomyces*-suvun hiivalajit ovat käytetyimpiä hiivalajikkeita viinin valmistuksessa, koska ne käyttävät ravintonaan useita sokereita ja niillä on korkea etanolitoleranssi. Kaikki viinihiivat eivät ole *Saccharomyces*-sukuisia. Ei-*Saccharomyces*-sukuisilla hiivoilla on todettu olevan muun muassa positiivinen vaikutus aistittaviin ominaisuuksiin. Hiivalaji vaikuttaa käymisaikaan, etanolin ja muiden alkoholien pitoisuuksiin, antosyaanien stabiilisuuteen sekä aistittavaan laatuun. Hiivalajien erojen tunteminen on tärkeää oikean lajin valitsemiseksi.

Työssä valmistettiin 11 viiniä käyttäen kahta eri mustaherukkalajiketta, Mikaelia (LUKE:n lajike 17) ja LUKE:n lajike 15:ta, sekä neljää kaupallista hiivatuotetta, Cross Evolution YSEO (*S. cerevisiae*), Lalvin ICV-K1 (*S. cerevisiae*), Lalvin C (*S. bayanus*) sekä Biodiva™ (*Torulaspora delbrueckii*). Viinit valmistettiin mukaillen kolmea kaupallista kotiviinireseptiä. Käymisen edistymistä seurattiin pH- ja °Brix-mittauksin sekä ominaispainoseurannalla. Alkoholit tunnistettiin GC-FID-laitteistolla. Antosyaanien analysoinnissa ja tunnistuksessa käytettiin ensin HPLC-DAD-laitteistoa ja lopullinen tunnistaminen tehtiin UHPLC-DAD-MS-laitteistolla.

Hiivan vaikutti käymisen alkamiseen sekä kestoon. Etanolin lisäksi viineistä tunnistettiin kolme sikuna-alkoholia, metanoli, isoamyylialkoholi sekä isobutanoli. *S. cerevisiae* –lajit tuottivat eniten etanolia ja *T. delbrueckii* tuotti eniten korkeita alkoholeja. Viineistä tunnistettiin neljä mustaherukalle tyypillistä antosyaania sekä kuusi muuta antosyaania, joista kaksi olivat asyloituneita antosyaniinia. Hiivalla huomattiin olevan enemmän vaikutusta lopulliseen antosyaanien pitoisuuteen kuin mustaherukkalajikkeella: *S. cerevisiaella* valmistetuissa viineissä lopullinen antosyaanipitoisuus oli korkeampi kuin *S. bayanuksella* valmistetuissa.

Asiasanat: alkoholi, alkoholikäyminen, antosyaani, hedelmäviini, hiiva, kromatografia, mustaherukka

Contents

Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
C_2H_5OH	Ethanol
$C_6H_{12}O_6$	Glucose
CO_2	Carbon dioxide
DA	Degree of acylation
DAD	Diode array detector
DAY	Dried active yeast
DNA	Deoxyribonucleic acid
E.g.	Exempli gratia
FID	Flame ionization detector
GC	Gas chromatography
Glu	Glucose
HCl	Hydrochloride
HPLC	High performance liquid chromatography
K1, K2, K3	Toxic proteins secret by *Saccharomyces cerevisiae*
MeOH	Methanol
MO	Microorganism
MS	Mass spectrometry
NAD^+	Nicotinamide adenine dinucleotide (oxygenator)
NADH	Nicotinamide adenine dinucleotide (reductor)
PC	Principal component
PCA	Principal component analyse
PDA	Photo diode array detector
RNA	Ribonucleic acid
SO_2	Sulphide dioxide
UHPLC	Ultra high performance liquid chromatography
UV	Ultraviolet
UV-Vis	Ultraviolet-Visible

1 Introduction

1.1 Wine

Men have been enjoying wine over 7000 years and the oldest discovered winery is over 5500 years old. Evidence of grapes (*Vitis vinifera*) have been found in both cases.[1] The European Union defines wine as a product made from whole or crushed grapes or juice pressed from grapes by partly or whole ethanol fermentation.[2] The most common yeast used to ferment wines is *Saccharomyces cerevisiae* and it is proved it has been used to ferment wine in Egypt 5000 years ago[3].

There are two main reasons why grapes have been used to process wine thousands of years. Primary reason is that all carbohydrates of the grapes are in soluble form unlike in almost every other fresh fruits and berries, which storage their carbohydrates as a pectin or starch. When carbohydrates are in soluble form, they usually are composing of mono- or disaccharides and yeast can easily use them. Yeasts can start fermentation of sugars fast and concentration of the ethanol increases, which reduce the risk of spoilage by other microorganisms (MO). The second reason is tartaric acid. Tartaric acid content in grapes is relatively high compared to other fruits. That is rare, because usually tartaric acid occurs only in other parts of the plant. Few MOs can metabolize tartaric acid. Wine remains sufficient acidic which limits the growth of spoilage microorganisms.[4]

The climate in Finland is too cold for grapevines to grow successfully and bear fruits because the plant prefers areas which annual isotherm is approximately between 10 and 20 °C. In practice, that means European countries in Mediterranean Sea area and southern parts of Germany, Poland and United Kingdom, which are the most important wine countries in Europea.[4] Fruit and berry wines are more common than grape wines in Finland. Finnish wines are made in 25 wineries and the annual yield is approximately 200 000 litres.[5]

1.2 Wine making

Winemaking in the winery is sophisticated and long process. It can include many unit operations, such as harvesting, pressing, fining and filtration (see fig. 1). Winemaking in a home can be a simpler process, because of the various commercial available ready-to-use ingredients and equipment. In this thesis, wine was made by home making. Nevertheless, the fermentation in yeast cells occur in the same way in the both conditions.

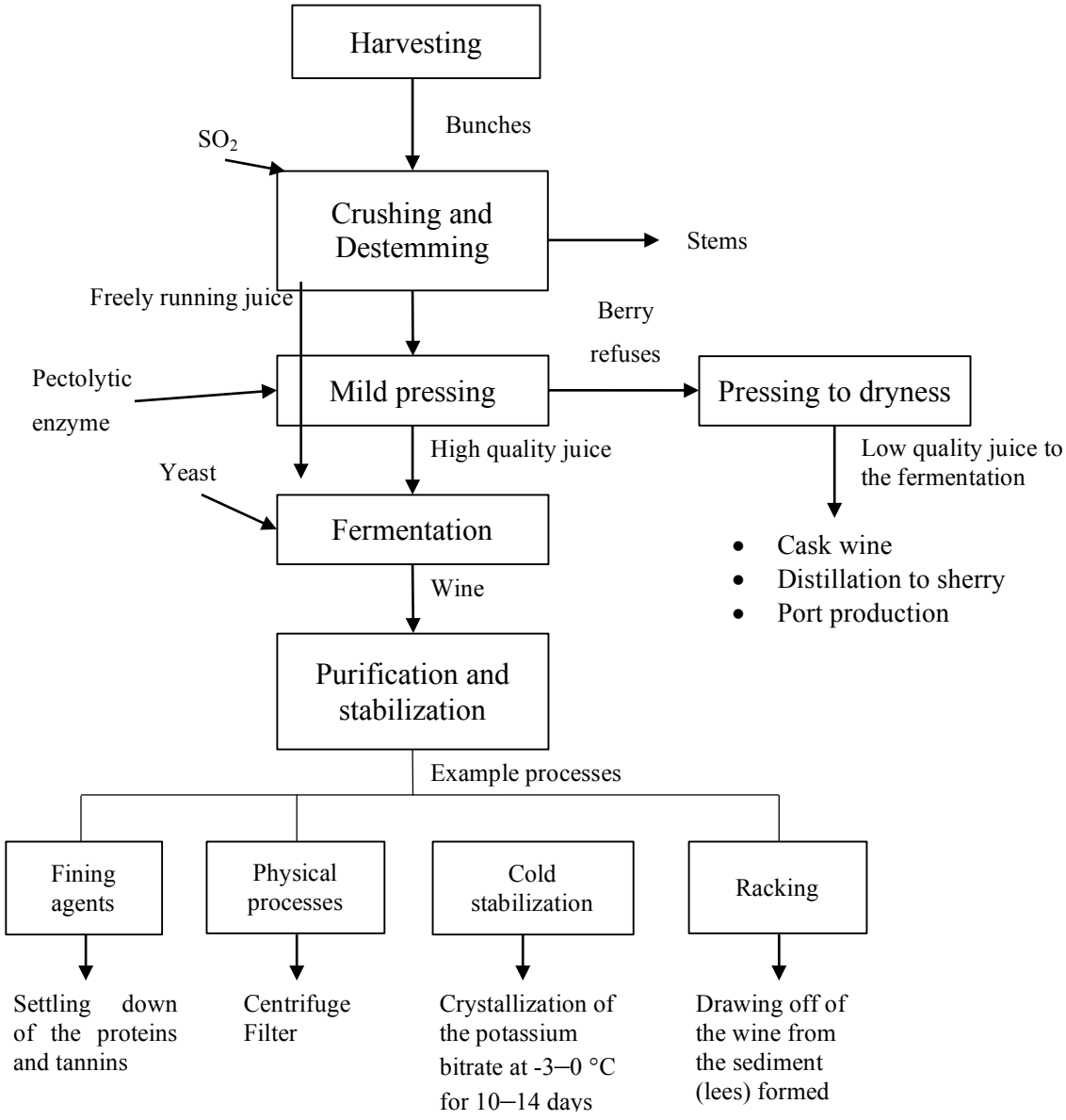


Figure 1 An example of winemaking process in the winery.[6]

Usually, wine is made from grapes, which are juiced immediately after harvesting[6]. When wine is made from non-grape-fruit materials, if they are not used immediately,

they should be frozen after harvesting, to reduce decomposition of the i.e. polyphenols.[7] It is important to schedule harvesting to the optimum moment when the grapes contents of the sugars, aroma compounds and acid are right for the wine. The development of the grapes is followed by monitoring pH, acid, flavor compounds and sugars. When the optimum levels are reached, the grapes are harvested.[6] The grapes can be treated with sulfur dioxide after harvesting to avoid the spoilage by MOs[8].

Grapes are harvested in the bunches. Before present technologies in the winemaking, the stems were left to the crushing and pressing, because they helped the retention of the grape juice by making drainage channels where juice could run freely. Nowadays the stems are removed before crushing, because they contain phenolic compounds which can produce more bitter and astringent taste to the wine. Some red grape varieties have notably low concentration of the phenolic compounds thus the stems are left to the must to increase the concentration. Usually the stemming and the crushing are made with the one equipment due to effectiveness and practicality. The first part of the crusher-stemmer removes the stems from the grapes and allows the grapes move through leaving the stems and leaves behind. The grapes bruise as little as possible.[8]

Crushing of the grapes happens immediately after the stemming, so is avoided oxidative and microbiological spoilage of broken grapes[8]. Before crushing the grapes can be treated with sulfur dioxide to decrease the amount of MOs in the final juice[6]. Usually crushing is made by pressing the grapes against a perforated wall or by the set-rollers. The set-rollers are usually turning in opposite directions they can have spiral ribbing or grooves with intercollecting profiles, which draw grapes through the crushing space. The spacing between rollers is adjustable, thus the same equipment can be used to crush different sized grapes. Crushing of the grapes can be also be done with the centrifuge, where the grapes are pressed to the wall by centrifugal forces. Supraextraction is a new no-crushing way to separate juice from the grapes before pressing. There the grapes are first cooled to -4 °C and warmed to +10 °C. Freezing causes cells to rupture and the skins to spli, which let the juice escape from the grape. Supraextraction increases sugar and phenolic contents and decreases total acids.[8]

After crushing, grape crush (the must) and separated juice is rested. This process is called maceration. During maceration, the released enzymes liberate and dissolve

bounded solids (e.g. phenolic and flavonoid compounds, flavors, tannins and nutrients) from the skin, flesh and seeds to the juice. Success of the maceration is dependent on time and temperature. For example, with short time and cool temperature can minimize oxidative browning and flavonoid uptake, which results in decreasesd the anstringency and bitterness. Maceration is more common and important for the red wines than for the white wines. The color of the red wine is formed during maceration: if the maceration time is short (for red wines <24 h), the result is rosé wine. Usually for red wines the time of the maceration is 3–5 days, but for long aging wines it can be as long as 3 weeks. 3–5 days is suitable time to colors and flavors extract to the juice, but tannins will still mostly remain in the skin and seeds.[8]

Before pressing, the freely running juice can be collected with a de-juicer. De-juicer is typically a columnar tank with a perforated basket. The crushed grapes are packed to the basket and the mass of the juice makes it flow to the tank above. The separation can be speeded up with carbon dioxide pressure. Rest of the juice is collected with pressing. Mild pressing is used to press high quality juice for the actual wine and after that the grapes are pressed to the dryness resulting in lower quality juice, which is used to sherries, porters and cask wines.[6] Pressing can be done to the whole grapes, the must or to the fermented must. There are four main press equipment: horizontal, vertical, pneumatic and continuous screw press. The press type should be chosen depending on aforementioned types of the press raw material. For example, the continuous screw presses work best with the fermented must and pneumatic presses with the non-fermented crushed or non-crushed grapes. The type of the press and the mildness or hardness of the pressing effect on sensory properties of the wine. The first fractions from the press have similar physico-chemical characteristics to the free-running fraction. Pressing time also effects on sensory properties of the wine: the longer time result in more dissolved tannins and other phenolic compounds. Pressing aids, such as rice hulls or cellulose, can be used and they can increase the yield of freely-running juice from 5 to 15%.[8]

Fruit and berry juices have to be treated with pectinolytic enzymes before fermentation, because their higher concentration of the pectin than grapes. Sometimes pectinolytic enzyme is used with grapes, too, because some grape varieties concentration of the pectin is high.[7,8] If the pectin structures are not degraded by enzymes, they can cause so

called “pectin haze” in wine[7]. Pectinolytic enzyme treatment decreases pectin concentration and viscosity, but it also improves the filtration of the juices and wines[8,9]. The enzyme can also be added to the must before pressing. Then it increases the yield of freely-running juice.[8] Commercial pectinase products are typically used to hydrolyze pectin and other polysaccharides structures in fruits[9]. Usually the used enzyme product contains is pectin lyase activity, which acts on methylated pectins, but does not release methanol. Another pectinolytic enzyme is pectin methyl esterase, which releases methanol to the wine.[8] After the hydrolyzation, pectin-protein complexes are formed and they flocculate. Bigger particles are easier to precipitate.[9]

When the grape juice ready, it is fermented (fig. 1). Almost all wines are batch-fermented, which means that the concentration of the nutrients is maximal at the beginning of the fermentation and the concentrations decrease during the fermentation. Some essential nutrients (e.g. long fatty acids and nitrogen source) can be added to ensure viability of the yeast. The aim of the fermentation is dry wine, which sugar content is lower than 1 g per liter.[8]

The specific yeast species is inoculated into the must and after lag phase (see chapter 1.3) fermentation starts. Yeast uses sugars from the must as an energy source for its own biological processes. Under anaerobic conditions, yeast produces ethanol, fusel alcohols and carbon dioxide as by-products in its own metabolism. However, they are the main products for the user. The chain of biochemical reactions involved in the ethanol production is shown in the figure 2. The reaction includes 12 enzymes. Yeast produces two moles both ethanol and carbon dioxide from one mole of sugar:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$ [6]

Yeast does not only produce ethanol and other alcohols during fermentation, but it is responsible for unique flavor and bouquet of wine[8] which are formed from e.g. organic acids, fusel alcohols, polyols and phenols[10].

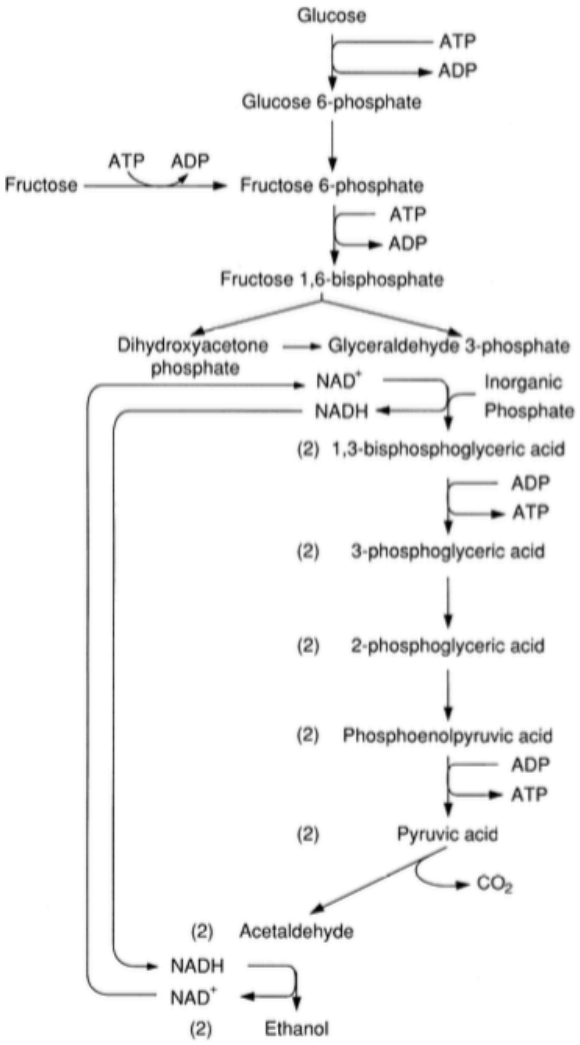


Figure 2 The chain of biochemical reactions involved in the ethanol production. [8]

Fermentation of wine has to be done in under carbon dioxide layer, because in aerobic conditions the yeast can convert the ethanol to water and carbon dioxide[6]. Fermentation time depends on the yeast, sugar and nutrition content of the must and fermentation conditions (e.g. temperature). Fermentation of wines takes approximately three weeks below 20 °C for white wines and at 20–24 °C for red wines.[11] After fermentation, small amounts of non-fermentable sugars, such as rhamnose, arabinose and xylose, can be found from the wine, but they do not effect on wine's sensory properties.

After fermentation is completed the yeast is killed, for example, with potassium sulfate-potassium sorbate mixture, which kills rapidly yeast cells and other MOs. Yeast has to be killed to discontinue the fermentation after bottling. Potassium sorbate is not only used in winemaking, but also to kill molds from table grapes. It has been proved to be

effective against many molds and fungi. It is not toxic to humans or environment and it is inexpensive. [12] Potassium sulfate can be added as a potassium bisulfite ($KHSO_3$) or a potassium metabisulfite ($K_2S_2O_5$). They both have the same effects on wine as a sulfur dioxide, which are explained below.[13]

The most common preservative used in wines is sulfur dioxide (SO_2)[14,15]. Sulfur dioxide has been used indirectly in wines and ciders as early as 15th century, but its intentional addition began in the early 20th century. Sulfur dioxide is a normal constituent of the wine, because yeast metabolizes it from sulfuric compounds of the raw materials.[15] Before addition of the sulfur dioxide the concentration can be between 10 and 30 mg/liter[13]. The concentration of SO_2 forming during fermentation depends on the yeast strain, fermentation temperature and sulfur content of the raw materials. The yeast strains of *Saccharomyces bayanus* are more prone to product SO_2 during fermentation than *S. cerevisiae* strains.[15] According to European Union, the final allowable maximum concentration of sulfur dioxide after fermentation and additional depends on wine type, but the maximal concentrations are between 125 and 400 mg/l. Sulfur dioxide has four characteristics why it is used so widely: it is antiseptic, antioxidant, antioxidasic and it binds ethanal. As an antiseptic agent it inhibits MOs development, but it works properly only in high concentrations and then it only destroys a percentage of MOs. SO_2 is more effective on bacteria than yeasts. During storage it complicates the development of all kinds of MOs. SO_2 antioxidants prevents chemical oxidation by binding dissolved oxygen, for example, it protects phenolic compounds from oxidation. The reaction is very slow and it needs catalyzers and it does not prevent enzymatic oxidations. Antioxidation properties of SO_2 helps develop wine aroma and taste during storage and aging by reducing oxidation-reduction potential. An antioxidasic agent, SO_2, inhibits oxidation enzymes, such as tyrosinase and laccase, which can be secreted by molds. The antioxidation properties of the SO_2 are useful during storage unlike the antioxidasic properties, which are useful during fermentation, because enzymatic oxidation is more common during fermentation than storage. Ethanal binding property of the SO_2 makes a flat character of the wine disappear.[13] The use of sulfur dioxide can be harmful to both wine (unwanted odors and flavors) and humans (allergic reactions). SO_2 can only be used in certain pH range, because SO_2 is antimicrobial molecule in the low pH. When the pH is 3–4, can only 5% of the SO_2 be in antimicrobial form.[14] Use of SO_2 as a

preservative is decreasing, because of its bad characteristics, and use of new preservatives is increasing.

Chitosan is one of the new preservatives in winemaking. It is produced from chitin by deacetylation. Chitin consists of 2-acetamido-2-deoxy- β-D-glucoside units which are attached by β(1 → 4) linkages and it is second abundant polysaccharide after cellulose in nature. When cellulose is produced by higher plants, chitin is synthesized by algae, fungi, insects and molluscs. Chitin resembles cellulose by its poor solubility and its only slight chemical reactivity.[16] Deacylation increases solubility, because solubility is highly effected on the degree of N-acetylation, which is decreased during deacetylation.[17] Chitosan is the N-deacetylated derivative of chitin.[16] Typically, chitin's degree of acylation (DA) is 0.90. DA indicates the presence of some amino groups and it is decreased during deacetylation. The DA of the chitosan is typically 0.35. Usually, naturally occurred polysaccharides are neutral or acid, but both chitin and chitosan are highly alkaline.[16] Chitosan kills bacteria, yeasts and moulds. Unlike sulphur dioxide, chitosan acts better on yeast than bacteria. It is safe to use by humans and it may have some health beneficial characteristics, such as it may decrease cholesterol levels and it may act as an antioxidant.[14]

Clarity of the wine is an important quality requirement of the consumers, which controls the buying decision. Turbidity of the wine causes Tyndall effect. In the Tyndall effect, the particles in suspension deflect light from its normal path: light scatters making wine look cloudy. Turbidity is unwanted and the meaning of fining and clarification are the elimination of it. The fining can also prevent haze formation by removal of proteins, which causes hazing, and some off-odors. It also decreases bitterness and astringent by aggregating phenolic compounds.[18] A success of the fining can be estimated by analyzing the turbidity.[19] Usually fining of the wine is done by spontaneous precipitation and sedimentation, but it can also be done by centrifugation or filtration. Because precipitation without any help would take months, the use of fining agents is come to use.[18,20] The fining agents were traditionally proteins of animal origin, such as gelatins (pork), casein (milk), isinglass (fish) and egg albumin. Inorganic fining agents (bentonite and siliceous earth) are also used.[19] The fining agents adsorb and bind to solid particles in wine. When particle size enlarges they precipitate quickly.

Occasionally precipitation does not work and centrifugation or filtration is needed to achieve crystal clear wine.

1.3 Wine yeasts

Yeasts are the simplest eukaryotes. They are a small (1%) group in the fungi kingdom. The size of a yeast cell is 3–5 µm in diameter. They reproduce by budding or sexually by forming ascospores. In the budding, a daughter cell extrudes from the mother cell. In wine and must environments, the duration of the budding is approximately 1–2 hours. Reproduction by ascosporing occurs when the environment is too hostile (for example, due to lack of sugar and nutrients) for yeast cells to reproduce. Cells transform to asci, sacs with thick cell wall which contains four haploid ascospores. In the wine fermentation, yeast cells rarely form ascospores, because the must is not propitious to the yeast sporulation.[13]

About half of yeast species are aerobic and the other half is facultative anaerobic. The latter is used in the alcohol fermentation. Because yeast cells have cell membrane, as well as the cell wall, they can tolerate the alcohols which they convert from the sugars. Optimal pH for almost all yeast species is 4.0–5.5, but for some of the yeasts optimal pH can drop as low as 1.5 and some can grow in wide pH range, between 3–10.

One of the most important decisions in winemaking is selection of the yeast species. Yeast effects on fermentation speed, secondary metabolites character and quality and aromatic properties.[13] It is common to use yeast from *Saccharomyces* genus in wine fermentation, because *Saccharomyces* species tolerate ethanol so well[21]. However, the use of non-*Saccharomyces* yeasts is increasing, because their ability to produce different and diverse aromatic compounds to wine[22]. *Saccharomyces* and non-*Saccharomyces* yeasts are usually used together, because non-*Saccharomyces* species do not tolerate the ethanol in equally high concentrations, which leads to incomplete fermentation[21].

Dried active yeasts (DAY) are widely used in winemaking. They differ from the instant yeasts with non-direct inoculation. That means they should first inoculate to small amount of liquid (reactivation) and after inoculation to the fermentation vessel is done.

The reactivation can be done in water or water-must mixtures. The temperature of the activating liquid depends on yeast strain, but temperature is usually between 25 °C and 40 °C. Also the stirring has to be gentle or no stirring at all.[23–27] DAY were developed, because winemakers were interested in improving wine quality and kinetics during fermentation. They have many beneficial characteristics for winemaking: It enables the choice of the yeast strain to match up requirements of the must, such as high sugar content, which, for example, increases average alcohol concentration of wines. It has also helped to eliminate apiculated yeasts. Apiculation means yeast cells which die shortly after the start of the fermentation.[23] When the yeast is woken up from dryness, rehydrate substrate can be used. Rehydration eases the adaption of the yeast cells and ensures healthy and strong fermentation.[28]

After inoculation reproduction of the yeast cells follow normal growth curve of MOs. Lag phase adapts yeast cells to the new environment.[8] Yeast cells do not reproduce in the lag phase, but they can grow in size and mass. They may also produce enzymes and the metabolic activity can increase. When the lag phase is over starts the logarithmic phase. In that phase, the yeast cells number increase exponentially. The length of the phase depends on the composition of the must, and reproduces time of the yeast species. Yeast cells reproduce as long as there are enough nutrients left and the ethanol concentration is not too high. When the nutrients levels decrease starts the stationary phase. In stationary phase yeast cells do not reproduce anymore, but they may produce more secondary metabolites than in any other phase. The last phase is death. Death occurs when wine can no longer support yeast cells and they die.[29]

1.3.1 *Saccharomyces cerevisiae and Saccharomyces bayanus*

Even though not until PCR technology made possible to analyze DNA of yeasts, for decades *Saccharomyces cerevisiae* and *Saccharomyces bayanus* have been considered to be different species. However, it is almost impossible to identify these two species from each other only with the physiological tests. Their most significant difference is the use of sugars: *S. cerevisiae* species can use D-galactose, sucrose, maltose and raffinose as energy an energy source whereas *S. bayanus* can use saccharose, maltose and raffinose. Two other differences exist between these *Saccharomyces* species: *S. bayanus* tolerate higher concentration of ethanol than *cerevisiae*, but its fermentation

time is also longer.[13] All the species in the *Saccharomyces* sensu stricto group are able to convert sugar into ethanol and carbon dioxide, but they are also widely used in brewing and bread making.[30] Both *Saccharomyces* species used in this research belong to the *Saccharomyces* sensu stricto group with *S. paradoxus*, *S. pastorianus* (natural hybrid of *S. cerevisiae* and *S. bayanus*), *S. cariocanus*, *S. mikatae* and *S. kudriavzevii* species.[13,31]

Cross Evolution YSEO used in this study is a new hybrid of *Saccharomyces cerevisiae* strain, which is developed by the Institute for Wine Biotechnology (Stellenbosch University, South Africa) and Lallemand Oenology. The development has aimed for increased availability of the nutrient and specificity. YSEO is desingned for white wines and rosé wines. Cross Evolution YSEO has high flavour release, its fruity and vegetative flavors are in balance. It does not require much of nutrients and it products prevails against other yeasts (killer phenomenon, see chapter 1.3.3). Cross Evolution YSEO produces a small amount of SO_2 bonding partners and its optimal fermentation temperature is 14–16 °C.

Lalvin ICV-K1 (V1116) used in this study is *Saccharomyces cerevisiae cerevisiae* strain. It is one of the yeasts producing more floral esters, if the fermentation temperature is below 16 °C and added nutrients are right. It is resistant to difficult fermentation conditions such as low fatty acid content, low turbidity and low temperature.[24] The yeast needs long fatty acids (e.g. palmitic acid C_{16} and stearic acid C_{18}) to synthetize essential steroids and build cell membranes. Also long fatty acids inhibits the synthetization of toxic mid-long (e.g. capric acid (C_{10}) and lauric acid (C_{12})) fatty acids.[8] Even though Lalvin ICV-K1 is used in the cold conditions, it can also grow in high temperatures, up to 35 °C. Lalvin ICV-K1 tolerate alcohol up to 18%, it has competitive factor K2. Its requirement in assimilable nitrogen is low to average, but its O_2 requirement is high. It produces a small amount of volatile acidity and the average amount of SO_2. It is the most suitable to produce white wines, but it can also be used to produce red and rosé wines.[24]

Lalvin C used in this study is a *Saccharomyces bayanus* strain, which is developed both for ferment basic and sparkling wines. It can be also used to restart the stuck fermentation.[25] The stuck fermentation means fermentation which has stopped for some

reason. The reasons are usually related to the non-optimum fermentation conditions, such as temperature is wrong or the nutrients are not right. All yeasts do not tolerate inoculation to the liquid which contains ethanol, so only certain yeast strains can be used.[23] Optimal temperature of Lalvin C is 15–30 °C, it tolerates up to 16% (v/v) of ethanol and it has high CO_2 tolerance (up to 15% by volume of alcohol), fermentation starts fast and main fermentation is fast, too, and it formats low amount undesirable by-products (*e.g.* SO_2, hydrogen sulphide and acetaldehyde) during fermentation.[25]

1.3.2 *Torulaspora delbrueckii*

Torulaspora delbrueckii was the first commercial non-*Saccharomyces* yeast species used in the commercial winemaking[32]. The winery is not normal niche for *T. delbrueckii*, so it cannot be found from the surfaces of the winery. Though, sometimes *T. delbrueckii* takes fermentation over from *S. cerevisiae* in spontaneous fermentation.[33] Many good oenological characteristics of *T. delbrueckii* favor use of the species. For example, it resists relatively high concentrations of the sugars and ethanol compared to other non-*Saccharomyces* species and it has low formation of volatile acids and higher formation of higher alcohols and fruity esters than *S. cerevisiae*. Though *T. delbrueckii* can resist ethanol to some extent, the resist is only up to 8–9% (v/v) and so some sugars remain in must. Therefore, it is usually used with some *S. cerevisiae* strain, which is added after *T. delbrueckii*'s fermentation and it ferments the "rest" of the sugars.[21,32,34,35] Obviously *T. delbrueckii* is either neutral for *S. cerevisiae* toxins (chapter 1.3.3) or it is killer yeast itself. Velázquez et al. (2015) reported effects of one killer *T. delbrueckii* strain which had eliminated *S. cerevisiae* from sterile wine must. *T. delbrueckii* strain was also able to dominate and complete the fermentation.[34]

Torulaspora delbrueckii strain used in this study was Biodiva™ (TD291). According to its Technical data sheet, it will enhance wine aromatic and mouthfeel complexity. Biodiva has moderate lag phase, in the high concentration of the sugars it is recommended to use yeast protectant, which protects it from osmotic pressure. The optimal fermentation temperature of *T. delbrueckii* is over 16 °C, it forms very low amount of volatile acids and it can be used with malolactic fermentation.[26]

In this research was used two different *Saccharomyces cerevisiae* strains, Cross Evolution YSEO and Lalvin ICV-K1 (V1116), one *S. bayanus* strain, Lalvin C and one *Torulaspora delbrueckii* strain, Biodiva.

1.3.3 Killer phenomenon

Saccharomyces cerevisiae was the first yeast which was identified with killer phenomenon[13,36,37]. Killer phenomenon occurs when is used one or more killer yeast strains in the fermentation. The killer yeast strains secrete proteinic toxins into the wine must during fermentation killing other, sensitive strains. Some yeast strains can be also neutral which means they do not secrete toxins, but they have a resistance to toxins.

S. cerevisiae is not the only killer yeast of the genus *Saccharomyces* and the killer yeast strains exist also in other genera, for example in *Hansenula*, *Torulopsis*, *Candida* and *Debaryomyces*[13]. Some *S. bayanus* strains occur dsRNA plasmids which are responsible for the toxins, but killer phenotype was not in any strain[36].

S. cerevisiae strains have three different killer toxins: K1, K2 and K3. K1 is a small protein, which is stable and active in narrow pH range (4.2–4.6). K2 is the most common toxin among the genus of *Saccharomyces*. It is a glycoprotein, which is active between pH range 2.8 and 4.8, with the maximum activity between 4.2 and 4.4. Both K1 and K2 attack by attaching to the glucan receptor in the cell wall. The toxins are transferred to a membrane receptros site from the glucan receptor. In the sensitive cell plasmic membrane toxins cause serious alterations (e.g. the acidification of the cellular contents and ATP and potassium leakage) to the cell functions and cell dies in 2–3 hours after contact with the toxins.[13] Some *S. cerevisiae* strains in wines have lost the ability to secrete toxins, but they remain resistant to them [36].

The killer toxins are made by virus-like particles in the cytoplasm (*e.g.* in *S. cerevisiae*) or by plasmids. The impact of the toxins vary depending on how they are made: the toxins from virus-like particles disrupt the plasma membranes' permeability or they damage them, whereas the toxins made by the plasmid bring about G1 arrest[37], which prevents sensitive yeast cell from further division[38]. The latter way results in log phase

cells being more sensitive to plasmid toxins than cells in the stationary phase. The killer toxins do not harm humans or animals.[39]

1.4 Anthocyanins in wines/grapes and berries

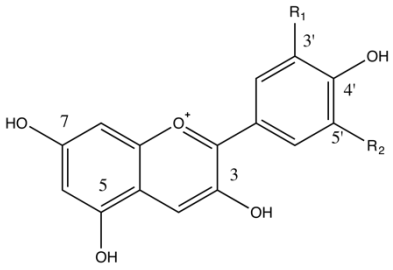
Word anthocyanin comes from Greece's words *anthos* (flower) and *kyanos* (dark blue). They belong to the family of flavonoids. Anthocyanins are secondary metabolites of the plants and entirely or partly responsible for many natural pigments in the colors of pink, red, purple, blue or orange.[40,41] Though anthocyanins belong to the flavonoid group they do not make wine flavor bitter or mouth feel astringent, because anthocyanins have only slight flavor and they are entirely odorless. Despite this anthocyanins can influence flavor by interacting with aroma substances.[42] The anthocyanins have many functions in the plants, which depends on the function of the plant tissue: in the photosynthesis tissue, the anthocyanins protect tissue from the UV radiation, whereas in the flower they lure pollinators and in the berries and fruits herbivorous to spread seeds. Anthocyanins occur in every part of the plants, but they are the most abundant in the flowers and the peels of the berries and fruits. Use of the peels in winemaking results more color in wine, because of the higher concentration of anthocyanins. Anthocyanins are dissolved uniformly in the cell's vacuolar solution.[43,44]

At Zafra-Stone's review (2007) is said anthocyanins have many health promoting characteristics, due to antioxidant activity and they scavenge free radicals. Their health benefits have been studied both *in vitro* and *in vivo* and also some human studies have been done. These researches show that anthocyanins have an influence on health of the eyes, ureteral and skin. The anthocyanins also decrease risk of cardiovascular disease, they protect the nervous system and may have anticarcinogenic and anti-diabetic characteristics.[45]

1.4.1 Chemical structures and properties of anthocyanins

More than 600 anthocyanins have been identified. Usually, anthocyanins form from one of the six common anthocyanidin (aglycons), cyanidin, peonidin, pelargonidin, malvidin, delphinidin and petunidin.[46] Aglycons differ from each other only by substituents of B ring (fig. 2). The substituents of the B ring are hydroxyls or methoxyls. Hydroxylation pattern of the B ring can directly effect on hue and colour

stability. If there are more hydroxyl groups in the B ring, the color is bluer, and with more methyl groups the color is redder.[42] Aglycons are not typically found in nature, because they are too unstable, but they may occur in foods. Linked to the C ring of the aglycon is sugar moiety, which can be mono-, di- or triglycosides. Sugar moiety is a wide group. For example, in the anthocyanins of the black currants sugars are glucoside, rutinoside, xyloside and arabinoside.[47–49] The glycosidic unit is usually attached to the 3rd carbon of anthocyanidin's B ring (fig. 3)[46]. In the grapes and grape juices, the most abundant anthocyanins contain mono- or diglucosides, which are attached to the 3rd (monoglucoside) or to the 3rd and 5th carbon (diglucoside)[50,51]. The linkage can be either α or β.[46]



Anthocyanide	R_1	R_2	λ_{max} (nm)	MW
Cyanidin	-OH	-H	506	287
Delphinidin	-OH	-OH	508	303
Pelargonidin	-H	-H	494	271
Peonidin	$-OCH_3$	-H	506	301
Petunidin	$-OCH_3$	-OH	508	317

Figure 3 Basic structure of aglycones. In the table are anthocyanidins identified from black currant, maximal wavelength of the anthocyanidins (aglycons) and their molecular weights[47,48,52].

Anthocyanin is acylated when organic acid is linked to the sugar moiety. The acyl substituent is typically esterified to the hydroxyl of the 6th carbon of the sugar.[52] The acid is usually either aliphatic (e.g. acetic, malic and malonic acids) or aromatic acid (cinnamic, caffeic or coumaroyl acid).[46,52] The type of acylation can be seen from UV-Vis spectra. An absorption band of the hydroxycinnamic acid is 310 nm when aliphatic acids do not have typical UV-Vis absorption band.[52] Some acylated anthocyanins, such as 3-*O*-(6''-*p*-coumaroyl)glucosides, have two stereoisomerism structure, the *cis*- and *trans*- isomers. In wine acylated anthocyanins are not very stable and they rapidly disappear after fermentation, usually in few months.[42]

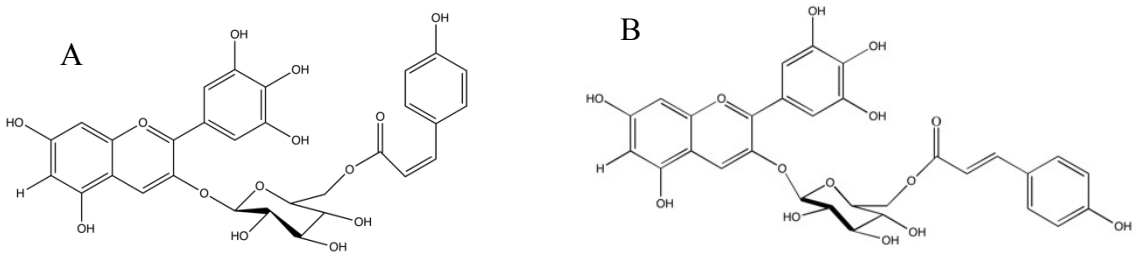


*Figure 4 Acylated anthocyanin delphinidin-3-*O*-(6''-*p*-coumaroyl glucoside)* cis *(A) and* trans *(B) forms*[42].

Anthocyanins are water and alcohol soluble compounds. Color of the anthocyanin solution is depending on pH. The pH changes the structure of the anthocyanins which alters the absorption of light and eventually the color: in the acidic solution anthocyanins are red and in the neutral solution they are blue and above that greenish and yellow.[43,44,46] pH effects also on the intensity of the color and stability of the structure. In the acid water solution, such as fruits and wines, anthocyanins are more stable than in the alkaline solution. Anthocyanins have four main equilibrium species in the aqueous solution (fig. 5): the flavylium cation, the quinonoidal base, the carbinol or pseudobase, and the chalcone C. The pH influences to the structure and amount of the particular anthocyanin. At low pH, the flavylium cation is the most abundant form and its colour is red. When pH increases the colour changes from red to blue, and flavylium cation changes form to the quinonoidal base and the pseudobases and chalcones which are colourless.[40]

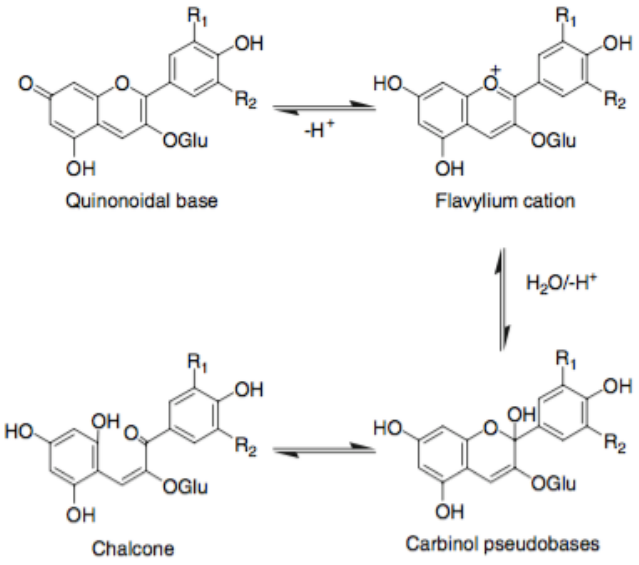


Figure 5 pH dependent chemical change of anthocyanins. Figure is adapted from de Pascual-Teresa and Sanchez-Ballesta (2008).[40]

1.4.2 Anthocyanins in black currant

In the fruits of the black currants anthocyanin content is high, 250 mg/100 g. There are four major anthocyanins in black currant: delphinidin-3-*O*-glucoside, delphinidin-3-*O*-rutinoside, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside. The percentage of 11 minor monomeric anthocyanins, which are shown in the table 1, is approximately 3% of total anthocyanins. [48,49] Two of the nine minor anthocyanins are acylated anthocyanins: delphinide-3-*O*-(6''-*p*-coumaroyl)glucoside (fig. 4) and cyanidin3-*O*-(6''-*p*-coumaroyl)glucoside [47].

Table 1 Anthocyanins found in black currant, their mass ratios and UV-Vis lambda maximums. Table is adapted from the article of Slimestad and Solheim (2002).[48]

Compound	[M]+ m/z	fragments, m/z	UV-vis, nm
Delphinidin-3-*O*-glucoside	465	303	278, 526
Delphinidin-3-*O*-rutinoside	611	303, 465	278,528
Cyanidin-3-*O*-glucoside	449	287	280, 519
Cyanidin-3-*O*-rutinoside	595	287, 449	281, 519
Petunidin-3-*O*-glucoside	479	317	278, 527
Petunidin-3-*O*-rutinoside	625	317, 479	278, 529
Cyanidin-3-*O*-arabinose	419	287	281, 519
Pelargonidin-3-*O*-glucoside	433	271	283, 507
Pelargonidin-3-*O*-rutinoside	579	271	278, 507
Peonidin-3-*O*-glucoside	463	301	280, 519
Peonidin-3-*O*-rutinoside	509	301, 463	2,81, 519
Malvidin-3-*O*-glucoside	493	331	278, 531
Malvidin-3-*O*-rutinoside	639	331, 493	278, 531
Delpinidin-3-*O*-(6"-coumaroyl)glucoside	611	303, 465	282, 315sh, 534
Cyanidin-3-*O*-(6"-coumaroyl)glucoside	595	287	285, 315sh, 524

Black currant (*Ribes nigrum*) is one of the 150 species in the genera *Ribes* and it is native to north and central Asia and northern Europe. Black currant does not need as much as other currants from niche and that is why it is so common. It can survive even though temperature decreases to -30 °C and even lower, but they do not tolerate extremely warm summers. They thieve in cold, well-watered and -fertiled, slightly acidic soils, in direct sun or in partial sun. Blackcurrant is a deciduous shrub which has stiff, up to 2 meters long upright branches. Black currant shrub flowers first time as a one-year-old. Usually flowers do not need cross-pollination. Berries are born in chains or strings and their sizes are approximately 1 cm. Flavour of the black currant is strong and it contains high amount of vitamin C.[53]

The fruits of black currant are known for their high content of anthocyanins (250 mg/100 g of fresh fruit). The main anthocyanins of the black currant are delphinidin-3-*O*-rutinoside, delphinidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and cyanidin-3-*O*-glucoside. Altogether the are 15 different anthocyanins identified from black currant.[48]

Annual black currant production is about 1017 tons in the word[54] and in Finland production is about 1.8 million kg/year in the years 2003–2005. Black currant is the second important cultivated berry as its crops yield as its area under the cultivation in Finland.

1.4.3 Anthocyanins in wines

Color of the wine is one of the most important characteristics, which effects on the quality. Like in the black currants, the grape and thus the wine color comes from the anthocyanins. In the young red grape wines, the concentration of free monomeric anthocyanins can be between 50–200 mg/100 ml.[42,55] The final anthocyanin concentration of the wine can vary on many factors, such as fruit or berry variety, ripeness, used manufacturing techniques, yeasts and maturity and aging of the wine.[56]

During fermentation and maturation, the monomeric anthocyanins undergo many reactions and new anthocyanin-derived pigments are formed and they can form polymeric compounds with proanthocyanins[56,57]. These reactions can take long time (e.g. formation of new pigments, such as pyranoanthocyanins) or shorter time (e.g. disruption of the co-pigmentation[55]). Formation of the new pigments is crucial for color stability, because monomeric anthocyanins are not stable enough to last the wine maturation.[42] Co-pigmentation means complex of the anthocyanins and metal cations (Al^{3+}, Fe^{3+}, Mg^{2+}, Cu^{2+}). Phenomenon does not much occur in the wines, because ethanol breaks down the bonds formed by the co-pigmentation.[55] All reactions decrease original concentration of the monomeric anthocyanins.[42] Still the color of the wine remains red[55]. Yeasts can produce secondary metabolites (e.g. acetaldehyde) which can prevent unwanted reactions between anthocyanins and other compounds or be precursors for reactions where the end-products are more stable anthocyanin complexes (e.g. malvidin-3-*O*-glucoside-acetaldehyde)[56].

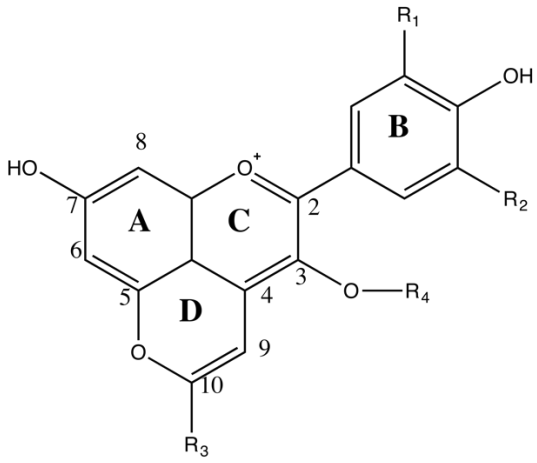


Figure 6 General structure of pyranoanthocyanin, which can occure in the red wine. The R_1 and R_2 could be H, OCH_3 and OCH_3, R_3 could be H, CH_3, COOH, (vinyl)flavanols and (vinyl)phenols and R_4 is a sugar moiety.[58]

The yeast strain has also an influence to the final anthocyanin concentration, because the cell wall of the yeast adsorbs anthocyanins. Strains difference from each other by composition and porosity of the cell wall. The strongest cell walls are able to adsorb acylated anthocyanins.[59]

So even though two wine batch could be done from same berry or fruit variety, the final anthocyanin profile can be quite different from each other, because some of the anthocyanins precipitate with tartaric salts and some of the anthocyanins reduced by fining or filtration. It is also possible that the level of anthocyanins do not correlate with the levels in the start material, because some processes, such as filtration, and the yeast species effect on anthocyanin concentrations.[42]

1.5 Alcohols in wine

1.5.1 Ethanol

Ethanol is a primary alcohol. Ethyl alcohol is after water the second most abundant substance in the wine. Yeasts produces ethanol from sugars as is shown in the figure 2. Ethanol is toxic to humans, affecting liver and nerve cells. LD_{50} value of ethanol is 1400 mg/body weight kg orally used. Ethanol is powerful dehydrant, which gives it some good properties: it helps flocculate hydrophilic colloids, such as polysaccharides and proteins, and it makes ethanol a disinfectant without maturation of the wines would be

impossible.[55] Even though ethanol is not an aromatic compound it effects on expression of the aromatic compounds in the wine by dissolving phenols from pomace[55,57].

1.5.2 Fusel alcohols

In addition to ethanol production yeast also forms fusel alcohols and aromatic compounds[60]. Some fusel alcohols are also known as higher alcohols, which means there are more than two carbon atoms in the alcohol.[57,61] Some chemical structures of fusel alcohols are shown in the figure 7. Fusel alcohols include both harmful and non-harmful alcohols. Harmful fusel alcohols are methanol and isopropanol, but the concentrations of these alcohols are typically below harmful concentrations. For example, concentration of the methanol is 30–35 mg/l and its LD_{50} is 350 mg/kg body weight.[55] Non-harmful fusel alcohols (e.g. mannitol) can cause off-flavors to the wine or can be aromatic alcohols (e.g. phenyl ethanol).[57,60] Fusel alcohols can be aliphatic or aromatic[62]. The most abundant aliphatic fusel alcohols are 2-methyl-1-propanol (isobutanol), 2-methyl-1-butanol (active amylalcohol), 3-methyl-1-propanol (isoamylalcohol), 1-propanol (n-propanol) and 1-butanol. 2-phenyl-1-ethanol is the most important aromatic alcohol in the wine.[57] All aforementioned alcohols have some kind of aroma which usually is very intense. Generally, aroma of the fusel alcohols can be 'fusel oily', 'solvent like' or 'malty'.[61,63] The effect of the higher alcohols on wine aroma depends on their concentrations: when concentration is less than 300 mg/l they contribute to complexity of the wine aroma and at higher levels, they tend to mask aroma of the wine.[61]

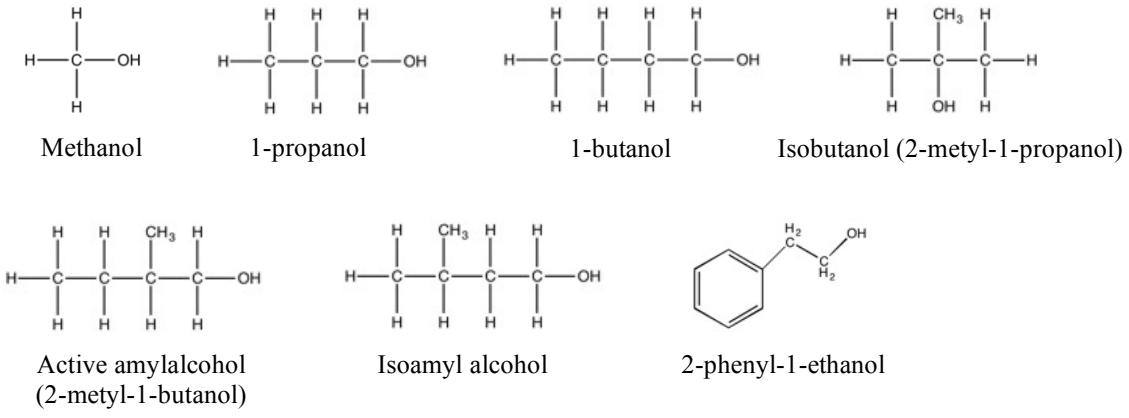


Figure 7 Examples of fusel alcohols in wine.

Formation of the fusel alcohols mostly depends on yeast and berry or fruit and fermentation conditions.[57] Methanol is formed by the same biochemical pathway than ethanol (fig. 2) from sugars and methanol can also be result from enzymatic hydrolysis of the pectin's methoxyl groups. The final methanol content almost only depends on pectin content of the must. Higher alcohols can be formed from both sugars and amino acids and it can happen in anabolic or catabolic conditions. Therefore, the final concentrations of higher alcohols are depending on the composition and concentration of amino acids of the berry. The supplement nitrogen and other nutrients are usually added to the wine during fermentation which can result in decreased formation of fusel alcohols, because yeast does not to use amino acids as nitrogen source.[57,60] Some amino acids are known to be directly linked to the specific fusel alcohol. Amino acids, which mostly effect on the formation of the fusel alcohols, are valine, leucine and isoleucine and for example concentration of the isoleucine effects on concentration of the isobutanol and phenylalanine effects on 2-phenyl-1-ethanol concentration.[57,60,64]

1.6 Aim of the thesis

The aim of this thesis was to use well known grape wine yeasts to ferment black currant wine and research how different yeast species would effect on chemical compositions of the wines. Research was concentrated on the anthocyanins and alcohols.

2 Materials and Methods

2.1 Materials

Two black currant species, Mikael (variety number 17) and LUKE's variety 15, were used in this research. Berries got from University of Turku storage. Four different yeast, Cross Evolution YSEO, Lalvin® ICV-K1, Lalvin® C and Biodiva™, were used in this research. Yeasts were received from Lallemand, Inc. (Canada).

2.2 Wine making

The method for winemaking modified based on the methods of three commercial wine making kits' methods and ingredients. Recipe is shown in the table 2. Wines were produced as is shown in the figure 8.

Table 2 Recipe used in winemaking.

Raw Materials		Mass (g)
	Black currant	960
	Sugar	1080
	Pectolase enzyme	0.5
	Rehydrate	1.8
	Nutrient supplement	1.5
	Yeast terminator (potassium sulfate-potassium sorbate mix, 1:1)	2.4
	Silic acid solution	3.6
	Wine fining agent	24
Yeast		
	Cross Evolution YSEO	1.5
	Lalvin® ICV-K1 (V1116)	1.8
	Lalvin® C	1.8
	Biodiva™*	1.5

* Because yeast Biodiva does not tolerate over 8–9% of ethanol, Cross Evolution YSEON –yeast is added also to the must to be sure about success of fermentation.

Lalvin C was used to make parallel wines with both black currant varieties and was also made one wine (15 Lalvin C (3)) which was not opened during the fermentation, so could be seen how the opening effects on wines.

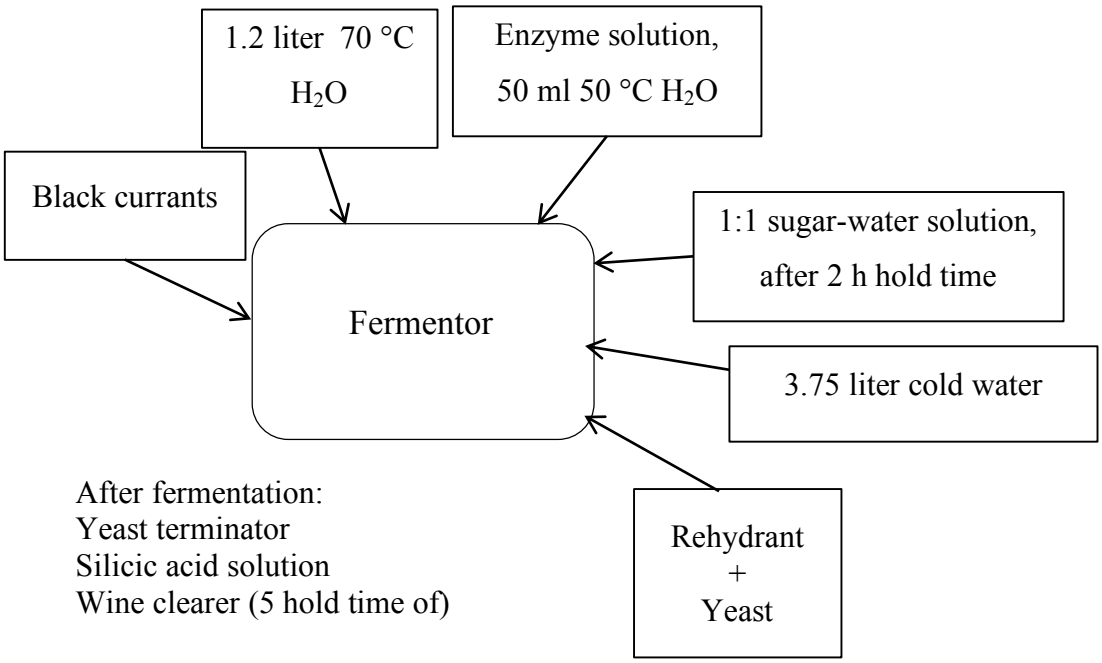


Figure 8 Diagram of wine making. Ingredients were added to the fermentor from left to right.

2.3 Sampling and sample naming

Wine samples were collected in days 0 (inoculation day), 1 and every other day after that. Good hygiene was maintained during sampling lest the wine would contaminate. After sampling samples were immediately frozen.

The sample wines are named after the name of the yeast in the results or in the PCA figures they are named by the number of the black currant variety, after that comes the name of the yeast and then is number of the parallel wine (only in Lalvin C wines). For example, 15 Lalvin C (1) is made from LUKE's variety 15 with Lalvin C yeast and it is the first wine of parallel 15 Lalvin C wines.

2.4 pH, °Brix and specific gravity

pH (Mettler Toledo, USA), °Brix and specific gravity were measured on days 0 and 1 and after that they were measured every other day. °Brix and specific gravity were also measured more often, if it was necessary, for example when the time of supplement adding was near or it was almost time to stop fermentation. °Brix was measured with an optical refractometer, which measuring range was between 0–32. Specific gravity was measured with a hydrometer.

2.5 Alcohol analysis

5% of 1-propanol was added to wine samples as an internal standard. Samples were filtered with 0.2 µm syring filters. Nine external standards were prepared same way: 5% ethanol (Etax B, Altia Oy, Finland), 5% 1-propanol (J.T. Baker, USA), 2% methanol (J.T. Baker, USA), 4% 1-butanol (2-methyl-1-propanol) (Riedel-Deltaën, Germany), 2% isoamylalcohol (3-methyl-1-propanol) (Sigma-Aldrich, Germany), 2% isobutanol (Merck, Germany), 2% tert-butanol (Merck, Germany), 1% pentanol and 1% 1-phenylethanol (Sigma-Aldrich, Germany). All standards were water alcohol mixtures.

Before analyses chromatographic parameters were optimized. In the table 3 is the final conditions and temperature program of GC-FID.

Table 3 Instrument information and chromatographic conditions of identification of the alcohols with GC-FID.

Instrument	
GC-FID	Shimadzu GC-2010Plus with AOC-20i Autoinjec tor / AOC-20s Autosampler, Flame ionization detector and LabSolutions software (Shimadzu corp., Kyoto, Japan)
Column	EC-WAX (Alltech, i.d. 0.53 mm, phase thickness 1.2 µm)
Run conditions	
Carrier gas	Helium, flow presure 22.8 kPa, flow rate 3.71 ml/min
Carrier gas linear velocity	30,0 cm/sec
Total flow	118.0 ml/min
Injection temperature	230 °C
Injection type	Split/Splitless, split ration 1:30
Injection volume	0.2 µl
Column start temperature	80 °C
Column's temperature program	80 °C , hold time 5 min, change 10 °C/min 240 °C, hold time 9 min
Analyse time	30 minutes
Detection temperature	250 °C

2.6 Anthocyanin analysis

2.6.1 Determination from wines

Samples were diluted 1:1 with methanol hydrochloric acid solution (99:1) and were filtered with 0.2 µm syringe filters. Anthocyanins were identified with high

performance liquid chromatography with diode array detector (HPLC-DAD). Instrument and chromatographic parameters are shown in the table 4.

Four major anthocyanins were identified with the external standards. Three external standards were chosen with literature and standards were delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside. Four-point dilution series were made from delphinidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside and five-point dilution series were made from cyanidin-3-*O*-glucoside for quantitative analysis.

Table 4 Instrument information and chromatographic conditions of identification of the anthocyanins with HPLC-DAD.

Instrument	
HPLC-DAD	Nexera, With two LC-30AD pumps, SIL-30AC autosampler, CTO-20AC column oven, SPD-M20A diode array detector (DAD), OBM-20A communication unit (Shimadzu corp., Kyoto, Japan)
Column	Kinetex (2.6 µm C18 100 Å. 100x4.60mm i.d., 2.6 µm)
Mobile phases	
Mobile phase A	Formic acid (5%)
Mobile phase B	Acetonitrile
Run conditions	
Gradient conditions of mobile phase B	0–1 min, 4–6%; 1–2 min, 6–8%; 2–6 min, 8–9%; 6–10 min, 9–10%; 10–14 min, 10–11%; 14–20 min, 11–18%; 20–21 min, 18–24%; 21–28 min, 24–80%; 28–29 min, 80–20%; 29–31 min, 20–4%; 31–35 min, 4%.
Changes of mobile phases	1.3–1.2 ml/min, 2–6 min; 1.2–1.1 ml/min, 6–7 min; 1.1 ml/min, 7–18 min; 1.1–1.3 ml/min, 18–22 min, 1.3 ml/min, 22–35 min
Run time (min)	35
Column temperature (°C)	25
Injection volume (µl)	10
PDA conditions	
Start wavelength (nm)	190
End wavelength(nm)	600
Sampling rate (spektrum/sec)	1
Resolution	1.2

2.6.2 Anthocyanin assay from berries and fermentation refuses

Anthocyanin extractions were performed as it is shown in the figure 9. The analysis was performed with the same HPLC-DAD than analysis of the wine samples. The column used to identify anthocyanins from the wine samples did not work with the berries and the fermentation refuses, because it did not hold the anthocyanins enough to achieve clear peaks. A few columns were tested and the best result was achieved with Phenomenex Aeris Peptide ((3.6 μ XB-C18 150x4.60 mm), USA) column.

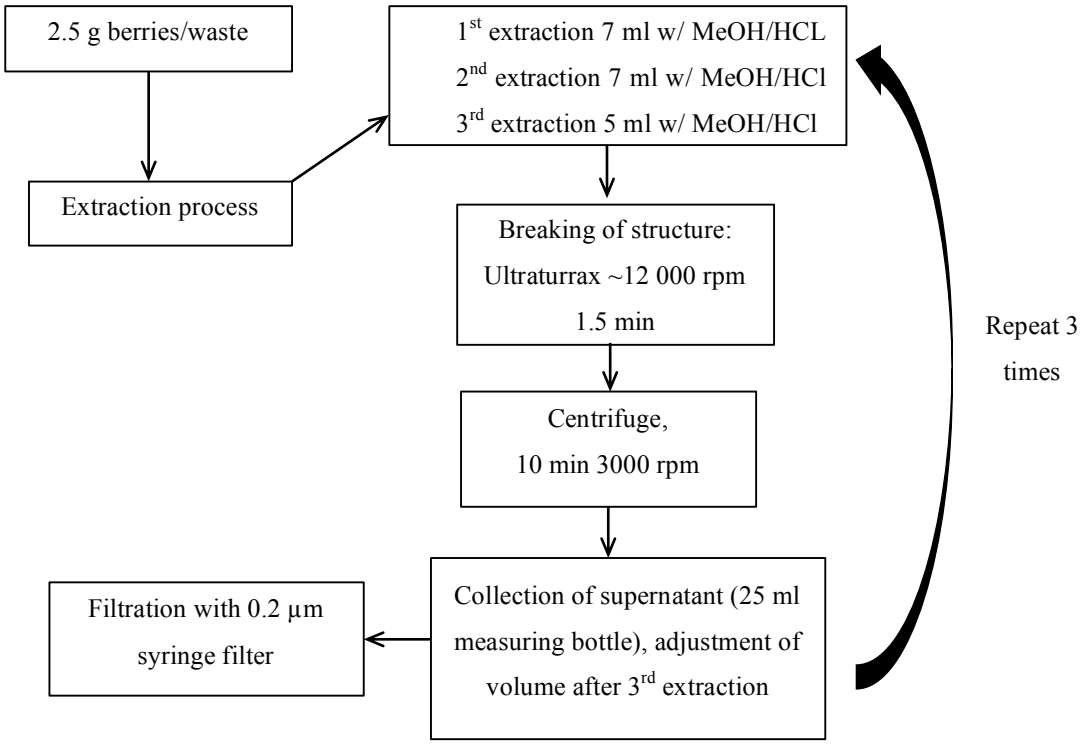


Figure 9 Extraction process of berry samples and fermentation wastes.

2.7 Analysis of anthocyanins with UHPLC-MS

In addition to identifications based on UV-vis spectra, identification of the anthocyanins was performed with ultra-high-performance liquid chromatography with diode array detector and triple quadrupole mass spectrometry (UHPLC-DAD-MS). Instrument and method information is shown in the table 5. Prior to analysis the wine samples were concentrated. 1 ml of the samples were evaporated to dryness under nitrogen stream in mild temperature and the concentrates were dissolved in 100 µl MeOH/HCl (99:1) solution.

Table 5 Instrument information and chromatographic conditions of identification of the anthocyanins with UHPLC-DAD-MS.

Instrument		
UHPLC-MS	Water Aquity UPLC Waters Quattro Premier Tandem Mass Spectrometry (ESI/APCI)	
Column	Phenomenex Aeris Peptide (3.6 µ XB-C18 150x4.60 mm)	
Mobile phases		
Mobile phases A	Formic acid (5%)	
Mobile phases B	Acetonitrile	
Wash solutions		
Week needle wash	Water-acetonitrile 8:2 (v/v)	500 µl
Strong needle wash	Methanol (100%)	200 µl
Seal wash	Water-methanol 8:2 (v/v)	
Run conditions		
Gradient conditions of mobile phase B	0–1 min, 4–6%; 1–2 min, 6–8%; 2–6 min, 8–9%; 6–7 min, 9%; 7–10 min, 9–10%; 10–14 min, 10–11%; 14–18 min, 11%; 18–20 min, 11–18%; 20–21 min, 18–24%; 29–31 min, 20–4%; 31–35 min, 4%.	
Run time	35 min	
Flow rate (ml/min)	1	
Flow rate at mass spectrometry (ml/min)	0.2	
Column temperature	RT	
Injection type	Full loop	
Seal wash interval (min)	2	
PDA conditions		
Start wavelength (nm)	190	
End wavelength (nm)	600	
Sampling rate (spectrum/sek)	1	
Resolution	1.2	
Mass spectrometer conditions		
Ionization type	ESI+	
Capillary voltage (kV)	325	
Cone voltage (V)	30	
Extractor voltage (V)	2	
RF lens voltage (V)	0	
Ion source temperature (°C)	150	
Desolvation temperature (°C)	400	
Desolvation gas flow rate (l/h)	47	
Mass range (m/z)	250–1000	

2.8 Statistical methods

Principal component analysis (PCA) was performed with The Unscrambler X 10.3 software (CAMO Inc., Norway).

3 Results and Discussion

3.1 Fermentation

Eleven wines were made from two black currant species and four yeasts. Lag phase (the yeast adapt itself to the new environment) of the yeasts was 3 or 4 days. Biodiva was the only yeast with the lag phase of 4 days. Duration of the lag phases could be seen from the sugar consumption of the yeast, which is shown in appendix 1 in the figures 17 and 18. Lag phase ends when the °Brix starts to go sharply down.

Fermentation time varied between 14 and 18 days. Only Lalvin C had the same fermentation time in all 5 wines and its fermentation time was the shortest, 14 days. Lalvin ICV-K1 (18 and 16 days) and Biodiva (17 and 16 days) had the longest fermentation times. Mikael wine fermented with Biodiva, did not succeeded same as others, so it is not taken into account in the all results.

3.2 Major alcohols in the wines

Alcohols were identified using selected low-molecular weight alcohols as the external standards. Five of the nine standards used were identified from the wine samples: methanol, ethanol, 1-propanol, isobutanol and isoamylalcohol. However, because 1-propanol concentrations were too low for reliable quantification. The concentrations of the alcohols were calculated with the known internal standard, 1-propanol. The correlation factors were calculated with external standards with the internal standard. The calculation is shown in the equation 1. The correlation coefficients are in the table 6.

Table 6 Correlation coefficients of the external standards.

Standard	**Correlation coefficient**
Methanol	0.42351032
Ethanol	0.66611464
Isoamyl alcohol	1.20727908
Isobutanol	1.14177728

Equation 1.

$$c_a = \frac{c_s A_a}{k_a A_s}$$

Concentrations of the identified alcohols (excluding 1-propanol) were calculated. Computational ethanol concentrations were also calculated to all wines. The results on ethanol concentration are in the appendix 2. All ethanol concentrations were lower than the computational concentrations. Reasons for that might be that the fermentation circumstances were not optimal for yeasts, lack of nutrients and air leakage during fermentation.

PCA were made between the samples and alcohols. Because first two PCs explain 88% of variability between samples and variables, only one PC figure is relevant to show. Samples were grouped by yeast species: Blue is *Saccharomyces cerevisiae*, green is *S. bayanus* and violet is *Torulaspora delbrueckii*. Numbers means black currant species (17 = Mikael and 15 = LUKE's variety 15) and numbers in the parenthesis are parallel sample numbers. PCA is shown in the figure 10.

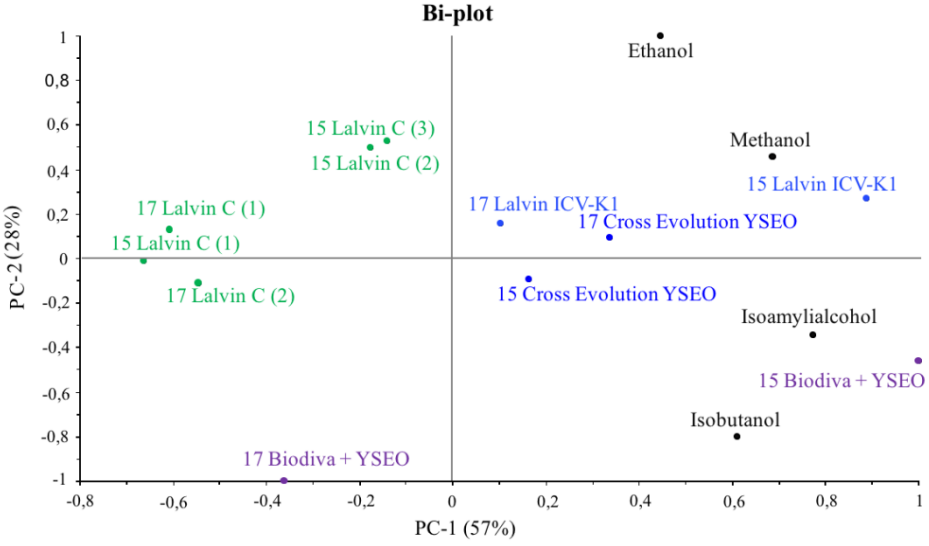


Figure 10 PCA of the finished wine samples. The samples are grouped by yeast species: blue is S. cerevisiae, *green is* S. bayanus *and violet is* T. delbrueckii *and* S. cerevisiae. *Numbers in front of the yeasts' names means black currant species: 17 = Mikael and 15 = LUKE's variety 15. Numbers in the parenthesis mean ordinals of the parallel wines.*

Black currant species does not have similar effect on alcohols in the wines than in anthocyanins. Instead, the yeast species has a much clearer effect on alcohols. PCA shows Lalvin C (*S. bayanus*) does not produce fusel alcohols as much as other yeasts, but it produces as much or more ethanol than Cross Evolution YSEO and Lalvin ICV-

K1 (*S. cerevisiae* species). Lalvin ICV-K1 seems to produce the highest concentration of methanol. Clear cap between *Saccharomyces cerevisiae* species (right side) and *S. bayanus* (left side) can be seen in the PCA, which means there is some variability between yeasts. Both *Saccahromyces* species are also grouped near each other, so there is not much variability between them. Biodiva forms the most fusel alcohols, but not that much ethanol than *Saccharomyces* yeasts.

3.3 Anthocyanins

3.3.1 Anthocyanins in wine samples

Samples were collected from the wines every other day and dissolve of the anthocyanins were analyzed. Four major anthocyanins of two example wines are shown in the appendix 2 in the figures 18 and 19. These figures show 3-*O*-rutinosides dissolve very quickly and they are more stable than 3-*O*-glucosides.

Four major anthocyanins were identified with the external standards. These anthocyanins were delphinidin-3-*O*-glucoside, delphinidin-3-*O*-rutinoside, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside, respectively. Other anthocyanins were identified with UHPLC-MS and the data was compared to the data of the table 1. Identified anthocyanins and their masses are in the table 7. One anthocyanin, cyaniding-3-*O*-arabinose, was identified with its lambda maximum. Example chromatograms from HPLC-DAD are shown in the appendix 4.

Table 7 The mass spectral and UV-Vis data of anthocyanin analyses.

Retentio time (min)	$[M]^+$ (m/z)	Fraction (m/z)	Compound
13.6	479	317	Petunidin-3-*O*-glucoside
15.4	626	479/317	Petunidin-3-*O*-rutinoside
16.6	579	271	Pelargonidin-3-*O*-rutinoside
20.1	609	301	Peonidin-3-*O*-rutinoside
22.9	611	303	Delphinidin-3-*O*-(6'-coumaroyl) glucoside
23.1	595	287	Cyanidin-3- *O*-(6'-coumaroyl) glucoside

Concentrations of four major anthocyanins were calculated with standard curves which were formed from the dilution series of the external standards. The figure 11 is one example of the four-point standard curves. Table 8 presents equations of the standard

curves and their correlation coefficients. From the correlation coefficients can be seen that all of the standard curves are very linear. An example calculation is shown in the appendix 5.

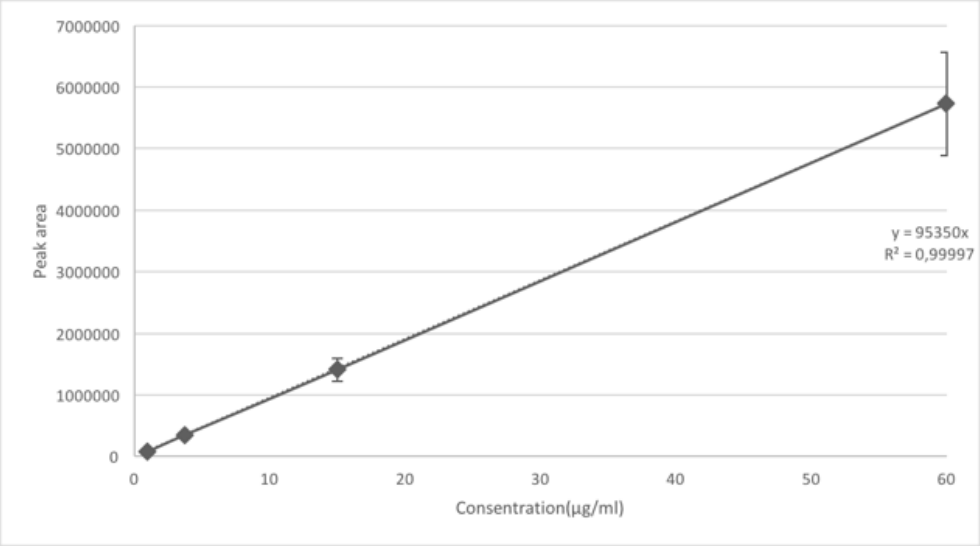
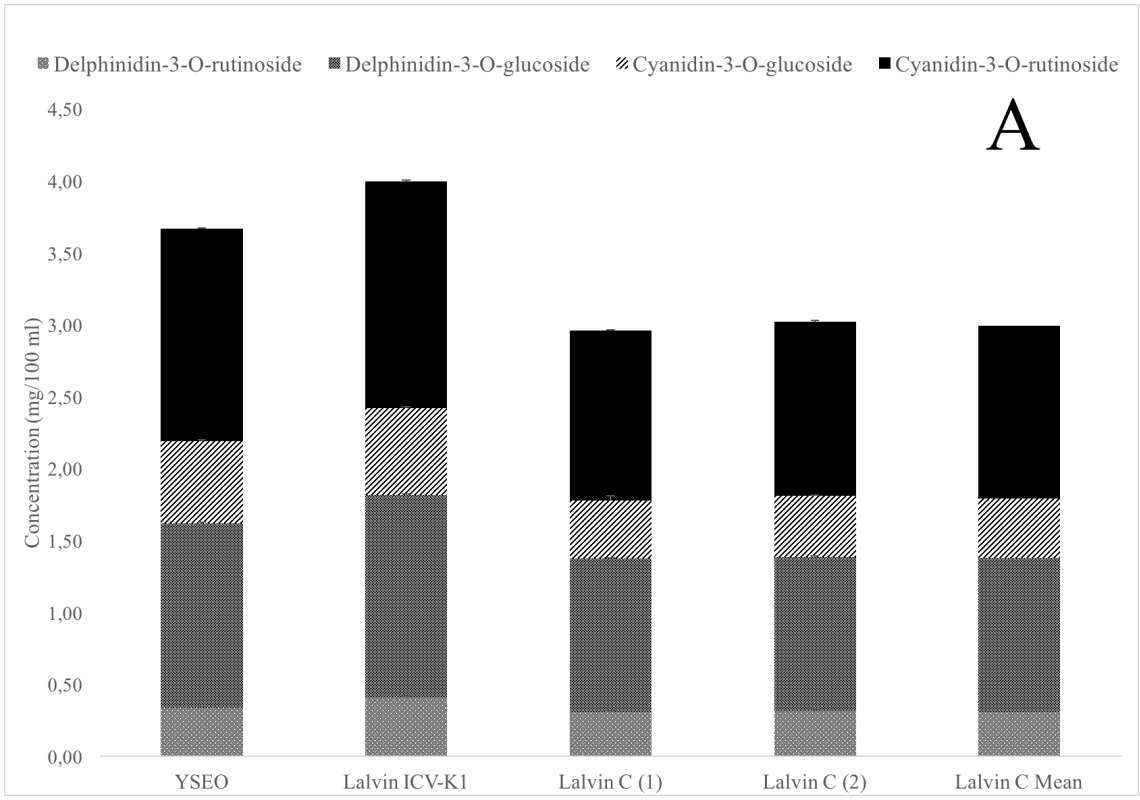


*Figure 11 Example delphinidin-3-*O*-glucoside standard curve with intercept fixed at zero.*

Table 8 Equations and correlation coefficients of trend lines of all standard curves.

Standard	**Slope**	**Correlation coefficient R^2**
Delphinidin-3-*O*-glucoside	y = 95350x	0.99997
Cyanidin-3-*O*-glucoside	y = 36510x	0.99976
Cyanidin-3-*O*-rutinoside	y = 139470x	0.99978

The concentrations of the four abundant anthocyanins in the wines are shown in the figure 12. It is hard to see any pattern between concentrations from these figures. Though it can be seen that there is a difference in the most abundant anthocyanin between these two species: in Mikael (variety 17) wines there is not that much difference between 3-*O*-rutinoside anthocyanins, but in the LUKE's variety 15 wines is more delphinidin-3-*O*-rutinoside than cyanidin-3-*O*-rutinoside. Lalvin C was used to make parallel wines. In the figure 12 is also calculated mean concentrations of four major anthocyanins in these parallel wines. Two wines made with Mikael (LUKE's 17 variety) and Lalvin C seem to have very similar amounts of anthocyanins, but wines made with LUKE's variety seem to all be different from each other.



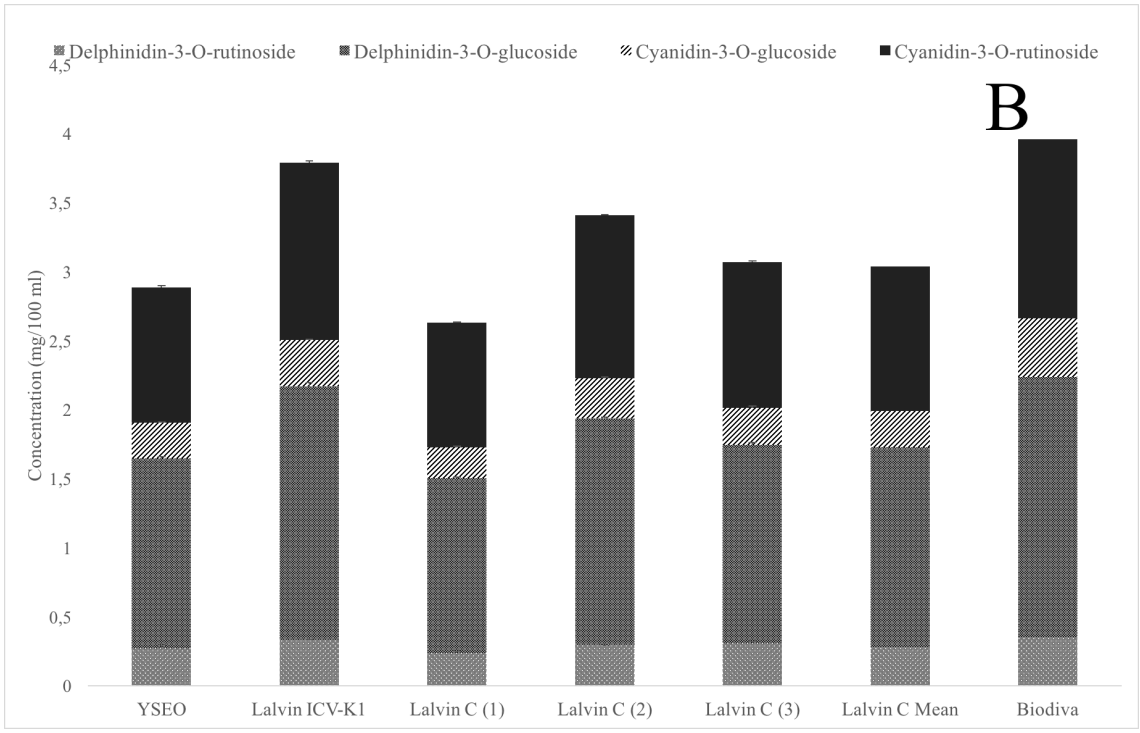


Figure 12 The concentrations of the four most abundant anthocyanins in the finished wines. Figure A shows concentrations from wines made from Mikael (variety 17) and figure B shows concentrations from wines made from LUKE's variety 15.

Principal component analysis (PCA) was made with the finished wine samples (n = 11) and their anthocyanin concentrations (X-variables n = 4, fig. 13). Because first two principal components (PC-1 and PC-2) explained most of the variability (97%) between samples and variables, they are the only PCs taking into account. First time the samples were grouped by the black currant species: blue is Mikael and red is LUKE's variety 15. Second time the samples were grouped by the yeast species: blue is *S. cerevisiae*, red is *S. bayanus* and green is *T. delbrueckii*. Because Mikael wine fermented with Biodiva wasn't successful, it is not taken account in results.

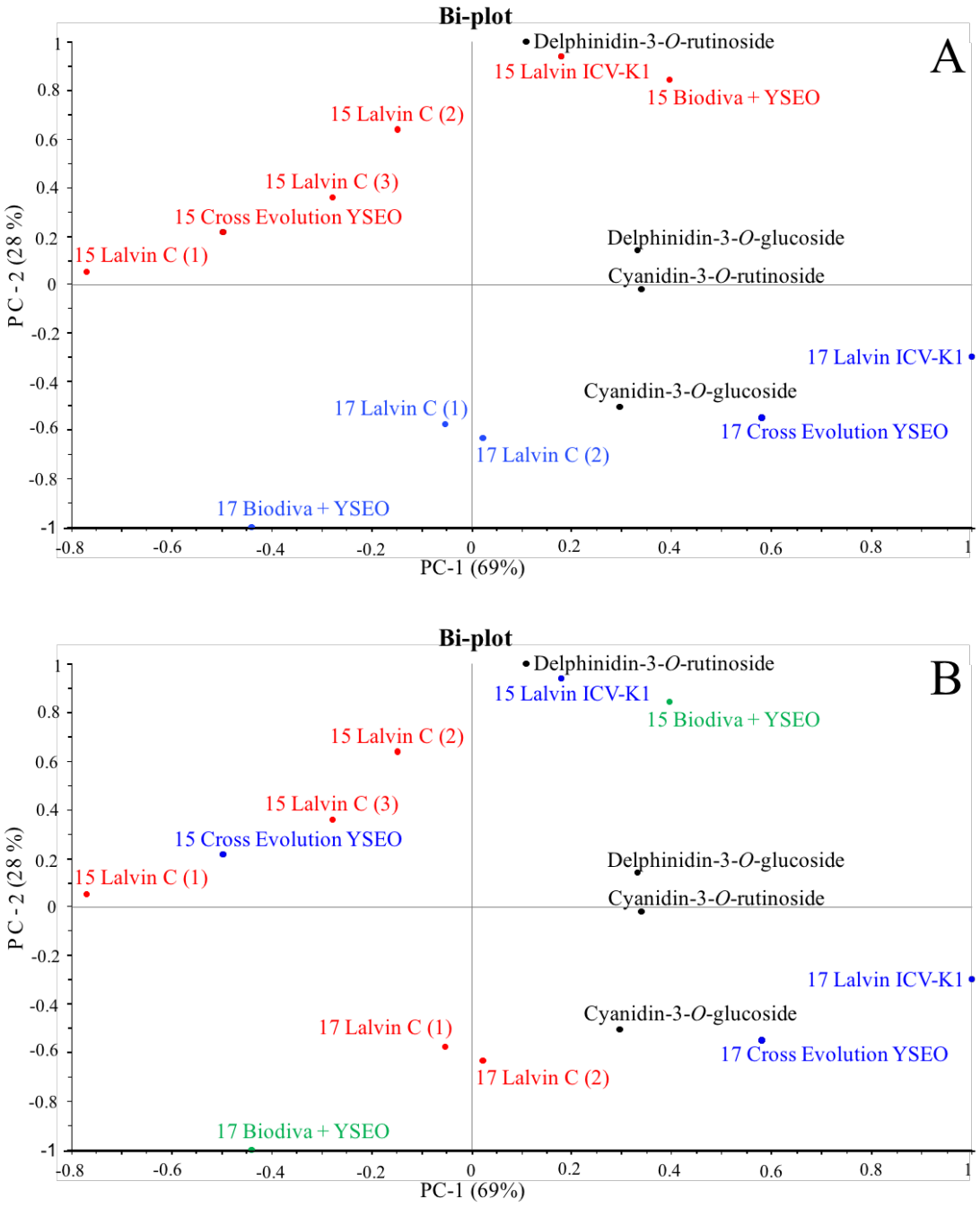


Figure 13 PCA of the finished wine samples (n = 11). The samples are grouped by black currant species in the figure A: blue = Mikael (17) and red = LUKE's variety 15 (15). Numbers in the parenthesis mean ordinals of the parallel wines. The samples are grouped by yeast species in the figure B: blue = S. cerevisiae, *red* = S. bayanus *and green* = T. delbrueckii *and* S. cerevisiae.

The first PCA shows well how yeast species have more effect on anthocyanins than black currant species (fig 13). The wines fermented with Lalvin ICV-K1 had the highest concentration of the anthocyanins in the both cases and in the wines fermented with Lalvin C had the lowest concentrations. The second PCA shows that yeast species are almost perfectly divided to different parts of the figure: *S. cerevisiae* species to the right side and *S. bayanus* to the left side. Biodiva seems to have almost same amount of

anthocyanins than Lalvin ICV-K1. Surprisingly wine from LUKE's variety 15 has same amount of anthocyanins than Mikael wines (fig. 12), because Mikael has originally more anthocyanins than LUKE's variety 15 (fig. 14). It can be caused by the different solubility of the anthocyanins. The PCAs (fig. 13) also show there is a clear difference between main anthocyanins in the wines: There are more delphinidin-3-*O*-rutinoside in the wines made from LUKE's variety 15 and more cyanidin-3-*O*-glucoside in the wines made from Mikeal (variety 17).

Lalvin C (*S. bayanus*) caused two extra peaks in the chromatograms next to the acylated anthocyanins (appendix 5, fig. 26). The extra peaks were identified with lambda maximum from HPLC and mass to charge ration from UHPLC-MS. The compounds seems to be delphidin and cyanidin aglycons, respectively. As seen from the figure 13, PCA of the samples of the finished wines there are less anthocyanins in wine made with Lalvin C. Also, the fermentation refuses of Lalvin C wines contain less anthocyanins left than other refuses (excluding the first wine made with LUKE's variety 15 and Lalvin C). These both results indicate Lalvin C yeast can produce enzymes able to break down anthocyanins.

3.3.2 Anthocyanins in berries and fermentation refuses

Anthocyanin profiles of two black currant species, Mikael and LUKE's variety 15, were determined with HPLC-DAD. Concentrations of anthocyanins are shown in the figure 14. As can be seen, Mikael contains more anthocyanins than LUKE's variety 15. LUKE's variety 15 has more delphinidin-3-*O*-rutinoside and less all other anthocyanins than Mikael.

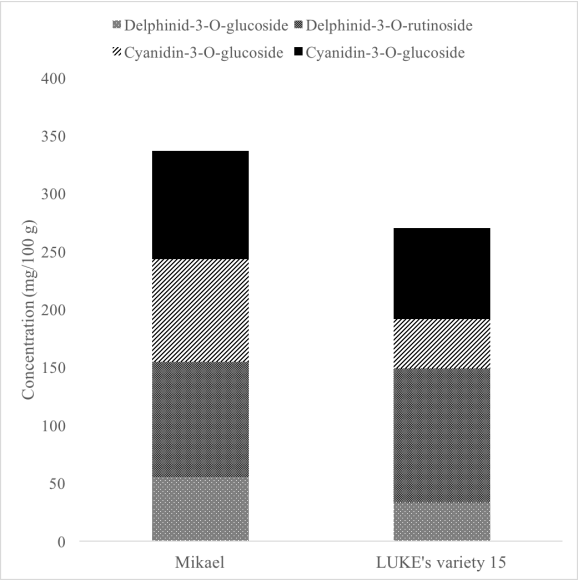


Figure 14 Concentrations of the four abundant anthocyanins in Mikael (variety 17) and LUKE's variety 15.

The fermentation refuses (excluded from wine made with Mikael and Lalvin C) were analyzed and concentrations were calculated the same way as with the wine samples and the berries. Concentrations are shown in the appendix 6 in the figures 27 and 28. The refuses of LUKE's variety 15 have less anthocyanins left, as expected, because there was originally less anthocyanins in LUKE's variety 15 (fig. 14). The percentages of the anthocyanin left in the fermentation refuses were calculated and the results are shown in the the tables 9 and 10. The concentrations of the 3-*O*-glucosides were smaller than 3-*O*-rutinosides in the wines, but the percentage of them are higher than 3-*O*-rutinosides. The results suggest that the glucosides do not dissolve as easily as the rutinosides during the fermentation. Same kind of anthocyanins dissolving pattern can be seen at the example figures (appendix 3, fig. 20 and 21). It can also be seen from the tables 9 and 10 there are the least anthocyanins in the fermentation refuses of Lalvin C wines (excluded the wine first made of the LUKE's variety 15).

Table 9 The percentage of remaining anthocyanins in the fermentation refuse of the wines made from Mikael compared to the original anthocyanin concentrations.

	Delphinidin-3-*O*-glucoside	Delphinidin-3-*O*-rutinoside	Cyanidin-3-*O*-glucoside	Cyanidin-3-*O*-rutinoside
Cross Evolution YSEO	64.5	44.8	53.7	38.4
Lalvin IVC-K1	77.1	40.2	60.8	31.7
Biodiva	69.0	43.4	55.8	35.2
Lalvin C (2)	56.3	32.2	46.6	27.9

Table 10 The percentage of remaining anthocyanins in the fermentation refuse of the wines made from LUKE's variety 15 compared to the original anthocyanin concentrations.

	Delphinidin-3-*O*-glucoside	Delphinidin-3-*O*-rutinoside	Cyanidin-3-*O*-glucoside	Cyanidin-3-*O*-rutinoside
Cross Evolution YSEO	38.6	28.4	28.2	21.1
Lalvin IVC-K1	41.5	26.6	30.4	19.3
Lalvin C (1)	46.9	35.3	36.3	27.2
Biodiva	45.8	30.5	16.7	37.7
Lalvin C (2)	25.9	20.8	8.7	25.0
Lalvin C (3)	23.0	15.4	7.4	18.9

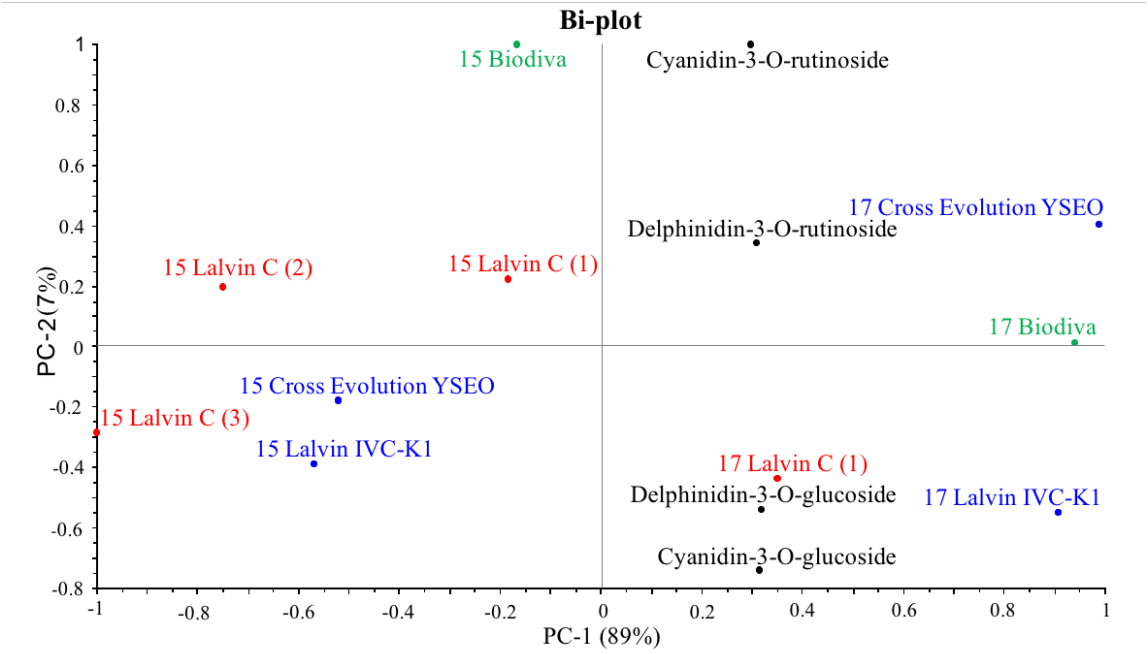


Figure 15 PCA of the anthocyanins of the fermentation refuses. Samples are grouped by yeast species: blue = Saccharomyces cerevisiae*, red* = S. bayanus *and green* = Torulaspora delbrueckii.

PCA was done with the fermentation refuses (n = 10) and concentrations of the anthocyanins (X-variables n = 4, fig. 15). The first two principal components (PC-1 and PC-2) explained most of the variability (96%) between samples and variables, thus they were the only PCs taken into account. PCA shows that there were less 3-*O*-rutinosides left than 3-*O*-glucosides. It can be also seen that there is not same kind of distribution of samples caused by yeast strain, but the clearest distribution is caused by black currant variety.

3.3.3 Impact of opening the fermentator

Wine 15 Lalvin C (3) was not opened during the fermentation. PCA (fig. 12) shows that residue of the sample had the lowest concentration of the anthocyanins, but at the same time the finished wine of 15 Lalvin C (3) did not have the highest amount of the anthocyanins of Lalvin C wines. The ethanol production in that wine can be an explanation for the little amount of the anthocyanins remaining in the fermentation refuses, because ethanol dissolves anthocyanins and in 15 Lalvin C (3) wine the ethanol concentration is the highest between all Lalvin C wine (appendix 2, fig. 19).

4 Conclusions

Eleven wines were made from two black currant varieties, Mikael and LUKE's variety 15 using four yeasts. Fermentation times varied between yeast a few days: *Saccharomyces bayanus* was the fastest with the 14 days and *Torulaspora delbrueckii* was the slowest with the 18 days. *S. bayanus* also has a short lag phase which is good characteristic, because the ethanol production would start quickly and growth of other microorganisms would be prevented. The short fermentation time is also more economical choice.

According to this study black currant varieties Mikael and LUKE's variety 15 do not effect on as much as yeast species on concentrations of the anthocyanins and the alcohols. The amount of ethanol produced would be the highest with *Saccharomyces cerevisiae* and the lowest with *Torulaspora delbrueckii*, which has the highest production of the higher alcohols, instead. Air leakage during fermentation can decrease both concentrations of the anthocyanins and the alcohols.

Fermentation with *S. bayanus* breaks down anthocyanins in the berries and in the wine, so it is not be a good choice to produce wines from anthocyanin-rich materials, such as currants, because color is one of the most important quality factors of the wine. However, due to the shorter fermentation time for a ready wine, compromises are needed in the selection of suitable yeasts. Some decomposition products can be colorless or may cause unwanted colors. It can also be that cell wall of *S. bayanus* adsorpts more anthocyanin than the others or it does not product as much metabolic precursors which would prevent the polymerization of the anthocyanins[65]

This research could have given more accurate results, if the fermentation circumstances had been more optimized for every yeast. For example, temperature is an important parameter of fermentation and too low or high temperature can inhibit the growth of the yeast. Then the production of ethanol would be compromised. Also, the fermenters in this research was not as air-tight as they should have been, because air in the fermenter compromises final concentration of ethanol and anthocyanins, which is a quality error in the wines.

Even though one wine was made in the fermenter, which was not opened during the fermentation, the ethanol and anthocyanin concentrations did not differ much from other wines.

References

1. Owen, J., 12, for N. G. N. J. & 2011. Earliest Known Winery Found in Armenian Cave. *National Geographic News* (2011). Available at: http://news.nationalgeographic.com/news/2011/01/110111-oldest-wine-press-making-winery-armenia-science-ucla/. (Accessed: 3rd June 2016)

2. European Union. Viini. (2008). Available at: http://eur-lex.europa.eu/legal-content/FI/TXT/HTML/?uri=URISERV:ag0001&from=FI. (Accessed: 3rd June 2016)

3. Cavalieri, D., McGovern, P. E., Hartl, D. L., Mortimer, R. & Polsinelli, M. Evidence for S. cerevisiae Fermentation in Ancient Wine. *J. Mol. Evol.* **57,** S226–S232 (2003).

4. Jackson, R. S. in *Wine Science (Second Edition)* 1–12 (Academic Press, 2000).

5. Suomen Viiniyrittäjät ry. Suomalaiset viinitilat. Available at: http://www.viinitilat.net/Suomen_viinitilat.pdf. (Accessed: 3rd June 2016)

6. Wansbrough, H., Sherlock, R., Barnes, M. & Reeves, M. Chemistry in Winemaking. *N. Z. Inst. Chem.*

7. Pinelo, M., Zeuner, B. & Meyer, A. S. Juice clarification by protease and pectinase treatments indicates new roles of pectin and protein in cherry juice turbidity. *Food Bioprod. Process.* **88,** 259–265 (2010).

8. Jackson, R. S. in *Wine Science (Second Edition)* 281–354 (Academic Press, 2000).

9. Lee, W. C., Yusof, S., Hamid, N. S. A. & Baharin, B. S. Optimizing conditions for enzymatic clarification of banana juice using response surface methodology (RSM). *J. Food Eng.* **73,** 55–63 (2006).

10. Ribéreau-Gayon, P., Glories, Y., Maujean, A. & Dubourdieu, D. *Handbook of Enology, The Chemistry of Wine: Stabilization and Treatments*. (John Wiley & Sons, 2006).

11. Belitz, H.-D., Grosch, W. & Schieberle, P. in *Food Chemistry* **2009,** 950–973 (Springer-Verlag, 2009).

12. Feliziani, E. *et al.* Preharvest Fungicide, Potassium Sorbate, or Chitosan Use on Quality and Storage Decay of Table Grapes. *Plant Dis.* **97,** 307–314 (2013).

13. Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B. & Lonvaud, A. in *The*

Microbiology of Wine and Vinification 193–221 (John Wiley & Sons, Ltd, 2006).

14. Bağder Elmaci, S. *et al.* Effectiveness of chitosan against wine-related microorganisms. *Antonie Van Leeuwenhoek* **107,** 675–686 (2015).

15. Jackson, R. S. in *Wine Science (Second Edition)* 232–280 (Academic Press, 2000).

16. Kumar, R. & Majeti, N. V. A review of chitin and chitosan applications. *React. Funct. Polym.* **46,** 1–27 (2000).

17. Pillai, C. K. S., Paul, W. & Sharma, C. P. Chitin and chitosan polymers: Chemistry, solubility and fiber formation. *Prog. Polym. Sci.* **34,** 641–678 (2009).

18. Jackson, R. S. in *Wine Science (Second Edition)* 355–433 (Academic Press, 2000).

19. Ribéreau-Gayon, P., Glories, Y., Maujean, A. & Dubourdieu, D. in *Handbook of Eonology: The Chemistry of Wine Stabilization and Treatment* 301–331 (John Wiley & Sons, Ltd, 2006).

20. Ribéreau-Gayon, P., Glories, Y., Maujean, A. & Dubourdieu, D. in *Handbook of Eonology: The Chemistry of Wine Stabilization and Treatment* 285–300 (John Wiley & Sons, Ltd, 2006).

21. Taillandier, P., Lai, Q. P., Julien-Ortiz, A. & Brandam, C. Interactions between Torulaspora delbrueckii and Saccharomyces cerevisiae in wine fermentation: influence of inoculation and nitrogen content. *World J. Microbiol. Biotechnol.* **30,** 1959–1967 (2014).

22. Azzolini, M. *et al.* Effects of Torulaspora delbrueckii and Saccharomyces cerevisiae mixed cultures on fermentation and aroma of Amarone wine. *Eur. Food Res. Technol.* **235,** 303–313 (2012).

23. Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B. & Lonvaud, A. in *Handbook of Enology: The Microbiology of Wine and Vinifications* **1,** 79–113 (John Wiley & Sons, Ltd, 2006).

24. Lallemand, Inc. Lalvin ICV-K1 (V1116).

25. Eaton Corporation. Lalvin C. (2013).

26. Lallemand, Inc. BiodivaTM (TD291). (2013).

27. Eaton Corporation. *Pure Fermentation, Cross Evolution YSEO*. 2 (2013).

28. Scott Laboratories. Easy Steps for Optimal Yeast Rehydration of Lallemand Yeast. *www.scottlab.com* (2010). Available at: http://www.scottlab.com/uploads/documents/downloads/59/YeastRehydration_Lallema

nd.pdf. (Accessed: 4th June 2016)

29. Fellows, P. J. in *Food Processing Technology : Principles And Practice* 11–95 (Woodhead Publishing, 2009).

30. Sicard, D. & Legras, J.-L. Bread, beer and wine: Yeast domestication in the Saccharomyces sensu stricto complex. *C. R. Biol.* **334,** 229–236 (2011).

31. Naumov, G. I., James, S. A., Naumova, E. S., Louis, E. J. & Roberts, I. N. Three new species in the Saccharomyces sensu stricto complex: Saccharomyces cariocanus, Saccharomyces kudriavzevii and Saccharomyces mikatae. *Int. J. Syst. Evol. Microbiol.* **50,** 1931–1942 (2000).

32. Michel, M. *et al.* Screening for new brewing yeasts in the non-Saccharomyces sector with Torulaspora delbrueckii as model. *Yeast* n/a–n/a (2016). doi:10.1002/yea.3146

33. Ciani, M. & Maccarelli, F. Oenological properties of non-Saccharomyces yeasts associated with wine-making. *World J. Microbiol. Biotechnol.* **14,** 199–203 (1997).

34. Velazquez, R., Zamora, E., Alvarez, M. L., Hernandez, L. M. & Ramirez, M. Effects of new Torulaspora delbrueckii killer yeasts on the must fermentation kinetics and aroma compounds of white table wine. *Front. Microbiol.* **6,** 1222 (2015).

35. Canonico, L., Agarbati, A., Comitini, F. & Ciani, M. Torulaspora delbrueckii in the brewing process: A new approach to enhance bioflavour and to reduce ethanol content. *Food Microbiol.* **56,** 45–51 (2016).

36. Ivannikova, Y. V., Naumova, E. S. & Naumov, G. I. Viral dsRNA in the wine yeast Saccharomyces bayanus var. uvarum. *Res. Microbiol.* **158,** 638–643 (2007).

37. Replansky, T., Koufopanou, V., Greig, D. & Bell, G. Saccharomyces sensu stricto as a model system for evolution and ecology. *Trends Ecol. Evol.* **23,** 494–501 (2008).

38. Bertoli, C. & de Bruin, R. A. M. Turning cell cycle entry on its head. *eLife* **3,** e03475 (2014).

39. Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B. & Lonvaud, A. *Handbook of Enology, The Microbiology of Wine and Vinifications*. (John Wiley & Sons, 2006).

40. Pascual-Teresa, S. de & Sanchez-Ballesta, M. T. Anthocyanins: from plant to health. *Phytochem. Rev.* **7,** 281–299 (2007).

41. Castañeda-Ovando, A., Pacheco-Hernández, M. de L., Páez-Hernández,

M. E., Rodríguez, J. A. & Galán-Vidal, C. A. Chemical studies of anthocyanins: A review. *Food Chem.* **113,** 859–871 (2009).

42. He, F. *et al.* Anthocyanins and Their Variation in Red Wines I. Monomeric Anthocyanins and Their Color Expression. *Molecules* **17,** 1571–1601 (2012).

43. de Pascual-Teresa, S., Moreno, D. A. & García-Viguera, C. Flavanols and Anthocyanins in Cardiovascular Health: A Review of Current Evidence. *Int. J. Mol. Sci.* **11,** 1679–1703 (2010).

44. Raghvendra *et al.* Chemical and Potential Aspects of Anthocyanins - a Water-soluble Vacuolar Flavonoid Pigments: Review. *Int. J. Pharm. Sci. Rev. Res.* **6,** 28–33 (2011).

45. Zafra-Stone, S. *et al.* Berry anthocyanins as novel antioxidants in human health and disease prevention. *Mol. Nutr. Food Res.* **51,** 675–683 (2007).

46. He, J. & Giusti, M. M. Anthocyanins: Natural Colorants with Health-Promoting Properties. *Annu. Rev. Food Sci. Technol.* **1,** 163–187 (2010).

47. Aneta, W., Jan, O., Magdalena, M. & Joanna, W. Phenolic profile, antioxidant and antiproliferative activity of black and red currants (Ribes spp.) from organic and conventional cultivation. *Int. J. Food Sci. Technol.* **48,** 715–726 (2013).

48. Slimestad, R. & Solheim, H. Anthocyanins from Black Currants (Ribes nigrum L.). *J. Agric. Food Chem.* **50,** 3228–3231 (2002).

49. Wu, X., Gu, L., Prior, R. L. & McKay, S. Characterization of Anthocyanins and Proanthocyanidins in Some Cultivars of Ribes, Aronia, and Sambucus and Their Antioxidant Capacity. *J. Agric. Food Chem.* **52,** 7846–7856 (2004).

50. Lingua, M. S., Fabani, M. P., Wunderlin, D. A. & Baroni, M. V. From grape to wine: Changes in phenolic composition and its influence on antioxidant activity. *Food Chem.* **208,** 228–238 (2016).

51. Oh, Y. s. *et al.* Characterization and Quantification of Anthocyanins in Grape Juices Obtained from the Grapes Cultivated in Korea by HPLC/DAD, HPLC/MS, and HPLC/MS/MS. *J. Food Sci.* **73,** C378–C389 (2008).

52. Giusti, M. M. & Wrolstad, R. E. Acylated anthocyanins from edible sources and their applications in food systems. *Biochem. Eng. J.* **14,** 217–225 (2003).

53. Myers, C. *Specialty and Minor Crops Handbook*. (UCANR Publications, 1998).

54. Hummer, K. E. & Dale, A. Horticulture of Ribes. *For. Pathol.* **40,** 251–263 (2010).

55. Ribéreau-Gayon, P., Glories, Y., Maujean, A. & Dubourdieu, D. in *Handbook of Eonology: The Chemistry of Wine Stabilization and Treatment* 141–203 (John Wiley & Sons, Ltd, 2006).

56. Morata, A. *et al.* Yeast influence on the formation of stable pigments in red winemaking. *Food Chem.* **197, Part A,** 686–691 (2016).

57. Nykänen, L. & Suomalainen, H. *Aroma of Beer, Wine and Distilled Alcoholic Beverages*. (Springer Science & Business Media, 1983).

58. He, F. *et al.* Anthocyanins and Their Variation in Red Wines II. Anthocyanin Derived Pigments and Their Color Evolution. *Molecules* **17,** 1483–1519 (2012).

59. Morata, A., Gómez-Cordovés, M. C., Colomo, B. & Suárez, J. A. Cell wall anthocyanin adsorption by different Saccharomyces. *Eur. Food Res. Technol.* **220,** 341–346 (2004).

60. Lilly, M., Bauer, F. F., Styger, G., Lambrechts, M. G. & Pretorius, I. S. The effect of increased branched-chain amino acid transaminase activity in yeast on the production of higher alcohols and on the flavour profiles of wine and distillates. *FEMS Yeast Res.* **6,** 726–743 (2006).

61. Ribéreau-Gayon, P., Glories, Y., Maujean, A. & Dubourdieu, D. in *Handbook of Eonology: The Chemistry of Wine Stabilization and Treatment* 51–64 (John Wiley & Sons, Ltd, 2006).

62. International Union of Pure and Applied Chemistry. *Compendium of Chemical Terminology - Gold Book*. (2014).

63. Cameleyre, M., Lytra, G., Tempere, S. & Barbe, J.-C. Olfactory Impact of Higher Alcohols on Red Wine Fruity Ester Aroma Expression in Model Solution. *J. Agric. Food Chem.* **63,** 9777–9788 (2015).

64. Stribny, J., Gamero, A., Pérez-Torrado, R. & Querol, A. Saccharomyces kudriavzevii and Saccharomyces uvarum differ from Saccharomyces cerevisiae during the production of aroma-active higher alcohols and acetate esters using their amino acidic precursors. *Int. J. Food Microbiol.* **205,** 41–46 (2015).

65. Carew, A. L., Smith, P., Close, D. C., Curtin, C. & Dambergs, R. G. Yeast Effects on Pinot noir Wine Phenolics, Color, and Tannin Composition. *J. Agric. Food Chem.* **61,** 9892–9898 (2013).

Appendix 1 °Brix changes during fermentation

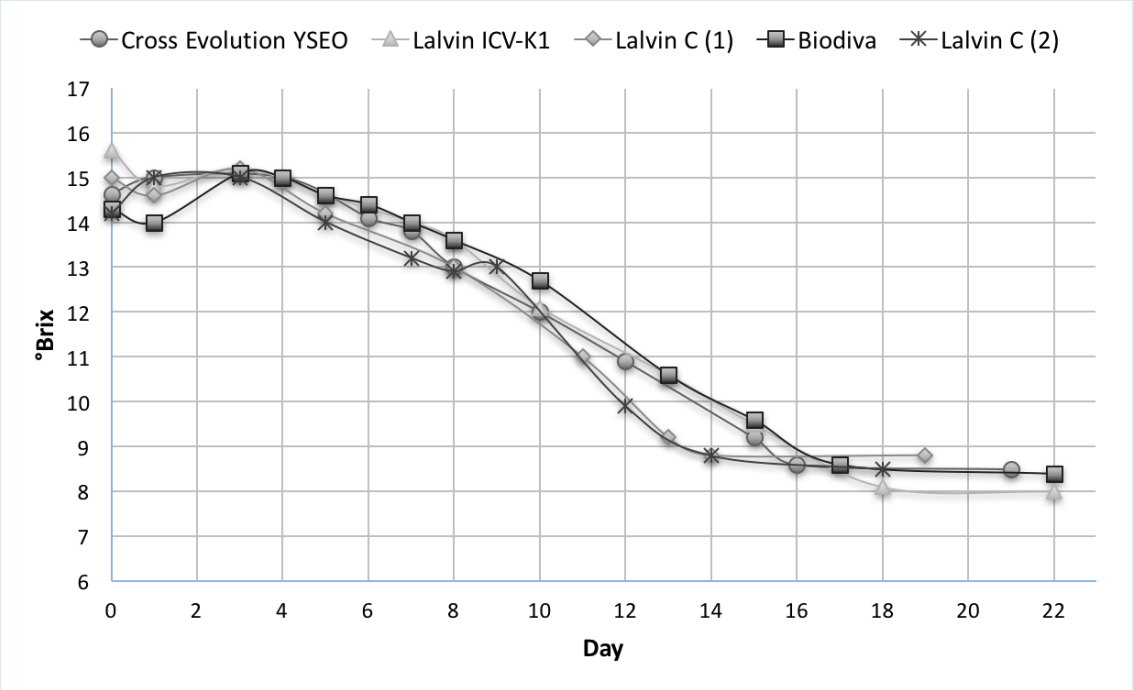


Figure 16°Brixs measured during fermentation of the wines produced from black currant variety Mikael (LUKE's variety 17)

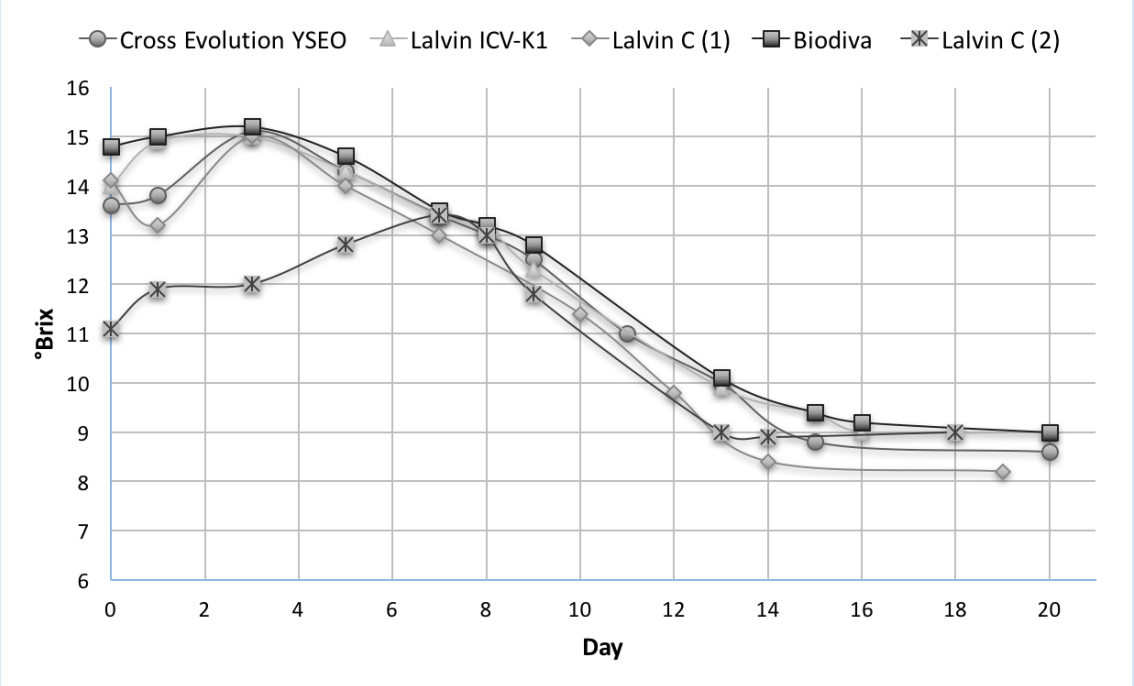


Figure 17 °Brixs measured during fermentation of the wines produced from black currant variety LUKE's variety 15.

Appendix 2 Computational and real ethanol concentrations in the finished wines

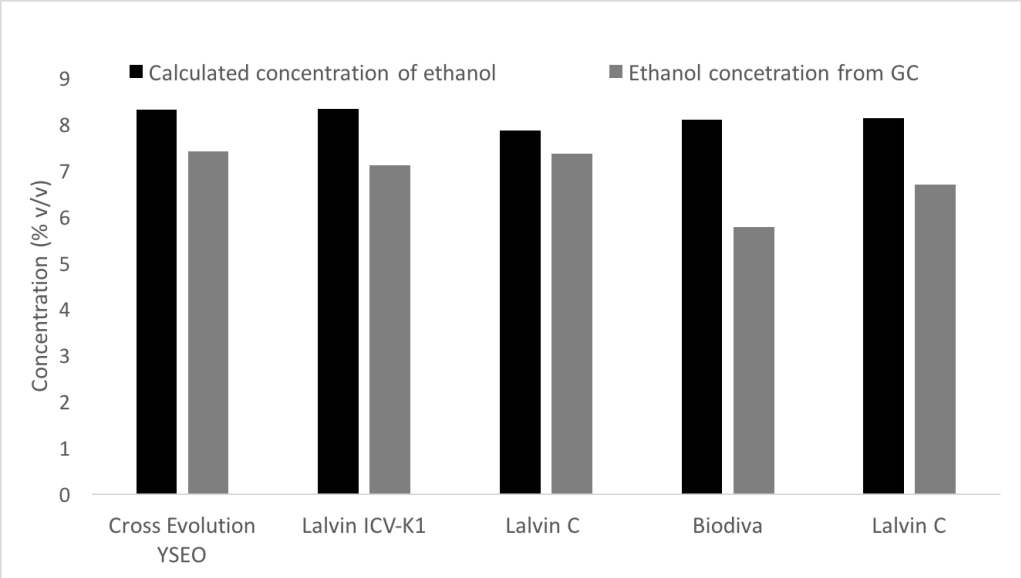


Figure 18 Computational and real ethanol concentrations in the wines made from Mikael.

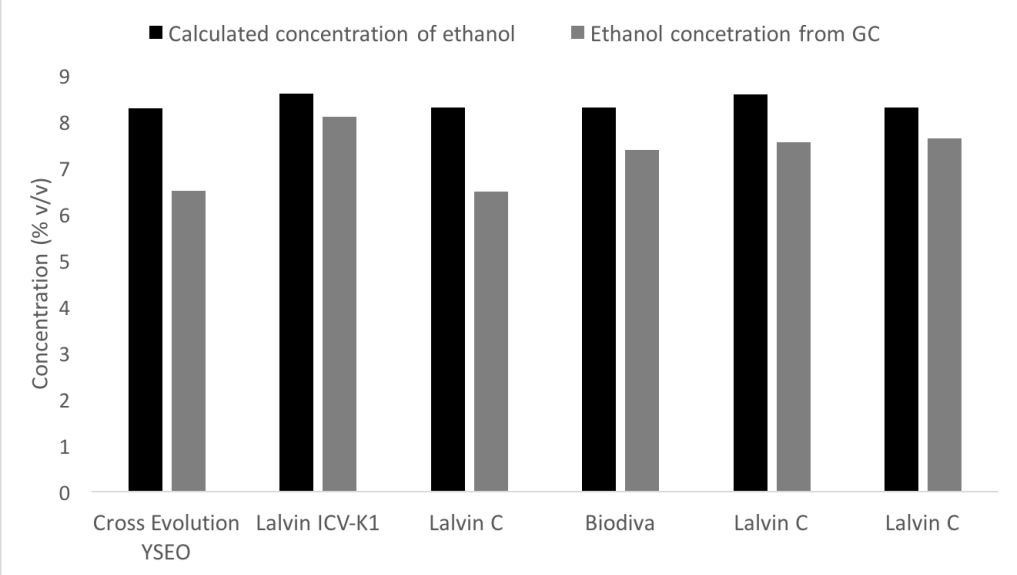


Figure 19 Computational and real ethanol concentrations in the wines made from LUKE's variety 15.

Appendix 3 The example pictures of dissolving patterns of anthocyanins

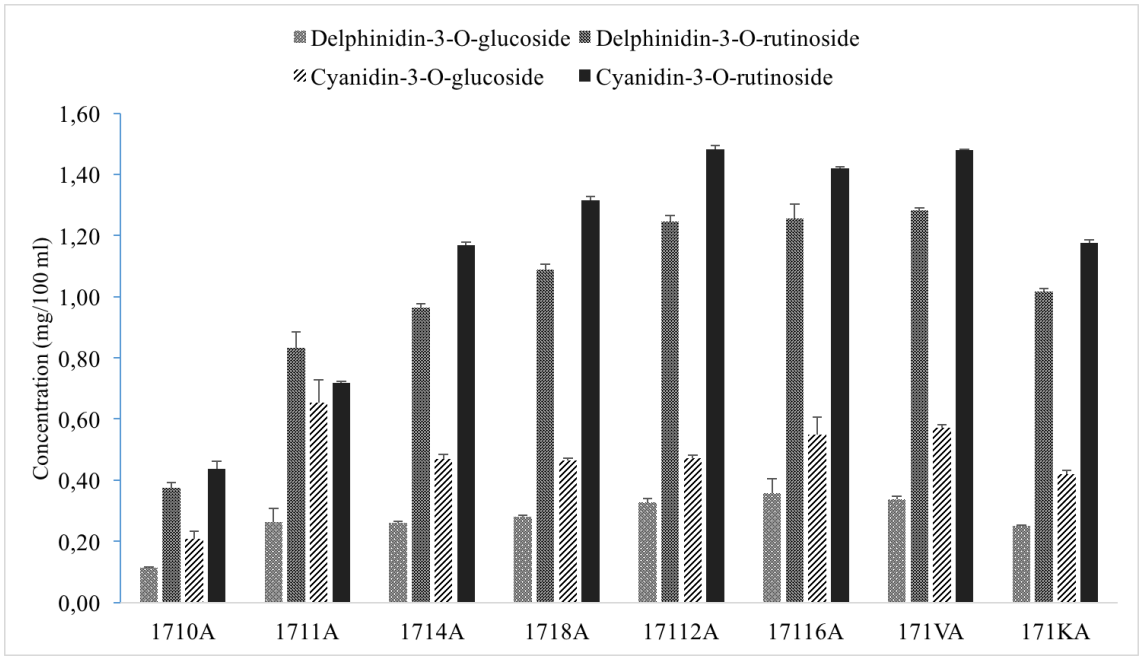


Figure 20 An example of dissolve of the anthocyanins during fermentation of wine made from Mikael (LUKE's variety 17).

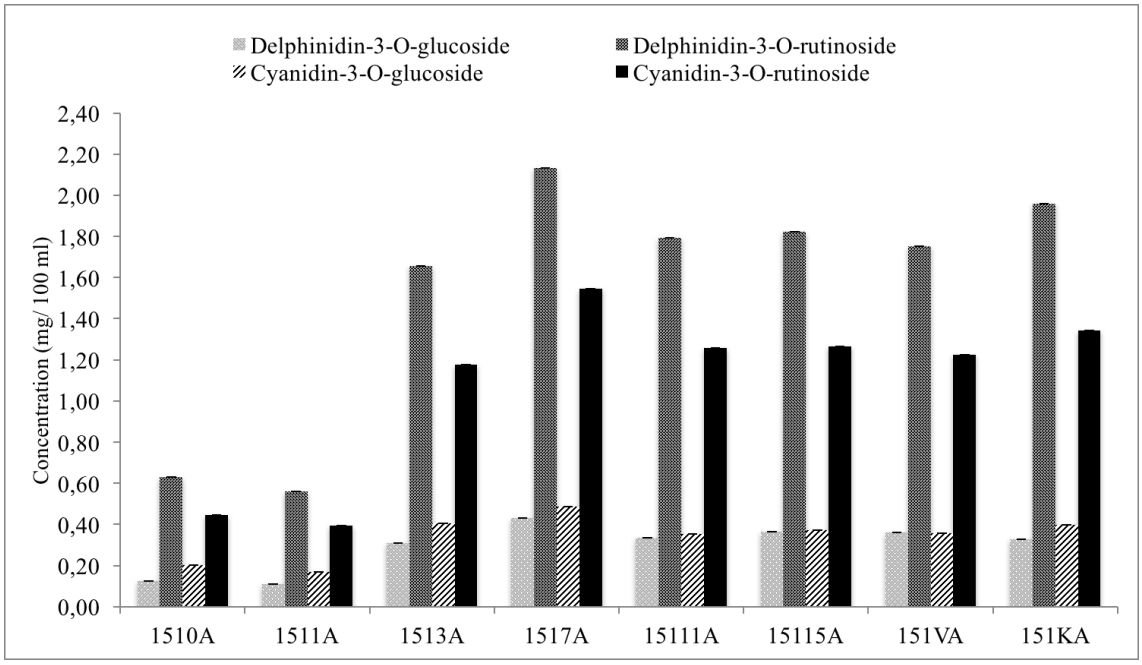


Figure 21 Figure 22 An example of dissolve of the anthocyanins during fermentation of wine made from LUKE's variety 15.

Appendix 4 An example for calculating concentration of the anthocyanin

An example for calculating concentration of delphinidin-3-O-glucoside

y_n = Area of the delphinidin-3-O-glucoside's peak in the sample = 167.622

x = Concentration of the delphinidin-3-O-glucoside (µg/ml)

y = 95350x = Equation to calculate the concentration of the delphinidin-3-O-glucoside

$$x = \frac{y_n}{95350} = \frac{167622}{95350} = 1.758\ \mu g/ml \qquad \textit{(Equation 3)}$$

Because sample was 1:1 wine and MeOH/HCl (99:1), the result was multiplied by two → 2x = 3.516 µg/ml.

Appendix 5 Example chromatograms from HPLC-DAD

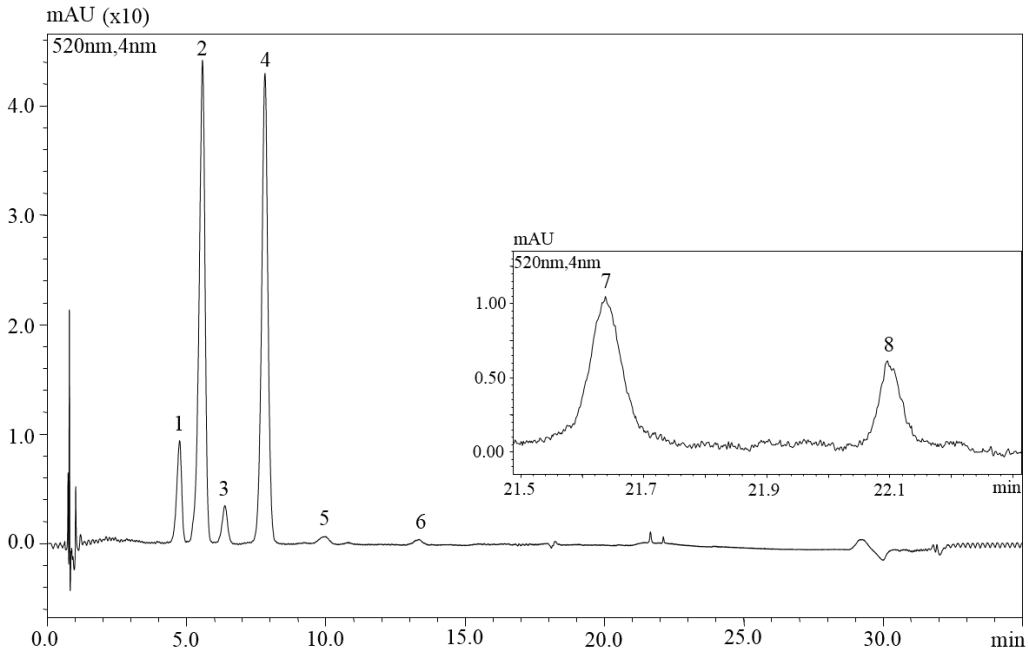


*Figure 23 Anthocyanin profile from LUKE's variety 15 fermented with Cross Evolution YSEO (*Saccharomyces cerevisiae*).*

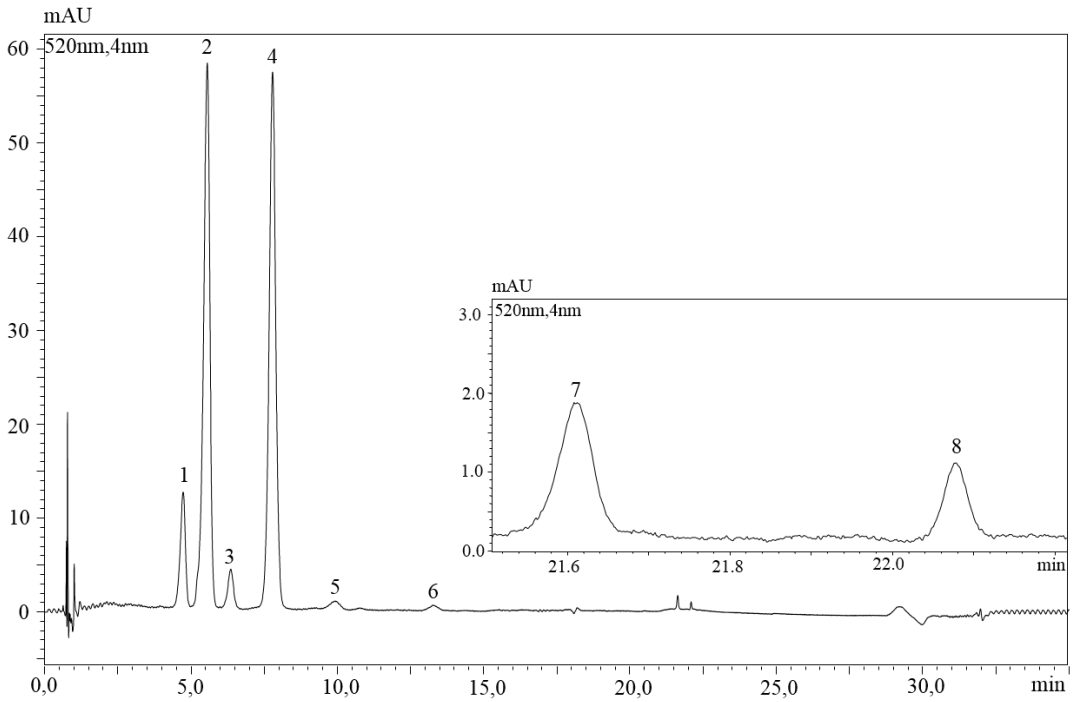


*Figure 24 Anthocyanin profile from LUKE's variety 15 fermented with Lalvin ICV-K1 (*S. cerevisiae cerevisiae*).*

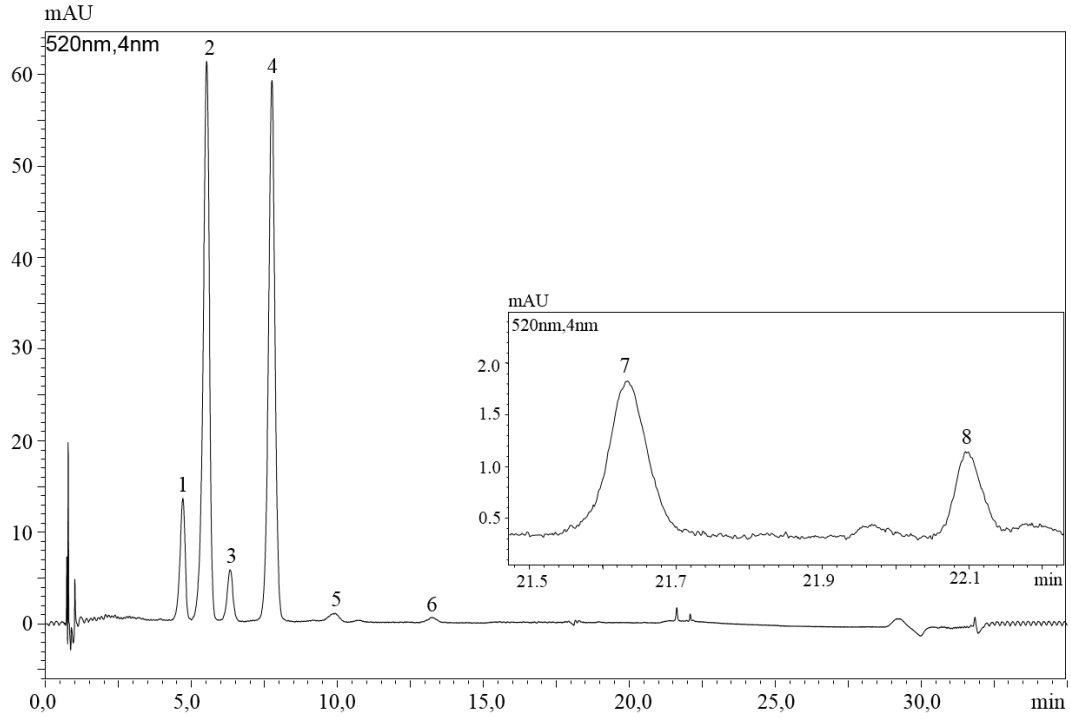


*Figure 25 Anthocyanin profile from LUKE's variety 15 fermented with Biodiva (*Torulaspora delbrueckii).

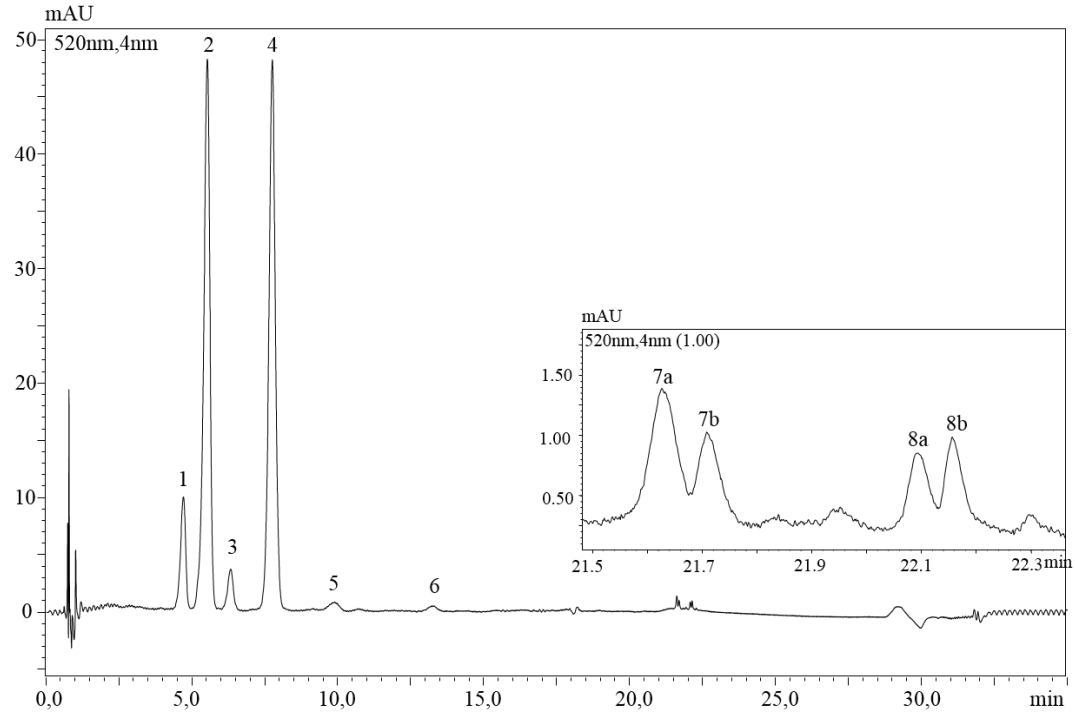


*Figure 26 Anthocyanin profile from LUKE's variety 15 fermented with Lalvin C (*S. bayanus*).*

Table 11 Compounds identified from the wines.

Peak number	Compound	Reference
1	Delphinidin-3-*O*-glucoside	
2	Delphinidin-3-*O*-rutinoside	
3	Cyanidin-3-*O*-glucoside	
4	Cyanidin-3-*O*-rutinoside	
5	Petunidin-3-*O*-glucoside	
6	Cyanidin-3-*O*-arabinose	
7/7a	Delpinidin-3-*O*-(6"-coumaroyl)glucoside	
7b	Delphinidin	
8/8a	Cyanidin-3-*O*-(6"-coumaroyl)glucoside	
8b	Cyanidin	

Appendix 6 Anthocyanin profiles of the fermentation refuses

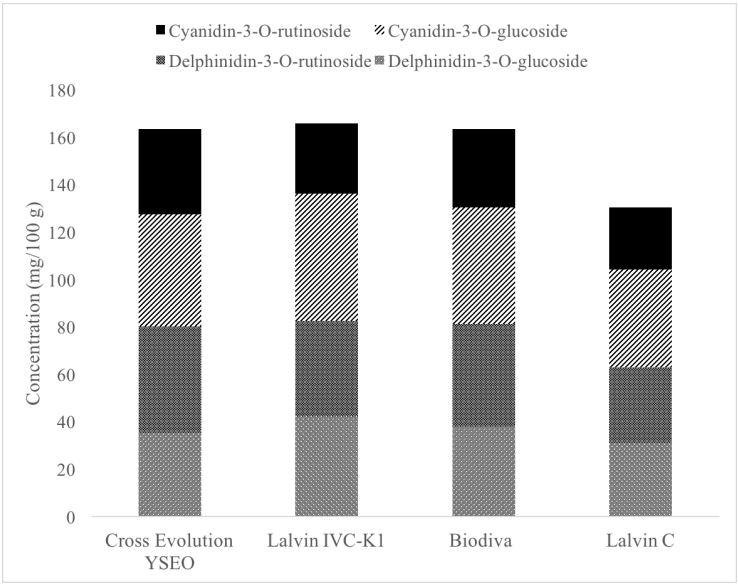


Figure 27 Anthocyanin profile of Mikael wine's fermentation refuses.

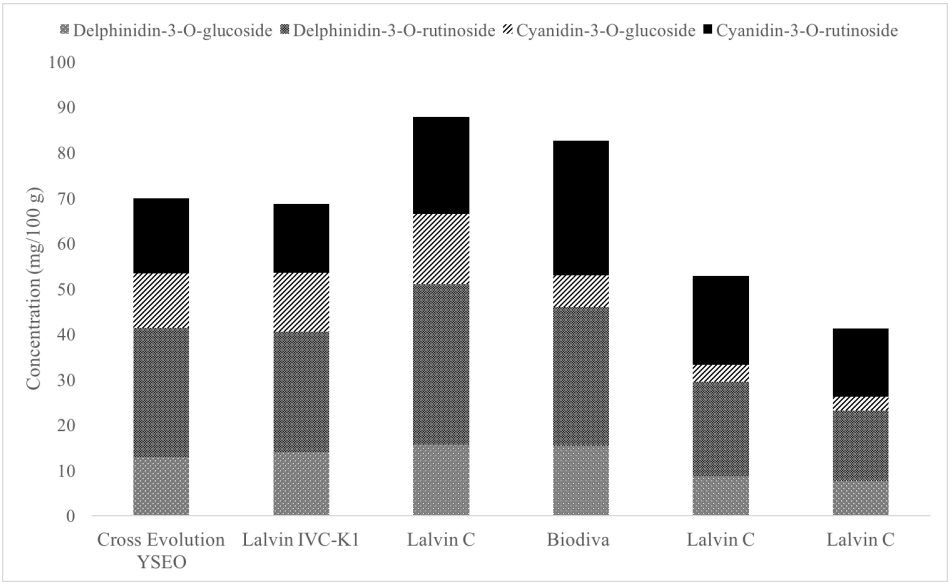


Figure 28 Anthocyanin profile of LUKE's variety 15 wine's fermentation refuses.